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Gennady Evtugyn

Biosensors: Essentials

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Biosensors: Essentials

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Preface

Over the past decades, biosensors have emerged from the laboratories into the everyday life of many millions of people around the world. Like some other sensors, they were first developed for the detection of particular low-molecular species, i.e., metabolites or disease biomarkers, which had a predominant importance for clinical diagnostics, pharmacy and the health care industry. To some extent, the peculiarity of biosensors was due to the application of biochemical items (enzymes, antibodies, nucleic acids) that played a role similar to that of litmus paper in pH measurement or mercury in the clinical thermometer. Biologists and medics who used to deal with such biological tissues easily adapted to the unusual functioning of living things as measuring devices. Contrary to that, chemists and physicians could not accept the "vitality" of biosensors, with their sensitive but not stable response and their high sensitivity to experimental conditions.

The situation has changed rather quickly. The broad dissemination of glucometers, mass production of warning devices for military purposes, and DNA diagnostics tools have transformed biosensors into a remarkable but rather habitual sign of the progress and made the representatives of science and technology more lenient to their lively temper. Time has flown, and nobody would now say that a biosensor is a kind of scientific trick with no sense in real life. The human genome project, environmental monitoring, even searching for live evidence in space needed hi-tech products based on biosensing principles, but they are far from the good old glucose electrode described by L. Clark in 1962.

About 10 % of current publications in analytical chemistry journals consider research related to biosensors. Many of them illustrate the indisputable point about the interdisciplinary approaches and convergence trends in modern natural sciences. However, the comprehension of this trend does not facilitate the study of biosensor development, which assumes the knowledge and skills from many relative disciplines such as biochemistry, medicine, materials sciences, electrochemistry, analytical chemistry, microbiology, etc. Most researchers involved in the development of biosensors began their careers as biologists. Sometimes they considered biosensors as a kind of applied by-product of their principal research interests in the basic sciences. However, the development of sophisticated signal

transducers and modern tendencies of miniaturization and automation call for a serious attention to other aspects of biosensors that is often beyond the scope of life sciences.

The interdisciplinary character complicates the consideration of biosensors as a subject of study. This book is addressed to the post-graduate students and young researchers who are specializing either in chemistry or biology but are also interested in biosensors. Here, the biosensors are considered to be a variety of combinations of biochemical recognition elements and physical signal transducers so that anyone can specify the chapter most appropriate for his/her own interest and basic level of knowledge. To provide the inside of biosensor functioning, some of the most popular and instructive examples are considered as a case study. Besides the general information, reviews and monographs on the special aspects of biosensors are referred to and summarizing tables are provided.

Most certainly, it is impossible to cover all the issues and application areas related to biosensor development. This book is mostly aimed at stirring interest and providing background for those who need some stimuli to begin working in this inexhaustible field of science.

And, last but not least, I would like to address my heartfelt thanks to my teachers who supported me all the way and to my patient and all-forgiving family.

Kazan, 2013 Gennady Evtugyn

Abbreviation List

Ab	Antibody
Ag	Antigen
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
ss-DNA	Single-stranded DNA
ds-DNA	Double-stranded DNA
DPV	Differential pulse voltammetry
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
e.m.f.	Electromotive force
EQCM	Quartz crystal microbalance with simultaneous electrochemical signal detection
$FAD/FADH_2$	Flavine adenine dinucleotide
FDA	Food and Drug Administration
FET	Field-effect transistor
ChemFET	Chemical sensor based on FET transducer
EnFET	Enzyme sensor based on FET transducer
ISFET	Ion-selective sensor based on FET transducer
FRET	Fluorescence resonant energy transfer
HIV	Human immuno deficiency virus
HRP	Horseradish peroxidase
ISE	Ion-selective electrode
LB film	Langmuir-Blodgett film
LbL	Layer-by-layer
LOD	Limit of detection
MEMS	Microelectromechanical system(s)
$NAD^+/NADH$	Nicotinamide adenine dinucleotide
ODN	Oligodeoxyribonucleotide
PAMAM	Polyaminoamine dendrimer
PNA	Peptide nucleic acid
PVC	Polyvinyl chloride

RNA	Ribonucleic acid
SAM	Self-assembled monolayer
SCE	Saturated calomel electrode
SERS	Surface enhanced Raman spectroscopy
SPR	Surface plasmon resonance
TTF	Tetrathiafulvalene
TCNQ	Tetracyanoquinodimethane
QCM	Quartz crystal microbalance

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Chapter 1
Introduction and Overview of History

For a long time, people used to check a biological response toward toxic species. Masses of fish perishing in winter or the leaves turning yellow in summer were interpreted as signs of the potential danger for living beings caused by water or soil contaminants. In the nineteenth century, canaries were used in coal mining as early warning systems to prevent poisoning with carbon monoxide, methane and other noxious gases accumulated in the coal mines. Until recently, behavioral or phenotypical disorders were considered to be biological indicators of chemical hazards in the environmental impact assessment. Besides toxic species, biological response was successfully applied to the testing of food and drinks, in medical diagnostics and other similar areas. What is typical for such approaches, the conclusion regarding the content and nature of the chemicals, is substituted by the decision about their biological effect. Being very sensitive, biological indications based on taste, smell, etc., undoubtedly do not meet the common requirements of the analytical systems. First and foremost, the result of a bioassay highly depends on the observer and cannot be easily quantified and/or expressed in terms acceptable for physical measuring devices, whether it is a medicinal thermometer or an NMR spectrometer.

A biosensor can be considered to be a direct descendant of the coal mine canary. Its signal originates from the biological component implemented in its assembly as a recognition element. The biological signal is transformed by a physical element (sensor, transducer) so that the output (current, potential, optical density) does not differ dramatically from that of conventional measurement devices and can be amplified, processed and quantified against an internal or external standard in a similar manner. A combination of the high sensitivity and biochemical "sense" exerted by the biological part with accuracy and certainty of the physical counterpart was the main idea of the biosensor development. A general scheme of the biosensor assembly is presented in Fig. 1.1.

Since the mid-1960s, the meaning of the term "biosensor" has changed several times, depending on the research area and problems to be solved. In physiology and medicine, biosensors are any system used for monitoring living systems, even pregnancy test strips or blood pressure tonometers. In biotechnology, a biosensor was defined as "a device that uses specific biochemical reactions mediated by

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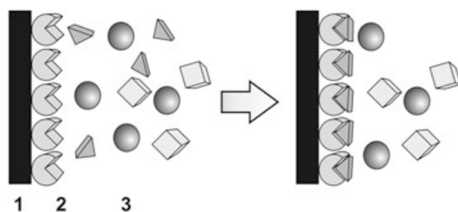

Fig. 1.1 Principal scheme of a biosensor assembly. *1*—Transducer; *2*—Biological recognition element; *3*—Sample

isolated enzymes, immune systems, tissues, organelles or whole cells to detect chemical compounds, usually by electrical, thermal or optical signals" (Nagel et al. 1992). Being very general in describing the functioning of biosensors, this definition has at least two limitations that differ from that of the modern point of view. First, nucleic acids and oligonucleotides are omitted from the list of biochemical components. Second, the term "mediation" can be used only for enzymes, tissues or whole cells as sources of enzyme activity. However, the reaction of analyte molecules with antibodies, or the formation of DNA–protein complexes, do not correspond to the mediation because such reactions do not take the catalytic path of the analyte conversion.

The description of a biosensor as any kind of device based on biochemical reactions has initiated many discussions. As a result of some of them, the following definition was suggested:

> An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element (Physical Chemistry and Analytical Chemistry Divisions of IUPAC 1999) (Thevenot et al. 1999).

Although the technical report mentioned (Thevenot et al. 1999) referred to electrochemical biosensors, it was assumed that the definition could then be extended to other types of signal transduction. What seems important is that the biosensors are clearly distinguished from the bioanalytical systems. They involve additional units or accessories, e.g., peristaltic pumps, pre-concentration columns, injection systems, etc. From this point of view, all the flow-through biosensors developed for wastewater control or blood analysis do not belong to the biosensor family. Besides, glucometers, which are often considered to be the essence of a biosensor idea, specifically in the area of commercialization, are not real biosensors either, because they are equipped with needles, erythrocyte separating membranes, etc.

An intimate contact of the biochemical part with a transducer was described as another indispensable feature of a biosensor. This has made it possible to draw a line between the compact devices and cumbersome technological apparatuses such as fermenters or wastewater treatment facilities that are equipped with their own sensors, e.g., oxygen sensors, pH meters, thermometers, or glucose biosensors.

At present, interest in the specific details of a biosensor definition is declining. In comparison with the late 1990s, the term "biosensor" has become more popular with analytical chemistry, molecular biology and biochemistry. The limitations related to the use of complex apparatuses or accessories are regarded with much less pedantry. Novel biochemical elements, e.g., aptamers and materials with molecular imprints, have been introduced in the biosensors assemblies. The family of DNA sensors has been added to the traditional biosensors with their own specific goals, like the detection of oligonucleotides, proteins and the low molecular compounds able to bind to DNA. At present, no one would refuse to accept the use of a biosensor term with respect to the commercial devices manufactured for routine clinical analysis or drug design purposes. Nevertheless, two main characteristics of a biosensor definition have remained, i.e., (1) the use of a biochemical recognition element, and (2) the rather simple and compact design that provides real time measurement. To emphasize the difference between integrated biosensors and more complicated devices that include some additional items or analysis steps, the terms "biosensing device" or "biosensor-related techniques" might be recommended.

Other common terms related to biosensors and their application were never a battlefield comparable to that around the biosensor definition. Nevertheless, let me give examples of some of the most suitable definitions:

- **Analyte** *is a substance to be determined.*
- **Chemical sensor** *is a device that transforms chemical information (concentration, sample content,* etc.*) into an analytically useful signal.*
- **Transducer** (*sensor, electrode,* etc.) *serves to transfer the output signal generated by biochemical reaction to the electrical signal which can be amplified and processed by appropriate equipment.*
- **Substrate** *is* (1) *a solid support used for the manufacture of transducer elements;* (2) *organic matter oxidized by bacteria;* (3) *a substance whose reaction is catalyzed by an enzyme.*
- **Receptor** *is a molecule or part of a complex molecule or supramolecular complex (biological membrane, cell subunit) that is responsible for the recognition and specific binding of the low-molecular counterpart.*
- **Biochemical recognition** *is the ability of a biomolecule (enzyme, antibody, nucleic acid, cell,* etc.*) or biochemical tissue to attach a counterpart molecule by a complementary shape due to non-covalent multi-point interactions.*
- **Biosensor signal (response)** *is a change of the transducer characteristic that is directly related to the biochemical recognition event and can be quantitatively or semi-quantitatively expressed.*

We can conclude that a biosensor differs from a chemical sensor only in the nature of its recognition element, i.e., implementation of the biochemical parts in

their assembly. And vice versa, a chemical sensor can be used as a transducer in the assembly of a biosensor to amplify the signal or improve its other characteristics. All the parameters required for the description of the performance of a chemical sensor are equally necessary for the biosensor characterization, i.e., selectivity, sensitivity of the response, lifetime, signal drift, reproducibility, interferences, etc.

Biosensors most certainly differ from chemical sensors. The use of the parts of living beings in a lifeless shell is exciting. One can note the reference to cyborgs from science fiction movies and to the incomprehensible miracle of life as well. The fact that a small piece of this miracle can be used and sold in a pharmacy does not diminish the mere feeling of something impossible, especially for those who have just begun working in this area. Even those who do not have any specific knowledge related to the development of a biosensor or its application can estimate great opportunities offered in such futuristic areas as biological computers, smart prostheses and artificial viscera, remote health care service and so on.

The goal of this book is first to give a general introduction to biosensors, on the way they are assembled and work, and what kind of information can be obtained with their assistance. The book is not aimed at substituting any monographs or special editions of journals intended for a professional audience; rather, it is aimed at graduate students and young researchers who are involved in the neighboring branches of chemistry and biochemistry and are interested in a general outlooks on the area described above.

1.1 Short Biosensor History

The development of the first biosensor is closely associated with the name of L. C. Clark, who first suggested the oxygen probe (1956) for measuring oxygen in the blood and then described the "enzyme electrode" consisting of this probe and two dialysis membranes carrying a small portion of the aqueous solution of glucose oxidase in between them (Fig. 1.2) (Clark and Lyons 1962). The enzyme catalyzed the oxidative conversion of glucose to gluconic acid. Thus, the oxygen content in the proximity of the electrode surface decayed proportionally to the

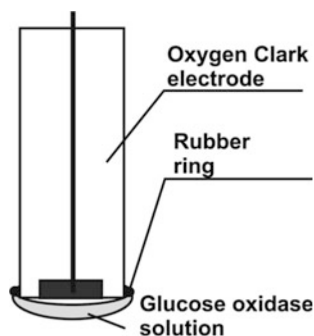

Fig. 1.2 First "enzyme electrode" with glucose oxidase solution entrapped in the sandwich-type membranes

glucose concentration. Later on, a similar device with glucose oxidase entrapped in the polyacrylamide gel was described by Updike and Hicks (1967). However, the first analytical application of the immobilized enzyme was made in 1962 (Guilbault et al. 1962). G. Guilbault had proposed the alarm system for the early detection of nerve agents. It consisted of two grid Pt electrodes with a porous tablet of sorbent containing the immobilized enzyme cholinesterase, a biological target for chemical warfare.

The progress in the development of biosensors can be illustrated by a kind of a timeline that can be further extended for each particular area of the biosensor application:

- 1969—the first potentiometric enzyme sensor based on the standard NH_3 selective electrode and urease for the determination of urea in urine (Guilbault and Montalvo 1969);
- 1975—the first immunosensor on a potentiometric transducer (Janata 1975);
- 1976—the first amperometric immunosensor (Aizawa et al. 1976);
- 1976—the microbial biosensor utilizing the rate of respiration as a specific signal on the total amount of oxidizable organic matter in water (Karube et al. 1977);
- 1976—the first enthalpiemetric biosensor (Mosbach and Danielsson 1974);
- 1970–1972—the first chemical sensors based on an ion-selective field-effect transistor (ISFET) for electrophysiological measurements to quantify the ion composition around nerve tissues (Bergveld 1972);
- 1976—the idea of combining ISFET with an enzyme (realization for penicillin detection in 1980 (Caras and Janata 1980));
- 1976—the electrochemical glucose sensor is introduced in the bedside artificial pancreas;
- 1981–1984—the new tools for biosensor development, including electropolymerization techniques, fiber-optic sensors (optodes) (Voelkl et al. 1980) and flow injection immunoassay (Worsfold, Hughes 1984);
- 1983—the backgrounds of the surface plasmon resonance (SPR) techniques for biosensing are described (Liedberg et al. 1983);
- 1983—the first gas phase biosensor based on the quartz crystal microbalance (QCM) (Guilbault 1983);
- 1986—the analytical application of QCM for the detection of antigen–antibody interactions (Muramatsu et al. 1986).

Some other important benchmarks of biosensor history can be found in reviews (Guilbault et al. 2004; Palchetti and Mascini 2010) and the historical aspects of glucose sensors in (Wang 2008).

The number of enzymes and antibodies used in biosensor assembly increased very rapidly, especially in combination with electrochemical transducers such as oxygen electrodes or pH-sensitive ISE. Most of the enzymes introduced in the investigations during the first period of the biosensor history were associated with particular protocols of biochemical assay already used in clinical analysis. The use of cholinesterase as a biochemical target for chemical warfare for a long period of time was one of the rare exceptions.

At that time, the development of biosensors significantly depended on the progress in the isolation and characterization of biochemical components. Many of the publications between 1980 and 2000 were devoted to a new enzyme or an enzyme source that was first introduced in an appropriate measuring device or immobilized by a newly elaborated protocol. The biochemicals used in such works were often obtained by a research group involved in developing a transducer and final assembling of the biosensor. Even though more and more enzymes, antibodies and cell cultures became commercially available, at first they required special treatment to be performed prior to their analytical application. For example, crude enzymes required purification or separation from ballast proteins, and nucleic acids were re-crystallized from phenol to reduce their enzymatic digestion, etc. The treatment of biochemicals made it necessary to characterize the purification products, i.e., to determine the specific activity and kinetic parameters of enzymes or residual amounts of protein in DNA (Byfield and Abuknesha 1994).

In the last decade, the situation had changed, and the focus of biosensor investigations had shifted to transduction systems. The progress of biotechnology and related areas improved the quality of the biochemicals to the extent sufficient for their direct use, "as they are", in biosensor systems. For enzymes, this relates not only to the content of the target protein and the highest specific activity achieved. The stabilizers prolong the lifetime of preparations and simplify their implementation in appropriate analytical devices. Such progress continuously increases the number of scientists involved in biosensor development. The new generation of researchers does not feel the demand in any specific biochemical knowledge and experimental skills and considers biochemical preparations to be a kind of analytical organic reagent. In accordance with such an opinion, biochemical components used in biosensor assembly are definitely less stable and more expensive than, for example, ionophores, but, to be honest, do not differ from them so much. Besides, some preparations can already contain the additives required for biosensor development, i.e., specific functional groups for enzyme labeling, solid supports for affine concentration and separation, etc. The availability of the half-finished products mentioned was initiated by the global commercial success of conventional bioassay techniques, such as immunoassay in medical diagnostics. Yet they are also suitable for biosensor development and significantly decrease labor expenditures and the cost of the device and of any single measurement.

On the other hand, the commercial preparations of common biochemical components of biosensors can sometimes inconspicuously restrict their operation. Thus, many researchers involved in investigating enzyme inhibition noted that the

modern enzyme preparation products that are commercially available are much more stable toward metal ions than the same enzymes were 15–20 years ago. New stabilizers added in trace amounts suppress the inhibiting activity of impurities so that the results obtained with “old-fashioned” enzymatic sensors give odds to modern analogs in sensitivity, especially towards metals.

This does not mean that the contribution of biochemistry to biosensor development has been waning. Besides a clear understanding of the biochemical reactions in the biosensors, there is a constant need to select measurement conditions that should maximally take into account the specific features of the biochemical elements. A physician or chemist who starts working with biosensors, but has no idea about how they function, can obtain a positive result, but the cost and effort required would be incomparable with those necessary for colleagues who understand the driving forces behind the processes initiated in the biosensors by addition of the biological target.

The progress in the biochemical aspects of the biosensors is mainly addressed to new forms or new types of previously known biochemical elements that are at present especially adapted to their application in biosensors. The aptamers (Šmuc et al. 2013), i.e., artificial oligonucleotides obtained by the combinatorial chemistry approach and selected against analyte molecules by affine chromatography, are a most exciting example of such an adaption. The application of gene expression for common enzyme production, point mutations at the enzyme active sites, binding or removing functional groups to reach a higher stability, or activity of enzymes are the other examples of biochemical approaches to the improvement of the biosensor performance, whether the increased sensitivity toward an analyte or a sufficiently lower cost of the enzyme preparation (Campàs et al. 2009; Boni et al. 2004).

In the “lifeless” part of a biosensor, most efforts are now concentrated on the demands related to its practical applications. They include not only developing more reliable measurement principles and appropriate devices that meet the specific requirements of the sensitivity and selectivity of response. Increasing attention is paid to the remote control devices that include implantable medical sensors (Vaddiraju et al. 2010) and alarm systems for chemical hazards in the environment (Badihi-Mossberg et al. 2007). Besides miniaturization problems, compatibility with biological tissues and reliability of the connections should be taken into account in such applications.

1.2 Biosensor Classification

The most frequently used classification of biosensors is based on the origin of their main parts mentioned above in accordance with a general definition (Table 1.1).

The definition of the biosensor class via the biochemical component used in its assembly is intuitively understandable and requires hardly any additional explanations. *Enzymatic biosensors* (also called *enzyme sensors*, or *enzyme electrodes*) exploit enzymes implemented in the surface layer of a transducer. In some cases, the number of individual enzymes can be specified (bi-enzymatic, tri-enzymatic

Table 1.1 Main components of the biosensors

Biochemical components	Transducer	Analytes
Enzymes	Electrochemical	Drugs and their metabolites
Antibodies	Optical	Disease biomarkers
Nucleic acids	Microelectromechanical	Vitamins and antioxidants
Receptors	Mass sensitive	Metabolites
Tissues	Enthalpiemetric	Environmental pollutants
Cells		Industrial chemicals

sensors) but only in the case where alternative constructions of biosensors exist. Thus glucose can be determined with enzymatic sensors including glucose oxidase, which catalyzes the oxidation of a substrate to gluconic acid. Meanwhile, the bi-enzyme sensor involves peroxidase as an auxiliary enzyme intended for the simplification of the signal measurement (see details in Chap. 4) (Zhu et al. 2007). Tri-enzyme sensors are described for the detection of starch (Hu et al. 1999), ATP (Cui et al. 2008) and lactate (Kwan et al. 2004).

Immunosensors utilize the components of immune interactions, i.e., antibodies or antigens, which form specific complexes on the sensor interface (Morgan et al. 1996). *Microbial sensors* presume the application of living or dead microorganisms (Lei et al. 2006).

The *DNA sensor* is a minor exception to this rule. Besides native or denatured DNA, the same term (DNA sensor or DNA biosensor) refers to the biosensors that involve elements derived from DNA, i.e., natural oligonucleotide sequences (DNA probes) and aptamers. A DNA probe is a short oligonucleotide sequence that reproduces a small part of a native biomolecule. The biosensors based on such sequences and intended to detect the DNA fragments specific for appropriate genes are also called *genosensors* (Yang and McGovern 1997). The aptamers also consist of the nucleotides but have no analogy in the native DNA structure. The aptamers are synthesized by the combinatorial approach and are selected by affine chromatography against a target analyte. The aptamers can be based on an RNA library, whereas native RNA molecules are not currently used in biosensors. The biosensors based on aptamers are called *aptasensors* (Famulok and Mayer 2011).

In addition to the primary biochemical component, the biosensors can involve auxiliary reagents that can participate in the generation or amplification of the signal or in some other miscellaneous functions, e.g., interference elimination, biosensor regeneration after the signal measurement, the analyte accumulation in a surface layer, etc. Thus, the immuno- and DNA sensors use enzymes as specific labels (Medyantseva et al. 2001; Pan 2007) that are similar to conventional immunoassay protocols such as Enzyme-linked immunosorbent assay (ELISA). Antigen–antibody interactions can be used for affine immobilization of enzymes or oligonucleotides on the transducer interface (Andreescu et al. 2006; Litos et al. 2009). In this case, the biosensor is specified in accordance with the primary biochemical process that leads to analyte detection. In some cases, an additional biochemical component is mentioned in the qualifying part of a class name. The DNA sensors with an

enzymatic amplification of the signal, or the DNA sensor with the immunochemical accumulation of the analyte, are examples of such a classification.

Deoxyribozymes (DNAzymes) are single-stranded oligonucleotides that exert catalytic activity similar to that of enzymes (Willner et al. 2008). Although the first DNAzyme was synthesized in 1994, they have recently been introduced in the biosensor assembly for the specific detection of organic species. DNAzymes are synthesized using the protocols developed for aptamers and are commonly intended for the hydrolysis of phosphodiester bonds or for mimicking peroxidase activity (Kosman and Juskowiak 2011).

Several decades ago, it was suggested that biological tissues could be used in biosensor assembly (Sidwell and Rechnitz 1986). In most cases, they were applied as a source of the specific enzyme activity. In this manner, they substituted enzyme preparations that were either more expensive or unavailable. Besides, the stability of the enzymes in biological tissues was even higher than in the isolated form due to the protective effect of the microenvironment. For example, minced horseradish root added to carbon paste acted as a peroxidase sensor (Wang and Lin 1989), cucumber slices contained ascorbate oxidase (Tomita et al. 2005), etc. The low reproducibility of the characteristics of such biosensors was the main drawback, seriously limiting their application in biosensors.

Later on, the use of biological tissues as the source of specific enzyme activity became insignificant. The success of biotechnology and enzymology significantly simplified the access to pure proteins and even provided some ready-made products combining the enzymes and supports (cellulose derivatives, silica particles, and magnetic beads).

Nevertheless, some aspects of biosensors based on biological tissues remain urgent, and two applications warrant special attention. First, biological tissues can provide a cascade of enzymatic reactions that include as many steps as are realized in a living being. The full reconstruction of the same path by separate preparations of enzymes seems too complicated and senseless. The photosystems I and II isolated from chloroplasts and used for the sensitive detection of the herbicides are an example of such a strategy (Giardi and Koblízek 2001). The second approach involves the use of cell cultures that mimic an organ or specialized biological tissue (skin, muscle, etc.), and the non-proliferated cell culture can be used for acute toxicity assessment (Polak et al. 1996). More recently, such systems have found growing interest in antitumor drug- screening and cancer diagnostics (Rasooly 2006).

Whole cells, whether microorganisms or cultivated tissues of multicellular organisms, have an intermediate rank between biomolecules (proteins and nucleic acids) and biological tissues. Due to a rather simple and cost-effective cultivation, microorganisms are used as an inexpensive source of appropriate enzyme activity (Su et al. 2011). The application of *microbial biosensors* is closely related to the main microbiological technologies producing starch, saccharides, ethanol, organic acids, etc. What is important is that the microbic industry is ready for biosensor approaches in the control of biotechnological processes because it does not dramatically differ from the target processes used (digestion, microbial respiration,

etc.). Besides individual compounds, microbial biosensors showed growing potential in the estimation of organic matter (Liu et al. 2010; Ponomareva et al. 2011). This parameter characterizes the water's quality, biochemical treatment efficiency, the pollution of surface water with degradable organic residuals, etc.

Similar to the biosensor classification presented above is distinguishing the biosensors between the enzyme and *affinity biosensors*. The latter group involves immunosensors and DNA sensors. The main difference in their behavior is related to the signal nature; enzyme sensors belong to the kinetic methods of analysis. The signal reflects the rate of substrate conversion that is maximal in the first minutes of the reaction and then decreases. Affinity biosensors exploit reversible biochemical interactions like antigen–antibody or DNA–protein. They reach maximal response corresponding to the equilibrium state. The difference in the time dependence of the response not only dictates the strategy of the measurement protocol but also presumes different influences of such mass transfer factors as the flow rate in flow-injection analysis or solution stirring in batch conditions. The biological tissues, receptors and whole cells have an intermediate rank in between enzyme and affinity biosensors in accordance with the particular aim of their use and approach to the signal measurement. It should be mentioned that the more complicated the biochemical component, the more variational the response it shows, so that the term "equilibrium" is hardly applicable to biological cells or receptors. In most cases, the signal independent on time is classified as stationary. The classification of biosensors in accordance with the biochemical component and analytes is outlined in Fig. 1.3.

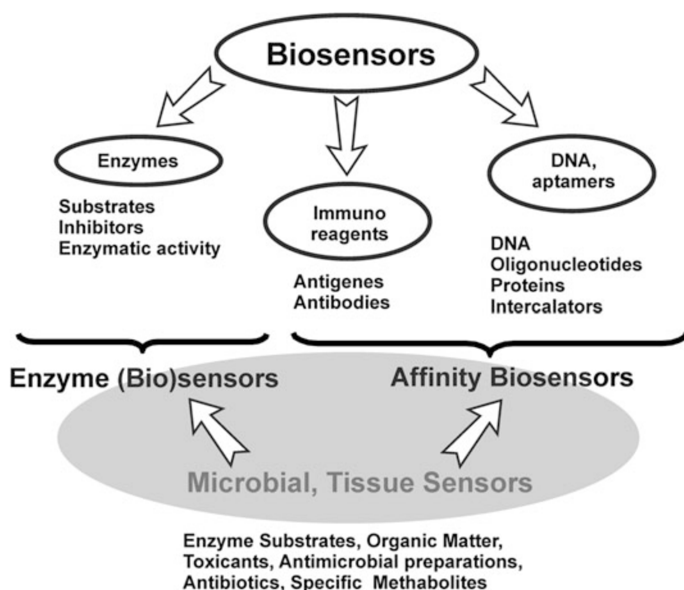

Fig. 1.3 Biochemical components used in the biosensor design and specific analyte determined

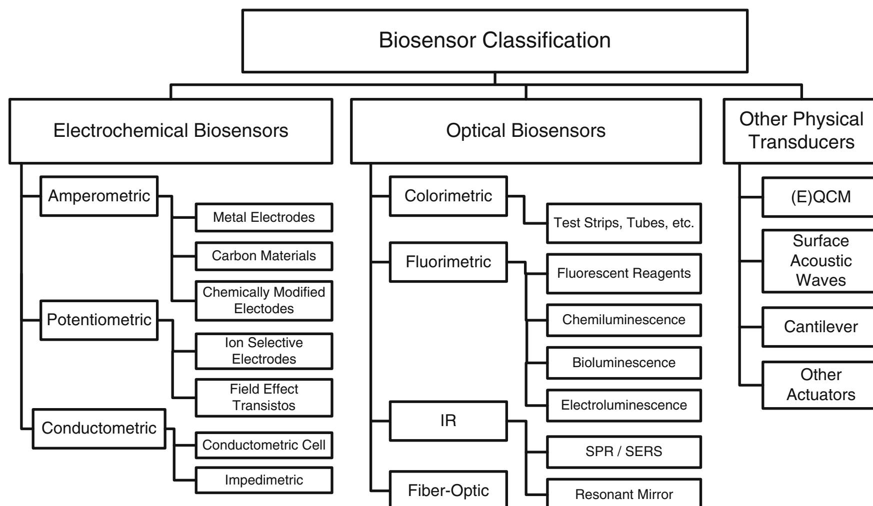

Fig. 1.4 Classification of the biosensors in accordance with the transducer/signal transduction principles

The transducers exploited in biosensors are listed in Fig. 1.4. For many reasons, the biosensors started with electrochemical transducers, as was already mentioned. Until recently, *amperometric biosensors* have been the most popular, especially in various commercial devices such as glucometers or lactate sensors (Ronkainen et al. 2010).

The principles of the electrochemical detection used in the biosensors will be considered in detail in Chap. 3. Here, some advantages of voltammetry should be mentioned, e.g., simple design, lack of limitations of the size and shape of the transducer, low cost, prospects for miniaturization, and field applications. To some extent, the popularity of amperometric biosensors can be referred to related techniques that were and remain common in medicine and environmental monitoring.

The equipment for the electrophysiology studies, for the detection of dissolved oxygen and heavy metals and for the measurement of some metabolites (nitrogen monoxide, dopamine, cysteine) can be easily adapted for recording the signal of the enzyme or the DNA sensor. Besides, the redox conversion of the analytes and/or products of their biochemical consumption is rather typical for biochemical processes and is often realized in complicated biochemical paths like the citric acid cycle, photosynthesis, etc.

A variety of electrode modifications with mediators of electron transfer promotes the use of such transducers in complex media as biological fluids or wastewaters due to the high selectivity achieved (Chaubey and Malhotra 2002). In most cases, direct current voltammetry or chronoamperometry are used for signal recording.

Meanwhile, the potentiometric transducers based on ion-selective electrodes (ISEs) developed in parallel with amperometric devices have lost most of their

applications. Mainly based on conventional glass pH electrodes, they were forced out of the biosensor applications by voltammetry which offered a faster and bigger signal linearly dependent on the analyte concentration. The semi-logarithmic plot presumed by the Nernst equation for the calibration curve of potentiometric sensors and biosensors provides a wider concentration range by a much lower sensitivity of the response and inferior metrological characteristics. The measurement is conducted in the open-circuit mode with no significant power consumption and does not require any complicated equipment.

Field effect transistors (FETs) are the only potentiometric transducers that find growing interest in the development of chemical sensors and biosensors. More precisely, chemically modified field effect transistors (ChemFETs) (Bergveld 2003) can be easily adapted for transducing the biochemical recognition event due to the selectivity of the signal achieved by covering the gate area with a thin oxide or polymer film with the appropriate selectivity of ion permeability or (bio)chemical interactions on the surface. Thus, the pH-sensitive ChemFETs can be manufactured by applying surface layers of Ta_2O_5, SiO_2 or Si_3N_4 which are reversibly protonated in an aqueous solution. Such devices belong to the family of metal oxide microelectronic units that are used in modern control systems including actuators, thermal sensors, electroacoustic devices, etc. The combination of such ChemFETs with the enzymes catalyzing the oxidation of organic substrates makes it possible to detect either the substrate concentration or the enzyme activity due to the quantification of the organic acids released in such biochemical reactions (Dzyadevych et al. 2006). Another enzyme, urease, produces ammonia ions and increases the pH of the surface layer. Although the enzyme sensors on this platform, called EnFETs, retain all the drawbacks typical for potentiometric biosensors, they show some advantages specific for microelectronic transducers, i.e., low cost in mass production, fast response, low expenses of biochemical components, small size, compatibility with conventional microelectronic controller systems, prospects on the remote control and full automation of the measurement cycle.

Conductometric devices can be subdivided into two parts. In conductometric cells, the resistance is measured at a rather low voltage frequency (Mikkelsen and Rechnitz 1989). Most of the biochemical reactions, especially the enzymatic conversion of the substrates, result in changing the number and mobility of the charge carriers that can be quantified and used as a measure of the amounts (concentration) of low-molecular substrate or that of the biochemical component. The techniques do not differ greatly from the conventional conductometers used, for example, for the assessment of distilled water quality. The miniaturization of the working cell and the special geometry of the electrodes make it possible to reduce the volume of the conductometric cell to several microliters. Besides, planar interdigitated electrodes have been proposed for conductometric measurements. In their assembly, strip electrodes are separated with thin insulating paths. The modifying layer is deposited onto this gap so that the resistance measured is directly addressed to the interactions in the microenvironment of the electrodes.

The increase in the frequency of the AC measurements results in the complication of the response. The frequency characteristic of the response is realized in

the electrochemical impedance spectroscopy (EIS), a powerful tool for the investigation of the processes that include charge carriers in the proximity of the electrode interface (Lisdat and Schäfer 2008). EIS measurements were first elaborated for the investigation of corrosion and ion mobility in porous membranes. At present, this method has found growing applications in biosensors. In such experiments, the effect of the surface films consisting of biochemical reagents and auxiliary components (immobilization supports, mediators, etc.) is estimated in accordance with the changes in the charge transfer resistance and capacitance of the surface layer. Measurements are commonly performed in the presence of a so-called redox buffer. Ferricyanide ions and ruthenium complexes are mostly used depending on the charge of the buffer species required. EIS provides a very sensitive tool for tuning the content of the surface layer in order to control the charge separation or attraction caused by biomolecular interactions or enzymatic reactions run on the electrode interface. Although the reproducibility of the results is a weak point of the EIS investigations, the information obtained is unique and in some cases cannot be obtained by other electrochemical techniques.

Optical biosensors have been developed together with first electrochemical devices but for a long time they did not receive adequate interest. Except for test strips, or indicating tubes with visual detection of the color change (Lodeiro et al. 2010), no other optosensors have gained recognition in the past. Some of the optical biosensing devices, such as surface plasmon resonance (SPR) biosensors, do not meet the definition of a biosensor but traditionally retain this name. This refers to reflectance detectors and most fluorometric sensors. More precisely, they can be referred to the compact equipment for the detection and quantification of biochemical recognition in homogeneous conditions. Recent progress in the development of reflectance Fourier-transformed infrared spectroscopy (FTIR) and surface-enhanced Raman spectroscopy (SERS), which can be used on a solid interface, increases interest in optical biosensors (David et al. 2010). This is particularly true for the use of nanosized particles and nanopore materials (artificial ion channels) that offer opportunities for an enormous increase in sensitivity, to the point of detection of a single DNA or protein molecule. The use of SPR (Gopinath 2010) provides a tunable control for the dynamic biochemical interactions that affect the total reflectance of the laser emission in the ultra-thin golden layer modified with biochemical receptor molecules. Contrary to many other spectroscopy methods, the light emission and measurement mode is positioned on the opposite side of the interface so that it cannot affect the interactions with an analyte solution. The first commercial device, called the Biacore SPR Biosensor, was produced in 1993. Until recently, the construction and performance of the SPR sensors were advanced to obtain compact, highly automated equipment suitable for field applications.

Chemi- and bioluminescence biosensors, as well as the hybrid approach of electroluminescence, provide a very sensitive detection of specific reagents that dissipate a portion of energy as emission in the UV–vis part of the spectrum (Kukoba et al. 2000; Roda et al. 2009; Wolfbeis 2004). Luminol oxidation catalyzed by peroxidase and DNA intercalation fluorescence can be mentioned as successful examples of the biosensors. Peroxidase is used as an enzyme label of

immunosensors and offers great sensitivity of the interaction of antigen–antibody complexation. DNA intercalators, such as ethidium bromide, produce an effective change in the luminescence intensity due to their involvement in the DNA helix or DNA damage; both approaches can be realized in the bioassay format and biosensor assembly. The only problem of luminescence detection of the biochemical signal corresponds to the selectivity of the detection system. It suffers from numerous interferences in light emission or luminescence quenching. Not all the biochemical systems used in biosensor assembly can be provided with the necessary luminophores, either. Together with the relative complexity of the detector equipment, this limits progress in this area. Meanwhile, the great sensitivity of fluorometric detection and the possibility to control the detection conditions by selecting the labels or reaction conditions offers good opportunities in the further use of such techniques in the biosensor format. The use of green fluorescent protein as a label in DNA encoding and DNA chips is only one of the existing indications of success.

Of the other physical principles of biochemical transduction, quartz crystal microbalance (QCM) has enjoyed the most interest (Marx 2007; Becker and Cooper 2011). The bulk acoustic waves are generated by applying electric power to a slice of a quartz crystal. For this purpose, thin metal electrodes are placed on both sides of the crystal slice for oscillation excitation. The basic oscillation frequency depends on the internal relaxation processes in the crystal and shifts with the change in the mass of a surface. This makes it possible to detect changes in the mass caused by the attachment of biochemical molecules in the nanogram scale. The mass shift is directly calculated from the frequency change. Like gravimetry in traditional chemical analysis, the QCM techniques do not need any standards or calibration for mass quantification and hence provide an absolute estimate of the quantities of the species deposited on the surface. QCM as an analytical tool began with detecting the gases and vapors adsorbed on the quartz or the exciting electrode surface. The measurements in liquid media assume the differentiation of the mass change against the crystal—air interface or against a pure solvent in the flow injection mode. This takes the solvent adsorption into account. In biosensors, the surface of the QCM sensors is modified by appropriate receptors, e.g., DNAs or antibodies to make the deposition of the analytes highly specific. The use of metal-exciting electrodes makes it possible to measure their electrochemical characteristics (EQCM protocols) simultaneously and, for example, control the polymerization of the organic species or the immobilization of the biochemical components via electrostatic adsorption or entrapment into the polymer film. A similar response is exerted by the sensors based on surface acoustic waves (Länge et al. 2008). In such equipment, the reversed piezo effect is used. The mechanical oscillation of the quartz crystal excites the alternating voltage between the pair of auxiliary electrodes printed onto the crystal surface by lithography or screen-printing techniques. Such construction makes it possible to avoid the necessity of two circuits for mechanical and electric alternating oscillations as required in conventional QCM sensors.

Both surface and bulk acoustic waves have some intrinsic limitations on the mass of the surface layer that should not exceed the limit established by frequency resonance maintenance. Otherwise, the quartz crystal is overloaded and the frequency of oscillations decays with no respect to the surface phenomena. The QCM techniques are mainly intended for the characterization of affinity biosensors developed for the detection of rather high-molecular compounds, e.g., oligonucleotides or proteins. The smaller the molecular mass of an analyte, the lower the sensitivity of its QCM detection. For this reason, the signals of enzyme sensors cannot seriously compete with those of fluorometric or voltammetric biosensors. The performance of such QCM biosensors can be improved by complication of the surface layer, i.e., the inclusion of metal nanoparticles or polymers that either increase the surface density of the binding sites or the mass of the products deposited onto the quartz crystal in the biorecognition event.

The microelectromechanical systems (MEMS) that are often mentioned among actuators are mainly presented by cantilevers in the biosensor family (Li 2010; Tamayo et al. 2013). As QCM sensors, they react to the changes in the mass of specific coating that is registered by the deviation of the tip of a specially designed tip. The movement is controlled by the angle of the laser beam reflection. Together with QCM techniques, MEMS belong to the universal transducers that can be easily combined with all kinds of biochemical reagents. And similar to QCM, they are privileged to be able to detect large and bulky complexes formed by the DNA probes and immunoreagents and are not applied in enzyme sensors. Cantilevers can also utilize the bulk acoustic waves, such as QCM, for detecting mass changes.

The short overview of the transduction systems that have been developed for biosensing purposes does not cover the entire variety of the biosensors already described or proposed for design in the near future. New prospects in the development of transduction systems are related to the requirements of the application of biosensors that presume a high level of automation and reliability of the device operation, especially in field conditions. It can be expected that current progress in the miniaturization of conventional instrumental analysis extends the number and performance of biosensors, especially in optosensors and MEMS. The same has been expected for a long time from microelectronics where FET-related devices can be easily integrated into existing circuits and microchips. Even though some of the examples of micro- and nanobiosensors are quite impressive, the real progress, especially in the lab-on-chip platform, is still a long way from the common consumer.

Among the specific terms based on the nature of the biochemical component and a transduction system, the biosensors can be classified in accordance with more general criteria. They mainly consider the measurement mode, i.e., the sample feed and temporal characteristics of the signal.

Most biosensors are operated in *batch conditions* (Fig. 1.5). The sample is added to the working solution that contains supporting electrolytes, a buffer system, stabilizers, and the other chemicals required for a stable reproducible signal. The solution is stirred with a magnetic stirrer to reach the homogeneous distribution of analyte molecules in solution and on the sensor interface. After that, signal is measured and the biosensor is removed from the solution for the following treatment: washing, regeneration with special reagents, etc.

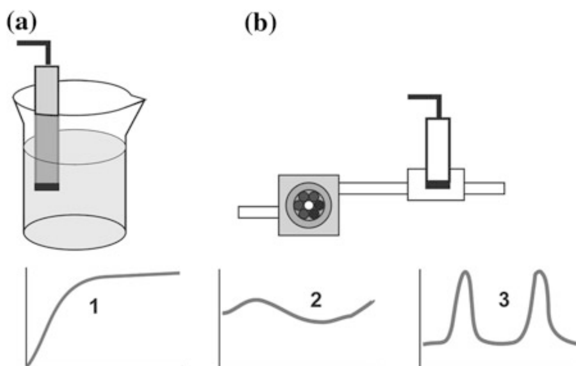

Fig. 1.5 Measurement mode: **a** stationary conditions; **b** flow measurements. *1*—The signal is changing to stationary value; *2*—The analyte is continuously present in flow; *3*—Repeated injection of the analyte solution in the flowing carrier

Such a measurement protocol does not imply any complicated systems for reagent injections and is fully compatible with conventional laboratory equipment (stirrers, automated pipettes, glass cells, thermostats, etc.). Similarly, the biosensors are used in a field where fresh water or drinks can be tested as such or preliminarily diluted with auxiliary solutions. Being very simple, measurements in batch conditions have some limitations related to the time required for the equalization of the biosensor environment. The dilution of the solutions, especially those containing very small amounts of the species, can require a rather long time. Besides, it is quite difficult to estimate whether the time is sufficient for the beginning of the signal record or not. Mostly, the time interval is estimated from the empirical information on the signal stabilization with no respect to its reason. This can be a source of some typical mistakes of the overestimation of the operational stability or washing efficiency, especially for enzyme sensors.

The relatively low rate of the signal measurement in batch conditions is not very important for biosensors that are based on conventional electrochemical techniques, which presume a rather long signal measurement, but it becomes critical for microelectronics providing sub-second time intervals for signal measurements.

Flow-through conditions are considered to be an alternative toward batch conditions of the measurements (Hartwell and Grudpan 2010). In this case, the biosensor is put into the liquid, which is pushed through the working cell with a constant flow rate. The analyte can be permanently present in the flow, or added periodically by a special sample injector. The backgrounds of flow-through biosensors go back to relative instrumentation techniques such as HPLC or flow analysis with photometric detectors. In flow systems, the mass transfer is significantly controlled by the geometry of the working cell and plastic tubing used for the mixing and delivery of reagents. The signal related to the analyte concentrations does not remain constant and reaches a limit as in the batch conditions. The signal can follow the concentration profile in proximity to the sensor surface. When an analyte is injected into the flowing liquid as a portion of the solution with a definite volume, the signal first reaches its maximal value—which depends on the mixing conditions. In some cases, the sample can substitute the flowing carrier

so that the maximum corresponds to the real concentration of the analyte. In other cases, the sample is dissolved with the carrier so that the maximal signal is below the values corresponding to batch conditions. This is compensated for by a much faster response and the possibility to control the mass transfer and biosensor regeneration to a much higher extent than that achieved in a simple working cell, as described above. After reaching its maximum, the signal decreases until it completely disappears, corresponding to pushing the analyte from the working cell by a new carrier portion.

Flow-through biosensors offer strict requirements for the durability of the sensing layer and stability of the biosensor signal. Some additional efforts for a complicated device and extra expenses for flowing liquids deserve special attention if the biosensor is intended for mass analyses, e.g., in clinical diagnostics or environmental monitoring. In medicine, there is another argument in favor of such a system, which is related to the high price paid for a mistake caused by the biological pollution of the sample and/or working cell. There are some transducers that preferably work in a flow stream due to internal limitations as well. A QCM biosensor intended for measurements in liquids is an example.

If the analyte is dissolved in the carrier, the signal changes with its concentration as fast as the mass transfer on the sensor interface and the rate of biochemical reactions that use an analyte molecules allow. In addition to durability and signal stability, such flow biosensors show a truly reversible behavior to change the response with alternating concentrations of an analyte. Also, continuous measurement is impossible for biosensors assuming periodic signal measurement with regeneration steps in between analyte additions. Such systems are demanded in the areas where temporal profiles of the analytes are required. The continuous monitoring of biomarkers and metabolites in the blood or toxic substances and organic matter in surface waters serve as examples.

The classification of biosensors on dynamic and stationary systems is very close to that in batch and flow conditions. In this case, the classification applies the temporal dependence of the response, irrespective of the driving forces of the changes. In *dynamic systems*, the signal changes with time, and the rate of this change is taken as a measure of the biochemical reaction with an analyte molecule. Thus, enzyme sensors often use the first time set after the substrate injection, which is much shorter than the time required for the stabilization of the signal. This corresponds to the maximal rate of the substrate conversion when the surface layer is saturated with the substrate. The signal changes approximately linearly and this simplifies the slope measurement. The dynamic regime of the measurement makes it possible to shorten the measurement time, especially in cases when the equalization requires a very long time. *Stationary conditions* assume the signal constant in the time period comparable with the measurement interval. For affinity biosensors, it corresponds to the true equilibrium on the biosensor surface. Enzyme sensors reach stationary conditions when the rate of the substrate conversion reaches the rate of mass transfer, so that the consumption and delivery of the substrate nearly equal each other. The behavior of enzyme sensors in such situations will be considered in more detail in Chaps. 2 and 3.

Specific areas of application can also classify biosensors in accordance with a definite protocol of application and information obtained. Let me give some examples from medicine: Glucose measurements can be performed directly in the bloodstream by the *implantable biosensor* or by sweat analysis directly on the skin with a *non-invasive biosensor* (Onuki et al. 2008). The detection of enzyme activity can be performed ex vivo with an initially separated enzyme preparation, in vitro in the sample taken from a human being, and in vivo, i.e., directly in the biological tissues or liquids by an implantable biosensor. The same terms describe the quantification of the toxic species present in the biological species or in the standard solutions. For industrial application, the terms *in-line*, *on-line* and *at-line* describe different sampling conditions related to continuous measurements in the flow, periodic sampling, and the analysis of the samples transported from the sampling point. The significance of the terms mentioned is beyond the scope of the biosensors and their use does not differ from that which is typical of chemical and physical sensors.

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Chapter 2
Biochemical Components Used in Biosensor Assemblies

Over the past years, biosensor research has revolutionized clinical diagnostics, especially in diabetes management, and has had a significant impact on laboratory research. As was shown in Chap. 1, this was due to the extremely high importance of biochemical components that determine both the interpretation of the signal and the possibility to specifically detect a variety of the species, including metabolites, drug residues, toxic hazards, proteins, etc. It should be mentioned that from the very beginning, the development of biosensors has been mainly concentrated in the groups of biologists and medical staff who are involved in the development of medical equipment or investigation of molecular backgrounds of human health. For this reason, the behavior of enzymes, nucleic acids and microorganisms was considered to be self-evident so that the main efforts had been concentrated on transducers and signal transduction principles. The situation has been changing, and many chemists and engineers begin their scientific careers in medical equipment and compact sensing devices for environmental monitoring, point-of-care medical diagnostics and treatment and related areas. They are not as familiar with the biochemical backgrounds of biosensor performance as their predecessors and need broadened information about the advantages and limitations of biochemical reagents in biosensor design. Below, the main biochemical functions of principal biochemical components are considered, with particular emphasis on those most important from the point of view of biosensor development. The description of proteins, nucleic acids and microbial cells does not claim to be exhaustive and can easily be extended by monographs and student books on biochemistry. However, this chapter contains the minimum of information necessary for the beginning of biosensor development for young researchers and graduate students who are not specializing in the life sciences. The information is classified in such a manner that the knowledge most important for biosensor development is summarized and presented in a way that is acceptable for quick search and use. In addition to the main information on the structure and biochemical functions of appropriate biomolecules, some useful data are placed in tables. The description is supported by examples related to the most common situations or decisions related to biosensor manufacture or operation.

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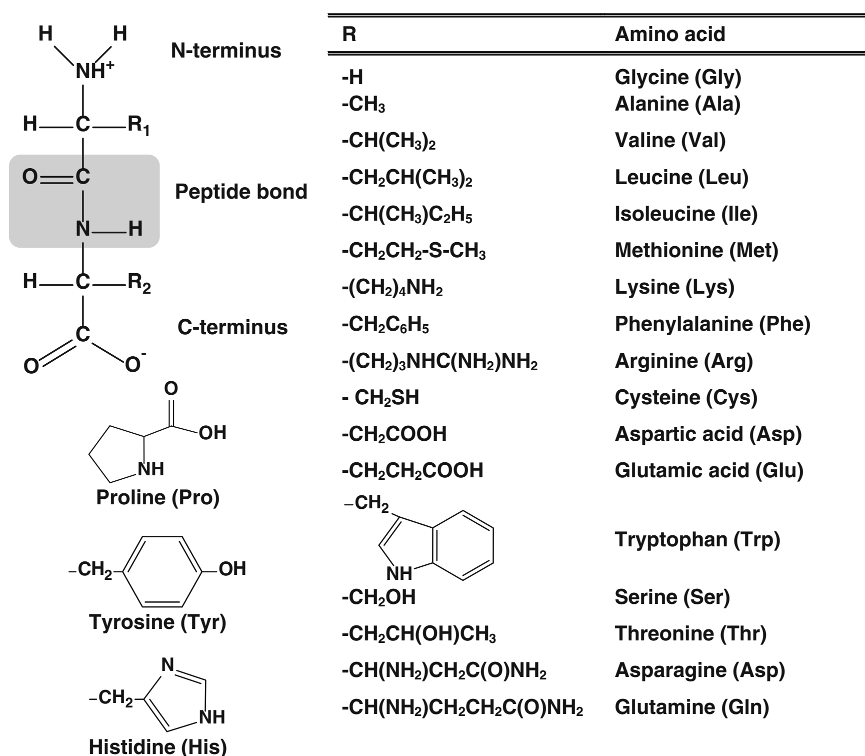
2.1 Enzymes

Let's start with enzymes that not only belong to the most common representatives of the biochemical systems of biosensors but were also the first to demonstrate the potential for the commercialization of biosensors and their application outside university laboratories.

Enzymes are proteins, i.e., biopolymers that consist of α-amino acid residues connected by amide linkages and which exert catalytic activity. In total, 20 α-amino acids are present in the protein structure (Fig. 2.1) (Copeland 2000).

All amino acids are important as building blocks of proteins and can take part in enzyme activity. But some of them are more frequently mentioned when the enzyme behavior or biosensor applications are discussed.

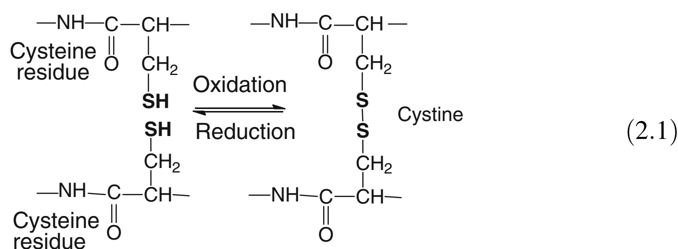
Thus, *cysteine* is the only amino acid that provides covalent disulfide bridging between protein sub-units or a protein and thiolated solid support. Cystine, a cysteine dimer, is important in establishing redox equilibrium (2.1). Cysteine residues are also used for the site-specific covalent attachment of enzyme molecules to the Au surface with covalent Au–S bonds simultaneously formed in the



R	Amino acid
-H	Glycine (Gly)
$-CH_3$	Alanine (Ala)
$-CH(CH_3)_2$	Valine (Val)
$-CH_2CH(CH_3)_2$	Leucine (Leu)
$-CH(CH_3)C_2H_5$	Isoleucine (Ile)
$-CH_2CH_2\text{-S-}CH_3$	Methionine (Met)
$-(CH_2)_4NH_2$	Lysine (Lys)
$-CH_2C_6H_5$	Phenylalanine (Phe)
$-(CH_2)_3NHC(NH_2)NH_2$	Arginine (Arg)
$- CH_2SH$	Cysteine (Cys)
$-CH_2COOH$	Aspartic acid (Asp)
$-CH_2CH_2COOH$	Glutamic acid (Glu)
$-CH_2$ NH	Tryptophan (Trp)
$-CH_2OH$	Serine (Ser)
$-CH_2CH(OH)CH_3$	Threonine (Thr)
$-CH(NH_2)CH_2C(O)NH_2$	Asparagine (Asp)
$-CH(NH_2)CH_2CH_2C(O)NH_2$	Glutamine (Gln)

Fig. 2.1 Dipeptide structure and some amino acids forming primary sequence of proteins. A three-letter code is given in *brackets* after the amino acid

presence of dissolved oxygen. Some of the reagents, i.e., cystamine or thiourea, affect the cysteine–cystine equilibrium and hence the efficiency of the enzyme attachment to the solid support.



(2.1)

"Acidic" amino acids (*asparagine* and *glutamine*) contain a carboxylic group in the side chain that is not involved in the peptide bond formation. The dissociation of the group makes the amino acid sequence negatively charged. Similarly, the "basic" amino acids (*lysine*, *arginine* and *histidine*) positively charge the protein domains by protonation of their amino groups. The equilibrium with the amino acids bearing proton donating and proton accepting groups specifies the total charge of a protein globule in an aqueous solution. The pH changes in the protein microenvironment alter the charge of the protein globule caused by such interactions. This is one of the reasons for the pH dependence of the enzyme activity.

The point of the recharging of the protein globule is called the *isoelectric point*. It is equal to the pH value corresponding to the zero charge of a protein. Isoelectric points are determined not only for proteins but also for individual amino acids in electrophoretic measurements (Westermeier 2005).

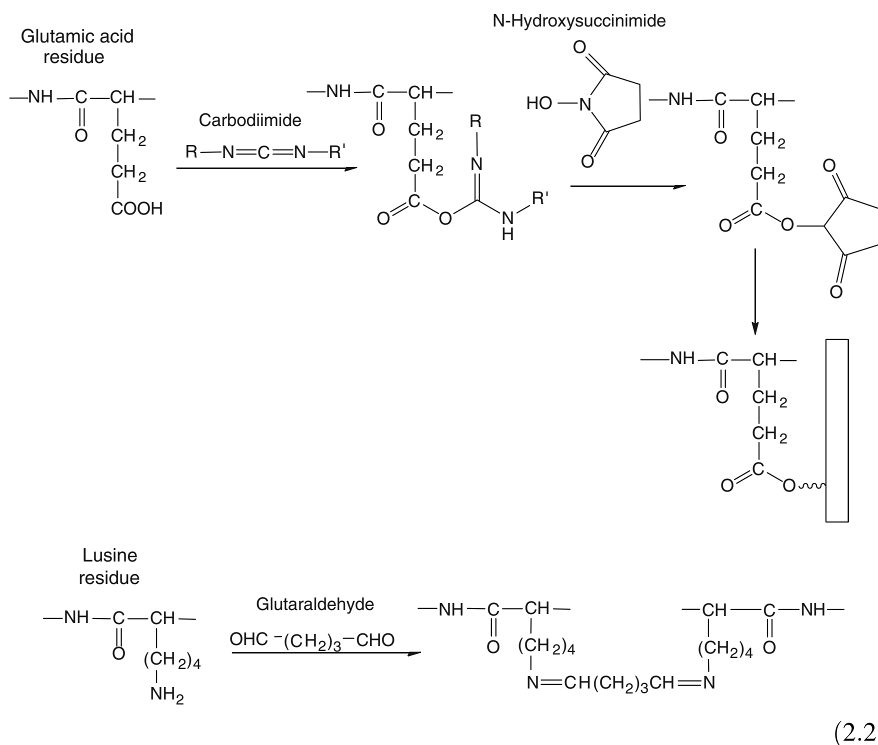
> Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. The direction and rate of the movement allow the charge of the particles to be determined.

If the pH of the solution is higher than the isoelectric point, the protein is negatively charged and, vice versa, if the pH is lower than the isoelectric point, the protein bears a positive charge.

Besides "acidic" and "basic" amino acids, the amino acids include other functional groups, e.g., hydroxyl (*serine*, *threonine*), aromatic (*tryptophan*, *tyrosine* and *phenylalanine*) or aliphatic (*glycine*, *alanine*, *valine*, *leucine* and *isoleucine*) units. As cysteine, the "acidic" and "basic" amino acids take part in the covalent binding with some species present in the solution.

Among others, there are the so-called *bi-functional reagents*. They are required for the modification of the enzyme molecules intended to vary their characteristics or covalent attachment to the solid carrier, e.g., the polymer support or transducer of a biosensor. The examples of such a covalent modification are presented below (2.2). The carbodiimide binding is commonly used for modification of carboxylic

and amino groups and can be performed in two stages. First, one azomethine fragment is attached to a carboxylic group to form an ester group. Then, *N*-hydroxysuccinimide is bonded to the second fragment. The final product is easily combined with aminated and carboxylic functional groups of proteins, polymeric supports, electrode materials, etc. The second stage accelerates the modification and increases its efficiency. The direct reaction of the second azomethine fragment with carboxylic groups without any *N*-hydroxysuccinimide treatment is rather slow for application in biosensor assembling (Cao 2006).



(2.2)

Glutaraldehyde can react with thiol, amine, carboxylic and hydroxide groups of the amino acid residues or plastic support. The relative reactivity of the fragments to be modified decreases in the above-mentioned order. The reaction results in cross-linking protein molecules and their attachment to the high molecular supports or the transducer surface. The reaction is complicated by polycondensation of the initial glutaraldehyde molecules and the reversibility of the Schiff base formation.

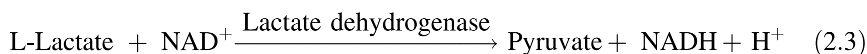
The sequence of appropriate amino acids in accordance with the way they bind is called a primary protein structure. It can be represented by a linear sequence of three letters (see Fig. 2.1) or single-letter codes assigned to each amino acid. The primary sequence of amino acids pre-determines the three-dimensional structure of a protein that depends on all kinds of interactions, including the variation of the

lipophilic–lipophobic balance, π-stacking interactions, donor–acceptor and electrostatic interactions and H-bonds. Besides amino acids, proteins can contain other species, e.g., carbohydrates in glycoproteins, covalently attached to the terminal amino acid residue.

Steric interactions highly depend on the metal ions and their complexes with organic ligands, on small organic molecules, and water kept in the protein globule but can, more or less freely, escape the globule. The presence of such small items is of primary importance for such biochemical functions as enzyme activity. This is especially true for enzymes catalyzing the redox conversion of a substrate. For them, the protein part is called an *apoenzyme*. The small molecule with its own redox activity that is responsible for the acceleration of the substrate oxidation is called a *cofactor* (Fig. 2.2).

The cofactors are subdivided into two groups, i.e., metal ions and organic molecules, *coenzymes*. The coenzymes, in turn, can dissociate from the enzyme molecule to act independently in a solution (*co-substrates*) or act in the protein globule (prosthetic groups). In the latter case, they are permanently associated with an enzyme, often by covalent bonds. The types of enzyme cofactors are presented in Fig. 2.3.

Nicotinamide adenine dinucleotide (NADH) is one of the most important co-substrates and is related to the functioning of more than 250 enzymes catalyzing the oxidation of organic species (Eq. 2.3). NADH is released in the solution and requires special efforts for re-oxidation to complete the cycle of the electron transfer of the target process. The phosphorylate derivative of NADH, NAD(P)H, is another common representative of co-substrates. In the reaction scheme, the co-substrates are recorded together with the enzyme substrate (see Eq. 2.3 as an example):



$$\text{L-Lactate} + \text{NAD}^{+} \xrightarrow{\text{Lactate dehydrogenase}} \text{Pyruvate} + \text{NADH} + \text{H}^{+} \quad (2.3)$$

Cofactor molecules, except for prosthetic groups, can be reversibly removed from the enzyme molecule. This results in the loss of its catalytic ability. The treatment of an apoenzyme with the cofactor molecule results in a partial or

Fig. 2.2 Reconstruction of an active enzyme from a protein part (apoenzyme) and a cofactor

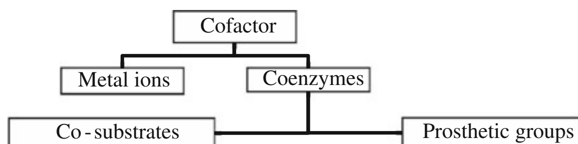
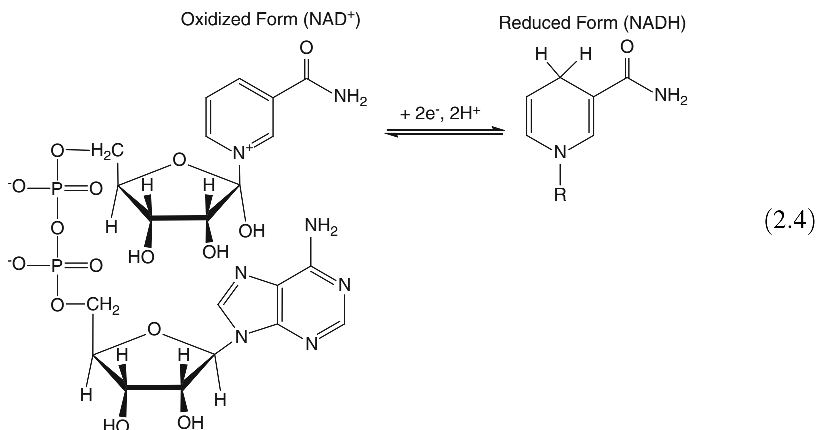


Fig. 2.3 Reconstruction of an active enzyme from a protein part (apoenzyme) and cofactor

full restoration of its catalytic activity. The enzyme obtained by interaction of an apoenzyme and a cofactor is called a *reconstructed enzyme* (*holoenzyme*).



(2.4)

The reaction of the apoenzyme with a cofactor is very specific and can be used for the selective detection of a cofactor. The inactivation of the enzyme caused by the removal of a metal cofactor is observed in some procedures of the enzyme isolation and purification assuming the use of such complexing agents as EDTA. This is an undesirable process that suppresses the catalytic efficiency and hence the sensitivity of the enzyme sensor toward the substrates. On the other hand, reversible removal of the cofactor can be necessary for establishing multi-enzyme cascades on the artificial support or for specific immobilization of the enzyme on appropriate support by covalent linkage incompatible with the cofactor. Many cofactors exhibit a reversible influence on the enzyme activity as well, which is used for the mild control of the chemical energy in metabolism.

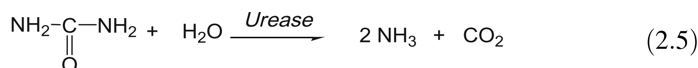
In addition to apoenzyme and the reconstructed enzyme, the term "*proenzyme* (*zymogen*)" can be found in the literature (Plainkum et al. 2003). This is an inactive enzyme precursor that does not exert any enzyme activity but can be "activated" by a specific biochemical reaction. Contrary to the interaction in the "apoenzyme–cofactor" pair, the activation of proenzyme is highly specific, and a specific enzyme system is needed. In many cases, the activation of proenzyme is achieved by the specific removal of terminal groups by selective hydrolysis of P–O–C bonds. The phosphate group is of primary importance in many biochemical reactions related to oxidative metabolism and ATF synthesis and simultaneously controls the enzyme activity especially in substrate cycles (glycolysis–gluconeogenesis, etc.). The high specificity and necessity of additional enzymes with their own substrates complicates the use of proenzyme activation in analytical systems although the substrate cycles were successfully used for the detection of lactic acid, glucose, and a few other metabolites.

As other proteins, enzymes can self-associate into the dimeric and tetrameric forms involving subunits, either different or similar in their enzymatic activity, i.e.,

the efficiency of the catalysis. Enzyme subunits are bonded by weak multi-point interactions and, sometimes, with disulfide S–S bridges. The ions of alkali and alkali earth metals promote the association of the enzyme subunits due to electrostatic interactions. Contrary to apoenzymes, the enzyme subunits in a dissociated state retain most of their catalytic activity. The process of di- and tetramerization is spontaneous and sterically specific. If the subunits differ in their specific activity, the enzyme can form various oligomeric structures in accordance with the number of the associated particular subunits. This can result not only in the various catalytic properties but also in specificity toward particular substrates, pH and temperature influence, etc. Thus the enzyme lactate dehydrogenase is a tetramer that consists of two types of subunits. Their content varies with environmental conditions and this is used for the biochemical adaptation of trout toward low water temperature in mountain lakes and rivers. Together with minor changes in the amino acid sequence near the enzyme-active site, this fact provides a variety of the enzyme characteristics observed not only for various biological species but also within individuals belonging to the same population.

For this reason, the description of the enzyme application, for example, in biosensor assembly, requires not only its definition in accordance with the biochemical function, but also of its origin and, to some extent, purification protocol. The latter requirement does not cover commercial preparations with rather standard characteristics, although, especially at the beginning of biosensor development, the enzyme characterization was often considered an indispensable part of the investigation with appropriate efforts in kinetics quantification and purity examination.

As common chemical catalysts, enzymes accelerate conversion of selected species called enzyme substrates due to the decrease of the reaction energy. Hence the enzymes do not affect the equilibrium constant of the substrate reaction and function by the specific binding of the substrates into a very unstable reactive product, i.e., an enzyme–substrate complex. The great efficiency of catalysis at rather low temperatures (commonly below 50 °C) and the physiological pH interval (pH = 5.0–8.0) is one of the reasons for the wide use of enzymes in the biosensor assembly. Thus, the enzyme urease accelerates the hydrolysis of urea (Eq. 2.5) by a factor of about 10^{14}.



(2.5)

The functional groups occurring in the reaction with a substrate molecule form an *active site* of the enzyme. As a result of such interaction, a new reactive compound, an *enzyme–substrate complex*, is formed. Later, the complex is converted to the final products with releasing the initial enzyme molecule that is able to bind to another substrate molecule. Each enzyme molecule can contain several active sites; their number usually corresponds to the number of subunits or cofactors. Thus the enzyme glucose oxidase is a dimer involving two identical subunits; each of them involves one cofactor, flavine adenine dinucleotide (FAD). Crude enzyme

preparations can contain the proteins partially dissociated into subunits with a various number of enzyme active sites and molecular mass of the fragments so that the specific enzyme activity is calculated against one active site but not one molecule of an enzyme. This is not very important for rather simple enzyme molecules but can have significance for newly isolated enzymes with the distribution of subunits not characterized and dependent on the isolation and purification procedures. The amino residues of the active site can stay rather far from each other in the primary protein sequence but are brought together in the spatial structure typical for the enzyme molecule. This means that any changes in the three-dimensional structure of the enzyme will affect the enzyme activity. Most such interactions in living beings are reversible and provide a flexible alteration of the enzyme activity in accordance with particular demands.

Apart from the active site, an *allosteric site* can be specified for some enzymes. It combines some functional groups that are able to specifically interact with particular species (Fig. 2.4).

Although such interaction does not block the access to the enzyme-active site, it affects the catalytic activity of an enzyme due to changes in the non-covalent interactions—hence, the spatial structure of the domain in the proximity of the enzyme active site. The species that influence the enzyme activity are called enzyme *effectors* and the variation of enzyme activity due to the allosteric site binding is called an *allosteric enzyme regulation* (Traut 2008). Together with changes in cofactor binding, allosteric regulation provides a specific way for directed changes of enzyme activity, i.e., its reduction (*inhibition*) or increase (*activation*). Allosteric regulators do not exhaust all the mechanisms of such changes in enzymatic catalysis. Thus, inhibition mechanisms involve the non-specific denaturation of the protein three-dimensional structure by high temperatures or strong acids (bases) as well.

A similar effect is exerted by compounds irreversibly modifying the functional groups of an active site. Activation can be achieved by the partial dissociation of an enzyme to separate subunits or by the addition of the excessive amounts of a cofactor. In some cases, enzyme–substrates or the products of their enzymatic conversion can be attached to allosteric sites and alter the enzyme activity. In most cases, this results in a decrease of the reaction rate (substrate/product inhibition). Not all the enzymes have an allosteric site in their structure, but they are all

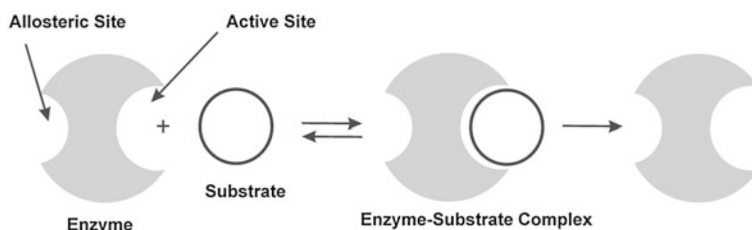


Fig. 2.4 Reconstruction of an active enzyme from a protein part (apoenzyme) and a cofactor

sensitive toward effectors, the number and specificity of the action of which depend on both the enzyme and substrate. In substrate detection, the addition of effectors in the reaction media provides stabilization of the signal and its increase against standard conditions. Allosteric inhibitors can be used for masking undesirable enzyme activity, for example, related to the competitive path of an analyte conversion. In a more complicated case, the effectors are obtained in accordance with the relative increase/decrease of the signal toward the constant concentration of a substrate. It should be noted that except in rare cases, the specificity of the response on an effector is much less than that on a substrate. This limits the application of such schemes in biosensor-based analysis.

The sequence of amino acid residues determines the three-dimensional structure of the protein due to numerous interactions by electrostatic and H-bonding, by van der Waals, hydrophobic and donor–acceptor interactions (Berg et al. 2007). The H-bonding between carbonyl and amide groups of amino acids results in the formation of regular domains, i.e., α-helix and the β-pleated sheet structures. Both of them determine the *secondary protein structure*. The α-helix is a spring-like structure with residues joined by a hydrogen bond between carbonyl oxygen and a nitrogen proton in peptide groups; about 3.6 amino acids are placed in each turn of a coil. The β-pleated sheet is stabilized by hydrogen bonds between peptide linkages to form distorted planes, one embedded in another one. The regular areas described are subdivided by amorphous parts so that the protein molecule usually has a rounded, sometimes elongated or kidney-shaped particle often called a globule.

The enzyme–substrate specificity is a most important feature of enzymatic catalysis. In accordance with the type of substrate conversion, six classes of enzymes are specified in the enzyme nomenclature approved by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Table 2.1).

Oxidoreductases catalyze the redox conversion of the substrates. This is the most popular enzyme class used in biosensor assembly because of the importance of

Table 2.1 Enzyme nomenclature

Class	Name	Examples of subclasses
EC 1	Oxidoreductases	EC 1.1 acting on the CH–OH group of donors
		EC 1.2 acting on the aldehyde or oxo group of donors
		EC 1.4 acting on the CH–NH_2 group of donors
		EC 1.6 acting on NADH or NADPH
		EC 1.11 acting on a peroxide as acceptor
EC 2	Transferases	
EC 3	Hydrolases	EC 3.1 acting on ester bonds
		EC 3.2 glycosylases
		EC 3.4 acting on peptide bonds (peptidases)
EC 4	Lyases	
EC 5	Isomerases	
EC 6	Ligases	

appropriate substrates that belong to metabolites, vitamins, disease indicators, etc. The biosensors based on oxidoreductases are demanded in biomedical diagnostics, food quality testing and other similar areas. The oxidoreductases are subdivided into several groups, depending on the nature of electron acceptors:

- *dehydrogenases* catalyze the reaction formally related to two hydrogen atoms transfer from the substrate to the acceptor differing from molecular oxygen (see reaction 2.3 for lactate dehydrogenase);
- *oxidases* catalyze a similar reaction of the hydrogen transfer to molecular oxygen with the formation of hydrogen peroxide (glucose oxidase);
- *monooxigenases* catalyze a similar reaction but water is obtained instead of H_2O_2;
- *peroxidases* catalyze the oxidation of the substrates with hydrogen peroxide or organic peroxides.

Transferases catalyze the transfer of a group (methyl, glycosyl, etc.) from one compound to another. *Hydrolases* promote the reaction of the hydrolysis of various bonds in aqueous media and the reverse reaction of esterification in predominantly organic media. Both processes have industrial and analytical applications especially regarding the detection of toxic species (organophosphorus nerve agents, carbamate pesticides) and derivates of organic acids (area, carbamates etc.). In accordance with their popularity for biosensor application, hydrolases are the second enzyme class after oxidorudectases. *Lyases* cleave to C–C, C–O, and C–N bonds by elimination. Eliminating a group can be specified in names like decarboxylase, aldolase, dehydratase (for eliminating carbon dioxide, aldehyde, or water). When important, the reverse reaction is expressed by the name *synthase*. *Isomerases* catalyze geometric or structural changes within one molecule. And finally, *ligases* are enzymes catalyzing two molecules joined together. The reaction is coupled with the hydrolysis of ATP or a similar triphosphate.

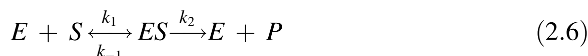
Each enzyme is uniquely specified with a code containing the information about the class and subclasses depending on the substrate converted and the reaction catalyzed. Thus, acetylcholinesterase, an enzyme widely used for the detection of organophosphate nerve agents, has the code EC 3.1.1.7. The code shows that the enzyme belongs to the class of hydrolases (EC 3), the subclass of enzyme catalyzing the hydrolysis of esters (EC 3.1), the subgroup of the one that hydrolyzes carboxylic esters (EC 3.1.1) and that related to choline esters hydrolysis (special number 3.1.1.7). The enzyme nomenclature combines the enzyme name from the substrate class and the reaction to be accelerated. For example, the glucose oxidase catalyzes the reaction of glucose oxidation, etc. Nevertheless, in the nomenclature, some common names, like urease (EC 3.5.1.5) or laccase (EC 1.10.3.2), are still used.

The urease and glucose oxidase mentioned above belong to the enzymes with individual substrate selectivity when only one compound acts as an enzyme substrate. More common is the situation where the enzyme catalyzes the conversion of a family of structurally relative compounds. Thus, tyrosinase and peroxidase catalyze the oxidation of a wide range of phenolic compounds and aromatic amines, and carboxyesterase accelerates the hydrolysis of uncharged esters. In some cases, the

enzymes can activate different substrates by various biochemical paths. Thus, cytochrome P_{450} catalyzes the reactions of *N*-demethylation and *C*-hydroxylation depending on the enzyme origin and substrate nature. It should be mentioned that cytochrome P_{450} is a complex of at least three different enzymatic centers that are involved in various stages of the substrate conversion, and their action differs depending on the substrate structure. To some extent, the substrate selectivity can also vary within one source of enzyme by changing the conditions of the reaction control. The attachment of the enzyme onto the solid support limits the steric access of bulky substrates in favor of smaller molecules. The pH and temperature changes can alter the contribution of various subunits in the resulting reaction rate. The use of lipophilic substances and hydrophobic solvents changes the equilibrium and can reverse the direction of the reaction against that in aqueous solution. All of these possibilities are compatible with the biosensor platform when the substrate specificity as such is insufficient for the detection of an analyte.

Table 2.2 summarizes the examples of the most common applications of various enzymes in the biosensor assembly. Besides substrate determination, the detection of inhibitors is also used by enzyme sensors even though the selectivity of the regulation of enzyme activity is inferior to the substrate detection (Byfield and Abuknesha 1994; Ispas et al. 2012).

The kinetics of enzymatic conversion of a substrate is formally described by the Michalis–Menten reaction (Eq. 2.6) (Keleti 1986).



$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P \quad (2.6)$$

Here E, S and P denote free enzyme, substrate and product molecules, respectively. The k_i are the rate constants of the elemental steps of the reaction. The first step involves the reversible formation of the enzyme–substrate complex *ES,* which then irreversibly decomposes to the product and initial enzyme molecule ready to bind another substrate molecule. The formal kinetics provides the following expression of the reaction rate (2.7) for the Eq. (2.6) known as the Michaelis–Menten equation.

$$\upsilon = \frac{\mathrm{d}c_P}{\mathrm{d}t} = \frac{\upsilon_{\max} c_S}{K_m + c_S} \quad (2.7)$$

K_m is the Michaelis constant, which has the dimension of a concentration that also corresponds to the substrate concentration giving the reaction rate equal to half of its maximal value ($\upsilon = 1/2\upsilon_{\max}$); $\upsilon_{\max} = k_2 c_E$ is the maximal rate of enzymatic reaction. In most enzymatic reactions, K_m varies from 10^{-1} to 10^{-7} M. The higher the K_m value, the lower the affinity of an enzyme-active site toward the substrate.

The relation k_2/K_m quantifies the efficiency of the substrate conversion. It has a theoretical upper limit of 10^8–10^9 M^{-1} s^{-1}. Besides the maximal rate, the efficiency of enzyme reaction can be quantified by *enzyme activity*.

The term “enzyme activity” can refer to the enzyme quantity (mass of the enzyme preparation, mass of the support with an enzyme attached) or to the

Table 2.2 Application of various enzymes in the biosensor assembly

Enzyme class	Enzyme name	Analyte	Application area
Oxidoreductases	Glucose oxidase	Glucose	Health industry, medicine, food testing, biotechnology
	Glucose-6-phosphate dehydrogenase	Glucose, phosphate	Medicine, environmental monitoring
	Lactate oxidase, lactate dehydrogenase	Lactate	Health industry, medicine, biotechnology
	Alcohol dehydrogenase	Ethanol	Beverage testing, blood tests, microbe industry
	Tyrosinase	Phenols	Environmental monitoring, pharmaceutics industry
	Peroxidase	H_2O_2, phenols, aromatic amines	Auxiliary enzyme for enzyme sensors based on other oxidoreductases, medicine, pharmaceutics and microbe industry, environmental monitoring
	Cholesterol oxidase	Cholesterol	Medicine, food industry, dietology
	Uricase	Uric acid	Medicine
	Xanthine oxidase	Hypoxanthine, xanthine	Medicine
Hydrolases	Acetylcholinesterase	Acetylcholine, nerve agents	Medicine, environmental monitoring, chemical warfare detection
	Urease	Urea	Medicine, agriculture chemistry
	Alkaline phosphatase	Organophosphate esters	Medicine
	Cholesterol esterase	Cholesterol ester	Medicine, food industry, dietology
	Organophosphate hydrolase	Organophosphates	Environmental monitoring, chemical warfare detection

volume of an enzyme solution. The enzyme activity is measured in units (U). One unit corresponds to the amount of enzyme that catalyzes conversion of one

> *Enzyme activity* is an amount of a substrate converted in a time in standard reaction conditions.

micromole of a substrate per minute. In addition to that, the turnover number is used. This is a maximal number of substrate molecules that can be converted by one active site of enzyme per time unit.

In many manipulations with enzymes required for biosensor development, the K_m value increases. This might be due to limitation in the substrate access or changes in native conformation of the enzyme globule. Similar relations are used for the quantification of the enzyme kinetics with immobilized enzymes if the rate of substrate transfer exceeds that of its biochemical conversion. Such investigations are necessary for the establishment of the influence of biosensor assembling on the reactivity and selectivity of substrate detection that depends predominantly on the biochemical characteristics of the reaction. Instead of the reaction rate,

derivative functions directly measured with appropriate transducers can be used. Thus, for amperometric sensors combined with oxidoreductases, the current recorded can be considered a measure of the rate of an enzymatic reaction. This allows estimating the Michaelis constant by the direct substitution of the υ values with the current in the Eq. (2.6) and appropriate plots mentioned above. The specificity of the heterogeneous reactions of enzymes will be considered in greater detail below in the section devoted to enzyme immobilization.

The Eq. (2.7) can be simplified by the assumption of $K_m \gg c_S$. For low substrate concentration, the rate of an enzymatic reaction linearly depends on its value (2.8).

$$\upsilon \approx \frac{\upsilon_{\max} c_S}{K_m} = \frac{\upsilon_{\max}}{K_m} c_S \tag{2.8}$$

Some other equations related to enzyme kinetics and estimation of kinetic parameters are presented in the Appendix. They can be useful for the characterization of the enzyme-substrate system and its optimization in the biosensor development and operation.

The linear piece of calibration curves of a substrate usually covers about 30 % of the maximal shift of the reaction rate. A linear relationship is used in simplified methods of the substrate analysis that are called "*fixed time*" and "*fixed concentration*" methods. In the first case, the concentration of the product is measured in a definite time interval after the start of the reaction. The reaction period is chosen within the linear piece of the calibration curve so that the concentration measured is directly proportional to the reaction rate. In the fixed concentration method, the time period related to constant conversion of the substrate is measured as a value inversely proportional to the rate of the reaction. The idea of such measurements is to substitute the estimation of the rate, which should be based on a set of measurements performed in different time intervals with a single measurement to be made on the assumption of the linear dependence of the signal and a constant degree of the substrate conversion. Such a measurement mode is especially compatible with semi-quantitative techniques of substrate determination and the colorimetric detections that are often developed for filed applications (test strips, indicator tubes, etc.). Thus the fixed concentration method assumes the measurement of the time period necessary to reach a definite color shade provided by the product of the enzymatic reaction. For enzyme sensors, the fixed time approach substantiates the choice of the measurement period, especially in cases when the enzymatic reaction is not limited by the substrate transfer.

In steady-state conditions of biosensor operation, the enzymatic conversion of the substrate is compensated for by the diffusion flux in the direction orthogonal to the transducer surface. These conditions can be expressed by the superposition of the second Fick's Law describing the diffusion rate and Michaelis–Menten kinetics yielding the Eq. (2.9).

$$\begin{aligned}\frac{\partial c_S}{\partial t} &= D_S \frac{\partial^2 c_S}{\partial x^2} - \frac{\upsilon_{\max} c_S}{K_m + c_S} \\ \frac{\partial c_P}{\partial t} &= D_P \frac{\partial^2 c_P}{\partial x^2} + \frac{\upsilon_{\max} c_S}{K_m + c_S}\end{aligned} \tag{2.9}$$

Here, D_S, D_P are the diffusion coefficients for the substrate and product, respectively, t is the reaction time, and x is the distance in orthogonal direction from the transducer interface to the bulk solution. If the concentrations of the reagents outside the surface layer are taken to be constant, the solution of equations results in the Eq. (2.10) describing the linear dependence between the surface and volume concentration of a substrate.

$$c_S = c_S^0 \exp\left(-\sqrt{\frac{\upsilon_{\max}}{K_m D_S}} t\right) \tag{2.10}$$

The enzyme sensors that meet the Eq. (2.10) function at a rather low substrate concentration corresponding to the linear piece of the Michaelis–Menten plots (see above). In this case, the diffusion inside the surface layer does not compensate for the decay of the substrate concentration due to enzymatic conversion. From the practical point of view, such behavior is typical for thin membranes with moderate enzyme activity. The substrate concentration profile within the surface layer is linear (Fig. 2.5a). In the opposite case, when the enzyme activity is low or the transport of the reactants is hindered by high viscosity (low permeability) of an enzyme support, all the active sites of an enzyme in the membrane remain saturated so that the signal of a biosensor depends on the rate of enzyme reaction but not on the concentration of a substrate in the bulk solution (Fig. 2.5b). These two extreme cases are also called the diffusional and kinetic regimes of biosensor functioning. In between, there is an area where both kinetics and mass transfer affect the substrate determination.

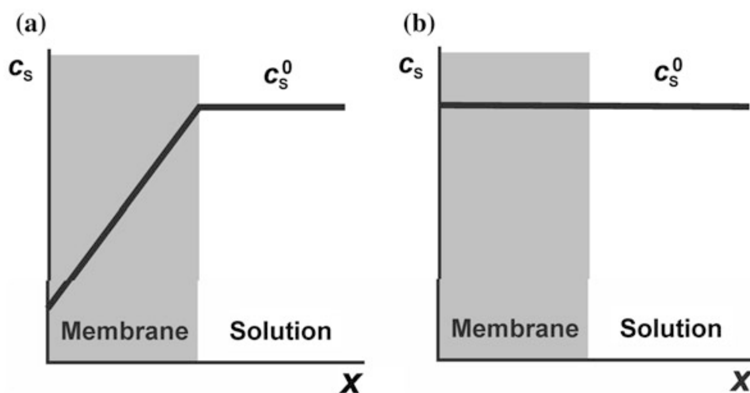


Fig. 2.5 Substrate concentration profiles related to diffusional (**a**) and kinetic (**b**) regimes of the enzyme sensor functioning and nature

Most of the calculations in the biosensor area originate from several assumptions. First, the kinetic parameters of enzymes implemented in the surface layer are supposed to be the same in solution. This might be true for gentle approaches to the formation of the surface layer that do not assume significant changes of the protein structure. However, this might not be true for extreme environments (hydrophobic supports, application of organic solvents for enzymatic membrane formation) or for the use of modified enzymes including specific tags and functional groups implemented for use in biosensor assembly. The second assumption involves the presentation of a surface layer as a uniform homogeneous media that differs from aqueous solution only by its higher viscosity. This is quite acceptable for ultra-thin layers, with enzymes directly attached to the transducer surface or for rather thick membranes with a low enzyme activity made from gelatin, chitosan, polysiloxanes, etc. Meanwhile, the use of nanosized carriers, lately very popular, complicates the behavior of biosensors. Carbon nanotubes, silver and gold nanoparticles provide different conditions for reactant transport, especially for charged species. In some cases, channels of increased permeability and pores that are different in size and accessibility to the substrate might appear.

Besides transport properties, the enzyme carrier can affect the kinetic parameters of an enzymatic reaction due to changes in the dielectric constant, acid–base equilibria regulation and electrostatic interactions. In some cases, the products of the substrate conversion can be accumulated in the layer and change its properties and possible influence on the substrate conversion. All of the factors presented make it very difficult to simulate the enzyme sensor behavior.

In addition to substrates, enzymes are affected by other species that do not take part in the reaction but influence its rate. In general, such compounds are called *enzyme effectors*. In nature, many enzyme effectors exert a reversible influence on the enzyme active site. Their contribution increases and decreases with their concentration and is observed only in the presence of a substrate, the reactions of which become faster or slower depending on the effector nature. The specificity of natural effectors and reversibility of their influence are due to their regulatory function in biochemical paths. Negative effectors, or inhibitors, decrease the enzyme activity. They can also protect living beings from pathogens or enemies such as predators or competitors for living space and other resources.

The interest in the detection of inhibitors by enzyme sensors is mainly related to the synthetic compounds belonging to xenobiotics. They have never been released from natural sources and appeared in the environment as a result of anthropogenic

> The inhibitor is a molecule that reacts with an enzyme-active site to prevent it from converting the substrate.

impacts. Pesticides are one of the most important examples of such analytes. Toxic metals and natural toxins can be determined by appropriate biosensors as well. The

Table 2.3 Application of various enzyme sensors for inhibitor determination

Analyte	Representative	Enzyme/substrate	Signal transduction
Pesticides	Organophosphates, carbamates	Acetylcholinesterase/acetyl(thio)choline) (Evtugyn et al. 1999; Arduini et al. 2010)	Amperometric, potentiometric, optic (visual detection)
		Tyrosinase/catechol (Campanella et al. 2007)	Amperometric
	Triazines	Tyrosinase/catechol (Hipolito-Moreno et al. 1998)	Spectrophotometric
	Dithiocarbamates	Aldehyde dehydrogenase/propanaldehyde (Noguer and Marty 1997)	Amperometric
Industrial pollutants	Cyanide	Peroxidase/ascorbic acid (Volotovsky and Kim 1998)	Field effect transistor
	Heavy metals	Urease/urea (Rodriguez et al. 2004; Yang et al. 2006; Ogonczyk et al. 2005)	Potentiometric, spectrophotometric, field effect transistor
		Peroxidase/catechol (Bogdanovskaya et al. 1994)	Amperometric
	Chlorophenols	Peroxidase/catechol (Solna et al. 2005)	Amperometric
	Fluoride	Tyrosinase/catechol (Asav et al. 2009)	Amperometric
Pharmaceuticals	Antidepressants	Monoamine oxidase/dopamine (Medyantseva et al. 2007)	Amperometric

list of the common inhibitors that can be determined with enzyme sensors is summarized in Table 2.3.

The mechanism of enzyme–inhibitor interaction depends on their nature. From a whole variety of inhibitors, some classes are specified. Thus, *specific inhibitors* have their structure adapted to the enzyme active site or some functional groups included in the active or allosteric site of an enzyme. They are subdivided in accordance with the reversibility of their interaction and the target group attacked. Non-specific inhibitors change the structure of a protein globule irrespective of the specific groups at the active site. To specify such species, the term "denaturation" is used instead of "inhibition." The non-specific inhibitors involve detergents, the compounds breaking disulfide bridges, polyelectrolytes, etc.

The classification of various inhibitors is presented in Fig. 2.6.

Irreversible inhibitors are highly toxic species that exert their influence on enzyme activity by direct interaction with an enzyme active site. To some extent, the structure of irreversible inhibitors is similar to that of the substrate. This provides the specificity of enzyme–inhibitor interaction which is commonly much higher than that of reversible inhibitors. In analogy to the *ES* complex, the reactivity of the *EI* (enzyme–inhibitor) complex formed by irreversible inhibitors is small enough to exclude a part of enzyme molecules from the catalytic cycle.

The mechanism of irreversible inhibition can be illustrated by acetylcholinesterase and organophosphate insecticides. The enzyme promotes the hydrolysis of acetylcholine, the neuromediator (2.11) (Skládal 1996).

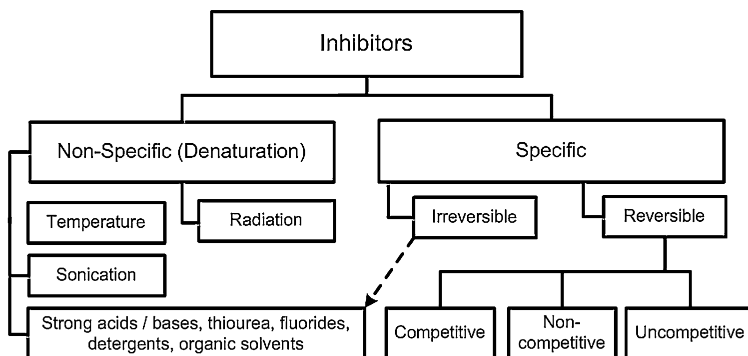
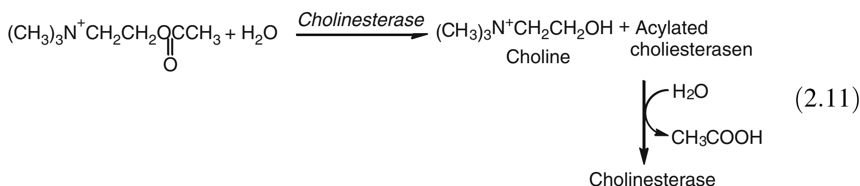


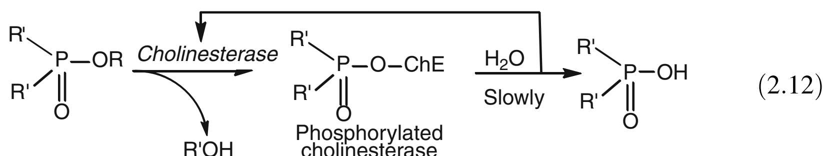
Fig. 2.6 Classification of enzyme inhibitors in accordance with the mechanism of action and nature



(2.11)

After the *ES* complex formation, the substrate breaks up into two parts, i.e., the acetic acid and choline. The latter remains in the active site due to the formation of the esteric bond with a hydroxide group in the serine residue of a protein. The intermediate product, called "acetylated cholinesterase," reacts with the final release of the acetic acid and regeneration of an active form of the enzyme. The reactivity of acetylated acetylcholinesterase is fast enough to establish a high turnover number of the enzyme.

Organophosphates are also able to form an esteric (phosphorylated) acetylcholinesterase so that the first step of the reaction is very similar to that of acetylcholine (2.12) (Giacobini 2000).



(2.12)

However, the hydrolysis of phosphorylated cholinesterase proceeds very slowly and the total rate of the substrate conversion decreases dramatically. The rate of the enzyme regeneration can be accelerated by special agents called *reactivators*, or antidotes. Thus, the reaction of the irreversible inhibitor with an enzyme is

followed by the partial destruction of its molecule. Therefore, the intermediate stage, a phosphorylated enzyme, cannot be reached from the side of the product, i.e., from alcohol R′OH, enzyme *E* and organophosphoric acid. This is one of the key features of irreversible inhibitors that distinguishes them from reversible inhibitors that meet the conditions of "true" chemical equilibrium.

The term "irreversible" in enzymology and biosensorics is also referred to the reactions that cannot be inverted in the time period comparable with the measurement duration. Such an empirical approach makes it possible to extend the number of "irreversible" inhibitors to those interacting with an enzyme reversibly but not flexibly. For example, mercury ions inhibit the enzyme activity of most enzymes due to their interaction with thiol groups of the proteins. The product of such interactions does not assume changes in the chemical structure of an inhibitor. However, the dissociation of the *EI* complex is extremely slow, so that the restoration of enzyme activity can be achieved only by treating the enzyme preparation with chelating agents. The kinetic consideration of irreversible inhibition differs from that the mechanism of which is implied. Below, a "true" irreversible inhibition with an inhibitor modification is described.

An irreversible inhibition can be monitored by the consecutive addition of an inhibitor and substrate to the enzyme preparation. The first step is called "incubation" and is performed in the absence of the substrate that diminishes the sensitivity of inhibitor detection. The inhibitory effect is quantified by the relative decay of enzyme activity. For the inhibitor concentration significantly exceeding that of the enzyme-active site, the Aldridge Eq. (2.13) is used (Aldridge 1950).

$$\ln \frac{\upsilon_o}{\upsilon_t} = k_{II} c_I \tau \tag{2.13}$$

Here, υ_o and υ_t are the rates of enzymatic reaction prior to and after the incubation step, c_I is the inhibitor concentration, and τ is the incubation time. The k_{II} bimolecular inhibition constant describes the efficiency of the enzyme-inhibitor interaction. This depends on the nature of the reactants and reaction conditions but not on the quantity of the enzyme and inhibitor as such. The bimolecular inhibition constant can be expressed by a combination of rate constants of various steps of the reaction.

The reaction (2.13) can be converted into the form (2.14), which is more convenient for the description of the biosensor response. It expresses the dependence of the remaining enzyme activity (a) on the inhibitor concentration.

$$\log a = \log 100 - \frac{k_{\mathrm{II}} \tau}{2.303} \tag{2.14}$$

In both cases, the inhibition effect depends on the product of the inhibitor concentration and the period of its interaction with an enzyme. An increase in the incubation period diminishes the concentration of an inhibitor exerting the same effect on enzyme activity. The incubation period is commonly limited by 20–30 min from taking into account the enzyme stability and the biosensor usability.

Besides the remaining enzyme activity and inhibition constants, some general characteristics of inhibition are used, e.g., the inhibition degree *I* % (2.15) and inhibitor concentration resulting in the decrease of the enzymatic activity by a half I_{50}. They are also applied for the description of reversible inhibition and even enzyme denaturation caused by such physical factors as sonication, radiation, or heating.

$$I\,\% = \frac{\upsilon_0 - \upsilon_t}{\upsilon_0} 100\,\% \qquad (2.15)$$

The irreversible character of inhibition can be detected by several experiments. Thus, inhibition increases with the incubation period and does not depend on the substrate concentration. The addition of the substrate to the inhibitor on the incubation step decreases the inhibition effect. The confirmation of the irreversible mechanism of inhibition is important for the selection of the working conditions of biosensor functioning. Specifically, it allows choosing a high substrate concentration and performing the incubation step with no substrate in the reaction media.

Reversible inhibitors exert a great variety of interaction mechanisms that all refer to the true equilibria of the stages with the enzyme participation (Copeland 2000). The kinetics of reversible inhibition as well as the approaches to the establishment of inhibition constants are given in the Appendix.

The implementation of enzymes into a solid support on the surface of the signal transducer affects the canonical dependence of inhibition on an analyte concentration. This might be for various reasons, some of which belong to the mass transfer conditions and others to the enzyme-inhibitor interaction. In short, the fewer the limitations of the substrate/inhibitor access, the higher the sensitivity of an inhibitor determination in other comparable conditions. Other effects, like the charge of enzyme carriers as such, or the accumulation of the reactants in the surface layer, are of lesser importance, at least for common signal transduction principles. Some other considerations for cholinesterase inhibitors detection are presented in Chap. 4.

In most cases, inhibition should be measured in conditions of the saturation of the enzyme with the substrate (the kinetic regime). This statement has direct application for reversible inhibition with the inhibitor solution added after the substrate to the reaction medium. Meanwhile, this is valid for irreversible inhibition as well, although the inhibitor commonly reacts with enzyme-active sites in the absence of a substrate (Fig. 2.7).

The kinetic regime of the signal measurement should be confirmed by a separate measurement series performed with the increasing substrate concentration. The following measurement of the reduced signal after the incubation step should be performed with the substrate concentration on the plateau of the calibration curve.

If the enzymatic membrane is not saturated with the substrate, the decrease in the number of active sites due to inhibition is compensated for by the involvement of other active sites that were occupied by the substrate molecules in the first signal measurement (Fig. 2.13). As a result, the inhibition degree calculated on the

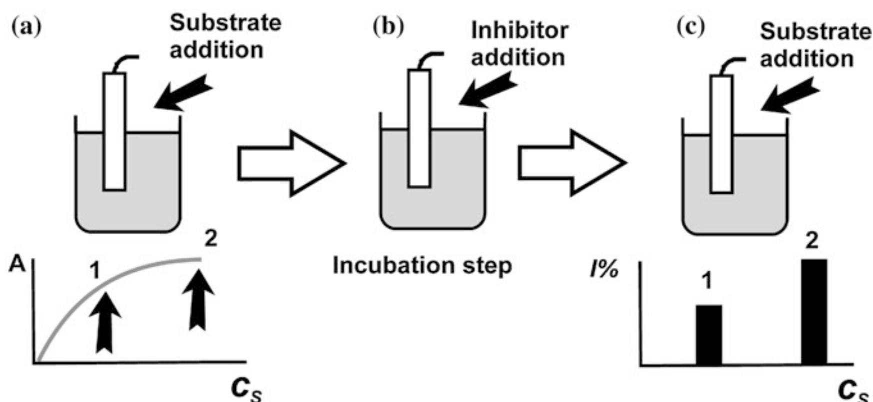


Fig. 2.7 Selection of the substrate concentration for irreversible inhibition measurement. **a** Signal measurement prior to the contact with an inhibition; **b** incubation step in the absence of the substrate; **c** the second measurement of the biosensor signal and calculation of inhibition degree *I* %. The choice of the substrate concentration on the progressive part of the substrate calibration *curve* (*1*) decreases the sensitivity towards the inhibitor in comparison with measurements with the saturating concentration of the substrate (*2*)

base of the biosensor signal shift will be lower than the actual shift of enzyme activity due to inhibition (Van Dyk and Pletschke 2011).

The use of the inhibition degree (*I* %) is a most popular approach of the biosensor signal processing, irrespective of the inhibition mechanism and biosensor functioning regime. The dependence of the inhibition degree on the inhibitor concentration is usually linearized in semi-logarithmic plots and commonly covers up to four orders of concentration magnitude. In some cases, the linear dependence I %—c_I is also described within one or two orders of magnitude. The proportional increase of the inhibition with the incubation time is observed for biosensors in a rather narrow interval of *I* %. If the inhibition exceeds 40–60 %, the influence of incubation becomes less than that expected from theoretical equations.

Although the inhibition degree is an experimental estimate of inhibition kinetics, it can be used for some conclusions about the mechanism of inhibition and the biosensor signal generation. Thus, for irreversible inhibitors, the increase in inhibition concentration should result in a 100 % inhibition, even though the inhibition is performed in the presence of a substrate. In some cases, the limited *I* % level is below 100 %. If the irreversible mechanism of inhibition has been confirmed by measurements with an enzyme solution, the underestimation of inhibition with an enzyme sensor can be due to the following reasons (Evtugyn et al. 2012):

- the chemosorption of an inhibitor on the enzyme support with a decrease in its concentration near the enzyme active site;

- the non-enzymatic reaction of the substrate conversion increasing the concentration of a product detected with the signal transducer irrespective of the enzyme activity;
- the reactivation of the inhibited enzyme active site promoted by a support component; and
- changes in the microenvironment of the enzyme in the surface layer suppressing the inhibitory effect of an analyte.

These changes might result from the pH shifts or the substrate/product accumulation in the surface layer.

In most cases, the decrease in the sensitivity of a biosensor signal toward the irreversible inhibitor caused by the above reasons can be overcome by introducing appropriate changes in the content of the surface layer and measurement protocol.

The reversible inhibition, vice versa, results in the limiting inhibition below 100 % and is theoretically independent of the measurement time. The latter condition is not obligatory and in many cases the biosensor should first be incubated in the reversible inhibitor solution prior to substrate addition. The obvious reason of such a step from the protocol of irreversible inhibition measurement is the promotion of steady-state conditions. Contrary to the theory, the reversible inhibition measured by the appropriate biosensor depends on the order of the reactant addition and is usually higher for preliminary inhibition than for the opposite order of reactant addition. For heavy metals, e.g., mercury, the behavior of an enzyme biosensor does not differ significantly from that of irreversible inhibition measurement.

For rather thin membranes, the inhibition degree can be regarded as a measure of the inhibited active sites of an enzyme. This allows the expression of inhibition parameters by the changes in the biosensor signal. Thus, for the amperometric detection of the current I related to the product of the substrate conversion, Eq. (2.16) can be used. The I_0 value corresponds to the maximal current reached with the enzyme sensor in the absence of an inhibitor, i.e., to the saturation of the enzyme with a substrate (Adeyoju et al. 1995).

$$I\,\% = \frac{c_{EI}}{c_{EI} + c_E} = \frac{c_I}{c_I + K_I} = \frac{\Delta I}{I_0} \tag{2.16}$$

The Eq. (2.32) assumes purely competitive inhibition, a most common mechanism of inhibition occurring in biosensor-based investigations. It should be taken into account that the kinetics of reversible inhibition determined for homogeneous conditions do not always coincide with those for immobilized enzymes. Moreover, the formal mechanism of inhibition observed for the biosensor signal can experience changes that depend on measurement conditions, e.g., the use of an organic solvent to increase the solubility of inhibitors.

The Eq. (2.34) can be linearized in the Scatchard plots $I\,\%/c_I$–$I\,\%$ (2.17) or by the Hill Equation (2.18) (Kurganov et al. 2001).

$$\frac{I\%}{c_I} = \frac{I\%}{K_I} + \frac{I_0}{K_I} \tag{2.17}$$

$$\frac{I\%}{100 - I\%} = \left(\frac{c_I}{K_I}\right)^x \approx \left(\frac{c_I}{I_{50}}\right)^x \tag{2.18}$$

In the Hill equation, the K_I value can be determined by the slope of the graph in double logarithmic plots. The Hill coefficient x is an empirical parameter introduced to take into account cooperative effects in the non-Michaelis–Menten kinetics description. The x value for the enzymes most popular in biosensor development and inhibition measurement is nearly one in aqueous solutions and can increase in the presence of polar organic solvents. Besides the Hill coefficient, the effect of measurement conditions can be quantified by the I_0/K_m value with the Michaelis constant determined from biosensor measurements.

2.2 Antibodies

Antibodies, also called *immunoglobulins*, or γ-globulins, are Y-shaped serum glycoproteins that are produced in the blood as a response to foreign substances called *antigens*. Mainly, antigens are related bacteria, viruses, and their parts (cell membrane fragments, deoxyribonucleic acid (DNA) (Blažková et al. 2009), carbohydrates (Huang et al. 2011), proteins (Minkstimiene et al. 2009), etc.). The interaction between an antigen (Ag) and antibody (Ab) is formally expressed by equilibrium (2.19) characterized by an association constant K_a, also denoted as an affinity constant.

$$\mathrm{Ag} + \mathrm{Ab} \overset{K_a}{\longleftrightarrow} \mathrm{Ag} - \mathrm{Ab} \tag{2.19}$$

The product of interaction, Ag–Ab, is called an *antigen–antibody complex*. Although the reaction is reversible, the equilibrium is shifted toward the complex so that one unbounded antigen molecule corresponds to 10^{15} and even more Ag–Ab complexes. The reaction (2.35) is very specific. Some antibodies can distinguish the enantiomers of the same organic molecule. For this reason, the immunochemistry approaches become popular in biomedical assays and other areas requiring a sensitive and specific detection of biologically active compounds. Both Ab and Ag molecules can be detected using immunoassay approaches, but more frequently the Ag molecules are considered a target.

It should be mentioned that the production, purification and characterization of antibodies remain an essential part of investigations on immunoassays and immunosensors. For this reason, many reports on immunosensors are devoted to the procedures related to the preliminary stages mentioned and the variety of target analytes as well as specific antibodies against them grows enormously. To some extent, the situation in the area of immunosensors development is similar to that

observed for enzyme sensors 30 years ago when the number of biochemical components increased faster than the number of specific techniques applied for signal transduction. Meanwhile, signal transduction in immunosensors is still mainly derived from conventional techniques applied in immunoassays developed for biomedical applications. Let's start with the consideration of biochemical backgrounds of immunoassays and immunosensors.

All antigens belong to high-molecular compounds. However, some of the low-molecular species called *haptens* can also initiate the production of antibodies and bind with them. The haptens first interact in living beings with some native biopolymers, such as proteins, to form a high-molecular adduct initiating the immune reaction. Such a mechanism of hapten action is taken into account in the production of specific antibodies against haptens and the signal measurement of appropriate biosensors.

The structure of an antibody is outlined in Fig. 2.8. It consists of four sub-units comprising two identical oligopeptides called "large" or "heavy" chains (H-chains), and two identical small, or "light" chains (L-chains). The heavy and light chains are held together by non-covalent forces and covalent disulphide bridges of cysteine residues. The carbohydrate residues in antibodies are covalently bonded to the *C*-terminal half of the molecule (Fc). Themolecular weights of H- and L-chains are 50 and 22 kDa, respectively. The total molecular weight of an antibody usually varies from 140 to 200 kDa (Berg et al. 2007).

The key part of the Ab molecule with the Ag binding sites is termed the Fab fragment. Thus, there are two Fab fragments in one Ab molecule and each one of them comprises an entire light chain and a segment of the heavy chain.

The binding versatility and specificity of antibodies toward the Ag molecules are achieved due to the variation in the chain region near the tips of a "Y-shaped" molecule (the *N*-terminal part of the sequences). Within these variable regions, three regions are hypervariable. They are called complementarity-determining regions. The opposite *C*-terminal end of the chain is constant for nearly all vertebrates. The L- and H-chains of Ab are folded into globular motifs of about 110 amino acids, formed by two β-pleated sheets. The L-chain contains variable light (V_L) and constant light (C_L) domains. The H-chain contains four domains

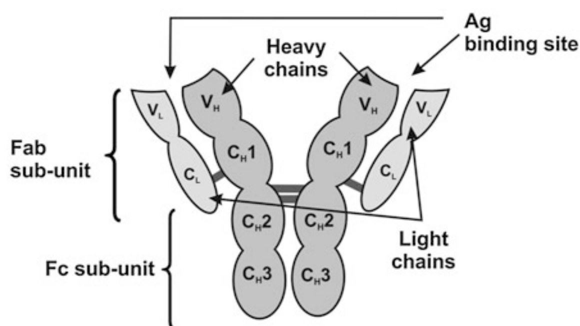


Fig. 2.8 The outline presentation of the antibody molecule

(denoted in Fig. 2.13 as V_H, C_H1, C_H2, C_H3) and the domains are assembled in pairs. The assembly of the variable domain of the H- and L-chains brings six complementarity-determining regions into a spatial proximity. They form the Ab combining site.

Antibodies are divided into five classes (IgG, IgA, IgM, IgD, and IgE) according to their heavy chain structure. Each class has some sub-classes determined by the structure of the H-chain domains. IgG is present in its highest concentration in the serum; IgM is the first to appear in the serum after the Ag exposure. IgA is the major Ab class in external secretions like saliva, tears, bronchial mucus, and intestinal mucus. IgE causes an allergic reaction, while the role of IgD is yet unknown.

Besides native Ab molecules, their derivatives can be used in the immunoassays and biosensor assembly. Thus, the treatment of Ab with dithiotreitol and some other reducing agents provides breaking disulphide bridges and formation of various subunits that retain the specificity of the Ag recognition (Stocklein et al. 1998). The thiol groups remaining on the *C*-regions of the sequences can be used for the attachment of the "half-Ab" molecules to gold or other supports. The Fab fragments can be isolated as well after treating the Ab molecules with papain. They can be used instead of the full molecules to recognize the same analytes with a lesser non-specific binding of interferences and the higher stability of immunopreparations.

The Ab production on a laboratory scale is mostly based on the immunization of a susceptible mammal (rabbit, goat, sheep, chicken, etc.) with a target Ag. For haptens, a conjugate of hapten with a high-molecular carrier is first prepared to increase the efficiency of immunization. Then the concentration of Ab in the blood gradually increases over several weeks. The blood is extracted from an animal and the Ab concentrated and purified from serum proteins. Various β-T-lymphocytes are activated by the same Ag to start producing Ab at the immunization step. Thus, the Ab pool (*polyclonal* Ab) is obtained with a different sensitivity towards various potential binding sites (*epitopes*) in the Ag molecule. Polyclonal antibodies exhibit an extended variability of the Ag binding that can vary from one animal (immunization step) to another. The Ab comprising the pool can also react to the molecules structurally relative to Ag and therefore decrease the specificity of the immunoassays. This ability is also called *cross-reactivity* and is quantified by the ratio of the concentrations of the target Ag and other species yielding the same relative binding of Ab in the sample. Cross-reactivity is highly dependent on the immunization protocol but can be altered to some extent on the stages of biosensor assembling and signal protocol optimization (Law 1996).

Another way to produce the Ab was provided by genotechnology. In this method, the cells producing antibodies are removed from the spleen and fused with tumor cells to form so-called "hybridoma cells." They can infinitely replicate and produce strictly the same Ab (called *monoclonal antibody*) as a parent cell. Being more expensive at the initial stages of research, monoclonal antibodies show a much better selectivity toward the target analytes and a lesser mass production cost than monoclonal antibodies. In a similar manner, recombinant Ab fragments (commonly Fab and single-chain variable fragments) are obtained from bacteria,

e.g., *Escherichia coli*, with an introduced specific gene (Rasmussen et al. 2007). This greatly facilitates the production and manipulation for biotechnological, medical or research applications.

Recombinant Ab technique is a third possibility for producing antibodies. In these protocols, the Ab genes are cloned, introduced and expressed in inexpensive and relatively simple organisms (yeast, plant and insect cells). *E. coli* is the most common host organism. The properties of antibodies obtained in various ways are summarized in Table 2.4 (Dankwart 2000).

A similar technique can be used for the production of the recombinant Ab fragments. Thus, the ScFv antibodies (molar mass about 27 kDa) consist of variable parts of heavy and light chains joined together with a peptide linker (Zeng et al. 2012). scFv is the smallest antibody fragment retaining the Ag binding specificity of the parent Ab. They can be genetically engineered to contain metal binding amino acids (cysteine, hystidine) involved in covalent immobilization of ScFv on metal surfaces. The linkers mentioned can participate in the formation of di-, tri- and tetrameric ScFv molecules with enhanced sensitivity of recognition.

The affinity of Ab is characterized by an equilibrium constant determined in a series of experiments using the different ratios of the Ab and Ag concentrations. Assuming the stoichiometry 1:1 of the Ag–Ab interaction, the following Scatchard equation can be derived from the equilibrium (2.20):

$$\frac{c_{\mathrm{Ag-Ab}}}{c_{\mathrm{Ag}}} = K_a c^0_{\mathrm{Ab}} - K_a c_{\mathrm{Ag-Ab}} \tag{2.20}$$

Table 2.4 Properties of polyclonal, monoclonal and recombinant antibodies

Properties	Polyclonal Ab	Monoclonal Ab	Recombinant Ab
Supply	Limited and variable	Unlimited production possible	Unlimited production possible, immunization not mandatory
Uniformity	Properties change with different sera	Constant properties	Constant properties, variation by genetic manipulations
Affinity	Mixture of antibodies with different affinities	Uniformity high or low, can be selected by testing	Uniformity high or low, can be selected by testing and can be modified
Cross-reactivity	Results from different affinities	Different, depending on individual Ab	Different, depending on individual Ab, can be modified
Demands on Ag	High purity required for specific antisera	Impure Ag or mixture of antigens can be used for immunization, pure Ag necessary for screening	Impure Ag or mixture of antigens can be used for immunization, pure Ag necessary for screening, immunization not mandatory
Cost	Low	High	Once established, low

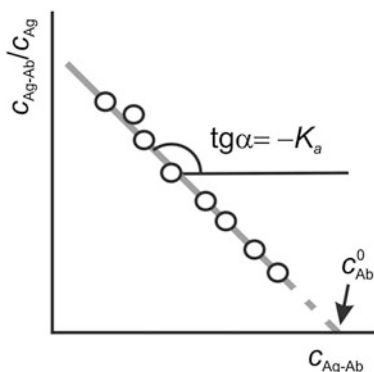


Fig. 2.9 Graphical presentation of the Scatchard equation

In accordance with (2.20), the ratio of the bonded Ag to free Ag linearly depends on the concentration of the Ag–Ab complex with a slope equal to the association constant K_a (Fig. 2.9). If Ab is bonded to *nAg* molecules, the Eq. (2.20) can be converted to (2.21):

$$\frac{c_{\mathrm{Ag-Ab}}}{c_{\mathrm{Ag}}}\frac{1}{c^0_{\mathrm{Ab}}} = nK_a - K_a\frac{c_{\mathrm{Ag-Ab}}}{c^0_{\mathrm{Ab}}} \tag{2.21}$$

In the plots of $\left(c_{\mathrm{Ag-Ab}}/c_{\mathrm{Ag}}\right)/c^0_{\mathrm{Ab}}$ against $c_{\mathrm{Ag-Ab}}/c^0_{\mathrm{Ab}}$, the linear graph intersects the x-axis at $x = n$.

Methods of the graphical presentation of the immune interactions based on the Scatchard equation are valid for the monoclonal antibodies and for the polyclonal ones with similar affinity toward Ag. If the affinity constant varies, groups of clone Ab can be specified from Scatchard graphs by specifying a narrow concentration range of the reactant corresponding to the high and low activity of antibodies. Besides, a full non-linear fitting of the progressive curve is used for assumed stoichiometry of interaction.

Although the reaction of Ab and Ag is reversible, the use of one of the reactants attached to the solid support does not guarantee a multiple use of the biosensor system. The dissociation of the Ag–Ab complex is normally too slow for spontaneous reactivation. The use of the concentrated solutions of inorganic salts with the addition of surfactants can accelerate the process. Of other measurement parameters, temperature is of particular interest. Commonly, the reaction of the sample with antibodies, either in solution or on a solid support, is performed at the ambient temperature or that close to the temperature of the host body (37 °C for human antibodies). Intermediate steps of washing and removal of excessive reagents can be performed at 40–60 °C. In general, immunoreagents are sensitive to the solution temperature to a lesser extent than the enzymes. The same applies to the presence of polar organic solvents that alter the affinity but at higher concentration and to a lesser extent than the activity of the enzymes. This might not be very important for the immunoassay format based on the measurement of enzyme

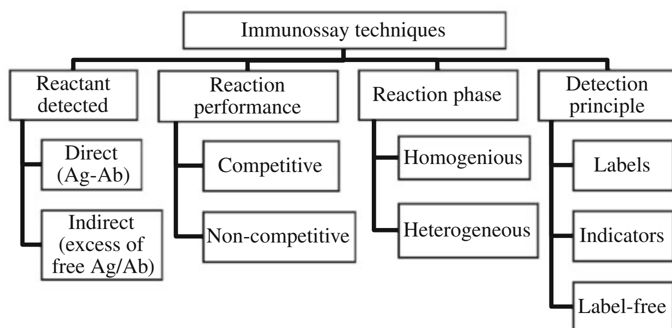


Fig. 2.10 Classification of immunoassay methods

activity like enzyme linked immuno sorbent assay (ELISA) but extends the opportunities of enzyme-free detection techniques.

All the methods of immunoassays are based on the detection of either the Ag–Ab complex or excessive amounts of reagents. The division on direct and indirect methods and homogeneous and heterogeneous types among other classification principles seems most important (Fig. 2.10).

Thus, the *direct* detection of Ag–Ab complexes can be performed by any mass-specific transducer, e.g., quartz crystal microbalances or cantilever-based techniques. Of other methods that are not compatible with the biosensor format, agglutination can be mentioned. In this method, the decrease of the solubility of the Ag–Ab complex against individual components is recorded by optical techniques. The sedimentation can also result in the surface accumulation of various species such as radioactive isotopes. Taking into account the sensitivity of appropriate detectors, radioimmunoassay belongs to one of the most sensitive detection methods with the femtomolar limits of detection. In *indirect methods*, the formation of Ag–Ab complexes is detected via the signal of auxiliary agents either covalently attached to the reactant or left free in reaction solution. In the first case, the compound or functional group measured by the appropriate transducer is termed a *label* and in the second case an *indicator*.

Previously, the term "markers" was used as well, but at present it is referred to as species indicating particular illnesses by the appearance or quantity changes in a human body. The reaction with the Ag bearing the label results in an increase of the signal due to the formation of immune complex.

Enzymes are the most popular 'labels' because of the possibility to detect their very low concentration by the amount of the product of the substrate conversion. The appropriate method involving the separation of the reactants on the solid support is termed ELISA. In addition to enzymes, colored particles, redox-active and fluorogenic compounds are used as labels and indicators.

Below are some of the most popular formats of heterogeneous immunoassay that are compatible with the biosensor format (Fig. 2.11) (Wild 2005).

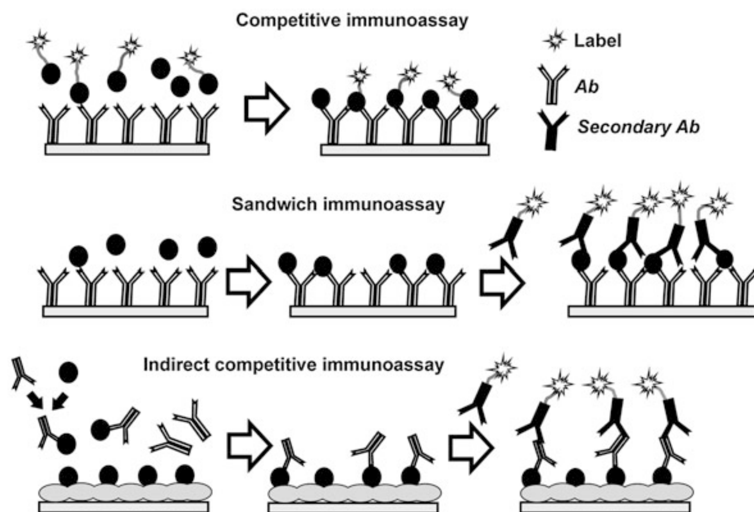


Fig. 2.11 Immunoassay formats

Direct competitive immunoassay. In this format, the sample to be tested is mixed with the solution of the appropriate immunoreagent bearing a label. The way of label introduction should be optimized to prevent its influence on the affinity of the labeled agent to its counterpart. Thus, for Ag detection, specific antibodies are attached to the solid support. The incubation with a mixture of labeled and non-labeled antigens results in their interaction with the layer of antibodies. The subsequent washing removes the non-bonded labeled reagent so that the signal of the label detected can be related only to the bonded label. Thus, the higher the signal, the lesser the content of non-bonded Ag in the sample tested. The direct competitive immunoassay is one of the simplest detection schemes that is quite suitable for the automated assay and immunosensor format. The measurement includes three steps (incubation, washing, and signal measurement) and hence a rather acceptable time interval is required. However, there are two principal limitations: First, the competition decreases the sensitivity of the Ag measurement because some of the binding sites are occupied by a labeled auxiliary agent. Second, at low target concentration, small changes of a maximal signal related to the label should be detected. This complicates the metrological characterization of the result and gives a high deviation of the signal against blank measurement.

Sandwich immunoassay. In this format, two types of antibodies are required. As in the previous case, the antibodies specific for Ag are attached to a solid support. The incubation with the samples allows accumulation of all the target analyte molecules on the solid support. After that, the support with the Ag–Ab complex attached is carefully washed and treated with secondary antibodies that bind to the analyte but not to the preliminary antibodies that remained unoccupied on the surface. Then antibodies are labeled so that the signal measured is directly

proportional to the number of the Ag–Ab complex onto the surface, i.e., to the molar concentration of a target in the sample.

In comparable conditions, the sandwich immunoassay is more sensitive than the competitive immunoassay. The detectable concentrations can differ up to ten times and even more. The minimal detectable levels depend on the signal-to-noise ratio but it is much simpler to observe a signal over the zero level than the decrease of maximal response in the competitive immunoassay. The only, yet significant limitation of sandwich immunoassay, consists of a multi-step protocol increasing the measurement time up to several hours and complicating its realization in the immunosensor format.

Indirect competitive immunoassay. This is a hybrid method that includes some steps performed on a solid support and others in the homogeneous solution. Indirect immunoassay was described for hapten detection because the traditional schemes of label-based immunoassay are not applicable to small molecules. In this case, the solid support is first covered with a high-molecular derivate of hapten; this might be the conjugate used for immunization (Ab production) or another one synthesized specifically for signal measurement. The potential carriers involve synthetic polyelectrolytes and proteins such as albumin from various sources or the soybean trypsin inhibitor. The sample is first mixed with an excess of specific anti-hapten Ab. After that, the mixture is placed onto the support bearing the hapten conjugate. It can react with the free Ab but not with the Ag–Ab complex present in the same solution. Thus the higher the concentration of the analyte, the lower the amount of Ab attached to the surface. The latter is detected with secondary antibodies bearing the label for sandwich immunoassay as described above.

The formation of a bulky Ag–Ab complex affects the optic and electric characteristics of the interface that can be recorded by appropriate techniques. Thus, total reflectance spectroscopy and surface plasmon resonance provide the information on the amount of the immunoreagent attached to the surface and the kinetics of the immune reaction. Being rather complex and cumbersome for mobile devices, such equipment deserved complementary estimates due to the high sensitivity and versatility of its response. And last but not least is that these measurement modes do not require any specific reagents, so that they are both label-free and reagent-free and can be used with minimal sample treatment.

The changes in the permeability of the surface layer caused by immunochemical reactions can also be traced by electrochemical techniques that provide an important tool for both the miniaturization and automation of the results. In the simplest way, the Ag–Ab complex limits the mass transfer of small ions like ferricyanides. This can be easily monitored by conductivity measurement and electrochemical impedance spectroscopy. In both methods, the changes in the content of the surface layer are quantified by the decreased rate of the charge transfer. In the case of the electrochemical impedance, the indicator ion is involved in the reversible electron transfer and the estimation of a mass transfer is supplemented by the influence of the charge distribution on the electrode interface. This increases the sensitivity of the method against the simple recording of the ion

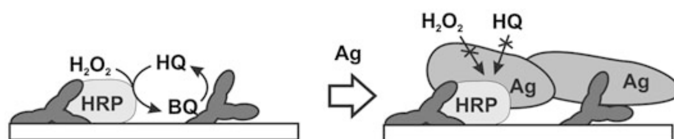


Fig. 2.12 Detection of Ag–Ab formation on the electrode by inhibition of the reaction of enzyme co-immobilized with specific antibodies. HRP horseradish peroxidase, HQ hydroquinone, BQ benzoquinone

distribution and allows drawing a number of conclusions on the steric rearrangement of the surface layer during the immunoreaction.

The use of enzymes as indicators of the mass transfer in the neighborhood of the Ab–Ag complex is reached by the co-immobilization of enzymes and antibodies onto the transducer surface (Fig. 2.12).

The binding of the Ag molecules suppresses the substrate access to the enzyme's active site. From the formal point of view, this phenomenon is described as similar to the competitive enzyme inhibition with a linear relationship between the relative decay of the enzyme activity and the Ag concentration. Acetylcholinesterase, alkaline phosphatase and peroxidase are mainly used both as labels in immunoassay and indicating enzymes in immunosensors. The high turnover number and the simple detection of enzyme activity based on optical or electrochemical transducers make them very attractive for the development of immunosensors. In Fig. 2.12, horseradish peroxidase (HRP) catalyzes the oxidation of hydroquinone (HQ) with hydrogen peroxide. The product of the reaction, benzoquinone (BQ), is reduced by the electrode to the initial HQ (Evtugyn et al. 2003).

Such a cycle, including the regeneration of a substrate in the electrode reaction, is called "the substrate recycling system;" it is analagous to similar biochemical paths, including the enzymes catalyzing the appropriate reactions of conversion and regeneration of a substrate.

The monitoring of the permeability of the surface layer, including Ab molecules, is less specific than the use of labeled immunoreagents as described above (see Fig. 2.11). Any changes in the surface layer, e.g., the non-specific adsorption of auxiliary reagents or sample components (serum proteins, lipids, nucleic acids for the assay of biological liquids) affects the permeability of the layer similarly to the target Ag–Ab interaction. The deterioration of the layer in the consecutive stages of washing, incubation and reagent addition decreases the sensitivity of the signal toward the Ag binding. This offers stricter requirements for assembling the surface layer, its durability and the stability of its main characteristics in the immunosensor manipulation.

ELISA-related immunoassay data are commonly quantified using empirical non-linear models, e.g., four-parameterlogistic-log, log-logtransforms, logistic-logtransforms, etc. The four-parameterlogistic-logmodel is used also for immunosensors based on immobilized immune reagents (Eq. 2.22) (Brady 2006)

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D \tag{2.22}$$

where x is the concentration of an analyte and y is the signal related to the Ag–Ab binding (absorbance for colorimetric end point determination, current for amperometric immunosensors, etc.). Certifications are determined for midpoint on the curve at a 50 % signal shift (IC_{50}), a maximum for the lower asymptote (A), and a minimum for the upper asymptote (D). B value corresponds to the slope of the mid-linear piece of the curve in semi-logarithmic plots, and C is a central point of this linear portion. The specific curve-fit constants vary from day to day and the accepted ranges of such variations should be determined. The reproducibility of the results for triplicate analysis is usually within 30 % but can be reduced to about 10 %, depending on the specific assay. The recoveries of positive controls typically range from 70 to 130 % or better. In similar conditions, the determination of haptens results in higher signal deviation than that of high-molecular compounds.

2.3 Protein/Peptide Receptors

Besides antibodies, some other proteins and peptides 'exert' the biorecognition properties demanded for the specific detection of biological targets, site-specific immobilization of biochemicals, or their labeling followed by signal transduction. Although most of them are still under study and will not 'assume' application in appropriate biosensors in the near future, the synthesis and characterization of protein-/peptide-based receptors is of increasing interest due to the opportunities they offer in the enhancement of the variety of biomolecular receptors and understanding their functioning both in living beings and in biosensor assemblies.

Synthetic peptides take 'intermediate' place between the amino acids and proteins. They are commonly synthesized by peptide synthesis from the randomized library of amino acids with separation of the target products by affine chromatography or solid-phase extraction (Tothill 2010). The progress in the chemistry and application of synthetic peptides is related to the attempts to avoid some of the problems often observed with antibodies and enzymes. They include low stability in extreme environment, poor performance in organic solvents, and high cost. The peptide receptors show increased affinity toward small organic molecules and are hence considered to be an alternative to antibodies in hapten analysis. The peptide-based biorecognition assumes multi-point interaction with an analyte molecule that is based on non-covalent interactions, e.g., hydrogen bonding, the formation of salt bridges, and hydrophobic and van der Waals interactions.

The peptide-based receptors consist of 5–10 amino acid residues. The short chain prevents winding the molecule and shielding its binding sites by self-aggregation. Although there are no strict limitations, most peptides applied to date include 20 essential amino acids that are similar to natural proteins. Several approaches have

been described for peptide synthesis and selection against target analytes, i.e., combinatorial library technique, phage display technology, rational design based on computational approach, and molecular imprinting. In combinatorial techniques, the diversity of peptides is first synthesized from a mixture of natural and unnatural amino acids, preferably on solid support. Then the peptides are tested for specific binding activity. Such approaches were successfully applied in the drug discovery. The screening for peptide receptors can be improved by the addition of target molecules that act as templates promoting the synthesis of the appropriate peptide that envelops the target (*molecular imprinting*). This can also be achieved by a combination of the primary synthesis of building blocks in aqueous media with their assembling on the interface in the presence of biological target followed by receptor isolation and stabilization by adsorption. *Computational modeling* predicts the molecular structure of the receptor and guides the selection of primary building blocks applied for peptide synthesis. On this stage, the interactions between a target analyte and potential receptor can be visualized and evaluated. In *phage display technology*, DNA fragments encoding random peptide sequences are inserted into bacteriophages. The peptides are expressed on the capsids and their affinity toward target molecules interest is tested by appropriate protocols.

Regarding the application of peptides in biosensors, the signal transduction with peptide receptors is mainly based on the same principles as conventional immunoassay. The binding of specific analytes can be detected by changes in optical or electrical properties of the interface or by direct mass changes measured with QCM technique. The list of analytes detected with peptide receptors involves mycotoxins (Tothill 2011), cAMP (Katayama et al. 2000) and dioxins (Mascini et al. 2004). The selection of peptides involved the formation of dipeptide combinatorial library, followed by the selection of most relevant dimers and their involvement in tetrameric structures.

A bioelectronic nose has been developed for the detection of seafood spoilage based on trimethylamine detection (Lim et al. 2013). A field effect transistor (FET—see details of signal transduction in Chap. 3) with the gap modified with single-walled carbon nanotubes was used as a signal transducer. Olfactory receptors that consisted of 15 residues were immobilized by self-assembling onto the surface of carbon nanotubes by preferably hydrophobic interactions of phenylalanine groups. The nose reacted on the triethylamine vapors by a significant shift of the conductance. The bioelectronics nose detected triethylamine on a femtomolar level and could distinguish the analyte from other similar amines. Besides seafood spoilage, the portable device can be applied to medical diagnostics and environmental pollution assessment.

Protein nanotubes are another example of the biorecognition elements derived from natural biopolymers (Seabra and Durán 2013). The synthesis of a new class of peptide receptors based on rationally designed eight alternating D- and L-amino acid residues of cyclo[-(D-Ala-Glu-D-Ala-Gln)$_2$-] was published in 1993. The internal pore diameter of the peptide nanotube is controlled by the size of the peptide subunit used, i.e., the number of amino acid residues within the cyclic structure. The cyclic peptide monomers can be modified with chromophore groups taking into

account their possible application in electronic and optical devices. Regular nanotubes can also be self-assembled from linear surfactants such as peptides enriched with aromatic amino acid residues. The peptide nanotubes are more reactive than carbon nanotubes, a conventional nanomaterial for biosensor development. Meanwhile they require additional modification to establish signal transduction, e.g., the introduction of metal porphyrines or nanocrystals (Matsui and MacCuspie 2001). Peptide nanotubes can be used as templates for the synthesis of silver nanowires (Reches and Gazit 2003) or copper nanospheres (Banerjee et al. 2003) and some other elements demanded in electronic devices. In sensor applications, the peptide nanotubes offer broad opportunities to construct nano-sized devices based on optical and electrical signals related to conformational changes of the peptide chains followed by alteration of the ionic transport (artificial ion channels) or optical properties of the interface (de la Rica and Matsui 2010).

Lectins are proteins of non-immune origin that interact with carbohydrates but do not modify them. First described in 1888 by Stillmark, lectins have received application a century later in cell biology as probes to investigate cell surface structure and functions (Rahaie and Kazemi 2010). Lectins are derived from plant, microbial and animal sources, and can be water-soluble or membrane-bound. The classification of lectins is based on their specific affinity toward various carbohydrates. Five groups are classified, i.e., lectins exerting affinity toward *N*-acetylglucosamine, galactose/*N*-acetyl-*D*-galactosamine, glucose/mannose, L-fructose and sialic acid. Concanavalin A is one of the most popular lectins in biosensor applications. It binds to polysaccharides and hence provides site-specific immobilization of glycoproteins to solid supports, e.g., signal transducers of a biosensor. In a similar manner, the specific binding of oligosaccharides in the walls of bacteria makes it possible to recognize them by mass sensitive sensors. Quartz crystal microbalance modified with lectin shows the reliable detection of about 1,000 cells in flow-injection mode for about 30 min. (Safina et al. 2008). Glycan-lectin interactions have been used for the sensitive detection of disease-related markers, e.g., cancer-associated T-antigens (β-D-Gal-[lf3]-D-GalNAc disaccharide) with electrochemical and fluorescent signal detection (Dai et al. 2006). Other analytical applications of lectin-based recognition involve glycosylation monitoring, glycoprotein separation and recognition (Bertok et al. 2013), drug delivery system assembling, (Gorityala et al. 2012) and the direct detection of cancer cells (Hu et al. 2013).

2.4 Nucleic Acids

DNA is the fundamental molecule of life. It is present in all living beings and determines the basic features of their reproduction and inheritance of the characteristics specific for appropriate biological species.

A single DNA molecule consists of a phosphate-sugar backbone bearing four different substituents at each deoxyribose ring of the chain (Fig. 2.13).

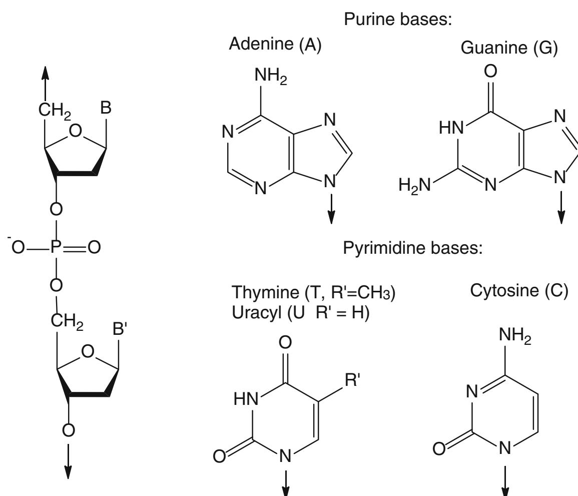


Fig. 2.13 Structure of the DNA backbone and of four DNA nucleic bases. *Arrows* indicate their bonds with the rest of the molecule

Uracil residue substitutes thymine in ribonucleic acid (RNA) and is present in aptamers, i.e., synthetic oligonucleotides obtained *de novo* by combinatorial chemistry. All the above-mentioned substituents are called *nucleic bases* (or simply *bases*) and are subdivided into two groups, i.e., purine and pyrimidine bases, which interact with each other by the formation of two and three hydrogen bonds (Berg et al. 2007).

The nucleic bases bonded to ribose (in RNA) or deoxyribose (in DNA) by *β*-glycosidic linkage are termed *nucleosides*. Their phosphorylation yields *nucleotides*. Thus, the DNA can be regarded as a polynucleotide and its fragment as an oligonucleotide. Although RNA and its fragments are very rarely used in biosensors, in some works, a more definite term, "*oligodeoxynucleotide*" (abbreviated "ODN") has been introduced. For the same reasons, the codes of nucleic bases and appropriate nucleotides (A, G, T and C for adenine, guanine, thymine and cytosine, respectively) are specified by dA, dG, dT and dC, where "d" means "deoxy." The necessity to distinguish between the derivatives of ribose and deoxyribose in the biosensor assembly is rather mild and can be easily omitted when the term "*DNA sensor"* is used. From this point of view, the short descriptions "poly(A)" and "poly(dA)" mean the same sequence consisting, only of adenine residues. Such sequences are called *homo-oligonucleotides* as well.

The sequence of deoxynucleotides bonded by phosphodiesteric bonds is a primary DNA sequence (chain, strand). Its direction proceeds from 5′- to 3′-ends, meaning the unoccupied positions of the terminal monomer units. In other words, a free hydroxyl group of ribose is placed at the beginning of the DNA chain and the monophosphoesteric group at its end (Fig. 2.14). The primary DNA sequence is recorded by codes referred to as nucleic bases. Thus, the oligonucleotide consisting of nine nucleotides is univocally described by the following record: 5′-ATTGCATTC-3′. It definitely differs from 5′-CTTACGTTA-3′ by its chain direction although the sequence of nucleic bases seems the same.

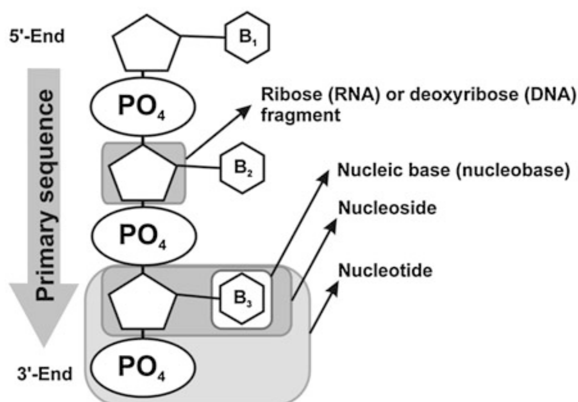
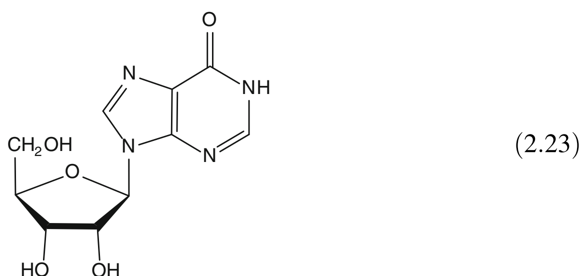


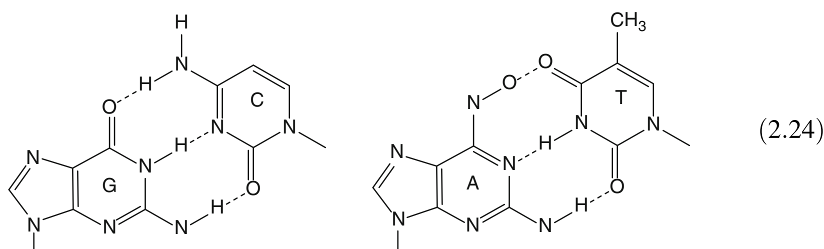
Fig. 2.14 Primary DNA sequence and its constituents

In synthetic analogs of native oligonucleotides, guanine can be substituted by inosine, which retains the ability to bind the cytosine fragment (Eq. 2.23). Some other nucleotide derivatives are known and used to increase the stability of the sequences demanded for biosensor preparation and operation.



(2.23)

A native DNA molecule consists of two antiparallel primary sequences wrapped around a central axis in the right-hand screw sense. From 10 to 12 base pairs are involved in each turn of the DNA helix. The spaces between the sequences are called *grooves*. One of them (the major groove) is broader than the other (the minor groove). The formation of the helix is due to specific interactions between all the nucleotides of each strand. Adenine interacts with thymine and guanine with cytosine with the help of hydrogen bonds (2.24).



(2.24)

The A–T and G–C base pairs fit exactly into the space between the backbones, so that the internal volume of the helix is filled with a parallel stack of the nucleic base pairs additionally stabilized by hydrophobic interactions of overlapping aromatic planes. The outer surface of the DNA has a high density of the negative charge of the phosphate groups.

Both nucleic base pairs and the grooves on the surface specifically coordinate some molecules. Thus, small aromatic molecules can penetrate the DNA helix (intercalation reaction). Proteins and polyelectrolytes are attached in the grooves of the molecule. Such interactions alter the volume and spatial structure of the DNA molecule and can be detected by appropriate changes in the charge distribution, optical properties, redox activity, etc.

The formation of the double-stranded (ds-) DNA from single sequences is called *hybridization*. In such a reaction, each nucleic base is bonded to a counterpart (A–T, G–C) in the second sequence. The feature of the hybridizing DNA strands to meet each other is called *complementarity*. The DNA sequences retain their ability for hybridization even if a few of the nucleic bases do not interact with each other. Such disturbance in full complementarity is called a *mismatch* (single-, double-mismatched DNA sequences, etc.). The selectivity of the DNA hybridization detection is characterized by the possibility to distinguish fully hybridized and mismatched sequences. In a simplified approach, homo-oligomeric nucleotides are used as fully mismatched sequences. In addition to mismatches in which the complementary oligonucleotide is substituted with another one, the *abasic sites* of DNA sequences are specified. This is a location in the DNA molecules in which there are neither purine nor pyrimidine bases. They are formed spontaneously or in the DNA damage (Lhomme et al. 1999). The detection of 'abasic' sites is one of the purposes of the DNA sensors on the potential hazards' estimation.

The reaction of DNA hybridization is reversible. Slow heating ("DNA melting") breaks the hydrogen bonds in the base pairs, and sharp cooling prevents the ss-DNA molecules from re-hybridization. Concentrated electrolytes, sonication and adsorption on the solid support result in partial de-hybridization as well. The ss-DNA molecules obtained have an amorphous structure; some of the pieces of the sequence can be hybridized to each other. This can result in the formation of loops, knots and some other unusual structures that affect the behavior of ss-DNA molecules in biosensor assembly.

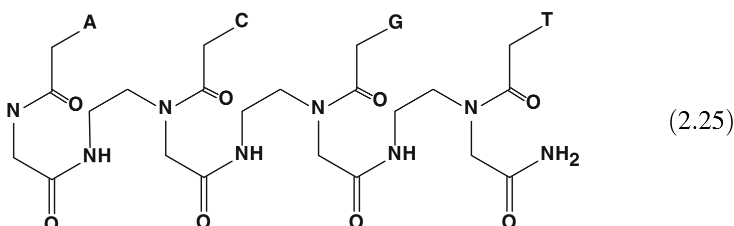
The ds-DNA helix described above exists in three major classes: DNA A, DNA B and DNA Z, which are different in their wrapping and screwing directions. The structure first analyzed by Watson and Crick belongs to the DNA B class (10 bp per turn). Native DNA is a rather flexible molecule in physiological conditions. Its geometry depends on the local nucleic sequence and microenvironment.

In DNA sensors, native DNA is not so often used because of its large size (molecular weight up to several million Da) and complications in reproducibility and specificity of the biosensor performance. More often, short sequences (ODNs, or simply oligonucleotides) are used. Many of them are selected from the polymerase chain reaction (PCR) of the DNA amplification used in a traditional DNA assay for establishing particular sequences specific for hosting organisms (pathogenic microorganisms or viruses). In analogy with the DNA assay techniques, such as ODNs are also termed *DNA probes*.

The ODN-based biosensors belong to the family of the DNA sensors. In some cases, the origin of oligonucleotides is mentioned ("gene related to sarcoma," or "HIV virus detection"). The length of the DNA probe does not exceed several dozen nucleotides. Modern technologies introduce modifying fragments into DNA probes, preferably by covalent modification of terminal groups. The list of modifiers involves fluorogenic labels like fluorescein and functional groups required for biosensor assembling (biotin, or thiol and amino groups).

Among native DNA molecules and derived oligonucleotides, some other biorecognition elements relative to DNA structure have become popular in the past decades.

Peptide nucleic acids (PNAs) are synthetic analogs of native DNA that have a similar distance between the nucleic bases and hence have hybridization ability. PNA consists of repeating *N*-(2-aminoethyl)-glycine units linked by amide bonds (2.25). PNAs do not contain any sugar moieties and phosphate groups and are much more stable toward hydrolysis and oxidation than DNA analogs (Wang 1998).



(2.25)

The application of PNAs in bioassay is based on their abilities to form hybridized PNA–DNA products similar to ds-DNA. In comparison with natural analogs, PNAs show excellent biological stability, higher binding affinity, and better specificity. Some of the advantages mentioned are related to the electrical neutrality of PNA molecules that excludes electrostatic repelling of negatively charged phosphate units of the DNA probe and target DNA sequence in

conventional hybridization event. The strong PNA–DNA duplexes result in a higher melting point, which is about 1 °C per base pair higher than that of corresponding ds-DNA duplexes. Single-base mismatches of the sequences exert a more destabilizing effect than that in DNA sensors. This makes it possible to detect them with high reliability and sensitivity. PNA probes are resistant to enzymatic degradation because the backbone of *N*-(2aminoethyl)-glycine is not recognized by nucleases and proteases. The same can be related to the stability of the PNA probes toward strong acids and high temperatures. The stability and high affinity of PNA–DNA interactions offers extended opportunities for application of the PNAs in sequence specific DNA detection and biomedical diagnostics (Singh et al. 2010). Besides, hybrid PNA–DNA, PNA–RNA duplexes can be used as building blocks for the assembling of biorecognition layers on the solid interface. The nanorods consisted of quadruplexes PNA_2–DNA_2 were reported in nanotechnology applications as building blocks for nanoscale bridges to conduct electrical charges (Armitage 2003).

Aptamers. Contrary to the DNA probes, *aptamers* are fully synthetic oligonucleotides obtained *de novo* from the DNA (RNA) library by a combinatorial chemistry approach (Mascini 2008; Song et al. 2008). The aptamers are selected from the randomly synthesized oligonucleotides by affinity chromatography, ultrafiltration, capillary electrophoresis, flow cytometry, centrifugation and other techniques against the target analyte. A chemically synthesized library consists of 10^9–10^{15} molecules of the ss-DNA, each possessing a random-sequence region positioned between specific primer sequences for amplification. For RNA aptamer selection, the primary ss-DNA library is first converted into a corresponding RNA library. The selected oligonucleotides are amplified by PCR for ss-DNA aptamers or reverse transcription-PCR for RNA aptamers. The properties of the aptamers

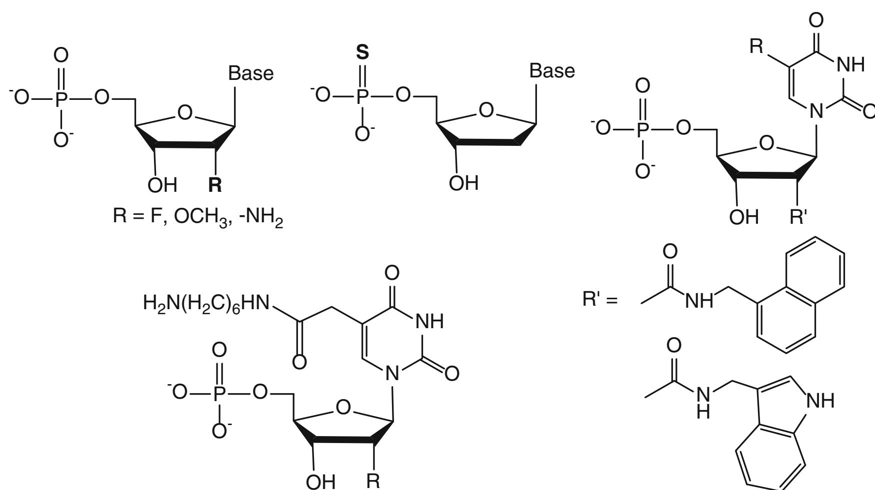


Fig. 2.15 Modified and unnatural nucleic acids used in the assembly of DNA/RNA aptamers

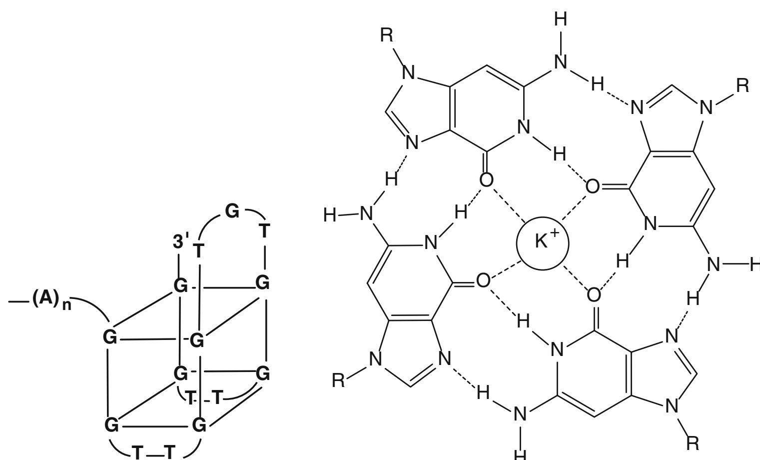


Fig. 2.16 Configuration of an aptamer against thrombin and the structure of G_4 quadruplex specifying three-dimensional structure of the aptamer. K^+ ions stabilize the quadruplex

can be sufficiently enhanced by the involvement of unnatural elements on the stages of synthesis of the primary library and product selection. The examples of modified natural and unnatural nucleic acids used for DNA\RNA aptamers are presented in Fig. 2.15.

The modification is directed to the covalent immobilization of aptamers via amino and carboxylic groups in the side-chains of the molecules as well as increased stability of aptamers toward hydrolysis and oxidation in aqueous environment and biosensor assembly. The incorporation of substituents to a 5′-position of uridine improved the selectivity of recognition of human proteins. The diversity of the response could be additionally increased by kinetic discrimination of binding (Gold et al. 2010). The technology based on such aptamers (Slow off-rate modified aptamer, SOMAmers) was successfully applied for biomarker discovery. The technique was tested on 813 proteins with the detection limits in picomolar range of 'conventration' and seven orders of magnitude of concentration determined (100 fM–1 mM). Fifty-eight proteins were identified as biomarkers of chronic kidney diseases.

The aptamers have a rather unusual primary and spatial structure that has no analogs in native DNA molecules. Thus, the aptamers can have loops of unhybridized sequences or guanine quadruplexes amalgamated in the stacks formed by the nucleotides belonging to the same or different sequences (Fig. 2.16) (Tucker et al. 2012).

The aptamers are also provided by linkers consisting of homo-oligonucleotides. They prevent a too-close position of the aptamer on the solid supports to avoid steric limitations of an analyte binding. The oligonucleotide linkers can form ds-DNA fragments that are rather rigid and duplicate the number of binding sites at

Table 2.5 Examples of aptamer applications in the assembly of aptasensors

Target analyte	Detection principle	Target analyte	Detection principle
Thrombin	Amperometric (Suprun et al. 2010; Degefa et al. 2009)	Interferon γ	Amperometric (Liu et al. 2010)
	Impedimetric (Radi et al. 2005; Li et al. 2008)	IgE	FET (Maehashi et al. 2007)
	QCM (Hianik et al. 2005)	Prostate cancer cell biomarker	Fluorescent (Farokhzad et al. 2004)
	Potentiometric (Numnuam et al. 2008)		
Adenosine	Electroluminescence (Zhu et al. 2011)	Cocaine	Amperometric (Zhang et al. 2011a)
Lysozyme	Amperometric (Cheng et al. 2007; Rodríguez and Rivas 2009	Glycoproteins	Amperometric (Xia et al. 2013)
HIV Tat protein	QCM (Minunni et al. 2004)	Adenosine	Amperometric (Feng et al. 2008)
Ochratoxin A	Amperometric (Bonel et al. 2011)	Trinitrotoluene	Fluorescent (Ehrentreich-Förster et al. 2008)
Acetamiprid	Impedimetric (Fan et al. 2013)	Breast cancer cells	Amperometric (Zhu et al. 2013)
K^+ ions	Amperometric (Radi and O'Sullivan 2006)	Chloramphenicol	Amperometric (Yan et al. 2012)
	Optical (Ho and Leclerc 2004)	Theophylline	Amperometric (Ferapontova and Gothelf 2009)

QCM quartz crystal microbalance; *FET* field effect transistor

the opposite ends of the sequences. Due to similarity of the geometry, it was suggested that such dimeric aptamers be called “aptabodies” (Hianik et al. 2008).

More than 200 aptamers are characterized against various ‘analytes’, but not all of them have been introduced in the biosensor assembly. The most common examples of aptamers applications into the biosensor format are presented in Table 2.5. They cover a great variety of the analytes from inorganic ions to large proteins. Certainly this does not mean the aptasensors could compete with the conventional techniques applied for potassium determination. This is proof of the concept confirming the great potential of aptasensors as universal measurement devices. Besides, the aptamers can be used as part of more complicated systems developed as prototypes of logic gates and other prototypes of biological computers.

DNAzymes and rybozymes are ds-ODNs which exert their own catalytic activity, i.e., oxidation or bond cleavage in DNA and RNA molecules, respectively (He et al. 2009; Kurata et al. 2000). Many of them have the structure of aptamers with a high affinity for the enzyme cofactor. Thus, the aptamer toward hemin exerts a peroxidase-like activity towards typical enzyme substrates (Yuan et al. 2011). Like aptamers, DNAzymes are selected from a randomized library with 10^{14}–10^{15} different sequences. The sensitivity of the target analyte detection is comparable or lower than that of antibodies with the lowest detection limits of about 45 fM.

DNAzymes were initially designed to detect Pb^{2+} and other divalent cations, but later on were adapted to other biomolecular targets (Zhang et al. 2011b). The interactions of aptamers very much depend on the microenvironment that promotes or hinders the required topology of the binding site. Thus, the attachment of the DNAzyme to the sd-DNA fragment switches the enzyme activity on, due to the folding of the aptamer side into the G_4 quadruplex followed by the hemin binding and the catalysis of the substrate oxidation. Thus, oligonucleotide 5′-ATT GGG AGG GAT TGG GTG GGC AC-3′ forms a stable G-quadruplex structure that binds hemin and then catalyzes oxidation of colorless 10-acetyl-3,7-dihydroxyphenoxazine to

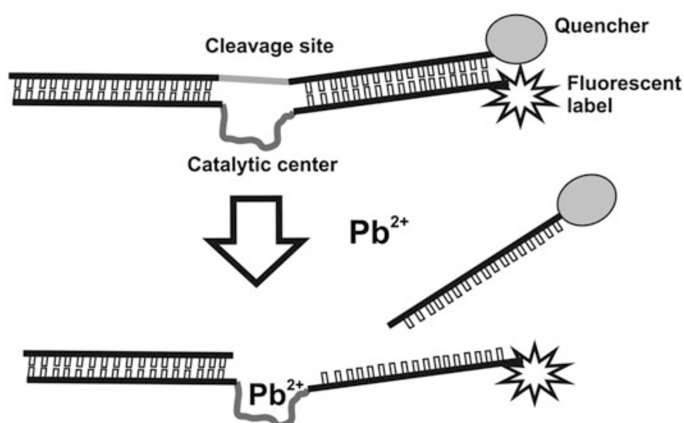


Fig. 2.17 Fluorescent detection of Pb^{2+} ions based on DNAzyme with fluorescent label

resorufin. The complex mimics the HRP activity and can easily be combined with glucose oxidase to detect glucose by the absorbance of the final product detected at 570 nm (Bo et al. 2013).

Other DNAzymes selectively cleave the sd-DNA sequences in the presence of selected metal ions. The principle of the signal detection with a fluorescent label is illustrated in Fig. 2.17. The Pb^{2+} ions activate the catalytic site of the ds-DNA-zyme and promote cleavage of the opposite part of the nucleotide sequence; this results in full dehybridization of the molecule. Prior to interaction, the terminal fluorescent label is shielded by a quencher group attached to the neighboring fragment of the sequence. Dehybridization results in removal of the quencher and initiation of the fluorescence measured by an appropriate detector. A similar approach can be used with electrochemically active labels for voltammetric or impedimetric detection of the target analyte (Xiao et al. 2007).

The DNAzymes have been successfully applied for the detection of infectious pathogens directly in the PCR products (Monsur Ali et al. 2011; Zagorovsky and Chan 2013). In these biosensing devices, the specific DNA sequence was used to assemble the catalytic site of the ds-DNA. This made it possible to switch on or switch off the fluorescence by the addition of catalytic sequences to the reaction mixture. Visual detection of the reaction is possible with the sequences labeled with Au nanoparticles.

2.5 Whole Cells as Biosensing Elements

The analytical application of whole cells in the biosensor assembly was begun in 1975. The determination of ethanol was described with *Acetobacter xylinum* immobilized in the cellulose membrane attached to the oxygen sensor. From this time, various approaches enhancing the number of analytes and improving microbial sensor performance have been elaborated, and they were recently summarized in reviews (Lei et al. 2006; Xu and Ying 2011; Ron 2007). Although many of them can be extended to other supramolecular structures, e.g., liposomes, organelles, mitochondria or fibroblasts, microbial biosensors have gained special attention due to the simplicity of operation with living cells and advantages related to their reproduction and self-maintenance in operational conditions.

To some extent, living cells can be considered to be a source of appropriate enzyme activity that can be utilized for the detection of specific substrates. In comparison with isolated enzymes, the use of living cells is complicated by the additional stage of the substrate transfer through the cell membrane. This makes the response of microbial sensors longer and less sensitive against conventional enzyme sensors. On the other hand, the target enzyme in a living cell exists in a natural environment including specific protecting and repairing systems and effective operation in a favorable medium. The selective transport of ions, especially that of H^+, specifies the functioning of enzymes bonded to the membranes or placed in periplasm and cytoplasm that remarkably differs from the conditions in

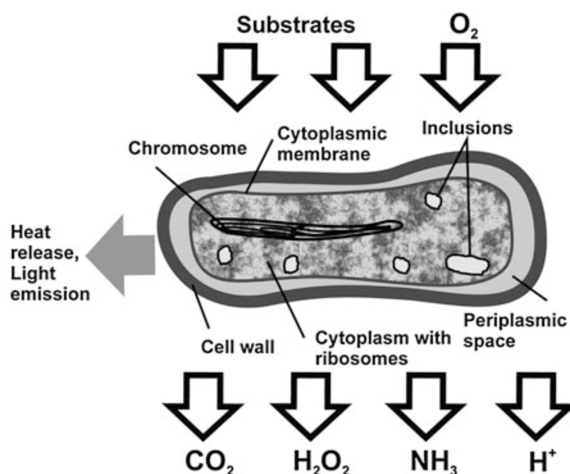


Fig. 2.18 Schematic representation of a bacterial cell and the material/energy fluxes in metabolic reactions

artificial membranes of biosensors. Genetic manipulations and gene fusion extend the biochemical paths of the substrate conversion and hence the ways to reach selectivity and sensitivity of detection in accordance with modern requirements of bioassay. Cells naturally provide cofactors and activators of enzymatic reactions that are normally added to enzyme sensors and are often unstable and rather expensive.

The detection of microbial reactions is based on general fluxes of the substances between a cell and the environment, as is schematically shown in Fig. 2.18.

The oxidation of the substrates results in oxygen consumption and release of the metabolic products, among which carbon dioxide corresponds to a full energy conversion. The efficiency of the destruction of the organic matter can be easily monitored by the O_2/CO_2 balance. In this respect, monitoring of microbial activity does not differ dramatically from oxidoreductase-based biosensors. In some cases, other products, e.g., ammonia for nitrate-/nitrite reducing microorganisms or H_2S for anaerobic species, can be detected to increase the specificity of the response. The pH shifts, as well as heat generation and biomass increase, also refer to general parameters utilizing the assessment of the bacterial activity both in suspensions, natural populations and in the biosensor assemblies. Their application for the measurement of biological oxygen demand (BOD) and antibacterial agents is described in more detail in Chap. 4. The examples of the substrate detection with microbial sensors are given in Table 2.6. Most of them are based on Clark-type oxygen sensors or pH shift measurements that are traditionally used in the monitoring of fermentation processes. Meanwhile, optic detectors have received increasing attention in the past decade. The measurement of light emission is achieved by specific proteins, e.g., luciferase or green fluorescent protein, which are displayed by the introduction of appropriate *lux* and *gfp* genes in the DNA of a host microorganism.

Table 2.6 Examples of microbial sensors for the detection of organic substrates

Substrate	Microorganisms	Detection principle
Ethanol, glucose	*Gluconobacter oxydans* (Tkac et al. 2002, 2003)	Amperometric (oxygen consumption)
Sucrose	*Gluconobacter oxydans/Saccharomyces cerevisiae* (Tkác et al. 1998)	Amperometric (oxygen consumption)
Paraoxon	Genetically engineered *Moraxella* sp. displaying organophosphate hydrolase (Mulchandani et al. 2006)	Amperometric (oxygen consumption)
Organophosphates	*Flavobacterium* sp. (Gäberlein et al. 2000)	Potentiometric
Formaldehyde	*Hansenula polymorpha* (Khlupova et al. 2007)	Amperometric (oxygen consumption)
Formate	*Cloatridium butyricum* (Ho and Rechnitz 1985)	Potentiometric
Phenolic compounds	*Pseudomonas putida* (Timur et al. 2003)	Amperometric (mediated oxidation)
Acetic acid	*Fusarium solani* (Subrahmanyam et al. 2001)	Amperometric (oxygen consumption)
Trichloroethylene	*Pseudomonasa eruginosa* JI104 (Han et al. 2002)	Potentiometric
	Pseudomonas putida (Hnaien et al. 2011)	Conductometric
Tetracyclines	Recombinant *Esherichia coli* with lux gene (Hansen and Sørensen 2000)	Luminescent

The determination of tetracyclines with bioluminescent microbial sensor is an example of such genetic engineering used for both enhancement of the selectivity and sensitivity of the assay (Hansen and Sørensen 2000). In this method, the tetracycline-related repressor is removed from *lux* or *gfp* genes fused in the DNA of *E. coli* so that the bioluminescence increases with the concentration of the target analyte contrary to inhibitory tests where the signal decays with the dose of toxic species. The application of microbial biosensors for the detection of toxic species is examined in Chap. 4.

The use of living cells instead of isolated enzymes complicates the interpretation of the signal and frequently shows moderate or low substrate selectivity, especially for oxidizable compounds like ethanol, glucose, organic acids, etc. 'Long' response affected by changes in the population of the cells on the biosensor transducer is another weak point of microbial sensors.

To some extent, the measurement time can be reduced by approaching the membrane-bonded enzymes, either in the whole cells or isolated together with a membrane piece. The influence of other enzymes, if necessary, can be suppressed by thermal treatment of the cell dispersion because membrane-bonded enzymes are more stable for such a procedure. However, the use of thermally killed cells deprives the biosensor of the advantages mentioned above, i.e., increased stability, repair mechanisms, etc. The immobilization of living cells and limitation of the transfer of auxiliary reagents (second substrates, cofactors) is another way to exclude the contribution of undesirable reaction of the target substrate.

2.6 Immobilization of Biochemical Elements of Biosensors

2.6.1 General Requirements for Immobilization Techniques

As was mentioned above, biosensor assembly involves a biochemical element closely attached to the surface of a signal transducer. The *immobilization* procedure involves the process of making the biochemical component insoluble and fixing it on an appropriate interface (solid support or transducer surface). Immobilization is a crucial stage of the biosensor development and it is required for the following reasons:

- Multiple uses of rather expensive biochemical reagents. This is particularly true for enzymes that can be repeatedly applied for the substrate determination without any intermediate regeneration. For DNA and immunosensors, the applicability of multiple application is not so obvious because of the problems related to additional treatment of the biosensors and changes in their characteristics;
- Stabilization of the biochemical components. At present, this purpose of immobilization seems even more important than multiple signal measurement. In many cases, immobilization improves the stability of the proteins and DNAs toward oxidants, organic solvents and digesting enzymes. This can be due to limited access of appropriate chemicals to the immobilized biomolecules in comparison with homogeneous conditions. The immobilization makes the three-dimensional structure more rigid as well, so that the enzymes or antibodies retain their biochemical function even when partially damaged;
- Involvement of the biomolecules in the assembly of a biosensor to meet specific requirements to signal transduction. In many cases, the immobilization is accompanied by changes in the native structure of a biomolecule. The product might be unstable in the solution but has some advantages over the parent molecule on the transducer surface. The Fab fragments mentioned in the Sect. 2.2 are an example of such an approach. They are produced by treating the antibodies with papain and stabilized by immobilization on the solid support, preferably by Au–S bonds. The product of immobilization is active in the binding of the Ag molecule; it is more stable and shows less cross-reactivity to the structures relative to an analyte in comparison with the initial Ab molecule. In other cases, the immobilization can be performed together with label implementation, partial opening of the protein globule, removal of the saccharide residues from the surface layer, etc. All of the operations are directed to reaching better conditions for signal transduction, i.e., accelerating the enzyme exchange between the cofactors of the enzyme and the electrode.

It should be mentioned that all the advantages of immobilization are not compulsory and no one could rely on the immediate success of this procedure without any special efforts in investigating the conditions and testing the products

of immobilization with primary attention to the signal transduction system to be applied for biosensor development. Generally, the following parameters of investigation are of primary importance:

The support (carrier) of the biomolecules. The immobilization assumes the use of polymeric or viscous materials that provide a mechanical support for the proteins or DNAs implemented in the biosensor assembly. At the beginning of the biosensor history, while the enzymes were mainly used, the supports provided the replacement of a biochemical component after its inactivation. This made the biosensor less expensive because the price of many transducers was rather high. Plastic membranes produced for separation or filtering of biochemicals were adapted for enzyme immobilization. The first supports were based on common materials such as cellulose acetate or nitrate. To some extent, this remains true at the moment, although the arguments referred to the replacement of biorecognition elements become less significant. It seems more valuable that the use of replaceable membranes diminishes the probability of the technical mistakes that are rather common in conventional protocols of enzyme immobilization. On the other hand, some biomedical enzyme sensors are so cheap that their being disposable is preferable to the substitution of any elements.

From the parameters of the carriers, the permeability toward low-molecular analytes and durability are of primary importance for the biosensor performance. As in many other aspects of biosensor development, the final choice of the materials for immobilization is a compromise between many features that depends on the area of biosensor application, requirements of analytical and operational characteristics and preferences of a researcher. Thus, cellulose nitrate is represented by a variety of commercial membranes widely used in membrane technologies, such as protein plotting and even water desalination. Meanwhile, the use of a cellulose nitrate for enzyme immobilization is limited by its rather low elasticity and fragility. This complicates fixing the membrane onto the transducer surface, especially if its geometry differs from that of a flat plane. Gelatin and other hydrophilic supports (agar, chitosan, and [to some extent] chitin, and, lately, clay and zirconium phosphate) exhibit remarkable stabilizing effects and compatibility with almost all transducers and measurement protocols. The enzymes implemented in a gelatin matrix can be stored for several years without any loss of activity. Meanwhile, they swell in aqueous solution and change the signal within several hours during the first measurements. The material of support indirectly affects many other characteristics of biosensors, e.g., the interference of detergents, the pH dependence of the response, the reproducibility of main characteristics in mass production, etc. In comparison with the first (mainly "enzymatic") period of biosensor development, the focus is shifted to the materials obtained "in situ" with the immobilization of a biochemical component onto the surface by a self-assembling procedure. These materials are much more compatible with miniaturization and mass production requirements than the conventional membranes based on commercially available membranes.

The content of membrane components. To some extent, this group of optimization criteria follows from the choice of the carrier. Most often, the amount of

the biochemical component is specified. For enzymes, its quantity is expressed by the specific activity that takes into account not only the mass of enzyme itself, but also the substrate access conditions and the efficiency of immobilization. For immunoreagents, the titer can be used for the same surface. It is denoted as a dilution of the Ab solution resulting in a pre-determined shift of the parameter indicating the formation of the Ag–Ab complex. Derived from spectrophotometric measurements, the titer is mainly related to the shift of the optic density of the solution by one unit. Both titer and enzyme activity do not refer to the absolute amounts of biochemicals but rather to their biochemical function (substrate conversion and Ag binding). Their use dates to times when the determination of real concentrations of biochemical components was rather difficult and not obligatory. In simple cases, the effect of the content of the membrane is expressed by the graphs or equations describing the signal of the biosensor as a function of any parameter related to the biocomponent quantity. Thus, it might be a volume of the aliquot of DNA/enzyme solution added to the polymer for membrane preparation or the mass of a membrane per surface unit. The results of such optimization are hardly extended to other conditions because they do not allow estimating the real quantities of biochemicals used for immobilization.

From a worldly point of view, the more enzyme/DNA in the surface layer, the better for the biosensor signal. This is not true ‘the more so that’ for all the biosensors the maximal signal corresponds to a definite range of the biochemicals in the surface layer. This is due to various reasons, and the balance between the rate of biochemical reaction and mass transfer is of great importance. Thus, a lower enzyme quantity can give a higher signal because it provides a thinner membrane with a faster substrate access in comparison with bigger enzyme loading. A too- dense placing of DNA probes limits the number of bulky molecules attached to the binding sites on the surface unit, etc.

Other reasons to limit the quota of biocomponents in the surface layer involve the worsening of immobilization efficiency, increasing the price of the final device, and shortening the lifetime of a biosensor.

Of course, other components of the surface layer also affect the performance of a biosensor but their contribution seems less important than the amount of the biocomponent. When the surface layer is formed directly on the transducer, the ratio of binding reagents and matrix is of interest. Thus, the reactions of polymerization and polycondensation yield products varied in porosity, permeability, charge and even thickness depending on the monomer ratio and the amount and nature of polymerization initiators. Sol–gel technologies and molecular imprinting are mostly mentioned regarding this aspect of immobilization.

Immobilization efficiency. The criteria of the final success are mainly determined by the requirements to a particular biosensor. Some of them should retain their characteristics during a rather long period of storage but can be quite moderate in the accuracy of signal measurement (biomedical enzyme sensors for substrate detection). Other biosensors are expected to show a remarkable stability of the response (flow-through biosensors, enzyme sensors for inhibitor determination)

with no specific requirements towards sensitivity. These and other similar parts predetermine the strategy of immobilization and the estimation of its results.

The stability of a signal during the longest period of its use is certainly encouraged for all the biosensors. Together with the minimal amount of biocomponents necessary to reach the desired signal, that is an advantage attained. As in many other attempts to accomplish a goal, the price of the result is of greatest importance. In many cases it is probably simpler to limit the stability of the response in order to obtain reliable immobilization protocol or to reach a higher sensitivity of analyte detection at the expense of the biosensor's lifetime. Incidentally, the sensitivity of the inhibitor determination is frequently higher for the lower long-term stability of the response toward the substrate. For DNA sensors and immunosensors, the long-term stability of the response is less important than the reproducibility of the signal, because such biosensors are often intended for a single use. Nevertheless, some general estimations of immobilization protocol can be given.

For enzyme sensors, the *remaining enzyme activity* can be used. This is the relative decay of enzyme activity caused by immobilization (80, 20 %, etc.). The decrease of enzyme activity during immobilization is due to the inactivation of the enzyme by organic solvents or other reagents necessary for immobilization, or due to a limited access of the substrate caused by the shielding effect of the carrier, or a disturbance in the native protein structure. The remaining enzyme activity does not distinguish between these mechanisms so that the real decay of activity can be lower than the shift of the response after immobilization. The relative enzyme activity was also suggested for the quantification of an enzyme inhibition. At present, the absolute signal of an enzyme biosensor and its stability are preferable instead of the remaining enzyme activity in the characterization of the immobilization efficiency.

For immunosensors and DNA sensors, the consideration of immobilization is much milder than that for enzyme sensors. Many of them utilize the approaches already elaborated for solid-phase bioassay (ELISA- and DNA-based hybridization detection). For this reason, the transfer to the biosensor format does not imply any significant changes in the immobilization techniques. The only exception is the use of microdevices like FETs, which have no macroscale analogs. In this case, as well as for other biosensors developed *ab ovo*, the stability of the response and the sensitivity of the analyte detection are often used to characterize the biomolecule attachment. The latter parameter is quantified either by the slope of the calibration curve or the concentration resulting in a 50 % shift of the signal against its maximal value.

The immobilization of living cells (microorganisms, microalgae, etc.) is mainly based on their adsorption on the solid support and implementation in the gels. The use of polysaccharides, e.g., Sepharose, agarose or cellulose nitrate, provides very strong adhesion of the cells that can usually be removed from the support only with simultaneous deformations of the tectorial membrane. The use of gelating materials is preferable because of the higher surface concentration of the cells and milder conditions for transfer of the oxygen and metabolites to the cells. Gelatin, agar, Ca alginate gel, and polyacrylamide copolymers are described for this purpose. It should be mentioned that the adsorption of living and death cells can differ dramatically even though the cells inactivated by thermal treatment exert

Table 2.7 Characteristics of the biocomponent immobilization methods for biosensor development

Type	Immobilization method	Advantages	Drawbacks
Physical	Physical adsorption	Simplicity, accessibility of the binding sites, low cost	Reversibility, limited operation period, dependence of immobilization efficiency on the support pre-treatment
	Sol–gel immobilization	One-step synthesis, hydrophilicity of the matrix, mild limitations of the analyte access	Moderate stability toward hydrolysis, rather high viscosity of the matrix, swelling in aqueous solution and compression of the films in dry conditions
	Entrapment into polymeric films	Improved storage and operation stability, one-stage deposition, variety of the matrices used	Limited access especially of bulky analytes, necessity in special reagents for polymerization, rather long response time
	Electropolymerization	One-step synthesis, controlled thickness and mass of the coated film	Partial denaturation of biopolymers, reversed leaching of the biocomponents, dependence of the operation stability on the ionic strength and pH
	Layer-by-layer composites	Easy control of thickness and charge, self-organization of selected layers, compatibility with LB-film technology and SAMs, preservation of the native structure of biocomponents	Reversibility, limited mechanical durability, dependence of the characteristics on the ionic strength of the solutions, temperature and biopolymer structure, necessity in monitoring layers integrity
	Affine	High efficiency and specificity of the DNA/protein binding (site-specific immobilization), mimicking natural processes of biopolymer functioning	Requirements of additional modification of biopolymers and/or specific reagents used for signal transduction
Chemical	Covalent binding	High efficiency and prolonged storage stability, compatibility with the protocols elaborated for protein chemistry, stability toward oxidative cleavage and hydrolysis, fixing biopolymer structure onto the solid template	Necessity in toxic reagents, low conformational lability of the biopolymers attached to the solid support, possible distortion of their native three-dimensional structure

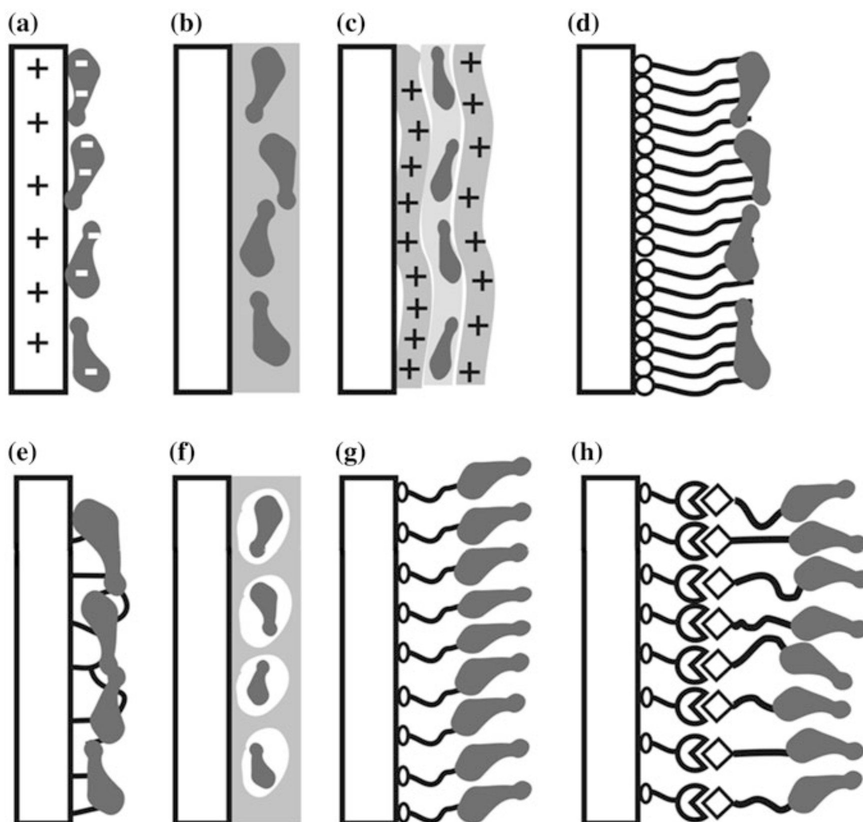


Fig. 2.19 Schematic presentation of immobilization protocols used for biosensor development. **a** Electrostatic adsorption. **b** Implementation in polymeric film. **c** Layer-by-layer immobilization in the polyelectrolyte complexes. **d** Non-covalent immobilization on the self-assembled monolayer. **e** Cross-linking by bi-functional reagents. **f** Immobilization in reversed micelles. **g** Covalent binding to the solid support (sensor surface); **h** Affinity immobilization

enzymatic activity for a certain period of time. This can be used for distinguishing living and dead cells in cytotoxicity biosensors. The immobilization of microorganisms is often followed by cell division so that the number and biomass of microorganisms changes within the period of the biosensor operation. The biomass changes can be controlled by the content of cultural medium, e.g., by decreasing the concentration of biogenic elements. However, such an approach affects the response of the microbial biosensor toward target analytes (commonly oxidized organic compounds).

All the immobilization protocols can be classified in accordance with the forces retaining the biomolecules on the surface and the way to the biorecogniton layer formation. The general principles of immobilization are presented in Table 2.7 and Fig. 2.19.

2.6.2 Physical Immobilization

Physical immobilization does not assume the formation of the covalent bonds between the biopolymer and the carrier (membrane material, mechanical support or transducer surface).

In the simplest case (immobilization via physical adsorption), the protein or DNA molecules are held on the solid surface due to multi-point interactions, preferably electrostatic (Pividori and Alegret 2005). The adsorption can affect the three-dimensional structure of the biopolymer, but normally such changes are negligible because the number of bonds providing the three-dimensional structure of a protein or DNA exceeds that of sites attached to the solid surface.

Protein globules are adsorbed as they are; the linear molecules of DNA probes are positioned along the surface plane. However, increasing density of the surface filling can result in a partial desorption of the molecule domains with formation of aggregates and even of multilayer coatings. In this case, the DNA oligonucleotides are differentiated by their strength of binding to the carrier, their spatial structure and hence the affinity toward a biotarget, e.g., complementary ss-DNA sequences or proteins.

The use of porous materials with pores that meet the size of biopolymers simplifies the adsorption and makes desorption less probable. The surface of the backsides of the pores or channels has the charge distribution, roughness and some other features that dramatically differ from those on the surface. Besides, the number of the bonds established by a single protein or DNA molecule with a carrier is much higher in pores than on the surface plane. This promotes the implementation of the bulky biopolymers even if they are weakly bonded to the surface of the carrier. Meanwhile, the implementation of the biopolymers in the internal volume of porous materials complicates their interaction with the analyte molecules. For this reason, these techniques are preferably used for the detection of small molecules (enzyme substrates and inhibitors, DNA intercalators, etc.). Even in this case, the regeneration of the sensor signal requires more effort than that of the same biocomponents placed on flat surfaces. The use of polar materials, e.g., carbon black, metal oxides, anodized alumina layers, etc., promotes the physical adsorption. It can also be amplified by the pre-treatment of the support to increase its charge or the number of polar functional groups by anodic oxidation of the electrode materials in the electrochemical sensors. Thus, the oxdative treatment of carbon materials results in the formation of hydroxyl, carbonyl and carboxylic groups on the surface. Electrochemical or chemical oxidation increases the internal volume of porous graphite and decreases the average size of carbon nanotubes. In most cases, this stabilizes the characteristics of biosensors and increases their signal due to the higher surface concentration of the biocomponents.

Self-regulation is one of the advantages of physical adsorption. The quantities of biopolymers attached to the surface are mainly determined by the real surface area and the number and distribution of the charged fragment in the binding. This means that the transducers treated in a similar manner adsorb approximately the

same amount of biopolymers regardless of their concentration in solution. This is especially convenient if the real concentration of the biocomponent is unknown and estimated by indirect parameters, such as the enzyme activity of the biological extracts. On the other hand, the same advantage offers very high requirements for the reproducibility of the surface used for physical adsorption. Any mechanical polishing, etching and treatment with a reactive species affects the roughness, pore distribution and polarity of the surface. This can alter the characteristics of physical adsorption, especially if no full coverage of the surface is reached.

The reversibility of physical adsorption is another drawback of this immobilization approach. Being placed in a solution with no biocomponent, the biosensor loses some part of the adsorbed components leaching from the surface layer by shifting the equilibrium of adsorption–desorption. This is a thermodynamically favorable process that cannot be stopped but can be slowed down. The higher the strength of the biopolymer adsorbed to the surface, the lower the desorption rate. In many cases, multi-point attachment, and especially immobilization in pores, makes desorption so slow—as compared with the biosensor lifetime—that it can be neglected. However, sometimes the rate of leaching suddenly increases. Thus, the increase in the ionic strength of the solution by sample injection can provoke the desorption of the proteins retained in the surface layer by electrostatic interactions. The same refers to any processes diminishing the charge of the carriers. For the groups participating in acid–base equilibria, the desorption can be initiated by a sharp pH shift. This was observed, for example, in hydrolases. The reaction of a substrate hydrolysis results in the formation of acids appearing in the microenvironment of the immobilized enzyme. Even though the absolute amount of the reaction product is usually small, the reaction with the carboxylate fragment of the carrier can sharply decrease the pH value in the close neighborhood of the enzyme and hence decrease the electrostatic forces between the enzyme and carrier.

Paradoxically, the physical adsorption of biopolymers calls for a specific treatment of the surface to increase its roughness and charge, whereas the reproducibility of the biosensor performance is better on minimally treated materials that have a very smooth surface established by some specific features of a crystallographic plane (grapheme, highly ordered pyrographite, doped diamond). For DNA sensors, there was an attempt to avoid this limitation by the application of polarizing voltage. Screen-printed electrodes made of a carbon paste have rather modest adsorptional capacity against other relative carbon materials but are well reproduced on a smooth working surface. The electrodes were immersed in the DNA solution and polarized at about 0.8–1.0 V to accumulate negatively charged DNA molecules on the working surface (Mascini et al. 2001). After that, the electrodes were moved to the analyte solution and the signal was measured. Such an approach was justified for the detection of hybridization, DNA damage, and even intercalation by small antitumor drug molecules. This makes the immobilization protocol very simple and reliable, especially taking into account the low cost of the transducers sufficient for their single use.

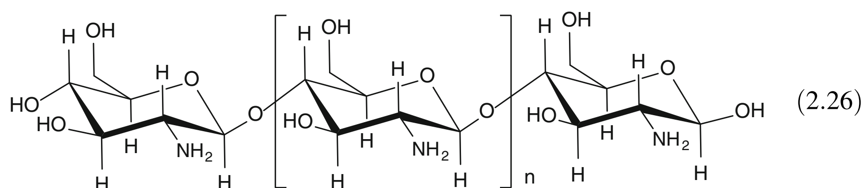
Two drawbacks limit the application of the approach described. First, the formation of the biorecognition layer is performed by the user, not the manufacturer.

This complicates the application of the biosensors, especially in field conditions. Second, any interruption of the circuit promotes desorption of a part of biopolymers. This also refers to the stage of transfer of the DNA sensor from the accumulating solution to the sample that has to be performed with the cell switched on. This can be performed only with several kinds of equipment without any consequences for the quality of the signal recorded.

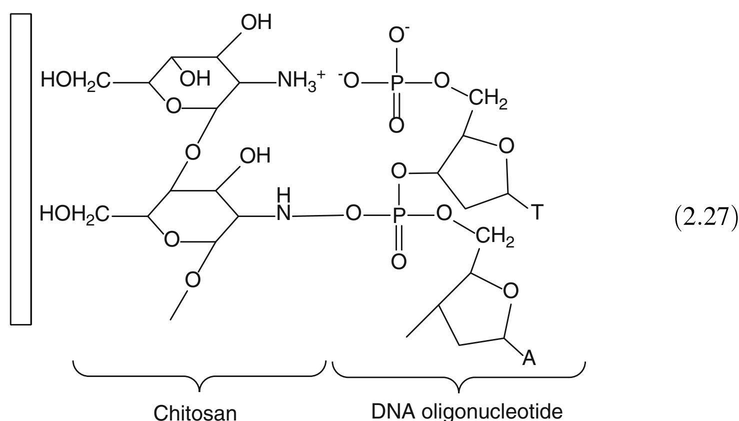
To avoid complications resulting from insufficient stability of the biorecognition layer obtained by physical adsorption, the biosensors can be covered with additional permeable membranes that mechanically prevent the leaching of the biomolecules from the surface area. The membranes are produced from polymers like cellulose nitrate and polycarbonate. Many of them are intended for electrophoresis and related laboratory techniques. The protective membranes can be also formed on the layer of adsorbed biomolecules by other methods described below (sol–gel technology, electropolymerization, chemical polymerization, and deposition from the organic solvent of the appropriate polymer). The protective membranes are widely used for commercial enzyme sensors where they offer some additional opportunities, e.g., selection of the substrates from interferences, mechanical protection, especially in flow, prevention of biofouling for invasive biosensors. The additional membranes should not decelerate the transfer of the analyte molecules and are applied mainly for enzyme sensors.

In some cases, the direct interaction of the carrier with biomolecules becomes irreversible and results in the formation of the chemical product of interaction. This process, called *chemosorption*, can be observed, for example, for proteins and thiolated oligonucleotides deposited on the gold nanoparticles or golden electrodes. The reaction of the Au with SH groups in the presence of dissolved oxygen is spontaneous and fast enough to strongly attach the biopolymer to the carrier. Previously, this way of immobilization was used only for the compact Au films obtained by electroplating (electrochemical transducers and optical devices based on transparent Au films). The application of nanoparticles obtained by the chemical reduction of $AuCl_4^-$ anions extended the application of chemosorption to other carriers such as carbon materials or even cellulose derivatives. First they are modified with Au nanoparticles by consecutive treatment with aurochloric acid and a reducer (citric or ascorbic acid) and then with biopolymers attached to the Au nanoparticles adsorbed on the carrier. The latter provides the stabilization of the Au nanoparticles as well and prevents their growth and amalgamation during deposition.

The difference between chemical and physical sorption in other cases is mild. Sometimes the rate of desorption is so slow that it cannot be observed in the time period comparable with the signal measurement duration. In other cases, the mechanism of adsorption can vary, depending on the particular conditions. Thus, chitosan (2.26) can electrostatically attract charged biomolecules such as DNA via protonated amino groups. Besides, the formation of esteric bonds has been discussed for the same molecules (2.27) (Mandong et al. 2007).



(2.26)



(2.27)

The *entrapment of the biomolecules into the polymeric film* is another way of physical immobilization, very close to that described above for adsorption followed by deposition of the upper polymeric film (Shtilman 1993).

The main difference is that the entrapment protocol assumes a uniform distribution of the biomolecules in the polymer. It was first suggested for the stabilization of enzyme activity. For this purpose, the enzyme-containing samples (extracts, tissue homogenates) are mixed with starch, gelatin or agar and left to gel. The product can be molded as a thin film by casting the viscous liquid on glass or placing it in the pores of an appropriate sorbent for better durability and mechanical stability. As mentioned, hydrophilic polymers swell in aqueous solutions and form rather thick membranes with extended response due to the slow substrate transfer. In dry conditions, they are fragile and thin but restore their size and shape after the contact with water. Similar materials based on polyacrylic acid, their copolymers and ethers are used in gel electrophoresis and related techniques developed for protein and DNA purification.

The mixing of biocomponents with a polymeric matrix is a critical stage of the immobilization. Except for materials that are able to gel, this can be achieved by the application of polar organic solvents. The polymers are first dissolved in them and then mixed with an aqueous solution of protein or DNA. If the solvent is continually mixed with water, this can result in the formation of a homogeneous system that becomes dense and rigid while the solvent and water are evaporated. If the polymeric arrangement of the protein is too dense and this prevents the contact

with a low-molecular reagent (enzyme substrate, inhibitor, etc.), additives exerting a pore-forming effect can be used.

The low compatibility of the biopolymers with polar organic solvents is a main drawback to the immobilization into polymer films. Although many enzymes are still active in non-polar solvents (Campanella et al. 2001; Zaks and Klibanov 1985), the use of the solvents mixing with water irreversibly decreases the activity of enzymes and the affinity of antibodies toward target molecules. The organic solvents "extract" water molecules from the inner area of the protein and disturb the three-dimensional structure of the protein globule. On the first stages, the decay of activity is reversible and the immobilization efficiency is increased by shortening the contact period of an enzyme with organic solvents. However, even in this case, the variation of the enzyme activity can be observed for immobilized preparation for a rather long time. For DNA molecules, the compatibility with organic solvents is higher than that with enzymes. Thus, DNA is purified by recrystallization from phenol. Besides, the detection of analytes with the DNA sensors assumes much stronger requirements toward the accessibility of the binding sites so that the entrapment of DNA probes in a polymeric carrier is not currently used for the DNA sensors' design.

Careful optimization of the immobilization conditions makes it possible to avoid the limitations related to enzyme inactivation caused by polar organic solvents. Thus, for some oxidoreductases it was observed that the use of high concentrations of solvents decreases the enzymatic activity to a much lower extent than the more diluted aqueous solutions (Sekretaryova et al. 2012). Moreover, acetonitrile even increased the activity of lactate oxidase, peroxidase, glucose oxidase and acetylcholinesterase, at least within the short period of time required for enzyme immobilization. The phenomenon observed extended the number of materials used for enzyme immobilization by Nafion, i.e., an ion exchangeable polymer soluble only in organic solvents (Dimcheva and Horozova 2005).

To some extent, the negative influence of organic solvents can be lessened by the application of protecting compounds. When dissolved in water, they decrease the inactivation of proteins by protecting the globule from water-leaching, or by the substitution of water molecules in hydrogen bonds, stabilizing the protein conformation. Hydrophilic water-soluble polymers, polyols (polysaccharides, sorbitol, glycerin), and ionogenic detergents can be applied as protectors. The fact that crude enzyme preparations and minced biological tissues are less sensitive to organic solvents than purified enzyme preparations is due to the protecting influence of native components. Although the concentration of the additives is small enough (several percent of the matrix polymer), they affect both the enzyme activity and its sensitivity toward inhibitors.

The low solubility of the polymers in water can result in the formation of microemulsions when mixed with aqueous polymer solution. As a result, the enzyme remains in microdrops preferably with an aqueous content which is distributed in a hydrophobic polymer. The structure of the final product of immobilization is similar to that realized in reversed (oil–water) emulsions applied in the synthesis of nanosized polymer particles or extraction intensification. If the relative content of

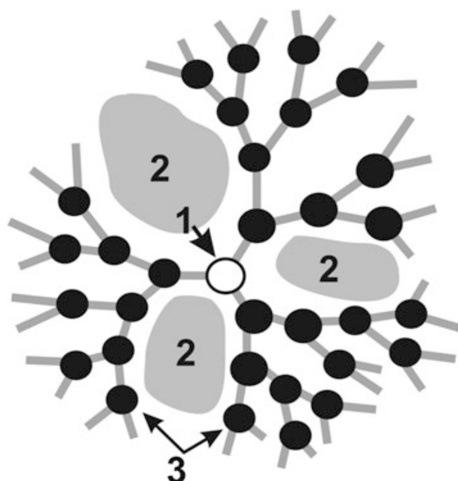


Fig. 2.20 Dendrimer molecule outline. *1* Core; *2* internal cavities; *3* branching points

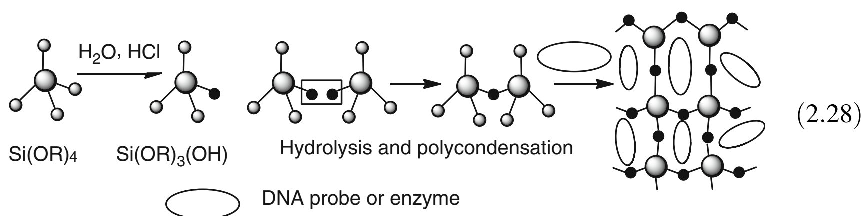
an enzyme-containing phase is high, the microdrops can exchange with the bulk solution by low-molecular compounds or ions. This provides both the functioning of an enzyme and the biosensor signal measurement. The low mechanical durability of the composite membranes and complications related to the formation of reproducible polymeric layers limit the use of such techniques in biosensor development.

The effect close to emulsification can be achieved by the application of *dendrimers*, hyperbranched polymers involving a core with repeated branching elements (Fig. 2.20) (Klajnert and Bryszewska 2001).

The dendrimer molecules are large enough to serve as "nanodrops," or molecular nanocontainers that keep the biomolecules in internal cavities. Besides, the electrostatic interaction with surface-charged groups and covalent attachments to them are realized. Polyamidoamine (PAMAM) dendrimers are mostly used (Satija et al. 2011). The generation of a dendrimer is determined as a number of "shells" (branching points) placed on increasing distance from the core. The molecules of the fourth generation provide the size and binding ability sufficient for interaction with most biopolymers. The formation of the dendrimer–protein (DNA) complexes is mostly spontaneous and can be controlled by varying the terminal functional groups of a dendrimer molecule (its generation) and reaction conditions. Like common polymers, dendrimer swells, but to a much lesser extent.

The entrapment of the biomolecules into the polymeric films has several variations more adapted to the biosensor design than the mechanical mixing of the component described.

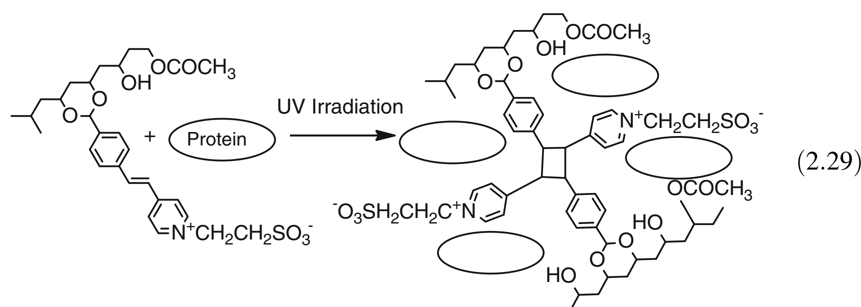
Sol–gel immobilization is based on the polycondensation performed directly on the surface of a sensor transducer in the presence of the biopolymer (Kandimalla et al. 2008). Organosilicates and siloxanes are mainly used for this purpose. The reaction is initiated with a minimal volume of water or aqueous solution of a strong acid added to the immobilization mixture (Reaction 2.28).



(2.28)

In a similar manner, the product of polycondensation can entrap small hydrophobic molecules required for signal transduction (mediators, fluorogenic or chromogenic agents, etc.). Leaching is prevented by their low solubility in water. The reaction rate and the characteristics of the product (viscosity, swelling, permeability for charge carriers, durability) depend on the concentration of the reactants, organic solvent and temperature. Organosilicate layers are quite compatible with glass and quartz, and for this reason are used with optical and piezometric detectors. For some materials, the formation of solid products is initiated by freezing the mixture (*cryogels*). Fully dried products (*xerogels*) can be mechanically dispensed and used in combination with any other polymer (Doretti et al. 1997). The organosilicate shell prevents the biopolymers from the negative influence of the other reagents mentioned. Similarly, inorganic polymers of hydrated metal oxides and zirconia phosphate can be obtained and applied in the DNA sensors' assembly.

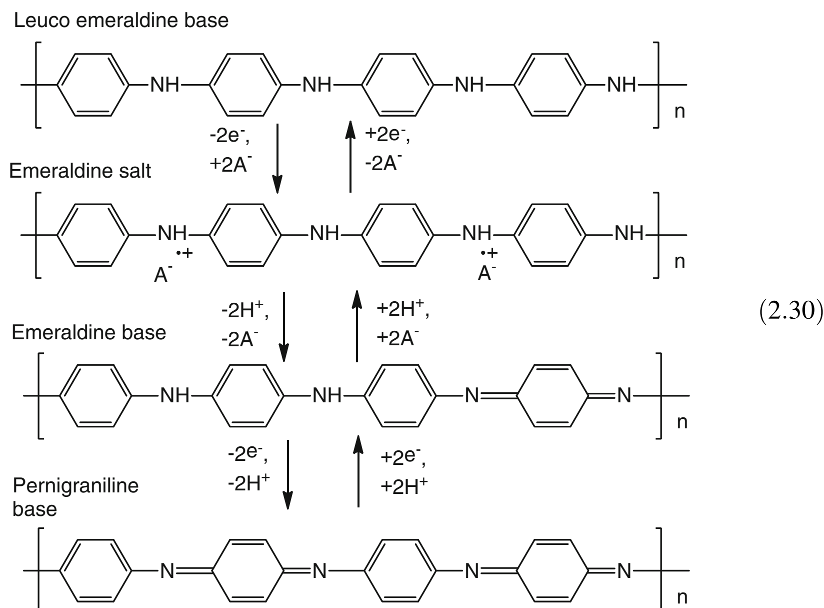
A similar effect is achieved by the application of photopolymerization (2.29). Contrary to sol–gel techniques, photopolymerization can be run directly in an aqueous solution of appropriate monomers in the presence of biopolymers under UV irradiation. Neither polycondensation nor photopolymerization involve the covalent binding of the biochemical components and hence preserve the native structure most favorable for the biorecognition of target analytes (Andreescu et al. 2002).



(2.29)

Electropolymerization is a process of the formation of insoluble oligomeric and polymeric products that deposit from the solution during the oxidation of monomers. If the electrolysis is performed in the presence of biopolymers, they are

entrapped in the growing polymer film and adsorb on the surface of the commonly positively charged final product. The advantages of the approach are the one-step protocol and simple regulation of the polymerization by choice of the potential and current. Some of the polymers show the electroconductivity comparable with that of semiconductors and even metals. This makes possible the electric wiring of the active sites of oxidoreductases and simplifies the signal transduction for other biopolymers. As an example, the synthesis of polyaniline provides the formation of several redox active forms of polyaniline, of which emeraldine salt exerts a rather high electroconductivity (Eq. 2.30) (Prakash 2002).



(2.30)

The electropolymerization of aniline requires strong mineral acids that inactivate enzymes. For this reason, the residual activity of immobilized enzymes does not exceed 2–3 %. In addition to polyaniline, polypyrrole and polythiophene also demonstrate electroconductivity but their polymerization is preferably conducted in polar organic solvents because of the low solubility of intermediates and initial species. Some other products of electropolymerization, such as polyphenazines and polyphenols, can serve as mediators of electron transfer.

The deposition of *polyelectrolyte complexes* was first suggested for the immobilization of bacterial cells on glass and then extended to other materials and biochemicals (Evtugyn and Hianik 2011).The immobilization is performed on the charged support by the consecutive addition of oppositely charged polyelectrolytes and removal of their non-bonded amounts by washing (Fig. 2.21). The protocol also called "Layer-by-Layer" (LbL) immobilization is especially suitable for the

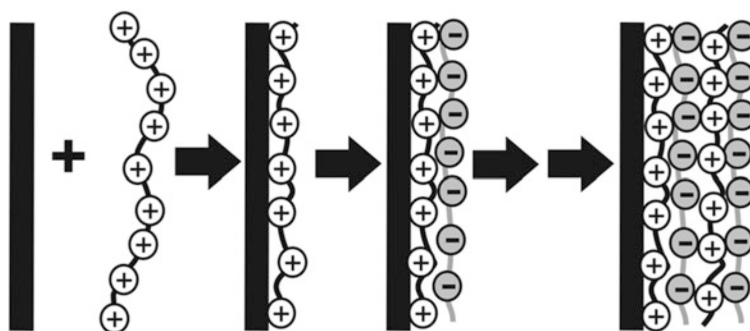


Fig. 2.21 Schematic representation of LbL immobilization

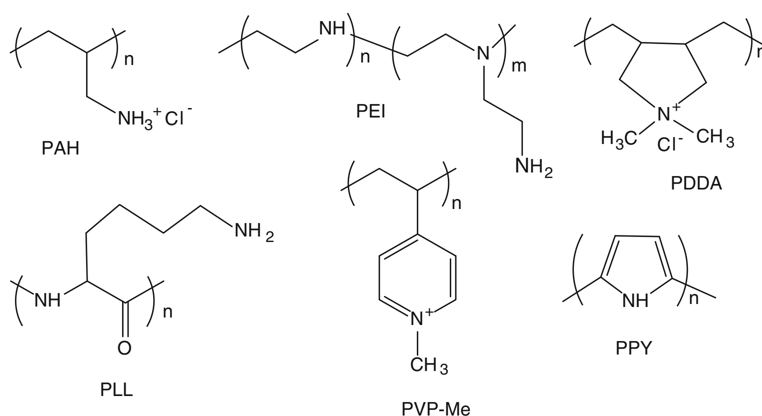


Fig. 2.22 Components of polyelectrolyte complexes used for biocomponent immobilization in biosensor design. *PAH* poly(allylamine hydrochloride), *PEI* poly(ethylene imine), *PDDA* poly(dimethyldiallylammonium chloride), *PLL* polylysine, *PVP-Me* partially methylated polyvinylpyridine, *PPY* polypyrrole

DNA oligonucleotides with a high density of charged phosphate groups of the backbone.

Synthetic polyelectrolytes typically implemented in the polyelectrolyte complexes are presented in Fig. 2.22.

Some of them become charged after protonation. Polypyrrole and some other products of electropolymerization change their charge during reversible oxidation on the electrode. Thus, assembling the complexes with such components can be controlled by external stimuli, i.e., pH changes or electrode polarization. The LbL immobilization can include positively charged chitosans (see Eq. 2.27) and negatively charged pre-oxidized carbon nanotubes as well.

The polyelectrolyte complexes have a definite number of layers easily regulated by the number of reagent additions. Weak, non-covalent interactions retain the

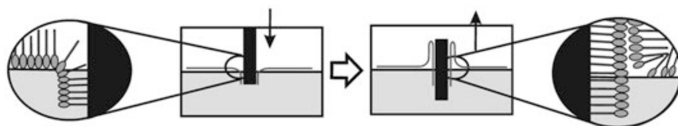


Fig. 2.23 The formation of a self-assembled monolayer and Langmuir–Blodgett film from the aqueous solution of amphiphilic compounds

access of small molecules that can move apart the ionic components while attaching the binding site of the biomolecule. This offers a fast and reproducible response toward specific analytes, e.g., intercalators or reactive oxygen species oxidizing the DNA molecule, or enzyme substrates and inhibitors (Decher and Schlenoff 2002).

The variation of polyelectrolytes manipulates the hydrophobic–hydrophilic balance on the inner interfaces of the composite layer and hence allows tuning the interactions of the DNA with various substances.

To some extent, other multilayered constructions are related to LbL composites. Thus, Langmuir–Blodgett (LB) films are formed by amphiphilic molecules consisting of a polar head bearing a long hydrophobic tail. They are self-assembled on the water–air interface to reduce the area of the contact of hydrophobic parts with a liquid phase. Then LB films can be gently collected from the surface and transferred to the solid support (Fig. 2.23) (Girard-Egrot and Blum 2007).

For this purpose, the spontaneous transfer of the monolayer from the water–air interface to the hydrophobic material is used. A thin plate is immersed in the solution and then vertically moved to pick up the film. Changes in the movement direction allow amplifying the number of layers deposited LbL on the solid support.

The DNA or protein molecules can be implemented in between the amphiphilic monolayers or positioned on the front of the LB-film if it contains charged groups. The low mechanical durability complicates the use of LbL constructions and LB-films in the biosensors, especially those intended for field applications. The defects in the structure of the mechanical support and the films themselves (cracks, ruptures, layer crossings) appearing during transportation or operation stages are self-repaired by hydrophobic forces. The use of supports capable of chemosorption or affinity interactions or implementation of the films in the pores of solid films improve the mechanical characteristics and stability of such films.

Self-assembled monolayers (*SAMs*) are a particular case of such constructions. They are formed by thiolated amphiphilic compounds that interact with Au as described above and form regular monolayers due to hydrophobic interactions between the long chains positioned in a way to minimize the contact with the aqueous environment (Chaki and Vijayamohanan 2002). The structure of SAMs is similar to half of the LB-film in which the ends of molecules are tightly fixed onto the surface by chemical bonds. Single- and multi-walled carbon nanotubes provide a mechanical support for supramolecular multilayers as well. Their efficiency is achieved by oxidative treatment resulting in the formation of negatively charged

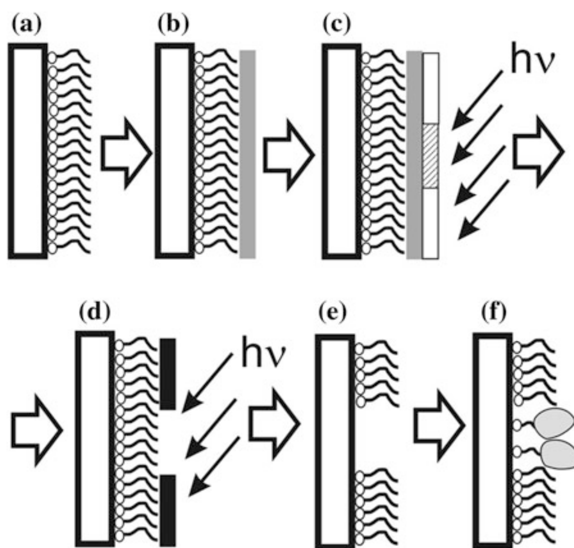


Fig. 2.24 Stepwise specific immobilization of biomolecules using SAM-based technology. **a** SAM formation; **b** photocurable polymer deposition; **c** mask deposition and UV irradiation; **d** removal of non-reacted components; **e** removal of SAM from the template sites (pore formation); **f** immobilization of thiolated biomolecule on naked Au spots

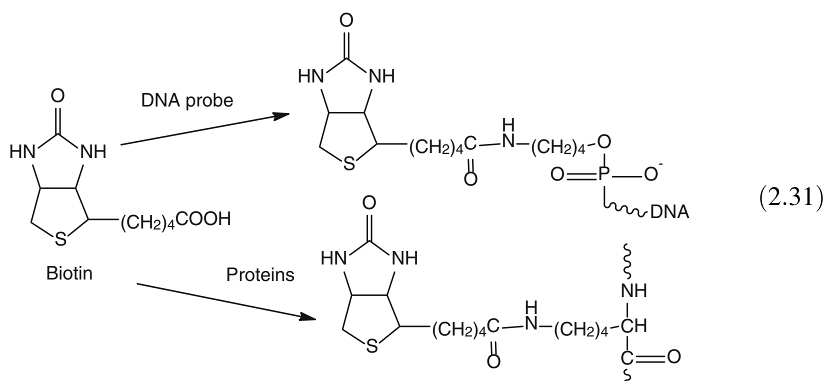
carboxylic groups at both ends of the tubes and on the defects of the side walls (Rahman and Umar 2009).

What is important for SAMs is that they provide a very accurate positioning of the biocomponents on the transducer surface and hence can be used for the preparation of miniaturized biosensors with dimensions comparable with those of microelectronic devices. Photolithography is applied for making the binding sites that are also called “spots.” The principal scheme of such a process is shown in Fig. 2.24.

First the SAM obtained on the Au surface is covered with a photocurable polymer. Then, the negative mask is plotted onto the transparent material and placed on the polymer layer. This is a drawing of the future biosensor surface with the spots for protein/DNA binding marked in black. The surface is irradiated from a UV source to harden the photocurable polymer. The area protected by the black sections of the mask remains soft and is removed together with the mask by an appropriate solvent; after that, the SAM is chemically removed from the future spots by oxidation with oxygen to open the bare gold surface. Then thiolated target molecules can be attached accurately into the spot areas. The size of several microns and the density of the thousands and thousands of spots per mm^2 can be obtained by such a process. Of other applications of the SAMs, the prevention of undesired adsorption of interferences on the transducer surface should be mentioned.

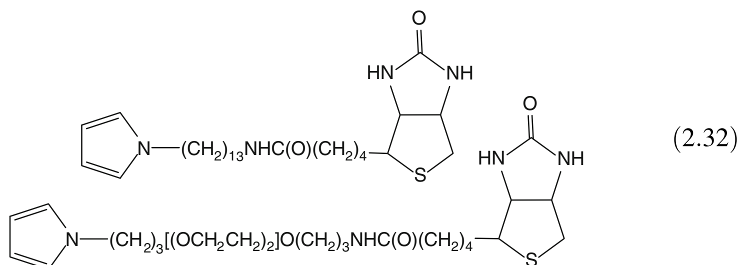
Affine immobilization is ranked as intermediate between physical and chemical immobilization protocols. The combination of biopolymers with a solid support is achieved by natural receptors providing a very specific and durable binding of the counterpart. Thus, biotin (Eq. 2.31) is able to specifically bind avidin or streptavidin with high efficiency (dissociation constant about 10^{-14}–10^{-15} M) (Dupont-Filliard et al. 2004). Due to the residue of valerian acid in the side chain, biotin can

be easily attached to the terminal phosphate groups of the DNA backbone or to the amino groups of the amino acid residues of the protein molecules, commonly via various spacers intended to provide a steric access for biological targets.



(2.31)

Both avidin and streptavidin bind up to four biotin molecules and hence serve as bridges to combine several biotinylated parts in the surface layer. Simple structure and modification protocols, as well as high stability, make it possible to introduce biotin into many commercially available biochemical molecules and supports. This simplifies the composition of the surface layers involving the DNA probes. Avidin or streptavidin can also be immobilized onto the solid support by other methods elaborated in protein chemistry, e.g., cross-linking with glutaraldehyde and implementation into the bovine serum albumin (BSA) matrix. Biotin can also be introduced into the electropolymerized layers by modification of the monomers used on this stage of modification. The examples of the molecules providing the formation of polypyrrole with covalently attached biotin are presented below (2.32) (Cosnier 1999).

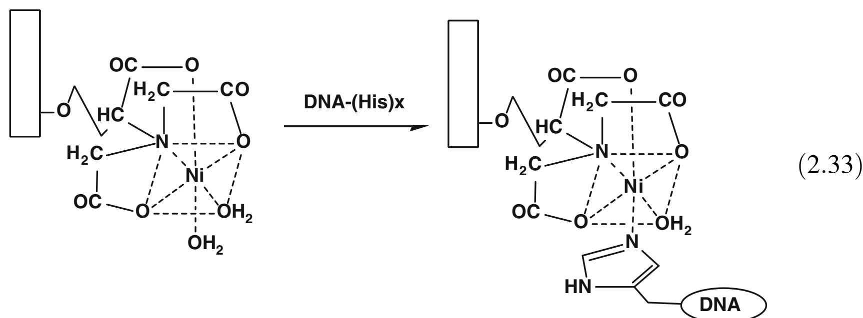


(2.32)

Long-chain linkers between the pyrrole moiety and biotin are required to give steric access of the target analyte to the binding site of a bioreceptor and retain the permeability of the biorecognition layer by charge carriers necessary for electrochemical signal transduction (Cosnier 2005).

The biotin–avidin binding is a very popular one but not the only way for the affine immobilization. Concanavalin A, a plant protein of the lectin family, can specifically bind carbohydrates; protein A binds immunoglobulines. Their application is mainly related to the immobilization of enzymes and antibodies.

The term "affine immobilization" is also used for artificial receptors like Ni or Cu chelate complexes. Thus they can be spontaneously formed by nitrilotriacetic acid derivatives and Ni(II) or Cu(II) ions in aqueous solution or on the solid interface. The complex coordinates histidine residues in the equatorial position of a complex plane (Eq. 2.33) (Baur et al. 2010).



(2.33)

The biomolecules are modified with histidine residues ("tags") at the terminal functional groups. Commonly, the 5–10 histidine fragments are attached to the DNA probe or protein molecule to reach the efficiency of the binding necessary for biosensor development. The silicate sorbent bearing nitrilotriacetic acid group is commercially available.

Affine immobilization is site-specific and hence exactly determines the structure of the final product. Single-point binding offers a maximal access of the biological targets to the active sites and a minimal influence on the conformation of the protein or DNA molecule.

Methods based on the immunoreagents also refer to the affine immobilization, but are called "immunoimmobilization" techniques (Jin et al. 2008). The chemistry of such processes does not dramatically differ from that considered above for immunoassay approaches. Immunoimmobilization is often used for attaching the bacteria cells to the solid supports followed by their investigation by optical or piezoelectrical methods. In a similar manner, aptamers covalently attached to the solid support can bind to receptors on the surface of the cell wall and hence bear microorganisms in the biorecognition layer of a biosensor. Peptide receptors are displayed on the bacteriophage surface. Although the latter case is not yet realized in biosensor assembly, it also shows the advantages of site-specific affine immobilization.

A very high efficiency of binding makes the affine immobilization similar to the covalent binding described below. Meanwhile, the reaction is reversed and the free DNA oligonucleotides or proteins can be removed from the support by a sharp pH change and/or electrolyte addition.

2.6.3 Covalent Immobilization

Covalent immobilization presumes the formation of covalent bonds between functional groups of a carrier and a biopolymer. Covalent bonds are much stronger toward any outer influence than electrostatic and donor–acceptor interactions and H-bonds, and provide maximal stability of the product. The covalent binding may affect the affinity of the bioreceptors due to the alteration of their spatial structure and steric DNA structure and the affinity toward target biomolecules, but in most cases such an influence is insignificant. To some extent, steric limitations can be avoided by the introduction of the spacers, i.e., long fragments between the support and biopolymer. The reagents used in covalent binding are called *bifunctional reagents* (Fig. 2.25).

Bifunctional reagents can bind protein molecules to each other (*cross-linking*). The product becomes insoluble and is precipitated on the sensor surface or plastic support.

Glutaraldehyde and carbodiimide binding is most often used (Dugas et al. 2004; Lucarelli et al. 2004). The appropriate reagents attack the amino and carboxylic groups, respectively. The mechanism of protein binding is considered in Sect. 2.1 [Eq. (2.2)]. Glutaraldehyde can chemically modify thiol, amino and hydroxyl groups of proteins and the same terminal groups of the DNA oligonucleotides. The reactivity of glutaraldehyde decreases in the above range and is complicated by the chemical instability of the glutaraldehyde in the reaction of polycondensation and partial cyclization during its storage and use. Up to 70 % of glutaraldehyde is present in the aqueous solution in these forms (Eq. 2.34) (Migneault et al. 2004). The polycondensation diminishes the efficiency of immobilization by glutaraldehyde and makes the properties of the product less predictable and reproducible.

The influence of polycondensation can be suppressed by the application of glutaraldehyde vapors instead of the aqueous solution. These techniques were first suggested for the preparation of enzyme sensors based on FETs and other microelectronic devices. Due to the small size of the working area, the amounts of

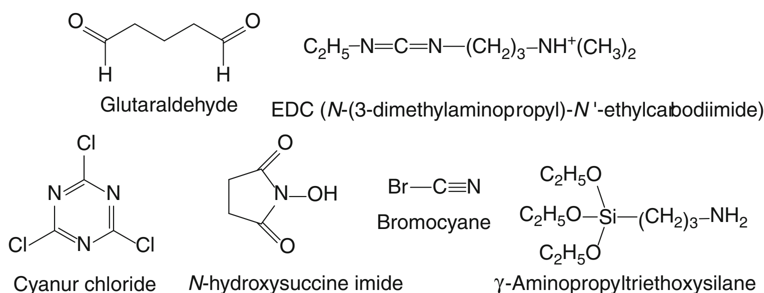
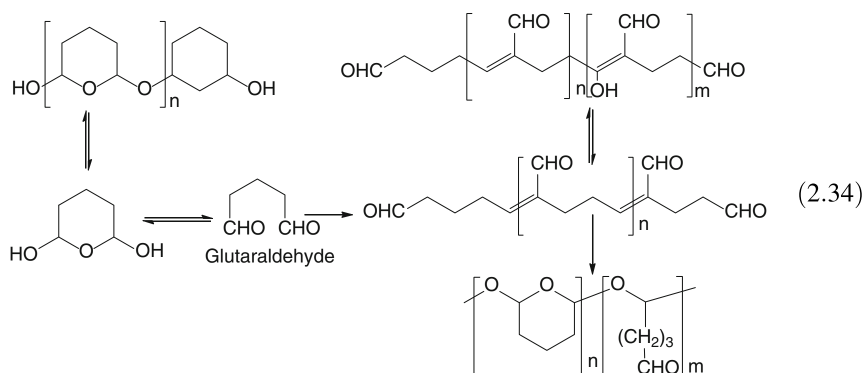


Fig. 2.25 Bifunctional reagents commonly used for the covalent immobilization of biomolecules

enzyme loaded onto the surface were much lower than those for conventional sensors. The FETs were fixed at the distance of several millimeters over the 5–15 % glutaraldehyde solution and the system was vacuumed.



(2.34)

The volatile monomer of glutaraldehyde crossed the gap between the solution and sensor surface and cross-linked the protein molecules as described. The period of treatment was certainly longer than that for the aqueous solution and the efficiency of immobilization is lower, but in many cases the result was adequate enough from the point of view of the signal stability and reproducibility.

The enzymes are mixed with inert proteins like BSA to decrease the loss of the enzyme activity often observed at cross-linking with bi-functional agents. The appropriate procedure is also called "implementation in the BSA matrix." It should be mentioned that both the amount of the enzyme and BSA are the subjects of careful optimization together with the glutaraldehyde concentration and treatment period, because these parameters determine the thickness of the protein film obtained and its permeability for the substrate or inhibitor of an enzyme. For carbodiimides, the decay of activity is not serious and no BSA or similar "thinners" are used. The same refers to the covalent immobilization of antibodies and the DNA oligonucleotides.

Both carbodiimide binding and treatment with glutaraldehyde of amino groups preferably result in the formation of Schiff bases with the >C = N- group sensitive to hydrolysis. This is not particularly important for the laboratory samples of biosensors but can be critical for commercial applications. Additional measures, e.g., the chemical reduction with $NaBH_4$ and the saturation of unreacted groups with glycine are often suggested to stabilize the immobilization products.

Some of the examples of covalent binding are given in Fig. 2.26. The carboxylated supports are easily prepared by a partial oxidation of carbon materials, i.e., glassy carbon and carbon black often used in electrochemistry. The oxidation of carbon nanotubes also results in the removal of terminal cups substituted with carbonyl and carboxylic functional groups. For multi-walled carbon nanotubes, the defects in the side walls are expected to give the hydroxyl, carbonyl and carboxylic groups active in covalent binding. From other sources, polyaminated and

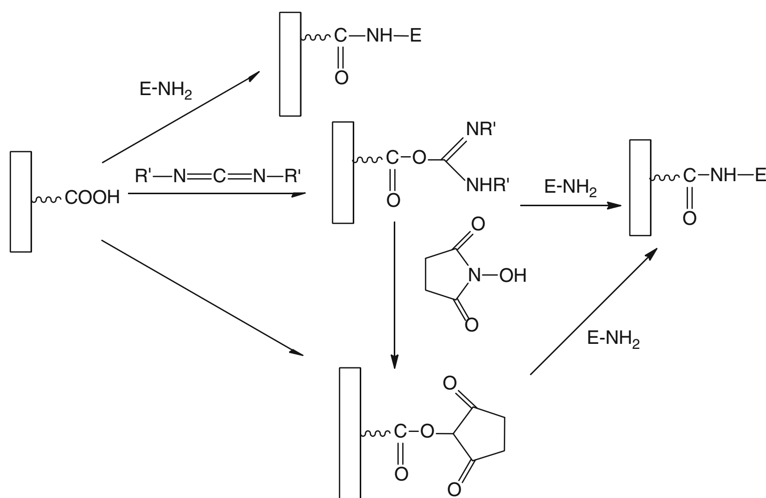
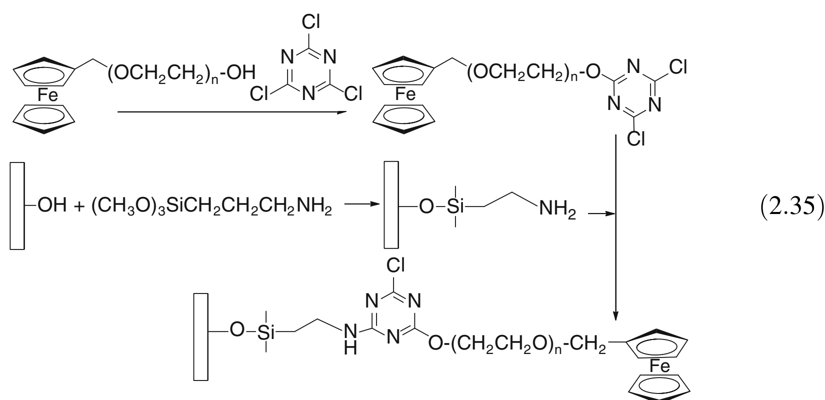


Fig. 2.26 Covalent immobilization of proteins (E-NH_2) on carboxylated carriers

carboxylated ion-exchange resin can be used. However, their efficiency in covalent immobilization is less than that of carbon materials due to the electrostatic repulsion and steric hindrance of biopolymer binding (Lucarelli et al. 2004).

Covalent immobilization is also used for the attachment of other auxiliary reagents, either to bioreceptors or to a solid support (Eq. 2.35). Thus, the mediators of electron transfer or fluorogenic substances can be fixed by covalent bonds for the following measurement of electrochemical and optic signals, respectively. Covalent immobilization is also applied for attaching the labels to the DNA or protein molecules already immobilized by the other protocols described. This makes covalent immobilization a universal tool for biosensor development.



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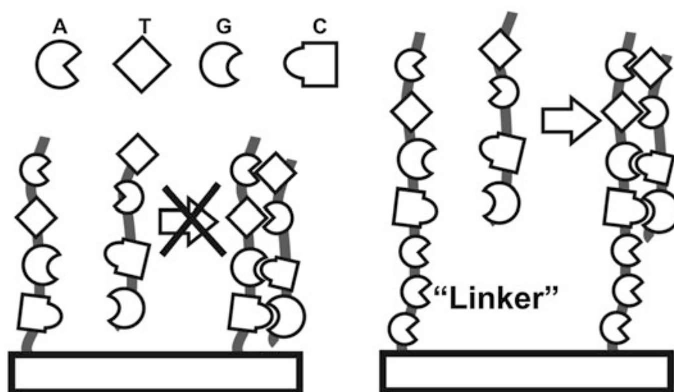

Fig. 2.27 The hybridization detection using a homo-ODN linker for avoiding spatial limitations of interactions

The optimization of covalent immobilization protocol is mainly directed to the minimization of the losses of biochemical functions, i.e., enzyme activity, affinity toward immunoreagents, etc. The formation of rather rigid constructs around the binding site by covalent interactions limits the flexibility of the immobilized biomolecule. Because of that, the immobilization product is less adjusted to an analyte structure than that in its native environment. For this reason, the rate of an appropriate biochemical reaction decreases, even though the quantity of the biomolecules in the surface layer of the biosensor remains constant.

As mentioned earlier, this limitation can be diminished if long-chain bridging molecules (also called *spacers* or *linkers*), are introduced in between the biopolymer and solid support. Besides alkyl radicals, polyoxyethelene and polyethylene imine linkers are used as shown in schemes (2.32, 2.35). In the case of the DNA probes, the functions of linkers are realized by a specific ODN sequence (Fig. 2.27). A homo-oligonucleotide strand is introduced between the recognition sequence and the terminal functional group so that the hybridization takes place not on the surface but spatially separated from the transducer. This scheme constrains the detection system but exhibits advantages of higher sensitivity and selectivity of the target sequence detection.

The use of linkers is a routine step in the covalent immobilization often combined with the SAM technology. The necessity of assembling monolayers with linked ODNs is related to the prevention of the DNA lodging. When DNA probes are placed along the support surface, the advantages of linkers become insignificant. Both linked and short sequences are expected to have a similar influence on steric factors while the hybridization of the target analyte takes place.

The choice and optimization of the linker is a part of the biosensor development that significantly depends on the signal transduction mode and the analyte nature. The bigger the analyte molecule, the more attention paid to this problem. Thus, the covalent modification of biorecognition elements is mostly considered in the

immunosensor development. The immobilization of the Ab cannot rely on the localization of the binding point because many of the protein functional groups are involved in the interactions with conventional bi-functional reagents. The application of Fab fragments and separation of the Ab molecules into identical parts, followed by their attachment on the golden supports, can help in reaching a high reproducible response.

For enzymes, covalent immobilization often results in the losses of their activity due to the limitations in the mobility of protein domains required for *E-S* complex formation. To suppress this unfavorable influence, the covalent immobilization can be performed in the presence of a substrate. While included in the active site of an enzyme, the substrate "freezes" the favorable spatial conformation of a protein globule. The same result can be reached by treating the enzyme with a reversible inhibitor. The covalent immobilization can be combined with preliminary implementation of an enzyme into an inert gel, e.g., gelatin, BSA or agar. The following treatment with carbodiimide or glutaraldehyde provides not only the necessary covalent binding but also decreases the swelling of the matrix and improves its storage stability especially at room temperature. The protecting effect of matrix components that interact with reactive species used in covalent immobilization is one of the reasons for the phenomenon previously observed in the enzyme sensor development. The use of crude enzyme preparations, e.g., extracts and homogenates, sometimes yielded better immobilization results than those after purification and isolation of the active components. For the same reason, the determination of inhibitors and substrates is affected by the stabilizers and antioxidants added to commercial products to prolong their storage periods. Certainly, this is not the only, and not even the primary reason for the immobilization influence on the biosensor performance, but it should be taken into account especially when a new enzyme source (or enzyme preparation) is considered for biosensor production.

Glycoproteins can be immobilized by the partial oxidation of the saccharide moieties yielding the aldehyde groups active in various condensation paths (Zaborcky 1974). Thus, peroxidase or glucose oxidase can be pre-oxidized by Os(VIII) oxide or periodate salt. The latter yields aldehyde groups that can react with aminated supports or even give oligomeric products of self-condensation similar to that described for glutaraldehyde. The oxidation changes the substrate specificity of the enzyme. Thus, the oxidation of glycolic fragments increases the rate of peroxidase oxidation of bulky organic substrates that have ready access to the hemin fragment deeply installed in the protein moiety. Besides enzymes, the oxidative immobilization is applied for the Ab immobilization.

As in the other cases of covalent immobilization, multi-layered films can be assembled on the transducer surface. Similar to the LbL immobilization, they have a regular and good reproducible structure of their inner layers but are more stable towards concentrated solutions of electrolytes and high temperatures that destroy the complexes coupled only with electrostatic interactions.

There is great evidence of the advantages of such an approach. As one example, the LbL immobilization of pre-oxidized glucose oxidase on the golden support is

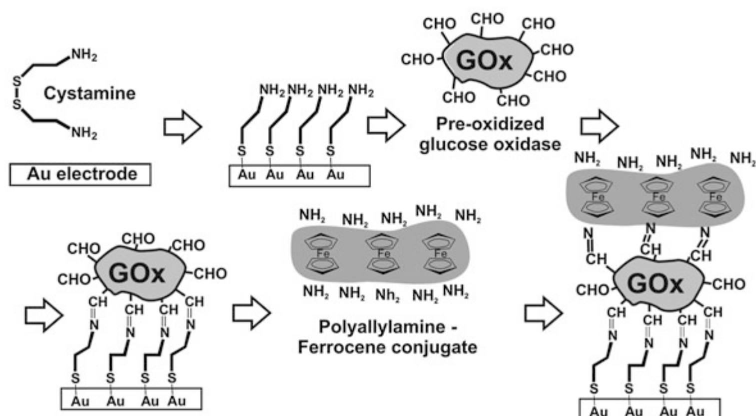

Fig. 2.28 Multilayered immobilization of pre-oxidized glucose oxidase on the cystamine modified Au electrode together with ferrocene containing polyallylamine

presented in Fig. 2.28 (Yang et al. 2004). The aldehyde groups of the enzyme are first coupled with the electrode modified by cystamine; then aminated ferrocene derivatives are attached to the protein layer. An alternate treatment makes it possible to build up a number of layers. Meanwhile, the electric wiring of the enzyme is retained through the whole construct due to intimate contact of the enzyme-active site and ferrocene moieties provided by the immobilization protocol.

The classification given here is rather arbitrary and many protocols, especially those recently developed, can exploit several approaches. Even simple cases, like the implementation of DNA probes on the zirconium phosphate layer, can be considered to be both the physical process of the mobility limitation and the affine reaction of phosphate groups of the DNA sequence with vacancies in the crystal net of the carrier. The interaction of DNA with chitosan, a natural polysaccharide obtained by partial hydrolysis of chitin, can be considered an electrostatic interaction and covalent binding with the formation of phosphate ester (see 2.27).

In other cases, different immobilization techniques are used on various stages of the formation of the biorecognition layer to reach the spatial separation of its components playing different roles in signal generation. An example of such hybrid immobilization techniques is given in Fig. 2.29 for DNA probe immobilization.

The electrode is first covered with a copolymer of pyrrole and its derivative-bearing biotin (Dupont-Filliard et al. 2004). Then the polymeric film obtained is consecutively treated with avidin as a bridging agent and a biotinylated DNA probe. This provides site-specific immobilization with a strictly controlled thickness and permeability of the surface layer. After hybridization detection, the DNA sensor is treated with a concentrated electrolyte solution to remove all the layers over the polypyrrole film and the deposition of another DNA probe can be performed on the same support.

Other similar schemes have been described to increase the sensitivity of the biological targets' detection. Thus, in addition to avidin–biotin bridges, the layers

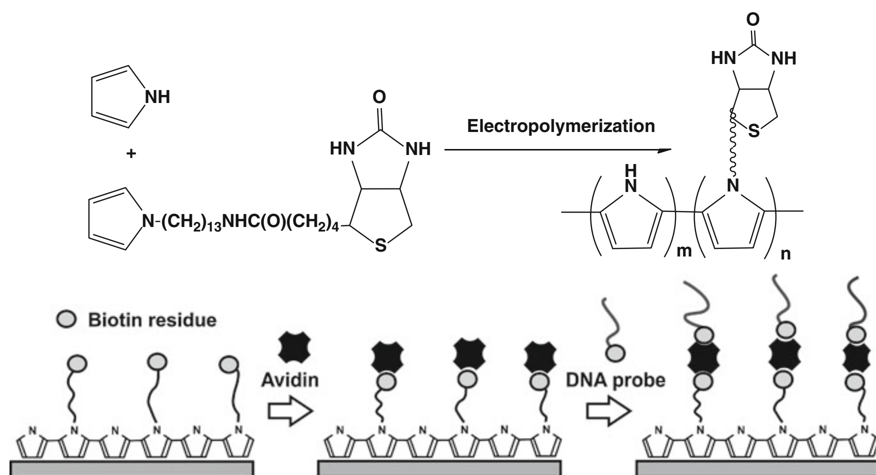

Fig. 2.29 Immobilization of the DNA probe by avidin–biotin binding coupled with covalent attachment of biotin residues to a polypyrrole layer

of biocomponents and auxiliary reagents can be coupled with Au nanoparticles and carbon nanotubes able to covalently bind thiol and amino functional groups, respectively. As in the previous case, regularity of the composition is provided by the order of the reactant addition and specificity of covalent binding.

In Fig. 2.30, alternating layers of carbon nanotubes and Au nanoparticles are obtained by intermediate oxidation and treatment of the reactants with cystamine. This provides the implementation of thiol groups on the sidewalls of the carbon nanotubes bearing carboxylate functions. The procedure makes it possible to deposit several dozen monolayers with their final assembling on thiolated DNA probes. As a result, both electric wiring and steric accessibility of the biotargets are achieved (Zhang et al. 2009).

The short summary of the immobilization techniques shows a great variety of possibilities available for each biocomponent. In many cases, different immobilization protocols result in quite comparable results, i.e., selectivity and specificity of the analyte detection. Some of the techniques acceptable in the laboratory are not convenient in mass production. And vice versa—most complicated protocols with careful maintenance of the order of reagent addition and intermediate washing and drying steps are better fulfilled in an automated regime developed for the biosensor manufacture than manually.

In many cases, the immobilization of biocomponents for biosensor development started from the methods that already existed in related areas, i.e., in affine chromatography, protein purification techniques, bioreactors and biofilm manufacture. Since first results are obtained, the protocols are modified to meet the specific requirements of biosensor performance. In most cases, they are directed to the longer lifetime and more stable signal within the whole biosensor operation.

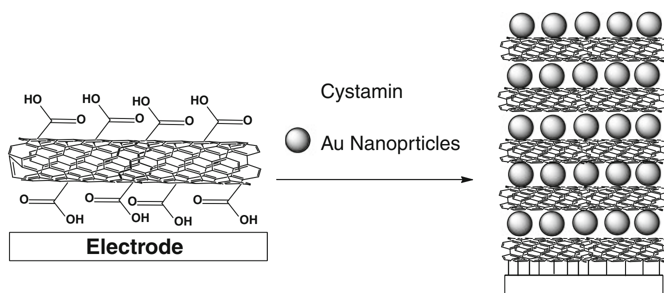

Fig. 2.30 Multiple assembling of the layers of carbon nanotubes and Au nanoparticles for the following immobilization of thiolated DNA probes

The influence of immobilization on the affinity of interactions and hence selectivity of the biosensor signal should also be taken into account.

It is interesting to note that microelectronics and micromachining techniques greatly influenced the development of new immobilization techniques in the past decade. Many of the technologies developed for the production of the microelectronic devices were adapted to biochemicals. In some cases, the success was obvious enough to be extended to a macro scale. Thus, the use of screen-printing techniques with a millimeter scale of the parts (conductor pads, electrode areas) can be considered to be a simplified realization of the photolithography methods used for the creation of multilayered structures based on silicon and metal oxide supports with micron resolution of linear dimensions of the structural elements (Albareda-Sirvent et al. 2000).

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Chapter 3
Biosensor Signal Transducers

3.1 Electrochemical Transducers

Electrochemical signal transduction is most widely applied in biosensor development due to the undisputable advantages it possesses (Wang 2006):

- Electrochemical biosensors utilize measurement technologies widely represented in most (bio)chemical laboratories and discussed in most monographs and student books. Thus, the potentiometric biosensors are compatible with pH- and ionometers and voltammetric (amperometric) biosensors with oximeters and voltammographs. The latter are used for heavy metals determination. Novel electrochemical methods, e.g., spectroscopy of electrochemical impedance and field effect transistors, require specialized equipment that has not yet become widespread. But even in this case, the commercial applications of analytical equipment cover most of the requirements specific for biosensors and involve very diverse devices for a moderate price and with acceptable characteristics
- Besides measurement equipment, some of the commercially available electrochemical sensors can be easily adapted to biosensor development. This is particularly true for Clark oxygen sensors and pH metric electrodes but is also applicable for specialized tools, i.e., conductometic cells or microelectronic systems (FETs, cantilevers for AFM, etc.)
- The principles of signal transduction realized in electrochemical signals are universal and can be easily applied for the enzymatic reactions, DNA and immunosensors, microbial sensors, etc. Popular detection modes (oxygen reduction, pH measurements, and the use of electron mediation techniques) make it possible to construct biosensors based on not various biorecognition elements but similar signal measurement mode. This simplifies the R&D stage of biosensor commercialization, and lowers the expenses and final price of the product
- The electrochemical methods do not assume any strict requirements for the geometry, shape or size of the transducers and can be used in flow-through and flow-injection modes and in automated analytical systems

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- Biologists and doctors are familiar with the basic concepts of electrochemistry and electroanalytical chemistry. Many of the biochemical paths, e.g., ATP synthesis, photosynthesis, glycolysis and citric acid cycle include redox reactions of numerous metabolites. This makes the perception of an idea of electrochemical biosensors and understanding the relationships between the biochemical and electrochemical reactions in signal measurement easier.

All the electrochemical transduction tools are based on the charge transfer processes that take place on the electrode-solution interface. Some reactants (mediators, enzyme substrates, electrolytes) are immersed in the solution, but the changes in the electrochemical characteristics (potential, current, even conductivity) followed by these additions are related to changes in the energy distribution, structure and chemical content of the interface. This is the biggest difference between the electrochemical and optic measurement techniques that are applied for homogeneous reactions. Meanwhile, both electrochemical and optic detectors are sometimes applied for recording the same reactions. Thus, the redox potential changes are recorded by appropriate sensors and redox indicators changing their color in accordance with the redox potential.

The heterogeneous nature of the signal has the advantages and drawbacks specific for electrochemical transducers. The localization of a biochemical receptor directly on the surface involving maximal changes of the characteristics to be recorded is an obvious advantage of electrochemical devices over optic systems. Adsorption and other simultaneous mass transfer reactions on the surface can significantly improve the sensitivity of the biosensor. The mass transfer stage in the signal generation offers additional prospects for the control of biosensor performance. Thus the use of additional membranes with pores or appropriate charge provides a selection of the molecules in accordance with their characteristics (polarity, molar mass) prior to or together with biochemical conversion. Besides, the low rate of mass transfer increases detection time and can decrease the absolute signal value and hence the accuracy of its measurement.

Similar to biosensors, all the electrochemical techniques can be subdivided into static and dynamic systems. In static systems, the signal does not depend on the measurement time and corresponds either to equilibrium or to stationary conditions. The use of static methods simplifies the measurement protocol and, to some extent, the description of the relationships between the signal and amount (mass, concentration) of an analyte. The minimal contribution of mass transfer steps to the signal is an advantage of static methods that involves potentiometry, conductometry and EIS. The signal generation does not assume any changes in the analyte content and is reversible irrespective of the processes on the sensor interface.

The dynamic systems are mainly represented by voltammetry and coulometry. The current recorded at appropriate potential indicates the oxidation, or, rather rarely, the reduction of species followed by the electron flux between the working and auxiliary electrodes. Thus, new chemicals appear in signal measurements, i.e., the products of electrode reactions. They do not refer to the biochemical

interactions and interfere with them in some cases. The electron transfer can initiate coupled chemical reactions of hydrogen/oxygen subtraction or addition. The final result can be presented as a series of consecutive or parallel electrochemical and chemical steps that can be rather complicated and determine both the charge passed through the system and hence the currents recorded.

Although the electrode reaction changes the content of the solution, the scale of such changes is normally very small and the concentrations of the species in bulk solution are assumed to be constant. The thin-layer cells with the high surface-to-volume ratio are an exception. However, minor changes in the chemical content can affect the biochemical interactions because they are both localized in a very narrow layer at the electrode surface. This refers to the losses of dissolved oxygen reduced on the electrode, the competitive electrode conversion of the enzyme substrates, pH shift, etc. The layer within the concentration of oxidizing/reducing species that is changed from the local one to that of the bulk solution is called *the reaction layer* (Bard and Faulkner 2001). In biosensors, the reaction layer is mostly localized within the surface film containing immobilized biopolymers.

At present, there are numerous methods of electrochemical analysis that differ by their signal generation mode and hence by the relative importance of electrode stages and mass transfer of the reactants. For this reason it is very important to realize how the electrochemical signal reflects the particular steps of biochemical reaction. Besides quite obvious cases of the reactions related to direct electrochemical oxidation or reduction of enzyme substrates and cofactors, there are many other systems that require a fine tuning of the surface layer content and the detection mode. They are intended for quantification of rather weak and less specific changes in charge distribution, equilibrium and association-dissociation reactions that follow biorecognition of DNA probes and immune reagents, i.e., electrochemically inactive biomolecules.

Later on, the basic principles of popular electrochemical techniques are discussed with particular emphasis on their application for biosensor development. Certainly, the real number of successful solutions in this area is enormous and only basic approaches could be chosen and discussed.

3.1.1 Potentiometric Sensors

In potentiometric sensors, the electromotive force (e.m.f.) generated in the *galvanic cell* consisting of two electrodes immersed in the electrolyte solution is measured by a high impedance voltmeter (Gründler 2007). The measurement is performed without any external polarization of the circuit at about zero currents. In accordance with the theory, the e.m.f. value is mainly determined by the difference in the potentials of the electrodes. One of them, the potential of which is assumed to be constant, is called a *reference electrode* and the other a *working electrode*. If the assembly of the working electrode provides the dependence of its potential on the concentration of the so-called *potential-determining,* or *primary ion*, it is called

an *ion-selective electrode* (ISE). In the cell consisting of the ISE and reference electrode, any e.m.f. changes can be referred to the shift of the content of the primary ion. Thus, potentiometry provides a simple way to quantify the concentration of the ions, whose nature is determined by the appropriate ISE used in the circuit.

The formation of the electrode potential is caused by thermodynamically spontaneous reactions directed to leveling the chemical potential of the species on the electrode interface. Thus, for a metal electrode immersed in the solution of the salt, the redox reaction (3.1) is caused by the difference in the chemical potential of the ion Me^{z+} in solution and in the points of a metal lattice.

$$Me^{z+} + z\,e^{-} = Me \tag{3.1}$$

In accordance with (3.1), some ions can leave the metal lattice and, vice versa, some ions can deposit from the solution on the electrode surface. The direction of the reactions depends on the relation between the free energy of the ions in both phases. As a result, the electrode interface becomes polarized. The potential generated is a measure of the charge transfer job. This makes it possible to express the potential by the parameters characterizing the nature and quantity of the primary ion known as the Nernst Equation (3.2).

$$E = E^0 + \frac{RT}{zF} \ln a_{\mathrm{Me}} \tag{3.2}$$

Here, E is the electrode potential, E^0 is a standard potential that depends on the nature of the ion and temperature, R is the universal gas constant, T is the Kelvin temperature, z the charge of the ion, and F the Faraday constant. For the thermodynamic behavior of the system, the activity a_{Me} of the Me^{z+} ion corresponds to its concentration. In the opposite case, the activity differs from the concentration by a factor γ called the *activity coefficient* (3.3).

$$a_{\mathrm{Me}} = \gamma c_{\mathrm{Me}} \tag{3.3}$$

For a low concentration of the ion, the activity coefficient can be estimated from the ionic strength I, a parameter, calculated from the total ion content in the solution (3.4).

$$I = \frac{1}{2} \sum_i c_i z_i^2 \tag{3.4}$$

In accordance with the Debye–Hückel Law (3.5),

$$\log \gamma = -0.509 z^2 \sqrt{I} \tag{3.5}$$

the Eq. (3.2) can be transformed into a more convenient form (3.6) for 25 °C.

$$E = E^0 + \frac{0.059}{z} \log a_{\mathrm{Me}} \tag{3.6}$$

The value 0.059 $V = 59$ mV is also called the Nernst slope of a calibration curve in the plots of E verus c_{Me}.

The reference electrode contains a low soluble salt on the surface of a metal wire. In this case, the potential of the electrode becomes sensitive to the counter ion concentration. If it is high enough, the potential of such an electrode becomes stable and does not depend on the changes in the ionic content of the solution. The reference electrodes are denoted by a metal symbol and an insoluble component on its surface. Two reference electrodes are most frequently used, i.e., the f/AgCl electrode and the Hg/Hg_2Cl_2 (saturated calomel electrode, SCE). The internal space of the electrodes can be separated from the external solution by additional filling and pore membranes to avoid the pollution of an internal filling (double-junction ISE). The Nernst equation for Ag/AgCl electrode is given below (3.7).

$$E = E^{\mathrm{o}}_{\mathrm{Ag/Ag^+}} + \frac{RT}{F}\ln a_{\mathrm{Ag^+}} = E^{o}_{\mathrm{Ag/Ag^+}} + \frac{RT}{F}\ln\frac{K_S^{\mathrm{AgCl}}}{a_{Cl^-}} = Const - \frac{RT}{F}\ln a_{Cl^-} \quad (3.7)$$

In electrochemistry, all the potentials are given against a standard reference electrode whose potential is assumed to be zero. This is a so-called *normal hydrogen electrode* (NHE). The design of NHE and Ag/AgCl electrodes are presented in Fig. 3.1.

The NHE consists of a Pt plate covered with finely dispersed platinum (“black platinum”) and immersed in a solution of hydrochloric acid with the activity of hydrogen ions equal to 1. A constant flow of gaseous hydrogen under pressure of 1 atmosphere is bubbled through the solution to saturate the black platinum with hydrogen. Of course, such an assembly is inconvenient for conventional measurements. The potentials determined against other reference electrodes can be recalculated to the potential versus NHE in accordance with the simple relations presented in Table 3.1 (Zoski 2007).

In aqueous solutions, Ag/AgCl and SCE reference electrodes are commercially available and mostly applied in biosensor-related investigations, and the electrodes have some limitations. Thus, the Ag/AgCl electrode is not recommended for use in the media with high amounts of chlorides and other compounds that form insoluble products with Ag^+ ions. The Ag/AgCl electrode is not very stable in water-

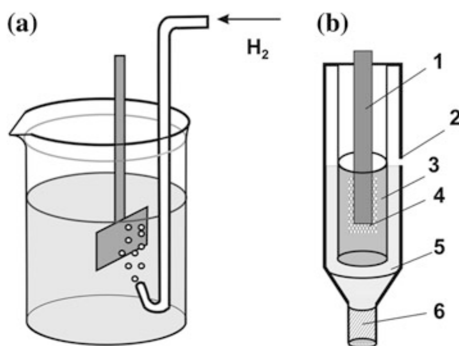


Fig. 3.1 The design of a normal hydrogen electrode (**a**) see description in the text and double-junction Ag/AgCl electrode (**b**) *1* silver wire; *2* filling hole; *3* KCl solution (internal filling); *4* AgCl film; *5* intermediate filling; *6* porous frit

Table 3.1 The potentials of some reference electrodes against NHE and SCE

Reference electrode	Potential at 25 °C	
	versus NHE	versus SCE
NHE: Pt/H_2, H^+ (a = 1)	0	−0.2412
Ag/AgCl, KCl (0.1 M)	0.2881	0.047
Ag/AgCl, KCl (3.5 M)	0.205	−0.036
Ag/AgCl, KCl (sat.)	0.1988	−0.042
SCE: Hg/Hg_2Cl_2, KCl (0.1 M)	0.3337	0.0925
Hg/Hg_2Cl_2, KCl (0.1 M)	0.250	0.009
Hg/Hg_2Cl_2, KCl (0.1 M)	0.2412	0
Hg/HgO, NaOH (1.0 M)	0.14	−0.101
Hg/Hg_2SO_4, H_2SO_4 (0.5 M)	0.682	0.441

miscible organic solvents. The Hg/HgO electrode is unstable in strong mineral acids. As regards SCE, it contains toxic mercury and offers some complications in calibration, especially for homemade samples.

Besides reference electrodes, the *pseudo-reference electrodes* have been developed for the application in the assemblies of miniaturized sensors. Thus, the thin film Ag/AgCl electrode can be prepared by lithography and screen-printing techniques by consecutive deposition of the Ag and Ag_2O–AgCl paste on the ceramic or plastic support. Such a design does not imply any internal filling and for this reason does not provide an absolute, but rather a relative stability of the potential in the changing media.

Most commercial ISEs belong to the family of the electrodes with a plastic membrane and internal filling (Fig. 3.1a). The membrane includes an *ionophore*, or an ion exchanger. This is a complexing agent responsible for selective binding and the transmembrane transfer of potential determining ion. In addition to the ionophore, the membrane material (mostly polyvinyl chloride, PVC) contains a plasticizer (e.g., octylphenyl ether, dioctylphthalate) and lipophilic salt.

The latter is necessary for the acceleration of the transfer of counter ions and reaching the reversible potential. The cation-selective ISEs contain the salt of lipophilic anions (e.g., *tetrakis* (*p*-clorophenyl) borate) and the anion-selective electrodes, the salt of lipophilic cation (tetradodecylammonium nitrate as an example). The internal filling contains the salt of the ion to be determined in contact with a metal conductor or an internal reference electrode.

The polymeric membranes of the ISEs described refer to quasi-liquid, or solidified membranes. In miniaturized electrodes made of a glass capillary or a thin plastic cap for pipettes (Fig. 3.2c), the membrane can be prepared from a small portion of organic solvent immiscible with water (nitrobenzene, etc.) and held in the tip by capillary forces. Such electrodes with very thin tips of several microns in diameter are used as transducers of implantable sensors and biosensors. Their low durability can be compensated for by inert fillers impregnated with membrane components and stuffed in the tip.

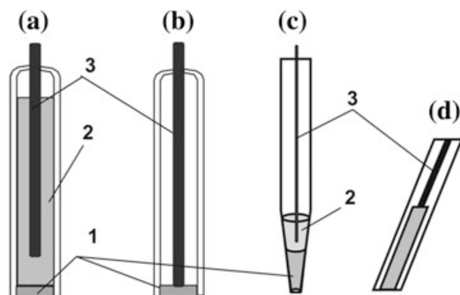


Fig. 3.2 The design of conventional ISEs. **a** Membrane ISE with internal filing; **b** solid-contact ISE; **c** microelectrode with a liquid membrane; **d** planar thin-film electrode. *1* ion-selective plastic membrane; *2* internal filling; *3* metal conductor or internal reference electrode

Glass ISEs selective for H^+ and Na^+ ions have a similar assembly, but the membrane is made of a special sort of glass that can exchange with the environment by hydrogen and sodium ions. The generation of the potential is mainly based on the reversible protonation of the surface silicate groups of glass for pH electrodes and the exchange of Na^+ ions for Na-selective ISEs.

Similarly, crystal ion selective membranes, like the fluoride selective electrode based on LaF_3, exchange the primary ions with those in solution. The selectivity of the response is due to the correspondence of the size and charge of the target ion to the vacancies in the crystal structure of the membrane. Besides fluoride-sensitive ISEs, crystal membranes are used for the detection of silver cations and halogenide anions (Ag_2O–AgBr or AgI membranes) and sulfides (Ag_2O–Ag_2S membranes).

Solid-contact electrodes (3.2b) have been developed to remove the internal filling from the electrode assembly (Bakker and Pretsch 2007). Instead, the membrane with an ionophore makes contact with special materials that establish electron-to-ion transduction, i.e., aligning the ion and electron transfer on the interface. Hence they can convert changes in the ionic content into the electron flow in the metal conductor. Such materials involve various mixtures of the oxides of transient metals that can reversibly oxidize/reduce each other with the simultaneous transfer of an oxygen atom within the layer. The chemical step that appears in an aqueous solution involves the transfer of water molecules or hydrogen ions so that most solid-contact electrodes have pH-dependent potential irrespective of the ionophore used in the covering membrane.

In the simplest way, the solid contact is obtained by thermal destruction of unstable salts deposited on the electrode surface or by oxidation of the metal electrodes. Some of the electrodes obtained with such a treatment serve for pH measurements [antimony (Sb/Sb_2O_5) or RuO_2 electrode (Kurzweil 2009)]. Being less sensitive toward pH than conventional glass electrodes, they can be produced without significant limitations in their sizes and shapes. But more often they are applied to the following deposition of membranes, the content of which is similar to that described for traditional ISEs with an internal filling. The membranes can be deposited by drop-casting and solvent evaporation (planar electrodes), or by immersing the wires into the viscous mixture followed by drying the layer left on the metal surface (coated wire electrodes). In both cases, the ionophore is placed on the material with the ion-to-electron transduction and specifies the selectivity of the potentiometric response.

Electroconductive polymers have attracted increasing attention in the potentiometric measurements (Inzelt 2008). They are synthesized by chemical or electrochemical oxidation of some aromatic compounds, i.e., aniline, thiophene and pyrrole and their derivatives. The product of oxidation exerts electroconductivity comparable with that of p-doped semiconductors and even metals. The mechanism of the redox reaction of polyaniline was presented in Chap. 2 [eq. (2.48)]. In brief, the pH sensitivity of the potential is related to the protonation of amino and imino groups and the involvement of H^+ ions in the reversible redox conversion of imine fragments. As a result, polyaniline has the pH-depending potential in two pH regions. In acidic media, the potential is shifted due to changes in the ratio of reduced and oxidized fragments of the polymer chain as described by Eq. (3.8).

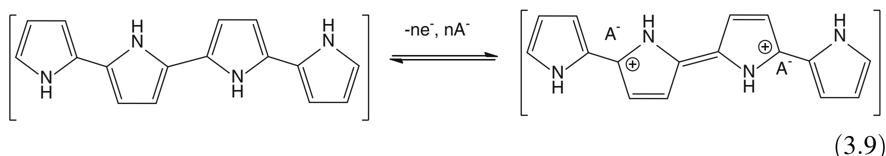
$$E = E^0 + \frac{0.059}{n} \log \frac{[\mathrm{Ox}]}{[\mathrm{Red}]} - 0.059 \frac{m}{n} \mathrm{pH} \tag{3.8}$$

[Ox] and [Red] denote the concentrations of the imino and amino structural units of the polymer and m and n the number of H^+ions and the electron transferred per one monomer unit of polyaniline.

In basic media, the potential is shifted due to the changes in the number of charged groups in the polymer chain. Polyaniline, such as in its deprotonated form, is non-conductive in these conditions.

The reaction of protonation and deprotonation of polyaniline is accompanied by the equivalent transfer of counter ions that affect the polymer permeability and the thickness of the surface layer. To some extent, this can be suppressed by the introduction of polyanionic species into the polymer layer. Nafion and polystyrol sulphonate made it possible to suppress the pH-sensitivity of polyaniline in favor of the potentiometric response toward other ions, in accordance with the ionophore used in the upper polymer layer. On the other hand, the interaction with acidic additives increases the pH sensitivity of polyaniline based-sensors. Thus, the addition of the strong water insoluble camphor sulfonic acid to the chemically synthesized polyaniline resulted in an increase of the sensitivity to 89 mV/pH, indicating the transfer of one excessive hydrogen ion per monomer unit (Karyakin et al. 1999).

The reactions of polypyrrole and polythiophene derivatives (3.9) do not dramatically differ from those of polyaniline, but the pH dependence of the potential is much milder because the oxidation does not assume the simultaneous transfer of the H^+ ions (Wallace et al. 2009).



(3.9)

As a result, the pH sensors based on polypyrrole and polythiophene require an additional pH sensitive ionophore placed on the polymer layer alone or in the PVC matrix. For this purpose, secondary amines with long-chain hydrophobic substituents are used.

The solid-state potentiometric sensors based on electroconductive polymers often include mediator systems, e.g., Prussian blue, hydroquinone derivatives, metal porphyrine complexes, transient metal oxides, and carbon nanotubes that promote the electron exchange within the surface layers and the reversibility of the response. These additives simplify the introduction of other components necessary for the improvement of the sensor performance or biomolecule immobilization as well.

Of special importance is the fact that the electropolymerization makes it possible to obtain various nanostructures of the polymers. They can have the shape of tubes, flowers, needles, etc. Their formation increases the internal volume of the surface layer and the specific surface of a redox material. The latter does not affect the stationary potential directly but accelerates the achievement of a stationary condition, especially in the microenvironment sharply changed. The nanostructured polymers adsorb and confine the biopolymers by multi-point electrostatic interactions in the pores and channels and establish intimate contact of the biomolecules necessary for a fast and reversible electron exchange.

The formation of the nanostructured polymers can be achieved by the template effect of porous plastic membranes or oxidized aluminum foil fixed on the electrode surface prior to polymerization and removed by appropriate solvents after the polymer deposition (Fig. 3.3). For this purpose, the special regime of electrolysis, including several steps that differ in the conditions of the polymer growth, has also been developed. Current pulses and the alternation of potentiostatic and galvanostatic electrolysis steps promote formation of the nanostructures (Wallace et al. 2009).

Besides combinations with conventional plastic membranes bearing ionophores, electropolymerized materials are applied as such for the immobilization of biomolecules and measurement of the changes in the charge distribution and redox

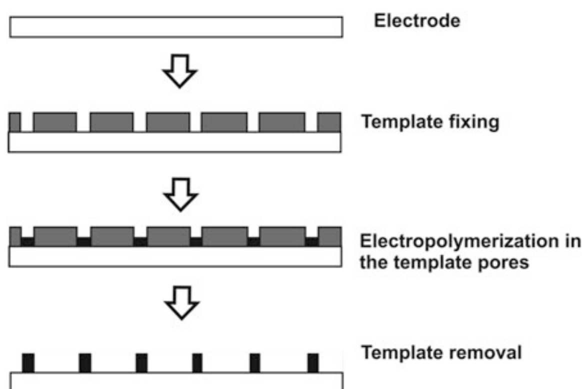


Fig. 3.3 The electrodeposition of the polymers on the electrode covered with template film

potential of the system. Such a non-selective response provides a universal tool for the design of immuno- and DNA-sensors, the reactions of which significantly alter the above parameters on the surface of a transducer. But the signal of such biosensors is preferably recorded in an impedimetric and even amperometric mode than in the potentiometric regime.

The *selectivity* of the signal of potentiometric sensors is quantified by the potentiometric selectivity coefficients introduced by the Nikolskii-Eisenman Eq. (3.10) (Bakker et al. 2000).

$$E = \text{Const} + \frac{RT}{z_A F} \ln\left(a_A + \sum_j k_{A,j} a_j^{z_A/z_j}\right) \tag{3.10}$$

The index 'A' belongs to the primary ion $A^{z\pm}$ and 'j' to the interfering ions. In accordance with (3.9), the presence of interfering ions does not affect the slope of the calibration curve but reduces the linear portion, i.e., the concentration range determined by the sensor. There are several methods approved by IUPAC for the estimation of the potentiometric selectivity.

The *fixed interference method* is one of those most frequently used. In them, the e.m.f. is measured for the solution of the constant activity of the interfering ion and the varying activity of the primary ion. The e.m.f. values obtained are plotted against the logarithm of the activity of a primary ion. The intersection of the extrapolated linear portion of the plot yields the a_A value inserted in the Eq. (3.11).

$$k_{A,j} = \frac{a_A}{a_j^{z_A/z_j}} \tag{3.11}$$

The *matched potential method* (MPM) does not depend on the Nicolskii-Eisenman equation. The potentiometric selectivity coefficient is defined as the ratio of the activities of the primary and interfering ions that give the same potential shift. At first, a known activity $a'_A$of the primary ion is added to the reference solution with a fixed activity a_A of the same ion, and the corresponding shift of the potential (ΔE) is recorded. Then, a solution of an interfering ion is added to the reference solution until the same potential shift (ΔE) is reached.

$$k_{A,j} = \frac{a'_A - a_A}{a_j} \tag{3.12}$$

The lower the $k_{A,j}$ value, the higher the selectivity of the ISE. For $a_A \gg k_{A,j}$the calibration graph is linear in semi-logarithmic plots with the slope of $RT/z_A F$. In the opposite case, the same relationship will be observed for the interfering ion. Although the potentiometric biosensors are mainly based on commercial ISEs with well-known selectivity, in some cases their selectivity has to be taken into account—and this is especially true for newly developed potentiometric transducers sensitive to organic species. Thus, the application of choline-sensitive electrodes in cholinesterase-based biosensors is limited by a rather high cross-sensitivity toward acetylcholine, i.e., a substrate. In the enzyme reaction, the concentration of choline

and acetylcholine changes in the opposite direction and the lack of selectivity affects the calibration curve and accuracy of the substrate determination. It is of interest to note that conventional K^+ selective ISEs provide a potentiometric response to choline and can be used in the assembly of cholinesterase sensors. The potentiometric selectivity of potentiometric transducers can also impose restrictions on the ionic content of the supporting electrolyte required for potentiometric measurements to maintain the constant ionic strength of the solution.

The whole calibration curve of an ISE in semi-logarithmic plots has two extremes and a mid-range linear portion corresponding to the Nernst equation (Fig. 3.4). The limit of detection (LOD) is the activity (or concentration) of the primary ion at the point of intersection of the extrapolated linear section and the low concentration level of the signal.

Usually the conventional ISEs make it possible to detect from 10^{-1} to 10^{-6} M of the primary ion. Careful adjustment of the potentiometric membrane content and some modification of the ISE assembly diminish the LOD down to the picomolar level. The reproducibility of the e.m.f. measurements is about 1–2 %, but semi-logarithmic linearization yields the 7–12 % variation of the primary ion concentration. This is significantly higher than the accuracy of the measurements in voltammetry or spectrophotometry where the signal linearly depends on the analyte concentration. The wide range of linear response (up to five orders of magnitude) and a constant relative deviation of the signal in the whole range of concentrations are the advantage of the potentiometric sensors.

Mostly the biosensors based on potentiometric transducers include the pH—sensitive ISEs. Most of the enzymatic reactions, applicable to biosensors, i.e., ester hydrolysis and oxidation of organic matter, release organic acids that can be quantified by the appropriate pH shift in the surface layer. It should be mentioned that the pH of the bulk solution remains approximately constant because of the very small total conversion of the substrate in a single measurement. Thus, the pH shift is measured against a similar pH sensitive transducer with no membrane. The differential signal obtained does not depend on any chemical reactions changing the pH or buffer capacity of the solution. This mode is especially suitable for miniaturized devices, i.e., FET-based enzyme sensors. A pair of FETs is manufactured as a single set on the same support. The dual construction diminishes the complications related to pseudo-reference electrodes as well.

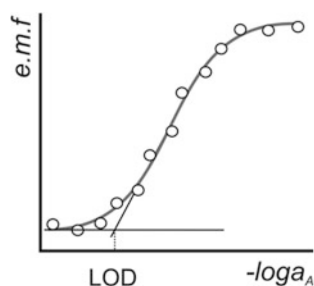


Fig. 3.4 Total calibration curve of potentiometric determination of the primary ion. The calculation of LOD is shown as the intersection of two lineal extrapolations

It should be mentioned that recording pH changes in an enzymatic reaction is contradictory itself. Indeed, the activity of an enzyme is always pH-dependent, with the maximum normally within about two pH units. For this reason, in a biochemical assay the enzymatic conversion of a substrate is usually conducted in buffered media to suppress the possible pH shift caused by accumulated products. After immobilization, the support, e.g., gelatin or BSA, exerts its own buffer capacity and is expected to additionally decrease the pH shift. In other words, all the efforts directed to stabilize and reproduce the signal of a biosensor will decrease the pH shift. This is inappropriate for potentiometric transducers.

As in many other cases typical for biosensor development, a compromise is necessary between the accuracy of the measurement and signal value. The buffer system cannot be fully excluded because of the low reproducibility of the enzyme activity in non-buffered media and significant interferences from the sample pH and matrix components. In basic media, the changes in pH of the solution can be due to the accumulation of CO_2 from the atmosphere. In acidic solutions, some substrates can be hydrolyzed. The potentiometric measurements are conducted in the presence of millimolar concentrations of the buffer. Besides, the storage solution can contain higher quantities of auxiliary reagents to stabilize the immobilized enzyme between biosensor operations.

The examples of the enzyme sensors based on the ISEs are presented in Table 3.2 (Janata 2009). Most of the examples refer to the pH sensors. The ISEs for ammonia and carbonate ions are also based on pH-sensitive electrodes covered

Table 3.2 Application of ISEs in the assembly of enzyme sensors

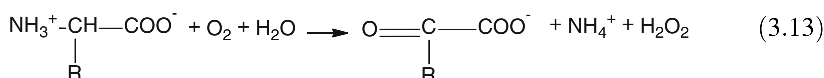
ISE (Primary ion)	Potential determining component	Enzyme	Analytes
H^+ (pH)	Organic acids released in enzymatic reactions	Hydrolases, oxidoreductases	Specific enzyme substrates and inhibitors
K^+	Choline	Cholinesterase	Acetylcholine, cholinesterase activity
NH_3/NH_4^+	Ammonia, primary amines	Urease, amineoxidase, amino acid oxidases, nitrate and nitrite reductases	Urea, selected amino acids or their total content, nitrates and nitrites
Choline	Choline	Cholinesterase	Acetylcholine, cholinesterase inhibitors
F^-	F^- (product of pentafluorophenol oxidation)	Peroxidase	Hydrogen peroxidase, peroxidase substrates
CO_3^{2-}	CO_2, organic acids	Cholinesterase, urease, tyrosine decarboxylase, carboanhydrase	Urea, acetylcholine, tyrosine, hydrolase substrates
CN^-	Cyanide	β-Glycosidase	Amigdalin
I^-	Iodide	Peroxidase	Peroxidase substrates

with selective membranes. The determination of choline is the only example of the use of potentiometric sensors specific for organic component.

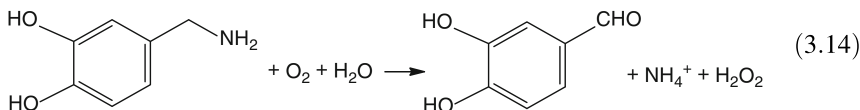
The urea biosensor with the enzyme immobilized onto the membrane of the NH_3 selective sensor has been developed for the detection of urea in urine and other biological fluids. The selectivity of the response is achieved by basic media in the internal filling of the electrode. This promotes both the reaction of urease, which has the maximum of the pH activity at pH = 8, and the transfer of the neutral NH_3 molecules through the membrane. Urease is also compatible enough with polyaniline and polypyrrole and can be immobilized on these polymers without any supports (Adeloju et al. 1996). The hydrolysis of urea (see Eq. (2.5) in Chap. 2) results in the formation of two ammonium and one carbonate ion so that the pH sensitivity of the signal is determined by relative acid-base properties of these particles. This reduces the slope of the calibration curve to about 22–30 mV versus theoretical 59 mV/pH for the biosensors based on pH sensitive transducers.

Some other enzymatic reactions resulting in the formation of ammonia are presented below (3.13–3.15).

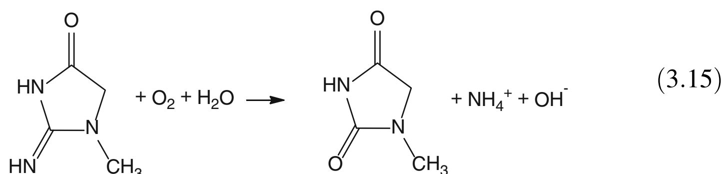
Amino acid oxdiase:



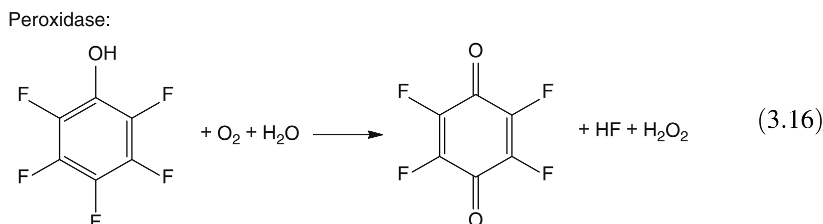
Amine oxdiase:



Creatinine deiminase:

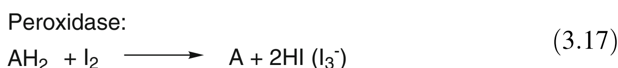


Two potentiometric peroxidase sensors were described. The use of fluoride ISE provides a very selective response toward the primary anion. The target reaction catalyzed by peroxidase includes the oxidation of pentafluorophenol to a quinoid product followed by release of one fluoride ion per substrate molecule (3.16) (Cowell et al. 1992).



(3.16)

The second approach assumes the detection of the potential related to the reduction of iodine as an artificial electron acceptor involved in the peroxidase reaction instead of molecular oxygen (3.17).



(3.17)

AH_2 is an organic substrate such as catechol, hydroquinone, ascorbic acid, phenothiazines, etc. Its peroxidase oxidation can be formally ascribed by a transfer of two hydrogen atoms. Appropriate sensors are mainly applied for the detection of hydrogen peroxide but can also be used for detecting peroxidase inhibitors.

The first urea biosensor was developed in 1969, seven years after the description of the first amperometric biosensor for glucose determination. For a rather long period of time, the development of potentiometric biosensors matched the amperometric devices "stride for stride". Later on, however, the interest in the potentiometric biosensors lessened due to the drawbacks related to the rather big size of conventional ISEs, the semi-logarithmic scale of the calibration plot, and the remarkable influence of the matrix component, especially the pH and buffer capacity for pH-sensitive sensors. The "renaissance" in the development of potentiometric biosensors began in the mid-1980s with the development of new transducers called ISFETs (Bergveld 2003). At first they were adapted for the pH measurements and development of appropriate enzyme sensors were considered earlier. Then, the family of the FET-related transducers was successfully applied for the detection of DNA sensors, especially in the framework of the research directed towards the biocomputer conception and application of nanomaterials, e.g., single-walled carbon nanotubes and nanoparticles of noble metals, in microelectronics and appropriate microsensors.

The FET-based sensors operate on principles that are different from those of canonical ISE. For this reason they do not belong to potentiometric transducers, but because of the similarity of behavior they are often discussed together.

The scheme of standard FETs and FET-based chemical sensors are outlined in Fig. 3.5.

The common FET (Fig. 3.5a) is made of a *p*-type substrate (body), e.g., doped silicon wafer. Two heavy *n*-doped regions, called source and drain, are created. Between them, a very thin (micrometers-thick) layer of SiO_2, an isolator, and a

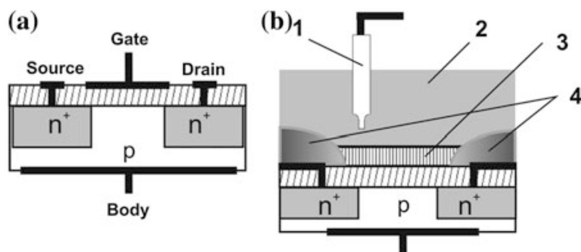


Fig. 3.5 Principal scheme of FET (**a**) and FET-based chemical sensor (**b**). *1* external reference electrode; *2* sample solution; *3* ion-selective membrane; *4* capsulation layer

metal film (gate) are deposited onto them. All the parts, i.e., source, drain, body and gate are provided with metal conductors. For the FET operation, the source and the body are grounded and voltage is applied between the gate and the source. This voltage repels the holes in the p-type substrate near the gate, resulting in the formation of a very thin n-channel of electron conductivity. The current recorded depends on the voltage applied. Thus, a small voltage can control the current flow between the source and drain. The FET described belongs to the family of MOSFET devices (metal—oxide—semiconductor).

In the chemical sensor based on FET (ChemFET, or ISFET for ion detection; see Fig. 3.5b), the metal conductor in the gate area is replaced with a membrane that is sensitive to the ion present in solution. The interaction results in the changes of the surface charge that resulted in the corresponding changes into the current between the drain and source. Thus the ISFET converts the changes in the charge of ions accumulated in the surface layer in the current recorded with an appropriate measuring device. Although the currents are very low (nano- and picoampers), modern amplifiers and current transducers make it possible to obtain a reliable analytical signal toward the primary ions present in the solution. The content of the membranes in ISFETs is generally the same as in conventional ISEs, but the thickness of the membrane is much less and the relative mass ratio of components (ionophore, plasticizer, etc.). In comparison with conventional ISEs, ISFETs show the slope of the calibration curve that is normally 10–15 % lower than the theoretical value. They are also inferior to ISEs in their lifetime, operational stability and metrological characteristics. However, miniaturization and the possibility to combine various reception systems and measurement modes are considered as the strength of ISFETs. The miniaturization and universal nature of the response based on recording the pH shifts during enzymatic reaction allowed assembling multi-analyte systems on a single chip or in a hybrid module. Thus, multi-enzyme EnFETs were developed for the simultaneous detection of glucose, sucrose and citric acid based on two FETs and for the detection of four species (urea, glucose, acetylcholine and *N*-acetyl-L-tyrosine ethyl ester) on an EnFET array.

The pH response of ISFET is achieved by applying oxides and nitrides of some elements that undergo reversible protonation. TiO_2, V_2O_5, Al_2O_3, Si_3N_4, and Ta_2O_5 are described for this purpose (Sandifer and Voychek 1999). The immobilization of enzymes onto the gate region has some limitations related to a lesser thickness of the protein layer and its accurate positioning, especially in the mass

production of the devices. The enzyme sensors utilizing ISFETs as signal transducers are called ENFETs. The list of enzymes used in EnFETs is about the same as that of the potentiometric biosensors described above. Urease has received the most attention, although penicillinase, glucose oxidase, glucose dehydrogenase, organophosphate hydrolase, and alcohol oxidase have also been used in EnFET assembly (van der Schoot and Bergveld 1987). Some recent technological solutions have been realized to solve the problems specified and improve the characteristics of EnFETs. Thus, additional charged membranes made of Nafion or poly(4-vinylpyridine-*co*-styrene) control the substrate/product diffusion and suppress the interfering influence of charged inhibitors and buffering components of the samples. Differential ISFET/EnFET sets were proposed for minimizing the influence of experimental factors on the signal generated by an enzymatic reaction (Fig. 3.6). pH-Static EnFETs were developed for recording the urease activity at constant pH of the surface layer. The exceeding amounts of H^+ ions are cathodically reduced to gaseous hydrogen. The charge required for this purpose is calculated in a real-time scale from the rate of the enzymatic reaction.

Similarly, the amplification of the signal related to glucose oxidase, the additional Pt microelectrode, was added to oxidize the H_2O_2 formed in enzymatic reaction with the formation of two additional H^+ ions releasing in the membrane of pH-ISFET based biosensor. All the modifications extended the linearity of the calibration graphs and increased the sensitivity of the pH-EnFETs toward the analytes.

The immunosensors based on FETs began their history together with EnFETs in 1979. When the enzymes are used as labels for detecting the *Ag*–*Ab* interaction, the assembly of immunoFETs does not significantly differ from that of EnFET utilizing the same enzyme. The direct (label-free) detection of the immunoreaction is of greater interest. In these devices, the *Ag* or *Ab* molecules are immobilized in the gate area, often inside the membranes. Since both molecules are charged, the interaction is expected to change the charge distribution to the extent allowing its reliable detection by a FET transducer. From the theory, a 10 mV response can be obtained for 10^{-7}–10^{-11} M of *Ag* molecules. This limit requires full monomolecular coverage of the gate area, highly charged *Ag* molecules and the low ionic strength of

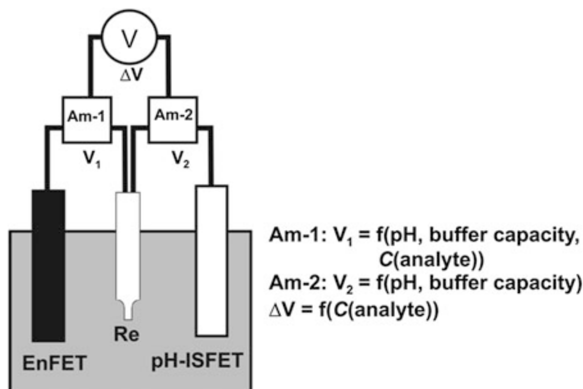


Fig. 3.6 Differential arrangement of ISFET and EnFET for elimination of the interferences related to the pH and buffer capacity of a sample tested. *Am-1* and *Am-2* amplifiers of the FET signals, *Re* reference electrode

the solution. Indeed, the size of the *Ab* molecule (about 10 nm) is much bigger than the thickness of the electrode double layer (1 nm in physiological conditions). For this reason, the largest part of the *Ab* molecule is located too far from the interface where the charge is shielded by small mobile counter ions from the solution. To some extent, the sensitivity can be increased by using the flow measurement with a sharp stepwise shift of the electrolyte concentrations.

DNA sensors based on FET transducers have been preferably developed for hybridization detection (Liu et al. 2010). In this biochemical reaction, two complementary DNA sequences interact with each other so that the density of the negative charge caused by a phosphate backbone significantly increases. This can be directly detected by FET with no specific membrane on an insulating SiO_2 layer. The concept was first proved with homo-oligonucleotides involving 20–1,000 single adenine bases.

A very interesting tool for the development of miniaturized immune- and DNA sensors has been developed with the FET based on a single carbon nanotube. The idea of the biosensor is illustrated in Fig. 3.7. The single-walled carbon nanotube is placed on the silicon platform. The drain and source areas are formed by an electroless plating. The treatment with mineral acids leads to the formation of carboxylic groups at the defects of the side wall. The immobilization of the aminated DNA probe or *Ab* molecules is performed by carbodiimide binding. The hybridization of DNA oligonucleotides as well as the *Ag*–*Ab* complex formation affects the semiconductor properties of the carbon nanotube similarly to the *n*-channel of conventional FET.

The pH ISFETs were successfully applied for the development of microbial biosensors as well. The vitality of the cells and the metabolism level are estimated by the relative acidification measured in the stop-flow regime with the suspension of the microorganisms pumped through a chamber with pH ISFETs or with the cell culture physically adsorbed on the gate area of the FET.

3.1.2 Voltammetric Sensors

Voltammetric experiments are carried out in an electrochemical cell consisting of a working electrode, a reference and an auxiliary (counter) electrode. As in the case of potentiometric devices, the amperometric biosensor can be a module of all

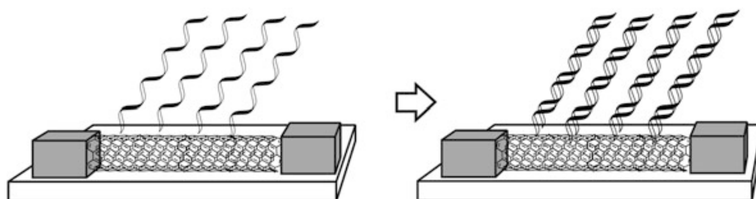


Fig. 3.7 DNA sensor based on FET on a single carbon nanotube

the electrodes assembled in one capsule. However, the biosensor more commonly plays a role of the working electrode. Thus, the measurement is performed in a cell with an analyte solution and two other immersed electrodes. As in potentiometry, the working cell contains a constant amount of the inorganic salts (KCl, Na_2SO_4) or mineral acids (HCl, H_2SO_4) inactive in the measurement conditions, called *supporting electrolytes*. This is necessary for the maintenance of the constant ionic strength and suppression of migration under the electrode polarization.

> *Migration (electrochemical migration)* is a movement of the charged particles in an electric field.

In accordance with the general principles of voltammetry, the working electrode is polarized in a way to maintain a constant or regularly changed (scanned) potential. The potential scanning is pre-determined by the parameters of the scan rate, initial and final (holding) potential. The control of the electrode polarization as well as current measurement is performed by a special analytical device called *potentiostat*, or *voltammograph*.

In modern voltammographs, the polarization of the working electrode is established against the auxiliary electrode, whereas its potential is recorded versus reference electrode. Such a mode, called a *three-electrode cell*, makes it possible to avoid the high current to be run through the reference electrode. If the currents are small enough, the counter electrode is unnecessary (*two-electrode cell*) and the functions of potential measurement and electrode polarization are combined. This refers to the ultra-small electrodes with the currents of several nanoamperes.

The current recorded in voltammetric experiments is directly related to the amount of the species oxidized or reduced on the electrode (3.18). Here, Ox denotes the oxidized form and Red the reduced form of the electroactive compound.

$$\begin{aligned} \mathrm{Ox} + \mathrm{ne}^- &\rightarrow \mathrm{Red} \\ \mathrm{Red} - \mathrm{ne}^- &\rightarrow \mathrm{Ox} \end{aligned} \tag{3.18}$$

The reactions (3.18) proceed on different electrodes, one on the working electrode and the other on a counter-electrode. The corresponding flow of ions takes places in the solution and the electron flow in metal conductors that close the circuit (Fig. 3.8).

Besides the electron transfer, the electrode reactions of organic species can include chemical steps, e.g., hydrogen/oxygen transfer, dimerization, cyclization, etc. Most of them are fast enough so that the product of the initial electron transfer can differ from that of the reversed reaction. The electrochemical and chemical steps are denoted with “E” and “C” letters, respectively, in accordance with their order in the full reaction of the analyte. Thus, the oxidation of hydroquinone is mostly an ECE process (3.19).

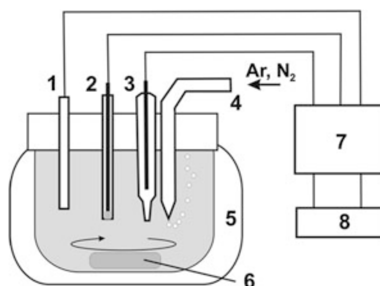
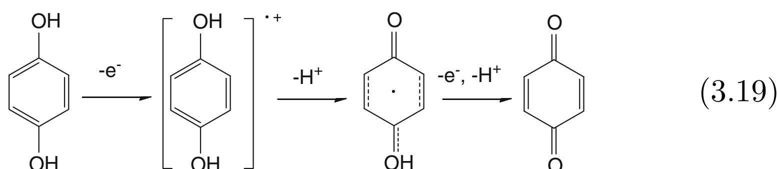


Fig. 3.8 Principal scheme of three-electrode cell for voltammetric measurements. *1* auxiliary electrode; *2* working electrode; *3* reference electrode; *4* deaerating system; *5* thermostating jacket; *6* magnetic stirrer; *7* voltammograph; *8* computer



(3.19)

The transfer of the second hydrogen ion does not affect the current value and is omitted from the mechanism notation. In amperometric biosensors, mostly oxidation of organic substances is used. The reduction of molecular oxygen and hydrogen peroxide is the only significant exception.

The relationship between the current I and electric charge is defined by Faraday's Law (3.20) (Wang 2006; Bard and Faulkner 2001).

$$I = nF\frac{\mathrm{d}N}{\mathrm{d}t}, \tag{3.20}$$

in which n is the number of the electrons passing, F is the Faraday constant (96485 C) and dN/dt is the oxidation/reduction rate, mole per second. The reaction rate can be expressed by the reactant flux j per unit of the electrode surface area S (3.21).

$$I = nFSj, \tag{3.21}$$

The Eqs. (3.20) and (3.21) describe the so-called *Faradaic currents*, which are related to the redox conversion of chemical compounds and hence provide information about the quantity of an analyte. The particular formulation of the equations depends on the conditions of mass transfer and the electrode geometry that specify the flux j. There are always non-Faradaic, or *background current* in addition to the Faradaic current. It is related to the double layer formation (charging current) and to the ohmic drop of the potential due to the impedance of the solution separating the working and auxiliary electrode. The background current does not describe the redox conversion of an analyte and very much depends on the electrode material, modifier layers and ionic content of the solution. The Faradaic currents are always determined against the background current.

The correction can be performed by special processing of the *I–E* curve performed manually (tangent lines plotting), or by the software (differentiation of the curves).

The contribution of non-Faradaic currents can be rather significant, especially in organic solvents and for the electrodes covered with low-permeable and non-conductive coatings. The immobilization of proteins and nucleic acids increases the charge of the electrode interface and decreases the rate of the small ions transfer. This decreases the ratio of Faradaic and charging currents. On the other hand, changes in the non-Faradaic currents can be applied to the quantification of the amounts of biopolymers immobilized or adsorbed on the electrode surface. Such an approach to the detection of biomolecules is not as sensitive and selective, but it was realized for some biomedical assays, especially those based on micro-sensors. In complicated cases, the contribution of non-Faradaic currents can be decreased by the modulation of the potential applied or by the inclusion of additional resistors in the circuit with the impedance close to that of the ohmic drop occurring in the system.

The ability of a substance to reduce or oxidize on the electrode depends on its standard redox potential, E_0. The higher the positive value, or the lower (more negative) its negative value, the higher the power of the compound as an oxidant and reducer, respectively. The E_0 values of individual compounds are summarized in handbooks on physical chemistry together with other thermodynamic constants, e.g., melting point, free energy of formation, etc. However, the potential necessary for the electron transfer in real conditions is mostly higher than that expected from the thermodynamic constant. The difference between the real and theoretical potential value is called *overvoltage* (*overpotential*) $\eta = |E - E_0|$. An additional energy is required for the removal of the solvation sphere of the ions discharged, for new phase formation (electrocrystallization of metals or evolving gas), and for positioning the complex organic species onto the electrode surface. In the latter case, the overpotential is required to reach close contact between the electrode and the functional group of organic molecules able to accept/give the electron. In accordance with the Marcus' Eq. (3.22) (Tachiya 1993).

$$k_{et} \sim \exp[-\beta(d - d_0)] \exp\left[-\frac{(\Delta G^0 + \lambda)^2}{4RT\lambda}\right] \tag{3.22}$$

the rate constant of the electron transfer k_{et} depends on the distance d of the electron transfer Gibbs energy ΔG^0 and the solvent reorganization energy λ (d_0 is the van der Waals distance denoting the size of the molecule). For example, bulky and rather rigid DNA molecules cannot provide a high number of the contact points while adsorbed on the solid electrode surface. For this reason, the signals of the direct oxidation of nucleotides are small and require special protocols for subtracting the charging current.

The standard redox potential determines the theoretical limit of the potential required for the redox reaction. The overpotential can be diminished by special auxiliary agents called *mediators of electron transfer* (or simply *mediators*). They can be added to the solution or attached to the electrode surface prior to or together

with the biochemical component of a biosensor. In some cases, mediators are implemented in the structure of biopolymers by covalent binding.

The amperometric detection of a target biochemical reaction is possible only if no other compounds are oxidized (reduced) in the potential area near the standard redox potential. To verify this requirement, the conditions of voltammetric detection should be investigated. Firstly, the potential window is marked. This is an area on the potential axis of the voltammogram in the *I–E* plots where only non-Faradaic currents are recorded in the supporting electrolyte solution. In other words, no other species are involved in the current generation. The potential window depends on the electrode nature and ionic content of the solution. Aqueous solutions are mainly used for biosensors, and chemically inert electrodes serve as signal transducers (silver, gold, platinum, glassy carbon). In the cathodic area, the working window of the potentials is mainly limited by the reduction of hydrogen ions (in acidic media) and water (3.23).

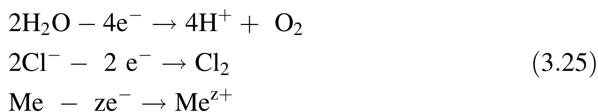
$$\begin{array}{l} \mathrm{Red} - ne^- \rightarrow \mathrm{Ox} \\ 2H_2O + 2e^- \rightarrow H_2 + 2OH^- \end{array} \tag{3.23}$$

Transient metals and dissolved oxygen can also interfere with target redox reactions (3.24).

$$\begin{array}{l} Me^{z+} + ze^- \rightarrow Me \\ O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \end{array} \tag{3.24}$$

The alkali and alkali-earth metals, as well as most inorganic anions, are stable at the cathodic potentials. The reduction of molecular oxygen is mainly observed on the Au and *Ag* electrodes. The chemosorption of the oxygen results in the formation of very thin layers of metal oxides that are reduced at appropriate potentials. On carbon materials, the reduction of oxygen is kinetically hindered and does not interfere with other electrode reactions.

In the anodic area, the oxidation of water molecules can take place. From supporting electrolytes, chlorides can be oxidized to chlorine, a reactive species that underwent chemical reactions with the organic species present in the sample. The high concentration of chlorides and the evolving oxygen can promote the anodic dissolution of metallic anodes (3.25).



$$\begin{array}{l} 2H_2O - 4e^- \rightarrow 4H^+ + O_2 \\ 2Cl^- - 2e^- \rightarrow Cl_2 \\ Me - ze^- \rightarrow Me^{z+} \end{array} \tag{3.25}$$

The overpotential of particular redox reactions as well as their mechanism depends on the electrode material and ionic content of the solution so that the working window of potentials varies. Usually, modern electrochemical systems allow recording currents at ± 2 V. If required, the window can be extended by the addition of organic solvents miscible with water and substitution of conventional

electrolytes with the salts of tetraalkylammonium, more stable at cathodic potentials.

Let us consider the reaction of heterogeneous electron transfer on a flat electrode, the size of which is high enough to disregard possible influence on its border. In this case, the mass transfer is described by the first Fick's Law (3.26).

$$j(x,t) = D\frac{\mathrm{d}[c(x)]}{\mathrm{d}x} \tag{3.26}$$

The equation assumes steady-state conditions and requires only one axis directed orthogonally to the electrode plane for the description of the spatial reactant distribution. The driving force of the transfer is the inequality of the reactant concentration in the proximity of the electrode interface and in the bulk solution. The model described is called a semi-infinite orthogonal diffusion to the flat electrode.

> Diffusion is a movement of a substance from the area of its high concentration to the area of lower concentration.

Migration and convection (mass transfer of dissolved particles with liquid flux) are alternative mechanisms of the mass transfer near the electrode surface. However, their contribution can be minimized. The migration is suppressed by the addition of excessive amounts of the supporting electrolyte and the convection is commonly realized only with external stimuli, such as solution heating or mechanical mixing.

The combination of (3.21) and (3.26) brings about:

$$I_d = nFSD\frac{\mathrm{d}c(x)}{\mathrm{d}x} \tag{3.27}$$

Here I_d is the current value, which is determined by diffusion (diffusional current). For the case under discussion, the changes in the reactant concentration can be regarded as linear within a thin layer at the electrode interface (reaction layer), the thickness of which is equal to δ (Fig. 3.9).

This assumption makes it possible to substitute the derivative $\mathrm{d}c/\mathrm{d}x$ by an algebraic fraction.

$$\frac{\mathrm{d}c(x)}{\mathrm{d}x} = \frac{c_0 - c_S}{\delta} \tag{3.28}$$

where c_0 and c_S are the concentrations of a reactant in the bulk solution and on the electrode surface, respectively. The maximum current is reached at the full exhaustion of the reactant on the electrode surface ($c_S = 0$). Each molecule reaching the electrode is immediately oxidized (or reduced) so that the difference in the numerator of the right part of the Eq. (3.28) becomes maximal (3.29).

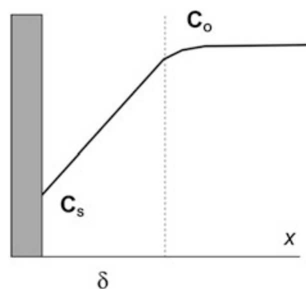


Fig. 3.9 The dependence of the reactant concentration on the distance to the electrode for semi-infinite orthogonal diffusion to a flat electrode

$$(I_d)_{\max} = \frac{nFSDc_0}{\delta} \tag{3.29}$$

In the theoretical consideration of amperometric sensors, the reaction layer δ is often associated with the membrane covering the electrode. For biosensors, this assumption is valid only if the surface layer does not participate in the formation or consumption of the redox active species. Thus, for enzyme sensors, the substrate is converted within the surface layer so that diffusion is counterbalanced by the rate of enzymatic reaction. This complicates the behavior of the amperometric enzyme sensors, the signal of which refers to the substrate consumption (molecular dioxygen for oxidoreductases) or product release (hydrogen peroxide in reactions of dehydrogenases, (see Eq. 2.13) for glucose oxidation as an example).

In other systems, the reaction layer depends on the particular conditions of the voltammetric measurement. In *chronoamperometry* the potential is stepwise changed from the E_1 value with no electrode reactions occurred to E_2 value near the maximal rate of the electron transfer. The current produced by the electrode reaction is recorded as a function of time (Fig. 3.10).

The reaction layer increases in time, due to the gradual exhaustion of the surface layer, which is due to the slower diffusion rate (3.30).

$$\delta = \sqrt{\pi D t} \tag{3.30}$$

The implementation of (3.30) in the Eq. (3.29) produces the *Cottrell* Eq. (3.31), describing the current run in the potentiostatic experiment.

$$I_d = nFSc_0\sqrt{\frac{D}{\pi t}} \tag{3.31}$$

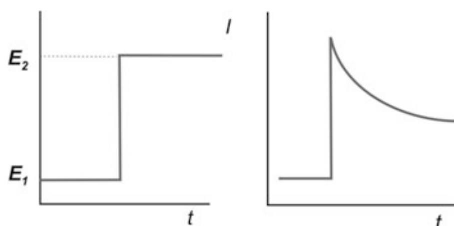


Fig. 3.10 The potential and current changes in the chronoamperometric signal measurement on a flat electrode at semi-infinite diffusion

Chronoamperometry is suitable for recording biochemical interactions, especially in flow-through biosensors. The detection of the current in a definite period of time after the analyte injection is a simple and popular approach that does not require any special software for its realization and provides the comparison of various biosensors with a different content of the surface layer but with similar thickness and permeability.

Meanwhile, dynamic methods are more suitable for the investigation of electrode reactions. In them, the potential is shifted in accordance with a pre-determined rule and the current is recorded as a function of the potential. Linear direct-current (linear sweep) voltammetry is an example of such a technique. The potential is swept from the initial E_1 potential to the final E_2 potential (Fig. 3.11). This process is also called *scanning*. In modern voltammographs, a staircase potential sweep is applied, instead of a true linear change. While reaching final value, the potential can be swept at the same rate in the reverse direction. This mode is called *a cycling voltammetry*. The scanning can be repeated with intermediate stopping the potential at extreme values—or continuously. The additional steps of a constant electrode polarization can be inserted in the triangle potential sweep for cleaning the electrode or for the accumulation of the electrolysis products.

The principal advantage of linear sweep voltammetry and especially of cycling voltammetry is that it provides information on both the nature of the redox reaction and the quantity of the species oxidized (reduced) on the electrode.

The useful scan rates vary from 1 to 1000 mV/s, although the rate over 10 V/s is technically available as well. Ultra-fast scanning is performed for the investigation of the electrode reactions and is rarely used in biosensor operations.

If the solution contains a particle able to undergo the electrode reaction within the potential window, the current on the *I–E* curve (*voltammogram*) first grows to reach a maximal value called the "peak current" and then slowly decays according to the Cottrell equation. Each peak on the voltammogram theoretically corresponds to a single electrode reaction, its position (peak potential $E_{p,a}$ and $E_{p,c}$ for anodic and cathodic peaks, respectively) indicates the nature of an oxidized (reduced) species and its height (peak currents $I_{p,a}$ and $I_{p,c}$) depends on the number of electrons transferred and the concentration of the species in bulk solution. If the electron transfer is faster than the mass transfer at the electrode interface and is not complicated with any slow chemical steps and chemosorption, the electrode

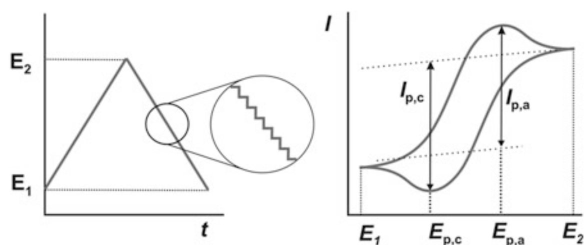


Fig. 3.11 The potential and correct shifts in cyclic voltammetry for a reversible process

reaction is electrochemically *reversible*. In this case, scanning the potential in the reverse direction (from E_2 to E_1) results in the formation of a symmetrical peak on the curve. This corresponds to the transfer of the same number of electrons as in the direct scan and to the formation of the initial compound (see reactions 3.18). The resolution of the anodic and cathodic peaks related to the reversible electrode reaction provides the number of electrons transferred in the rate-limiting step (3.30). The half-sum of the peak potentials is equal to the standard potential of the redox reaction. The latter assumes the equality of the diffusion coefficients of oxidized and reduced forms.

$$\begin{aligned} \Delta E_p &= \left|E_{p,a}\right| - \left|E_{p,c}\right| = 2.303\frac{RT}{nF} \\ E^0 &\approx \frac{E_{p,a} + E_{p,c}}{2} \end{aligned} \tag{3.32}$$

The peak potential separation should not vary with the concentration of the reactant and scan rate. The concentration of the reactant in case of a reversible diffusional current can be determined by *the Randles-Sevčik* Eq. (3.33) (Bard and Faulkner 2001).

$$I_p = 2.69 \times 10^5 n^{3/2} S c_0 D^{1/2} v^{1/2}, \tag{3.33}$$

in which S is the active electrode surface area, m^2, c_0 the concentration of the electroactive species in the bulk solution, mol m^{-3}, D is its diffusion coefficient, $m^2\ s^{-1}$, n is the number of electrons transferred, and v is the scan rate, V s^{-1}.

If the non-electrochemical stages become significant for the current generation, the reaction becomes semi-reversible and irreversible. In comparison with reversible processes, irreversible voltammograms are characterized by smoothened peaks with elongated current growth and a less pronounced maximum. For semi-reversible reactions, the peak separation is larger than the theoretical 59 mV (one electron exchange at 25 °C). The symmetry of anodic and cathodic peaks remains as in the reversible reaction. The irreversible process is characterized by a decrease of the peak on the reversed branch of the cyclic voltammogram and a high (more than 100 mV) separation of the peak potentials. For irreversible reactions with a slow electron transfer, the Eq. (3.33) is transformed into Eq. (3.34) in the absence of other complicating factors.

$$I_p = 2.99 \times 10^5 n (\alpha n)^{1/2} S c_0 D^{1/2} v^{1/2} \tag{3.34}$$

The empirical coefficient α, called the transfer coefficient, can vary from 0 to 1 and reflects the contribution of the direct electron transfer in the total current value. It can be interpreted as a measure of the symmetry of the energy barrier of the electron transfer. For all the overpotentials, the ideal α magnitude is 0.5 but it can actually vary. The determination of α is required for the quantification of an irreversible electrode reaction (determination of the rate of electron transfer, the number of the electrons transferred, etc.).

The number of electrons transferred in an irreversible electrochemical reaction can be also determined from the dependence of the peak potential on the scan rate (3.35).

$$E_p = E^0 - \frac{RT}{\alpha nF}\left[0.78 - \ln\frac{k_{et}}{\sqrt{D}} + \ln\left(\frac{\alpha nFv}{RT}\right)^{1/2}\right] \tag{3.35}$$

The slope of the curve in the plots E—$\ln(v)$ is equal to:

$$\frac{\mathrm{d}E_p}{\mathrm{d}\ln v} = \frac{1}{2}\frac{RT}{\alpha nF} \tag{3.36}$$

If the electrochemical substance is involved in the electron exchange when adsorbed on the electrode surface, the morphology of the peaks is significantly changed (Fig. 3.12). The peak becomes bell-shaped, with the final current decaying back to the background value. The peak current linearly depends on the scan rate in accordance with (3.37), in which Γ is the surface coverage (concentration of the analyte per electrode square unit). The surface coverage can be determined from the charge Q passing (3.38) calculated by integrating the peak after the subtraction of the background current.

The description of the heterogeneous rate constant of the electron transfer k_{et} was described by Laviron (Laviron 1995). In accordance with his approach, the peak current is plotted versus $\log(v)$ and the slope (3.39) is calculated. Then k_{et} is determined from (3.38).

$$I_p = \frac{n^2F^2}{4RT}vS\Gamma \tag{3.37}$$

$$\Gamma = \frac{Q}{nFS} \tag{3.38}$$

$$\frac{\mathrm{d}E}{\mathrm{d}\log v} = \frac{2.303RT}{\alpha nF} \tag{3.39}$$

$$\log k_{et} = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\left(\frac{RT}{nFv}\right) - \frac{\alpha(1-\alpha)nF\eta}{2.303RT} \tag{3.40}$$

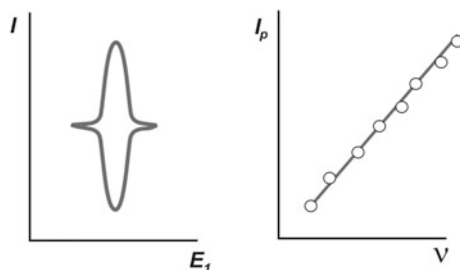


Fig. 3.12 Cyclic voltammogram and dependence of the peak current on the scan rate for a surface electron exchange

The determination of electron transfer rate is important because it becomes possible to compare the efficiency of the electron transfer in various modifications of the electrodes used for biosensor development. The analysis of the peak shape and calculations of the number of electrons transferred are also applied for confirming the mechanism of the signal generation and its dependence on the content and structure of the surface layer.

It should be mentioned that the electron exchange between the reduced and oxidized species can restore a more familiar peak shape with mild current decay after reaching maximal value even though the redox species remain fixed at the electrode surface. Such a mechanism was realized for the SAMs containing terminal ferrocene groups and for the electrodes covered with non-conductive polymers bearing ferrocene units.

The sensitivity of linear sweep voltammetry is limited by the relationships between the Faradaic and background current. The idea of the reduction of this influence involves a different behavior of the charging and Faradaic currents while the potential is sharply changed. The Faradaic current decays with $t^{-1/2}$ for diffusion-controlled reactions, and the charging current decays exponentially with time. Accordingly, the sampling current at the end of each potential pulse can make the contribution of the charging current insignificant. In this way, the sensitivity of voltammetry can be substantially increased. The modern pulse techniques realized in voltammographs differ in the pulse form and in the way the current is sampled.

Some popular modes of pulse voltammetry are briefly considered below (Fig. 3.13).

In *normal pulse voltammetry* [Fig. (3.13a)], a series of potential pulses with constant width and increased amplitude are applied. The potential returns to its initial value after each pulse. The duration of each pulse is from 1 to 200 ms; the interval between the pulses is several seconds, and the current is measured at a certain period at the end of each pulse. The current value is described by the modified Cottrell Eq. (3.41).

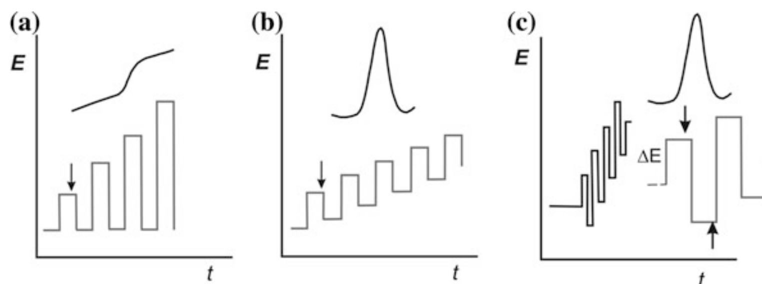


Fig. 3.13 Potential modulation and *I–E* curve shape in normal pulse (**a**), differential pulse (**b**), and square-wave voltammetry (**c**)

$$I = nFSc_0\sqrt{\frac{D}{\pi t_1}} \quad (3.41)$$

Here, t_1 is the lag time from the pulse start to the recording point.

In *differential pulse voltammetry* [DPV, Fig. (3.13b)], small pulses of constant amplitude (10–100 mV) are superimposed on a staircase-wave form. The current is measured twice in each pulse, first at the beginning and then at the end of the same pulse. The difference in the current measured is plotted against the applied potential and the graph is called a differential pulse voltammogram. The symmetrical peak corresponds to the electrode reaction, with the maximum proportional to the concentration of a redox species (3.42).

$$I_p = nFSc_0\sqrt{\frac{D}{\pi t_1}\left(\frac{1-\sigma}{1+\sigma}\right)},\ \sigma = \exp\left(\frac{nF}{RT}\frac{\Delta E}{2}\right) \quad (3.42)$$

In square-wave voltammetry [Fig. (3.13c)], two pulses are superimposed in opposite potential directions in each measurement cycle. The amplitude of each pulse is much higher than that considered in other pulse techniques so that the product of the electrode reaction is involved in the electron transfer at the second pulse. The current is recorded twice, at the end of the first (direct) pulse and at the end of the second (reversed) pulse. The difference in the currents obtained in each measurement cycle is plotted against the potential as described for the DPV.

Amperometric sensors modified with electrocatalysts. For voltammetric sensors and biosensors, efforts are mostly concentrated on the decrease of the working potential and increase of the signal sensitivity. The decrease of the working potential is directed toward the improvement of the biosensor selectivity, particularly the possibility of measuring signal in the presence of other electrochemically active substances in the sample. Both goals are attained by inclusion of the species able to accomplish the reversible electron exchange near the overpotential of target analyte reactions. They are commonly called *electrocatalysts*. Their efficiency can be related either to inclusion in the electron exchange chain or to the promotion of the redox conversion of the analyte itself. In the first case, the signal of the modifier is increased due to the electron exchange with the analyte molecule, also called a substrate. The signal of the analyte proper can remain constant, or even no special signal can occur. Such modifiers are called *mediators of electron transfer*. In the second case, the signal of the substrate is shifted to the lower potentials against those obtained on an unmodified electrode. The peak current increases with the substrate and electrocatalyst concentrations. The promotion of the analyte oxidation (reduction) is attained in this case by its surface accumulation or stabilization of intermediate products, by suppression of non-desired adsorption and some other reasons related to the decrease of the energy barrier of the electron transfer. Thus, such electrocatalysts increase the heterogeneous reaction rate of the electron transfer from/to the substrate molecule (Kano and Ikeda 2000).

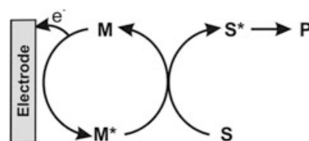


Fig. 3.14 Scheme of electrode reaction with mediated electron transfer, *M* mediator, *S* a substrate, *M** and *S** products of electron transfer, *P* final product

The principal scheme of the mediator functioning is presented in Fig. 3.14. Here, the substrate involves the compound that is oxidized or reduced on the electrode modified with a mediator *M*. The latter is fixed on the electrode surface and cannot leave the electrode, at least within the measurement duration.

The symmetrical peak typical of the reversible reaction of adsorbed mediator (see Fig. 3.12) changes after the substrate addition. The peak current of the initial *M* form increases whereas the opposite peak related to the product *M** of the electron transfer becomes lower. The changes grow with the substrate concentration down to the full disappearance of the *M** peak. This simplified scheme of the electrode reaction is complicated when the substrate *S* participates in non-mediated oxidation (reduction) on the electrode and the homogeneous electron transfer between *M** and *S* has the efficiency different within the surface layer and in bulk solution. The contribution of the mass transfer outside the mediator layer can decrease the efficiency of immobilized mediators. In this case, another mediator is added to the solution to establish the wiring of the immobilized mediator and a substrate molecule. This is often observed for biosensors based on the immobilized mediators and biomolecules that are not mobile enough for electron shuttling (Fig. 3.15).

The number of mediator systems can exceed two, but in most cases the scheme described is sufficient for reaching a high efficiency of electron mediation. In oxidoreductases, the cofactor is involved in the chain of electron exchange like a common mediator. For hydrolases, the mediated oxidation of the product of enzymatic reaction is frequently used. Some examples will be given below.

The choice of the mediator most suitable for a particular substrate and biosensor system is an important goal of the design of the electrochemical biosensor. The following criteria of mediator selection were specified (Chaubey and Malhotra 2002):

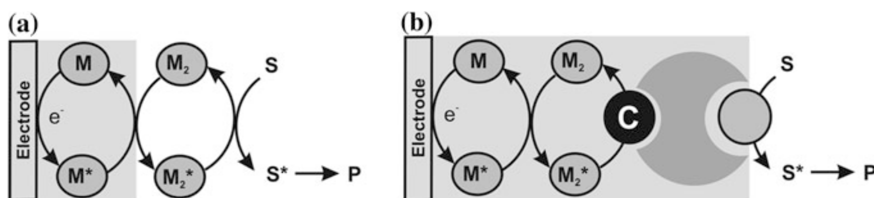


Fig. 3.15 Mediated electrode reactions with inclusion of diffusionally free mediator system M_2. **a** substrate conversion; **b** biosensor with oxidoreductase immobilized in the surface layer, enzyme cofactor; **c** participating in the electron transfer

1. *Thermodynamic criterion*. The standard redox potential of the mediator system should be higher than that of the substrate:

$$E^0_{M/M*} > E^0_{S/S*}$$

This provides a thermodynamically spontaneous electron transfer between the reactants. The same requirement is valid for the arbitrary number of intermediate mediators:

$$E^0_{M/M*} > E^0_{M_1/M_1^*} > E^0_{S/S*}$$

It is important that this limitation be related to thermodynamic redox potentials but not overpotentials. In the voltammogram, the peak of the mediator always precedes the peak of a substrate. The overpotential rarely exceeds 500–600 mV; this limits the difference between the peak potentials recorded for a mediator and a substrate in a non-mediated process.

2. *Kinetic criterion*. The rate of mediated electron transfer should be higher than the rate of direct electron transfer between the substrate and electrode. For this reason, the use of organic complexes of transient metals has an advantage over the aqua-ions. The oxidation of the complexes is not accompanied by any significant changes in the hydrate sphere of the ion. The rate of electron transfer is additionally increased if the organic ligands participate in the electron transduction (bipyridine, phenanthroline ligands, etc.).
3. *Chemical criterion*. The oxidized and reduced forms of a mediator should be stable in the measurement conditions and not interact with any components present in the solution. Thus, the stability of metal complexes is limited with their partial hydrolysis and the stability of organic mediators by their oxidation with dissolved oxygen and oligomerization. The pH-sensitivity of the mediator potential is considered its drawback, especially for enzyme sensors. The target biochemical reaction can shift the pH on the electrode surface by 1–2 units. This affects the redox activity of the pH-depending mediator and hence the electrocatalytic response.

The electron exchange can rarely be limited by the associated transfer of counter ions. Thus, the reaction of mixed metallocyanates is K^+ sensitive (Nguyen et al. 2009). The redox-reaction of Ni(II) ferrocyanide is presented below.

$$KNi[Fe(CN)_6] + K^+ \overset{e^-}{\leftrightarrows} K_2Ni[Fe(CN)_6] \quad (3.43)$$

The limitation of the redox activity of polyaniline by the limited access of counter ions is another example of such a mechanism. This can be used for analytical purposes if the mediation activity of the olymer is estimated in the presence of growing amounts of DNA molecules. The shielding of the redox centers suppresses the catalytic current of polyaniline on the voltammogram.

The number of mediators used in the assembly of biosensors is extremely high. Most substrates converted in mediated reactions involve the co-factors of the oxidoreductases (NAD^+/NADH, $NADP^+$/NADPH, FAD/$FADH_2$, NAD^+/NADH, PQQ), oxygen as the substrate, and hydrogen peroxide as the substrate/product of oxidoreductases. The mediated reactions are used in DNA sensors based on electrochemically active intercalators. The most popular examples of mediators used in the biosensor assembly are outlined in Fig. 3.16, and the targets of their application are given in Table 3.3. The references given dot exhaust the variety of application and are examples of approaches.

Most mediators can be easily deposited from the precursor solution followed by thermal treatment. Thus, the metal oxides are precipitated in hydrolysis and oxidation by oxygen and high temperature. Prussian blue is formed by mixing iron

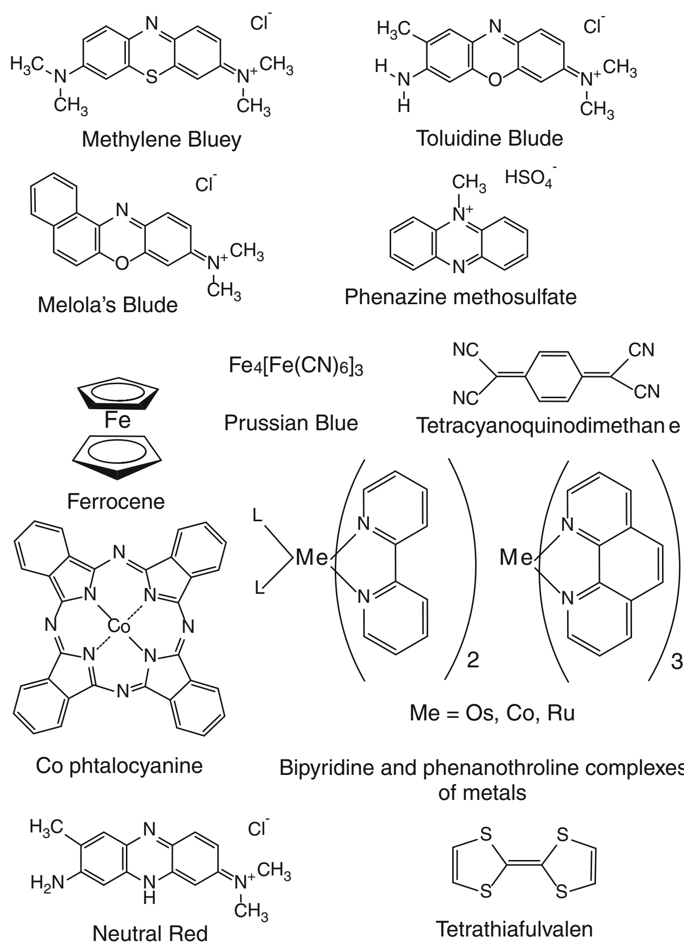


Fig. 3.16 Mediators frequently used in electrochemical biosensors

Table 3.3 Application of mediators in the electrochemical biosensors

Mediator	Reactant	Biochemical component	Target analyte
Metallocyanates	Hydrogen peroxide (Varma et al. 2006)	Oxidoreductases	Enzyme substrates and effectors
	Thiols (Suprun et al. 2005)	Acetylcholinesterase	Acetylthiocholine, organophosphate inhibitors
Phenazines	NADH (Karyakin et al. 1999)	NAD dependent oxidoreductases	Enzyme substrate
Phenothiazines	Hydrogen peroxide (Liu et al. 1999)	Oxidoreductases	Enzyme substrates
	DNA (Yabuki and Mizutani 2004)	DNA	DNA intercalators, DNA damaging factors, hybridization detection
Phtalocyanine and bipyridine complexes of metals	DNA (Wang et al. 2011)	DNA	
MnO_2, CeO_2	Oxygen, hydrogen peroxide (Dontsova et al. 2011)	Oxidoreductases	Enzyme substrates
	Oxygen, hydrogen peroxide (Lu et al. 2011)	Microorganisms	Metabolites, antimicrobial preparations
Tetracyano-quinodimethane	Hydrogen peroxides, NADH (Cano et al. 2008)	Oxidoreductases	Enzyme substrates
Ferrocene derivatives	$FADH_2$ (Sato and Okuma 2008)	Glucose oxidase	Glucose
	Hydrogen peroxide (Boujtita et al. 1996)	Lactate oxidase	Lactate
Ferricyaninde	Hydrogen peroxide (Ojani et al. 2009)	Oxidoreductases	Enzyme substrates
	Thiols (Neufeld et al. 2000)	Acetylcholinesterase	Organophosphate inhibitors
	Methylene blue (Li et al. 2007)	DNA	Hybridization detection

(III) and ferricyanide salts. Recently, the three-dimensional structures with the mediators entrapped in gels and polymeric layers were obtained by gelation and polymerization performed in the presence of a mediator suspension. Covalent linking to biopolymers or functional groups of supports provides a single point binding required for a more effective electrode transfer to the enzyme cofactors and DNA sequences. In other words, all the approaches to the immobilization of biocomponents are applicable for mediator binding.

Over the past decade, the site-specific binding of organic mediators is of special note due to the advantages of the directed modification of biomolecules and the progress expected in developing molecular receptors with an ultra-high sensitivity of the redox reactions. The consecutive complication of the molecular assemblies created can be illustrated by schemes in Fig. 3.17.

Firstly (Fig. 3.17a), the carboxylated mediator *M* can be covalently attached to aminated surfaces. The SAMs technology can be applied to establish close contact with an electrode. The golden electrode is first treated with cystamine to obtain a monolayer with the terminal amino group. Then the electrode is covered with a film of immobilized enzyme that retains the ability toward the electron exchange with the electrode via the mediator system (Zayats et al. 2008).

Secondly (Fig. 3.17b), the mediator can be covalently attached to the cofactor excluded from the enzyme. After that, the apoenzyme is combined with the cofactor modified with the modifier to restore the enzyme activity (Willner and Willner 2001). The same approach is used for site-specific binding of the enzyme to the electrode. And finally (Fig. 3.17c), the mediator can be implemented in a protein matrix formed by glutaraldehyde.

The use of mediators can affect the mechanism of the redox conversion of the substrate in comparison with reactions on a bare electrode. This can be due to changes in the hydrophilicity of the transducer surface. But most frequently, the changes are related to the intermediate chemical steps of the reaction.

The oligomerization of intermediates is often suppressed as well as the relative contribution of competitive paths of their reaction (dimerization versus hydrogen removal). The pH maximum of the current recorded can shift be for the same reasons.

This means that the introduction of mediators calls for optimization of the measurement conditions usually performed prior to the biocomponent immobilization. The amounts of mediators and the protocol of modification are considered in the presence of potential species to be oxidized (reduced) in the biosensor functioning and the working potential and efficiency of mediation are determined with particular emphasis on the compatibility of the measurement conditions with those of biocomponent functioning.

Besides organic compounds and complexes and oxides of transient metals, other species can play the role of mediators. Thus, Au nanoparticles exhibit the electrocatalytic current of the metal oxidation/oxide reduction that is substantially suppressed by the limitation of the substrate access due to biochemical interactions.

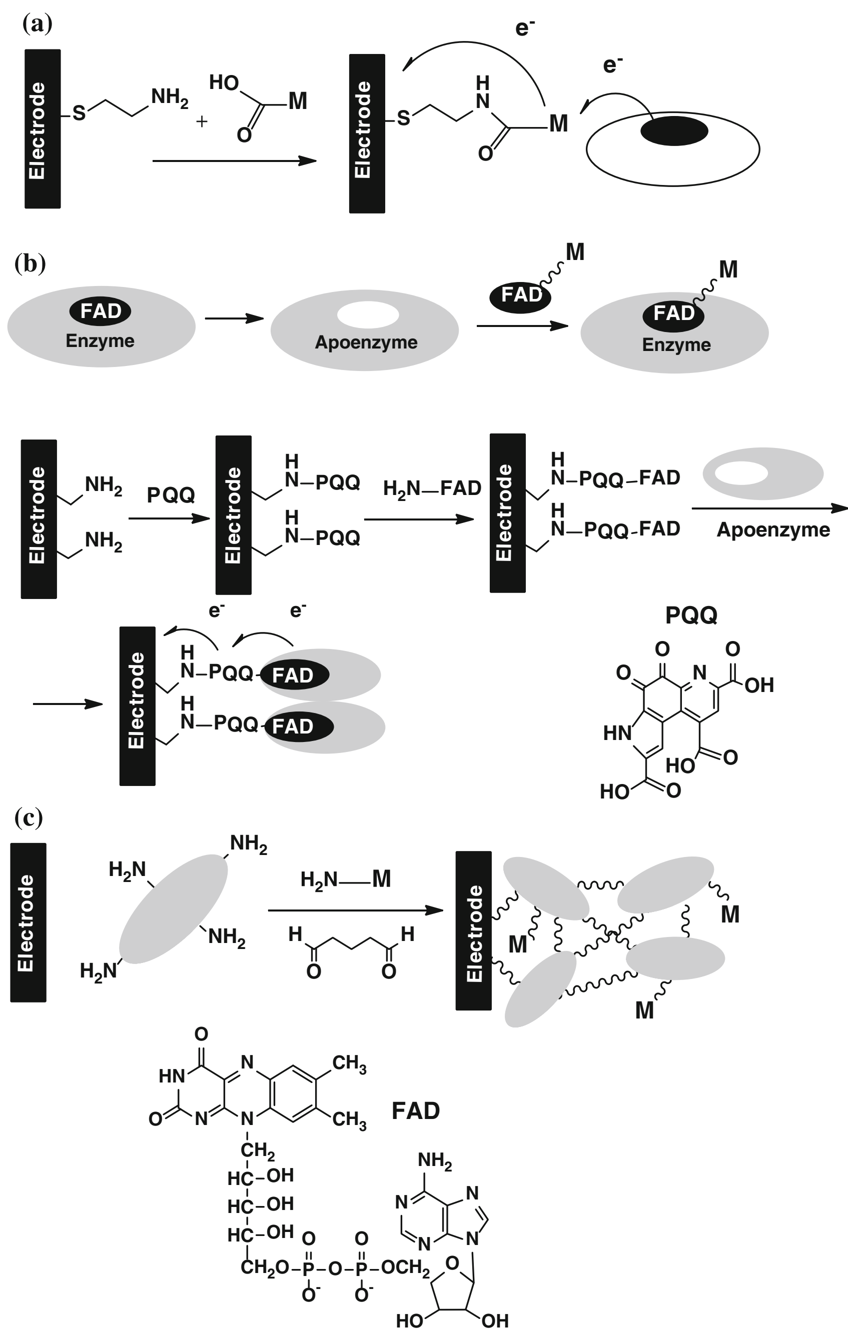


Fig. 3.17 Covalent attachment of mediator *M* in the enzyme sensor assembly

The immunosensor based on this principle of the signal measurement consists of an electrode covered with the Au nanoparticle suspension with the covalently attached *Ab* molecules. The addition of specific *Ag* diminishes the amperometric signal measured by direct current voltammetry (Zhang et al. 2009).

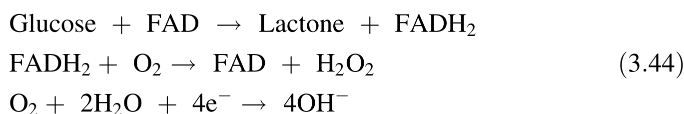
Hemoproteins, e.g., hemin, hemoglobin, cytochrome *c*, and myoglobin, also participate in the mediated electron transfer although their efficiency is lower than that of low-molecular compounds. They contain a flat porphyrin ring with the iron central atom involved in electron exchange reactions (Gursahani et al. 2008).

To some extent, mediation activity is ascribed to carbon nanotubes and even amorphous carbon, which are used as either protein supports or signal transduction. All the nanosized mediators are rather compatible with the "traditional" systems described. Their joint use is aimed at better compatibility with the biochemical components of biosensors and stimulation of the electron transfer, especially in extreme conditions (unusual pH area, hydrophobic microenvironment, and presence of organic solvents). The carbon materials bearing low-molecular components can be introduced into the carbon paste and the mixture for the screen-printing of planar electrodes. In some cases, the enzymes and DNA molecules are also added to the paste. The renewal of the surface is achieved by cutting a thin surface layer of the paste followed by the mechanical polishing of the cut.

Amperometric enzyme sensors. Voltammetry is mainly applied for the detection of the signals generated by oxidoreductases. In accordance with the signal mode, three generations of the enzyme sensors have been classified.

The *first generation* involves the direct reduction of an oxygen, i.e., natural electron acceptor participation in the majority of the oxidoreductase reactions (see Fig. 1.2 in Chap. 1). The *second generation* (Scheller et al. 1991) assumes the use of mediators of the electron transfer instead of oxygen, and the *third generation* covers the biosensors with direct electron transfer between the electrode and enzyme cofactor. No diffusionally free mediators are involved in the reaction although the use of heterogeneous mediators is allowed.

For glucose oxidase, i.e., an FAD-dependent oxidoreductase, the reaction can be recorded by the following set of the reactions (3.44).



$$\begin{aligned} &\text{Glucose} + \text{FAD} \rightarrow \text{Lactone} + \text{FADH}_2 \\ &\text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2 \\ &\text{O}_2 + 2\text{H}_2\text{O} + 4\text{e}^- \rightarrow 4\text{OH}^- \end{aligned} \tag{3.44}$$

The FAD cofactor (the structure is given in Fig. 3.17) participates in the glucose oxidation that formally corresponds to the transfer of two hydrogen atoms. Then the active form of the enzyme is recovered by the oxidation of $FADH_2$ with molecular oxygen. This results in the decrease of the oxygen concentration that is proportional to the concentration of $FADH_2$, i.e., the rate of the enzymatic process. The first glucometers were graduated in the units of the partial oxygen pressure. Nevertheless, such biosensors very rapidly lost their significance. The compensation of oxygen consumption by its transfer from the atmosphere, the dependence

of the oxygen solubility in water on the temperature and ionic strength of the solution, and alternative oxygen reactions are the main drawbacks of the first generation of biosensors.

At present, measurements based on oxygen consumption have retained their importance mainly in microbial sensors in which the oxygen intake indicates the level of microbial metabolism, as well as in the photosynthesis measurements. Instead of the rather expensive Clark oxygen electrode with a Pt transducer, various metal oxide electrodes are used. Oxygen consumption makes it possible to detect toxic species and the level of metabolite production (ethanol, penicillin, starch, carbohydrates, vitamins, etc.). Of other biosensors using oxygen reduction, those based on lactate oxidase can be mentioned (Yashina et al. 2010).

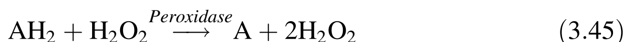
The progress in the development of enzyme sensors started from the attempts to substitute oxygen detection with the hydrogen peroxide oxidation. However, the high overpotential did not allow detecting the enzyme reaction in the presence of many common components of biological liquids, such as uric acid, bilirubin, etc. For this reason, the mediated reduction of hydrogen peroxide has been introduced. The potential of this process can be significantly decreased to very small values. Together with the high catalytic efficiency of many mediators, this offered high sensitivity of the analyte detection. In Table 3.4, the enzymes and their substrates that were used in the biosensors based on the H_2O_2 detection are shown.

The mediated reduction of hydrogen peroxide can be performed with phenazine derivatives, polyaniline and polythionine, MnO_2, CeO_2, NiO(OH), Co phtalocyanine and many other transient metal complexes. The best characteristics of the electrocatalytic reduction of H_2O_2 have been obtained with Prussian blue (Karyakin et al. 2007). Its electrodeposition on partially blocked glassy carbon made it possible to obtain nanostructured coating functioning as a microelectrode array with a very high sensitivity of the H_2O_2 detection with about seven orders of magnitude. Besides, the working potential of H_2O_2 detection on the electrode covered with Prussian blue is about zero *V*. The low stability of the modifier and the substantial drift of the signal can be eliminated by additional coverage of Ni ferricyanide and Nafion film.

Table 3.4 Enzymes used for the detection of organic substrates based on cathodic reduction of hydrogen peroxide

Analyte	Enzyme	Analyte	Enzyme
Glucose	Glucose oxidase	Phenol	Tyrosinase, peroxidase
Uric acid	Uricase	Amino acids	*L*-, *D*-amino acid oxidases
Hypoxanthine	Xanthine oxidase	Amines	Monoamine oxidase
Cholesterol	Cholesterol oxidase, cholesterol esterase	Formaldehyde	Formate oxidase
Lactate	lactate oxidase	Malate	Malate oxidase
Oxalate	Oxalate oxidase	Ethanol	Alcohol oxidase
NADH	NADH oxidase	Glycerol	Glycerol oxidase

Some proteins, such as hemoglobin and peroxidase, can also mediate the reduction of hydrogen peroxide. Horseradish peroxidase has found great popularity in developing oxidoreductase-based biosensors. This enzyme catalyzes the oxidation of various substrates by hydrogen peroxide in accordance with a formal Eq. (3.45).



$$AH_2 + H_2O_2 \overset{Peroxidase}{\longrightarrow} A + 2H_2O_2 \qquad (3.45)$$

The activity of peroxidase as well as the concentration of its substrate can be easily determined by the current of the product reduction. Thus, in the case of hydroquinone, the benzoquinone obtained in the biochemical path is easily reduced to initial hydroquinone. This creates a substrate cycle near the electrode surface that improves the sensitivity of the hydrogen peroxide detection and the stability of the response. The signal generation realized in such a bi-enzyme sensor is similar to that described earlier for two mediators (Fig. 3.15b). The oxidoreductase first catalyzes the H_2O_2 formation, which is immediately catalytically converted with peroxidase into a water molecule in the same layer. An equal amount of the product of peroxidase oxidation is reduced to the initial substrate (Fig. 3.18).

Besides the molecular oxygen and hydrogen peroxide detection, the third universal approach to the signal detection in enzyme sensors is the redox conversion of the cofactors. This mostly refers to NAD(P)H [see the structure in Chap. 2, (2.4)], $FADH_2$ and PQQ (Fig. 3.17) and some hemoproteins.

More than 250 NAD-dependent oxidoreductases are known and many of them can be applied for the detection of specific substrates in the biosensor format. The interest in the electrochemical reactions of cofactors is stimulated by the possibility of realizing a full cycle with biochemical conversion and electrode regeneration of the cofactor on the electrode and hence of achieving a “reagent-free” biosensor that does not require any additional reactants other than an analyte.

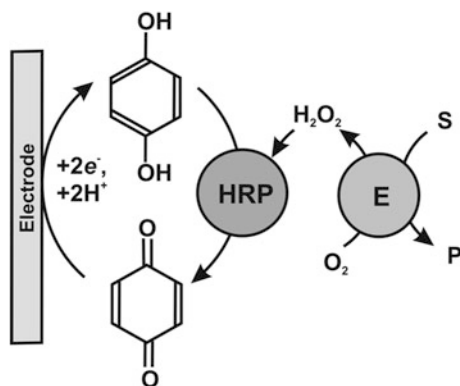


Fig. 3.18 Bi-enzyme sensor including oxidoreductase *E* and horseradish peroxidase *HRP*. The biosensor signal is generated due to cathodic reduction of benzoquinone, a product of HRP-aided oxidation of hydroquinone

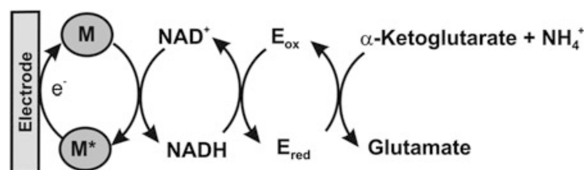
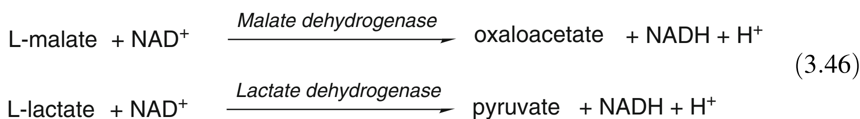


Fig. 3.19 Determination of ammonium ion with electrochemical biosensors based on mediated reduction of NAD^+ cofactor of glutamate dehydrogenase

The number of enzymes that have already found application in biosensors based on cofactor regeneration is rather wide. Some examples have already been mentioned (lactate dehydrogenase, glucose oxidase).

Of other interesting systems, the amperometric biosensor for ammonium determination can be mentioned (Prieto-Simón et al. 2007). The reaction of glutamate dehydrogenase is mediated by Meldola's blue, which is implemented in the polysulfonated carrier on the surface of the electrode (Fig. 3.19).

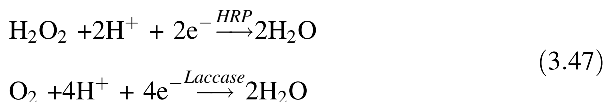
L-malate and L-lactate are determined with the help of a screen-printed carbon electrode modified with co-immobilized NAD^+ and malate and lactate dehydrogenase. The diffusionally free hexacyanoferrate anion is used as a mediator for the cofactor recovery (3.46) (Katrlik et al. 1999).



$$\begin{aligned} &\text{L-malate} + NAD^+ \xrightarrow{\textit{Malate dehydrogenase}} \text{oxaloacetate} + NADH + H^+ \\ &\text{L-lactate} + NAD^+ \xrightarrow{\textit{Lactate dehydrogenase}} \text{pyruvate} + NADH + H^+ \end{aligned} \tag{3.46}$$

The efficiency of the mediation of $NADH/NAD^+$ conversion depends on the mediator system. Neutral red was found to be most effective for NADH. The mediated reduction of the $NADP^+$ system is necessary for the development of biosensors based on cytochrome P_{450}. This enzyme complex catalyzes complex reactions of oxidation and N-methylation of xenobiotics in the liver cells and hence protects the organism from their toxic effect. The activity of cytochrome P_{450} determines the susceptibility of a human being to drug addiction and intolerance to some medications. The development of biosensors based on cytochrome P_{450} was hampered by the ineffective system of NAPD regeneration. For this purpose, a coupled reaction of glucosophosphate dehydrogenase was used, with NAD^+ as a cofactor. This system was unstable and rather expensive for use in the biosensor format. Direct regeneration of NADPH has recently been detailed with the enzymes implemented in an LbL polyelectrolyte complex (Sadeghi et al. 2012) and carbon nanotube adducts (Vatsyayan et al. 2011).

Among cytochrome P_{450}, the direct electron transfer was realized for other enzymes [laccase (Yaropolov et al. 1994), peroxidase (Ferapontova et al. 2001), and glucose oxidase (Yang et al. 2008)]. For laccase and peroxidase, the current value corresponded to the amounts of hydrogen peroxide and oxygen reduced in biocatalytic reactions (3.47).



$$\begin{aligned}&H_2O_2 + 2H^+ + 2e^- \xrightarrow{HRP} 2H_2O\\&O_2 + 4H^+ + 4e^- \xrightarrow{Laccase} 2H_2O\end{aligned} \tag{3.47}$$

A direct electron transfer can be performed on the electrodes modified with mediators or with enzymes immobilized in the polymers bearing redox-active centers. In this case, mediators participate in the electron exchange and the term "direct" rather means that there is no necessity for the additional soluble mediator systems used. The rate of the direct electron transfer is commonly lower than that of conventional mediated processes, but it can be accelerated by the partial unfolding of the enzyme globule or its chemical modification. Thus oxidation of saccharide fragments shielding the peroxidase active site by periodate treatment increases the rate of direct electron transfer as well as the use of genetically engineered enzymes with no covering carbohydrate shell. To some extent, a similar phenomenon has been observed while an enzyme is adsorbed on polycrystalline gold and other materials.

The direct electron transfer between the electrode and the enzyme redox site can be described by (3.48), where k_2 is the rate of the enzymatic formation of the product (see Michaelis–Menten Eq. (2.6),

$$\begin{aligned}\frac{dc_S^{Ox}}{dt} &= -\frac{I}{F} + k_2 c_S^0 c_E^{\mathrm{Red}}\\ \frac{dc_S^{\mathrm{Red}}}{dt} &= \frac{I}{F} - k_2 c_S^0 c_E^{\mathrm{Red}}\end{aligned} \tag{3.48}$$

Here, $c_S^0$is the bulk concentration of a substrate, $c_E^{\mathrm{Ox}}$and $c_E^{\mathrm{Red}}$are the surface concentrations of oxidized and reduced forms of the enzyme. The solution of the equation makes it possible to express the dependence of the current on two terms, i.e., I_1 describing the Nernst dependence of the conversion of adsorbed enzyme and the other, I_2, the contribution of the catalytic reaction of an immobilized enzyme with a substrate present in the solution. For cyclic voltammetry

$$\begin{aligned}&\left(c_E^S = c_E^{\mathrm{Ox}} + c_E^{\mathrm{Red}}\right),\\ &I_1 = Fc_E^s \frac{Fv}{RT} \frac{\exp\left[-\frac{F}{RT}(E - E^o)\right]}{\left\{1 + \exp\left[-\frac{F}{RT}(E - E^o)\right]\right\}^2}\\ &I_2 = \frac{Fc_E^S k_2 c_S^0 [S_o]}{1 + \exp\left[-\frac{F}{RT}(E - E^o)\right]}\end{aligned} \tag{3.49}$$

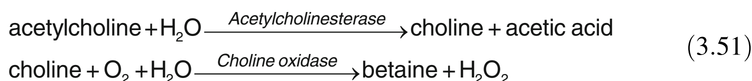
The catalytic contribution does not depend on the scan rate and has its limiting value reached with the increasing overpotential (3.50).

$$I_2^{\lim} = Fk_2 c_E^S c_S^0. \tag{3.50}$$

The use of peroxidase together with oxidoreductases to amplify the signal related to hydrogen peroxide is only one example of the *bi-enzyme sensors*. The

use of several enzymes complicates both the immobilization and sensor operations and should be compensated for by the obvious advantages this assembly provides. The following typical cases can illustrate the approaches described:

Consecutive enzymatic conversion of a substrate. The product of the first enzymatic reaction is utilized as a substrate in the second reaction. Lack of diffusional loss is an advantage of this reaction scheme. However, the enzymes can differ by their pH optimum and this complicates the optimization of the measuring conditions. Consecutive reactions are used when the reactant of a target process are electrochemically insensitive and cannot be easily detected by electrochemical transducer. The conversion of acetylcholine, i.e., a neurotransmitter, to choline followed by the oxidation of choline to betaine is an example (3.51) (Kok et al. 2002).



$$\begin{aligned}&\text{acetylcholine} + H_2O \xrightarrow{\textit{Acetylcholinesterase}} \text{choline} + \text{acetic acid}\\&\text{choline} + O_2 + H_2O \xrightarrow{\textit{Choline oxidase}} \text{betaine} + H_2O_2\end{aligned} \qquad (3.51)$$

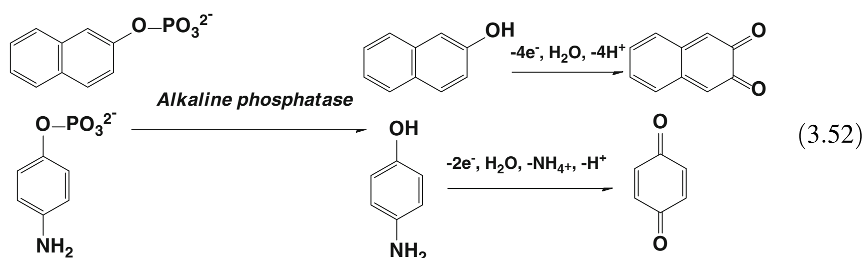
Coupling glucose oxidase with invertase and mutorotase makes it possible to determine sucrose; the combination of the enzymes with amylase provides starch detection. The determination of total cholesterol required first the hydrolysis of its esters catalyzed by cholesterol esterase and then the oxidation of cholesterol with cholesterol oxidase. In all of these reactions, both enzymes are immobilized together but it is admitted to leave one of them in the solution. This is usually made for increasing the sensitivity of the signal. Thus, in the reactions (3.51) acetylcholinesterase is often used in a soluble form to amplify the signal related to its inhibition with irreversible inhibitors. The idea is that inhibition does not allow a repeated use of the enzyme, but free enzyme is more sensitive toward an inhibitor than the immobilized one. After the measurement, the solution of acetylcholinesterase that is changed in the inhibitor measurement is performed with the same sensor with immobilized choline oxidase. This protocol makes it possible to avoid the problems related to the significant difference in the specific activity of acetylcholinesterase and choline oxidase and in the pH dependence of their activity.

If the product of the second reaction coincides with the substrate of the first one, the substrate cycling takes place. It is similar to that already discussed for the combination of biochemical and electrochemical conversion of the reactants (see Fig. 3.18), but both reaction paths are controlled by enzymes. Such reactions are utilized in humans in various biochemical cycles where they are spatially separated and controlled by different effectors. The oxidation of glucose in glycolysis and its release from glycogen is an example of such a biochemical cycle. Similarly, in the Cori cycle, lactate is converted into glucose in gluconeogenesis in liver and glucose produces two lactate molecules in anaerobic glycolysis in muscles.

The substrate recycling in biosensors has been introduced for amplifying the signal. Thus, for the glucose detection, a combination of glucose oxidase and glucose dehydrogenase can be employed. For lactate, the same result is achieved with lactate dehydrogenase and lactate oxidase.

Parallel enzymatic reactions can be used for increasing the selectivity of the target biochemical path. Thus, the use of hydrogen peroxide for the detection of the oxidoreductase signal can be limited by the so-called endogenous hydrogen peroxide present in biological fluids. Its influence is suppressed by the immobilization of catalase that decomposes the H_2O_2 molecule into oxygen and water. The glucose is transferred through the catalase layer and converted to lactone and hydrogen peroxide, which is reduced on the electrode while the external flux of H_2O_2 is terminated in the catalase layer (Fig. 3.20) (Krysteva and Yotova 1992).

The voltammetric transducers can be employed for the detection of hydrolase substrates and effectors. Contrary to oxidoreductases, the presence of electrochemically active components of the enzymatic reaction is not obligatory. This limits the use of electrochemical techniques for the detection of native substrates. As shown earlier, the problem can be solved by coupling the hydrolase reaction with another one producing an electrochemically active substance. This was shown for acetylcholinesterase combined with choline oxidase. Of other systems, the determination of the alkaline phosphatase is of significance. This is related to the application of this enzyme, in addition to peroxidase and acetylcholinesterase, as a label in immunosensors and DNA-based sensors (Kwon et al. 2008). These enzymes meet the principal requirement of the labels, i.e., a very low limit of the detection of enzyme activity. For alkaline phosphatase, this is achieved by the electrochemical detection of the products formed in hydrolysis of *p*-aminophenylphosphate and naphtylphosphate (3.52).



(3.52)

The combination of alkaline phosphatase with peroxidase and tyrosinase with phenylphosphate as a substrate made it possible to reach the LOD of 1.4 fM of alkaline phosphatase (Ruan and Li 2001). The principal scheme of the reactions is represented in Fig. 3.21. Phenol generated in the step of the cascade is oxidized by tyrosinase to catechol, which is then transformed in *o*-quinone. The latter reaction is catalyzed by peroxidase. The cathodic current of the reduction of *o*-quinone is a

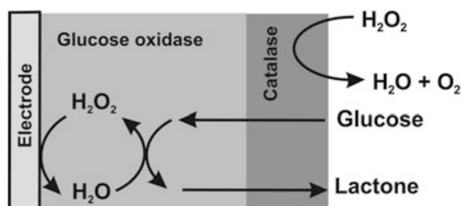


Fig. 3.20 Glucose oxidase—catalase biosensor for determination of glucose in the presence of endogenous hydrogen peroxide

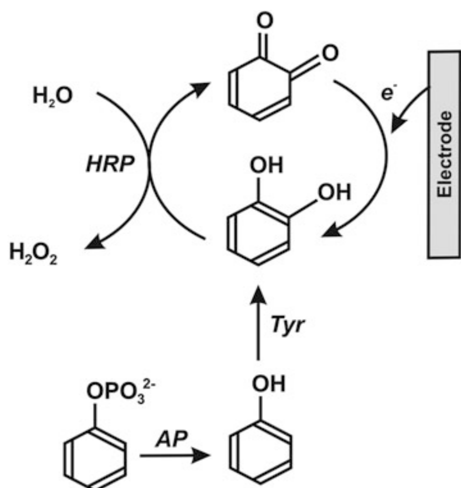
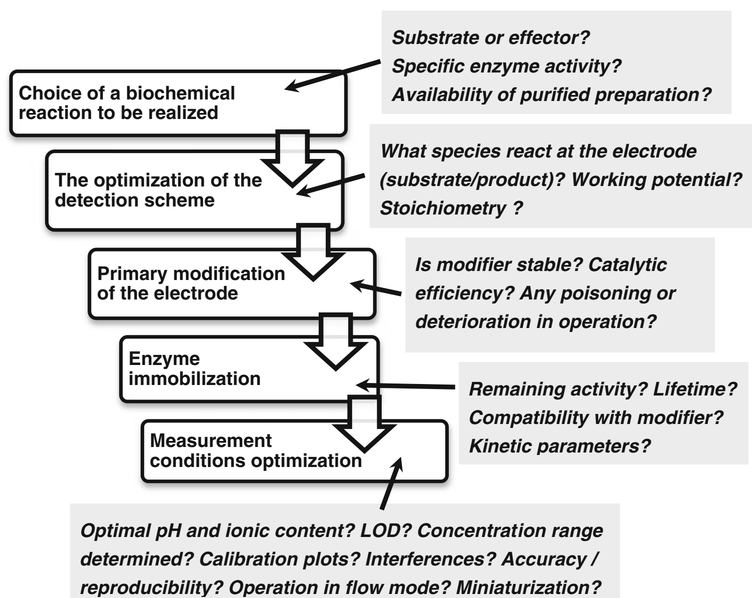


Fig. 3.21 Measurement of alkaline phosphatase (*AP*) activity with bi-enzyme sensor based on peroxidase (*HRP*) and tyrosinase (*Tyr*)

signal of the biosensor, which is proportional to the activity of alkaline phosphatase.

Summarizing the principles of the functioning of mediated electrochemical biosensors, the following plan can be suggested to those who want to design a biosensor for a particular analyte. This is, *sui generis*, a roadmap for the investigations. In this scheme, the steps of the research are provided with questions to be answered.

Some of the questions have answers in the literature and previous experience of researchers; others, such as the kinetic characterization of immobilized enzymes or the determination of the number of electrons and hydrogen ions transferred, are not obligatory on the preliminary steps of investigations. The fulfillment of the research program at one step might require going back to the previous one. But on the whole, this roadmap improves the chances for final success and might be useful for inexperienced researchers.



Analytical characteristics of voltammetric enzyme sensors are mainly determined by the kinetic characteristics of the enzymes. The calibration graphs are linearized in the plots of I vs. c_S within two orders of magnitude. The additional surface accumulation of the substrate diminishes the lower limit of quantification by 10^{-7} M. Thus, if the signal is based on oxygen reduction, hydrophobic membranes or the inclusion of hydrophobic materials (Teflon) in the elelectrode material can be recommended. In many cases, the mechanism of the electrode reaction and the establishment of the working conditions is performed in the linear sweep mode, whereas the following detection of the analytes is made by the DPV or square-wave voltammetry. This allows decreasing the LOD and detectable concentration by one more order of magnitude. The chronoamperometric regime at a constant potential is frequently applied for flow-through biosensors. In comparison with conventional techniques, this results in a moderate decrease of the signal sensitivity. The recording of the I–t curve is also used when no resolved peaks but a gradual increase in the current are observed on a voltammogram. To distinguish measurements at a constant potential from other techniques, the term "amperometric biosensor" is used, contrary to voltammetric biosensors using other modulations of the potential. The list of parameters determined in the optimization of the enzyme assembly is rather common and includes the linearity portion of the calibration graph (range of concentration, or dynamic range of concentration), LOD, drift of the signal within the operation, the signal time, the biosensor lifetime, the sensitivity of detection (slope of a calibration plot), metrological characteristics (repeatability or accuracy, reproducibility, recovery characteristics). What is different from conventional electrochemical sensors is that the changes in the characteristics of biochemical components should be

quantified within the period of the biosensor use. The interferences are considered from the two points of view, i.e., substances that could interfere with the biochemical conversion of the analyte and those interfering with the signal generation mode, e.g., electrochemically active matrix components and surface active compounds able to block ("poison") the mediator or significantly change its functions. As the number of such species can be very high, those present in common samples to be analyzed are considered first. This might be the components of biological fluids, the medication often used in therapeutic treatment, etc. The influence of the interference is estimated either by their contribution to the signal toward a standard concentration of an analyte or by the current value generated in the presence of the interference and absence of an analyte. The influence of interferences on the slope of the calibration plot of an analyte is also important.

The number of parameters optimized after the proof of the concept, when the possibility of the analyte detection is proved, is rather limited and includes the content of any modifying layer (mediator, enzyme, support, auxiliary reagents), the protocol of the electro modification, the specific enzyme activity (or, enzyme quantity), and the pH and ionic content of the working solution. In addition, the parameters of immobilization considered in Chap. 2 are varied (the period of treatment with the binding agent, the concentration of a reactant, and the protocol of immobilization as such). No exact results of such an optimization are expected, and comparable results are attained in significantly different conditions. The final choice depends on the goals of the biosensor development, the specificity of the sample and the personal user preferences.

Voltammetric DNA sensors. As was stated above, hybridization detection is a primary goal for most of the DNA sensors developed and used. The detection of hybridization is mainly based on three strategies (Drummond et al. 2003):

- direct DNA electrochemistry (oxidation of nucleotides);
- application of electrochemically active intercalators;
- label-based techniques.

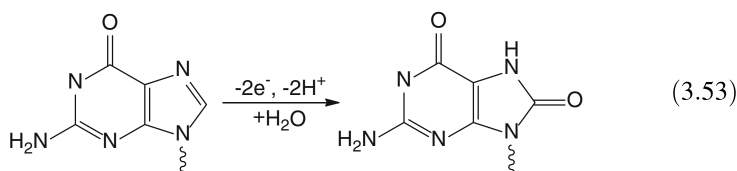
> *DNA intercalation* is the insertion of a small ligand or fragment between two adjacent base pairs in the DNA strand, forming stable sandwich-like structures.

The *direct detection* of the DNA nucleotides is mainly based on their oxidation onto the electrodes. The reactions are hindered by steric factors. The DNA helix does not provide the physical contact of the nucleotides with the electrode necessary for the electron transfer. The reaction can be detected on carbon electrodes by special measurement techniques, e.g., stripping chronopotentiometry or DPV. In the first method, the electrode is polarized in the galvanostatic regime and the decay of the potential is recorded in time. After that, the curve is differentiated and drawn in the plots of the time t versus dE/dt. The oxidation process is represented

by a bell-shaped peak. The square of the peak is proportional to the quantity of the species oxidized on the electrode. Any changes in the ds-DNA structure resulted in the simplification of guanine oxidation. This is used for the detection of the intercalation, hybridization abnormalities and the DNA damage. The hybridization results in shielding guanines involved in the complementary interactions and hence in the decrease of their signal. The changes in the guanine signal are often followed by the appearance of a new current peak related to the primary product of the guanine conversion, i.e., 8-oxoguanine (Palecek 2002).

The hybridization is detected by repeated measurement of the signal observed at a high anodic potential (about 0.8–1.0 V) and assigned to the guanine oxidation (3.53) prior to and after the contact of the biosensor with the sample.

The anodic oxidation of guanine residues can be mediated by diffusionally free mediators added to the solution, e.g., Ru(II) bipyridine or Co(II) phenanthroline complexes, or by heterogeneous mediators placed on the electrode surface (carbon nanotubes or metallocyanate complexes).



(3.53)

The use of specific intercalators belongs to the so-called non-labeled systems. They have become an advantage due to their simpler design, higher sensitivity of the signal in comparison with the direct DNA oxidation, and compatibility with the DNA assay protocols elaborated for other purposes (PCR analysis). The application of intercalators is mainly based on the treatment of the DNA sensor with a low molecular compound that can penetrate the DNA helix and change their electrochemical characteristics in this process called *intercalation*. The use of the term "intercalator" in DNA biosensors is rather mild and also includes the compounds that are partially inserted in the DNA helix, so that a part of the molecule is left outside (mainly in the area of the minor groove), so that its electrochemical activity remains constant. In some cases, DNA can be considered to be a kind of specific sorbent that accumulates the low-molecular compounds and increases their redox signals. Most of the electrochemically active intercalators are toxic, and many of them are used as antitumor drugs.

The use of intercalators is based on a rather obvious and intuitively understandable statement that they can intercalate only the ds-DNA but not ss-DNA probe (Fig. 3.22). This means that the intercalator signal will significantly change in the hybridization event. At first, the intercalators used in the DNA assay belonged to fluorogenes (ethidium bromide, Hoechst stain family). Some of them exert electrochemical activity, but at rather high anodic potentials. The electrochemically active intercalators are presented in Fig. 3.23.

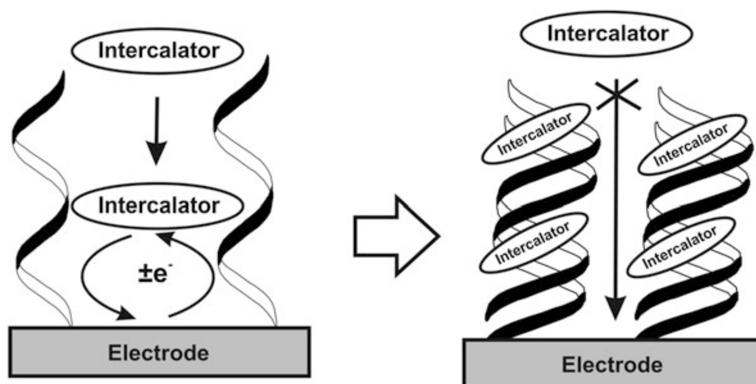


Fig. 3.22 Detection of the hybridization event based on electrochemically active intercalator signal

Methylene blue belongs to the phenothiazine dye family and is especially actively used in the detection of the hybridization event. It has a photo sensitizing activity and promotes oxidation of ribose fragments of the DNA when illuminated. For this reason it is used for treating skin cancer cells and as an algaecide in aquaria. Methylene blue is mainly present in its oxidized form that bears positive charge promoting its interaction with ss-DNA. For this reason, the signal of Methylene blues is higher by 30–40 % on the electrode covered with a DNA probe than on bare glassy carbon. After hybridization, the signal decays due to the intercalation of the Methylene blue and the limitations in the electron exchange with the electrode (Erdem et al. 2001).

Daunomycin and adriamycin are the anthracycline drugs recommended for leukemia treatment; they accumulate in the ds-DNA. Intercalation decreases the signal after the hybridization event. In early research, daunomycin and adriamycin were used for the investigation of the DNA damage mechanism. Their signals on the DPV voltammograms correlated with DNA damage signs (guanine and 8-oxoguanine currents). The high potential of oxidation is one of the drawbacks of the application of anthracycline preparations in electrochemical DNA sensors (Huska et al. 2009).

Phenanthroline and bipyridyl complexes of Os, Ru and Co partially intercalate the DNA helix by aromatic ligand planes and promote the partial oxidation of the guanine residues due to electron exchange between the central atom, nucleic bases and sugar residues of the DNA helix. The mechanism is affected by the stereochemistry of the complexes, in which flat aromatic ligands are swung against each other like in a propeller.

Ferrocenyl naphtyl diimide (3.54) is a rare case of intercalators whose signal increases after penetrating the DNA helix (Takenaka et al. 2000).

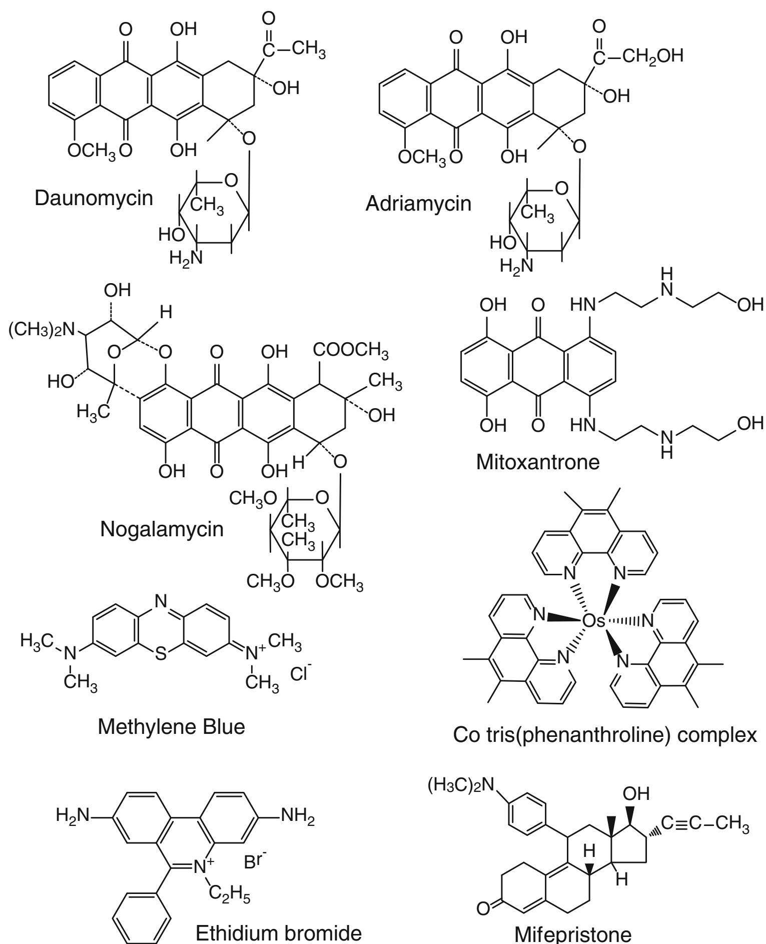
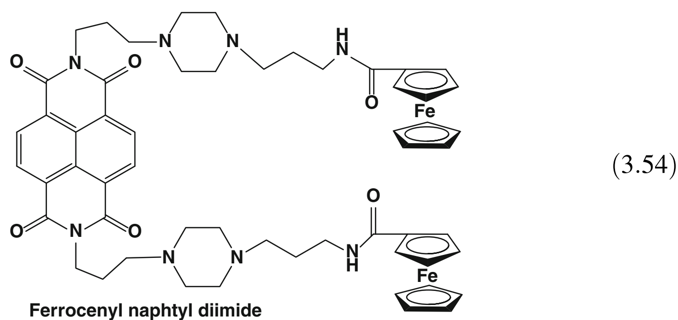


Fig. 3.23 Redox active intercalators and related compounds used for electrochemical detection of the hybridization event with DNA sensors



(3.54)

The long-chain linkers separate the ferrocene units from the central flat part of the molecule in the reaction with the ds-DNA. Amplification of the signal resulted in a significant decrease in the LOD of specific ODN detection. Thus, for the dA_{20} and the yeast choline transport gene sequence, subpicomolar levels were achieved. Later on, the protocol was modified by including adamantyl fragments instead of ferrrocene in the intercalator core. After the hybridization and intercalation with adamantyl derivate, the DNA sensor was treated with a ferrocenyl β-cyclodextrin complex. The macrocycle interacted with adamantyl groups positioned close to the ds-DNA surface and allowed ferrocene units to exchange electrons with the electrode.

The application of specific indicators presumes a multi-stage measurement protocol because the hybridization is detected by the difference in the signal measured prior to and after the contacts of the DNA sensor with an ODN target sequence. Washing and additional steps make the measurement rather long and dependent on the non-specific adsorption of the reactants.

The interference caused by non-specific adsorption can be prevented by blocking the free surface with SAMs or by treating the biosensor with BSA and other proteins.

Label-based methods are based on specific groups or enzyme molecules introduced into the DNA probe by covalent linking. The reaction of the labeled DNA with the target ODN on the sensor surface results in fixing a label near the electrode and hence promotion of the electron exchange. The efficiency of signal detection depends on the density of the DNA probe positioned on the sensor surface. For the low surface concentration of the DNA probe, the signal measured might be too low and interfere with the non-specific adsorption of the labeled sequences without the help of the DNA probe. For high surface coverage, the steric access of a biological target is limited and this also affects the sensitivity of its detection.

Previously, the development of DNA sensors started from rather short (up to ten bases) sequences. This allowed avoiding the spontaneous formation of partially hybridized structures inaccessible for the analyte recognition. At present, the length of the DNA probe has increased up to 20–25 nucleotides. This makes the hybridization assay more reliable but requires preliminary de-hybridization of the DNA probes by heating (DNA melting). Longer DNA sequences also increase the irregularity of the surface layer package because of the spontaneous adsorption.

The requirements for labels do not differ dramatically from those in the immunoassay. In addition to the high sensitivity of detection, they should be chemically stable in a rather wide pH range and easily introduced in the DNA probe.

Enzyme labels are of special interest. The catalytic conversion of the substrate amplifies the signal and increases the sensitivity of the analyte detection by several orders of the concentration magnitude. Two enzymes, i.e., peroxidase from horse serum and alkaline phosphatase, are very popular in such systems. The measurement protocol includes the treatment of the DNA sensor after the attachment

of the labeled sequence with the specific enzyme substrate followed by measuring the signal related to the quantities of the product formed near the electrode surface. The reaction can be performed with the help of mediators covalently attached to the carriers in the proximity of the DNA probes or even in the absence of organic substrates of peroxidase by direct electron transfer. Alkaline phosphatase catalyzes the hydrolysis of the of *p*-aminophenylphosphate followed by the oxidation of p-aminophenol as described earlier [Eq. (3.52)]. This made it possible to reach femtomolar levels of detectable concentrations. Moreover, both enzymes can be used for labeling different oligonucleotides immobilized together on the same support. The signal discrimination is achieved by the addition of an appropriate substrate due to the high specificity of enzymatic reactions. The methodology of enzyme labeling is similar to that previously developed for ELISA and other immunoassay techniques. This is rather typical for the DNA sensors that have inherited many of the approaches of immunoassay.

For detecting real samples, various protocols have been detailed that are similar to those described for the immunoassay. In a *competitive assay*, labeled complementary oligonucleotides are added to the sample. As a result, they compete with biological targets for the same binding sites of the DNA probe. Assuming that the affinity constant does not significantly differ after the label introduction, the ratio of labeled and non-labeled oligonucleotides captured by the DNA probe is proportional to their ratio in the solution. In other words, the higher the label signal, the lesser the content of a biological target in the solution.

In *non-competitive protocols*, two different DNA probes are used. The first sequence is immobilized on the solid support (capture strand). It reacts with a biological target but part of the sequence remains free for the reaction with the second DNA probe bearing a label. In this mode, the higher the signal, the greater the amount of the biological target in the sample (sandwich immunoassay as an analog).

Some other alternatives of the two DNA-probe employment have been proposed. Thus, the DNA probe attached to the support and the target ODN can both react with an auxiliary sequence to anchor the biological target to the sensor. The linker between the capture strand and the probe strand can differ in the flexibility and length to reach the maximal affinity of the hybridization event. The reaction of three oligonucleotides can be designed to reach the rigid conformation of the product providing the coordination of the label on the sensor surface (Fig. 3.24).

A similar format was realized in bioelectronic DNA chip arrays developed by Clinical Micro Sensors, Inc. (now a part of Motorola Life Sciences) with ferrocene as a label. The automated assay based on a chip made it possible to simultaneously measure the signal on 36 electrodes obtained by photolithography on the same platform. The eSensor 4800 system can analyze 48 chips at a time. The eSensor described is a part of the point-of-care diagnostics tool that can involve micro-PCR chambers, microfluidics systems, etc., as well (Lee and Hsing 2006).

The use of nanomaterials extends the operational and analytical characteristics of the DNA sensors. Thus, the application of the Au nanoparticles covalently modified with short DNA sequences multiplies the number of DNA spots for

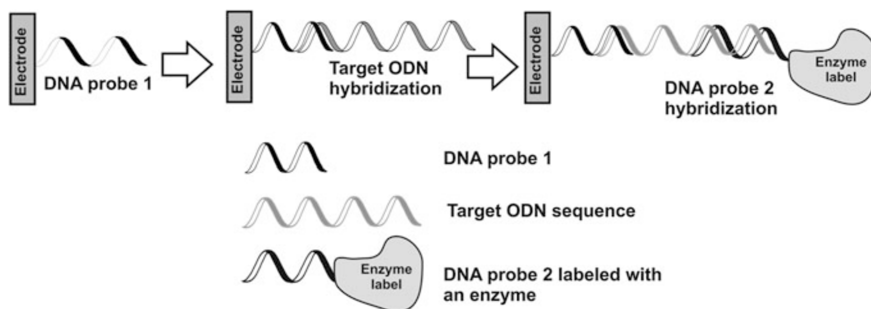


Fig. 3.24 Detection of DNA sequence with a second DNA probe labeled with enzyme

labeling with no loss of permeability of the surface layer. This decreases the minimal detectable concentration to 10^{-15}–10^{-18} M. Similar results were obtained by a combination of the DNA probes and aptamers with antibodies applied for the direct linking of the label, e.g., ferrocene or enzyme. Some of the examples of the DNA sensors with labeled sequences are given in Table 3.5. They do not completely cover the full number of the assemblies described in the literature but give some overview of the approaches and quantification levels achieved.

The extension of the sandwich assay methodology resulted in the development of the DNA sensors based on hairpin strands bearing terminal labels (E-sensors). The rigid structure of the loop is maintained by a short ds-sequence near the sensor surface (a leg). The proximity of the label to the transducer results in the detection of a remarkable signal that can be used for an independent control of the sensor operability. Ferrocene and Methylene blue were used as labels. The reaction with a biological target results in the formation of a rather rigid stem and separation of the label from the electrode (Fig. 3.25) (Ricci and Plaxco 2008).

To improve the reversibility of the response, the measurement is conducted in the presence of electron acceptors added to the solution (ferricyanide ions or hydrogen peroxide). In comparison with the common DNA sensors, the E-sensors described provide a one-stage detection of an analyte without any washing steps, addition of auxiliary reagents, or second DNA probes.

DNA sensors for the detection of low-molecular compounds. The DNA sensors for hybridization detection described above are influenced by many low molecular compounds. Intercalators, positively charged species, and oxidants can interact with the DNA molecules and change their characteristics in accordance with the specific mechanism of interaction, DNA structure and the signal transduction used. Some of these interactions can be used for quantification of low molecular compounds.

Table 3.5 Examples of label-based detection of the hybridization event

Biological target	Immobilization protocol	Label/Signal measurement	Concentration range, LOD
Pneumoniae(Martínez-Paredes et al. 2010)	Au–S assembling on Au nanoparticles	Alkaline phosphatase/indoxyl phosphate,	50–750 pM, LOD 20–25 pM
Aflatoxigenic *Aspergillus* species (Snevajsova et al. 2010)	Physical adsorption on carbon paste electrode surface	Alkaline phosphatase/1-naphtylphosphate	0–5 μg/mL, LOD 1 ng in 5 μL drop
Enterohemorrhagic *Escherichia coli*(Liao and Ho 2009)	Au–S assembling on screen-printed electrode	Liposomes encapsulating $Ru(NH_3)_6^{3+}$ ions, competitive assay	$1–10^6$ fmol, LOD 0.75 amol (5 μL of 0.15 pM solution)
Escherichia coli DNA fragments (326 bases) (Loaiza and Campuzano 2008)	Streptavidin–biotin binding on magnetic beads deposited onto electrode	Peroxidase/tetratiafulvalene, H_2O_2, sandwich assay	LOD 3.75 fmol
Cystic fibrosis (Gao et al. 2008)	Au–S assembling on Au bare electrode	Multi-walled carbon nanotubes bearing Ag nanoparticles, DPV mode (Ag oxidation current)	3.1×10^{-4}–1.0×10^{-11} M, LOD 10 fM
Human immunodeficiency virus (HIV) (Baur et al. 2010)	Affine attachment via histidine tags to polypyrrole film bearing nitrilotriacetic acid	Biotinylated glucose oxidase/glucose	7.7×10^{-12} mol/cm^2
PCR products related to *Vibrio cholera* O1 and O139 (Choo et al. 2013)	Au–S assembling on screen-printed electrode	Alkaline phosphatase labeled via Ag–Ab binding, α-naphtol oxidation	0–8 nM, 8–183 pM
Thrombin (Rahman et al. 2009)	Carbodiimide binding of aminated aptamer to the polymer of terthiophen-3-carboxylic acid	Ferrocene attached to anti-thrombin antibodies via streptavidin–biotin binding, sandwich immunoassay mode	5–2000 ng/L, LOD 5 ng/L (0.14 pM).

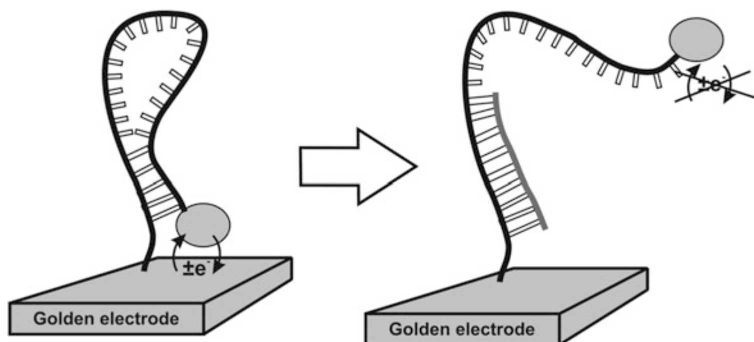


Fig. 3.25 E-Sensor based on hairpin DNA probe labeled with Methylene Blue or ferrocene

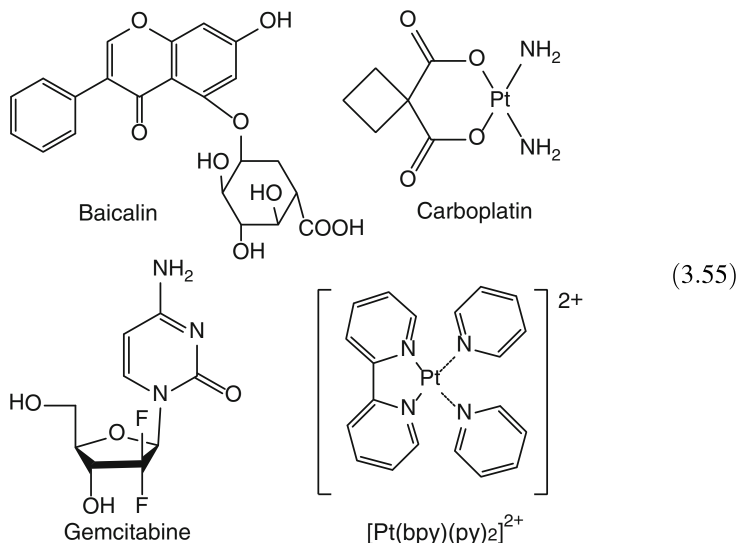
The variety of interactions and a large number of potential binding sites make such detection much less specific as compared with the hybridization detection, but they have generated increasing interest in the past decade in solving problems related to oncology, food quality testing and environmental monitoring.

All the DNA sensors devoted to the detection of low molecular compounds can be classified in accordance with the target analyte, i.e., drugs, DNA damage and antioxidants.

A native DNA is a biological target for antitumor drugs that have been designed to specifically damage cancer cell DNAs followed by their apoptosis or lysis. Anthracycline derivatives (see Fig. 3.22) specifically intercalate the DNA at the G–C pairs. Their signals of anodic oxidation are detected at high potentials (about +0.8… +1.2 V) and are frequently followed by the appearance of guanine and 8-oxoguanine signals. The reaction with DNA is performed either on the electrode or in solution. The adducts can be adsorbed on the electrode prior to measurement. Thus, direct detection of DNA in neuroblastoma cells has been described by treating them with adriamycin and the detection of coupled peaks related to the catalytic oxygen reduction and adenine oxidation on cyclic voltammograms.

In other cases, the dissolved oxygen plays an important role in the DNA damage started by intercalation. Its presence promotes the formation of 8-oxo-guanine, an indicator of oxidative DNA damage, and increases the signals related to DNA-drug interaction.

Some other examples of the drug-DNA interactions are described as regards biosensor development (3.55). Gemcitabine inhibits DNA synthesis from tri-phosphate nucleosides. It forms stable complexes with the ds-DNA followed by suppression of its own electrochemical signal.



(3.55)

Baicalin exerts antitumor, antimicrobial and immunomodulation effects and hinders DNA replication of HIV. Contrary to previous examples, it can react both with the ss- and ds-DNA and diminishes its activity recorded at moderate potentials (0.30 V). The cisplatin ($PtCl_2(NH_2)_2$), carboplatin, and bipyridyl-pyridynyl complex of Pt(II) stimulates the chemical and electrochemical oxidation of guanine and adenine residues in the DNA molecule recorded at 0.95 V on the Pt electrode. For cisplatin, the catalytic hydrogen escape is observed in cathodic reduction (Sun et al. 2004).

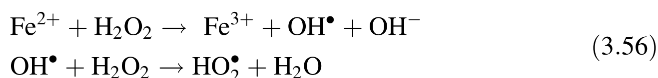
The DNA damaging factors involve physical treatment (heating, ionizing emission, ultrasound) and chemical reagents that exert any disturbances in the primary DNA sequence and the three-dimensional structure of the DNA if they prevent biochemical functioning. The estimate of DNA damage is related both to screening new antitumor drugs and testing genotoxic compounds in industry and environment. Contrary to the previously considered compounds, genotoxic compounds have never been used in medicine. The screening of DNA-damaging factors is commonly examined from the point of view of the genotoxicity screening and the risk assessment related to oncology and long-term effects of environmental pollution. Such an approach has some obvious limitations, e.g., difficulties in modeling the metabolic activation of genotoxic substances and in the DNA repair mechanism. Antioxidants are described together with genotoxic species because their quantification is based on the protective effect exerted on model genotoxicants in their interaction with native DNA.

The DNA damage is mainly detected by the changes in the guanine signals, sometimes proved by the 8-oxoguanine detection. The appropriate oxidation currents are recorded prior to and after treating the electrodes with toxic species.

Rather small polyaromatic compounds (anthraquinone and naphthalene derivatives) decrease the guanine oxidation, whereas polychlorobiphenyles increase the currents by 30–40 % due to the partial unwinding of the DNA helix. Besides individual toxicants, screen-printed electrodes with physically adsorbed DNA were used for preliminary testing of the wastewaters and soil extracts (Ovádeková and Labuda 2007).

Metabolic activation is responsible for the DNA damage caused by many chemicals capable of being biochemically converted in living beings. This can be imitated by UV irradiation or treatment of the sample with oxidative species or oxdioreductases. The number of hazards tested is rather wide and includes polyaromatic compounds, their aminated derivatives, *s*-triazine herbicides, heavy metal ions, niclosamides, styrene oxide, etc. The sensitivity of the response can be increased by using Co and Ru complexes, which both promote oxidative damage and generate the biosensor signal as described above.

The *estimation of antioxidant capacity* is based on the estimation of the DNA damage after its contact with the Fenton reaction (3.56). Similar schemes are known for Cu(I/II), Co(II/III), and Ru(II) complexes mixed with hydrogen peroxide.



$$\begin{aligned} &Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \\ &OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \end{aligned} \quad (3.56)$$

DNA damage is quantified by the guanine currents and some intercalator signals. The greater the DNA damage, the more changes in the above signals. The use of Ru complexes with bipyridyl ligands makes it possible to combine the electrochemical reduction with the luminescence measurement to amplify the DNA damage detection.

The efficiency of the DNA damage detection depends on the DNA source and immobilization protocol. Physical adsorption is preferable due to the better restoration of a native DNA helix on the sensor surface. Electrostatic adsorption on positively charged polymer-bearing Os complexes participating in DNA oxidative damage and adsorption on a polarized screen-printed electrode were used in most studies.

The antioxidants are added together with the Fenton reagents and the signal measured is compared with that obtained in the absence of protecting compounds. Flavonoids used as food additives, industrial antioxidants and those isolated from species, foodstuffs, and medicinal herbs are characterized by a high protective effect that depends on their concentration, contact period and the signal measurement mode. Examples of the individual antioxidants investigated are given in Fig. 3.26.

The protecting effect was obtained with 0.1 mM–0.1 μM antioxidants and it correlated with the changes of the standard redox potentials of the compounds. In addition to individual compounds, the antioxidant capacity was estimated for herbal extracts, polysaccharides of yeast fungus, flavors, and flavored waters and spices.

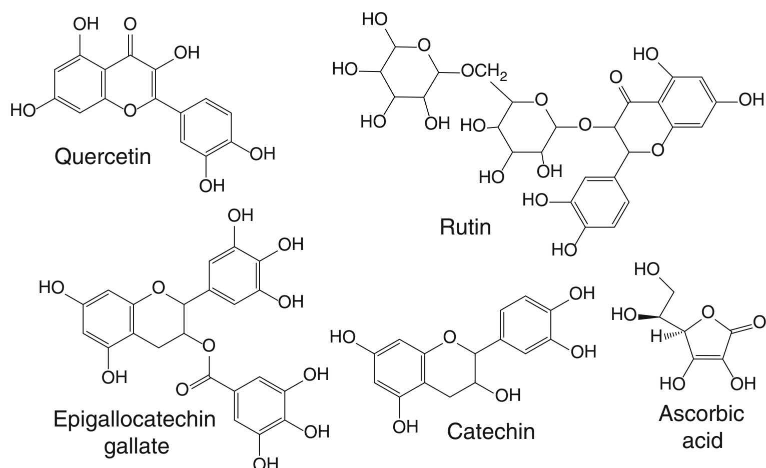


Fig. 3.26 Antioxidants investigated with electrochemical DNA sensors

Voltammetric immunosensors. The strategies for electrochemical immunosensor design are close to those developed for DNA sensors and have an internal relation to the solid phase immunoassay with the electrode substituting the support used for *Ab* immobilization. In accordance with that, all the electrochemical immunosensors can be subdivided into redox-labeled and non-labeled techniques (Skládal 1997).

Redox labeled immunosensors. The requirements for the labels introduced in one of the immunoreagents do not differ from those of conventional immunoassay. The labels should be easily detected with great sensitivity and selectivity and chemically and electrochemically stable. The use of redox labels is complicated by very strict requirements to the sensitivity of the immunoassay. The LODs of the nanomolar and picomolar concentration range is normally necessary and they are below the sensitivity of conventional electrochemical techniques. In non-amplified systems, the detection of one *Ag* (*Ab*) molecule generates one equivalent of the label detected. For this reason, the LOD of such systems is determined by the characteristics of the label detection, which are far from the limits declared.

The amplification of the signal of an immunosensor can be achieved by the use of electrocatalysis and enzyme labels, which offer a much higher sensitivity of detection. As was shown earlier, the activity of alkaline phosphatase can be detected on the zeptomolar level and the activity of horseradish peroxidase on the nanomolar level. Of other approaches, the use of free or immobilized enzymes for the recycling of ferrocene attached as a label to the *Ag–Ab* complex on the electrode surface can be mentioned. Glucose oxidase, PQQ-dependent glucose dehydrogenase and laccase were described for this purpose (Warsinke et al. 2000). The enzymes themselves are often used as labels in the conjugates with immunoreagents. Depending on the immunoassay format, they can either be implemented in the surface layer or applied in homogemeous solutions at the stages assuming the

Table 3.6 Examples of enzyme-labeled electrochemical immunosensors

Enzyme/substrate	Analyte	Transducing system	Immunoassay format
HRP/*o*-Aminophenol (Liu et al. 2001)	Complement C_3	Carbon paste electrode	Competitive
HRP/Hydroquinone (Ivanov et al. 2008)	Azinphos-methyl	Glassy carbon	Indirect competitive
HRP/Polyaniline (Grennan et al. 2003)	Atrazine	Screen-printed electrode	Indirect competitive
HRP/*o*-anisidine (Rosales-Rivera et al. 2012)	Human immunoglobulin A	Au coated with SAM	Sandwich
HRP/Tetramethylbenzidine (de Ávila et al. 2013)	Human Troponin T	Au screen-printed electrode/magnetic separation	Sandwich
HRP/H_2O_2 (direct electron transfer) (Dias et al. 2013)	Dengue virus NS 1protein	Screen-printed electrode/carbon nanotubes	Sandwich
Alkaline phosphatase/1-napthyl phosphate (Ammida et al. 2004)	Aflatoxin B_1	Screen-printed electrode	Indirect competitive
Alkaline phosphatase/Ag^+, indoxylacetate (Neves et al. 2012)	anti-gliadin *Ab*	Screen-printed electrodes/carbon nanotubes/nano Au	Sandwich
Glucose oxidase/glucose (Guana and Chen 2004)	α-Fetoprotein	Screen-printed electrode/Prussian blue	Sandwich
Glucose oxidase/glucose/Ag^+(Qu et al. 2011)	PDGF	Au electrode/SAM	Sandwich
Glucose oxidase/ferrocene (Zhuo et al. 2011)	ProGRP	Au/TiO_2, grapheme oxide	Sandwich
Alkaline phosphatase, tyrosinase/phehylphosphate (Carralero et al. 2007)	Progesterone	Colloid Au/Teflon/Carbon	Competitive
Laccase/oxygen (Milligan and Ghindilis 2002)	Insulin	Glassy carbon	Sandwich

PDGF platelet derived growth factor, *ProGRP* progestin releasing peptide

binding target molecules of *Ag* or *Ab*. The appropriate schemes of the immunoassay are given in Chap. 2. Some examples of various enzymes involved in the immunosensor assembly are shown in Table 3.6.

The sensitivity of electrochemical immunoassay can be additionally increased by the use of special geometry of the electrode setup. Thus, in interdigitated electrodes, the reaction of oxidation and reduction of the substances takes place in the very thin (several micrometers) gaps between the electrodes (Fig. 3.27a). The total current recorded depends on the turnover of the redox substance and its concentration. Due to the multiple cycling, the signal sensitivity is higher than that of a macroelectrode. The low selectivity of the amperometric signal is the

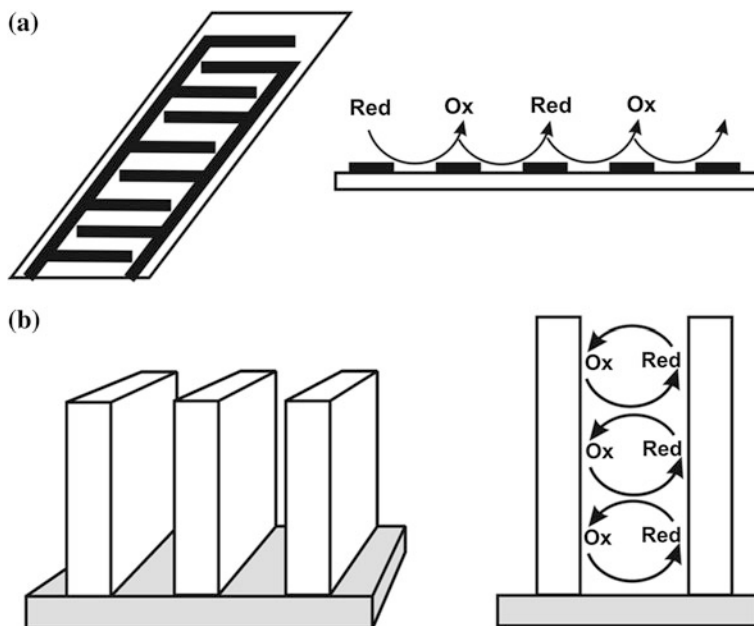


Fig. 3.27 Multi-electrode formats of transducers for the amplification of the signal of electrochemical immunosensors. **a** interdigitated electrodes, **b** 3D comb electrodes

drawback of the system required. The working potential of the redox species detected should be low enough to eliminate interfering redox reactions because the measurement format does not allow controlling the working potential of the electrode. To some extent, the signal is similar to that measured in electroconductive microcells as described in the next section.

In a similar manner, the so-called 3D electrodes have been manufactured and used in the electrochemical immunoassay based on alkaline phosphatase and 4-aminpophenyl phosphate (Fig. 3.27b) (Honda et al. 2005). The “comb shape” of the surface is produced by a combination of electrolysis and template electroless deposition of Ni. The combs are high enough to involve most of the reaction product in the cycled redox conversion, resulting in the signal amplification versus a plain electrode.

Label-free immunoassay techniques are commonly realized in impedimetric biosensors in which the changes in the resistance and the charge distribution are monitored while the *Ag–Ab* complex is formed on the electrode interface. Of other approaches, the substrate elimination methods can be mentioned. The enzyme and *Ab* are immobilized together on the solid support. The interaction with *Ag* brings about a steric hindrance for the substrate access resulting in formal inhibition of the enzymatic reaction. Peroxidase and cholinesterase were tested in the detection of anti-DNA antibodies produced in blood in autoimmune diseases (Fig. 3.28).

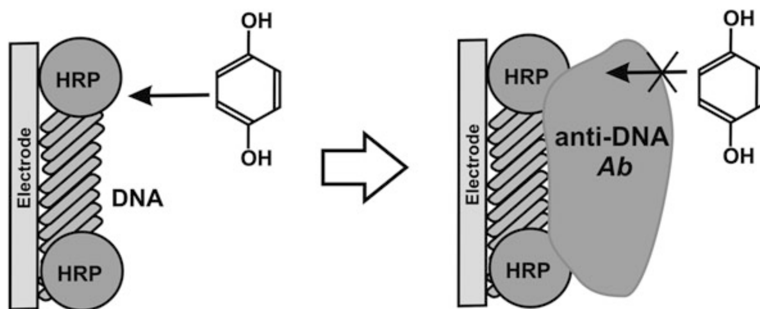


Fig. 3.28 The detection of DNA—anti-DNA Ab interaction based on the activity of co-immobilized enzyme

Separation-free techniques assume a stepwise addition of the reagents with no intermediate washing steps. Such a format not only accelerates the immunoassay but also improves the reliability of the results and can be realized in a flow-injection mode. One of the approaches suggests the modification of the electrode with a label (ferrocene as an example) by covalent binding to the Au electrode covered with the cystamine monolayer and *Ag* by electrostatic deposition.

The following reaction with *Ab* added to the solution results in a dramatic decay of the ferrocene signal because of the suppression of electron exchange abilities caused by a denser nonconductive layer formed by the *Ag*–*Ab* complex (Blonder et al. 1996). In addition to ferocene, microperoxidase and hemin, as well as a few other mediators, can be used in relative schemes of the enzyme signal amplification in the immunosensor design. The idea of separation of the electron transfer chain can be realized for diffusionally free mediators as well. Thus the pathogens related to *Listeria monocytogenes* and *Bacillus cereus* were determined in the electrochemical ELISA with ferricyanide ion as a mediator (Susmel et al. 2003). The signal decreased due to the delayed transfer of the ferricyanide ion to the electrode surface. The use of a mediator is often combined with the parallel investigation of the electrode reactions with electrochemical impedance spectroscopy (EIS). Of other substrates, thionine has found an interesting application for carcinoma antigen 125 detection. The thionine was both introduced into the carbon paste as heterogeneous mediator and added to the solution as a diffusionally free component of the reaction. The Au nanoparticles stabilized the redox characteristics of the electrode materials as thionine carriers. The Ab was adsorbed on the electrode surface, and the thionine signal decreased with the antigen concentration in the sample tested (Tang et al. 2006). Human chorionic gonadotropin was determined with a similar sensor based on the alternating layers of Au nanoparticles and Methylene blue (Chai et al. 2008).

Most of the solutions directed towards enhancing the immunosensor performance are related to the conditions of the label detection. For enzymes, the electrodes can first be modified with metal nanoparticles, carbon nanotubes and electrochemically active metal oxides that not only accelerate the electron

transduction but also increase the relative surface area and hence the current recorded. Au nanoparticles have some indisputable advantages in the list of modifiers. They can be either deposited on the electrode or first modified with thiolated agents and then proteins to amplify the number of potential binding sites and/or label molecules attached to the electrode via the *Ag–Ab* complex formation. It should be mentioned that contrary to enzyme sensors, the use of nanomaterials in the assembly of immunosensors often requires diffusionally free mediators to establish the electric wiring of the label and the electrode. This is necessary because of a rather large volume of the *Ag–Ab* complex that is separated from the electrode surface of the distance eliminating the direct electron transfer.

The Au nanoparticles can serve as a catalyst in the reactions mimicking the enzyme activity on the electrode surface. Thus, immunoglobulin G was detected by sandwich immunoassay with the Au nanoparticles attached to appropriate antibodies and the hydroquinone/benzoquinone catalytic cycle. The electrode was modified with ferrocene implemented in the SAM, and the $NaBH_4$ was used as an external reducer. The reaction was very sensitive to the Au nanoparticles bearing the Ab molecules. The LOD of 1.6 fM of a target analyte was about 100 times lower than that achieved in the conventional immunoassay format (Das et al. 2006).

To some extent, carbon nanotubes and ferritin can play a similar dual role of an electroconductive carrier. The latter consists of a protein core of 12.5 nm in diameter with an internal cavity that can accumulate up to 4,500 iron atoms. Additionally, ferritin can be easily modified by other mediators, such as ferricyanide complexes or phosphates of transient metals (Liu and Lin 2007). The nanoparticles of mediators can also be dispersed in nonconductive carriers, e.g., dendrimers (Giannetto et al. 2011) and silica nanoparticles (Hong et al. 2009). The components of biorecognition layers used in electrochemical immunosensors are summarized in Table 3.7.

3.1.3 Conductometric and Impedimetric Devices

The conductivity of liquids results from the dissociation of the electrolytes, i.e., mineral salts, acids and bases. The ions formed in the dissociation are able to migrate in the electrical field to the oppositely charged electrodes. The current recorded in the cell with two electrodes immersed in the electrolyte will depend on this movement, i.e., on the migration and diffusion of the main ions. In accordance with Ohm's Law (3.57),

$$E = IR \tag{3.57}$$

where E is the applied potential and R is the resistance of the solution. The higher the concentration of ions, the lower the R. The distance between the electrodes, their surface area and ion nature also affect the resistance of the solution. The

Table 3.7 Auxiliary components in the electrochemical immunosensors

Component	Function	Component	Function
Dextran, cyclodextran	Ab (enzyme) stabilization	Metal nanoparticles	Ab (Ag) immobilization and electric transduction
	Accumulation of redox active reactants	Ferrite particles	Magnetic separation followed by electrochemical detection
ZnO, zirconia phosphate nanoparticles nanoparticles	Immobilization of immunoreagents, increase in their surface concentration	Liposomes	Immunoreagents release in the reaction area
Quantum dots, TiO_2	Electrochemiluminescence initiation, Ab immobilization	Transient metals complexes and oxides	Mediation of the label indicator redox conversion
Carbon nanotubes	Ab (Ag) immobilization and electric transduction	Electroconductive polymers	Electrostatic Ab immobilization, signal amplification
Graphene oxide	Mediation of the label indicator redox conversion	Ferrocene and ferrocene-bearing polymers	Signal amplification

reciprocal of the solution is called *conductance, or conductivity S*, which is measured in *siemens* (S or Ω^{-1}). The *specific conductivity* κ is expressed by the conductivity S, the electrode area A, and the distance between the electrode d (3.58).

$$S = \frac{1}{R} = \kappa \frac{A}{d} \tag{3.58}$$

The ratio d/A is determined by the design of a particular measurement cell and is called a *cell constant*.

The conductivity depends both on the number of ions and their ability to move in aqueous media. The latter is referred to as *mobility u* (3.59).

$$u = \frac{|z|e}{6\pi\eta R} \tag{3.59}$$

Here z is the ion charge, R its solvated radius, η the viscosity of the solvent, and e the elementary charge constant. The conductivity can be written in terms of mobilities of all the ions present in the solution (3.60).

$$\kappa = F\sum_i |z_i|u_i c_i = F\sum_i |z_i|\left(\frac{|z|e}{6\pi\eta R}\right)c_i \tag{3.60}$$

Since the conductivity depends on the ion concentration, it can be referred to one unit of the ions' quantity (*molar conductivity* Λ) (3.61).

$$\Lambda = \frac{\kappa}{c} \tag{3.61}$$

In other words, molar conductivity is defined as the solution conductivity normalized by the total concentration of the ions $c = \sum_i c_i$.

The measurements in conductometry are based on the dependence of the conductivity and the specific conductivity on the ion concentration.

For strong electrolytes, the molar conductivity ideally does not depend on their concentration. However, most electrolytes are partially dissociated so that they behave differently in different concentrations. The *limiting molar conductivity* Λ^0 is mostly used for the comparison of conductivities. This refers to the value hypothetically corresponding to the limit of the infinite dilution of the electrolyte. In practice, the Λ^0 value is determined from Kohlrausch's Law (3.62):

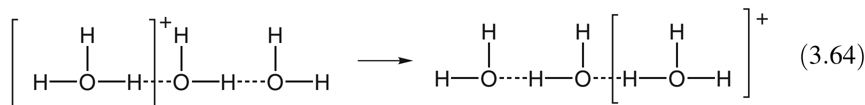
$$\Lambda = \Lambda^0 - Const\sqrt{c} \tag{3.62}$$

The Λ^0 for any electrolyte can be expressed as the sum of independent contributions of constituent cations and anions (3.63)

$$\Lambda^0 = v_+\lambda_+^0 + v_-\lambda_-^0 \tag{3.63}$$

where the v_+ and v_- represent stoichiometric coefficients for the cation and anion in the electrolyte and λ^0 is the limiting ionic molar conductivity*, or ion molar mobility* in an infinitely diluted solution (see values for common inorganic ions in Table 3.8).

Although the limiting ionic molar conductivities range from 50 to 70 Ω^{-1} cm^2 mol^{-1}, there are two important exceptions, i.e., the hydrogen cation and hydroxide anion. For them, the charge transfer is conducted via the re-distribution of the hydrogen bonds based on the water ionization mechanism (3.64) and does not assume the physical movement of the hydrogen (hydroxide) particle as such.



(3.64)

The temperature dependence of the conductivity is expressed by the temperature coefficient $\alpha = d\lambda/dt$, which is equal to about 0.02 deg^{-1}(0.014 and 0.018 deg^{-1} for H^+ and OH^- ions, respectively). The ion mobility can be re-calculated to a temperature different from the standard of 25 °C by (3.65).

$$\lambda_{i,t}^0 = \lambda_{i,25}^0\left[1 + \alpha\left(t - 25\,^0\mathrm{C}\right)\right] \tag{3.65}$$

Table 3.8 Ion mobility in infinitely diluted solution at 250 °C

Cation	λ_{+}^{0}, Ω^{-1} cm^2 mol^{-1}	Anion	λ_{-}^{0}, Ω^{-1}cm^2 mol^{-1}
H^+	349.8	OH^-	198.3
NH_4^+	74.6	Cl^-	76.4
K^+	73.5	NO_3^-	71.5
Na^+	50.1	CO_3^{2-}	69.3
Ca^{2+}	59.5	$H_2PO_4^-$	36.0
Fe^{3+}	68.0	SO_4^{2-}	80.0
$[Co(NH_3)_6]^{3+}$	102.3	$[Fe(CN)_6]^{3-}$	100.9

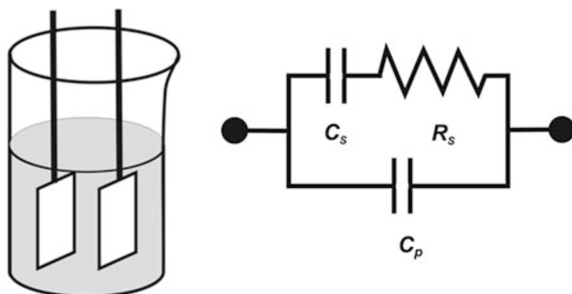


Fig. 2.29 Conventional two-electrode cell and a simplified equivalent circuit

Most conductance measurements are performed with a two-electrode cell. The electrodes of the same size are positioned parallel to and facing one another and separated with a fixed gap. The equivalent electric circuit of the cell is presented in Fig. 3.29. Here, the capacitance and resistance of the electric connectors are ignored.

The R_S signifies the solution resistance, and C_S and C_P denote combined series and parallel capacitances, respectively. In modern equipment, the sinusoid or pulse potential modulation is used. Short pulses with a high frequency allow avoiding the problems related to Faradaic processes on the electrodes and the influence of the electrode interface on the resistance measured. The main challenge of the conductance measurements is to separate the contribution of the capacitive elements of the circuits. This can be achieved by the waveform interrogation that involves several galvanostatic and potentiostatic techniques.

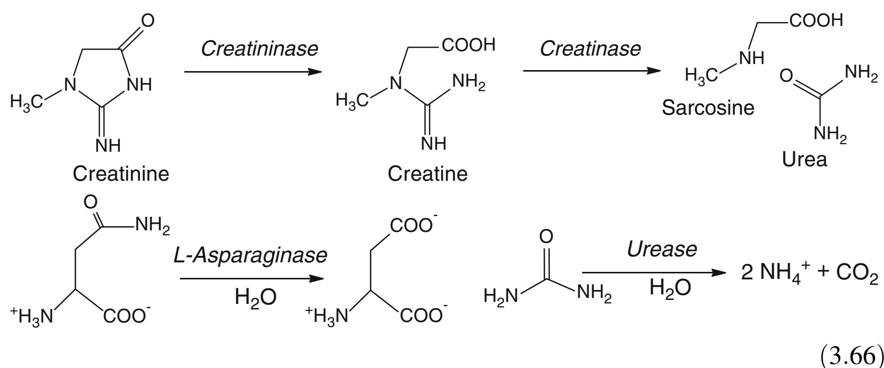
Conductometry first found application in measuring enzyme activity. In 1960 it was suggested to quantify urea by means of a urease solution in weakly basic media in the 0.1 μM to 2 mM range. Somewhat later (1965), a differential setup was described for the same purpose for the first time. Two pairs of electrodes were each placed in their own measuring cells; one was with urease and the other without, and the difference in conductivities was proportional to the concentration of urea. The differential scheme eliminated interferences related to temperature, the difference in buffer capacity in the series of the solutions, etc. Both approaches, i.e., the detection of the reactions related to the consumption or the release of

highly mobile H^+, or OH^- ions and the differential scheme of measurement, have found increasing application in the biosensor format beginning in the early 1980s.

As in pH-metric ISEs, the signal of conductometric transducers is mainly dependent on the concentration of highly mobile H^+ ions. However, the measurement is performed in a two-electrode cell and does not require any reference electrode. Moreover, urease-based conductometric biosensors show a higher sensitivity toward an analyte than any potentiometric devices. The slope of the NH_3-sensitive ISE is equal to 29 mV and is two times lower than that of pH measurements. Meanwhile, the hydrolysis of each urea molecule results in binding two hydrogen ions into the ammonium cation so that the sensitivity of the detection is higher. The same refers to other reactions yielding the NH_3 (amino oxidase, oxidases of amino acids, deaminases, etc.). The conductivity can be influenced by the enzyme reaction due to some other reasons, i.e., charge separation (dehydrogenases and decarboxylases), ion migration (esterases), changes in the associations of the ionic groups (kinases). But, the pH shifts have become very attractive, due to the higher and well-pronounced signal.

Modern conductometric biosensors are far from the two-electrode cell used for measuring the conductivity of solutions. They are represented by mucro-fabricated devices, with planar elements positioned on the solid support by micromachining or lithography techniques. Some examples of such equipment are outlined in Fig. 3.30.

The first biosensor for urea was based on the silicon waver covered with a pair of gold interdigitated and serpentine electrodes. The concentration range of 0.1–10 mM of urea was achieved at pH 7.5. Multisensor setups provide the simultaneous measurement of several biochemical reactions. The immobilization of creatinase, creatininase and L-asparaginase together with urease in appropriate areas of the multisensor made it possible to detect urea, L-asparagine and creatinine (3.66) (Cullen et al. 1990).



(3.66)

Acetylcholinesterase and alkaline phosphatase were used for the conductometric detection of organophosphate and carbamate inhibitors and heavy metals, alcoholoxidase for formaldehyde, tyrosinase for 4-chlorophenol and triazine

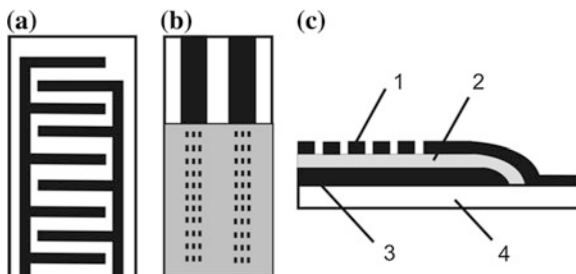


Fig. 2.30 Conductometric cells as biosensor transducers. **a** two interdigitated electrodes; **b** with additional capsulation layer; **c** multilayered electrode setup. *1* reticulated electrode, *2* conducting polymer layer, *3* auxiliary electrode, *4* nonconductive support

pesticides. The changes in the conductivity can be stimulated by the mediator-based acceleration of the enzymatic reaction. The nitrate reductase from *Aspergillus niger* was cross-linked with glutaraldehyde in the presence of nafion and metilviologen (Xuejiang et al. 2006).

Impedimetric biosensors. Electrochemical impedance measurements are similar to those of conductivity except that the main attention is focused on the capacitance and resistance on the electrode interface. For this reason, the equivalent circuits similar to those in Fig. 3.29 are used to model the electric phenomena in the electrochemical cell; the signal processing is directed to distinguish the capacitance and resistance contribution, but not to subtracting them as in conductometric techniques.

The term impedance is used in the sense analogous to the resistance toward the alternating potential/current that becomes sensitive to the frequency of alteration. This is stressed by the term "electrochemical impedance spectroscopy," which appeared attractive during the past decade (Orazem and Tribolett 2008).

The working electrode is polarized by the sinusoidal potential E_t (3.67).

$$E_t = E_0 \sin(\omega t) \tag{3.67}$$

where E_t and E_0 are the potentials at the time t and amplitude and $\omega = 2\pi f$ is the radial (angular) frequency; f is the ordinary frequency expressed in Hertz (Hz).

The current response I_t to the potential is recorded by (3.68). It has the same frequency but is shifted against the potential alteration by a phase angle ϕ.

$$I_t = I_0 \sin(\omega t + \phi) \tag{3.68}$$

In analogy with Ohm's Law, the impedance Z of the system is expressed as (3.69), i.e., in terms of a magnitude Z_0 and a phase shift ϕ, which define a vector in the plane of the complex variable (Fig. 3.31).

$$Z = \frac{E_t}{I_t} = \frac{E_0 \sin(\omega t)}{I_0 \sin(\omega t + \phi)} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \phi)} \tag{3.69}$$

In accordance with the data presentation, the vector can be described either by its length and angle to the axis or by its projections to the axis. The first description is realized in the so-called "Bode diagram," which plots $\log|Z|$ and ϕ as a function of $\log f$.

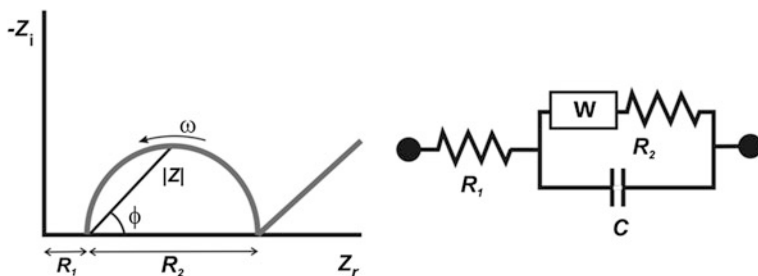


Fig. 3.31 Presentation of the impedance as a function of complex variable (the Nyquist diagram) and the corresponding equivalent circuit

In the second case, the Laplas transformation is used and the impedance is presented in plots of real and imaginary components of the function as projections in the in-phase and out-of-phase axes (3.70).

$$Z = Z_r + jZ_i; \; j = \sqrt{-1} \tag{3.70}$$

The impedance vector is graphically presented in the in-phase and out-of-phase axes representing real and imaginary components of the function. The equivalent circuit of Fig. 3.31 is called the *Randles circuit*. It has an intuitively clear interpretation in terms of double layer structure. Thus R_1 corresponds to the resistance of the solution, R_2 (R_{et} or R_{ct} are more common abbreviations) to the resistance of the charge transfer and C to the capacity of the surface layer. W is the Warburg impedance, which reflects the contribution of the counter ions moving near the electrode interface, the charge of the polymeric films attached to the electrode, etc., to the charge carriers. There are many other equivalent circuits described in literature that take into account a more complicated behavior of ions at the electrode interface, e.g., their transport through the charged polymeric layer or pores, the unequivalent conditions within the surface layer, etc.

In the Nyquist diagram, the semicircle corresponds to the limitation of the charge transfer in the impedance value where the 45° line indicates the limitation of the diffusion of charge carriers toward the electrode surface.

The appropriate characteristics of the resistance can be calculated from the location of the semi-circle as is shown in Fig. 3.31. The capacitance of the surface layer is determined from the maximum point at the semicircle and the Warburg impedance from the intercept obtained by the extrapolation of the linear portion of the curve to the real axis (3.71).

$$\begin{aligned} \omega &= \frac{1}{R_2 C} \\ W &= R_1 + R_2 - 2\frac{CRT}{n^2 F^2 \sqrt{2}}\left(\frac{1}{\sqrt{D_O c_O}} + \frac{1}{\sqrt{D_R c_R}}\right) \end{aligned} \tag{3.71}$$

The behavior of the electrode interface frequently differs from that ascribed by the above equations. Taking this into account, the so-called "constant phase element" (CPE) is introduced instead of the capacity in the Randles circuit. Its value depends on two parameters, i.e., Q and α, which determine the particular expression of its impedance, Z_{CPE} (3.72).

$$Z_{CPE} = \frac{1}{Q(j\omega)^{\alpha}} \tag{3.72}$$

In this case, Q has the numerical value of the admittance 1/|Z| at$\omega = 1$ Rad/s. The phase angle of the CPE impedance is independent of the frequency and has a value of $-(90 \times \alpha)$ degrees. This gives the CPE its name.When $\alpha = 1$, Q has units of capacity (μF/cm^2) and the equation is the same as for the impedance of a capacitor, in which $Q = C$.

$$Z_{CPE} = \frac{1}{j\omega C} \tag{3.73}$$

When α is close to 1.0, the CPE resembles a capacitor, but the phase angle is not 90°; it is constant and somewhat less than 90° at all frequencies. The case $\alpha = 0$ describes a full resistor. The non-ideal behavior of the electrode interface is commonly related to the disturbance in the surface layer properties occurring in the direction that is orthogonal to the electrode plane.

In impedimetric biosensors, the biocomponent is immobilized on the electrode and the interaction with an analyte molecule is detected. The measurement conditions are chosen in such a way that the impedance of the biosensor controls the overall impedance. This is usually achieved by using the counter electrode with a much smaller area and a supporting electrolyte (0.01–1 M) to adjust the solution resistance so as not to limit the current response.The redox-active compounds are commonly added to the solution, resulting in a well-defined charge transfer resistance R_2. If the redox-active compound is omitted or the electrode is insulated with a nonconductive layer, the capacitive impedance behavior is observed (R_2 becomes very large). In an intermediate case, both parameters are discussed in terms of the reactions at the electrode. Thus, for the impedimetric DNA sensors with the surface covered with the SAM and DNA probe, the hybridization results both in the changes of the charge distribution (capacitance changes) and the permeability of the surface layer for the redox indicator (resistance changes) (Katz and Willner 2003). Ferricyanide salts are commonly used for detection. They bear the negative charge as the DNA strands and experience the electrostatic repulsion increasing with the hybridization event. Besides ferricyanides, anthraquinone disulphonic acid, positively charged Ru(II) hexamine (chloride salt) and neutral hydroquinone/benzoquinone, redox indicators are used (Prodromidis 2010). In some cases, the redox probes can interact with the constituents of the surface layer. Thus, ferricyanide ions can damage the SAMs on gold and reduce the protein activity (Rickert et al. 1996).

The EIS measurements can be performed in various modes. Most frequently, the direct current potential of the biosensor is fixed (the potentiostatic mode in a three-electrode cell). The equilibrium potential is specified to avoid the influence of Faradaic processes, The ferricyanide pair (equal amounts of ferricyanide (II) and (II) salts, also called the *redox probe* and defined as $[Fe(CN)_6]^{3-/4-}$), is determined as the half-sum of the peak potentials on the cyclic voltammogram. It is assumed that no electron exchange reactions take place within the surface layer. The other approaches involve the measurement at the "open circuit" potential (a two-electrode cell with the measurement conditions similar to potentiometric sensors) and the galvanostatic regime in a three-electrode cell.

The idea of most impedimetric biosensors is illustrated in Fig. 3.32 in the example of DNA hybridization detection.

One can see that the bigger the analyte molecule and the denser the electrode coating, the higher the sensitivity of the signal detection. The signal is hence very close to that realized for an intercalator-based detection of DNA hybridization (see Fig. 3.22 for comparison). However, changes in the permeability of the surface layer are much less selective than DNA-intercalator interactions. This offers strict requirements for surface layer assembling that are directed to the suppression of non-specific adsorption of proteins from the biological samples, or non- complementary ODN sequences in the case of DNA sensors. The examples of the biorecognition layer organization applied on the impedimetric biosensors are presented in Table 3.9.

To avoid the above-mentioned interferences, the BSA and polyelectrolytes are commonly added to the solution prior to the analyte contact with the biosensor.

As regards enzymatic reactions, impedimetric sensors are less appropriate because of the rather minor changes in the surface layer. The formation or partial dissolution of insoluble products is an exception. Thus, the peroxidase oxidation of 4-chloronaphtol yields the precipitation of the nonconductive polymeric product and the following increase in the charge transfer resistance. The reaction was successfully applied for the detection of cholera toxin in sandwich immunoassay

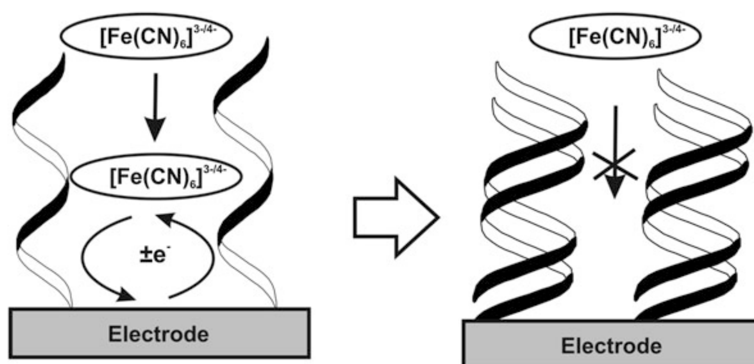


Fig. 3.32 Impedimetric detection of a DNA hybridization event with ferricyanide redox probe

Table 3.9 Examples of assemblies of impedimetric biosensors

Biocomponent	Modifying layer/redox probe or blocking agent	Analyte
Ag (transglutaminase) (Balkenhohl and Lisdat 2007)	Polystyrolsulfonate/BSA	Transglutaminase Ab
Ani-Aflatoxin M_1Ab—colloidal Au conjugate (Vig et al. 2009)	BSA	Aflatoxin M_1
Anti-atrazine single-chain Fab Ab (Ionescu et al. 2010)	Polypyrrole/BSA	Atrazine
Ochratoxin A Ab (Radi et al. 2009)	4-Carboxyphenyl SAM	Ochratoxin A
ODN related to HIV sequence (Baur et al. 2010)	Polypyrrole/Hydroquinone	HIV-related ODN
Partially hybridized ds-ODNs (Lin et al. 2011)	SAM on Au	Hg^{2+}, Pb^{2+}, Ag^{+}
Native ds-DNA (Ziyatdinova et al. 2008)	Multi-walled carbon nanotubes/ DNA LbL layer	DNA damaging factors, antioxidants
ODN probe (Zhu et al. 2010)	Multi-walled carbon nanotubes/ dendrimer LbL layer	Complementary DNA sequence
Aptamer toward thrombin (Porfireva et al. 2010)	Multi-walled carbon nanotube/ poly(Methylene blue)	Thrombin
ODN probe (Shervedani and Pourbeyram 2011)	SAM on Au/Zr(IV) phosphate	Complementary DNA sequence

(Alfonta et al.2001). For the same purpose, the soluble 3-amino-9-ethylcarbazole was converted in peroxidase to catalyze the reaction into insoluble 3-azo-9-ethylcarbazole (Balkenhohl and Lisdat 2007). Alkaline phosphatase was proposed for the amplification of the impedimetric signal on the *Ag–Ab* interaction caused by the precipitation of the insoluble product obtained by the hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate (Ruan et al. 2002).

In the EIS measurements, the Nyquist diagram is most often used because of the visual data presentation. If the impedance measurements are aimed at the optimization of the surface layer or the confirmation of the layer deposition in a stepwise modification of the transducer, the quantitative consideration of increased semi-circles is quite sufficient. Figure 3.33 represents changes in the Nyquist diagram occurring at the electrode modified with a polyfunctional macrocyclic carrier bearing an aptamer toward thrombin and Neutral red as signal amplifier. Impedance measurements were performed in the presence of a ferricyanide probe. The addition of increasing amounts of thrombin increases the radius of the semicircle, indicating the binding of the bulky nonconductive analyte molecule in the surface layer.

The quality of the equivalent circuit chosen can be estimated in accordance with the shape of the semi-circles, and quantitatively by the fitting parameters determined by the software used in modern impedimetric analyzers. Most manufacturers offer several types of models, some of which coincide with specific requirements for the biosensor signal interpretation.

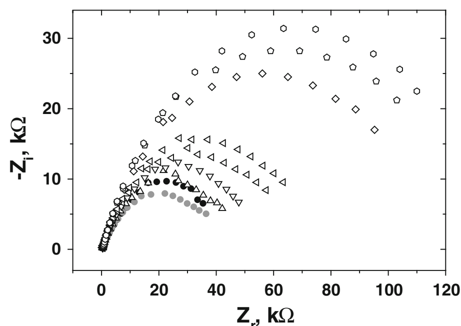


Fig. 3.33 The Nyquist diagram recorded at the electrode covered with macrocyclic modifier bearing Neutral red (*gray circles*) and aptamer against thrombin (*black circles*). Series of open circles correspond to increasing concentrations of thrombin from 1 nM to 1 μM

Besides the simplest and most popular Randles circuit, a dual scheme is often considered (Fig. 3.34). It consists of two semi-circles that correspond to the inner (electrode-biorecognition layer) and outer (surface layer-bulk solution) interfaces involved in the charge transfer. Such a presentation of data is of less importance for analytical purposes but can specify the influence of the structure of the biorecognition layer on the sensitivity of the signal detection. The dual scheme can be easily extended to more complicated structures of the surface layer, e.g., for a separate description of the transfer of the charge carriers along the layer thickness and in the pores (channels) different in charge and diffusion rate from the main layer material.

The consideration of various equivalent circuits that are different in the description of the processes in the surface layer are of greater significance for the selection of an optimal structure of the biorecognition layer and some mechanism details (the rate-limiting step, the nature of the main charge carrier providing impedance changes, etc.) that are necessary rather in the discussion of biosensor behavior than for analyte quantification. In most cases, Randles circuit gives quite a reliable result of impedance estimation.

The EIS biosensors often involve electroconductive polymers, e.g., polyaniline or polypyrrole. They can react with the ferricyanide indicator and change their electroconductivity in accordance with their redox-status. The reaction of an analyte binding suppresses the access of the ferricyanide ion and hence changes in the resistance of the charge transfer. This amplifies the signal of the impedimetric

Fig. 3.34 The Nyquist diagram corresponding to a “dual” equivalent circuit describing the charge transfer on the electrode covered with the layer of a finite thickness

sensors based on conventional transducers. The use of various nanosized materials, i.e., metal particles, carbon materials, zirconia phosphates, etc., not only increases the density of the binding sites per electrode area, but also enhances the thickness of the layer in which the charge and permeability change due to the target analyte binding. This shifts the plane of biorecognition from the electrode toward the solution, and to some extent eliminates the contradiction between the size of the biochemical element (*Ab*, the ODN probe) and the distance of the direct electron transfer. As was mentioned above in the discussion of the FET-based immunosensors, the size of the *Ab* molecule is large enough to span the double layer on the electrode interface. This decreases the sensitivity of the charge distribution changes to the biorecognition event. The same can be referred to the DNA sensors, especially those including the SAMs on the bare electrode. Nano- sized particles and the carriers with the pores and channels of appropriate dimensions amplify the signal of impedimetric sensors.

The resistance of the charge transfer remains the principal parameter used for the quantification of the analytes. The capacity of the surface layer is less useful because of the lesser reproducibility and a greater effect of ionic species in the solution. Moreover, changes in the three-dimensional structure of the biopolymers involved in the surface reactions affect the capacity. This is due to minor changes in the capacity of the inner area filled with a protein or the DNA that resulted from a biochemical interaction with a target.

The impedance measurements, especially those based on electropolymerized materials, can be performed in an open circuit with no redox indicator. The interdigitated electrodes for conductivity measurements described above can serve this purpose. The impedance |Z| can be recorded instead of charge transfer resistance. Of other possibilities, the use of multiplied and signal perturbation pulses of the potential can be mentioned. The capacitance is determined from the dependence of the transient current response on time.

3.2 Optical Transducers

3.2.1 Optosensors

Most optosensors are based on optical fibers modified with various auxiliary chemicals and biocomponents. Optical fiber is a flexible transparent fiber (core) made of glass (silica, plastic) and covered with a cladding that is different in the light refraction from the core material. Due to the *total internal reflection* phenomenon, optical fibers transmit light. The light increases along a core in the refractive index of a core n_1, which is larger than that of a cladding (n_2). When the ray of light strikes the boundary interface between the core and the cladding, and the angle of incidence is larger than the critical values determined by the Shell

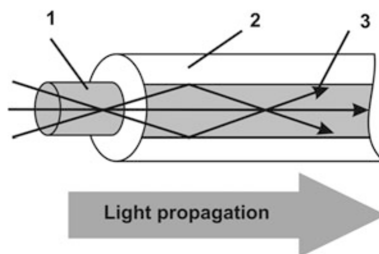


Fig. 3.35 Optical fiber. *1* core, *2* cladding, *3* direction of light

Law (3.74), it is totally reflected and hence increased through the fiber (Fig. 3.35) (Marazuela and Moreno-Bondi 2002).

$$\theta_c = \frac{1}{\sin(n_2/n_1)} \tag{3.74}$$

In the simplest manner, the analyte is optically active, and its concentration can be expressed by the Beer-Lambert Law (3.75).

$$A = \varepsilon cl \tag{3.75}$$

The A (absorbance) is defined as a logarithm of the ratio of the light intensity I_0 (the incident light) to I (light passed through the sample), i.e., $A = \log(I_0/I)$. The molar absorptivity ε is the analyte characteristic, and l is the optical path length. In an optical fiber mode, a classical spectrophotometer scheme can be emulated with two optical fibers for illumination and travelling light detection. In one fiber mode, a mirror can be used near the tip of the fiber to return the light through the same fiber.

Alternatively, a part of cladding can be removed and the losses of the light due to the incomplete total internal reflectance are measured. It should be mentioned that the detection equipment of a conventional spectrophotometer is rather large and does not fully correspond to the sensor requirements, i.e., small dimensions, cheap and simple operation, mobility and possibility of being used in field conditions. For this reason, the tungsten lamps, lenses, prism or grating monochromators are replaced with *light-emitting diods* (LEDs), photodiode arrays and *charge coupled devices* (CCDs) as photodetectors. The setup is often directed to the requirements of a specific analyte detection without recording a full spectrum and with the measurement time not larger than 10 ms. The facilities of optical fibers as miniaturized spectrophotometers are substantially enhanced by the use of modifiers immobilized on the padding or in the core tip. In most cases, the modifier used generates a color or luminescing species in a reversible reaction related to the analyte. The detection of pH with an insoluble pH indicator is an example. Such modifiers are easily combined with biocomponents, e.g., enzymes producing the pH shift resulting in the changes of the indicator color. The immobilization can be performed by all the methods described for biosensors, but the matrix formed on the tip of the fiber should not interfere with optical measurements.

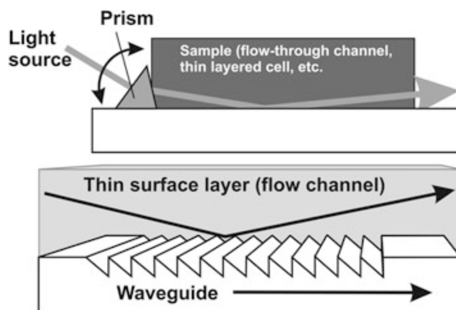


Fig. 3.36 Grating coupler sensor for fluorescence measurements

The reactions of fluorescence quenching are described by the Eq. (3.76).

$$\frac{I_0}{I} = 1 + K_q R_0 c \tag{3.76}$$

Here, K_q is the equilibrium constant of the quenching reaction, c is the concentration of a quencher, and R_0 is the quantum yield of the reaction. Molecular oxygen is often detected as a quencher of the fluorescence of many organic dyes. This offers potentials for the detection of biochemical reactions resulting in the oxygen consumption, e.g., respiratory measurements with the cell culture, the oxidoreductase catalyzed oxidation of the organic substrates, etc.

In addition to optical fibers, planar sensors are also described. In *grating coupler sensors* (Fig. 3.36), laser beams are coupled to the planar medium by diffraction at the grating with a precise angle of incidence. Light is totally reflected many times at the interface because of the low thickness of the surface layer, and hence the signal is multiplied. The coupling of light into a waveguide by means of a grating coupler occurs at certain angles determined by (3.77) (Gründler 2007).

$$n_{\text{eff}} - n_a \sin \alpha = \frac{k\lambda_0}{\Lambda} \tag{3.77}$$

where n_{eff} is the effective refractive index of the waveguide mode excited by the input coupling. The refractive index of the ambient, vacuum wavelength and grating constant are denoted by n_a, λ, and Λ, respectively. The diffraction order is denoted by k, and the n_{eff} value changes depending on the concentration of particles within a sample.

Going back to the total internal reflectance phenomenon, we can assume that at the reflection interface, the intensity of the light does not decay to zero. A small portion of the light penetrates the reflecting medium. This electromagnetic field, called the evanescent wave, has the intensity that decays exponentially with the distance orthogonally to the reflection interface. The penetration distance depends on the wavelength of the light and the angle of incidence, and it increases while matching the closer refraction index. The evanescent wave interacts with the molecules located within the penetration depth, producing an energy flow across the reflecting surface to maintain the evanescent field and hence the attenuation in

the reflectance. Based on these phenomena, absorption sensors on evanescent waves, called *attenuated total reflection sensors,* can be developed. If the evanescent light excites an ionophore placed near the reflectance surface, the fluorescence is emitted back into the fiber and guided to the detection (*total internal reflection fluorescence*, TIRF sensor).

Optical fiber biosensors can be used in combination with various spectroscopic techniques, e.g., absorption, fluorescence, Raman, and surface plasmon resonance (SPR). (The latter will be discussed in the following sections.) The biocomponent of the biosensor is immobilized close to the optical fiber or on its surface. Additional fibers can be used to guide the light from the source to the sensor tip and the emerging irradiation to the detector. Many of the biopolymers exert their own fluorescence at certain conditions, but fluorescence biosensors are commonly combined with artificial labels. Thus, many of the immunoreactions can be detected by flourescence quenching. The selectivity of the signal transduction is additionally attained by the illumination and excitation wavelengths. Many reactions of oxidoreductases can be monitored by the decay in the oxygen consumption measured by the decrease of the luminescence of special dye molecules, which is produced by these molecules. In bio- and chemiluminescent sensors, analyte molecules produce the light emission due to interaction of the analyte molecules with a bioreceptor.

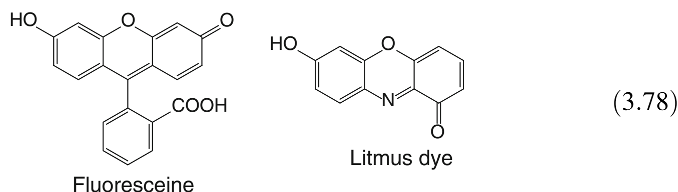
The use of optical fibers in the biosensor assembly has some advantages, such as:

- each kind of analyte can be determined by appropriate spectrometric techniques;
- fibers can transmit the light over rather long distances and do not strictly require close contact with bioreceptor molecules. This enables remote monitoring and non-invasive formats of biomedical sensors;
- the light can be guided at different wavelengths at the same time in different directions. This offers multi-analyte determinations with a single fiber; and
- optical fibers are insensitive toward electrical and magnetic interferences and do not require any reference electrodes contrary to electrochemical biosensors.

Meanwhile, some drawbacks of the fiber-optic biosensors should be mentioned. They involve interference with ambient light, the possible photobleaching of dyes and other auxiliary components, the high background absorbance or fluorescence of the fiber, a rather long measurement time, and a limited availability of commercial accessories.

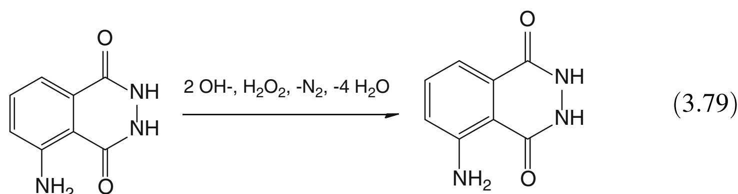
Fiber-optic enzyme sensors. Like electrochemical enzyme sensors, the optodes are mostly utilized oxidoreductases with the signal dependent on molecular oxygen or NAD^+, and hydrolases (H^+/NH_3 detection). The detection of oxygen is based on the quenching luminescence of some indicators, such as Ru(II) phenanthroline and diphenylphenanthroline complexes. They show a high photostabilitiy, a long excited state lifetime and high quantum yields. NAD-dependent oxidoreductases are monitored by the fluorescence of the NADH produced in the enzymatic conversion of an analyte.

The enzyme-based fiber-optic sensor can utilize the polyaniline and polypyrrole layers that change their absorbance characteristics in the reaction of protonation (detection of esterases) and deprotonation (detection of NH_3 releasing enzymes, e.g., urease, monoamine oxidase, amidases, oxidases of amino acids, etc.). Of the more common fluorescent pH indicators, fluorescein derivatives and litmus dyes (3.78) can be mentioned. Bromothymol blue and chlorophenol red can also be used for the development of absorbance biosensors (Rich and Myszka 2002).



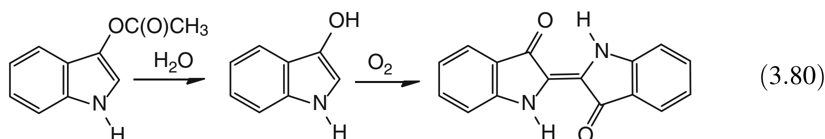
(3.78)

The indication of hydrogen peroxide is mainly based on the chemiluminescent reaction of luminol oxidation (3.79).



(3.79)

The examples of the enzymatic reaction employed in optic fiber-based biosensors are presented in Table 3.10.



(3.80)

Rather rarely, specific substrates are employed to detect the fluorescence related to the products of enzymatic reaction. Thus, acetylcholinesterase catalyzed the hydrolysis of the esters of 1-naphtol and indoxyl that resulted in the formation of the appropriate product either able to fluoresce or to convert in such species. The reaction of indoxyl acetate is given as an example (3.80) (Díaz and Peinado 1997).

Immuno- and DNA sensors based on optodes. The use of optical detection principles for the specific binding of immuno reagents and DNA complementary sequences is mainly based on the label techniques described in Chap. 2 and

Table 3.10 Examples of enzyme application in optical fiber-based enzyme sensors (summarized from (García-Campaña and Baeyens 2001) and other sources)

Enzyme	Analyte	Optically active component	Indicator/Reagent
Glucose oxidase	Glucose	Oxygen	Ru phenanthroline complexes
Penicillinase	Penicillin	pH	Aminofluorescein
Urease	Urea	pH	Aminofluorescein, polypyrrole
Acetylcholinesterase	Enzyme inhibitors	pH	Bromothymol blue, Litmus dye, Chlorophenol red
Alcohol dehydrogenase	Ethanol	NADH	–
Alkaline phosphatase (Chen et al. 1996)	Enzyme inhibitor (Paraoxon)	–	Chloro-3-(4-methoxy spiro[1,2-dioxetane-3-2′-tricyclo-[3.3.1.1]-decan]-4-yl) phenyl phosphate
HRP	Chlorophenols	H_2O_2	Luminol
Lactate oxidase	Lactate	H_2O_2	Luminol
Toluene monooxygenase (Zhong et al. 2011)	Toluene	Oxygen	Ru fluorescent dye
Choline oxidase	Choline	H_2O_2	Luminol

appropriate electrochemical sensors in Chap. 3. Instead of electrochemically active labels, the fluorogenic substances, e.g., fluorescein derivatives, are implemented in the *Ag* (*Ab*) molecules or the ODN sequences. Both the competitive and sandwich formats are used. The optical detectors are compatible with enzyme labels, e.g., peroxidase and alkaline phosphatase. In the first case, luminol oxidation is used as an indicator reaction. Additionally, some chromogenic substrates, e.g., tetramethylbensidine, *o*-dianisidine and 4-aminoantipirine can be used. For alkaline phosphatase, the fluorescence related to 1-naphtol, i.e., the product of the hydrolysis of the appropriate phosphate ester, can be registered with a high sensitivity.

The refractometric techniques can be used for the detection of specific analytes of the immuno- and DNA sensors. Thus, in the Mach–Zehnder interferometer, the light traveling in the waveguide is split into two arms, the sensing and the reference arms. Then two beams are recombined again in a second Y-junction producing their interference. For biosensing, the protective cladding is removed from a part of the sensing arm providing the asymmetric interferometric configuration. Any change of the refractive index in the sensing area generated by biochemical interactions affects the effective refractive index of the guided mode inducing a phase difference between the light beams travelling in both arms (Fig. 3.37).

Such a phase difference will result in the intensity I_T variation at the output of the device (3.81).

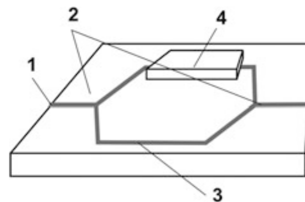


Fig. 3.37 Mach-Zender interferometer. *1* input; *2* Y-junction; *3* reference arm; *4* sensing area

$$I_T(t) = I_S + I_R = 2\sqrt{I_S I_R}\cos(\Delta\varphi_S(t)),$$
$$\Delta\varphi_S(t) = 2\pi\frac{L}{\lambda}(n_S(t) - n_R) \tag{3.81}$$

where I_S and I_R are the intensities of light in the sensing and reference arms, n_S and n_R are the effective refractive indices of the guided modes in the sensing and reference arms, respectively, λ is the wavelength of light, and L is the length of the sensing area. The refractometric immunosensor was successfully applied for the detection of the human growth factor (Dante et al. 2012).

3.2.2 Bioluminescence and Electrogenerated Luminescence Devices

Chemiluminescence refers to one of the most sensitive methods of analytical chemistry. Light can be emitted in the ultraviolet, visible or infrared region. However, visible light that is emitted is the most common and convenient from the point of view of instrumentation and applicability in the sensor format.

> Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction.

The chemiluminescent reactions can be classified in accordance with the nature of the reactions initiating light emission (Marazuela and Moreno-Bondi 2002):

- Chemical reactions of synthetic compounds involving strong oxidants, e.g., peroxide or Ce(IV) salt are commonly termed *chemiluminescent reactions*
- Light-emitting reactions arising from a living being (firefly or jellyfish) are commonly termed *bioluminescent reactions*. The terms also involve related artificial systems utilizing the natural substrate, i.e., luciferin, and the enzyme, i.e., luciferase, and genetically engineered microorganisms with a luciferase gene (*lux*)
- Light-emitting reactions that take place with the electric current are called *electrochemiluminescent reactions*.

The efficiency of chemiluminescence as a detection method directly depends on the number of the photons of light generated per one oxidizing molecule. Each chemiluminescent compound (or group) produces not more than one photon of light. The generation is quantified by the quantum yield Φ_{CL}, i.e., the ratio of the photons emitted per molecule reacted (3.82).

$$\Phi_{CL} = \Phi_{CE}\Phi_{F}\Phi_{R} \tag{3.82}$$

The *chemiexcitation quantum yield* Φ_{CE} is the probability of generating an electronic excited state ($0 < \Phi_{CE} < 1$). The common chemiluminescent reactions used in analytical chemistry have a Φ_{CE} greater than 10^{-3}. The *fluorescence quantum yield* Φ_F is the probability of the excited state emitting a photon by fluorescence. This property is frequently at least 0.1. The *reaction quantum yield* Φ_R is the fraction of starting molecules that undergo the luminescent reaction rather than a side reaction. This value is usually about 1. The chemiluminescence can be amplified by the application of the so-called fluorescent acceptor. The excitation energy is then transferred from the primary excited state compound to the fluorescent acceptor/emitter. The chemiluminescence quantum yield is then determined by the Eq. (3.83):

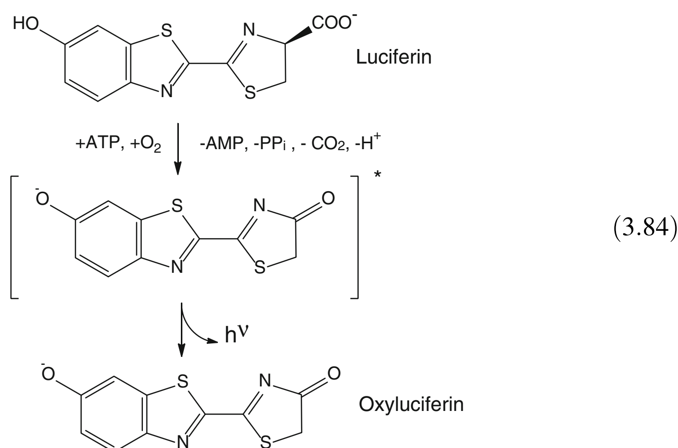
$$\Phi_{CL} = \Phi_{CE}\Phi_{R}\Phi_{ET}\Phi_{F}' \tag{3.83}$$

The *energy transfer quantum yield* Φ_{ET} expresses how efficient the conversion of the primary excited state formed in the reaction with an analyte in the excited state of the acceptor is. The fluorescence quantum yield of the acceptor $\Phi_F{}'$ should be about 1.

Bioluminescence occurs when chemical excitation of light emission takes place in living beings. It occurs in almost all zoological kingdoms except the higher vertebrates. In all organisms, the reaction of the substrate oxidation with molecular oxygen catalyzed by specific enzymes takes place. Luciferase and luciferin are generic terms referring to the reactants mentioned. The oxidation of luciferin results in the formation of a peroxy derivative, the breakdown of which provides the energy of excitation. In "in vitro" conditions, the quantum yield of the reactions is approximately 0.9. The maximum of the light emitted is often in the range of 460–560 nm; this corresponds to the color ranging from red (worms) to deep blue (most marine organisms). The color depends on the luciferase structure. In bacteria, the chromophores of the proteins associated with luciferase serve as alternative emitters, e.g., the yellow fluorescent protein (YFP) and the green fluorescent protein (GFP). Both are now used as reporter genes and cellular markers in the bioluminescent assay, preferably of gene expression.

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product (proteins, ribosomal and transport RNAs).

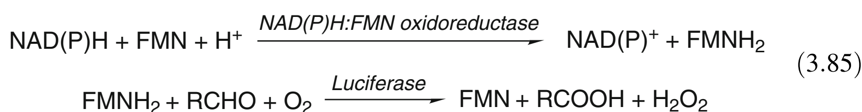
At first, the firefly (*Photinus pyralis* and related species) luciferase found analytical applications. This enzyme catalyzes the ATP-dependent oxidation of luciferin in the presence of the Mg^{2+} ions (3.84) (Roda et al. 2009).



(3.84)

The reaction sequence is ATP-dependent, and this made it possible to detect all the reactions affecting the production of ATP in microorganisms and tissues. The detection of bacterial contamination of subcutaneous wounds and the assessment of bactericides are obvious application areas. Besides, express tests based on genetically engineered organisms, such as *Escherichia coli* with the luciferase gene (*lux* and *luc*), have attracted increasing attention in environmental microbiology, the food industry, agriculture and environmental monitoring. They provide the high sensitivity of the detection of pollutants affecting natural microbial populations. Gene expression factors offer an extremely sensitive, fast and reliable detection of genotoxicants. The appropriate measurements are provided with rather compact equipment and can be performed in the field, using commercial instrumentation and text kits. Besides whole cells, the extracts and homogenates of biological tissues can be used.

Bacterial luminescence systems consist of two enzymes, i.e., NAD(P)H:FMN oxidoreductase and a luciferase-emitting light at 490 nm in the presence of FMN, NAD(P)H and a long-chain aliphatic aldehyde and molecular oxygen (3.85).



(3.85)

Whole luminescent bacteria are preferably used for the detection of environmental pollution. Their luminescence is a sensitive indicator of xenobiotic toxicity, including the cancerogenic effect mentioned above for the *lux*-tagged microorganisms.

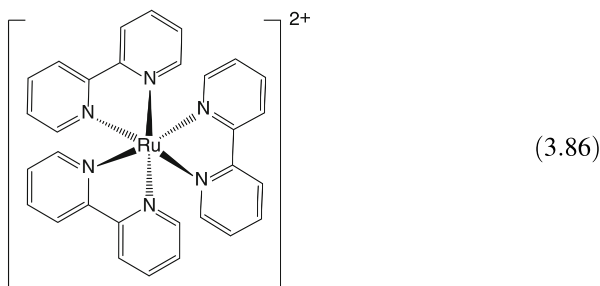
Although most of the commercial kits and instrumentation are directed toward the use of bacteria suspensions, there are examples of immobilized bioluminescent systems. The immobilization was first applied for the stabilization of rather unstable firefly luciferase and then for the development of multiple-use systems, flow-through analyzers, and test strips. The bacteria immobilization stabilizes the preparation; the entrapment in the metacrylate gels and adsorption on nylon are preferably used for this purpose. The flow-injection analysis of aspartate, glutamate, phenylalanine, oxaloacetate, bile acids, lactate dehydrogenase and creatine kinase activity has been reported. All the methods make it possible to detect picomoles of the substrates and the concentrations starting from 0.1–10 μM within two orders of magnitude. The determination of pyrophosphate anion with pyrophosphatase co-immobilized with luciferase can be mentioned as a rather unusual example.

Of other bioluminescent systems, the GFP deserves special attention (Lippincott-Schwartz and Patterson 2003). The wild type GFP absorbs blue light at 395 and 475 nm and emits green light with a maximum at 509 nm. The GFP is resistant to thermoinactivation and does not require any cofactor and substrate. Besides the wild protein, mutational analysis has generated a variety of the GFP with increased brightness and differences in stability and excitation conditions. One of the main applications of the GFP is a reporter gene, which has been expressed in many organisms for monitoring gene expression. Due to nondestructive visualization GFP, it is also used for investigating the organelle structure. It is frequently used for mapping DNA sequences in DNA arrays. Some of the applications of bioluminescence in environmental monitoring and the DNA chips are examined in Chap. 4.

Electrogenerated luminescence is another format of chemiluminescent methods that has become more and more attractive in modern bioanalytical chemistry.

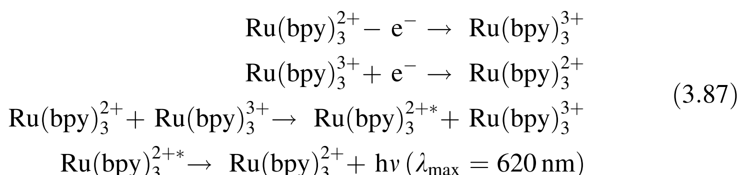
> Electrogenerated luminescence (ECL, electrochemiluminescence) is the process in which the light emission is produced by electrochemical reactions.

Although there are several ECL systems that have been investigated, the analytical application of electroluminescence is mainly related to 2,2′-bipyridil Ru(II) complex (3.86).



(3.86)

The $Ru(bpy)_3^{2+}$ undergoes the electron transfer with a central atom and organic ligands. As mentioned above, this reaction is responsible for, among others, the oxidative DNA damage. The ligands of the complex partially intercalate the DNA and participate in the electron transfer chain from the DNA nucleotides to the electrode. The luminescence is achieved by the reaction sequence (3.87) (Sun and Zhang 2012).



$$\begin{aligned} Ru(bpy)_3^{2+} - e^- &\rightarrow Ru(bpy)_3^{3+} \\ Ru(bpy)_3^{3+} + e^- &\rightarrow Ru(bpy)_3^{2+} \\ Ru(bpy)_3^{2+} + Ru(bpy)_3^{3+} &\rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{3+} \\ Ru(bpy)_3^{2+*} &\rightarrow Ru(bpy)_3^{2+} + h\nu\,(\lambda_{max} = 620\,nm) \end{aligned} \tag{3.87}$$

The oxidation of the $Ru(bpy)_3^{2+}$ ion can also be performed by chemical oxidants, e.g., the $S_2O_8{}^{2-}$ anion. One of the first analytical applications of the ECL was related to the determination of oxalate ion. The list of analytes, which might be of interest from the point of view of potential biosensor applications, also involves ascorbic acid, pyruvate ions (in the presence of Ce(IV) ions), tertiary amines, and NADH. Due to the latter, the ECL was successfully applied for the detection of the substrates of the NAD dependent oxidoreductases, e.g., ethanol and glucose. The oxidation of hydrogen peroxide has been used in the enzymatic immunoassay in the sandwich format with a peroxidase-labeled immuno reagent. The $Ru(bpy)_3^{2+}$ proper can be determined in the presence of excessive amounts of amines at subpicomolar levels (Arora et al. 1007). This offers good prospects in the DNA and immunosensor developments. At present, some commercial instrumentation is available (Perkin-Elmer and Boehringer Mannheim GmbH, now a division of Roche Diagnostics). Thus, in immunomagnetic separation, the magnetic beads modified with streptavidin, and then Ab molecules via biotin-bridging, are first separated on the electrode by the external magnetic field (Ding et al. 2010). Then $Ru(bpy)_3^{2+}$ labeled secondary Ab is attached to the surface in sandwich immunoassay format and the luminescence is electrochemically generated in the presence of tripropylamine as an amplifier. The instrumentation was used for the diagnosis of thyroid diseases, tumors, infectious diseases, anemia, cardiological function, pregnancy, and sexual

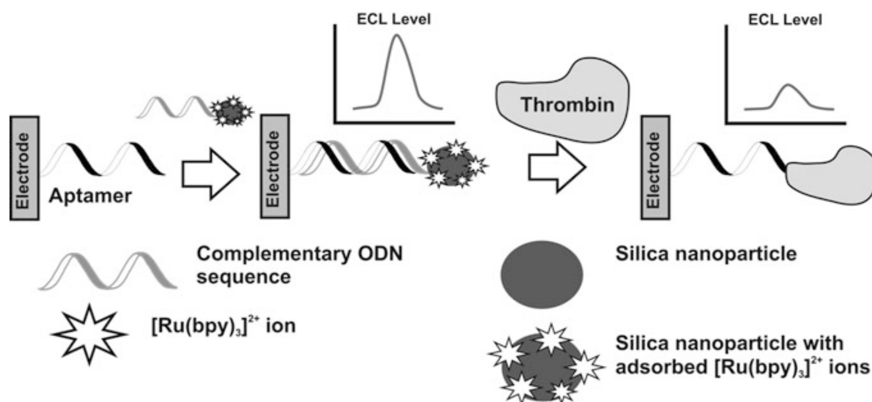


Fig. 3.38 ECL detection of thrombin based on displacement protocol with ODN labeled with silica nanoparticles bearing $Ru(bpy)_3^{2+}$ complex

dysfunction. Similar ECL-based immunoassays were applied for the detection of virulent bacteria with the labeled specific *Ab* (*Bacillus antrax*, *Escherichia coli* O157:H7, and *Salmonella typhimurium*) (Knight 2001).

Besides magnetic separation, some other assemblies of ECL-based sensors have been proposed. Commonly, the stages of electrochemical conversion are conducted on plane transducers, e.g., screen-printed carbon or Au electrodes, in flow regime with the subsequent measurement of the luminescence layer with a diode detector. The Ru complex is dissolved in the solution but can also be immobilized on the transducer surface by electrostatic adsorption in a nafion matrix or mechanical entrapment in the graphite paste prepared by mechanical mixing of the components. The use of transparent ITO electrodes (indium–tin oxides) makes it possible to measure electrochemical and luminescence response simultaneously (Fang et al. 2008). It should be mentioned that the stability of such systems with the immobilized $Ru(bpy)_3^{2+}$ is insufficient and most of its activity is lost within a working day.

The sensitivity of the DNA sensors based on the ECL can be extended by the entrapment of the $Ru(bpy)_3^{2+}$ ions in silica nanoparticles. The latter were obtained by a water-in-oil microemulsion method in the presence of an indicator. The Au electrode was consecutively treated with a thiolated aptamer and the partially complementary DNA sequence bearing silica nanoparticles. After that, the thrombin competed with the labeled ODN sequences and displaced them in the DNA probe binding. This resulted in the decay of the electrochemical luminescence (Fig. 3.38). The biosensor made it possible to detect 10 fM – 10 pM thrombin (Wang et al. 2007).

In a similar manner, the platelet-derived growth factor was bonded to an appropriate aptamer molecule immobilized by avidin-biotin bridging on the surface of the Au nanoparticles. The $Ru(bpy)_3^{2+}$ complex was subsequently bonded to the analyte via the secondary Ab bearing the label (Chai et al. 2011).

Of other ECL systems, Tb and Eu complexes have found some application in the immunoassay and luminol/H_2O_2 systems in the detection of peroxidase activity and related biochemical reactions.

3.2.3 Surface Plasmon Resonance and SERS

Surface plasmon resonance is a charge-density oscillation that occurs at the interface of two media separated by a thin metal layer in the conditions of total internal reflectance. In a resonance phenomenon, some part of the light energy "disappears" in the metallic film (Homola et al. 1999). The loss depends on the angle of the incident light and is maximal at the angle θ_{spr} when the intensity of the light reflected reaches a minimum. This surface plasmon resonance is a consequence of the oscillation of mobile electrons (surface plasmons) on the metal surface. When the wave vector of the incident light matches the wavelength of the surface plasmons, the resonance takes place followed by a loss of the energy in the form of an evanescent wave of the electric field distributed at a short distance. The penetration depth on the metal–water interface depends on the wavelength of the incident light and the metal nature. Thus, the depth for gold is about 25–30 nm and for that in the dielectric phase it is 162 and 400 nm at $\lambda = 630$ and 850 nm, respectively. The resonant frequency of the surface plasma wave (and thus θ_{spr}) depends on the refractive index of the medium. The aqueous buffer system has the refractive index μ of about 1.0, whereas that of the proteins is 1.33. Thus, the adsorption of proteins will increase the refractive index detected by a shift in the θ_{spr}. Photo-detector arrays are used in SPR instruments to measure very small changes in the θ_{spr}. This makes this method very sensitive to any process at the metal interface. Commercial SPR biosensors are able to detect 1 pg/mm^2 of the adsorbed analyte. Besides this parameter, the detectable refractive index unit (RIU) change is also reported. Typically it varies from 1×10^{-5} to 1×10^{-6} RIU. What is important is that the reactions on the metal surface and the interaction with the light take place on the opposite sides of the metal film. Thus, the detection is not affected by the optical characteristics of the solution. Further progress in the SPR biosensors is related to the integration of the components, e.g., detectors, control electronics, etc., and sensitivity enhancement.

The SPR measurements can be realized with any type of equipment developed for refractometric measurements, but most commonly the so-called Kretchmann configuration is used (Fig. 3.39).

Being reliable, this approach is not amenable to miniaturization and integration. For this reason, alternative constructions based on optical fibers and waveguide structures are examined. In early SPR microsensors, the tip was polished and metallized so that either the light reflected back from the fiber or the diffracted light from the polished end was collected. The use of microfabrication techniques provided new opportunities for the tip processing to fashion the shape of the cone or microprism.

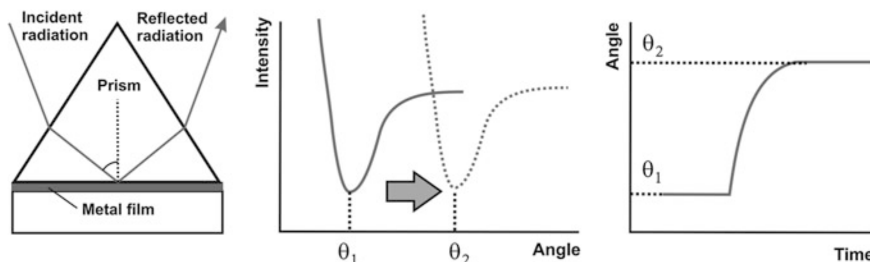


Fig. 3.39 SPR measurements by SPR Kretchman interferometer and the resonance shift in reflected light spectrum

The angle of the microprism on the fiber tip can be changed for operating in different spectral regions. Thus the measurements in the near IR range offer a 10-time sensitivity of the response. The polarization-maintaining fiber is another improvement providing more accurate and stable measurement. In other instruments, part of the fiber cladding is removed and the core surface metallized. Planar waveguide analyzers are represented by differential schemes relative to the Mach–Zender interferometer (Fig. 3.36), with the sensing area additionally covered with a thin metal layer (Fan et al. 2008).

In a standard experiment, the *Ab* or ODN molecules are immobilized via thiol terminal groups directly on the Au film or in the assembly of the SAM together with the thiolated long chain molecules necessary for avoiding the non-specific adsorption of interferences. Then the dependence of the refraction on the incident angle is measured and the minimum corresponding to the resonance condition is specified. The graph in the plots of the reflected light intensity on the refraction angle is called a sensogram. Then the sample is added to the solution and the shift of the angle is determined either in the course of time or in steady-state conditions. The first experiment yields the kinetics of the binding, whereas the second makes it possible to establish the amount of an analyte bonded to the biocomponents of the SPR biosensor. A differential scheme can be applied in the flow-through regime, in which the shift of the critical angle is measured against the same parameter corresponding to the blank sample or the same SPR sensor with no biocomponent immobilized on the Au surface. And vice versa—the recording of the temporal dependence of the critical angle after reaching its maximal shift makes it possible to estimate the dissociation of the complex formed. It should be mentioned that although modern SPR sensors are much more compact and reliable than the first commercial Biacore biosensor, this method is still mainly used for the investigation of fundamental backgrounds of biochemical interactions and, to a lesser extent, for the quantification of the analytes especially in real samples (Hoa et al. 2007).

Surface-enhanced Raman spectrometry (***SERS***) belongs to the so-called localized SPR techniques utilizing conductive nanoparticles instead of a metal film. Similarly to the SPR techniques described above, the main attention is paid to *Ag* and Au particles. The size of the particles should be comparable with the length of the illumination light.

Raman spectroscopy is a spectroscopic technique used to study vibrational, rotational, and other low-frequency modes in a system on the basis of inelastic scattering (Raman scattering) of mono- chromatic light, usually from a laser.

The laser light interacts with molecular vibrations resulting in the energy of the laser photons shifted up or down. The shift in energy gives information about the vibrational modes in the system (Petryayeva and Krull 2011).

$$\Delta\lambda_{\max} = m\Delta n\left[1 - \exp\left(-\frac{2d}{l_d}\right)\right] \tag{3.88}$$

Here m is the bulk refractive index response of a nanoparticle (sensitivity factor, nm/RIU), d is the effective thickness of the surface layer, and i_d is the characteristic decay of the electromagnetic field. The Eq. (3.88) is a background for the use of the shifts in the refractive index or wavelength in the SERS for biochemical assay purposes.

In canonical Raman spectroscopy, only about 10^{-6} of incident photons are scattered. This makes these techniques best suitable for structural analysis rather than for quantitative analysis of traces. In the SERS, the Raman signal for molecules adsorbed on the nanoparticle surface is enhanced due to two phenomena, i.e., the electromagnetic (the so-called “EM factor”) and the chemical. The EM factor results from the coupling of electromagnetic fields (incident and scattered) with the SERS substrate. It influences the molecules within about 10 nm. Besides, the interaction of a molecule adsorbed with the nanoparticle material can lead to some perturbation in the electronic structure and hence to the induced polarizability. In such processes, the electromagnetic field is enhanced, and the Raman scattering scales with the fourth power of the field enhancement.

The signal intensity in the SERS is proportional to the cross-section of scatter, the intensity of the radiation source and the density number of molecules. The magnitude of enhancement in the cross-section of the scatter is a function of the roughness of the surface, and the chemical and optical properties of the adsorbed molecules. In addition, the shape and aggregation of the nanoparticles affect Raman spectra. Thus, aggregated nanoparticles exert greater influence on the measurement than on separated particles of the same size.

The nanoparticles of Au and Ag for the SERS can be obtained on the transparent plate by chemical reduction from precursor salts ($AgNO_3$ or $HAuCl_4$ and

$NaBH_4$ or citrate as reducers). The lithography techniques and vapor deposition through template membranes are applied as well. The templates with a regular distribution of the pores of appropriate size can be obtained by the sol-gel technology or by burning holes in a polymeric layer with the electron beam or laser ablation (electron beam or nano-imprint lithography). The ultrathin metal layer can be deposited on silica nanoparticles or dendrimer molecules.

The instrumentation for the SERS measurements involves a light source (laser), the sample and detection. The Raman-scattered light is collected and processed to separate the energy bands of scattered radiation. The SERS mapping is achieved with a camera with the CCD chip providing a two-dimensional spectrum. The application of multiple wavelength light sources leads to a hyper-spectral imaging. In a three-dimensional image, two dimensions define the pixilated area and the third one the wavelength of the light source.

The detection of biochemical interactions is based on the consideration of the changes in the scattering spectrum caused by specific interactions on the surface of the nanoparticles. For this purpose, the metal surface is first modified with receptor structures (proteins or DNA), preferably by Au–S bonds or avidin–biotin binding. After that, the biotarget molecules interact with them to form a new layer different from the initial one by its refractive and scattering properties. The sensitivity of the detection depends on the shape of the nanoparticles (aspect ratio for particles of a complex shape) and the effective distance of the biochemical interaction from the particle surface. As in the case of SPR, the greatest attention is focused on the monitoring of the temporal characteristics of specific biochemical interactions, e.g., association–dissociation constants, affinity of interaction, etc. Meanwhile the SERS is very compatible with immunoassay techniques, especially the sandwich assay with a high sensitivity of the signal detection.

All the SERS-based biosensing approaches can be classified as label-free techniques and applications of SERS labels. In the first case, the analyte SERS spectra are recorded in conditions of the full coverage of nanoparticles bearing specific biochemical receptors. The identification of the analyte molecules is achieved either by a specific receptor applied for nanoparticle modification, or by the Raman vibrational mode specific for an analyte. The latter approach does not assume any specific receptors that are substituted by nanoparticles to generate the SERS spectra. The sensitivity of label-free techniques can be enhanced by increasing the roughness of the metal surface. This can be reached by a specific treatment of the metal particles (repeated oxidation-reduction cycles) or nucleation control in the template deposition methods. The reproduction of the metal nanoparticles as the SERS signal source is the main challenge of this group of methods.

In the second approach, a nanoparticle with adsorbed indicator molecules exerting the SERS spectrum is used as a label in various modes of competitive and sandwich-like protocols. The generation of the signal involves close approaching but no obligatory intimate contact between the metal nanoparticle and the SERS label. This makes it possible to perform bioassay both on the solid support in the ELISA-like format and in the solution. Thus, Fig. 3.40 schematically represents two SERS-based DNA biosensors, i.e., the sandwich format with an intermediate

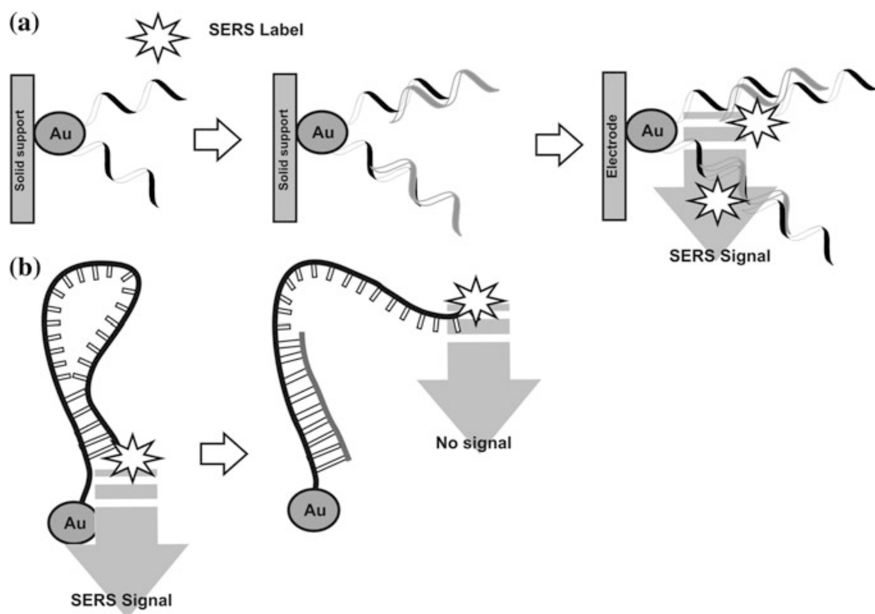


Fig. 3.40 DNA biosensing in SERS using (**a**) sandwich format and (**b**) hairpin DNA probe

separation of the product of hybridization and the hairpin DNA probe with one-step signal determination in homogeneous solution. In the second case, the hybridization of the target DNA sequence uncouples both the Au nanoparticle and SERS label, making signal generation impossible.

Cucurbituril (Taylor et al. 2011), rodhamine derivatives (Huh et al. 2009), diazo (Bizzarri and Cannistraro 2007), fluorescein (Li et al. 2008) and a few other organic dyes were used as the SERS labels. The single-walled carbon nanotubes provide high amplification of the SERS signal due to the enormous Raman scattering cross-section (10^{-21} cm^2 sr^{-1}molecule^{-1}) (Chen et al. 2008). The stability of nanoparticles with adsorbed labels can also be improved by the additional deposition of protective layers capsulating the surface with the SERS agents. Organosilicates and polymers have been described for this purpose.

The SERS provides extremely high enhancement of the weak inelastic scattering effect of photons. This is especially obvious for nanoscale probes where the routes to the detection of a single biomolecule are explored. The concept of SERS can be extended to two-photon excitation (surface-enhanced hyper-Raman scattering, SEHRS), offering additional prospects of optical methods for structural biology and bioorganic chemistry. However, the application of these techniques to the biosensor format is still limited by the rather high cost of the equipment and fewer possibilities for quantification of the analytes in the sample (Kneipp et al. 2008).

3.3 Other Techniques

3.3.1 Quartz Crystal Microbalances

The Quartz crystal microbalances (QCM) is a method of the measurement of the mass of a thin surface coating that is based on the so-called “piezoelectric effect.”

> The piezoelectric effect is assumed to be the linear electromechanical interaction between the mechanical and the electrical state in crystalline materials with no inversion symmetry.

The piezoelectric effect is observed when the pressure applied to a dielectric material deforms its crystal lattice. The mechanical force causes the separation of the cationic and anionic centers and hence changes the dipole moment of a molecule. If electrical contacts are applied to the sides of a thin slice of a piezoelectric material, a current will flow through an external circuit (*direct piezoelectric effect*). And, vice versa, the alternating voltage applied to the electrodes attached will initiate mechanical oscillations within the crystal lattice (*reversed piezoelectric effect*). Each piezoelectric material is characterized by its natural resonant frequency f_0 (also called *basic*, or *intrinsic* resonant frequency), which can vary when some films are deposited on the surface or the chemical substances adsorbed. To record the frequency changes, the crystal is incorporated into the feedback loop of an oscillator circuit. The most popular commercial QCM crystals are made of the AT-cut quartz discs of 10–15 mm diameter and 0.1–0.2 mm thickness (Fig. 3.41). The disk is covered with the Au or Ag thin-film electrodes. The suppliers offer quartz crystals with the resonant frequency of 6–20 MHz. The QCM sensors described are also called thickness-shear mode (TSM) sensors and refer to *bulk acoustic wave* (BAW) sensors (Bard and Faulkner 2001).

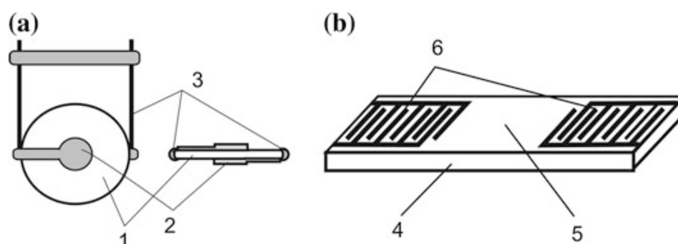


Fig. 3.41 Transducers based on the piezoelectric effect. (**a**) BAW sensor based on a Tcutofquartz, (**b**) SAW sensor based on intedigitated electrodes *1* quartz crystal slice; *2* electrodes; *3* holder; *4* piezo material slab; *5* sensing area; *6* interdigitated electrodes

In the *surface acoustic wave* (SAW) sensors, the wave increases along the surface of the solid support. There are several constructions of such sensors that are based on various types of acoustic waves. The planar sensors with interdigitated electrodes are the simplest. In this sensor, two pairs of interdigitated electrodes are formed by lithography on the surface of a slab of piezoelectric material. The alternating voltage between the electrodes of the first pair generates an acoustic wave oscillation that excites an appropriate repeated voltage alternation between the electrodes of the second pair. The surface between the electrodes is covered with a film containing receptor molecules providing specific accumulation of the target analyte and hence changes in the frequency of mechanical oscillation and sensor signal. Such a scheme does not require any oscillators besides the attenuation of two circuits related to the electrode pairs. A dual mode with the SAW sensors described in which one is provided with a sensitive layer and the other is applied as a reference is also described to exclude temperature and other measurement characteristics from analyte quantification.

In QCM sensors, the frequency of oscillation always decays with an additional mass deposition on the piezo material. The relative shift of the frequency is proportional to the mass deposited on the quartz (3.89) (Skládal 2003).

$$\Delta f / f_0 \sim \Delta m \tag{3.89}$$

The Eq. (3.87) is better known as the "Sauerbray equation" (3.90), which is valid for the quartz-air interface and for a thin solid layer deposited on the electrode. The mass changes should not exceed 2 % of the quartz crystal mass.

$$\Delta f = -\frac{\Delta m f_0^2}{A\sqrt{\mu \rho_q}} = C_f \Delta m \tag{3.90}$$

Here Δf is the shift of the basic resonance frequency f_0, Δm in the mass change, A is the electrode area (0.196 cm^2 in many applications with standard quartz crystals), μ is the shear modulus (2.95×10^{11} dyn/cm^2), and ρ_q is the density of quartz (2.65 g/cm^3). The C_f, an integrated QCM sensitivity, is about 0.903 Hz/ng for the 9 MHz crystal.

At first, the QCM biosensors used the Sauerbray equation for the measurements of the mass changes related to biochemical reactions with proteins or the DNA localized on the electrode surface. The protocol was called "deep-and-dry." This means that the biosensor was first wetted with an analyte solution and then washed and dried prior to the frequency measurement. Such an experiment resulted in a rather high deviation of the signal caused not only by the differences in the remaining humidity of the surface layer but also by the thermal instability of the biochemical and partial losses of the film washed out from the surface in the intermediate measurement stages.

The Sauerbray equation is not applicable in the liquid phase because the liquid density and viscosity affect the oscillation frequency. This might be ascribed either to inelastic mass change with the dissipation of energy, or to elastic mass shift combined with liquid density–viscosity changes. The viscous coupling of the

solution to the crystal results in the addition of the solution to the mass component at the oscillating crystal (Marx 2003). This phenomenon can be expressed by the Kanazawa Eq. (3.91),

$$\Delta f = -f_0^{3/2}\sqrt{\frac{\rho_L \eta}{\pi \mu \rho_q}} \tag{3.91}$$

where ρ_L and μ are the density and viscosity of the solvent. In a two-layer system, i.e., the rigid mass layer and a viscous liquid, the combination of (3.88) and (3.91) is appropriate (3.92).

$$\Delta f = -f_0^2\left(\frac{\Delta m}{A\sqrt{\mu \rho_q}} + \sqrt{\frac{\rho_L \eta}{f_0 \pi \mu \rho_q}}\right) \tag{3.92}$$

Thus, the total decrease in the frequency cannot be split into the contributions related to mass shift and a viscous media. Nevertheless, in the series of measurements performed in solutions of approximately constant concentration of electrolytes (standard analyte solutions in the presence of a supporting electrolyte, artificial serum samples, etc.), the decay of the frequency of the QCM sensor is assumed to be proportional to the mass of the species accumulated on the electrode. Some additional information can be extracted from more complex experiments with the QCM transducer combined with impedance analyzer.

The QCM sensors have found increasing interest for the investigation of the surface processes, e.g., electroplating, electropolymerization, immobilization of biochemicals, and their interaction with target analytes. If the mass deposition is related to the charge passed through the electrodes, the simultaneous monitoring of the frequency and *I–E* curves make it possible to attribute the mass deposition to particular peaks on voltammograms and hence to identify the nature of the product. For example, Fig. 3.42 shows the cyclic voltammogram obtained in the solution of a phenazine dye, i.e., Neutral red, which undergoes the electrochemical polymerization initiated by the irreversible oxidation at 600–800 mV versus Ag/AgCl.

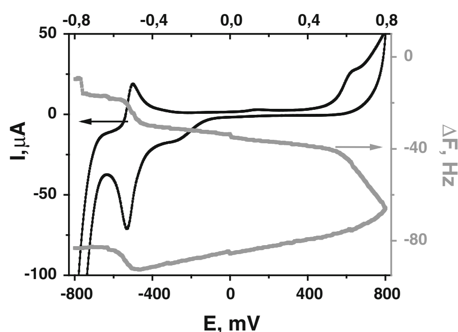


Fig. 3.42 EQCM measurements in the 0.4 mM Neutral red solution in 0.025 M phosphate buffer, pH 7.4. QCM sensor with Au electrodes, basic frequency 7 MHz

On sensogram, the electropolymerization is indicated by an appropriate decay of the frequency by about 50–60 Hz. The reversible peaks pair at −500 mV corresponding to the equilibrium of the charge transfer between the neutral (reduced) and positively charged (oxidized) forms of the dye. The appropriate changes in the frequency of the QCM sensor can be attributed to the transfer of a counter ion or electrostatic adsorption-repulsion of a monomeric form of the Neutral red, which are electrochemically oxidized/reduced at the same potentials as the polymeric dye. In a similar manner, the QCM sensor can specify the potentials of the adsorption of the oxygen on Au and the hydrogen on Pt. What is important is that the integration of the peaks on voltammograms yields the charge passed and hence absolute mass of the product formed on the electrode. The comparison of the result with the mass measurement by the QCM module makes it possible either to prove the mechanism of the electrode reaction or to estimate the non-Faradic reactions on the electrode (adsorption, chemosorption, chemical oxidation, etc.). The same approach was found to be very attractive in the investigations of protein adsorption. The combination of the QCM and electrochemical measurements is called EQCM (electrochemical quartz crystal microbalance).

The QCM biosensors are entirely compatible with the protocols of the biopolymer polymerization that provides regular thin layers of a certain mass and thickness. The monolayers are obtained on the Au and Ag electrodes by the self-assembling of thiolated acids and amines followed by the carbodiimide binding of proteins and the aminated ODNs. The LB-films and polyelectrolyte complexes obtained by the consecutive deposition of the oppositely charged polyionic components offer similar prospects for biosensor assembling.

In case of the immuno- and DNA sensors, the mass shift related to binding the analyte molecule can be directly measured by the QCM sensor. The sensitivity of such detection depends on the mass of the analyte molecule and the density of the binding sites on the surface of the QCM transducers. As mentioned, the latter contribution is limited by the necessity of monolayer filling of the transducer surface. For this reason, the analytical characteristics of the direct formats of the piezoelectric detection are rather moderate and cannot compete with those of electrochemical and optosensors. Thus, the detection of hybridization can be performed with the LODs of about 10^{-8} to 10^{-9} M against the femtomolar ranges reached by electrochemical and fluorescent labels.

For this reason, amplification of the QCM biosensor signal is necessary (Cooper and Singleton 2007). This can be performed in two ways, i.e., by increasing the number of the biorecognition elements and by increasing the mass of the product of the biochemical reaction with an analyte. The first way involves the application of multifunctional carriers that provides a high regularity and reproducibility of the coating. For Au nanoparticles and dendrimer molecules, the amplification depends on the relations between the surface square of the nanoparticles and the size of the bioreceptor attached. Carbon nanotubes and the avidin–biotin binding provide a definite number of new bonds established with the terminal functional group of the ODN or immunoglobulin. The steric limitations of the following biochemical interactions can be decreased by the choice of an appropriate linker separating the

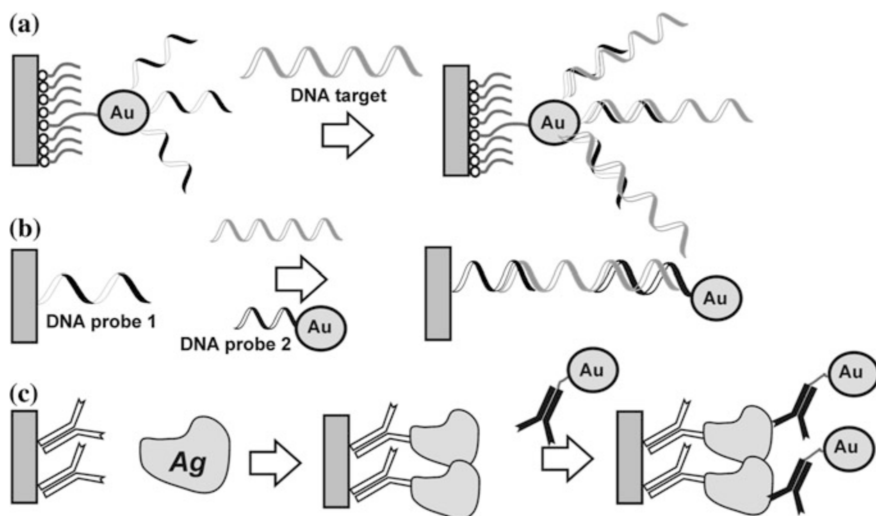


Fig. 3.43 Amplification of the signal of QCM biosensors. **a** the use of nanosized carriers; **b** DNA dual probe sensors with Au labels; **c** sandwich immunoassay with the secondary Ab conjugates bearing Au nanoparticles

carrier and reaction centers. The use of multilayered QCM coatings not only makes the requirements of the reproducibility stricter, but also decreases the sensitivity of mass change detection because of the greater contribution of the viscosity factor.

The use of heavy labels is another strategy of QCM signal amplification. In this approach, the mass of an analyte is additionally increased by attaching such bulky or heavy labels as Au, ferrite, and silica nanoparticles. This is mainly based on the sandwich format. For immunosensors, secondary *Ab* conjugates with colloidal gold are bonded to the primary *Ag–Ab* complex on the transducer surface. In the DNA sensors, the ODN sequences partially complemented to the DNA probe and target sequence form triple complexes bearing the label. The efficiency of labeling for mass sensitive transducers can be additionally increased by the accumulation of some auxiliary species after the biorecognition effect. Thus, the mass of Au nanoparticles can be additionally increased by the selective cathodic deposition of silver. The DNA adsorbs heavy metal ions. In some sensors, the polymerization of aniline adsorbed in the biochemical components on the transducer was initiated. The summary of some common amplification protocols is outlined in Fig. 3.43.

The enzymes are less compatible with the QCM signal transduction. Changes in the reaction with low-molecular substrates do not provide significant mass changes, even though insoluble products can be produced. The peroxidase-based disruption of the polymeric substrate involved in the surface layer is a rare exception (Karousos et al. 2002). In the biosensor, 3.3′-diaminobenzidine was enzymatically converted into an insoluble product. The reaction was applied for the

determination of cholinesterase inhibitors in a three-enzyme sensor (acetylcholinesterase-choline esterase-peroxidase).

Similarly, the mass losses of the DNA adsorbed on the QCM transducer have been monitored as an estimate of DNA damaging factors (Rawle et al. 2008). In addition, the influence of DNA damage on the hybridization efficiency can be recorded together with electrochemical signals of selected nucleotides.

3.3.2 Calorimetric Biosensors

The calorimetric (enthalpiemetric) biosensors measure heat evolution (enthalpy change) following biochemical reactions. Although the detection principles are universal, most calorimetric biosensors utilize the enzymes that are either free in solution or immobilized on the carrier placed on the thermistor surface or in the reactor, i.e., a tube with a porous carrier with an immobilized enzyme and a substrate solution flowing through the reactor and the thermistor cell at a constant rate (Danielsson and Mattiasson 1996; Yakovleva et al. 2013). The response is related to the local temperature change ΔT which depends on the molar enthalpy of the product formation ΔH, the stoichiometry coefficient n and the thermal capacity of the system C_p (3.93).

$$\Delta T = -\frac{\Delta H \times n}{C_p} \tag{3.93}$$

The thermal effects of enzymatic reactions are rather high because of the catalytic nature of the process. The molar enthalpies of some enzymes' catalytic reactions are presented in Table 3.11.

Table 3.11 Molar enthalpies of some enzyme-catalyzed reactions

Substrate	Enzyme	−ΔH (kJ/mol)
Cholesterol	Cholestgerol oxidase	53
Esters	Chemotrypsin	4–16
Glucose	Glucose oxidase	80
Glucose	Hexokinase	75
Hydrogen peroxidase	Catalase	100
Penicillin G	β-lactamase	67
Pyruvate	Lactate dehydrogenase	47
Proteins	Trypsin	10–30
Starch	Amylase	8
Sucrose	Invertase	20
Urea	Urease	61
Uric acid	Uricase	49

The temperature shift is measured with the help of *thermistors*. These are resistors made of semiconducting ceramics on the basis of Mn, Ni, Co, Cu, Fe and U oxidases with a high negative temperature coefficient of the electric impedance. The linear size of the modern thermistors does not exceed several millimeters. The temperature dependence of the thermistor resistance R is expressed by the empirical Steinhart–Hart Eq. (3.94).

$$\frac{1}{T} = A + B(\ln R) + C(\ln R)^3 \tag{3.94}$$

Here T is the Kelvin temperature and A, B, and C are empirical parameters. For narrow temperature ranges, the Eq. (3.95) is transformed into an exponential function (3.95),

$$\ln\left(\frac{R_1}{R_2}\right) = B\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{3.95}$$

where R_1 and R_2 are the resistance values at the temperatures T_1 and T_2, respectively. It can be expressed from (3.95):

$$\frac{R_1}{R_2} = \exp\left[Const\left(\frac{1}{T_1} - \frac{1}{T_2}\right)\right] \tag{3.96}$$

Taken $e^x \approx 1+x$:

$$R_1 = R_2\left[1 + B\left(\frac{T_2 - T_1}{T_1 T_2}\right)\right] \tag{3.97}$$

And for small temperature changes $T_1 \approx T_2 \approx T$:

$$\frac{\Delta R}{R} = -\left(\frac{B}{T^2}\right)\Delta T \tag{3.98}$$

Thus, the relative shift of the thermistor resistance $\Delta R/R$ is proportional to the temperature shift ΔT. The coefficient of proportionality ($-B/T^2$) is commonly about –4 % grad^{-1}. The changes of the temperature are recorded by the Wheatstone bridge. Modern amplifiers make it possible to reach the sensitivity of temperature measurement of about 100 mV/0.001 K. Commonly used full-scale sensitivities are in the range of 0.01–0.05 °C. This is sufficient for the determination of 0.01–100 mM substrates in the enzyme catalyzed reactions. Catalase is the only exception to this sensitivity estimate. This enzyme provides detection down to 0.1 μM of hydrogen peroxide. The same LODs were obtained for bi-enzyme reactions including the catalase-based decomposition of H_2O_2 (glucose oxidase-catalase as an example).

The maintenance of thermostating the bridges' resistances is one of the crucial problems of thermometry. If the temperature misbalance of the bridge arms is

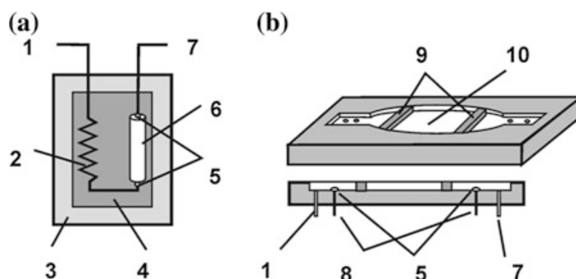


Fig. 3.44 Flow reactor (**a**) and thin film (**b**) enthalpiemetric cells. *1* flow carrier inlet; *2* 0heat exchanger; *3* external thermo jacket; *4* aluminum box; *5* input and output thermistors; *6* enzyme reactor; *7* flow carrier outlet; *8* electric contacts of thermistors; *9* filters; *10* cell with an enzyme solution

equal to 1°, the deviation of the ΔT measurement is about 0.01°. This error source is eliminated by using a massive aluminum thermostat containing all the equipment, including the cells or columns with the enzyme preparation, flow channels, thermistors, etc.

The common enthalpiemetric sensor consists of the thermostatic block of at least 10 cm high, which involves input (reference) and output thermistors and a flow cell (Fig. 3.44a). The common flow rates are about 0.1–2 mL/min and the volume of the enzyme reactor is about 10 mL. Under proper conditions, about 80 % of the evolving heat is converted into the temperature shift measured. For the reaction heat of about 100 kJ/mol, the enzyme conversion of 1 mmol of a substrate yields the increase of the temperature by 0.02 °C. This is quite sufficient for the detection of the rate of the enzymatic reaction because modern thermistors make it possible to detect up to 10^{-4} °C. The sampling frequency is about 10 measurements per hour (Ramanathan et al. 1998).

Miniaturized thin-film cells (Fig. 3.44b) for enthalpiemetric measurements are prepared from plastics or ceramics on a silicon plate and involve a system of miniaturized channels, mixers and valves establishing the regime of the reactant distribution. This offers new opportunities for the development of multi-enzyme analyzers for the simultaneous or consecutive detection of several substrates or for a deeper conversion of the substrate in the multi-enzyme conversion.

Besides glucose oxidase-catalase systems, other multi-enzyme sensors have been used for the amplification of the response of enthalpiemetric biosensors. Thus, lactate and pyruvate can be determined in the substrate recycling system including lactate dehydrogenase and lactate oxidase (3.97). In such a multi-enzyme system, a 5000-fold amplification of the signal can be reached.

The enthalpiemetric biosensors have been used for the determination of more than 50 enzyme substrates, among them ethanol, glucose and other carbohydrates, lactate, cellobiose, cholesterol and cholesterol ester, penicillins, and triglycerides. The measurements can be conducted in the undiluted blood, serum, urine, or fermentation vats.

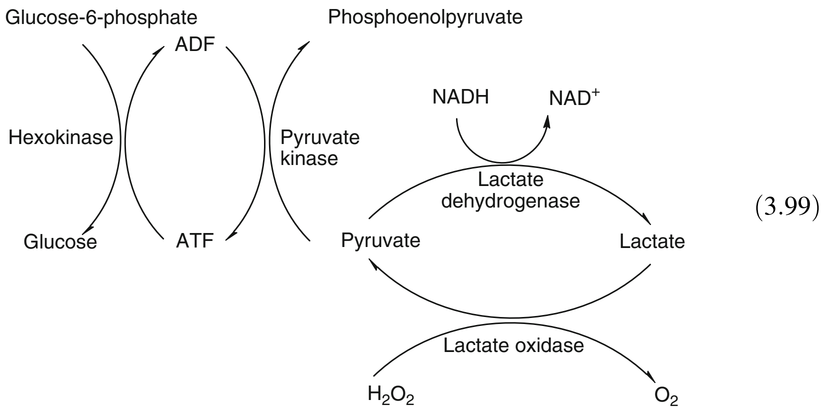
Enthalpiemetric systems have been described for monitoring the enzyme reactions in organic solvents. The thermal capacity of organic solvents is much lower than that of water and this increases the sensitivity of the enzyme substrate detection. This was confirmed by the determination of the activity of lipoprotein lipase in the binary system water—toluene, of the peroxidase activity in toluene and that of chymotrypsin in a 10 % aqueous dimethylformamide. In the latter case, the reaction of esterification catalyzed by chymotrypsin in such a medium is endothermic so that the decrease in the temperature was monitored contrary to the majority of other enzymatic reactions.

Besides substrates, some enzyme inhibitors were detected with the biosensors described. The microgram ranges of heavy metals were detected using the urease reactor in the flow-injection analysis. The inhibiting activity of organophosphates on acetylcholinesterase was quantified using microthermistor systems.

The potential of enthalpiemetric sensors in the determination of enzyme reactions has also been used in the immunoassay utilizing the enzymes and labels. The thermometric enzyme-linked immunosorbent assay (TELISA) is based on the use of the flow immunoreactors with the Ab immobilized on the porous carrier (glass beads, Sepharose, etc.) (Ramanathan and Danielsson 2001).

In a competitive assay, the sample is mixed with the *Ag* conjugate-bearing enzyme and after the immune reaction, the substrate is injected. After the measurement, the reactor can be regenerated by a glycine solution. The sensitivity of the response can be increased by additional application of the substrate recycling scheme described above (3.99). For this purpose, lactate dehydrogenase, lactate oxidase and catalase were co-immobilized with the human pro-insulin in the reactor and the alkaline phosphatase/phosphoenolpyruvate system was employed for the signal generation (Xie et al. 1999).

The enthalpiemetric measurement of the DNA melting in microchambers and on the plastic supports can be used in microsensors for detecting the DNA–protein interactions and DNA intercalation. Although such experiments cannot provide the sensitivity of the response comparable with that of conventional methods, they are rather interesting from the point of view of nanoelectronics and MEMS systems. This is due to great, sharp changes in the DNA properties, e.g., viscosity and electroconduction observed during the melting. The reversibility of the DNA melting procedures offers possibilities to control the biofilm properties by very small external stimuli (Nasef et al. 2010).



(3.99)

3.3.3 MEMS Systems

The term MEMS (*microelectromechanical systems*) denotes the devices of a micro- and millimeter scale (linear dimensions from 20 μm to several millimeters), which are produced by microfabrication technology. The MEMS devices employ the motion of separate parts that are driven by electric stimuli, or, vice versa, produce electric signals by such a movement or deformation. Besides MEMS, the terms *micromachined devices* and *microsystems technology* are used for such devices and their processing. The MEMS devices include miniaturized structures, sensors, actuators, and microelectronics.

An actuator is a type of motor for moving or controlling a mechanism or system. It is usually operated by an electric current, hydraulic fluid pressure or pneumatic pressure.

Besides microsensors, the MEMS technology is used for the production of micropumps, microinjectors (syringes), valves and switches that are necessary for the development of various automated systems including microfluidic devices and sensor arrays necessary in medicine (lab-on-chip concept), industrial control and related technology areas.

Of special importance is that the MEMS structures are merged on the common silicon substrate together with integrated circuits (microelectronics). They are produced with the help of similar technological processes that edge away parts of the silicon waver and add new structural layers to form mechanical and

electromechanical devices. This simplifies the manufacture process and avoids many problems of connections, isolation and control integration, which are rather typical for conventional macro-scale analogs.

The microsensors and microbiosensors are objects of special attention among the MEMS technologies due to the prospects of their application. Their small size, low cost in mass production, compatibility with conventional microelectronics and a wide range of the tasks to be solved, e.g., artificial internals and prostheses, self-controlled industrial processes and chemical reactors, etc., (Lavrik et al. 2004) are of great advantage.

In this section, only one but the most interesting example of the MEMS biosensors is considered, i.e., the cantilever biosensors.

> A cantilever is a beam anchored at only one end.

The cantilevers are prepared from silicon or a silicon nitride slab by a lithography technique coupled with selective etching to obtain a single-clamped suspended beam of various shape and thickness. A cantilever array can be represented as a row of miniaturized strips separated by gaps and joined to the general block. Besides cantilevers, a double-clamped suspended beam (a "bridge") is used for the same purposes. The cantilever-based sensors and biosensors can be operated in two modes, i.e., the beam bending (deflection mode) and the resonant frequency of mechanical oscillation (resonance mode) that can be measured separately (Hwang et al. 2009).

In static conditions, the entire beam structure bends according to the amount of the analytes attached to one side of the cantilever, i.e., to the stress on the beam.

In static conditions, the deflection of the cantilever tip can be expressed by (3.100), assuming that the cantilever radius of the curvature is much greater than the length of a beam,

$$\Delta z = \frac{3l^2(1 - v)}{Et^2}\Delta\sigma, \tag{3.100}$$

where Δz is the tip deflection, l is the length of the beam, v is the Poisson's ratio, E is the Young modulus of the substrate, t is the thickness of the beam and $\Delta\sigma$ is the differential stress, i.e., the difference between the surface stresses on the top and bottom surfaces of the cantilever. The Poisson's ratio is determined as a ratio of the contraction over the extension or of the transverse and axial strains of the material pressed or stretched. The Young's modulus characterizes the stiffness of the material and is defined as the ratio of the uniaxial stress over the uniaxial strain in the range of stress.

The Eq. (3.91) assumes a proportionality of the linear shift of the tip lever to the surface stress that provides the quantification of the amount of the analyte deposited on one side of the cantilever. The so-called optical lever technique is

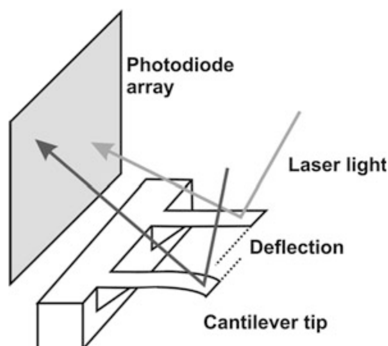


Fig. 3.45 Cantilever deflection measurement with an optical lever system

used for such measurement (Fig. 3.45). The deflection is measured by the shift of the laser light spots detected by a position-sensitive photo detector system. To improve the detection, the tip of the cantilever can be covered with a metal layer reflecting light (typically gold). The same layer is used for the immobilization of biocomponents for the biosensor development. In the cantilever array, the cantilevers can be illuminated by different lasers that are switched on and off at a recessing rate of several milliseconds (Fritz 2008).

The interference between a reference laser beam and that reflected off the cantilever can be used with the optical readout system as well (Pedersen et al. 2010). The modification of the cantilever construction offers other possibilities for deflection measurements. Thus, the use of doped silicon as a piezoelectric material makes it possible to estimate the piezoresistance by the Wheatstone bridge with four resistances installed in the arms of the bridge. Each cantilever includes two resistors placed on different ends of the beam. One cantilever is used as a reference, whereas the other one involves receptors on its tip. The piezoresistive method detects the deflection of 1 nm, whereas the optical systems detects that of 10 nm.

In the resonance mode, the frequency of an oscillating cantilever is determined as a function of the mass (3.101).

$$f_0 = \frac{1}{2\pi}\sqrt{\frac{k}{m}}, \tag{3.101}$$

where k is the spring constant and m is the effective mass of the cantilever.

Assuming that k remains constant during the experiment and the mass is uniformly distributed, the loading mass can be estimated from (3.102).

$$\Delta m = \frac{k}{\pi^2}\left(\frac{1}{f_2^2} - \frac{1}{f_1^2}\right), \tag{3.102}$$

where f_2 and f_1 are the resonance frequencies after the mass loading and in the blank experiment, respectively. The measurements can be performed using

external electrostatic and magnetic actuating or by the direct measurement of the piezoelectric effect (Raiteri et al. 2001).

Similarly to the QCM biosensors, the application of cantilevers in the biosensor assembly is based on the direct mass loading measurements. The static measurement mode can be used for aqueous sensors, whereas dynamic sensors are preferably used in the air or in vacuum. The immobilization of the biocomponents is preferably achieved by covalent and affine binding on golden films at the tip of the cantilever. The suppression of the thermal fluctuations increasing the background signal can be performed by the application of additional thin polymer layers deposited on the cantilever prior to their biochemical attachment. Some examples of the cantilever biosensor applications are given in Table 3.12.

As QCM sensors, the micro- and nanocantilever biosensors do not require any application of labels and any modifications of the biochemicals applied in their assembly. Nevertheless, the theory of cantilever-based detection of biochemical interactions is rather complicated due to a variety of factors affecting both the dynamics of interactions and signal measurement. The fact that most sensitive platforms operate on the air interface limits their applications in the real sample assay. Meanwhile, the miniaturization and integration with the microelectronic circuit mentioned provide good prospects in the automated and semi-automated systems that can be directed to the mass scale assay, e.g., in gene expression investigations, pharmaceuticals screening, etc. The sensitivity of the cantilevers very much depends on their structure and measurement modes. In the best of cases, the sensitivity of 10^{-14} g/Hz has been achieved (piezoresistance measurements).

Table 3.12 Application of cantilever biosensors

Analyte	Biocomponent	Cantilever system
Prostate-specific Ag	Ab	Static (Wu et al. 2001), dynamic (Hwang et al. 2004)modes
Myoglobin and creatine kinase (acute myocardial infarction biomarkers)	Ab/BSA	Dual mode cantilever with differential deflection measurement (Hwang et al. 2006)
12-mer ODN sequence (single nucleotide polymorphism study)	DNA probe	Optical beam deflection measurement (Fritz et al. 2000)
Gene specific sequences for gene expression study (interferon, aldolase A)	DNA probes	Cantilever array, static mode (Zhang et al. 2006)
Esherichia coli	Ab	Dynamic mode, measurements in the air (single cell detection) (Ilic et al. 2001)
Vaccinia virus	Ab	Dynamic mode, measurements in the air, thin layered construction (Gupta et al. 2004)
Oestradiol	Human estradiol receptor	Piezoresistance measurements (Mukhopadhyay et al. 2005)
Mussel gluing protein, insulin, polydT	Ab, poly(dA) probe	Piezoresistance measurements (Lee et al. 2007)

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Chapter 4
How Does It Work? Case Studies

In this chapter, we deal mainly with the aspects of biosensor development related to specific areas of their application. It is definitely clear that biosensors that perform well in the laboratory can be utterly inappropriate outside the university walls. The gap between the laboratory pattern and a commercial product is intuitively understandable. The end-users want to receive a device that is robust, cost-effective, simple to operate, and as sensitive as required for its purpose. As regards biosensors, the following aspects are imperative for the various stages of commercialization:

- *Stability of the biological component*. The satisfactory working period estimated from the stability of the calibration parameters begins after six months. This is a rather mild estimate accepted by the medical services that are used to maintaining the unstable biochemicals that are necessary for conventional bioassays. In other areas, e.g., industry or environmental monitoring, the requirements can be stricter.
- *Operation/functioning convenience*. To some extent, this is implied from the previous statements. A limited period of operation simply means the replacement of the sensing elements performed by an unqualified staff or an inexperienced buyer. In addition, mass production is hardly compatible with a complicated, multi-stage protocol of measurement. The simpler (faster, cheaper), the better. The producer should take into account that the customer might not be familiar with the specific conditions of biochemical reactions and will make very simple mistakes that a biochemist can't possibly make (buffers, order of reagents addition, temperature regime, etc.).
- *Analytical characteristics and metrology*. The more complicated the sensor, the more difficult to establish the expected accuracy of the measurement. Concerning real samples assay, the main problems are focused on selectivity and signal drift. The biochemical recognition is sensitive enough to compete with most of the conventional instrumentation techniques, and this is one of the undisputable benefits always mentioned in research related to biosensor development. However, in real sample testing, the selectivity of the transducer can seriously limit the prospects of biochemical recognition. Careful consideration

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of the matrix effect eliminates, at least partially, such sources of possible errors. For implantable sensors, this covers the problems of biocompatibility, including the possible biofouling of the biosensor. The correct metrological characterization of the biosensor signal, in order to be true, does not refer to the specific problems of biosensor development; this is a common requirement for any analytical technique. But facing the problems of the signal instability, many researchers are tempted to "color the truth" by rather legal measures. Some of the most popular are the ways that the statistics can contribute to the "data improvement": the use of 2:1 signal-to-noise ratio instead of 3:1 for the LOD estimation; the application of logarithmic or semi-logarithmic plots for the analyte quantification; the attribution of the deviation calculated from the measurement replications to the signal, but not to the analyte concentration; the extrapolation of the linear portion of the calibration curve to lower concentration in the LOD estimation; and the attribution of the relative deviation of the signal determined for median value to those near the lowest detectable signals. Many of them are quite justifiable in research articles but need to be seriously reconsidered in the development of commercial products.

Instead of considering all of the above statements, we have chosen to suggest a kind of "case study". In this way, the problems of biosensor application are considered according to the examples of the particular biosensors developed for the solution of very common and typical problems, whether the quantification of an important analyte or the general assessment of the sample based on some integral characteristics or biochemical impact. It is assumed that the principles of biochemical recognition and signal transduction have been examined in the previous chapters.

4.1 The Biggest Success: Glucose Meters

As mentioned above, biosensors for glucose measurement, also called "glucose meters" are the first and most convincing example of the commercialization prospects in the area considered. Diabetes mellitus is a worldwide public health problem. It mainly results from insulin deficiency caused hyperglycemia. The diagnosis and management of the illness require monitoring the glucose level in the blood. In accordance with the WHO estimates, about 10 % of the adult population can expect problems related to increased glucose levels. According to the International Diabetes Federation, there were 246,000,000 diabetics worldwide in 2005, and the number is expected to reach 3,800,000,000 by 2025.

There are three types of diabetes. Type 1 diabetes (10 % of patients) usually affects the young and occurs when the pancreas no longer produces any (or very little) insulin. Type 2 diabetes (90 % of patients) affects middle-aged or older patients and occurs when the pancreas does not produce enough insulin or when the organ cannot effectively use the insulin produced. Gestational diabetes is a

temporary condition that occurs during pregnancy. It affects 2–4 % of all pregnancies, with an increased risk of developing diabetes for both mother and child.

Millions of diabetics test their blood glucose levels daily. This makes glucose analysis not only very common but also very attractive for the development of compact devices suitable for use outside the hospitals (the so-called "point-of-care diagnostics"). The frequent self-testing provides information on trends in glucose control, the factors causing high or low glucose levels, the optimization of therapeutic treatment, etc.

For this reason, glucose sensors have become a subject of very energetic efforts since 1962, when Clark and Lyons first proposed the initial concept of the enzyme sensor with glucose oxidase as a biorecognition element. A variety of approaches have been explored and many glucose meters compete at present on the market of biomedical equipment. In addition, the glucose assay is necessary in some other areas, e.g., the food and microbial industries.

The first glucose sensor was based on the biocatalytic oxidation of glucose to gluconolactone spontaneously hydrolyzed to gluconic acid. The glucose oxidase accelerating this reaction exerts a high substrate specificity that allows measuring glucose in the presence of other carbohydrates. The reaction [see (Eq. 3.37)] is followed by the conversion of molecular oxygen into hydrogen peroxide. The first glucose sensors, which were later considered to be the first generation of enzyme sensors, utilized the current of the oxygen (hydrogen peroxide) reduction. After that, mediated electron transducers were used and some other enzyme systems and transducing principles developed. A brief history of the glucose meters is presented in Table 4.1 (Wang 2008).

The amperometric measurements of dissolved oxygen and hydrogen peroxide have been confronted with serious difficulties mainly related to the matrix effect. Thus, some endogenous reducing species, e.g., ascorbic and uric acids, as well as pharmaceuticals (acetaminophen) are electroactive at the working potential and interfere with glucose measurements. The intensive efforts in the 1980s minimized this effect due to the use of perm selective coatings limiting the access of such constituents to the electrode. The selection of the transport was achieved by the size, charge and mobility of interferences. The auxiliary films also excluded the

Table 4.1 History of the first stages of glucose sensors development

Year	Event
1962	The first glucose enzyme electrode
1973	Glucose enzyme electrode based on hydrogen peroxide detection
1975	Launching of the first commercial glucose sensor
1982	Development of ferrocene mediators
1984	The first mediated glucose sensor based on a ferrocene derivative
1987	Launching of the first personal glucose meter (MediSense)
1987	Electric wiring of enzyme
1992	Launching of the first hand-held blood analyzer (i-STAT)
1999	Launching of a commercial in vivo glucose sensor
2000	Introduction of a wearable noninvasive glucose monitor

influence of surfactants, and to some extent the biofouling. Various materials, e.g., electropolymerized phenylene diamine, overoxidized polypyrrole, Nafion, the Kodak AQ ion-exchange resin, and cellulose acetate and nitrate were used for these purposes. Regarding oxygen detection, the fluctuation of the signal was related to the oxygen exchange of the solution with the atmosphere. The rather low solubility of oxygen in water limited the upper concentration of the glucose measured. To some extent, the problem was solved by a special cylindrical form of the electrode increasing the surface-to-volume ratio and the rate of the oxygen access to the enzyme and electrode proper. The rate of the oxygen transport could also be increased by the inclusion of some hydrophobic materials, e.g., fluorocarbons, into the electrode and films covering the transducer.

The subsequent improvement of the performance of the glucose sensor has been achieved by the introduction of artificial electron acceptors instead of oxygen (the *second generation* glucose sensors). The signal of such mediated glucose sensors becomes largely independent of the oxygen concentration and can be recorded at lower potentials than the signal of the first-generation biosensors described. At the first stages of the investigation, diffusionally free mediators were used (ferrocyanide ions, ferrocenecarboxy acid, phenothiazine and phenazine dyes). After that, mediators were implemented in the surface layer together with the glucose oxidase (TTF-TCNQ organic salt, polymers bearing Os complexes). The ferrocene derivatives appeared most attractive due to low redox potential, high chemical and electrochemical stability, the reversibility of the response, and the pH-independent signal.

In addition to the mediated glucose oxidation catalyzed by glucose oxidase, the PQQ-dependent glucose dehydrogenase and the NAD-dependent glucose dehydrogenases can be used in glucose meters. The enzymes are oxygen-independent and rather effective in glucose oxidation, but are less stable and comparatively costly.

Home glucose self-testing with glucose meters is recommended for individuals prone to hypoglycemia, for those in intensive treatment (insulin infusion devices or multiple daily injections), and in cases of pregnancy complicated by diabetes mellitus, etc. The glucose level monitored is commonly within a 3.9–10.0 mM interval. The acceptable error of the single-point measurement should not exceed 5 % (Oliver et al. 2009). The use of glucose meters at home can be realized using two strategies, i.e., the application of single-use biosensors that are thrown wawy after their contact with blood, or the multiple use of biosensors with special procedures for blood removal, washing and biosensor recovery. The latter approach had been successfully realized in the glucose biosensors used in hospitals when the first commercial biosensor for personal use was launched. However, they were too complicated, too bulky and too expensive for personal care.

The situation changed in 1987. The first personal glucose sensor used the ferrocene derivatives that were co-immobilized together with the enzyme and the stabilizer in a thin layer formed on the planar electrodes. The construction of the sensing part (disposable strip) was made of a plastic slab with the conductive tracks produced by screen printing techniques. The covering layer eliminated the

access of red blood cells and electroactive interferences. The content of the sensing layer and exact ratio of the components have not yet been revealed and probably vary from manufacturer to manufacturer.

The first product had a pen-style design (ExacTech^{TM}, MediSense Inc., 1987). About 90 % of the markets of the glucose meters for self-testing are shared by LifeScan, Roche Diagnostics, Abbott, and Bayer. In all cases, the patient pricks the finger, places the small blood droplet on the tip of the strip and reads the glucose concentration on the display within 5–10 s. The strip used is replaced with a new one. Modern investigations in the area of in vitro glucose measurements are directed at the decrease in the volume of the blood droplet required, and the improvements in the sampling systems for a less painful prick. Thus, the FreeStyle Lite^{TM} glucose meter (Abbott) requires only 0.3 μL of blood which can be taken from the hand, the forearm, the upper arm, the thigh or the calf. The internal memory of devices stores up to 400 measurement results. Other finger prick-type glucose sensors exert comparable characteristics.

The in vivo glucose meters are mostly for use in a hospital setting under the control of experienced staff. A tighter glycemic control is desired for proper therapeutic treatment of severe cases of hypo- and hyperglycemia and for monitoring blood contents in professional sports. The glucose sensors are mainly parts of the closed-loop glycemic control systems and operate according to the flow-through system; the suitable devices include heparin injection systems to prevent blood clotting. The use of mediators in the sensing layer is limited because of its toxicity and possible leaching into the blood. Thus the detection is mainly based on the H_2O_2 oxidation recorded in the two-electrode system. The implantable biosensors for subcutaneous monitoring are made on a golden needle with the tip filled in with the enzyme and mediators commonly placed into a viscous polymeric mass consisting of a mixture of constituents providing access of the analyte to the enzyme, the mechanical durability of the layer and the hydrophilicity of the layer necessary for establishing the electric contact in the measuring system and preventing biofouling. The lifetime of such sensors is limited to several days.

Besides the amperometric glucose sensors described, some other approaches have been detailed. Thus, for glucose detection, the FET based on a single-walled carbon nanotube with the glucose oxidase immobilized via the hydrophobic interactions of the pyrene group covalently attached to the protein globule was described (Besteman et al. 2003). The current from the source to the drain of the device increased with the substrate concentration. Conductometric microdevices with the complex of the glucose oxidase with polyaniline were developed (Forzani et al. 2004). The signal was amplified by an additional inclusion of the TTF molecules into the electron transfer chain (Bartlett and Birkin 1994).

Non-invasive glucose sensing is the ultimate goal of glucose monitoring. Cygnus Inc. (now subsidiary of Johnson & Johnson) has developed a wearable glucose sensor (GlucoWatch^{TM}) that uses the iontophoretic accumulation of glucose through the skin (transdermal transfer). In this method, called "reverse iontophoresis," a low electric current is applied across the skin between two electrodes. This stimulates the charged and uncharged species to pass across the dermis at rates

significantly greater than passive permeability, with the ions moving across the skin to maintain neutrality. The skin is negatively charged at the physiological pH and so it is selectively permeable to cations, mainly to sodium. The flow induced by reverse iontophoresis carries neutral molecules, including glucose, from anode to cathode. An alarm capability is included to alert the wearer when there is a low or high glucose level. The glucose level detected by the GlucoWatch sensor depends on the blood level with a lag from 2 to 40 min. The low-frequency ultrasound was also used to increase skin permeability for glucose measurements. Other non-invasive methods of glucose analysis are based on optical spectroscopy.

Various clinical situations require a simultaneous detection of glucose and some other species, e.g., the lactate, cholesterol, triglicerides, insulin or ketone bodies (Pickup 2007). For this purpose, dual enzyme sensors were developed on the basis of appropriate enzymes co-immobilized on various transducers, preferably of the needle-type and screen-printed platform (Palmisano et al. 2000; Newman and Turner 2005). The detection of ketone bodies was performed with a standard glucose meter (MediSense), additionally provided with an electrode with an immobilized NAD-dependent hydroxybutyrate dehydrogenase (Chiu et al. 2002). The insulin was detected by the Ru-modified chemical sensor, and the multi-analyte detection is performed by some commercial devices. Thus, the Precision Xtra™ biosensor (Abbott) measures both the blood glucose and the ketone bodies in a real-time scale with two types of test strips fitted into the same instrument. This is important because millions of diabetics are at risk of developing diabetic ketoacidosis, a life-threatening condition that can result in coma or death.

It is expected that progress in glucose biosensors will result in the significant miniaturization of the devices and in their integration into the more complex analyzers demanded of clinical medicine and point-of-care diagnostics.

4.2 From Chemical Weapons Toward Pesticides: Anticholinesterase Sensing

Cholinesterases are the key enzymes in nerve impulse transduction. Their irreversible inhibition, caused by chemical warfare or pesticides, may result in severe consequences for human beings, involving muscular tremor, respiratory impairment, slow heartbeat, and death. The synthesis of nerve gases, e.g., sarin and soman, with a highly acute toxicity toward cholinesterases, called for the development of adequate analytical devices. At first, they were devoted to the early detection of such compounds during wartime as a part of individual bio/chem protection gears. After that, the cholinesterase biosensors found their application in agriculture for detecting the residuals of organophosphate and carbamate pesticides in soil, vegetables and ground waters. Nevertheless, the threats of chemical terrorism demand alarm devices that operate automatically (Miao et al. 2010). This, as well as a rather

high scale of anticholinesterase pesticide production, maintains interest in the development of new cholinesterase biosensors with a high sensitivity and stability of their signal. To avoid the time-consuming sample treatment, the cholinesterase biosensors should detect sub-ppb levels of pesticides and effectively eliminate interference with the matrix component (Marty et al. 1998).

The cholinesterases are rather similar for various vertebrates and show a remarkable specific activity that simplifies their immobilization, irrespective of the enzyme source. In accordance with the rate of the substrate hydrolysis, they are divided into two major groups—acetylcholinesterase ("true cholinesterase") and butyrylcholinesterase (serum cholinesterase). These groups differ in their substrate and inhibitor specificities. Acetylcholinesterase preferentially hydrolyzes acetylcholine [see Eqs. (2.16) and (3.44)], a natural neurotransmitter, whereas butyrylcholinesterase is more active in the hydrolysis of butyrylcholine.

From the biochemical point of view, the measurement of the irreversible inhibition is very simple. The biosensor is incubated in the sample, then the substrate is added and the enzyme activity measured. The relative decay in the activity depends on the amount and nature of the inhibitor and the incubation period. The details of inhibition kinetics were detailed in Chap. 2. Here, it is important to recall some of the information necessary for the application of the enzyme assay for real sample testing (Evtugyn et al. 1999):

- The relative decay of the enzyme activity does not depend on the substrate nature and concentration. The choice of measurement conditions should be directed to the establishment of the most accurate measurement of the signal to reach the minimal LOD.
- The addition of a substrate and an inhibitor together suppresses the inhibition because the irreversible inhibitor cannot access the *E–S* complex. This phenomenon is called the "protecting effect". It also occurs when a reversible inhibitor is present in the solution of an irreversible inhibitor. All the antidotes used in the case of poisoning by anticholinesterases exert reversible inhibition.
- The cholinesterase activity is influenced by a variety of factors including the alkali and alkali-earth metal ions, biogenic amines, surfactants, polar organic solvents, fluorides, etc. The inhibiting effect of the potassium ions on acetylcholinesterase is most important for the testing of the plant and soil extracts. All of these factors are nonspecific and reversible. Hence, they exert a protecting effect on irreversible inhibition.
- Strong irreversible inhibitors do not allow for multiple uses of cholinesterases; the *E–I* complex is very slowly reactivated.

First and foremost, the development of cholinesterase biosensors is directed toward the increase of the signal sensitivity. Let us briefly consider the main factors affecting sensitivity and directions of the improvement of the biosensor assembly.

Cholinesterase immobilization. As mentioned, the detection of irreversible inhibition does not require a high signal toward a substrate. What is much more convenient is that the accuracy of the signal be maximal. The increased loading of

the enzyme in the immobilization decreases the sensitivity of the inhibitor detection due to many factors, e.g., the compensatory effects of the substrate transfer and the non-enzymatic conversion of an inhibitor on the surface layer material. In this respect, ultra-thin enzyme layers obtained by site-specific affine immobilization and covalent attachment to the transducer surface have become somewhat attractive. The use of histidine-tagged cholinesterases, concanavalin A and carbodiimide binding showed a remarkable improvement in the inhibitor sensitivity in comparison with a rather traditional entrapment in gelatin gels, the BSA matrix or cellulose derivatives (Andreescu and Marty 2006). The sol–gel techniques and polyelectrolyte assembling were successfully applied for acetylcholinesterase immobilization as well. The above-mentioned methods preserve the native conformation of the enzyme globule and minimally affect the transport of low-molecular inhibitors to the enzyme active site.

The use of nanosized materials, e.g., Au nanoparticles, carbon nanotubes and dendrimers, also improves the performance of the inhibitor biosensors that combine satisfactory inhibitor sensitivity with a rather high signal toward the substrate. It is important that the immobilization also affect the changes in the sensitivity of the immobilized enzyme toward irreversible inhibitors within the period of biosensor storage. This might be due to the leaching of the less-bonded enzyme molecules from the matrix, the partial dehydration of the enzyme molecules stored in dry conditions and the changes in the accessibility of the cholinesterase active site due to various reorganizations of the three-dimensional structure of the surface layer. For this reason, the immobilization methods are compared with freshly prepared cholinesterase sensors with the following estimation of the temporal dependence of the inhibition degree for a standard inhibitor solution. There is some evidence that the hydrophobicity of the enzyme support can improve the sensitivity of the inhibitors' detection due to their accumulation on the material. The non-enzymatic factors affecting the analytical characteristics of the inhibitor determination will be considered below in greater detail.

Signal detection mode. As mentioned above, the establishment of a high accuracy of the measurement is the only, but rather strict requirement for the detection system applied in cholinesterase assays. The summary of the transduction systems used in cholinesterase biosensors is presented in Table 4.2.

The electrochemical methods have priority owing to their compact transducer design and rather positive characteristics. Optical detectors, even though they have comparable sensitivity, show obvious limitations in the testing of colored and turbid solutions. Among the voltammetric techniques, the choice between the bi-enzyme system of acetylcholinesterase and choline oxidase and a mono-enzyme cholinesterase sensor with acetylthiocholine, is most interesting. Both approaches demonstrate excellent sensitivity characteristics. The application of an additional enzyme, i.e., choline oxidase, makes it possible to use a natural substrate, i.e., acetylcholine. From this point of view, the results obtained coincide better with toxicity tests on vertebrates (acute toxicity on mice and rats, a conventional test in toxicology). In some assemblies, only choline oxidase is immobilized on the electrochemical transducer, whereas acetylcholinesterase is added to the solution.

Table 4.2 Signal transduction systems employed in cholinesterase biosensors for irreversible inhibition measurements

Detection system	Modifier/transducer	Detecting product of enzymatic reaction
Voltamperometric (amperometric)	Mediators (TCNQ (de Oliveira Marques et al. 2004), Co phtalocyanine (Arduini et al. 2006), carbon nanotubes (Joshi et al. 2005))/carbon materials	Thiocholine
	Choline oxidase/Mediator of H_2O_2 reduction (Prussian Blue, Co phtalocyanine)/glassy carbon, screen-printed electrodes (Amine et al. 2006)	H_2O_2
Potentiometric	Polyaniline (Ivanov et al. 2003)/EnFET (Dzyadevych et al. 2004)	pH shift
Galvanostatic	Polyurethane foam (Goodson et al. 1973)	Thiocholine/iodide
Thermal lens spectroscopy	5,5-dithio-bis-2-nitrobenzoic acid (Pogacnik and Franko 1999)	5-thio-2-nitrobenzoate
Fluorescence spectroscopy	Measurements in homogeneous conditions with indoxyl acetate and naphtyl acetate as enzyme substrates (Diaz et al. 1997)	3-hydroxy-indole, 2-naphthol
Optic fiber	LB-film (Choi et al. 2001)	*o*-Nitrophenol

This makes it possible to avoid the problems related to the various conditions of the immobilization of both enzymes. The inhibited cholinesterase is then replaced and the choline oxidase sensor can be used with a novel portion of the enzyme/substrate solution.

On the other hand, the use of the mono-enzyme system is simpler to assemble and operate. For field applications, it is important that acetylthiocholine be much more stable toward non-enzymatic hydrolysis than acetylcholine, which should be used within several hours after dissolution in a weak, basic working buffer. The amperometric signal of thiocholine oxidation is higher than that of choline oxidase sensors in comparable conditions and can be rather easily separated from the electroactive components of the matrix by the use of the mediators of electron transfer. The rather low working potentials (150–250 mV vs. Ag/AgCl) and lack of electrode poisoning typical for Pt make carbon electrodes very convenient for cholinesterase biosensor production.

Other measurement conditions. Most of the laboratory investigations related to cholinesterase biosensors are performed with organophosphates, i.e., real cholinesterase inhibitors. Some examples are given here:

Most of these compounds are no longer used as pesticides at present due to moderate toxicity for warm-blooded organisms. Instead, their triphosphate analogs are applied. They differ from their phosphoryl analogs by the thionic sulphur atom ($P = S$ group instead of $P = O$). In insects, organic thiophosphates are easily converted into phosphoryl compounds, the anticholinesterase effect of which is a thousand times higher. The reaction is catalyzed by mixed oxidases whose activity in human beings is much lower, For this reason, such pesticides referred to as the "second generation of insecticides" are much safer for agricultural workers and do not cause the persistence of pests. In laboratory experiments, thionic pesticides

should first be oxidized to phosphoryl analogs by bromine, *N*-bromosuccinimide, or other oxidants (Dondoi et al. 2006). If the irreversible effect is caused by non-oxidized preparations, this means that the standard sample was old enough to be oxidized by oxygen. This certainly decreases the robustness of the results obtained.

Incidentally, there is a rather reliable way to detect thionic derivatives. They exert a reversible inhibition on cholinesterase and hence cannot give a 100 % inhibition of the biosensor signal, even at high concentrations. If the calibration curve has a upper limit under 90 %, this is the result of the influence of reversible inhibitors on the cholinesterase reaction. To some extent, the conversion of thionic pesticides into phosphoryl compounds takes place in the environment so that the laboratory experiments can be considered to be an estimation of the total level of pollution. Carbamates do not require any metabolic activation, so that the inhibition caused by carbaryl, aldicarb, propoxur, and other common carbamates can be easily extrapolated to the field testing conditions.

The incubation time varies from 5 to 30 min. The irreversible inhibition observed increases with the incubation period up to 100 %, i.e., the full enzyme inactivation. If an upper level appears, mixed inhibition with the contribution of reversible inhibitors takes place. A long contact of cholinesterase with organophosphates results in a phenomenon of aging. It is assumed that aging results in the methylation of the serine group participating in the active site; this makes the reactivation of the enzyme impossible. The aging does not seem very important from the point of view of pesticide detection, but it should be taken into account that this phenomenon disturbs the theoretical relations based on inhibition kinetics, e.g., the Aldridge Eq. (2.13). In some cases, no special incubation stage is used, and both the enzyme and substrate are simultaneously added to the biosensor. This is acceptable, especially for the rapid preliminary testing of potential hazards. The inhibition observed corresponds to the formal kinetics of competitive inhibition. But, the LOD achieved in such experiments might be tenfold larger than that attained with the preliminary incubation with no substrates. Thus, such a simplified approach is applicable for rather strong inhibitors detected at rather low concentrations.

The content of the solution affects both the substrate signal and inhibition, but to a lesser extent than the incubation time or inhibitor concentration. However, some typical conditions should be fulfilled. Therefore the concentration of alkali and alkali-earth metals should be the same in the series of standard solutions used for the calibration of the biosensor response. K^+ ions exert a minor reversible inhibition effect on acetylcholinesterase but not on butyrylcholinesterase. In most publications related to the use of acetylcholinesterase, Mg^{2+} and Ca^{2+} ions are added to the solution in a millimolar range of their concentrations. The ionic strength of the inhibitor solution should be similar to that of the working buffer. This improves the stability of the signal and inhibition reproducibility. There is some evidence that the buffer system can affect the irreversible inhibition. Thus, the TRIS (tris-hydroxymethylaminomethane hydrochloride) buffer exerts a minor

reversible inhibition, whereas the borate buffer increases the inhibition caused by organophosphates and the Ca^{2+} yields a synergetic effect in these conditions. Such results deserve special consideration, but it should be mentioned that they are not always reproduced when an inhibitor or immobilization protocol is changed. For the same reason, the experiments with free enzymes and homogeneous conditions of inhibitions are difficult to be extrapolated to biosensor behavior.

The *use of organic solvents* is necessary if the extracts of pesticides from soil or plants are tested. The evaporation of organic solvents complicates the experiment by eliminating most of the attractive advantages of biosensors, i.e., easy and fast operation. However, most organic solvents inhibit cholinesterases, both free and immobilized. The effect is more pronounced for water-miscible polar solvents. To some extent, the short contact of the biosensor with hexane or CCl_4 solutions of a pesticide does not interfere with inhibition measurements. In other studies, the synergetic effect of the hexane and paraoxon inhibition (Turdean and Turdean 2008) and acetonitrile and chlorpyrifos (Evtugyn et al. 1999) was reported. The introduction of the enzyme into the hydrophobic matrix protects the enzyme from its inactivation in preferably organic media, but the sensitivity of pesticide determination seems lower than that in standard pesticide solutions. Of other common components of plant extracts, the so-called “natural cholinesterase inhibitors” should be mentioned. Biogenic amines and substituted phenols reversibly inhibit cholinesterase and diminish the influence of pesticide residues. Their content is maximal in some vegetables, grass and leaves. Nevertheless, cholinesterase-based protocols for the detection of anticholinesterase pesticides in tomatoes, apples, oranges, banana, barley, wheat, lettuce, grape, wine and winery products, honey, and milk have been developed.

Regeneration or single use? In the development of biosensing devices for filed applications based on cholinesterase inhibition, there are two strategies: The first assumes the regeneration of the acetylcholinesterase after its contact with organophosphates or carbamates. If the inhibition does not exceed 30 %, several consecutive measurements can be performed with no reactivation. The calculation of the inhibition degree is performed using the result of a previous measurement as a reference. In second strategy, treatment of the inhibited cholinesterase with aldoxime derivatives makes it possible to regenerate the initial signal (Gabrovska et al. 2008); this regeneration extends the life of the biosensor up to 10–15 measurements. Indeed, re-activation decreases the sensitivity of the enzyme toward an irreversible inhibitor so that the inhibition degree measured in the series of experiments with the same inhibitor concentration gradually decreases. This is probably due to the inactivation of the most accessible enzyme molecules, which increases the stability of the remaining active sites toward an inhibitor. Reactivation requires carefully washing the biosensor after treatment; otherwise the irreversible inhibition in the following incubation will be underestimated due to the protecting effect of a reactivator. In many cases, it is recommended to eliminate this stage in favor of the substitution of the sensing element. Thus, the

immobilization of acetylcholinesterase on magnetic beads allows removing and restoring the bioreceptor coating of the electrode by switching the external magnetic field on and off (Gan et al. 2010). This makes it possible to perform measurements in the automated regime and in the lab-on-chip format. The latter includes electroosmotic micropumps for the reagents' delivery and system washing (Llopis et al. 2009).

Screen-printed electrodes are an alternative to the application of stationary and flow-through transducers with the periodical regeneration of the inhibited enzyme, due to the advantages of cheap and mass production and the rather good reproducibility of the main characteristics (Hart and Wring 1997). Commonly, screen-printed electrodes are prepared from carbon paste on plastic or ceramic supports, with some additives improving the mechanical stability of the layers printed. Metal conductors can be first deposited by vacuum deposition or from appropriate pastes. The enzyme charge does not exceed 0.1 units of the enzyme activity per electrode, so that even a single use established a very moderate price of the analysis. The screen-printed transducers vary in metal conductors, in the shape and size of the working area and the geometry of the setup. Commonly, the working electrode of 1–10 mm^2 is assembled with a pseudo-reference Ag/AgCl electrode printed from its paste with plasticizers and stabilizers. The screen-printing technique is compatible with various immobilization protocols including sol–gel and affine immobilization. They can be implemented in flow systems or used in batch conditions with a manual addition of reagents (Sassolas et al. 2012).

Selectivity and multi-analyte assay. The kinetics of irreversible inhibition does not allow selecting the contribution of certain pesticides to the total inhibition degree. The signal of a cholinesterase biosensor can be distinguished by the difference in the spontaneous reactivation and the sample pre-treatment. Thus, the oxidation stage mentioned above for thiophosphates can separate the signals of thionic pesticides and carbamates. The incubation of the sample in basic media increases the inhibition caused by trichlorfon converted into the more toxic dichlorvos (see Fig. 4.1). Both methods are insufficient for a complex medium containing a number of unknown inhibitors, especially those rather similar in the inhibition constant. Thus, application of cholinesterase biosensors for real sample testing calls for some approaches to the data quantification:

- *Standard toxicant concept*. In this approach, the total inhibitory effect of the sample is expressed as the concentration of a standard inhibitor exerting the same decay of the enzyme activity. Paraoxon is most commonly used for this purpose.
- *Enzyme source variation*. The first experiments on cholinesterase biosensors were mainly performed with butyrylcholinesterase from horse serum and acetylcholinesterase from an electric eel. Even for this pair, the selectivity toward individual inhibitors significantly varied. Recently, a family of genetically engineered cholinesterases was obtained and characterized. Some of the products showed excellent sensitivity toward organophosphates. The combination of various cholinesterases with multivariate analysis of the response provides the

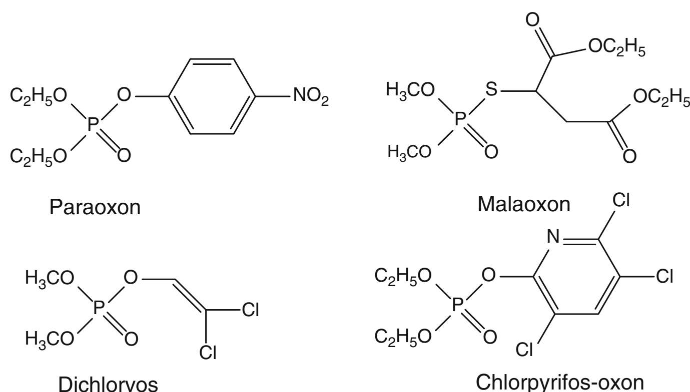


Fig. 4.1 Organophosphate pesticides, real cholinesterase inhibitors

quantification of each inhibitor present in the mixture (Alonso et al. 2012). Although the concept has been proved only for artificial mixtures of a limited number of pesticides, this approach seems very promising for environmental monitoring and food safety assessment.

- *Toxicity assay*. In this approach, the inhibition degree is compared with the results obtained with conventional toxicity assay techniques, i.e., acute toxicity on *Daphnia magna* or the Ames test of genotoxicity (Evtugyn et al. 1997). This makes it possible to avoid the necessity of the information on the chemical content of the sample. Instead, the potential hazard is estimated in terms of "safe"—"unsafe", or "toxic"—"nontoxic". This approach is rather common in bioassay techniques but is limited by the small number of toxicants detected by cholinesterase biosensors.

Sensitivity control. An impressive success in the development of a highly sensitive cholinesterase biosensor does not answer the question of why the sensitivity of irreversible inhibition changes. Indeed, as stated above, the inhibition determination is similar to the titration of the enzyme active sites. This means that the substrate response should not significantly affect the sensitivity of the inhibition measurement except for the signal accuracy influence. The theoretical LOD can be estimated from the Aldridge equation by assuming a minimal change in the reaction rate reliably detected. In most cases, this change is limited by 10 %, although 15 % seems a more reliable estimate. From (4.1)

$$c_I = \frac{1}{k_{\mathrm{II}}\tau} \ln \frac{v_o}{v_t}, \tag{4.1}$$

the LOD corresponding to the 15 % inhibition is equal to $0.13/(k_{\mathrm{II}} \times \tau)$. In other words, it depends on the enzyme/inhibitor system (the bimolecular inhibition constant k_{II}) and incubation time τ. Neither the nature of the substrate nor the measurement mode affects the irreversible inhibition. This contradicts the

Table 4.3 The LOD of paraoxon determination with acetylcholinesterase based biosensors

Enzyme source	Detection mode	LOD/Incubation time
Electric eel	Screen printed electrode with carbon nanotubes as mediator	0.5 nM/30 min (Arduini et al. 2006)
Electric eel	Screen printed electrode modified with TCNQ	3.6 nM/10 min (Schulze et al. 2002)
Electric eel	Screen printed electrodes covered with Nafion, measurements in the presence of 5 % acetonitrile	19 nM/10 min (Andreescu et al. 2002)
Electric eel	FET, BSA matrix	0.5 μM /20 min (Dzyadevych et al. 2004)
Electric eel	Conductometric, Pt planar interdigitated electrodes	0.5 mM/15 min gd(Dzyadevych et al. 2005)
Electric eel	Voltammetric, Pt electrodes, flow-through regime	10 nM/30 min (Jeanty and Marty 1998)
Drosophila melanogaster	Screen printed electrode modified with TCNQ	2.0 nM/10 min (Bucur et al. 2004)
Drosophila melanogaster	Screen-printed electrode modified with Ni/NiO nanoparticles	1.0 pM/20 min (Ganesana et al. 2011)

experimental works on the significant effect of the measurement conditions. In Table 4.3, the results of paraoxon determination are given as an example.

The reasons for such a difference—of approximately five orders of magnitude—are not obvious. For their discussion, the following aspects of biosensor performance can be considered:

- *Substrate/inhibitor distribution*. The Alrdridge equation assumes quasi-homogeneous reaction conditions in the surface layer. A non-uniform structure can offer some privilege to inhibitors in accordance with their size, charge, affine interactions, etc. The inclusion of polyelectrolytes and hydrophilic polymers amplifies the possible influence of the distortions in the reactant distribution inside the surface layer.
- *Metrological characteristics*. The inhibition quantification assumes repeated measurement of the signal separated by the incubation stage. This results in an increase of the inhibition deviation against that of a substrate measurement. Some researchers attribute the deviation estimated for the substrate detection to the inhibition result. This is especially important for non-linear and semi-logarithmic calibration plots where the confidence interval changes non-linearly from the median value of an argument. This does not mean that all the advantages in the LODs can be assigned to the chemometrics artifacts, but this calls for a more careful description of the metrological aspects of the investigations.
- *Enzyme loading*. Modern transduction systems based on nanosized carriers decrease the amount of an enzyme taken for immobilization. The relative decay of enzyme activity directly depends on the initial enzyme activity.

- *Non-enzymatic paths*. The hydrophobicity of some supports, like dendrimers, was mentioned as a possible reason for the sensitivity improvement. From this point of view, the higher the granulation of the surface, the better the inhibition signal. Limitations in the linearity of the calibrations graphs as well as the maximal inhibition degree can also be caused by some side reactions unfavorable to biosensor performance.

4.3 Immunosensors Versus Test Strips: Hapten Determination

The universal nature of an immune reaction makes it possible to produce antibodies for most of the chemical compounds and hence to develop the immunoassay protocol with a universal readout and measurement equipment. Meanwhile, the advantages of biosensor approaches for these techniques are not as obvious as in other bioassay formats. First and foremost, the long duration of the immunoassay steps, i.e., incubation and washing, diminish the importance of the short signal measurement provided by the biosensor. Standard 96-well plates are more convenient for mass analyses than immunosensors because all of the steps mentioned are performed when the average time required for the assay of one sample seems not as long as that for a single measurement with an immunosensor. For this reason, the efforts in the further progress of immunosensors are directed to the development of one-step protocols of the measurement or flow-through techniques that could compete with the ELISA and related immunoassay techniques. In this chapter, some of these approaches are discussed in the example of hapten detection.

Many analytes important for the food, safety, and health of the population, e.g., pesticides, surfactants, vitamins, etc., are too small (molecular mass $< 10{,}000$ D) to elicit the Ab formation. First they must be converted to immunogenic compounds to induce a specific Ab response. This typically involves three steps:

- designing a hapten that preserves typical elements of an analyte in its structure;
- synthesis of the hapten; and
- coupling the hapten with a macromolecule, such as protein.

The relationship between an analyte, hapten and a hapten conjugate is shown in Fig. 4.2. The hapten design affects the selectivity and sensitivity of the resulting Ab. The linker for binding the protein molecule should be introduced distally to the functional groups (determinants) of an analyte responsible for the Ab recognition. The resulting hapten-carrier conjugate is an immunogenic compound used to stimulate the immune system for the specific Ab production.

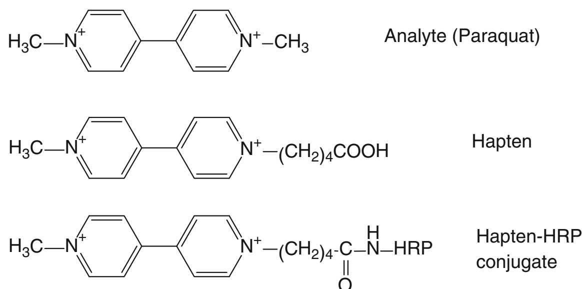
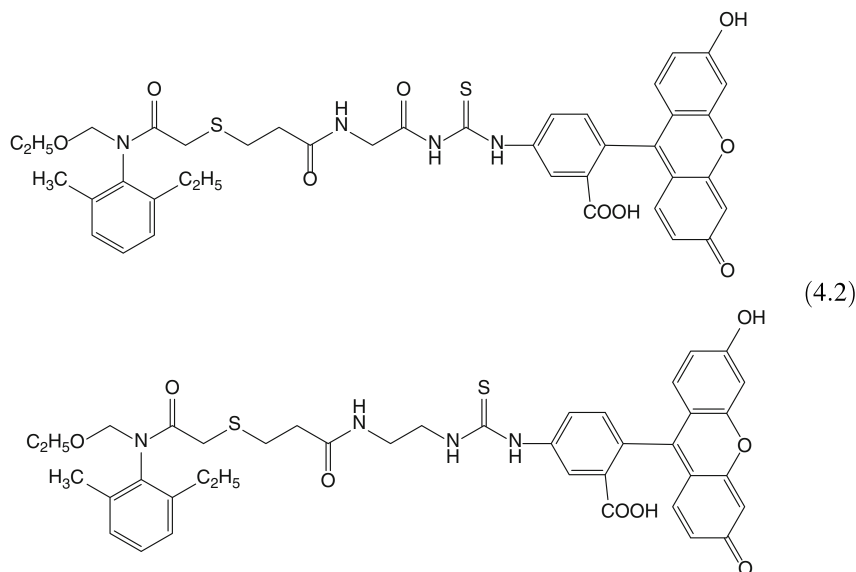


Fig. 4.2 Relationship between an analyte, a hapten and a hapten conjugate in the example of paraquat determination

The design of haptens and hapten conjugates refers to one of the most important tasks of immunoassay development. The length of the linkers, their connection to the main structural domains of the molecule, the flexibility of their structure, and the accuracy of the single-point attachment to the protein determine both the uniformity of the Ab pool obtained and the efficiency of the following biorecognition of an analyte. Even small differences in the hapten structure can cause significant changes in the immunoassay characteristics. The fluorescent detection of acetochlor is one example (Deryabina et al. 2004). Two haptens obtained by derivatization of the analyte with glycylaminofluorescein and ethylenediaminofluorescein (4.2) showed a remarkable difference in the selectivity and sensitivity of the assay. In optimized conditions, the detection limit of acetochlor of 10 ng/ml was reached. The optimal design of haptens and linkers makes it possible to develop the immunoassay for individual compounds and for the classes of, for example, sulfanilamide drugs or triazine pesticides. This might be useful for the direct assessment of the environment contamination or therapeutic treatment optimization.

The position of the linkers against the functional groups of the analyte affects the cross-reactivity of the product. Thus, for a specific assay of an individual compound, the unique substituents in its structure should be spatially separated from the linker. And vice versa—if a class-selective assay is desirable, the linker should be located at or near a position that differentiates the members of the class and exposes the features common for the class.



(4.2)

In the hapten conjugate synthesis, the hapten/protein ratio, the ease of synthesis, stability of the final products and purification requirements should be taken into account. The binding is mostly attained by carbodiimide reagents (haptens with terminal carboxylic groups) or succinic anhydride (haptens with amino groups).

The following assay is mainly performed in an indirect competitive format. The support is first covered with the so-called coating hapten. The sample is mixed with a definite concentration of specific antibodies and then the mixture is added on the hapten coating. After incubation, the system is washed and the hapten-enzyme conjugate is added to measure the signal. (The immunoassay formats are described in more detail in Chap. 2). The concentrations of the reagents are determined to reach minimal LOD, and a wide range of concentrations determined. For preliminary experiments, the estimation of the IC_{50}, a concentration yielding a 50 % decrease of the label signal, is used for a rough estimate. The following optimization includes determining the optimum temperature, incubation times and the effect of potential interferences.

In most experiments, homolithic immunoreagents are used. This means the hapten coating and hapten-protein conjugate are obtained from the analyte molecule. In some cases, the use of structural analogs of an analyte for hapten-conjugate synthesis provides some advantages in the immunization efficiency (Sardinha et al. 2002).

Since 1995, more than 250 individual low molecular compounds have been determined with immunoassay techniques. Most of them represent various classes of pesticides and food contaminants (drugs, steroids, hidden allergens, vitamins, etc.). The reviews of some recent developments can be found in (Watanabe 2011;

Jiang and Fan 2012; Meng and Xi 2011). The most important are phenoxyacetic acid derivatives, *s*-triazines, sulfonylurea herbicides, polychlorinated biphenyls, surfactants, nonylphenol and veterinary drugs.

Displacement immunoassay techniques. The development of the displacement techniques is related to the attempts to avoid the multi-step treatment and time-consuming washing in the immunoassay formats. In this group of methods, the analyte substitutes the hapten-conjugate in the Ag–Ab complex formed on the solid support, e.g., the permeable polymeric membrane, the affine column or the nanoparticles on the transducer surface (Fig. 4.3). As a result, the conjugate is substituted by a non-labeled analyte molecule so that the surface concentration of a label on the support decreases proportionally to the analyte concentration. The relative decay of the label signal depends on the dissociation of the Ag–Ab complex and hence on the relative stability of the complex formed by a conjugate against that of an analyte. This decreases the sensitivity of the analyte detection and the appropriate LOD value by one order of magnitude in comparison with the conventional techniques described.

Meanwhile, the one-step measurements can be performed in the flow-through or stop-flow mode with reduced time, and possibilities of the recovery by the inverse saturation of the surface layer with a conjugate after each measurement. The displacement method was first successfully applied for the detection of explosives (Whelan et al. 1993) and then extended to a variety of analytes, e.g., toxins (Lates et al. 2012), alkaloids (Hinds et al. 1984), drugs (Kaptein et al. 1997), and many other analytes (Ngo 2005). In most cases, the label signal changes in inverse direction to the analyte concentration. However, measurements of the conjugates pushed along from the complex are also described. The displacement immunoassay is compatible with most optical measurement techniques described for the conventional immunoassay and immunosensors, e.g., spectrophotometry, fluorescence, electroluminescence, etc.

Immunofiltration is a concentration procedure that utilizes the carriers modified with specific antibodies (Morais et al. 1999). The filtering of large volumes of the sample enables the specific accumulation of the trace amounts of analyte molecules in the Ag–Ab complex. After that they can be either extracted from the complex by specific reagents or detected in situ with sandwich-like immunoassay formats. The technique described is similar to the affine chromatography (immunochromatography) and some other sampling techniques utilized in medicine for detecting pathogens and disease biomarkers. The immunofiltration is mostly

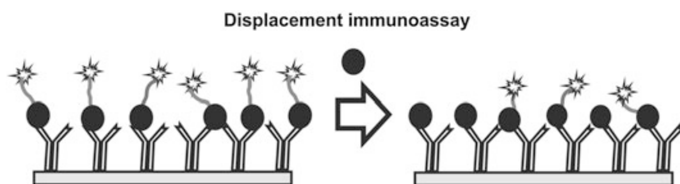


Fig. 4.3 Principal scheme of displacement immunoassay

performed in microcolumns (tubes, cartridges) or permselective plastic membranes in flow-through or stopped flow modes. Gel immunofiltration has also been described for the protein assay (Grant and Everall 1965). Immunofiltration is compatible with the immunosensor concept and can be implemented in flow or stationary immunosensing devices to increase their sensitivity toward analytes. Variation in the Ab pool used in filters, and the multi-analyte detection with the same transducer and label signal is possible.

Lateral flow tests are simple devices intended to detect the presence (or absence) of a target analyte in a sample without any specialized equipment (Weller 2000). These tests are typically used for medical diagnostics either at home (point-of-care testing) or in the laboratory. A widely used home pregnancy test is an example of such a device. The technology is based on a set of the capillary beds made of special paper or sintered polymers (Fig. 4.4) (Zhang et al. 2008).

Each of these elements has the capacity to transport a fluid spontaneously by capillary forces. The first element (the sample pad) holds an excess of sample fluid. Once soaked, the fluid migrates to the second element (conjugate pad) in which dried particles containing specific antibodies in a salt-sugar matrix are stored. While flowing through the porous carrier, the sample fluid dissolves the matrix and mixes with an immunoreagent. While migrating further through the third capillary bed, the analyte molecules bind to the particles. This material has one or more areas (often called stripes, or lines) where the secondary Ab molecules are immobilized. By the time that mixture reaches these stripes, the analyte has been bound on the strip area by the capture molecules. After a while, when more and more fluid has passed the stripes, particles accumulate and the stripe area changes color. There are at least two stripes: one (the control) that captures any particle and thereby shows that reaction conditions and technology have worked well, and the

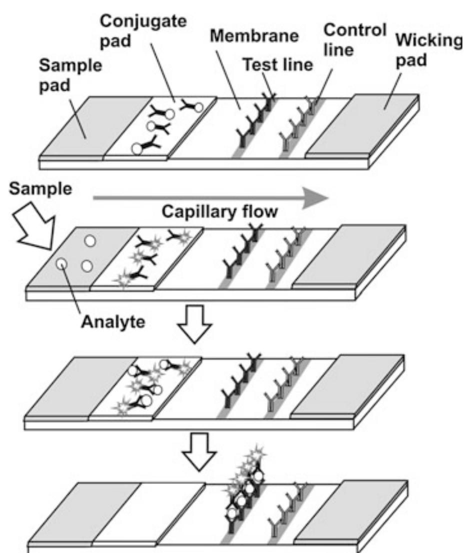


Fig. 4.4 Principal scheme of the flow lateral test operation

other one that contains a specific capture molecule and only captures those particles onto which an analyte molecule has been immobilized. After passing these reaction zones, the fluid enters the final porous material—the wick—which basically acts as a waste container. The lateral flow tests can operate either as competitive or sandwich assays. They can be combined with simple and inexpensive readout systems based on optical comparators with visual detection.

The lateral flow tested can be developed for many of the analytes but they are especially convenient for field applications with no universal analytical instrumentation. In many cases, the field testing is directed to the discovery of environmental pollutants or food contaminants with the semi-quantitative estimation of their content. Pesticide residuals are the most common analytes for such tests (Kim et al. 2011). The principles of flow lateral testing were successfully applied in the IMASS (integrated multiplex assay and sampling system) devices developed by British scientists for military purposes. Developed at the Defense Science and Technology Laboratory (DSTL), the device can collect samples of powder, water, or air and simultaneously detect eight substances belonging to: explosives, chemical and biological warfare, toxins, pathogenic microorganisms and viruses.[1]

4.4 DNA Chips: The Human Genome Project and More

DNA arrays (also called DNA chips) belong to one of the most intensively progressing areas of biosensors. The Human Genome Project was formally begun in 1990 and coordinated by the U.S. Department of Energy and the National Institutes of Health (NIH) and successfully completed in 2003 (Brown and Botstein 1999). The primary goals of the Project were:

- to identify all of the approximately 20,000–25,000 genes in human DNA,
- to determine the sequences of the 3 billion chemical base pairs that make up human DNA,
- to store this information in databases,
- to improve tools for data analysis,
- to transfer related technologies to the private sector, and
- to address the ethical, legal, and social issues (ELSI) that may arise from the project.

> DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA.

[1] http://www.bbigold.com/IMASS_Device_535.aspx

The solution of the encoding problems has been achieved by the development of absolutely novel technical decisions based on the parallel processing of rather short nucleotide sequences. Briefly, the complementary structures were synthesized in a manner similar to the polymerase chain reaction (PCR).

> The PCR is a technology of the ODN amplification. The method is based on thermal cycling, consisting of the repeated heating and cooling of the reaction directed to DNA melting and enzymatic replication.

A short 20–30-mer ODN was used as a template and a complementary sequence was synthesized with a one-by-one addition of nucleotides. The process was terminated by the application of dideoxynucleotides (ddT, ddC), which terminated the sequence elongation. Thus, the distribution of the sequences obtained in accordance with their mass indicated the position of appropriate nucleotides in the template. The electrophoretic separation with the fluorescence detection of the terminal dideoxynucleotides provided a colored map that corresponded to the exact sequence of nucleotides in the DNA sequence. This is of course a rather simple description of a much more complicated automated process successfully applied for the identification of several billion nucleotides in a human DNA sequence.

Similarly, the *DNA microarrays* called *DNA chips* use a great variety of the short ODN sequences (20–100-mer), which detect the complementary pieces of the target DNA related to appropriate genes or gene-specific pieces in a target DNA molecule. Indeed, the DNA chips do not belong to any analytical devices in the pure sense of the word because they are intended for the qualitative description of the sequence but not for the determination of its quantity. Then, the DNA chips analyze the PCR amplification products that significantly differ from the raw biological material by their content and relative quantities of DNA nucleotides. Nevertheless, the DNA chips have found increasing application in the investigations related to the detection of similar DNA sequences, e.g., gene expression, single nuclear polymorphism in genes, etc. They are expected to apply for new drug screening and genetic diseases diagnostics.

The idea of the DNA chips is similar to the sandwich assay protocol. Thousands of individual capture probes are positioned on the solid support (wafer, nylon or glass) with a high density, and they are incubated with the PCR products. The hybridization reactions with individual probes are conducted in parallel. This means that the decision about matching or mismatching of the sequences can be made simultaneously for thousands of potential targets. After that, the hybridization product is detected by the label fluorescence induced by the laser beam (Fig. 4.5).

The first DNA chip designed by Affimetrix under the GeneChip trademark contained the DNA probes that synthesized *de novo* onto the quartz plate after micromachining the positions by the photolithography process. At present, the technologies for direct positioning of DNA probes have been sufficiently extended

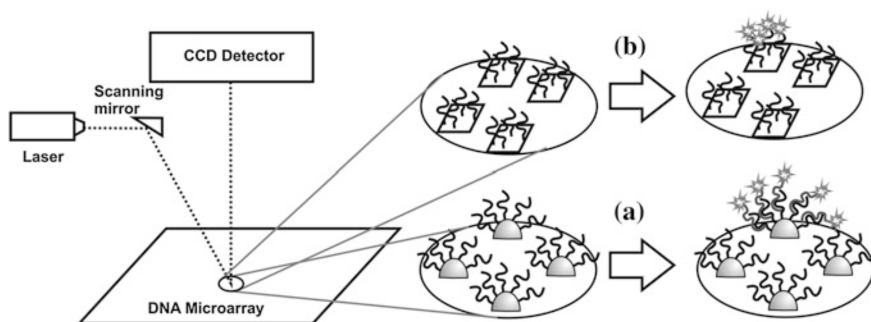


Fig. 4.5 Principal scheme of a DNA microarray operation. The ODN probes are printed on the 2D spots (**a**) and 3-D hydrogel nanodrops (**b**)

and include inkjet printing, electrophoretic deposition, and even electropolymerization based approaches. Some of the characteristics of the modern methods of DNA chips manufacture are summarized in Table 4.4.

Robotic microprinting uses a special print head with a set of thin pins. The head is positioned with a three-dimensional motion control, and the pins are then dipped into a tray of a probe solution consisting of pre-synthesized ODNs, which is then transferred to a pre-determined position on a solid substrate. The amount transferred depends on the geometry of the printhead. The size of each spot is approximately 100–150 μm in diameter. The use of semispherical drops of hydrogels increases the density of the ODN probes at the same surface and hence the resolution and intensity of the signal recorded (Rubina et al. 2004).

The *photolithography* process has already been described in the sections on the FETs and the SAM immobilization of biopolymers. It is based on special polymeric layers that are UV irradiated to form the holes filled with the ODN probes. The size and location of the spots are determined by special templates protecting part of the surface from irradiation. The resolution of the template details limits the minimal number of the spots. In turn, it is defined by the optical range used for hardening the surface layers. The nanolithography technique based on the X-radiation and electron flow provide the spots of several nanometers in diameter. But high energy sources damage the ODN structure and can result in a false detection of the hybridization events. *Inkjet printing* is the working principle similar to that of an inkjet printer; the “ink” (the ODN solution) is sprayed onto

Table 4.4 The characteristics of different DNA chips manufacture approaches

DNA probe positioning	Spatial resolution	Cost	DNA probe length	Ease of use
Robotic microprinting	Poor	Cost effective	Not restricted	Require PCR or cloning steps
Photolithography	High	High	Less than 25-mer	Protected by Affimetrix patent
Inkjet printing	Medium	Middle	5–75-mer	Equipment requires strict maintenance

the array surface. The coordination of the print head and the spots' positioning are achieved by the piezoelectric effect. The volume dispensed is about 50 pL and the spot size 100–200 μm (Wallace 1996).

Over 400,000 ODN probes can be placed on a glass plate of 1.28 × 1.28 cm in size. Actually, the number of unique sequences is much smaller due to replications, negative controls, etc. In addition, to avoid possible mistakes, one gene is detected by several (up to 40) probes containing various pieces of the gene-related code. On the average, from 5,000 to 20,000 targets are screened on each chip. The length of the DNA probe depends on the manufacturer. The Affimetrix provides 26-mer sequences, whereas the Agilent ("LabChip"™) gives 50–100-mer.

DNA arrays make it possible to solve the *sequencing problem* described above for the Genome Project. For this purpose, a collection of overlapping ODN probes is assembled together on a chip (Drmanac and Drmanac 1990). Partially mismatched probes can form hybridization products but they are removed from the chip by careful melting and washing due to a less binding efficiency. The labeling of the target materials is commonly performed on the stage of the PCR amplification in which these are the fluorescent labels (cyanine, fluorescein derivatives, GFP) (Pease et al. 1994). Since the wavelengths of the excited and emitted lights are different, the fluorescence detector can filter the excitation wavelengths. Radioactive isotopes are also used as labels.

Besides DNA sequencing, there are many other areas of DNA chip application. *Gene expression profiling* is necessary to monitor gene expression in diseased and normal cells (Schena et al. 1995). The technology enables measurements of thousands of genes in a single RNA sample. The RNA molecules are labeled with fluorescent dyes and hybridized on the DNA chip. Hybridization between the sample RNA molecules and the reference RNA pool under different environmental conditions provides the information necessary for the new drugs' development and environmental pollution assessment. The biomarkers that are present only in diseased cells can be discovered and used for diagnostics (Lockhart et al. 1996). For cancer diagnostics in particular, the identification of so-called "proto-oncogenes," which are converted into tumor-inducing substances directly into any cells can be discovered; they are not found in healthy cells. Therefore these genes can lead to new therapeutic approaches to cancer treatment. The GeneChip (Affymetrix) and the HyX (Gene Discovery Modules) are available on the market for particular diagnostics purposes (cardiovascular diseases, etc.).

Single nucleotide polymorphism is a variation of a single nucleotide in the DNA sequence related to an appropriate gene. Point mutations are very rare (0.1 % chance per single base pair within a human DNA molecule). The point mutations can affect the immune status and result in genetic diseases, e.g., cystic fibrosis and sickle-cell anemia. The DNA chips analyze multiple point mutations within individuals and provide information on the relations between the mutation areas and disease susceptibility or drug response. The p53 GeneChip (Affimetrix) detects a single nucleotide polymorphism of the p53 tumor suppressor gene. The HIV GeneChip is used for detecting mutations in the HIV-1 protease and the virus reverse transcription genes.

4.5 Toxicity Bioassay: Microtox® Related Systems

The increasing need in the risk assessment related to a large number of organic and inorganic substances, both natural and released in the environment by human activities, calls for the development of biosensors directed to the general estimation of potential hazards related to contamination. These biosensors differ from the majority of bioassay devices by the signal interpretation. While most of the biosensors determine the quantity of an analyte that is specifically recognized by a biological component, the toxicity biosensors detect the effect of all the chemicals present in the sample tested irrespective of their content and chemical nature. To some extent, this approach is similar to that considered above for inhibitor biosensors whose signal (inhibition degree) cannot be derived from the concentrations of individual inhibitors in the mixture. Similarly, toxicity biosensors do not provide any information on the nature and source of toxicants directly, but alert the user to the dangers related to chemical contamination.

Although all of the toxic effects can be related to the initial damage of particular enzymatic systems (biological targets), the use of enzyme sensors was found to be ineffective for the toxicity assessment because of the high specificity of the response. Even cholinesterase, i.e., the enzyme with the widest range of chemicals affecting its activity, cannot be used for the detection of some dangerous species, such as atrazine pesticides, DDT or chlorophenols. The total inhibition of cholinesterases is affected by reversible inhibitors protecting the enzyme active site. Inhibition is also influenced by the ionic content, the temperature, the presence of organic solvents and the temperature. Other enzymes show a rather small number of toxicants inhibiting their activity, although there were attempts to use some oxidoreductases and urease for the estimation of the total amount of heavy metals (Guascito et al. 2009; Brack et al. 2000).

The use of microorganisms for toxicity assessment extends the possibilities of toxicity biosensors because of the more complex nature of the response. The metabolic activity of microorganisms depends both on the transmembrane transfer of toxicants in the cell and its influence on the main biochemical paths. To some extent, the response of microorganisms in such toxicity tests mimics the toxic effect of the contaminants on multicellular organisms except for some specific protecting mechanisms (immune reactions) and the repair capacity. Besides, microorganisms are easy to cultivate and they can be adapted to the specific chemicals by the selection of cultivation conditions and even by genetic engineering. A rather short alternation of microorganisms' generations makes it possible to monitor the long-term negative effects of hazards on reproduction.

Bacterial toxicity tests are well known in applied microbiology and toxicology. They are mainly based on the cultivation of colony-forming microorganisms in the presence of toxicants and on the estimation of the total antibacterial effect by

the relative decay in the colony growth. In the thickened media (agar, gelatin), the colonies are observed as small spots of appropriate color and shape in Petri dishes on the surface of the film. The toxicity is estimated by counting the number of colonies prior to and after the addition of a toxicant to the growth media (Fig. 4.6). In other protocols of the same method related to gel permeation chromatography, the microorganism colonies are counted after the injection of the toxicant solution in the center of the dish. Besides the aqueous solution, the paper strip soaked in a toxicant can be applied. The toxicant molecules diffuse from the center to the brink of the film. While moving, they decrease the concentration until the limit of toxicity is reached. This reflects the formation of a symmetric empty area with no colonies ("dead zone"). The radius of the zone reflects the concentration of a known toxicant or the potential danger of a chemical taken at its certain concentration. The variety of the culture media, growth conditions (temperature, pH) and the regime of the contaminant incubation alter the results of the toxicity assay. This offers additional capacities to characterize the toxic species in accordance with their stability, mechanism of action, ability of bioaccumulation and some other properties. The modification of the test described is used in genotoxicity testing with mutant bacteria strains (Ames test; see below). Instead of the visual estimation, the acute toxicity can be estimated by pH or electroconductivity changes, with the biofilm deposited on the interdigitated electrodes.

The information on the acute toxicity (death of microorganisms) can be supplemented by other conclusions related to the specific toxication symptoms (changes in color and specific shape of colonies, colony growth retardation also called "bacteriostasis").

In a suspension of microorganisms (Fig. 4.6b) prepared in a so-called "broth", the changes in the population of microorganisms can be monitored spectrophotometrically by the turbidity of the suspension. Changes in the number of microorganisms are called the "cultivation curve". The simplified description of

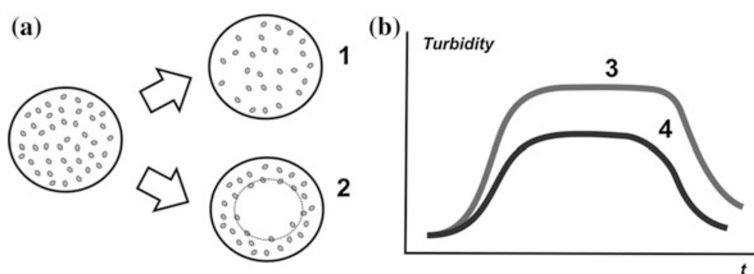


Fig. 4.6 Bacterial toxicity tests in gel (**a**) and liquid (**b**) growth media. Petri dish is filled with gelatin gel and a uniform distribution of the colonies is observed. The addition of a toxicant in the growth media decreases the number of colonies with their uniform distribution. *1* The injection of the toxicant solution in the center *2* results in the formation of an empty area ("death zone"), the radius of which is proportional to the toxicant concentration. In suspension of microorganisms (**b**) the toxicants alter the growth curve (*3* for blank experiments and *4* in the presence of the toxicant)

the curve involves four stages: The lag phase corresponds to the constant number of the organisms; the growth phase reflects the prevalence of cell doubling over their death; the third phase (plateau) corresponds to the upper maximum of the population; and the fourth stage (death) results in a rather sharp decrease in the number of cells due to the exhaustion of the nutrients and accumulation of waste products. The shape of the curve described is typical for appropriate microorganism and cultivation conditions (nutrients, temperature, electrolytes, etc.). The addition of toxicants results in the suppression of the culture growth, the lower limit and faster decay of the population, in comparison with the blank experiment. Instead of recording the full curve, the number of microorganisms is commonly determined within the first linear piece of the curve, the so-called “log phase” of the growth. This corresponds to the maximal activity of microorganisms and hence to the more sensitive detection of hazards.

Being visual, monitoring of cell numbers by the methods described is rather labor-consuming and provides the detection of a relatively high concentration of the antibacterial compounds. Instead of the counting approaches, the activity and vitality of microorganisms can be detected using an enzyme produced during their life cycle and excreted from the cell into the environment.

Microbial biosensors based on immobilized cells have been paid increasing attention due to the problems of contamination of the surface water with organic matter. The microorganisms that are present in the environment destroy organic matter, resulting in oxygen consumption. If the pollution is too intensive, the lack of dissolved oxygen can cause severe consequences for the aerobic organisms. For this reason, the organic matter available for natural consortiums of microorganisms is monitored in surface waters, as well as during the wastewater treatment using a so-called “biological oxygen demand (BOD)” parameter. This is the amount of oxygen necessary for the oxidation of organic matter present in the water sample. A standard technique of BOD determination includes a long incubation period of the water samples in the tightly closed vessels provided with oxymeters to record the oxygen consumption. Routine experiments take up to 21 days; for municipal wastewaters that do not contain species toxic for microorganisms, a 5-day test is used.

The biosensor format can significantly decrease this time period. The microorganisms are immobilized onto the surface of an oxygen electrode so that the reaction of oxidation directly affects the amount of oxygen reduced at the electrode (Su et al. 2011). The BOD parameter is estimated from the slope of the current recorded in the first period of time after the biosensor’s contact with wastewaters, or from the steady-state value of the current recorded in a galvanostatic regime. The flow-injection modes and the bioreactors connected to the oxygen sensor are rather common, especially in wastewater treatment plants (Oota et al. 2010) (Fig. 4.7). The miniaturized BOD sensor has been developed from the planar screen-printed electrode with interdigitated working and auxiliary electrodes covered with a biofilm-containing bacteria. The measurements were done in the presence of the hydroquinone/benzoquinone mediation system (Liao et al. 2001).

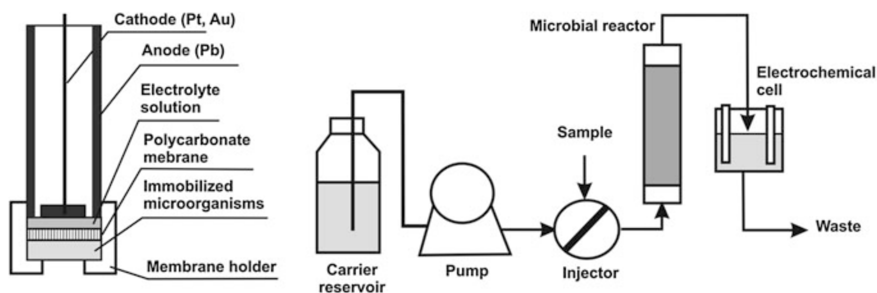


Fig. 4.7 Stationary and flow BOD sensors for the detection of organic contamination

The response time of the BOD biosensor can be reduced against conventional protocol to about 30 min. The determination of individual compounds can be performed within 3–5 min. It should be noted that this period does not cover the time required for bacteria cultivation and biofilm conditioning, which can be quite time- and labor-consuming.

The performance of BOD biosensors depends on the microorganisms' oxidizing of organic contaminants. Three types of cells can be introduced in biosensor assembly:

- single organisms, e.g., baker's yeast, *Bacillus subtilis* and *Serratia marcescens*;
- mixed cultures and consortiums, e.g., activated sludge applied for the biological treatment of the wastewaters; and
- genetically engineered microorganisms.

Single organisms can metabolize a limited range of contaminants and hence lead to an inaccurate BOD estimation. The activated sludge is more appropriate because of the obvious application of the results for the optimization of the wastewater treatment (Kumlanghan et al. 2008). Besides oxygen consumption, the CO_2- and NH_3-sensitive sensors are applied for detecting the active sludge damage caused by toxic wastes and effluents.

The main problem related to BOD sensors based on this component is their low sensitivity to small amounts of contaminants. The activated sludge is rather difficult to calibrate and standardize, which complicates the comparison of the results obtained by various research groups. The application of genetically engineered organisms is directed toward the use of some other detection principles, based on the fluorescence or bioluminescence measurement caused by gene expression. All the BOD sensors are calibrated with standard solutions of glucose and some other readily oxidizable compounds.

Measurements of toxicity with the BOD biosensors are mainly performed at biological wastewater treatment facilities to prevent the damage caused by activated sludge (Codina et al. 1994). The soil and sediment contamination can be estimated directly with their aqueous suspensions or indirectly with the extracts performed in different ways, taking into account the distribution of chemicals between the sample

parts (solid matter, moisture, exchangeable cations of toxic metals, etc.). The toxic compounds exerting antibacterial activity suppress the metabolism of bacteria placed in the biofilm of the BOD biosensor. This results in the decrease of oxygen consumption and hence in the increase of the current related to cathodic oxygen reduction. The toxic effect can be monitored in continuous flow conditions assuming the constant content of oxidizable organic compounds within the measurement period. Otherwise, the biosensor is first incubated in the sample tested, which is usually mixed with nutrients and other chemical compounds necessary for bacteria activity. After that, the response is measured in the standard solution of organic compounds in the absence of any toxicant. The flow-injection regime is also possible but its sensitivity is low enough because of the rather long period of the biosensor response.

Most of the common industrial pollutants, e.g., heavy metals, pesticides and surfactants, exert a reversible effect on the oxygen consumption at their low concentrations. The response of the BOD biosensor increases while the toxicants' content decreases and vice versa. Highly toxic wastes show an irreversible decay of the signal. Although BOD biosensors can operate for several months, their lifetime in toxicity measurements is much shorter even though the low reversible effects are monitored. The bacteria in biofilm adapt to the chemical content of the sample media so that the sensitivity of toxicity detection decreases. This phenomenon can be applied for the selective detection of specific pollutants. Thus, yeast cultivated in the presence of a low phenol concentration can enhance the metabolic activity in the contaminated waste waters due to the involvement of the toxicant as an organic carbon source in metabolic paths. Genetic engineering offers additional possibilities for diversifying the responses to various contaminants. The detection of oil contamination with the bacteria adapted to the oxidation of long-chain carbohydrates is but one of the most impressive examples.

The first commercial BOD biosensor was produced by the Nisshin Denki (Electric) in 1983. Since then, several more BOD biosensors have been commercialized by the DKK Corp. (Japan), Autoteam FmbH (Germany); Prufgeratewerk Medingen GmbH (Germany), Dr. Lange GmbH (Germany), STIP Isco GmbH (Germany), Kelma (Belgium), LAR Analytik and Umweltmesstechnik GmbH (Germany), Bioscience, Inc. (USA), and USFilter (USA) (Lei et al. 2006).

Monitoring oxygen as an indicator of viability and metabolism level is applied for other organisms. In biology, this group of methods is called a "respiration test". Infusorian, soil organisms, and land plants are frequently considered in the ecological risk and human impacts assessments. However, they are much less compatible with the biosensor format. Nevertheless, there were some successful attempts to incorporate the biological tissues and organelles in the biosensors to obtain a signal related to the toxic effect of the particular group of toxicants. Thus, the use of thylakoid membranes with the Photosystem II offers broad possibilities for herbicide detection. They suppress the photosynthesis detected by the value of the photocurrent generated by chlorophyll under illumination (Piletskaya et al. 1999; Bettazzi et al. 2007). The same results are achieved by the direct measurement of chlorophyll fluorescence (Merz et al. 1996). Photosynthesis activity

can be easily measured with microalgae in flow or on the surface of appropriate transducer (Farré and Barceló 2009). To simplify the immobilization and replacement of the cells, the cells of *Chlorophylla vulgaris* were preliminarily covered with magnetite nanoparticles (Zamaleeva et al. 2011). All the biosensors based on the estimation of photosynthetic activity require an illumination, either constant or alternating with a dark period. In the latter case, the photocurrent recorded corresponded to the difference in the electron chain activity in the light and dark photosynthesis stages.

Genotoxicity testing. The detection of DNA damage caused by chemical contaminants can be done by the mutant strains of *S. typhimurium*. The test was developed in the 1970s by B. Ames (University of California, Berkeley).

> A strain is made up of the descendants of a single isolation in a pure culture and usually is made up of a succession of cultures ultimately derived from an initial single colony—*Bergey's Manual of Systematic Bacteriology*

Ames suggested that the strains of the *S. typhimurium* that carry mutations in genes are responsible for the synthesis of histidine. As a result, the bacteria can be cultivated only in the presence of this amino acid. The mutation is unstable, and some bacteria return the capacity to synthesize histidine in the next generations (reversion of the mutation). The rate of reversed mutation is constant for each strain and is increased in the presence of mutagenic substances. To increase the reliability of the testing, the sample can also be oxidized by a mitochondrial fraction of the liver that bears the P_{450} cytochrome complex that is responsible for the so-called metabolic activation of the mutagens in a living being. The detection of mutagens was first based on the colony counting similar to that described for the toxicity testing above. The number of colonies increased with the concentration of mutagenic species because of the increased rate of reverse mutations. The initial Ames test is now significantly modified, but its main idea has remained. The genetically engineered *E. coli* strains are used in addition to the *S. typhimurium*; some of the mutants are tryptophan-dependent. The Ames test is widely used for the preliminary screening of mutagenic factors, including the chemical contaminants and radioactive isotopes that can cause carcinogenesis or affect the reproduction functions of a living being. The adoption of the test by the FDA resulted in a substantial decrease of the number of synthetic food additives (dyes, preservatives, stabilizers) that were found to be dangerous.

The umu-test is also a standardized and validated method, based *on S. typhimurium* (Oda et al. 1984). Genotoxicity is detected by measuring the transcription of the SOS-response genes, whose code for enzymes is involved in DNA repair. The fusion of the SOS-response genes with the β-galactosidase-encoding reporter gene enables the colorimetric detection of genotoxic compounds. The SOS-chromotest, based on the same principles, applies *E. coli* as a test species.

It shows a high sensitivity to certain groups of pollutants, e.g., chlorinated pesticides and chlorophenols (Logar and Vodovnik 2007).

> • *A fusion gene* is a hybrid gene formed from two previously separate genes.
> • *The SOS response* is a global response to DNA damage in which the DNA repair is induced. The system involves the *RecA* protein that inactivates the *LexA* repressor followed by inducing the response. The activation of the *RecA* protein is activated by the ss-DNA.

The rate of reversed mutations can also be determined by microbial biosensors utilizing the mutant strains and detecting the reversion of the mutations by oxygen consumption. The contact of the culture with the sample can be performed prior to or after the immobilization in gel films on the oxygen sensor surface. To exclude the interferences with the response to the measurement conditions, a differential scheme is used. One biosensor is provided by the mutant strain of the *S. typhimurium*, whereas the other one is provided by a wild culture.

Microbial biosensors based on bioluminescence. Bioluminescence tests can be applied to assess the toxicity in water, sediments, soil, foodstuffs and industrial products. Due to high sensitivity towards toxicants, and preferably xenobiotics and universal response, they have become intensively used during the past decades. The luminescence tests can employ two kinds of microorganisms:

- Original marine bioluminescent bacteria (*Vibrio fischeri,* formerly known as *Photobacterium phosphoreum*). They emit light as a result of normal metabolic process and reduce luminescent ability during exposure to contaminants.
- Microorganisms isolated from the environment for testing and transformed in luminescent strains by genetic manipulation. The most frequently reported tests include genetically modified bacteria with artificially constructed fusions of particular regulatory systems (native promoter) with the reporter genes (*lux* gene coding for luciferase or the *gfp* gene coding for the GFP). The expression of the reporter genes correlates with the luminescence level that increases with the stress factor.

The famous Microtox® test illustrates the first approach. It contains lyophilized luminescent bacteria that are re-hydrated and incubated with the sample diluted in various ratios with water or with a series of contaminant solutions of different concentrations. The results of the measurement, i.e., luminescence intensity, can be recorded within 5–30 min of incubation. The results are presented as a dependence of the relative decay of light intensity on the dilution or contaminant concentration. The toxicity of the sample is estimated by the minimal dilution eliminating toxicity (luminescence suppression) or by the IC_{50}, i.e., concentration resulting in 50 % of luminescence decay. The sensitivity of the Microtox culture is validated with the help of a standard toxicant, i.e., phenol. First developed for

surface water testing, Microtox was then successfully applied for other subjects, preferably for sediments and soils. Together with the Ames test, the Microtox establishes the safety of most food additives permitted for use.

The application of genetically engineered microorganisms with bioluminescent response is directed toward the detection of any stress factors able to affect metabolism (temperature shock, xenobiotics or heavy metals). *E. coli* and *S. typhimurium* are mainly used as host organisms. The test organisms are adapted to the presence of an effector (non-specific stressor, such as the DNA damaging agents, heat shock, oxidative stress, toxic metals, or organic environmental pollutants) resulting in the transcription and translation of fused target genes, generating recombinant proteins that produce some measurable response. The ability for luminescence can be associated with a specific promoter to be detected selectively with high sensitivity.

The toxicity can also be detected by the factors related to the luciferase reaction. Thus, the nitrification inhibition has been evaluated in wastewater with a recombinant *Nitrosomonas europaea* bearing the luciferase gene. The inhibition of the ammonia monooxygenase activity in *Nitrosomonas* results in a rapid decay of the NADH/ATP concentration followed by a reduction of the light emission intensity.

All the above-mentioned bioluminescence tests utilize cell suspensions to be mixed with the sample of toxicant solutions. Besides, immobilized preparations and microbial biosensors have been described for the same purposes. Agarose, collagen, epoxy metacrylate and nylon have proved to be very effective carriers for chemical immobilization applied to the luciferase enzyme. Bacterial cultures are mainly entrapped in hydrogels, and should be stored at 4 °C to preserve their activity for several weeks. Below are some illustrations of biosensor-based approaches to the bioluminescence assay.

Genotoxicant, i.e., mitomycin C, was detected by a "dark" strain of the *P. phosphoreum* (A2), immobilized in the agar-gel membrane on the tip of the optic fiber linked with a bioluminometer (Sun et al. 2004). A LOD of 0.1 mg/L was achieved. The microfluidic MEMS system was fabricated by immobilizing bioluminescent bacteria in the polyvinylalkyl-styrylpyridinium (PVA-SbQ) (Yoo et al. 2007). The biosensor increased light that was emitted in the presence of hydrogen peroxide as a response to oxidative stress. Bioluminescent strains have been immobilized in a microfluidic chip assembly in agarose gel. The device was made of the sandwich prepared from a silicon plate placed between two poly(dimethylsiloxane) films with microchannels connecting perforated microwells on the silicon substrate. Bacteria mixed with the agarose were injected into the channels and left gelling in the microwells. The induction of the luminescence emission occurred when channels were filled with the mutagen solution (mitomycin C) (Tani et al. 2004).

In addition to the numerous components of light-emitting systems, i.e., enzymes, bacterial strains, substrates, some ready-to-use kits and bioluminescence, biosensing systems are commercially available (Girotti et al. 2008). Besides the Microtox (Strategic Diagnostic Inc., Carlsbad, CA, USA) already mentioned, its portable version, Deltatox, is intended for the field testing of drinking water quality. The MUTATOTOX® reagent of the same manufacturer is based on a dark

variant of *V. fisheri*. Genotoxicity is indicated by the recovery of luminescence caused by the base-pair mutations, frameshift, the DNA intercalation and the DNA synthesis inhibitors. The analog based on another dark strain is produced by AZUR Environmental (Carlsbad, CA, USA). The *BioTox*™ *Rapid Toxicity Testing System* and the "*Biotox Flash Test*" are based on *V. fisheri*; the latter automatically corrects the turbidity/color interference (Hidex Oy, Turku, Finland). The average measurement time is approximately a few seconds. LUMITOX® (Lumitox Gulf LC River, Ridge, LA, USA) is the only portable system based on eukaryotic cells. It is believed that they better model toxicity to a human being than microorganisms do. In a field-portable system with mutant luminescent dinoflagellate cells, ppb levels of pollutants can be detected.

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Chapter 5
Biosensor Prospects: Quo Vadis? (Conclusion)

The overview of the approaches to biosensor development and application presented in the previous chapters shows that biosensors have successfully emerged from the laboratories. In most areas, they compete with conventional analytical devices, e.g., chromatographs or spectrophotometers. Their compact design, their user-friendly measurement protocol and their low cost are indisputable advantages in chemical analysis, especially if it assumes any field applications. This is especially true for various substances that are important from the point of view of their potential hazard (environmental pollutants, food contaminants, genotoxic chemicals, etc.) or human health protection (metabolites, nutrients, biomarkers of diseases, or development pathologies). In this chapter, some general considerations of the trends of biosensor progress in specific areas are considered. Probably, some of the statements are disputable and can be rejected by researchers specializing in particular areas; nevertheless, they coincide with the global feeling of future changes in the methodology, research facilities and approaches to biosensor design and its applications.

Enzyme biosensors. After the great success of glucose meters, a rapid commercialization of other similar enzyme sensors was expected. The analysis of lactate, urine, and uric acid, biomarkers of common illnesses are demanded in hospitals and biochemical laboratories all over the world; nevertheless, only lactate and urea biosensors were introduced in the biomedical assay praxis, but the scale of sales and variety of the commercial products are far from those observed for glucose meters. To some extent, this relative lack of success is related to the different importance of the biochemical indices determined. Indeed, the glucose level is essential for diabetics, but the concentration of lactate in blood is of interest mainly to professional sportsmen and probably to fitness fans. The relationships between one's general health status and lactate levels are not so very rigorous and most people are not ready to pay high prices for them.

The situation described is typical for many other biosensors developed for point-of-care diagnostics. Many of them are actually very close to commercial implementation, but this process is seriously limited by low market expectations. As regards equipment developed for hospitals, the advantages of biosensors are counterbalanced with some limitations in the frequency of measurements,

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accuracy and reliability of the results, which are often far from the conventional techniques used. Instead of compact individual devices, there are various stationary analyzers. They are more complicated, bulkier, and much more expensive than the personal glucose meters that everyone can buy in the pharmacy. From the formal point of view, modern biochemical analyzers do not belong to the biosensor family, even though they exploit the same signal measurement principles. They are often provided with automated samplers and sample treatment systems, including chromatographic columns, extraction and mixing boxes, etc. And, last but not least, the auxiliary equipment—which is often as expensive as the measurement unit itself—is easily adapted to other biochemical assay necessities. To some extent, the high specialization of enzyme sensors mostly intended for the detection of a single analyte becomes a drawback that limits their further use in medicine.

Metabolomics is another promising growing field for medical biosensor applications. The study of metabolite profiles as fingerprints of specific cellular processes and health monitors has received enormous attention over the past few decades. The biosensor-based analysis of biological liquids or exhaled air is considered to be a rather inexpensive alternative to HPLC and mass spectroscopy techniques.

The enzyme sensors developed for nonmedical purposes remain too complex and unreliable for use by any unqualified users. Medics and laboratory personnel are prepared to work with biochemicals, whereas engineers, drivers, and householders are not. Attempts to manufacture compact devices for the early detection of pesticides or estimation of soil contamination proved not very impressive. The insufficient stability of the cholinesterase and multi-step measurement protocol were not effective enough to win the attention of the end-users outside the specialized laboratories involved in environmental monitoring and the farming industry.

In many potential application areas, the enzyme sensors expect pressure from immunoassay techniques, especially immunotests. Disposable immunostrips and tubes exert at least the same or even higher specificity of the response but do not require a substrate addition and careful consideration of the measurement conditions. For the same reasons, the immunoassay methods stopped the progress and then fully pushed out chromatography from biomedical assays. The development of immunostrips for pesticide detection is an example of such a tendency. Previously, the cholinesterase biosensors were the only devices providing the detection of sub-ppb levels of organophosphates, i.e., below their threshold values. This was sufficient to prevent poisoning and incidents with pesticides; however, the test strips with specific antibodies can perform the same analysis faster and simpler. They do not allow detecting the anticholinesterase effect of several compounds and this is the only advantage of the cholinesterase sensors over the immunoassay technique. However, only one or two anticholinesterase pesticides are applied at a time in the farming industry, and this is quite convenient for the immunoassay. The prevalence of the immunoassay methods in the areas where numerous samples are tested, and a limited number of components detected, is common beyond the biosensors area, too.

The above does not mean that past efforts in the development of enzyme sensors were fruitless. The following prospects of the enzyme sensors seem quite probable:

- *Miniaturization and integration.* Of enzyme sensors into multi-analyte platforms with the general circuits of the fluids and sample injection. A multiplex analysis format with several biomarkers, metabolites or drug residues determined from one sample at a time coincides well with personalized medicine, a medical model assuming customization of healthcare being tailored to the individual patient. The prototypes of wireless implantable biosensors that have recently appeared make it possible to monitor several biochemical and physiological parameters, i.e., blood gases, pH and metabolites, over several months. However, calibration stability, biocompatibility and operation reliability issue challenges have to be solved prior to broad dissemination of the technologies (Kotanen et al. 2012).
- *Application of new biocomponents.* The number of natural enzymes included in the biosensors tends to be limited. However, the genetic engineering can significantly influence the characteristics of the enzymes, i.e., the affinity toward a substrate and the substrate/inhibitor specificity. The experiments with insect cholinesterases and mutant enzymes produced by genetically modified microorganisms showed remarkable results in the control of the selectivity of inhibitor determination. To a lesser extent, genetic manipulations are used for the improvement of enzyme stability or its immobilization by affine interactions. The enzymes from engineered microorganisms are expected to dominate among other sources, although plant proteins are less studied at the moment. The modification of enzymes, either by gene manipulation or chemical treatment, can be extended by the selection of the domains with the active site. The simplification of the protein structure similarly to the use of the Fab fragments in an immunoassay can improve the stability of the enzymes and simplify their attachment to the carrier/transducer surface.
- *New application areas.* The analytes determined with the enzyme sensors are mainly derived from the nature of biochemical agents. This list can be largely extended by the genetic modification of enzymes. Together with improved stability and development of appropriate immobilization protocols, this can result in the sufficient extension of the traditional applications of both the usual and newly developed enzyme sensors. The application of biosensors for the discovery of life forms in the solar system (Kounaves 2003) is an example of such approaches. Other extreme conditions for the enzyme sensor operation include the analysis of predominantly non-aqueous media (oil products, organic solvents, extracts from plants and soil), the direct continuous functioning in atmosphere and industrial gas pipelines (the industrial volatile, the detection of breath components in medicine, the analysis of permanent gases, and the greenhouse effect control) (Dong and Wang 2002). In both cases, serious efforts are required to establish a long-term stable biochemical response.

- *New-old analytes.* The improved performance of the enzyme sensor can return its attraction to the traditional areas of application. Thus, the sensitivity of modern enzyme sensors does not meet the requirements of environmental monitoring because the detection limits of most pollutants—except anticholinesterases—are higher than their threshold values. The determination of phenolics in wastewaters and biogenic amines in foodstuffs are most promising applications of enzyme sensors in the future. The further increase in the sensitivity can be achieved either by in-line pre-concentration of the substrates by solid-phase or immunochemical extraction or by manipulation with the enzyme structure. Thus, the removal of polysaccharide shells from peroxidases increases the sensitivity of the substrate/inhibitor detection as well as electron wiring with an amperometric transducer. The benefits of such an approach are not obvious because of the losses in the long-term stability of the enzyme. Reconstructed oxdioreductases obtained from apoenzymes and cofactors that can be preliminarily modified or immobilized on appropriate supports also offer additional possibilities of improving the sensitivity of the response.
- *Enzyme sensors in integrated assay platforms.* The investigations in the enzyme sensors are stimulated by the efforts in the development of integrated measurement systems (microfluidics, biochips, lab-on-chip concepts). The detection of the specific enzyme activity is required in ELISA similar formats of the immunoassay and the DNA sensors with enzyme labels. This is also one of the most intensively investigated classes of the biomarkers of various human pathologies.

Immunosensors. Contrary to the enzyme sensors that have exhausted the initial extensive way of development, immunosensors are still on this path. A significant increase in the number of the *Ab*-related recognition elements (peptide receptors, Fab fragments) as well as their modification by various methods is expected. The new targets for immunoassay arise from the modern investigations of the regulation processes. The contribution of regulatory proteins, both known and discovered in the investigation of the immune response and DNA functioning, can be expected in the very near future. As for the enzymes, genetic manipulation will be directed at the improvement of biosensor assembling and specificity of the Ab–Ag interaction. However, traditional polyclonal Ab preparations will also be applied in an increased scale due to progress in the immunization protocols and the new tasks that appear, mostly in medicine. The intensive investigations of biomarkers offer new target analytes the detection of which will be established by immunoassay techniques.

The variety of analytes softens the requirements for the elaboration of new detection techniques so that most of the newly developed immunosensors will be based on conventional measurement principles slightly modified in accordance with the specific goals of the analysis (sample volume, efficiency of assay, regeneration of immunosensors, etc.). The miniaturization and automation of the immunoassay, especially in a traditional biomedical assay, will promote the substitution of the enzymatic assay, e.g., in the assay of regulatory enzymes and their substrates. New detection formats are rather possible for optic immunosensors

related to fluorescence spectroscopy. The fluorescence resonant energy transfer (FRET) between two chromophore groups in the bioreceptor molecules enhanced or quenched due to biospecific interaction increases the sensitivity of the immunoassay to the detection of several molecules (Medintz et al. 2003). The concept has found numerous proofs in the investigation of various biomolecules. Its application for real targets is as yet limited by the high cost of the reagents and of the measurement equipment and complications of the design of appropriate conformation of active states that are able to FRET. Together with the extra-high background signal typical for many fluorescent assay techniques, this limits the use of the FRET to selected systems isolated from the cell. Anyway, the progress in this area leaves enough room for optimism.

DNA sensors. The number of publications on the development of DNA sensors now exceeds those on other kinds of biosensors. Their success for the detection of hybridization is indisputable and stimulates the development of commercialization products that are, or can appear on the market in the very near future. Together with diagnostics purposes, such DNA sensors can solve other problems, including those not connected to medicine, but of interest for other human activities. For example, the detection of pork in minced meat and other meat products is important for people adhering to dietary limitations for ethnic or religious reasons (*kashrut, halal*), and a prompt identification of humans by their biological materials is necessary for crime investigation, etc.

Some other applications of DNA sensors overlap the importance of hybridization detection. First and foremost, human gene expression mapping is meant. This project is comparable to the Human Genome Project with its labor-intensiveness and importance. The fulfillment of the projects gives a genetic basis of the variation in gene expression. Technically it is based on DNA microarrays. Early studies of gene expression provided a detailed map of expressed genes in various tissues and diseases, and the great volume of gene expression data revealed that the expression levels of many genes differ among individuals. Identifying the normal variation in human gene expression stimulates a fruitful merger of human genetics and genomics. Progress in this area is closely related to the achievements of nanotechnology providing new transducers and manufacturing facilities providing denser positioning of the ODN sequences, and a much lower detection limit of target sequences.

An increasing interest in other DNA sensing elements, e.g., aptamers and DNAzymes, is maintained by unique recognition functions and possibilities of the detection of the DNA–protein interactions whose importance for the biochemical functions only begins to be valued (Willner et al. 2008). DNAzymes, or ribozymes, exert catalytic function and hence combine the properties of the biorecognition element and of the label. Contrary to common enzyme labels, they catalyze much more complicated changes in the three-dimensional structure of DNA that can find application for the self-assembling and self-regulation processes in the prototypes of biological computers and nanomachines. The molecular PCR nanoreactor, the DNA repair and molecular logic gates based on DNAzymes and their conjugates with aptamers are described. The following complication of the structures can

contribute to the development of nanorobots demanded in medicine for addressed drug delivery systems, tissue repair and other functions better known from science fiction.

Microbial sensors. The continuing progress in this field is mainly related to genetic manipulations and gene fusion. The modified microorganisms can be applied as living containers of uncommon enzymes that are additionally stabilized by natural protecting systems and self-reproduced if inactivated or damaged. Besides common areas of bioassay, the protein libraries can be obtained in a similar manner for use in protein chips and microfluidic systems for screening pharmaceuticals or metabolomics purposes.

Nanomaterials and biosensors. Most of the current publications devoted to biosensors are based on the use of the new nanosized particles or nanostructured materials. With the progress in their synthesis and investigation with probe microscopy, the variety of such materials stimulates their involvement in biosensor assembling and signal generation (Merkoçi 2010). The main focus is on *golden and silver nanoparticles*, alone or combined with hyper-branched polymers, carbon nanotubes, graphene, electropolymerized films, etc. Besides unique electrochemical and optical properties, the nanoparticles of noble metals are quite compatible with biocomponents that are easily immobilized on their surface via thiol groups. Simple synthesis and possibilities of careful control of size distribution make the metal nanoparticles ideal for microsensors and the automation of the sensor production. The combination of nanoparticles with a SERS response increases the sensitivity of the detection of biorecognition events, e.g., in DNA mapping and the development of DNA microarrays. *Magnetic beads*, which are now commercially available, provide a separation and concentration of the analytes or their complexes with the biocomponents of biosensors. They are used for the capsulation of biochemicals, from immobilized enzymes in the biosensors to the whole cells. This preserves their activity and vitality for a period sufficient for toxicity assay, gene expression control, and others. Such approaches offer new prospects for the development of biosensors based on whole cells and their use (Fakhrullin et al. 2012).

The interest in *carbon nanotubes* and, more recently, *grapheme* (Liu et al. 2012), is mainly related to miniaturization prospects and the development of non-silicon microelectronics. The carbon nanotubes are utilized as semiconductors and the structural elements of the transistors and logic gates. Like DNA, the structure of carbon nanotubes involves hydrophobic and hydrophilic domains' interaction with various analytes by electrostatic and hydrophobic interactions. They can serve as nano containers for the delivery of ultra-small amounts of low-molecular compounds to the target regions. The progress in the application of carbon nanotubes depends on the technologies of their purification and standardization especially based on the chirality that determines electroconductivity and has other purposes that are important for the operation of micro biosensors. Although the technological solutions have existed for two decades, they are too complicated and do not provide a sufficient scale for the production required for manufacture and biosensor design. The use of carbon nanotubes in "macro" biosensors has advantages related to the

higher surface density of biorecognition elements, which are less resistant to the charge transfer. Meanwhile, the electrocatalytic activity of this material seems overestimated and does not contribute to significant benefits over conventional mediator systems.

Quantum dots. (CdTe, CdSe) are nanometer-scale semiconductor crystals with enhanced luminescence capabilities that are advantageous for the development of novel chemical sensors and biosensors (Frasco and Chaniotakis 2009). Quantum dots typically have very broad continuous absorption spectra, at wavelengths extending from the ultraviolet to the visible, depending on particle size. The broad excitation and narrow size-tunable emission spectra (usually 20–40 nm full width at half maximum intensity), negligible photobleaching, and high photochemical stability are some of their remarkable properties. Although quantum dots are mostly used in optical biosensing devices, electrochemical sensors based on such nanomaterials as enzyme and ODN carriers are also described.

Lab-on-chips. Are integrated devices that combine the sensors with several laboratory functions (separation, concentration, purification of the samples prior to analysis, sensor regeneration, etc.). Lab-on-chips have dimensions of several millimeters and utilize microliter volumes of fluids per measurement. They belong to the MEMS family of devices and are often denoted as "micro total analysis systems (μTAS)" (Xu et al. 2009). The chips are mainly produced from sandwiched structures consisting of several thin plates made of plastic, ceramics or glass, with the channels and chambers providing the connections and cells for mixing, reactions and measurement operations. Micro osmotic and electrophoretic pumps are used for fluid flow and volume dosing. Most of the μTAS systems utilize optical signal detection, although enthalpiemetric and electrochemical devices are described. The development of the systems is directed both to the miniaturization system and simplification of the measurement protocol, which is fully automated and avoids the errors due to manual operations typical for conventional biomedical diagnostics devices. It should be mentioned that lab-on-chip development is a rather technological problem that utilizes known solutions in the area of nanotechnologies and biosensor design. Although most of the devices described are quite costly and have not yet found mass application, they are in demand and will certainly be applied to the growing scale after the solution of some problems limiting their dissemination in their present state.

In finalizing future prospects for biosensor development, it should be noted that many of the approaches mentioned or expected to be detailed in the very near future do not belong to the biosensor concept, in the purest sense of the term; they deal with the biochemicals that are either present in natural structural elements (biological membranes, cell organelles, etc.) or dissolved in liquids. Signal transduction assumes stages that require the time and effort comparable to, or even exceeding those of the readout stage. To some extent they are much closer to the conventional approaches of "wet" chemistry and therewith utilize sophisticated equipment. Nevertheless, integration tendencies and miniaturization trends provide hope for their future conversion to biosensor format and promise benefits that far exceed the present facilities of biosensors.

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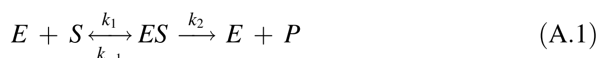
Appendix A
Enzyme Kinetics

As a catalyst, an enzyme accelerates the reaction of a substrate but does not affect equilibrium. The increase in the substrate concentration results in increasing the rate of the reaction estimated by the accumulation of the final product. Contrary to common chemical reactions, the rate of the enzymatic process reaches saturation, indicating a complicated mechanism of the conversion. Numerous investigations and discussions about possible mechanisms ended at the turn of the nineteenth and twentieth centuries when the German biochemist L. Michaelis and the Canadian physician M. Menten suggested the simplest empirical description of the dependence of the rate of an enzymatic reaction on the substrate concentration for invertase. Later on, in 1925, the integral form of the Eq. (A.1) was suggested (Keleti 1986).



$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P \qquad \text{(A.1)}$$

E, S and P denote free enzyme, substrate and product molecules, respectively. The k_i are the rate constants of the elemental steps of the reaction. The rate of the formation of the product P of reaction (A.1) is expressed by the Michaelis–Menten Eq. (A.2).

$$\upsilon = \frac{dc_P}{\mathrm{d}t} = \frac{\upsilon_{\max} c_S}{K_m + c_S} \qquad \text{(A.2)}$$

The reaction rate υ is commonly determined as a slope of a tangent line drawn to the kinetic curve c_S (c_P) $- t$ at the reaction beginning ($t = 0$) (Fig. A.1).

The Eq. (A.2) follows from the steady-state assumption where the rate of the ES complex formation is equal to its conversion, so that the concentration of the enzyme-substrate complex remains about constant and time-independent. For general reaction (A.1), rearrangement yields an expression (A.3) for Michaelis constant K_m (so-called Briggs-Haldane conditions).

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad \text{(A.3)}$$

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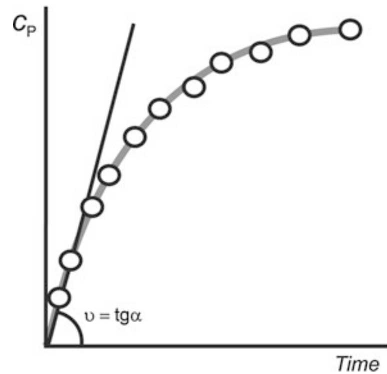

Fig. A.1 Kinetic curve of the accumulation of the product *P* of enzymatic reaction

If the total enzyme reaction is limited by the breakdown of the *ES* complex ($k_{-1} \gg k_2$, Michaelis–Menten conditions), the K_m is equal to the dissociation constant K_S of the enzyme-substrate complex. The K_m corresponds also to the substrate concentration giving the reaction rate equal to half of its maximal value ($\upsilon = 1/2\upsilon_{\max}$). In homogeneous conditions, K_m is an upper limit of the substrate concentration that can be determined from the linear piece of calibration curve in υ–C_S plots.

The Eq. (A.2) can be simplified by the assumption of $K_m \gg c_S$. The rate of an enzymatic reaction linearly depends on the substrate concentration in the range of its low values (A.4).

$$\upsilon \approx \frac{\upsilon_{\max} c_S}{K_m} = \frac{\upsilon_{\max}}{K_m} c_S \tag{A.4}$$

This makes it possible to determine the substrate concentration by the rate of its conversion.

The Eq. (A.2) gives a hyperbolic shape of the reaction curve (Fig. A.2), which coincides well enough with most experimental data. The Eq. (A.2) is often transformed to obtain a linear dependence of the experimental parameters that simplify the calculation of the main kinetic parameters of the reaction.

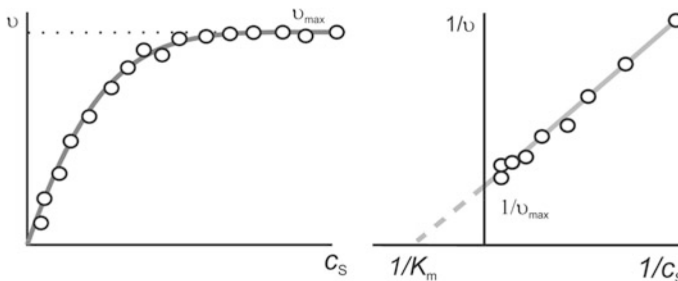

Fig. A.2 The graphs corresponding to the Michaelis–Menten kinetics and its graphical representation on a double reciprocal plot

Table A.1 Linearization of the experimental data and determination of kinetic parameters of the enzyme reaction

Method	Linearization equation x–y	Slope	Axes intercepts	
			x-	y-
Lineweaver-Burk plot	$\frac{1}{c_S} - \frac{1}{\upsilon}$	$\frac{K_m}{\upsilon_{max}}$	$-\frac{1}{K_m}$	$\frac{1}{\upsilon_{max}}$
Eadie-Hofstee plot	$\frac{1}{\upsilon} - \frac{\upsilon}{c_S}$	$-\frac{1}{K_m}$	υ_{max}	$\frac{\upsilon_{max}}{K_m}$
Hanes-Wolf plot	$c_S - \frac{c_S}{\upsilon}$	$\frac{1}{\upsilon_{max}}$	$-K_m$	$\frac{K_m}{\upsilon_{max}}$

A double reciprocal plot (Line weaver-Burk plot) is most often used for both the graphical representation of enzyme kinetics and their quantification. Though rather popular, the Line weaver-Burk plot does not provide the most accurate estimation of the K_m and υ_{max} values because of the non-linear transformation of experimental data. Instead, some other approaches can be recommended, e.g., Eadie-Hofstee or Hanes-Wolf plots. In all these methods, the kinetic parameters are determined by the *x*- and *y*-intercepts and the slope of the linear piece of the curve. These values are presented in Table A.1.

In addition to steady-state kinetics, the K_m value can be derived from the progress curve indicating temporal changes of the substrate concentration. In this case, the Eq. (A.2) is transformed to (A.5), or, in an integral form, to (A.6).

$$\upsilon = -\frac{dc_S}{dt} = \frac{\upsilon_{max} c_S}{K_m + c_S} \tag{A.5}$$

$$K_m \ln\frac{c_S^0}{c_S^t} + \left(c_S^0 - c_S^t\right) = \upsilon_{max} t, \tag{A.6}$$

in which c_S^0 and c_S^t are the substrate concentration at the time points 0 (zero) and *t*, respectively. For graphical presentation, the Eq. (A.6) is rearranged into (A.7) (Taylor 2002).

$$\frac{1}{t}\ln\frac{c_S^0}{c_S^t} = \frac{\left(c_S^0 - c_S^t\right)}{K_m t} + \frac{\upsilon_{max}}{K_m}, \tag{A.7}$$

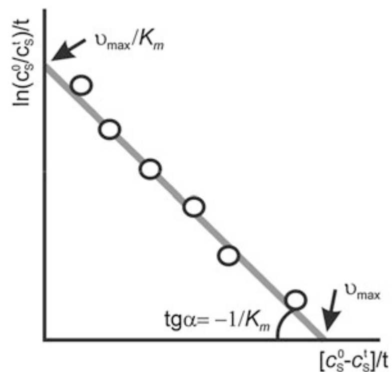

Fig. A.3 Determination of kinetic parameters υ_{max} and K_m from a progressive curve

The kinetic parameters of the reaction are determined from the straight line in the plots $t^{-1}\ln\left(c_S^0/c_S^t\right)$ versus $\left[c_S^0 - c_S^t\right]/t$ (Fig. A.3).

All the equations describing the Michaelis–Menten kinetics are based on the simplest scheme of the reaction with a single substrate and a single active site in the enzyme molecule that interact in the 1:1 ratio. Multi-substrate reactions, as well as allosteric enzymes, often exhibit a formally similar behavior, at least within a limited range of substrate concentration. This allows calculating the kinetic parameters called an effective (or experimental) Michaelis constant and maximal reaction rate. They can be expressed by a combination of the constant rates of elemental stages, which is more complicated than that in Eq. (A.3).

Appendix B
Inhibition Kinetics

Formal kinetics offers rather simple and reliable protocols for establishing the mechanism of inhibition. As other kinetic approaches, they are valid for a certain set of primary conditions (concentrations of a substrate, inhibitor and enzyme active sites). Changes in the conditions of the experiments as well as immobilization of an enzyme can shift the relative rate of the stages of the complex reaction scheme so that the criteria of the mechanisms would be not valid or contradict with the chemical backgrounds of the process. Nevertheless, kinetic analysis of inhibition is very useful to establish the limits of the inhibitor concentrations that are determined by appropriate enzymes in ideal conditions. The mechanism of inhibition determines the requirements of the optimal conditions of inhibition detection as the substrate concentration and necessity in intermediate incubation and/or washing immobilized enzyme preparations.

An *irreversible inhibition* can be monitored by the relative decay of enzyme activity. For the inhibitor concentration significantly exceeding that of the enzyme active site, the Aldridge Eq. (B.1) is used (Aldridge 1950).

$$\ln \frac{\upsilon_0}{\upsilon_t} = k_{\mathrm{II}} c_{\mathrm{I}} \tau \tag{B.1}$$

Here, υ_0 and υ_t are the rates of enzymatic reaction prior to and after the incubation step, c_I is the inhibitor concentration, and τ is the incubation time. The k_{II}, bimolecular inhibition constant, describes the efficiency of the enzyme-inhibitor interaction. This depends on the nature of the reactants and reaction conditions but not on the quantity of the enzyme and inhibitor as such. The bimolecular inhibition constant can be expressed by a combination of rate constants of various steps of the reaction.

Reversible inhibitors exert a great variety of interaction mechanisms, all of which refer to the true equilibria of the stages with enzyme participation (Keleti1986). For Michaelis-Menten conditions (one *ES* complex, 1:1 interaction of an enzyme active site and substrate molecule) the general scheme of reversible inhibition can be presented as follows (B.2):

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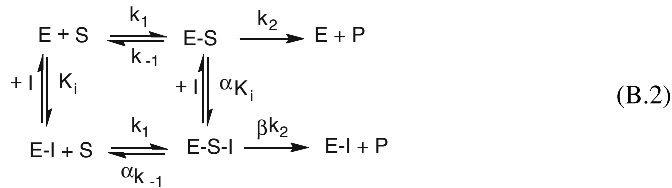


(B.2)

The scheme takes into account the formation of an enzyme-inhibitor complex and a triple enzyme-substrate-inhibitor *E-S-I* complex. Contrary to irreversible inhibition, such complexes retain their possibility of participating in the formation of the final product *P*. This implies that the inclusion of an inhibitor does not fully block the active site that can interact with a substrate even though the efficiency of such an interaction decreases. Empirical coefficients α and β account for the influence of an inhibitor on the target reaction of the substrate conversion. Here, capital letters K_I and αK_I correspond to equilibrium constants, whereas lower case letters k_1, k_{-1}, k_2, αk_{-1}, and βk_2 involve the rate constants of the appropriate stage of the reaction.

The scheme (B.2) is described by the Eq. (B.3). However, its application to real cases of inhibition is complicated by many parameters.

$$\frac{\upsilon_i}{\upsilon_0} = \frac{c_S(\alpha K_I + \beta c_I)}{c_S c_I + \alpha(K_I c_S + K_m c_I + K_I)} \tag{B.3}$$

The rate of enzymatic reaction in the absence and the presence of an inhibitor is denoted as υ_i and υ_0, respectively. The K_m value also corresponds to the zero concentration of the reversible inhibitor. Usually the $\alpha > 1$ and $\beta < 1$ values are typical for reversible inhibitors.

Although the reaction scheme (B.2) involves accurate development, some simplified cases are popular. They are classified in accordance with relation between various parameters and are called *competitive*, *non-competitive* and *uncompetitive* inhibition (Leskovac 2004).

For *competitive inhibition*, $\alpha \to \infty$. This results in the following reaction scheme (B.4).

(B.4)

The inhibitor and substrate both compete for the same active site of an enzyme and the enzyme-inhibitor complex cannot catalyze the substrate conversion. For such a reaction, the following equation can be given for the reaction rate (B.5):

$$\upsilon_\mathrm{i} = \frac{\upsilon_{\max} c_S}{K_m(1 + c_I/K_I) + c_S} \tag{B.5}$$

The comparison of the Eq. (B.5) with the Michaelis-Menten equation makes it possible to conclude that the presence of competitive inhibitors increases the experimental K_m value proportionally to the inhibitor concentration. Meanwhile, the formal expression of the dependencies remains the same in the competitive

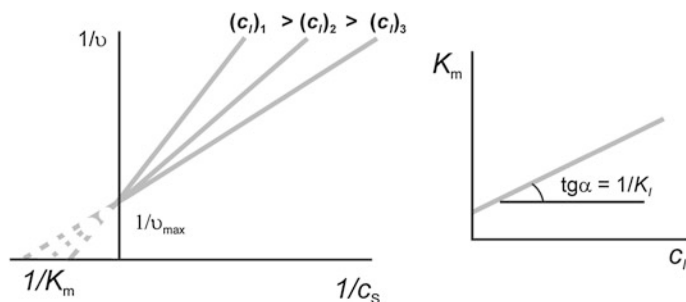


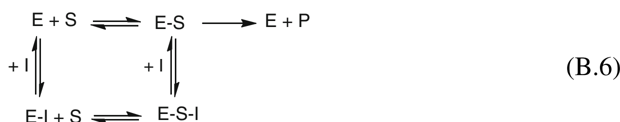
Fig. B.1 Kinetic description of competitive inhibition in Line weaver-Burk plot and graphical determination of K_I constant

inhibition and cannot be confirmed by the kinetics analysis. An example of appropriate linear curves is given in Fig. B.1.

The maximal rate of the reaction, $\upsilon_{\max}$, does not depend on the concentration of the competitive inhibitor. It corresponds to the saturation of enzyme-active sites with a substrate when all the inhibitor molecules are supplanted by a substrate.

It should be noted that competitive inhibition describes the interaction of a fully irreversible inhibitor with an enzyme active site in the presence of the substrate.

Non-competitive inhibition ($\alpha = 1$, $\beta = 0$) corresponds to the case where the substrate and inhibitor do not interfere with each other in enzyme binding, and the interaction with an inhibitor affects the affinity of an enzyme towards a substrate but not the reactivity of the *ES* complex. In this case, the general scheme (B.2) is reduced to (B.6).



(B.6)

From the theoretical point of view, there are three possible mechanisms of non-competitive inhibition:

- inhibitor and substrate binding sites of the enzyme are different;
- inhibitor and substrate bind to the same binding site but via different functional groups; or
- inhibitor does not bind with an active site of an enzyme but affects the protein confirmation. This changes the charge distribution and/or acid-base properties of functional groups involved in the ES complex formation.

In non-competitive inhibition, the K_m constant remains the same as in the absence of the inhibitor, but the maximal reaction rate decays proportionally to the inhibitor concentration (B.7).

$$\upsilon_{\mathrm{i}} = \frac{\upsilon_{\max}}{(1 + c_I/K_I')}\frac{c_S}{K_m + c_S} \tag{B.7}$$

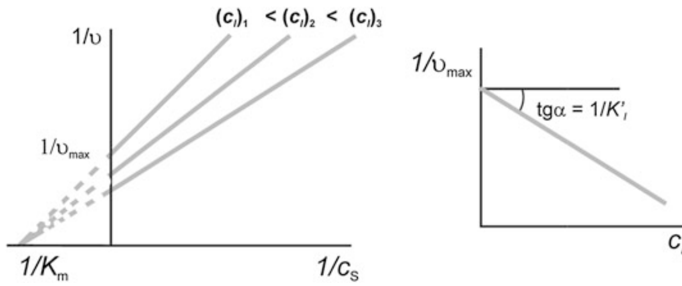
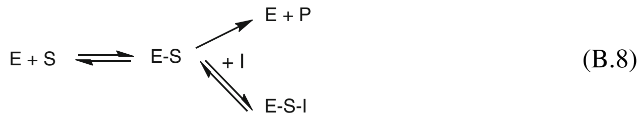


Fig. B.2 Kinetic description of non-competitive inhibition in Line weaver-Burk plot and graphical determination of K_I constant

The appropriate graphic illustrations of non-competitive inhibition are presented in Fig. B.2.

Uncompetitive inhibition ($\alpha = \beta$; $\alpha, \beta < 1$) assumes the interaction of an inhibitor with the *E–S* complex, but not with a free enzyme active site (B.8). In this case, both K_m and $\upsilon_{\max}$ experience a change, so that the curves in the Line weaver-Burk plot obtained for various inhibitor concentrations form parallel lines (Fig. B.3). The appropriate equation is given in (B.9).



(B.8)

$$\upsilon_{\mathrm{i}} = \frac{\upsilon_{\max}}{(1 + c_I/K_I'')} \frac{c_S}{K_m(1 + c_I/K_I'') + c_S} \tag{B.9}$$

The Eq. (B.9) can be used for the estimation of the inhibition constant and confirmation of the mechanism of uncompetitive inhibition. It can be shown that the $\upsilon_{\max}$ and K_m change proportionally and to a degree that depends on the inhibitor concentration and inhibition constant (B.10).

$$\frac{(\upsilon_{\max})_{\exp}}{(\upsilon_{\max})_{c_I=0}} = \frac{(K_m)_{\exp}}{(K_m)_{c_I=0}} = \frac{\alpha\left(K_I' + c_I\right)}{\alpha K_m + c_I} \tag{B.10}$$

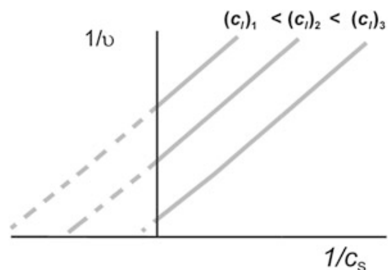


Fig. B.3 Kinetic description of uncompetitive inhibition in the Line weaver-Burk plot and the graphic determination of K_I constant

Mixed inhibition (α, $\beta \neq 1$) describes the dependence of the enzymatic activity on substrate/inhibition concentration as a superposition of the special cases described above. Thus, the changes in the reaction rate in many cases can be presented as a combination of competitive and non-competitive inhibition (B.11) with K_I and K'_I corresponding to the inhibition constants describing competitive and non-competitive inhibition, respectively. The total inhibition can be expressed in this case by the so-called reduced inhibition constant $\overline{K}_I$ (B.12).

$$\frac{1}{\upsilon_{\mathrm{i}}} = \frac{K_m}{\upsilon_{\max} c_S}\left(1 + \frac{c_I}{K_I}\right) + \frac{1}{\upsilon_{\max}}\left(1 + \frac{c_I}{K'_I}\right)$$
$$\frac{\upsilon_0}{\upsilon_{\mathrm{i}}} = \frac{K_m(1 + c_I/K_I) + c_S\left(1 + c_I/K'_I\right)}{K_m} \tag{B.11}$$

$$\overline{K}_I = \frac{K_I K'_I}{K_I + K'_I} \tag{B.12}$$

In all the mechanisms of reversible inhibition, the increase in the inhibition constant decreases the sensitivity of an enzyme towards an inhibitor. The value of a constant corresponds to the inhibition concentration resulting in a 50 % decrease of the rate of the enzymatic reaction. This rule makes it possible to compare the relative strength of inhibitors that react with an enzyme by different reaction paths. For the same reasons, other empirical estimates based on simplified experiments involve a reduced number of measurements. Thus for simple cases, I_{50} (concentration of an inhibitor yielding a 50 % decrease in the rate of enzymatic reaction) represents the K_I value. To some extent, this refers to other similar variables like I_{90}. The only, but serious, limitation of such empirical characteristics is that they do not provide any information on the possible mechanism of inhibition.

For "true" inhibition constants, the conclusions about possible mechanisms are made together with the inhibition constants' calculation from a series of experiments with varied concentrations of the substrate/inhibitor. As could be seen from Figs. B.1–B.3, the x- and y-intercepts, as well as line concurrence points, are of most importance. The appropriate examples are given for the Line weaver-Burk plot but can be easily extended to other linearization methods mentioned above (see Table B.1).

Table B.1 The dependence of reversible inhibition on the substrate/inhibitor concentration

Inhibition mechanism	Relative decay of the rate of enzymatic reaction
Purely competitive	$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{K_m}{K_I(K_m + c_S)} c_I$
Purely non-competitive	$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{c_I}{K'_I}$
Purely uncompetitive	$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{c_S}{\alpha K''_I(K_m + c_S)} c_I$
Mixed	$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{\alpha K_m + c_S}{\alpha \overline{K}_I(K_m + c_S)} c_I$

The description of the mechanisms of reversible inhibition presented here is to some extent the idealized presentation of much more complicated reactions. Some of the restrictions on the enzyme/substrate/inhibitor interactions specifying a particular reaction scheme can be non-obligatory. Thus, instead of the full inactivation of some intermediates, they can decrease their reactivity. For this reason, apart from the "pure", or "complete" mechanisms presented above, *partially* non-reversible, uncompetitive, etc., mechanisms are denoted. They are certainly more complicated in formal kinetics than those presented here but do not change the general criteria and empirical rules necessary for establishing the specificity of inhibitor action.

The relation between the K_m and c_S is the principal criterion of the substrate influence on the inhibition measurement. For example, competitive inhibition offers the reciprocal dependence of the inhibition degree and substrate concentration for $c_S \gg K_m$. For this case,

$$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{K_m}{K_I}\frac{c_I}{c_S} \tag{B.13}$$

so that the lower the substrate concentration, the lower the inhibitor concentration corresponding to the same inhibition degree. However, the opposite relation ($c_S \ll K_m$)

$$\frac{\upsilon_0 - \upsilon_i}{\upsilon_i} = \frac{c_I}{K_I} \tag{B.14}$$

makes the inhibition independent of the substrate concentration similarly to non-competitive inhibition and uncompetitive inhibition in the case of $c_S \gg K_m$.

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