Capillary Electrophoresis in Polymer Analysis

H. Engelhardt, O. Grosche

Institute of Instrumental and Environmental Analysis, University of the Saarland,
66123 Saarbrücken, Germany
e-mail: iaua@rz.uni-sb.de

Abstract. Capillary electrophoresis has demonstrated its enormous potential for the separation of biopolymers for several years. For the separation of proteins and carbohydrates, and to an even larger extent in DNA sequencing, new buffer systems and capillary coatings as well as derivatization agents have been developed. Recently, synthetic water-soluble polymers, for example, polyethylene glycols, or polyelectrolytes, such as polystyrenesulfonic acid or polyvinylypyridinium hydrochloride, have been successfully separated. This article introduces the principles of capillary electrophoresis, capillary gel electrophoresis, as well as giving an overview of the recent literature. Finally, it provides a strategy as to how to optimize an individual separation system.

Keywords. Capillary electrophoresis, Gel electrophoresis, Polystyrenesulfonate, Biopolymer, DNA

Advances in Polymer Science, Vol. 150

List of Abbreviations

η	dynamic viscosity
ζ	zeta potential
ε	dielectric constant
ξ_b	blob size for the entangled polymer solution
μ	electrophoretic mobility; mobility in free solution
μ_1	mobility of the closest solute
μ_{EOF}	mobility of the electroosmotic flow
a	Mark-Houwink constant
b	peak width in half height
c	gel or polymer concentration
c^*	entanglement concentration of the polymer solution
D	diffusion coefficient
e	elementary charge
K	Mark Houwink constant
K_0, K_{rep}	constants
K_r	retardation coefficien
L_{eff}	effective length of the capillary
L_{tot}	total length of the capillary
M, M_w	molecular weight
N	number of theoretical plates
N_P	molecular size of the solute
r	radius of the solvated molecule
R	resolution
R_g	radius of gyration
r_s	thickness of the polymer strand of the buffer additive
T	temperature
t, t_m	migration time
U	applied Voltage
u	electrophoretic velocity
z	effective charge of the solute

1 Introduction

Capillary electrophoresis (CE), also designated with the acronym HPCE for High Performance Capillary Electrophoresis, unites the classical separation technique of electrophoresis on plates with the instrumental methods of chromatography with respect to direct detection of the solutes separated in the capillary and their ready identification and quantification [1–3]. As a rapid and reliable separation system it is available for ionic compounds from the smallest cation (the lithium ion) up to polyanions with molecular weights ranging in the millions, such as DNA molecules. The concept of capillary electrophoresis encompasses various separation techniques. In capillary zone electrophoresis (CZE) the capillary is filled only with an appropriate electrolyte, and the separation is based solely on the mobility differences of the solutes. The method resembles elution chromatography in that the zones migrate at different velocities through the separation system and, in the optimum case, are separated from each other by buffer electrolyte. Always superimposed onto the electrophoretic migration is a more or less strong electroosmotic flow (EOF) that contributes passively to the transport of the solutes zones, but not to their separation. This EOF depends strongly on the pH value of the buffer and the surface properties of the capillary.

Due to the dissociation of the surface silanol groups in most buffers, negative charges exist on the surface of the fused silica (FS) capillary. They induce a positive counterion layer in the solution adjacent to the wall. As a result, the EOF

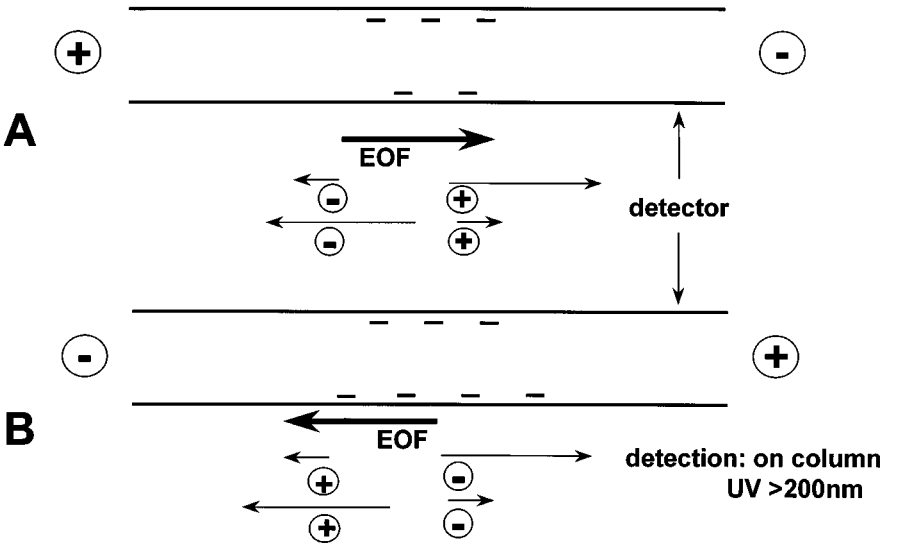

Fig. 1. Separation scheme for capillary electrophoresis

moves in the direction of the cathode. Therefore, the detector is located in the vicinity of the cathode compartment in the usual instrumental configuration. The EOF can be so large that not only neutral molecules, but even negative ions, can be transported to the detector in an opposite direction to their electrophoretic mobility. A schematic presentation of the system with the indication of the EOF direction is given in Fig. 1.

Biopolymers as well as synthetic polymers, as polyanions or polycations, exist with the same surface/charge ratio and migrate with almost identical velocity and cannot be separated in a normal electric field without an auxiliary aid. Since these polymers differ greatly in size, however, a gel can be used to influence the mobility of a large polymer more than that of a smaller one. In capillary gel electrophoresis (CGE), the electrophoretic migration of macromolecules is hindered by the gel matrix. The transport of the solutes through the capillary is based on the charge of the macromolecules, but the separation is dependent on the molecular size. It is easy to understand that with this technique EOF should be eliminated because otherwise the gel is extruded from the capillary.

2
Mobility and Diffusion

2.1
Electrophoretic Migration

In an electrical field ions move at constant velocity. The velocity u increases with the potential U and consequently the field strength E. The electrophoretic mobility μ relates the velocity and the field strength as:

$$u = \mu \cdot E = \frac{L_{eff}}{t} \tag{1}$$

The velocity of the ion is determined by dividing the traversed path length (capillary length from inlet to detector) by the migration time of the zone t. It should be pointed out that the field decreases over the total capillary length L_{tot} between the two buffer vials, whereas the solutes traverse only the effective capillary length L_{eff} during the migration time. The migration mobility can be determined by the following equation:

$$\mu = \frac{L_{eff}}{t \cdot E} = \frac{L_{eff} \cdot L_{tot}}{t \cdot U} \tag{2}$$

The mobility is determined by the equilibrium of the driving electrical force and the frictional force approximated by Stokes' law:

$$\mu = \frac{z \cdot e}{6 \cdot \pi \cdot \eta \cdot r} \tag{3}$$

where z is the effective charge of the solute ion, e the elementary charge, η the dynamic viscosity of the electrolyte, and r the radius of the solvated molecule. The effective charge of a solute ion is the charge of that ion minus the fractional charge of the surrounding oppositely charged ions (in the rigid double layer model). During migration the ion drags this portion of the double layer with it and therefore migrates more slowly than would correspond to its actual charge. This is called the electrophoretic effect and is largest for thin, diffuse double layers surrounding the ion. This characteristic double layer can be calculated from the Debye–Hückel theory and is inversely proportional to the square root of the electrolyte concentration. It can be shown experimentally that the effective charge and therefore the migration velocity decreases with increasing ionic strength.

For large molecules of similar composition and sizes larger than the double layer, the mobilities are independent of the size, which makes their separation by electrophoresis difficult. Thus the migration velocity of polyelectrolytes like DNA molecules and proteins denatured with sodium dodecyl sulfate (SDS) is almost identical in pure electrolytes. Separations are attained only if their migration is modified by exclusion or sieving effects.

2.2 Electroosmotic Flow

In CE, the electroosmotic flow is in most cases superimposed on the electrophoretic migration. The EOF depends on the distribution of charges in the proximity of the capillary surface. Nearly all surfaces carry a charge. These are negative charges from the dissociation of surface silanol groups in the case of FS capillaries. In solution these surface charges are counterbalanced by oppositely charged ions (counterions). If an electrical field is applied parallel to the surface, the field pulls the counterions from the mobile layer along its axis and thus moves the entire liquid in the capillary along with it. In FS capillaries with an enrichment of positive ions in the boundary layer, the EOF is induced to move to the cathode. An extremely flat (piston-shaped) flow profile is produced, which is the reason for the narrower peaks obtained in CE in comparison to the pressure-driven (hydrodynamic) flow in liquid chromatography (LC) where the Hagen–Poiseuille force is the reason for peak broadening.

The migration velocity u of the EOF can be described in simplified form by means of the Helmholtz equation:

$$u = \frac{\varepsilon \cdot E \cdot \zeta}{4\pi \cdot \eta} \tag{4}$$

The EOF is inversely proportional to the viscosity η of the electrolyte, proportional to its dielectric constant ε, the applied field strength E and the ζ-potential (zeta-potential). For FS capillaries, the EOF diminishes with increasing electrolyte concentration, and increases with the degree of dissociation of the surface

silanol groups, i. e. with the pH value. The addition of organic components to the electrolyte also has a significant influence on the magnitude of the EOF. However, no consistency in the behavior of the EOF was found on the addition of organic solvents, and no correlation with the buffer viscosity detected. In general, the addition of methanol leads to a decrease in the EOF, whereas with acetonitrile in the buffer an increase is observed. Tetrahydrofuran has little effect on the EOF, whereas isopropanol produces a drastic reduction of the EOF.

EOF appears in all electrophoretic separation modes because surface charges cannot be completely eliminated. On the one hand, it can lead to convective mixing of the electrophoretic zones but, on the other, it also plays an essential and decisive role in the transport of the zones through the capillary. The detector in CE is usually placed on the cathode side because of the ever present EOF. Cations move with the EOF (comigration), resulting in very short analysis times for this group of components. Even anions that migrate opposite to the direction of the EOF (countermigration) are transported to the detector (on the cathode site) if their migration velocity is lower than the velocity of the EOF. Only the anions that migrate faster than the flow velocity of the EOF migrate into the anode compartment and escape detection.

Through chemical modification of the capillary surface the EOF can be controlled, nullified, or even reversed. When working with gel-filled capillaries, the EOF should be completely suppressed, otherwise the gels will be flushed out of the capillary. By modifying the capillary surface, interactions between the sample components and the wall can be suppressed. Sorption effects of sample components (especially of positively charged solutes) at the walls always degrade the efficiency (resistance to mass transfer) and, in particular in the separation of proteins, lead to a significant reduction in the plate numbers and/or loss of sample through irreversible adsorption.

2.3 Modification of Capillary Surfaces

By adding long-chain cationic detergents, such as cetyltrimethylammonium salts, to the buffer the EOF can be reversed. By adsorption on the surface silanols a double layer is formed, with positive charges directed toward the electrolyte. Polymers like Polybren™, a polyamine with quaternary groups, or lower polyamines like spermine can, in part, be adsorbed so strongly that they are not flushed out during a buffer change. Their application is limited to neutral or medium-acidic pH values. These modifications, as well as the adsorption of surfactants, are termed "dynamic coatings". They reduce or reverse the EOF and the irreversible adsorption of positively charged biopolymers, e. g. proteins.

The EOF can also be controlled by chemical modification of the capillary surface. Various options initially developed for modification of silica in high performance liquid chromatography (HPLC) and for coating capillaries in gas chromatography (GC) are available for the chemical coating of capillaries. The surface silanols at the capillary wall can be reacted with silanes introducing anchor

groups containing olefinic groups such as γ-methacryloxypropyltrimethoxysilane which can be copolymerized in a second step with, for example, acrylamide in aqueous solution [4,5]. These coatings with linear polyacrylamide completely suppress the EOF and are stable in the pH-range 2.5–8. Their instability at high pH values becomes noticeable by a reappearance of the EOF and fluctuations in migration times.

On account of their simple preparation and commercial availability there is a wide variety of applications for linear polyacrylamide coatings. These involve not only the separation of proteins but also include biomolecules such as DNA fragments. They are also used for the preparation of gel-filled capillaries.

Higher hydrolytic stability is exhibited by capillaries based on vinyl coatings as primary anchor group, where, in the second step, vinyl acetate is polymerized and subsequently hydrolyzed with sodium methylate in methanol [6]. Such capillaries are stable up to a pH value of 10. Polyvinyl alcohol has also been thermally immobilized onto the capillary surface [7]. With such stable layers in the acidic and neutral region no measurable EOF is present and the stability of the capillaries is very good.

Many types of adsorptive and permanent coatings have been described in the literature. In this review only well described and/or commercially available coated capillaries have been included. Table 1 summarizes the possibilities of modifying the EOF.

Table 1. Possibilities to modify the EOF

Change in separation system	Effect on EOF	Comments
pH value of buffer	EOF increases with pH	May effect charges of analytes
Buffer concentration	EOF increases with decreasing concentration	High concentrations lead to high currents, low ones to overloading
Temperature	Viscosity changes	Selectivity may be affected
Organic solvents	Affect EOF and buffer viscosity	Complex changes in separation system
Surfactants as buffer additives	Drastic changes of EOF through adsorption on wall	Anionic surfactants increase EOF; cationic reduce or reverse it
Ionic polymers	Adsorption on capillary wall	With positively charged polymers, flow reversal
Neutral polymers	Adsorption on capillary wall	Reduction of EOF and solute adsorption
Covalently bonded coatings	Affect EOF; reduce wall adsorption	Stability problems
Additional radial electric field	Change in EOF	Limited applicability

2.4 Band Broadening

The well-known parameters of chromatography, such as the number of theoretical plates, N, have been adapted to describe the zone dispersion in CE. Analogous to chromatography, the plate numbers are calculated from the migration time t and peak width b in half height:

$$N = 5.54 \cdot \left(\frac{t}{b} \right)^2 \tag{5}$$

If all other causes of band broadening are neglected, the plate number is directly proportional to the electric field E and inversely proportional to the diffusion coefficient D of the solute in the electrolyte. The relationship is given by:

$$N = \frac{\mu U}{2D} \tag{6}$$

Only the longitudinal diffusion term has to be considered. The main contribution to band broadening in chromatography stems from the restricted mass transfer in the mobile phase, which is unimportant in CE due to the plug-like flow profile. In contrast to LC the plate number increases with the decreasing diffusion coefficient of the solute. From this it can be deduced that CE is particularly suited for the separation of polymers because they predominately possess low diffusion coefficients which decrease with increasing molecular weight. It has been shown [8] that at room temperature and over a wide range of molar masses the equation for the plate number can be approximately reduced to:

$$N \cong 20 \cdot z \cdot U \tag{7}$$

where z is the effective charge of the solute in the buffer. With potentials up to 30,000 V and effective charges between 1 and 10, up to 10 million theoretical plates are attainable. These high plate numbers have actually been verified for DNA molecules in gel-filled capillaries. This demonstrates that CE is superior to HPLC in respect to possible separation efficiency. However, DNA molecules represent a special case as because of their large number of negative charges they do not interact with the capillary surface, and as elongated molecules they possess a very low diffusion coefficient in the gel matrix.

Other effects may also contribute to band broadening causing reduced achievable plate counts. Besides the already-mentioned wall adsorption, temperature effects (Joule heating) may reduce plate numbers. Sample application can have a strong influence on plate count, especially when large volumes and/or high sample concentrations are injected. Mobility differences between buffer constituents and analyte ions lead to asymmetric (triangular) peaks caused by electrodispersion, which is extremely noticeable with smaller molecules. Differ-

ences in the liquid levels of the buffer vials may introduce a hydrodynamic flow with the corresponding flow profile also resulting in reduced plate counts.

3 Principles of Optimization in Capillary Electrophoresis

Before discussing the separation of polyions, the principle optimization strategies in CZE will be discussed. During the entire separation the buffer, its pH value, and the field strength remain constant. Only solutes differing in mobility can be separated. The solutes are introduced in a mixture as a concrete zone at the beginning of the capillary and reach the detector as discrete zones. The buffer has the function of holding the pH constant and to assure the passage of the current. The buffer pH selected determines the charge of the analyte molecule and hence its direction of migration. The buffer concentration and its pH affect the EOF. The selectivity is optimized by choosing a suitable pH, and the analysis time depends strongly on the buffer concentration, the capillary length and the applied potential.

The resolution R in CZE can be determined from the following equation:

$$R = \frac{\sqrt{N}}{4} \cdot \frac{\Delta u}{\bar{u}} \tag{8}$$

The equation is similar to that used in chromatography [8] if the relative migration differences are inserted. These are proportional to the relative observed mobilities that include the contribution of the EOF:

$$\frac{\Delta u}{\bar{u}} = \frac{\mu_1 - \mu}{\mu + \mu_{EOF}} \tag{9}$$

It is evident that increasing the mobility simultaneously raises the EOF, and the relative velocity for each pair of zones is reduced along with the resolution. On the other hand, if the analyte migrates in an opposite direction to the EOF, the resolution increases because the residence time in the capillary is prolonged and the effective migration distance is lengthened, so that even components with very small differences in mobility can be separated from each other.

3.1 Influence of pH

The mobility of the ions is determined by their extent of dissociation in the carrier electrolyte and therefore by its pH. The largest migration differences are obtained when the buffer pH lies between the pK values of the sample components. The pH range between 2 and 12 can be exploited. At lower and higher pH values the current transport is overtaken by hydrogen or hydroxide ions, respectively. Due to their very high mobilities, only very low buffer concentrations can be

used in these ranges. The magnitude of the EOF in FS capillaries is, as mentioned above, also influenced by the pH. At pH values below 2, the EOF becomes negligible and may even be reversed due to protonation of the silanol groups.

3.2 Effect of Buffer Concentration

The buffer concentration selected should be high enough to maintain the pH constant during the analysis and to keep overloading effects to a minimum, while still permitting rapid analysis via the EOF but preventing the appearance of band broadening through thermal effects. The conductivity in the system should not be affected by sample introduction, therefore the buffer concentration should be higher than that of the ions in the sample solution. For the most frequently used capillaries with 75 μm internal diameter (i. d.), buffers of 10 to 50 mmol (mM) concentrations are most commonly employed.

Increasing the buffer concentration:

1. increases the current and consequently Joule heating,
2. increases the danger of band broadening by thermal convection,
3. decreases the EOF and, consequently,
4. increases analysis time,
5. decreases the danger of overloading phenomena,
6. decreases the danger of electrodispersion, and
7. decreases the danger of wall adsorption, e. g. with proteins.

3.3 Type of Buffer

As discussed above a certain buffer concentration is required to perform optimal analyses. The minimum ionic strength required determines the current and Joule heating. This effect can be measured as a deviation from Ohm's law. With organic buffers the conductivity is much smaller for a given ionic strength. Consequently organic zwitterionic buffers, or at least buffers with counterions of low mobility, should be preferred especially when long capillaries have to be used.

During the electrophoretic run, electrolysis takes place in the buffer vials, which are usually very small in modern instrumentation. A sufficiently large buffer capacity is required to achieve reproducible analyses because of the generated protons and hydroxide ions in the buffer compartments. The buffer is transported via the EOF into the capillary. Therefore, only high capacity buffers should be used and/or the buffer solutions in the vials should be frequently replenished.

4
Separation of Biopolymers

CE has found wide application in the analysis of proteins and in nucleic acid analysis in combination with the human genome project. DNA-analyzers based on CE have been introduced recently. Many biopolymers have identical electrophoretic mobilities because of the constant charge to size ratio. Consequently they cannot be separated in free solution. Some sort of sieving matrix has to be used. Generally, gels like cross-linked polyacrylamides, common in slab-gel electrophoresis, are also used in CE. One of the main disadvantages of these gels when prepared within the capillary is possible shrinkage during polymerization, air bubble formation (causing current disruption), drying out during injection, and degradation during use. These problems can be prevented when so-called "liquid" gels are used, consisting of more or less concentrated solutions of water-soluble polymers, which can be easily replaced in the capillaries. It was found that these "entangled polymer solutions" are comparable to cross-linked gels.

The theoretical treatment by CE of biopolymers has been established for nucleic acids, consequently in the following the fundamentals are described for this example.

4.1
Properties of Sieving Media

Initially, gels were prepared within the capillary by copolymerization of acrylamide with bisacrylamide [9] as in slab gel electrophoresis. The capillary wall was coated with an acrylate as described above to remove the EOF. Thus the gel could also be chemically fixed on the wall. Agarose which can be thermally mobilized has also been used [10]. With these gels the problem discussed above arose. Therefore liquid gels were introduced [11] after they had already been proposed for classical electrophoresis [12]. Some remarks on the properties of the sieving media will be given as the information found in the literature can be very confusing.

Generally, gels are somewhat intermediate between a solid and a liquid. Under deformation this shows elasticity, but it keeps a permanent memory of its form. Water and dilute solutions do not show any elasticity, they flow under pressure and are purely viscous (Newtonian liquids). In between both viscous-elastic fluids are located. These solutions have the property of a viscous solution and of a solid, depending on the forces applied and on the time scale. Under fast deformation they are elastic, keep a memory of their shape, but under slow deformation they behave like viscous solutions.

In dilute solutions of polymers the chains are hydrodynamically isolated from each other and the solutions behave as a liquid. When the concentration of the polymer in the solution is increased the polymer chains become entangled, forming a transient network of obstacles. This entanglement takes place above

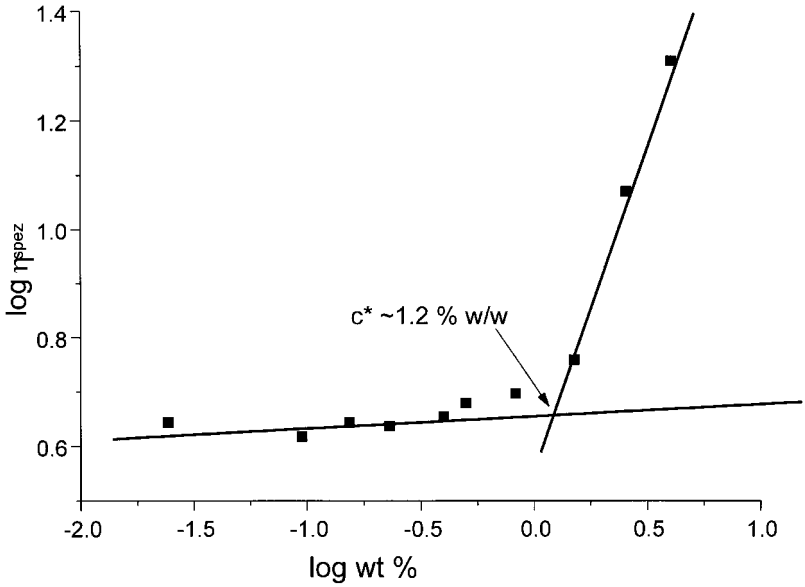

Fig. 2. Determination of the overlap threshold of dextran T 2000 as buffer additive in phosphate 50 mmol/l (pH 2.0) with the CE instrument. Conditions: capillary length 50–60 cm; pressure 1.5 psi

the overlap threshold c^*. It can be determined for each polymer experimentally by measuring the viscosity of the polymer solution at different concentrations. The point of deviation from linearity is defined as the overlap threshold [13]. It can be estimated experimentally in the CE instrument [14], as shown in Fig. 2.

The threshold concentration can also be calculated [15] from the Staudinger index or intrinsic viscosity $[\eta]$ according to Eq. (10):

$$c^* \cong 1.5\left[\eta\right]^{-1} \tag{10}$$

The intrinsic viscosity is a measure of the ability of a polymer to enhance the viscosity of a solution. It is related to the molecular weight of the polymer by the Mark–Houwink equation:

$$\left[\eta\right] \cong KM_w^a \tag{11}$$

where K and a are characteristic constants for a given polymer solvent combination. Characteristic values for some polymers used in CGE are summarized in Table 2 (Data taken from [16]).

For separations in CGE the "pore size" of such a polymer solution is interesting. The chain segment between two points of entanglement can be regarded as an independent subunit which can undergo random walk per se. The volume en-

Table 2. Mark–Houwink constants of some polymers used in CGE

Polymer	K (ml g^{-1})	a
Polyacrylamide	6.31×10^{-3}	0.80
Polydimethylacrylamide	2.32×10^{-2}	0.81
Polyethylene glycol	1.25×10^{-2}	0.78
Hydroxyethyl cellulose	9.53×10^{-3}	0.87
Methyl cellulose	0.316	0.55
Dextran	4.93×10^{-2}	0.60

closed by this chain segment is called a "blob". This "blob size" ξ_b should be used as "pore size" [15]. In DNA analysis, it is calculated by the following equation:

$$\xi_b = 1.43 R_g \left(\frac{c}{c^*} \right)^{-\frac{3}{4}} \tag{12}$$

It can be shown that the "pore size" of an entangled polymer solution does not depend on the degree of polymerization, only on its concentration. This means also that two solutions of the same type of polymer and with the same concentration but different molecular weights may have the same "pore size" as long as they are entangled. However, the viscosity of the two solutions is different because viscosity depends on the molecular weight. This has important consequences for the choice of the appropriate polymer solution. The pressure with CE instrumentation needed to displace the buffer solutions is limited, therefore the viscosity should not exceed a certain value. Explicit descriptions can be found in literature [16] for the selection of appropriate polymers and their concentrations for DNA analysis.

Above concentrations of 10% w/w one speaks of "concentrated solutions" whose properties are still poorly understood. They have little use in CE, partially because of their high viscosity.

4.2 Migration Theories for DNA Molecules in Capillary Gel Electrophoresis

DNA is a stiff polyelectrolyte. The collision of the migrating DNA and the separation media have different quantitative and even qualitative effects on DNAs of different size. Different conformations of the analytes exist during the separation, depending on the pore size of the gel and the length of the biopolymer. These conformations are ultimately responsible for the different mobilities and the apparent irregularities. The different conformations reproduced in Fig. 3 were observed by fluorescence microscopy [17,18].

The observed phenomena and anomalies lead to different migration models [19]. It is obvious that a compact or a relaxed conformation develops a different

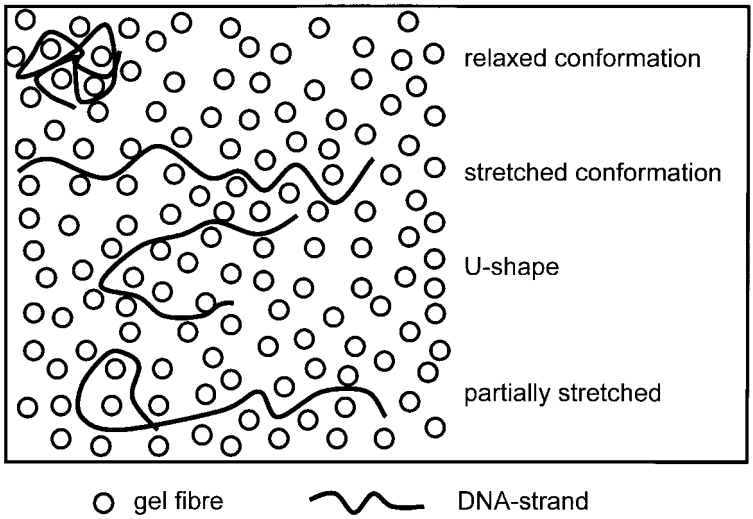

Fig. 3. Dependence of the mobility of a biopolymer on its conformation in a separation gel

mobility than, for example, an extended form. Separation is determined by the interaction of the analytes of increasing molecular weight with the polymer chains and the "pores" formed by them. In general, the mobility of an analyte in an entangled polymer solution is found to vary with respect to its size in the way depicted in Fig. 4. Four different regions can be distinguished in this curve. In the first part of the curve, the mobility changes very little with increasing analyte molecular weight or its length. The separation selectivity of the gel is very low for the smaller fragments. The "pore size" is much larger than the size of the molecule. The molecules can migrate through the gel without great resistance. A theoretical model describing the migration in this region has been developed by Ogston [20].

Using geometrical considerations, the mobility μ of a solute in the polymer solution is related to that in free solution μ_0 and the concentration c of the sieving polymer by:

$$\log\mu = \log\mu_0 - K_O(r_s + R_g)^2 c \tag{13}$$

The retardation coefficient K_r is a constant and depends on the thickness r_s of the polymer strand of the additive and the radius of gyration of the analyte:

$$K_r = K_O(r_s + R_g)^2 \tag{14}$$

It is important that the field intensity is low enough so that the DNA molecule retains a coiled conformation. Gels with narrower "pore size", i.e. larger cross-linking or higher polymer concentrations, must be selected for this separation

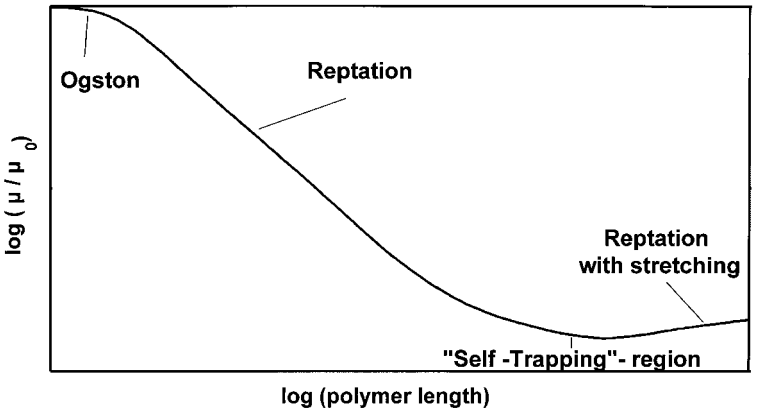

Fig. 4. Simulation of the log(analyte mobility) vs. log(analyte segment length) at a constant pore size of the separation matrix

region to improve the separation selectivity. The plot log μ vs. c, known as the Ferguson plot, should be a straight line with the slope of the retardation coefficient. The linearity of this plot is a necessity but not a condition to infer that the solute follows the Ogston model.

The greatest dependence of the analyte mobility on its chain length and therefore the greatest selectivity is found in the so-called reptation region. The size of the DNA molecule is much larger than the average pore size of the gel. It cannot fit into a single pore and must deform during its migration, especially if the field strength is not negligible. The analytes move through the gel via a snake-like movement (reptation). The largest interactions of the analyte with the gel matrix are possible, reflecting the maximum selectivity for separation according to chain length. The reduced electrophoretic mobility in this region is inversely proportional to the molecular weight.

In the reptation region the electrophoretic mobility depends on the solute's molecular size N_P and on the field strength E according to the biased reptation model [13]:

$$\mu = K_{rep}\left(\frac{1}{N_P} + b\left(\frac{E}{T}\right)\right)^2 \tag{15}$$

where K_{rep} is a constant and b is a function of the solute and the polymeric network.

It is evident from this equation that the mobility is inversely proportional only to the analyte chain length when the second term is negligible. This holds, however, only when relatively low field strengths (less than 200 V cm^{-1}) are used for CE. The relationship between mobility and the reciprocal of the chain length should become clearer at elevated temperatures. This effect can be seen in Eq. (15).

The higher the field strength and the lower the temperature of the separation system, the less dependent is the mobility on the reciprocal of the chain length.

For intermediate size DNA molecules a region can be observed where the mobility reaches a minimum. The mobility no longer depends on molecular weight in this abnormal migration area, where even a reversal ("band inversion") of migration may occur. One explanation for this phenomenon is that both ends of the DNA molecule migrate in the same direction and become strongly entangled with the polymer strands (*self*-trapping). The trapping cannot occur with small molecules as they can free themselves very quickly from this conformation. The probability of extremely large molecules reaching this status is very small. These effects remain the focus of scientific discussions [17] because they can also be explained assuming tertiary structures with DNA molecules. It has also been observed that the field strength, temperature, and gel concentration also exert strong influences on the expected migration sequence and on the mobilities. In the cases of inversion (larger molecules migrate faster than smaller ones) they can generally be eliminated by reducing the applied field strength, raising the temperature, decreasing the gel concentration, incorporating buffer additives such as ethidium bromide as DNA intercalates, and using pulsed electric fields.

Anomalous migration data where the mobility decreases very rapidly with molecular size have been described by an entropic trapping of the molecules within the gel matrix. This effect should be noticeable when the pore size of the polymer matrix corresponds to the radius of gyration of the analyte. However, the electric forces reduce the entropic effect; consequently it should contribute only at extremely low field strength. It does not seem relevant under normal CGE conditions, and cannot improve separations.

4.3 Separation of DNA

A typical separation of DNA restriction fragments is shown in Fig. 5 with a capillary filled with a linear polyacrylamide (LPA). The concentration of the acrylamide was 3% (3%T, 0% C in the standard nomenclature, where C is the crosslinker and T the monomer concentration). Since all fragments in the range between 34 base pairs and 622 base pairs are present in an equimolar ratio, the larger fragments have more absorption units in the molecule. As a result, the peak area increases with chain length. Deviations from this general trend are caused by insufficient separation or by inversion of migration order. The short fragments appear as small peaks and, because of their low resistance in the gel, they are first in the electropherogram. Between 600,000 and 1 million plates/m have been generated with this separation.

In Fig. 6 the dependence of the relative mobility vs. the number of base pairs (chain length) is given. It can be seen that the theoretical model discussed above is able to describe the experimental data. Temperature influence has already been discussed in the previous section.

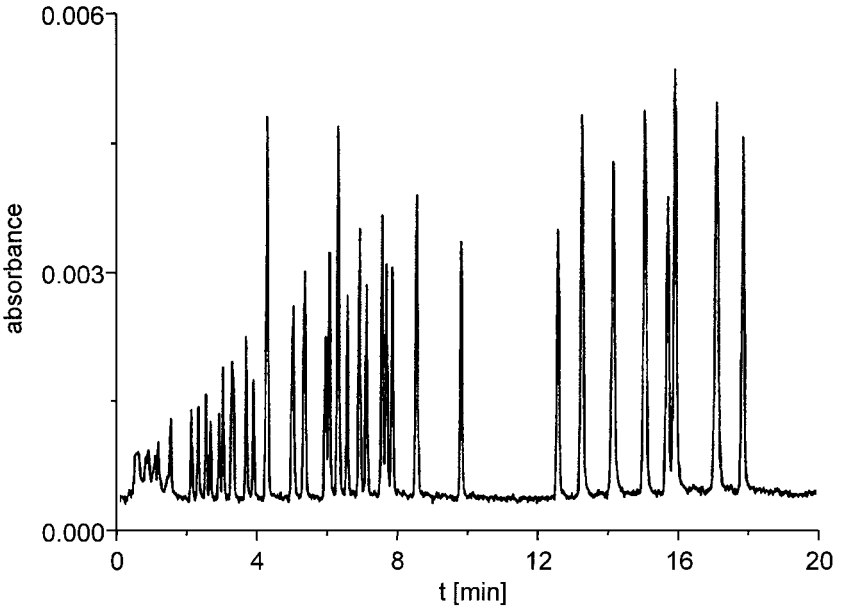

Fig.5. Separation of DNA restriction fragments in a capillary filled with linear polyacrylamide. Separation conditions: L=40–47 cm, E=150 V cm^{-1}, T=50 °C; buffer: 0.1 M TBE, pH 8.3 with 3% T, 0% C LPA; detection: 254 nm; sample: pBR 322 MSP I, pBR322 Hae III (peak assignment right to left: number of base pairs (bp): 622, 582, 540, 527, 504, 458, 434, 404, 309, 267, 242, 238, 234, 217, 213, 201, 192+190, 184, 180, 160+160, 147+147, 123+123+124, 110, 104, 89+90, 80, 76, 67, 64, 57, 51, 34+34)

Separation of DNA molecules is also possible in uncross-linked polymer solutions at concentrations well below the polymer overlap threshold [21]. Working with dilute solutions the molecular weight of the polymer is a very important factor for the efficiency of DNA separations with these systems. Low-molecular-weight hydroxyethyl cellulose (HEC) will provide good separations of small DNA fragments well below the threshold concentration. Increasing the concentration does not result in improved separation of longer fragments. However, a high-molecular-weight HEC in dilute solutions is advantageous for the separation of these molecules.

In contrast, with entangled solutions the molecular weight of the sieving polymer is not an important factor in determining the "pore size" of the polymer network and should, therefore, not strongly influence the DNA separation. Once the overlap threshold has been passed, the pore size of the network should only depend on the total polymer concentration. As the viscosity of the polymer solution depends strongly on the polymer molecular weight, it seems to be advantageous to select a polymer which is large enough to be well entangled at the concentration required for high resolution, but small enough to yield a manageable viscosity.

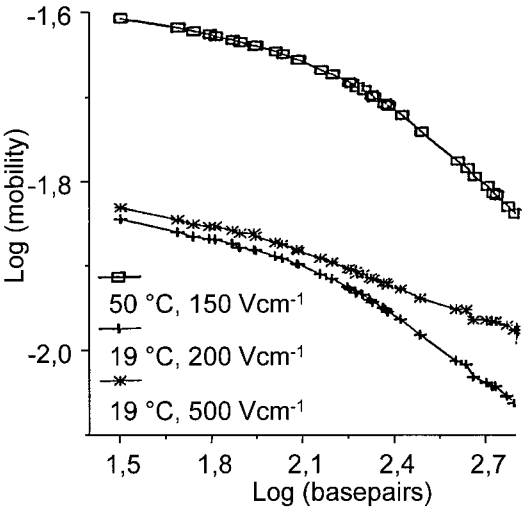

Fig. 6. Plot of log(mobility) of a mixture of DNA restriction fragments against log(number of base pairs). Separation conditions: L=40–47 cm; buffer: 0.1 M TBE, pH 8.3 with 3% T, 0% C LPA; various field strengths and temperatures

Recently, it has been shown [22] that the separation capability in DNA sequencing can be improved by using two different LPAs as sieving matrix. When a 2% solution of a high-molecular-mass LPA (MW 9 mio Da) was used, high efficiency could only be obtained in the 700 base pairs region (14 million plates/m), whereas with smaller fragments (49 base pairs) only low efficiency (3 million plates/m) and reproducibility could be achieved. In adding 0.5% of low-molecular-mass LPA to the electrolyte buffer, the separation efficiency was improved to determine short and long DNA fragments with equal efficiency.

4.4 Separation of Proteins

Proteins are mostly separated by CZE. Strong interactions between the analyte molecules and the capillary wall that are predominately electrostatic in nature have a strong influence on separation efficiency. By the use of buffer additives like amines or the use of dynamically or permanently coated capillaries, highly efficient separation of proteins in CZE is achievable. Here, the native proteins with their tertiary structure are separated. Denatured proteins as SDS complexes can be separated in gels. Advantageous are polysaccharide-based polymers, because they permit UV detection at low wavelength (214 nm), impossible with acrylamide-based gels. A separation of SDS-denatured protein standards in a dextran gel is shown in Fig. 7.

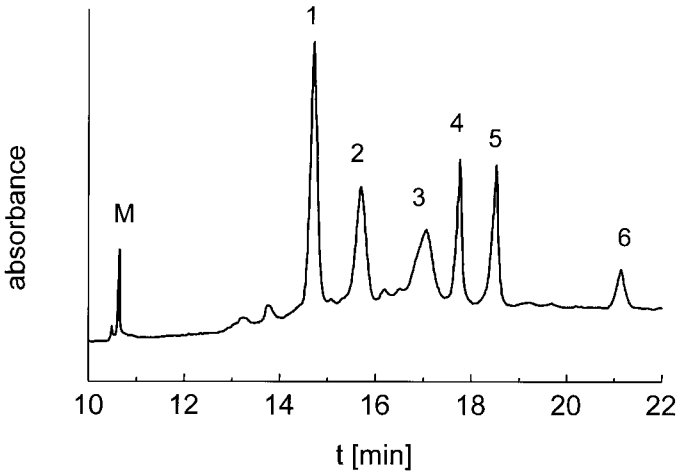

Fig. 7. Separation of SDS protein complexes with an exchangeable dextran polymer solution. Separation conditions: L=30–37 cm, E=300 V cm^{-1}, T=20 °C; (Beckman SDS protein kit); detection: 214 nm; analytes: Orange G (*M*), carboanhydrase (*1*), ovalbumin (*2*), bovine serum albumin (*3*), phosphorylase B (*4*), β-galactosidase (*5*), myosin (*6*)

5
Separation of Synthetic Polyelectrolytes

CE presents a great advantage over conventional chromatographic techniques in characterizing polyelectrolytes by operating in a single aqueous phase system thus excluding any unwanted interaction with a solid stationary phase. It allows investigation of the behavior of ionic polymers under conditions close to their area of application at high speed and high resolving power.

5.1
Polystryrenesulfonates

Polystryrenesulfonates (PSSs) can be analyzed easily by CE in plain silica capillaries, because they do not interact with the surface and are commercially available with low dispersities and different molecular masses. As early as 1992 Poli and Schure [23] showed the advantages of CE separation compared to conventional techniques like SEC. The behavior of PSSs in CE has also been studied by Minárik et al. [24], Cottet et al. [25] and Clos et al. [26].

Polystryrenesulfonates are already fully dissociated even at low pH values, where the EOF in unmodified fused silica capillaries is strongly suppressed. Consequently, it is possible to separate them in a counter electroosmotic way in free solution at pH values around or below 2.5. It is only possible to separate PSSs in free solution up to a molecular mass of 8000. Schure et al. [23] discussed wall adsorption because they could not separate PSSs at a pH value of 5. It seems

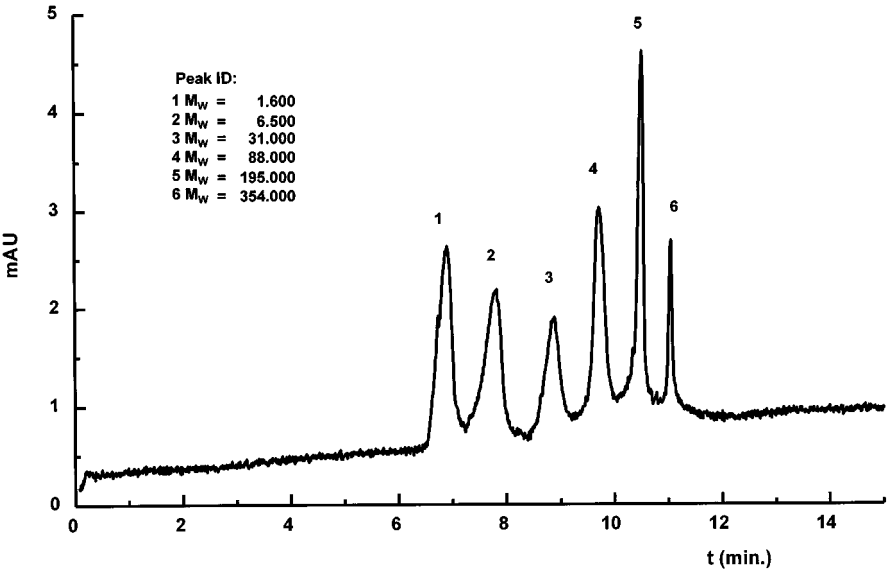

Fig. 8. Separation of polystyrole-4-sulfonates at pH 2.5 with dextran T 2000 as buffer additive. Separation conditions: fused silica L=50–57 cm, i.d.=75 μm; buffer 50 mM phosphate, pH 2.5+2% w/w dextran T 2000; E=–300 V cm^{-1}; injection: t=5 s, p=0.5 psi; detection: UV 254 nm

more likely that at this pH the EOF and the migration were balancing each other. Consequently, the overall mobility of the PSS was zero. Wall adsorption of negative PSS at the negatively charged capillary wall seems unlikely and has not been observed by others for PSS and for DNA molecules. Cottet [25] determined the mobilities of PSS in free solution with uncoated as well as with PEG (polyethylene glycol) coated capillaries and did not observe significant adsorption of the analytes at the wall of uncoated capillaries. Even at non-dissociated silanols (at pH 2.5) no wall adsorption was noticed [26].

When using sieving polymer solutions, it is more favorable to diminish the influence of the sieving media to the EOF (wall adsorption, changes in viscosity) with coated capillaries in order to avoid changes in migration times and, thus, separation efficiencies. Besides PEG coatings [25], polyacrylamide (PAA) coatings [24] and polyvinyl alcohol coatings [26] have been used. However, uncoated capillaries can also be used at low pH values. A separation of PSS standards at low pH values and with uncoated capillaries is shown in Fig. 8. The separation efficiency is highest in the molecular mass range below 100,000. The peak broadening of the higher-molecular-mass standards (e.g. 354,000) is extremely small compared with that of the standards with molecular mass below 100,000. This demonstrates that the sieving efficiency of the dextran used here is limited to the lower-molecular-mass range, where standard polydispersity is causing the broad peak observed.

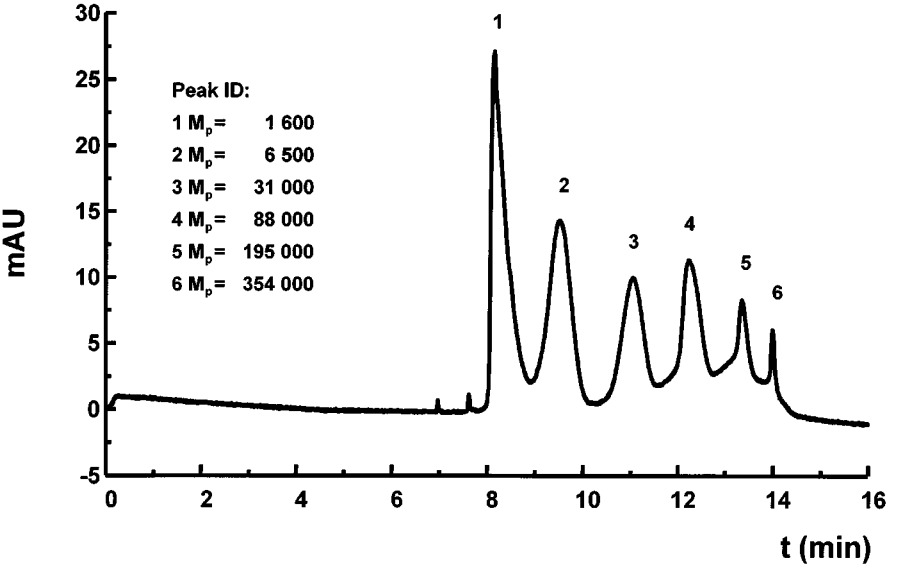

Fig. 9. Separation of polystyrole-4-sulfonates at pH 8.8 in a PVA-coated capillary with dextran T 2000 as buffer additive. Separation conditions: PVA capillary L=50–57 cm, i.d.=75 µm; buffer 60 mM AMPD/phosphoric acid, pH 8.8+2% w/w dextran T 2000; E=–300 V cm^{-1}; injection: t=20 s, p=0.5 psi; detection: UV 254 nm

Hydroxyethyl cellulose (HEC) was initially used as the sieving medium [23]. The differences in migration of PSS in HEC and PEG were studied [25]. The separation potential of PEG was inferior to that of HEC, and the PSS adsorption at the PEG coating increased with increasing PEG concentration in the buffer.

The problem with HEC solutions are their high viscosities. Therefore, dextrans differing in molecular mass have been used as sieving media [26]. These solutions can easily be replenished due to their low viscosities and exhibit good separation properties.

PSSs with molecular masses between 1 and 1000 kDa were studied by various authors. Their experimental results in references 23–25 are comparable to separations in dextran solutions. A separation of PSS standards with dextran (T 2000) in a coated capillary at pH 8.5 is shown in Fig. 9. There is no significant difference to the separation shown in Fig. 8. This demonstrates that uncoated capillaries can also be used if the polymer contains a strongly acidic group.

When plotting the log of the reduced mobility vs. the log of the molecular mass of the analyte the typical sigmoidal curves known from DNA analysis are also obtained. The concentration of the sieving polymer has to be beyond its entanglement threshold concentration c^*. A typical plot is shown in Fig. 10, where different concentrations of dextran T 2000 have been added to the running buffer. Also included are the reduced mobilities of the PSS in the plain buffer. As can be seen, the sieving properties are improved with increasing dextran concentra-

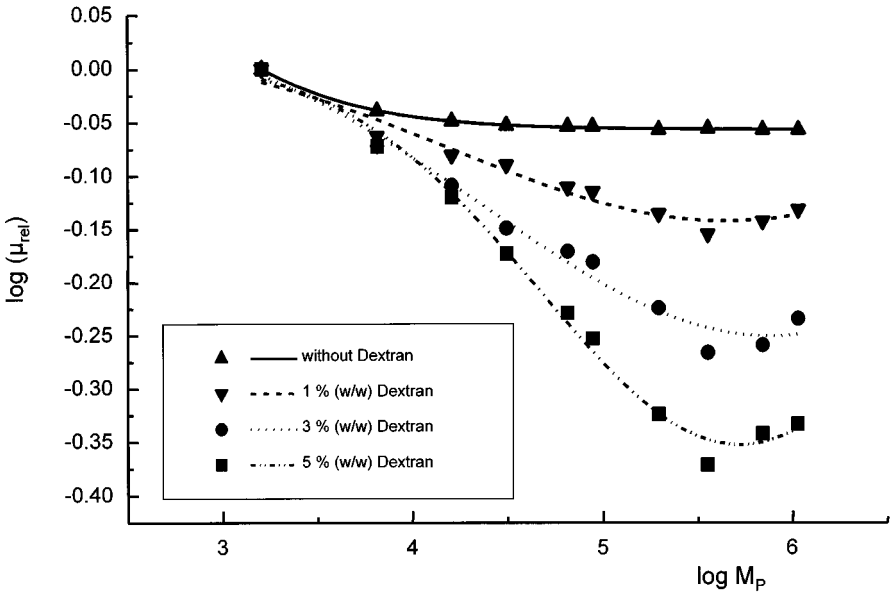

Fig. 10. Influence of the polymer additive concentration. Separation conditions: PVA capillary L=50–57 cm, i.d.=75 μm; buffer 60 mM AMPD/phosphoric acid, pH 8.8+2% w/w dextran T 2000; E=–300 V cm^{-1}; injection: t=20 s, p=0.5 psi; detection: UV 254 nm

tion, because the mesh size of the sieving media is decreased. The mass resolution improves in the reptation region.

The Ogston model can be applied at low molecular masses. According to Poli and Schure [25], this region extends up to molecular masses of 100,000. However, the region where the greatest mobility differences are observed belongs certainly to the reptation region. Here, the average mesh size in the polymer solution is smaller than the radius of gyration of the analyte. According to the discussions for DNA migration, the sigmoidal curve of the slope should be –1 at the point of inflection when a pure reptation mechanism is responsible for the separation. This, however, was not observed for PSS solutions. The slope depends on the sieving polymer concentration and its average weight and is smaller than –1. This deviation has been explained by an overlap of Ogston and reptation mechanisms [25]. The loss of resolution at high PSS molecular masses can be explained by increasing orientation of the sieving polymer and small relaxation times of the matrix pore structure caused by the size of the analytes.

It should always be kept in mind that the analysis time increases with increasing concentration of the sieving media. The regime where a separation is no longer possible seems to drift to lower molecular masses. This has been observed with PEG and dextrans (cf. Fig. 10).

As already discussed for DNA separation, the pore size of the sieving media should only be dependent on its concentration, and not on its molecular mass.

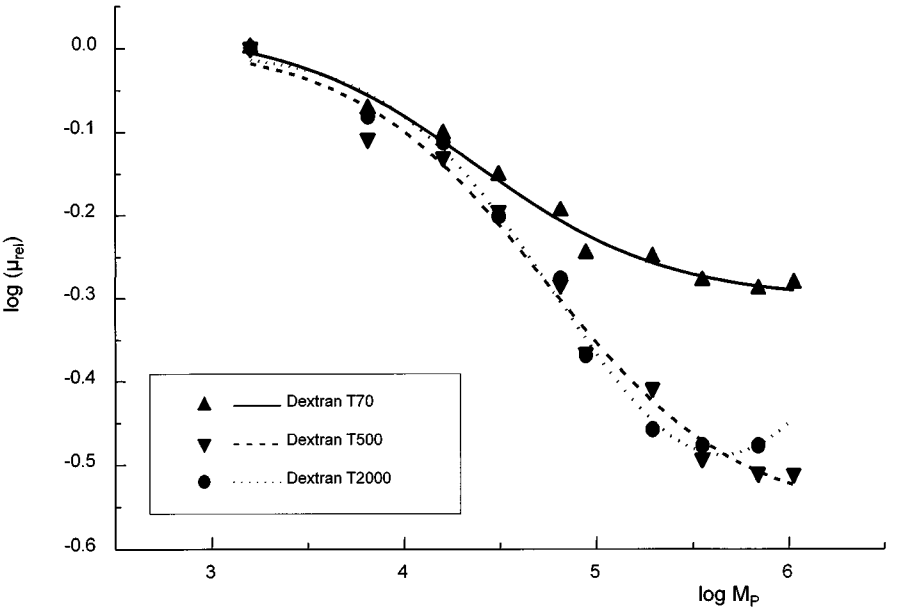

Fig. 11. Influence of the molecular weight of the polymer additive. Separation conditions: PVA capillary L=50–57 cm, i.d.=75 µm; buffer 60 mM AMPD/phosphoric acid, pH 8.8 +10% w/w dextran; E=–300 V cm^{-1}; injection: t=20 s, p=0.5 psi; detection: UV 254 nm

In Fig. 11 the influence of the molecular mass of the sieving medium is demonstrated. There is no difference between dextran T 500 and T 2000 at identical concentration. Less efficient separations are only observed with dextran T 70. The mass concentration here is just around the overlap threshold. For practical reasons it is advisable to use the sieving polymer which gives the lowest viscosity in the buffer solution at the required concentration.

The increasing rigidity of the pore structure with increasing molecular mass of the sieving polymer may be the reason for better separation efficiency in the case of the high-molecular-weight solutes [24]. The mesh structure of the sieving media with higher molecular masses may be deformed by the analyte to a lesser extent.

The selection of the sieving polymer follows to a great extent the rules applied in DNA separations. Studies have revealed [27] that the efficiency of the sieving polymer depends on its chain stiffness and its hydrophobicity. The higher the flexibility of the polymer and the smaller its gyration radius due to hydrophobic interactions, the higher the concentration in the buffer has to be in order to achieve comparable separation efficiencies.

When comparing different sieving polymers, it seems reasonable to keep the mesh size in the solution constant. As shown, beyond the threshold concentration the mesh size seems to be independent of the molecular mass of the polymer. Consequently, it is only important to compare the sieving properties as a

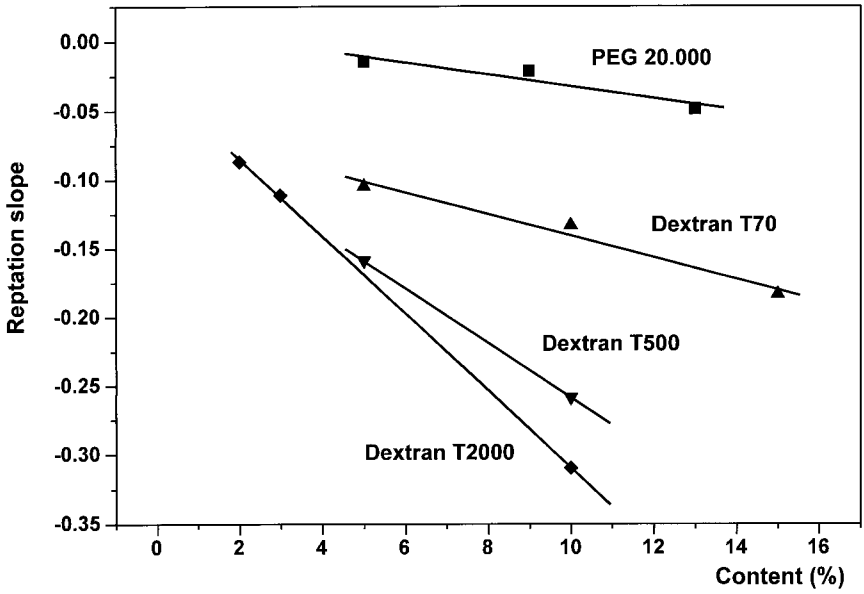

Fig. 12. Quantification of the sieving performance of a polymer solution. The slope at the point of inflection of the log μ/log M_w diagram is plotted vs. the polymer concentration

function of the concentration. This is shown in Fig. 12, where PEG 20,000 is compared with dextrans of different molecular masses. As a measure of efficiency the slope in the reptation region was taken [28]. From this it can be seen that the best sieving polymer in the separation of PSS is dextran T 2000. The comparison of sieving polymer solutions of identical viscosity [24] is only of practical relevance. HEC has good sieving properties in already very dilute solutions. The efficiency of a 0.3% solution corresponds almost to that of a 7% dextran T 2000 solution. However, it takes much longer to fill the separation capillary with this solution. The superiority of HEC over PEG at identical pore structure has been described [25]. It should be mentioned that in this case the PEG concentration was just below the threshold concentration.

Optimization of CE Conditions. As found for DNA separations, the separation efficiency decreases with increasing field strength. The selectivity seems to be optimal [24] at an extremely low field strength of 190 V cm^{-1}. It is feasible that the matrix becomes oriented at higher field strength, resulting in a displacement of the inversion range to lower molecular masses. Low field strength also means higher analysis time. Consequently, for each system, the optimum between analysis time and sufficient selectivity has to be found.

The mobilities of the PSS molecules decrease [25] with increasing ionic strength of the buffer. This can be explained by a shielding effect of the negative charges of the PSS by the higher concentration of the counterions. The reduction

of the intramolecular ionic repulsion forces by the increasing counterion concentration should lead to a reduction of the radius of gyration, resulting in a mobility increase. This effect does not seem to be important here.

The molecular size of the PSS depends on the type of the buffer counterion. The effective charge of the PSS decreases in the series $Li^+/Na^+/K^+/Cs^+$ due to the increasing affinity to the sulfo groups. The decrease in the effective charge leads to a decrease in the radius of gyration of the PSS due to a decrease in ionic repulsion. The consequent increase of the mobility has been observed with PSS with uncoated capillaries in the counter electroosmotic mode [25].

5.2 Separation of Cationic Polyelectrolytes

Cationic polyelectrolytes like Polybren are widely used in CE as buffer additives to reverse the EOF. They are strongly adsorbed at the negatively charged surface silanol groups. Only surface-coated fused silica capillaries can be used for their separation. The problems are similar to those discussed for protein separation.

With PVA-coated capillaries [6], it was possible to separate at pH 2.5 the polyelectrolyte 2-polyvinylpyridinium hydrochloride (2-PVP) in the molecular mass range between 1.5 and 1730 kDa with dextran as sieving matrix [26]. A separation of standards is depicted in Fig. 13. Comparing the efficiencies of the

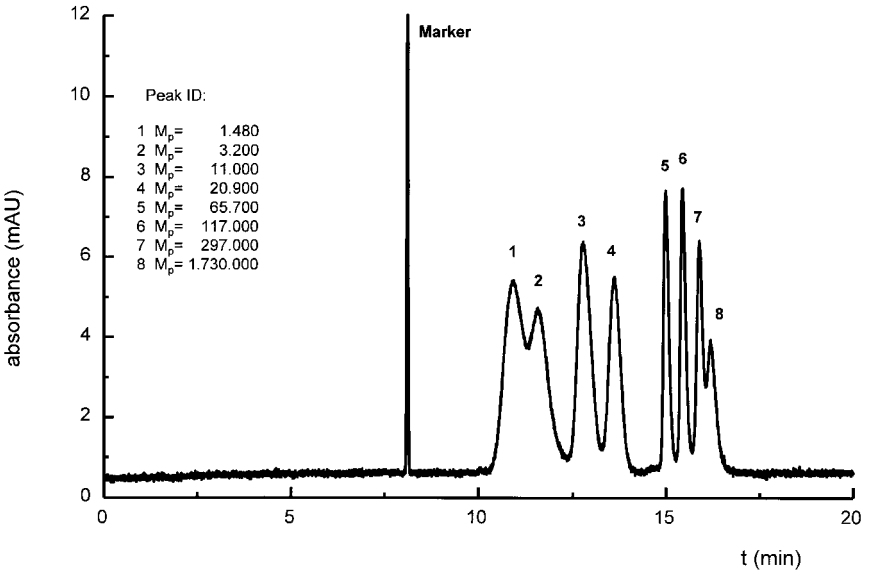

Fig. 13. Separation of polyvinylpyridinium hydrochloride at pH 2.5 in a PVA-coated capillary with dextran T 70 as buffer additive. Separation conditions: PVA capillary L=50–57 cm, i.d.=75 μm; buffer 50 mM phosphate, pH 2.5+5% w/w dextran T 70; E=–300 V cm^{-1}; injection: t=20 s, p=0.5 psi; detection: UV 254 nm

monomolecular marker *p*-aminopyridine with the broad peaks of the standards shows that the polydispersity of the standards is the cause of the observed broad peaks. Reptation dominates ranging from 20 up to 300 kDa. The slope in this range is also smaller than –1, demonstrating also a combined Ogston and reptation separation mechanism.

The hydrophobicity and hence the radius of gyration of PVP derivatives can be changed. Despite their increased molecular masses, the alkylated PVP derivatives migrate faster than the starting PVP. This can be explained by intramolecular hydrophobic interactions in the aqueous buffer resulting in a smaller radius of gyration. Also, the effective ionization can be different. It was possible to separate the ethyl and the benzyl derivative from the underivatized PVP with identical contour length.

5.3 Other Water-Soluble Polymers

Most of the water-soluble natural or synthetic polymers, like oligosaccharides or PEG, have neither a charge nor a chromophore. Because their separation in LC

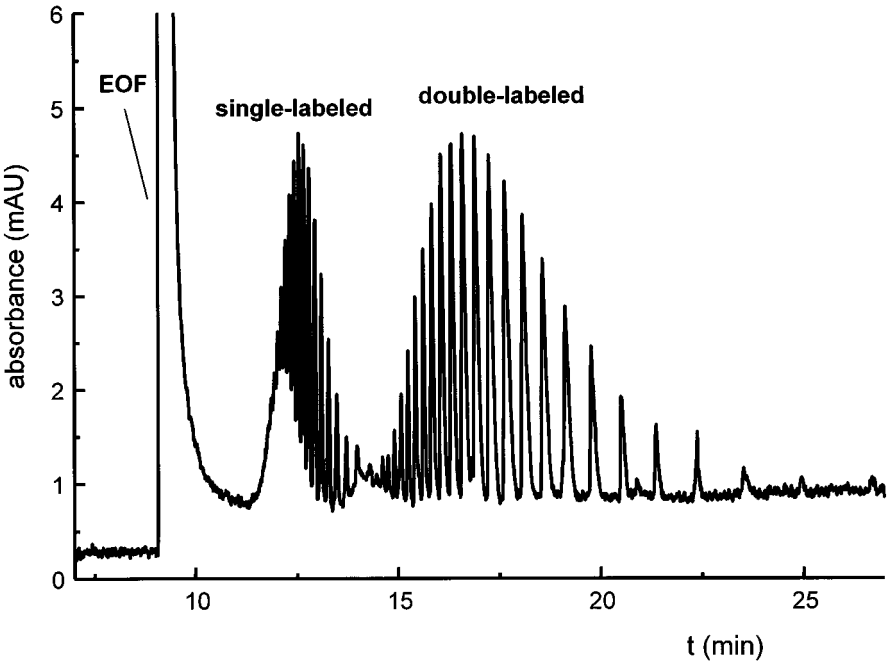

Fig. 14. Oligomeric distribution of PEG 1000 after derivatization with phthalic anhydride. Separation conditions: Fused silica capillary L=50–57 cm, i.d.=75 µm; buffer 110 mM Tris/boric acid, pH 8.3+5% in 20% v/v ethanol; E=526 V cm^{-1}; injection: t=10 s, p=0.5 psi; detection: UV 254 nm

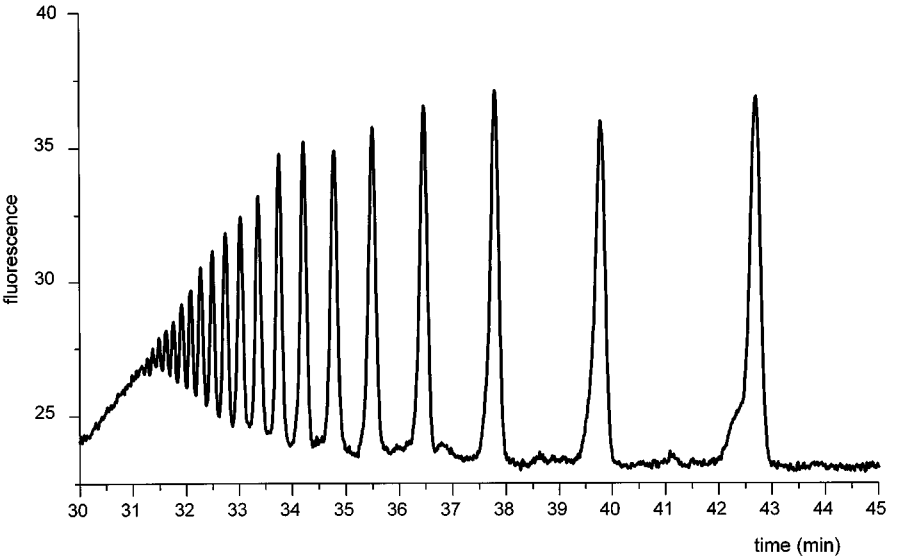

Fig. 15. CGE of a dextran ladder (derivatives with 2-aminoanthracene). Separation conditions: fused silica capillary L=106–120 cm, i.d.=50 μm; buffer 0.3 M borate, pH 10.5+3% w/w dextran T 10; E=250 V cm^{-1}; injection: t=10 s, p=0.5 psi; fluorescence detection: λ_{ex}=263 nm, λ_{em}=496 nm

imposes several problems, the use of CE for their characterization has also been described. Most of the separations in the literature are in the oligomeric range (molecular mass 10,000). For better detection and/or improved mobilities, various derivatization reagents have been applied.

In the separation of oligosaccharides, sulfonic acids (mono-, di- or tri-) of aminonaphthalene have been used [29]. Polyuronic acids like hyaluronic acid have also been characterized by CE [30].

Tensides based on derivatives of PEGs can also be separated by CE [31]. They may already contain charged groups, so that their migration is not problematic, only their detection. Here, indirect detection techniques with creatinine as background electrolyte have also been applied for samples up to molecular masses of 4000.

Phthalic acid anhydride reacts readily with alcoholic end groups of PEG. Besides an appropriate chromophore, charges are introduced simultaneously. Single- and double-labeled molecules are formed when two possible alcoholic end groups are available. The separation of derivatives of low molecular weight, such as PEG 1000, is shown in Fig. 14. Here, no sieving matrix was added. The homologues of the double labeled derivatives were completely separated [32].

Using sieving polymer solutions as buffer additives can enhance resolution [33,34]. However, the mass range of the solutes is far below that necessary for the high resolving reptation regime.

Polysaccharides can be derivatized by reductive amination at the reducing end group. With highly fluorescing labels like 2-aminoanthracene (2-AA), dextrans up to a molecular mass of 20 kDa can be visualized and hence analyzed by CE [35]. The separation of the AA derivative (only one chromophore introduced) of a synthetic dextran (4–6 kDa) is shown in Fig. 15. This opens new possibilities in the characterization of polysaccharides.

6 Conclusions

Synthetic polyelectrolytes can be separated by capillary electrophoresis applying the same rules derived for the electrophoresis of biopolymers. In the reptation regime, determination of the molecular mass and polydispersity of the polyelectrolytes is possible. Introduction of chromophores facilitates the detection of non-UV-absorbing polymers. Indirect detection techniques can probably be applied when analytes and chromophores of similar mobilities are available.

References

1. Li SFY (1992) Capillary electrophoresis. Elsevier, Amsterdam
2. Weinberger R (1993) Practical capillary electrophoresis. Academic Press, New York
3. Engelhardt H, Beck W, Schmitt T (1996) Capillary electrophoresis, methods and potentials. Vieweg, Braunschweig
4. Hjerten S (1985) J Chromatogr 347:191
5. Hjerten S, Ellenbring K, Kilar F, Liao J (1987) J Chromatogr 403:47
6. Engelhardt H, Cunat-Walter MA (1995) J Chromatogr 716:27
7. Gilges M, Kleemiß MH, Schomburg G (1994) Anal Chem 66:20
8. Giddings JC (1991) Unified separation science. Wiley, New York
9. Cohen AS, Karger BL (1987) J Chromatogr 397:409
10. Motsch SR, Kleemiß M, Schomburg GV HRC 14:629
11. Guttman A, Cohen AS, Heiger DN, Karger BL (1990) Anal Chem 62:137
12 Bode HJ (1977) Anal Biochem 83:204
13. Grossman BD, Soane DS (1991) Biopolymers 31:1221
14. Bello MS, Rezzonico R, Righetti PG (1994) J Chromatogr A 659:199
15. Viovy JL, Duke T (1993) Electrophoresis 14:322
16. Heller C (ed.) (1997) Analysis of nucleic acids by capillary electrophoresis. Vieweg, Braunschweig
17. Schwartz D, Koval M (1989) Nature 338:520
18. Carlsson C, Larsson A, Jonsson M (1997) In: Heller C (ed) Analysis of nucleic acids by capillary electrophoresis. Vieweg, Braunschweig
19. Slater GW (1997) In: Heller C (ed.) Analysis of nucleic acids by capillary electrophoresis. Vieweg, Braunschweig
20. Ogston AG (1958) Trans Faraday Soc 54:237
21. Barron AE, Soane DS, Blanch HW (1993) J Chromatogr 652:3
22. Salas-Solana O, Carrilho E, Kotler L, Miller AW, Götzinger W, Sisic Z, Karger BL (1998) Anal Chem 70:3996
23. Poli JB, Schure MR (1992) Anal Chem 64:896
24. Minárik M, Gas B, Kenndler E (1997) Electrophoresis 18:98
25. Cottet H, Gareil P (1997) J Chromatogr A 772:369
26. Clos H, Engelhardt H (1998) J Chromatogr A 802:149

27. Barron A, Sunada WM, Blanch HW (1996) Electrophoresis 17:744
28. Dolník V (1999) Capillary electrophoresis in sieving matrices: what governs the separation?. Poster Presentation at the HPCE'99 conference, Palm Springs, USA
29. Paulus A, Klockow A (1997) J Chromatogr A 772:369
30. Hong M, Sudor J, Stefansson M, Novotny M (1998) Anal Chem 70:568
31. Bullock J (1993) J Chromatogr A 645:169
32. Clos H (1998) Charakterisierung synthetischer ionischer und nichtionischer Polymere mittels Kapillarelektrophorese. PhD Thesis, University of the Saarland, Saarbrücken, Germany
33. Wallingford RA (1996) Anal Chem 68:2541
34. Barry JP, Radtke DR, Carton WJ, Anselmo RT, Evans JV (1998) J Chromatogr A 800:13
35. Grosche O (1998) Kapillarelektrophorese von polymeren Kohlenhydraten nach reduktiver Aminierung. Masters Thesis, University of the Saarland, Saarbrücken, Germany

Received: February 1999