POLYMER-PROTEIN BIOCONJUGATE MATERIALS

By

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CHAPTER I

INTRODUCTION

Bioconjugate materials in medicine

The term “biocongugate materials” refers to the hybrid materials composed of biological molecules, such as antibodies, nucleic acids, lipids, carbohydrates, chemically joined with synthetic molecules, such as drugs, toxins, or fluorophores. In this class of materials, polymer-protein conjugates particularly stand out due to a variety of applications in the modern science and technology.[1] For example, polypeptides immobilized on an inert solid support are used in sensors[2] and biocatalyst systems (immobilized enzymes).[3] In medicine, polymeric bioconjugates are studied as prospective systems for tissue engineering and controlled drug release.[4]

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physico-chemical factors to improve or replace biological functions. Bioconjugate materials in this area are utilized in the form of implantable scaffolds, where biodegradable polymers, such as poly(L-lactic acid), polyglycolic acid, polycaprolactone, or polyphosphazenes are functionalized with growth factor proteins for the restoration or regeneration of living tissues.[5,6] There are two strategies in application of the scaffolds: the closed system and the open system strategy. In a closed system, scaffolds are encapsulated using a semipermeable membrane that

protects cells from host immune response and allows nutrient transport and waste removal. In the open system, scaffolds are precultured *in vitro* before implantation. The required properties of scaffolds are biocompatibility, bioactivity, biodegradability and non-toxic nature of the products of degradation. Biocompatibility is support of cellular activity without inducing negative effect, such as tissue rejection.[7] Bioactivity is the ability of the scaffold to favor cell attachment, proliferation and differentiation.[8] Controlled biodegradability is an important factor as well, because the rate of degradation of scaffold should be consistent with the formation of new tissue. The use of biodegradable scaffolds alone with cells and growth factors has shown a success in regeneration of skin, blood vessels, bone and heart valves.[1]

In controlled drug release, metabolically degradable composition of the synthetic polymer covalently linked to a biologically active compound is employed. Slow decomposition of the conjugate enables the administration of the drug over an extended period of time, thus ensuring a constant concentration of the active ingredient in the bloodstream and the prolonged therapeutic effect. The controlled release mechanism also provides protection for the active molecules, especially peptide and nucleic acid based agents, from degradation and unwanted interaction. Protection is especially necessary if a medicine is administered orally, because the drug must pass a series of biological and physiological barriers before reaching its target.

Two basic mechanisms are implemented in controlled drug release approach. In the first mechanism, the slow metabolic degradation of the matrix polymer releases the active compound. Poly(L-lactic acid),[9] poly(ethylene oxide)-co-(lactic acid),[10] polyanhydrides[11] and polyphosphazenes[12] are used for this purpose. For example, poly(L-

lactic acid) and polyglycolic acid undergo hydrolysis under normal physiological conditions. Lactic and glycolic acids are released, and the results of such hydrolysis are normal products of human metabolism which do not possess an adverse toxicological effect. The half-life of degradation for poly[(lactic acid)-*co*-(glycolic acid)] (PLGA) normally ranges from 10 to 20 weeks, depending on composition of the copolymer.[13] Addition of the drug affects the degradation rate of the polymer matrix. Thus, PLGA copolymer loaded with 2 wt% of diazepam decomposed by 35% in 20 days, releasing the corresponding fraction of the active ingredient.[14]

The second mechanism of the controlled release involves cleavage of the linker between the matrix and the active molecule. The linker is usually an oligopeptide that is subjected to enzymatic degradation in the body. For example, Seliktar *et al.*[15] employed a protease-sensitive 8-peptide linker to attach the vascular growth factor to the poly(ethylene glycol) hydrogel in the preparation of a conjugate material aimed at blood vessel wall restoration. Leach and Schmidt[16] have demonstrated the use of hyaluronic acid as a linker between the polymer scaffold and the protein. Hyaluronic acid (or hyaluronan) is a natural disaccharide, distributed widely throughout connective, epithelial, and neural tissues. In both examples the linking molecules were slowly digested during metabolism. At the present time poly(ethylene glycol) is the most common material in bioconjugate systems because it is soluble in water and biologically inert.[16-21]

Functionalization methods in bioconjugate chemistry

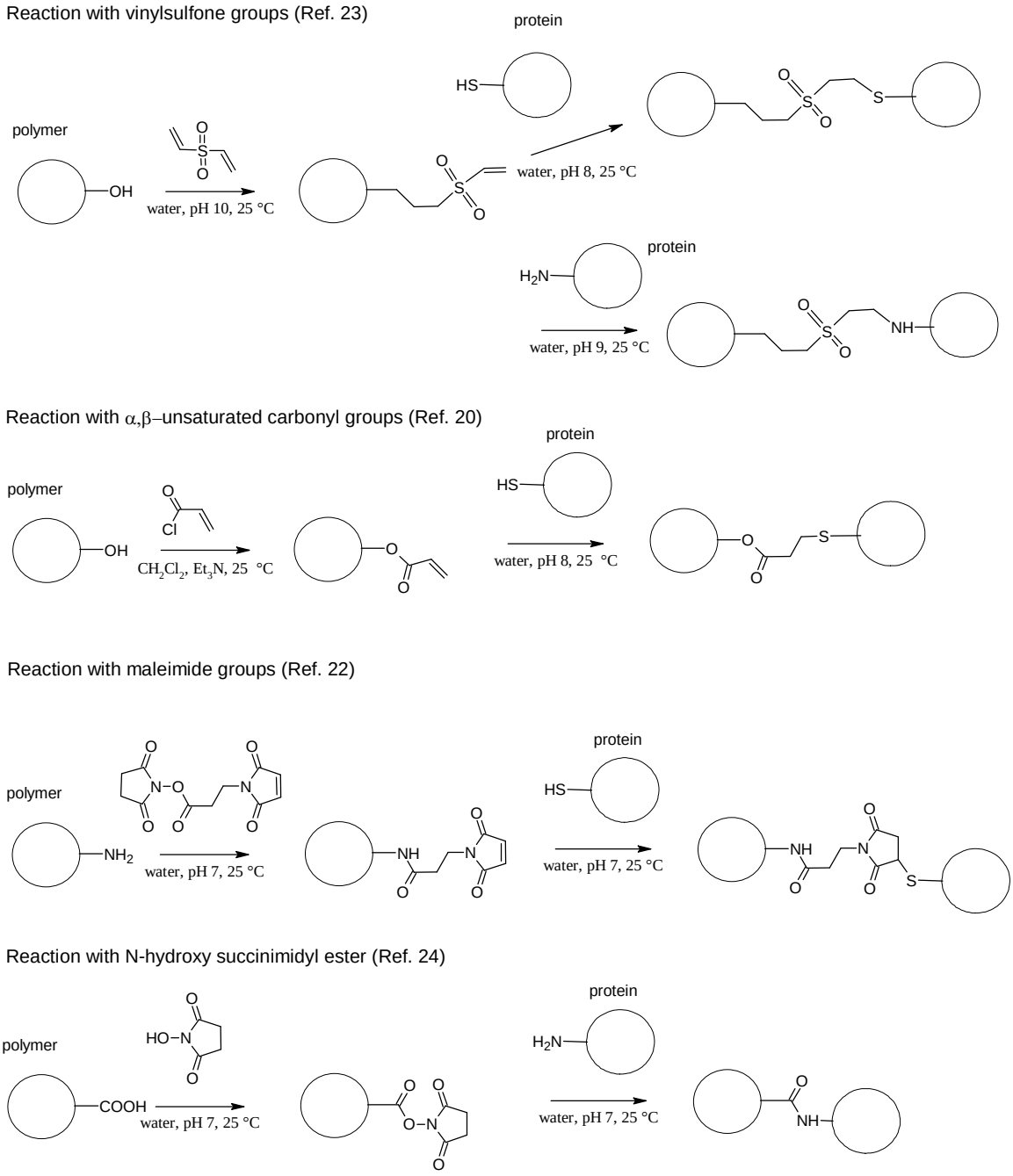
Scheme 1 represents common conjugation schemes.[20,22-24] Functionalization of poly(ethylene glycol) or poly(hydroxyethyl methacrylate) with divinyl sulfone in aqueous solution at pH 10 introduces vinyl sulfone and α,β-unsaturated carbonyl functionalities to the polymer.[23] Both groups can easily react with thiols at pH 8 or amines at pH 9 via Michael addition, resulting in conjugation with proteins.[23] Another method of introducing unsaturated carbonyls to the polymer is esterification of its hydroxyls with acryloyl chloride, which is performed in dichloromethane at room temperature.[20]

Conjugation of proteins to the amine containing matrix polymers can be also performed via maleimide-thiol reaction. Maleimide groups are introduced to the polymer by reaction with maleimide-N-succinimidyl crosslinking reagents.[22] N-hydroxy succinimidyl ester (NHS-ester) chemistry is a convenient route for conjugation of the carboxyl-terminated polymers. Esterification of carboxyl groups with N-hydroxy succinimide results in the formation of "active esters",[24, 25] which are highly reactive toward primary amines of the corresponding protein.

Preliminary functionalization of both protein and polymer with the "click" chemistry-enabled functional groups is an effective although less common method. For example, in the work of Dirks *et al.*[26] the target oligopeptide was first treated with N-propyl maleimide to attach alkyne groups, which further reacted with the azide-terminated polystyrene to yield the oligopeptide-polystyrene complex. In another example, by Heredia *et al.*,[17] the ketone functionalities were introduced to bovine serum albumin, enabling the conjugation with the various aminooxy-terminated acrylic

polymers. The primary factor that should be taken in account is choosing the conjugation technique is the ability of the immobilized protein to retain its biological activity.

Scheme 1. Common conjugation reactions.



CHAPTER II

SCOPE AND OBJECTIVE OF THE RESEARCH

Hemoperfusion

Hemoperfusion has been used for many years for removal of toxic substances from the patient's blood.[27,28] Hemoperfusion is essential for the treatment of intoxicated or poisoned patients in emergency situations, for removal of waste products from the blood of the patients with acute renal failure, as well as a supportive treatment before and after liver transplantation.[27, 28] In this extensive care procedure the blood passing through the purification apparatus directly perfuses the particles that bind specific toxins. Originally, charcoal was used as a filtration media, but recently polymeric beads with immobilized drugs and antibodies for removal of toxins have been introduced.[28-30] For example, polymyxin-functionalized fibrous columns are commonly used to prevent sepsis in surgical procedures,[29] and to treat inflammatory response syndrome.[31] Polymyxin B is an antibiotic that is highly efficient in binding and inactivation of endotoxin and is also bactericidal for gram negative bacteria, which defines the use of this drug in filtration media for hemoperfusion. Another example of a bioconjugate material for blood treatment is given in the work of Azhari,[30] that describes a material for emergency

treatment of patients poisoned with herbicide Paraquat. Specific antiparaquat antibodies were covalently bound to cross-linked agarose-polyacrolein microsphere beads. The material removed Paraquat from the bloodstream of the dog during hemoperfusion session 4 times faster than a regular charcoal column.

Objective of the project and research strategy

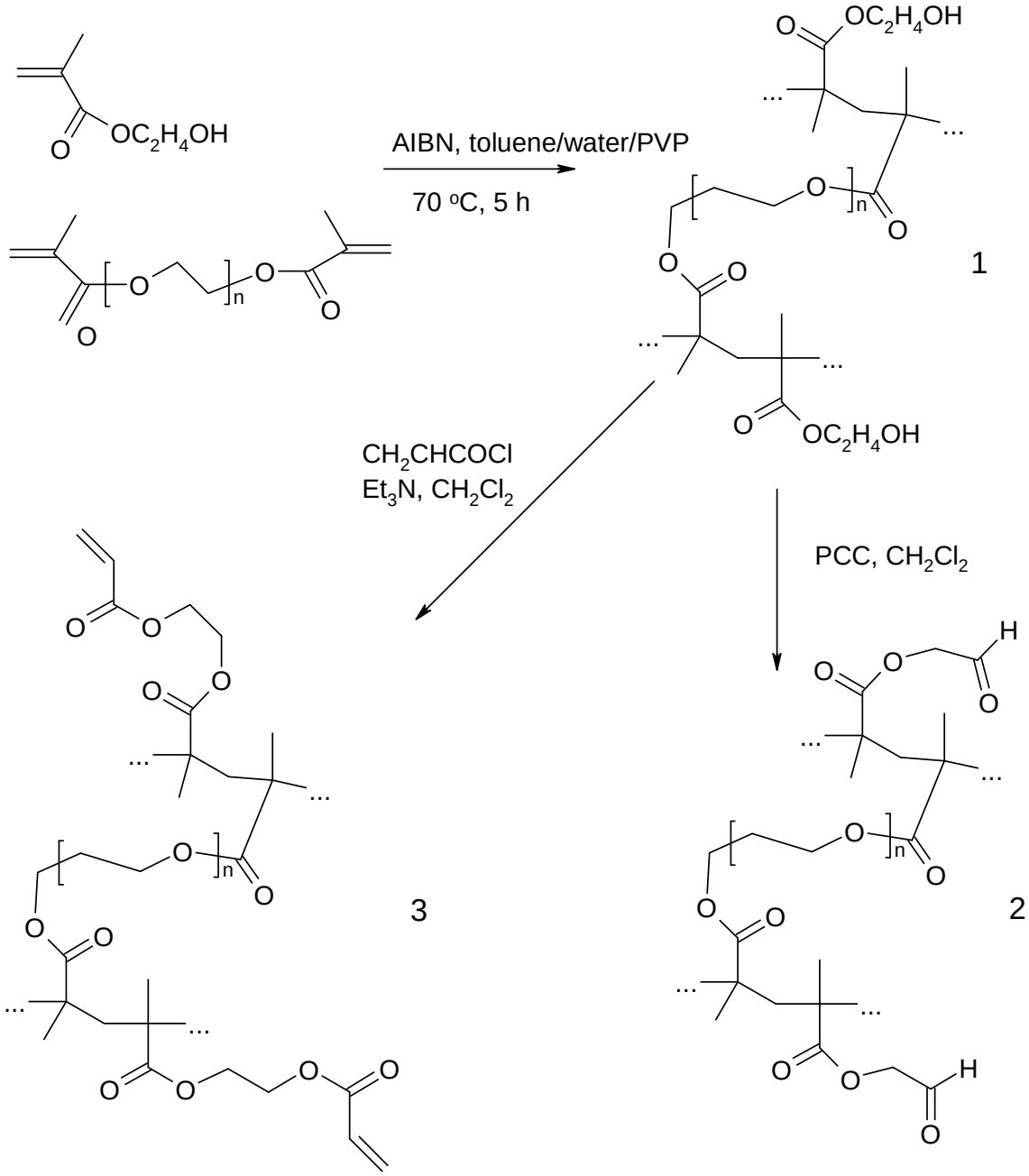
The objective of the current research project was to develop a synthetic bioconjugation method that could be potentially utilized to produce active filtration materials for hemoperfusion. The novelty of this work is the use of acrylic ester polymers as the material of choice for immobilization of the biologically active compounds. As it was mentioned above, charcoal has been the most common filter in hemoperfusion methods, while conjugated polymers are being introduced only recently. Acrylic polymers are chemically and biologically inert, insoluble, have a low degree of swelling in water and are already used for producing various parts of blood purification equipment. Copolymerization of different monomers with specific functional groups allows modification of the surface of the resulted polymeric particles for further functionalization with the required moieties, using well established techniques of bioconjugate chemistry.

In the present work, crosslinked copolymers poly(hydroxyethyl methacrylate-*co*-poly(ethylene glycol) dimethacrylate) (poly(HEMA-*co*-PEGDMA)) and poly(carboxyethyl acrylate-*co*-poly(ethylene glycol) dimethacrylate) (poly(CEA-*co*-PEGDMA)) were used. The surface of the polymer particles was modified by different

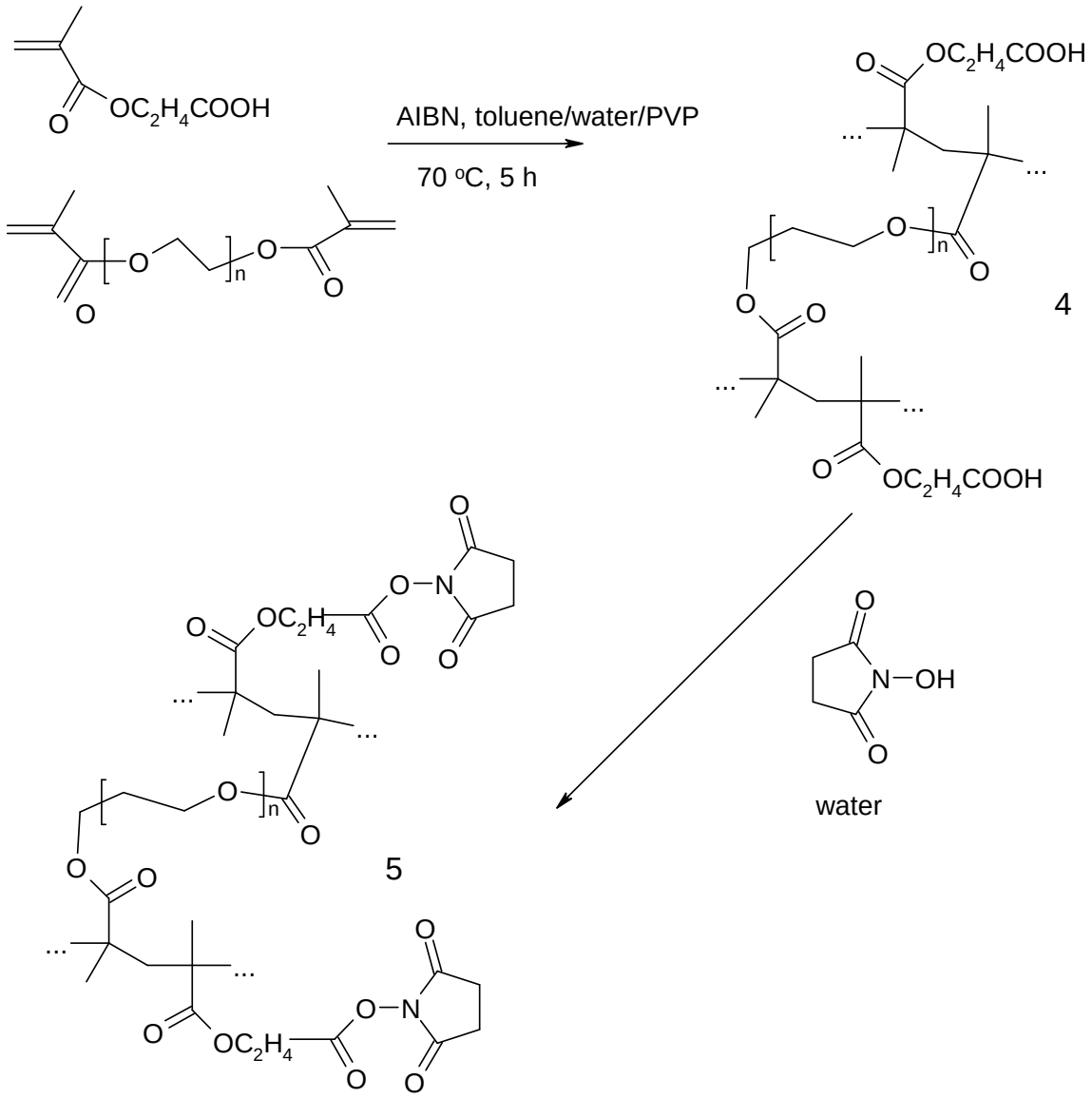
methods in order to chemically attach biomolecules. Schemes 2 and 3 outline the synthetic procedures for the preparation and surface functionalization of poly(HEMA-*co*-PEGDMA) and poly(CEA-*co*-PEGDMA) correspondingly.

To test the conjugation techniques, L-glutathione (Glutamyl-Cysteinyl-Glycine) in its reduced and oxidized forms and L-carnosine were used as examples of polypeptides. Conjugation was performed by three methods. In the first method, the -OH groups of HEMA were oxidized to aldehydes, which further reacted with –SH groups of L-glutathione resulting in thioacetal bond formation between polypeptide and copolymer. In the second method the copolymer was acrylated via reaction of -OH groups of HEMA with acryloyl chloride. This procedure introduced α,β-unsaturated carbonyls to the surface of the polymer which further reacted with -SH groups of the polypeptide.[20] This reaction has been used previously for the PEG-peptide conjugates.[16,18-21] In order to investigate the efficacy of reaction of the cysteine -SH groups vs. amine groups of protein, the cysteine-free oligopeptide L-Carnosine (*beta*-alanyl-L-histidine) was tested. The third conjugation method was based on reaction of the N-hydroxysuccinimide-functionalized polymer with amine groups of the protein.[24]

Scheme 2. Synthesis and modification of poly(2-hydroxyethyl methacrylate-*co*-poly(ethylene glycol) methacrylate).



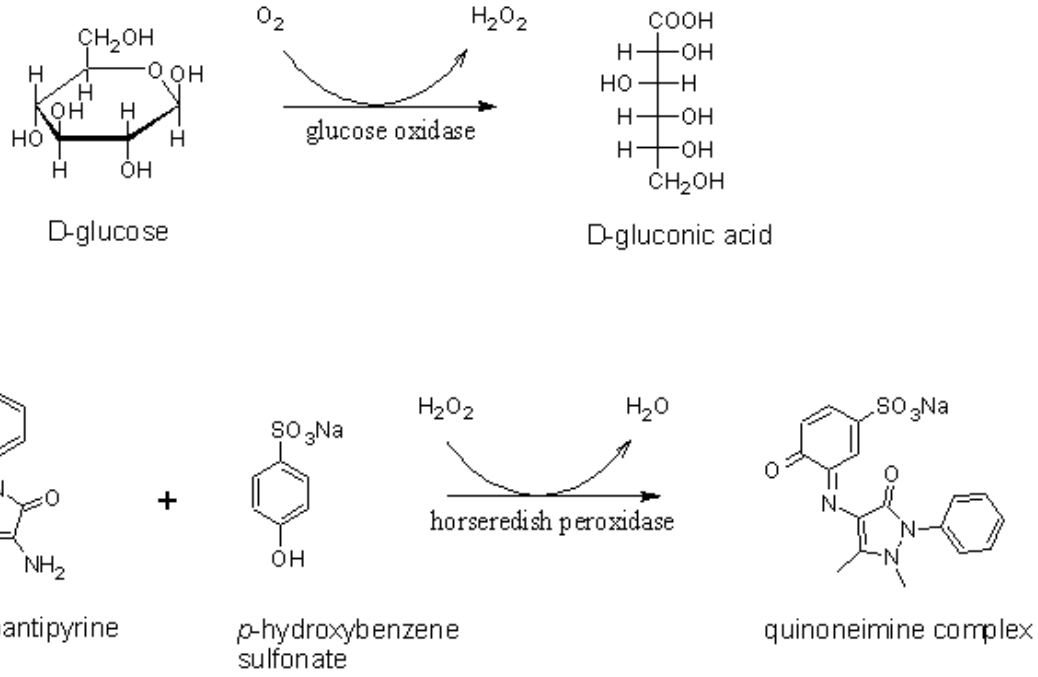
Scheme 3. Synthesis and modification of poly(2-carboxyethyl acrylate-*co*-poly(ethylene glycol methacrylate).



In order to examine the biological activity of the immobilized protein, glucose oxidase has been chosen due to simplicity of the activity determination for this enzyme. Two cross-linked polymers, poly(HEMA-co-PEGDMA) and poly(CEA-co-PEGDMA), were used as a support. In the first polymer the surface -OH groups were oxidized to aldehydes and in the second polymer carboxylic groups were esterified with N-hydroxysuccinimide. Activity of the polymer-bonded enzyme was studied by the modified Stanbio enzymatic glucose assay with free Horseradish Peroxidase (Hp).[32]

Scheme 4 shows the reactions involved in the analysis of enzymatic activity. In the first step glucose oxidase reacts with glucose yielding gluconic acid and hydrogen peroxide.[33] The amount of hydrogen peroxide evolving during this step is directly proportional to the activity of the enzyme. In order to estimate this amount, the color reaction with Stanbio assay, containing Horseredish peroxidase, 4-aminoantipyrine, and p-hydroxybenzenesulfonate, is employed. Oxidation of 4-aminoantipyrine with hydrogen peroxide, catalyzed by Horseredish peroxidase in the presence of p-hydrozybenzene sulfonate, produces the red-colored quinoneimine complex. The amount of the complex, measured by UV absorption at 500 nm, is proportional to the concentration of hydrogen peroxide and, therefore, to the activity of glucose oxidase.

Scheme 4. Chemical reactions involved in determination of the enzymatic activity of glucose oxidase by Stanbio assay.



CHAPTER III

EXPERIMENTAL SECTION

Materials

All the chemicals were purchased from Aldrich. Poly(ethylene glycol) dimethacrylate with average M_n of the poly(ethylene glycol) chains of 300 Da (PEGDMA) and 2-hydroxyethyl methacrylate (HEMA) monomers were purified from inhibitor by passing through basic alumina. All other chemicals were used as received. Double processed sterile deionized water (pH=6.7) was used for all the experiments that involved oligopeptides or glucose oxidase.

Instruments

The ^{13}C NMR spectra were taken on a 300 MHz Bruker DSX solid state NMR spectrometer. FTIR analysis was performed on a Varian 800 FTIR Spectrometer. UV-VIS spectra were acquired on a CARY 5000 UV-VIS-NIR spectrometer. The pH of the buffer solutions was measured on Mettler-Toledo pH-meter. The elemental analysis of the enzyme conjugated polymers was performed by Galbraith Laboratories, Inc., Knoxville TN.

Synthesis of the crosslinked poly(HEMA-*co*-PEGDMA)

For the experiment, 1 g of polyvinylpyrrolidone was dissolved in 115 ml of water and the solution was transferred into a 500 ml 3-neck round bottom flask equipped with nitrogen inlet and outlet, mechanical stirrer, and reflux condenser. The solution was purged with nitrogen for 10 min to eliminate oxygen. A mixture of 25 ml (200 mmol) of HEMA, 3.1 ml (10 mmol) of PEGDMA, and 0.5 g (3.05 mmol) of 2,2′-azobis(2-methylpropionitrile) (AIBN) in 25 ml of toluene was added to the flask and the reaction mixture was stirred at 70 ^{0}C for 5 h in a nitrogen atmosphere. The resulting suspension was filtered through the Whatman #54 paper filter and the solid was washed consecutively with excess water, acetone, and methanol, yielding the crosslinked copolymer **1** (Scheme 2). The sample of the product for the FTIR and NMR spectroscopy was dried in vacuum at room temperature for 24 h.

Oxidation of poly(HEMA-*co*-PEGDMA)

1.5 g of pyridinium chlorochromate (PCC) was dissolved in 60 ml of dichloromethane and transferred into a 100 ml two-neck round flask under nitrogen atmosphere. 2.8 g of polymer **1**, taken immediately after the synthesis and purification, was washed with dichloromethane and added to the flask, and the mixture was stirred at 45 ^{0}C under nitrogen until the solution color changed to brown-green. The solid was filtered through the paper filter and washed with dichloromethane until the filtrate was clear, yielding polymer **2**. The sample for spectroscopy was dried under vacuum at room temperature for 24 h.

Conjugation of the oxidized poly(HEMA-*co*-PEGDMA) with oligopeptides

Three different oligopeptides: L-carnosine, and L-glutathione in both reduced and oxidized form, were used for this experiment. Reaction was performed both in deionized water (pH 6.7) and in phosphate buffer saline (pH 8.2). Copolymer **2** in the amount of 1.8 g was added to the 40 ml of the 20 mg/ml solution of the oligopeptide in a 100 ml beaker and the mixture was stirred at room temperature for 2 h. The resulting product was filtered, rinsed with water, and dried under vacuum at room temperature for 24 h.

Acrylation of poly(HEMA-*co*-PEGDMA)

The synthesis was performed according to the procedure described previously for acrylation of PEG.[21] 30 g of polymer **1** taken immediately after the synthesis and purification was washed with dichloromethane and transferred into a 500 ml round-bottomed flask containing 300 ml of dichloromethane. The mixture was cooled to 0 °C in an ice bath. 13.1 ml of triethylamine and 15.8 ml of acryloyl chloride were added to the flask, and the reaction mixture was stirred for 12 h at 0 °C followed by stirring for 12 h at room temperature under nitrogen atmosphere. The resulting product was filtered and washed with dry dichloromethane and tetrahydrofuran, yielding polymer **3** (Scheme 2). The sample for spectroscopy was dried in vacuum at room temperature for 24 h.

Conjugation of acrylated poly(HEMA-*co*-PEGDMA) with oligopeptides

Three different oligopeptides: L-carnosine, and L-glutathione in both reduced and oxidized form, were used for this experiment. 0.84 g of peptide was dissolved in 20 ml of the phosphate buffer solution (K_2HPO_4/KH_2PO_4, pH=8.14) and 1.6 g of polymer **3** was

added to the solution and mixed immediately. The reaction was stirred for 2 h at room temperature. The resulting glutathione–copolymer conjugate was washed and dried under vacuum at room temperature for 24 h.

Conjugation of oxidized poly(HEMA-*co*-PEGDMA) with glucose oxidase

Glucose oxidase (GOx) in the amount of 0.18 g was dissolved in 4 ml of water in a test tube and 1.51 g of polymer **2** was added to the solution. Reaction was carried out at 37 °C for 30 min for enzyme activation followed by additional 2 h at 3 °C. The resulting conjugate was washed by centrifugation several times until no GOx was detected in the supernatant by UV-VIS spectroscopy. The solid product was dried at room temperature under vacuum for 4 h and stored at – 30 ^{o}C.

Synthesis of poly(2-carboxyethyl acrylate-co-poly(ethylene glycol) dimethacrylate) (poly(CEA-co-PEGDMA))

1 g of polyvinylpyrrolidone was dissolved in 115 ml of water and the solution was transferred into a 500 ml 3-neck round bottom flask equipped with a nitrogen inlet and outlet, mechanical stirrer, and reflux condenser. The solution was purged with nitrogen for 10 min to eliminate oxygen. A mixture of 23.7 ml (200 mmol) of carboxyethyl acrylate, 3.1 ml (10 mmol) of PEGDMA, and 0.5 g (3.05 mmol) of AIBN in 25 ml of toluene was added to the flask and the reaction mixture was stirred at 70 ^{0}C for 5 h in a nitrogen atmosphere. The resulting suspension was filtered and the solid was washed consecutively with excess water, acetone, and methanol, yielding the crosslinked copolymer **4** (Scheme 3).

Functionalization of poly(CEA-*co*-PEGDMA) with N-hydroxysuccinimide

N-hydroxysuccinimide (NHS) in the amount of 16.48 g was dissolved in 50 ml of deionized water and 8.2 g of poly(CEA-co-PEGDMA) was added to the solution. The mixture was stirred for 30 min. The NHS-modified polymer **5** was filtered and dried under vacuum at room temperature for 24 h.

Conjugation of the NHS-modified poly(CEA-co-PEGDMA) with glucose oxidase

Glucose oxidase (GOx) in the amount of 0.147 g was dissolved in 3 ml of water in a test tube and 0.483 g of polymer **5** was added to the solution. Reaction was carried out at 37 °C for 30 min for enzyme activation followed by additional 2 h at 3 °C. The resulting conjugate was washed by centrifugation several times until no GOx was detected in the supernatant by UV-VIS spectroscopy. The solid product was dried at room temperature under vacuum for 4 h and stored at – 30 ^{o}C.

Enzymatic activity of the glucose oxidase conjugated with crosslinked copolymers

The measurements of the GOx activity were performed by the modified Stanbio glucose assay. The assay reagent was prepared by dissolving 152.4 mg of 4-aminoantipyrine, 174 mg of p-hydroxybenzene sodium sulfonate and 5 mg (100 units) of Horseradish peroxidase in 50 ml of double processed sterile deionized water (pH=6.7). In a typical experiment, 33 mg of the GOx-conjugated polymer was placed in a test tube, 2 ml of the 1.92 mg/ml solution of glucose was added and the mixture was incubated at 37 ^{o}C for 30 or 60 min. In the control experiment, 7 mg of GOx was used instead of the polymer. After incubation, 2 ml of the assay reagent was added to the test tube and after

15 min of incubation the absorption of the solution at 500 nm was measured. The mixture of 2 ml of the 1.92 mg/ml glucose solution and 2 ml of the assay reagent was used as a blank sample for the UV-VIS measurements.

Lifetime enzymatic activity

After enzymatic activity experiment the sample of the GOx-conjugated polymer was separated by centrifugation, washed with water, and placed in the freezer at -30 ^{o}C. After 24 hours the sample was retrieved and one more enzymatic activity test was performed, after which the sample was recovered and stored in the freezer. Such a cycle was repeated for 6 days, until no activity toward oxidation of glucose was observed.

CHAPTER IV

RESULTS AND DISCUSSION

Synthesis and functionalization of the crosslinked copolymers

The synthetic procedures are outlined in Schemes 2 and 3. Mild oxidation with pyridinium chlorochromate is a well established synthetic method for conversion of alcohols to aldehydes.[34] Reaction of aldehydes with amines is widely used for cross-linking proteins.[25] The FTIR spectra of poly(HEMA-*co*-PEGDMA) before and after oxidation with PCC are presented in Figure 1. Appearance of the new peak at 1630 cm^{-1} corresponding to the vibrations of C=O bonds in aldehyde groups indicates the oxidation of the alcohols on the poly(HEMA) chains to aldehydes. Low intensity of the peak suggests that the polymer particles poorly swelled in dichloromethane and only the hydroxyl groups exposed on the surface oxidized.

Cross-linking of proteins via reaction of sulfhydryl or amine groups of proteins with acrylates is another effective method for preparation of bioconjugated materials.[35] Figure 2 shows the FTIR spectra of poly(HEMA-*co*-PEGDMA) (**1)** and acrylated poly(HEMA-*co*-PEGDMA) (**3).** The new peaks at ca. 1650 cm^{-1} and 810 cm^{-1}, corresponding to the stretching and bending vibrations of the C=C double bonds appear on the spectrum confirming the introduction of the acryl groups along the polymer chain.

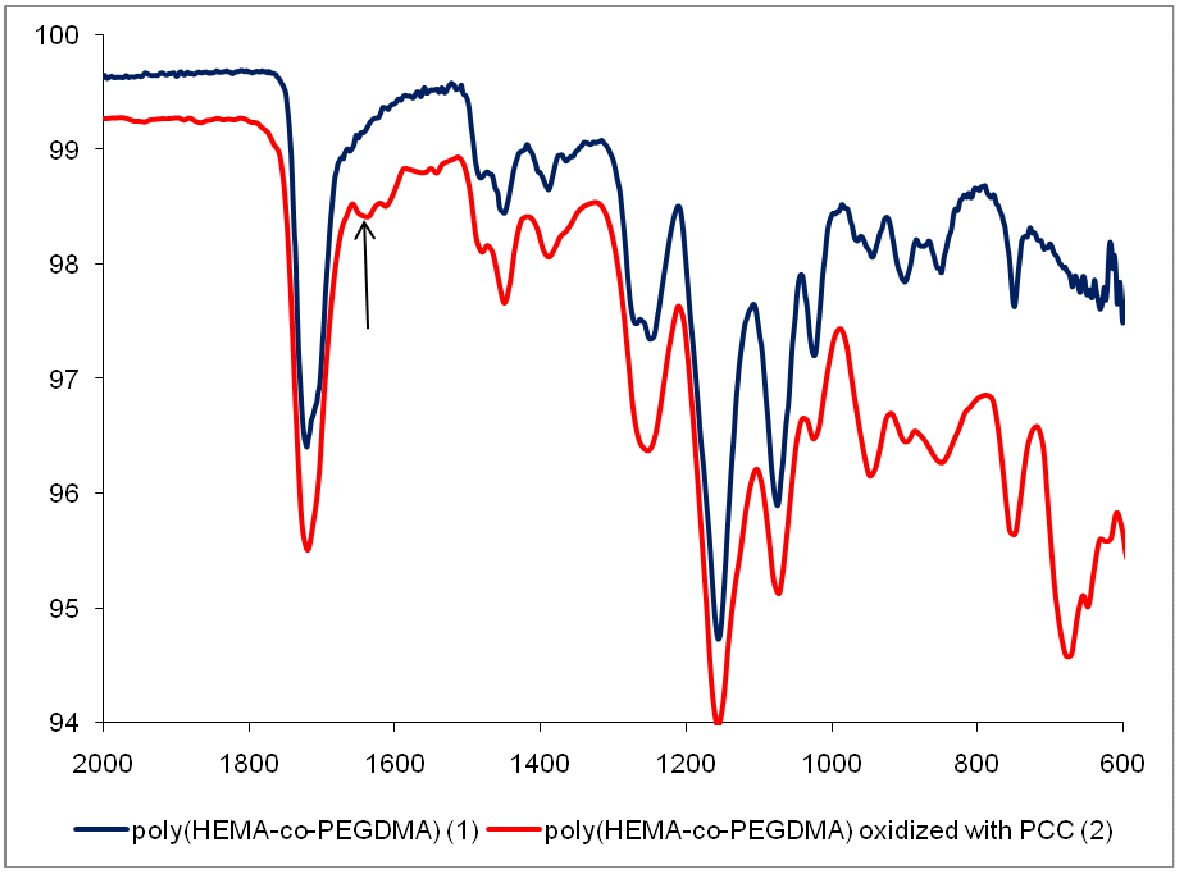


Figure 1. FTIR spectra of the poly(HEMA-*co*-PEGDMA) after the synthesis (1) and after oxidation with PCC (2). The arrow indicates the new peak at 1630 cm^{-1}.

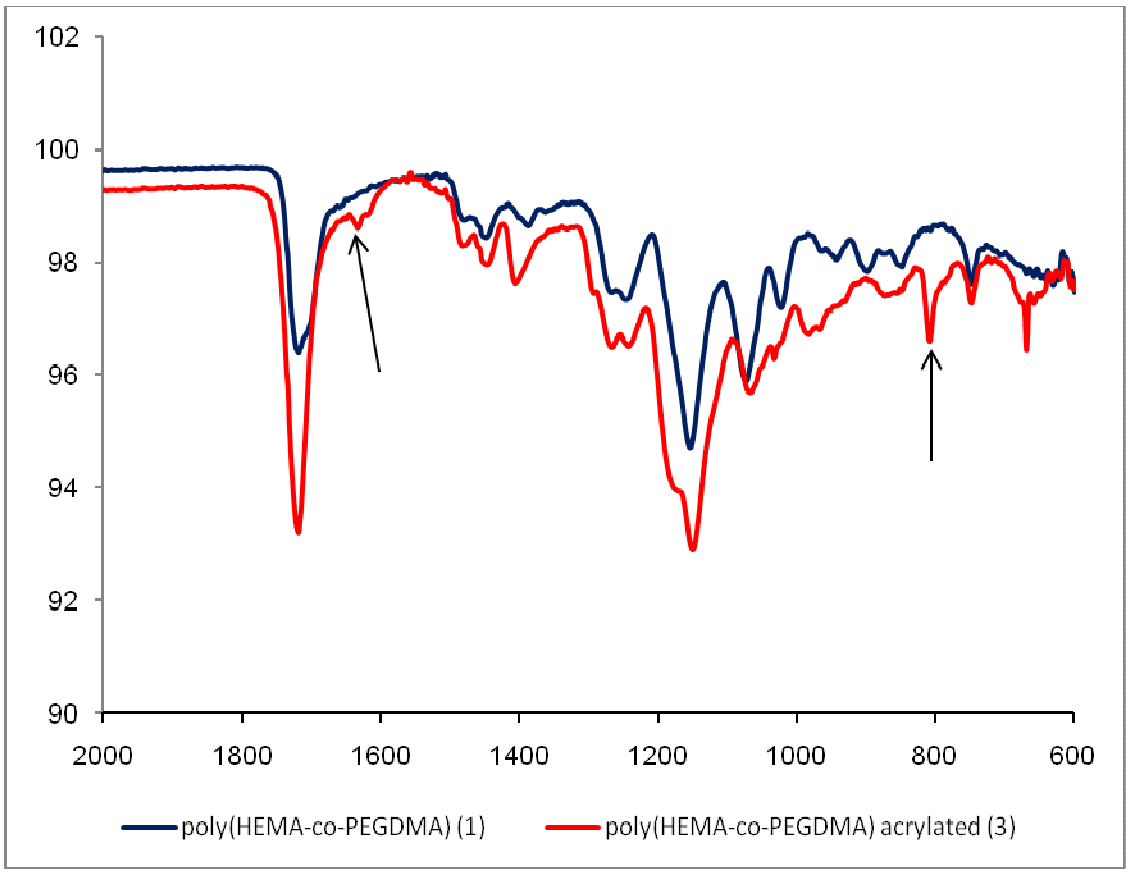


Figure 2. FTIR spectra of poly(HEMA-*co*-PEGDMA) before (1) and after (3) reaction with acryloyl chloride. Arrows indicate the new peaks at 1650 and 810 cm^{-1}.

Conjugation of the crosslinked copolymers with oligopeptides

In this experiment the oligopeptides were used as a model of proteins in order to develop techniques for conjugation of proteins. For that purpose, reaction in neutral or slightly basic conditions had to be tested, so that the protein conformation would not be destroyed. The structures of the oligopeptides are shown in Scheme 5. The synthetic procedures for conjugation of glutathione with acrylated and oxidized poly(HEMA-*co*-PEGDMA) are presented in Scheme 6. The FTIR spectrum of polymer **2** conjugated with reduced L-glutathione is shown in Figure 3. A series of new peaks between 1500 and 1650 cm^{-1} appearing in this spectrum, compared to that of the polymer before the reaction, indicates the presence of amines and amides due to the conjugation of glutathione to the surface of the copolymer particles. At the same time, the –S-H thiol peak of the reduced glutathione at 2550 cm^{-1} cannot be seen in the spectrum, suggesting that thiols were engaged in the reaction with aldehydes. When the polymer **2** was allowed to react with dimerized (oxidized) glutathione and with L-carnosine, both of which lack thiol groups, no changes were observed in the FTIR spectra after reaction. All of these facts suggest that thiols, not amines, react with aldehydes on the surface of the polymer particles, resulting in the formation of the thioacetal bonds.

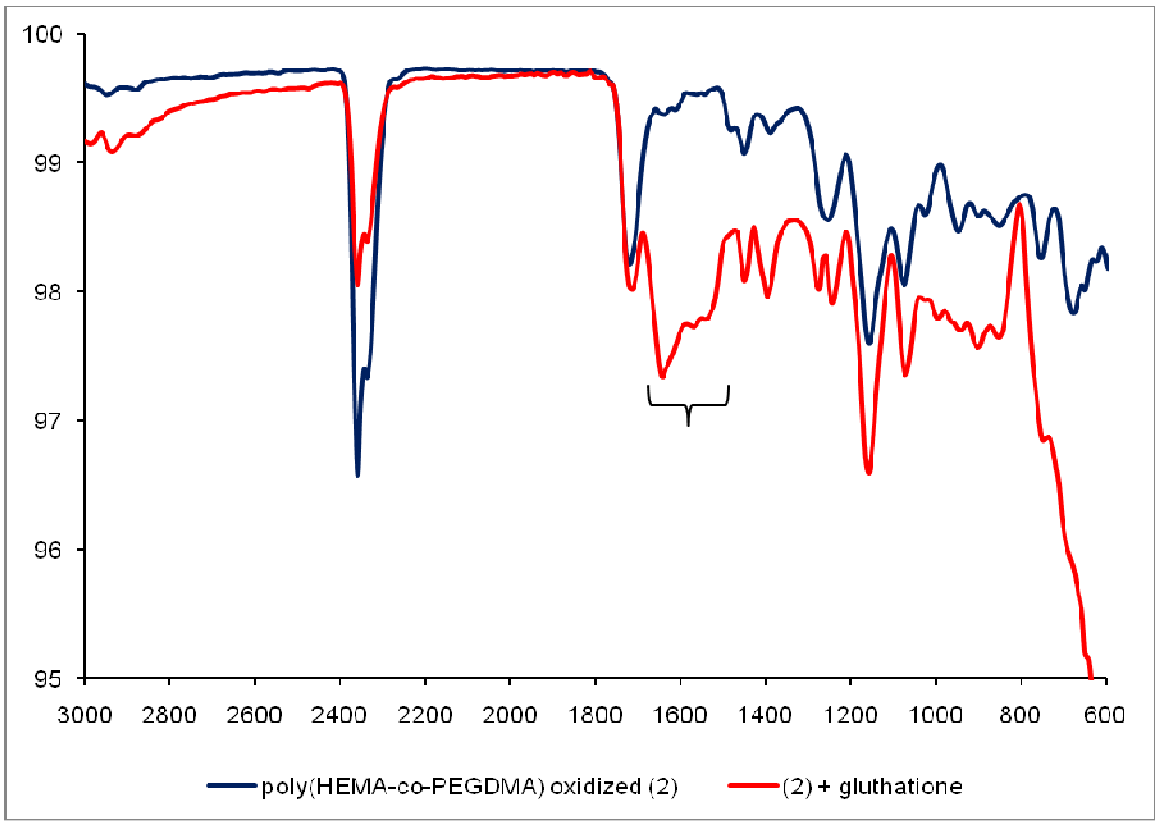
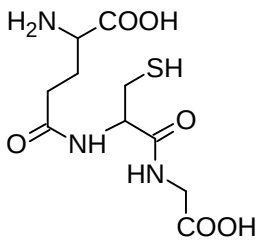
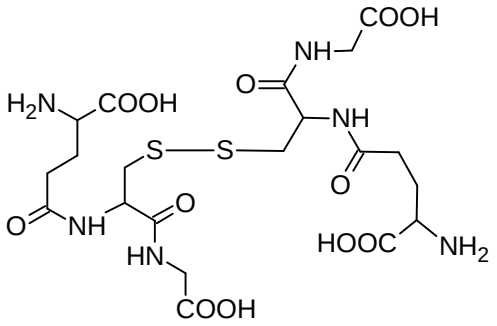


Figure 3. FTIR spectrum of the aldehyde modified polymer 2 conjugated with reduced L-glutathione. The bow indicates new peaks corresponded to glutathione.

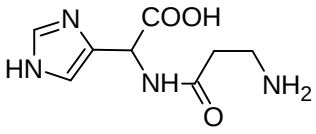
Scheme 5. Structures of oligopeptides.



L-glutathione, reduced form

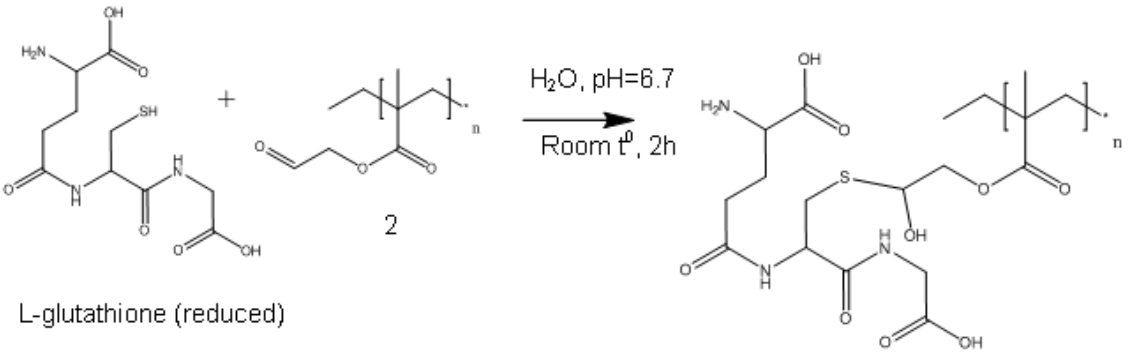


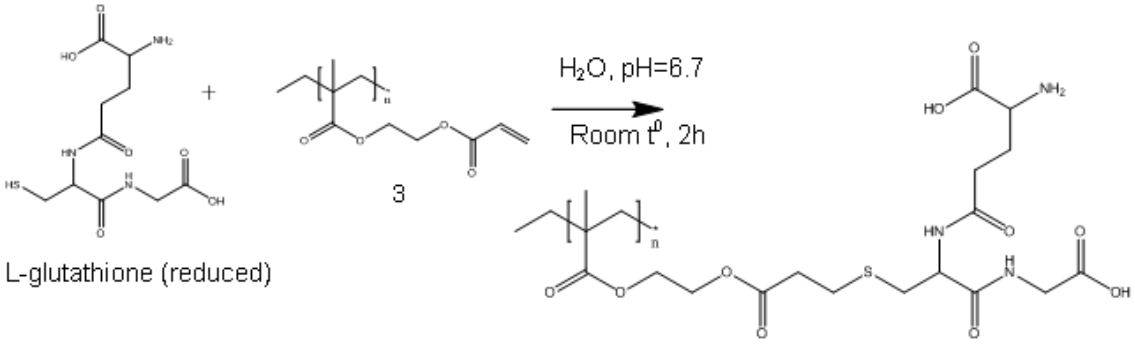
L-glutathione dimer (oxidized form)



L-carnosine (beta-alanyl-L-histidine)

Scheme 6. Reaction of L-glutathione with oxidized and acrylated poly(HEMA-*co*-PEGDMA)





Results of the mass change in polymer **2** and polymer **3** after reaction with oligopeptides are presented in Table 1. In the blank experiment a sample of polymer was stirred in water for 2 h followed by drying in vacuum for 24 h at room temperature. Negative numbers can be explained by removal of the residue of either PCC or acrylic acid and triethylamine not removed in the previous steps, or removal of water under vacuum. The table shows that the reaction of the aldehyde functionalized polymer and reduced glutathione resulted in the highest degree of attachment of the oligopeptide, so this polymer would be the most effective for the conjugation of proteins. Reaction with aldehydes at pH 6.7 was more effective than at pH 8.2. These results are in agreement with the FTIR spectroscopy (Fig. 3), that also shows the presence of glutathione in the aldehyde-modified copolymer.

Aldehyde-thiol reaction has not been used in bioconjugate chemistry before. However, formaldehyde and glutaraldehyde are routinely used in histology and electron microscopy for fixation of specimens, the process aimed to preserve a sample of biological material for the examination.[36] It is believed that during fixation aldehydes react with the amine at the end of the side-chain of lysine and with the nitrogen of a peptide linkage, crosslinking the protein molecules into the insoluble structure.[37] However, one should also expect a nucleophilic addition of the thiol arising from cysteine residue of the protein to the carbonyl group of glutaraldehyde, resulting in formation of thiohemiacetals. It was anticipated that aldehyde groups on the surface of the polymer beads would react with oligopeptides the same way. Contrary to our expectations, carbonyls on the surface of the oxidized poly(HEMA-co-PEGDMA) did not react with amines of glutathione and carnosine. The conjugation took place only with the reduced

form of glutathione that possessed -SH groups. Probably, due to a low amount of aldehydes on the surface and hence low reaction rate, the reaction with sulfhydryl groups, as being stronger nucleophiles, becomes prevailing.

Table 1. Mass change in crosslinked polymers after reactions with oligopeptides.

Copolymer	Grafted with:	Mass change (w/w) %
Poly(HEMA-co-PEGDMA) oxidized (**2**)	Blank	- 6.7
	L-Glutathione (reduced) in DI water (pH 6.7)	14.6
	L-Glutathione (reduced) in pH 8.2 buffer	10.2
	L-Glutathione (oxidized) in pH 8.2 buffer	- 5.1
	L-Carnosine in pH 8.2 buffer	-3.1
Poly(HEMA-co-PEGDMA) acrylated (**3**)	Blank	- 2.8
	L-Glutathione (reduced) in pH 8.2 buffer	2.7
	L-Glutathione (oxidized) in pH 8.2 buffer	- 2.5
	L-Carnosine in pH 8.2 buffer	4.8

The gravimetric results for the acryloyl chloride modified copolymer have shown only a small increase in weight after reaction with carnosine and glutathione (reduced). Since the FTIR spectroscopy has not revealed the presence of the oligopeptides in these materials, we cannot confirm that the conjugation has taken place for the acrylated copolymer. Acryloyl chloride chemistry as a bioconjugation method has been developed primarily for attaching of proteins to the PEG-based polyhydrogels. Hydrogels are highly water permeable, which makes all the functional groups available for the reaction.

Conjugation to the insoluble polyHEMA particles, in contrast, is possible mostly on the surface. As we can see from the experimental results, aldehyde-thiol chemistry presents a great advantage for this type of systems.

Conjugation of the crosslinked copolymers with glucose oxidase

For this experiment, aldehyde-modified poly(HEMA-co-PEGDMA) and NHS-modified poly(CEA-co-PEGDMA) were chosen. Esters of N-succinimide are used for bioconjugation[24] due to their high activity toward primary amines. In the present approach, glucose oxidase served as a model of a biologically active protein due to a well established method of determination of activity for this enzyme. Elemental analysis was used to estimate the degree of attachments of GOx in each polymer. Table 2 shows the weight fraction of GOx in each conjugate, calculated from the elemental analysis results. Based on the detected content of sulfur and nitrogen, which comes only from the protein, the fraction of the attached glucose oxidase was found to be 25% by weight in poly(HEMA-co-PEGDMA) and 7.7% by weight in poly(CEA-co-PEGDMA).

Table 2. Elemental analysis results for the conjugated crosslinked polymers.

Sample	Element	Content by elemental analysis, %	Calculated GOx content, %
Glucose Oxidase (GOx)	S	0.06	100
	N	7.28	100
Poly(HEMA-co-PEGDMA)+GOx	S	0.0137	27.4
	N	1.24	23.4
Poly(CEA-co-PEGDMA)+GOx	S	0.0046	7.7
	N	0.51	7.7

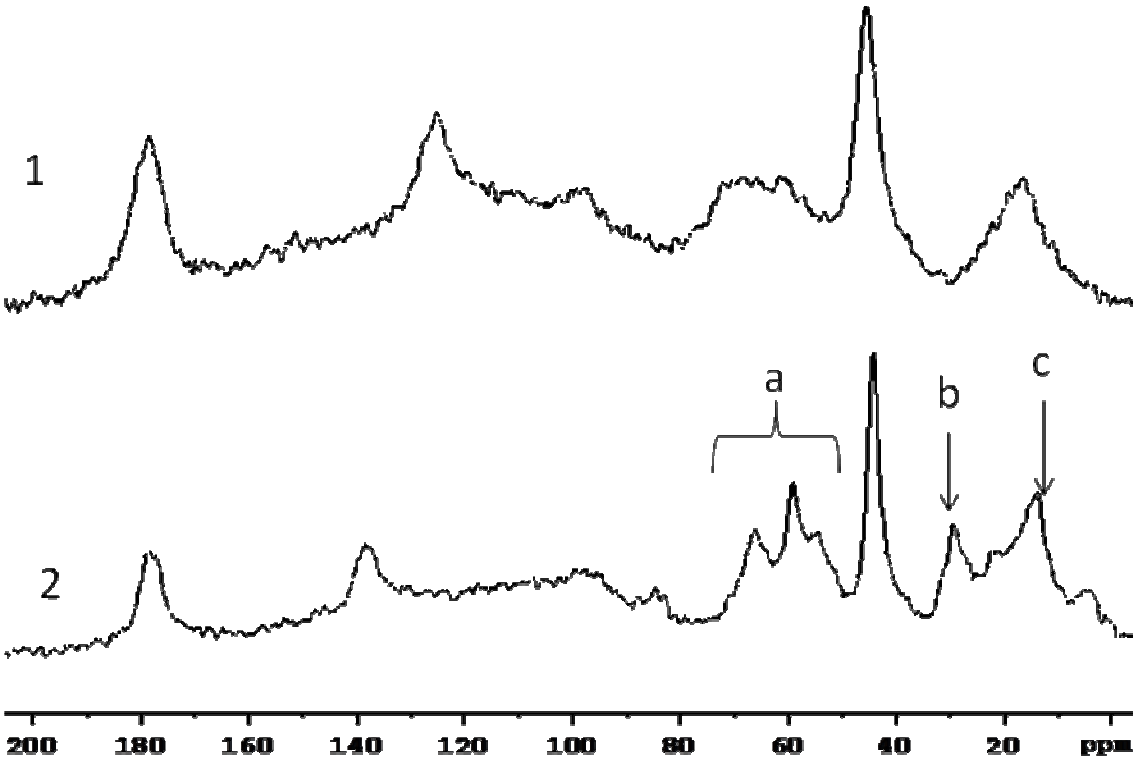


Figure 4. ^{13}C solid state NMR spectra of poly(HEMA-co-PEGDMA) before (1) and after (2) conjugation with glucose oxidase. The spinning rate of the probe was 4000 Hz and 3000 Hz for the samples before and after conjugation, correspondingly. The marked peaks in the spectrum 2: a (55, 60 and 65 ppm, peptide bond carbons); b (30 ppm, methylene carbons); c (15 ppm, methyl carbons).

Figure 4 shows the ^{13}C solid state NMR spectra of the aldehyde-modified crosslinked copolymer-peptide conjugate system. A series of new peaks arises in the sample after conjugation (spectrum 2): peaks at 55 – 65 ppm corresponding to the

-**C**-CO-, -**C**O-NH-, and -NH-**C**- carbons in the peptide bonds, and the peaks at 30 and 15 ppm, arising from methylene and methyl carbons of the protein respectively. The carboxyl and amide carbons contribute to the ester carbon peak at 175 ppm with the spinning sideband at 138 ppm in the spectrum of the conjugated material.

Table 3 presents the results of the enzymatic activity test for each sample. Absorbance of the quinone complex formed during the analysis is directly proportional to the enzymatic activity of glucose oxidase in the system. The glucose oxidase immobilized on poly(CEA-co-PEGDMA), despite its much lower content, has shown significantly higher activity compared to that conjugated to poly(HEMA-co-PEGDMA). According to the literature,[38] the active site of the glucose oxidase is comprised of flavin adenine dinucleotide (FAD) and 3 amino acid residues, tyrosine (Tyr 515) and two histidines (His 516, His 559), located in a funnel-shaped cavity. There are 3 cysteine residues in the structure of the enzyme, 2 of which (Cys 187, Cys 228) are involved in the disulphide bridge, leaving the single thiol group of Cys 543 available for binding to the aldehyde-functionalized polymer. It is possible that the attachment of the glucose oxidase to poly(HEMA-co-PEGDMA) results in obstruction of the active site or distortion of its conformation. In addition, the protein may be partially destructed or "poisoned" during the conjugation with this particular polymer. All of these factors will lead to the lower activity of the enzyme. To clarify this issue, more study should be conducted. Conjugation to poly(CEA-co-PEGDMA), on the other hand, proceeds via reaction of the NHS ester with the primary amines of arginine and lysine residues of the enzyme. Given 23 arginines and 15 lysines in the structure of glucose oxidase, there is a large variety of

possible enzyme conformations on the surface of the polymer and therefore less chance for obstruction or distortion of the active site.

Table 3. Relative enzymatic activity of Glucose Oxidase grafted polymers

Sample	Mass of sample, mg	Content of GOx, %	Time of incubation, min	Absorbance at 500 nm, Au
poly(HEMA-co-PEGDMA)-GOx	33.0	25 %	60	0.0321
			30	0.034
Poly(CEA-co-PEGDMA)-GOx	33.0	7.7 %	60	0.3837
			30	0.3921
GOx	7.0	100 %	60	1.0706
			30	0.8063

Lifetime enzymatic activity of the polymer immobilized glucose oxidaze

Enzymes, as natural catalysts, promote the biochemical reactions without consuming themselves in the process. The advantage of the enzyme immobilized on the polymer support would be the ability of the multiple use of such material. Lifetime enzymatic activity experiment was performed to see how the immobilized enzyme will tolerate the conditions of the multiple uses. After each test the material was recovered and stored in the freezer until the next day, when the same test was repeated on it.

Figure 5 shows the absorption of the Stanbio assay solution in two series of the experiments performed with the two enzyme-conjugated polymers. A much higher absorption in the case of the poly(CEA-co-PEGDMA)-GOx complex corresponds to the higher activity of the enzyme in this polymer. Moreover, after five consecutive experiments glucose oxidase was still viable. The poly(HEMA-*co*-PEGDMA)-GOx complex, in contrast, has revealed very low activity toward oxidation of glucose, which was undetectable already in the third cycle. The loss of activity of the enzyme could be attributed to the growth of bacteria on the surface of polymer particles that feed on the protein, resulting in its total destruction.

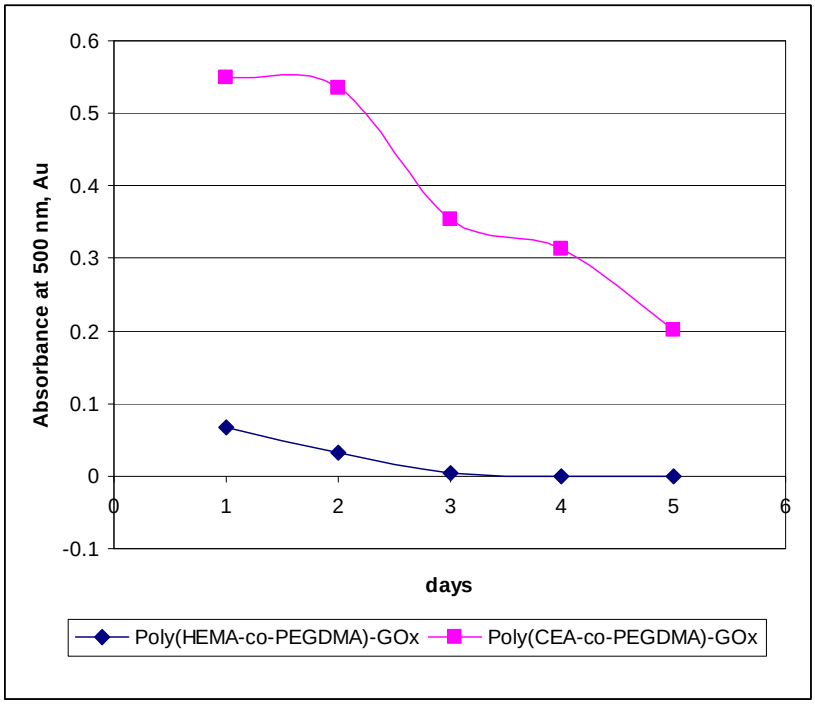


Figure 5. Lifetime enzymatic activity of glucose oxidase grafted to the polymers.

CHAPTER V

CONCLUSIONS

In the present work a novel method of immobilization of biologically active molecules on the solid inert support has been developed. The reactions of aldehyde and NHS-ester groups on the surface of polymer particles with thiols and amines of the polypeptides provided an effective way of conjugation of proteins to polymers. In particular, the aldehyde-thiol chemistry, which has been introduced here for the first time as a bioconjugation technique, was found to be the most efficient towards chemical attachment of polypeptides. Insoluble poly(HEMA) based microspheres are preferable as a filtration media for the continuous flow systems employed in hemoperfusion, due to their low swelling in water. At the same time, unlike in hydrogels, the conjugation of this material is possible only on the surface of the particles, so the standard techniques, such as acryloyl chloride chemistry, may not work for this type of systems. Therefore, aldehyde-thiol conjugation method can provide a good substitution for the preparation of new biomedical materials.

The study of the enzymatic activity of glucose oxidase conjugated with polymer particles has shown that the activity of the enzyme attached via aldehyde-thiol chemistry was significantly impaired compared to that of the NHS-conjugated material. Presumably the attachment to thiols either distorts the conformation of the protein or obstructs the active site, or affects in other way the viability of the enzyme, which greatly lowers its

activity. At this moment it is not clear whether such an effect is specific to this particular enzyme or to the aldehyde-thiol conjugation chemistry. In order to understand this problem better, reactions of the aldehyde-modified polymer with different enzymes should be studied.

Addition to the NHS-esters, due to reaction only with the primary amine groups of the protein, preserves its 3-dimensional structure and retains the biological activity. The enzyme attached on the polymer support is not consumed during the biochemical reaction, so that it can be used as a catalyst multiple times, and also can be employed in the continuous flow systems. This important property opens the possibility for utilizing the developed method for producing new bioconjugate materials for medical applications such as hemodyalysis or hemoperfusion.

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Scope and Method of Study:

The purpose of this research was to develop a synthetic bioconjugation method that could be potentially used to produce active materials for biomedical applications, such as hemoperfusion. Cross linked polyHEMA-based copolymers were used as a support, where the surface was chemically modified to introduce aldehydes and N-hydroxysuccinimide (NHS) esters to attach polypeptides. Glucose oxidase was used as an example of a biologically active material to graft to the polymer particles. Enzymatic activity analysis was utilized to evaluate activity of the immobilized glucose oxidase.

Findings and Conclusions:

Introduction of aldehyde and NHS-esters to the surface of polymer particles allowed chemical bonding of proteins to the polymer, which was confirmed by FTIR and NMR spectroscopy as well as by gravimetry and elemental analysis. Experiments with the model oligopeptides have shown that aldehydes of the polymer preferred reacting with thiols rather than with amines of the oligopeptides. Grafting of glucose oxidase was more effective to the aldehyde functionalized polymer than to the NHS-ester functionalized polymer. The latter polymeric conjugate has shown a higher activity of the immobilized enzyme despite its lower weight fraction in the material. The covalently attached biomolecule was not lost during the reaction with glucose and activity of the conjugate material lasted through 5 consecutive experiment performed with the same sample.

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