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Functional and reactive polymethacrylates suitable for preparation of peptide/protein–polymer conjugates

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ABSTRACT

Three reactive polymers, poly(para-nitrophenoxycarbonyloxyethyl methacrylate-*co*-methyl methacrylate) P(*p*-NPCEMA-*co*-MMA) [**rCoP1**], poly(methyl methacrylate-*co*-butyl methacrylate-*co*-allyl methacrylate-*co*-phenoxycarbonyloxyethyl methacrylate) P(MMA-*co*-BMA-*co*-AMA-*co*-PCEMA) [**rCoP2**] and poly(methyl methacrylate-*co*-dimethylaminopropyl methacrylate-*co*-dodecyl methacrylate-*co*-phenoxycarbonyloxyethyl methacrylate) P(MMA-*co*-DMAPMA-*co*-DMA-*co*-PCEMA) [**rCoP3**], respectively, were prepared following two synthetic concepts. Following the first concept, *p*-NPCEMA and PCEMA were prepared starting with commercially available HEMA and were copolymerized via ATRP with MMA, BMA and AMA. According to the second concept phenoxycarbonyloxy decorated polymethacrylates were obtained via polymer analogous reaction of a HEMA containing functional polymethacrylate, P(MMA-*co*-DMAPMA-*co*-DMA-*co*-HEMA) [**fCoP**], obtained via a cascade reaction of enzymatic transacylation and free radical polymerization. The highly reactive poly(methacrylate)s and silk peptides, hen egg-white lysozyme and *Candida antarctica* lipase B were used for the preparation of protein/peptide-poly(methacrylate) bioconjugates.

1. Introduction

Peptide/protein–polymer conjugates are hybrid materials, which covalently combine one or more copies of a peptide sequence or protein with a synthetic polymer [1,2] and are either designed to benefit from the behaviour of both components or to overcome shortcomings inherent to the components alone. To date, the most well known representatives of this class of materials are conjugates of peptides/proteins with poly(ethylene glycol) (PEG). PEGylation of proteins is established as a powerful strategy to improve their *in vivo* properties [3]. Over the past three decades PEGylation has developed into a well-established technology to modify therapeutic proteins and improve their stability and solubility, enhance circulation half-life, reduce immunogenicity and antigenicity, and prevent proteolytic degradation.

The first reports on peptide/polymer conjugates go back to the early 1950s [4]. In the 1970s, Davis and Abuchowski found that covalent attachment of PEG to bovine serum albumin (BSA) and bovine liver catalase was a useful strategy to reduce or eliminate the immunogenicity of these proteins and increase their blood circulation times [5,6]. Further, in the mid-1980s, Maeda et al., studying the pharmacokinetics of conjugates [7,8] discovered an anticancer agent bearing both hydrophilic and hydrophobic parts by combining the antitumor protein neocarzinostatin and poly(styrene-*co*-maleic anhydride). Other early examples of peptide/protein–polymer conjugates include a variety of hybrid di- and triblock copolymers prepared by ring – opening polymerization of α–amino acid *N*-carboxyanhydrides (NCA's) using appropriate synthetic polymer macroinitiators [9].

Peptide/protein–polymer conjugates have attracted interest for a number of reasons. First of all, the peptide/protein segment can provide these materials with unique self-assembly properties and can induce the formation of hierarchically organized nanoscale structures, both in solution and in the solid state, with higher level of complexity as compared to block copolymers. In several instances, the sensitivity of the protein secondary structure to environmental parameters such as temperature, pH or ion strength allows to reversibly manipulate the nanoscale structure formation of these hybrid materials. The literature on this subject is vast and has been summarized in various review articles [10–12]. The properties and the multifunctionality of the peptide/polymer–conjugates makes them very attractive for usage in drug and gene delivery applications [13–15], in modulation of the mechanical properties of synthetic polymers [16], in hydrogels [17–19] as well as therapeutic proteins [20–22].

The synthesis of the conjugates can be performed using (i) a convergent [23–27] or (ii) a divergent synthetic strategy [27–29].

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The convergent synthesis (i) involves the direct coupling of a functionalized peptide/protein with a complementary functionalized synthetic polymer. In the case of divergent synthesis (ii), two main routes can be distinguished: polymerization of synthetic monomers using peptide/protein macroinitiators or synthesis of a peptide segment on a soluble or solid-supported synthetic polymer.

For the convergent synthesis of peptide/protein–polymer conjugates, either via selective modification of a suitable end-reactive polymer or via functionalization of appropriate reactive side chains, a broad range of reactions is available. A detailed overview of these coupling conditions has been published in a recent review [25]. Table 1 lists some of the functional groups potentially available in proteins and the functionalities of the required polymers [30].

Side-chain functional polymers may be prepared by a variety of (controlled) radical polymerization methods. Within this approach, monomers bearing reactive and functional side-groups are polymerized both in their protected or unprotected form, and subsequently reacted with the functional groups in the peptide/protein. As an example, *p*-nitrophenyl chloroformate activation of the pendant hydroxyl groups of poly[(ethylene glycol) methacrylate] and poly(2-hydroxyethyl methacrylates) has been used to introduce peptide sequences [31–33]. In this concept attention has to be paid on eventual conformational or functional changes in the protein induced by the conjugation with the polymer [34].

In our previous work we described the syntheses of different multifunctional polymethacrylates via a cascade of enzymatic and chemical reactions. By this procedure hydrophilic, hydrophobic and ammonium groups were introduced in the polymer backbone besides reactive phenyl carbonate groups [35]. These reactive groups are known to interact with nucleophiles such as amines without side reactions [36–39]. In the present study we report preliminary results obtained for the convergent synthesis of the peptide/protein–polymer conjugates using the reaction of different proteins like: silk peptides, lysozyme and *Candida antarctica* lipase B (CALB) with different multifunctional polymethacrylates bearing *p*-nitrophenyl carbonate or phenyl carbonate groups in the side-chain. These reactions were performed either in *N*,*N*-dimethylformamide (DMF) solution or in the case of CALB also in miniemulsion. By preparing particles of reactive copolymers, a large surface of the polymer is exposed for the interactions with the functional groups of proteins. Protein concentration of the conjugates was determined via the Bio-Rad DC protein assay which is similar to the well-documented Lowry assay [40]. When CALB was used, the activity of the enzyme–polymer conjugate was determined via active site titration using *p*-nitrophenyl laureate as a substrate.

2. Materials and methods

2.1. Materials

Methyl methacrylate (**MMA**, 99+%, Fluka), butyl methacrylate (**BMA**, 99+%, Merck) and allyl methacrylate (**AMA**, 98%, Aldrich) were purified via column chromatography on Al_2O_3 (Fluka, type 5016A basic). 2-Hydroxyethyl methacrylate (**HEMA**, 95+%, Fluka), phenyl chloroformate (97+%, Fluka), 4-nitrophenyl chloroformate (96%, Aldrich), butyl acetate (**BuAc**, 99.5%, Fluka), azobisisobutyronitrile (**AIBN**, 98+%, Fluka), ethyl 2-bromoisobutyrate (**EBrIB**, 98%, Aldrich), **CuBr** (98%, Fluka), 2,2′-bipyridine (**Bpy**, 99+%, Aldrich), 3-dimethylamino-1-propanol (**DMAP**, 99%, Aldrich), 1-dodecanol (**D-ol**, 98.5+%, Fluka), pyridine (**Py**, 99.5%, KMF Laborchemie), 4-dimethylamino pyridine (99%, Aldrich), dichlormethane (99.5%, Fluka), hen egg-white lysozyme [lyophilized powder 105,000 U/mg, 2.185 mg protein/4.1 mg powder (53% protein), Fluka], silk peptide [1.676 mg protein/2.8 mg compound (60% protein)] were used as received. *C. antarctica* lipase B (**CALB**, lyophilized powder 10,000 U/g, Fluka) was purified prior use on a Sephadex G25 chromatography column using a 25 mM natrium phosphate buffer solution as eluent at a pH 7. A commercial lipase, Novozyme 435 (Lipase B from *C. antarctica* immobilized onto macroporous acrylic resin, 10,000 U/g Novo Nordisk) was dried in vacuum at room temperature for 24 h and stored under nitrogen before it was used as a biocatalyst for the transacylation reactions.

All reactions were carried out under nitrogen atmosphere. Nitrogen (Linde, 5.0) was passed over molecular sieves (4 Å) and finely distributed potassium on aluminium oxide before use.

Table 1
Functional groups in proteins and functionalities of the required polymers (30).

Amino acids (functional groups)	Polymers/materials/surfaces
Lys (–NH_2)	Carboxilic acid, active ester, epoxy
Cys (–SH)	Vinyl sulfone, maleimide, pyridil disulfide
Asp and Glu (–COOH)	Amine, epoxy
Ser and Thr (–OH)	Epoxy

2.2. Measurements

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively, using deuterated chloroform ($CDCl_3$) and tetramethylsilane (TMS) as internal standard.

Size exclusion chromatography analyses (SEC) were carried out using THF (with addition of 250 mg/L 2,6-di-tert-butyl-4-methylphenol) and DMAc (with 1 g/L LiCl) as eluting solvents. For THF as eluting solvent a pump (ERC Model 6420), a refractive index visco-detector (WGE Dr. Bures ETA-2020) and an UV detector (Jasco UV-2075Plus) were used at 35 °C with a flow rate of 1.0 mL/min. Four columns with MZ-SD Plus gel were applied. The length of each column was 300 mm, the diameter 8 mm, the diameter of the gel particles 5 μm, and the nominal pore widths were 50, 10^2, 10^3 and 10^4 Å. Calibration was achieved by using poly(methyl methacrylate) (PMMA) standards. For DMAc as eluting solvent a high pressure liquid chromatography pump (Bischoff HPLC) and a refractive index detector (Waters 410 Millipore) were used at 80 °C with a flow rate of 0.8 mL/min. Three columns with MZ-DVB gel were applied. The length of each column was 300 mm, the diameter 8 mm, the diameter of the gel particles 5 μm and the nominal pore widths were 10^2, 10^3 and 10^4 Å. Calibration was achieved using poly (methyl methacrylate) (PMMA) standards.

The Bio-Rad DC protein assay was used in order to determine the protein concentration of the isolated enzymes and of the protein–polymer conjugates using bovine serum albumin 99% purchased from Sigma–Aldrich as a standard. This assay is a colorimetric test for protein concentration similar to the well-documented Lowry assay. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: the reaction between protein and copper in an alkaline medium; and the subsequent reduction of Folin reagent by the copper-treated protein. The blue colour development is primarily due to the amino-acids tyrosine and tryptophan, and to a lesser extent to, cysteine, cystine and histidine. Thus the protein concentration was determined measuring the absorbance of the solution at 750 nm with a Varian Cary 100 Bio spectrophotometer after a calibration with bovine serum albumin was performed.

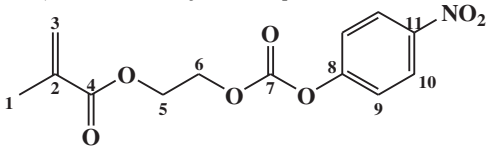
For the activity tests, the isolated enzymes as well as protein–polymer conjugates were weighed into safe-lock tubes and layered with a 0.1 M Tris–HCl buffer solution (1.9 mL, pH = 8) and the substrate solution (0.1% *para*-nitrophenyl laurate in acetonitrile, 20 mL) was added. After 20 min at 37 °C the amount of

para-nitrophenol produced was determined by measuring the extinction of the solution at 410 nm with a Varian Cary 100 Bio spectrophotometer. The reported values are an average of three measurements.

2.3. Syntheses

2.3.1. First concept

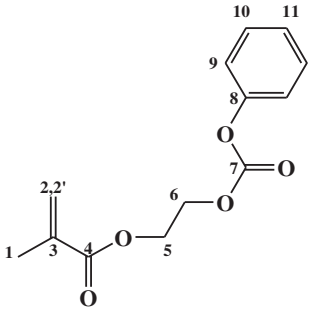
2.3.1.1. Synthesis of para-nitrophenoxycarbonyloxyethyl methacrylate (p-NPCEMA). To a solution of para-nitrophenyl chloroformate (4.65 g, 23 mmol) in dry CH_2Cl_2 (30 mL) was added a solution of dry pyridine (1.823 g, 23 mmol) in dry CH_2Cl_2 (30 mL). To this mixture, a solution of 2-hydroxyethyl methacrylate (HEMA, 3 g, 23 mmol) in dry CH_2Cl_2 (30 mL) was added dropwise at a temperature of 5 °C. The reaction mixture was stirred for 40 h at room temperature (rT). The product was washed once with distilled water, twice with HCl 1 M, twice with brine and dried on Na_2SO_4. The product obtained after evaporation of the solvent was a yellowish liquid. Recrystallization from hexane yielded *p*-NPCEMA (3.135 g, 47%) as a white crystalline powder.



^{1}H NMR ($CDCl_3$): δ = 1.97 (s, H^1); 4.49–4.54 (m, H^5, H^6); 5.64 (s, br, $H^{3'}$); 6.18 (s, br, H^3); 7.4 (d, H^9); 8.3 (d, H^{10}) ppm.

^{13}C NMR ($CDCl_3$): δ = 18.26 (C^1); 61.88 (C^5); 66.9 (C^6); 121.78 (C^{10}); 125.35 (C^9); 126.46 (C^3); 135.73 (C^2); 145.5 (C^{11}); 152.42 (C^7) 155.41 (C^8); 167.04 (C^4) ppm.

2.3.1.2. Synthesis of phenoxycarbonyloxyethyl methacrylate (PCEMA)[39]. To a solution of phenyl chloroformate (12.03 g, 80 mmol) in dry CH_2Cl_2 (60 mL) a solution of dry pyridine (Py, 9.11 g, 115 mmol) in dry CH_2Cl_2 (60 mL) was added. To this mixture, a solution of 2-hydroxyethyl methacrylate (HEMA, 10 g, 80 mmol) in dry CH_2Cl_2 (60 mL) was added dropwise at a temperature of 5 °C. The reaction mixture was stirred for 70 h at rT. The product was dissolved in CH_2Cl_2 (100 mL), washed twice with a 5% HCl solution and once with brine and dried on Na_2SO_4. The product obtained after evaporation of the solvent was a yellowish liquid. Yield of PCEMA: 17.22 g (90%).



^{1}H NMR ($CDCl_3$): δ = 1.96 (s, H^1); 4.46 (m, H^5, H^6); 5.59 (s, $H^{2'}$); 6.16 (s, H^2); 7.16–7.36 (m, phenyl and H^9, H^{10} and H^{11}).

^{13}C NMR ($CDCl_3$): δ = 18.26 (C^1); 62.15 (C^5); 66.23 (C^6); 120.98 (C^9); 126.17 (C^{11}); 126.34 (C^2); 129.58 (C^{10}); 135.82 (C^3); 151.06 (C^8) 153.54 (C^7); 167.00 (C^4).

*2.3.1.3. Synthesis of P(MMA-co-p-NPCEMA) (**rCoP1**).* Typical copolymerization procedure: In a Schlenk tube were mixed under nitrogen CuBr (29 mg, 0.2 mmol), bipyridine (Bpy, 62 mg, 0.4 mmol), *p*-NPCEMA (2.95 g, 10 mmol), MMA (1 g, 10 mmol), ethyl 2-bromoisobutyrate (EBrIB, 39 mg, 0.2 mmol) and butyl acetate (BuAc, 4 mL). The polymerization was started by placing the Schlenk tube in a thermostated oil bath at 90 °C. After a reaction time of 4 h the mixture was cooled to room temperature and quenched by addition of CH_2Cl_2 followed by stirring in air until the copper complex was completely oxidized. The copper complex was removed by washing with a solution of 5% HCl. The polymer was isolated by precipitation in pentane. Yield of the P(MMA-*co*-*p*-NPCEMA), (**rCoP1**): 1.2 g (31%). SEC-THF: M_n = 12,000 g/mol and M_w/M_n = 1.5.

^{1}H NMR ($CDCl_3$): MMA repeating units: δ = 0.88/1.05/1.26 (*rr*/*mr*/*mm*, s, 3 H, C***H*****$_3$**); 1.85 (br, 2 H, C***H*****$_2$**-backbone); 3.55 (s, br, 3 H, –O–C***H*****$_3$**); *p*-NPCEMA repeating units: δ = 0.88/1.05/1.26 (*rr*/*mr*/*mm*, s, 3 H, C***H*****$_3$**); 1.85 (br, 2 H, C***H*****$_2$**-backbone); 4.27/4.48 (s, br, 2 H/2 H, –O–C***H*****$_2$**–C***H*****$_2$**–O–); 7.4–8.28 (br, 4 H, aromatic).

*2.3.1.4. Synthesis of P(MMA-co-BMA-co-AMA-co-PCEMA) (**rCoP2**) [39].* Typical copolymerization procedure: In a Schlenk tube were mixed under nitrogen CuBr (0.49 g, 3.4 mmol), bipyridine (Bpy, 1.07 g, 6.8 mmol), PCEMA (10 g, 40 mmol), MMA (3 g, 30 mmol), BMA (2.84 g, 20 mmol), AMA (1.26 g, 10 mmol), ethyl 2-bromoisobutyrate (EBrIB, 0.667 g, 3.42 mmol) and butyl acetate (17.1 mL). The polymerization was started by placing the Schlenk tube in a thermostated oil bath at 90 °C. After a reaction time of 1 h the mixture was cooled to room temperature and quenched by addition of THF followed by stirring in air until the copper complex was completely oxidized. The copper complex was removed via column chromatography on aluminium oxide. The copolymer was isolated by precipitation in MeOH/H_2O (1/3 volume ratio). Yield of the copolymer P(MMA-*co*-BMA-*co*-AMA-*co*-PCEMA), (**rCoP2**): 7.2 g (41%). SEC-THF: M_n = 11,000 g/mol and M_w/M_n = 1.6.

^{1}H NMR ($CDCl_3$): MMA repeating units: δ = 0.9/1.03/1.4 (*rr*/*mr*/*mm*, s, –CH_3); 1.85 (br, –CH_2-backbone); 3.59 (s, –O–CH_3); BMA repeating units: δ = 0.9/1.03/1.4 (*rr*/*mr*/*mm*, s, –CH_3); 1.6 (br, –CH_2-backbone); 3.94 (s, br, –O–CH_2–); AMA repeating units: δ = 0.9/1.04/1.26 (*rr*/*mr*/*mm*, s, –CH_3); 1.9 (br, –CH_2-backbone); 4.46 (s, br, –O–CH_2–); 5.23–5.31 (m, –CH = C**H$_2$** allyl); 5.9 (broad signal, –C**H** = CH_2 allyl); PCEMA repeating units: δ = 0.9/1.03/1.4 (*rr*/*mr*/*mm*, s, –CH_3); 1.85 (br, –CH_2-backbone); 4.2–4.4 (s, br, –O–CH_2–CH_2–O–); 7.2–7.4 (br, phenyl).

2.3.2. Second concept

2.3.2.1. Procedure for lipase-catalyzed transacylation. Stock solutions of DMA/MMA and DMAPMA/MMA were prepared from MMA, 1-dodecanol and 3-dimethylamino-1-propanol with Novozyme 435 as catalyst according to Ref. [35].

*2.3.2.2. Preparation of P(MMA-co-DMA-co-DMAPMA-co-PCEMA), (**rCoP3**) [35].* P(MMA-*co*-DMA-*co*-DMAPMA-*co*-HEMA), (**fCoP**), SEC-DMAc: M_n = 59,000 g/mol and M_w/M_n = 2 was reacted with phenyl chloroformate according to reference 35 producing P(MMA-*co*-DMA-*co*-DMAPMA-*co*-PCEMA), (**rCoP3**) with SEC-DMAc: M_n = 49,000 g/mol and M_w/M_n = 2.5.

2.4. Representative procedure for synthesis of polymer–protein conjugates

2.4.1. Reaction with silk peptide, lysozyme and CALB

The **rCoP3** (80 mg, with 0.09 mmol PCEMA repeating units), soluble fraction from silk protein (104 mg) and 4-dimethylamino pyridine (DMAP, 11 mg, 0.09 mmol) were stirred in DMF (1 mL) for 48 h at 60 °C. At certain time intervals aliquots (10 μL) were withdrawn from this mixture and analyzed by SEC, using DMAc as eluent. The product was isolated by precipitation in diethylether. All other reactions were performed according to this procedure (Table 2). For the reactions 2, 3 and 4 protein loading assays were performed.

Table 2
Polymer–protein conjugates: starting materials and reaction conditions.

No.	Polymer (mg)	Protein (mg)	DMAP (mg/mmol)	DMF (mL)	Conditions
1	**rCoP3** (80)	Silk peptide (104)	11/0.09	1	T = 60 °C t = 48 h
2	**rCoP1** (80)	Silk peptide (80)	25.67/0.21	3	T = 60 °C t = 23 h
3	**rCoP1** (14)	Lysozyme (14)	1/0.008	1	T = 24 °C t = 43 h
4[a]	**rCoP1** (5)	CALB (4.3)	1.3/0.01	0.8	rT t = 4 h

[a] Enzymatic activity of the bioconjugate was determined.

2.4.2. Reaction with CALB in miniemulsion

The **rCoP2** (0.2 g with 0.46 mmol PCEMA) and hexadecane (100 mg) were dissolved in toluene (2.2 g). This mixture and a 1.0 wt.% Lutensol AT50 solution in water (10.0 g, surfactant solution) were vigorously stirred for 1 h at 45 °C. The miniemulsion was prepared by ultrasonicating the mixture during 120 s at 90% amplitude (Branson sonifierW450 digital). A solution of CALB (3 mL with 19 mg/mL protein, 25 mM sodium phosphate buffer, pH 7) was added to the miniemulsion and stirred at ambient temperature. After 19 h the mixture was freeze dried, and the solid was washed with ethanol and with water. The washed conjugate was dried in vacuo for 24 h and then the protein concentration (1.2 mg protein/10.5 mg bioconjugate) was determined.

3. Results and discussion

The goal of the present work is to prepare bioconjugates starting with polymethacrylates bearing phenyl carbonate reactive groups and silk peptides, lysozyme and *C. antarctica* lipase B. From the polymer perspective, the attachment of a protein may provide a synthetic polymer with unique functional and structural properties. One of the strategies for covalent linkage of proteins/peptides to a synthetic polymer comprises reaction of the ε-amino group of lysine residues in the protein with phenyl carbonate groups in the side chains of the polymer (lysines can make up to 6% of the overall amino acid sequence [41,42]). In general, the ε-amino group of lysine is known to react with electrophilic reagents such as activated esters, aldehydes or ketones and isocyanates/isothiocyanates.

One of the approaches to synthesize functional and reactive poly(methacrylate)s makes use of 2-hydroxyethyl methacrylate (HEMA) which can be functionalized with *p*-nitrophenyl chloroformate or phenyl chloroformate. The obtained reactive monomers para-nitro phenoxycarbonyloxy ethyl methacrylate (*p*-NPCEMA) and phenoxycarbonyloxy ethyl methacrylate (PCEMA) were copolymerized with different methacrylates via free radical polymerization or controlled radical polymerization techniques. The atom transfer radical polymerization (ATRP) [43–45] of *p*-NPCEMA

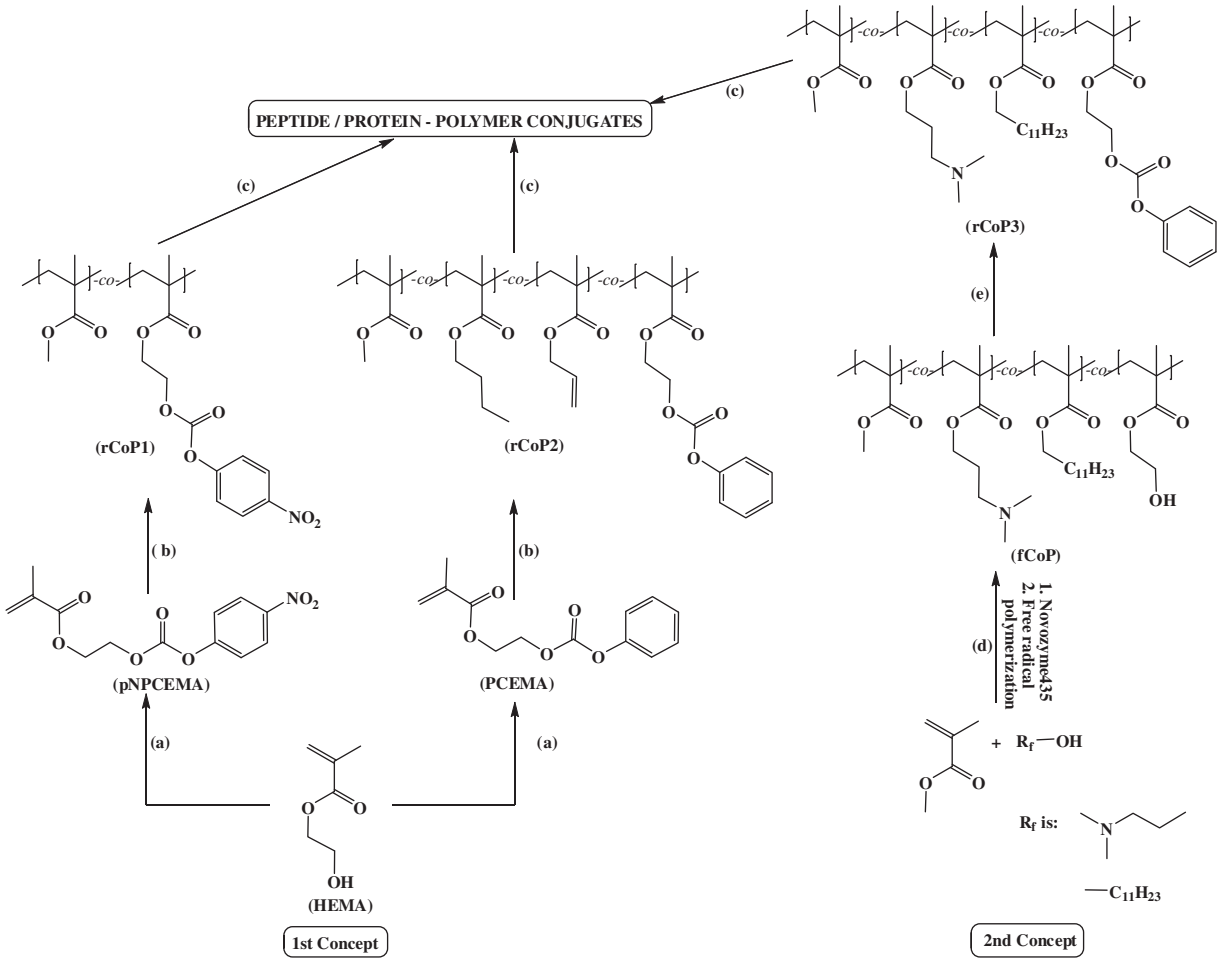


Fig. 1. Schematic representation for the preparation of peptide/protein–polymer conjugates. 1st Concept: (a) preparation of reactive monomers; (b) copolymerization via ATRP to result in reactive polymers; (c) reaction with the peptide/protein. 2nd Concept: (d-1) enzyme-catalyzed transacylation; (d-2) copolymerization of the monomer mixture by FRP obtaining functional polymers; (e) polymer analogous reaction to result in reactive polymers.

with MMA as well as of PCEMA with MMA, BMA and AMA are presented (Fig. 1, 1st concept).

An alternative to the chemical monomer synthesis was previously reported and makes use of a cascade reaction comprising a sequence of enzymatic transacylation and free radical polymerization [35]. Thus, highly functional poly(methacrylate)s presenting besides hydrophilic, hydrophobic and ammonium groups, also reactive groups exemplified by phenyl carbonate, were prepared (Fig. 1, 2nd concept).

3.1. Synthesis of the reactive poly(methacrylate)s

3.1.1. First concept

The synthesis of the monomer PCEMA was described in a previous work [39], and implies the commercially available 2-hydroxyethyl methacrylate (HEMA) as starting material. During this work we used dry pyridine as acid scavenger and the yield of the product was enhanced from 75% (when triethyl amine was used) to 90%. The commercially available HEMA was used as starting material also for the synthesis of the monomer *p*-NPCEMA. A solution of HEMA in dry CH_2Cl_2 was allowed to react with *p*-nitrophenyl chloroformate in the presence of dry pyridine as acid scavenger, at a temperature not higher than 25 °C. The whole system has to be free of water, because *p*-nitrophenyl chloroformate reacts with water. All characteristic signals of the monomer are observed in the ^1H and ^{13}C NMR spectra with no impurities detected (Fig. 2A). Both reactive monomers were stored under nitrogen in the dark in order to avoid adventitious polymerization.

In the first polymerization experiments, *p*-NPCEMA was copolymerized with MMA in a molar ratio of 1:1, via ATRP. ^1H NMR analysis of the resulting copolymer revealed that the two monomers are incorporated into the polymer in a ratio of 1:1 as was expected from the ratio in the feed (Fig. 2B) (compare intensity of signal A and signals 5 and 6). Based on the integration of the repeating unit signals and the integration of the methylene group of the initiator (a), a molecular weight of 16,000 g/mol was calculated for **rCoP1**.

From SEC analysis using THF as the eluting solvent a molecular weight of M_n = 12,000 and a polydispersity of M_w/M_n = 1.5 was determined. The discrepancy between the molecular weight determined by NMR spectroscopy and SEC chromatography can be explained by the calibration protocol based on PMMA standards, by the accuracy of ^1H NMR measurement and by poor signal resolution. Moreover, the symmetry of the SEC traces using UV and RI detectors confirm a random distribution of the UV active compound within the copolymer, Fig. 3.

Further, a multifunctional copolymer, **rCoP2**, was prepared via ATRP using MMA, BMA, AMA and PCEMA in a 30/20/10/40 mol% ratio as described elsewhere [39]. While the ratio of MMA and BMA defines the bulk properties, the percentage of PCEMA defines the degree of potential functionalization of the copolymer, the concentration of the allyl group was 10 mol% since such a concentration is sufficient for cross-linking and hence for surface structuring via cross-linking [46]. The SEC-THF analysis shows a M_n = 11,000 g/mol and a monomodal molecular weight distribution with a polydispersity of M_w/M_n = 1.6.

The PDI value is relatively high for a controlled polymerization and might be due to the side reaction caused by AMA and by the carbonate side-groups, since it is known that phenyl carbonates and *p*-nitrophenyl carbonates are activated in the presence of tertiary amines for nucleophilic substitution, and the –C,C– double bonds in the allyl side chains at high monomer conversions leads to branched and cross-linked structures. In addition, the symmetry of the SEC-UV/RI traces reveals a random distribution of the aromatic side chains in the copolymer illustrated by Fig. 4.

3.1.2. Second concept

In order to prepare functional methacrylates we investigated the transacylation of MMA as substrate and different functional alcohols (DMAP and D-ol) as reagents with Novozyme 435 as catalyst. Under the applied conditions this transacylation leads to the formation of a mixture of two methacrylates (DMAPMA or DMA and MMA) in two alcohols (DMAP or D-ol), and CH_3OH, respectively. It is worth mentioning that all the transacylation reactions were performed in bulk. An exhaustive study of these transacylations has been recently presented [35]. ^1H NMR spectroscopy served as tool for determination of the concentration of dimethylamino propyl methacrylate (DMAPMA) and dodecyl methacrylate (DMA) using the signals of the protons attached to sp^2 hybridized

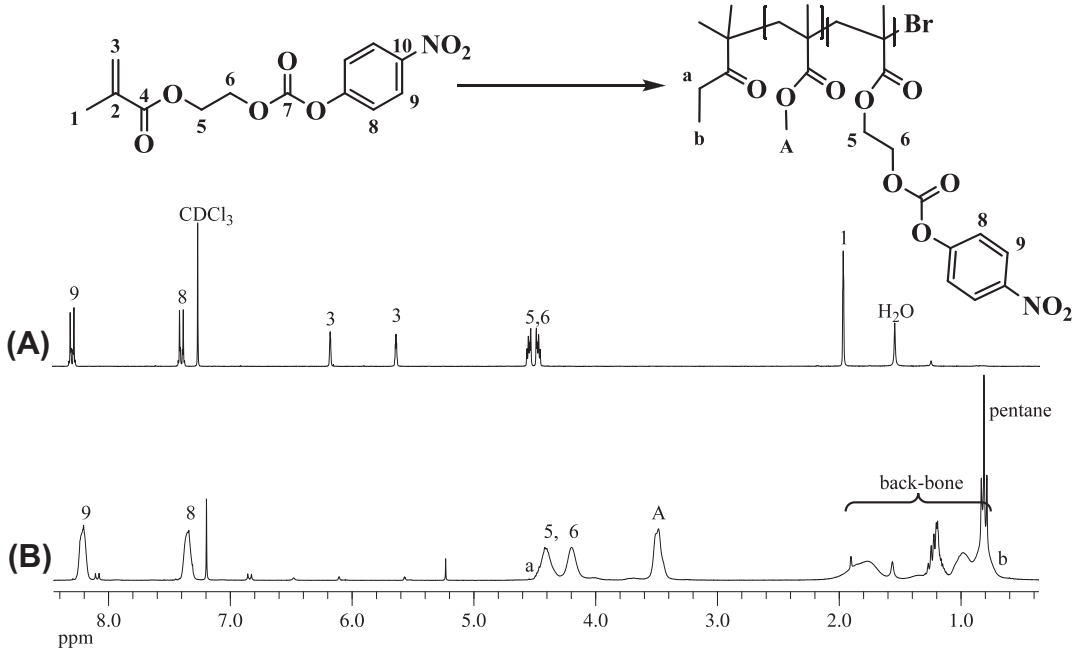


Fig. 2. ^1H NMR spectrum of (A) *p*-NPCEMA and (B) of the P(MMA-*co*-*p*-NPCEMA) (**rCoP1**).

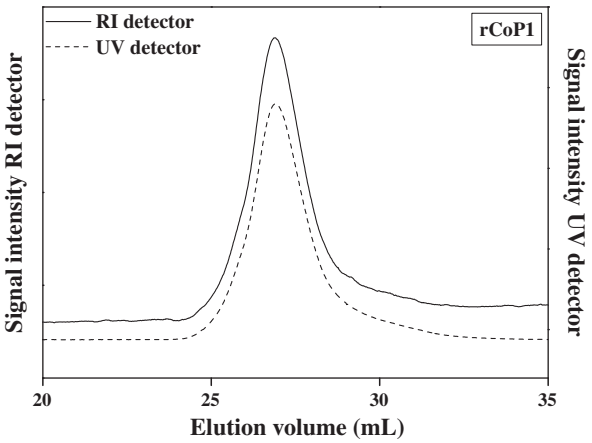


Fig. 3. SEC traces (solvent THF) using UV/RI detectors for the **rCoP1**.

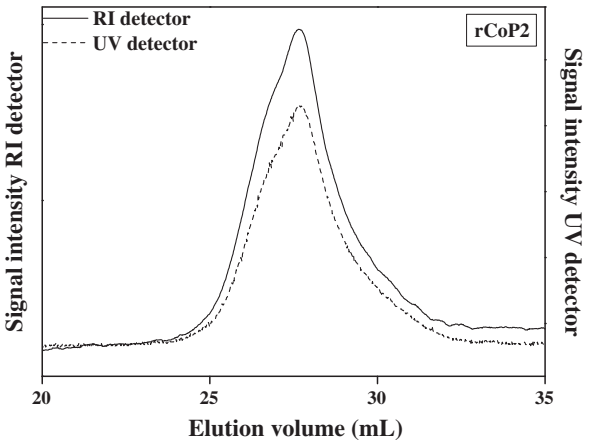


Fig. 4. SEC traces (solvent THF) using UV/RI detectors for the **rCoP2**.

carbon atoms of the product and MMA at δ = 5.5–6.1 ppm as a reference, and the signal corresponding to the methylene group of DMA at δ = 4.13 ppm and of δ = 4.20 ppm for DMAPMA.

The concept of cascade reactions was applied for the synthesis of multifunctional polymers: starting with MMA and a functional alcohol ROH, in the first step a transacylation was performed resulting a mixture of two monomers (MMA and RMA), which, in the second step, after the removal of the enzyme by filtration and after addition of 2-hydroxyethyl methacrylate (HEMA) – was subjected to free radical polymerization. The ratio of monomers was determined by means of ^{1}H NMR spectroscopy. Usually the signals adjacent to the functional group were selected to quantify the ratio of repeating units. ^{1}H NMR analyses show characteristic signals for each repeating unit, and based on the integration of these signals the composition of copolymers was determined. Fig. 5A depicts the NMR peaks used for the determination of the copolymer composition: for MMA δ = 3.59 ppm (signal 1); for DMAPMA δ = 4.01 (signal 2), 2.2 (signal 5) and 2.3 ppm (signal 4); for DMA δ = 3.9 (signal 6) and 1.27 ppm (signal 8) and for HEMA δ = 3.8 ppm (signal 10). Based on the integration of the signals of protons of the repeating units the composition of the **fCoP** was determined $(MMA)_{51}$-*co*-$(DMAPMA)_{17}$-*co*-$(DMA)_{12}$-*co*-$(HEMA)_{20}$ with the indices representing the molar ratio of repeating units.

Via cascade reaction we obtained multifunctional polymers with hydrophobic, hydrophilic, tertiary amine and hydroxy groups (**fCoP**). The hydroxy groups can be esterified with phenyl chloroformate and the tertiary amine groups are prone for quaternization. The preparation of the reactive copolymer **rCoP3** will be discussed as an example. **fCoP** was successfully converted in methylene chloride solution in the presence of pyridine as acid scavenger into the respective ester **rCoP3**. The course of reaction was followed by means of ^{1}H NMR spectroscopy (Fig. 5B). The conversion of HEMA repeating units into PCEMA repeating units is noticed by the characteristic signals of the protons of the phenyl

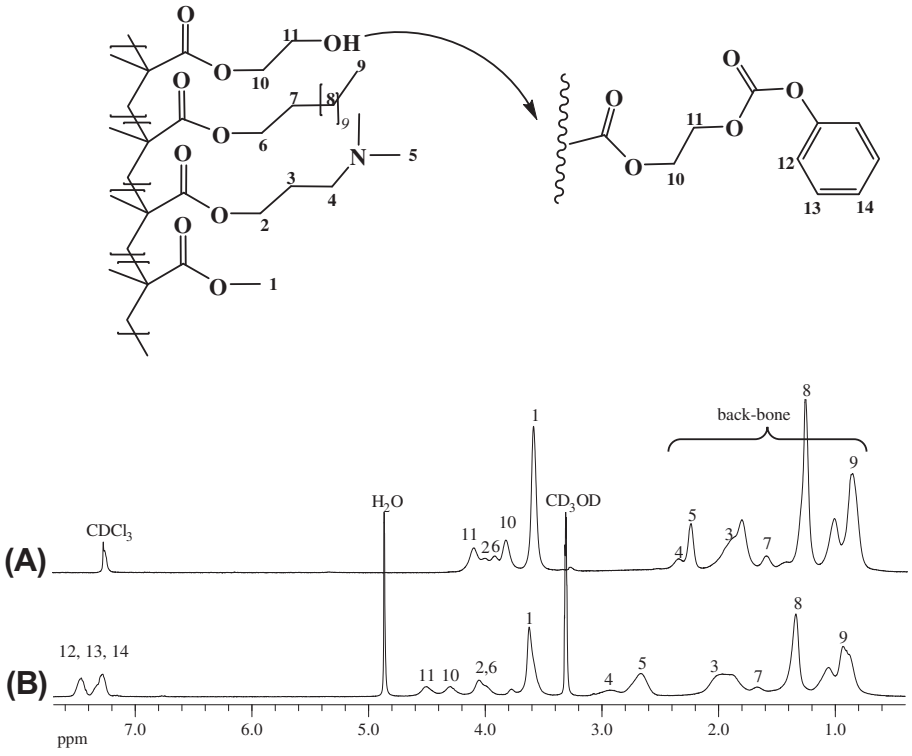


Fig. 5. ^{1}H NMR spectrum of (A) **fCoP**, the copolymer P(MMA-*co*-DMAPMA-*co*-DMA-*co*-HEMA) and (B) **rCoP3** bearing the reactive carbonate group.

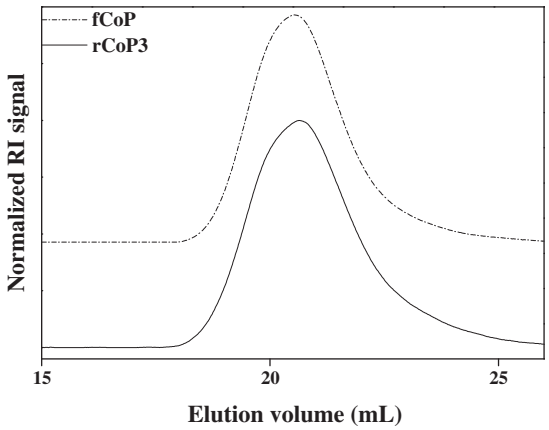


Fig. 6. SEC traces (solvent DMAc) of the functional copolymer (**fCoP**) and the reactive copolymer (**rCoP3**).

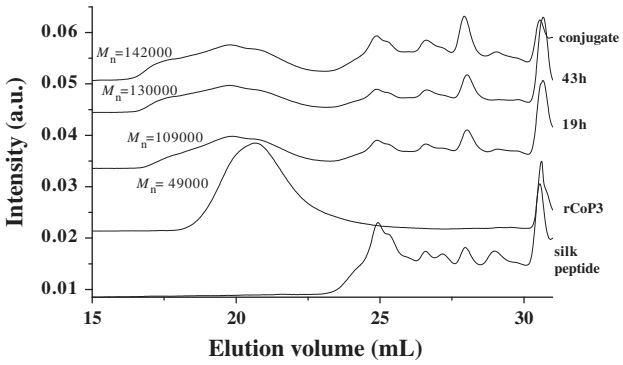


Fig. 7. SEC traces (solvent DMAc) of the silk peptide, of the reactive copolymer **rCoP3** and of different samples taken from the reaction between **rCoP3** and the silk peptide.

carbonate groups which appear in the aromatic region of the spectra at δ = 7.2–7.4 ppm as well as from the shift of the signals of the ethylidene protons from δ = 3.9–4.0 ppm in HEMA repeating units to δ = 4.2–4.4 ppm in PCEMA repeating units.

Fig. 6 depicts the SEC traces of the functional copolymer (**fCoP**) as well as of the reactive copolymer (**rCoP3**) using DMAc as eluent. The decrease of the molecular weight from 59,000 g/mol of the functional copolymer to 49,000 g/mol for the reactive one might be explained by the differences of the hydrodynamic radius of the copolymers in DMAc solvent, while the increase of the polydispersity from 2 (**fCoP**) to 2.5 (**rCoP3**) can be ascribed to the interactions of the polymer with the column leading to a bimodal distribution.

3.2. Synthesis of polymer–protein conjugates

All the reactive copolymers (Table 3) prepared via the two concepts presented in this paper were purified and were reacted with different proteins in order to investigate the potential application of these materials for synthesizing bioconjugates or even for enzyme immobilization.

The first experiment between a polymer and a protein was performed using the reactive copolymer, **rCoP3** and the soluble fraction of silk peptide. The soluble fraction of silk peptide contains different amino-acids suitable for covalent linking. Lysine, histidine and arginine are convenient for conversion of the free amino group with the reactive side chains of the copolymer.

The polymer dissolved in dimethylformamide (DMF) was added to the peptide solution in DMF. 4-Dimethylaminopyridine (DMAP) was used in a catalytic amount, since the reaction requires the presence of a base. The mixture was stirred at 60 °C for various times, and samples were analyzed via size exclusion chromatography (SEC) using dimethyl acetamide (DMAc) as eluent. It was observed that at the beginning of the reaction the peptide was not soluble; after 19 h a viscose suspension is obtained. Fig. 7 shows the DMAc-SEC eluograms for starting materials: the silk peptide and the functional copolymer (**rCoP3**) as well as the samples taken at different times of the reaction. The bioconjugate was isolated by precipitation in diethyl ether and SEC analysis revealed a M_n = 142,000 g/mol. It can clearly be seen that part of the silk protein was preferentially linked to the polymer since the molecular weight of the hybrid fraction is different from that of the polymer fraction.

In order to increase the reaction rate instead of phenyl carbonate group, *p*-nitro phenyl carbonate was chosen as leaving group as described in the literature [32].

The reactive copolymer, **rCoP1**, with a concentration of 54 mol% *p*-NPCEMA was used for the reactions with silk peptide, lysozyme and CALB. All these reactions were performed in DMF as solvent and are presented in Table 4.

In the reaction the polymer **rCoP1**, silk peptide and DMAP were dissolved in DMF and stirred at 60 °C for 23 h. Due to solubility problems SEC analysis was not performed. At the end of the reaction the bioconjugate was isolated by precipitation in diethyl ether and dried under vacuum (10^{-3} mbar). The protein content in the isolated conjugate was determined as described in Section 2 and compared with the pure silk peptide's protein content. Thus, for the reaction between **rCoP1** and silk peptide, for 7 mg conjugate a protein content of 1.373 mg was determined using the Bio-Rad DC protein assay. The efficiency of bioconjugation was determined from the values obtained from the protein content test reported to the amount of the polymer and protein in the feed. The same procedure was applied for the reaction 3, between the reactive copolymer **rCoP1** and lysozyme.

Table 3
Overview of the reactive poly(methacrylate)s used.

Name	Repeating units in the copolymer (mol%)			
	MMA	*p*-NPCEMA/PCEMA	BMA/DMAPMA	AMA/DMA
rCoP1	46	*p*-NPCEMA 54	–	–
rCoP2	30	PCEMA 39	BMA 22	AMA 9
rCoP3	50	PCEMA 18	DMAPMA 16	DMA 11

The composition (mol%) was calculated from the ^{1}H NMR spectrum using the characteristic signals of each repeating unit as described in Section 3 of this paper.

Table 4
Peptide/protein–polymer bioconjugates. Protein content and activity results.

No.	Bioconjugate	Mass bioconjugate[a] (mg)	Mass protein[a] (mg)	Yield[b] (%)	CALB activity[c]
1[d]	**rCoP3-Silk**	n.d.	n.d.	n.d.	n.d.
2	**rCoP1-Silk**	7	1.373 (20%)	65%	n.d.
3	**rCoP1-Lyz**	4.5	1.07 (24%)	89%	n.d.
4	**rCoP1-CALB**	3	0.31 (10%)	22%	0.437
5[e]	**rCoP2-CALB**	10.5	1.2 (11%)	51%	1.253

[a] Determined using Bio-Rad DC protein assay, as described in Section 2.
[b] Efficiency of bioconjugation was determined from the values obtained from the protein content test reported to the amount of the polymer and protein in the feed.
[c] CALB activity determined as described in Section 2. All the values represent the extinction at 410 nm. The pure enzyme has an extinction of 1.859 for 60 μg CALB. For reaction 4 the extinction was determined for 6 μg CALB, while for reaction 5 the extinction was determined for 60 μg CALB.
[d] Protein content and activity tests not determined. Reaction followed by SEC.
[e] Reaction performed in miniemulsion.

For reaction (4) besides the protein content in the bioconjugate, activity tests were also performed using 0.1 wt.% *p*-nitrophenyl laurate (pNPL) as substrate. The samples were incubated for 20 min at 37 °C, and then the extinction was determined at a wave length of 410 nm measuring the formation of *p*-nitrophenol. For the pure CALB (60 μg CALB) an extinction of 1.859 was determined while for the bioconjugate **rCoP1-CALB** was calculated an extinction of 0.437 (6 μg CALB, due to the low amount of bioconjugate).

In general, the difficulty of this approach lies, on one hand, in the reduced accessibility of functional groups within the polymer which can limit the conversion, and on the other in the isolation of the desired conjugate from the reaction mixture. Therefore we used a new strategy for covalently linking CALB with a functional and reactive copolymer. In order to do so, a miniemulsion of the copolymer **rCoP2** in water was firstly prepared, according to the literature [47]. To this mixture was added a sodium phosphate buffer solution with protein and stirred for 19 h. After removing the water, washing the solid with ethanol and water for removing the free enzyme and the buffer, the white solid was dried and analyzed for the protein content and the activity. A protein content of 11% in the bioconjugate was determined, which is slightly higher than the value obtained for the **rCoP1** copolymer and can be explained by the composition of the two copolymers. However, in the miniemulsion reaction the efficiency of bioconjugation is clearly higher than in the solution reaction and might be explained by the large surface of the polymer which is exposed for the interactions with the functional groups of the enzyme.

4. Conclusions

Highly functional and reactive polymethacrylates were synthesized via two concepts. We exemplify the first concept with the synthesis of two reactive methacrylates, which were subjected to controlled radical polymerization (ATRP) with MMA, BMA and AMA. The second strategy for obtaining multifunctional and reactive poly(methacrylate)s makes use of the cascade reaction concept. Transacylation of methyl methacrylate as a substrate with 3-dimethylamino-1-propanol and dodecanol in the presence of Novozyme 435 leads to a mixture of monomers, which in a subsequent step are copolymerized with HEMA via free radical polymerization resulting in polymethacrylates with predefined functionalities. The hydroxyl groups of HEMA are later converted with phenyl chloroformate into reactive PCEMA repeating units. An interesting application of these multifunctional and reactive polymethacrylates is their ability to react with different peptides/proteins. This work explores the covalent binding of different proteins like silk peptide, lysozyme and CALB to the reactive groups attached to the polymethacrylate backbone. This way different bioconjugates were prepared. Although in the case of silk peptide and lysozyme it was measured a protein content of 20% and 24%, in the case of CALB no more than 11% of protein was determined in the bioconjugate. The bioconjugates of CALB and different polymers exhibit also enzyme activity. An interesting approach for the formation of peptide/protein–polymer bioconjugates was achieved by using a miniemulsion for the reaction of the polymer with peptides.

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