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RESEARCH PAPER

Synthesis and In Vitro Characterization of a Poly(Acrylic Acid)-Homocysteine Conjugate

Andreas Bernkop-Schnürch,[1,*] Verena Leitner,[2] and Verena Moser[2]

[1]Institute of Pharmacy, Department of Pharmaceutical Technology, Leopold-Franzens-University Innsbruck, Innsbruck, Austria
[2]Center of Pharmacy, Institute of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Vienna, Austria

ABSTRACT

It was the aim of this study to improve our knowledge on thiolated polymers by the synthesis and in vitro characterization of a poly(acrylic acid)-homocysteine conjugate. Mediated by a carbodiimide, homocysteine was therefore covalently attached to poly(acrylic acid) via the formation of an amide bond. The isolated conjugate displayed 930 μmol ± 83 μmol sulfur atoms per gram polymer. Of these thiol groups, 80.1% were oxidized to disulfide bonds during the coupling reaction. In aqueous solutions the conjugate was rapidly oxidized by the formation of disulfide bonds at pH 8, whereas it remained stable at pH 7 and below during the observation period of 4 hours. Due to the immobilization of thiol groups on the polymer, the mucoadhesive and cohesive properties of poly(acrylic acid) were strongly improved. Furthermore, the thiolated polymer exhibited a significantly ($p < 0.05$) improved permeation enhancing effect in comparison to the unmodified polymer. Because of these features the poly(acrylic acid)-homocysteine conjugate seems to represent a promising novel tool, which might be useful in particular for aqueous formulations based on thiomers.

Key Words: Thiomers; Homocysteine; Poly(acrylic acid); Mucoadhesion; Permeation enhancement.

*Correspondence: Andreas Bernkop-Schnürch, Institute of Pharmacy, Department of Pharmaceutical Technology, Leopold-Franzens-University Innsbruck, Josef Moeller Haus, Innrain 52, 6020 Innsbruck, Austria; Fax: ++43-512-507-2933; E-mail: andreas.bernkop@uibk.ac.at.

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INTRODUCTION

The concept of mucoadhesion was pioneered two decades ago. Since then, numerous attempts have been undertaken in order to improve the adhesive properties of polymers. These attempts are based on approaches such as the development of polymer-adhesion conjugates providing a specific binding to epithelia (e.g., Refs. [1,2]), the use of adhesion promoters like poly(ethylene glycols),[3] and the use of in situ gelling polymers.[4] All these systems are based on the formation of non-covalent bonds such as hydrogen bonds, van der Waal's forces, and ionic interactions providing only weak adhesion of the drug delivery system to mucosal membranes.

Recently, however, a new generation of mucoadhesive polymers has been established, which is capable of forming covalent bonds with the mucus gel layer.[5] The most commonly bridging structure in biological systems, the disulfide bond, has thereby been discovered for the adhesion of polymers to the mucus layer. *Thio*lated poly*mers* or the so-called *thiomers* are able to form disulfide bonds with cysteine-rich subdomains of mucus glycoproteins. Because of the immobilization of thiol groups to poly(acrylic acid) and chitosan, for instance, the mucoadhesive properties of these polymers were improved 100-fold and 250-fold, respectively.[6,7] Apart from these excellent mucoadhesive properties, which should provide a prolonged residence time of the delivery system on mucosal membranes, thiomers also exhibit high cohesive properties and a strong permeation enhancing effect.[6,8,9] Among all thiolated polymers developed so far, poly(acrylic acid)-cysteine conjugates turned out to be the most promising. They exhibit the comparatively highest mucoadhesive and permeation enhancing properties.[10] In addition, as poly(acrylic acid) is not absorbed from mucosal tissues[11] and is not biodegradable, the toxicological studies for thiolated poly(acrylic acids) as a new pharmaceutical excipient can be reduced to a minimum.

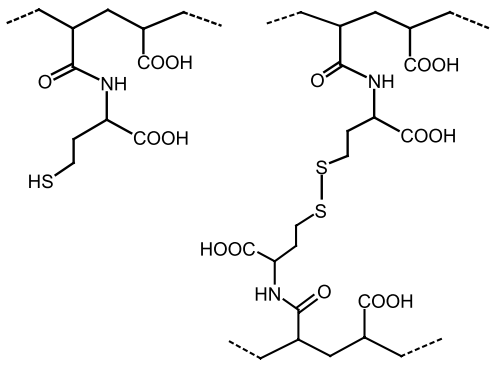

Figure 1. Presumptive substructures of the poly(acrylic acid)-homocysteine conjugate.

In order to improve our knowledge on thiolated polyacrylates, it was the aim of this study to synthesize and characterize a novel thiolated poly(acrylic acid) derivative. For cationic thiolated polymers, it was shown that a comparatively less reactive thiol substructure led to higher mucoadhesive properties.[7] As an analogy, a thiolated polyacrylate bearing less reactive sulfhydryl groups than poly(acrylic acid)-cysteine conjugates should also display comparatively higher mucoadhesive properties. Furthermore, the stability of the polymer towards oxidation in aqueous solutions should be improved. As homocysteine has a less reactive thiol group than cysteine, it was chosen as a ligand for poly(acrylic acid). Accordingly, a poly (acrylic acid)-homocysteine conjugate (PAA-HC conjugate) as shown in Fig. 1 was synthesized and evaluated in vitro with regard to its mucoadhesive properties and stability. In addition, essential further features such as swelling behavior, cohesive properties, and its permeation enhancing effect were analyzed.

MATERIALS AND METHODS

Synthesis of Polymer-Homocysteine Conjugates

The covalent attachment of homocysteine to poly(acrylic acid) (exclusively linear, MM: 450 kDa in average; PAA_{450}; Sigma, St. Louis, MO) was achieved by the formation of amide bonds between the primary amino group of the amino acid and a carboxylic acid group of the polymer. First, 0.5 g of PAA_{450} was hydrated in 40 mL demineralized water and the pH value of the PAA_{450} solution was adjusted to 5 by the addition of 5 M NaOH. Then, the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma, St. Louis, MO) was added in a final concentration of 150 mM in order to activate the carboxylic acid moieties of hydrated polymer. After 20 min of incubation under stirring at room temperature, 0.4 g homocysteine (Sigma, St. Louis, MO) was added and the pH was readjusted to 5. The reaction mixture was incubated for 5 h at room temperature under stirring. The resulting conjugate was isolated by dialysis according to the method previously described for PCP-Cys.[12] A control polymer was prepared and isolated in the same way as described for the PAA_{450}-HC conjugate, however, the carbodiimide (EDAC) was omitted during the

coupling reaction. After dialysis, the pH value of the polymers was readjusted to 5 and frozen aqueous polymer solutions were dried by lyophilization at −30° C and 0.01 mbar (Christ Beta 1–8 K; Osterode am Harz, Germany). The conjugate and control were stored at 4° C until further use.

Determination of Thiol Groups and Disulfide Bonds Within the Polymer

The degree of modification, i.e., the amount of thiol groups immobilized on the polymer, was determined photometrically with Ellman's reagent quantifying free thiol groups. First, 0.5 mg each of the conjugate and control was hydrated in 500 μL of 0.5 M phosphate buffer pH 8.0 and then 500 μL of Ellman's reagent [3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 10 mL of 0.5 M phosphate buffer pH 8.0] were added. The samples were incubated for 2 hours at room temperature. Thereafter, 300 μL of each sample was transferred into a microplate and the absorbency was measured at a wavelength of 450 nm with a microplate reader (Anthos reader 2001; Salzburg, Austria). L-Homocysteine standards were used to calculate the amount of thiol groups immobilized on the polymer.

The disulfide content of the polymer was measured after reduction with $NaBH_4$ and addition of Ellman's reagent, as described by Habeeb.[13] Orientating studies performed with various disulfide-bearing compounds such as cystine showed that under these test conditions, $NaBH_4$ is capable of quantitatively reducing disulfide bonds.

Disulfide Bond Formation Within the PAA-HC Conjugate

The PAA_{450}-homocysteine conjugate was hydrated in demineralized water in a final concentration of 0.25% (m/v) at pH 5. The pH of the solution was adjusted to 5, 6, 7, and 8, respectively. All samples were incubated at 37° C under continuous shaking. At predetermined time points aliquots were withdrawn and the amount of remaining thiol groups was determined with Ellman's reagent as described above.

Preparation of Tablets

Lyophilized PAA-HC conjugate and control were compressed (Hanseaten Type El, Hamburg, Germany) into 5.0-mm-diameter flat-faced tablets of 30 mg. The compaction pressure was kept constant during the preparation of all tablets.

Evaluation of the Swelling Behavior

The water absorbing capacity was determined by a gravimetric method as described previously.[14] Tablets were fixed to a needle and immersed in 100 mM phosphate buffer pH 6.8 at 37° C. At predetermined time points the hydrated tablets were taken out of the incubation medium, excess water was removed, and the amount of water uptake was determined gravimetrically.

Disintegration Studies

The disintegration behavior of the tablets in 100 mM phosphate buffer pH 6.8 at 37° C was analyzed with a disintegration test apparatus according to the European Pharmacopeia. The oscillating frequency was adjusted to 0.5 s^{-1}.

In Vitro Evaluation of the Mucoadhesive Properties

Tensile Studies

Tensile studies were carried out on native porcine intestinal mucosa. Each tablet as described above was glued to a stainless steel flat disc (5 mm in diameter), which was attached to a laboratory stand with a nylon thread (15 cm). The porcine mucosa was fixed to a glass support using a cyanoacrylate adhesive (Loctite, Henkel, Austria) placed in a beaker and completely immersed with 0.1 M phosphate-buffered saline pH 6.8. The beaker was placed on a balance and carefully raised by a mobile platform until the mucosa came in contact with the tablet. After an incubation time of 30 min at 25° C, the mucosa was pulled down from the tablet at a rate of 0.1 mm/s. Data points were collected every second by a personal computer (WINWEDGE software; TAL Technologies Inc., Philadelphia, PA) connected to the balance. The total work of adhesion (TWA) representing the area under the force/distance curve and the maximum detachment force (MDF) were calculated with EXCEL 97 (Microsoft, Vienna, Austria).[14]

Rotating Cylinder Method

Polymer tablets were attached to freshly excised intestinal porcine mucosa, which had been attached to a stainless steel cylinder (diameter: 4.4 cm, height 5.1 cm; apparatus 4-cylinder, USP XXVI) using a cyanoacrylate adhesive. The cylinder was placed in the dissolution apparatus according to the USP, fully immersed with 0.1 M phosphate buffer pH 6.8 at 37° C,

and agitated at 125 rpm. The detachment, disintegration, and/or erosion of test tablets was monitored over a 48-h time period.[12]

Permeation Studies

Permeation studies were carried out in Ussing type chambers displaying a volume of 1 mL (=1 cm^3) in the donor- and acceptor-compartment and permeation area of 0.64 cm^2. To mimic the intestinal fluid, an incubation medium was prepared containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose, and 50 mM $NaHCO_3$ buffered with 40 mM HEPES, pH 7.4.

Immediately after sacrificing the guinea pig 15 cm of the small intestine (duodenum) were excised and mounted in the Ussing chamber. All experiments were performed at least five times in an atmosphere of 95% O_2 and 5% CO_2 at 37° C. After 15–20 minutes of preincubation with the artificial intestinal fluid, the incubation medium of the donor compartment was substituted by either PAA-HC conjugate (0.5% m/v) containing 0.5% (m/v) of the permeation mediator reduced glutathione (GSH) or the corresponding unmodified polymer. Furthermore, each sample contained 0.001% (m/v) Na-Flu as model compound. Samples of 100 µL were withdrawn from the acceptor compartment every 30 minutes over a time period of 3 hours. Samples were immediately replaced by 100 µL artificial intestinal fluid equilibrated at 37° C. The amount of permeated Na-Flu was determined using a fluorimeter (SLT, Spectra Fluor; Tecan, Austria). Cumulative corrections were made for the previously removed samples. The apparent permeability coefficients (P_{app}) for Na-Flu were calculated according to the following equation:

$$P_{app} = Q/(A*c*t)$$

where P_{app} is the apparent permeability coefficient (cm/sec), Q is the total amount permeated within the incubation time (µg), A is the diffusion area of the Ussing chamber (cm^2), c is the initial concentration of the marker in the donor compartment (µg/cm^3), and t is the total time of the experiment (sec). Transport enhancement ratios (R) were calculated from P_{app} values according the following equation

$$R = P_{app}(PAA - HC)/P_{app}(PAA)$$

Statistical Data Analysis

Statistical data analysis was performed using Student's t-test with $p<0.05$ as the minimal level of significance. Calculations were done utilizing the software Xlstat version 5.0 (b8.3).

RESULTS

Basic Characterization of Poly(Acrylic Acid)-Homocysteine Conjugate

The poly(acrylic acid)-homocysteine conjugate has been synthesized in the same way as poly(acrylic acid)-cysteine conjugates. The new conjugate showed 930 µMol ± 83 µMol sulfur atoms per gram polymer. Of these thiol groups, 80.1% were oxidized to disulfide bonds during the coupling reaction. A control prepared in the same way as the poly(acrylic acid)-homocysteine conjugate but with the carbodiimide omitted during the coupling reaction displayed only a negligible amount of remaining traces of homocysteine. The conjugate appeared as a white, odorless powder of fibrous structure.

Stability studies of the conjugate in aqueous solutions demonstrated that at pH 7 and below, the thiol groups were no longer subject to the oxidation process. In contrast, at pH 8 and above, a rapid formation of disulfide bonds took place. The results of this study are shown in Fig. 2. The poly(acrylic acid)-homocysteine conjugate is therefore comparatively more stable in aqueous solutions than the poly(acrylic acid)-cysteine conjugate, which is already rapidly oxidized at pH 7.[15] These findings are in good agreement with the theory that

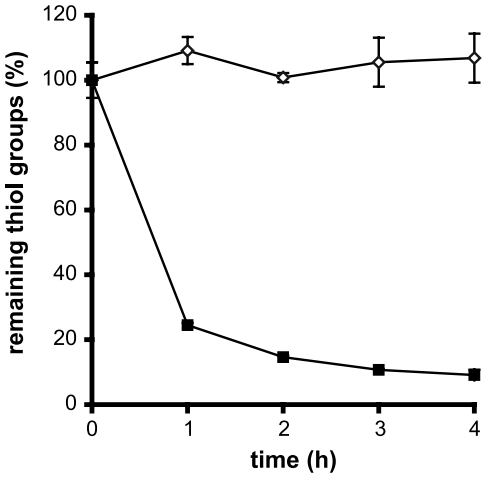

Figure 2. Decrease in thiol groups of 0.25% (m/v) poly (acrylic acid)-homocysteine conjugate in aqueous solutions at pH 7 (◇) and pH 8 (■); indicated values are means of three experiments ± SD. (*View this art in color at www.dekker.com.*)

less reactive thiol groups are to a lower degree subject to the oxidation process. The oxidation of the poly(acrylic acid)-homocysteine conjugate at pH values above 7 leads to a crosslinking of the polymer and an increase in viscosity, which was recently shown in detail for poly-(acrylic acid)-cysteine conjugates.[6]

Swelling Behavior and Cohesive Properties

The mucoadhesive and cohesive properties of polymers are strongly influenced by their swelling behavior. On the one hand, a rapid swelling of mucoadhesive polymers favors the interdiffusion process between the polymer and the mucus layer. Due to a more pronounced interdiffusion, relatively more interactions between the polymer and the mucus layer can take place leading to stronger adhesive properties. On the other hand, a rapid swelling behavior is in many cases responsible for limited cohesive properties, causing a failure in the mucoadhesive bond rather within the polymeric network itself than between the polymer and the mucus gel layer.[10] Water uptake studies demonstrated that the covalent attachment of homocysteine to poly(acrylic acid) has a significant influence on the swelling behavior of the polymer. Control tablets showed a partial disintegration and erosion already within 10–20 min, whereas tablets comprising the poly(acrylic acid)-homocysteine conjugate exhibited an almost constant water uptake over a 3-hour period. Moreover, no erosion or disintegration of these tablets could be observed within this time period, demonstrating also the high cohesive properties of the polymer. In contrast to most other mucoadhesive polymers, the poly(acrylic acid)-homocysteine conjugate seems therefore to combine both features: rapid swelling and high cohesive properties. Results of the study are shown in Fig. 3. These

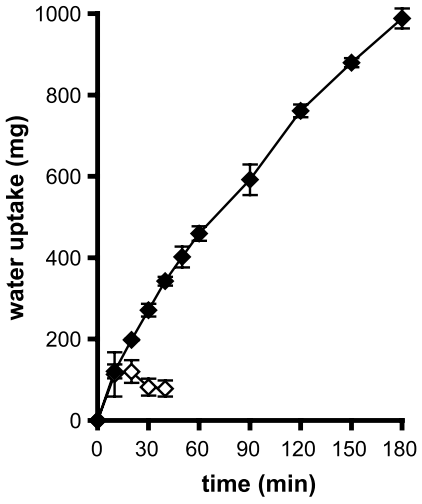

Figure 3. Swelling behavior of tablets comprising poly (acrylic acid)-homocysteine conjugate (◆) in comparison to that of tablets comprising unmodified poly(acrylic acid) (◇) in 100 mM phosphate buffer pH 6.8 at 37° C; indicated values are means of three experiments ± SD.

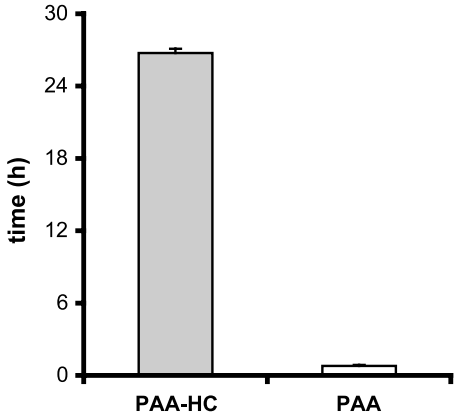

Figure 4. Disintegration studies of polymer tablets in 100 mM phosphate buffer pH 6.8 at 37° C. Grey bar: tablets comprising the poly(acrylic acid)-homocysteine conjugate; white bar: tablets containing unmodified poly(acrylic acid); indicated values are means of three experiments ± SD.

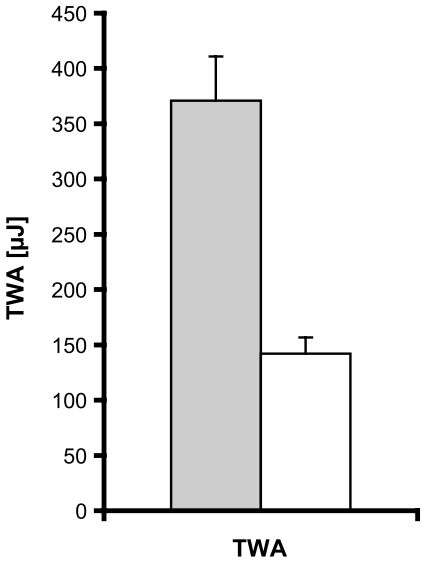

Figure 5. Tensile studies performed in 100 mM phosphate buffer pH 6.8 at 37° C. Grey bar: tablets comprising the poly(acrylic acid)-homocysteine conjugate; white bar: tablets containing unmodified poly(acrylic acid); indicated values are means ± SD of the total work of adhesion (TWA); n = 3.

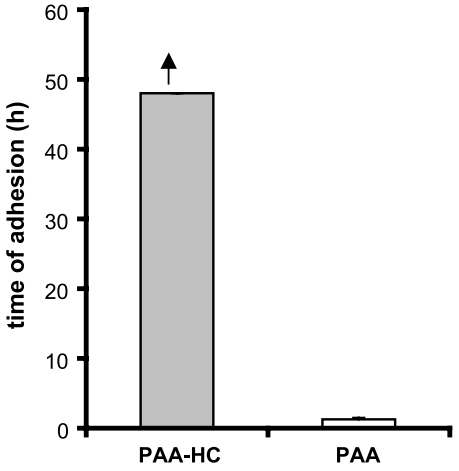

Figure 6. Mucoadhesion studies according to the rotating cylinder method; studies were performed in 100 mM phosphate buffer pH 6.8 at 37° C. Grey bar: tablets comprising the poly(acrylic acid)-homocysteine conjugate; white bar: tablets containing unmodified poly(acrylic acid); indicated values are means of three experiments±SD.

results were in good agreement with disintegration studies performed with tablets comprising the poly(acrylic acid)-homocysteine conjugate and the corresponding unmodified polymer, respectively. As shown in Fig. 4, the cohesive properties of the thiolated polymer were significantly higher than that of the control. Even after one day the swollen carrier matrix of the conjugate tablets remained stable and in their original shape without any erosion process.

Mucoadhesive Properties

Tensile studies carried out with unmodified poly(acrylic acid) and the corresponding poly(acrylic acid)-homocysteine conjugate revealed a significant influence of the immobilized thiol groups on the mucoadhesive properties of the polymer. Results of this study are shown in Fig. 5. The maximum detachment force (MDF) did not increase with increasing total work of adhesion (TWA), but was almost in the same range of 5–12 mN for both polymers (data not shown).

Results of mucoadhesion studies performed according to the rotating cylinder method, which is supposed to be closer to the in vivo situation than simple tensile studies, were in good agreement with the total work of adhesion of both polymers determined via tensile studies. The results are shown in Fig. 6. In the case of tablets based on the poly(acrylic acid)-homocysteine conjugate, even after two days on the rotating cylinder neither erosion nor partial disintegration of the swollen polymer matrices occurred.

Permeation Studies

Thiolated polymers are known to exhibit a strong permeation enhancing effect for the paracellular uptake of drugs.[8] This effect can even be improved in the presence of glutathione, which seems to mediate this permeation enhancing effect.[9] Whether or not a thiolated polymer displaying comparatively fewer reactive thiol groups, as is the case for the poly(acrylic acid)-homocysteine conjugate, is still capable of enhancing the paracellular uptake of drugs, however, is questionable. Therefore, in order to evaluate this effect of the poly(acrylic acid)-homocysteine conjugate, permeation studies were performed in Ussing chambers using sodium fluoresceine as the paracellular marker compound. Results demonstrated a significant permeation enhancing effect of this comparatively less reactive polymer, as illustrated in Fig. 7. In addition, no damage of the tissue could be observed by staining with trypan blue at the end of permeation studies (data not shown).

The P_{app} values achieved by the addition of 0.5% (m/v) PAA-HC conjugate with 0.5% (m/v) GSH and 0.5% (m/v) unmodified poly(acrylic acid) were determined to be $59.1 \pm 19.7\ 10^{-7}$ cm/sec and $20.1 \pm 13.6\ 10^{-7}$ cm/sec, respectively. According to this, the enhancement ratio gained by the immobilization of homocysteine to poly(acrylic acid) and the addition of glutathione was 2.4.

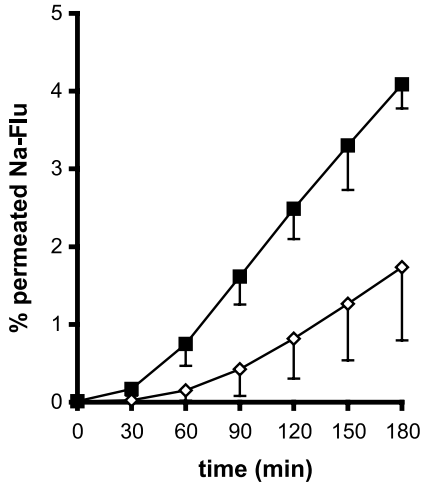

Figure 7. Permeation studies; the permeation enhancing effect of poly(acrylic acid)-homocysteine conjugate (0.5%; m/v) (■) in comparison to that of unmodified poly(acrylic acid) (◇); indicated values are means of three experiments±SD.

DISCUSSION

The activity of thiol groups is characterized by their pKa value. Lower pKa values correlate with higher concentrations of thiolate anions representing the reactive form of sulfhydryl groups. Thiolate anions are responsible for thiol/disulfide exchange reactions via a nucleophilic attack of the original disulfide bond. Futhermore, thiolate anions are subject to the oxidation process. Based on the chemical structure of the modified polymers, the pKa value of the thiol groups on the poly(acrylic acid)-cysteine conjugate was determined to be 8.5, whereas it was determined to be 10.5 for the poly(acrylic acid)-homocysteine conjugate using the ACD-software (Toronto, Canada). According to these calculations, there should be fewer thiolate anions available on the poly(acrylic acid)-homocysteine conjugate than on the poly(acrylic acid)-cysteine conjugate. This calculated difference in activity was in good correlation with stability studies. The poly(acrylic acid)-homocysteine conjugate was stable at pH 7 and below, as the thiolate anion concentration at these pHs was already $\leq 0.1\%$, whereas the poly(acrylic acid)-cysteine conjugate was determined to be stable at pH 5 and below.[15] As on mucosal tissues such as the ocular, buccal, and intestinal mucosa, the thiolated polymer faces a pH above 5, a higher stability in the pH range between 5–7—as is the case for the poly(acrylic acid)-homocysteine conjugate—seems to be advantageous. Comparatively more free thiol groups should remain available for thiol/disulfide exchange reactions with the mucus gel layer, consequently leading to higher mucoadhesive properties of the thiomer. On the other hand, if the thiol groups on the polymer are too inactive, the mucoadhesive properties will not increase, as no disulfide bonds between the thiomer and the mucus are formed. A comparison of the mucoadhesive features of poly(acrylic acid)-homocysteine conjugate and poly-(acrylic acid)-cysteine conjugate, which has been evaluated previously,[6] revealed similar mucoadhesive properties of both thiomers. The improved stability of the poly(acrylic acid)-homocysteine conjugate seems therefore to be compensated by the much lower activity of the thiol groups, leading in sum to similar mucoadhesive properties as the poly(acrylic acid)-cysteine conjugate, which is less stable and more reactive.

The higher stability of the poly(acrylic acid)-homocysteine conjugate exhibiting similar mucoadhesive, cohesive, and permeation enhancing properties as the poly(acrylic acid)-cysteine conjugate,[6,9] renders the new polymer advantageous over the poly(acrylic acid)-cysteine conjugate. In the case of aqueous formulations, for instance, a higher stability of the thiomer is needed. Although aqueous thiomer solutions can be stabilized towards oxidation by an inert sealing of the vessel with aluminium foil, this protection cannot be maintained in multiunit dosage forms. In eyedrops, for instance, thiomers seem to be highly beneficial because of their mucoadhesive, permeation enhancing, and in situ gelling properties.[6,16] Once the inert sealing of the bottle is opened, the thiomer in the aqueous solution should remain stable for 4 weeks. Using the poly(acrylic acid)-cysteine conjugate, stability for this time period cannot be guaranteed. However, stability should be provided by using the new polymer developed within this study exhibiting a comparatively higher stability in aqueous solutions. A similar situation can be expected for mucoadhesive vaginal and buccal aqueous gel formulations based on thiomers.

Whether used in liquid, semisolid, or solid formulations, a controlled drug release out of delivery systems based on thiolated polymers can be achieved (e.g., Ref. [17]). This effect can be based on ionic interactions and/or hydrogen bonds between the active ingredient and the polymer or on a simple diffusion process. Accordingly, a controlled drug release out of poly(acrylic acid)-homocysteine conjugates can be expected as well.

In summary, a new thiolated polymer, namely a poly(acrylic acid)-homocysteine conjugate, has been synthesized and characterized in vitro. The mucoadhesive, cohesive, and permeation enhancing properties of this novel thiomer were in the same range as those of the poly(acrylic acid)-cysteine conjugate, which is so far known as the most mucoadhesive polymer displaying comparatively strong permeation enhancing properties. In contrast to the poly(acrylic acid)-cysteine conjugate, however, the poly(acrylic acid)-homocysteine conjugate displays a much higher stability in aqueous solutions. The new thiomer is therefore advantageous over the thiolated polymers synthesized so far, in particular when the formulation is based on aqueous solutions, as in the case of multiunit eyedrops or mucoadhesive aqueous gels for vaginal or buccal use.

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Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016