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Research paper

Thiolated polymers: Evaluation of the influence of the amount of covalently attached L-cysteine to poly(acrylic acid)

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Abstract

It was the aim of this study to investigate the influence of the amount of thiol groups being covalently attached to poly(acrylic acid) 450 kDa on its properties. Five different PAA_{450}–L-cysteine conjugates (PAA_{450}–Cys) were synthesized bearing 53.0 (PAA I), 113.4 (PAA II), 288.8 (PAA III), 549.1 (PAA IV) and 767.0 (PAA V) μmol immobilized thiol groups per gram polymer. Mucoadhesion studies utilizing the rotating cylinder method, tensile studies and disintegration studies were performed. Self-crosslinking properties were measured by the increase in viscosity. Permeation studies were performed on rat small intestine and Caco-2 monolayers using sodium fluorescein as model drug. Following residence times on the rotating cylinder could be identified: PAA I 3.1; PAA II 5.2; PAA III 22.0; PAA IV 33.8; PAA V 53.7; control 1.3 [h]. The disintegration time of all PAA_{450}–Cys tablets was strongly dependent on the degree of thiolation of the polymer. Self-crosslinking studies showed that the different PAA_{450}–Cys conjugates (3% m/v) in phosphate buffer, pH 6.8, formed intramolecular disulfide bonds. In case of Caco-2 monolayer transport studies following P_{app}-values could be identified: PAA I 9.8; PAA II 10.1; PAA III 11.1; PAA IV 8.9; PAA V 8.2; control 6.4 [$P_{app} \times 10^{-6}$, cm s^{-1}]. Mucoadhesive and self-crosslinking properties are strongly dependent on the degree of thiolation of the polymer and with respect to transport studies, an optimum amount of covalently attached L-cysteine could be identified.

Keywords: Poly(acrylic acid); Thiomer; Mucoadhesion; Disintegration; Self-crosslinking; Permeation enhancement

1. Introduction

Thiolated polymers – designated thiomers – have been introduced to pharmaceutic literature within the last years. Although they also serve as excipients in nasal drug delivery, the main focus of administration is the oral route. It has been demonstrated that hydrophilic model drugs such as peptides show improved bioavailability due to the use of thiomers. Different sulfhydryl ligands such as L-cysteine, iminothiolan, cysteamine and glutathione, respectively, were linked to polymer backbones, like chitosans, poly(acrylic acids), celluloses and alginates. By this modification certain properties of well-established polymeric excipients are strongly improved. It has been proven that thiomers exhibit improved mucoadhesive properties due to covalent bonds of thiol moieties of the polymer with cysteine rich subdomains of mucus glycoproteins [1]. Furthermore, thiolated polymers show improved cohesive properties, because of their ability to form intrapolymeric disulphide bonds [2]. These disulphide bonds are the result of an oxidation process in aqueous media at physiological pH in the upper part of small intestine. A permeation-enhancing effect in comparison to unmodified polymers was described as well. This effect can be explained by the ability of thiomers to open tight junctions [3].

Recently, thiomers have been well investigated towards various influences like molecular mass, type of polymer, type of sulfhydryl bearing molecule and influence of pH.

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However, the influence of the amount of covalently attached thiol groups to the polymeric backbone has not been investigated so far. It was the aim of this study to fill this gap to further improve our knowledge on thiolated polymers. As model drug poly(acrylic acid) 450 kDa–L-cysteine (PAA_{450}–Cys) conjugates were used. This thiomer was chosen because it is a well-investigated poly(acrylic acid), it is not crosslinked and therefore L-cysteine rich conjugates could be synthesized. Conjugates with different amounts of covalently attached L-cysteine were characterized. Therefore, the thiomers were tested towards their mucoadhesivness, self-crosslinking properties and permeation-enhancing effects. The results are intended to further improve thiomers and to contribute important information about the knowledge of thiolated polymers.

2. Materials and methods

2.1. Materials

Poly(acrylic acid) with an average molecular weight 450,000 Da (PAA_{450}), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), Ellman's reagent (DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)), picrylsulfonic acid solution 5% (w/v) (TNBS), sodium fluorescein, L-glutathione reduced form (GSH) and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes) were obtained from Sigma–Aldrich, St. Louis, MO.

Cell culture medium was prepared by using MEM powder 9.66 g/l, 2.2 g/l sodium bicarbonate, 2 mM L-glutamine, penicillin/streptomycin solution (100 U penicillin and 0.1 mg of streptomycin per liter medium) and 20% fetal calf serum (FCS). All substances were purchased at Sigma–Aldrich, St. Louis, MO, except FCS, it was obtained from Gibco, Carlsbad, CA. Corning costar© transwell© – clear, 12 mm diameter, 0.4 μm pore size, clear polyester membranes were purchased at Corning, Acton, MA. All other chemicals were of reagent grade and obtained from Sigma, St. Louis, MO, as well.

2.2. Methods

2.2.1. Synthesis of polymer–L-cysteine conjugates

The poly(acrylic acid) 450 kDa–cysteine conjugates (PAA_{450}–Cys) were synthesized according to a method described previously by our research group [4]. In brief, the covalent attachment of L-cysteine to neutralised PAA_{450} was achieved by the formation of amide bonds between the primary amino group of cysteine and the carboxylic acid group of the polymer. Therefore, the carboxylic acid moieties of the polymer were activated for conjugation by the addition of different amounts of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). After dialysis protected from light at 4 °C to avoid oxidation of thiol moieties, the resulting polymer–cysteine conjugates were lyophilized by drying frozen aqueous polymer solutions at −75 °C condenser temperature at 4×10^{-4} mbar (Virtis, Gardiner, NY). Previously to freeze drying, the polymer was frozen at −70 °C (Refco, Knoxville, TN). The PAA_{450}–Cys conjugates were stored at 4 °C until further use. Control polymers were prepared in the same way, however, without submitting EDC to the coupling reaction.

2.2.2. Degree of thiolation of PAA_{450}–Cys conjugates

The amount of free thiol groups immobilized on the PAA_{450} backbone, i.e. the degree of modification, was determined photometrically with Ellman's reagent quantifying free thiol groups. First, 0.5 mg of both the conjugates and control was hydrated in 500 μl of 0.5 M phosphate buffer, pH 8.0, then 500 μl Ellman's reagent (3 mg dissolved in 10 ml of 0.5 M phosphate buffer, pH 8.0) was added. The samples were incubated for 2 h at room temperature protected from light. Thereafter, 300 μl of each sample was transferred into a microplate and the absorbancy was measured at a wavelength of 450 nm using a microplate reader (FluoStar Galaxy, BMG, Offenburg, Germany) [5]. The total amount of sulfhydryl groups fixed on the polymer, represented by the summation of free thiol groups and of oxidized thiol moieties available in form of disulphide bonds, was quantified after reduction with $NaBH_4$ [6]. The quantity of remaining unbound L-cysteine in the PAA_{450}–Cys conjugate solutions was determined with TNBS. TNBS reacts with the primary amino groups of cysteine in a nucleophilic aromatic substitution, developing an orange dye. The absorbance was measured at 450 nm using the Fluostar microplate reader [7].

2.2.3. Tablet manufacture

The different PAA_{450}–Cys conjugates, as listed in Table 1, and unmodified control polymers were compressed utilizing a hydraulic press (Paul Weber, Remshalden-Grunbach, Germany). The resulting tablets were flat-faced, 5.0 mm in diameter and they weighed 30 mg, compaction force was 5 kN.

2.2.4. Tensile studies

Tensile studies were carried out on freshly excised porcine intestinal mucosa. PAA_{450}–Cys and control tablets were glued to a stainless steel flat disc (8 mm in diameter, 0.3 g of weight in the system), which was hung by a nylon thread (15 cm) from a laboratory stand. The porcine mucosa was fixed on a glass support, placed in a beaker, and totally immersed with 400 ml of 0.1 M phosphate buffer, pH 6.8. The beaker was placed on a balance and carefully lifted by a mobile platform until the mucosa came in contact with the tablet. The contact was determined when the nylon thread holding the tablet became bent. After an incubation time of 30 min, 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 (Windwedge software; TAL Technologies Inc., Philadelphia, PA) linked to the balance. The total work of adhesion (TWA) representing the area under the force/distance was determined.

Table 1
Amount of L-cysteine covalently attached to poly(acrylic acid) 450 kDa and amount of EDC used for synthesis of each thiomer

Polymer	Final conc. of EDC (mM)	Percentage of acrylic acid units thiolated (%)	Free thiol groups per g polymer (μmol)	Amount of disulphide groups per g polymer (μmol)
PAA_{450}–Cys I	50	0.38	53.0 ± 1.8	12.0 ± 2.5
PAA_{450}–Cys II	50	0.83	113.4 ± 1.6	13.4 ± 11.9
PAA_{450}–Cys III	100	2.15	288.8 ± 9.7	34.4 ± 1.2
PAA_{450}–Cys IV	150	4.23	549.1 ± 4.2	89.6 ± 9.0
PAA_{450}–Cys V	200	6.08	767.0 ± 14.6	89.5 ± 19.9
Control	–	–	–	–

Free thiol groups were determined by the Ellman's test, determination of the total amount of thiol groups on the polymer was performed with Ellman's test as well, but pretreated with sodium borohydrid to cleave disulphide bonds ($n = 3$, ±SD).

2.2.5. In vitro mucoadhesion studies with the rotating cylinder method

PAA_{450}–Cys and control tablets were attached to freshly excised intestinal porcine mucosa, which has been attached to a stainless-steel cylinder (diameter 4.4 cm; height 5.1 cm; apparatus 4-cylinder, USP). Thereafter, the cylinder was placed in the dissolution apparatus according to the USP, entirely immersed with 900 ml of 0.1 M phosphate buffer, pH 6.8 at 37 °C and agitated with 125 rpm. The detachment of the test tablets was determined visually during an observation time of 180 h [8].

2.2.6. Evaluation of the disintegration behaviour

The stability of PAA_{450}–Cys and control tablets was investigated with the disintegration apparatus according to European Pharmacopoeia at an oscillating frequency of 0.5 s^{-1}. Studies were performed in 100 mM phosphate buffer at pH 6.8 at 37 °C.

2.2.7. Self-cross-linking properties

The PAA_{450}–Cys conjugates were hydrated in 50 mM phosphate buffer, pH 6.8, to get a 3% (m/v) solution. These gels were incubated at 37 °C under continuous stirring. At predetermined timepoints, aliquots of 1 ml were transferred to a cone-plate viscosimeter (Physica Rheolab MC1, Paar Physica, Graz, Austria) and allowed to equilibrate at 24 °C on the plate for 3 min before rheological measurements. Apparent viscosity was determined at a controlled shear rate of $D = 50\ s^{-1}$.

2.2.8. Permeation studies utilizing a Caco-2 cell culture monolayer system and rat intestine in Ussing-type chambers

Caco-2 cells were maintained in the media described above at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The media were changed daily and cells were split twice a week. The following experiments were conducted during passages 80–90. Cells were plated directly after splitting in a density of 1×10^5 cells onto the membrane inserts of 12-well plates. The cells were allowed to grow and differentiate for 24 days, during this time the media mentioned above were changed every 48 h. Transepithelial electrical resistance (TEER) of the monolayers was measured with the EVOM instrument (World Precision Instruments, Sarasota, FL).

Permeation studies were carried out in the transwell monolayer system, displaying a volume of 1 ml of both donor and acceptor chambers and a permeation area of 4.52 cm^2. The pH of the prepared incubation medium 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 was adjusted to 6.8. All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. After 1 h of preincubation with the artificial intestinal fluid, the media of the donor compartment were substituted by the different PAA_{450}–Cys conjugate solutions (0.5%, w/v) and control containing 0.5% (w/v) of the permeation-enhancing mediator reduced glutathione (GSH). The hydrophilic model drug sodium fluorescein was used as model compound in a final concentration of 0.001% (w/v). Over 3 h incubation time, aliquots of 200 μl were taken from the acceptor compartment every 30 min, and the volume was substituted by 200 μl incubation medium pre-equilibrated at 37 °C. The amount of permeated sodium fluorescein was determined fluorimetrically at an emission wavelength of 514 nm and an excitation wavelength of 490 nm. Cumulative corrections were made for the previously removed samples.

Permeation studies in Ussing-type chambers were carried out by mounting rat small intestine (duodenum) in the chambers. Right after sacrificing the rat, the first 15 cm of the small intestine (duodenum) was excised and mounted in the Ussing chamber. The chambers were displaying a volume of 1 ml (1 cm^3) of both donor and acceptor chambers and a permeation area of 0.64 cm^2. The pH of the prepared incubation medium 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 was adjusted to 6.8. All experiments were performed at 37 °C and carbogen (5% CO_2 in O_2) was continuously bubbled through the donor and acceptor compartments. After 60 min of preincubation with the artificial intestinal fluid, the media of the donor compartment were substituted by the five different PAA_{450}–Cys conjugates (0.5% w/v) containing 0.5% (w/v) of the permeation-enhancing mediator GSH. The corresponding unmodified polymer (0.5% w/v) was used as control. Sodium fluorescein was used as model compound in a final concentration of 0.001% (w/v). Over 3 h

of incubation time, aliquots of 200 μl were taken from the acceptor compartment every 30 min, and the volume was substituted by 200 μl incubation medium pre-equilibrated at 37 °C. The amount of permeated sodium fluorescein was determined as described above.

The apparent permeability coefficients (P_{app}) for sodium fluorescein were calculated according to Eq. (1):

$$P_{app} = Q/(A * c * t) \quad (1)$$

where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount permeated throughout the incubation time (mg), A is the diffusion area of the transwell inserts (cm^2), c is the initial concentration of the marker in the donor compartment (mg/cm^3), and t is the total time of the experiment(s). Transport enhancement ratios (R) were calculated from P_{app} values by Eq. (2):

$$R = P_{app}(PAA_{450}\text{–Cys})/P_{app}(PAA_{450}\text{–control}) \quad (2)$$

2.2.9. Statistical data analysis

Statistical data analyses were performed using the Student's t test with $p < 0.05$ as the minimal level of significance.

3. Results

3.1. Characterization of the PAA_{450}–Cys conjugates

In order to evaluate the influence of the amount of covalently attached L-cysteine to PAA_{450} polymer backbones, EDC was submitted in different concentrations to activate the polymer to form amide bonds with L-cysteine. The amount of covalently attached thiol groups to the polymer is listed in Table 1. The amount of EDC influenced the amount of thiol groups per gram polymer, when EDC was omitted during reaction, no L-cysteine – polymer amide bonds were formed. This control polymer seemed not to be changed in molecular weight due to modification, because the original PAA_{450} and the control polymer were both tested on their viscosity at pH 6.8. A 3% (m/v) solution of the original PAA_{450} and the PAA_{450} treated with EDC resulted in an apparent viscosity of 0.19 ± 0.03 m Pas and 0.20 ± 0.02, respectively. A total amount of 767.0 μmol/g polymer could maximally be achieved. These results are in agreement with former studies [9,10]. Since the amount of free unbound L-cysteine also influences the polymer properties, the concentration was determined [1]. Therefore, TNBS was used to quantify primary amino groups photometrically and the results were substracted from the amount of free thiol groups per gram polymer and disulphide groups, respectively. The lyophilized thiomers and controls contained between 1 and 4 μmol unbound L-cysteine groups per gram polymer (data not shown). A low amount of free L-cysteine has been described to be essential for good mucoadhesive properties [1]. The freeze dried polymer-conjugates were white, odorless sponges, which had a pH of 4 when swollen in distilled water after production. Under the storage conditions mentioned above, it was shown that there is no change in the amount of free thiol moieties [11].

3.2. Mucoadhesion studies

Tensile studies represent the most widely employed in vitro test method for the assessment of the adhesive strength of mucoadhesives. In the present study, the total work of adhesion (TWA) was low for PAA_{450} controls compared to PAA_{450}–Cys conjugates, demonstrating a higher affinity of the thiolated polymers for mucosal tissue. As depicted in Fig. 1, TWA of PAA_{450}–Cys tablets was increased up to 3.25-fold in comparison to the control. It is obvious that the higher the amount of thiol groups on the polymer, the higher the TWA. However, it has to be mentioned that there is no advantage of thiomers towards the unmodified control at low coupling rates. These results were in the same range compared to the results of other publications [12,9]. In order to confirm the results of tensile studies, mucoadhesion studies were also carried out with the rotating cylinder method. Results from the rotating cylinder method were in good correlation with results obtained by tensile studies. The higher the amount of sulfhydryl moieties on the polymer, the longer is the residence time on the mucosa. A long residence time stands for good mucoadhesive properties. Fig. 2 shows that for the polymer bearing 767 μmol thiol groups per gram polymer, the improvement ratio compared to the control is 40.5 and enhanced mucoadhesion was observed at thiomers bearing few thiol moieties as well. The improvement ratio was

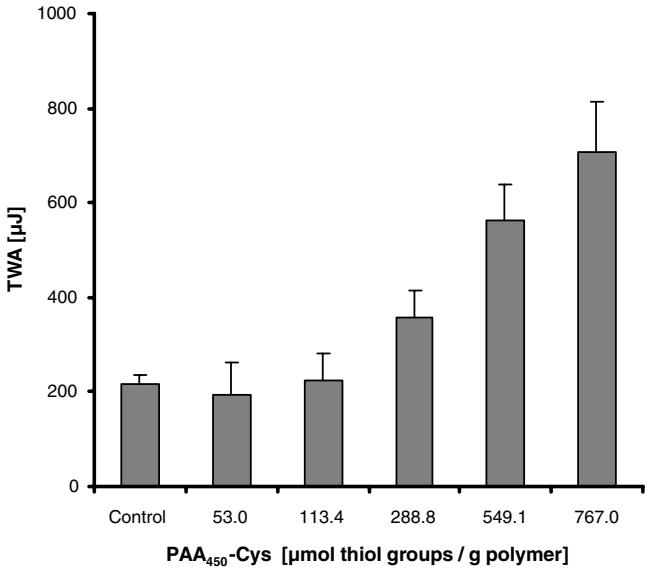

Fig. 1. Comparison of the mucoadhesive properties of PAA_{450}–Cys conjugates and control determined by tensile studies. Presented values ($n = 3$, ±SD) are means of the total work of adhesion (TWA). Thirty milligrams tablets were attached to porcine mucosa in 100 mM phosphate buffer pH 6.8 and the force necessary to remove the tablets was acquired.

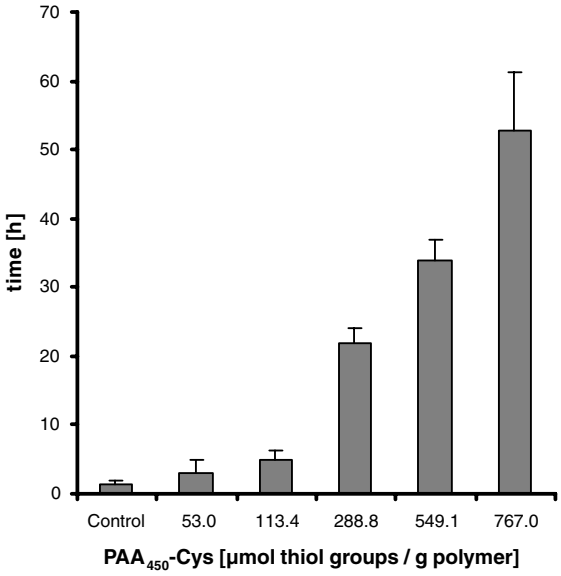

Fig. 2. Comparison of the mucoadhesive properties of PAA_{450}–Cys conjugates with different amounts of L-cysteine attached to the polymer determined by the rotating cylinder method. Thirty milligrams of PAA_{450}–Cys conjugate tablets and control tablets was attached to excised porcine mucosa, which was spanned on a vertical cylinder rotated with 125 rpm in 100 mM phosphate buffer pH 6.8 at 37 °C. The indicated time of adhesion represents the average of three experiments (±SD).

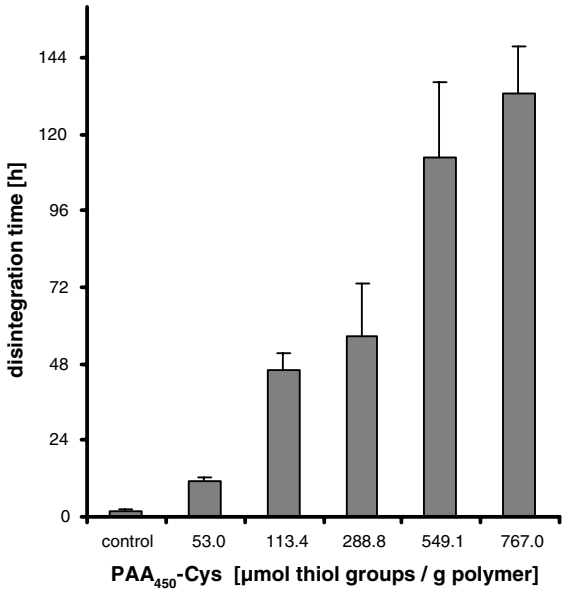

Fig. 3. Disintegration behaviour of PAA_{450}–Cys conjugates and control tablets. Studies were carried out with a disintegration apparatus according to the European Pharmacopoeia in 100 mM phosphate buffer pH 6.8 at 37 °C ($n = 3$, ±SD).

calculated by the adhesion time of conjugates versus adhesion time of the control.

3.3. Self-crosslinking properties

Disintegration studies were carried out with PAA_{450}–Cys and unmodified PAA_{450} tablets in physiological medium. Polymer tablets bearing 549.1 and 767.0 thiol groups per gram polymer were almost stable for 1 week (Fig. 3). These tablets are stable, because they can form intramolecular disulphide bonds at pH 6.8. As expected, control tablets disintegrated within 1.5 h, lacking the possibility to form intramolecular disulphide bonds. The formation of intra- and intermolecular disulphide bonds is strongly dependent on the amount of free thiol groups available which can be observed at mucoadhesion studies as well. Thus, polymers with high amounts of thiol groups succeeded the control tablets by a factor of 102.

To confirm the results obtained by disintegration studies, additionally the increase of viscosity of 3% (m/v) PAA_{450}–Cys conjugates in 100 mM phosphate buffer, pH 6.8, was measured as a function of time. Control gel was not submitted to these rheological studies, since there is no change in viscosity due to the lack of the ability to form intramolecular bonds. These studies confirmed the theory of intramolecular disulphide bond formation, as the increase in viscosity correlates with the amount of sulfhydryl moieties on the polymer. Fig. 4 shows the time dependent increase in viscosity and that the increase in viscosity is dependent on the amount of covalently attached L-cysteine to the polymer as well. These results were in good correlation with the results obtained by Marschütz and Bernkop-Schnürch [9].

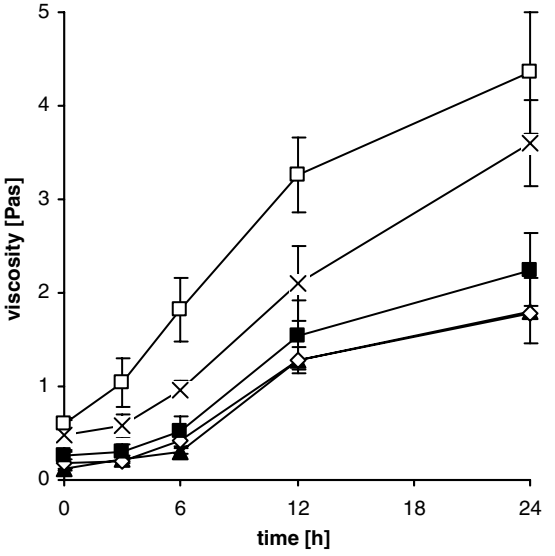

Fig. 4. Time dependent increase in viscosity measured at 50 s^{-1} shear rate of 3% (m/v) PAA_{450}–Cys conjugates in 100 mM phosphate buffer pH 6.8 incubated at 37 °C. PAA_{450}–Cys I (▲), PAA_{450}–Cys II (◇), PAA_{450}–Cys III (■), PAA_{450}–Cys IV (×), PAA_{450}–Cys V; ($n = 3$, ±SD).

3.4. Permeations studies

Poly(acrylic acids) such as PAA_{450} were shown to exhibit a strong permeation-enhancing effect on the paracellular drug uptake, however, the influence of the amount of thiol

moieties has not been evaluated so far [10,13]. Results of permeation studies on Caco-2 monolayer and rat small intestine are depicted in Figs. 5 and 6, respectively. Several thiomers admitted to transport studies could enhance the permeation rate in comparison to control. In both transport systems, Caco-2 monolayer and rat intestine, the PAA_{450}–Cys conjugate bearing 288.8 μmol thiol groups per gram polymer could achieve the highest enhancement ratio 1.76 and 1.73, respectively. At both systems, the PAA_{450}–Cys conjugate with the highest amount of thiol groups did not exhibit the best permeation enhancement. P_{app} values are summarized in Table 2 and for all polymers they are higher when permeated through the rat small intestine [14].

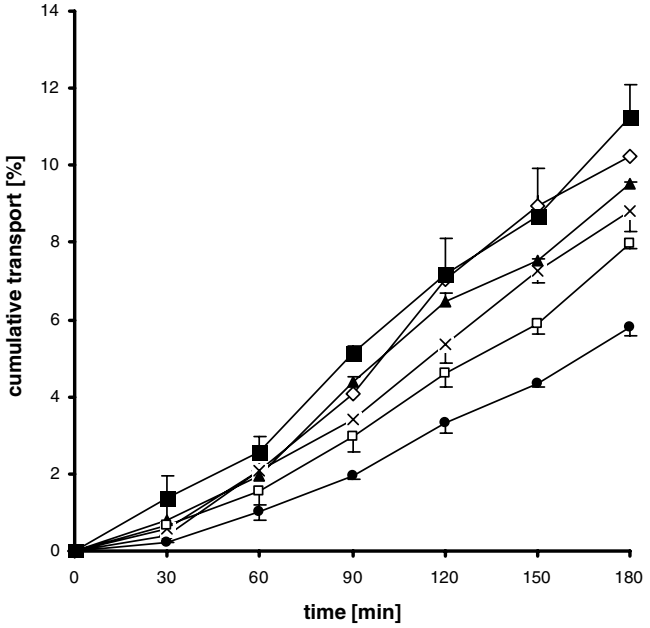

Fig. 5. Permeation studies carried out with 0.5% (m/v) PAA_{450}–Cys conjugates and control in presence of 0.5% (m/v) GSH. Data represent the transport of sodium fluorescein across Caco-2 monolayers in percent. PAA_{450}–Cys I (▲), PAA_{450}–Cys II (◇), PAA_{450}–Cys III (■), PAA_{450}–Cys IV (×), PAA_{450}–Cys V (□), control polymer (●); ($n = 3$, ±SD).

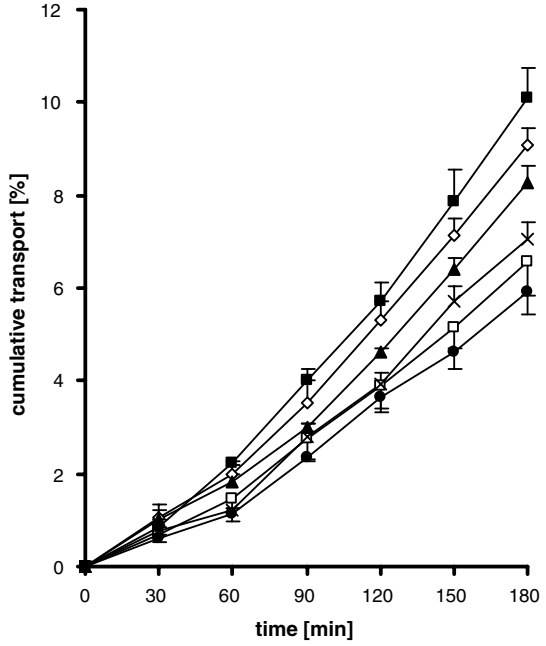

Fig. 6. Permeation studies carried out with 0.5% (m/v) PAA_{450}–Cys conjugates and control in presence of 0.5% (m/v) GSH. Data represent the transport of sodium fluorescein across rat small intestine in percent. PAA_{450}–Cys I (▲), PAA_{450}–Cys II (◇), PAA_{450}–Cys III (■), PAA_{450}–Cys IV (×), PAA_{450}–Cys V (□), control polymer (●); ($n = 3$, ±SD).

In case of the Caco2-cell culture model, TEER measurements were performed and it revealed that TEER decreased to 30% of the initial value due to the preincubation with the incubation media containing HEPES. After the transport studies, it recovered to 80% in average of the initial value which means that the cells were not irreversibly damaged by the PAA_{450}–Cys conjugates or the control polymer. Additionally, there was no significant difference detectable in TEER between the various thiomers and the control (data not shown).

4. Discussion

The interaction of polymers such as poly(acrylic acid) derivates or celluloses has been described to be based on ionic interactions and van der Waals forces or physical interpenetration of the polymer with the mucus layer [15,16]. Thiomers, however, are able to form covalent bonds by building disulphide bonds between the thiol groups of the polymer conjugate and thiol groups of the mucus [1]. Mucoadhesion experiments within this study were performed at pH 6.8. At this pH disulphide bonds could easily be formed due to oxidation processes [17]. Within this study, it was detected that an increasing amount of thiol groups on the polymer backbone improves the mucoadhesive properties of thiomers. In addition, it has been demonstrated that the swelling behaviour has an impact on the adhesive properties of polymers [18]. Marschütz and Bernkop-Schnürch showed that the water absorbing capacity for a PAA_{450}–Cys conjugate with 511.6 μmol thiol groups per gram polymer is higher than a conjugate bearing 90.5 μmol thiol groups per gram polymer [9]. This effect has been explained by Duchene and Ponchel: mucoadhesive polymers are assumed to take water from the underlying mucosal tissue, leading to a considerably strong adhesion [19]. The PAA_{450}–Cys conjugates and the control polymer were lyophilized from a solution of pH 4.0 and with respect to Guggi et al. a low pH of the conjugates is also essential to achieve high levels of mucoadhesion [20]. Grabovac et al. confirmed these findings and detected that freeze dried polymers were superior to precipitated polymers [12]. Thus, it might be concluded that PAA_{450}–Cys conjugates exhibit the highest mucoadhesion if they were adjusted to pH 4, freeze dried and bearing a high as possible amount of covalently attached thiol groups.

Table 2
Comparison of the apparent permeability coefficients of the different PAA_{450}–Cys conjugates and control on the permeation of sodium fluorescein in the presence of 0.5% (m/v) GSH

PAA_{450}–Cys (μmol thiolgroups/g polymer)	Transport studies in Caco-2 monolayer system		Transport studies in Ussing-type chambers on rat small intestine	
	$P_{app} \times 10^{-6}$ (cm s^{-1})	Enhancement ratio	$P_{app} \times 10^{-6}$ (cm s^{-1})	Enhancement ratio
53.0 ± 1.8	9.8 ± 0.2	1.53	10.19 ± 0.68	1.19
113.4 ± 1.6	10.1 ± 0.1	1.57	13.15 ± 0.62	1.54
288.8 ± 9.7	11.1 ± 0.7	1.73	14.61 ± 1.16	1.71
549.1 ± 4.2	8.9 ± 0.4	1.39	11.96 ± 0.62	1.40
767.0 ± 14.6	8.2 ± 0.2	1.28	9.47 ± 1.02	1.11
Control	6.4 ± 0.2	–	8.56 ± 0.84	–

Enhancement ratios are related to control ($n = 3$, ±SD).

Self-crosslinking properties are related to the mucoadhesive features, because disulphide bonds are not just formed with the mucosa, but also intramolecular. PAA_{450}–Cys conjugate tablets are longer stable due to the formation of intramolecular disulphide bonds. According to the results obtained within this study, the increasing stability of the PAA_{450}–Cys conjugates may be due to the increasing amount of thiol groups which are able to form disulphide bonds. Marschütz and Bernkop-Schnürch described the correlation between the time dependent decrease in thiol groups and the increase in viscosity of two different PAA_{450}–Cys conjugates [9]. They showed that the decrease of free thiol groups in percent is the same for different PAA_{450}–Cys conjugates. That might lead to the conclusion that disulphide bond formation occurs with the same speed and if there are more thiol groups, there is a higher level of self-crosslinking at a pH where thiol groups are being oxidized.

The five different PAA_{450}–Cys conjugates were evaluated concerning their permeation-enhancing effect. First, polymers itself have a permeation-enhancing effect due to their ability to interpenetrate the mucous layer [15]. For hydrophilic compounds such as sodium fluorescein it is essential that the tight junctions are opened for paracellular transport. GSH is also a compound which is facilitating paracellular transport: GSH has a high affinity to the enzyme protein tyrosine phosphatase (PTP) which is responsible for closing tight junctions [21] and consequently inhibition of PTP by GSH must lead to improved permeability. It has been demonstrated that 0.4% GSH and polycarbophil-cys improved the transport of sodium fluorescein by a factor of 1.86 and 2.04, respectively. Polycarbophil-cys in combination with GSH led to an enhancement ratio of 2.93 and oxidized glutathione (GSSG) showed a comparatively low permeation enhancement [22]. This means that the thiolated polymer and reduced glutathione are essential for effective permeation enhancement. GSH is oxidized on the cell surface and so chemical reduction of GSSG would be favourable, but GSSG is reduced by GSSG reductase only to a very small extent [23,24]. In the presence of thiomers GSH remains stable in its reduced form due to its higher pK_a [22]. Figs. 5 and 6 show that the most improved permeation enhancement was not achieved by the thiomer with the highest coupling rate, but that the best effect was achieved by the thiomer with 288.8 μmol/g thiol groups per gram polymer. The effect of limiting permeation enhancement due to highly thiolated PAA might be explained by a combination of following factors: firstly, a very high level of self-crosslinking and viscosity due to a very high level of thiolation, which leads to reduced interpenetration of the thiomer. Secondly, there might be less than 383.5 μmol/g L-cysteine attached to the polymer (0.5% m/v PAA_{450}–Cys V conjugate) enough to entirely maintain GSH, being available in a concentration of 16.3 mM, in its reduced form on the apical side of the monolayer.

5. Conclusion

This study examined the influence of the amount of covalently attached L-cysteine to poly(acrylic acid) 450 kDa. The PAA_{450}–Cys conjugates were investigated towards their mucoadhesive properties which were strongly dependent on the amount of sulfhydryl groups on the polymer backbone. Additionally, disintegration and self-crosslinking properties are improved when PAA_{450}–Cys conjugates with high amounts of thiol groups are admitted to the experiments. This leads to the conclusion that a maximum achieveable amount of thiol groups on the polymer backbone is favourable for applications at which mucoadhesive properties are in the foreground. PAA_{450} itself also arises as the polymer with the best properties in comparison to other hydrophilic polymers, additional features of importance seem to be that the polymer is lyophilized at a pH around 4. With respect to transport studies, an optimum amount of covalently attached L-cysteine could be identified, it seems that a high coupling rate is not favourable for high permeation enhancement. This study contributes to further optimize thiolated polymers. It suggests PAA_{450}–Cys conjugates as ideal hydrophilic anionic polymeric excipient, because they are determined to have the ideal size regarding all the mucoadhesive, crosslinking and permeation-enhancing properties since their thiol group content is optimized.

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