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Research paper

Thiolated polymers: evidence for the formation of disulphide bonds with mucus glycoproteins

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Abstract

Disulphide bonds between thiolated polymers (thiomers) and cysteine-rich subdomains of mucus glycoproteins are supposed to be responsible for the enhanced mucoadhesive properties of thiomers. This study set out to provide evidence for these covalent interactions using poly(acrylic acid)–cysteine conjugates of 2 and 450 kDa (PAA_2–Cys, PAA_{450}–Cys) displaying 402.5–776.0 μmol thiol groups per gram polymer. The effect of the disulphide bond breaker cysteine on thiomer–mucin disulphide bonds was monitored by (1) mucoadhesion studies and (2) rheological studies. Furthermore, (3) diffusion studies and (4) gel filtration studies were performed with thiomer–mucus mixtures.

The addition of cysteine significantly ($P < 0.01$) reduced the adhesion of thiomer tablets to porcine mucosa and G'/G'' values of thiomer–mucin mixtures, whereas unthiolated controls were not influenced. These results indicate the cleavage of disulphide bonds between thiomer and mucus glycoproteins. Diffusion studies demonstrated that a 12.8-fold higher concentration of the thiomer (PAA_2–Cys) remains in the mucin gel than the corresponding unmodified polymer. Gel filtration studies showed that PAA_2–Cys was able to form disulphide bonds with mucin glycoproteins resulting in an altered elution profile of the mucin/PAA_2–Cys mixture in comparison to mucin alone or mucin/PAA_2 mixture. According to these results, the study provides evidence for the formation of covalent bonds between thiomer and mucus glycoproteins.

Keywords: Mucin; Poly(acrylic acid); Thiomers; Rheology; Disulphide bonds

1. Introduction

A new generation of mucoadhesive polymeric drug carrier systems has been established in the past years [1]. These thiolated polymers or so-called thiomers are hydrophilic polymers such as poly(acrylates) [2], chitosan [3] or deacetylated gellan gum [4] derivatised with thiol groups on their side chains. Due to the formation of inter- and/or intrachain disulphide bonds, these conjugates show strongly improved cohesive properties. The high stability of thiolated polymer matrix tablets, for instance, resulted in an almost zero order release of the model drug calcitonin for at least 8 h [5]. Further advantages of thiolated polymers are their enzyme inhibitory [6] and permeation enhancing effects, especially in combination with glutathione [7]. Together with the excellent mucoadhesive properties of thiomers these features seem to be of highest practical relevance rendering them useful excipients also for the challenging non-invasive application of peptide drugs. For example, with orally administered salmon calcitonin tablets based on thiolated chitosan approximately a 10% reduction of the plasma calcium level of rats could be achieved, which was equivalent to a pharmacological efficacy of 1.3% vs. i.v. administration [5]. The oral administration of insulin–PEG being incorporated in thiolated polymer microtablets resulted in a significant reduction of the blood glucose level of diabetic mice. The pharmacological efficacy of the oral formulation vs. s.c. injection was determined to be 7% [8].

At the molecular level, the enhanced mucoadhesive properties of thiomers are believed to be based on an interaction of thiolated polymers with the mucus layer [1]. This

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layer protects the gastrointestinal epithelia against mechanical and chemical damage and consists mainly of glycoprotein chains with cysteine-rich subdomains [9]. These subdomains are involved in the linking of mucin monomers into oligomers via disulphide bonds forming thereby a three-dimensional network [10]. As sulphhydryls such as mercaptoethanol or dithiothreitol are covalently bound to mucin glycoproteins leading to a collapse of the gel [11–13] other thiol bearing substances such as thiomers should also be able to form covalent bonds with the mucus.

It was therefore the aim of this study to provide evidence for the formation of disulphide bridges between thiolated polymers and cysteine-rich subdomains of mucus glycoprotein. In Fig. 1 two suggested mechanisms of thiomer–mucin interaction are shown: thiol/disulphide exchange reactions (A) [14] and a simple oxidation process (B) [15]. Within this study, different methods to prove the existence of covalent bonds between thiomers and mucin glycoproteins are described. According to the theory of disulphide bond formation between poly(acrylic acid)–cysteine (PAA–Cys) and mucin, the disulphide bond breaking agent cysteine should be able to cleave these bonds, as illustrated in Fig. 2. (1) The mucoadhesive properties of PAA–Cys matrix tablets should therefore be strongly reduced in the presence of cysteine. (2) Additionally, changes in the rheological properties of PAA_{450}–Cys/mucin mixtures in the presence of cysteine should take place, as polymer–mucin interactions in such mixtures can be evaluated by viscosity measurements [16]. Furthermore, (3) diffusion studies and (4) gel filtration tests with thiolated polymers–mucin incubates were performed to monitor the formation of thiomer–mucin conjugates.

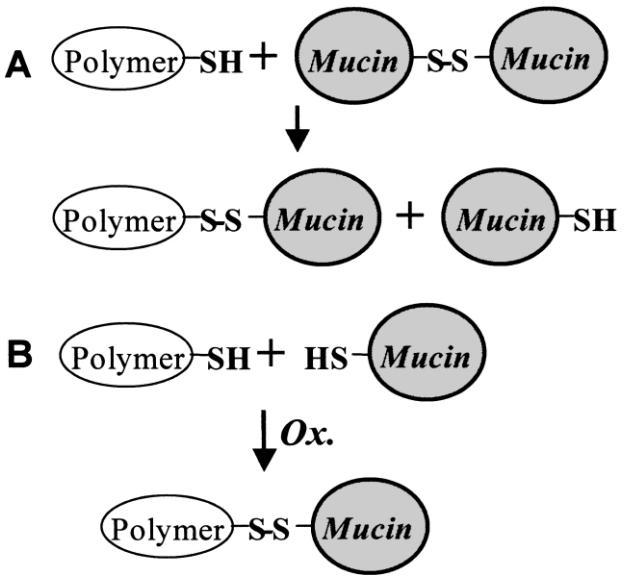

Fig. 1. Hypothetical schema representing about the formation of covalent bonds between thiolated polymers and mucin glycoproteins (A) via thiol/disulphide exchange reaction (B) via an oxidation process.

2. Materials and methods

2.1. Materials

PAA of 2 and 450 kDa and cysteine hydrochloride hydrate were purchased from Sigma-Aldrich, Steinheim, Germany. Two PAA–Cys conjugates with a molecular mass of 2 kDa (PAA_2–Cys I and II) and 450 kDa (PAA_{450}–Cys I and II) derivatised with different amounts of cysteine were supplied by MucoBiomer (Leobendorf, Austria). Sepharose 2B was obtained from Pharmacia (Uppsala, Sweden). Porcine gastric mucin (type II: crude), 2,4,6-trinitrobenzenesulphonic acid (TNBS), ethylene-diaminetetraacetic acid (EDTA, disodium salt), and trypsin–chymotrypsin inhibitor (Bowman-Birk Inhibitor, BBI) were provided by Sigma, St Louis, MO.

2.2. Preparation and characterisation of polymers and mucin solution

All PAA–Cys conjugates and unthiolated controls were completely hydrated in demineralised water and the pH of the polymer solutions was adjusted to 5 with 5 M HCl. They were dialysed in order to remove remaining traces of acrylic acid and organic solvents according to a method previously described for a polycarbophil-cysteine conjugate [17]. Afterwards, the pH of all samples was adjusted to 5 with 1 M NaOH and the frozen polymer solutions were lyophilised at −30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany). All polymers were stored at 4°C until further use. The amount of thiol groups immobilised on the polymer–cysteine conjugates was determined photometrically with Ellman's reagent as described previously [1].

Porcine gastric mucin for rheological studies and gel filtration was hydrated overnight at 4°C in 0.1 M phosphate buffer pH 6.8. The pH of the solution was adjusted to 6.8 with 1 M NaOH, diluted to a final concentration of 8% (m/v) and stored at 4°C no longer than 24 h. Disulphide and thiol content of mucin was measured after reduction with $NaBH_4$ and addition of 5,5′-dithiobis(nitrobenzoic acid), as described by Habeeb [18]. Free thiols were determined in the same way, but the reduction step was omitted.

2.3. Preparation of PAA_{450}–Cys tablets

To evaluate the influence of free unbound cysteine in the polymer tablet on mucoadhesion, cysteine (1%, m/m) was mixed with fully hydrated PAA_{450}–Cys I or unthiolated PAA_{450} control polymer. The pH of all samples was readjusted to 5 with 1 M NaOH and the polymer solutions were dried by lyophilisation. Conjugates and controls with and without free unbound cysteine were compressed (Hanseaten Type El, Hamburg, Germany) into 5.0 mm diameter flat-faced discs of 30 mg. The compaction pressure was kept constant during the preparation of all tablets.

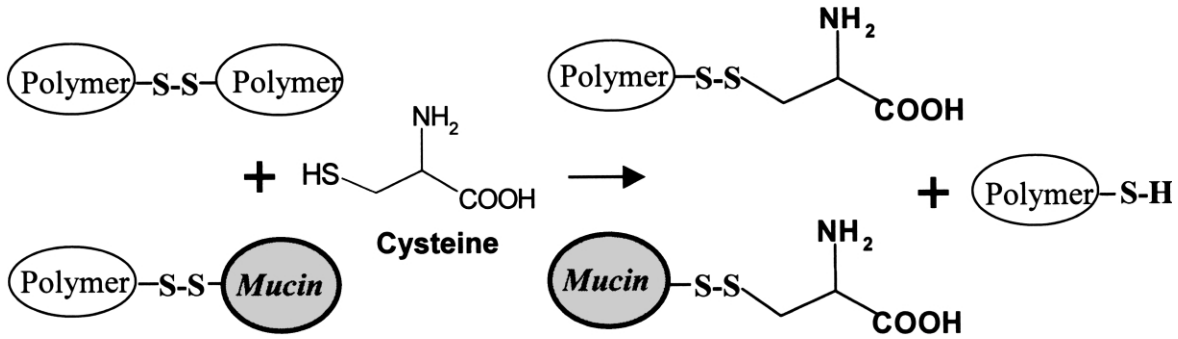

Fig. 2. Schematic presentation of the cleavage of disulphide bonds by the addition of cysteine.

2.4. Mucoadhesion studies

Test tablets of polymer and control were attached to freshly excised porcine intestinal mucosa, which had been glued to a stainless steel cylinder (diameter: 4.4 cm, height 5.1 cm; apparatus four-cylinder, USP XXVI) with a cyanoacrylate adhesive (Loctite, Henkel, Austria). 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 24 h time period ($n = 3$) [17].

2.5. Rheological studies

Rheological measurements were performed with a cone-plate (C35/2°) rheometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). PAA_{450}–Cys II conjugates and unthiolated controls were fully hydrated in demineralised water and diluted with an equal volume of 0.1 M phosphate buffer pH 6.8 to give a concentration of 6% (m/v). The polymer solutions were added to an equal volume of the 8% (m/v) solution of porcine gastric mucin, mixed with a spatula and the pH of the mixture was adjusted to 7 with 2 M NaOH. After an incubation period of 20 min at room temperature, 1% (m/v) of solid cysteine was added under stirring and the pH was adjusted to 7. After incubation for another 20 min, the polymer–mucin incubates were transferred to the viscometer and allowed to equilibrate on the plate for 3 min at 20 ± 0.5°C. Dynamic oscillatory tests within the linear viscoelasticity region were performed at 1 Hz frequency. Frequency sweep measurements were also carried out with a frequency varying from 0.1 to 10 Hz. The parameters obtained thereby were the storage modulus (G') and the loss modulus (G''). As references, polymer–mucin mixtures were prepared in the same way but without adding cysteine. Additionally, 3% (m/v) solutions of PAA_{450}–Cys II and PAA_{450} were analysed with and without incubation with 1% cysteine. All experiments were repeated three times.

2.6. Mucus preparation for diffusion studies

Fresh porcine small intestine stored in ice-cold saline was provided by the local slaughterhouse. Native mucus was isolated according to the method described by Mantle and Allen [19], but slightly modified. In brief, the intestine was sectioned into short lengths, flushed through with cold running water, opened at the mesenteric border and unfolded. The superficial mucus layer was gently scraped off with a glass slide, collected in a small beaker and homogenised by stirring vigorously. In order to minimise proteolysis of the isolated mucus, the proteinase inhibitors EDTA (0.7 mg/ml) and trypsin–chymotrypsin inhibitor (1.0 mg/ml) were added. The mucus was immediately frozen in aliquots and kept at −20°C until further use. The dry weight of the mucus was determined by lyophilisation of small portions (0.3 g) in open glass vials ($n = 3$).

2.7. Diffusion studies

For these studies, native porcine mucus was exhaustively dialysed (dialysis tubings, molecular weight cut-off (MWCO) 12 kDa, Sigma, St Louis, MO) against purified water at 4°C in the dark to remove fractions smaller than 12 kDa. Afterwards, the mucus was centrifuged for 15 min at 24,000 × g, the supernatant discarded and the mucus pellet redispersed with demineralised water to its initial volume. The binding studies were carried out in Ussing type chambers displaying a diffusion area of 0.64 cm^2. Between the donor and the acceptor compartments (volumetric capacity: 1.0 ml) a cellulose membrane with a MWCO of 12 kDa was mounted. PAA_2–Cys I and unthiolated PAA_2 (2 mg) were mixed with 1.0 ml of mucus and the pH was adjusted to 7 with 1 M NaOH. The mixtures were incubated for 1 h at 37°C under continuous shaking and transferred into the donor compartment. To get a reference solution for analysis, diffusion tests were also carried out with 1 ml of purified mucus pH 7 being treated in the same way as the polymer–mucus mixtures. The acceptor compartment was filled with 1.0 ml of demineralised water. The Ussing chambers were placed in an oscillating water bath at 37°C. After a time

period of 24 h, the content of the acceptor chamber was centrifuged for 15 min at 24,000 × *g*. The supernatant was mixed with 0.2 g of a cation exchanger (Dowex 50 WX8, Sigma, Steinheim, Germany) to remove small mucus fragments and centrifuged again. Thereafter, the supernatant was analysed photometrically (Lambda 16; Perkin–Elmer, Vienna, Austria) by measuring the absorbance at 215 nm against the reference ($n = 3$). The amount of polymer in the acceptor compartment was calculated using a standard curve obtained by the measurement of a series of solutions with increasing amounts of PAA_2–Cys I and PAA_2, respectively.

2.8. Gel filtration

Gel filtration was performed on a column (40 cm × 1.5 cm) packed with Sepharose 2B. PAA_2–Cys II or PAA_2 control (30 mg) was mixed with 0.1 ml of the 8% (m/v) solution of porcine gastric mucin. After the pH of the mixture was adjusted to 7, it was incubated at 37°C for 1 h under continuous shaking. To quench any non-covalent interactions, urea and sodium dodecyl sulphate were added in a final concentration of 9 and 0.125% (m/v), respectively. Thereafter, the sample was loaded on the column and eluted with 5 mM sodium phosphate buffer pH 6.8. Fractions of 2.5 ml were collected until a total volume of 140 ml was reached. The mucin glycoproteins were quantified with TNBS reagent developing an orange dye with primary amino groups with an absorbance maximum at 450 nm. As reference, 8% gastric mucin containing urea and sodium dodecyl sulphate, prepared as described above was also loaded on the column.

2.9. Statistical data analysis

Statistical data analyses were performed using the Student's *t*-test with $P < 0.01$ as the minimal level of significance, unless indicated otherwise. Calculations were done using the software Xlstat version 5.1 v1.

3. Results

3.1. Characterisation of PAA–Cys conjugates and porcine gastric mucin

The lyophilised polymers appeared as white powder of fibrous structure. The amount of covalently attached cysteine on the polymers was quantified via Ellman's test. On an average 776.0 ± 47.1 and 402.5 ± 58.2 μmol thiol groups were immobilised on 1 g PAA_2–Cys I and PAA_2–Cys II, respectively. PAA_{450}–Cys I and PAA_{450}–Cys II exhibited 472.7 ± 24.7 and 712.4 ± 34.7 μmol thiol groups per gram polymer, respectively.

Porcine gastric mucin contained 99.2 ± 5.0 nmol of total thiols per mg (–S–S–/–SH) including 2.4 ± 0.39 nmol of free thiols per mg (–SH) (means ± SD, $n = 3$).

3.2. Mucoadhesion studies

Mucoadhesion of polymer tablets with and without free cysteine added to the polymer was determined. As unbound cysteine acts as a disulphide bond breaking agent, it may cleave disulphide bonds formed between thiolated polymer and mucin as illustrated in Figs. 1 and 2. Accordingly, a decrease in the mucoadhesive properties of these cysteine containing thiomer tablets can prove the existence of disulphide bonds between thiomer and the mucus layer. The mucoadhesive properties of PAA_{450}–Cys and PAA_{450} control tablets, either with 1% (m/m) of free cysteine in the polymer matrix or without it are illustrated in Fig. 3. The presence of free cysteine in the PAA_{450}–Cys matrix tablets resulted in a significant ($P < 0.001$) decrease in their time of adhesion, whereas the mucoadhesion of unthiolated polymer tablets was not influenced by cysteine at all.

3.3. Rheological studies

Within this study, oscillatory measurements were performed within the linear viscoelasticity region. Results provide information about the dynamic properties, the elastic modulus G' and the viscous modulus G'' of the analysed gels. G' and G'' of thiolated polymer and corresponding control polymer gels (3%; m/v) as well as mixtures of these gels with 4% (m/v) mucin were determined. Rheological studies were performed with commercially available mucin instead of native mucus as

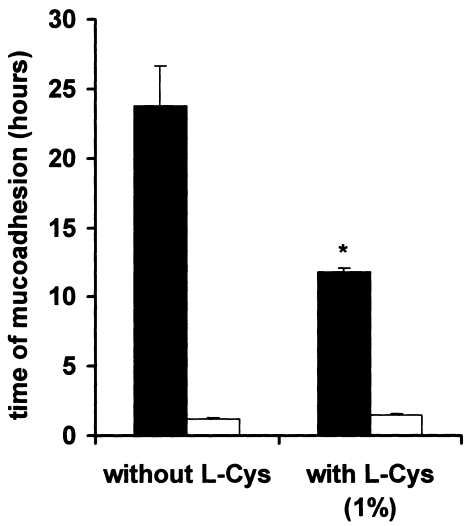

Fig. 3. Influence of cysteine on mucoadhesion of matrix tablets determined via the rotating cylinder method. The black bars indicate the time of mucoadhesion of PAA_{450}–Cys tablets, the white bars the time of mucoadhesion of PAA_{450} control tablets with and without the addition of 1% free cysteine (means ± SD, $n = 3$). *, Differs from corresponding polymer without cysteine, $P < 0.001$.

described by Rossi et al. [20] giving more reproducible and comparable results. To evaluate the influence of the disulphide bond breaking agent cysteine on covalent interactions between polymer and mucin, viscosity measurements were performed with and without 1% (m/v) cysteine. Only if disulphide bonds between the thiolated polymer and mucus glycoproteins are formed, cysteine will have a strong influence on the viscosity of mucus–thiomer mixtures. Results of this study are shown in Figs. 4 and 5. Due to the addition of cysteine to PAA_{450}–Cys and PAA_{450}–Cys/mucin, the elastic and viscous moduli of the mixtures decreased significantly ($P < 0.01$). In contrast, no significant change in G' and G'' of the unthiolated control polymer and polymer–mucin mixtures could be detected after addition of cysteine.

Frequency sweep measurements of PAA_{450}–Cys/mucin mixtures showed only little frequency dependence of elastic and viscous moduli throughout the frequency range assessed (0.1–10 Hz). After incubation with cysteine, both moduli became strongly frequency dependent with a large decrease in the measured values particularly at lower frequencies (data not shown).

3.4. Diffusion studies

These studies were performed with incubates of PAA_2–Cys I or PAA_2 with native porcine intestinal mucus

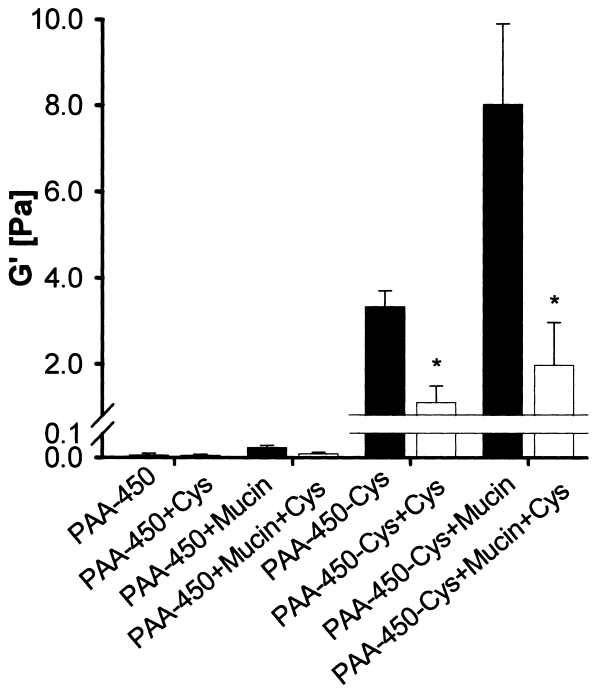

Fig. 4. Elastic modulus G' of 3% (m/v) PAA_{450}–Cys, PAA_{450} control and their mixtures with 4% (m/v) mucin. Measurements were performed without (black bars) and with (white bars) 1% free cysteine added to all samples. Oscillatory measurements were carried out at 1 Hz frequency at room temperature after an incubation period of 20 min. All indicated values are means (±SD) of at least three experiments. *, Differs from corresponding polymer without cysteine, $P < 0.01$.

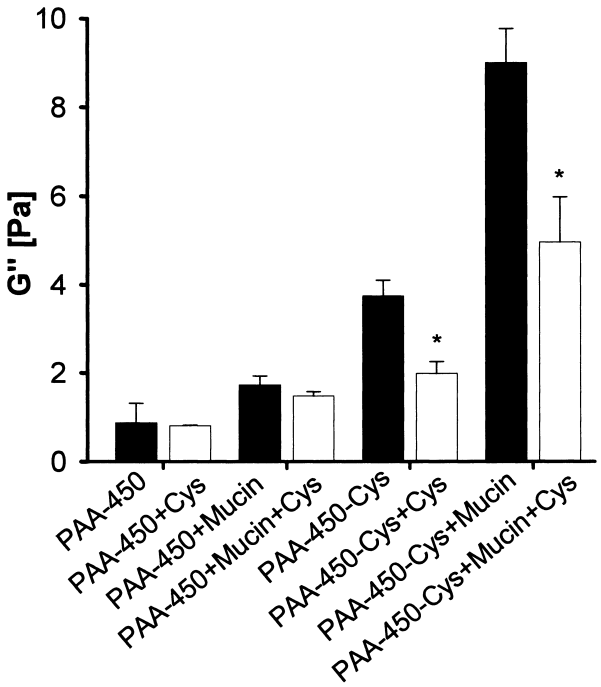

Fig. 5. Viscous modulus G'' of 3% (m/v) PAA_{450}–Cys, PAA_{450} control and their mixtures with 4% (m/v) mucin. Measurements were performed without (black bars) and with (white bars) 1% free cysteine added to all samples. Oscillatory measurements were carried out at 1 Hz frequency at room temperature after an incubation period of 20 min. All indicated values are means (±SD) of at least three experiments. *, Differs from corresponding polymer without cysteine, $P < 0.01$.

(11 ± 0.3% m/m dry weight), purified via exhaustive dialysis. PAA_2–Cys I was used for this study because it displays a comparatively higher amount of covalently bound cysteine and is therefore more likely to form –S–S– linkages. In theory, if PAA_2–Cys is able to form covalent bonds with mucus glycoproteins, only the unbound fraction of the thiolated polymer should diffuse through the membrane into the acceptor chamber. The unmodified control polymer, however, should be retained in the donor chamber to a comparatively much lower extent as it can only interact non-covalently with mucus glycoproteins. The diffusion process of the 2 kDa polymers through the membrane (MWCO 12 kDa) turned out to be very slow, therefore the study was carried out within a time period of 24 h. As shown in Table 1, a 12.8-fold higher concentration of the thiomer remained in the mucin gel compared to the unthiolated control. Diffusion studies of PAA_2–Cys I and PAA_2 solutions performed without any mucus did not show a significant difference in the diffusion behaviour of the two polymers (data not shown).

3.5. Gel filtration

For this study the low molecular weight PAA_2–Cys II was used, as it exhibits on an average only one thiol moiety per molecule in comparison to PAA_2–Cys I with

Table 1
Diffusion studies: concentrations (means ± SD, $n = 3$) of PAA_2–Cys and PAA_2 control in the acceptor chamber after an incubation period of 24 h in an oscillating water bath at 37°C

Test compound	Polymer concentration in the acceptor chamber (% of total amount)	SD
PAA_2 control	8.43	0.7
PAA_2–Cys I	0.66	0.2

approximately two thiol moieties per molecule. Due to this one thiol group, PAA_2–Cys II is supposed to act as a mucolytic agent similar to cysteine, whereas polymers with two or more thiol groups per molecule are supposed to lead to additional crosslinks between mucus glycoproteins. In theory, if thiol/disulphide exchange reactions between thiolated polymers and disulphide bonds within the mucus take place, a thiolated oligomer displaying only one thiol group (PAA_2–Cys II) will break mucus glycoprotein –S–S–mucus glycoprotein bonds leading to comparatively smaller mucus glycoprotein–S–S–oligomer and mucus glycoprotein–SH units (Fig. 1A). In order to verify this formation of smaller mucin units, PAA_2–Cys II was incubated with an 8% dispersion of porcine mucin at 37°C for 1 h. Then, size exclusion chromatography was performed to determine changes in the molecular weight of mucus glycoproteins. The glycoproteins were analysed with TNBS reagent developing an orange dye with an absorbance maximum at 450 nm. Fig. 6 shows the elution profiles of porcine intestinal mucin alone (A), and the profiles of PAA_2–Cys II/mucin and PAA_2/mucin incubates (B). The altered elution profile of mucins after incubation with PAA_2–Cys II demonstrates their partial breakdown into smaller sized units, as described also by Hutton et al. [21] due to the addition of mercaptoethanol.

4. Discussion

The interactions between mucoadhesive polymers and mucus glycoproteins have so far been described to be based on the formation of non-covalent bonds such as hydrogen bonding, ionic interactions and van der Waals forces or physical interpenetration effects of polymer chains and mucus [22,23]. These interactions are weak in comparison to covalent bonds being not influenced by factors such as ionic strength and pH. Functional groups being able to form covalent bonds are overall thiol groups. The resulting disulphide bond is a bridging structure most commonly encountered in biological systems. The formation of disulphide bonds between thiolated polymers and mucus glycoproteins is supposed to be responsible for the enhanced mucoadhesive properties of these polymers [1], yet no full evidence has been supplied.

Within this study, different methods have been employed to prove the covalent nature of the thiomer–mucin interaction. All experiments were performed at a pH above 6 as the concentration of thiolate anions, $-S^-$, representing the reactive form in oxidation and nucleophilic attack increases at higher pH [14]. In mucoadhesion studies, the mucolytic agent cysteine added to the polymer formulation led to a strongly decreased adhesion time of thiomer tablets without influencing the mucoadhesion of the control. If a degradation of the mucus layer, described e.g. for dithiothreitol [13] or mercaptoethanol [12] was the reason for this decrease in mucoadhesion, the adhesion of the control tablet containing cysteine would also have been influenced. As this was not the case, a disruption of thiomer–mucin disulphide bonds by cysteine can be assumed. The addition of cysteine also significantly ($P < 0.01$) reduced the rheological parameters of thiomer–mucin mixtures. Partly, this effect may be explained by the cleavage of disulphide

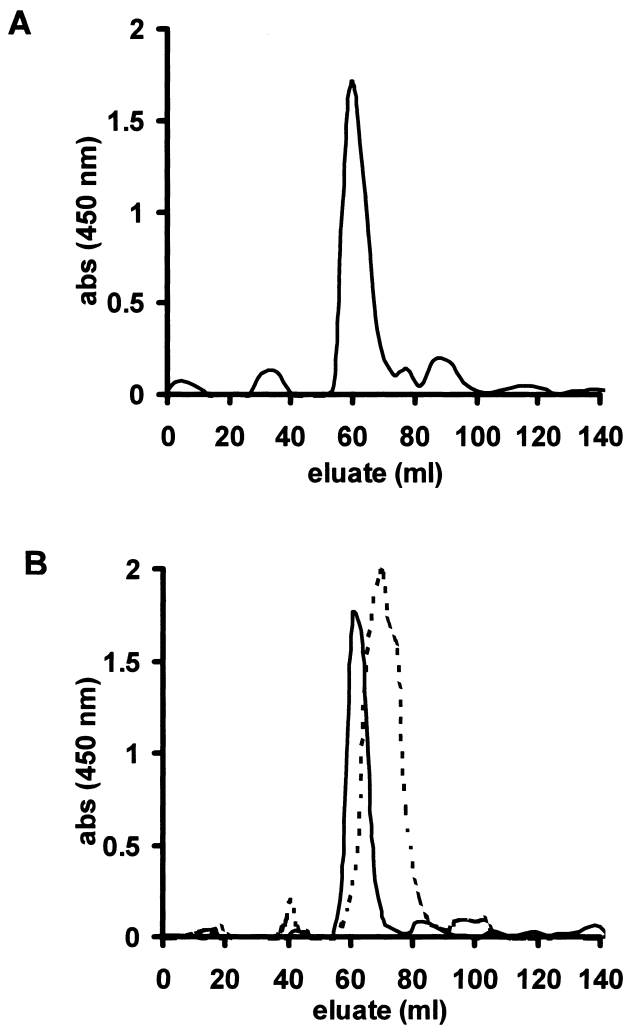

Fig. 6. Gel filtration on Sepharose 2B (40 cm × 1.5 cm). (A) Porcine intestinal mucin, pH 7, incubated for 1 h at 37°C. (B) Porcine intestinal mucin homogenated with PAA_2–Cys (---) and PAA_2 control polymer (—), respectively, and incubated for 1 h at 37°C, pH 7. Fractions of 2.5 ml were assayed for free amino groups in the glycoproteins using TNBS reagent.

bonds within the thiolated polymer and within porcine mucin itself. However, these reactions alone cannot explain the strong decrease of G'/G'' of thiomer–mucin mixtures. Therefore, also bondings between mucin and the thiomer must have been formed, which were cleaved by cysteine. Hence, the formation of disulphide bridges is suggested to be important in the formation of a strengthened mucus gel network. A similar rheological method was used to prove the existence of hydrogen bonds between Carbopol and mucus glycoproteins. The addition of the hydrogen bond breakers KCNS and urea in high concentrations also resulted in a significant decrease in viscosity [24].

Polymer/mucus diffusion studies were carried out with native mucus as this component was described to be the most suitable preparation for this purpose [25]. Due to the relatively high molecular mass (2 kDa) and linearity of the polymer, the diffusion process was very slow. However, it was not the aim of this study to reach the equilibrium, i.e. equal concentrations of unbound polymer in both compartments [26], but to determine the relative difference in the concentrations of thiolated polymer and unmodified polymer. As a 12.8-fold higher concentration of the thiomer than of the unmodified polymer remained in the mucin gel, it can be concluded that the thiomer was retained in the gel by forming covalent bonds with glycoproteins. These results are in good agreement with previously published results of our research group reporting that thiolated polymers but not the corresponding unthiolated control polymers were able to bind to commercially available mucin on incubation for 2 h at 37°C. While the mucin could be completely removed from unmodified polymer–mucin incubates by centrifugation and removing the supernatant, it remained bound to the thiomer and could only be washed out after addition of the disulphide bond breaker dithiothreitol. In this study, however, the amount of polymer-bound mucin could not be quantified because of the heterogeneity of the used mucin [1].

In gel filtration studies, the ability of low molecular weight PAA_2–Cys bearing approximately one thiol group per molecule to cleave mucin disulphide bridges was tested. Incubation of this polymer with mucin resulted in an altered elution profile in comparison to mucin alone or mucin/PAA_2 mixture. The shift of material into the included volume indicates that polymer-bound cysteine was able to bind covalently to mucin glycoproteins thereby forming smaller subunits. Similar gel filtration profiles with not clearly separated species were reported by Fogg et al. [27], who analysed purified pig colonic mucin before and after reduction with 0.2 M mercaptoethanol.

In summary, this study describes four different methods to substantiate the formation of disulphide bonds between thiolated polymers and mucin glycoproteins. The results obtained from this study give strong evidence for these thiomer–mucin disulphide bridges, a very important factor in mucoadhesion, and contribute valuable basic information to the knowledge in the field of thiomers.

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