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Synthesis and characterisation of mucoadhesive thiolated polymers

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Abstract

This study examined various factors influencing the mucoadhesive properties of thiolated polymers (thiomers), which are capable of forming covalent bonds with thiol sub-structures of the mucus glycoprotein. Mediated by a carbodiimide, L-cysteine was therefore covalently bound to polycarbophil (PCP) and to carboxymethylcellulose (CMC). The resulting polymer conjugates displayed 12.3 and 22.3 μmol thiol groups per gram, respectively. Whereas the swelling behaviour of tablets based on CMC was not markedly influenced by the immobilisation of cysteine, it was improved significantly ($P < 0.05$) in case of PCP. Tensile studies carried out with the unmodified and thiolated polymers of pH 3, 5 and 7, respectively, revealed that only if the polymer displays a pH-value of 5, the total work of adhesion can be improved significantly due to the covalent attachment of thiol groups. These results were in good agreement with a new mucoadhesion test system described here taking also the cohesiveness of the delivery system into account. The results represent helpful basic information in order to improve the mucoadhesive properties of thiolated polymers.

Keywords: Thiomers; Mucoadhesion; Cohesiveness; Polycarbophil; Carboxymethylcellulose

1. Introduction

In the 1980s and 1990s the concept of mucoadhesion has received a significant degree of attention. Because of a lengthened period of time in which the dosage form should be in contact with the absorbing membrane versus a standard dosage form, an enhanced drug bioavailability is expected. The use of mucoadhesive delivery systems should thereby reduce the frequency of dosing, leading to an improved patient-compliance. In addition, based on the intensified contact to the mucosa, various mucoadhesive polymers such as poly(acrylic acid) derivatives and chitosan are able to increase the epithelial permeability for many drugs (Borchard et al., 1996). Moreover, the intensified contact to the absorbing mucosa should also exclude an enzymatic degradation of perorally given (poly)peptide drugs on the way between the delivery system and the mucosa (Bernkop-Schnürch, 1999). Hence, considerable

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intensive research work and development both in academia and in industry have been carried out in this field. Nevertheless, mucoadhesive polymers could so far in many cases not really convince as a 'pharmaceutical glue' (Khosla and Davis, 1987; Lehr, 1996).

Recently, however, a new type of mucoadhesive excipients has been established (Bernkop-Schnürch et al., 1999a,b). In contrast to all so far used mucoadhesive polymers, at which the attachment to the mucus layer has been achieved by exclusively non-covalent bonds such as ionic interactions, hydrogen bonds and van der Waal's forces (Peppas and Mikos, 1990), these novel polymers are capable of forming covalent bonds. The disulfide bond representing the most commonly bridging structure in biological systems has thereby been discovered for the adhesion of polymers to the mucus layer. Thiolated polymers, or so-called thiomers, are supposed to interact with cysteine-rich subdomains of mucus glycoproteins (Gum et al., 1992) thereby forming disulfide bonds. Although it could already be demonstrated that a polycarbophil–cysteine conjugate displays more than twofold higher adhesive properties than the corresponding unmodified polymer (Bernkop-Schnürch et al., 1999a,b), further improvements seem to be feasible. A broad understanding of various effects as shown in Fig. 1 being involved in this mechanism of mucoadhesion will thereby be essential in order to achieve such improvements. According to this, it was the objective of this work to carry out basic studies, which should contribute substantial information to our knowledge in this new research field.

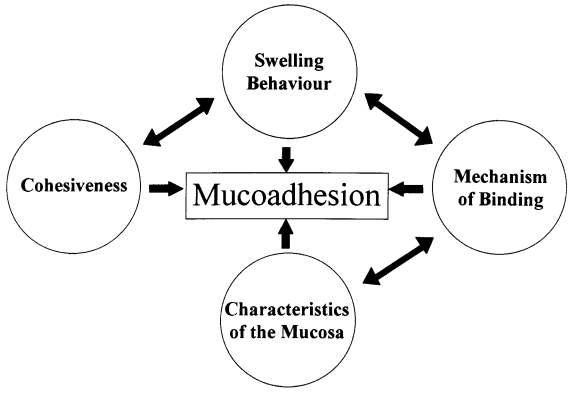

Fig. 1. Schematic presentation of effects influencing mucoadhesion.

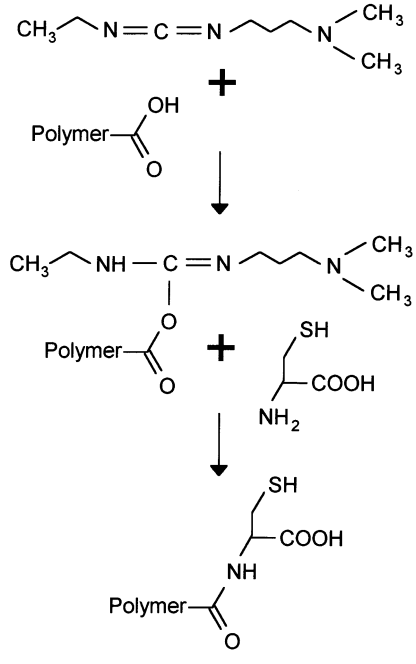

Fig. 2. Synthetic pathway for the covalent attachment of L-cysteine to polycarbophil and carboxymethylcellulose mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

2. Material and methods

2.1. Synthesis of polymer–cysteine conjugates

The covalent attachment of cysteine to sodium carboxymethylcellulose (NaCMC) and polycarbophil (PCP) 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 according to the synthetic pathway as shown in Fig. 2. Polycarbophil (mol. wt. >700 kDa; Noveon AA1, BF Goodrich, Brecksville, OH) was neutralised with NaOH as described previously by our research group (Bernkop-Schnürch and Göckel, 1997). The carboxylic acid moieties of hydrated, neutralised polycarbophil and hydrated sodium carboxymethylcellulose (mol. wt. ~ 1000 kDa; Kwizda, Vienna, Austria)

Table 1
Synthesis of polymer–cysteine conjugates[a]

Polymer	Polymer + Cys	EDAC	Thiol groups (μmol) per g polymer
PCP–Cys conjugate	2 g + 0.25 g/500 ml	50 mM	12.3
PCP control	2 g + 0.25 g/500 ml	–	0.0
CMC–Cys conjugate	2 g + 0.0156 g/100 ml	50 mM	22.3
CMC control	2 g + 0.0156 g/100 ml	–	0.0

[a] Carboxylic acid moieties of polycarbophil (PCP) and carboxymethylcellulose (CMC) were activated for 45 min with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in aqueous solutions; after the addition of cysteine (Cys), reaction mixtures were incubated for 3 h at pH 4 and room temperature

as listed in Table 1 were activated for 45 min by adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St Louis, MO) in a final concentration of 50 mM. L-Cysteine hydrochloride (Sigma, St Louis, MO) was added in amounts as listed in Table 1 and the pH-value of reaction mixtures was adjusted to 4. The molar ratio of EDAC to L-cysteine was thereby 50:3.2 and 50:1 for coupling reactions with polycarbophil and carboxymethylcellulose, respectively. In case of coupling reactions with sodium carboxymethylcellulose the pH-value had to be kept constant by continuously adding 1 N HCl. Reaction mixtures were incubated for 3 h at room temperature. The resulting polymer–cysteine conjugates were isolated by dialysing at 10°C in the dark against 1 mN HCl containing 2 μM EDTA, two-times against the same medium but additionally containing 1% of NaCl and then exhaustively against 1 mN HCl. Samples being prepared and isolated in exactly the same way as polymer–cysteine conjugates but omitting EDAC during the coupling reaction served as controls for the following analytical studies. Samples were lyophilised by drying frozen aqueous polymer solutions at −30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany). Polymer–cysteine conjugates and controls were stored at 4°C until use.

2.2. Determination of the thiol group content

The amount of thiol groups on the polymer–cysteine conjugates and controls was determined by iodometric titration. First, 10.00 mg of each polymer were hydrated in 3.0 ml of demineralised water. The pH-value was then adjusted to 2–3 by adding 1 N HCl. After the addition of 150 μl of aqueous starch solution (1%), samples were titrated with an aqueous iod solution 1.00 mM until a permanent light blue colour became visible.

2.3. Water-uptake studies

Firstly, 30 mg of lyophilised polymer–cysteine conjugates and controls were compressed (Hanseaten Type EI, Hamburg, Germany) into 5.0 mm diameter flat-faced discs. The compaction pressure was kept constant during the preparation of all discs. Test discs were placed on a water permeable membrane serving as the bottom of a plastic tube with a diameter of 16 mm. The tube was then set in a vessel containing demineralised water of 20°C. At predetermined time points the amount of water-uptake was calculated by re-weighing the tubing and content after removing the unbound water.

2.4. Tensile studies

Thirty milligrams of lyophilised polymer–cysteine conjugates, controls and unmodified neutralised polymers were compressed to flat-faced discs as described above. Following this, tensiometer studies with these test discs were carried out on native porcine intestinal mucosa. Test discs were therefore attached to the mucosa with a force of 2.5 mN. After a contact time between test disc and mucosa of 30 min in 100 mM Tris–HCl buffered saline (TBS) pH 6.8 at 25°C, the mucosa was pulled at a rate of 0.1 mm s^{-1} from the disc.

The total work of adhesion (TWA) representing the area under the force/distance curve and the maximum detachment force (MDF) were determined using the WINWEDGE software (TAL Technologies, Inc., Philadelphia, PA) in combination with EXCEL 5.0 (Microsoft).

2.5. In vitro mucoadhesion studies

In order to evaluate the binding to the mucosa as well as the cohesiveness of the tablet, an appropriate new method has been established. Tablets as described above were thereby attached to freshly excised intestinal porcine mucosa, which has been spanned on a stainless steel cylinder (diameter: 4.4 cm; height: 5.1 cm; apparatus 4-cylinder, USP XXII). Thereafter, the cylinder was placed in the dissolution apparatus according to the USP containing 100 mM TBS pH 6.8 at 37 ± 0.5°C. The experimental set up is illustrated in Fig. 3. The fully immersed cylinder was agitated with 250 rpm. The detachment, disintegration and/or erosion of test tablets was observed within a time period of 10 h.

2.6. Statistical data analysis

Statistical data analysis were performed using the t test with $P < 0.05$ as the minimal level of significance.

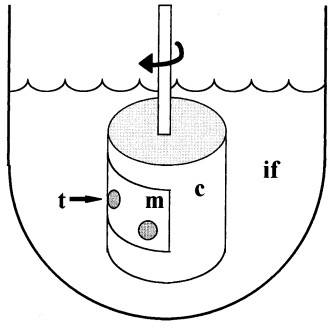

Fig. 3. Schematic presentation of the test system used to evaluate the mucoadhesive properties of tablets based on various polymers. c, cylinder; if, intestinal fluid; m, porcine mucosa; t, tablet.

3. Results

3.1. Chemical characterisation

Poly(acrylic acid) and sodium carboxymethylcellulose are in the rank order of mucoadhesiveness among the highest adhesive polymers (Smart et al., 1984). In order to demonstrate that the mucoadhesive properties even of these polymers can be improved by the covalent attachment of thiol groups, the poly(acrylic acid) derivative polycarbophil and sodium carboxymethylcellulose were chosen as basic polymers. Although it could already be shown that the amount of polymer attached cysteine increases with decrease in the proton concentration during the coupling reaction (Bernkop-Schnürch et al., 1999a,b), a pH-value of 4 was chosen for the reaction in order to exclude thereby the formation of any disulfide bonds. The resulting thiol group content of polymer–cysteine conjugates is shown in Table 1. Although polycarbophil displays at least 2.6-fold more carboxylic acid groups per gram polymer than CMC, which are essential for the covalent attachment of cysteine, the thiol group content for the CMC conjugate was even higher. A reason for this observation might be seen in the additional binding of cysteine to CMC via ester bonds, which would also explain the continuously raising pH-value during coupling reactions with this polymer. The efficacy of the purification method described here could be verified by corresponding controls. Omitting EDAC during the coupling reaction led to negligible amounts of remaining cysteine. In addition, a quantification of remaining primary amino groups on the polycarbophil–cysteine conjugate and control, carried out with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the method described previously by our research group (Bernkop-Schnürch and Krajicek, 1998), showed no remaining free unconjugated cysteine in both samples. The fact that the polycarbophil–cysteine conjugate displays on the one hand no primary amino groups, but on the other hand numerous thiol moieties, gives strong evidence for the formation of amide bonds between the polymer and cysteine. Both polymer–cysteine conjugates remained stable after lyophilisation as well

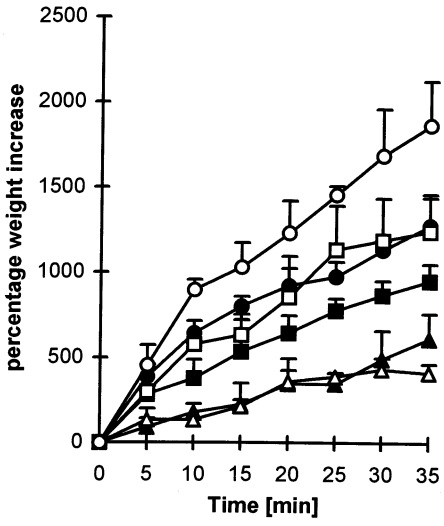

Fig. 4. Comparison of the water-uptake of test discs (30 mg) consisting of polycarbophil (▲, ■, ●) and thiolated polycarbophil (△, □, ○) of pH 3.0 (▲, △), pH 5.0 (■, □) and pH 7.0 (●, ○); indicated values are means ± S.D. of at least three experiments.

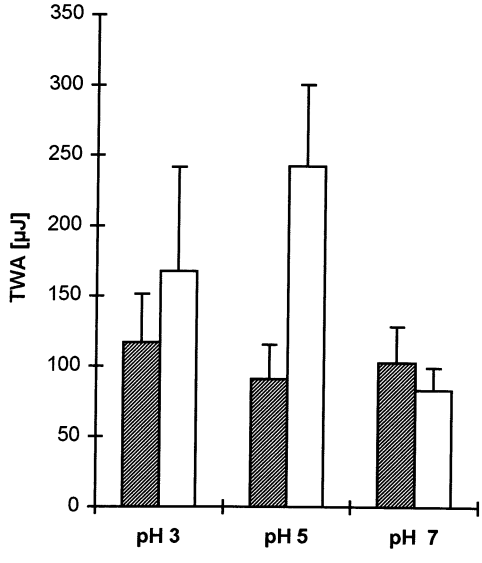

Fig. 5. Comparison of the TWA of test discs (30 mg) consisting of polycarbophil (hatched bars) and thiolated polycarbophil (blank bars) displaying different pH-values; indicated values are means ± S.D. of at least three experiments.

as in aqueous solutions of pH 5.0 and lower. The dried thiomers had the appearance of a white powder, which could be compressed easily to tablets.

3.2. Water-uptake studies

As the swelling behaviour of mucoadhesive polymers has a great influence on their adhesive properties, water-uptake studies were carried out with polycarbophil and CMC with and without covalently attached thiol groups. Whereas the immobilisation of cysteine had no significant influence on the swelling behaviour of CMC (data not shown), it was markedly improved in case of polycarbophil. The results as shown in Fig. 4 demonstrate that the influence of covalently bound cysteine increases with decrease of the proton concentration on the polymer. Whereas the swelling behaviour was not improved significantly at pH 3.0 due to the cysteine moieties, it was comparatively higher at pH 5.0 and 7.0. As the proton concentration on the polymer directly correlates with the amount of carbonic acid groups, a higher concentration of $-COO^-Na^+$ moieties on the polymer will improve the influence of covalently attached cysteine on the swelling behaviour. This observation is in good accordance with results obtained for the CMC conjugate, displaying a comparatively much lower amount of $-COO^-Na^+$ moieties per gram polymer and therefore no significant influence of covalently attached cysteine.

3.3. Mucoadhesion studies

Tensile studies carried out with the polycarbophil–cysteine conjugate and the CMC–cysteine conjugate revealed a strong pH-dependence of the mucoadhesive properties. For polymers exhibiting a pH-value of 7.0, no marked improvement in their mucoadhesive properties could be achieved due to the immobilisation of thiol groups on the polymers. In contrast, at pH 5.0 the TWA was for thiolated polycarbophil as well as thiolated CMC significantly higher than that of the corresponding unmodified polymers. A further increase in the proton concentration on the polymer, however, led only in case of CMC to a further improvement in mucoadhesive properties. Results of this study are shown in Figs. 5 and 6. The maximum detach-

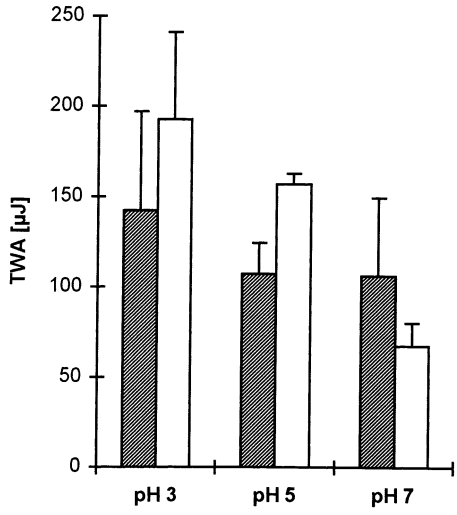

Fig. 6. Comparison of the TWA of test discs (30 mg) consisting of NaCMC (hatched bars) and thiolated NaCMC (blank bars) displaying different pH-values; indicated values are means ± S.D. of at least three experiments.

ment force was thereby in good correlation with the total work of adhesion.

In order to be able to take also the cohesiveness of the polymers into account, a new in vitro test model has been established being much closer to the in vivo situation than simple tensile studies as described above. The use of the dissolution apparatus according to the Pharm. Eur. and the USP in combination with a standard steel cylinder, which is also described in the USP, makes this test model to a very reproducible evaluation system. Thereby obtained results demonstrated improved adhesive properties due to the immobilisation of thiol groups as well. In particular, tablets based on the polycarbophil–cysteine conjugate pH 5.0 remained even after 10 h of incubation very stable attached to the mucosa. The corresponding control, however, detached the mucosa within the half of this time. These results were in good agreement with the results obtained from simple tensile studies. They are listed in Table 2 including also observations concerning the cohesiveness of the test systems. In contrast to tablets based on unmodified polycarbophil as well as CMC, and with the exception of the PCP–cysteine conjugate pH 3.0, neither erosion nor disintegration could be observed for tablets based on thiolated polymers.

4. Discussion

Within this study we could confirm by two different test systems recently published results which suggested the improvement of mucoadhesive properties of polymers due to the immobilisation of thiol groups. The pH-value of thiolated polymers has thereby an important influence. Only if the polycarbophil–cysteine conjugate and the CMC–cysteine conjugate exhibit a pH-value of 5.0, a significantly higher TWA could be determined. A reason for this observation can be seen in the fact that thiolated polymers do not only form disulfide bonds with the mucus gylcoprotein but also inter- and/or intrachain disulfide bonds within the polymer itself (Bernkop-Schnürch et al., 1999a,b). In aqueous solutions, this crosslinking process within the polymer takes place very

Table 2
Comparison of the mucoadhesive properties of polymers with and without thiol groups[a]

Polymer	pH 3		pH 5		pH 7	
PCP control	7.5 ± 2.0	dis.	4.8 ± 1.35	det.	4.6 ± 1.39	dis.
PCP–Cys	7.55 ± 1.15	dis.	>10		2.25 ± 0.87	det.
CMC control	2.0 ± 0.35	det.	2.5 ± 0.5	dis.	1.5 ± 0.91	det.
CMC–Cys	3.9 ± 1.02	det.	3.0 ± 0.35	det.	1.7 ± 0.57	det.

[a] Test discs consisting of indicated polymers were attached to excised porcine mucosa, which has been spanned on a cylinder and agitated with 250 rpm in 100 mM TBS pH 6.8 at 37 ± 0.5°C. The indicated time of adhesion represents the mean ± S.D. of at least three experiments. Failure of adhesion was either caused by the detachment (det.) of the whole test disc from the mucosa or disintegration (dis.) of the adhering disc.

rapidly, if the polymer displays a pH-value of above 5 being closer to the pK_a-value of cysteine with 8.35 (Roth et al., 1990). Disulfide exchange reactions between thiol groups of the polymer and disulfide bonds within the mucus layer are thereby strongly reduced, as most thiol groups of the polymer are already oxidised before they can interact with the mucus. In contrast, if the polymer exhibits a pH-value of below 5, most thiol groups of the polymer remain stable, as the concentration of thiolat anions, $-S^-$, representing the reactive form for nucleophilic attack is very low (Snyder, 1987). Hence, also thiol/disulfide exchange reactions between the polymer and the mucus layer can almost be excluded. According to this, the best compromise between a too rapid and a too slow reaction of thiol groups on the polymer can be seen at pH 5, leading to the strongest increase in adhesive properties.

Aside from the pH-value of the polymer itself, also the pH-value of the intestinal fluid has an influence on the mucoadhesive properties of thiolated polymers which could already be verified by our research group (Bernkop-Schnürch et al., 1999a,b). Further studies carried out with matrix tablets comprising carbomer and CMC, respectively, demonstrated a high buffer capacity of the hydrated carrier matrix. The effect can be explained by these anionogenic polymers per se, which can function as ion exchange resins. In particular, tablets based on the neutralised poly(-acrylic acid) derivative were capable of maintaining a pH-value of above 5.5 in the delivery system for 60 min even in a simulated gastric fluid (Bernkop-Schnürch and Gilge, 1999). According to this, a pH-value of 5.0, which is highly beneficial in order to improve mucoadhesion of thiomers as described here, should be maintained even for a longer period of time, as the pH-value of the intestinal fluid is also in this pH-range. In particular, as the pH-value of the intestinal fluid in the duodenum is around 5 (Wissenschaftliche Tabellen Geigy, 1983), mucoadhesion of thiomers should be the relatively highest in this segment of the gut.

Comparing the improvement in mucoadhesive properties due to the covalent attachment of cysteine on CMC and polycarbophil, demonstrates a much stronger effect of thiol moieties bound to the latter ones. A reason for this observation can be seen in the additional formation of ester bonds between CMC and cysteine leading to covalently attached primary amino groups on this polymer. The combination of primary amino groups on a polymer causes strongly reduced mucoadhesive properties as, e.g. already shown for chitosan–EDTA conjugates (Bernkop-Schnürch and Krajicek, 1998) and will at least partially mask the effect of the covalently attached cysteine in this case.

Besides the mechanism of adhesion, also the cohesiveness of the dosage form has a great influence on mucoadhesion (Fig. 1). The highest adhesive properties of a dosage form to the mucus layer are worthless, if binding fails within the delivery system itself. Results as shown in Table 2 revealed, that most tablets based on unmodified polycarbophil and CMC are not very stable. These results are in good agreement with studies carried out by Kaiho et al. (1996) demonstrating a fast disintegration of tablets based on carbomer. Tablets based on thiolated polycarbophil and CMC, however, displayed a comparatively high stability which can be explained by the formation of inter- and/or intramolecular disulfide bonds within these polymers. The combination of strong mucoadhesive properties and high cohesiveness — as displayed by thiomers — seems therefore to be highly beneficial for adhesive dosage forms.

According to the theory, that rapidly swelling polymers will also quickly interact with the mucin, a good swelling behaviour should contribute to mucoadhesion (Fig. 1) (Mortazavi and Smart, 1993). A correlation between the swelling behaviour of investigated polymers and their adhesive properties, however, could not be found. Whereas the swelling behaviour of polycarbophil pH 7.0, for instance, was the comparatively highest, its mucoadhesive properties were the very lowest. Previous studies demonstrated that the swelling behaviour of thiolated polycarbophil depends also on the amount of covalently bound cysteine. The more of the sulfhydryl compound was bound to the polymer, the higher was the swelling behaviour of the conjugate (Bernkop-Schnürch et al., 1999a,b). Although the test sys-

tem was the same for both studies, we had to realise that the water-uptake values could not be directly compared with each other. Only if all parameters such as the compaction pressure and remaining traces of water in the polymer are exactly the same, the swelling behaviour will become comparable between different test series.

Aside from its strong adhesive and cohesive properties, thiomers display additional highly beneficial features. Clausen et al. (1999), for instance, could demonstrate an improved penetration enhancing effect of polycarbophil due to the immobilisation of cysteine, whereas the same concentration of unbound cysteine and polycarbophil did not display this effect. Moreover, sulfhydryl compounds including cysteine are well known for their complexing properties towards Zn-ions. Hence, various Zn-dependent proteases of the gut can be inactivated by the deprivation of this essential cation out of the enzyme structure (Bernkop-Schnürch, 1998). Aminopeptidase N, for example, could be strongly inhibited by *N*-acetylcysteine (Bernkop-Schnürch and Marschütz, 1997). According to this, thiomers might display a protective effect for embedded peptide drugs towards an enzymatic attack in the intestine rendering them probably useful as tool for peroral peptide delivery systems.

In summary, within this study the improvement of the mucoadhesive properties of anionogenic polymers due to the immobilisation of cysteine could be confirmed for polycarbophil and CMC by two different in vitro test systems. In addition, due to the formation of disulfide bonds within these thiolated polymers, their cohesiveness could also be greatly improved. Because of these features, thiolated polymers seem to represent a promising new generation of mucoadhesive polymers which should provide a much more prolonged residence time of drug delivery systems on various mucosal tissues compared to well established polymers.

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