Polymers with Thiol Groups: A New Generation of Mucoadhesive Polymers?

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Purpose. To improve the mucoadhesive properties of polycarbophil by the introduction of sulfhydryl groups.

Methods. Mediated by a carbodiimide, cysteine was covalently bound to polycarbophil (PCP) forming amide bonds between the primary amino group of the amino acid and the carboxylic acid moieties of the polymer. The amount of covalently attached cysteine and the formation of disulfide bonds within the modified polymer were determined by quantifying the share of thiol groups on the polymer conjugates with Ellman's reagent. The adhesive properties of polycarbophil-cysteine conjugates were evaluated *in vitro* on excised porcine intestinal mucosa by determining the total work of adhesion (TWA).

Results. Depending on the weight-ratio of polycarbophil to cysteine at the coupling reaction, e.g., 16:1 and 2:1, 0.6 ± 0.7 μmole and 5.3 ± 2.4 μmole cysteine, respectively, were covalently bound per g polymer. The modified polymer displayed improved internal cohesive properties due to the formation of interchain disulfide bonds within the polymer in aqueous solutions at pH-values above 5. Adhesion studies revealed strongly improved adhesive properties. Whereas the TWA was determined to be 104 ± 21 μJ for the unmodified polymer, it was 191 ± 47 μJ for the polymer-cysteine conjugate 16:1 and 280 ± 67 μJ for the polymer-cysteine conjugate 2:1.

Conclusions. Polymers with thiol groups might represent a new generation of mucoadhesive polymers displaying comparatively stronger adhesive properties.

KEY WORDS: mucoadhesion; cohesion; cysteine; polycarbophil; disulfide bonds.

INTRODUCTION

Since the concept of bioadhesion has been introduced into the pharmaceutical literature, many attempts in academia as well as industry have been undertaken to improve bioadhesive properties of various polymers. These attempts include the neutralization of ionogenic polymers (1), the precipitation of polymers in organic solvents and air drying instead of lyophilization (2), and the development of polymer-lectin conjugates (3,4), as well as, polymer-bacterial adhesin conjugates (5) focusing on a specific binding to epithelia. All these systems, however, are based on the formation of non-covalent bonds such as hydrogen bonds and ionic interactions. They are therefore able to provide only a weak adhesion being in many cases insufficient to guarantee the localization of a drug delivery system at a given target site. According to this, polymers capable of forming covalent bonds—even if it is only to the mucus layer—should display comparatively much higher adhesive properties.

The mucus layer covering GI-epithelia consists mainly of mucus glycoproteins which have a central region heavily laden with O-linked oligosaccharide chains and two flanking cysteine-rich subdomains on either side. These cysteine-rich subdomains containing over 10% Cys in their primary structure are involved in the linking of mucin monomers into oligomers via disulfide bonds, building up the three-dimensional network of the mucus gel layer (6). The mucolytic activity of thiols such as N-acetylcysteine is based on disulfide exchange reactions (7) between mucin glycoproteins in mucus and the mucolytic agent. Due to exchange reactions such as illustrated in Fig. 1, intra- as well as intermolecular disulfide bridges within the glycoprotein-structure are cleaved leading to a breakdown of the mucus. Based on the observation, that the mucolytic agent is thereby covalently bound to mucin glycoproteins in mucus, also other thiol bearing compounds in particular polymers with thiol groups should be covalently bound to the mucus (Fig. 1). Apart from this disulfide exchange reactions, the oxidative formation of additional disulfide bridges between thiol groups of the mucin glycoprotein and the polymer could be expected representing the principle of covalent chromatography for (poly)peptides on resins with thiol groups (9).

In order to verify this working hypothesis, it was the objective of this study to generate a polymer bearing thiol substructures and to demonstrate an improved mucoadhesion based on the formation of disulfide bonds between the modified polymer and the mucus gel layer. Cysteine was therefore covalently bound to polycarbophil (PCP) representing one of the most bioadhesive polymers (10). The mucoadhesive properties of the resulting polymer-cysteine conjugates should then be evaluated by different adhesion studies *in vitro*.

MATERIALS AND METHODS

Synthesis of Polymer-Cysteine Conjugates

The covalent attachment of cysteine to polycarbophil 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. Polycarbophil (Noveon AA1, BF Goodrich, Brecksville, Ohio, was neutralized with NaOH as described previously by our research group (11). Sixteen grams of neutralized polycarbophil (NaPCP) were hydrated in 4 L of demineralized water. The carboxylic acid moieties of the polymer were activated for 45 min by adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, Missouri, in a final concentration of 50 mM. In order to avoid oxidation of sulfhydryl groups by atmospheric oxygen, the pH-value was adjusted to 4–5 by adding 5 N HCl and the reaction mixture was gassed with nitrogen for 15 min. Increasing amounts of L-cysteine (Sigma, St. Louis, Missouri, as shown in Table 1 were added to 250 mL aliquots and reaction mixtures were incubated for 3 h at room temperature under nitrogen. According to the weight-ratio of polycarbophil to cysteine during this coupling reaction, the resulting polymer-cysteine conjugates were called 32:1 up to 1:4 as listed in Table 1. The

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ABBREVIATIONS: EDAC, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; MDF, maximum detachment force; TWA, total work of adhesion; NaPCP, polycarbophil neutralized with NaOH; TBS, Tris-HCl buffered saline (0.9% NaCl).

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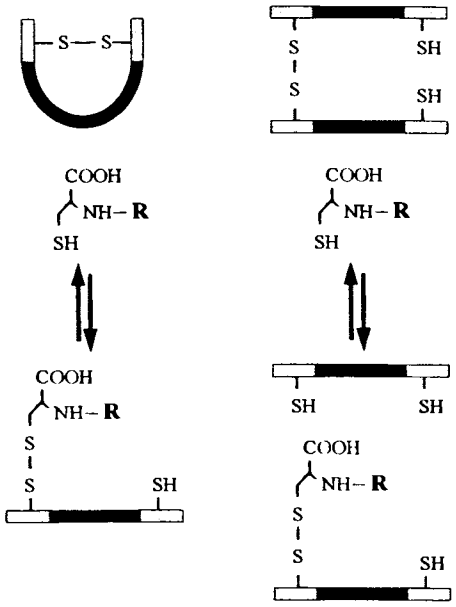

Fig. 1. Schematic presentation of disulfide exchange reactions between a (poly)peptide and a cysteine derivative according to G. H. Snyder (8). The (poly)peptide stands here for a mucin glycoprotein of the mucus and the cysteine derivative is a polymer-cysteine conjugate (R = polycarbophil).

conjugates were isolated by dialyzing at 10°C in the dark against 1 mN HCl containing 2 μM EDTA, two-times against the same medium but containing 1% NaCl and then exhaustively against 0.5 mN HCl. Samples being prepared and isolated in exactly the same way as polycarbophil-cysteine conjugates but omitting EDAC or cysteine during the coupling reaction served as control A and control B for the following analytical studies. The pH value of dialyzed polymer-cysteine conjugates and controls was adjusted to pH 5 with 2 N NaOH and samples were lyophilized by drying frozen aqueous polymer solutions at −30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany). Polymer-cysteine conjugates as well as controls were stored at 4°C until evaluation.

Determination of the Thiol Group Content

The degree of modification was determined by measuring the amount of thiol groups of polycarbophil-cysteine conjugates and corresponding controls using Ellman's reagent (DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid), Sigma, St. Louis, Missouri). Nine milligrams of each conjugate were swelled for 2 h at room temperature in 1 mL of 100 mM phosphate buffer pH 8.2, 50 mN HCl and 4% NaCl. 100 μL of 0.5 N NaOH were added and aliquots of 200 μL transferred in the first wells of a microtitration plate (96-wells, not binding). After incubation for 45 min at room temperature with 100 μL of 0.4% (m/v) DTNB dissolved in 0.5 M phosphate buffer pH 7.1, absorbance at 405 nm was measured (Anthos Reader 2001, Salzburg, Austria). The amount of thiol groups was calculated using a standard curve obtained by the sulfhydryl group determination of a series of solutions containing unmodified polycarbophil and increasing amounts of cysteine.

Water-Absorbing Capacity

Thirty milligrams of lyophilized polycarbophil-cysteine conjugates and unmodified neutralized polycarbophil 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 demineralized 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.

Disulfide Bond Formation Within the Polymer Conjugate

First, 20 mg of polycarbophil-cysteine conjugate 1:2 which had not been brought to pH 5 after dialyzing was hydrated in 1.6 mL of demineralized water for 12 h at 4°C. The pH-value of aliquots (0.8 mL) was then adjusted to pH 5.0 and pH 6.8, respectively, demineralized water was added in order to obtain a final volume of 1 mL and samples were incubated at 37°C under permanent shaking. At predetermined time points, aliquot

Table 1. Concentrations of Reagents Used for Reaction Mixtures in Order to Form Polycarbophil-Cysteine Conjugates with Increasing Amounts of Thiol Groups

Polycarbophil-cysteine conjugate	Polycarbophil (g/250 mL)	Added cysteine (g)	EDAC (mM)	Thiol groups (μMole per gram polymer); means ± S.D. n = 6–8
PCP-Cyst. 1:4	1	4	50	142.2 ± 38.0
PCP-Cyst. 1:2	1	2	50	12.4 ± 2.3
PCP-Cyst. 2:1	1	0.5	50	5.3 ± 2.4
PCP-Cyst. 4:1	1	0.25	50	3.2 ± 2.0
PCP-Cyst. 8:1	1	0.125	50	2.9 ± 1.4
PCP-Cyst. 16:1	1	0.0625	50	0.6 ± 0.7
PCP-Cyst. 32:1	1	0.03125	50	0.3 ± 0.5
Control A	1	0.03125 up to 4 g	—	0.0 ± 0.0
Control B	1	—	50	n.d.

Note: The degree of modification was determined using Ellman's reagent.

volumes of 150 µL were transferred to a microtitrationplate, the pH-value was adjusted to 8.2 with 1 N NaOH and 0.5 M phosphate buffer pH 8.2 was added in order to obtain a final volume of 200 µL. The amount of remaining thiol groups was then determined with Ellman's reagent as described above. In addition, the increase in viscosity due to the formation of interchain disulfide bonds was determined by measuring viscosity of the gel (ΔD = 10 s^{-1}/min; RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) immediately after starting the reaction and after 8 h and 24 h of incubation at 37°C.

Mucin Binding Studies

First, 5 mg of porcine mucin (Sigma, St. Louis, Missouri) were dissolved in 1.0 mL of demineralized water. After the addition of 5 mg of the polycarbophil-cysteine conjugate 1:2 and unmodified neutralized polycarbophil, respectively, the pH-value was adjusted to 7.8 with 1 N NaOH and samples incubated for 2 h at 37°C while shaking. Samples were centrifuged for 10 min at 30,000 g and the supernatants containing unbound mucin discarded. The remaining pellets were diluted 1:10 with 50 mM Tris-HCl pH 7.8 containing 2% NaCl, again centrifuged and the supernatant removed. This purification step was repeated five times. Thereafter the amount of polymer bound mucin was spectrophotometrically (Lambda 16; Perkin-Elmer, Vienna, Austria) investigated by measuring the absorption shoulder at 280 nm.

In Vitro Evaluation of the Adhesive Properties

Tensile Studies with Dry Polymer Compacts

Thirty milligrams of lyophilized polycarbophil-cysteine conjugates, controls and unmodified neutralized polycarbophil were pressed to flat-faced discs as described above. The compaction pressure was kept constant during the preparation of all discs. 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 50 mM Tris-HCl buffered saline (TBS) pH 6.8 with and without 1% (m/v) dithiothreitol or 100 mM glycine-HCl pH 3.0 containing 0.9% NaCl 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 in combination with EXCEL 5.0 (Microsoft).

Tensile Studies with Hydrated Polymers

In order to minimize the influence of an 'adhesion by hydration,' tensile studies were also carried out with hydrated polymers in a slightly modified way as described previously by Robinson and co-workers (12). 150 µL of aqueous gels of 2.5% (m/v) lyophilized NaPCP and polycarbophil-cysteine conjugate 8:1 were spread in a uniform monolayer over excised porcine intestinal mucosa which had been fixed on a flat surface (10 mm i.d.) exhibiting a relative weight of 0.26 g in system. In 100 mM TBS pH 6.8 at 25°C, the polymer was brought in contact with a second porcine mucosa. The TWA was then determined as described above.

Statistical Data Analysis

Statistical data analysis was performed using the *t* test with $p < 0.05$ as the minimal level of significance.

RESULTS

Synthesis of Polycarbophil-Cysteine Conjugates

For synthesis of polycarbophil-cysteine conjugates it was essential to avoid air oxidation of thiol groups. The coupling reaction was therefore carried out under nitrogen at a pH-value of 4–5. In order to remove Cu^{2+} -ions, which would catalyze an oxidation, EDTA was added in the first step of dialysis. Results demonstrated a good correlation between the polymer to cysteine ratio at the coupling reaction and the amount of covalently attached cysteine. The more cysteine was added to the polymer, the more covalently attached thiol groups could be determined in the resulting conjugate. The efficacy of the purification method described here has been verified by controls A. Omitting EDAC during the coupling reaction led to polymers exhibiting a negligible amount of cysteine. Results of this study are shown in Table 1. All polymer-cysteine conjugates were easy swellable in aqueous solutions at a pH-value above 5, thereby forming transparent gels of highly viscoelasticity. They are stable towards air oxidation as dry powders as well as in aqueous solutions at a pH-value below 5.

Swelling Behavior of Polymer-Cysteine Conjugates

Based on the theory of 'adhesion by dehydration' (13) the water uptake of the polymer-cysteine conjugate might also influence mucoadhesion. Water uptake studies, however, demonstrated no significantly quicker swelling behavior of the polymer-cysteine conjugates 32:1 up to 2:1. Merely the polycarbophil-cysteine conjugates 1:2 and 1:4 displayed a significantly higher water uptake in comparison to the unmodified polymer. Results of this investigation are shown in Fig. 2.

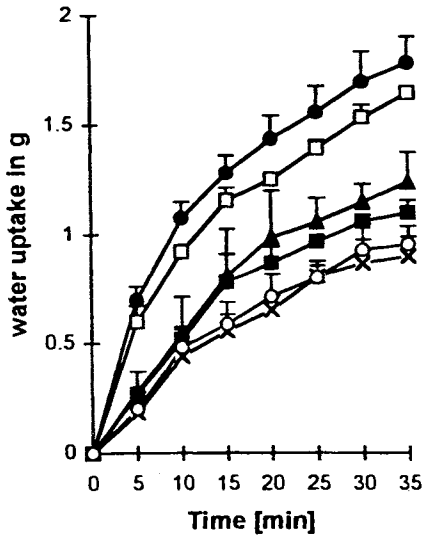

Fig. 2. Comparison of the water uptake of compacts (30 mg) of polycarbophil-cysteine conjugate 1:4 (●), polycarbophil-cysteine conjugate 1:2 (□), polycarbophil-cysteine conjugate 2:1 (▲), polycarbophil-cysteine conjugate 8:1 (■), polycarbophil-cysteine conjugate 32:1 (X), and unmodified neutralized polycarbophil (○). Represented values are means (±S.D.) of at least three experiments.

Formation of Disulfide Bonds Within Polymer-Cysteine Conjugates

In aqueous solutions at pH-values above 5 the thiol groups of polycarbophil-cysteine conjugates are not stable any more. They are continuously oxidized thereby forming disulfide bonds. The decrease in sulfhydryl groups at pH 6.8 is illustrated in Fig. 3. Due to the high density of carbonic acid moieties within poly(acrylic acid) derivatives, these polymers can also function as ion exchange resins. Hydrated matrix tablets based on such polymers are able to maintain a previously adjusted pH-value even in GI-fluids over several hours (data not shown). According to this, the formation of disulfide bonds within polymer-cysteine conjugates might be controllable by a priori adjusting the pH-value of the system. Whereas the amount of thiol groups decreases, viscosity of the polymer conjugate increases. Corresponding investigations demonstrated a viscosity of 2907 ± 193, 3228 ± 154 and 3394 ± 149 mPa*s (means ± S.D.; n = 4–5) after 0, 8 and 24 h of incubation at 37°C. This markedly increase in viscosity can be explained by the formation of interchain disulfide bonds leading to an improved cohesion of the polymer network. Adhesion of many quick swelling polymers is limited by an insufficient cohesion of the polymer resulting in a break within the polymer network rather than between the polymer and mucus layer. Although polycarbophil-cysteine conjugates are rapidly hydrated, they are able to form highly cohesive and viscoelastic gels due to the formation of additional disulfide bonds. Compacts of polycarbophil-cysteine conjugates, which were actually pressed for tensile studies, displayed high mechanical stability as well as elasticity without any erosion even after several days of incubation with 50 mM TBS pH 6.8. In contrast, compacts of unmodified polycarbophil disintegrated within several hours. Especially for polymer conjugates of high cysteine dotation the formation of an over-hydrated slippery mucilage can therefore be completely excluded.

Mucin Binding Studies

The mucin is composed largely of flexible glycoprotein chains, which are crosslinked by disulfide bonds. Due to these disulfide bonds and/or remaining thiol moieties of the glycoprotein, it should be bound to polymers exhibiting sulfhydryl groups. Although a detailed quantification of the amount of mucin bound to tested polymers was impossible because of the heterogeneity of the used mucin, this theory could nevertheless be verified. Results demonstrated the mucin was effectively bound to the tested polymer-cysteine conjugate, whereas it was not at all bound to unmodified neutralized polycarbophil. Moreover, due to the addition of 1% (m/v) dithiothreitol already bound, mucin could be completely removed from the polycarbophil-cysteine conjugate.

Tensile Studies

Tensile studies with dry compacts of polymer-cysteine conjugate 32:1, 16:1, and 8:1 demonstrated a clear correlation between the amount of polymer-linked cysteine and the adhesive properties. The more cysteine was bound to the polymer, the higher were its adhesive properties. At the polymer-cysteine conjugates 8:1, 4:1, and 2:1 mucoadhesion reached a plateau phase displaying a more than twice as high total work of adhesion (TWA) than the unmodified polymer. A further increase in the amount of covalently linked sulfhydryl groups, however, lead to a comparatively lower TWA. A reason for this observation can be seen in a too strong modification of the original polymer leading also to a significantly higher swelling behavior as shown in Fig. 2. Results of adhesion studies are shown in Fig. 4. Whereas the maximum detachment force (MDF) of all conjugates and controls was in very good correlation with the total work of adhesion, it was comparatively higher at the polymer-cysteine conjugate 1:4. Tensile studies carried out at pH 3.0 instead of pH 6.8 revealed a significant decrease in the TWA of the polymer-cysteine conjugate displaying only a negligible amount of active thiolate anions at this pH-value.

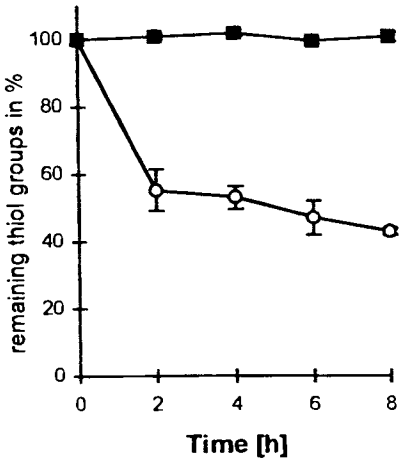

Fig. 3. Disulfide bond formation within a gel of 1% (m/v) polycarbophil-cysteine conjugate 1:2 at pH 6.8 (○) and pH 5.0 (■) at 37°C. Indicated values are means (±S.D.) of at least four experiments.

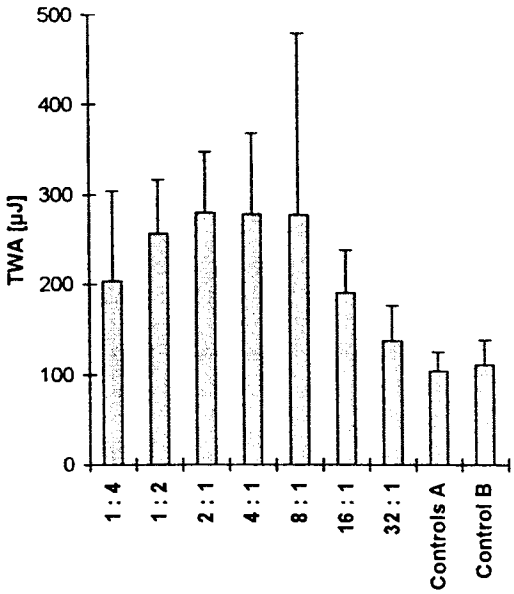

Fig. 4. Comparison of the adhesive properties of polycarbophil-cysteine conjugates and controls which were generated according to the scheme as listed in Table 1. Represented values are means ±S.D. (n = 3–8) of the TWA determined in tensile studies at pH 6.8 with dry compacts of indicated test material.

Whereas the increase in TWA of the polycarbophil-cysteine conjugate 2:1 was determined to be 2.69 ± 0.65-fold compared to unmodified polycarbophil at pH 6.8 (mean ± S.D.; n = 3), it was only 1.36 ± 0.71-fold at pH 3.0 (mean ± S.D.; n = 5). Furthermore, the increase in TWA of the same polycarbophil-cysteine conjugate compared to the unmodified polymer was also only 1.55 ± 0.23-fold at pH 6.8 (mean ± S.D.; n = 4) due to the addition of 1% dithiothreitol inhibiting the formation of disulfide bonds between the polymer and the mucus. The difference in TWA between the unmodified polymer and the polycarbophil-cysteine conjugate 2:1 was therefore neither at pH 3.0 nor in the presence of dithiothreitol of significance.

Tensile studies carried out with hydrated polymers demonstrated also an approximately twice as high TWA for the tested polycarbophil-cysteine conjugate. Results are shown in Table 2. As at this type of adhesion test, the break occurred more within the polymer itself than between the polymer and the mucus layer, it was impossible to differentiate between polymer adhesion and cohesion. Both factors, however, are essential for a long-term attachment of dosage forms to the mucosa.

DISCUSSION

According to our working hypothesis, the mucoadhesive properties of polymers should be improved due to the introduction of thiol groups leading to covalent bonds between the polymer and the mucus layer.

On the one hand this theory could be confirmed (I) by the effective immobilization of isolated mucin to the polymer-cysteine conjugate, whereas it was not at all bound to the unmodified polymer. (II) Tensile studies carried out with dry compacts of polymers demonstrated that the mucoadhesive properties of polycarbophil can be raised for more than 100% due to the immobilization of cysteine. (III) In contrast to tensile studies carried out at pH 6.8, the adhesive properties of the tested polycarbophil-cysteine conjugate 2:1 were strongly reduced at pH 3.0. At this pH-value the formation of disulfide bonds as well as disulfide exchange reactions can be almost excluded due to a negligible amount of negative thiolate anions, $-S^-$, representing the reactive form of cysteine in oxidation and nucleophilic attack (8). (IV) The comparably lower adhesive properties due to the addition of dithiothreitol suppressing the formation of disulfide bonds could also substantiate our working hypothesis.

However, we had to realize the improved adhesive properties of polycarbophil-cysteine conjugates cannot exclusively be explained by the formation of disulfide bonds between the polymer and the mucus layer. As the mechanism of mucoadhesion is even for well established mucoadhesive polymers not yet fully understood, the exact explanation of an additional mechanism turns out to be much more complex and difficult. In contrast to unmodified polycarbophil, for instance, the also quickly hydrated polycarbophil-cysteine conjugates remain very cohesive due to the formation of interchain disulfide bonds within the swelling polymer. The approximately twice as high TWA of the hydrated polycarbophil-cysteine conjugate 8:1 compared to the hydrated unmodified polymer has to be seen as the result of higher cohesive properties of the polymer conjugate, as the adhesive bond of both polymers failed more within the polymer itself. These results are in good accordance with earlier investigations demonstrating that the detachment of hydrated poly(acrylic acid) discs from a mucosa depends on interfacial phenomena as well as viscoelastic properties (14).

So far the use of quick swelling polymers was limited by an over-hydration leading to a slippery mucilage. Using such polymers the break occurred rather within the polymer than between the polymer and the mucus layer. In contrast, polycarbophil-cysteine conjugates display both high cohesive properties, which could be demonstrated in tensile studies carried out with hydrated polymers, and a quick swelling behavior. The results of this study revealed also a significantly improved swelling behavior of polycarbophil-cysteine conjugates 1:2 and 1:4 compared to the unmodified polymer. According to the theory, rapidly swelling polymers will also quickly interact with the mucin thereby providing good adhesion, the quicker water uptake of these polycarbophil-cysteine conjugates should also be taken into consideration as an additional effect for improved adhesive properties. However, in comparison to the polymer-cysteine conjugate 8:1 up to 2:1, which did not display a significantly improved swelling behavior, the adhesive properties of these two conjugates were even lower.

In summary, the high adhesive properties of polycarbophil-cysteine conjugates have therefore to be seen as a result of various factors. The influence of factors such as the formation of disulfide bonds, hydration and internal cohesion on the mucoadhesive properties of modified polymers can only be evaluated in connection with each other and not apart.

Table 2. Adhesive Properties of 2.5% (m/v) NaPCP and the Polymer-Cysteine Conjugate 8:1 at pH 6.8 According to the Method Described by Robinson and Co-workers (12)

Tested Polymer	Total work of adhesion (TWA) in μJ	± Standard deviation (n = 4–8)
PCP-Cysteine Conj. 8:1	8.81	2.94
NaPCP	4.05	1.27

CONCLUSIONS

The covalent attachment of cysteine to polycarbophil leads to polymer conjugates displaying strongly improved adhesive as well as cohesive properties. Being aware of the mucus turnover and peristaltism, these features should nevertheless render polycarbophil-cysteine conjugates useful as excipients for drug delivery systems such as tablets, pellets and microparticles providing a more prolonged residence time on various mucosal tissues compared to well established polymers.

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REFERENCES

1. M. J. Tobyn, J. R. Johnson, and P. W. Dettmar. Factors affecting *in vitro* gastric mucoadhesion II. Physical properties of polymers. *Eur. J. Pharm. Biopharm.* **42**:56–61 (1996).

2. A. Bernkop-Schnürch, C. Humenberger, and C. Valenta. Basic studies on bioadhesive delivery systems for peptide and protein drugs. *Int. J. Pharm.* **165**:217–225 (1998).
3. B. Naisbett and J. Woodley. The potential use of tomato lectin for oral drug delivery. Lectin binding to rat small intestine *in vitro*. *Int. J. Pharm.* **107**:223–230 (1994).
4. J. M. Irache, C. Durrer, D. Duchêne, and G. Ponchel. Bioadhesion of lectin-latex conjugates to rat intestinal mucosa. *Pharm. Res.* **13**:1716–1719 (1996).
5. A. Bernkop-Schnürch, F. Gabor, M. P. Szostak, and W. Lubitz. An adhesive drug delivery system based on K99-fimbriae. *Eur. J. Pharm. Sci.* **3**:293–299 (1995).
6. J. R. Gum, Jr., J. W. Hicks, N. W. Toribara, E.-M. Rothe, R. E. Lagace, and Y. S. Kim. The human *MUC2* intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. *J. Biol. Chem.* **267**:21375–21383 (1992).
7. G. H. Snyder, M. K. Reddy, M. J. Cennerazzo, and D. Field. Use of local electrostatic environments of cysteines to enhance formation of a desired species in a reversible disulfide exchange reaction. *Biochim. Biophys. Acta* **749**:219–226 (1983).
8. G. H. Snyder. Intramolecular disulfide loop formation in a peptide containing two cysteines. *Biochemistry* **26**:688–694 (1987).
9. Methods for covalent chromatography using Activated Thiol Sepharose 4B or Thiopropyl Sepharose 6B. In, *Affinity Chromatography: Principles and Methods*, Pharmacia, Uppsala, 1996, pp. 106–111.
10. C.-M. Lehr, J. A. Bouwstra, E. H. Schacht, and H. E. Junginger. *In vitro* evaluation of mucoadhesive properties of chitosan and some other natural polymers. *Int. J. Pharm.* **78**:43–48 (1992).
11. A. Bernkop-Schnürch and M. Kraijcek. Mucoadhesive polymers for peroral peptide delivery: synthesis and evaluation of chitosan-EDTA conjugates. *J. Contr. Rel.* **52**:1–16 (1998).
12. H. S. Ch'ng, H. Park, P. Kelly, and J. R. Robinson. Bioadhesive polymers as platforms for oral controlled drug delivery II: Synthesis and evaluation of some swelling, water-insoluble bioadhesive polymers. *J. Pharm. Sci.* **74**:399–405 (1985).
13. S. A. Mortazavi and J. D. Smart. An investigation into the role of water movement and mucus gel dehydratation in mucoadhesion. *J. Contr. Rel.* **25**:197–203 (1993).
14. G. Ponchel, F. Lejoyeux, and D. Duchêne. Bioadhesion of poly-(acrylic acid) containing-systems. Thermodynamical and rheological aspects. *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **18**:111–112 (1991).