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Research Paper

Synthesis and *in Vitro* Evaluation of a Novel Chitosan–Glutathione Conjugate

Krum Kafedjiiski,[1] Florian Föger,[1] Martin Werle,[2] and Andreas Bernkop-Schnürch[1,3]

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Purpose. It was the aim of this study to synthesize and characterize a novel chitosan–glutathione (GSH) conjugate providing improved mucoadhesive and permeation-enhancing properties.
Methods. Mediated by carbodiimide and *N*-hydroxysuccinimide, glutathione was covalently attached to chitosan via the formation of an amide bond. The adhesive properties of chitosan–GSH conjugate were evaluated *in vitro* on freshly excised porcine mucosa via tensile studies and the rotating cylinder method. The cohesive properties and stability of the resulting conjugate were evaluated by disintegration test and by oxidation experiments, respectively. The permeation-enhancing effect of the chitosan–GSH/GSH system was evaluated in Ussing chambers by using rhodamine 123 as model compound.
Results. The obtained conjugate displayed 265.5 μmol immobilized free thiol groups and 397.9 μmol disulfide bonds per gram polymer. Because of the formation of disulfide bonds within the polymer, the stability of matrix tablets could be strongly improved. In tensile studies, the total work of adhesion of the conjugate was determined to be 9.9-fold increased in comparison to unmodified chitosan. Results from the rotating cylinder method showed more than 55-fold increase in the adhesion time of thiolated chitosan *vs.* unmodified chitosan. In addition, the conjugate in combination with GSH displayed a 4.9-fold higher permeation-enhancing effect compared with unmodified chitosan.
Conclusions. Because of the improved mucoadhesive and cohesive properties, and the strong permeation-enhancing effect of the chitosan–GSH conjugate/GSH system, the novel thiolated chitosan seems to represent a promising multifunctional excipient for various drug delivery systems.

KEY WORDS: chitosan; glutathione; mucoadhesion; permeation enhancement; thiolated chitosan.

INTRODUCTION

Over the past few years, a series of novel thiolated polymers have been introduced in the pharmaceutical literature, exhibiting strongly improved mucoadhesive, controlled release, permeation enhancing, and enzyme inhibitory properties (1–3). Thiolated polymers or so-called thiomers are hydrophilic macromolecules containing free immobilized thiol groups on the polymeric backbone (4,5). Permeation studies carried out with model drugs across intestinal mucosa demonstrated that the combination of thiolated polymers with reduced glutathione (GSH) as low molecular mass permeation mediator led to a significantly improved permeation-enhancing effect of thiomers (6,7). For instance, the system of polycarbophil–cysteine (0.5%) with GSH (0.4%) led to an enhancement ratio up to 2.93 for the model substance Na-Flu in comparison to the control buffer. In contrast, permeation studies with polycarbophil–cysteine (0.4%) demonstrated an enhancement ratio of 1.88. In another study, a similar enhancement of rhodamine 123 uptake was reached by using the system chitosan–4-thiobutylamidine (chitosan–TBA) with 5% GSH. Results showed a 3-fold higher permeation-enhancing effect of the system in comparison to unmodified chitosan. The mechanism responsible for this permeation-enhancing effect of thiomers involves the reduction of oxidized glutathione. In such a way, the concentration of reduced glutathione on the absorption membrane is increased. Different studies were carried out to optimize the thiomer/GSH system by increasing the concentration of GSH or thiomer, respectively, or by means of combining the system with permeation enhancers acting in different way (8). Unfortunately, no further improvement was achieved.

A new strategy to combine the properties of the two-component system in an "all-in-one system" would be the direct immobilization of free GSH on chitosan, which might lead to a new generation of thiomers with highly improved permeation-enhancing properties. The reasons for such expected advantageous features of the novel chitosan–GSH conjugate are based on the unique ligand GSH itself (9). Focusing on the structure of GSH as a new ligand, the presence of thiol group in the tripeptide structure, and its high negative redox potential, a novel chitosan–glutathione derivative might exhibit higher permeation-enhancing properties among the rest of thiomers. The strategy of immobilizing GSH on the polymer backbone takes also into consideration the impact of gastrointestinal track conditions on thiomer/GSH permeation-enhancing system. Observing

[1] Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef Möller Haus, A-6020 Innsbruck, Austria.
[2] ThioMatrix GmbH, Research Center, Mitterweg 24, 6020 Innsbruck, Austria.
[3] To whom correspondence should be addressed. (e-mail: andreas.bernkop@uibk.ac.at)

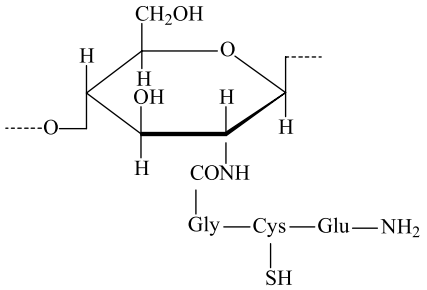


Fig. 1. Presumptive chemical substructure of chitosan–GSH conjugate.

in vivo environment, a subsequent dilution of free glutathione cannot be excluded. Consequently, the permeation-enhancing effect of free glutathione in the system thiomer/GSH might be lost. In contrast, the polymer has a high molecular weight to be absorbed and remains concentrated on the absorption membrane. In addition, a toxicologically harmless profile of chitosan–GSH conjugate is highly advantageous. To verify this working hypothesis, it was the aim of this study to synthesize a novel chitosan–glutathione conjugate by modifying chitosan with glutathione as shown in Fig. 1. Apart from the chemical modification, essential polymer features such as *in vitro* mucoadhesive properties and permeation-enhancing effect were investigated. In addition, its cohesive properties and stability toward oxidation were analyzed.

MATERIALS AND METHODS

Materials

Chitosan (medium molecular mass, 400 kDa; degree of deacetylation, 83–85%) and rhodamine 123 were obtained from Fluka Chemie (Buchs, Switzerland). L-Glutathione reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and 5,5′-dithiobis(2-nitrobenzoic acid) were all purchased from Sigma (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) was obtained from Acros (Geel, Belgium). All chemicals were of analytical grade.

Synthesis of Chitosan–Glutathione Conjugate

The covalent attachment of reduced glutathione to chitosan was achieved via the formation of amide bonds between carboxylic acid moieties of glutathione and amine groups of chitosan.

First, 1 g of chitosan was hydrated in 8 mL of 1 M HCl and then dissolved by the addition of demineralized water to obtain a 1% (w/v) polymer solution. The pH was adjusted to 6.0 by the addition of 5 M NaOH. Afterwards, 5 g of reduced glutathione in 10 mL demineralized water was added under stirring. Then, EDAC dissolved in 5 mL demineralized water was added in a final concentration of 200 mM. Thereafter, NHS dissolved in 5 mL demineralized water was added into the reaction mixture under vigorous stirring in a final concentration of 200 or 500 mM as listed in Table I. The pH was readjusted to 6.0 with 5 M NaOH. The reaction mixture was incubated for 7 h at room temperature under permanent stirring. The resulting polymer conjugate was dialyzed in tubings (molecular weight cutoff 12 kDa) first against 5 mM HCl, twice against 5 mM HCl containing 1% NaCl, and finally two times against 1 mM HCl. Controls were prepared in the same way but omitting EDAC and NHS during the coupling reaction. The complex between chitosan and glutathione was obtained according to the method described in a patent (10). Finally, the frozen aqueous polymer solutions (samples, controls, and complex) were lyophilized at −50°C and 0.01 mbar (Lyolab B; Inula, Austria) and stored at 4°C until further use.

Determination of the Thiol Group and Disulfide Bond Content

The amount of thiol groups immobilized on chitosan–GSH conjugate was determined spectrophotometrically using Ellman's reagent quantifying free thiol groups as described previously (1).

Disulfide content was determined after reduction with $NaBH_4$ and addition of Ellman's reagent as described by Habeeb (11).

Decrease in the Thiol Group Content within the Polymer Conjugate

Chitosan–GSH conjugate was hydrated in demineralized water in a final concentration of 0.5% (w/v), and the pH values of the solutions were adjusted to 5 and 6, respectively. All samples were incubated at 37°C under permanent shaking. At predetermined time intervals, aliquots of 200 μL were withdrawn and 50 μL of 1 M HCl was added to stop any further reactions. The amount of remaining thiol groups was determined via Ellman's reagent.

Preparation of Matrix Tablets

Lyophilized chitosan–GSH conjugate and controls were compressed into 30-mg, 5.0-mm-diameter flat-faced tablets (single-punch eccentric press, Korsch EK, Berlin, Germany).

Table I. Amount of Thiol/Disulfide Groups Immobilized on Ch–GSH Conjugate

No. of the conjugate	Chitosan (g/100 mL)	GSH (g)	EDAC (mM)	NHS (mM)	Thiol/disulfide groups (μmol/g polymer ± SD; $n = 3$)
Ch–GSH 1	1.0	5.0	200	–	127.8 ± 5.8/154.1 ± 10.7
Ch–GSH 2	1.0	5.0	200	200	265.5 ± 7.8/397.9 ± 28.2
Ch–GSH 3	1.0	5.0	200	500	254.2 ± 23.2/345.7 ± 21.3
Control	1.0	5.0			10.1 ± 0.7/9.7 ± 1.3

The compaction pressure was kept constant during the preparation of all tablets.

Evaluation of the Swelling Behavior

The water-absorbing capacity was determined by a gravimetric method. Thirty milligrams each of the polymer conjugate and controls was compressed (Hanseaten Type EI, Hamburg, Germany) to 5.0-mm-diameter flat-faced tablets. The compaction pressure was kept constant during the preparation of all tablets. Test tablets were fixed to a needle and incubated in a 0.1 M phosphate buffer, pH 6.8, at 37°C. At scheduled time intervals, the hydrated test tablets were taken out of the incubation medium, excess water was removed, and the amount of water uptake was determined gravimetrically (12).

Disintegration Studies

The disintegration behavior of the polymer tablets and the corresponding control tablets in 0.1 M phosphate buffer, pH 6.8, at 37°C was performed with the disintegration test apparatus according to the European Pharmacopoeia. The oscillating frequency was set to 0.5 s^{-1}.

In Vitro Evaluation of the Mucoadhesive Properties

Tensile Studies

Tensile studies were carried out on a freshly excised 4-mm-thick porcine intestinal mucosa. Thirty milligrams of lyophilized chitosan–GSH conjugate and controls was compressed to tablets. Then, the tablet was 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 using a cyanoacrylate adhesive, placed in a beaker, and totally immersed with 400 mL of 0.1 M phosphate buffer, pH 6.8, at 37°C. The beaker was placed on a balance and was 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 at 25°C, the mucosa was pulled down from the tablet at a rate of 0.1 mm/s. Data points were collected every second by a personal computer (Windwedge software; TAL Technologies Inc., Philadelphia, PA, USA) linked to the balance. The total work of adhesion (TWA) representing the area under the force/distance curve and the maximum detachment force (MDF) representing the force needed for detachment were calculated with Excel 97 (Microsoft, Redmond, WA, USA) (12).

In Vitro Mucoadhesion Studies with the Rotating Cylinder Method

Thirty milligrams of thiolated chitosan and control tablets was attached to a 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 500 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 (13).

Permeation Studies

Permeation studies were carried out in Ussing-type chambers 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 6.0.

Right after sacrificing the rat, the first 15 cm of the small intestine (duodenum) was excised and mounted in the Ussing chamber. All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. After 20 min of preincubation with the artificial intestinal fluid, the media of the donor compartment was substituted by either chitosan–GSH conjugate (0.5% w/v) or chitosan–GSH conjugate (0.5% w/v) containing 5% (w/v) of the permeation-enhancing mediator reduced glutathione (GSH). The corresponding unmodified polymer (0.5% w/v) was used as control. The exclusively cationic fluorescence compound rhodamine 123 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 rhodamine 123 was determined using a microtitration plate reader (Fluostar Galaxy, Offenburg, Germany). Cumulative corrections were made for the previously removed samples. The apparent permeability coefficients (P_{app}) for rhodamine 123 were calculated according to the following equation:

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

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

$$R = P_{app}(\text{Ch} - \text{GSH})/P_{app}(\text{Chitosan})$$

Statistical Data Analysis

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

RESULTS

Synthesis of Chitosan–Glutathione Conjugate

Novel chitosan–GSH conjugate was synthesized by the amide bond formation between glycine carboxylic acid groups of glutathione and amine groups of chitosan. The most

comprehensive NMR-based studies on GSH conformation revealed that at pH 7, the Gly residue is particularly mobile, and the Glu part of the GSH molecule is the most rigid (9). EDAC catalyze the formation of amide bonds by activating carboxyl to form an *O*-urea derivative. This derivative reacts readily with amine nucleophiles or with water molecule to regenerate the original COOH. When EDAC is used in the coupling reaction, the fast hydrolysis of *O*-urea derivative reduced the yield of thiol moieties—sample Ch–GSH 1 (Table I). Results showed that the addition of NHS to EDAC-mediated coupling reaction significantly improved the coupling yields. *N*-Hydroxysuccinimide esters hydrolyze very slowly compared with their rates of reaction with primary amino groups. The general reaction scheme is as follows (Fig. 2): (a) activation of carboxylic acid groups of glutathione by EDAC to give *O*-acylisourea groups; (b) conversion of the *O*-acylisourea groups into a NHS-activated carboxylic acid group; (c) yielding a so-called zero-length cross-link between activated carboxylic acid groups and amine groups of chitosan (14). The by-products during cross-linking of EDAC/NHS are water-soluble and can be easily removed by rinsing. The content of immobilized thiol groups strongly depends on the EDAC/NHS concentration and the molar ratio used. The Grabarek–Gergely protocol described the use of molar ratio EDAC/NHS 1:2.5—sample Ch–GSH 3 (Table I). The same result was obtained when the molar ratio was 1:1. Other parameters such as the weight ratio polymer/glutathione, pH, and reaction time were also studied. The optimal coupling conditions were defined: polymer/GSH ratio of 1:5, pH 5.5–6.0, and a reaction time of 7 h. The efficacy of the purification method used here could be verified by controls that were prepared in exactly the same way as the polymer conjugate but omitting EDAC/NHS during the reaction, resulting in a negligible amount of thiol groups. The new conjugate exhibited 265.5 μmol immobilized free thiol groups and 397.9 μmol disulfide bonds per gram polymer. The conjugate appeared as a white, odorless powder of fibrous structure. It was easily hydratable in aqueous solution forming thereby a solution of initially low viscosity.

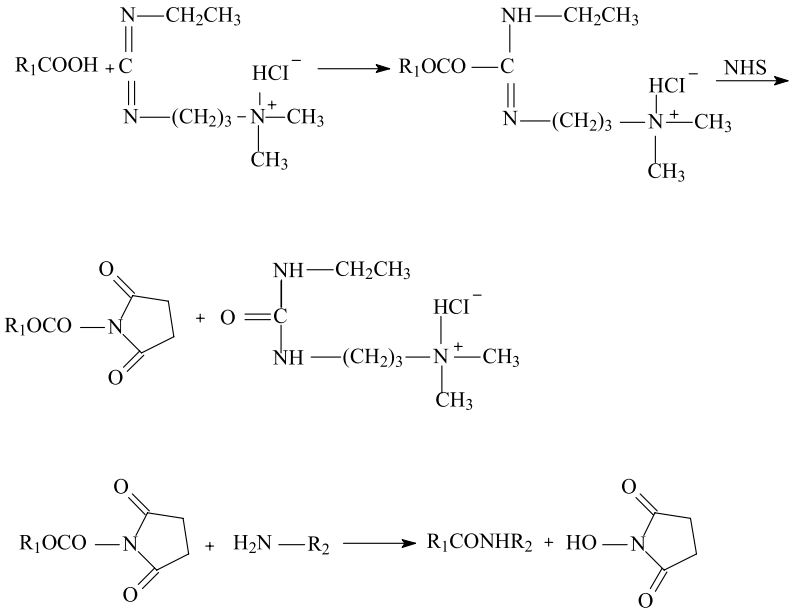
Oxidation of Thiol Groups and Cohesive Properties

Thiol groups of the polymer, as other thiols, can be oxidized to disulfides in aqueous solutions by either an intra- or intermolecular reaction. The results of this study are shown in Fig. 3. A significant decrease in the thiol group content can be observed within the first hour of the oxidation process. At pH 6, the cross-linking process takes place more rapidly. Within 6 h, the viscosity of the thiomer solution is increased, but at least 40% of thiol groups remained still stable. This oxidation behavior can be explained by the pK_a of the thiol moieties and by the stearic factor (12). Thiol groups being located closely to each other can form disulfide bonds more rapidly than remaining isolated thiol groups.

Disintegration studies in physiological medium revealed that the matrix tablets of chitosan–GSH conjugate were stable within 48 h, and no erosion was observed over that period of time. In contrast, control tablets disintegrated within 9 h. The long disintegration time of the conjugate demonstrated its higher cohesive properties in comparison to unmodified chitosan. The oxidation process already takes place within the conjugate network during disintegration of matrix tablets in physiological medium. This leads to the formation of stabilizing disulfide bonds and cross-linking of the thiomer (15).

Swelling Behavior

It is well known that the swelling behavior of mucoadhesive polymers is a part of mechanisms, which are responsible



Abbreviation: R_1: Glutathione
R_2: Chitosan

Fig. 2. Scheme of synthetic pathway of Ch–GSH conjugate.

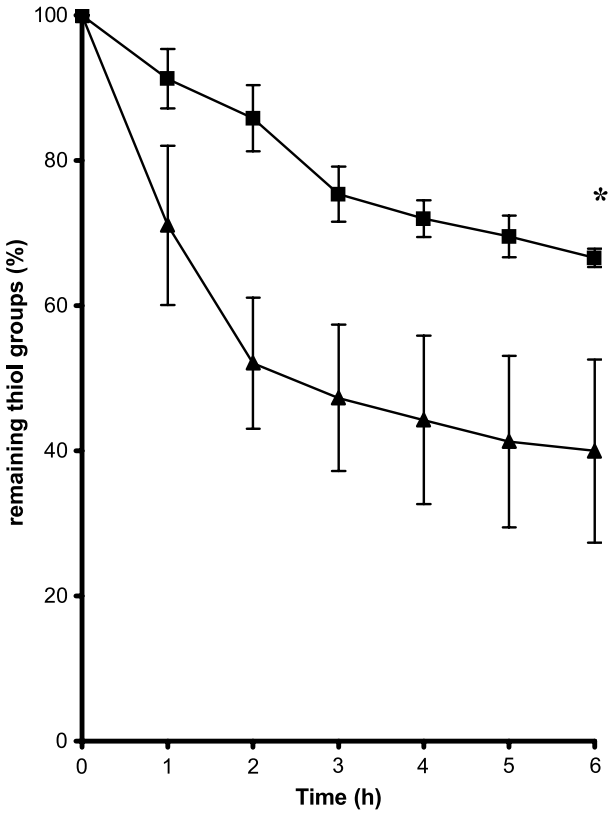


Fig. 3. Decrease of the thiol group content within aqueous 0.5% (w/v) Ch–GSH conjugate solutions at pH 5 (■) and pH 6 (▲), respectively, at 37°C. Indicated values are means (±SD) of at least three experiments; * differs from conjugate at pH 6, $p < 0.02$.

for their adhesive and cohesive properties, stability during disintegration, and drug release. The processes of absorbing, swelling, and capillarity explain the adhesion between the polymer and the mucus layer (16). As shown in Fig. 4, water uptake studies revealed that the covalent attachment of glutathione has no significant influence on the swelling behavior of chitosan (two-sample t test, $p > 0.05$). The obtained dependence was in the same range as other thiolated chitosans. A slow swelling is useful to avoid the formation of an overhydrated form of conjugate that may lose its mucoadhesive properties before reaching the target. On the other hand, many authors have indicated the water uptake as the major cause of activation of the disintegration mechanism (17). At the end of the experiment, no erosion or dissolving of the tablets was observed. Therefore, the slow swelling process favors the high cohesive properties of the conjugate.

Mucoadhesive Properties *in Vitro*

Two different experimental setups were used to investigate the influence of the ligand glutathione on the mucoadhesive properties of Ch–GSH conjugate. In addition, the complex formed between chitosan and glutathione was compared to the conjugate and the control, respectively.

Results from the tensile studies are shown in Fig. 5. Total work of adhesion of chitosan–GSH conjugate (sample Ch–GSH 2) was determined to be 319 μJ, or 9.9-fold improvement was obtained in comparison to unmodified chitosan and the complex. In addition, the complex did not exhibit any enhanced mucoadhesive properties. The maximum detachment force (MDF) of all tested polymers correlated well with TWA (data not shown).

The mucoadhesive efficacy of chitosan–GSH conjugate was also confirmed by another mucoadhesion tests system—the rotating cylinder method (Fig. 6). This method is supposed to correlate better with the *in vivo* conditions than simple tensile studies, as it concurrently imitates the adhesion and cohesiveness of the polymer in physiological medium. The adhesion time or the duration of adhesion of chitosan–GSH was around 166 h, which means that more than 55-fold increase in the adhesion time was achieved in comparison to unmodified chitosan and the complex. The results also demonstrated a correlation between the degree of modification and the adhesion time. Compared with other thiolated chitosans such as chitosan–thioethylamidine conjugate (chitosan–TEA) (18) and chitosan–thioglycolic derivatives (maximum 10-fold increase in adhesion time), the time of adhesion of this new generation of chitosan conjugates was greatly improved. Only the adhesion time of chitosan-4-thiobutylamidine conjugate (chitosan–TBA) was in the same range (19).

In Vitro Permeation Studies

Thiolated chitosans were shown to exhibit a strong permeation-enhancing effect on the paracellular drug

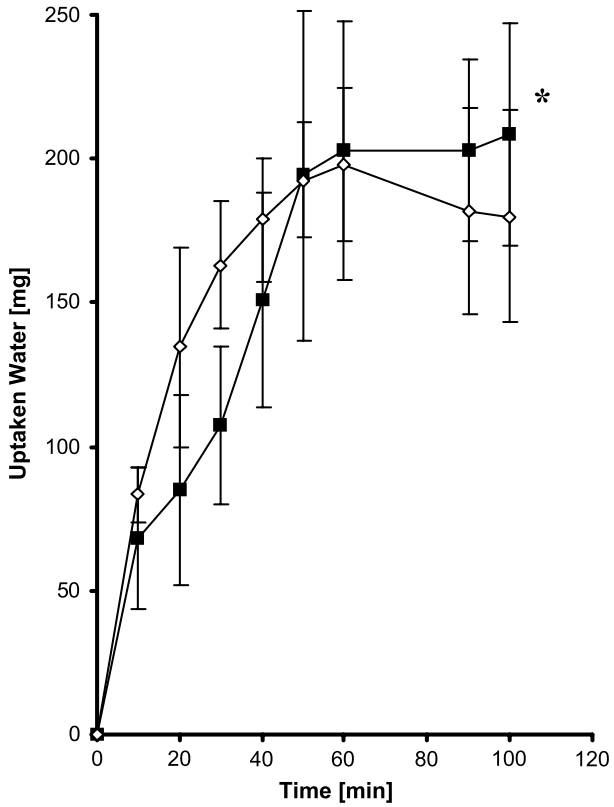


Fig. 4. Swelling behavior of tablets comprising Ch–GSH (■) and unmodified chitosan (◇) in 0.1 M phosphate buffer, pH 6.8, at 37°C; indicated values are means (±SD) of at least three experiments; * differs from unmodified chitosan, $p < 0.32$.

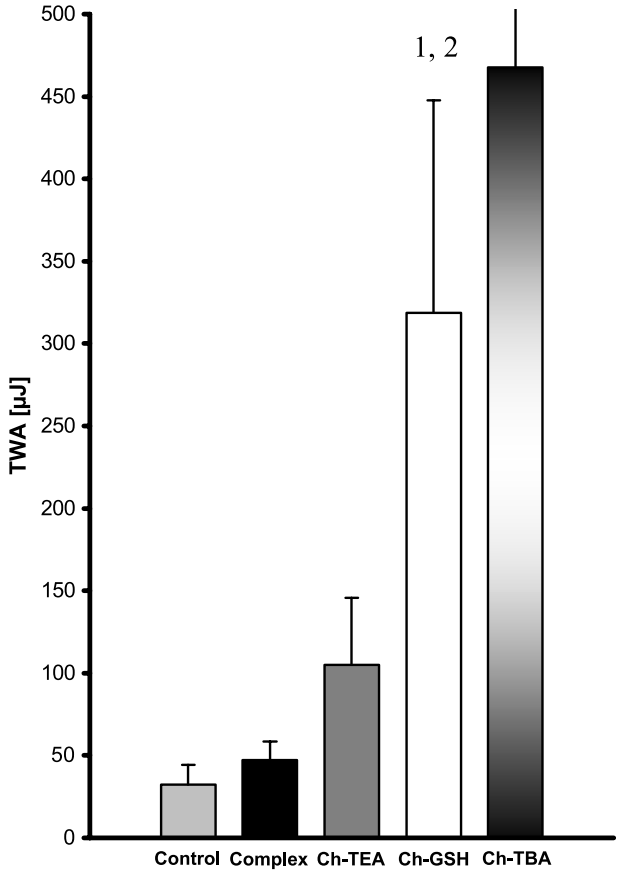


Fig. 5. Comparison of the adhesive properties of Ch–GSH conjugate with unmodified chitosan, complex, Ch–TEA (18), and Ch–TBA conjugate (19). Represented values are means (±SD; $n = 3-5$) of the total work of adhesion (TWA); [1]Differs from control, $p < 0.0003$; [2]differs from complex, $p < 0.0002$. Studies were performed in 0.1 M phosphate buffer, pH 6.8, at 37°C.

uptake (7). In this study, the optimized system thiomer/glutathione was used for the transport of the cationic marker compound rhodamine 123, proven in previous studies. Among all thiomers, chitosan–TBA/GSH system reached a maximum 3-fold higher permeation-enhancing effect in comparison to unmodified chitosan. Results of these studies carried out under the same experimental conditions are shown in Fig. 7. The P_{app} values achieved by the addition of 0.5% (w/v) chitosan–GSH conjugate with 5% (w/v) GSH (sample Ch–GSH 2), 0.5% (w/v) chitosan–GSH conjugate, and 0.5% (w/v) unmodified chitosan were determined to be 3.23×10^{-7}, 2.06×10^{-7}, and 0.66×10^{-7} cm/s, respectively. Accordingly, the transport enhancement ratio (R) in the presence of GSH was calculated to be 4.9. When chitosan–GSH conjugate was applied without the addition of GSH, the enhancement ratio was also high, determined to be 3.1, which is comparatively higher in comparison to the rest of thiomers. The complex exhibited a permeation-enhancing effect in the range of the unmodified chitosan. So far, it represents the highest improvement in the permeation-enhancing effect of the generated thiolated chitosans. Chitosan–GSH conjugate achieved this goal because of the unique reducing properties of the ligand glutathione in biological systems.

DISCUSSION

So far, studies on derivatization of GSH on the carboxylic group have not been reported. At present, only the formation of salts between chitosan and glutathione is known (10). Novel chitosan–glutathione conjugate was synthesized by the amide bond formation between glycine carboxylic acid groups of glutathione and amine groups of chitosan. Additional studies indicated that the unintended oxidation of thiol groups during synthesis could be avoided by performing the reaction under inert conditions. More strict control on the disulfide bonds formation can be achieved by introducing an additional step in the conjugate synthesis like treatment of the obtained product with suitable reducing agents. Several different reducing agents were employed, but the most promising results were obtained by using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in a final concentration of 5 mM TCEP at pH 4–5. For instance, the disulfide bonds content in the treated samples was half-decreased in comparison to untreated conjugates. Studies carried out with chitosan–GSH conjugate demonstrated its remarkable properties, which are prerequisite for the development of optimized mucoadhesive drug delivery systems. First, the conjugate displayed a prolonged adhesion time on mucosa, which is a consequence of balanced cohesive and mucoadhesive properties. The above-mentioned improvement in the conjugate features can be explained by the combination of the following effects:

(I) Nature of ligand. The main mechanism of mucoadhesion of thiomers is based on the formation of disulfide bonds between the polymer and cysteine-rich subdomains of

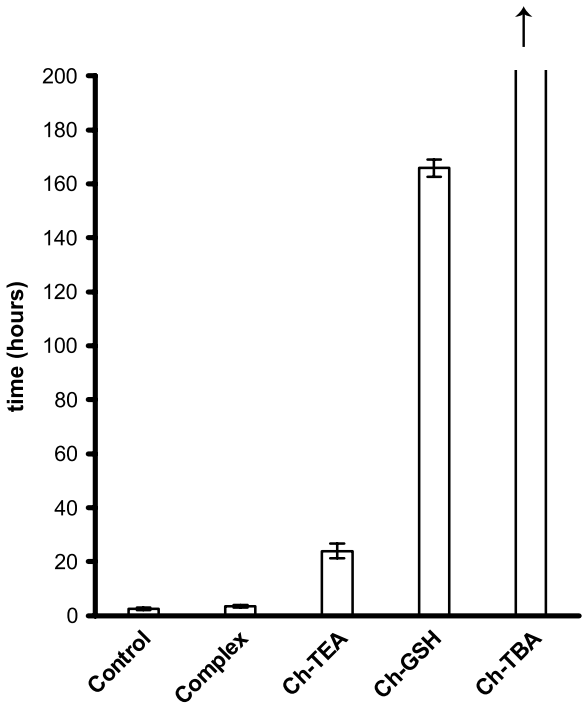


Fig. 6. Comparison of the adhesion time of Ch–GSH conjugate with unmodified chitosan, complex, Ch–TEA (18), and Ch–TBA conjugate (19) on the rotating cylinder. Indicated values are means (±SD) of at least three experiments.

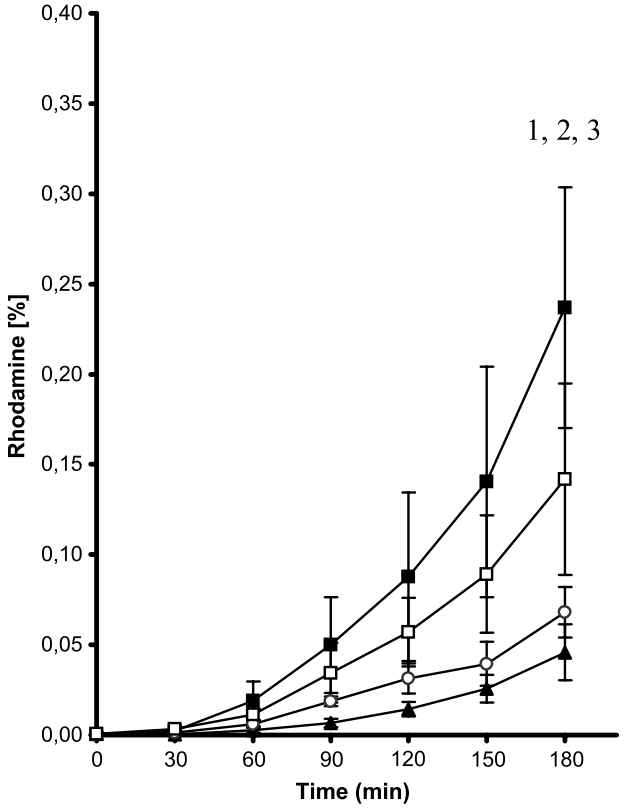


Fig. 7. Permeation-enhancing effect of 0.5% (w/v) Ch–GSH conjugate with 5% (w/v) GSH (■) in comparison to that of 0.5% (w/v) Ch–GSH conjugate (□), 0.5% (w/v) unmodified chitosan (▲), and the complex (◇) on the permeation of rhodamine 123 across freshly excised small intestinal mucosa. Indicated values are means (±SD) of at least seven experiments; [1]differs from control, $p < 0.00003$; [2]differs from complex, $p < 0.003$; [3]differs from Ch–GSH, $p < 0.02$.

mucus glycoproteins. The primary role in the exchange reaction plays the activity of the thiol groups of the conjugate. Their activity is determined from the chemical structure and the corresponding pK_a, which is favorable for the formation of sufficient concentration of thiolate anions in the physiological medium. The selection of glutathione ligand was based on its thiol pK_a value (8.7) and the rate of solubility.

(II) The lyophilized polymer conjugate exhibits a pH value of 4. Therefore, most thiol groups of the polymer remain stable and free to react with mucin. The oxidation process within the polymer also takes place very slowly. The stability of Ch–GSH conjugate toward autoxidation at physiological conditions is also an important feature for its potential clinical application. Results from the test showed that within an incubation period of 6 h, at least 40% of the thiol groups still remained stable.

(III) Degree of modification. As a result of the established optimal reaction conditions, the obtained degree of modification was higher than 250 μmol/g, which certainly provides a sufficient concentration of thiol groups on the polymer. Results demonstrated a direct correlation between the thiol groups content and the adhesion time.

(IV) Nature of chitosan. Chitosan has been shown to display mucoadhesive properties due to molecular attractive forces formed by electrostatic interactions between positively charged chitosan and negatively charged mucosal surfaces. Chitosan with medium molecular mass of 400 kDa was used. This type of chitosan is known to exhibit the best cohesive properties and ability for interpenetration, which are essential for high mucoadhesive properties.

(V) Cohesive properties. The cohesiveness increases immediately in the aqueous medium due to a running cross-linking process within the polymer. Thus, it supports the integrity and stability of the polymer matrix during adhesion.

Chitosan is also known for its permeation-enhancing effect (20). Former studies demonstrated a 3-fold higher permeation-enhancing effect of chitosan–TBA conjugate/GSH system in comparison to unmodified chitosan. But when the thiomer was used without the addition of GSH, the highest enhancement ratio was 2.0. At present, the novel chitosan–glutathione conjugate gained an enhancement ratio of 3.1. The obtained enhancement ratio in the presence of GSH was even significantly increased to 4.9. To evaluate the influence of different ligands and the nature of polymers on their permeation-enhancing effect, the experimental data were compared with the previous studies (Table II). The underlying mechanism of the permeation-enhancing effect of thiomers is still not satisfactory explained. The likely mechanism being responsible for the increased permeability in the presence of chitosan–GSH conjugate is based on the inhibition of the enzyme protein tyrosine phosphatase (PTP) by the reduced form of GSH (6). Studies with glutathione showed that Cys 215 of PTP was able to react with glutathione and thus formed a mixed disulfide causing an inactivation of PTP (24). This enzyme dephosphorylates tyrosine residues of occludin, which is supposed to influence the opening of the tight junctions. Therefore, the inhibition of PTP by reduced glutathione will lead consequently to a phosphorylation and opening of the tight junctions. Due to an autoxidation of GSH, the amount of active GSH decreases thereby leading to lower concentrations, resulting in a reduction of the permeation enhancement. Accordingly, the presence of thiolated polymer is essential, as it prevents the oxidation of glutathione on the surface of mucosa. The permeation-enhancing effect of the chitosan–GSH/GSH

Table II. Permeation-Enhancing Properties of Thiomers in Comparison to the Corresponding Unmodified Polymers Tested on Freshly Excised Intestinal Mucosa

Permeation enhancer	Test compound	Enhancement ratio (P_{app} thiomer/P_{app} unmodified control polymer)	Ref.
Chitosan–GSH	Rhodamine	3.1	–
Chitosan–GSH/GSH	Rhodamine	4.9	–
Chitosan–TBA	Rhodamine	2.0	(21)
Chitosan–TBA/GSH	Rhodamine	3.6	(21)
Chitosan–Cys	Bac–FITC	signif.	(22)
PCP–Cys	Na-Flu	1.6	(23)
PCP–Cys/GSH	Na-Flu	2.9	(6)

bac-FITC, Fluorescein–isothiocyanate labeled bacitracin; Na-Flu, sodium fluorescein; rhodamine, rhodamine 123.

system depends on the reduction property of the conjugate. The new chitosan–GSH conjugate displayed itself a high permeation-enhancing effect. Hence, it might be suggested that the immobilized GSH on chitosan activates an additional mechanism responsible for the permeation enhancement. A direct interaction between thiol groups of the conjugate and PTP might take place. To verify this hypothesis, future detailed studies will be carried out. The hypothesis of the interaction of immobilized GSH with intestinal enzymes is based simultaneously on the structural conjugate features, the special characteristics of the ligand GSH, and of the general properties of thiomers. The inhibitory effect of reduced glutathione toward aminopeptidase N and other enzymes present on mucosa is shown by Langoth (25). The inhibition of aminopeptidase N seems to be based on the deprivation of Zn ions from the enzyme due to the ability of GSH of forming complexes with Zn. It is also shown that the complex formation between GSH and Zn is based on the sulfhydryl substructure (26). The reaction was found to take place via two different mechanisms depending on the degree of protonation of GSH. The fact that oxidized glutathione exhibits no enzyme-inhibiting properties confirms the theory that the complex between GSH and Zn is achieved via this functional group. Thiomers like polycarbophil–cysteine also showed an enzyme inhibitory effect toward brush border membrane bound aminopeptidase N. Polycarbophil itself has a strong inhibitory effect toward aminopeptidase N, which is enhanced by the covalent attachment of cysteine (3). According to this, it might be expected that the new conjugate could also exhibit enzyme inhibitory properties.

It should be also considered that although an enhancer may greatly increase intestinal permeability *in vitro*, it cannot be guaranteed that this effect will be completely retained *in vivo*. The volume of fluid present in the gastrointestinal tract, gastric emptying, and intestinal mobility will affect the dilution and residence time at any site. In contrast to permeation enhancers of low molecular size like GSH, thiomers can remain concentrated on the delivery system because of their ability to form disulfide bonds with the mucus. Dilution effects as well as systemic side effects can be subsequently avoided.

The results confirmed the clear advantage of the new strategy for the development of the thiomer/GSH permeation-enhancing system being, on the one hand, based on the immobilization of GSH on the polymer backbone to circumvent dilution of unbound GSH in the gastrointestinal track and, on the other hand, development of a new type of thiomer with stronger reduction capability. Reduction properties of chitosan–GSH conjugate are determined by the nature of ligand. The tripeptide GSH has a potent electron-donating capacity, linked to its thiol group. The presence of a γ-peptidic bond between Glu and Cys residues is the most distinct structural feature of glutathione as it is supposed to protect GSH from intracellular aminopeptidases (9). Another distinct feature is the high conformational flexibility of GSH, which is especially important for its interactions. This explanation is probably related with the results evaluating the activity of chitosan–glutathione conjugate to reduce oxidized GSH. Accordingly, the new conjugate demonstrated the highest permeation-enhancing effect among thiomers by now.

CONCLUSION

Within this study, chitosan–glutathione conjugate has been synthesized and characterized for the first time. The new strategy for optimizing chitosan–GSH permeation-enhancing system led to a unique type of thiolated chitosan, which exhibited improved mucoadhesive and cohesive properties, and the highest permeation-enhancing effect among other thiomers. Glutathione as ligand was found to have a dramatic effect on the permeability of model compound rhodamine 23 across small intestine. Because of these features, the novel polymer seems to represent a promising new generation of permeation-enhancing thiolated polymers for the noninvasive administration of hyldrophilic macromolecular drugs.

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REFERENCES

1. A. Bernkop-Schnürch, V. Schwarz, and S. Steininger. Polymers with thiol groups: a new generation of mucoadhesive polymers? *Pharm. Res.* **16**:876–881 (1999).
2. A. Bernkop-Schnürch, C. E. Kast, and D. Guggi. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. *J. Control. Rel.* **93**:95–103 (2003).
3. A. Bernkop-Schnürch, H. Zarti, and G. F. Walker. Thiolation of polycarbophil enhances its inhibition of soluble and intestinal brush border membrane bound aminopeptidase *N. J. Pharm. Sci.* **90**:1907–1914 (2001).
4. V. M. Leitner, G. F. Walker, and A. Bernkop-Schnürch. Thiolated polymers: evidence for the formation of disulphide bonds with mucus glycoproteins. *Eur. J. Pharm. Biopharm.* **56**:207–214 (2003).
5. A. Bernkop-Schnürch, M. Hoffer, and K. Kafedjiiski. Thiomers for oral delivery of hydrophilic macromolecular drugs. *Expert Opin. Drug Deliv.* **1**(1):87–98 (2004).
6. A. E. Clausen, C. E. Kast, and A. Bernkop-Schnürch. The role of glutathione in the permeation enhancing effect of thiolated polymers. *Pharm. Res.* **19**:602–608 (2002).
7. A. Bernkop-Schnürch, D. Guggi, and Y. Pinter. Thiolated chitosans: development and *in vivo* evaluation of a mucoadhesive permeation enhancing oral drug delivery system. *J. Control. Rel.* **94**:177–186 (2004).
8. D. Guggi and A. Bernkop-Schnürch. Improved paracellular uptake by the combination of different types of permeation enhancers. *Int. J. Pharm.* **288**:141–150 (2005).
9. A. Krezel and W. Bal. Structure–function relationships in glutathione and its analogues. *Org. Biomol. Chem.* **1**:3885–3890 (2003).
10. R. F. Hebert. US 2002/ 0002136 A_1 (2002).
11. A. Habeeb. A sensitive method for localization of disulfide containing peptides in column effluents. *Anal. Biochem.* **56**: 60–65 (1973).
12. C. E. Kast and A. Bernkop-Schnürch. Thiolated polymers–thiomers: development and *in vitro* evaluation of chitosan–thioglycolic acids conjugates. *Biomaterials* **22**:2345–2352 (2001).
13. A. Bernkop-Schnürch, M. Hornof, and T. Zoidl. Thiolated polymers–thiomers: synthesis and *in vitro* evaluation of chitosan-2-iminothiolane conjugates. *Int. J. Pharm.* **260**:229–237 (2003).
14. Z. Grabarek and J. Gergely. Zero-length crosslinking procedure with the use of active esters. *Anal. Biochem.* **185**:131–135 (1990).
15. D. Guggi, M. K. Marschütz, and A. Bernkop-Schnürch. Matrix tablets based on thiolated poly(acrylic acid): pH-dependent

variation in disintegration and mucoadhesion. *Int. J. Pharm.* **274**:97–105 (2004).
16. S. A. Mortazavi and J. D. Smart. An investigation into the role of water movement and mucus gel dehydration in mucoadhesion. *J. Control. Rel.* **25**:197–203 (1993).
17. F. Ferrari, M. Bertoni, M. C. Bonferoni, S. Rossi, A. Gazzaniga, U. Conte, and C. Caramella. Influence of porosity and formula solubility on disintegration in tablets. *STP Pharma Sci.* **5**:116–121 (1995).
18. K. Kafedjiiski, A. H. Krauland, M. H. Hoffer, and A. Bernkop-Schnürch. Synthesis and *in vitro* evaluation of a novel thiolated chitosan. *Biomaterials* **26**:819–826 (2005).
19. M. Roldo, M. Hornof, P. Galiceti, and A. Bernkop-Schnürch. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and *in vitro* evaluation. *Eur. J. Pharm. Biopharm.* **57**:115–121 (2004).
20. P. Artursson, T. Lindmark, S. S. Davis, and L. Illum. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm. Res.* **11**:1358–1361 (1994).
21. A. Bernkop-Schnürch, D. Guggi, and Y. Pinter. Thiolated chitosans: development and *in vivo* evaluation of a mucoadhesive permeation enhancing oral drug delivery system. *J. Control. Rel.* **94**:177–186 (2004).
22. A. Bernkop-Schnürch, U. M. Brandt, and A. E. Clausen. Synthesis and *in vitro* evaluation of chitosan–cysteine conjugates. *Sci. Pharm.* **67**:196–208 (1999).
23. A. E. Clausen and A. Bernkop-Schnürch. *In vitro* evaluation of the permeation-enhancing effect of thiolated polycarbophil. *J. Pharm. Sci.* **89**:1253–1261 (2000).
24. W. C. Barrett, J. P. DeGnore, S. Konig, H. M. Fales, Y. F. Keng, Z. Y. Zhang, M. B. Yim, and P. B. Chock. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* **38**:6699–6705 (1999).
25. N. Langoth. Development of buccal drug delivery systems for peptide drugs. *Thesis, University of Vienna* (2003).
26. L. A. Dominey and K. Kustin. Kinetics and mechanism of Zn (II) complexation with reduced glutathione. *J. Inorg. Biochem.* **18**:153–160 (1983).