Thiolated Chitosans: *In Vitro* Comparison of Mucoadhesive Properties

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Received 23 March 2011; accepted 10 September 2011
DOI 10.1002/app.35622
Published online 11 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: The aim of this study was to compare the mucoadhesive properties of thiolated chitosans with regard to their molecular mass and type of immobilized thiol ligand. Mediated by a carbodiimide, aromatic- and aliphatic-thiol-bearing compounds were covalently attached to low- and medium-molecular-mass chitosan. All synthesized conjugates displayed on average 320 ± 50 μmol of immobilized free thiol groups per gram of polymer. The rheological synergy was observed by the mixture of equal volumes of polymer with mucin solution. Because of the increase in viscosity of the conjugate/mucin mixture, the self-crosslinking properties and the interaction of thiomers with the mucus layer could be confirmed. Further mucoadhesion of the chitosan conjugates was evaluated *in vitro* with the rotating cylinder method and tensile studies on excised porcine intestinal mucosa. The results show a significantly enhanced residence time ($p < 0.05$) on the mucosa of all thiolated chitosans compared to the unmodified polymer. Among all of the conjugates tested, the following rank order of mucoadhesion could be determined: Chitosan–thiobutylamidine > Chitosan–4-mercaptobenzoic acid > Chitosan–glutathione > Chitosan–6-mercaptonicotinic acid > Chitosan–*N*-acetyl cysteine > Chitosan–thioglycolic acid > Unmodified chitosan. The charge, pK_a, and reactivity of the attached compounds were found to be important factors influencing the mucoadhesive potential of the polymer. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 5046–5055, 2012

Key words: adhesion; conjugated polymers; modification; rheology

INTRODUCTION

Within recent years, there has been an increased interest in mucoadhesive drug-delivery systems, which promise several advantages for biomedical applications. Particularly, the gastrointestinal tract is a highly suitable site for bioadhesive formulations and has been the subject of intense research on the use of mucoadhesive polymeric excipients.[1] Because of enhanced adhesion, gastroretentive formulations stay longer at the absorption site and advance the uptake of a drug into systemic circulation. Consequently, the bioavailability of medical agents is benefited, and administration frequency can be reduced.[2] Bioadhesive applications could also be used as therapeutic materials to coat and protect damaged tissues or to act as lubricating agents.[3]

Mucoadhesive polymers are synthetic or natural macromolecules that are able to adhere to mucosal tissues for an extended period of time through their physical and/or chemical interactions with mucin glycoproteins.[4] Chitosan has attracted scientific interest because of its nontoxic, biocompatible, and biodegradable properties and, particularly, because of its mucoadhesive nature at physiological pH.[5] The amino groups and both primary and secondary hydroxyl groups within the D-glucosamine and *N*-acetyl-D-glucosamine units of chitosan can be grafted to gain additional mucoadhesive properties. A number of well-established chitosan derivatives, such as *N*-trimethylated chitosan,[6] carboxylated chitosan,[7] *N*-arylated chitosan,[8] and acylated chitosan,[9] have been synthesized to optimize the biological profile of this cationic biomaterial.

A further improvement was established by the coupling of complexing agents, such as ethylenediamine tetraacetic acid, to chitosan; this generated excellent mucoadhesiveness and provided a strong enzyme inhibitory effect in the gastrointestinal system, which are beneficial for orally administered peptides.[10] The development of β-cyclodextrin conjugated chitosan resulted in synergistic effects of the transport properties of cyclodextrin and mucoadhesive characteristics of chitosan.[11] In addition, several microparticles,[12] nanoparticles,[13] and gelling systems[14] of chitosan have been devised to intensify the contact between the polymeric excipient and the mucosa.

Nevertheless, most of these derivatives and formulations have been based on the formation of noncovalent bonds (e.g., hydrogen bonds, van der Waal's forces, ionic interactions), which show relatively insufficient adhesion to the mucus. The mechanism of

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TABLE I
Amounts of Reagents Used for the Reaction Mixtures and Degrees of Modification Determined with Ellman's Reagent

Polymer	Ligand	Added ligand (g)	EDAC (m*M*)	Reduced thiol groups (μmol/g of polymer; Mean ± Standard deviation)	Total thiol groups (μmol/g of polymer; Mean ± Standard deviation)
Low-molecular-mass chitosan	NAC	4.0	200	327 ± 34	650 ± 60
Medium-molecular-mass chitosan	NAC	4.0	200	270 ± 27	742 ± 62
Low-molecular-mass chitosan	TBA	0.2	—	347 ± 31	508 ± 50
Medium-molecular-mass chitosan	TBA	0.2	—	285 ± 28	402 ± 38
Low-molecular-mass chitosan	GSH	2.5	200	270 ± 21	712 ± 56
Medium-molecular-mass chitosan	GSH	2.5	200	359 ± 25	630 ± 35
Low-molecular-mass chitosan	TGA	0.5	125	370 ± 14	776 ± 25
Medium-molecular-mass chitosan	TGA	0.5	125	351 ± 21	637 ± 36
Low-molecular-mass chitosan	4-MBA	0.6	150	284 ± 20	480 ± 51
Medium-molecular-mass chitosan	4-MBA	0.6	150	338 ± 16	489 ± 75
Low-molecular-mass chitosan	6-MNA	0.5	50	276 ± 26	340 ± 12
Medium-molecular-mass chitosan	6-MNA	0.5	50	291 ± 9	415 ± 39

mucoadhesion could be markedly augmented by the immobilization of thiol moieties on the polymeric backbone of various polymers. Because of the formation of disulfide bonds with mucus glycoproteins, thiomers have emerged as highly effective drug-delivery systems.[15] So far, this enhanced concept of mucoadhesion caused by the introduction of sulfhydryl-bearing moieties has already been verified for chitosan in formulations for buccal, nasal, gastrointestinal, vaginal, and colonic use in drug and gene delivery.[16]

Because the concept of the covalent bonding of polymers on mucosal membranes has revealed excellent bioadhesion, further attempts have been undertaken to advance the formation of chemical bonds between intestinal tissue and auxiliary materials. A novel type of polymer also capable of forming covalent linkages was reported by Davidovich-Pinhas and Bianco-Peled.[17] Here, poly(ethylene glycol) diacrylate was capable of reacting with thiol groups of glycoproteins in the mucosa through Michael addition.[17] However, information on the cytotoxicity was omitted, and the characterization of mucoadhesion remains incomplete. In contrast, thiomers provide a convenient synthesis technology, nontoxicity, and additional qualities, such as permeation enhancement and *in situ* gelling effects. Thiolated chitosans can be broadly classified into two groups, namely, chitosan derivatized with alkyl thiol compounds and aromatic thiol compounds on their side chains. Depending on the chemical structure of the immobilized compounds, thiomers show different adhesive characteristics. Accordingly, the aim of this study was to analyze the impact of the molecular mass of chitosan and the type of attached mercaptane on mucoadhesion.

In vitro tests are by far the most common experimental assays for the evaluation of the mucoadhesive strength of polymer systems. Herein, two direct test techniques, namely, the rotating cylinder method and tensile studies, were applied to measure the force and time required to detach a tablet from mucus, respectively. Another focus was on the indirect determination of the interaction between mucin and chitosan by measurement of the rheological synergy.[18] The obtained results should provide an overview and profound information about thiolated chitosans, which have been synthesized during recent years.

EXPERIMENTAL

Materials

N-Hydroxysuccinimide (NHS) and low-molecular-mass chitosan (150 kDa, degree of deacetylation = 75–85%) were purchased from Fluka (Vienna, Austria). Medium-molecular-mass chitosan (400 kDa, degree of deacetylation = 75–85%), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)], dioxane, reduced-form glutathione (GSH), 2-iminothiolane HCl (Trauts reagent), 4-mercaptobenzoic acid (4-MBA), 6-mercaptonicotinic acid (6-MNA), tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), *N*-acetyl-L-cysteine (NAC), thioglycolic acid (TGA), and porcine gastric mucin (type II, crude) were all obtained from Sigma-Aldrich (Vienna, Austria). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was purchased from Carbosynth (Compton, United Kingdom). All other chemicals, reagents, and solvents were analytical grade and were received from commercial sources.

Synthesis of the chitosan conjugates

Chitosan conjugates were synthesized as previously described by our research group.[19–24] In brief, 500 mg of chitosan was hydrated in 4 mL of 1*M* HCl and dissolved by the addition of demineralized water to obtain a 1% (w/v) polymer solution. Then, EDAC was added to the chitosan solutions in final concentrations as listed in Table I. After 20 min, different amounts of thiol-bearing ligand (see Table I)

were slowly added to the reaction mixtures under stirring. In the case of chitosan–thiobutylamidine (TBA), 2-iminothiolane HCl was added to the polymer under the omission of EDAC.

Some syntheses required further reagents to optimize the thiol content. The addition of NHS to the coupling reaction of glutathione to chitosan significantly improved the thiol yields. In the case of 4-MBA and 6-MNA, both water-insoluble compounds were solved in a dioxane–water mixture (80 + 20 mL). Furthermore, coupling reactions with aromatic ligands required TCEP in a final concentration of 10 m*M* as a reducing agent.

The pH of each solution was adjusted to 5 by the addition of 1*M* NaOH, and the reaction was allowed to proceed at room temperature under vigorous stirring for 5 h. To isolate chitosan conjugates, the resulting polymer solutions were dialyzed in tubing (molecular mass cutoff = 12 kDa) at 10°C in the dark. After dialysis, the pH values of all samples were readjusted to 4. The frozen aqueous polymer solutions were freeze-dried at −77°C and 0.01 mbar (Virtis bench-top freeze drier, Bartelt, Graz, Austria) and stored at 4°C until further use. All syntheses were performed with both the low- and medium-molecular-mass chitosans. To verify the purification steps, the controls of each polymer were prepared according to the same procedure but without EDAC.

Determination of the thiol group and disulfide bond content

The amount of thiol groups immobilized on chitosan was determined photometrically with Ellman's reagent to quantify free thiol groups, as described previously.[24] Initially, 0.5 mg of each conjugate and control were hydrated in 250 μL of dematerialized water. Then, 250 μL of 0.5*M* phosphate buffer at pH 8.0 and 500 μL of Ellman's reagent [3 mg of DTNB dissolved in 10 mL of 0.5*M* phosphate buffer at pH 8.0] were added. The samples were incubated for 2 h and protected from light at room temperature. The supernatant was separated from the precipitated polymer by centrifugation at 13,400 g for 5 min (Minispin, Eppendorf, Vienna, Austria). Afterward, 200 μL of each sample was transferred into a microtitration plate, and the extinction was measured at a wavelength of 450 nm with a microplate reader (Infinite M200, Tecan, Grödig, Austria). The amount of free thiol groups was calculated according to a standard curve obtained by chitosan solutions with increasing concentrations of the particular attached ligand or cysteine HCl prepared in exactly the same way as the samples.

The disulfide content was measured after reduction with $NaBH_4$ and the addition of DTNB, as described previously by Habeeb.[25]

Tablet manufacture

Lyophilized chitosan conjugates and unmodified chitosan were compressed into 30.0-mg flat-faced tablets 5.0 mm in diameter (single-punch eccentric press, Paul Weber, Remshalden, Germany). The compaction pressure was kept constant during the preparation of all discs (15 kN).

Evaluation of the swelling behavior

The water-absorbing capacity was determined by a gravimetric method. Tablets (30 mg) of unmodified chitosan and thiolated chitosan were fixed on a needle and submersed in a test tube containing 100 m*M* phosphate buffer solution (pH 6.8) at 37 ± 0.5°C. At predetermined time intervals, the hydrated test tablets were taken out of the incubation medium, excess water was removed, and the tablets were weighed. The amount of absorbed water was calculated by subtracting the original weight of the tablet before the test, and the weight of the tablet taken at scheduled times.

In vitro evaluation of the mucoadhesive properties

Tensile studies

Tensile studies with tablets were carried out on porcine intestinal mucosa. Each polymer tablet was carefully glued with cyanoacrylate glue (Loctite, Vienna, Austria) to a stainless steel, flat disc attached to a 15-cm nylon string. The other end of the nylon string was fixed to a laboratory stand. Freshly excised porcine small intestinal mucosa was thawed and cut in small pieces with an area of approximately 9 cm^2. The mucosa was glued on the lower support, which was placed in a beaker containing 400 mL of 0.1*M* phosphate buffer at pH 6.8.

The beaker was placed on a balance and carefully raised by a mobile platform until the mucosa had contact with the tablet. The mucosa was incubated in buffer for 30 min at 25°C. Afterward, the platform was pulled down from the tablet at a rate of 0.1 mm/s. Data points were registered every second by a personal computer connected to a balance with WINWEDGE Software (TAL Technologies, Inc., Philadelphia, PA). The maximum detachment force (MDF) and the total work of adhesion (TWA), given by the area under the force–displacement curve, were calculated with Microsoft Excel.[26]

Rotating cylinder method

Polymer conjugates and unaltered polymers were attached to freshly excised intestinal porcine mucosa, which was glued to a stainless steel cylinder (diameter = 4.4 cm, height = 5.1 cm; four-cylinder apparatus, United States Pharmacopeia (USP)) with

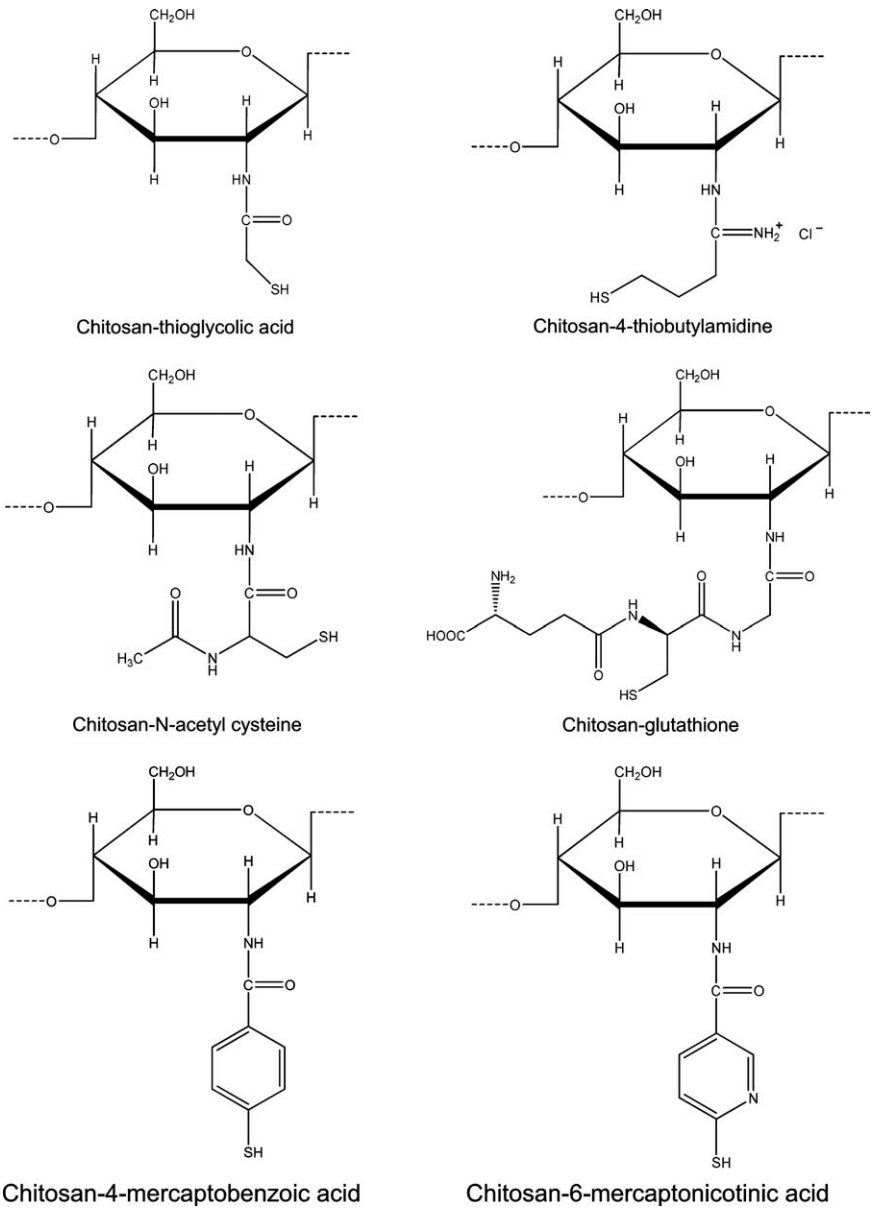

Figure 1 Overview of the substructure of the thiolated chitosans.

cyanacrylate glue (Loctite, Vienna, Austria). The cylinder was immersed in the dissolution apparatus according to USP, containing 100 m*M* phosphate buffer at pH 6.8 and 37°C. The velocity of the cylinder was adjusted to 100 rpm. The detachment, disintegration, and erosion of tablets were registered over 200 h via a camera. This test was conducted at least five times for each polymer.

Rheological evaluation of the polymer/mucin mixtures

First, 2.5 g of porcine mucin (type II, crude, Sigma, Vienna, Austria) was dissolved in 12.5 mL of demineralized water under continuous stirring. Subsequently, the pH of the solution was adjusted to 6.5 by the addition of 1*M* NaOH and diluted to a final volume of 25 mL with 0.1*M* phosphate buffer at pH 6.5. The resulting 10% (m/v) mucin stock solution was kept at 4°C.

Chitosan conjugates and unthiolated controls were hydrated in demineralized water to obtain a concentration of 3% (m/v). After complete hydration, the polymer solutions were mixed with aliquots of 10% mucin stock solution, and the pH was adjusted to 6.5 with 1*M* NaOH. After an incubation period of 20 min at room temperature, 0.7 mL of the polymer/mucin mixture was transferred to a cone–plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). The samples were allowed to equilibrate on the plate for 3 min at 37 ± 0.5°C. Then, dynamic oscillatory tests within the linear viscoelasticity region were performed, as described by Marschutz and Bernkop-Schnurch.[27]

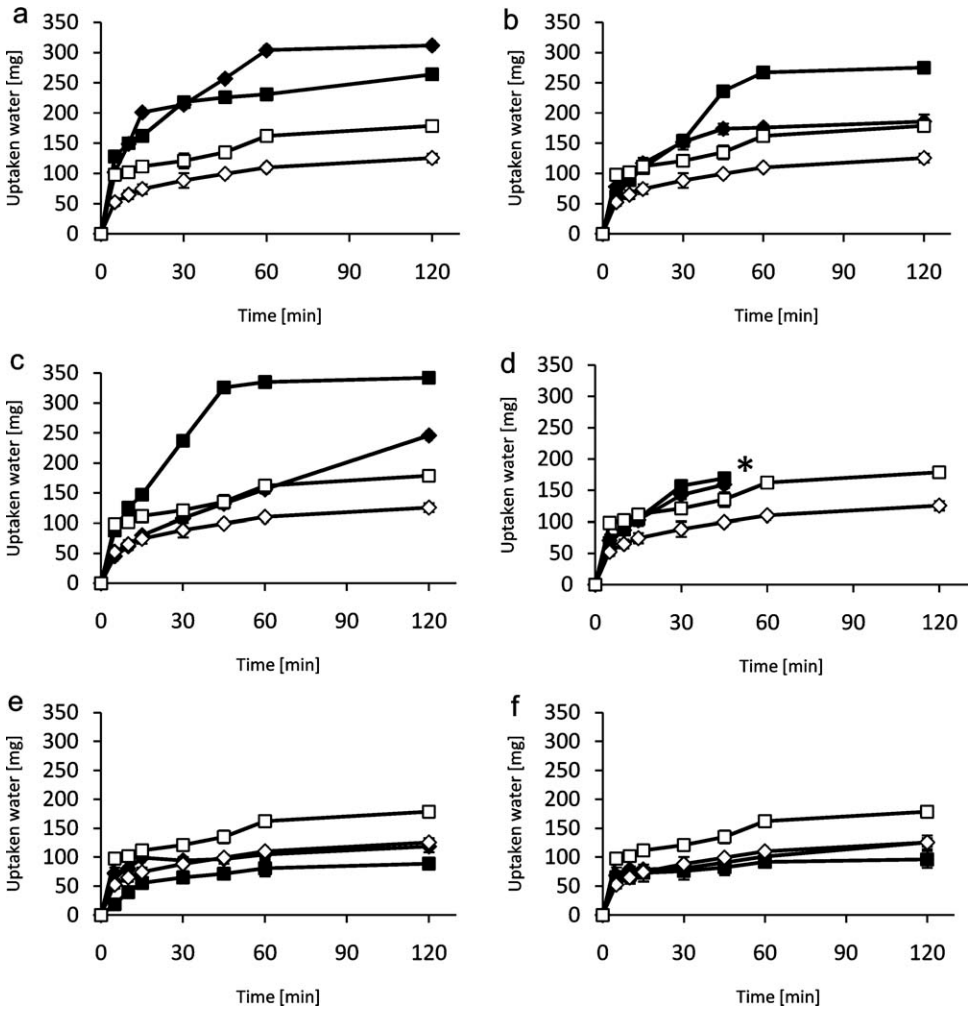

Figure 2 Swelling behavior of 30-mg tablets composed of thiolated chitosans [(◆) low molecular mass (LMM) and (■) medium molecular mass (MMM)] and unmodified chitosans [(◇) LMM and (□) MMM] in 100 m*M* phosphate buffer at pH 6.8 and 37°C. The water uptake is expressed in milligrams over a time period of 2 h. An asterisk indicates that the chitosan–TGA tablets disintegrated after 45 min. Thiomers: (a) chitosan–TBA, (b) chitosan–GSH, (c) chitosan–NAC, (d) chitosan–TGA, (e) chitosan–4-MBA, and (f) chitosan–6-MNA. The indicated values are the means plus or minus the standard deviation of at least five experiments.

RESULTS

Synthesis and characterization of the chitosan conjugates

TGA, 2-iminothiolane HCl, NAC, glutathione, 4-MBA, and 6-MNA were covalently attached to the primary amino groups of chitosan under the formation of amide bonds according to the methods described previously (Fig. 1).[19–24] The carboxylic acid moieties of sulfhydryl ligands were activated by EDAC to form an *O*-acylurea derivative as an intermediate product; this reacted with the primary amino groups of chitosan. Some synthesis required further additives to optimize the thiol content. The addition of NHS to the coupling reaction of glutathione to chitosan significantly improved the thiol yield. In the case of chitosan–TBA, the addition of EDAC was not necessary because 2-iminothiolane (Trauts reagent) served as a coupling reagent and offered a simple, one-step reaction.

The amount of free thiol groups was determined via Ellman's reagent and revealed 320 ± 50 μmol of free thiol groups per gram of polymer. To create comparable mucoadhesive systems, all of the conjugates exhibited amounts of free thiol groups in a close range, as shown in Table I. The efficacy of the purification method for the resulting polymers could be verified by the corresponding controls, which were prepared in the same way but without EDAC during the coupling reaction. The total amount of thiol groups located in the control samples was negligible (data not shown). The thiolated chitosan powders were stable toward air oxidation when stored at 4°C.

Swelling behavior

An important factor influencing the mucoadhesive strength of polymers is their capability to absorb water. Accordingly, polymers need to assimilate

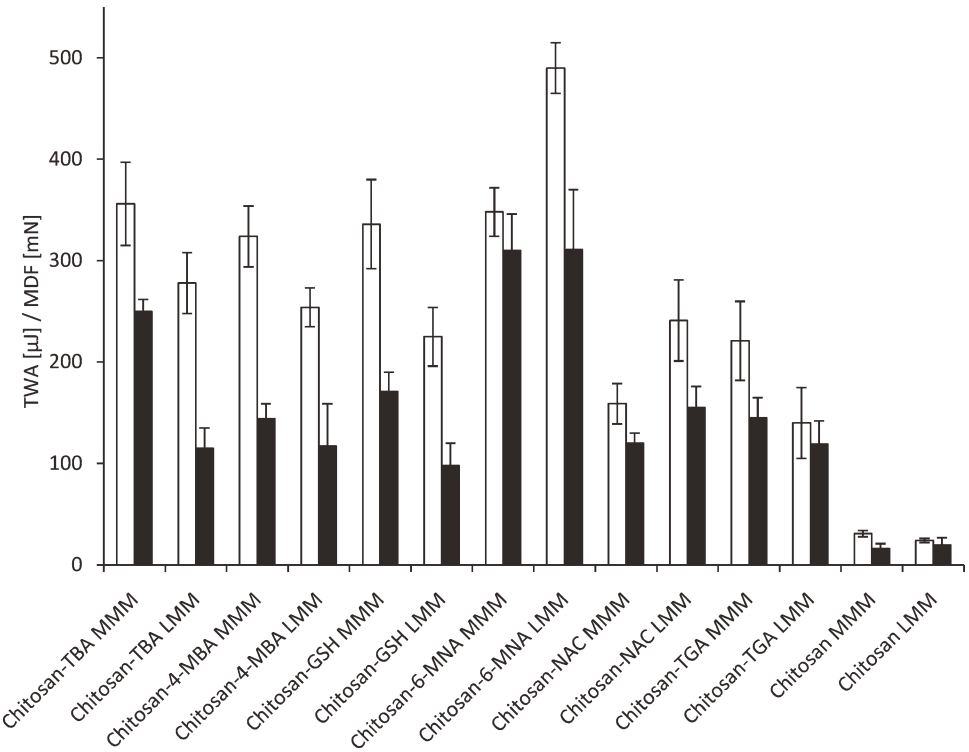

Figure 3 Comparison of the mucoadhesive properties of the test discs (30 mg) based on conjugated and unmodified chitosan (LMM & MMM) determined by tensile studies. TWA is represented in white, and MDF is displayed in black. The indicated values are the means of at least five experiments plus or minus the standard deviation.

liquid from underlying mucosal tissues by absorption, swelling, and capillary effects.[28] A sufficient amount of water appears necessary to hydrate and expand the dosage form, which favors interdiffusion between the polymer and mucosa and which results in bond formation and, therefore, stronger mucoadhesion. However, rapid swelling behavior is associated with rapid drug release, reduced stability, and the formation of overhydrated forms. A slow swelling process seems to be favorable for mucoadhesive formulations to avoid a loss of adhesion before the target is reached.

As represented in Figure 2, water-uptake studies revealed that the covalent attachment of aliphatic ligands showed a tendency to assimilate more liquid compared to unmodified chitosan or chitosan possessing aromatic compounds. The marked lipophilic character of the immobilized mercaptanes could elucidate the minor swelling behavior of chitosan–4-MBA and chitosan–6-MNA. A substantial difference in the enhanced water uptake between the low- and medium-molecular-mass conjugates could not be observed.

Mucoadhesion studies

Tensile studies

The adhesive strength between the polymer and mucosa can be measured by quantification of the force required to detach the tablet from the mucosal surface through the application of an external force. The results are usually presented as TWA and MDF. The applied test technique carried out with the unmodified chitosan and chitosan conjugates revealed a significant influence of immobilized thiol groups on the mucoadhesive properties of chitosan. TWA and MDF were in good correlation to each other. As shown in Figure 3, the MDF increased proportionally with increasing TWA values.

Chitosan–6-MNA exhibited the highest TWA and MDF values, followed by chitosan–TBA, chitosan–4-MBA, and chitosan–GSH. Chitosan–TGA and chitosan–NAC showed less adhesive properties, but their values were still sufficient compared to the unaltered polymer. Furthermore, the results of this assay indicate enhanced mucoadhesion with the use of medium-molecular-mass chitosan.

Rotating cylinder method

To confirm the findings of the tensile studies, a second mucoadhesion test system was applied. The rotating cylinder method determines the ability of mucoadhesive formulations to maintain contact with the mucosal surface under shear forces. This method is supposed to have more similarity to *in vivo* conditions than the tensile studies described previously because it simulates the adhesion and cohesiveness of the polymer in the physiological environment.

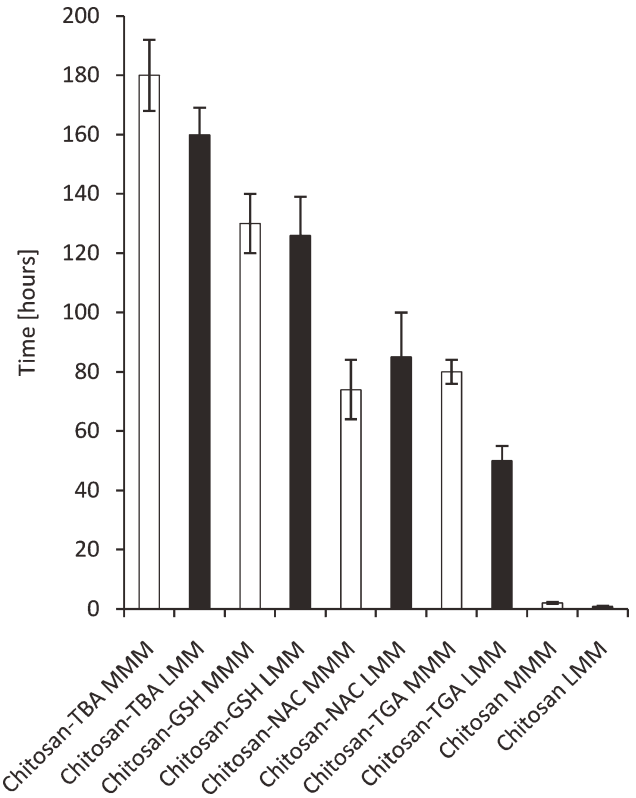

Figure 4 Influence of the molecular mass and type of immobilized thiol ligand on the mucoadhesive properties of the chitosan conjugates. Comparison with the rotating cylinder method on porcine small intestine mucosa in 100 m*M* phosphate buffer at pH 6.8 and 37°C. The white bars show medium-molecular-mass (MMM) chitosans, and the black bars display low-molecular-mass (LMM) chitosans. The indicated values are the means of at least five experiments plus or minus the standard deviation.

The results of the mucoadhesion studies, which are shown in Figures 4 and 5, conformed to the TWA and MDF values determined via tensile studies.

All of the tested thiomers showed comparatively higher adhesive properties, which were verified by mucoadhesion times from 50 to 180 h. As opposed to this, the unmodified chitosans detached from the mucosa after approximately 2 h. With respect to the immobilized sulfhydryl ligand, it could be demonstrated that NAC and TGA led to reduced adherence on the intestinal mucosa of chitosan. TBA- or 6-MNA-functionalized chitosan showed greatly improved adherence on the mucosa. By comparing chitosan with an identical ligand of different molecular mass, we revealed that all medium-molecular-mass chitosan conjugates, except chitosan–NAC, showed prolonged mucoadhesion.

Viscosity studies

Mucoadhesion is favored by key elements, including polymer entanglements and interdiffusion and chemical interactions. When mucin is added to a solution of a bioadhesive polymer, the same phenomenon can be observed. The increase in the viscosity of the polymer/mucin mixtures directly correlates with the mucoadhesion of the concerned polymers.[29] Therefore, a so-called rheological synergy measurement was performed to evaluate the force of interaction involved in mucoadhesion. Commercially available mucin was used instead of native mucus to obtain more reproducible and comparable results.

Figure 6 displays the rheological properties of the chitosan conjugates, the unmodified chitosan, and their mixtures with mucin. After 20 min of incubation, the polymer solutions showed low or insignificant alterations in the viscosity at the concentration applied (1.5% m/v). The nonconjugated chitosan demonstrated low viscous properties in the absence and presence of mucin, whereas the alkyl thiolated polymers exhibited significantly increased viscosity values when in combination with the mucus. In case of aromatic thiomers, the augmentation of viscosity was less pronounced. All medium-molecular-mass conjugates displayed a stronger enhancement in viscosity compared to low-molecular-mass conjugates.

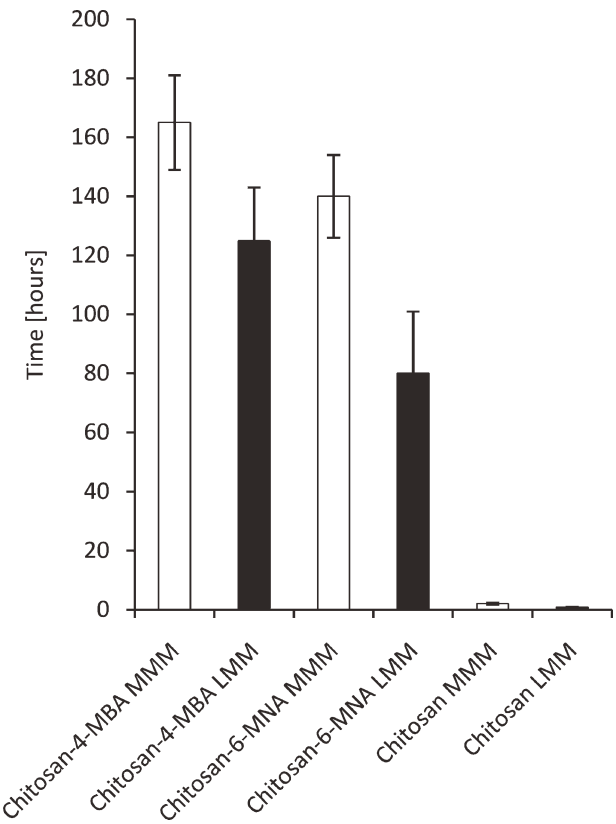

Figure 5 Comparison of the mucoadhesion of chitosan conjugated with aromatic ligands (LMM & MMM) and unmodified chitosans (LMM & MMM) with the rotating cylinder method on porcine small intestine mucosa in 100 mM phosphate buffer pH 6.8 at 37°C. The white bars show medium-molecular-mass chitosans, and the black bars display low-molecular-mass chitosans. The indicated values are the means of at least five experiments plus or minus the standard deviation.

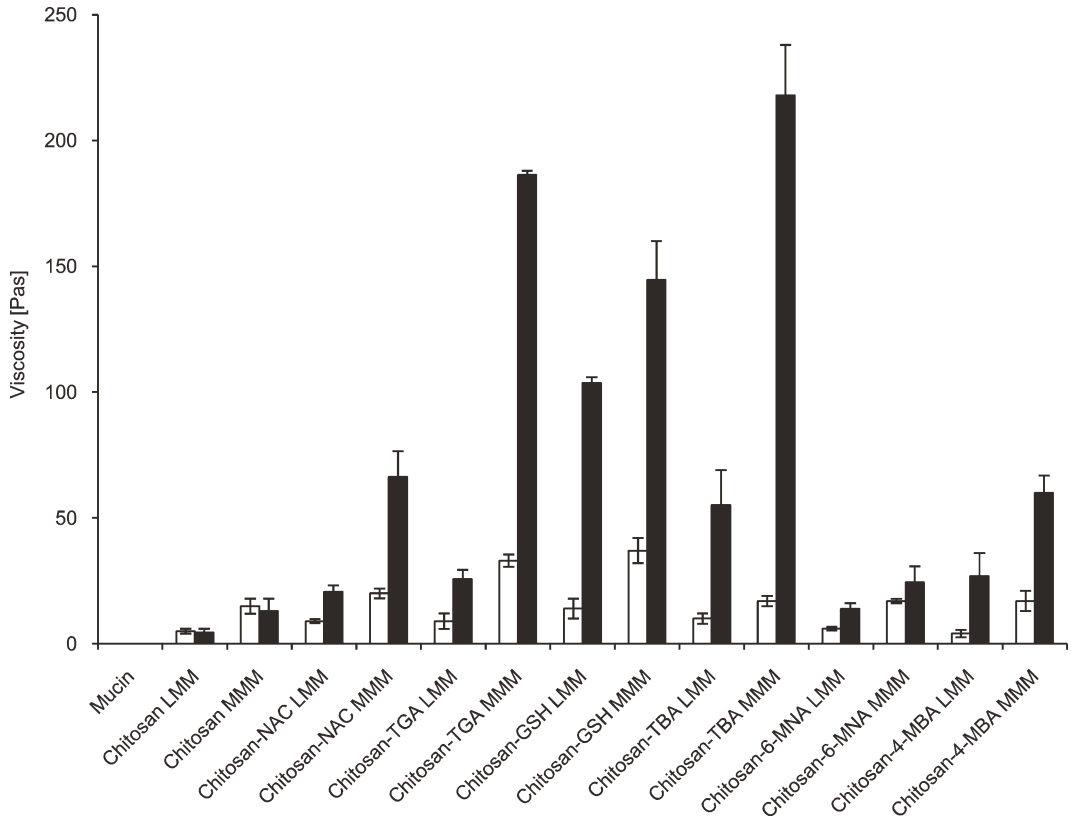

Figure 6 Viscosity measurements for the 1.5% (m/v) conjugates and unthiolated chitosan (white bars) and their corresponding mixtures with mucin (black bars) in 100 m*M* phosphate buffer at pH 6.8. Oscillatory measurements were performed at 37°C after an incubation period of 20 min. All values are the means of five experiments plus or minus the standard deviation.

DISCUSSION

The mucoadhesive process can be divided into three steps: (1) preliminary swelling of the polymer to initiate contact with biological tissues, (2) interpenetration of bioadhesive polymer chains and entanglement of polymer and mucosa chains, and (3) formation of chemical bonds between entangled chains.[30] On the basis of these assumptions, the tensile force, shear force, and viscosity[18] values of all of the mentioned thiomers were determined to provide a comprehensive overview.

The success and degree of mucoadhesive bonding are influenced by polymer-dependent properties, such as the molecular weight, degree of crosslinking, and presence of various functional groups.[4] To analyze the obtained results, it was crucial to include chemical and physical properties, such as pK_a values, charge, lipophilicity, and hydrophilicity of the attached thiol-bearing ligand (Table II, Fig. 1). The modification of chitosan with 2-iminothiolane generated not only a thiol group but also an amidine substructure that presented a cationic charge. This charge was responsible for ionic interaction with anionic substructures, such as sialic acid or sulfonic acid, of the mucus and supported the mucoadhesion[31] of chitosan–TBA. Considering the constitution of TBA, we assumed that longer thiol chains seemed to favor the formation of disulfide bridges within the polymer itself because of enhanced entanglement.

Glutathione led to improved bioadhesive qualities because it possessed various functional groups within the tripeptide structure, such as amine or carboxylic acid moieties. Accordingly, mucoadhesion was not only based on the disulfide exchange reaction of the thiol group but also on secondary noncovalent bonding. Polymers that exhibit a high density

TABLE II
Summary of the Chemical and Physical Properties of the Immobilized Thiol Ligands

		Theoretical pK_a value of the thiol groups	
	Molecular weight (g/mol)	Free molecule	Attached to chitosan
TGA	92.12	9.86	9.45
NAC	163.19	10.05	9.96
TBA	119.20	10.15	10.15
GSH	307.32	9.96	9.96
4-MBA	154.19	6.21	6.04
6-MNA	155.17	7.51	7.43

The theoretical pK_a values were calculated by the Marvin software, Chem Axon, Budapest, Hungary.

of hydrophilic groups for hydrogen-bond formation are able to interact more intensively with mucin glycoproteins.[32] In contrast, the tethering of hydrophobic ligands, such as TGA or NAC, resulted in uncharged amide bonds without any supporting features. Additionally, it was obvious that comparatively short spacers of up to two carbon atoms between the sulfhydryl group and polymeric backbone did not show a sufficient gap for crosslinking; this resulted in lower adhesion times. Chitosan itself showed low mucoadhesion, which was only based on the electrostatic interactions of chitosan with the negatively charged mucin.

Recently, the adhesive properties of alkyl thiolated polymers were improved by the introduction of a second generation. Therefore, 4-MBA–functionalized chitosan was generated to accomplish an enhanced affinity to mucin-containing surfaces. The introduced hydrophobic entity was supposed to exhibit a higher reactivity because of the low pK_a value of sulfhydryl functional groups.[22] Aromatic thiols exhibited pK_a values of 5–7, whereas alkyl mercaptanes showed pK_a values from 8 to 10 (Table II).[33] The theoretical results reveal a pK_a value of 6.04 for the thiol group of 4-MBA. At intestinal pH values from 5.5 to 7.5, aromatic thiol groups were present in the reactive form of thiolate anions, which facilitated the formation of disulfide bonds.[34] In contrast, aliphatic thiolated chitosans partially expressed the anionic form because of their higher pK_a values. Outcomes of this study proved a satisfying mucoadhesion of aryl thiolated chitosans, although the aromatic benzene ring displayed a hydrophobic and less flexible structure compared to chitosan–TBA. A further improvement could be achieved by the immobilization of 6-MNA to chitosan's backbone because the thiol group also possessed a lower pK_a compared to the aliphatic polymers. In addition, the 6-MNA polymers exhibited a pH-independent formation of disulfide bonds because of their ability to form tautomeric structures.[21]

The chain lengths of the polymers correlated with their molecular masses and were, therefore, a crucial parameter, which affected the strength of the mucoadhesive interaction. A sufficient chain length of chitosan is necessary for its flexibility and ability to form entanglements. However, excessively long polymer chains lose their capability to interpenetrate into the highly entangled network of mucus.[35] Among the used polymers in this study, chitosan at 400 kDa provided stronger cohesive properties and more sufficient interpenetration with the mucus compared to the low-molecular-mass chitosan.

CONCLUSIONS

In this study, conjugates were synthesized and characterized, and their mucoadhesive properties were evaluated *in vitro* by three different methods. Among all of the conjugates tested, a rank order of mucoadhesion of aliphatic and aromatic thiolated chitosans was established. The strongest adhesive properties on mucosa were obtained by the utilization of chitosan–TBA and chitosan–GSH, followed by the aryl thiolated chitosans. The findings of this study highlighted the optimal physicochemical properties that sulfhydryl ligands should possess for strong mucoadhesiveness and defined the direction for future development of novel and even more bioadhesive thiolated polymers. Additionally, this comparison of chitosan derivatives should present detailed information on their mucoadhesive properties by the application of standardized conditions because different amounts of thiol groups and intestinal mucosa of various animals in previous studies caused a marked variability of data. This study revealed comparable results, which should facilitate the production of novel dosage forms and provide a prolonged residence time on certain mucosal tissues.

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