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Research paper

Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation

Marta Roldo[a], Margit Hornof[a,b], Paolo Caliceti[c], Andreas Bernkop-Schnürch[a,*]

[a]*Institute of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Vienna, Austria*
[b]*Croma Pharma GmbH, Leobendorf, Austria*
[c]*Department of Pharmaceutical Sciences, University of Padova, Padova, Italy*

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Abstract

The aim of the present study was to evaluate the influence of the degree of modification and the polymer chain length on the mucoadhesive properties and the swelling behavior of thiolated chitosan derivatives obtained via a simple one-step reaction between the polymer and 2-iminothiolane. The conjugates differing in molecular mass of the polymer backbone and in the amount of immobilized thiol groups were compressed into tablets. They were investigated for their mucoadhesive properties on freshly excised porcine mucosa via tensile studies and the rotating cylinder method. Moreover, the swelling behavior of these tablets in aqueous solutions was studied by a simple gravimetric method. The obtained results demonstrated that the total work of adhesion of chitosan-TBA (= 4-thio-butyl-amidine) conjugates can be improved by an increasing number of covalently attached thiol groups; a 100-fold increase compared to unmodified chitosan was observed for a medium molecular mass chitosan–TBA conjugate exhibiting 264 μM thiol groups per gram polymer. Also, the polymer chain length had an influence on the mucoadhesive properties of the polymer. The medium molecular mass polymer displayed a fourfold improved adhesion on the rotating cylinder compared to the derivative of low molecular mass. These results contribute to the development of new delivery systems exhibiting improved mucoadhesive properties.

Keywords: Chitosan; Thiomers; Mucoadhesion; Traut's reagent

1. Introduction

In order to improve the targeting and delivery of orally administered drugs to the stomach and the small intestine, the interest in mucoadhesive polymers has greatly increased in the last two decades. The use of these polymers as carriers is supposed to enhance the bioavailability of orally given drugs because of a lengthened contact time of the drug with the gastrointestinal (GI) mucosa [1]. Moreover, an enzymatic degradation of perorally given (poly)peptide drugs en route to the absorption membrane by secreted proteases should be excluded utilizing a mucoadhesive delivery system [2,3]. When mucoadhesive polymers also exhibiting permeation enhancing properties are used, the intensified contact with the mucosa should provide the prerequisite for an increased epithelial permeability for many drugs mediated by the polymeric carrier system [4]. Furthermore, the frequency of dosing might be reduced by a prolonged GI-residence time of the mucoadhesive delivery systems, leading to an improved patient compliance.

Because of these advantages, numerous attempts have been undertaken in order to improve the mucoadhesive properties of polymeric excipients. The mucoadhesive properties of the first studied polymers were based on the formation of non-covalent bonds such as hydrogen bonds and ionic interactions with the mucus layer [5,6]. Later on, it has been demonstrated that polymers capable of forming covalent bonds with the mucus layer display more pronounced mucoadhesive properties. This new generation of mucoadhesive polymers is based on so-called thiomers. Thiomers are polymers bearing thiol groups that lead to the formation of disulfide bonds with cysteine-rich subdomains

* Corresponding author. Institute of Pharmaceutical Technology and Biopharmaceutics, Center of Pharmacy, University of Vienna, Althanstr. 14, 1090 Vienna, Austria. Tel.: +43-1-4277-55413; fax: +43-1-4277-9554.
E-mail address: andreas.bernkop-schnuerch@univie.ac.at (A. Bernkop-Schnürch).

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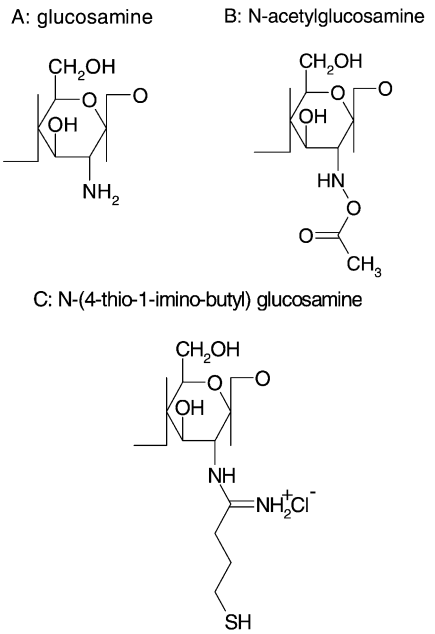

Chitosan MM	A*	B*	C*
Low	681.3	151.4	23.4
Medium	1915.8 – 1989.6	390	16.9 – 63.7
High	3022.4	490	85.6

*reported numbers of monomers /molecule are average values calculated on the basis of the declared molecular mass

Fig. 1. Presumptive monomers on which chitosan–TBA derivatives are based.

of mucus glycoproteins, the main constituents of the mucus layer covering gastro-intestinal epithelia [7].

Recently, it has been shown that chitosan-TBA (4-thio-butyl-amidine) conjugates, depicted in Fig. 1, can lead to a 140- and 42-fold improvement in mucoadhesion compared to the unmodified polymer and the previously investigated chitosan–thioglycolic acid conjugates, respectively [8,9]. These promising results can be explained by the fact that this new generation of thiolated chitosan combines two different mechanisms of mucoadhesion: (a) improved ionic interactions between the strengthened cationic groups of modified chitosan and the anionic moieties provided by sialic acid and sulfonic acid substructures within the mucus layer; and (b) the formation of disulfide bonds due to the introduction of thiol groups by reaction of chitosan with 2-iminothiolane.

In order to further improve the mucoadhesive properties of chitosan–TBA conjugates, it was the aim of this study to evaluate the influence of key parameters, i.e. the number of attached thiol groups and polymer chain length, on mucoadhesiveness. Therefore, various chitosan–TBA derivatives were synthesized according to the protocol optimized in a previous study [8] varying the molecular mass of the polymer backbone and the amounts of immobilized thiol groups. The swelling behavior and mucoadhesive properties of the resulting polymer conjugates were investigated.

2. Materials and methods

2.1. Determination of the degree of deacetylation

Initially, 2 g of chitosan (low molecular mass: 150 kDa, medium molecular mass: 400 kDa and high molecular mass: 600 kDa, respectively; Fluka Chemie, Buchs, Switzerland) was dissolved in 400 ml of 1% acetic acid by stirring the mixture for 1 h at room temperature. To 1 ml of this 0.5% (m/v) chitosan solution, demineralized water was added to obtain a final volume of 100 ml. The absorbance of this solution was measured at 202 nm (Lambda 16; Perkin-Elmer, Vienna, Austria). The amount of acetylic groups bound to the polymer was calculated from a standard curve obtained by solutions with increasing concentrations of *N*-acetyl-D-glucosamine (NAG, Sigma-Aldrich, Steinheim, Germany) [10].

2.2. Modification of chitosan with 2-iminothiolane

The coupling reaction of chitosan with 2-iminothiolane was performed according to a protocol optimized in a previous study [8]. To obtain a 1% (m/v) solution 500 mg of chitosan (molecular mass; MM: 150, 400 and 600 kDa, respectively) was dissolved in 50 ml of 1% acetic acid by stirring the mixture for 1 h. The pH was adjusted to a value of 7 with 5 M NaOH. According to the scheme outlined in Table 1, 2-iminothiolane HCl (Traut's reagent; Pierce, Oud Beijerland, NL) was added. After 24 h of incubation at room temperature under continuous stirring, the resulting polymer conjugates were dialyzed against 5 mM HCl, twice against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. Samples prepared in the same way but omitting the addition of 2-iminothiolane HCl served as controls. Thereafter, the polymers were freeze-dried at −30 °C and 0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4 °C until further use.

2.3. Determination of the thiol group content

Ellman's reagent was used to quantify the amount of thiol groups on modified chitosan as described previously [11]. First, 5 mg of each conjugate were dissolved in 2.5 ml of demineralized water. To aliquots of 250 μl of the conjugate solutions, 250 μl of 5 M phosphate buffer pH 8.0 and 500 μl of Ellman's reagent [3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma, St. Louis, MO) dissolved in 10 ml of 0.5 M phosphate buffer pH 8] were added. The reaction was allowed to proceed for 2 h at room temperature. After removing the precipitated polymer by centrifugation (24,000 × *g*; 5 min), 300 μl of the supernatant fluid was transferred to a microtitration plate and the absorbance was immediately measured at 450 nm (microtitration-plate reader, Anthos Reader, Salzburg, Austria). The amount of thiol moieties was calculated from a standard curve obtained from solutions with increasing

Table 1
Influence of the molecular mass (MM) of chitosan (I), the degree of deacetylation (II) and the weight-ratio polymer to 2-iminothiolane during the coupling reaction (III) on the amount of immobilized thiol groups on the polymer (± S.D, $n = 3$)

Polymer conjugates	MM of Chitosan (kDa)	Degree of deacetylation (%, m/m)	Weight-ratio polymer: 2-iminothiolane	Immobilized thiol groups (μM/g)
Low MM chitosan–TBA 270	150	79.5	10:6	269.7 ± 21.7
Medium MM chitosan–TBA 54	400	80.2	10:1	53.79 ± 19.6
Medium MM chitosan–TBA 208	400	80.2	10:2	207.5 ± 25.7
Medium MM chitosan–TBA 264	400	80.2	10:4	263.8 ± 47.4
High MM chitosan–TBA 235	600	83.4	10:4	235.2 ± 33.6

concentrations of L-cysteine hydrochloride hydrate (Sigma-Aldrich, Steinheim, Germany).

2.4. Evaluation of the swelling behavior

The water-absorbing capacity was determined by a gravimetric method. Thirty milligrams each of the thiolated chitosans and the corresponding unmodified chitosans, which were used as controls, were compressed (Hanseaten Type EI, Hamburg, Germany) into 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 for 40 min in 100 mM phosphate buffered saline pH 6.0 at 37 ± 0.5 °C. At scheduled time intervals the swollen test tablets on the needle were taken out of the incubation medium and the water uptake was determined gravimetrically.

2.5. Tensile studies

Tensile studies were performed according to a method described in detail previously [12]. In brief, 30 mg each of lyophilized thiolated chitosan and controls were compressed to test tablets as described above. The tablets were attached to the porcine mucosa in the following manner. One tablet was attached to a stainless steel flat disc (5 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 to a glass support using a cyanoacrylate adhesive. The support with the fixed tissue was completely immersed in 500 ml of 100 mM phosphate-buffered saline pH 6.0. The beaker containing the excised mucosa was placed on a balance, then carefully raised by a mobile platform until the mucus came in contact with the tablet. The contact was determined when the nylon thread holding the tablet become bent. After 30 min of incubation 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) connected to the balance and collaborated using an EXCEL 97 (Microsoft, USA) datasheet. The force/distance curve, i.e. the total work of adhesion (TWA) and the maximum detachment force (MDF) were calculated.

2.6. In vitro mucoadhesion studies

In order to evaluate the adhesion time of thiolated chitosan to the mucosa, a slightly modified method as described previously was used [13]. Freshly excised intestinal porcine mucosa was fixed on a stainless steel cylinder (diameter: 4.4 cm; height 5.1 cm; apparatus 4-cylinder, USP XXVI) and tablets prepared as described above were applied to it. Thereafter, the cylinder was placed in the dissolution apparatus as described by the USP, containing 1 l of 100 mM phosphate buffered saline pH 6.0 at 37 ± 0.5 °C. The fully immersed cylinder was agitated at 125 rpm according to the USP protocol. The detachment of the test tablets was determined during an observation time of 14 days.

2.7. Statistical data analysis

Statistical data analysis was performed using the Mann–Whitney test with $P < 0.05$ as the minimum level of significance. Calculations were performed with the software Xlstat version 5.1 v1 (Addinsoft).

3. Results and discussion

3.1. Modification of chitosan

2-Iminothiolane (Traut's reagent) is a common reagent used to immobilize thiol groups to primary amino groups of proteins [14]. In this work it has been used for the modification of chitosan (Fig. 1). Commercially available chitosan of three different chain lengths was modified by the addition of various amounts of 2-iminothiolane as listed in Table 1. Prior to the coupling reaction, the degree of deacetylation of each polymer was determined. Results of this study are listed in Table 1.

All coupling reactions were carried out at pH 7. This pH value was a compromise between an acidic environment in which chitosan is more soluble and an alkaline solution that

enhances the reactivity of 2-iminothiolane. As demonstrated by previous studies [8], the addition of reducing agents, such as 2-mercapoethanol, had no influence on the coupling rate (data not shown). Coupling reactions performed with different ratios of chitosan and Traut's reagent showed that the amount of immobilized thiol groups on the polymer can be varied by the concentration of 2-iminothiolane during the reaction. As shown in Table 1, as more 2-iminothiolane was added to medium MM chitosan, more thiol groups were immobilized on the polymer.

The lyophilized chitosan-TBA (4-thio-butyl-amidine) conjugates appeared as a white and odorless powder of fibrous structure. They were soluble in acidic solutions and formed transparent gels of high viscosity. They were stable towards air oxidation when stored at 4 °C.

The described pathway for the synthesis of chitosan–TBA conjugates is so far the easiest way to obtain polymer derivatives bearing thiol groups. The method leads to desired derivatives via a simple one-step reaction with no need of adding any other reagents. In contrast, the addition of a carbodiimide is necessary in order to activate the carboxylic acid moieties of the sulfhydryl ligands for the synthesis of chitosan–cysteine and chitosan–thioglycolic acid conjugates [9,15].

3.2. Swelling behavior

The adhesive properties and cohesiveness of mucoadhesive polymers are generally affected by their swelling behavior [16]. Mucoadhesive polymers are supposed to take water from the underlying mucosal tissue by absorbing, swelling, and capillary effects, leading to a considerably stronger adhesion [17]. On the other hand, an excessive water uptake will transform the mucoadhesive dosage forms into an over hydrated slippery mucilage that completely loses its adhesiveness. Mucoadhesive drug delivery systems (DDS) can be applied in their dry form to buccal, nasal or vaginal mucosa. In contrast, when a mucoadhesive DDS is directed to the small intestine, it reaches its target already in partially hydrated form [18]. Therefore, slow swelling is a requisite to avoid the formation of an over hydrated form that loses its mucoadhesive properties before reaching the target. In order to evaluate this effect, water uptake studies were carried out with tablets made by both controls and chitosan–TBA conjugates.

The relation between the degree of modification and the swelling behavior was investigated with medium MM chitosan conjugates. The modification of medium MM chitosan with thiol groups led to a significant increase in water uptake in case of chitosan–TBA 54 and 208. An inverse correlation between increasing amounts of immobilized thiol groups on medium MM chitosan and the velocity in the swelling process is shown in Fig. 2.

A correlation between the molecular mass of the unmodified polymers and their swelling behavior was not observed. As depicted in Fig. 3, the three control samples

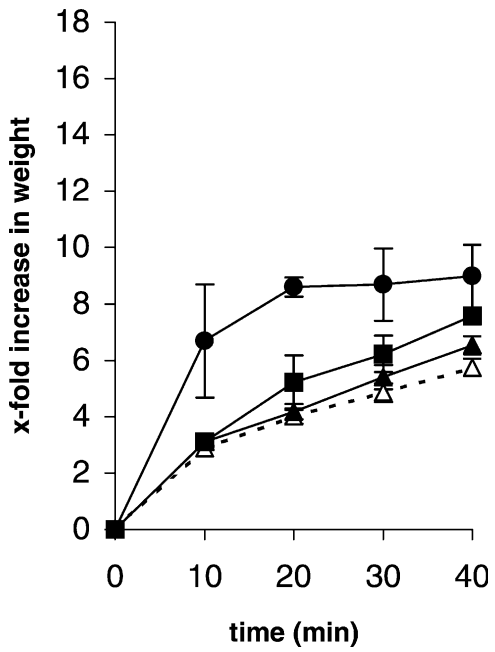

Fig. 2. Swelling behavior of tablets (30 mg) based on medium molecular mass chitosan control (△), medium molecular mass chitosan–TBA 54 (●), medium molecular mass chitosan–TBA 208 (■) and medium molecular mass chitosan–TBA 264 (▲) in 100 mM phosphate buffered saline pH 6.0 at 37 °C; indicated values are means (± S.D.) of at least three experiments.

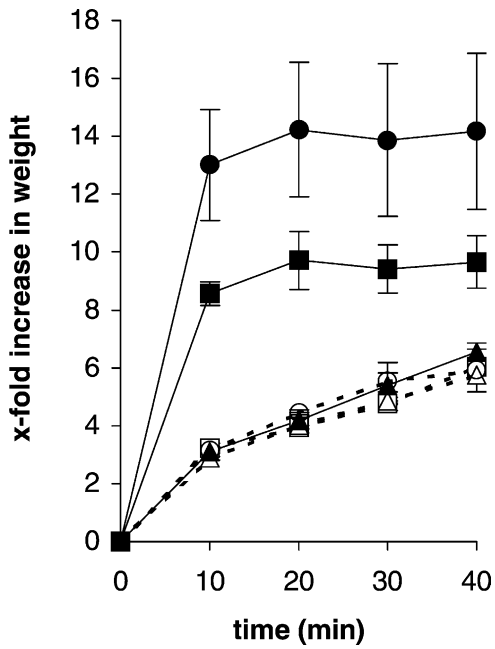

Fig. 3. Swelling behavior of tablets (30 mg) based on low molecular mass chitosan control (□), low molecular mass chitosan–TBA 270 (■), medium molecular mass chitosan control (△), medium molecular mass chitosan–TBA 264 (▲), high molecular mass chitosan control (○) and high molecular mass chitosan–TBA 270 (●) in 100 mM phosphate buffer solution, 0.9% NaCl pH 6.0 at 37 °C; indicated values are means (± S.D.) of at least three experiments.

reached a sixfold increase in their weight irrespective of their molecular mass. On the other hand, the molecular mass seems to play a role in the swelling behavior of the conjugates with the same average amount of immobilized thiol groups: the maximum increase for the medium MM chitosan–TBA 264 was reached after 2 h, whereas conjugates of low and high MM reached a plateau phase in water uptake already after 20 min. The longer the polymer chain length, the higher was the maximum increase in weight (9.66-, 10.68- and 14.17-fold for low, medium and high MM, respectively) for the corresponding tablets during an observation time of 2 h (data not shown).

3.3. Mucoadhesion studies

Tensile studies were performed with chitosan–TBA conjugates to evaluate the influence of both degree of modification and molecular mass of chitosan on the adhesive properties of the polymer. Focusing on the degree of modification, a significant influence of the thiol concentration on the polymer on its mucoadhesive properties could be observed. Results of these studies carried out with the medium MM conjugates bearing increasing amounts of covalently linked thiol groups demonstrated a clear correlation between the amount of immobilized thiol groups and the mucoadhesiveness of the polymer (Fig. 4). The higher the number of thiol groups covalently attached to the polymer backbone was, the higher was the total work of adhesion (TWA) of the corresponding tablets (Fig. 4). The TWA of medium MM chitosan–TBA 54 was increased 1.3-fold compared to unmodified chitosan. A significant improvement of the mucoadhesive properties was obtained with medium MM chitosan–TBA 208 and 264 that showed a 39- and 118-fold increase in the TWA, respectively. The maximum detachment force (MDF) of all tested polymers correlated with the TWA (data not shown). Compared to chitosan–thioglycolic acid derivatives the TWA of this new generation of chitosan conjugates is greatly increased: the previously studied derivatives showed a maximum 10-fold increase in the TWA compared to unmodified chitosan. In contrast, the maximum improvement of the conjugates described here was more than 100-fold. It represents, so far, the greatest improvement in the mucoadhesive properties of a polymer by the covalent attachment of thiol moieties. Chitosan–TBA conjugates could reach this goal because of a synergic contribution of non-covalent and covalent bridges: ionic interactions and disulfide bonds, respectively. The raised cationic character of the polymer very likely leads to the formation of intensified ionic interactions between the polymer and the anionic moieties of mucin. Moreover, the thiol groups covalently linked to the polymer form disulfide bonds with the cysteine-rich subdomains of mucus glycoproteins [7].

Apart from the degree of modification, the molecular mass of chitosan also seems to play an important role in the mucoadhesive properties of the derivatives. Within this study, conjugates of different chain length with a similar degree of modification (on average 256 μM thiol groups per gram polymer) were compared with each other. The conjugate displaying the highest TWA is the medium MM chitosan–TBA 264 (Table 2). This compound had a 12.5-fold improved TWA compared to the low MM chitosan–TBA 270. On the other hand, high MM chitosan–TBA 235 showed only a slight improvement in mucoadhesion.

In order to confirm the results of tensile studies by another mucoadhesion test system, mucoadhesion studies were also carried out with the dissolution apparatus according to the USP in combination with a standard steel cylinder and freshly excised porcine mucosa. The obtained results were in good accordance with the results of tensile studies described above (Table 3). The contact time of

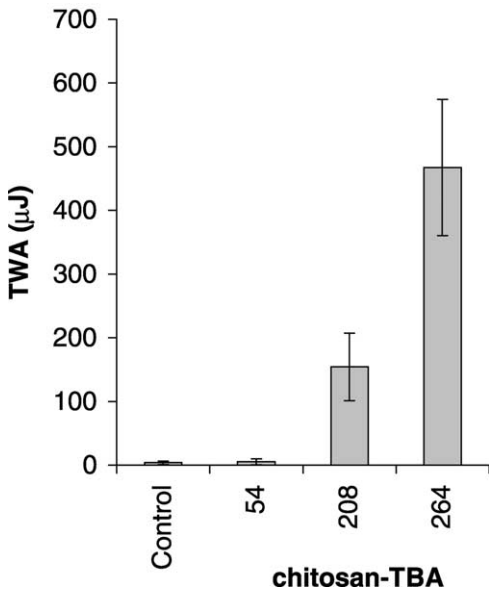

Fig. 4. Influence of the degree of modification on the mucoadhesive properties of chitosan–TBA conjugates. Represented values are means (± S.D.; $n = 3–5$) of the total work of adhesion (TWA) determined in the tensile studies at pH 6.0 with tablets of indicated test material.

Table 2
Influence of the molecular mass on the mucoadhesion properties of chitosan controls and chitosan–TBA conjugates

Polymer	TWA (μJ)	MDF (mN)
Low MM chitosan control	1.5 ± 0.8	0.1 ± 1.3
Low MM chitosan–TBA 270	37.2 ± 13.4	26.6 ± 7.8
Medium MM chitosan control	4.0 ± 2.5	6.0 ± 1.0
Medium MM chitosan–TBA 264	467.7 ± 107.1	255.9 ± 29.3
High MM chitosan control	1.3 ± 0	2.3 ± 1.5
High MM chitosan–TBA 235	45.2 ± 22.1	55.6 ± 30.4

Represented values are means (± S.D.; $n = 3–5$) of the total work of adhesion (TWA) and the maximum detachment force (MDF) determined in the tensile studies at pH 6.0 with tablets of indicated test material.

Table 3
Comparison of the mucoadhesive properties of polymers with and without thiol groups

Polymer	Time (h)	Improvement ratio
Low MM chitosan control	1.3 ± 0	1
Low MM chitosan–TBA 270	152.5 ± 185.8	70.4
Medium MM chitosan control	1.3 ± 0	1
Medium MM chitosan–TBA 54	2.7 ± 5.0	2.1
Medium MM chitosan–TBA 208	6.7 ± 9.9	5.2
Medium MM chitosan–TBA 264	> 360	> 277
High MM chitosan control	1.3 ± 0	1
High MM chitosan–TBA 235	0.08 ± 0.1	0.06

Test discs consisting of indicated polymers were attached to excised porcine mucosa, which has been spanned on a cylinder and agitated at 125 rpm in 100 mM phosphate buffered saline pH 6.0 at 37 ± 0.5 °C. The indicated time of adhesion represents the mean (± S.D.) of at least three experiments. The improvement ratio is calculated by adhesion time of conjugates versus adhesion time of controls.

medium MM chitosan–TBA conjugates on mucosal tissue increased with the increasing amount of thiol groups covalently attached to the polymer from 2.7 h up to more than 360 h. The results of studies comparing conjugates of different chain length with a similar degree of modification (on average 256 μM thiol groups per gram polymer) confirm the results of tensile studies. High molecular mass chitosan–TBA 236 did not show any improvement in mucoadhesive properties compared with unmodified chitosan. The most valuable improvement was reached with medium molecular mass chitosan–TBA 264 that shows an improvement ratio of more than 277. A reason for this observation might be, on the one hand, the insufficient cohesive properties of low molecular mass chitosan leading to a break-up in the adhesive bond within the polymeric network itself rather than between the polymer and the mucus gel layer. On the other hand, if the polymer chains are too long, the extent of interpenetration, which is essential for high mucoadhesive properties [19], is strongly reduced. The results are in good agreement with mucoadhesion studies performed with thiolated poly(acrylic acids) of different molecular mass, where thiolated poly (acrylic acid) of medium molecular mass displayed the highest mucoadhesive properties [20].

Surprisingly, the most adhesive thiolated polymer exhibited the slowest water uptake as shown in Fig. 2. The determined difference between the swelling of medium MM chitosan–TBA 264 and unmodified chitosan was of no statistical significance demonstrating that the swelling behavior is not responsible for the improved mucoadhesive properties of the conjugates. Generally, the higher the water uptake was, the weaker were the mucoadhesive properties of the thiolated polymers.

The efficacy of chitosan–TBA conjugates could meanwhile be demonstrated in various in vivo studies. The oral administration of calcitonin, for instance, led to a significant reduction in the blood calcium level of rats when the peptide drug was embedded in a chitosan–TBA conjugate matrix tablet. In contrast, no effect was seen using unmodified chitosan as drug carrier matrix [21,22].

4. Conclusions

Within this study the influence of the molecular mass and the amount of immobilized thiol groups on the mucoadhesive properties of chitosan–TBA conjugates were investigated. The greater the level of 4-thio-butyl-amidine substructures immobilized on the polymer, the higher were its mucoadhesive properties. In addition, thiolated chitosan of medium molecular mass was identified as comparatively the most mucoadhesive polymer. The evaluation of these parameters led to the optimization of chitosan–TBA conjugates with regard to their mucoadhesive properties. As a result, a polymer thus far exhibiting the so far highest mucoadhesive properties of all chitosan derivatives could be identified.

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