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Thiomers: Preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system

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Abstract

It was the aim of this study to develop a mucoadhesive nanoparticulate delivery system. Nanoparticles were generated by in situ gellation of the thiomer chitosan-4-thiobutylamidine (chitosan-TBA) with tripolyphosphate (TPP) followed by stabilization via the formation of inter- and intrachain disulfide bonds by oxidation with H_2O_2 in various concentrations. Afterwards TPP was removed by exhaustive dialysis at pH 1–2. Incorporation of the model compound fluorescein diacetate (FDA) was achieved by incubation of this fluorescence marker, dissolved in acetonitrile, with aqueous particle suspensions for 1 h at room temperature. Mucoadhesion studies were performed on porcine intestinal mucosa.

Results showed that the preparation method described above leads to nanoparticles of a mean diameter of 268 ± 15 nm and a FDA load of 2%. Due to the removal of the anionic crosslinker TPP, the zeta potential of the nanoparticles was raised from 4 ± 1 up to 19 ± 2 mV without loosing stability of the nanoparticles. The more H_2O_2 was added to the particles, the more inter- and intrachain disulfide bonds were formed. The more thiol groups were oxidized within the particles, however, the lower was the improvement in mucoadhesive properties. Nevertheless, even when 91% of all thiol groups on the nanoparticles were oxidized, their mucoadhesive properties were still twice as high as the mucoadhesive properties of unmodified nanoparticles.

Thiolated chitosan nanoparticles show a two-fold higher zeta potential (I), improved stability (II) and more than doubled mucoadhesive properties (III) than corresponding unmodified chitosan nanoparticles. Therefore, they seem to be advantageous over ionically crosslinked chitosan nanoparticles.

Keywords: Nanoparticles; Thiomers; Mucoadhesion; Crosslinking; Chitosan

1. Introduction

For non-invasive drug administration particulate delivery systems offer the advantage of providing a prolonged residence time on mucosal membranes (Coupe et al., 1991) and the possibility to reach greater mucosal surface areas leading to a comparatively higher drug uptake (Ponchel and Irache, 1998). Although the efficacy of such systems has already been demonstrated in various clinical trials (Kubik et al., 2005), it is believed that the full potential of non-invasive micro- and nanoparticulate delivery systems has so far not been reached. Nanoparticulate delivery systems for the non-invasive administration are based on various polymers, such as polyacrylates (Kriwet et al., 1998), PLGA (Ribeiro et al., 2005) or chitosans (Agnihotri et al., 2004). Among these polymers chitosans offer the advantage of high mucoadhesive properties due to ionic interactions between the positive charged primary amino groups on the polymer and negatively charged sialic acid and sulfonic acid substructures of the mucus (Hassan and Gallo, 1990). These mucoadhesive properties of chitosans were even significantly further improved by the immobilization of thiol groups on the polymer. Roldo et al. (2004), for instance, showed that the mucoadhesive properties of chitosan were 140-fold improved due to the immobilization of thiol groups on the polymer. These strongly improved mucoadhesive properties are based on the formation of disulfide bonds between the thiolated polymer and cysteine-rich subdomains of the mucus gel layer (Leitner et al., 2004). Accordingly, thiolated chitosan nanoparticles should display

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comparatively higher mucoadhesive properties than unmodified chitosan nanoparticles and should therefore be of advantage for the mucosal administration of various drugs.

In order to make use of the high mucoadhesive properties of thiolated chitosans on the one hand and nanoparticulate delivery systems on the other hand, it was the aim of this study to develop a nanoparticulate drug carrier system based on thiolated chitosans. To achieve this goal, thiolated chitosan nanoparticles were prepared via a well-established in situ gellation technique with TPP followed by intra- and intermolecular disulfide bond formation and subsequent removal of the ionic crosslinker. The mucoadhesive properties of thiolated chitosan nanoparticles in comparison to unmodified chitosan nanoparticles were determined within this study. Moreover, the impact of the amount of thiol groups on the mucoadhesive properties of such nanoparticles was evaluated.

2. Materials and methods

2.1. Preparation of low molecular weight chitosan

First, 2 g of chitosan (medium molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland) were dissolved in 100 ml of acetic acid (6%, v/v). To this solution, 80 mg of sodium nitrite dissolved in 10 ml of demineralized water were added and the reaction mixture was incubated for 1 h under continuous stirring. Afterwards, chitosan was precipitated by the addition of 4 M NaOH until pH 9 was reached. The resulting precipitate was filtered and washed with cold acetone. The residue was resolubilised in 15 ml of 0.1 M acetic acid and exhaustively dialyzed against demineralized water. The dialyzed product was concentrated partially under vacuum followed by lyophilisation at −30 °C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA).

2.2. Synthesis of thiolated chitosan

Thiolated chitosan was synthesised according to a method described previously (Roldo et al., 2004). In brief, 500 mg of low molecular weight chitosan, prepared as described above, were dissolved in 50 ml acetic acid (1%, v/v). After adjusting the pH to 6.5 with 1 M NaOH, 200 mg of 2-iminothiolane HCl (Traut's reagent; Pierce, Oud Beijerland, NL, USA) were added. The reaction mixture was incubated for 12 h at room temperature under stirring. The resulting polymer conjugate was dialyzed in five steps, which lasted 24 h each. First, the polymer was dialyzed against 5 mM HCl, followed by two dialyze steps against 5 mM HCl containing 1% NaCl. Subsequent the polymer was dialyzed against 5 mM HCl and finally against 0.4 mM HCl. Thereafter, the polymer was freeze-dried at −30 °C and 0.01 mbar (Benchtop 2K, VirTis) and stored at 4 °C until further use.

2.3. Determination of thiol/disulfide groups

The amount of thiol groups on thiolated chitosan was evaluated via iodometric titration as described previously (Kast and Bernkop-Schnürch, 2001). Disulfide content was measured after reduction with $NaBH_4$ and iodometric titration.

2.4. Preparation of nanoparticles

Nanoparticles were prepared via in situ gellation of (thiolated) chitosan with TPP in aqueous solution according to a method described previously (Greimel, 2005). In brief, thiolated and unmodified low molecular weight chitosan were dissolved in 0.05% (w/v) acetic acid solution at a final concentration of 0.25% (w/v) and the pH was adjusted to 5.5 by the addition of a 0.5% (w/v) NaOH solution. Subsequently, a 0.2% (w/v) TPP solution in demineralized water was added to the low molecular weight chitosan solution in volume ratios of 3:1, 4:1, 5:1 and 6:1 leading to the spontaneous formation of nanoparticles.

In the following step, 2 ml of the particle suspension were partially oxidized via the addition of H_2O_2 in final concentrations, as listed in Table 1. During the oxidation process, which lasted 24 h, the polymer solution was stirred continuously at room temperature. Thereafter, TPP and H_2O_2 were removed by exhaustive dialysis against 0.1 M HCl for 5 days. Optionally the model compound fluorescein diacetate (FDA) was incorporated in obtained particles as following. First, 1.0 ml of dialyzed nanoparticle suspensions prepared as described above was transferred to 1.0 ml of a 0.1% (w/v) FDA solution in acetonitrile. The suspension was then incubated on a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 25 °C for 1 h. In order to avoid aggregation of particles during the following centrifugation for 10 min at 13,400 rpm, trehalose was added to the suspension in a final concentration of 5% (w/v). After centrifugation, the supernatant fluid was removed and particles were resuspended in 2 ml of 0.05% (v/v) acetic acid solution. For determining the FDA load particles were resuspended in 5 M

Table 1
Amount of thiol groups and disulfide bonds immobilized on the basic thiomer chitosan-4-thiobutylamidine (chitosan-TBA) and nanoparticles after ionic gelation with tripolyphosphate (TPP) and different oxidation with H_2O_2, respectively

	H_2O_2 [mmol]	–SH [μmol/g]	–S–S–[μmol/g]	Σ–SH [μmol/g]
Chitosan-TBA	–	208	83	374 ± 12
Chitosan-TBA (+TPP)	–	136	117	369 ± 9
Chitosan-TBA (ox1)	2.65	116	128	372 ± 17
Chitosan-TBA (ox2)	5.29	80	148	375 ± 21
Chitosan-TBA (ox3)	10.59	33	169	371 ± 13

NaOH and incubated for 20 min at 37 °C while shaking in order to quantitatively hydrolyse FDA to the fluorescent sodium fluorescein (Albrecht et al., in press), which was quantified with a microplate reader (Fluostar Galaxy, BMG, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

2.5. Particle characterisation

The amount of thiol groups on particles was determined via iodometic titration (1 mM iodine; indicator: starch) at pH 1–2. Remaining traces of TPP were determined spectrophotometrically (DU Series 600 spectrophotometer, Beckman Instruments, Fullerton, USA) after the addition of ammonium molybdate in sulfate solution and subsequent reduction with ascorbic acid by measuring the absorbance of the resulting molybdene blue at 830 nm (Kakac and Vejdelek, 1977). Size distribution and zeta potential of particles were determined with a particle sizer (Zeta Potential/Particle Sizer, Nicomp^{TM} 380 ZLS, Tokyo, Japan). Shape of particles was monitored with an in-column energy filter transmission electronic microscope (ZEISS 902, Zeiss AG, Oberkochen, Germany). Particles were photographed using global imaging and inelastic imaging with a selected energy loss of 50 eV.

2.6. Mucoadhesion studies

Mucoadhesion studies of thiolated and unmodified polymer nanoparticles were performed on porcine intestinal mucosa using the experimental set-up established by Rango Rao and Buri (1989). An analytical method to quantify the amount of remaining marker being embedded in the polymer particles was developed by our research group (Albrecht et al., in press).

In brief, freshly excised porcine intestinal mucosa was mounted on a half pipe and placed in an angle of 45° in an incubation chamber providing 100% humidity and a temperature of 37 °C. The mucosa was then continuously rinsed with 100 mM phosphate buffer pH 6.5, which served as artificial intestine fluid. To humidify the mucosa, an equilibration period of 5 min was maintained before administering the particles. During the whole experiment, the temperature of the phosphate buffer was kept at 37 °C. A constant flow rate of 1 ml/min was provided by utilizing a peristaltic pump. After the equilibration period, 15 mg of lyophilised particles were transferred on the mucosa in dry form and continuously rinsed with the phosphate buffer pH 6.5. After 1, 2 or 3 h, the mucosa with the remaining marker on it was incubated in 25 ml of 5 M NaOH for 20 min at 37 °C under shaking in order to quantitatively hydrolyse FDA to sodium fluorescein (NaFlu). After centrifugation (13,400 rpm; 5 min) fluorescence of each sample was measured as described above.

2.7. Statistical data analyses

Statistical data analyses were performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat Version 5.0 (b8.3).

3. Results and discussion

3.1. Preparation and characterisation of thiolated low molecular mass chitosan

Low molecular mass chitosan exhibiting a mean molecular mass of 10 kDa has been prepared according to a method described previously (Vauthier, 2004). On this low molecular mass chitosan, thiol groups in form of 4-thiobutylamidine substructures were immobilized. Orientating toxicity studies performed with such chitosan-4-thiobutylamidine conjugates demonstrated no significant alterations in the toxicological profile of chitosan due to the immobilization of thiol groups (Guggi et al., 2004). Analyses of thiol groups showed that in total 374 ± 12 μmol thiol groups were immobilized per gram chitosan. During the preparation process, 44% of all sulfhydryl groups were already oxidized. Previous studies, however, demonstrated that thiol groups being covalently attached to chitosan are stable towards oxidation in dry form (Bernkop-Schnürch et al., 2002) and in aqueous solutions below pH 5 (Bernkop-Schnürch et al., 2000).

3.2. Particle preparation

Within this study, thiolated chitosan nanoparticles were prepared via gelation with TPP followed by the formation of intra- and intermolecular disulfide bonds within the particles and the removal of the ionic crosslinker TPP. The ratio of polymer to TPP had a great impact on the formation of nanoparticles. In case of unmodified chitosan, it turned out that the higher the share of chitosan is the larger are the resulting nanoparticles. In case of thiolated chitosan, it was shown that by mixing 0.25% chitosan-TBA solution with 0.2% TPP solution at ratios of 3:1 and 4:1, nanoparticles were formed spontaneously. Results of these studies are shown in Fig. 1. Formation of disulfide bonds was achieved by oxidation due to the addition of increasing amounts of H_2O_2. Results demonstrated that the formation of disulfide bonds could be well controlled in this way. The more H_2O_2 was added, the more disulfide bonds were formed within the particles. Results of this study are shown in Table 1. Generally, the size of ionically crosslinked particles was comparatively smaller than that of covalently crosslinked particles, which is illustrated in Fig. 2. Lamprecht et al. (2001) investigated the mucoadhesive properties of fluorescent particles with a size of 100 nm, 1 μm and 10 μm in the gastrointestinal tract of rats. Results of this study showed the highest mucoadhesive properties for 100 nm particles. According to these results, chitosan-TBA nanoparticles should be advantageous in terms of a prolonged gastrointestinal residence time, as they have been determined to be of a mean diameter between 150 and 270 nm, as listed in Table 2. Because of the covalent crosslinking, particles became stable at low pH levels. Ionically crosslinked particles disintegrated in aqueous solution at pH 2 rapidly, whereas the same particles remained stable when they were covalently crosslinked via disulfide bonds. In contrast to ionically crosslinked chitosan nanoparticles, covalently crosslinked particles will not disintegrate in the acidic milieu of the stomach because of their

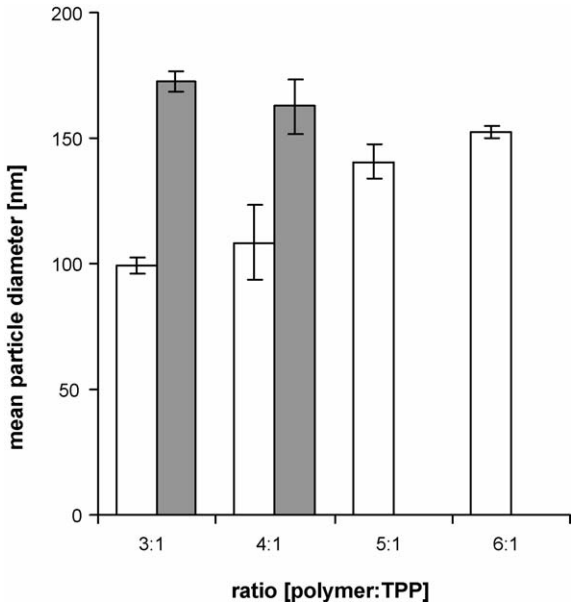

Fig. 1. Influence of the ratio of 0.25% (w/v) chitosan-TBA solution to 0.2% (w/v) TPP solution on the generation of nanoparticles: mean diameter of particles based on either chitosan (white bars) or chitosan-TBA (grey bars) prepared by ionic gelation. Indicated values are means of at least three experiments (±S.D.).

improved stability. Because of this strongly improved stability of thiolated chitosan nanoparticles via covalent crosslinking, the ionic crosslinker TPP was not necessary anymore. Due to the removal of the ionic crosslinker, the zeta potential increased significantly. As shown in Table 2, a more than four-fold increase in the zeta potential was achieved when TPP was removed from the particles. Quantification of the remaining amount of TPP

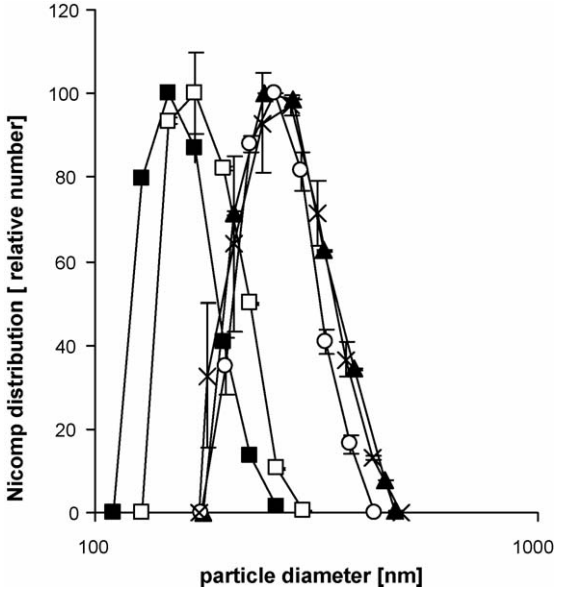

Fig. 2. Size distribution of ionically crosslinked nanoparticles obtained by ionic gelation with TPP based on chitosan (0.25% chitosan to 0.2% TPP ratio 6:1, ■) and chitosan-TBA nanoparticles (0.25% chitosan-TBA to 0.2% TPP ratio 3:1, □), as well as covalently crosslinked nanoparticles based merely on thiolated chitosan obtained by oxidation with H_2O_2 (ox1, ▲; ox2, ◯; ox3, ×). Indicated values are the means of at least three experiments (±S.D.).

Table 2
Mean particle diameter and zeta potential of chitosan-4-thiobutylamidine (chitosan-TBA) nanoparticles obtained by ionic gelation with TPP and followed by different oxidation with H_2O_2, respectively

Nanoparticles	Mean particle diameter [nm]	Zeta potential [mV]
Ionically crosslinked		
Chitosan (6:1)	152.5 ± 2.6	9.9 ± 0.6
Chitosan-TBA (3:1)	172.7 ± 3.8	4.3 ± 1.2
Covalently crosslinked		
Chitosan-TBA (ox1)	261.6 ± 0.1	20.3 ± 4.1
Chitosan-TBA (ox2)	259.0 ± 2.5	19.3 ± 2.8
Chitosan-TBA (ox3)	267.7 ± 15.3	18.7 ± 1.8

Indicated values are the means of at least three experiments (±S.D.).

after dialysis showed that less than 1% of the ionic crosslinker remained within the particles. As the permeation enhancing properties of chitosan are likely induced by its positive charges (Thanou et al., 2001), relative more positively charged nanoparticles should exhibit higher permeation enhancing properties. Moreover, due to the immobilization of thiol groups the permeation enhancing properties of chitosans are significantly further improved (Bernkop-Schnürch et al., 2004).

Electronic microscopic investigations revealed that all particles were of spheric shape and had a smooth surface. Results are shown in Fig. 3.

Within this study, particles were loaded with a marker compound instead of a therapeutic agent for analytical reasons. The incorporation of the marker was achieved via a simple diffusion process making use of the solubility of FDA in organic solvents and its insolubility in aqueous solutions. Once FDA has been embedded in the nanoparticles the organic solvent guaranteeing solubility of the marker was removed leading to a temporary fixation of this compound within the particles in aqueous solutions. Utilizing this technique, a FDA load of 2% was achieved. Raising the concentration of FDA during the incorporation process should lead to even higher amounts of FDA within these particles. This technique seems therefore appropriate for drugs being poorly soluble in aqueous solutions, such as class II and IV drugs, according to the biopharmaceutical classification scheme. Such drugs can be incorporated in thiolated chitosan nanoparticles utilizing an organic solvent. After removal of this solvent, a sustained drug release from these nanoparticles can be achieved in a solubility-controlled manner. The incorporation of solubility enhancing auxiliary agents in these particles might allow adjustment to demanded release profiles.

3.3. Mucoadhesion studies

Mucoadhesion studies revealed that thiolated chitosan nanoparticles display significantly higher mucoadhesive properties than the corresponding unmodified chitosan nanoparticles. In Fig. 4, a comparison in the residence time of FDA as such, FDA being incorporated in chitosan nanoparticles and in thiolated chitosan nanoparticles on the small intestinal mucosa is provided. Due to the incorporation in chitosan nanoparticles and in thiolated chitosan nanoparticles the residence time of FDA on

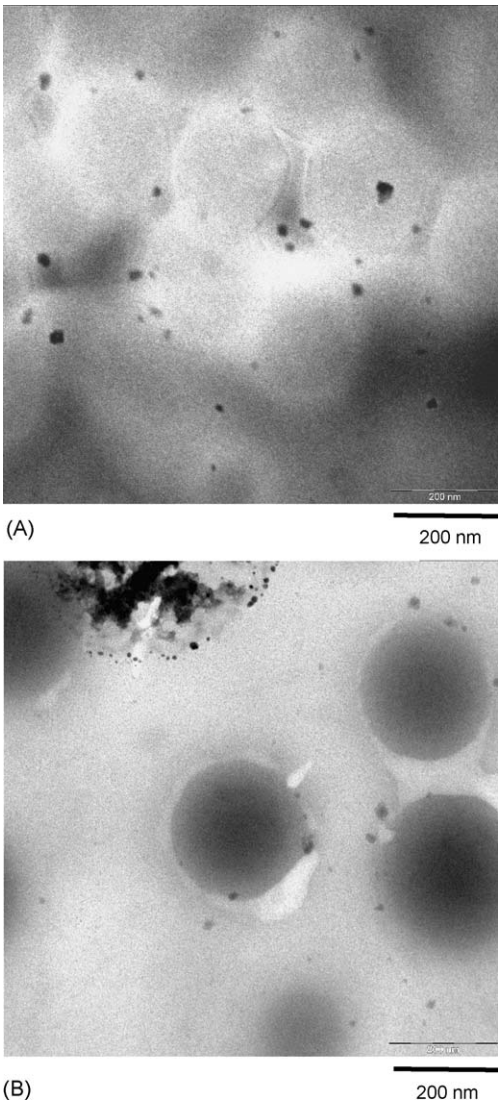

Fig. 3. TEM micrographs of covalently crosslinked nanoparticles (ox1) based on chitosan-TBA. Particles were obtained by ionic gelation with TPP and subsequent oxidation by the addition of 2.65 mM H_2O_2. (A) Inelastic imaging with a selected energy loss of 50 eV and (B) global imaging. Bar represents 200 nm.

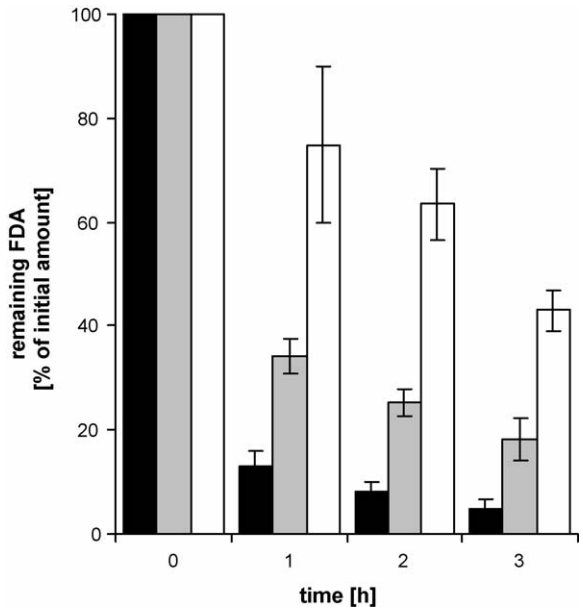

Fig. 4. Amount of FDA remaining on excised porcine small intestinal mucosa. FDA was applied without any excipients (black bars) or incorporated in chitosan nanoparticles (grey bars) or chitosan-TBA nanoparticles (blank bars). Indicated values are the means of at least three experiments (±S.D.).

the mucosa was three-fold and even nine-fold improved, respectively. The more free thiol groups were available for the formation of disulfide bonds with cysteine-rich subdomains of mucus glycoproteins, the higher were the mucoadhesive properties of particles, which is shown in Table 3. Results provide evidence for improved mucoadhesive properties due to the immobilization of thiol groups on nanoparticles. Apart from improved mucoadhesive properties mediated via thiol groups, the raised zeta potential will also contribute to the comparatively higher mucoadhesion. As the mucoadhesive properties of chitosan are likely based on ionic interactions of the positively charged polymer with negatively charged moieties within the mucus, such as sulfonic and sialic acid substructures, more positively charged particles will consequently be relatively more mucoadhesive. So far, however, the full mucoadhesive potential of chitosan nanoparticles could not be reached, as the stability of chitosan particles is in most cases provided by the addition of polyanionic excipients, such as tripolyphosphate (Calvo et al., 1997), sulfate (van der Lubben et al., 2001) or hyaluronic acid (Lim et al., 2001) leading to an ionic crosslinking of chitosan. Due to the addition of polyanions, the positive charges of chitosan, however, are neutralized resulting in a loss of the mucoadhesive properties (Luessen et al., 1996).

Results revealed that due to the immobilization of thiol groups on chitosan nanoparticles, their mucoadhesive properties can be doubled. In comparison to all other in vitro test systems, the improvement in mucoadhesion due to the immobilization of thiol groups on chitosan was less pronounced. The same thiomer as used here showed in tensile studies and on the rotating cylinder 117-fold and 140-fold higher mucoadhesive properties than unmodified chitosan (Roldo et al., 2004). A reason for this observation might be seen in the comparatively much lower molecular mass of chitosan-TBA used here. In tensile studies and on the rotating cylinder chitosan with a molecular mass of 400 kDa was used, whereas in this study chitosan-TBA of an average molecular mass of 10 kDa was utilized. Tobyn et al. (1996)

Table 3
Comparison of the amount of remaining fluorescence marker being incorporated in thiolated chitosan nanoparticles with different amounts of free thiol groups, on excised porcine small intestinal mucosa after 3 h of continuous rinsing

Nanoparticles	Remaining marker [%]	Improvement ratio
Chitosan	18.10 ± 4.13	1
Chitosan-TBA (ox1, 116 μmol/g −SH)	43.03 ± 3.88	2.38
Chitosan-TBA (ox2, 80 μmol/g −SH)	38.93 ± 0.50	2.15
Chitosan-TBA (ox3, 33 μmol/g −SH)	36.73 ± 1.59	2.03

Improvement ratio = remaining marker within chitosan-4-thiobutylamidine (chitosan-TBA) particles/remaining marker within chitosan particles. Indicated values are the means of at least three experiments (±S.D.).

showed that the smaller the chain length of a mucoadhesive polymer is the lower are its mucoadhesive properties. Therefore, the comparatively lower improvement in mucoadhesion might be explained by the lower molecular mass of the thiomer used within this study. But using thiolated chitosan of comparatively higher molecular mass led to much greater particles in the range of above 1000 μm (data not shown). A further improvement in the mucoadhesive properties of nanoparticles seems therefore to be more likely feasible via a further increase in the content of thiol groups on the particles.

4. Conclusions

Within this study, thiolated chitosan nanoparticles being covalently crosslinked via disulfide bonds were prepared. Due to this covalent crosslinking, particles were more stable than the corresponding ionically crosslinked particles. Because of the presence of thiol groups on the nanoparticles their mucoadhesive properties were more than two-fold improved. In addition, drugs can be easily incorporated in such particles. According to these properties, nanoparticles described here might be promising delivery systems for therapeutic agents being administered via mucosal routes of application.

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