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Synthesis and characterization of a chitosan-*N*-acetyl cysteine conjugate

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Abstract

The aim of the present study was to synthesize and characterize a novel thiolated polymer by covalent attachment of *N*-acetyl cysteine to chitosan.

The obtained conjugate was characterized *in vitro* by quantification of immobilized thiol groups and their pH dependent oxidation, swelling behaviour in artificial intestinal fluid at pH 6.8, rheological properties and evaluation of its mucoadhesive properties on freshly excised porcine mucosa.

The chitosan-*N*-acetyl cysteine conjugate was synthesized via a carbodiimide mediated coupling reaction displaying up to 325.5 ± 41.8 μmol of immobilized thiol groups per gram polymer. 79% of the total amount of thiol groups was oxidized to disulfide groups during the coupling reaction. Adhesion studies on the mucosa indicate that the resulting polymer shows a 50-fold longer residence time on the mucosa and 8.3-fold higher total work of adhesion necessary to detach a flat-faced polymeric tablet from the mucosa in comparison to unmodified chitosan. Swelling properties at pH 6.8 were rather limited displaying only 5% of increment in weight after 2 h of experiment. Within 1 h the viscosity of an aqueous chitosan-*N*-acetyl cysteine conjugate mixture at 37 °C, pH 5.0 decreased by 35% after addition of hen white egg lysozyme demonstrating its biodegradability.

Because of these features chitosan-*N*-acetyl cysteine seems to represent a promising novel tool, which might be useful in particular for the development of mucoadhesive and biodegradable formulations.

Keywords: Chitosan-*N*-acetyl cysteine; Thiomer; Mucoadhesion; Cohesive properties; Biodegradability; Rheology

1. Introduction

Mucoadhesion and the potential of polymers to express mucoadhesive properties has been focus of intense research. Mucoadhesive polymers are in many cases advantageous drug delivery systems since they have proven the ability to prolong the residence time of drugs on various mucosa and consequently to enhance the absorption rate of incorporated drugs (Dodou et al., 2005). Within the last decade, the concept of mucoadhesive polymeric excipients has gained a new dimension by introducing thiolated polymers at the pharmaceutical arena (Bernkop-Schnürch, 2005). The mechanism of improved mucoadhesion of thiomers is based on the formation of disulfide bonds between thiol bearing side chains of the polymer and cysteine-rich subdomains of mucus glycoproteins (Leitner et al., 2003). This new concept has already been verified for various thiolated anionic polymers and chitosan derivates as exclusive representatives for cationic polymers. The positive charges are responsible for ionic interactions with anionic substructures such as sialic and sulfonic acid of the mucus layer providing its mucoadhesiveness (Lehr et al., 1992). Since chitosan is a polysaccharide consisting of glucosamine and *N*-acetylglucosamine subunits its modification is based on the immobilization of thiol groups on the primary amino groups at the 2-position of the glucosamine subunits. As such the ligands immobilized on the surface of the hydrophilic polymers do not only introduce a thiol substructure, but they also form a hydrophobic component of the carrier matrix (Bernkop-Schnürch et al., 2003a,b). Recently it was shown that the mucoadhesive properties of chitosan are strongly improved by the covalent attachment of thioglycolic acid (Kast

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et al., 2003), 2-iminothiolane (Roldo et al., 2003), thioethylamidine (Kafedjiiski et al., 2006) or glutathione (Kafedjiiski et al., 2005). For example, a covalent attachment of thioglycolic acid resulted in conjugates exhibiting up to 10 times and that of 2-iminothiolane up to 140 times improved mucoadhesion. This study is focused on the modification of chitosan by *N*-acetylcysteine as thiol bearing ligand in order to improve the mucoadhesive properties of unmodified chitosan due to the immobilization of thiol groups on the polymeric backbone. *N*-acetyl cysteine reacts with chitosan as shown in Fig. 1, resulting in an uncharged amide bond linkage.

2. Materials and methods

2.1. Materials

Chitosan (medium molecular mass: 400 kDa; degree of deacetylation: 83–85%) and *N*-acetyl-L-cysteine were purchased from Fluka Chemie (Buchs, Switzerland). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) 5, 5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) and hen egg white lysozyme were purchased from Sigma (Austria). All chemicals were of analytical grade.

2.2. Methods

2.2.1. Synthesis and characterization of chitosan-N-acetyl cysteine conjugates

As shown in Fig. 1, the covalent attachment of *N*-acetyl cysteine to chitosan was achieved by the formation of amide bonds between the primary amino group of the chitosan and a carboxylic acid group of the sulfhydryl compound. Five hundred milligrams of medium molecular weight were dissolved in 1% aqueous hydrochloric acid and adjusted to pH 5 with 1 M NaOH. Additionally 4 g of *N*-acetyl cysteine were dissolved in 50 ml demineralized water. The carboxylic acid moieties of the *N*-acetyl cysteine were activated for 20 min by the addition of EDAC (see Table 1). The pH was adjusted within the range of 4–5 and was maintained during the whole experiment. Finally, reaction mixtures were united and incubated for 6 h under permanent stirring at room temperature. The resulting chitosan-*N*-acetyl cysteine conjugate was isolated in the dark by dialyzing at 10 °C as previously described (Clausen and Bernkop-Schnürch, 2000). All samples were frozen and lyophilized −78 °C and 0.08 bar (Virtis Benchtop Freeze Dryer, Bartelt, Graz, Austria). Table 1 outlines the composition of all synthesized conjugates.

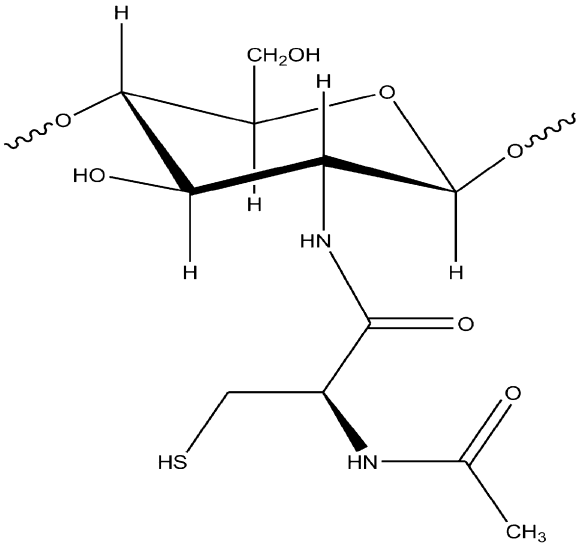

Fig. 1. Schematic diagram of the substructure of chitosan-*N*-acetyl cysteine.

The degree of modification, i.e. the amount of thiol groups immobilized on the chitosan-*N*-acetyl cysteine conjugate, was determined photometrically with Ellman's reagent quantifying free thiol groups. First, 0.5 mg each of the conjugate and control was hydrated in 500 μl of 0.5 M phosphate buffer pH 8.0 and then 500 μl of Ellman's reagent (3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 10 ml of 0.5 M phosphate buffer pH 8.0) were added. The samples were incubated for 2 h at room temperature. Thereafter, 300 μl of each sample was transferred into a microplate and the absorbency was measured at a wavelength of 450 nm with a microplate reader (Fluostar Galaxy; BMG-Labtech, Offenburg, Germany). Cysteine standards were used to calculate the amount of thiol groups immobilized on the polymer.

The amount of disulfide bonds within the polymer was tested according to the following test. First, 0.5 mg of the conjugate was hydrated in 1 ml of 50 mM phosphate buffer pH 8.0 for 30 min. A 3% sodium-borohydride solution was freshly prepared, 600 μl were added to the polymer solution, and the mixture was incubated for 2 h in an oscillating waterbath at 37 ± 0.5 °C. Thereafter, 500 μl of 1 M HCl were added in order to destroy the remaining sodium-borohydride. After the addition of acetone (100 μl) the mixture was agitated for 5 min. Then, 1 ml of 1 M phosphate buffer pH 8.5 and 200 μl of a 0.5% (m/v) DTNB dissolved in 0.5 M phosphate buffer pH 8.0 were added. After incubation for 15 min at room temperature aliquots of 200 μl were transferred to a 96-well microtitration plate and the free sulfhydryl groups were determined as described above. The amount of disulfide bonds was calculated by subtracting the quantity of free thiol groups as determined by the method described above from the totality of thiol moieties present on the polymer. Chitosan-*N*-acetyl cysteine conjugates were hydrated in 0.1 M acetate buffer pH 5.0 and in 0.1 M phosphate buffer pH 6.0 in a final concentration of 0.5% (w/v). Samples were incubated at 37 °C under continuous shaking. At predetermined time points, aliquots of 50 μl were withdrawn and frozen at −20 °C in order stop any further oxidation. The amount of remaining thiol groups was determined via Ellman's reagent as described above.

2.2.2. Evaluation of the swelling behaviour

The water-absorbing capacity was determined by a gravimetric method. Thirty milligrams of each of the thiolated chitosan and of unmodified chitosan were compressed to 5.0 mm diameter flat-faced tablets. The compaction force of 4 kN was kept constant during the preparation of all tablets. Test tablets were fixed to a needle and placed in a beaker containing 100 mM phosphate-buffered saline pH 6.8 at 37 ± 0.5 °C. At scheduled

Table 1
Characterization of the thiolated chitosan

Conjugate	Aqueous polymer solution (g/80 ml)	EDAC (final concentration mM)	*N*-acetyl cysteine (g)	Free thiol groups (μmol/g polymer) mean ± S.D.; $n=4$	Total thiol groups (μmol/g polymer) mean ± S.D.; $n=4$
Chitosan-NAC I	0.5	50	4	62.4 ± 1.2	298.4 ± 34.1
Chitosan-NAC II	0.5	150	4	325.5 ± 41.8	571.4 ± 48.6
Control	0.5	–	–	–	–

time intervals the swollen test tablets were taken out of the incubation medium, excess water was removed and the water uptake was determined gravimetrically.

2.2.3. Degradation of chitosan and chitosan-N-acetyl cysteine conjugates by lysozyme

First, 150 mg of chitosan was hydrated in 2.4 ml of 1 M HCl and 7.6 ml of demineralized water was added in order to obtain a 1.5% (m/v) solution of chitosan. In addition, a 1.5% (m/v) solution of the lyophilized chitosan-*N*-acetyl cysteine conjugate was prepared. Both polymer solutions were adjusted with 0.1 M acetate buffer to pH 5.0. Thereafter, hen egg white lysozyme was added in a final concentration of 1.5 mg/ml to each sample. At predetermined time points the viscosity of 1 ml aliquots of the mixtures was measured at 25 ± 0.5 °C with a cone-plate viscometer (Rheolab MC1, Paar Physica GmbH, Germany) connected to a personal computer for setting the analysis parameters and for processing and recording the data with the *Rheo Win Pro 2.64* program. Polymer solutions (1.5%) without lysozyme were evaluated under the same conditions and served as controls. The change of viscosity at time t was normalized by the viscosity at time zero (values before the addition of enzyme solution). The percent viscosity loss was calculated from the following equation:

$$\text{Percent}\,\eta_1 = \left(\frac{\eta_0 - \eta_t}{\eta_0}\right) \times 100$$

where percent η_1: percent viscosity lost after time t; η_t: final viscosity at time t and η_0: initial viscosity of solution.

2.2.4. In vitro evaluation of mucoadhesive properties

2.2.4.1. Mucoadhesion studies via rotating cylinder. Time of adhesion of compressed discs to the porcine small intestinal mucosa was determined by the rotating cylinder as follows. Discs were attached to freshly excised porcine intestinal mucosa (approximately 12 cm^2), prior spanned onto a stainless steel cylinder (diameter 4.4 cm, height 5.1 cm; apparatus 4-cylinder, Ph. Eur. 4.0). Thereafter the cylinder was placed into the dissolution apparatus (Ph. Eur. 4.0) and completely immersed into the 0.1 M phosphate-buffered saline of pH 6.8 at 37 ± 0.2 °C. The cylinder was rotated at speed of 125 rpm. Every 30 min the changes in the test system were determined visually and registered until all of the discs were detached from the mucosa.

2.2.4.2. Tensile studies. Tensile studies were carried out on native porcine intestinal mucosa. First, 30 mg of polymer were compressed into 5.0 mm diameter flat-faced discs. The compaction force of 4 kN was kept constant during the preparation of all discs. Each polymer disc was carefully glued with cyanacrylate glue (Loctite®) to a stainless steel flat disc attached to a 15 cm nylon thread. The other end of the nylon string was fixed to a laboratory stand. Freshly excised porcine small intestinal mucosa was cut in small pieces with an area of approximately 9 cm^2. The mucosa was glued to a glass tissue mount which was set in a beaker and completely immersed into 0.1 M phosphate-buffered saline pH 6.8. Thereafter, the beaker was placed on a balance and carefully raised by a mobile platform until the mucosa came in contact with the disc. The mucosa was allowed to hydrate in buffer for 30 min at 25 °C. After incubation the platform was lowered at the rate of 0.1 mm/s. Data points were registered every second by a personal computer connected to the balance (Sartorius BL 1500S with integrated interface), using 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, was calculated using EXCEL (Microsoft).

2.3. Statistical data analysis

Statistical data analysis was performed using the student t-test with $p < 0.05$ as the minimal level of significance. Calculations were performed using software Origin version 7.0.

3. Results

3.1. Basic characterization of chitosan-N-acetyl cysteine conjugate

The chitosan-*N*-acetyl cysteine conjugate has been synthesized following the preparation procedures as applied for thiolated polyacrylates (Bernkop-Schnürch et al., 2003a,b). Fig. 1 shows the schematic diagram of a thiolated substructure of the newly formed conjugate. Two batches of the conjugate, differing in the amount of immobilized free thiol groups, were prepared according to Table 1. The chitosan-*N*-acetyl cysteine conjugate I (chitosan-NAC I) showed 62.4 ± 1.2 μmol thiol groups per gram polymer. 43.1% of the total amount of thiol groups was oxidized to disulfide groups during the coupling reaction, whereas chitosan-*N*-acetyl cysteine conjugate II (chitosan-NAC II) showed 325.5 ± 41.8 μmol thiol groups per gram polymer. For conjugate II, 79.1% of the total amount of thiol groups was oxidized to disulfide groups. According to these results 1.2 and 6.3% of all primary amino groups on chitosan were derivatized with *N*-acetyl cysteine in case of conjugate I and II, respectively. A control being prepared in the same way as the

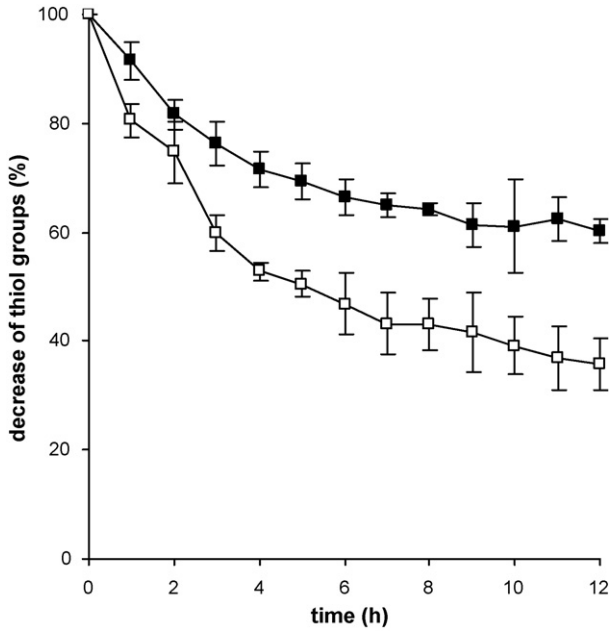

Fig. 2. Oxidation of thiol groups immobilized on the chitosan-*N*-acetyl cysteine conjugate. The decreasing amount of thiol groups is expressed in percent over a time period of 12 h. Data are means of at least three experiments ± standard deviation. (■) Decrease of thiol groups at pH 5, (□) decrease of thiol groups at pH 6.

chitosan-*N*-acetyl cysteine conjugate but omitting the carbodiimide during the coupling reaction displayed only a negligible amount of remaining traces of cysteine. After lyophilization, the conjugate appeared as white odourless cotton-like matrix.

Stability studies of the conjugate in aqueous solutions at pH 5 and 6 demonstrated that at both pHs thiol groups were subject of an oxidation process leading to a rapid pH dependant formation of disulfide bonds. The results of this study are shown in Fig. 2. The chitosan-*N*-acetyl cysteine conjugate is comparatively more stable at pH 5 where around 40% of the amount of thiol groups was decreased in comparison to a rapid decrease of nearly 65% of all thiol groups at pH 6. These findings are in good agreement with the theory that less reactive thiol groups are to a lower degree subject of an oxidation process.

3.1.1. Swelling behaviour and cohesive properties

The mucoadhesive and cohesive properties of polymers are strongly influenced by their swelling behaviour. On the one hand, a rapid swelling of mucoadhesive polymers favours the interdiffusion process between the polymer and the mucus layer, providing stronger adhesion than in the case of poorly swelling polymers (Smart, 2005; Lehr, 1996). On the other hand a rapid swelling behaviour is in many cases responsible for a too rapid drug release and limited cohesive properties causing a failure in the mucoadhesive bond rather within the polymeric network itself than between the polymer and the mucus gel layer (Bernkop-Schnürch, 2000). Water uptake studies as shown in Fig. 3, demonstrated that the covalent attachment of *N*-acetyl cysteine to chitosan had no significant influence on the swelling behaviour of the polymer according to student *t*-test with $p < 0.05$. Although less significant, the modified chitosan showed lower water uptake beyond 50 min, which might be explained by the lower number of primary amino groups on the thiolated polymer in comparison to unmodified chitosan. Control and test tablets showed no disintegration or erosion within 2 h. An almost linear water uptake during the first 50 min was observed for both formulations, before reaching a steady state.

3.2. Rheological studies

The viscosity measurements performed at room temperature, comparing chitosan and chitosan-*N*-acetyl cysteine conjugate showed that upon a testing period of 1 h, both polymers barely changed in viscosity. Results of the study are shown in Fig. 4.

However, by incubating control and test polymers with hen white egg lysozyme revealed differing degradation profiles for both formulations. The viscosity of chitosan decreased dramatically within 1 h, to reach a minimum of only 40% in comparison to the starting point. The curve describing the degradation of chitosan-*N*-acetyl cysteine conjugate showed a less pronounced decrease in viscosity. However, even the thiolated polymer was degraded by 35% after 1 h.

3.3. Mucoadhesive properties

Results of mucoadhesion studies performed according to the rotating cylinder method, which is supposed to be closer to the *in vivo* situation than simple tensile studies, were in good agreement with the total work of adhesion of both polymers determined via tensile studies. The results are shown in Fig. 5. In case of tablets being based on the chitosan-*N*-acetyl cysteine

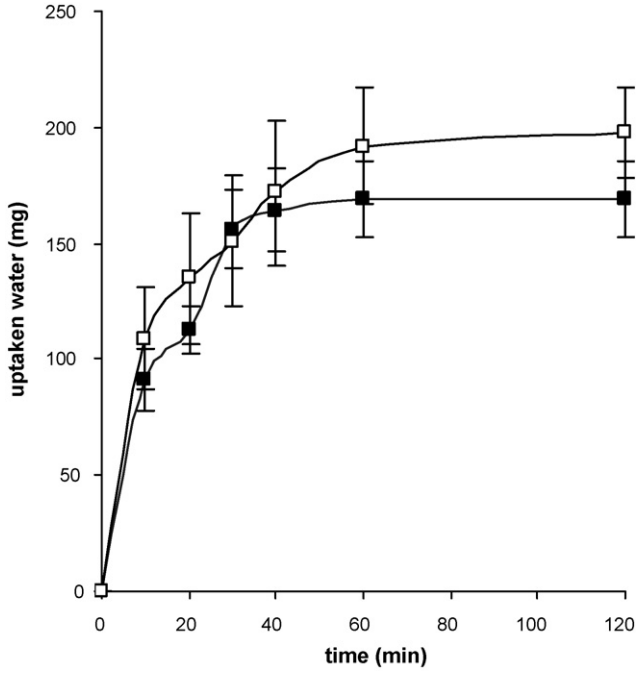

Fig. 3. Evaluation of the swelling behaviour of chitosan-*N*-acetyl cysteine conjugate II in comparison to unmodified chitosan. The water uptake is expressed in mg over a time period of 2 h. Data are means of $n = 6 \pm$ standard deviation. (■) Chitosan-*N*-acetyl cysteine, (□) chitosan.

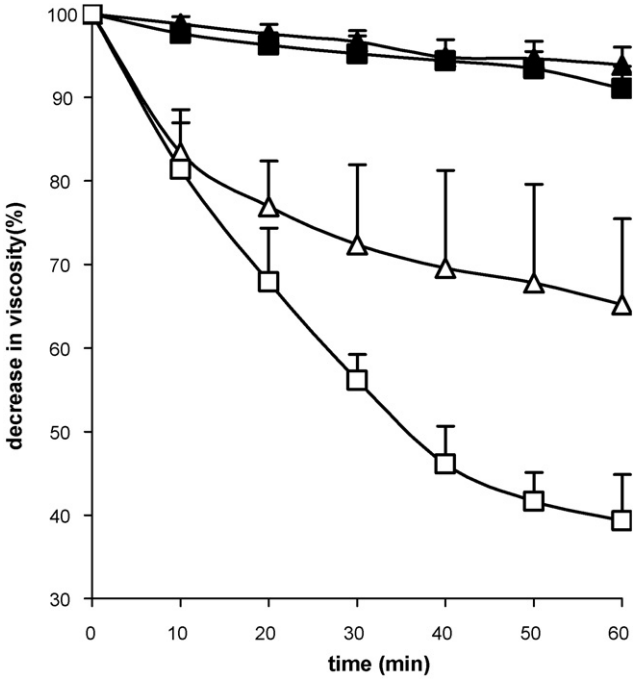

Fig. 4. Rheology studies of chitosan and chitosan-*N*-acetyl cysteine conjugate II performed at 25 °C. The viscosity expressed in percent was investigated for both polymers in presence and in absence of lysozyme over a time period of 60 min. Legend: (▲) chitosan-*N*-acetyl cysteine, (■) chitosan, (△) chitosan-*N*-acetyl cysteine + lysozyme, (□) chitosan + lysozyme. Data are means of at least five experiments.

conjugate even after 4 days on the rotating cylinder neither erosion nor partial disintegration of the swollen polymer matrices occurred. We further observed a definite correlation between an increasing amount of immobilized thiol groups on the polymer and the time of adhesion on the mucosa. While chitosan-NAC I lead to a 2.25-fold improvement in adhesion time, chitosan-NAC II exhibited a 50-fold improvement of the adhesion time in comparison to the unmodified chitosan.

Tensile studies carried out with unmodified chitosan and the corresponding chitosan-*N*-acetyl cysteine conjugate revealed as well a significant influence of the immobilized thiol groups on the mucoadhesive properties of the polymer. Results of this study are shown in Fig. 6. In comparison to chitosan as control, chitosan-NAC I displayed a 1.6-fold higher total work of adhesion, while chitosan-NAC II showed a 8.3-fold higher total work of adhesion. The maximum detachment force (MDF) increased proportionally with increasing total work of adhesion (TWA) to reach a maximum range of 30 mN for chitosan-NAC II.

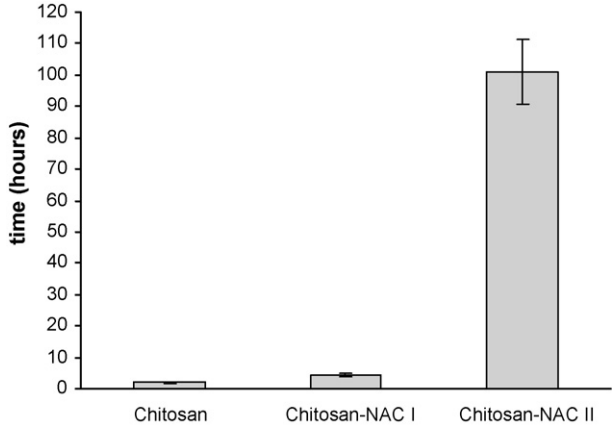

Fig. 5. Mucoadhesion studies via rotating cylinder method. Tablets of chitosan are compared with tablets formed of both chitosan-*N*-acetyl cysteine conjugates. Time of adhesion on the mucosa of pig intestine is expressed in hours. Data are means of at least three experiments ± standard deviation.

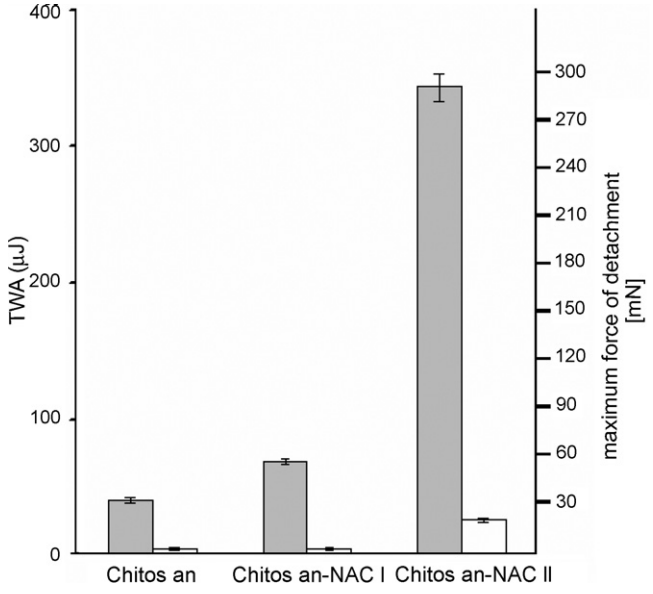

Fig. 6. Tensile studies performed with chitosan and the two different conjugates of chitosan-*N*-acetyl cysteine. The total work of adhesion is expressed in µJ. The secondary axis represents the maximum force of detachment expressed in kN. Data are means of at least three experiments ± standard deviation.

4. Discussion

In recent years, polymer based technologies have found wide biomedical applications (Valenta, 2005; Prego et al., 2005; Ludwig, 2005). Polymers, whether synthetic (e.g. polylactide-co-glycolide or PLG) or natural (e.g. alginate, chitosan, etc.), have gained a lot of scientific interest due to their wide selection of physicochemical and biopharmaceutical properties and in particular for their potential to optimize either localized drug delivery or systemic delivery by retaining a formulation in intimate contact with the absorption site (Smart, 2005). However, several factors affecting mucoadhesive properties of polymers have been traditionally reported, including molecular weight, chain flexibility, cross-linking density, swelling, and the presence of chemical groups, charge and ionization (Edsman and Hagerstrom, 2005). Despite a continuous progress, one disadvantage often lies within the unsatisfying cohesive properties of suggested polymeric scaffolds. One approach to improve this attribute is offered by the introduction of defined substructures into well-established biodegradable, cationic hydrophilic polymers like chitosan. These substructures should offer on the one hand a hydrophobic component like an acyl residue, which is able to undergo hydrophobic interactions and decrease the swelling behaviour of the polymeric educt in aqueous solu-

tions, while on the other hand it is advantageous to introduce a sulfhydryl group for cross-linking via the formation of intra- and intermolecular disulfide bonds. The presented study follows this strategy by the covalent attachment of a carbodiimide activated *N*-acetyl cysteine residue onto a polyaminosaccharide structure. The resulting conjugate was investigated thoroughly for its cohesive and mucoadhesive properties. For well-established polymers such as carbomers and chitosans the mechanisms of mucoadhesion is the result of a number of different physicochemical properties. If these materials are incorporated into pharmaceutical formulations, drug absorption by mucosal cells may be enhanced or the drug may be released at the site of action for an extended period of time. In comparison to buccal or nasal delivery, the gastrointestinal tract is proving a more difficult site because of the rapid turnover of mucus, and relatively constant transit time (Woodley, 2001). Therefore, strong mucoadhesive properties are desired in order to guarantee a tight contact between the formulation and the mucosa leading to a steep concentration gradient, necessary for adequate absorption (Haas and Lehr, 2002). Thiolated polymers have shown to be promising excipients, exhibiting beside these mucoadhesive properties also permeation enhancing and enzyme inhibitory properties (Bernkop-Schnürch et al., 2004; Bilicic et al., 2005). The rotating cylinder method, used to evaluate the mucoadhesive and cohesive properties of chitosan-*N*-acetyl cysteine conjugate, revealed significantly improved properties in comparison to chitosan as control. Further on these results gain in importance, as latest evaluations based on gamma scintigraphy seriously question the gastric-retentive mucoadhesion of unmodified chitosan formulations in humans (Sakkinen et al., 2006). Results of tensile studies showed a defined correlation between the total work of adhesion and the amount of thiol groups immobilized on the polymer surface. The rank order obtained for adhesion time was in agreement with the rank order obtained for total work of adhesion. Although various factors such as the pH of the polymer and the drying method were identified as important parameters influencing the mucoadhesive potential of polymers, a former comparative study by our group revealed that among all tested polymers a thiolated version of chitosan, namely chitosan-thiobutylamidine exhibited the highest mucoadhesion (Grabovac et al., 2005). Mucoadhesive properties which have been expected for the chitosan derivate introduced in this study could be confirmed, placing chitosan-*N*-acetyl cysteine conjugate in the class of most mucoadhesive polymers such as chitosan-thiobutylamidine. It can be deduced that the concentration of thiolate anions, which represent the reactive form of sulfhydryl groups and which are responsible for thiol/disulfide exchange reactions via a nucleophilic attack of the original disulfide bond, is relatively high. Therefore chitosan-*N*-acetyl cysteine conjugate exhibits a high susceptibility to oxidation process, as confirmed by the results of Fig. 2, showing that the highly reactive thiol groups immobilized on the polymer are oxidized in a significant number at pH 5 and above.

Although unmodified chitosan is used as a natural absorbable medical film material which is easy biodegradaded, we performed rheology studies to define if thiolation affects the biodegradability of the freshly formed conjugate. Cao et al. demonstrated that cross-linked chitosan films prepared with glutaraldehyde, epichlorhydrine or hexamethylene diisocyanate as cross-linking agents, showed a significant decrease in the degradation rate in lysozyme solution when compared with non-cross-linked chitosan films (Cao et al., 2005). We observed a less pronounced decrease in viscosity for a polymer solution of chitosan-*N*-acetyl cysteine incubated with lysozyme in comparison to control after one hour of experiment. However, our results suggest that the basic characteristics of chitosan, which is highly biodegradable and which is not accumulated in the body, were transferred onto the chitosan-*N*-acetyl conjugate (Onishi and Machida, 1999). Additionally unbound *N*-acetyl cysteine is a potent antioxidant presently undergoing clinical trials as an enhancer of the immune system in persons with acquired immuno deficiency disorder (Treitinger et al., 2000; Spada et al., 2002). In consideration of its non-toxic profile, *N*-acetyl cysteine is as a perfect reactant to form scaffold material with chitosan.

In summary, a new biodegradable thiolated polymer, namely a chitosan-*N*-acetyl cysteine conjugate has been synthesized and characterized *in vitro*, showing strong mucoadhesive and cohesive properties. Therefore the new thiomer seems to be a promising hydrophilic cationic polymeric matrix which practical application as biodegradable structure forming material is beneficial in a variety of pharmaceutical and cosmetic formulations.

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