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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.

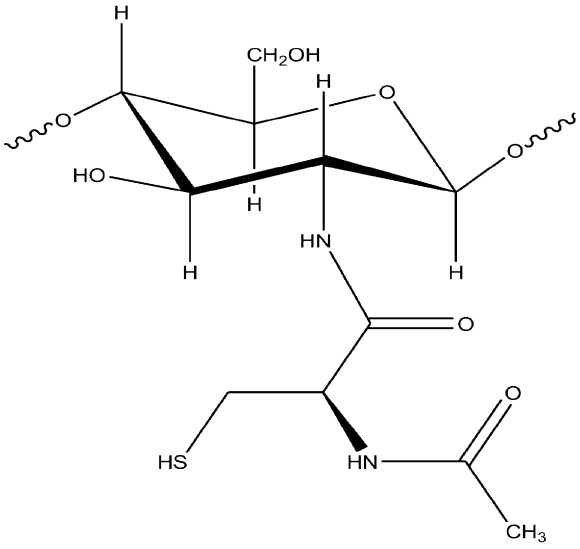

Fig. 1. Schematic diagram of the substructure of chitosan-*N*-acetyl cysteine.

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