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S-protected thiolated chitosan: Synthesis and in vitro characterization

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ABSTRACT

Purpose of the present study was the generation and evaluation of novel thiolated chitosans, so-named S-protected thiolated chitosans as mucosal drug delivery systems. Stability of all conjugates concerning swelling and disintegration behavior as well as drug release was examined. Mucoadhesive properties were evaluated in vitro on intestinal mucosa. Different thiolated chitosans were generated displaying increasing amounts of attached free thiol groups on the polymer, whereby more than 50% of these thiol groups were linked with 6-mercaptonicotinamide. Based on the implementation of this hydrophobic residue, the swelling behavior was 2-fold decreased, whereas stability was essentially improved. Their mucoadhesive properties were 2- and 14-fold increased compared to corresponding thiolated and unmodified chitosans, respectively. Release studies out of matrix tablets comprising the novel conjugates revealed a controlled release of a model peptide.

Accordingly, S-protected thiomers represent a promising type of mucoadhesive polymers for the development of various mucosal drug delivery systems.

1. Introduction

As the uptake of most drugs from mucosal membranes is controlled by a passive diffusion process, it is essential to provide a concentration gradient as steep as possible on the mucosa. In order to achieve that goal, the delivery system has to be kept as long as possible in contact with the absorption membrane, which can be guaranteed by mucoadhesive polymers. They can increase the intimate contact time with mucus surfaces resulting in increased drug concentration at the site of absorption and consequently improved overall bioavailability. In contrast to 'conventional' polymers, which are quite insufficient in order to guarantee this effect and whose mucoadhesive properties are only based on non-covalent bonds, thiolated polymers (thiomers) are capable of forming covalent bonds with cysteine-rich subdomains of the mucus gel layer via disulfide exchange reactions (Bernkop-Schnürch, Schwarz, & Steininger, 1999). Mucoadhesive, permeation enhancing and efflux pump inhibitory properties of poly(acrylic acid) and chitosan, were strongly improved by thiolation (Bernkop-Schnürch, Hornof, & Zoidl, 2003; Marschütz & Bernkop-Schnürch, 2002). Besides all these advantages, however, thiomers show comparatively low stability in solutions, as they are subject of thiol oxidation at pH ≥6 unless sealed under inert conditions (Bernkop-Schnürch et al., 2003). This hindrance limits their permeation enhancement and mucoadhesive features in body regions, where pH is raised. The design and development of thiomers being stable in liquid and semisolid formulations would therefore be highly advantageous, opening the door for numerous additional applications. According to this, it was the aim of the present study to generate thiolated polymers, whose thiol groups are protected by already formed disulfide bonds, which offer the advantage of not being subject of oxidation. The hypothesis to achieve this goal is based on covalent chromatography, where, for instance, peptides and proteins are very efficiently linked to thiol bearing resins, when they are preactivated via pyridyl substructures (Carlsson, Drevin, & Axén, 1978; Norris & Brocklehurst, 1976). Pyridyl disulfides react very rapidly and quantitatively with sulfhydryl groups over a broad pH range to form disulfide bonds. During this reaction, a disulfide exchange occurs between the molecule's —SH group and the pyridyl thiol group, i.e. the thiomer forms disulfide bonds with cysteine-rich subdomains of mucus glycoproteins with the pyridyl thiol moiety as leaving group. The pyridyl thiol leaving group, however, is from the toxicological point of view problematic. Utilizing nicotinamide (vitamin B3) instead of the pyridyl group excludes such toxic effects. In order to apply this novel concept for thiolated chitosans, it was the aim of this study to synthesize and characterize an S-protected thiolated chitosan regarding its application in mucosal drug delivery systems.

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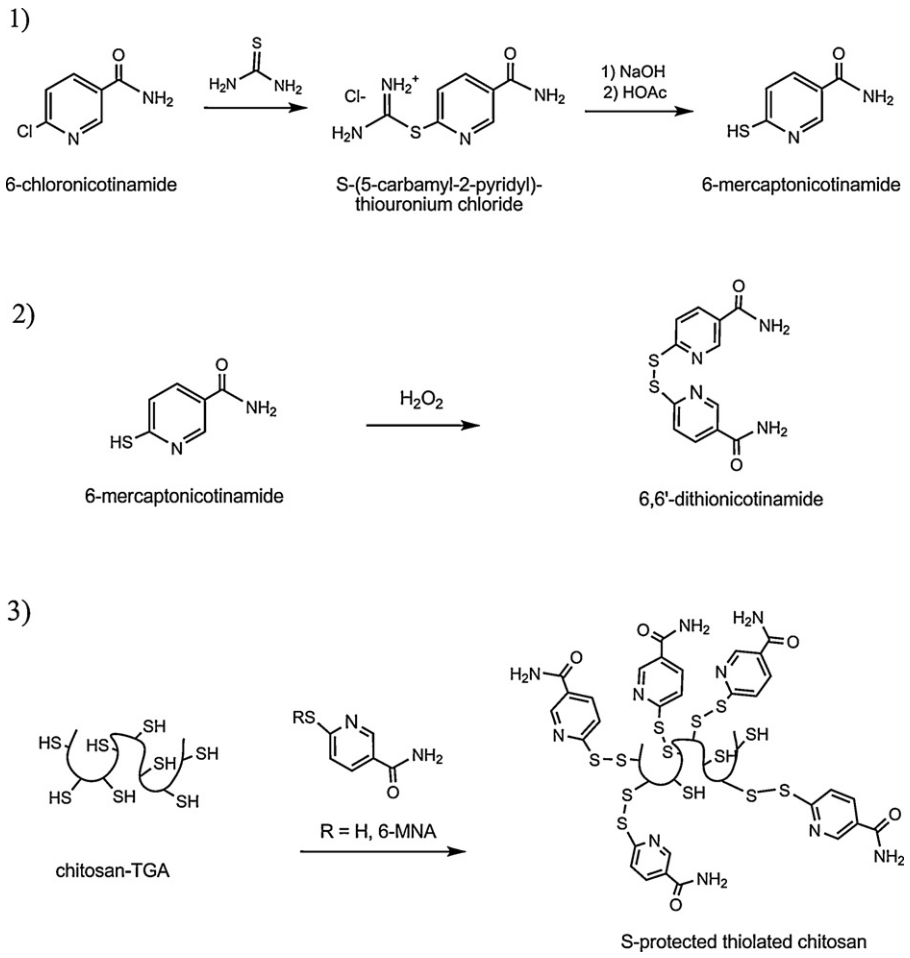

Fig. 1. Modification of thiolated chitosan leading to S-protected thiolated chitosan. 6-Chloro-nicotinamide is first reacted with thiourea to get 6-mercaptonicotinamide (6-MNA) (1). This product is then oxidized to 6,6′-dithionicotinamide (6,6-DTNA) (2). Both reagents (6-MNA and 6,6-DTNA) were added to chitosan-TGA to form S-protected thiolated chitosan (3).

2. Materials and methods

2.1. Materials

Low viscous chitosan with an average molecular weight of 150 kDa and a deacetylation degree of 85% was obtained from Sigma Aldrich, Austria. Dimethyl sulfoxide (DMSO), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent), sodium borohydride ($NaBH_4$), hydrogen peroxide (H_2O_2), dialysis tubes (MW cutoff 12 kDa), thioglycolic acid (TGA), thiourea, 6-chloronicotinamide, glutathione (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), porcine gastric mucin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma Aldrich, Austria. Leuprolide was supplied from Bachem AG, Switzerland. Caco-2 cells were kindly donated by Prof. Pfaller, Institute of Physiology, Medical University of Innsbruck. All other chemicals were of analytical grade.

2.2. Synthesis of thiolated and S-protected thiolated conjugates

The synthesis of S-protected thiolated chitosan is a tow-step procedure. The first modification involved the covalent attachment of thioglycolic acid (TGA) to chitosan (CS) due to the formation of amide bonds between the primary amino groups of the polymer and carboxylic acid groups of TGA as described previously (Kast & Bernkop-Schnürch, 2001). For the second modification, the aromatic ligands 6-mercaptonicotinamide (6-MNA) as well as its dimer 6,6′-dithionicotinamide (6,6-DTNA), which are commercially not available, had to be synthesized according a method developed by Forrest and Walker (1948). The second modification step was then achieved by disulfide bond formation between free thiol groups of the thiolated chitosan and the aromatic ligand (Fig. 1).

2.3. Assessment of cytotoxic effects

To evaluate potential cytotoxic effects of these S-protected thiolated chitosans, MTT assay was performed on a Caco-2 monolayer (Mosmann, 1983). Cells were cultured on 24-well plates at a density of 1×10^5 cells/ml in 500 μl minimum essential medium (MEM) for 14 days at 37 °C and 5% CO_2. Thereafter, cells were incubated with 0.5% (m/v) of S-protected thiomers, thiomers or unmodified chitosan control. Furthermore, nicotinamide (vitamin B_3) as well as the monomeric and dimeric reagent were tested for their influence on cell viability. Untreated cells served as positive control, whereas a 10% solution of Triton X-100 was used as negative control. After 3 and 24 h of incubation at 37 °C cells were washed with PBS and medium was replaced by 500 μl of MTT assay. Cells were incubated for 3 h along with MTT solution at 37 °C. Absorbance of the dye was measured at a wavelength of 570 nm. Cell viability was calculated