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Evaluation of functional characteristics of preactivated thiolated chitosan as potential therapeutic agent for dry mouth syndrome

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ABSTRACT

Purpose: The objective of this study was to investigate preactivated thiomers for their potential in the treatment of dry mouth syndrome.
Methods: Chitosan–thioglycolic–mercaptonicotinamide conjugates (chitosan–TGA–MNA) were synthesized by the oxidative S–S coupling of chitosan–thioglycolic acid (chitosan–TGA) with 6-mercaptonicotinamide (MNA). Test disks were compressed out of unmodified chitosan, chitosan–TGA (thiomers) and chitosan–TGA–MNA conjugates to investigate cohesive properties, cytotoxicity assays and mucoadhesion studies.
Results: Immobilizing the MNA achieved higher swelling and cohesive properties of chitosan–TGA–MNA conjugates compared to unmodified chitosan. Rotating cylinder studies displayed a 3.1-fold improvement of mucoadhesiveness of chitosan–TGA–MNA conjugates compared to thiolated polymers. Findings in tensile strength were in good agreement with rotating cylinder ones. Furthermore, preactivated thiomers exhibit higher stability. All conjugates were found non-toxic against Caco-2 cells.
Conclusion: Preactivated thiolated chitosan could be a promising system for the treatment of dry mouth syndrome where mucosa requires lubrication and mucoadhesiveness.

1. Introduction

Conventional mucoadhesive materials such as carbopol, polycarbophil or sodium carboxymethylcellulose are to a large extent hydrophilic compounds containing numerous hydrogen bond forming groups [1]. Conventional polymers form non-covalent bonds with mucus substructures which result in mainly weak mucoadhesion. However, functionalized polymers bearing sulfhydryl ligands on the polymeric backbone or designed thiomers show the ability to form covalent bonds with cysteine-rich subunits of mucus. By virtue of these features, both benefits of prolonged cohesion and strong mucoadhesion are met in the case of designated thiomers. The interactions of mucus and thiomer are, however, likely to be most intense in the deprotonated state of sulfhydryl groups [2]. Almost all oral degenerative diseases, including tooth decay, are associated with a low pH (acidic) in the body [3,4]. People with a dry mouth have mouth acidity caused by saliva thickening or dying up because of various medications such as anticholinergics, sympathomimetics, anti-HIV drugs as well as bezodiazepines [5] and dehydration [6,7]. Since xerostomia goes hand in hand with a leaky or even lacking mucus gel layer covering intraoral epithelia and consequently reduced properties of the mucosa to keep sufficient moisture on its surface [8,9], one strategy to increase salivary secretion could be using mucoadhesive polymers such as cellulose derivates as lubricants. Thiomers, however, are unstable toward oxidation and aqueous thiomer solutions are unstable. Therefore a novel second generation of thiomers, the so-called preactivated thiomers have been developed exhibiting stability toward oxidation as well as improved mucoadhesion [10]. To overcome these limitations preactivated thiomers bearing an additional ligand with a heteroaromatic structure reacting in a pH independent manner were designed and evaluated within this study. The thiol groups are protected via disulfide bonds due to the additional aromatic ligand 6-mercaptonicotinic amid (MNA). Furthermore chitosan–TGA–MNA is protected against early oxidation. Prevention of an early oxidation before coming into contact with the mucus layer occurs. Hence, more active thiol groups are

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available for a long intimate contact with mucosal membranes. To date, however, neither thiomers nor preactivated thiomers were investigated regarding their potential for dry mouth syndrome. According to this, it is the aim of this study to evaluate the positive influence of thiomers and especially preactivated thiomers on mucoadhesion for their potential as therapeutic agents for dry mouth syndrome.

2. Material and methods

2.1. Materials

Chitosan (low molecular mass 50–100 kDa; degree of deacetylation, 83–85%) was obtained from Fluka Chemie (Buchs, Switzerland). Ellman's reagent [5,5′ dithiobis(2-nitrobenzoic acid) (DTNB), reduced-form glutathione (GSH), tris(2-carboxylethyl)-phosphine hydrochloride (TCEP), 6-chlornicotinamide, thiourea and thioglycolic acid, 5,5-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Aldrich, Steinheim, Germany. All other chemicals, reagents and solvents were of analytical grade and were received from commercial sources.

2.2. Synthesis of chitosan thioglycolic acid conjugates

Thioglycolic acid was covalently attached to chitosan as previously described by our research group [11]. Thiolated chitosan was synthesized by the covalent attachment of thioglycolic acid to chitosan which formed amide bonds in between the primary amino group of chitosan and the carboxylic acid moieties of the sulfhydryl agent. Briefly, 500 mg of chitosan (low molecular mass 50–100 kDa; degree of deacetylation, 83–85%) was dissolved in 1 M HCl by adding demineralized water in order to obtain a 1% solution of chitosan hydrochloride. In the next step the coupling reaction was mediated by EDAC in a final concentration of 100 mM in order to activate the carboxylic acid moieties of the subsequently added 500 mg of TGA. The reaction mixture was incubated for 3 h at room temperature under constant stirring. Samples prepared by applying the exact same method with the exception of omitting EDAC during the coupling reaction served as controls for the analytical studies. The reaction mixtures were dialyzed in tubings in order to eliminate unbound TGA and to isolate polymer conjugates. The dialysis process is a washing process of the synthesized mixture. For this purpose the synthesized polymer was transferred into dialysis tubing (Cellulose hydrate, cut-off 12.000 Da, Carl Roth, Karlsruhe, Germany). For purifying reasons as well as to get rid of the unbound ligand, the polymer mixture was three times dialyzed against demineralized water containing 1% NaCl and twice against demineralized water. Afterward, samples and controls were lyophilized by drying frozen aqueous polymer solutions at −30 °C and 0.01 mbar (Christ Beta; Germany) (primary drying conditions of 23 h, beginning with 3 h at −30 °C, augmenting to −15 °C for 3 h , up to −10 °C for 2 h, following 17 h at 0 °C; secondary conditions of 8 h, starting with 1 h at +20 °C and 7 h at +25 °C) and finally stored at 4 °C until further use.

2.3. 6-mercaptonicotinamide (MNA) and 6,6′-dithionicotinamide (6,6′-DTNA) synthesis

2.3.1. 6-mercaptonicotinamide- synthesis and purification

6-mercaptonicotinamide was synthesized during a two-step synthesis [12]. For this synthesis, a suspension of 6-chlornicotinamide and thiourea in ethanol was generated and refluxed for 6 h at boiling temperature. After the suspension turned yellow, the mixture was stirred for 12 h at room temperature. In this first step the compound S-(5-carbamyl-2-pyridyl) thiuronium chloride was obtained. This was filtered and dried. By adding 2 M NaOH in the second proceeding step the compound was precipitated. Subsequently, it was stirred at room temperature for 45 min. In the following procedure, the pH was adjusted to 4, where the mixture turned into dark yellowish color. By this adjustment, mercaptonicotinamide (MNA) as the synthesized compound was obtained. This was purified by filtration, washed with acetone and dried.

2.3.2. 6,6′-Dithionicotinamide (6,6′-DTNA)- synthesis and purification

A suspension of 6-MNA was adjusted to a pH of 7, afterward H_2O_2 solution was added. This oxidizing reaction was stirred for 1 h at room temperature. During this reaction the yellow colored compound rendered into the white compound 6,6-dithionicotinamide (6,6′-DTNA). 6,6′-DTNA was filtered, washed with water and dried as mentioned above.

2.4. Synthesis of chitosan–thioglycolic acid–mercaptonicotinamide

The aromatic ligand was covalently attached by disulfide bond formation. 200 mg of chitosan–TGA was dissolved in a 50 mL mixture of demineralized water/DMSO (3:7) under stirring. Next, 50 mg of 6,6′-DTNA dimer dissolved in 50 mL of DMSO was added and the pH adjusted to 6.2 with 1 M NaOH. The mixture was stirred for 6 h at room temperature. In order to separate the conjugate from the unbound MNA dimer, the reaction solution was dialyzed seven times against 5 L of demineralized water under stirring in the dark at 10 °C. The dialysate was replaced every 12 h. The obtained product was freeze dried for 31 h as mentioned above and stored dry at 4 °C.

2.5. Analyses of thiolated and preactivated chitosan

2.5.1. Spectrophotometric quantification of the thiol groups

Immobilization of thiol groups on the chitosan thioglycolic acid backbone was quantified by Ellman's reaction which determines the free thiol groups [13]. Polymers and thiomers were hydrated in 0.5 M phosphate buffer pH 8.0. Ellman's reagent (5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was dissolved in 0.5 M phosphate buffer pH 8.0) and added to the polymer solutions. After an incubation time of 120 min protected from light, the absorbance of each sample was measured with a microplate reader (Tecan Fluostar; Galaxy BMG, Offenburg, Germany). The quantity of thiol groups being immobilized on the chitosan thioglycolic acid conjugate was determined by referring to cysteine standards [14].

2.5.2. Characterization of 6-MNA and 6,6′-DTNA

6-MNA and 6,6′-DTNA were soluble in DMSO. UV-spectrometer measurements were performed with a UV–VIS spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) [15]. 6-MNA revealed a peak at 307 nm and 6,6′-DTNA showed two peaks, one expressive at 297 nm and a second minor one at 253 nm. Furthermore DSC thermograms were evaluated, according to the literature melting points of around 266 °C and 264 °C are expected [16]. For DSC studies, approximately, 2 mg of sample was taken in an aluminum pan, sealed with an aluminum cap and kept under nitrogen purging (atmosphere). Samples were scanned from 0 °C to 300 °C with the scanning rate of 10 °C/min using differential scanning calorimeter (DSC-60, Shimadzu, Japan).

2.5.3. FT-IR spectra of the 6-MNA, dimer 6,6′-DTNA and the polymer conjugates

In order to verify the syntheses FT-IR analyses of 6,6′-DTNA, MNA and conjugates were undertaken. Characterization of 6-MNA and 6,6′-DTNA was conducted with attenuated-total-reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The spectrum was recorded by a Perkin Elmer Spectrum 100