Synthesis and Characterization of pH Tolerant and Mucoadhesive (Thiol–Polyethylene Glycol) Chitosan Graft Polymer for Drug Delivery

SABINE HAUPTSTEIN,[1] SONJA BONENGEL,[1] JULIA GRIESSINGER,[2] ANDREAS BERNKOP-SCHNÜRCH[1]

[1]Center for Chemistry and Biomedicine, Center for Molecular Biosciences, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innsbruck 6020, Austria
[2]ThioMatrix GmbH, Research Center Innsbruck, Innsbruck 6020, Austria

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ABSTRACT: The objective of this study was to generate a water-soluble thiolated chitosan to enable the permeation-enhancing effect of chitosan at pH of at least 5.5 without losing the advantages of improved mucoadhesive properties. Therefore, the thiol-bearing polyoxyethylene ligand {O-(3-carboxylpropyl)-O′-[2-[3-mercaptopropionylamino)ethyl]-polyethyleneglycol} was conjugated via amide bond formation to the amino group of chitosan. Resulting novel chitosan derivative (Chito–PEG–SH) exhibited 250 μmol free thiol groups per gram polymer. By the attachment of the thiol-bearing PEG ligand, an improvement of permeation-enhancing effect on rat intestine (2.7-fold improvement) as well as on a Caco-2 monolayer model (1.9-fold improvement) could be found. Cytotoxicity studies on Caco-2 cells revealed no change in biocompatibility. Mucoadhesion was improved 3.1-fold by the formation of disulfide bonds with mucus glycoproteins. The mucoadhesive effect of Chito–PEG–SH turned out to be similar to thiolated chitosan and more pronounced than mucoadhesive properties of unmodified chitosan. The graft polymer is soluble in water and aqueous solutions over a broad pH range. In aqueous media, the novel polymer does not precipitate at pH of 8.6 or less. According to these results, Chito–PEG–SH might show potential as auxiliary agent in oral drug delivery where its solubility even up to pH 8 is likely beneficial. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:594–601, 2014

Keywords: pegylation; water soluble chitosan; thiomers; toxicity; mucoadhesion; polymeric drug carrier; mucosal drug delivery; formulation vehicle

INTRODUCTION

Chitosan is a biocompatible, nontoxic biopolymer being of interest in different fields of application. In pharmaceutical research, it found its entries in the early 1990s and it is still subject of great interest because of certain remarkable properties. The polymer exhibits mucoadhesive properties, a permeation-enhancing effect for different kinds of drugs, efflux pump inhibition, transfection enhancing properties, and sustained release.[1–4] However, besides these promising features chitosan offers, there are some drawbacks hampering its use in drug delivery. The probably most interfering property of chitosan is the limited solubility. In aqueous environment, it is only soluble at pH values lower than its p*K*s (≤pH 5.5–6.5) when amino groups of the polysaccharide chain are protonated.[5] Being not completely soluble under physiological conditions in the intestine, it is not possible to take full advantage of its properties. Kotzé et al.[6] reported that chitosan failed to improve permeation on Caco-2 cell monolayer at pH values of at least 7.2, whereas water-soluble quaternized chitosan was able to decrease transepithelial electrical resistance (TEER) and increase permeability of mannitol. These findings were confirmed by *in vivo* studies in rats and pigs.[7–9] Besides quaternized chitosan, pegylation of chitosan led to improved water solubility from pH 1.0 to 11 depending on the degree of modification.[5,10,11] Additionally, reduced cytotoxicity compared with unmodified chitosan could be shown for pegylated chitosan with an even improved ability to enhance permeation of macromolecular substances.[12,13]

Modification of chitosan with thiol-bearing ligands led to improved mucoadhesion, sustained release, permeation enhancing, and efflux pump inhibition in several studies compared with unmodified chitosan.[14–17] Considering this, the aim of this study was to combine the advantages of pegylated and thiolated chitosan developing a novel soluble, biocompatible, permeation-enhancing chitosan derivative with improved mucoadhesion compared with unmodified chitosan. For this purpose, chitosan was modified by covalent attachment of the thiol-bearing polyethylene glycol–ligand O-(3-carboxylpropyl)-O′-[2-[3-mercaptopropionylamino)ethyl]–polyethylene glycol (COOH–PEG–SH) to the primary amino group of chitosan. This newly synthesized chitosan derivative was investigated in terms of solubility, permeation-enhancing properties across Caco-2 cell monolayer and freshly excised rat intestine, mucoadhesion, and cytotoxicity.

MATERIALS AND METHODS

Material

Low-viscous chitosan (average molecular weight 150 kDa, deacetylation degree of 85%), 5,5′dithiobis(2-nitrobenzoic acid) (Ellman's Reagent), resazurin, ethanol, acetone, dimethyl sulfoxide (DMSO), Triton-X 100, dialysis cellulose membrane

Correspondence to: Andreas Bernkop-Schnürch (Telephone: +43-512-507-58601; Fax: +43-512-507-58699; E-mail: andreas.bernkop@uibk.ac.at)

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tubes (molecular weight cut-off, MWCO, 12 kDa), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), and O-(3-carboxylpropyl)-O′-[2-[3-mercaptopropionylamino)ethyl]–polyethylene glycol (COOH-PEG-SH) (3000 Da) were purchased from Sigma–Aldrich (Vienna, Austria). Flouresceinisothiocyanate-dextran (FD4, 4400 Da) was supplied by TdB Consultancy AB (Uppsala, Schweden). Professor Pfaller, Institute of Physiology, Medical University of Innsbruck kindly donated Caco-2 cells. Transwell and well plates were purchased from BioGreiner (Kremsmünster, Austria). All other cell culture care supplies were obtained from PAA (Pasching, Austria). All chemicals were of analytical grade and used as received.

Preparation of Porcine Mucus

Small intestine from pigs was obtained from a local slaughter and transported on ice to the laboratory. The mucus was collected from the tissue using a scraper. To homogenize and purify the collected mucus, it was mixed with a sodium chloride solution (0.1 M) and stirred for 1 h on ice. Next, the mixture was centrifuged at 4°C and 10400 g for 2 h. The supernatant and granular material on the bottom was discarded and homogenized; purified mucus was collected for immediate use.

Synthesis of Chito–PEG–SH

To prepare the thiol group bearing pegylated chitosan, COOH–PEG–SH was chemical bound to chitosan (Chito–PEG–SH) via amide bound formation between the primary amino group of chitosan and the carboxyl group of the ligand. The reaction was mediated by a carbodiimid (EDAC) and N-hydroxysuccinimide (NHS). Therefore, 500 mg of chitosan were dissolved at pH 3 (adjusted with HCl) in 25 mL of water. Next, 200 mg of COOH–PEG–SH were dissolved in 25 mL demineralized water; EDAC and NHS were added in a concentration of 100 mM. The pH was adjusted to 5, and mixture was stirred for 30 min. Then, the solutions were combined and stirred overnight. To purify the product of unbound ligand, EDAC, and NHS, the reaction mixture was diluted and dialyzed against water pH 3 (adjusted using 5 M HCl) for 3 days using Spectra/Por® 3 membranes (MWCO 12 kDa). The dialysate medium of 5 L was replaced every 12 h; during the 2nd day of dialysis, 1% NaCl was added. To avoid disulfide bond formation, purification occurred at 10°C in the dark. Finally, the product was frozen at −80°C and lyophilized for 2 days under reduced pressure.

Synthesis of Chitosan–Thioglycolic Acid

For comparison, chitosan–thioglycolic acid (TGA), a thiolated but not pegylated chitosan derivative was prepared. Synthesis of the thiomer with a similar degree of substitution was performed by a method described by Kast and Bernkop–Schnürch; the ligand was bound via amide bound formation between the carboxylic acid groups of TGA and primary amino groups of chitosan.[18] The resulting product was lyophilized after purification via dialysis. Additionally, chitosan was treated the same way omitting EDAC and NHS.

Determination of Degree of Substitution

The amount of bound COOH–PEG–SH and TGA was determined by quantifying the thiol groups spectrophotometrically using Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid)] according to a method described previously.[19] For the calibration curves, COOH–PEG–SH and TGA were employed.

Determination of Solubility

To investigate the solubility of Chito–PEG–SH in comparison to nonpegylated thiomer and unmodified chitosan, mixtures of aqueous buffer and polymer [0.5% (m/v)] were prepared and stirred overnight. To examine solubility in aqueous environment at different pH values acetate buffer 0.1 M (pH 4 and 5), phosphate buffer 0.1 M (pH 6, 7, and 8), and carbonate buffer 0.1 M (pH 9 and 10) were used. Further experiments with acetone, ethanol, and DMSO were carried out. To confirm findings of this study, Chito–PEG–SH, chitosan–TGA and unmodified chitosan were dissolved in a concentration of 0.5% (m/v) at pH 4. By adding 1 M NaOH stepwise to the solution, the pH was raised until the polymer precipitated. The pH values were measured until precipitation of the polymer occurred.

Cytotoxicity Studies—Resazurin Assay

To investigate the influence of the derivatives on cell viability, a resazurin assay was performed. The assay is based on the reducing environment of viable cells. Resazurin, a blue nonfluorescent substance is converted into resorufin, a red fluorescent substance in the presence of metabolic active cells.[20] For the assay, Caco-2 cells were cultured in a 24-well plate for 10 days at 37°C in 5% CO_2 environment. The minimum essential medium with Earls salts containing fetal calve serum (FCS) was replaced every 2nd day. Five-hundred microliter of the prepared polymer solutions 0.05% (m/v) of different conjugates and unmodified chitosan was added to each well. Negative control minimal essential medium without phenol red and FCS was employed, whereas Triton X® 100 4% (v/v) served as positive control. The experiment was carried out in quadruplicates. The cells were incubated with samples for 24 h. Thereafter, samples were removed and cells were washed two times with prewarmed (37°C) phosphate-buffered saline. Then, 250 µL of a 2.2 µM resazurin solution were added to each well and incubated for another 2 h. The fluorescence of the supernatant was measured at 540 nm with background subtraction at 590 nm with Tecan infinite, M200 spectrometer, Grödig, Austria.[21,22]

Permeation Studies

Permeation Across Caco-2 Monolayer

The permeation-enhancing properties of Chito–PEG–SH were evaluated in comparison to unmodified chitosan and chitosan–TGA. Therefore, a Caco-2 cell monolayer model was employed.[23] Caco-2 cells were kept under conditions as described above. For permeation studies, cells were cultivated on 24-transwell plates in a density of 1×10^5 cells/mL. To achieve a complete monolayer and totally differentiated cells, cells have been cultivated for 21 days. During these times, the medium was replaced every 48 h. The transwell system (Greiner bio one®) had a diffusion area of 0.33 cm^2 and the pore size of the pPolyethylene terephthalate membrane was 0.4 µm. TEER was measured during the 21 days of cultivation and directly before, during, and after the experiment using epithelial voltohmmeter (World Precision Instruments, Berlin, Germany) with a pair of side-by-side electrodes. Monolayers employed for transport studies exhibited a TEER value between 500 and 600 Ωs/cm^2. Before the experiment, cells were washed with phosphate-buffered saline. Then, 600 µL of minimal essential medium (MEM) without phenol