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Chitosan-catechol: A polymer with long-lasting mucoadhesive properties

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ABSTRACT

Numerous mucoadhesive polymers have been exploited for prolonging the residence time of formulated drugs or pharmaceuticals at specific delivery sites. However, it has been difficult to achieve satisfactory mucoadhesive properties. The two major modification strategies such as thiolation or lectin functionalization have been extensively studied, but disulfide bond reversibility in the case of thiolation and the toxicity of lectins have been problems. Thus, approaches for further improvement of mucoadhesive properties need to be developed. With an overwhelming library of mucoadhesive polymers, one practical way to improve mucoadhesion is chemical modification of existing mucoadhesive polymers. In other words, the method is based on utilizing the cooperative effect that might be achieved by chemical tethering of a small adhesive moiety to an available mucoadhesive polymer. Here, we conjugated catechols derived from mussel adhesive proteins to chitosan, which is a widely known mucoadhesive polymer. We demonstrated that the gastrointestinal (GI) tract retention of chitosan-catechol was improved compared to unmodified chitosan, which is due to the formation of irreversible catechol mediated-crosslinking with mucin. The results indicate that catechol modification of mucoadhesive polymers may possibly lead to a new generation of mucoadhesive polymers for mucosal drug delivery.

1. Introduction

Ever since mucoadhesive polymers were introduced for sustained drug release, numerous attempts have been made to develop novel mucoadhesive polymers [1–5]. Polymers that exhibit excellent mucoadhesive properties include poly(acrylic acid), carboxyl methyl cellulose, scleroglucan, and chitosan. Chitosan has been extensively studied due to its strong interaction with the mucin layer by numerous inter-molecular hydrogen bonds and electrostatic interactions with the negatively charged sialic acid and sulfate residues in mucin [2,6–8]. Furthermore, chitosan is considered to be suitable for clinical use due to biocompatibility and degradability.

Although chitosan is itself mucoadhesive, numerous attempts have been made to further improve its adhesion through chemical modifications. Thiolation is considered to be a highly effective method for enhancing mucoadhesion [8–12] because tethered thiols have the unique property of forming disulfide bonds (dissociation energy of 251 kJ/mol) with the cysteine residues in mucin [10,13–18]. However, the disulfide bridges between the mucin and thiolated polymers are reversible. Thus, the *in vivo* retention of thiol groups on mucosal surfaces is typically short lived. Therefore, thiolation may not be the ultimate solution for increasing the mucoadhesion of existing polymers. Lectin modification can also be used to enhance mucoadhesive properties. Lectins are naturally occurring proteins that play a role in biological recognition. Bacteria use lectins to attach themselves to the cells of the host organism by way of receptor-mediated adhesion. However, lectins bind reversibly to cell surfaces, are immunogenic [19] and highly toxic [20].

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Thus, identification of a novel functional group that can strongly improve binding to mucosal layers remains a challenging task. Functional group identification is critical because mucoadhesive properties can be synergistically enhanced by chemical conjugation of the functional group to existing mucoadhesive polymers.

One good candidate is catechol. It is found in nature as a side chain of L-3,4-dihydroxyphenylalanine (DOPA), an amino acid crucial to the robust underwater adhesion of mussels [21,22]. One advantage of using catechol is that catechol derivatives are commercially available for bioconjugation to amine or carboxylic acid group [23,24]. Furthermore, it has the ability to form irreversible covalent bonds with thiols and amines via formation of o-quinone by catechol oxidation [25–29]. Several previous studies investigated the mucoadhesive properties of catechol. In the 1990s, it was reported that purified mussel adhesive proteins are mucoadhesive *in vitro* [30,31]. Later, conversion from a non-mucoadhesive polymer, poly(ethylene glycol) (PEG), to a mucoadhesive polymer was achieved by catechol end-functionalization, but the mucoadhesion properties were only evaluated *in vitro* [32]. In 2012, a mucoadhesive hydrogel was prepared by mixing chitosan and catechol derivatives [33]. However, none of the previous studies have investigated catechol as a mucoadhesion enhancer and studied its mucoadhesive properties *in vivo*.

Mucoadhesion occurs in two steps, through a contact stage and a consolidation stage [34,35]. The charge interaction between chitosan and mucin is reversible, yet able to rapidly initiate contact with mucin, thus facilitating the contact stage. We hypothesized that the formation of catechol-mediated interactions would provide irreversible anchorage to mucin in the consolidation stage, providing a cooperative two-step mechanism. In this study, we investigated the enhancement of the mucoadhesive properties of chitosan by catechol conjugation and studied the mucin interaction with catechol-tethered chitosan (Chi-C). The residual amounts of Chi-C that were detected by Surface Plasmon Resonance (SPR) spectroscopy showed more than four-fold enhancement in mucoadhesion compared to unmodified chitosan and the *in vivo* retention of Chi-C was substantially longer than chitosan and poly(acrylic acid) (Mw = 450 kDa), which were rarely detected after 3 h (h) after oral administration. However, we observed enhancement of *in vivo* retention up to 10 h in the case of orally administered Chi-C and observed controlled release of insulin from the Chi-C containing capsules. Furthermore, we confirmed that Chi-C forms catechol-mediated covalent crosslinks with mucin and verified that it is non-cytotoxic. This study indicates that the intrinsic mucoadhesive properties of previously known polymers can be further increased by conjugation with catechol moieties.

2. Materials and methods

2.1. Materials

Chitosan 100 (802 kDa according to hyalyronic acid standard [23], 80% deacetylated provided by the vendor) was purchased from Wako Pure Chemical Co. (Tokyo, Japan). 3,4-dihydroxy hydrocinnamic acid (HCA) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchaseed from Sigma-Aldrich (St. Louis, USA.). Ethanol was purchased from Merck Chemicals, (Darmstadt, Germany). MWCO 3500 dialysis membrane used for Chi-C synthesis was purchased from Spectrum Laboratories (Rancho Dominguez, U.S.A.). Mucin from porcine stomach type II (partially purified, bound sialic acid <1.2%), fluorescein isothiocyanate isomer I (FITC), fluorescein-5-thiosemicarbazide (FTSC), N-Ethylmaleimide (NEM), and poly(acrylic acid) (Mw 450 kDa) were purchased from Sigma-Aldrich. Dithiothreitol (DTT) was purchased from Duchefa Biochemie (Haarlem, The Netherlands). A SIA Kit Au used for the BIACORE analysis was purchased from GE Healthcare (Little Chalfont, U.K.). A 20 nm of titanium and 100 nm gold were sequentially deposited onto silicon wafers to produce gold wafers used for atomic force microscope (AFM) imaging. A mouse sonde was purchased from Daehan biolink (Chungbuk, Korea). BALB/c mice and Wistar/ST rats were purchased from Central Lab Animal Inc., (Seoul, Korea). The recombinant human insulin (h-insulin) and human insulin ELISA kit used to test bioavailability were purchased from Sigma-Aldrich (St. Louis, USA) and The Cell Counting Kit-8 assay for cell viability assay was purchased from Dojindo Molecular Technologies, Inc. (Rockville, USA) and Lactate Dehydrogenase (LDH) activity assay kit was purchased from Biovision (Milpitas, USA). Caco-2 cells and Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the American Type Culture Collection (ATCC) (Rockville, USA) and cultured in Endothelial Cell Growth Media (EGM) (Lonza, USA) and Eagle's Minimum Essential Medium (ATCC) containing 10% Fetal Bovine Serum, respectively. Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium was purchased from Gibco/Life Technologies (NY, USA). All other chemicals were of analytical grade and used as received.

2.2. Synthesis of chitosan derivatives

2.2.1. Synthesis of Chi-C

Chi-C was synthesized as we described in the previous reports [23,27]. Briefly to synthesize Chi-C with 12% degree of conjugation, 3.25 mmol chitosan was hydrated in 5 mL of 1 N HCl (aq) solutions, 45.5 mL of distilled and deionized water (DDW) was sequentially added to the solution, and the pH of the solution was adjusted to 4.5 with 5 N NaOH. Then, 3.25 mmol of 3,4-dihydroxy hydrocinnamic acid dissolved in a 3 mL DDW was added. To the reaction mixture, 6.49 mmol of EDC dissolved in the 50 mL of DDW and ethanol solution (1:1, v/v) was added drop-wise. The reaction mixture was stirred vigorously at room temperature for 4 h and the pH of the reaction solution was maintained between 4.5 and 5.0. Dialysis (MWCO: 3,500, SpectraPor, USA) was carried out in acidified DDW (pH 5.0, HCl) for 2 days, in PBS buffer for 4 h, and in DDW for 1 day while the dialysis solvent was changed every 3–6 h. The same procedure was repeated with 6.49 mmol of HCA with 6.49 mmol of EDC for a higher degree of catechol conjugation (DOC) and 3.25 mmol of HCA with 3.25 mmol EDC for lower degree of catechol conjugation. The final product was lyophilized and kept in a moisture-free desiccator. The DOC of Chi-C was determined using ^{1}H NMR (Bruker Avance, 400 MHz) by the method previously described [23,36].

2.2.2. Fluorescence labeling of the chitosan, Chi-C and poly(acrylic acid)

Chitosan and Chi-C_{12} were modified with FITC, and poly(acrylic acid) (PAA) was modified with fluorescein-5-thiosemicarbazide (FTSC). To synthesize FITC modified chitosan (Chi-FITC), 0.65 mmol of chitosan was hydrated in 5 mL of 1 N HCl (aq) solutions, 45.5 mL of DDW was sequentially added to the solution, and the pH of the solution was adjusted to 4.5 with 5 N NaOH. To the chitosan dissolved solution, 8.45 μmol of FITC in 1 mL ethanol was added. The solution was stirred vigorously for 4 h and dialyzed in pH-adjusted DDW (pH 3) for two days, once in phosphate buffer solution, and twice in pH-adjusted DDW. To synthesize FITC conjugated Chi-C_{12} (Chi-C-FITC), 0.65 mmol of Chi-$C_{12}$was dissolved in DDW (pH 4.5) directly and 8.45 μmol of FITC in 1 mL ethanol was added. The reaction time was 4 h and the product was dialyzed in the solutions previously described. Similarly, 8.45 μmol of FTSC was conjugated to 0.65 mmol of PAA to synthesize FTSC conjugated PAA (PAA-FTSC). The conjugation of fluorescent molecule was confirmed using In Vivo Imaging System Lumina (IVIS Lumina) from Xenogen (Alameda, U.S.A.).

2.3. SPR investigation of mucoadhesive polymers

Chi-C with 7.2% DOC (Chi-$C_{7.2}$), and 20.5% DOC (Chi-$C_{20.5}$) were dissolved in PBS buffer (pH 2 and at pH 7.4) at 1 mg/mL. The pH of the PBS buffer solutions was adjusted to 2 and 7.4 using 5 N HCl. Although the PBS solution does not have the buffering capacity at pH 2, it was chosen to mimic the salts *in vivo*. Measurements were performed using SPR (BIACORE 3000, GE Healthcare) and glycerol 70% (GE healthcare) was used to normalize a gold sensor chip prior to all measurements. The mucin solution was adsorbed onto a sensor chip at a flow rate of 10 μL/min. The mucin-coated surface was thoroughly washed with DDW before chitosan derivatives were tested. All the samples were filtered with a 0.45 μm filter. For polymer association, 1 mg/mL of the filtered chitosan, Chi-$C_{7.2}$, or Chi-$C_{20.5}$ was injected at a flow rate of 30 μL/min. PBS (pH 7.4) was used for washing in between injections.

2.4. Turbidimetric titration

Mucin was dissolved in pH-adjusted PBS buffer (pH 2 and pH 7.4 using 1 M HCl) at 1 mg/ml, and sonicated for 15 min with pulse (1 s (s) sonication with 1 s interval). The size and zeta potential of mucin particles were measured after 5 min using a zeta-potential and particle size analyzer (ELSZ-1000, Otsuka electronics, Osaka, Japan). Unmodified chitosan and Chi-$C_{20.5}$ dissolved in either pH 2 or pH 7.4 PBS buffer solutions (10 mg/mL) were each added to the prepared mucin solutions (1 mg/mL). The samples were left in room temperature for 10 s before each measurement. The turbidity of the mixture solutions was measured at 600 nm using UV spectrophotometer (HP8453, Hewlett Packard, USA) (n = 3 for each point).

2.5. Analysis of mucin/polymer complexes

Chi-$C_{20.5}$ (1 mg/mL) was dissolved in the pH-adjusted PBS buffer solutions (pH 2). 20 uL and 40 uL of Chi-$C_{20.5}$ were each added to PBS solution at pH 7.4. or 1 mL mucin solution at pH 2 or pH 7.4. The absorbance was measured immediately after complexation using UV–Vis spectrophotometer (Agilent Technologies 8453, USA).

The size of the complex was measured by AFM (XE-100, Park Systems, Suwon, Korea). In detail, 10 μL of the mucin, mucin/chitosan, and mucin/Chi-$C_{20.5}$ complexes