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Research paper

Hydrocaffeic acid–chitosan nanoparticles with enhanced stability, mucoadhesion and permeation properties

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ABSTRACT

Catechol-containing molecules, such as hydrocaffeic acid (HCA) have been shown to increase the mucoadhesion of several polymers. We report here a simple and bioinspired approach to enhance chitosan (CS) mucoadhesion and stabilize it in nanoparticulate form by preparing HCA–CS conjugates. HCA–CS conjugates containing 6 and 15 mol% HCA were synthesized and characterized by FT-IR, ^{1}H NMR and UV–vis spectrophotometry. HCA–CS nanoparticles prepared by ionic gelation with sodium tripolyphosphate (TPP) ranged in size between 100 and 250 nm depending on the polymer and TPP/CS weight ratio. In contrast to CS nanoparticles, which aggregate at pH > 6.5, HCA–CS nanoparticles did not show any sign of aggregation or precipitation over the 4–10 pH range and maintain their size. Unexpectedly, HCA–CS nanoparticles also maintained their size and polydispersity index at pH 7.4 and NaCl concentrations of up to 500 mM. Partial oxidation of HCA resulted in nanoparticle cross-linking and improved stability at pH < 4. HCA–CS mucoadhesion to rabbit small intestine was 6 times higher than unmodified CS. CS and HCA–CS nanoparticles were able to induce reversible tight junction opening in Caco-2 cell monolayers. Tight junction opening facilitated the permeability of a model hydrophilic molecule, fluorescein isothiocyanate-labeled dextran (FD4) and was 3 times higher in the cells treated with HCA–CS 15% nanoparticles compared to control groups. HCA–CS conjugates were found to be excellent candidates for stable nanodelivery systems with enhanced oral absorption of hydrophilic molecules.

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1. Introduction

Bioadhesive polymers that adhere to biological interfaces offer great potential in maximizing drug efficacy and localization for ocular, vaginal and oral administration. They enhance drug bioavailability through the intimate contact with the tissue allowing improved absorption, sustained local delivery and reduced dosage and dosing frequency.

Among the inherently bioadhesive polymers, chitosan (CS) is the most widely studied for drug delivery and tissue engineering, due to its biocompatibility, biodegradability, and ability to enhance absorption [1,2]. Although CS is approved for dietary applications in Japan, Italy and Finland and as wound dressing, it is not yet approved for drug delivery applications [3]. CS nanoparticles polyanionically cross-linked by sodium tripolyphosphate (TPP) enhance drug bioavailability, drug stability and controlled release, and have shown promising results as delivery systems for anticancer drugs, proteins, siRNA and DNA [4–7]. However, instability of CS nanoparticles under neutral and alkaline conditions and its only limited bioadhesion in wet conditions greatly restricts its utility [2,8].

Various modifications have been made to CS to improve mucoadhesion such as thiolation and partial quaternization to form trimethyl chitosan (TMC) [9–11]. TMC has as better solubility in neutral and alkaline media compared with unmodified CS, and allows faster permeation of a model hydrophilic macromolecule through porcine cheek epithelium [12]. Trimethyl chitosan–cysteine (TMC-Cys) nanoparticles show enhanced mucoadhesion, increased insulin transport through rat intestine and better uptake in Caco-2 cells compared to TMC nanoparticles [13]. However, TMC

Abbreviations: HCA, hydrocaffeic acid; CS, chitosan; TPP, sodium tripolyphosphate; FD4, fluorescein isothiocyanate-labeled dextran; TMC, trimethyl chitosan; TMC-Cys, trimethyl chitosan-cysteine; DOPA, 3,4-dihydroxy-L-phenylalanine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; BSA, bovine serum albumin; FITC, Fluorescein isothiocyanate; NPs, nanoparticles; DLS, dynamic light scattering; RT, room temperature.

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was shown to be cytotoxic to L929 mouse fibroblasts and caused an irreversible opening of the tight junctions of Caco-2 cells. Further modifications of TMC such as pegylation were suggested to enhance its biocompatibility profile [14].

In order to improve CS nanoparticles mucoadhesion in wet conditions, stabilize them under physiological conditions and enhance their permeation properties, we looked to nature for inspiration and adapted strategies used by aquatic organisms, such as mussels to adhere to almost any surface. Mussels secrete proteinaceous material that mediates firm attachment of the organisms to a wide variety of organic and inorganic objects, such as rocks, metal ship hulls, and wood structures [15]. The key component responsible for this impressive adhesion is 3,4-dihydroxy-L-phenylalanine (DOPA), an unusual amino acid naturally present in marine mussel foot proteins. A key functional group of DOPA is the ortho-dihydroxyphenyl group (catechol), which forms strong bonds with various surfaces (e.g., inorganic, organic or metallic) [16,17]. Inspired by the mussel adhesion mechanism, several new bioadhesive materials have been reported [8,18–20]. For instance, hydrocaffeic acid (HCA)–CS/pluronic were formulated into hydrogels and used as tissue adhesives and hemostatic materials. The hydrogels showed strong adhesiveness to soft tissues and mucous layers and had superior hemostatic properties [8]. However, none of these studies addressed the issues of limited CS nanoparticle mucoadhesion, instability at physiological conditions and absorption enhancement properties, which we set about to explore.

In a previous study, we have shown that a physical mixture of catechol-containing compounds and CS formed a hydrogel with enhanced mucoadhesion [21]. Rapid release of the catechol from the hydrogel necessitates the development of more stable catechol-CS conjugates. We describe herein the synthesis and characterization of catechol-CS conjugates to enhance CS nanoparticles mucoadhesion and improve their stability. Further, the utility of these nanoparticles in enhancing the oral absorption of hydrophilic drugs was evaluated using Caco-2 monolayers. Catechol-CS conjugates were synthesized by amide bond formation between CS and a catechol-containing compound, hydrocaffeic acid (HCA) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) as a coupling agent. The conjugates were formulated into nanoparticles by ionotropic gelation with TPP and characterized by different techniques. *Ex vivo* mucoadhesion of HCA–CS nanoparticles was evaluated using rabbit small intestine and their ability to enhance the absorption of a model hydrophilic molecule was tested in Caco-2 cell monolayers.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (M_W 50,000–190,000 Da, degree of deacetylation 75–85%), sodium periodate, EDC, TPP, bovine serum albumin (BSA), fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4400 Da (FD4), trehalose and HCA were purchased from Sigma–Aldrich (St. Louis, MO). The weight average molecular weight of chitosan was determined to be 115,000 ± 5000 Da [22]. Fluorescein isothiocyanate (FITC) was purchased from Alfa Aesar (Ward Hill, MD). Glacial acetic acid (99.98%, Fisher Scientific (Rancho Dominguez, CA), hydrochloride acid (HCl, 36.5–38.0%, Chimiques ACP Chemicals, Canada), sodium hydroxide (NaOH, ACROS) were reagent grade and used as received. Pierce BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL). Rabbit small intestine was obtained from rabbits sacrificed in accordance with McGill University animal ethics protocol and stored at −20 °C within 2 h of animal sacrifice. Before being used for experiments, samples were thawed at room temperature, followed by gentle removal of the non-digested matter with deionized water.

2.2. Synthesis of hydrocaffeic acid–chitosan (HCA–CS) conjugates

HCA–CS conjugates were synthesized using standard EDC chemistry as depicted in Scheme 1. Briefly, chitosan (0.6 g, ~3.0 mM amine groups) was suspended in 60 mL of double distilled water and the pH was adjusted to 2.5 by 0.1 N HCl addition. The suspension was stirred overnight until complete dissolution of chitosan was achieved. Subsequently, HCA (0.28 g, 1.5 mM) and EDC (0.71 g, 3.7 mM) were dissolved in 30 mL of 1:1 v/v mixture of water and ethanol and were gradually added to the chitosan solution. The pH of the reaction mixture was adjusted to 5.5 by 0.1 N NaOH and the reaction mixture was continuously stirred for 24 h. The pH of the reaction solution was maintained at 5.5 by the addition of 0.1 N HCl. The reaction mixture was dialyzed (MWCO: 3500, SpectraPor) against pH 4.0 water for 3 days. The HCA–CS conjugates were separated by freeze-drying using a Virtis freeze-dryer (Bench-Top K, VirTis, Canada). ^{1}H NMR spectra of HCA–CS conjugate and unmodified CS solutions in D_2O containing 1% v/v DCl (5 mg/mL) were recorded at 25 °C using a Varian spectrometer operating at 400 MHz. UV–vis spectroscopy measurements of the conjugate solution in deionized water (3.6 mg/mL) were recorded using a Cary 5000UV–Vis–NIR Spectrophotometer (Agilent Technologies). HCA content of the conjugates was calculated by ^{1}H NMR and UV–vis spectroscopy measurements. Reaction yield and coupling efficiency were calculated using the following equations:

$$\text{Reaction yield } (\%) = \frac{\text{actual yield} \times 100}{\text{theoritical yield}} \quad (1)$$

$$\text{Coupling efficiency } (\%) = \frac{\text{HCA in conjugates} \times 100}{\text{HCA used initially}} \quad (2)$$

FT-IR spectra of the conjugates and unmodified CS were recorded using a Bruker Tensor 27 FT-IR spectrometer (Bruker Optics Inc.).

2.3. Synthesis of FITC-labeled CS and HCA–CS

FITC-CS conjugates were made based on a reaction between the isothiocyanate group of FITC and the primary amino group of chitosan (Scheme 2) [23]. FITC solution in methanol (10 mL, 0.8 mg/mL) was slowly added to CS or HCA–CS solution in 0.1 M acetic acid (10 mL, 10 mg/mL). The reaction mixture was stirred in the dark for 3 h at room temperature. FITC–CS conjugates were precipitated in 0.1 N NaOH and separated by centrifugation at 5000 RPM for 5 min. The pellet was suspended in deionized water, vortexed and FITC–CS was separated by centrifugation as above. The washing/separation cycle was repeated until no FITC was detected in the supernatant. FITC–CS conjugates were suspended in deionized water and separated by freeze drying. Because of the solubility of HCA–CS in 0.1 N NaOH, FITC-labeled HCA–CS conjugates were purified by dialysis against deionized water for 4 days. The product was separated by freeze drying. To determine the labeling efficiency, FITC–CS solution in 0.1 M acetic acid (2 μg/mL) was diluted 500 times in phosphate buffer (10 mM, pH 8.0) and its fluorescence intensity was recorded on Infinite F200 filter-based detection system (Tecan, Austria). Standard FITC solutions in phosphate buffer pH 8.0 diluted from a FITC stock solution in methanol (100 μg/mL) were used to construct the calibration curve. The curve was linear for FITC concentrations of 0.002–0.032 μg/mL and the r^2 was >0.999. Labeling efficiency was calculated as the mole percent of FITC to chitosan repeating units and found to be 0.14%, 1.70% and 0.40% for FITC–CS, FITC–HCA–CS 6% and FITC–HCA–CS 15%, respectively.

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