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Positively and negatively surface-charged chondroitin sulfate-trimethylchitosan nanoparticles as protein carriers

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ABSTRACT

Positively and negatively surface-charged nanoparticles (NPs) were prepared with chondroitin sulfate (ChS) and trimethylchitosan (TMC). NP size, surface charge, formation yield, and water content were investigated as a function of weight ratio and concentration. Size and zeta potential were controlled by varying the ChS/TMC mass ratio. FTIR spectra revealed interactions among composite NP constituents. TEM images showed that the NPs were nearly spherical, with an average size of ~300 nm. Encapsulation efficiency increased in positively charged NPs with increases in fluorescein isothiocyanate–bovine serum albumin concentration. Negatively charged NPs had only 10–20% encapsulation efficiency. The release profile, release kinetics and mechanism of positively charged ChS-TMC NPs were studied in vitro. NP cytocompatibility and uptake were verified ex vivo. Both types of NPs were taken up and retained in cells. A549 cells took up more positively charged (49.4%) than negatively charged (35.5%) NPs.

1. Introduction

Chondroitin sulfate (ChS) is a key structural component in connective tissues and cartilage. It binds to a core protein to produce highly absorbent aggrecan, a major structure inside cartilage that acts as a shock absorber, or produces a cell receptor syndecan and interacts with adhesion proteins, cells, and the extracellular matrix. ChS is an anionic polyelectrolyte consisting of repeating disaccharide units of β-1,3-linked D-glucuronic acid and β-1,4-linked *N*-acetyl galactosamine with a molecular weight of 20–50 kDa. When the linear chain of these disaccharide structural units is sulfated at either the 4- or 6-position of *N*-acetyl galactosamine, the anionic polysaccharides are called chondroitin sulfate A and C, respectively. These compounds are synthesized intracellularly from glucose or glucosamine precursors (Mikami & Kitagawa, 2013) and are secreted by chondrocytes as a macromolecular complex for release into the extracellular matrix or to remain localized at the cell surface. ChS reportedly has anti-oxidation (Muller, Letelier, Galleguillos, Molina-Berrios, & Adarmes, 2010), and anti-inflammation (Iouv, Dumais, & Du Souich, 2008) properties and functions in immunoregulation (Sakai et al., 2006), regulating cell adhesion, and morphogenesis (Mizuguchi et al., 2003). In Europe, ChS is marketed as a symptomatic, slow-acting drug treatment for osteoarthritis and is widely used to relieve pain in arthritic diseases. In the United States, ChS is recognized as a dietary supplement. ChS also plays key roles in the wound healing process (Zou et al., 2009). It reduces inflammatory reactions at injury sites by accelerating the metabolism of cells and maintaining a normal microenvironment for cell growth. A number of recent studies have used ChS as a biomaterial for cartilage repair (Chang, Cheng, Ho, Huang, & Lee, 2009), tissue engineering (Lee, Kung, & Lee, 2005), and growth factor release (Cai, Liu, Shu, & Prestwich, 2005). However, the water-soluble ChS is unable to produce a sustained drug release; one of the solving methods is complex with a cationic polyelectrolyte to form nanoparticles.

Chitosan (CS), a cationic polyelectrolyte, is obtained via chitin deacetylation and is the second most abundant naturally occurring polysaccharide. Composed of *N*-acetylglucosamine and glucosamine residues, CS is particularly attractive for biological and clinic applications due to its nontoxicity, biodegradability, and biocompatibility and muco-adhesive and antimicrobial activity. CS has been studied as a paracellular permeation enhancer for hydrophilic

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molecules and macromolecules because it alters tight junction expression. Other applications have also been investigated, including stimulation of cell differentiation and growth and use as an antimicrobial agent. However, practical use of CS is limited due to poor aqueous solubility at physiological pH.

N,*N*,*N*-Trimethyl chitosan (TMC), the simplest form of quaternized CS, is generally synthesized by reacting CS with excess methyl iodide under strong alkaline conditions with *N*-methyl-2-pyrrolidone as the solvent and sodium iodide as the catalyst (Sieval et al., 1998). It can also be synthesized by treating CS with formaldehyde and formic acid to generate dimethyl chitosan, which is then reacted with methyl iodide at a lower temperature (Verheul et al., 2008). TMC retains the key qualities of CS but has an improved solubility profile, enhanced muco-adhesive properties (Cardile et al., 2008), a significant absorption-enhancing effect over a wide pH range (Hamman, Stander, & Kotze, 2002), and superior antibacterial activity (Jia, Shen, & Xu, 2001).

CS nanoparticles (NPs) were first prepared by Calvo, Remunan-Lopez, Vila-Jato, and Alonso (1997) based on the ionic gelation of CS with sodium tripolyphosphate anions. The hydrophilic CS NPs generally have a long circulation in the blood. Such systems allow control of the rate of drug administration, prolonging the duration of the therapeutic effect, and help deliver drugs to specific sites. Later reports have indicated that entire CS NPs can be taken up by human cells (Yeh, Cheng, Hu, Huang, & Young, 2011), thus significantly enhancing the bioavailability of the encapsulated bioactive molecules.

TMC reportedly forms NPs with various anionic polyelectrolytes such as sodium tripolyphosphate (Paliwal, Paliwal, Agrawal, & Vyas, 2012), alginate (Martins et al., 2013), and heparin (Martins, Pereira, Fajardo, Rubira, & Muniz, 2011). Notably, TMC NPs are central in the drug delivery field due to their excellent physicochemical properties. They deliver insulin (Jin et al., 2012), influenza subunit antigen (Amidi et al., 2007), hepatitis B virus surface antigen (Subbiah et al., 2012), tetanus toxoid (Sayin et al., 2008), peptides (Wang, Jiang, Ma, Hu, & Zhang, 2010), monoclonal antibodies (Vongchan et al., 2011), DNA (Zhao et al., 2010), and RNA (Zhang, Tang, & Yin, 2013).

In this study, we developed a new type of hydrophilic NP with positive (+) or negative (−) surface charge from ChS and TMC in neutral aqueous solution. We investigated the formation zone of these NPs under various addition processes and measured their particle size, zeta potential, formation yield, and water content at various ChS/TMC weight ratios and concentrations. We then evaluated the efficacy of oppositely charged ChS-TMC NPs for the entrapment and controlled release of fluorescein isothiocyanate–conjugated bovine serum albumin (FITC-BSA) in vitro. The ex vivo cytocompatibility and cellular uptake of oppositely charged ChS-TMC NPs were also investigated.

2. Materials and methods

2.1. Materials

CS (viscosity: 3.6 mPa s [5 g/L]; degree of deacetylation: 93.8%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chondroitin 4-sulfate sodium salt from bovine trachea was provided by Fluka (Buchs, Switzerland). FITC, FITC-BSA, and sodium iodide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium, antibiotic solution (100 IU/mL penicillin and 100 μg/mL streptomycin), fetal bovine serum, and phosphate-buffered saline (PBS; pH 7.4) were supplied by Gibco BRL (Corning, NY, USA). Methyl iodide, sodium hydroxide, and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained with Milli-Q equipment (Millipore, Billerica, MA, USA).

2.2. TMC synthesis

TMC was synthesized via a two-step methylation method according to a previous method (Sieval et al., 1998) with minor modification. In step one, 12 g CS, 28.8 g sodium iodide, and 66 mL 15% aqueous sodium hydroxide were mixed in 250 mL *N*-methylpyrrolidinone in a 60 °C water bath. Then, 69 mL methyl iodide was added, and the reaction was carried out with a Liebig's condenser for 90 min. The polymer was collected via precipitation with ethanol and thereafter isolated via centrifugation. The final product was washed with acetone on a glass filter and dried under vacuum.

In step two, the polymer obtained in step one was mixed with 28.8 g sodium iodide and 66 mL 15% sodium hydroxide in 250 mL *N*-methylpyrrolidinone at 60 °C. Methyl iodide (42 mL) was added to the mixture with rapid stirring, and the reaction was carried out with a Liebig's condenser for 60 min. An additional 12 mL methyl iodide and 3.6 g sodium hydroxide pellets were added, and the stirring continued for 1 h. The product was precipitated, washed, and dried as described above.

For the ion-exchange step, the products prepared above were dissolved in 240 mL 10% (w/v) sodium chloride solution (to exchange the iodide ion with chloride) and precipitated with ethanol. The products were repeatedly dissolved in 240 mL water and precipitated with ethanol to remove the remaining sodium chloride. The final products were dried in a vacuum chamber for at least 12 h before further characterization.

To determine the degree of quaternization of the TMC polymers, we obtained ^{1}H nuclear magnetic resonance (NMR) spectra with an NMR spectrometer (AV-500, Bruker, Switzerland) by dissolving samples of the polymers in deuterium oxide at 80 °C with suppression of the water peak. The degree of quaternization was calculated with ^{1}H NMR data according to Eq. (1):

$$\%DQ = \{[(CH_3)_3]/[H] \times 1/9\} \times 100 \quad (1)$$

where %DQ is the degree of quaternization as a percentage, $[(CH_3)_3]$ is the integral of the chemical shift of the trimethyl amino group at 3.3 ppm, and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

2.3. Synthesis of FITC-conjugated TMC (f-TMC)

f-TMC was synthesized according to a method described by Huang, Ma, Khor, and Lim (2002) with minor modification. Briefly, TMC was dissolved in ultrapure water. FITC dissolved in dimethyl sulfoxide was slowly added to the TMC aqueous solution under continuous stirring. The reaction was carried out overnight at room temperature in the dark. The resulting solution was poured into an excess of acetone and centrifuged for 10 min at 3000 rpm. The pellet was washed several times with fresh acetone until no FITC fluorescence was observed in the washing solution. The pellet was then dissolved in water and dialyzed against water with a dialysis cassette (Slide-A-Lyzer®, G2, 2000 MWCO, 70-mL capacity) for 3 days while protected from light. The f-TMC was then lyophilized. To determine the labeling efficiency, we dissolved a specified amount of f-TMC in ultrapure water and measured the fluorescence intensity with a spectrofluorometer (Fluoroskan Ascent, Thermo, Finland) at λ_{exc} 485 nm and λ_{emi} 520 nm. The spectrofluorometer was calibrated with standard solutions of 0.0625–1 mg/mL FITC in the same solution.

2.4. Investigation of ChS-TMC NP aggregation zones

The charged ChS-TMC NPs were prepared with a method based on the polyelectrolyte complexation (PEC) of anionic polyelectrolyte ChS and cationic polyelectrolyte TMC. Preliminary