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Fabrication and characterization of a self-crosslinking chitosan hydrogel under mild conditions without the use of strong bases

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ABSTRACT

Self-crosslinking chitosan hydrogels are a highly suitable material for biomedical applications owing to their biodegradability and biocompatibility. However, strong bases, such as sodium hydroxide, which are often used in the preparation of such hydrogels, are known to affect biocompatibility and even destroy the bioactive factors or drug payload of the hydrogel. In the present study, strong bases were replaced by sodium chloride (NaCl) and phosphate buffer saline (PBS, pH = 7.4), which were used as gelling solutions for hydrogel fabrication via the freeze-melting-neutralization method. Non-cytotoxicity was showed in MTT assay for hydrogel. Our findings suggest that hydrogel microstructure and physical properties may be adjusted by modifying parameters, such as concentration, temperature, and pH, during the gelling process. Furthermore, the present hydrogel was found to exhibit pH-and ionic strength-responsive properties and may be utilized as a stimulus-responsive material for biomedical applications such as controlled drug release.

1. Introduction

Chitosan, derived from the partial deacetylation of natural chitin, is a linear, semi-crystalline polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units (Rinaudo, 2006). Chitosan exhibits remarkable intrinsic properties, such as biocompatibility, biodegradability, and sterilization, which make it an outstanding candidate for biomedical applications (Bhattarai, Gunn, & Zhang, 2010; Domard, 2011; Sashiwa & Aiba, 2004; Silva, Juenet, Meddahi-Pellé, & Letourneur, 2015; Zhang et al., 2012). Chitosan hydrogels, which are networks of cross-linked three-dimensional polymers, are the most widely used biomaterials for drug delivery, gene delivery, and tissue engineering. For example, chitosan/glycerophosphate thermosensitive hydrogels (Hui, Ling, Pei, Li, & Xi, 2015) and chitosan/tripolyphosphate micro/nanogels (Abd-Allah, Kamel, & Sammour, 2016) have been widely studied and improved for local drug delivery and tissue engineering. Bulk chitosan hydrogels/scaffolds have also been studied for biomedical applications. In particular, they have been used to study the effect of hydrogel structure and properties on cell behavior in vitro and in vivo, with the ultimate goal of tissue regeneration. (Berger, Reist, Mayer, Felt, Peppas et al., 2004; Drury & Mooney, 2003)

Chitosan hydrogels have been fabricated in three different ways: via physical association (ionic crosslinking), coordination with metal ions, or irreversible/chemical cross-linking between chitosan and the crosslinker (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Xu et al., 2013; Yang et al., 2012). In particular, chitosan hydrogels fabricated by physical association, without an external cross-linker, exhibit enhanced biocompatibility. The primary aliphatic amines (pK_a = 6.3) of chitosan may be protonated under acidic conditions, which renders the molecule fully soluble (Bhattarai et al., 2010). Consequently, the chitosan solution forms entangled hydrogels via secondary interactions (Van der Waals interactions and hydrogen bonds) resulting from the increase in pH (Berger, Reist, Mayer, Felt, & Gurny, 2004; Domard, 2011; Ho et al., 2004a, 2004b; Hsieh et al., 2007; Silva et al., 2015). For instance, bulk hydrogels are formed in a gelation solution consisting of ethanol (C_2H_5OH) and sodium hydroxide (NaOH), at a temperature below the freezing point of frozen chitosan solution, in order to induce gelation(Ho et al., 2004b). Furthermore, multilayered bulk hydrogels are fabricated by exploiting the pH-dependent solubility of chitosan via periodic neutralization of the chitosan solution with NaOH (Dash et al., 2011; Ladet, David, & Domard, 2008; Montembault, Viton, & Domard, 2005). Such multilayered hydrogels may be used to encapsulate molecules for the co-delivery of multiple drugs, pulse-like

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delivery of a specific payload, or as chondrocytic cell bioreactors (Suh & Matthew, 2000). In our previous work, multilayered chitosan hydrogels were prepared via a controlled freeze-melting-neutralization method (Xu, Han, & Lin, 2016; Yongxiang, Jianmin, & Hong, 2016).

Strong bases such as NaOH and ammonium hydroxide (NH_4OH) were often used as neutralization solutions to increase pH in the preparation of bulk hydrogels. However, there are two main limitations associated with the use of strong bases: (1) strong bases are difficult to wash out completely and the presence of residual base results in a decrease in biocompatibility of the hydrogel; (2) strong bases are known to destroy the bioactive factors or drug payload. Consequently, the aim of this study is to fabricate a chitosan-based physical hydrogel by utilizing the pH- and ionic strength-dependent solubility of chitosan, without the use of strong bases. The influence of the preparation conditions on hydrogel structure, properties, and release of bioactive protein are examined.

2. Material and methods

2.1. Materials

Chitosan was purchased from Beijing HWRK Chem Co., LTD, China. Acetic acid (CH_3COOH), NH_4OH, bromothymol blue, phosphate buffer saline ($1\times$ PBS: 0.068 g of disodium phosphate dodecahydrate ($NaH_2PO{\cdot}12H_2O$) + 0.126 g of sodium hydrogen phosphate dehydrate ($Na_2HPO{\cdot}2H_2O$) in 100 mL de-ionized water, pH adjusted with 0.1 M hydrochloric acid (HCl) or 0.1 M NaOH), sodium acetate (CH_3COONa), sodium chloride (NaCl), and bovine serum albumin (BSA) were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd, China. All other reagents and solvents were analytical grade and used without further purification.

2.2. Purification and characterization of chitosan

Briefly, a chitosan solution was prepared in CH_3COOH aqueous solution and sequentially filtered through membranes. The filtrate was precipitated with dilute NH_4OH and centrifuged, then repeatedly rinsed with de-ionized water and centrifuged until a neutral pH was achieved before dispersion in water and freeze-drying.

The average viscosimetric molecular weight ($\bar{M}_\eta$) of chitosan was 187 kDa, as determined using an Ubbelohde viscometer with the Mark-Houwink-Sakurada equation: $[\eta] = 0.0843\bar{M}_\eta^{0.78}$. The degree of de-acetylation of the chitosan was 89.8%, as measured by ATR-FTIR spectroscopy using a Nicolet iN10 spectrometer (Thermo Scientific) (Brugnerotto et al., 2001).

2.3. Preparation of chitosan hydrogel

Chitosan was dispersed in de-ionized water and a stoichiometrically equivalent amount of acetic acid was added. After complete dissolution, the solution was left to stand for 24 h without stirring for degassing at 4 °C. The solution was injected into a cylindrical mold (φ 9.0 × 9.0 mm) and frozen at −20 °C for 24 h. The frozen chitosan solution was then immersed in gelling solution at 4 °C for 48 h. The gelling solution was composed of PBS (pH = 7.4), NaCl, or both PBS and NaCl.

2.4. Gelation of chitosan hydrogel

In order to examine the gelation process, bromothymol blue was added to the chitosan solution as a pH indicator. After freezing, the bromothymol blue-containing chitosan solution was fully immersed in the gelling solution in a rectangular quartz cuvette. Images were captured from the side of the cuvette.

The gelation time was determined by morphologic observation of samples at different time points. Briefly, one sample was removed at each time point and then cut in half to observe the morphology and color of the whole sample. A change in morphology from solution to gel indicated complete salting-out of the gel. A change in color from yellow to blue indicated complete neutralization of the gel.

2.5. Structure of chitosan hydrogel

Images of hydrogels forming under various conditions were captured using a digital camera (EOS 5D Mark II, Canon, Japan). Furthermore, the liquid-nitrogen fracture surfaces of the hydrogels were characterized using a Scanning Electron Microscope (SEM, EVO 18, Zeiss, Germany).

2.6. Physico-mechanical characterization of chitosan hydrogel

In order to investigate material performance under pseudo-physiological conditions, the chitosan hydrogel was washed three times with $1\times$ PBS (pH = 7.4) and then stored in $1\times$ PBS (pH = 7.4) for 24 h before further testing. All experiments were repeated three times.

2.6.1. Water content (W_c)

The weight of the chitosan hydrogel (W_h) was measured using an electronic balance after rapidly drying the surface with a filter paper. The weight of the dry hydrogel (W_d) was measured after lyophilization, according to the following equation:

$$W_c = W_h - W_d$$

2.6.2. Young's modulus (E)

The mechanical properties, i.e., Young's modulus (E), of the chitosan hydrogel were determined via compression testing, at a rate of 5 mm/min and a temperature of 23 ± 2 °C, using a universal material testing machine (model 5543A, Instron, USA).

2.6.3. Stimulus-responsive properties

The stimulus-responsive properties of the hydrogel were studied. Each sample was immersed in 10 mL $1\times$ PBS solution with various pH values (pH = 3.0, 5.5, 7.4, and 9.0) and solutions with varying ionic strengths (de-ionized water, $1\times$ PBS + 0.9% NaCl) for 24 h. Next, the changes in W_c and E were measured.

2.7. Cytotoxicity assays

The cytotoxicity of the hydrogels was evaluated using hydrogel extracts and L929 fibroblast cells (ATCC, Manassas, VA, USA) following ISO 10993-5:2001 standards. The test hydrogel extract was prepared with amounts of 0.5, 1.0 and 1.5 mg of chitosan in 1 mL of MEM at 37 °C for 24 h. The L-929 cells (ATCC) were cultured at a density of 1×10^5 cells/well in MEM with 10% FBS at 37 °C and 5% CO_2 for 24 h. The culture medium was then removed and replaced with medium containing the extract. After 24 h, 50 μL of MTT solution (1 mg/mL in PBS) was added to each well, and the cells were incubated at 37 °C for 2 h. The optical intensity was then measured at a wavelength of 570 nm using a microplate reader (Model 680, Bio-Rad, USA).

The blank culture medium and 10% DMSO were used as negative and positive controls, respectively. The cytotoxicity of the hydrogel was expressed as% cell viability, which was calculated from the ratio between the number of cells treated with the polymer solutions and that of non-treated cells (control). The experiments were performed in triplicate.