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Preparation of nanosize alginate gel using pluronic-based nano-carrier as a template

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ABSTRACT

Nanosize (~50 nm) alginate gel was made by loading alginate into the pluronic nano-carrier, followed by adding calcium ions to induce calcium ion-induced crosslinking inside the nano-carrier without using any organic solvent during the preparation. The alginate gel formation inside the nano-carrier did not alter the size or the surface charge of the nano-carrier, and the residence of alginate inside the nano-carrier in physiological buffer was greatly increased by calcium ion complexation. Also, this complex nanogel could be re-dispersed after lyophilization without aggregation. In addition, proteins were efficiently loaded into the alginate gel-filled nano-carrier by simple co-incubation at 4 °C, followed by increase to 37 °C, and this alginate gel-filled nano-carrier showed prolonged release of proteins over one month while preserving the bioactivity of proteins. Therefore, pluronic-based nanogel was effective for easy preparation of nanosize alginate gel in it. The presence of pluronic shell of the composite nanogel did not hinder the loading or sustained release of positively charged proteins from negatively charged alginate gel inside, but rather provided the neutralization of surface charge and enhanced stability of the nanosize alginate gel.

1. Introduction

Polymeric nanogels are useful in a variety of nanotechnology fields such as drug delivery (protein and gene delivery), nanobiomaterials, and biomedical applications due to a wide range of nanosized structure with dual features as nanoparticle and hydrogel [1]. Nanogels are nanosized hydrogels formed by physical or chemical crosslinking [2], and have many advantages including excellent biocompatibility, escaping phagocytosis, controlled pore size by crosslinking density, and preservation of bioactivity of loaded biomacromolecules, as well as characteristics of nanosized materials such as enhanced permeation and retention (EPR) effect, a preferential accumulation of nanomaterials in tumor tissue than normal tissue via a leaky vascular structure in tumor tissue, and large surface to volume ratio [3].

Despite the benefits of polymeric nanogels, bioapplications of polymeric nanogels have some limitations due to the methods for making nanogels. For examples, emulsion method, the most common method to make nanogels as well as nanoparticles and microparticles, employs water–oil mixture or excess amount of surfactants to form nanosized structure of polymers or polymer precursors [4–11]. Because of strong interaction between the nanogels and the organic phases, removal of surfactants and oil from the nanogels in emulsion method is difficult. Furthermore, the presence of organic-water interface and the purification of nanogels to remove surfactants and oil phase usually lead to the loss and reduction of drug amount and especially the bioactivity of biomolecular drugs (proteins and genetic materials). Nanoprecipitation method, based on the different polymer solubility between water and water miscible solvent to

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form the nanosized structure [12–15], is another approach to make nanogels that can avoid remaining organic solvent in produced nanogel. To maintain the nanogel structure during the removal of water miscible solvent by repetitive washing, nanoprecipitation method requires chemical cross-linking. However, to get a small size and narrow distribution of nanogel, polymers or polymerization precursors should be prepared in a very dilute condition. Also, mixing of water and water-miscible organic solvent and chemical crosslinking reaction induce the problem in maintaining bioactivity of biomolecular drugs similar to emulsion method. Therefore, the existing methods of making polymeric nanogels have intrinsic limitations for bioapplications, specifically for biomolecular drug delivery due to the use of organic solvents and chemical reaction that can easily denature the biomolecules.

Alginate is an anionic polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid [16]. Alginate hydrogel, formed by ionic crosslinking in the presence of divalent cations such as calcium ion, have been used widely for biomedical applications due to their advantages such as non-toxic and non-immunogenic characteristic, and relatively easy and mild fabrication condition without using organic solvent to prevent the loss of biological activity of encapsulated proteins and cells [17–20]. Selective binding of divalent ions into guluronic acid units in alginate strands, known as an egg-box structure [21], induces the gel formation. Alginate gels are degraded in the presence of chelating agent or media without divalent cations such as physiological situations. Using the negatively charged state of alginate gels, sustained release of positively charged proteins or drugs can be obtained while keeping their bioactivities [17,22,23]. As a result, alginate gels have been applied for protein delivery [24]. However, in most cases, alginate gels are prepared as microbeads [25–28], thus do not keep the advantages as a nanogel such as EPR effect. It is not easy to prepare nanosized alginate gel, so few approaches using quite complicate processes have been suggested [29,30].

In this study, we have employed a pluronic-based nano-carrier as a template to make alginate gel in it. The pluronic-based nanocarrier, formed by photo-crosslinking diluted diacrylated pluronic, shows a large volume expansion by lowering the temperature [31] and has many advantages as a delivery vehicle including such as serum stability and good biocompatibility as well as long blood circulation time *in vivo* [31–33]. We have demonstrated a simple method of preparing nanosized alginate gel inside this pluronic-based nano-carrier in all aqueous environment without chemical reaction, and characterized the composite nanogel as a protein delivery system.

2. Materials and methods

2.1. Materials

Pluronic F127 (PEO100PPO65PEO100, MW 12.6 kDa) was a kind donation from BASF Corp. (Seoul, Korea). Acryloyl chloride, triethylamine, anhydrous toluene, calcium chloride, 1,9-dimethyl methylene blue (DMMB), potassium phosphate monobasic, sodium phosphate dibasic, potassium chloride, sodium chloride, sodium azide, and lysozyme from chicken egg whites were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anhydrous diethyl ether was purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA). Ethanol absolute for analysis EMSURE® was purchased from Merck KgaA. (Darmstadt, Germany). 4-(2-hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure 2959) was purchased from Ciba Specialty Chemicals Inc. (Basel, Switzerland). Ultrapure sodium alginate (PRONOVA UP VLVG, from *Laminaria hyperborean,* whose guluronic/mannuronic acid (G/M) ratio is more than 1.5 and molecular weight is less than 75 kDa) was purchased from FMC BioPolymer AS/NovaMatrix (Sandvika, Norway). Citric acid monohydrate was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Phosphate buffered saline (PBS) was prepared to have 1.47 mM potassium phosphate monobasic, 10 mM sodium phosphate dibasic, 2.7 mM potassium chloride, 137 mM sodium chloride, and 2 mM sodium azide (pH 7.4) in de-ionized water (DIW). 0.2 μm cellulose acetate syringe filter was purchased from Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). 0.2 μm nylon and 0.45 μm polypropylene (PP) syringe filter were purchased from Whatman International Ltd. (Florham Park, NJ, USA). A cellulose ester dialysis bag (MWCO 300,000) was purchased Spectrum (Houston, TX, USA).

2.2. Preparation of pluronic-based nano-carrier

Pluronic-based nano-carrier was synthesized by photo-crosslinking a diluted diacrylated pluronic solution, as previously described [31]. Diacrylated pluronic F127 was prepared by reacting pluronic with acryloyl chloride [34,35]. First, 5 g of dried pluronic were acrylated by reacting with a 10-fold molar excess of acryloyl chloride and triethylamine in 100 ml of anhydrous toluene with stirring under argon overnight. After reaction, this polymer was precipitated in cold anhydrous diethyl ether, filtered, and dried under vacuum. The degree of substitution of acrylation in pluronic was over 98% by comparing the acryl protons (═CH_2, 5.7–6.4 ppm) and methyl protons (—CH_3, 1.1 ppm) in 400 MHz ^{1}H NMR spectroscopy (D_2O, JNM-ECX-400P, JEOL, Japan). Next, diacrylated pluronic was dissolved in DIW at 10 wt% with filtering through 0.2 μm cellulose acetate syringe filter, and then was diluted with DIW to 0.77 wt% of diacrylated pluronic. A photoinitiator solution was prepared by mixing Irgacure 2959 and 70 v/v% ethanol with filtering through 0.2 μm nylon syringe filter. This photoinitiator solution was added to the diluted diacrylated pluronic solution to make 0.05 wt% photoinitiator concentration. Finally, this diluted diacrylated pluronic solution containing photoinitiator was UV-irradiated by using an unfiltered UV lamp (VL-4.LC, 8 W, Vilber Lourmat, France) for 15 min with 1.3 mW cm^{-2} intensity for nano-carrier formation.

2.3. Preparation of alginate gel-filled nano-carrier

Alginate gel-filled nano-carrier was prepared, as described in Fig. 1. First, alginate was dissolved in DIW to make 1 w/v% solution. 14 mg of lyophilized nano-carrier was added into 1 ml of alginate solution, mixed, and then