Polymer 115 (2017) 261–272

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Cross-linked polypeptide-based gel particles by emulsion for efficient protein encapsulation

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ARTICLE INFO

Article history:
Received 12 January 2017
Received in revised form
16 March 2017
Accepted 21 March 2017
Available online 22 March 2017

Keywords:
Polypeptide-based microgel/nanogel
Protein encapsulation/delivery
Stimuli-responsive

ABSTRACT

We report the preparation of cross-linked poly(L-lysine) (PLL), polyethylene glycol-*block*-poly(L-lysine) (PEG-*b*-PLL), and poly(L-lysine)-*block*-polysarcosine (PLL-*b*-PSar) gel particles with size ranged between 100 and 250 nm by emulsion and their evaluation as carriers/encapsulants for protein encapsulation/delivery. Their size and solution property were depended upon polymer molecular weight, composition, and cross-linking degree. These gel particles exhibited not only excellent colloidal stability at a wide range of solution pH but also enhanced biocompatibility due to the incorporation of neutral polymer segments and genipin cross-link. We also showed that a variety of protein can be efficiently encapsulated in gel particles and the encapsulated protein still exhibited bioactivity. The *in vitro* protein release study showed that reduction-responsive gel particles can be prepared by incorporating disulfide bond in gel network. This study demonstrated that versatile gel particles comprising various types of polymers can be prepared and are potentially useful as protein carriers and encapsulants.

1. Introduction

Hydrogels, which exhibited polymeric networks stabilized through physical or chemical cross-links, are promising materials and widely investigated in many biomedical fields [1–7]. Recently, efforts have been devoted to prepare gel particles, which are gel-like sub-micro-/nano-entities, by combining the features of hydrogel and nanoparticle (NP) and explore their potential applications [8–11]. These gel particles, which are also called microgels or nanogels, were mainly prepared by heterogeneous polymerization of monomers, self-assembly of amphiphilic copolymers, or physical or chemical cross-linking of hydrophilic polymers in a non-continuous heterogeneous phase. Chemically cross-linked gel particles were found to be structurally stable as comparing to non-covalent bond associated counterparts. Gel particles exhibited advantages including excellent biocompatibility, high water content and porosity, and tunable swelling properties, making them ideal for encapsulation of bioactive agents such as drugs and proteins. While a variety of biocompatible natural and synthetic polymers have been utilized to prepare gel particles [4,6,8,10,12], synthetic polymers exhibited the flexibility to tune the properties of gels via versatile macromolecular chemistry.

Due to the requirement of biocompatibility/biodegradability for biomedical applications, gel particles comprising natural building blocks including saccharides and amino acids can be one way to meet the requirement. The preparation of polypeptide-based gel particles has been mainly focused on the self-assembly of amphiphilic block or graft copolymers via hydrophobic interactions and the polyionic complexation of charged polypeptides via electrostatic interactions [13–17]. Both approaches are based on phase separation during the preparation procedure to obtain preformed NPs, followed by cross-linking. This would lead to the cross-link only at the surface of the NPs due to the inaccessible of the cross-linking agent to the interior of the NPs. Using emulsion can circumvent this drawback to obtain polypeptide-based gel particles with homogeneous cross-linking since the cross-linking reaction occurred without phase separation during the preparation procedure [12,18]. Additionally, the homogeneous, hydrophilic domains resided in gel particles can afford the efficient encapsulation of bioactive molecules such as proteins.

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http://dx.doi.org/10.1016/j.polymer.2017.03.055
0032-3861/

Herein, we report the feasibility of preparing cross-linked gel particles based on polypeptides by emulsion and their evaluation as protein carriers/encapsulants. A series of polypeptide-based polymers including poly(L-lysine) (PLL), polyethylene glycol-*block*-poly(L-lysine) (PEG-*b*-PLL), and poly(L-lysine)-*block*-polysarcosine (PLL-*b*-PSar) were synthesized via ring-opening polymerization (ROP) and used for the preparation of genipin cross-linked gel particles. Genipin, which is a natural, non-toxic crosslinking agent found in gardenia fruit extract, was selected to cross-link PLL in order to improve the stability and biocompatibility of gel particles, which would be advantageous for protein encapsulation. Previously, we have shown that chemically cross-linked polypeptide hydrogels exhibiting biocompatibility and biodegradability can be prepared by genipin cross-link and the incorporation of hydrophobic segment can significantly enhance the mechanical properties and enzymatic degradability of hydrogels [19,20]. It is expected that the incorporation of neutral, hydrophilic segment such as PEG or PSar can not only influence the solution properties and molecular structure of the as-prepared gel particles but also provide non-cross-linked, hydrophilic domains for protein encapsulation, which might effectively retain their bioactivity. The encapsulation of protein in cross-linked gel particles via emulsion can afford high encapsulation efficiency without causing the denaturalization of proteins. The influence of polymer chain length, composition, and cross-linking degree on the size, gel network, and solution properties of the gel particles was investigated. The biocompatibility of these gel particles was evaluated through investigating their cytotoxicity toward fibroblast 3T3 cells. To demonstrate the feasibility of using these gel particles for protein encapsulation, three proteins, which are bovine serum albumin (BSA), myoglobin (Mb), and lectoferrin (LTF) were encapsulated in these gel particles using emulsion. The bioactivity of the encapsulated protein was evaluated to demonstrate their feasibility as protein encapsulants. For drug/protein delivery, reduction-responsive gel particles have been designed for intracellular cargo release since the cytoplasm and cell nucleus exhibited a high level of glutathione (GSH) [18,21–23]. In this study, reduction-responsive gel particles were also prepared by using a cross-linking agent containing a disulfide moiety and their feasibility as protein carriers was evaluated by investigating the *in vitro* protein release upon cleaving the disulfide bond.

2. Experimental section

2.1. Materials

N_ε-Z-L-lysine (~99%), sarcosine, methoxypolyethylene glycol amine (PEG—NH_2, molecular weight: 2000 and 5000 g/mol) and hexyl amine were used as received from Sigma-Aldrich, as well as hydrogen bromide (HBr, 33 wt% in acetic acid), triphosgene (98%), and anhydrous dimethylformamide (DMF). Trifloroacetic acid (TFA, 99%) was purchased from Alfa Aesar. Tetrahydrofuran (THF, ACS Reagent, Merck) and hexane (ACS Reagent, ECHO) were dried using Na metal (99.95%, in mineral oil, Aldrich) and calcium hydride (95%, Aldrich), respectively.

2.2. Synthesis of Z-L-lysine (ZLL) and sarcosine (Sar) N-carboxyanhydrides (NCA)

Z-L-lysine *N*-carboxyanhydrides (ZLL NCA) was synthesized by following the literature reported procedures [24]. Sarcosine (Sar) NCA was synthesized by following the literature reported procedures with modified procedures [25,26]. Specifically, sarcosine (3.08 g, 34.6 mmol) and triphosgene (3.64 g, 12.3 mmol) were dried separately under vacuum for at least 2 h. Anhydrous THF (73 mL) and (+)-limonene (7.34 mL, 45.8 mmol) were added to sarcosine in a glove box. Then triphosgene was dissolved in anhydrous THF (18 mL) and added to the suspension. The reaction mixture was taken out of the glove box and heated to 65 °C under stirring. The colorless suspension turned to a clear solution after 2 h of stirring. The solvent was evaporated under reduced pressure, yielding a brownish oil. The oil was heated to 80 °C and dried under reduced pressure to obtain a brownish solid. The crude product was dissolved in anhydrous THF (15 mL) and precipitated with anhydrous hexane (100 mL). This mixture was cooled to −20 °C overnight to complete precipitation. The solid was filtered under argon atmosphere and dried under reduced pressure. The solid was sublimated at 85 °C under vacuum and the product was collected from the sublimation apparatus. The purified product were handled in a glove box and immediately used for polypeptide synthesis.

2.3. Synthesis of poly(L-lysine) (PLL) and polyethylene glycol-block-poly(L-lysine) (PEG-b-PLL)

Poly(Z-L-lysine) (PZLL) was synthesized using the zero valent nickel initiator 2,2-bipyridyl-Ni(1,5-cyclooctadiene) (BpyNiCOD) to polymerize ZLL NCA by following the literature reported procedures [17,27]. PEG-*b*-PZLL block copolymers were synthesized by using PEG—NH_2 as the macroinitiator and the feed molar ratios of the macroinitiator to NCA were to set to be 1:25 and 1:50. From the given ZLL NCA/PEG—NH_2 molar ratio, ZLL NCA and PEG—NH_2 were weighted out and dissolved in anhydrous DMF in a glove box. The PEG—NH_2 solution (0.013 M) was added to the ZLL NCA solution (0.32 M). The mixture was taken out of the glove box and stirred for 3 days at room temperature. The resulting solution was then placed in a cellulose membrane dialysis tube (MWCO 3500 g/mol) and dialyzed against methanol for 1 day and subsequent against deionized (DI) water for 3 days. The block copolymer was freeze-dried to give a white solid. The Z protecting group on PZLL and PEG-*b*-PZLL was removed by HBr [28]. PZLL or PEG-b-PZLL (0.3 g) was dissolved in TFA (30 mL) in a 100 mL flask. The excess 33 wt% HBr in acetic acid (5: 1 HBr/Z-Lys molar ratio) was then added to the solution via syringe. The solution was left to stir for 20 min and then diethyl ether (80 mL) was added to precipitate the polypeptide. The polypeptide was collected via centrifugation, dried in vacuum, and dissolved in DI water (40 mL). Once the polypeptide was in DI water, the solution was transferred to a dialysis tubing cellulose membrane (MWCO 3500 g/mL) and the water was exchanged two to three times per day over the next 3 days. The solution was freezed using liquid nitrogen and then placed on a freeze dryer to yield the product as a white spongy material.

2.4. Synthesis of poly(L-lysine)-block-polysarcosine (PLL-b-PSar)

PZLL-*b*-PSar block copolypeptides were synthesized by sequential polymerization of ZLL and Sar NCAs using hexyl amine as the initiator. ZLL NCA and hexyl amine were weighted out and dissolved in anhydrous THF in a glove box. The hexyl amine solution (6.6 mM) was added to the ZLL NCA solution (0.26 M). The mixture was taken out of the glove box and stirred for 3 days at room temperature. Then Sar NCA was weighted out and dissolved in anhydrous THF in a glove box. The Sar NCA solution (0.26 M) was added to the reaction mixture and stirred for additional 3 days at room temperature. The PZLL-*b*-PSar block copolypeptide was isolated by adding diethyl ether to the reaction mixture, causing precipitation of the polypeptide. The polypeptide was dried in vacuum to give a white solid. The Z protecting group on PZLL-*b*-PSar was removed by HBr [28]. The block copolypeptide was completely dissolved in TFA followed by addition of excess 33 wt% HBr in acetic acid (1.05: 1 HBr/Z-Lys molar ratio) and the reaction mixture was left to stir for another 12 h [25]. Then the PLL-*b*-PSar