A novel microgel and associated post-fabrication encapsulation technique of proteins

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Abstract

A novel negatively thermo-sensitive and biodegradable microgel was prepared by combination of macromer synthesis and inverse suspension polymerization. A new post-fabrication encapsulation technique based upon this kind of intelligent microgel was developed. Model proteins (hemoglobin, bovine serum albumin and insulin) were encapsulated into the microgels at 4 °C and released in vitro at 37 °C. Relatively high loading levels and sustained release profiles demonstrate the feasibility of the encapsulation strategy. Since the encapsulation of proteins was performed at low temperature and after the preparation of microgels, organic solvent and high temperature were completely avoided in drug encapsulation. FTIR, Raman and circular dichroism measurements confirmed that the ordered structure of proteins was not destroyed during encapsulation and after release. Thus, the post-fabrication encapsulation technique in this paper is much unique and beneficial for controlled release of biomacromolecular drugs.

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1. Introduction

In recent years, microparticles have been one of the most important drug carriers. Although much progress has been made in the field of pharmaceutics, the protein drug delivery is still quite challenging. The biomacromolecules such as proteins are sensitive to their environment and easily degraded; thus, topical parenteral delivery system is the main way so far. A principal advantage of entrapment of these biomacromolecules in microparticles is that microparticles enable administration via injection and fabrication from a biodegradable polymer eliminates the need for surgical removal. The commonly used materials are poly(L-lactic acid) (PLLA) and its copolymers with D-lactic acid or glycolic acid, which provide a wide range of degradation periods from weeks to years [1–4]. As usual, researchers utilize solvent evaporation, or modification thereof, to prepare micro-
particles in the field of controlled release [5–7]. However, most of present microparticle preparation methods may jeopardize proteins by exposing proteins to potentially damaging conditions, such as organic solution, elevated temperature, vigorous agitation and detergents.

Hydrogels have been intensely investigated as protein drug vehicles for recent decades. Compared with hydrophobic biomaterials such as poly(D,L-lactide-co-glycolide) (PLGA) [8], hydrogels more closely resemble natural living tissues due to their high water contents, soft and rubbery consistency, and minimize irritation to surrounding tissues [9–11]. Furthermore, a highlight of hydrogel is that the drug loading and release can be controlled by environment stimuli in the so-called intelligent hydrogels [12–15]. Microgels, i.e. microhydrogels combining the advantages of microparticles and hydrogels as drug delivery systems, may enable the release of proteins in a specified area. So far, many publications can be found about biomaterials for drug release, which, nevertheless, either were just biodegradable hydrogels [16,17] or just intelligent microparticles [18,19]. It is noticeable that there are rare reports about a material combining the properties of intelligence, injectability, degradability and biocompatibility [20–22].

Besides biomaterials, the loading strategy of proteins is also much important. It is well known that drugs can be included by forming a gel in the presence of the protein or by soaking a gel in a protein solution.

Fig. 1. Schematic presentation of a post-fabrication encapsulation strategy of protein drugs based upon an “intelligent” and “biodegradable” microgel. Microgels were obtained from cross-linking the pre-designed macromers. After microgel preparation, drug loading was performed by soaking aqueous solution of proteins with microgels at 4 °C (below volume phase transition temperature). The gel microparticles collapsed after drying and thus entrapped proteins in the microgel network. When released in PBS at human body temperature, striking early release of protein was decreased because of the shrinkage of microgels at 37 °C with respect to at 4 °C. The drug release proceeded with hydrolysis of microgels and underlying diffusion. All of proteins were eventually released due to complete biodegradation of the polymeric network.
The former method may denature proteins caused by impurities or byproducts from gel formation. As the latter approach is concerned, proteins are, due to size-exclusion, usually excluded from gel networks [24]. Due to this limitation, the loading levels achieved by the latter method are often less than 0.1 wt.% [25] as summarized in literature [23,26]. On the other hand, the burst release is very serious if the post-fabrication loading is easy.

In order to avoid protein-denaturing factors such as organic solvent and high temperature in most of the present microparticle techniques and meanwhile to resolve the dilemma of loading and release in hydrogel encapsulation techniques, a novel negatively thermo-sensitive and biodegradable microgel was prepared by inverse suspension polymerization in this work. The volume phase transition temperature is between 4 °C (around the refrigerator temperature) and 37 °C (human body temperature). Proteins were encapsulated into the network of the microgels at 4 °C, which led to highly swollen hydrogels for relatively high loading and possibility of preserving spatial structures of proteins during encapsulation. At human body temperature, protein was entrapped into relatively less swollen hydrogels, which might avoid very striking burst release. The complete drug release may be achieved by degradation of the network.

The microgel and associated post-fabrication encapsulation is schematically presented in Fig. 1. To our knowledge, such a microgel and underlying post-fabrication encapsulation technique based upon this kind of hydrogel microparticles have not yet been reported in literature. This work provides a novel carrier for protein drugs and a unique way for controlling the loading and release of protein drugs.

2. Experimental

2.1. Materials

L-Lactide was obtained from Purac Company and was recrystallized from ethyl acetate. Acryloyl chloride was synthesized by ourselves and distilled before use. Ammonium persulfate was recrystallized before use. Pluronic F127, a triblock copolymer of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) is a Sigma product with chemical formula of EO99–PO65–EO99 (the number in the subscript denotes the associated averaged degree of polymerization). Stannous octoate (Aldrich) was used as purchased. All other chemicals were of reagent grade and were used without further purification. Hemoglobin (Hb), bovine serum albumin (BSA) and insulin were obtained from Sigma Chemical Co. Phosphate buffer saline (PBS) solution is composed of the following: 8.5 g/l of NaCl, 0.2 g/l of KCl, 2.85 g/l of Na$_2$HPO$_4$·12H$_2$O, 0.27 g/l of KH$_2$PO$_4$ in deionized water.

2.2. Preparation of microgels

The microgels were synthesized from the thermo-sensitive and biodegradable macromer. We adopted the method of Sawhney et al. [27] to prepare the Pluronic-LA$_2$-DA macromer. The difference of our macromer is that the central part in our work is Pluronic F127 instead of PEG. Therefore, our macromer and the resulting microgels are thermo-sensitive. The chemical structure of the macromer in this work is shown in Scheme 1.

Briefly, the macromer was synthesized in a two-step process, ring opening polymerization and diacrylation. The macromer products were preserved at low temperatures (around 4 °C).

Scheme 1. Chemical structure of the F127-LA$_2$-DA macromer. The central part of Pluronic F127 is a triblock copolymer of PEO-PPO-PEO. LA$_2$ indicates that there are, on average, about two lactic-acid repeating units at each end of the F127 block copolymer based upon the feed molar ratio. DA denotes di-acryloyl groups, namely, each acryloyl group at one end of F127-LA$_2$ block copolymer.
temperature under argon. The macromer structure was characterized by Fourier transform infrared spectrometer (FTIR, Nicolet Magna-550) and 500-MHz proton NMR spectrometer (Bruker, DMX500). The degree of polymerization (DP) of lactic acid (2) shown in Scheme 1 is based on the feed molar ratio. The actual value is 1.9 as determined by $^1$H NMR.

The inverse suspension polymerization was employed to prepare microgels. The reactions were performed in an inert atmosphere of nitrogen in a 250-ml four-neck flask fitted with a reflux condenser and a mechanical Teflon stirrer. The continuous phase comprised 120 ml of heptane and 4.2 g emulsifier Span 60. The dispersed phase was 5 ml of 16.7% w/w of the macromer aqueous solution with ammonium persulfate (APS) and ascorbic acid (AA) as the initiator system. The flask with continuous phase was heated to $70 \degree C$ using an oil bath, the aqueous dispersed phase was added dropwise at a rate of 1–2 drops/s into the flask. (3 wt.% APS and 2.3 wt.% AA based on the amount of the macromer were dissolved in the macromer solution 30 s before adding the dispersed phase into the flask.) The reaction proceeded for 1 h at a stirring speed of 400 rpm. The resulting microgels were separated from the solvent with a standard sieve. The sieved microgels were washed firstly with acetone, followed by deionized water. After drying, the microparticles were preserved in a desiccator at 4 $\degree C$.

2.3. Thermo-sensitivity of microgels

The morphology of the swollen microgel was observed in an optical microscope equipped with a temperature-controlling device (Olympus BX51). The microgels were put into water in a glass vessel and kept at the predetermined temperature for 3 min and then the temperature was raised at a rate of 5 $\degree C$/min. The volumes of the microgels were calculated from the images, which were taken by a digital camera. The volume change was used to evaluate the thermo-sensitivity.

2.4. In vitro degradation of microgels

About 20 mg of the dried microgels were suspended in 5 ml of PBS at pH 7.2 and placed into an incubator at 37 $\degree C$ with 100 rpm shaking speed. At predetermined time intervals, the samples were separated from medium with a polysulfone ultrafiltration membrane (molecular weight cutoff 50,000) and dried under vacuum until constant weight was reached. Mass loss was then examined gravimetrically. Each datum was collected from three independent samples, and shown as mean value and standard deviation. 100% weight loss was the point at which the microgel was completely degraded into water-soluble products [27].

2.5. Loading of protein drugs in the microgels

A certain amount of proteins were loaded into about 15 mg of the dried microgels by incubating the latter with 2 ml of a 12–25 mg/ml protein PBS solution for 72 h at 4 $\degree C$. Insulin was dissolved in phosphate buffer saline solution followed by addition of a small amount of 0.1 N HCl. After protein loading, the microgels were separated with standard sieves and were dip-rinsed in 5 ml PBS at 4 $\degree C$ for a few seconds in order to wash off proteins adsorbed on the surface of the microgels. After separation, the microgels were dried at 4 $\degree C$ in a desiccator at normal pressure until constant weight was reached.

2.6. Release of proteins

To release the loaded proteins, the dried microgels were incubated at 37 $\degree C$ with 5 ml PBS (pH=7.2) in tubes. The samples were placed on a shaker at 100 rpm. At intervals until degradation proceeded completely, 0.5 ml of buffer was withdrawn and replaced by 0.5 ml of fresh buffer to maintain 5 ml of incubation volume. The protein concentration was evaluated with Coomassie Brilliant Blue method at 595 nm in a UV/VIS spectrophotometer (Lambda 35, Perkin Elmer). All measurements were performed in triplicate. The cumulative release of protein was calculated. The complete release amount $M_N$ was used to calculate drug loading defined as $M_N/M_p$, where $M_p$ was the mass of the dried gel loaded with protein. Blank microgels without protein loading were also performed according to the same procedure. No absorption bands were found in the experiment of the blank microgels, which demonstrated that the degradation products did not interfere with the Coomassie Brilliant Blue experiment.
2.7. Structure characterization of proteins

Native hemoglobin, blank microgels and microgels loading hemoglobin were characterized by FTIR (Magna-550, Nicolet) and micro-Raman spectrograph (LabRam-1B, Dilor). Samples for FTIR and Raman measurements were first washed with deionized water and then lyophilized. For FTIR analysis, KBr tablets were prepared by grinding the sample with KBr and compressing the mixture into a transparent tablet. In Raman spectrographic experiments, He–Ne laser (632.8 nm) was used as the excitation source and the radiation power was 6 mW.

The structure of native hemoglobin before encapsulation and the hemoglobin released from microgel were also characterized by circular dichroism (CD) in a Jasco Spectropolarimeter (Jasco J-715). The concentration of native hemoglobin was 100 \( \mu \)g/ml; the concentration of released protein was obtained again by the Coomassie Brilliant Blue method. A molar ellipticity scale on a hemoglobin basis was used.

3. Results and discussion

3.1. Thermo-sensitivity of microgels

“Intelligence” of the microgel was taken advantage of for the controlled delivery of protein drugs in this paper. The term “intelligence” herein refers to a microgel that has negative temperature sensitivity, i.e., the gel swells at low temperatures and contracts at temperatures above the volume phase transition temperature of the microgel.

Fig. 2 shows the volume change of the microgels with temperature, where the volume at 4 °C was taken to perform normalization. The microgels were swollen most at 4 °C and exhibited a significant decrease at temperatures around 23 °C. The average volume at 4 °C was almost twice of that at 37 °C. Hydrogen bonding between hydrophilic segments of the polymer chain and water molecules dominates at low temperatures, leading to enhanced dissolution in water of the microgels. As the temperature increases, hydrophobic interactions among hydrophobic segments especially PPO segments are strengthened, leading to further association and shrinking of the microgels. The solute diffusivity in microgels must be closely related to the swelling volume, determined by concentration of polymer, cross-linking degree, temperature and the other surrounding environment factors. The loading process was conducted at 4 °C, which provided maximum swelling volume and possibility of including proteins. It is expected that size exclusion can be reduced and drug loading be improved. On the other hand, the release temperature (37 °C) provided lower swelling volume, which might prevent the loaded protein from leaching out quickly and thus control the drug release behavior.

3.2. In vitro degradation of microgels

As shown in Scheme 1, the macromer contains the hydrolytically labile ester bonds, and thus the resulting microgel is biodegradable. When exposed to water, the breaking of linking bonds within the network leads to the mass loss of network, and thus the increase of volume and the acceleration of drug release. Fig. 3 shows degradation profiles of microgels made from the macromer F127-LA₂-DA. The main degradation products of microgels, in analogy to analysis in the literature [27], are presumably Pluronic F127, oligomeric lactic acids and oligomeric acrylic acids. The high swelling degree due to the presence of hydrophilic Pluronic F127 lowers the concentration of acidic species within the cross-linked networks and allows efficient removal of degradation products from the system [28]. The microgel might be
suitable for drug release carriers, as the chemical composition of the biomaterial is concerned.

3.3. In vitro protein release from microgels

Three proteins were examined with different molecular weights and charges, namely, Hb with molecular weight (MW) 67,000 and isoelectric point (PI) 6.9, BSA with MW 66,000 and PI 4.9, and insulin with MW 5700 and PI 5.3. Fig. 4 shows the mass release profiles of the above three proteins from the microgels. The release of Hb and BSA were dominated by a relatively small burst at the initial stage and followed by a steady release. The burst from hydrogels might be caused by the protein adsorption onto the hydrogel surface or uneven distribution across the hydrogel during the process of drying [29]. As the lactide linkages were hydrolyzed and cross-links were gradually broken, the swelling volume increased, which resulted in the increase of drug release. At final stage, the drug was released completely along with disappearance of the microgel network. The protein in the microgel is released by the combination of both a diffusion and a degradation mechanism. Comparing the degradation profile (Fig. 3) with the release kinetics (Fig. 4) indicates that diffusion prevails in the first stage. Degradation may be more important later.

Release behavior of the blank microgels is also given in Fig. 4, which indicates that the degradation products do not interfere with the measurement of the protein amount.

Drug loading level in this paper was relatively high, about 87 mg/g dry gel for Hb, 97 mg/g dry gel for BSA and 70 mg/g dry gel for insulin. So far, few papers in the literature reported drug loading in microgels using post-fabrication encapsulation technique and most of publications concern bulk hydrogel. For example, on a dry basis, loading levels below 0.1 wt.% of α-amylase and lysozyme were achieved by soaking poly(2-hydroxyethyl methacrylate) in solutions of these two proteins [25]. Gehrke et al. greatly improved the loading of ovalbumin (270 mg/g polymer) and α-amylase (67 mg/g polymer) into the dextran hydrogels using the principles of aqueous two-phase extraction [26]. But drug was completely released from dextran hydrogel before degradation occurred, and the release was swelling-controlled or diffusion-controlled instead of degradation controlled. Some papers do not directly state the loading level. For example, Ref. [30] just indicates that, using an organic solvent, protein loading of the gels swollen in ethanol was enhanced by formation of hydrophobic ion-paired complexes of proteins with sodium dodecyl sulfate.

The dilemma of high loading level and sustained release was overcome in our paper by the negative thermo-sensitivity of the microgel, namely, more swollen at low temperatures to achieve high loading.
and relatively less swollen to achieve sustained release at high temperatures. When the loaded microgels were immersed into 37 °C after drying, the swelling volume and thus mesh size of the microgels were not sufficiently large for Hb and BSA to leach out freely. Therefore, the release of Hb and BSA was mainly regulated by the degradation of the microgels.

The striking burst effect of Insulin might come from its small size in comparison with Hb and BSA. However, we do not think that the size effect is the only factor to control the protein release even in this nonionic polymeric network. The interactions between proteins and microgels must be considered. In the case of uncharged thermo-sensitive microgels in this work, the hydrophobic interaction between proteins and microgels may affect the release behavior to a large extent. The nature and extent of these interactions might control the protein diffusion in the gradually degraded network, which will be investigated in our future work.

We also investigated the structure of loaded hemoglobin in microgels by FTIR and Raman techniques. Native hemoglobin solution before encapsulation and hemoglobin released from the microgels were further measured by CD, a powerful tool in the study of spatial structures of ordered biomacromolecules with high sensitivity. The experimental measurements are shown in Figs. 5, 6 and 7.

The FTIR spectra of the microgels absorbing hemoglobin show the bands of blank microgel plus hemoglobin (Fig. 5). The bands at 1542 cm⁻¹ and 1655 cm⁻¹ of loaded microgels and those at 1544 cm⁻¹ and 1657 cm⁻¹ of native Hb were attributed to the N–H bending vibration and C=O stretching vibration of the peptide, respectively. The Raman spectra (Fig. 6) of the loaded microgel are almost the same as of the native hemoglobin. The four structure-sensitive bands (1364 cm⁻¹, 1429 cm⁻¹, 1581 cm⁻¹ and 1621 cm⁻¹) in the loaded microgel are very close to those of heme in the native hemoglobin (1366 cm⁻¹, 1428 cm⁻¹, 1584 cm⁻¹ and 1623 cm⁻¹) [31,32]. These results indicated that hemoglobins did penetrate into the microgels and the structure was not destroyed.

The conclusion is strengthened further by CD measurement. The CD spectrum of native hemoglobin in the far-UV range shows a typical α helix fingerprint with two negative cotton peaks at 208 nm and 222 nm (Fig. 7a), while the CD spectra of Hb in the Soret band shows one cotton peaks at 412 nm (Fig. 7b). As reported in literature [33,34], far-UV range and Soret band in CD spectra are sensitive to the secondary
structure, tertiary and quaternary structures of hemoglobin, respectively. The CD spectra of the released hemoglobin from the microgel in far-UV region and Soret band region are almost the same as those of native hemoglobin in both cotton peak profiles and peak values. Therefore, combination of FTIR, Raman and CD measurements demonstrate that the spatial structure of hemoglobin does not undergo any significant change after loading and release.

4. Conclusions

A new post-fabrication encapsulation technique for increasing the loading of the bioactive molecules and for achieving sustained release was developed using the intelligence of a novel negatively thermo-sensitive and biodegradable microgel. Hemoglobin, BSA and insulin were used as model proteins to evaluate the feasibility of this technique. Relatively high loading levels of proteins were achieved. The loaded and released hemoglobins preserve their native structure, as characterized by FTIR, Raman and CD. The release of larger proteins, Hb and BSA is predominantly controlled by the combination of diffusion and degradation with relatively small burst effect. Thus, we have successfully demonstrated that the post-fabrication encapsulation technique is able to decrease size exclusion of hydrogels to macromolecular drugs and meanwhile to avoid protein-denaturing factors such as organic solvent and high temperature in drug encapsulation.

This paper provides a novel intelligent and biodegradable hydrogel microparticle and confirms the feasibility of associated post-fabrication encapsulation technique for protein drugs. However, some questions are still open such as the interactions between proteins and polymer gels. Further investigations are meaningful.

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