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Preparing polymer-based sustained-release systems without exposing proteins to water–oil or water–air interfaces and cross-linking reagents

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Abstract

We report a method to load proteins into polymer-based sustained-release systems without exposing them to water–oil or water–air interfaces, factors known to denature proteins. By dispersing a dextan solution containing a protein into a PEG solution containing small amount of alginate, a stable aqueous–aqueous "emulsion" was formed. The poly-anionic alginate generated a diffuse double layer around each dextran droplet to prevent them from contacting with each other and fusing to a block phase. Proteins distributed in the stabilized dextran droplets by preferential partition favoring dextran. Freeze-drying this emulsion resulted in protein-loaded dextran particles, 1–2 μm in diameter and 1.6 g/cm^3 in density. The particles were harvested by washing the lyophilized powder using organic solvents to remove the PEG continuous phase. An activity assay of encapsulated β-galactosidase indicated that protein activity was preserved during the particle-forming process including the step of sonicating the particles in organic solvents. The dextran particles also improved release profile and integrity of proteins when encapsulated in degradable polymer sustained-release systems. The aqueous–aqueous emulsion offers a convenient way to prepare solvent-resistant protein–polysaccharide particles that can easily be incorporated in a variety of polymer-based pharmaceutical dosage forms and medical devices such as microspheres, scaffolds and drug-eluting stents for sustained-release protein delivery.

Keywords: Protein; Sustained-release; Stability; PLG/PLA; Microspheres; Stent

1. Introduction

While the concept of using polymer-based sustained-release delivery systems to maintain therapeutic concentration of protein drugs for extended periods of time has been well accepted for decades [1–3], there has yet to be a single product in this category successfully commercialized to date despite emerging clinical and market demands. To develop a polymer-based sustained-release system for proteins, the fragile high structure of these macromolecules must be protected from water–solvent or water–air interfacial tension and from hydrophobic environment of polymeric matrix, the known causes for protein denaturing, aggregating and immunogenicity. Converting proteins into solid particles prior to encapsulation into polymeric systems has been reported as an effective approach to endow proteins with immobility and resistance to organic solvents used to dissolve polymers [4–6]. However, the particle-forming process itself often associates with hazardous conditions to proteins. For examples, complexation with bivalent metal ions successfully used to prepare particles of human growth hormone (hGH) [6] resulted in aggregation of erythropoietin (EPO) [7]. Spray drying [8], in situ polymerization [9] or in situ cross-linking [10] exposes proteins to water–air interface or reactive reagents, respectively. While a method to form protein particles for sustained-release microencapsulation by freezing-induced protein precipitation in a polyethylene

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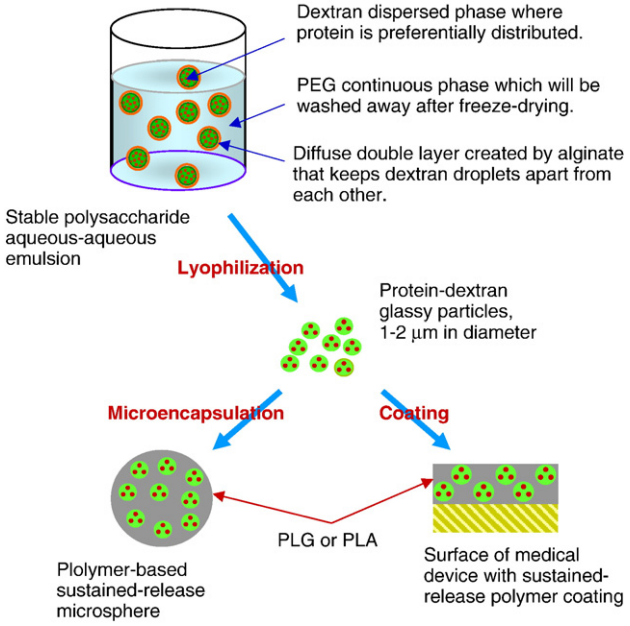

Fig. 1. Protein formulation strategy using self-standing polysaccharide aqueous–aqueous emulsion.

glycol (PEG) solution was demonstrated [5], loading unprotected bare protein particles in hydrophobic polymer matrix caused incomplete release up to 40% of protein loadings [5]. In addition, some researchers reported protein aggregation in concentrated PEG solution [11].

Gustavsson et al. demonstrated a method to prepare starch (amylopectin) particles to load proteins through repeated cooling–heating cycles across the sol–gel transition temperature of the materials around 55 °C [12]. In addition to exposing proteins to such a hazardous temperature, particle sizes prepared using this method were in the range of 20–80 μm, too large to be encapsulated in composite microspheres and thin layer-coatings of medical devices for burst-free sustained-release. Absorbing proteins into pre-cross-linked amylopectin nanoparticles is another approach to load proteins into polysaccharide particles without water–oil or water–air interface tension [13]. Preparation of the cross-linked starch particles involves a series of chemical reactions, which is more laborious and alter the native structure of amylopectin.

To circumvent the protein stability issues discussed above, we have developed a unique system, stabilized aqueous–aqueous "emulsion", of which both the dispersed and continuous phases are aqueous solutions. The system consists of a PEG continuous phase and a dextran dispersed phase which is stabilized from fusion by adding poly-anionic polysaccharide (sodium alginate) to form a diffuse double layer around each dextran droplet. Water soluble proteins can be loaded into the dextran droplets by preferential partition favoring the polysaccharide phase, similar as the so called "aqueous two-phase system" comprising a dextran and a PEG block phases [14]. Upon lyophilizing this emulsion, the protein-loaded dextran droplets are solidified to fine glassy particles, 1–2 μm in diameter. The polysaccharide particles prepared via the stabilized aqueous–aqueous emulsion will be named as AuqeSpheres below. Once loaded in the polysaccharide particles (AuqeSpheres), proteins are immobilized in the dextran matrix and endowed with resistance to organic solvents used in various formulation processes involving hydrophobic polymer solutions. The concept of this stabilized aqueous–aqueous emulsion and its usages in formulating protein sustained-release systems are described schematically in Fig. 1.

2. Materials and methods

2.1. Reagents

Poly(DL-lactic-co-glycolic) acid (PLG) and poly(DL-lactic) acid (PLA) of various average molecular weights were supplied from Lakeshore Biomaterials (Cincinnati, OH). Dextran (MW=70 kD), poly(ethylene glycol) (PEG) 8000, sodium alginate (medium viscosity), trehalose, myoglobin, β-galactosidase, *o*-nitrophenyl β-D-galactopyranoside (ONPG), bovine serum albumin (BSA) and micro-BCA protein assay kit (QuantiPro-BCA) were purchased from Sigma (St. Louis, MO). LysoSensor™ Yellow/Blue dextran, (10,000 MW) was obtained from Invitrogen (Eugene, OR).

2.2. Preparation of aqueous–aqueous emulsion

A dextran solution (Solution A, 20% w/w, containing soluble proteins or fluorescent pH sensor) was mixed with a PEG solution (Solution B, 20% w/w, containing 1.0–1.5% w/w sodium alginate), followed by stirring for 20–30 s. The volume ratio of solution A to solution B was 1/5–1/20 depending on experiments.

2.3. Micro-Bicinchronic Acid (micro-BCA) protein concentration assay

A micro-BCA working reagent was prepared by mixing 25 parts of reagent "QA" (mixture of sodium carbonate, sodium tartate, and sodium bicarbonate in 0.2 M NaOH with pH of 11.25), 25 parts of reagent "QB" (4% w/v bicinchoninic acid solution, pH=8.5) and 1 part of reagent "QC" (4% w/v copper (II) sulfate, pentahydrate solution). Protein sample (250 μl) was added into 250 μl of the mixed working reagent and was incubated at 60 °C for 1 h. The protein concentration of the sample was determined based on absorption at 562 nm (by comparison to a standard curve).

2.4. Measurement of partition coefficient of proteins between PEG and dextran phases

To measure partition coefficient of proteins between the two aqueous phases, protein-containing aqueous–aqueous emulsions prepared as above were centrifuged to two block phases, the dextran-rich phase (at the bottom) and the PEG-rich phase (at the top). The two phases were separated and measured for volume change (due to water transfer from one phase to the other) and protein content. Protein concentration of each phase was determined using micro-BCA method.

2.5. Preparation of protein-loaded fine dextran particles and pure myoglobin particles

The aqueous–aqueous emulsion loaded with proteins as above was frozen at −20 °C for 3 h or longer, followed by lyophylization. The lyophylized powder was re-suspended and sonicated in dichloromethane or acetonitrile, followed by centrifugation to remove PEG continuous phase dissolved in the supernatant. The re-suspension–centrifugation procedure was repeated for three times, and the pellet was evaporated in vacuum to remove solvent residues. For most of samples, the mass ratio of protein/dextran varied from 1/2 to 1/10 depending on experiments, and the volume ratio of dextran solution/PEG solution was 1/5.

Pure myoglobin particles were prepared by a freezing-induced precipitation process reported previously [5]. In brief, a co-solution containing 0.4% w/w myoglobin and 2% w/w PEG (MW = 8000) was frozen at −20 °C and lyophilized. The resulting powder was re-suspended in dichloromethane or acetonitrile and centrifuged to remove PEG dissolved in the supernatant.

For comparison, β-galactosidase-loaded dextran particles were prepared via water-in-oil emulsification. A protein-containing dextran solution (20% w/w in concentration) was emulsified into mineral oil, 5 times of the volume of the dextran solution. This emulsion was poured into acetone of 15–20 times in volume to extract water from the dextran droplets and wash away the oil.

2.6. Activity assay of β-galactosidase

The assay was carried out according to a protocol from Sigma [15]. Briefly, a solution of the protein, original or reconstituted, after each preparation step, was diluted to approximately 1 U/ml using 100 mM PBS buffer. Then, 0.1 ml of the protein solution was mixed with 2.6 ml PBS buffer (pH = 7.3); 0.1 ml of 30 mM $MgCl_2$ solution; 0.1 ml, 3.36 M 2-mercaptoethanol solution; and 0.1 ml, 68 mM ONPG (the substrate). The mixed solution was allowed to react at 37 °C for 5 min, then immediately cooled to 0 °C. Protein activity was determined by absorbance of the reaction product of ONPG at 420 nm. Protein concentrations were calculated based on a millimolar extinction coefficient of 3.5 (provided by Sigma).

2.7. Density estimate for dextran particles

To estimate the density of the dextran particles prepared via aqueous–aqueous emulsion, the particles were suspended in a series of organic solvents (which do not dissolve dextran particles) of known densities. Methylene chloride (density = 1.32), chloroform (density = 1.49) and carbon tetrachloride (density = 1.59) were used for the density estimate.

2.8. Differential scanning calorimetry (DSC) of dextran particles

To determine the state of the dextran particle matrix, the particles were loaded to a TA.CO-Q10 DSC system and heated to 250 °C in a nitrogen steam at the rate of 10°/min. The DSC diagrams were recorded during the heating.

2.9. X-ray diffraction (XRD) pattern of dextran particles

XRD patterns of the dextran particles were recorded on a Rigaku D/MAX2000 XRD system equipped with Cu-Kα radiation source (40KV, 20 mA). Samples were loaded on the quartz samples-holder and scanned from 5° to 70° at a rate of 6°/min.

2.10. Preparation of composite PLG microspheres

Dextran glassy particles (containing proteins or fluorescent sensors), or pure myoglobin particles, were suspended in a 20% w/w PLG solution (in dichloromethane) with desired dextran to PLG ratio (1/5–1/10, w/w). After vigorous stirring, this suspension was injected into an aqueous buffer containing 1% polyvinyl alcohol (PVA) and 5% w/v NaCl, 5 times in volume, under stirring to form a solid-in-oil-in-water emulsion. Embryonic composite PLG microspheres formed in this emulsion was immediately poured into a cold (<5 °C) NaCl solution (5%, 160 times in volume) for hardening. The sample was gently stirred for approximately 2–3 h until the solution reached room temperature, and the hardened PLG microspheres were rinsed with water and freeze-dried before storage.

To assay protein contents in microspheres, PLG microspheres of given weight were re-suspended and sonicated in dichloromethane or acetonitrile, followed by centrifugation and removal of the supernatant. This washing–centrifugation process was repeated four times, and dextran particles were recovered and reconstituted. The amount of proteins in the recovered dextran particles (or as pure protein particles) was determined using micro-BCA method. Protein loading efficiency was defined by the ratio of recovered protein/added protein × 100%. For the myoglobin-loaded composite microspheres prepared in the present study, protein encapsulation efficiency was in the range of 80%–90%.

2.11. Protein sustained-release from PLGA microspheres

Protein-loaded PLG microspheres (10 mg) were suspended in 1 ml of 20 mM PBS buffer (pH = 7.4) and shaked at 37 °C. The supernatant was collected and replenished at each sampling date. The protein contents were measured using micro-BCA method.

2.12. Estimate of local acidity change in PLGA microspheres

Fine dextran particles loaded with LysoSensor™ Yellow/Blue dextran through aqueous–aqueous emulsion were encapsulated in PLG microspheres as described above. Microspheres loaded with the pH indicator, 10 mg, were incubated in 1 ml of 20 mM PBS buffer at 37 °C. The samples were examined using a fluorescent microscope (Olympus, IX71-32PH) each day. Fluorescent images were taken using filters of 521 nm and 452 nm in wavelength, respectively, and pH was estimated by

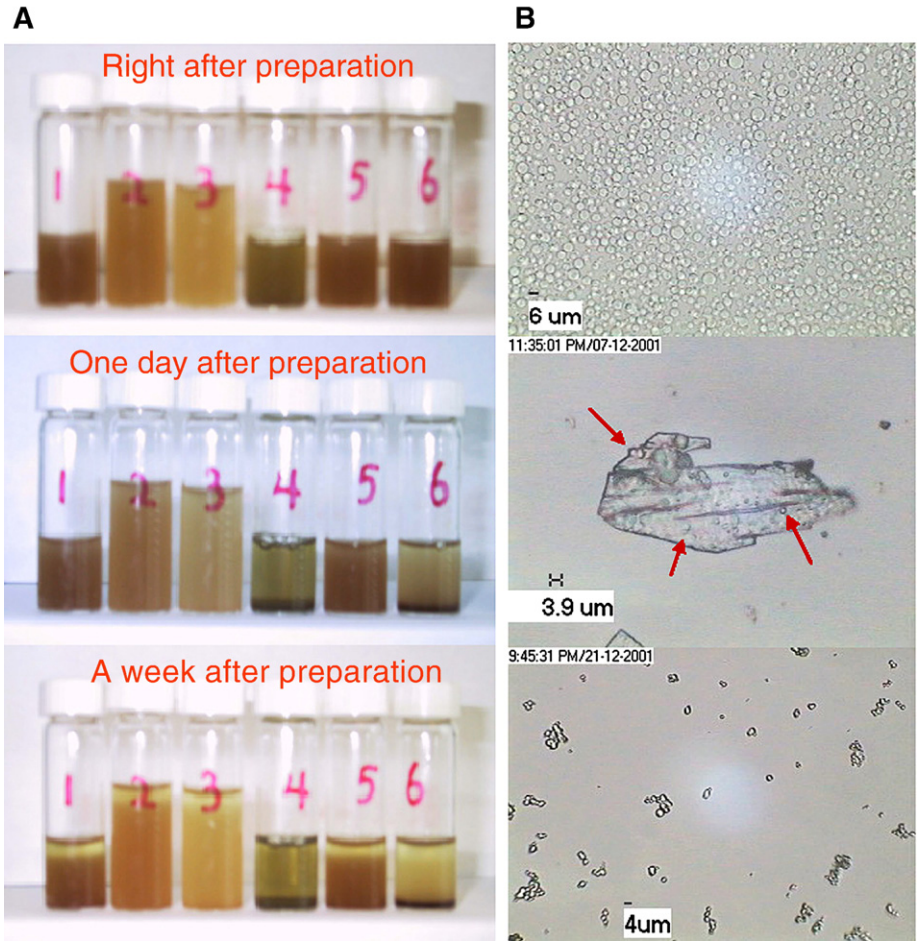

Fig. 2. A: Photo images of aqueous–aqueous emulsion right after preparation, a day after preparation and a week after preparation. Samples 1, 2, 3 were prepared by mixing a solution 20% dextran containing 2% myoglobin with 20% PEG containing 1.5% alginate at volume ratios of 1:5, 1:10 and 1:15, respectively; Sample 4 was same to sample 1 but without sodium alginate; samples 5 and 6 were same to sample 1 but with 10 mM and 100 mM NaCl, respectively. B: microscopic images of sample 1 (upper), lyophilized sample 1 (middle); and harvested dextran particles from sample 1 (lower).

comparing the ratio of the fluorescent intensity through the two filters (I_{521}/I_{452}) taken for the sample with that taken for a series of standard samples of known pH. Fluorescent intensity was analyzed using software, WASABI V.1.4.

2.13. Protein coating on to stent surfaces

Bovine serum albumin (BSA)-loaded dextran glassy particles (containing 20% protein) were suspended in a 10% PLA solution (in dichloromethane) with particle to PLA ratio ranging from 1/5 to 1/10. After vigorous stirring, this suspension was painted to the surface of a stent using a spraying pen. After evaporation of the solvent, the stent surface was painted with another layer of pure PLA to reduce burst release. For a stent (wire surface area was approximately 1 cm^2) 2.5 to 10 mg of PLA was coated.

2.14. Protein sustained-release from stents

A stent coated with a PLA layer containing BSA-dextran particles was suspended in 0.5 ml of 20 mM PBS buffer (pH = 7.4) and shaked at 37 °C. The supernatant was collected and replenished at each sampling date. The protein contents were measured using a (micro-BCA) method.

3. Results and discussion

3.1. Stable polysaccharide aqueous–aqueous emulsion

Fig. 2 shows photographs of some polysaccharide stable aqueous–aqueous emulsion samples. The samples were prepared by mixing a myoglobin-containing dextran solution (solution A) with a sodium alginate-containing PEG solution (solution B) as described in Materials and methods section. These mixed solutions were photographed immediately (Fig. 2A, upper panel), after one day (middle panel), and after one week (lower panel) of mixing. Myoglobin was selected as the model protein for its rusty color (easy to photograph) and for its low cost. The two solutions were mixed at different ratios of A to B, including 1:5 (Fig. 2A, lane 1), 1:10 (Fig. 1A, lane 2) and 1:15 (Fig. 2A, lane 3), and were labeled 1, 2, and 3, respectively. To examine formation of a diffuse double layer by

sodium alginate, solution B without sodium alginate (lane 4) or with the addition of sodium chloride (10 mM for lane 5 and 100 mM for lane 6) were also prepared for comparison (A:B ratio = 1:5 for lane 4 to lane 6). The mixed solutions labeled 1, 2, 3, and 5 formed stable emulsions in which the dextran dispersed phase did not fuse and form a block phase for at least a week. Although some sedimentation was observed in all samples after a week because of gravity (shown by the rusty color, Fig. 2), the dextran droplets remained constant sizes around 5 μm in diameter, indicating that they did not fuse with each other (Fig. 2B, upper panel). In the sample without the polyelectrolyte, sodium alginate (lane 4), and the sample with sodium alginate and 100 mM sodium chloride (an agent that destroys diffuse double layer) (lane 6), the dispersed phase readily fused together and formed a block phase at the bottom within one day after mixing. These results suggest that stability of the dispersed dextran phase is due to a diffuse double layer created by sodium alginate surrounding the dextran droplets. This diffuse double layer prevented the dextran droplets from approaching to each other to fuse.

3.2. Polysaccharide glassy particles prepared from aqueous–aqueous emulsion

After freezing this emulsion (sample 1 in Fig. 2A), followed by lyophilization, the dextran droplets (Fig. 2B, upper panel) were solidified to fine spherical particles of uniform sizes around 1–2 μm in diameter and dispersed in the matrix of large irregular PEG particles (Fig. 2B, middle panel). These dextran particles were harvested by rinsing the lyophilized powder with dichloromethane or acetonitrile to remove PEG which is soluble in the solvents (Fig. 2B, lower panel). Direct lyophilizing a dextran solution did not result in such spherical particles of uniform sizes but particles of irregular shape and scattered sizes.

To examine morphology of the dextran particles prepared through the aqueous–aqueous emulsion, density of the particles was estimated by suspending the particles in a series of organic solvents of known density. The particles readily sank to the bottom of the container when added to methylene chloride (density = 1.32 g/cm^3) and chloroform (density = 1.49 g/cm^3) but suspended in carbon tetrachloride (density = 1.59 g/cm^3). Taking surface tension between the fine hydrophilic particles and the hydrophobic solvents into account, the density of the dextran particles should be at least 1.59 g/cm^3. For comparison, dextran powders prepared by direct lyophilization of a dextran solution were also suspended in the same solutions and found to be floating on the surface of chloroform, suggesting a density lower than 1.49 g/cm^3. Although this density estimate may not be accurate, the result is evident enough to conclude that the dextran particles prepared through the stable aqueous–aqueous emulsion are dense and non-porous.

The matrix state of the dextran particles prepared using the aqueous–aqueous emulsion were further characterized using differential scanning calorimetry (DSC) and X-ray diffraction (XRD), and the results were shown in Fig. 3A and B,

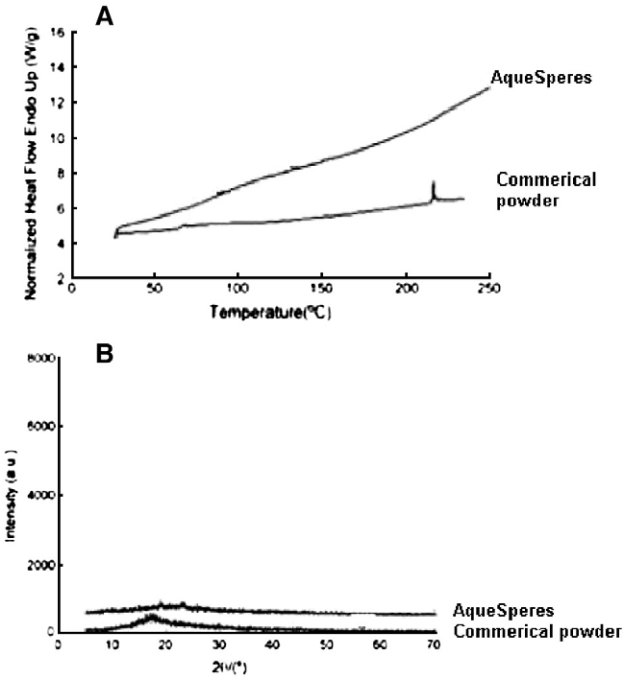

Fig. 3. Differential scanning calorimetry (DSC) diagrams and X-ray diffraction (XRD) patterns of dextran particles prepared using present method and purchased from Sigma. A: DSC diagrams; B: XRD patterns.

respectively, as compared with the dextran powders supplied by Sigma. The dextran powder from Sigma showed an endothermic peak around 215 °C on the DSC diagram, representing melting point of crystalline dextran existing in the sample. For AqueSpheres, however, there was no such a melting point peak but a bear-type bending of the DSC curve around 190 °C, suggesting a phase transition from glassy to gel state (Fig. 3A). The XRD patterns in Fig. 3B was consistent with the DSC result in that the commercial dextran particles showed a broad diffraction peak centered at 17°, while AqueSpheres showed no diffraction patterns. The good agreement between DSC, XRD results and density measurement described above strongly suggests that AqueSpheres are dense glassy particles.

In terms of how the alginate added in the PEG solution distributed in the two phases during the particle-forming process, it is not easy to determine the amount of alginate recovered with the dextran particles or the PEG phase after washing with organic solvents. However, since alginate possesses similar chemical structure to dextran (both are polysaccharides) and is insoluble in the organic solvents used, we assume that majority of alginate are associated with dextran particles. The negative charge of alginate backbone should push it to be able to reach the surface of each dextran droplet. We also speculate that during the sustained-release process, some of the alginate enriched at the surfaces of the dextran particles may deposit on the inner surface of the PLG matrix due to its hydrophobicity in an acidic environment. As PLG start to degrade, the surfaces of the inner wall inside the PLG matrix (see Fig. 1) may become acidic.

Table 1
Partition of myoglobin between dextran dispersed phase and PEG continuous phase

Solution A	Solution B	Initial volume ratio (A/B)	Equilibrium volume ratio (A/B)	K_P	Proteins in dextran phase
20% dextran 1.5%myoglobin	20% PEG 1.3% alginate	1/5	1/8	0.01	92%
20% dextran 1.5%myoglobin	20% PEG 1.3% alginate	1/5	1/8	0.02	88%

3.3. Partition of proteins between the dextran and the PEG phases

Table 1 summarizes partition coefficients of myoglobin and BSA between the dextran and the PEG phases. When one part of the protein-containing dextran solution (solution A) was mixed with five part of the alginate-containing PEG solution (solution B), water was extracted partially from the dextran phase to the PEG phase to reach an equilibrium at which the volume ratio changed from 1/5 to 1/8. The partition coefficient of myoglobin between the two phases ($K_P = C_{PEG\ phase}/C_{dextran\ phase}$) was 0.01, and the protein recovered from the dextran phase was 92% of the total protein added to the sample. This partition coefficient value ($K_P = 0.01$) suggests that the protein-in-dextran phase is thermodynamically more stable than that in PEG phase for about 2.8 kcal/mol in free energy ($\Delta G = -RT\ln K_P$). This energy value is comparable to reported energy barriers for protein conformation changes such as unfolding (5–7 kcal/mol) [16], suggesting that the free energy lowering for proteins due to partitioning in the polysaccharide phase than in PEG phase is considerable for maintaining protein native state. For BSA, partition coefficient was 0.02 and protein loaded in dextran was 88%.

3.4. Preservation of protein activity during polysaccharide particle-forming processes

To examine how protein activity is preserved during the particles forming process and how the dextran glassy matrix protect proteins from organic solvents, β-galactosidase, an enzyme with quaternary structure and a molecular weight over 500 kD, was loaded into the system following the aforementioned protocol and sonicated in methylene chloride. Trehalose (5% the mass of dextran), was added in solution A as a lyophilizing protector for its ability to protect enzymes during the drying process [17]. The enzymatic activity of β-galactosidase in cleaving *o*-nitrophenyl group (a species absorbing 420 nm light) from ONPG (the substrate) was assayed after each formulation step: before freeze-drying, after freeze-drying, and after sonicating in methylene chloride to remove the PEG continuous phase. In this assay, *o*-nitrophenyl formation is first order to the enzyme concentration, thus can be used to assay the enzymatic activity changes quantitatively [15]. Fig. 4 shows the protein activity after each of the formulation step. The baseline was defined as the activity before the freeze-drying process. The lyophilized powder and particles obtained after washing (sonicating) with dichloromethane were weighed and reconstituted for ONPG assay. Almost 100% of β-galactosidase activity was retained after freeze-drying and 95% after sonicating the freeze-dried powder in dichloromethane to remove the continuous phase (Fig. 4). This indicates that the protein was well protected by the polysaccharide phase during sonication in organic solvents. As a control, β-galactosidase-loaded dextran particles were prepared by emulsifying a β-galactosidase containing dextran solution in mineral oil, followed by washing with

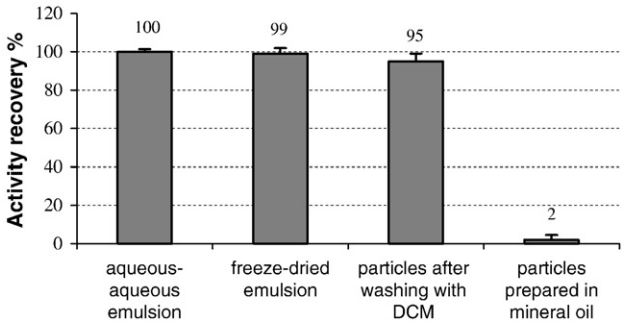

Fig. 4. Catalytic activity of β-galactosidase in hydrolysis of ONPG after aqueous–aqueous emulsification, lyophilization and sonication in dichloromethane.

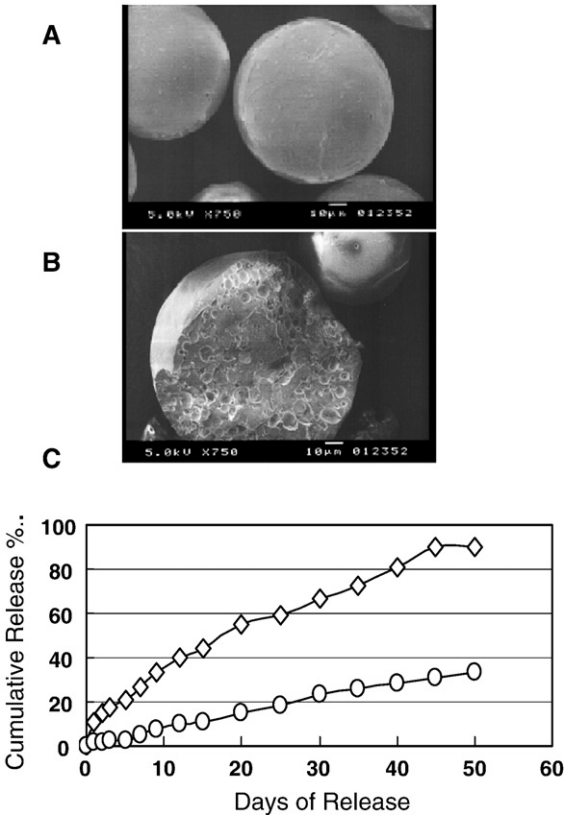

Fig. 5. A: Scanning electron microscopic (SEM) image of the surface of AqueSphere-loaded PLGA microspheres and B: SEM of the interior of the same microspheres C: Release profiles of myoglobin from PLGA microspheres loaded with AqueSpheres (◇) and those loaded with pure myoglobin particles (○).

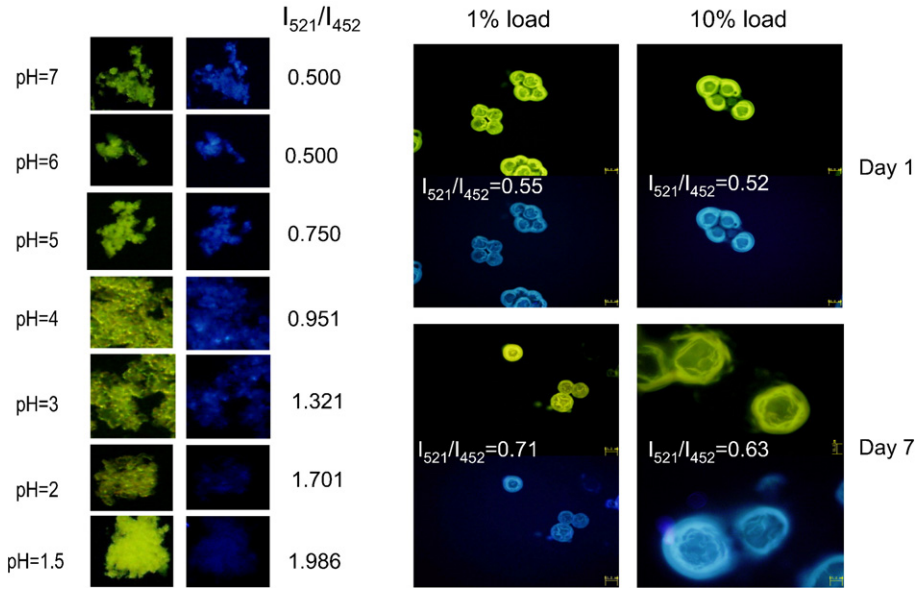

Fig. 6. Fluorescent images of PLGA microspheres loaded with Lyso Sensor AuqeSpheres. Left: standard images; Right: samples loaded with 1% and 10% AqueSpheres.

acetone to remove the oil. Same activity assay of β-galactosidase reconstituted from these particles showed an enzymatic activity only 2% of the baseline (Fig. 4). This result clearly indicates that the polysaccharide glassy phase formed under a mild condition (without water–oil interface tension) effectively protects proteins against organic solvents.

3.5. Release profile of proteins from composite PLG microspheres

Fig. 5A and B shows, respectively, the scanning electron microscopic images of exterior and interior morphology of composite PLG microspheres prepared using the present method with dextran fine particles dispersed in the microsphere matrix. The microspheres possess a smooth surface and polysaccharide particles evenly disperse in the PLG matrix.

Blending hydrophilic polymers into PLG microspheres was reported as a method to reduce on-polymer adsorption and incomplete release of proteins from the microspheres due to increased hydrophilicity of the PLG matrix [16]. It is, therefore, reasonable to expect that the dextran particles loaded with proteins and dispersed in the matrix of PLG microspheres may also improve protein release profiles. To examine effect of the blended dextran particles on protein release kinetics, myoglobin-loaded PLG microspheres were subjected to an in vitro release assay. As a model protein, myoglobin possesses a molecular weight (16 kD) similar to many clinically available protein therapeutics, such as growth hormone, interferon and granulocyte macrophage colony stimulating factor (GM-CSF). Its rusty color offers a convenient indication for protein leaks during a solid-in-oil-in-water (S–O–W) microencapsulation process.

The release profiles of myoglobin encapsulated in composite PLG microspheres as protein-in-dextran particles and as a pure protein particles are compared in Fig. 5C. For the composite PLG microspheres loaded with pure myoglobin particles, the cumulative release reached only about 38% of the total loadings over 45 days (Fig. 5C). Along the course of sustained-release incubation, the light rusty microspheres changed to dark brown pieces, suggesting formation of insoluble myoglobin aggregates or myoglobin deposits on the polymer. For the composite PLG microspheres loaded with protein-in-dextran particles (protein/dextran ratio = 1/5 and dextran particle/PLG ratio = 1/10), however, about 90% of myoglobin loadings were released over the same period of time (Fig. 5C), indicating significantly reduced on-PLG protein adsorption or insoluble protein aggregation. No colored pieces were observed at the end of sustained-release incubation.

As compared with PEG or sugars blended in the matrix of PLG microspheres as protein stabilizers [18,19], blending the dextran fine particles in microspheres led to a complete release of the protein (90%) without causing severe burst (Fig. 5C). This is probably due to that the dispersed polysaccharide was retained in the PLG matrix and protected proteins for longer time during the sustained-release period. Kisser et al. micro-encapsulated fluorescent dextran in PLG microspheres and found that dextran released from the microspheres was slower than EPO [20]. To improve protein release profile by blending hydrophilic protein stabilizers in PLG microspheres, burst and incomplete release are often two contradictory concerns. While blending hydrophilic protein stabilizers in degradable polymer microspheres may reduce incomplete release, the same approach may also cause burst release [18]. Since burst release caused by blending hydrophilic substances in microspheres is related with amount of the blending, one probable way to achieve complete release with mitigated initial burst may be to select a protein stabilizer that could effectively protect proteins during the entire period of sustained-release with less amount.

Pre-loading proteins in dextran fine particles prior to micro-encapsulation may be an idea solution since the polysaccharide dispersed phase can be retained in the microsphere matrix longer than proteins during sustained-release [19].

It has been reported that a reversed thermal gelling system made of PLG–PEG–PLG tri-block co-polymer may offer a convenient way to form sustained-release depot after subcutaneous injection without organic solvents [21]. For delicate proteins such as erythropoietin (EPO), however, protein aggregated in the thermal gel more significantly than in PLG microspheres [11]. Thomas et al. attributed protein aggregation to incompatibility of EPO with the PEG domains of the system [11]. This argument consists with our protein partition experiments that water soluble proteins dislike PEG phase (Table 1), as well as the observation that concentrated PEG solution caused delicate water soluble proteins to become thermodynamically unstable [22]. It is therefore reasonable to assume that delicate proteins may better be protected in polysaccharide microenvironment within a PLG microspheres than the PEG domains in the aqueous thermal gelling systems.

3.6. Acidity trapped inside PLGA microspheres during release incubation

Acid generated from PLG degradation may accumulate inside the microspheres and lead to protein denaturation [23,24]. The acidity inside the PLG matrix can be visualized by loading fluorescent pH indicators in the microspheres [24]. In order to examine the effect of the dispersed dextran particles on preventing the acidity trapping, we loaded a pH indicator (LysoSensor™ Yellow/Blue dextran) into dextran particles, then encapsulated the fluorescent particles in PLG to form composite microspheres. If the pH decreases, the fluorescent emission in green–yellow range (through a 521 nm filter) increases while that in blue range (through a 452 nm filter) decreases [25]. A pH decrease can therefore be indicated by the increase in the ratio of fluorescent emission at 521 nm over that at 452 nm (I_{521}/I_{452}). Fig. 6 shows the fluorescent images of microspheres loaded with 1% and 10% dextran particles containing LysoSensor™, respectively. The microspheres were incubated at 37 °C in 10 mM PBS buffer (pH=7.4), and fluorescent images were taken at day 1 and day 7 (the time that the PLG starts to degrade and generate acidity). The rationales of this measurement are that when drug particles are dispersed in a controlled-release polymer matrix at low loading, each drug particle is considered as isolated dot and the drug will be release by diffusion across the polymer matrix [26,27]. As the drug loadings increases, drug particles start to touch to each other and form diffusion channels at hydrated state [26,27]. For 10% particle loading in the present case, the fluorescent-dextran particles were hydrated and expended in volume during the course of incubation, so that they touch each other to form diffusion channels inside the PLG microspheres. For the microspheres of 1% loading of fluorescent-dextran particles, however, most of the particles were isolated from each other so that such drug-formed diffusion channels were not expected. The acids generated by PLG degradation may therefore be trapped in the microspheres. For the microspheres with 1% loading fluorescent-dextran particles, the I_{521}/I_{452} ratio increased from 0.55 to 0.71, corresponding to a pH decrease from 7 to 5 (Fig. 5). Whereas for the microspheres loaded 10% fluorescent-dextran particles, the I_{521}/I_{452} ratio increased from 0.52 to 0.63 (corresponding to a pH drop roughly from 7 to 6). This result suggests that blending dextran particles in PLGA matrix may be an effective way to release the acidity generated from PLG degradation.

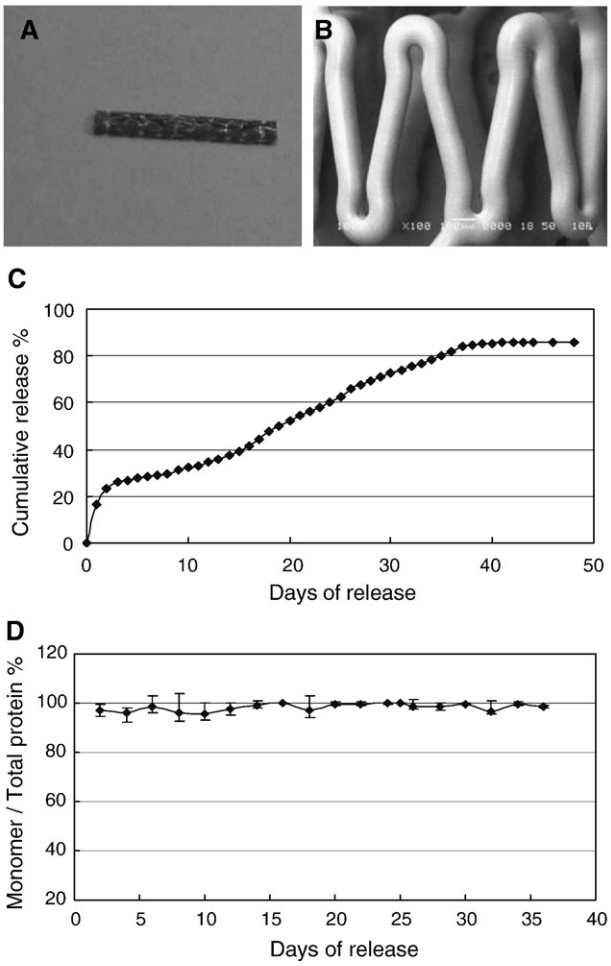

Fig. 7. Protein eluting stent. A: optical microscopic image of stent coated with composite PLA layer in which BSA-dextran particles are dispersed; B: Scanning electron microscopic image of A; C: cumulative release profile of BSA from A; D: ratio of BSA monomer/total BSA released from A each day and measured using SEC-HPLC.

3.7. Sustained-release profiles from protein-eluting stent

Since the chemical drugs used on currently available drug-eluting cardiovascular stents to prevent restenosis inhibit healing of vessel endothelium damaged during stent installation, protein drug-eluting stent has been proposed for their therapeutic specificity [28]. However, to coat sufficient amount of proteins on a stent and achieve a sustained-release delivery without protein denaturing is a challenging task. For example, impregnating a polymer-coated stent in a protein solution, a

well used method to load therapeutic proteins on stents, could only immobilized 18 μg proteins per stent [29]. In addition, adsorption on a hydrophobic polymer surface often results in protein denaturing. These issues were addressed in the present study by pre-loading proteins into the solvent-resistant dextran particles prior to dispersing in a polymer solution for stent coating (Fig. 1). Proteins may therefore be protected in the polysaccharide phase against organic solvents used for formulation process and against the hydrophobic microenvironment inside the polymer. Fig. 7A and B shows the optical and electronic microscopic images, respectively, of a stent coated with a PLA layer in which protein-containing particles were dispersed. The composite polymer layer (Fig. 7B) was loaded with up to 5% bovine serum albumin (BSA) and had a smooth surface. For a polymer coating 40 μm in thickness, 250 μg protein can be loaded on a stent (the surface area of the wires of a stent is approximately 1 cm^2, and PLA density is 1.25 g/cm^3).

The sustained-release profile of BSA from a protein-eluting-stent prepared by our method is shown in Fig. 7C. A fairly linear release profile with minimal burst (<20%) and incomplete release (~10%) was achieved. The BSA released each day was assayed using SEC-HLPC for possible protein aggregation, and the result is summarized in Fig. 7D by plotting the ratio of BSA monomer over total BSA against days of release. The fraction of BSA monomer varied randomly between 94% and 100%, a value slightly higher than that of the original BSA sample as purchased from Sigma (90%), suggesting that the system did not cause protein aggregation. This unique protein formulation method can also be used for medical devices other than coating layers. For example, for stents all made of a polymer, protein drugs may easily be loaded by dispersing the protein-dextran particles in the matrix of the polymer scaffold.

4. Conclusion

By using a stabilized polysaccharide aqueous–aqueous emulsion, proteins can be loaded into dense polysaccharide glassy particles of uniform sizes without exposing them to water–oil or water–air interfaces (as in water/oil emulsification or spray-drying) and particle-solidification reagents. Once loaded in the fine polysaccharide particles, delicate proteins gain resistance to organic solvents and can therefore be easily incorporated in various polymer-based pharmaceutical dosage forms and medical devices without aggregation. The protein-loaded polysaccharide particles dispersed in matrix of sustained-release polymers may also improve protein release kinetics by reducing incomplete release resulted from protein aggregation and adsorption on the polymer. The stable aqueous–aqueous emulsion system may offer a simple yet effective solution for the series of formidable difficulties in developing sustained-release technologies for proteins.

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