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Characterization of Reservoir-Type Microcapsules Made By the Solvent Exchange Method

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Yoon Yeo,[1,2] and Kinam Park[1]

[1]Departments of Pharmaceutics and Biomedical Engineering, Purdue University, West Lafayette, IN 47907
[2]Present address: Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

ABSTRACT

The purpose of this research was to characterize and optimize the properties of microcapsules produced by the solvent exchange method, a new microencapsulation technique. Reservoir-type microcapsules containing lysozyme as a model protein were produced using a coaxial ultrasonic atomizer under various formulation and instrument settings, and characterized with respect to in vitro release kinetics and stability of the encapsulated protein. The solvent exchange method could encapsulate protein drugs with high efficiency under an optimized condition and was mild enough to preserve the integrity of the encapsulated lysozyme during the process. In vitro release studies showed that the microcapsules could release proteins in a controllable manner. The solvent exchange method is a mild and simple microencapsulation method that could encapsulate lysozyme, maintaining its functional integrity.

KEYWORDS: microencapsulation, solvent exchange, ultrasonic atomizer, reservoir-type microcapsules, protein stability.

INTRODUCTION

Protein release from traditional polymeric microparticles is typically triphasic.[1] The 3 phases are (1) an initial burst-release of surface-bound and poorly encapsulated protein, (2) a second phase consisting of diffusional release and/or an induction period that does not release protein, and (3) release due to the degradation of the polymer matrix.[1] On the other hand, it has been noticed that most proteins undergo inactivation events such as degradation and aggregation within the microparticles during the manufacturing process as well as the release period.[2,3] For this reason, the protein release is typically incomplete despite substantial degradation of the polymer. Protein inactivation in the microparticle system is largely due to extensive exposure of the protein to damaging environments, such as large interfacial area between aqueous and organic phases (w/o), hydrophobic polymers, and their acidic degradation products. Various formulation strategies were employed to overcome these problems.[4,5] One of the recent examples includes encapsulating proteins in hydrogels prior to polymeric microencapsulation, which was found to be effective in preserving the protein stability and controlling the release rate.[6] In addition to the formulation approaches, improvement of microencapsulation techniques has also been attempted. The anhydrous microencapsulation process is one of the examples.[7]

Recently, a new microencapsulation technique called the solvent exchange method has been developed in an attempt to address the above problems.[8,9] Briefly, the new method produces reservoir-type microcapsules by inducing collision between drug-loaded aqueous drops and polymer-dissolved organic solvent drops. The microscaled liquid drops can be generated by different equipment such as ink-jet nozzles[8] and ultrasonic atomizers.[9] In particular, a coaxial ultrasonic atomizer is able to produce microdrops and allow their collision in a simple and highly efficient manner.[9] The potential advantage of the new method is that it does not involve most sources that have been known to induce inactivation of the encapsulated proteins for the following features. First, in reservoir-type microcapsules the w/o interfacial area generated during the encapsulation process is relatively small as compared with conventional multinuclear microcapsules, so it is expected that there is less protein inactivation due to its exposure to the interface. Second, due to reduced contact area between the aqueous and polymer phases, the unfavorable interaction between encapsulated proteins and the hydrophobic environment can be minimized. Third, physical stress that the ultrasonic atomizer generates is relatively mild,[10] so protein stability will barely be affected during the fabrication process. If these features successfully cooperate to maintain structural stability and biological activity of the encapsulated protein, the microcapsules generated by the new method will display improved release kinetics and stability profile as compared with conventional microparticles.

The present study was performed in order to validate these expectations. The in vitro protein release profile and stability of the released protein were examined, and the impact of the new microencapsulation process on such properties was investigated. Encapsulation efficiency is an important quality of microcapsules, especially when it comes to costly therapeutics such as proteins. Thus, another objective of this study was to optimize the encapsulation efficiency of the new method.

Corresponding Author: Kinam Park, Purdue University, School of Pharmacy, 575 Stadium Mall Drive, Room G22, West Lafayette, IN 47907-2051. Tel: 765-494-7759. Fax: 765-496-1903. Email: kpark@purdue.edu.

Materials and Methods

Materials

Poly (lactic-co-glycolic acid) (PLGA; lactide:glycolide ratio (L:G) = 50:50, intrinsic viscosity = 0.59 dL/g, weight average molar mass (mw) = 44 kd) was purchased from Birmingham Polymers, Inc (Birmingham, AL). PLGA (L:G = 50:50, intrinsic viscosity = 0.15 dL/g, mw = 13 kd, Medisorb 5050DL2A) was obtained from Alkermes (Cambridge, MA). *Micrococcus lysodeikticus*, lysozyme, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Ethyl acetate (EA) was obtained from Mallinckrodt Baker, Inc (Phillipsburg, NJ). Polyvinyl alcohol (PVA; 98.0%-98.8% hydrolyzed; mw ~195 000) was purchased from Fluka (Milwaukee, WI). The bicinchoninic acid (BCA) assay agent and microBCA assay agent were obtained from Pierce (Rockford, IL).

Preparation of Microcapsules

Microcapsules were produced as described elsewhere.[9] Briefly, a PLGA solution in EA (PLGA-EA) and an aqueous solution were separately fed into an ultrasonic atomizer through coaxial cables. The polymer concentration and the content of the aqueous solution were varied according to the purpose of the experiment. The 2 solutions were delivered using syringe pumps at controlled flow rates. Typically, the flow rates for the aqueous solution (Q_{Aq}) and the polymer solution (Q_{Pol}) were 0.2 mL/min and 2 mL/min, respectively, unless specified otherwise. Upon the onset of atomizer vibration at 60 kHz, both liquids were fragmented into microdrops and then collected in a water bath for 2.5 minutes. The collection bath was typically 200 mL of a 0.5% PVA solution. Microcapsules were left in the collection bath with gentle stirring for 2.5 hours and then centrifuged at 4000 rpm for 2 minutes. The microcapsules were washed at least 3 times with distilled water. Portions of the microcapsules were washed once with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.4, ionic strength = 150 mM) containing 0.02% sodium azide and then suspended in 3 mL of fresh HEPES buffer for a release study. The remaining portions were frozen in a –25°C freezer and then lyophilized using a LyoStar II Tray Dryer (Kinetic Systems, Inc, Stone Ridge, NY). Primary drying was conducted at –25°C for at least 30 hours, and secondary drying was conducted at 25°C for at least 3 hours.

Scanning Electron Microscopy

The surface of dried microcapsules was examined by scanning electron microscopy (SEM). The samples were attached to specimen stubs using double-coated tape and sputter coated with gold palladium in the presence of argon gas using a Hummer I sputter coater (Anatech Ltd, Union City, CA). Microcapsules were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc, Peabody, MA) using a 4- to 5-kV accelerating voltage, a 28-mm working distance, a 70-μm objective aperture, and a probe current of 6×10^{-11} amps.

Determination of Actual Protein Content and Encapsulation Efficiency

The actual protein content in the microcapsules was determined using the dimethylsulfoxide/sodium hydroxide/sodium dodecyl sulfate (DMSO/NaOH/SDS) method modified from the literature.[11] Less than 10 mg of freeze-dried microcapsules were precisely weighed and put into a microcentrifuge tube. DMSO 0.2 mL was added into the tube to dissolve the polymer portion of the microcapsules. Then, 0.8 mL of 0.05 N NaOH solution containing 0.5% SDS was added to the tube and gently mixed. After sonication for 90 minutes at 25°C using a Branson 5200 (Danbury, CT), samples were centrifuged at 10 000 rpm for 5 minutes. Clear supernatant, in 25-μL aliquots, was pipetted into the 96-well microplate and analyzed using the BCA assay method. The actual protein content and the encapsulation efficiency were defined as follows:

Actual protein content (%) = 100 × (Encapsulated protein/Microcapsule weight)

Theoretical protein content (%) = 100 × (Protein used for encapsulation/Microcapsule weight)

Encapsulation efficiency, EE (%) = 100 × (Encapsulated protein/Protein used for encapsulation) = 100 × Actual protein content / Theoretical protein content

Cloud Point Titration for Solubility of PLGA in EA

The solubilities of PLGA polymers in ethyl acetate were compared using cloud points as described in the literature.[12] Solutions of 2.5% PLGA in EA were titrated against methanol until a sustained turbidity was obtained. The cloud point was defined as the volume of methanol required to precipitate the least soluble fraction in the polymer solutions and produce sustained turbidity.

In Vitro Release Profile

The washed microcapsules, without drying, were suspended in 3 mL of 10 mM HEPES buffer (pH 7.4, ionic strength = 150 mM) containing 0.02% sodium azide. Alternatively, 40 to 60 mg of freeze-dried microcapsules were precisely weighed and placed in a polystyrene culture tube. A 3-mL amount of HEPES buffer was added to each tube. The tubes were then stored in a 37°C incubator. In order to simulate an in vivo situation in which the microcapsules are implanted within skin or muscle, the tubes were kept still. At selected time intervals, 1 mL of the release buffer was withdrawn and

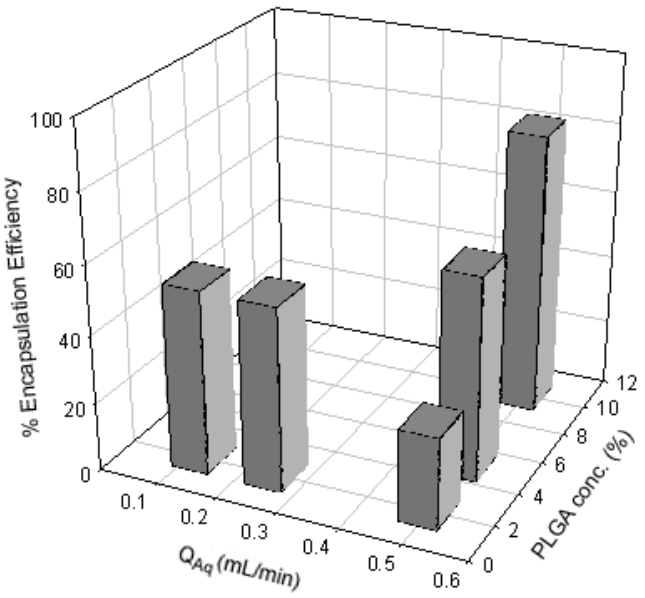

Figure 1. Dependence of the encapsulation efficiency on the polymer concentration and Q_{Aq} /Q_{Pol} ratio (Q_{Aq} varied from 0.125 to 0.5 mL/min with fixed level of Q_{Pol} at 3 mL/min) (n = 2). Encapsulated aqueous solution was 1% BSA in distilled water containing 0.5% Pectin.

replaced by fresh HEPES buffer. Protein concentration was determined using the BCA or microBCA protein assay method. The amount of protein released in each interval was divided by the total amount encapsulated in the microcapsules (= % Actual protein content × Microcapsule weight) to determine the percentage of released protein. The percentage of cumulative released protein was defined as the sum of the percentage of released protein by the specified time point.

Nonreducing SDS-Polyacrylamide Gel Electrophoresis

Released lysozyme was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under a nonreducing condition. Briefly, 150 µL of lysozyme solution samples and 50 µL of a sample buffer consisting of 0.25 M Tris hydrochloride (Tris-HCl) buffer (pH 6.8), 10% SDS, and 4 mM EDTA were mixed and heated at 50°C for 15 minutes. The solution was cooled to room temperature and mixed with 20 µL of a loading buffer containing 0.1% bromophenol blue, 70% glycerol, and 62.5 mM Tris-HCl buffer (pH 6.8). Prepared samples were then loaded into the 15% polyacrylamide gel, and electrophoresis was performed using a Mini-PROTEAN 3 cell/PowerPac 300 system (Bio-Rad, Hercules, CA). The gel was stained using Coomassie blue.

Freeze-Dry Microscopy

The morphological changes of the microcapsules during the freezing/thawing and freeze-drying process were directly observed using freeze-dry microscopy. A polarized light microscope (Olympus Model BH-2, Melville, NY) equipped with a microscope stage (Model BCS 196, Linkam Scientific Instruments, Tadworth, Surrey, UK), liquid nitrogen cooling (Model LNP, Linkam Scientific Instruments, Tadworth, Surrey, UK), and a temperature controller (Model TMS 93, Linkam Scientific Instruments, Tadworth, Surrey, UK) was used for the observation, following the reported method.[13] Approximately 5 µL of the suspension was placed on a 13-mm diameter glass dish and was cooled to –25°C at the rate of 10°C/min. The temperature was held at –25°C, and vacuum was then applied. When the ice surrounding the microcapsules disappeared, the temperature was increased to room temperature at the rate of 10°C/min. Alternatively, the frozen samples were thawed to room temperature at the rate of 10°C/min. Evaporation of water vapor and freezing/thawing of the suspension were observed through the microscope, and photomicrographs were taken with a Nikon Coolpix 995 digital camera (Melville, NY).

RESULTS

Encapsulation Efficiency

Encapsulation efficiency was dependent on various formulation parameters. Figure 1 shows that encapsulation efficiency increased with decreasing flow rate ratio of the aqueous solution (Q_{Aq}) to the polymer solution (Q_{Pol}) (Q_{Aq}/Q_{Pol} ratio) and increasing polymer concentration. The encapsulation efficiency was also affected by the molecular weight (mw) of the polymer used. The encapsulation efficiency was significantly higher when a relatively high mw PLGA polymer (44 kd) was used instead of 13-kd PLGA polymer. It is likely that the difference was related to the solubility of each PLGA polymer in ethyl acetate. When the methanol cloud point (C_s) of the polymer (as a relative estimation of the solubility) was compared,[12] 13-kd PLGA had a significantly higher cloud point (1.4 mL) than 44-kd PLGA (0.6 mL) indicating that the 13-kd PLGA was more soluble in ethyl acetate.

Volume and composition of the collection bath that accommodated a fixed amount of microcapsules also seemed to be influential factors. Figure 2A shows dependence of encapsulation efficiency on bath size. Though the difference between 100 mL and 200 mL was insignificant, overall trend was that increasing bath size increased encapsulation efficiency. Figure 2B shows that increasing PVA concentration resulted in decreasing encapsulation efficiency.

In Vitro Release Profile

At first, microcapsules were made using 44-kd PLGA (L:G ratio = 50:50). A release test was performed by 2 different methods. First, the microcapsules were suspended in a

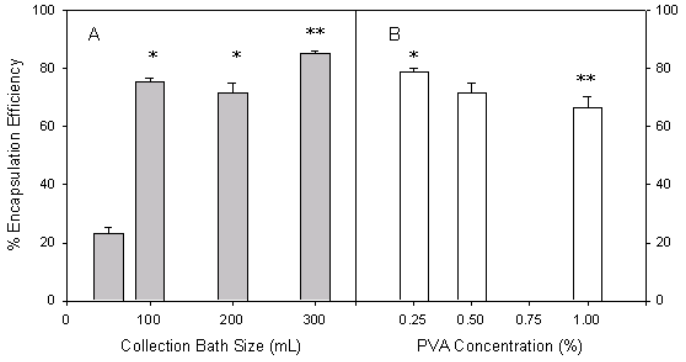

Figure 2. Dependence of encapsulation efficiency on (A) the size of the collection bath (0.5% PVA) and (B) the concentration of PVA in the bath (200 mL) (n = 3). * and ** indicate significance of statistical difference at $\alpha = 0.05$. A 5% PLGA-EA solution and 3% lysozyme in 0.1M phosphate buffer (pH 2.5) were used as a polymer solution and an aqueous solution, respectively. The PLGA polymer was a 50/50 blend of 44-kd and 13-kd polymers. Q_{Pol} = 2 mL/min and Q_{Aq} = 0.2 mL/min. Microcapsules were collected for 2.5 minutes in PVA baths of indicated volumes and concentrations.

release medium (HEPES buffer, pH 7.4) without drying ("undried"); second, the microcapsules were freeze-dried and reconstituted in a release medium ("dried"). As shown in Figure 3, the microcapsules displayed different release profiles depending on whether they had been dried (Figure 3, 100/0 D) prior to the release test or not (Figure 3, 100/0 UD). Here, the initial burst-release was defined as the percentage of lysozyme released from microcapsules for 24 hours. From the dried microcapsules, ~70% of loaded lysozyme was released in the first few days, and no subsequent release followed. On the other hand, the undried microcapsules showed a smaller initial burst (22.7%) and slow release (0.46% of the total encapsulated lysozyme per day, $r^2 = 0.9947$) over 35 days, followed by slightly accelerated release for the next 25 days. This result suggested that the freeze-drying process compromised the physical integrity of the microcapsules. As expected, SEM observation revealed that the freeze-dried microcapsules had visible cracks on the surface (Figure 4).

Since the drying process appeared to induce damage, the release profile of the following microcapsules was first examined without drying. Previously, molecular weight of the polymer was 44 kd, which usually takes 50 days for 50% reduction in molecular weight.[14] In an attempt to increase the release rate, a low-mw PLGA polymer (mw = 13 kd) was blended with 44-kd PLGA by 50 weight-in-weight percentage (% w/w). Addition of the fast-degrading polymer modified the release profile successfully. The initial burst was significantly suppressed from 22.7% ± 2.1% to 3.4% ± 0.1% (n = 3). The release rate increased to 2.8% of the total encapsulated lysozyme per day ($r^2 = 0.9836$) for 35 days and 100% of the loaded lysozyme was released in 50 days (Figure 3, 50/50 UD).

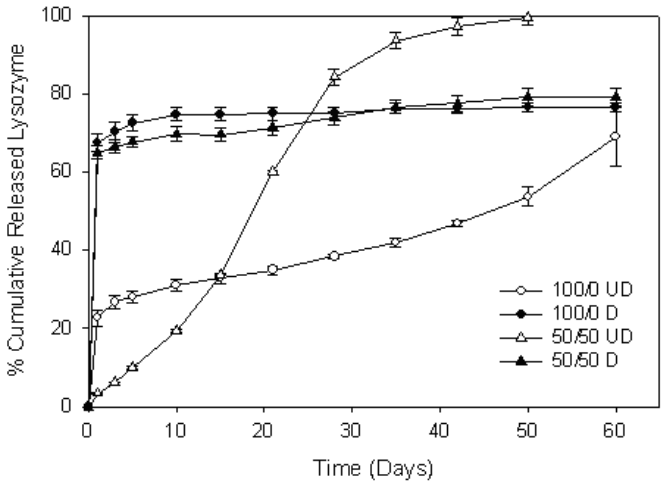

Figure 3. Comparison of release profiles (n = 3). A 5% PLGA-EA solution, 3% lysozyme in 0.1 M phosphate buffer (pH 2.5), and 0.5% PVA solution were used as the polymer solution, aqueous solution, and collection bath, respectively. The PLGA polymer was either a 44-kd polymer ("100/0") or a 50/50 blend of 44-kd and 13-kd polymers ("50/50"). Q_{Pol} = 2 mL/min and Q_{Aq} = 0.2 mL/min. Microcapsules were collected for 2.5 minutes in a 200-mL collection bath. "UD" and "D" indicate undried microparticles and dried microparticles, respectively. The release profile of 50/50 UD was adapted from reference 9.

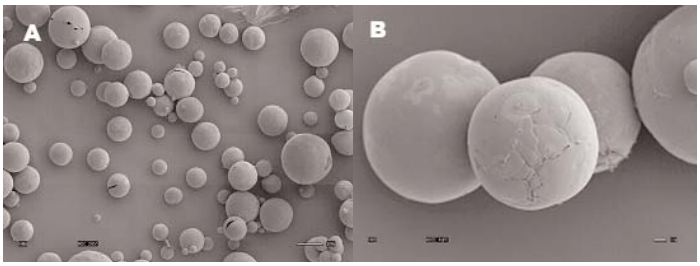

Figure 4. SEM images of freeze-dried microcapsules. Scale bars = (A) 100 μm and (B) 10 μm.

Two methods were used to determine if the encapsulated lysozyme remained intact throughout the release period: (1) measuring enzymatic activity of the released lysozyme and (2) nonreducing SDS-PAGE. The activity assay showed that the enzymatic activity was well preserved throughout the fabrication and the release period (data not shown). The SDS-PAGE performed under a nonreducing condition showed that the band patterns of the released lysozyme solutions were equivalent to that of native lysozyme (Figure 5). It is thought that the broad bands were due to the relatively high ionic strength of the sample buffer, which might have reduced the net charge and, therefore, changed the mobility of the protein. The bioactivity test and nonreducing SDS-PAGE, taken together, indicated that the integrity of the enzyme was preserved during the microencapsulation process and residence within the microcapsules.

The release profile of microcapsules made of 50/50 polymer blends was then examined after freeze-drying. Interestingly, the release profile of dried microcapsules (Figure 3, 50/50 D)

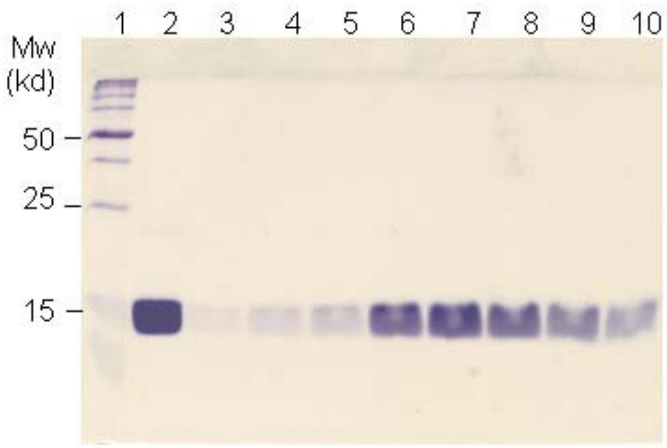

Figure 5. Nonreducing SDS-PAGE pattern of the released lysozyme: lane 1, size marker; lane 2, standard lysozyme; lane 3 to lane 10, released lysozyme on days 1, 5, 10, 21, 28, 35, 42, and 50.

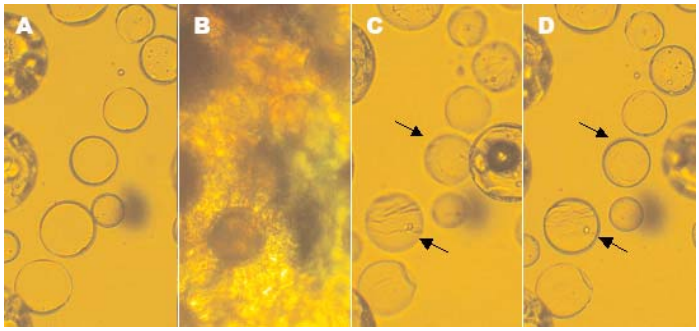

Figure 6. Crack formation during freezing: microcapsule suspensions in water (A) before freezing, (B) frozen at –30°C, and (C and D) thawed at 25°C. The arrows indicate the grooves formed on the membrane during freezing.

was not much different from the one obtained with the high-mw polymer (Figure 3, 100/0 D). In other words, the drug release out of dried microcapsules was persistently slow and incomplete, regardless of the molecular weight of the employed polymer.

In an attempt to understand the role of the freeze-drying process in exhibition of large initial burst and incomplete release, the microcapsules during the freeze-drying were directly observed using a light microscope. The first stage of freeze-drying is freezing the sample suspension. In order to examine the influence of freezing on the microcapsules, the temperature was decreased to –30°C to freeze the suspension and raised back to room temperature to thaw the suspension. Though it was not clearly observed because of the surrounding ice, the frozen microcapsules became dark upon freezing (Figure 6B). When the frozen suspension was thawed, grooves were observed on the surface of the membrane (Figure 6C and D). It is supposed that the grooves formed primarily due to the volumetric expansion of water during freezing. The second stage of freeze-drying is vacuum-drying of the frozen suspension. Here, the specimen stage was evacuated after freezing the microcapsule suspension at –25°C to simulate the primary drying condition. Figure 7 shows changes during the process. It was observed that the surrounding ice dried first and then the encapsulated ice evaporated. The ice from the microcapsules having visible cracks dried noticeably faster than the others, as indicated by the changes in darkness of the microcapsule images (Figure 7D and E). The ice within those microcapsules having relatively intact membranes did not completely dry in a given time and melted as the temperature increased to 25°C.

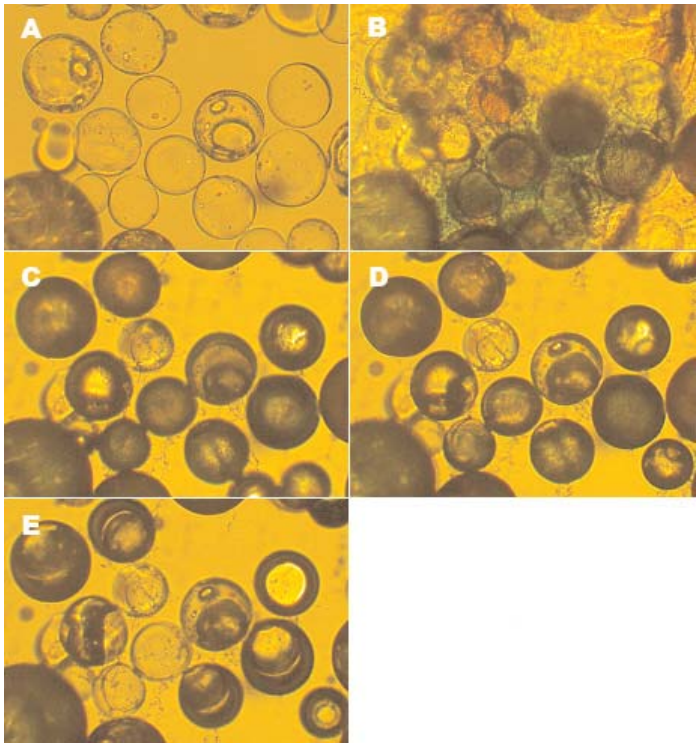

Figure 7. Crack formation during freeze-drying: microcapsule suspensions in water (A) before freezing, (B) frozen at –25°C, (C and D) during primary drying at –25°C, and (E) during secondary drying at 25°C. The arrows indicate the grooves/cracks formed on the membrane during freezing.

Discussion

In order to optimize the new microencapsulation method, various formulation parameters were examined. Encapsulation efficiency was used as a primary criterion in evaluating the quality of the microcapsules. Influential variables were found to be the volume ratio of the component liquids that participated in microencapsulation (Q_{Aq}/Q_{Pol} ratio), concentration of the polymer solution, molecular weight of the polymer, and composition and volume of the collection bath.

In order to explain the dependence on the Q_{Aq}/Q_{Pol} ratio, it is necessary to understand the microencapsulation mechanism of this new method: The microcapsules form by collision and coalescence of multiple droplets of the component liquids,

which are produced by the coaxial ultrasonic atomizer.[9] The high encapsulation efficiency at a low Q_{Aq}/Q_{Pol} ratio results from the preferential encapsulation of aqueous drops by polymer drops. At relatively low Q_{Aq}/Q_{Pol} ratios, the polymer drops are generated in larger quantity and tend to aggregate into bigger drops that can easily engulf the aqueous drops upon collision. At relatively high Q_{Aq}/Q_{Pol} ratios, the aqueous drops tend to engulf the polymer drops; thus, the majority of the aqueous phase is exposed to the surface of the microcapsules and eventually diluted into the collection bath. For this reason, the encapsulation efficiency decreases with increasing Q_{Aq}/Q_{Pol} ratios. It is interesting to note that the decrease of Q_{Aq} from 0.25 mL/min to 0.125 mL/min did not bring about any significant increases in the encapsulation efficiency. This means that below 0.25 mL/min, the population density of the aqueous drops was sufficiently diluted that the collision among aqueous drops could not compete with the collision between aqueous and polymer drops.

The influence of polymer concentration is attributed to its effect on viscosity and solidification rate of the polymer phase. First, increased viscosity of the polymer solution delayed drug diffusion through the polymer membrane. Second, highly concentrated polymer solution solidified rapidly because it did not require a large amount of nonsolvent for precipitation. It is generally believed that once the polymer is solidified, the encapsulated drugs do not easily escape from the polymer and thus the encapsulation efficiency remains high.[12,15,16] In the present study, however, the use of high polymer concentrations was practically limited because rapid precipitation of the polymer often resulted in formation of a large polymer precipitate. Thus, the polymer concentration used in the subsequent study was maintained at 5%. Polymer molecular weight also seemed to influence the encapsulation efficiency by influencing the solidification rate of the polymer phase as well as viscosity of the solution. The low-mw PLGA had a higher solubility in ethyl acetate and, thus, required longer time for solidification of the polymer phase upon contact with a nonsolvent. During the extended solidification, the encapsulated drugs might have been lost through the unsolidified membrane.

It is likely that the bath size was important because it determined the final solvent concentration in the collection bath. Here, a 5% PLGA-EA solution was sprayed at the rate of 2 mL/min for 2.5 minutes, which made the total volume of EA added to the bath ~5 mL. In a 50-mL bath, the EA concentration (density = 0.895 g/mL) in the bath exceeded the saturation solubility (8% w/w) since 5 mL of EA in 50 mL = 8.95% w/w. Then extraction of EA was significantly delayed leading to drug loss. In contrast, a large bath provided a high concentration gradient of solvent across the phase boundary facilitating the solvent exchange, which resulted in fast solidification of the microcapsules. In terms of encapsulation efficiency, a larger bath would therefore be preferable. However, in this case, it would be hard to stir the entire bath efficiently, which was necessary for efficient introduction of the incipient microcapsules into the bath. For this reason, the optimal bath size was found to be 100 to 200 mL for collecting microcapsules equivalent to 5 mL EA.

The effects of PVA concentration on the characteristics of microparticles are generally ascribed to its influence on particle size in different studies.[17,18] Decrease in particle size means an increased surface area through which the drug can be exposed to the exterior. In order to examine if this explanation applied to our observation as well, the size distributions of microcapsules collected in different PVA concentrations were compared. Interestingly, the particle size distribution was not significantly affected by the PVA concentration (data not shown). A possible explanation is that increasing viscosity of the PVA solution caused a portion of the polymer layer to separate from the microcapsules and caused drug loss into the collection bath.

Based on the above study, the optimum conditions that would result in high encapsulation efficiency were found to be Q_{Aq}/Q_{Pol} ratio = 1/10; PLGA concentration = 5%; collection bath = 0.25%-0.5% PVA solution; and bath size = 200 mL for collecting microcapsules equivalent to 5 mL polymer solution. The release study was performed with the microcapsules fabricated under these conditions.

For characterization of the release profile, lysozyme was chosen as a model protein for 2 reasons. First, enzyme activity is highly dependent on the tertiary structure of the enzyme and, thus, makes a good indicator of the functional integrity of the protein. Lysozyme is a well-characterized enzyme, and it is relatively simple to measure its activity.[19] For this reason, lysozyme has been used in many other microencapsulation studies.[20-22] Second, lysozyme is more susceptible to the physical and chemical stresses such as emulsification than BSA, which has been a typical model protein in most microencapsulation studies.[23] Therefore, lysozyme is an appropriate model protein for examining the effect of the fabrication process on stability of the protein as well as on the state of released protein.

When the microcapsules were tested without drying, the encapsulated lysozyme was continuously released at a rate that could be controlled by modifying the polymer composition. Addition of a low-mw PLGA (13 kd) to a high-mw PLGA (44 kd) resulted in an increased release rate and a reduced initial burst as compared with that of a 44-kd PLGA alone. Although the suppression of initial burst is not well understood, the increased release rate seems to be due to the presence of the fast-degrading low-mw PLGA. From this result, it is expected that the overall drug release can further be controlled by blending different mw polymers in various ratios. The stability of the released lysozyme was confirmed by the retained enzymatic activity and the protein band pattern that was equivalent to

that of native lysozyme. The release profile and the stability data suggested that the microencapsulation process itself did not present damaging effects to the protein stability, and the encapsulated protein could be completely retrieved before the microcapsules went through the drying process.

When the microcapsules were dried by freeze-drying, however, they exhibited high initial bursts and incomplete release. SEM, freeze-dry microscopy, and measurement of the protein retrieved after the freezing and/or drying processes indicate that cracks formed on the surface of the microcapsules were mainly responsible for the initial burst. The polymer membrane was primarily damaged due to the expansion of water during freezing. Freeze-dry microscopy shows that the expansion of freezing water caused visible grooves on the membrane.

The slow and incomplete release from the dried microcapsules also seems to be related to the freeze-drying. The slow release rate during the first few days may be due to the time required for rehydration of the dried microcapsules. It was expected, however, that once the microcapsules were rehydrated, the drug release would follow a similar rate as that of undried microcapsules. On the contrary, virtually no additional release followed the initial burst, and the protein release was incomplete after a significant time. This result suggests that the encapsulated protein might have been damaged and become unavailable for release during freeze-drying. Freeze-drying of the microcapsules could have affected the protein stability in different ways. First, freeze-dry microscopy shows that when the membrane remained relatively intact throughout the drying cycle, the evaporation was not complete in a given time, and the residual ice melted in the microcapsules as the temperature increased back. If the water inside the microcapsules is not removed completely during the freeze-drying for the same reason, the encapsulated protein and the residual water together may make a concentrated protein solution within the microcapsules. The extended residence in a highly concentrated state can damage the protein stability[24]; therefore, the incomplete drying can be a potent source of protein inactivation. Second, freeze-drying itself is not necessarily a stress-free process. During freezing, water in the protein solution forms ice crystals, making the solution more concentrated. When combined with cold denaturation, the freeze concentration can increase both the rate and extent of protein aggregation, as well as chemical reactions.[25] During drying, when the temperature is allowed to rise above the glass transition temperature of the maximally concentrated solute, the water entrapped in the glassy state can increase the molecular mobility of the protein and promote various deleterious reactions.[26]

In this context, it is worthy to note the existing argument on the incomplete release issues. Traditionally, the incomplete release has been ascribed to the denaturation of protein by the microencapsulation process and the unfavorable microenvironments generated during the release period, which include low pH caused by the accumulation of acidic degradation products and hydrophobicity of the encapsulating polymer.[2,27] On the other hand, the present example shows that the fabrication process itself does not damage protein stability, and the entire payload could be retrieved preserving the bioactivity unless the microcapsules are dried. In this case, the deterioration of the release profile seems to be due more to the drying process rather than to traditional causes. The drying process has barely been appreciated as a potential source for incomplete protein release, and only recently the effect of a drying process on the release profile began to be noticed.[28] Drying is an essential process for handling and long-term shelf storage of the microcapsules; therefore, it is necessary to engineer a protective drying method. To prevent crack formation in our case, plasticizers can be included in the polymer phase to make the membrane more flexible, or one can use polymers having inherently low glass transition temperatures. To protect the protein during both freeze-drying and storage, the freezing rate and primary/secondary drying conditions should be optimized first. In addition, it might be necessary to include lyoprotectants and cryoprotectants along with the encapsulated protein. Several sugars and amino acids are known to effectively protect protein stability during the freezing and/or drying cycle.[26] In fact, our recent studies have indicated that the microcapsules produced by the solvent exchange method can be dried by freeze-drying without damaging the PLGA membrane. Alternatively, in order to avoid the complications related to freeze-drying; it is also conceivable to preserve the microcapsules in an undried state. The hydrolytic degradation of the polymer can be delayed by using a nonaqueous liquid as a storage medium. The microcapsules can be preserved as an oil suspension until the time of application and can then be directly injected into the body.

CONCLUSION

Reservoir-type microcapsules were successfully produced using the solvent exchange method based on a coaxial ultrasonic atomizer. The in vitro release study using undried microcapsules demonstrated that the release profile could be controlled by the composition of the polymer phase. The integrity of the encapsulated protein was preserved throughout the fabrication process and the release incubation, validating the mildness of the new microencapsulation method and the advantage of the microcapsule design. On the other hand, the drying process following microencapsulation remains to be optimized in order to preserve the advantages of the solvent exchange method.

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REFERENCES

1. Hora MS, Rana RK, Nunberg JH, Tice TR, Gilley RM, Hudson ME. Release of human serum albumin from PLGA microspheres. *Pharm Res*. 1990;7:1190-1194.

2. Schwendeman SP. Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems. *Crit Rev Ther Drug Carrier Syst*. 2002;19:73-98.

3. Lu W, Park TG. Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J Pharm Sci Technol*. 1995;49:13-19.

4. Blanco MD, Alonso MJ. Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. *Eur J Pharm Biopharm*. 1997;43:287-294.

5. Zhu G, Mallery SR, Schwendeman SP. Stabilization of proteins encapsulated in injectable PLGA. *Nat Biotechnol*. 2000;18:52-57.

6. Capan Y, Jiang G, Giovagnoli S, Na K-H, DeLuca PP. Preparation and characterization of poly(D,L-lactide-co-glycolide) microspheres for controlled release of human growth hormone. *AAPS PharmSciTech*. 2003;4(2):E28.

7. Perez C, Castellanos IJ, Costantino HR, Al-Azzam W, Griebenow K. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. *J Pharm Pharmacol*. 2002;54:301-313.

8. Yeo Y, Basaran OA, Park K. A new process for making reservoir-type microcapsules using ink-jet technology and interfacial phase separation. *J Control Release*. 2003;93:161-173.

9. Yeo Y, Park K. A new microencapsulation method using an ultrasonic atomizer based on interfacial solvent exchange. *J Control Release*. 2004; In press.

10. Berger HL. *Ultrasonic Liquid Atomization*. 1st ed. Hyde Park, NY: Partridge Hill Publishers; 1998.

11. Sah H. A new strategy to determine the actual protein content of PLGA microspheres. *J Pharm Sci*. 1997;86:1315-1318.

12. Mehta RC, Thanoo BC, DeLuca PP. Peptide containing microspheres from low molecular weight and hydrophilic poly(d,l-lactide-co-glycolide). *J Control Release*. 1996/9 1996;41(3):249-257.

13. Chongprasert S, Knopp SA, Nail SL. Characterization of frozen solutions of glycine. *J Pharm Sci*. 2001;90:1720-1728.

14. O'Hagan DT, Jeffery H, Davis SS. The preparation and characterization of PLGA microspheres: III. Microparticle/polymer degradation rates and the in vitro release of a model protein. *Int J Pharm*. 1994;103:37-45.

15. Rafati H, Coombes AGA, Adler J, Holland J, Davis SS. Protein-loaded PLGA microparticles for oral administration: formulation, structural and release characteristics. *J Control Release*. 1997;43:89-102.

16. Li X, Deng X, Yuan M, et al. Investigation on process parameters involved in preparation of polylactide-poly(ethylene glycol) microspheres containing *Leptospira interrogans* antigens. *Int J Pharm*. 1999;178(2):245-255.

17. Hsu Y-Y, Hao T, Hedley ML. Comparison of process parameters for microencapsulation of plasmid DNA in poly(D,L-lactic-co-glycolic) acid microspheres. *J Drug Target*. 1999;7:313-323.

18. Yang Y-Y, Chung T-S, Ping Ng N. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *Biomaterials*. 2001/2 2001;22(3):231-241.

19. Ghaderi R, Carlfors J. Biological activity of Lysozyme after entrapment in PLGA-microspheres. *Pharm Res*. 1997;14:1556-1562.

20. Park TG, Lee HY, Nam YS. A new preparation method for protein loaded poly(,-lactic-co-glycolic acid) microspheres and protein release mechanism study. *J Control Release*. 1998;55:181-191.

21. Jiang G, Woo BH, Kang F, Singh J, DeLuca PP. Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(d,l-lactide-co-glycolide) microspheres. *J Control Release*. 2002;79:137-145.

22. Aubert-Pouessel A, Bibby DC, Venier-Julienne M-C, Hindre F, Benoit J-P. A novel in vitro delivery system for assessing the biological integrity of protein upon release from PLGA microspheres. *Pharm Res*. 2002;19:1046-1051.

23. Sah H. Protein instability toward organic solvent/water emulsification: implications for protein microencapsulation into microspheres. *PDA J Pharm Sci Technol*. 1999;53:3-10.

24. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm*. 1999;185:129-188.

25. Nail SL. Formulation of proteins and peptides. In: Park K, ed. *Controlled Drug Delivery Challenges and Strategies*. Washington, DC: ACS Professional Reference Book; 1997:185-203.

26. Pikal MJ. Freeze-drying of proteins: process, formulations, and stability. In: Cleland JL, Langer R, eds. *Formulation and Delivery of Proteins and Peptides*. Vol 567. Washington, DC: ACS; 1994:121-133.

27. van de Weert M, Hennink WE, Jiskoot W. Protein instability in PLGA microparticles. *Pharm Res*. 2000;17:1159-1167.

28. Kim TH, Park TG. Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres. *Int J Pharm*. 2004;271:207-214.