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Reservoir-Type Microcapsules Prepared by the Solvent Exchange Method: Effect of Formulation Parameters on Microencapsulation of Lysozyme

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Received October 2, 2005

Abstract: A new microencapsulation technique based on the solvent exchange method was implemented using an ultrasonic atomizer system to encapsulate a protein drug in mild conditions. The reservoir-type microcapsules encapsulating lysozyme as a model protein were prepared by inducing collisions between the aqueous droplets containing lysozyme and the droplets of organic solvent with dissolved poly(lactic acid-*co*-glycolic acid) (PLGA).The main focus of the study was to examine formulation variables on the size and the encapsulation efficiency of the formed microcapsules. The formulation variables examined were concentrations of mannose in the aqueous cores, NaCl in the aqueous collection medium, and PLGA in organic solvent. The mean diameter of the microcapsules ranged from 40 μm to 100 μm. Smaller microcapsules showed lower encapsulation efficiencies. The resulting microcapsules released native lysozyme in a sustained manner, and the release rate was dependent on the formulation conditions, such as the concentration and molecular weight of the polymer used. The solvent exchange method does not induce lysozyme aggregation and loss of its biological activity. The solvent exchange method, implemented by the ultrasonic atomizer system, provides an effective tool to prepare reservoir-type microcapsules for delivering proteins.

Keywords: Microencapsulation; microcapsules; mannose; protein delivery; solvent exchange method; PLGA; osmotic pressure

Introduction

For the past few decades, recombinant DNA technology has made remarkable advances in developing a number of protein therapeutics for treating many diseases. Nevertheless, most protein drugs have suffered from their short half-lives and susceptibility to hydrolytic enzymes in a biological environment. To realize the clinical potentials of such protein drugs, it is necessary to develop effective delivery systems that can improve the stability of protein drugs against enzymatic degradation and to sustain the therapeutic effect. Biodegradable microspheres have been used extensively as sustained protein delivery systems.[1−3] Their applications, however, have been limited because current methods of preparing microspheres involve exposure of protein drugs to harsh environments, such as organic/aqueous interfaces, high temperature, and strong physical stresses. Proteins can also be denatured by an acidic local environment formed by degradation of PLGA microspheres. Such undesirable conditions are known to induce conformational changes of protein drugs, resulting in loss of their biological activity.[4−6]

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(1) Bartus, R. T.; Tracy, M. A.; Emerich, D. F.; Zale, S. E. Sustained delivery of proteins for novel therapeutic agents. *Science* **1998**, *281*, 1161−1162.

(2) Sinha, V. R.; Trehan, A. Biodegradable microspheres for protein delivery. *J. Controlled Release* **2003**, *90*, 261−280.

(3) Tamber, H.; Johansen, P.; Merkle, H. P.; Gander, B. Formulation aspects of biodegradable polymeric microspheres for antigen delivery. *Adv. Drug Delivery Rev.* **2005**, *57*, 357−376.

10.1021/mp050083w CCC: $33.50
Published on Web 12/22/2005

In an attempt to minimize protein denaturation during the manufacturing process of microspheres, several approaches have been developed by modifying the conventional encapsulation methods or varying formulation parameters. Anhydrous microencapsulation processes have been developed to remove the water/organic solvent interface,[7−9] and surface-eroding polymers, including polyanhydrides and poly(ortho esters), have been suggested because they do not create acidic environments within the microspheres.[10,11] In some cases, proteins were first encapsulated using hydrophilic polymers followed by coating with hydrophobic polyesters,[12] or basic excipients (e.g., $Mg(OH)_2$ and $MgCO_3$) were added into the microsphere to prevent a pH drop during degradation of microspheres.[13,14]

In an effort to alleviate unfavorable conditions in the microencapsulation of proteins, we have developed a novel method, based on interfacial solvent exchange, as a potential alternative to the existing techniques.[15−18] This method produces reservoir-type microcapsules by inducing collisions between droplets of aqueous drug solution and organic polymer solution. Initially, the microsized liquid droplets were generated by a dual microdispenser system, consisting of two ink-jet nozzles.[15,16] Recently, a more efficient system based on a coaxial ultrasonic atomizer has been used to produce microcapsules.[17,18] The ultrasonic atomizer system generates large quantities of microdroplets under mild physical stresses.[17] The average diameter of microcapsules produced by this method was on the order of 100 μm, and we were interested in reducing the microcapsule size. In this study, we examined the effect of formulation variables, such as composition of the aqueous core, collection medium, and polymer solution, on the particle size and encapsulation efficiency of the microcapsules. Selected microcapsules were further characterized for their morphology, lysozyme release behavior, and the biological activity of the encapsulated lysozyme, which was used as a model protein.

Materials and Methods

Materials. High molecular weight poly(lactic acid-*co*-glycolic acid) (HPLGA, lactic/glycolic acid = 50/50, MW = 80 000) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Low molecular weight PLGA (LPLGA, lactic/glycolic acid = 50/50, MW = 13 000) was obtained from Alkermes, Inc. (Wilmington, OH). Poly(vinyl alcohol) (PVA, 98% hydrolyzed, MW = 195 000), purchased from Fluka (Milwaukee, WI), was used as an emulsifier. Fluorescein isothiocyanate labeled dextran (FITC-dextran, MW = 42 000), nile red, and lysozyme were purchased from Sigma (St. Louis, MO). The bicinchoninic acid (BCA) and microBCA protein assay kits were obtained from Pierce (Rockford, IL). All other chemicals were analytical grades and used without further purification.

Preparation of Microcapsules. Microcapsules were generated by using a coaxial ultrasonic atomizer (Sono-Tek Corp, Milton, NY), as described previously.[17] A PLGA solution in ethyl acetate and an aqueous solution, containing lysozyme as a model protein, were separately fed into an ultrasonic atomizer through a coaxial nozzle at defined flow rates. In most experiments, the flow rates of the polymer solution and the aqueous solution were fixed to 2 and 0.2 mL/min, respectively. The concentrations of polymer (HPLGA/LPLGA = 50/50) and lysozyme were 5 and 3 w/v %, respectively, unless specified otherwise. Microdroplets were produced by atomizing the solutions at a vibration frequency of 60 kHz, and they were then immediately collected in a 0.5 w/v % PVA solution for 2.5 min. The resulting solutions were left with gentle stirring for 1 h to allow solidification

(4) Lu, W.; Park, T. G. Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J. Pharm. Sci. Technol.* **1995**, *49*, 13−19.
(5) Putney, S. D.; Burke, P. A. Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* **1998**, *16*, 153−157.
(6) Morlock, M.; Kissel, T.; Li, Y. X.; Koll, H.; Winter, G. Erythropoietin loaded microspheres prepared from biodegradable LPLG-PEO-LPLG triblock copolymers: protein stabilization and in-vitro release properties. *J. Controlled Release* **1998**, *56*, 105−115.
(7) Sanchez, A.; Gupta, R. K.; Alonso, M. J.; Siber, G. R.; Langer, R. Pulsed controlled-released system for potential use in vaccine delivery. *J. Pharm. Sci.* **1996**, *85*, 547−552.
(8) Tobio, M.; Nolley, J.; Guo, Y.; McIver, J.; Alonso, M. J. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm. Res.* **1999**, *16*, 682−688.
(9) Perez, C.; Castellanos, I. J.; Costantino, H. R.; 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.
(10) Heller, J.; Barr, J.; Ng, S. Y.; Shen, H. R.; Schwach-Abdellaoui, K.; Einmahl, S.; Rothen-Weinhold, A.; Gurny, R. Poly(ortho esters)−their development and some recent applications. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 121−128.
(11) Heller, J.; Barr, J.; Ng, S. Y.; Abdellauoi, K. S.; Gurny, R. Poly-(ortho esters): synthesis, characterization, properties and uses. *Adv. Drug Delivery Rev.* **2002**, *54*, 1015−1039.
(12) Tobio, M.; Schwendeman, S. P.; Guo, Y.; McIver, J.; Langer, R.; Alonso, M. J. Improved immunogenicity of a core-coated tetanus toxoid delivery system. *Vaccine* **1999**, *18*, 618−622.
(13) Zhu, G.; Mallery, S. R.; Schwendeman, S. P. Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). *Nat. Biotechnol.* **2000**, *18*, 52−57.
(14) Zhu, G.; Schwendeman, S. P. Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives, *Pharm. Res.* **2000**, *17*, 351−357.
(15) Yeo, Y.; Basaran, O. A.; Park, K. A new process for making reservoir-type microcapsules using ink-jet technology and interfacial phase separation. *J. Controlled Release* **2003**, *93*, 161−173.
(16) Yeo, Y.; Chen, A. U.; Basaran, O. A.; Park, K. Solvent exchange method: a novel microencapsulation technique using dual microdispensers. *Pharm. Res.* **2004**, *21*, 1419−1427.
(17) Yeo, Y.; Park, K. A new microencapsulation method using an ultrasonic atomizer based on interfacial solvent exchange. *J. Controlled Release* **2004**, *100*, 379−388.
(18) Yeo, Y.; Park, K. Characterization of reservoir-type microcapsules made by the solvent exchange method. *AAPS PharmSciTech* **2004**, *5*, Article 52.

of microcapsules, followed by washing with distilled water. The morphology of obtained microcapsules was observed using an optical microscope (NiKon, Labophot-2, Japan). The microcapsule size was measured using a particle size analyzer (Microtrac, Inc., Montgomeryville, PA). For comparison, microspheres were also prepared using a double emulsion solvent extraction method, as described previously.[15,16]

Characterization of Microcapsules. The encapsulation efficiency of lysozyme was determined using the dimethyl sulfoxide (DMSO)/sodium hydroxide (NaOH)/sodium dodecyl sulfate (SDS) method in the literature[19] with slight modification. The microcapsules, after solidification and washing in distilled water, were freeze-dried and stored at 4 °C, prior to the measurement. Microcapsules (5−10 mg) were placed into a test tube to which DMSO (0.2 mL) was added 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 allow solubilization of the protein and hydrolysis of PLGA. The resulting mixture was sonicated for 90 min at 25 °C and centrifuged at 10 000 rpm for 5 min. The clear supernatant (30 μL) was transferred into a 96-well microplate. The protein concentration was measured using the BCA assay. To make a calibration curve, a series of protein solutions was prepared using DMSO/NaOH/SDS as the solvent. The encapsulation efficiency (E) was defined as follows:

$$E = [(\text{amount of encapsulated protein})/(\text{total amount of feed protein})] \times 100$$

The internal structure of the microcapsules was examined using a laser scanning confocal imaging system (model MRC-1024, Bio-Rad, Hercules, CA) equipped with a krypton/argon laser and a Nikon Diaphot 300 inverted microscope. For the sample preparation, microcapsules were produced using 0.02% FITC-dextran in aqueous solution and 0.003% nile red in the polymer solution. Before the measurement, they were thoroughly washed with distilled water. The excitation wavelengths for FITC-dextran and nile red were 488 and 568 nm, respectively.

In Vitro Release Study. The prepared microcapsules (~20 mg) were washed with distilled water and then dispersed into 1.5 mL of phosphate buffered saline (PBS, pH 7.4). This solution was immediately placed in an incubator at 37 °C and was shaken at a frequency of 100 rpm. At predetermined time intervals, the microcapsule suspension was centrifuged at 10 000 rpm to collect 500 μL of the supernatant. Fresh PBS (500 μL) was then added, prior to the resuspension of the microcapsules. The amount of lysozyme, released from the microcapsules, was quantified using the BCA or microBCA protein assay. After the release test, the microcapsules were freeze-dried and the remaining amounts of lysozyme in the microcapsules were quantified using the DMSO/NaOH/SDS method, as described above.

SDS−Polyacrylamide Gel Electrophoresis. The solutions of lysozyme released from microcapsules were analyzed using SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The lysozyme solution (150 μL) was mixed with 50 μL of a 0.25 M Tris-HCl buffer (pH 6.8) containing 10% SDS and 4 mM EDTA. After being heated for 15 min at 50 °C, the solution was cooled to room temperature and mixed with 20 μL of a loading buffer (0.1% bromophenol blue, 70% glycerol, 62.5 mM Tris-HCl, pH 6.8). The sample solution was loaded into the 15% polyacrylamide gel, and electrophoresis was carried out using the Mini PROTEAN 3 cell/PowerPac 300 system (Bio-Rad, Hercules, CA).

Biological Activity Assay. To determine biological activity of the released lysozyme, turbidity change of a *Micrococcus lysodeikticus* bacterial cell suspension was measured using a UV/vis spectrophotometer.[20] The lysozyme solution (100 μL), obtained from the releasing medium, was mixed with 2.9 mL of a *M. lysodeikticus* suspension (0.25 mg/mL in HEPES buffer, pH 7.4). The changes in turbidity of the cell suspensions were monitored for 3 min to measure the rate constant for the decrease in transmittance (k, min^{-1}), and the percentage of the retained biological activity of lysozyme was estimated by comparing it with the results for standard lysozyme solutions.

Results

The effect of excipients on the microcapsule size and the lysozyme encapsulation efficiency was examined by incorporating different amounts of mannose in the aqueous cores of the microcapsules and NaCl in the aqueous collection medium (Figure 1). For the control sample prepared in the absence of mannose and NaCl, the particle size and encapsulation efficiency were 109.6 ± 9.3 μm and 69.1 ± 4.3%, respectively. Both mannose and NaCl significantly influenced the particle size and encapsulation efficiency, depending on their concentrations. The presence of 50% mannose and 1.12 M NaCl reduced the particle size to 72.4 ± 7.9 μm and 65.4 ± 8.9 μm, respectively. On the other hand, encapsulation efficiency decreased to 53.2 ± 3.8% at 10% mannose, and lysozyme loaded into the microcapsules was not detectable at mannose concentrations higher than 20%. The increase in the NaCl concentration up to 0.30 M did not significantly affect the encapsulation efficiency. At higher NaCl concentrations, however, a remarkable decrease in encapsulation efficiency was observed; as a consequence, only less than 20% of lysozyme was loaded into the microcapsules at 1.12 M NaCl.

The combined effect of mannose and NaCl was also evaluated, in which the mannose concentration was fixed at 10% (Figure 2). The increase in the NaCl concentration

(19) Sah, H. A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres. *J. Pharm. Sci.* **1997**, *86*, 1315−1318.

(20) Ghaderi, R.; Carlfors, J. Biological activity of lysozyme after entrapment in poly(d,l-lactide-co-glycolide)-microspheres. *Pharm. Res.* **1997**, *14*, 1556−1562.

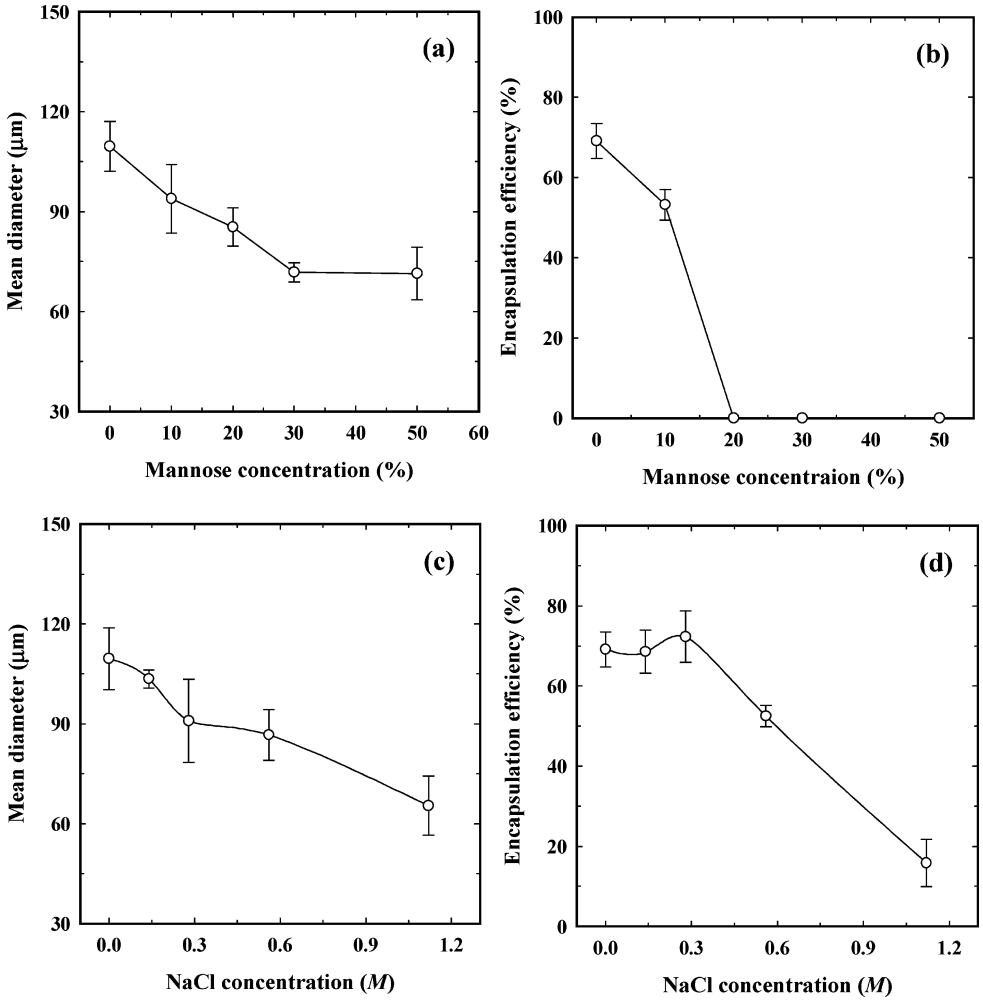

Figure 1. The mean diameter and encapsulation efficiency of microcapsules as a function of the concentration of mannose in the aqueous core of microcapsules (a, b) or the concentration of NaCl in the aqueous collection medium (0.5 w/v % PVA in distilled water) (c, d). The HPLGA/LPLGA (50/50) solution (5 w/v % in ethyl acetate) and the aqueous solution (3 w/v % lysozyme in distilled water) were separately fed into the ultrasonic atomizer system at flow rates of 2 and 0.2 mL/min, respectively ($n = 3$).

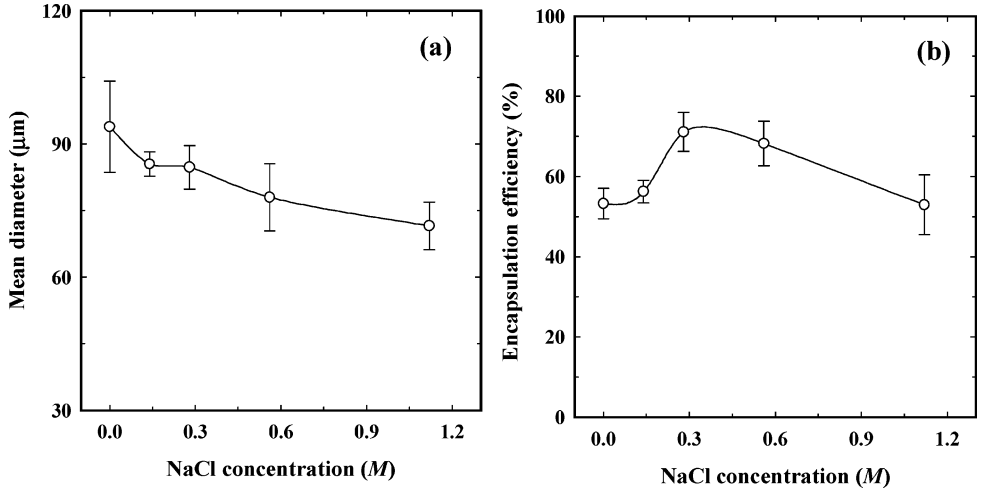

Figure 2. Combined effect of mannose and NaCl on the microcapsule size (a) and lysozyme encapsulation efficiency (b). The aqueous solution containing 10% mannose was atomized into the 0.5% PVA solution with different concentrations of NaCl ($n = 3$).

produced smaller microcapsules, and the mean diameter was as small as 71.6 ± 5.4 μm at 1.12 M NaCl. Interestingly, encapsulation efficiency reached a maximum at 0.28 M NaCl (71.1 ± 4.9%). Further microencapsulation experiments were carried out using 10% mannose and 0.28 M NaCl to examine the effects of the polymer concentration and composition.

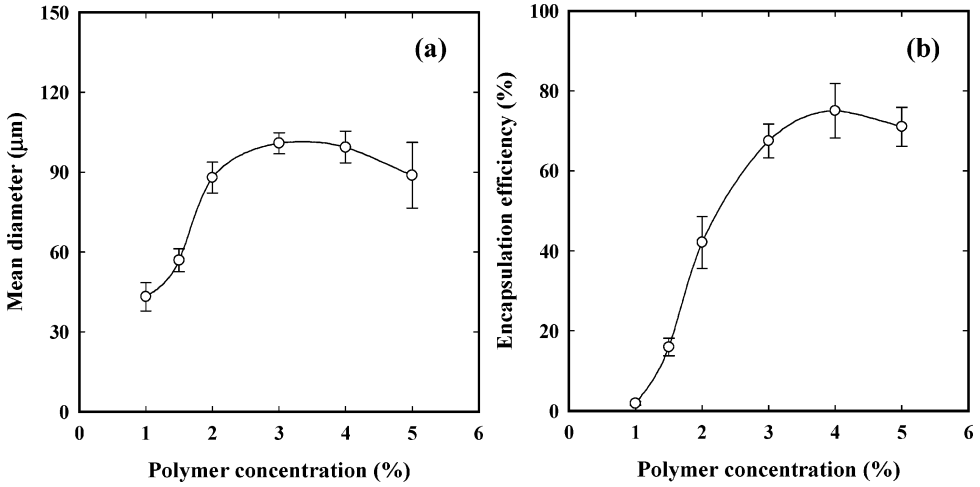

Figure 3. Effect of the polymer concentration on the microcapsule size (a) and lysozyme encapsulation efficiency (b). The HPLGA/LPLGA (50/50) solutions were prepared in different concentrations, followed by atomization with the aqueous solution containing 10% mannose into the 0.28 M NaCl collection medium ($n = 3$).

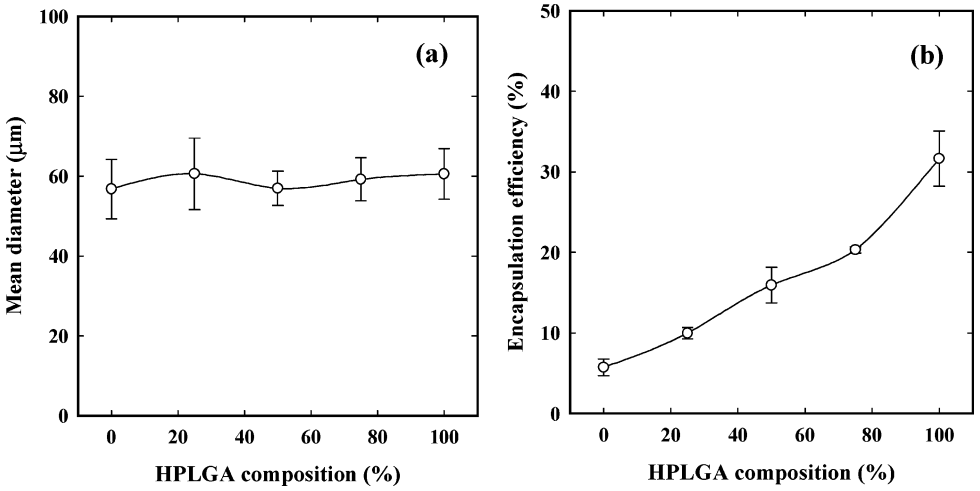

Figure 4. Effect of the polymer composition on the microcapsule size (a) and lysozyme encapsulation efficiency (b). The composition of HPLGA (MW = 80 000) and LPLGA (MW = 13 000) was varied while the total polymer concentration was maintained at 1.5 w/v %. The polymer solutions were atomized with the aqueous solution containing 10% mannose into the 0.28 M NaCl collection medium ($n = 3$).

Figure 3 shows the effect of polymer concentration on the microencapsulation of lysozyme. When the polymer concentration was in the range of 2−5%, there was no significant difference in the microparticle size. However, at lower polymer concentrations, smaller microcapsules were produced. The mean diameters, prepared in 1% and 1.5% polymer solutions, were 43.2 ± 5.4 and 56.9 ± 4.3 μm, respectively. For encapsulation efficiency, polymer concentrations less than 2% could not effectively entrap lysozyme. Using a 1.5% polymer solution resulted in encapsulation of only 16% of lysozyme.

The effect of polymer composition on the microcapsule size and encapsulation efficiency was examined using different amounts of HPLGA and LPLGA at the polymer concentration of 1.5% (Figure 4). The mean diameter of the microcapsule was in the range of 55−60 μm and independent of the polymer composition. The encapsulation efficiency, however, was significantly affected by the ratio of HPLGA in the blend. An increase in the relative concentration of HPLGA improved encapsulation efficiency.

The internal structure of the microcapsules was examined using confocal microscopy (Figure 5). For this experiment, the aqueous solution was labeled with FITC-dextran, whereas the polymer solution was visualized by nile red, a lipophilic fluorescence dye. As shown in Figure 5a, microspheres produced by the conventional double emulsion−solvent extraction method were composed of multiple aqueous inner cores embedded in the polymer matrix. On the contrary, the microcapsules prepared in the absence of mannose and NaCl by the solvent exchange method had a single aqueous core which was covered by the thin polymer membrane (Figure 5b). A similar cross-sectional morphology was obtained from the microcapsules, prepared in the presence of 0.28 M NaCl (Figure 5c). At a higher NaCl concentration of 0.56 M, a

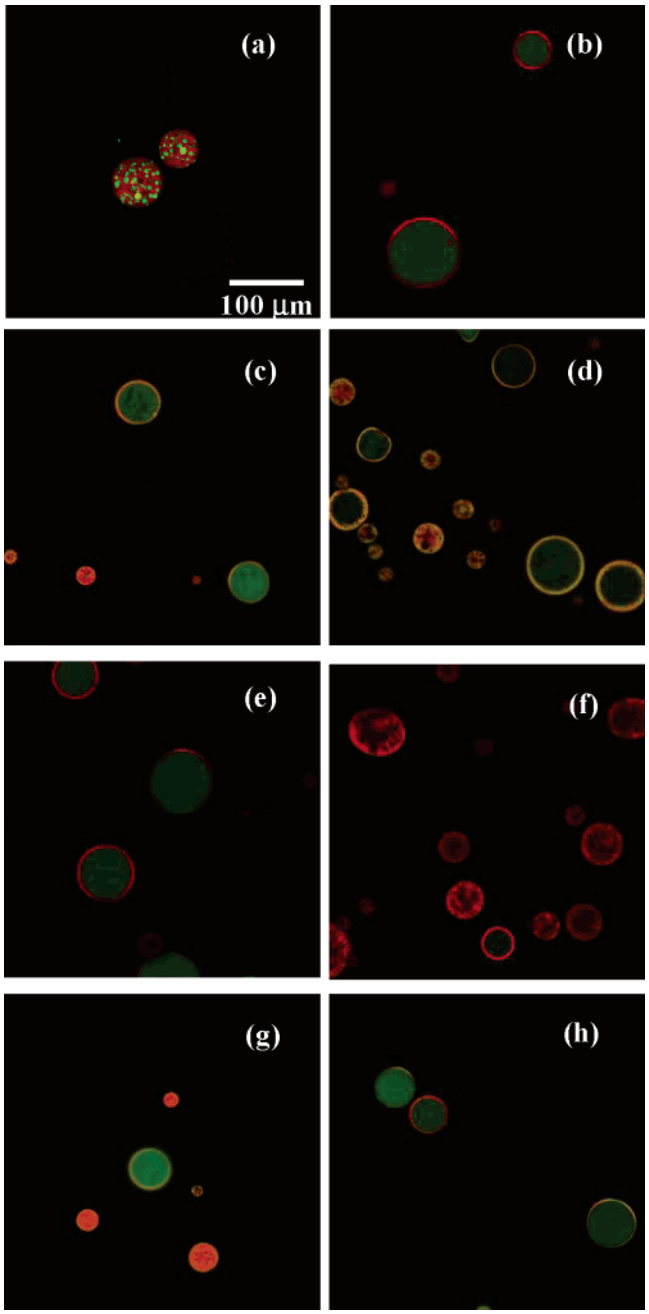

Figure 5. Confocal fluorescence microscopic images of microparticles produced by a double emulsion−solvent evaporation method (a) and by the solvent exchange method (b−h). Microcapsules were prepared using 5 w/v % HPLGA/LPLGA (50/50) and 3 w/v % lysozyme solutions in the absence of mannose in an aqueous solution and NaCl in the collection medium (b). Microcapsules were collected in a PVA solution with 0.28 M NaCl (c) or 0.56 M NaCl (d). Microcapsules were prepared using an aqueous solution containing either 10% mannose (e) or 30% mannose (f). Microcapsules were prepared using 1.5 w/v % polymer solutions composed of HPLGA/LPLGA (50/50) (g) or HPLGA (h) solutions.

significant portion of microcapsules was not mononuclear reservoir-type and entrapped a smaller amount of lysozyme, as judged by the pale green color (Figure 5d). For microcapsules prepared by incorporating mannose as an aqueous excipient, the morphology of reservoir-type microcapsules was preserved when 10% mannose was included in the aqueous core (Figure 5e). When the mannose concentration was 30%, microcapsules were formed but lysozyme was not encapsulated and the polymer solution appeared to be diffused into the bulk of the particle (Figure 5f). At a diluted polymer concentration (1.5%), HPLGA produced the reservoir-type microcapsules in an efficient manner (Figure 5g,h). At a diluted polymer concentration (1.5%), reservoir-type microcapsules were more effectively produced from HPLGA (Figure 5h), compared with the HPLGA/LPLGA (50/50) blend (Figure 5g).

Table 1. Characteristics of Reservoir-Type Microcapsules Prepared by Three Different Formulations

	polymer solution			
formulation[a]	composition (%)[b]	concn (%)	microcapsule size (μm)	encapsulation efficiency (%)
A	50	5.0	84.8 ± 4.9	71.1 ± 4.9
B	50	1.5	56.9 ± 4.3	15.9 ± 2.2
C	100	1.5	60.5 ± 6.4	31.7 ± 3.5

[a] All microcapsules were prepared using a 10% mannose solution as an aqueous core and 0.5% PVA solution with 0.28 M NaCl as the collection medium. The protein concentration in the aqueous solution was fixed at 3 w/v %. [b] Weight percentage of HPLGA in the HPLGA/LPLGA blend.

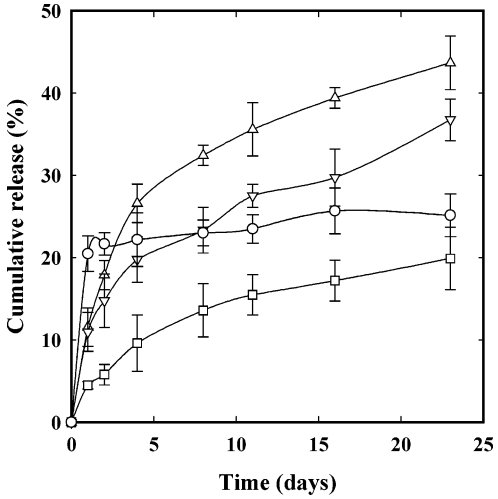

Figure 6. In vitro release profile of lysozyme from microspheres (○) and microcapsules at 37 °C in a PBS solution (pH 7.4). Microcapsules were prepared by formulation A (□), B (△), or C (▽) listed in Table 1 ($n = 3$).

Three representative formulations for microcapsule preparation were chosen for further experiments, and the characteristics of the resulting microcapsules are listed in Table 1. The in vitro release profile of lysozyme was evaluated as a function of time in a PBS (pH 7.4) solution. As shown in Figure 6, the release behavior of lysozyme was dependent on the formulation conditions. All the formulations, except for microspheres prepared by the double emulsion method, exhibited low initial burst for day 1 (<12%), followed by sustained release of lysozyme. In general, microcapsules

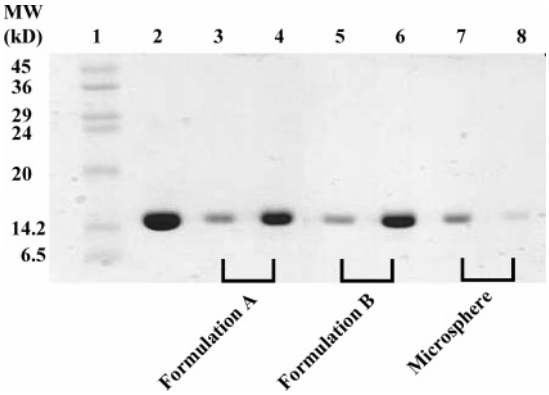

Figure 7. SDS−PAGE patterns of lysozyme released from reservoir-type microcapsules (lanes 3−6) and microspheres (lanes 7−8). Lanes 1 and 2 are size markers and native lysozyme, respectively. The release media were withdrawn after day 1 (lanes 3, 5, and 7) and day 30 (Lanes 4, 6, and 8) for each formulation.

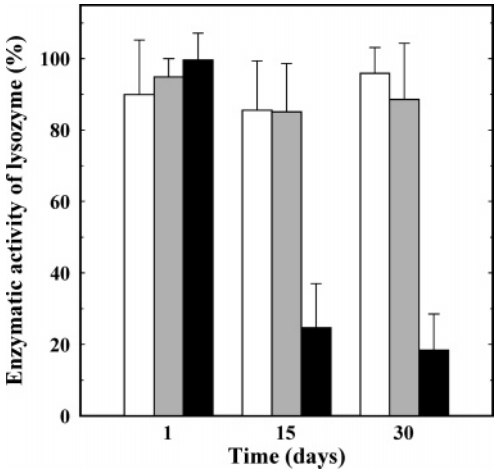

Figure 8. Enzymatic activity of lysozyme released from reservoir-type microcapsules and microspheres (black). Microcapsules were prepared by either formulation A (white) or B (gray).

prepared using a high polymer concentration (5%) showed the lowest initial burst (4.5%) as well as the slowest release of lysozyme. For formulations based on 1.5% polymer concentration, the release rate of lysozyme was higher for the microcapsule generated from HPLGA/LPLGA (50/50) blend, compared to that of HPLGA.

The integrity and biological activity of lysozyme, released from microspheres and microcapsules in a PBS (pH 7.4) solution, were evaluated as shown in Figures 7 and 8. The integrity of lysozyme was evaluated from SDS−PAGE results (Figure 7). For both microcapsules and microspheres, the band patterns of the lysozyme released for 30 days were equivalent to that of native lysozyme, indicating that lysozyme released is not the aggregated or hydrolyzed form. The biological activity test was performed by measuring transmittance change of *M. lysodeikticus* bacterial cell suspension (Figure 8). At 1 day after release, lysozyme from both microspheres and microcapsules showed biological activity comparable to that of native lysozyme. It is interesting to note that, as the release time increased, the biological activity of released lysozyme from microspheres prepared by the conventional double emulsion method was significantly attenuated from 99.6 ± 7.6% at day 1 to 18.3 ± 10.2% at day 30. In contrast, lysozyme released from microcapsules was fully active for the entire period of the release kinetic study.

Discussion

The solvent exchange method, implemented by an ultrasonic atomizer system, involves atomization of aqueous and organic solutions, spreading of an organic solution on the aqueous microdroplet core, mutual mass transfer of solvents at the interface between aqueous and organic solutions (i.e., solvent exchange), and solidification of the polymer shell in a collection medium. In this process, the final size and morphology of microcapsules may be dependent on a number of formulation parameters, such as the presence of excipients in the solutions, the polymer concentration, and the molecular weight of the polymer used. Microparticle size is of high importance because it determines the release rate, biodistibution, and allowable administration route of the encapsulated drug.[22−24]

The solvent exchange method provides several advantages over the conventional protein microencapsulation methods: (i) Since the solvent exchange method does not involve an emulsification procedure or high temperature, microcapsules are produced under the mild conditions and the exposure of protein to the w/o interface is limited only to the outer layer of the single aqueous core; (ii) the relatively smaller surface area of the polymer membrane layer in microcapsules alleviates hydrophobic interactions between protein and the polymer; and (iii) microspheres prepared by the conventional methods are principally prepared under strong physical stresses such as sonication and mechanical vortexing,[25−27] whereas the current method using an ultrasonic atomizer produces microcapsules with energy less than a few watts, which is far below a damaging level.[28] Formation of mononuclear reservoir-type microcapsules by the solvent exchange method was clearly supported by the confocal microscopy results (Figure 5).

In this study, we were interested in controlling the particle size. The effects of the composition of the aqueous core,

(21) Berkland, C.; King, M.; Cox, A.; Kim, K.; Pack, D. W. Precise control of PLG microsphere size provides enhanced control of drug release rate. *J. Controlled Release* **2002**, *82*, 137−147.
(22) Berkland, C.; Kim, K.; Pack, D. W. Fabrication of PLG microspheres with precisely controlled and monodisperse size distributions. *J. Controlled Release* **2001**, *73*, 59−74.
(23) Berkland, C.; Kim, K.; Pack, D. W. PLG microsphere size controls drug release rate through several competing factors. *Pharm. Res.* **2003**, *20*, 1055−1062.
(24) Berkland, C.; Kipper, M. J.; Narasimhan, B.; Kim, K. K.; Pack, D. W. Microsphere size, precipitation kinetics and drug distribution control drug release from biodegradable polyanhydride microspheres. *J. Controlled Release* **2004**, *94*, 129−141.

collection medium, and polymer solution on the particle size and encapsulation efficiency were examined. Selected particles were evaluated for their morphology, release kinetics, and stability of released proteins.

According to the theoretical prediction, the diameter of liquid droplets produced by the ultrasonic atomizer can be estimated by the following equation:[28]

$$d = 0.34(8\pi s/\rho f^2)^{1/3}$$

where s and ρ are the surface tension and density of the liquid, respectively, and f is the frequency of vibration. Since s and ρ are determined by the solvent used, the size of the liquid droplet is controlled by adjusting the vibration frequency. For example, the ultrasonic atomizer produces water droplets with a mean diameter of 25−80 μm, corresponding to the vibration frequency (range: 25−120 kHz). Therefore, it is expected that the microcapsule size can readily be controlled by the choice of the vibration frequency. However, our preliminary study showed that the vibration frequency does not affect the final size and morphology of microcapsules. This suggests a possibility that the water droplets produced are merged into large ones in the air because of the high spatial density near the atomization nozzle.[17]

Once the polymer solution covers the microdroplets of the aqueous core, the flexible polymer membrane layer is formed by the mutual mass transfer of the solvents.[17] The incipient microcapsules tend to form temporary aggregates which are subsequently separated into smaller ones under magnetic stirring in the collection medium, accompanied with solidification of the polymer via the extraction of the residual solvent. In this procedure, the rates of solvent exchange and solidification appear to play a critical role in breaking up the temporary aggregates of incipient microcapsules. Formulation variables that affect this procedure determine the final size of the resulting microcapsules. As a formulation variable, mannose was incorporated in an aqueous core. The reasoning was that mannose can hold a large amount of water due to its high aqueous solubility of 2.5 g/mL, and thus it may slow down the solvent exchange rate and allow the temporary aggregates to break down to smaller microcapsules. Furthermore, the presence of mannose inside the microcapsules was expected to enhance protein stability, as reported by other groups who demonstrated that sugars can stabilize the protein during its microencapsulation and freeze-drying.[26,29−31] The presence of mannose in the aqueous cores significantly reduced the particle size without deterioration of the microcapsule morphology (Figures 1 and 5). However, the excess amount of mannose (>20%) disturbed formation of reservoir-type microcapsules (Figure 5f) and decreased the encapsulation efficiency (Figure 1).

Addition of NaCl into the collection medium substantially reduced the microcapsule size down to 65.41 ± 8.9 μm. A similar trend was also reported for microspheres manufactured by double emulsion−solvent evaporation methods.[32,33] In general, the high osmotic pressure, occurring from the NaCl addition, led to the formation of a dense internal structure for microspheres and improved encapsulation efficiency.[33,34] However, for microcapsules, it was found that the high NaCl concentration decreased the encapsulation efficiency (Figure 1). The difference might be due to the different morphological characteristics. In contrast to microspheres prepared by the double emulsion method that have a continuous polymer matrix in the bulk which can inhibit protein extraction, microcapsules in this study have only a thin layer (1−2 μm) of polymer membrane through which a portion of lysozyme may be released from the aqueous core to the collection medium under the high osmotic pressure before the polymer is completely solidified.

The microcapsule size and encapsulation efficiency were also influenced by combination of mannose in the aqueous core and NaCl in the collection medium. This combined formulation allowed controlling the microcapsule size in the range of 70−90 μm with high encapsulation efficiency (>50%). In particular, matching of molar concentrations between two aqueous phases resulted in the highest encapsulation efficiency (71.10 ± 4.9%). These results reinforce that the solvent exchange rate and osmotic pressure are primarily responsible for the final microcapsule size, morphology, and encapsulation efficiency.

(25) Perugini, P.; Genta, I.; Pavanetto, F.; Modena, T.; Maculotti, K.; Conti, B. Evaluation of enzyme stability during preparation of polylactide-co-glycolide microspheres. *J. Microencapsulation* **2002**, *19*, 591−602.

(26) Perez-Rodriguez, C.; Montano, N.; Gonzalez, K.; Griebenow, K. Stabilization of alpha-chymotrypsin at the CH2Cl2/water interface and upon water-in-oil-in-water encapsulation in PLGA microspheres. *J. Controlled Release* **2003**, *89*, 71−85.

(27) Kang, F.; Singh, J. Conformational stability of a model protein (bovine serum albumin) during primary emulsification process of PLGA microspheres synthesis. *Int. J. Pharm.* **2003**, *260*, 149−156.

(28) Berger, H. L. *Ultrasonic liquid atomization*; Partridge Hill Publishers: New York, 1998.

(29) Liao, Y. H.; Brown, M. B.; Nazir, T.; Quader, A.; Martin, G. P. Effects of sucrose and trehalose on the preservation of the native structure of spray-dried lysozyme. *Pharm. Res.* **2002**, *19*, 1847−1853.

(30) Liao, Y. H.; Brown, M. B.; Quader, A.; Martin, G. P. Protective mechanism of stabilizing excipients against dehydration in the freeze-drying of proteins. *Pharm. Res.* **2002**, *19*, 1854−1861.

(31) Imamura, K.; Ogawa, T.; Sakiyama, T.; Nakanishi, K. Effects of types of sugar on the stabilization of protein in the dried state. *J. Pharm. Sci.* **2003**, *92*, 266−274.

(32) Deng, X. M.; Li, X. H.; Yuan, M. L.; Xiong, C. D.; Huang, Z. T.; Jia, W. X.; Zhang, Y. H. Optimization of preparative conditions for poly-DL-lactide- polyethylene glycol microspheres with entrapped Vibrio cholera antigens. *J. Controlled Release* **1999**, *58*, 123−131.

(33) Pistel, K. F.; Kissel, T. Effects of salt addition on the microencapsulation of proteins using W/O/W double emulsion technique. *J. Microencapsulation* **2000**, *17*, 467−483.

(34) Jiang, G.; Thanoo, B. C.; DeLuca, P. P. Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres. *Pharm. Dev. Technol.* **2002**, *7*, 391−399.

The polymer concentration was also varied to control the microcapsule size. Small microcapsules (<60 μm) were produced by atomizing the diluted polymer solution (<2%) which is slowly solidified because a large amount of water is required for polymer precipitation.[18] However, this allowed lysozyme in an aqueous core to escape into the collection medium, resulting in low encapsulation efficiency (<20%). On the other hand, it should be noted that the low encapsulation efficiency was substantially improved by increasing the percentage of HPLGA in the polymer solution (Figure 4). This might be due to the increased viscosity of the polymer solution by HPLGA that plays a role as a barrier for the lysozyme diffusion.

The reservoir-type microcapsules released lysozyme in a sustained manner without the significant burst effect (<12%), as shown in Figure 6. This release behavior was different from that with the microspheres: Approximately 20% of lysozyme encapsulated in the microspheres was released in day 1, after which no substantial release was found for the rest of the release study. In fact, other groups have shown that the proteins entrapped in the microspheres undergo aggregation and attachment to the polymer matrix via hydrophobic interaction, resulting in the limited release to the surrounding medium.[9,35,36] For microcapsules, the release rate of lysozyme is governed by the polymer membrane layer that plays a role as a diffusion barrier. Therefore, the membrane compactness and molecular weight of the polymer may determine the release behavior. The result also implies that the low degradation rate of HPLGA is responsible for the slower release than that for the blends containing LPLGA.

From the results of SDS−PAGE and the transparency change of the bacterial cell suspension, it was confirmed that lysozyme released from microcapsules preserved its native structure as well as biological activity (Figures 7 and 8). On the contrary, the biological activity of lysozyme released from microspheres was deteriorated as a function of the releasing time, although SDS−PAGE results indicated that the released lysozyme was neither aggregated nor hydrolyzed. This may be due to the long-term exposure of lysozyme to a large area of the acidic (pH <3) microclimate inside the microsphere.[5] For microcapsules, however, the acidic fragments generated from microcapsule shells can be diffused rapidly out of the microcapsule, which enables lysozyme to maintain its native structure as well as biological activity. The solvent exchange method provides an opportunity to prepare reservoir-type microcapsules with different particle sizes that can release a protein in its native form.

Conclusion

This study explored the solvent exchange method, implemented by an ultrasonic atomizer, to prepare reservoir-type microcapsules as a potential carrier of protein drugs. Various formulation parameters, such as the presence of excipients and polymer composition, affected the size and encapsulation efficiency of microcapsules. Lysozyme encapsulated was released from the microcapsules in a sustained manner and maintained its biological activity during the manufacturing process and the releasing period of time.

Acknowledgment. This study was supported in part by the National Institutes of Health through Grants GM67044 and EB003584.

MP050083W

(35) Blanco, D.; Alonso, M. J. Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. *Eur. J. Pharm. Biopharm.* **1998**, *45*, 285−294.

(36) van de Weert, M.; Hennink, W. E.; Jiskoot, W. Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm. Res.* **2000**, *17*, 1159−1167.