A Novel Biodegradable System Based on Gelatin Nanoparticles and Poly(lactic-*co*-glycolic acid) Microspheres for Protein and Peptide Drug Delivery

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Abstract □ Gelatin nanoparticle–poly(lactic-*co*-glycolic acid) (PLGA) microsphere composites were prepared by encapsulating protein-loaded gelatin nanoparticles in PLGA microspheres. This encapsulation was conducted by using a phase separation method and a solvent extraction method. The average diameter of the gelatin nanoparticle–PLGA microsphere composites is between 160 and 175 μm. Protein loading efficiency is 93.2% for the nanoparticle–microsphere composite prepared by the phase separation method, while it is 31.31% for the composite prepared by the solvent extraction method. Protein release experiments indicate that this new composite system possesses sustained release characteristics. This system also demonstrates the capability of preventing the denaturation of protein drugs.

Introduction

Some physicochemical and biological properties of protein and peptide drugs are different from those of conventional ones. These properties may include molecular size, biological half-life, conformational stability, solubility, and dose requirement and administration. The delivery systems that function well for conventional drugs may not be suitable for protein and peptide drugs.[1] Therefore, design and manufacturing of truly unique protein and peptide drug delivery systems is desired.

To meet this desire, many nonconventional systems have been developed and tested. These systems may be classified into two types: nondegradable and biodegradable. Devices based on poly(ethylene-*co*-vinyl acetate) are typical examples of the nondegradable protein/peptide drug delivery systems.[2,3] Examples of biodegradable systems for protein/peptide drug delivery are injectable microspheres based on poly(lactic-*co*-glycolic acid) (PLGA).[4−6] The biodegradable systems have many advantages over their nondegradable counterparts. For instance, if the drug delivery system is administered by implantation, use of the biodegradable polymers can avoid retrieval of the matrix from the site of administration after drug is depleted. Consequently, this avoidance of matrix retrieval can improve the patient's compliance and lower the therapeutic cost.

The biodegradable polymeric systems may be further categorized into hydrophilic polymeric systems, such as gelatin nano- and microparticles,[7] and hydrophobic polymeric systems, such as PLGA microspheres.[8−10] Neither the hydrophilic nor the hydrophobic system is ideal for protein/peptide drug delivery. They each have their own advantages and disadvantages. For example, the hydrophilic polymeric systems are biocompatible with the protein/peptide drugs, but have difficulty achieving sustained drug release.[11] When the systems absorb water and swell, protein/peptide molecules will rapidly diffuse out. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of protein/peptide molecules; therefore, the protein/peptide drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems.[12,13] To promote the advantages and overcome the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, we combined a hydrophilic system, gelatin nanoparticles, with a hydrophobic polymeric system, PLGA microspheres. This combination creates a new biodegradable system for protein and/or peptide drug delivery.

Hydrophilic gelatin nanoparticles containing a model protein drug, bovine serum albumin (BSA), first were prepared, and then BSA-loaded gelatin nanoparticles were encapsulated in the hydrophobic PLGA microspheres to create nanoparticle−microsphere composites. This encapsulation was conducted using a phase separation method and a solvent extraction method, separately. The average diameter of the gelatin nanoparticle−PLGA microsphere composites is between 160 and 175 μm. Protein loading efficiency is 93.2% for the nanoparticle−microsphere composite prepared by the phase separation method, while it is 31.31% for the composite prepared by the solvent extraction method. Protein release experiments indicate that this new composite system possesses sustained release characteristics. This system also demonstrates the capability of preventing the denaturation or the molecular integrity loss of protein drugs.

Experimental Section

Materials—PLGA (molar ratio of D,L-lactic to glycolic acid, 75:25; inherent viscosity, 0.58 dL/g in $CHCl_3$ at 30 °C) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Gelatin (bloom strength, 225; p*I*, 4.7) was obtained from Sigma Chemical Co. (St. Louis, MO). BSA (MW, 66 000) was also supplied by Sigma Chemical Co. Corn oil (Mazola, 100% pure) was obtained from CPC International, Inc. (Englewood Cliffs, NJ). Methylene chloride was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Silicon oil (350 cs) was obtained from Dow Corning Corp. (Midland, MI).

Preparation of Gelatin Nanoparticles—The preparation of gelatin nanoparticles was reported in our previous work.[11] Briefly, aqueous gelatin solution (7% w/v), 1 mL, containing 30 mg of BSA was added to 90 mL of corn oil preheated to 40 °C in a water bath.

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The biphasic system was homogenized at a speed of 10 000 rpm for 5 min to form a water-in-oil emulsion by using a homogenizer (Model M 122, Biospec Products Co., Barhesville, OK). The emulsion was then cooled to 4 °C in a refrigerator. When the temperature of the emulsion fell down below the gelling point of the gelatin solution, the globules of the gelatin solution containing BSA were converted to gelatin hydrogel particles, while the emulsion was changed to a suspension. To reduce the viscosity of the suspension, methylene chloride was added. The diluted suspension was then vacuum filtered using a Nylon membrane filter having a pore size of 100 nm (Micron Separations, Inc., Westboro, MA). The filtrant was washed using methylene chloride to remove the residual corn oil. The collected gelatin nanoparticles were vacuum dried overnight. A white, flowing powder was obtained. The average size of the collected gelatin nanoparticles is around 800 nm.[11]

Preparation of Gelatin Nanoparticle−PLGA Microsphere Composite Using a Phase Separation Method—A 10% (w/w) polymer solution was prepared by dissolving PLGA in methylene chloride. Gelatin nanoparticles (150 mg) containing 25 mg of BSA were suspended in 5 mL of the PLGA solution. The suspension was stirred at 200 rpm. Silicon oil (6 g) was then progressively added to the suspension at a speed of 1 g/min to form an oil-in-oil emulsion. Once the droplets of the PLGA solution containing gelatin nanoparticles reached the desired size (100−200 μm), the emulsion was transferred with stirring to a quenching tank containing 2 L heptane. The quenching tank was stirred for 3 h to harden the gelatin nanoparticle-loaded PLGA microspheres. Finally, the microspheres were collected by filtration, washed with heptane, and dried in a vacuum container for 24 h.

PLGA microspheres containing BSA without gelatin nanoparticles were also prepared for the purpose of comparison. BSA (25 mg) was dissolved in 0.5 mL of phosphate-buffered saline (PBS; pH 7.4). This aqueous solution was emulsified in the PLGA solution using a homogenizer to form a water-in-oil emulsion. Then, following the same steps as described above, PLGA microspheres containing only BSA were obtained.

Preparation of Gelatin Nanoparticle−PLGA Microsphere Composite Using a Solvent Extraction Method—The same amount and the same type of BSA-loaded gelatin nanoparticles as described above (150 mg) were suspended in 5 mL of the aforementioned PLGA solution of methylene chloride (10% w/w). The suspension was poured into 60 mL of aqueous poly(vinyl alcohol) solution (1% w/w) saturated with methylene chloride. The mixture was stirred at 200 rpm to produce an oil-in-water emulsion. When the diameter of the droplets reached 100−200 μm, the emulsion was transferred to a mixture of ice and distilled water (2 L, 5 °C) to harden the droplets. The water was stirred for 3 h to extract the solvent from the microspheres containing the BSA-laden gelatin nanoparticles. The hardened microspheres were then collected by filtration and dried in a vacuum container for 24 h.

Morphology Observation—A Nikon Optiphot biological microscope (Model M-35S) was used to examine the microscopic characteristics of the gelatin nanoparticle−PLGA microsphere composites. Polaroid pictures were taken from the microscope during the examinations.

Size and Size Distribution Measurement—Each of the two gelatin nanoparticle−PLGA microsphere composites (5 mg) was suspended in 15 mL of distilled water by sonicating the suspension for 10 s using a probe sonicator (Model 2000 U, B. Braun Biotech, Inc., Allentown, PA). The suspension was then evaluated using a particle sizer (Accusizer, Model 770, Particle Sizing Systems, Santa Barbara, CA). Average size and size distribution of the composite were obtained from this study.

Protein Loading Efficiency Determination—The amount of protein (BSA) loaded in the gelatin nanoparticle−PLGA microsphere composites was determined directly by recovering the protein from the composites. The experiments were conducted in triplicate. A dried composite (20 mg) was accurately weighed and transferred to a separatory funnel. Methylene chloride (2 mL) was added to the composite sample. After the composite was dissolved, phosphate buffer (4 mL) was added to the separatory funnel. The funnel was capped with a stopper and the mixture was agitated in a shaker for 2 days. Then, the separatory funnel was left hanging on a metal ring until the aqueous phase and the organic phase separated completely. After the phase separation, the aqueous layer was collected for assay of the protein. A capillary electrophoresis apparatus (Spectra, Model 1000-CE00, Thermo Separation Products, San Jose, CA) was employed to determine BSA concentration in the aqueous extractant using phosphate buffer (pH 10.4) as a mobile phase. A UV detector (wavelength, 200 nm) was used to quantitate the BSA. The protein loading efficiency was calculated by using the following formula

$$\text{loading efficiency} = \frac{M_{\text{actual}}}{M_{\text{theoretical}}} \times 100\%$$

where M_{actual} is the actual amount of BSA in each composite determined by the above experiment and $M_{\text{theoretical}}$ is the theoretical amount of BSA in each composite calculated from the quantity added in the fabrication process.

In Vitro Protein Release Study—In vitro BSA release studies were performed in triplicate. The gelatin nanoparticle−PLGA microsphere composite prepared by the phase separation method (50 mg) was dispersed in 2 mL of PBS. The dispersion was incubated at 37 °C in a shaking water bath with shaking speed of 36 rpm. At predetermined time intervals, 1 mL of the supernatant was taken and 1 mL of fresh PBS was added. The withdrawn supernatant was analyzed using the same capillary electrophoresis apparatus described above for quantitation of the protein released and also for assay of the protein integrity. The cumulative amount of the protein released from the composite is defined by the following equation

$$\text{cumulative amount released} = \frac{\sum_{t=0}^{t} M_t}{M_{\text{actual}}} \times 100\%$$

where M_t is the amount of BSA released at time t, and M_{actual} is the actual amount of BSA loaded in the composite defined above.

Results and Discussion

Microscopic Characteristics—Figure 1 shows optical micrographs of a typical population of the gelatin nanoparticle−PLGA microsphere composites prepared by the phase separation method and the solvent extraction method. From these micrographs, one can see that the PLGA microspheres containing the protein-laden gelatin nanoparticles (i.e., the composites) are spherical in shape and uniform in size.

Size and Size Distribution—Size and size distribution are important properties of microparticulate drug delivery systems. One reason is that injectable microparticles cannot be too large to pass through a syringe needle. The other reason is that the size of the microparticles has a great effect on drug release characteristics.

Figure 2 shows the size distribution of the gelatin nanoparticle−PLGA microsphere composites. This figure tells us that the composites possess a normal or Guassian size distribution. The average diameter of the composite prepared by the phase separation method is approximately 160 μm, while that prepared by the solvent extraction method is approximately 175 μm. The preparation methods (phase separation and solvent extraction) have little effect on the composite size.

The stir rate of the emulsion apparatus in the preparation process is a critical factor in determining the size of the composites.[14] In our experiments, the average diameter of the collected composites is around 170 μm with a stir rate of approximately 200 rpm. However, when the stir rate increases to 300 rpm, the average diameter of the collected composites becomes smaller than 100 μm.

Protein Loading Efficiency—BSA loading efficiency of the gelatin nanoparticle−PLGA microsphere composite prepared by the phase separation method is 93.2%, and that prepared by the solvent extraction method is 31.31%. This difference comes from the difference between these two methods. Every step of the phase separation method is

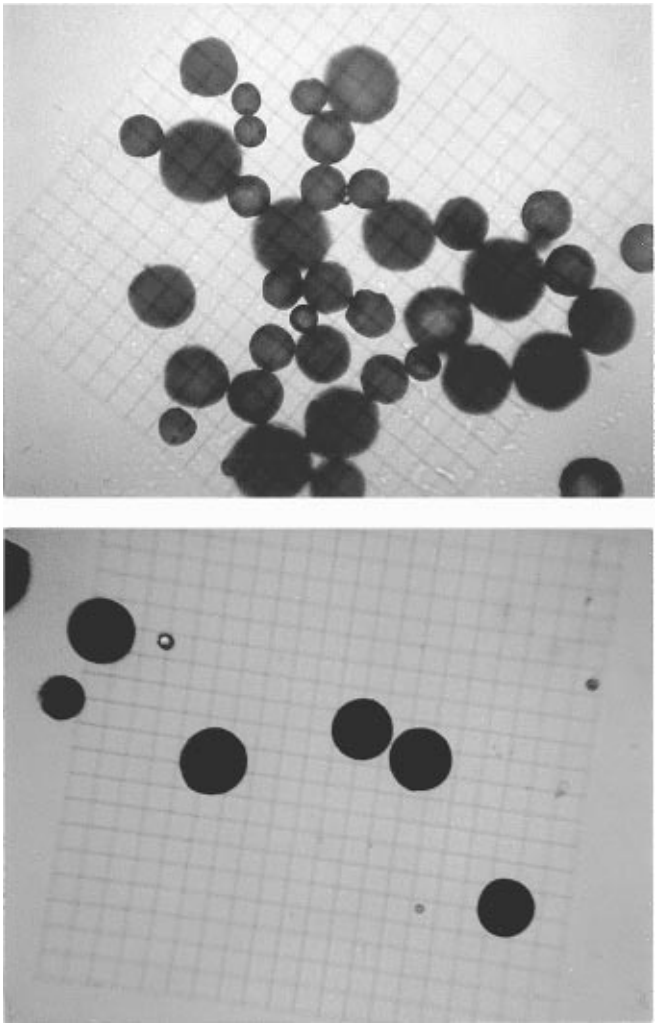

Figure 1—Optical micrographs of the gelatin nanoparticle–PLGA microsphere composites: (A, top) prepared by the phase separation method and (B, bottom) prepared by the solvent extraction method.

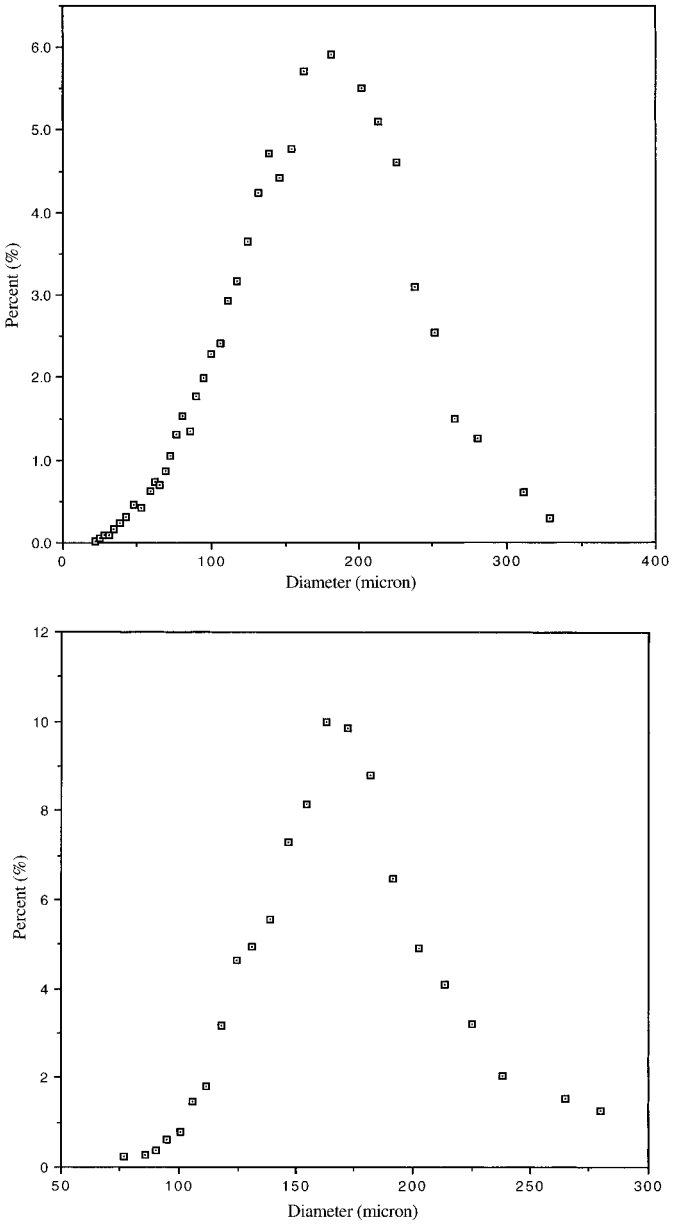

Figure 2—Size distribution of the gelatin nanoparticle–PLGA microsphere composites: (A, top) prepared by the phase separation method and (B, bottom) prepared by the solvent extraction method.

conducted in an organic system. There is no aqueous phase involved in this method. In contrast, for the solvent extraction method, the composite is prepared in an aqueous system, particularly the composite is washed in water for 3 h to harden the composite or to extract the solvent from the composite. During this washing process, the gelatin nanoparticles might uptake water and swell, resulting in the diffusion of BSA molecules out of the gelatin nanoparticles. Furthermore, the BSA molecules might diffuse out from the unsolidified composite. To take the loading efficiency into consideration, the phase separation method is recommended for the preparation of PLGA microspheres for encapsulation of water-soluble substances.

Protein Release—Figure 3 shows BSA release curves of the gelatin nanoparticle−PLGA microsphere composite and the PLGA microspheres containing only BSA, prepared by the phase separation method. The BSA release profile of the composite presents two phases: fast release (17.37% of the protein encapsulated) and sequential slow release. The fast release may be due to some gelatin nanoparticles sitting on or imbedded in the PLGA microspheres surface or imperfection of the composite surface. The mechanism of slow release is relatively complicated. It may involve the following aspects: (1) water permeation through the PLGA matrix and absorption by the gelatin nanoparticles, (2) gelatin nanoparticle swelling, (3) diffusion of BSA molecules through the swollen gelatin nanoparticles, (4) further diffusion of BSA molecules through a tortuous, water-filled path in the PLGA microsphere matrix, and (5) PLGA degradation.

In comparison with the gelatin nanoparticle−PLGA microsphere composite, the PLGA microspheres containing protein only release the protein very fast. In approximately 3.5 days, 90% of the protein has been released. This burst release retards application of PLGA microspheres to protein drug delivery.[15] However the new gelatin nanoparticle−PLGA microsphere composite releases the protein slowly. In 18 days, only 66% of the loaded protein molecules are released. After 40 days, protein can still be released from the composite, but the released rate is very low. Retardation of the burst release or the slow release characteristic of the composite makes this new biodegradable system a good carrier candidate for prolonged protein/peptide drug delivery.

Protein Stability—Protein drugs are fragile or unstable in comparison with the conventional synthetic drugs. Therefore, protein drugs are easily denatured or easily lose their biological activity. The denaturation of protein drugs may

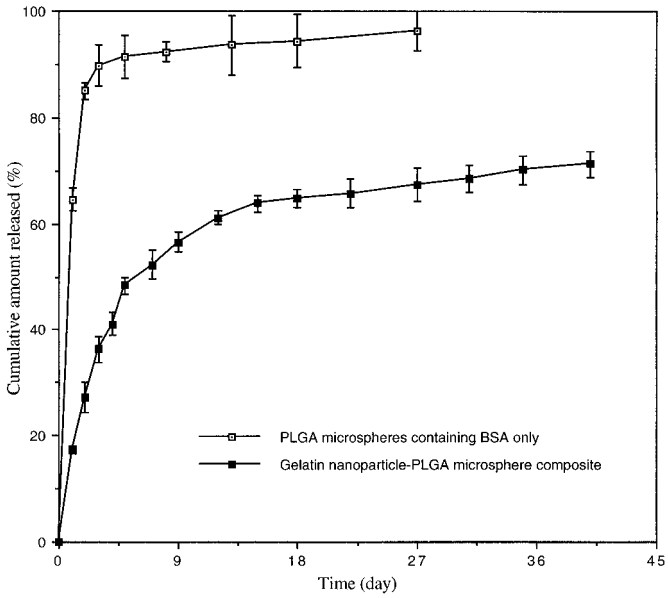

Figure 3—In vitro release of BSA from the gelatin nanoparticle–PLGA microsphere composite and the PLGA microspheres containing only BSA, prepared by the phase separation method.

include the following aspects: aggregation, degradation, and/or unfolding. All of these denaturation aspects can differentiate the protein molecules from their native counterpart conformationally or structurally. The conformational or structural change may be detected instrumentally.

Capillary electrophoresis has many potential uses, such as analyzing purity and structure conformation of a substance. The separation mechanism of capillary electrophoresis is based on the difference in the charge-to-mass ratio of individual analytes. The difference in the charge-to-mass ratio results in the different electrophoretic mobility of individual analytes. The migration rate of an analyte is influenced not only by its electric charge but also by the bulk flow of the mobile phase. This flow is known as electroosmotic flow, which is a result of the movement of a counteranion at the wall toward the cathode. For the fused silica capillary, the electroosmotic flow is strong and has great influence on the migration rate of the analyte. The capillary electrophoresis system used in this experiment has the anode on the injection side. In the mobile phase of pH 10.3, the BSA is negatively charged. Under the applied high voltage field, the negatively charged BSA is pulled toward the anode. Hence, the negatively charged species with higher charge-to-mass ratio will present a longer retention time, while those with lower charge-to-mass ratio will display a shorter retention time.

During preparation and storage of the gelatin nanoparticle–PLGA microsphere composite, harmful conditions that BSA molecules encountered may cause protein denaturation, such as aggregation, degradation, and/or unfolding. These harmful conditions may include contact with organic solvents, exposure to the hydrophobic and acidic microenvironment of the PLGA matrix, and mechanical agitation. The protein denaturation changes the mass-to-surface charge ratio, which results in distinctly different capillary electrophoretic peaks from their parent product.

Figure 4 shows electropherograms of BSA. Electropherogram A is from a native BSA dissolved in PBS. Electropherogram B is from BSA released from the gelatin nanoparticle–PLGA microsphere composite prepared by the phase separation method, and electropherogram C is from BSA released from the PLGA microspheres containing only the protein. As one can see, the BSA released from PLGA microspheres containing

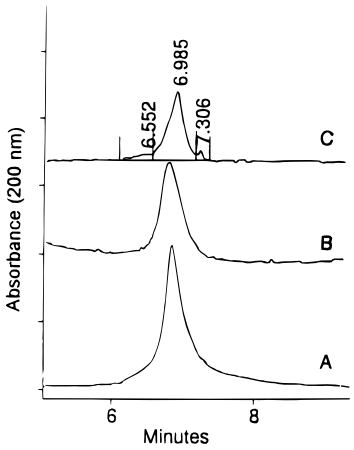

Figure 4—Electropherograms of BSA released from the gelatin nanoparticle–PLGA microsphere composite and the PLGA microspheres containing only BSA: (A) native BSA dissolved in PBS, (B) BSA released from the gelatin nanoparticle–PLGA microsphere composite, (C) BSA released from the PLGA microspheres containing only BSA.

Table 1—Integrity Changes of Protein Released from the Gelatin Nanoparticle–PLGA Microsphere Composite and the PLGA Microspheres Containing Only BSA

Time (day)	% Integrity Change of BSA	
	Released from Composite	Released from PLGA Microspheres Containing Only BSA
5	0	2.6
8	0	7.1
11	0	10.1
14	0	22.6
20	0	31.8

only BSA presents deformative peaks at the retention time of 6.55 and 7.30 min. These peaks are an indication of formation of a new mass-to-surface charge ratio or formation of a new molecular weight species, which probably means that the BSA has been denatured to some extent. The peak at 6.55 min indicates an aggregated species because they possess a lower charge-to-mass ratio, which results in greater electrophoretic mobility, whereas the peak at 7.30 min may be due to degraded species, because they have a higher charge-to-mass ratio which results in lower electrophoretic mobility. In contrast, BSA released from the gelatin nanoparticle–PLGA microsphere composite displays a normal BSA peak, which is the same as that of the native BSA. Table 1 further shows the percentage of the integrity change of BSA as a function of the protein release time. The percentage of integrity change is calculated from the peak area of the shoulder peaks (such as those at 6.55 and 7.30 min in Figure 4) divided by the area of the total peaks. From Table 1, one can find that more BSA molecules lose their integrity as the release time increases. In comparison, the BSA molecules protected by the gelatin nanoparticles do not show any deformative peaks, even after 20 days of release. Therefore, it may be concluded that the gelatin nanoparticles in the PLGA microspheres show some potential to protect BSA from integrity loss or denaturation during the microsphere preparation, storage, and release.

Summary

A novel biodegradable system for prolonged or controlled release of protein/peptide drugs has been developed. This new system is a combination of a hydrophilic polymer (gelatin) and a hydrophobic polymer (PLGA). The gelatin is formulated as

nanoparticles while the PLGA is formulated as microspheres. The nanoparticles are then encapsulated in the microspheres. The experimental results show that the BSA-loaded gelatin nanoparticles were successfully encapsulated in the PLGA microspheres. The encapsulation was conducted by a phase separation method and a solvent extraction method, separately. This encapsulation practice creates a new composite system. The average diameter of the gelatin nanoparticle−PLGA microsphere composites is between 160 and 175 μm. BSA loading efficiency is 93.2% for the composite prepared by the phase separation method, while it is 31.31% for the composite prepared by the solvent extraction method. Release experiments show that only 66% of BSA is released from the gelatin nanoparticle−PLGA microsphere composite in 18 days, which indicates that this new system possesses sustained release characteristics for protein drugs. This new system also demonstrates the capability of preventing protein drugs from integrity loss or denaturation.

References and Notes

1. Siegel, R. A.; Langer, R. Controlled release of polypeptides and other macromolecules. *Pharm. Res.* **1984**, *1*, 2−10.
2. Bawa, R.; Siegel, R.; Marasca, B.; Karel, M.; Langer, R. An explanation for the controlled release of macromolecules from polymers. *J. Controlled Release* **1985**, *1*, 259−267.
3. Miller, E. S.; Peppas, N. A.; Winslow, D. N. Morphological changes of ethylene/vinyl acetate-based on controlled delivery systems during release of water-soluble solutes. *J. Membr. Sci.* **1983**, *14*, 79−92.
4. Hora, M. S.; Rana, R. K.; Nunberg, J. H.; Tice, T. R.; Gilley, R. M.; Hudson, M. E. Release of human serum albumin from poly-(lactide-*co*-glycolide) microspheres. *Pharm. Res.* **1990**, *7* (11), 1190−1194.
5. Jeffery, H.; Davis, S. S.; O'Hagan, D. T. The preparation and characterization of poly(lactide-*co*-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique. *Pharm. Res.* **1993**, *10* (3), 362−368.
6. Ogawa, Y.; Okada, H.; Yamamoto, M.; Shimamoto, T. In vivo release profiles of leuprolide acetate from microcapsules of polylactic acid or copoly(lactic/glycolic) acid and in vivo degradation of these polymers. *Chem. Pharm. Bull.* **1988**, *36*, 2576−2581.
7. Tabata, Y.; Ikada, Y. Synthesis of gelatin microspheres containing interferon. *Pharm. Res.* **1989**, *6*, 422−427.
8. Ruiz, J. M.; Benoit, J. P. In vivo peptide release from poly(DL-lactic acid-*co*-glycolic acid) copolymer 50/50 microspheres. *J. Controlled Release* **1991**, *16*, 177−186.
9. Heya, T.; Okada, H.; Ogawa, Y.; Toguchi, H. Factors influencing the profiles of TRH release from copoly(d,l-lactic/glycolic acid) microspheres. *Int. J. Pharm.* **1991**, *72*, 199−205.
10. Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L. H.; Langer, R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm. Res.* **1991**, *8*, 713−720.
11. Li, J. K.; Wang, N.; Wu, X. S. Gelatin nanoencapsulation of protein/peptide drugs using an emulsifier-free emulsion method. *J. Microencapsulation*. In press.
12. Sluzky, V.; Tamada, J. A.; Klibanov, A. M.; Langer, R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9377−9381.
13. Johnson, E. R.; Lanaski, L. A.; Gupta, V.; Griffin, M. J.; Gaud, H. T.; Needham, T.; Zia, H. Stability of atriopeptin III encapsulated in poly(D,L-lactide-*co*-glycolide) microcapsules. *Pharm. Res.* **1990**, *7*, S-181.
14. Wu, X. S. Preparation, characterization, and drug delivery applications of microspheres based on biodegradable lactic/glycolic acid polymer. In *Encyclopedic Handbook of Biomaterial and Bioengineering*; Wise, D. L.; Trantolo, D. J.; Altobelli, D. E.; Yaszemski, M. J.; Gresser, J. D.; Schwartz, E. R., Eds.; Marcel Dekker: New York, NY, 1995; pp 1151−1250.
15. Wang, H. T.; Palmer, H.; Linhardt, R. J.; Glanagan, D. R.; Schmitt, E. Degradation of poly(ester) microspheres. *Biomaterials* **1990**, *11*, 679−685.

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