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A novel approach to stabilization of protein drugs in poly(lactic-co-glycolic acid) microspheres using agarose hydrogel

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Abstract

A novel approach has been taken to stabilize protein drugs in poly(lactic-co-glycolic acid) (PLGA) microspheres. This approach creates a new protein drug delivery system, which is based on the combination of agarose hydrogel particles and PLGA microspheres. This combination produces a heterogeneously structured polymeric composite. The protein drug molecules are encapsulated in the agarose hydrogel particles and the drug-containing agarose hydrogel particles are further dispersed in the PLGA microspheres. One PLGA microsphere may contain many agarose hydrogel particles to form a PLGA–agarose composite microsphere. The PLGA–agarose composite microspheres have spherical shape and a smooth surface. They possess a normal or Gaussian size distribution and an average diameter of 150 μm. The PLGA–agarose composite microspheres have higher protein loading efficiency than that of the conventional PLGA microspheres. The hydration of the PLGA–agarose composite microsphere matrix is faster than that of the conventional PLGA microspheres. Protein drugs can be slowly released from the PLGA–agarose composite microspheres. The agarose hydrogel particles can stabilize protein drugs in the PLGA matrix, which is the major advantage of this novel protein drug delivery system over the conventional PLGA microspheres.

Keywords: Protein stabilization; Poly(lactic-co-glycolic acid); Microspheres; Agarose; Hydrogel; Heterogeneous/domain structure

1. Introduction

Since the first publication of protein delivery using poly(lactic acid) microspheres by Chang

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(1976), the microspherical drug delivery system based on poly(lactic acid), poly(glycolic acid), or their co-polymers has been extensively investigated due to many advantages of this drug delivery system (Kaetsu et al., 1987; Cha and Pitt, 1989; Jalil, 1990; Cohen et al., 1991; Shah et al., 1993; Mehta et al., 1994; McGee et al., 1995). Examples of the advantages of this drug delivery system include biocompatibility, controllable biodegradability, absorbability and low toxicity of the degradation end products, sustained release potential, and ease of administration (Wu, 1995).

On the other hand, there are some problems for this polymeric drug delivery system. One of the problems is structural or conformational change of proteins during preparation of, storage of, and release from this type of drug delivery system (Tabata et al., 1993; Lu and Park, 1995). One reason for the protein structural or conformational change, or integrity loss, may be the harsh preparation or formulation conditions (Lu and Park, 1995; Uchida et al., 1996). During formulation, two immiscible liquid phases (such as organic and aqueous phases) are usually involved and interfaces are created, e.g. an aqueous protein solution is usually dispersed in an organic polymer solution by using a high-speed homogenizer or a sonicater yielding a water-in-oil micro-emulsion (Yan et al., 1994; Lu and Park, 1995). Such a micro-emulsion possesses extremely high interfacial area. Proteins, which can be surface active, tend to migrate to the interface between the aqueous phase and the organic phase. The protein molecules at the interfaces may unfold and, consequently, the structure or conformation may change. Another reason for causing the protein structural or conformational change may be the hydrophobicity and/or acidity of the lactic and/or glycolic acid polymers (LGAP). During the post-preparation storage, the hydrophobic LGAP matrix may induce protein unfolding and/or aggregation. The acidity of the LGAP matrix may lead to degradation of the protein molecules. In addition, during drug release from the LGAP matrix, protein may be adsorbed at the interface of the LGAP matrix and the release medium (Mehta et al., 1994). The adsorption may also induce unfolding of the protein molecules. To reduce or balance the hydrophobicity of the LGAP matrix, to protect the protein from integrity loss during preparation of, storage of, and release from the LGAP matrix, we combine the hydrophobic LGAP with a hydrophilic polymer, agarose. The combination is conducted in such a way that a heterogeneous or domain structure is produced, i.e. the hydrophilic agarose is formulated as particles, and the agarose hydrogel particles are dispersed in the LGAP microspheres to form a composite. One LGAP microsphere may contain many agarose hydrogel particles. These agarose hydrogel particles form domains or islands in the LGAP microspheres due to the special technique employed for the preparation of the LGAP microsphere–agarose hydrogel particle composite. This novel approach can stabilize proteins from structural or conformational change in LGAP microspherical delivery systems.

Insulin is used as a model protein drug. It is loaded in this novel composite by first entrapping the drug molecules in the agarose hydrogel particles and then loading the insulin-containing particles in the LGAP microspheres. The new heterogeneously structured composite is characterized with regard to morphology, product size and size distribution, water absorption, drug loading efficiency, drug release, and efficiency of insulin stabilization.

2. Materials and methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA), (lactic to glycolic acid molar ratio, 75:25; inherent viscosity, 0.58 dl/g in $CHCl_3$ at 30°C) was purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). Insulin (bovine pancreas, 28.5 USP units/mg), agarose, trifluoroacetic acid (TFA, protein sequencing grade), and acetonitrile (HPLC grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Silicone oil (viscosity, 350 cSt) was purchased from Dow Corning Corp. (Midland, MI, USA). All other organic solvents were HPLC grade and from Spectrum Chemical Manufacturing Corp. (Gardena, CA, USA).

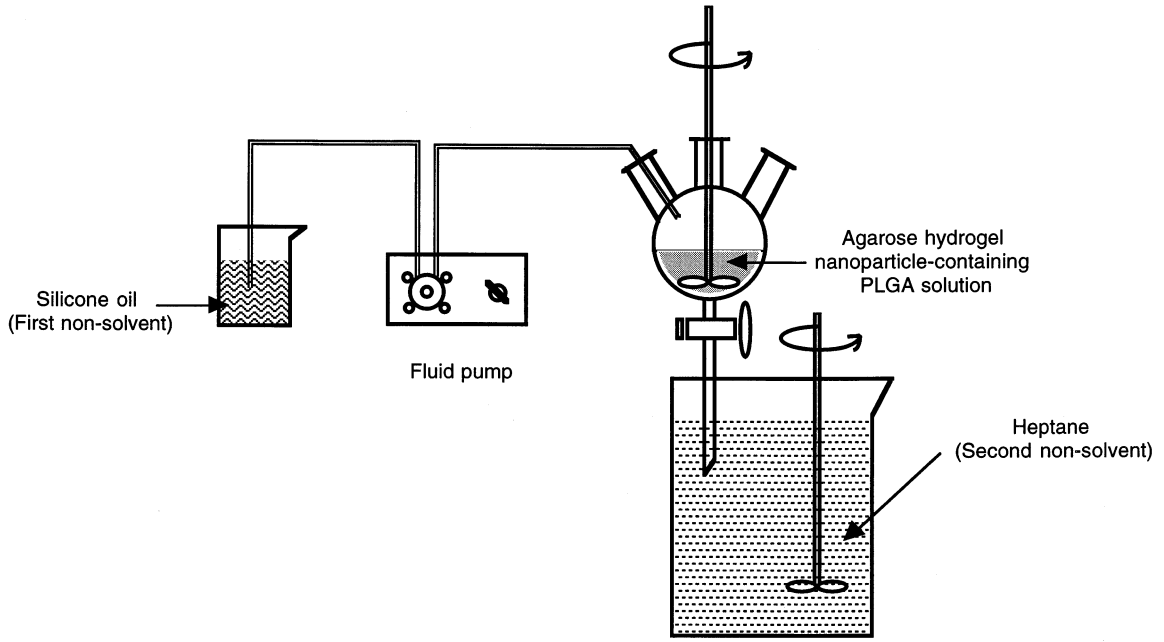

Fig. 1. Schematic drawing of the apparatus used for the preparation of the PLGA–agarose composite microspheres.

2.2. Preparation of the composite of PLGA microspheres and agarose hydrogel particles

The PLGA-agarose composite microspheres were prepared by using a modified phase separation method. The PLGA (1 g) was dissolved in 9 g methylene chloride to make a 10 wt.% polymer solution. The polymer solution was heated to 40°C in a water bath. Agarose powder, 50 mg, was dissolved in 2 ml phosphate-buffered saline (PBS, 20 mM, pH 7.5) at 90°C and then cooled to 40°C. Insulin powder, 30 mg, was added to 1 ml PBS to make a suspension. The suspension was warmed to 40°C in the water bath. The agarose solution and the insulin suspension were mixed and then transferred into the warm PLGA solution at 40°C. The mixture was homogenized to a fine emulsion using a homogenizer (Model M 122, Biospec Products Co., Barhesville, OK, USA). The homogenization was conducted at a speed of 10000 rpm for 60 s at 40°C. The resulting emulsion was cooled in a refrigerator ($\sim$ 5°C) to convert the aqueous droplets of the insulin-containing agarose solution into agarose hydrogel particles having a size between 1 and 5 μm (The approximate size of the agarose hydrogel particles was obtained using an optical microscope having an eyepiece micrometer disc that was pre-calibrated by an objective micrometer.) The agarose hydrogel particles were formed when the temperature of the emulsion was reduced below the gelation temperature of the agarose solution (approximately 36°C). The insulin molecules were entrapped inside the agarose hydrogel particles during the gelation process. The formed suspension (agarose hydrogel particles suspended in the PLGA solution) was transferred into the apparatus shown in Fig. 1. The suspension was stirred using an overhead stirrer while 4 g silicone oil was gradually added. The addition of the silicone oil was conducted using a peristaltic pump at a speed of 1 g/min. The silicone oil induced phase separation in the PLGA solution and PLGA microspheres were formed, entrapping the insulin-containing agarose hydrogel particles inside. The composite microspheres were slowly transferred into 1 l heptane with stirring. The composite microspheres were washed in the heptane for 2 h and then collected by filtration and dried in a vacuum desiccator. White, freely flow-

ing powder was obtained and used for further investigations.

Conventional PLGA microspheres containing only insulin with no agarose added were also prepared using the same procedure described above. The microspheres containing only insulin were used in the control experiments for the purpose of comparison.

Placebo microspheres were also prepared for the *water absorption determination* experiments. The first placebo sample, called PLGA–agarose composite microsphere placebo, was comprised of PLGA–agarose composite microspheres with no insulin added. The second placebo sample, called conventional PLGA microsphere placebo, was comprised of PLGA microspheres with no agarose and insulin added.

2.3. Morphology study

The insulin-loaded PLGA–agarose composite microspheres and conventional PLGA microspheres were examined using an Olympus optical microscope (Olympus, Tokyo, Japan) with a Polaroid camera attached (Model MF-10, Newton, MA, USA). Photographs were taken during examination.

2.4. Size and size distribution measurement

The PLGA–agarose composite microspheres and the conventional PLGA microspheres were suspended in distilled water with 1% Tween 80 added. The Tween 80 was added to wet and easily suspend the microspheres. Each microsphere suspension was then analyzed using a NICOMP 770 particle sizing system (Particle Sizing Systems Inc., Santa Barbara, CA, USA). The mean size and size distribution of the microspheres were obtained.

2.5. Water absorption determination

In this experiment, two sample groups were prepared. One sample group was prepared using the PLGA–agarose composite microsphere placebo, and another sample group was prepared using the conventional PLGA microsphere placebo. Each sample group consists of 10 samples of microspheres. In each sample, 50 mg of placebo microspheres were suspended in 4 ml distilled water in a test tube. The test tubes were incubated in a shaking bath (37°C) at the speed of 30 rpm. At predetermined time intervals, one sample of the PLGA–agarose composite microsphere placebo and one sample of the conventional PLGA microsphere placebo were taken out of the shaking bath, respectively, and filtered using a 0.1-μm filter membrane. The filter membrane had been saturated with distilled water and then weighed using a weighing bottle. The filter membrane with placebo sample on it was sealed in a weighing bottle and weighed. The water contents of the PLGA–agarose composite microsphere placebo and the conventional PLGA microsphere placebo were calculated by the following equation:

$$\text{water uptake } (\%) = \frac{W_T - W_M - W_D}{W_T - W_M} \times 100$$

where W_T is weight of the wet filter membrane with wet placebo sample, W_M is weight of the wet membrane, and W_D is weight of the dry PLGA–agarose composite placebo or the conventional PLGA microsphere placebo.

2.6. Loading efficiency determination

Accurately weighed 10-mg samples of the PLGA–agarose composite microspheres and the conventional PLGA microspheres were each dissolved in 1 ml acetonitrile in test tubes. After the samples were completely dissolved, 4 ml PBS was added into each test tube and thoroughly mixed. For the conventional PLGA microspheres sample, 1 ml supernatant was immediately withdrawn and filtered through a 0.22-μm Teflon syringe filter. However, for the PLGA–agarose composite microsphere sample, the test tube was placed in a 37°C shaker bath overnight to completely extract insulin out of the agarose hydrogel particles. After this extraction, 1 ml supernatant was withdrawn and filtered through a 0.22-μm Teflon syringe filter. The clear filtrates were analyzed using a reverse-phase HPLC system equipped with a Photo Diode Array (PDA) detector (Thermo Sep-

aration Products, Inc., Riviera Beach, FL, USA). A Delta-pak C_{18} column (Waters Corporation, Milford, MA, USA) was used and maintained at 27°C. The mobile phase was a mixture of solution A (0.1% TFA in H_2O) and solution B (90:10 CH_3CN:H_2O, containing 0.1% TFA) at a ratio of 100:0 initially, and programmed for gradient elution linearly over 10 min to a ratio of 50:50 of solution A and solution B. The detecting wavelength was 214 nm and the flow rate was 1 ml/min. The loading efficiency was calculated by the following equation:

$$\text{loading efficiency (\%)} = (L_A/L_T) \times 100$$

where L_A is the actual loading of insulin in the PLGA–agarose composite microspheres or the conventional PLGA microspheres, which were determined above experimentally. L_T is the theoretical loading of insulin in the PLGA–agarose composite microspheres or the conventional PLGA microspheres based on the amount used in the preparation of the microspheres.

2.7. Insulin in vitro release study

The PLGA–agarose composite microspheres and the conventional PLGA microspheres, 50 mg of each, were separately suspended in 1 ml PBS in test tubes. The PBS used in this set of experiments contained 0.25% phenol, which served as a preservative. The test tubes were placed in a shaking water bath set at 37°C with a 30-rpm shaking speed. At predetermined time intervals, 0.5 ml of supernatant was accurately withdrawn using a HPLC syringe and 0.5 ml fresh PBS was added to maintain sink conditions. The samples were filtered through a 0.22-μm Teflon syringe filter. The filtrates were analyzed using the same reverse-phase HPLC system under the same operating conditions described in the *loading efficiency* experiment. The in vitro release experiments were conducted in triplicate. The cumulative amount of insulin released is calculated using the following equation:

$$\text{cumulative amount of insulin released (\%)} = (M_t/M_\infty) \times 100$$

where M_t is amount of insulin released at time t and M_∞ is total amount of insulin released at time infinity, which is the actual loading of insulin determined in loading efficiency experiment.

2.8. Assay of insulin stabilization efficiency

The PLGA–agarose composite microspheres and the conventional PLGA microspheres, 30 mg of each, were separately added to test tubes containing 2 ml PBS in each test tube. The test tubes were placed in a shaking water bath. The incubating temperature and the shaking speed were the same as those used in the experiment for the insulin in vitro release study. After an 18-h incubation, the incubating medium was withdrawn and filtered through 0.22-μm Teflon syringe filters. The clear solutions were analyzed using a size exclusion HPLC system equipped with the same PDA detector described above. A size exclusion chromatography column (Alltech Macrosphere GPC column, 7 μm, 250×4.6 mm, Alltech Associates, Inc., Deerfield, IL, USA) with a 300-Å pore size was used and maintained at 40°C. The mobile phase was a pH 7.0 buffer solution containing 0.05 M KH_2PO_4 and 0.15 M Na_2SO_4. The flow rate was 0.8 ml/min. The detecting wavelength was 214 nm. A pure insulin solution was used as a reference. The insulin stabilization efficiency was expressed by the percentage of insulin denatured, which was defined by the following equation:

$$\text{percentage of insulin denatured (\%)} = \frac{\sum A_{deg} + \sum A_{agg}}{A_o + \sum A_{deg} + \sum A_{agg}} \times 100$$

where A_{deg} is the peak area of the degraded insulin and A_{agg} is that of aggregated insulin, while A_o is that of native or unattacked insulin.

3. Results and discussion

3.1. Preparation of the PLGA–agarose composite microspheres and their morphology

The phase separation method is widely used for preparation of polymeric microspheres (Kondo, 1979; Donbrow, 1992; McGee et al., 1995; Wu, 1997). A typical organic phase separation method involves a polymer–organic solvent solution. Drugs, either water soluble or water insoluble, can be encapsulated in a polymer matrix by using this method. For encapsulation of protein drugs, the drugs are usually dissolved in an aqueous solution and then micro-emulsified in the polymer–organic solvent solution. Then, phase separation of the polymer solution is induced, leading to microsphere formation.

In the experiment for preparation of the PLGA microsphere–agarose hydrogel particle composite, the protein drug (insulin) is mixed in the agarose solution and then the insulin-containing agarose solution is emulsified in the PLGA solution. Before formation of the PLGA microspheres, the agarose solution has been gelled, i.e. the agarose hydrogel particles have been formed in the PLGA solution, entrapping and protecting the insulin inside. The presence of the agarose hydrogel particles influences the formation of the PLGA–agarose composite. It is found from the experiments that the following two parameters are main concerns. The first parameter is the volume ratio of the agarose aqueous solution to the PLGA organic solvent solution. This volume ratio should be less than approximately 30% to assure formation of the agarose hydrogel particle containing PLGA composite microspheres. The second parameter is the concentration of the agarose solution. If the concentration of agarose in the aqueous solution is higher than 2%, it is difficult to produce submicron-size agarose hydrogel particles by using a homogenizer. The presence of large agarose hydrogel particles affects the formation of the agarose hydrogel particle-containing PLGA microspheres. It is difficult for the PLGA microspheres to entrap large agarose hydrogel particles. In addition, the large agarose hydrogel particles in the PLGA microsphere composite generate higher osmotic pressure so that water can easily penetrate into the PLGA microsphere composite matrix and cause a fast or burst release of insulin.

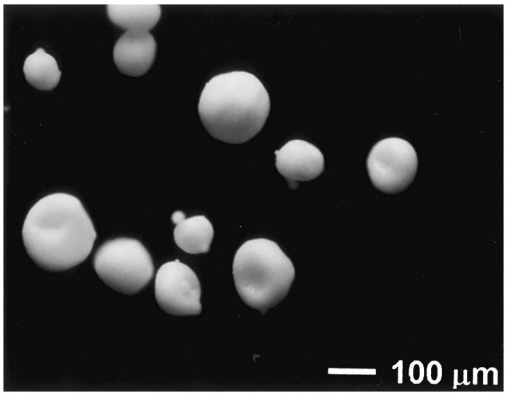

Fig. 2. Micrograph of the PLGA–agarose composite microspheres (scale bar:- = 100 μm).

In addition to the effect on composite formation, the agarose hydrogel particles influence the quality of the composite microspheres. If the aforementioned two parameters are well controlled the heterogeneously structured composite of PLGA microspheres and agarose particles can be prepared with consistent quality. Fig. 2 shows an optical micrograph of the PLGA–agarose composite microspheres, while Fig. 3 is an optical

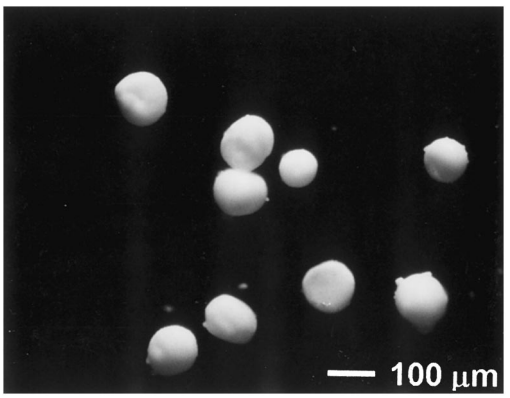

Fig. 3. Micrograph of the conventional PLGA microspheres (scale bar:- = 100 μm).

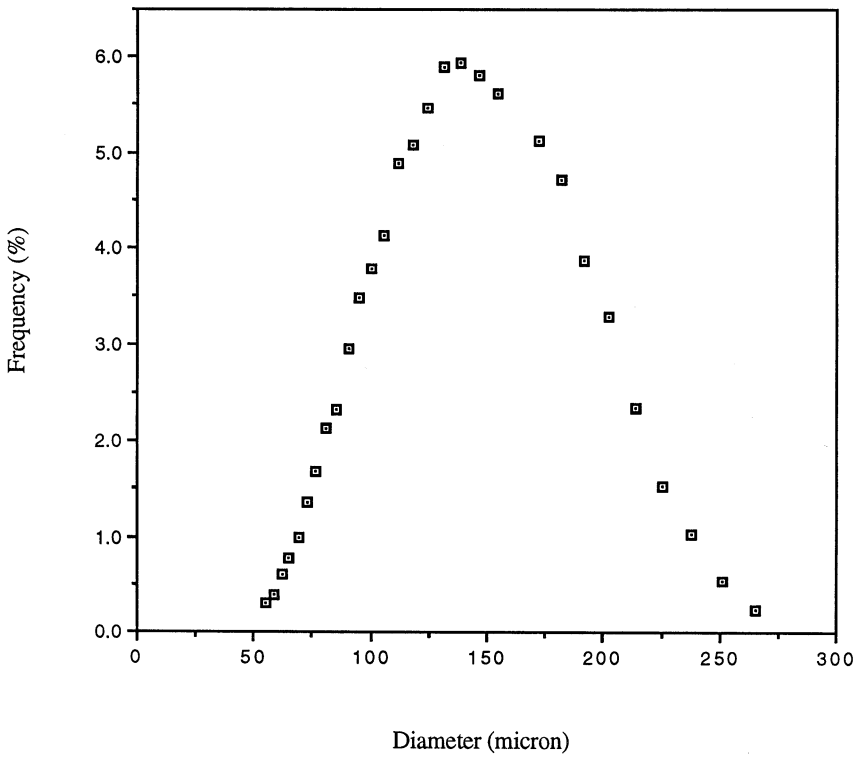

Fig. 4. Size distribution of the PLGA–agarose composite microspheres.

micrograph showing the conventional PLGA microspheres. As one can see, the PLGA–agarose composite microspheres are discrete and have a spherical shape and a smooth surface. There are no agarose particles observed on the surface of the composite microspheres. In comparing Fig. 2 with Fig. 3, the composite microspheres are almost identical to the control experiment sample, the conventional PLGA microspheres, in terms of appearance and morphology.

3.2. Size and size distribution

It is known that the size distribution of a microspherical system is affected by many factors such as stir rate, concentration of matrix forming polymer, pH, concentration of electrolytes, and surfactants or emulsifiers added (Kondo, 1979; Douglas et al., 1984; Fong, 1988; Malaiya and Vyas, 1988; Wu, 1997). For the method used in our experiments, the main factors are the stir rate and the concentration of the matrix-forming polymer. The higher the stir rate or the lower the concentration of the polymer solution, the smaller the size of the microspheres. The size distribution of a microspherical system is important because of its influence on the drug release characteristics and ease of passing through a syringe needle if administrated by injection.

Fig. 4 is the size distribution of the heterogeneously structured PLGA–agarose composite microspheres, whereas Fig. 5 shows that of the conventional PLGA microspheres. The size distribution of both samples ranges from 50 to 295 μm. The volume mean diameter of the composite microspheres is 151 μm, while that of the conventional PLGA microspheres is 160 μm.

3.3. Water absorption

Water absorption is of importance to the PLGA microspherical drug delivery system. Both

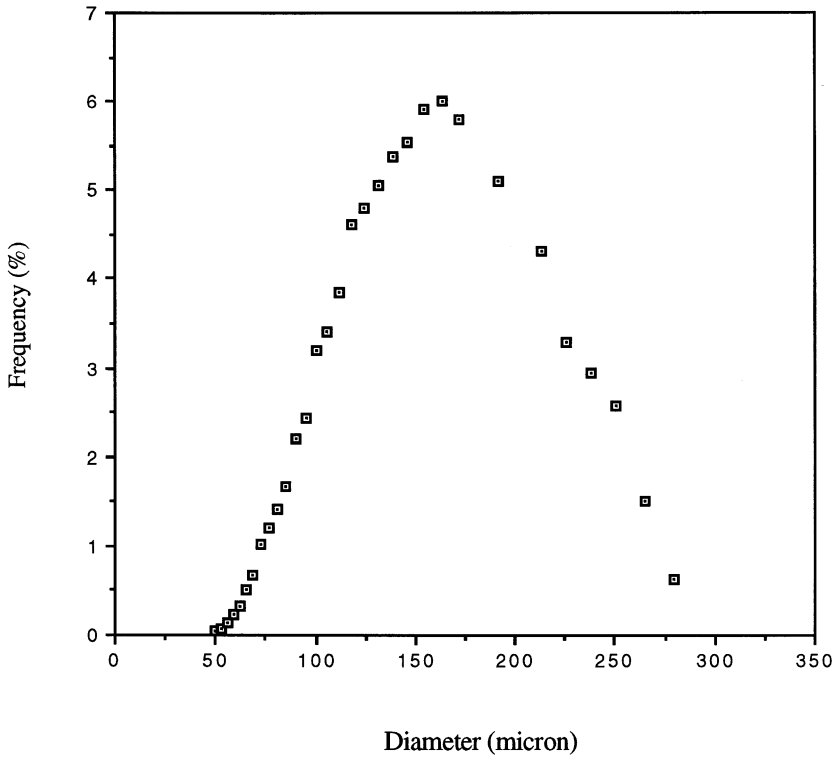

Fig. 5. Size distribution of the conventional PLGA microspheres.

the drug release pattern and the release rate are affected by water absorption. High molecular weight drugs, such as proteins, have significantly low diffusivity in polymeric matrices (Baker, 1987). Most protein drugs are not released by diffusion through a polymeric matrix. Instead, they are released by diffusion through medium filled pores and/or channels in the polymeric matrix (Siegel and Langer, 1983). Therefore, the amount of water absorbed by the PLGA microspheres has a significant effect on the drug release.

For a biodegradable drug release system, such as PLGA microspheres, hydration of a polymer matrix is the first step in biodegradation (Wu, 1995). Therefore, water absorption also affects biodegradation of the microsphere. The biodegradation will enlarge the pores and channels within the matrix and, consequently, accelerate drug release rate from the microsphere.

Because a hydrophilic material, agarose, is introduced into the PLGA microspheres, water uptake into the PLGA–agarose composite microspheres is different from that of the conventional PLGA microspheres. Fig. 6 shows water uptake of the PLGA–agarose composite microspheres and the conventional PLGA microspheres as a function of time. As shown in this figure, hydration of both PLGA–agarose composite microspheres and conventional PLGA microspheres follows the same pattern. They absorb water very fast at the beginning and then reach an approximate steady state. At the steady state, the water content of the PLGA–agarose composite microspheres is approximately 12% higher than that of the conventional PLGA microspheres. The higher water content of the PLGA–agarose composite microspheres is most likely due to the presence of the hydrophilic agarose polymer.

3.4. Loading efficiency

The insulin loading efficiency of the PLGA–agarose composite microspheres is $88.6 \pm 10\%$, and that of the conventional PLGA microspheres

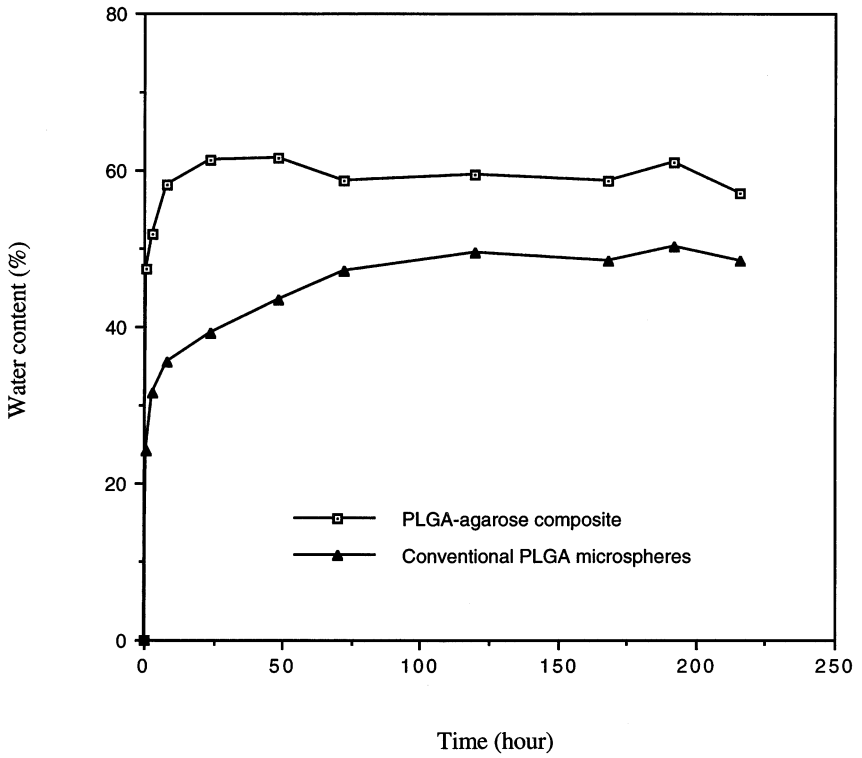

Fig. 6. Water uptake of the PLGA–agarose composite microspheres and the conventional PLGA microspheres as a function of time at 37°C.

is $74.6 \pm 11\%$. The loading efficiency data were based on five experimental measurements. The loading efficiency of the PLGA–agarose composite microspheres is higher than that of the conventional PLGA microspheres. The difference may be explained as follows. During the preparation of the conventional PLGA microspheres, the insulin molecules were denatured by unfolding. The unfolding may be induced by the presence of the interface of the water (insulin solution)-in-oil (PLGA solution) emulsion. The unfolded insulin molecules may have been washed away from the PLGA microspheres in the hardening process as shown in Fig. 1. Since the agarose hydrogel particles have a protection function for the insulin molecules, there is less possibility for the insulin molecules to be unfolded and then washed away from the PLGA–agarose composite microspheres.

3.5. Insulin in vitro release

Fig. 7 is the cumulative amount of insulin released from the PLGA–agarose composite microspheres and the conventional PLGA microspheres as a function of time. The insulin release rate of the PLGA–agarose composite microspheres is higher than that of the conventional PLGA microspheres. This higher release rate may be explained by the following reason. In order for insulin molecules to be released, the PLGA matrix has to be hydrated first. Because of the extremely low diffusivity of protein molecules in polymeric matrices, the insulin molecules cannot diffuse out until the buffer front has diffused into the matrix and reached the insulin molecules. So the water content and hydration rate have a significant effect on the drug release rate at the initial period.

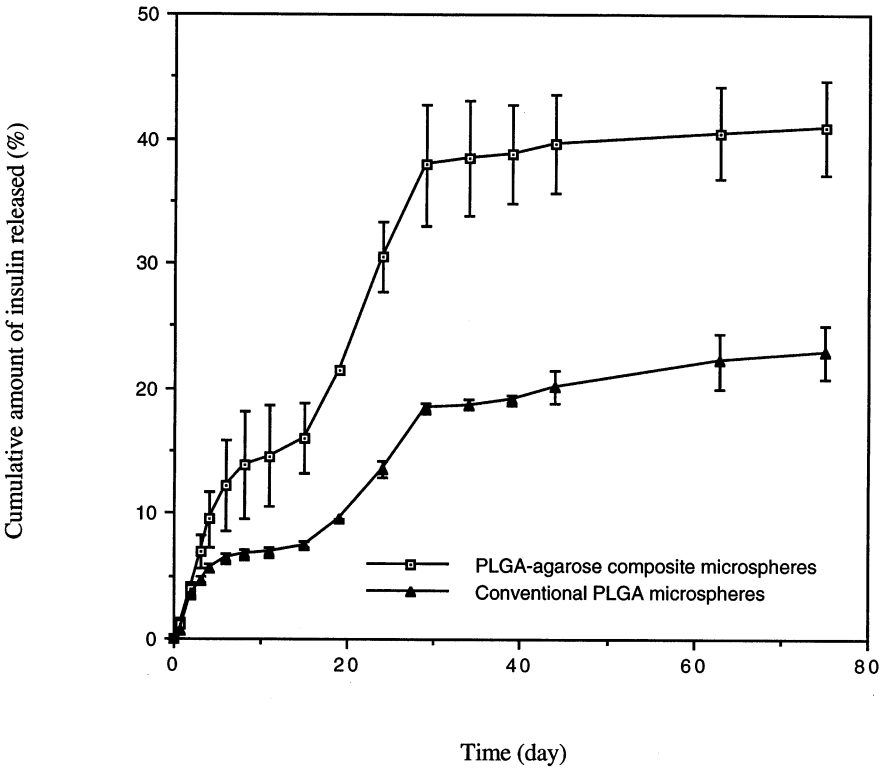

Fig. 7. Cumulative amount of insulin released from the PLGA–agarose composite microspheres and the conventional PLGA microspheres as a function of time at 37°C.

The hydration rate of the PLGA–agarose composite microspheres is found to be higher than that of the conventional PLGA microspheres (Fig. 6), because of the presence of the hydrophilic agarose hydrogel particles. The agarose hydrogel particles increased the hydrophilicity of the PLGA–agarose composite microspheres, which accelerated their water uptake and consequently the insulin release from them.

The in vitro release of protein and peptide drug from PLGA microspheres involves two different mechanisms (Wu, 1995). One is the diffusing of drug molecules through aqueous pores or channels formed either during microsphere preparation or after dissolving and releasing of certain drug domains. The drug released by this mechanism usually exists on and/or near the surface of the microspheres. The second mechanism involves degradation of the PLGA matrix. The drug molecules completely entrapped in the PLGA matrix cannot be released until the polymer matrix starts losing its integrity, the drug molecules are then accessible to aqueous release medium and diffuse out.

The aforementioned two release mechanisms can be observed in Fig. 7 for both PLGA–agarose composite microspheres and conventional PLGA microspheres. In the first 3 days, the release of insulin has a relatively high release rate. Since there is no burst release observed, this period mainly corresponds to the release of insulin molecules near the surface of the microspheres or any insulin molecules that are accessible to the release medium through pores and channels formed during preparation of the microspheres. Between 3 and 15 days, the release of insulin gradually declines. This decrease of release might be caused by exhaustion of the insulin molecules near the surface of the microspheres or those accessible to the release medium through pores

and channels. Starting from day 15 to day 29, the insulin release increases again. In this release period, the loss of polymer integrity occurs. New pores or channels start to form due to integrity loss of the polymer matrix. Many insulin molecules that are previously not accessible to release medium can diffuse out and be released at this period, which causes the second increase in the insulin release rate.

3.6. Insulin stabilization efficiency

Previous studies have shown that protein drugs are usually denatured to some extent by direct encapsulation in PLGA microspheres (Pitt, 1990; Lu and Park, 1995; Uchida et al., 1996; Li et al., 1997). The denaturation is probably caused by harsh encapsulation conditions which include using a large amount of organic solvent and the existence of aqueous–organic interfaces (Sluzky et al., 1991; Lu and Park, 1995). The hydrophobicity and acidity of the PLGA matrix may also have negative effects on the protein drug stability (Uchida et al., 1996). The denaturation of protein drugs caused by the harsh microencapsulation process and the PLGA hydrophobic and acidic matrix may include unfolding, aggregation, and/or degradation.

Saccharides have shown the capacity to stabilize proteins (Back et al., 1997; Manning et al., 1989). Agarose is a polysaccharide derived from red-purple seaweed. It has been widely used in the biomedical and pharmaceutical fields (Selby and Wynne, 1973; Upadrashta et al., 1993; Haglund et al., 1994; Lindenbaum et al., 1995; Wang and Wu, 1997). For example, agarose was used to immobilize xenogeneic islets for insulin therapy of type I diabetes and shown capable of prolonging xenograft survivals (Iwata et al., 1994).

To stabilize protein drugs during the microencapsulation process, and in the hydrophobic and acidic PLGA matrix, we use agarose as a stabilizer for protein drugs. In this approach, the protein drug molecules are entrapped in the agarose particles so that they have less or no chance to meet with the organic solvents. They also have little chance to migrate to the aqueous–organic interfaces. In addition to protecting protein drugs during the microencapsulation process, the agarose hydrogel particles can also protect the protein drugs inside the PLGA microsphere matrix. The size exclusion chromatography results shown in Fig. 8 indicate the protection effect of the agarose hydrogel on the insulin. The top chromatogram (A) is from insulin released from the conventional PLGA microspheres. The middle chromatogram (B) is from insulin released from the heterogeneously structured PLGA–agarose composite microspheres. The bottom chromatogram (C) is from native insulin in the insulin solution. The native insulin possesses only one peak at the retention time near 4.1 min. However, chromatogram A, as one can see, has three peaks. One occurs at approximately 4.1 min, as seen with native insulin (approximately at 4.1 min), and the other two are at retention times of 3.6 and 5.0 min. In contrast, chromatogram B is almost identical to that of the native insulin, except for a small shoulder peak at 3.6 min. According to the separation mechanism of size exclusion chromatography, the peak at 3.6 min represents a species having a higher molecular weight than that of native insulin, which means that it is an aggregation product of insulin. The peak at 5.0 min represents a species having a molecular weight lower than that of native insulin, which corresponds to a degradation product of insulin. Taking the ratio of the peak area of the aggregation product and the degradation product to the total peak areas, including the peak area of the native insulin, the aggregation product and the degradation product, one can obtain a comparison of the percentage of insulin denatured for these three insulin samples (Table 1). From this table, one can clearly see that the agarose hydrogel particles can protect protein drugs during the microencapsulation process and can isolate them from the surrounding hydrophobic and acidic polymeric micro-environment.

4. Conclusions

A novel approach has been taken to stabilize protein drugs in PLGA microspheres. This approach creates a new protein drug delivery sys-

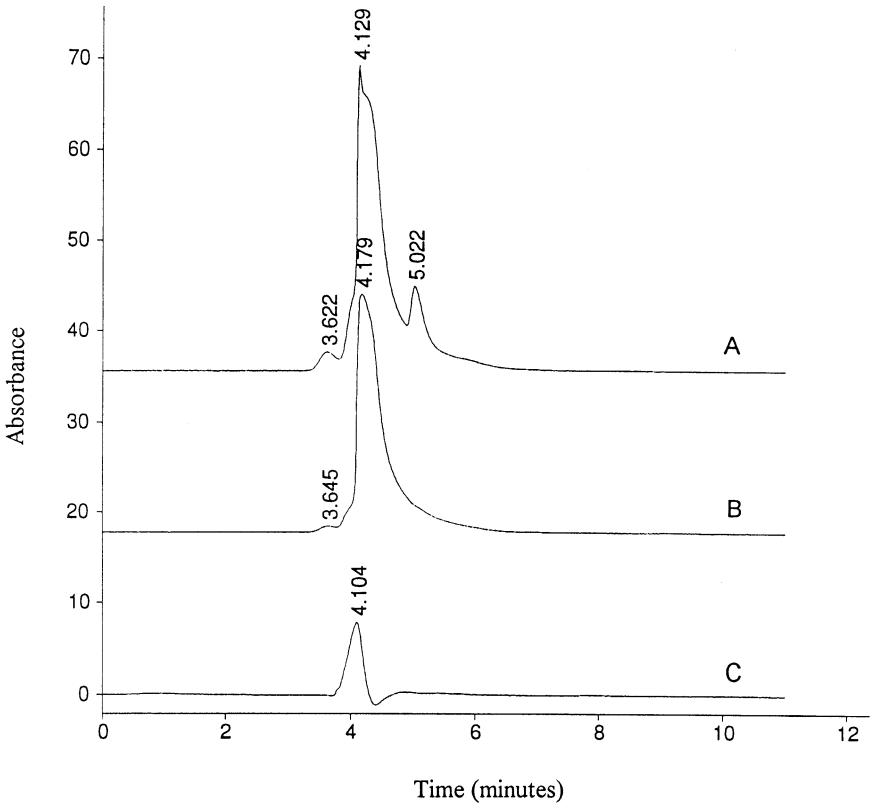

Fig. 8. Comparison of denaturation of insulin released from the PLGA–agarose composite microspheres and the conventional PLGA microspheres using size exclusion chromatography. Top curve (A), insulin released from the conventional PLGA microspheres; middle curve (B), insulin released from the PLGA–agarose composite microspheres; bottom curve (C), insulin solution.

tem, which is based on the combination of agarose hydrogel particles and PLGA microspheres. This combination produces a heterogeneously structured polymeric composite. The protein drug molecules are encapsulated in the agarose hydrogel particles and the drug-containing agarose hydrogel particles are further dispersed in the PLGA microspheres. One PLGA microsphere may contain many agarose hydrogel particles to form a PLGA–agarose composite microsphere. The PLGA–agarose composite microspheres have spherical shape and smooth surface. They possess a normal or Gaussian size distribution and have an average diameter of 150 μm. The PLGA–agarose composite microspheres have higher protein loading efficiency than that of the conventional PLGA microspheres. The hydration of the PLGA–agarose composite microsphere matrix is faster than that of the conventional PLGA microspheres. Protein drugs can be slowly

Table 1
Comparison of denaturation of insulin released from different matrices

Sample	Insulin solution	Insulin released from the PLGA–agarose composite microspheres	Insulin released from the conventional PLGA microspheres
Insulin denatured (%)	0	1.12 ± 0.11	18.82 ± 3.61

released from the PLGA–agarose composite microspheres. The agarose hydrogel particles can stabilize protein drugs in the PLGA matrix, which is the major advantage of this novel protein drug delivery system over conventional PLGA microspheres.

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