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Poly(vinyl alcohol) nanoparticles prepared by freezing–thawing process for protein/peptide drug delivery

Jia Kui Li[a], Nuo Wang[a], Xue Shen Wu[a,b,*]

[a]*Division of Pharmaceutics and Industrial Pharmacy, Arnold and Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, Brooklyn, New York 11201, NY, USA*

[b]*Herman F. Mark Polymer Research Institute, Polytechnic University, Brooklyn, New York 11201, NY, USA*

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Abstract

Poly(vinyl alcohol) (PVA) hydrogel nanoparticles have been prepared by using a water-in-oil emulsion technology plus cyclic freezing–thawing process. The PVA hydrogel nanoparticles prepared by this method are suitable for protein/peptide drug delivery since formation of the hydrogel does not require crosslinking agents or other adjuvants and does not involve any residual monomer. Particularly, there is no emulsifier involved in this new method.

Bovine serum albumin (BSA), as a model protein drug, is incorporated into the PVA hydrogel nanoparticles. The PVA hydrogel nanoparticles possess a skewed or log-normal size distribution. The average diameter of the PVA hydrogel nanoparticles is 675.5±42.7 nm. Protein drug loading efficiency in the PVA hydrogel nanoparticles is 96.2±3.8%. The PVA hydrogel nanoparticles swell in an aqueous solution and the swelling degree increases with the increase of temperature. In vitro release studies show that the BSA release from the nanoparticles can be prolonged to 30 h. The BSA release follows a diffusion-controlled mechanism. The number of freezing–thawing cycle and release temperature both influence BSA release rate considerably. Less freezing–thawing cycle or higher release temperature leads to faster drug release. The BSA is stable during preparation of the PVA hydrogel nanoparticles.

Keywords: Poly(vinyl alcohol); Hydrogel; Nanoparticles; Freezing–thawing process; Protein/peptide drug delivery

1. Introduction

With rapid advancements in the field of biotechnology/genetic engineering, a growing number of protein/peptide drugs have been produced by recombinant DNA technology [1]. These new biological products are exact chemical replicas of the natural ones. Although they show potent and specific physiologic actions in small doses, most of them are difficult to administer clinically. After oral administration, these drugs are easily degraded by proteolytic enzymes in the gastrointestinal tract. If administered parenterally, repeated injections are often needed because of their extremely short biological half-life. Daily multiple injections are highly risky to administer without close medical supervision and also difficult for most patients to accept. Hence, developing viable delivery systems to improve the systemic bioavailability of protein/peptide drugs is highly desirable [2].

We have developed a poly(vinyl alcohol) (PVA)-based formulation that can increase the effects of the

*Corresponding author.

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protein/peptide drugs by prolonging their release duration. The PVA is formulated as hydrogel nanoparticles that can be administered parenterally. A model protein drug, bovine serum albumin (BSA), has been incorporated into the injectable PVA hydrogel nanoparticles.

PVA hydrogels have been used in a number of biomedical applications including soft contact lenses [3], implants [4], and artificial organs [5]. This is because of their inherent non-toxicity, non-carcinogenicity, good biocompatibility, and desirable physical properties such as rubbery or elastic nature and high degree of swelling in aqueous solutions. The PVA hydrogels have also gained wide pharmaceutical applications as drug-delivery matrices [6–9] or in the form of powders added to mixtures of other excipients for tablet formation [10].

PVA is a linear polymer so that it has to be crosslinked into hydrogels for the application of drug delivery. Traditional methods for crosslinking PVA use irradiation or bifunctional group-containing chemical agents such as glutaraldehyde or sodium borate and boric acid. The traditional methods, particularly the crosslinking agents added during the hydrogel preparation, may have side effect on the drugs loaded. This is especially true when protein and peptide drugs are incorporated in the hydrogel. In addition, the chemical method may leave residual substances in the hydrogels formed.

N.A. Peppas' research group [11] and others [12] have developed a physical crosslinking method for PVA hydrogels. This method is based on freezing–thawing cycles. However, this method is most suitable for preparing macro-size hydrogels such as films and cylinders. To prepare particulate PVA hydrogels using this cyclic freezing–thawing method, N.A. Peppas' group used the emulsion technique together with the freezing–thawing process, i.e., the PVA solution was emulsified in corn oil and then the emulsion was frozen at −4°C and consequently thawed at room temperature [13]. Spherical PVA hydrogel beads having size from 150 μm to greater than 1.4 mm were successfully prepared. However, this method can not be used for preparing nano-sized PVA particles. The freezing temperature of −4°C may not be low enough to freeze the oil phase as well as the drug containing PVA solutions due to freezing point depression of the solutions. In order to stabilize the emulsion during freezing–thawing cycle, a large amount of emulsifier has to be used to stabilize the emulsion, particularly when the droplets in emulsion are in nano-size range. The presence of such emulsifier(s) may be an obstacle to protein/peptide drugs if the hydrogel nanoparticles are prepared for protein/peptide drug delivery. To overcome these problems, we have developed a new technology to prepare nanoparticulate drug (protein)-containing PVA hydrogels. This new technology is based on the emulsifier-free emulsion technique [14,15] plus cyclic freezing–thawing process. The freezing temperature is −20°C, at which the emulsion is frozen and stable. The average size of the prepared PVA hydrogel particles by this new method can be as small as 675.5 nm.

The prepared PVA hydrogel nanoparticles resulting from this improved technology were evaluated regarding morphology, size and size distribution, drug loading efficiency, degree of swelling, in vitro protein release and protein stability.

2. Materials and methods

2.1. Materials

PVA (average molecular weight: 70 000–100 000) was purchased from Sigma Chemical Co. (St. Louis, MO). BSA (MW: 68 000) was supplied by Boehringer Mannheim Corp. (Indianapolis, IN, USA). Silicone oil (350 cs) was obtained from Dow Corning Corp. (Midland, MI, USA). Acetone was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA, USA).

2.2. Preparation of PVA hydrogel nanoparticles

PVA (500 mg) was dissolved in 3 ml phosphate buffer (20 mM, pH 7.4) by heating at a temperature near 100°C for 15 min. Then, the PVA solution was cooled down to room temperature. Meanwhile, BSA (125 mg) was dissolved in 2 ml of the same phosphate buffer at room temperature. The above solutions were mixed thoroughly. This aqueous solution was added to 100 ml silicone oil. The mixture was homogenized (10 000 rpm) for 5 min to form a water-in-oil (w/o) emulsion by using a

homogenizer (Model M 122, Biospec Products Co., Bartlesville, OK). The prepared w/o emulsion was then frozen at −20°C for 20 h and then allowed to thaw for 4 h. Three such freezing–thawing cycles were performed for each sample, which resulted in conversion of the emulsion to a suspension of PVA hydrogel nanoparticles in silicone oil. After three of these cycles, the PVA hydrogel nanoparticles in silicone oil was extracted using acetone (1:10 volume ratio of the suspension to acetone). The PVA hydrogel nanoparticles were then collected by filtering the acetone phase through a filter membrane having a pore size of 100 nm (Micron Separations, Inc., Westboro, MA). The filtrant was washed with acetone to remove silicone oil residue. The collected PVA hydrogel nanoparticles were vacuum dried and kept in a 4°C refrigerator for further investigations.

2.3. Microscopic observation

Morphology of the dried PVA hydrogel nanoparticles was investigated by using a Hitachi scanning electron microscope (SEM, Hitachi, Ltd., Tokyo, Japan) regarding the shape and surface characteristics.

2.4. Determination of size and size distribution

The BSA-containing PVA hydrogel nanoparticles prepared by using three freezing–thawing cycles (4 mg) were suspended in 2 ml heptane by hand shaking. The suspension (1 ml) was taken and evaluated using a NICOMP submicron particle sizer (Model 370, Particle Sizing System, Inc., Santa Barbara, CA). Intensity-weighted average size and size distribution were obtained from this assay. The nanoparticles from five different batches were measured separately. The particle size was calculated as the average of these five measurements.

2.5. Measurement of protein loading efficiency

The dry PVA hydrogel nanoparticles (2 mg) were accurately weighed, and transferred into test tubes. The phosphate buffer (3 ml) was added. The samples were then incubated in a shaking water bath at 37°C. After 48 h, the samples were centrifuged for 5 min. One milliliter of the supernatants was withdrawn, and the BSA concentration in the supernatants was analyzed by using a capillary electrophoresis apparatus (Spectra, Model 1000-CE00, San Jose, CA). A sodium tetraborate buffer (20 mM, pH 9.4) was used as the mobile phase. Wavelength of the UV detector was 210 nm. The operating temperature was 25°C. The protein loading efficiency was calculated by using the following equation:

$$\text{Loading efficiency (\%)} = \frac{M_{\text{actual}}}{M_{\text{theoretical}}} \times 100$$

where M_{actual} is the actual loading amount of BSA in the PVA hydrogel nanoparticles determined by the above experiment and $M_{\text{theoretical}}$ is the theoretical loading amount of BSA calculated from the quantity added during the preparation.

2.6. Assay of swelling degree

Degree of swelling of the PVA hydrogel nanoparticles was measured at 15°C, 24°C, 37°C, and 50°C. The BSA-containing PVA hydrogel nanoparticles (10 mg) were dispersed in 5 ml distilled water. Each sample was placed in a water bath (Model 2095, Masterline Forma Scientific, Marietta, OH) for 5 h at each of the above-mentioned temperatures. At each temperature, a Nylon filter membrane (pore size: 100 nm) was moistened with distilled water and weighed using a weighing bottle which had an air-tight cap to prevent evaporation of water during the weighing process. The sample was then filtered using the same filter membrane at the designated temperature. The filter membrane with the swollen PVA hydrogel nanoparticles on it was weighed using the same weighing bottle. The degree of swelling was calculated using the following equation:

$$\text{Degree of swelling} = \frac{W_{\text{w}}}{W_{\text{d}}} = \frac{W_{\text{t}} - W_{\text{m}} - W_{\text{d}}}{W_{\text{d}}}$$

where W_{w} is the weight of distilled water absorbed by the PVA hydrogel nanoparticles, W_{d} is the dry weight of the PVA hydrogel nanoparticles, W_{t} is the total weight of the moistened filter membrane, the swollen PVA hydrogel nanoparticles, and the weighing bottle, W_{m} is the total weight of the moistened filter membrane and the weighing bottle.

2.7. Study of in vitro protein release

The BSA-loaded PVA hydrogel nanoparticles, 4 mg, were placed in test tubes containing 2 ml phosphate buffer (20 mM, pH 7.4). The test tubes were shaken (35 strokes/min) in a shaking water bath. At designated time intervals, the supernatant (1 ml) from each test tube was withdrawn through a syringe filter, and 1 ml fresh phosphate buffer was added. The BSA concentration in the released samples was analyzed by using the capillary electrophoresis described above. The sodium tetraborate buffer (20 mM, pH 9.4) was used as the mobile phase. Wavelength of the UV detector was 210 nm. The operating temperature was 25°C.

The cumulative amount of BSA released from the PVA hydrogel nanoparticles was calculated using the following equation:

$$\text{Cumulative amount released (\%)} = \frac{M_t}{M_{\text{actual}}} \times 100$$

where M_t is the amount of BSA released from the PVA hydrogel nanoparticles at time t and M_{actual} is the actual amount of BSA loaded in the PVA hydrogel nanoparticles.

The release experiments were performed at both room temperature (24°C) and 37°C. For the purpose of comparison, release studies were also performed for PVA hydrogel nanoparticles prepared by using only one freezing–thawing cycle.

The cumulative amount of BSA released is plotted versus incubating time. Each data point is calculated based on three measurements. The error bars were the standard deviation of the three measurements.

2.8. Analysis of protein stability

The PVA nanoparticles (10 mg) were incubated in 2 ml of distilled water overnight in a water bath at 37°C. After incubation, 1 ml supernatant was withdrawn and filtered through a syringe filter having pore size of 0.22 μm. The clear filtrate was analyzed by using a size exclusion HPLC system equipped with a photo diode array detector. A size exclusion chromatography column (Alltech Macrosphere GPC column, 7 μm, 250×4.6 mm, Alltech Associates, Inc., Deerfield, IL, USA) with 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 280 nm. A pure BSA solution was used as the reference.

3. Results and discussion

3.1. Morphology and shape

In Fig. 1 a scanning electron micrograph showing shape, size, and surface characteristics of the PVA hydrogel nanoparticles is shown. The shape of the PVA hydrogel nanoparticles is nearly spherical. The deviation from the sphere (or the imperfect spherical geometry) is probably due to the vacuum filtration of the formed PVA nanoparticles. From the micrograph, one may also see roughness of the surface of those nanoparticles. The roughness of the surface may be due to the shrinkage of the PVA hydrogel during the drying process when water is evaporated.

3.2. Size and size distribution

The PVA hydrogel nanoparticles possess a skewed or log-normal size distribution as shown in Fig. 2. The intensity-weighted average diameter of the nanoparticles is 675.5±42.7 nm based on five batch measurements.

Many process parameters can influence the mean

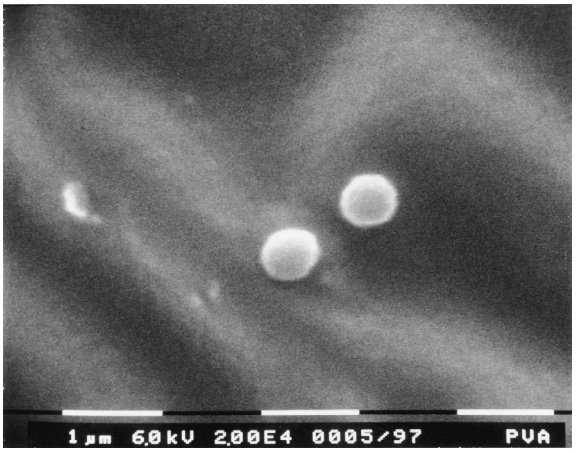

Fig. 1. A scanning electron micrograph of the PVA hydrogel nanoparticles showing the shape and the surface characteristics.

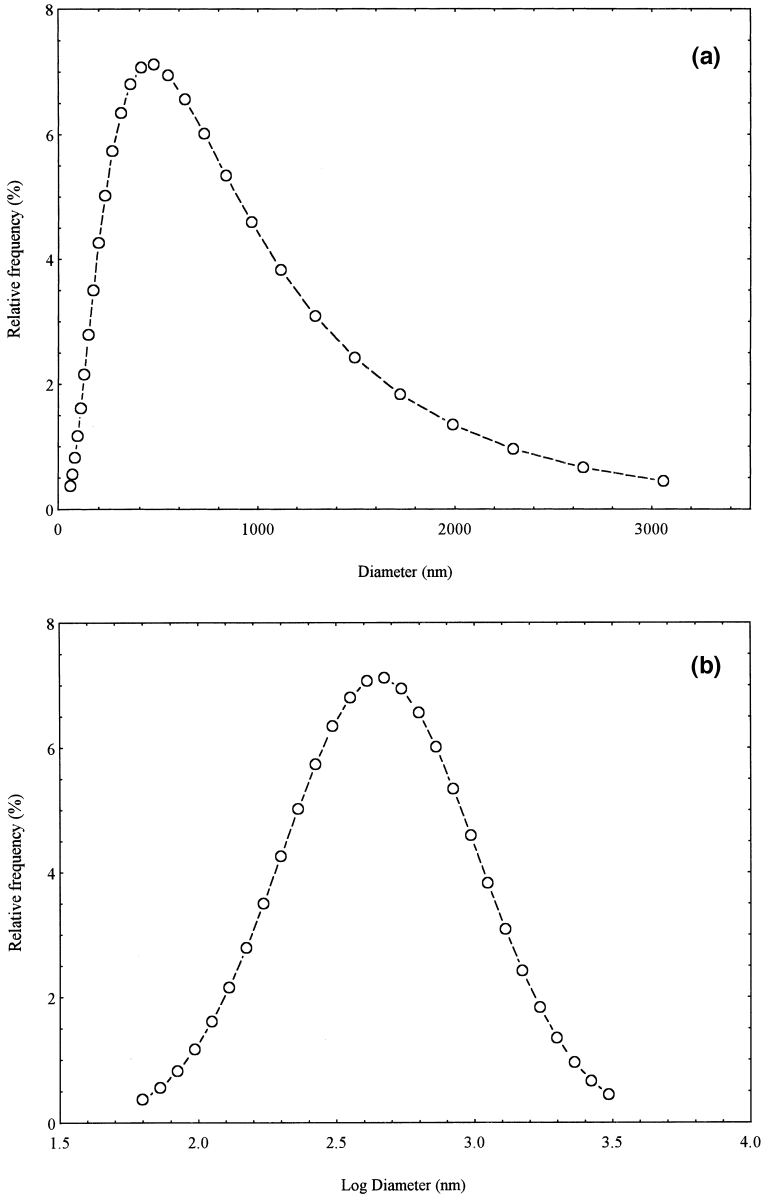

Fig. 2. Intensity-weighted particle size distribution of the PVA hydrogel nanoparticles showing (a) skewed size distribution and (b) log-normal size distribution.

particle size of a final product [16]. In the experiments, we find that the volume ratio of the aqueous phase (the buffer solution containing PVA and BSA) to the organic phase (silicone oil), the concentration of PVA in the aqueous solution, and the homogenization speed are critical factors affecting the particle size distribution of the end product. The greater the volume ratio and the higher the PVA concentration, the greater the particle size. Increase of the homogenization speed brings about a decrease in particle size.

No emulsifier is employed in the preparation of the PVA hydrogel nanoparticles. Nevertheless, the water-in-oil emulsion still shows good stability dur-

ing the whole freezing–thawing cycles. This may be due to high viscosity and good thermal stability of the silicone oil. It may be also due to the nature of the PVA. The PVA itself has been used as an emulsifier for preparation of microspheres [17,18]. At the freezing temperature of −20°C in our experiments, the emulsion is frozen, which can also stabilize the emulsion.

3.3. Protein loading efficiency

Measurement of protein loading efficiency was conducted in triplicate. An appealing advantage of the formulation and technology used for preparation of the PVA hydrogel nanoparticles is that the protein loading efficiency can be as high as 100% (96.2±3.8%). The reason for this high loading efficiency may be that the continuous phase of the emulsion is silicone oil in which the water-soluble protein (BSA) is insoluble. Therefore, almost all protein molecules are entrapped in the PVA hydrogel nanoparticles.

3.4. Temperature effect on swelling degree

The swelling degree of the PVA hydrogel nanoparticles (prepared by three cycles) was investigated under different temperatures, 15°C, 24°C, 37°C, and 50°C. In Fig. 3 the fact that swelling degree increases with increasing temperature is shown. This may be explained by the crosslinking density change of PVA hydrogel. PVA network is formed by physical crosslinking. Three basic models have been proposed to explain the mechanisms of PVA hydrogel formation [19]. These models are the hydrogen bond formation between polymer chains, the polymer crystallite formation of polymer chain segments, and/or the phase separation of polymer-rich region from polymer-poor region. At higher temperature, the polymer chain segments obtain more thermal energy and move faster, which may destroy some of the crystallites and result in dissociation of hydrogen bonds. The temperature increase may even influence the phase separation between the polymer-rich region and the polymer-poor region. This phenomenon brings about a decrease in crosslinking density. The lower crosslinking density results in higher swelling degree. The degree of crosslinking will also influence the drug release rate.

The fact that higher temperature results in higher swelling degree due to the decrease in crosslinking density is also supported by the work published by F. Urushizaki et al. [20]. They found a similar pattern

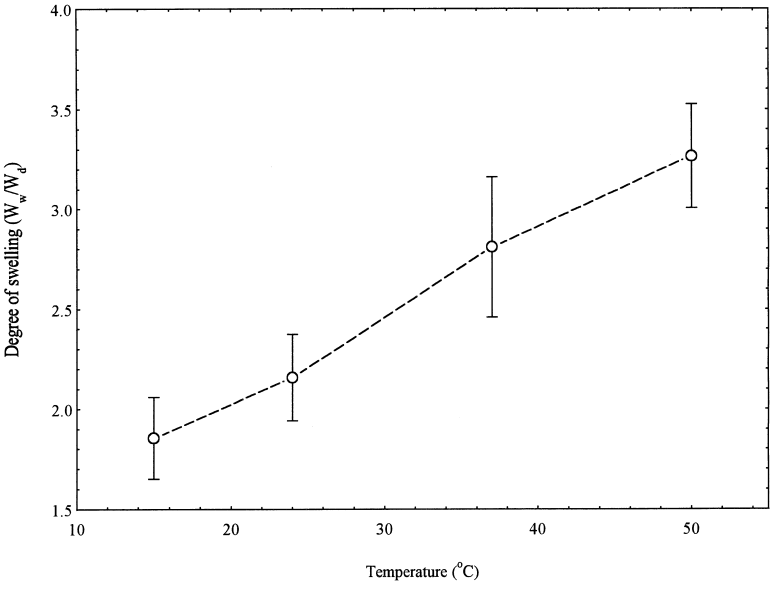

Fig. 3. Temperature dependency of swelling degree of the PVA hydrogel nanoparticles.

of swelling phenomena of a PVA hydrogel prepared by using the freezing–thawing cycles. They have shown that water uptake increases with the increase of temperature.

3.5. In vitro protein release

In Fig. 4 the BSA release profiles from the PVA hydrogel nanoparticles prepared by three freezing–thawing cycles are shown. The top curve shows the BSA release at 37°C while the bottom one at room temperature (24°C). The BSA release at 37°C is much faster than that at 24°C. After 9 h, 71.1% BSA has been released at 37°C while only 48.42% BSA has been released at room temperature. Temperature has influence on the drug release from the PVA nanoparticles. This can be explained from the following two aspects. Firstly, higher temperature increases the diffusion coefficient of BSA in the inert matrix, which accelerates the BSA release. Secondly, temperature has a significant effect on the swelling degree. A higher temperature induces a higher swelling degree and, consequently, leads to a higher permeability of the PVA nanoparticles so that a faster BSA release is yielded.

The number of freezing–thawing cycle also has an effect on the BSA release from the PVA hydrogel nanoparticles. This is demonstrated in Fig. 5. The results shown in this figure are from the release experiments performed at room temperature. The release rate of BSA is found to be higher from the one-cycle PVA nanoparticles than from the three-cycle PVA nanoparticles. This result is probably due to the effect of the number of freezing-thawing cycle on the hydrogel crosslinking density. During the freezing process, a significant change occurs in the PVA hydrogel structure due to formation of the crystallite and supermolecular structure. The supermolecular structure forms with each freezing–thawing cycle and undergoes an increase in diameter [19]. The more freezing–thawing cycles, the more contribution to the hydrogel network formation. Therefore, degree of crosslinking increases with increasing number of cycles of freezing and thawing. Because of this special gelling mechanism of the PVA hydrogel, the more freezing–thawing cycles make the PVA hydrogel nanoparticles have higher crosslinking density. Therefore, the PVA hydrogel nanoparticles produced by the three freezing–thawing cycles have higher crosslinking density than those by the one freezing–thawing cycle. The increase of the crosslinking density of the hydrogel retards the drug release from the PVA hydrogel nanoparticles due to the decrease of hydrogel matrix permeability.

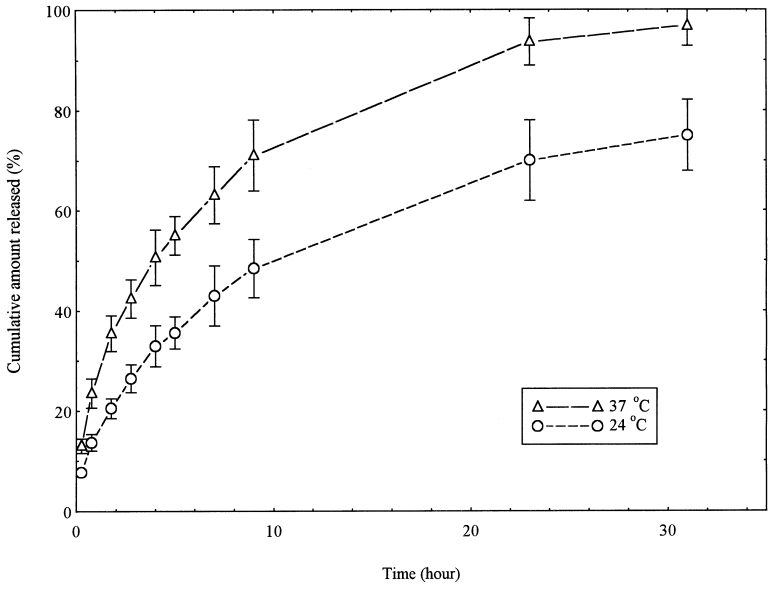

Fig. 4. Release of BSA from the PVA hydrogel nanoparticles as a function of time at two different temperatures. (Δ) 37°C, (○) 24°C.

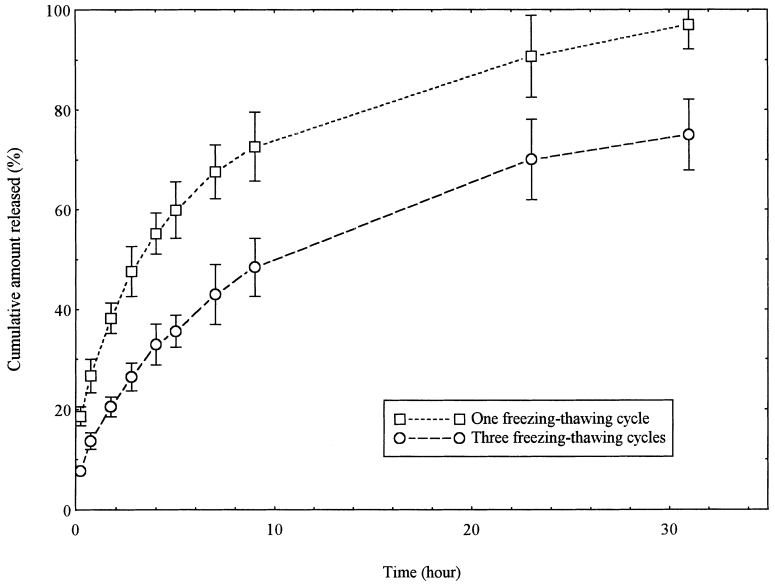

Fig. 5. Effect of freezing–thawing cycle on the release of BSA from the PVA hydrogel nanoparticles. (□) one freezing–thawing cycle and (○) three freezing–thawing cycles.

In Figs. 4 and 5 the fact that the PVA hydrogel nanoparticles possess prolonged released characteristics is shown. The release of BSA from the PVA hydrogel nanoparticles can last up to 30 h. These two figures also show that no initial burst release is present for the PVA hydrogel nanoparticles. This phenomenon suggests that the BSA molecules are all encapsulated inside the PVA hydrogel nanoparticles.

In Fig. 6 cumulative release of BSA from the PVA hydrogel nanoparticles versus the square root of time for the first 9 h is shown. Observed is the nearly linear relationship between the cumulative release of BSA and the square root of time. This square root of time order release suggests that BSA release from the PVA hydrogel nanoparticles follows a diffusion controlled mechanism [21].

3.6. Protein stability

In Fig. 7 two size exclusion chromatograms of BSA are shown. Chromatogram A is from the native BSA, while Chromatogram B is from the BSA released from the PVA hydrogel nanoparticles. As one can see in Fig. 7, there is only a single peak in Chromatogram B and this peak has the same retention time as that in Chromatogram A. This result suggests that BSA did not undergo any integrity change during the preparation and the storage of the PVA hydrogel nanoparticles. In other words, the BSA is stable in the PVA hydrogel nanoparticles. The use of low temperature (−20°C) and organic liquids (silicone oil and acetone) during preparation did not cause any integrity loss of the BSA.

4. Conclusions

An injectable nanoparticulate drug delivery system based on PVA hydrogel has been developed by using the emulsifier-free emulsion technique plus the freezing–thawing process. The PVA hydrogel nanoparticles prepared by this method are suitable for protein/peptide drug delivery since this method does not involve any emulsifier or hydrogel crosslinking agents. In addition to this, there is no residual monomer presented. The PVA hydrogel nanoparticles possess a skewed or log-normal size distribution. The intensity-weighted average diameter of the PVA hydrogel nanoparticles is 675.5±42.7 nm. The protein drug loading efficiency is as high as 96.2±3.8%. The PVA hydrogel nanoparticles swell in an aqueous solution and the swelling degree increases with the

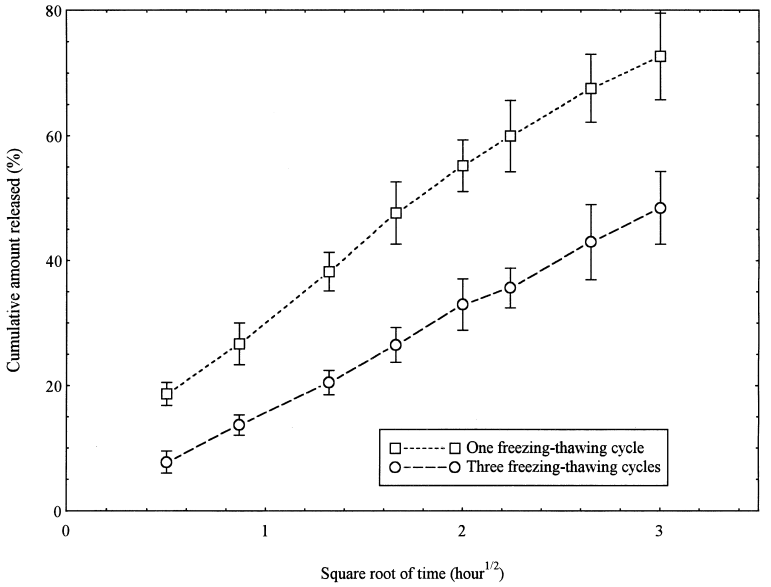

Fig. 6. Release of BSA from the PVA hydrogel nanoparticles as a function of square root of time. (□) one freezing–thawing cycle and (○) three freezing–thawing cycles.

increase of temperature. The in vitro drug release studies show that the PVA hydrogel nanoparticles have prolonged released characteristics. The experimental results show that the BSA release from the PVA hydrogel nanoparticles follows diffusion controlled mechanism. The incubating temperature has great influence on the BSA release by affecting the protein diffusivity and the crosslinking density of the PVA hydrogel matrix. The number of freezing–thawing cycles affects the BSA release considerably. Different drug release rates may be obtained by changing the number of freezing–thawing cycles. The BSA is stable during preparation and in the PVA hydrogel nanoparticles.

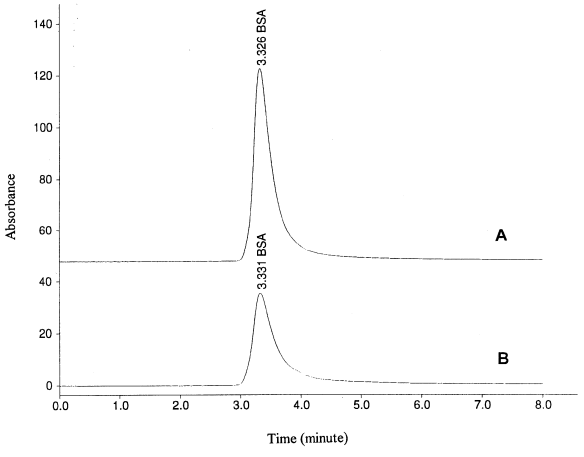

Fig. 7. Size exclusion chromatograms of BSA. (Chromatogram A: BSA solution and chromatogram B: BSA released from the PVA hydrogel nanoparticles).

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