Synthesis and Characterization of a Amphiphilic Pluronic-Poly(D,L-lactide-*co*-glycolide) Copolymer and Their Nanoparticles as Protein Delivery Systems

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ABSTRACT: A new amphiphilic Pluronic (F68)-PLGA copolymer, which can be used to prepare the stealth or long-circulating nanoparticles, was synthesized with Pluronic (F68), DL-Lactide, and glycolide. The structures of Pluronic (F68)-PLGA copolymer as the products were characterized with infrared spectrometry, nuclear magnetic resonance and their molecular weights were determined by gel permeation chromatography. Two methods, double emulsion (DE) and nanoprecipitation (NP), were employed to fabricate the polymeric nanoparticles. Bovine serum albumin (BSA) was loaded into nanoparticles as a model protein. Influence of the preparation conditions on the nanoparticles size, encapsulation efficiency, and release profile *in vitro* was investigated. They showed the entrapment efficiency of 42.9–63.4% and the average diameter of 119.1–342.8 nm depending on the fabrication technique of nanoparticles and the type of copolymer. The stability maintenance of BSA in the nanoparticle release *in vitro* was also measured via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism (CD), and fluorescence spectrometry. The results showed that the new copolymer could load BSA effectively and BSA kept stable after it was released from the nanoparticles. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 1118–1128, 2008

Key words: Pluronic (F68); poly(lactide-*co*-glycolide); amphiphilic copolymer; nanoparticles; bovine serum albumin; protein delivery system

INTRODUCTION

Nanoparticles or colloidal particles have been widely used for targeted drug delivery and other biomedical applications.[1,2] Numerous investigators have shown that the biological distribution of drugs, proteins, or DNA can be modified, both at the cellular and organ levels, using micro/nanoparticles delivery systems.[3] The nanometer size ranges of these delivery systems offer distinct advantages for drug delivery. To achieve this objective, several micro/nanosized biodegradable particles such as poly(lactide-*co*-glycolide) (PLGA), poly(lactic acid) (PLA), and poly(caprolactone) (PCL) particles have been developed.[4] The degradation rate and therefore the drug release rate can be controlled by varying the composition ratio and the molecular mass of the graft or block copolymers.[5–8] The capture of water-soluble drugs such as proteins in the nanoparticle carrier system can be carried out through various approaches.

In the past few years, there has been an increasing interest to develop stealth nanoparticles as drug carrier systems. One of main methods for preparation of stealth nanoparticles or long-circulating nanoparticles is to modify their surface with a hydrophilic, flexible and nonionic polymer, poly(ethylene glycol) (PEG).[9–12] The biodegradable PEG-coated nanoparticles have been found to be important potential therapeutic applications as injectable colloidal systems for the controlled release of drugs and site-specific drug delivery.[13–15] The stealth nanoparticles compared with other long-circulating systems showed better shelf stability and ability to control the release of the encapsulated compounds.[9,14] Tobío et al. studied PEGylated polylactic acid (PEG-PLA) nanoparticles as tetanus toxoid (TT) carriers for nasal administration, and their results showed a character of extending its half-life and changing its biodistribution in rats.[10] Recently, PEGylated poly(lactic-*co*-glycolic acid) (PEG-PLGA) has been reported as some hydrophobic and hydrophilic drug carriers,[9,16]

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but information of the stealth nanoparticles prepared by Pluronic-PLGA used to deliver water-soluble drug such as proteins has not been published.

Pluronics, water-soluble ABA triblock copolymers of poly ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO), are commercially available nonionic macromolecular surface active agents. They contain hydroxyl functional groups at the end of the chains.[17–20] PEO-PPO-PEO block copolymers are an important class of surfactants. Depending on the ratio of PEO to PPO and the molecular weight of block copolymer, Pluronics have been used for specialized applications such as in pharmaceutics for the solubilization and controlled release of drug. Illum et al. demonstrated that it was possible to alter significantly the *in vitro* interaction with isolated macrophages and the biodistribution of model polystyrene nanospheres after coating the particles surface with PEO-PPO-PEO block copolymers.[18,19] In addition, the presence of PEO on the surface of nanospheres reduced the extent of phagocytosis by mouse peritoneal macrophage cells *in vitro*. Furthermore, Pluronics series also have found interests because of their temperature-dependent micellization and gel formation of aqueous PEO-PPO-PEO block copolymer solutions. Therefore, many studies have been conducted about the driving force or mechanism of the phenomena.[20]

Recent trends in drug delivery technology have focused on biodegradable polymers requiring no surgical removal once the drug supply is depleted. PLGA is a well-known biodegradable and biocompatible material with a hydrophobic character. In addition, it was reported that copolymers of DL-lactide and glycolide with other material such as PEG degraded much more rapidly than PLGA homopolymer.[16,21,22]

The aim of the present work was to assess the merits of Pluronic-PLGA nanoparticles as water-soluble drug carriers. For this purpose, the copolymer Pluronic-PLGA (Fig. 1) was synthesized with Pluronic (F68 is chosen as a model of Pluronic series) and PLGA. The structure of Pluronic (F68)-PLGA copolymer was confirmed with ^{1}H-NMR, TGA, and Fourier transform infrared (FTIR) spectra. Molecular weight was determined by gel permeation chromatography (GPC). An examination of protein encapsulation efficiency and release from biodegradable micro particles (nanoparticles) has often been performed with bovine serum albumin (BSA) because of its extensive characterization, ease of assay, lost cost, and general availability.[11,23] BSA was encapsulated within nanoparticles made of Pluronic (F68)-PLGA with the double emulsion (DE) and nanoprecipitation (NP) methods. The particles were characterized in terms of size, zeta potential, entrapment efficiency, and *in vitro* release of the protein.

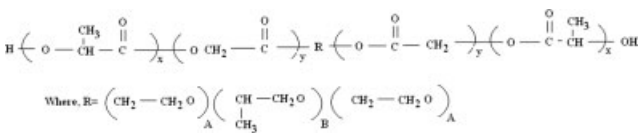

Figure 1 Structure of the Pluronic-PLGA copolymer.

EXPERIMENTAL

Chemicals and materials

Pluronic (F68) was purchased from Sigma-Aldrich. DL-Lactide and glycolide were purchased from PURAC (The Netherlands) and used without further purification. Stannous 2-ethylhexanoate was purchased from Sigma Chemical Co. (St. Louis, MO) and was used as received. BSA was purchased from Sigma (St. Louis, MO). Poly(vinyl alcohol) (PVA), 30–70 kDa, was purchased from Tianjin Kermeo Chemical Reagent Center (China). All other reagents and solvents were of analytical grade.

Synthesis and purification of Pluronic-PLGA copolymer

Pluronic (F68)/PLGA copolymers were synthesized by ring opening polymerization of DL-Lactide and glycolide monomers in the presence of PEO-PPO-PEO triblock copolymer, Pluronic (F68), using stannous 2-ethylhexanoate as a catalyst. Under nitrogen atmosphere, Pluronic (F68) was dried in a three-necked flask under vacuum and stirring at 120°C for 2h. A total of 50 g of DL-lactide, glycolide plus Pluronic (F68) were used for the polymerization. Copolymerization in the bulk state was carried out with various molar ratios of DL-lactide/glycolide (6/1, 10/1, 15/1) and the weight ratio of Pluronic (F68) was adjusted to 30% (w/w). Stannous 2-ethylhexanoate (0.2%, w/w) was added into a dried polymerization tube followed by the addition of DL-lactide, glycolide, and Pluronic (F68). Then the tube was sealed under vacuum. The sealed tube was immersed and kept in an oil bath thermostated at 150°C. After 8 h, the reaction product was cooled to ambient temperature. The obtained viscous material was dissolved with CH_2Cl_2 and then precipitated with ether/petroleum (1 : 1, v/v) to remove unreacted DL-lactide, glycolide monomers. That was extracted in an excessive amount of methanol to remove Pluronic (F68), collected by filtration and washed several times with CH_2Cl_2. The resulting product was dried in a vacuum oven at 40°C for 3 days.

Characterization of Pluronic (F68)-PLGA copolymer

The structure of block copolymer composed of PEO-PPO-PEO and PLGA was confirmed by Fourier transform infrared (FTIR, spectrum one-FT-IR,

Perkin–Elmer). For FTIR analysis, KBr tablets were prepared by grinding the polymer sample with KBr and compressing the whole into a transparent tablet.

The ^{1}H-NMR spectra was measured in $CDCl_3$ on a Bruker AM 400 MHz spectrometer.

Gel permeation chromatography (GPC) was performed on a Waters 2410 GPC apparatus (USA). Molecular weight and molecular weight distribution of the copolymer were calculated using polystyrene as the standard.

Thermogravimetric analysis (TGA) was also carried out with a 2960SDT (USA) apparatus in the temperature range from 30 to 900°C at a heating rate of 20°C/min in a flow of N_2.

Fabrication of the copolymer nanoparticles in the DE method

The double emulsion (DE) method was used to fabricate nanoparticles as described by Rodrigues etal.[24] with a few modifications. Briefly, 0.5-mL BSA solution with 10 mg/mL concentration was emulsified in 5 mL of DCM (dichloromethane) containing Pluronic (F68)-PLGA (100 mg) by homogenization at 5000 rpm in an ice bath for 3 × 10 s (Bailing, Model DS-200, China). Thereafter, this first emulsion was poured into 50 mL of the PVA aqueous solution (0.4%, w/v) and homogenized at 10,000 rpm in an ice bath for 3 × 15 s (Bailing Model DS-200, China). The double emulsion was diluted in 150 mL PVA solution (0.1%, w/v) and the DCM was rapidly eliminated by evaporation under reduced pressure. Finally, the nanoparticles were collected by centrifugation at 25,000 × *g* for 25 min at 4°C (Beckman Model J2-21) and washed twice with water. The nanoparticles were diluted with 2 mL of 5% glucose and stored at 4°C.

Fabrication of the polymer nanoparticles in the NP method

A nanoprecipitation (NP) technique[25] was developed for comparison with the DE method. Briefly, 5 mL of the polymer solution (10 mg/mL) in acetone was added dropwise to 10 mL of water with BSA or without BSA at a rate of 0.5 mL/min using a syringe pump (74,900 series multichannel syringe pumps, Cole-Parmer Instrument Company, China) under magnetic stirring. Acetone was eliminated by evaporation under reduced pressure. The nanoparticles were recovered by ultracentrifugation and treated as above in the DE method.

Physicochemical characterization of the nanoparticles

The morphological examination of nanoparticles was performed using a transmission electron microscope (TEM, Hitachi, H-600) following negative staining with sodium phosphotung state solution (2%, w/w).

Nanoparticle sizes were determined on BI-90 Plus Particle Size Analyzer/ξ potential Analyzer, Brookhaven Instruments Corp. The experiment was performed at 25°C using the samples appropriately diluted with distilled water. The ξ potential of the polymer nanoparticles was measured when the blank sample was diluted with 0.1*M* NaCl solution to a constant ionic strength. For each sample, the mean value of three determinations was established and the value reported here was the mean value of two replicate samples.

Determination of the BSA encapsulation efficient (EE%)

The amount of nonentrapped BSA in aqueous phase was determined by the Lowry-Peterson protein assay in the supernatant obtained after ultracentrifugation of nanoparticles.[26] This procedure permits analysis of very dilute protein solutions with removal of most interfering substance. The amount of BSA entrapped within nanoparticles was calculated by the difference between the total amount used to prepare nanoparticles and the amount of BSA present in the aqueous phase. Each sample was assayed in triplicate.

In vitro release of BSA from the Pluronic (F68)/PLGA nanoparticles

About 25 mg of the dried BSA-loaded nanoparticles were suspended in 5 mL of PBS (pH 7.4), stabilized with 0.2% NaN_3 (w/v), and then incubated at 37.5°C under shaking at the rate of 80 rpm on the SHZ-88 (constant temperature water-bath shaker, China). At predetermined time intervals, 3 mL samples were withdrawn and centrifuged at 25,000 × *g* for 20 min. The supernatant was assayed for protein release and replaced by 3 mL of fresh release medium. The amount of BSA in the release medium was determined by the Lowry-Peterson protein assay. Each nanoparticle batch was analyzed in triplicate.

Stability of BSA released from NPs

The free BSA in the supernatant after its release of 20 days from the Pluronic (F68)/PLGA nanoparticles was stored at −20°C. The integrity (the soluble-aggregation and degradation) of BSA was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE). The BSA in supernatant and contrast SDSPAGE analysis were performed under nonreducing conditions using a Bio-Rad electrophoresis system.[27]

TABLE I
Composition and Molecular Weight Distribution of Pluronic (F68)[a]/PLGA Block Copolymers

Sample	Copolymer	Molecular weight of copolymer		Polydispersity (M_w/M_n)
		M_w (kDa)[b]	M_n (kDa)[b]	
1	PLGA-Pluronic68- PLGA(6/1)	15846	9781	1.62
2	PLGA-Pluronic68- PLGA(10/1)	18404	12188	1.51
3	PLGA-Pluronic68- PLGA(15/1)	23327	15977	1.46

[a] Pluronic: PEO-PPO-PEO block copolymer: Pluronic68, M_n = 8400, weight portion PEO block in total = 80%.
[b] Measured by GPC relative to polystyrene standards.

The secondary structure of released BSA and BSA control was determined by measuring circular dichroism spectra. The circular dichroism measurements of free BSA in the supernatant and control BSA solutions in PBS buffer were performed on the Jasco-810 Spectropolarimeter (JASCO, Tokyo, Japan) at room temperature using the matched 5-mm path length quartz cells. Each sample solution was scanned in the range of 190–300 nm. A circular dichroism spectrum was generated as the average value of three scans. The measured circular dichroic signals were converted to mean residue ellipticity.[28]

The tertiary structure of protein samples was analyzed by measuring the intrinsic fluorescence emission spectra. Fluorescence emission spectra (300–440 nm for BSA) were obtained on a fluorescence instrument (Perkin–Elmer, USA) with a 1 cm cell. The excitation wavelength for BSA was set to 285 nm. The slits of excitation and emission monochromators were adjusted to 3 nm.

RESULTS AND DISCUSSION

Synthesis and characterization of the Pluronic (F68)/PLGA copolymer

Pluronic (F68) /PLGA copolymers were prepared by a ring-opening mechanism of the DL-Lactide and glycolide unit in the presence of Pluronic (F68), using stannous 2-ethylhexanoate as a catalyst. Polymerization of DL-Lactide and glycolide can be effected by at least four different mechanisms categorized as anionic, cationic, coordination, and radical. Among them, coordination polymerization is one of the most versatile methods for preparing PLGA and its copolymers, affording high molecular weights and conversions.[28] In the present polymerization system, metal species are believed to function as a catalyst and the hydroxyl end group of Pluronic (F68) serves as an initiator.

Stannous 2-ethylhexanoate has the advantage of having been used to prepare polymers for which substantial toxicological data are now available.[29] The active hydrogen atom at one end of the Pluronic chains acted as an initiator and induced a selective acyl-oxygen cleavage of DL-Lactide and glycolide. The molecular weights, DL-Lactide/glycolide molar ratio and polydispersity indexes of the copolymers are shown in Table I.

The hydrophobicity of the copolymer increases in the order PLGA-Pluronic-PLGA (6/1), PLGA-Pluronic-PLGA (10/1), PLGA-Pluronic-PLGA (15/1) by increasing the molar ratio of DL-Lactide/glycolide in the PLGA segment because DL-Lactide moiety is more hydrophobic than glycolide.

Figure 2(A,B) exhibited the structure and a typical ^{1}H-NMR spectrum of Pluronic (F68)/PLGA block copolymer. The sharp singlet at $\sim$ 3.68 ppm is due to protons of CH_2CH_2 units of PEO block in Pluronic (F68).The doublet at $\sim$ 1.13 ppm belongs to the CH_3 protons in the PPO block, each characteristic peak at $\sim$ 3.4 and $\sim$ 3.5 ppm come from the CH and CH_2

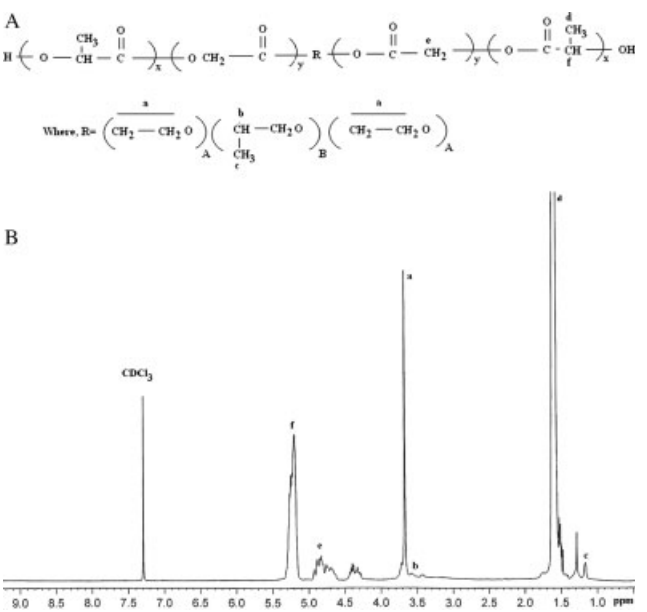

Figure 2 Structure (A) and ^{1}H-NMR spectrum (B) of the Pluronic (F68)-PLGA copolymer (Pluronic (F68)-PLGA (6/1)).

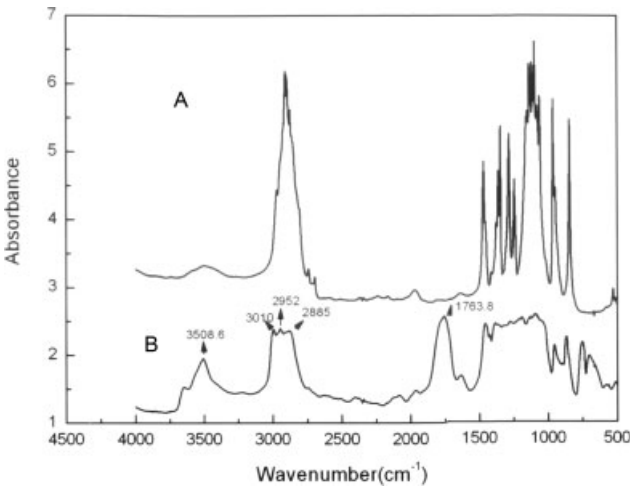

Figure 3 IR spectra of Pluronic68 (A) and Pluronic68-PLGA (10/1) copolymer (B).

units of PPO block in Pluronic (F68). Overlapping doublets at ~ 1.55 ppm are attributed to the methyl groups of the D- and L-lactic acid repeat units. The multiplets at ~ 5.2 and ~ 4.8 ppm correspond to the lactic acid CH and the glycolic acid CH_2, respectively, with the high complexity of the peaks resulting from different D-lactic, L-lactic, glycolic acid sequences in the polymer backbone (Structure of the Pluronic-PLGA copolymer shown in Fig. 1).

The FTIR spectrum is consistent with the structure of the expected copolymer (Fig. 3). It shows that absorption band at 3508.6 cm^{-1} is assigned to terminal hydroxyl groups in the copolymer. The bands at 3010 and 2952 cm^{-1} are due to C—H stretch of CH_3, and 2885 cm^{-1} due to C—H stretch of CH_2. A strong band at 1763.8 cm^{-1} is assigned to C=O stretch.

Figure 4 illustrated the GPC chromatogram of sample (Pluronic (F68)/PLGA (6/1)). In GPC analysis, we confirmed the disappearance of elution volume peak due to Pluronic and the appearance of a new and single peak. The new peak was attributed to the Pluronic (F68) /PLGA block copolymers. It also indicated that Pluronic (F68)/PLGA copolymer was produced without PLGA homopolymer or any impurities.

In addition, the thermal properties of Pluronic (F68)/PLGA copolymer were examined by TGA measurement. Figure 5 shows the TGA thermogram of Pluronic (F68) and Pluronic (F68)/PLGA copolymer over the temperature range from 30 to 900°C, at a heating rate of 10°C/min in a flow of N_2. The thermogravimetric trace of Pluronic (F68) shows a single mass loss [see Fig. 5(A)]. The weight loss temperature (6.1% loss) of virgin Pluronic (F68) was 320°C, exhibiting an outstanding thermal stability. Two decomposition events were observed for Pluronic (F68)/PLGA copolymer [see Fig. 5(B)]. The first decomposition occurs, from 220 to 320°C. This is attributed to the decomposition of the PLGA block. The second decomposition step, beginning at about 390°C, corresponds to the Pluronic (F68), PEO-PPO-PEO copolymer. These results on TGA traces are in good agreement with the NMR or GPC results for Pluronic(F68)/PLGA copolymer.

Preparation techniques for the copolymer nanoparticles (NPs) Effect of copolymer composition on particle size and EE

Data are shown in Table II. Regarding to particle size, the higher Mw of PLGA contributes to the viscosity increase of copolymer solution and the decrease of Pluronic relative contents, both factors resulting in particle size increase. The copolymer composition has no obvious effect on EE because an opposite effect exists. The increased viscosity can decrease the diffuse of BSA, which is in favor of

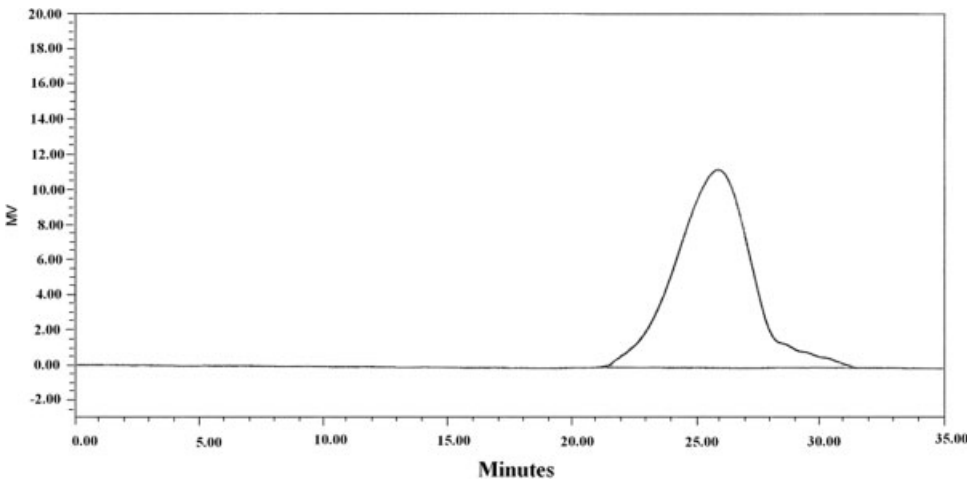

Figure 4 GPC chromatogram of the Pluronic (F68)-PLGA copolymer (Pluronic (F68)/PLGA (6/1)).

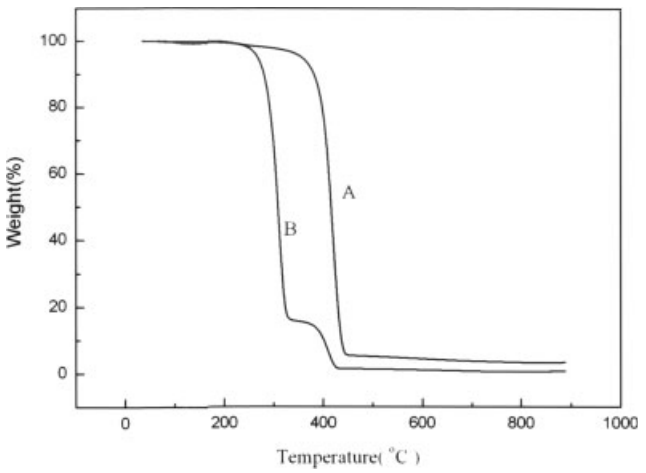

Figure 5 Weight loss of Pluronic68 (A) and Pluronic (F68)/PLGA (6/1) copolymer (B) by thermogravimetric analysis.

enhancing EE. On the contrary, the interaction between Pluronic and BSA may decrease with the lowering of Pluronic relative contents, which is unfavorable for enhancing EE.

Effect of fabrication method on particle size and EE

DE method

Because of the high solubility of the protein in water, DE (double emulsion) method was chosen as the most appropriate method. The particular data are shown in Table III.

Effect of BSA concentration in the inner aqueous phase on particle size and EE

With the increasing of the protein concentration in the inner aqueous phase, the particle size became smaller and the polydispersity was slightly lower, which was also reported by Verrachia et al.[30] Becasue the particle size is related to a great extent to the stability of the first emulsion, they claimed indeed that serum albumin could act as a surfactant by stabilizing the first emulsion and consequently hampering the fast coalescence of the droplets. EE decreased with higher protein concentration in the inner aqueous phase. The difference in osmotic pressure between the internal and external aqueous phases could be responsible for the decrease in entrapment efficiency. The osmotic pressure difference did in fact rise with increased BSA loading and promote an exchange between the internal and external aqueous phases, with a consequent loss of BSA.[23]

Effect of inner aqueous phase volume on particle size and EE

An increase in the internal aqueous phase volume of the same concentration led to a decrease of nanoparticles' average size. Coalescence of droplets could be prevented by a large quantity of internal phase. An increase in the internal aqueous could lead to an increase in EE. The precipitation of the polymer solution phase was accelerated and the hardening time was shortened with the increase of inner aqueous. As a result, much BSA could be held in the NPs and EE increased.

Effect of polymer concentration in organic phase on particle size and EE

An increase in the polymer concentration resulted in an increase of the particle average size from $\sim$ 186 to 302 nm with a broadened particle size distribution. The reason is that a high viscosity holds back the shear forces of emulsion. While the EE increased when the polymer concentration in organic phase was higher because high viscosity avoids the leak of BSA.

Effect of PVA concentration in external aqueous phase on particle size and EE

An increase in external aqueous concentration of PVA from 1 to 5% (w/v) resulted in an increased BSA EE% and a decreased particle size. The tight surface was formed from PVA macromolecules of high concentration, which increased diffusion resistance of BSA from the internal aqueous phase and

TABLE II
Effect of Copolymer Composition on EE and Particle Size[a,b]

Fabrication method	Copolymer composition	Encapsulation efficiency (%)	Mean diameter (nm)	Polydispersity index
DE	PLGA-Pluronic68- PLGA(6/1)	58.1 ± 3.6	221.7	0.131
DE	PLGA-Pluronic68- PLGA(10/1)	60.1 ± 2.4	246.2	0.092
DE	PLGA-Pluronic68- PLGA(15/1)	63.4 ± 3.1	261.5	0.145
NP	PLGA-Pluronic68- PLGA(6/1)	46.3 ± 2.4	119.1	0.063
NP	PLGA-Pluronic68- PLGA(10/1)	44.63 ± 1.8	143.2	0.089
NP	PLGA-Pluronic68- PLGA(15/1)	42.9 ± 1.9	163.1	0.071

[a] For DE method, 30 mg BSA was dissolved in 1mL of water in inner phase, 50 mg of the copolymer Pluronic (F68)-PLGA was dissolved in 1 mL of dichloromethane, PVA concentration was 3%; For NP method, BSA concentration was 10 mg/mL and copolymer concentration was 5 mg/mL.
[b] Mean value ± standard deviation ($n = 4$).

TABLE III
The Influence of Processing Factors on EE and Particle Size Using DE Technique[a]

Batch	BSA concentration (mg/mL)	Internal aqueous phase volume (mL)	Polymer concentration (mg/mL)	PVA concentration (w/v %)	Encapsulation efficiency (%)	Mean diameter (nm)	Polydispersity index
1	10	0.2	50	3	61.2% ± 2.5	286.2	0.141
2	30	0.2	50	3	58.1 ± 3.6	221.7	0.135
3	50	0.2	50	3	51.5 ± 2.3	212.6	0.138
4	30	0.1	50	3	49.1 ± 3.1	220.1	0.162
5	30	0.3	50	3	60.6 ± 1.2	178.5	0.128
6	30	0.2	25	3	47.5 ± 2.7	186.6	0.133
7	30	0.2	75	3	62.1 ± 1.8	302.3	0.172
8	30	0.2	50	1	57.4 ± 2.1	342.8	0.131
9	30	0.2	50	5	62.8 ± 3.4	170.5	0.116

[a] Pluronic (F68)- PLGA (6/1) was used for all conditions.

stabilized the emulsion. But too much PVA was suspectable in the NPs because it could not be totally biodegraded *in vivo*. On the one hand, the decrease in NP size was very small when PVA concentration was above 5% of PVA, similar results were reported by Ferdous et al.[31] On the other hand, too much PVA was difficult to remove.

NP method

NP (nanoprecipitation) method was a relative mild technique when compared with DE method because sonicators were not used by NP method. This technique also allowed for the preparation of large quantities of NPs because sonicators and homogenizers were not used in the process.[32] Briefly, NP technique was to precipitate the NPs through the use of an organic solvent that is entirely miscible with water. The formation of NPs with NP method could be carried out without any surfactant due to the amphiphilic properties of copolymers. Both the particle size and EE increase with the increasing of copolymer concentration in acetone, whereas the particle size and EE decrease with the increasing BSA concentration in water. These can be explained as above-mentioned. The results are listed in Table IV. The above data showed that the fabrication method had a significant effect on the particle size and EE. We could prepare NPs with particle size of 170.5–342 nm and EE of 47.5–63.4% using DE method. Meanwhile, we could fabricate NPs with particle size of 119.1–188.3 nm and EE of 42.9–48.7% using NP method at various conditions. On the one hand, DE technique fabricates NPs with a multinanoreservoir structure,[24] while NP method could get micelles. On the other hand, the larger particle size is in favor of enhanced EE. This is why a larger particle size and enhanced EE are achieved using DE method.[33]

The structure preservation of BSA was also considered during its nanoencapsulation. Proteins are heat-sensitive and sonication is an exothermic operation. Therefore, the ice bath was used during primary emulsion.

Other experimental technique should be paid attention to during encapsulation. A rotation evaporator was used to reduce the evaporation time to avoid the protein release during stirring at room temperature. Moreover, organic solvent must be evaporated completely because the remained organic solvent will cause caking during centrifugation. At last, glucose was added to overcome NPs aggregation and allowed the resuspension of the NPs after freeze-drying.[34]

TABLE IV
The Influence of Processing Factors on EE and Particle Size Using NP Technique [a,b]

Batch (No)	Polymer concentration (mg/mL)	BSA concentration (mg/mL)	Encapsulation efficiency (%)	Mean diameter (nm)	Polydispersity index
1	2	30	43.6 ± 3.4	130.5	0.081
2	5	30	45.2 ± 2.6	159.6	0.073
3	10	30	48.7 ± 3.1	188.3	0.096
4	5	10	47.9 ± 1.9	183.1	0.065
5	5	50	43.1 ± 4.1	150.9	0.078

[a] Pluronic (F68)- PLGA (15/1) was used for all conditions.
[b] Mean value ± standard deviation ($n = 4$).

TABLE V
ξ-Potential of the Blank Pluronic (F68)- PLGA Nanoparticles

Materials	Fabrication method	ξ-potential (mV)
PLGA-Pluronic68- PLGA(6/1)	DE	−16.6
PLGA-Pluronic68- PLGA(6/1)	NP	−20.5

ξ potential

The blank PLGA nanoparticles had a large negative ξ potential due to the presence of the ionized carboxyl groups on the nanospheric surface. As found from the data in Table V, the $|\xi|$ values of the Pluronic (F68)-PLGA nanospheres were lower than those of PLGA nanospheres, indicating that the Pluronic (F68)-PLGA nanoparticles possessed less negative charge because no free carboxylic group existed on the Pluronic (F68)-PLGA copolymers. The $|\xi|$ potential of the nanoparticles prepared in the DE method was lower than that in the NP method the PVA was difficult to be removed from the nanoparticle surfaces and the residue PVA could decrease the negative potential of the nanoparticles.[35] Theoretically, the nanoparticles aggregation easily occurred when ζ potential was reduced, leading to the formation of large nanoparticles.[36] However, not much aggregation was observed in our experiments. The hydrophilic Pluronic (F68) moieties of the copolymer Pluronic (F68)-PLGA act as a steric barrier on the surface of the nanoparticles.

Morphology

The photographs shown in Figure 6 indicated that the NPs appeared to be fine spherical shapes and no aggregation or adhesion occurred among the NPs made by both NP and DE methods. The diameter observed in TEM was a little smaller than that detected on the Particle Size Analyzer. Because the nanoparticles for the TEM observation were air-dried and those for the detection of the hydrodynamic diameter were in the aqueous medium, the outer water-solvated layer of the air-dried nanoparticles will be thinner so that the smaller diameter is displayed on the TEM.

In vitro release experiment

The release profiles of BSA from the Pluronic (F68)-PLGA were displayed in Figure 7. In all of the curves, a little burst effect was observed and a slow continuous release phase was followed. Generally, the drug release from Pluronic (F68)-PLGA could be controlled by both drug diffusion and polymer degradation. The initial burst release indicated that the initial release of protein occurred predominantly by its diffusion through the aqueous pores generated in the dosage form. However, the protein within the body of the polymer matrix could not be released until the polymer degradation took place.

Selecting appropriate NPs fabrication method could potentially control BSA release from NPs. As for the fabrication methods, it was found that the BSA released faster from the NPs with the NP method than with the DE method. When the polymers are not soluble in water, drug molecules dissolved in water may be very close to the outer NPs surface, forming a layer of molecules, susceptible to be easily and rapidly released.[37] In addition, more burst release was observed from NPs fabricated with NP technique than those from DE method. This is because different methods led to various distributions of BSA molecules in the NPs. The fabrication method determines the amount of protein existing near the surface of NPs. Using DE method, most BSA molecules were encapsulated within the NPs as the multinanoreservoir systems. Using NP method, NPs were formed as the multimolecular polymeric

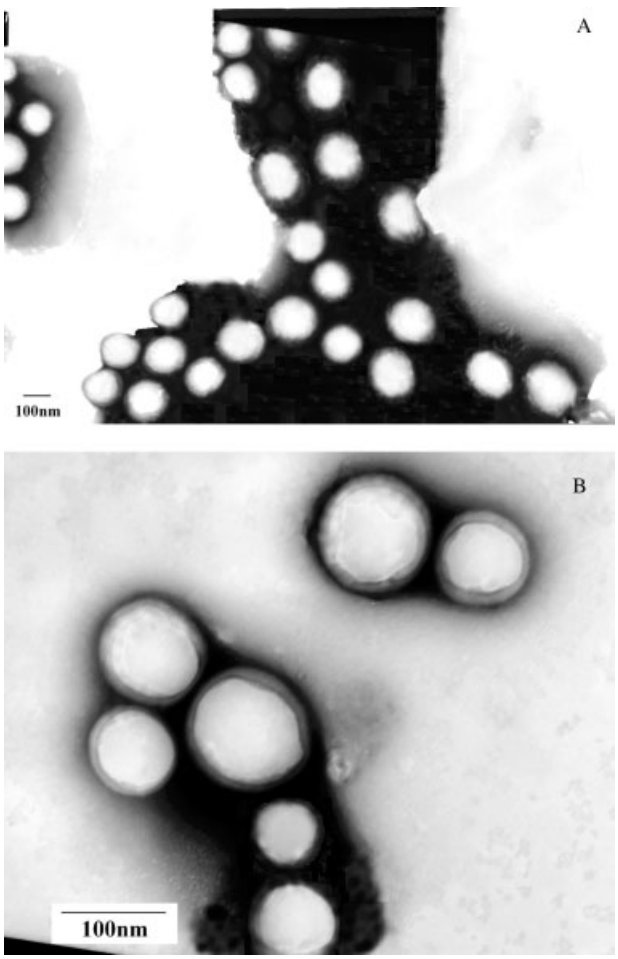

Figure 6 TEM of micrograph of the Pluronic68-PLGA (10/1) nanoparticles loading BSA in the DE (A) and NP method (B).

micelles trapping BSA molecules near their outer layers.[24] The initial release burst corresponded to the diffusion of protein located near the NPs surface. Although, burst release is always considered as an undesirable property of protein-loaded NPs, it may be useful as vaccine delivery system to obtain the primary immunity.

Stability of BSA released from the Pluronic (F68)-PLGA

Both direct and indirect methods could be employed to detect the BSA stability in the nanoparticles. In the indirect method as mentioned in the experiment part, only the released BSA from the nanoparticles was detected. In terms of practical application of protein delivery system in clinic, the data from the indirect method was more valuable to evaluate effectiveness of a protein delivery system. So the indirect method was used in our laboratory. The SDS-PAGE results of BSA released from nanoparticles after 20 days are shown in Figure 8. The nonreducing conditions employed for this analysis will preserve all aggregates linked by disulfide bonds.[27] Neither degradation nor soluble aggregation takes place with the Pluronic (F68)-PLGA nanoparticles fabricated by both methods, suggesting that the Pluronic (F68) moiety attributes to the preservation of BSA integrity. Many factors such as exposure of a protein to organic solvents, high temperature and shearing strength could make a protein deactivated during an encapsulation process.[33] Otherwise, the deleterious micro-environmental factors for the protein stability in the polymeric nanoparticles were acidic pH and the hydrophobic polymer surface.[38] To preserve BSA activity during its encapsulation, more moderate condition was employed such as acetone as water miscible solvent,[39] sonication in an ice bath and ultracentrifugation at a low temperature as well as the use of the mechanical forces as low as possible. $Mg(OH)_2$ was successfully used to increase the microenvironmental pH and to prevent BSA from structural loss and aggregation for over 1 month.[38,40] The Pluronic (F68) moiety possessed many hydrophilic hydroxyl groups that could form hydrogen bonds with the BSA molecule. An adaptive microenvironment for BSA was constructed from the polymer with hydrophilic hydroxyl groups to keep BSA stable. So the BSA activity could be preserved in the Pluronic (F68)-PLGA nanoparticles.

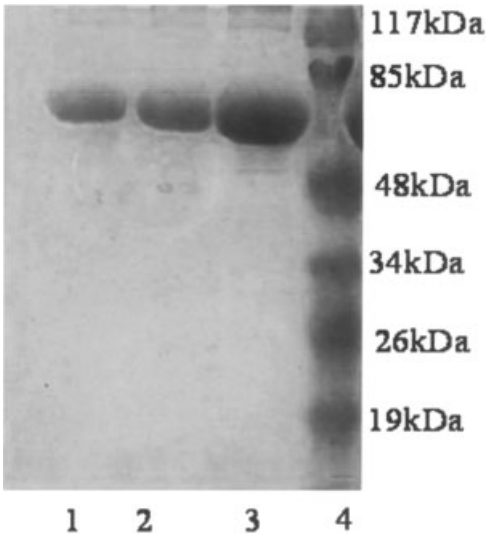

Figure 8 SDS-PAGE results of BSA released from the nanoparticles after 20 days in PBS. Lane 1, BSA standard; lane 2, Pluronic (F68)-PLGA (10/1) (DE); lane 3, Pluronic (F68)-PLGA (10/1) (NP); lane 4, MW standard.

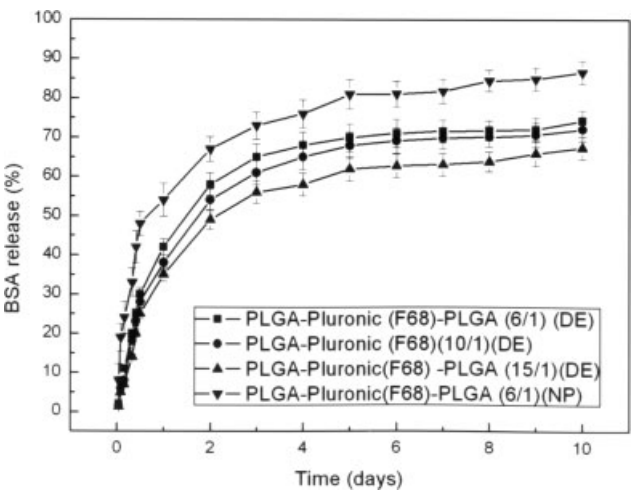

Figure 7 *In vitro* release profiles of BSA from Pluronic (F68)-PLGA nanoparticles. (■) PLGA-Pluronic (F68)-PLGA (6/1) (DE); (●) PLGA-Pluronic (F68)-PLGA (10/1) (DE); (▲); PLGA-Pluronic (F68)-PLGA (15/1) (DE); (▼) PLGA-Pluronic (F68)-PLGA (6/1) (NP).

Circular dichroism spectroscopy is a common method to analyze the secondary structure of a protein with high reliability. In the CD spectrum of the native BSA in PBS (pH 7.4), there were two extreme valleys at 209 and 220 nm.[41] The CD spectra of the free BSA in the supernatant from the release test after 10 days were measured and shown in Figure 9. Obviously, two extreme valleys at 209 and 221 nm occurred without any significant difference from those of the native BSA. The result indicated that the released BSA remained its original structure. The intensities of the double minimums reflected the helicity of BSA as more than 50% of α-helical structure.[42] The slightly declined intensity of the double minimums implied that the extent of α-helicity of the protein decreased a little. Because NP method fabricated nanoparticles in a more mild way. As for the Pluronic (F68)-PLGA nanoparticles, the secondary structure of the released BSA from the

nanoparticles fabricated by the NP method remained more stable than by DE method. The Pluronic (F68)-PLGA nanoparticles of a protein might be promising as a nasal delivery system, because the biological response of proteins encapsulated in some biodegradable nanoparticles was significantly greater than those in the microparticles when administered intranasally.[43]

Likewise, tertiary structure of BSA was similar to BSA standard (Fig. 10). The fluorescence of aqueous tryptophan-containing proteins arises almost from their tryptophanyl residues.[44] Pluronic (F68) may interact with the protein molecules exposed upon unfolding. Therefore, introduction of Pluronic (F68) into polymer contributed to secondary and tertiary structure maintenance of protein release from its NPs.

CONCLUSIONS

In conclusion, the results obtained in the present study showed that Pluronic (F68)-PLGA could be synthesized by ring-opening polymerization of the lactide and glycolide in the presence of Pluronic (F68). Structural analyses such as ^{1}H-NMR, FTIR, GPC, and TGA were carried out to confirm the preparation of Pluronic (F68)-PLGA copolymers. The nanoparticles loading BSA could be prepared by double emulsion and nanoprecipitation technique.The entrapment efficiency was 42.9–63.4%, and particle size 119–324 nm depending on the fabrication technique of nanoparticles and the type of copolymer. BSA release from the stealth nanoparticles showed an initial burst release and then sustained release phase. These results showed that Pluronic (F68)-PLGA nanoparticles could be an effective carrier for drug delivery. Conjugating of Pluronic (F68) onto PLGA chain was found to be a feasible approach for the fabrication of NPs for protein delivery with following promising properties: Besides the copolymer composition, fabrication methods (DE and NP techniques) had significant influence on particle size, EE. and release profile.

Therefore, we can conclude that Pluronic (F68)-PLGA amphiphlic copolymeric nanoparticles could be useful as a drug carrier for protein drug delivery system. To obtain more information about the properties of Pluronic (F68)-PLGA copolymeric nanoparticles and investigate their feasibility as a vehicle for various protein and peptide drugs, further studies on the loading of bioactivity protein and peptide into copolymeric nanoparticles, examine *in vitro* and *in vivo* bioactivity, release behaviors of drug and cytotoxicity are underway in our laboratory.

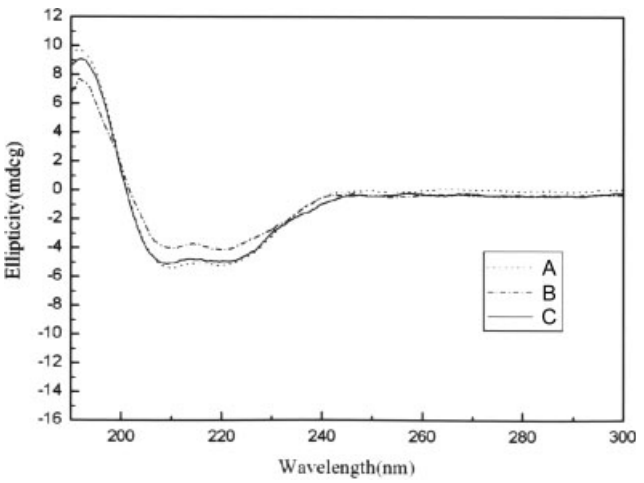

Figure 9 Circular dichroism spectra of the BSA solutions. (A) Native BSA, (B) BSA released after 10 days from the Pluronic (F68)-PLGA (10/1) nanoparticles made by the DE method, (C) BSA released after 10 days from the Pluronic (F68)-PLGA (10/1) nanoparticles made by the NP method.

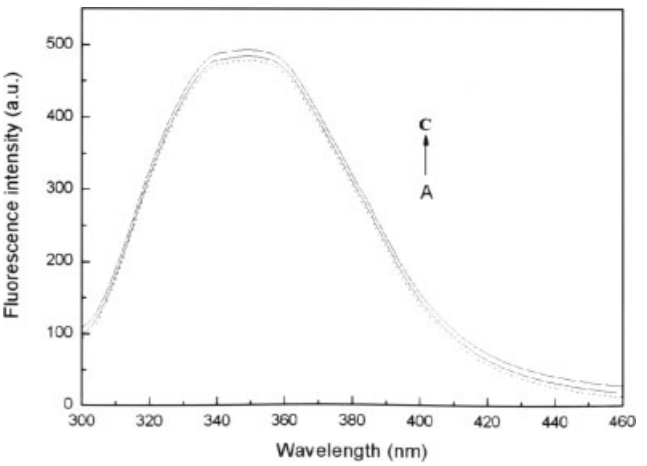

Figure 10 Fluorescence emission spectra of the BSA solutions. (A) Native BSA, (B) BSA released after 20 days from the Pluronic (F68)–PLGA (10/1) nanoparticles made by the NP method, (C) BSA released after 20 days from the Pluronic (F68)–PLGA (10/1) nanoparticles made by the DE method.

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