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Surfactant-Free Microspheres of Poly(ε-caprolactone)/Poly (ethylene glycol)/Poly(ε-caprolactone) Triblock Copolymers as a Protein Carrier

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The aim of this study is to prepare biodegradable microspheres without the use of surfactants or emulsifiers for a novel sustained delivery carriers of protein drugs. A poly(ε-caprolactone)/poly(ethylene glycol)/poly(ε-caprolactone) (CEC) triblock copolymer was synthesized by the ring-opening of ε-caprolactone with dihydroxy poly (ethylene glycol) to prepare surfactant-free microspheres. When dichloromethane (DCM) or ethyl formate (EF) was used as a solvent, the formation of microspheres did not occur. Although the microspheres could be formed prior to lyophilization under certain conditions, the morphology of microspheres was not maintained during the filtration and lyophilization process. Surfactant-free microspheres were only formed when ethyl acetate (EA) was used as the organic solvent and showed good spherical microspheres although the surfaces appeared irregular. The content of the protein in the microsphere was lower than expected, probably because of the presence of water channels and pores. The protein release kinetics showed a burst release until 2 days and after that sustained release pattern was showed. Therefore, these observations indicated that the formation of microsphere without the use of surfactant is feasible, and, this the improved process, the protein is readily incorporated in the microsphere.

Key words: Surfactant-free, Emulsifier, Biodegradable triblock copolymer, Protein carrier Microsphere

INTRODUCTION

During the past three decades, there has been an increasing interest in developing microspheres cr nanoparticles as drug delivery systems for peptides or protein drugs (Alonso *et al.*, 1994; Jeffery *et al.*, 1993; Yeo *et al.*, 2001). For the development of protein drug delivery systems, biodegradable polyesters such as poly-(lactide-co-glycolic acid)(PLGA) or poly-(ε-caprolactone) and their copolymers with poly-(ethylene glycol) have been extensively used as protein carriers in controlled-release drug delivery system (Boury *et al.*, 1997; McGee *et al.*,1994; Mehta *et al.*,1996; Yang *et al.*, 2001). Dunn *et al.* (Dunn *et al.*, 1995), Tipton *et al.* (Tipton *et al.*, 1991), and Radomsky *et al.* (Radomsky *et al.*, 1993) proposed an alternative approach where the biodegradable system forms situ upon injection.

The double-emulsion solvent evaporation technique such as W/O/W is the most commonly used method to encapsulate proteins or peptide drugs into polymeric microspheres, which are the key considerations in designing a microsphere delivery system (Alonso *et al.*, 1994; Boury *et al.*, 1997; Ciftci *et al.*, 1996; Jeffery *et al.*, 1991; Kreitiz *et al.*, 1997; Leach *et al.*,1998; Ogawa *et al.*, 1988; Scholes *et al.*, 1993; Spenlehauer *et al.*, 1989; Venier-Julienne *et al.*, 1996; Yeh et al; 1995; Yeo *et al.*, 2001). In these methods, a large quantity of surfactants or emulsifiers is required to stabilize the dispersed particles. In particular, poly-(vinyl alcohol) (PVA) as a stabilizing agent is most frequently used to prepare microspheres (Lee *et al.*, 1999; Rafati *et al.*,1997; Shakesheff *et al.*, 1997). However, PVA has certain limitation in the formulation in that PVA remains at the surface of the particles, which makes it

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difficult to remove. Presence of residual PVA on the surface of micro- or nanoparticles is known to change the biodegradability, biodistribution, and drug release behavior of the drug carrier (Lavelle *et al.*, 1995; Landry *et al.*, 1996; Landry *et al.*, 1997). Furthermore, when PVA attached to micro- or nanosphere were given to the body, the alcohol may be eventually detached from the microsphere surfaces *in vivo*. However, PVA is likely to circulate for a long time without any desirable action (Yamaoka *et al.*, 1995a; Yamaoka *et al.*, 1995b) and may even be carcinogenic (Hueper, 1971). Other types of surfactants such as the Span series or Tween series, poly-(ethylne oxide) (PEO), and poloxamer (PEO-poly-(propylene oxide) block copolymer) have also been used to prepare and stabilize particles (Sjostrom *et al.*, 1993a; Sjostrom *et al.*, 1993b). Almost all these types of surfactants are non-biodegradable and non-digestible. In addition, these surfactants may lead to an allergy-like reaction in human. There is no effective means to remove these surfactants after the preparation of micro- or nanospheres.

Several authors have investigated surfactant-free particulate systems for almost a decade (Carrio *et al.*, 1995; Fess *et al.*, 1989; Govender *et al.*, 1999; Jeong *et al.*, 2001). For example, surfactant-free nanocapsules of poly-(DL-lactide) (PLA) based on a nanoprecipitation technique were developed by Fessi *et al.* (Fessi *et al.*, 1989) and a nanoprecipitation technique for preparing nanoparticles has been extensively employed by other research groups. It was reported that PLGA or PLA microspheres can be prepared by using PLA oligomers (Carrio *et al.*, 1995) or PLA-poly-(ethylene glycol) diblock copolymers (Bouillot *et al.*, 1999a; Bouillot *et al.*, 1999b), which have an amphiphilic surfactant-like structure and behavior. Thus, the use of these agents appears just a replacement of conventional surfactants. Since di- or triblock copolymers consisting hydrophobic and hydrophilic block have the potential to self-aggregation in an aqueous environment, nanoparticles (Gref *et al.*,1994) or microspheres-(Bouillot *et al.*, 1999a) may be prepared from polylactide/PEO or poly-(ε-caprolactone)/PEO block copolymers without the use of surfactants or emulsifiers. Moreover, since one of the problems with poly(ε-caprolactone) is the very slow degradation rate, the presence of PEG hydrophilic segments may induce an increase in the degradation rates of microspheres

The aim of this study, therefore, was to study the feasibility of the preparation of microspheres of poly-(ε-caprolactone)/poly-(ethylene glycol)/poly-(ε-caprolactone) (CEC) triblock copolymers without the use of surfactants or emulsifiers. In addition, we have studied whether the microspheres contained a protein drug could be prepared as a novel protein carriers using bovine serum albumin (BSA) as a model protein. To study the formation of surfactant-free microspheres of CEC, a W/O/W double emulsion method was used and several formulation variables such as series of organic solvents, the content of the polymers in the organic phase, and the content of BSA in the initial water phase of the W/O/W double emulsion studied.

MATERIALS AND METHODS

Materials

PEG (M.W. = 2,000 g/g mole, PEG 2K) and the bovine serum albumin (BSA) were purchased from Sigma Chemical Co. U.S.A.. The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratory. U.S.A.. The ε-caprolactone, dichloromethane (DCM), ethyl formate (EF), ethyl acetate (EA), methanol, and diethyl ether were purchased from Aldrich Chemical Co., U.S.A.. All other chemicals and reagents were used as extra reagent grade at all of the experiments.

Synthesis of CEC triblock copolymer

The CEC triblock copolymers were synthesized by the non-catalyzed ring opening polymerization of ε-caprolactone in the presence of PEG (Cerrai *et al.*, 1989), as shown in Fig. 1. PEG and ε-caprolactone were mixed in a round-bottomed flask under vacuum. The mixture was cooled and degassed with a vacuum pump. The round-bottomed flask was sealed and placed in an oil bath at 185°C. After the polymerization was complete, the resultant product was cooled at room temperature and dissolved in dichloromethane. The solution was precipitated by an excess amount of cold methanol and filtered to remove the unreacted PEG homopolymers and ε-caprolactone monomers. The precipitates were then washed with diethyl ether three times and then dried in a vacuum oven for 3 days.

^{1}H-NMR spectroscopy measurement

^{1}H-NMR spectra of the copolymer were measured in $CDCl_3$ to estimate the copolymer compositions and the molecular weights of the PCL blocks, using a 300 MHz NMR spectrometer (FT-NMR, Bruker AC-300F, 300 MHz). As the number-average molecular weight of PEG (2,000) is known, one can estimate the number-average molecular weights of the PCL block and the copolymer composition calculated from the peak intensities in the spectrum assigned to both polymers.

Preparation of microspheres

The double-emulsion solvent evaporation method was employed to fabricate the microspheres containing BSA. BSA (20, 50, and 100 mg, respectively) dissolved in 0.5 mL of deionized water was emulsified into 3 ml of organic solvent (DCM, EF, or EA) containing the polymer (0.1, 0.2,

or 0.5 g) using a probe type sonifier (Sonic & Materials Inc., Danbury, CT. USA) at an output power of 50 W for 30 s on ice (5 s×6). The W_1/O was poured into 150 mL of the aqueous solution without any surfactants or emulsifiers to produce the $(W_1/O)/W_2$ emulsion. This solution was stirred for 1 h at room temperature under reduced pressure. The microspheres were filtered and washed three times with deionized water, and freeze-dried for 2 days. The final products were stored at +4°C in a desiccator.

Determination of BSA contents in microspheres

20 mg of the microspheres containing BSA were dissolved in 1 mL of DCM, followed by the addition of 8 mL deionized water. The resulting mixture was stirred for 24 h in a shaking incubator at 100 rpm. After separating the two phases, 1 mL of the water phase was sampled and analyzed by a Bio-Rad protein assay, based on the method of Bradford (Bradford, 1976), using a UV spectrophotometer (UV-1200, Shimadzu Co. Japan) at the wavelength of 595 nm. The equations to calculate the drug contents and the loading efficiency are as follows:

$$\text{Drug contents} = \frac{\text{Amount of BSA in microspheres}}{\text{Weight of microspheres}} \times 100$$

$$\text{Residual amount of BSA in microsphers} = \frac{\text{Residual amount of BSA in microsphers}}{\text{Feeding amount of BSA in microspheres}} \times 100$$

In vitro BSA release study

3 mg of the microspheres was added to an eppendorf tube containing 1 mL phosphate buffered saline (PBS, 0.1 M, pH 7.4) and 0.03% sodium azide. The tubes were placed in a shaking incubator (Vision scientific co., Korea) with a rotating speed of 100 rpm at 37°C. The tubes were, then, centrifuged at 10,000×g for 10 min at specific time intervals and 0.1 mL of the supernatant was collected for protein analysis using a Bio-Rad protein assay by UV spectrophotometry (UV-1200, Shimadzu Co. Japan) at 595 nm.

Particle size analysis

The size of the microspheres was estimated using a Shimadzu SALD-2001 (Shimadzu Co., Japan) Laser Diffraction Particle Size Analyzer.

Scanning electron microscope (SEM)

The morphologies of the microspheres containing albumin were observed using a JSM 35 CF (Jeol, Japan) scanning electron microscope (SEM) at 25 kV. The samples were placed on metal stubs with double sided tape, and coated with gold (30 mA for 5 min).

RESULTS AND DISCUSSION

The CEC triblock copolymers were prepared by the ring-opening polymerization of an ε-caprolactone monomer in the presence of monomethoxy PEG without any other catalysts according to the reported method by Cerrai *et al.* (Cerrai *et al.*,1989), where an active hydrogen atom at one end of the chains of the PEG homopolymers act as an initiator, which induces selective acyl-oxygen cleavage of the ε-caprolactone (Fig. 1). The PCL homopolymer is a semicrystalline polymer and has hydrophobic characteristics with a long degradation time in vivo. The introduction of ε-caprolactone into PEG to form the PCL and PEG block copolymer may reduce the degradation time and produce better physicochemical properties and processibilities. In particular, block copolymers composed of PCL and PEG can easily be prepared into microspheres or nanoparticles as biodegradable carriers of various drugs. Changing the molar ratio of PEG homopolymer/ε-caprolactone monomer and their molecular weights can be used to control the molecular weights CEC triblock copolymer. The composition of the CEC triblock copolymer was determined by ^1H NMR spectroscopy. The unit ratio of PEG and ε-caprolactone was obtained from the peak intensities of the methylene proton of the PEG chain and the methylene proton in the ε-caprolactone units, respectively. Since the signals at approximately 3.7 ppm and 4.13 ppm were assigned to these protons, respectively, the molecular weight of the PCL block was readily calculated to be 12,040 for the PCL block and 14, 040 for the CEC triblock (viz, M.W. of PEG is 2,000 g/mol) respectively.

It is well known that various formulation factors affect the characteristics of the protein-encapsulated biodegradable microspheres. These factors include the stirring speed, the size of the microspheres, the PVA concentration, the viscosity of the polymer solution, the internal (W_1) water phase, series of organic solvents, the volume ratio of the oil to external (W_2) water phase, the protein contents, and the protein distribution status into the microspheres. (Bodmier *et al.*,1992; Boury *et al.*,1997; Carrio *et al.*,1995; Celebi *et al.*, 1996; Cho *et al.*, 2000; Jeffery *et al.*, 1993; Kreitiz *et al.*, 1997; Leach *et al.*, 1998; O'Hagan *et al.*, 1994; Pradhan *et al.*, 1994). Among them the PVA con-

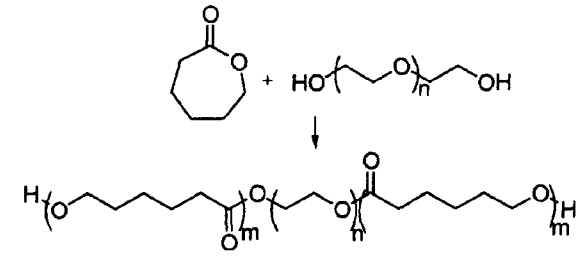

Fig. 1. Synthesis scheme of the CEC triblock copolymer.

Table I Characterization of surfactant-free microparticles of CEC triblock copolymers

Batch	Polymer weight (mg)	Organic solvent	BSA weight (mg)	Drug contents (%, w/w)	Loading efficiency (%, w/w)	Particle size (μm)	State
1	100	DCM	20	-	-	-	disintegrate
2	200	DCM	20	-	-	-	disintegrate
3	200	DCM	50	-	-	-	irregular
4	500	DCM	50	-	-	-	irregular
5	500	DCM	100	-	-	-	disintegrate
2	200	DCM	20	-	-	-	disintegrate
6	200	EF	20	-	-	-	disintegrate
7	200	EF	50	-	-	-	disintegrate
8	200	EA	20	-	-	40.5	Microparticle
9	500	EA	50	1.1	11.3	114.0	Microparticle
10	500	EA	100	1.9	9.5	114.2	Microparticle
11[a]	500	DCM	50	-	-	-	disintegrate
12[a]	500	EA	50	-	-	-	irregular

* Basic formulation variable is described in experimental section.
* DCM dichloromethane, EA : ethyl acetate, EF : ethyl formate.
[a] Stirring speed was 700 rpm

centration in the water phase is ignored in this surfactant-free microsphere system.

To investigate whether the CEC microspheres could be prepared without the use of additional surfactants or emulsifiers, various formulation variables were studied. This include the protein content in the W_1 phase, the content of the CEC triblock copolymer in the organic solvent, the stirring speed, and the series of solvent as shown in Table I. When DCM was used as the organic solvent, microspheres were not readily formed by changing the contents of the polymer or BSA. Furthermore, the particles disintegrated after freeze drying, as shown in Table I (batch 1-2). When the content both of the polymer and BSA was increased (at batch 3-5), spherical microparticles were obtained in batch 3 and 4 before freeze-drying (data not shown). However, the microspheres disintegrated after free-drying, as shown in Fig. 2(a) and (b). The mechanism for the instability of the spheres with the use of DCM is not clear. However, it appears possible that the weak mechanical force of microspheres, the instability of the organic solvent droplets, and/or a reduced hydrophobic/hydrophilic balance of the microsphere surfaces may be contributed to the rapid disintegration. Additional studies with the use of EF indicated that microspheres were not formed at any conditions (Table I). In contrast, EA showed a potential to form microspheres without surfactants or emulsifiers as shown in batch 8. The resulting microspheres exhibited a nice spherical shape, as shown in Fig. 3(a) and (b). However, the surface of the

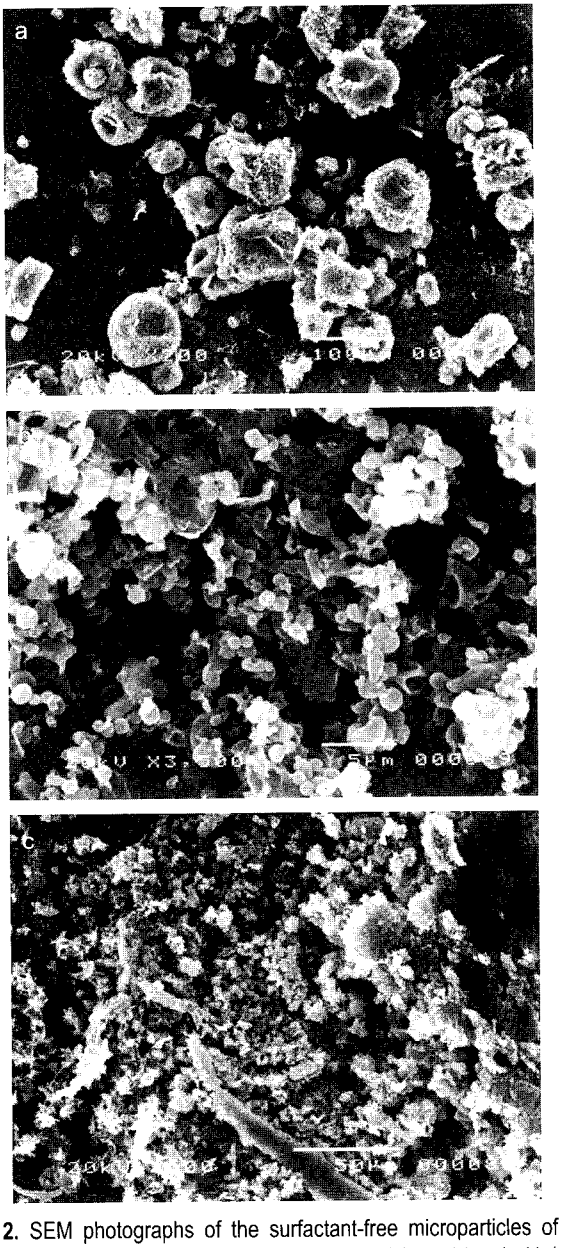

Fig. 2. SEM photographs of the surfactant-free microparticles of the CEC triblock copolymers. Batch 3 (a), batch 4 (b), and batch 11 (c) in Table I.

particles appeared irregular. When the stirring speed was increased from 400 rpm to 700 rpm, the microspheres showed an irregular morphology after lyophilization, as shown in Table I (batch 11 and 12). As shown in Fig. 2(c) and Fig. 3(c), the morphology of microspheres in batch 11 was fully disintegrate (DCM) and formed irregular shapes with rough surfaces in batch 12 (EA).

The protein contents in the microsphere prepared without surfactants were found to be 1-2% (w/w) and the loading efficiency was about 10% (w/w), values lower than the expected. The underlying mechanism(s) for the low loading efficiency was not directly investigated in this study.

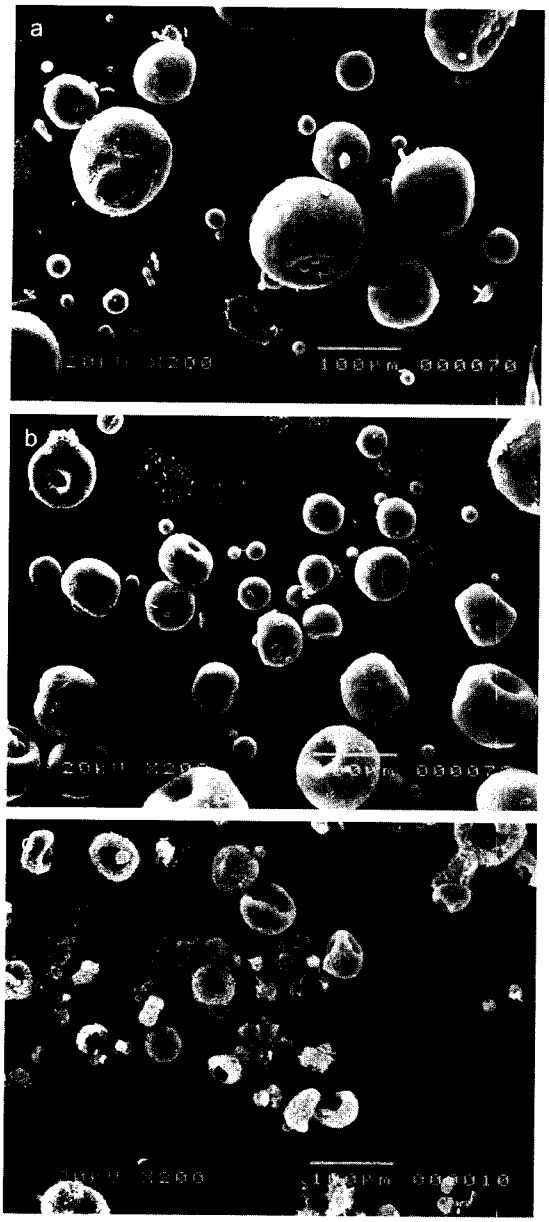

Fig. 3. SEM photographs of the surfactant-free microparticles of the CEC triblock copolymers. Batch 9 (a), batch 10 (b), and batch 12 (c) in Table I.

However, since the CEC triblock copolymer has a hydrophilic PEO chain, formation of a water channel may have contributed to the low efficiency. This aspect of the encapsulation process may warrant further investigation. can be formed more easily than in normal microspheres such as poly-(lactide-co-glycolide) (PLGA).

Fundamental understanding about the relationship between microsphere characteristics and release mechanisms is essential to yield practical formulation since the release kinetics of the protein depends on the polymer properties, microsphere morphology, and drug distribution within the microspheres. Protein release from biodegradable

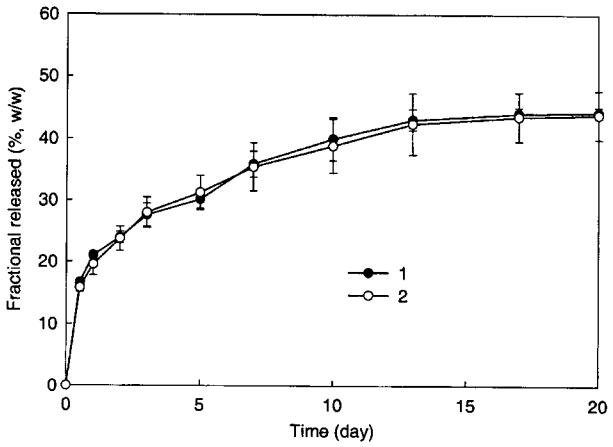

Fig. 4. BSA release from the surfactant-free microparticles of the CEC triblock copolymer. Batch 9 (a), batch 10 (b) in Table I.

microsphere is thought to occur by a combination of pore diffusion through the water-filled channels and release from the device by diffusion as a function of polymer degradation and erosion. The initial protein release from the microspheres is characterized by a rapid burst release caused by the drug being located close to or on the surface of the microspheres. After a lag phase following this pore diffusion phase, drug release recommences when mass loss of the devices becomes prominent (erosion-phase) (Bodmer *et al.*, 1992). Initial pore diffusion is thought to be mainly affected by parameters such as the protein/polymer ratio (loading), the particle size and the wettability of the carrier (Siegel *et al.*, 1991).

The lag and erosion phase are predominantly influenced by polymer degradation (Shah *et al.*, 1992). Factors, such as crystallinity, molecular weight distribution as well as the co-monomer composition influence the drug release in the erosion phase. Fig. 4 shows the release kinetics of the BSA from the surfactant-free microspheres of the CEC triblock copolymer. As shown in Fig. 4, a burst release phase appeared during the first 2 days and a sustained release pattern was shown thereafter. A number of reasons (e.g., rapid release of protein from the surface, water channels by the homopolymers of poly-(ε-caprolactone), polylactide, or PLGA, and/or ready formation of inner pore) may participate for the burst release of the microspheres in this study.

CONCLUSION

Preparation of surfactant-free microspheres of CEC-triblock copolymer appeared feasible. When DCM or EF was used as the solvent, the microspheres was not readily formed probably a rapid disintegration during the preparation. In addition, irregular surface of microsphere may be obtained after the lyophilization process in certain

cases. Surfactant-free microspheres were only formed when EA was used as the organic solvent. These microspheres showed an acceptable spherical shape with some degree of surface irregularity. The protein release kinetics showed a burst release for the first 2 days and a sustained release pattern was observed thereafter.

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