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The preparation of BSA-PLLA microparticles in a batch supercritical anti-solvent process

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ABSTRACT

Poly(L-lactic acid) (PLLA) microparticles loaded with a model protein (bovine serum albumin: BSA) have been prepared using a novel batch supercritical anti-solvent process. BSA-PLLA microparticles were characterized as regards their morphologies, BSA content and *in vitro* release profile. Scanning electron microscopy observations showed that BSA-PLLA microparticles possessed irregular and elliptical morphology with smooth surface. Fourier-transformed infrared (FTIR) assessment and differential scanning calorimetry (DSC) measurement indicated the successful encapsulations of BSA into PLLA during the process. *In vitro* release of BSA-PLLA microparticles showed an initial burst release of about 40% in 30 min and a prolonged release of the protein over a 24 h period at 37 °C. This work shows that our novel process can be used to efficiently encapsulate solid protein particles and get sustained-release solvent-free microparticles, without any structural alteration of the BSA protein.

1. Introduction

Many proteins and peptides possess a biological activity that makes them have potential therapeutics, such as growth factors in bone regeneration (Carano & Filvaroff, 2003; Ghannam, 2005; Hsiong & Mooney, 2006), anticancer agents (Froidevaux & Eberle, 2002), peptide inhibitors of angiogenesis (Figg, Kruger, Price, Kim, & Dahut, 2002; Kerbel & Folkman, 2002) and so on. These proteins and peptides agents not only provide the osteoinductive action of new bone formation (Celeste et al., 1990; Erlebacher, Filvaroff, Ye, & Derynck, 1998; Urist, 1965; Wozney et al., 1988) but also treat tumors and inhibit the growth of tumors (Asselin, 1999). However, the use of proteins and peptides as therapeutic agents is hampered by their rapid elimination, enzymatic degradation, and a danger of developing an immune response. Numerous approaches to overcome rapid elimination and enzymatic degradation of drugs have been developed and can be adapted for the delivery of proteins and peptides (Langer & Folkman, 1976; Santini, Cima, & Langer, 1999; Veronese & Harris, 2002). Among these approaches, microparticulate carriers might represent a valid alternative, including liposomes, micelles, and polymer microparticles. The biodegradable polymeric microparticles essentially allows the impregnated proteins or peptides to release at a desirable rate and concentration, and to linger at targeting sites for a sufficient time to stimulate tissue healing processes and inhibit the growth of tumors. Polymers are attractive drug delivery carriers, as they can protect proteins and peptides from proteolysis, resulting in a prolonged retention of the protein activity *in vivo*.

Microencapsulation into polymer microparticles of these bioactive drugs is usually carried out by a water-in-oil-in-water (W/O/W) emulsion method or spray drying (Blanco, Sastre, Teijón, Olmo, & Teijón, 2006). Early studies with microparticles prepared by this process showed partial loss of biological activities (Oldham et al., 2000; Zhu, Mallery, & Schwendeman, 2000) induced from large amounts of organic solvent, emulsifiers and high temperature.

To address these issues, supercritical fluids technique, a new green technique, is extensively attracted. Supercritical fluids with liquid-like solvent power and gas-like transport properties provide a 'clean' and effective alternative to traditional methods, while circumventing many of the problems associated with traditional techniques (Foster et al., 2003). Supercritical carbon dioxide is the most widely used supercritical fluid, due to the favorable critical parameters (T_c 31.1 °C, P_c 73.8 bar), cost and non-toxicity (Kazarian, 2000).

In our previous studies (Kang et al., 2008a,b), we have successfully preformed supercritical CO_2 fluid technique (SEDS process) to encapsulate small molecular drug into a PLLA microparticle. However, in the microencapuslation of proteins by the supercritical fluid method, it is often difficult to find a solvent that can not only dissolve the proteins without influencing its stability but also has good miscibility and supercritical CO_2, as many proteins can dissolve only in water. Therefore, it is hard to co-precipitate proteins and polymer in one solvent by the previous SEDS process (Kang et al., 2008a,b). To solve this problem, in this study, a modified process based on batch supercritical anti-solvent technique was

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developed. A model protein, bovine serum albumin (BSA), was suspended in the polymeric organic solution to produce drug-loaded microparticles in this bath supercritical anti-solvent process. To our knowledge, there is not any report about this novel batch supercritical CO_2 anti-solvent process encapsulating BSA drug into PLLA microparticles for controlled release. The morphology, protein content and encapsulation properties were investigated. *In vitro* drug release property was also studied.

2. Materials and methods

2.1. Materials

PLLA (M_w 250 kDa), was purchased from the Medical Polymer Institute of Shandong (Jinan, China). Bovine serum albumin (BSA) was gifted by West China Center of Medical Sciences. CO_2 with a purity of 99.9% was supplied by Chengdu Tuozhan Gas Co. Ltd. (Chengdu, China). Dichloromethane (DCM) was of analytical purity.

2.2. Encapsulation of BSA in a batch supercritical CO_2 process

In this study, the description and schematics of operation procedure is different from that published in our previous studies (Kang et al., 2008a,b). Firstly, 100 mg PLLA was dissolved in 20 mL DCM in a glass tube and then 100 mg BSA particles was suspended into the solution. After the glass tube was placed in the high-pressure vessel, CO_2 was fed by a high-pressure pump through one inlet (valve 1) into the particle formation vessel (Fig. 1). The high-pressure vessel was simultaneously incubated in a gas bath to keep the temperature constant and the pressure was monitored by a manometer. When the desired pressure of the high-pressure vessel and temperature were both stabilized (P: 120 bar and T: 33 °C), valve 1 was closed and simultaneously valve 2 was immediately opened (Fig. 1). Thus then CO_2 was directly delivered into BSA-PLLA suspension through the nozzle. At the same time, the flow rate of CO_2 was rapidly increased three times by changing the frequency of piston of high-pressure pump to increase the mass transfer rate between DCM solution and supercritical CO_2. In this study, the nozzle was extended and plugged into the suspension, and the flow rate of supercritical CO_2 significantly increased comparable to previous rate, which made the mass transfer rate faster and the organic solvent quickly extracted into the supercritical CO_2, resulting in the immediate formation of solid microparticles in the glass tube. After 15 min, fresh CO_2 was still continued to flow for 10 min to remove the residual organic solvent in the products. When the washing process was completed, the CO_2 flow was stopped and the high-pressure vessel was slowly depressurized to atmospheric pressure. The products were collected from the glass tube.

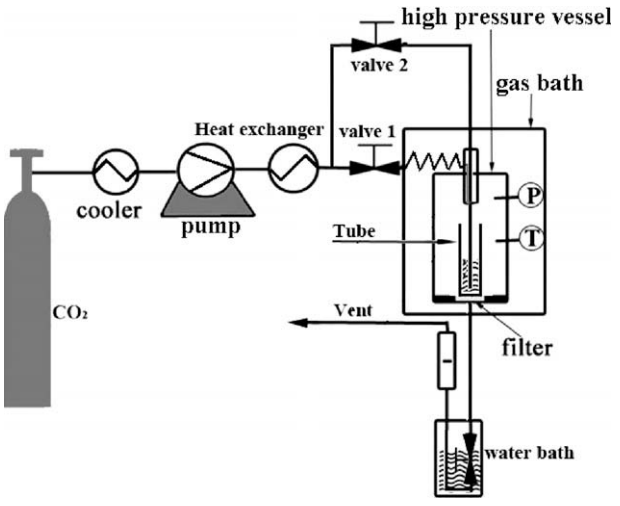

Fig. 1. The schematic graph of the bath supercritical anti-solvent process.

2.3. Characterization of microparticles

The surface morphologies of BSA-PLLA microparticles were investigated by scanning electron microscope (SEM) (JSM-5900LV, Japan). Powder X-ray diffraction (XRD) was carried using a Philips X'Pert MDP diffractometer. The measurement was performed in the range of 5–45° with a step size of 0.02° in 2θ using Cu Kα radiation as the source.

Fourier-transformed infrared (FTIR) was performed using a NEXUS spectrometer 670 (Thermo Nicolet, USA). Approximate 1 mg of microparticles was ground with KBr and pressed into a thin tablet. Four sets of spectra were collected: the native BSA, blank PLLA microparticles, BSA-loaded microparticles, and BSA + PLLA in a physical mixture.

Differential scanning calorimetry (DSC, STA449C, Netzsch, Germany) was used to measure the effect of the supercritical CO_2 process on the thermal properties of drug-loaded microparticles. Approximately 10 mg samples were heated in a sealed aluminum pan under nitrogen atmosphere (investigated temperature range: 20–300 °C, heating rate: 5 °C/min).

2.4. The determination of BSA content

The BSA content in the BSA-PLLA microparticles was determined by the concentration of BSA in PBS after *in vitro* completed release for 28 days (Dos Santos, Richard, Pech, Thies, & Benoit, 2002). BSA-PLLA microparticles were immersed in PBS, which led to a complete release of BSA. The amount of BSA in PBS was measured by BCA (Bicinchoninine acid) assay method (Smith et al., 1985). Each experiment and measurement was carried out in triplicate.

2.5. Drug release studies

20 mg sample of BSA-PLLA microparticles was put into the sterilized tea bag, and the tea bag was hung in a centrifuged tube with 10 ml phosphate buffered solution (PBS, pH 6.8), and then incubated in a water-bath shaker at 37 °C at 60 rpm. In designed time interval, the released solution was periodically removed and BCA method was employed to measure the concentration of released BSA. Then, 10 ml fresh PBS was periodically added to continue release. Release profiles were calculated in terms of cumulative release percentage of BSA (wt%) with incubation time. Each experiment was carried out in triplicate.

3. Results and discussion

3.1. Surface morphology

Fig. 2a–c shows the SEM images of original BSA, PLLA and BSA-PLLA microparticles, respectively. The PLLA microparticles exhibit irregular shape and smooth surface. PLLA microparticles with obvious aggregation are obtained. Morphology of the microparticles is worse than that of microparticles produced in the SEDS process (Kang et al., 2008a,b). Similarly, irregular micron-metric aggregated particles of BSA-PLLA are also obtained. The particle size of drug-loaded microparticles from SEM is almost less than 2.5 μm. The morphology and particle size are dominated by the possible

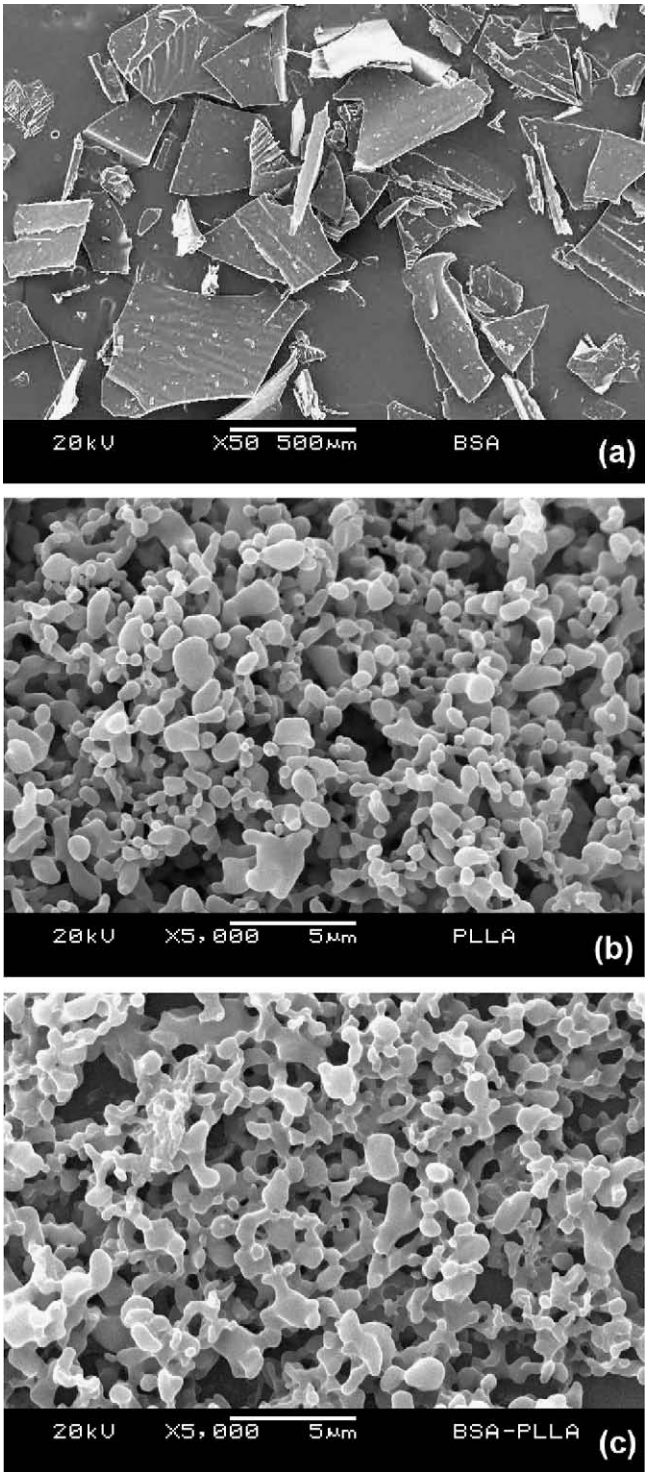

Fig. 2. The SEM morphologies of original BSA particles (a), PLLA microparticles (b), and BSA-PLLA microparticles (c).

mechanisms that evaporation of the solvent into the anti-solvent phase and diffusion of the anti-solvent into the organic solvent (Mukhopadhyay & Dalvi, 2004). In this batch supercritical anti-solvent process, to increase the evaporation and diffusion rate, high rate of supercritical CO_2 was performed to improve the mass transfer between supercritical phase and organic solvent phase. Because large amounts of solution filled in the tube were placed into the high-pressure vessel at the beginning of the experiments, it was difficult for supercritical CO_2 to expand the organic solvent for crystallizing solutes at the previous CO_2 rate. In the previous SEDS process, a droplet of organic solution was delivered into the high-pressure vessel filled large amounts of supercritical CO_2 fluids, which made the mass transfer between two phases fast. Therefore, in this work, a small rate of supercritical CO_2 fluids was conducted to obtain the required temperature and pressure at the beginning, and then a higher rate of supercritical CO_2 fluids (increasing three times) was delivered to organic solution for fast diffusion of two phases and for particles nucleation. Furthermore, the BSA particles might act as host particles, which lead to easy encapsulation of BSA by the precipitation of PLLA and thus form drug-loaded microparticles. In these conditions, drug-loaded microparticles with small size can be successfully obtained.

3.2. Properties of BSA-PLLA microparticles

To investigate the crystalline changes of microparticles after supercritical anti-solvent process, XRD analysis was performed. Fig. 3 shows the diffraction patterns of semi-crystalline PLLA, BSA, BSA + PLLA microparticles in physical mixture, and BSA-PLLA microparticles, respectively. The main peak at about 16.48° provides the presence of semi-crystalline domains of PLLA specimens, and there are broad characterization peaks of BSA. After the supercritical anti-solvent process, the crystallization of PLLA in the BSA-PLLA microparticles is slightly suppressed, but it is insignificant, compared to the BSA + PLLA in physical mixture (the ratio of two compositions was according to the BSA content). Furthermore, broad characterization peaks of BSA in the BSA-PLLA microparticles can be also observed at the corresponding positions of BSA. These results implied that this supercritical process did not strongly affect the crystalline state of PLLA and BSA in the BSA-PLLA microparticles produced in the supercritical process.

Amide I band (1690–1620 cm^{-1}) of FTIR spectra, which reflects an almost pure vibrational character, has been used extensively in the characterization of chemical composition and conformational studies of proteins, since it consists mainly of the carbonyl stretching vibration mode of the peptide bond. Therefore, it has a well-recognized potential in protein secondary structure studies (Ferrer, Bosch, Yantorno, & Baran, 2008; Surewicz, Mantsh, & Chapman, 1992). To test whether the encapsulation of BSA in the supercritical fluids process produced possible conformational changes, amide bands of FTIR spectra of this protein and their drug-loaded microparticles were analyzed. We used amide I region data

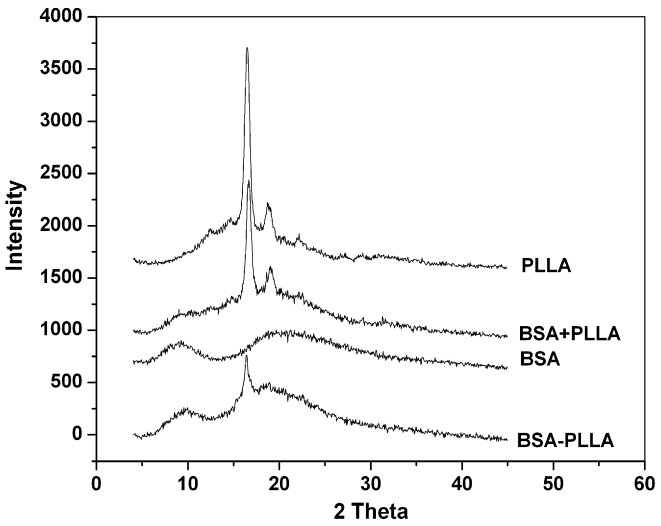

Fig. 3. The XRD patten of original BSA, PLLA, BSA + PLLA in physical mixtures and BSA-PLLA microparticles in the supercritical process.

(1600–1700 cm^{-1}) for this study because the polymer does not have a strong signal in this region and thus does not interfere with the FTIR band of BSA. The C=O stretching vibration (1758.18 cm^{-1}) of the polymer is well separated from the amide I vibration (1653.43 cm^{-1}) of the BSA. As shown in Fig. 4, a band at around 1653 cm^{-1}, which was assigned to a-helix conformation (1658–1650 cm^{-1}) (Rawel, Rohn, Kruse, & Kroll, 2002), can both be observed in free BSA (1653.43 cm^{-1}), BSA-PLLA microparticles (1652.06 cm^{-1}), and physical mixture BSA + PLLA microparticles (1652.43 cm^{-1}). The peak positions of amide band I have not significantly changed in BSA-PLLA microparticles prepared by this process, compared to that of physical mixture BSA + PLLA microparticles. An amide II band around 1539 cm^{-1} is ascribed the C—N stretch coupled with N—H bending mode, which can also be observed at the corresponding position band in the BSA-PLLA microparticles (1639.33 cm^{-1}) and physical mixture BSA + PLLA microparticles (1639.34 cm^{-1}). This qualitative analysis indicated that the interaction of BSA and PLLA in the supercritical fluid process did not cause strong perturbation in the BSA secondary structure that is involved in the relevant biological activities.

Fig. 5 shows the DSC curves of PLLA, original BSA, and BSA-PLLA microparticles. Original PLLA is observed as a semi-crystalline polymer with a melting temperature (T_m) of about 179.5 °C. The DSC curve of BSA shows clearly a denaturation temperature at 76.4 °C and a T_m at 224.2 °C. The curve of BSA-PLLA microparticles shows three endothermic peaks at 60.5, 179.2, and 223.4 °C, representing the denaturation temperature of BSA, T_m of PLLA and BSA, respectively.

Generally, BSA goes through a reversible structural transformed stage when heat-treated (Kuznetsow, Ebert, Lassmann, & Shapiro, 1975; Lin & Koenig, 1976; Oakes, 1976). In the reversible structural stage, some of the alpha-helices are transformed to random coils and beta-sheets were formed when BSA protein was heated above 65 °C (Wetzel et al., 1980), or 70 °C (Clark, Saunderson, & Suggett, 1981; Lin & Koenig, 1976). The temperature of the reversible conformation change was defined as the denaturation temperature of BSA, which was found to be 62 °C by DSC (Ruegg, Moor, & Blanc, 1977). Wetzel et al. (1980) found low concentration of BSA would decrease the denaturation temperature. In this study, the denaturation temperature of BSA was shifted from 76.4 to 60.5 °C, implying that the relatively low content of BSA in the BSA-PLLA microparticles, compared with the native BSA, might decrease the denaturation temperature that is related to the alpha-helices transformation and beta-sheet formation.

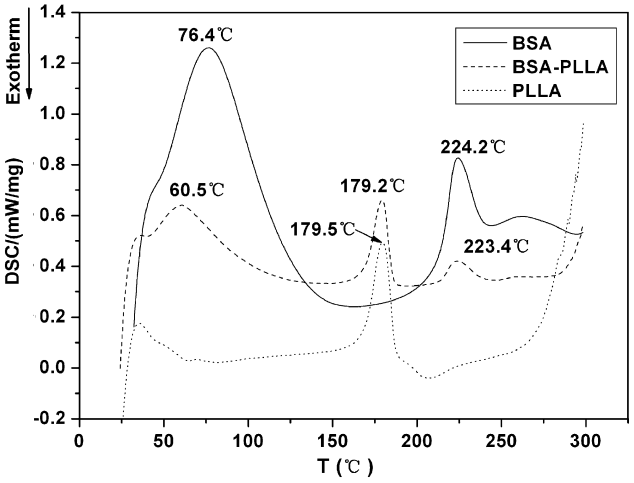

Fig. 5. DSC curves of original BSA, PLLA, and BSA-PLLA microparticles in the supercritical process.

The melting temperatures of BSA and PLLA in the BSA-PLLA microparticles are not significantly shifted to lower temperature, indicating that supercritical process did not affect the crystallization of BSA and PLLA. These results together with XRD show that the short preparation time of the supercritical process did not significantly affect the crystalline changes of BSA and PLLA.

3.3. BSA content and in vitro release

In this study, 17.11% of BSA content in the BSA-PLLA microparticles was obtained. The complete release method employed in this work made it possible to keep the structural integrity of BSA from the BSA-PLLA microparticles for further BSA concentration assays, though the BSA encapsulated within PLLA possibly indicated an incomplete release. Therefore, this obtained BSA content should be lower that that of actual BSA content in the BSA-PLLA microparticles. In fact, the other method such as the extraction method (Lee,

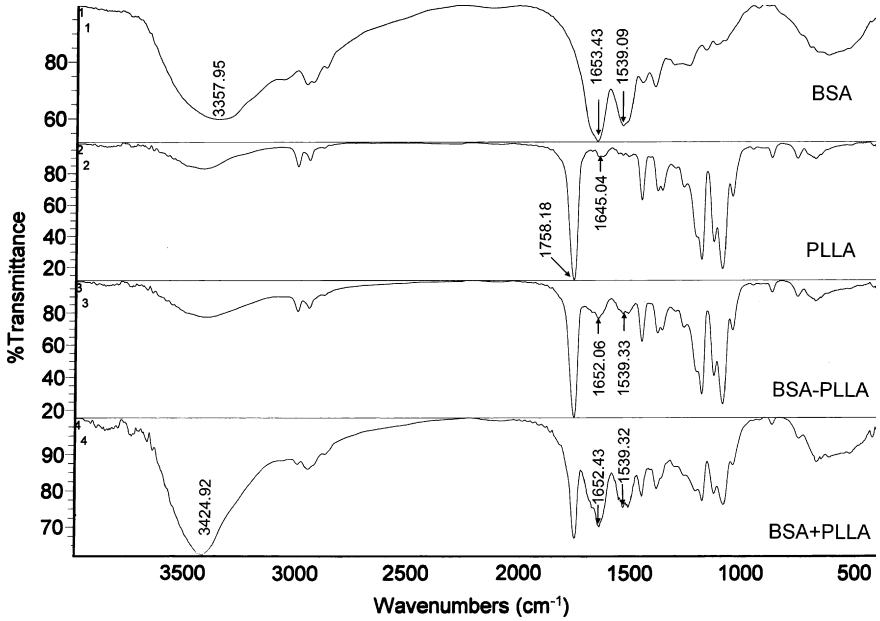

Fig. 4. FTIR spectrum of original BSA, PLLA, BSA + PLLA in physical mixtures and BSA-PLLA microparticles in the supercritical process.

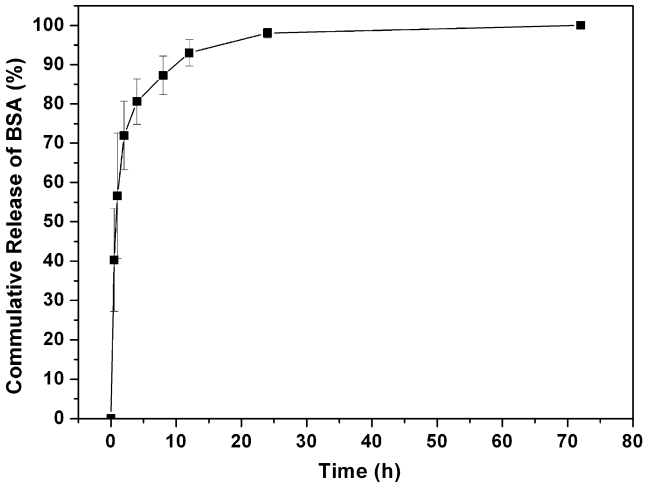

Fig. 6. *In vitro* release profile of BSA from BSA-PLLA microparticles.

Zhang, & Feng, 2007) can not also overcome this problem due to the extraction recovery efficiency. Furthermore, the extraction method would possibly destrupt the structural integrity of protein.

In this study, the BSA particles were suspended in the DCM solution of PLLA, thus encapsulating BSA into PLLA in the process. For this process, an advantage was that proteins needed not to be dissolved in water and thus this avoided the interface of water-oil that may affect the structure of protein. Additionally, supercritical technique can produce drug-loaded microparticles with a very low residual DCM (Kang et al., 2008a, b). This is another advantage of the supercritical fluid technique over conventional spray drying and double emulsion technique. In this process, the preparation of BSA-PLLA microparticles can be achieved in very short time. The rapid removal of organic solvent from microparticles without high temperature or extensive downstream purification further satisfies the requirement of pharmaceutics of protein and peptide drug. Fig. 6 shows the *in vitro* release kinetics of BSA-PLLA microparticles. Release kinetic profile of samples shows an initial burst release of about 40% in 30 min and a prolonged release of the protein over a period of more than 24 h. For comparison, the dissolution rate of BSA in the physical mixture BSA + PLLA microparticles was investigated. Results show the BSA rapidly dissolved in the released medium (PBS) in the first 30 min (data not shown), while BSA in the BSA-PLLA microparticles released in a sustainable manner.

4. Conclusion

In this work, a new batch supercritical fluid technique was successfully performed to encapsulate model protein BSA into PLLA. The microparitcles loaded with BSA have smooth surface with small particle size. FTIR investigation and DSC measurement confirmed the encapsulation of BSA in the process without any structural alteration of the protein. Supercritical fluid technique is an apparently clean and effective technique of encapsulating peptide or protein drugs into polymer microparticles due to mild condition. These results manifested the promising potentials of producing protein or peptide pharmaceutical microparticles for sustained-release using this new supercritical fluid process.

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