Study of Lysozyme-Polymer Composite Microparticles in Supercritical CO_2

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ABSTRACT: Lysozyme-loaded polymeric composite microparticles were successfully coprecipitated by solution-enhanced dispersion by supercritical CO_2 (SEDS), starting with a homogeneous organic solvent solution of lysozyme/poly(L-lactide)/poly(ethylene glycol) (lysozyme/PLLA/PEG). The effects of different drug loads (5, 8, and 12% w/w), PLLA Mw (10, 50, 100, and 200 kDa), PEG contents (0, 10, 30, and 50% PEG/(PLLA+PEG) w/w), and PEG Mw (400, 1000, and 4000 kDa) on the surface morphology, particle size, and drug release profile of the resulting composite microparticles were investigated. The results indicate that the size of the microparticles decreased and the rate of drug release increased with an increase in drug load, PEG content, or PEG Mw; the particle size first increased and then decreased with an increase in PLLA Mw, and the drug release was controlled by both particle size and PLLA Mw. The Fourier transform infrared spectrometer analysis and circular dichroism spectra measurement reveal that no significant changes occurred in the molecular structures during the SEDS processing, which is favorable to the production of protein–polymer composite microparticles for a protein drug delivery system. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 3175–3183, 2012

Key words: lysozyme; PEG; PLLA; microparticles; Supercritical CO_2

INTRODUCTION

Proteins and other biomacromolecular drugs have been widely used in the treatment of numerous diseases due to their incredible selectivity and their ability to provide effective and potent action. However, several challenges are still encountered, including a relatively short half-life within the body, and low oral and transdermal bioavailabilities. They are thus currently administered by frequent injection or infusion, which is less user friendly than the options available for small-molecule drugs and could eventually limit the widespread use of protein therapeutics by physicians and patients.[1–3]

Hence, a new drug delivery system, which can protect protein drugs from enzymatic degradation and provide a sustained-release effect, is a promising approach for prolonged retention and biological activity of the drugs within the body. An appropriate delivery carrier, which can meet the above requirements and has been accepted for the delivery of protein drugs, is polymeric microparticles.[4–10]

However, in the process of the conventional methods[11–15] for producing such polymeric microparticles, the strong shearing force, temperature, pH, and the interface between the oil and water phases may affect and/or alter the structure of the bioactive agents. Moreover, an additional drying step is required to reduce the residual solvents, since removal of residual solvents to very low concentrations is important to ensure a safe and stable microparticle product.

A supercritical CO_2 technique, in particular the supercritical antisolvent (SAS) process, has become a good choice for the development of polymeric microparticles due to its great advantages, such as a mild operating temperature, very low/no organic solvent residue, efficient separation, and a benign effect on the environment, which avoid most of the drawbacks of the conventional methods.[16–20]

In the SAS process, the organic solution is sprayed into supercritical CO_2 to cause a rapid contact between the two media. This generates a higher supersaturation ratio in the solution, resulting in fast nucleation and growth, and consequently creates

Additional Supporting Information may be found in the online version of this article.

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microparticles.[21] When an active substance and a polymer dissolved in an organic solvent are sprayed together or separately in an antisolvent, the antisolvent expands the solvent, which leads to the formation of active substance-loaded polymer microparticles. The solution-enhanced dispersion by supercritical CO_2 (SEDS) process is a modified version of the SAS process, in which the liquid solution and supercritical fluid are sprayed together using a specially designed coaxial nozzle. The supercritical CO_2 is used as both an antisolvent because of its chemical properties and a "spray enhancer" because of its mechanical effect. The spontaneous contact of high-speed streams of a liquid solution and a supercritical fluid generates a finely dispersed mixture and a prompt particle precipitation.[22–24]

Both polymers such as poly(L-lactide) (PLLA)[24] and proteins such as lysozyme[25–29] have been widely studied separately in supercritical CO_2. In this study, we attempted to prepare lysozyme-loaded PLLA microparticles using the SEDS process, and poly(ethylene glycol) (PEG) was also used in the process to increase the shelf-stability and biocompatibility of the protein-loaded microparticles.

MATERIALS AND METHODS

Materials

PLLA with a molecular weight of 10, 50, 100, and 200 kDa was purchased from the Department of Medical Polymers, Shandong Institute (Jinan, China). *Lysozyme and PEG* were purchased from the *Chengdu* Kelong Chemical Reagent Factory (Chengdu, China). The bicinchoninic acid (BCA) protein assay kit was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). CO_2 with a purity of 99.9% was supplied by the Chengdu Tuozhan Gas (Chengdu, China). All other compounds were of analytical purity.

Methods

Preparation of lysozyme–polymer composite microparticles

Lysozyme was dissolved in dimethyl sulfoxide (DMSO) and then mixed with the PLLA solution in dichloromethane (DCM) (DMSO : DCM = 1 : 3) to obtain a homogenous solution.

The apparatus[20] for the SEDS process consists of three major components: a CO_2 supply system, an organic solution delivery system, and a high pressure vessel with a volume of 500 mL.

In running an experiment, the CO_2 fed from a CO_2 cylinder was cooled down to around 0°C by using a cooler to ensure the liquefaction of the gas and also to prevent cavitations. Then a high pressure meter pump was used to deliver liquefied CO_2 to the high pressure vessel. After leaving the pump head, the liquefied CO_2 was preheated to the desired operating temperature by using a heat exchanger. The high-pressure vessel was incubated in a gas bath to keep the temperature constant during the experiment. When the desired pressure of the high-pressure vessel was reached, a steady flow of CO_2 was maintained, and the system pressure was controlled by adjusting a downstream valve and monitored by a pressure gauge to keep the pressure constant. When the desired pressure and temperature were stabilized, the solution was delivered into the high-pressure vessel through a stainless steel coaxial nozzle by using an high performance liquid chromatography (HPLC) pump at a flow rate of 1 $mL \cdot min^{-1}$. During the process, the pressure, temperature, and flow rate of CO_2 were kept at 12 MPa, 33°C, and 14 $NL \cdot h^{-1}$), respectively. When the spraying was finished, fresh CO_2 was used continuously for about 30 min to wash the products to remove the residual organic solvent. During the process of washing, the system operating conditions were maintained as described before. After washing, the high-pressure vessel was slowly depressurized, and the products were collected for characterization.

The lysozyme–polymer composite microparticles with different drug loads, different PLLA Mw, different PEG contents, and different PEG Mw were prepared using the above process.

Surface morphology and particle-size distribution characterizations

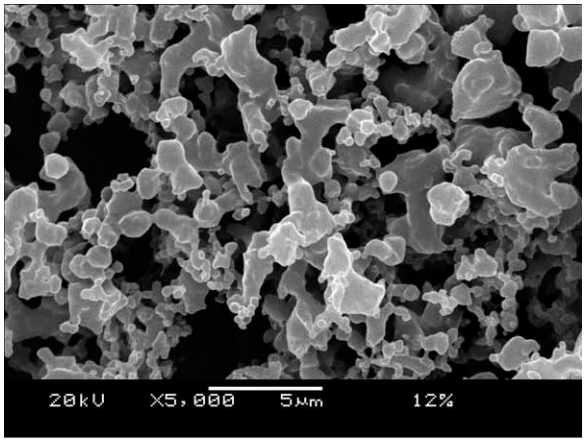
The surface morphological examination of the lysozyme–polymer composite microparticles was performed using a scanning electron microscope (JSM-5900LV, Japan). The particle size and particle-size distribution of the samples were determined using a laser diffraction particle size analyzer (Rise-2008, Jinan Runzhi technology, Shandong).

Study of drug release profiles

Approximately 20 mg of lysozyme–polymer composite microparticles, accurately weighed, was replaced in the teabag, put into a bottle with 20 mL of phosphate-buffered saline (PBS, pH 6.8), and incubated in a shaking water bath at 37°C at 60 rpm. Five milliliters of solution was periodically removed, and the amount of lysozyme was analyzed using the BCA protein assay kit.[30] To maintain the original PBS volume of 20 mL, 5 mL of fresh PBS was periodically added. Release profiles were calculated in terms of the cumulative release percentage of lysozyme (%, w/w) with incubation time. (All the Figures of drug release curves are shown in the "Supporting Information".)

FTIR analysis

Fourier transform infrared spectrometer (FTIR) spectra for the samples were obtained on an FTIR



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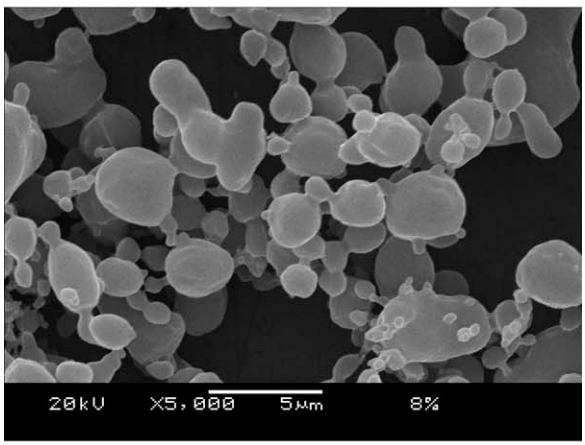

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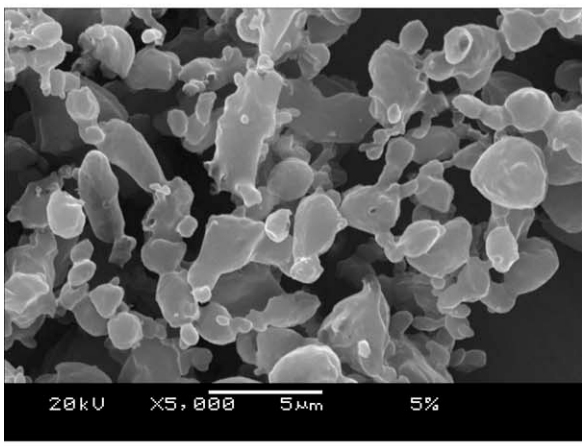

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Figure 1 SEM photographs of lysozyme–PLLA composite microparticles with different drug loads: (a) 12%, (b) 8%, and (c) 5%.

Perkin-Elmer 1720 (Perkin-Elmer) in the transmission mode with the wavenumber ranged from 4000 to 400 cm^{-1}. KBr pellets were prepared by gently mixing the sample powder with KBr.

CD spectra measurement

Circular dichroism (CD) spectra were recorded on a Jasco-500 Spectropolarimeter (JASCO, Tokyo, Japan) at 25°C under a constant flow of nitrogen gas. Typically, a cell with a 0.02-cm path length was used for spectra recorded between 190 and 250 nm.

Activity assay of lysozyme before and after supercritical processing

The activity assay of lysozyme before and after supercritical processing was performed by using *Micrococcus lysodeikticus* (Sigma, St. Louis) as a substrate for the lysozyme. Solutions of lysozyme of the same concentration before and after supercritical processing were added to the *Micrococcus lysodeikticus* suspension. The change in turbidity of the mixture was immediately measured every 15 s for 5 min at 450 nm. The rate of decrease in the turbidity in the first 90 s was calculated as the activity of lysozyme.

RESULTS AND DISCUSSION

Effect of drug load

In the SEDS process, the lysozyme and PLLA (0.5%, wt/v) composite solution was sprayed into the supercritical CO_2; the solvents DCM and DMSO were extracted by the supercritical CO_2, and this drop produced a spontaneous supersaturation of lysozyme and PLLA which generated the coprecipitation of lysozyme and PLLA and consequently the formation of the composite microparticles.

Figure 1(a–c) shows the surface morphologies of lysozyme–PLLA (100 kDa) composite microparticles with different drug loads: (a) 12%, (b) 8%, and (c) 5%, respectively; their corresponding particle-size distributions are shown in Table I. Compared with the PLLA microparticles,[24] the lysozyme–PLLA composite microparticles show a heavier aggregation and a poorer sphericity; this is probably due to the addition of DMSO, which would result in a slower mutual diffusion into and out of the drop with supercritical CO_2, as the solubility of DMSO in

TABLE I
Particle-Size Distribution and Mean Size of Lysozyme–PLLA Composite Microparticles with Different Drug Loads

Drug load (%)	D10	D50	D90	D3	D97	D_{av} (μm)
12	1.233	1.946	3.223	0.989	4.182	2.120
8	1.568	2.548	5.414	1.256	8.568	3.148
5	1.601	2.600	5.520	1.286	8.583	3.203

TABLE II
Drug Release Data of Lysozyme–PLLA Composite Microparticles with Different Drug Loads

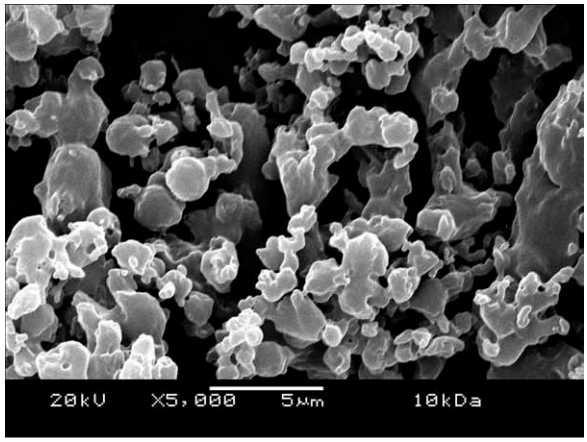
Drug loads	12%	8%	5%
Release of lysozyme after 0.5 h (%)	22.9	15.2	5.2
Release of lysozyme after 240 h (%)	93.3	87.2	82.2

supercritical CO_2 is lower than that of DCM. The results also indicate that the size of the lysozyme–PLLA composite microparticles decreased with the increase in drug load. An explanation for this is that the higher content of lysozyme generated a higher supersaturation when the antisolvent process occurred, and this resulted in a faster nucleation, which also led to an easier coprecipitation of lysozyme and PLLA, consequently resulting in a smaller size. Moreover, the increase in the concentration of lysozyme would more significantly increase the saturation as the DCM would act as a nonsolvent for lysozyme, which would reduce the particle size significantly in the SEDS process.[31]

In general, when the other parameters are kept constant, microparticles with a higher drug load release the drug more rapidly, as do the microparticles of a smaller size, due to the shorter diffusion path into the medium. The results also validate the above phenomena, namely that the lysozyme–PLLA composite microparticles with a higher drug load and smaller size released the drug more rapidly. However, no burst release effect was found in the first 0.5 h (<30%), and all the microparticles could release the drug completely in 240 h (>75%); the key data of the drug release profiles of lysozyme–PLLA composite microparticles with different drug loads are given in Table II.

Effect of PLLA Mw

Figures 2(a,b), 1(b), and 2(c) show the surface morphologies of lysozyme–PLLA (drug load 8%, PLLA 0.5% wt/v) composite microparticles prepared with different PLLA Mw: (a) 10 kDa, (b) 50 kDa, (c) 100 kDa, and (d) 200 kDa, respectively; their corresponding particle-size distributions are shown in Table III. It was found that the particle size of the resulting composite microparticles first increased and then decreased with the increase in PLLA Mw. The PLLA Mw actually has two opposite effects on the particle size and particle-size distribution. At the same



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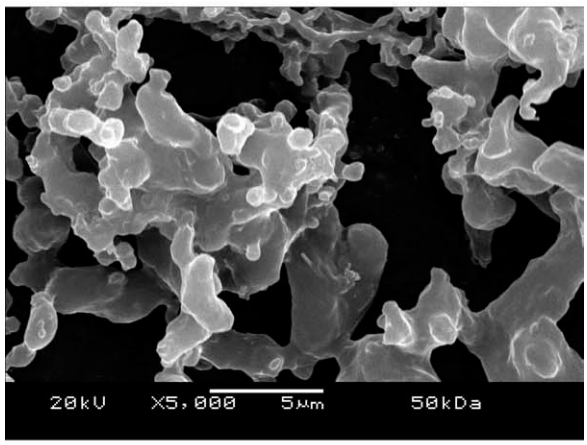

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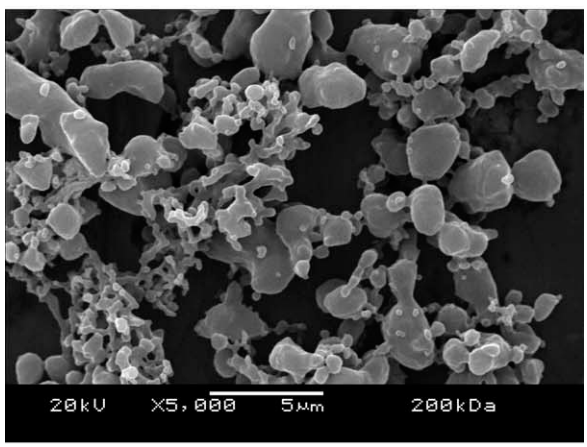

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Figure 2 SEM photographs of lysozyme–PLLA composite microparticles with different PLLA Mw: (a) 10 KDa, (b) 50 KDa, and (c) 200 KDa.

TABLE III
Particle-Size Distribution and Mean Size of Lysozyme–PLLA Composite Microparticles with Different PLLA Mw

PLLA Mw	D10	D50	D90	D3	D97	Dav (μm)
10 KDa	1.692	2.862	6.441	1.355	9.763	3.585
50 KDa	1.779	3.356	8.201	1.338	11.901	4.287
100 KDa	1.568	2.548	5.414	1.256	8.568	3.148
200 KDa	1.273	2.011	3.330	1.015	4.314	2.190

TABLE IV
Drug Release Data of Lysozyme–PLLA Composite Microparticles with Different PLLA Mw

PLLA Mw	10 KDa	50 KDa	100 KDa	200 KDa
Release of lysozyme after 0.5 h (%)	25.3	8.0	15.2	14.2
Release of lysozyme after 240 h (%)	91.0	82.2	87.2	87.0

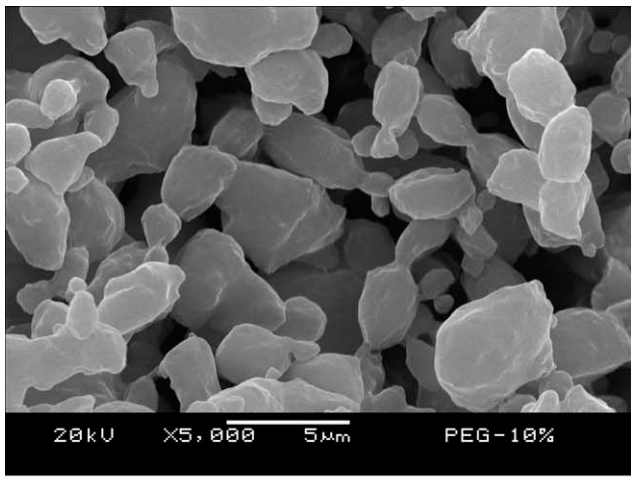
concentration, the higher the Mw of PLLA, the higher the viscosity of the PLLA solution. The PLLA solution with the higher viscosity reaches supersaturation more readily, and it generates a faster nucleation of PLLA in supercritical CO_2; however, the higher viscosity also has a stabilizing effect on the jet and yields bigger droplets, which will decrease the mass transfer performance and tend to produce a bigger particle size due to the longer time for crystal growth.[23,30]

The drug release data in the first 0.5 h and 240 h are shown in Table IV. It was found that the PLLA Mw and the particle size play important roles in the drug release rate. When the other parameters are kept constant, the composite microparticles with a higher PLLA Mw have a more solid and stronger network structure, which will delay the drug release from the matrix, and the composite microparticles with a bigger particle size will release the drug more slowly due to the longer diffusion path. The results show that the composite microparticles (10 kDa group) with a bigger particle size and the smallest PLLA Mw released the drug most quickly, which indicates that the polymeric network structure is the prevailing factor during its drug release. The composite microparticles (50 kDa group) with the biggest particle size and a smaller PLLA Mw released the drug most slowly, which indicates that the length of the diffusion path is the prevailing factor during its drug release. Because of the balance of the polymeric network structure and the length of the diffusion path, the 100 kDa and 200 kDa groups, which possess a more solid and stronger polymeric network structure and a shorter diffusion path, both released the drug in a moderate way with no significant differences.

Effect of PEG content

PEG is a kind of amphiphilic polymer, which is soluble in water, DCM, and DMSO. When PEG is added to the formulation of the lysozyme–PLLA solution, the aqueous lysozyme will be partitioned in the PEG-rich liquid phase due to its highly hydrophilic character; during the coprecipitation, the amphiphilic PEG will work as a bridge to combine the lysozyme and PLLA to form composite microparticles, avoiding the phase separation between the hydrophilic drug and the hydrophobic polymer.

Figures 1(b) and 3(a–c) show the surface morphologies of lysozyme–PLLA with PEG 1000 (drug load 8%, (PLLA+PEG) 0.5% wt/v) in different contents



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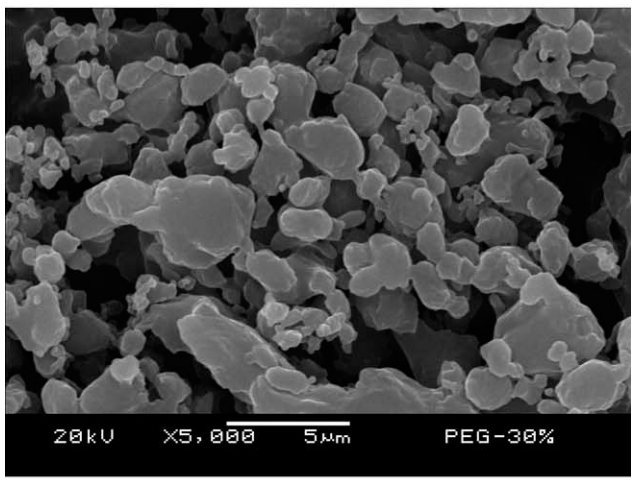

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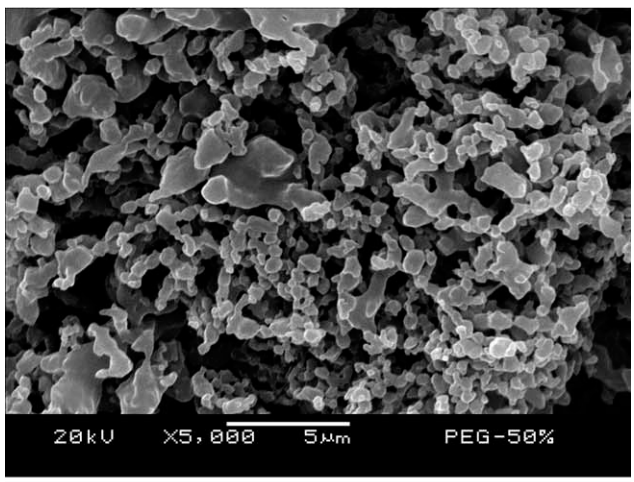

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Figure 3 SEM photographs of lysozyme–PLLA composite microparticles with different PEG contents: (a) 10%, (b) 30%, and (c) 50%.

TABLE V
Particle-Size Distribution and Mean Size of Lysozyme–PLLA Composite Microparticles with Different PEG Contents

PEG content (%)	D10	D50	D90	D3	D97	Dav (μm)
0	1.568	2.548	5.414	1.256	8.568	3.148
10	2.063	3.437	5.619	1.625	7.029	3.682
30	1.582	2.569	5.493	1.271	8.630	3.177
50	1.178	1.883	3.017	0.952	3.952	1.998

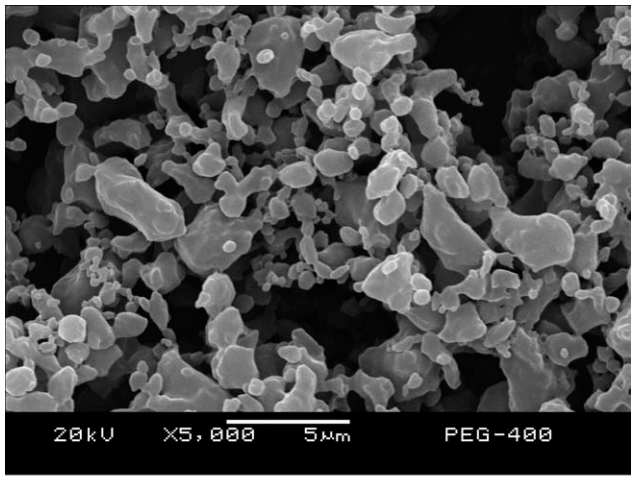
(PEG/(PEG+PLLA) × 100%): (a) 0%, (b) 10%, (c) 30%, and (d) 50%, respectively; their corresponding particle-size distributions are shown in Table V. It was found that the addition of PEG affected the particle size and drug release greatly; when the PEG content reached 10%, the particle size decreased significantly with the increase in PEG content, and the release of lysozyme, which was controlled by a diffusion mechanism, also became faster as the PEG content was increased. Similar phenomena were also found in the literature,[32,33] which explained that this behavior was attributable to the PEG effect on spray atomization, particle nucleation, and growth rate. The presence of PEG can slow down both solute and particle diffusion during the precipitation step, processes which are responsible for the particle growth and agglomeration. While the increase in the protein release rate is mainly attributed to the pore and channel formation in the PLLA microparticles caused by PEG dissolution, the higher PEG content will generate more or larger pores in the PLLA microparticles, resulting in a faster drug release by diffusion. Furthermore, the reduction in particle size also accelerates the drug release due to the shorter diffusion path. When the PEG content reaches 50%, as shown in Table VI, a burst release effect is observed; that is, more than 30% of the drug is released in the first half an hour. This indicates that adjusting the PEG content would be helpful in the effective design of the dosage form with a desired drug release rate.

Effect of PEG Mw

Figures 1(b), 4(a), 3(b), and 4(b) show the surface morphologies of lysozyme–PLLA with PEG (drug load 8%, (PLLA+PEG) 0.5% wt/v, PEG content 30%) of different Mw: (a) without PEG, (b) PEG 400, (c) PEG 1000, and (d) PEG 4000, respectively; their corresponding particle-size distributions are shown in Table VII. When PEG was added, the PLLA microparticle size decreased as the PEG Mw increased, particularly when the PEG Mw reached 4000, when the particle size reduced significantly. As with the increase in PEG content, the drug release also became faster with the increase in PEG Mw. When mixing the hydrophobic PLLA and amphiphilic PEG polymers with a high Mw, the enthalpy effect of the polymer–polymer interactions

TABLE VI
Drug Release Data of Lysozyme–PLLA Composite Microparticles with Different PEG Contents

PEG contents	0%	10%	30%	50%
Release of lysozyme after 0.5 h (%)	15.2	13.2	27.2	37.1
Release of lysozyme after 240 h (%)	87.2	90.0	90.8	92.4



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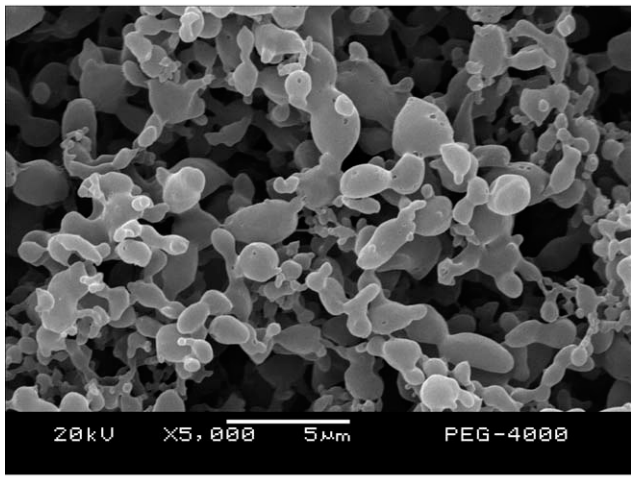

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Figure 4 SEM photographs of lysozyme–PLLA composite microparticles with different PEG Mw: (a) PEG 400 and (b) PEG 4000.

TABLE VII
Particle-Size Distribution and Mean Size of Lysozyme–PLLA Composite Microparticles with Different PEG Mw

PEG Mw	D10	D50	D90	D3	D97	Dav (μm)
Without PEG	1.568	2.548	5.414	1.256	8.568	3.148
PEG 400	1.606	2.617	5.598	1.288	8.771	3.238
PEG 1000	1.582	2.569	5.493	1.271	8.630	3.177
PEG 4000	1.492	2.310	3.810	1.186	4.875	2.517

will dominate the combinatorial entropy of mixing and therefore promote a PLLA-rich and PEG-rich phase segregation. Moreover, the PLLA could precipitate more readily due to the faster extraction of DCM relative to DMSO in the SEDS process; the resulting delay in the precipitation of the PEG and drug could lead to precipitation on the surface of PLLA microparticles or as separated microparticles, consequently reducing the particle size and accelerating the drug release. From Table VIII, it can be seen that a burst release effect was also found when the PEG Mw reached 4000. For low Mw PEGs, despite the influence of the hydroxyl end-group, the entropy of mixing could become dominant and therefore form a homogeneous PLLA/PEG solution, which tends to generate a coprecipitation. This means that lower Mw PEGs can be more easily entrapped in the PLLA network, which favors a slow and prolonged drug release for the hydrophilic drug loaded in the PLLA network.[32,33]

FTIR analysis

To investigate the changes in the molecular structures of the protein and PLLA before and after the supercritical CO_2 treatment, FTIR measurements of the lysozyme–PLLA composite microparticles and the physical mixture of lysozyme and PLLA with the same ratio were carried out. As shown in Figure 5, the major peaks of 1759.1 cm^{-1} of the PLLA spectrum, 1758.9 cm^{-1} of the lysozyme+PLLA mixture spectrum, and 1759.1 cm^{-1} of the lysozyme–PLLA composite microparticles spectrum are attributed to the stretching vibration from the carbonyl group of PLLA; the major peaks of 1653.0 cm^{-1} of the lysozyme spectrum, 1652.9 cm^{-1} of the lysozyme+PLLA mixture spectrum, and 1653.1 cm^{-1} the lysozyme–PLLA composite microparticles spectrum are the characteristic peaks of amide group in lysozyme, which are attributed to the secondary structures of α-helix and β-sheet. The results show that the lysozyme was successfully loaded in the PLLA microparticles, and almost no changes occurred in the molecular structures of lysozyme and PLLA before and after SEDS processing, which reveals that the SEDS process would be potentially useful in the application of a drug delivery system for protein–polymers.

TABLE VIII
Drug Release Data of Lysozyme–PLLA Composite Microparticles with Different PEG Mw

	Without PEG	PEG 400	PEG 1000	PEG 4000
Release of lysozyme after 0.5 h (%)	15.2	25.2	27.2	36.0
Release of lysozyme after 240 h (%)	87.2	90.0	90.8	92.0

CD spectra measurement

To further determine the effect of the SEDS process on the secondary structure of lysozyme, the CD spectra measurements of the lysozyme before and after the SEDS process were also carried out. From Figure 6, it can be seen that the lysozyme after the SEDS process showed a slight decrease of the signals, which demonstrates a subtle decrease of the α-helical structure and consequently subtle changes in the secondary structure of the lysozyme.[34,35] Combining the results of the FTIR and CD spectra measurements, it was found that although the molecular structure of lysozyme had not changed, a minor change in the secondary structure occurred during the supercritical processing.

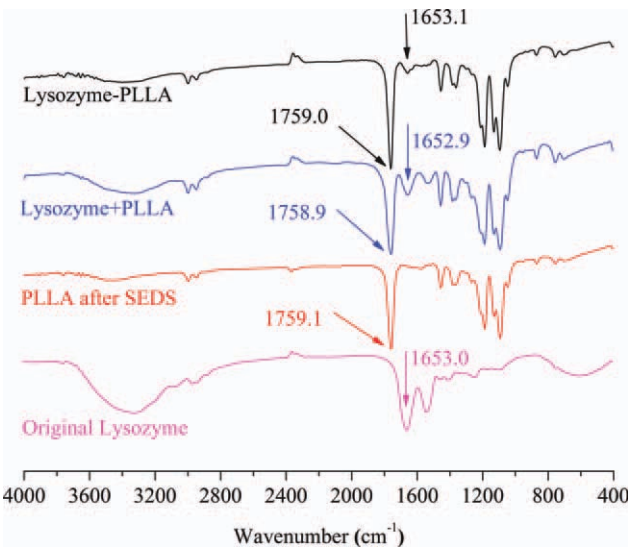

Figure 5 FTIR spectra of lysozyme, PLLA, a lysozyme+PLLA mixture, and lysozyme–PLLA composite microparticles.[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

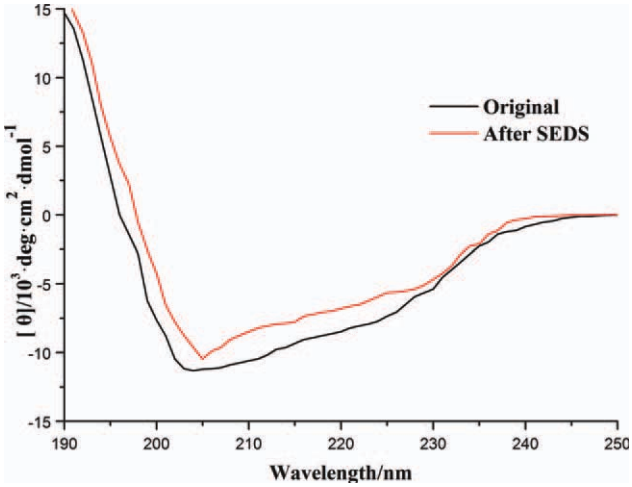

Figure 6 CD spectra of the original lysozyme and lysozyme after the SEDS process. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Activity assay of lysozyme before and after supercritical processing

Figure 7 shows the relationship between absorbance and time of the *Micrococcus Lysodeikticus* suspension added to the original lysozyme and to the lysozyme after SEDS processing, respectively. It can be seen that a faster reaction occurred after the supercritical processing; the specific activity of the lysozyme before and after supercritical processing was 8892.9 and 13446.5 $U \cdot mg^{-1}$, respectively. This is probably due to the minor change in the secondary structure of lysozyme confirmed by the CD spectra measurement, which may lead to a better combination of the active groups of lysozyme and the substrate; the supercritical processing may also have had a purification effect on the lysozyme, which would also increase the specific activity of the lysozyme.

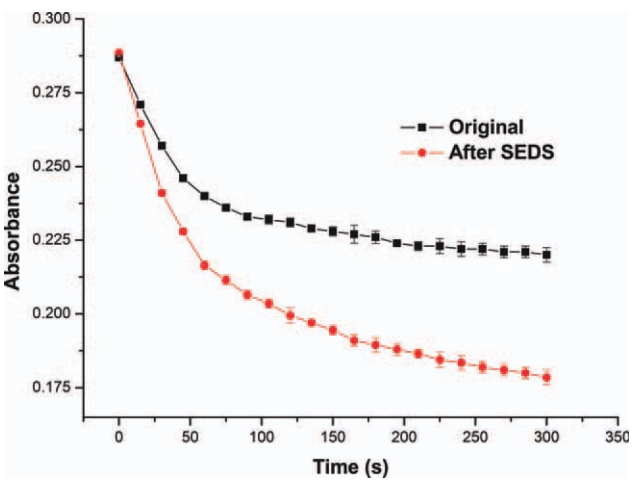

Figure 7 Relationship between absorbance and time of a *Micrococcus Lysodeikticus* suspension added with original lysozyme and to lysozyme after SEDS processing, respectively.[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

The hydrophilic lysozyme-loaded composite microparticles consisting of hydrophobic PLLA and amphiphilic PEG were successfully prepared in the SEDS process, avoiding the changes in the secondary structure of the protein. The drug release from the composite microparticles by a diffusion mechanism was controlled by the length of the diffusion path and the polymeric network structure. Adding PEG to the lysozyme–polymer formulation was found to play an important role in the particle size and polymeric network structure of the composite microparticles, which consequently affected the drug release rate significantly. PEG with a lower Mw can be more efficiently entrapped in the polymeric network, therefore embedding the drug inside the polymeric composite microparticles and creating greater sustained-release efficiency. The increase in either PEG content or PEG Mw reduced the size of the particles and increased the drug release rate; a burst release effect was obtained once the PEG content or PEG Mw increased beyond a certain extent. The results indicate that the SEDS process has potential in the application of protein–polymer drug delivery, and the addition of PEG with a suitable content and Mw would be helpful to obtain the desired drug release rate.

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