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Journal of Controlled Release 91 (2003) 407–416
journal of controlled release
www.elsevier.com/locate/jconrel

W/O/W double emulsion technique using ethyl acetate as organic solvent: effects of its diffusion rate on the characteristics of microparticles

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Received 27 February 2003; accepted 22 May 2003

Abstract

Monomethoxypoly(ethylene glycol)-b-poly(DL-lactide) copolymer (PELA) microparticles loading lysozyme were prepared through a modified W/O/W double emulsion-solvent diffusion method using ethyl acetate (EA) as organic solvent. The modified process was divided into five steps: (1) primary emulsification (W_1/O), (2) re-emulsification ($W_1/O/W_2$), (3) pre-solidification, (4) solidification and (5) purification. The pre-solidification step was carried out in the modified process to control the diffusion rate of EA from oil phase into outer aqueous phase, in order to prevent the wall polymer from precipitation, which usually occurred when the diffusion rate was too fast. The adequately rapid solidification of microparticle caused by controlled fast diffusion of EA and the use of amphiphilic copolymer PELA as wall material, facilitated a high protein entrapment (always above 94%) and full preservation of bioactivity of entrapped lysozyme. It was found that the volume of the outer aqueous phase in the re-emulsification step and the shear stress in the pre-solidification step had a significant effect on the diffusion rate of EA from the droplets into outer aqueous solution, and thereby on the characteristics of the resultant microparticles. With the volume or the shear stress increasing, the removal rate of EA increased, resulting in rapid solidification of the microparticles. This result led to a lower burst effect and a slower lysozyme release from the microparticles. This study suggests that the modified W/O/W double emulsion-solvent diffusion method with EA as organic solvent is a prospective technique to prepare biodegradable microparticles containing water-soluble sensitive agents.

Keywords: Protein delivery; Stability; Solvent diffusion method; Ethyl acetate; Microparticle

1. Introduction

Preparation of biodegradable microparticles containing water-soluble bioactive agents, such as proteins, peptides, viral and bacterial antigens, has received much attention in recent years, due to their numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience [1].

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0168-3659/03/$ – see front matter
doi:10.1016/S0168-3659(03)00273-6

Various techniques are available to entrap water-soluble bioactive agents into biodegradable microparticles. These techniques include double emulsion, organic phase separation, supercritical fluid and spray drying techniques [2,3]. Among them, the W/O/W double emulsion technique is the most popular method, as developed by Ogawa et al. [4]. The typical W/O/W double emulsion process consists of four steps: (1) primary emulsification: an aqueous solution of the active agent (internal water phase, W_1) is emulsified into an organic solution containing the biodegradable polymer (oil phase, O); (2) re-emulsification: the primary emulsion (W_1/O) is further emulsified into a second aqueous phase containing a stabilizer (external water phase, W_2) to form a $W_1/O/W_2$ double emulsion; (3) solidification: the organic solvent is removed by evaporation or extraction and then solid microparticles are formed; and (4) separation and purification: microparticles are collected by centrifugation or filtration and subsequently lyophilized.

A successful microencapsulation should yield a high entrapment efficiency, a desired particle size, and an effectively sustainable release property. The stability of the entrapped substance should be maintained. This is especially important for protein encapsulation. If the protein is denatured during encapsulation, it will be therapeutically inactive, and may cause unpredictable side effects, such as immunogenicity or toxicity [5]. Thus, optimization of preparative process of protein-loaded microparticles should be primarily focused on preservation of the native protein structure during preparation.

Although a W/O/W double emulsion process is a relatively mild method to prepare microparticles containing proteins, there are still many factors that possibly denature proteins, such as mechanical stress and interactions between proteins and organic solvent, etc., during the microentrapment process [6]. The most often used organic solvents in double emulsion technique are methylene chloride (MC) and ethyl acetate (EA). Compared to the more hydrophobic MC, EA usually plays a less deteriorative effect on bioactivity of the entrapped proteins [7–9]. So far, only protein C has been found more sensitive to EA than to MC [10]. However, most researchers in this field still choose MC as the organic solvent because of its desirable physical properties, such as ability to dissolve large amounts of biodegradable polymers, low solubility in water (2.0%, w/v) and low boiling point convenient to be removed by evaporation (39.8 °C). In contrast, the relatively high solubility in water (8.7%, w/v) and the high boiling point (76.7 °C) of EA limited its application in W/O/W double emulsion technique. The relatively high solubility of EA in water usually gives rise to a fast diffusion of EA from oil droplets into the outer aqueous phase during the re-emulsification and solidification, which easily leads to polymer precipitation rather than the formation of microparticles [11]. On the other hand, the higher boiling point indicates a longer removal time to solidify microparticles by evaporation, which means a longer contact time between proteins and ethyl acetate, resulting in more loss in protein bioactivity. Due to these reasons, in recent years there were only a few reports in the literature that used EA as organic solvent in the double emulsion process to prepare microparticles loading proteins. Alonso et al. [12] prepared tetanus toxoid-loaded PLA nanoparticles with EA as organic solvent, and the removal of EA was carried out by evaporation at reduced pressure. Freytag et al. [13] and Tinsley-Bown et al. [14] prepared microparticles with EA as organic solvent, and the solidification of microparticles was carried out by directly adding the W/O/W double emulsion into excess water. Both these groups achieved a higher protein entrapment efficiency when EA was used as organic solvent instead of MC. Indeed, the fast diffusion rate of EA from oil phase into outer aqueous phase benefits rapid solidification of microparticles, which results in improved protein entrapment efficiency. However, the microparticle yields were not presented in these papers.

A W/O/W double emulsion is a thermodynamically unstable system, thus a shorter proceedure time in this stage, that is, a rapid solidification of the double emulsion droplets, will undoubtedly favor a higher microentrapment efficiency [15–18]. Moreover, rapid solidification of double emulsion droplets means short contact time between proteins and organic solvent, which favors the stability of the entrapped proteins [18]. Additionally, rapid solidification can efficiently reduce coalescence of the inner aqueous droplets within oil droplets, forming a less interconnecting channel. Thus, a low initial burst and a constant drug release can be expected. Therefore, using EA as the organic solvent in W/O/W double

emulsion technique is still an attractive method if the drawback that high diffusion rate of EA easily leads to polymer precipitation can be overcome.

Kim et al. [19] tried to slow down the diffusion rate by saturating the outer aqueous phase with EA prior to re-emulsification. However, the additional EA prolonged the solidification time of the embryonic microparticles, which was unfavorable for the stability of the entrapped protein. In this study, we slow down the rate by decreasing the volume of the outer aqueous phase, and solidifying the embryonic microparticles step by step, i.e. introducing a preliminary solidification (pre-solidification) step before the complete solidification during W/O/W double emulsion process. Briefly, the $W_1/O/W_2$ double emulsion was poured into a small amount of aqueous solution (W_3) to partially solidify the embryonic microparticles at first, then the suspension was poured into an excess aqueous solution to completely solidify the embryonic microparticles.

The objectives of this study were to develop a modified W/O/W double emulsion process using ethyl acetate as organic solvent to prepare protein-loaded microparticle with high microparticle yield, high protein entrapment efficiency, and high bioactivity preservation of entrapped protein, as well as to investigate the influence of process parameters (the volume of the re-emulsification solution and the shear stress of pre-solidification) on the properties of the resultant microparticles. For these purposes, lysozyme, whose bioactivity is easy to be measured, was chosen as the model protein. The copolymer monomethoxypoly(ethylene glycol)-b-poly-DL-lactide (PELA) was used as wall material because of its ideal physical properties, biocompactibility and biodegradability [20]. The microparticles loading lysozyme were characterized in terms of surface morphology, particle size, protein entrapment efficiency, release behavior and bioactivity of the released proteins.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white and *Micrococcus lysodeikticus* cells was purchased from Sigma (St. Louis, USA). D,L-Lactic acid (85%) was supplied by Chemical Reagent Co. (Beijing, China). Methoxypoly(ethylene glycol) (MPEG) of M_w 2000 Da was obtained from Fluka (Switzerland). Stannous octoate was purchased from Sigma (Germany). Poly(vinyl alcohol) (PVA M_w 22 000 Da, ca. 88% hydrolyzed) was purchased from Across (Belgium). All other reagents were of analytical grade.

Methoxypoly(ethylene glycol)-b-poly-DL-lactide (PELA) was synthesized as described by Lucke et al. [20], in which the methoxypoly(ethylene glycol) block has a molecular weight of 2000 Da. Mean number molecular weight (M_n) of PELA was estimated to be 45 000, with a polydispersity (M_w/M_n) of 1.75 by gel permeation chromatography (GPC) in THF at 25 °C on the basis of a calibration with polystyrene standards, using a PL-GPC 210 (UK).

2.2. Microparticle preparation

PELA microparticles were prepared using the W/O/W emulsion technique described by Freytag et al. [13] with some modifications. Briefly, 10 mg of lysozyme was dissolved into 0.1 ml of distilled water (internal aqueous phase, W_1) and then added to 1 ml of ethyl acetate containing 50 mg PELA (oil phase, O). The primary W_1/O emulsion was prepared by a homogenizer (Hermo Research Institute, Beijing) at 11 000 rpm for 20 s. The primary emulsion was re-emulsified with the external aqueous phase containing 1% of PVA (w/v) and 0.9% of NaCl (re-emulsification solution, W_2), using the homogenizer at 3600 rpm for 40 s. The volume of re-emulsification solution (W_2) was varied as 6, 8, and 10 ml. Afterwards, the resulting $W_1/O/W_2$ double emulsion was poured quickly into another solution containing PVA (1%, w/v) and NaCl (0.9%, w/v) (pre-solidification solution, W_3) and stirred with either the homogenizer (3600 rpm) or magnetic stirrer (100 rpm) to preliminarily solidify the double emulsion droplets; the combined total volume of W_2 and W_3 was always maintained at 16 ml. After 2 min, the suspension containing embryonic microparticles was poured into 200 ml of 0.9% NaCl solution (solidification solution) under magnetic stirring at 400 rpm to completely solidify the embryonic microparticles. After 4 min, the microparticles were isolated by centrifugation, washed three times with 0.9% NaCl solution and lyophilized overnight. Finally, the mi-

croparticles were stored under dry conditions at −20 °C.

In order to investigate the effects of amount of re-emulsification solution and shear stress in pre-solidification on the characteristics of the microparticles, four experiments were designed as summarized in Table 1.

2.3. Determination of microparticle size and surface morphology

The volume-mean diameter of lysozyme-loaded microparticles was measured by a Coulter Multisizer (Coulter LS230, USA). The surface morphology of microparticle was observed with a scanning electron microscope (SEM) (KYKY 2800, Beijing) after coating with gold film.

2.4. Determination of protein entrapment efficiency

Eight to ten milligrams of freeze-dried microparticles, accurately weighted, was dissolved in 4.0 ml 0.1 M NaOH containing 2% (w/v) SDS, and then neutralized with 0.4 N HCl solution. Afterwards, the sample was diluted to 16 ml with distilled water and its protein content was determined by Peterson–Lowry method. From this result, the percentage (w/w) of lysozyme entrapped per dry weight of microparticles, i.e. protein loading, was determined. Each microparticle batch was assayed in triplicate. The entrapment efficiency was calculated by:

$$\mathrm{EE} = \frac{\text{Measured protein loading}}{\text{Theoretical protein loading}} \times 100$$

2.5. In vitro protein release studies

Eight to ten milligrams of freeze-dried microparticles, accurately weighed, was suspended in 1.5 ml of PBS buffer, pH 7.4 (8 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4, 1.81 g $Na_2HPO_4 \cdot H_2O$, 0.5 g NaN_3, 0.1 g Tween-20 and 1000 ml distilled water). The samples were agitated in a 37 °C incubator-shaker at 120 rpm. At defined time intervals, 1.0 ml of supernatant was collected by centrifugation and 1.0 ml fresh PBS was added back. The amount of released lysozyme was determined by the Peterson–Lowry method. Release profiles were calculated in terms of cumulative release (%) with incubation time. Each sample was assayed in duplicate.

2.6. Biological activity assay

The rate of lysis of *M. lysodeikticus* cells by lysozyme was used to estimate the bioactivity of the enzyme as described by Gu et al. [21]. Briefly, The absorbance (450 nm) of *M. lysodeikticus* suspension in 0.1 mol/l potassium phosphate buffer, pH 6.2, was first adjusted to 1.3. Fifty microliters of lysozyme sample was added to 2.5 ml of the above suspension (25 °C). The decrease in absorbance was monitored at 450 nm during a total period of 2 min. The activity was calculated from the slope of linear region of the $\Delta A_{450\ \mathrm{nm}}$/time curve, assuming that one unit of enzyme activity will reduce the $\Delta A_{450\ \mathrm{nm}}$ by 0.001/min under the conditions employed. Specific activity is defined in terms of units of activity per milligram of protein (U/mg). All samples were assayed in triplicate.

3. Results and discussion

When a W/O/W double emulsion with an oil phase composed of polymer and ethyl acetate (EA) is directly poured into a large amount of aqueous solution, the fast diffusion of EA from oil droplets to the outer aqueous phase will cause the droplets to

Table 1
Microparticle preparative parameters

Batch	Volume of W_2 (ml)	Volume of W_3 (ml)	Pre-solidification
A	6	10	Homogenization at 3600 rpm
B	6	10	Magnetic stirring at 100 rpm
C	8	8	Magnetic stirring at 100 rpm
D	10	6	Magnetic stirring at 100 rpm

W_2, the outer aqueous phase used in re-emulsification; W_3, the aqueous solution used in pre-solidification.

agglutinate and coagulate. Consequently, some polymer agglomerates are formed in the resulting suspension. A similar effect has been reported by Groothuis et al. [22] and Jeffreys et al. [23]. Both these groups observed that coalescence was promoted if a solute was being transferred from the dispersed phase to continuous phase. Therefore, to successfully prepare microparticles containing water-soluble drugs without the formation of polymer agglomerates, the W/O/W double emulsion should be preliminarily partially-solidified by adding a little amount of outer aqueous solution to slowly extract the EA out of the oil phase, and then the embryonic microparticles are completely solidified by addition of excess aqueous solution to further remove the residual EA within the semi-solid microparticles. This approach effectively restricted the coagulation of oil droplets and almost all of the wall polymers transformed into microparticles without agglomerates in the resulting suspension. Unquestionably, the volume of the re-emulsification aqueous solution as well as the shear stress in pre-solidification governed the rates of solvent removal and polymer solidification, and subsequently determined the characteristics of the resultant microparticles.

3.1. Surface morphology of the microparticles

The surface morphology of the microparticles was observed by scanning electron microscopy (SEM), and is shown in Fig. 1. The microparticles prepared with high shear stress during pre-solidification (Batch A, Table 1) show an elliptical and slender appearance (Graph A1) with a very smooth surface (Graph A2). The microparticles prepared with low shear stress (Batch B) appear regularly spherical (Graph B1) with some pits on the surface (Graph B2). Obviously, the shear stress during pre-solidification has a pronounced effect on the surface morphology of microparticles. During pre-solidification in Batch A, the high shear stress of 3600 rpm pulled long W_1/O oil droplets, whose viscosity had been increased with the diffusion of EA into the outer aqueous phase (W_2) in the previous re-emulsification step. At the same time, the high speed fluid of the external aqueous phase outside the interface of the deformed oil droplets accelerated the diffusion of the EA near the surface layer, resulting in a rapid solidification of the polymer on the surface layer. Consequently, most of the microparticles prepared by Batch A exhibited a cylindrical appearance. In contrast, the mild shear stress of 100 rpm in Batch B did not deform the W_1/O oil droplets, so the resultant microparticles were spherical.

The microparticles prepared with various volumes of aqueous re-emulsification are shown as Graphs B, C and D in Fig. 1. When the volume was 6 or 8 ml, the resultant microparticles had a regular spherical shape with a slightly pitted surface (Graphs B and C). However, when the re-emulsification aqueous solution volume increased to 10 ml (Batch D), the resultant microparticles appeared irregularly cylindrical (Graph D1), and the surfaces were very rough with numerous saliencies (Graph D2). This result was agreement with that of Sah [11], who reported that some microspheres prepared with 8 ml of EA and 80 ml of water solution displayed surface defects and irregularity. The cylindrical shape may be attributed to the deformation of the oil droplets resulted from the high shear stress of 3600 rpm during re-emulsification together with the rapid solidification of the deformed oil droplets, because a relatively large amount of re-emulsification aqueous phase was used. As described by Janssen et al. [24] and Tsakalos et al. [25], when a suspended oil droplet is subjected to a suddenly increased shear force, it stretches rapidly into a long cylindrical thread, which subsequently disintegrates due to the growth of interfacial tension. Because of the comparatively high solubility of EA in water (8.7%, w/v), most of the 1 ml EA (ca. 0.9 g) in Batch D was quickly extracted from the oil phase into the external aqueous phase (10 ml) during re-emulsification, resulting in rapid solidification of the deformed oil droplets in a long cylindrical-thread shape free from the subsequent disruption. Due to the very fast diffusion of EA in the surface layer of the deformed oil droplets, PELA polymers on the surface solidified so quickly that the internal aqueous droplets near the oil droplet surface were promptly ‘frozen’ on the surface, leading to the formation of saliences on the surface of microparticle (Graph D2).

3.2. Microparticle size and distribution

The volume-mean sizes and size distributions of

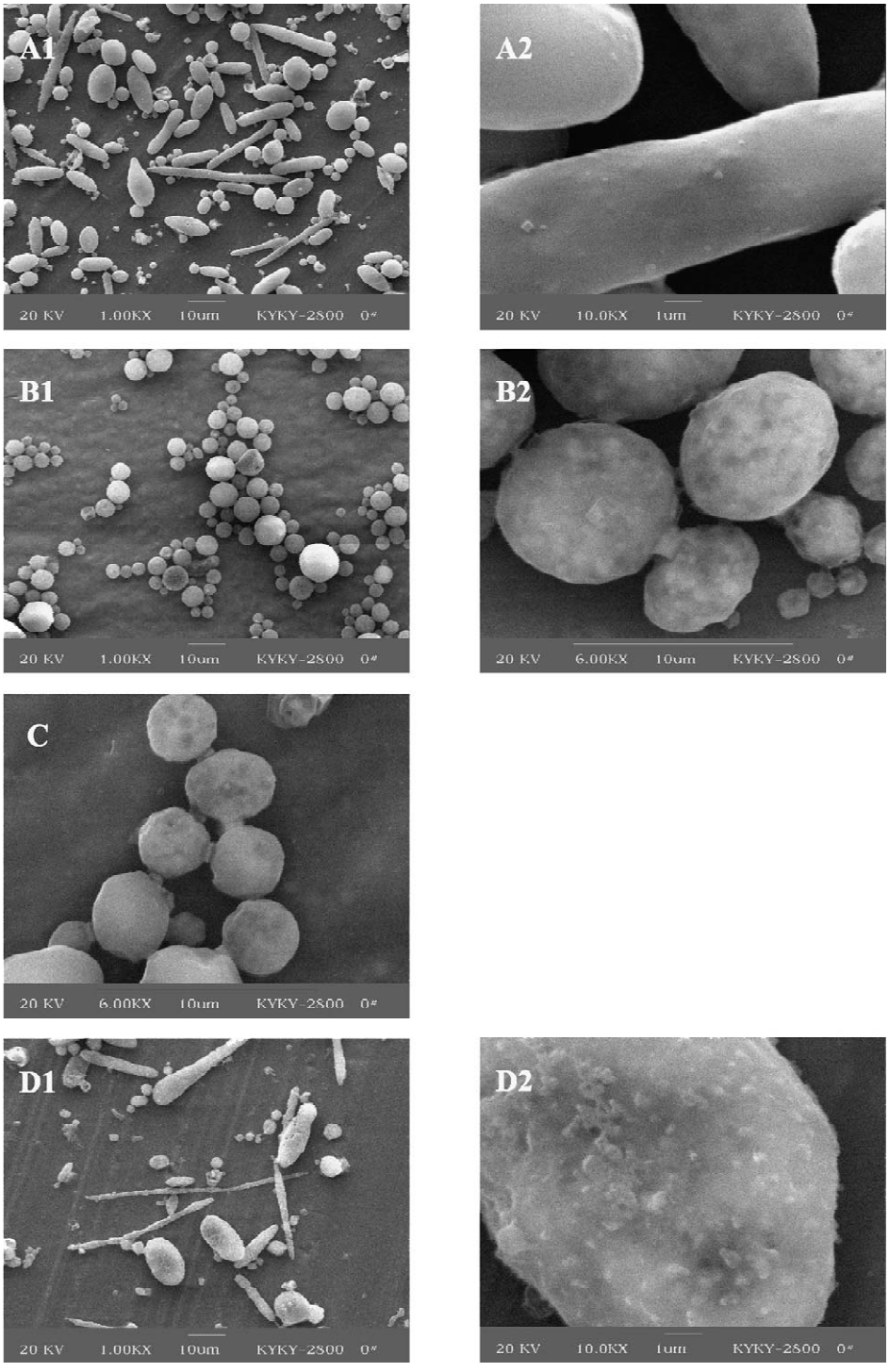

Fig. 1. SEM photographs showing size distribution (1) and surface morphology (2) of lysozyme-loaded PELA microparticles. For key legend see Table 1.

Table 2
Characteristics of microparticles

Batch	Protein loading (%±S.D.)	Entrapment efficiency (%±S.D.)	Microparticle size (μm)	
			Volume mean diameter	Standard deviation
A	16.3±0.5	97.6±3.0	9.46	4.1
B	15.9±0.4	95.2±2.4	6.59	2.8
C	15.7±0.4	94.0±2.4	4.65	2.0
D	16.0±0.6	95.8±3.6	11.28	5.2

the microparticles are shown in Table 2 and Fig. 2. The microparticles prepared with high shear stress during pre-solidification (Batch A) exhibit a larger volume-mean size and a wider particle size distribution than those with mild shear stress (Batch B). Apparently, the right shift and the wider distribution of Profile A compared to Profile B (Fig. 2) were caused by the deformation of the microparticles resulted from the high shear stress during pre-solidification.

The distinct difference in the volume-mean sizes and size distributions of the microparticles prepared with Batches B, C and D reveals that the volume of re-emulsification solution (W_2) plays a key role on the resultant microparticle size. It is easy to understand that the large volume-mean size (11.28 μm) and wide size distribution (Profile D) of the microparticles in the case of Batch D, where the volume of W_2 is 10 ml, according to the explanation of the formation of cylindrical microparticles, in the foregoing text. That is, the particle was solidified before being further disrupted. However, it is difficult to understand that the volume-mean size decreased from 6.59 to 4.65 μm as the volume of re-emulsification solution increased from 6 to 8 ml. Usually, when the volume of the external aqueous phase, i.e. re-emulsification solution (W_2) increases, there will be a larger amount of EA diffusing from the oil phase into the external aqueous phase, and the oil droplet will become more viscous and difficult to be disrupted into smaller droplets, resulting in a larger size. Nevertheless, the routine explanation fails as applied to the results in this study. We regard this surprising result as the cooperative consequence of the rapid diffusion of EA in the oil phase together with the transient-breakup situation of the oil droplets under high shear stress. A similar effect has been reported by Jassen et al. [24] and Tjahjadi et al. [26]. Compared to less viscous oil drops, more viscous drops sometimes can stretch thinner and longer before the break, producing very small fragments. That is, the drop can stretch thinner and longer before being broken when 8 ml of W_2 is used, compared with the case of 6 ml. Therefore, the microparticles prepared with 8 ml of re-emulsification solution are smaller than those with 6 ml. The detailed explanation is given in Tjahjadi's study [26]. Additionally, another possible reason for the surprising result was that a larger volume of W_2 (8 ml) allowed more EA to diffuse from the oil phase into the outer aqueous phase, resulting in an increase in the concentration of PELA in the oil phase. The higher concentration decreased the tension of O/W_2 interface, and thereby the size of emulsion droplets. Therefore, smaller microparticles were prepared with 8 ml of W_2 than those with 6 ml.

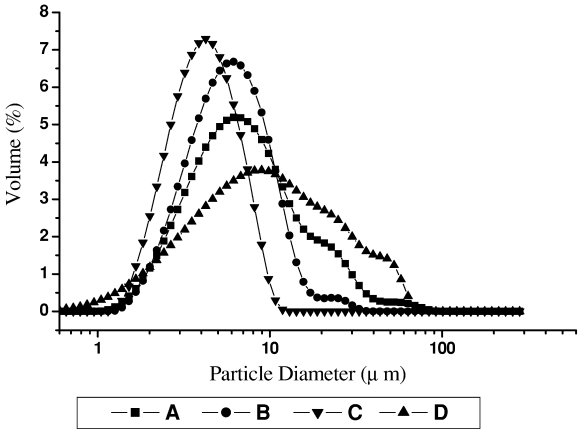

Fig. 2. Particle size distribution of lysozyme-loaded PELA microparticles. For key legend see Table 1.

3.3. Entrapment efficiency of lysozyme

It has been recognized that stabilization of primary emulsion achieved by amphiphilic polymer [27,28] and rapid solidification of microparticles [15–18] are favorable for improving protein entrapment efficiency in W/O/W double emulsion technique. In this study, the use of amphiphilic polymer PELA and EA performed perfectly in the both strategies, so the efficiencies of lysozyme entrapment for all four batches attained as high as 94% (Table 2).

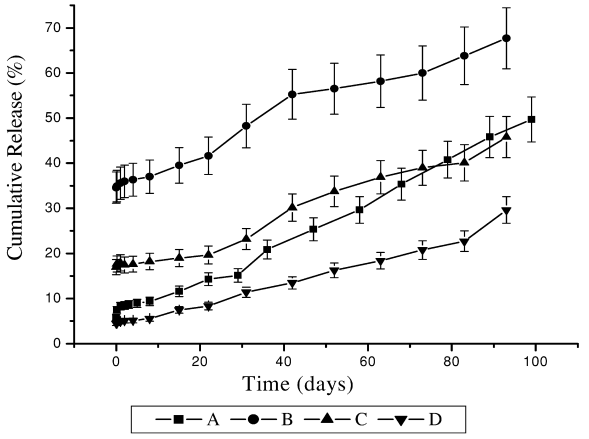

Fig. 3. Cumulative release profiles of lysozyme from PELA microparticles produced from Batches A, B, C, and D. The data are presented as mean±S.D. ($n=2$).

3.4. In vitro release behavior of the microparticles

The release profiles of the microparticles prepared with all the batches in Table 1 are shown in Fig. 3. The lysozyme release profiles were biphasic, showing an initial burst followed by nearly constant release of lysozyme. The measured values for the initial burst and release rate are summarized in Table 3. The results showed that microparticles prepared by Batch A displayed a lower initial burst (8%) than those prepared by Batch B (35%). This result suggests that a rapid diffusion of EA from embryonic microparticles may produce a compact polymer layer on the microparticle surface, as outlined by Crotts et al. [29], which results in a low burst effect. This explanation was conformed by the burst effects exhibited in the microparticles of Batches B, C, and D (Table 3). With the re-emulsification aqueous volume increasing (6 ml, 8 ml to 10 ml), the burst effect decreased (35%, 18% to 5%, Table 3). The increased volume accelerated solvent removal from embryonic microparticle surface and produced a concentrated and viscous polymer layer, which went on to form a more compact polymer shell on the microparticle surface, resulting in a lower burst effect.

Table 3
Burst effect and release rate of lysozyme from microparticles

Batch	Burst release[a] (%±S.D.)	Release rate[b] (%±S.D.)
A	8±0.5	0.43±0.01
B	35±3.0	0.36±0.02
C	18±1.1	0.31±0.01
D	5±0.3	0.24±0.01

[a] Burst release was measured at 6 h.
[b] Release rate is expressed as % of lysozyme released/day. Release rate was calculated by linear regression on all data points.

In addition, it can be seen from Table 3 that the release rate of lysozyme slowed down with increasing of re-emulsification solution volume. A possible explanation is that a higher solvent removal rate caused by larger re-emulsification solution volume efficiently reduced coalescence of the inner aqueous droplets within oil droplets, thus forming a less interconnecting channel, resulting in a slower release.

Due to the great disparity in the initial bursts displayed by the microparticles prepared by Batches A and B, the comparison between the subsequent lysozyme release rates is unavailable. Nevertheless, the fact that the microparticle prepared by Batch A released lysozyme faster than that prepared by Batch D (both the microparticles displayed comparable initial burst, 5% and 8%, respectively) confirmed that a faster solvent removal defined a slower release behavior. In Batch D, the removal of EA had been almost completed after the re-emulsification step due to the sufficient amount of outer water phase (10 ml). In contrast, the basic completion of solvent removal in Batch A occurred in the pre-solidification step. Therefore, the solvent removal rate in Batch D was faster than that in Batch A.

The release study clearly demonstrated that the initial burst and release rate was able to be controlled by adjusting the removal rate of EA, which can be achieved by varying the volume of W_2 and the shear stress in pre-solidification. Obviously, Batches A and D are better formulations from the viewpoint of suppressing the initial burst.

3.5. Bioactivity of the released lysozyme

Release bioactivity profiles of the model protein lysozyme from PELA microparticles are presented in Fig. 4. The profile O represents the change of free lysozyme bioactivity with the incubation time. Apparently, the profiles A–C were in good agreement

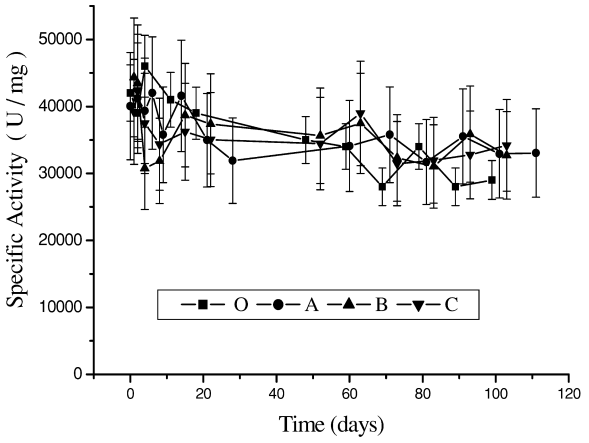

Fig. 4. Released bioactivity of lysozyme-loaded microparticles prepared by Batches B, C and D. O, free lysozyme; A, B, C: see Table 1. The data are presented as mean±S.D. ($n=3$).

with the profile O. The results show that the microentrapment process has a less detrimental effect on the bioactivity of the entrapped lysozyme due to the short contact time between lysozyme and EA, and that PELA copolymer has no obvious effect on the entrapped lysozyme.

4. Conclusions

The microparticles containing model protein lysozyme have been successfully prepared by the modified W/O/W double emulsion-solvent diffusion method with ethyl acetate as organic solvent. The tendency of wall polymer precipitation resulting from the fast diffusion rate of EA can be successfully overcome through preliminary solidification of the W/O/W double emulsion with a small amount of outer aqueous solution. Due to the comparatively high water solubility of ethyl acetate, the distinguishing feature of the modified process for the preparation of microparticles is the rapid solvent removal. This leads to the resultant microparticles possessing some attractive and anomalous characteristics different from those produced by a typical process using methylene chloride as organic solvent. The solvent removal rate can be controlled by adjusting the aqueous volume of the re-emulsification as well as the shear stress in pre-solidification. With the volume or the shear stress increasing, the diffusion rate of ethyl acetate from embryonic microparticles to outer aqueous phase increased, resulting in rapid solidification of the microparticles. This result led to a lower burst effect and a slower drug release of the microparticles.

The modified W/O/W double emulsion process can achieve a high protein entrapment efficiency (above 94%), and maintain a good bioactivity of protein, independent on the volume of re-emulsification aqueous solution and the shear stress of pre-solidification.

Acknowledgements

The authors are thankful to Natural Science Foundation of China for financial support to this work (grant no. 20136020).

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