ELSEVIER

International Journal of Pharmaceutics 178 (1999) 245–255

international journal of pharmaceutics

Investigation on process parameters involved in preparation of poly-DL-lactide-poly(ethylene glycol) microspheres containing *Leptospira Interrogans* antigens

Xiaohong Li [a,*], Xianmo Deng [a], Minglong Yuan [a], Chengdong Xiong [a], Zhitang Huang [b], Yanhua Zhang [c], Wenxiang Jia [c]

[a] *Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Chengdu 610041, People's Republic of China*
[b] *Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, People's Republic of China*
[c] *West China University of Medical Sciences, Chengdu 610041, People's Republic of China*

Received 7 August 1998; received in revised form 10 November 1998; accepted 11 November 1998

Abstract

Block copolymer, poly-DL-lactide-poly(ethylene glycol) (PELA) with 11.5% of poly(ethylene glycol) (PEG) content was prepared by bulk ring-opening polymerization using stannous chloride as initiator. PELA microspheres with entrapped *Leptospira Interrogans* antigens, outer membrane protein (OMP) were elaborated by solvent extraction method based on the formation of multiple w/o/w emulsion, and the resulting microspheres were characterized with respect to particle size, OMP entrapment and morphology characteristics. The purpose of the present study is to perform the optimization of preparative parameters for OMP-loaded PELA micropsheres to control particle size and improve the OMP encapsulation efficiency. Of all the parameters investigated, the polymer concentration of organic phase and the external aqueous phase volume play major roles on particle size, while the organic phase volume, internal aqueous phase volume and the addition of surfactant into the internal aqueous phase display considerable effects on OMP loading efficiency. A small volume of internal aqueous phase and intermediate volumes of organic phase and external aqueous phase were favorable to achieve micropsheres with a size of 1–2 μm and high antigen encapsulation efficiency (70–80%). In vitro OMP release profiles from PELA microspheres consist of a small burst release followed by a gradual release phase. The OMP release rate shows some relations with the porous and water-swollen inner structure of the microspheres matrix. The presence of surfactant in microspheres accelerates OMP release, but the OMP entrapment within microspheres shows limited effects on the release profile.

Keywords: Poly-DL-lactide-poly(ethylene glycol); Outer membrane protein; Preparative parameter; Encapsulation efficiency; in vitro release

* Corresponding author. Tel.: +86-28-5223847; fax: +86-28-5223978.

1. Introduction

Recent scientific advances have provided information relevant to the design of vaccines for use against a variety of infectious agents. In particular, numerous vaccine antigens are being identified and produced in the form of subunits, synthetic peptides and proteins expressed through recombinant genetics. Although such formations offer advantages in the selection of antigenic epitopes and safety, they are in many cases weakly immunogenic and require boosters to elicit protective immunity. Thus, it creates an acute need to identify pharmaceutically acceptable delivery systems for these antigens to enhance their immunogenicity, simplify the vaccination procedures and promote their stability. Controlled-release microspheres prepared from biodegradable polymers, such as polylactide (PLA) and polylactide-co-glycolide (PLGA) have been extensively evaluated with the object of extending the duration of drug or antigen release (Uchida and Goto, 1994; Jeffrey et al., 1996). Controlled-release vaccines encapsulated into PLGA microspheres have recently been shown to be effective for induction of long term immune responses following a single injection (O'Hagan et al., 1991), oral (Eldridge et al., 1991) and intranasal (Yan et al., 1996) administration. In addition, the vaccine is dispersed within the PLGA matrix of the microspheres in a dry state, thus providing extended shelf life without the need of stabilizer or a cold chain. The choice of PLGA as the matrix for vaccine formulations is based on its long-term safety in humans, its biodegradability, and the commercial availability of a variety of polymers of different molecular weights and monomer ratios (Tabata and Ikada, 1988).

Nevertheless, PLGA and PLA show some drawbacks, resulting from their hydrophobic nature. The difference in physico–chemical properties between hydrophilic antigens and hydrophobic polymer matrix leads to a lower antigen encapsulation efficiency within microspheres, and a higher burst effect of antigen release from microspheres. Furthermore, during the initial protein release phase in vivo, the hydrophobic PLGA or PLA prevents the penetration of water into the center of microspheres, thus forming an acidic microenvironment due to the accumulated acidic breakdown products, such as lactic and glycolic acid end groups (Kenley et al., 1987). The acid environment, combined with the elevated temperature and hydrophobic surfaces may provide conditions in which antigens are unlikely to survive for a long time.

To overcome these disadvantages resulting from the hydrophobic nature of PLA, the second component poly(ethylene glycol) (PEG), which has been widely used to improve the biocompatibility of the blood contacting materials, is introduced to form ABA block copolymer poly-DL-lactide-poly(ethylene glycol) (PELA) (Deng et al., 1990). This combination and the biocompatible nature of PELA (Wang et al., 1995) create a new biodegradable system for peptide, protein and antigen delivery. The hydrophilic PEG domains of PELA acting as a protein stabilizer or surface modifier of hydrophobic PLA network, could promote the stability of antigens, increase antigen encapsulation efficiency and decrease the amount of emulsifier used in PELA microspheres preparation (Li et al., 1997; Li et al., 1998). Investigation into the influence of physico–chemical characteristics of microspheres on the immune response to associated antigens have suggested the importance of such factors as polymer compositions (Coombes et al., 1996), surface properties (Rafati et al., 1997) and particle size. Particle size, for example, has been shown to play a major role in being targeted to the immunization-related tissues (Eldridge et al., 1990). From the viewpoint of the limited efficiency of microspheres to be phagocytosized and transported by macrophages, it may be beneficial to improve the antigen encapsulation efficiency to promote the immune response.

In a previous report from this group, PELA microspheres with 1.25 μm of average diameter containing *Vibrio Cholera* antigens ware prepared by using a double emulsion w/o/w based on solvent extraction method. Through investigation the polymer composition, regulating the solvent components of the oil phase, adding stabilizer into the internal water phase, adjusting the pH values and salt concentrations of the external water phase,

antigen loading efficiency was impressively improved (Deng et al., 1998). Consequently, this paper describes the preparation of PELA microspheres with entrapped outer membrane protein (OMP) of *L. Interrogans*. The effect of process parameters, such as the volumes of internal water phase, oil phase and external water phase, and the addition of surfactants into the internal water phase, on particle size, surface morphology and OMP encapsulation efficiency are investigated in details. The different release profiles in vitro of resultant microspheres are also included.

2. Materials and methods

2.1. Materials

L. Interrogans antigen, OMP (molecular weight, 39 K/Da) was granted from West China University of Medical Sciences. DL-lactide (85%) was produced in the Chemical Factory of Hubei University, China. PEG (average molecular weight, 6 K/Da) was from Guangzhou Chemical Reagents Department, China. All other chemicals and solvents were of reagents grade or better.

2.2. Synthesis of PELA copolymers

Copolymer PELA was prepared by bulk ring-opening polymerization of lactide and PEG using stannous chloride as initiator (Deng et al., 1990). Briefly, the pretreated lactide and PEG were transferred to a dry and clean ampoule, and freshly prepared stannous chloride solution in ethyl acetate was added. The bottle was evacuated and displaced with dry nitrogen gas for three times before placing in an oven with a temperature controlling system. The obtained crude product was dissolved in methylene chloride and precipitated by adding the solution into precipitated medium, a mixture of ethyl ether and petroleum ether by vigorous stirring.

The purified copolymer had a yield of over 95%. The ABA block structure (A refers to polylactide, and B to PEG) was characterized by carbon nuclear resonance (^{13}C-NMR) as described previously (Li et al., 1997). PELA with 11.5% of the actual PEG content, calculated from the integral height of hydrogen shown in ^{1}H-NMR(Varian FT-80A), and the intrinsic viscosity $\eta = 0.39$, evaluated with an Ubbelohde viscometer on 0.4 g/dl solution in tetrahydrofuran (THF) at 30°C was chosen as the matrix polymer for microspheres preparation. The weight-average molecular weight (Mw) and polydispersity (Mw/Mn) of PELA, determined with gel permeation chromatography (GPC, Waters ALC/GPC 244), were 58.9 K/Da and 2.70, respectively.

2.3. Preparation of PELA microspheres containing OMP

OMP-encapsulated PELA microspheres were prepared by a previously reported w/o/w solvent extraction procedure (Li et al., 1998). Briefly, an aqueous OMP solution was added to PELA dissolved in methylene chloride. The mixture was emulsified with magnetic stirrer for 2 min to form the primary w/o emulsion. This emulsion was then added to the external water phase containing stabilizers and was emulsified again by a high-speed homogenizer. The organic solvent was extracted by adding 100 ml of 6% isopropanol and the mixture was stirred at a moderate speed at ambient temperature for 5–6 h. The OMP-loaded microspheres were centrifuged at $5000 \times g$ for 8 min and washed three times with double distilled water. The microspheres were then lyophilized overnight and stored at 4°C.

The present work was designed to assess the effect of formulation variables on microsphere characteristics, i.e. particle size, surface morphology and OMP entrapment. Unless otherwise stated, microspheres were prepared using 0.6 ml OMP solution (protein concentration, 25 mg ml^{-1}), 5.0 ml of PELA solution (6%,w/v) and 20 ml of external aqueous phase.

2.4. Microspheres characterization

Scanning electron micrography (SEM, Amray) was utilized to observe the topography and surface morphology. The microspheres size was determined with laser diffraction method using particle size analyzer (Shimadzu SALD-2009,

Japan), and expressed as volume mean diameter (D). The level of residual methylene chloride within microspheres was detected by gas chromatography (GC, Shanghai Analytical Instrument, China), and compared with a set of standard samples with known amount of methylene chloride.

Core loading of OMP in microspheres was determined by extracting the protein from microspheres and assaying the protein content of the extracted solution. In brief, a known amount of microspheres ($\sim$ 100 mg) was dissolved in 500 μl of methylene chloride and extracted three times with 600 μl of double distilled water. The OMP content of the extracted solution was determined using Bradford's method (Bradford, 1976), compared with a standard curve of data obtained by assaying known concentrations of OMP solutions. The tests were conducted in triplicate for each sample. The actual OMP entrapment was calculated from the initial microspheres weight and the amount of OMP encapsulated, and the loading efficiency from the initial imputing OMP and the amount of OMP encapsulated in microspheres.

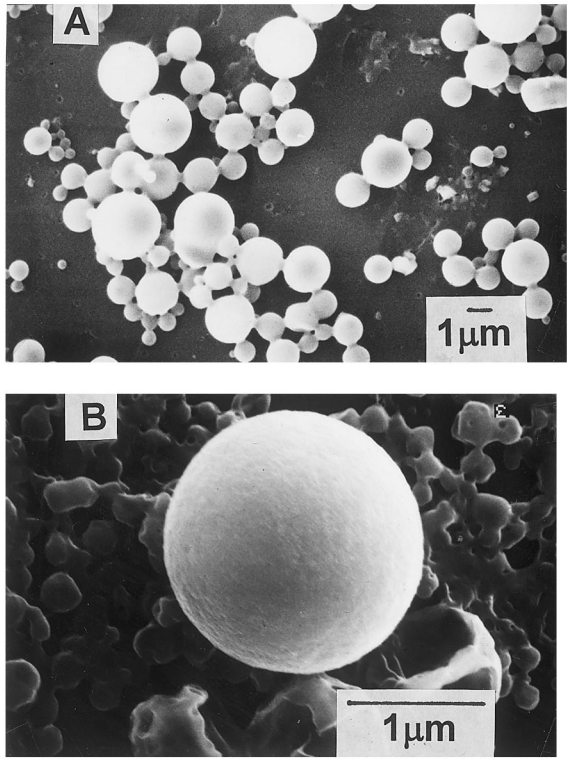

Fig. 1. The dispersion pattern (A) and surface morphology (B) of PELA microspheres with entrapped OMP.

2.5. In vitro OMP release test

Selected batches of the obtained microspheres were evaluated for in vitro release. Briefly, 500 mg sample of microspheres was incubated into a test tube containing 20.0 ml of 0.02 M phosphate buffer saline (PBS, pH 7.4). The tubes were kept in a thermostatic shaking water bath (Jiangsu Taichang Medical Apparatus, China) that was maintained at 37°C and 60 cycles/min. At predetermined intervals, three tubes for each sample were withdrawn, and 1.5 ml of the supernatant was collected by centrifugation while the same volume of fresh PBS was added back to the tubes. The OMP content was determined by Bradford's protein assay, and the mean of three values for each sample was calculated.

3. Results and discussion

3.1. Characteristics of microspheres

We previously investigated the effect of PELA with varied PEG contents from 0 to 50% on the particle size and protein loading efficiency, and the maximum loading efficiency was observed for copolymer with 10% of PEG content (Deng et al., 1998). In the present paper, PELA with 11.5% of PEG content was synthesized and set as matrix polymer. The resulting microspheres entrapping OMP displayed smooth, spherical surface structure, with no evidence of collapsing (Fig. 1). Each of the batches of microspheres had a mean particle size of less than 3 μm, which is suitable for parental vaccination due to their efficient prevention of capillary clogging. OMP-loaded PELA microspheres with the size 1–5 μm could be targeted to the immunization-related tissues, such as the intestinal peyer's patches, liver and spleen following oral administration (Eldridge et al., 1990), which is known as a more convenient and safer vaccination way. The residual methylene

Table 1
Effect of polymer concentration on particle size and OMP entrapment

Weight of polymer (mg)	Concentration of polymer solution (%, w/v)	D (μm)	S.D.	OMP entrapment (%, w/w)	Loading efficiency (%, w/w)
100	2.0	0.96	0.237	3.84	25.6
200	4.0	1.54	0.269	3.68	49.1
300	6.0	1.87	0.263	3.65	72.9
400	8.0	2.65	0.252	3.17	84.5
500	10.0	4.32	0.304	2.58	86.1

chloride presented in microspheres was below 20 ppm evaluated by gas chromatography, which was lower than the limit according to the US-PXXIII requirement (i.e. 500 ppm for methylene chloride) (Bitz and Doelker, 1996). To enhance the immune response, the purpose of the present study is to perform the optimization of preparative parameters for PELA microspheres containing OMP to control the particle size and improve the antigen encapsulation efficiency. For each variable investigated, batches of micropsheres were prepared in duplicate, and the results of particle characteristics obtained for each batch showed limited batch-to-batch variation.

3.2. Effect of the polymer concentration and organic phase volume on particle size and OMP entrapment

The polymer concentration in the oil phase of w/o/w was regulated two ways, one through changing the weight of PELA dissolved in a fixed volume of methylene chloride, and the other through dissolving a fixed weight of PELA in various volumes of methylene chloride. Tables 1 and 2 list the effect of polymer concentration and organic phase volume on particle size and OMP entrapment, respectively. Increasing the weight of PELA dissolved in a fixed volume of methylene chloride resulted in a distinct increase in the particle size (Table 1). The average diameter of microspheres prepared from 500 mg of PELA dissolved in 5.0 ml of methylene chloride as the organic phase showed ~ 4.5 times that of microspheres prepared from 100 mg of PELA. The higher concentration of polymer in the oil phase may lead to an increased frequency of collisions, resulting in fusion of semi-formed particles. This would produce an overall increase in the size of microspheres. In addition, the increase in the concentration of dissolved polymer also contributes to a viscous organic phase, which may have reduced the efficiency of stirring of the solution, and led to forming microspheres with larger size.

An alternative method of increasing polymer concentration, dissolving a fixed amount of PELA (300 mg) in decreasing volumes of methylene chloride caused a slight increase in particle size (Table 2). The organic phase volume was reduced from 15.0 to 3.0 ml, and the obtained microspheres showed a 21.7% increase in particle size. This may be due to the increased viscosity of organic phase, which would cause a reduction of efficiency of disruption of oil phase, resulting in larger size. As noted above, it can be concluded that the actual amount of polymer dissolved in organic phase affects particle size to a greater extent than simple variation of polymer concentration through changing the organic phase volume.

As shown in Tables 1 and 2, the OMP-encapsulation efficiency into PELA microspheres matrix increased with the increase in the polymer concentration by any of the two ways. In the preparation of antigen loading microspheres by w/o/w emulsion solvent evaporation or extraction process, the expulsion of the internal aqueous phase (internal droplets containing antigens) to the external aqueous phase is unavoidable during the second emulsification, but the extent of expulsion determines the antigen loading efficiency. The high OMP loading efficiency of the resulting microspheres from an increase in polymer concentration is probably contributed to the combination

of three factors: (1) small mass transfer area due to the large size of obtained microspheres; (2) high mass transfer resistance within the more viscous medium; and (3) the high precipitation rate of the polymer organic phase into microspheres due to the relatively lower amount of solvent. Due to the use of a larger weight polymer to increase the polymer concentration, the actual OMP entrapment within PELA microspheres slightly decreased, though the encapsulation efficiency increased with the increase in polymer concentration (Table 1). Therefore, for antigen delivery system, it is beneficial to prepare microspheres with similar particle size and higher antigen entrapment by dissolving polymer into smaller volume of organic solvent.

3.3. Effect of the external aqueous phase volume on particle size and OMP entrapment

Table 3 shows that an increase in the volume of the external aqueous phase leads to an increase in both the particle size and OMP entrapment. The increase in particle size is related to the reduced mixing or dispersion efficiency during the second emulsification process due to the larger volumes, resulting in formation of microspheres with larger sizes. The increase in OMP entrapment is owing to the increased particle size, which provides an increase in particle volume and enable more OMP to be incorporated into microspheres. In addition, the small mass transfer area of microspheres with large size would result in lower amount of OMP in the internal aqueous phase expelled into the external aqueous phase during the second emulsification process.

3.4. Effect of the internal aqueous phase volume on particle size and OMP entrapment

A small decrease in particle size and an evident increase in OMP entrapment were observed following an decrease in the internal aqueous phase volume (Table 4). The decreased internal aqueous phase volume results in a well-dispersed and stable w/o emulsion, which could probably reduce the frequency of collisions during the formation of microspheres in the second emulsification procedure, leading to an overall decrease in particle size. In contrast to the results of Jeffery et al. (1993), who reported an increased OVA entrapment into PLGA 50/50 microspheres when the inner aqueous phase volume was increased, the use of small volume as an internal water phase, in the present work, produced a comparatively high OMP entrapment and encapsulation efficiency.

As noted above, in the preparation of protein-loaded microspheres by w/o/w emulsion solvent evaporation or extraction process, the loading efficiency is associated with the amount of protein expelled from the inner water phase into the external water phase during the second emulsification procedure. The larger the internal aqueous phase with fixed volume of organic phase is, the thinner the oily layer (methylene chloride phase) is expected to be. Due to this oily PELA layer acting as a barrier through which internal OMP is diffused to the external aqueous phase, a thinner oily PELA is expected to give rise to larger diffusion. In addition, due to the hydrophilicity of PELA, a lower precipitation rate of copolymer solution into microspheres is probably presented due to the increased amount of water in the

Table 2
Effect of organic phase volume on particle size and OMP entrapment

Volume of organic phase (ml)	Concentration of polymer solution (%, w/v)	D (μm)	S.D.	OMP entrapment (%, w/w)	Loading efficiency (%, w/w)
3.0	10.0	2.03	0.307	4.08	81.6
5.0	6.0	1.87	0.263	3.65	72.9
7.5	4.0	1.81	0.240	3.14	62.7
15.0	2.0	1.75	0.229	2.57	51.5

Table 3
Effect of the external aqueous phase volume on particle size and OMP entrapment

Volume of external water phase (ml)	D (μm)	S.D.	OMP entrapment (%, w/w)	Loading efficiency (%, w/w)
10	1.71	0.299	3.56	71.3
20	1.87	0.263	3.65	72.9
40	2.15	0.244	3.74	74.8
70	3.17	0.246	3.98	79.7
100	4.17	0.271	4.07	81.4

internal phase. Accordingly, the large internal aqueous volume, perhaps as a result of low mass transfer resistance and slow precipitation the polymer phase, would result that protein molecules had more chance to diffuse out from the inner w/o emulsion. Therefore, the probability of OMP leakage from the internal aqueous phase to the external phase would decrease with the reduction of internal phase volume, which offers some advantages in elaborating microspheres with high OMP entrapment and loading efficiency. The resulting microspheres showed round and smooth surface, observed by SEM spectrum, except that the surface of microspheres prepared with higher internal aqueous phase volume (1000 μl) appeared pitted and some of the particles had collapsed (results not shown).

For purely practical reasons, lyophilized OMP powders rather than solution were dispersed into organic phase to prepare OMP-loaded microspheres. However, it appeared that particulate antigens were less efficiently encapsulated than the corresponding aqueous solutions. In the preparation process, it was observed that a fine-dispersed and stable w/o emulsion was difficult to achieve when the OMP powder was directly dispersed into organic phase than the use of OMP solution. This may lead to less amounts of OMP being entrapped during the precipitation of organic phase into microspheres matrix. Alonso et al. (1993) reported that the microsphere population was irregular, showing some broken fissures when the *Tetanus Toxoid* powder was directly used in preparation PLGA 50/50 microspheres. However, the resultant microspheres in the present study appeared to be smooth and there was no evidence of collapsed particles. This may be due to the existence of hydrophilic PEG segments in the polymer chain, which could achieve a better or easier dispersion of PELA in the external water phase to form microspheres with smoother surface and less defect.

3.5. Effect of surfactant in the internal aqueous phase on microspheres characteristics and OMP release profile

We reported previously (Li et al., 1998) that the addition of 0.1–2.0%(w/v) of Span-80 into the organic phase led to an increase in protein entrapment in PELA microspheres. In the present study, the effect of surfactant in the internal aqueous phase on the microspheres characteristics was investigated, and results are provided in Table 5. A reduction in particle size and an increase in OMP entrapment were observed with an increase in Tween-80 concentration in the internal water phase. The addition of Tween-80 is expected to promote the stability of the inner w/o emulsion, and the increased concentration of Tween-80 could achieve a better stabilization of the inner w/o emulsion. As mentioned above, this would result in an overall reduction in particle size. A stable w/o emulsion provides a higher mass transfer resistance, thus decreasing the amount of OMP diffused into the external aqueous phase during the second emulsification. In addition, during the precipitation of polymer solution into microspheres, the surfactant could promote the compatibility of the hydrophilic protein and hydrophobic polymer network, which is preferential for protein to locate within microsphere matrix, resulting in a higher loading efficiency. However, no apparent increase in OMP entrapment was

observed when the Tween-80 concentration in the internal aqueous was beyond 0.5%(w/v). This may be due to the decreasing particle size, resulting in a large mass transfer area.

Fig. 2 shows the effect of Tween-80 concentration on the OMP release from PELA microspheres. A significant increase in the release rate of OMP by a higher concentration of surfactant was observed during the incubation time, and the amount of total released protein from microspheres also increased with the increase in surfactant concentration. Unlike protein-loading microspheres prepared with PLA, which displayed a large initial burst release followed by a lag phase characterized as no protein release, the protein-loaded PELA microspheres present a small burst effect and then a gradual release profile (Deng et al., 1998). In the present work, following the initial burst, a steady release of OMP over 30 days was observed (Fig. 2).

The burst release of protein is associated with those protein molecules dispersing close to the microspheres surface, which diffuse out in the initial incubation time. Thus, the small burst effect of PELA microspheres is due to the preferential location of protein molecules within the deep sections of microspheres matrix. The similar profile of burst release phase of the three PELA microspheres (Fig. 2) is owing to the similar dispersion state of OMP near the microsphere surface, which is allowed to concluded that the incorporation of Tween-80 into the internal aqueous phase has promoted the preferential location of OMP in microspheres matrix and shows limited effect on the dispersion state of OMP close to microspheres surface.

However, different profiles were achieved in the subsequential gradual release phase of the investigated microspheres. It is indicated that the gradual release of protein from PELA microspheres is due to the swollen inner structure formed by contact with the aqueous release medium due to the existence of hydrophilic PEG domains in polymer matrix, and the protein diffusion through the swollen phase. A higher release rate and larger amount of total protein was detected for microspheres prepared with higher Tween-80 concentration in the internal water phase. This is probably due higher concentrations of surfactant making microspheres porous, as surfactant molecules would be released quicker from microspheres owing to their small sizes. Additionally, the coordinated action of the surfactant would result in easier diffusion of protein molecules out from microspheres matrix.

As seen from Fig. 2, 20–40% of OMP was remained in microspheres after the 1-month test, which may be due to the low diffusion rate caused by low protein loading during the later incubation time, and the electrical interactions between the basic amino acid of protein and the acidic carboxyl group of the degraded polymer (Ogawa et al., 1988). Accordingly, following incubation, a larger porous inner structure would be present in a microspheres matrix prepared with higher surfactant concentration, resulting in a lower amount of protein remaining in microspheres after 1-month of incubation.

Table 4
Effect of the internal aqueous phase volume on particle size and OMP entrapment

Internal aqueous phase volume (μl)	OMP concentration (%, w/v)	D (μm)	S.D.	OMP entrapment (%, w/w)	Loading efficiency (%, w/w)
[a]	[a]	1.79	0.314	1.91	38.3
150	10.0	1.75	0.247	3.77	75.4
300	5.0	1.87	0.263	3.65	72.9
600	2.5	1.96	0.289	3.24	64.8
750	2.0	1.99	0.303	2.59	51.7
1000	1.5	2.04	0.309	2.37	47.5

[a] 150 mg of lyophilized OMP powder was directly dispersed in organic phase.

Table 5
Effect of surfactant in the internal aqueous phase on particle size and OMP entrapment

Surfactant concentration (%, w/v)	D (μm)	S.D.	OMP entrapment (%, w/w)	Loading efficiency (%, w/w)
0	1.87	0.263	3.65	72.9
0.2	1.71	0.250	3.79	75.9
0.5	1.66	0.255	3.96	79.2
1.0	1.62	0.261	3.95	79.0
1.5	1.54	0.249	3.93	78.6

3.6. Effect of OMP entrapment on the release profile

In the protein release from PLA or PLGA microspheres, it was known that higher protein entrapment should result in higher release rate due to the higher driving force derived from the protein concentration difference between the polymer matrix and the aqueous release medium. In the present study, we compare the release profiles of resulted microspheres with different OMP entrapment prepared with different organic phase volumes. Table 2 lists the characteristics of obtained microspheres, showing close particle size (1.8–2 μm) but different OMP entrapment from 2.5 to 4.1% (w/w). Fig. 3 displays the influence of OMP entrapment on the release rate. The higher loading levels of 4.08 and 3.65% yield an in vitro release rate, close to 3.14% and slightly higher than 2.57%. According to the release mechanism of PELA microspheres described above, it is suggested the existence of interactions between the polymer matrix and the protein entrapped in microspheres. It is possible that the viscosity of the swollen inner structure was increased by the encapsulated protein, resulting in relative a lower diffusion rate of protein within microspheres with higher protein entrapment.

4. Conclusion

The evaluation of the particle size and antigen encapsulation efficiency as a function of different preparation parameters was studied. The particle size is associated with the mixing efficiency of emulsification procedure and stability of the primary w/o emulsion. An increase in the viscosity of polymer organic solution and/or the external aqueous phase volume leads to reduction in the

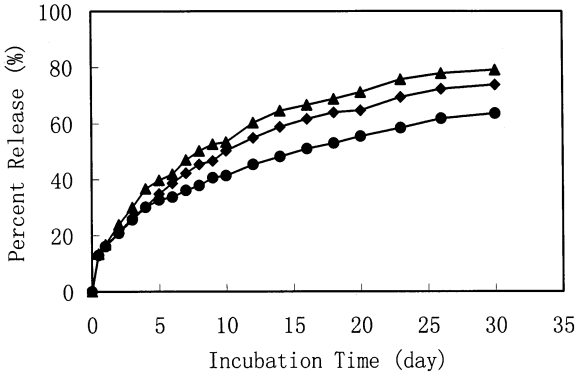

Fig. 2. Effect of surfactant concentration of 0 (●), 0.5% (◆) and 1.0% (▲) in the internal aqueous phase on the release of OMP from PELA microspheres.

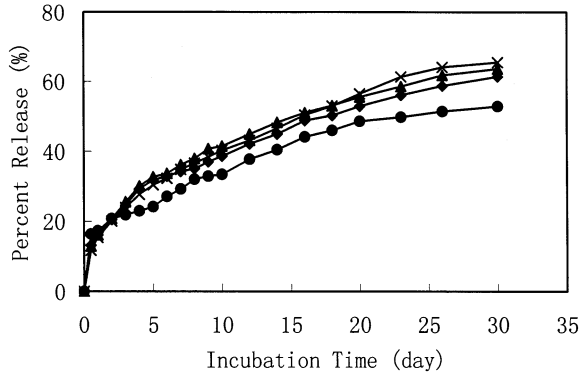

Fig. 3. In vitro release of OMP as a function of OMP entrapment in PELA microspheres with the size and OMP entrapment as follows: 2.03 μm and 4.08% (X), 1.87 μm and 3.65% (▲), 1.81 μm and 3.14% (◆), 1.75 μm and 2.57% (●).

mixing efficiency. The amount of protein expelled from the internal aqueous phase to the external aqueous phase during the second emulsification determines the antigen loading efficiency. The small mass transfer area (due to large size), high mass transfer resistance (due to stable inner w/o emulsion, viscous organic phase and thick oily polymer layer) and high precipitation rate of polymer solution into microspheres (due to less solvent) are beneficial to elaborate microspheres with high antigen loading efficiency. Of all the parameters investigated, the polymer concentration in organic phase and the external aqueous phase volume play major influences on particle size, while the organic phase volume, internal aqueous phase volume and the addition of surfactant in the internal phase have considerable effects on OMP loading efficiency. The OMP release profiles from PELA microspheres consist of a small burst release phase followed by a gradual release phase. The OMP release rate shows some relations with the porous and water-swollen inner structure of microspheres matrix. The presence of surfactant in microspheres accelerates the OMP release, and the OMP entrapment within microspheres exhibits limited effect on the release profile.

As a novel matrix polymer for drug delivery system, copolymer PELA has shown some advantages over PLA and PLGA, especially for hydrophilic drugs, peptides, proteins and antigens. However, it is clear that more detailed investigations are necessary to clarify the effect of manufacturing parameters on microsphere characteristics, the effect of matrix polymer on protein stability and antigen immunogenicity during microspheres preparation and antigen releasing procedure, the different degradation and drug release profiles of PELA compared with PLA or PLGA delivery systems.

Acknowledgements

The authors are thankful to the National Natural Science Foundation of China for financial support to this work (grant no. 29774034, 39670304).

References

Alonso, M.J., Cohen, S., Park, T.G., Gupta, R.K., Siber, G.R., Langer, R., 1993. Determinants of release rate of tetanus vaccine from polyester microspheres. Pharm. Res. 10, 945–953.

Bitz, C., Doelker, E., 1996. Influence of the preparation method on residual solvents in biodegradable microspheres. Int. J. Pharm. 131, 171–181.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Coombes, A.G.A., Lavelle, E.C., Jenkins, P.G., Davis, S.S., 1996. Single dose, polymeric, microparticle-based vaccines: the influence of formulation conditions on the magnitude and duration of the immune response to a protein antigen. Vaccine 14, 1429–1438.

Deng, X.M., Li, X.H., Yuan, M.L., Xiong, C.D., Huang, Z.T., Jia, W.X., Zhang, Y.H., 1998. Optimization of preparative conditions for poly-DL-lactide-poly(ethylene glycol) microspheres with entrapped *Vibrio Cholera* antigens. J. Control. Rel. (in press).

Deng, X.M., Xiong, C.D., Cheng, L.M., 1990. Synthesis and characterization of block copolymer from lactide and polyethylene glycol. J. Polym. Sci., Polym. Lett. 28, 411–416.

Eldridge, J.H., Hammond, C.J., Meulbroek, J.A., Staas, J.K., Gilley, R.M., Tice, T.R., 1990. Controlled vaccine release in the gut-associated lymphoid tissues. 1. Orally administered biodegradable microspheres target the paper's patches. J. Control. Rel. 11, 205–214.

Eldridge, J.H., Staaas, J.K., Meulbroek, J.A., McGhee, J.R., Tice, T.R., Gilley, R.M., 1991. Biodegradable microspheres as a vaccine delivery system. Mol. Immunol. 28, 287–294.

Jeffery, H., Davis, S.S., O'Hagan, D.T., 1993. The entrapment of a model protein using a water-in-oil-in-water emulsion solvent evaporation technique. Pharm. Res. 10, 362–368.

Jeffrey, L., Cleland, L.B., Phillip, W.B., Ann, D., Tim, G., Amy, L., Joann, V., Terri, W., Michael, F.P., 1996. Development of a single-shot subunit vaccine for HIV-1. 2. Defining optimal autoboost characteristics to maximize the humoral immune response. J. Pharm. Sci. 85, 1346–1349.

Kenley, R.A., Lee, M.O., Mahoney, T.R., Sanders, L.M., 1987. Poly(lactide-co-glycolide) decomposition kinetics in vivo and in vitro. Macromolecules 20, 2398–2403.

Li, X.H., Yuan, M.L., Xiong, C.D., Deng, X.M., Huang, Z.T., 1998. Preparation of poly-DL-lactide-poly(ethylene glycol) microspheres encapsulating human serum albumin. Acta Polym. Sin. (in press).

Li, X.W., Xiao, J., Deng, X.M., Li, X.Y., Wang, H.L., Jia, W.X., Zhang, W.B., Men, L., Yang, Y., Zheng, Z.X., 1997. Preparation of biodegradable polymer microspheres encapsulating protein with micro size. J. Appl. Polym. Sci. 66, 583–590.

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T., Shimamoto, T., 1988. A new technique to efficiently entrap leuprolide acetate into microencapsules of polylactide acid or copoly(lactide/glycolide) acid. Chem. Pharm. Bull. 36, 1095–1103.

O'Hagan, D.T., Rahman, D., McGee, J.P., Jeffery, H., Davis, M.C., Williams, P., Davis, S.S., 1991. Biodegradable microparticles as antigen delivery system. Immunology 73, 239–242.

Rafati, H., Lavelle, E.C., Coombes, A.G.A., Stolnik, S., Holland, J., Davis, S.S., 1997. The immune response to a model antigen associated with PLG microparticles prepared using different surfaces. Vaccine 15, 1888–1897.

Tabata, Y., Ikada, Y., 1988. Macrophage phagcocytosis of biodegradable microspheres composed of L-lactide acid/glycolic acid homo- and copolymers. J. Biomed. Mater. Res. 22, 837–858.

Uchida, T., Goto, S., 1994. Particle size studies for subcutaneous delivery of poly(lactide-co-glycolide) microspheres containing ovalbum as vaccine formation. J. Pharm. Pharmacol. 47, 556–560.

Wang, D.G., Zheng, H., Hu, Y.J., Qiu, S.M., 1995. Study on biocompatibility of degradable PELA copolymer. Beijing Shengwu Yixue Gongcheng (in chinese) 14, 173–177.

Yan, C.H., Rill, W.L., Malli, R., Hewetson, J., Naseem, H., Tammariello, R., Kende, M., 1996. Intranasal stimultion of long-lasting immunity against aerosol ricin challenge with ricin toxoid vaccine encapsulated in polymeric microspheres. Vaccine 14, 1031–1038.