Effects of Solvent Selection and Fabrication Method on the Characteristics of Biodegradable Poly(lactide-co-glycolide) Microspheres Containing Ovalbumin

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To demonstrate the effect of formulation conditions on the controlled release of protein from poly(lactide-co-glycolide) (PLGA) microspheres for use as a parenteral drug carrier, ovalbumin (OVA) microspheres were prepared using the W/O/W multiple emulsion solvent evaporation and extraction method. Methylene chloride or ethyl acetate was applied as an organic phase and poly(vinyl alcohol) as a secondary emulsion stabilizer. Low loading efficiencies of less than 20% were observed and the in vitro release of OVA showed a burst effect in all batches of different microspheres, followed by a gradual release over the next 6 weeks. Formulation processes affected the size and morphology, drug content, and the controlled release of OVA from PLGA microspheres.

Key words: PLGA, Ovalbumin, Microsphere, Solvent evaporation, Solvent extraction

INTRODUCTION

There have recently been a variety of attempts to improve the efficacy of currently available vaccines by using novel adjuvants or antigen delivery systems. This is because alum salts, the sole adjuvant which is currently approved for humans has a few disadvantages, including variability between batches, the need for refrigeration during storage, poor adjuvant effect, etc. (Uchida *et al.*, 1994). One approach of considerable interest is the use of poly(lactide-co-glycolide) (PLGA) microparticles with entrapped antigens as controlled release vaccines (Arshady, 1991; Thomasin *et al.*, 1996; Jeffery *et al.*, 1993). The use of these polymers for vaccine development may also provide prolonged antigen release over several months and overcome the primary drawbacks of the currently available vaccines, i.e., the need for repeated booster doses and the drop out rates from individuals receiving the first dose. The controlled release of vaccines prepared from PLGA polymers following a single immunization may obviate the need for booster doses (O'Hagan *et al.*, 1991).

In general, the selection of solvent and preparation methods are the most important parameters affecting the characteristics of PLGA microspheres (Cohen *et al.*, 1991; Yan *et al.*, 1994). The most commonly used microencapsulation technique using these polymers is based on the concept of solvent evaporation, employing methylene chloride and water as dispersed and continuous phases, respectively. A variety of methods that rely on this concept are well documented in numerous publications (Sah *et al.*, 1994; Yeh *et al.*, 1995; Yan *et al.*, 1994; Yeh *et al.*, 1996). However, the use of halogenated alkanes including methylene chloride is not desirable from the viewpoints of environmental and human safety since they are suspected as a carcinogen and mutagen (Sah, 1997). For this reason, ethyl acetate is considered a better solvent than dichloromethane due to its good miscibility to water. However, the effects of ethyl acetate on microsphere preparations have not yet been fully reported in the current literature.

The solvent evaporation method leads to decreased microencapsulation efficiencies of drugs (Benoit *et al.*, 1996). The solvent extraction method induces the formation of a polymeric barrier at the organic solvent-continuous phase interface, slowing the diffusion of the drug from the organic phase to the aqueous external phase and preventing crystal formation of the active principle (Cowsar *et al.*, 1985). Therefore, we have focused on the development of a microencapsulation process of a model protein, ovalbumin, utilizing ethyl acetate instead of dichloromethane as a dispersed solvent. Moreover, the

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preparation methods of solvent evaporation and solvent extraction via W/O/W multiple emulsification were also investigated.

MATERIALS AND METHODS

Materials

50:50 poly(DL-lactide-co-glycolide) (PLGA; molecular weight 40,000), poly vinyl alcohol (PVA; molecular weight 13,000-23,000, 87-89% hydrolyzed) and ovalbumin (OVA; chicken egg, grade V) were supplied by Sigma-Aldrich, USA. Sodium dodecyl sulfate (SDS) from Bio-rad, USA. Dichloromethane (DCM) and ethyl acetate (EA) were supplied by Fischer, USA. Micro-BCA assay reagent was supplied by Pierce, USA. All materials were used as received.

Preparation of PLGA microspheres containing ovalbumin

PLGA microspheres were prepared by the solvent evaporation method modified from a previous report (Jeffery *et al.*, 1993). Briefly, a solution of OVA in pH 7.4 phosphate buffered solution (PBS; internal aqueous phase) containing 2% PVA as a stabilizer was emulsified with an organic phase, dichloromethane or ethyl acetate containing 6% (w/v) PLGA polymer, using a homogenizer (X-520D, CAT, Germany) at 13,000 rpm in an ice bath for 5 min. The resulting water-in-oil emulsion was then emulsified by applying a magnetic stirrer at 600 rpm with a PVA solution to produce a W/O/W emulsion; it was stirred magnetically overnight at room temperature under reduced pressure to allow solvent evaporation. The microspheres were isolated by centrifugation, washed three times with distilled water, and freeze dried. The final product was stored in a desiccator at below 25°C. OVA and polymer ratio was fixed at 1:5(w/w) and the viscosity of the external aqueous phase was varied by dissolving PVA at different concentrations of 1, 5 and 10 w/v%.

On the other hand, microspheres were also prepared by the solvent extraction method modified from a previous report (Yan *et al.*, 1994). After W/O/W emulsion was produced as described above, the emulsion was diluted to an aqueous isopropyl alcohol solution (5v/v%) and agitated for 10 min. The solvent was eliminated rapidly by extraction and the microspheres formed were then collected by centrifugation, washed and freeze dried.

Optical microscopy

In order to observe the dynamic shrinkage phenomena during microsphere formation, photomicrographs were taken with a Leica DMLS microscope. This was preformed for each preparation at the time of preparation and overnight after preparation.

Particle size determination

Lyophilized microspheres were dispersed in pH 7.4 PBS by bath sonication in the presence of surfactant to prevent aggregation and then sized by laser diffractometry using a Malvern sizer/E (Malvern Ins., UK). Particle size is expressed as volume-surface mean diameter (d_{vs}) in micrometers.

Surface morphology

The surface appearance of the different PLGA microspheres were observed by scanning electron microscopy (SEM ; JEOL 35CF, USA). Freeze dried microspheres were mounted onto metal slubs using double sided adhesive tape, dried under vacuum and coated with gold-palladium.

Ovalbumin loading in microspheres

The protein contents of the microspheres were assayed by a previously reported method (Hora *et al.*, 1990). Briefly, 20 mg of lyophilized microspheres were digested in 5 ml of a 5% sodium dodecyl sulfate (SDS), 0.1N NaOH solution by shaking overnight on a Vibrax shaker (IKA, Germany) until the complete dissolution of the microspheres. The sample was centrifuged and bichinchoninic acid (BCA) protein microassay was used to determine the OVA concentration in the supernatant. From this result, the weights of OVA entrapped per dry weight of microspheres were determined. Each sample was assayed in triplicate. The loading efficiency (%) was expressed as the actual OVA loading to the theoretical OVA loading.

In vitro release of protein from PLGA microspheres

Accurately weighed lyophilized microspheres were dispersed in pH 7.4 PBS and retained in an orbital shaking incubator (JeioTech, SI900R, Korea) at 37°C with successive shaking at 250 rpm. At appropriate time intervals, the samples were centrifuged and the supernatant was assayed for released protein. Protein concentration in the release medium was analyzed by a micro-BCA assay. Release profiles were expressed in terms of the cumulative amount released with incubation time.

RESULTS AND DISCUSSION

Effects of processing conditions on the size and protein loading of microspheres

Photomicrographs of microspheres prepared by DCM or EA as an organic solvent displayed different behaviors in response to the time passed. The microspheres prepared with DCM (Figs. 1 and 2, upper pictures) were of a large particle in the first stage owing to the immiscibility in the water and followed by a size reduction in great part due

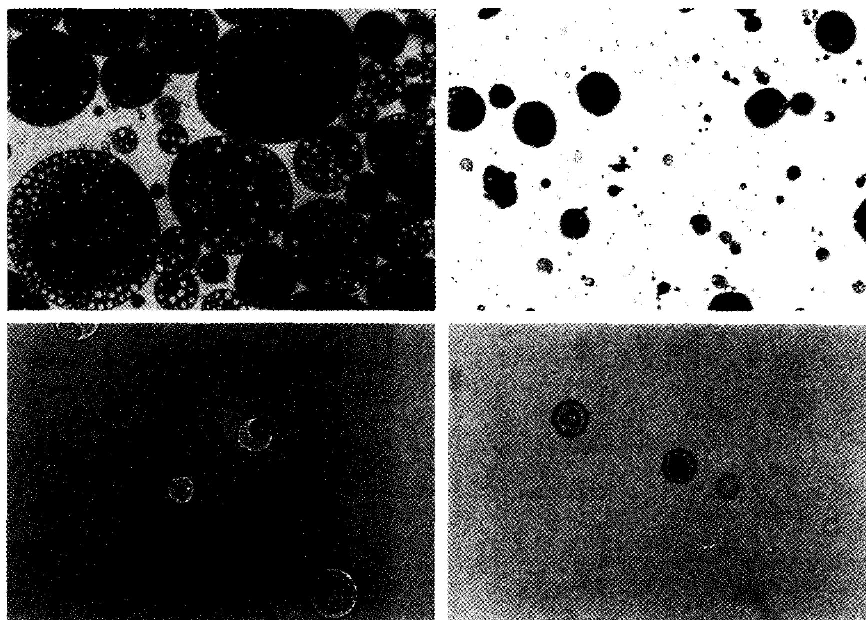

Fig. 1. Photomicrographs(×400) of PLGA microspheres prepared by the solvent evaporation method using dichloromethane(upper) or ethyl acetate(lower). The left panel represents the time immediately after preparation and the right panel the overnight standing.

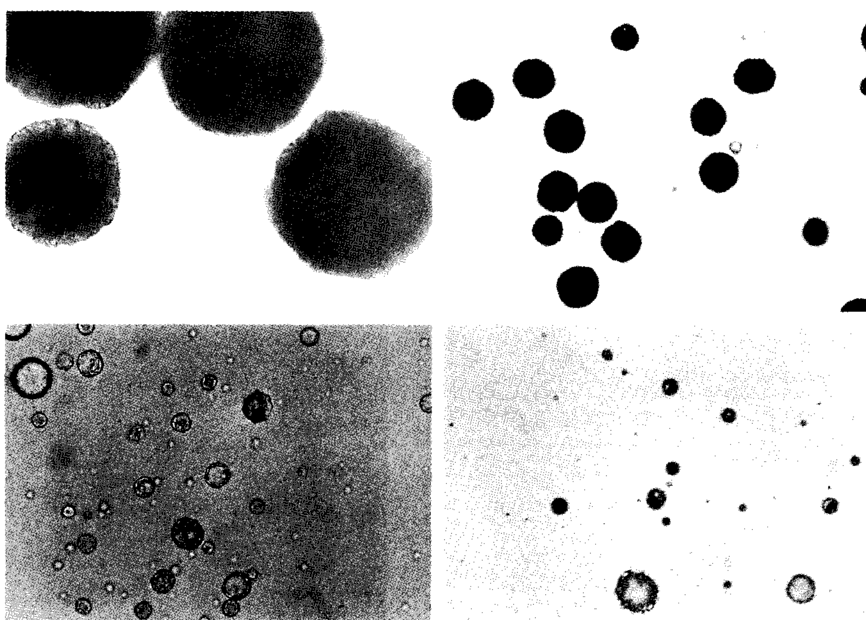

Fig. 2. Photomicrographs(×400) of PLGA microspheres prepared by the solvent extraction method using dichloromethane(upper) or ethyl acetate(lower). The left panel represents the time immediately after preparation and right panel the overnight standing.

to the diffusion of solvent to the continuous phase. However, the microspheres prepared with EA (Figs. 1 and 2, lower pictures) showed a smaller size in the initial stage due to excellent diffusion of the solvent to the continuous phase. This also revealed that the evaporation of DCM led to a significant shrinkage of the microspheres, although removal of EA was less prone to a reduction in particle size. In particular, the microspheres prepared with EA had a smaller size distribution and greater loading efficiency and protein loading than DCM microspheres in the same PVA concentration (Table I).

There was a size reduction by the increase of PVA

Table I. Physical properties of PLGA microspheres

PVA[a)] (%)	Microspheres[b)]	Protein loading (μg/mg)	loading efficiency (%)	Particle size (d_{vs}, μm)
1%	DEV	17.68 ± 0.19	11.20 ± 0.12	48.33
	DEX	7.16 ± 0.40	4.19 ± 0.01	20.11
	EEV	23.52 ± 0.82	14.47 ± 0.45	14.98
	EEX	26.28 ± 0.18	16.53 ± 0.10	15.42
5%	DEV	11.80 ± 0.59	7.46 ± 0.40	15.17
	DEX	17.58 ± 0.98	10.48 ± 0.40	15.16
	EEV	30.85 ± 0.35	19.34 ± 0.32	8.99
	EEX	27.31 ± 0.46	17.39 ± 0.26	14.36
10%	DEV	17.92 ± 1.13	10.96 ± 0.51	8.69
	DEX	17.45 ± 0.12	10.72 ± 0.21	4.38
	EEV	34.36 ± 3.21	19.96 ± 0.98	3.56
	EEX	19.48 ± 1.79	12.34 ± 1.14	2.33

[a)]represents PVA concentrations in W_2 phase.
[b)]D or E indicates the solvent used, dichloromethane or ethyl acetate, respectively; EV or EX indicates the preparation method, evaporation or extraction, respectively.

concentration, irrespective of the preparation method and solvent used, because PVA was used as an emulsion stabilizer by reducing the interfacial tension between the aqueous and the organic phases (Fig. 3). At proper concentrations, the emulsion stabilizing molecules diffuse well to the emulsion droplet/aqueous phase interface, resulting in a greater presence of stabilizer at the surface of the emulsion droplet formed during the preparative process. As the solvent evaporates from the system, these droplets harden to form the microparticles. Therefore, the final size of the microparticles is dependent on the dynamic size and stability of the emulsion droplets formed during agitation. At low PVA concentrations, small emulsion droplets are not stable, and the resulting microparticles are larger in size than those prepared with higher PVA concentrations. On the other hand, in order to evaluate protein denaturation during the microencapsulation process, size exclusion chromatography(SEC), SDS-PAGE, isoelectric focusing (IEF) and circular dichroism(CD) were examined. The structural integrity of OVA was unaffected by the processing condition (data is not shown).

SEM examination showed different morphological characteristics at various time periods during *in vitro* degradation. The microspheres showed an intact outer surface immediately after preparation (Fig. 4A). After 15 days in the releasing medium, small pores were generated (Fig. 4B). This was interpreted as surface erosion in which the microspheres adsorbed the water when it was immersed in the releasing medium. The pore size was increased with time and, at 30 days (Fig. 4C), the microspheres showed a greater erosion with a highly porous structure although they maintained their spherical shape. At 45 days, there was a collapse in the structure and the microspheres did not keep their spherical shape (Fig. 4D).

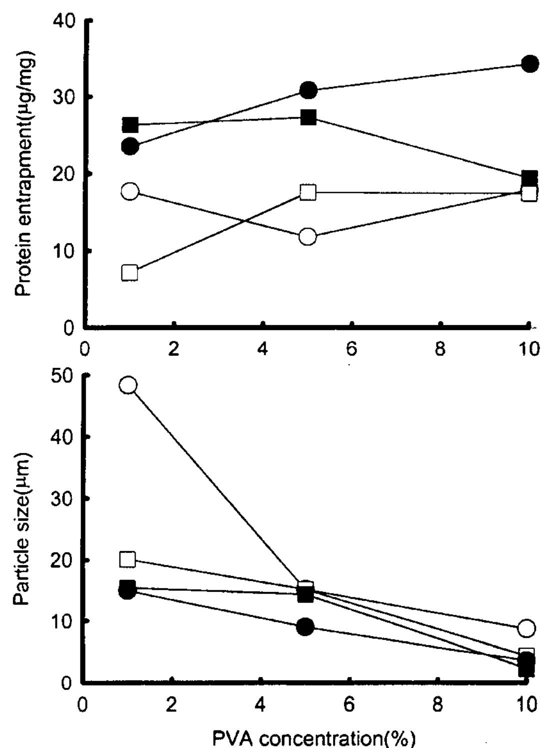

Fig. 3. PVA concentration dependency of PLGA microspheres on protein loading and particle size: ○, DEV; □, DEX; ●, EEV; ■, EEX

Protein release from biodegradable PLGA microspheres

Microspheres showed an initial burst release in the range of 10% to 40% and gradual release over the next 6 weeks (Fig. 5). This release study demonstrates that the drug release rate may be controlled by selecting the method of preparation and adjusting the particle size.

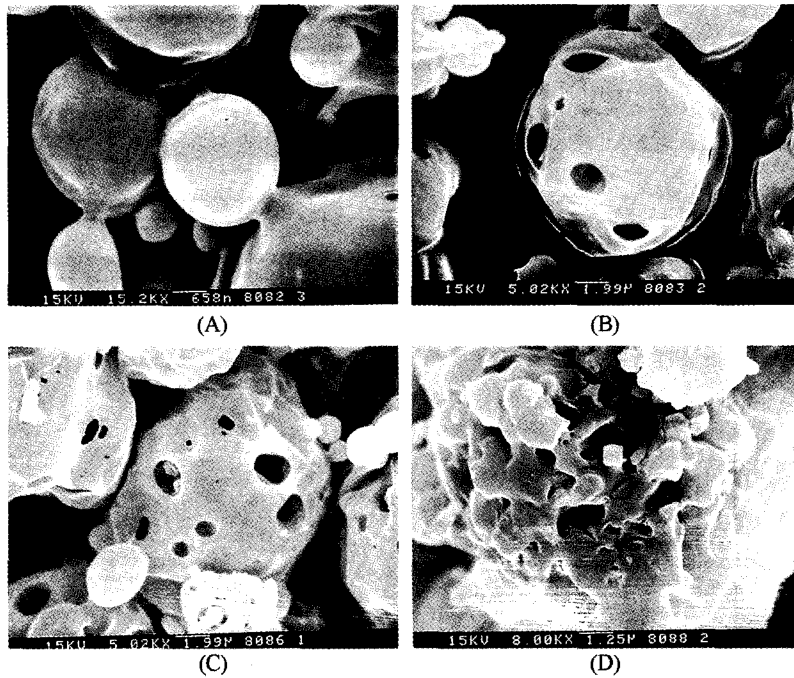

Fig. 4. Scanning electron microscopy(SEM) of PLGA microspheres at different degradation states: Immediately after preparation(A); After 15(B), 30(C), and 45(D) days in releasing medium at 37°C.

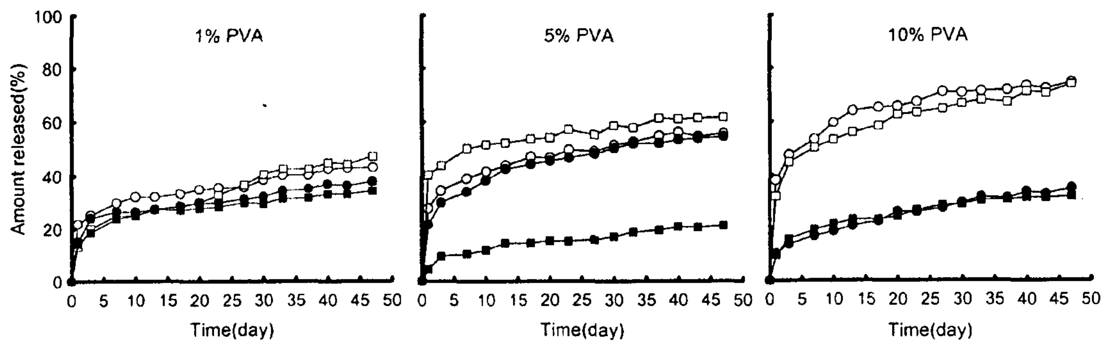

Fig. 5. Cumulative release profile of OVA from PLGA microspheres with various PVA concentrations: ○, DEV; □, DEX; ●, EEV; ■, EEX

Typically, the protein is released from PLGA microspheres in three phases: an initial burst, diffusion controlled release, and erosion controlled release (Creland and Jones, 1996; Cohen *et al.*, 1991). The initial burst phase is a rapid release (within a few days) of protein at or near the surface of the microspheres. The diffusion controlled release phase consists of protein diffusion through pores or channels in the microspheres and erosion controlled release is produced by polymer erosion (hydrolysis). Although the microspheres represent the typical release pattern of a bioerodible matrix system, there is a lack of additional release in the erosion state that might be due to low antigen loading or interaction of the PLGA polymer with ovalbumin. These phenomena are documented very well in numerous publications. Lai *et al.*(1993) reported 70% of isoprenaline release within a few hours. Takahiro *et al.*(1994) also reported that PLGA 50/50 microspheres with a small diameter(1.2 μm) showed an 80% burst release within one day, although additional release was very low as time passed.

CONCLUSION

Biodegradable PLGA microspheres have been successfully prepared by W/O/W emulsion solvent evaporation or extraction method with ethyl acetate. The results described in the present study show that the fabrication method, including solvent selection and PVA concentration, can influence the size, morphology, drug content, and the release of protein from PLGA microspheres. In the future,

these microspheres need to be extensively characterized *in vitro* and *in vivo* before being used parenterally as an antigen delivery system. Further studies will focus on the structural integrity of OVA during the preparation process and their immunogenicity in small animal models *in vivo*.

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