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The mechanism of surface-indented protein-loaded PLGA microparticle formation: the effects of salt (NaCl) on the solidification process

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The purpose of this study was to evaluate ovalbumin (OVA) leakage pathways and to explore the mechanism of the surface-indented microparticle formation in the preparation of OVA-loaded microparticles. OVA-loaded poly (D,L-lactic-*co*-glycolic acid) (PLGA) microparticles were prepared by a water-in oil-in water (w/o/w) solvent evaporation method associated with varied NaCl (NaCl) concentrations and adjusted with urea at 1240 mOsm kg^{-1} in the external aqueous phase. To evaluate dichloromethane (DCM)-related OVA leakage, three stirring rates, 600, 800, 1000 rpm at 25°C were carried out during the solvent evaporation stage. Both DCM and OVA levels in the external phase medium and total dispersion were sampled and measured. The time course of particle characteristics was evaluated by microscopy or SEM photography. The surface adsorptive capacities of the prepared microparticles were measured by using bovine serum albumin conjugated with fluorescein isothiocyanate (FITC-BSA). The findings were that the DCM-related OVA leakage accounted for ~34% of the total leakage. By combining NaCl in the external phase, a faster solidifying crust-like structure was formed as a barrier to remarkably reduce OVA loss and improve OVA content from 40.1 to 72.8 μg mg^{-1}. The yield and OVA content for formulations containing NaCl were much improved by the ionic effect, in addition to the osmotic effect. The total entrapment efficiency was also highly increased from 43 to 72%. The formations of the crust-like surface structure of the microparticle were affected by entrapped drugs, salt content in the external phase and aqueous volume in the inner phase. A scheme was proposed to interpret the formation mechanism of the surface-indented microparticles. In comparison to the surface-smooth microparticles, the surface adsorptive capacities of the surface-indented microparticles were highly improved from 26.6 to 87.0%, determined by the adsorption of FITC-BSA.

Keywords: PLGA, indented surface, salt effect, protein leakage, dichloromethane evaporation.

Introduction

Polylactic acid (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA) have been extensively investigated as delivery systems in therapeutic proteins and vaccines (Jiang and Schwendeman 2001, Yeh *et al.* 2002a). The approach of multiple

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emulsion (water-in oil-in water; w/o/w) combined with a solvent evaporation method is widely used to encapsulate drugs in PLGA or PLA matrices for preparing drug-loaded microparticles (Coombes *et al.* 1998, Yeh *et al.* 2002b). By using PLGA or PLA microparticle delivery systems, carried drugs are either adsorbed on the surface region or encapsulated in the whole polymeric matrix of microparticles (Herrmann and Bodmeier 1995, Yeh *et al.* 2002a, c). The limitations of protein-encapsulated microparticles are drug stability and loading capacity. The applied organic solvents and mechanic agitation usually impact the bioactivity of the loaded protein during the preparation process (Witschi and Doelker 1998). Alternatively, the release of adsorbed protein is rapid and transient to be applied for delivering protein drugs in a short time. Also, the surface adsorbed method could minimize the stability problem of protein drugs in organic solvent or under mechanic stress. Microparticles with an indented surface due to higher specific surface area could provide a stronger carrying capacity for drug adsorption than microparticles with a smooth surface.

For improving protein-loading of PLGA microparticles, a simple method has been applied to prepare protein delivery microparticles by combining sodium chloride (NaCl) in the external phase (Thomasin *et al.* 1997, Pean *et al.* 1998). Recently, a modified method was reported by combining NaCl in the external aqueous phase for preparing ovalbumin (OVA)-loaded PLA microparticles. The OVA content was greatly improved to achieve 96% of the theoretical content (Chen *et al.* 2002). The OVA distribution is predominately dispersed in the inner core region of the microparticles using 3% or higher NaCl. In addition, the prepared microparticle with an indented surface could adsorb a significant amount of the drug to provide its initial release. The preparation method might be potentially applied to therapeutic proteins or vaccine-delivery systems for achieving therapeutic effects.

The purpose of the study was to explore the effects of NaCl on the solidification process of the microparticles using OVA as a model drug and to evaluate the pathway of protein loss during drug encapsulation. In addition to OVA, sodium fluorescein and insulin were also studied and prepared as drug-loaded microparticles for comparison. Their surface characteristics were investigated to further explore the formation mechanism of surface-indented microparticles.

Materials and methods

Materials

Ovalbumin (grade V; OVA), insulin (human recombinant insulin), bicinchoninic acid solution (BCA), polyvinylpyrolidone (PVP; MW 40 000), NaCl, sodium hydroxide, urea and bovine serum albumin conjugated with fluorescein isothiocyanate (FITC-BSA) were ordered from Sigma (St. Louis, MO, USA). Sodium fluorescein was ordered from Merck (Darmstadt, Germany). Poly(D,L-lactic-*co*-glycolic acid) (PLGA) with a ratio of 50:50 (RESOMER RG503, MW ~ 9000, intrinsic viscosity 0.4 dl g^{-1} in chloroform) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Dichlormethane (DCM) was purchased from BDH (Poole, UK).

Microparticle characterization

OVA was encapsulated in PLGA microparticles by modifying previously reported methods using the w/o/w solvent evaporation technique (Yeh *et al.* 2002c). Four types of microparticles were produced. Their formulations are shown in table 1.

About 8–10 mg of freeze-dried microparticles were accurately weighed and treated with 3.0 ml of swelling agent (containing 0.1 M NaOH, 5% w/v sodium dodecyl sulphate) overnight in a 37°C shaking water bath. The supernatant was obtained after centrifugation to determine OVA content using the BCA method (Sigma, St. Louis, MO, USA).

Zeta potentials of prepared microparticles were measured by using a zeta potential analyser (ZetaPlus, Brookhaven, New York, USA).

Evaluation of microparticle solidification process.

Evaluation of solidification time during solvent evaporation. After the w/o/w multiple emulsion was prepared, then maintained at 25°C and stirred at 800 rpm using a magnetic stirrer to evaporate DCM. One millilitre of the dispersion was sampled and immediately centrifuged at 3200 *g* for 1 min and then redispersed using an ultrasonicator (Bransonic B2200R-1, Branson Ultrasonics, CT, USA, 125-W) for 30 s. The suspended sample, 0.5 ml, was withdrawn and diluted with 3 ml of water. The prepared sample was measured at 600 nm using a spectrophotometer (UV-160, Shimadzu, Japan) to evaluate the redispersity of samples by the absorbance. The extent of redispersity (%) was determined as follows (Chen *et al.* 2002):

$$\text{relative redispersity (\%)} = \text{absorbance}_t/\text{absorbance}_{60\,\text{min}} \times 100\% \qquad (1)$$

Determining dichloromethane (DCM) by gas chromatography (GC). Samples were spiked with an internal standard carbon tetrachloride and determined by a GC system (GC-17A, Shimadzu, Tokyo, Japan). In the analysis, nitrogen gas was used as a carrier gas. The GC system was equipped with a headspace sampler (HSS-4A, Shimadzu, Tokyo, Japan), a polyethylene glycol column (SGE, 25 m × 0.22 mm) and a flame ionization detector connected to a computer. The operating temperatures were set as follows: column oven: 80°C; injection port: 250°C; detector: 200°C; vial heater: 80°C; and syringe: 80°C. Analytical time was set at 6 min with a pre-heat time of 4 min (Wang and Schwendeman 1999).

Sampling methods in measuring protein leakage and DCM levels. During the period of DCM evaporation, two different volumes of total mixtures, 0.5 and 0.2 ml, were sampled. The 0.2 ml sample was analysed by the GC method to determine the DCM level in the total mixture. The 0.5 ml sample was centrifuged, then the supernatant samples were analysed by GC method and BCA method to determine the DCM and OVA levels in the external phase medium. Each sample was run in triplicate.

Microparticle shrinkage during solvent evaporation stage. During the DCM evaporation stage, the prepared w/o/w multiple emulsion was examined by using a microscope (BX-40, Olympus, Tokyo, Japan) connected with a digital camera (model: 1.5.0, Diagnostic Instrument Inc., USA) at suitable time intervals.

Table 1. Formulations, contents and physical properties of OVA-loaded PLGA microparticles prepared at 800 rpm and 25°C.

Formulations[a]	External phase (H_2O, 30 ml)				Yield (%)	Entrapment efficiency (%)	Dvs[b] (μm)	Protein[c] adsorption (μg mg^{-1})	Zeta potential (mV)
	Urea (%)	PVP (%)	NaCl (%)	Content (μg mg^{-1})					
F1	0	5	0	40.1	69.7	30.7	12.5	20.7	−15.4 ± 1.3
F2	10.3	5	0	51.3	76.1	42.9	16.5	26.2	−16.8 ± 1.0
F3	4.1	5	3	60.8	85.8	57.4	19.3	31.8	−23.8 ± 1.5
F4	0	5	5	72.8	89.5	71.6	15.6	38.7	−25.1 ± 3.8

[a] In four formulations, composed of the same primary emulsion, OVA 30 mg in 1 ml water as inner aqueous phase and 300 mg PLGA in 5 ml DCM as organic phase; [b] Dvs: volume-surface mean diameter; [c] Surface adsorption of BSA-FITC on OVA-loaded PLGA microparticle.

Adsorption of FITC-BSA

Bovine serum albumin (BSA) conjugated with fluorescein isothiocyanate (FITC-BSA) was dissolved in water to obtain a solution with concentration 400 μg ml^{-1}. Prepared microparticles, each 10 mg, were separately incubated in 2 ml of FITC-BSA solution at 25°C for 2 h. After centrifugation, the supernatant was measured by a spectrofluorophotometer (RF-5301PC, Shimadzu, Tokyo, Japan) with excitation and emission wavelengths of 515 and 528 nm.

Results and discussions

DCM evaporation and protein leakage

This study used the F1 formulation (without NaCl and urea in the external phase) to investigate the mechanism of protein-loading under four agitating rates of 400, 600, 800 and 1000 rpm to cause different DCM evaporative rates. Except for 400 rpm, blank or drug-loaded microparticles were successfully prepared in another three agitating rates. DCM evaporative rates from the external aqueous phase were calculated from the slopes of the lines by plotting the remaining DCM amount vs time (figure 1). As shown in table 2, the DCM evaporative rate increased as increasing the agitating rate. The DCM saturated concentration in the external phase had been determined to be 0.22 ± 0.01 M ($n = 3$ at 25°C). Determined C_m (0.20–0.22 M; table 2) approached the saturated level for the three agitating rates during DCM evaporation stage. The results suggested that the effluent rate of DCM from the organic droplet across the interface (between the internal phase or organic droplet and the external phase) was faster than the evaporative rate in the air/liquid interface, during the initial evaporation stage to maintain about a constant level of C_m. Thus, the evaporation of DCM followed a zero-order kinetic (figure 1). The rate limiting step of solvent evaporation was more likely in the air/water interface. The increase of DCM evaporative rate was caused by increasing interfacial area of air/liquid using different agitating rates.

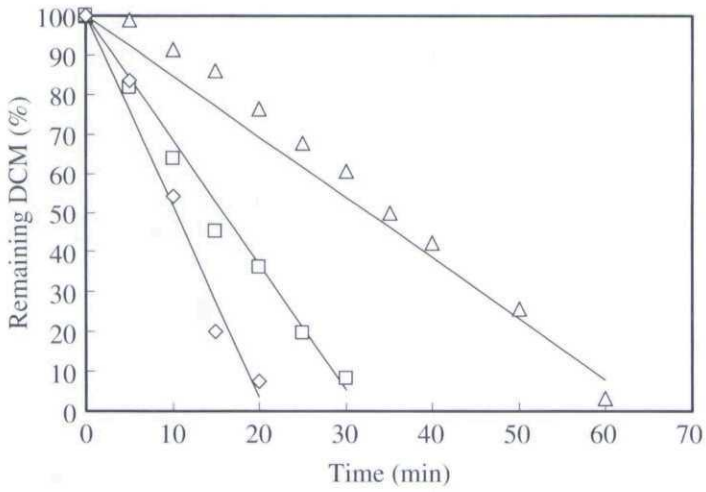

Figure 1. Remaining DCM in the total mixture during solvent evaporation stage at 25°C by using varied stirring rates for F1 formulation (containing 5% w/v PVP in the external phase). Key: (△) 600 rpm; (□) 800 rpm; (◇) 1000 rpm.

Table 2. The effects of stirring rate on the DCM evaporative rate and permeability during the period for extracting DCM from the w/o/w multiple emulsion (without NaCl or urea in the external phase) of F1.

Stirring rate (rpm)	Evaporative rate (mmole min^{-1})	A[a] (cm^2)	C_m[b] (M)	P[c] × 10 000 ($cm\,s^{-1}$)	OVA leakage rate ($\mu g\,min^{-1}$)	OVA leakage at 60 min (mg)
600	0.86	19.6	0.21	34.5	425	18.6
800	1.84	27.4	0.22	51.0	500	18.8
1000	2.02	31.2	0.20	54.5	525	20.1

[a] A: interfacial area of air/liquid during stirring; [b] C_m: DCM concentration in external aqueous medium; [c] P: Permeability coefficient was calculated by the following equation: P = evaporative rate/A/C_m.

One might further assess the relationship between DCM evaporative rate (R_{DCM}) and OVA leakage rate (R_{OVA}). A good linear relationship was found in the following equation:

$$R_{OVA}\ (\mu g\,min^{-1}) = R_{DCM}(mmole\,min^{-1}) \times 83.3\ (\mu g\,mmole^{-1}) + 352.3\ (\mu g min^{-1})$$
$$(r^2 = 0.99, p < 0.05) \quad (2)$$

According to equation (2), it was implicated that the DCM-related OVA leakage was 83.3 μg per millimole DCM evaporation. Then the total amount of DCM-related OVA leakage was calculated as 6.37 mg which accounted for ~34% of the total OVA leakage (OVA total leakage amount 18.78 mg at 60 min) in the solidification stage.

The relationship of OVA leakage, DCM evaporation and microparticle solidification

During the evaporation stage, as the DCM content of the organic phase diminished to a certain level, consequently the PLGA polymer was precipitated to encapsulate test agents to form microparticles. The formed solid particle showed a good redispersion but the non-solid dispersion had a tendency to merge or bind together during centrifugation. The degree of relative redispersity was used as an index to evaluate the solidification of microparticles. Figure 2 shows the time course of redispersity, DCM levels of the total mixture and external medium and protein leakage for F1 during the evaporation stage at an agitating rate of 800 rpm. The complete solidification time of the formulation was ~40 min. During the initial 0 ~ 15 min, the evaporation of DCM from the total mixture was described by a zero-order kinetic with an evaporative rate of 1.84 mmole min^{-1}.

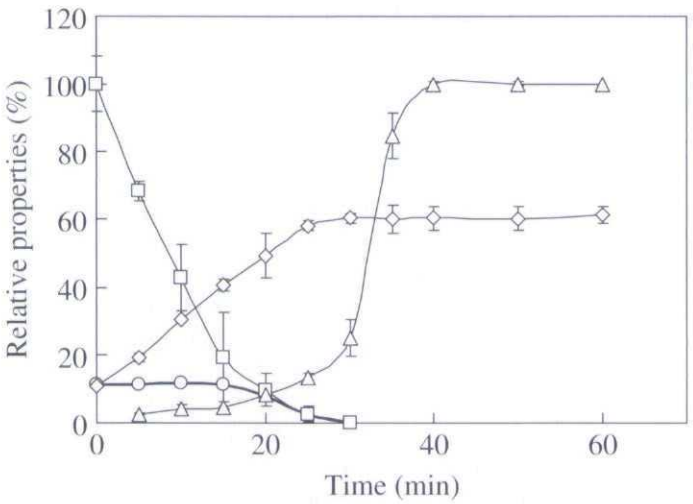

Figure 2. Relative properties of protein leakage, redispersity, DCM content in the total mixture and the external medium during the solvent evaporation process for F1 formulation with a stirring rate 800 rpm at 25°C. Key: (○) DCM content of the external medium (initial DCM content of the total mixture as 100%); (□) DCM content of the total mixture (initial DCM content of the total mixture as 100%); (△) redispersity (absorbance$_{60\,min}$ as 100%); (◇) protein leakage (the total leakage, 18.63 mg as 100%).

At 35 ~ 40 min, DCM concentrations in the total mixture and the external medium were below the detectable limit and coincided with the solidification time of F1. The OVA leakage from the PLGA dispersion into the external aqueous phase was also following a zero-order kinetic with a constant rate of 500.0 μg min^{-1} during 0 ~ 25 min (figure 2). After solidification, the OVA leakage was dramatically reduced. Thus, the curve for the cumulative amount of OVA leakage vs time gradually approached a plateau with a rate of 27 ± 8 μg min^{-1}.

Osmotic pressure and ionic effects on ovalbumin-loaded PLGA microparticle

The physical properties of OVA-loaded PLGA microparticles are summarized in table 1. Formulation F1 (without NaCl and urea in the external aqueous phase) was used as a control. In the external aqueous phase, F2 contained urea for adjusting osmolarity (1,240 mOsm kg^{-1}) but without NaCl, F3 contained 3% NaCl and 4.1% urea and F4 contained 5% NaCl. Formulations with NaCl providing higher ionic strengths dramatically improved the yield rate and OVA content from 76% and 51 μg mg^{-1} (F2) to 89% and 73 μg mg^{-1} (F4). The total entrapment efficiency was also increased from 43 to 72%. When comparing F1 and F2 to evaluate the osmotic pressure effect, the yield rate and OVA content were increased from 70 to 76% and from 40 to 51 μg mg^{-1}, respectively.

The mechanism of surface-indented OVA-loaded microparticle formation

Formulation components were regarded as important factors to form surface-indented microparticles. These included polymer (PLGA), protein (OVA or insulin), osmotic agent (NaCl or urea) and water content of the inner phase. To evaluate these factors, microparticles were also prepared using water only (blank), sodium fluorescence (F5) and an insulin aqueous solution (F6) for the inner phase, while combining 5% NaCl and 5% PVP for the external aqueous phase (same as F4). The insulin-loaded and OVA-loaded PLGA microparticles produced indented surface, but blank and sodium fluorescence-loaded microparticles formed smooth surface characteristics (figures 3 and 4). Yeh *et al.* (2002a) have reported that the *Vibrio Cholerae*-loaded PLGA microparticles prepared in a similar external phase containing 5% NaCl were smooth surface. From these results, it was found that microparticles with indented surface characteristics were successfully prepared only in certain conditions, such as using OVA and insulin as entrapped drugs associated with higher salt or urea in the external phases. The approaches for preparing an indented structure of drug-loaded microparticles were further discussed. Uchida *et al.* (1995) also prepared OVA-loaded PLA microparticles using 5% NaCl and PVP solution as an external continuous phase. A smooth instead of indented surface of the OVA-loaded PLA microparticle was obtained in their studies. Pistel and Kissel (2000) also reported a similar result. The major difference between Uchida *et al.*'s study and this report was that the former used 50 μl of the inner aqueous phase but it was 1 ml in this study. This suggests that the amount of water is an important factor in preparing surface-indented microparticles. Both OVA and insulin were surface active proteins that potentially interacted with PLGA polymer to stabilize the o/w interface. The polymer dispersion could be easily precipitated in a high concentration of salt or urea to form a crust-like structure. After the formation of a crust-like structure, the remaining liquid (water and DCM) flowed out, consequently the structure

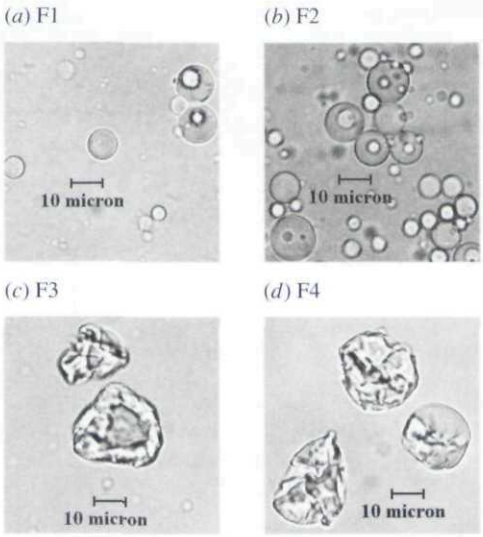

Figure 3. Transformations of surface morphology in four formulations of OVA-loaded PLGA microparticles during solvent evaporation stage at 15 min. Key: (*a*) F1, (control), 0% of NaCl and urea; (*b*) F2, 0% NaCl and 10.3% urea; (*c*) F3, 3% NaCl and 4.1% urea; (*d*) F4, 5% NaCl and 0% urea.

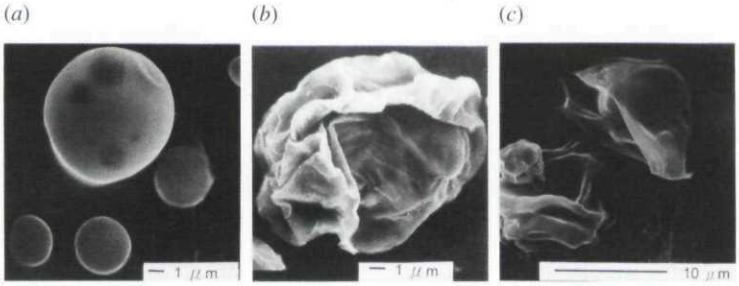

Figure 4. SEM photograms of OVA and insulin-loaded microparticles. Key: (*a*) OVA-loaded microparticle, 0% of NaCl and urea; (*b*) OVA-loaded microparticle, 5% NaCl and 0% urea; (*c*) insulin-loaded microparticle, 5% NaCl and 0% urea.

of the surface was contracted to become surface-indented or osmotic collapsed microparticles. An illustration for interpreting the formation of surface-indented microparticle is shown in figure 5.

Influcing OVA leakage by crust-like structures

The crust-like structure of the nascent microparticles also impeded the penetration of OVA (figure 6) and DCM (F4 in figure 7) from the inner aqueous phase across the interface into the external phase. The initial leakage of OVA at the end of the homogenization stage (0 min in figure 5) was 10–15% of the total leakage amount in the study. Thus, the major OVA leakage occurred in the DCM evaporation stage. The results for the impediment of OVA leakage by

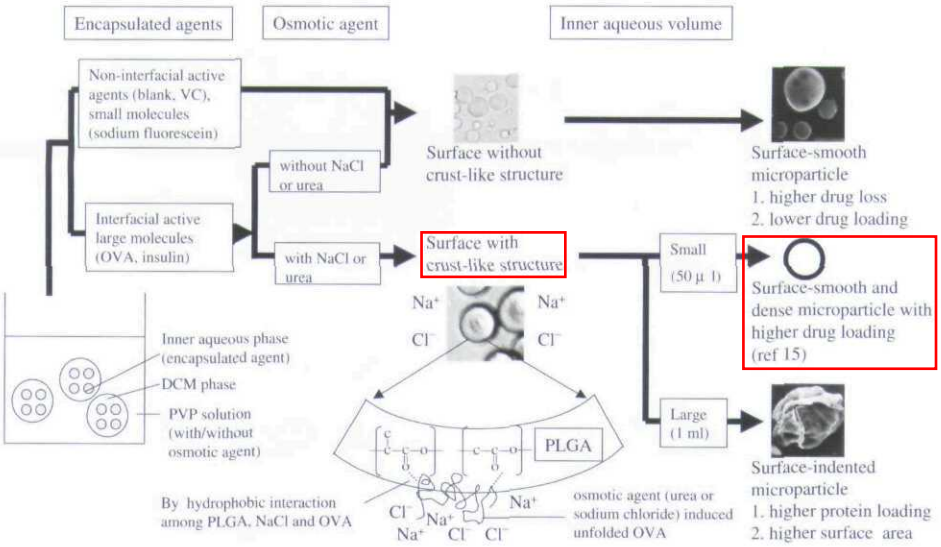

Figure 5. The mechanism of surface-indented OVA-loaded PLGA microparticle formation.

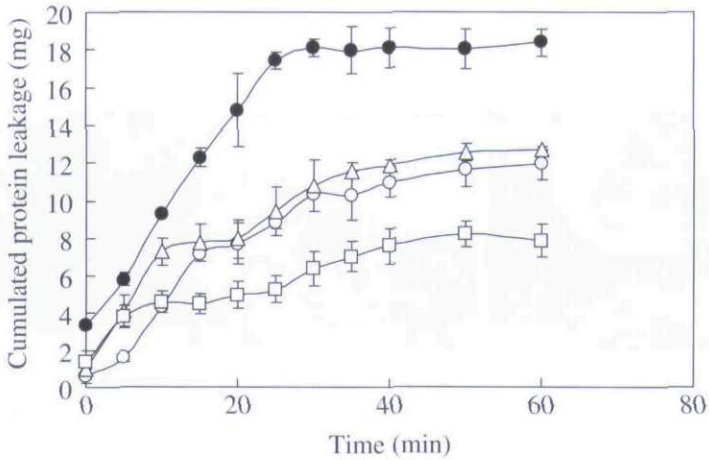

Figure 6. Cumulated OVA leakage during solvent evaporation stage with a stirring rate 800 rpm at 25°C, the external aqueous phase containing 5%w/v PVP and varied concentrations of NaCl and urea. Key: (●) F1 (control), 0% of NaCl and urea; (○) F2, 0% NaCl and 10.3% urea; (△) F3, 3% NaCl and 4.1% urea; (□) F4, 5% NaCl and 0% urea.

the crust-like structure were also concordant with the previous stated results for increasing OVA-loaded efficiency and microparticle yield rate by combining of NaCl and urea.

Adsorption of FITC-BSA on the surface of microparticles

In comparison, the osmotic effect for F2 (0% NaCl with urea) to F1 (0% NaCl without urea), the surface adsorbed FITC-BSA was increased from 20.7 to

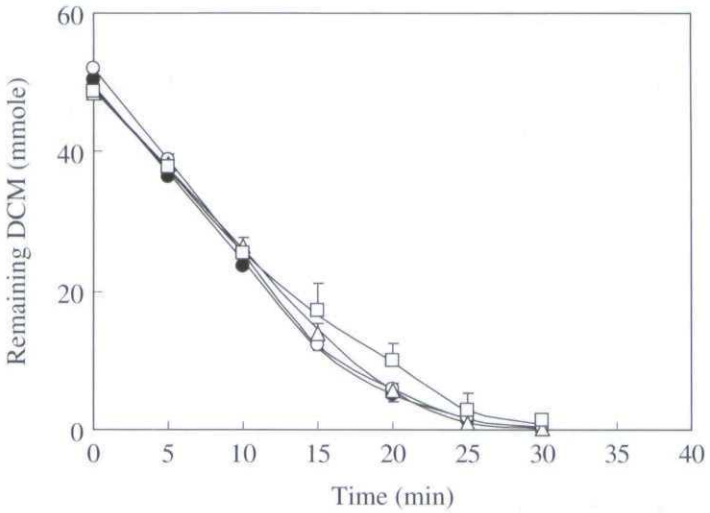

Figure 7. Remaining DCM in the total mixture during solvent evaporation stage with a stirring rate of 800 rpm at 25°C, the external aqueous phase containing 5%w/v PVP, and varied concentrations of NaCl and urea. Key: (●) F1 (control), 0% NaCl and urea; (○) F2, 0% NaCl and 10.3% urea; (△) F3, 3% NaCl and 4.1% urea; (□) F4, 5% NaCl and 0% urea.

26.2 μg mg^{-1}. To evaluate the non-osmotic effect of NaCl on the surface adsorption of OVA loading microparticles, formulations were investigated combining 0 ~ 5% NaCl and adjusting to an equal osmolarity (1240 mOsm kg^{-1}) with urea as the external phase. The adsorption capacities of OVA-loaded microparticles for FITC-BSA increased from 26.2 to 38.7 μg mg^{-1}, as increased the NaCl level of the external phase from 0 to 5% for F2 and F4 (table 1).

Conclusion

Protein leakage reduced the entrapment efficiency of protein-loaded PLGA microparticles. The major protein leakage was not occurring in the homogenization stage but in the DCM evaporation stage. By combining NaCl or urea in the external phase, the surface of PLGA polymeric dispersion rapidly formed a crust-like structure to impede OVA leakage and enhance drug loading and yield rate. After the remaining liquid outflow, the surface of the PLGA polymeric dispersion indented to characterize higher protein adsorptive capacity.

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