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RESEARCH ARTICLE

Effect of Osmotic Pressure in the Solvent Extraction Phase on BSA Release Profile from PLGA Microspheres

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ABSTRACT

*This study investigated the influence of osmotic pressure in the organic solvent extraction phase on release profile of bovine serum albumin (BSA) from poly(lactide-*co*-glycolide) (PLGA) microspheres. BSA-loaded PLGA microspheres with a target load of 10% were prepared by a double emulsion phase separation method. All the microsphere batches were fabricated in the same conditions except that in the organic solvent (CH_2Cl_2) evaporation step. Different concentrations of NaCl (0, 1.8, and 3.6%) or sucrose (20%) were used to generate a range of osmotic pressures in the extraction aqueous phase. These microspheres were characterized for incorporation efficiency, surface and internal morphology, particle size, protein stability, and in vitro release. The microspheres were spherical with particle size ranging from 16.8 to 27.8 μm. Higher osmotic pressure resulted in a denser internal structure although similar nonporous surface morphology was observed with all batches. No significant difference in encapsulation efficiency existed from batch to batch (87–94%). Sodium dodecyl sulfate–polyamide gel electrophoresis showed that BSA integrity was well retained. The release profile of the batch prepared with only water as the continuous (solvent extraction) phase exhibited a 79% burst release in the first 24 hr followed by a plateau and then a little release after 21 days. In the presence of NaCl or sucrose, the burst effect significantly decreased with increase in osmotic pressure in the extraction aqueous phase, which was then followed by sustained release for 35 days. A mass balance was made when the*

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release terminated. Therefore, in the organic solvent extraction and evaporation step, increasing the osmotic pressure in the aqueous phase both reduced the burst release from the microspheres and improved the subsequent sustained release profile.

Key Words: *BSA; Microsphere; Osmotic pressure; Release; Solvent extraction*

INTRODUCTION

In recent years, controlled release of therapeutic peptides and proteins from biodegradable polymeric devices has been extensively studied.[1–4] Injectable poly(lactide-*co*-glycolide) (PLGA) or poly-lactide microspheres are being utilized as a sustained release carrier due to their proven record of good biodegradability[5] and biocompatibility.[6] Controlled release dosage forms can be used both to reduce the total drug amount required and to increase the patient compliance due to fewer doses. In many of the reported microsphere formulations, immediately after placement in the release media, an initial large amount of drug is liberated prior to a sustained and controlled release profile.[7–9] This so called "burst effect" results in a high initial drug delivery, which could be either advantageous or detrimental depending on the desired application. For example, in certain situations, high initial rates would be favorable if a rapid onset of pharmacological effect is desired, such as the drug administration strategy for wound treatment[10] or immediate testosterone suppression from chemical castration of GnRH antagonists.[11]

In most cases, the burst release is considered a negative consequence because firstly, it reduces the effective lifetime of the device due to lack of effective utilization of the initially released drug, and then, more importantly, the initial high release rates could result in drug levels near or exceeding toxic threshold levels in vivo; whose occurrence could lead to considerable or even detrimental side effect in therapeutic applications.[12] Moreover, burst release is often unpredictable, and even when desired, the amount of burst cannot be well controlled.[13]

Currently, PLGA microspheres seem to present a greater challenge to deliver proteins. In many studies dealing with the encapsulation of protein in PLGA microspheres, the protein release is often characterized by a marked burst immediately followed by a plateau or an extended period of slow continuous release.[14–16] Often, the total amount of protein released from microspheres is incomplete.[17–19] This warrants the need to better understand the release behavior of such compounds to optimize the formulation. The double-emulsion solvent extraction/evaporation technique is one of the most popular methods to encapsulate protein drugs. The release pattern depends on polymer nature, morphology, drug distribution within the microsphere, and release conditions. Generally, porous microspheres have a large surface area and hence a high initial burst.[20] The method and rate of solvent removal, dispersed-phase/continuous-phase ratio, polymer concentration, and polyvinylalcohol (PVA) concentration in the inner aqueous phase are critical factors in determining the morphology and ultimately influencing the drug release.[21,22] Understanding how the solvent dilution/extraction process affects the release performance of microspheres will be an asset in formulation development.

The objective of this study was to investigate a formulation parameter, osmotic pressure in the solvent extraction phase, on the protein profiles from PLGA microspheres. This information is essential for microsphere optimization because it provides an easy and potential approach to achieve a desired release pattern with controlled burst, which may be practical in scale-up production. To achieve this objective, bovine serum albumin (BSA) was selected as a model protein for encapsulation into PLGA microspheres by a double-emulsion solvent extraction/evaporation method.

MATERIALS AND METHODS

Materials

PLGA was obtained from Boehringer Ingelheim (PLGA 50/50, Resomer RG 503H, Ingelheim, Germany). PVA with a M_w range 30,000–70,000 was purchased from Sigma Chemical Company, St. Louis, MO. MicroBCA Kit was supplied by Pierce, Rockford, IL. Sodium chloride, BSA, and sucrose were all purchased from Sigma. Other reagents were of analytical grade.

Preparation of Microspheres

BSA-loaded microspheres were produced according to a W/O/W emulsion solvent extraction method. Fifty

milligrams of BSA were dissolved in 250 μL water and emulsified in an organic solution (20% PLGA in CH_2Cl_2) by vortexing. The emulsion was injected into 50 mL of 6% PVA, which was saturated with CH_2Cl_2 and maintained at 4°C. A double W/O/W emulsion was formed by homogenization at 4000 rpm for 2 min. While overhead stirring, the resultant emulsion was transferred to 1 L of solvent extraction phase, in which different concentrations of NaCl (0, 1.8, 3.6%) or sucrose (20%) were dissolved in deionized water to generate osmotic pressure. The extraction process was maintained at 4°C for 3 hr with mechanical stirring. Finally, the microspheres were filtered on a 0.45-μm filter, washed 3 times with 2 L deionized water, and freeze-dried to obtain a free-flowing powder. The dried particles were stored at 4°C.

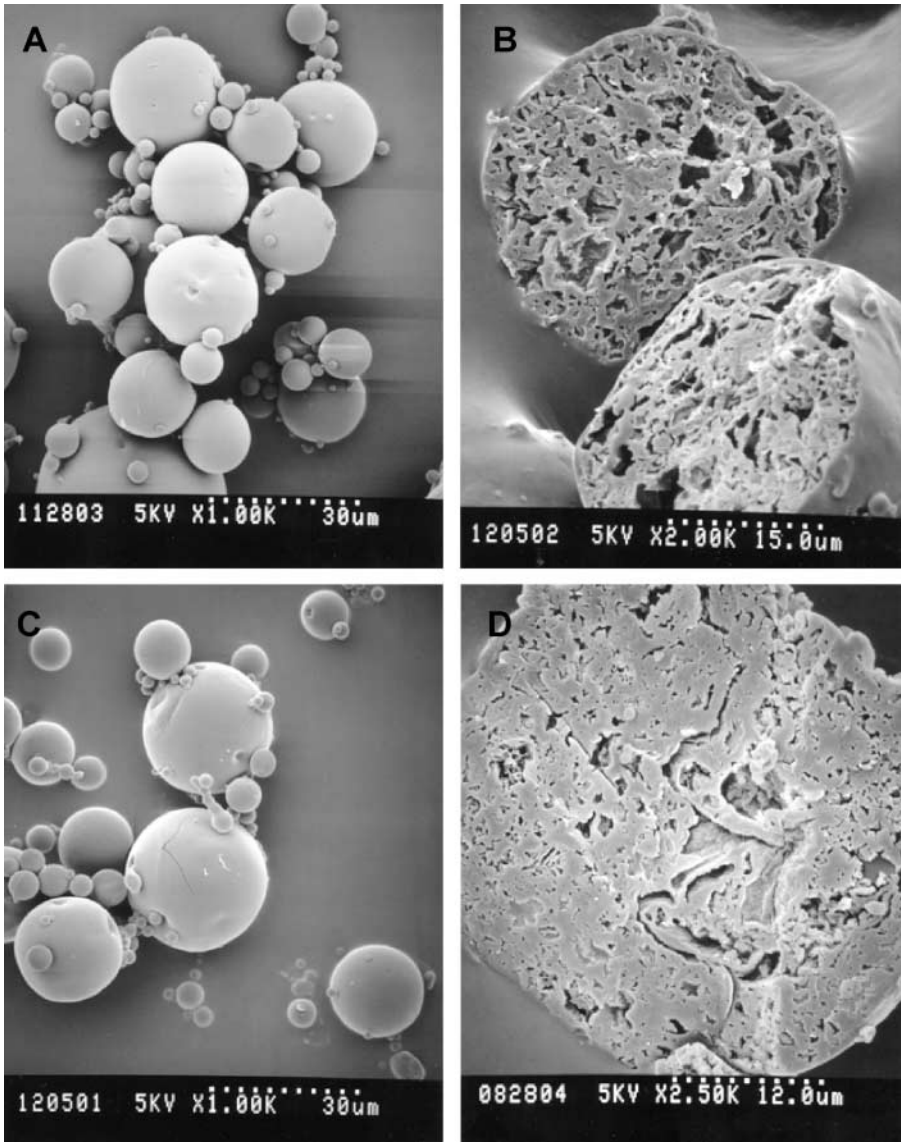

Figure 1. Scanning electron micrographs: Surface (A) 1000 × magnification and cross-section, (B) 2000 × magnification of microspheres prepared with water as organic solvent extraction phase; Surface (C) 1000 × magnification and cross-section, (D) 2500 × magnification of microspheres prepared with 3.6% NaCl in solvent extraction phase.

Table 1

Characterization of BSA-Loaded Microspheres

Batch #	Extraction Phase	Target Loading (%)	Encapsulation Efficiency (%)	Size (μm)			Initial BSA Burst (%)
				D (v, 0.1)[a]	D (v, 0.5)[a]	D (v, 0.9)[a]	
1	Water	10	88.8	5.5	16.8	49.8	78.7
2	20% sucrose	10	87.0	5.0	16.9	54.0	52.4
3	1.8% NaCl	10	94.0	6.3	21.9	65	40.6
4	3.6% NaCl	10	92.9	7.7	27.8	74.4	32.9

[a] D (v, 0.5): average diameter, D (v, 0.1): the diameter at which 90% particles are larger, D (v, 0.9): the diameter at which 90% particles are smaller.

Determination of BSA Content in the Microspheres

The BSA encapsulation efficiency was determined by a microsphere digestion method. Dried microspheres (10 mg) were dissolved in 1 mL of 1 *N* NaOH, and 1 mL of 1 *N* HCl was added to neutralize the solution. The resulting solution was diluted with PBS for MicroBCA protein assay to quantify BSA content. Incorporation efficiency was expressed as the ratio of actual and theoretical BSA loading.

In Vitro BSA Release Study

In vitro release tests were carried out in duplicate at 37°C. Dried microspheres (10 mg) were suspended in microcentifuge tubes containing 1.5 mL of PBS and rotated. At predetermined time intervals, the supernatant from each tube was removed after centrifugation and replaced with fresh buffer. The BSA content of the supernatant was analyzed by MicroBCA method.

Mass Balance Study

When BSA release study was terminated at day 35, the remaining microspheres were collected and free dried. The BSA in the residual microparticles was determined as described in "Determination of BSA Content in the Microspheres." The sum of the residual and released protein was calculated and compared with the actual drug content.

Particle Size Analysis

Dried microspheres were suspended in 0.1% Tween 80 and size distribution was measured with a forward laser light scattering sizer (Malvern Instruments, Worcestershire, England).

Sodium Dodecyl Sulfate–Polyamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE of 9% polyacrylamide gel was performed under voltage mode of 150 V. A low M_w protein marker (Sigma) was used as a molecular weight standard. The technique to extract protein from microspheres was modified from that reported by Blanco and Alonso.[23] In brief, 5–7 mg of microspheres were mixed with sample buffer and boiled at 100°C for 3 min. The PLGA microsphere melted and BSA released and interacted with SDS. The solution was cooled to room temperature and spinned down to precipitate PLGA. The supernatant was loaded onto the gel and after electrophoresis, the gel was stained with Coomassie Blue.

Scanning Electron Microscopy

Surface and internal morphology of the prepared microspheres was examined by scanning electron microscopy (SEM) (Hitachi Model S800, Hitachi Naka City, Ibaragi, Japan).

RESULTS

Spherical microspheres were produced in each batch with relatively nonporous surfaces. The introduction of NaCl in the solvent extraction phase significantly lowered the inner pore size, but did not substantially alter the surface morphology (Fig. 1). For example, without salt or sucrose, PLGA microparticles exhibited a thin skin shell surrounding a porous matrix. Numerous internal cavities

existed between clumps of polymer and the cavity size was in a range between 0.3 and 3.5 μm. Some cavities were inter-connected with channels extending from near the surface to the inner core of the microspheres. In Fig. 1d, the microspheres appeared denser and harder with the addition of 3.6% NaCl. Cavity space was smaller and the number of cavities was also reduced.

Table 1 shows that high encapsulation efficiencies of 87–94% were achieved and the average size of the microspheres ranged from 16.8 to 27.8 μm, which was syringeable with a #20 gauge hypodermic needle. The presence of NaCl or sucrose in the organic solvent extraction phase seemed to result in larger microspheres.

The protein size was assessed by SDS-PAGE (Fig. 2). The intact form of BSA in each batch was observed at the 66K position, suggesting that protein stability was retained in all cases.

Figure 3 shows the release profiles from the different batches of BSA microspheres. The addition of either sucrose or salt reduced the intensity of the burst effect from 79 to 52% over the first 24 hr for the sucrose batch and from 79 to 41 and 33% for the 1.8 and 3.6% NaCl batches, respectively. As shown in Fig. 3, the release profile of the batch prepared with simply water in the extraction media exhibited a 79% burst release in the first day followed by a plateau and then gradual release after 21 days. The inclusion of NaCl or sucrose during solvent removal decreased the burst release. The first day release with 20% sucrose, 1.8% NaCl, and 3.6% NaCl batches, were 52, 41, and 33%, respectively. After the initial burst, the subsequent release was essentially linear although a slight enhancement of release occurred after 21 days. The profiles in the batches prepared with sucrose and NaCl are essentially the same after the first day.

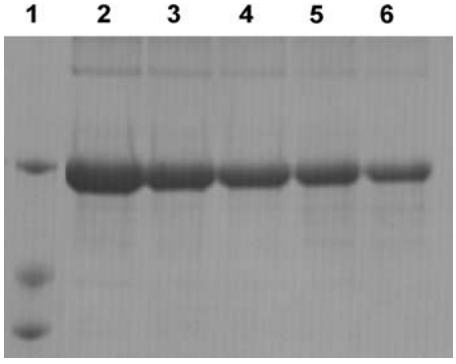

Figure 2. BSA stability analysis by SDS–PAGE. Lane 1, MW marker (BSA 66,000, ovalbumin 45,000, glyceral-3-phosphate dehydrogenase 36,000). Lane 2, BSA standard. Lanes 3, 4, 5, and 6 are BSA from microspheres Batch #1, #2, #3, and #4, respectively.

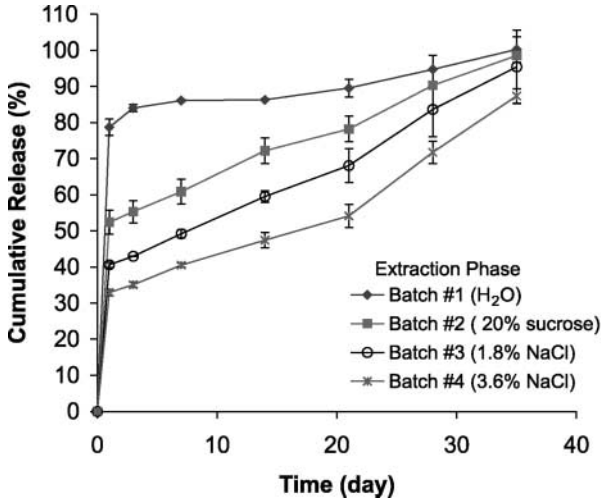

Figure 3. The effect of organic solvent extraction phase during the microsphere preparation on the BSA release profiles.

Good mass balance (Fig. 4) in the range 99–113% of the loaded BSA amount was obtained upon the termination of the study.

DISCUSSION

Increasing the osmotic pressure in the aqueous continuous phase during solvent extraction and evaporation reduced protein burst release from PLGA microspheres and improved the sustained release profile. The extent of the decrease in initial burst release seemed to be more significant with the higher osmotic pressure in the bulk aqueous phase.

There have been attempts to investigate the burst release and prevent it technologically, such as post coating with gelatin film,[24] complexation of drug with cyclodextrin,[25] and reducing drug particle size.[26] In this research, a simple approach of adjusting osmotic pressure provides an alternate way to control burst. The physical states of a PLGA solution were divided into three transitional phases by Li et al.,[27] as shown in Fig. 5, the solution, gel, and glass states. The solution–gel transition is called the viscous boundary according to Wijmans et al.[28] This model shows that when a W/O primary emulsion is transferred to the bulk aqueous phase, the physical states of the polymer phase are coupled with mass transfer of solvent to determine the progress of the microsphere formation. Based on Li's

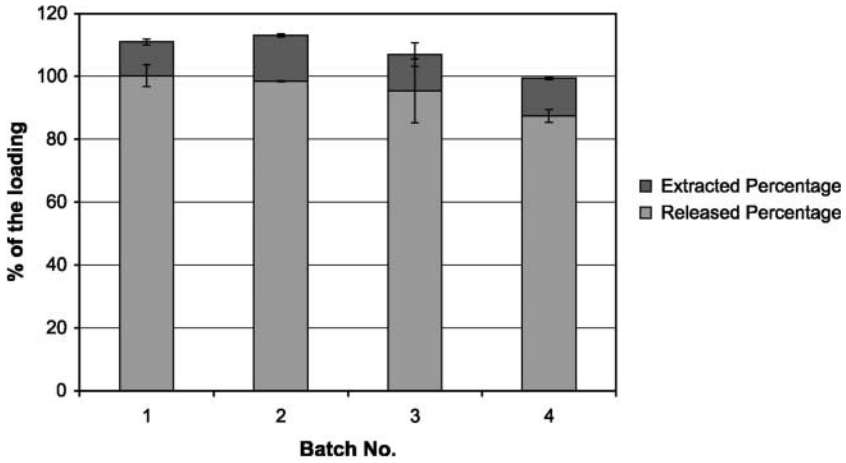

Figure 4. Mass balance of BSA upon termination of release study on day 35.

transitional state model, a proposed mechanism is shown in Fig. 6 to explain the in vitro release and size distribution.

In the W/O/W double emulsion, the polymer solution in CH_2Cl_2 forms a barrier between the inner and outer aqueous phases. Since CH_2Cl_2 has about 2% solubility in water, water molecules were able to partition into this organic phase and subsequently move to the outside continuous phase. Large protein molecules, however, were restricted in their diffusion through this organic phase because of their size and their low miscibility. Additionally, the highly charged NaCl was restricted in its diffusion through the organic polymer barrier. Therefore, the polymer phase actually acted as a semipermeable membrane between the inner aqueous phase and outer continuous phase. When NaCl is added to the outside bulk aqueous phase, an osmotic pressure gradient is generated between the continuous phase (W_2) and inner aqueous phase (W_1). The high osmotic pressure in W_2 causes water to flow from the inner aqueous phase to the outside. In this way, the NaCl batches will have smaller inner cavities thereby contributing to a lower burst release. This expectation was consistent with the release profile as well as with the internal morphology of the microspheres shown in Fig. 2. In contrast, if the continuous phase were simply water, the volume of the inner aqueous phase would not decrease due to the lack of osmotic gradient and therefore, these large aqueous droplets may interconnect when the O/W_2 interface, i.e., the boundary of polymer phase, condenses inward. In fact, if W_1 has a high concentration of protein, it may result in a higher osmotic pressure in W_1 compared to W_2, which could result in an inward water flow and make the inner W_1 droplet larger. The resulting high matrix porosity enhances the burst due to the easier accessibility of protein molecules by the dissolution medium.

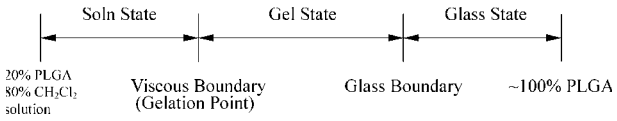

Figure 5. Physical states of polymer solution (extracted from Ref. [27] and slightly modified).

In the microsphere preparation, with the progression of solvent (CH_2Cl_2) removal, the polymer solution is more concentrated. Consequently, the flow of water through the more viscous polymer phase slows down and, therefore, the solvent removal rate will also have an impact on water diffusion. The addition of NaCl to W_2 increases the polarity and thus, causes CH_2Cl_2 to be less soluble in W_2. This will result in slower solvent removal and allow the microsphere to remain softer for a longer period of time. Therefore, water in W_1 had sufficient time to diffuse through the polymer phase to W_2, making the microsphere denser. In contrast, faster solvent removal led to an increasing and more widely distributed pore size.[29]

The 20% sucrose batch reduced the burst to 52 from 79% in the water batch, which could also be explained by the osmotic effect. The less burst reduction from sucrose compared to NaCl could be attributed to the lack of ionization. Nevertheless, the sucrose molecules may still diffuse through the semipermeable barrier of the polymer

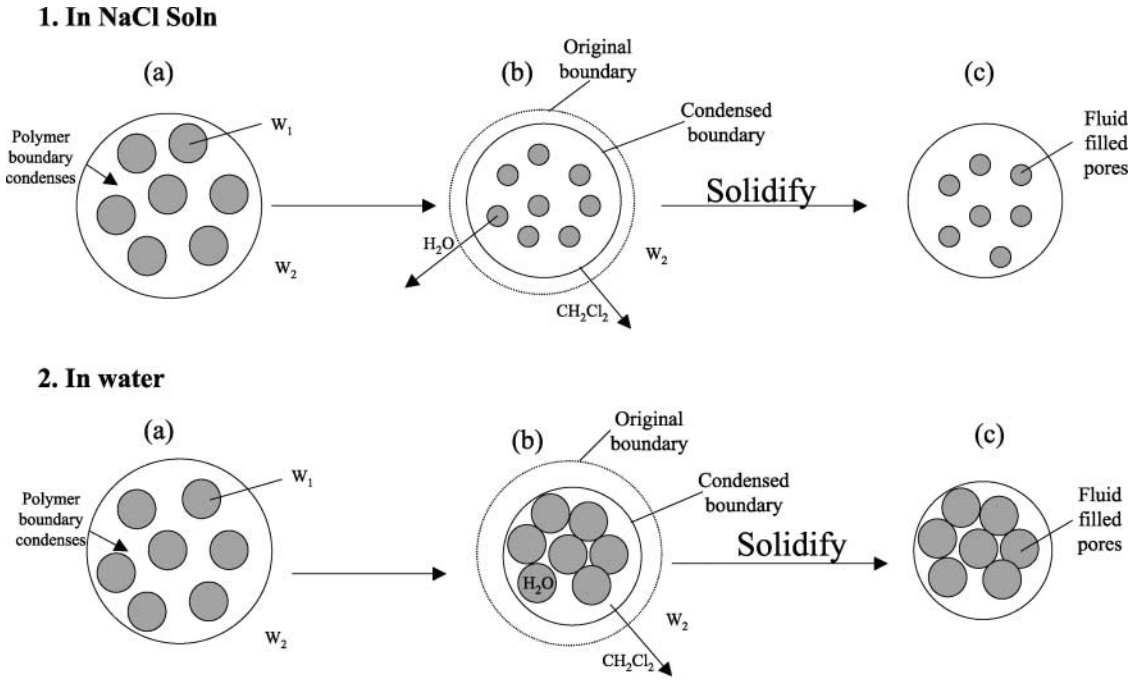

Figure 6. Proposed mechanism resulting in different release and particle size by the addition of NaCl in W_2. 1. In NaCl solution. (a) $W_1/O/W_2$ double emulsion is formed. Polymer boundary condenses slowly with slow solvent removal. (b) H_2O in W_1 flows out due to the osmotic pressure gradient. (c) Polymer boundary solidified, resulting in denser and bigger microspheres. 2. In water. (a) $W_1/O/W_2$ double emulsion is formed. Polymer boundary condenses rapidly with rapid solvent removal. (b) No H_2O outflow due to the lack of osmotic pressure gradient. (c) Polymer boundary solidified, resulting in more porous and smaller microspheres.

phase, even in small amounts sufficient to reduce the osmotic pressure gradient.

The addition of either sucrose or NaCl caused a larger size particle than the water batch. The particle size difference could also be ascribed to the rate of organic solvent extraction and solidification of PLGA. When the double emulsion was transferred to the large volume of solvent extraction phase, CH_2Cl_2 partitions into the water, thereby condensing the polymer phase. The boundary of the whole droplet then moves inward as shown in Fig. 6. In the presence of NaCl, CH_2Cl_2 is expected to have lower solubility and slower extraction into the NaCl solution. Slower CH_2Cl_2 removal results in slower inward movement of the W_1/O interface, i.e., the shell of polymer phase, which in turn, gives rise to larger microspheres when the shell or cortical layer finally solidifies. With respect to the sucrose batch, a size increase was not observed, since sucrose did not change the polarity of W_2 as substantially as NaCl.

Péan et al.[20] used 10% NaCl in the extraction medium to prepare nerve growth factor (NGF)-loaded PLGA microspheres. Even though a remarkably reduced burst effect was achieved, unfortunately, the high salt induced a parallel pronounced NGF denaturation. The NGF release profile also quickly plateaued after the burst. More recent work of Péan et al.[30] found that at the same salt concentration in the extraction medium, NGF stability was improved by co-encapsulation of PEG 400. In this work, the BSA stability was well retained in the prepared microspheres at NaCl concentrations of 1.8 and 3.6%. Release of BSA was also sustained over a one-month period following the burst and culminated in almost complete exhaustion of the protein. In Uchida's work,[31] it was found that the addition of 10% (w/v) NaCl to the external aqueous phase reduced insulin loading efficiency compared to the case where no NaCl was added. In our study, BSA encapsulation efficiency was in fact slightly higher with the NaCl batches than the batch with water. Two reasons for the difference are a lower NaCl concentration and the size difference of the protein employed. In our case, the results suggested that a lower concentration of salt could be used for the purpose of reducing the burst without adversely affecting protein stability or loading efficiency. Alternatively, for very sensitive proteins, sugars could be used instead of salt to control the burst since sugars have

been well known for their protective effect on protein stability.

CONCLUSION

In the organic solvent extraction and evaporation step of microsphere preparation, increasing the osmotic pressure in the aqueous continuous phase reduced the protein burst release from PLGA microspheres, as well as improving the sustained release profile and retaining BSA integrity.

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