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Prevention of structural perturbations and aggregation upon encapsulation of bovine serum albumin into poly(lactide-*co*-glycolide) microspheres using the solid-in-oil-in-water technique

Ingrid J. Castellanos, Wanda L. Cuadrado and Kai Griebenow

Abstract

Bovine serum albumin (BSA) was encapsulated into poly(lactide-*co*-glycolide) (PLG) microspheres by a solid-in-oil-in-water (s/o/w) technique. We tested whether perturbations in BSA secondary structure could be minimized during encapsulation by using trehalose and how this would influence BSA aggregation and release. BSA secondary structure was monitored non-invasively by Fourier-transform infrared spectroscopy. When BSA was co-lyophilized with trehalose, lyophilization-induced structural perturbations were significantly reduced. The formulation obtained (BSA-Tre) was encapsulated into PLG microspheres and, by optimizing critical encapsulation parameters, a loading efficiency of 85 % was achieved. However, due to the loss of the excipient in the o/w emulsion step, the structure of BSA-Tre was more perturbed than before encapsulation. Excipient-loss and encapsulation-induced structural perturbations could be prevented by saturating the aqueous phase in the o/w step with trehalose and by using the organic solvent chloroform. This in turn reduced the formation of soluble BSA aggregates. BSA was released from PLG microspheres using the improved formulations with an initial release in 24 h of not more than 22 %, followed by a sustained release over at least 2 weeks. In summary, optimization of the encapsulation conditions in the s/o/w procedure resulted in the encapsulation of BSA without procedure-induced structural perturbations and minimized the release of aggregated protein. This demonstrates that the s/o/w technique is an excellent alternative to the most common encapsulation procedure, namely the water-in-oil-in-water technique.

Department of Chemistry, University of Puerto Rico, Río Piedras Campus, PO Box 23346, San Juan, PR 00931-3346, USA

Ingrid J. Castellanos, Wanda L. Cuadrado, Kai Griebenow

Correspondence: K. Griebenow, Department of Chemistry, University of Puerto Rico, Río Piedras Campus, PO Box 23346, San Juan, PR 00931-3346, USA.

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Introduction

The preparation of protein-loaded microspheres has attracted much attention in recent years to enable the sustained delivery of pharmaceutical proteins to humans and animals (Schwendeman et al 1996). In this context, the development of formulations that prevent detrimental changes to proteins upon encapsulation into the polymer devices, and release from such polymers is of the utmost importance, because the preparation of protein-loaded microspheres is associated with various conditions imposing chemical and physical stresses on labile pharmaceutical proteins (e.g. lyophilization, shearing forces, exposure to hydrophobic interfaces, polymer adsorption) (Schwendeman et al 1996; Griebenow et al 1999). The most commonly used water-in-oil-in-water (w/o/w) encapsulation technique has been associated with severely diminishing the bioactivity of many proteins

(Johnson et al 1991; Lu & Park 1995a, b; Chen et al 1997). This effect can be largely attributed to the formation of the first w/o emulsion, in which the protein is exposed to a water–organic solvent interface, which causes unfolding and aggregation of proteins (Schwendeman et al 1996; Morlock et al 1997; Sah 1999; Li et al 2000; Van de Weert et al 2000; Pérez & Griebenow 2001). An alternative encapsulation procedure is the solid-in-oil-in-water (s/o/w) technique, which uses dehydrated protein powders to create a suspension in an organic solvent, followed by emulsification in an aqueous solution to afford microsphere creation and hardening. This eliminates the w/o emulsion step and thus the water–organic solvent interface present in the w/o/w technique and this, in turn, might increase protein stability upon encapsulation (Castellanos et al 2001). Previously, we encapsulated bovine serum albumin (BSA) into poly(lactide-*co*-glycolide) (PLG) microspheres using the s/o/w technique (Castellanos et al 2001) and encapsulation-induced structural perturbations in BSA were reduced compared with those observed when using the w/o/w technique (Fu et al 1999). Although the s/o/w technique has been used successfully to encapsulate hydrophobic drugs, it has often been associated with low encapsulation efficiency when hydrophilic drugs were encapsulated (Wang et al 1991; Castellanos et al 2001). In the development of sustained-release formulations for proteins, it is important to achieve high drug-loading to ensure a prolonged therapeutic effect and to increase cost-efficiency of production. One goal of this work was to test whether the improved encapsulation efficiency reported for lyophilized BSA (Castellanos et al 2001) could be extended to excipient-containing formulations. Furthermore, the effects of process parameters, such as the organic solvent used and the presence of excipient, on the release kinetics and structural integrity of the encapsulated protein were investigated. FTIR spectroscopy was used to determine protein integrity through the different encapsulation steps and when encapsulated in PLG microspheres. This technique allows the non-invasive examination of the secondary structure of proteins encapsulated into PLG microspheres (Griebenow et al 1999; Fu et al 1999; van de Weert et al 2000; Carrasquillo et al 2001; Castellanos et al 2001). The quality of BSA released initially within 24 h was studied by size-exclusion (SEC)-HPLC for the presence of soluble BSA aggregates. By combining the results of both techniques, we were able to select formulations and conditions leading to the minimization of perturbations in BSA secondary structure and formation of soluble aggregates. Thus, we were able to determine how excipients can stabilize BSA structure during encapsulation and subsequent release in the s/o/w technique.

Materials and Methods

Chemicals

Bovine serum albumin (BSA; $\geqslant 96\%$ albumin, essentially fatty acid free), poly(D,L-lactic-*co*-glycolide) (PLG; copolymer ratio 1:1, MW 40000–75000), and D(+)trehalose were from Sigma. Poly(vinyl alcohol) (PVA; 87–89% hydrolysed, MW 13000–23000), CH_2Cl_2, ethyl acetate and $CHCl_3$ were from Aldrich. The solvents were all of ACS HPLC grade. All other chemicals were form various commercial suppliers and were of analytical grade or better.

Protein and microsphere lyophilization

For trehalose-containing samples, trehalose was co-dissolved at a 1:4 mass ratio (BSA/trehalose) in the aqueous buffer before lyophilization. The aqueous solutions of BSA (10 mg mL^{-1}) at pH 7.3 or microspheres obtained were rapidly frozen in liquid nitrogen and then lyophilized using a Labconco FreeZone 6L lyophilizer (model 77530) at a condenser temperature of –45°C and a pressure of < 60 μm Hg for at least 48 h (Castellanos et al 2001).

Microsphere preparation

Microspheres were prepared as described previously (Castellanos et al 2001). In brief, the following protocol was defined as the standard protocol, which was varied systematically by changing one parameter at a time. BSA (20 mg) without excipient or BSA (100 mg) co-lyophilized with trehalose was suspended in 1 mL CH_2Cl_2 containing 80 mg of dissolved PLG by homogenization with a VirTis Tempest homogenizer at 5000 rev min^{-1} for 30 s. The resulting s/o suspension was added to 100 mL of a 1% (w/v) aqueous PVA solution. The o/w emulsion was formed by homogenization at 5000 rev min^{-1} for 2 min. CH_2Cl_2 was dissolved/evaporated for 3 h at room temperature under stirring with a magnetic stirrer. The microspheres formed were collected by filtration (through a 0.45-μm cellulose acetate filter), washed with distilled water, and lyophilized.

Table 1 Effect of the variation in processing parameters in the s/o/w technique on microsphere properties using trehalose as excipient.

Sample	Homogenization speed (rev min^{-1})		PLG:BSA (w/w)	PVA concn (%, w/v)	Aqueous phase volume (mL)	Microsphere diameter[a] (μm)	Encapsulation	
	s/o	w/o					efficiency[a] (%)	yield[a] (%)
C-1	5000	5000	4:1	1	100	29±7	8±2	4±3
C-2	20000	20000	4:1	1	100	9±4	15±3	7±2
C-3[b]	N/A	N/A	4:1	1	100	11±6	27±2	17±2
C-4	5000	5000	10:1	1	100	32±5	15±3	6±2
C-5	5000	5000	4:1	10	100	12±5	15±2	7±2
C-6	20000	20000	10:1	10	100	10±2	38±2	28±3
C-7	20000	20000	10:1	10	50	7±4	85±5	43±4
C-8[c]	20000	20000	10:1	10	50	13±5	53±2	30±3
C-9[d]	20000	20000	10:1	10	50	8±3	80±2	45±2
C-10[e]	20000	20000	10:1	10	50	2±1	81±2	46±5

[a]The ± values are the standard deviations calculated based on measuring 10–15 microspheres for the diameter, and 3–4 batches to determine the encapsulation efficiency and yield. Actual loading of the microspheres in mg BSA/20 mg of microspheres: C-1, 0.32±0.07; C-2, 0.61±0.08; C-3, 1.08±0.07; C-4, 0.3±0.05; C-5, 0.59±0.07; C-6, 0.77±0.03; C-7, 1.71±0.08; C-8, 1.05±0.01; C-9, 1.61±0.03; C-10, 1.63±0.03. Theoretical loading: C1–C3, C5, 20%; C4, C6–C10, 10%. The theoretical loading was changed to achieve the PLG:BSA ratios of 4:1 and 10:1, respectively (Castellanos et al 2001). [b]The s/o and o/w steps were carried out by sonication for 30 s at 50 W. [c]Microspheres prepared using ethyl acetate as the solvent. [d]Microspheres prepared using chloroform as the solvent. [e]Aqueous phase in the o/w emulsion step saturated with trehalose (BSA-Tre-Tre).

Determination of the protein loading of microspheres

Actual protein loading of microspheres was determined as described previously (Castellanos et al 2001). In brief, 20 mg of lyophilized microspheres were added to 2 mL CH_2Cl_2 and PLG dissolved by agitation for 30 min. The solid protein was pelleted by centrifugation for 15 min at 5000 rev min^{-1}, the supernatant discarded, and the pellet dissolved in 5 mL 10 mM phosphate buffer at pH 7.4. The BSA concentration in the resulting clear solution was determined from its absorbance at 280 nm using a BSA standard calibration curve (Castellanos et al 2001).

Encapsulation efficiency and yield

The encapsulation efficiency of BSA in the PLG microspheres was calculated from the actual protein loading with respect to the theoretical loading of BSA (%, w/w) in the PLG microspheres (Castellanos et al 2001). BSA encapsulation yield (%) was calculated from the total BSA content of the microspheres obtained and the total amount of BSA initially used (Castellanos et al 2001).

Protein release studies

BSA in-vitro release studies were conducted as described previously (Castellanos et al 2001). The concentration of the released protein was used to construct cumulative release profiles. Release experiments were performed at least in triplicate, the results averaged, and the standard deviations calculated.

Scanning electron microscopy (SEM)

SEM of microspheres was performed using a Jeol 5800 LV. The samples were coated with gold (200–500 Å) using a Denton Vacuum DV-502A. The size of the microspheres was determined from saved images using the program picture publisher from Micrografx. For each sample, the dimensions of 10–15 microspheres were determined, averaged, and the standard deviations calculated.

FTIR spectroscopy

FTIR studies were performed using a Nicolet Magna-IR System 560 optical bench (Carrasquillo et al 1998, 2001; Castellanos et al 2001). The spectra of BSA in aqueous solution (40 mg mL^{-1}), in amorphous dehydrated powders, and encapsulated in PLG microspheres were obtained as described in detail in the literature (Prestrelski et al 1993; Carrasquillo et al 1998, 1999, 2001) and corrected for the background in an interactive manner (Carrasquillo et al 1998, 1999, 2001).

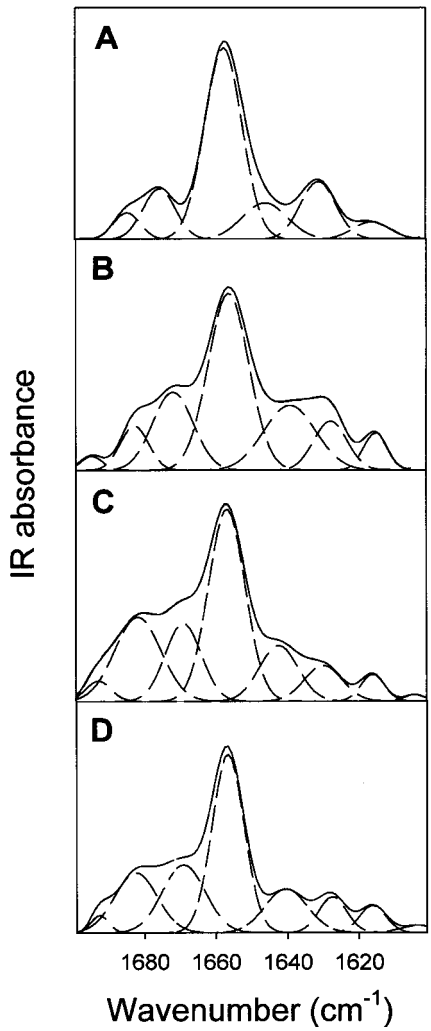

Figure 1 Fourier self-deconvoluted (FSD) amide I FTIR spectra and their Gaussian curve-fitting of bovine serum albumin (BSA). A. Aqueous solution at pH 7.3; B. BSA co-lyophilized with trehalose (BSA-Tre); C. BSA-Tre encapsulated into PLG microspheres using condition C-7 (Table 1); D. BSA-Tre encapsulated into PLG microspheres using condition C-10 (Table 1). Solid lines represent superimposed the amide I FSD spectra and the results of the Gaussian curve-fitting; dashed lines represent the individual Gaussian bands fitted to the spectra.

All spectra were analysed for the number and position of components in the amide I region (1700–1600 cm^{-1}) by calculation of the second derivative spectra (Griebenow & Klibanov 1996, 1997). The secondary structure of BSA under various conditions was calculated from the amide I IR spectra by Gaussian curve-fitting analysis (Carrasquillo et al 1998, 2001) for at least four independently obtained spectra. The values were averaged, and the standard deviations calculated.

SEC-HPLC

The amount of soluble BSA aggregates in each sample was assayed by SEC-HPLC using a TSK-GEL G2000SWXL column. The column was developed at a flow-rate of 0.5 mL min^{-1} using a mobile phase of 50 mM sodium phosphate containing 0.3 M sodium chloride, pH 7.0, on a Perkin Elmer HPLC system. BSA was detected by measuring its absorbance at 280 nm. The amount of soluble BSA aggregates was determined from the integrated chromatogram peaks using the software program Turbochrom from Perkin Elmer.

Results and Discussion

The s/o/w technique presents a valuable alternative to common w/o/w encapsulation procedures (Castellanos et al 2001), because dehydrated proteins are less susceptible to procedure-induced structural perturbations (Griebenow & Klibanov 1996). Accordingly, detrimental changes in BSA in addition to those afforded by the lyophilization process are prevented upon encapsulation into PLG microspheres by the s/o/w technique (Castellanos et al 2001). In this study, we investigated whether BSA could be encapsulated having an unperturbed secondary structure while preventing lyophilization-induced structural changes. The well known excipient, trehalose, which is capable of minimizing lyophilization-induced structural changes to BSA, was used (Carrasquillo et al 1998, 2001).

Optimization of processing parameters in the s/o/w technique

An important criterion to enable the structural analysis of proteins encapsulated in PLG microspheres by FTIR spectroscopy is sufficient protein loading. To this end, we used the approach developed for lyophilized excipient-free BSA powder (Castellanos et al 2001). When we encapsulated BSA co-lyophilized with trehalose to achieve a 1:4 mass ratio of BSA/trehalose (BSA-Tre), the microspheres obtained had a diameter of 29 ± 3 μm and the encapsulation efficiency was only 8 ± 2% (Table 1). By adjusting key parameters in the encapsulation procedure according to our previous findings (Castellanos et al 2001), the encapsulation efficiency was increased 10-fold to 85 ± 5%. The results obtained in this work under identical conditions to those in our previous work (C-1 to C-7; Castellanos et al 2001) produced very similar outcomes with respect to microsphere diameter, encapsulation efficiency and encapsu-

lation yield. Thus, the procedure developed is probably useful in the encapsulation of a variety of proteins and formulations by the s/o/w method into PLG microspheres.

Secondary structure of BSA

FTIR spectroscopy was used to verify whether BSA secondary structure was influenced by the encapsulation procedure. The aqueous spectrum of BSA is dominated by a sharp band with a maximum at 1656 cm^{-1} as it is typical for proteins with a high α-helix content (Figure 1A). Lyophilization caused a substantial reduction in the α-helix content from 51 to 23% and an increase in the β-sheet content from 13 to 28% (Table 2). Co-lyophilization of BSA with trehalose largely reduced the spectral (Figure 1B) and structural (Table 2) changes. However, when BSA-Tre was encapsulated into PLG microspheres using the optimized conditions, some spectral changes occurred (cf. Figure 1C and Figure 1B). Quantitative data showed a reduction in the α-helix content from 43 to 39% and an increase in the β-sheet from 14 to 18%. When we analysed the FTIR spectra of BSA-Tre for the presence of trehalose IR bands (Belton & Gil 1994), we were unable to detect any. For example, an IR band at 998 cm^{-1} visible in PLG microspheres containing trehalose was absent. Thus, the small hydrophilic molecule, trehalose, was dissolved into the aqueous phase in the o/w emulsion step, possibly explaining the presence of some encapsulation-induced structural perturbations for BSA-Tre. To prevent any loss of trehalose, the aqueous PVA solution was saturated with trehalose (BSA-Tre-Tre). Investigation of the FTIR spectra revealed the presence of trehalose IR absorption bands in the PLG microspheres obtained. Accordingly, the FTIR spectrum of BSA encapsulated in the PLG microspheres (Figure 1D) appears more similar to that of BSA in aqueous solution (Figure 1A) than that of BSA-Tre does (Figure 1C) and the α-helix and β-sheet contents of BSA-Tre-Tre were the same as those of BSA-Tre before encapsulation (Table 2).

Effect of the solvent

The aforementioned experiments demonstrated that leaching of trehalose into the aqueous phase occurred during the o/w step when using CH_2Cl_2 as the solvent. This indicates that at some point in the s/o/w procedure water comes into contact with trehalose and thus BSA, somewhat counterproductive to the initial goal of minimizing the contact of the protein with water to maintain the protein restricted in its conformational mobility (Griebenow & Klibanov 1996). Thus, we explored whether different solvents could minimize the contact of the protein with water. We hypothesized that BSA microspheres prepared using a solvent with low water solubility would result in less loss of trehalose into the aqueous phase and thus lead to better preservation of BSA secondary structure during the encapsulation. To test this, BSA-Tre was encapsulated into PLG micro-

Table 2 Secondary structure and formation of soluble aggregates after encapsulation of BSA in PLG microspheres using the s/o/w technique under various conditions.

Sample/state	Secondary structure content (%)			Aggregates (%)[a]
	α-Helix	β-Sheet	Unordered	
Aqueous solution at pH 7.3	51 ± 3	13 ± 1	36 ± 1	
Lyophilized BSA	23 ± 1	28 ± 1	49 ± 1	
BSA-Tre[b]	43 ± 2	14 ± 1	43 ± 2	
Microspheres prepared with CH_2Cl_2[c]				
BSA in PLG microspheres	36 ± 3	22 ± 2	42 ± 3	27
BSA-Tre in PLG microspheres	39 ± 1	18 ± 3	43 ± 2	20
BSA-Tre-Tre in PLG microspheres (C-10)	42 ± 3	15 ± 1	43 ± 3	13
Microspheres prepared with chloroform[d]				
BSA-Tre in PLG microspheres	42 ± 2	9 ± 2	49 ± 3	14
Microspheres prepared with ethyl acetate[e]				
BSA-Tre in PLG microspheres	36 ± 2	21 ± 3	43 ± 2	29

[a]Formation of soluble aggregates was determined by SEC-HPLC and is expressed as a percentage of the total BSA detected during one run. [b]BSA was co-lyophilized with trehalose at a 1:4 weight ratio.[c]Condition C-7 (Table 1); [d]condition C-9 (Table 1); [e]condition C-8 (Table 1). Values are mean ± s.d.

spheres using the optimized condition (C-7; Table 1) using the solvents ethyl acetate (water solubility 8 g/100 mL) and chloroform (0.8 g/100 mL) to allow for comparison with CH_2Cl_2 (1.32 g/100 mL).

The change of solvent influenced the encapsulation efficiency and yield, the microsphere size (Table 1) and morphology. When ethyl acetate was used as the solvent, the encapsulation efficiency dropped to 53% (Table 1; C-8). This could be due to the high solubility of water in this solvent, allowing more trehalose and BSA to leach into the outer aqueous phase. The FTIR data for BSA-Tre in the PLG microspheres are in accord with this hypothesis. The α-helix content was 36% lower and the β-sheet content higher (21%) than for BSA-Tre-Tre in PLG microspheres (Table 2). FTIR experiments showed that this was due to loss of the excipient trehalose.

When the solvent chloroform was used, the encapsulation efficiency of BSA-Tre was 80% (C-9; Table 1). No significant structural alterations occurred upon encapsulation into PLG microspheres (Table 2) due to prevention of the loss of the excipient trehalose during the o/w step. The FTIR spectrum of the microspheres showed a trehalose band at 998 cm^{-1}.

Stability of released BSA

Having determined that the details of the encapsulation procedure influence the secondary structure of BSA in PLG microspheres, we were interested to know how this would relate to the stability of the protein upon encapsulation. We evaluated the aggregation state of BSA by using SEC-HPLC after 24 h of in-vitro release. BSA is an exceptionally good model protein in this context, because it aggregates substantially via thiol–disulfide interchange when exposed to conditions relevant to the sustained release, for example upon exposure to moisture (Costantino et al 1997).

We evaluated the method and performed SEC-HPLC with freshly prepared BSA solution. The chromatogram (Figure 2A) is comparable with those reported in the literature and shows peaks corresponding to the monomer (80%), dimer (17%), and small oligomers (3%) (Crotts & Park 1997; Costantino et al 1997). When PLG microspheres containing lyophilized BSA prepared without any excipients were suspended for 24 h in release buffer, the amount of water-soluble aggregates determined from the HPLC chromatogram (Figure 2B) was 27% (Table 2). For BSA-Tre, 20% of insoluble aggregates were determined from the HPLC chromatogram (Figure 2C), substantially less than for BSA. This effect was even more pronounced for BSA-Tre-Tre, where only 13% of aggregates were found (Figure 2D; Table 2). Similarly, for BSA-Tre encapsulated into PLG microspheres using chloroform, the HPLC chromatograms (Figure 2E) revealed a low amount of aggregation (14%). In contrast, the HPLC chromatogram for BSA-Tre encapsulated into PLG microspheres using ethyl acetate (Figure 2F) showed clear signs of the presence of aggregates (29%). When we plotted the α-helix content as a solid structural parameter (Griebenow & Klibanov 1996, 1997) against the amount of soluble aggregates formed (Figure 3), a clear correlation emerged with a high correlation coefficient. Thus, perturbations in the secondary structure of BSA in the s/o/w technique cause the formation of soluble BSA aggregates. When the structural perturbations are reduced during the

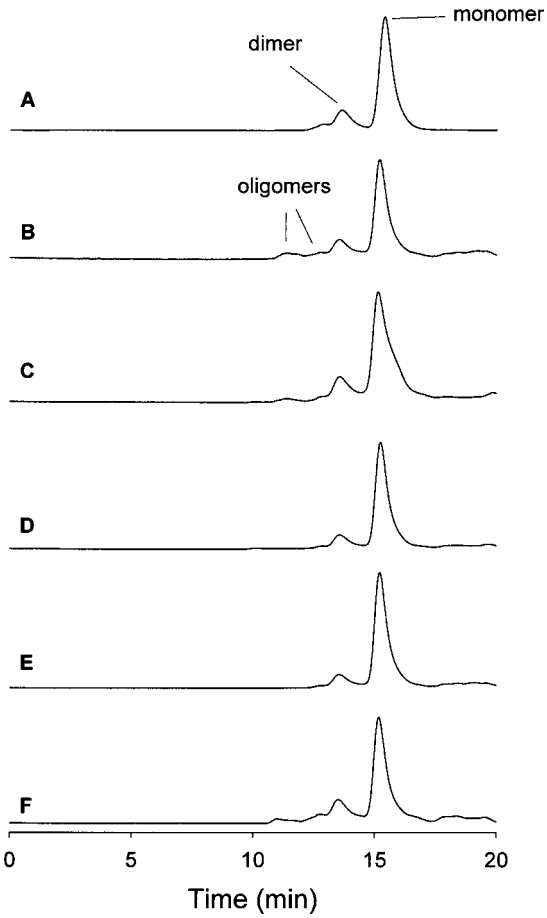

Figure 2 SEC-HPLC chromatograms of various bovine serum albumin (BSA) samples (detected at $\lambda_{abs} = 280$ nm). A. Commercially available BSA dissolved in buffer; B. BSA released from PLG microspheres prepared with CH_2Cl_2 after 24 h; C. same as B, but using BSA-Tre; D. same as C, but external aqueous phase in the o/w step saturated with trehalose (BSA-Tre-Tre); E. same as C, but using chloroform as organic solvent; F. same as C, but using ethyl acetate as the organic solvent.

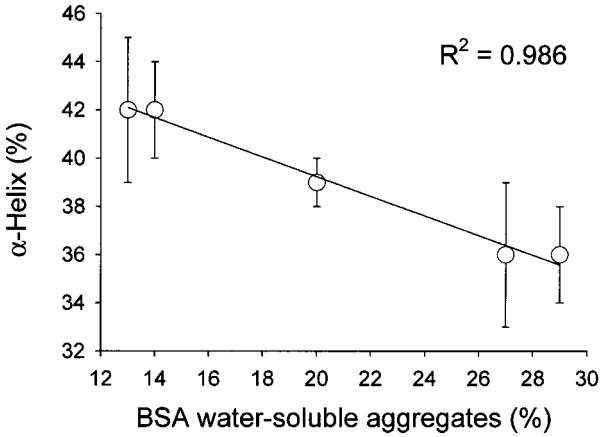

Figure 3 Relationship between the α-helix content found for various preparations of bovine serum albumin (BSA) encapsulated in PLG microspheres and the amount of soluble BSA aggregates formed after 24 h of in-vitro release (data from Table 2).

encapsulation of BSA, formation of soluble aggregates is reduced to the level typical of that found in commercial preparations.

In-vitro release studies

The in-vitro release properties of the microspheres obtained using the various optimized protocols (conditions C-7 to C-10; Table 1) were also determined. Figure 4A shows the release profiles of PLG microspheres prepared with CH_2Cl_2 using the three different formulations (BSA, BSA-Tre, and BSA-Tre-Tre). In accordance with the finding that trehalose leaches into the aqueous phase in the o/w step, the release profiles obtained for BSA and BSA-Tre were similar. The initial burst release within 24 h was $< 20\%$ for both preparations and the cumulative release was 96% in 25 days. In contrast, PLG microspheres containing BSA-Tre-Tre released the protein in a substantially different manner, as was expected, due to the presence of the excipient. After an initial burst release of 16% of the protein and a gradual release period similar to that observed for the two aforementioned preparations, release significantly increased after approximately 10 days.

A profound effect on the in-vitro release profiles was observed when microspheres were prepared with ethyl acetate (Figure 4B) and chloroform (Figure 4C) because the choice of solvent influenced microsphere morphology. Figure 4B compares the in-vitro release profiles of BSA and BSA-Tre from microspheres prepared using ethyl acetate. Both profiles showed a low initial burst release of 22% for BSA and 18% for BSA-Tre. Surprisingly, the release profiles were different. Thus, even though trehalose was removed from the PLG microspheres for the BSA-Tre formulation, the release profile was still influenced. The initial presence of trehalose in the encapsulation procedure must exert some influence on microsphere morphology. Figure 4C compares the release profiles obtained for BSA and BSA-Tre from microspheres prepared using chloroform. A low initial burst release of 18% was followed by diffusion release of the protein. At approximately 200 h, a lag phase of 120 h was observed. Associated with the degradation of the microspheres, a second faster release followed. The

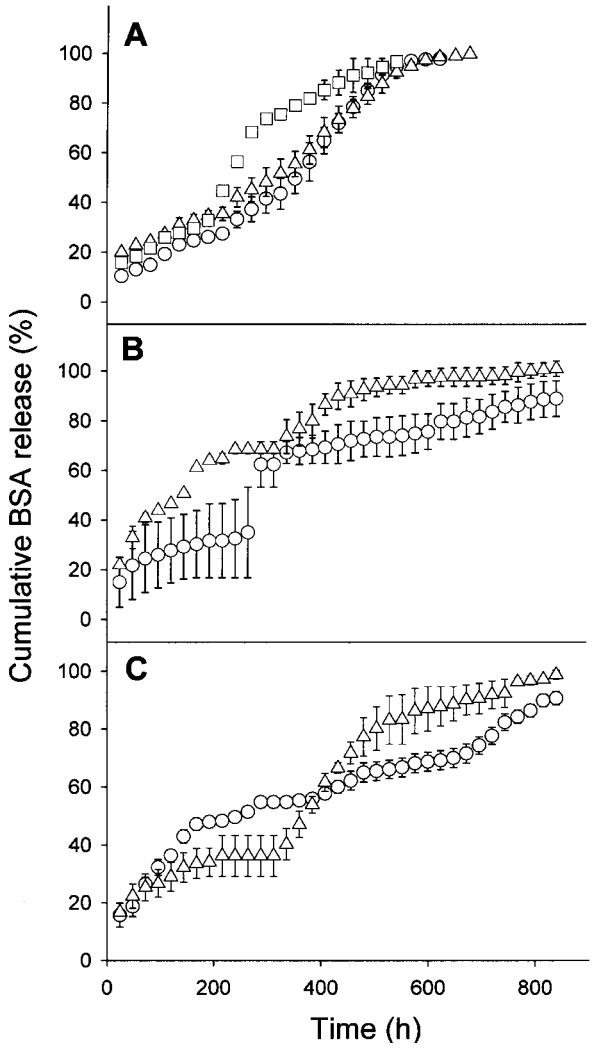

Figure 4 Cumulative in-vitro release of bovine serum albumin (BSA; △), BSA-Tre (○) and BSA-Tre-Tre (□) from PLG microspheres. Microspheres were prepared using CH_2Cl_2 (A), ethyl acetate (B), and chloroform (C) as the organic solvent in the o/w step using conditions C-7 to C-10 (Table 1).

amount of the released protein was 95% over 840 h. The microspheres prepared with BSA-Tre showed an initial burst release similar to that of microspheres containing BSA (18%) followed by a continuous release due to both diffusion and microsphere degradation. These release profiles showed that the protein was better distributed in the matrix of the microspheres prepared with trehalose than those prepared without trehalose. The amount of the released protein was 90%.

In conclusion, all microspheres prepared by the s/o/w technique showed a low initial burst release followed by a sustained release. The exact profiles observed for the individual preparations varied depending on the presence of the excipient and other microsphere properties caused by the different solvents used.

Microsphere morphology

Differences in microsphere morphology for BSA-Tre encapsulated into PLG microspheres were investigated by SEM (typical examples are shown in Figure 5) and are particularly visible when comparing the images of lyophilized microspheres obtained initially (Figure 5A) and after 24 h of in-vitro release (Figure 5B). Under all conditions, microspheres initially were spherically shaped without visible pores on the surface. After 24 h of in-vitro release, pores were visible due to initially released protein and excipient. In general, we found that the higher the solubility of water in the organic solvent, the more pronounced the pores formed after 24 h release. A more porous matrix should increase protein release by diffusion and also increase microsphere erosion because a larger surface area comes into contact with water. Accordingly, microspheres eroded faster when prepared with ethyl acetate than when CH_2Cl_2 or chloroform was used as the solvent. In particular in the latter case, microspheres appeared quite compact after 2 weeks of in-vitro release, in good agreement with the slow and continuous release pattern observed (Figure 4C).

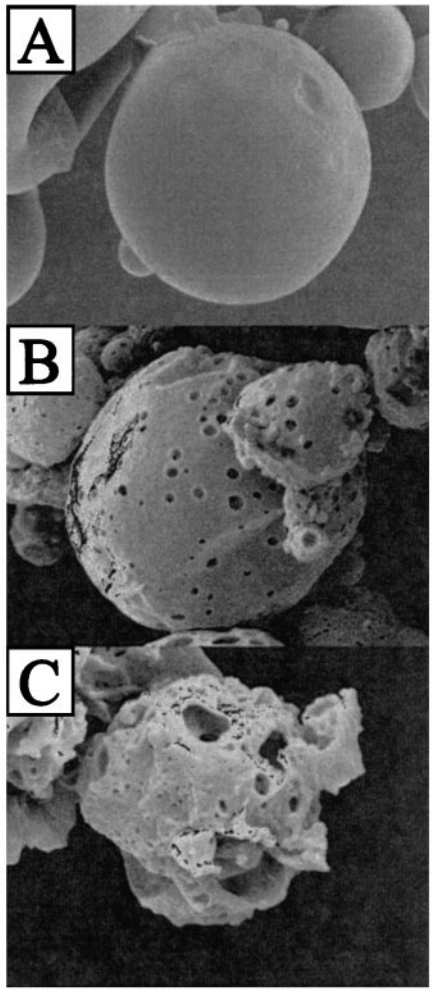

Figure 5 Scanning electron microscopy images of PLG microspheres prepared with BSA-Tre using condition C-7 (Table 1) at various times after in-vitro release. A. Microspheres immediately after preparation; B. microspheres after 24 h exposure to the release buffer; C. microspheres after 2 weeks of in-vitro release. The width of all images corresponds to 14.7 μm.

Conclusions

The model protein BSA was encapsulated in PLG microspheres using the s/o/w technique. Structural perturbations in BSA were minimized throughout the encapsulation procedure when two conditions were fulfilled: BSA powder was obtained by co-lyophilization with trehalose, and loss of trehalose in the o/w emulsion step was prevented. Under these conditions, the formation of soluble BSA aggregates was also prevented. The results obtained by SEC-HPLC and FTIR spectroscopy show a clear correlation between prevention of perturbations in the secondary structure and aggregation of encapsulated BSA. Thus, the non-invasive analysis of BSA secondary structure by FTIR spectroscopy should, in the future, enable the prediction of conditions leading to the prevention of deleterious aggregate formation.

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