JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 167–178

Received August 9, 2000
Accepted October 13, 2000
ISSN 0022-3573

# Encapsulation of bovine serum albumin in poly(lactide-*co*-glycolide) microspheres by the solid-in-oil-in-water technique

Ingrid J. Castellanos, Karen G. Carrasquillo, Jesiebel De Jésus López, Mariali Alvarez, and Kai Griebenow

**Abstract**

Non-aqueous protocols to encapsulate pharmaceutical proteins into biocompatible polymers have gained much attention because they allow for the minimization of procedure-induced protein structural perturbations. The aim of this study was to determine if these advantages could be extended to a semi-aqueous encapsulation procedure, namely the solid-in-oil-in-water (s/o/w) technique. The model protein bovine serum albumin (BSA) was encapsulated into poly(lactide-*co*-glycolide) (PLG) microspheres by first suspending lyophilized BSA in methylene chloride containing PLG, followed by emulsification in a 1% aqueous solution of poly(vinyl alcohol). By variation of critical encapsulation parameters (homogenization intensity, BSA:PLG ratio, emulsifier concentration, ratio of organic to aqueous phase) an encapsulation efficiency of $> 90\%$ was achieved. The microspheres obtained showed an initial burst release of $< 20\%$, a sustained release over a period of about 19 days, and a cumulative release of at least 90% of the encapsulated BSA. Different release profiles were observed when using different encapsulation protocols. These differences were related to differences in the microsphere erosion observed using scanning electron microscopy. Release of BSA was mainly due to simple diffusion or to both diffusion and microsphere erosion. Fourier-transform infrared studies were conducted to investigate the secondary structure of BSA during the encapsulation. Quantification of the $\alpha$-helix and $\beta$-sheet content as well as of overall structural changes showed that the secondary structure of encapsulated BSA was not more perturbed than in the lyophilized powder used initially. Thus, the encapsulation procedure did not cause detrimental structural perturbations in BSA. In summary, the results demonstrate that the s/o/w technique is an excellent alternative to the water-in-oil-in-water technique, which is still mainly used in the encapsulation of proteins in PLG microspheres.

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

Ingrid J. Castellanos, Karen G. Carrasquillo, Jesiebel De Jésus López, Mariali Alvarez, Kai Griebenow
**Correspondence:** K. Griebenow, Department of Chemistry, University of Puerto Rico, Río Piedras Campus, PO Box 23346, San Juan, PR 00931–3346, USA.
**Acknowledgements and funding:** We thank Gabriel Cruz for valuable technical assistance with the SEM, and Dr. Reinhard Schweitzer-Stenner for comments on the paper. This work was supported by grants from the National Institutes of Health MBRS programme (S06 GM08102-26S1), the GAANN programme of the US Department of Education (K.G.C. graduate fellowship), and institutional funds of the University of Puerto Rico (FIPI programme).

## Introduction

The delivery of protein pharmaceuticals to patients is not an easy task, largely due to their inherent physical and chemical instability (Schöneich et al 1997). Their sustained release from biodegradable and biocompatible polymers, such as poly-(lactide-*co*-glycolide) (PLG), could circumvent many of the stability problems (Cleland & Langer 1995). However, it has become increasingly apparent that the most commonly used protein encapsulation procedure, the formation of microspheres by the water-in-oil-in-water (w/o/w; Figure 1) technique, often leads to unwanted protein aggregation (Tabata et al 1993; Alonso et al 1994; Lu & Park

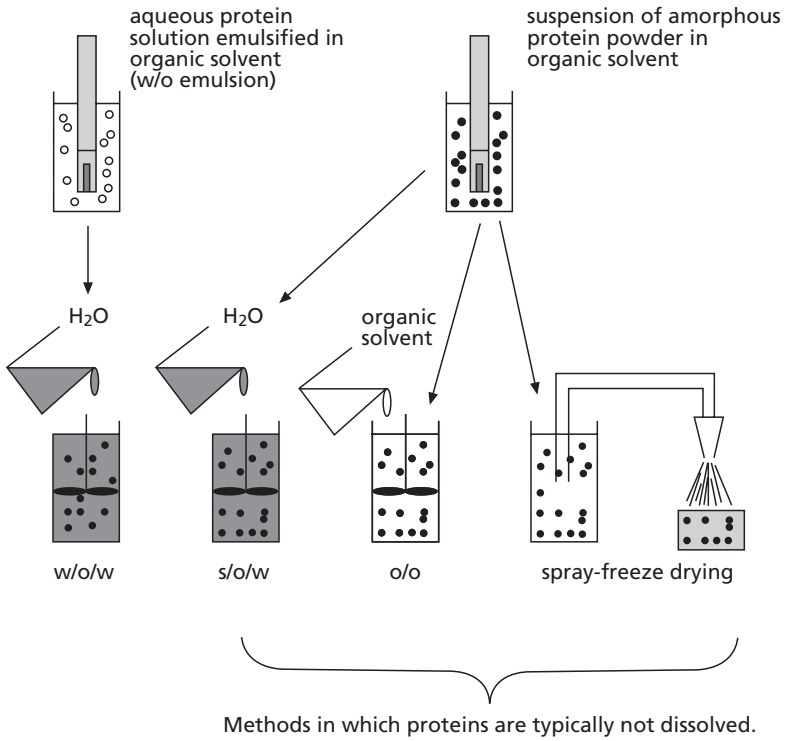


**Figure 1** Schematic representation of the main methodologies used to encapsulate proteins in hydrophobic polymers such as poly(lactide-*co*-glycolide).

1995a, b). In particular, the formation of the first emulsion where an aqueous protein solution is emulsified into a PLG solution in methylene chloride leads to protein aggregation at the aqueous–organic interface (Sah 1999).

Several research groups have started developing new encapsulation strategies that avoid the exposure of hydrated proteins to physical stress factors (Carrasquillo et al 1998, 1999, 2001; Schwendeman et al 1998; Griebenow et al 1999a). The hypothesis behind these approaches is that dehydrated proteins are less susceptible to denaturation because conformational changes are kinetically prohibited (Griebenow & Klibanov 1996). It has been shown that protein structural alterations can be minimized upon encapsulation in PLG when dehydrated protein powders are suspended in organic solvents (Griebenow et al 1999a; Carrasquillo et al 1998, 1999). Furthermore, protein structural perturbations could be minimized in the production of PLG microspheres using an oil-in-oil procedure (Carrasquillo et al 2001). Thus the development and improvement of alternative encapsulation procedures offers the possibility of avoiding procedure-induced protein denaturation. However, there are also problems associated with oil-in-oil encapsulation procedures. Potentially dangerous chemicals, such as silicon oil and heptane, are used and are difficult to remove from the microspheres (Thomasin et al 1996), and microspheres produced by an oil-in-oil technique showed a large initial burst release of approximately 60 % (Carrasquillo et al 2001).

In this work we investigate the potential of the solid-in-oil-in-water (s/o/w) technique to obtain protein-loaded microspheres. The dehydrated protein powder is suspended in an organic solvent containing the dissolved polymer (Wang et al 1991; Cleland & Jones 1996; Atkins 1997; Maa & Hsu 1997). This suspension is then emulsified in an aqueous solution (Figure 1). After microsphere hardening by dissolving or evaporating the organic solvent, they are finally washed and lyophilized. Because the protein should be exposed to little water in the s/o/w technique (the solubility of water in methylene chloride is less than 2 %), we hypothesized that it might be possible to extend the minimization of protein structural perturbations found in oil-in-oil techniques (Carrasquillo et al 2001) to this technique. Thus far no data have been presented on the structural consequences

of encapsulating BSA (or any other protein) by the s/o/w method in polymer microspheres.

One problem hampering the use of the s/o/w technique is the frequently low encapsulation efficiency achieved (Fong et al 1986; Wang et al 1991; Atkins 1997). For example, Atkins (1997) reported an efficiency of only up to 14.5% when encapsulating BSA into various polymers using the s/o/w method. In the first systematic study addressing this issue, Maa & Hsu (1997) established that the particle characteristics (size and shape) of the solid protein powder are important for achieving higher encapsulation efficiency; the smaller the particles, the higher the encapsulation efficiency. However, that work used solid fine-particulate BSA powders obtained by spray-drying and solvent precipitation methods and any effect of the encapsulation process itself (formation of s/o suspension and o/w emulsion) on the protein powder particle size was not studied. Furthermore, processing conditions were not related to encapsulation efficiency, microsphere morphology, and no release data were presented for the microspheres obtained. In addition, very limited data have been published on the characteristics of PLG microspheres obtained by the s/o/w method in general, in particular concerning the erosion characteristics.

The aim of this study was to investigate the influence of the s/o suspension and the o/w emulsion processing parameters on encapsulation efficiency and yield, and to develop reproducible protocols leading to high values for both. The release profile of the microspheres obtained was characterized and the release mechanism investigated using scanning electron microscopy (SEM) in combination with in-vitro release studies. We also tested the hypothesis of whether the advantages of the strictly non-aqueous encapsulation protocols in minimizing protein structural perturbations could be extended to the s/o/w technique.

## Materials and Methods

### Chemicals

Bovine serum albumin (BSA; $\geqslant 96\%$ albumin, essentially fatty acid free) and PLG (50:50 copolymer ratio, MW 40 000–75 000) were from Sigma. Poly(vinyl alcohol) (PVA; 87–89% hydrolysed, MW 13 000–23 000) and dichloromethane (99.9%, ACS HPLC grade) were from Aldrich. All other chemicals were from various commercial suppliers and were of analytical grade or higher.

### Protein and microsphere lyophilization

An aqueous solution of BSA (10 mg $mL^{-1}$) at pH 7.3, or microspheres obtained were rapidly frozen in liquid $N_2$ and then lyophilized using a Labconco FreeZone 6L lyophilizer at a condenser temperature of $-45°C$ and a pressure of $< 60$ $\mu$mHg for at least 48 h.

### Microsphere preparation

The following protocol was defined as the standard protocol, and was systematically varied by changing one parameter at a time. Lyophilized BSA powder (20 mg protein) was suspended in 1 mL methylene chloride containing 80 mg of dissolved PLG by homogenization with a VirTis Tempest homogenizer (Gardiner, NY) using a 10-mm shaft equipped with a micro-fine rotor/stator generator 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. Methylene chloride 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-$\mu$m cellulose acetate filter, washed with approximately 50 mL distilled water, and lyophilized for storage.

### Determination of the protein loading of microspheres

Actual protein loading ($L_A$) of the microspheres was determined as previously reported (Maa & Hsu 1997). Lyophilized microspheres (15–20 mg) were added to 2 mL methylene chloride 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 adjusted to 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 (Tabata et al 1993).

### Encapsulation efficiency and yield

The encapsulation efficiency (Ee) of BSA in the PLG microspheres was defined as:

$$\text{Ee}\ (\%) = (L_A/L_T) \times 100$$

where $L_A$ is the actual loading and $L_T$ is the theoretical loading of BSA (%, w/w) in the PLG microspheres (Atkins & Peacock 1996). BSA encapsulation yield (%) was calculated from the total BSA content of the micro-

spheres obtained and the total amount of BSA initially used. Determination of the encapsulation yield provides information on the overall loss of protein that will not necessarily affect the encapsulation efficiency. For example, because the yield of microsphere formation typically is not 100% and some PLG will be lost during the procedure, the value for the encapsulation yield will be lower than that for the encapsulation efficiency. The encapsulation yield is an important parameter for cost efficient production of microspheres when using expensive pharmaceutical proteins.

### BSA in-vitro release studies

Microspheres (40 mg) were placed in 3 mL 10 mM phosphate buffer at pH 7.3 and incubated at 37°C. At predetermined times (typically every 24 h) the samples were subjected to centrifugation (500 rev $min^{-1}$ for 1 min in a Beckman J-21B centrifuge) to pellet the insoluble components. The supernatant was removed and the concentration of released protein determined as described above. The buffer was completely replaced to maintain sink conditions in the release experiments and to avoid protein denaturation by changes in the pH due to microsphere erosion (Park et al 1995). We noted a small (but significant) contribution to the absorption at 280 nm due to microsphere erosion. Therefore, blank microspheres were obtained under identical conditions and subjected to in-vitro release in parallel with BSA-loaded microspheres. Blank microspheres were harvested as described above, the absorption at 280 nm determined, and subtracted from those obtained with BSA-loaded microspheres. The concentration of the released protein was plotted directly against time and also used to construct cumulative release profiles (Tabata et al 1993). The release profiles (Figure 6) demonstrate that the correction of the absorption was appropriate because after prolonged release no protein absorption at 280 nm was evident even though microspheres (and thus absorption at 280 nm due to microsphere components) were still present. Release experiments were at least performed in triplicate, the results averaged, and the standard deviations calculated.

### 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.

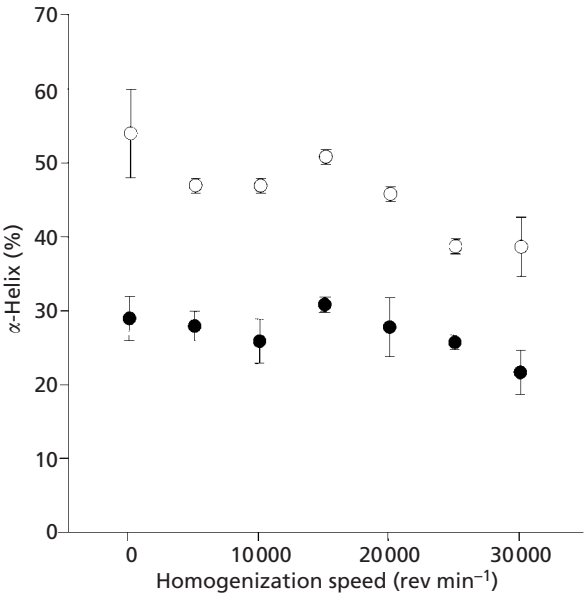


**Figure 2** $\alpha$-Helix content of BSA lyophilized from buffer (●) or co-lyophilized with trehalose at a 1:4 ratio (w/w) (○) after suspension in methylene chloride at various homogenization intensities and subsequent drying.

### Fourier-transform infrared (FTIR) spectroscopy

FTIR studies were performed using a Nicolet Magna-IR System 560 optical bench (Carrasquillo et al 1998). 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 elsewhere (Carrasquillo et al 1998, 1999, 2001), and corrected for the background in an interactive manner using the Nicolet OMNIC 3.1 software (Carrasquillo et al 1998, 2001). The proper subtraction of the PLG background from FTIR spectra obtained for BSA in microspheres was performed as described in detail by Carrasquillo et al (1998, 1999, 2001) and Fu et al (1999).

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 (Prestrelski et al 1993). The secondary structure content 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. As an

**Table 1** Comparison of the secondary structure of lyophilized BSA samples and those after suspension in methylene chloride using the spectral correlation coefficient (r).

| Sample | Homogenization speed (rev $min^{-1}$) | r ± s.d. (x(ii)) | x(i) − x(ii) | $t$(pooled)$((n_1+n_2)/(n_1 \times n_2))^{\frac{1}{2}}$[a] | Null hypothesis[b] |
|---|---|---|---|---|---|
| BSA lyophilized from buffer | | | | | |
| | 5000 | 0.950 ± 0.001 | 0.001 | 0.002 | Accepted |
| | 10000 | 0.947 ± 0.001 | 0.002 | 0.002 | Accepted |
| | 15000 | 0.945 ± 0.000 | 0.004 | 0.003 | Rejected |
| | 20000 | 0.954 ± 0.005 | 0.005 | 0.002 | Rejected |
| | 25000 | 0.948 ± 0.002 | 0.001 | 0.001 | Accepted |
| | 30000 | 0.951 ± 0.001 | 0.002 | 0.002 | Accepted |
| BSA : trehalose co-lyophilizate 1 : 4 (w/w) | | | | | |
| | 5000 | 0.980 ± 0.001 | 0.02 | 0.02 | Accepted |
| | 10000 | 0.951 ± 0.008 | 0.01 | 0.01 | Accepted |
| | 15000 | 0.952 ± 0.005 | 0.01 | 0.01 | Accepted |
| | 20000 | 0.945 ± 0.002 | 0.015 | 0.016 | Accepted |
| | 25000 | 0.970 ± 0.004 | 0.01 | 0.01 | Accepted |
| | 30000 | 0.975 ± 0.001 | 0.015 | 0.018 | Accepted |

If the experimental difference is less than or equal to the computed value, then the null hypothesis is that the samples are not different and cannot be rejected. The degrees of freedom (DF = 7) for finding $t$ is $n_1 + n_2 - 2$, where $n_1 = 6$ (number of samples) and $n_2 = 3$ (number of measurements averaged to obtain one data point with standard deviation). [a]The $t$ value is at a 95% confidence interval. [b]The average correlation coefficient (x(i)) used to determine the difference of two means is 0.949 ± 0.003 for all the correlation coefficients determined individually, in the case of lyophilized BSA, and 0.96 ± 0.02 in the case of BSA co-lyophilized with trehalose.

alternative measure of overall protein structural perturbations, we also calculated the spectral correlation coefficient between BSA in aqueous solution and in the various amorphous states from the amide I second derivative spectra (Prestrelski et al 1993; Griebenow et al 1999b; Carrasquillo et al 2000). A spectral correlation coefficient of 1 shows spectral (and thus structural) identity, lower numbers indicate perturbations in the secondary structure.

## Results and Discussion

### Impact of the s/o suspension conditions on BSA secondary structure

The first step of the s/o/w methodology involves the suspension of the lyophilized protein powder in an organic solvent capable of dissolving PLG, typically methylene chloride (Figure 1). Even though it has been established that various proteins are not denatured upon suspension in organic solvents (Griebenow & Klibanov 1996, 1997), including BSA (Carrasquillo et al 1998, 2000), the effect of the homogenization speed on the structure of proteins has not been systematically explored. The structure of BSA was assessed by FTIR spectroscopy before homogenization and after homogenization in methylene chloride for 1 min followed by a 24-h drying period with a vacuum of $< 60$ $\mu$mHg. We selected the $\alpha$-helix content as a solid parameter for structural integrity (Figure 2) and also calculated the correlation coefficient (Table 1) to characterize overall secondary structure changes. For the lyophilized BSA powder the $\alpha$-helix content did not change significantly, irrespective of the homogenization speed. For the BSA powder obtained by co-lyophilization with trehalose, the $\alpha$-helix content indicated a slight change in the secondary structure of the protein at high homogenization speeds of 25000 and 30000 rev $min^{-1}$. The correlation coefficients (Table 1) were not statistically different, with the exception of the points using homogenization speeds of 15000 and 20000 rev $min^{-1}$ for BSA without trehalose at the various homogenization intensities from the average of all correlation coefficients

using the *t*-test and 95% confidence interval. For the two points failing the *t*-test hypothesis the $\alpha$-helix content did not reveal any changes (Figure 2). From these results we conclude that suspension of lyophilized BSA in methylene chloride at various homogenization speeds and subsequent drying does not significantly perturb its secondary structure, regardless of whether trehalose as an excipient was present or not. However, because we observed a slight decrease in the $\alpha$-helix content at higher homogenization intensities, in the further investigation a maximum homogenization speed of 20 000 rev $min^{-1}$ was used.

### Particle size after s/o suspension

It has been reported that the encapsulation efficiency depends on the particle size of the primarily used protein powder (Maa & Hsu 1997). To assess this, we suspended lyophilized BSA in methylene chloride at the minimum (5000 rev $min^{-1}$) and maximum (20 000 rev $min^{-1}$) homogenization speeds used later in this work for 30 s, followed by methylene chloride evaporation under a stream of nitrogen gas. SEM analysis revealed that the lyophilized powder was characterized by particles of various shapes and sizes (Figure 3A). Homogenization in general reduced the size of the BSA powder particles. However, irrespective of the homogenization speed, resulting particles were still very heterogeneous in size and shape and did not appear different (Figure 3B, C). When compared with the size of the microspheres obtained finally (Table 2), the size of the powder particles was quite large. This should have resulted in low encapsulation efficiencies (Maa & Hsu 1997), as we indeed observed under non-optimized conditions.

### Optimization of processing parameters in the encapsulation of BSA into PLG

The microspheres obtained following the standard protocol had a diameter of approximately 30 $\mu$m and the encapsulation efficiency for BSA was approximately 7% (C-1; Table 2). The encapsulation efficiency is in good agreement with the low encapsulation efficiency reported elsewhere (Fong et al 1986; Wang et al 1991; Atkins 1997). We therefore focussed on improving the encapsulation efficiency by systematically changing the critical parameters in the encapsulation protocol.

Higher homogenization intensity in the o/w step results in a reduction in the size of the oil droplets and thus an increased o/w surface area. The resulting accelerated methylene chloride removal should then lead to increased encapsulation efficiency because of fast BSA entrapment in the polymer matrix. In addition, higher homogenization intensities could also lead to a reduction in size of the protein BSA particles in the s/o or o/w step and this should improve encapsulation efficiency (Maa & Hsu 1997). To test this, we systematically varied the homogenization intensities used in the s/o and o/w

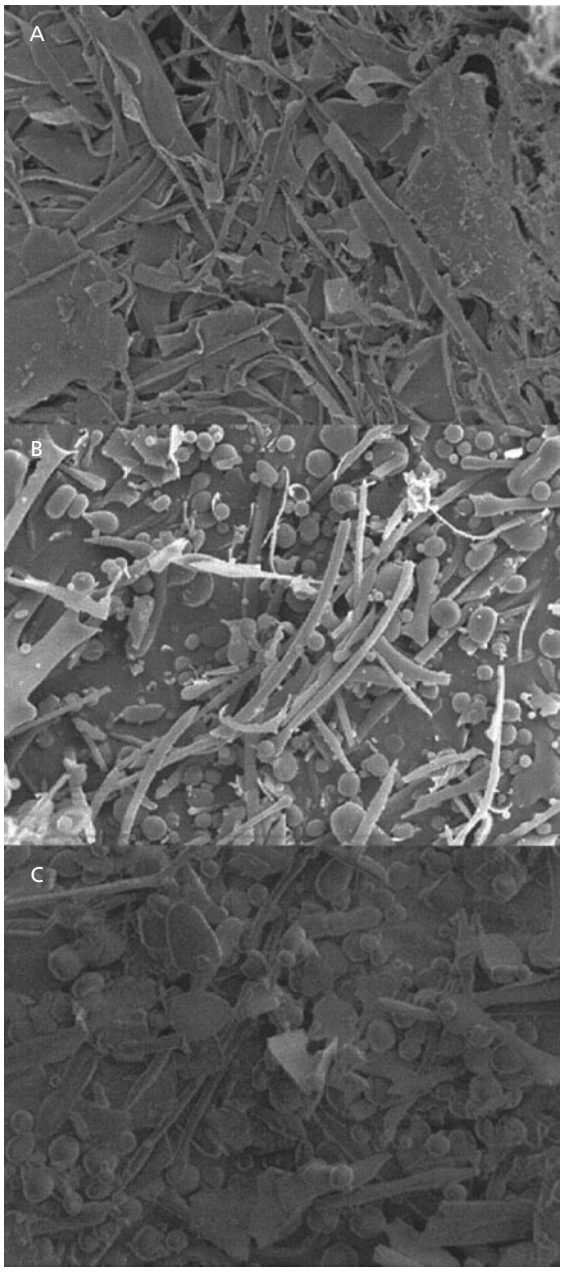


**Figure 3** SEM images of BSA lyophilized from buffer (pH 7.3) (A), the BSA powder after suspension with homogenization in methylene chloride at 5000 (B) and 20 000 rev $min^{-1}$ (C) followed by subsequent drying (the width of the images corresponds to 66 $\mu$m).

**Table 2** Effect of variation in processing parameters in the solid-in-oil-in-water technique on microsphere properties.

| Sample | Homogenization speed (rev min$^{-1}$) | | PLG:BSA (w/w) | PVA concn (%, w/w) | Aqueous phase volume (mL) | Microsphere diameter (μm) | Encapsulation efficiency (%) | Encapsulation yield (%) |
|---|---|---|---|---|---|---|---|---|
| | s/o | w/o | | | | | | |
| C-1 | 5000 | 5000 | 4:1 | 1 | 100 | 33±6 | 7±5 | 3±2 |
| C-2 | 20000 | 5000 | 4:1 | 1 | 100 | 30±13 | 10±2 | 4±2 |
| C-3 | 20000 | 10000 | 4:1 | 1 | 100 | 25±19 | 15±3 | 12±4 |
| C-4 | 20000 | 15000 | 4:1 | 1 | 100 | 12±6 | 17±4 | 11±5 |
| C-5 | 20000 | 20000 | 4:1 | 1 | 100 | 10±3 | 22±2 | 9±2 |
| C-6[a] | N/A | N/A | 4:1 | 1 | 100 | 14±3 | 36±2 | 21±2 |
| C-7 | 5000 | 5000 | 10:1 | 1 | 100 | 36±6 | 18±4 | 8±3 |
| C-8 | 5000 | 5000 | 4:1 | 10 | 100 | 15±6 | 21±3 | 9±3 |
| C-9 | 20000 | 20000 | 10:1 | 10 | 100 | 12±3 | 63±3 | 40±3 |
| C-10 | 20000 | 20000 | 10:1 | 10 | 50 | 9±3 | 93±2 | 68±5 |

Parameters that were kept constant in all experiments: volume of the methylene chloride phase (1 mL); total amount of BSA and PLG (100 mg); stirring for 3 h at approximately 600 rev min$^{-1}$ with a magnetic stirrer for solvent removal; and microsphere collection, washing, and lyophilization procedure. [a]Suspension and emulsification performed by probe sonication at 50 W output power.

steps (Table 2). Comparison of C-1 (homogenization speed 5000 rev min$^{-1}$ in the s/o step) and C-4 (homogenization speed 20000 rev min$^{-1}$ in the s/o step) showed that microsphere size and encapsulation efficiency did not change. This result is in agreement with the SEM results showing that the particle size in the BSA powder did not change significantly in the s/o step. In contrast, changing the homogenization intensity in the w/o emulsion step had a profound influence on microsphere size and encapsulation efficiency (C-2 to C-5; Table 2). The higher the homogenization intensity, the smaller the microspheres diameter and the higher the encapsulation efficiency. Thus, the homogenization intensity in the w/o emulsion step likely influences the BSA particle size and thus encapsulation efficiency and microsphere size.

We also tested emulsification by probe sonication (C-6; Table 2). Again, the smaller microsphere size (14 $\mu$m) correlated with higher encapsulation efficiency (36%). From this experiment it is evident that there is not a linear relationship between encapsulation efficiency and microsphere diameter. Even though microsphere size obtained by sonication or homogenization at 20000 rev min$^{-1}$ was comparable, encapsulation efficiency was significantly higher using probe sonication. This likely indicates a better disruption of the BSA powder particles by probe sonication. However, because probe sonication of BSA is reported to cause procedure-induced structural alterations (Carrasquillo et al 1998), we focussed on optimizing the emulsification conditions using homogenization.

Increasing the amount of the polymer under otherwise constant conditions (in particular the oil-to-water ratio) should result in an increase in the encapsulation efficiency because the entrapment of the protein in the polymer matrix should be faster. Accordingly, when the ratio of polymer to BSA was increased from 4:1 to 10:1 (w/w), the encapsulation efficiency more than doubled (compare C-1 and C-7; Table 2). The size of the microspheres obtained was not significantly affected in this experiment because the total amount of PLG and BSA was kept at 100 mg mL$^{-1}$ methylene chloride in all experiments.

The concentration of the emulsifying agent (PVA) should also influence the encapsulation efficiency because the emulsifier exerts a distinct influence on the characteristics of the emulsion formed. An increase in the emulsifier concentration should lead to smaller emulsion droplets formed because a larger o/w surface can be occupied and thus stabilized (Jeffery et al 1993). The smaller oil droplet size and thus larger surface area should lead to a faster microsphere hardening and thus result in less leaching of BSA into the aqueous phase. Results obtained with 1 and 10% PVA in the aqueous phase under otherwise identical conditions support this view: the microspheres formed at the higher PVA concentration were 15 $\mu$m in diameter (C-8; Table 2) and thus smaller than those formed with 1% PVA (C-1; Table 2). The encapsulation efficiency was increased to 21% (Table 2).

The final parameter to be optimized was the volume of the aqueous phase. To select suitable final encapsulation conditions, this procedure was carried out using

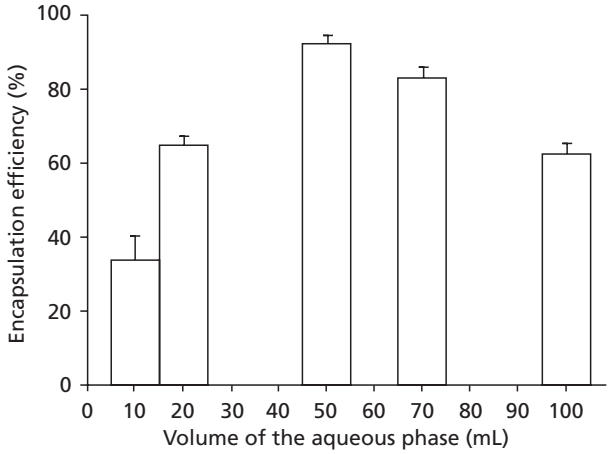


**Figure 4** Dependence of the encapsulation efficiency of BSA into PLG microspheres on the volume of the aqueous phase in the oil-in-water emulsification step at otherwise constant parameters.

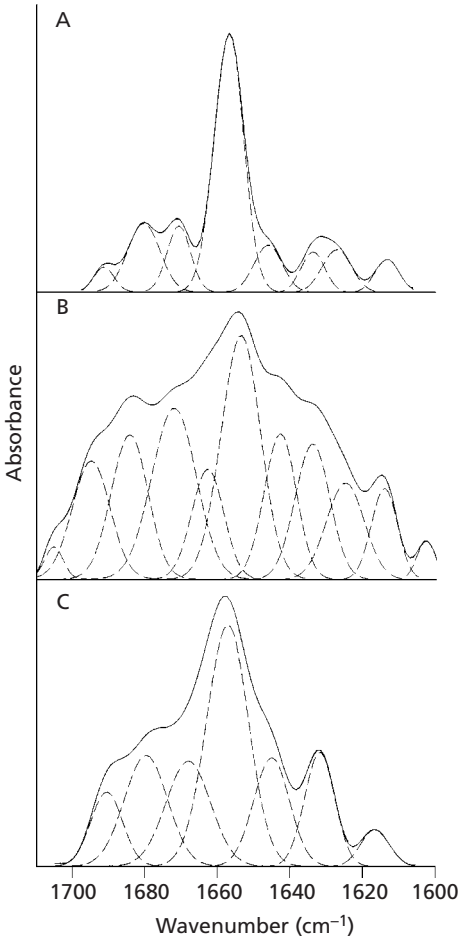


**Figure 5** Amide I FTIR spectra of BSA after resolution-enhancement by Fourier-self-deconvolution (FSD) and Gaussian curve-fitting. A, aqueous solution at pH 7.3; B, powder obtained by lyophilization from buffer (pH 7.3); C, powder encapsulated in PLG microspheres using condition C-10 in Table 2. The solid line represents the spectra after FSD overlaid with the results of the curve-fitting procedure. The broken lines are the individual Gaussian bands fitted to the spectra.

all optimum parameters identified thus far. Using 100 mL of aqueous phase, the resulting microspheres had an average diameter of 12 $\mu$m and the encapsulation efficiency increased to 63 % (C-9; Table 2). It is difficult to predict the optimum volume of the aqueous phase. However, increasing the volume of the aqueous phase should result in a decreased emulsion droplet size because collisions of methylene chloride droplets resulting in fusion should occur less frequently. Homogenization intensity should decrease with increasing volume, resulting in larger emulsion droplets. Figure 4 summarizes the results obtained. Optimum encapsulation results were achieved when the aqueous phase was adjusted to 50 mL. The microspheres obtained had an average diameter of 9 $\mu$m and the encapsulation efficiency was 93 % (C-10; Table 2). The yield of BSA encapsulation was 68 %, also much higher than for all other conditions, an important point when encapsulating expensive pharmaceutical proteins in PLG.

We conclude that the efficiency of BSA encapsulation into PLG using the s/o/w method can be influenced by two different strategies. Firstly, the particle size of the BSA powder used initially can be minimized as described by Maa & Hsu (1997). Secondly, the follow-up procedure can be systematically optimized, as outlined in this work.

### Secondary structure of BSA in PLG microspheres

An important factor in the encapsulation of proteins into biocompatible polymers is to assess the magnitude of procedure-induced protein structural perturbations (Schwendeman et al 1996). We assessed the impact of the encapsulation procedure on the structure of BSA using FTIR spectroscopy (Carrasquillo et al 1998, 2001).

As previously reported (Carrasquillo et al 1998), lyophilization drastically altered the structure of BSA. The FTIR spectrum of the lyophilized powder (Figure 5B) in the structurally-sensitive amide I region is broadened compared with that of the aqueous solution (Figure 5A), indicating significant changes in the secondary structure of BSA (Carrasquillo et al 1998). When

**Table 3** Secondary structure of BSA at different stages during the encapsulation into PLG microspheres by the solid-in-oil-in-water technique.

| Sample/state | Secondary structure (%) | | Spectral correlation coefficient |
|---|---|---|---|
| | $\alpha$-helix | $\beta$-sheet | |
| Aqueous solution (pH 7.3) | $54 \pm 5$ | $7 \pm 2$ | $0.980 \pm 0.001$[a] |
| Lyophilized powder | $29 \pm 3$ | $30 \pm 2$ | $0.617 \pm 0.001$ |
| In PLG microspheres (s/o/w) | $36 \pm 3$ | $22 \pm 2$ | $0.839 \pm 0.005$ |
| In PLG microspheres (w/o/w)[b] | $21 \pm 2$ | NR | NR |

The secondary structure of BSA was determined from the resolution-enhanced amide I protein vibrational spectra by Gaussian curve-fitting. [a]The spectral correlation coefficient given was calculated using one representative amide I second derivative spectrum of BSA in aqueous solution vs other spectra of BSA in aqueous solution. Thus, the value is indicative for the error of the methodology. [b]Data from Fu et al (1999); NR, not reported.

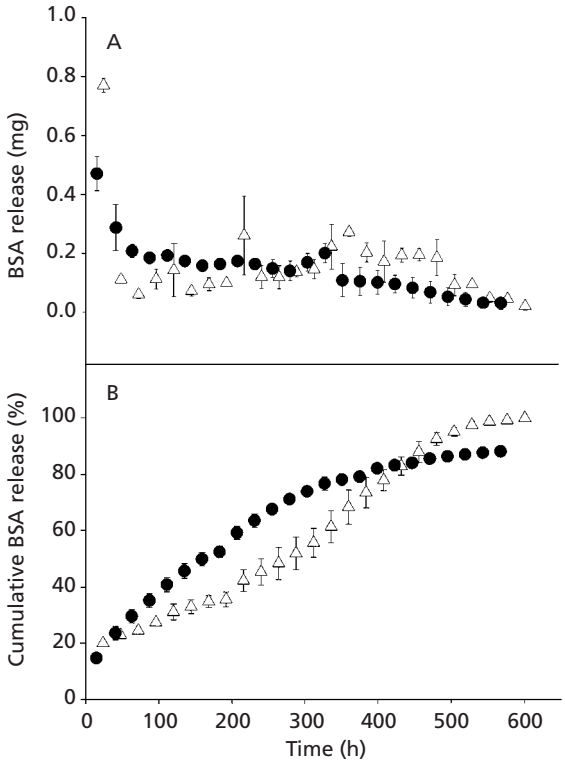


**Figure 6** Absolute (A) and cumulative (B) release profiles of BSA encapsulated in PLG microspheres using non-optimized conditions (●) (C-1; Table 2) and optimized conditions (Δ) (C-10; Table 2). Error bars represent the s.d. calculated from three release studies.

the secondary structure content was estimated by Gaussian-curve fitting, a drastic decrease in the $\alpha$-helix content from 54 to 29%, and increase in the $\beta$-sheet content from 7 to 30%, was observed upon lyophilization (Table 3). The spectral correlation coefficient also dropped significantly indicating lyophilization-induced structural perturbations in BSA (Table 3). We have already described that the suspension of this powder in methylene chloride, and subsequent drying, resulted in no further structural perturbations under the conditions used. To assess the impact of the follow-up procedure on the secondary structure of BSA in microspheres, FTIR spectra were obtained for BSA encapsulated in PLG using optimized conditions (C-10; Table 2). FTIR analysis of BSA secondary structure in microspheres obtained under non-optimum conditions (C-1; Table 2) could not be performed because of the very low loading of the microspheres of 1.4% BSA in PLG (w/w). The resulting FTIR spectra could not be properly corrected by subtraction of the PLG polymer background.

The FTIR spectrum shown in Figure 5C for BSA encapsulated in PLG microspheres clearly shows that the follow-up procedure did not induce further protein structural perturbations in addition to those caused by the initial lyophilization procedure. The spectrum even appeared to be somewhat more similar to that of the aqueous solution shown in Figure 5A. Quantitative data support this observation (Table 3). The $\alpha$-helix content was slightly higher, and the $\beta$-sheet content slightly lower, than in the lyophilized BSA powder and thus closer to the values found for BSA in aqueous solution. In addition, the spectral correlation coefficient is significantly higher than for the lyophilized BSA powder. Because this result was unexpected, FTIR data were measured for an independently prepared batch of PLG microspheres obtained under identical conditions. The secondary structure was within the same error ($34 \pm 2$% $\alpha$-helix, $19 \pm 1$% $\beta$-sheet) as reported in Table 3, and the spectra were also strikingly similar. It might be that the exposure to the aqueous solution in the emulsification step (somewhat opposed to our initial expectations) led to some increased structural mobility of BSA. This in turn might have allowed BSA–polymer interactions to occur leading to partial refolding of BSA. In addition PVA might interact with BSA and influence its structure. Whatever the explanation, BSA structure is less perturbed in PLG microspheres obtained by the s/o/w emulsion technique than in those obtained by the w/o/w technique (Fu et al 1999).

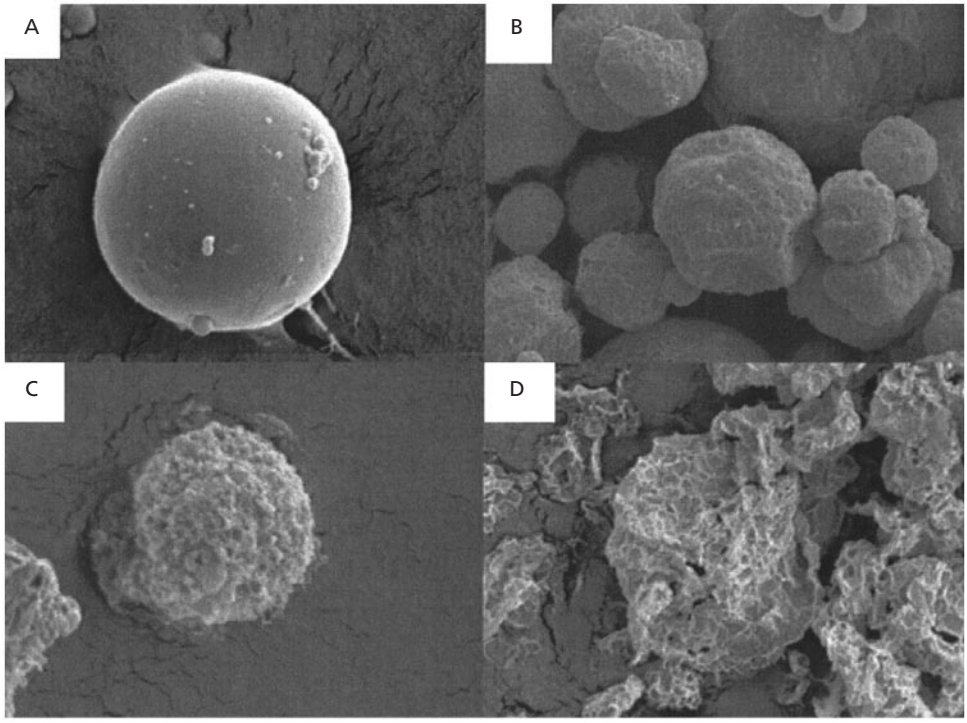


**Figure 7** SEM images of microspheres obtained under optimized conditions (C-10) at various times after in-vitro release. A, BSA-loaded microspheres immediately after preparation (width of the image corresponds to 14.7 $\mu$m); B, after 24-h exposure to the release buffer (width of the image corresponds to 14.7 $\mu$m); C, after 1 week release (width of the image corresponds to 13.2 $\mu$m); D, after 2 week release (width of the image corresponds to 26.4 $\mu$m).

### In-vitro release of BSA from selected microsphere preparations

The release of BSA from the microspheres obtained under non-optimized conditions (C-1; Table 2) and optimized conditions (C-10; Table 2) was also investigated. The initial burst release was approximately 15% for BSA encapsulated under non-optimized conditions and only slightly higher (approx. 20%) for BSA encapsulated under optimized conditions (Figure 6B). Since the actual loading of the microspheres obtained under optimum conditions was higher (9.2% BSA:PLG, w/w) than under non-optimized conditions (1.4% BSA:PLG, w/w), and the diameter was smaller (9 $\mu$m vs 33 $\mu$m; Table 2), this result was expected because of the larger surface exposed BSA. However, the quite small difference in the values indicates the better distribution of the BSA powder in the microsphere matrix resulting in a more efficient encapsulation of the protein when using optimized conditions (C-10). The smaller BSA powder particle size required in this instance is in line with the initial smaller microsphere size, and the small size of the porous cavities left after release of encapsulated BSA (Figure 7D). Further indication for this fact can be derived from the release profiles between days 2 and 8. The release from microspheres produced under optimized conditions (C-10) significantly lagged behind that from microspheres produced under non-optimized conditions (C-1), indicating more efficient BSA encapsulation in the former.

Another important feature of the release of BSA from both preparations is that BSA was released for an extended period of time at a nearly constant rate (approx. Days 3 to 14 for microspheres obtained under non-optimized conditions (C-1) and Days 2 to 21 for those obtained under optimized conditions (C-10); Figure 6A). Eventually, both microsphere preparations are depleted of protein as indicated by the high cumulative BSA release (nearly 90 and 100%; Figure 6B). This shows that only insignificant amounts of insoluble BSA aggregates could have been formed during the microsphere preparation preventing complete release of the protein.

### Microsphere morphology after different release times

To evaluate the differences in the release profiles observed for microspheres prepared using conditions C-1 and C-10, they were characterized by SEM after various times of in-vitro release. The microspheres obtained under both conditions were initially characterized by a smooth surface and were spherical in shape (Figure 7A). Exposure to release buffer for 24 h led to some characteristic changes in the surface structure. Microspheres obtained under condition C-1 showed very fine pores on the surface, probably caused by polymer swelling and release of surface exposed protein (not shown). The lack of significant changes in the surface, and the formation of very fine pores, explains the low amount of initial burst release. In contrast, microspheres obtained under condition C-10 also showed some morphological changes and appeared less regularly shaped (Figure 7B). This could have been caused by the loss of non-encapsulated protein, and the somewhat more pronounced burst release when compared with microspheres obtained under condition C-1 would be in line with this explanation. Pores formed by this preparation were extremely fine, explaining the lag phase in the cumulative release profile. Exposure of microspheres obtained under condition C-1, for 1 and 2 weeks, to the release buffer led to a significant increase in the pore size. These results explain the release profile, which is consistent with BSA release mainly due to diffusion (Göpferich & Langer 1995; Batycky et al 1997). In contrast, microspheres obtained under condition C-10 showed significant signs of erosion (Alonso et al 1994; Chen et al 1997) after 1 and 2 weeks of release (Figures 7C and D, respectively). Again, these results relate to the release profile observed (Figure 6B). Initially, the release was characterized by a lag phase due to limited diffusion of BSA from the microspheres (Göpferich & Langer 1995; Batycky et al 1997). After approximately 200 h of exposure to release buffer (corresponding to approx. 8 days) microsphere erosion becomes significant and BSA is released more efficiently (Göpferich & Langer 1995; Batycky et al 1997). The differences in the microsphere degradation found for both preparations are probably related to the different actual loading with BSA.

Another important observation concerns the shape and size of the cavities of microspheres obtained under condition C-10 after prolonged release (Figure 7D). The small cavities formed by release of BSA (and perhaps polymer erosion) are typically of sub-micrometer size and somewhat irregularly shaped. It is evident that the size of the encapsulated BSA particles must have been lesser or equal to the size of the formed cavities. This result strongly supports the notion that the o/w emulsion step is important in breaking down the BSA powder particles to a size much smaller and more homogeneous than during formation of the s/o suspension (compare with the image of particles obtained by homogenization at 20000 rev $min^{-1}$ shown in Figure 3C).

### Conclusions

The model protein BSA was encapsulated into PLG microspheres by a s/o/w technique. Adjustment of critical encapsulation parameters, in particular during the o/w emulsion step, allowed an encapsulation efficiency of $> 90\%$ to be achieved, and an encapsulation yield of approximately 70%. The secondary structure of BSA did not show any signs of further structural perturbations caused by the encapsulation procedure in addition to those caused by the initial lyophilization step. Release profiles were characterized by a low initial burst release and a cumulative release of $> 90\%$. Taken together, the results demonstrate that the s/o/w technique is an attractive alternative to the commonly used w/o/w double emulsion technique in protein encapsulation procedures.

## References

Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R., Langer, R. (1994) Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine* **12**: 299–306

Atkins, T. W. (1997) Fabrication of microspheres using blends of poly(ethylene adipate) and poly(ethylene adipate)/poly(hydroxybutyrate-hydroxyvalerate) with poly(caprolactone): incorporation and release of bovine serum albumin. *J. Biomater. Sci. Polymer Ed.* **8**: 833–845

Atkins, T. W., Peacock, S. J. (1996) The incorporation and release of bovine serum albumin from poly-hydroxybutyrate-hydroxyvalerate microcapsules. *J. Microencapsul.* **13**: 709–717

Batycky, R. P., Hanes, J., Langer, R., Edwards, D. A. (1997) A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. *J. Pharm. Sci.* **86**: 1464–1477

Carrasquillo, K. G., Cordero, R. A., Ho, S., Franquiz, J. M., Griebenow, K. (1998) Structure-guided encapsulation of bovine serum albumin in poly(DL-lactic-*co*-glycolic)acid. *Pharm. Pharmacol. Commun.* **4**: 563–571

Carrasquillo, K. G., Costantino, H. R., Cordero, R. A., Hsu, C. C., Griebenow, K. (1999) On the structural preservation

of recombinant human growth hormone in a dried film of a synthetic biodegradable polymer. *J. Pharm. Sci.* **88**: 166–173

Carrasquillo, K. G., Sanchez, C., Griebenow, K. (2000) Relationship between conformational stability and lyophilization-induced structural changes in chymotrypsin. *Biotechnol. Appl. Biochem.* **31**: 41–53

Carrasquillo, K. G., Aponte Carro, J. C., Alejandro, A., Diaz Toro, D., Griebenow, K. (2001) Reduction of structural perturbations in BSA by non-aqueous microencapsulation. *J. Pharm. Pharmacol.* **53**: 115–120

Chen, L., Apte, R. N., Cohen, S. (1997) Characterization of PLGA microspheres for the controlled delivery of IL-1a for tumor immunotherapy. *J. Contr. Rel.* **43**: 261–272

Cleland, J. L., Jones, A. J. S. (1996) Stable formulations of recombinant human growth hormone and interferon-$\gamma$ for microencapsulation in biodegradable microspheres. *Pharm. Res.* **13**: 1464–1475

Cleland, J. L., Langer, R. (1995) Formulations and delivery of proteins and peptides: design and development strategies. In: Cleland, J. L., Langer, R. (eds) *Formulation and Delivery of Proteins and Peptides*. ACS Symposium Series 567. ACS Books, Washington DC, pp 1–19

Fong, J. W., Nazareno, J. P., Pearson, J., Maulding, H. V. (1986) Evaluation of biodegradable microcapsules prepared by solvent evaporation process using sodium oleate as emulsifier. *J. Contr. Rel.* **3**: 119–130

Fu, K., Griebenow, K., Hsieh, L., Klibanov, A. M., Langer, R. (1999) FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. *J. Contr. Rel.* **58**: 357–366

Göpferich, A., Langer, R. (1995) Modeling monomer release from bioerodible polymers. *J. Contr. Rel.* **33**: 55–69

Griebenow, K., Klibanov, A. M. (1996) On protein denaturation in aqueous-organic but not in pure organic solvents. *J. Am. Chem. Soc.* **118**: 11695–11700

Griebenow, K., Klibanov, A. M. (1997) Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents? *Biotechnol. Bioeng.* **53**: 351–362

Griebenow, K., Castellanos, I. J., Carrasquillo, K. G. (1999a) Application of FTIR spectroscopy to probe and improve protein structure in sustained release devices. *Internet J. Vib. Spect.* www.ijvs.com 3, 5, 2

Griebenow, K., Santos, A. M., Carrasquillo, K. G. (1999b) Secondary structure of proteins in the amorphous dehydrated state probed by FTIR spectroscopy. Dehydration-induced structural changes and their prevention. *Internet J. Vib. Spect.* www.ijvs.com 3, 1, 3

Jeffery, H., Davis, S. S., O'Hagan, D. T. (1993) The Preparation and characterization of poly(lactide-co-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique. *Pharm. Res.* **10**: 362–368

Lu, W, Park, T. G. (1995a) In vitro release profiles of eristostatin from biodegradable polymeric microspheres: protein aggregation problem. *Biotechnol. Prog.* **11**: 224–227

Lu, W., Park, T. G. (1995b) Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J. Pharm. Sci. Technol.* **49**: 13–19

Maa, Y.-F., Hsu, C. C. (1997) Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process. *J. Microencapsul.* **14**: 225–241

Park, T. G., Lu, W., Crotts, G. (1995) Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D,L-lactic acid-co-glycolic acid) microspheres. *J. Contr. Rel.* **33**: 211–222

Prestrelski, S. J., Tedischi, N., Arakawa, T., Carpenter, J. F. (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* **65**: 661–671

Sah, H. (1999) Protein behavior at the water/methylene chloride interface. *J. Pharm. Sci.* **88**: 1320–1325

Schöneich, C., Hageman, M. J., Borchardt, R. T. (1997) Stability of peptides and proteins. In: Park, K. (ed.) *Controlled Drug Delivery, Challenges and Strategies*. ACS Professional Reference Book. American Chemical Society, Washington DC, pp 205–228

Schwendeman, S. P., Cardamone, M., Brandon, M. R., Klibanov, A. M., Langer, R. (1996) Stability of proteins and their delivery from biodegradable polymer microspheres. In: Cohen, S., Bernstein, H. (eds) *Microparticulate Systems for the Delivery of Proteins and Vaccines*. Marcel Dekker, New York, pp 1–49

Schwendeman, S. P., Tobio, M. Joworowicz, M., Alonso, M. J., Langer, R. (1998) New strategies for the microencapsulation of tetanus vaccine. *J. Microencapsul.* **15**: 299–318

Tabata, Y. Gutta, S. Langer, R. (1993) Controlled delivery systems for proteins using polyanhydride microspheres. *Pharm. Res.* **10**: 487–496

Thomasin, C., Johansen, P., Adler, R., Bemsel, R., Hottinger, G., Altorfer, H., Wright, A. D., Wehrli, G., Merkele, H. P., and Gander, B. (1996) A contribution to overcoming the problem of residual solvents in biodegradable microspheres prepared by coacervation. *Eur. J. Biopharm.* **42**: 16–24

Wang, H. T., Schmitt, E., Flanagan, D. R., Linhardt, R. J. (1991) Influence of formulation methods on the in vitro controlled release of proteins from poly(ester) microspheres. *J. Contr. Rel.* **17**: 23–32