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Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly(lactide-*co*-glycolide) microspheres results in release of native protein

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Abstract

Encapsulation of the model protein bovine serum albumin (BSA) into poly(D,L lactide-*co*-glycolide) (PLG) microspheres was performed by a non-aqueous oil-in-oil (o/o) methodology. Powder formulations of BSA obtained by spray-freeze drying were first suspended in methylene chloride containing PLG followed by coacervation by adding silicon oil and microsphere hardening in heptane. The secondary structure of BSA was determined at relevant steps of the encapsulation procedure by employing Fourier-transform infrared (FTIR) spectroscopy. This fast and non-invasive method demonstrated the potential to rapidly screen pharmaceutically relevant protein delivery systems for their suitability. Structural perturbations in BSA were reduced during the spray-freeze drying step by employing the excipient trehalose. The protein was then encapsulated into PLG microspheres under various conditions without inducing significant structural perturbations. BSA released from these microspheres had a similar monomer content as unencapsulated BSA and also the same secondary structure. Upon blending of a poloxamer (Pluronic F-68) with the polymer phase, in vitro release was characterized by a small initial release and a prolonged and continuous sustained phase. In conclusion, the developed o/o methodology coupled with FTIR spectroscopic monitoring of protein structure is a powerful approach for the development of sustained release microspheres. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Microspheres; Non-aqueous encapsulation; Poly(lactide-*co*-glycolide); Protein aggregation; Spray-freeze drying

1. Introduction

One of the most promising methods of protein delivery is their sustained release from biocompatible polymer devices, such as poly(lactide-*co*-glycolide) (PLG) microspheres, which, once injected, slowly release the protein drug by diffusion and polymer erosion [1,2]. However, the chemical and, in particular, the physical instability of proteins constitutes a major challenge because their encapsulation into such polymer devices involves harsh conditions that pose challenges even to quite stable proteins such as

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hen egg-white lysozyme [3–6]. Specifically, most protein encapsulation procedures are based on the double-emulsion solvent evaporation (a.k.a., water-in-oil-in-water (w/o/w)) technique [7]. The creation of the first water-in-oil emulsion has been identified as one major factor contributing to protein inactivation and aggregation in this procedure [3,5–10]. Emulsification leads to the creation of a large water/organic solvent interface. Because of their amphiphilic character, proteins adsorb to such interfaces [8–10], unfold [5], and aggregate [3] or undergo other undesired changes leading to biological activity loss [3,5]. For many proteins, the formation of medically unacceptable aggregates occurs as a consequence of such harsh encapsulation methods [4,11–15].

As a direct result, the development of alternative encapsulation methods has gained much attention from various researchers [16–25]. The approach to strictly avoid water during encapsulation is based on many arguments supporting that dehydrated protein powders often exhibit extraordinary stability in neat organic solvents. For example, dehydrated proteins function as efficient catalysts in organic solvents [26] and exhibit properties of molecular memory [27] and extended thermostability [28] compatible with the notion of drastically reduced conformational mobility under such conditions. The latter is due to the fact that restrictions in conformational mobility kinetically lock protein structure upon suspension in organic solvents [29]. Therefore, the thermodynamically favored event of unfolding in the organic solvent is prohibited by severe restrictions in the conformational mobility of the proteins. It has been shown that this increased structural rigidity can be utilized to minimize procedure-induced protein structural changes upon non-aqueous casting encapsulation [19,20] and o/o microencapsulation [21] into PLG.

These non-aqueous techniques employ dehydrated protein powder formulations. Dehydration is known to perturb protein structure (for review, see Dong et al. [30] and Griebenow et al. [31]) making proteins susceptible to detrimental phenomena such as solid-phase aggregation [32,33]. Excipients, such as trehalose, are usually required to prevent these dehydration-induced structural perturbations [34,35], and stability can be maintained by protein encapsulation under strictly non-aqueous conditions [19–21]. Furthermore, such non-aqueous microencapsulation by o/o procedures also requires powder formulations exhibiting extremely small (i.e., sub-micron) particle size upon suspension in the first organic phase to assure minimization of initial release [36]. This can ideally be achieved by spray-freeze drying leading to very porous and fragile protein powders that disintegrate into such small particles upon suspension in an organic solvent [36].

Despite recent advances in non-aqueous o/o encapsulation by various researchers [16–25], many critical pieces of information are still missing. For instance, it has not been shown that minimization of structural perturbations upon non-aqueous microencapsulation into PLG [19–21] indeed results in the release of structurally unmodified proteins. Thus, the predictive value of a spectroscopic method allowing for the non-invasive investigation of protein structure within PLG microspheres, such as FTIR spectroscopy [4,21,37,38], has not been demonstrated thus far. Herein, it is investigated whether minimization of protein structure perturbations during encapsulation probed by non-invasive FTIR spectroscopy would facilitate the development of suitable formulations leading to the release of native, non-aggregated protein.

2. Materials and methods

2.1. Chemicals

Dichloromethane (99.9%, ACS HPLC grade) and heptane (99%, anhydrous) were from Aldrich. Poly(dimethylsiloxane) (silicone oil) (3000 mPas inherent viscosity) was from Lancaster Synthesis. Poly(D,L-lactide-*co*-glycolide) (PLG) with a copolymer ratio of 50:50 was from Boehringer Mannheim (resomer RG502H, $M_w \sim 10$ kDa) and Aldrich ($M_w \sim 50$–75 kDa). Poloxamer (Pluronic F-68) was from Sigma.

2.2. Spray-freeze drying

An aqueous solution of BSA with or without co-dissolved trehalose was atomized using a two-

fluid nozzle in a stainless steel chamber; the processing conditions were chosen to minimize the particle size after the first suspension step in the encapsulation procedures (for details, see Ref. [36]). The frozen slurry was collected in stainless steel beakers and poured into glass dishes, which were lyophilized after pre-cooling the shelves to −40°C. Drying was carried out at a chamber pressure of 300 mTorr and a shelf temperature of 10°C for excipient free protein. For formulation with trehalose, primary drying at −26°C shelf temperature and 96 mTorr for about 3 d was followed by secondary drying at 20°C and 10–20 mTorr for 2 d (this formulation is referred to as BSA-Tre below). Dried powders were stored at −20°C.

2.3. Microsphere preparation

A sample of 40 mg of solid BSA was suspended in 2 ml of methylene chloride containing 200 mg of dissolved PLG with a VirTis Tempest homogenizer (Gardiner, NY, USA) using a 10-mm shaft equipped with a micro-fine rotor/stator type generator at ca. 20,000 rpm for 1 min. In the case of employing BSA-Tre, the mass of the added solid material to PLG was kept constant. In the case of PLG/poloxamer blends, appropriate amounts of poloxamer were added to the polymer solution in CH_2Cl_2 and co-dissolved in order to obtain the desired PLG-to-poloxamer mass ratio. After protein suspension, the coacervating agent poly(dimethylsiloxane) was added at a rate of 2 ml min^{-1} while still homogenizing at the same speed to allow for homogeneous dispersion of the coacervating agent. The coacervating mixture containing the microspheres was then poured into an Erlenmeyer flask containing 50 ml heptane under constant agitation and stirred for 2 h at room temperature to allow for hardening of the microspheres. Microspheres were collected by filtration using a 0.22 μm pore nylon filter, washed twice with heptane, and dried for 24 h at a vacuum of <60 μm of Hg at room temperature. The actual BSA loading in the microspheres was 5% for BSA in PLG, 3.3% for BSA-Tre in PLG, 2.2% for BSA-Tre in the poloxamer–PLG blend at a 1:20 mass ratio, and 1.4% for BSA-Tre in the poloxamer–PLG blend at a 1:1 mass ratio.

2.4. Protein secondary structural analysis

FTIR spectra were acquired using a Magna-IR System 560 optical bench and corrected for excipients, PLG background, and water vapor as described [19–21,39]. The feasibility to obtain an artifact-free amide I (1700–1615 cm^{-1}) IR spectrum for protein encapsulated in PLG by subtracting the PLG background has been established in the literature [4,19,20,37,38]. However, because tailing of the PLG band at ca. 1750 cm^{-1} into the protein amide I band occurs, wrong subtraction might in principle cause some spectral alterations in the high frequency region (above ca. 1675 cm^{-1}) of the protein amide I IR spectrum [4,37,38]. Thus, the strategy developed in the past [4,19,20] was employed to carefully assure that the protein amide I IR spectrum was not affected by the subtraction procedure. Herein, the PLG background was subtracted from the measured spectrum using different subtraction factors (deliberately achieving over- and under-subtraction) and the protein amide I IR spectrum analyzed for the secondary structure composition by Gaussian curve-fitting. No significant variation was found for the secondary structure content determined using various subtraction factors, excluding a significant effect of the PLG subtraction procedure on the shape of the amide I band under the conditions employed in this work.

All spectra were analyzed by second derivatization in the amide I region for their component composition and BSA secondary structure quantified by Gaussian curve-fitting after Fourier self-deconvolution of the corrected spectra [19–21,39].

2.5. In vitro release profiles

Ten milligrams of microspheres were placed in 2 ml of 10 mM phosphate buffer adjusted to pH 7.3 and incubated at 37°C. Every 24 h, microspheres were centrifuged for 1 min at 500 rpm. The supernatant was removed and the protein concentration determined as described [40]. Microspheres were then resuspended in 2 ml of fresh buffer to maintain sink conditions and control the pH [41]. Total protein concentration values were used to construct cumulative release profiles. Data are presented as the

average with the standard deviations for experiments performed in triplicate.

2.6. Size-exclusion HPLC

SEC-HPLC was conducted using a G2000SW-XL1 TSK Gel Column (TosoHaas, PA, USA) and followed by UV detection at 280 nm. Typically, 50 μl of each filtered protein sample after release was loaded and the column developed at a flow rate of 0.5 ml/min. The mobile phase consisted of 0.3 M NaCl, 50 mM sodium phosphate, pH 7.0. Data are reported as percent BSA monomer for samples following 24 h of release.

2.7. Circular dichroism studies

CD spectra were recorded on an Aviv Instruments circular dichroism spectrometer model 202. Data were collected at 25°C using a bandwidth of 0.5 nm, a time constant of 100 ms, a step size of 0.5 nm, and an average time of 0.1 s. The CD spectra were collected from 195 to 300 nm using 0.5 cm quartz cells and corrected for the phosphate buffer signal contribution measured under identical conditions.

3. Results and discussion

3.1. Reduced protein structural perturbations in non-aqueous microencapsulation procedures

BSA was employed as the model protein in this study to allow for building on our previously published data concerning non-aqueous encapsulation procedures [19,21]. It should be noted that BSA is a good model system in such investigations because it easily forms covalent aggregates by thiol–disulfide interchange in the solid state, and thus is an excellent sensor to allow monitoring of protein aggregation as the result of encapsulation procedures [33,42].

Non-aqueous o/o protein encapsulation procedures are based on the suspension of a dehydrated protein formulation in a suitable organic solvent able to dissolve the chosen polymer (e.g., CH_2Cl_2), followed by emulsification in a non-solvent for the polymer (e.g., silicone oil) to achieve polymer hardening, and therefore microsphere formation [43–45]. The necessary initial dehydration step is known to cause protein structural perturbations [30,31,34,35,46], also reported for freeze-dried [19] and spray-freeze dried [21,36] BSA. FTIR spectroscopy has demonstrated that spray-freeze drying induces structural perturbations under the conditions employed in this work (Fig. 1A,B). Significant spectral changes occurred in the amide I band (1700–1615 cm^{-1}), which is sensitive to protein structure [47,48]. Analysis of the spectra by Gaussian curve-fitting revealed a decrease in the α-helical content from 54 to 31% and an increase in β-sheet content from 8 to 22% (Table 1). When the BSA powder obtained was encapsulated into PLG microspheres, only minor additional spectral changes were observed (Fig. 1C); the secondary structure was within the error the same as for the powder prior to encapsulation (Table 1). Thus, decreased conformational mobility of BSA in the dehydrated powder prevented substantial encapsulation-induced structural changes.

Structural perturbations occurring in the spray-freeze drying step were reduced when trehalose was co-dissolved with BSA at a 4:1 mass ratio prior to the spray-freeze drying procedure. (The resulting powder is referred to below as BSA-Tre.) The secondary structure (44% α-helix and 12% β-sheet) determined from the amide I IR spectrum was more similar to that of BSA in aqueous solution than that of BSA spray-freeze dried without excipient (Table 1). When encapsulating BSA-Tre by the o/o procedure into PLG (resomer RG502H, $M_w \sim 10$ kDa) microspheres, the spectrum (Fig. 1D) and secondary structure (Table 1) of BSA-Tre encapsulated in PLG microspheres were similar to the spectrum of BSA-Tre in aqueous solution (Fig. 1A). The excipient containing powder exhibited stability towards the encapsulation stress. PLG microspheres were also blended with a poloxamer (Pluronic F-68) by co-dissolving PLG and poloxamer in CH_2Cl_2 in the encapsulation procedure. When BSA-Tre was encapsulated in poloxamer/PLG microspheres employing a poloxamer/PLG mass ratio of 1:20, the secondary structure was the same as for BSA-Tre in PLG microspheres (Table 1). Poloxamer did not have a detrimental effect on protein structure during the encapsulation.

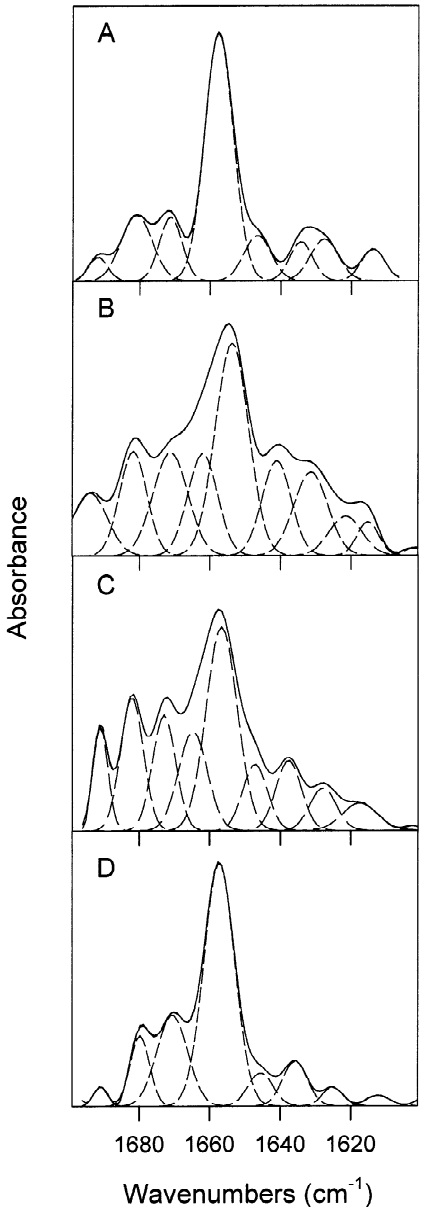

Fig. 1. Resolution-enhanced amide I FTIR spectra of BSA under various conditions and their Gaussian curve-fitting. (A) BSA in aqueous solution pH 7.4; (B) BSA powder obtained by spray-freeze drying from buffer; (C) powder as in (B) microencapsulated in PLG (M_w 10,000) microspheres; (D) BSA powder obtained by co-spray-freeze drying with trehalose at a 1:4 weight ratio of BSA/trehalose and microencapsulated in PLG (M_w 10,000) microspheres. The solid lines are the resolution-enhanced amide I FTIR spectra overlaid with the result of the Gaussian curve-fitting (both practically coincide); the broken lines are the individual Gaussian bands fitted to the spectra.

3.2. Minimization of BSA structure perturbations upon encapsulation reduces aggregation

No study thus far has addressed the question of how the structure of proteins encapsulated in PLG microspheres by o/o protocols is related to the amount of protein aggregation upon release. If protein secondary structure, non-invasively determined by FTIR spectroscopy, could be used to reasonably predict the quality of the released protein, it would allow for a more rapid screening of appropriate conditions. To investigate this, we monitored the amount of protein aggregates after 24 h of in vitro release by size exclusion (SEC) HPLC [49,50]. The working hypothesis was that minimization of structural perturbations for encapsulated BSA should result in less aggregation.

For BSA as supplied by the commercial source, a monomer content of 95% was determined by performing size-exclusion (SEC) HPLC experiments (Fig. 2), a value quite typical for commercial samples [33]. When BSA was spray-freeze dried under the conditions employed in this work, an additional drop in the monomer content by ca. 10% occurred [36]. BSA released within 24 h from PLG microspheres exhibited pronounced formation of soluble aggregates. The monomer content for the initially released protein dropped to only 36% (Table 1). A likely explanation is that since BSA structure was significantly perturbed in the powder formulation employed, aggregation occurred because of exposed thiol and disulfide groups.

In contrast, when employing trehalose as the excipient in the spray-freeze drying procedure, the monomer percentage for non-encapsulated BSA-Tre re-dissolved in buffer was 93% (Table 1) and thus similar to that in aqueous solution. Trehalose prevented formation of soluble BSA aggregates. Furthermore, when BSA-Tre was encapsulated into PLG or poloxamer/PLG microspheres, BSA was released predominantly as monomer (88–94%, Table 1). BSA was protected from aggregation during the initial spray-freeze drying step and subsequently during the encapsulation procedure and the initial release period. This is likely due to the preservation of BSA structure upon spray-freeze drying and subsequent encapsulation by trehalose. The α-helix content for all trehalose-containing samples was much higher,

Table 1
BSA secondary structure and monomer content upon encapsulation and release from PLG microspheres

Sample/state	Secondary structure (%)			Monomer content (%)[a]	Microsphere diameter (μm)
	α-Helix	β-Sheet	Other		
BSA					
Aqueous solution pH 7.4	54±6[b]	8±3[b]	38±1[b]	95	n.a.[c]
Spray-freeze dried powder	31±3	22±3	47±3	n.d.[c]	n.a.
Microencapsulated in PLG	32±4	17±2	51±2	36	11±8
BSA-Tre[d]					
Spray-freeze dried powder	44±3	12±3	45±2	93	n.a.
Microencapsulated in					
PLG	45±3	13±2	42±3	88	15±6
Poloxamer/PLG, 1:20 (w/w)	46±2	13±3	41±2	90	36±25
Poloxamer/PLG, 1:1 (w/w)	[e]	[e]	[e]	94	53±25

[a] Determined after 24 h release from PLG microspheres.
[b] These data have been reported before by Carrasquillo et al. [21] and are given here to allow for comparison.
[c] n.a., not applicable; n.d., not determined.
[d] BSA-to-trehalose at a 1:4 mass ratio, see Methods section for details.
[e] The secondary structure could not be determined for this sample because of interference of poloxamer with the amide I spectral region. Due to the high poloxamer content the background could not be subtracted adequately.

and the structure more native-like than for BSA without the excipient (Table 1).

These results confirm the stabilizing effect of trehalose on protein stability upon encapsulation and release from PLG microspheres prepared by o/o coacervation protocols. For example, a tetanus toxoid formulation achieved by co-lyophilization with trehalose was released with about 85% of its initial biological activity in contrast to less than 10% when released from PLG microspheres prepared by w/o methods [23].

After having found a relation between BSA secondary structure in PLG microspheres and the aggregation state of initially released BSA, it was also interesting to explore whether BSA-Tre would be released having a native structure and the circular dichroism (CD) spectra of BSA released within a 24 h period were measured to test this (Fig. 3). It was found that in those instances where BSA secondary structure was preserved upon microencapsulation and protein was released mainly as a monomer, the CD spectra were not significantly different from that of an aqueous solution of commercially available BSA. All spectra exhibit minima at ca. 208 and 222 nm indicative of predominantly α-helical secondary structure and were quite comparable in their spectral shape. It is concluded that BSA was released having a native-like secondary structure from the microspheres.

3.3. BSA release from PLG microspheres

BSA release from PLG microspheres was governed by an asymptotic profile in which >80% of the protein was released rapidly within only 2 days (Fig. 4). The same release profile was noted for PLG microspheres loaded with BSA-Tre. Neither the presence of the excipient nor differences in the actual loading (see Methods section) exerted any noticeable influence on BSA release. The complete release in both cases also rules out the significant formation of insoluble BSA aggregates for both formulations.

It is obvious that the release profiles obtained were undesirable. In order to improve the release of BSA from PLG microspheres, BSA-Tre loaded microspheres consisting of blends of low M_w PLG (~10 kDa) and the nonionic surfactant poloxamer [51] were tested. Both were co-dissolved at various ratios in the methylene chloride phase prior to suspension of BSA-Tre to afford blending. Poloxamer is a triblock copolymer composed of polyoxyethylene and polyoxypropylene [52]. In w/o/w protocols such polymers produce a coating because the hydrophobic polyoxypropylene chains interact with the

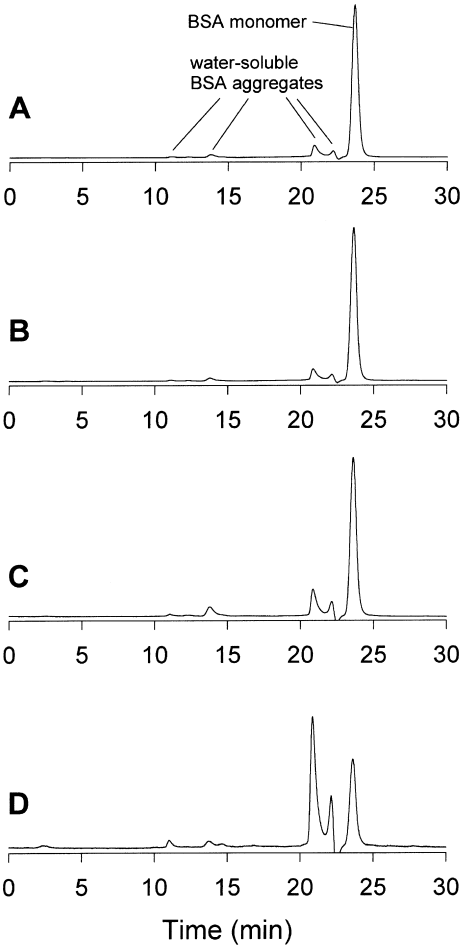

Fig. 2. Size-exclusion HPLC chromatograms (detection at $\lambda =$ 280 nm) of (A) BSA as commercially supplied dissolved in phosphate buffer at pH 7.3; (B) BSA spray-freeze dried from phosphate buffer containing trehalose at a 1:4 BSA-to-trehalose weight ratio and re-dissolved in phosphate buffer at pH 7.3; (C) powder as in (B) encapsulated into PLG (M_w 10,000) microspheres after 24 h of in vitro release; (D) BSA spray-freeze dried from buffer alone encapsulated into PLG (M_w 10,000) microspheres after 24 h of in vitro release.

PLG polymer, while the more hydrophilic polyoxyethylene chains protrude into the surrounding aqueous medium [52–54]. The arrangement of such molecules in microspheres produced by o/o encapsulation procedures is not known, but it seems likely that poloxamer is more evenly distributed in the microsphere matrix. In general, poloxamer/PLG systems have the potential to reduce the lag-phase,

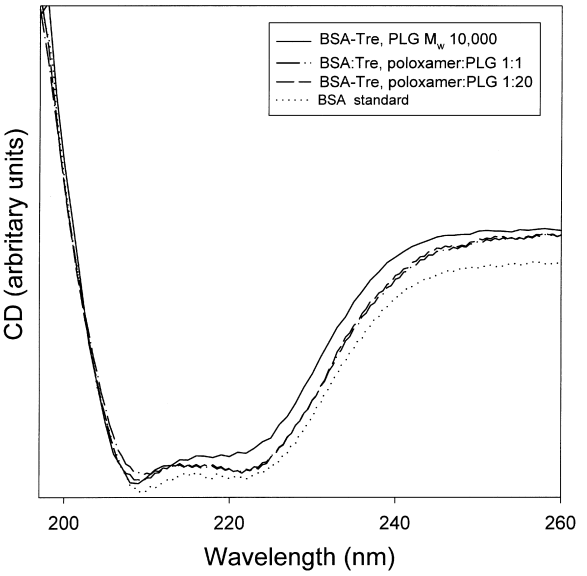

Fig. 3. Circular dichroism spectra of BSA as supplied commercially dissolved in phosphate buffer at pH 7.3 (· · ·) and of BSA co-spray-freeze dried with trehalose and encapsulated under various conditions in PLG microspheres after 24 h of in vitro release. The spectra were normalized to compensate for concentration differences.

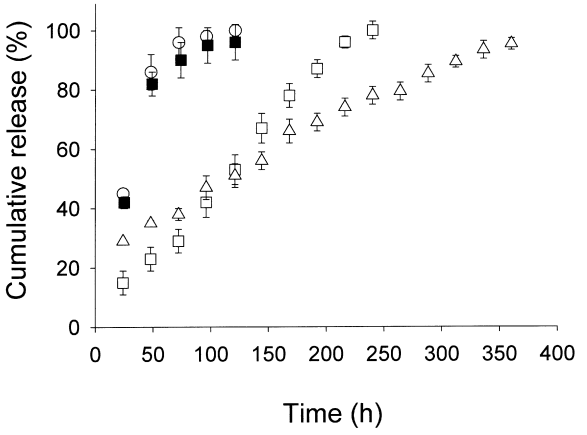

Fig. 4. Cumulative release of (■) spray-freeze dried BSA and (○) BSA-Tre from PLG (M_w 10,000) microspheres, (□) spray-freeze dried BSA-Tre from poloxamer/PLG (M_w 10,000) microspheres (1:20 poloxamer/PLG mass ratio), and (△) spray-freeze dried BSA-Tre from poloxamer/PLG (M_w 10,000) microspheres (1:1 poloxamer/PLG mass ratio).

and therefore lead to a more continuous release [51,55].

Blending of PLG with poloxamer had a dramatic effect on the in vitro release of BSA, specifically an increase in the release duration (Fig. 4). A possible explanation is that the microsphere diameter was significantly increased by the presence of poloxamer in the o/o procedure (Table 1). This is supported by the observation that the burst was decreased from 45% to a minimum of 15% with poloxamer added (Fig. 4), likely due to a lower amount of BSA powder particles in direct contact with the microsphere surface. It is unlikely that the lower actual loading of the poloxamer/PLG microspheres (2.2% for the 1:20 ratio and 1.44% for the 1:1 ratio) contributed significantly to the extended in vitro release and reduction in the initial burst release, because the differences in the actual loading did not influence the release of BSA/BSA-Tre from PLG microspheres.

The effect of a larger PLG polymer (M_w 50–75 kDa) on release was also evaluated. As expected [55], triphasic BSA release was observed (data not shown). After initial surface protein was released, there was an extended lag period characterized by a very slow release rate. When poloxamer was blended with this PLG, the release profile exhibited a biphasic pattern, primarily due to a reduced lag phase. Also for high M_w PLG, blending with poloxamer exerted an improved release profile.

4. Conclusions

It is concluded that a structure-guided encapsulation approach for protein encapsulation by polymer casting can be further extended to protein encapsulation into PLG microspheres by an o/o methodology. The degree of BSA structural perturbations upon microencapsulation is a good indicator of subsequent BSA stability and formation of soluble BSA aggregates. Using this methodology it was possible to produce microspheres which released the protein in substantially native, monomeric form. FTIR spectroscopy proved to be an effective non-invasive probe to identify suitable protein formulations for sustained release. The addition of poloxamer to the polymer phase substantially improved release profiles under all conditions tested.

Acknowledgements

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