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Effect of the molecular weight of poly(ethylene glycol) used as emulsifier on α-chymotrypsin stability upon encapsulation in PLGA microspheres

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Abstract

Poly(ethylene glycol) (PEG) was used as emulsifier to prepare α-chymotrypsin-loaded poly(lactic-*co*-glycolic) acid (PLGA) microspheres by a solid-in-oil-in-water (s/o/w) technique. The effect of the molecular weight of PEG on protein stability was assessed by the determination of the amount of insoluble aggregates, the activity loss and the magnitude of structural perturbations. In addition, the effect of the molecular weight of PEG on the encapsulation efficiency, microsphere characteristics and release kinetics was investigated. X-ray photoelectron spectroscopy (XPS) was employed to characterize the surface chemistry of the microspheres. Microspheres were prepared using PEG with molecular weight of 6000, 8000, 10 000, 12 000 and 20 000. The results indicate that PEG 20 000 was the most effective emulsifier when producing α-chymotrypsin-loaded microspheres with respect to protein stability. The aggregate formation was decreased from 18% to 3%; the protein inactivation and the encapsulation-induced structural perturbations were largely prevented. XPS confirmed that PEG was largely located on the surface of microspheres. The molecular weight of PEG affected the microspheres' characteristics and release kinetics. Microspheres prepared with PEG 20 000 showed improved encapsulation efficiency (80%) and a continuous release (for 50 days) with the lowest amount of initial release. It is demonstrated that the selection of the optimum molecular weight of PEG when used as emulsifier in the preparation of microspheres is a critical factor in the development of sustained-release formulations for the delivery of proteins.

Introduction

Significant challenges still remain in the encapsulation of protein pharmaceuticals in biodegradable microspheres, such as maintaining protein integrity during encapsulation and release. The search for stabilization strategies has been intensified in an attempt to optimize the existing controlled-delivery systems (Pérez et al 2001). Some research has focused on minimizing protein instability during encapsulation by using a solid-in-oil-in-water (s/o/w) technique instead of the more common water-in-oil-in-water (w/o/w) technique (Morita et al 2000; Castellanos et al 2001a, b; Takada et al 2003). In s/o/w procedures the protein is first dehydrated and then suspended in the organic solvent to form a solid/organic solvent suspension. Proteins have a drastically reduced conformational mobility under these conditions and large structural changes are prohibited because the proteins are kinetically trapped in their conformation (Griebenow & Klibanov 1996; Griebenow et al 2001). Nevertheless, encapsulation of proteins by the s/o/w procedure also induces inactivation and aggregation (Castellanos et al 2001a, b, 2002). As in w/o/w encapsulation, it has been established that the organic solvent/water emulsion step is largely responsible for protein instability (Castellanos et al 2003).

Poly(ethylene glycol) (PEG) has been widely used to stabilize proteins upon encapsulation using different techniques (Péan et al 1999; Meinel et al 2001; Al-Azzam et al 2002; Castellanos et al 2002, 2003; Pérez-Rodríguez et al 2003). The use of PEG as an excipient during the s/o/w encapsulation process largely prevented γ-chymotrypsin instability (Castellanos et al 2002). It was also found that PEG is a potent alternative to the most commonly used emulsifying agent, poly(vinyl alcohol) (PVA) (Castellanos

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et al 2003). Since PVA is listed in the 1976 Register of Toxic Effects of Chemical Substances and has also been implicated as being carcinogenic when introduced parenterally into animals, some attempts to replace it with other emulsifiers have been reported (Coombes et al 1998; Bezemer et al 2000; Feng & Huang 2001). We found that PEG was more effective than PVA in stabilizing different model proteins upon encapsulation and was able to stabilize the emulsion droplets forming microspheres (Castellanos et al 2003). In addition, when PEG was used as an emulsifying agent it also stabilized proteins upon encapsulation with different organic solvents (Castellanos & Griebenow 2003). To further optimize the microsphere formulation using PEG as an emulsifier, the effect of the molecular weight of PEG on α-chymotrypsin stability upon s/o/w encapsulation was investigated. We hypothesized that PEG with high molecular weight would be superior in improving protein stability compared with PEGs with lower molecular weight by acting as a better spacer molecule, preventing direct contact of the protein with hydrophobic interfaces during encapsulation. To investigate this, PLGA microspheres were prepared using PEG with different molecular weights (6000, 8000, 10 000, 12 000 and 20 000). Following encapsulation, the amount of insoluble aggregates, the specific activity and the secondary structure of α-chymotrypsin were determined. In addition, the effect of the molecular weight of PEG on microsphere properties, such as morphology, surface chemistry, size, encapsulation efficiency and release kinetics, was also investigated.

Materials and Methods

Materials

α-Chymotrypsin, poly(vinyl) alcohol (PVA; 87–89% hydrolysed, MW 13 000–23 000) and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from Sigma-Aldrich. PEGs with a molecular weight of 6000, 8000, 10 000, 12 000 and 20 000 were from Fluka. Poly(D,L-lactic-*co*-glycolic) acid (PLGA) with a copolymer ratio of 50:50 and an average molecular weight of 60 000 was a generous gift from Alkermes Inc. (Cambridge, MA, USA). Methylene chloride (99.9%, ACS HPLC grade) and ethyl acetate were from Fischer.

Protein lyophilization

α-Chymotrypsin (dissolved in de-ionized water at a concentration of 10 mg mL^{-1}, pH 7.8) was frozen in liquid nitrogen and lyophilized for 48 h using a Labconco FreeZone 6L freeze drier at a condenser temperature of -45°C and a pressure of < 60 μmHg. Lyophilized protein powders were kept at -20°C until used in the experiments.

Microsphere preparation

Microsphere preparation followed the procedure developed by Castellanos et al (2001a). In brief, lyophilized α-chymotrypsin (40 mg) was suspended in 2 mL of methylene chloride containing 360 mg of PLGA by homogenization with a VirTis Tempest homogenizer using a 10-mm shaft at 20 000 rev min^{-1} for 30 s. The resulting suspension was added to 50 mL of an aqueous solution (10% w/v) of the emulsifying agent, PEG, with different molecular weights. The oil-in-water emulsion was formed by homogenization (20 000 rev min^{-1}, 2 min). Microsphere hardening was achieved by stirring of the emulsion for 3 h and the microspheres were collected by filtering through a 0.45-μm pore size cellulose acetate filter, washing with 100 mL of distilled water and drying for 24 h at < 60 μm Hg.

Determination of enzyme activity

Activity of α-chymotrypsin was determined using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate. The reaction was carried out in 1 mL 0.1 M Tris-HCl buffer containing 0.6 μg enzyme (protein), 0.35 mM substrate and 0.01 M $CaCl_2$ at pH 7.8. Specific activity was defined as mM of substrate converted into product per minute per mg of protein (DelMar et al 1979). The time-dependent absorbance changes at 410 nm were measured and the linear portion of the graphs was used to obtain the initial velocities (V_0) (Castellanos et al 2003). The experiments were performed in triplicate and the results averaged. Error bars in the figures are the calculated standard deviations.

Determination of actual protein loading of microspheres and encapsulation efficiency

The actual protein loading of microspheres was determined following the method developed in our laboratory (Castellanos et al 2002). Protein aggregates formed during the encapsulation process are taken into account in this procedure. To determine the amount of non-soluble protein aggregates, protein-loaded microspheres were suspended in 1 mL of ethyl acetate to dissolve the PLGA, followed by centrifugation. The supernatant was discarded and the pellet was vacuum dried. Next, 1 mL of phosphate buffer was added to the protein powder to dissolve the soluble protein fraction. The buffer-insoluble protein fraction was treated with 1 mL of 6 M urea solution to completely dissolve the insoluble aggregates. All aggregates could be dissolved with urea. The protein concentration in the clear solutions obtained was determined from the UV absorbance at 280 nm using a standard calibration curve. The encapsulation efficiency of protein in the microspheres was calculated from the actual loading with respect to the theoretical loading of protein (%, w/w) in the microspheres. The experiments were performed in triplicate, the results averaged and the standard deviations calculated.

Scanning electron microscopy (SEM)

SEM of α-chymotrypsin-loaded PLGA microspheres and lyophilized protein was performed using a JEOL 5800LV scanning electron microscope at a voltage of 20 kV. The

samples were coated with gold (200–500 Å) using a Denton Vacuum DV-502A. The size of microspheres was determined from saved images using the program picture publisher from Micrografx. For each sample, the dimensions of 100 microspheres were determined, averaged and the standard deviation calculated.

FTIR spectroscopy

FTIR studies were performed using a Nicolet Magna-IR 560 optical bench (Castellanos et al 2001b, 2002, 2003). The spectra of protein in aqueous solution, in amorphous dehydrated powders and encapsulated in PLGA microspheres were obtained as described in detail in the literature and corrected for the background (Castellanos et al 2001b). The proper subtraction of the PLGA background from FTIR spectra obtained for α-chymotrypsin in microspheres was performed as described in detail (Carrasquillo 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 (Griebenow & Klibanov 1996). The band assignments in the amide I region followed those in the literature (Carrasquillo et al 2000). The secondary structure content of protein under various conditions was calculated from the amide I IR spectra by Gaussian curve-fitting analysis for four independently obtained spectra. Spectral correlation coefficients to quantify procedure-induced protein structural perturbations were calculated as described using the amide I second derivative spectra (Prestrelski et al 1993).

Surface chemistry

The X-ray photoelectron spectroscopy (XPS, Physical Electronics PHI 5600 ESCA system) was utilized for analysing the surface chemistry of the microspheres. A large window spectrum (0–1200 eV) was taken for each sample using pass energy of 188 eV to assure that no contamination was present. Then, the spectra for C1s, N1s, O1s were obtained with higher energetic resolution; for O1s the pass energy was 58.70 eV. The analysis was performed with a take off angle of 45°. Peak curve fitting of the O1s (atomic orbital 1s of oxygen) envelope was performed using the program GRAMS 386 from Galactic Industries.

In-vitro release studies

Microspheres (30 mg) of α-chymotrypsin were placed in 1 mL of 10 mM phosphate buffer at pH 5.0 (to prevent autolysis) and incubated at 37°C (Castellanos et al 2002). At predetermined times the samples were subjected to centrifugation (5000 rev min^{-1} for 5 min) to pellet the insoluble components. The supernatant was removed and the concentration of released protein was determined by UV absorbance spectroscopy at 280 nm. These samples were also used to measure the specific activity of encapsulated α-chymotrypsin after in-vitro release. The buffer was completely replaced to maintain sink conditions in the release experiments. Since there was a small (but significant) contribution to the UV absorbance at 280 nm due to microsphere erosion, blank microspheres were subjected to in-vitro release in parallel with α-chymotrypsin-loaded microspheres. The UV absorption at 280 nm was determined and subtracted from those obtained with α-chymotrypsin-loaded microspheres. The concentration of the released protein was plotted directly against time and also used to construct cumulative release profiles. Release experiments were performed in triplicate, the results averaged and the standard deviations calculated.

Thermal denaturation experiments

Fluorescence spectra of α-chymotrypsin were monitored on a computerized Cary Eclipse fluorescence spectrophotometer (Varian, Walnut Creek, CA, USA) in the temperature range of 30–70°C using a quartz cuvette with 10-mm pathlength. The temperature was adjusted with a microprocessor programmable controller. The thermal denaturation of α-chymotrypsin (0.3 mg mL^{-1}) in the presence or absence of PEG 6000 and 20 000 was measured in 10 mM sodium phosphate at pH 5.0. Each sample was excited at 281 nm, and the emission spectra recorded from 290 to 450 nm. Changes in the fluorescence emission maximum were recorded and plotted versus the temperature. The melting temperature (T_m) values were determined from the maximum of the inverted first derivatives of the thermal denaturation curves (Carrasquillo et al 2000). Thermal denaturation experiments were performed in triplicate, the results averaged and the standard deviations calculated.

Statistical methods

Statistical analysis of the effect of PEG molecular weight (6000, 12 000 and 20 000) on the cumulative release and specific activity of α-chymotrypsin at each time period was performed. In the thermal denaturation experiments, the effect of the PEG molecular weight on the maximum emission wavelength for α-chymotrypsin at each temperature was compared for different treatments (in the absence of PEG, PEG 6000 and 20 000). The differences in the % of α-helix, % of β-sheet and SCC (spectral correlation coefficient; measure of spectral changes of amide I region) for different treatments (α-chymotrypsin before encapsulation, after encapsulation using PEG 6000, 12 000 and 20 000) were statistically analysed. In all cases, statistics analysis were performed using the Kruskal–Wallis test and the individual differences between the various molecular weight values was then examined using a post-hoc test (Nemenyi's test). $P < 0.05$ denoted significance in all cases. All experiments were performed in triplicate except for the differences in the secondary structure content, which was performed in quadruplicate.

Results and Discussion

Stability of α-chymotrypsin upon encapsulation in PLGA microspheres

It has been demonstrated that protein aggregation and inactivation upon s/o/w encapsulation largely stem from the

organic solvent/water emulsion step (Castellanos & Griebenow 2003; Castellanos et al 2003). Even though the suspended protein in the organic phase is in the solid state during emulsion formation, the protein could migrate to the interface. It has been reported that large protein particles tend to migrate faster to the surface of the droplet near to the organic solvent/water interface than small particles because of their higher terminal velocities during emulsification (Maa & Hsu 1997). At the interface the protein particle will have some contact with water, which could increase its conformational mobility in the presence of the organic solvent (Griebenow & Klibanov 1996). In addition, water diffusing into the organic droplets could rehydrate the protein molecules (Castellanos & Griebenow 2003). As a result, the protein will be more susceptible to unfolding and subsequent aggregation. Thus, the prevention of protein adsorption to the organic solvent/water interface, either on the surface of embryonic microspheres or inside of the forming microspheres, should avoid protein instability upon encapsulation. We hypothesized that an increase of PEG molecular weight (chain length) used as emulsifying agent would improve protein stability by acting as a better spacer molecule, preventing direct contact of the protein with hydrophobic interfaces during microsphere preparation. The stability of α-chymotrypsin upon encapsulation was assessed by determining the amount of insoluble aggregates, and the loss of enzyme activity. When the molecular weight of PEG was plotted versus the amount of insoluble aggregates formed upon encapsulation, the data could be fitted by linear regression ($R = 0.93$) and the P value was generated using the analysis of variance (Figure 1A). For the formulation using PEG 20 000 the amount of insoluble aggregates was decreased to 3%. The molecular weight of PEG also has a significant effect on the enzyme activity upon encapsulation, with the formulation using PEG 20 000 exhibiting the highest retention of the specific activity of encapsulated α-chymotrypsin (Figure 1B). One advantage of PEG is that it not only possesses amphiphilic properties, but it can be dissolved in both the organic and water phase. In addition, it has been suggested that the hydrophobic nature of PEG is size dependent, with the larger one assuming a greater degree of hydrophobicity (Forcittini et al 1991). To test this, a methylene chloride/water emulsion was formed, the aqueous phase containing PEG 6000 or PEG 20 000. The emulsions were allowed to separate well in two phases for various times and centrifuged. It was observed that PEG 20 000 was better distributed in the organic polymeric phase during the preparation of microspheres (15% more than PEG 6000). Thus, it would be expected that PEG 20 000 produces a higher-viscosity organic phase, which may allow lower movement of solid protein particles towards the o/w interface on the surface of the droplets upon emulsification and, as a consequence, less protein denaturation.

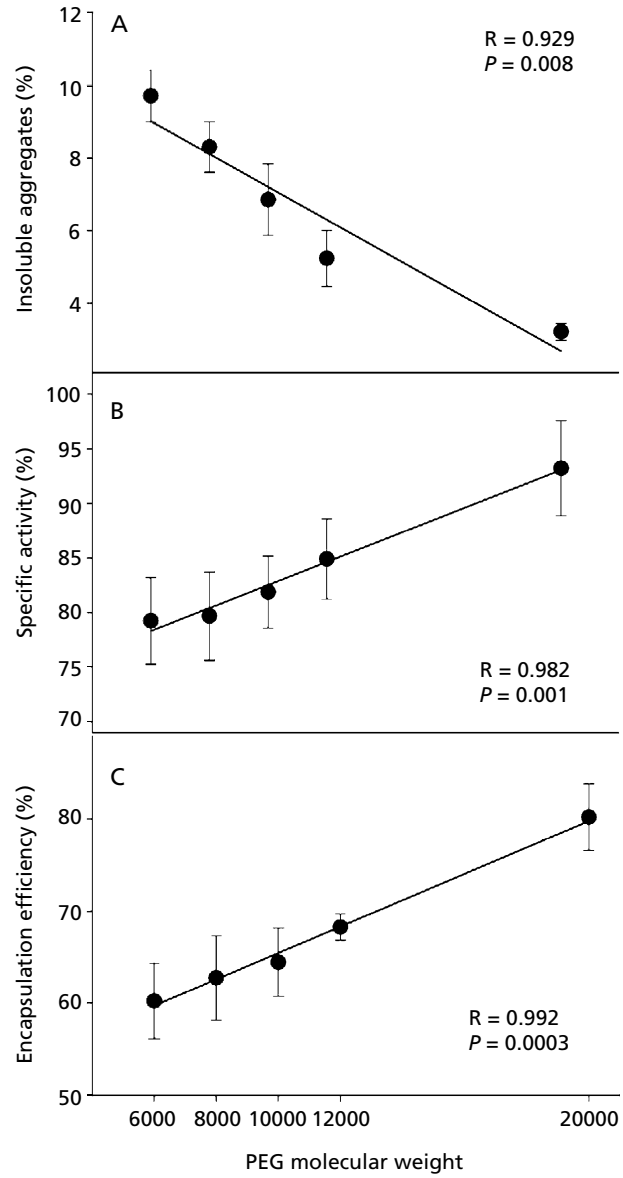

Figure 1 Effect of the molecular weight of PEG as emulsifier on the formation of insoluble aggregates (A), the specific activity (B) and the encapsulation efficiency (C) of α-chymotrypsin. Data are means $\pm$ s.d., $n = 3$.

Secondary structure of α-chymotrypsin in PLGA microspheres

The secondary structure of α-chymotrypsin was investigated by FTIR spectroscopy to assess both the magnitude of procedure-induced structural perturbations and aggregation employing a non-invasive methodology. Two analysis methods were employed, Gaussian curve-fitting of the Fourier self-deconvoluted amide I spectra and a strictly mathematical method. The latter involved the calculation of the spectral correlation coefficient (Prestrelski et al 1993) for the amide I second derivative spectrum of native α-chymotrypsin in aqueous solution at pH 7.8 and the spectrum of the protein in different formulations. Utilizing the first method, the secondary structure content of α-chymotrypsin in aqueous solution was determined to be $43 \pm 2\%$ β-sheet and $12 \pm 2\%$ α-helix structure, in agreement with previous data (Carrasquillo et al 2000).

Lyophilization caused an increase in the β-sheet content to 51 ± 1% and an apparent increase in the helix content to 18 ± 1%, relative to α-chymotrypsin in aqueous solution at pH 7.8 (Table 1, Figure 2B) (Carrasquillo et al 2000). Overall structural perturbations due to the lyophilization process caused a drop in the value of the spectral correlation coefficient to 0.86 (Table 1). The secondary structure content of α-chymotrypsin after encapsulation in PLGA microspheres was then investigated for some formulations. The spectra for the formulations using PEG 6000 and 12 000 showed the occurrence of a new IR band at ca. 1625–1627 cm^{-1} (Figure 2C, D; labelled β_{agg}). A band at this frequency is typically assigned to intermolecular aggregation β-sheets (Dong et al 1995; Castellanos et al 2002, 2003). It is apparent from the spectra shown in Figures 2C and 2D that encapsulation using PEG as an emulsifying agent with molecular weight of 6000 and 12 000 did not completely prevent formation of intermolecular β-sheets, in agreement with the data of insoluble aggregates isolated from microspheres (Figure 1A). The spectrum for the formulation using PEG 20 000 did not show the band at 1625 cm^{-1} (Figure 2E), indicating that the formation of insoluble aggregates was largely prevented. The α-helix content and SCC values obtained for α-chymotrypsin after encapsulation using the formulations containing PEG 12 000 and 20 000 were statistically not significantly different from those obtained for the lyophilized protein before encapsulation (Table 1). However, the β-sheet content for the formulation using PEG 20 000 was the only one not statistically different when compared with the lyophilized protein before encapsulation. Thus, PEG of high molecular weight used as an emulsifying agent provides a protein with a more preserved secondary structure upon encapsulation.

Microsphere characterization

Table 1 Secondary structure content of α-chymotrypsin under various conditions

Sample	α-helix (%)	β-sheet (%)		SCC[b]
		intra	inter[a]	
Aqueous solution at pH 7.8	12 ± 2	43 ± 2	0	0.99 ± 0.01
Lyophilized from pH 7.8	18 ± 1	51 ± 1	0	0.86 ± 0.01
In microspheres				
PEG 6000	16 ± 1	46 ± 1	5 ± 1	0.78 ± 0.02
PEG 12 000	17 ± 1	47 ± 2	4 ± 1	0.81 ± 0.01
PEG 20 000	22 ± 1	51 ± 2	0	0.88 ± 0.01

[a]The area of the band at 1625–1627 cm^{-1} was assigned to intermolecular β-sheet. [b]SCC was determined by comparison of the spectrum of α-chymotrypsin in aqueous solution with that of α-chymotrypsin under different conditions.

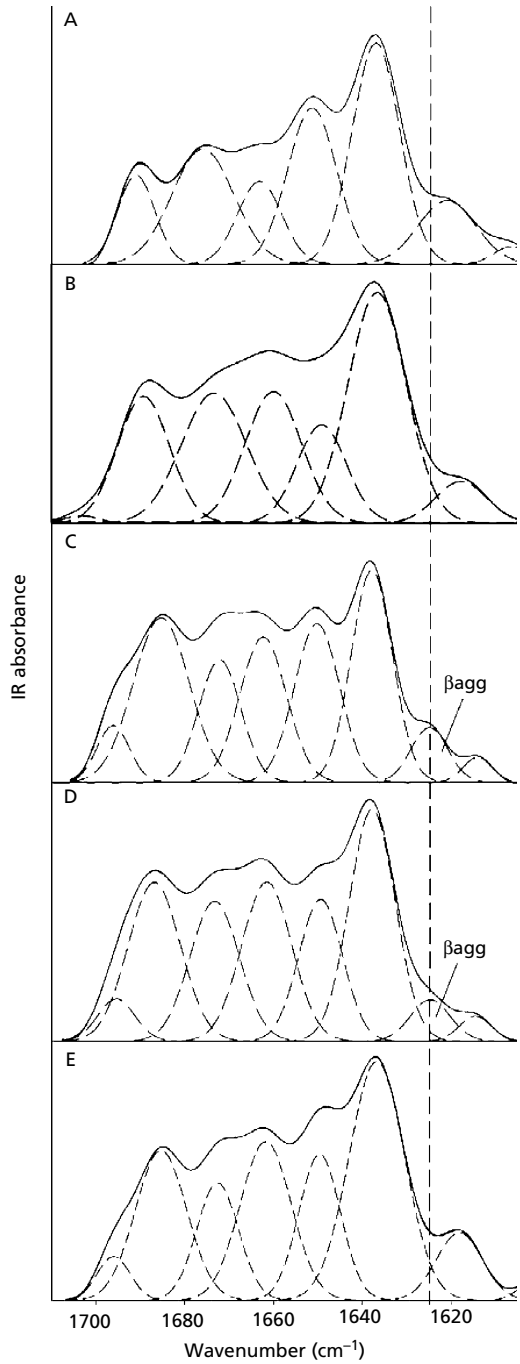

Figure 2 Resolution-enhanced amide I IR spectra of α-chymotrypsin in aqueous solution at pH 7.8 (A), lyophilized powder (B) and after encapsulation in PLGA microspheres using PEG 6000 (C), PEG 12 000 (D) and PEG 20 000 (E) as emulsifier.

The surface chemistry of α-chymotrypsin-loaded PLGA microspheres was analysed by X-ray photoelectron spectroscopy (XPS). Protein is the only substance that contains nitrogen in the microspheres. The results of the XPS show the existence of N1s (atomic orbital 1s of nitrogen) core level signal, indicating that nitrogen could be detected on the surface of the microspheres. There was

no significant difference in the content of nitrogen for the microspheres prepared using PEG with different molecular weights (i.e., 6000 and 20 000) as emulsifier (Table 2). Figure 3 shows the XPS spectra of PLGA (A), PEG (B) and PLGA microspheres prepared using PEG 6000 (C) and PEG 20 000 as emulsifying agent (D). The decomposition of the O1s envelopes reveals the presence of two types of oxygen (O1: O=C at 531 eV and O2: O-C at 532.9 eV) from the PLGA chains. The XPS spectrum of PEG shows the O1s peak at 532.2 eV due to the presence of C-O in PEG. The binding energy spectrum of the surface of PLGA microspheres prepared with PEG (Figure 3C, D) is clearly distinguished from the pure PLGA spectrum (Figure 3A). The O1s spectra of PLGA microspheres shows a large C-O peak and small C=O peak. The area percentage contribution from the deconvolution analysis of O1s peak envelope varied for the PLGA microspheres emulsified with PEG 6000 and 20 000 (Table 2). The formulation using PEG 20 000 shows a similar O1s envelope contribution to those of pure PEG, thus confirming the major presence of PEG chains on the surface of microspheres, which supports our initial hypothesis that PEG should accumulate at the organic solvent/water interface and thus be at the microsphere surface.

Figure 4 shows scanning electron micrographs of α-chymotrypsin-loaded PLGA microspheres for some formulations. The porosity of the microspheres decreased with an increase in the molecular weight of PEG. The presence of pores on the microsphere surface could be attributed to both the leaching of PEG towards the aqueous phase (Cleek et al 1997) or dissolution of solid protein particles that came in contact with the microspheres' outer surface during microencapsulation. To test the latter, α-chymotrypsin was suspended in methylene chloride by homogenization to get an idea of the size of incorporated protein particles in microspheres. SEM pictures showed that the protein particles had a very heterogeneous size and shape, which made it difficult to measure the size using our methodology. However, we were able to identify a population of protein particles having a size (0.7–3 μm) corresponding to the size of the pores observed in the microspheres, which were in the range of 1–4 μm. Therefore, it is possible that the dissolution of these solid protein particles is responsible for the presence of pores on the microsphere surface. Microspheres prepared with PEG 20 000 were perfectly spherical and had a smaller size ($21 \pm 10\,\mu$m) than those prepared with PEG 6000 ($27 \pm 13\,\mu$m), as statistically demonstrated by the t-test for a 95% level of confidence (Figure 4C). The fact that PEG 20 000 largely covered the surface of the microspheres (XPS) indicates that it worked as a better

Table 2 XPS analysis and the area percent contribution for the deconvolution of O1s peak envelope of PLGA microspheres prepared using PEG with different molecular weight as emulsifier

Samples	Elemental composition (%)			O1s area (%)	
	C	O	N	C-O	C=O
PLGA	62.89	37.11	0.00	57.75	42.25
PEG	71.00	29.00	0.00	100.00	—
Microspheres with PEG 6000	69.28	25.85	4.87	85.07	14.93
Microspheres with PEG 20 000	68.48	26.89	4.62	97.53	2.47

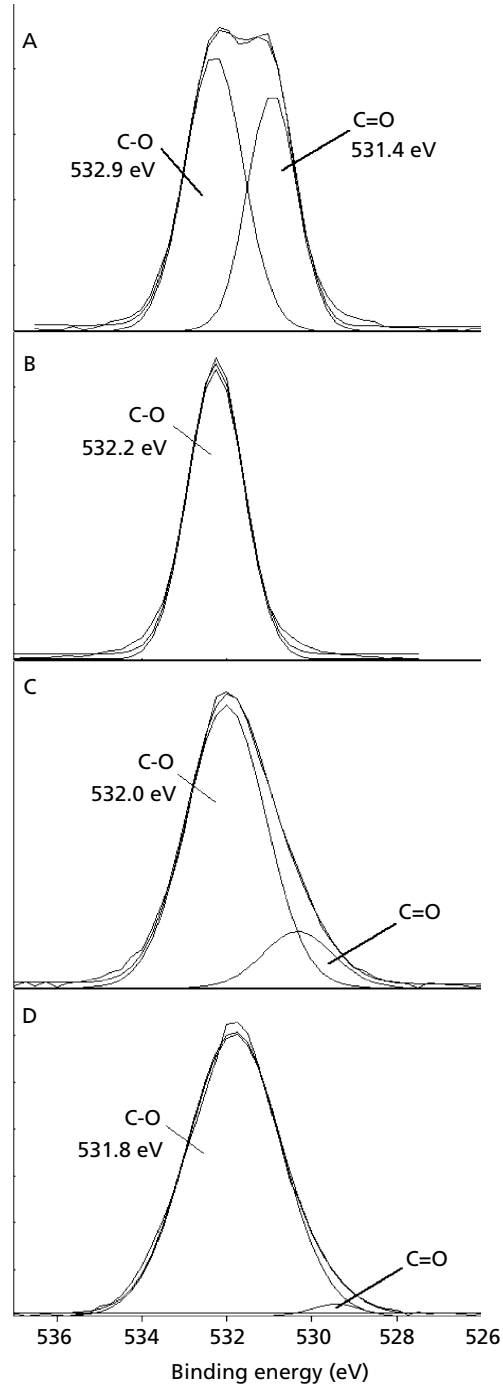

Figure 3 Surface chemistry analysis of O1s with X-ray photoelectron spectroscopy of PLGA (A), PEG (B) and PLGA microspheres prepared using PEG 6000 (C) and PEG 20 000 as emulsifying agent (D).

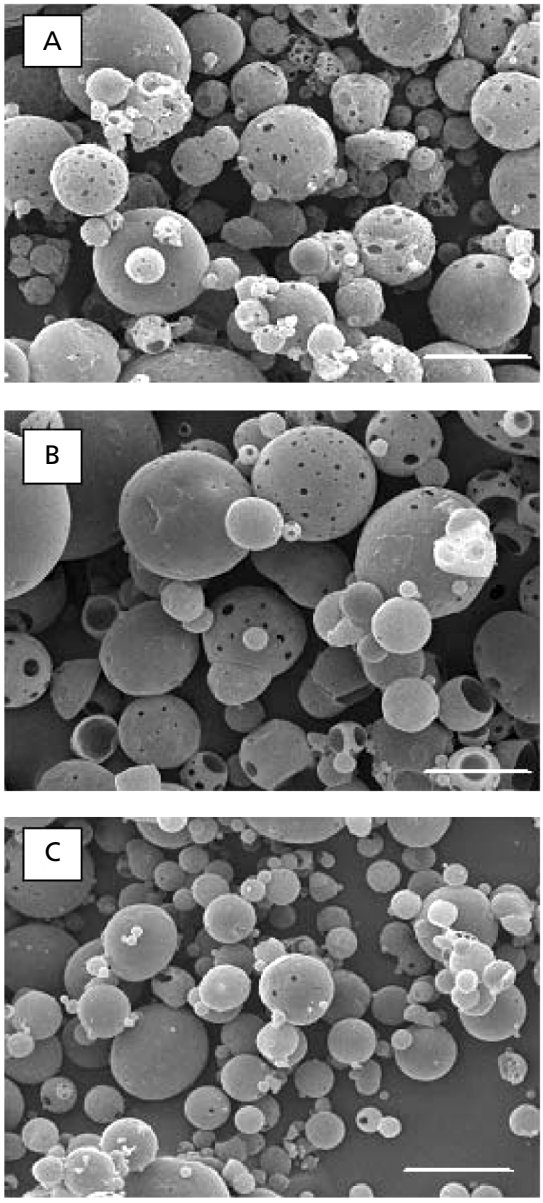

Figure 4 SEM pictures of PLGA microspheres using PEG 6000 (A), PEG 12 000 (B) and PEG 20 000 (C) as emulsifier. The width of the scale bars and the images corresponds to 50 and 264 μm, respectively.

emulsifier at the interface between the organic and aqueous phases, thus stabilizing the emulsion during the formation of microspheres. This phenomenon might also account for the high encapsulation efficiency of the microspheres observed using this formulation (Figure 1C). An interesting observation is that even the formulation using PEG 6000 showed a higher encapsulation efficiency than the microspheres prepared using PVA as emulsifier (54%). An increase of the molecular weight of PEG in the PLGA microsphere formulation significantly improved the encapsulation efficiency. The high encapsulation efficiency (80%) for the formulation using PEG 20 000 was very surprising, since in this study the solid protein used was not micronized before encapsulation. It is normally difficult to achieve high encapsulation efficiency for α-chymotrypsin without micronization of protein by co-lyophilization with PEG (Castellanos et al 2002). However, herein the encapsulation efficiency was very similar to that obtained for formulations using micronized protein by co-lyophilization with PEG (Castellanos et al 2003). Therefore, the high encapsulation efficiency achieved by micronization of proteins through co-lyophilization with PEG may not only be attributable to the small size of the protein particles but also to other factors (e.g. adsorption of PEG to the interface, increase in the viscosity of the organic phase, etc.).

In-vitro release studies

The incorporation of PEG in the microspheres as blends with PLA and PLGA (Cleek et al 1997; Jiang & Schwendeman 2001) and as additive (Morita et al 2000) usually results in continuous release profiles. This has been attributed to the increased hydrophilicity of the matrix by the PEG, which results in a more rapid entry of water into the microspheres, eventually accelerating the release of proteins. The release of α-chymotrypsin from microspheres was biphasic, showing an initial burst effect followed by a continuous release for all the formulations prepared (Figure 5A). The initial amount of the protein released increased with the molecular weight of PEG and it was statistically different for the formulations using PEG 6000 and PEG 20 000. This initial release is related to both the presence of protein molecules located at or near to the microsphere surface and the presence of pores and channels formed during the preparation of the microspheres (Figure 4A) (Cleek et al 1997). Since XPS demonstrated that the same amount of protein was located on the surface of the microspheres for both formulations the lower initial release could be due to the fewer amount of pores on the surface as shown in Figure 4C. One of the most important observations is that the release profile for all formulations was linear after the initial release. For the formulation using PEG of low molecular weight most of the protein has already been released in the first phase (65%) and the small portion remaining was released continuously over approximately 30 days (Figure 5). Microspheres prepared with PEG 20 000 had a more sustained release than the microspheres with PEG of low molecular weight because a smaller percent of the loaded protein released in the initial phase (48%) and there was higher loading. Statistical analysis revealed that for some periods of time (first 33 days and 41–47 days) the release profiles were different as demonstrated by the Kruskal–Wallis test, although in the last stage of the release profile (after 49 days) the formulations were not statistically different. Nemenyi's test revealed that the cumulative release for the formulations using PEG 6000 and 20 000 were the only statistically different for the first 33 days. However, from days 41 to 47 there was a significant difference between the formulations using PEG 6000 and 12 000. The amount of non-released protein correlated with the amount of insoluble protein determined upon encapsulation for all

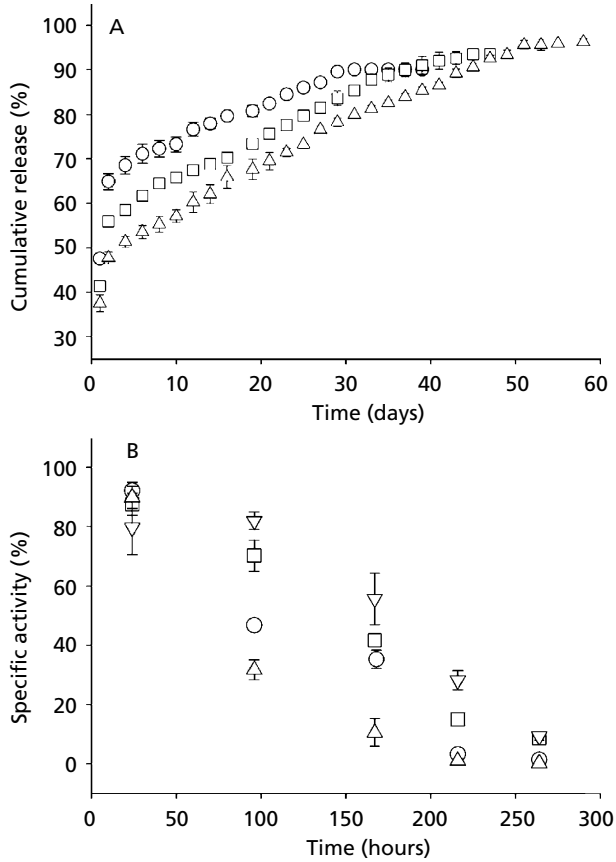

Figure 5 A. Cumulative in-vitro release of α-chymotrypsin from PLGA microspheres prepared using PEG 6000 (O), PEG 12 000 (□) and PEG 20 000 (△) as emulsifier. B. Specific activity of α-chymotrypsin after various times of in-vitro release from PLGA microspheres prepared using PEG 6000 (O), PEG 12 000 (□) and PEG 20 000 (▽) as emulsifier; △, control.

formulations, suggesting that in-vitro release did not cause further insoluble aggregation as it has been previously demonstrated (Castellanos et al 2002). In general, the high initial release observed for all microsphere formulations could be attributed to the large size of the encapsulated protein particles as previously reported (Castellanos et al 2003). Thus, the micronization of protein before encapsulation could minimize the initial release observed for the formulations.

Activity of α-chymotrypsin during in-vitro release

Figure 5B shows the specific activity of α-chymotrypsin released from PLGA microspheres for the different formulations upon in-vitro release in comparison with the commercial protein dissolved in buffer under the release conditions as the control. The activity of protein released for the different formulations within 24 h was not statistically different to that of the control. Statistical analysis revealed that throughout release there was no significant difference in the specific activity for α-chymotrypsin of the control and of the formulation using PEG 6000. After four days of incubation, the enzyme activity decreased for all formulations, although the drop was statistically significantly lower for formulations using PEG 12 000 and 20 000. It is known that loss of activity is observed during prolonged incubation of the protein at 37°C (Castellanos et al 2002). The stabilizing effect for α-chymotrypsin by PEG 12 000 and 20 000 upon in-vitro release is not clear but it has been attributed to different factors, such as prevention of the adsorption of protein to the polymer matrix upon in-vitro release (Péan et al 1999) and the increased water content in the formulation (Jiang & Schwendeman 2001). In addition, a stabilizing effect as a result of binding between PEG and α-chymotrypsin could not be discarded, as it has been reported for other proteins (Lee & Lee 1987; Lotwin & De Bernardez 1999; Nerli et al 2001). We determined whether the presence of PEG of different molecular weights caused conformational changes of α-chymotrypsin. Thermal denaturation of α-chymotrypsin was monitored in the absence and presence of PEG 6000 and 20 000 at a constant concentration of PEG under conditions relevant to the release. Figure 6 shows thermal unfolding curves for α-chymotrypsin in the presence and in the absence of PEG. In the absence and presence of PEG 6000 and 20 000 the T_m for α-chymotrypsin was 50.6 ± 0.5, 49.7 ± 0.7 and 50.1 ± 0.3°C, respectively. Statistical analysis revealed that there was not a significant statistical difference between the maximum emission wavelength at each temperature for the different formulations. Therefore, the results indicate that the presence of PEG does not affect the T_m and consequently no significant conformational changes of α-chymotrypsin occurred in the presence of PEG. Future experiments will be directed toward gaining more information on protein stability during release from microsphere formulations using PEG as emulsifier.

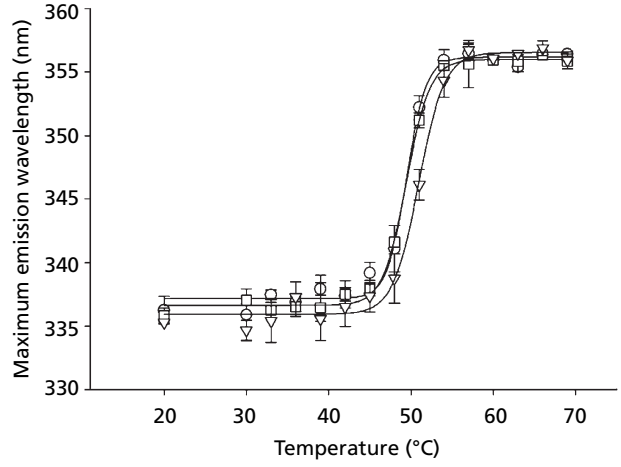

Figure 6 Thermal denaturation curves for α-chymotrypsin monitored by fluorescence spectroscopy in the absence (▽) and in the presence of PEG 6000 (□) or PEG 20 000 (O). Data are means ± s.d., n = 3.

Conclusions

The results clearly indicate that PEG with high molecular weight (long chain) used as an emulsifier provides a better protection of the protein against stress encountered during encapsulation than PEG with low molecular weight. PEG 20 000 prevented aggregation and inactivation of α-chymotrypsin during encapsulation. PEG used as an emulsifier had a significant influence on the microsphere characteristics, which largely determined the release kinetics of α-chymotrypsin from PLGA microspheres. Together the results demonstrate that PEG with high molecular weight can act as an efficient emulsifier in the preparation of PLGA microspheres for controlled protein and peptide delivery purposes.

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