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Preservation of lysozyme structure and function upon encapsulation and release from poly(lactic-*co*-glycolic) acid microspheres prepared by the water-in-oil-in-water method

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Abstract

When proteins are encapsulated in bioerodible polymers by water-in-oil-in-water (w/o/w) encapsulation techniques, inactivation and aggregation are serious drawbacks hampering their sustained delivery. Hen egg-white lysozyme was employed to investigate whether stabilizing it towards the major stress factors in the w/o/w encapsulation procedure would allow for the encapsulation and release of structurally unperturbed, non-aggregated, and active protein. When it was encapsulated in poly(lactic-*co*-glycolic) acid (PLGA) microspheres without stabilizing additives, lysozyme showed substantial loss in activity and aggregation. It has been shown that by co-dissolving various sugars and polyhydric alcohols with lysozyme in the first aqueous buffer, interface-induced lysozyme aggregation and inactivation can be minimized in the first emulsification step [J. Pharm. Pharmacol. 53 (2001) 1217]. Herein, it was found that those excipients, which were efficient in preventing interface-induced structural perturbations, were also efficient in minimizing lyophilization-induced structural perturbations (e.g. lactulose). The efficient excipients identified also reduced structural perturbations upon lysozyme encapsulation in PLGA microspheres and this led to reduced lysozyme inactivation and aggregation. However, the data obtained also show that later steps in the encapsulation procedure are detrimental to lysozyme activity. Lysozyme inactivation was completely prevented only by employing the efficient excipients in the second aqueous phase also. In summary, protein aggregation and inactivation were minimized by rationally selecting excipients efficient in stabilizing lysozyme against the major stress factors of w/o/w encapsulation.

Keywords: Protein aggregation; Protein delivery; Protein stability; Sustained release; Water–oil interface

1. Introduction

Delivery of protein and peptide drugs from bioerodible polymers, such as poly(lactic-*co*-glycolic) acid (PLGA), is a promising approach to prevent and cure diseases (Langer, 1990, 2000). However, protein encapsulation into such poly-

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mers presents a major problem because of the physical and chemical instabilities inherent in protein molecules (Manning et al., 1989; Wang, 1999; Van de Weert et al., 2000a). Most methods employed in creating protein-loaded polymer microspheres involve the exposure of protein to aqueous–organic interfaces, organic solvents, mechanical stress, and dehydration. This frequently causes protein aggregation and inactivation (Sah, 1999a,b,c; Van de Weert et al., 2000b; Wang, 2000; Pérez and Griebenow, 2001; Meinel et al., 2001; Pérez et al., 2002). This is pharmaceutically unacceptable because it poses danger to patients due to increased immunogenicity of aggregated protein.

The most commonly employed method to achieve protein encapsulation into the matrix of bioerodible polymers is the so-called water-in-oil-in-water (w/o/w) technique. Amongst a variety of other processing steps, the formation of the first water-in-oil (w/o) emulsion exposing protein to a water–organic solvent (typically methylene chloride) interface is particularly considered a cause for protein inactivation and aggregation (Crotts and Park, 1998; Kim and Park, 1999; Morlock et al., 1997; Sah, 1999a,b,c; Van de Weert et al., 2000a,b; Pérez and Griebenow, 2001; Pérez et al., 2002). For lysozyme, the model protein chosen in this study, the aggregates formed were non-covalent in nature (Van de Weert et al., 2000b; Pérez and Griebenow, 2001). In addition, activity loss occurred for non-aggregated lysozyme (Pérez and Griebenow, 2001). The principal cause for the deleterious events is protein adsorption to the CH_2Cl_2 interface. It is believed that proteins first move into the interfacial area. Once in this area, interaction with the oil phase is energetically favorable with sometimes no apparent activation energy reported (Middelberg et al., 2000). Protein adsorption to the oil interface likely occurs via exposed hydrophobic areas on the protein surface (Middelberg et al., 2000). This adsorption causes substantial protein structural perturbations (Sah, 1999b), presumably due to the reduction in thermodynamic protein stability due to a reduction in the strength of the hydrophobic effect stabilizing the protein core. This leads to what is commonly referred to as a protein turning inside-out, e.g. as observed when proteins are exposed to aqueous–organic mixtures (Griebenow and Klibanov, 1996). In addition, the mechanical forces employed in the creation of the w/o emulsion might also cause irreversible aggregation (Meinel et al., 2001). Lastly, microspheres are dehydrated to prolong storage stability, a process causing protein structural perturbations (Prestrelski et al., 1993; Griebenow and Klibanov, 1995), which often cause irreversible aggregation upon exposure of proteins to moisture (Costantino et al., 1994, 1995; Wang, 2000). The stress factors outlined above have been reported to cause perturbations in the secondary structure (Fu et al., 1999) and loss in activity (Ghaderi and Carlfors, 1997) of lysozyme after encapsulation in PLGA microspheres by w/o/w methodologies. For many other proteins aggregation and loss in biological activity have also been reported upon encapsulation in PLGA microspheres by the w/o/w method or upon release from the devices (Alonso et al., 1994; Nihant et al., 1994; Lu and Park, 1995; Morlock et al., 1997; Kim and Park, 1999; Pérez et al., 2002). Only a few pharmaceutical proteins have been delivered in an unaltered state using the w/o/w encapsulation method (for recent reviews see Van de Weert et al., 2000a; Pérez et al., 2002). Because of this situation, alternative encapsulation procedures avoiding formation of an aqueous–organic solvent interface, such as solid-in-oil-in-oil encapsulation (Thomasin et al., 1998; Carrasquillo et al., 2001a,b), have gained much attention (Pérez et al., 2002). However, these procedures also have drawbacks. For example, it is technically complex to assure the homogeneous distribution of protein powder particles in the microspheres, and in many protocols protein powder particles have to be micronized prior to encapsulation (Maa and Hsu, 1997; Costantino et al., 2000). Because many of these problems are avoided in w/o/w encapsulation procedures, development of rational strategies to stabilize proteins upon w/o/w encapsulation is still urgently required.

There is excellent evidence that the first emulsification step in the w/o/w technique is a principal cause for protein denaturation and aggregation (see e.g. Sah, 1997, 1999a,b,c; Van de Weert et al., 2000b; Pérez and Griebenow, 2001). However, no

work has been reported showing that sustained release of protein from microspheres created by the w/o/w method can be substantially improved by structurally stabilizing the protein in the o/w emulsion step (see Van de Weert et al., 2000a and references therein). This is because data are still largely missing on protein instability upon release as the result of protein structural perturbations during encapsulation (Pérez et al., 2002). In addition, protein structural and activity data thus far have not been collected in a systematic manner at various stages of the encapsulation process. Even though many studies have employed lysozyme as model protein in w/o/w encapsulation (see e.g. Ghaderi and Carlfors, 1997; Fu et al., 1999; Van de Weert et al., 2000c), thus far no study has been reported to completely prevent lysozyme inactivation and aggregation in this process. Only the chemical modification of lysozyme with PEG afforded excellent stability upon encapsulation in PLGA microspheres (Diwan and Park, 2001).

In a recent publication, we investigated strategies ameliorating the unfolding and aggregation of the model protein lysozyme at the o/w interface by employing various polyhydric alcohols and sugars as excipients (Pérez and Griebenow, 2001). In this work, we investigated whether the strategies developed could be used to rationally design a process assuring lysozyme stabilization upon encapsulation and release from PLGA microspheres prepared by the w/o/w methodology. The effects of excipients on the structural stabilization, activity retention, and prevention of aggregation of lysozyme during microencapsulation and subsequent release were assessed. Based on these results, efficient excipients were selected and were utilized to assure encapsulation and release of largely intact lysozyme from PLGA microspheres.

2. Materials and methods

2.1. Materials

Lysozyme from hen egg-white (crystallized and lyophilized, EC 3.2.1.17), fructose, lactose, lactulose, maltose, sorbitol, sucrose, trehalose, anhydrous CH_2Cl_2 (purity 99.8%), and poly (vinyl alcohol) (PVA, 87–89% hydrolyzed) were from Sigma–Aldrich Chemical Co. (St. Louis, MO). Poly(D,L-lactic-*co*-glycolic) acid (50:50, M_W 10 kDa) was kindly supplied by Alkermes, Inc., Cambridge, MA. All other chemicals were of analytical grade and from various commercial suppliers.

2.2. Microsphere preparation

Protein loaded microspheres were prepared by a w/o/w technique as described by Sah (1997) because the procedure has been reported to result in high encapsulation efficiencies, essential for allowing the investigation of the structure of encapsulated protein by FTIR spectroscopy. Briefly, a sample of 15 mg of lysozyme was dissolved in 0.3 ml of phosphate buffer (pH 5.10) and 0.9 g of PLGA was dissolved in 7 ml of methylene chloride. The two solutions were emulsified with a VirTis Tempest homogenizer (Gardiner, NY) using a 10-mm shaft equipped with a micro-fine rotor/stator type generator at approximately 12 000 rpm for 1.5 min to form the first w/o emulsion. Excipients were co-dissolved with lysozyme at a 1:37 molar ratio of lysozyme-to-additive, when desired. The primary emulsion was then poured into a solution of 4% polyvinyl alcohol (PVA) in distilled water (300 ml) under stirring at 470 rpm to obtain the second emulsion. After 30 min, an additional 700 ml of 10 mM phosphate buffer was added slowly over a period of 30 min and the emulsion stirred for 1 h at room temperature to afford microsphere hardening. In some cases excipients were added to the second aqueous phase at a concentration of 10 mg ml^{-1}. Microspheres were then collected by filtration using a 0.22 μm Nylon filter, washed with nanopure water (18 MΩ resistance), and dried for 48 h at a vacuum of <60 μm of Hg at room temperature.

2.3. Lyophilization

Lysozyme was dissolved in 10 mM potassium phosphate buffer at pH 5.10 to obtain a concentration of 10 mg ml^{-1}. When needed, excipients were added to this solution to achieve a 1:37 molar

ratio of lysozyme-to-excipient. These samples 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 μm of Hg for at least 48 h.

2.4. Lysozyme activity assay

Lysozyme activity was determined using freeze-dried cells of *Micrococcus lysodeikticus* as the substrate as described (Pérez and Griebenow, 2001).

2.5. Protein loading of microspheres

Fifteen milligrams of dry microspheres were dissolved in 2 ml of methylene chloride and agitated for 30 min followed by centrifugation at 5000 rpm for 20 min. The supernatant was discarded and the protein was dissolved in 1 ml of 10 mM phosphate buffer at pH 5.1. Insoluble lysozyme aggregates and lysozyme adsorbed to PLGA were taken into account in the calculation of the actual protein loading of the microspheres (see Section 2.6 for details). The protein concentration was determined by measuring the absorbance at 280 nm using an UV–Vis spectrometer (Shimadzu 1601 PC) and by the method of Lowry et al. (1951). The encapsulation efficiency was determined as described (Castellanos et al., 2001a).

2.6. Isolation of insoluble lysozyme aggregates

During determination of the protein loading, a white precipitate was observed at the bottom of the centrifugation tube. This precipitate was dried under vacuum (< 60 μm of Hg) for 24 h and used directly in FTIR experiments as a KBr pellet preparation. Aggregated lysozyme was completely dissolved in buffer containing 6 M urea and the concentration determined from the absorbance at 280 nm. Remaining insoluble components showed no detectable protein when measured by FTIR spectroscopy after the urea treatment and solely consisted of some residual PLGA (for details see Section 3). A control experiment has been performed using lyophilized lysozyme and it was found that the procedure induced < 1% lysozyme aggregation, less than the standard deviations (S.D.) given for the amounts of aggregated lysozyme determined in this work.

2.7. Protein release studies

Microspheres (20 mg) were placed in 2 ml of a 10 mM phosphate buffer at pH 7.3 at 37 °C. Typically every 24 h the samples were subjected to centrifugation (500 rpm for 1 min in a Beckman J-21B centrifuge). The supernatant was removed and the concentration of released protein determined. The buffer was completely replaced to maintain sink conditions. The concentration of the released protein was plotted directly versus time and also used to construct cumulative release profiles. Release experiments were performed at least in triplicate, the results averaged, and the S.D. calculated.

2.8. Size-exclusion (SEC) HPLC

SEC-HPLC was conducted using a G2000SW-XL1 TSK Gel Column (TosoHaas, PA) and followed by measuring the UV absorbance at 280 nm. Typically, 50 μl of a filtered lysozyme sample was loaded onto the column, which was developed at a flow rate of 0.5 ml min^{-1}. The mobile phase consisted of 0.3 M sodium chloride, and 50 mM sodium phosphate at pH 7.0.

2.9. Surface electron microscopy

Scanning electron microscopy of microspheres was performed using a JEOL 5800 LV scanning electron microscope. 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 at least 25 microspheres were determined, averaged, and the S.D. calculated.

2.10. FTIR measurements of lysozyme after encapsulation

FTIR studies were conducted using a Nicolet Magna-IR 560 optical bench. A total of 256 scans at 2 cm^{-1} resolution using Happ–Ganzel apodization were averaged to obtain each spectrum. The spectra of lysozyme in aqueous solution (50 mg ml^{-1}), in amorphous dehydrated powders, and encapsulated in PLGA microspheres were acquired as described (Carrasquillo et al., 1998, 1999, 2001a,b). Protein powders and proteins encapsulated in microspheres were measured as KBr pellets (1 mg protein per 200 mg KBr) (Prestrelski et al., 1993; Griebenow and Klibanov, 1995). Each sample was measured at least four times. All spectra were corrected for the background in an interactive manner using the NICOLET OMNIC 3.1 software to obtain the protein vibrational spectra (Carrasquillo et al., 1999). Subtraction of the PLGA spectrum from spectra of protein encapsulated in PLGA was performed as described (Carrasquillo et al., 1998, 1999, 2001a,b; Fu et al., 1999; Yang et al., 1999; Van de Weert et al., 2000c; Castellanos et al., 2001a,b). Although the polymer band with a maximum at approximately 1760 cm^{-1} is well separated from the amide I band (1700–1600 cm^{-1}), we were careful in excluding spectral artifacts caused by the polymer subtraction procedure. The PLGA background was subtracted from various lysozyme samples encapsulated in PLGA employing different subtraction factors (e.g. 0.5025, 0.4631, 0.4208 and 0.3692 for lysozyme encapsulated in PLGA and 0.3191, 0.3870, 0.4141 and 0.5473 for lysozyme with sucrose as excipient and encapsulated in PLGA). For each of the spectra obtained, we calculated the secondary structure by performing Gaussian curve-fitting analysis. For the case of the lyophilized lysozyme encapsulated in PLGA, the α-helix contents determined were 27, 25, 26 and 26% for the four situations tested. For the case of the lysozyme/sucrose formulation encapsulated in PLGA, the α-helix contents determined were 25, 26, 26 and 24%, respectively. The values were the same within the error of the method, excluding a significant influence of the PLGA background subtraction procedure on the amide I protein spectrum in our case.

The corrected protein vibrational spectra were analyzed in the amide I region (1700–1600 cm^{-1}) by calculation of the second derivative spectra for their component composition (Griebenow and Klibanov, 1995). Second derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}). Fourier self-deconvolution (FSD) was applied to the corrected spectra to enable the quantification of the secondary structure in the amide I region by Gaussian curve fitting (Pérez and Griebenow, 2000; Griebenow and Klibanov, 1996) using the program OMNIC 3.1. The parameters chosen for FSD were 27 for the full-width at half-maximum (FWHM) and $K = 2.4$ for the enhancement factor (Pérez and Griebenow, 2001). The frequencies and numbers of the bands identified by in the second derivative spectra were used as starting parameters for the Gaussian curve-fitting (performed using the program GRAMS 386 from Galactic Industries, Inc.). The secondary structure contents were calculated from the areas of the individual assigned bands and their fraction to the total area in the amide I region for at least four spectra, the values were averaged, and the S.D. were calculated. The assignment of the individual secondary structural elements was carried out for the amide I region according to Fu et al. (1999). Spectral correlation coefficients (SCC) were calculated between the amide I second derivative spectrum of lysozyme in aqueous solution and that of lysozyme at various stages of processing as described by Prestrelski et al. (1993). SCC values are a measure for overall perturbations in the protein structure. Identical spectra (and thus structures) result in a value of 1. SCC values < 1 show the magnitude of overall perturbations in the protein's secondary structure.

3. Results and discussion

3.1. Preservation of lysozyme structure upon lyophilization and emulsification by excipients

As introduced, two steps in the encapsulation of proteins in PLGA microspheres by the w/o/w

method are reported to cause protein structural perturbations: the initial emulsification step (Morlock et al., 1997; Crotts and Park, 1998; Kim and Park, 1999; Sah, 1999a,b,c; Van de Weert et al., 2000b; Pérez and Griebenow, 2001) and the final dehydration step (Prestrelski et al., 1993; Griebenow and Klibanov, 1995; Dong et al., 1995; Griebenow et al., 1999a,b). In a previous work, Pérez and Griebenow (2001) tested various polyhydric alcohols and sugars for their ability to prevent lysozyme inactivation and aggregation at the water–CH_2Cl_2 interface. Such excipients have the advantage that, in addition to being potentially efficient in preventing interface-induced protein denaturation (Cleland and Jones, 1996), they also have potential in preventing dehydration-induced protein structural changes (Prestrelski et al., 1993; Griebenow and Klibanov, 1995; Griebenow et al., 1999a). We recently hypothesized that those additives efficient in preventing lysozyme inactivation and aggregation at the w/o interface in the first emulsification step might also be efficient lyoprotectants (Pérez et al., 2002). To test this, eight excipients (polyhydric alcohols and sugars) employed by us to study their effect on lysozyme stability in the first w/o emulsification step (Pérez and Griebenow, 2001) were co-dissolved with lysozyme at the same molar ratio employed previously (1:37, lysozyme-to-additive) and lyophilized. Lysozyme secondary structure in the co-lyophilizates was determined from the FTIR-spectra collected (Table 1). As repeatedly found in the past (Prestrelski et al., 1993; Carrasquillo et al., 1998; Costantino et al., 1998), some excipients were efficient in minimizing lyophilization-induced structural changes, while others were not. For example, co-lyophilization of lysozyme with sucrose caused an even more substantial drop in the α-helix content to 20% than in the absence of the additive (25%) and also the SCC showed a more pronounced drop. On the contrary, co-lyophilization with lactulose substantially reduced the magnitude of lyophilization-induced structural perturbations. The initial hypothesis was tested by plotting the α-helix contents (Fig. 1A) and SCC values (Fig. 1B) determined for the lyophilized lysozyme samples (Table 1) versus the amount of soluble lysozyme after w/o emulsification (taken from Table 2 of the paper by Pérez and Griebenow, 2001). The statistical analysis by performing a linear regression resulted in correlation coefficients of 0.95 (Fig. 1A) and 0.93 (Fig. 1B). An *F*-test was performed to test whether the association between the variables is statistically significant. The *P*-values calculated were 0.0008 and 0.0026 and thus there is a statistically significant association between the variables for a confidence level of 95%. This suggests that the mechanism of prevention of lyophilization-induced structural changes and the amelioration of interface-induced aggregation are indeed related for lysozyme. However, the generality of this concept still has to be established for other proteins. Important in the context of this work is that excipients have been identified that were efficient in both, prevention of interface-induced lysozyme aggregation and inactivation as well as dehydration-induced structural perturbations.

3.2. Preservation of structure and activity of lysozyme upon encapsulation in PLGA microspheres by a w/o/w method

Next, we investigated whether lysozyme stabilization at the w/o interface and upon lyophilization could be utilized to afford lysozyme stabilization upon encapsulation in PLGA microspheres by the w/o/w methodology and subsequent in vitro release. Thus, lysozyme was encapsulated in PLGA microspheres in the absence and presence of various excipients. In this context, additives efficient (lactulose and lactose) and inefficient (sucrose and trehalose) in minimizing interface-induced lysozyme inactivation and aggregation were employed (Table 1). FTIR spectroscopy was used to non-invasively probe the structure of encapsulated lysozyme (Carrasquillo et al., 1998, 1999, 2001a,b; Fu et al., 1999; Yang et al., 1999; Van de Weert et al., 2000c; Castellanos et al., 2001a,b). For lysozyme encapsulated into PLGA microspheres by the w/o/w technique in the absence of any stabilizing excipients FTIR data indicated severe structural perturbations (Fig. 2A). The low SCC-value of 0.6 calculated for the spectrum of lysozyme in aqueous solution (Fig. 2A, broken line) and that of encapsulated lyso-

Table 1
Secondary structure of lysozyme in aqueous solution and in the dried state after lyophilization with or without various excipients

Sample/excipient	Secondary structure content (%)[a]			SCC[b]
	α-Helix	β-Sheet	Unordered[c]	
Aqueous solution, pH 5.10	36±1	14±2	50±1	1
Lyophilized powder, none[d]	25±2	24±1[e]	51±6	0.72±0.02
Lysozyme co-lyophilized with[e]				
Sorbitol	27±1	18±1[e]	55±1	0.79±0.08
Trehalose	23±4	18±1[e]	58±4	0.68±0.03
Lactose	28±4	17±1[e]	55±1	0.81±0.05
Fructose	29±4	17±4[e]	54±2	0.81±0.04
Lactulose	31±3	16±4[e]	51±8	0.87±0.01
Maltose	30±2	10±1[e]	60±1	0.76±0.05
Sucrose	20±2	18±3[e]	61±3	0.64±0.04

[a] The secondary structure of hen egg white lysozyme was calculated by Gaussian curve-fitting of the Fourier self-deconvoluted amide I spectra.
[b] Spectral correlation coefficient (SCC) values were determined using the inverted second derivative spectra of lysozyme in aqueous solution at pH 5.1 (e.g. see Fig. 2) and the lyophilized lysozyme samples.
[c] Unordered structures include random coil, extended chains, and turns.
[d] Lysozyme was lyophilized from an aqueous solution adjusted to pH 5.1 at a concentration of 25 mg ml^{-1}.
[e] Additives were added to a solution of lysozyme prior to lyophilization at a 1:37 molar ratio.

zyme (Fig. 2A, full line) demonstrated substantial overall structural perturbations. Analysis of the spectra by Gaussian curve-fitting revealed a decrease in the α-helix content from 36 to 26% (Table 2) in agreement with data of Fu et al. (1999). When lysozyme was encapsulated with the two excipients that were inefficient in stabilizing lysozyme at the w/o interface and upon lyophilization, structural perturbations were even more pronounced. Specifically, when trehalose and sucrose were employed, the α-helix contents (19±3 and 24±2, respectively) and the SCC-values were the same or less (0.44 and 0.52, respectively) than for lysozyme encapsulated without excipients (Table 2 and Fig. 2). Trehalose has been found to be an inefficient excipient before upon encapsulation of lysozyme in PLGA microspheres by a w/o/w procedure (Fu et al., 1999).

The opposite was observed when lysozyme was encapsulated with lactulose and lactose (Table 1). In the case of lactulose, the secondary structure (Table 2) was much more similar to that of native lysozyme. The inverted second derivative spectra (Fig. 2C) were also similar and the SCC-value increased to 0.81. Using lactose during the encapsulation procedure also led to improvements in the structure of encapsulated lysozyme (Table 2). Thus, those excipients efficient in stabilizing lysozyme at the w/o interface and upon dehydration were efficient stabilizers upon encapsulation of lysozyme in PLGA microspheres by the w/o/w method. Excipients inefficient in stabilizing lysozyme at the w/o interface and upon lyophilization did not prevent structural perturbations upon encapsulation. These results are consistent with the idea that the main events destabilizing lysozyme structure upon encapsulation are indeed the formation of the first emulsion and the final dehydration step. For the first time it has been demonstrated that protein structural changes can be minimized by using efficient excipients during encapsulation in PLGA microspheres following a w/o/w methodology.

3.3. Lysozyme activity

Once structural preservation of the protein upon encapsulation was achieved, the hypothesis was tested that the minimization of lysozyme structural perturbations upon encapsulation would also cause the release of functionally improved protein. It has been suggested that data obtained for

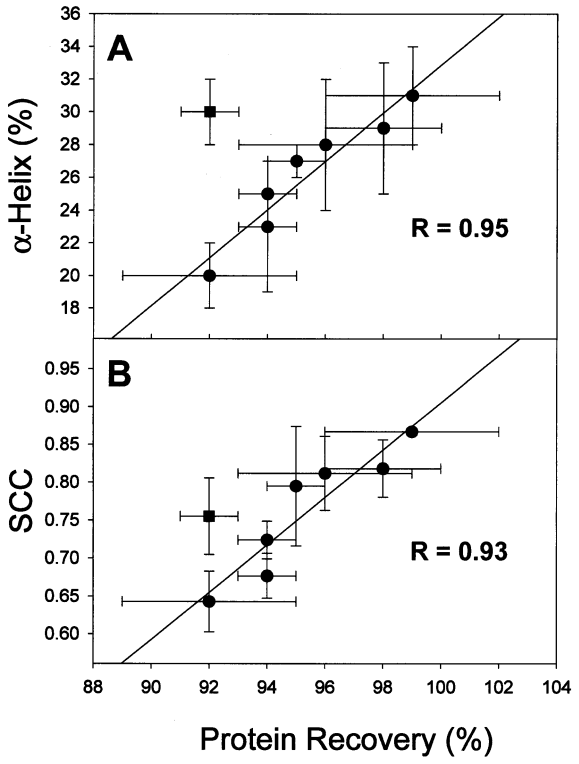

Fig. 1. Relationship between recovery of soluble (non-aggregated) lysozyme (%) after o/w emulsification and the α-helix content (A) and SCC (B) of lysozyme after lyophilization. The error bars are the S.D.s calculated for experiments performed at least in triplicate. The linear regression was calculated for the data shown as ●. One sample (that for the additive maltose, ■) clearly did not follow the trend and when it was included in the calculation of the linear regression in (A) the R-value dropped to 0.61.

initially released protein are a good measure for encapsulation-induced protein inactivation (Cleland and Jones, 1996). Thus, the specific activity of lysozyme after 24 h of in vitro release was assessed. The specific activity of dissolved lysozyme dropped substantially to 43% when it was encapsulated in PLGA microspheres without excipient (Table 2). This drop in activity was likely due to the formation of permanently unfolded lysozyme (Pérez and Griebenow, 2001) because formation of soluble protein aggregates was excluded. When we employed SEC-HPLC, a sensitive method to monitor the formation of such aggregates (Carrasquillo et al., 2001b; Castellanos et al., 2001a,b), we found no peaks other than that of the lysozyme monomer. Employing the excipients inefficient in stabilizing lysozyme against interface-induced inactivation (trehalose and sucrose) during the encapsulation gave only marginal improvements in the specific enzyme activity (Table 2). In contrast, when co-dissolving lactose and lactulose with lysozyme prior to encapsulation, the released lysozyme had an increased specific activity of 78 and 81%, respectively (Table 2). Thus, preservation of lysozyme structure during encapsulation improved its specific activity; but it was still lower than prior to encapsulation. To further improve the functionality of released lysozyme, a strategy introduced by Fu et al. (1999) was employed and the excipients were also added to the second aqueous phase. As the result, lysozyme activity improved to 90% for the excipient lactose and to 99% for lactulose. Thus, employment of the excipients in the second aqueous phase completely prevented loss in activity during encapsulation and 24 h of in vitro release. To shed some light into the possible mechanism, the activity for the soluble lysozyme fraction prior to any release directly after the microsphere preparation was also determined. For lysozyme co-encapsulated with the respective excipient, the specific activity was 82% for trehalose, 88% for lactose, and 98% for lactulose (note that no excipients were employed in the second aqueous phase in these instances). Thus, loss in activity indeed occurred for these samples after the exposure to release conditions (Table 2). For the three excipients the specific activity dropped by about 13% as the result of exposure to 24 h of in vitro release conditions. This was prevented when excipient-loss during the second emulsification step was prevented by dissolving the excipients in the second aqueous phase. It is possible that the drop in lysozyme activity upon release was related to reversible polymer adsorption leading to irreversible lysozyme unfolding. Such irreversible unfolding has been observed when lysozyme was exposed to the water–methylene chloride interface (Pérez and Griebenow, 2001). Lysozyme contact with the hydrophobic polymer matrix might lead to similar inactivation processes once encapsulated lysozyme becomes exposed to the aqueous release medium.

Table 2
Secondary structure and stability parameters for various lysozyme formulations

Sample/excipient	Secondary structure content (%)[a]			Stability parameters	
	α-Helix	β-Sheet	Others[b]	Activity (%)[c]	Aggregates (%)[d]
Aqueous solution, pH 5.10	36±1	14±2	50±1	95±1[e]	0
Lyzozyme encapsulated, none[f]	25±2	24±1	50±4	43±1	48±10
Lysozyme encapsulated with[g]					
Trehalose	19±3	18±2	63±4	69±1[h]	37±13
Sucrose	24±2	17±1	59±1	42±7	n.d.
Lactulose	34±1	15±1	51±4	81±7[h]	21±1
Lactose	30±1	12±1	58±3	78±7[h]	17±2
Lactulose[i]	[j]	[j]	[j]	99±11	16±12
Lactose[i]	[j]	[j]	[j]	90±1	13±10

[a] The secondary structure of hen egg white lysozyme was obtained by Gaussian curve-fitting of the Fourier self-deconvoluted amide I spectra.
[b] Other secondary structures include random coil, extended chains, and turns.
[c] Activity (%) values refer to the specific activity for lysozyme encapsulated into PLGA microspheres under various conditions after 24 h of in vitro release with respect to that of lysozyme as supplied by the commercial supplier freshly dissolved in the same buffer.
[d] Aggregates refer to the percentage of lysozyme after encapsulation in PLGA microspheres that could not be dissolved in buffer with respect to the total amount of lysozyme encapsulated in the microspheres. The values do not distinguish between aggregated and PLGA-adsorbed lysozyme.
[e] Incubation of lysozyme in release buffer under release conditions for 24 h did not cause a substantial decrease in the specific activity.
[f] Lysozyme was lyophilized from an aqueous solution adjusted to pH 5.1 at a concentration of 25 mg ml^{-1}.
[g] The additives were co-dissolved with lysozyme at a 1:37 molar ratio (lysozyme-to-excipient) prior to the formation of the first (w/o) emulsion in the encapsulation procedure.
[h] When the activity was determined directly after encapsulation, the values were 82±2% for trehalose, 88±7% for lactose, and 98±10% for lactulose. Thus, 13±3% of activity was lost when the microspheres were exposed to in vitro release conditions.
[i] For the preparation of these samples the excipients were dissolved in the second aqueous solution at a concentration of 10 mg ml^{-1} to prevent excipient loss.
[j] We repeatedly prepared protein loaded PLGA microspheres using the excipients in the second aqueous phase to obtain FTIR spectra. Unfortunately, the spectra were characterized by a very low signal-to-noise ratio disabling to obtain protein structural information.

Even though we did not find a statistically significant correlation between structural and activity data (e.g. α-helix content vs. specific activity), qualitatively it is apparent that when lysozyme structure was preserved in the PLGA microspheres (Table 2), a better retention of the specific activity was found.

3.4. Insoluble lysozyme aggregates

As another stability parameter the amount of insoluble lysozyme aggregates formed as the result of the encapsulation procedure was determined. The procedure used herein to determine aggregated protein employed dehydrated microspheres. The PLGA was dissolved using methylene chloride, followed by centrifugation (to harvest all encapsulated protein), drying of the protein powder pellet thus obtained, and re-dissolving of this powder in aqueous buffer. The pellet after the final centrifugation step is associated with aggregated protein, but might contain residual amounts of polymer. However, the procedure has the advantage over extraction methods (see e.g. Atkins and Peacock, 1996) that exposure of protein to a methylene chloride–water interface, which might produce protein aggregation itself, is avoided. FTIR spectroscopy was necessary and useful in investigating the chemical composition of the pellets obtained during isolation of aggregated protein. It is important to note that the above and other procedures do not allow to distinguish

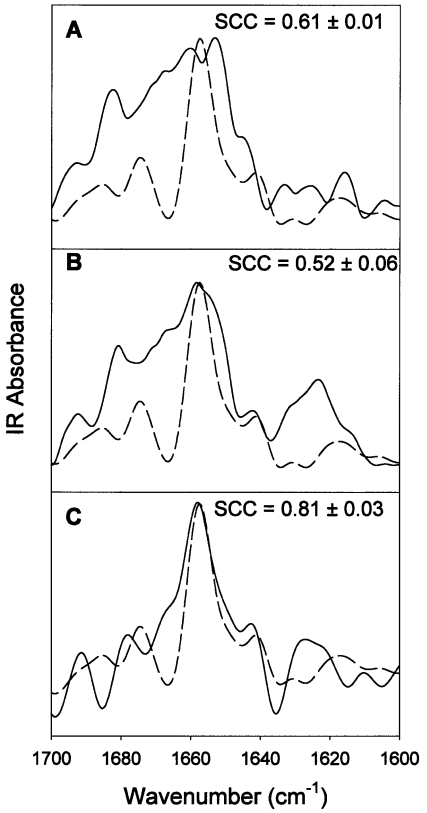

Fig. 2. Inverted second derivative amide I infrared spectra of lysozyme in PLGA microspheres under various conditions (full lines) compared with that of lysozyme in aqueous solution at pH 5.10 (broken line). (A) Lysozyme encapsulated in PLGA microspheres without additives; (B) lysozyme encapsulated with sucrose as excipient; (C) lysozyme encapsulated with lactulose as excipient.

between aggregated lysozyme and lysozyme that is adsorbed to the PLGA polymer. Both are referred to in the following as aggregated lysozyme.

The FTIR spectra of the pellet obtained after performing the above procedure revealed the presence of lysozyme (amide I band visible at 1600–1700 cm^{-1}) and PLGA (sharp band with a maximum at ca. 1760 cm^{-1}). When this pellet was treated with a 6 M urea solution, FTIR spectra revealed the complete removal of protein from the polymer. The FTIR measurements allowed for the precise quantitative determination of aggregated lysozyme by assuring that remaining insoluble components in the pellets were solely due to PLGA. Furthermore, the data demonstrate that lysozyme aggregation and the possible interaction between lysozyme and the PLGA polymer were non-covalent in nature and mainly due to hydrophobic interactions, in agreement with data published by various groups (Kim and Park, 1999; Nam and Park, 1999; Van de Weert et al., 2000c; Pérez and Griebenow, 2001; Pérez et al., 2002).

3.4.1. *Structure of aggregated lysozyme*

The FTIR spectra of the pellets also revealed some information on the secondary structure of aggregated lysozyme (Fig. 3). The second derivative IR spectrum of lysozyme in aqueous solution is dominated by a strong peak at 1658 cm^{-1}, which is due to α-helix secondary structure. The spectrum of aggregated lysozyme is quite different from that of native lysozyme. In particular, new IR bands at approximately 1627 and 1695 cm^{-1} are significant in amplitude. Such bands are typically associated with the formation of intermolecular β-sheets (Dong et al., 1995; Van de Weert et al., 2000b,c; Pérez and Griebenow, 2000, 2001). When compared with intermolecular β-sheets created using poly-L-lysine at pH 12 after heating, a classical example for such a structure (Prestrelski et al., 1993), it is obvious that the frequency of the 1627 cm^{-1} component is too high to allow to assign it to typical intermolecular β-

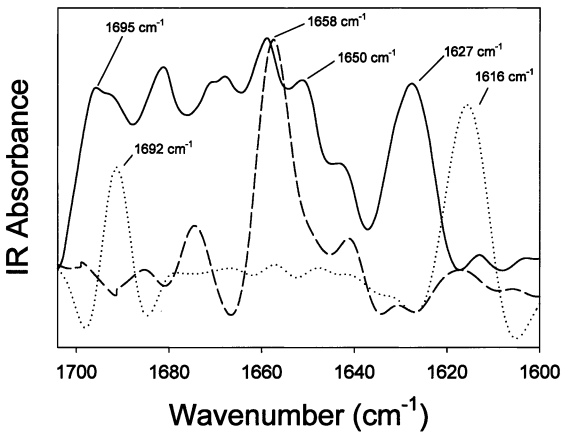

Fig. 3. Inverted second derivative amide I infrared spectra of 'insoluble' lysozyme after extraction from PLGA microspheres (full line) in comparison to lysozyme in aqueous solution at pH 5.1 (broken line) and aggregated poly-L-lysine (dotted line).

sheet structure and resembles more that of regular antiparallel β-sheet structure (Griebenow et al., 1999a). Thus, the structural organization of the protein-protein contacts in lysozyme aggregates likely resembles more that of regular β-sheet structure than that of extended ('aggregation') β-sheets formed when homopolypeptides are used. However, more importantly, contributions in the spectrum of aggregated lysozyme between the two IR bands at 1695 and 1627 cm^{-1} are not associated with intermolecular protein aggregates. These bands likely arise from structurally perturbed, but non-aggregated lysozyme. It is interesting that a band at 1650 cm^{-1} is clearly visible. Bands at such frequencies are due to non-repetitive ('random coil') secondary structure (Griebenow et al., 1999a). An intensity increase in this band frequently is interpreted as an unfolding event and thus the structure of a fraction of the insoluble lysozyme in the pellet is more unfolded than that of native lysozyme. This makes sense as it is expected that adsorption of lysozyme to the hydrophobic PLGA polymer should cause unfolding of the protein.

In conclusion, the insoluble lysozyme fraction isolated from PLGA microspheres seems to be heterogeneous in nature consisting of aggregated lysozyme and structurally severely perturbed and partially unfolded lysozyme.

3.4.2. Amount of aggregated lysozyme

Since all aggregated lysozyme was completely dissolved when exposed to 6 M urea solution, the amount could be determined (Table 2). Lysozyme encapsulated in PLGA microspheres in the absence of a stabilizing excipient showed severe aggregation (48%). In contrast, co-dissolving of lactulose in the lysozyme solution prior to encapsulation resulted in substantial reduction of lysozyme aggregation. Thus, the additive that was most efficient in reducing encapsulation-induced structural perturbations in lysozyme, was the most efficient one in preventing the detrimental loss of protein due to aggregation. As expected, formulation with lactose, an excipient also efficient in minimizing dehydration-induced structural perturbations and interface-induced unfolding and aggregation, was also efficient in reducing lysozyme aggregation upon encapsulation (Table 2). On the contrary, trehalose was inefficient in this context. The employment of the excipients lactose and lactulose also in the second aqueous phase did not reduce the amount of lysozyme aggregates substantially. Thus, most of the aggregation of lysozyme seems to occur during the formation of the first w/o emulsion. This is in contrast to the activity, which was also significantly affected by later steps in the encapsulation.

3.5. In vitro release

Even though the main focus of this work is a systematic dissection of events causing lysozyme inactivation and aggregation, the release profiles for selected preparations were also determined. The properties of the microspheres obtained are summarized in Table 3. Lysozyme release from PLGA microspheres was governed by an asymptotic profile in which up to 80% of the protein was released rapidly within only 150 h (Fig. 4). Incomplete release after this time was due to the formation of lysozyme aggregates during the encapsulation procedure because the amount of non-released protein (Fig. 4) equalled that of aggregated lysozyme after encapsulation (Table 2). Thus, no aggregation was caused by the in vitro release conditions. Procedure-induced aggregation has been implicated in incomplete delivery of proteins from polymeric delivery systems previously (Morlock et al., 1997, 1998; Crotts and Park, 1998; Kim and Park, 1999; Van de Weert et al., 2000b). The release profile showed a substantial burst release of 60–70% within the first 24 h. It is obvious that this was undesirable for practical applications. However, it is important in the context of this work that most of the protein was released within 24 h, assuring that the stability related studies were performed with a significant fraction of the encapsulated protein. Future efforts will be directed to improve the release profiles by employing PLGA polymers of higher molecular weights and also by employing blends of PLGA and the nonionic surfactant poloxamer (Carrasquillo et al., 2001b). This will then allow studying the long-term stability of lysozyme in PLGA microspheres for cases where encapsulation-in-

Table 3
Microspheres properties of lysozyme encapsulated with various excipients

Sample/excipient	Encapsulation efficiency (%)	Microsphere diameter (μm)[a]	Soluble lysozyme (%)[b]
None	65±1	86±16	52±10
Lysozyme encapsulated with			
Trehalose	49±1	122±25	63±13
Sucrose	72±2	107±17	n.d.
Lactulose	58±3	102±24	79±1
Lactose	47±1	94±17	83±2
Lactulose[c]	65±1	n.d.	83±12
Lactose[c]	47±4	n.d.	87±11

[a] Data have been obtained from scanning electron micrographs, see Section 2 for details.
[b] Fraction (%) of the encapsulated lysozyme that could be dissolved in buffer in the procedure used to determine the actual protein loading.
[c] See footnote i in Section 2.

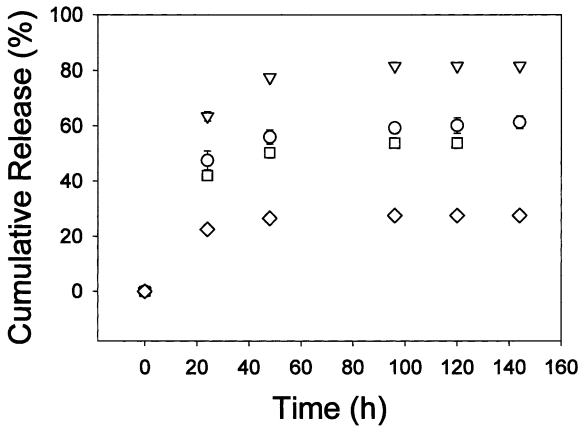

Fig. 4. Cumulative release profiles of lysozyme from PLGA microspheres for different formulations. Additives co-dissolved with lysozyme in the aqueous solution used in the first w/o emulsification step: ▽, lactulose; ○, none; □, lactose; and ◇, trehalose. The error bars were omitted in cases where they were smaller than the symbols shown.

duced structural perturbations and aggregation have been reduced.

4. Conclusions

The major disadvantage of protein encapsulation in PLGA microspheres for their sustained release by w/o/w methods is the exposure of protein in aqueous solution to an organic solvent interface. The stabilization of lysozyme in the first emulsification step by excipients (in particular lactulose) resulted in the reduction in encapsulation-induced structural perturbations. This in turn led to a reduction in encapsulation-induced lysozyme aggregation and inactivation. However, also later stages in the procedure can give rise to detrimental events. Some activity loss occurred after exposure of lysozyme to in vitro release conditions. This loss in activity was prevented when excipients were also dissolved in the aqueous PVA solution used in the second emulsification step. There was a relationship between structural preservation and prevention of lysozyme aggregation as well as between structural preservation and prevention of loss in activity. In general, the better the structure of lysozyme was preserved during the encapsulation procedure, the better the structural and functional properties of lysozyme were during release.

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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., Peacock, S.J., 1996. The incorporation and release of bovine serum albumin from poly-hydroxybutyrate–hydroxyvalerate microcapsules. J. Microencaps. 13, 709–717.

Carrasquillo, K.G., Cordero, R.A., Ho, S., Franquiz, J.M., Griebenow, K., 1998. Structure-guided encapsulation of bovin serum albumin in poly(D,L- 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., 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.

Carrasquillo, K.G., Stanley, A.M., Aponte Carro, J.C., De Jésus, P., Costantino, H.R., Bosques, C.J., Griebenow, K., 2001. Non-aqueous encapsulation of excipient-stabilized spray freeze-dried BSA into poly(lactide-*co*-glycolide) microspheres results in release of native protein. J. Control. Release 76, 199–208.

Castellanos, I.J., Carrasquillo, K.G., De Jésus López, J., Alvarez, M., Griebenow, K., 2001. Encapsulation of bovine serum albumin in poly(lactide-*co*-glycolide) microspheres by the solid-in-oil-in-water technique. J. Pharm. Pharmacol. 53, 167–178.

Castellanos, I.J., Cuadrado, W.L., Griebenow, K., 2001. Prevention of structural perturbations and aggregation upon encapsulation of bovine serum albumin into poly(lactide-*co*-glycolide) microspheres using the solid-in-oil-in-water technique. J. Pharm. Pharmacol. 53, 1099–1107.

Cleland, J.L., Jones, A.J.S., 1996. Stable formulations of recombinant human growth hormone and interferon-γ for microencapsulation in biodegradable microspheres. Pharm. Res. 13, 1464–1475.

Costantino, H.R., Langer, R., Klibanov, A.M., 1994. Solid phase aggregation of proteins under pharmaceutically relevant conditions. J. Pharm. Sci. 83, 1662–1669.

Costantino, H.R., Griebenow, K., Mishra, P., Langer, R., Klibanow, A.M, 1995. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. Biochim. Biophys. Acta 1253, 69–74.

Costantino, H.R., Carrasquillo, K.G., Cordero, R.A., Mumenthaler, M., Hsu, C.C., Griebenow, K., 1998. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone (rhGH). J. Pharm. Sci. 87, 1412–1420.

Costantino, H.R., Firouzabadian, L., Hogeland, K., Wu, C., Beganski, C., Carrasquillo, K.G., Cordova, M., Griebenow, K., Zale, S.E., Tracy, M.A., 2000. Protein spray-freeze drying. Effect of atomization conditions on particle size and stability. Pharm. Res. 17, 1374–1383.

Crotts, G., Park, T.G., 1998. Protein delivery from poly(lactic-*co*-glycolic acid) biodegradable microspheres; release kinetics and stability issues. J. Microencaps. 15, 699–713.

Diwan, M., Park, T.G., 2001. Pegylation enhances protein stability during encapsulation in PLGA microspheres. J. Control. Release 73, 233–244.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

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. Control. Release 58, 357–366.

Ghaderi, R., Carlfors, J., 1997. Biological activity of lysozyme after entrapment in poly(D,L-lactide-*co*-glycolide) microspheres. Pharm. Res. 14, 1556–1564.

Griebenow, K., Klibanov, A.M., 1995. Lyophilization-induced reversible changes in the secondary structure of proteins. Proc. Natl. Acad. Sci. USA 92, 10969–10976.

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

Griebenow, K., Santos, A.M., Carrasquillo, K.G., 1999a. Secondary structure of proteins in the amorphous dehydrated state probed by FTIR spectroscopy. Dehydration-induced structural changes and their prevention. Internet J. Vibr. Spec. [http://www.ijvs.com], 3, 1, 3.

Griebenow, K., Santos, A.M., Carrasquillo, K.G., 1999b. Application of FTIR spectroscopy to probe and improve protein structure in sustained release devices. Internet J. Vibr. Spec. [http://www.ijvs.com], 3, 5, 2.

Kim, H.K., Park, T.G., 1999. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation, stability and incomplete release mechanism. Biotechnol. Bioeng. 65, 659–667.

Langer, R., 1990. New methods of drug delivery. Science 249, 1527–1533.

Langer, R., 2000. Biomaterials in drug delivery and tissue engineering: one laboratoty's experience. Acc. Chem. Res. 33, 94–101.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265.

Lu, W., Park, T.G., 1995. 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. Microencaps. 14, 225–241.

Manning, C., Patel, K., Borchardt, R.T., 1989. Stability of protein pharmaceuticals. Pharm. Res. 6, 903–918.

Meinel, L., Illi, O.E., Zapf, J., Malfanti, M., Merkle, H.P., Gander, B., 2001. Stabilizing insulin-like growth factor-I in

poly(D,L-lactide-*co*-glycolide) microspheres. J. Control. Release 70, 193–202.

Middelberg, A.P.J., Radke, C.J., Blanch, H.W., 2000. Peptide interfacial adsorption is kinetically limited by thermodynamic stability of self association. Proc. Natl. Acad. Sci. USA 97, 5054–5059.

Morlock, M., Knoll, H., Winter, G., Kissel, T., 1997. Microencapsulation of rherythropoietin, using biodegradable poly(lactide-*co*-glycolide): protein stability and the effects of stabilizing excipients. Eur. J. Pharm. Biopharm. 43, 29–36.

Morlock, M., Kissel, T., Li, Y.X., Koll, H., Winter, G., 1998. Erythropoietin loaded microspheres prepared from biodegradable LPLG-PEO-LPLG triblock copolymers: protein stabilization and in-vitro release properties. J. Control. Release 56, 105–115.

Nam, Y.S., Park, T.G., 1999. Protein loaded biodegradable microspheres based on PLGA–protein bioconjugates. J. Microencaps. 16, 625–637.

Nihant, N., Schugens, C., Grandfils, C., Jérôme, R., Teyssié, P., 1994. Polylactide microparticles prepared by double emulsion/evaporation technique. I. Effect of primary emulsion stability. Pharm. Res. 11, 1479–1484.

Pérez, C., Griebenow, K., 2000. Fourier-transform infrared spectroscopic investigation of the thermal denaturation of hen egg-white lysozyme dissolved in aqueous buffer and glycerol. Biotechnol. Lett. 22, 1899–1905.

Pérez, C., Griebenow, K., 2001. Improved activity and stability of lysozyme at the water/methylene chloride interface: enzyme unfolding and aggregation and its prevention by polyols. J. Pharm. Pharmacol. 53, 1217–1226.

Pérez, C., Castellanos, I.J., Constantino, R.C., Al-Azzam, W., Griebenow, K., 2002. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J. Pharm. Pharmacol. 54, 301–303.

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., 1997. A new strategy to determine the actual protein content of poly(lactide-*co*-glycolide) microspheres. J. Pharm. Sci. 86, 1315–1318.

Sah, H., 1999. Protein instability toward organic solvent/water emulsification: Implications for protein microencapsulation into microspheres. PDA J. Pharm. Sci. Technol. 53, 3–10.

Sah, H., 1999. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. J. Control. Release 58, 143–151.

Sah, H., 1999. Protein behaviour at the water/methylene chloride interface. J. Pharm. Sci. 88, 1320–1325.

Thomasin, C., Ho, N.T., Merkle, H.P., Gander, B., 1998. Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical considerations. J. Pharm. Sci. 87, 259–268.

Van de Weert, M., Hennink, W.E., Jiskoot, W., 2000. Protein instability in poly(lactic-*co*-glycolic acid) microparticles. Pharm. Res. 17, 1159–1167.

Van de Weert, M., Hoechstetter, J., Hennink, W.E., Crommelin, D.J., 2000. The effect of a water/organic solvent interface on the structural stability of lysozyme. J. Control. Release 68, 351–359.

Van de Weert, M., van't Hof1, R., van der Weerd, J., Heeren, R.M., Posthuma, G., Hennink, W.E., Crommelin, D.J., 2000. Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques. J. Control. Release 68, 31–40.

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129–188.

Wang, W., 2000. Lyophilization and development of solid protein pharmaceuticals. Int. J. Pharm. 203, 1–60.

Yang, T.-H., Dong, A., Meyer, J., Johnson, O.L., Cleland, J.L., Carpenter, J.F., 1999. Use of infrared spectroscopy to assess secondary structure of human growth hormone within biodegradable microspheres. J. Pharm. Sci. 88, 161–165.