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Nanoprecipitation Versus Emulsion-based Techniques for the Encapsulation of Proteins Into Biodegradable Nanoparticles and Process-related Stability Issues

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ABSTRACT

The goal of this study was to investigate the entrapment of 3 different model proteins (tetanus toxoid, lysozyme, and insulin) into poly(D,L-lactic acid) and poly(D,L-lactic-*co*-glycolic acid) nanoparticles and to address process-related stability issues. For that purpose, a modified nanoprecipitation method as well as 2 emulsion-based encapsulation techniques (ie, a solid-in oil-in water (s/o/w) and a double emulsion ($w_1/o/w_2$) method) were used. The main modification of nanoprecipitation involved the use of a wide range of miscible organic solvents such as dimethylsulfoxide and ethanol instead of the common acetone and water. The results obtained showed that tetanus toxoid and lysozyme were efficiently incorporated by the double emulsion procedure when ethyl acetate was used as solvent (>80% entrapment efficiency), whereas it was necessary to use methylene chloride to achieve high insulin entrapment efficiencies. The use of the s/o/w method or the formation of a more hydrophobic protein-surfactant ion pair did not improve protein loading. The nanoprecipitation method led to a homogenous population of small nanoparticles (with size ranging from ~130 to 560 nm) and in some cases also improved experimental drug loadings, especially for lysozyme (entrapment efficiency > 90%). With respect to drug content determination, a simple and quick matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method provided results very close to those obtained by reverse phase-high-performance liquid chromatography. With respect to protein stability, the duration and intensity of sonication were not a concern for tetanus toxoid, which retained more than 95% of its antigenicity after treatment for 1 minute. Only a high methylene chloride:water ratio was shown to slightly decrease toxoid antigenicity. Finally, no more than 3.3% of A21 desamido insulin and only traces of covalent insulin dimer were detected in nanoparticles. In conclusion, both the double emulsion and nanoprecipitation methods allowed efficient protein encapsulation. MALDI-TOF MS allowed accurate drug content determination. The manufacturing processes evaluated did not damage the primary structure of insulin.

INTRODUCTION

The entrapment of proteins into nanoparticles still remains a difficult task, since each protein is characterized by essential properties (eg, molecular weight, hydrophilicity, stability) that might be somehow different with respect to another protein. This situation often hampers protein formulation, because each protein becomes the subject of a case study. The choice of a correct formulation strategy is mainly driven by solubility and molecular stability considerations. In this context, the water-in oil-in water ($w_1/o/w_2$) double emulsion method was often used for successfully encapsulating a wide range of proteins in microparticles, mostly because it allowed hydrophilic proteins to be dissolved in an aqueous w_1 phase before the encapsulation process. However, proteins might be altered to some extent by interfaces and agitation stress with this method. Moreover, proteins that are insoluble in water (eg, because of an isoelectric point close to the neutral pH) should rather be formulated by solubilizing them in a nonaqueous solvent or by suspending them in an appropriate medium. In this respect, a benefit of protein suspension over the solution is that proteins can maintain their secondary and tertiary structure.[1] The solid-in oil-in water (s/o/w) method is used from time to time as an alternative to the double emulsion method, in order to avoid the water-organic solvent interface during the first emulsification step. Recently, a modified nanoprecipitation method was optimized so as to allow hydrophilic and hydrophobic compounds to be entrapped within small nanoparticles by using miscible solvents and in the absence of water.[2] In this method, hydrolytic degradation pathways can be minimized and other factors of instability such as interfaces and high shearing rates are avoided. Moreover, it has been reported, for

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instance, that lysozyme and insulin, which were precipitated from DMSO, partially unfolded but recovered biological activity after reconstitution in aqueous media.[1] Another strategy to promote stability is to form a protein-surfactant complex by means of an ion-pairing technique. Based on the interaction of an anionic amphiphilic molecule and a positively charged protein, ion-pairing takes place at surfactant concentrations below the critical micelle concentration (CMC) and at a pH below the isoelectric point of the protein.[3,4] Ion-pairing is expected to be an interesting means for rendering a protein more hydrophobic, for increasing its solubility in organic solvents, and thus for improving its loading into nanoparticles.[5,6]

The main goal of this study was to systematically assess the performance of nanoprecipitation and emulsion-based encapsulation processes with respect to nanoparticle size and final drug loading. The traditional nanoprecipitation method was modified so as to favor protein encapsulation. Actually, a wide range of miscible organic solvents such as dimethylsulfoxide (DMSO) and ethanol were used instead of commonly used acetone and water.[2,7-9] Two emulsion-based encapsulation techniques (ie, a solid-in oil-in water [s/o/w] and a double emulsion [w_1/o/w_2] method) were also used.

Three model proteins, tetanus toxoid (TT), lysozyme, and insulin, were chosen for this comparative encapsulation investigation on the basis of their molecular weight (150 kDa, 14 300 Da, and 5777 Da, respectively) and their solubility in the solvents used. Indeed, TT is a large protein with a complex molecular structure, whereas lysozyme and insulin are rather small in comparison. Both TT and lysozyme are soluble in pure water, whereas insulin is only soluble in acid solutions and in solvents such as DMSO. Therefore, all these proteins were good candidates for the double emulsion method. Among these 3 proteins, only TT could not be encapsulated by nanoprecipitation because of a lack of solubility in DMSO. In addition, lysozyme and insulin were ion-paired with sodium oleate and sodium dodecylsulfate (SDS), respectively, in order to determine if the resulting complexes could be more efficiently entrapped into nanoparticles by the above-mentioned methods than the native proteins (ion-pairing with TT is not possible).

Some additional stability assays were also performed to evaluate the effect of these encapsulation methods on protein stability. For this purpose, some specific analytical methods were selected according to the protein investigated. Thus, TT, the main function of which is to induce an immune response without pathogenic effects, was evaluated with respect to its antigenic properties through an indirect enzyme-linked immunosorbent assay (ELISA). This test was performed after exposure of TT to the ultrasounds and organic/aqueous interfaces that are both degradation factors involved during emulsion-based encapsulation procedures. Concerning insulin, the chemical degradation due to the encapsulation procedure was tracked using reverse-phase high performance liquid chromatography (RP-HPLC) (detection of deamidated insulin by-products) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (detection of covalent insulin dimers [CID]). As a matter of fact, a new MALDI-TOF mass spectrometry procedure was developed for both the quantification and the analysis of the primary structure of insulin, without extensive deleterious extraction of the protein, as discussed previously.[10-12] It is indeed of utmost importance to analyze encapsulated proteins in mild conditions so as to avoid additional stress for the analyte. This issue has been already discussed, for instance regarding the analysis of protein secondary structure by Fourier transform infrared (FTIR) spectroscopy.[13] It should be noted here that the therapeutic effect of both TT and insulin is strongly dependent on the molecular stability of these molecules. This is the main reason why it is important to perform qualitative analysis. On the contrary, the stability of lysozyme was not assessed here, since it is a far less interesting protein from the therapeutic point of view.

Finally, protein release kinetics and protein stability during release were not assessed, since this study only focused on the effect of the encapsulation process on the entrapped proteins. Although the release of the protein in an active form is an important issue, this study primarily focused on the value of the sample preparation for analysis and an analytical method mass spectrometry (MS) able to discriminate between process-related or release-related protein instability.

MATERIALS AND METHODS

Materials

Tetanus toxoid (batch S2545, Pasteur Mérieux, Lyon, France), specific activity of 205 Limes factor (Lf/mg) protein, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was a gift from Claire-Ann Siegrist, PhD (Geneva, Switzerland). Porcine insulin (batch S69941607. LGA, Novo Nordisk) was a gift from Keith Rose, PhD (Geneva, Switzerland). Freeze-dried lysozyme, human arginsulin, sodium dodecyl sulfate (SDS), sodium oleate, and α-cyano-4-hydroxycinnammic acid (α-CHCA) were obtained from Sigma-Aldrich Co (Buchs, Switzerland). The TT010 monoclonal antibody and guinea-pig antitetanus toxoid purified IgG were made available from NIBSC (National Institute for Biological Standards and Control, Hertfordshire, UK). Goat anti-guinea-pig IgG-horseradish peroxidase (HRP) conjugate (A7289) was obtained from Sigma-Aldrich Co. Copolymers of D,L-lactide and glycolide (PLGA), 50:50 molar ratio, with capped and uncapped

carboxylic end groups (respectively, Resomer RG 503 and RG 503 H, molecular weight [MW] gel permeation chromatography [GPC] of 34 kDa), as well as a capped homopolymer of D,L-lactide (PLA) (Resomer R 203, MW [GPC] of 28 kDa) were purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). A PLA with free carboxylic end groups (Medisorb PLA 100 DL 4A, MW [GPC] of 57 kDa) was provided by Alkermes (Cincinnati, Ohio). Poly(vinyl alcohol) (PVAL, Mowiol 4–88) of MW 31 kDa, 88% hydrolyzed, was provided by Clariant Gmbh (Frankfurt am Main, Germany). Poloxamer 407 (Lutrol F127) was obtained from BASF (Ludwigshafen, Germany) and povidone K30 was purchased from Fluka Chemie (Buchs, Switzerland), together with acetonitrile (MeCN, high-performance liquid chromatography [HPLC] grade) and trifluoroacetic acid (TFA). All other chemicals used were of reagent grade.

Preparation of Protein-surfactant Complexes by Ion-pairing

Ion-pairing was in part performed as previously described by Yoo et al.[14] Briefly, lysozyme was dissolved in a pH 8.7 Tris buffer, whereas insulin was dissolved in a pH 2.5 HCl solution. The final concentration of the solutions was of 3 mg/mL. Lysozyme and insulin were ion-paired (and thus precipitated) at room temperature with sodium oleate and SDS, respectively, by adding the counter-ion (previously dissolved in the same media) in the above protein solutions. The protein:surfactant molar ratio was 1:6 in both cases, owing to the available positively charged residues at the above-mentioned pH. After equilibration for 5 hours, the suspensions were centrifuged twice for 5 minutes at 530g, and the cakes were washed with water to remove the excess of salts. Then, the aqueous suspensions of the complexes were freeze dried at 0.03 mbar and at –60°C for 24 hours (LSL Secfroid, Lyolab BII, Aclens-Lausanne, Switzerland). In order to determine the composition of complexes (ie, the experimental protein:surfactant molar ratio), the oleate-lysozyme and SDS-insulin complexes were dissolved in a 1M sodium hydroxide solution and analyzed by spectrophotometry at 281 and 291 nm, respectively (model 8453, Hewlett Packard, Boeblingen, Germany).

Nanoparticle Preparation

Double Emulsion-solvent Evaporation Method (w₁/o/w₂)

Protein-loaded nanoparticles were prepared in triplicate at 2% nominal drug loading by a $w_1/o/w_2$ double emulsion-solvent evaporation method as described previously.[15] Briefly, a protein-containing aqueous phase was first poured into a 20% organic solution of polymer (Resomer RG 503, unless otherwise stated). Only insulin, which was insoluble

in pure water, was dissolved in a 0.1M HCl solution. The volumes of these solutions were 100 μL and 2 mL, respectively. Ethyl acetate (EtOAc) and methylene chloride (Me₂Cl₂) were selected as organic solvents. Primary (w_1/o) emulsion was obtained with a 10-second sonication at 65 W (Probe sonicator, model VC 50T, Sonic and Materials Inc, Danbury, CT). Then, 2 mL of a 2% aqueous PVAL solution was poured into the primary emulsion and sonicated (15 seconds, 65 W) to form the $w_1/o/w_2$ double emulsion. The final preparation was then magnetically stirred overnight at room temperature to evaporate the organic solvent. The resulting nanoparticles in suspension were then centrifuged 4 times for 15-minute cycles at 15 000g and washed with distilled water to remove the free PVAL from the batches. Freeze-drying of the batches was performed at 0.03 mbar and at –60°C for 24 hours.

Solid-in Oil-in Water Method (s/o/w)

Native or ion-paired proteins were directly suspended in ethyl acetate or methylene chloride and sonicated to obtain a finely dispersed solid-in oil suspension (s/o). All the subsequent steps of nanoparticle production were the same as those for the double emulsion method (see Double Emulsion-solvent Evaporation Method ($w_1/o/w_2$)).

Modified Nanoprecipitation Method

As for those prepared by the emulsion-based methods, nanoparticles were prepared in triplicate and at 2% nominal drug loading. The polymer (~200 mg) and the protein drug (~4 mg) were dissolved either in DMSO or in *N*-methylpyrrolidone (NMP) (~8 mL). This phase was added by means of a syringe by inserting the needle directly into the dispersing phase (~40 mL), which was magnetically stirred. Nanoprecipitation occurred as soon as the protein solution was in contact with the dispersing phase. The dispersing phase was constituted of a solvent in which the polymer is insoluble (water, methanol, ethanol, or propanol) and contains, if needed, poloxamer 407 or polyvinyl-pyrrolidone K30 as a surfactant. For the sake of clarity, it should be reminded that only water is traditionally used as dispersing phase during nanoprecipitation. Then, the suspensions of nanoparticles were centrifuged 4 times for 15-minute cycles at 15 000g and washed with distilled water, in order to remove the dispersing organic medium and to replace it gradually with water for subsequent freeze-drying.

Size Determination

Particle size and polydispersity were determined by photon correlation spectroscopy using a Zetasizer 5000 (Malvern

Instruments Ltd, Worcestershire, UK). Nanoparticles prepared by double emulsion were appropriately diluted just after preparation with double-distilled and filtered water (0.22 μm Millipore filter, Millipore, Billerica, MA), whereas those prepared by nanoprecipitation were diluted with the nonsolvent. Mean size and polydispersity were measured 3 times for each batch.

Nanoparticle Morphology

The nanoparticle surface and shape were observed by scanning electron microscopy (SEM). Samples were prepared by finely spreading concentrated nanoparticle suspensions over slabs and by drying them under vacuum. The samples were then coated in a cathodic evaporator with a fine gold layer and observed by SEM using a JSM-6400 scanning electron microscope (JEOL, Tokyo, Japan).

Protein Loading Determination

Aliquots (20 mg) of TT-loaded nanoparticles and of those containing the lysozyme-oleate complex were first hydrolysed by a 1M sodium hydroxide solution, and the resulting solutions were spectrophotometrically measured at 291 nm. The protein content of all other nanoparticle batches was determined after dissolution of the carriers in DMSO. The medium was stirred at room temperature until complete transparency and the solution absorbance measured at 281 nm. The residual absorbance of blank nanoparticles at this wavelength was taken into account for the calibration. Insulin-loaded nanoparticles were also analyzed by RP-HPLC. Accurately weighed 20-mg aliquots of nanoparticles were first dissolved under mild agitation (vortex) in 300 μL of acetonitrile in test tubes. Then, 700 μL of 0.1% TFA were added dropwise to the above vortexed mixture to dissolve insulin completely. The remaining undissolved PLGA and PVAL were then separated by centrifugation at 2000g for 2 minutes. The clear supernatants were then withdrawn, filtered through a 0.45-μm syringe filter, and analyzed using a RP-HPLC Waters Corp system (LC Module 1 plus, Milford, MA) equipped with a UV-visible detector. Insulin and A21 desamido insulin were separated at room temperature on a Nucleosil C_{18} column (25 mm × 4.6 inner diameter [id], Macherey-Nagel, Oensingen, Switzerland) packed with 5-μm particles of 100 Å pore size. The mobile phase was an acetonitrile:water mixture (32:68) containing 0.1% TFA. The flow rate was 0.9 mL/min and the detecting wavelength was 214 nm. The amount of A21 desamido insulin detected in the samples was expressed as

$$A21 \; desamido \; insulin \; (\%) = \frac{A_{A21 \; desamido-insulin}}{A_{native \; insulin} + A_{A21 \; desamido-insulin}} \times 100 \quad (1)$$

where A is the peak area of the considered molecule.

Each sample was assayed in triplicate. The entrapment data were expressed as described in a previous study.[16] Briefly, drug loading (DL) refers to the amount of drug used with respect to the amount of both polymer and drug, whereas entrapment yield (EY) refers to the amount of recovered drug in the final batch with respect to the amount initially used. Nanoparticle yield (NY) is the total weight yield, which is calculated when all nanoparticles are recovered after freeze-drying. Entrapment efficiency (EE) is the ratio of the final drug loading to the nominal one.

Tetanus Toxoid Antigenicity Measurements

An ELISA assay was used for estimating TT antigenicity after exposure to stresses caused by ultrasound and organic/aqueous interfaces. A monoclonal antibody (TT010) was used as the capture antibody. Briefly, wells of flat-bottomed microtiter plates (Nunc-Immuno Plate Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μL of 1 μg/mL monoclonal antitetanus IgG in 0.05M carbonate buffer (pH 9.6) and incubated at 4°C overnight. Nonspecific sites were blocked with 200 μL of 1% gelatin in PBS (PBSG), and the plates were incubated for 1 hour. The temperature was set at 37°C for each incubation step, and the plates were washed at least 3 times with 300 μL of a 0.05% Tween 20 solution of NaCl 0.9% after each incubation. Serial dilutions of reference TT and samples were prepared in a PBS solution containing both 0.2% gelatin and 0.05% Tween 20 (PBSTG). The wells were filled with 100 μL of the TT reference solutions or of the samples and incubated for 2 hours. Guinea-pig IgG (4.5 μg/mL) was added to each well in 100 μL of PBSTG and followed by another 2-hour incubation step. Then, 100 μL of goat anti-guinea-pig IgG-HRP conjugate (1/5000 dilution in PBSTG) were added to each well, and plates were incubated for an additional 1 hour. Finally, 100 μL of a 1 mg/mL HRP substrate ABTS solution containing 30.5 mM citric acid and 0.01% H_2O_2 were added to the wells for the enzymatic reaction to occur. Color was allowed to develop, and the end-point optical density was measured at 405 nm after a 1-hour incubation at room temperature and protection against light.

MALDI-TOF MS Sampling and Preparation of Targets

Reference solutions of increasing concentrations of insulin were prepared in a mixture of water, acetonitrile, and TFA (50%, 50%, and 0.1%, respectively). For nanoparticle sampling, 2 mg of insulin-loaded nanoparticles were first put in 500 μL of a 0.2% TFA acetonitrile solution for complete polymer dissolution. Then, 500 μL of water were added to this solution, allowing the polymer to precipitate. Quantification was made possible by adding the same amount of an internal standard (arg-insulin) to each reference solution

as well as to each unknown sample. One microliter of each solution was then deposited on a plate and the solvent was allowed to evaporate under vacuum, making the drop turn into a solid spot. One microliter of the matrix (α-CHCA) in solution (10 mg/mL in a 0.1% TFA and 50% acetonitrile solution) was applied on each sample spot and allowed to dry again in the same manner. The plate was then inserted into the mass spectrometer. MALDI-TOF MS experiments were conducted on a MALDI-TOF instrument (Voyager Super STR; Applied Biosystems, Foster City, CA) using a 337-nm nitrogen laser. Spectra were acquired in positive ion linear mode (instead of reflector mode) (acceleration voltage 20 kV or 25 kV). A total of 40 shots per location (thus up to 560 shots were averaged per spot) was programmed. One spectrum per spot was obtained and each spot was analyzed 3 times. The ratio of the relative peak height of insulin on the peak height of arg-insulin was plotted against insulin concentration and a linear calibration curve was generated as reported elsewhere.[10,11] Only the intensity values of the dominant $[M+H]^+$ peak (5777 Da) of the MS spectra was considered for the calibration. External mass calibration was performed on the protonated $[M+H]^+$ ion using native porcine insulin.

RESULTS AND DISCUSSION

Tetanus Toxoid-loaded Nanoparticles

TT was shown to be easily entrapped within PLGA particles (Table 1). Both ethyl acetate and methylene chloride enabled entrapment of over 70% of the initial amount of protein, which means with entrapment efficiencies close to 95%. From this point of view, the double emulsion method was superior to the s/o/w method. This finding is certainly owing to a promoted drug leakage toward the outer aqueous phase when the protein is used in the solid state. As is often the case with the double emulsion procedure, the nanoparticle yield was very elevated, reaching values as high as 85%. High TT entrapment efficiencies and yields were also observed when microspheres of 10 to 25 µm made of polymers having different PLA:PGA ratios (50:50 and 75:25) or made of PLGA-polyoxyethylene-PLGA triblocks were produced.[17] Such results were also obtained with microspheres produced by spray-drying and coacervation techniques. Moreover, it was also reported that, when TT was used in suspension rather than in solution, the entrapment was diminished as demonstrated here (batch TT 3).[18] The nanoparticle batch TT 1 displayed the smallest mean size value, which was to be expected since the use of ethyl acetate always leads to smaller particles relative to methylene chloride.[16,19] Actually, in the latter case, the preparation also contained larger particles, as shown in Figure 1 (batch TT 2). Such a wide range of nanoparticle size in a same population (due to methylene chloride), along with notable antigen loading performance, might be an advantage if a pulsed or a sustained release provided by the larger particles is sought. Moreover, this kind of preparation might be mixed with very large microspheres in order to favor both an immediate and a prolonged release over months. Finally, antigenic adjuvancy of encapsulating TT cannot be excluded, as previously reported, with similar or even better effect than alum-based vaccines.[20-22]

Effect of Ultrasounds and Aqueous/Organic Interfaces on TT Antigenicity

In order to evaluate the resistance of TT to sonication, the antigen was dissolved in PBS and submitted to various sonication durations between 5 seconds and 2 minutes

Table 1. Protein-loaded Nanoparticles Produced by s/o/w and w/o/w Methods*

Batch	Emulsion Method	Organic Solvent	EE ± SD§ (%)	EY ± SD (%)	NY ± SD (%)	Size ± SD (nm)	PI ± SD
TT 1	w/o/w	EtOAc	96.5 ± 3.9	72.3 ± 4.9	74.9 ± 4.2	353 ± 15	0.13 ± 0.13
TT 2	w/o/w	Me₂Cl₂	93.4 ± 6.4	80.2 ± 7.3	85.8 ± 2.0	1153 ± 248	0.36 ± 0.13
TT 3	s/o/w	Me₂Cl₂	44.3 ± 3.4	38.1 ± 3.1	85.9 ± 1.2	796 ± 108	0.34 ± 0.17
LYS A1	w/o/w	EtOAc	84.4 ± 4.4	56.3 ± 11.8	66.8 ± 13.1	459 ± 49	0.18 ± 0.07
LYS A2†	s/o/w	EtOAc	88.0 ± 6.0	63.4 ± 5.0	72.0 ± 1.3	369 ± 14	0.16 ± 0.02
LYS A3	w/o/w	Me₂Cl₂	112.3 ± 1.5	101.3 ± 3.6	90.4 ± 3.1	967 ± 39	0.78 ± 0.21
INS A1	w/o/w	EtOAc	24.1 ± 9.5	18.4 ± 7.4	76.5 ± 2.5	425 ± 44	0.49 ± 0.21
INS A2‡	w/o/w	EtOAc	25.0 ± 5.6	19.7 ± 5.1	78.6 ± 4.6	468 ± 72	0.37 ± 0.29
INS A3	w/o/w	Me₂Cl₂	78.7 ± 10.3	69.6 ± 8.8	88.6 ± 4.3	1402 ± 584	0.66 ± 0.05
INS A4	s/o/w	Me₂Cl₂	73.1 ± 7.7	53.7 ± 5.8	73.4 ± 0.9	1425 ± 580	0.74 ± 0.23
INS A5†	s/o/w	Me₂Cl₂	87.1 ± 17.1	68.6 ± 14.2	79.0 ± 6.3	1038 ± 126	0.74 ± 0.24

* EE indicates entrapment efficiency; SD, standard deviation ($n = 3$); EY, entrapment yield; NY, nanoparticle yield; PI, mean polydispersity index is expressed using a 0 to 1 scale ($n = 3$); TT, tetanus toxoid; LYS, lysozyme; and INS, insulin. All batches were produced at 2% of nominal drug loading.
† Batches Lys A2 and INS A5 were produced with the lysozyme-oleate and the SDS-insulin complexes, respectively.
‡ Batch INS A2 was produced with a 3-second sonication for the first emulsification step and a 15-second sonication for the second emulsification step.
§ All values are ±SD ($n = 3$). The entrapped proteins were quantified by UV spectrophotometry.
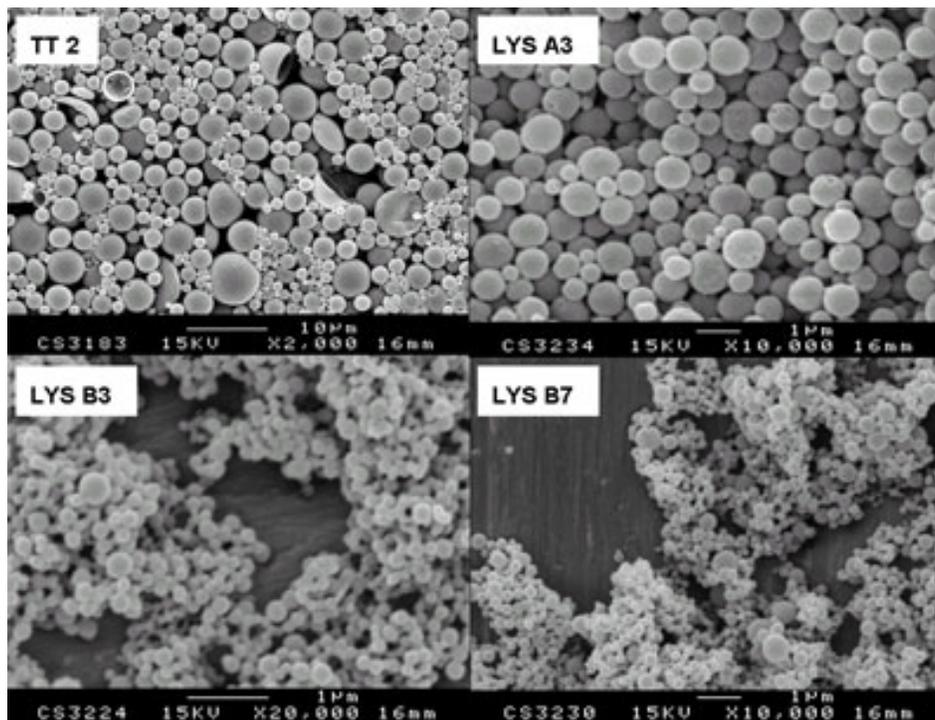
Figure 1. Scanning electron photomicrographs of batch TT 2, batch LYS A3, batch LYS B3, and batch LYS B7.

(intensity:65W). ELISA tests showed that the antigen remained particularly unaffected after 1- minute sonication and that a slight loss of antigenicity was detectable only after a 2-minute exposure (Figure 2). There was also no difference between low and high sonication intensities, since sonicated TT at 25 W during 25 seconds and 60 seconds remained antigenically stable (95% ± 1% and 101% ± 3%, respectively) as for samples treated at 65 W. Such an insensitivity was also observed with lysozyme, the specific activity of which was only slightly affected by sonication.[23] When a protein comes into contact with a hydrophobic environment such as an aqueous/organic interface, the
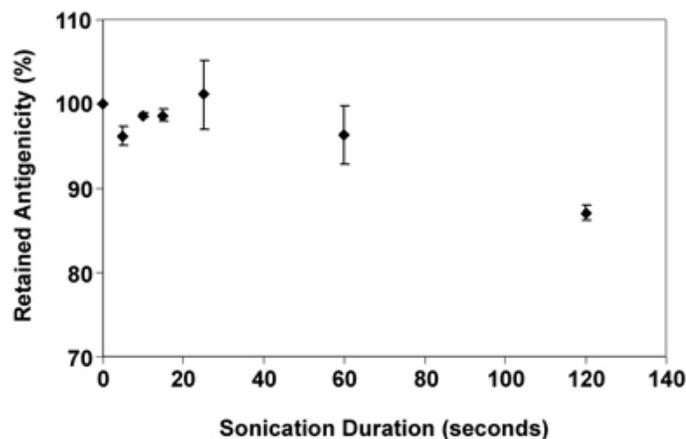

Figure 2. Effect of ultrasound on residual antigenicity of TT dissolved in water as determined by an Elisa assay. The sonication power input was set at 65 W.

molecule adopts a destabilized conformation, since hydrophobic side chains, which are normally buried in the molecule core, are reversed and exposed toward the interface. This new conformation is often responsible for subsequent aggregation and specific activity loss of the denatured protein. Raghuvanshi et al[24] have also shown that sonication alone led to only a slight loss of immunoreactivity, whereas sonication along with emulsification with methylene chloride was very harmful to TT. The authors mostly ascribed this effect to the contributing deleterious effect of the water/methylene chloride interface, which provoked protein precipitation and thus concomitant antigenicity loss. However, emulsification of TT with either ethyl acetate or methylene chloride after 2-minutes of vortexing led to very limited negative effect (Figure 3), showing that emulsification alone cannot be held responsible for TT antigenicity loss. It should also be pointed out that, according to Tobio and colleagues,[22,25] TT inactivation is mostly due to the interaction of TT with PLGA and its acidic degradation products. In another study, Alonso et al,[21] who related protein aggregation with antigenicity reduction, proved that emulsification did not cause more than 14% aggregation of TT and that ethyl acetate was superior to methylene chloride in this respect, which is consistent with the results obtained in Figure 3, but contrasts with Raghuvanshi's work.[24] For the sake of comparison, TT antigenicity was totally destroyed when dissolved in a 0.1M sodium hydroxide solution (results not shown). Although complete alkaline digestion enables a precise protein content determination without underestimating the
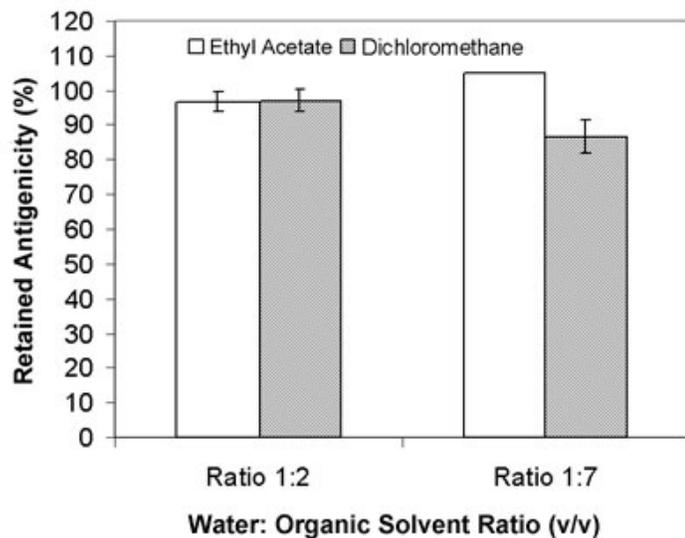
Figure 3. Effect of aqueous/organic solvent interfaces on residual TT antigenicity as determined by an Elisa assay (mean ± SD, n = 3).

TT recovered amount,[26] this method is not suitable for antigenicity determination, nor is acid digestion, which destroyed ~30% of TT antigenicity as reported elsewhere.[27] The alkaline digestion method was shown to be deleterious for diphtheria toxoid also.[28]

Lysozyme-loaded Nanoparticles

Lysozyme is a small, 129-residue protein (MW:14 300 Da). It is a water soluble protein, and in this respect lysozyme might suffer from drug leakage into the external aqueous phase during encapsulation by the w/o/w double emulsion method. Despite this known negative effect, lysozyme was quite well retained within nanoparticles when this method was used (batches LYS A1 and A3, Table 1; Figure 1). Nevertheless, in order to minimize even more drug leakage, it is possible to modify protein solubility by an ion-pairing technique.[4] In substance, the electrostatic interaction between a positively charged peptide or protein and an anionic detergent renders the drug more hydrophobic. Ion-pairing was shown to increase the solubility of proteins such as neurotensin, bradykinin, or insulin in various organic solvents.[3,29] Lysozyme was previously successfully ion-paired with sodium oleate, as reported by Yoo et al.[14] It was claimed that the resulting complex was more easily dissolved in polar organic solvents such as DMSO than free lysozyme and hence that increased loading could be achieved when formulated through a modified nanoprecipitation method. Moreover, it was shown that complexed lysozyme exhibited improved conformational stability in organic solvents and at elevated temperatures. It was postulated that the ion-paired oleate molecules were able to diminish protein

mobility and therefore to prevent lysozyme unfolding to some extent. It should be noted that preventing protein unfolding might also avoid the exposure of buried hydrophobic regions and therefore reduce nonspecific protein adsorption onto polymer surface, as discussed elsewhere.[30] In the present work, a lysozyme-oleate complex was used in a s/o/w method to study the effect on the protein final loading and compared with free lysozyme when processed via a w/o/w method. Indeed, it was thought that the hydrophobic complex should be less prone than free lysozyme to leak out into the external aqueous phase. The complex contained 87% ± 1% (wt/wt) of lysozyme (which is very close to the theoretical 89% value expected for a protein/surfactant ratio of 1:6) and high powder recovery after freeze-drying was achieved, reaching 83%. As shown in Table 1 (batch LYS A2), however, this strategy had no real influence on entrapment efficiency. Contrasting with the s/o/w suspension method, direct dissolution of ion-paired leuprolide in the organic phase by means of a cosolvent such as NMP enabled the entrapment efficiency to increase from 75% to 96%.[6]

Aside from the emulsion-based methods mentioned above, a modified nanoprecipitation technique was also used for lysozyme entrapment.[2] Among the solvents investigated, DMSO and methanol were reported as being well adapted for lysozyme dissolution,[1,31] but preliminary dissolution testing showed that only DMSO accurately dissolved this protein (results not shown). This discrepancy is generally ascribed to the way the commercially available protein was freeze-dried. Actually, the protein solubility in some polar organic solvents can be increased if the pH of the protein solution prior to freeze-drying is set at a value far from the protein isoelectric point. Since lysozyme was soluble in DMSO and insoluble in many alcohols, we attempted to entrap it by using DMSO as solvent (ie, the diffusing phase) and an alcohol as nonsolvent (ie, the dispersing phase). Most of the time, the entrapment efficiencies were better than when the nonsolvent was water (Table 2) despite lower nanoparticle yields. Similar loadings but different nanoparticle mean sizes were achieved with methanol, ethanol, and propanol as nonsolvents (batches LYS B2, B3, and B7; Figure 1). This effect of the nonsolvent on the mean size confirmed the results already observed in a previous study with blank nanoparticles.[2] As often observed with the double emulsion method employed for protein entrapment, the use of polymers with uncapped carboxylic groups favors high entrapment efficiencies (batches LYS B4 and B6 vs batches LYS B3 and B5), suggesting that ionic interaction between lysozyme and polymer plays an important role in the protein loading even in this anhydrous protocol. The PLGA copolymer was superior in terms of final loading to the more hydrophobic PLA homopolymer (batches LYS B4 vs LYS B6). In fact, batch LYS B4

Table 2. Protein-loaded Nanoparticles Produced by Nanoprecipitation*

Batch	Polymer[‡]	Nonsolvent	EE ± SD[§] (%)	EY ± SD (%)	NY ± SD (%)	Size ± SD (nm)	PI ± SD
LYS B1[†]	PLGA	Water	40.2 ± 1.7	36.4 ± 0.02	90.7 ± 1.5	279 ± 17	0.19 ± 0.08
LYS B2	PLGA	Methanol	47.4 ± 5.0	34.7 ± 4.6	72.7 ± 4.6	137 ± 3	0.21 ± 0.01
LYS B3	PLGA	Ethanol	56.0 ± 1.9	37.9 ± 2.6	67.4 ± 6.6	229 ± 3	0.13 ± 0.01
LYS B4	PLGA H	Ethanol	91.2 ± 4.4	52.9 ± 2.8	58.6 ± 5.7	202 ± 5	0.14 ± 0.01
LYS B5[†]	PLA	Ethanol	34.6 ± 5.3	16.8 ± 1.5	42.6 ± 5.0	351 ± 26	0.07 ± 0.01
LYS B6	PLA H	Ethanol	59.9 ± 13.8	30.0 ± 4.3	58.5 ± 8.6	237 ± 7	0.15 ± 0.02
LYS B7[†]	PLGA	Propanol	43.3 ± 4.9	27.2 ± 7.2	62.4 ± 13.0	567 ± 140	0.29 ± 0.29
INS B1[†]	PLGA	Water	22.8 ± 5.5	9.3 ± 3.1	40.0 ± 4.3	133 ± 8	0.14 ± 0.02
INS B2[†]	PLGA	Ethanol	13.5 ± 5.0	1.5 ± 4.0	11.5 ± 3.4	173 ± 10	0.11 ± 0.01
INS B3[†]	PLGA	Ethanol	< 2	< 1	29.2 ± 3.6	169 ± 19	0.14 ± 0.02
INS B4	PLA H	Ethanol	17.6 ± 3.2	9.7 ± 4.0	55.3 ± 10.3	302 ± 6	0.18 ± 0.04

* EE indicates entrapment efficiency; SD, standard deviation (n = 3); EY, entrapment yield; NY, nanoparticle yield; PI, mean polydispersity index is expressed using a 0 to 1 scale (n = 3); LYS, lysozyme; INS, insulin; All batches were produced at 2% of nominal drug loading.

[†] The Solvent used for nanoparticle production was DMSO, except for batch INS B3 (*N*-methylpyrrolidone). Batches LYS B1, LYS B5, INS B1, and INS B2 required 1% poloxamer 407 dissolved in the nonsolvent, whereas povidone K30 (2%) was necessary for batch LYS B7.

[‡] PLGA indicates poly(D,L-lactic-*co*-glycolic acid); PLGA H stands for PLGA copolymer containing uncapped carboxylic end groups; PLA for poly (D,L-lactic acid); and PLA H for PLA with uncapped carboxylic end groups. The polymers used were Resomer RG 503 (PLGA); Resomer R 203 (PLA); Resomer RG 503 H (PLGA H); and Medisorb PLA 100 DL 4A (PLA H).

[§] All values are ±SD (n = 3). Entrapped proteins were quantified by UV spectrophotometry.

displayed the best loading characteristics. Moreover, these nanoparticles are very small and show narrow size distribution. It should also be noted that some of the batches produced required no surfactants (except for batches LYS B1, B5, and LYS B7). In comparison, a different method, involving first lysozyme dissolution in a methylene chloride/ DMSO mixture containing also PLGA, then the emulsification of this solution into an aqueous phase, led to microspheres (~ 10 μm) with entrapment efficiencies increasing along with increasing DMSO proportion.[32]

Insulin-loaded Nanoparticles

Insulin is a small protein composed of 2 polypeptide chains linked by 2 disulfide bridges. The A-chain is made of 21 amino acids and the B-chain contains 30 amino acids. Insulin is only sparingly soluble in water and for it to be entrapped by the $w_1/o/w_2$ or by the nanoprecipitation method, it is mandatory to set the aqueous pH below the insulin isoelectric point (pI) (pI = 5.5), where the basic amino groups become protonated. An acidic environment is also required for ion-pairing to take place with all 6 basic functionalities of insulin being positively charged (at pH 2.5). These groups can thus fully interact with the anionic part of SDS at a protein/surfactant ratio of 1:6.[33] The obtained complex contained 82% ± 1% (wt/wt) of insulin (theoretical value = 78%), and powder recovery after freeze-drying reached 75%. In contrast to lysozyme, insulin was not efficiently entrapped in nanoparticles when ethyl acetate was used during the double emulsion procedure (batches INS A1 and A2, Table 1), whereas methylene chloride was able to improve protein loading

(batch INS A3). The duration of sonication had no effect on entrapment efficiency, as reported in a previous study concerning bovine serum albumin entrapment.[16] Entrapment efficiencies for insulin reported in other studies using emulsion-based methods vary from 14% to 97% depending on the manufacturing conditions. Generally, using methylene chloride, PLA, a small internal w_1 phase, or low initial drug loading leads to better results.[34-36] When insulin was entrapped in large microparticles (~200 μm), very high insulin entrapment efficiencies (near 100%) could be obtained.[37,38] Concerning the s/o/w method, free insulin was not interesting for augmenting the loading level (batches INS A4 and A5) and the more hydrophobic SDS-insulin ion-pair was inefficient too, despite an improved solubility in some solvents such as 1-octanol.[5]

By using nanoprecipitation as described by Yoo et al,[14] insulin entrapment efficiency was comparable to the level obtained with double emulsion and when using ethyl acetate as solvent (batch INS B1, Table 2). Classical nanoprecipitation (with acetone as diffusing solvent) was shown to produce nanoparticles containing approximately the same amount of insulin.[9] When small nanoparticles are required (and hence, when it is necessary to use an entrapment method such as nanoprecipitation), improving insulin loading still remains a difficult task.[39] Using ethanol, in which insulin is also insoluble, instead of water as dispersing medium did not give a satisfactory result (batch B2). With respect to the solvents, a trial with NMP in place of DMSO, resulted in a significant negative impact on protein loading (batch INS B3). However, a polymer with free carboxylic end groups (batch INS B4) enabled similar entrapment as

for batch INS B1. This last example illustrates again that ionic interactions might promote protein entrapment in nanoprecipitation, as observed above with lysozyme (batch LYS B6). In this respect, it is noteworthy that PLGA and PLA H exhibited in both cases (with lysozyme as well as with insulin) the same capacity of protein retention. As expected, the size of the resultant carriers is comparatively smaller and more monodisperse than when nanoparticles were obtained by double emulsion, confirming the results previously obtained with unloaded nanoparticles.[2]

Insulin chemical stability was also investigated. Insulin mainly suffers from 2 major degradation pathways.[40] In acid media (pH ~ 2–3), some amino acids undergo deamidation. Statistically, the last amino acid of the A-chain (asparagine A21) preferentially deamidates, leading to the so-called A21 desamido insulin. In less acid environments (pH ~ 4–5), CIDs are formed, this degradation pathway being favored with respect to deamidation.[41,42] Usually, the deamidated by-products are separated from insulin by RP-HPLC, whereas CID are detected and quantified by size exclusion HPLC.[43] In this work, A21 desamido insulin was analyzed with a classical RP-HPLC method, but a new MALDI-TOF MS method was used to check for CID in nanoparticles.[10] In fact, this method was shown to be an outstanding analytical tool that is able to provide accurate qualitative information along with precise drug quantification (Table 3), provided that an appropriate internal standard is added in the final mixture to counteract signal variability. Sampling is rapid, easy and is performed in rather mild conditions, which simply require dissolution of the carrier in an adapted medium. The amount of A21 desamido insulin measured in nanoparticles ranged between 1.1% and 2.1% and complies with data from

literature.[37] Only batch INS B4 contained a slightly higher amount of deamidated insulin (3.3 ± 1.0%). The small amounts of deamidated products and the detected traces of CID might be the result of the slow rate of by-product formation, as reported elsewhere.[37,40] As already mentioned, insulin needed to be dissolved in an acid solution during the w/o/w procedure, but the solution was used immediately and such working conditions were therefore not damaging enough to induce formation of high percentages of A21 desamido insulin. As a matter of fact, since even deamidated insulin retains more than 90% of its native potency, it is possible to claim that entrapped insulin within nanoparticles could be considered as fully biologically active, but only provided insulin does not suffer from fibrillation. Indeed, it should be mentioned that insulin is liable to be inactivated through physical degradation such as fibrillation. However, this aspect was not investigated here, since this study only focused on insulin chemical degradation.

CONCLUSIONS

The results obtained from these studies revealed that nanoprecipitation can sometimes be a good alternative to the classical and widely used double emulsion method. This is a valuable method for protein entrapment (especially for lysozyme) leading to small and highly loaded nanoparticles. In this connection, the use of solvents such as DMSO (instead of acetone or ethanol) and the use of nonsolvents such as methanol or ethanol (instead of water) certainly promote the formation of a narrow population of nanoparticles. It has also been demonstrated that MALDI-TOF mass spectrometry is an outstanding analytical method for both drug content determination and molecular stability study. The accuracy of the method matched that obtained with liquid chromatography and sampling did not require long and damaging steps.

Table 3. Entrapment Efficiencies of Insulin in Nanoparticles Obtained From UV Spectroscopy, RP-HPLC and MALDI-TOF MS*

Batch[†]	EE ± SD[‡](%)		
	UV Spectroscopy	RP-HPLC	MALDI-TOF MS
INS A1	24.1 ± 9.5	20.1 ± 4.1	25.9 ± 4.9
INS A2	25.0 ± 5.6	20.2 ± 4.5	23.2 ± 2.7
INS A3	78.7 ± 10.3	74.9 ± 6.2	72.5 ± 6.1
INS A4	73.1 ± 7.7	60.7 ± 8.6	65.4 ± 12.2
INS B2	13.5 ± 5.0	8.1 ± 2.7	11.0 ± 2.2
INS B3	< 2	< 1	nd
INS B4	17.6 ± 3.2	13.9 ± 1.9	13.0 ± 2.0

* RP-HPLC indicates reverse phase-high-performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; EE, entrapment efficiency; INS, insulin; and nd, not detected.
[†] Batches: A, obtained by an emulsion-based method; B, obtained by nanoprecipitation.
[‡] All values are ±SD ($n = 3$).

REFERENCES

1. Stevenson CL. Characterization of protein and peptide stability and solubility in non-aqueous solvents. *Curr Pharm Biotechnol.* 2000; 1:165–182.

2. Bilati U, Allémann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nano-particles. *Eur J Pharm Sci.* 2005;24:67–75.

3. Meyer JD, Manning MC. Hydrophobic ion pairing: altering the solubility properties of biomolecules. *Pharm Res.* 1998;15:188–193.

4. Quintanar-Guerrero D, Allemann E, Fessi H, Doelker E. Applications of the ion-pair concept to hydrophilic substances with special emphasis on peptides. *Pharm Res.* 1997;14:119–127.

5. Powers ME, Matsuura J, Brassel J, Manning MC, Shefter E. Enhanced solubility of proteins and peptides in nonpolar solvents through hydrophobic ion pairing. *Biopolymers.* 1993;33:927–932.

6. Choi SH, Park TG. Hydrophobic ion pair formation between leuprolide and sodium oleate for sustained release from biodegradable polymeric microspheres. *Int J Pharm.* 2000;203:193–202.

7. Quintanar-Guerrero D, Allémann E, Fessi H, Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. *Drug Dev Ind Pharm.* 1998;24:1113–1128.

8. Fessi H, Puisieux F, Devissaguet J-Ph, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. *Int J Pharm.* 1989;55:R1–R4.

9. Barichello JM, Morishita M, Takayama K, Nagai T. Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nano-precipitation method. *Drug Dev Ind Pharm.* 1999;25:471–476.

10. Bilati U, Pasquarello C, Corthals GL, Hochstrasser DF, Allémann E, Doelker E. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for quantitation and molecular stability assessment of insulin entrapped within PLGA nanoparticles. *J Pharm Sci.* 2005; 94:688–694.

11. Na DH, DeLuca PP, Lee KC. Direct determination of the peptide content in microspheres by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry. *Anal Chem.* 2004;76:2669–2673.

12. Jiang G, Qiu W, DeLuca PP. Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres. *Pharm Res.* 2003;20:452–459.

13. Capan Y, Jiang G, Giovagnoli S, Na KH, DeLuca PP. Preparation and Characterization of Poly(D,L-lactide-co-glycolide) Microspheres for Controlled Release of Human Growth Hormone. *AAPS PharmSciTech.* 2003;4:E28.

14. Yoo HS, Choi HK, Park TG. Protein-fatty acid complex for enhanced loading and stability within biodegradable nanoparticles. *J Pharm Sci.* 2001;90:194–201.

15. Bilati U, Allémann E, Doelker E. Sonication parameters for the preparation of biodegradable nanocapsules of controlled size by the double emulsion method. *Pharm Dev Technol.* 2003;8:1–9.

16. Bilati U, Allemann E, Doelker E. Protein-loaded nanoparticles prepared by the double emulsion method - processing and formulation issues for enhanced entrapment efficiency. *J Microencapsul.* 2005;22:205–214.

17. Jung T, Koneberg R, Hungerer KD, Kissel T. Tetanus toxoid microspheres consisting of biodegradable poly(lactide-co-glycolide)- and ABA-triblock-copolymers: immune response in mice. *Int J Pharm.* 2002;234:75–90.

18. Thomasin C, Corradin G, Men Y, Merkle HP, Gander B. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response. *J Control Release.* 1996;41:131–145.

19. Nicoli S, Santi P, Couvreur P, Couarraze G, Colombo P, Fattal E. Design of triptorelin loaded nanospheres for transdermal iontophoretic administration. *Int J Pharm.* 2001;214:31–35.

20. Men Y, Thomasin C, Merkle HP, Gander B, Corradin G. A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. *Vaccine.* 1995;13:683–689.

21. Alonso MJ, Gupta RK, Min C, Siber GR, Langer R. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine.* 1994;12:299–306.

22. Tobio M, Nolley J, Guo Y, McIver J, Alonso MJ. A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle. *Pharm Res.* 1999;16:682–688.

23. Kang F, Jiang G, Hinderliter A, DeLuca PP, Singh J. Lysozyme stability in primary emulsion for PLGA microsphere preparation: effect of recovery methods and stabilizing excipients. *Pharm Res.* 2002; 19:629–633.

24. Raghuvanshi RS, Goyal S, Singh O, Panda AK. Stabilization of dichloromethane-induced protein denaturation during microencapsula-tion. *Pharm Dev Technol.* 1998;3:269–276.

25. Tobio M, Alonso MJ. Study of the inactivation process of the tetanus toxoid in contact with poly(lactic/glycolic acid) degrading microspheres. *STP Pharma Sci.* 1998;8:303–310.

26. Gupta RK, Chang AC, Griffin P, Rivera R, Guo YY, Siber GR. Determination of protein loading in biodegradable polymer micro-spheres containing tetanus toxoid. *Vaccine.* 1997;15:672–678.

27. Chang AC, Gupta RK. Stabilization of tetanus toxoid in poly(DL-lactic-co-glycolic acid) microspheres for the controlled release of antigen. *J Pharm Sci.* 1996;85:129–132.

28. Johansen P, Tamber H, Merkle HP, Gander B. Diphtheria and tetanus toxoid microencapsulation into conventional and end-group alkylated PLA/PLGAs. *Eur J Pharm Biopharm.* 1999; 47:193–201.

29. Falk R, Randolph TW, Meyer JD, Kelly RM, Manning MC. Controlled release of ionic compounds from poly(L-lactide) micro-spheres produced by precipitation with a compressed antisolvent. *J Control Release.* 1997;44:77–85.

30. Jiang G, Woo BH, Kang F, Singh J, DeLuca PP. Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly (D,L-lactide-co-glycolide) microspheres. *J Control Release.* 2002;79:137–154.

31. Chin JT, Wheeler SL, Klibanov AM. On protein solubility in organic solvents. *Biotechnol Bioeng.* 1994;44:140–145.

32. Park TG, Lee HY, Nam YS. A new preparation method for protein loaded poly(D,L-lactic-*co*-glycolic acid) microspheres and protein release mechanism study. *J Control Release.* 1998;55:181–191.

33. Matsuura J, Powers ME, Manning MC, Shefter E. Structure and stability of insulin dissolved in 1-octanol. *J Am Chem Soc.* 1993; 115:1261–1264.

34. De Rosa G, Iommelli R, La Rotonda MI, Miro A, Quaglia F. Influence of the co-encapsulation of different non-ionic surfactants on the properties of PLGA insulin-loaded microspheres. *J Control Release.* 2000;69:283–295.

35. Yeh MK. The stability of insulin in biodegradable microparticles based on blends of lactide polymers and polyethylene glycol. *J Microencapsul.* 2000;17:743–756.

36. Uchida T, Nagareya N, Sakakibara S, et al. Preparation and characterization of polylactic acid microspheres containing bovine insulin by a w/o/w emulsion solvent evaporation method. *Chem Pharm Bull (Tokyo).* 1997;45:1539–1543.

37. Shao PG, Bailey LC. Porcine insulin biodegradable polyester microspheres: stability and in vitro release characteristics. *Pharm Dev Technol.* 2000;5:1–9.

38. Uchida T, Yagi A, Oda Y, Nakada Y, Goto S. Instability of bovine insulin in poly(lactide-co-glycolide) (PLGA) microspheres. *Chem Pharm Bull (Tokyo).* 1996;44:235–236.

39. Kawashima Y, Yamamoto H, Takeuchi H, Fujioka S, Hino T. Pulmonary delivery of insulin with nebulized DL-lactide/glycolide copolymer (PLGA) nanospheres to prolong hypoglycemic effect. *J Control Release.* 1999;62:279–287.

40. Brange J, Langkjaer L. Insulin structure and stability. In: Wang JY, Pearlman R, eds. *Stability and Characterization of Protein and Peptide Drugs.* New York, NY: Plenum Press; 1993:315–350.

41. Darrington RT, Anderson BD. Effects of insulin concentration and self-association on the partitioning of its A-21 cyclic anhydride intermediate to desamido insulin and covalent dimer. *Pharm Res.* 1995;12:1077–1084.

42. Darrington RT, Anderson BD. Evidence for a common intermediate in insulin deamidation and covalent dimer formation: effects of pH and aniline trapping in dilute acidic solutions. *J Pharm Sci.* 1995; 84:275–282.

43. Hvass A, Hach M, Jars MU. Complementary analytical HPLC methods for insulin-related degradation products. *Am Biotechnol Lab.* 2003;21:8–10.