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Research paper

Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres

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Abstract

Proteins were precipitated to ensure their stability upon subsequent encapsulation within PLGA microspheres. Spherical, nanosized protein particles were formed by the addition of a salt (sodium chloride) and a water-miscible organic solvent (glycofurol) to protein solutions. Various process parameters were modified to optimize the precipitation efficiency of four model proteins: lysozyme, α-chymotrypsin, peroxidase and β-galactosidase. As monitored by enzymatic activity measurement of the rehydrated particles, conditions to obtain more than 95% of reversible precipitates were defined for each protein. The study of the structure of the rehydrated particles by absorbance spectroscopy, fluorescence spectroscopy and circular dichroism showed an absence of structural-perturbation after precipitation. Protein particles were then microencapsulated within PLGA microspheres using s/o/w technique. The average encapsulation yield was around 80% and no loss of protein activity occurred after the encapsulation step. Additionally, a lysozyme *in vitro* release study showed that all of the released lysozyme was biologically active. This method of protein precipitation is appropriate for the encapsulation in PLGA microspheres of various proteins without inactivation.

Keywords: Protein precipitation; Protein stability; Microspheres; Poly(lactic-co-glycolic acid) (PLGA); Solid-in-oil-in-water (s/o/w) encapsulation procedure

1. Introduction

Although the production of various proteins has become possible with the recent advances in biotechnology, their use for therapeutic purposes has been limited due to physical and chemical instability. Due to relatively high enzymatic susceptibility and short half-life, much attention has been paid to their delivery from systems controlling local release [1]. Numerous studies have reported their delivery from injectable, biodegradable microspheres made of poly(D,L-lactide-co-glycolide) (PLGA) [2–5]. Among the techniques used for the preparation of PLGA microspheres loaded with protein, the water-in-oil-in-water (w/o/w) encapsulation procedure has been the most often employed. In the first step of this formulation process, a protein solution is emulsified in an organic phase containing the polymer. This can result in critical protein stability problems. Indeed, the protein dissolved in the aqueous

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phase can aggregate at the water/organic solvent interface, adsorb to the hydrophobic polymer and unfold because of the shear stress used for the formation of the emulsion. Many studies have suggested formulation optimization in order to improve protein stability during classical w/o/w procedure [2,3,6–10]; even so, the complete stabilization of any protein following this type of processing remains a formidable challenge. Therefore, a different encapsulation approach without this first emulsification step was explored and the dispersion of a solid-state protein in the organic phase was considered. Moreover, in the absence of water, protein conformational mobility is reduced, so that the stability of proteins in contact with the organic solvent is enhanced. Consequently, the encapsulation methods involving no protein/water contact may be relevant: i.e. the solid-in-oil-in-water (s/o/w) emulsion-evaporation method [11–14], phase separation [15,16] or spray-drying [17].

As traditional drying processes such as freezing or spray-drying potentially cause harmful stress to proteins [12,18–21], the aim of the study was to develop a non-denaturing method to prepare protein particles. For subsequent encapsulation in PLGA microspheres, a mean diameter of the solid particles much lower than the desired mean microsphere size is required; a particle/microsphere size ratio of 1/10 or less is appropriate [11]. Since the microspheres are typically 15–100 μm in diameter, the protein particle size has to be in the order of a few micrometers or less for good dispersion. The method developed should also be suitable for any protein and easy to adapt. To meet all these requirements, protein precipitates were formed from aqueous solution by the addition of a water-miscible organic solvent. Glycofurol was chosen because it is particularly advantageous for protein encapsulation in PLGA microspheres. Indeed, glycofurol is a non-toxic solvent of PLGA and could be used for microsphere formulation [22,23]. In this study, glycofurol was only used to precipitate proteins and more current solvents were employed for the formulation of the PLGA microspheres to first assess the behaviour of a solid-state protein. As the production of recombinant proteins by genetic engineering is costly, a process without protein loss is needed. To increase the efficiency of the protein precipitation, sodium chloride was used in conjunction with glycofurol. Four model proteins differing by their physical and chemical properties were precipitated: lysozyme, α-chymotrypsin, peroxidase and β-galactosidase. Enzymes were chosen in order to easily evaluate their biological activity during the process. The optimum conditions to obtain reversible particles of these proteins were determined. The protein particles obtained were subsequently encapsulated in PLGA microspheres using an s/o/w technique to control their stability upon encapsulation. The *in vitro* release profile was studied for lysozyme whose physical and chemical properties are similar to those of growth factors such as NGF (nerve growth factor) or GDNF (glial cell line-derived neurotrophic factor). These growth factors have great potential in tissue-engineering for cell survival and differentiation and their encapsulation in PLGA microspheres is attractive [3,6].

2. Materials and methods

2.1. Materials

Proteins: lysozyme (chicken egg-white), α-chymotrypsin (bovine pancreas), peroxidase (horseradish) and β-galactosidase (*Aspergillus oryzae*) and their respective substrates, i.e.: *Micrococcus lysodeikticus*, *N*-benzoyl-L-tyrosine ethyl ester (BTEE), pyrogallol and *o*-nitrophenyl β-D-galactopyranoside (ONPG), were all purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Glycofurol (tetraglycol or α-[(tetrahydro-2-furanyl) methyl]-ω-hydroxy-poly (oxy-1, 2-ethanediyl) and buffer compounds were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Capped 75/25 PLGA, provided by Phusis (Saint-Ismier, France), had a mean molecular weight of 27,000 Da (Polydispersity index, $I = 1.9$) as determined by size-exclusion chromatography (standard: polystyrene). Polyvinyl alcohol (Mowiol® 4-88) was from Kuraray Specialities Europe (Frankfurt, Germany) and micro-BCA protein assay reagent from Pierce (Bezons, France).

2.2. Protein precipitation

2.2.1. Precipitation and isolation of the protein

Protein, glycofurol, and sodium chloride were used without further purification. Experiments were carried out at room temperature. The overall mixture was prepared directly inside a centrifugation tube as follows. The protein powder was first dissolved in a non-buffered aqueous solution of sodium chloride and this solution was introduced into glycofurol. Thirty-minutes later, the protein particles were recovered by centrifugation (10,000*g*, 30 min). Mixing and centrifugation times of 30 min were selected in order to optimize the quantity of precipitated protein.

2.2.2. Experimental design

To define the optimum conditions of precipitation, an experimental design was used. Three parameters influencing protein precipitation were modified: the ionic strength, the ratio between the volumes of aqueous phase and of glycofurol, and the mass of protein. To study these three variables, a Doehlert matrix was chosen because it represents an optimal design for the spherical domain defined by the factors and it allows a sequential approach [24]. The experimental domain is described as follows:

- ionic strength of the aqueous phase (U_1): 0.01–0.59 M (5 levels),
- volume of the aqueous phase (U_2): 25–155 μl (7 levels),
- protein quantity (U_3): 0.1–0.9 mg (3 levels).

The volume of glycofurol was the complement for 1 ml of suspension.

Experiments required for this design are described in Fig. 1a. It consists of a set of 13 distinct experiments uniformly distributed in a cubo-octahedron (Fig. 1b). The central point of the experimental domain, represented by experiment 13, was repeated three times to perform a statistical analysis (experiments 14 and 15).

The measured responses were the precipitation efficiencies of four proteins (Table 1):

- lysozyme (Y_1),
- α-chymotrypsin (Y_2),
- peroxidase (Y_3),
- β-galactosidase (Y_4).

The same experiments were performed for each protein; this allowed a direct comparison of responses.

NEMROD® W software (2000, LPRAI, Marseille) was used for the generation and exploitation of the statistical experimental design.

The precipitation efficiency (Y_1, Y_2, Y_3, Y_4) was considered as the percentage of active protein recovered after precipitation and rehydration. The reference was the initial activity, i.e. the activity measured for the initial protein mass whose activity was 100%. The protein suspensions in glycofurol were centrifuged, the supernatant eliminated and the pellet of protein particles rehydrated. Lysozyme was rehydrated in Tris–HCl 0.01 M buffer, pH 7.4, α-chymotrypsin in Tris–HCl 0.08 M buffer, pH 7.8, peroxidase in potassium phosphate buffer, pH 6.0 and β-galactosidase in water. The biological activity of lysozyme was determined by measuring the turbidity change in a *M. lysodeikticus* bacterial cell suspension as reported by Aubert-Pouëssel et al. [25]. The enzymatic activity of α-chymotrypsin was evaluated with *N*-benzoyl-L-tyrosine ethyl ester as substrate [26]. The enzymatic activity of peroxidase was determined with pyrogallol as substrate [27].

Table 1
Molecular weight and isoelectric point of the studied proteins

	Molecular weight (Da)	Isoelectric point
Lysozyme	14,300	11.0
α-Chymotrypsin	25,000	9.1
Peroxidase	40,000	7.2
β-Galactosidase	105,000	4.7

The ability of β-galactosidase to hydrolyse *o*-nitrophenyl β-D-galactopyranoside was also determined [28].

Each set of experiment was repeated two times (for lysozyme, peroxidase and β-galactosidase) or three times (for α-chymotrypsin) to evaluate the repeatability of the measurements. The mean response for each experiment is reported in Table 2.

2.2.3. Characterization of the protein particles

2.2.3.1. Morphology and size. The particle size of each protein suspended in glycofurol was determined by light diffraction (Mastersizer® 2000, Malvern Instruments, Worcestershire, UK). Their morphology was investigated using scanning electron microscopy (SEM) (JSM 6310F, JEOL, Paris, France). Particles were mounted on glass slides fixed on metal stubs and coated with Au film (1–5 nm) using a MED 020 (Bal-Tec, Balzers, Lichtenstein).

2.2.3.2. Structure. Three different spectroscopic techniques were used to detect possible changes in the structure of the proteins after precipitation:

UV-spectroscopy in the fourth derivative mode. Baseline-corrected absorbance spectra in the range of 250–305 nm were recorded at 37 °C with a Cary 3E spectrophotometer (Varian Inc.) characterized by a high spectral reproducibility (SD ± 0.02 nm). Data acquisition was in steps of 0.1 nm with an acquisition time of 1 s per data point. The fourth

a

N°Exp	Ionic strength U_1 (M)	Aqueous phase volume U_2 (µl)	Protein quantity U_3 (mg)
1	0.59	90.0	0.50
2	0.01	90.0	0.50
3	0.44	155.0	0.50
4	0.16	25.0	0.50
5	0.44	25.0	0.50
6	0.16	155.0	0.50
7	0.44	111.7	0.90
8	0.16	68.3	0.10
9	0.44	68.3	0.10
10	0.30	133.4	0.10
11	0.16	111.7	0.90
12	0.30	46.6	0.90
13	0.30	90.0	0.50
14	0.30	90.0	0.50
15	0.30	90.0	0.50

b

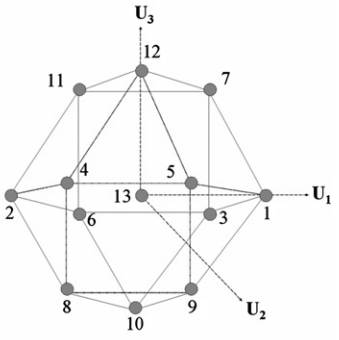

Fig. 1. Optimization of the protein precipitation: (a) experimental design and (b) its spatial representation. Table (a) represents the values of each studied factor (U_1, U_2, U_3) for the fifteen experiments. These values vary in a defined experimental domain which is illustrated in (b). In the 3D view of the experimental domain, the directions of variation of the factor values are represented by arrows and the experiments of the table by dots.

Table 2
Experimental design results: percentage of protein refolded into a biologically active form (Y), for each experiment and each protein

No. Exp	Solid active lysozyme Y_1 (%)	Solid active α-chymotrypsin Y_2 (%)	Solid active peroxidase Y_3 (%)	Solid active β-galactosidase Y_4 (%)
1	39	46	29	110
2	0	10	102	100
3	38	13	32	110
4	104	75	63	90
5	99	71	44	111
6	66	6	52	99
7	94	19	32	109
8	23	99	51	109
9	25	16	51	109
10	7	47	29	101
11	94	22	79	104
12	102	57	48	108
13	80	41	38	91
14	87	44	35	95
15	88	59	36	100
	$n = 2$ $S^2 = 88.2$ 17 df	$n = 3$ $S^2 = 169.5$ 30 df	$n = 2$ $S^2 = 25.3$ 17 df	$n = 2$ $S^2 = 71.9$ 17 df

derivatives of the UV spectra were calculated with the optimized spectral shift method as described previously [29] with an automated transformation program designed in the laboratory. Briefly, this technique improves the low resolution of zero-order spectra and provides information about structural changes of proteins in the local environment of tyrosine and tryptophan residues [29].

Fluorescence spectroscopy. Spectra were recorded on a spectrofluorimeter (Fluoromax 23 Jobin-Yvon-Spex) at 25 °C. Each spectrum was an average of three measurements (from 310 to 400 nm) with a resolution of 1 nm. The chosen wavelength for excitation was 297 nm. Slits of 4 nm were used for excitation and emission.

Circular dichroism spectroscopy. Spectra were recorded on JASCO 810 at 25 °C. Triplicate scans were obtained for each formulation (from 260 to 310 nm) with a resolution of 2 nm and correction for background dichroism was made by subtracting the CD-spectra recorded for the respective vehicle solutions.

2.3. Encapsulation of the solid-state protein

2.3.1. Microsphere preparation

Protein-loaded PLGA microspheres were prepared using s/o/w emulsion solvent extraction-evaporation process adapted from Pean et al. [2]. Briefly, 0.9 mg of protein particles (0.6% w/w with respect to the amount of PLGA) were prepared in the optimum conditions of precipitation and collected as described above. They were then carefully dispersed in an organic solution (2 ml; 3:1 methylene chloride:acetone) containing 150 mg of PLGA. The resulting organic suspension was then emulsified in a poly(vinyl alcohol) aqueous solution (30 ml, 6% w/v) maintained at 1 °C and mechanically stirred at 550 rpm for 1 min (Heidolph RZR 2041, Merck Eurolab, Paris, France). After the addition of 100 ml of deionized water and stirring for 10 min, the resulting s/o/w emulsion was added to deionized water (500 ml) and stirred for a further 20 min to extract the organic solvent. Finally, the formed microparticles were filtered on a 0.45 μm filter (HVLP type, Millipore SA, Guyancourt, France), washed five times with 100 ml of deionized water and freeze-dried. The average volume diameter and the size distribution of the resulting microspheres were evaluated using a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Roissy CDG, France).

2.3.2. Protein encapsulation efficiency

2.3.2.1. Total protein. The total protein content (active + inactive) in the microspheres was measured by micro-BCA protein assay [30]. Briefly, protein-loaded microspheres (10 mg) were dissolved in 1 ml of acetone (3 batches, 2 experiments per batch). After 1 h, they were centrifuged (10,000*g*, 15 min) and the organic phase was removed. The precipitates were then dispersed with freshly added acetone to eliminate the excess PLGA. Rinsing was carried out three times. After evaporation for 2 h, the collected protein fraction was dissolved in water for protein content measurement using mBCA reagent.

For lysozyme, the percentage of entrapped lysozyme was evaluated by encapsulating ^{125}I-labelled lysozyme and by counting the radioactivity within the microspheres (3 batches). Lysozyme was labelled by a chemical method using a fixed oxidant: Iodogen® (1, 3, 4, 6-tetrachloro-3α-6α-diphenylglycouril). This method was described by Aubert-Pouëssel et al. [25].

2.3.2.2. Active protein. Protein encapsulation yield was also determined considering the biologically active entrapped protein. For peroxidase and β-galactosidase, the microparticles (10 mg, 2 batches) were dissolved in acetone (see above) and the collected protein was dissolved in an appropriate buffer to determine its enzymatic activity in the presence of its substrate.

For lysozyme, another technique to extract the protein was used as the previous one may inactivate lysozyme. Lysozyme PLGA microspheres (10 mg, 3 batches) were dissolved in 0.9 ml DMSO in silanized glass tubes. After 1 h, 3 ml of 0.01 M HCl was added. The solution was left to stand for one more hour, and then incubated with *M. lysodeikticus* suspension for lysozyme activity determination.

For α-chymotrypsin, neither of these methods to quantify the entrapped active protein could be used because it was inactivated by acetone and DMSO.

2.4. In vitro release profile of lysozyme from microspheres

The *in vitro* release profile of lysozyme from PLGA microspheres was determined using a continuous-flow system previously developed in our laboratory [25]. Microspheres (10 mg, $n = 2$) were placed in an HPLC tube fitted with 0.5 μm frits and connected with 1.6 mm o.d.

HPLC tubing at each end. Eluent (Tris–HCl 0.01 M buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) was supplied to the column inlet at 5 μl min^{-1} by syringe pump. Heating of the chamber at 37 °C was achieved by the immersion of the column assembly in a water bath. Tubing from the column outlet led into a refrigerated chamber (4 °C), allowing the collection of samples without protein degradation. The percentage of released ^{125}I-lysozyme was determined by measuring radioactivity and the percentage of released biologically active lysozyme was measured by enzymatic assay.

3. Results

3.1. Protein precipitation

3.1.1. Formation of fine protein particles and their characterization

An excess of water-miscible organic solvent, glycofurol, and sodium chloride was conjunctively added to protein solutions to induce the formation of fine protein particles. By centrifugation of the homogeneous protein suspension, collection of the protein particles was possible. This easy process presented the advantage of being reversible as the precipitates could be dissolved. Four model proteins (lysozyme, α-chymotrypsin, peroxidase, β-galactosidase) were precipitated as described. As no effect of temperature (between 4 °C and room temperature) on β-galactosidase precipitation efficiency was observed, the experiments were carried out at room temperature. Fig. 2 shows the morphology and the size distribution of the four types of protein particles. For all proteins studied, nano-scale spheroids (around 150 nm) were obtained. Some of these particles were agglomerated in clusters (around 1 μm).

3.1.2. Optimization of protein precipitation efficiency

As the efficiency and reversibility of precipitation vary with the process conditions, the composition of the medium was varied in a defined experimental domain represented by a Doehlert matrix, at room temperature. Precipitation optima for four model proteins were investigated. They corresponded to the recovery of more than 95% of the initial protein mass in its biologically active state. The experimental results are reported in Table 2. The Doehlert matrix allows the use of a mathematical model to predict the response within a studied experimental domain. In this case, the coefficients of the model are estimated from the experimental results by the least square regression method. In the present study, one or more precipitation optima were determined for the four proteins but the response within the studied experimental domain could not be predicted. For β-galactosidase, the precipitation efficiency was above 90% for all the experiments. This high activity value all over the experimental domain caused the low variation of the experimental results; the regression was not significant and the validation of a proper model was not possible. For the other proteins, the investigated zone was too large to propose a validated model and predict the responses in the whole experimental domain. Therefore, the exploitation of the data was limited to the experimental results which exhibited, nevertheless, a good response in the domain investigated for each of the four proteins as the four tested proteins were successfully precipitated with the technique developed and recovered in a biologically active form (Fig. 3). For instance, reversible precipitates of peroxidase were obtained for experiment number 2. The conditions of this experiment correspond to the addition of 90 μl of an aqueous solution containing 500 μg peroxidase and 10 mM sodium chloride to 910 μl of glycofurol.

It was thus demonstrated that different proteins could be precipitated without inactivation; even so they behave differently. Indeed, their optima were located in different zones of the experimental domain. The easier precipitation of β-galactosidase could be attributed to its higher molecular weight which could justify its preferential hydration and high sensitivity to the modification of the dielectric constant and ionic strength. Nevertheless, many other structural factors influence protein solubility and the combined effect of structural flexibility, amphipathic structure, hydrophobicity, charge density and secondary structure of proteins has also to be considered.

It is well known that the salt electrostatic screening favours protein precipitation in the presence of organic sol-

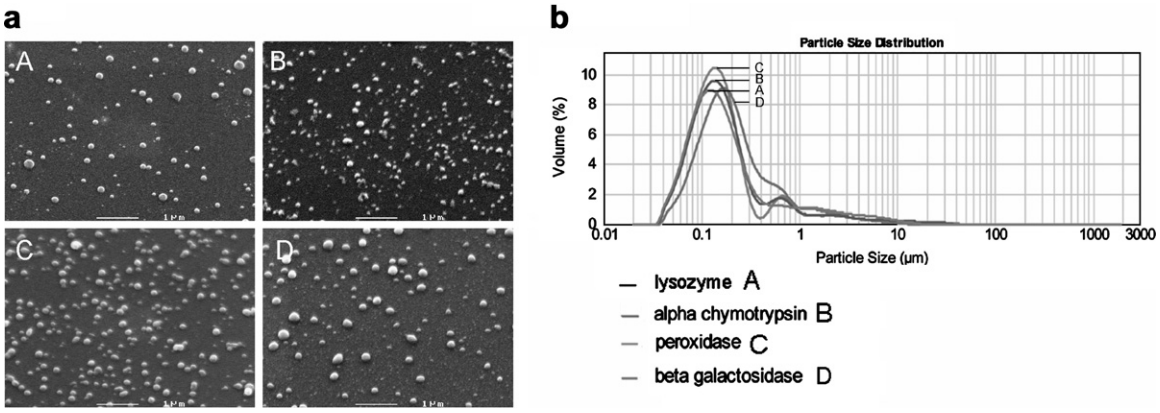

Fig. 2. Characterization of the four protein particles: morphology analysed by SEM (a) and size distribution determined by light diffraction (b).

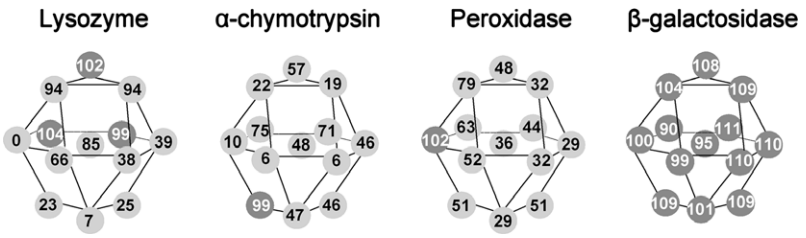

Fig. 3. Optimization of the protein precipitation: spatial representation of the experimental design results. Numbers in the circles refer to the percentage of protein recovered under a biologically active form, after precipitation and dissolution, for each experiment.

vent. Thus, for a fixed amount of lysozyme and aqueous phase volume, precipitation efficiency of lysozyme and α-chymotrypsin increased with sodium chloride concentration (from 10 to 590 mM) (experiments number 1 and 2 of the experimental design). On the contrary, the precipitation efficiency of peroxidase under the same conditions decreased with increasing sodium chloride concentration. Despite the use of a low chaotropic salt, peroxidase unfolding with the increase of the ionic strength is hypothesized.

The amount of protein and the volume of aqueous phase (for a fixed total volume in the system) also greatly influence protein precipitation and should be taken into consideration as well. Combined analysis of these two parameters, i.e. protein concentration, underlines that lysozyme precipitation optima correspond to higher protein concentrations (>5.55 mg/ml) (experiments number 4, 5, 7, 11, and 12) but this observation is not transposable to the other studied proteins, even though a higher protein concentration will favour phase separation. Concerning the glycofurol concentration in the water–glycofurol–salt mixtures, the experimental results confirm that glycofurol in excess is preferential (above 80%w/w). This correlates with a reduction of the dielectric constant when increasing the organic solvent concentration.

3.1.3. Integrity of the protein particles after dissolution

To know whether the protein precipitates did refold in their native conformation, several assays were performed. The structure of the proteins after precipitation (and dissolution of the pellet in an appropriate solvent) was compared to that of the native protein using spectroscopic methods (Fig. 4). After protein precipitation, the fourth derivative UV–visible spectra did not show any shift of maxima in the area corresponding to tyrosine and tryptophan residues (284 and 290 nm, respectively) (Fig. 4A). With fluorescence spectroscopy, spectra did not differ either before or after precipitation (and rehydration) for lysozyme, α-chymotrypsin and β-galactosidase (Fig. 4B-a, b, d). These results were confirmed using near-UV CD spectroscopy, indicating that the micro-environment of tyrosine and tryptophan residues of these proteins were maintained (spectra not shown). For peroxidase (Fig. 4B-c), a reduced level of fluorescence intensity was observed, but no change in peak maximum. This suggests that the precipitation caused a quenching of the fluorescence, rather than changes in the secondary or tertiary structures.

3.2. Encapsulation of the solid-state protein

The obtained protein particles were suspended in the organic phase containing the polymer and encapsulated in a solid-state within PLGA microspheres. Whatever the encapsulated protein, the microspheres were smooth and spherical, the mean particle size was 27 ± 11 μm (volume weighted) (Fig. 5), and the mean encapsulation efficiency was around 80% for the four proteins as determined by micro-BCA protein assay (Table 3). This result was confirmed by entrapped ^{125}I-lysozyme count, which does not require a previous extraction step of the protein from the microspheres. Furthermore, to detect any loss in biological activity during the encapsulation process, the amount of active protein extracted from microspheres was determined for lysozyme, peroxidase and β-galactosidase. For α-chymotrypsin, this value could not be quantified because of interferences. For these three proteins, the relative ratio (in percent) between active entrapped protein and total entrapped protein was determined: *ca.* 95% of the effectively encapsulated proteins was active without any stabilizer. It seems that protein precipitation using glycofurol and sodium chloride efficiently preserves protein activity during the microsphere preparation process without requiring the use of any additives.

3.3. In vitro release study of lysozyme from microspheres

Microspheres containing lysozyme particles prepared in optimum conditions were incubated *in vitro*. The state of released lysozyme was assessed and the release profile was determined. The encapsulation of radio-labelled lysozyme combined with a biological activity assay allowed us to confirm that all the released protein was biologically active (Fig. 6). The release profile was characterized by a marked initial protein release, with 10% of the protein released over the first 24 h, followed by low, continuous release.

4. Discussion

Since a solid-state protein exhibits restricted conformational flexibility, non-aqueous encapsulation approaches

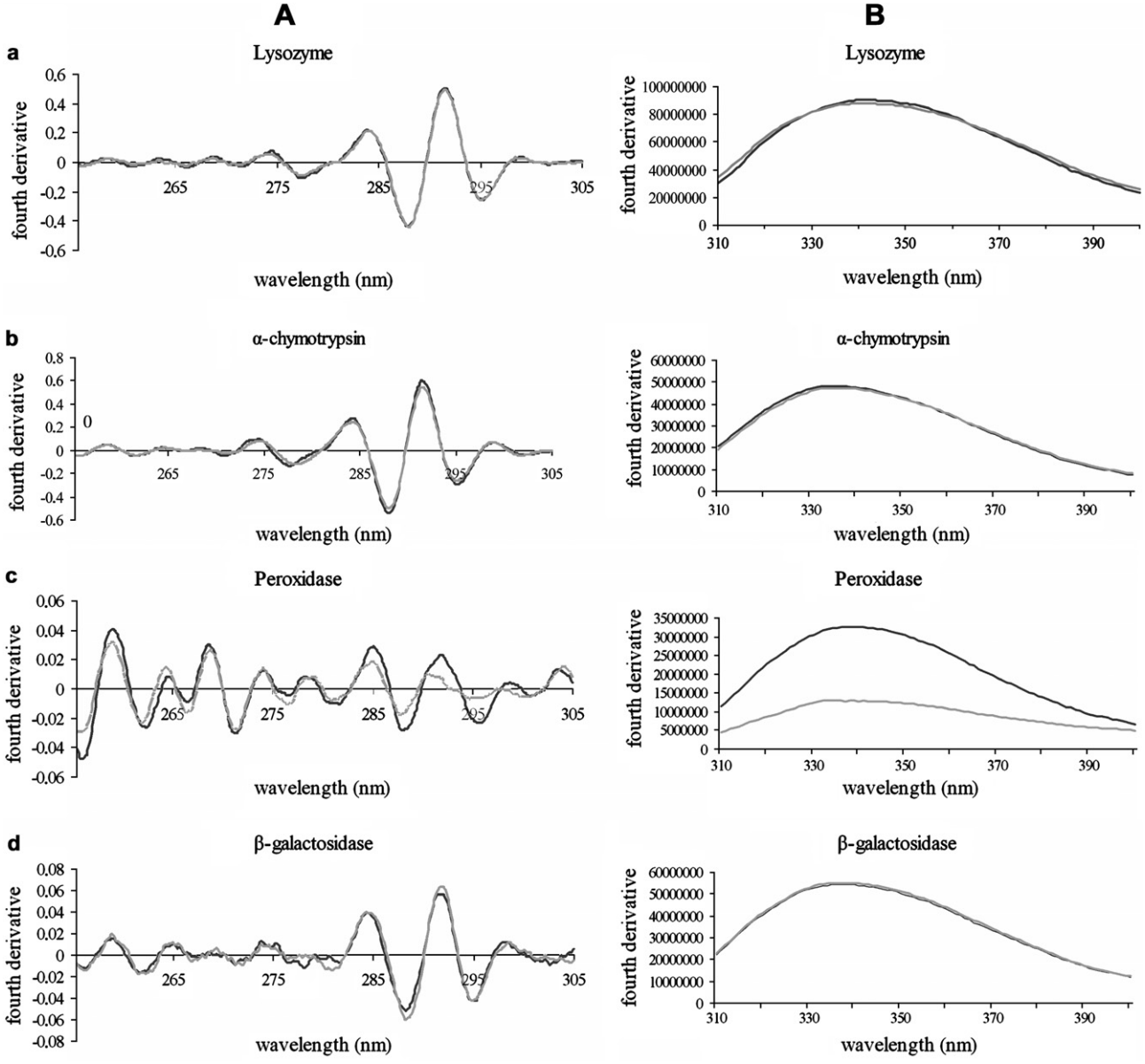

Fig. 4. Results of spectroscopic studies before (black curve) and after protein precipitation (grey curve). Fourth derivative UV (A) and fluorescence (B) spectra are represented for lysozyme (a), α-chymotrypsin (b), peroxidase (c) and β-galactosidase (d).

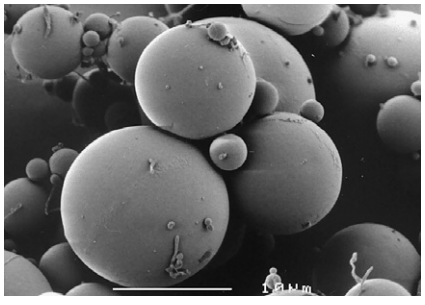

Fig. 5. Lysozyme-loaded microspheres analysed by SEM.

Table 3
Protein encapsulation efficiencies

	Total[A] (%)	Active (%)	Active/total (%)
Lysozyme	$72 \pm 5^{(a)}$ $72 \pm 7^{(b)}$	71 ± 4	99
α-Chymotrypsin	$94 \pm 3^{(a)}$	Interferences	–
Peroxidase	$89 \pm 7^{(a)}$	82 ± 5	92
β-Galactosidase	$84 \pm 6^{(a)}$	78 ± 1	93

[A] As determined by mBCA (a) or radioactivity count (b).

have emerged to ensure protein stability upon encapsulation within biodegradable polyester microspheres [7]. Various methods like spray-drying or spray-freeze drying have been reported for the preparation of small protein particles. Although these methods can generate protein particles, they present some drawbacks for microencapsulation: they are technically complex; they lead to low protein recovery; and they may denature proteins [19,20].

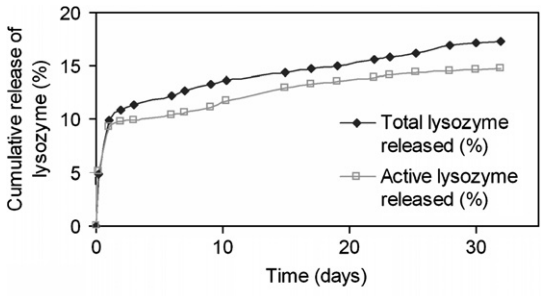

Fig. 6. *In vitro* release profile of lysozyme from PLGA microspheres.

Freeze-drying has often been used to obtain protein particles without protein loss but it induces the formation of large particles. To obtain fine particles and preserve protein integrity, proteins have been freeze-dried with poly(ethylene glycol) (PEG) which induces a two-phase separation [31,32]. This interesting approach can be applied to various proteins and has been used for protein microencapsulation by s/o/w [13,33] and s/o/o techniques [34]. However, the remaining amount of PEG in the freeze-dried protein product leads to an important initial burst (20% in 1 h) upon release from PLGA microspheres, and so an adaptation of the process was necessary [13,35].

In order to obtain protein particles without these disadvantages, a chemical rather than a mechanical processing was employed in this study. From an aqueous solution, protein precipitation can be induced via isoelectric precipitation, the reduction of the dielectric constant by the addition of water-miscible organic solvents, the reduction of the protein charge by changing the pH, and the addition of polymers or salts. The formulation of the protein at its isoelectric point for its encapsulation in PLGA microspheres has been previously studied with limited success on protein stability during encapsulation [36]. Moreover, since this approach favors protein dissolution in organic solvents by a charge neutralization effect, protein molecules may be more sensitive to the deleterious environment. The addition of polymers such as PEG was not retained either because the residual amount can enhance the burst effect. Salt-induced precipitation is an extensively used method in biotechnology for separating target proteins from multi-component protein solutions as the first purification step [37–39]. However, the increase of ionic strength required to induce salting-out is often limited by salt solubility or protein stability issues [40,41]. Thus, when the protein has become unfolded by salt-induced precipitation, it may either remain incorrectly folded or form insoluble aggregates upon redissolution. In this study, an organic solvent was employed by preference to induce the formation of fine protein particles. Rather than using ethanol, whose detrimental effect on protein integrity is well known [42], nanosized protein particles were formed by the addition of excessive glycofurol and sodium chloride to aqueous solutions of globular proteins. By this precipitation process driven by hydrophobic interactions, spherical particles of uniform size were formed. The similarity of size and morphology of the prepared protein particles is particularly advantageous for the preparation of protein formulations meeting pharmaceutical criteria of controlled size and good dispersion capacity. Glycofurol was chosen as the precipitant as it possesses interesting properties. Firstly, it is a non-toxic PLGA solvent which could be used to prepare PLGA microspheres [23]; moreover, it is a protic solvent containing hydrogen attached to oxygen so that it is able to form hydrogen bonds or to donate a proton such as the stabilizing PEG. The presence of glycofurol induces a liquid–liquid phase separation resulting in a protein-rich phase and a protein-poor phase. The addition of salt was indispensable to collect the maximum amount of protein precipitates. Sodium chloride reduced the electrostatic repulsive interactions between the charged proteins, promoting attractive hydrophobic interactions. The selection of sodium chloride was related both to its possible use in parenteral pharmaceutical formulations and to its intermediate location in the lyotropic series [43]. Sodium chloride decreases protein solubility very little with minimal denaturant effects.

For therapeutic purposes, the precipitation conditions need also to be very efficient and non-denaturing. Therefore, an optimization of the precipitation efficiency was designed by varying the ionic strength of the aqueous protein solution, its volume and the amount of protein present. As the protein is only efficient if it is active, the amount of the active fraction recovered after precipitation and dissolution was quantified and the percentage of reversible precipitates was calculated. Four physically and chemically different enzymes were submitted to various precipitation conditions. For each one, optimum conditions (efficiency of 95% or more) were successfully determined (Fig. 3). The structural integrity of the rehydrated protein particles was studied and showed that, under selected optimal conditions (when enzymatic active site is preserved), the protein structure remains unchanged for the four proteins.

These results suggest that this technique is of interest to prepare protein particles for microencapsulation by the s/o/w encapsulation procedure. As described above, the encapsulation of a solid-state protein proved to enhance its stability during encapsulation in PLGA microspheres [36]. However, complete success of this approach has been limited by formulation issues (protein loss, the use of additives, a complex manufacturing process). In this study, complete and reversible protein precipitation was associated with the preservation of protein stability during the encapsulation step without the addition of stabilizers in the formulation.

It is also important to maintain protein stability during release. Generally, if a protein becomes denatured during the encapsulation step, it will remain in that same state during the release step. But, it is not obvious that when activity is preserved within microspheres, that it is also preserved upon *in vitro* release. For example, protein co-lyophilisation with methyl-β-cyclodextrin preserved the stability of an entrapped protein but, upon *in vitro* release, a loss of

activity was observed [21]. The inactivation was supposed to be due to inactivation during prolonged incubation at 37 °C. Therefore, the choice of an appropriate release medium which approached physiological conditions and prevented protein denaturation prior to analysis in the release medium was taken into account as previously proposed [25] and lysozyme *in vitro* release was studied. Lysozyme is a good model to predict the behaviour of growth factors such as NGF and GDNF whose therapeutic potential is well known because of their similar physical and chemical properties [3,25]. Moreover, its enzymatic assay is sensitive (in the ng/ml order); so, among the four studied proteins, lysozyme was the only one for which the total and active released fractions could be compared. Furthermore, although lysozyme is known as a relatively stable protein, it may be inactivated upon release. A previous study made in similar conditions (polymer type, encapsulation yield, *in vitro* release conditions) showed that lysozyme inactivation occurred upon release (69% of activity loss) if no $Mg(OH)_2$ was added, despite protein stabilization during encapsulation by a w/o/w technique and the choice of an appropriate release medium [25]. In this study, protein precipitation with glycofurol/sodium chloride in optimum conditions allowed stabilization during the encapsulation process and the release of an active mass. Only optimum conditions to precipitate 900 μg of protein were selected. As neither hydrophilic polymer nor carrier was necessary in the formulation to assure protein stability; the burst effect was minimized [13]. The size of the particles was also prominent, as several experimental results indicated a reduction in burst if the protein particle size was reduced [44]. Despite these concerns on the release profile, incomplete release from the PLGA microspheres could not be avoided. Indeed, after the first day, the *in vitro* release levelled off and the cumulative release amount remained below 20%. This classical problem is generally attributed to many destabilizing mechanisms occurring during the incubation period such as moisture-induced aggregation, adsorption onto the polymer surface (especially for proteins positively charged at neutral pH such as lysozyme) and acid-induced aggregation due to polymer degradation [45]. The formulation should now be adjusted to overcome this phenomenon [7].

5. Conclusion

Numerous studies reported the encapsulation of a solid-state protein to enhance protein stability during encapsulation process. Common methods to obtain protein particles might be complex, denaturing and wasteful. So, the problem of protein preformulation before its encapsulation in PLGA microspheres by the s/o/w encapsulation procedure was addressed and the development a more appropriate preformulation process based on phase separation was considered. Reversible, nanosized protein particles were obtained with good efficiencies, for four model proteins differing in terms of physical and chemical properties. The interest of this simple preformulation process was emphasized by the successful encapsulation of these proteins in PLGA microspheres. Good yields were achieved without protein activity loss. It is interesting to underline that no stabilizer was needed. Finally, lysozyme activity was retained upon release from the carrier.

The incomplete protein release will now have to be overcome by avoiding non-covalent aggregation and hydrophobic PLGA–protein interactions. The encapsulation of a therapeutic protein will also be considered.

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