European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 298–305

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Integrity characterization of myoglobin released from poly(ε-caprolactone) microspheres using two analytical methods: UV/Vis spectrometry and conductometric bi-enzymatic biosensor

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ARTICLE INFO

Article history:
Available online 1 February 2011

Keywords:
Myoglobin
Multiple emulsion
Protein integrity
Conductometric biosensor
UV/Vis spectrometry
Microparticles

ABSTRACT

Myoglobin (Mb)-loaded poly(ε-caprolactone) (PCL) microparticles were prepared by multiple emulsion with solvent extraction/evaporation method under more or less deleterious operating conditions. The protein integrity was monitored using both UV/Vis absorbance ratio method at specific wavelengths and a conductometric bi-enzymatic biosensor based on proteinase K and pronase. Under standard operating conditions, Mb remained in native conformation, while different degrees of protein denaturation were observed by changing the encapsulation conditions. It was shown that solvent elimination under reduced pressure and in a lower extent addition of a higher molecular weight PCL led to protein alteration. In the first case, the loss of protein integrity can be attributed to residual solvent entrapped in particles whose solidification was accelerated. In the second case, denaturation may be explained by an increase in the protein exposure time at water/organic solvent interface due to an increase in organic phase viscosity.

1. Introduction

In the last decade, the rise of biotechnology industry, the advances in DNA recombinant technologies, and the development of controlled delivery systems based on therapeutic proteins allowed the treatment of a great number of diseases and the improvement of the patient daily life [1,2]. However, these macromolecules are fragile, often hydrophilic, sensitive to environmental conditions, and they possess a specific and complex architecture (such as secondary, tertiary, and quaternary structures maintained by several non-covalent interactions) [1,3]. The disruption of these structures by degradation, aggregation or precipitation, or modification of side chains during the encapsulation process [1,4] can destabilize the protein and lead to the loss of protein biological activity [2,3]. The monitoring of native protein 3D conformation is a key element to ensure the success of protein formulations and their therapeutic efficiency [5,6]. Encapsulation by multiple emulsion method is one of the most common techniques to preserve stability and activity of proteins inside biodegradable microspheres. However, several factors can compromise the protein stability during the encapsulation process and it is necessary to control the protein integrity in microparticles.

In a previous work, we used a conductometric biosensor associated with the determination of protein concentration for the analysis of the conformation of bovine serum albumin (BSA) released from poly(ε-caprolactone) (PCL) microspheres. A mixture of native and denatured protein was detected after 24 h release, and it was shown that the protein released at the beginning of the experiment was denatured, whereas after 5 h, the protein conformation was the native one [7]. In the present work, the same microencapsulation process was used to prepare myoglobin-loaded microspheres.

Myoglobin (Mb) was used as model protein for the monitoring of protein conformational changes due to its small size (16.95 kDa) [8], its spherical shape, and overall, its sensitivity under some deleterious conditions (organic solvent, unfolding agent, acidic pH, etc.) [4], which is higher than that of BSA or lysozyme [9]. The analysis of the conformation of released Mb required the development of a new conductometric biosensor based on 2 enzymes, proteinase K and pronase. In parallel, Mb was analyzed by UV/Vis spectrometry and Bradford protein assay method.

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doi:10.1016/j.ejpb.2011.01.013

Thereby, the main objectives of this work were to prove the pertinence of two rapid and reliable methods (UV/Vis spectrometry and conductometric biosensor associated with Bradford method) to monitor protein integrity after the encapsulation process. A reference formulation, known to maintain native protein conformation [2,7], was firstly used to evaluate and compare the potential of the two analytical methods. Then, these methodologies were applied to other Mb-loaded microparticles prepared under conditions assumed to be more or less denaturing, such as high organic phase viscosity, low polymer concentration, and the change in solvent removal conditions. The final objective was to correlate particle properties (mean size, inner morphology, surface topology, and encapsulation efficiency) to protein integrity.

2. Materials and methods

2.1. Reagents

Horse heart myoglobin, >90%, PhastGel electrophoresis essentially salt-free, lyophilized powder (Mb), as model protein (16.95 kDa), proteinase K (EC 3.4.21.64, freeze-dried from *Tritirachium album*), bovine serum albumin (BSA) (67 kDa), glycerol (99%), and Bradford reagent were purchased from Sigma Chemical Co.

Pronase (*Streptomyces griseus*), freeze-dried, 7 U/mg, was purchased from Roche. It is an enzymatic preparation exhibiting a non-specific protease activity. This preparation contains endo- and exopeptidases and in particular chymotrypsin, trypsin, carboxypeptidase, and aminopeptidase. Its optimum pH is in the 7–8 range. Glutaraldehyde (GA, grade II, 25% aqueous solution) was purchased from Sigma–Aldrich Chemical GmbH. All chemicals and solvents were of analytical grade. Ultrapure water used throughout for the preparation of solutions was obtained from an ELGA UHQ II System (resistivity 18.2 M $\Omega \cdot$ cm).

Two poly (ε-caprolactone) (PCL) grades (M_w = 14,000 and 65,000 g $\cdot$ mol^{-1}) were supplied by Aldrich Chemical Co. Methylene chloride (MC) was used as the polymer solvent, and isopropanol (IPr) was from Carlo Erba. Poly(vinylalcohol) or Mowiol® 4–88 (PVA) hydrolyzed 86.7–88.7 mol%, from Fluka, was used as a stabilizer in the external phase (M_w = 31,000 g mol^{-1}). Sucrose was obtained from Merck and was used to equilibrate the osmotic pressure. The phosphate-buffered saline tablets (0.01 M PBS, KCl 0.0027 M + NaCl 0.137 M, pH 7.4) were obtained from Fluka.

2.2. Microparticle preparation

Microparticles were prepared by a multiple emulsion $W_1/O/W_2$ with solvent extraction/evaporation method at room temperature (22–25 °C). This method was previously described by Ruffin et al. [7]. All process and formulation conditions used for the preparation of the reference formulation were those recommended by the authors to ensure best particle properties and to respect the protein integrity [2,7]. Then, the protein conformation was affected by modifying the organic phase viscosity and solvent removal conditions in order to evaluate the potential of the two monitoring methods.

Briefly, the protein in an aqueous solution of 0.1% PVA (0.3 mL) was emulsified in an organic solution (O) composed of 5 mL MC and 2.5 or 1 g PCL (i.e., 50% or 20% w/v of one PCL only or a mixture of 2 PCL grades) by means of a homogenizer (Ultra-Turrax® T25, Ika) at 16.000 rpm for 15 s. The rotor–stator homogenizer type was proved to be more suitable to limit the protein aggregation than vortex or sonicator, especially during the first emulsification [10]. The primary emulsion (W_1/O) was subsequently poured into a small volume (50 mL) of outer aqueous phase (W_2) made up of 1% PVA and 0.43 g sucrose. The multiple emulsion ($W_1/O/W_2$) was prepared by means of a homogenizer (Ultra-Turrax® T25, Ika) at 24.000 rpm for 15 s at room temperature. Then, 150 mL of IPr/H_2O (10:140) was added to the resultant multiple emulsion ($W_1/O/W_2$) and maintained under gentle mechanical stirring at 300 rpm for 3 h to extract MC and to allow the particle solidification by PCL precipitation (liquid surface area was about 60 cm^2 for all experiments).

This step was replaced by a direct evaporation under reduced pressure below MC saturated vapor pressure (i.e., 474 mBars at 20 °C) for 75 min by decreasing the pressure from 590 mBars to 90 mBars (Rotavapor R-114® with water bath B-490®, Büchi) for a complete elimination of MC in a round-bottom flask at 25 °C (liquid surface area was about 50 cm^2 for this experiment). Finally, particles were separated and purified by filtration (0.45 μm PVDF filters, membrane Durapore®) and water washing (200 mL three times), before to be lyophilized (Usifroid® SMH50, freeze dryer) in order to eliminate residual water and stored in hermetically closed vials at 25 °C. Four microparticle formulations named MI–MIV were prepared in various conditions, as reported in Tables 1 and 2.

2.3. Protein characterization

2.3.1. Protein integrity

2.3.1.1. Conductometric biosensor analysis. Sensor design: The conductometric transducers, consisting of two identical pairs of gold interdigitated thin film electrodes (thickness: 150 nm), were fabricated by vacuum deposition on a ceramic substrate (5 × 30 mm) (sensor chip) at the Lashkaryov Institute of Semiconductor Physics (Kiev, Ukraine). A 50 nm-thick intermediate chromium layer was used for better gold adhesion. The dimension of each interdigital space and digit was 20 μm, and the length of the digits was about 1.0 mm. The sensitive area of each pair of electrodes was about 1 mm^2 [11,12].

Biosensor preparation: A three-step procedure was followed to prepare the bi-enzymatic biosensor. As a differential experimental setup was used, 0.2 μL of a 20 mM phosphate buffer (PBS, pH 7.2) containing 6% (m/v) BSA, 10% (m/v) glycerol and 4% of pronase was first deposited on the working pair of electrodes, while on the reference pair of electrodes only a mixture containing 10% (m/v) BSA and 10% (m/v) glycerol was applied. Then, the sensor chip was placed for 15 min in a saturated GA vapor atmosphere. A second layer was then deposited following the same protocol except that pronase was replaced by proteinase K in the enzymatic solution deposited on the working pair of electrodes. In the last step of the procedure (immobilization of enzymes), both pairs of electrodes were placed again for 15 min in a saturated GA vapor atmosphere. After exposure, membranes were kept in air at room temperature for 30 min in order to complete the cross-linking process. Biosensors were then stored at 4 °C in a 20 mM phosphate buffer solution (PBS, pH 7.2) for equilibration until measurements. They were stable for at least one month [12].

Conductometric measurements: Microelectrodes were placed in a glass cell filled with 5 mL of a 5 mM phosphate buffer (PBS, pH 7.2). The solution was vigorously stirred. Measurements were then taken at 23 ± 2 °C by applying to the differential pairs of electrodes an alternating voltage (10 mV amplitude, 100 kHz frequency) generated by a low-frequency wave-form generator (SR830 Lock-in amplifier from Stanford Research Systems). These conditions of alternative voltage allowed reducing faradic processes, double-layer charging, and concentration polarization at the microelectrode surface [11]. After stabilization of the differential output signal, small aliquots (5–50 μL) of a concentrated substrate solution were added in order to achieve final concentrations between 1 and 9 mg L^{-1}. The biosensor was calibrated before use [12]. Associated with the classical Bradford protein assay, this biosensor was able to provide information about the native or

Table 1
Key absorbance ratios of Mb released from different PCL microparticle preparations during 48 h in PBS pH 7.4 at 25 ± 1 °C, with in bold all native ratios (native protein structure) and in italics non-native conformation.

Samples	Process parameters	Formulation parameters	Time (h)	A_{409}/A_{280}	A_{280}/A_{505}	A_{280}/A_{635}	A_{409}/A_{505}	A_{505}/A_{635}	Biosensor response ($\mu S\ mg^{-1}\ L$)	Protein integrity
MI	Extraction/ evaporation	50% w/v $PCL_{14,000}$	1	3.1	5.3	21.1	**16.5**	4.0	3.5	*Non-native*
			2	2.5	6.4	23.6	**15.9**	3.7	3.2	*Non-native*
			4	3.4	5.2	15.6	**17.6**	**3.0**	3	*Mixture of native + non-native*
			15	**5.0**	**3.5**	**8.9**	**17.8**	**2.6**	**0.7**	**Native**
			24	**4.8**	**3.6**	**10.6**	**17.2**	**3.0**	**0.7**	**Native**
			48	**4.9**	**3.6**	**9.9**	**17.7**	**2.7**	**0.7**	**Native**
MII	Evaporation at 590 mBar	50% w/v $PCL_{14,000}$	2	2.5	22.4	7.1	**17.5**	3.2	3.6	*Non-native*
			4	2.9	22.0	6.0	**17.6**	3.7	3.4	*Non-native*
			15	4.3	15.0	4.2	**18.1**	3.6	2.2	*Mixture of native + non-native*
			24	4.4	3.9	**10.4**	**16.9**	**2.7**	2.1	*Mixture of native + non-native*
			48	**4.8**	**3.4**	13.1	**16.5**	3.8	1.98	*Mixture of native + non-native*
MIII	Extraction/ evaporation	37.5% w/v $PCL_{14,000}$ + 12.5% w/v $PCL_{65,000}$	2	1.1	11.6	14.1	13.2	1.2	3.6	*Non-native*
			4	1.4	9.6	72.1	13.7	7.5	3.5	*Non-native*
			15	3.0	6.0	18.7	**17.7**	**3.1**	*2.9*	*Mixture of native + non-native*
			24	4.4	4.0	**9.5**	**17.6**	2.4	*2.8*	*Mixture of native + non-native*
			48	**5.3**	**3.3**	7.9	**17.4**	2.4	*2.8*	*Mixture of native + non-native*
MIV	Extraction/ evaporation	20% w/v $PCL_{65,000}$	3	4.0	4.3	12.6	**17.3**	**2.9**	*2.8*	*Mixture of native + non-native*
			15	4.0	3.8	**10.2**	15.2	**2.7**	*2.7*	*Mixture of native + non-native*
			48	5.9	3.7	**8.9**	21.8	2.4	3.5	*Non-native*
Values of absorbance ratios for native Mb in aqueous solution of PBS (pH 7.4) at 25 °C				**4.7–5.5**	**2.8–3.6**	**8.5–10.7**	**15.8–18.1**	**2.6–3.1**	<1	**Native**

non-native conformation of the protein through the ratio between the biosensor response and myoglobin concentration.

2.3.1.2. UV/Vis spectrometric analysis. Mb properties: Myoglobin (Mb) is a small globular hemoprotein, like hemoglobin (Hb). Contrary to Hb, Mb is a monomeric protein made of a globular part or *globin* and a molecular part called *heme* entrapping an iron atom linked to a porphyric cycle [13]. Mb absorbs in UV domain at 280 nm as most proteins, due to the presence of *aromatic amino acid* or *AAA residues* (i.e., Tyrosine, Tryptophan, and Phenylalanine). Mb hemic part corresponds to protein active site (or oxygen-binding site) that also confers absorbance or photodynamic properties in visible domain, depending on the iron oxidation state [8]. Three specific wavelengths are characteristic of the heme: a first intense absorbance band close to 400 nm corresponding to SORET band and other bands of lesser intensity named *Q* bands α and β between 500 and 700 nm. The position of these three bands allows the determination of the iron oxidation state. Therefore, UV/Vis spectrophotometric analysis allows the detection of environment changes close to AAA residues or to hemic iron or oxidation state modifications [13]. Information about the heme loss and/or the heme interactions with the rest of protein is also available [13–15].

Measurements: Absorbance spectra were obtained from 600 μL protein solution aliquots into a quartz cell (ℓ = 10 mm) by using a spectrophotometer with a photodiode array (8453A UV/Visible spectrophotometer®, from Agilent). The UV/Vis method used was based on the calculation of absorbance ratios at protein specific wavelengths as proposed by Jovanović et al. [14] and Oliveira et al. [15], who recommended the monitoring of one absorbance ratio (A_{409}/A_{280}). This concept was extended to other absorbance ratios to take into account the four UV/Vis wavelengths specific to native Mb under oxidized form (Fe^{3+}) or *Metmyoglobin* (Fig. 1): 280, 409, 505, and 635 nm. Except for A_{409}/A_{635}, all ratios can be used to check the protein integrity. From these ratios, we could distinguish independently of Mb concentration which part of the protein was affected during the encapsulation process and determine whether operating conditions were more or less denaturing compared with standard conditions.

2.3.2. Protein quantification

Native myoglobin: UV/Vis spectrometry offered the advantage to provide qualitative and quantitative data at once. Protein concentration was calculated using a calibration curve performed in phosphate buffer saline (pH 7.4) aqueous solution at 409 nm in the

Table 2
Particle properties of formulations MI–MIV, where the surface is defined by poorly (+) and highly porous (++).

Samples	Preparation conditions		Particle properties				
	Process parameters	Formulation parameters	Mean size (±SD, $n = 3$) (μm)	EE (±SD, $n = 2$) (%)	Inner morphology	Layer thickness (μm)	Surface topology
MI	Extraction/ evaporation	50% w/v $PCL_{14,000}$	14.4 ± 0.3	37 ± 1	Core–shell	0.9	Smooth (+)
MII	Evaporation at 590 mBar	50% w/v $PCL_{14,000}$	9.1 ± 0.1	32		1.6	Smooth (+)
MIII	Extraction/ evaporation	37.5% w/v $PCL_{14,000}$ + 12.5% w/v $PCL_{65,000}$	17.6 ± 1	7		ND[a]	Rough (+)
MIV	Extraction/ evaporation	20% w/v $PCL_{65,000}$	11.2 ± 0.3	16 ± 2		2.2	Rough (++)

[a] ND = Non-determined.

0.025–0.25 × 10^3 mg · L^{-1} range. Standard protein quantification through absorbance at 280 nm may also be used but is less precise than absorbance at 409 nm, which is maximal in the case of Mb.

Altered myoglobin: In the case of the denatured protein, UV/Vis spectrum was affected and the quantification at 409 nm became unusable. Therefore, the Bradford assay method was used for the quantification of denatured protein, after encapsulation in deleterious conditions, or extraction procedures since it can be applied independently of the 3D protein structure [7]. This method involves the binding of Coomassie Brilliant Blue G-250 to proteins (especially with Arginine and Lysine residues), causing a shift in the maximal absorption of the dye from 470 to 595 nm. This assay is very reproducible and rapid; the binding process being complete in approximately 10 min, and the coloration stable for about 45 min. The protein quantification was achieved in standard conditions using 100 μL protein solution aliquots mixed with 3 mL of Bradford reagent into a glass cell (ℓ = 10 mm). Three replicate measurements were taken at each concentration level. Protein content extracted from microparticles was determined at 595 nm using a calibration curve built in the 0.01–0.15 × 10^3 mg · L^{-1} range.

2.4. Particle physicochemical characterization

Microparticles were characterized by their particle mean size, their encapsulation efficiency (EE), their surface topology, their inner porosity, and their release profiles.

2.4.1. Particle size distribution

Microsphere particle size distribution was measured by laser light scattering (Coulter® LS230, Beckman Coulter) on a dispersion of lyophilized microspheres in deionized water under mechanical stirring. The particle size was expressed as volume-mean diameter in micrometers.

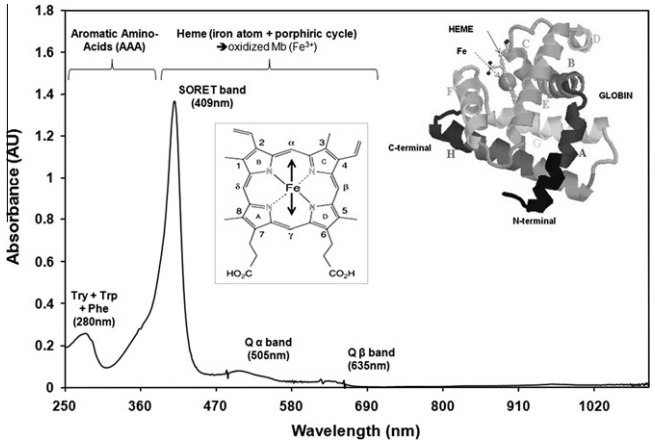

Fig. 1. Specific absorbance spectrum of native oxidized Mb in PBS pH 7.4 solution at 25 ± 1 °C.

2.4.2. Particle morphology

Morphology of freeze-dried microspheres was studied by scanning electron microscopy (SEM) (FEG Hitachi® S800). Microspheres were fixed on metal pieces using a double-side adhesive tape. Subsequently, they were coated with a thin gold layer (100–150 Å), vacuum-dried, and observed by SEM at 10 kV or 15 kV.

2.4.3. Protein encapsulation efficiency (EE)

Protein encapsulation efficiency (EE) was determined after protein extraction from microparticles according to a protocol described by Ruffin et al. [7]. Briefly, 100 mg of freeze-dried microparticles were incubated in 1 mL of MC during 2 h to dissolve the polymer. Then, 2 mL of PBS (pH 7.4) solution were added for the recovery of the protein into an aqueous phase after 24 h under gentle agitation at room temperature (25 ± 2 °C). The supernatant was centrifuged for 10 min at 2700*g* (Eppendorf Centrifuge® model 5417C). The extracted protein solution was clarified by means of syringe filters RC (regenerated cellulose, 0.45 μm) and then analyzed by standard Bradford protein assay. Protein EE was obtained by calculating the ratio of the protein amount encapsulated in microspheres to the amount of protein initially introduced in the process.

2.4.4. Protein release kinetics

The release of protein from microspheres of formulations MI–MIV was measured as a function of time. The protein quantification was carried out with 143 mg of dried microparticles dispersed in 1 mL of phosphate saline buffer solution (PBS, pH 7.4) by vortexing for 20 s at 10 Hz. All samples were maintained at 25 ± 1 °C under magnetic agitation to allow protein release from PCL microspheres. Protein concentration in the release medium was regularly measured by means of the three protein assay methods. Each sample was clarified by filtration (0.45 μm) before analysis.

3. Results and discussion

3.1. Analytical characteristics of the proteinase K/pronase biosensor

Three different biosensors based on pronase, proteinase K, or a mixture of pronase and proteinase K were first evaluated. Whatever biosensor considered (Fig. 2), Mb injection into the measurement cell caused a rapid and significant increase in the conductivity due to the enzymatic hydrolysis of the protein. Equilibrium between the production of ions due to the enzymatic reaction inside the membrane and the influx of ions into the membrane

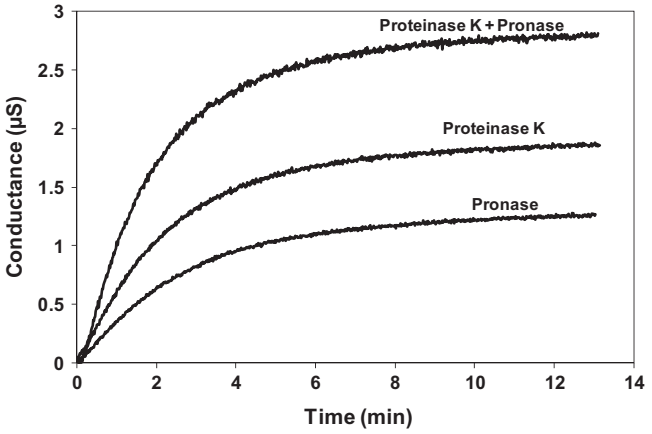

Fig. 2. Typical response curve of the conductometric biosensor based on different protease enzymes and 4.5 mg L^{-1} of native Mb.

via a mediated transport mechanism, with buffer species acting as a carrier, was achieved within 10 min. The steady-state response time, defined as the time required to reach 90% of the steady-state signal, was close to 5 min. Proteinase K + pronase (pkp) biosensor yielded the highest signal. Indeed, pronase composition and its association with proteinase K confer to the biosensor the capability to catalyze all the hydrolysis sites contained in Mb.

The relationship between the pkp biosensor response and myoglobin concentration was examined in the 0–9 mg · L^{-1} range. For that, nine standard solutions were used and five measurements were taken at each concentration level (Fig. 3). A lack of fit test (LOF test) at the 5% level proved that the biosensor response is linear up to 7 mg · L^{-1} Mb. The correlation coefficient and the sensitivity were 0.9974 and 0.6913 µS mg^{-1} · L (α_1), respectively. The limit of detection (LOD), calculated for a signal to noise ratio of 3, was 0.3 mg · L^{-1}.

The variation coefficient obtained from five measurements taken within one day with the same sensor was very satisfying since comprised between 5.5% and 6.4% in the concentration range studied.

3.2. Influence of solvent and thermal treatments of myoglobin on biosensor response

To evaluate the effect of protein conformation on biosensor response, Mb was firstly altered by boiling at 100 °C for 20 min and secondly by contacting the protein with organic solvent (MC) for 2 h under gentle agitation. It was observed that the biosensor response toward non-native Mb was much higher than toward native Mb (Fig. 3). The slopes of the curves were α_2 = 2.55 µS · mg^{-1} · L ($\alpha_2 \approx 3.7 \times \alpha_1$) and α_3 = 3.52 µS · mg^{-1} · L ($\alpha_3 \approx 5 \times \alpha_1$) for the thermal and solvent denatured protein, respectively. The low value observed for native Mb may be attributed to the globular structure of this protein, which can hamper the accessibility of proteinase K and pronase to some cleaving sites. The diffusion of Mb into the enzymatic biofilm may also be hindered. The highest sensitivity was obtained for the solvent-treated protein, the accessibility of proteinase K and pronase to Mb sites being increased. An intermediate response obtained for thermal denaturation indicated an altered conformation of the protein whose structure is different from that of solvent-treated Mb. These results were consistent with UV/Vis analysis showing altered spectra with the loss of the heme in the case of Mb in MC (results not shown).

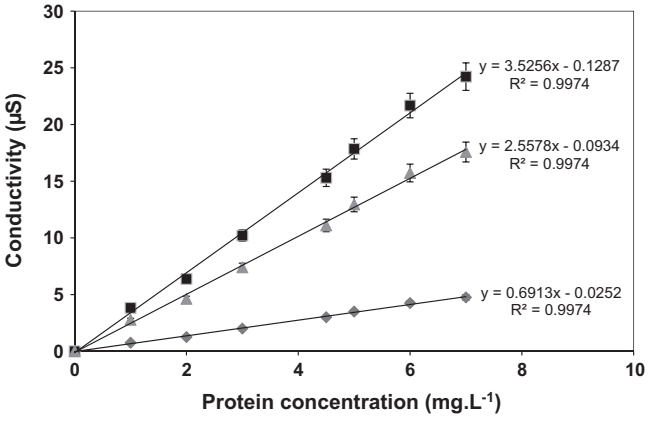

Fig. 3. Calibration curves of the conductometric biosensor for native and non-native Mb: denatured Mb by organic solvent (■), denatured Mb by ebullition (▲), and native Mb (♦).

3.3. Mb characterization from reference formulation microspheres (MI)

3.3.1. Protein integrity

The integrity of Mb released from microspheres was checked by UV/Vis spectrometric and conductometric measurements.

The five absorbance ratios A_{409}/A_{280}, A_{280}/A_{505}, A_{280}/A_{635}, A_{409}/A_{505}, and A_{505}/A_{635}, reported in Table 1 allowed assessing protein conformation during the release studies. If all ratios corresponded to native Mb conformation, protein was considered as unaffected by the encapsulation process. Reciprocally, if at least one ratio was out of the range defined with native Mb, protein conformation was altered. For reference formulation MI, the model protein was totally denatured in the first 4 h of release. After 4 h, the global state of the protein evolved, passing through denatured to non-denatured state beyond 15 h. The intermediate state could be explained by the presence of a mixture of altered and native protein in the release medium. In the first hours, the Mb released corresponded to "surface protein" adsorbed on polymeric matrix surface [7]. This protein is less deeply entrapped within the particles and consequently more exposed to denaturing conditions [16]. However, this corresponds to a low fraction of protein, less than 10% of the particle content. After 15 h and until the end of the experiment (i.e., 48 h), the protein remained in native conformation. These release studies were limited to 48 h since a loss of Mb stability was observed after 48 h in PBS solution.

The evolution of Mb conformation deduced from UV/Vis measurements was confirmed by conductometric results (Table 1). The conductivity/concentration ratio decreased from 3.5, close to the slope of the calibration curve obtained for solvent-treated Mb (α_3 = 3.5 µS mg^{-1} L), to 0.7 µS mg^{-1} L, which corresponds to native Mb (Fig. 3). Thus, these two different characterization methods proved that the protein encapsulated in particle core in the reference formulation conditions was not affected by the process.

3.3.2. Release kinetics

Mb release kinetics from PCL microspheres were investigated by absorbance spectra at (i) 280 nm (classical protein quantification method), (ii) maximal absorbance wavelength (409 nm), and (iii) by Bradford analysis (595 nm) (Fig. 4).

As shown in Fig. 4, the three quantification methods led to very similar release profiles. Results obtained from the two latter methods were not significantly different (<1%), while data obtained at 280 nm were quite higher. This could be explained by a mixture of native and denatured proteins observed in this reference formulation. Conformation of Mb released during the first hours was altered, and as shown in Table 1, the absorbance at 280 nm was significantly modified as all ratios containing A_{280} were not in the standard confidence interval. On the contrary, one ratio (A_{409}/A_{505}) was not altered at all; therefore, the hemic part of protein

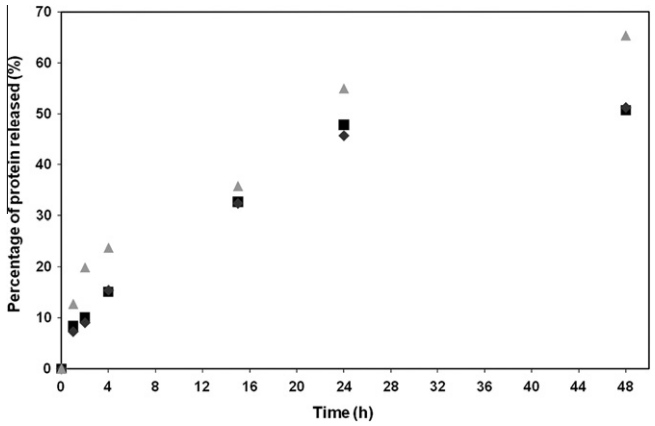

Fig. 4. Percentage of Mb released from MI formulation determined at different wavelengths, by Bradford protein assay at 595 nm (■) and 2 standard protein assays at 409 nm (♦) and at 280 nm (▲).

remained stable for all the experiment duration. This also explains that quantification at 409 nm and Bradford assay led to the same results. Here, it appeared that the maximal absorbance wavelength (409 nm) was more appropriate than AAA bands to quantify Mb released, due to the alteration in aromatic amino acid residues of the protein adsorbed at the particle surface. These results are in agreement with several authors, who used preferentially the SORET band for oxidized Mb [15,17]. Bradford protein assay was not biased, because this method is independent of the structural conformation of the protein.

PCL microspheres displayed a classical two-stage release profile (Fig. 4) characterized by a poor initial release for MI formulations (16% released after 4 h) followed by a nearly linear release and a "plateau". This profile is in good agreement with literature data, the release mechanism being governed by diffusion phenomena [7,18–20]. Nevertheless, the initial Mb release from MI formulation was slower than that reported in other studies [7,21,22]. For the same formulation conditions [7], 38% of another protein (BSA) was released during the first 4 h. Several factors can influence the initial release: the protein molecular weight [9], its diffusion coefficient, and the particle structure. Mb molecular weight is smaller than that of BSA (respectively, 16.95 kDa and 67 kDa), so this protein was probably better entrapped and the fraction of surface protein was smaller. After 48 h, the percentage of protein released was not complete, about 53% for MI. We did not investigate longer times because of the lack of protein stability in PBS after 48 h. As frequently observed in PCL microspheres encapsulation studies, protein release can either continue for a long time or be incomplete [23,24].

For the other formulations (from MII to MIV), Mb lost its native conformation as shown by the results developed in the next paragraph; therefore, their in vitro release kinetics were not presented.

3.4. Application to other formulations

To evaluate the potential of our analytical methods, Mb microspheres were prepared in conditions known to alter the protein integrity, such as MC elimination under reduced pressure (MII formulation) or an increase in the organic phase viscosity (MIII and MIV formulations).

Table 1 shows that all these experiments led to an alteration in protein conformation. Absorbance ratios and conductometric analysis demonstrated that Mb was denatured in the case of MII and MIII formulations. This result can also be due to a mixture of native and altered proteins, in unknown proportions [7]. For MII formulation, as already observed with MI, one ratio (A_{409}/A_{505}) remained constant during the whole experiment, suggesting that the SORET band was less affected than the other bands. After 15 h, the number of non-conform ratios decreased so a modification of protein structural state occurred. After 48 h, only two ratios were not conform, namely A_{280}/A_{635} and A_{505}/A_{635}. Then, the 635 nm band seemed to be more altered than the other bands, indicating a modification of the heme environment or a beginning of heme extraction.

The ratio between biosensor response and Mb concentration decreased from 3.6 corresponding to a denatured state to about 2 after 15 h, showing a mixture between native and denatured proteins with a non-native content higher than that obtained with MI formulation. Direct evaporation under reduced pressure accelerates solvent diffusion rate reducing particle solidification duration. Smaller and denser particles were obtained with a greater thickness of the polymeric layer as shown in Table 2 (1.6 μm for MII versus 0.9 μm for MI). Therefore, residual organic solvent may be entrapped inside the particles inducing the protein alteration.

MIII formulation also resulted in a denatured state, but protein seemed to be more altered than in MII formulation. Indeed, no absorbance ratios were consistent before 15 h and only three ratios were standard at 48 h. The biosensor response decreased from 3.6 to 2.9 after 15 h and then remained quite high. These results proved that the increase in organic phase viscosity altered the protein integrity. This can be explained by a decrease in MC extraction/evaporation rate and an increase in protein exposition to organic solvent.

Finally, the last formulation (MIV) corresponded to the most deleterious conditions of encapsulation. Only one absorbance ratio (A_{280}/A_{635}) corresponded to native protein at the end of experiment (48 h). However, at the early hours of release (before 15 h), 2 ratios were standard, suggesting a mixture of native and denatured proteins.

For all experiments, the results obtained with the bi-enzymatic conductometric biosensor confirmed the UV/Vis spectrophotometric results. Furthermore, it should be mentioned that the biosensor response can provide information about a degree of denaturation. However, with absorbance ratio method, one can determine which part of the protein is altered (heme or globin) (Fig. 5). Here, the use of several absorbance ratios appeared as useful to detect protein alteration independently of its concentration. If only one ratio was considered (namely A_{409}/A_{280}), protein could be considered as native at the end of release study for MII and MIII. However, other ratios and also biosensor responses showed that protein was non-native.

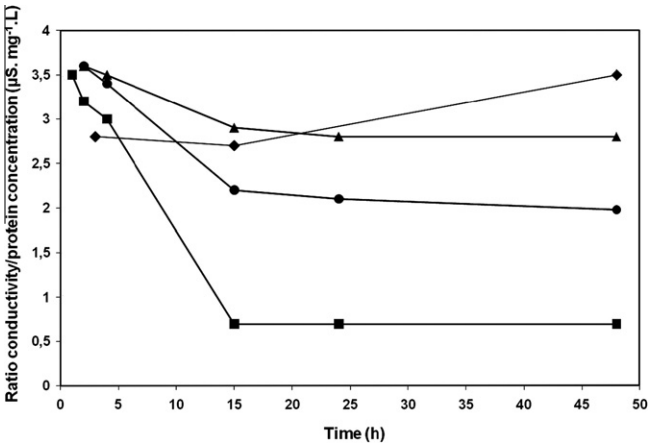

Fig. 5. Ratio conductivity/protein concentration as a function of release time, with MI (■), MII (●), MIII (▲), and MIV (♦).

3.5. Particle physicochemical characterization

Several particle properties namely: size distribution, encapsulation efficiency (EE), and particle morphology (surface porosity and inner polymeric network, Fig. 6) were also studied for the four formulations. The results are reported in Table 2.

3.5.1. Particle mean size

Particle mean sizes were measured in triplicate. Solvent evaporation under reduced pressure led to smaller particles compared with reference formulation (MI), respectively, 9.1 ± 0.1 μm and 14.4 ± 0.3 μm (see Table 2). This can be attributed to a higher rate of solvent removal due to lower pressure. This result is in agreement with the work of Chung et al. [25], where albumin was encapsulated in PLA microspheres obtained by multiple emulsion solvent evaporation method.

The use of $PCL_{65,000}$ influenced particle mean size. The mixture of 2 PCL grades in MIII formulation (12.5% w/v $PCL_{65,000}$) increased the particle mean size (17.6 ± 1 μm) in relation with the increase in organic solution viscosity, unlike MIV formulation (11.2 ± 0.3 μm) where the polymer amount is reduced for a similar MC volume [2,21,26].

3.5.2. Particle morphology

All particles were spherical with a more or less porous surface and a core–shell type structure (or capsule). As shown in Fig. 6 (MI–MIV), the particle surface topology of reference formulation was quite smooth (i.e., MI formulation). The particles prepared under reduced pressure, such as MII formulation, did not exhibit any significant surface alterations, with a particle surface as smooth as MI formulation.

However, for MIII and MIV formulations prepared with $PCL_{65,000}$, the surface topology was more porous and rough. These results may be explained by a change in organic phase viscosity and then in MC removal rate. The mixture of polymers used in MIII (e.g., 37.5% w/v $PCL_{14,000}$ + 12.5% w/v $PCL_{65,000}$), may also result in heterogeneous precipitation due to the different polymer molecular weight inducing surface roughness. Whereas for lower polymeric solution viscosity (MIV with 20% w/v $PCL_{65,000}$), MC diffusion was accelerated and the first emulsion stability was affected inducing pore formation.

3.5.3. Protein encapsulation efficiency (EE)

The EE_{MI} calculated for MI formulation was 37 ± 1% (n = 2). This result was close to those obtained by Ruffin et al. [7] with bovine serum albumin (BSA) (EE_{BSA} = 45%). The use of $PCL_{65,000}$ for particle preparation (i.e., 12.5% w/v for MIII and 20% w/v for MIV) reduced significantly the EE by increasing the roughness and pore formation on particle surface (EE_{MIII} = 7% and EE_{MIV} = 16 ± 2% (n = 2) versus EE_{MI} = 37 ± 1%).

In addition, protein was denatured in these formulations. However, the modification of solvent removal conditions slightly

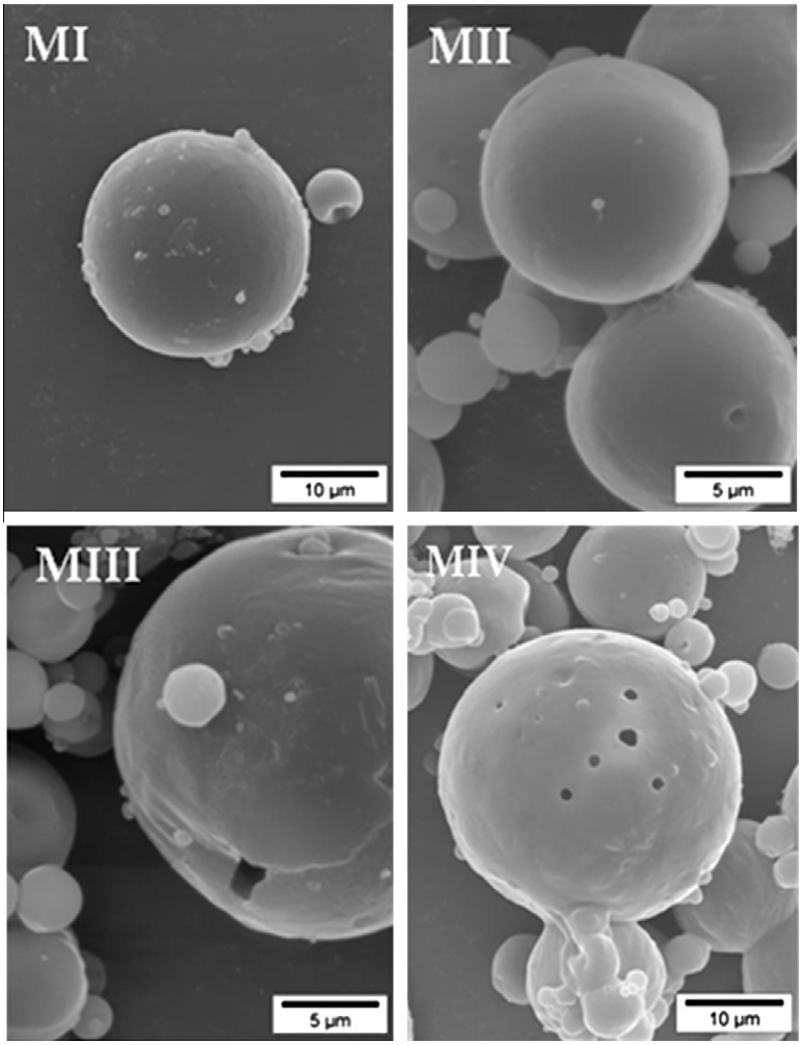

Fig. 6. Scanning electron micrographs of MI–MIV formulations.

decreased the EE (EE_{MII} = 32% versus EE_{MI} = 37 ± 1%) and it can be concluded that this step did not influence the EE, but the protein was denatured. All results are summarized in Table 2.

4. Conclusion

The protein conformation can be altered by changing the microencapsulation process or formulation parameters, and the use of two monitoring methods for protein integrity was shown to be pertinent to detect the protein integrity modifications. Indeed, the UV/Vis spectrometry or conductometric biosensor measurements associated with protein quantification method allowed checking the preservation of native protein conformation in reference conditions and distinguishing a degree of denaturation in two other formulations. Our results showed that the use of higher molecular weight polymer even at a low concentration may induce protein denaturation. This can be explained by a prolonged exposition of Mb to organic solvent inside the particles (MIII formulation) or an instability of primary emulsion in a low viscous organic solution (MIV formulation). The modified solvent removal method was less denaturing than the change in organic phase viscosity. These results could be explained by a faster MC elimination rate than for other encapsulation conditions studied. In addition, the two analytical methods used in this work led to similar results concerning the release kinetics from microspheres.

The UV/Vis spectrometry is a simple, rapid, and non-invasive method indicating which part of Mb was affected, but was not suitable for the quantification of altered protein. Absorbance spectra analysis was simplified and more sensitive by the use of absorbance ratios especially in the case of similar absorbance profiles. Moreover, by considering several ratios instead of one as recommended by several authors [14,15], one can detect protein distortion inducing no significant changes in the SORET band.

The biosensor associated with a protein quantification method allowed determining a conductivity/concentration ratio whose value could reach several levels correlated with a denaturation degree of the protein structure. This method is invasive but very rapid for protein solution analysis and is not limited by protein size like NMR or by AAA presence like fluorescence and UV/Vis spectrometry. Although NMR allows obtaining information on alterations of both tertiary and secondary structures, its detection efficiency requires very concentrated samples with a high isotope percentage and a protein size below 30 kDa. On the contrary, the monitoring of BSA integrity with a size upper 30 kDa (i.e., 67 kDa) was made using the developed biosensor. However, the use of biosensor is not efficient to establish the presence of soluble aggregates compare with SEC or SDS–PAGE with a convenient standard molecular markers range.

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