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Research paper

Effect of various additives and polymers on lysozyme release from PLGA microspheres prepared by an s/o/w emulsion technique

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ABSTRACT

Incomplete protein release from PLGA-based microspheres due to protein interactions with the polymer is one of the main issues in the development of PLGA protein-loaded microspheres. In this study, a two-dimensional adsorption model was designed to rapidly assess the anti-adsorption effect of formulation components (additives, additives blended with the polymer or modified polymers). Lysozyme was chosen as a model protein because of its strong, non-specific adsorption on the PLGA surface. This study showed that PEGs, poloxamer 188 and BSA totally inhibited protein adsorption onto the PLGA37.5/25 layer. Similarly, it was emphasised that more hydrophilic polymers were less prone to protein adsorption. The correlation between this model and the *in vitro* release profile was made by microencapsulating lysozyme with a low loading in the presence of these excipients by a non-denaturing s/o/w encapsulation technique. The precipitation of lysozyme with the amphiphilic poloxamer 188 prior to encapsulation exhibited continuous release of active lysozyme over 3 weeks without any burst effect. To promote lysozyme release in the latter stage of release, a PLGA–PEG–PLGA tribloc copolymer was used; lysozyme was continuously released over 45 days in a biologically active form.

1. Introduction

Protein delivery from microparticles made of poly(D,L-lactide-co-glycolide) (PLGA) biodegradable polymers is very interesting in order to avoid proteolysis and to ensure sustained protein release. Nevertheless, the successful delivery of proteins from PLGA microparticles has still not been achieved. The most important hurdles are related on the one hand to protein stability issues during the formulation process and on the other hand during the release period [1–5]. While protein stabilisation during the formulation process is being achieved, thanks to the use of additives [6,7] or to the encapsulation of a solid-state protein [8], protein release with a zero-order profile and without denaturation has scarcely been reported [9–11].

The release of small hydrophobic molecules from PLGA microspheres is governed by drug diffusion through the matrix in the initial phase and by polymer degradation at later stages. For proteins, an initial massive release (burst effect) followed by an incomplete release is frequently observed due to instability problems [12]. Moisture-induced aggregation and ionic interactions are supposed to occur in the initial phase of the microsphere hydration. Later, during polymer erosion, non-specific protein adsorption onto the degrading surface area and covalent/non-covalent aggregation due to the formation of acidic PLGA degradation products are reported as factors responsible for this incomplete release [13].

The aim of this study was to provide insight into the improvement of protein release from PLGA microspheres. As protein interactions with the polymer are one of the main causes of incomplete protein release, a two-dimensional model was developed to quantify protein adsorption onto the PLGA surface. These interactions are particularly severe for positively charged proteins at neutral pH level [14,15]. Therefore, lysozyme, whose isoelectric point is superior to 7, was an appropriate model to study protein interaction with PLGA carboxylic acid end groups. In this model, the anti-adsorption effect of formulation components (additives in solution, additives blended with the polymer, or modified polymers) was studied. The most efficient additives were then selected to prepare PLGA microspheres, whose release profile was studied over 3 weeks. Microspheres were prepared by a non-denaturing solid-in-oil-in-water (s/o/w) emulsion evaporation/extraction technique based on a preliminary step of protein precipitation.

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Nanosized protein particles were thus formed by the addition of a salt (sodium chloride) and a water-miscible organic solvent (glycofurol) to a protein solution. They were then recovered by centrifugation for their subsequent encapsulation in PLGA microspheres without activity loss. A low level of protein loading was chosen in order to approach the conditions for microencapsulation of therapeutic proteins, effective at low concentrations. For the late release period, complementary strategies were tested as additional stability issues occur when the polymer starts to degrade.

2. Materials and methods

2.1. Materials

Uncapped (free carboxylic acid group at the terminal end) PLGA37.5/25 (Mn 14,000 Da) and PLA50 (Mn 22,700 Da) were provided by Phusis (Saint-Ismier, France). Uncapped PLGA25/50 (RG502H), PLGA25/50–PEG (RGP d 50105), PLGA25/50–PEG–PLGA25/50 (RGP t 50106) (t-LG25/50-PEG9) were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Other non-commercialised tribloc copolymers: PLA96–PEG–PLA96 (24% PEG 20,000, Mn total 85,000) (t-L96-PEG24), PLA50–PEG–PLA50 (23% PEG 20,000, Mn total 88,000) (t-L50-PEG23), PLA50–PEG–PLA50 (8% PEG 20,000, Mn total 254,000) (t-L50-PEG8) were obtained from IBMM-CRBA (Montpellier, France) [16]. The physical characteristics of the studied polymers are listed in Table 1.

Lysozyme (chicken egg white) and its substrate *Micrococcus lysodeikticus,* glycofurol (tetraglycol or α-[(tetrahydro-2-furanyl) methyl]-ω-hydroxy-poly (oxy-1, 2-ethanediyl)), additives and buffer compounds were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). 2-Hydroxylpropyl-β-cyclodextrin (HP-β-CD) was kindly given by Roquette Frères (Lestrem, France). Polyvinyl alcohol (Mowiol® 4-88) was obtained from Kuraray Specialities Europe (Frankfurt, Germany). Poloxamer 188 and poly(ethylene imine) were kindly supplied by BASF (Levallois-Perret, France).

2.2. Methods

2.2.1. Lysozyme quantification

The amount of active lysozyme was determined by measuring the turbidity change in a *M. lysodeikticus* bacterial cell suspension as previously reported [17]. Hundred microlitre of a lysozyme solution was added to 2.9 mL of a 0.015% w/v *M. lysodeikticus* suspension in TRIS–HCl (0.01 M, pH 7.4) buffer solution. After incubation (37 °C, 4 h), the absorbance was measured at 450 nm. The amount of active protein was calculated thanks to a standard curve.

2.2.2. Lysozyme/polymer interaction study

Lysozyme/polymer interactions were indirectly investigated in a two-dimensional model by quantifying the amount of protein adsorbed onto a polymer layer. To obtain the polymer layer, a PLGA 37.25/25 solution in chloroform (10% w/v) was placed in the wells of a polystyrene 24-well plate (Nunclon™ Delta) (700 μL per well), and, in each well, 600 μL of the polymer solution was immediately drawn up. The initial addition of a large volume allowed the sides of the wells to be coated. After overnight evaporation under a hood, the plate was placed under vacuum in a freeze-dryer for 12 h. Three hundred microlitre of lysozyme solution (0.5 μg/mL) in TRIS–HCl (0.01 M, pH 7.4) buffer was then added in each polymer-coated well. After 2 h of incubation at room temperature, the "free" lysozyme (not adsorbed by the polymer) was quantified and the amount of adsorbed lysozyme was deduced by mass balance.

The effects of additives (in solution or blended with PLGA 37.5/25) and polymer type on lysozyme/polymer interactions were studied. The additives were respectively co-dissolved with lysozyme in the TRIS–HCl buffer prior to incubation with the polymer, or they were co-dissolved with the polymer itself in chloroform for blending. The additive/polymer ratio was chosen according to the literature. The effect of modifying the polymer type was also studied. Simultaneously, a control without protein was performed to assess the ability of the additives to induce lytic activity on a *M. lysodeikticus* suspension. The experiments were duplicated for each additive.

2.2.3. Microsphere preparation

2.2.3.1. PLGA microspheres. Microspheres were prepared using an s/o/w emulsion solvent extraction/evaporation process adapted from Péan et al. [6]. An organic solution (2 mL; 3:1 methylene chloride/acetone) containing 150 mg of polymer was emulsified in a poly(vinyl alcohol) aqueous solution (90 mL, 4%w/v) maintained at 1 °C and mechanically stirred for 1 min (Heidolph RZR 2041, Merck Eurolab, Paris, France). After the addition of 100 mL of deionised water and stirring for 10 min, the resulting o/w emulsion was added to deionised water (500 mL) and stirred at 550 rpm for a further 20 min to extract the organic solvent. Finally, the formed microparticles were filtered on a 5-μm filter (HVLP type, Millipore SA, Guyancourt, France), washed with 500 mL of deionised water and freeze-dried before storage at +4 °C. 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).

2.2.3.2. Microspheres loaded with lysozyme. The protein loading was 0.6% w/w with respect to the amount of polymer. Firstly, NaCl and glycofurol, a water-miscible non-solvent of proteins, were used to precipitate lysozyme. Then, at room temperature, 45 μL of a 0.3 M NaCl solution containing 900 μg of protein was added to glycofurol to form a 1-mL suspension. 30 min later, the protein particles were recovered by centrifugation (10,000*g*, 30 min). They were then

Table 1
Physical characteristics of the studied polymers.

Code name	Polymer	Copolymer ratio (L-LA/GA%)	% PEG in mass	Mn PEG	Mn polyester	Mn total	Supplier
PLGA37.5/25	PLGA	37.5/25			14,000[a]	14,000[a]	Phusis
PLA	PLA50				22,700[a]	22,700[a]	Phusis
PLGA25/50	PLGA	25/50			7900[b]	7900[b]	Boehringer
PLGA–PEG	PLGA–PEG	25/50	10	5000	44,000	49,000[c]	Boehringer
t-LG25/50-PEG9	PLGA–PEG–PLGA	25/50	9	6000	58,000	64,000[c]	Boehringer
t-L96-PEG24	PLA96–PEG–PLA96		24	20,000	32,500	85,000[c]	IBMM-CRBA
t-L50-PEG23	PLA50–PEG–PLA50		23	20,000	34,000	88,000[c]	IBMM-CRBA
t-L50-PEG8	PLA50–PEG–PLA50		8	20,000	117,000	254,000[c]	IBMM-CRBA

[a] Given by SEC in THF.
[b] Given by the supplier for the studied batch.
[c] Determined by using the integration ratio of resonances due to PEO blocks at 3.6 ppm, PGA blocks at 4.76 ppm and to PLA blocks at 5.19 ppm in the ^{1}H NMR spectra.

carefully dispersed in the organic solution and the microspheres were prepared as described above.

2.2.3.3. Microspheres loaded with lysozyme/additive. When the effect of the co-encapsulated excipients was tested, they were added to the protein solution before mixing with glycofurol in an additive to protein ratio of 50:1 (w/w) for PEG 8000 and 10:1 (w/w) for poloxamer 188 and BSA.

2.2.3.4. Microspheres made of a different matrix. In some preparations, additives (PEG 8000 and poloxamer 188) were dissolved with the polymer in the organic solution (1:2 (w/w) additive/polymer) to assess the influence of additive/polymer blends on the release profile. In the same way, the PLGA 37.5/25 reference was replaced by other polymer types (PLGA 25/50, t-PLGA25/50-PEG9, PLGA–PEG, t-L96-PEG24, t-L50-PEG23, t-L50-PEG8). To elicit a better comparison of the results from *in vitro* release studies, the formulation parameters were unchanged except for the stirring speed which was adjusted (from 550 to 850 rpm) for a similar microsphere diameter.

2.2.4. Protein encapsulation efficiency

Protein encapsulation yield was determined considering the biologically active entrapped protein. Lysozyme PLGA microspheres (10 mg, 3 batches) were dissolved in 0.9-mL DMSO in a silanised glass tube. After 1 h, 3 mL of 0.05 M HCl was added. The solution was left to stand for one more hour and was then incubated with *M. lysodeikticus* suspension for lysozyme activity determination.

2.2.5. In vitro release study

The *in vitro* release profile of lysozyme from PLGA microspheres was determined by adding 500 μL of 0.05 M TRIS–HCl buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl to 10 mg of microspheres, into the centrifugation tubes. The tubes were closed, incubated in a shaking water bath (37 °C, 125 rpm). At determined time intervals, the tubes were centrifuged for 5 min at 3000 rpm. The 500 μL of the supernatant was collected for analysis and replaced by fresh buffer. The percentage of biologically active released lysozyme was measured by enzymatic assay.

2.2.6. Effect of poloxamer 188

2.2.6.1. Lysozyme–FITC distribution within microspheres. Lysozyme–FITC was prepared as defined by Bezemer et al. [18]. Microspheres were loaded with lysozyme–FITC or with lysozyme–FITC/poloxamer 188 as described above. A laser-scanning confocal imaging system (Olympus light microscope Fluoview FU300, Paris, France) was used to observe lysozyme–FITC distribution within the microspheres. Dry microspheres were dispersed on a glass slide; fluorescence images of cross sections were taken by optical sectioning. All the images were obtained using a single resolution.

2.2.6.2. Poloxamer quantification. The poloxamer content in the formulation was determined by a colorimetric method based on the formation of a colour complex between polyethylene oxide segments of poloxamer and cobalt(II) thiocyanate. The method was adapted from Mao et al. [19] and Ghebeh et al. [20]. The cobalt thiocyanate reagent was prepared by dissolving 3 g of cobalt nitrate and 20 g of ammonium thiocyanate in 100 mL water. Forty microlitre of standard poloxamer, 600 μL of cobalt thiocyanate reagent, 100 μL of ethyl acetate and 200 μL of absolute ethanol were mixed well in a tube and centrifuged (1 h, 13,360*g*). After centrifugation, the supernatant was carefully removed. The sediment was washed three times with 100 μL of ethanol. The sediment was dissolved in 1 mL of acetone and the absorbance was measured at 624 nm. A standard curve was constructed, and the concentration of poloxamer in the unknown samples was accordingly determined. Absorbance measurements were carried out in triplicate. The detection limit of this method was assessed at 0.1 mg/mL.

2.2.6.3. Raman micro-spectroscopy measurement. To look for the possible molecular interactions of lysozyme with poloxamer, the molecular mixtures of these compounds were analysed by Raman micro-spectroscopy.

The Raman spectra were recorded on a LabRam (Jobin Yvon, Horiba) confocal scanning Raman microspectrometer equipped with an Olympus BX 41 microscope and an internal, air-cooled, helium–neon laser source providing radiation at 632.8 nm. The Raman scattering was excited and collected through a ×50 objective. The powder samples were analysed directly, without any special preparation. The aqueous solutions were deposited onto a microscope glass slide and dried under a hood at ambient conditions (ca. 20 °C). No sample degradation was observed under the conditions used (2 mW laser power on the sample, nine scans of 20 s). A computer with LABSPEC 4.04 software was used for data acquisition and treatment. The spectra presented are the averages of at least three independent measurements, corrected for fluorescence background with a spline function.

The improved spectral resolution obtained in Raman makes this technique more suitable than IR to analyse the mixtures. However, to detect protein vibrations from the PLGA microparticles with relatively weak protein content, FTIR spectroscopy appeared more sensitive than Raman. Therefore, we monitored the conformation of the encapsulated lysozyme through the software-assisted analysis of the vibrational bands in the Amide I region (1700–1600 cm^{-1}) of the FTIR spectra.

2.2.6.4. FTIR spectroscopy. The secondary structure of lysozyme with or without poloxamer 188 inside the microspheres was determined by FTIR spectroscopy. Microspheres loaded with 5% w/w of protein (with respect to the amount of PLGA) were studied in order to detect the protein. FTIR studies were conducted with a Brücker IFS 28 equipped with a DTGS detector. Five hundred scans (4000–400 cm^{-1}) at 2 cm^{-1} resolution were averaged to obtain each spectrum. Lyophilised microspheres were measured as KBr pellets (4–5 mg of microspheres per 200 mg of KBr). All spectra were analysed in the amide I region (1700–1600 cm^{-1}) using the version 2.0 OPUS program. In all cases, a linear baseline between 2000 and 1800 cm^{-1} was subtracted. The infrared band position and the number of bands in the amide I region were calculated by using the Levenberg–Marquardt algorithm using the OPUS program. The secondary structure contents were calculated from the area of the individual assigned bands and their fraction of the total area in the amide I region.

2.2.7. Contact angle measurements

In order to study the hydrophilicity of the polymer surfaces, the dynamic contact angles were measured with a Krüss Processor Tensiometer K100 by immersing polymer-coated glass in deionised water. The contact angle data for each sample were averages of three individual measurements. The measurements were performed at 26 °C.

3. Results

3.1. Lysozyme/polymer interaction study

To achieve better, sustained protein release from PLGA microspheres, formulation components are often modified [21–23]. The choice of appropriate excipients is generally the result of an experimental observation of their effect on the *in vitro* release pro-

file, after their microencapsulation. In the present study, a two-dimensional model was designed to better predict the effect of additives on the release profile; *i.e.* lysozyme adsorption onto a PLGA surface was quantified and its inhibition by numerous additives was rapidly evaluated.

Since protein concentration, temperature, pH and ionic strength influence protein interactions with hydrophobic surfaces, they were controlled during these experiments [24,25]. The values of these parameters were fixed at levels where protein adsorption to the PLGA 37.5/25 surface is the most important and the most rapid. Accordingly, with a low protein concentration of 0.5 μg/mL, a temperature of 37 °C and a buffer where lysozyme is stable, 72% of the initial protein amount was adsorbed onto the PLGA 37.5/25 layer within 2 h (Fig. 1-1A).

Thanks to the model developed, the effect of formulation components to limit lysozyme adsorption was determined: the effi-

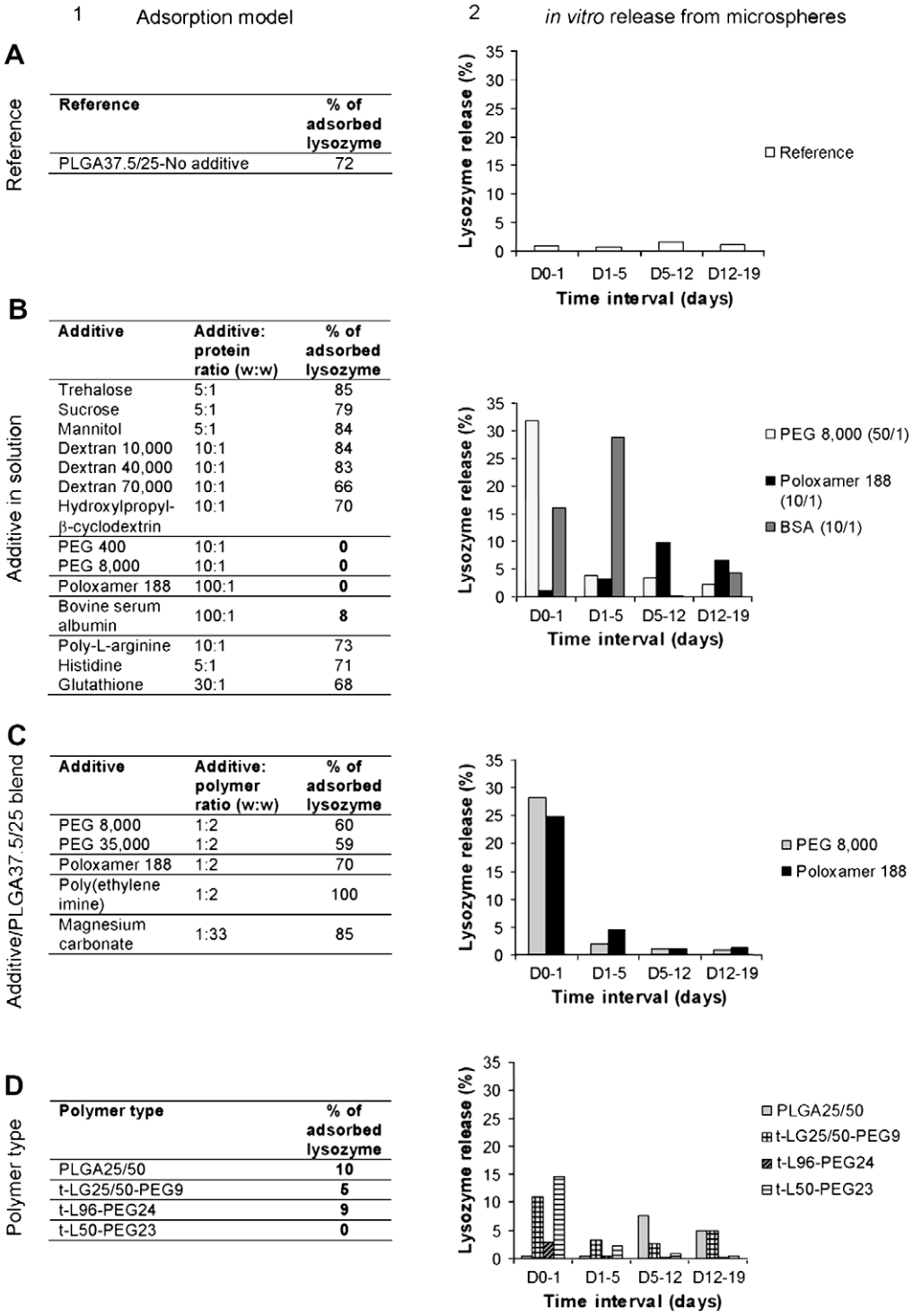

Fig. 1. Effect of formulation components on lysozyme adsorption onto a polymer surface (1) and on *in vitro* lysozyme release profiles from microspheres over 19 days (2). Release is expressed as a percentage of released lysozyme during the interval time period. (A) The reference corresponds to a formulation of lysozyme without additive and to PLGA37.5/25, (B) additives were co-dissolved with lysozyme, (C) additives were blended with the polymer within the layer, and (D) polymer type was modified.

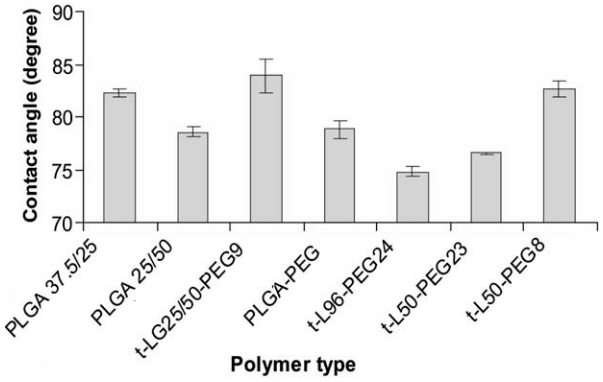

Fig. 2. Contact angles of the studied polymers.

ciency of additives in solution, additives blended with PLGA37.5/25 or the polymer type, was monitored (Fig. 1-1). In a first part of the study, additives were co-dissolved with the protein prior to contact with the PLGA 37.5/25 surface. The additives most commonly employed in PLGA microspheres were tested: polyalcohols (trehalose, sucrose, mannitol, dextran) [26–29], hydrophilic polymers (PEGs [7]), a surfactant (poloxamer 188 [14,30]), a protein (Bovine Serum Albumin (BSA) [31]) and amino acids (poly-L-arginine, histidine, glutathione). As shown in Fig. 1-1B, polyalcohols and amino acids were unable to prevent lysozyme adsorption on the PLGA surface. It is noticeable that they had no effect, even when their concentration was enhanced or when they were first incubated with the PLGA layer. On the contrary, PEGs, poloxamer 188 and BSA considerably reduced lysozyme adsorption: the percentage of adsorbed lysozyme dropped from 72% to 0–8%. Otherwise, PEGs and poloxamer were blended with PLGA 37.5/25 within the layer. Results showed that, in this form, they did not shield lysozyme from adsorption (Fig. 1-1C). Even when a higher molecular weight PEG (35,000) was used, lysozyme was still adsorbed to the PLGA surface. Malzert et al. showed that PEG segments could desorb from the interface, allowing lysozyme molecules to adsorb to the hydrophobic polymer [32]. Assuming that positive charges on PLGA surface could keep away positively charged lysozyme, a cationic polymer, poly(ethylene imine), was blended with PLGA. However, the expected effect did not occur and the lysozyme was still adsorbed onto the surface (100% adsorption). As basic salts are often employed to improve protein release from PLGA devices when a polymer starts to degrade (by counteracting the local pH drop) [33], magnesium carbonate was added to the PLGA within the layer to detect any additional effect on protein–polymer interactions during the initial release period. No beneficial effect on protein adsorption was noticed.

In a further study, the polymer type was modified. The reference polymer (PLGA 37.5/25) was replaced by a PLGA 25/50 and di or tribloc copolymers (Table 1). With these polymers, lysozyme adsorption did not occur (10% of adsorbed lysozyme or less) (Fig. 1-1D). Contact angle measurements showed that these polymers are mainly more hydrophilic than PLGA 37.5/25 (Fig. 2). This feature could explain the lower protein adsorption on the surface of these polymers.

3.2. Effect of formulation components on the in vitro release of lysozyme over 3 weeks

3.2.1. In vitro release from microspheres

PLGA 37.5/25 microspheres loaded with lysozyme were prepared by an s/o/w technique. As shown in Fig. 1-2A, lysozyme release over 19 days from these microspheres was very low. The above data suggest that protein in solution tends to adsorb to PLGA and that additives inhibiting protein adsorption could improve the protein release profile at least during early-stages when lysozyme release is mainly controlled by adsorption phenomena [15]. Hence, lysozyme-loaded microspheres were also prepared with selected additives and with various polymer types. The mean particle size of the microspheres was 60 ± 23 μm (volume weighted) and the mean encapsulation efficiency was 73 ± 5% as determined by lysozyme activity measurement. Regarding the lysozyme release profile, marked differences were observed (Fig. 1-2). Therefore, when BSA, PEG 8000 and poloxamer 188 were co-dissolved with lysozyme prior to its precipitation and subsequent encapsulation, its release was markedly increased (Fig. 1-2B). Nevertheless, this effect was different according to the hydrophilic nature of the excipients. Hence, BSA and PEG 8000, which are hydrophilic, induced a higher lysozyme release during the first day (16% and 32%, respectively) but low subsequent release. They diffused rapidly out of the microspheres and did not assure further lysozyme protection [26]. On the contrary, poloxamer 188, which is amphiphilic and resisted initial hydration, exhibited lysozyme-sustained release over almost 20 days. As expected from the above results, when PEG or poloxamer 188 was blended with PLGA, sustained release was not achieved despite their frequent use [34–37] (Fig. 1-2C). They favoured initial protein release by forming pores in the PLGA matrix but they did not inhibit lysozyme adsorption on the matrix. Finally, the polymer constituting the microspheres was modified and more hydrophilic polymers were used (Fig. 1-2D). When PLGA 25/50 was employed, an increased release rate actually occurred between Day 5 and Day 19 (D5–12; D12–19). However, a time lag was observed before the fifth day as almost no lysozyme was released. The use of PLA–PEG–PLA (t-L96-PEG24 and t-L50-PEG23) was not beneficial to favour lysozyme-sustained release; indeed, protein retention within the microspheres was still observed after the first day. Nevertheless, the triblock copolymer PLGA–PEG–PLGA (t-LG25/50-PEG9) displayed encouraging results with an almost continuous release over time.

3.2.2. Effect of poloxamer 188

In a previous study carried out by our team [38], it was shown that spherical, nanosized protein particles were formed by the addition of a salt (sodium chloride) and a water-miscible organic solvent (glycofurol) to protein aqueous solution. Poloxamer is practically insoluble in glycofurol (0.87 ± 0.08 mg/mL, 24 h under magnetic stirring at room temperature). However, its solubility increases (×10) in the medium used for protein precipitation (mix of sodium chloride aqueous solution and glycofurol). Consequently, after protein precipitation, a part of the poloxamer was dissolved in the supernatant; the residual poloxamer/protein ratio in the sediment was estimated at 1:1 (w/w) (1.6:1 mol/mol).

A study of fluorescent protein (lysozyme–FITC) distribution within the microspheres by confocal microscopy revealed that these protein particles were packed within the matrix in the absence of an additive (Fig. 3A). When poloxamer 188 was added to in the formulation, the size of the protein particles remained unchanged, but the protein was well distributed within the polymer matrix (Fig. 3B).

For Raman analysis, the poloxamer and lysozyme were mixed (1:1 and 5:1 w/w ratio) in aqueous solutions complemented with 0.3 M NaCl. These samples (analysed after drying) exhibited Raman spectra with recognisable contributions from bands of both lysozyme and poloxamer, without significant band perturbation compared to the corresponding reference spectra of these molecules in powder (Fig. 4). The fitting of the Raman spectra recorded from different microscopic regions of the same dried sample (1:1 w/w ratio) revealed heterogeneous distribution of the two molecules:

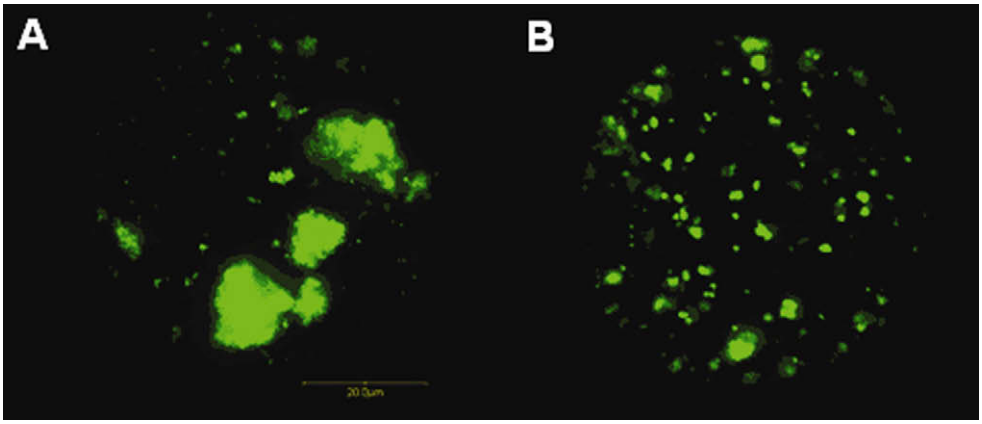

Fig. 3. Lysozyme–FITC distribution within PLGA37.5/25 microspheres without (A) or with poloxamer 188 (B). The bright zones represent the lysozyme–FITC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Secondary structure of lysozyme under various conditions (as determined by FTIR spectroscopy).

Conditions	% α-Helix	% β-Sheet
Native lysozyme (powder)	24.5	37.9
Lysozyme in PLGA microspheres	24.9	34.9
Lysozyme + poloxamer188 (1/10) in PLGA microspheres	23.9	20.2

the fitting coefficients were comprised between 0.15/0.85 and 0.79/0.21 for poloxamer/lysozyme contributions. With the 5:1 w/w poloxamer/lysozyme ratio, the molecular repartition was also heterogeneous (data not shown). The absence of Raman band perturbation and heterogeneous molecular distribution both indicated no interaction between lysozyme and poloxamer 188 in the aqueous solution. This conclusion was confirmed by the data of surface plasmon resonance (not shown).

To characterise the protein encapsulated in the presence of poloxamer 188, the secondary structure of encapsulated lysozyme was characterised by Fourier transform infrared (FTIR) spectroscopy. The analysis of the Amide I region of the FTIR spectra (summarised in Table 2) demonstrates that the encapsulation in the presence of poloxamer-induced protein structural perturbations in the β-sheet content.

3.3. Effect of the matrix composition on the in vitro release of lysozyme- poloxamer 188 after 3 weeks

Whereas protein encapsulation with poloxamer 188 was found to be interesting to limit protein adsorption in early-stage release, it was insufficient to achieve complete protein release, and protein release levelled off after the first 20 days (2.76% of release between Day 21 and Day 35) (Fig. 5A). Among the potential causes of this phenomenon, acid-induced protein aggregation was reported to be significant [39,40]. Indeed, during PLGA degradation, acidic degradation products are formed which cause a pH drop within the matrix. To counteract the formation of this acid-induced aggregation, blends of the polymer with additives within the matrix as well as less-denaturing polymers were tested.

Firstly, in order to favour the release of the protein and of the acidic degradation products, PEG was blended with PLGA 37.5/25 in lysozyme-poloxamer 188 microspheres. No positive effect on lysozyme release was induced between Days 21 and 35, similar

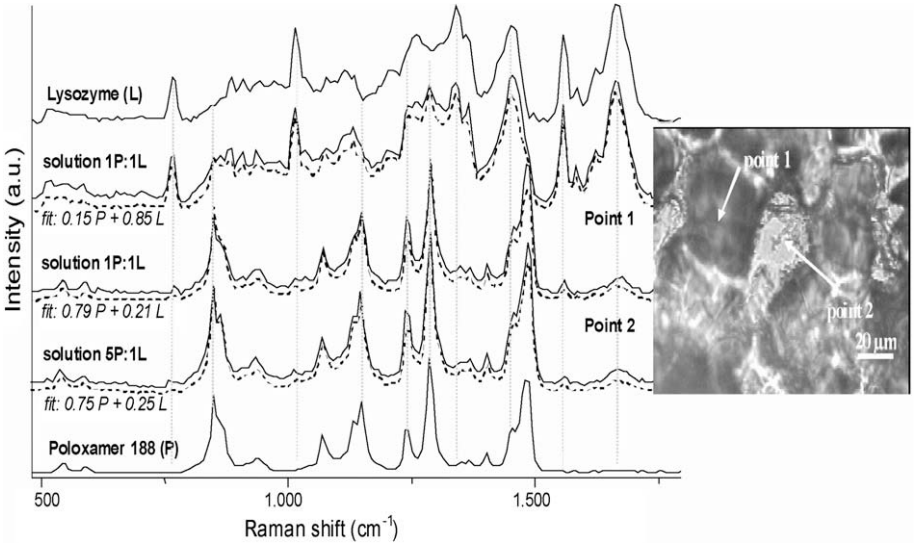

Fig. 4. Typical Raman spectra (solid lines) of poloxamer 188 and lysozyme alone (reference spectra from powder) and in mixture prepared at 1:1 and 5:1 w/w ratio in aqueous solutions complemented with NaCl (spectra from different points of dried samples). Dashed lines show the fit of the mixture spectra with a proportional contribution of the reference spectra. Insert: microscopic image illustrating the heterogeneity of a dried sample corresponding to a 1:1 poloxamer/lysozyme molecular mixture.

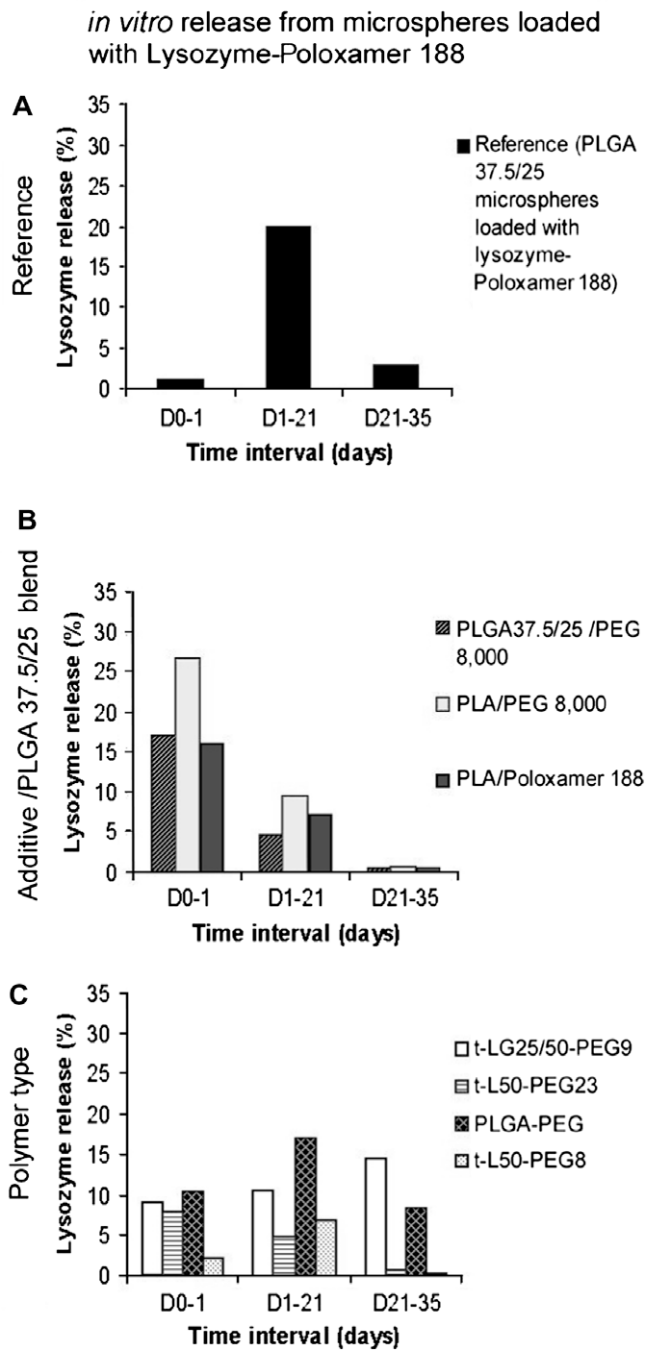

Fig. 5. Optimisation of lysozyme *in vitro* release from microspheres after Day 21. The reference corresponds to microspheres of PLGA 37.5/25 encapsulating lysozyme–poloxamer 188. Release is expressed as a cumulative percentage of released lysozyme during each time interval.

to what was observed during the first 3 weeks (Fig. 5B). Microspheres made of a slow-degrading PLA were also prepared to assess the effect of delaying the hydrolytic cleavage of ester bonds. To counterbalance this slow degradation and to perform sufficient release of the protein, poloxamer 188 and PEG 8000 were each blended with PLA. The release profile was not improved for later stage release (Fig. 5B). The beneficial effect of co-encapsulated poloxamer 188 was maintained during the first 3 weeks, but later, protein retention was observed. When the PEG polymer was covalently conjugated to the PLA, a plateau was also reached after 3 weeks of release (Fig. 5C). These profiles may be related to the

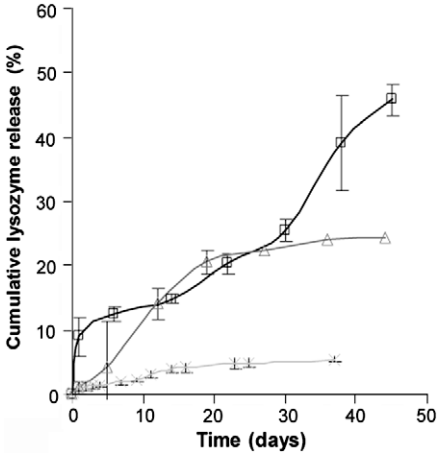

Fig. 6. *In vitro* release profile of lysozyme (mean ± SD) from PLGA microspheres without poloxamer 188 (×) (n = 2 batches, twice) and with poloxamer 188 (△) (n = 3 batches) and from PLGA–PEG–PLGA microspheres containing poloxamer 188 (□) (n = 7 batches).

destabilising environment of the hydrophobic polymer. Thus, in order to further increase the delay of polyester degradation and of the outbreak of acidic compounds, a high molecular weight copolymer (t-L50-PEG8) was tested. This strategy was unsuccessful. Interestingly, the use of a tribloc PLGA–PEG–PLGA copolymer and of a diblock PLGA–PEG copolymer allowed active lysozyme continuous release over at least 35 days (Fig. 5C). Fig. 6 represents the cumulative lysozyme release from PLGA–PEG–PLGA microspheres. Lysozyme release continued over 45 days and almost 50% of active lysozyme was continuously released after a limited burst of about 10%. Some batches, followed after Day 45, showed that the release then levelled off. At this stage, lysozyme–FITC was trapped into the polymer matrix and only 1% of the protein was still active by Day 50.

4. Discussion

Protein adsorption to PLGA is a critical factor affecting protein release kinetics from PLGA microspheres [15,41,42], and no universal strategy currently exists to avoid this phenomenon. That is the reason why we chose to design a two-dimensional model to select formulation components able to circumvent this event. Previous studies on protein–PLGA interactions for the development of protein-loaded PLGA microspheres considered the influence of the release medium [41] and of pegylating the protein [42] but, to our knowledge, none compared the effect of formulation components. In this study, the adsorption duration was reduced to 2 h, and the anti-adsorption effect of numerous excipients was rapidly screened. This was achieved by working with a low protein concentration (0.5 μg/mL) and a sensitive protein-quantification method. Indeed, at low concentrations, the protein fraction adsorbed to a hydrophobic surface is higher, and changes in protein concentrations in the supernatant are more easily detectable [42]. To create favourable conditions for lysozyme adsorption in this model, the temperature was set to 37 °C during the experiment since adsorption increases with the temperature [25,41]. Jiang et al. reported that lysozyme was more prone to adsorption at pH 7.4 [41]. So, to set up conditions whereby maximum amounts of lysozyme adsorb to PLGA, and to approach the most suitable physiological conditions, a medium pH level of 7.4 was used. Under these conditions, rapid lysozyme adsorption on the PLGA surface was observed. Interestingly, these conditions (low amount of protein, 37 °C, TRIS–HCl 0.01 M, pH 7.4 buffer) are sim-

ilar to those of the *in vitro* release study and are presumed to fit lysozyme behaviour during release from PLGA microspheres. By using PLGA-coated microplates, the rapid screening of numerous excipients, in various forms, could be achieved and certain reported results could be explained. In particular, it was previously described that trehalose, dextran, HP-β-CD and L-arginine incorporated in PLGA microspheres were inefficient to sustain protein release [27,29]; our results actually showed that they could not inhibit protein adsorption to PLGA. It was namely described earlier that sugars were ineffective in blocking protein adsorption (at a w/o interface) [43]. Therefore, the model is useful to eliminate some candidates for the enhancement of protein release. Only BSA, PEGs and poloxamer 188 were able to block lysozyme adsorption; this is consistent with their properties at interfaces. Indeed, the surface-active properties of albumins have been widely documented [43,44]. Poloxamers are also frequently used to minimize protein adsorption onto hydrophobic surfaces due to their active surface properties and their biocompatibility [45–47]. They are triblock copolymers of the type PEO–PPO–PEO, where PEO and PPO are poly(ethylene oxide) and poly(propylene oxide). Hydrophobic PPO centre blocks attach to the surface, and longer PEO tails build a steric barrier between the surface and the protein molecules. PEG can also sterically displace the protein from its interface with the polymer surface [7]. Nevertheless, among the excipients identified as inhibiting protein adsorption, poloxamer 188 was the only one which allowed a sustained release of lysozyme from the PLGA microspheres, presumably because of its stronger interactions with hydrophobic surfaces (with the PPO segment) [48].

Previous studies have reported the co-encapsulation of protein with poloxamer 188 with limited success. By the w/o/w process, the co-encapsulation of a surfactant led, in most cases, to a faster release of the encapsulated protein during Day 1, but not to a further increase in the release rate because of the rapid loss of the surfactant. Similarly, when poloxamer 188 was blended with PLGA, Sanchez et al. [49] showed an increase in the initial release but no subsequent release, as confirmed in the present study. The only study which reported on continuous protein release in the presence of poloxamer 188 dealt with the structure of reservoir-type microparticles made by a 'polymer-alloys technique' [30]. In the present study, the effect of poloxamer on protein release from PLGA microspheres was quite different because of its co-precipitation prior to encapsulation. Only a small fraction of poloxamer 188 (1/10 of the initial amount) was precipitated with the protein, but the final poloxamer/lysozyme ratio (1:1 w/w) was sufficient to favour protein release from the microspheres. According to vibrational spectroscopy data obtained in this study (both by Raman and FTIR techniques), the poloxamer has no strong molecular interaction with lysozyme; in any case, this interaction is not strong enough to significantly modify the protein conformation. Poloxamer 188 could be of interest for several reasons. On the one hand, poloxamer prevents self-association of the protein particles (even at low concentrations) [50] and allows a more homogeneous protein distribution within the microspheres. On the other hand, by hydrophilisation of the polymer surface, poloxamer contributes to a reduction in non-specific adsorption of the protein on the PLGA surface [51] during microsphere hydration. Moreover, the gelling properties of poloxamer may also play a key role during the release period. Although poloxamer 188 concentration in aqueous solution prior to particle formation is below the CMC at room temperature [52,53], a liquid–crystalline phase transition of poloxamer may occur by desolvating with glycofurol, suggesting the formation of a gel-like structure inhibiting aggregation between protein molecules.

The presence of poloxamer 188 in the formulation exhibited a continuous release of lysozyme over 3 weeks. Afterwards, a plateau was reached because of additional destabilising mechanisms concomitant with polymer degradation. Therefore, we chose to modify the degradation behaviour of the biodegradable matrix. By using a PLGA–PEG–PLGA triblock copolymer (t-LG25/50-PEG9), continuous release was obtained. The reason for such an effect cannot be restricted to a more hydrophilic feature. Indeed, as shown in Fig. 2, PLGA–PEG–PLGA presented quite similar contact angles as PLGA37.5/25; besides, polymers which were more hydrophilic (PLGA25/50, t-L96-PEG24, t-L50-PEG23) were unable to prolong the release. The favoured release profile with PLGA–PEG–PLGA was rather a consequence of a microphase separation and the formation of a physically, cross-linked biodegradable hydrogel upon contact with water [54,55]. Because of enhanced water uptake, the onset of an erosion period was shorter and the lag phase, observed for example with PLGA 25/50, was avoided [56]. Moreover, the PLGA–PEG–PLGA degradation was faster than that of PLGA and PLA–PEG–PLA copolymers [57]. Due to the swollen pore structure, a rapid exchange of ions might be feasible leading to reduce the aggregation and denaturation of the protein. The PEO block itself also promotes the stability of proteins lowering protein aggregation [58]. Kissel and coworkers reported EPO release from similar systems for 2 weeks [59].

In this study, by co-precipitating lysozyme with poloxamer and encapsulating it within PLGA–PEG–PLGA microspheres, a continuous release over 45 days with limited initial burst (9%) was observed. Interestingly, diblock copolymers exhibited a similar effect. Incomplete protein release due to protein destabilisation will still have to be clarified. We can assume that after 45 days, considering the wettability of the polymer, the poloxamer and the PEG segments are leached, thereby limiting their protective function. The hydration kinetics of the microspheres had to be adapted for optimal protein release. Therefore, the optimisation of the copolymer composition, the type and proportion of poloxamer co-precipitated with the protein, is now in progress.

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