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Research paper

Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres

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Abstract

The goal of this work is to develop poly(lactide-co-glycolide) (PLGA) nanospheres designed to deliver proteins for extended periods of time. To accomplish this goal a water-in-oil-in-water emulsion technique was conveniently modified. A study was performed to evaluate how the solvent elimination procedure, the copolymer type (different molecular weight and containing either free or esterified carboxyls) and the surfactant Poloxamer 188 affected the properties of the nanoparticles. The size of the nanospheres become larger (from 300 to 600 nm) by increasing the copolymer molecular weight and by the incorporation of Poloxamer 188. The protein loading efficiency varied from 40 to 90%, reaching the maximum values for the formulations made of PLGA with free carboxyls. However, the co-encapsulation of Poloxamer 188 reduced the protein loading. The in vitro protein release rate, was fairly constant after an initial burst release. The release rate was significantly reduced for the copolymer with terminal free carboxyls but was enhanced by the incorporation of Poloxamer 188 in the nanoparticles. No significant aggregation or fragmentation of the encapsulated protein was observed after incubation for 1 month. Consequently, these nanoparticles can be proposed as new controlled release protein delivery systems. © 1997 Elsevier Science B.V.

Keywords: Biodegradable nanospheres; Poly(lactide-co-glycolide); Multiple emulsion; Protein delivery; Protein stability

1. Introduction

At present, protein delivery is a very promising area of research, due to the recognised necessity of improving the in vivo efficacy of the newly developed therapeutic as well as antigenic proteins. Among the protein delivery systems intended for parenteral administration, the poly(lactide-co-glycolide) (PLGA) microspheres, have been shown to have an important potential because of their ability to control the release of model and antigenic proteins [1,2]. Furthermore, these microspheres have also been revealed as promising carriers for the transport of antigens through the nasal and oral epithelia [3–5]. In this latter sense, several authors [6,7] have shown, a number of years ago, that the size of the microspheres is a crucial parameter since it determines the uptake of the encapsulated antigen by the immune system. More specifically, their studies evidenced that microspheres less than 10 μm were preferable to those of a larger size in terms of their improved uptake by the Peyer's patches and further antigen presentation to the immune system. More recently, Jani et al. [8–10] and Jenkins et al. [11] published an important work dealing with the oral uptake and in vivo distribution of polystyrene particles. These authors showed that the uptake of the nanospheres is much greater than that of the microspheres. In addition, they observed that the in vivo distribution of the particles which were taken up by the Peyer's patches was highly affected by their size. These results indicate that nanospheres are expected to disseminate systemically whereas microspheres are ex-

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pected to remain and deliver their content in the Peyer's patches. Despite this information, which shows the great potential of the nanospheres as protein delivery systems for mucosal administration, it is surprising that there is no relevant published work dealing with the encapsulation and controlled release of hydrophilic proteins from PLGA nanospheres. In this context, it is worthwhile to mention that the techniques described in the literature for the production of PLGA nanoparticles involve the dissolution of the active compound in an organic solvent, a method that is not applicable, in general, for the encapsulation of proteins [12].

On the other hand, looking at the factors that govern the release of proteins from PLA and PLGA microspheres, it has been generally accepted that the polymer degradation rate substantially affects the release rate of the entrapped protein [13–17]. However, almost no attention has been paid to the protein–polymer affinity and its consequences on the in vitro protein release rate. Our group has, very recently, reported that the use of new PLGA copolymers with free carboxylic end groups may drastically modify the in vitro release of the entrapped protein [18]. These results show the necessity of further investigation of the mechanisms and factors which control the release of proteins from PLGA-based systems.

Taking into account this information, the primary goal of this work has been to create PLGA nanospheres with an important capacity for the association and controlled release of proteins. Another purpose of this work was to improve the knowledge of the complex mechanisms that govern the release of proteins from PLGA systems. With this objective in mind, we used the bovine serum albumin (BSA) as a model protein and various PLGA copolymers differing in their molecular weight and also in the esterification of the terminal carboxyl groups of the polymer chain.

2. Materials and methods

2.1. Materials

BSA and fluorescent labeled bovine serum albumin (FITC-BSA) were purchased from Sigma Chemical (Madrid, Spain). D,L-PLGA 50/50 (lactic acid/glycolic acid) copolymers (Resomer® RG 502, RG 503 and 503H) were purchased from Boehringer (Ingelheim, Germany). The difference between the PLGA types called Resomer® RG 502 and 503 lies in their molecular weight (MW) and thus in their viscosity in chloroform (0.2, 0.4 dl/g, respectively), whereas the difference between the PLGA called Resomer® RG 503 and 503H lies in the higher number of carboxyl groups in the 503H type. The emulsifying agents polyvinyl alcohol (PVA) (molecular weight 30 000–70 000) and Poloxamer 188 (Pluronic® F68) were obtained from Sigma Chemical (Madrid, Spain) and BASF AG (Ludwigshafen, Germany), respectively. The solvents ethyl acetate and isopropyl alcohol were purchased from Panreac (Madrid, Spain).

2.2. Nanoparticle preparation

The formation of BSA loaded-nanospheres was achieved by conveniently adjusting the multiple emulsion (w/o)/w technique, previously applied to the encapsulation of proteins into microspheres [17]. The adjustment was based on the use of ultrasounds in the two-step emulsification process, thus reducing significantly the size of the dispersed globules. Briefly, 2 mg of BSA were dissolved in 50 μl of distilled water and then emulsified in a 1 ml solution of PLGA in ethyl acetate (200 mg/ml) by sonication (Branson 250, Sonifier®) for 15 s (15 W) in an ice bath. Then, 2 ml of an aqueous PVA solution (1% w/v) were added to this first emulsion and the resulting (w/o)/w emulsion was sonicated again for 15 s (15 W). The double emulsion was diluted in 100 ml PVA solution (0.3% w/v) and the solvent was rapidly eliminated either by evaporation under vacuum or by extraction with 100 ml of an aqueous isopropyl alcohol solution (2% v/v). Finally, the nanospheres were isolated (by centrifugation at 10 000 × g for 15 min) and washed three times with water.

The effect of several variables on the characteristics of nanospheres was evaluated. The variables included: type of copolymer, solvent elimination procedure and incorporation of a protein stabiliser (Poloxamer 188) (Table 1). Poloxamer 188 and BSA were dissolved in the same amount in the inner water phase (1% BSA/1% Poloxamer with respect to the total amount of PLGA).

2.3. Physicochemical properties of nanospheres

The morphological examination of nanospheres containing BSA was performed using transmission electron microscopy (TEM) (CM12 Philips). The samples were placed on copper grids with Formvar® films and stained with 2% (w/v) phosphotungstic acid for TEM viewing.

Measurements of particle size and zeta potential of the nanospheres were performed, respectively, by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA) using a Zetasizer® III (Malvern Instruments). Each determination was performed on, at least, three samples from different batches.

2.4. Determination of protein content in nanospheres

The amount of protein entrapped within PLGA nanospheres was determined by two alternative techniques: one involved the dissolution of the polymer in

Table 1
Processing conditions in the preparation of BSA-PLGA nanospheres and results of mean average particle size and zeta potential as determined by PCS and LDA

Formulation	Copolymer	S.E.[a]	Surfactant[b]	Mean size ± S.D.[c] (nm)	ζ Potential ± S.D.[c] (mV)
A	Resomer® RG 502	Extraction	Poloxamer 188	347 ± 4 (0.163)[d]	−24 ± 1
B	Resomer® RG 502	Evaporation	Poloxamer 188	323 ± 2 (0.164)	−28 ± 1
C	Resomer® RG 502	Extraction	—	320 ± 2 (0.114)	−29 ± 1
D	Resomer® RG 503	Extraction	Poloxamer 188	506 ± 12 (0.249)	−27 ± 1
E	Resomer® RG 503	Evaporation	Poloxamer 188	514 ± 4 (0.263)	−25 ± 1
F	Resomer® RG 503	Extraction	—	457 ± 2 (0.240)	−29 ± 1
G	Resomer® RG 503 H	Extraction	Poloxamer 188	531 ± 54 (0.303)	−32 ± 1
H	Resomer® RG 503 H	Evaporation	Poloxamer 188	521 ± 7 (0.263)	−32 ± 1
I	Resomer® RG 503 H	Extraction	—	398 ± 5 (0.217)	−30 ± 2

[a] Solvent elimination procedure: extraction vs. evaporation.
[b] Surfactant in the inner aqueous phase containing the protein.
[c] ($n = 3$).
[d] Polydispersity.

an organic solvent and further extraction of the protein into an aqueous medium (extraction technique) and the other involved the accelerated polymer hydrolysis (hydrolysis technique).

In the extraction technique, 20 mg of protein (FITC-BSA or BSA) loaded-nanoparticles were dissolved in 1 ml of ethyl acetate and, the protein was extracted into 3 ml phosphate buffer (PB) containing 0.02% (w/v) polysorbate 80, by vortexing for 15 min. Thereafter, the aqueous phase containing the protein was separated from the organic phase containing the PLGA by centrifugation at 10 000 × *g* for 15 min.

In the hydrolysis technique, 20 mg of FITC-BSA loaded-nanospheres were incubated in 5 ml of 0.1 N NaOH containing 5% (w/v) sodium dodecyl sulphate (SDS) at room temperature until the complete dissolution of the nanospheres.

Afterwards, the protein (BSA) was assayed by high performance liquid chromatography (HPLC) or, in the case of FITC-BSA, by spectrophotometry (494 nm). The HPLC conditions were: column TSK-GEL® G3000SW (Pharmazia); column temperature: 25°C; mobile phase: phosphate-buffer (pH 7.4) containing NaCl 0.2 M; flow rate: 0.8 ml/min; wavelength: 280 nm.

Results of encapsulation efficiency obtained by the extraction and the hydrolysis techniques are reported as 'EE-extraction' or 'EE-hydrolysis' respectively. Both terms represent the percentage of protein encapsulated with respect to the total amount of protein used in the encapsulation process.

2.5. Stability and integrity of BSA in PLGA nanospheres

The integrity and stability of BSA encapsulated in the nanospheres were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC techniques. The separation of the protein from the nanospheres, freshly prepared or previously incubated for 4 weeks at 37°C, was achieved by alkaline hydrolysis in the conditions specified above. For the electrophoretic analysis, samples of BSA and several protein standards were solubilized with Laemmli buffer, boiled for 30 min and loaded into a 12.5% SDS-polyacrylamide gel (Phast System, Pharmazia). After electrophoresis, the gel was stained with silver to be able to visualise the protein. The conditions for the HPLC technique were as specified above.

2.6. In vitro release studies

Samples of 20 mg of nanospheres were placed in 5-ml tubes containing 4 ml of PB (pH 7.4) and incubated, at 37°C for 4 weeks. At appropriate intervals, the samples were collected and centrifuged for 15 min at 10 000 × *g* (Centrifuge 2-15, Sigma, Spain). Two ml of supernatant was assayed for protein release and replaced by 2 ml of a fresh PB. BSA concentration in the release medium released was determined by the microBCA protein assay (Pierce, IL, USA).

2.7. Determination of the PLGA average molecular weight

Molecular weight distributions of PLGA before and after different incubation times were determined by gel permeation chromatography (GPC). The conditions were: lineal column (Phenomenex); column temperature: 35°C; mobile phase: chloroform; flow rate: 1 ml/min; detection: refractive index (Shodex RI SE-31, Spectra Physics). At the appropriate intervals, after centrifugation and lyophilization, nanospheres were dissolved in chloroform and injected into the GPC equipment. The weight average molecular weight was

calculated with reference to the polystyrene standards (Teknokroma, Spain).

3. Results and discussion

Several techniques have been proposed for the encapsulation of proteins and antigens in PLGA microspheres, including the double emulsion [13–17], phase separation [19] and spray-drying [20]. Nevertheless, to our knowledge, no method for the encapsulation and control release of hydrophilic proteins from PLGA nanospheres has been reported until now. The only reference we found in this field dealing with this subject describes the encapsulation of a water-soluble peptide in PLGA nanospheres by a coacervation technique [21]. Several disadvantages of these reported nanospheres could be: (i) their size is quite large (more than 500 nm) (ii) the technique involves the use of important amounts of oils and solvents (iii) the ability of these nanospheres to control the release of hydrophilic molecules was not presented. On the other hand, Almeida et al. [3] proposed the association of proteins to previously formed blank PLA nanospheres (0.8 μm) by a simple adsorption process. The main limitation of this approach is that the protein is only physically bound to the surface of the nanospheres, a fact that limits their protein loading capacity and their control release properties. In order to develop a new procedure for optimizing the incorporation of proteins within PLGA nanospheres we chose to adjust the water-in-oil-in-water solvent evaporation technique previously reported for the preparation of microspheres. More specifically, we modified this original procedure by minimizing the amount of organic solvents and using sonication in both emulsification steps, thereby reducing the size of the droplets and, consequently, obtaining submicron particles. The batch yield of this preparation procedure (defined by the weight of nanospheres recovered after lyophilization with respect to the initial weight of the polymer) reached values higher than 80%. Our next goal was to evaluate the possibilities of modulating the size of the nanospheres as well as their loading capacity and control release properties by analyzing systematically several process variables (Table 1). In this sense, the procedure for the elimination of the organic solvent was expected to be a relevant parameter since it determines the polymer precipitation rate and also the organic solvent environment for the protein to be encapsulated. On the other hand, the various PLGA types used in this study differ in their molecular weight and hydrophilicity, properties that could theoretically influence the protein–polymer interaction and protein release rate. The MW of the different copolymers, determined by GPC, were 15 000 and 43 000 Daltons approximately for Resomer® RG 502 and Resomer® RG 503, respectively. Concerning the hydrophilicity, Resomer® RG 503 differs from Resomer® RG 503 H by the presence of free carboxylic-end groups in the polymer chain and it has a MW of 50 000 Da. Finally, the selection of the surfactant Poloxamer 188 was based on the previous information about its ability to reduce protein aggregation [22] and protein adsorption onto hydrophobic surfaces [23].

3.1. Physicochemical characterisation of the BSA-loaded nanospheres

Results in Table 1 indicate that the mean particle size distribution and polydispersity of the nanospheres are mainly affected by the polymer molecular weight (MW) and by the incorporation of Poloxamer 188 in the protein aqueous solution. The significant influence of these parameters was confirmed by a two-way ANOVA ($\alpha < 0.01$). The minimum size (319 nm) was observed for formulation C made of the low MW PLGA in the absence of Poloxamer. The influence of the copolymer MW was interpreted by the relationship between MW and viscosity of the organic polymer solution. Low viscosity polymer solutions can be dispersed in the external aqueous phase to a greater extent than high viscosity solutions, thus leading to a reduction in the size of the nanospheres. The same effect was previously observed during the preparation of PLGA microspheres [24]. On the other hand, the enlarged size of the Poloxamer-containing nanospheres could be attributed to two previously reported observations: (i) the diffusion of the Poloxamer from the aqueous to the organic phase leading to the plasticization of the PLGA [25] (ii) the complex interaction between BSA–PLGA–Poloxamer, a phenomenon which has been shown to have a deleterious effect on the stability of the inner emulsion [26]. In this sense, other authors who used the double emulsion technique for the preparation of microspheres

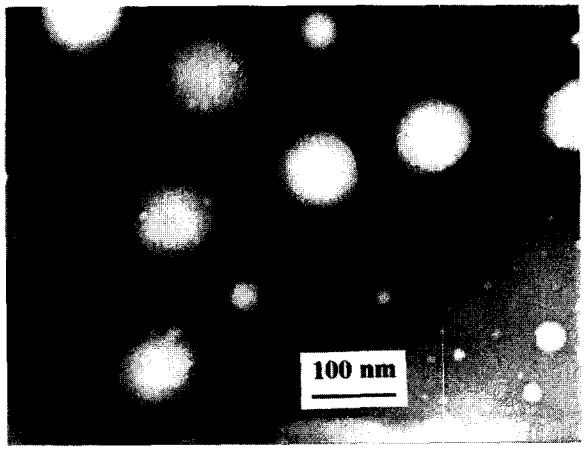

Fig. 1. A TEM photograph of nanospheres containing BSA. Formulation A.

Table 2
Encapsulation efficiency of protein-loaded nanospheres as determined by extraction of the protein in PB containing 0.02% polysorbate 80 (EE-extraction) or by hydrolysis (EE-hydrolysis)

Formulation	EE-extraction (%)		EE-hydrolisis (%)
	HPLC	Spectrophotometry	Spectrophotometry
A	19.56 ± 0.15[a]	31.80 ± 0.93[b]	33.65 ± 2.58[b]
B	19.58 ± 0.71	26.80 ± 1.73	27.55 ± 1.77
C	38.85 ± 1.39	56.70 ± 1.23	53.64 ± 0.75
D	15.64 ± 3.35	20.41 ± 0.82	54.61 ± 1.39
E	17.60 ± 9.13	25.00 ± 6.33	43.29 ± 6.20
F	15.37 ± 0.57	46.80 ± 1.52	63.89 ± 4.71
G	39.00 ± 0.64	57.50 ± 1.38	72.81 ± 5.17
H	40.82 ± 2.79	59.65 ± 0.74	67.24 ± 4.37
I	56.79 ± 0.75	78.46 ± 4.79	88.38 ± 4.06

Protein concentration was assayed by PHLC and spectrophotometry ($\lambda = 494$ nm).
[a] $n = 2$.
[b] $n \geq 3$.

observed a correlation between the size of the droplets of the inner aqueous phase and the microspheres [24].

The TEM microphotograph shown in Fig. 1 displays a typical image of the nanospheres. All formulations developed presented the same appearance, however, the size of the nanospheres varied depending on the formulation conditions. The values of the diameter of the particles correlate with those values obtained by PCS (Table 1) although, in all cases, the size appeared to be smaller in the microphotographs.

The mean zeta potential values obtained for all the formulations were negative and quite similar. Nevertheless, the statistical analysis of these data indicated a significant influence (ANOVA, $\alpha < 0.01$) of the polymer hydrophilicity on this parameter. As mentioned above, Resomer® RG 503 H has carboxylic-end groups in the polymer chain which are negatively ionised upon contact with water, whereas classical PLGA copolymers have the carboxylic-end groups sterified and thus are not susceptible to ionisation.

3.2. Protein encapsulation efficiency and stability

The accurate determination of the levels of proteins entrapped in microspheres is a difficult issue. In early studies these levels were determined by the Lowry or related techniques following the dissolution of the microspheres in methylene chloride and further extraction of the protein into an aqueous phase [14]. More recently, it was reported that this procedure may lead to an underestimation of the protein loading efficiency, whereas the technique based on the hydrolysis of the polymer in an alkaline medium may give more accurate data [27]. In our study we compared both procedures and we determined the amount and the integrity of the protein extracted by direct absorbance of FITC-BSA at 494 nm and also by HPLC. Table 2 compares the encapsulation efficiency values obtained by the extraction and the hydrolysis techniques. Results indicate that the EE-extraction values varied, for each formulation, depending on the analytical technique (spectrophotometry vs. HPLC), the largest difference being observed for the formulations with the highest loading (C, F and I). In this respect, it should be mentioned that by measuring direct absorbance it is not possible to discriminate between the monomeric or aggregated protein, whereas using HPLC only the monomeric protein was taken into account for the determination of the loading. Therefore, these differences could be attributed to the formation of soluble aggregates which were visible in the chromatograms but not taken into account for the calculation of the amount of protein entrapped in the nanospheres. It was also interesting to compare the EE-extraction and EE-hydrolysis values determined by the same protein analytical technique (FITC-BSA absorbance). As shown in Table 2, these values are quite similar for the formulations made of Resomer® RG 502, but they differ substantially for the formulations made of Resomer® 503 and 503 H. In the latter cases, the EE-hydrolysis values were higher than the EE-extraction values, thus indicating that the protein extraction technique was not accurate for the determination of protein loading in these particular formulations. This could be attributed to a favourable interaction of the BSA with these polymers which would hinder the extraction of the protein from the organic phase into the aqueous phase. In the case of the Resomer® 503 H, its interaction with the protein could be favored by the free carboxyls in the polymer chain, whereas the more important interaction with 503 with respect to 502 could be related to the viscosity of the polymer solution and/or to the MW dependent protein–polymer attraction forces. The same behavior was observed for other proteins i.e. tetanus toxoid (results not shown).

One conclusion from this comparative study is that the hydrolysis of the polymer microspheres and analysis of the protein in the monomer mixture enables a good quantitative determination of the BSA encapsulation efficiency. Nevertheless, the extraction of the protein into an external aqueous medium and further analysis by HPLC provides interesting information on the amount monomeric protein and permits the identification of soluble aggregates.

Looking at the effects of the processing conditions on the protein encapsulation efficiency, Table 2 shows that this parameter is highly influenced by the PLGA MW and hydrophilicity and by the presence of Poloxamer; the highest encapsulation values being observed for the hydrophilic polymer Resomer® RG 503 H in the absence of Poloxamer. This could be partially justified by

the larger size of these nanospheres but mainly by a favourable interaction of BSA with this particular polymer. Certainly, nanospheres made of Resomer® RG 503 and Resomer RG® 503 H had the same size but the BSA encapsulation efficiency was higher for those made of the hydrophilic polymer RG 503 H. Consequently, the presence of free carboxyl groups in the polymer chain appears to be an interesting feature for increasing the protein encapsulation efficiency. On the other hand, the more important loadings achieved for Resomer® 503 with respect to 502 could be, as indicated above, in relation with the higher viscosity and/or a more prononced protein/polymer afinity. The negative effect of the Poloxamer on the protein encapsulation efficiency could be interpreted as a result of the complex interaction mechanism between BSA–Poloxamer–PLGA that leads to the destabilization of the inner emulsion as was mentioned above [26].

In order to determine the nature of the aggregates detected by HPLC we also analyzed the encapsulated protein by SDS-PAGE. The single line, corresponding to the MW of BSA, observed in the gels (results not shown) revealed that the BSA aggregates detected by HPLC were destroyed by the treatment with NaOH and SDS required for the electrophoretic analysis (see Section 2). Consequently, these results suggest the non-covalent nature of the BSA aggregates, although the formation of disulfide bridges cannot be ruled out.

Among the various protein inactivation sources, there is great concern that PLGA encapsulated proteins will undergo an aggregation process due to the decreasing pH inside the microspheres, this drop of pH being caused by the accumulation of the polymer degradation products [1]. In order to investigate this phenomenon, we checked the formation of aggregates following the incubation of the nanospheres in PB for one month, at 37°C, and observed a single band corresponding to the entrapped BSA. Consequently, there is evidence to conclude that the entrapped BSA did not suffer a significant covalent aggregation or fragmentation during the nanospheres degradation process.

3.3. In vitro protein release and polymer degradation studies

Fig. 2 illustrates the in vitro release profiles obtained for the formulations developed by representing the % of BSA release with respect to the amount of BSA encapsulated. The common feature in these profiles is the fast release (1st day) of a certain amount of protein which was poorly entrapped in the polymer matrix, followed by a slow and continuous release. Similar patterns have been previously observed for the release of BSA from microspheres, the burst effect frequently being related to the size of the microspheres [14–16]. In our study, it was surprising that the initial burst of release was not related to the size of the nanospheres but to the polymer hydrophilicity. Indeed, the minimum burst was observed for formulation I (20%) composed of nanospheres of 398 nm in size, whereas an important burst

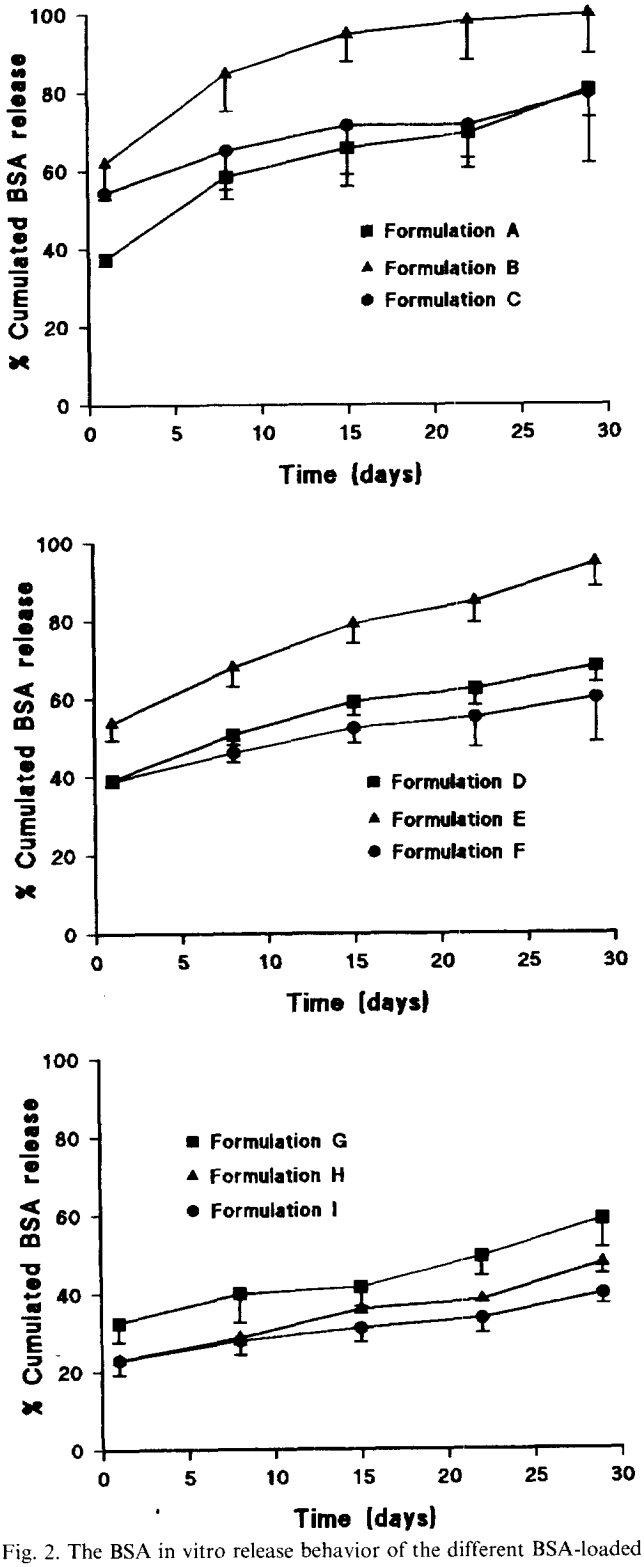

Fig. 2. The BSA in vitro release behavior of the different BSA-loaded nanospheres.

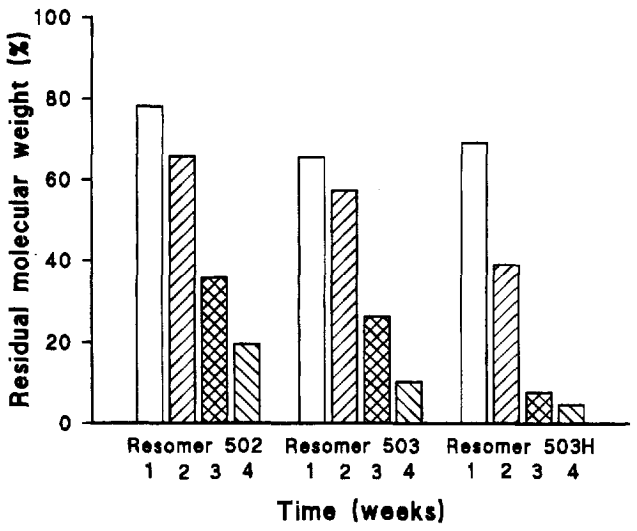

Fig. 3. The in vitro degradation profiles of the various types of PLGA used to prepare the nanospheres.

of release (60%) was obtained for formulation B composed of nanospheres of 323 nm. These data indicate that the most efficient encapsulation of the BSA was attained for nanospheres made of the hydrophilic polymer Resomer RG® 503 H. The low burst effect observed for this formulation could be explained by the carboxylic end groups in the polymer chains and their favourable interaction with the BSA. On the other hand, the burst effect of the nanospheres made of the esterified polymers (RG 502 and RG 503) was affected by the solvent elimination technique (ANOVA $\alpha < 0.01$), indicating a more efficient entrapment of the BSA in the nanospheres prepared by the solvent extraction procedure. This could be justified by a faster precipitation of the polymer when the solvent was extracted, thereby preventing the migration of the BSA molecules from the inner to the external aqueous phase.

The second phase of the release profiles corresponds to the release of the entrapped protein. It has been suggested that the release of proteins from PLGA microspheres is determined by their inner structural morphology and also by the polymer degradation rate [14,15]. Thus, to investigate the mechanism of protein release from the nanospheres we followed the evolution of the polymer MW during the in vitro release process. Results in Fig. 3 indicate that the evolution of the residual MW (%) of the polymer is not dependent on the initial copolymer MW but is highly affected by the polymer hydrophilicity. Hence, the presence of free carboxyl groups in the polymer chain increases the degradation rate of the polymer dramatically. Since protein release profiles (cumulative % of protein released vs. time) and polymer degradation profiles (residual MW (%) vs. time) were fairly linear, the slopes of these profiles were taken as the BSA constant release rate and the PLGA constant degradation rate, respectively (Table 3). These data indicate that the BSA release rate was not dependent on the copolymer MW but on the polymer hydrophilicity, the release being slowed down for the copolymer with terminal carboxyl groups. This could be explained by the previously mentioned affinity of the BSA for this polymer which holds back the BSA, hindering its release into the aqueous medium. In addition, the incorporation of Poloxamer 188 in the formulation enhanced the release of the BSA from the hydrophilic nanospheres (formulations G, H and I). This could very logically be attributed to the reduced interaction of the BSA with the PLGA caused by the presence of the surfactant. On the other hand, the similar release rate of BSA from the nanospheres made of copolymers of different MW (RG 503 and RG 502) can be understood in terms of their similar polymer degradation rate. It is, however, important to point out that no relation between the BSA release rate and PLGA degradation rate was observed for the formulations made of Resomer® RG 503 and RG 503 H. Actually, despite the fast degradation rate of the RG 503 H, it could be accepted that the affinity of this polymer for the BSA hampers its release in vitro. This information led us to the conclusion that the release of proteins from PLGA nanospheres is not only governed by the degradation rate of the polymer, as previously reported, but also by the affinity of the protein vs. the polymer.

4. Conclusions

We have succeeded in developing protein-loaded PLGA nanospheres using the double emulsion technique conveniently modified. The size of these nanospheres as well as the protein encapsulation efficiency

Table 3
The 'in vitro' constant release rate of BSA from PLGA nanospheres (% BSA released·day^{-1}) and the 'in vitro' degradation constant rate of PLGA (% MW reduction·week^{-1})

Formulation	BSA release rate ± S.D[a]	PLGA degradation rate ± S.D[a]
A	1.43 ± 0.24 (0.9193)[b]	N.D.
B	1.38 ± 0.32 (0.8487)	N.D.
C	1.08 ± 0.45 (0.9163)	−20.56 ± 1.57 (0.9756)[b]
D	1.02 ± 0.15 (0.9533)	N.D.
E	1.45 ± 0.18 (0.9775)	N.D.
F	0.85 ± 0.39 (0.9749)	−19.70 ± 1.66 (0.9574)
G	1.00 ± 0.18 (0.9585)	N.D.
H	0.87 ± 0.10 (0.9766)	N.D.
I	0.59 ± 0.05 (0.9762)	−30.71 ± 1.73 (0.9998)

[a] $n = 4$.
[b] r^2.
N.D., not determined.

and release rate can be modulated by adjusting the formulation conditions. A remarkable fact is that the new PLGA copolymers with free terminal carboxyl groups were shown very efficient in improving the protein encapsulation efficiency and slowing down the protein release rate. The same approach was recently used by our group for the encapsulation of L-asparaginase (article in preparation) clearly showing the potential of these nanospheres as efficient protein delivery systems.

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