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Stability of BSA encapsulated into PLGA microspheres using PAGE and capillary electrophoresis

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Abstract

Bovine serum albumin (BSA, M_w 66200 Da) has been encapsulated as a model protein drug within poly(D,L-lactide-co-glycolide) (PLGA 50:50) microspheres using a w/o/w double emulsion method. The microspheres prepared were smooth and spherical with a mean particle size of 1.32 μm. The total protein loading and surface-associated protein were 8.61 and 16.60%, respectively. The microspheres showed a triphasic in vitro release profile with an initial burst effect due to the release of the protein adsorbed on the microsphere surface, a second sustained release phase due to protein diffusion through the pores or channels formed in the polymer matrix, and a third phase due to polymer bioerosion. The purpose of this paper was to evaluate the effect of the microencapsulation process on the integrity of the entrapped protein using polyacrylamide gel electrophoresis and capillary electrophoresis. The stability of the protein released during in vitro assays was also assessed. The results obtained showed that there was no apparent effect of the drastic encapsulation conditions (contact with dichloromethane (DCM), probe sonication, and vigorous shaking) on the structural integrity of the protein. On the other hand, it was found that after 1 week of incubation the protein released from the microspheres starts to hydrolyze to smaller fragments, probably due to a significant decrease in the medium pH as a result of the accumulation of the polymer degradation products.

Keywords: Bovine serum albumin; Poly(D,L-lactide-co-glycolide); Microsphere; Polyacrylamide gel electrophoresis; Capillary electrophoresis; Stability

1. Introduction

The preparation of protein-loaded microspheres has received much attention in recent years (Göpferich et al., 1994; Cohen and Bernstein,

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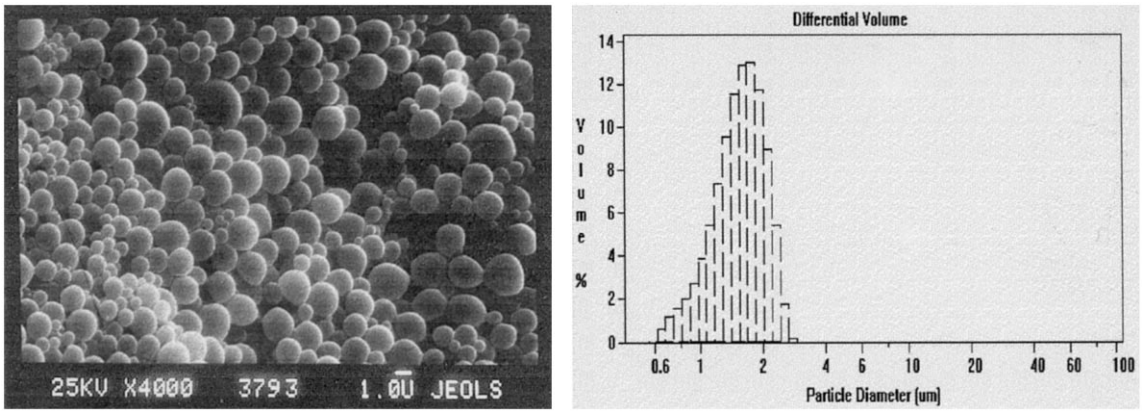

Fig. 1. SEM photograph and size distribution of PLGA microspheres.

1996). Especially, sustained-release microspheres using biodegradable polymers such as poly(D,L-lactide-co-glycolide) (PLGA) have been investigated and satisfactory results have been obtained (Jeffery et al., 1991; Igartua et al., 1997a; Okada, 1997).

Nowadays, several microencapsulation methods are available for the preparation of sustained-release delivery systems (Pavanetto et al., 1992; Guo, 1994). They are essentially based on phase separation and emulsion–evaporation techniques. More recently, a technique based on a water-in-oil-in-water (w/o/w) double emulsion has been proposed for the encapsulation of hydrophilic drugs, in order to improve the loading efficiency of microspheres (Uchida et al., 1996). During the microsphere formulation, the protein has often been exposed to various unfavourable conditions. In particular, probe sonication and exposure to an organic solvent (dichloromethane) are problematic factors in the solvent evaporation process (Jeyanthi et al., 1997). In addition, different molecular deteriorations of the protein such as denaturation, aggregation, chemical degradation and adsorption onto the polymer surface may result from the creation of an acidic environment within microspheres, due to polymer degradation during the release studies. So far, there have been few systematic studies on the stability of proteins encapsulated into PLGA microspheres. Most studies have dealt with protein release kinetics without examining the stability of the protein encapsulated within the microspheres and the protein molecules in the release medium (Wang et al., 1991; Soriano et al., 1995).

In this study, bovine serum albumin (M_w 66200 Da) has been encapsulated into PLGA microspheres using a double emulsion method. Two analytical techniques: polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE) were employed to examine the stability of BSA within microspheres and in the release medium up to 6 weeks in release experiments.

2. Materials and methods

2.1. Materials

The polymer poly(D,L-lactide-co-glycolide) (PLGA) (Resomer® RG 506) with a copolymer ratio of 50:50 (lactic/glycolic (%)) and an intrinsic viscosity of 0.8 dl/g was supplied by Boehringer

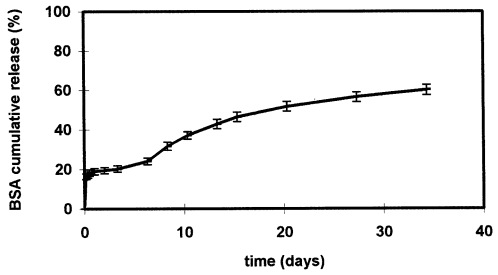

Fig. 2. Release of BSA from PLGA 50/50 microspheres.

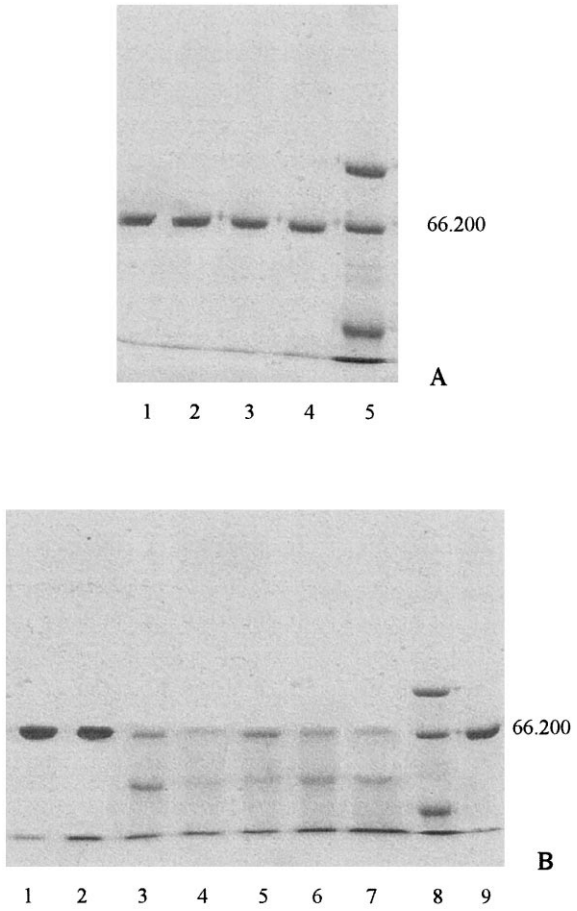

Fig. 3. SDS–PAGE results of different BSA samples. Gel A: lane 1, native BSA; lane 2, sonicated BSA; lane 3, BSA emulsified with dichloromethane; lane 4, BSA extracted from the microspheres; lane 5, M_w markers. Gel B: lanes 1 and 9, native BSA; lanes 2–7, BSA released from microparticles at 1, 7, 15, 21, 28 and 42 days of the in vitro release assay; lane 8, M_w markers.

Ingelheim K.G. (Ingelheim, Germany). Bovine serum albumin (BSA, M_w 66200 Da), polyvinylalcohol (PVA) (average M_w 30000–70000; 88% hydrolyzed), acrylamide, *N*,*N*′-methylene-bis-acrylamide, ammonium persulphate, Coomassie brilliant blue R, *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine and β-mercaptoethanol were supplied by Sigma Chemical Co. (St. Louis, MO). A protein assay kit (micro BCA) was purchased from Pierce. All other chemicals were analytical grade and were purchased from Probus S.A. (Barcelona, Spain).

2.2. Microsphere preparation

PLGA microspheres containing BSA were prepared from PLGA 50:50 (Resomer® RG 506) using a double emulsion method previously described (Igartua et al., 1997b). Briefly, 100 mg PLGA were dissolved in 2 ml dichloromethane and emulsified with 100 μl of a 10% BSA aqueous solution by probe sonication for 30 s at 50 W (Branson® sonifier 250). The resulting emulsion was poured into 10 ml of an aqueous solution of PVA 8% and mixed for 5 min at 9500 rpm using

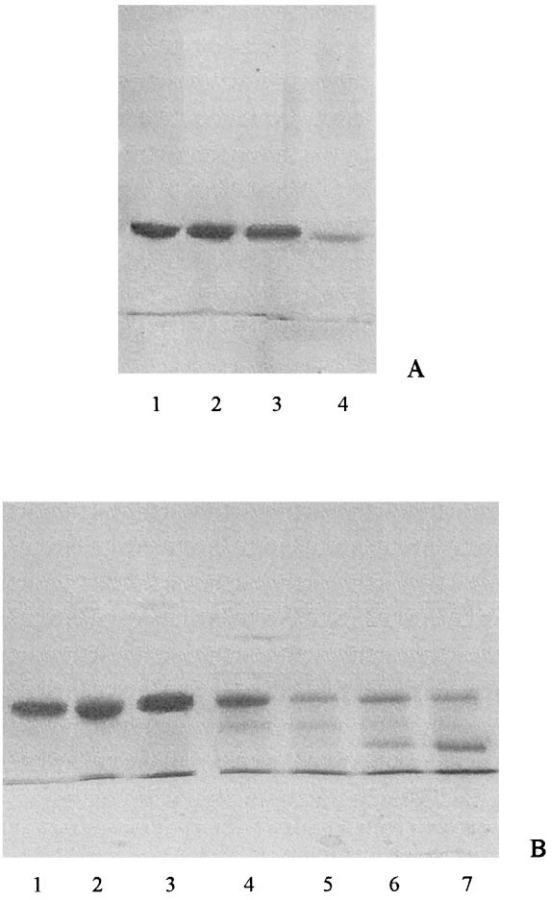

Fig. 4. SDS–PAGE results of different BSA samples, run under non-reducing conditions. Gel A: lane 1, native BSA; lane 2, sonicated BSA; lane 3, BSA emulsified with dichloromethane; lane 4, BSA extracted from the microspheres. Gel B: lane 1, native BSA; lanes 2–7, BSA released from microparticles at 1, 7, 15, 21, 28 and 42 days of the in vitro release assay.

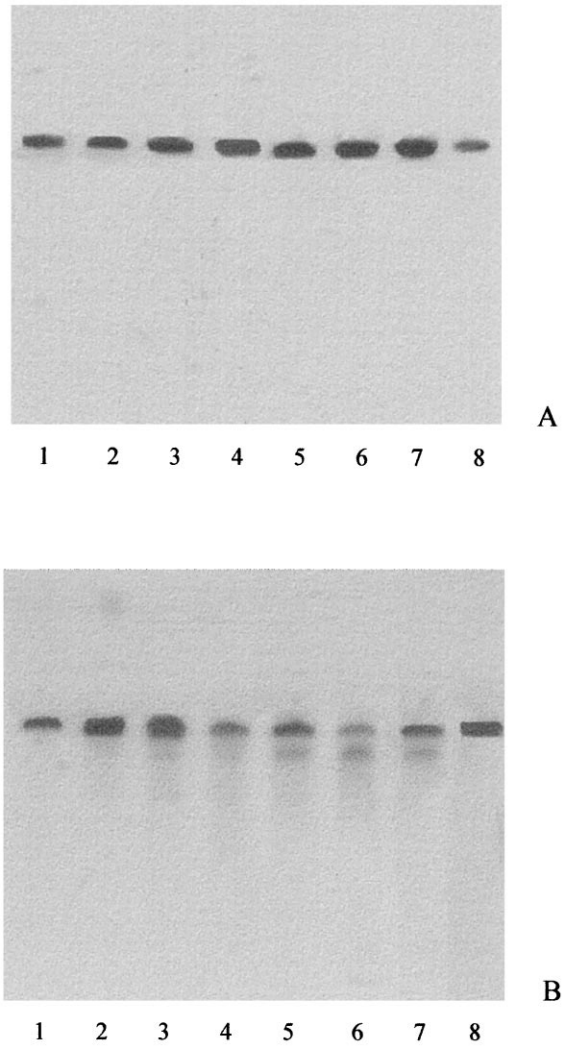

Fig. 5. Native PAGE results of different BSA samples. Gel A: lanes 1 and 5, native BSA; lanes 2 and 6, sonicated BSA; lanes 3 and 7, BSA emulsified with dichloromethane; lanes 4 and 8, BSA extracted from the microspheres; lane 5, M_w markers. Gel B: lanes 1 and 8, native BSA; lanes 2–7, BSA released from microparticles at 1, 7, 15, 21, 28 and 42 days of the in vitro release assay; lane 8, M_w markers.

a turbine homogenizer (Ultraturrax T-25). Finally, 20 ml of a 2% isopropanol solution was added and the system was maintained under mechanical stirring for 1 h. The microspheres were collected by centrifugation at $10000 \times g$, washed and lyophilized (Cooling HetoTrap® HETO).

2.3. Characterization of the microspheres

Microsphere size, morphology and surface appearance were examined by scanning electron microscopy (SEM) (Jeol JSM-35 CF) and laser diffractometry using a Coulter Counter LS130 particle size analyzer (Washington, 1992).

2.4. Albumin quantification

Total protein loading was estimated using the Lowry assay (Lowry et al., 1951) after disruption of the microspheres with a 2.5% sodium dodecyl sulphate (SDS)/0.2 M NaOH solution (Jeffery et al., 1993). Sodium hydroxide catalyzes the hydrolysis of the PLGA and SDS ensures the complete solubilization of the protein during the polymer hydrolysis. The resulting solution was then neutralized to pH 7 by stepwise addition of 1 M HCl. The amount of surface-associated protein was assessed by suspending the microparticles in PBS for 15 min, centrifuging the samples to sediment the microparticles and then analyzing the supernatant for protein by performing the micro BCA protein assay (Pierce, Rockford IL) (Smith et al., 1985).

2.5. In vitro release studies

In order to carry out the protein release studies, 20 mg of microspheres were suspended in 2 ml phosphate buffer 20 mM, pH 7.4, and were incubated at 37°C under continuous orbital rotation. At certain time intervals the samples were collected by centrifugation at $10000 \times g$ for 10 min. The supernatant was replaced with fresh buffer and assayed for protein release using the micro BCA assay. Release experiments were done independently in triplicate.

2.6. Polyacrylamide gel electrophoresis (PAGE)

The structural integrity of extracted and released BSA was first analyzed with SDS–PAGE using the method described by Laemmli (1970). BSA under the encapsulation operating conditions (emulsified with DCM and sonicated for 30

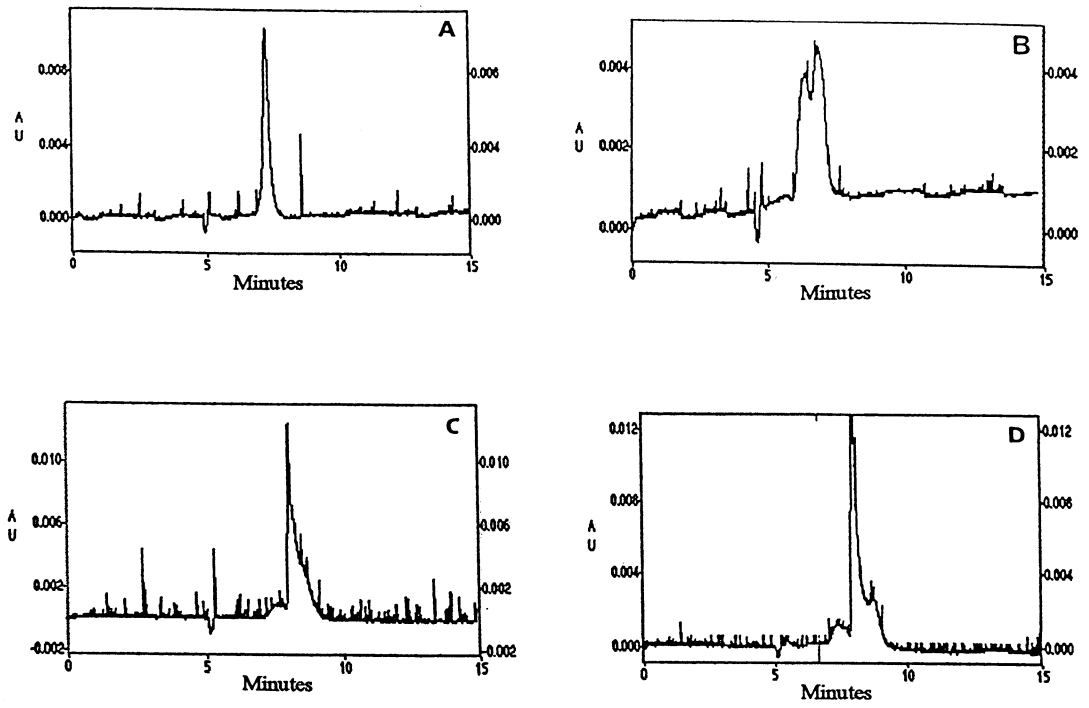

Fig. 6. Electropherograms of native BSA (A) and under different denaturing agents. (B) High temperatures; (C) SDS; and (D) β-mercaptoethanol.

s), extracted from the microparticles and released during in vitro assays was compared with native BSA and low-molecular weight reference markers (20–94 kDa). Protein samples were diluted with a Tris-buffer (pH 6.8) with 2% SDS. Electropherograms of samples were performed at a constant voltage of 200 V in a Tris/glycine/SDS buffer using a Bio-Rad Mini-Protean II electrophoresis system. However, the above SDS–PAGE analysis does not allow us to ensure the absence of BSA aggregates, since we use a reducing agent (β-mercaptoethanol) that breaks up disulfide bonds and an anionic detergent (SDS) which dissociates protein aggregates in a non-covalent fashion (Goers, 1993). Thus, we carried out a PAGE under non-reducing conditions and a native PAGE to confirm the absence of protein aggregates.

After migration, gel sheets were stained with Coomassie Blue in methanol–acetic acid–water (2.5:1:6.4) to reveal protein, destained and dried (Cook, 1994).

2.7. Capillary electrophoresis (CE)

In order to confirm the stability of the albumin, a determination by capillary electrophoresis was performed in addition to the PAGE. The analysis of the protein by this technique allows us to know whether the albumin showed the same electrophoretic pattern as the native BSA or whether additional peaks shown are due to the denaturation or self-aggregation of the protein. CE analyses were carried out using a Hewlett-Packard capillary electrophoresis system equipped with a diode-array detector. A fused-silica capillary with an effective length of 50 cm, 58.5 cm total length and 75 μm I.D. was used for the separation. Samples were introduced into the capillary by hydrodynamic injection for 5 s. UV detection was employed at 190 nm (Pande et al., 1992). The whole apparatus was computer-controlled with the HP 3D-CE ChemStation software for the storage and handling of the data. Before the analysis, the capillary was rinsed under pressure

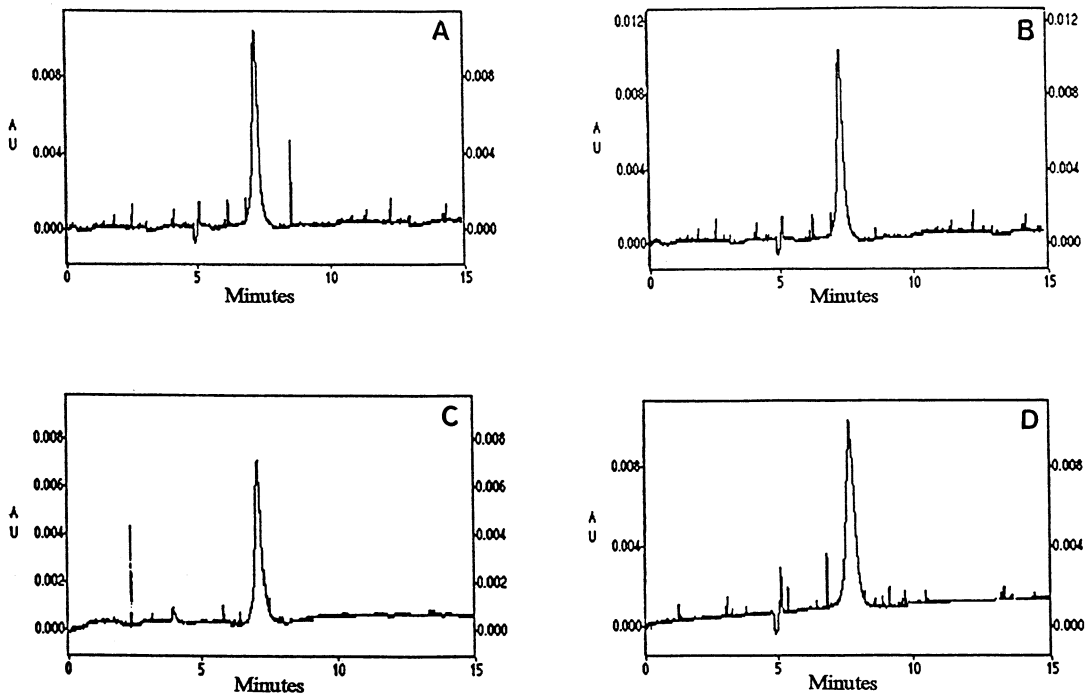

Fig. 7. Electropherograms of different BSA samples under the conditions of the microencapsulation process: (A) native BSA; (B) sonicated for 30 s; (C) emulsified with dichloromethane; (D) BSA extracted from the microspheres.

with 0.1 M NaOH for 5 min, and then equilibrated with the running buffer for 3 min (50 mM borate buffer, pH 8.5).

3. Results and discussion

3.1. Characterization of microspheres

When observed under SEM the microspheres used in the study appear smooth and spherical with a mean particle size of 1.32 μm (Fig. 1). The total protein loading and surface-associated protein were 8.61 and 16.60%, respectively.

3.2. In vitro release studies

The release of BSA from the microspheres showed a triphasic profile as shown in Fig. 2. The microspheres showed an initial burst release followed by a very slow release phase due to the channels and pores formed in the microspheres and the bioerosion of the polymer. The burst release was 20% of the total protein loading, and would be due to poorly encapsulated or surface-bound protein (16.60%), as suggested by several authors (Blanco et al., 1994; Hampl et al., 1996). An additional 5% of the protein was released during the first week of the study and, finally, about 60% of the drug was released during the rest of the assay.

3.3. Bovine serum albumin structural integrity

Proteins in their native state possess a complex internal structure that provides the molecule its unique biological activity (Rodwell, 1993). This spatial configuration or native conformation of the protein comprised a combination of irregular structures, such as random coils, and of regular structures, such as α-helices and β-sheets associated at four interrelated levels.

Proteins may become inactive by chemical alteration, denaturation and aggregation. The term stability, as it relates to proteins, is often used in a variety of ways (Manning et al., 1989). Chemi-

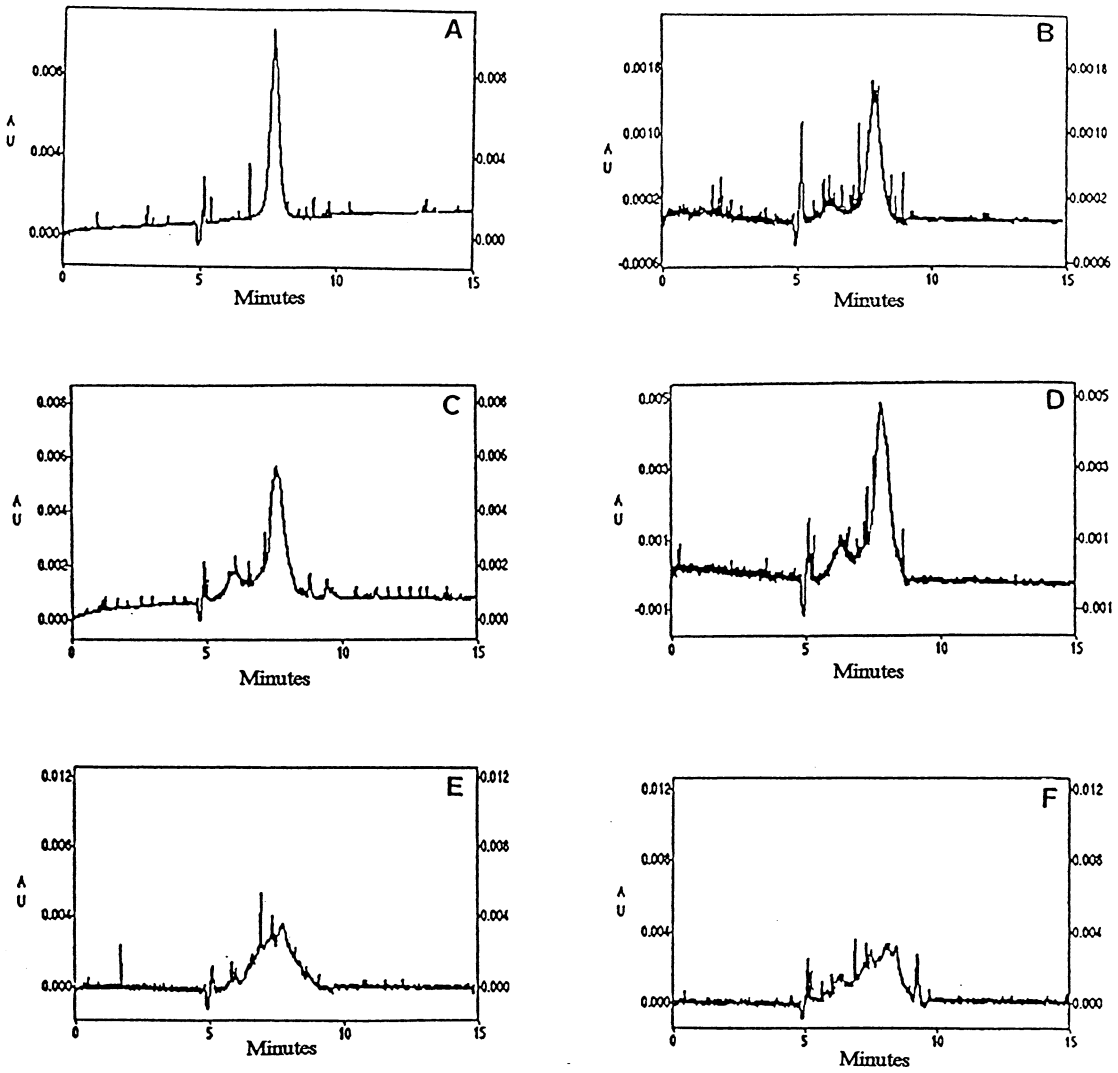

Fig. 8. Electropherograms corresponding to the BSA samples obtained at different time intervals of the in vitro release assay: (A) 1 day, (B–F) 1, 2, 3, 4 and 6 weeks, respectively.

cal stability involves the reactivity of the side chains and lability of the peptide bonds. However, we define conformational or physical stability of a protein as the ability of the protein to retain its tertiary structure. Proteins may be denatured by a number of different causes including extremes in pH, elevated temperatures ($>60-70°C$), exposure to surfactants or high concentrations of selective denaturants (e.g. urea and guanidine hydrochloride). Denaturation includes any alteration of the native structure of a protein which can change the physical, chemical or biological properties of the protein (Devlin, 1992). It is usually accompanied by an increase in molecular dimensions, viscosity, and aggregation, and a decrease in ordered structures (α-helix and β-sheets), solubility and solvation. Proteins can adopt a variety of conformations from native-like states, such as the so-called 'molten globule' state, conformers or aggregates, to highly unfolded states that are apparent at high concentrations of denaturants. These structures can also coexist with the native

form. However, although the secondary, tertiary and quaternary structure can be lost in the denatured form of a protein, the covalently bonded amino acid sequence is maintained.

During preparation of microspheres, the use of organic solvents, probe sonication, vigorous shaking and lyophilization, are processes able to inactivate proteins (Marchais et al., 1996). On the other hand, during release studies incubation, the reduction in pH (resulting from the formation of new carboxylic acid ends groups of the polymer), as well as the creation of water-soluble polymer fragments and monomers, are potential sources of protein inactivation (Park et al., 1995).

Undoubtedly, in order to successfully develop a protein–PLGA delivery system, it is essential that the biological activity of the protein be retained throughout encapsulation, storage and duration of release.

3.3.1. Polyacrylamide gel electrophoresis (PAGE)

Fig. 3 shows SDS–PAGE results of BSA exposed to the unfavourable encapsulation conditions that could affect the stability of the protein (contact with dichloromethane, probe sonication and vigorous shaking), the protein extracted from the microspheres following the method used to determinate the total protein encapsulated into the microspheres, and the samples obtained at different time intervals of the in vitro release studies.

The results obtained for the BSA under the drastic conditions encountered during the encapsulation procedure confirm the integrity of the protein, with a single band corresponding to its M_w (66200 Da), as can be seen in Fig. 3A.

Fig. 3B presents the SDS–PAGE profile of the BSA released in PBS during in vitro assays. It can be observed that, up to 1 day of incubation, the released protein maintains its structural integrity (lane 2). However, for 1 week incubation, it can be seen that the BSA is hydrolyzed with the generation of small molecular weight fragments (lanes 3–7). This can be due to the acidified medium, which contains water-soluble polymer degradation products, mainly lactic and glycolic acid, which participate in the protein hydrolysis. Similar results were obtained by Crotts and Park (1997). These authors determined the lactic acid concentration and the pH of the microparticle release media and observed that the pH value decreases over time.

The above analytical conditions, however, do not completely rule out the presence of protein aggregates linked by covalent or non-covalent bonds. The presence of a reducing agent (β-mercaptoethanol) would break up all disulfide-bonded aggregates, and the addition of the anionic surfactant SDS dissociates non-covalent aggregates. This detergent binds both to the hydrophobic residues and to the peptide backbone of proteins approximately once every two amino acids, causing complete unfolding of native protein structure while imparting a constant negative charge per unit length of peptide chain. The proteins assume a random coil shape, roughly a sphere, the size of which depends on the molecular weight of the protein. Therefore, we performed a PAGE under non-reducing conditions, as previously suggested by Crotts and Park (1997) and a native or non-denaturing PAGE to confirm the absence of protein aggregates.

SDS–PAGE results of different samples of BSA, run under non-reducing conditions, are shown in Fig. 4A,B. In this case, it can be seen that the samples prepared under the encapsulation conditions (Fig. 4A) do not have any structural alteration in their integrity, whereas those from the in vitro assays exhibit hydrolyzed fragments that appear as additional bands below the molecular weight of the albumin (66200 Da).

Finally, we carried out a non-denaturing PAGE which can preserve either covalently or noncovalently bonded aggregates. Fig. 5 shows the electrophoretic results of BSA samples prepared in order to evaluate the effect of the microencapsulation process on the stability of the protein and the BSA released at various sampling intervals of the in vitro assay.

As it was expected from the results previously obtained using SDS–PAGE run under reducing and non-reducing conditions, the gel sheets displayed in Fig. 5 showed that there is no aggregation of the protein under the experimental conditions studied.

3.3.2. Capillary electrophoresis (CE)

Capillary electrophoresis is a relatively new analytical method which has seen a great development in recent years because of its multiple advantages. In addition to automation and rapid analysis times, the advantages of CE include minimal sample volume requirements, high efficiency, reproducibility and resolution, and low cost (Calvo et al., 1996a). The separation mechanism in CE is the same as that of conventional electrophoresis. Charged compounds, such as peptides and proteins, are separated by differential migration in a buffer solution when applying an electric field, based on their mass-to-charge ratio.

CE has been successfully applied in pharmaceutical analysis and has been utilized in separation of macromolecules, control of purity, formulation analysis and chiral separations (Calvo et al., 1996b). Recently, it has been applied to study the stability of the orosomucoid protein adsorbed into polyisobutylcyanoacrylate nanoparticles in the presence of serum (Olivier et al., 1994).

In contrast to the polyacrylamide gel electrophoresis, which is probably the most frequently used technique for the analysis of proteins but requires laborious sample preparation procedures and long analysis times (Hames and Rickwood, 1990), CE offers a simple method development and it is possible to achieve good results without previous sample preparation and using simple buffer solutions (Calvo et al., 1996c).

The electropherograms displayed in Fig. 6 show the different mobilities of the BSA under several denaturing agents (high temperatures, SDS and β-mercaptoethanol). It can be seen that native BSA could be distinguished from closely related degradation products by the presence of an additional peak due to some structural changes in the native molecule. As indicated above, the analysis of the protein by this technique allows us to know whether the albumin showed the same electrophoretic pattern as the native BSA or additional peaks are shown due to the denaturation or self-aggregation of the protein during the microencapsulation process and in vitro release studies.

Fig. 7 shows the electropherograms of the albumin samples under different conditions: probe sonication (Fig. 7B) and emulsification with DCM (Fig. 7C), in order to evaluate the effect of these parameters in the stability of the encapsulated protein. Electropherograms A and D correspond to the native BSA and the protein extracted from the microspheres.

As can be seen in Fig. 7, the migration times obtained for the native protein, BSA sonicated for 30 s, emulsified with DCM and extracted from the microspheres are reproducible, as are the area and shape of the peaks. These results suggest that the encapsulation procedure does not affect the stability of the protein as we have previously reported using PAGE.

Finally, Fig. 8 shows the electopherograms obtained for the different sampling times of the in vitro release studies. Comparing the electropherograms displayed in this figure, from 1 week incubation, we can see an additional small peak whose size increases as the incubation time increases due to the presence of small fragments of the hydrolyzed protein. Similar results were obtained using PAGE where an additional band was observed below the molecular weight of the albumin (66200 Da).

In summary, the study of the stability of the encapsulated BSA using polyacrylamide gel electrophoresis and capillary electrophoresis techniques has demonstrated that no structural or conformational damage of the protein occurs during the formulation process and the protein released from microspheres has an intact homogeneity. However, after prolonged incubation in the buffered solution, small hydrolyzed fragments appear in the acidic medium. It is of particular interest to note that the importance of this degradation on the induction of a long-lasting immune response should be evaluated by performing in vivo immunization studies.

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