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Biodegradable monodispersed nanoparticles prepared by pressure homogenization-emulsification

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Abstract

The aim of the present work was to investigate the preparation of nanoparticles (NP) as potential drug carriers for proteins. The hydrophilic protein bovine serum albumin (BSA) was chosen as the model drug to be incorporated within NP. Owing to the high solubility of the protein in water, the double emulsion technique has been chosen as one of the most appropriate method. In order to reach submicron size we used a microfluidizer as a homogenization device with a view to obtaining NP with a very high grade of monodispersity. Two different biodegradable polymers, poly[D,L-lactic-co-glycolic acid] 50/50 (PLGA) and poly[ε-caprolactone] (PCL) has been used for the preparation of the NP. The drug loading has been optimized by varying the concentration of the protein in the inner aqueous phase, the polymer in the organic phase, the surfactant in the external aqueous phase, as well as the volume of the external aqueous phase. The BSA encapsulation efficiency was high ($>80\%$) and release profiles were characterized by a substantial initial burst release for both PLGA and PCL NP. A higher release was obtained at the end of the dissolution study for PLGA NP (92%) compared with PCL NP (72%).

Keywords: Nanoparticles; Encapsulation; w/o/w emulsion; Bovin serum albumin; Poly[lactic-co-glycolic acid]; Poly[ε-caprolactone]

1. Introduction

Modern drug carrier systems play an important role in controlled delivery of the pharmacological agent to its target at a therapeutically optimal rate and dose. Among various colloidal drug delivery systems nanoparticles (NP) represent a very promising approach to this aim (Maincent et al., 1992; Kreuter, 1994; Nakada et al., 1996). Their properties as protein protection and controlled release behavior by the polymeric matrix seem to be useful for a drug delivery system. Especially this is of interest due to the particle size which

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allows a transport of carriers across biological membranes and specific targeting by a possible surface modification (Bourel et al., 1988; Alyautdin et al., 1997).

Several encapsulation techniques have already been developed to prepare particulate sustained drug delivery systems. The selection of the preparation method is primarily determined by the solubility of the protein and the coating polymer.

In earlier works the protein loaded NP were obtained by adsorbing the protein at the surface of the particles (Velge-Roussel et al., 1996; Armstrong et al., 1997). The main limitation of this process is that the protein is only physically bound to the surface of the NP, a fact that limits their controlled release properties as does the risk of an uncontrolled desorption of the drug from the NP surface in dependence of environmental influences. That is the reason why it seems to be advantageous to incorporate the drug into the NP.

For the incorporation of hydrophilic compounds into NP, different polymerization techniques have been used. For NP prepared by polymerization two major risks are being left: (i) residues in the polymerization medium, e.g. monomers, oligomers, initiators of the polymerization, can be more or less toxic; and (ii) activated monomer molecules may interact with the protein during the polymerization process, leading to the denaturation and inactivation of the protein drug. Therefore new encapsulation techniques were developed by using preformed polymers to avoid these problems. For the incorporation of peptides into NP based on preformed polymers a w/o phase separation technique has already been described (Niwa et al., 1995). Most of the particles prepared by this method had a relatively large diameter up to 1 μm.

Many authors (Alex and Bodmeier, 1989; Iwata and McGinity, 1992) have previously shown that the double emulsion technique was the most appropriate method to encapsulate hydrophilic drugs and proteins within microparticles. This technique has been later applied to NP (Blanco and Alonso, 1997; Lemoine and Préat, 1998; Tobío et al., 1998). In these cases the homogenization of the double emulsion was performed by sonication (Blanco and Alonso, 1997; Tobío et al., 1998) or by Ultra-turrax (Lemoine and Préat, 1998). However, in all cases the polydispersity of the NP size was relatively high.

Our first aim was to prepare NP with a lower polydispersity of the size by using a pressure homogenization device for the double emulsion. Secondly, the encapsulation efficiency has been optimized by varying the concentration of the protein in the inner aqueous phase, the polymer in the organic phase, the concentration of the surfactant as well as the volume of the external aqueous phase. Bovine serum albumin (BSA) was chosen as the model protein.

NP were prepared with two biodegradable polymers, poly[D,L-lactic-co-glycolic acid] 50/50 (PLGA) and poly[ε-caprolactone] (PCL) and compared in terms of size, polydispersity, surface potential, encapsulation efficiency and protein release.

2. Materials and methods

2.1. Materials

The two biodegradable polymers, PLGA (MW 40 000 Da) and PCL (MW 42 000 Da) were, respectively, purchased from Medisorb Technologies International L.P. (Welmington, DE, USA) and Aldrich (Steinheim, Germany).

Polyvinylalcohol (PVA; MW 30 000 Da, 88% hydrolyzed) was supplied by Sigma (St. Louis, MO, USA) and chosen as a surface active agent for the second emulsion. BSA with a mean molecular weight of 65 000 Da was purchased from Sigma and used as the model drug. All other chemical reagents were obtained from Sigma and Prolabo (Strasbourg, France) and were of analytical grade.

2.2. Preparative method

2.2.1. Nanoparticles preparation

The preparation of the NP was achieved by adjusting the multiple emulsion (w/o/w) technique, previously applied to the preparation of

both micro- and nanoparticles (Alex and Bodmeier, 1989; Iwata and McGinity, 1992; Blanco and Alonso, 1997; Lemoine and Préat, 1998). The adjustment was based on the use of a homogenizer in the two-step emulsification process, thus reducing considerably the size of the dispersed droplets. Briefly, the BSA previously dissolved in 1 ml of distilled water was emulsified in 10 ml of methylene chloride containing the polymer (PLGA and PCL) under magnetic stirring at 1500 rpm. This first emulsion was thereafter poured into the PVA aqueous solution and homogenized in a homogenizer (AML 2, Guérin, Mauze, France) in an ice bath for 3 min. After evaporation of the methylene chloride under reduced pressure, the polymer precipitated and the NP were isolated by centrifugation at $20\,000 \times g$ for 25 min (Beckmann Model J2-21) and washed twice before lyophilization. The effect of several variables on the characteristics of the NP was evaluated, including the amount of BSA (25, 50, 100, 200, 300 and 400 mg), PVA (0.05, 0.1, 0.2 and 0.4%) and polymer (0.25, 0.5, 1, 1.5 and 2 g) as well as the volume of the external aqueous phase (100, 200 and 300 ml).

2.3. Analytical methods

2.3.1. Determination of the BSA loading

The amount of BSA entrapped within the polymer NP was determined by measuring the amount of non-entrapped protein with the Lowry–Peterson protein assay (Peterson, 1977) in the supernatant recovered after ultracentrifugation and washing of the NP. This procedure permits analysis of very dilute protein solutions with removal of most interfering substances, especially interferences with possible non-ionic and cationic detergents. Each sample was assayed in triplicate.

2.3.2. Determination of the particle size and the surface potential

The NP were analyzed for their size distribution and their surface potential using a Zetasizer II® (Malvern Instruments, UK). The results were all normalized with respect to a polystyrene standard solution (Malvern Instruments, UK). Each sample was measured in triplicate.

2.3.3. NP characterization by atomic force microscopy (AFM)

The characterization of unloaded and BSA containing NP was performed with a commercial AFM (Dimension 3000™ Scanning Probe Microscope, Digital Instruments, Sta. Barbara, USA). The AFM was only used in the non-contact mode. NP samples were placed on a standard glass surface by dropping 200 μl of the suspension onto the glass surface and removing the water under reduced pressure.

2.3.4. In vitro release experiments

Lyophilized NP (120 mg) prepared with 250 mg of each polymer and 25 mg of BSA were resuspended in a 100 ml of phosphate buffer (pH 7.4) flask containing sodium azide and incubated into a bath at 37°C under gentle magnetic stirring at 300 rpm. At appropriate intervals, 5 ml samples were removed and centrifugated for 25 min at $20\,000 \times g$. The supernatant was assayed for protein release and replaced by 5 ml of fresh buffer. The amount of BSA in the release medium was determined by the Lowry–Peterson protein assay. Each NP batch was analyzed in triplicate.

3. Results and discussion

The choice of a particular method of encapsulation is usually determined by the solubility characteristics of the drug. In our approach, the double emulsion-evaporation process was adopted since it is known to be superior to other incorporation methods in terms of stability of proteins (Tabata et al., 1993). Encapsulation by the solvent evaporation method involves two major steps, the formation of stable droplets of the first emulsion and the subsequent removal of solvent from the droplets of the second emulsion. The BSA encapsulation efficiency and the particle size have been optimized by varying the amount of BSA, polymer and surfactant, as well as the volume of the external aqueous phase. Fig. 1 shows that NP appear spherical with a relatively monodispersed size and the polydispersity was much lower than 0.2 (Tobío et al., 1998) especially for PLGA NP (Table 1).

3.1. Effect of BSA concentration

As reported in Table 1, NP size and polydispersity were only slightly affected by the presence of the drug since no significant differences were observed for both loaded and unloaded PLGA and PCL NP. It was observed by Verrachia et al. (1995) that the PLA and PLA-PEG NP size decreased with increasing BSA concentrations in the internal aqueous phase. Since the particle size is related to a great extent to the stability of the first emulsion, they claimed indeed that serum albumin can act as a surfactant by stabilizing the first emulsion and consequently hampering the fast coalescence of the droplets. Moreover, a very small concentration of BSA in water promotes a sharp decrease in the H_2O/CH_2Cl_2 interfacial tension (Nihant et al., 1994), involving a higher

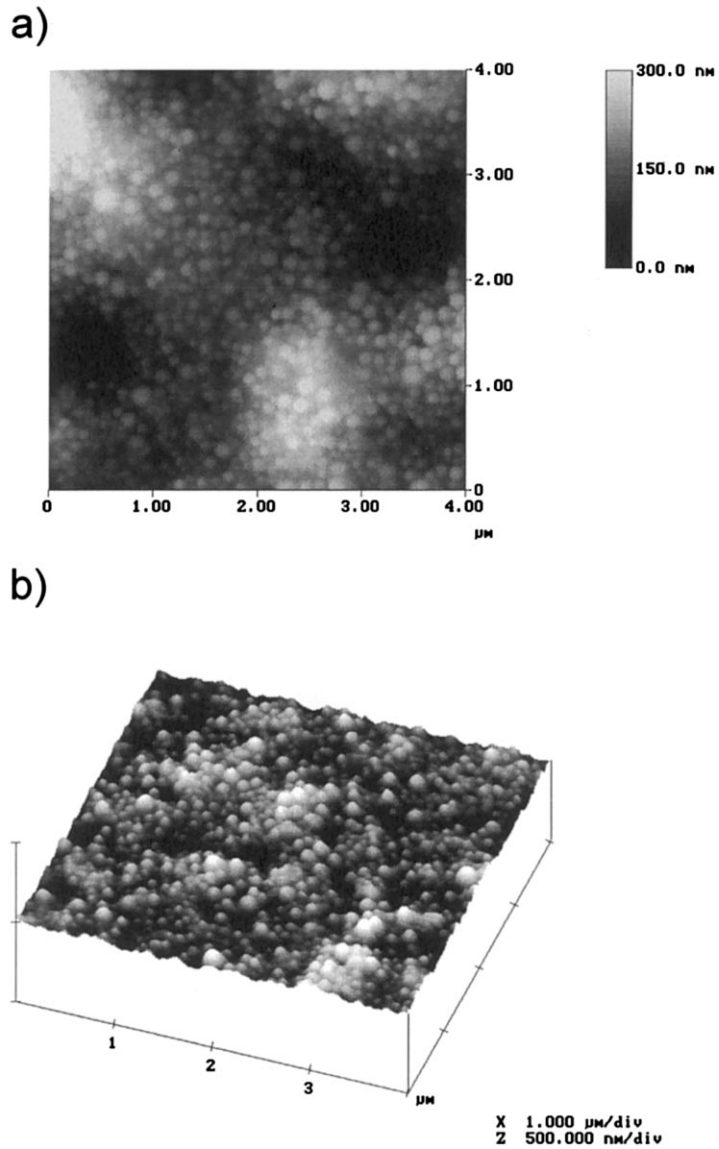

Fig. 1. Images of NP taken by AFM (non-contact mode).

Table 1
Influence of the BSA concentration in the inner aqueous phase on the particle size and the polydispersity[a]

BSA (mg/ml)	PLGA		PCL	
	Size	Polydispersity	Size	Polydispersity
0	264 ± 5	0.024 ± 0.013	276 ± 4	0.030 ± 0.015
25	262 ± 9	0.069 ± 0.020	298 ± 6	0.026 ± 0.040
50	278 ± 7	0.081 ± 0.030	308 ± 10	0.103 ± 0.017
100	273 ± 6	0.064 ± 0.028	299 ± 13	0.091 ± 0.041
200	278 ± 8	0.063 ± 0.031	302 ± 10	0.099 ± 0.028
300	270 ± 7	0.070 ± 0.030	299 ± 3	0.091 ± 0.017
400	272 ± 10	0.078 ± 0.029	299 ± 8	0.110 ± 0.019

[a] Data are shown as mean ± SE obtained from three formulations.

stability of the emulsion. In our study, the stability of the size of the NP could probably be explained by a weak adsorption of the BSA onto the NP. This hypothesis is strengthened by the fact that a small difference in surface potential was observed between blank and BSA loaded NP, especially for PLGA NP (Fig. 2). AFM was also used to display any difference of the external surface of the NP. However, the too low sensitivity of the microscope did not allow to detect any adsorption (data not shown). It could consequently be concluded that the protein is to a greater extent rather encapsulated than adsorbed. On the other hand, after the second emulsion, the organic solvent starts to evaporate, and the polymer precipitates at the interface between the external aqueous phase and the organic one. When the polymer does not precipitate rapidly at the outer surface, diffusion of BSA into the aqueous phase before the formation of NP can take place (Bodmeier and McGinity, 1988). In spite of its low solubility in the organic polymer solution, this phenomenon could involve a part of the loaded protein to relocate at the NP surface (Schugens et al., 1994).

Fig. 3 shows that the concentration of BSA in the internal aqueous phase has a non-negligible influence on the encapsulation efficiency. The results show firstly that the optimal encapsulation efficiency reached 85 and 84% for both PLGA and PCL NP when 25 mg of BSA were used, and secondly, that it decreased down to a plateau with increasing BSA concentration. It should also be noted that even at low loading BSA concentration, a fraction of the protein was not encapsulated during the preparation of NP. There are several possible explanations of the loss of BSA which was not entrapped within NP. Among them, the difference in osmotic pressure between the internal and external aqueous phases could be responsible for the decrease in entrapment efficiency. The osmotic pressure difference did in fact increase with increased BSA loading, leading to a rupture of the lipophilic droplets, and an exchange between the internal and external aqueous phases, with a consequent loss of BSA. Moreover, it is also possible that the polymer does not precipitate quickly enough and a loss of BSA occurs. Indeed, Bodmeier and McGinity (1988)

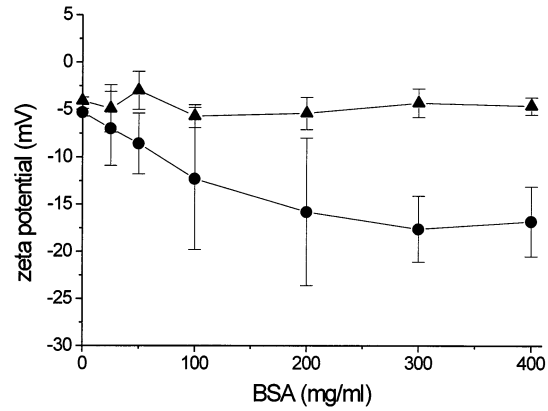

Fig. 2. Change of the zeta potential versus the BSA concentration in the inner aqueous phase for PLGA NP (●) and PCL (▲) NP.

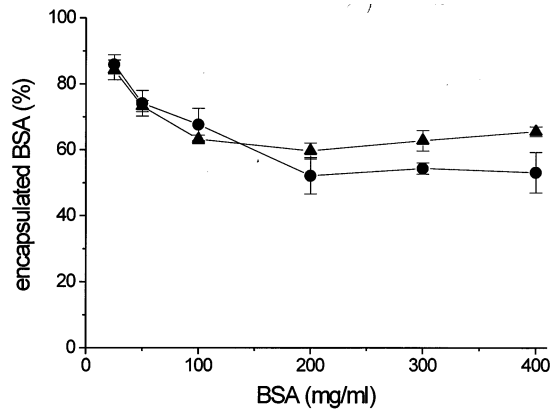

Fig. 3. Entrapment efficiency of BSA loaded PLGA (●) and PCL (▲) NP versus the amount of BSA dissolved into the internal aqueous phase (1 ml). Data are shown as mean ± SE.

reported that the successful entrapment of drugs within particles is dependent on the fast precipitation of the coating polymer from the organic phase. In addition, theoretically, during the formation of the particles by the double emulsion/solvent evaporation method, the polymer solution acts as a barrier between the internal and external aqueous phases. Nevertheless, an exchange between the two aqueous phases might occur as a consequence of the instability of the primary emulsion, and particularly because of protein solubility in the outer aqueous phase.

3.2. Effect of polymer concentration

An increase in the concentration of polymer in the organic phase led to an increase of both the size and the polydispersity of the NP (Fig. 4 (a, b)). In addition, when the polymer/BSA ratio was increased by increasing the amount of polymer in the organic phase, an increase in BSA entrapment within NP was observed (Fig. 5). These results are correlated with those of Ogawa et al. (1988). Indeed, they stated that an increase in polymer concentration led to an increase of the viscosity of the first emulsion and consequently to a reduction in the partitioning of the protein into the external aqueous phase, resulting in an increase in protein entrapment efficiency. However, beyond 1.0 g of polymer, PLGA and PCL NP appeared completely aggregated after lyophilization. It could be considered that at high polymer/BSA ratio, the viscosity was consequently increased, involving the collapse of the particles (Jeffery et al., 1993).

3.3. Effect of the volume of the external aqueous phase

An increase in the volume of the external aqueous phase led to an increase in both BSA entrapment efficiency and NP size (Fig. 6). The increase in particle size was probably attributed to a reduction of the shear during the homogenization process, because of a decrease in mixing efficiency associated with larger volume, involving an increase of the size of the emulsion droplets and consequently of the NP.

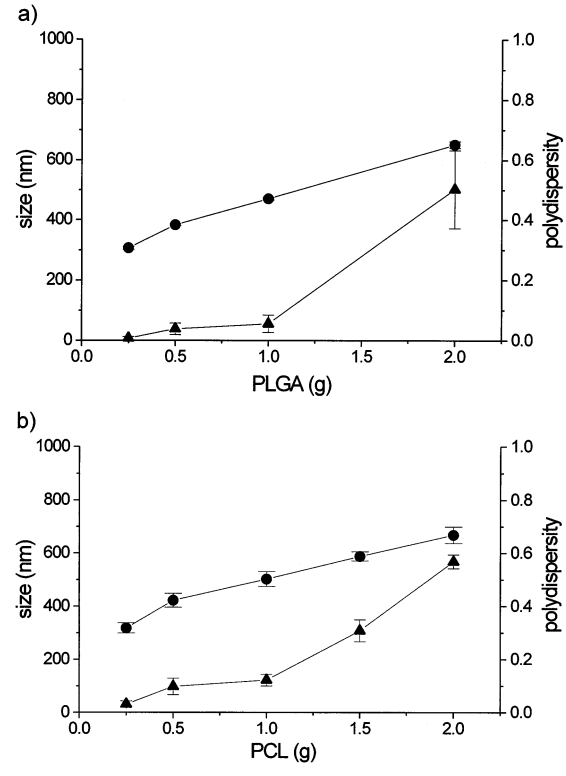

Fig. 4. Size (●) and polydisperisty (▲) of PLGA (a) and PCL (b) NP prepared by the double emulsion technique. Data are shown as mean ± SE obtained from three formulations.

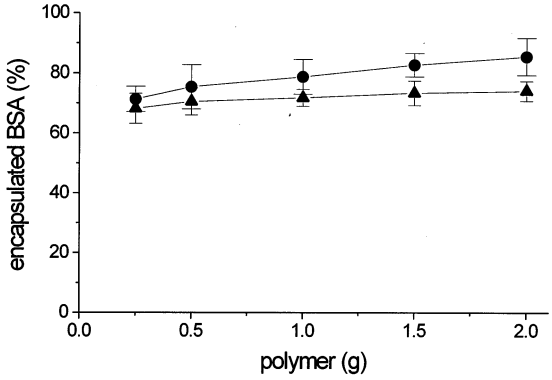

Fig. 5. Entrapment efficiency of BSA loaded NP prepared by the double emulsion technique versus the amount of the polymer in the organic phase (PLGA (●) and PCL (▲) NP). Data are shown as mean ± SE.

3.4. Effect of PVA in the external aqueous phase

Since exchanges between the internal and external aqueous phases should be kept to a minimum during the second emulsification step, the stability of the second emulsion is also critical. Furthermore, during the solvent evaporation process, there is a gradual decrease in the volume and subsequent increase in the viscosity of the dispersed droplets. This affects the droplets size equilibrium, involving the coalescence and the agglomeration of the droplets during the early step of the solvent removal (Jalil and Nixon, 1990; Arshady, 1991). This problem has been improved

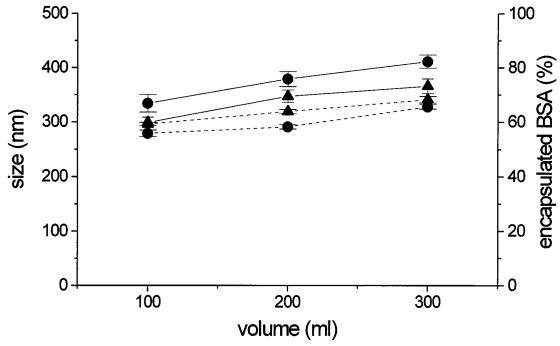

Fig. 6. Effect of the external aqueous phase volume on the mean diameter (PLGA = —▲—, PCL = ---▲---) and the protein content of, respectively PLGA (—●—) and PCL (---●---) NP. Data are shown as mean ± SE.

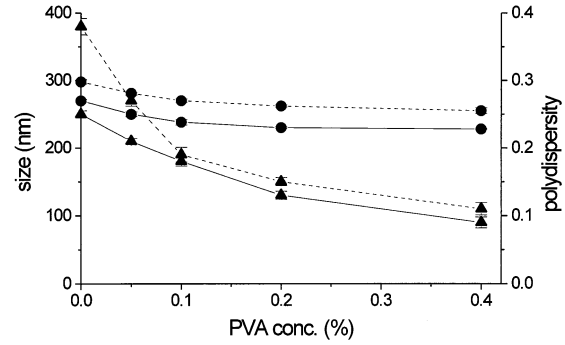

Fig. 7. Effect of the PVA concentration in the external aqueous phase on the size (PLGA = —●—), PCL = ---●---) and the polydispersity of, respectively PLGA (—▲—) and PCL (---▲---) NP. Data are shown as mean ± SE.

by adding a surfactant into the continuous phase, providing a thin protective layer around the droplets and hence reducing their coalescence. While maintaining a constant volume for the external aqueous phase (200 ml), the amount of PVA was varied. As reported in Fig. 7, the size and the polydispersity of both PLGA and PCL NP decrease with increased PVA concentrations. It could thus be concluded that the more the PVA concentration increased, the more the PVA molecules overlayed the surface of the droplets, involving an improvement in the protection of the droplets from coalescence and resulting consequently to smaller emulsion droplets than at lower PVA concentrations. Since NP were formed from the emulsion droplets after the solvent evaporation, their size is dependent upon the size and the stability of the emulsion droplets.

3.5. In vitro protein release

Fig. 8 illustrates the in vitro release profiles obtained for each formulation, by representing the percentage of BSA release with respect to the amount of BSA encapsulated. For all NP, BSA release occured in three phases: (i) a first initial burst release, owing to the drug particles adsorbed onto the wall of the NP which would be immediately released during the initial stage; (ii) after the initial burst, the drug release profiles displayed a plateau for an extended period, resulting from the

only diffusion of the drug dispersed into the polymer matrix; and finally (iii) a sustained release of the drug over 60 days resulting from the diffusion of the protein through the polymer wall as well as the erosion of the polymers. On the other hand, the results also showed that a higher and faster BSA release was observed for PLGA NP than those of PCL. There are several possible hypothesis to explain this difference. Among them, the difference in affinity between BSA and the two polymers, with a stronger affinity between BSA and PCL, involving a greater dispersion of the protein into the PCL matrix. Furthermore, the greater hydrophobicity of PCL compared to PLGA could also elucidate this event.

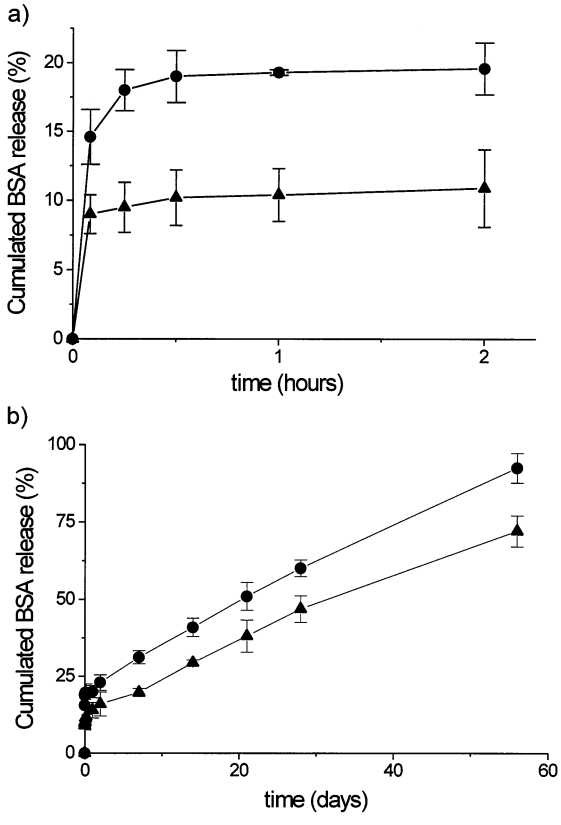

Fig. 8. Release profiles of BSA and PLGA (●) and PCL (▲) NP in phosphate buffer at 37°C and pH 7.4 after 2 h (a) and 60 days (b). Data are shown as mean ± SE.

4. Conclusion

The present NP can be expected to be used as carriers of hydrophilic compounds prepared by a w/o/w extraction method. Furthermore, the present NP are attractive for parenteral or transmucosal application because of their monodispersed submicron-sized structure and their biodegradability. Moreover, the preliminary protein release test from the PLGA and PCL NP in vitro proved that the present NP had the properties of a sustained release form.

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