J Mater Sci: Mater Med (2008) 19:1703–1711
DOI 10.1007/s10856-007-3253-9

Engineering of poly(ε-caprolactone) microcarriers to modulate protein encapsulation capability and release kinetic

Valentina Coccoli · Alessia Luciani · Silvia Orsi · Vincenzo Guarino · Filippo Causa · Paolo Antonio Netti

Received: 1 June 2007 / Accepted: 2 August 2007 / Published online: 4 October 2007

Abstract Drug delivery applications using biodegradable polymeric microspheres are becoming an important means of delivering therapeutic agents. The aim of this work was to modulate the microporosity of poly(ε-caprolactone) (PCL) microcarriers to control protein loading capability and release profile. PCL microparticles loaded with BSA (bovine serum albumin) have been de novo synthesized with double emulsion solvent evaporation technique transferred and adapted for different polymer concentrations (1.7 and 3% w/v) and stabilizer present in the inner aqueous phase (0.05, 0.5 and 1% w/v). SEM (scanning electron microscope) and CLSM (confocal laser scanning microscope) analysis map the drug distribution in homogeneously distributed cavities inside the microspheres with dimensions that can be modulated by varying double emulsion process parameters. The inner structure of BSA-loaded microspheres is greatly affected by the surfactant concentration in the internal aqueous phase, while a slight influence of polymer concentration in the oil phase was observed. The surfactant concentration mainly determines microspheres morphology, as well as drug release kinetics, as confirmed by our in-vitro BSA release study. Moreover, the entrapped protein remained unaltered during the protein encapsulation process, retaining its bio-activity and structure, as shown through a dedicated gel chromatographic analytical method.

V. Coccoli · A. Luciani · S. Orsi · V. Guarino · P. A. Netti (✉)
Interdisciplinary Research Centre in Biomaterials (CRIB), University of Naples Federico II, Piazzale Tecchio 80, Naples, 80125, Italy
e-mail: nettipa@unina.it

V. Coccoli · A. Luciani · S. Orsi · V. Guarino · P. A. Netti
Italian Institute of Technology (IIT), Via Morego 30, Genova, Italy

F. Causa
Department of Experimental and Clinical Medicine, University of Magna Graecia, University Campus Salvatore Venuta, Germaneto, Catanzaro, 88100, Italy

1 Introduction

There are several known problems associated with protein based drug therapeutic applications; amongst them the short drug in vivo half-life and the adverse side-effects caused by the multiple and high-dose injections, necessary for the attainment of the desirable drug concentration in plasma [1–3], are considered as the most prominent.

Conventional oral drug administration on the other hand quite often does not assure drug rate controlled release or target specificity. Hence, drug delivery may result in a sharp increase of local drug concentration, relatively short periods at the expected therapeutic level followed by a sharp drop-off and diminution until drug re-administration. On the other hand, at present the desired drug release profile can be achieved by implanted biodegradable polymers containing dispersed medication [4]. For this purpose controlled drug release systems based on biodegradable microspheres and their application as smart drug delivery devices has already been the subject of extensive research. By delivering therapeutic moieties at a controlled rate over a prolonged period of time, such devices sustain optimal drug concentrations [5–8], protect and stabilize the bioactive compound [9], whereas these contribute towards the patient compliance by the effective reduction of the drug administration frequency. More specific, microspheres can be easily injected and, therefore their surgical removal is not required. Moreover, drug release rates can be controlled through the manipulation of the particle size

[10–13], the polymer degradation or/and erosion rates [13, 14], and polymer erosion mechanism (bulk versus surface erosion), amongst other determinative factors. Since protein based drugs delivery kinetics primarily depend upon polymer nature, morphology and drug topology within the microspheres, the fundamental understanding of the relationship amongst these key parameters and the drug release mechanisms is a prerequisite for useful products [15] engineering. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL) and their copolymers are the primary polymeric candidates for the development of microparticles as drug delivery systems, because they are biodegradable and biocompatible [16, 17]. PCL is ideally suitable for long term sustained drug delivery extending over a period longer than one year [18], due to its relatively low degradation rate when compared to PLA, PGA and their copolymers. Consequently, PCL has been applied in the preparation of different delivery systems in the form of microspheres [16, 18–21] and nanospheres [18, 22].

PCL microspheres have been prepared by various methods such as phase separation [23], emulsion solvent evaporation [15], emulsion solvent extraction [24], spray drying [25], and melt encapsulation [26]. The successful encapsulation of proteins and peptides requires high drug loading in the microspheres, prevention of protein degradation by the encapsulation method, and predictable release of the drug compound from the microspheres. Among the different encapsulation techniques, the multiple emulsion solvent evaporation method, previously described by Ogawa et al. [27], is generally considered as one of the most appropriate ways to obtain a high encapsulation efficiency of water soluble drugs [28].

According to this method an aqueous solution of hydrophilic compound is emulsified into an organic solution of the hydrophobic polymer. The primary water-in-oil emulsion (w/o) is then dispersed in an external aqueous phase accompanied by the formation of a double water-oil-water emulsion [29–31]. The volatile solvent is removed as it diffuses into the aqueous medium and evaporates at the air/water interface during the second emulsification step. The polymer precipitates out during the removal of the solvent, thereby immobilizing and encapsulating the inner aqueous phase within a spherical polymeric matrix [32].

The starting point for the work in this paper was the application of the double emulsion solvent evaporation technique—as outlined above—for the de novo production of PCL microspheres loaded with Bovine Serum Albumin (BSA). BSA was selected and used as a model bioactive compound, since it is well characterised and readily available [19, 33].

It is well known that PCL is used for long lasting delivery due to its slow degradation rate [18]. However, besides the release activated by the slow polymer degradation process a faster release mechanism from this material is also present and evident at early stage. This latter mechanism is strongly influenced by loading and microstructure of PCL microspheres. Our study focused mainly on this early stage mechanism and aimed to establish the microstructure parameters to modulate protein encapsulation capability as well as release kinetic.

It was soon unveiled that by varying process parameters such as polymer solution concentration and stabilizer concentration in the inner aqueous phase it is possible to modulate the size and distribution of microspheres inner cavities, rendering possible the tailored design and engineering of PCL microcarriers with a predictable release of drug compounds.

2 Materials and methods

2.1 Materials

Poly(ε-caprolactone) (65,000 MW) was supplied by Aldrich. Polyvinyl alcohol (PVA) (Mw 13,000–23,000, 87–89% hydrolyzed), obtained from Aldrich, was used as emulsion stabilizer. Dichloromethane, used as polymer solvent, was purchased from Sigma-Aldrich. Bovine serum albumin (BSA, Sigma Aldrich) was encapsulated in microspheres as a model drug. Rhodamine labelled bovine serum albumin was from Sigma-Aldrich. Other chemicals were reagent grade and used as supplied.

2.2 Preparation of protein loaded microspheres

Protein loaded microspheres were prepared using emulsification processes followed by the evaporation of the solvent. BSA was dispersed in 1 ml Phosphate Buffer Saline (PBS) solution containing different percentages of PVA (0.05, 0.5 and 1% w/v). This internal aqueous phase was emulsified for 30 s with 9 ml of dichloromethane with different amounts of PCL (1.7 and 3% w/v) using a sonicator (GRANT ULTRASONIC BATH XB3, 50–60 Hz, 60W) for this purpose. The resulting first emulsion (W/O) was administered dropwise into 250 ml of 0.5% (w/v) PVA/PBS solution as external aqueous phase. The mixture was then stirred magnetically at 400 rpm (FALC Instruments mod. F60) for 30 min to produce a double W/O/W emulsion. In order to allow the evaporation of the organic solvent a PVA/PBS solution was added dropwise continuously. Solid microparticles were separated by filtering and washed three times with de-ionized water. The dried particles prepared by double emulsion were then stored at 4 °C.

2.3 Microspheres morphology

The surface morphological and inner structure examination of the microspheres was carried out by means of a scanning electron microscope (SEM) (LEIKA S400). In particular, for the surface analyses purposes the microparticles were mounted onto metal stubs using double sided adhesive tape and then coated with gold-palladium under an argon atmosphere. For the analysis of the inner structure microspheres were frozen in liquid nitrogen and cross-sectioned. Finally, these were gold–palladium coated and viewed under the scanning electron microscope.

An estimation of the pore sizes have been carried out by image analysis on SEM micrographs of cross-section surfaces by using a specific imaging software (ImageJ 1.36b, NIH freeware). Preventively, a manipulation of high resolution images was realized and, in detail, the adequate definition of the threshold level allowed defining the optimal contrast and brightness to the calculation of the pore features. Then, the 8-bit conversion allowed obtaining a monochromatic image with 256 grey levels where the level 0 corresponds to pure black and the highest level 255 to pure white. Means and standard deviations of pore surfaces have been determined from limited number of micrographs of each sample.

The topographic distribution of BSA-rhodamine inside the microspheres was monitored by confocal laser scanning microscopy (CLSM 510, Zeiss). More specific, with CLSM the signal from a fluorochrome at a specified single focal plain within the spheres was recorded. Rejection of out-of-focus light allowed optical sectioning of the intact spheres. The laser set to 30% of full power at a wavelength of 488 nm was used as the excitation source. Light emitted from the BSA-rhodamine moieties passed through a 570 nm filter and was amplified by a photomultiplier. The spheres were viewed at the horizontal optical plain.

2.4 Particles size analysis: Coulter counter

The microspheres' mean diameter and size distribution were determined by laser light scattering (Coulter LS 100Q, USA) on a dispersion of sonicated microspheres in 0.2% w/v aqueous PVA. Particle size was expressed as mean volume diameter, the average of values collected from three different batches. The polydispersity index (PI) for each batch was calculated as the mean volumetric diameter and the mean numeric diameter ratio.

2.5 Protein encapsulation efficiency

The extraction of BSA from microspheres was performed according to the technique patented by Tice and Gilley [34]. The microspheres were first dissolved in dichloromethane under magnetic stirring. Then 2 ml of a phosphate buffer solution (PBS, pH 7.4) was added and the system was vigorously mixed. The mixtures were then centrifuged for 10 min at 13,000 rpm and the aqueous phase was transferred into a vial. After extraction the encapsulated amount of protein was determined by measuring the absorbance at 280 nm in a multi-well plate spectrophotometer (PERKIN-ELMER WALLAC 1421).

The drug incorporation efficiency was expressed as the percentage of measured drug content over the theoretical drug content.

Drug Incorporation Efficiency = 100 × (Measured drug content/Theoretical drug content)

2.6 Spectrophotometric in vitro BSA release study

In vitro release tests were carried out in triplicate. A 50 mg aliquot of dried microspheres was placed in a 2 ml microfuge tube and incubated in 500 μl of de-ionized water at 37 °C. Tubes were continuously shaken and a 100 μl aliquot of supernatant aliquot was periodically taken from each tube after being centrifuged for 60 min at 13,000 rev/min using an Eppendorf Centrifuge (Model 5415C) and then replaced with an equal volume of fresh water. Concentration of BSA in the supernatant was determined via a standard curve by measuring the absorbance at 280 nm in a multi-well plate spectrophotometer (PERKIN-ELMER WALLAC 1421).

2.7 Analysis of protein's native state stability in PCL microspheres

In order to verify if microspheres preparation procedure leads to the denaturation of BSA, a gel filtration chromatography method was used. Gel filtration is the protein purification method which takes advantage of the differences in molecular size and conformational shape of proteins. A gel filtration column is composed of beads with molecular size pores which small proteins can enter and large ones can not. A 500 μl aliquot of BSA solution was analysed by AKTA system (Amersham Pharmacia Biotech) with a chromatographic column for gel filtration at flow rate of 0.3 ml/min.

3 Results and discussion

Drug delivery applications using biodegradable polymeric microspheres are becoming an important means of delivering therapeutic agents since the latter offer facile,

non-invasive administration via injection, protection of encapsulated drugs and sustained drug release over periods from hours to months or even years [35, 36].

Our results clearly demonstrate that it is possible through our novel high precision particle fabrication technique to accurately and reliably investigate factors that influence drug release from tailored polymeric microspheres.

More specific, a microencapsulation technique based on the w/o/w multiple emulsion solvent evaporation process was transferred and adapted for two polymer concentrations (1.7% and 3% w/v) and stabilizer present in the inner aqueous phase (0.05%, 0.5% and 1% w/v); both of these factors have been shown to affect microspheres morphology as well as drug release kinetics.

Eventually, analyses of microspheres surface morphology and inner structure in conjunction to the measured encapsulation efficiency values allowed the interpretation of protein release mechanisms.

3.1 Microstructure of protein-loaded microspheres

The surface analysis of drug-loaded microparticles prepared by w/o/w emulsion-method revealed that the microparticles were spherical and polydispersed, with porous surface (Fig. 1A and B).

The same appearance was observed for all formulations.

Cross section—SEM analyses of the freeze-fractured PCL microspheres revealed a porous inner structure (Fig. 2). A decrease of average pore surface at increasing PVA concentration is evaluated by a quantitative estimation performed by image analysis (Fig. 3). The oil drops of the double emulsion contain several aqueous droplets, resulting from the primary emulsion, which, in turn, are the precursor of pores. The inner structure of BSA-loaded microspheres was greatly affected by the surfactant (PVA) concentration in the internal aqueous phase that stabilizes the primary emulsion. By improving the stability of the primary emulsion microspheres inner cavities were reduced in size and showed a more homogenous distribution, as confirmed in scanning laser confocal micrographs (Fig. 4).

In particular, high PVA concentrations (e.g., 1%) yielded microspheres with small and homogeneously distributed cavities, while at low PVA concentration (e.g., 0.05%) the pores in the inner structure were much bigger. Therefore, the presence of PVA stabilizes the inner water droplets impeding their coalescence.

Moreover, SEM and CLSM images showed that polymer concentration also affected the inner structure of microparticles. In particular, microspheres with lower polymer concentration showed larger inner cavities compared to microspheres with higher polymer concentration (Fig. 2–4). A tentative interpretation of the observed phenomenon was that at a low polymer concentration, resulting in a less viscous polymer solution, the probability of coalescence of the inner aqueous droplets is higher; this, in turn, results in microspheres with enlarged pores. On the other hand, high polymer concentrations lead to a more viscous solution which can set faster in the secondary emulsion, inhibiting the coalescence of the water droplets.

3.2 Particle size analysis

BSA loaded microparticles were prepared using different concentrations of PCL and different amounts of PVA in the inner aqueous phase in order to investigate particle size variation under these conditions. In particular, by increasing the weight of PCL in a fixed volume of dichloromethane, an increase in the mean particles' size was witnessed (Fig. 5). This could be the manifestation of an increase in the emulsion viscosity caused by the higher polymer concentration. For this reason, it is more difficult to break up the solution into smaller droplets when applying equal mixing power giving rise to larger size microspheres.

Furthermore, it was observed that increasing PVA concentration in the inner aqueous phase resulted in a slight increase in the mean microparticles size (Fig. 5). This, in turn, was attributed to the evident difficulties encountered when emulsifying a solution of higher viscosity.

Therefore, in the light of the above results, it can be safely concluded that the microspheres' size can be

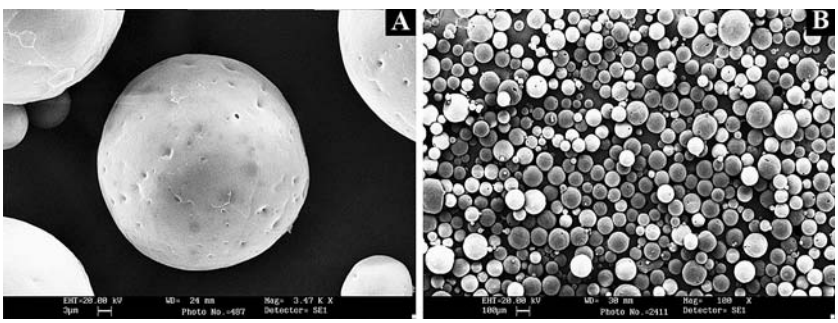

Fig. 1 Scanning electron micrograph of BSA-loaded PCL microspheres at different magnifications (**A**) 3.47 Kx; (**B**) 100×

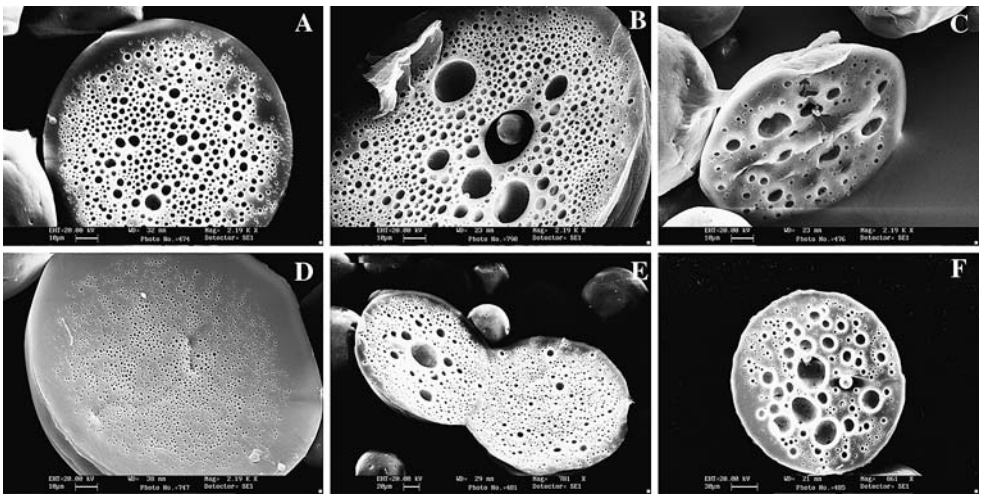

Fig. 2 Freeze-fractured particles observed by SEM. Influence of PCL concentration and stabilizer in the internal aqueous phase on the microspheres inner structure (**A**) 1.7% PCL–1% PVA, (**B**) 1.7% PCL–0.5% PVA, (**C**) 1.7% PCL–0.05% PVA; (**D**) 3% PCL–1% PVA, (**E**) 3% PCL–0.5% PVA, (**F**) 3% PCL–0.05% PVA

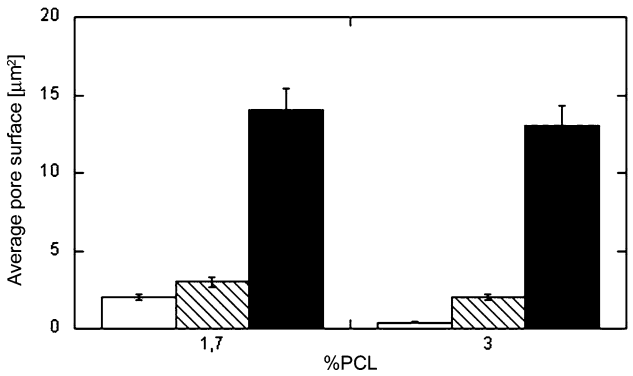

Fig. 3 Average pore surface of microspheres inner porosity as a function of PVA concentration in the inner aqueous phase and PCL concentration in the oil phase. □ 1% PVA, ▨ 0.5% PVA, ■ 0.05% PVA

modulated by the polymer concentration and surfactant concentration in the inner aqueous phase, primarily through the variation of the polymeric emulsion viscosity.

3.3 Protein encapsulation efficiency

In order to prevent the waste of expensive drugs and sustain drug dosage over an extended treatment period it is necessary to increase or control the encapsulation efficiency (EE) in the polymeric microspheres. Towards that end model experimentation has been carried out using BSA and the encapsulation efficiency measured for the novel microspheres fabricated by the double emulsion solvent evaporation method are reported in Table 1.

These results demonstrate that PCL concentration and the amount of PVA in the inner aqueous phase have a significant impact upon BSA entrapment efficiency. More specific, it was observed that an increase of PVA concentration (from 0.05 to 1% w/v) resulted in an increase of BSA encapsulation efficiency. This result could be attributed to the interaction between the surfactant and the protein, which can protect BSA from the solvent and prevent its dissolution in the external aqueous phase. Furthermore, it was shown that by increasing the concentration of polymer in the organic phase (oil phase) an increase of BSA encapsulation efficiency is obtained. This could be due to an increase in the emulsion viscosity that prevented BSA diffusion towards the external aqueous phase. Moreover, a more viscous solution could lead to a faster setting that prevented BSA diffusion.

3.4 In vitro release of BSA from PCL microspheres

PCL is a semi-crystalline polymer [18] and, therefore, water can easily penetrate only into the amorphous part of the polymer matrix, thus facilitating the release of the water soluble drug by diffusion. In this manner, the encapsulated drug can be released through polymer amorphous regions as well as from the inner cavities created during the first emulsion step.

The drug release profiles illustrated in Fig. 6 were obtained at two PCL contents, 1.7 and 3%, respectively, whereas the amount of PVA in the inner aqueous phase was varied from 0.05% to 1%.

In particular, for all the microsphere batches investigated at start a burst effect was observed followed by a low rate release profile. The burst effect, which corresponded to a rapid initial drug delivery phase, was attributed to the

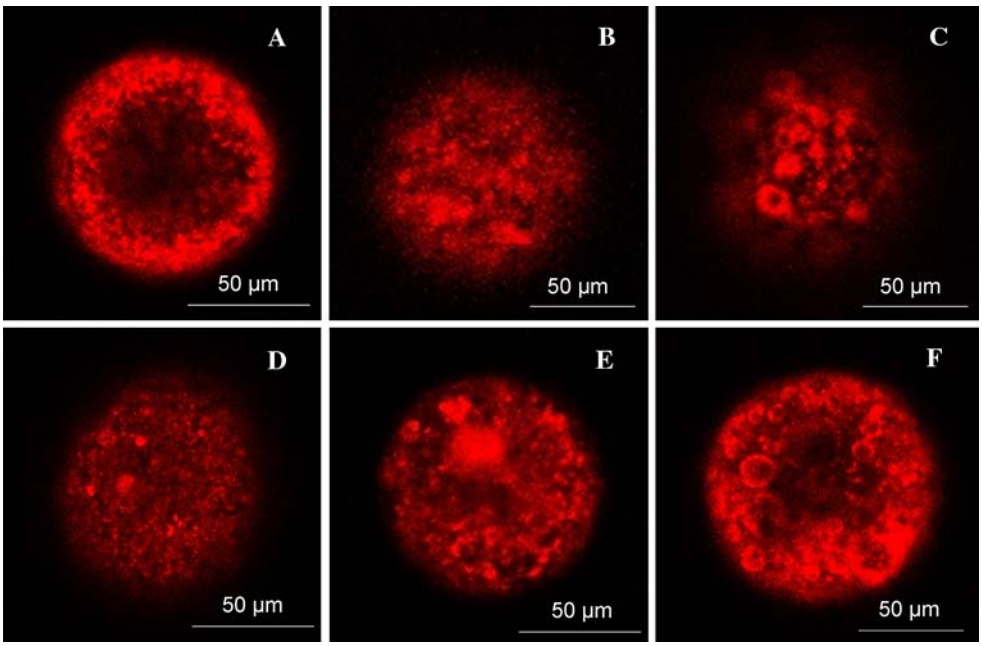

Fig. 4 Distribution of BSA-Rhodamine in PCL microspheres observed by CLSM. Influence of PCL concentration and stabilizer concentration in the internal aqueous phase on the microspheres inner structure (**A**) 1.7% PCL–1% PVA, (**B**) 1.7% PCL–0.5% PVA, (**C**) 1.7% PCL–0.05% PVA; (**D**) 3% PCL–1% PVA, (**E**) 3% PCL–0.5% PVA, (**F**) 3% PCL–0.05% PVA

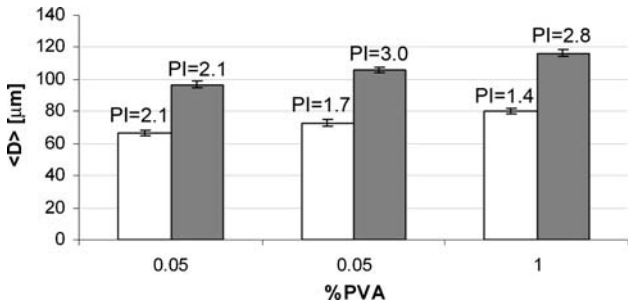

Fig. 5 Microspheres mean diameter (<D>) and polydispersity index (PI) as a function of polymer concentration (1.7% white, 3% grey) and stabilizer (PVA) concentration in the inner aqueous phase

Table 1 BSA encapsulation efficiency in PCL microspheres as a function of polymer concentration and stabilizer (PVA) amount in the inner aqueous phase

% PVA	% PCL	Encapsulation efficiency (%)
0.05	1.7	29.1
0.5	1.7	40
1	1.7	59.9
0.05	3	48.2
0.5	3	51.6
1	3	62.9

release of BSA from the external pores on the surface of the microspheres. The amount of BSA released during this burst phase for all six formulations surveyed was less than 50% of the total BSA quantity encapsulated in the microspheres. After the burst effect a remarkable decrease in drug release rate has been observed. Although there is a progressive release approaching to a zero order kinetic this could be assessed by a further investigation on longer release time.

PVA concentration in the inner aqueous phase was shown to influence the BSA release profiles as illustrated in Fig. 6. Microspheres produced using a high concentration of PVA showed a low BSA release profile most probably due to their specific inner morphology. In actual terms, it was observed that at a high PVA concentration microspheres produced bared small and homogeneously distributed cavities as showed in Fig. 3. This, in turn, resulted in high tortuosity of the BSA route, since the protein should cross the amorphous regions of PCL, then the inner cavity of microspheres and eventually the amorphous region of PCL once more, this sequence of steps was repeated several times in order to cross the full thickness of the microsphere. On the other hand, a low concentration of PVA in the inner aqueous phase led to the fabrication of microspheres with larger pores in their inner structure; this meant, however, that the protein had to cross less amorphous regions of PCL to transverse the full thickness of the microsphere and a concomitant increased BSA release rate was, therefore, recorded.

Moreover the data showed a slightly influence of polymer concentration in the oil phase on the release profiles of protein. A low polymer concentration yields microspheres with larger cavities when compared to microspheres

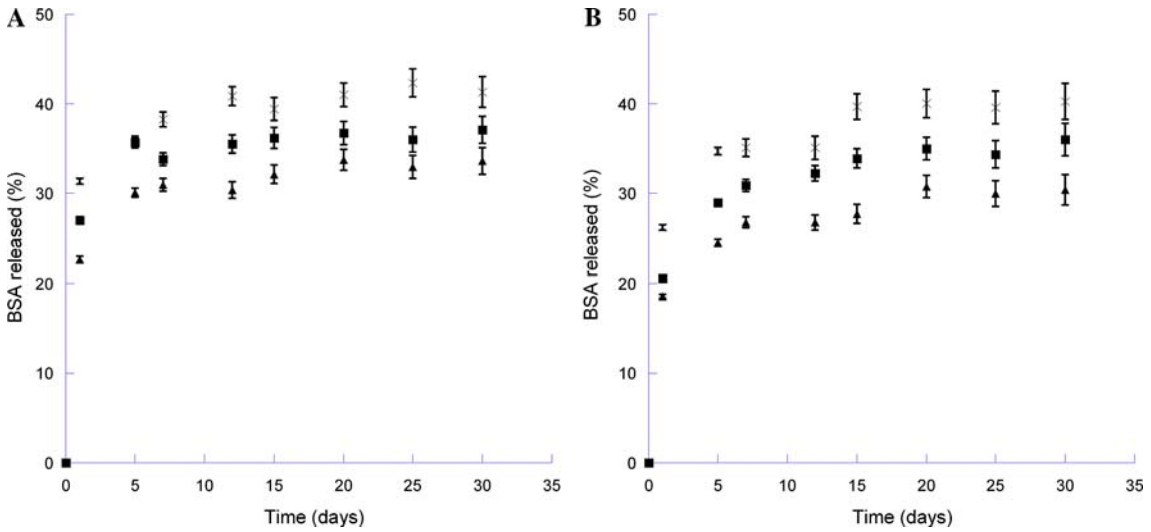

Fig. 6 Release profiles as a function of PVA concentration in the inner aqueous phase: (**A**) 1.7% PCL, (**B**) 3% PCL; **x** 0.05% PVA, ■ 0.5% PVA, ▲ 1% PVA

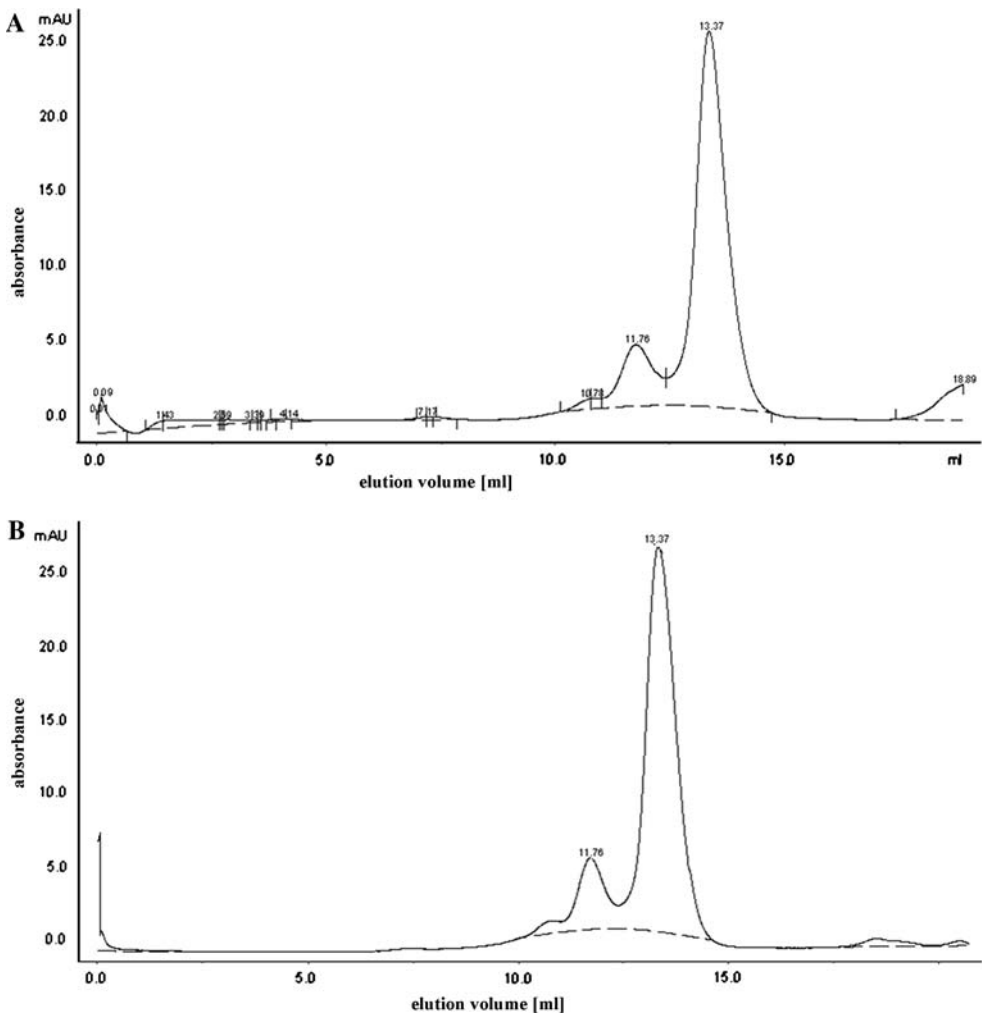

Fig. 7 Gel filtration chromatographic profiles recorded for the BSA released from the PCL microspheres (**A**) and untreated (**B**) protein moieties

fabricated using a higher polymer concentration. As already mentioned, the BSA release profile was directly affected by the inner morphology of microspheres. Consequently, the microspheres produced at a low polymer concentration showed an accelerated drug release profile.

As discussed before, a secondary stage of protein release was attended as consequence of polymer degradation. However, a further investigation on long lasting release will be reported in a following paper since the aim of the current work was focused on the study of the role of microcarriers morphology on protein loading capability and release profile resulting from diffusion-driven mechanisms.

3.5 Analysis of protein's native state stability in PCL microspheres

During microparticles preparation the protein was exposed to potentially harsh conditions, such as vigorous mechanical agitation and contact with organic solvents. These conditions could have resulted in irreversible protein drug denaturation and then the loss of biological activity. In order to estimate if the BSA is in its native or denatured state, a gel filtration chromatography analysis was performed on the native protein and on the BSA released from microparticles. This analysis has been accomplished by graphing the absorbance values of the BSA, before and after the emulsion process, versus its elution volumes from the gel filtration column and using the elution profile of the native protein to estimate BSA conformational changes. We have observed that the BSA elution profile after the emulsion process was equal to the native BSA pattern (Fig. 7). From the afore mentioned results graphically illustrated in Fig. 7 it was deduced that the BSA didn't undergo denaturation during the emulsion process and it preserved its structural and conformational integrity as well as its molecular weight.

4 Conclusions

PCL-microparticles loaded with BSA were de novo synthesized with the transfer and adaptation of the double emulsion solvent evaporation technique; the product novel microparticles were spherical and polydispersed with a porous surface.

Moreover, we have systematically investigated and quantified the effects of the amount of PVA in the inner aqueous phase along with the PCL concentration in the oil phase upon the novel PCL microspheres size, inner structure architecture, encapsulation efficiency and protein release. Along these research lines a most significant finding of our work has been that, despite the low diffusion rate of macromolecules in PCL, it is possible to fabricate PCL microspheres with controlled encapsulation capability capable of delivering various amounts of proteins by diffusion at different kinetics; the latter new devices will, therefore, serve in the near future as multifunctional "smart" drug delivery platforms.

References

1. D. BLANCO and M. J. ALONSO, *Eur. J. Pharm. Biopharm.* **45** (1998) 285
2. S. P. SCHWEDERMAN, M. CARDAMONE, M. R. BRANDON, A. KLIBANOV and R. LANGER, in "Microparticulate Systems for the Delivery of Proteins and Vaccines" (Marcel Dekker, New York, 1996) p. 1
3. T. KISSEL, R. KONEBERG, in "Microparticulate Systems for the Delivery of Proteins and Vaccines" (Marcel Dekker, New York, 1996) p. 51
4. S. FREIBERG and X. X. ZHU, *Intern. J. Pharm.* **282** (2004) 1
5. M. CHIBA, J. HANES and R. LANGER, *Biomaterials* **18** (1997) 893
6. E. MATHIOWITZ, D. KLINE and R. LANGER, *Scanning Microsc.* **4** (1990) 329
7. E. MATHIOWITZ, W. M. SALTZMAN, A. DOMB and R. LANGER, *J. Appl. Polym. Sci.* **35** (1988) 755
8. J. TAMADA and R. LANGER, *J. Biomater. Sci. Polym. Ed.* **3** (1992) 315
9. I. J. CASTELLANOS, W. O. CUADRADO and K. GRIEBENOW, *J. Pharm. Pharmacol.* **53** (2001) 1099
10. S. COHEN, T. YOSHIKA, M. LUCARELLI, L. H. HWANG and R. LANGER, *Pharm. Res.* **8** (1991) 713
11. R. NARAYANI and K. PANDURANGA RAO, *Int. J. Pharm.* **143** (1996) 255
12. P. SANSDRAP and A. J. MOES, *J. Control. Release.* **43** (1997) 47
13. J. M. BEZEMER, R. RADERSMA, D. W. GRIJPMA, P. J. DIJKSTRA, C. A. VAN BLITTERSWIJK and J. FEIJEN, *J. Control. Release.* **67** (2000) 249
14. M. A. TRACY, K. L. WARD, L. FIROUZABADIAN, Y. WANG, N. DONG, R. QIAN and Y. ZHANG, *Biomaterials* **20** (1999) 1057
15. YI.-Y. YANG, T.-S. CHUNG and N. PING, *Biomaterials* **22** (2001) 231
16. M. A. BENOIT, B. BARAS and J. GILLARD, *Intern. J. Pharmaceutics* **184** (1999) 73
17. D. L. WISE, T. D. FELLMAN, J. E. SANDERSON and R. L. WENTWORTH, in "Drug Carriers in Medicine" (Academic Press, New York, 1979) p. 237
18. V. R. SINHA, K. BANSAL, R. KAUSHIK, R. KUMRIA and A. TREHAN, *Int. J. Pharm.* **278** (2004) 1
19. B. BOTTI, C. YOUAN, T. L. JACKSON, L. DICKENS, C. HERNANDEZ and G. O. ABABIO, *J. Control. Release* **76** (2001) 313
20. C. G. PITT, in "Biodegradable Polymers as Drug Delivery Systems" (Marcel Dekker New York 1990), p. 71
21. M. D. DHANARAJU, D. GOPINATH, M. R. AHMED, R. JAYAKUMAR and C. VAMSADHARA, *J. Biomed. Mater. Res.* **76A** (2006) 63
22. M. VERGER, L. FLUCKIGER, Y. KIM, M. HOFFMAN, P. MAINCENT, *Eur. J. Pharm. Biopharm.* **46** (1998) 137
23. B. K. KIM, S. J. HWANG, J. B. PARK and H. J. PARK, *J. Microencapsul.* **22** (2005) 193

24. S. ZHOU, X. DENG and H. YANG, *Biomaterials* **24** (2003) 3563
25. R. L. SASTRE, M. D. BLANCO, C. TEIJÓN, R. OLMO and J. M. TEIJÓN, *Drug Dev. Res.* **63**(2) (2004) 41
26. S. R. JAMEELA, N. SUMA and A. JAYAKRISHNAN, *J. Biomater. Sci. Polym. Ed.* **8** (1997) 457
27. Y. OGAWA, H. OKADA, M. YAMAMOTO and T. SHIMAMOTO, *Chem. Pharm. Bull.* **36** (1988) 2576
28. J. H. KIM and Y. H. BAE, *Eur. J. Pharm. Sci.* **23** (2004) 245
29. N. NIHANT, C. SCHUGENS, C. GRANDFILS, R. JEROME and P. TEYSSIE, *J. Colloid Interface Sci.* **173** (1995) 55
30. M. N. VRANCKEN and D. A. CLAYS, United States Patent Office, Patent No 3,526,906, (1970)
31. N. C. DE JAEGER and B. H. TAVERNIER, British Patent No 1,405,108 (1971)
32. G. CROTTS and T. G. PARK, *J. Control. Release.* **35** (1995) 91
33. T. PETERS, *Adv. Prot. Chem.* **37** (1985) 161
34. T. R. TICE, R. M. GILLEY, US patent No 5,407,609 (1993)
35. D. L. WISE, D. J. TRANTOLO, R. T. MARINO and J. P. KITCHELL, *Adv. Drug Deliv. Rev.* **1** (1987) 19
36. B. H. WOO, J. W. KOSTANSKI, S. GEBREKIDAN, B. A. DANI, B. C. THANOO and P. P. DELUCA, *J. Control. Release.* **75** (2001) 307