Colloid Polym Sci (2005) 283: 480–485
DOI 10.1007/s00396-004-1173-5

ORIGINAL CONTRIBUTION

Sai Li
Yi He
Cao Li
Xiaobo Liu

In vitro release of protein from poly(butylcyanoacrylate) nanocapsules with an aqueous core

Received: 22 November 2003
Accepted: 16 April 2004
Published online: 24 July 2004

S. Li · Y. He · C. Li · X. Liu (✉)
Chengdu Institute of Organic Chemistry,
Chinese Academy of Sciences,
610041 Chengdu, Sichuan, China
E-mail: liuxb.cioc@163.com
Tel.: +86-28-85238656
Fax: +86-28-85223978

Abstract Factors influencing the in vitro release of bovine serum albumin (BSA) from poly(butylcyanoacrylate) (PBCA) nanocapsules, such as the pH value, BSA loading, the polymeric nanocapsule walls and protein molecular weight, were investigated in detail. The BSA release rate was affected by the degradation rate of the polymeric wall and protein loading. For low molecular weight proteins, the initial burst release was faster than that of high molecular weigh proteins and got to equilibrium quickly. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis results showed that BSA encapsulated within PBCA nanocapsules did not suffer covalent aggregation or fragmentation during the initial days of in vitro incubation. For nanocapsules prepared by interfacial polymerization in water-in-oil microemulsions, these findings were useful as a foundation for the development of nanocapsules with desired properties.

Keywords Nanocapsules · Protein release · Poly(butylcyanoacrylate) · Degradation

Introduction

In the last 20 years, many synthetic and natural polymers have been examined in drug delivery applications [1]. Poly(alkylcyanoacrylate) (PACA) nanocapsules have gained extensive interest as drug carriers because of the biocompatibility and biodegradability of the polymer and the simplicity of the polymerization process [2, 3, 4]. Poly(butylcyanoacrylate) (PBCA) nanocapsules are easily absorbed through the intestinal lumen via the paracellular route as a result of their small size and also move transcellularly through the M cells which line the Peyer's patch region [5]. Secondly, nanocapsules protect peptides from degradation by proteolytic enzymes that would otherwise severely hinder their oral bioavailability [6].

To our knowledge, two techniques are available to prepare biodegradable nanocapsules:

1. Interfacial polymerization of alkylcyanoacrylate monomers [7]. In this process the cyanoacrylate monomer and lipophilic drugs are dissolved in a mixture of oil and ethanol. The organic solution is then added slowly into water or a buffer solution (pH 3–9) containing surfactants such as poloxamers or phospholipids. Nanocapsules are formed spontaneously by anionic polymerization of the cyanoacrylate in the oily phase.
2. Interfacial deposition of preformed polymers [8]. In this process, the lipophilic drug, oil, polymer and optionally phospholipids are dissolved in a water-miscible solvent (e.g., acetone). This solution is then poured under stirring into an aqueous solution containing a nonionic surfactant (e.g., poloxamer). Nanocapsules are formed instantaneously by the fast diffusion of the solvent into water, which provokes the spontaneous emulsification of the oily solution in the form of nanodroplets where the desolvated polymer will form a film around the oily nanodroplets that contain the drug.

However the nanocapsules prepared by these two methods both have an oily core that is not suitable for hydrophilic protein and peptide drugs.

Recently, Lambert et al. [9] proposed a simple method for the preparation of biodegradable PACA nanocapsules having an aqueous core. This kind of nanocapsule could deliver hydrophilic protein and peptide drugs to their target sites. But up to now factors influencing the in vitro release profiles of protein from the PACA nanocapsules have not been investigated intensively. Using bovine serum albumin (BSA) as a model protein, the aim of this work was to determine the effect of various factors on the in vitro release of protein from PBCA nanocapsules having an aqueous core. In addition, the stability of an encapsulated protein was also investigated.

Experimental

Materials

Butyl 2-cyanoacrylate was purchased from Suncon Medical Adhesive Co., Beijing, China. Insulin, ovalbumin, BSA and urease were purchased from Biolife Science & Technology Co., Shanghai, China. Polysorbate 80 and glycerol trioleate were obtained from Chemical Reagent Company, Shanghai, China, and were used without further purification. Distilled water was used throughout. All other reagents were of analytical grade.

Preparation of polymeric nanocapsules

Nanocapsules were prepared according to a published method [9]. In brief, 0.2 mL ethanol was added to 0.8 mL phosphate buffer solution (PBS, pH 7.4) containing BSA at various concentrations from 0 to 10 mg/mL. The solution was added to an organic phase containing 16 g glycerol trioleate and 3 g polysorbate 80, and then the solution was emulsified by ultrasonication. Butyl 2-cyanoacrylate (0.1 g) was added slowly to the emulsion with stirring at 600 rpm. Two hours later, the PBCA film around the water droplets containing BSA was formed. The nanocapsule suspension was collected by ultracentrifugation at 15,000 rpm for 30 min. The nanocapsule pellets were washed three times with distilled water, and were resuspended in 1 mL distilled water by sonication, resulting in nanocapsules dispersed in an aqueous medium with an aqueous core containing the BSA.

Characterization of nanocapsules

Particle size measurement

The size of the nanocapsules was determined by laser diffraction (Mastersizer, Malvern Instruments, UK). For particle size analysis, the nanocapsule dispersion was diluted with water to obtain the desired obscuration. Measurements were carried out at room temperature.

Nanocapsule wall measurement

Examination of the PBCA nanocapsule wall was performed using a transmission electron microscope following negative staining with phosphotungstic acid solution (0.2%). Colloids were dispersed in the staining solution for 1 h at room temperature, put on a copper grill covered with nitrocellulose, dried under vacuum at least 24 h and observed (×100,000) by transmission electron microscopy (JEM-100CX).

Gel permeation chromatography

The polymer molecular weight was measured using gel permeation chromatography (GPC). The GPC system used tetrahydrofuran (Merck 8101, 99.5% high-performance liquid chromatography grade) as the mobile phase with a flow rate of 1 mL/min through two Polymer Laboratories Mixed-E columns at 40 °C connected to a reflective index detector. The nanocapsules were centrifuged for 30 min at 15,000 rpm. The pellet was freeze-dried and was then dissolved in tetrahydrofuran. The solution was filtered using a 0.5-μm Miller-SR filter. The sample was injected into the GPC system.

In vitro release of BSA from PBCA nanocapsules

About 10 mL of the nanocapsules containing BSA was immersed in 4mL of a suspension medium consisting of pH 7.4 PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 10 mM NaCl, 3 mM KCl, 0.01% Tween 20). The samples were incubated in a thermostated beaker (37 °C). At preset time intervals, samples were collected by centrifugation (18,000 rpm) and the BSA concentration in the aqueous supernatant was determined by the Folin–phenol method. Fresh PBS was added at each sampling point and incubation was continued. All experiments were carried out in triplicate.

Determination of BSA loading

The amounts of BSA loaded per unit weight of the nanocapsules were determined by an extraction method. Dried nanocapsules (10 mg) were dissolved in 1 mL methylene chloride for 20 min and 1.0 mL PBS (pH 7.4) was then added. The mixture was vigorously shaken for 2 min in order to extract BSA into PBS from the organic solution. After centrifuging, the aqueous solution was withdrawn and the BSA content of the solution was analyzed using the Folin–phenol method.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The structural integrity of BSA extracted from nanocapsules and released in the in vitro assay was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and was compared with native BSA and reference marker protein samples, which were diluted with tris(hydroxymethyl)aminomethane buffer (pH 6.8) with 2% SDS. Electrophoresis of samples was performed at a constant voltage of 200 V using a Mini-Protein II electrophoresis unit from Bio-Rad. The gel sheet was stained with 0.1% Coomssie brilliant blue to visualize the protein, destained with an aqueous solution of 20% ethanol and 10% acetic acid, then dried overnight.

Results and discussion

Effect of pH on the release profiles of BSA

The release of profiles of BSA from nanocapsules for different pH values is shown in Fig. 1. All release patterns showed a burst release of 8–20% followed by a second phase of slower release. Release of BSA from PBCA nanocapsules was suppressed in an acidic release medium (pH 2.5). When the pH of the release medium was changed to 8.6, the release rate of BSA decreased markedly. The release of BSA from PBCA nanocapsules was dependent on the degradation of the polymer. The mechanism of in vitro degradation of PBCA was proposed by Leonard et al. [10] to be a reverse Knoevenangel reaction resulting in hydrolysis of the polymer chain and the formation butyl 2-cyanoacrylate and formaldehyde. During the degradation of PBCA, BSA diffused into the release medium from the interspaces of the polymer chains and the core of the bulk polymer. The initial burst release was probably due to protein desorption from the nanocapsule surface over the first 10–30 min of release. All the release profiles had an incomplete release, which might be attributed to polymer–protein interaction such as static adsorption.

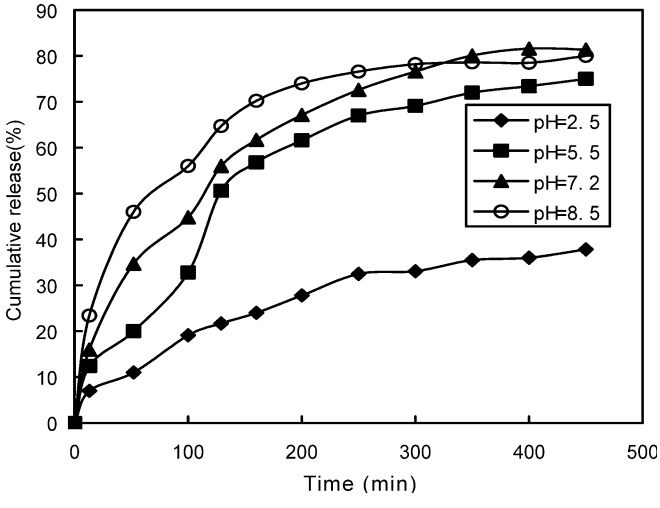

Fig. 1 Effect of pH on the release of bovine serum albumin (*BSA*). Molecular weight of poly(butylcyanoacrylate) (*PBCA*) 7,500)

Te nanocapsule diameter distribution during BSA release in the pH 7.2 buffer medium is shown in Fig. 2. It demonstrates that as time increased the average nanocapsule diameter showed a definite decrease (from 160 to 75 nm). At the same time, the diameter distribution reduced with time. In this work, an immediate decrease in nanocapsule diameter was noted after 30 min, and the diameter and the molecular weight of the nanocapsules decreased gradually with time. These results were consistent with the GPC analysis (as shown in Table 1). Therefore a surface erosion mechanism was presumed. However it should be pointed that because of gravity during the preparation of the nanocapsules, the position of the cores of some PBCA nanocapsules was not exactly in the center. So the wall thickness of these

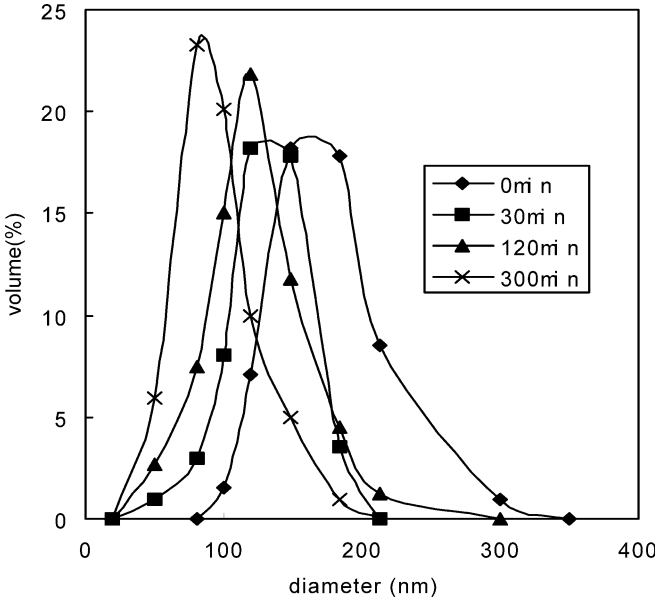

Fig. 2 Nanocapsule diameter distribution during BSA release

Table 1 Molecular weight and the average size of poly(butylcyanoacrylate) nanocapsules

Time (min)	Average size (nm)	Molecular weight (M_p)
0	160	8,310
30	134	5,520
120	120	4,700
300	75	2,430

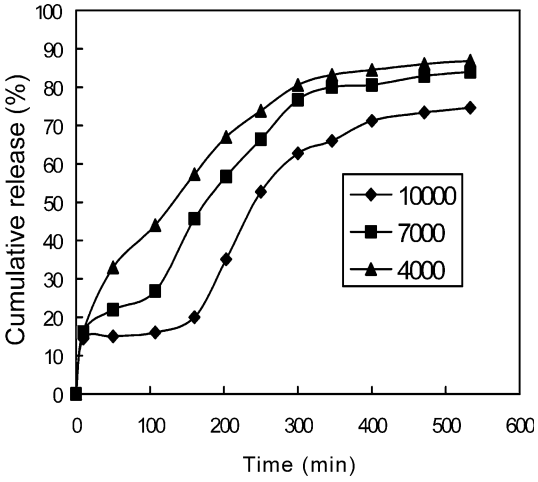

Fig. 3 Release of BSA from PBCA nanocapsules with different molecular weights

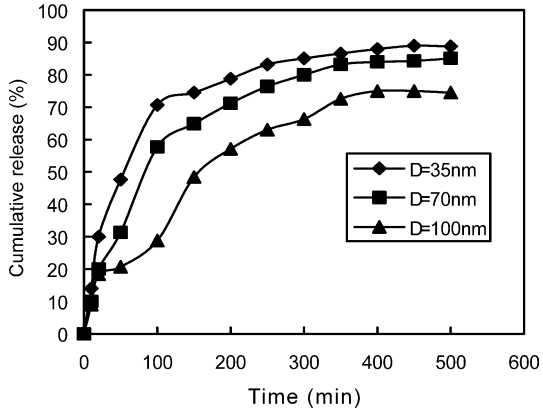

Fig. 4 Release of BSA from PBCA nanocapsules with different wall thicknesses

nanocapsules was not symmetric. The release profiles of the protein were the statistical results.

Effect of molecular weight of polymer on the release of BSA

Three different molecular weight PBCA nanocapsules formulations were prepared using different masses of the monomer. With low molecular weight PBCA ($M_n = 4{,}000$), a biphasic release profile was observed, whereas with high molecular weight PBCA, sigmoidal curves with a lag release were observed (Fig. 3). There was no obvious difference in the initial burst protein releases among different molecular weight polymeric nanocapsules. This indicated that the high molecular weight PBCA was difficult to degrade compared with low molecular weight PBCA. This may be due to the formation of nanocapsules having a thicker polymer wall. At the same time, the mean diameter of these three different PBCA nanocapsule formulations ranged from 35 to 100 nm with increasing mass of monomer used for the polymerization. The BSA release profiles are shown in Fig. 4, and are consistent with the results of Fig. 3.

The polymer with a thicker wall needed a period time to degrade to reduce the thickness of the wall. Therefore the BSA release rate was slower than that of polymer nanocapsules with thin walls. The results of Fig. 3 and 4 also validated that the degradation mechanism of PBCA nanocapsules was a surface erosion mechanism.

Effect of BSA loading on the release profiles

The influence of the loading on the BSA release from 180-nm PBCA nanocapsules is indicated in Fig. 5. With

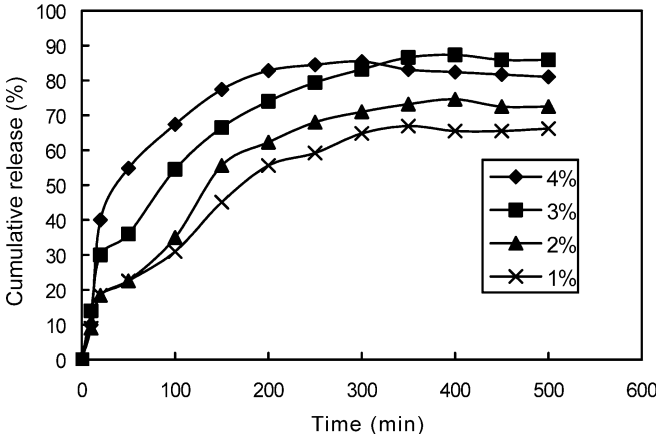

Fig. 5 Effect of BSA loading (w/w) on the release profiles

an increase in the BSA loading, a higher BSA release rate was achieved. This was due to the fact that there was a large BSA concentration gradient between the inner nanocapsules and the outer water phase when the BSA loading was high. Since the gradient was the driving force for BSA diffusion, 4% BSA loading led to a high initial burst and a rapider release rate.

Effect of protein molecular weight on the release profiles

Four hydrophilic proteins were encapsulated in PBCA in order to determine how the protein molecular weight affected the release from the nanocapsules. These results are shown in Fig. 6.

The initial burst release of the proteins increased with decreasing molecular weight. For instance insulin (molecular weight 5,800) had the fastest burst release (40%), whereas ovalbumin, BSA and urease, whose

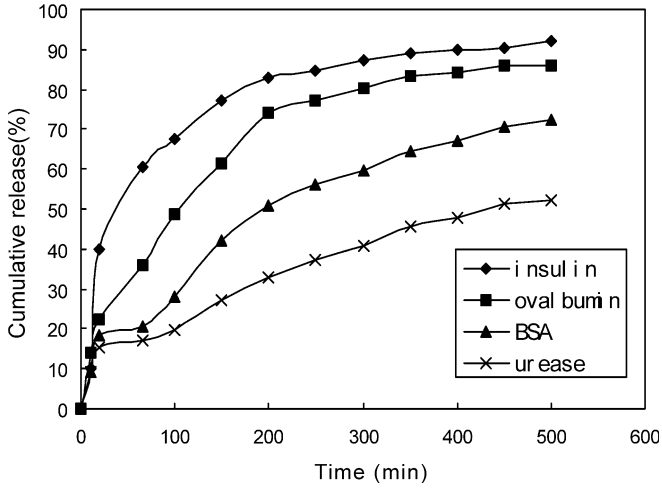

Fig. 6 Effect of protein molecular weight on the release profiles (insulin 5,800; ovalbumin 45 kDa; BSA 65 kDa; urease 483 kDa)

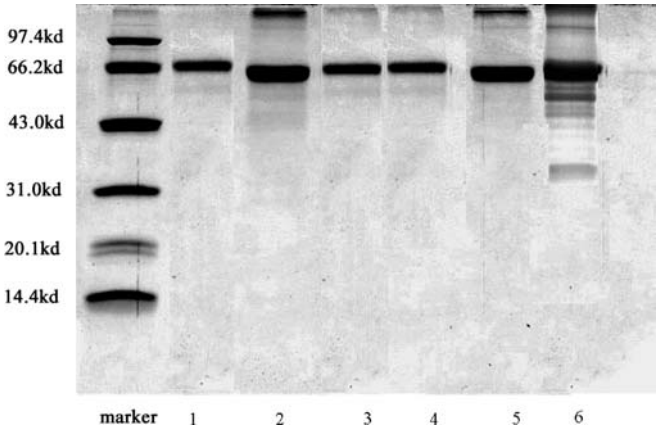

Fig. 7 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of different BSA samples. (Native BSA *lane 1*; BSA extracted from PBCA nanocapsules *lane 2*; BSA released from PBCA nanocapsules after 1, 2, 3 and 7 days of in vitro release *lanes 3–6*)

molecular weights were 45, 65 and 483 kDa respectively, had slower burst releases (15–20%). However, the next slow release rate increased with increasing protein molecular weight. It would therefore appear that low molecular weight proteins, together with water, may diffuse through the forming polymeric wall at the initial stage of the polymerization and were solubilized in the water droplets of the microemulsion outside the nanocapsules, and were absorbed onto the surface of the nanocapsules. During the protein release, the adsorbed protein desorbed into the release medium and hence resulted in the faster burst release. Higher molecular weight proteins were difficult to diffuse through the polymeric wall. They were most likely released through the pores that were caused by the polymer degradation. So the release of higher molecular weight proteins would continue for a much longer period of time.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Nonreducing SDS–PAGE of BSA extracted and released from PBCA nanocapsules was performed to investigate the BSA structural integrity (Fig. 7). It could be seen that the sample extracted from PBCA nanocapsules using organic solvent, showed apparent dimeric and higher-order aggregates with exposure to an water/oil interface [11].

BSA released from PBCA nanocapsules showed no additional peak of high and low molecular weight BSA during the initial first and second days. This suggested that no chemical polymerization, noncovalent aggregation and molecular hydrolysis occurred during the initial release process. BSA-released PBCA nanocapsules after 3 and 7 days exhibited obvious trace hydrolyzed fragments and drastic aggregates. This meant that during the process of encapsulating BSA within the nanocapsules the exposure of BSA to these conditions did not lead to irreversible aggregation or cleavage of the proteins. It also indicated that the encapsulated BSA did not suffer covalent aggregation or fragmentation during the initial days of in vitro incubation. This meant that the nanocapsules with an aqueous core were a promising delivery system for hydrophilic protein and peptide drugs.

Conclusions

BSA-encapsulated PBCA nanocapsules with an aqueous core were prepared by interfacial polymerization in a water-in-oil emulsion. Factors influencing the in vitro release of BSA, such as the pH value, BSA loading, the polymeric nanocapsule walls and protein molecular weight, were investigated in detail. The BSA release rate was affected by the degradation rate of the polymeric wall and protein loading. For low molecular weight proteins, the initial burst release was faster than that of high molecular weigh proteins and got to equilibrium quickly. SDS–PAGE results showed that BSA encapsulated within PBCA nanocapsules did not suffer covalent aggregation or fragmentation during the initial days of in vitro incubation. For nanocapsules prepared by interfacial polymerization in water-in-oil microemulsions, these findings were useful as a foundation for the development of nanocapsules with desired properties.

Acknowledgements This study was sponsored by the ''Outstanding Young Scientist Fund of Sichuan Province'' and the ''Hundreds of Talent Program'' of the Chinese Academy of Sciences.

References

1. Langer R (1998) Nature 392:5
2. Hideki M, Masao K, Hirofumi T, Yoshiaki K (1999) Int J Pharm 187:143
3. Takeuchi H, Yamamoto H, Kawashima Y. (2001) Adv Drug Delivery Rev 47:39
4. Li S, Qian Z, Liu XL, Liu XB (2003) Acta Polym Sinica 5:620 (in Chinese)
5. Fattal E, Vauthier C, Aynie I, Nakada Y, Lambert G, Malvy C, Patrick C (1998) J Controlled Release 53:137
6. Alexaner KA, Lendon GP (1998) Adv Drug Rev 34:155
7. Fresta M, Cavallaro G, Wehrli E, Puglisi G (1996) Biomaterials 17:751
8. Fessi H, Puisieux F, Devissaguet J P, Ammoury N, Benita S (1989) Int J Pharm 55:R14
9. Lambert G, Fattal E, Pintoalphandary H, Couvreur P (2000) Pharm Res 17:707
10. Leonard F, Kulkarni R, Barnades G, Nelson J (1966) J Appl Polym Sci 10:259
11. Vranckx H, Demoustier M, Deleers M (1996) Eur J Pharm Biopharm 42:345