Ethylenediamino Bridged Bis(β-cyclodextrin)/ Poly(DL-lactic-*co*-glycolic acid) Nanoparticles Prepared by Modified Double Emulsion Method: Effect of Polyvinyl Alcohol on Nanoparticle Properties

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ABSTRACT: This study continues long-standing efforts to develop protein delivery systems based on cyclodextrin-conjugated polyester in our laboratory. The crude products of ethylenediamino bridged bis(β-cyclodextrin)-conjugated poly(DL-lactic-*co*-glycolic acid) were used in this study to make full use of unreacted reactant. With bovine serum albumin (BSA) as a model protein, the encapsulation effects (the encapsulation efficiency and particle size) of nanoparticles were similar to those of using pure conjugated products. Besides, a water-in-oil-in-water emulsification technique was conveniently modified. By adding polyvinyl alcohol (PVA) in the internal aqueous phase, a more stabilized emulsion was formed. Consequently, less PVA (∼ 0.05%) was needed in the outer aqueous phase and less PVA (0.14 g/g nanoparticles) remained in the nanoparticles. This modification resulted in improved encapsulation efficiency (∼ 89–94%) of BSA and an enlarged particle size (340–390 nm). Furthermore, the burst release of BSA at the 1st day was less pronounced (7–12% of the encapsulated amount) than that of nanoparticles with no PVA added in the internal aqueous phase. Degradation studies using transmission electron microscope and gel permeation chromatography suggested that the mechanism for protein release was mainly through nanoparticles erosion. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 571–576, 2008

Key words: protein; nanoparticles; cyclodextrin; poly(DL-lactic-*co*-glycolic acid)

INTRODUCTION

The selection of a particular method of encapsulation is usually determined by the solubility characteristics of the drug.[1] Water-in-oil-in-water ($W_1/O/W_2$) emulsion technique is commonly used to encapsulate the water-soluble proteins into polymeric nanoparticles.[2–4] However, when particles are prepared by the $W_1/O/W_2$ method, water-soluble drugs exhibit a tendency to migrate to the outer aqueous medium, resulting in a poor protein entrapment in the polymer nanoparticles[5] and an obvious burst release.[6] The importance of an enhanced nanoparticles drug incorporation efficiency has been emphasized.[7,8] On the other hand, nanospheres could not achieve precisely controlled drug release because of the initial burst of drug on the surface. To overcome these problems, different approaches have been employed to enhance the encapsulation efficiency and reduce the burst release, such as alteration of the nanoparticles fabrication method,[9,10] reduction in encapsulated particle size[11] usage of the hydrogel nanoparticles,[12,13] the polymer mixture,[14,15] and stabilizing excipients.[16–20] Yang et al.[3] have added PVA to the internal water phase to enhance the protein encapsulation of microspheres and decrease the initial release. In this study, we extend this method for nanoparticles fabrication and then determine the effects of dose PVA in W_1 on encapsulation efficiency and release profiles of protein.

A series of conjugates of amino cyclodextrin with polyester[7,21,22] have been synthesized in our group. Polymer fraction method has been used to purify the products. Nevertheless, fraction of copolymer always leads to a relatively low yield (38–43%). In the conjugating reaction, amino cyclodextrin was usually used excessively to ensure that one amino cyclodextrin conjugated to one PLA chain. Cyclodextrins have been reported as useful additives to change the drug release kinetics,[7,22,23] stabilize proteins against methylene chloride/water interface induced denaturation and aggregation,[24] improve protein conformational stability[25–27] and increase protein recovery.[28] In this study, we try to make full use of the excess amino cyclodextrins by using the crude conjugated products for nanoparticle fabrication. The properties of crude

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products such as encapsulation efficiency and particle size were investigated in comparison with the pure conjugated products. The influence of PVA on nanoparticle characteristics (particle size, residual amount of surfactant, degradation, and protein release) was also studied.

MATERIALS AND METHODS

Materials

Poly(DL-lactic-*co*-glycolic acid) (PLG) copolymers with free acid end of composition (50/50 and 75/25) and molecular weight of 18 kDa were purchased from Durect Company. β-CD was obtained from Aldrich Chemical Company (Cupertino, CA). Bovine serum albumin (BSA, M_w 66,200 Da) (Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences, Tianjin, China). Poly(vinyl alcohol) (PVA) (average M_w 30,000–70,000; 88% hydrolyzed) (Tianjin Kermeo Chemical Reagent Center, China). *N,N′*-Dimethylformamide (DMF) was dried over calcium hydride for 2 days and then distilled under a reduced pressure before use.

Conjugating reaction of PLG with ethylenediamino bridged bis(β-cyclodextrin)

The synthesis of ethylenediamino bridged bis(β-cyclodextrin)-conjugated poly(DL-lactic-*co*-glycolic acid) (BCDenPLG) was according to a previously report, using *N,N′*-Dicyclohexycarbodiimide (DCC) as conjugating agent.[21] However, the purification procedure was changed as following. After solid dicyclohexylurea (DCU) was filtered out, the filtrate was evaporated under vacuum to obtain the crude products. Then the products were dissolved in acetone and centrifuged. The pallet was discarded and the supernatant was evaporated under vacuums. This product was named as BCDen/PLG, while the pure product was named as BCDenPLG. The numeral marked following the name of products represented of lactic acid (LA) in PLG.

Fabrication of the biodegradable nanoparticles in the modified double emulsion method

The double emulsion (DE) method was employed to fabricate nanoparticles as described by Gref and coworkers[29] with a few modifications. Typically, 50 mg of the copolymer BCDen/PLG (or BCDenPLG) was dissolved in 2 mL of dichloromethane/acetone (1:1). BSA (20 mg) was dissolved in 200 μL of PVA (0.1%, w/v) aqueous solution. After the aqueous phase was dropped into the copolymer solution under sonication (VCX400 Sonics and Materials, USA) at 30 W for 0.5 min in an ice bath, a W_1/O emulsion was formed. Then 4 mL of the aqueous PVA solution (0.05% for BCDen/PLG, 0.5% for PLG) was added under sonication at 30 W for another 0.5 min in an ice bath. The organic solvent was removed by evaporation under reduced pressure and the nanoparticles were recovered by the centrifugation at 21,000 rpm for 20 min and washed twice with water.

Determination of encapsulation efficiency and particle size

After centrifugation of nanoparticles, the encapsulation efficiency was determined by measuring the BSA concentration in the supernatant according to the blue coomassie G250 protein assay.[30] Nanoparticles size was measured through BI-90 Plus Particle Size Analyzer (Brookhaven Instruments Corp.) at 25°C in distilled water.

Morphology observation of the polymer nanoparticles

The morphology of the produced BSA-loaded nanoparticles before and after degradation was investigated using transmission electron microscope (TEM) (Tecnai, G^2 20 S- TWIN, Philips Co., Netherlands). A drop of nanoparticles suspension (0.1 mg/mL) was placed on copper grids with a carbon film and allowed to dry at room temperature before being loaded in the TEM.

Residual PVA content

Residual amount of PVA associated with nanoparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule.[31,32] In brief, 2 mg of lyophilized nanoparticle sample was hydrolyzed with 2 mL of 0.5*M* NaOH for 15 min at 60°C. The solution was then neutralized with 900 μL of 1*N* HCl and the volume was adjusted to 5 mL with distilled water. To each sample, 3 mL of a 0.65*M* solution of boric acid, 0.5 mL of I_2/KI (0.05/0.15*M*) solution, and 1.5 mL of distilled water were added. Finally, the visible spectra absorbance of the samples was measured at 690 nm (U-3010 spectrophotometer, HITACHI, Japan) after 15 min incubation. A standard plot of PVA was prepared under identical conditions. In addition, Panyam et al.[33] have shown that the oligomers (lactic and glycolic acid), as the degradation products of nano- and microparticles, do not interfere in the PVA assay.

Nanoparticles degradation and protein release

Polymer degradation release tests and evaluation on the released protein integrity were determined as described in Ref. 21.

RESULTS AND DISCUSSION

Conjugating reaction and purification

The fraction procedure to purify the crude products was omitted after the conjugating reaction. During the fractioning of the copolymer, some pure products, unreacted PLG and BCDen were lost. Nevertheless, PLG copolymer has been widely used for protein deliver. Moreover, BCDen has many advantages as mentioned above and can serve as emulsifier due to its amphiphilicity. Thus, we developed an alternative method of acetone precipitation to eliminate insoluble compounds of DCU and excessive unreacted BCDen. This posttreatment method could get BCDen/PLG75 and BCDen/PLG50 with a yield of 86.5% and 89.4%. A broaden polydispersity index (M_w/M_n) of 1.6 and 2.1 were observed in GPC [Fig. 1(a)]. The broaden phenomenon identified that the crude products composed of BCDenPLG and PLG. A small peak presented in GPC indicating the presence of BCDen.

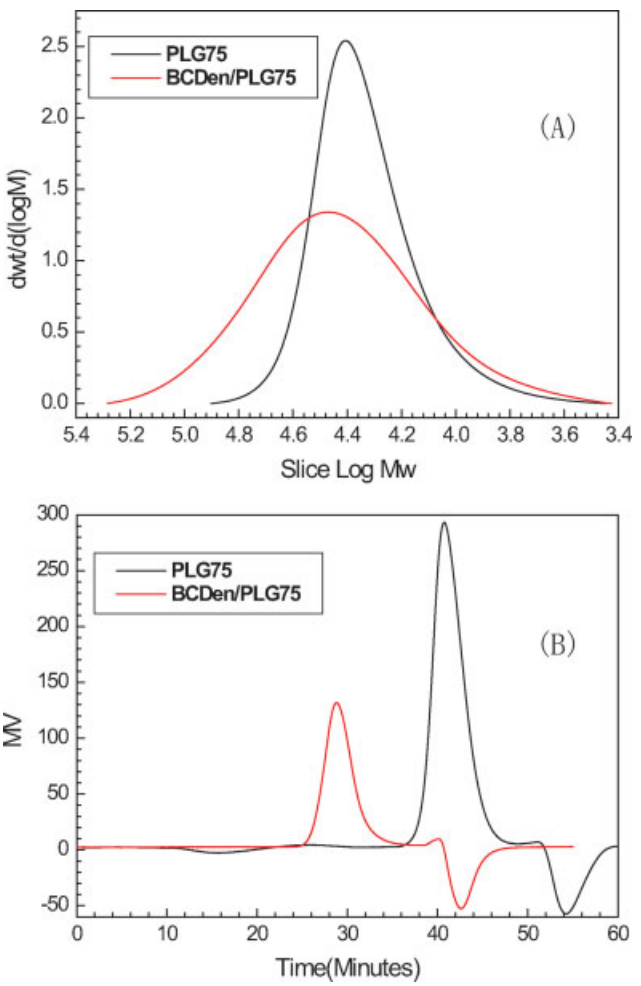

Figure 1 GPC curves of PLG75 and BCDen/PLG75. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I
Nanoparticles Characterization of BCDen/PLG Compared with BCDenPLG

Composition	Encapsulation efficiency (%)	Mean diameter (nm)	Polydispersity index
BCDenPLG50	93.9	302.4	0.041
BCDenPLG75	90.6	362.1	0.072
BCDen/PLG50	89.3	343.2	0.033
BCDen/PLG75	92.6	393.8	0.087

Some excessive BCDen were found in the products due to its entanglement with PLG although BCDen has lower solubility in acetone.

Nanoparticles characterization of BCDen/PLG compared with BCDenPLG

Table I revealed that there is no difference in encapsulation efficiency and mean diameter in the case of BCDen/PLG and BCDenPLG when they are used for fabrication nanoparticles. This confirmed that the crude products could be used for protein encapsulation. In addition, as expected, the copolymers with higher GA content had a smaller mean diameter, which attributed to a higher hydrophilic/hydrophobic balance value of GA is higher than that of LA.

Effect of PVA on the nanoparticle fabrication

PVA concentration in the internal water phase

Increasing PVA concentration of the internal water phase (W_1) resulted in an increased encapsulation efficiency (Table II). The difference in osmotic pressure between the internal (0.1% PVA) and external (0.05% PVA) aqueous phases could be responsible for the increase in entrapment efficiency. In addition, a high PVA concentration in the internal water phase could lead to an increase in the viscosity of the internal phase, preventing BSA from migrating out and consequently enhance encapsulation efficiency. On the other hand, it was more difficult to break up the solution with high viscosity into smaller droplets at the same power of mixing, resulting in slightly larger nanospheres (Table I). These results are related with the reports of microspheres.[3]

PVA concentration in the external water phase

In the present work, the nanospheres of BCDen/PLG75 could be fabricated at PVA concentration of 0.05%, but utilizing PLG75, large uncontrolled precipitates were present when the concentration of PVA was below 0.5%. The reason is that the amphiphilic properties of BCDen/PLG copolymers in addition to

TABLE II
Effect of PVA on the Nanoparticle Fabrication

Composition[a]	PVA concentration in W_1 (%, w/v)	Encapsulation efficiency (%)	Mean diameter (nm)	Polydispersity index	Residue PVA (g/g nanoparticles)
PLG50	0.05	59.9	369.5	0.064	1.9
PLG75	0.1	68.6	416.2	0.098	1.7
BCDen/PLG75	0.05	81.9	365.8	0.087	0.12
BCDen/PLG75	0.1	92.6	406.6	0.077	0.16

[a] For PLG 75, PVA concentration in the external aqueous phase (W_2) is 0.5; for BCDen/PLG75, PVA concentration in the external aqueous phase (W_2) is 0.05.

the PVA effect act as a stabilizer for the double emulsion. By lowering the interfacial tension of the emulsion during nanoparticles preparation, BCDen/PLG solutions led to finer emulsions than those of PLG.

Residue PVA

A fraction of PVA remained in association with the nanoparticles despite washing repeatedly because PVA formed an interconnected network with the polymer at the interface.[31] An improved protection of the droplets from coalescence was obtained at higher PVA concentrations, leading consequently to smaller emulsion droplets than at lower PVA concentrations.[34] Nevertheless, PVA is nonbiodegradable and potentially carcinogenic.[35] Therefore, its amount in the particles should be reduced as much as possible. As shown in Table II, residual PVA content in BCDen/PLG nanoparticles was much less (0.14 g/g nanoparticles) compared with that in PLG nanospheres (1.8 g/g nanoparticles) of similar diameter (around 400 nm). Much less PVA in W_2 was needed to stabilize the nanoparticles, as mentioned above. Moreover, the presence of the BCDen at the surface hindered the adsorption of PVA.

Nanoparticles degradation and protein release

Nanospheres erosion and polymer degradation

TEM photograph (Fig. 2) showed a spherical shape and smooth surface of the nanoparticles. No aggregation or adhesion among the nanoparticles was found. Moreover, the particle size was in exact agreement with the result of Particle Size Analyzer. After being left for 14 days *in vitro*, pores were formed due to extensive degradation/erosion although the particles were still spherical.

The variation molecular weight of BCDen/PLG with PVA in the W_1 (A), as compared with that without PVA in the W_1 (B), was determined by GPC after immersion in 0.1*M* phosphate buffer solution (pH 7.2) at 37°C. As shown in Figure 3, the degradation of A nanospheres is almost linear. Initially, A nanoparticles degraded faster than B nanospheres. More PVA residues existed on the surface of B nanoparticles. These residues are resistant to the infiltration of buffer solutions that trigger the hydrolysis. However,

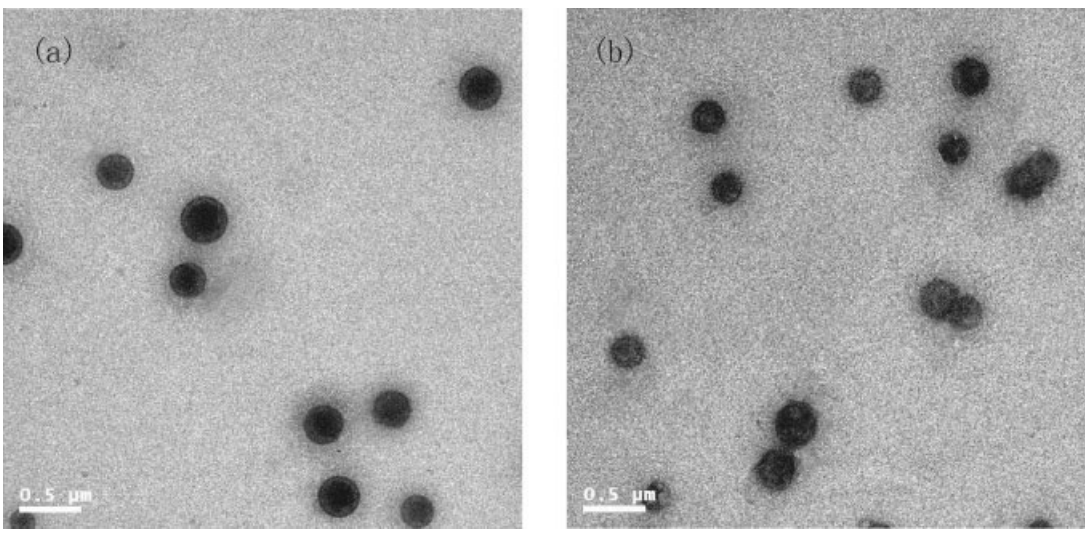

Figure 2 TEM images of BSA-loaded BCDen/PLG75 NPs: (a) before degradation; (b) degradation for 14 days.

after 7 days, degradation of B increased because the residual PVA associated with B nanoparticles could form a barrier to the outward diffusion of the acidic oligomers and monomers. Thus, the acidic degradation products were accumulated, which catalyzed the hydrolysis of the PLG nanoparticles. The rate of the degradation also depended on the GA molar ratio in the copolymer, which affected the hydrophilic/lipophilic balance. The degradation rate increased with higher GA molar ratio. These results suggested that both the polymer composition and residue PVA had a significant impact on the erosion rate.

In vitro release studies

Figure 4 reveals the release profiles of BSA from the different types of spheres. The BSA release from A nanoparticles exhibited a near-constant release. While the release from B showed two phase release, a burst release and a followed sustained release. It is believed that the protein burst was due to protein release from the nanoparticles surface. During the sonication process for the second emulsification, the protein could easily migrate through the nanometer-sized organic droplet into the continuous aqueous phase, thereby concentrating on the surface of the particles and involving the burst effect.[5] Moreover, the burst release could also be explained by the imperfect encapsulation of the drug inside nanoparticles, resulting from the unstable nature of the emulsion droplets during the solvent removal step. This potential instability might cause a part of the loaded drug to relocate at the nanoparticle surface, thereby rapidly released.[36,37] Adding PVA into the inner aqueous phase could significantly arrested BSA migrating to the nanoparticles surface and consequently decreases the burst release. The near-constant release of BSA from A nanoparticles agreed with the near-linear decrease of A nanoparticles in polymer molecular weight and the fast progression in pore formation, suggesting the protein release in A systems was controlled mainly by matrix erosion.

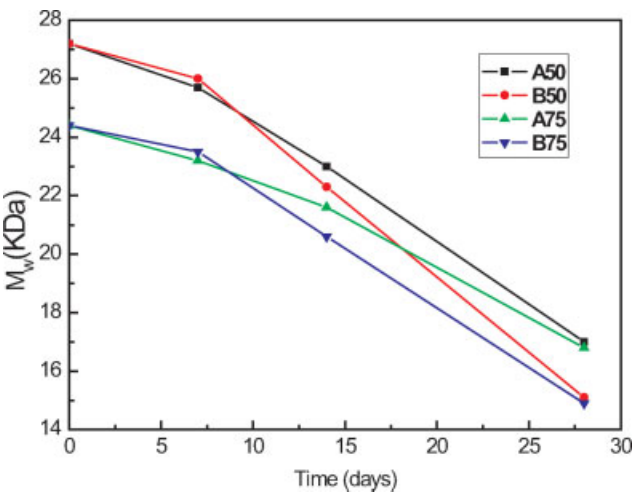

Figure 3 Weight molecular weight changes of the degrading CDen/PLG nanoparticles in the presence (A) and absence (B) of PVA in the internal phase as a function of incubation time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

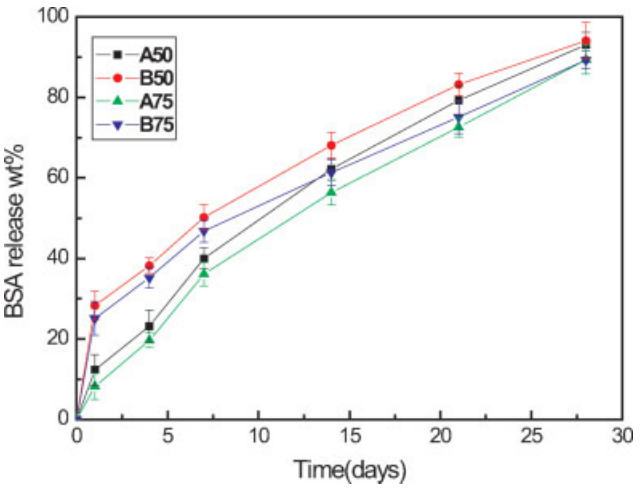

Figure 4 *In vitro* release profiles of BSA from BCDen/PLG nanoparticles (A, adding PVA in the internal phase; B, no PVA in the internal phase). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BSA remained its structure integrity in the release medium as determined by SDS-PAGE (data not shown). One of the important mechanism of protein instability in nanoparticles was due to the generating acid degradation products (oligomers and monomers), which participated in the protein hydrolysis.[38] It has demonstrated that introducing of CDs into polymers formed a more neutral and hydrophilic microenviroment, which was contributed to the BSA stability in our previous work.[20] Apart from that, the un-reacted BCDen decrease the contact of BSA with the interface of oil/water. The presence of PVA in the inner aqueous phase further limits the contact of BSA with organic phase. Moreover, they all contribute to a stable w/o emulsion formation, and thus less PVA was needed as emulsifier in the outer aqueous phase, which, in turn, less PVA will facilitate the diffusion of acid degradation products out of nanoparticles. Therefore, the accumulation of acid microenvironment can be decreased significantly.

CONCLUSIONS

The results revealed that crude conjugated products (BCDen/PLG) nanoparticles could be an effective carrier for protein delivery. Moreover, the encouraging preliminary results reflected the potential of the double emulsion modified by adding PVA into the inter-

nal aqueous phase. BSA was encapsulated efficiently in BCDen/PLG nanopaticles, and released in a more continuous way. Further studies will be focused on loading hydrophilic vaccines into these biodegradable nanoparticles.

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