Reverse micelles based on biocompatible \( \beta \)-cyclodextrin conjugated polyethylene glycol \( \text{block} \) polylactide for protein delivery

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A series of linear and star-shaped amphiphilic polyethylene glycol \( \text{block} \) polylactide (PEG-\( \beta \)-PLA) and \( \beta \)-cyclodextrin (CD) conjugated PEG-\( \beta \)-PLA (PEG-\( \beta \)-PLA-CD) copolymers were synthesized. Bovine serum albumin (BSA) aqueous solution was emulsified in the copolymer organic solutions to fabricate reverse micelles (RMs), and was then further transferred into ethyl oleate (EO), a pharmaceutically acceptable vehicle, by the RMs. As identified by \( ^1 \text{H} \) NMR, the RMs were formed with a hydrophilic core of PEG and CD, covered with a hydrophobic corona of PLA moiety, and were spherical in shape, as observed by a scan electronic microscope. Compared with the PEG-\( \beta \)-PLA RMs, the PEG-\( \beta \)-PLA-CD RMs presented higher encapsulation efficiency. The release of BSA was influenced by the copolymer composition and architecture. BSA stability in the release aqueous phase was confirmed by circular dichroism spectroscopy. The oil-based formulation fabricated from biodegradable copolymers with high drug loading showed a great potential for protein delivery.

Introduction

At present, more than 130 different proteins or peptides are approved for clinical use by the US Food and Drug Administration (FDA), and many more are in development. Protein therapeutics already plays a significant role in almost every field of medicine. The best example of trends in the production and use of protein therapeutics is found in the history of insulin in the treatment of diabetes mellitus type I and type II.\(^1\) Although peptides and proteins have been studied and proposed as therapeutic agents, considerable hurdles need to be overcome before their practical use, e.g., their chemical and enzymatic instability, poor absorption through biological membranes, rapid blood clearance, peculiar dose-response curves, and immunogenicity.\(^2\) A variety of systems, such as polymeric microspheres/nanoparticles, liposomes, and solid lipid nanoparticles, etc., have been used for the encapsulation of proteins and peptides to improve drug accumulation inside target cells, and to protect them from denaturation, although most systems involve the aqueous phase.\(^3\) Recently, oil-based formulations have attracted significant attention in terms of the construction of drug delivery systems, which can form a continuum with other lipid barriers in the body, such as skin lipids and cell membranes.\(^4\) Reverse micelles (RMs), consisting of a hydrophilic core surrounded by a hydrophobic corona, were constructed in nonpolar solvents and have been applied to sequester hydrophilic guest molecules.\(^5\)\(^–\)\(^7\) However, the research on RMs in biomedical applications is very limited, probably owing to some degree to the toxicity of organic solvents.

Polyethylene glycol \( \text{block} \) polylactide (PEG-\( \beta \)-PLA) nanoparticles are one of the most promising assemblies in drug delivery systems (DDS), because of their high biocompatibility, biodegradability, nontoxicity, low immunogenicity, and good mechanical properties. These unique properties make it suitable for controllable drug releasing devices.\(^8\) Jiang et al.\(^9\)\(^–\)\(^11\) designed novel drug carriers for brain delivery with cationic bovine serum albumin (BSA) or albumin conjugated PEG-\( \beta \)-PLA nanoparticles. Wang et al.\(^12\) reported cationic lipid-assisted PEG-\( \beta \)-PLA nanoparticles as siRNA carriers prepared by a double emulsion – solvent evaporation technique. Zheng\(^13\) and Zhu et al.\(^14\) presented a formulation with a prodrug incorporated into PEG-\( \beta \)-PLA nanoparticles for enhanced antitumor efficacy or theranostic drug delivery systems. Besides these, a number of systems based on PEG-\( \beta \)-PLA nanoparticles have been explored for drug delivery.\(^15\)\(^–\)\(^18\)

Cyclodextrins with a hydrophobic central cavity have shown great ability to bind different molecules, including peptides and proteins, to form stable inclusion complexes through host-guest complexation.\(^19\)\(^–\)\(^21\) Therefore, cyclodextrins have been widely used as drug carriers,\(^22\) fluorescent sensors,\(^23\) molecular-
recognition,24 and recycling extractors.25 In our previous study,26 beta-cyclodextrin (CD) was coupled to PLA to give tadpole-shaped copolymers, which could load BSA efficiently, owing to the interaction between CD and BSA. In this paper, we expected that the introduction of CD could increase the encapsulation efficiency (EE) and loading capacity (LC) of PEG-b-PLA nanoparticles.

Herein, a series of linear and 4-arm PEG-b-PLA and CD-conjugated PEG-b-PLA (PEG-b-PLA-CD) copolymers were synthesized. Though PEG-b-PLA derivatives and CD-conjugated PLA-b-PEG copolymers have been previously synthesized in order to obtain micelles for hydrophobic drugs,27,28 their application to deliver hydrophilic drugs in the oil phase has not been studied so far. In this study, BSA aqueous solution was emulsified into both PEG-b-PLA and PEG-b-PLA-CD organic solutions to fabricate RMs (Scheme 1), and was then further transferred into the oil phase by the RMs. The EE, LC, and in vitro release of BSA from PEG-b-PLA-CD RMs, as well as the secondary structure of BSA released from the RMs, were investigated by comparing with their counterpart, i.e., PEG-b-PLA. A biocompatible oil-based polymeric formulation with high BSA loading was expected to be obtained in the present study.

**Experimental**

**Materials and methods**

Stannous octoate (Sn(Oct)2), dicyclohexylcarbodiimide (DCC), and 4-dimethylamino-pyridine (DMAP) were purchased from Sigma Aldrich Co. Ltd. (Shanghai, China). Monomethoxy PEG (MPEG) (Mₙ = 8 kDa) and 4-arm PEG (Mₙ = 10&20 kDa) were obtained from Seebio Biotech Inc. (Shanghai, China). D,L-lactide was purchased from Daigang Co. Ltd. (Shandong, China). Bovine serum albumin (BSA) was bought from Aladdin Chem-istry Co. Ltd. (Shanghai, China). All other reagents were purchased from Tianjin Chemical Reagent Co. Ltd. (Tianjin, China). Prior to use, dimethylformamide (DMF) and ethanedi-ol (C₂H₅OH) were distilled.

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**Synthetic procedures**

**Synthesis of PEG-b-PLA.** Hydroxy-terminated PEG-b-PLA was synthesized by the ring-opening polymerization of D,L-lactide using MPEG as a linear initiator (or 4-arm PEG as a star-shaped initiator) and Sn(Oct)₂ as a catalyst according to literature reports (Scheme 2).29,30 Briefly, after appropriate amounts of D,L-lactide, MPEG, 4-arm PEG and 0.5 wt% Sn(Oct)₂ were added to a tube, the tube was evacuated under vacuum and filled with pure nitrogen three times, and then sealed under vacuum. The polymerization reaction was maintained at 130 °C for 12 h. The solid product was dissolved in DCM and precipitated in anhydrous ethyl ether three times. The resulting precipitate, i.e., PEG-b-PLA, was filtered and dried in vacuum at 40 °C for 48 h.

**Synthesis of PEG-b-PLA-CD.** Carboxylated PEG-b-PLA (PEG-b-PLA-COOH) was prepared by the esterification of PEG-b-PLA with succinic anhydride (SA) using DMAP and triethyl-amine (TEA) as catalysts (Scheme 2).31,32 PEG-b-PLA, SA, DMAP, and TEA were dissolved in anhydrous chloroform and stirred for 24 h at room temperature. After removing most of the solvent, the residue was precipitated in anhydrous ethyl ether. Then, the crude product was dissolved in DCM, filtered to remove the unreacted SA, and then precipitated in ethyl ether twice. The precipitate was collected and dried in vacuum at 40 °C for 48 h to yield a white powder of PEG-b-PLA-COOH. The carboxyl content of PEG-b-PLA-COOH was determined by non-aqueous titrations using sodium hydroxide as a base. In brief, a 1.2 g sample of PEG-b-PLA-COOH was dispersed in toluene and titrated with standardized ethanol sodium hydroxide using phenolphthalein as an indicator.33 The carboxyl content of PEG-b-PLA-COOHs was determined as ca. 60%.

**Synthesis of PEG-b-PLA-CD.** The CD-modified PEG-b-PLA-COOH (PEG-b-PLA-CD) copolymers were synthesized via coupling reactions between mono[6-(ethylendiamine)-6-deoxy]-CD (CDen) and PEG-b-PLA-COOH using DCC and DMAP as the coupling agents (Scheme 2). For this purpose, CDen was synthesized as reported previously.34 To obtain final PEG-b-PLA-CD copolymers, PEG-b-PLA-COOH, CDen, DCC and DMAP were added to a flask, followed by evacuation under vacuum, and then filled with dry nitrogen. Then, the anhydrous DMF was
injected and the reaction system was stirred at room temperature for 24 h. The by-product was removed by filtration, and the mixture solution was dialyzed using a dialysis membrane (molecular weight cut-off of 7 kDa) against water for 2 days, and the final product was obtained by lyophilization.

Construction of BSA-loaded RMs composed of PEG-b-PLA or PEG-b-PLA-CD

The RMs were prepared in DCM at first and then transferred into EO. The PEG-b-PLA or PEG-b-PLA-CD copolymers (4 mg) were dissolved in DCM (4 mL), and BSA aqueous solution (40 μL, 50 mg mL⁻¹) was added to this solution. The mixture was sonicated in an ice bath at 100 W (Sonic and Materials, USA) for 4 min, until a visibly clear solution was obtained. EO (0.5 mL) was added under stirring, and the volatile DCM was removed in vacuum to yield oleaginous RMs. To calculate the encapsulation efficiency (EE) and the loading capacity (LC), BSA was recovered utilizing acetone to disassemble the RMs. The copolymer fragments were removed by centrifugation and then the mixed solution was air-dried to obtain BSA. The BSA content was determined using the Coomassie blue method on a UV-Vis spectrophotometer. The EE and LC were calculated according to the following equations:

\[
EE(\%) = \frac{\text{Final loading}}{\text{Initial loading}} \times 100\%
\]

\[
LC(\%) = \frac{\text{Mass of loaded guest}}{\text{Mass of nanoparticles}} \times 100\%
\]

BSA release study

BSA-loaded RMs in EO were prepared as described above. The BSA-loaded RMs in EO (2 mL) were mixed with distilled water (2 mL) in a vial, and then placed in an orbital shaker water bath (100 rpm) at 37 °C. At pre-decided intervals, 1 mL of distilled water was withdrawn and replaced with 1 mL of fresh water. The released BSA concentration was determined by the Coomassie blue method. All the experiments were performed in triplicate.

Results and discussion

Scheme 1 describes the synthetic procedure of the amphiphilic PEG-b-PLA-CD copolymers. Monohydroxy-terminated PEG-b-PLA was synthesized via a ring-opening polymerization and the PEG-b-PLA-COOH copolymers were prepared by the acylation of PEG-b-PLA end-hydroxyl with succinic anhydride.
Compared to Fig. 2(i) and (ii), peaks ring-opening reaction was performed in a controlled manner. A series of PEG-b-PLA and PEG-b-PLA copolymers were synthesized, and the molecular weights calculated from $^1$H NMR and measured by GPC are listed in Table 1. The FT-IR evidenced the functionality of the copolymers (Fig. 1). As for MPEG8K-b-PLA10K-CD, the absorption peak at 1638 cm$^{-1}$ was assigned to the stretching vibration of carbonyl C=O in the amide group. The peak intensity at 3445 cm$^{-1}$ assigned to the OH group of PEG-b-PLA was relatively low, and a similar spectrum is shown in the literature. However, after the introduction of CD, the peak increased significantly, indicating the introduction of CDs. The above characterization suggested that the amino groups of CDen were coupled with the carboxylic group successfully.

The representative $^1$H NMR spectra of CDen and the copolymers are demonstrated in Fig. 2. Fig. 2(ii) exhibits the spectrum of MPEG8K-b-PLA10K in CDCl$_3$. Peaks “d” at 1.48 ppm and “e” at 5.19 ppm arise from the protons of the PLA segment, respectively. Peaks “a” at 3.40 ppm and “b” at 3.62 ppm are the protons of PEG. The peak ratio of “c” and “b” indicate that the ring-opening reaction was performed in a controlled manner. Compared to Fig. 2(i) and (ii), peaks “1” at 4.82 ppm and “2–6” at 3.60 ppm in Fig. 2(iii) could be assigned to the protons on H1 and on H2–H6 of the CD units, indicating that CD had conjugated with the PEG-b-PLA copolymers. The reaction efficiency of the CD conjugation was ca. 50–60%, as calculated from the integral area ratio of peaks “1” and “a”.

PEG-b-PLA nanoparticles were widely used as micellar carriers in DDS in aqueous solution. Most of the PEG-b-PLA DDSs were constructed with two types of topological structures, i.e., drug-loading$^{27,37,38}$ and drug-conjugated.$^{9–11,39}$ However, no report was found about the construction of RMs based on PEG-b-PLA. CD-conjugated PLA-b-PEG copolymers$^{27}$ could undergo self-assembly into micelles in aqueous solution, due to the hydrophobic interaction of the PLA segment. Similarly, the PEG-b-PLA-CD could self-assemble into RMs in non-polar solvents, owing to the hydrophilic PEG segment. The core–shell structure of the RMs from the amphiphilic copolymers could be verified by the $^1$H NMR spectrum. DMSO is a good solvent for all the blocks of the copolymers, and all the proton signals appeared when using DMSO-$d_6$ as the solvent in the $^1$H NMR measurement (Fig. 2(iii)). After the emulsion of CDCl$_3$ with small amounts of D$_2$O (Fig. 2(iv)), the specific signals of the PEG segment were weakened compared with its $^1$H NMR spectrum in DMSO-$d_6$, (integration ratio changed from 6.44 to 2.28), indicating that the MPEG8K-b-PLA10K-CD RMs were formed with a hydrophilic core of a PEG segment and a hydrophobic corona layer of the PLA segment. Moreover, the peak of H-1 (4.87 ppm) in CD disappeared, indicating that the CD units were located in the hydrophilic core or the core–shell interface after the formation of RMs.

The particle sizes and polydispersity index (Pdi) of the RMs were measured by DLS (Table 2). Most of the diameters were ca. 150–300 nm, and narrow size distributions were obtained. The hydrocarbon chains are not taken into account in the oil continuum because they are not detectable, due to similarities in the refractive indices (hydrocarbon chain and oil continuum). As expected, the particle size decreased with an increased hydrophilic segment. It has been reported that in aqueous solutions, the hydrophobic segment could promote the core compactness of normal micelles, and an enlarged
hydrophobic portion could result in the formation of smaller particles.\(^{40}\) Similarly, the larger hydrophilic portion could promote the compactness of the RM cores in organic solutions. Therefore, an increased hydrophilic segment results in reduced particle size. On the other hand, as CD was coupled with the PEG-\(b\)-PLA copolymers, the diameters decreased, because the introduction of CD increased the hydrophilicity of the copolymers. Interestingly, only when the \(M_n\) of the PLA block equals to that of the PEG block, could the BSA be encapsulated into the RMs in EO, indicating that a proper hydrophilic–hydrophobic balance was important for the RMs to remain stable before transferring into the EO.

The morphology of the RMs was spherical in shape, as observed by SEM (Fig. 3). The mean diameters of 4-arm PEG20K-\(b\)-PLA10K and 4-arm PEG20K-\(b\)-PLA10K-CD RMs were ca. 130 \(\pm\) 23 nm and 190 \(\pm\) 31 nm, respectively, and a little smaller than that as determined by DLS. This can be explained from the fact that the RMs for the SEM were air-dried and those for the DLS measurement were measured in solvation.

BSA was used as a water-soluble model protein to evaluate the feasibility of the RMs as soluble drug delivery carriers. The EE and LC of BSA are listed in Table 2. The EE and LC of BSA in the RMs fabricated from the PEG-\(b\)-PLA-CD copolymers were much improved in comparison with the counterpart (PEG-\(b\)-PLA). Especially for the MPEG8K-\(b\)-PLA5K-CD and MPEG8K-\(b\)-PLA10K-CD, the increase was remarkable. In fact, MPEG8K-\(b\)-PLA5K and MPEG8K-\(b\)-PLA10K could encapsulate BSA. However, the quality reports from the DLS measurement were poor, indicating that the RMs have a poor monodispersity, and thus, the data are not listed in Table 2. The inclusion complexes could be formed from the accessible residues of BSA with the hydrophobic cavity of CD moiety when the size of the aromatic and alkyl groups existing on the BSA molecule could fit within the cavity of CD. Moreover, it was observed that the RMs from the amphiphilic copolymers with longer PLA chains could encapsulate more BSA. The BSA loading was enhanced as a result of a relatively strong hydrophobic interaction between the copolymers and BSA.\(^{41}\) It was notable that the degree of copolymer branching also influenced the EE and LC. RMs comprising star-shaped copolymers were capable of accommodating more BSA compared with that of the liner copolymers. 4-arm PEG20K-\(b\)-PLA20K-CD exhibited the highest EE and LC. This might be explained by the larger space volume within the aggregates formed by the star-shaped copolymers.\(^{42}\)

The release profiles of BSA from the RMs in EO were investigated (Fig. 4). It was reported that drug release from PLA was generally controlled by both drug diffusion and polymer erosion.\(^{43}\) As for the nanoparticles in the aqueous phase, an initial phase could be observed where the release of protein occurs predominantly by diffusion of the drug through aqueous pores generated in the dosage form, and then, the drug within the body of the delivery matrix could be released with the

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**Table 2** Characterizations of the copolymer RMs in DCM and EO

<table>
<thead>
<tr>
<th>Copolymers</th>
<th>DCM</th>
<th>EO</th>
<th>DCM</th>
<th>EO</th>
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</thead>
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<tr>
<td></td>
<td>Diameter (nm)</td>
<td>PdI</td>
<td>Diameter (nm)</td>
<td>PdI</td>
</tr>
<tr>
<td>MPEG8K-(b)-PLA5K</td>
<td>281.8</td>
<td>0.056</td>
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<td>MPEG8K-(b)-PLA10K</td>
<td>326.5</td>
<td>0.029</td>
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<td>—</td>
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<td>4-arm PEG10K-(b)-PLA20K</td>
<td>288.4</td>
<td>0.031</td>
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<td>—</td>
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<tr>
<td>4-arm PEG20K-(b)-PLA10K</td>
<td>249.3</td>
<td>0.046</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MPEG8K-(b)-PLA5K-CD</td>
<td>224.6</td>
<td>0.023</td>
<td>216.1</td>
<td>0.094</td>
</tr>
<tr>
<td>MPEG8K-(b)-PLA10K-CD</td>
<td>268.7</td>
<td>0.050</td>
<td>298.8</td>
<td>0.257</td>
</tr>
<tr>
<td>4-arm PEG10K-(b)-PLA20K-CD</td>
<td>226.6</td>
<td>0.094</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4-arm PEG20K-(b)-PLA10K-CD</td>
<td>210.7</td>
<td>0.078</td>
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</tbody>
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*Fig. 3* SEM images of the 4-arm PEG20K-\(b\)-PLA10K-CD RMs (I) and 4-arm PEG20K-\(b\)-PLA10K RMs (II) loading with BSA in DCM.

*Fig. 4* Release profiles of BSA from the copolymer RMs in EO.
its original structure.

Degradation of the copolymers, which was associated with the generation of microcopolymers in the degradation and an enhanced water uptake. In Fig. 4(a) and (b), the release of BSA was in an approximately linear fashion in the initial 6 h, before reaching a plateau at 8 h. As a contrast, the BSA released from RMs without CDs (Fig. 4(c)) showed a different profile, one that maintained a sustained release during the whole release process. In general, the RMs composed of the PEG-PLA-CD copolymers released BSA faster than those composed of the PEG-PLA copolymers, which is probably due to the channels of CD being more favorable for the BSA diffusion.

Circular dichroism spectroscopy was used to determine whether BSA molecules were denatured after 7 days from the RMs (Fig. 5). The far-UV-CD band at 209 nm is primarily ascribed to a α-helix structure, while that at 222 nm is for the β-sheet. In this study, the [Φ]209/[Φ]222 ratios for standard BSA and released BSA from the 4-arm PEG10K-b-PLA20K-CD and 4-arm PEG10K-b-PLA20K were 1.20, 1.11, and 1.07, respectively. There was no significant difference between those released from RMs and from native BSA, indicating that the released BSA retained its original structure.

Conclusions

RMs based on linear and star-shaped PEG-PLA copolymers were fabricated by an emulsion method in organic/apolar solvents with a defined core–shell structure and a particle size of 150 to 300 nm. These RMs demonstrated the ability to solubilize BSA in organic/apolar solvents, and were further transferred into ethyl oleate (EO). The factors affected the particle size, and the drug loading and release, and were extensively studied. The present study provides the scientific foundation for further rational design of RMs. Both the hydrophilic–hydrophobic balance and the CDs introduction influence the RMs dispersion in EO. The introduction of CDs can aid the dispersion of RMs in EO. In both DCM and EO solutions, RMs composed of PEG-PLA-CD showed higher EE and LC than those composed of PEG-PLA. These novel RMs with improved EE and LC could be of great potential for protein delivery.

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Notes and references