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Reverse micelles based on biocompatible β-cyclodextrin conjugated polyethylene glycol block polylactide for protein delivery

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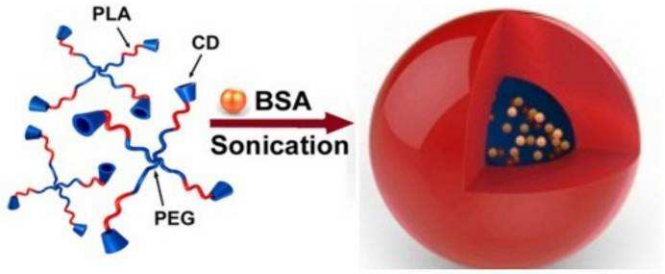
A series of linear and star-shaped amphiphilic polyethylene glycol block polylactide (PEG-*b*-PLA) and β-cyclodextrin (CD) conjugated PEG-*b*-PLA (PEG-*b*-PLA-CD) copolymers were synthesized. Bovine serum albumin (BSA) aqueous solution was emulsified in the copolymer organic solutions to fabricate reverse micelles (RMs), and further transferred into ethyl oleate (EO), a pharmaceutically acceptable vehicle, by the RMs. As identified by ^{1}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 scan electronic microscope. Compared with the PEG-*b*-PLA RMs, the PEG-*b*-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.

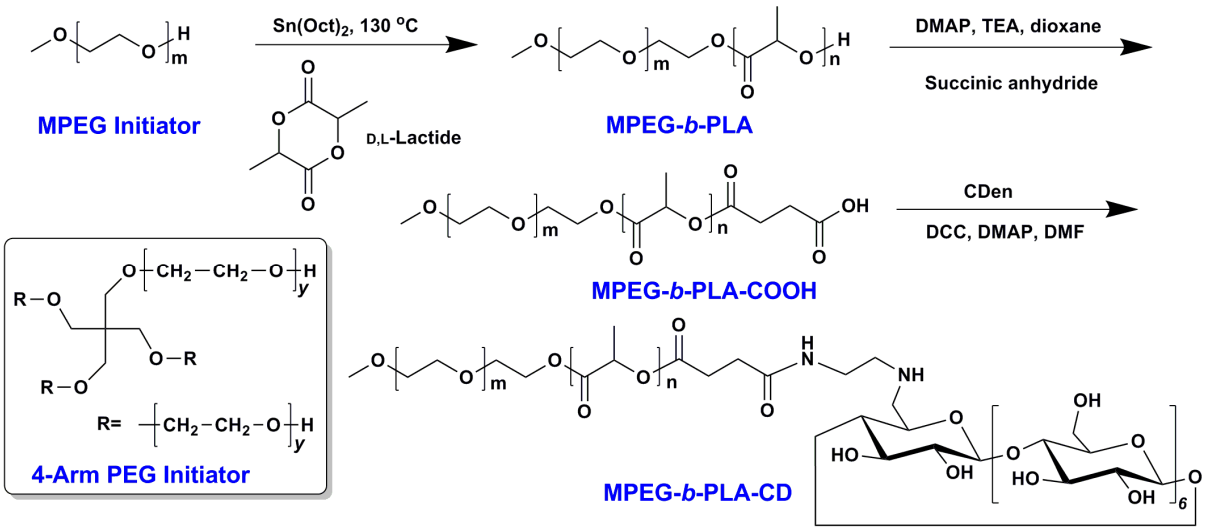
Introduction

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 should be 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 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 protect them from denaturation, but mostly are conducted in aqueous phase.[3] Recently, oil-based formulations have attracted much 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 hydrophobic corona, were constructed in nonpolar solvents and have been applied to sequester hydrophilic guest molecules.[5-7] However, the research of RMs in biomedical applications is very limited probably owing to some degree of toxicity of organic solvents.

Polyethylene glycol block polylactide (PEG-*b*-PLA) nanoparticles have been 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 or albumin conjugated PEG-*b*-PLA nanoparticles. Wang et al. [12] reported cationic lipid assisted PEG-*b*-PLA nanoparticles as the siRNA carriers prepared by a double emulsion–solvent evaporation technique. Zheng [13] and Zhu et al. [14] presented a formulation with prodrug incorporated into PEG-*b*-PLA nanoparticles for enhanced



Scheme 1. Schematic representation of BSA-loaded RMs from 4-arm PEG-*b*-PLA-CD.



Scheme 2. Synthesis of MPEG-*b*-PLA-CD and 4-arm PEG-*b*-PLA-CD copolymers.

antitumor efficacy or theranostic drug delivery systems. Besides these, a number of systems based on PEG-*b*-PLA nanoparticles have been explored for the drug delivery.[15-18]

Cyclodextrins with a hydrophobic central cavity have shown great ability of binding 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 bovine serum albumin (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 polyethylene glycol block polylactide (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] application to deliver hydrophilic drug in 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 further transferred into 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 is 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 Chemstry Co., Ltd. (Shanghai, China). All other reagents were purchased from Tianjin Chemical Reagent Co., Ltd. (Tianjin, China). Prior to use, dimethylfomamide (DMF) and ethanediamine (EDA) were distilled.

The Fourier transform infrared (FT-IR) spectra were recorded on a Bio-Rod 6000 spectrometer (Thermo Electron, USA). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AV-400 spectrometer (Freemont, CA). Samples were dissolved in deuterium oxide (D_2O), dimethylsulfoxide-d_6 (DMSO-d_6), or deuterated chloroform ($CDCl_3$). Molecular weight (Mn) and molecular weight distribution (Mw/Mn) of the copolymers were detected by Gel permeation chromatography (Shoko Scientific, Japan) at 40 ºC with THF as mobile phase at a flow rate of 0.5 mL/min. The morphology of the RMs was observed using a scanning electron microscope (SEM) collected on a JSM 6700F instrument (JEOL, Japan). All the samples were prepared by dropping the RMs solution (0.1 mg/mL) on coverslip and dried at room temperature for 24 h. Mean particle diameters and size distribution of RMs were also assessed in dichloromethane (DCM) and ethyl oleate (EO) at 20 °C by dynamic light scattering (DLS) on a Zetasizer Nano ZS90 instrument (Malvern, UK). The circular dichroism measurements of free BSA in the supernatant and control BSA solutions in water were performed on a Jasco-715 spectropolarimeter at 20 °C using the matched 10-mm path length quartz cells. Each sample solution was scanned three times in the range of 190-250 nm.

Synthetic procedures

Synthesis of PEG-*b*-PLA. Hydroxy-terminated PEG-*b*-PLA was synthesized by ring-opening polymerization of D,L-lactide using MPEG as linear initiator (or 4-arm PEG as 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-COOH. Carboxylated PEG-*b*-PLA (PEG-*b*-PLA-COOH) was prepared by the esterification of PEG-*b*-PLA with succinic anhydride (SA) using DMAP and triethylamine

Table 1. The characterization of the copolymers.

Copolymers	Mn (kDa)[a]	Mn (kDa)[b]	Mn (kDa)[c]	M_w/M_n[c]	CD-conjugated copolymers
MPEG8K-*b*-PLA5K	12	12.8	11.9	1.28	MPEG8K-*b*-PLA5K-CD
MPEG8K-*b*-PLA10	16	17.4	14.0	1.73	MPEG8K-*b*-PLA10K-CD
4-arm PEG10K-*b*-PLA20K	30	31.8	34.4	1.68	4-arm PEG10K-*b*-PLA20K-CD
4-arm PEG20K-*b*-PLA10K	30	26.6	11.5	2.60	4-arm PEG20K-*b*-PLA10K-CD
4-arm PEG20K-*b*-PLA20K	40	37.1	12.1	1.34	4-arm PEG20K-*b*-PLA20K-CD

Note: [a] Theoretical value calculated from feeding ratio, [b] Calculated from the ^{1}H NMR (integral area ratio of peak "c" and "b" in Fig. 2), [c] Measured by GPC.

Table 2. The characterizations of copolymer RMs in DCM and EO.

Copolymers	DCM		EO		DCM		EO	
	Diameter (nm)	PdI	Diameter (nm)	PdI	LC (wt%)	EE (wt%)	LC (wt%)	EE (wt%)
MPEG8K-*b*-PLA5K	281.8	0.056	—	—	—	—	—	—
MPEG8K-*b*-PLA10K	326.5	0.029	—	—	—	—	—	—
4-arm PEG10K-*b*-PLA20K	288.4	0.031	—	—	15.6	31.2	—	—
4-arm PEG20K-*b*-PLA10K	249.3	0.046	—	—	9.4	18.8	—	—
4-arm PEG20K-*b*-PLA20K	260.9	0.014	216.1	0.094	33.7	67.3	6.1	12.2
MPEG8K-*b*-PLA5K-CD	224.6	0.023	—	—	9.4	18.7	—	—
MPEG8K-*b*-PLA10K-CD	268.7	0.050	298.8	0.257	12.5	24.9	12.7	25.5
4-arm PEG10K-*b*-PLA20K-CD	226.6	0.094	—	—	17.0	34.0	—	—
4-arm PEG20K-*b*-PLA10K-CD	210.7	0.078	—	—	12.2	48.9	—	—
4-arm PEG20K-*b*-PLA20K-CD	174.1	0.069	270.1	0.251	36.5	72.9	7.9	15.9

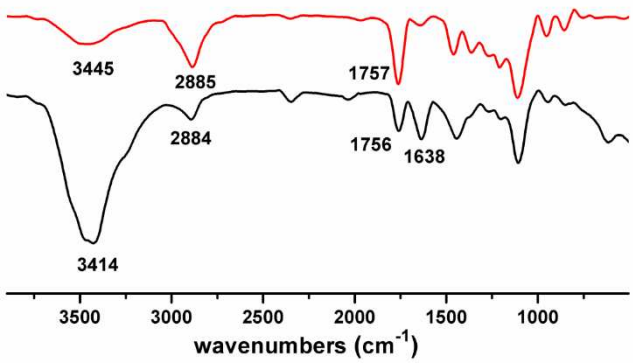


Fig. 1 FT-IR spectra of MPEG8K-*b*-PLA10K (red) and MPEG8K-*b*-PLA10K-CD (black).

(TEA) as catalysts (Scheme 1).[31,32] PEG-*b*-PLA, SA, DMAP and TEA were dissolved in anhydrous chloroform and stirred for 24 h at room temperature. After removing 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 precipitated in ethyl ether twice. The precipitate was collected and dried in vacuum at 40 °C for 48 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. Briefly, 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-(ethylenediamine)-6-deoxy]-CD (CDen) and PEG-*b*-PLA-COOH using DCC and DMAP as coupling agents (Scheme 1). 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 filled with dry nitrogen. Then the anhydrous DMF was injected and the reaction system was stirred at room temperature for 24 h.[35] The by-product was removed by filtration, and the mixture solution was dialyzed using a dialysis membrane (molecular weight cut-off 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 firstly prepared in DCM 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) was added to the solution. The mixture was sonicated in an ice bath at 100 W (Sonics and Materials, USA) for 4 min, until visibly clear solution was obtained. Under stirring, EO (0.5 mL) was added and the volatile DCM was removed in vacuum to yield oleaginous RMs. To calculate encapsulation efficiency (EE) and loading capacity (LC), BSA was recovered utilizing acetone to disassemble RMs. The copolymer fragments was removed by centrifugation and then air-dried the mixed solution to obtain BSA. The BSA content was determined using coomassie blue method on a UV-Vis spectrophotometer.[36] The EE and LC were calculated according to the following equations:

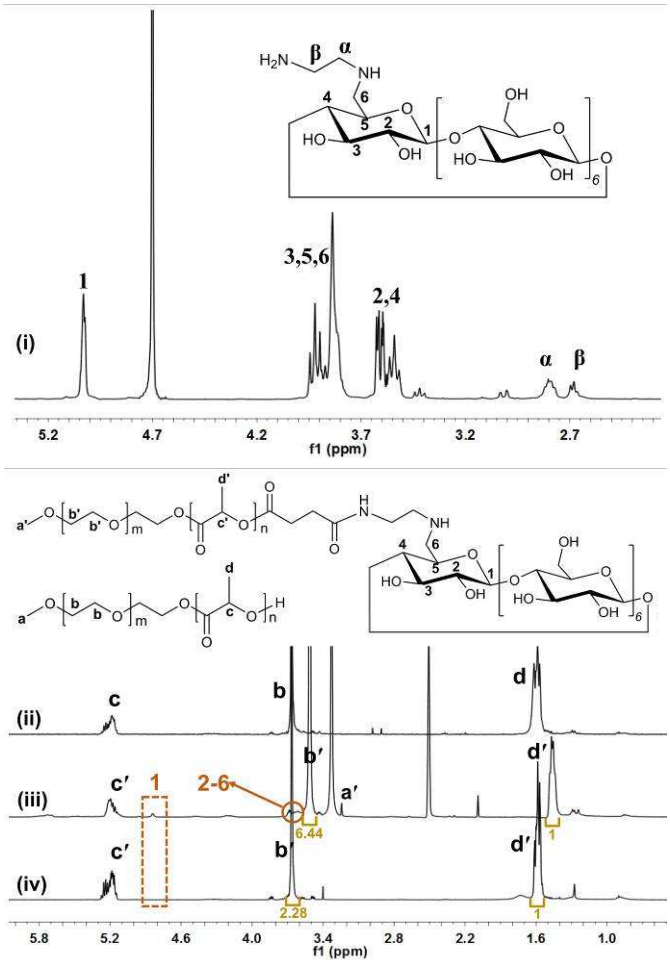


Fig. 2 ^{1}H NMR spectra of CDen in D_2O (i), MPEG8K-*b*-PLA10K in $CDCl_3$ (ii), MPEG8K-*b*-PLA10K-CD in DMSO-d_6 (iii), and MPEG8K-*b*-PLA10K-CD RMs in $CDCl_3$ (iv).

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

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

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 placed in an orbital shaker water bath (100 r/min) 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 coomassie blue method.[36] All 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* the ring-opening polymerization and the PEG-*b*-PLA-COOH copolymers were prepared by the acylation of PEG-*b*-PLA end-hydroxyl with succinic anhydride. Consequently, the PEG-*b*-PLA-CD copolymers were prepared by conjugation of CDen onto the PEG-b-PLA-COOH. A series of PEG-*b*-PLA and PEG-*b*-PLA-CD copolymers were synthesized, and the molecular weights

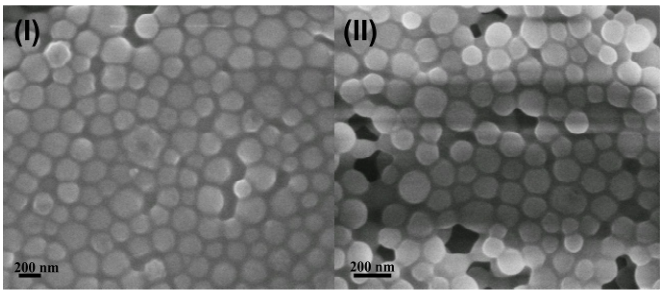


Fig. 3 SEM images of the 4-arm PEG20K-*b*-PLA10K-CD RMs (I) and 4-arm PEG20K-*b*-PLA10K RMs (II) loading with BSA in DCM.

calculated from ^{1}H NMR and measured by GPC were 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 spectra was showed in the literature.[37] While after the introduction of CD, the peak was 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 $CDCl_3$. The peaks "d" at 1.48 ppm and "c" at 5.19 ppm arose from the protons of the PLA segment, respectively. The peaks "a" at 3.40 ppm and "b" at 3.62 ppm were the protons of PEG. The peak ratio of "c" and "b" indicated the ring-opening reaction was performed in the controlled manner. Compared to Fig. 2(i) & Fig. 2(iii), the 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 PEG-*b*-PLA copolymers. The reaction efficiency of the CD conjugation was ca. 50-60% calculated from the integral area ratio of peak "1" and "a".

PEG-*b*-PLA nanoparticles were widely used as micellar carriers in DDS in aqueous solution. Most of PEG-*b*-PLA DDSs were constructed with two types of topological structures, *i.e.*, drug-load[17,18,27] and drug-conjugated[9-11,38]. However, no report was 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 PLA segment. Similarly, the PEG-*b*-PLA-CD could self-assemble into RMs in non-polar solvent owing to the hydrophilic of PEG segment. The core-shell structure of the RMs from the amphiphilic copolymers could be verified by ^{1}H NMR spectrum. DMSO is a good solvent for all the blocks of the copolymers, and all proton signals appeared using DMSO-d_6 as solvent in the ^{1}H NMR measurement (Fig. 2(iii)). After emulsion of $CDCl_3$ with small amount of D_2O (Fig. 2(iv)), the specific signals of PEG segment were weakened as compared with its ^{1}H NMR spectrum in DMSO-d_6 (from 6.44 to 2.28), indicating that the MPEG8K-*b*-PLA10K-CD RMs were formed with a hydrophilic core of PEG segment and a hydrophobic corona layer of the PLA segment.[39] Meanwhile, the peak of H-1 (4.87 ppm) in CD disappeared, indicating that the CD units 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 increased hydrophilic segment. It has been

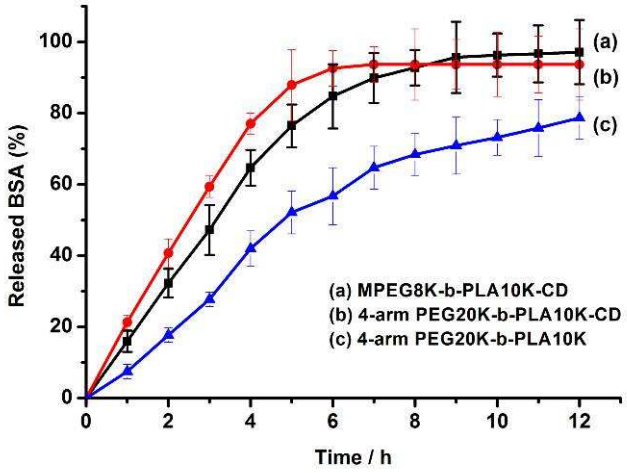


Fig. 4 Release profiles of BSA from the copolymer RMs in EO.

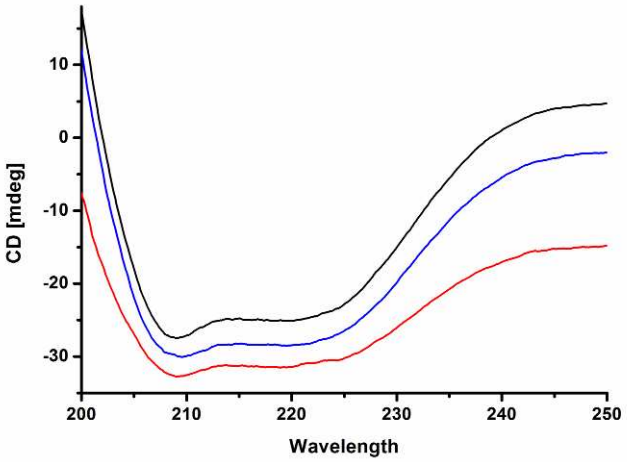


Fig. 5 Circular dichroism spectra of BSA released after 7 days from the 4-arm PEG10K-b-PLA20K-CD (red), BSA released after 7 days from the 4-arm PEG10K-b-PLA20K (blue), and native BSA (black).

reported that, in aqueous solutions, the hydrophobic segment could promote the core compactness of normal micelles, and enlarged amount of hydrophobic portion could result in the formation of smaller particles.[40] Similarly, the larger hydrophilic portion can promote the compactness of the RM cores in organic solutions. Therefore, an increased hydrophilic segment resulted 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 PLA block equals to that of PEG block, could the BSA be encapsulated into RMs in EO, indicating that the proper hydrophilic-hydrophobic balance was important for the RMs to keep stable after transferring into the EO.

The morphology of the RMs was spherical in shape as observed by SEM (Fig. 3). The mean diameter of 4-arm PEG20K-*b*-PLA10K and 4-arm PEG20K-*b*-PLA10K-CD RMs was ca. 130±23 and 190±31 nm, respectively, and a little smaller than that determined by DLS. It can be explained from the fact that the RMs for the SEM were air-dried and those for the DLS measurement were 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. Actually, MPEG8K-*b*-PLA5K and MPEG8K-*b*-PLA10K could encapsulate BSA. However, the quality reports of DLS measurement was poor, meaning that the RMs have a poor monodispersity, thus the data were 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 exist 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 chain could encapsulate more BSA. The BSA loading was enhanced as a result of a relatively strong hydrophobic interaction between copolymers and BSA.[41] It was notable that the degree of copolymer branching also influenced the EE and LC. The RMs comprised of star-shaped copolymers were capable to accommodate 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 a 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 aqueous phase, an initial phase could be observed that 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 degradation of the copolymers, which was associated with generation of micropores in the degradation and enhanced water uptake.[44] In Fig. 4(a) and 4(b), the release of BSA was in an approximate linear fashion in the initial 6 h, and reached a plateau at 8 h. As a contrast, the BSA released from RMs without CDs (Fig. 4(c)) showed a different profile that maintaining a sustained release during the whole release process. Generally, the RMs composed of the PEG-*b*-PLA-β-CD copolymers released BSA faster than that composed of the PEG-*b*-PLA copolymers, which could probably be owing to that the channels of CD was favorable for the BSA diffusion.

Circular dichroism spectroscopy was used to determine whether BSA molecules were denatured after the RMs formation (Fig. 5). The far-UV-CD band at 209 nm primarily ascribes to the α-helix structure, while that at 222 nm was for the β-sheet.[45] In this study, the[Φ]209/[Φ]222 ratio 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 wasn't significant difference between those released from RMs and the native BSA, indicating that the released BSA remained its original structure.

Conclusions

The RMs based on linear and star-shaped PEG-*b*-PLA-CD and PEG-*b*-PLA copolymers were fabricated by emulsion method in organic/apolar solvent with a defined core-shell structure and a particle size of 150 to 300 nm. These RMs demonstrated the ability to solubilize BSA in the organic/apolar solvent, and were further transferred into ethyl oleate (EO). The factors affect the particle size, the drug loading and release were extensively studied, which will provide the scientific foundation for rational design of RMs. Both the hydrophilic/hydrophobic balance and CDs introduction influence the RMs dispersion in EO. The introduction of CDs can help the dispersion of RMs in EO. In both DCM and EO solutions, RMs composed of PEG-*b*-PLA-CD showed higher EE and LC than those composed of PEG-*b*-PLA.

These novel RMs with improve EE and LC could be a great potential for protein delivery.

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Notes and references

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Table of Content

for

Reverse micelles based on biocompatible β-cyclodextrin Conjugated polyethylene glycol block polylactide for protein delivery

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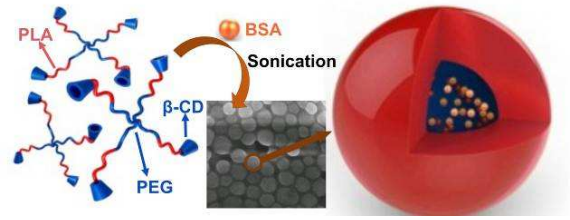

The oil based formulation fabricated from biodegradable PEG-*b*-PLA-CD copolymers with high drug loading showed great potential for protein delivery.