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Biodegradable Polymersomes from Four-Arm PEG-b-PDLLA for Encapsulating Hemoglobin

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ABSTRACT: A series of four-arm star block copolymers poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) with different hydrophobic length were synthesized by ring-opening polymerization of lactide and characterized using ^{1}H NMR and gel permeation chromatography (GPC). These copolymers could self-assemble in aqueous solution to form the vesicle structure with controlled size and morphologies. Transmission electron microscopy (TEM) and DLS show the polymersomes are spherical with diameter of 70∼500 nm. The polymersomes made by direct hydration of copolymer thin films in water exhibit the controllable ability of encapsulating hemoglobin under mild condition. The hemoglobin content in the polymersomes could reach to 35 wt %. More importantly, the encapsulated hemoglobin keeps its own bioactivity and is capable of binding oxygen. This hemoglobin-encapsulated four-arm PEG-PLA polymersomes could have the potential to be applied as an artificial oxygen carrier for transfusion. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40433.

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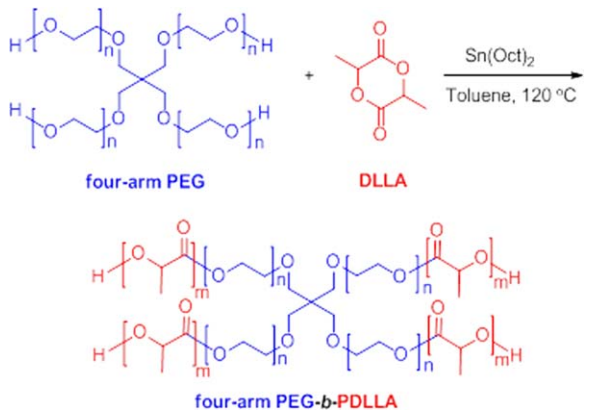
INTRODUCTION

Polymersomes or polymeric vesicles have been proven to have superior drug, protein, and gene delivery capacities, enhanced structural stability, and low permeability compared with liposomes.[1–3] Polymersomes of amphiphilic block copolymers could encapsulate water-soluble cargos such as pharmaceutical molecules and biomolecules within the water-filled inner compartment.[4,5] Nowadays, most of the polymersomes used for drug delivery are made of linear copolymers.[6,7] However, the development of other amphiphilic structures offers us enhanced control over the architecture, size, shape, and surface functionality of the nanoparticles compared with linear copolymers.

The multiarm star polymers have attracted attention to utilize their various block copolymers as hydrogels or micelles in biomedical fields because of their smaller hydrodynamic size and low solution viscosity compared with linear polymers.[8–10] In fact, the multiarm star copolymers have shown superior vesicle-forming ability compared with the linear diblock structure.[11] More recently, Percer et al. found that the dendrimersomes formed by multiarm or branched macromolecules are incredibly robust, very monodisperse, and more useful; they are suitable for further chemical functionalization on the surface.[12,13] However, the polymersomes prepared by star-shaped copolymers have been studied rarely in the literature.[14] Herein, we report on the preparation of polymersomes from four-arm PEG-*b*-PDLLA (poly(ethylene glycol)-block-poly(D,L-lactide)) by direct hydration of copolymer thin films in water and their application in hemoglobin encapsulation.

Interest in human blood substitutes has lasted for more than 100 years.[15] This is because the blood supply based on blood donation is limited, suffering from limited availability of blood donors, difficulty in blood type matching, and limited storage time. During the last three decades, biodegradable polymers were widely used in pharmaceutical field. The polymersomes made from amphiphilic block polymer have much better mechanical strength than liposomes. For example, Chang et al. used PEG-PLA copolymers as the membrane of the hemoglobin vesicles to prolong the circulation time.[16] We hypothesized that the multiarm amphiphilic copolymers can self-assemble into polymersomes and used as the hemoglobin carriers.



Scheme 1. Synthesis of four-arm PEG-*b*-PDLLA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MATERIALS AND METHODS

Materials

Four-arm PEG-OH (M_W = 2000) was purchased from Creative PEGWorks. Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, 90% in 2-ethylhexanoic acid) was purchased from Strem Chemicals. Rhodamine B, sodium ascorbate (NaVc), and sodium dithionite ($Na_2S_2O_4$) were purchased from Aladdin Co. Ltd. Bovine Hemoglobin (M_w 64,500) was purchased from Shanghai Kayon Biological Technology Co. Ltd. and stabilized under CO atmosphere to afford carbonylated hemoglobin (COHb). CO (99.95%) was from Dalian Date Gas Co. Ltd. Toluene was purified by distillation from sodium with benzophenone. Other solvents were analytical grade and used as received.

Characterization

^{1}H NMR spectra were recorded with a Bruker AV400 spectrometer at 25°c. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC instrument equipped with a Waters Styragel HT6E column and a differential refractometer detector. DMF was used as eluent at a flow rate of 1 mL min^{-1} at 35°C. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 KV. UV spectra were recorded on a UV-vis spectrophotometer (Shimadzu UV-2450) at room temperature.

Synthesis of Four-Arm PEG-PDLLA

The four-arm PEG-*b*-PDLLA was synthesized using ring-opening polymerization (ROP) of D,L-lactide in the presence of four-arm PEG as a macroinitiator, with stannous octoate as a catalyst. First, four-arm PEG was added to a reaction flask and dried by toluene azeotropic distillation, then the calculated LA were added together into the above flask, and the flask was argon-purged several times. $Sn(Oct)_2$ in toluene was added through a glass syringe. The reaction mixture was then heated to 120°C in an oil bath and stirred at this temperature for 12 h. The polymerization was stopped by removing the flask from the oil bath and cooled to room temperature. Purification was performed by dissolving the reaction mixture in a small amount of chloroform and pouring it into an excess of methanol with stirring. The product was collected and dried under vacuum. ^{1}H NMR (400 M, $CDCl_3$, δ ppm): δ 5.0–5.3 (1H, DLLA, -CH-CH_3); δ 3.6 (4H, PEG, -CH_2-CH_2-); δ 1.6 (3H, DLLA, CH-CH_3).

Preparation of Polymersomes

Polymersomes were prepared using direct hydration of copolymer thin films in water.[17,18] The copolymer solution in chloroform (0.5 mg/mL) was slowly evaporated to form a thin film, which was further dried under high vacuum. It was stirred for 12 h after adding water to the dry film. The polymersomes were obtained by the above method. It was subjected to lyophilization for 48 h and stored at −20°C before use.

Preparation of Polymersome-Encapsulated Hemoglobin

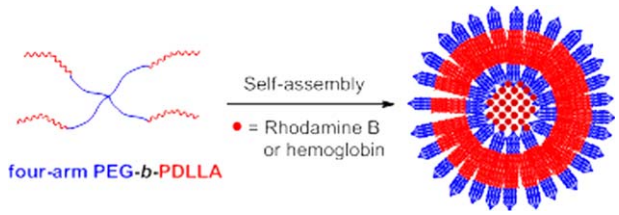
The polymersome-encapsulated hemoglobin was prepared in line with the method described by H. Agashe et al.[19] with some modifications. To encapsulate hemoglobin into the polymersome, a typical procedure was described as follows: 10 mg of dried polymersome was added into a vial that had been purged with CO for 30 min, then 20 mL of carbonylated hemoglobin (COHb) solution (0.9% saline) was added using a syringe. The solution was stirred for 2 h at 60°C, and then cooled to 30°C and kept stirring for another 24 h. Free hemoglobin was removed by diafiltration through a 500 kDa hollow fiber (HF) membrane. The filtrate was assayed for the absence of hemoglobin through UV-vis spectroscopy. To the retentate was added 5 mg of NaVc, and it was then purged with CO for 1 h. Finally, the vial was sealed and stored at 4°C for future use.

Hemoglobin Encapsulation Efficiency

The hemoglobin encapsulation efficiency was measured indirectly using the centrifugation method (10,000 rpm × 30 min). The amount of entrapped hemoglobin was determined by measuring the difference between the initial amount of hemoglobin (Hb_{total}) and the amount of free hemoglobin in the supernatant (Hb_{free}). The encapsulation efficiency of hemoglobin was calculated by the following equation. The hemoglobin concentration was assayed by UV-vis spectroscopy using the cyanomet hemoglobin method detailed elsewhere.[20]

Table I. Characterization of the Four-Arm Amphiphilic Copolymers Synthesized

		M_n of PDLLA	M_n		
Copolymer	Composition	^{1}HNMR	^{1}H NMR	GPC	PDI
1	4-PEG_{2k}-$PDLLA_{4k}$	4000	24,000	22,000	1.05
2	4-PEG_{2k}-$PDLLA_{7k}$	7000	36,000	26,000	1.11
3	4-PEG_{2k}-$PDLLA_{10k}$	10,000	48,000	34,600	1.12



Scheme 2. Preparation of polymersomes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$$\text{Encapsulation efficiency (\%)} = \frac{Hb_{total} - Hb_{free}}{Hb_{total}} \times 100$$

Gas-Binding Capacity

The O_2 binding and release ability of hemoglobin at different oxygen partial pressures could be monitored by UV-vis spectrophotometry. To prevent methemoglobin (metHb) formation, COHb was used during the encapsulation process. The encapsulated COHb was converted to O_2-binding hemoglobin (oxyHb) by exposing the polymersome-encapsulated hemoglobin solution to visible light under an O_2 atmosphere, while the deoxygenated hemoglobin (deoxyHb) state was obtained by exposing the solution to visible light under N_2 atmosphere with the addition of a trace amount of $Na_2S_2O_4$.

RESULTS AND DISCUSSION

Synthesis of Four-Arm PEG-*b*-PDLLA

Multiarm star copolymers can be synthesized by two main strategies: (1) the core-first approach, living polymerization on the basis of a multifunctional initiator core, and (2) the arm-first approach, polymerization on the foundation of a functional initiator arm.[21] The four-arm PEG-*b*-PDLLA was synthesized using ring-opening polymerization of D,L-lactide (DLLA) in the presence of stannous octoate using four-arm PEG as macroinitiators (Scheme 1).

Although many factors could influence the resulting structure of assembly from block copolymers such as temperature, solvent, block length, pH, and ionic strength, the major factor influencing morphologies is the mass fractions of hydrophilic segment in the copolymers.[22,23] Amphiphilic block copolymers with hydrophilic mass fractions ranging from 0.20 to 0.40 have been shown to form polymersomes in aqueous solution.[24,25] In our work, the molecular weight of hydrophilic four-arm PEG is 2000. The molecular weight of hydrophobic PDLLA could be controlled by the molar ratio of DLLA to PEG. The PEG-PDLLA copolymers with three different M_n were made, namely $PDLLA_{4K}$, $PDLLA_{7K}$, and $PDLLA_{10k}$. The molecular weight of PDLLA was calculated according to ^1H NMR spectra (see Table I and Figure S1). GPC results showed that a single and sharp peak with a low polydispersity of ~1.10 for these copolymers

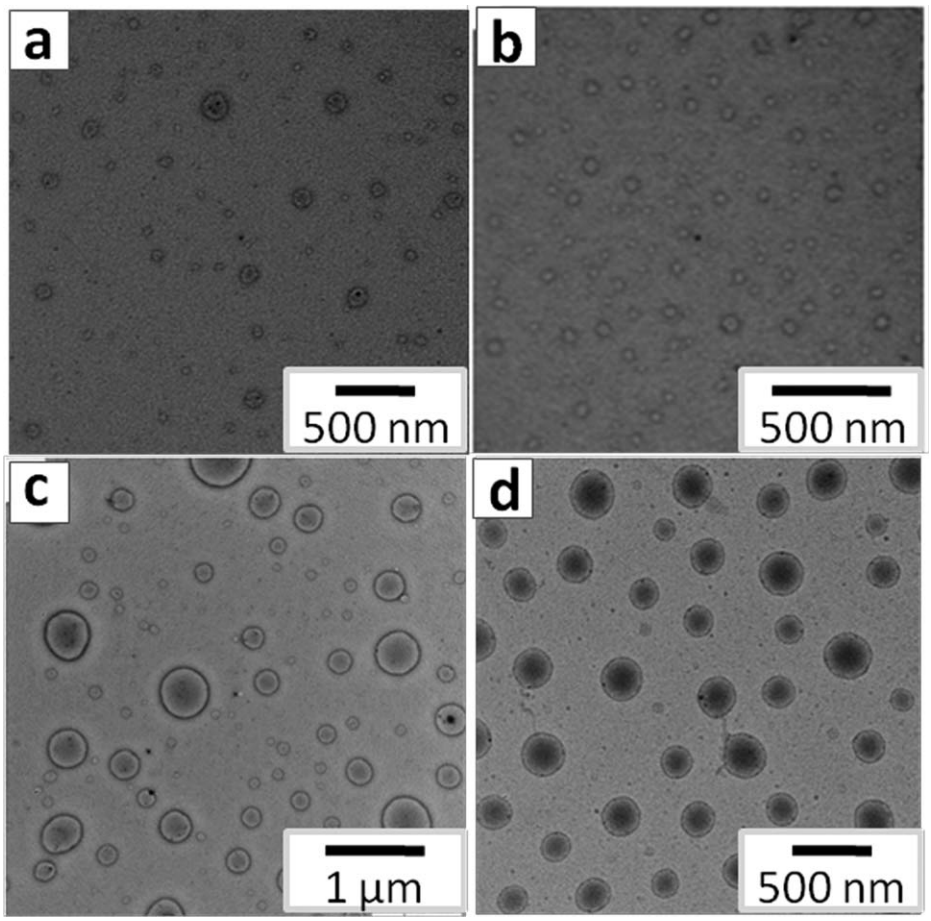


Figure 1. TEM images of polymersomes from 4-PEG_{2k}-$PDLLA_{4k}$ (a), 4-PEG_{2k}-$PDLLA_{7k}$ (b), 4-PEG_{2k}-$PDLLA_{10k}$ (c), and 4-PEG_{2k}-$PDLLA_{10k}$ encapsulating rhodamine B (d). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Characterization of the Polymersomes Formed by Four-Arm Amphiphilic Copolymers

Polymersomes	Copolymer	$f_{PEG}M_{w\text{-}PEG}/M_{w\text{-}copolymer}$	Diameter (nm) TEM	Hemoglobin content (wt %)
1	4-PEG_{2k}-$PDLLA_{4k}$	0.33	~70	34.5
2	4-PEG_{2k}-$PDLLA_{7k}$	0.22	~70	22.0
3	4-PEG_{2k}-$PDLLA_{10k}$	0.17	~450	15.8

(Figure S2). It implies that PEG had reacted with DLLA successfully and no homopolymerization of DLLA occurred.

Characterization of Polymersomes

Polymersomes were made through the film hydration of four-arm PEG-*b*-PDLLA in aqueous solution (Scheme 2) at room temperature. The size and morphology of the formed polymersomes were examined using transmission electron microscope (TEM). TEM image showed vesicular morphology. The diameter of the polymersomes made from copolymer 1 and 2 was about 70 nm (Figure 1a and b). When the molecular weight of PDLLA block reaches to 10,000 (copolymer 3), the average diameter reaches to 450 nm (Figure 2c).

Interestingly, the contrast of TEM image of polymersomes also increased after rhodamine B-loading (Figure 2d), which might be attributed to the high electron density of rhodamine B aggregated in the core of polymersomes. Similar result has been observed in micellar system.[26] Rhodamine B encapsulated in the polymersomes could be visualized using confocal laser scanning microscopy (CLSM), which clearly showed that rhodamine B was concentrated inside the polymersomes (Figure 2). This fact further confirmed the vesicular nature of these particles.

Encapsulation of Hemoglobin

Blood substitutes, also named artificial oxygen carriers, have attracted close attention during the past 30 years. Although there are still some key problems or obstacles for clinical practice, great progress was achieved by using amphiphilic copolymers in physical encapsulation or chemical conjugation of hemoglobin.[27–30] Our groups have reported several different oxygen carriers using chemistry conjugate or physical encapsulation.[31–33] Herein, hemoglobin was used as a model drug to evaluate the encapsulating capability of the polymersomes.

The content of hemoglobin was assayed via UV-vis spectroscopy using the cyanomethemoglobin method.[28,29] The difference between the original amount of hemoglobin and the total amount of free hemoglobin in the supernatants was considered to be the encapsulated amount of hemoglobin inside polymersomes. The encapsulation efficiency of hemoglobin was about 10%, and the hemoglobin content in the polymersomes was from 15 to 35 wt % with different f_{PEG} (0.17~0.33, Table II). It showed that hemoglobin encapsulation was more efficient with copolymers containing lower molecular weight hydrophobic blocks. This is probably due to the relatively thin membrane of these polymersomes, which results in the availability of more internal core volume for hemoglobin encapsulation. But the polymersomes with higher molecular weight PDLLA offer advantages in suppressing nonspecific protein interactions with the polymersome surface.[34]

It is well known that free hemoglobin molecules in CO-binding state can be converted to deoxyHb and oxyHb by irradiating the polymersome solution with visible light. The same procedure was used to examine these transforms. The polymersomes containing hemoglobin was directly used for measurement of UV-vis. As shown in Figure 3, the absorption peak of COHb at

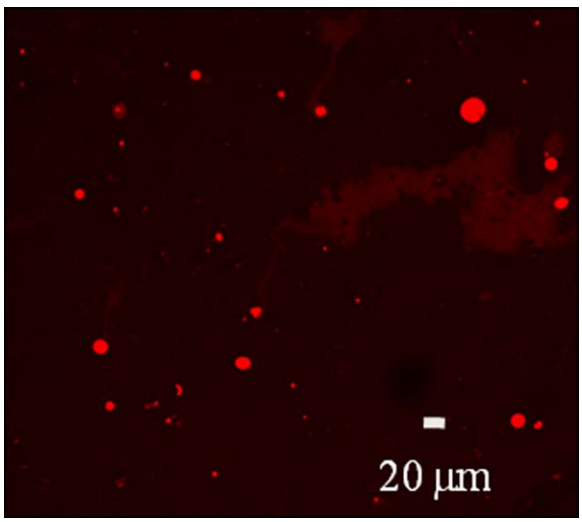


Figure 2. Confocal fluorescence microscopy images of the polymersomes encapsulating rhodamine B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

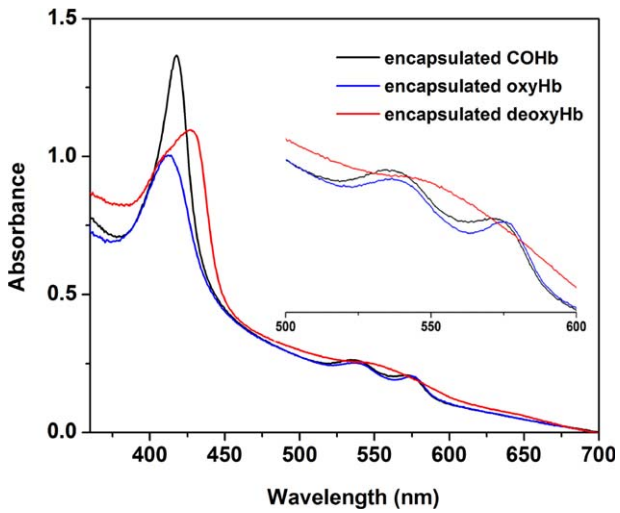


Figure 3. UV-vis spectra of encapsulated Hb in different gas-binding states (CO, O_2, and N_2). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

419 nm disappears, and the absorption peak of deoxyHb appears at 430 nm after degassing with nitrogen. When the deoxyHb was exposed to air, the absorption peak of oxyHb appears at 415 nm. This result also indicates that the hemoglobin encapsulated in the four-arm PEG-*b*-PDLLA polymersomes can bind and release oxygen reversibly, and retain its own bioactivity after encapsulation.

CONCLUSION

In summary, a series of four-arm PEG-*b*-PDLLA with different mass ratio of hydrophobic block to hydrophilic block were prepared. The biodegradable polymersomes were prepared by self-assembling four-arm PEG-*b*-PDLLA copolymers in water. The polymersomes containing water soluble Rhodamine B and/or hemoglobin were prepared in their own solution. The hemoglobin content in polymersomes could be controlled by using different polymers with proper hydrophilic mass fractions. The hemoglobin encapsulated in the polymersomes keep their bioactivity such as transforming from deoxygenated to oxygen-binding state. The four-arm PEG-PDLLA polymersomes showed a promising future to be used as artificial oxygen carriers and other protein carriers.

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