Synthesis and Characterization of Amphiphilic Lipopolymers for Micellar Drug Delivery

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The objective of this study was to design lipopolymers for hydrophobic drug delivery. Poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD) lipopolymers were synthesized and characterized by 1H NMR, FT-IR,GPC, and DSC. The critical micelle concentration (CMC) of PEG-PCD micelles was around $10^{-8}$ M and decreased with increasing length of hydrophobic block. PEG-PCD micelles could efficiently load a model drug embelin into its hydrophobic core and significantly improve its solubility. The loading capacity was dependent on the polymer core structure, but the length of hydrophobic core had little effect. PEG-PCD formed both spherical and cylindrical micelles, which were dependent on the copolymer structure and composition. PEG-PCD lipopolymers with various hydrophobic core lengths showed similar drug release profiles, which were slower than that of poly(ethylene glycol)-block-poly(2-methyl-2-benzoxy carbonyl-propylene carbonate) (PEG-PBC) micelles. Embelin loaded PEG-PCD micelles showed significant inhibition of C4-2 prostate cancer cell proliferation, while no obvious cellular toxicity was observed for blank micelles.

1. Introduction

Many anticancer agents are poorly soluble in water, which can cause serious problems for their clinical application. Dimethyl sulfoxide (DMSO), Ctenophore EL, Tween 80, or other surfactants are commonly used to solubilize these drugs. However, these solubilizing agents are harmful to the liver and kidney and cause dose-dependent hemolysis and acute hypersensitivity reactions. In addition, because of high critical micelle concentration (CMC) values associated with low molecular weight surfactant micelles, they are not stable after systemic administration and thus result in the precipitation of solubilized drugs.

Poly(ethylene glycol)-PEG-lipid conjugates, such as PEG-phosphatidylethanolamine (PEG-PE) have been used for micellar drug delivery. The use of PEG-lipid conjugates for drug delivery are superior to conventional small molecular weight surfactants, because PEG-lipid conjugates have lower critical micelle concentration (CMC: $\sim 10^{-3}$ M) than conventional surfactants (CMC: $\sim 10^{-2}$ M), suggesting PEG-lipid conjugate micelles are more stable than those formed by conventional surfactants. A typical PEG-lipid conjugate structure includes a hydrophilic PEG corona and a hydrophobic lipid core, which, in turn, is composed of two lipid chains varying from C12 to C18 in chain length. Although we could conjugate PEG with different lipids to make different PEG-lipid conjugates, the flexibility in the design of hydrophobic core is still limited.

Alternatively, amphiphilic copolymers have become promising materials for micellar drug delivery. Pluronic is a triblock copolymer composed of a central hydrophobic chain of poly(propylene oxide) (PPO) flanked by two hydrophilic chains of poly(ethylene glycol) (PEG) and has been widely used for drug delivery. It not only increases drug solubility, but also overcomes drug resistance in cancer. The CMC of Pluronic copolymers ranges from $5.3 \times 10^{-3}$ to $2.8 \times 10^{-6}$ M, depending on the molecular weight and composition of copolymers. However, the use of Pluronic for drug delivery is somewhat problematic because they are nonbiodegradable. Therefore, biodegradable copolymers such as PEG-PLLA-PEG were designed to facilitate the elimination of polymers from the body. In addition, the structure and size of hydrophobic core block could be properly designed to improve the micelle stability and drug loading. A significantly reduced CMC (10$^{-6}$ to 10$^{-7}$ M) is observed in a diblock copolymer, which is 10 to 100 folds lower than PEG-lipid conjugates. For examples, the CMC of PEG$_{5700}$-PLA$_{5400}$ was around $3 \times 10^{-6}$ M, as determined by Danquah et al. Similar results were also reported by Yasugi et al. who showed that the CMC of PEG$_{5500}$-PLA$_{4500}$ was around $3 \times 10^{-7}$ M. The low CMC of micelles formed by amphiphilic copolymers indicates that they are more stable than PEG-lipid conjugate micelles and can maintain the integrity of micelles upon dilution. In addition, we and others have showed that the drug solubility has been significantly improved by properly designing the hydrophobic core structure to improve the compatibility between the hydrophobic core and drugs.

For an example, PEG-b-poly(N-alkyl stearate l-aspartamide), a lipid conjugated polymer, has been used to improve the solubility of a hydrophobic drug, amphoterin B, and demonstrated the possibility of using lipopolymers for solubilization of lipidlike drugs.

In this study, we synthesized a novel amphiphilic lipopolymer, poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD). The polymer synthesis procedures were optimized and the properties of polymers were characterized. PEG-PCD lipopolymers could form micelles and effectively incorporate embelin and improve its water solubility. PEG-PCD lipopolymers were compared with similar polymers with different core structures to investigate the effect of core structure on micelle properties, such as stability, drug loading, particle size and morphology, and in vitro drug release. In addition, we also investigated the effect of hydrophobic core size on the properties of PEG-PCD lipopolymer micelles.
Finally, the anticancer activities of embelin loaded micelles were determined in vitro with prostate cancer cells.

2. Experimental Section

2.1. Materials. Hydroxybenzotriazole (HOBT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dodecanol, triethylamine (TEA), stannous 2-ethylhexanoate (Sn(Oct)2), diethyl zinc (Et2Zn), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 2,2-bis(hydroxymethyl)propionic acid, methoxy poly(ethylene glycol) (mPEG, $M_n = 5000$, PDI = 1.03), and all other reagents were purchased from Sigma Aldrich (St. Louis, MO) and used as received.

2.2. Synthesis of Poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD). PEG-PCD lipopolymer was synthesized using the following two methods (Figure 1):

Method I. (A) Synthesis of Poly(ethylene glycol)-block-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate) (PEG-PBC). Monomer 2-methyl-2-benzoxycarbonyl-propylene carbonate (MBC) was synthesized as described by Danquah et al.12 Briefly, a mixture of 0.168 mol 2,2-yl-2-benzyloxycarbonyl-propylene carbonate (MBC) was synthesized in toluene in a dried polymerization flask under the protection of a nitrogen atmosphere. To optimize the reaction condition, the reaction mixture was heated to 80°C for 3 and 16 h under stirring, respectively. (2) Et2Zn (10 mol % relative to mPEG) was added to the mixture of given amounts of mPEG and MBC dissolved in anhydrous CH2Cl2 and reacted at RT for 3 h under stirring. (3) Et2Zn (10 mol % relative to mPEG) was added to the mixture of given amount of mPEG and MBC dissolved in anhydrous toluene in a dried polymerization flask under the protection of nitrogen atmosphere and reacted at 80°C for 16 h under stirring. At the end of reaction, the reaction mixture was dissolved in CHCl3 and reacted under reduced pressure to get poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD).

(B) Hydrogenation of PEG-PBC. To remove protective benzyl groups, PEG-PBC was subjected to hydrogenation. Briefly, 1 g of PEG-PBC was dissolved in 12 mL of tetrahydrofuran (THF) and methanol mixture (1:1) containing 200 mg palladium on charcoal (Pd/C). The reaction flask was purged with N2三次 and charged with H2 using a balloon. The reaction was carried out for 18 h with stirring. At the end of reaction, Pd/C was removed by centrifugation. The solvent was removed under reduced pressure to get poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate) (PEG-PC).

(C) Lipid Conjugation to PEG-PCD. For lipid conjugation to PEG-PCD, 300 mg PEG-PCC, 260 mg dodecanol, 223 mg hydroxybenzotriazole (HOBT), and 317 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were dissolved in 10 mL of DMF and 340 µL of triethylamine (TEA) was then added to the mixture with stirring. After reaction for 18 h, products were precipitated with a large amount of cold isopropyl alcohol twice and diethyl ether once and then dried under vacuum at room temperature to afford poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD).

Method II. (A) Synthesis of 2-Methyl-2-carboxyl-propylene Carbonate (MCC). MCC was synthesized by hydrogenation of MBC, as described previously.12 Briefly, 1 g of monomer MBC was dissolved in 10 mL of ethyl acetate containing 100 mg Pd/C. The reaction flask was purged with N2 three times and charged with H2 to 45 psi. The reaction was carried out for 3 h. Then, Pd/C was removed by centrifugation. The solvent was removed under reduced pressure to give MCC-OH as a white crystal.

(B) Synthesis of 2-Methyl-2-dodecanoxycarbonyl-propylene Carbonate (MDC). To synthesize MDC, 480 mg MTC-OH, 465 mg dodecanol, 506 mg HOBT, and 720 mg EDC were dissolved in 20 mL of DMF and 490 µL of TEA was then added to the mixture with stirring for 18 h. The reaction mixture was diluted with ethyl acetate (20 mL), washed with water, and dried over MgSO4. The solvent was removed under reduced pressure to yield a crude product, which was further purified by column chromatography to get MCC as a white powder.

(C) Synthesis of PEG-PCD from Copolymerization of mPEG with MCC. DBU (40 µL) was added to the mixture of a given amount of mPEG, and MDC was dissolved in anhydrous CH2Cl2 and reacted under stirring for 3 h. The reaction mixture was dissolved in CHCl3, and the product was recrystallized from ethyl acetate to give white crystals. The residue was dissolved in 150 mL of ethyl acetate, washed with water, and dried over MgSO4. The solvent was removed to yield a crude product, which was recrystallized from toluene to give pure benzyl 2,2-bis(methylol)propionate. Then, 0.05 mol benzyl 2,2-bis(methylol)propionate was dissolved in 150 mL of CH2Cl2 containing 25 mL of pyridine, and the solution was chilled to 78°C. A solution of CH2Cl2 containing 25 mmol triphosgene was dripped into the solution over 1 h. The reaction mixture was stirred for an additional 2 h at the room temperature before quenched with 75 mL of saturated aqueous NH4Cl. Subsequently, the organic layer was sequentially washed with aqueous HCl (1 M, 300 mL) and saturated aqueous NaHCO3 (300 mL), dried with Na2SO4, and evaporated to give 2-methyl-2-benzoxycarbonyl-propylene carbonate (MBC) as a crude product, which was further recrystallized from ethyl acetate to give white crystals. Three methods with different catalysts were investigated in this study to synthesize PEG-PBC from copolymerization of mPEG with monomer MBC. (1) Sn(Oct)2 (10 mol % relative to mPEG) was added to the mixture of given amounts of mPEG and MBC dissolved in anhydrous toluene in a dried polymerization flask under the protection of nitrogen atmosphere. To optimize the reaction condition, the reaction mixture was heated to 80°C for 3 and 16 h under stirring, respectively. (2) DBU (40 µL) was added to the mixture of a given amount of mPEG and MBC dissolved in 10 mL of anhydrous CH3Cl, and reacted at RT for 3 h under stirring. (3) Et2Zn (10 mol % relative to mPEG) was added to the mixture of given amount of mPEG and MBC dissolved in anhydrous toluene in a dried polymerization flask under the protection of nitrogen atmosphere and reacted at 80°C for 16 h under stirring.

Figure 1. Synthesis of PEG-PCD lipopolymer. Conditions: (IA) DBU, CH2Cl2, RT, 3 h. (IB) Pd/C (10%), H2, THF/methanol (1:1), RT, 18 h. (IC) EDC, HOBT, TEA, DMF, RT, 18 h. (IIA) Pd/C (10%), H2, EtOAc, RT, 3 h. (IIIB) EDC, HOBT, TEA, DMF, RT, 18 h. (IIC) DBU, CH2Cl2, RT, 3 h.
product was precipitated with a large amount of cold isopropyl alcohol and diethyl ether and dried under vacuum at room temperature to get PEG-PCD lipopolymer.

2.3. Polymer Characterization. 2.3.1. Nuclear Magnetic Resonance (NMR). $^1$H NMR spectra were recorded on a Varian (500 MHz) using deuterated chloroform (CDCl$_3$) as a solvent, unless otherwise noted. The chemical shifts were calibrated using tetramethylsilane as an internal reference and given in parts per million.

2.3.2. Infrared (IR) Spectra. The composition of synthesized polymers was also confirmed with infrared (IR) spectra using a Perkin-Elmer IR spectrometer.

2.3.3. Gel Permeation Chromatography (GPC). The weight ($M_w$) and number ($M_n$) average molecular weight and polydispersity index (PDI) of synthesized polymers were determined by a Waters GPC system equipped with a GPC column (AM Gel 10$/3$) and a differential refractive index detector. THF was used as an eluent at a flow rate of 1 mL/min. A series of narrow polystyrene standards (700–40 000 g/mol) were used for calibration.

2.3.4. Differential Scanning Calorimetry (DSC). The thermal properties, including glass transition temperature ($T_g$), melting temperature ($T_m$), and crystalline temperature ($T_c$) of the synthesized polymers were determined by differential scanning calorimetry (DSC; TA Instrument DSC Q2000 module). Samples were placed in aluminum pans under nitrogen heated from 25 to 100 °C, cooled to −70 °C to remove thermal history, and heated from −70 to 100 °C at a rate of 5 °C/min.

2.4. Critical Micelle Concentration. Critical micelle concentration (CMC) was determined with fluorescent spectroscopy using pyrene as a hydrophobic fluorescent probe, as previously described.$^{11}$ Briefly, 40 µL of pyrene stock solution (2.4 × 10$^{-3}$ M) in acetone was added to 40 mL of water to prepare a saturated pyrene aqueous solution. Polymer samples were dispersed in water with concentration ranges from 0.5 to 4.8 × 10$^{-2}$ mg/mL and mixed thoroughly with above pyrene solution at a volume ratio of 1:1. The fluorescent intensity was recorded with a UV spectrophotometer based on UV absorbance at 310 nm. All experiments were performed in triplicate and the data reported as the mean of the three individual experiments. The release profiles were assessed as described previously$^{16}$ using a similarity factor (f2). The percentage of cumulative drug release was calculated with the following equation:

$$\text{cumulative drug release(%) = \frac{\text{cumulative amount of released drug}}{\text{total amount of drug added}} \times 100\%}$$

2.5. Preparation of Polymeric Micelles. Polymeric micelles were prepared with a film dispersion method as previously reported with some modifications.$^{15}$ Briefly, 15 mg polymer and a given amount of embelin were dissolved in 0.5 mL of CH$_3$Cl$_2$, and then the solvent was removed under reduced pressure. The resulting film was hydrated in 3 mL of PBS (pH 7.4) and sonicated for 1 min. Then, the residue-free drug was removed by centrifugation at 12 000 rpm for 5 min. The supernatant was filtered using a 0.22 µm filter.

2.6. Drug Loading and Encapsulation Efficiency. To determine drug loading, 10 µL of embelin-loaded micelle solution was diluted with methanol. Then drug concentration was determined using a UV spectrometer at 310 nm (Thermo Spectronic). Embelin concentration was calculated based on the UV absorbance using a standard curve. Drug loading and encapsulation efficiency were then determined using the following equations, respectively.

$$\text{drug loading(%) = \frac{\text{amount of loaded drug}}{\text{amount of polymer}} \times 100\%}$$

$$\text{drug encapsulation efficiency(%) = \frac{\text{amount of loaded drug}}{\text{amount of drug added}} \times 100\%}$$

$$\text{theoretical loading(%) = \frac{\text{amount of drug added}}{\text{amount of polymer}} \times 100\%}$$

Theoretical loading is equal to drug loading when the loading efficiency is 100%.

2.7. Morphology and Particle Size. The particle size of polymeric micelles was determined by dynamic light scattering (Malvern Nano ZS). The intensity of scattered light was detected at 90°. The particle size measurement was repeated three times and the data were reported as the mean ± SD. The morphology of polymeric micelles were observed using a transmission electron microscope (TEM, JEM-100S) using an acceleration voltage of 60 kV. Micelles was loaded on a copper grid and stained with 1% uranyl acetate. The grid was visualized under the electron microscope with magnifications of 75 000.

2.8. In Vitro Drug Release. The dialysis technique was employed to determine the release of embelin from polymeric micelles in PBS (pH 7.4) with 0.1% Tween-80. The micelles used for in vitro release study have a polymer concentration of 5 mg/mL. A total of 800 µL of micelles with 5% drug loading were used to compare the release profiles of PEG-PCD with different PCD length. In addition, 2 mL micelles with 2% drug loading were also used to compare the release profiles of PEG$^{14}$PCD$^{30}$ and PEG$^{14}$PBC$^{30}$. Embelin-loaded micelles were placed into a dialysis tube with a molecular weight cutoff of 3500 Da and dialyzed against 50 mL of release medium in a thermocontrolled shaker with a stirring speed of 180 rpm at 37 °C. At specified time, 1 mL release medium was withdrawn and embelin concentration was determined with a UV spectrophotometer based on UV absorbance at 310 nm. All experiments were performed in triplicate and the data reported as the mean of the three individual experiments. The release profiles were assessed as described previously$^{16}$ using a similarity factor (f2). The percentage of cumulative drug release was calculated with the following equation:

$$\text{cell viability(%) = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100\%}$$

3. Results

3.1. Synthesis and Characterization of Polymers. As summarized in Figure 1, the target lipopolymer PEG-PCD was synthesized by two approaches. In the first approach, PEG was copolymerized with MBC to afford poly(ethylene glycol)-block-poly(2-methyl-2-benzoxy carbonyl-propylene carbonate) (PEG-PBC). Then, the pendant benzyl group was removed by hydrogenation to expose carboxyl groups to get poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate) (PEG-PCC). Finally, poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD) lipopolymer was synthesized by conjugating dodecanol to the carboxyl groups. In the second approach, PEG was copolymerized with a lipid modified monomer 2-methyl-2-dodecanoxy-carbonyl-propylene carbonate (MDC). MCC and MDC were...
characterized with \( ^1H \) NMR (Figure S1) and ESI-MS. MCC: \( ^1H \) NMR (500 MHz, DMSO) \( \delta \) 13.35 (s, 1H), 4.57 (d, 2H), 4.32 (d, 2H), 1.18 (s, 3H). MS (ESI) \( m/z \) 159 [M-H]\. MCC: \( ^1H \) NMR (500 MHz, CDCl3) \( \delta \) 4.50 (d, 2H), 4.2 (d, 4H), 1.65 (m, 2H), 1.4–1.2 (m, 21H), 0.85 (t, 3H). MS (ESI) \( m/z \) 351 [M + Na]\. Although we could synthesize the lipopolymers with both approaches, the second approach involves column purification of MDC, which is a time-consuming process. Therefore, the first approach was selected to generate lipopolymer PEG-PCD.

To achieve the optimal polymerization efficiency, three different catalysts were tested for copolymerization of PEG and MBC, as summarized in Table 1. Among three catalysts tested, DBU was the best, as the conversion rate was 80–88% at the molar ratio of monomer and PEG ranging from 10 to 34. Both Sn(oct)\textsubscript{2} and Et\textsubscript{2}Zn were used at 80 °C and the degree of polymerization (DP) was increased with prolonged reaction time from 3 to 16 h for Sn(oct)\textsubscript{2}. However, both of these two catalysts gave less DP under the reaction conditions described in Table 1. Therefore, DBU was selected in the current study.

The structures of PEG-PBC, PEG-PCC, and PEG-PCD were confirmed by \(^1H\) NMR (Figure 2). From Figure 2A, the following peaks were observed for PEG-PBC polymer at \( \delta \) 1.2 (CH\textsubscript{3} in BC unit), 3.6 (CH\textsubscript{2} in PEG), 4.3 (CH\textsubscript{2} in BC main chain), 5.2 (CH\textsubscript{2} in BC side group), and 7.3 (phenyl ring). All signals are assigned as methoxy poly(ethylene glycol) (mPEG) and polymerized BC units. In addition, the success of polymerization was confirmed by the disappearance of signal at \( \delta \) 4.2 and 4.6 in the MBC monomer and appearance of a new peak at \( \delta \) 4.3. The degree of polymerization and molecular weight of the polymers was estimated based on the peak areas of PEG CH\textsubscript{2} groups at \( \delta \) 3.6 and those of CH\textsubscript{3} in BC main chain at \( \delta \) 4.3.

The benzyl groups were removed via Pd/C catalyzed hydrogenation. The peaks at \( \delta \) 5.2 (CH\textsubscript{2} in BC side group) and \( \delta \) 7.3 (phenyl ring) disappeared after hydrogenation (Figure 2B), which indicates the complete removal of pendant benzyl group. In addition, a peak at \( \delta \) 13 was observed, which belongs to the exposed carboxyl group after removing protective benzyl group. Dodecanol was conjugated onto the polymer backbone using EDC/HOB\textsubscript{T} chemistry. The success of dodecanol conjugation to PEG-PCC carboxyl group was confirmed by the appearance of new peaks at \( \delta \) 4.1, 1.6, 1.2–1.4, and 0.9, which could be assigned to different groups in the lipid as shown in Figure 2C.

The FT-IR of PEG-PBC, PEG-PCC, and PEG-PCD also confirmed the success of the reaction (Figure 3). In FT-IR spectra of PEG-PBC, there were peaks at 736 and 696 cm\textsuperscript{-1}, which were due to the CH vibrations of benzyl groups. These two peaks were absent in the FT-IR spectra of PEG-PCC and PEG-PCD, indicating the removal of the pendant benzyl groups during hydrogenation. In addition, we also observed a broad OH stretch band, which was centered at 3450 cm\textsuperscript{-1} and partially overlapped with C–H stretch. This further confirmed the presence of pendant COOH groups. Besides, we also observed absorbance bands due to the C=O stretching vibration at 1749 cm\textsuperscript{-1} (PEG-PBC), 1748 cm\textsuperscript{-1} (PEG-PCC), and 1755 cm\textsuperscript{-1} (PEG-PCD), respectively. The C–H stretch band was observed at 2866 cm\textsuperscript{-1} for PEG-PBC, 2874 cm\textsuperscript{-1}, and 2856 cm\textsuperscript{-1} for PEG-PCD, indicating the presence of pendant long alkanes.

The characteristics of PEG-PBC, PEG-PCC, and PEG-PCD polymers are summarized in Table 2. The molecular weight of PEG-PBC and PEG-PCC were calculated based on the peak areas of PEG CH\textsubscript{2} groups at \( \delta \) 3.6 and those of CH\textsubscript{3} in BC main chain at \( \delta \) 4.3 in the \(^1H\) NMR spectrum. In addition, the

Table 1. Effect of Different Catalyst on the Polymerization of PEG-PBC Copolymer

<table>
<thead>
<tr>
<th>Catalyst\textsuperscript{a}</th>
<th>[MBC]/[PEG-OH]\textsuperscript{b}</th>
<th>( M_{n,target} )</th>
<th>DP\textsubscript{MBC}\textsuperscript{c}</th>
<th>( M_n )</th>
<th>Conversion (%)\textsuperscript{d}</th>
</tr>
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<tbody>
<tr>
<td>DBU\textsuperscript{b}</td>
<td>10</td>
<td>7500</td>
<td>8</td>
<td>7000</td>
<td>80</td>
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<tr>
<td>DBU\textsuperscript{b}</td>
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<td>10000</td>
<td>17</td>
<td>9250</td>
<td>85</td>
</tr>
<tr>
<td>Sn(oct)\textsubscript{2}\textsuperscript{c}</td>
<td>34</td>
<td>13500</td>
<td>30</td>
<td>12500</td>
<td>88</td>
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<tr>
<td>Sn(oct)\textsubscript{2}\textsuperscript{c}</td>
<td>34</td>
<td>13500</td>
<td>17</td>
<td>9250</td>
<td>50</td>
</tr>
<tr>
<td>Et\textsubscript{2}Zn\textsuperscript{c}</td>
<td>34</td>
<td>13500</td>
<td>22</td>
<td>10500</td>
<td>65</td>
</tr>
<tr>
<td>Et\textsubscript{2}Zn\textsuperscript{c}</td>
<td>20</td>
<td>10000</td>
<td>6</td>
<td>6500</td>
<td>30</td>
</tr>
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</table>

\textsuperscript{a} DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; Sn(oct)\textsubscript{2}, stannous 2-ethylhexanoate; Et\textsubscript{2}Zn, diethy zinc. \textsuperscript{b} Reaction condition: RT, 3 h. \textsuperscript{c} Reaction condition: 80 °C, 3 h. \textsuperscript{d} Reaction condition: 80 °C, 16 h. \textsuperscript{e} Molar ratio of monomer (MBC) to PEG in the feed. \textsuperscript{f} \( M_{n,target} = [\text{MBC}]/[\text{PEG-OH}] \times M_{\text{monomer unit}} (250) + M_{\text{PEG-OH}} (5000) \). \textsuperscript{g} DP: degree of polymerization, determined by \(^1H\) NMR. \textsuperscript{h} \( M_n = DP \times M_{\text{monomer unit}} (250) + M_{\text{PEG-OH}} (5000) \). \textsuperscript{i} Determined by \(^1H\) NMR, conversion (%) = DP/[\text{MBC}]/[\text{PEG-OH}] \times 100\%.

Figure 2. \(^1H\) NMR of polymers: (A) PEG\textsubscript{114}-PBC\textsubscript{30} in CDCl\textsubscript{3}, (B) PEG\textsubscript{114}-PCC\textsubscript{30} in DMSO, and (C) PEG\textsubscript{114}-PCD\textsubscript{29} in CDCl\textsubscript{3}.

Figure 3. FT-IR spectra of polymers: (A) PEG\textsubscript{114}-PBC\textsubscript{30}, (B) PEG\textsubscript{114}-PCC\textsubscript{30}, and (C) PEG\textsubscript{114}-PCD\textsubscript{29}.
percentage of conjugation and molecular weight of the lipopolymer was calculated by the peak areas at δ 4.3 (belongs to polymer backbone) and at δ 4.1 (belongs to the conjugated pendant lipid) in the 1H NMR spectrum. The \( M_w \) determine by 1H NMR were 12500 g/mol for PEG114-PBC30, 9800 g/mol for PEG114-PCC30, 13561 g/mol for PEG114-PCD29, 10248 g/mol for PEG114-PCD16, and 6968 g/mol for PEG114-PCD8, respectively. GPC was also used to determine the apparent weight \( M_w \) and number \( M_n \) average molecular weight of polymers and their polydispersity index. A good correlation was observed between the molecular weight estimated from NMR and \( M_w \) or \( M_n \) determined by GPC. All of the polymers showed narrow distribution as revealed by the small polydispersity index (1.06–1.23). A representative plot comparing the GPC chromatograms for PEG114-PCC30, PEG114-PBC30, and PEG114-PCD29 was shown in Figure 4.

DSC was used to determine the thermal properties of the synthesized block copolymers. The thermograms of diblock copolymers with different core structures are shown in Figure 5. PEG114-PBC30 has a \( T_g = -35.7 \) °C, \( T_c = 6.3 \) °C, and \( T_m = 49.5 \) °C, respectively. PEG114-PCC30, which is a transparent viscous liquid, showed a \( T_g \) of \( -30.7 \) °C, but no obvious \( T_c \) or \( T_m \). PEG114-PCD29 showed a \( T_g \) of \( -35.7 \) °C, but no obvious \( T_c \) or \( T_m \). The different thermal properties observed with DSC suggesting different properties of the copolymers. Only \( T_g \) was observed in PEG114-PCC16, indicating it is an amorphous polymer. PEG114-PCD29 has a high degree of crystallinity, because there was no \( T_g \) observed. The observed \( T_g \), \( T_c \), and \( T_m \) in PEG-PBC indicate the existence of both amorphous domain and crystalline domains in PEG114-PBC30, which is a semicrystalline polymer. We also calculated the degree of crystallinity based on the DSC peak areas, which was 57.1%. Different degrees of crystallinity of PEG114-PCC16, PEG114-PBC30, and PEG114-PCD29 might be due to the different intermolecular forces among these three polymers with different hydrophobic core structures. We also determined the effect of hydrophobic core size on the \( T_m \) of PEG-PCD. As shown in Figure 5B, the \( T_m \) for PEG114-PCD29, PEG114-PCD16, and PEG114-PCD6 were 51.7, 53.6, 55.3 °C, respectively.

The polymers were further characterized by fluorescence spectroscopy to determine the CMC (Figure 6). PEG114-PBC30 and PEG114-PCD29 had low CMC values of \( 4.0 \times 10^{-4} \) g/L and \( 3.6 \times 10^{-3} \) g/L, respectively. In contrast, the CMC of PEG114-PCC30 was \( 2.2 \times 10^{-2} \) g/L, which was around 50 folds higher than those of PEG114-PBC30 or PEG114-PCD29. The low CMC indicates the good stability of micelles prepared from PEG114-PBC30 and PEG114-PCD29. The effect of hydrophobic core size on PEG-PCC micelle stability was also determined. The CMC of PEG-PCC micelles decreased significantly from \( 1.4 \times 10^{-3} \) g/L to \( 3.6 \times 10^{-4} \) g/L when the DP(n) increased from 6 to 29 (Figure 6B).

### Table 2. Characteristics of PEG Copolymers with Different Core Structure

<table>
<thead>
<tr>
<th>polymer</th>
<th>( M_w ) (NMR)</th>
<th>( M_m ) (GPC)</th>
<th>( M_n ) (GPC)</th>
<th>PDI</th>
<th>CMC (g/L)</th>
<th>CMC (M)</th>
<th>crystallinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG114-PBC30</td>
<td>12500</td>
<td>11140</td>
<td>9703</td>
<td>1.15</td>
<td>4.0 \times 10^{-4}</td>
<td>3.2 \times 10^{-8}</td>
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</tr>
<tr>
<td>PEG114-PCC30</td>
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<td>6563</td>
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<td>13342</td>
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<tr>
<td>PEG114-PCD16</td>
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<td>9590</td>
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<td>6203</td>
<td>1.06</td>
<td>1.4 \times 10^{-3}</td>
<td>2.0 \times 10^{-7}</td>
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*PEG-PBC, poly(ethylene glycol)-block-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate); PEG-PCC, poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol); PEG-PCD, poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate). 1 M_w and \( M_n \) were apparent molecular weight determined by GPC; PDI = (\( M_w/M_n \)) \times 100%. 2 CMC (g/L) normalized with \( M_w \) from 1H NMR.
However, the drug loading increased from 5 to 6 and 7% for PEG-PCD micelles, when theoretical loading increased from 5 to 10 and 15%, with decrease in loading efficiency from 100 to 60 and 50%, respectively. There was no significant difference among three PEG-PCD lipopolymers with different hydrophobic core lengths.

3.2.2. Core–Shell Structure Formation. 

$^1$H NMR spectroscopy was used to demonstrate the formation of core–shell of PEG-PCD lipopolymer micelles (Figure 8). In CDCl$_3$, peaks from both the hydrophilic PEG block (3.6 ppm) and the hydrophobic core block (4.3, 4.1, 1.6, 1.2–1.4, and 0.9 ppm) was observed. In D$_2$O, all the peaks corresponding to the hydrophobic core block were significantly diminished in contrast to the strong peaks for hydrophilic PEG block (Figure 8A). This is consistent with a core–shell structure with a collapsed hydrophobic core. In addition, we also compared $^1$H NMR spectra of embelin-loaded PEG-PCD lipopolymer in CDCl$_3$ and D$_2$O. Although the specific peaks for embelin at 6 and 2.4 ppm was observed when dissolved in CDCl$_3$, they disappeared when they formed micelles in D$_2$O. These results indicate that PEG-PCD lipopolymer will form micelles in water with a PEG hydrophilic shell and a hydrophobic core, and embelin is incorporated into the hydrophobic core.

3.2.3. Physical Appearance and Particle Size Distribution. 

The physical appearance of blank and drug-loaded micelles is shown in Figure 9A. Both PEG-PCD and PEG-PCD micelles were prepared at 5, 10, and 15% of theoretical drug loading. (C) Encapsulation of embelin by PEG-PCD micelles as a function of polymer concentration.

However, the drug loading increased from 5 to 6 and 7% for PEG-PCD micelles, when theoretical loading increased from 5 to 10 and 15%, with decrease in loading efficiency from 100 to 60 and 50%, respectively. There was no significant difference among three PEG-PCD lipopolymers with different hydrophobic core lengths.

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3.2.3. Physical Appearance and Particle Size Distribution. 

The physical appearance of blank and drug-loaded micelles is shown in Figure 9A. Both PEG-PCD and PEG-14-PBC$_{30}$ formed a translucent micelle solution, which was stable at room temperature and showed no precipitation on storage at the room temperature for one week. In contrast, there was a milk-like suspension formation of PEG$_{114}$-PCC$_{30}$ copolymer micelles and precipitation was observed after overnight storage of these micelles. Also, the drug-loaded micelles showed a pink color
due to the solubilized embelin (EM). The color observed in PEG114-PCD29 micelles was darker than those of PEG114-PBC30 micelles prepared at the same theoretical loading (5%), and this was consistent with the high drug loading in PEG114-PCD29 micelles. We also determined the particle size and size distribution of blank and embelin-loaded micelles by dynamic light scattering (Figure 9B). The blank and embelin-loaded PEG114-PBC30 micelles had the mean particle size of 74 and 64 nm, respectively. The particle size of blank and embelin-loaded PEG114-PCD29 micelles was 129 and 110 nm, respectively. There was no increase in the mean particle size after embelin loading.

In contrast, micelles formed by PEG114-PCC30 showed a particle size of 204 nm. We also determined the morphology of embelin loaded micelles prepared from PEG114-PCD6, PEG114-PCD16, and PEG114-PCD29. As shown in Figure S2, the loading of embelin at 5% in PEG-PCD and 2% at PEG114-PBC30 had no effect on the morphology of micelles.

3.2.4. Morphology of Micelles. The morphology of micelles formed by PEG-PBC and PEG-PCD was determined by transmission electron micrography (TEM; Figure 10). PEG-PCD formed micelles with different morphologies and sizes, depending on the ratio of hydrophobic core (Figure 10A–C). PEG114-PBC30 formed spherical micelles (Figure 10D). PEG114-PCD29 formed both spherical and cylindrical micelles with a diameter less than 50 nm, and the cylindrical micelle was the dominant form. The contour length of the cylindrical micelles ranged from several hundred nanometers to several micrometers. However, PEG114-PCD6 and PEG114-PCD16 formed only spherical micelles. We also determined the morphology of embelin loaded micelles prepared from PEG114-PCD6, PEG114-PCD16, PEG114-PBC30, and PEG114-PCC30. As shown in Figure S2, the loading of embelin at 5% in PEG-PCD and 2% at PEG114-PBC30 had no effect on the morphology of micelles.

3.2.6. In Vitro Drug Release. The cumulative percentage of embelin released from polymeric micelles with different PCD core lengths at drug loading of 5%. (B) Release profiles of PEG114-PCD29 and PEG114-PBC30 micelles at drug loading of 2%. *P < 0.05, +P < 0.01, compared with PEG114-PBC30 micelles using Student’s unpaired t-test. Results were expressed as mean ± SD (n = 3).
micelles could be enhanced by cross-linking of the shell, circulation upon systemic administration. The stability of into tumor requires the micelles remain stable during blood assembly into a core

Embelin-loaded PEG114-PCD29 micelles showed significant blank PEG114-PCD29 polymer showed negligible effect on cell in C4

by MTT assay at 72 h post treatment. Results were expressed as mean ± SD (n = 4).

Figure 12. Inhibition of C4-2 cell proliferation by blank and PEG114-PCD29 micelle formulated embelin. The weight ratio of embelin and polymer was 4% (embelin/polymer). Blank micelles with same polymer concentration were served as control. Cell viability was determined relative to the complexity of cross-linking process, possible effects on the structure and properties of loaded drugs, and problems associated with drug release. Alternatively, the stability of micelles could also be improved by engineering the structure of hydrophobic block by introducing aromatic moieties.

We have successfully synthesized PEG-PCD lipopolymer with two different approaches (Figure 1). However, the first approach was selected due to the relative simplicity in the synthesis process, which is critical for its application. In this synthesis approach, PEG-PBC was synthesized by ring-opening polymerization of MBC in the presence of PEG carrying a hydroxyl end group with one of the following catalysts: DBU, Sn(oct)2, and EtZn (Table 1). Relatively lower reaction efficiency was observed with Sn(oct)2 and EtZn; this is due to the high reaction temperature and long reaction time required for these two catalysts, which is usually associated with the potential polymer degradation due to transesterification or backbiting side reaction.24,26 In addition, the use of these metallic catalysts is also associated with safety issues due to the presence of trace metal residue in the polymer. Therefore, DBU was used in the current study, which is purely organic and suitable for the synthesis of biomaterials. Besides, the reaction was carried out in solution and at room temperature, which is superior to bulk reaction that usually results in broad polydispersity due to the inefficient mixing of reactants. To optimize the reaction time, molecular weight of the polymer was monitored during the reaction. We found that the reaction was almost completed after 3 h and the DP of polymerization decreased after reaction for 24 h. This may be due to the occurring of transesterification of polymer, which becomes dominant when the monomer is consumed.26 Therefore, the reaction time was set to 3 h to achieve optimal polymerization efficiency and polydispersity.

Subsequently, the benzyl groups in the PEG-PBC were removed via hydrolysis to expose COOH function group. This reaction was also carried out under at the room temperature to avoid the potential degradation of polymer backbone, while still maintaining the high reaction efficiency to completely remove benzyl groups. The dodecanol was conjugated to the COOH groups in the polymer backbone through EDC/HOBt coupling chemistry with conjugation efficiency of around 95%. This is much higher than the previous report, where less than 70% of conjugation efficiency was achieved in conjugating fatty acids to poly(ethylene glycol)-block-poly(β-benzyl-L-aspartate) through a link molecule.27

We have successfully synthesized PEG-PCD lipopolymer synthesized in the current study has the polycarbonate backbone and dodecanol was conjugated to this backbone with an ester bond. Therefore, this is a biodegradable polymer, which is expected to degrade in vivo through hydrolysis of the ester linkages. The hydrolysis of polycarbonate backbone was facilitated at the presence of esterase such as lipase or cholesterol esterase.28,29 In addition, the final degradation product was CO2, dual alcohol, dodecanol, and PEG5000, which have less effect on pH change in vivo compared to poly(D,L-lactide) and thus are less likely to cause inflammation.30 These degradation products are nontoxic and could be eliminated from the body.

PEG-PCD lipopolymer has multiple dodecanol lipid chains attached to the polycarbonate backbone, which mimics the structure of interface cross-linked PEG-lipids (Figure 13) and could enhance the hydrophobic interaction among the hydrophobic cores. Because the cross-linking of micelles are known to improve stability, it is not surprising to observe significantly reduced CMC values for PEG-PCD lipopolymer compared to conventional PEG-lipid conjugates. Due to its low CMC value (∼10−8 M), the concentration of PEG-PCD in the bloodstream is expected to be much higher than the CMC value of PEG-
PCD after systemic administration, which indicates its good in vivo stability. Therefore, PEG-PCD lipopolymer micelles can serve as an alternative to cross-linked PEG-lipid micelles for in vivo drug delivery.

Among PEG-PCD series, we found that the CMC decreased with increasing the number of lipid in the hydrophobic core (Figure 6B). This is due to the increased length of hydrophobic block, which is known to enhance the hydrophobic interaction among the core block. The effects of core structure were also investigated and the results showed that the CMC of PEG-PCD or PEG-PBC was around 50-fold lower than corresponding PEG-PCC (Figure 6A). This is because of the presence of hydrophobic aromatic rings in PEG-PBC and lipid chains in PEG-PCD, which will all facilitate the hydrophobic interaction within the core of micelles and promote the self-assembly of polymeric micelles. However, PEG-PCC has a higher CMC due to the presence of COOH group which significantly reduced the hydrophobicity of the core block. Comparable CMC values were observed for PEG-PBC and PEG-PCD, though the structure of PBC and PCD are significantly different (Figure 6A). Because the backbone is the same for PEG-PBC and PEG-PCD, the close similarity in CMC values are due to comparable contributions by the pendant phenyl rings in the PBC and the pendant dodecanol carbon chain in PCD to the core block hydrophobicity.

Spherical, cylindrical, and vesicular assemblies are three commonly observed morphologies of micelles prepared from amphiphilic block copolymers. Recently, interest has shifted from spherical to cylindrical micelles due to their longer systemic circulation time compared to spherical micelles. The cylindrical micelles prepared from poly(ethylene glycol)-b-poly(caprolactone) (PEG-PCL) showed significantly longer circulation time than that of spherical micelles prepared from the same polymer and was persistent up to several days after intravenous injection. In addition, paclitaxel-loaded cylindrical micelles were more effective to induce apoptosis and reduce the tumor size in tumor bearing mice. Several approaches have been investigated to control the morphology of micelles, including kinetic control, the use of interfacial instability, aromatic oxidation, and the use of nanopore extruder. The most important parameter that determines the morphology and architecture of micelles is the copolymer composition and volume ratio between insoluble and soluble blocks. Sumeet et al. have studied the effect of molecular size and composition using poly(ethylene glycol)-b-poly(1,2-butadiene) (PEG-PB) as a model diblock copolymer. These authors found that PEG-PB form spheres, cylinders, and bilayers in water, sequentially, with decreased ratio of PEG block. This could explain the transition from spherical to cylindrical micelles with the increased hydrophobic length of PEG-PCD. In the current study, the use of PEG-PCD29 with a PEG ratio of 0.37 produced cylindrical micelles, while PEG-PCD6 (PEG ratio: 0.49) and PEG-PCD16 (PEG ratio: 0.72) produced only spherical micelles. Although PEG-PCD29 formed cylindrical micelles, PEG-PBC30, a polymer with similar hydrophobic core length did not form cylindrical micelles, but instead formed spherical micelles (Figure 10). This indicates that not only the PEG ratio but also the structure of hydrophobic core determines the morphology of micelles. Thus, the morphology of micelles can be controlled by changing the length and structure of the hydrophobic core of the polymer. The preparation of micelles of different morphologies will help us to understand the effect of micelles morphology and their in vivo biodistribution and bioactivities.

The compatibility of core-forming block and the drug to be loaded is critical for drug loading capacity. One objective of this study was to design an engineered polymeric micelle for improved solubilization and delivery of embelin based on the polymer–drug compatibility. PEG-PCD showed significantly high embelin loading capacity compared with PEG-PBC (Figure 7), although these two polymers have similar CMC value and comparable thermodynamic stability. The compatibility is highly dependent on the three-dimensional arrangement and conformation of different groups in the structure of drug and polymer core. Figure 14 illustrates the structure of embelin and the repeating unit of the hydrophobic core.
of PCL blocks in PEG-PCL polymer.\(^{40}\) This may not be applicable for PEG-PCD, which has a highly branched structure in contrast to the linear structure of PEG-PCL. In fact, a good drug loading was observed in PEG-lipid conjugates with a much shorter hydrophobic core length,\(^{3}\) indicating that the further increase in hydrophobic length might not be able to improve the drug loading of lipopolymers with similar structure as PEG-PCD. Similarly, we also observed there was no significant difference in the in vitro drug release profiles among these PEG-PCD polymers with different block lengths (Figure 11A). The drug release profiles of polymeric micelles are influenced by several factors including strength of the interaction between drug and hydrophobic core, physical state of the core, the amount of drug loaded, the length of the hydrophobic core, and the location of the drug inside the micelle core.\(^{31}\) In addition, the highly branched structure in the hydrophobic core of PEG-PCD may have contributed to the observation in this study. We also found that the release of embelin from PEG-PCD was much slower than that from PEG-PBC (Figure 11B). This might due to the fact that the interaction between PEG-PCD and embelin was stronger than those of PEG-PBC and embelin.

5. Conclusion

We have successfully synthesized a novel lipopolymer poly(ethylene glycol)-block-poly(2-methyl-2-carboxypropylene carbonate-graft-dodecanol) (PEG-PCD) for delivery of a hydrophobic anticancer drug, embelin. PEG-PCD lipopolymer could form micelles in water through self-assembly and significantly improved embelin solubility by loading drugs inside its hydrophobic core. The drug loading and encapsulation efficiency were dependent on the length and structure of the polymer hydrophobic core block. The CMC of PEG-PCD micelles was around \(10^{-8}\) M and decreased with increasing the length of hydrophobic block, indicating good thermodynamic stability of PEG-PCD micelles. The particle size and morphology of micelles were also dependent on the structure and length of the hydrophobic core. Embelin-loaded PEG-PCD micelles showed significant inhibition of C4-2 prostate cancer cell proliferation in a dose-dependent manner, while no obvious cellular toxicity was observed with blank micelles themselves. Thus, these lipopolymer have the potential to be used as vehicles for anticancer hydrophobic drug delivery.

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Supporting Information Available. (1) \(^1\)H NMR of 2-methyl-2-carboxypropylene carbonate (MCC) in DMSO and 2-methyl-2-dodecanoxy carbonate-propylene carbonate (MDC) in CDCl\(_3\), (2) Transmission electron microscopy (TEM) of embelin-loaded micelles (A) PEG114-PCL\(_6\), (B) PEG114-PCL\(_{16}\), (C) PEG14-PCL\(_{28}\), and (D) PEG14-PBC\(_{30}\). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes


