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Construction of reverse vesicles from pseudo-graft poly(glycerol methacrylate)s via cyclodextrin-cholesterol interaction†

Wen-Xing Gu,a Ying-Wei Yang,b Jijie Wen,a Hongguang Lu,a Hui Gao*a

A new type of reverse vesicles was successfully constructed from pseudo-graft amphiphilic copolymers in dichloromethane, by dint of the host-guest inclusion complexation between β-cyclodextrins and cholesterol. Pseudo-graft copolymers were constructed from β-cyclodextrin conjugated linear or star-shaped poly(glycerol methacrylate)s (PGMAs) with different molecular weight and a cholesterol-ended linear polylactide. The Z-average diameter of reverse vesicles was in the range of 150-350 nm with an ideal narrow polydispersity, and could be adjusted by molecular weights and branched type of backbone PGMAs. Interestingly, these reverse vesicles could transformed into organogels under specified conditions, i.e. the concentration of reverse vesicles was >1.5 g/L, and DCM-H2O ratio (v/v) was 8:1. Extraction of Congo red from aqueous phase to organic phase showed good cargo encapsulation capability of reverse vesicles, demonstrating great potentials as carriers or nanoreactors.

Introduction

Reverse vesicles, as the counterparts of normal vesicles formed in aqueous solution, are spherical containers consisting of a hydrophobic core surrounded by a reverse bilayer, whose hydrophobic portions are exposed to nonpolar solvents both in the core and in the exterior.1 In 1991, Kunieda et al.2 firstly reported on reverse vesicle formed in a nonionic surfactant system in dodecane. After nearly a decade in development, research fell into “an ice age” until more available techniques for the characterization of reverse vesicles appeared. Then, several novel reverse vesicular systems were discovered by some pioneers in the field.3-6 However, compared with the extensive knowledge on normal vesicles, from formation mechanisms7 to applications, in particular theranostic,9 catalytic reactor,9 drug delivery10 and water remediation,11 research on reverse vesicles is still in its infancy.

Supramolecular self-assembly, emerged as a powerful and versatile tool, has been applied to the fabrication of novel types of vesicles.12-14 Being one of the most widely used supramolecular hosts, cyclodextrins (CDs) are donut-shaped cyclic oligosaccharides, and the relatively hydrophobic interior cavity endows CD the ability to complex with a wide range of lipophilic guest molecules possessing suitable polarity and dimension characteristics,15 e.g., adamantane,16 cholesterol,17 cinchona alkaloids,18 organic dyes,19 ferrocene,20 azobenzene21-23 and fullerenes.24 To date, numerous kinds of vesicles have been prepared not only from conventional block copolymers,25-27 but also based on these host-guest interactions.28-32 However, to our best knowledge, studies on the construction of reverse vesicles with supramolecular copolymers remain largely unexplored, and no examples on self-assembly behavior of pseudo-graft amphiphilic copolymer-based reverse vesicles via β-CD-cholesterol inclusion complexation has been reported.

Herein, we firstly developed a facile synthesis of pseudo-graft amphiphilic copolymer reverse vesicles based on poly(glycerol methacrylate) (PGMA)33 via β-CD-cholesterol host-guest interactions. Star-shaped or linear β-CD conjugated PGMAs (PGMA-CDs) were synthesized as the hydrophilic backbone,34 while cholesterol (Chol)-ended linear polylactide (PLA-Chol) was employed as hydrophobic side chain of...
pseudo-graft amphiphilic copolymers. All the building blocks, i.e., β-CD, poly(l-lactide), cholesterol, and PGMA have been proved to be biocompatible. Through a “dissolve-dialysis” method, the pseudo-graft amphiphilic copolymers self-assembled into reverse vesicles, and then transformed into gel after equilibration with water. In term of extensive applications of normal vesicles, we firmly believe that these unique reverse vesicles hold great potentials in bio-related fields.

**Experimental Section**

**Materials and methods**

Glycidyl methacrylate (GMA), 2-bromoisoobutyryl bromide, bipyridyl and CuBr were purchased from Adamas Reagent Co., Ltd. (Shanghai, China). D,L-lactide was purchased from Daigang Co., Ltd. (Shandong, China). Cholesterol was purchased from Best Co., Ltd. (Chengdu, China). Tin (II) bis(2-ethylhexanoate) (Sn(Oct)$_2$) was purchased from Alfa Co., Ltd. (Stoughton, MA). 8-anilino-1-naphthalene sulfonate (ANS) was purchased from Heowns Co., Ltd. (Tianjin, China). All the other reagents were obtained from Tianjin Chemical Reagent Co., Ltd. (Tianjin, China). Prior to use, tetrahydrofuran (THF) and ethanediamine (EDA) were dried over sodium, and distilled with benzophenone serving as a dryness indicator.

Proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a 400 MHz NMR spectrometer (Bruker AV-400). Samples were dissolved in deuterium oxide (D$_2$O), dimethylsulfoxide-d$_6$ (DMSO-d$_6$), or deuterated chloroform (CDCl$_3$). Gel permeation chromatography (GPC) measurements were performed at 40 °C with THF as mobile phase at a flow rate of 0.5 mL/min. Thermal analysis were studied using differential scanning calorimetry (DSC) on the NETZSCH F3 209 instrument. Nitrogen was used as the purging gas. Specimens (4 mg) were encapsulated in aluminum pans and heated to 160 °C at a heating rate of 10 °C/min; cooled to -20 °C at a cooling rate of 10 °C/min and kept at -20 °C for 3 min; the samples were heated again at a heating rate of 10 °C/min to 160 °C. The DSC thermograms of samples were recorded during the second heating run process. The mean diameters of the assemblies were measured using dynamic light scattering (DLS) at 25 °C on a Malvern Zetasizer Nano ZS90 instrument. The morphology of reverse vesicles was observed using transmission electron microscopy (TEM) on a JEOL JEM-200 CX instrument. The samples were prepared by dropping solutions (0.2 mg/mL) onto carbon-coated copper grids, stained with phosphotungstic acid (2 wt%), and dried at room temperature for 24 h. The morphology of the freeze-dried floccule of organogels was observed using a scanning electron microscope (SEM) collected on a JEOL JSM 6700F instrument. The fluorescence was evaluated on a Hitachi FLT4500 instrument. The excitation wavelength was 370 nm, while the emission wavelength was recorded from 390 to 700 nm. Gelatinization was observed in tubes, and gel settled at the bottom of the tube when the sample tubes were inverted.

**Synthesis of 5-arm initiator, linear and 5-arm PGMA**

With five functional hydroxyl groups, α-α-glucose was used as the precursor of 5-arm initiator: 1,2,3,4,6-penta-O-isobutyryl bromide-α-α-glucose (yield: 48%). $^1$H NMR (CDCl$_3$, with 0.05% v/v TMS, 400 MHz; Figure S1, see ESI): δ$_H$ 1.90-1.99 (m, 30H), 4.04-4.11 (m, 1H), 4.43-4.44 (d, 2H), 5.28-5.40 (m, 2H), 5.43-5.70 (t, 1H), 5.85-5.87 (d, 1H). Linear PGMA (LPGMA) and 5-arm PGMA (SPGMA) were synthesized by atom transfer radical polymerization (ATRP) according to our...
Scheme 2 Synthesis of LPGMA and SPGMA from linear and 5-arm initiator respectively.

Scheme 3 Synthesis of LPGMA-CD

Synthesis of PGMA-CD (Hydrophilic part) and PLA-Chol (Hydrophobic part)

PGMA-CD was prepared from PGMA and mono(6-(ethanediamine)-6-deoxy)-β-CD (CD-EDA) (Scheme S1 and Figure S3, see ESI†) by the ring-opening reaction (Scheme 3). Typically, LPGMA (0.1 g) and CD-EDA (1.8 g) were dissolved in DMF (30 mL). The mixture was heated at 70 °C for 24 h. Then, EDA (10 mL) was added to the flask and reacted for another 12 h. The crude mixture solution was purified by dialysis (cut-off molecular weight 12-14 kDa) against deionized water for 48 h at room temperature, then freeze-dried to obtain purified LPGMA-CD. SPGMA-CD was synthesized using the same procedure. To obtain the hydrophobic part, PLA-Chol was synthesized through the ring-opening reaction of ε-lactide using cholesterol as an initiator as described in the literature (Scheme S2, see ESI†).

Results and Discussion

Extraction of Congo red with reverse vesicles

Extraction of Congo red (CR) were experimented in the presence of reverse vesicles constructed with different CD/cholesterol molar ratio (3:1 and 1:1). Reverse vesicles (2 mg/mL, 2 mL) were mixed with the CR aqueous solution (gradient concentration, 2 mL) by slight shaking for 2~3 minutes, and then allowed to stand for 2 days.

LPGMA with number-averaged molecular weight (Mn) of 10 kDa (Mw/Mn = 1.32) or 20 kDa (Mw/Mn = 1.45), and SPGMA with Mn of 10 kDa (Mw/Mn = 1.15) or 19 kDa (Mw/Mn = 1.42) were obtained. Their molecular weights were determined by GPC, and their chemical structures were verified by $^1$H NMR (Figure S2, see ESI†). PGMA-CDs were prepared from PGMA and CD-EDA by the ring-opening reaction (yield: 90-95%). The typical $^1$H NMR spectra (Figure 1) confirmed the successful synthesis of LPGMA10K-CD. The peaks at δ$_h$ 5.02, 3.90 and 3.81 ppm are assigned to the protons of CD and the peaks at δ$_i$ 3.59, 2.77, 1.98 and 0.82 ppm are attributed to the protons of the PGMA backbone. PLA-Chol (Mn = 7 kDa) was synthesized through the ring-opening reaction of ε-lactide (yield: 70-80%). Reverse vesicles were prepared through a “dissolve-dialysis” method at different CD/cholesterol molar ratio. For example, to prepare reverse vesicles at 3:1 molar ratio of the CD/cholesterol units, PLA-Chol (9 mg, 1.3 mmol of cholesterol units) in DMSO (3 mL) was added dropwise into DMSO (3 mL) containing LPGMA-CD (9 mg, 3.9 mmol of CD units). The mixture was stirred for 12 h, followed by 48 h dialysis against dichloromethane (DCM) to remove DMSO using a dialysis membrane (cut-off molecular weight 12-14 kDa). A stable dispersion was obtained, and no precipitation was observed after storing for four weeks.
method, and denoted as LPC10K-g-LC, LPC20K-g-LC, SPC10K-g-LC, and SPC19K-g-LC, where S and L represent star and linear respectively, PC is for PGMA-CD, LC is for PLA-Chol, 10K/19K/10K/20K represent the Mn of PGMA. To illustrate the necessity of CD/cholesterol interactions on the formation of reverse vesicles, PLA was used to complex LPGMA10KTCD using the same protocol as above. However, flocculent precipitation was observed during dialysis (Figure S5, see ESI†), and the quality report from DLS was poor, suggesting that reverse vesicles cannot be obtained in the absence of guest polymer PLA-Chol. Therefore, the host-guest inclusion interaction was the key factors of reverse vesicles formation. We thus proposed the possible process to form vesicles (Scheme 1). Reverse vesicles composed of hydrophobic component (PLA) as outer and inner membrane, and hydrophilic component (CDs) as central layer.

The morphology and size of inclusion complexes were studied by SEM, TEM and DLS. As shown in images (Figure 2a and 2b; Figure S7, see ESI†), the thickness of reverse vesicles was ca. 22-30 nm with an average diameter of ca. 150 nm. The DLS results exhibited that the copolymers were able to form stable particles from LPC10K-g-LC at the molar ratio of CD/cholesterol ranged from 1 to 10 (Table S1, Figure S8, see ESI†). Typically, the Z-average diameter of all reverse vesicles, with 3:1 molar ratio of the CD/cholesterol units, was in the range of 150-350 nm with an ideal narrow polydispersity (PDI) (Table 1). Reverse vesicles prepared from star-shaped PGMA were slightly larger than that from linear ones. When the Mn of backbone PGMA increased, the diameter of reverse vesicles prepared from SPGMA became larger, while that from LPGMA remained constant, indicating Mn of the PGMA influenced more on the size of highly branched structure than that of linear one. Consequently, the diameters of reverse vesicles could be fine tuned by changing the branching and the molecular weight of backbone to meet different kinds of application requirements.

The inclusion formation of CD-cholesterol was explained by means of 2D-NMR, DSC and fluorescent experiments. The typical 2D $^1$H-$^1$H NOESY spectrum (Figure S6, see ESI†) of LPC10K-g-LC showed that the signals of cholesterol protons were correlated with the signals of inner protons (peak ‘c’ and ‘e’) of β-CD. This result demonstrated the successful assembly between CD and cholesterol moieties. DSC thermograms were recorded from 30-80 °C (Figure 3). The glass transition temperature ($T_g$) of LPC10K-g-LC reverse vesicles is 45.4 °C, while that of physical mixture of LPGMA10KTCD/PLA-Chol is

![Figure 2](image_url)  
Figure 2. (a) (b) TEM images of LPC10K-g-LC reverse vesicles stained with phosphotungstic acid (2 wt%) in DCM, (c) SEM image of freeze-dried floccule of organogels transformed from LPC10K-g-LC reverse vesicles, (d) photograph of organogels transformed from LPC10K-g-LC reverse vesicles in an inverted tube. (Note: Reverse vesicles was prepared with molar ratio of CD/cholesterol units 3:1, if no special instructions).

<table>
<thead>
<tr>
<th>Samples</th>
<th>CD grafted ratio (%)</th>
<th>Z-average diameter (nm)</th>
<th>PDI</th>
</tr>
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<tbody>
<tr>
<td>SPC10K-g-LC</td>
<td>14.9</td>
<td>251</td>
<td>0.087</td>
</tr>
<tr>
<td>SPC19K-g-LC</td>
<td>13.0</td>
<td>341</td>
<td>0.123</td>
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<tr>
<td>LPC10K-g-LC</td>
<td>14.3</td>
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<td>0.078</td>
</tr>
<tr>
<td>LPC20K-g-LC</td>
<td>13.1</td>
<td>157</td>
<td>0.085</td>
</tr>
</tbody>
</table>

Note: a Calculated from integral area of peak ‘b’ and peak ‘h’ in $^1$H NMR spectrum (Figure 1), b Determined by DLS.

![Figure 3](image_url)  
Figure 3 DSC thermograms of (a) LPC10K-g-LC reverse vesicles, (b) the physical mixture of LPGMA10KTCD/PLA-Chol, (c) PLA-Chol, and (d) LPGMA10K-CD.
Figure 4 Fluorescence spectra of ANS in the presence of (a) LPGMA10K-CD (green) and LPC10K-g-LC reverse vesicles with concentration of 1000 µg/mL (black), the inserted image is magnified curve (red) for ANS in water, (b) LPC10K-g-LC reverse vesicles with increased concentration. The concentration of ANS is 9 µg/mL. The excitation wavelength is 370 nm.

Figure 5 $^1$H NMR spectra of (a) LPC10K-g-LC in CDCl$_3$ and (b) CDCl$_3$ containing trace amounts of D$_2$O.

Figure 6 Encapsulation of CR in the presence of reverse vesicles (a) LPC20K-g-LC(3:1), (b) SPC10K-g-LC(3:1), (c) LPC10K-g-LC(3:1), (d) SPC19K-g-LC(3:1), and (e) LPC10K-g-LC(1:1); photograph of LPC10K-g-LC reverse vesicles in DCM (A) before and (B) after extraction of CR.

44.0 °C. The higher $T_g$ of LPC10K-g-LC reverse vesicles suggested that the possible existence of interactions between CDs and cholesterol assisted the formation of vesicles. As shown in Figure 4a, the fluorescent chromophore ANS showed remarkably low intensity in blank water (red), and the emission maximum was 507 nm. In the presence of LPGMA10K-CD (green), the fluorescence intensity increased and the peak shifted from 507 nm to 475 nm. ANS molecules could move into CD cavities to form the inclusion complex. Similar conclusions were reported by Jiang et al.$^{43}$ Compared to host polymer LPGMA10K-CD, corresponding reverse vesicles with the same number of CDs molecules had lower fluorescence intensity. It is reasonable that a part of CDs was occupied by cholesterol, resulting in a lower ability to accommodate ANS. As shown in Figure 4b, the intensity of the ANS emission increased with the concentration of vesicles. These reverse vesicles could transform into organogels (Figure 2c and 2d; Figure S9, see ESI†) upon addition of water under specific conditions, i.e. the concentration of reverse vesicles was > 1.5 g/L, and DCM-H$_2$O ratio (v/v) was 8:1. In other conditions, gelatinization was incomplete, or could not be formed. The formation mechanism of organogels was evidenced by $^1$H NMR. In CDCl$_3$, CDs in hydrophilic PGMA-CD existed in central layer, shielded by the outer PLA membrane, thus the signals ascribed to the protons of CD cannot be observed (Figure 5a). When trace amounts of D$_2$O (1/8 volume to CDCl$_3$) was added, the signals at $\delta_H$ 4.8 and 3.6 ppm appeared (Figure 5b), probably due to the out-migration of CDs. PLA-Chol chains rearranged in ways of accessing into blank CDs cavities near the surface of reverse vesicles, thus formed physical cross-linking.

To reveal the cargo encapsulation and release capability of reverse vesicles, extraction of a model molecule, CR, from aqueous phase to organic phase was demonstrated. The hydrophilic CR could not be dissolved in organic solvents such as chloroform and DCM, but can be transferred into DCM in the presence of reverse vesicles. Figure 6A and 6B show the photographs of CR extraction from the aqueous layer (upper) to...
DCM (bottom), where a majority of CR was extracted into DCM. As shown in Figure S10 (see ESIF), the morphological image of emulsion (bottom) observed by inverted microscopy was spherical with an average diameter of 20 µm. At low dye concentrations (< 0.5 g/L), all reverse vesicles were able to quantitatively extract CR from the aqueous layer. The amount of CR extracted from aqueous phase increased with the CR concentration (Figure 6). Interestingly, reverse vesicles with the 1:1 molar ratio of the CD/cholesterol units yielded significantly less loading efficiencies. The possible explanation for such a phenomenon is that the free CDs (not occupied by cholesterol) are favorable for dye extracting because of the host-guest interactions between CD and CR. Interestingly, no CR could be released back into the aqueous phase, probably owing to the physical cross-linking of the organogels.

Conclusions

In summary, a new kind of reverse vesicles based on pseudo-graft amphiphilic copolymers from PGMA-CD and PLA-Chol via interaction between β-CD and cholesterol was obtained upon dialysis against DCM. The morphology and size of reverse vesicles were studied by TEM and DLS. The possible complexation formation of CD-cholesterol was evidenced by 2D-NMR, DSC and fluorescent experiments. These reverse vesicles could be transformed into organogels when adding trace amounts of H2O. A possible gelation mechanism was proposed based on 1H NMR experiments. CR extraction experiment demonstrated the excellent loading capacities of reverse vesicles, showing great potential as carriers or nanoreactors.

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Notes and references


