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Preparation and self-assembly of amphiphilic triblock copolymers with polyrotaxane as a middle block and their application as carrier for the controlled release of Amphotericin B

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ABSTRACT

A kind of novel amphiphilic triblock copolymers containing polyrotaxane (PR) as a central block was synthesized *via* the ATRP of poly(ethylene glycol) methyl ether methacrylate (PEGMA) initiated with polypseudorotaxanes made from a distal 2-bromopropionyl end-capped Pluronic F127 with a varying amount of β-CDs in the presence of Cu(I)Cl/*N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA) at 25 °C in aqueous medium. The structure of the resulting copolymers was characterized in detail by ^{1}H NMR, 2D ROESY NMR, GPC, DSC and WAXD analyses. The degree of polymerization of PEGMA oligomers appeared to be adjustable. As a typical sample, F-30β-CD-60 with a molar feeding ratio of Pluronic F127 to β-CD to PEGMA holding 1:30:60 was found to self-assemble into nano-sized aggregates in water. Its critical aggregation concentration was assessed by fluorescence probe technique. The corresponding hydrodynamic radius and radius of gyration were also determined by dynamic and static light scattering measurements. The transmission electron microscopy images further revealed that the sizes of the polymeric micelles of the selected copolymer were in nano-scale and smaller than those of the blank brush-like block copolymer. These nano-sized particles showed great potential to be used as carrier for the controlled release of Amphotericin B (AmB) holding 8.7% drug-loading content and 87% drug-loading efficiency with the cumulative release profile substantially longer than that of the blank brush-like block copolymer.

1. Introduction

Cyclodextrins (CDs), a family of cup-shaped hollow oligosaccharides including α-, β- and γ-CDs, can include geometrical compatible polymer chains to construct polyrotaxanes (PRs) holding a necklace-like molecular structure with the intriguing characteristics of free sliding and/or rotating of the threaded cyclodextrins [1–3]. Due to their supramolecular architecture, PRs show the promise to be used as smart biomedical materials [4,5], such as thermo-/pH-sensitive hydrogels [6,7], vesicles and micelles as carriers for the controlled drug release [8–10], supramolecular gene carrier [11], insulated molecular lines [12], etc. As for the PR preparation, a vast variety of strategies have been developed so far [13,14]. However, there were a few studies devoted to polymeric oligomers as end stoppers for PRs *via* the atom transfer radical polymerization (ATRP) and especially to their self-assembly behavior in aqueous medium [15–17].

Poly(ethylene glycol) methyl ether methacrylate (PEGMA) is a typical macromer for biomedical applications due to the non-adhesive nature to proteins and cells as well as biocompatible property of its polymers [18,19]. Usually it is used to synthesize amphiphilic brush-like block copolymers which are able to self-assemble into micelles or hydrogels in aqueous medium showing tremendous interest not only for their basic research significance, but also for their applications in the biomedical fields [20,21]. As is well known, the self-assembly is the key to the design and fabrication of smart nanometer-scale particles and devices for biomedical purposes [22]. Hence exploring the preparation and self-assembly behavior of PR containing block copolymers are indispensable for their application as carriers for the controlled drug release. To this end a kind of amphiphilic triblock copolymers comprising PR as a central block was synthesized *via* the ATRP of PEGMA using 2-bromopropionyl end-capped Pluronic F127-β-CD polypseudorotaxane (PPR) as macroinitiator at 25 °C in aqueous medium. Rather than the traditional rigid PRs yielded as crystalline precipitates from aqueous solution or physically crosslinked hydrogels

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in situ formed from the self-aggregation of PRs [23,24], these triblock copolymer with a PR central block flanked by two hydrophilic brush-like PEGMA oligomers would self-assemble into nano-sized particles with the unique core–shell structure in aqueous medium, in which the PEGMA oligomers create a dense non-adhesive hydrophilic coating around the PR central block capable of suppressing protein and cell adhesion and extending residence time of the particles as carrier for the controlled drug release in biological milieu.

Although supramolecular hydrogels or self-aggregates constructed from CDs involving inclusion complexes (ICs) have been intensively investigated not only for their theoretical merits but also for their potential biomedical applications [5,9,25–28], the self-assembly of amphiphilic Pluronic F127-β-CD-PR-based triblock copolymers has not been used as carrier for the controlled drug release. Amphotericin B (AmB) is a broad spectrum chemotherapy, e.g. in cancer therapy and organ antifungal therapy for the treatment of systemic transplantation, but an aliphatic water-insoluble drug [29]. In this study, AmB was selected as a model drug to evaluate the potential of the resulting amphiphilic PR containing triblock copolymers as carrier for its controlled release. Surprisingly, the nano-sized micelles self-assembled from a selected PR-derived copolymer sample were found to effectively solubilize AmB with high drug-loading content and drug-loading efficiency as well as long sustainable release profile without initial burst. In fact administration of AmB encapsulated by long-circulating liposomes has been considered to be a promising approach to increase therapeutic index of AmB at low dose, low risk of toxicity, and low cost in comparison with AmB lipid complex (Abelcet) and conventional liposomal AmB (AmBisome) [29–31]. Evidently, the entrapment into the self-aggregates of amphiphilic PR-derived triblock copolymers can protect AmB molecules from hydrolysis, aggregation and precipitation by minimizing contact with the bulk aqueous phase. As for the higher stability of polymeric micelles, the AmB formulation based on the PR-derived triblock copolymers holds distinct advantages over those lipid-based AmB formulations.

2. Experimental section

2.1. Materials

β-CD was supplied by Sinopharm Chemical Reagent Company, China, and recrystallized three times before use. Pluronic F127 comprising a central block of 65 PPO units and two PEO blocks of 100 units ($M_n = 12\,600$), poly(ethylene glycol) methyl ether methacrylate (PEGMA) ($M_n = 1100$ g/mol), *N*-phenyl-1-naphthylamine (PNA) and *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma, USA. PEGMA was passed over a short basic alumina column to remove the inhibitor before polymerization. Both 2-bromopropionyl bromide and 4-dimethylaminopyridine (DMAP) were available from Alfa Aesar, USA. Amphotericin B (AmB) was Amerasco's product. Triethylamine (TEA) and sodium dodecyl sulfate (SDS) were supplied by VAS Chemical Reagents Company, Tianjin, China. TEA was refluxed with *p*-toluenesulfonylchloride and distilled. The resultant free TEA was stored over CaH_2. Copper(I) chloride (Cu(I)Cl) was prepared from $CuCl_2$, purified by stirring in acetic acid, washed with methanol and finally dried under vacuum prior to use. *N*,*N*-Dimethylformamide (DMF) was stirred with CaH_2 and distilled under high vacuum. All other solvents and reagents were of analytical grade.

2.2. Synthesis of 2-bromopropionyl terminated Pluronic F127 (BrP-F127-PBr)

Pluronic F127 was converted to the corresponding ATRP macroinitiator by the end-capping reaction with a four-fold molar excess of 2-bromopropionyl bromide in CH_2Cl_2. In brief, in a 100 ml three-neck round-bottom flask, Pluronic F127 (12.6 g, 1 mmol) was dissolved in distilled CH_2Cl_2 (20 ml). Then DMAP (122 mg, 1 mmol), TEA (0.42 ml, 3 mmol) and 10 ml dry CH_2Cl_2 containing 2-bromoisobutyryl bromide (0.92 g, 4 mmol) were added dropwise under nitrogen. The reaction continued for 2 h at 0 °C and for another 24 h at room temperature under stirring. Finally the mixture was filtered to remove the precipitated salt. The product was purified by precipitation into 500 ml anhydrous ether at 5 °C. The sequence was repeated three times. ^{1}H NMR analysis was used to determine the degree of esterification (higher than 95%). ^{1}H NMR (DMSO-d_6): δ 4.23–4.24 (s, 4H, –CH_2–O–C(=O)–), 1.02–1.04 (d, 210H, –O–CCH_3–C–O–), 2.08 (d, 3H, CH_3–C–Br), 1.71–1.73 (d, 1H, –CH–Br), 3.40–3.50 (m, CH_2CH_2O of PEG and CH_2CHO of PPG) ppm.

2.3. Preparation of PPEGMA-b-F127-b-PPEGMA

A typical protocol for the ATRP of PEGMA by using BrP-F127-PBr as macroinitiator and Cu(I)Cl/PMDETA as catalyst in aqueous medium was as follows: In a sealable Pyrex reactor, BrP-F127-PBr (0.13 g, 0.01 mmol) was dissolved in 2 ml distilled water to which PEGMA (0.66 g, 0.6 mmol) dissolved in 2 ml water was added before PMDETA (4.20 mg, 0.024 mmol) was added. The system was quenched in the liquid nitrogen to which Cu(I)Cl (2.0 mg, 0.02 mmol) was added. The reactants in the reactor were degassed three times by purging with nitrogen. The reactor was sealed under vacuum and then the reaction started and maintained for 6 h at 25 °C under stirring. The polymerization stopped after breaking the Pyrex reactor, the product was dialyzed using a cellulose membrane (MWCO 3500) for 48 h changing water every 12 h, and the whole content was freeze-dried. The crude product was dissolved in DMF and fractionally precipitated with anhydrous ether. The purified product was finally dried under vacuum, yield 48.7%. ^{1}H NMR (DMSO-d_6): δ 1.02–1.04 (d, 210H, –$OCCH_3$–CO–), 0.78–0.94 (s, 3*77.8, methyl of PPEGMA attached to the main chain), 3.24 (s, 3*77.8, methyl at the end of PPEGMA), 3.40–3.50 (m, CH_2CH_2O of PEG and CH_2CHO of PPG) ppm.

2.4. Synthesis of amphiphilic PR-based triblock copolymer via ATRP of PEGMA

A typical protocol for amphiphilic PR-based triblock copolymer synthesis *via* the ATRP of PEGMA was as follows. In a sealable Pyrex reactor, an aqueous solution containing a predetermined amount of β-CD was added to 1 ml aqueous solution of BrP-F127-PBr (0.13 g, 0.01 mmol), followed by vigorous stirring at room temperature for 24 h to form PPR. PEGMA (0.66 g, 0.6 mmol) and PMDETA (4.20 mg, 0.024 mmol) were then added to the resulting suspension of PPR. After quenched in the liquid nitrogen, Cu(I)Cl (2.0 mg, 0.02 mmol) was added, followed by three times of degassing using a nitrogen purge. The reactor was sealed under vacuum and the reaction started and maintained for 6.0 h at 25 °C. The polymerization stopped after breaking the Pyrex reactor. The crude product was directly freeze-dried before dissolved in 15 ml DMF, and then the solution was dialyzed using a dialysis bag (MWCO 3500) for 48 h with water changing every 12 h, in which the whole dialyzing bag was put into a 60 °C water bath for 16 h. All the content was freeze-dried. The crude product was again dissolved in DMF and fractionally precipitated with anhydrous ether. The purified product was dried under vacuum, yield 41.6%.

For the convenience of expression, the obtained polyrotaxane containing triblock copolymers were designated as F-*n*β-CD-*m*, where F means Pluronic F127, *n* stands for the feed molar ratio of β-CDs to Pluronic F127 and *m* represents the feed molar ratio of PEGMA to Pluronic F127, respectively.

2.5. Preparation of the polymeric micelles

The polymeric micelles were prepared by directly dissolving the polymer in water and allowing it to stay overnight to ensure complete dissolution. The resulting solution was then diluted to the desired concentration with proper amount of water. For LS measurements, the solution was filtered through a 0.45 μm Whatman PVDF filter into a dust-free vial.

2.6. Preparation of AmB-loaded polymeric micelles

AmB was loaded into the self-assembled polymeric micelles (PMs) by the dialysis method. In a typical procedure, the selected PR containing copolymer sample (25 mg) was dissolved in 1.0 ml DMF, and subsequently to this solution was added 2.5 mg AmB. The solution was stirred at room temperature for 1.5 h and then dialyzed against 1 L water for 24 h using a dialysis bag (MWCO 3500). The resulting solution was filtered through a 0.45 μm Whatman membrane to eliminate unincubated AmB. The loading amount of AmB was determined by UV–vis analysis after dissolving freeze-dried AmB-loaded polymeric micelles in DMSO. The drug-loading content (DLC %) and efficiency (DLE %) were calculated by the following equations, respectively.

$$\mathrm{DLC}\,\% = \frac{\text{amount of AmB in PMs}}{\text{amount of AmB loaded PMs}} \times 100\% \quad (1)$$

$$\mathrm{DLE}\,\% = \frac{\text{amount of AmB in PMs}}{\text{amount of AmB used for PM preparation}} \times 100\% \quad (2)$$

2.7. In vitro release of AmB from polymeric micelles

A release study was performed at 37 °C in a Guo Hua THZ82 incubator shaker. A dialysis bag (MWCO 3500) containing 3 ml AmB-loaded nanoparticle solution was placed into a flask containing 30 ml distilled water. The flask was shaken at 100 rpm at 37 ± 0.5 °C. At selected time intervals, 3 ml distilled water outside the dialysis bag was removed for UV–vis analysis and replaced with the same amount of deionized water. The AmB concentration was determined based on the absorbance intensity at 409 nm according to the standard line obtained from a series of solutions with different AmB concentrations.

2.8. Measurements

^{1}H NMR (400 MHz) spectra were recorded on a Bruker ARX-400 spectrometer at room temperature using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. The 2D ROESY experiment was recorded on a Bruker AV 600 MHz proton NMR spectrometer with D_2O as solvent.

Gel permeation chromatographic (GPC) measurements were carried out at 30 °C on a Waters 2410 instrument which was equipped with a Waters 2410 refractive index detector, a Waters 515 HPLC pump, and three Waters Styragel columns (HT2, HT3, and HT4). DMF + 1 wt% SDS was used as the eluent at a flow rate of 1.0 ml/min. SDS was added to suppress the association of PR-derived and blank copolymers. All the GPC data were calibrated with polystyrene (PS) standards.

Differential scanning calorimetry (DSC) measurements were run on a NETZSCH DSC 204 differential scanning calorimeter with a scanning temperature range from −20 to 120 °C at a scanning rate of 10 °C/min. A typical DSC sample size was about 4 mg. The samples were encapsulated in hermetically sealed aluminum pans, and the pan weights were kept constant. The following protocol was used for each sample: heating from room temperature to 120 °C at 10 °C/min, holding at 120 °C for 5 min, then cooling from 120 °C to −20 °C at 10 °C/min, and finally reheating from −30 °C to 120 °C at 10 °C/min. Data were collected during the second heating run. Transition temperatures were taken as peak maxima.

Fluorescence spectra of PNA in aqueous solution were measured at 25 °C as a function of polymer concentrations using the Varian Cary Eclipse fluorescence spectrophotometer. Emission spectra were measured in the range of 350–600 nm. Most of the spectra were collected with the following parameters: the excitation wavelength was 340 nm for emission spectra; excitation slit width was 5 nm; the optical path was 1 cm; scan speed, 120 nm/min. UV spectra were performed on a Hitachi U-2800 spectrophotometer.

Light scattering measurements were performed using a Brookhaven Instrument equipped with BI-200SM goniometer and a BI-TurboCorr digital correlation. A solid-state laser polarized at the vertical direction (CNI Changchun GXL-III, 532 nm, 100 mW) operating at 532 nm was used as light source. The light scatting measurements were carried out at 25 °C. The aqueous solution of the sample was prepared at a concentration of 0.3 mg/ml and purified by passing through a hydrophilic filter (Millipore, 0.45 μm) to remove dust. The hydrodynamic radius (R_h) was evaluated according to the following equation.

$$R_h = \frac{k_B T}{6\pi\eta_s D} \quad (3)$$

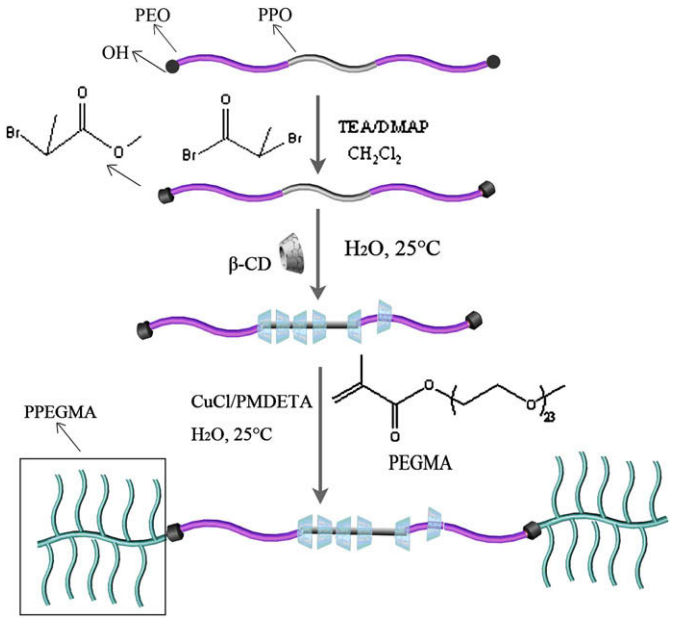
where k_B, T and η_s are the Boltzmann constant, the absolute temperature, and the viscosity of the solvent, respectively. And the diffusion coefficient (D) of the polymeric self-assemblies was calculated from $\Gamma = Dq^2$, where Γ is the relaxation rate ($\Gamma = 1/\tau$, τ is the relaxation time) and q is the scattering vector determined by the scattering angle θ ($q = 4\pi n/\lambda \sin\theta/2$, where n is the refractive index of the liquid medium and λ is the wavelength of the laser). The SLS measurements were carried out at different scattering angles (20–120°) to determine the radius of gyration (R_g) of the self-aggregates. By measuring the optical constant (K) and the excess Rayleigh ratio (ΔR_θ) in an infinite dilution, R_g can be determined by the extrapolation of $Kc/\Delta R_\theta$ to zero angle and zero concentration according to the following relation.

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_w}\left[1 + \frac{16\pi n^2}{3\lambda^2} R_g^2 \sin^2\left(\frac{\theta}{2}\right)\right] + 2A_2 c \quad (4)$$

where K is the optical constant, which depends on the refractive index increment (dn/dc) of the polymer solution $K = 4\pi^2 n^2 (dn/dc)^2/N_A\lambda^4$, where N_A is Avogadro's number, n is the refractive index of the liquid medium, and λ is the wavelength of the laser, and ΔR_θ is the excess Rayleigh ratio [$\Delta R_\theta = R_\theta(\text{solution}) - R_\theta(\text{solvent})$], respectively.

High-resolution digital electron micrographs were acquired on a JEM-2010 electron microscope using Morada Soft Imaging System CCD (3700–2500 pixels). The sample was prepared by dropping a few microliters of the aqueous solution (0.5 mg/ml) of the sample onto the copper grid coated with a sustaining film. After vacuum drying, the sample was visualized by TEM. After decreasing the resolution of the obtained micrographs, a higher contrast will be obtained.

The wide-angle X-ray diffraction (WAXD) experiments were carried out using a Philips X'Pert Pro diffractometer with an X'celerator detector in a reflection mode. The X-ray sources (CuKα) were provided by 3 kW ceramic tubes, and the diffraction peak positions were calibrated with silicon powder ($2\theta > 15°$) and silver



Scheme 1. Synthetic pathway of PR-based triblock copolymers *via* ATRP of PEGMA in aqueous medium.

behenate ($2\theta < 10°$). The voltage was set to be 40 kV and the current 40 mA. Powder samples were mounted on a sample holder and scanned between $2\theta = 5°$ and 35° at a speed of 5°/min. The samples were prepared by freeze-drying the self-assemblies.

3. Results and discussion

3.1. ATRP of PEGMA initiated with Br end-capped Pluronic F127-β-CD PPRs

The synthetic pathway of amphiphilic triblock copolymers with PR as a middle block *via* the ATRP of PEGMA initiated with PPRs self-assembled from BrP-F127-PBr with varying amounts of β-CDs is shown in Scheme 1. The theoretical/found feeding compositions and yields after thorough purification are summarized in Table 1. For improving the controllability of the ATRP conducted in aqueous solution, Cu(I)Cl instead of Cu(I)Br together with PMDETA was used as catalyst according to the principle of halogen exchange [32,33]. The feed molar ratio of BrP-F127-PBr to Cu(I)Cl to PMDETA was controlled at 1:2:2.4.

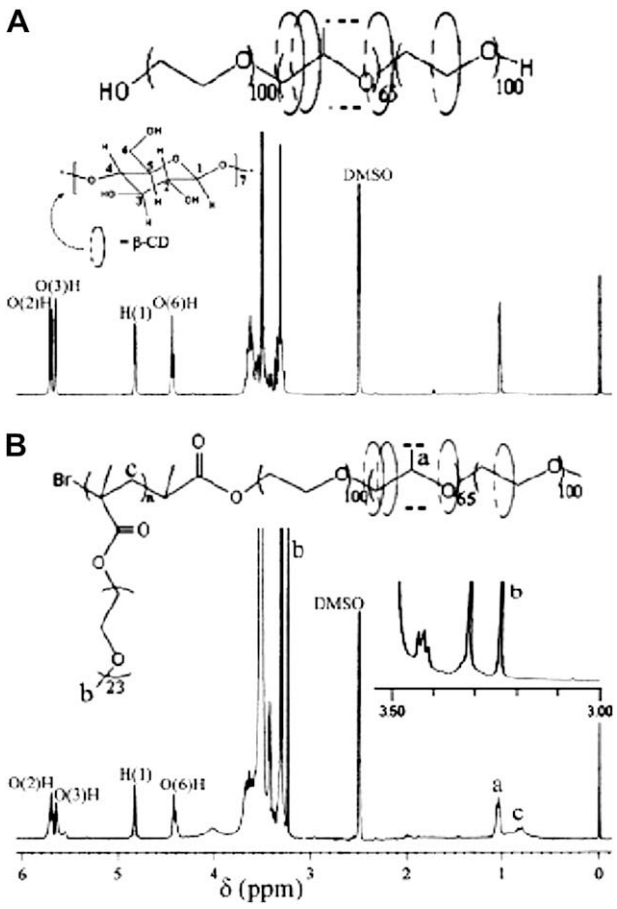


Fig. 1. ^{1}H NMR spectra of F-30β-CD PPR (A) and F-30β-CD-60 PR (B).

As illustrated in Table 1, when the feed molar ratio of PEGMA to BrP-F127-PBr was changed from 40 to 100 while that of β-CD to BrP-F127-PBr was kept at 30, the degree of polymerization (DP) of PEGMA oligomers attached to two ends of PPRs was in a range of 51.1–87.0. When the molar feed ratio of β-CD to BrP-F127-PBr was varied from 20 to 40 while that of PEGMA to BrP-F127-PBr was kept at 60, the number of β-CD trapped onto the F127 main chain was changed from 14.7 to 36.6. The results suggested that both the threaded number of β-CD and the DP of PEGMA oligomers appear to be adjustable to some extent in the ATRP of PEGMA carried out in the conditions as mentioned above, although there exists the

Table 1
Compositions and yields of PR-based triblock copolymers synthesized via ATRP of PEGMA.

Entry	Name	Molar composition of [BrP-F127-PBr]:[β-CD]:[PEGMA]		Molecular weight and polydispersity index			Monomer conversion[d] (%)	Yield[e] (%)
		Feed value[a]	Found value[b]	$M_n \times 10^{-3}$ [b]	$M_n \times 10^{-3}$ [c]	M_w/M_n [c]		
1	F-0β-CD-60	1:0:60	1:77.8	98.6	102.1	1.31	50.6	48.7
2	F-30β-CD-40	1:30:40	1:20.8:51.1	92.8	118.9	1.50	46.8	34.0
3	F-30β-CD-60	1:30:60	1:20.0:70.2	112.9	131.3	1.49	52.6	41.6
4	F-30β-CD-80	1:30:80	1:17.8:75.0	115.9	133.4	1.53	49.2	42.2
5	F-30β-CD-100	1:30:100	1:15.3:87.0	126.0	148.8	1.50	56.2	49.0
6	F-20β-CD-60	1:20:60	1:14.7:63.2	99.2	119.0	1.42	46.3	40.4
7	F-40β-CD-60	1:40:60	1:36.6:49.8	116.1	138.2	1.63	34.0	34.7

[a] The reactions were carried out at 25 °C in aqueous medium.
[b] Determined by ^{1}H NMR in DMSO-d_6.
[c] Determined by GPC with DMF and SDS as a mixing eluent at 1.0 ml/min using PS standards.
[d] Obtained according to the gravity.
[e] Calculating by the total weight of the feedings.

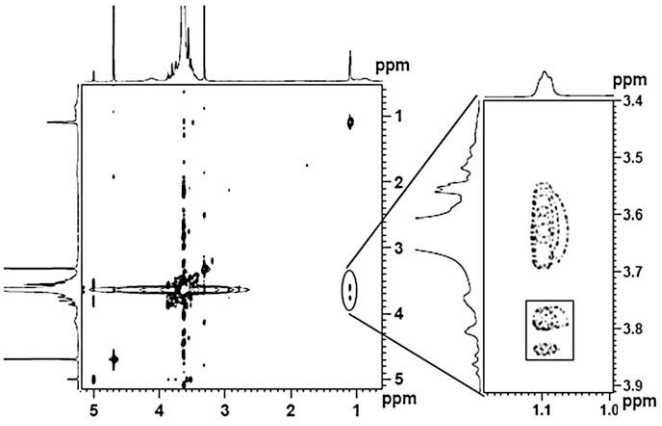


Fig. 2. 600 MHz 2D ROESY NMR spectrum of F-30β-CD-60 PR. The correlation peaks between the protons H_3 and H_5 of inner annular β-CD and the methyl protons (CH_3) of Pluronic F127 are shown in the rectangle in the magnified spectrum.

competitive effect between PEGMA and Pluronic F127 for inclusion complex with β-CDs as previously highlighted [34,35].

3.2. NMR analysis

For comparison, the ^{1}H NMR spectra of the F-30β-CD PPR and F-30β-CD-60 PR are outlined in Fig. 1. The inset is the magnified spectrum of the PR sample from 3.0 to 3.5 ppm, clearly distinguishing the end methyl peak of PPEGMA from the other peaks. As can be seen, O(2)H, O(3)H and O(6)H of β-CD in F-30β-CD-60 were obviously broadened and shifted about 0.06 ppm to the downfield compared with its F-30β-CD precursor owing to the decrease in conformational flexibility upon forming a polyrotaxane [36]. And the methyl proton peak of Pluronic F127 was also shifted to the lower field and broadened as previously reported [37]. These findings definitely confirmed that the amphiphilic triblock copolymers containing polyrotaxane as a middle block were successfully synthesized *via* the ATRP of PEGMA in aqueous medium.

Because the peak of methyl group for F127 (peak *a*) partially overlapped with the methyl of PPEGMA (peak *c*) which is directly attached to the main chain, for the calculation of the number of incorporated PEGMA monomer, it was needed to subtract the integration area of pendant methyl peak of PPEGMA (peak *b*) in Fig. 1(B) from the integration area of the whole methyl resonance peak area of Pluronic F127 (peak *a*) and the methyl of PPEGMA attached to the main chain (peak *c*) because these two peaks have the same integration area. The number of β-CD trapped onto the F127 main chain was deduced from the integration area ratio of the H-1 proton resonance peak of β-CD to that of the methyl protons of PPG block for F127 (peak *a*).

The inclusion complex structure of Pluronic F127-β-CD in F-30β-CD-60 was further verified by 2D ROESY experiment in D_2O as shown in Fig. 2. The correlation peaks between the protons H_3 and H_5 of inner annular β-CD and the methyl protons (CH_3) of Pluronic F127 provided solid evidence that β-CDs are threaded onto the F127 chain rather than on the PEGMA brushes. Another proof was presented by the following DSC analyses as shown in Fig. 4.

3.3. GPC measurement

As depicted in Fig. 3A and B, the GPC measurements of the resulting PR-derived triblock copolymers along with their controlling sample were carried out by using DMF and SDS (1 wt%) as the eluent. SDS was added to strongly suppress the association of both PR-derived and controlling copolymers. All the traces exhibited a nearly symmetrical and unimodal peak with a relatively lower polydispersity index (PDI) range of 1.49–1.63. It further revealed that the triblock copolymers containing PR as a central block with tunable PD of PEGMA oligomers were successfully synthesized.

As shown in Fig. 3C-3, the GPC trace of pure SDS with pure DMF as eluent exhibited its peak at ca. 21 min. The GPC trace of F-20β-CD-60 in pure DMF gave two peaks with distinct molecular weights but no peak appeared at ca. 21 min confirming no low molecular impurity such as β-CD or PEGMA existing in the PR-based copolymer sample after dialyzing in 60 °C water, and the PDI of the first peak only ca. 1.05, indicating the possible association of PRs in DMF [38]. However, after adding SDS into the eluent, the GPC trace of this sample still showed two peaks, but the outflow time was significantly changed, and the outflow time of the second peak was ca. 21 min clearly caused by the outflow of SDS in the eluent. It meant that the first peak in Fig. 3C-1 corresponded to the PR-based triblock copolymer and the second one was attributed to SDS. In comparison with the trace prior to adding SDS to the DMF eluent, the outflow time of the sample was shortened. It was likely due to the fact that adding SDS substantially suppresses the association of the sample in pure DMF and endows it holding the extended polymeric chain conformation with the increasing hydrodynamic volume.

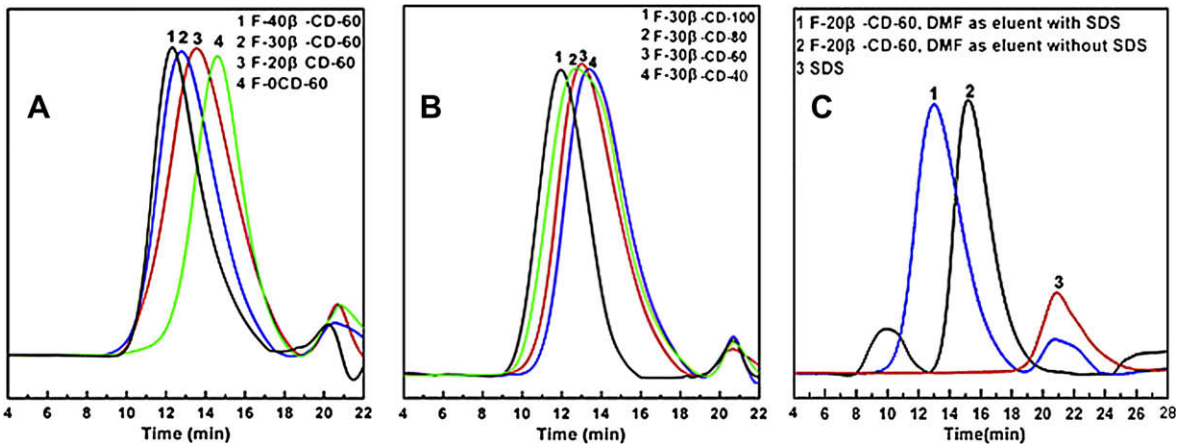


Fig. 3. GPC traces of F-40β-CD-60, F-30β-CD-60, F-20β-CD-60 and F-0β-CD-60 in (A) and F-30β-CD-100, F-30-β-CD-80, F-30β-CD-60 and F-30β-CD-40 in (B) with DMF + 1 wt% SDS as eluent, and GPC traces of F-20β-CD-60 and pure SDS in (C) with pure DMF or DMF + 1 wt% SDS as eluent.

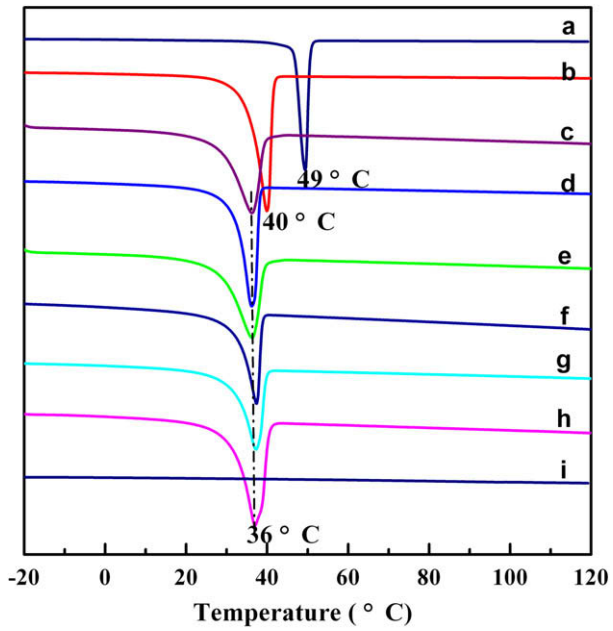


Fig. 4. DSC curves of BrP-F127-PBr (a), F-0β-CD-60 (b), F-30β-CD-40 (c), F-20β-CD-60 (d), F-30β-CD-60 (e), F-40β-CD-60 (f), F-30β-CD-80 (g), F-30β-CD-100 (h) and F-30β-CD PPR (i).

Furthermore, the molecular weight of all the obtained amphiphilic PR-based triblock copolymers was increased with increasing the feed molar ratio of PEGMA to macroinitiator and this trend was also true with increasing the amount of β-CDs. However, the molecular weight determined by GPC was generally not coherent with that by ^{1}H NMR analysis. This discrepancy was most likely due to the PS calibration standards used, as DMF is only a marginal solvent for PS [39]. Moreover, Pluronic F127 included into the cavity of β-CDs is significantly extended in comparison with free Pluronic F127 or PS chains, making a significant contribution to overestimated molecular weights.

3.4. DSC assessment

Fig. 4 depicts the DSC curves of BrP-F127-PBr, F-0β-CD-60 and the resulting PR-based triblock copolymers. For comparison, the F-30β-CD PPR sample was also presented here. An endothermic peak was observed in BrP-F127-PBr at 49 °C corresponding to the melting temperature (T_m) of crystallized PEG segments. As for F-0β-CD-60, the T_m of crystallized PEG appeared at 40 °C, lower than that of its macroinitiator precursor. This may be due to the interaction between the main chain PEG blocks and the side chain PEGMA blocks. According to the previous research, when β-CDs are threaded onto the Pluronic F127 backbone, not all the β-CDs stay on the PPG block, some of them can reside on PEO blocks, which actually prevents the PEO blocks from aggregating to form the crystalline phase [37,40]. Consequently there is no PEO melting peak appearing in the F-30β-CD PPR (i). However, all the triblock copolymers containing PR as a central block presented their own melting peaks at about 36 °C, which is considered as the melting peak of the PEGMA oligomers attached to the two ends of the amphiphilic PR-based triblock copolymers. It definitely indicated that β-CDs stayed on the Pluronic F127 chain rather than on the brush-like PEGMA domains. It also showed that novel amphiphilic triblock copolymer comprising a PR central block flanked by two brush-like blocks were successfully prepared *via* the ATRP of PEGMA in aqueous medium.

Table 2
Characterization of the self-assemblies formed by F-30β-CD-60 and F-0β-CD-60.

Sample code	DLC[a] (%)	DLE[b] (%)	R_h[c] (nm)	$R_{g,app}$ (nm)	R_g/R_h[d]	CAC × 10^{-3} [e] (mg/ml)
F-0β-CD-60	4.6 ± 0.2	46 ± 2.0	188.0	171.0	–	80.3
F-30β-CD-60	8.7 ± 0.3	87 ± 2.5	79.0	97.0	1.23	8.9

[a] DLC: drug-loading content.
[b] DLE: drug-loading efficiency.
[c] Sizes of the self-assembly determined by the dynamic light scattering (DLS) measurements with CONTIN analysis after the extrapolation to zero angle.
[d] Calculated while no unassociation PRs exist in the solution.
[e] Determined by the fluorescence probe method.

Moreover, these obtained amphiphilic triblock copolymers were analyzed by means of solid-state ^{13}C CP/MAS NMR, FTIR and TGA as referred in Supporting Information.

3.5. Critical aggregation concentration in aqueous medium

As a typical sample, F-30β-CD-60 was selected to demonstrate the self-assembly behavior of the amphiphilic PR-based triblock copolymer. When dissolved in deionized water, its aqueous solution at 0.5 mg/ml showed a blue tint, indicating the occurrence of aggregation. Furthermore, its critical aggregation concentration (CAC) was determined using PNA as a fluorescence probe as described in previous reports [41–43]. In comparison, the self-assembly properties of its controlling sample F-0β-CD-60 were also investigated. The self-assembly properties of both F-30β-CD-60 and F-0β-CD-60 are summarized in Table 2.

As PNA strongly emits fluorescence in a non-polar solvent or hydrophobic environment with regard to in polar media, it has been widely used to shed light on the self-aggregation character of surfactants and amphiphilic block copolymers as a molecular probe [44,45]. The emission spectra of the F-30β-CD-60 sample at different sample concentrations are shown in Fig. 5. As can be seen, at a low concentration, the emission spectra of PNA were hardly changed as compared with in pure water. However, as the concentration of the sample was increased, the intensity of the emission spectra (I) was evidently increased in a small range of

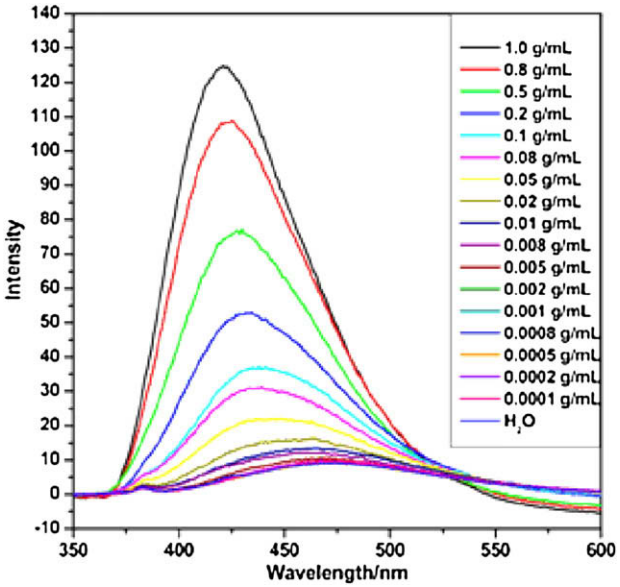


Fig. 5. Fluorescence emission spectra of PNA at different concentrations of F-30β-CD-60 in water. [PNA] = 2.0 × 10^{-6} mol/l; C [mg/ml]; λ_{exc} = 340 nm.

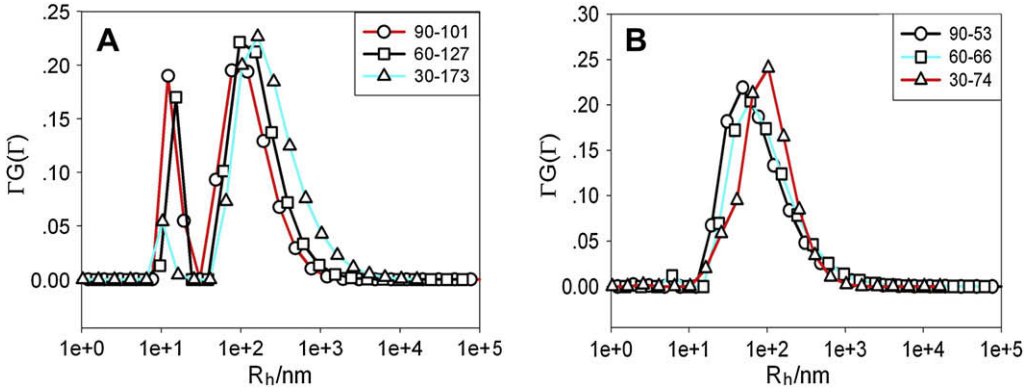


Fig. 6. Particle size distribution (intensity distribution) from DLS with the CONTIN analysis at observation angles 30°, 60° and 90° for F-0β-CD-60 (A) and F-30β-CD-60 (B).

concentrations. The abrupt increase of the I/I_0 values indicated that the PNA molecules begin to be removed from water bulk to the hydrophobic microdomains of the aggregates and the self-aggregation occurs. Meanwhile, the wavelength of maximum fluorescence emission of PNA was blue shifted. The calculation of the CAC value was specified in Supporting Information. As a result, the CAC value for the F-30β-CD-60 sample was 8.9×10^{-3} mg/ml, lower than that of its controlling sample F-0β-CD-60 at 80.3×10^{-3} mg/ml.

3.6. Sizes and size distribution of self-aggregates in aqueous medium

The sizes and size distribution of self-aggregates of both F-30β-CD-60 and F-0β-CD-60 were determined by DLS measurement with CONTIN analysis. As shown in Fig. 6A, a bimodal size distribution was evidenced in the blank brush-like block copolymer. It suggested that there coexist polymer aggregates and unassociated single polymer chain in the aqueous solution. However, Fig. 6B corresponding to F-30β-CD-60 solution shows almost no unassociated single polymer chain. After the extrapolation to zero angle, the R_h value of the self-aggregates of F-30β-CD-60 was around 79 nm, while for F-0β-CD-60, the R_h values of the fast and slow modes were about 18 and 188 nm, corresponding to the unimer and self-assemblies of the blank brush-like block copolymer, while the size distribution of the aggregates was 2.2 and 2.0 for F-30β-CD-60 and F-0β-CD-60, respectively. In conclusion, the entrapping of β-CDs onto the F127 chain contributes not only to the unimodal size distribution but also to the decrease in the size of self-aggregates (Fig. 6).

As the aggregates of F-30β-CD-60 exhibited a monomodal size distribution as depicted in Fig. 6B, the R_g/R_h value can provide insight into the morphological structure of its aggregates.

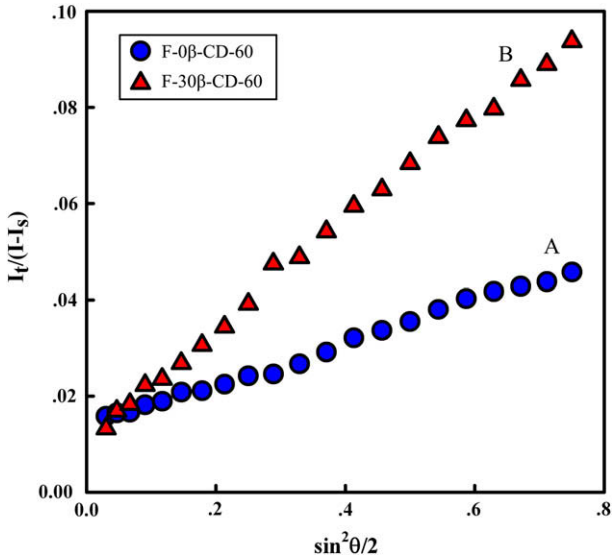


Fig. 7. Angular dependence of the scattered intensity of F-0β-CD-60 (A) and F-30β-CD-60 (B).

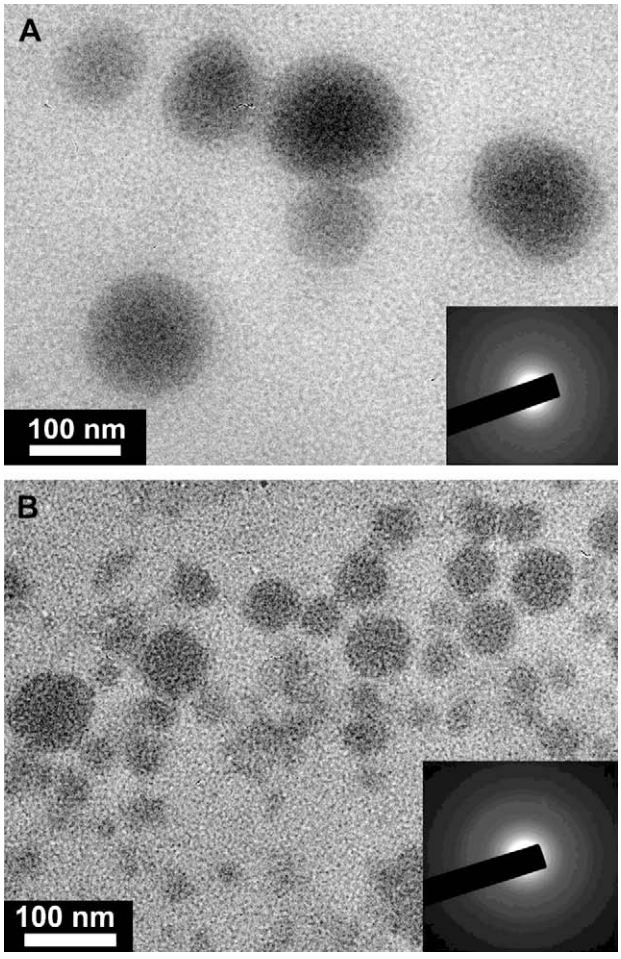


Fig. 8. TEM images of the self-assemblies of F-0β-CD-60 (A) and F-30β-CD-60 (B).

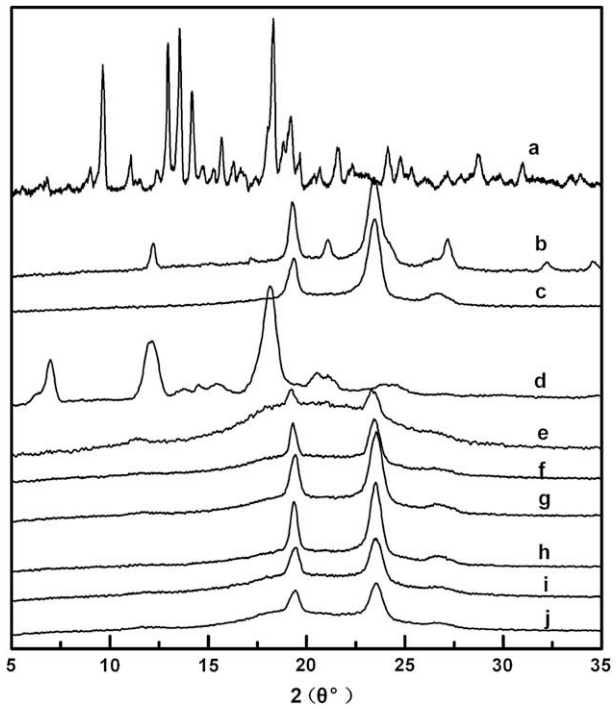


Fig. 9. X-ray diffraction patterns of pure β-CD (a), BrP-F127-PBr (b), F-0β-CD-60 (c), PPG 2000-30β-CD PPR (d), F-30β-CD-40 (e), F-30β-CD-60 (f), F-30β-CD-80 (g), F-30β-CD-100 (h), F-20β-CD-60 (i) and F-40β-CD-60 (j).

Consequently, SLS analysis was carried out to give rise to an apparent gyration radius ($R_{g,app}$) of around 97 nm for the F-30β-CD-60 aggregates (Fig. 7). Theoretically, for a uniform homogeneous solid sphere, a nondraining thin shell vesicle and a random polymer coil, the corresponding ratios of R_g/R_h are around 0.774, 1.0, and 1.5–1.8, respectively [46–48]. For the typical PR-based triblock copolymer, its R_g/R_h was found to be 1.23. In combination with the configuration of the polymer chains as well as the SAED images as shown below, it was deduced that the random polymer coil with some hollow cavities in the core was shaped by this selected copolymer. For the blank brush-like block copolymer, its R_g/R_h value was useless due to the bimodal size distribution of the aggregates in aqueous medium.

3.7. TEM observation of self-assemblies

Given the fluorescence and DLS analytical data as mentioned above, it can be concluded that the attachment of the PEGMA oligomers to the Pluronic F127 chain ends not only affects the chain conformation but also induces the aggregation of the novel triblock copolymers. This was further verified by the TEM image as shown in Fig. 8. The diameter of the spheres observed was ca. 100–150 nm for F-0β-CD-60 and ca. 70–90 nm for F-30β-CD-60, and both of them were smaller than that obtained from DLS analyses. It may be caused by the shrinkage of the particles on the grids during the drying process. Additionally there is probably another reason that the PEO segments could not be observed by TEM without staining. As a result, the observed spheres might be the core of self-assembled nanoparticles. The insets in the figures exhibit the selected area electronic diffraction (SAED) pattern of the self-assembled nano-sized particles, representing an amorphous assembly of both F-30β-CD-60 and F-0β-CD-60 [15]. The WAXD has demonstrated the similar results as shown in Fig. 9. All the PR-derived triblock copolymers exhibited the main peaks at

Scheme 2. Schematic diagram of self-assemblies formed by the PR-based triblock copolymer in aqueous medium.

19.37° and 23.45° corresponding to the crystalline peaks of the PPEGMA blocks but almost no channel-type crystalline peaks appearing at 12.08° and 18.11°. An amorphous assembly instead of characteristic channel-type crystalline assembly was formed by this selected copolymer sample most likely due to the fact that the PPEGMA brushes sterically hinder the PR central block to aggregate to form ordered crystalline structure. On the basis of these discussions, we schematically proposed the structure of the aggregates as shown in Scheme 2.

3.8. Assessment on the drug carrier of AmB and controlled release behavior in vitro

AmB is a broad spectrum water-insoluble chemotherapy and a number of micellar systems are used to improve its solubility and controlled release [29]. As nano-sized particles presented in aqueous medium, F-30β-CD-60 was testified as carrier for the controlled release of AmB. According to the literatures [49], the DLC and DLE values for AmB were determined by UV–vis spectroscopy. As summarized in Table 2, F-30β-CD-60 held 8.7% drug-loading content and 87% drug-loading efficiency for AmB, higher than those of F-0β-CD-60, well in accordance with its relatively lower CAC value and smaller particle sizes.

The controlled release profiles of both F-30β-CD-60 and F-0β-CD-60 as carriers for AmB in aqueous medium were compared and are illustrated in Fig. 10. Importantly, there was nearly no initial burst to occur for both samples. After 7 days about 90% encapsulated AmB was released from the F-0β-CD-60 self-aggregates while only about 54% encapsulated model drug was released for F-30β-CD-60. And the cumulative release time for F-30β-CD-60 was much longer than that for F-0β-CD-60. We speculated that encapsulated AmB may have some interaction with threaded β-CDs, giving longer

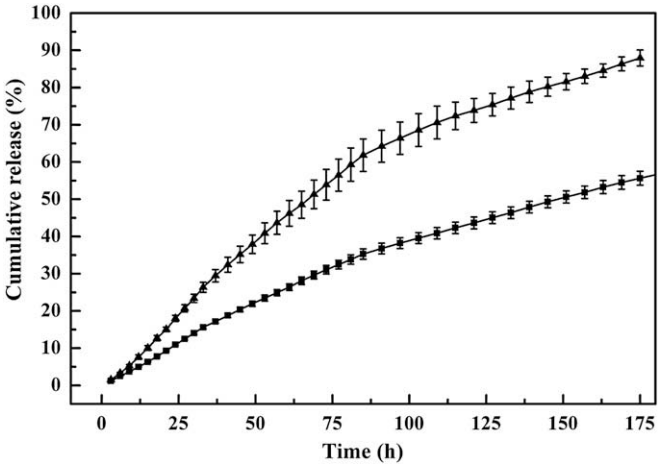


Fig. 10. Release profiles of AmB in vitro within 7 days: (■) F-30β-CD-60 and (▲) F-0β-CD-60 in aqueous medium. The standard deviation is shown by the error bars, $n = 3$.

sustained release time compared with F-0β-CD-60 without any β-CDs. Moreover, as this kind of amphiphilic triblock copolymers is characteristic of a rigid CD-covered central block flanked by two hydrophilic brush-like PEGMA oligomers, the dense non-adhesive hydrophilic coating around the central block would impart AmB "stealth" properties and long residence time in biological milieu. The PR induced self-aggregate AmB formulation should have distinct advantages over AmB lipid complex (Abelcet) and conventional liposomal AmB (AmBisome). More biological evaluations are ongoing in our laboratory.

4. Conclusions

Novel amphiphilic triblock copolymers with polyrotaxane as a central block have been prepared *via* the ATRP of PEGMA initiated with polypseudorotaxanes formed from 2-bromopropionyl end-capped Pluronic F127 with varying amounts of β-CDs in the presence of Cu(I)Cl/PMDETA at 25 °C in aqueous medium. The PD of PEGMA oligomers appeared to be tunable to some extent. The CAC value of one selected sample of the resulting PR-based triblock copolymers was determined with the fluorescence probe technique. The DLS and SLS measurements and TEM observations further confirmed that the selected sample enables to self-assemble into nano-sized particles. These particles showed the potential to be used as AmB-controlled delivery carrier with 8.7% drug-loading content and 87% drug-loading efficiency as well as longer sustained release time. This PR self-aggregate formulation might provide a long-circulating nanodepot for AmB capable of releasing drug slowly over time in vivo. More biological evaluations are our ongoing investigation.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2009.07.006.

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