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Supramolecular flower micelle formation of polyrotaxane-containing triblock copolymers prepared from macro-chain transfer agents bearing molecular hooks

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Keywords

Polyrotaxane; Triblock copolymer; RAFT polymerization; Polymeric micelle;

Abstract

Polyrotaxane (PRX)-containing triblock copolymers are unique modality of supramolecular polymers. In combination with the intrinsic property of polymer chains, the supramolecular properties of PRXs, such as freely mobile character of threading α-cyclodextrins (α-CDs) and the intermolecular hydrogen bonding, can be utilized as biomaterials. However, it is difficult to synthesize well-defined PRX-containing triblock copolymers with regulated polymeric chain length and the number of threading α-CDs. Herein, we described the precise synthetic method of PRX-containing triblock copolymers via reversible addition-fragmentation chain transfer (RAFT) polymerization using a novel PRX macro-chain transfer agent (CTA) with terminal hooks of phenylalanyl groups. The terminal phenylalanyl groups of PRX macro-CTA act as a hook to inhibit the dethreading of α-CDs during polymerization to succeed the regulation of molecular weight of the polymer chains with maintaining the number of threading α-CDs in PRX segments. Additionally, we first prepared self-assembled polymeric micelles with the outermost PRX layer using the PRX-containing triblock copolymers. The hydroxyethyl groups-modified triblock copolymers composed of PRX and hydrophobic poly(benzyl methacrylate) (PBzMA) were found to form polymeric micelles with 47 nm in diameter and narrow size distribution. The supramolecular polymeric micelles show a core-shell-type structure comprising a core of hydrophobic PBzMA surrounded by the PRX flower loops. This micelle formation allows the incorporation of water-insoluble anticancer drugs within the PBzMA core as well as biological ligands into α-CDs of the PRX flowers toward receptor proteins of target cell membranes. Finally, it is concluded that the obtained polymeric micelles with the outermost PRX layer is expected to apply for a supramolecular drug carrier.

Introduction

Polyrotaxanes (PRXs) are a modality of supermolecules comprising a linear polymer threading into the cavity of cyclic molecules capped with bulky stopper molecules.[1-4] Especially, cyclodextrin (CD)-threaded PRXs have been widely investigated in various fields including biomaterials.[1-5] There is no non-covalent bond between a polymer axle and cyclic molecules, therefore, the threading CDs in PRXs can freely rotate or mobile along the polymer axle.[6,7] We have found that the saccharide-installed PRXs showed the enhanced binding affinity to targeted lectins, because the sliding motion of ligand-installed α-CDs resolved the steric mismatch in multivalent interaction.[6] The binding constant between the saccharide-installed PRXs and lectins is found to be approximately 200-fold higher than the saccharide-installed linear polymers.[8] For utilizing the potentiality of such PRXs as biomaterials, it is recognized that the construction of movable biointerfaces based on PRXs is an essential issue. In this regard, we have designed PRX-immobilized surfaces and investigated the effect of the mobility of PRXs on protein adsorption and cell adhesion.[9-13] The PRX surfaces with movable cell-adhesive RGD (Arg-Gly-Asp) peptides can induce rapid cell binding compared to conventional RGD-immobilized surfaces.[13] Accordingly, it has been suggested that the movable PRX surfaces can provide a promising modality of biointerfaces.

Another possible application of performing PRX biointerfaces is the surface bearing of movable PRXs on nanoparticles such as polymeric micelles.[14] The polymeric micelles with the outermost ligand-installed PRXs are expected to enhance the binding ability to targeted molecules and cells. In order to prepare polymeric micelles with the outermost PRX surface, the self-assembly of PRX-containing triblock copolymers comprising an intermediate PRX segment and hydrophobic polymer chains will be a promising way. To date, various PRX-containing triblock copolymers have been developed.[9-13,15-20] For example, Feng and coworkers have synthesized PRX-based triblock copolymers bearing hydrophilic polymers, such as poly(*N*-hydroxypropylmethacrylamide),

poly(ethylene glycol) methyl ether methacrylate (PEGMA) and temperature-responsive poly(*N*-isopropylacrylamide).[15-18] Otherwise, Huang coworkers have developed supramolecular micelles and vesicles based on the self-assembly of inclusion complexes.[21,22] However, these PRX-based triblock copolymers bearing hydrophilic polymer chains can self-assemble into polymeric micelles with the outermost hydrophilic polymer layers, because the PRX segments are assembled via intramolecular hydrogen bonding between the threaded CDs to form the core of micelles.[16,17,19,20] In these cases, the PRXs are definitely located at the opposite side from interfaces with biological circumstance (cores of the micelles), thus, it is controversial in the use of the mobility of the PRXs as a molecular recognition moiety.

Particularly, the PRX-containing triblock copolymers were synthesized via living radical polymerization, such as atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization from pseudopolyrotaxane (PPRX)-based macro-initiaters or macro-chain transfer agents (CTAs).[9-13,15-19] Although the use of living radical polymerization is valuable to synthesize well-defined triblock copolymers, there is a predestinate issue that the threading CDs are dissociated from the PPRX macro-initiators or macro-CTAs in the process of polymerization, due to their thermodynamically unfavorable nature (Figure 1A). This means that the regulation of the number of threading CDs in PRX-based triblock copolymers has been hardly established. Since the number of threading CDs in PRX is one of the predominant factors to determine the property of PRXs, an alternative method which can regulate the number of threading CDs in PRX-based triblock copolymers is indispensable for further investigation and the optimization of molecular design.

In this regard, we developed the rational synthetic method of PRX-containing triblock copolymers via RAFT polymerization using a PRX-based macro-CTA with terminal hooks of phenylalanyl groups (Figure 1B). The terminal hooks of the macro-CTA inhibits the dethreading of α-CDs during

the polymerization. This method enables the regulation of both the molecular weight of polymer chains and the number of threading α-CDs in PRX segments. Additionally, we further studied the formation of self-assembled supramolecular flower micelles comprising a core of hydrophobic polymers surrounded by loops of PRXs and demonstrated their potential application as a drug delivery carrier.

Experimental section

Materials

α-Cyclodextrin (α-CD) was obtained from Ensuiko Sugar Refining (Tokyo, Japan). Poly(ethylene glycol) (PEG-OH) (M_n = 9,810, M_w/M_n = 1.02), 1,1'-carbonyldiimidazole (CDI), 4-cyanopentanoic acid dithiobenzoate (CPDTB), and pyrene were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-Hydroxyethylamine (HEA), phenylalaninol, and triethylamine (TEA) were obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). Methanesulfonyl chloride (MsCl) was obtained from Kanto Chemicals (Tokyo, Japan). 4,4'-Azobis(4-cyanopentanoic acid) (V-501), benzyl methacrylate (BzMA), sodium hydride (NaH), and paclitaxel were obtained from Wako Pure Chemical Industries (Osaka, Japan). BzMA was purified by passing through an inhibitor removal column (Sigma-Aldrich) just before use. Other solvents were obtained from Kanto Chemicals.

Characterization of polyrotaxanes

Size exclusion chromatography (SEC) was carried out on a HLC-8120 system (Tosoh, Tokyo, Japan) equipped with a combination of TSKgel α-4000 and α-2500 columns (Tosoh), eluted with dimethylsulfoxide (DMSO) containing 10 mM LiBr at a flow rate of 0.35 mL/min at 60 ºC. The $M_{n,SEC}$ and M_w/M_n were calculated based on the PEG calibration standard (Agilent Technologies,

Wilmington, DE, USA). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany).

Synthesis of PRX macro-chain transfer agents (PRX macro-CTAs)

PEG-OH (20.0 g, 2.0 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 100 mL), and TEA (4.3 mL, 30.6 mmol) and MsCl (1.6 mL, 20.4 mmol) were successively added to the reaction mixture.[23] After reaction for 5 h at room temperature, the solution was filtered with celite, and the filtrate was poured into diethyl ether to precipitate the polymer. The recovered polymer was dried under reduced pressure to obtain α,ω-bismesyl-PEG (PEG-Ms) (18.4 g, 92.5% yield). The functionality of mesyl group in PEG-Ms was determined to be 96.1% by ^{1}H NMR. SEC (DMSO containing 10 mM LiBr) M_n = 12,700, M_w/M_n = 1.03; ^{1}H NMR (500 MHz, DMSO) δ = 3.12 (s, 3H, -CH_2O-$SO_2C\boldsymbol{H_3}$), 3.67 (m, 905H, PEG backbone), 4.41 (t, 2H, -$C\boldsymbol{H_2}O$-SO_2CH_3).

PEG-Ms (2.0 g, 0.2 mmol) was dissolved in anhydrous DMF (10 mL) under nitrogen atmosphere. Separately, NaH (191 mg, 4.0 mmol) and phenylalaninol (300 mg, 2.0 mmol) were allowed to react in DMF for 30 min to form sodium alkoxide.[24] This was added to the PEG solution and the mixture was stirred for 24 h at room temperature. Then, the solution was filtered with celite, and the filtrate was poured into diethyl ether to precipitate the polymer. The recovered polymer was dried under reduced pressure to obtain α,ω-bisphenylalanyl-PEG (PEG-Phe-NH_2) (1.46 g, 71.3% yield). The functionality of phenylalanyl group in PEG-Phe-NH_2 was determined to be 92.0% by ^{1}H NMR. SEC (DMSO containing 10 mM LiBr) M_n = 7,400, M_w/M_n = 1.05; ^{1}H NMR (500 MHz, DMSO) δ = 3.52 (m, 905H, PEG backbone), 7.21 (t, 2H, aromatics of phenylalanyl group), 7.29 (t, 3H, aromatics of phenylalanyl group).

A saturated solution of α-CD was prepared by dissolving α-CD (3.0 g, 3.08 mmol) in water (20.7 mL). PEG-Phe-NH_2 (1.0 g, 971 μmol) dissolved in small aliquot of water was added to the α-CD

solution, and the mixture was stirred for 24 h at room temperature. After the reaction, the system was freeze-dried for 1 day to obtain a pseudopolyrotaxane as powder. Then, CPDTB (1.36 g, 4.87 mmol), DMT-MM (1.35 g, 4.87 mmol), and the pseudopolyrotaxane were allowed to react in methanol (10 mL) for 24 h at room temperature. Then, the precipitate was collected by centrifugation and dissolved in DMSO. The recovered solution was freeze-dried to obtain PRX-Phe-DTB macro-CTA (1.94 g, 48.5% yield based on PEG mol%). The number of threaded α-CDs PRX-Phe-DTB macro-CTA was determined by ^{1}H NMR spectra in DMSO-d_6. SEC (DMSO containing 10 mM LiBr) M_n = 29,500, M_w/M_n = 1.26; ^{1}H NMR (500 MHz, DMSO) δ = 3.2-3.8 (m, PEG backbone and H_2, H_3, H_4, H_5, and H_6 protons of α-CD), 4.45 (m, OH_6 of α-CD), 4.80 (m, H_1 of α-CD), 5.49 (m, OH_3 of α-CD), 5.65 (m, OH_2 of α-CD), 7.20 (t, aromatics of phenylalanyl group), 7.25 (t, aromatics of phenylalanyl group), 7.53 (t, aromatics of DTB group), 7.71 (t, aromatics of DTB group), 7.92 (t, aromatics of DTB group).

Synthesis of PBzMA-*b*-Phe-PRX-Phe-*b*-PBzMA triblock copolymers

PBzMA-*b*-Phe-PRX-Phe-*b*-PBzMA triblock copolymers (PRX-Phe-PBzMAs) with various M_n of PBzMA were synthesized by varying the feed [BzMA]/[DTB groups in PRX] molar ratio. Briefly, the PRX-DTB macro-CTA (400 mg, 10.8 μmol), BzMA (110 μL, 649 mmol), and V-501 (1.21 mg, 4.32 μmol) were dissolved in DMSO (2 mL), followed by bubbling with nitrogen for 30 min to deoxygenate the reaction mixture. The reaction mixture was stirred for 24 h at 70 °C. After the reaction, the polymer was purified by dialysis against DMSO for 3 days, followed by water for 3 days (Spectra/Por 6, molecular weight cut-off (MWCO) of 8,000) (Spectrum Laboratories, Rancho Dominguez, CA). The recovered solution was freeze-dried to obtain a PRX-Phe-PBzMA (417 mg, 79.9% yield). The number of threaded α-CDs, the degree of repeating units of PBzMA, and M_n of the PRX-Ph-PBzMA triblock copolymer were determined by ^{1}H NMR spectra in DMSO-d_6. SEC

(DMSO containing 10 mM LiBr) M_n = 34,100, M_w/M_n = 1.31; ^{1}H NMR (500 MHz, DMSO) δ = 0.5-0.9 (m, -CH(-C***H₃***)-CH_2- of PBzMA), 1.5-2.0 (m, -CH(-CH_3)-C***H₂***- of PBzMA), 3.2-3.8 (m, PEG backbone and H_2, H_3, H_4, H_5, and H_6 protons of α-CD), 4.45 (m, OH_6 of α-CD), 4.81 (m, H_1 of α-CD), 4.87 (m, -C***H₂***-Phe of PBzMA), 5.49 (m, OH_3 of α-CD), 5.65 (m, OH_2 of α-CD), 7.29 (m, aromatics of PBzMA).

Synthesis of hydroxylated PBzMA-*b*-Phe-PRX-Phe-*b*-PBzMA (HE-PRX-Phe-PBzMA)

The PRX-Phe-PBzMA (100 mg, 2.07 μmol, 55.9 μmol of α-CD) (run 2 in Table 1, M_n of PBzMA = 5,650) and CDI (91 mg, 560 μmol) was dissolved in anhydrous DMSO (6 mL), and the reaction mixture was stirred for 24 h at room temperature.[25] Then, HEA (167 μL, 2.8 mmol) was added to the reaction mixture, and the mixture was stirred for a further 24 h at room temperature. After the reaction, the polymer was purified by dialysis against water for 3 days (MWCO 8,000). The recovered solution was freeze-dried to obtain an HE-PRX-Phe-PBzMA (105 mg, 85.0% yield). The number of modified HE groups and $M_{n,NMR}$ of the the HE-PRX-Phe-PBzMA were determined from ^{1}H NMR spectra in DMSO-d_6. SEC (DMSO containing 10 mM LiBr) M_n = 44,500, M_w/M_n = 1.55; ^{1}H NMR (500 MHz, DMSO) δ= 0.5-0.9 (m, -CH(-C***H₃***)-CH_2- of PBzMA), 1.5-2.0 (m, -CH(-CH_3)-C***H₂***- of PBzMA), 3.05 (m, -O-C(=O)-NH-C***H₂***-CH_2-OH), 4.63 (m, OH_6 of α-CD), 4.87 (m, H_1 of α-CD and -C***H₂***-Phe of PBzMA), 5.4-6.2 (m, OH_2 and OH_3 of α-CD), 6.8-7.1 (m, -O-C(=O)-N***H***-CH_2-CH_2-OH), 7.28 (m, aromatics of PBzMA).

Preparation and characterization of polymeric micelles and paclitaxel-loaded polymeric micelles

The HE-PRX-Phe-PBzMA was dissolved in DMSO at various concentrations (0.1 to 1.0 mg/mL), and the solutions were dialyzed against distilled water for 24 h at room temperature (MWCO 3,500).

When the preparation of paclitaxel-loaded polymeric micelles, The HE-PRX-Phe-PBzMA (5 mg) and paclitaxel (0.25 to 2.5 mg) were dissolved in DMSO (2 mL), and the solutions were dialyzed against distilled water for 24 h at room temperature (MWCO 3,500).[26] The insoluble paclitaxels were removed by filtration (0.45 μm) to obtain paclitaxel-loaded HE-PRX-Phe-PBzMA polymeric micelles. The loading efficiency and the content of paclitaxel in the polymeric micelles were determined from ^{1}H NMR spectra in DMSO-d_6.[27]

The critical micelle concentration of the polymeric micelles was determined by fluorescent measurements using pyrene as a probe, according to the previous report.[28] The diameter of the polymeric micelles was determined on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4 mW He–Ne laser (633 nm) at 25 °C at a detection angle of 173°. The obtained autocorrelation functions were analyzed by the cumulant method to determine averaged diameters and polydispersity index of the micelles. The transmission electron microscopic (TEM) images of the polymeric micelles were obtained on a Hitachi H-7100 (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 keV. A 10 μL of the micelle solution (50 μg/mL) was placed on a 400 mesh copper grid coated with a formvar thin film, and dried at room temperature. The sample was stained with 1.5% uranyl acetate for 2 min at room temperature. The diameter of the polymeric micelles were determined using Image J software ver. 1.45 s (National Institutes of Health, Bethesda, MD).

Cytotoxicity of paclitaxel-loaded polymeric micelles

HeLa cells derived from human cervical carcinoma were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) and grown in minimum essential medium (MEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco) in a humidified 5% CO_2 atmosphere at 37 °C. HeLa cells were seeded on a 96-well plate at a density of 1.0×10^4 cells/well and incubated

overnight. After the medium was exchanged for 90 μL of fresh medium, 10 μL of the samples were applied. After incubation for further 24 h, 10 μL of Cell Counting Kit-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added and incubated for 2 h 37 °C. The absorbance at 450 nm was measured on a Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, MA). The cellular viability was calculated relative to the untreated cells.

Results and discussion

Synthesis of PRX macro-CTA and PRX-containing triblock copolymers

For the synthesis of PRX containing ABA-type triblock copolymers, the RAFT polymerization of benzyl methacrylate (BzMA) was performed using a conventional pseudopolyrotaxane (PPRX) macro-CTA in THF at 70 ºC for 24 h (Figure 1A). Since the THF is poor solvent for PPRX, the polymerization was conducted in inhomogeneous condition. The polymerization was impossible to conduct in good solvent such as DMSO, because the threading α-CDs in PPRX are immediately dethreaded from PEG chain due to the lack of terminal stoppers. Figure 2A shows the SEC chart of PBzMA-*b*-PRX-*b*-PBzMA (PRX-PBzMA) synthesized using the PPRX macro-CTA. Although the polymerization was performed in inhomogeneous condition, the peak of PRX-PBzMA was shifted to high molecular weight region in comparison with PEG-DTB, which is an axle polymer for the PPRX macro-CTA, indicating that the polymerization of BzMA successfully proceeded from the PPRX macro-CTA. However, the dethreading of the α-CDs from PPRX macro-CTA was observed at the elution volume of 14.9 mL after polymerization (Figure 2A). Although the number of threading α-CDs in PPRX macro-CTA was 23, the number of threading α-CDs in PRX-PBzMA was reduced to 7, as determined by ^{1}H NMR. It is obvious that most of the threading α-CDs were dethreaded from PPRX in the process of polymerization even in poor solvent, and it is difficult to reduce α-CD dethreading in PPRX macro-CTA method.

To establish the precise synthetic method of PRX-containing triblock copolymers without the dethreading of α-CDs during polymerization, a novel PRX macro-CTA with terminal hooks of phenylalanyl groups (PRX-Phe-DTB) was designed (Figure 1B). In this system, PEG bearing terminal moderately bulky phenylalanyl groups were utilized for the preparation of PPRX, followed by the capping with chain transfer agents (CPDTB) (Scheme 1). ^{1}H NMR spectrum of resulting PRX-Phe-DTB (PRX macro-CTA) clearly showed the peaks assignable to threading α-CDs (the number of threading α-CDs was 23), terminal DTB groups, and phenylalanyl groups (Figure 3A). We have found that phenylalanyl group between the PEG end and CPDTB act as a hook to inhibit the dethreading of α-CDs. Since the PRX macro-CTA could maintain its supramolecular structure even in their good solvent (DMSO), it is widely beneficial that we can perform RAFT polymerization of BzMA using the PRX-Phe-DTB even in homogenous condition (in DMSO). After polymerization of the BzMA in DMSO using PRX-Phe-DTB, the peak top of PBzMA-*b*-Phe-PRX-Phe-*b*-PBzMA (PRX-Phe-PBzMA) was slightly shifted to high molecular weight region, and the α-CD dethreading was scarcely observed in SEC chart (Figure 2B). It is clearly confirmed that the peak assignable to PBzMA was observed in ^{1}H NMR spectrum (Figure 3B). The number of threading α-CDs in PRX-Phe-PBzMA was 25.2 (Figure 3B), which is roughly consistent to the number of threading α-CDs in the PRX macro-CTA (the number of threading α-CDs was 23) (Figure 3A).

Next, we challenged to regulate the molecular weight of PBzMA in the PRX containing triblock copolymers using the PRX macro-CTA. The molecular weight of PBzMA segments was proportionally increased with the feed [monomer]/[PRX macro-CTA] ratios (Figure 4), and the resulting polymers were found to show narrow molecular weight distributions (M_w/M_n was approximately 1.3) (Table 1). The number of threading α-CDs in the resulting PRX-Phe-PBzMA triblock copolymers was almost constant values at these experimental conditions (Table 1),

indicating that the PRX macro-CTA method allows the regulation of molecular weight of the polymers with retaining the number of threading α-CDs. In our previous study, the number of threading α-CDs in PRX can be well controlled by conditions of PPRX preparation such as feed α-CDs/PRX ratio and solvent composition in capping reaction.[29-31] Although it is considered that the moderately bulky phenylalanyl terminal group in PEG-Phe-NH_2 might affect the PPRX formation, we have confirmed that the number of threading α-CDs in PRX-Phe-DTB can be controlled in the range of 23 (20%) to 59 (52%) by varying feed α-CDs/PRX ratio and reaction time. This range of the number of threading α-CDs is roughly consistent with our previous investigations.[29,30] Therefore, we believe that this synthetic method can develop far-reaching consequences of preparing a variety of PRX-containing triblock copolymers.

Preparation and characterization of PRX-Phe-PBzMA polymeric micelles

The amphiphilic block or graft copolymers composed of hydrophilic and hydrophobic polymers are known to be self-assembled into polymeric micelles with outermost palisade layer of hydrophilic polymers.[14] The triblock copolymers bearing hydrophilic polymers and PRX also form polymeric micelles by the intramolecular association of PRX segments through hydrogen bonding.[16,17,19,20] Herein, we tried to prepare supramolecular flower micelles comprising the outermost PRX layer and segregated hydrophobic polymer core using the PRX-Phe-PBzMA (Figure 5A). However, since the PRX segment is not soluble in aqueous solution due to the intra- and intermolecular hydrogen bonding among threading α-CDs, the PRX-Phe-PBzMA was precipitated in aqueous solution (Figure 5B). To increase the solubility of the PRX segments to aqueous media, hydrophilic hydroxyethyl (HE) groups were introduced at the threading α-CD moieties of PRX-Phe-PBzMA (run 2 in Table 1, M_n of PBzMA = 5,650) (Scheme 1).[25] The number of modified HE groups were determined to be 189 in PRX-Phe-PBzMA by ^{1}H NMR, which is sufficient number to solubilize PRX in aqueous

solution.[25] HE groups-modified PRX-Phe-PBzMA (HE-PRX-Phe-PBzMA) were dissolved in DMSO, followed by dialysis against water to obtain polymeric micelles. The obtained solution was transparent and the precipitation of HE-PRX-Phe-PBzMA was scarcely observed, suggesting the formation of polymeric micelles (Figure 5B). Transmission electron microscopic (TEM) observation revealed that HE-PRX-Phe-PBzMA formed uniform spherical nanoparticles in aqueous solution with 15.3 ± 1.9 nm in diameter and the coefficient of variation of 12.4% (n = 211) (Figure 5C, D). This HE-PRX-Phe-PBzMA had a critical micelle concentration at the concentration of 15.1 μg/mL, indicating that the observed nanoparticles were polymeric micelles formed from the self-assembly of PRX-containing triblock copolymers.

To further investigate the condition for preparing HE-PRX-Phe-PBzMA polymeric micelles, the effect of concentration on the diameter and polydispersity index of the resulting micelles were studied by dynamic light scattering (DLS) measurements (Figure 5E). The diameter of the micelles showed the smallest diameter (47.5 ± 0.1 nm) and the polydispersity index (μ_2/Γ^2 = 0.097 ± 0.004) at the concentration of 2.5 mg/mL. The diameter of the obtained micelles were smaller than previously reported polymeric micelles with interior PRX core, indicating that the strongly hydrophobic PBzMA forms tightly associated core compared to previously reported supramolecular micelles formed from the association of PRXs (more than 100 nm).[16,17,19,20] Above this optimal concentration, the diameter and the polydispersity index value increased with concentration, indicating that the association number of polymers increased or the micelles were aggregated by intra-particle bridging via triblock copolymers.[32-35] At the dilute concentration, HE-PRX-Phe-PBzMA polymeric micelles are thought to show negligible aggregation and intra-micellar bridging to provide small diameter, narrow distribution, and dispersion stability. The micelles prepared at optimal concentration is sufficiently stable in physiological ionic strength condition and retained its diameter at least for several months.

It is known that the ABA-type triblock copolymers with intermediate hydrophilic segments form the polymeric micelles comprising a core of hydrophobic segments and surrounded by loops of hydrophilic segments.[32-35] To validate the formation of HE-PRX-Phe-PBzMA polymeric micelles with the outermost PRX layers, ^{1}H NMR spectrum of the micelles were measured in D_2O (Figure 6). In DMSO, the peaks corresponding to both threading α-CD and PBzMA were observed. In sharp contrast, the peaks corresponding to PBzMA was diminished in D_2O. However, H_1 protons of α-CD and ethylene protons derived from HE groups were still observed in D_2O, although OH_2, OH_3, and OH_6 protons were diminished due to proton exchange. This result supports our hypothesis that the HE-PRX-Phe-PBzMA forms supramolecular flower micelles with the outermost PRX layers.

Loading of hydrophobic drugs into the core of PRX-Phe-PBzMA polymeric micelles

To investigate the potential of HE-PRX-Phe-PBzMA polymeric micelles as a drug delivery carrier, the loading of hydrophobic anticancer drug was demonstrated. Paclitaxel was used as a model anticancer drug, and the loading of paclitaxel into the polymeric micelles was performed by a conventional dialysis method.[26] By varying the feed loading content (wt%) of paclitaxel to the micelles, the loading content of paclitaxel was reached maximal value at the feed loading content of 10 wt% (Figure 7A). The maximum loading content of paclitaxel to the HE-PRX-Phe-PBzMA polymeric micelles were 8.8 wt%. The loading efficiency decreased with increasing the feed loading content of paclitaxel. The diameter and polydispersity index values of paclitaxel-loaded HE-PRX-Phe-PBzMA polymeric micelles (50.9 ± 0.3 nm, μ_2/Γ^2 = 0.095 ± 0.002) were almost comparable to the empty HE-PRX-Phe-PBzMA micelles (51.8 ± 0.5 nm, μ_2/Γ^2 = 0.08 ± 0.01). Figure 7B shows the viability of HeLa cells after 24 h incubation with paclitaxel and the paclitaxel-loaded HE-PRX-Phe-PBzMA micelles. The half maximal inhibitory concentration (IC_{50}) of paclitaxel and the paclitaxel-loaded HE-PRX-Phe-PBzMA micelles to HeLa cells were

determined to be 29.7 ± 1.2 ng/mL and 291 ± 16 ng/mL, respectively. Although the paclitaxel-loaded HE-PRX-Phe-PBzMA micelles could induce cytotoxic effect of HeLa cells, they showed lower toxic effect than free paclitaxel. This result indicates that most of the paclitaxel was remained in the micelles. Conversely, it is considered that the HE-PRX-Phe-PBzMA micelles can stably incorporate paclitaxel in the core of the micelles. Since the stable encapsulation of drugs is of importance to deliver the drugs to targeted tissue, the HE-PRX-Phe-PBzMA micelles is expected to apply for systemic delivery of anticancer drugs to the targeted tumor tissues.

We have reported the intracellular delivery of plasmid DNA (pDNA), small interfering RNA (siRNA) and proteins through the polyelectrolyte complexation with amino groups-modified PRXs.[30,36-38] In these complexes, the freely mobile nature of α-CDs along the axle is thought to be restricted by the electrostatic interactions. By contrast, the supramolecular flower micelles has appealing property to incorporate hydrophobic drugs within the core of micelles as well as the utilization of freely mobile PRX segments as a movable ligand moiety. Since this dynamic ligand surface is expected to drastically enhance the binding ability to the targeted receptors,[6,8,13] it is considered that the polymeric micelles with the outermost PRX layers is advantageous as a targetable nanocarrier with superior ability in molecular recognition.

Conclusions

In this study, the precise synthetic method of PRX-containing triblock copolymers was established using a novel PRX macro-CTA with terminal hooks of phenylalanyl groups. This method allows the regulation of molecular weight of the polymer chain at the distal ends with maintaining the number of threading α-CDs in PRX-containing triblock copolymers. This PRX-based synthetic approach would be readily applied for the other polymerization methods such as ATRP. The installation of HE groups at the α-CD moieties gave the sufficient water solubility to PRX segments in

PRX-Phe-PBzMA triblock copolymers to form stable self-assembled polymeric micelles in aqueous media, which were composed of a core of hydrophobic PBzMA surrounded by loops of PRXs. The hydrophobic core of the micelles allows the incorporation of hydrophobic anticancer drugs, and it can load 8.8 wt% of paclitaxel. The paclitaxel-loaded HE-PRX-Phe-PBzMA polymeric micelles showed the anticancer effect against HeLa cells in vitro. The outermost PRX layer of the polymeric micelles has potentiality to enhance the target recognition via the installation of ligand molecules and would be applicable for active targeting of the drugs.

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Table 1. Characterization of PRX-PBzMA triblock copolymers with various PBzMA chain lengths.

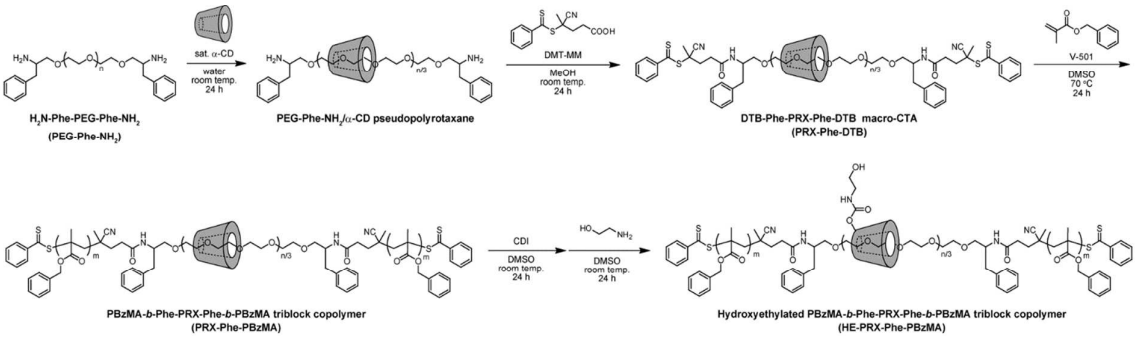
Run	In feed		PRX-PBzMA triblock polymer			
	[BzMA]/[DTB group] molar ratio[a]	Theor. M_n of PBzMA[b]	M_n of single PBzMA[c]	M_n of PRX-PBzMA[c]	M_w/M_n[d]	Number of threading α-CDs[c]
1	40 (20)	3,520	2,700	42,500	1.30	25.2
2	60 (30)	5,290	5,650	48,300	1.31	25.7
3	100 (50)	8,810	11,800	60,600	1.28	22.1

[a] values in the parenthesis are feed [BzMA]/[PRX macro-CTA] molar ratio.

[b] theoretical molecular weight of single PBzMA chain.

[c] Determined by ^{1}H NMR in DMSO-d_6.

[d] Determined by SEC in DMSO.



Scheme 1. Synthesis of PRX-Phe-PBzMA triblock copolymers and the following hydroxyethyl group modification.

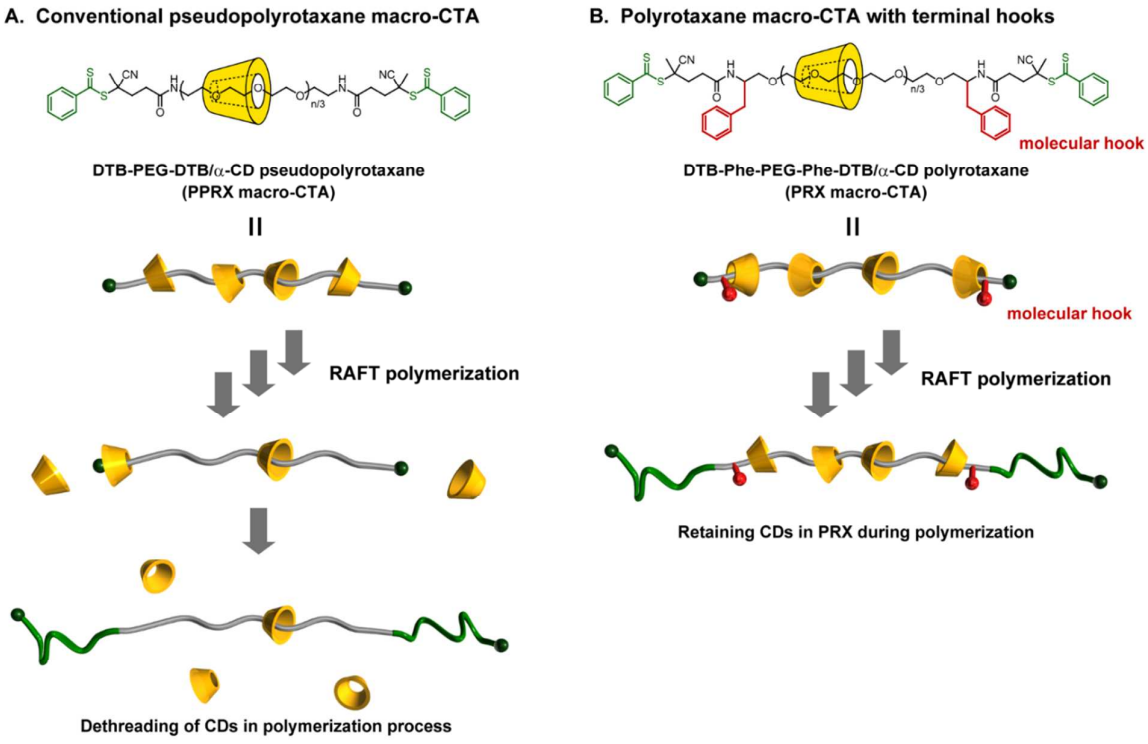


Figure 1. Synthetic method of PRX-containing triblock copolymers by macro-CTA with conventional terminals (A) and terminal molecular hooks (B).

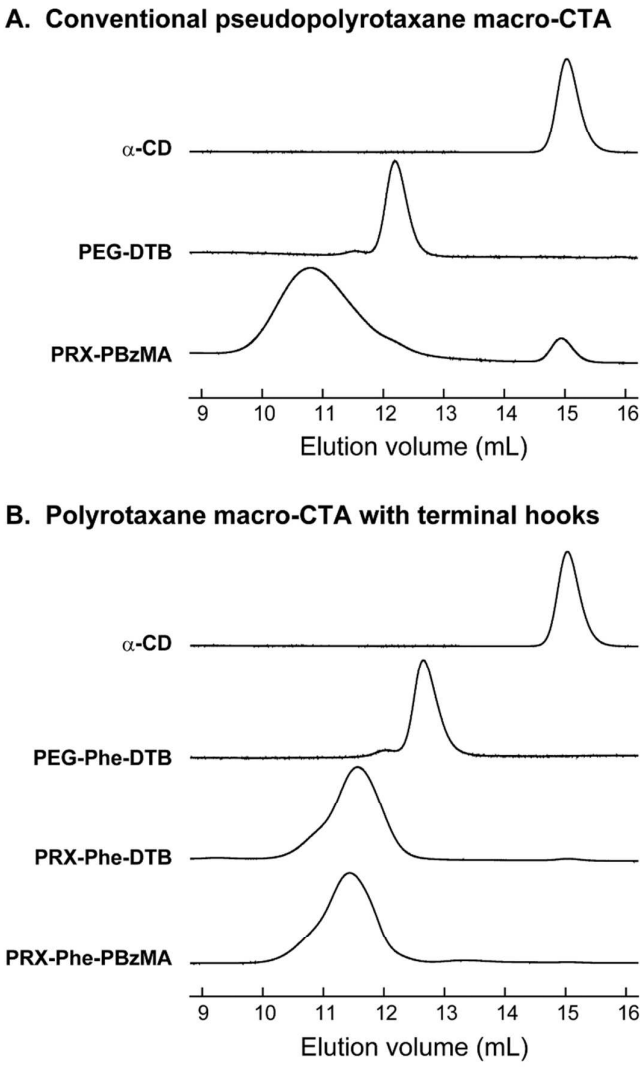


Figure 2. (A) SEC charts of α-CD, PEG-DTB, and PRX-PBzMA synthesized from pseudopolyrotaxane macro-CTA with conventional terminals. (B) SEC charts of α-CD, PEG-Phe-NH_2, PRX-Phe-DTB macro-CTA with terminal phenylalanyl groups, and PRX-Phe-PBzMA (run 1 in Table 1) synthesized by PRX macro-CTA method (B).

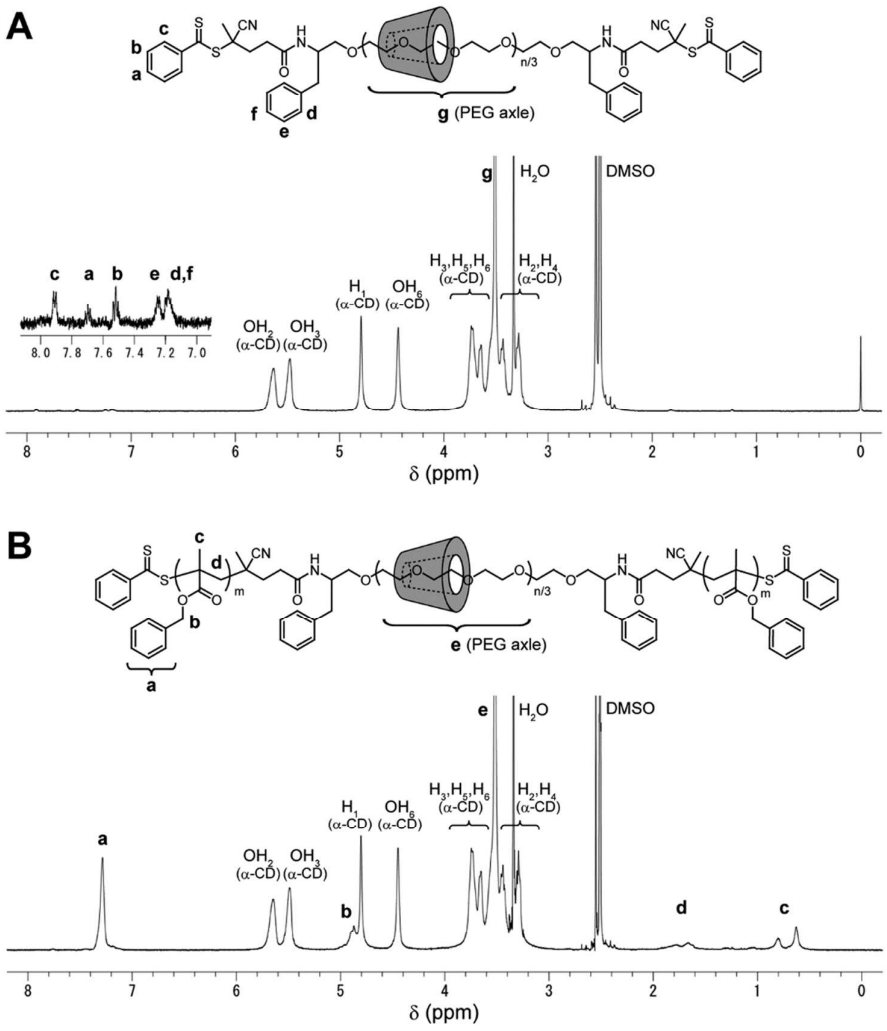


Figure 3. ^{1}H NMR spectra of PRX-Phe-DTB macro-CTA (A) and PBzMA-*b*-Phe-PRX-Phe-*b*-PBzMA (B, run 1 in Table 1).

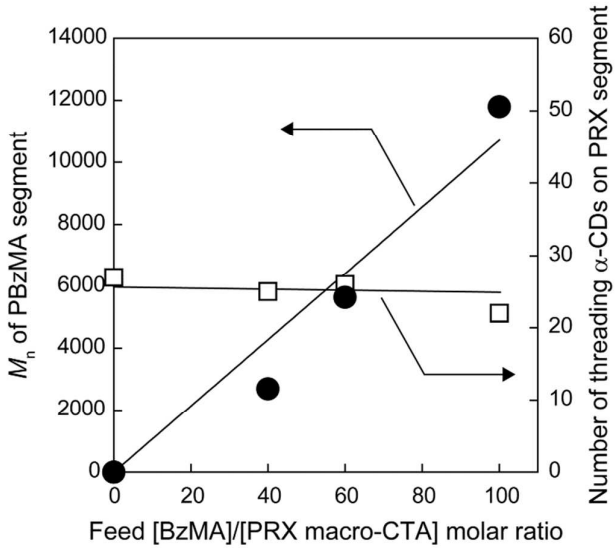


Figure 4. Change in the M_n of single PBzMA chain and the number of threading α-CDs in PRX-Phe-PBzMA triblock copolymers synthesized at various feed [BzMA]/[PRX macro-CTA] molar ratios.

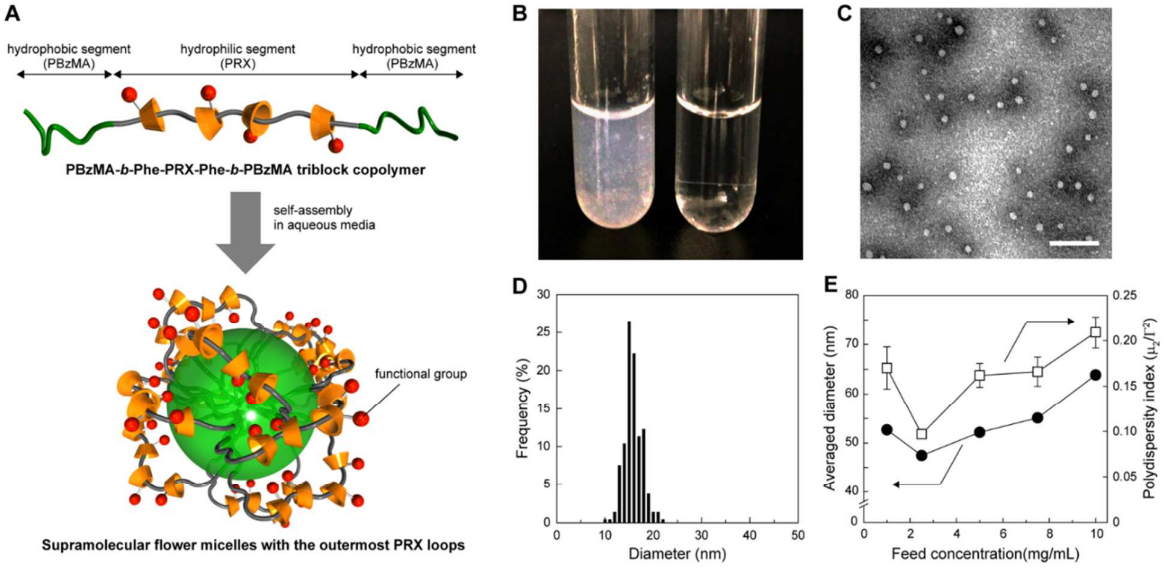


Figure 5. (A) Schematic illustration of the supramolecular flower micelle formation of PRX-Phe-PBzMA triblock copolymers with the outermost loops of PRX segments. (B) Photograph of aqueous solutions of PRX-Phe-PBzMA (left) and HE-PRX-Phe-PBzMA (right) after dialysis from DMSO. (C) TEM image of HE-PRX-Phe-PBzMA micelles (scale bar: 100 nm). (D) Size distribution of HE-PRX-Phe-PBzMA micelles determined from TEM images (n = 211). (E) Averaged diameter (closed circles) and polydispersity index (open squares) variations of HE-PRX-Phe-PBzMA micelles prepared at various concentrations determined by DLS.

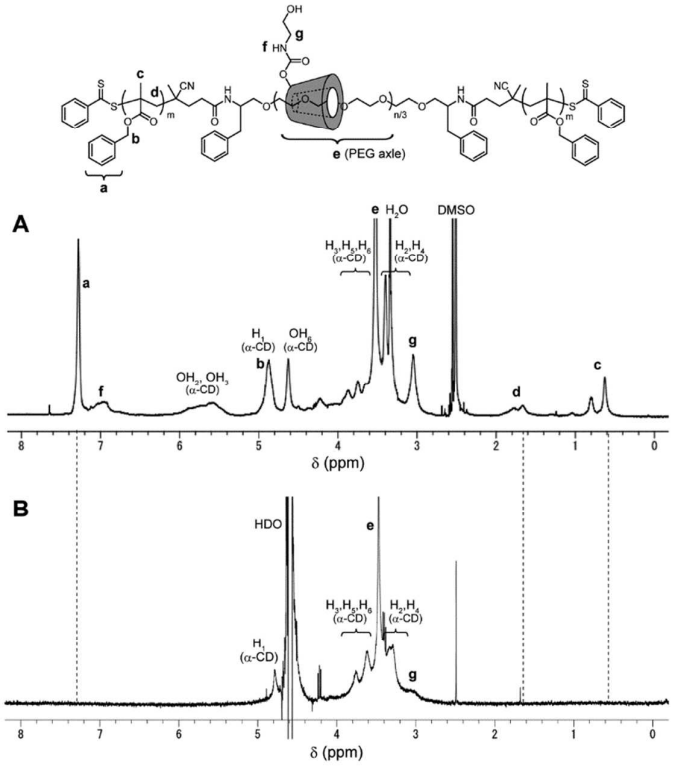


Figure 6. ^{1}H NMR spectra of HE-PRX-Phe-PBzMA in DMSO-d_6 (A) and D_2O (B).

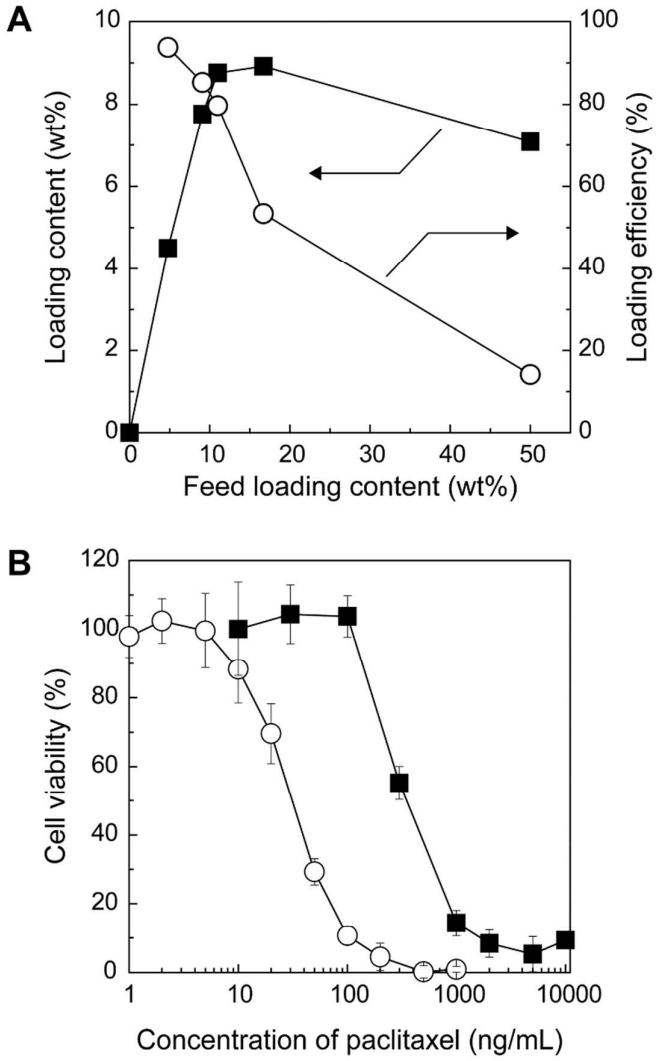
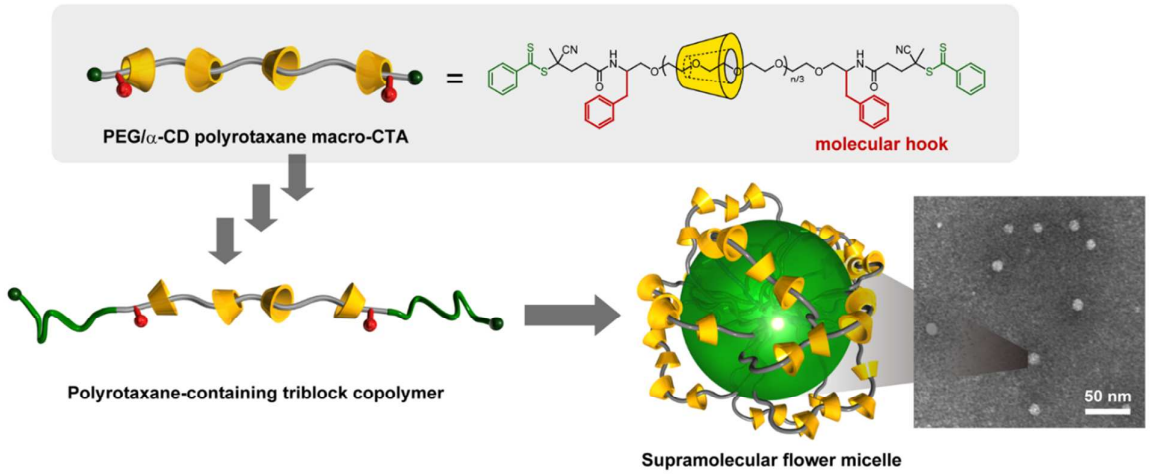
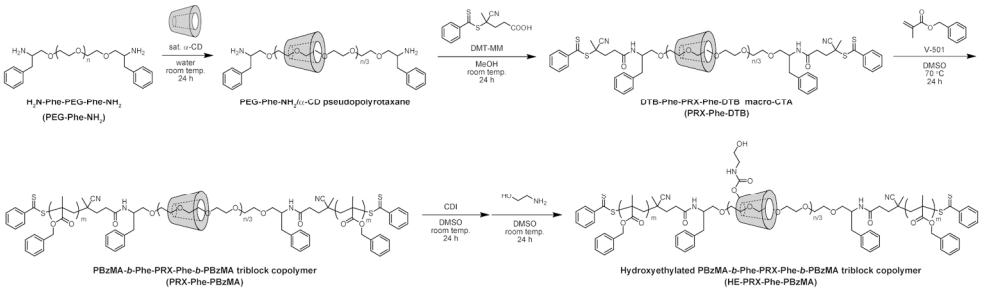


Figure 7. (A) Loading content (closed squares) and loading efficiency (open circles) of paclitaxel into HE-PRX-Phe-PBzMA micelles prepared at various feed loading contents. (B) Relative viability of HeLa cells treated with paclitaxel (open circles) and paclitaxel-loaded HE-PRX-Phe-PBzMA micelles (closed squares, paclitaxel loading content of 8.8 wt%) at various concentrations for 24 h.

Graphical abstract



The precise synthetic method of polyrotaxanes (PRX)-containing triblock copolymers was achieved using a PRX macro-chain transfer agent with terminal hooks. Also, their self-assembly behavior to form polymeric micelles with the outermost PRX layer were investigated.



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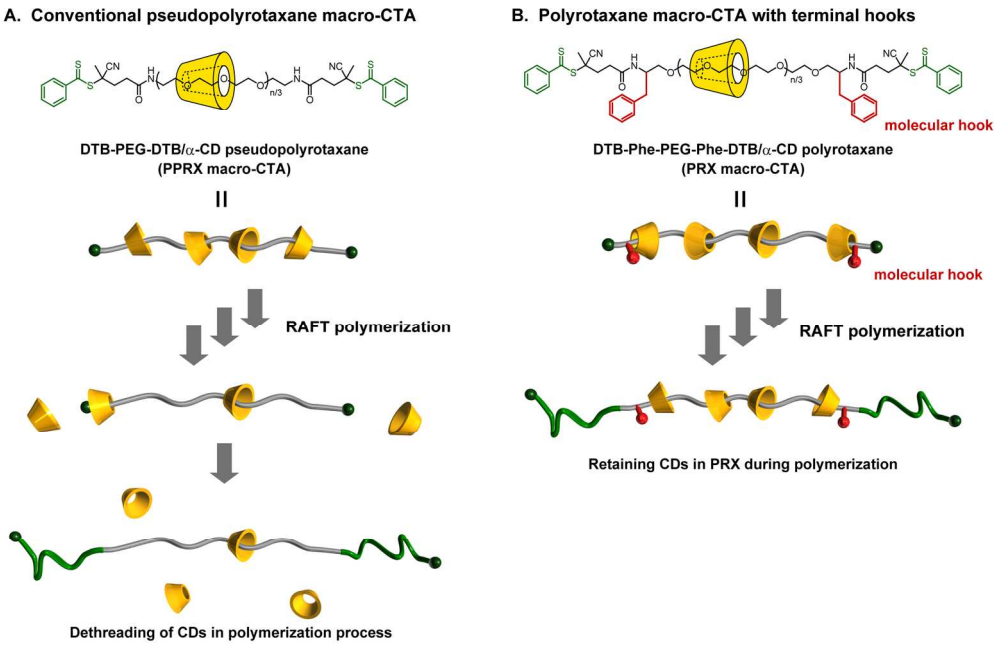

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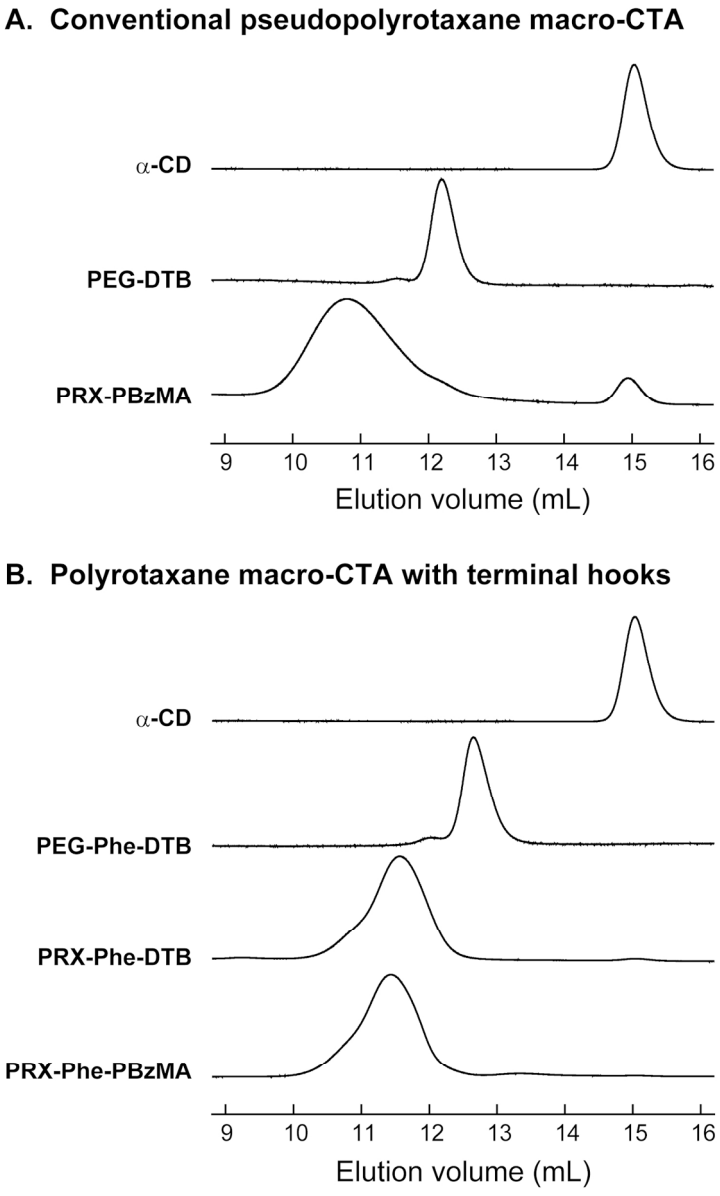

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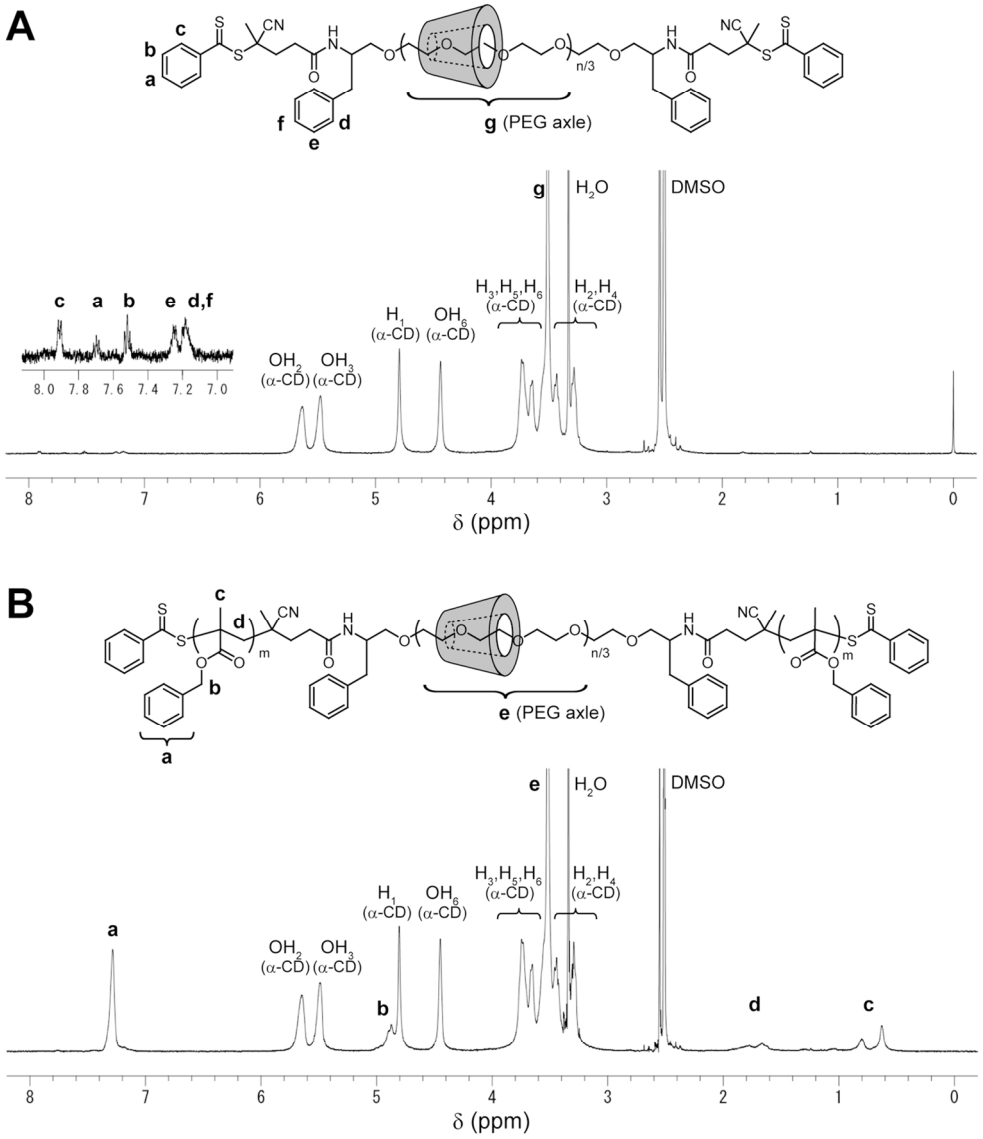

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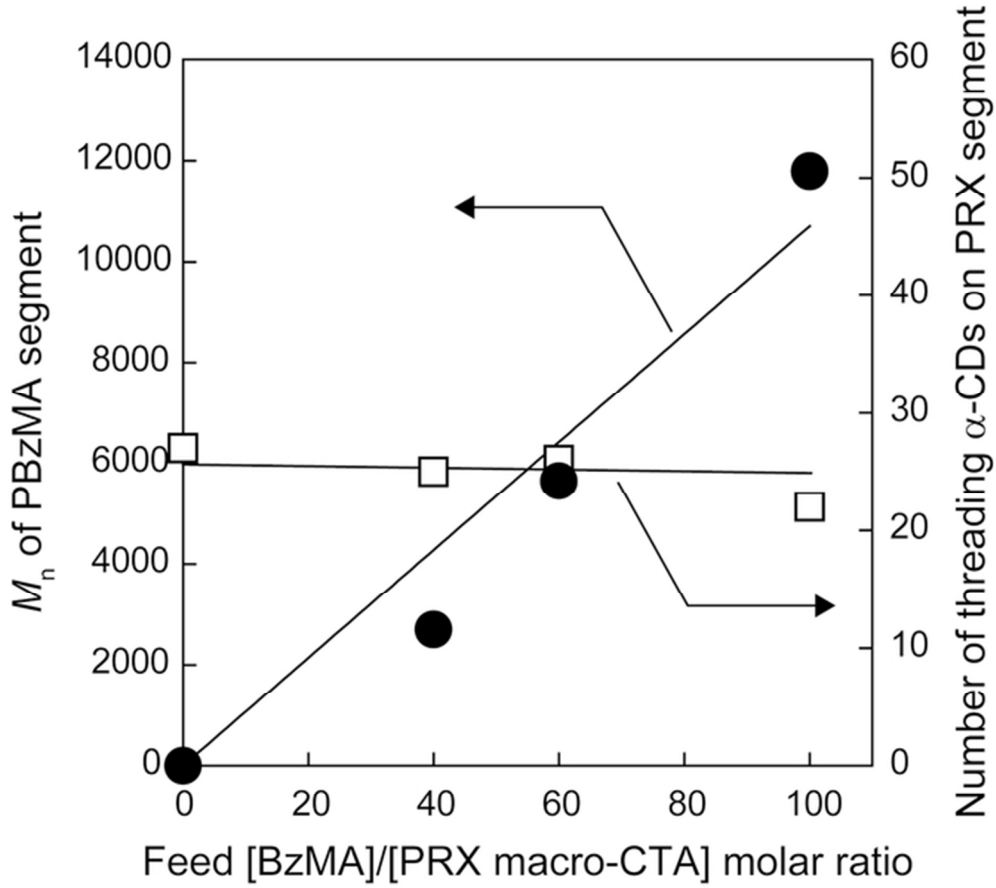

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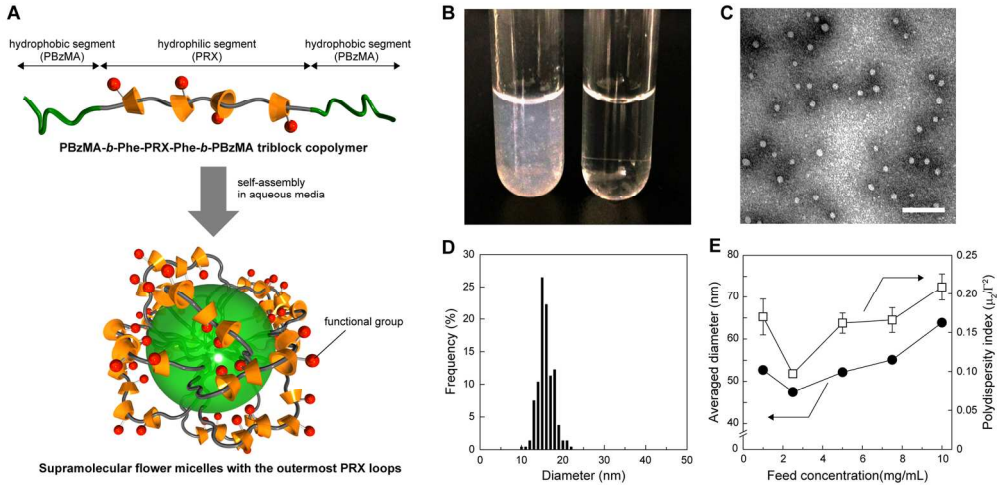

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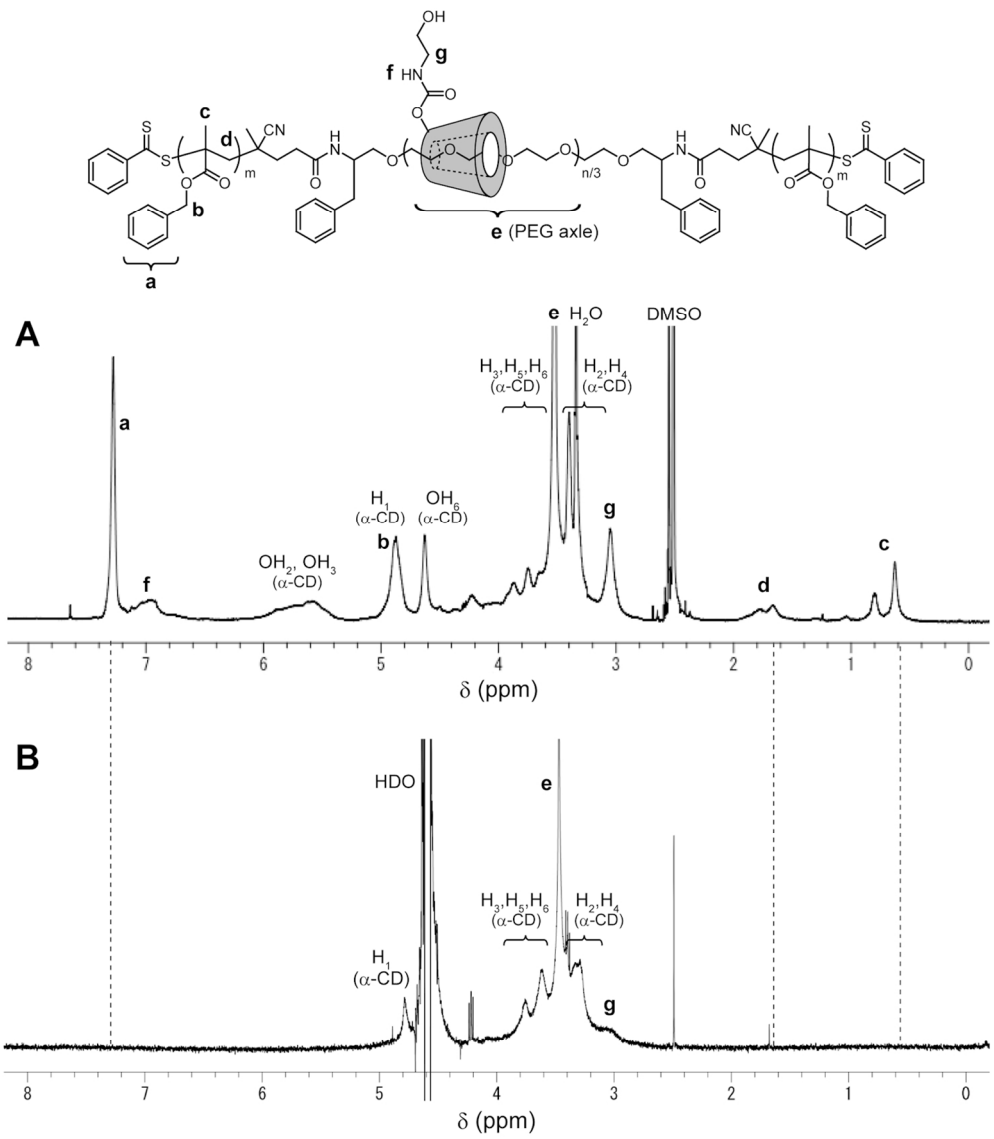

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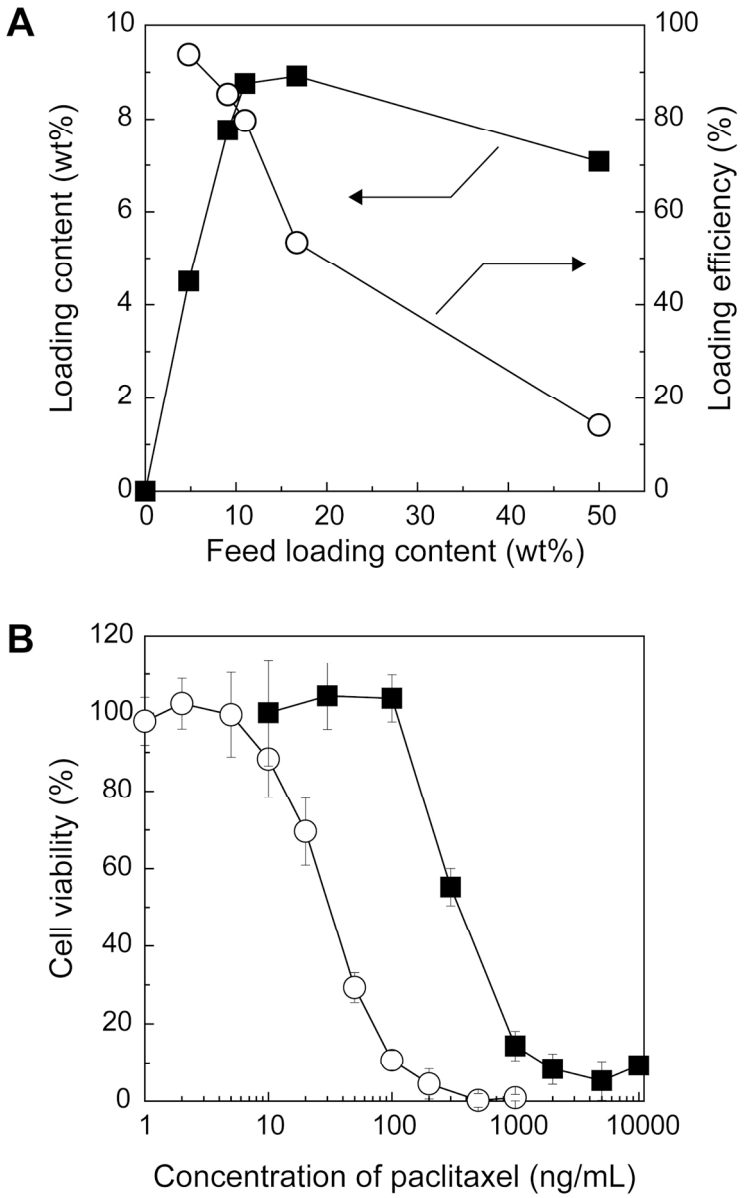

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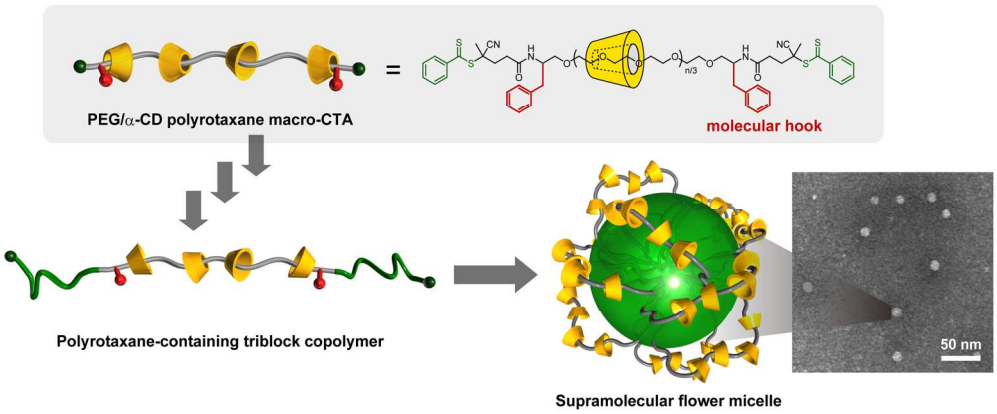

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