Synthesis of β-Cyclodextrin Containing Copolymer via "Click" Chemistry and Its Self-Assembly in the Presence of Guest Compounds

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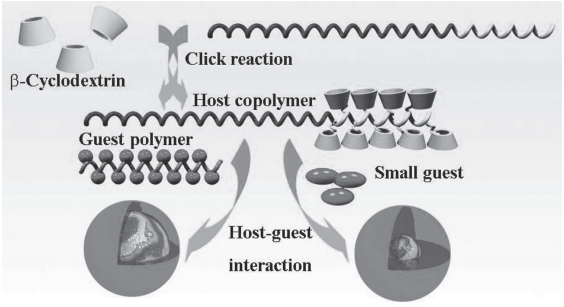
We report the synthesis of a hydrophilic copolymer with one polyethylene glycol (PEG) block and one β-cyclodextrin (β-CD) containing block by a "click" reaction between azido-substituted β-CD and propargyl flanking copolymer. ^{1}H NMR study suggested a highly efficient conjugation of β-CD units by this approach. The obtained copolymer was used as a host macromolecule to construct assemblies in the presence of hydrophobic guests. For assemblies containing a hydrophobic polymer, their size can be simply adjusted by simply changing the content of hydrophobic component. By serving as a guest molecule, hydrophobic drugs can also be loaded accompanying the formation of nanoparticles, and the drug payload is releasable. Therefore, the copolymer synthesized herein can be employed as a carrier for drug delivery.



1. Introduction

Nano- and microstructured polymer assemblies have attracted tremendous attention in recent years for their wide applications such as drug delivery, gene therapy, diagnostics, and imaging.[1–4] Among these diverse assemblies including micelles, vesicles, toroidal aggregates, and polymer nanofibers, core–shell structured polymer micelles have been intensively studied for the delivery of hydrophobic drugs during the last two decades.[1,3,5] Polymeric micelles are generally assembled in an aqueous solution utilizing the hydrophobic interactions between core-forming segments of amphiphilic copolymers. Macromolecular amphiphiles with various molecular architectures have been employed to construct micellar formulations.[6] On the other hand, it has been well demonstrated that noncovalent forces such as electrostatic, hydrogen-bonding, and coordination interactions can be adopted to assemble micelles, which are useful for the delivery of a broad spectrum of compounds, including genes, proteins, low molecular weight drugs and imaging agents.[3,4,7]

More recently, there is an increasing interest to develop functional materials via host–guest interactions that involve a complementary stereoelectronic arrangement of binding sites in host and guest molecules.[8,9] As well-documented, introducing host molecules into assemblies may conveniently enable additional surface functionalization.[10,11] Furthermore, chemical-responsive biosensing,

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release or therapy can be easily achieved due to the reversibility of host–guest interactions.[12–16] For the core-shell structured nano-assemblies derived from the guest molecules mediated self-assembling of host polymers, the core attributes can be conveniently tailored by changing the guest component and its content.[15,17] The delivery profiles can, therefore, be modulated with great flexibility. Cyclodextrins (α, β, or γ-CDs) and cucurbiturils are two broadly investigated host systems.[18] Various entities at different scales, such as nanomicelles,[19,20] nanoparticles,[12,21] vesicles,[22] nano- and microcapsules,[23] hydrogels,[13,24] and hybrid systems,[14,25] have been developed based on the molecular recognition for diverse applications, using either cyclodextrins or cucurbiturils. We have observed the formation of polymer assemblies by a double hydrophilic copolymer (PEG-*b*-PCD) with one polyethylene glycol (PEG) block and one β-cyclodextrin (β-CD) containing segment in the presence of guest compounds.[8] The involved guest compounds can vary from lipophilic small molecules to macromolecules. For the synthesis of PEG-*b*-PCD copolymers, a nucleophilic reaction between excess 6-monotosyl β-CD and a copolymer containing flanking amino groups was employed.[19] However, this reaction is time consuming, and more often results in low conjugation efficiency. This is especially true with a longer PCD chain. On the other hand, due to the advantages of quantitative and rapid reaction, mild reaction conditions, as well as functional group tolerance, the copper(I)-catalyzed Huisgen [3 + 2] dipolar cycloaddition between alkyne and azide, referred to the click reaction,[26] has been broadly adopted as a powerful method to synthesize functional polymers of diverse architectures.[27] More recently, this efficient reaction has been employed to synthesize cyclodextrin-containing functional materials for various applications.[28] Herein, a "click" chemistry approach has been adopted to synthesize the copolymer with one PEG block and one β-CD flanking block. Self-assembly behaviors of the newly prepared copolymer in the presence of guest molecules were examined as well.

2. Experimental Section

2.1. Materials

L-Aspartic acid β-benzyl ester was purchased from Sigma (USA). Triphosgene was obtained from Fisher (USA). α-Methoxy-ω-amino-polyethylene glycol (MPEG-NH_2) with an average molecular weight ($\overline{M}_w$) of 5000 was purchased from Laysan Bio, Inc. (USA), and used without further purification. Propargyl amine (PPA) and Amberlite® GT73 were purchased from Sigma (USA) and used as received. β-Cyclodextrin (β-CD, ≥98%) and dexamethasone (DMS) were purchased from Sigma–Aldrich Co. (USA) and used as received. Benzyl alcohol (BA) was obtained from J&K Scientific Ltd.

2.2. Synthesis of Mono-6-azido-β-CD

The method established by Baussanne et al. was employed to synthesize 6-monotosyl β-CD.[29] Mono-6-azido-β-CD was synthesized by the nucleophilic substitution of 6-monotosyl β-CD using 1.2-fold molar excess of sodium azide in DMF at 70 °C for 6 d. After the reaction solution was filtered through a 0.22 μm syringe filter, the product was precipitated from acetone.

2.3. Synthesis of Polyethylene Glycol-Block-Poly(β-benzyl L-aspartate)

β-Benzyl L-aspartate *N*-carboxyanhydride (BLA-NCA) was synthesized according to literature.[30] The reaction was performed by suspending 10 g of L-aspartic acid β-benzyl ester in 100 mL tetrahydrofuran (THF), heating the mixture to 50°C, and then adding 6.3 g triphosgene in 80 mL THF. The reaction mixture was stirred magnetically for 2 h at 50 °C under nitrogen atmosphere. The transparent solution was concentrated under reduced pressure and the obtained white oil was recrystallized three times from a mixture of THF/petroleum ether and dried at room temperature under vacuum. The polyethylene glycol-block-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) copolymer was synthesized as reported by Harada and Kataoka.[31] In brief, BLA-NCA was polymerized in DMF at 40 °C by the initiation of amino-terminated PEG (MPEG-NH_2) to obtain PEG-*b*-PBLA.

2.4. Synthesis of Polyethylene Glycol-Block-Polyaspartamide Containing PPA Units

A modified quantitative aminolysis reaction was employed to prepare polyethylene glycol-block-polyaspartamide containing PPA units [PEG-*b*-P[Asp(PPA)]] from PEG-*b*-PBLA.[32] In brief, 1.0 g PEG-*b*-PBLA was dissolved in dry dimethyl sulfoxide (DMSO) at 30 °C, into which twofold molar excess of PPA was added. After 48 h of reaction, the solution was dialyzed against deionized water, and the final aqueous solution was lyophilized to obtain yellow powder.

2.5. Synthesis of Polyethylene Glycol-Block-Polyaspartamide Containing β-CD Units (PEG-*b*-P[Asp(PPA-CD)]) by "Click" Chemistry

The Cu(I)-catalyzed azide–alkyne cycloaddition reaction was adopted to conjugate β-CD onto the side chain of P[Asp(PPA)] block of PEG-*b*-P[Asp(PPA)]. To this end, 100 mg PEG-*b*-P[Asp(PPA)] and 2 g mono-6-azido-β-CD was dissolved into 15 mL DMSO. Twenty-six milligram hydrated copper sulfate was added to this solution. After subsequent dropwise addition of a freshly prepared aqueous solution of sodium ascorbate (40 mg in 10 mL water), the mixture was stirred briefly at 70 °C to promote dispersion and was then stirred at room temperature until the solution became turquoise-blue (after about 6 h). The reaction mixture was then dialyzed against water. The crude product was further purified using Amberlite® GT73, a strong cation exchange resin. After lyophilization, a buff powder was obtained.

2.6. Synthesis of Poly(β-benzyl L-aspartate)

Poly(β-benzyl L-aspartate) (PBLA) was synthesized according to a reference.[33] In brief, 1.5 g BLA-NCA was dissolved in 30 mL anhydrous dioxane, into which appropriate amount of *n*-hexylamine was added to achieve a 20:1 molar ratio of monomer to initiator. Polymerization was performed at room temperature (22 °C) for 5 d. After being precipitated from diethyl ether, the polymer was dissolved in dichloromethane and precipitated from diethyl ether again. The resultant powder was dried under vacuum. The number-average molecular weight determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometer was about 2000.

2.7. Preparation of Host–Guest Assemblies Based on PEG-*b*-P[Asp(PPA-CD)]

Assemblies based on PEG-*b*-P[Asp(PPA-CD)] and PBLA were prepared by a dialysis method. Briefly, a mixture of PBLA and PEG-*b*-P[Asp(PPA-CD)] with a weight ratio of 6:15 was co-dissolved in DMSO at room temperature with a final copolymer concentration of 10 mg mL^{-1}. This solution was placed into a dialysis tubing (MWCO 6-8 kDa) for dialysis against deionized water for 24 h at 25 °C. The outer aqueous solution was renewed every 30 min for the first 2 h, and then every 5 h for the remaining period of time. The obtained assemblies were analyzed without additional treatment. For assemblies based on PEG-*b*-P[Asp(PPA-CD)] and a hydrophobic drug DMS, the same procedure was employed. For the DMS-containing assemblies, further characterization was performed after the dialysis solution was filtered through a 0.22 μm syringe filter.

2.8. In Vitro Release Study

Lyophilized assemblies containing DMS were dissolved into deionized water (10 mg mL^{-1}), from which 0.5 mL was placed into dialysis tubing and then immersed into 30 mL 0.01 M phosphate buffered saline (PBS) (pH 7.4). At predetermined time intervals, 4.0 mL of release medium was withdrawn, and fresh PBS was added. The DMS concentration in the release buffer was determined through UV spectroscopy at 265 nm.

2.9. Measurements

^1H, ^{13}C, and ^1H-^1H Reosy NMR spectra were recorded on a Varian INOVA-400 spectrometer operating at 400 MHz. Fourier transform infrared (FTIR) spectra were recorded on a Perkin-Elmer FTIR spectrometer (Spectrum GX). Gel permeation chromatography (GPC) measurement was performed on a Waters model 1515, equipped with a Waters 2414 refractive index detector. Deionized water containing 0.1% NaN_3 was used as the mobile phase at a flow rate of 1.0 mL min^{-1}. Molecular weights of polymers were calibrated with PEG standards. The MALDI-ToF mass measurement was performed with a Waters Micromass TofSpec-2E run in linear mode. Dynamic light scattering (DLS) measurements for the assemblies in aqueous solutions were performed with a Malvern Zetasizer Nano ZS instrument at 25 °C. Transmission electron microscopy (TEM) observation was carried out on a JEOL-3011 high resolution electron microscope operating at an acceleration voltage of 300 kV. Samples were prepared at 25 °C by dipping the grid into the aqueous solution of assemblies and the extra solution was blotted with filter paper. After the water was evaporated at room temperature for several days, the samples were observed directly without staining. Formvar-coated copper grids, stabilized with evaporated carbon film, were used. SEM images were taken on a field emission scanning electron microscope (XL30 FEG, Phillips) after a gold layer was coated using a sputter coater (Desk-II, Denton vacuum Inc., USA) for 100 s. Samples were prepared by coating aqueous solution of assemblies onto freshly cleaved mica, and water was evaporated at room temperature under normal pressure. Differential scanning calorimetry (DSC) measurement was performed on a TA Q2000 calorimeter under a conventional modulated DSC model. DSC curves were obtained from the first heating run at a rate of 2 °C min^{-1} under a nitrogen flow of 50 mL min^{-1}.

3. Results and Discussion

3.1. Polymer Synthesis

As shown in Scheme S1 (in Supporting Information), PEG-*b*-PBLA was synthesized using amino group initiated ring-opening polymerization of BLA-NCA. The ^1H NMR spectrum of this copolymer and the related signal assignments are illustrated in Figure 1a. The degree of polymerization (DP) of PBLA was calculated to be 70 based on this ^1H NMR spectrum. As well demonstrated by Kataoka's group, the flanking benzyl groups of PBLA can undergo a quantitative aminolysis reaction with various primary amino compounds.[32,34,35] Herein, a similar aminolysis reaction was employed to synthesize a diblock copolymer with one PEG block and one block containing propargyl groups. Figure S1 (Supporting Information) shows the FTIR spectra of PEG-*b*-PBLA and PEG-*b*-P[Asp(PPA)]. The disappearance of carbonyl absorption (at 1741 cm^{-1}) from benzyl ester groups as well as the appearance of amide carbonyl absorbance at 1665 cm^{-1} suggests the nearly complete aminolysis of PBLA block in the presence of PPA. In addition, the absorption at 2122 cm^{-1}, corresponding to the alkynyl, suggests the successful conjugation of propargyl groups. Figure 1b shows the ^1H NMR spectrum of PEG-*b*-P[Asp(PPA)]. The selected assignments of proton signals are also illustrated in this figure. The disappearance of the proton signal at 7.42 ppm due to aromatic protons suggest that almost all the benzyl ester groups were subjected to aminolysis reaction. Comparison of the intensity ratio of methylene protons from propargyl groups to ethylene protons from the PEG block indicated that the DP of the PPA-containing block was about 41. This is consistent with the GPC measurement which gave weight-averaged molecular weight ($\overline{M}_w$) of 13 kDa (Figure S2a, Supporting Information). The decreased DP of the PPA-containing segment

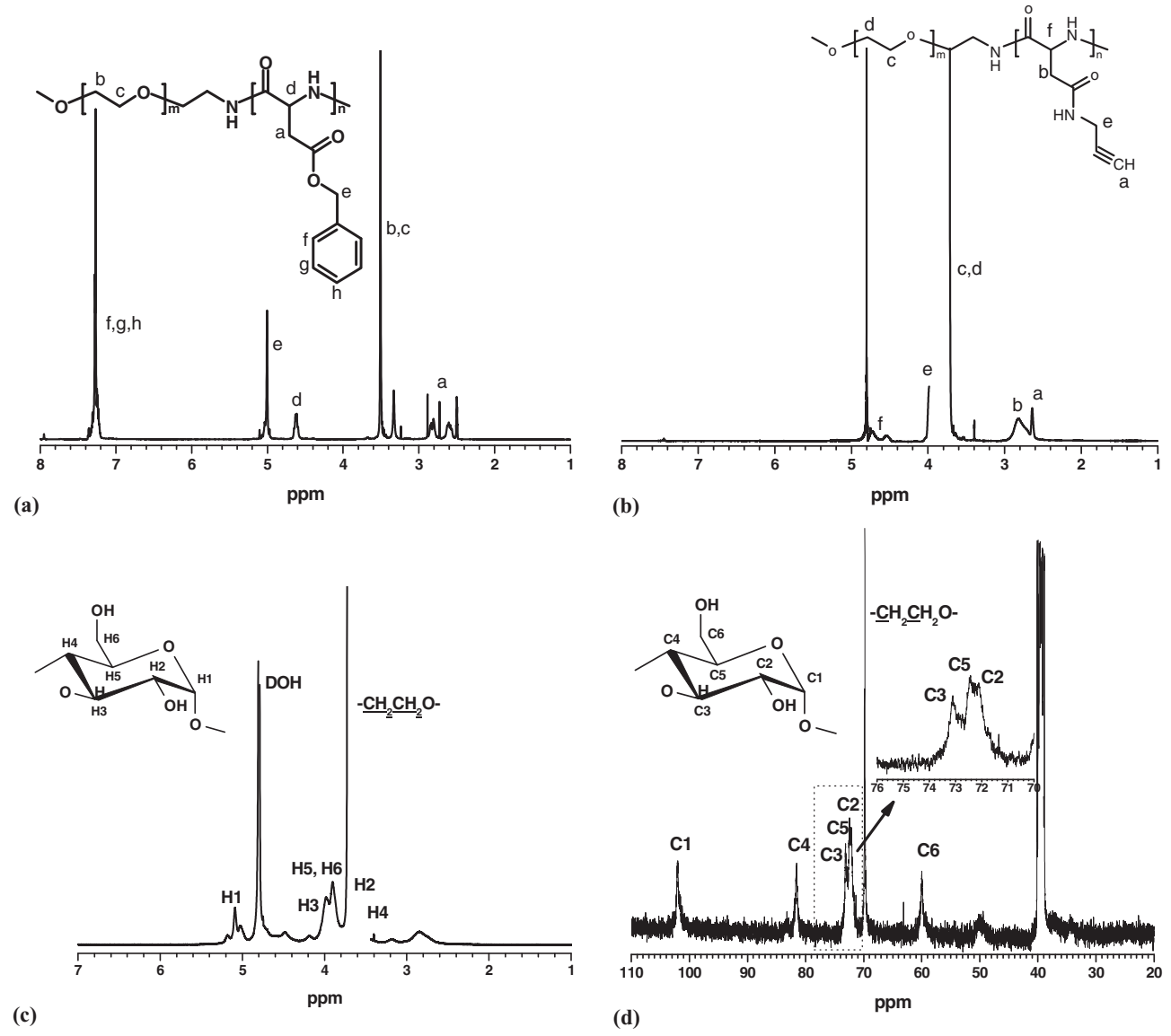

Figure 1. ^{1}H NMR spectra of (a) PEG-*b*-PBLA in DMSO-d_6, (b) PEG-*b*-P[Asp(PPA)] in D_2O, and (c) PEG-*b*-P[Asp(PPA-CD)] in D_2O; (d) ^{13}C NMR spectrum of PEG-*b*-P[Asp(PPA-CD)] in DMSO-d_6.

in PEG-*b*-P[Asp(PPA)] compared with that of the PBLA block revealed the presence of PBLA homopolymer in PEG-*b*-PBLA copolymer.[19] As demonstrated by Kataoka's group, aminolysis of PBLA by amino compounds under mild conditions has no dramatic contribution on the backbone degradation of PBLA.[35] Furthermore, no significant PBLA degradation due to aminolysis was observed in a separate experiment, where PBLA homopolymer was aminolyzed in the presence of propargyl amine under the same conditions as that employed for the PEG-*b*-PBLA aminolysis.

β-CD units were then conjugated onto the side chains of the P[Asp(PPA)] block via the copper(I)-catalyzed "click" reaction between the mono-6-azido-β-CD and PPA group. Figure 1c shows the ^{1}H NMR spectrum of the purified product. The presence of strong proton signals from β-CD units suggest the successful conjugation. ^{13}C NMR spectrum and the assignments shown in Figure 1d further confirmed the formation of the desired copolymer (a larger-scale image of the same ^{13}C NMR spectrum is illustrated in Figure S3, Supporting Information). In addition, calculation based on ^{1}H NMR spectrum (Figure 1c) revealed that the DP of the β-CD-containing block was 40. The GPC profile shown in Figure S2b (Supporting Information) also revealed the dramatically increased molecular weight, with an of about 45 kDa. Of note, the side peaks in the GPC curve of PEG-*b*-P[Asp(PPA-CD)] might be due to the presence of unconjugated or unreacted β-CD derivatives because excess amount of mono-6-azido-β-CD was

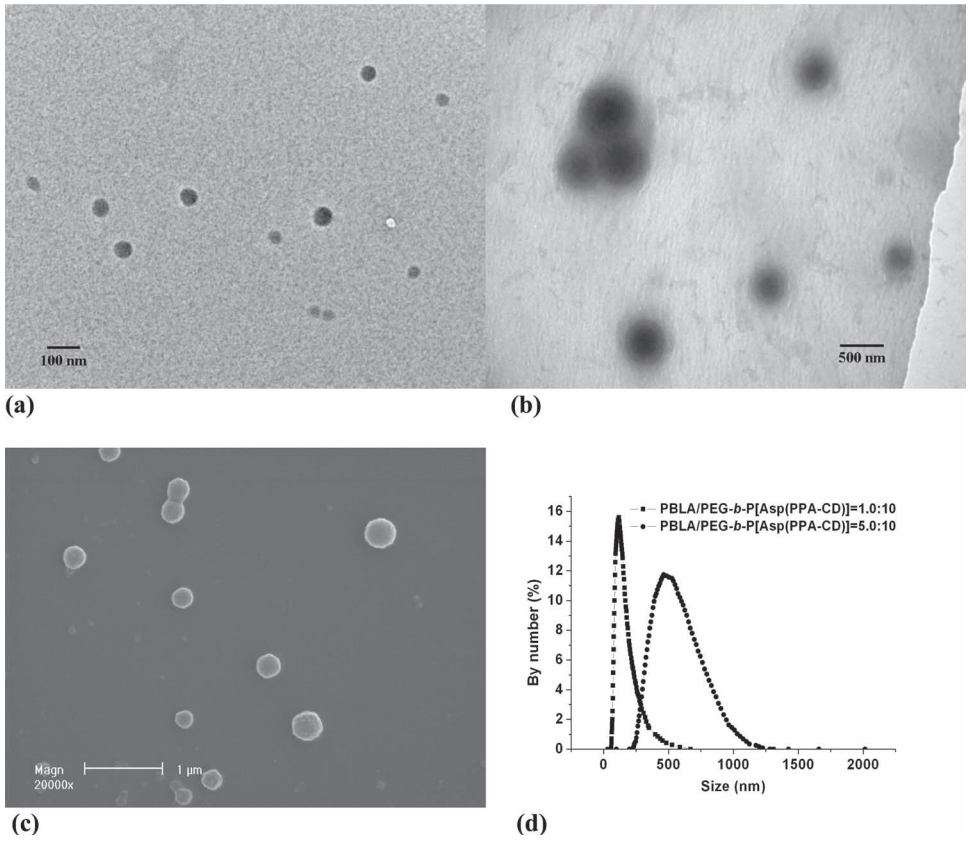

Figure 2. TEM images of assemblies based on PBLA and PEG-*b*-P[Asp(PPA-CD)] of various weight ratios (a) 1:10 and (b) 5:10; (c) SEM image showing the assemblies derived from a formulation with PBLA/PEG-*b*-P[Asp(PPA-CD)] of 5:10. (d) Size distribution of assemblies originated from PBLA and PEG-*b*-P[Asp(PPA-CD)].

used during "click" process. However, further experiments are necessary to convincingly elucidate this point. A comparison of the DP of the β-CD and PPA containing segment suggested that an almost completely quantitative reaction occurred. This further demonstrated that the employed "click" chemistry approach is highly efficient for the synthesis of the β-CD-containing block copolymer PEG-*b*-P[Asp(PPA-CD)].

3.2. Assemblies Containing Hydrophobic Polymer

Copolymer PEG-*b*-P[Asp(PPA-CD)] can be easily dissolved in water. DLS determination suggests that this copolymer cannot undergo self-association in an aqueous solution, which is independent of polymer concentration (Figure S4, Supporting Information). This agrees with previous study that cyclodextrin-containing polymers are generally highly water-soluble macromolecules other than amphiphilic ones.[10,36] This may be due to the fact that the hydrogen-bonding interaction between hydroxyl groups of β-CD units and water molecules is stronger than that among the hydroxyl groups in β-CD groups. As a preliminary study, a hydrophobic polymer PBLA was employed to explore the guest macromolecule-mediated self-assembling profiles of the newly synthesized PEG-*b*-P[Asp(PPA-CD)]. A dialysis procedure was performed to fabricate the assemblies containing both PBLA and PEG-*b*-P[Asp(PPA-CD)]. Figure 2a and b show the TEM images of assemblies prepared with various PBLA/PEG-*b*-P[Asp(PPA-CD)] ratios. Clearly, spherical assemblies can be achieved independent of PBLA content. Assemblies with a PBLA/PEG-*b*-P[Asp(PPA-CD)] ratio of 5:10 were also observed by SEM, as shown in Figure 2c. This result is consistent with TEM observation in terms of both size and morphology. However, the size of assemblies increased significantly when the PBLA content was increased as revealed by images shown in Figure 2a and b. DLS determination indicated the mean sizes to be 142 and 530 nm for assemblies prepared with PBLA/PEG-*b*-P[Asp(PPA-CD)] ratios of 1:10 and 5:10, respectively (Figure 2d). All these results demonstrated the formation of spherical assemblies by PEG-*b*-P[Asp(PPA-CD)] in the presence of hydrophobic PBLA. The driving force for the formation of these types of assemblies should be host–guest interactions between the CD units of the copolymer and the benzyl groups on the PBLA side chains. As shown in Figure 3a, a clear up-field shift of proton signals corresponding to the

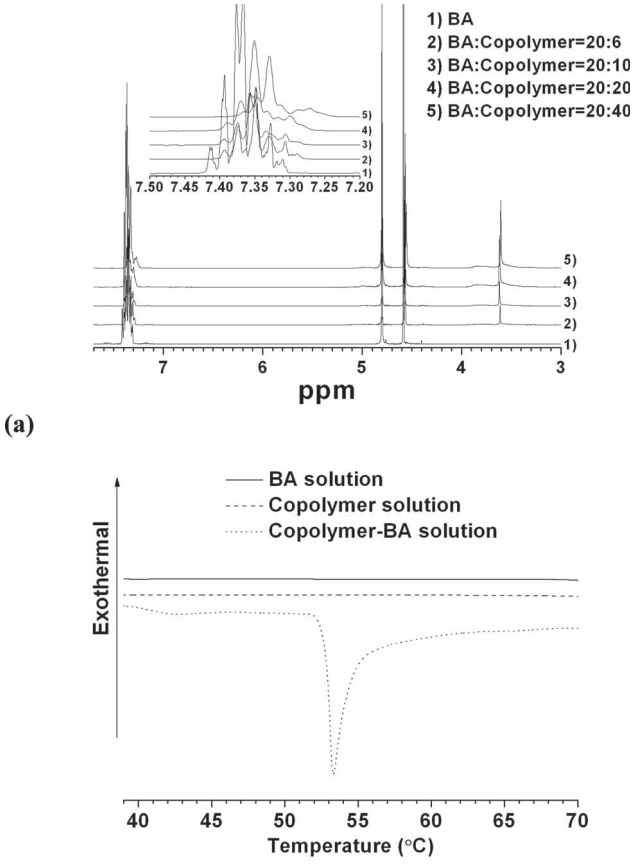

Figure 3. (a) ^{1}H NMR spectra of benzyl alcohol (BA) in the presence of various contents of copolymer PEG-*b*-P[Asp(PPA-CD)], and the weight ratios of BA/copolymer are listed in the inset. All the spectra were acquired at room temperature using D_2O as solvent. (b) DSC curves of BA, PEG-*b*-P[Asp(PPA-CD)], or the mixture of BA and copolymer in deionized water. In the BA-copolymer mixture, its weight ratio was 1:1. It should be noted that the BA or copolymer solution contained the same amount of BA or copolymer as that in the BA-copolymer mixture.

aromatic protons of benzyl group could be discerned when the content of PEG-*b*-P[Asp(PPA-CD)] was increased. This change in the chemical shift of guest molecules was demonstrated to be related to the inclusion complexation in the presence of cyclodextrins.[37] This can be further substantiated by the correlation signals between BA and the β-CD unit in the copolymer, as illustrated in the ^{1}H-^{1}H Roesy spectrum of Figure S5 (Supporting Information). Additional evidence was provided by the DSC curves shown in Figure 3b. Whereas no obvious endo- or exothermal processes could be found for either BA or copolymer solution, a significant endothermal peak appeared in the case of BA-copolymer mixture. These results strongly support the presence of inclusion interactions between BA and PEG-*b*-P[Asp(PPA-CD)] copolymer. For the PBLA/PEG-*b*-P[Asp(PPA-CD)] assemblies, PBLA should be the main component of the cores due to its lipophilic nature, while the hydrophilic segments, especially the PEG blocks would serve as a hydrophilic shell to endow the nanoparticles with colloidal stability.

Information on the microviscosity of the inner core of assemblies was provided by ^{1}H NMR spectra. After PBLA/PEG-*b*-P[Asp(PPA-CD)] assemblies fabricated by dialysis were lyophilized, the dried sample was dissolved into D_2O, and the ^{1}H NMR spectrum was acquired. The same sample was subjected to ^{1}H NMR measurement after it was lyophilized and dissolved in DMSO-d_6. As shown in Figure S6 (Supporting Information), no proton signals corresponding to PBLA can be observed for assemblies in D_2O. Even the proton signals from CD units were weakened in this case, suggesting that the CD-containing block should also participate in the core formation. However, signals at 7.3 and 5.0 ppm that are characteristic peaks of protons related to benzyl group were evident in DMSO-d_6. This reveals that the cores of the mentioned assemblies are mainly comprised PBLA chains with limited mobility, and therefore these assemblies possess a rigid core.

3.3. Formation of Assemblies Mediated by Small-Molecule Drug

The above results demonstrated the formation of assemblies by PEG-*b*-P[Asp(PPA-CD)] in the presence of a hydrophobic guest polymer. To show whether assemblies can also be formed in the presence of small molecules, we selected DMS, a steroidal anti-inflammatory drug, as the model compound. Again, the dialysis method was employed to prepare the assemblies containing DMS. As shown in Figure 4a, the nanoparticle formation was confirmed by TEM observation. DLS determination illustrated in the inset of Figure 4a showed the number-averaged size of assemblies to be 33 nm. UV measurement indicated the DMS content to be 6.4%. These results demonstrated that the drug loading and nanoparticle formation could be simultaneously achieved using the copolymer PEG-*b*-P[Asp(PPA-CD)]. As well documented, inclusion interactions exist between β-CD and DMS.[38] This host–gust recognition should be responsible for the formation of nanoparticles. By interacting with DMS molecules, PEG-*b*-P[Asp(PPA-CD)] may became a pseudo-amphiphile, which in turn results in self-assembly in an aqueous solution. Additional DMS molecules can be encapsulated via the hydrophobic interactions.

For a drug delivery system, release of the loaded therapeutics is an important parameter. An in vitro release study was carried out to examine whether the drug payload can be released. Figure 4b shows the release kinetics of the DMS assemblies. A biphasic profile with one rapid release stage followed by a sustained phase can be observed. The first stage may result from the rapid release

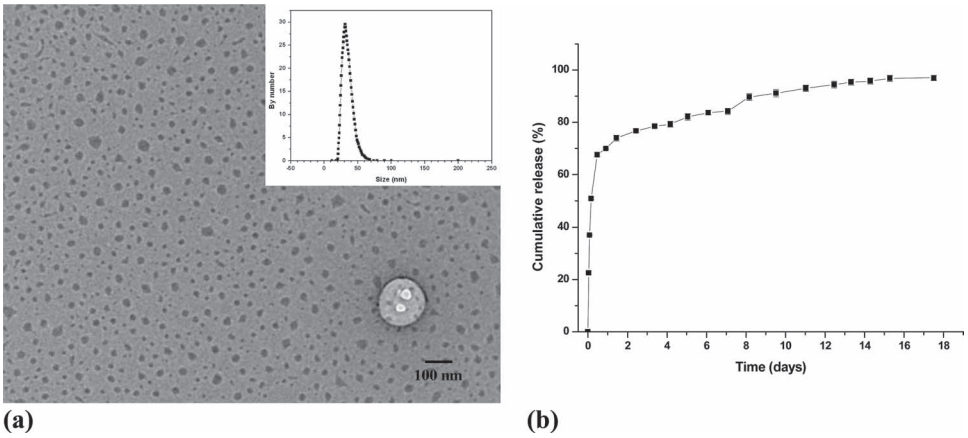

Figure 4. (a) TEM image of DMS containing assemblies based on PEG-*b*-P[Asp(PPA-CD)], and the inset showing the size distribution of the same assemblies in an aqueous solution determined by DLS. (b) In vitro release of DMS-containing assemblies. DMS loading content was 6.4%.

of drug molecules associated in the nanoparticles by hydrophobic interaction alone. The release of molecules interacting with CD units may contribute to the sustained release stage.

4. Conclusions

A double hydrophilic copolymer with both PEG and β-CD containing blocks was synthesized via a "click" chemistry approach. Analysis based on ^{1}H and ^{13}C NMR, FTIR, and GPC demonstrated the high-efficiency conjugation of CD units onto the side chain of the PPA containing block. The copolymer prepared was employed as a host polymer to fabricate assemblies containing various guest compounds (either a polymer or a small molecule). TEM and SEM observation combined with DLS determination showed the formation of nanoscaled assemblies in all of these cases. Using a hydrophobic drug as the guest molecule, we also demonstrated that the payload can be released, indicating the potential applications of the newly synthesized copolymer for drug delivery.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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