Synthesis and Micellar Characterization of Novel Amphiphilic A−B−A Triblock Copolymers of *N*-(2-Hydroxypropyl)methacrylamide or *N*-Vinyl-2-pyrrolidone with Poly(ϵ-caprolactone)

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ABSTRACT: Two novel A−B−A type amphiphilic triblock copolymers, namely poly(*N*-(2-hydroxypropyl)-methacrylamide)-*block*-poly(ϵ-caprolactone)-*block*-poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA-*b*-PCL-*b*-PHPMA) and poly(*N*-vinyl-2-pyrrolidone)-*block*-poly(ϵ-caprolactone)-*block*-poly(*N*-vinyl-2-pyrrolidone) (PVP-*b*-PCL-*b*-PVP), were synthesized and characterized. These polymers were prepared by free radical polymerization of *N*-(2-hydroxypropyl)methacrylamide and *N*-vinyl-2-pyrrolidone in the presence of a novel biodegradable, macromolecular chain-transferring agent, α,ω-poly(ϵ-caprolactone) dithiol (HS-PCL-SH). All triblock copolymers self-assembled in aqueous solutions to form supramolecular aggregates of 30−200 nm size. The critical aggregation concentration of the polymers ranged from 1 to 4 mg/L. The partition equilibrium constant (K_v) of pyrene in the hydrophobic core of micelles was comprised between 2.5×10^5 and 4.2×10^5. The triblock copolymer micelles were loaded by a dialysis procedure with 1−4% (w/w) of two model poorly water-soluble drugs, i.e., doxorubicin and amphotericin B. These triblock copolymers could prove useful as nanocarriers for the solubilization and transport of hydrophobic drugs. The bifunctional macromolecular chain-transferring agent reported in this work can also find application in the synthesis of a variety of novel A−B−A type biodegradable triblock copolymers.

Introduction

Polymeric micelles based on block copolymers comprising hydrophilic biocompatible and hydrophobic biodegradable segments are attracting much attention as nanosized carriers of poorly water-soluble drugs. These micelles can facilitate the solubilization of poorly water-soluble drugs, increase their circulation time, and possibly target them passively to tumoral tissues.[1−3] Several amphiphilic diblock copolymers, including monomethoxy poly(ethylene glycol)-*block*-poly(DL-lactide) (MPEG-*b*-PDLLA),[4] monomethoxy poly(ethylene glycol)-*block*-poly(ϵ-caprolactone) (MPEG-*b*-PCL),[5] and monomethoxy poly(ethylene glycol)-*block*-poly(β-benzyl L-aspartate) (MPEG-*b*-PBLA),[6] have been studied for micellar drug delivery. Among the different drug molecules that have been loaded in block copolymer micelles, one can cite paclitaxel,[7] testosterone,[8] indomethacin,[9] FK 506,[10] L-685, 818,[10] dihydrotestosterone,[11] amphotericin B,[12] doxorubicin,[13] and KRN.[14] In some cases, the incorporation of drugs into polymeric micelles has resulted in increased efficacy or decreased side effects.

To date, PEG has been the preferred choice for the hydrophilic segment imparting colloidal stability to block copolymer micelles in water. However, under certain conditions, PEG can promote the aggregation of nanoparticles after freeze-drying.[15] Besides, structural variation of outer hydrophilic shells to produce micelles that can interact with many different biological environments is highly desirable. Recently, Benahmed et al.[16] reported novel poly(*N*-vinyl-2-pyrrolidone)-*block*-poly(DL-lactide) (PVP-*b*-PDLLA) micelles, which take advantage of the PVP shell being both lyoprotectant[17] and cryoprotectant.[18] Also PVP, owing to its amphiphilic nature, is capable of interacting with a variety of compounds.[19a,b] On the other hand, Jeong et al.[20a,b] reported the use of poly(2-ethyl-2-oxazoline) (PEtOz) as the shell-forming polymer in poly(2-ethyl-2-oxazoline)-*block*-poly(DL-lactide) (PEtOz-*b*-PDLLA), poly(2-ethyl-2-oxazoline)-*block*-poly(ϵ-caprolactone) (PEtOz-*b*-PCL), and poly(2-ethyl-2-oxazoline)-*block*-poly(1,3-trimethylene carbonate) (PEtOz-*b*-PTMC). The hydrophilic shells in the above-described micelles form hydrogen-bonded complexes with poly(acrylic acid) that can dissociate above pH 3.9.

Poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) is a hydrophilic, nonimmunogenic, and biocompatible polymer.[21] It has been demonstrated that anticancer drugs conjugated to PHPMA can exhibit stronger antitumor effects than free drugs. Indeed, PK1 and PK2 are doxorubicin-conjugated PHPMA prodrugs that are now in clinical trials.[21] In view of these results, amphiphilic block copolymer micelles based on PHPMA and biodegradable polymers would be desirable. In fact, free PHPMA has been used as one of the components of poloxamer micelle-based chemotherapy liquid compositions.[22] Moreover, block and graft copolymers of PHPMA with poly(L-lysine)[23]and poly(2-(dimethylamino)ethyl methacrylate)[24] have been described for gene delivery applications. Diblock copolymers of PHPMA with poly(*N*-isopropylacrylamide)[25] and poly(butyl methacrylate)[26] have also been reported.

Most of these block copolymers comprising PHPMA were synthesized by conjugation reaction between carboxyl and amine functionalized polymers. Generally, this procedure leads to poor conjugation yields, and the elimination of unreacted polymers is not trivial.[27] Block copolymerization of HPMA using poly(butyl methacrylate) macroinitiator resulted in poor monomer conversion and high polydispersity due to the competition of PHPMA amide nitrogen atom with the added ligands,

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for Cu(I) catalyst, used in atom transfer radical polymerization (ATRP). Moreover, PHPMA is insoluble in tetrahydrofuran, the solvent suitable for polymer-initiated anionic ring-opening polymerization of lactide, glycolide, and ϵ-caprolactone. In addition, at high temperatures, the reactivity of secondary hydroxyl groups in the PHPMA pendant chain should not be neglected. Indeed, Breitenbach and Kissel[28] reported grafting of poly(DL-lactide) and poly(DL-lactide-*co*-glycolide) chains onto poly(vinyl alcohol) (PVA) under melt polymerization conditions via PVA-initiated ring-opening polymerization of DL-lactide and glycolide.

To the best of our knowledge, block copolymers of PHPMA with biodegradable polymers have not been reported so far. In this work, we report the synthesis and micellar characterization of two novel A−B−A type triblock copolymers, namely poly(*N*-(2-hydroxypropyl)-methacrylamide)-*block*-poly(ϵ-caprolactone)-*block*-poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA-*b*-PCL-*b*-PHPMA) and poly(*N*-vinyl-2-pyrrolidone)-*block*-poly(ϵ-caprolactone)-*block*-poly(*N*-vinyl-2-pyrrolidone) (PVP-*b*-PCL-*b*-PVP). These triblock copolymers were synthesized by radical polymerization of the respective monomers in the presence of a novel macromolecular chain-transferring agent, i.e. α, ω-poly(ϵ-caprolactone) dithiol (HS-PCL-SH).

Experimental Section

Materials. 3,3′-Dithiobis(propionic acid) (DTPA), poly(ϵ-caprolactone)diol (HO-PCL-OH) (M_n ca. 2000), dicyclohexyl carbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), 1,4-dithiothreitol (DTT), *N*-vinyl-2-pyrrolidone (VP), 2,2′-azobis-(isobutyronitrile) (AIBN), pyrene, tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), *N*,*N*-dimethylacetamide (DMAC), and dichloromethane (DCM) were purchased from Aldrich Chemical Co. Inc. (Oakville, ON, Canada). *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was obtained from Polysciences Inc. (Warrington, PA). Amphotericin B and doxorubicin were procured from Sigma (Oakville, ON, Canada). Spectra/Por dialysis membranes of 6000−8000 molecular weight cutoff were from Spectrum Laboratories (Rancho Dominguez, CA). VP was passed through a silica gel column to remove the inhibitor sodium hydroxide. AIBN was recrystallized from ethanol. THF was freshly distilled over sodium and benzophenone before use. All other chemicals were used as received.

Instrumentation. *^{1}H NMR Spectroscopy*. ^{1}H NMR spectra of all the compounds synthesized in this work were obtained on a Bruker (Milton, ON, Canada) spectrometer operating at 300 MHz, using deuterated dimethyl sulfoxide (DMSO-d_6) and chloroform ($CDCl_3$).

Molecular Weight Measurements. Weight-average (M_w) and number-average (M_n) molecular weights of polymers were determined by gel permeation chromatography (GPC) on a Waters Alliance GPCV 2000 chromatograph (Waters, Milford, MA) equipped with a differential refractive index detector and the Millennium software program, under the following conditions. Three columns HT1, HT2, and HT3 with a very high to low molecular weight separation range were used in series. Polymer samples were dissolved in DMF (4 mg/mL) and injected in the system. The mobile phase was DMF containing 50 mM LiBr. Flow rate and temperature were set at 0.8 mL/min and 40 °C, respectively. Molecular weights by relative analysis were obtained from comparison of the retention times of synthesized triblock copolymers with those of PEG standards.

Micelle Size Measurements. Micelle size in aqueous solutions was measured by dynamic light scattering at a 90° angle to the incident beam and at 20 °C on a Coulter N4 Plus particle size analyzer (Coulter, Miami, FL) equipped with software for differential size distribution intensity analysis. The aqueous solution of polymer (1 mg/mL) was passed through 0.22 μm filter before size measurement.

Fluorescence Spectroscopy. The apparent critical aggregation concentration (cac) of the polymers was estimated by a steady-state pyrene fluorescence method,[20a] which is based on the shift in the (0,0) band of pyrene in the excitation spectra from 333 to 336 nm due to its incorporation in the hydrophobic core of the micelles. A 2 mL aqueous solution of pyrene (2×10^{-7} M) was added to a 2 mL polymer solution with increasing concentrations (0.15−5000 mg/L). These solutions were kept at 4 °C in the dark and stirred gently for 16 h. Their excitation spectra were recorded on an AMINCO Bowman series 2 luminescence spectrometer (Thermo Spectronic, Rochester, NY) at $\lambda_{em} = 393$ nm (band-pass 4 nm). The cac was determined from the intersection of two straight lines (the horizontal line with an almost constant value of the ratio $I_{336\ nm}/I_{333\ nm}$ and the vertical line with a steady increase in the ratio value) on the graph of the fluorescence intensity ratio $I_{336\ nm}/I_{333\ nm}$ vs log polymer concentration.

Synthesis of α,ω-Poly(ϵ-caprolactone)-di(3,3′-dithiobis-(propionate)) (DTPA-PCL-DTPA). Two grams of HO-PCL-OH (1 mmol) and 1 g of DTPA (5 mmol) were added to 10 mL of THF. The reaction mixture was stirred to obtain a clear solution. To this, 0.8 g of DCC (4 mmol) and 20 mg of DMAP dissolved in 5 mL of THF were added in one portion. The reaction mixture was stirred at room temperature for 2 days. It was filtered to remove dicyclohexylurea (DCU). The clear solution was concentrated in vacuo. One hundred milliliters of DCM was then added and allowed to stand for 1 h to precipitate the remaining traces of DCU. The DCM solution was filtered and extracted with 3 × 50 mL 5% aqueous NaCl. The organic layer was dried on anhydrous magnesium sulfate. DCM was evaporated under reduced pressure to isolate DTPA-PCL-DTPA. The product was dried in vacuo for 16 h. Yield: 2 g (90%). ^{1}H NMR ($CDCl_3$): 4.17 δ, t, 4H (central $-CH_2-O-CO-$ of PCL (e)); 4.00 δ, t, 27H ($-CH_2-O-CO-$ of PCL main chain (a)); 3.64 δ, t, 3.6H (central CH_2-O-CH_2- of PCL (f)); 2.87 δ, m, 7H ($-CH_2-S-S-CH_2-$ of DTPA (g)); 2.72 δ, m, 8H ($CH_2-CO-O-$ of DTPA (h)); 2.25 δ, t, 27H ($-CH_2-CO_2-$ of PCL carbonyl (d)); 1.59 δ, m, 59H ($-CH_2-$ of PCL methylene groups (b)); 1.32 δ, m, 29H ($-CH_2-$ at the middle of CL units (c)).

Synthesis of HS-PCL-SH. Two grams of DTPA-PCL-DTPA was dissolved in 10 mL of DMF. DTT (0.616 g, ca. 4-fold excess over disulfide groups) was added, and the reaction mixture was stirred at room temperature for 24 h. The DMF solution was poured in cold water (1 L) under stirring to precipitate HS-PCL-SH. The product was isolated by filtration, washed thoroughly with water, and dried in vacuo. Yield: 0.86 g (43%). ^{1}H NMR ($CDCl_3$): 4.19 δ, t, 4H (central $-CH_2-O-CO-$ of PCL (e)); 4.02 δ, t, 38H ($-CH_2-O-CO-$ of PCL main chain (a)); 3.6 δ, t, 4.25H (central $-CH_2-O-CH_2-$ of PCL (f)); 2.88 δ, m, 4.27H ($HS-CH_2-CH_2-CO-$ of DTPA (g)); 2.7−2.72 δ, m, 6.74H ($-CH_2-CO_2-$ of DTPA (h)); 2.27 δ, t, 40H ($-CH_2-CO-O-$ of PCL carbonyl (d)); 1.61 δ, m, 79H ($-CH_2-$ of PCL methylene groups (b)); 1.34 δ, m, 43H ($-CH_2-$ at the middle of CL units (c)).

Synthesis of Triblock Copolymers. *Synthesis of PHPMA-b-PCL-b-PHPMA*. A typical procedure for PHPMA-*b*-PCL-*b*-PHPMA (1:0.33) is described below (the numbers in brackets represent the HPMA:CL molar feed ratio). In a three-neck round-bottom flask equipped with a magnetic stirring bar and reflux condenser, HS-PCL-SH (0.80 g, 0.36 mmol), HPMA (3 g, 20.9 mmol), and AIBN (0.034 g, 0.209 mmol) were dissolved in DMF (10 mL). The solution was purged with nitrogen for 30 min at room temperature and immersed in an oil bath preheated to 80 °C. Polymerization proceeded for 16 h under continuous nitrogen purging. The DMF solution was poured in diethyl ether (800 mL) to precipitate the polymer, which was dissolved in 100 mL of water and dialyzed against 2 L of water at 4 °C for 2 days. The water was replaced every 12 h. The aqueous polymer solution was centrifuged at 4000*g* for 5 min to separate any unreacted HS-PCL-SH (very small amount observed). Then, the solution was decanted, filtered through 0.45 μm filter, and lyophilized to obtain PHPMA-*b*-PCL-*b*-PHPMA. Yield: 2.5 g (65%). Typical ^{1}H NMR (DMSO-d_6) spectral data: 7.23 δ, s, ($-NH-CO-$ of PHPMA *not*

shown); 4.64 δ, s (−C*H*(OH)− of PHPMA(l)); 4.03 δ, t; 3.90 δ, t; 3.59 δ, s, (−O*H* of PHPMA (n)); 2.81 δ, s, (HO(CH)−CH_2−NH− of PHPMA (k)); 2.19 δ, t; 1.44 δ, m, (−CH_2− backbone of PHPMA (i) + PCL methylene groups (b)); 1.21 δ, m; 0.73−0.94 δ, m, (−CH_3 of PHPMA backbone (j) + pendant −CH_3 of PHPMA (m)).

All polymers were synthesized according to the above-described procedure with an increasing molar ratio of CL to HPMA in the feed. Yields were 60−70%.

Synthesis of PVP-b-PCL-b-PVP. These polymers were synthesized with increasing molar feed ratio of CL to VP, following the procedure described for PHPMA-*b*-PCL-*b*-PHPMA. Typical ^{1}H NMR (DMSO-d_6) spectral data: 4.10 δ, t; 3.97 δ, t; 3.59−3.74 δ, d, (−CH_2− VP ring (q) + −C*H*− PVP main chain (r)); 3.14 δ, s, (−CH_2−CO− into VP ring (o)); 1.28−2.26 δ, m, (−CH_2−PVP main chain (s) + −CH_2- into VP ring (p) + PCL methylene groups protons (b, c, d)). Polymer yields after purification were in the range 50−60%.

Ellman's Assay for the Detection of Free −SH Groups in Triblock Copolymers. Ellman's reagent was prepared by dissolving 100 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 20 mL of 0.1 M sodium phosphate (pH 9). One milliliter of Ellman's reagent was added to 10−50 μg of polymer dissolved in 1 mL of 0.1 M sodium phosphate (pH 9). The solution was allowed to stand at room temperature for 20 min, and the increase in the absorbance at 412 nm was measured on a Hewlett-Packard spectrophotometer (model 89090A, Palo Alto, CA). For comparison, Ellman's test was also performed on 0−25 μg of 2-mercaptoethanol and on 50 μg of polymer added to 0−25 μg of 2-mercaptoethanol.

Drug Loading in Triblock Copolymer Micelles. Typically, 5 mg of doxorubicin or amphotericin B and 50 mg of polymer were dissolved in 5 mL of DMAC. In the case of doxorubicin, 4-fold molar excess of triethylamine over the drug was added. The clear solution of polymer and drug was allowed to stand at room temperature for 30 min. Then, 0.5 mL of water was added in portions of 0.1 mL. This solution was placed in a dialysis membrane bag of 6000−8000 molecular weight cutoff and dialyzed against 2 L of water for 24 h at room temperature. The water in the outer chamber was replaced every 12 h. The solution in the dialysis bag was passed through 0.22 μm filters and lyophilized to obtain drug-loaded micelles. Drug loading was estimated by spectrophotometry after dissolving the micelles in DMAC and measuring the absorbance at 486 and 412 nm for doxorubicin and amphotericin B, respectively.

Results and Discussion

Because of the problems mentioned in the Introduction, our strategy was to avoid the conjugation chemistry of the two macromolecules, PHPMA-initiated ring-opening polymerization of lactones either in solvent or under melt polymerization conditions, and ATRP of HPMA. Since free radical polymerization of HPMA is well-established, it was decided to synthesize the block copolymers by this process in the presence of a biodegradable, macromolecular chain-transferring agent. HO-PCL-OH was selected as the biodegradable segment owing to its reactive −OH end groups that could be derivatized to free thiol groups. We wish to mention here that block copolymers have been synthesized earlier using thio-functionalized polymers as chain-transferring agents. Sato et al.[29] synthesized a variety of A−B and A−B−A type block copolymers by free radical polymerization of vinyl monomers, such as vinyl acetate, methyl methacrylate, *N*,*N*-dimethylacrylamide, and acrylic acid, in the presence of mono- or dithiol-terminated PEG, poly(propylene glycol), poly(methyl methacrylate), poly(vinyl alcohol), and poly(styrene). Inoue et al.[30] synthesized A−B type block copolymer micelles by radical polymerization of acrylic acid in the presence of thiol-terminated oligo(methyl methacrylate). However, to our knowledge, no such attempts with biodegradable polymers have been reported so far.

Choice of Thiolation Chemistry. Carrot et al.[31] reported the synthesis of thiol-terminated PCL by reacting it first with 2,4-dinitrophenylthioacetic acid and subsequently deprotecting the end ester group by treatment with 2-mercaptoethanol and triethylamine. Sato et al.[29] described the synthesis of thiol-terminated poly(methacrylate)s wherein the monomer, e.g., methyl methacrylate, is polymerized in the presence of thiol lactic acid. The resulting polymer is treated with NaOH for hydrolysis of the end ester group to obtain the free thiol-containing polymer. The same authors also described the synthesis of α,ω-poly(oxyethylene)dithiol by first reacting PEG with tosyl chloride. PEG-tosylate was refluxed with thiourea in ethanol and, finally, with NaOH.[32] These reaction conditions are, however, relatively harsh for a biodegradable polymer. Moreover, since we intend to use the proposed triblock copolymers in drug delivery, we decided to exploit the standard chemistry of thiol group introduction in proteins, i.e., derivatization of HO-PCL-OH with 3,3′-dithiobis(propionic acid) followed by the reduction of disulfide bonds by DTT at room temperature.[33] The reaction scheme for the synthesis of HS-PCL-SH is shown in Figure 1. Further, it was decided to use the bifunctional chain-transferring agent in free radical polymerization of HPMA and VP to obtain the triblock copolymers as represented schematically in Figure 2.

Synthesis and Characterization of HS-PCL-SH. *HO-PCL-OH.* The ^{1}H NMR spectrum (in $CDCl_3$) of HO-PCL-OH is shown in Figure 3. The spectral data obtained are as follows: 4.27 δ, t, 4H (central −CH_2−O−CO− of PCL (e)); 4.05 δ, t, 40H (−CH_2−O−CO− of PCL main chain (a)); 3.71 δ, m, 8H (terminal −CH_2−OH of PCL (a′) + central −CH_2−O−CH_2− of PCL (f)); 2.31 δ, t, 44H (−CH_2−CO_2− of PCL carbonyl (d)); 1.63 δ, m, 91H (−CH_2− of PCL methylene groups (b)); 1.42 δ, m, 45H (−CH_2− at the middle of CL units (c)). The peak at 3.71 δ for 8H is comprised of 4H that correspond to end hydroxymethyl groups (a′) of PCL and 4H that correspond to methylene groups in central ether moiety (f) in the PCL chain, used as the initiator. These NMR data for PCL proton peaks are in accordance with literature reports.[31]

DTPA-PCL-DTPA. This compound was synthesized by the DCC-mediated coupling of HO-PCL-OH with excess of DTPA. Figure 4 shows the ^{1}H NMR spectrum of DTPA-PCL-DTPA with two new peaks that correspond to the conjugated DTPA moiety. There is no significant shift in peak positions of the other main-chain PCL protons. The new peaks at 2.88 δ and 2.72 δ correspond to −CH_2−CO_2− (h) and −CH_2−S−S−CH_2− (g) of the DTPA moiety conjugated at both ends, respectively. The ratios of PCL central −CH_2−O−CO− (e) protons to DTPA protons (g and h) as well as PCL central −CH_2−O−CH_2− ether protons (f) to DTPA protons (g and h) are close to their respective theoretical values, as shown in Figure 4. Considering the polydispersity of commercial (1.3−1.5) PCL, the data indicate almost complete esterification of HO-PCL-OH with DTPA.

HS-PCL-SH. The product was obtained after DTT-mediated reduction of disulfide bonds in DTPA-PCL-DTPA. Figure 5 shows the ^{1}H NMR spectrum of HS-PCL-SH with the peak at 2.88 δ for integrating 4H that corresponds to the −CH_2−CO_2− (h) moiety in DTPA.

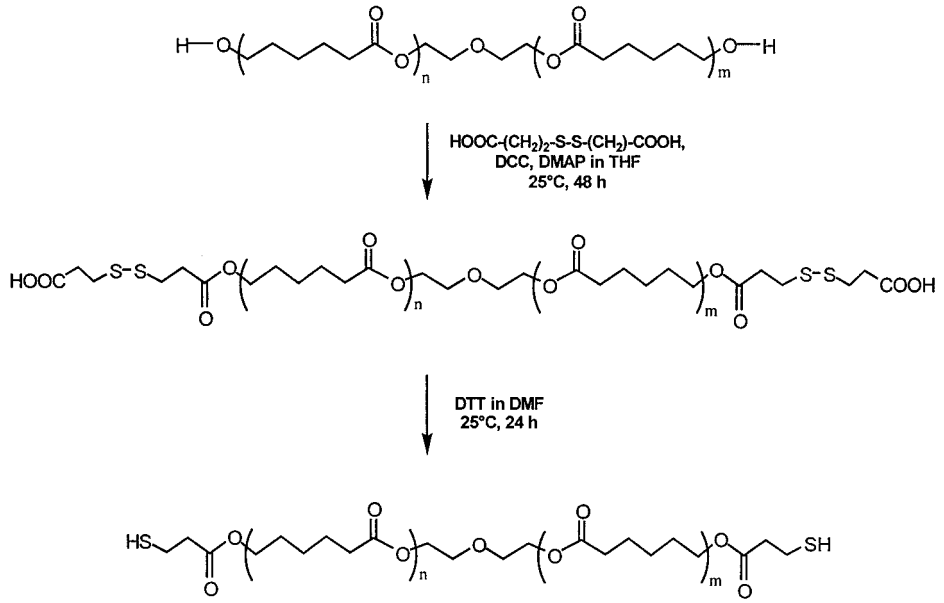


Figure 1. Reaction scheme for the synthesis of α,ω-poly(ϵ-caprolactone)dithiol (HS-PCL-SH).

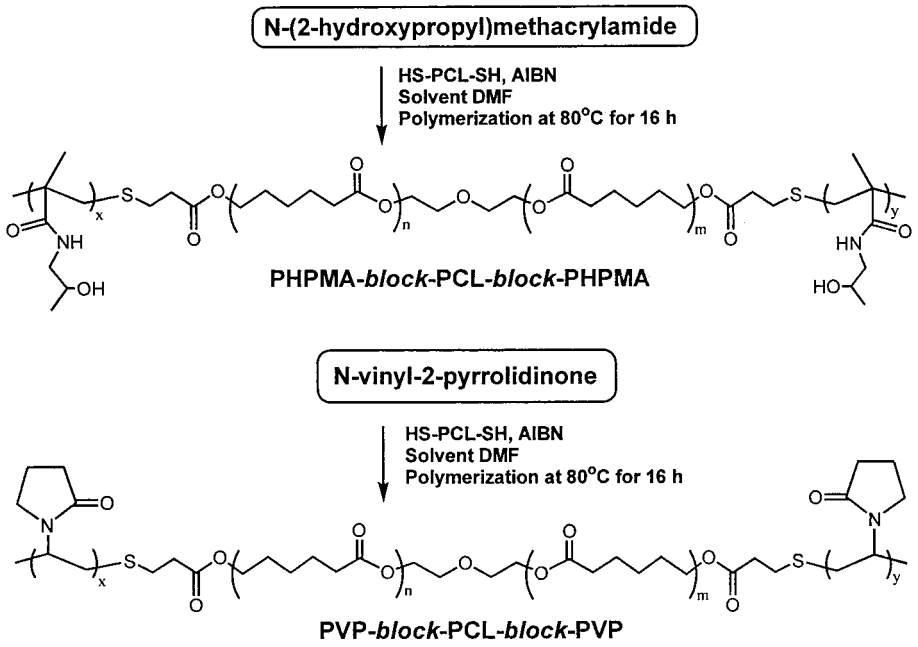


Figure 2. Reaction scheme for the synthesis of triblock copolymers.

There is a decrease in the number of $-CH_2-CO_2-$ protons to half its original value (8H) in DTPA-PCL-DTPA. This clearly indicates the loss of one molecule of 3-thiopropionic acid from each end of the PCL chain due to disulfide bond reduction. Also, Figure 5 shows that proton ratios e/f, e/g, h/e, and h/g are quite close to their respective theoretical values. These data clearly support the quantitative reduction of disulfide bonds by DTT and the formation of HS-PCL-SH. Although PEG thiols are sensitive to air oxidation, hydrophobic macromolecular thiols, e.g. thiol-functionalized poly(methyl methacrylate) (HS-PMMA), was found to be stable.[29] Also in the present work, we did not observe the formation of disulfide linkages due to air oxidation during the handling of HS-PCL-SH and during its storage in the freezer at −20 °C.

Synthesis and Characterization of PHPMA-*b*-PCL-*b*-PHPMA. As described in the Experimental Section, we polymerized HPMA using AIBN and HS-PCL-SH as the initiator and the chain transferring agent, respectively. In this procedure, AIBN initiated radical polymerization should form a small amount of PHPMA. However, after the proton abstraction by growing PHPMA chains and/or AIBN from HS-PCL-SH, the high reactivity of thio radicals is expected to initiate new chains and result in A−B−A type triblock copolymer. This is described in the following sections.

Polymers with increasing molar feed ratios of CL to HPMA (0.25−0.96) were synthesized and purified as described earlier. A typical ^{1}H NMR spectrum of PHPMA-*b*-PCL-*b*-PHPMA (1:0.33), in Figure 6, shows well-separated peaks for $-CH_2$ (k) and $-CH(OH)-$ (l) in HPMA at 2.81 δ and 4.64 δ, respectively. Also, the peak for central $-CH_2-O-CO-$ (e) methylene groups in PCL is still seen at 4.03 δ, which is used for the determination of M_n by ^{1}H NMR. The peaks for other moieties in the triblock copolymer that are merged with one another, i.e., (i) and (j + m), are also seen in the spectrum.

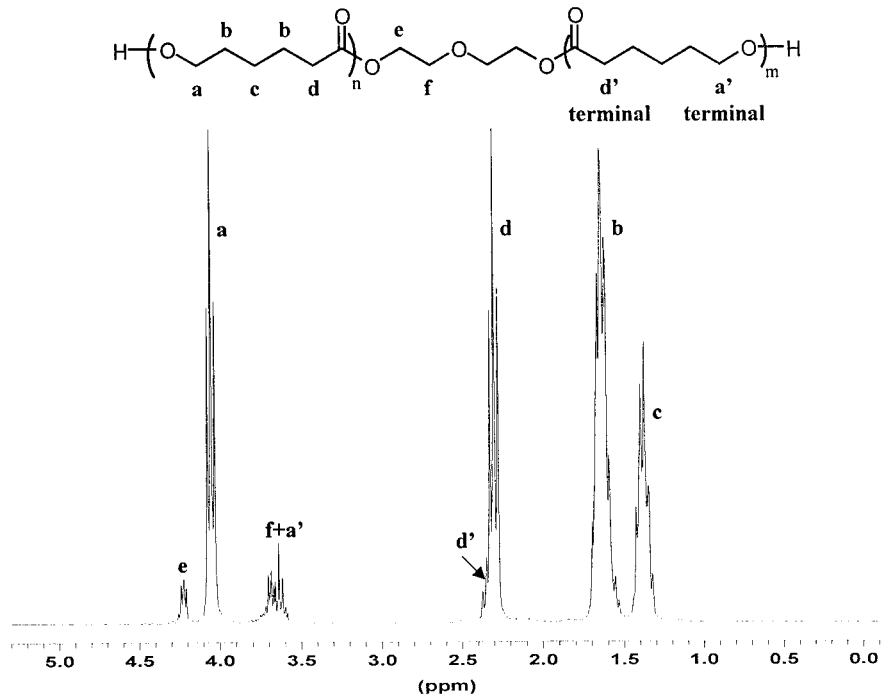
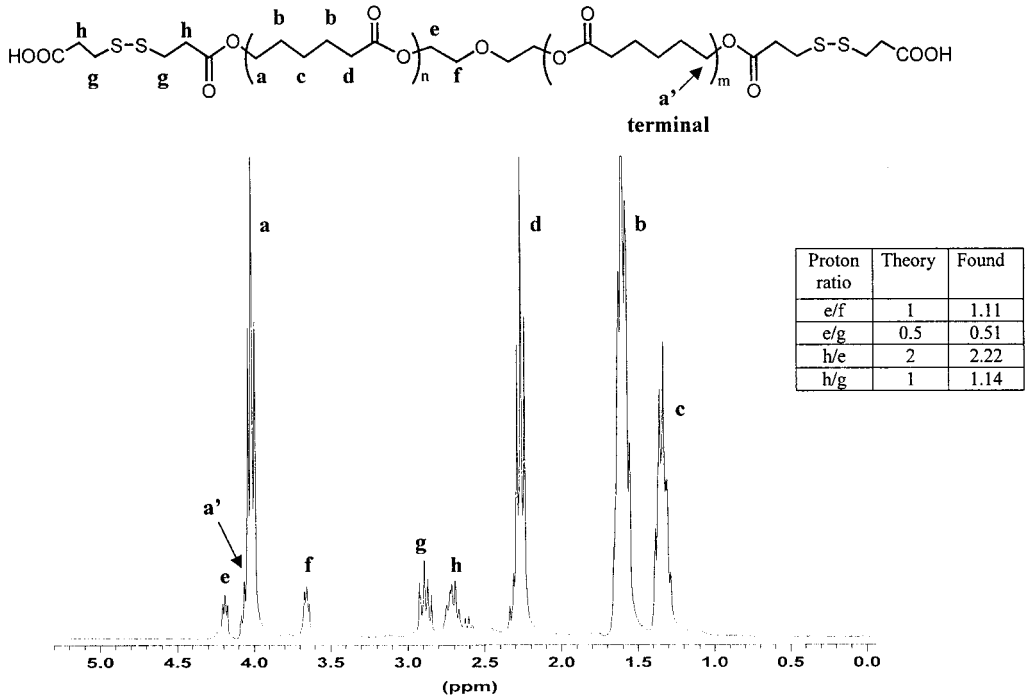


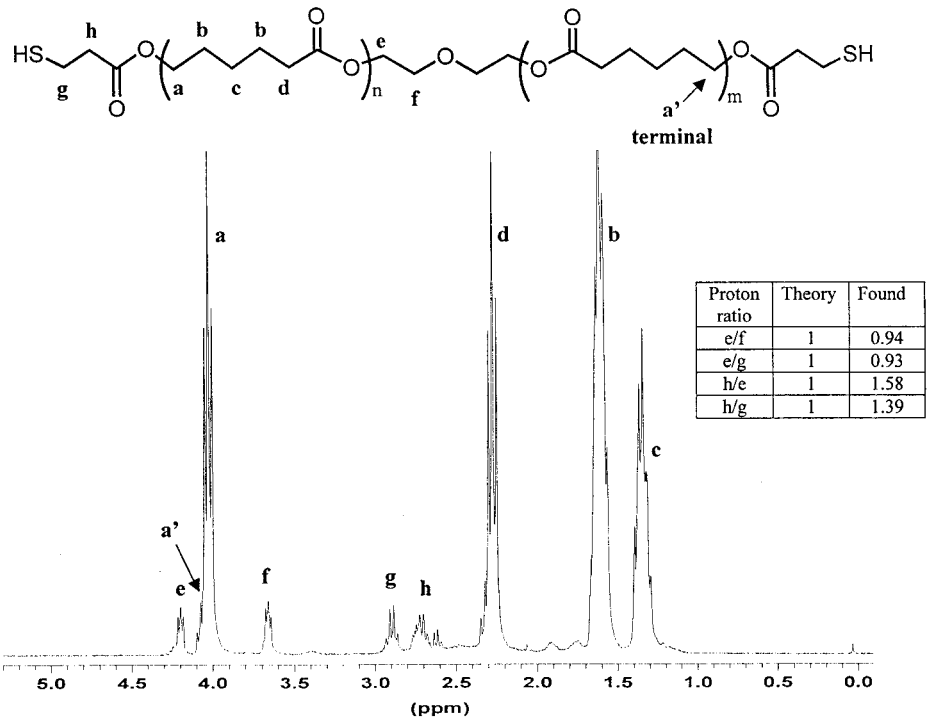
Figure 3. ^{1}H NMR spectrum of α,ω-poly(ϵ-caprolactone)diol (HO-PCL-OH).



Proton ratio	Theory	Found
e/f	1	1.11
e/g	0.5	0.51
h/e	2	2.22
h/g	1	1.14

Figure 4. ^{1}H NMR spectrum of α,ω-poly(ϵ-caprolactone)-di(3,3′-dithiobis(propionate)) (DTPA-PCL-DTPA).

The proportion of CL (mol %) incorporated in the triblock was estimated from the ratio of the number of protons under the peaks characteristic of PCL and PHPMA blocks in ^{1}H NMR spectra of the polymers. Table 1 shows that 0.20−0.90 molar ratio of CL/HPMA was incorporated in the triblocks, which is about 80−93% of that used in the feed. Molecular weights of the polymers were estimated by GPC relative to PEG standards and by ^{1}H NMR. M_w ranging from 5600 to 12 100 were obtained as the amount of macromolecular chain-transferring agent decreased in the feed. Polydispersity indices of the polymers ranged between 1.55 and 1.8. The M_n of polymers estimated by ^{1}H NMR (Table 1) were 2-fold higher than the corresponding values obtained from GPC analysis. Here, we wish to mention that for M_w determination we used PEG standards which are hydrophilic but structurally much different from PHPMA. Thus, analysis relative to PEG standards is a rough approximation of actual molecular weight of the triblock copolymer. Molecular weights of all polymers were well below the renal threshold limit (about 40 000), which would allow elimination of the polymers after PCL block degradation and/or dissociation of micelles in the body.[34] All polymers dissolved in water and did not form precipitates, indicating the absence of free/unreacted PCL. The polymers also dissolved easily in solvents like ethanol and methanol in which PCL is insoluble. This highlights the changed



Proton ratio	Theory	Found
e/f	1	0.94
e/g	1	0.93
h/e	1	1.58
h/g	1	1.39

Figure 5. ^{1}H NMR spectrum of α,ω-poly(ϵ-caprolactone)dithiol (HS-PCL-SH).

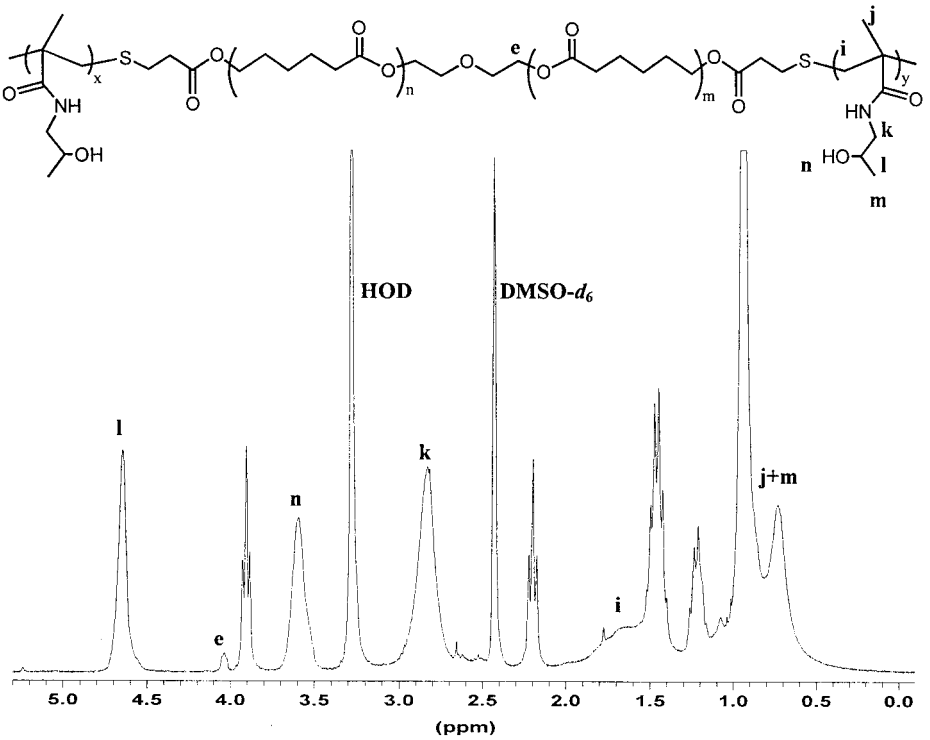


Figure 6. ^{1}H NMR spectrum of PHPMA-*b*-PCL-*b*-PHPMA (1:0.33).

physical properties of PCL block by incorporation in triblock copolymers.

Ellman's Assay To Detect Free Thiol Groups in Triblock Copolymers. The absence of free −SH groups in the triblock copolymers was confirmed quantitatively by reacting the polymers with 5,5′-dithiobis-(2-nitrobenzoic acid) and monitoring the increase in the absorbance at 412 nm (Δ_{abs} 412 nm). Figure 7 shows the standard Ellman's assay for 2-mercaptoethanol with a pronounced Δ_{abs} 412 nm due to the reaction of −SH groups with Ellman's reagent and subsequent liberation of 5-thiol-2-nitrobenzoic acid. When the assay was performed using PHPMA-*b*-PCL-*b*-PHPMA (1:0.33), there was practically no Δ_{abs} 412 nm. This indicates the absence of free −SH group in the polymer; i.e., −SH groups in HS-PCL-SH were consumed in transferring the chain to the growing PHPMA radical. These data strongly support the formation of A−B−A triblock copolymers. As an additional control, 50 μg of polymer was mixed with 2-mercaptoethanol standards. In this case, a slightly higher Δ_{abs} 412 nm was observed over that given by 2-mercaptoethanol alone, which could be

Table 1. Characterization and Drug Loading Data for A−B−A Triblock Copolymers

entry no.	polymer[a]	CL/HPMA or VP molar ratio in purified product[b]	M_w[c]	M_w/M_n[c]	M_n by ^{1}H NMR[d]	cac[e] (mg/L)	$K_v \times 10^5$	micelle size[f] (nm)	drug loading after dialysis (w/w%)[g]		entrapment efficiency (%)	
									doxorubicin[h]	amphotericin B[i]	doxorubicin	amphotericin B
1	PHPMA-*b*-PCL-*b*-PHPMA (1:0.25)	0.20	12 100	1.80	14 700	3.8 ± 0.8	2.6 ± 0.3	36 ±7 (62%), 154 ± 87 (38%)	1.9	0.32	21.1	1.9
2	PHPMA-*b*-PCL-*b*-PHPMA (1:0.33)	0.22	10 700	1.75	13 400	3.5 ± 1	2.6 ±0.2	34 ± 2 (58%), 100 ± 11 (42%)	2.4	1.6	26.6	9.5
3	PHPMA-b-PCL-*b*-PHPMA (1:0.51)	0.35	7 700	1.60	9 100	2 ± 0.1	2.5 ± 0.3	31 ± 1 (80%), 147 ± 36 (20%)	1.5	1.9	16.6	11.5
4	PHPMA-b-PCL-*b*-PHPMA (1:0.96)	0.90	5 600	1.55	4 700	1.3 ± 0.2	2.3 ± 0.2	37 ± 10 (20%), 220 ± 70 (80%)	1.1	ND[j]	12.2	ND[j]
5	PVP-*b*-PCL-*b*-PVP (1:0.20)	0.17	13 300	2.89	13 700	3 ± 1	3.8_± 0.2	51 ± 1 (15%), 198 ± 24 (85%)	0.84	1.0	9.3	6.0
6	PVP-b-PCL-*b*-PVP (1:0.26)	0.20	13 400	2.91	11 400	1.8 ± 1	4.2 ± 0.6	<3 (29%), 47 ±1 (41%), 245 ± 50 (30%)	1.1	0.91	12.2	5.5
7	PVP-*b*-PCL-*b*-PVP (1:0.40)	0.26	6 200	2.0	9 500	2 ± 0.5	3.2 ± 0.5	47 ± 10 (20%), 186 ±28 (80%)	1.6	3.9	17.6	23.4

[a] Numbers in the parentheses represent molar feed ratios of monomer:CL. [b] Determined by ^{1}H NMR from the ratio of the number of protons under the peaks characteristic of HPMA or VP and PCL, respectively. [c] Determined by GPC using PEG standards. [d] Calculated from the number of protons under the peaks characteristic of HPMA or VP. The average M_n of PCL was assumed to be 2000. [e] The value is the average of two experiments. [f] Measured by dynamic light scattering using aqueous polymer solutions at 1 mg/mL. The values reported are the average of three measurements. Numbers in the parentheses represent the percentage population of micelles of a particular size. [g] Drug loading based on w/w drug/(polymer + drug). [h] Initial drug loading 9% (w/w). [i] Initial drug loading 16.6% (w/w). [j] Not detectable.

Figure 7. Ellman's assay showing the absence of free thiol groups in PHPMA-*b*-PCL-*b*-PHPMA (1:0.33).

attributed to the increased nucleophilicity of the reaction medium by amide bonds in the polymer. However, all polymers synthesized in this work, when tested alone, were negative for this assay.

Synthesis and Characterization of PVP-*b*-PCL-*b*-PVP Triblock Copolymers. To demonstrate the general applicability of HS-PCL-SH in synthesizing block copolymers comprising biodegradable and biocompatible segments, we polymerized increasing amounts of VP in its presence and synthesized a family of novel triblock copolymers, PVP-*b*-PCL-*b*-PVP. As an example, the ^{1}H NMR spectrum of PVP-*b*-PCL-*b*-PVP (1:0.40) is shown in Figure 8. The spectrum reveals well-separated peaks for the central $-CH_2-O-CO-$ (e) and main-chain $-CH_2-O-CO-$ (a) methylene groups in PCL at 4.10 δ and 3.9 δ, respectively. Also, peaks for PVP backbone protons $-CH-$ (r) and ring $-CH_2-$ (p) are seen around 3.6 δ and 3.1 δ, respectively. The remaining methylene group peaks for PCL and the PVP signals are merged in a broad multiplet (s + p). Such a broad multiplet has also been reported in the ^{1}H NMR spectrum of PVP-*b*-PDLLA.[16] The proportion of CL incorporated in the triblock was estimated from the ratio of the number of protons under the peaks characteristic of PCL and PVP blocks in ^{1}H NMR spectra of the polymers. Table 1 shows 0.17−0.26 molar ratio of CL/VP was incorporated in the triblocks, which is about 65−85% of that used in the feed.

Molecular weights of the polymers estimated by GPC relative to PEG standards were in the range 6200−13 400. Polydispersity indices for these polymers ranged between 2.0 and 2.9, which is higher than that for PHPMA-*b*-PCL-*b*-PHPMA copolymers. The M_n calculated by ^{1}H NMR was 2−3-fold higher than that determined by GPC. These polymers also exhibited a negative Ellman's test, indicating the absence of free −SH groups in the polymers.

Micellar Characterization of Triblock Copolymers. *Determination of cac of Polymers.* Figure 9 shows the excitation spectra of pyrene in various concentrations of PHPMA-*b*-PCL-*b*-PHPMA (1:0.33). As the polymer concentration increased, the (0,0) band in pyrene excitation spectra shifted from 333 to 336 nm. This can be attributed to the partitioning of pyrene in the hydrophobic core of supramolecular aggregates (presumably micelles). Aqueous solutions of PVP-*b*-PCL-*b*-PVP copolymers also exhibited a similar shift for the

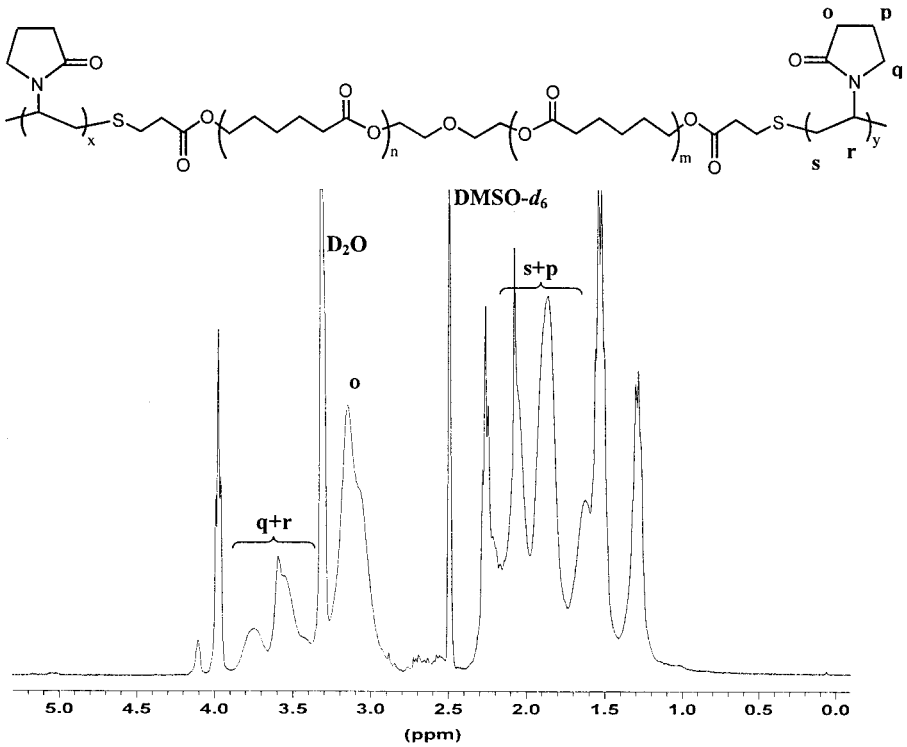


Figure 8. ^{1}H NMR spectrum of PVP-*b*-PCL-*b*-PVP (1:0.40).

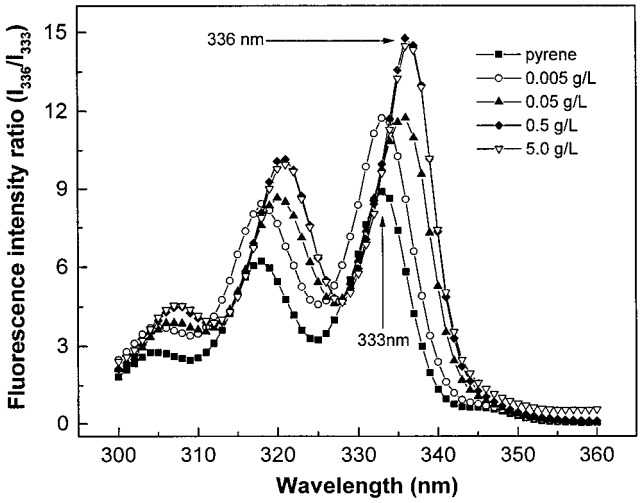


Figure 9. Excitation spectra of pyrene (2×10^{-7} M) with increasing concentrations of PHPMA-*b*-PCL-*b*-PHPMA (1:0.33).

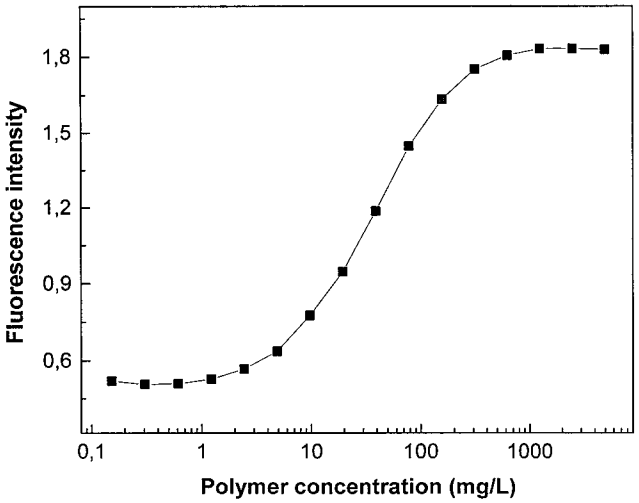


Figure 10. Graph of pyrene fluorescence intensity ratio $I_{336\ nm}/I_{333\ nm}$ vs polymer concentration for PHPMA-*b*-PCL-*b*-PHPMA (1:0.33).

(0,0) band in pyrene excitation spectra. Lee et al.[20a] have reported shift in the (0,0) band of pyrene to 335 nm in micelles of PEtOz-*b*-PCL. Thus, the red shift from 333 to 336 nm was used for cac determination of all the triblock copolymers synthesized in this work.

As an example, Figure 10 shows a graph of pyrene fluorescence intensity ratio $I_{336\ nm}/I_{333\ nm}$ vs PHPMA-*b*-PCL-*b*-PHPMA (1:0.33) concentration. The ratio $I_{336\ nm}/I_{333\ nm}$ is almost constant at low polymer concentrations, but from a certain concentration, it starts to increase steadily, indicating the incorporation of pyrene into the hydrophobic core of the micelles. The apparent cac was determined from this crossover point at a low concentration range. With further increase in the polymer concentration, the $I_{336\ nm}/I_{333\ nm}$ ratio increased to >1.5 and then finally reached a plateau. The cac for the triblock copolymers decreased from 4 to 1 mg/L as the proportion of incorporated hydrophobic PCL increased (Table 1). The cac values of these triblock copolymers are extremely low and comparable to those of other polymeric amphiphiles reported in the literature.[35−40]

Partition of Pyrene Probe in the Hydrophobic Core of Micelles. The partition equilibrium constant (K_v) of pyrene in the hydrophobic core of triblock copolymer micelles was estimated according to the method reported by Wilhelm et al.[35] and Lee et al.[20a] Binding of pyrene to the micelles is assumed to result from simple equilibrium between the micellar phase of volume (V_m) and the water phase of volume (V_w). Thus, the ratio of pyrene in the micellar phase to the water phase [Py_m]/[Py_w] can be expressed as the volume ratio of the two phases according to eq 1:

$$[Py_m]/[Py_w] = K_v V_m/V_w \quad (1)$$

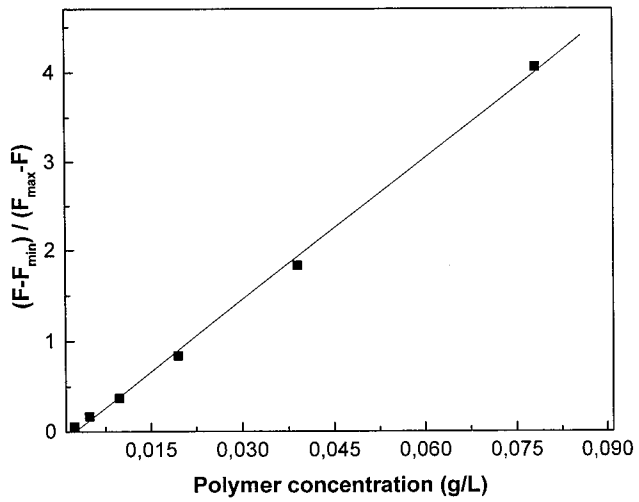


Figure 11. Determination of the partition equilibrium constant K_v of pyrene from the graph of $(F - F_{min})/(F_{max} - F)$ vs polymer concentration for PVP-*b*-PCL-*b*-PVP (1:0.26).

Note that $[Py_m]$ refers to the concentration of micelle-bound pyrene averaged over the entire sample volume. If the cac lies well below the polymer concentration (C), eq 1 can be rewritten as

$$[Py_m]/[Py_w] = K_v x_{PCL} C/1000\rho \qquad (2)$$

where x_{PCL} is the weight fraction of hydrophobic PCL in the block copolymer and ρ is the density of the PCL core of micelles, which is assumed to take the same value as bulk PCL (=1.146).[19a]

For polymer concentration above cac, where there is a substantial increase in the fluorescence intensity ratio $I_{336\ nm}/I_{333\ nm}$, $[Py_m]/[Py_w]$ can be written as

$$[Py_m]/[Py_w] = (F - F_{min})/(F_{max} - F) \qquad (3)$$

where F_{min} is the average value of the intensity ratio $I_{336\ nm}/I_{333\ nm}$ in the low polymer concentration region, F_{max} is the average value of the ratio $I_{336\ nm}/I_{333\ nm}$ in the very high polymer concentration region, and F is the intensity ratio $I_{336\ nm}/I_{333\ nm}$ in the intermediate polymer concentration region.

By combining eqs 2 and 3, one can write

$$(F - F_{min})/(F_{max} - F) = K_v x_{PCL} C/1000\rho \qquad (4)$$

Thus, we can plot a straight line graph of $(F - F_{min})/(F_{max} - F)$ vs polymer concentration (g/L), as shown in Figure 11 for PVP-*b*-PCL-*b*-PVP (1:0.26), and obtain the value of K_v from the slope of the graph. K_v values are reported in Table 1. It can be seen that they do not differ significantly within the set of PHPMA-*b*-PCL-*b*-PHPMA copolymers of increasing PCL content ($K_v = (2.3-2.6) \times 10^5$). This is also observed for PVP-*b*-PCL-*b*-PVP copolymers ($K_v = (3.2-4.2) \times 10^5$), which could be attributed to the fact that for all polymers prepared the molecular weight of PCL block is the same. The reported K_v values for pyrene in micelles of sodium dodecyl sulfate,[35] poly(ethylene oxide)-*block*-poly(styrene),[40] and PEtOz-*b*-PCL[20a] are 1.2×10^5, 3.0×10^5, and 5.4×10^5, respectively.

Determination of Micellar Size by Dynamic Light Scattering. Table 1 shows the mean sizes of triblock copolymer micelles in aqueous solutions at 1 mg/mL. The micelles exhibited a distinct bimodal size distribution (Figures 12 and 13). In PHPMA-*b*-PCL-*b*-PHPMA copolymers (except for (1:0.96)), 60−80% population had a mean diameter of about 30 ± 10 nm, and the remaining population exhibited larger size aggregates of about 150 ± 50 nm (Figure 12). The trend is opposite

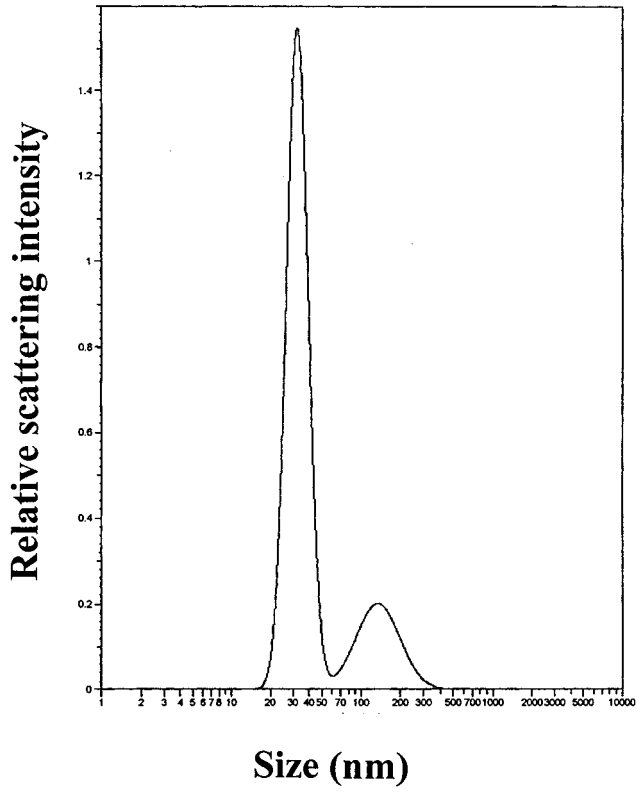


Figure 12. Bimodal size distribution of PHPMA-*b*-PCL-*b*-PHPMA (1:0.51) micelles.

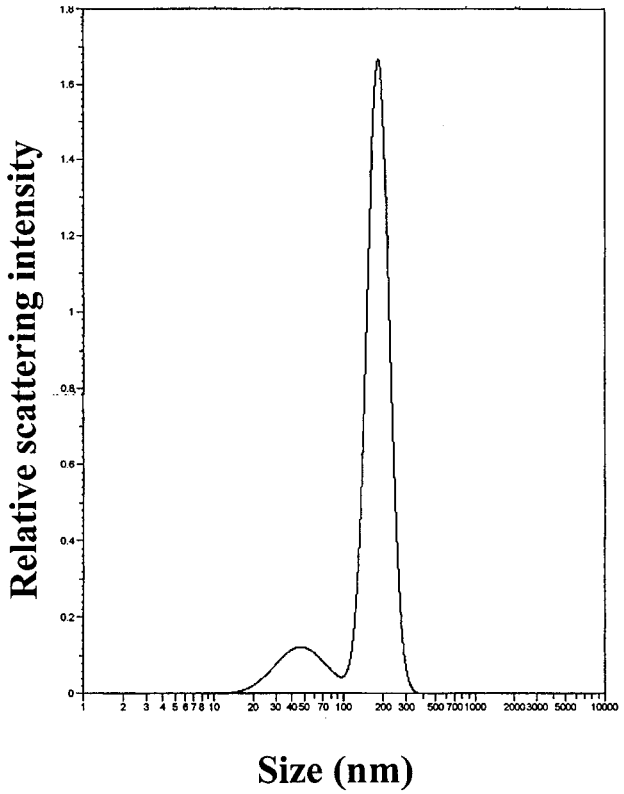


Figure 13. Bimodal size distribution of PVP-*b*-PCL-*b*-PVP (1:0.20) micelles.

for PVP-*b*-PCL-*b*-PVP copolymers with 80% of the population exhibiting large size aggregates of about 200 ± 50 nm and the remaining population exhibiting micelles of 50 nm size (Figure 13). The bimodal size distribution was also reported for PVP-*b*-PDLLA[16] and PEG-*b*-PCL[10] micelles. Large size aggregates of micelles were suggested to form due to hydrophobic association between exposed cores.[10] This secondary aggregation mainly depends on the concentration of the polymer solution used. More diluted solutions should favor the smaller size micelles. In the present study, we could not go below 1 mg/mL polymer concentration due to the sensitivity limits of the instrument. We believe that secondary aggregation might be favored strongly when PVP is used as the hydrophilic block because of its amphiphilic nature.[19a,b]

Drug Loading in A−B−A Triblock Copolymer Micelles. The ability of triblock copolymers to solubilize hydrophobic drug molecules was evaluated. Doxorubicin and amphotericin B were entrapped in triblock copolymer micelles by a dialysis procedure. Table 1 shows a comparatively better entrapment of doxorubicin (1−2.5% w/w) by PHPMA-*b*-PCL-*b*-PHPMA and of amphotericin B (1−4% w/w) by PVP-*b*-PCL-*b*-PVP. Although overall drug loading is relatively low, the solubility of these drugs can be increased significantly by entrapment in micelles. Drug loading could be further improved using a variety of other efficient loading methods reported in the literature and/or by fine-tuning the polymer compositions. This issue is presently addressed in our laboratory.

Conclusion

Two novel amphiphilic A−B−A triblock copolymers, namely PHPMA-*b*-PCL-*b*-PHPMA and PVP-*b*-PCL-*b*-PVP, were synthesized by free radical polymerization of HPMA and VP in the presence of HS-PCL-SH. This novel, biodegradable and macromolecular chain-transferring agent could find application in the synthesis of a variety of novel A−B−A type biodegradable triblock copolymers. The A−B−A triblock copolymers synthesized in this study self-assembled in supramolecular aggregates of 30−200 nm size in aqueous solutions and exhibited cac's ranging from 1 to 4 mg/L. Polymeric micelles also exhibited 1−4% w/w loading of poorly water-soluble drugs such as doxorubicin and amphotericin B. Such micelles could prove useful for the solubilization and targeting of various hydrophobic drugs.

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