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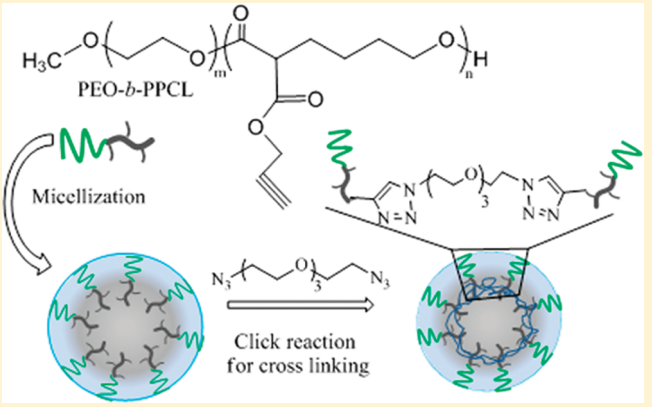
Application of Click Chemistry in the Preparation of Poly(ethylene oxide)-*block*-poly(ε-caprolactone) with Hydrolyzable Cross-Links in the Micellar Core

Shyam M Garg,[†] Xiao-Bing Xiong,[†] Changhai Lu,[†] and Afsaneh Lavasanifar*[,†,‡]

[†]Faculty of Pharmacy and Pharmaceutical Sciences and [‡]Faculty of Chemical and Material Engineering, University of Alberta, Edmonton, Alberta, Canada

Supporting Information

ABSTRACT: The aim of this study is to develop degradable core-cross-linked polymeric micelles based on poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) structures using click chemistry. Substituted monomer, that is, α-propargyl carboxylate-ε-caprolactone, was synthesized by anionic activation of ε-caprolactone and further treatment with propargyl chloroformate. Ring-opening polymerization of α-propargyl carboxylate-ε-caprolactone with methoxy PEO (5000 g/mol) as initiator and stannous octoate as catalyst was used to prepare PEO-*b*-poly(α-propargyl carboxylate-ε-caprolactone) (PEO-*b*-PPCL) block copolymer. The block copolymers were found to spontaneously associate in aqueous solution forming well-defined micelles. Stabilization of the micelles was obtained by cross-linking the core via click reaction between the azide group of tetraethylene glycol (bis)azide reagent and the alkyne group on the PPCL block in the presence of copper catalyst at room temperature. Successful cross-linking was evidenced by ^{1}H NMR, IR spectroscopy, and X-ray photoelectron spectroscopy (XPS). This was followed by the characterization of micellar morphology and size by transmission electron microscopy and light-scattering. Extent of bovine serum albumin (BSA) adsorption for cross-linked and non-cross-linked micelles illustrated the better stability of cross-linked micelles against protein adsorption. Finally, paclitaxel (PTX) was physically encapsulated in the micellar cores, where a similar PTX encapsulation and in vitro release profile for PTX from non-cross-linked and cross-linked micelles was observed. The results pointed to the increased stability of prepared cross-linked micelles and their potential in drug delivery.



INTRODUCTION

Over the last few decades, block copolymers have emerged as an interesting class of biomaterials because of their versatile applications in pharmaceutical science and drug delivery. Of particular interest are amphiphilic block copolymers, which self-assemble into polymeric micelles with core−shell architectures above the critical micelle concentration (CMC). Polymeric micelles are currently under investigation as nanodelivery systems for depot drug release and targeted drug delivery.[1−3] The use of polymeric micelles for the mentioned applications, however, has been hampered by the poor in vivo stability of most micellar structures upon administration to systemic circulation, which leads to micellar dissociation and premature release of encapsulated drugs.

Considerable research has focused on increasing the stability of polymeric micelles by preventing their dissociation in the extreme dilution conditions of the bloodstream upon intravenous administration. Some of the strategies currently under study for the stabilization of polymeric micelles include chemical modification of the hydrophobic block,[4−6] introduction of crystallinity or stereocomplex formation,[7,8] covalent attachment of hydrophobic drug,[9,10] formation of glassy core,[11] and cross-linking of the micellar core or shell. Cross-linked micelles can stay in the micellar form at concentrations below CMC of their block copolymer; thus avoiding rapid drug release.[12−15] Cross-links are preferred to degrade in response to internal or external stimuli, ensuring the release of incorporated drug in the vicinity of cellular or molecular drug targets and at the same time resulting in the biological elimination of the colloidal carrier after drug release.

One of the earliest examples of cross-linking of the micellar shell was reported by Wooley and coworkers in 1996.[16] Since then, this strategy has been investigated by many other groups.[17−21] Core-cross-linking was first reported by Tuzar

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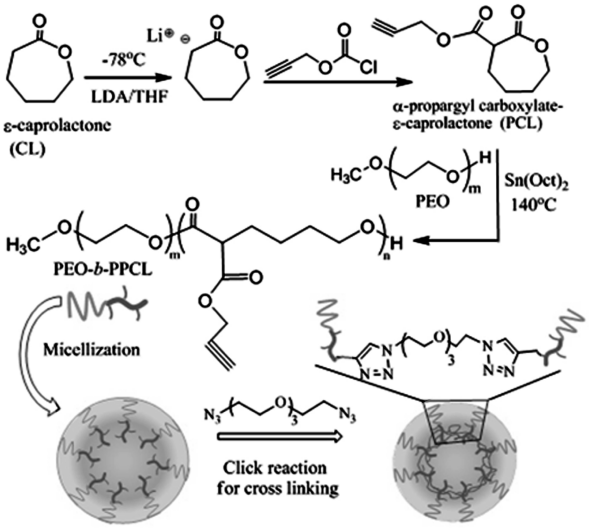
and coworkers in 1979[22] and 1982,[23] in which poly(styrene/butadiene/styrene) micelles with cores consisting of polybutadiene blocks were stabilized by UV radiation in the presence of a photoinitiator. Similar strategies involving cross-linking of the hydrophobic core by either thermal,[24] photoinduced polymerization,[25,26] or conventional chemical reactions in the micellar core have been carried out.[24−31] More recently, a doxorubicin methacrylamide derivative bearing a hydrolytically sensitive hydrazone linker was covalently incorporated into the cross-linked core of polymeric micelles composed of mPEO-*b*-poly[*N*-(2-hydroxypropyl)methacrylamide-lactate] diblock copolymers by free radical polymerization.[32] However, most of these strategies included harsh conditions during the procedures and have other disadvantages. For example, photo-cross-linkable materials are conventionally unstable when exposed to light.[33] Besides, high temperatures during thermal polymerization can cause decomposition of the incorporated biomolecules.

Click chemistry has emerged as a highly efficient technique, providing attractive possibilities for the synthesis of polymers with different architectures. It offers advantages including ambient reaction conditions, quantitative yields, easily obtained starting materials, and, in particular, high specificity, which make the reaction doable for molecules bearing extra functional groups, avoiding the need for the protection/deprotection reactions.[34−37] In 2001, Sharpless and coworkers[35] introduced the concept of click chemistry reactions, of which the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction between terminal alkynes and azides is one of the most popular and commonly used click reactions.[38,39] In previous studies, click chemistry has been used to prepare the shell as well as core-cross-linked micelles.[34,40−42]

In this Article, we report a unique strategy leading to the introduction of hydrolyzable cross-links to the core of poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) micelles using click chemistry. Copolymers of PEO-*b*-PCL have been extensively explored in drug delivery. In our research group, both the core and shell block of PEO-*b*-PCL micelles have been engineered to achieve depot or targeted drug and siRNA delivery.[43−53] Here we report the successful synthesis and characterization of core-cross-linked PEO-*b*-PCL micelles by click chemistry. Toward this, block copolymers of PEO-*b*-PCL having pendent propargyl carboxylate groups on the PCL block, that is, PEO-*b*-PPCL, were synthesized via ring-opening polymerization of α-propargyl carboxylate ε-caprolactone using PEO as the initiator. Consequently, the block copolymer self-associated into micelles in aqueous solution. The micelle core was then cross-linked via reaction between the azide group of tetraethylene glycol (bis)azide reagent and the alkyne group on the PPCL block in the presence of copper catalysts at room temperature. The formation of cross-linked micelles was confirmed and characterized by ^{1}H NMR and IR spectroscopy, transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), and dynamic light scattering (DLS). The encapsulation and in vitro release of paclitaxel (PTX) in cross-linked micelles was compared with these properties for micelles without cross-links. The results from this study point to the enhanced stability of cross-linked micelles under diluted conditions without any negative impact on the encapsulation and in vitro release of PTX in the presence of cross-links.

To the best of our knowledge, this is the first Article that reports the use of click chemistry to develop core-cross-linked polymeric micelles that contain biocompatible PEO "crosslinks" that are attached to the poly(ester) core of polymeric micelles via "degradable" ester bonds. This is also the first report on the application of click chemistry to prepare core-cross-linked micelles made up of highly used PEO-*b*-PCL diblock copolymers.

Scheme 1. Synthetic Scheme for the Preparation of Core-Crosslinked Micelles



EXPERIMENTAL SECTION

Materials. Methoxy-poly(ethylene oxide) (PEO) (average molecular weight of 5000 g/mol), diisopropylamine (99%), propargyl chloroformate, sodium (in kerosin), butyllithium (Bu-Li) in hexane (2.5 M solution), tetraethylene glycol ditosylate, and bovine serum albumin (BSA) powder were purchased from Sigma (St. Louis, MO). PTX (purity >99.5) was purchased from LC Laboratories (Woburn, MA). ε-Caprolactone was purchased from Lancaster Synthesis (Heysham, U.K.). Stannous octoate was purchased from MP Biomedicals (Eshwege, Germany). All other chemicals were reagent grade. Tetraethylene glycol (bis)azide was synthesized from tetraethylene glycol ditosylate and sodium azide according to literature procedures and confirmed by ^{1}H NMR.[54]

Synthesis of α-Propargyl Carboxylate-ε-Caprolactone. The method used for the synthesis of α-propargyl carboxylate-ε-caprolactone is shown in Scheme 1. In brief, Bu-Li (24 mL) in hexane was slowly added to dry diisopropylamine (8.4 mL) in 50 mL of dry THF in a three-necked round-bottomed flask at −30 °C under vigorous stirring with continuous argon supply. The solution was cooled to −78 °C. ε-Caprolactone (3.42 g) was dissolved in 10 mL of dry THF and added to the above-mentioned mixture slowly, and the reaction was allowed to continue for 45 min. Propargyl chloroformate (3.55 g) was added, and the temperature was allowed to increase to 0 °C. The reaction was allowed to continue for 2 h and was quenched with 5 mL of saturated ammonium chloride solution. The reaction mixture was diluted with water and extracted with ethyl acetate (110 mL). The combined extracts were dried over Na_2SO_4 and evaporated. The dark yellowish oily crude mixture was purified twice over a silica gel column, and the purity of the compound was confirmed with thin-layer chromatography (TLC) using hexane/ethyl acetate (2:1) as the mobile phase. The chemical structure was analyzed by ^{1}H NMR, ^{13}C NMR, and IR.

Synthesis of PEO-*block*-poly(α-propargyl carboxylate-ε-caprolactone) (PEO-*b*-PPCL). Block copolymers of PEO-*b*-PPCL were synthesized by ring-opening polymerization of α-propargyl

carboxylate-ε-caprolactone using PEO as initiator and stannous octoate as catalyst (Scheme 1). In brief, PEO (M_w: 5000 g/mol) (1.5 g), α-propargyl carboxylate-ε-caprolactone (1.5 g), and stannous octoate (0.002 equiv of monomer) were added to a 10 mL previously flamed ampule, nitrogen purged, and sealed under vacuum. The polymerization reaction was allowed to proceed for 4 h at 140 °C in oven. We terminated the reaction by cooling the product to room temperature. The ^{1}H NMR spectrum of α-propargyl carboxylate-ε-caprolactone bearing block copolymer in $CDCl_3$ at 300 MHz was used to assess the conversion of α-propargyl carboxylate-ε-caprolactone monomer to PPCL comparing the intensity of the $-O-CH_2$ (δ 4.25)-related peak for α-propargyl carboxylate-ε-caprolactone monomer to the intensity of the same protons for PPCL (δ 4.05). The number-average molecular weight of PEO-*b*-PPCL was determined from the ^{1}H NMR spectrum comparing peak intensity of PEO ($-CH_2CH_2O-$, δ 3.65) to that of PPCL ($-C\equiv CH$, δ 2.55), considering a 5000 g/mol molecular weight for PEO. The IR spectrum was obtained by dissolving the block copolymers in dichloromethane and preparing a thin film on NaCl disk.

The polydispersity of prepared block copolymer was assessed by gel permeation chromatography (GPC). In brief, 20 μL of polymer solution (20 mg/mL in THF) was manually injected into a 7.8 × 300 mm Styragel HMW 6E column (Waters, Milford, MA) which was attached to an HP 1100 pump. The column was eluted with 1 mL/min THF. The elution pattern was detected by refractive index (model 410; Waters) and DLS detectors (PD 2000 DLS; Precision Detectors, Franklin, MA) using polystyrene standard of two molecular weights (M_w) (9580 and 13 700 g/mol).

Micelle Formation and Core-Cross-Linking. First, micellization was achieved by solvent evaporation method. In brief, the synthesized block copolymer of PEO-*b*-PPCL (60 mg) was dissolved in acetone (1 mL). This solution was added to 6 mL of doubly distilled water in a dropwise manner under moderate stirring at room temperature, followed by the evaporation of acetone under vacuum. The prepared micellar solution was then centrifuged to remove any aggregates.

Micellar solution of block copolymers was cross-linked using the Huisgens 1,3-dipolar cycloaddition (azide−alkyne Click chemistry) reaction.[38,39] In brief, to the prepared PEO-*b*-PPCL micelle solutions, tetraethylene glycol (bis)azide (15.86 mg; 0.065 mmol) was added under vigorous stirring, followed by the addition of sodium ascorbate (2.57 mg; 0.013 mmol) and copper sulfate (0.21 mg; 0.0013 mmol). The reaction mixture was stirred for 16 h and then purified by dialysis against water and freeze-dried. The resulted polymer was subjected to ^{1}H NMR and IR analysis. The schematic representation of the preparation method for core-cross-linked micelles is shown in Scheme 1.

Characterization of Micelles. The particle size and distribution of prepared micelles were estimated by DLS using a Malvern Zetasizer 3000 at a polymer concentration of 10 mg/mL in water at 25 °C. Morphology of the micelles was investigated by TEM. An aqueous droplet of micellar solution (20 μL) with a polymer concentration of 1 to 2 mg/mL was placed on a copper-coated grid. The grid was held horizontally for 20 s to allow the colloidal aggregates to settle. A drop of 2% solution of phototungstic acid (PTA) in PBS (pH 7) was then added to provide the negative stain. After 1 min, the excess fluid was removed by filter paper. The samples were then air-dried and loaded into a Hitachi H 700 TEM. Images were obtained at a magnification of 18 000× at 75 kV.

A change in the fluorescence excitation spectra of the hydrophobic pyrene in the presence of varied concentrations of PEO-*b*-PPCL was used to measure the critical micellar concentration (CMC) of the prepared block copolymer according to a method previously described.[55] The fluorescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia). Emission wavelength and excitation/emission slit were set at 390 and 5 nm, respectively. The intensity ratio of peaks at 338 nm to those at 333 nm was plotted against the logarithm of copolymer concentration. The CMC was measured from a sharp rise in intensity ratios (I_{338}/I_{333}) at the onset of micellization.

XPS measurements were conducted on freeze-dried sample of cross-linked micelles using an Axis-165 spectrometer (Kratos Analytical, Chestnut Ridge, NY) with a monochromatic Al Kα X-ray source at 1486.6 eV. The analyzer was operated in constant resolution mode at a pass energy of 20 eV and charge referencing was accomplished by setting the C 1s line of adventitious hydrocarbon on the specimen surface at 284.8 eV.

Evaluation of Protein Adsorption on Micelles. The measurement of the amount of protein adsorbed on the surface of the micelles was carried out according to a method previously described.[56] In brief, non-cross-linked and cross-linked micellar solutions (8 mg/mL) were mixed with equal volume of BSA solution (45 g BSA/L in 0.01 M PBS) and incubated for 4 h at 37 °C. Micellar solutions with equal volume of PBS were used as control and incubated for 4 h at 37 °C. After incubation, solution samples of 20 μL were injected into a gel permeation chromatography (GPC) system with a hydrogel column (Waters, Milford, MA) at 25 °C. The elution pattern was detected at 35 °C by light scattering detector (model 410, Waters). We used 0.01 M PBS (pH 7.4) (1 mL/min) as eluent. Eluate containing the micellar fraction was collected, and the concentration of protein in the eluate was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Preparation of PTX-Loaded Micelles. Encapsulation of PTX in non-cross-linked PEO-*b*-PPCL was accomplished as reported before.[53,57] In brief, the block copolymer and PTX were both dissolved in *N*,*N*-dimethyl formamide (DMF) as the organic solvent. This solution was added to water in a dropwise manner, followed by dialysis of the solution against water to remove DMF. For PTX loading in cross-linked micelles, two different methods were used. In the first method (method I), PTX, PEO-*b*-PPCL, and the cross-linking agent (tetraethylene glycol (bis)azide) were all added to DMF, and this solution was added to water containing sodium ascorbate and $CuSO_4$. In the second method (method II), PTX-encapsulated micelles were prepared as described above. The cross-linking agent and other reagents were then added to micellar aqueous solution. The applied drug to polymer weight ratio was 20 for all formulations. After dialysis, the solution was centrifuged at 11 600*g* for 5 min to remove any precipitate, and an aliquot (100 μL) of the micellar solution was diluted with acetonitrile. The solution was analyzed for PTX content using HPLC. Reversed phase chromatography was carried out using a Varian Prostar 210 HPLC system equipped with a Microsorb-MV 5 μm C18-100 Å column (4.6 mm × 250 mm), and Varian 335 photodiode array HPLC detector (Mulgrave, Australia). We injected 20 μL of sample in a gradient elution using 0.1% trifluoroacetic acid aqueous solution and acetonitrile at a flow rate of 1.0 mL/min at room temperature. The proportion of acetonitrile was 40% at time 0 and increased with elution time up to 100% within 15 min.[53,58] The detection was performed at 227 nm. The level of PTX loading (w/w %) and encapsulation efficiency was calculated using the following equations

$$\text{PTX loading}\ (\%) = \frac{\text{amount of physically loaded PTX in mg}}{\text{amount of copolymer in mg}} \times 100\%$$

$$\text{encapsulation efficiency}(\%) = \frac{\text{amount of physically loaded PTX in mg}}{\text{amount of PT added in mg}} \times 100\%$$

Release of PTX from Polymeric Micelles. Release of PTX from non-cross-linked and cross-linked micelles was determined in 0.01 M phosphate buffer (pH 7.4) containing 2 M sodium salicylate at 37 °C.[53,59,60] The experiment was initiated by the addition of free or

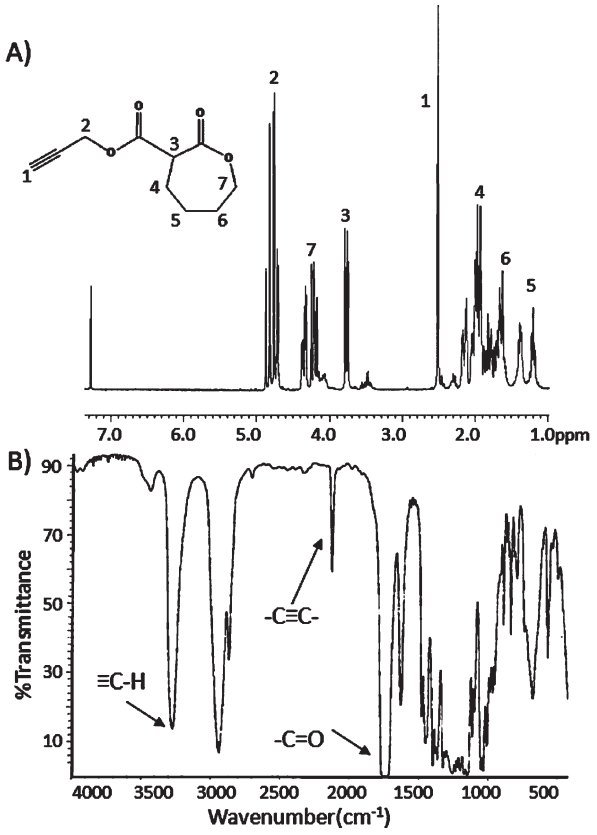


Figure 1. (A) ^{1}H NMR spectrum of α-propargyl carboxylate-ε-caprolactone (substituted monomer) in $CDCl_3$ and peak assignments. (B) IR spectrum of α-propargyl carboxylate-ε-caprolactone. Arrow indicates the presence of characteristic groups.

micellar PTX solution to the buffer. The PTX-loaded non-cross-linked and cross-linked micelles were prepared at 20 μg/mL PTX concentration according to the previous mentioned method. Then, 10 mL (containing 200 μg PTX) of the micellar solutions was transferred to a dialysis bag (Spectraphor, M_w cutoff 3500 g/mol). The dialysis bags were placed in 500 mL of 0.01 M phosphate buffer (pH 7.4). The release study was performed at 37 °C in a Julabo SW 22 shaking water bath (Seelbach, Germany). At selected time intervals, the whole release media was replaced with fresh one, and aliquots of 200 μL were withdrawn from the inside of the dialysis bag for HPLC analysis. The amount of PTX released was calculated by subtracting the amount of PTX that remained in the dialysis bag from the initially added PTX. The release profiles were compared using similarity factor, f_2, and the profiles were considered to be significantly different if $f_2 < 50$.[61]

$$f_2 = 50 \times \log\left(\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n}|R_j - T_j|^2\right]^{-0.5} \times 100\right)$$

where n is the sampling number and R_j and T_j are the percent released of the reference and test formulations at each time point j.

RESULTS AND DISCUSSION

Synthesis and Characterization of Block Copolymers. Block copolymers of PEO-*b*-PPCL were developed by ring-opening polymerization of α-propargyl carboxylate-ε-caprolactone monomer using PEO as initiator and stannous octoate as catalyst.[62] Our research group and others have previously reported on the preparation of substituted lactone monomers such as α-benzyl carboxylate-ε-caprolactone,[52] α-propargyl-δ-valerolactone,[63] α-allyl-δ-valerolactone,[64] α-propargyl-ε-caprolactone,[65,66] and α-iodo-ε-caprolactone.[67] For this purpose, anionic activation of ε-caprolactone monomer was performed using freshly prepared non-nucleophilic strong base LDA to extract a methylene proton from the α-position (−CH_2−C═O). The generated lithium carbanion was then quenched with propargyl chloroformate to obtain α-propargyl carboxylate-ε-caprolactone (Scheme 1).[68] After column chromatography, α-propargyl carboxylate-ε-caprolactone was isolated as a slightly yellow thick oily liquid. The product produced a single spot at R_f value of 0.37 in TLC. The yield of reaction was 49.2%. The structure was confirmed by combined analysis of ^{1}H NMR and IR. In 300 MHz ^{1}H NMR spectroscopy in $CDCL_3$, corresponding proton peaks were observed at δ: 1.20−2.20 (m, 6H); δ: 2.50 (s, 1H); δ: 3.75 (d, 1H); δ: 4.15−4.40 (m, 2H); δ: 4.70−4.85 (q, 2H) (Figure 1A). The peak at 3.75 ppm for α-propargyl carboxylate-ε-caprolactone, which corresponds to a single proton instead of two protons of ε-caprolactone monomer, indicates the successful substitution of the propargyl carboxylate on ε-caprolactone at the α-position. The presence of two negative peaks for carbonyl at 168.10 and 171.38 ppm and the generation of a new characteristic positive peak at 50.65 ppm in the ^{13}C NMR spectrum also confirm the chemical structure of the reaction product (Supporting Information Figure S1). The presence of strong peak in the IR spectrum (Figure 1B) at 1725 cm^{-1} corresponds to the carbonyl groups in lactone and propargyl carboxylate. The presence of strong peak at 3270 cm^{-1} and weak peak at 2120 cm^{-1} corresponds to the alkyne group in the monomer.

In 300 MHz ^{1}H NMR spectroscopy conducted on PEO-*b*-PPCL in $CDCL_3$, corresponding proton peaks for the product were observed at δ: 1.20−2.15 (m, 6H); δ: 2.50 (s, 1H); δ: 3.30−3.45 (s, 3H; m, 1H); δ: 3.65 (s, 4H); δ: 4.05−4.25 (m, 2H); δ: 4.75 (s, 2H) (Figure 2A). The presence of peaks at 2.50 and 4.75 ppm, which are due to the alkyne and methylene protons of the propargyl carboxylate group, respectively, confirms the polymerization of α-propargyl carboxylate-ε-caprolactone and the presence of alkyne groups in the structure of block copolymer. Furthermore, the characteristic downfield shift of the methylene protons (−OCH_2− of α-propargyl carboxylate-ε-caprolactone) from 4.30 to 4.15 and O═C−CH− proton from 3.75 to 3.30 in the ^{1}H NMR spectra (Figures 1A and 2A) strongly indicates the ring-opening polymerization of the monomer and formation of block copolymers. The characteristic terminal alkyne peak at 3270 cm^{-1} and the −C≡C− peak at 2120 cm^{-1} in the IR spectrum of PEO-*b*-PPCL indicates the presence of terminal alkyne group in the block copolymer (Figure 2B).

The molecular weight of prepared PEO-*b*-PPCL block copolymer, measured by comparing the peak intensities of four methylene protons of PEO (δ: 3.65) and one alkyne proton of PPCL (δ: 2.50) in the ^{1}H NMR spectrum, was calculated to be 8800 g/mol (equal to a degree of α-propargyl carboxylate-ε-caprolactone polymerization of 19, that is, PEO_{114}-*b*-$PPCL_{19}$). The resulting block copolymer showed a broad polydispersity (M_w/M_n = 1.50) when measured by GPC analysis.

Micellization of Block Copolymers and Core-Cross-linking. Synthesized block copolymers were assembled to polymeric micelles by a cosolvent evaporation method as previously described.[52,62] To prevent the disassembly of micelles under the high dilution conditions of the bloodstream, we must prevent

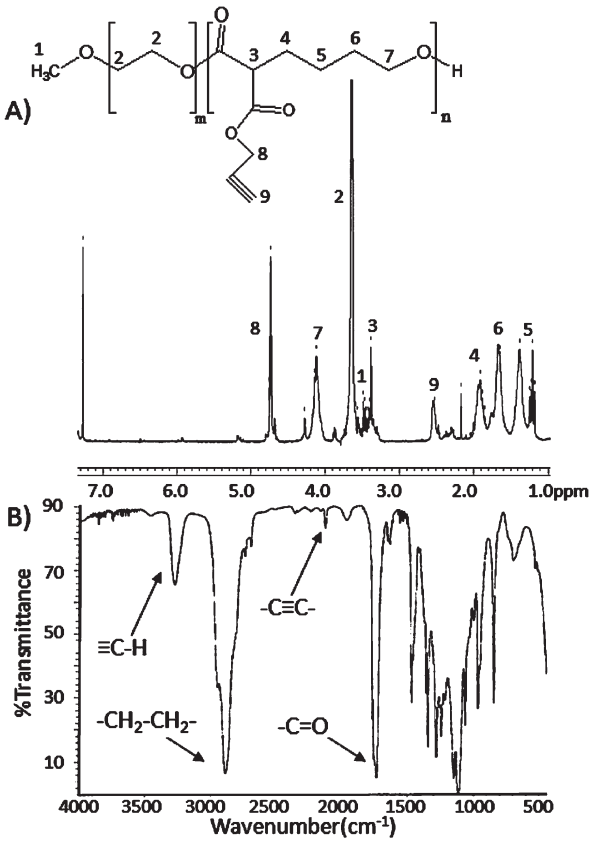


Figure 2. (A) ^{1}H NMR spectrum of PEO-*b*-PPCL in $CDCl_3$ and peak assignments. (B) IR spectrum of PEO-*b*-PPCL.

the dissociation of the micellar core. This can be achieved by cross-linking the micelles at the core, which has proven to be an effective method in the past.[24−26] Cross-linking of the PPCL core was carried out using bifunctional tetraethylene glycol bis(azide) via copper-catalyzed azide−alkyne cycloaddition (CuAAC) click chemistry reaction, which has been previously used for the preparation of block copolymers or polyesters with various functional groups.[69,70]

The presence of terminal alkyne group and azide group is necessary for the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction to take place. Cu(I) acts as a catalyst for the reaction. Cu(I) is prepared in situ by the addition of Cu(II) $CuSO_4$, and ascorbic acid, which acts as a reducing agent and reduces Cu(II) to Cu(I).[38] Preparation of cross-linked micelles by this method has several major advantages to those previously reported: The reaction is known to be carried out under ambient conditions and is highly specific. The Cu(I), ascorbic acid, and unreacted azide can be removed by dialysis after completion of the reaction. Finally, this method provides means for the introduction of cross-links at desired density through potentially degradable ester bonds to the poly(ester) core of micelles ensuring ultimate removal of the micellar carrier from the biological system upon degradation.

The size and morphology of the micelles were studied by DLS and TEM (Figure 3). The average diameter for micelles was shown to decrease from 97.9 ± 0.6 nm for non-cross-linked micelles to 82.6 ± 0.3 nm for cross-linked micelles. The micellar population showed a similar distribution in both cases (PI = 0.43). The decrease in size can be a result of the packing at the core due to covalent bond formations. The TEM picture for both micelles shows the formation of true spherical-shaped colloidal particles having a clear boundary (Figure 3A,B). The average diameter in the dry state based on TEM images was shown to decrease from 19.5 nm for non-cross-linked micelles to 15.4 nm for cross-linked micelles, which shows a similar trend to the DLS data. The difference in size measured by these two methods (97.9 vs 19.5 nm for non-cross-linked micelles and 82.6 vs 15.4 nm for cross-linked micelles) is attributed to the acquirement of the TEM images under a dry state as opposed to DLS that measures particles in a hydrated state in aqueous solutions.[53,71,72]

To further verify the success of core-cross-linking, we added equal volume of acetone to a 500 μg/mL micellar solution. DLS measurements were performed to compare the stability of the cross-linked and non-cross-linked micelles (Figure 3C,D). The cross-linked micelles did not dissolve in acetone, thus confirming cross-linking of the core.[73] The average size and polydispersity of the cross-linked micelles were slightly larger in acetone, which can be due to swelling of the core of the cross-linked micelles as a result of being penetrated by solvent molecules, in this case acetone.[29,31] The non-cross-linked micelle, however, dissociated and did not give any reading on the DLS. Samples maintained at 25 °C were analyzed by DLS after 21 days (data not shown). The cross-linked micelles still maintained their nanostructure, as opposed to the non-cross-linked micelles, but with slightly larger size. This further verifies the stability of the core-cross-linked micelles.

^{1}H NMR spectroscopy was performed on the freeze-dried micelles in $CDCl_3$ (Figure 4). $CDCl_3$ is a good solvent for the PEO-*b*-PPCL block copolymer. The signal for the hydrophobic PCL block is clearly visible in the non-cross-linked micelles (1.20−2.00 ppm) (Figure 4A). However, the signal has weakened considerably in the case of the cross-linked micelles (Figure 4B). As the PCL segment is present in the micellar core, the covalent bond formed because of cross-linking results in the rigidity of the hydrophobic core and maintenance of micellar structure in organic solvents like $CDCl_3$. Because the solvent is unable to penetrate the interior, the signal corresponding to the intensity of the PCL block at the interior is weakened because of lack of mobility of core segments.[31] This confirms the stability of cross-linked micelles over the non-cross-linked micelles.

The IR spectrum confirmed that a click chemistry reaction has taken place (Figure 5). The absence of peaks at 3270 cm^{-1} and weak peak at 2120 cm^{-1} corresponding to the alkyne group in the PPCL core along with the appearance of sharp peak at 810 cm^{-1} corresponding to =C−H bending (characteristic of trisubstituted alkene) indicates the reaction between alkyne groups in the PPCL core and diazide cross-linker producing the triazole ring.

The elemental analysis by XPS is shown in Table S1 (Supporting Information). The mass concentration of nitrogen in the sample calculated by XPS was found to be 3.65%. Figure S2 (Supporting Information) shows a broad N1s peak near 400 eV. By multipeak fitting, the peak can be separated into two peaks at 399.9 and 401.4 eV, which corresponds to the triazole ring. The absence of a peak at ∼405 eV indicates the absence of azide group in the sample.[74−76] This indicates the removal of any excess azide from the cross-linked micelles. In addition, XPS revealed an extremely small Cu2p peak denoting the presence of residual Cu^{2+} ions, with a mass concentration of 0.20% in the cross-linked micelles. This indicates that Cu ion could not be completely removed from the sample, which may be due to the

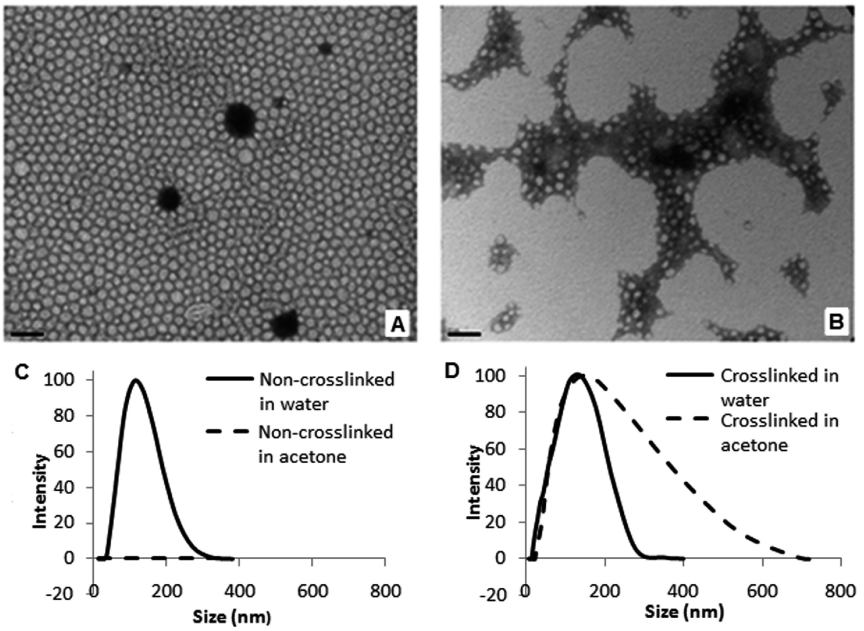


Figure 3. TEM picture of (A) non-cross-linked micelles and (B) cross-linked micelles (magnification 18 000×). Particle size distribution of (C) non-cross-linked micelles and (D) cross-linked micelles by DLS in water and in acetone.

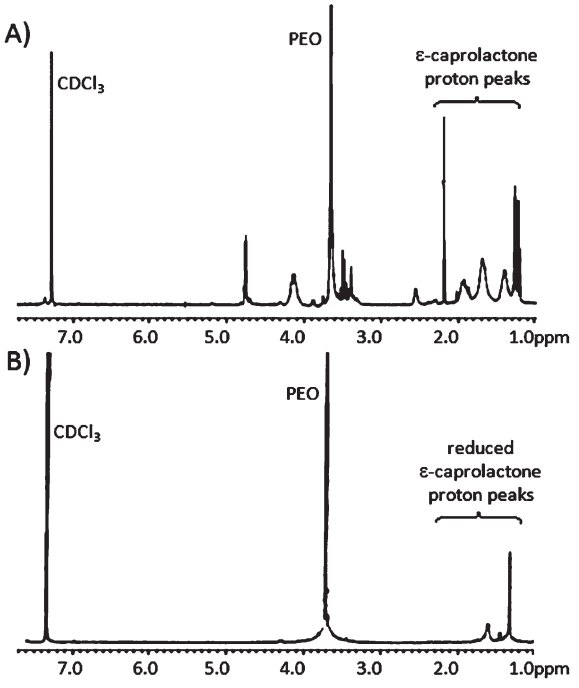


Figure 4. ^{1}H NMR spectrum and peak assignments of (A) non-cross-linked micelles and (B) cross-linked micelles in $CDCl_3$.

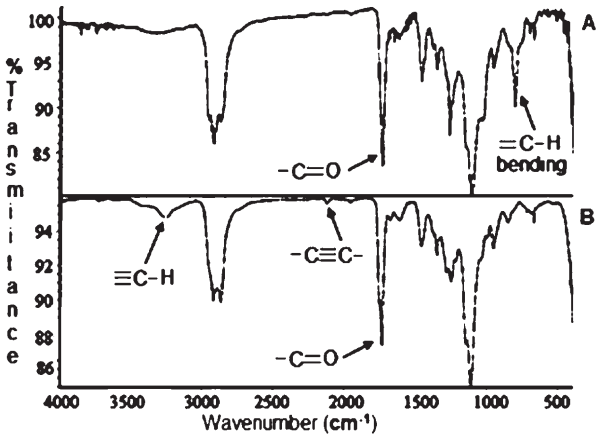


Figure 5. IR spectrum of (A) cross-linked micelles and (B) non-cross-linked micelles.

fact that the dialysis was not carried out in the presence of 0.02 M ethylenediaminetetraacetic acid disodium (EDTA).[74]

The CMC of PEO-*b*-PPCL block copolymer was determined by fluorescence spectroscopy using pyrene as the fluorescent probe. Pyrene is a strong hydrophobic probe with very low water solubility. Because of its hydrophobicity, it preferentially partitions into the hydrophobic domain of the micellar core at concentrations above CMC, resulting in a change in its photophysical properties. This property is used to measure the CMC of block copolymers. A sharp increase in the intensity ratio of peaks at 338 nm to those at 333 nm from the excitation spectra of pyrene indicates the onset of micellization. Using this method, the average CMC of PEO_{114}-*b*-$PPCL_{19}$ block copolymer was found to be 0.305 ± 0.025 μM. This value was found to be higher than that obtained for PEO_{114}-*b*-$PBCL_{19}$ (0.182 μM; previously carried out in our laboratory).[52] The increase in the CMC of PEO_{114}-*b*-$PPCL_{19}$ compared with PEO_{114}-*b*-$PBCL_{19}$ is attributed to the lower hydrophobicity of the core-forming block in PEO-*b*-PPCL. With regards to cross-linked micelles, the concept of CMC is not applicable because they have a covalently attached micellar structure, and hence the CMC assessment for cross-linked micelles was not carried out.[73]

Protein Adsorption on Micelles. Interaction with serum proteins is one of the factors that influences the fate of drug

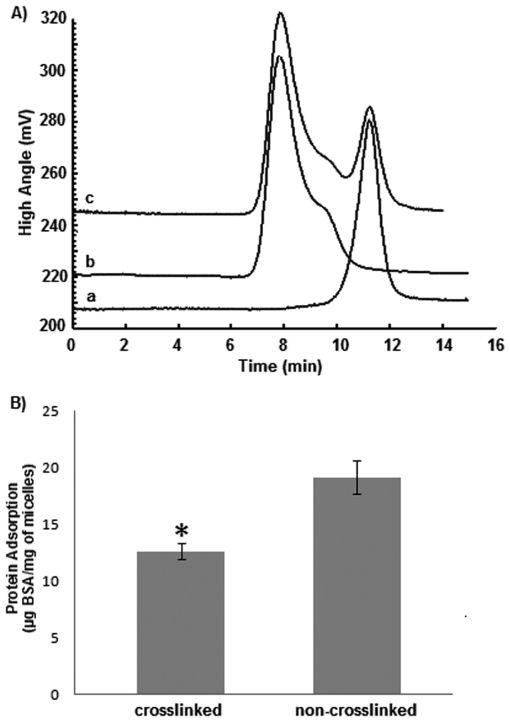


Figure 6. (A) Gel permeation chromotagram of (a) BSA solution, (b) cross-linked micelles, and (c) mixture of cross-linked micelles and BSA after incubation at 37 °C for 4 h. (B) Protein adsorption of cross-linked and non-cross-linked micelles (μg BSA/mg of micelles) (n = 3).

delivery vehicles like liposomes,[77] nanoparticles,[78] and micelles[56] in the body. The amount of protein adsorbed on the surface of the non-cross-linked and cross-linked micelles after incubation in a BSA solution prepared at physiological concentration was assessed according to a previously published method using gel permeation chromatography for the separation of BSA adsorbed micelles from free BSA, followed by Bio-Rad protein assay on the eluted sample containing BSA adsorbed micelles.[56,77] As shown in Figure 6A, cross-linked micelles were found to elute from the column at 7−10 min. Non-cross-linked micelles were found to have a similar elution profile as cross-linked micelles (data not shown). The elution time of BSA was found to be 10−12 min. After incubation for 4 h with BSA, the elution peak for cross-linked and non-cross-linked micelles remained the same. The protein binding values for cross-linked and non-cross-linked micelles were 12.6 ± 0.7 and 19.1 ± 1.4 μg BSA/mg of micelles (n = 3), respectively. Both cross-linked and non-cross-linked micelles showed insignificant adsorption of BSA, suggesting that the hydrophilic PEO block provides sufficient coverage of the hydrophobic core of the micelles.[56] Cross-linking of the micelles, however, was shown to have caused a significant decrease (p < 0.05) in the adsorption of proteins on the micellar surface. This can be due to the fact that the core of cross-linked micelles is in a fixed and more compact state when compared with non-cross-linked micelles. This may result in a higher density and extension of the PEO chains in the micellar shell, leading to a better steric effect by the hydrophilic shell in the case of cross-linked micelles. Also, the cross-linking block (tetrathylene glycol) is hydrophilic in nature, which might be decreasing the hydrophobicity of the core/shell interface region, leading to less protein adsorption. These results imply better in vivo stability of cross-linked micelles as compared with non-cross-linked micelles in terms of preventing protein adsorption and further opsonization in the biological system.

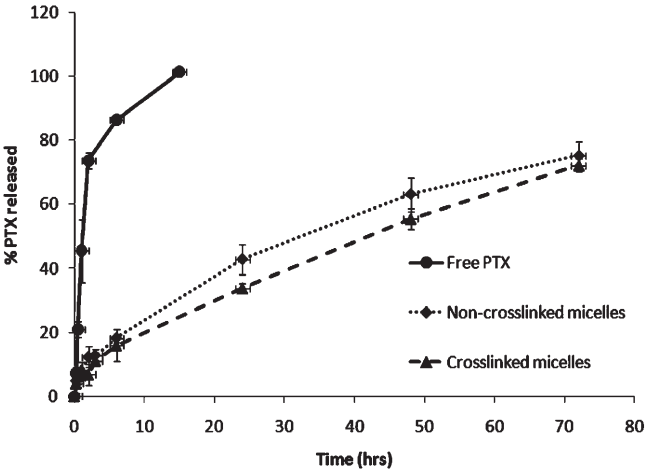


Figure 7. In vitro release profile of physically encapsulated PTX from different micellar formulations in phosphate buffer (pH 7.4) containing 2 M sodium salicylate at 37 °C. Each point represents mean ± SD (n = 3).

Preparation and Characterization of Polymeric Micelles Containing Physically Encapsulated PTX. A maximum PTX solubility of 20.99 μg/mL was achieved with non-cross-linked PEO-*b*-PPCL micelles (Table 1). The PTX encapsulation for the PEO-*b*-PPCL micelles was lower than that obtained by PEO-*b*-PCL micelles,[53] which may be due to an increase in the rigidity of the core with propargyl side chain in PEO-*b*-PPCL micelles, the presence of shorter hydrophobic backbone in PEO-*b*-PPCL micelles studied here, or both.

For PTX loading in cross-linked micelles, better solubility was achieved when the cross-linking agent was added in DMF containing polymer and drug (method I, PTX solubility of 21.48 μg/mL) as opposed to the method in which cross-linking agent was added to micellar solution of drug in water (method II, PTX solubility of 1.66 μg/mL). This may be due to leaking out of the PTX from the micellar core during the cross-linking step after the preparation of non-cross-linked micelles. Drug loading was expected to decrease in core-cross-linked micelles as a result of a reduction in the free volume of the micellar core; however, this was not the case for the cross-linked micelles prepared by method I.

The results of assessments on the in vitro release of PTX from non-cross-linked and cross-linked micelles and free PTX in phosphate buffer (pH 7.4, 0.01 M) containing 2 M sodium salicylate at 37 °C is illustrated in Figure 7. The maximum concentration of PTX in the medium was 0.4 μg/mL, whereas the solubility of PTX in 2 M sodium salicylate medium was 333.1 μg/mL,[60] and thus sink conditions were respected in the release study condition. Free PTX was released from the dialysis bag at a rapid rate, which means that the transfer of PTX through dialysis membrane to buffer solution is not a restricting factor and the release of PTX from the micellar formulation is the rate-limiting step in the process. Both non-cross-linked and cross-linked micelles showed much slower release profiles when compared with free PTX. Owing to a decrease in free volume in the micellar core as a result of cross-linking, we expected to see a slower release of PTX from the core-cross-linked micelles. In reality; however, the release profile of PTX at concentrations above the CMC of polymers was similar for both structures. Similar release

Table 1. Characteristics of PTX-Loaded Copolymer Micelles When DMF Was Used As the Solvent for Micellization (n = 3)

micelles	PTX loading content (%) ± SD: PTX/polymer (mol %)	PTX loading content (%) ± SD: PTX/polymer (wt%)	encapsulation efficiency (%)	average diameter (nm) (empty)	average diameter (nm) (PTX loaded)	PDI[a]	PTX released after 72 h (%)[b]
non-cross-linked	9.37 ± 0.13	0.92 ± 0.01	18.34 ± 0.25	57.1 ± 0.6	56.9 ± 0.3	0.41	75.17 ± 4.43
cross-linked	9.60 ± 0.12	0.94 ± 0.01	18.80 ± 0.23	57.5 ± 0.5	56.4 ± 0.4	0.38	72.20 ± 2.01

[a] Polydispersity index of micellar size distribution. [b] Release study was performed in phosphate buffer (pH 7.4) containing 2 M sodium salicylate.

profiles of PTX between non-cross-linked and cross-linked micelles were also seen in studies carried out previously by Kissel and group.[29] This could be due to the fact that although the core is stabilized by core-cross-linking, the drug is easily diffusible from the micellar structure. The observation may also imply the localization of PTX in core/shell interface rather than the micellar core in micellar structure. Further investigations are needed to define the possible reason behind this observation. Even without a difference in release, the stabilization of micelles by cross-linking is expected to lead to lower rate and extent of drug release in vivo because dissociation of micellar structure is now prevented by cross-linking. This hypothesis is under further investigation in our research group through in vivo studies comparing the pharmacokinetics of PTX formulated using cross-linked and non-cross-linked micelles.

CONCLUSIONS

Diblock copolymers of PEO and terminal alkyne bearing α-propargyl carboxylate-ε-caprolactone were successfully synthesized via ring-opening polymerization. The process of self-association of block copolymer to micelles was followed by cross-linking of the core using bifunctional tetraethylene glycol bis(azide) via the Cu(I)-catalyzed 1,3-Huisgen cycloaddition click reaction. The cross-linked micelles were characterized by ^{1}H NMR and IR spectroscopy, XPS, DLS, and TEM, which confirmed the formation of stable cross-linked micelles by the mentioned procedures. Protein adsorption study revealed low adsorption of BSA on cross-linked micelles implying better in vivo stability of these structures against opsonization. The cross-linking did not influence the size distribution, loading, and in vitro release of PTX from the micelles significantly. Overall, the results point to the suitability of prepared core-cross-linked polymeric micelles for use as more stable nanodelivery vehicles.

ASSOCIATED CONTENT

Supporting Information. ^{13}C NMR and XPS data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +1-780-492-2742. Fax: +1-780-492-1217. E-mail: alavasanifar@pharmacy.ualberta.ca.

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REFERENCES

(1) Rapoport, N. *Prog. Polym. Sci.* **2007**, *32*, 962–990.
(2) Mahmud, A.; Xiong, X. B.; Aliabadi, H. M.; Lavasanifar, A. *J. Drug Targeting* **2007**, *15*, 553–84.
(3) Adams, M. L.; Lavasanifar, A.; Kwon, G. S. *J. Pharm. Sci.* **2003**, *92*, 1343–1355.
(4) Lavasanifar, A.; Samuel, J.; Kwon, G. S. *Colloids Surf., B* **2001**, *22*, 115–126.
(5) Letchford, K.; Zastre, J.; Liggins, R.; Burt, H. *Colloids Surf., B* **2004**, *35*, 81–91.
(6) Gaucher, G.; Dufresne, M. H.; Sant, V. P.; Kang, N.; Maysinger, D.; Leroux, J. C. *J. Controlled Release* **2005**, *109*, 169–188.
(7) Kang, N.; Perron, M. E.; Prud'homme, R. E.; Zhang, Y.; Gaucher, G.; Leroux, J. C. *Nano Lett.* **2005**, *5*, 315–319.
(8) Zhang, J.; Wang, L. Q.; Wang, H.; Tu, K. *Biomacromolecules* **2006**, *7*, 2492–2500.
(9) Bae, Y.; Kataoka, K. *Adv Drug Deliv Rev* **2009**, *61*, 768–784.
(10) Yokoyama, M.; Fukushima, S.; Uehara, R.; Okamoto, K.; Kataoka, K.; Sakurai, Y.; Okano, T. *J. Controlled Release* **1998**, *50*, 79–92.
(11) Sun, X.; Rossin, R.; Turner, J. L.; Becker, M. L.; Joralemon, M. J.; Welch, M. J.; Wooley, K. L. *Biomacromolecules* **2005**, *6*, 2541–2554.
(12) Rosler, A.; Vandermeulen, G. W. M.; Klok, H. A. *Adv. Drug Delivery Rev.* **2001**, *53*, 95–108.
(13) Yoshida, E.; Tanaka, M.; Takata, T. *Colloid Polym. Sci.* **2005**, *284*, 51–57.
(14) Harada, A.; Kataoka, K. *Prog. Polym. Sci.* **2006**, *31*, 949–982.
(15) Riess, G. *Prog. Polym. Sci.* **2003**, *28*, 1107–1170.
(16) Thurmond, K. B.; Kowalewski, T.; Wooley, K. L. *J. Am. Chem. Soc.* **1996**, *118*, 7239–7240.
(17) Murthy, K. S.; Ma, Q. G.; Clark, C. G.; Remsen, E. E.; Wooley, K. L. *Chem. Commun.* **2001**, *8*, 773–774.
(18) Wooley, K. L.; Zhang, Q. *Abstr. Pap. Am. Chem. Soc.* **1999**, *218*, U488.
(19) Butun, V.; Billingham, N. C.; Armes, S. P. *J. Am. Chem. Soc.* **1998**, *120*, 12135–12136.
(20) Yang, T. F.; Chen, C. N.; Chen, M. C.; Lai, C. H.; Liang, H. F.; Sung, H. W. *Biomaterials* **2007**, *28*, 725–734.
(21) Nystrom, A. M.; Wooley, K. L. *Tetrahedron* **2008**, *64*, 8543–8552.
(22) Procházka, K.; Baloch, M. K.; Tuzar, Z. *Makromol. Chem.* **1979**, *180*, 2521–2523.
(23) Tuzar, Z.; Bedná, B.; Koák, e.; Kubín, M.; Svobodová, t.; Procházka, K. *Makromol. Chem.* **1982**, *183*, 399–408.
(24) Iijima, M.; Nagasaki, Y.; Okada, T.; Kato, M.; Kataoka, K. *Macromolecules* **1999**, *32*, 1140–1146.
(25) Jiang, J. Q.; Qi, B.; Lepage, M.; Zhao, Y. *Macromolecules* **2007**, *40*, 790–792.

(26) Kim, J. H.; Emoto, K.; Iijima, M.; Nagasaki, Y.; Aoyagi, T.; Okano, T.; Sakurai, Y.; Kataoka, K. *Polym. Adv. Technol.* **1999**, *10*, 647–654.
(27) Tao, J.; Liu, G. J. *Macromolecules* **1997**, *30*, 2408–2411.
(28) Tao, J.; Liu, G. J.; Ding, J. F.; Yang, M. L. *Macromolecules* **1997**, *30*, 4084–4089.
(29) Shuai, X. T.; Merdan, T.; Schaper, A. K.; Xi, F.; Kissel, T. *Bioconjugate Chem.* **2004**, *15*, 441–448.
(30) Rijcken, C. J.; Snel, C. J.; Schiffelers, R. M.; van Nostrum, C. F.; Hennink, W. E. *Biomaterials* **2007**, *28*, 5581–5593.
(31) Jamroz-Piegza, M.; Walach, W.; Dworak, A.; Trzebicka, B. *J. Colloid Interface Sci.* **2008**, *325*, 141–148.
(32) Talelli, M.; Iman, M.; Varkouhi, A. K.; Rijcken, C. J.; Schiffelers, R. M.; Etrych, T.; Ulbrich, K.; van Nostrum, C. F.; Lammers, T.; Storm, G.; Hennink, W. E. *Biomaterials* **2010**, *31*, 7797–7804.
(33) Narita, T.; Terao, K.; Dobashi, T.; Nagasawa, N.; Yoshii, F. *Colloids Surf., B* **2004**, *38*, 187–190.
(34) Joralemon, M. J.; O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2005**, *127*, 16892–16899.
(35) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004.
(36) van Dijk, M.; Rijkers, D. T. S.; Liskamp, R. M. J.; van Nostrum, C. F.; Hennink, W. E. *Bioconjugate Chem.* **2009**, *20*, 2001–2016.
(37) Lecomte, P.; Riva, R.; Jerome, C. Synthesis of Functionalized Aliphatic Polyesters by the "Click" Copper-Catalyzed Alkyne−Azide Cycloaddition. In *New Smart Materials via Metal Mediated Macromolecular Engineering*; Khosravi, E., Yagci, Y., Savelyev, Y., Eds.; Springer: Dordrecht, The Netherlands, 2009; pp 77−91.
(38) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 2596–2599.
(39) Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057–3064.
(40) Jiang, X.; Zhang, G.; Narain, R.; Liu, S. *Langmuir* **2009**, *25*, 2046–2054.
(41) Jiang, X. Z.; Zhang, J. Y.; Zhou, Y. M.; Xu, J.; Liu, S. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2008**, *46*, 860–871.
(42) O'Reilly, R. K.; Joralemon, M. J.; Hawker, C. J.; Wooley, K. L. *New J. Chem.* **2007**, *31*, 718–724.
(43) Falamarzian, A.; Lavasanifar, A. *Colloids Surf., B* **2010**, *81*, 313−320
(44) Falamarzian, A.; Lavasanifar, A. *Macromol. Biosci.* **2010**, *10*, 648–656.
(45) Mahmud, A.; Patel, S.; Molavi, O.; Choi, P.; Samuel, J.; Lavasanifar, A. *Biomacromolecules* **2009**, *10*, 471–478.
(46) Mahmud, A.; Xiong, X. B.; Lavasanifar, A. *Eur. J. Pharm. Biopharm.* **2008**, *69*, 923–934.
(47) Xiong, X. B.; Ma, Z.; Lai, R.; Lavasanifar, A. *Biomaterials* **2010**, *31*, 757–768.
(48) Xiong, X. B.; Mahmud, A.; Uludağ, H.; Lavasanifar, A. *Biomacromolecules* **2007**, *8*, 874–884.
(49) Xiong, X. B.; Mahmud, A.; Uludağ, H.; Lavasanifar, A. *Pharm. Res.* **2008**, *25*, 2555–2566.
(50) Xiong, X. B.; Uludağ, H.; Lavasanifar, A. *Biomaterials* **2010**, *31*, 5886–5893.
(51) Xiong, X. B.; Uludağ, H.; Lavasanifar, A. *Biomaterials* **2009**, *30*, 242–253.
(52) Mahmud, A.; Xiong, X.; Lavasanifar, A. *Macromolecules* **2006**, *39*, 9419–9428.
(53) Shahin, M.; Lavasanifar, A. *Int. J. Pharm.* **2010**, *389*, 213–222.
(54) Susumu, K.; Uyeda, H. T.; Medintz, I. L.; Pons, T.; Delehanty, J. B.; Mattoussi, H. *J. Am. Chem. Soc.* **2007**, *129*, 13987–13996.
(55) Zhao, C. L.; Winnik, M. A.; Riess, G.; Croucher, M. D. *Langmuir* **1990**, *6*, 514–516.
(56) Liu, J. B.; Zeng, F. Q.; Allen, C. *J. Controlled Release* **2005**, *103*, 481–497.
(57) Letchford, K.; Liggins, R.; Wasan, K. M.; Burt, H. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 196–206.
(58) Forrest, M. L.; Yanez, J. A.; Remsberg, C. M.; Ohgami, Y.; Kwon, G. S.; Davies, N. M. *Pharm. Res.* **2008**, *25*, 194–206.
(59) Cho, Y. W.; Lee, J.; Lee, S. C.; Huh, K. M.; Park, K. *J. Controlled Release* **2004**, *97*, 249–257.
(60) Hu, F. Q.; Ren, G. F.; Yuan, H.; Du, Y. Z.; Zeng, S. *Colloids Surf., B* **2006**, *50*, 97–103.
(61) Costa, P.; Sousa Lobo, J. M. *Eur. J. Pharm. Sci.* **2001**, *13*, 123–133.
(62) Aliabadi, H. M.; Mahmud, A.; Sharifabadi, A. D.; Lavasanifar, A. *J. Controlled Release* **2005**, *104*, 301–311.
(63) Parrish, B.; Breitenkamp, R. B.; Emrick, T. *J. Am. Chem. Soc.* **2005**, *127*, 7404–7410.
(64) Parrish, B.; Quansah, J. K.; Emrick, T. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 1983–1990.
(65) Darcos, V.; El Habnouni, S.; Nottelet, B.; El Ghzaoui, A.; Coudane, J. *Polym. Chem.* **2010**, *1*, 280–282.
(66) Darcos, V.; Tabchi, H. A.; Coudane, J. *Eur. Polym. J.* **2011**, *47*, 187–195.
(67) Habnouni, S. E.; Darcos, V.; Coudane, J. *Macromol. Rapid Commun.* **2009**, *30*, 165–169.
(68) Ponsart, S.; Coudane, J.; Vert, M. *Biomacromolecules* **2000**, *1*, 275–281.
(69) Binder, W. H.; Sachsenhofer, R. *Macromol. Rapid Commun.* **2007**, *28*, 15–54.
(70) Lecomte, P.; Riva, R.; Jérôme, C.; Jérôme, R. *Macromol. Rapid Commun.* **2008**, *29*, 982–997.
(71) Hans, M.; Shimoni, K.; Danino, D.; Siegel, S. J.; Lowman, A. *Biomacromolecules* **2005**, *6*, 2708–2717.
(72) Yao, J. H.; Mya, K. Y.; Li, X.; Parameswaran, M.; Xu, Q. H.; Loh, K. P.; Chen, Z. K. *J. Phys. Chem. B* **2008**, *112*, 749–755.
(73) Hu, X.; Chen, X.; Wei, J.; Liu, S.; Jing, X. *Macromol Biosci* **2009**, *9*, 456–463.
(74) Tang, Y.; Liu, G.; Yu, C.; Wei, X.; Zhang, G. *Langmuir* **2008**, *24*, 8929–8933.
(75) Devadoss, A.; Chidsey, C. E. *J. Am. Chem. Soc.* **2007**, *129*, 5370–5371.
(76) Vestberg, R.; Malkoch, M.; Kade, M.; Wu, P.; Fokin, V. V.; Sharpless, K. B.; Drockenmuller, E.; Hawker, C. J. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 2835–2846.
(77) Allen, C.; Dos Santos, N.; Gallagher, R.; Chiu, G. N. C.; Shu, Y.; Li, W. M.; Johnstone, S. A.; Janoff, A. S.; Mayer, L. D.; Webb, M. S.; Bally, M. B. *Biosci. Rep.* **2002**, *22*, 225–250.
(78) Moghimi, S. M.; Pavey, K. D.; Hunter, A. C. *FEBS Lett.* **2003**, *547*, 177–182.