

MIT Open Access Articles

Enhanced Stability of Polymeric Micelles Based on Postfunctionalized Poly(ethylene glycol)-b-poly(γ -propargyl L-glutamate): The Substituent Effect

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Zhao, Xiaoyong, Zhiyong Poon, Amanda C. Engler, Daniel K. Bonner, and Paula T. Hammond. Enhanced Stability of Polymeric Micelles Based on Postfunctionalized Poly(ethylene Glycol)-b-poly(γ -propargyl L-glutamate): The Substituent Effect. *Biomacromolecules* 13, no. 5 (May 14, 2012): 1315-1322.

As Published: <http://dx.doi.org/10.1021/bm201873u>

Publisher: American Chemical Society

Persistent URL: <http://hdl.handle.net/1721.1/79570>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Terms of Use: Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.





Published in final edited form as:

Biomacromolecules. 2012 May 14; 13(5): 1315–1322. doi:10.1021/bm201873u.

Enhanced Stability of Polymeric Micelles Based on Post-functionalized Poly(ethylene glycol)-*b*-Poly(γ -propargyl ϵ -glutamate): the Substituent Effect

Xiaoyong Zhao, Zhiyong Poon, Amanda C. Engler, Daniel K. Bonner, and Paula T. Hammond*

Department of Chemical Engineering, Massachusetts Institute of Technology and the Koch Institute for Integrative Cancer Research at MIT, Cambridge, Massachusetts 02139, United States

Abstract

One of the major obstacles that delay the clinical translation of polymeric micelle drug delivery systems is whether these self-assembled micelles can retain their integrity in blood following intravenous (IV) injection. The objective of this study was to evaluate the impact of core functionalization on the thermodynamic and kinetic stability of polymeric micelles. The combination of ring-opening polymerization of *N*-carboxyanhydride (NCA) with highly efficient “click” coupling has enabled easy and quick access to a family of poly(ethylene glycol)-block-poly(γ -R-glutamate)s with exactly the same block lengths, for which the substituent “R” is tuned. The structures of these copolymers were carefully characterized by ^1H NMR, FT-IR and GPC. Using pyrene as the fluorescence probe, the critical micelle concentrations (CMCs) of these polymers were found to be in the range of 10^{-7} - 10^{-6} M, which indicates good thermodynamic stability for the self-assembled micelles. The incorporation of polar side groups in the micelle core leads to high CMC values; however, micelles prepared from these copolymers are kinetically more stable in the presence of serum and upon SDS disturbance. It was also observed that these polymers could effectively encapsulate paclitaxel (PTX) as a model anticancer drug and the micelles possessing better kinetic stability showed better suppression of the initial “burst” release and exhibited more sustained release of PTX. These PTX-loaded micelles exerted comparable cytotoxicity against HeLa cells as the clinically approved Cremophor® PTX formulation while the block copolymers showed much lower toxicity compared to the Cremophor-ethanol mixture. The present work demonstrated that the PEG-*b*-PPLG can be a uniform block copolymer platform toward development of polymeric micelle delivery systems for different drugs through the facile modification of the PPLG block.

Keywords

Drug delivery; Polymeric micelles; Paclitaxel; Poly(amino acid); *N*-carboxyanhydride (NCA)

*Corresponding author: Paula T. Hammond, hammond@mit.edu.

SUPPORTING INFORMATION. Synthesis and characterization (^1H NMR and ^{13}C NMR) of azide-functionalized compounds, ^1H NMR of **P2**, **P3**, **P4** and **P5**, FTIR of **P1–P6**, ATR-FTR of PTX-loaded micelle from **P6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Polymeric micelles are nanometer scale (10–200 nm) colloidal particles that possess a hydrophobic core and a hydrophilic shell structure.^{1–5} The unique ability to customize both the core and shell properties has made them attractive as delivery vehicles for a variety of payloads from small molecule anticancer drugs, imaging probes to large biomacromolecules.^{6,7} To date, there are several anticancer agent-encapsulated systems based on polymeric micelles that are under clinical evaluations.^{8,9} Among them, micelles fabricated from poly(ethylene glycol)-b-poly(amino acid) block copolymers have shown great promise as effective delivery nanocarriers to increase the overall efficacy of the encapsulated anticancer agents.^{10–12}

Such block copolymers can be readily synthesized by ring-opening polymerization of N-carboxyanhydrides of natural amino acids in the presence of α -methoxy-amino poly(ethylene glycol)s.¹³ These block copolymers are of excellent biocompatibility and the core-forming polypeptide blocks can be fully degraded by enzymes *in vivo*.¹² In addition, the versatility of functional groups derived from the amino acid library, such as carboxyl, amino and thiol groups, enables easy chemical modification of the polypeptide core to accommodate various drugs.¹⁴ Kataoka et al¹⁵ were the first to show that chemical modification of the polyaspartate block by 4-phenol-1-butanol can lead to effective and stable encapsulation of paclitaxel, one of the broadly used anticancer drugs to treat various cancers. Gwon et al¹⁶ have also reported that chemical modification of the polyaspartate block with saturated fatty acid esters can lead to enhanced encapsulation of amphotericin B, an antifungal agent, in the micelle core compared to the benzyl core. These results have highlighted the importance of tailoring the structure of the core-forming block to match the specific chemical properties of different drugs in achieving stable micellar delivery systems.

Recently, we developed a clickable block copolymer platform, poly(ethylene glycol)-b-poly(γ -propargyl-glutamate) (**PEG-b-PPLG**), which by itself can self-assemble in aqueous solutions to form spherical micelles.^{17,18} The pendant propargyl group can be readily reacted with an azide by the alkyne-azide cycloaddition click reaction. We have shown that high density PEG-grafted poly(amino acid)s can be synthesized in high yields using a click reaction and thus bypassing the need for protection and deprotection steps commonly required to modify poly(amino acids)s.¹⁷ Using this synthetic strategy, we have further demonstrated that the core of this block copolymer can be functionalized with various amine moieties, which provides pH responsive properties to these micelles.¹⁹

The goal of the current research is to apply this convenient and efficient synthetic strategy to synthesize a series of PEG-b-polypeptides with different core functionalities, and evaluate the impact of noncovalent interactions on the drug encapsulation and stability of polymeric micelles. To do so, we modified the PPLG block of the **PEG-b-PPLG** with a series of 4-azido-butyl-benzenes bearing different functionalities using copper-catalyzed alkyne-azide cycloaddition reaction (“click chemistry”) (scheme 1). These functional groups on the core block are expected to interact with encapsulated drugs upon micellization through single or multiple noncovalent interactions, i.e. hydrophobic, π - π stacking and hydrogen-bonding. We further investigated the effect of incorporating these functional groups into the hydrophobic block of these PPLG-based copolymers on the ability of these micelles to encapsulate paclitaxel (PTX, water solubility: 0.3 μ g/mL) as a model anticancer drug in a biologically relevant environment. To accomplish the study, drug release profiles from PTX-loaded micelles prepared from polypeptide copolymers with various substituents were studied in a simulated physiological condition using phosphate buffered saline (PBS) with pH 7.4 at 37 °C. The kinetic stability of PTX-loaded micelles was evaluated in 20 vol% fetal bovine serum (FBS) by size exclusion chromatography and also probed in the presence of

sodium dodecyl sulfate using dynamic light scattering. We have also investigated the cytotoxicity of polymer micelles and PTX-loaded micelles against the HeLa cell line.

EXPERIMENTAL SECTION

Materials and Characterization

All chemicals were used as received. Anhydrous 99.8% DMF, purchased from Sigma Aldrich was used for polymerization and click reactions. PEG-NH₂ was purchased from Laysan Corporation. ¹H NMR and ¹³C NMR were recorded on Bruker 400 MHz spectrometers. Fourier transform infrared (FTIR) spectra were recorded on a Thermo Nicolet NEXUS 870 series spectrophotometer. Gel permeation chromatography (GPC) measurements were carried out using a Water Breeze 1525 HPLC system equipped with two Polypore columns operated at 75 °C, series 2414 refractive index detector, series 1525 binary HPLC pump, and 717plus autosampler. Waters' Breeze Chromatography Software Version 3.30 was used for data collection as well as data processing. DMF was used as the eluent for analysis and as solvent for sample preparation. The average molecular weight of the sample was calibrated against narrow molecular weight poly(methyl methacrylate) standards.

Synthetic details of six azide-functionalized compounds were provided in the Supporting Information. The alkyne containing monomer, γ -propargyl-L-glutamate N-carboxyanhydride (NCA) was synthesized following our previously reported method.¹⁷

Synthesis of Poly(ethylene glycol)-b-poly(γ -propargyl L-glutamate) (PEG-b-PPLG)

A typical procedure is as follows. A Schlenk flask charged with the monomer (1.5 g, 7.1 mmol) was added anhydrous DMF (10 ml) under argon protection. Then PEG-NH₂ (Laysan®, M_n = 5,000) (710 mg, 0.14 mmol) dissolved in 5 ml of DMF was added. The reaction mixture was stirred for 48–72 hours at room temperature. The polymer was isolated by pouring the reaction mixture into 150 ml of ethyl ether and further purified by repeated precipitation. GPC (DMF, 70 °C): M_n = 14,390, PDI = 1.09. ¹H NMR (400 MHz, DMF-d₇): 8.63 (br, s, -NH), 4.93 (s, 2H), 4.26 (s, 1H), 3.93-3.59 (m, 12.5H, PEG), 3.55 (s, 1H, terminal alkyne), 2.94 (br, s, 1H), 2.72 (br, s, 1H), 2.54 (br, s, 1H), 2.36 (br, s, 1H).

A typical procedure for copper-catalyzed alkyne-azide reaction

In a Schlenk flask, 100 mg of PEG₁₁₄-b-PPLG₃₅ (0.0092 mmol, for propargyl group: 0.0092 × 35 = 0.322 mmol) and 4-azidobutylbenzene (**2**) (74 mg, 0.42 mmol) were mixed in 5 ml anhydrous DMF. The solution was degassed with argon for 30 minutes. Copper (I) bromide (4.5 mg, 0.031 mmol) was added under the protection of argon, followed with the addition of N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA) (6.8 μ l, 0.031 mmol). The reaction solution was then stirred at room temperature in the dark for 12–15 h. The reaction mixture was poured into 50 ml of ethyl ether to remove the excess of 4-azidobutylbenzene. The crude product was then collected by centrifugation and dissolved in 15 ml of D. I. water. The resulting solution was dialyzed against 2 L of D.I. water, which was acidified by adding 2 ml of HCl (2 M) for 3 days. During the dialysis, the water was changed every 12 h. The final product was obtained by lyophilizing the solution after dialysis.

CMC measurement

Critical micelles concentrations (CMCs) of **P1–P6** in de-ionized (18.2 M Ω) water were determined using the standard pyrene procedure.³ A stock solution of pyrene in acetone (6.5 × 10⁻⁵ M) was prepared. Aliquots of pyrene (10 μ L) were added to vials and the acetone was allowed to evaporate. Polymer solutions at varied concentrations were added into the

vials and left to equilibrate for overnight. The final concentration of pyrene in each sample was 6.5×10^{-7} M. The excitation spectra were scanned from 280 nm to 350 nm by monitoring the emission at $\lambda_{em} = 370$ nm.

Micelle preparation and drug loading determination

Paclitaxel (PTX)-loaded block copolymer micelles were prepared as follows. 10 mg of **P1-P6** and 2 mg of PTX were dissolved in DMSO (0.5 ml) and vortexed for 15 minutes. The polymer-drug solution was then injected into PBS buffer (10 ml) at a speed of 0.05 ml/h controlled by a multi-syringe infusion pump (Model: KDS 220, KD Scientific Inc.). Following the completion of the addition, the micelle solutions were dialyzed against a huge excess (2 L) of PBS buffer for 4 hours to get rid of DMSO. The resulted micelle suspension was then sonicated in a bath sonicator (Branson 2510, Branson Ultrasonics Corporation, 40 KHz) for 30 minutes. To avoid heating up the micelle solution, the sonication process was divided into 3 cycles of sonication (10 minutes) and 10 minutes' interval. The white precipitate (unencapsulated PTX) was removed by centrifugation (15 minutes at 5000 rpm) and the supernatant was collected and filtrated through a 0.45 μ m filters. The micelle solutions were kept at 5 °C and used directly for other studies. To determine the drug loading, 0.2 ml of the PTX-loaded micelle solution was diluted with 0.8 ml of acetonitrile and then analyzed by high performance liquid chromatography (HPLC). The drug loading content (DLC) was calculated using the following equation:

$$DLC\% = \frac{\text{amount of loaded drug}}{\text{amount of loaded drug} + \text{amount of polymer}} \times 100\%$$

Dynamic light scattering (DLS)

Dynamic light scattering measurements were performed using a Delsa™ particle size analyzer (Beckman Coulter Inc.). All the measurements were carried out at 25 °C. The sample solutions were purified by passing through a Millipore 0.45 μ m filter. The scattered light of a vertically polarized He-Ne laser (632.8 nm) was measured at an angle of 165 ° and was collected on an autocorrelator. The hydrodynamic diameters (d_h) of micelles were calculated by using the Stokes-Einstein equation $d_h = k_B T / 3\pi\eta D$ where k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient. The polydispersity factor (PI), represented as μ_2/Γ^2 , where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width, was calculated from the cumulant method.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed on a JEOL 200CX, operating at an acceleration voltage of 200 kV. To observe the size and distribution of micellar particles, a drop (2 μ L) of sample solution was placed onto a 200-mesh copper grid coated with carbon and air-dried.

In vitro drug release

Release of PTX from a dialysis tube was measured using a Spectra/PorR-4 dialysis membrane with a molecular weight cut-off of 3,500 Da. One milliliter of PTX-loaded micelle solution was placed in a dialysis bag and immersed in the medium (100 ml PBS, pH 7.4 and 37 °C). At certain time intervals (1, 2, 3, 6, 12, 24 h), aliquots (1 ml) of the medium were withdrawn, and the same volume of fresh medium was added. The sample solution was analyzed by reverse-phase HPLC. All experiments were performed in triplicate.

HPLC Analysis

The concentration of PTX was determined by an isocratic reverse-phase HPLC (Agilent 1200 series, Agilent Technologies Inc.) using a Discovery® C18 column (Sigma Aldrich Co.) at 25 °C. The mobile phase consists of acetonitrile/water (50/50, v/v) with a flow rate of 1.0 ml/min. PTX was detected at 230 nm. A calibration curve was generated by plotting the weight of PTX in µg versus the peak area in the HPLC chromatogram using a series of PTX standards. A linear response was obtained in this concentration range between 0.2 – 100 µg/ml. For the release samples, 200 µl was injected and the actual weight of PTX was determined from the calibration curve.

In vitro stability

The kinetic stability of the PTX-loaded polymer micelles was probed by two independent methods. The stability of PTX-loaded micelles in serum-like medium was investigated by size exclusion chromatography (SEC).^{20,21} PTX-loaded micelles (4 ml) were mixed with 1 ml of fetal bovine serum (100%, Invitrogen), which was then incubated at 37 °C. At certain time intervals (0, 1, 3, 6, 12 and 24 h), aliquots (100 µL) of the mixtures were withdrawn and analyzed by SEC (Superose 6 10/300 GL, GE HealthCare, exclusion limit: 1×10^7 Dalton) connected with a UV detector. Samples were run at a flow rate of 0.5 mL/min by using $1 \times$ PBS (pH 7.4) as the mobile phase.

The second method is to measure the scattered light intensity in the presence of SDS as a destabilizing agent as reported by others.^{22,23} The PTX-loaded micelle solutions (3 ml, concentration: 0.5 mg/ml) were added 90 µl of sodium dodecyl sulfate (SDS, 50 mg/ml). The resulting mixture was then monitored using dynamic light scattering.

Cell culture

HeLa cells were cultured in Minimum Essential Media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified 37 °C atmosphere at 5% CO₂.

Cytotoxicity

HeLa cells were seeded into 96-well flat-bottomed tissue-culture plate at a density of 25,000 cells per well, and incubated for 48 h in a humidified atmosphere of 5 % CO₂ at 37 °C before use. The concentration of polymeric micelles was diluted with MEM medium to obtain a concentration range from 20 to 250 µg/mL. After incubation for 48 h, the cell culture medium was replaced with 100 µL of a 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution in the same medium. The plate was incubated for 3 h at 37 °C, allowing viable cells to reduce the MTT into purple formazan crystal. Then the medium was carefully removed and 200 µL of a mixture of SDS (0.5 M) : DMF (v/v, 1/1) was added to dissolve the formazan crystals. The absorbance of individual wells was measured at 540 nm by a microplate reader (Tecan infinite M200 PRO).

RESULTS AND DISCUSSION

Polymer Synthesis and Characterization

Scheme 1 shows the synthesis of the parent polymer **PEG-b-PPLG** and the subsequent modification of the PPLG block with different molecules using azide-alkyne “click” chemistry. As previously described,¹⁷ N-carboxyanhydride (**1**) was synthesized by reacting γ -propargyl L-glutamate with triphosgene in a moderate yield. **PEG-b-PPLG** was prepared by ring opening polymerization in dimethylformamide (DMF) at 40 °C for 3 days by initiating from PEG-NH₂ (M_n = 5000). The successful polymerization of monomer **1** was

confirmed by the shift of the evolution peak to higher molecular weight in gel permeation chromatography (GPC). The molecular weight was determined to be 14,390 Dalton with a polydispersity of 1.1 based on narrow poly(methyl methacrylate) standards. The good solubility of **PEG-b-PPLG** in common organic solvents, such as chloroform and DMF allows independent determination of the degree of polymerization (DP). By comparing the ratio of integration at 4.81 ppm (**PPLG** block) and 3.59 ppm (**PEG** block), the DP of PPLG was calculated to be 35.

Six different 4-azido-butyl-benzene based moieties carrying a variety of functionalities from aliphatic alkyl (C₁₂H₂₅) chains to phenyl and phenol groups were attached to the parent **PEG-b-PPLG** of the same molecular weight through the 1,3-cycloaddition chemistry. GPC was used to confirm that the propargyl groups of PPLG segments reacted with different azido-modified compounds. Relative to the parent **PEG-b-PPLG** prior to functionalization, the GPC traces shift to higher molecular weights while maintaining a monomodal distribution, indicating that the reactions proceeded in a clean and efficient fashion (see Figure S1 for GPC traces.). The polymer structures were further confirmed using ¹H NMR spectra. Representative ¹H NMR spectra of the dodecyl and phenol substituted **PEG-b-PPLG** along with the spectra of **PEG-b-PPLG** are shown in Figure 1. The reaction efficiencies were nearly quantitative for different side chain modifications, as indicated by the disappearance of the PPLG alkyne peak (δ , 3.42 ppm) and the appearance of a new methylene group peak (δ , 4.38–4.42 ppm) and the triazole ring peak (δ , 8.18 ppm). The characteristic peaks of alkyl chains (0.89 ppm, 1.27 and 1.88 ppm) of **P1** and aromatic protons at δ 6.74 ppm (d, J = 8.14 Hz) and δ 6.97 ppm (d, J = 8.28 Hz) of **P6** are also consistent with the substitution of the PPLG block with dodecyl and phenol groups, respectively. ¹H NMR spectra of the remaining block copolymers with other side chains can be found in the ESI.

The complete functionalization of the alkyne groups of **PEG-b-PPLG** was also confirmed by Fourier transfer infrared spectrum (FTIR). Figure 2 shows the FTIR spectra of **PEG-b-PPLG**, **P1** and **P6**. The absorption at 1110 cm⁻¹ in the spectra can be assigned to the C-O-C antisymmetric stretching of PEG ether. All polymers exhibit strong IR absorptions at 3292 cm⁻¹, 1655 cm⁻¹ and 1549 cm⁻¹ characteristic of a polypeptide backbone (Figure S2). The alkyne antisymmetric stretch at 2124 cm⁻¹, present in the spectrum of **PEG-b-PPLG**, disappears completely in the spectra of **P1** and **P6**, suggesting very high consumption of the alkyne group during post functionalization. As **P1–P6** were synthesized from the same parent polymer **PEG-b-PPLG** by clicking on different functional molecules, these diblock copolymers have PEG and poly(*L*-glutamate) segments of the same length. Thus, this synthetic approach enables us to evaluate the effect of core functionality of the block copolymer on the drug encapsulation, drug release and micelle stability independent of other factors such as molecular weight and molecular weight distribution.

Polymer Micellization and PTX Encapsulation

The micelle formation of functionalized **PEG-b-PPLG** in PBS was studied by fluorescence microscopy using pyrene as a fluorescent probe. The excitation and emission spectra of pyrene are known to be dependent on the local polarity.³ Figure 3a shows the representative excitation spectra of **P6** of concentrations ranging from 0.5 mg/ml to 1.2×10⁻⁴ mg/ml. Upon micellization, the excitation band at 334 nm shifts to 339 nm, indicating pyrene molecules preferably partition into the less polar hydrophobic core of **P6**. The critical micelle concentrations (CMC) of different polymers were then determined by plotting the intensity ratios (I₃₃₉/I₃₃₄) versus logarithm concentrations, as shown in Figure 3b. The CMC values of these polymers are in the range of 0.01 –0.03 mg/mL, corresponding to values of 10⁻⁷ to 10⁻⁶ M. These values are close to the CMC values reported for polylactide-based (PLA)

diblock copolymers²⁴ and lower than CMCs of Pluronic copolymers^{25,26} and PEG-lipid conjugates,²⁷ which suggests that micelles formed from these polymers should be expected to exhibit good stability. In addition, it was observed that **P5** and **P6**, which bear polar groups (phenylacetamide and phenol), exhibited higher CMCs (1.6×10^{-6} M and 1.7×10^{-6} M) compared with other polymers, **P1**, **P2**, **P3** and **P4**, featuring solely hydrophobic side chains, such as alkyl or phenyl functionalities with less polar groups (methoxy or acetyl). The CMC values for **P1–P4** are very similar at about 0.01 mg/mL or 5.3 to 6.0×10^{-7} M, suggesting that micelles prepared from **P1**, **P2**, **P3** and **P4** are more thermodynamically stable and can better maintain micellar integrity upon dilution.

The blank and PTX-loaded polymeric micelles were then prepared using a method known as nanoprecipitation. Briefly, a polymer or a mixture of a polymer and PTX was completely dissolved in a good solvent (dimethyl sulfoxide, DMSO). The resulting solutions are then added to a 20× volume of PBS buffer (pH 7.4) in a precisely controlled fashion as described in the experimental section to minimize variations among different batches. We have used dynamic light scattering to determine the sizes of both blank and PTX-loaded micelles prepared from different polymers. As an example, the size distribution of micelles prepared from **P6** is shown in Figure 4a. A hydrodynamic radius (d_h) value of 59 nm was determined with a relatively low polydispersity ($\sigma = 0.289$). TEM (Figure 4b) reveals that the shape of the micelles is primarily spherical. The size of the air-dried micelles shown in this figure is around 20 nm with a narrow size distribution, as shown in Figure 4b. Compared to the hydrodynamic diameters of the same micelles measured while in solution, the sizes of these air-dried micelles are significantly smaller, which can be assigned to the hydration of the PEG shell of the polymer micelle in solution.

Table 1 summarizes the sizes of micelles prepared from the series of polymers based on dynamic light scattering. In general, both the blank micelles and PTX-loaded micelles exhibit sub-200 nm sizes, which is an important size threshold for *in vivo* imaging and/or delivery applications for which the enhanced permeation and retention effect is relevant in passive tumor targeting.⁸ Interestingly, there are three trends observed by comparing the sizes of these blank and PTX-loaded micelles from different polymers. First, the hydrodynamic diameters (d_h) of the blank micelles of **P5** ($d_h = 43$ nm) and **P6** ($d_h = 59$ nm) are significantly smaller than those of micelles prepared from other polymers, **P1–P4** ($R_h = 109 - 163$ nm); Second, d_h increases upon PTX encapsulation for all polymer formulations but with a large 2–3 fold increase in d_h observed for **P5** and **P6**; Third, the micelles become more narrowly distributed after PTX encapsulation, as evident from the decreased polydispersity indexes of PTX-loaded micelles. These observations suggest there exist additional hydrogen-bonding (H-bonding) interactions within the micelle core of **P5** and **P6**, leading to smaller micelle sizes in comparison to those of micelles formed through solely hydrophobic interactions between the modified PPLG blocks. As a result, partitioning of PTX in the micelle cores disrupts the reversible intermolecular H-bonding interaction among the polymer chains of **P5** and **P6**, causing the large expansion of the micelle sizes. Furthermore, the improved size distributions of PTX-loaded micelles suggest that the polymer-PTX interaction leads to more regular particle sizes; this may be enabled because PTX can stabilize the polymer micelles and thus minimize the micelle dissociation and aggregation typical of micellar systems.⁵

***In Vitro* Drug Release**

In contrast to the observed size difference as described above, using the nanoprecipitation method, the PTX loading amounts are 5 to 7 wt% for all polymers. There was no statistical difference among the **PEG-b-PPLG** copolymers bearing different side chains. The *in vitro* PTX release from the micelles was then evaluated in PBS (pH 7.4) at 37 °C. The starting

micelle concentrations used in the release studies were 0.50 mg/mL, which is far above the CMCs of the polymers. Since polypeptide based block copolymers have been shown to be chemically robust and undergo relatively slow enzymatic degradation *in vivo*,¹² the *in vitro* PTX release of these micelles is simply dependent on the diffusion of the drug from the micelles. Figure 5a shows the cumulative percentage of PTX released from micelles prepared from **P1–P6**. For all polymers, an initial “burst” release followed by a prolonged release was observed. The drug release rates were slower for **P4, P5** and **P6** as compared to **P1, P2** and **P3**. The amount of PTX released at 3 h was above 40% of total loading for **P1, P2** and **P3** and below 30% for **P4, P5** and **P6**, respectively. Among the latter group of polymers, **P6** exhibited the best performance in suppressing the initial burst release and extending the second-stage prolonged release. Figure 5b shows a TEM image of cast, air-dried PTX-loaded micelles prepared from **P6** after desalting the micelles using a PD-10 column (GE HealthCare). Consistent with the DLS studies on hydrated micelles, the PTX-loaded micelles (average micelle size = ~ 100 nm) are larger in the hydrated state than as dehydrated micelles; the micelles are also much larger than the unloaded versions observed in TEM (average micelle size = ~ 20 nm).

Kinetic Stability

Following intravenous injection, these self-assembled micelles have to confront non-specific interactions with various proteins in a process known as opsonization.²⁸ These plasma proteins can adsorb on the surfaces of micelles, which in turn leads to dissociation and aggregation. To test the stability of micelles formed with **PEG-b-PPLG** with different side groups, these PTX-loaded micelles (0.5 mg/mL) were incubated with 20 vol% fetal bovine serum (FBS) at 37 °C. At different time intervals, the micelle solutions were analyzed by SEC equipped with a Superose 6 10/300 GL column. Figure 5c shows the SEC traces for micelles of **P6** sampled at different incubation times in the presence of 20 vol% FBS. The peak at ~ 15 minutes corresponds to the micelles, while the peaks within 29 – 35 minutes arise from different proteins present in the FBS. There was a minimal reduction of peak area at 15 minutes for **P6**, suggesting micelles were not largely dissociated following incubation with FBS. The PTX-loaded micelles of the other polymers also eluted out of the column at ~15 minutes, suggesting that the micelles are similar in size regardless of core functionalization (data not shown). To compare the micelle stability for different polymers, the peaks corresponding to micelles from **P1** to **P6** were integrated and the normalized peak areas were graphed versus time, as shown in Figure 5d. Micelles prepared from **P6** are most stable, as evident from the small change (< 10%) of the peak area even after 24 h incubation in FBS at 37 °C. In strong contrast, micelles formulated with the other polymers exhibit a much lower kinetic stability. Since all micelles, regardless of the polypeptide block, present a PEG outer layer, this result highlights the importance of fine-tuning the core block structure to improve the micelle stability in a serum-containing medium and the related opportunity to increase the micelle half-life in the blood stream.

Micelle kinetic stability was also studied by dynamic light scattering in the presence of sodium dodecyl sulfate (SDS) as a destabilizing agent, as described by others.^{22,23} Limited by the solubility of **P1**, which contains dodecyl side chains, the micelle concentrations for the different polymers was maintained at 0.5 mg/mL. An aliquot (90 µL) of SDS solution (50 mg/mL) was added to the micelle solution (3 mL), affording an SDS concentration of 1.5 mg/mL. The scattered light intensities of micelle solutions prepared from different polymers were then monitored at different time intervals (Figure S3). Upon addition of SDS, all micelles exhibited a significant decrease in the scattered light intensity, suggesting the dissociation of a large percentage of micelles caused by interactions between SDS and the block copolymer chains. In consistent with the SEC results, compared to other polymers, micelles prepared from **P6** showed more resistance to SDS destabilization as a slower

decrease in the scattered light intensity was observed and the micelles were able to maintain ~40% of its original value even after 24 hours' incubation.

The above results are consistent with the micelle properties and release characteristics of PTX-loaded micelles from **P6**, suggesting that the additional H-bonding interaction between phenol-substituted **PEG-b-PPLG** and PTX enhanced the micelles' resistance toward dissociation by SDS. The H-bonding interaction between **P6** and PTX was further proven by the observed shift of the amide I absorption in the FTIR spectra of PTX-loaded micelles after lyophilization compared with the absorption of **P6** (Figure S4).

Cytotoxicity of Blank Micelles and PTX-loaded Micelles

The cytotoxicity of modified PEG-b-PPLGs was evaluated against HeLa cells in comparison with the Cremophor EL (CrEL) and dehydrated ethanol (1/1, v/v) (10 vol% in PBS) used in current clinical formulations of Taxol®.^{29,30} Figure 6 shows that the PEG-PPLG modified block copolymers do not have any significant cytotoxicity up to 0.25 – 0.5 mg/ml in contrast to the CrEL-EtOH mixture. Importantly, it is clear that the modification of the PAA-based backbone with different chemical functionality does not introduce any additional cytotoxicity as there is no difference for the different polymers.

Figure 7 shows the viability of HeLa cells after 48 hour of incubation with PTX-loaded micelles prepared from different polymers and a formulation with CrEL-EtOH. The cell viability was concentration-dependent. The IC₅₀ value of **P6** is ~ 0.13 µg/mL, which is larger than those of **P1** (0.02 µg/mL) and CrEL-based formulation (0.018 µg/mL). In addition, the IC₅₀ values of other polymers (0.09 µg/mL, 0.05 µg/mL, 0.04 µg/mL and 0.085 µg/mL for **P2**, **P3**, **P4** and **P5**, respectively) are comparable and lie between **P1** and **P6**. The lower potency of micelles prepared from **P6** as compared to micelles with **P1** and CrEL-EtOH can be ascribed to the slower release of PTX from more stable micelles, as similar observations have been reported by others.^{23,31,32} The enhanced stability of micelles from **P6**, and its in vitro cytotoxicity suggest that **P6** is a good candidate for controlled delivery of paclitaxel.

CONCLUSION

In this work, we present the synthesis and characterization of a series of amphiphilic block copolymers consisting of a hydrophilic PEG block and a poly(γ -propargyl-glutamate) block modified with different side groups through the alkyne-azide cycloaddition “click” reaction. All the polymers form micelles in PBS through a simple self-assembly process and can effectively encapsulate paclitaxel as a model anticancer drug. Our results indicated that incorporation of different functional groups in the core has a pronounced effect on the size and the CMC of the micelles. As indicated by the CMC values, copolymers featuring more polar side groups, **P5** and **P6**, are thermodynamically less stable compared to other polymers (**P1–P4**) with simple hydrophobic chains; however, blank micelles prepared from **P5** and **P6** show much smaller sizes in solution, indicating strong intermolecular interactions exist within in the hydrophobic core. Furthermore, the kinetic stability was greatly enhanced for PTX-loaded micelles prepared from **P6**, which can be ascribed to the additional stabilization by H-bonding interaction between the core block of **P6** and the encapsulated PTX. As a result, more sustained PTX release under simulated physiological conditions (pH 7.4, 37 °C) was observed. These PTX-loaded micelles showed dose-dependent cytotoxicity against the HeLa cell line while the blank micelles exerted minimal cytotoxicity. A good deal of research effort has been focused on synthesizing block copolymers with potentially lower CMC values for nanoparticle delivery; the current work highlights the importance of improving kinetic stability of polymeric micelles by incorporating multiple noncovalent

interactions. We are currently investigating the *in vivo* stability of polymeric micelles prepared from **P6**, encouraged by its superior *in vitro* stability and promising release properties compared to other polymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the National Institutes of Health (NIH) NIBIB Grant R01-EB008082, an ARRA grant. X. Y. Z. acknowledges the award from the MIT-Harvard Center for Cancer Nanotechnology Excellence (CCNE Grant No. 1 U54 CA119349) for support. We also thank the Institute for Soldier Nanotechnology (ISN) and Center for Material Science Research (CMSE) for use of facilities. X. Y. Z. is also grateful to Huimeng Wu for help with the TEM imaging.

References

1. Kataoka K, Kwon GS, Yokoyama M, Okano T, Sakurai Y. *J. Controlled Release*. 1993; 24:119–132.
2. Allen C, Maysinger D, Eisenberg A. *Colloid Surf. B-Biointerfaces*. 1999; 16:3–27.
3. Jones MC, Leroux JC. *Eur. J. Pharm. Biopharm.* 1999; 48:101–111. [PubMed: 10469928]
4. Batrakova EV, Kabanov AV. *J. Controlled Release*. 2008; 130:98–106.
5. Mikhail AS, Allen C. *J. Controlled Release*. 2009; 138:214–223.
6. Khemtong C, Kessinger CW, Gao JM. *Chem. Commun.* 2009:3497–3510.
7. Yoon HJ, Jang WD. *J. Mater. Chem.* 2010; 20:211–222.
8. Davis ME, Chen Z, Shin DM. *Nat. Rev. Drug Discov.* 2008; 7:771–782. [PubMed: 18758474]
9. Matsumura Y, Kataoka K. *Cancer Sci.* 2009; 100:572–579. [PubMed: 19462526]
10. Matsumura Y. *Adv. Drug Deliv. Rev.* 2008; 60:899–914. [PubMed: 18406004]
11. Bae Y, Kataoka K. *Adv. Drug Deliv. Rev.* 2009; 61:768–784. [PubMed: 19422866]
12. Lavasanifar A, Samuel J, Kwon GS. *Adv. Drug Deliv. Rev.* 2002; 54:169–190. [PubMed: 11897144]
13. Hadjichristidis N, Iatrou H, Pitsikalis M, Sakellariou G. *Chem. Rev.* 2009; 109:5528–5578. [PubMed: 19691359]
14. Gauthier MA, Gibson MI, Klok H-A. *Angew. Chem. Int. Ed.* 2009; 48:48–58.
15. Hamaguchi T, Matsumura Y, Suzuki M, Shimizu K, Goda R, Nakamura I, Nakatomi I, Yokoyama M, Kataoka K, Kakizoe T. *Br. J. Cancer.* 2005; 92:1240–1246. [PubMed: 15785749]
16. Lavasanifar A, Samuel J, Kwon GS. *J. Controlled Release*. 2001; 77:155–160.
17. Engler AC, Lee HI, Hammond PT. *Angew. Chem.-Int. Edit.* 2009; 48:9334–9338.
18. Engler AC, Shukla A, Puranam S, Buss HG, Jreige N, Hammond PT. *Biomacromolecules*. 2011; 12:1666–1674. [PubMed: 21443181]
19. Engler AC, Bonner DK, Buss HG, Cheung EY, Hammond PT. *Soft Matter*. 2011; 7:5627–5637.
20. Yokoyama M, Sugiyama T, Okano T, Sakurai Y, Naito M, Kataoka K. *Pharm. Res.* 1993; 10:895–899. [PubMed: 8321859]
21. Lu J, Owen SC, Shoichet MS. *Macromolecules*. 2011; 44:6002–6008. [PubMed: 21818161]
22. Kang N, Perron ME, Prud'homme RE, Zhang YB, Gaucher G, Leroux JC. *Nano Lett.* 2005; 5:315–319. [PubMed: 15794618]
23. Kim SH, Tan JPK, Nederberg F, Fukushima K, Colson J, Yang CA, Nelson A, Yang YY, Hedrick JL. *Biomaterials*. 2010; 31:8063–8071. [PubMed: 20705337]
24. Yasugi K, Nagasaki Y, Kato M, Kataoka K. *J. Controlled Release*. 1999; 62:89–100.
25. Kabanov AV, Batrakova EV, Alakhov VY. *J. Controlled Release*. 2002; 82:189–212.
26. Kabanov AV, Batrakova EV, Alakhov VY. *Adv. Drug Deliv. Rev.* 2002; 54:759–779. [PubMed: 12204601]

27. Lukyanov AN, Torchilin VP. *Adv. Drug Deliv. Rev.* 2004; 56:1273–1289. [PubMed: 15109769]
28. Alexis F, Pridgen E, Molnar LK, Farokhzad OC. *Mol. Pharm.* 2008; 5:505–515. [PubMed: 18672949]
29. Gelderblom H, Verweij J, Nooter K, Sparreboom A. *Eur. J. Cancer.* 2001; 37:1590–1598. [PubMed: 11527683]
30. Hennenfent KL, Govindan R. *Ann. Oncol.* 2006; 17:735–749. [PubMed: 16364960]
31. Shuai XT, Ai H, Nasongkla N, Kim S, Gao JM. *J. Controlled Release.* 2004; 98:415–426.
32. Li YL, Zhu L, Liu ZZ, Cheng R, Meng FH, Cui JH, Ji SJ, Zhong ZY. *Angew. Chem.-Int. Edit.* 2009; 48:9914–9918.

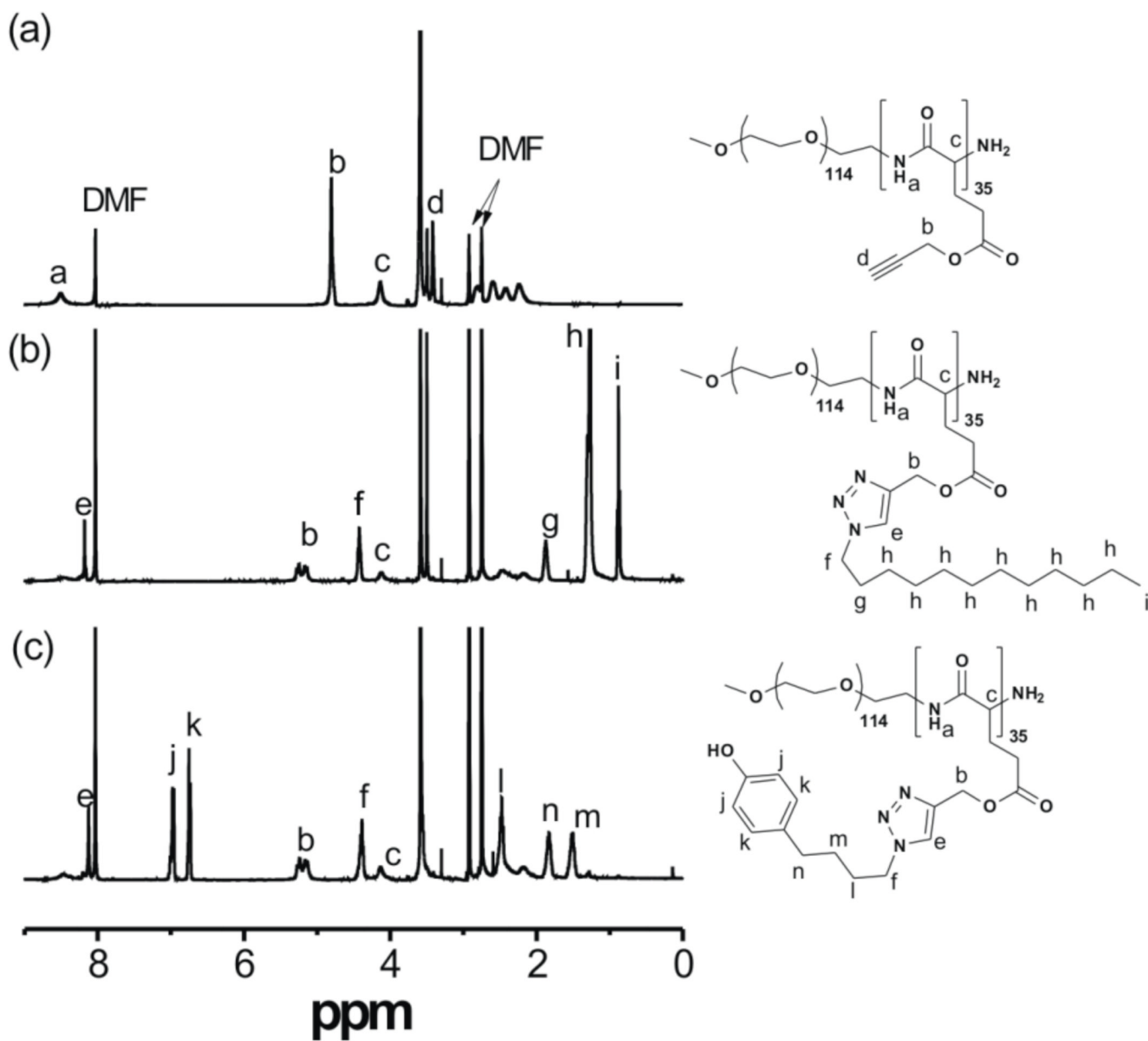


Figure 1.
 ^1H NMR spectra of (a) PEG-b-PPLG, (b) P1 and (c) P6.

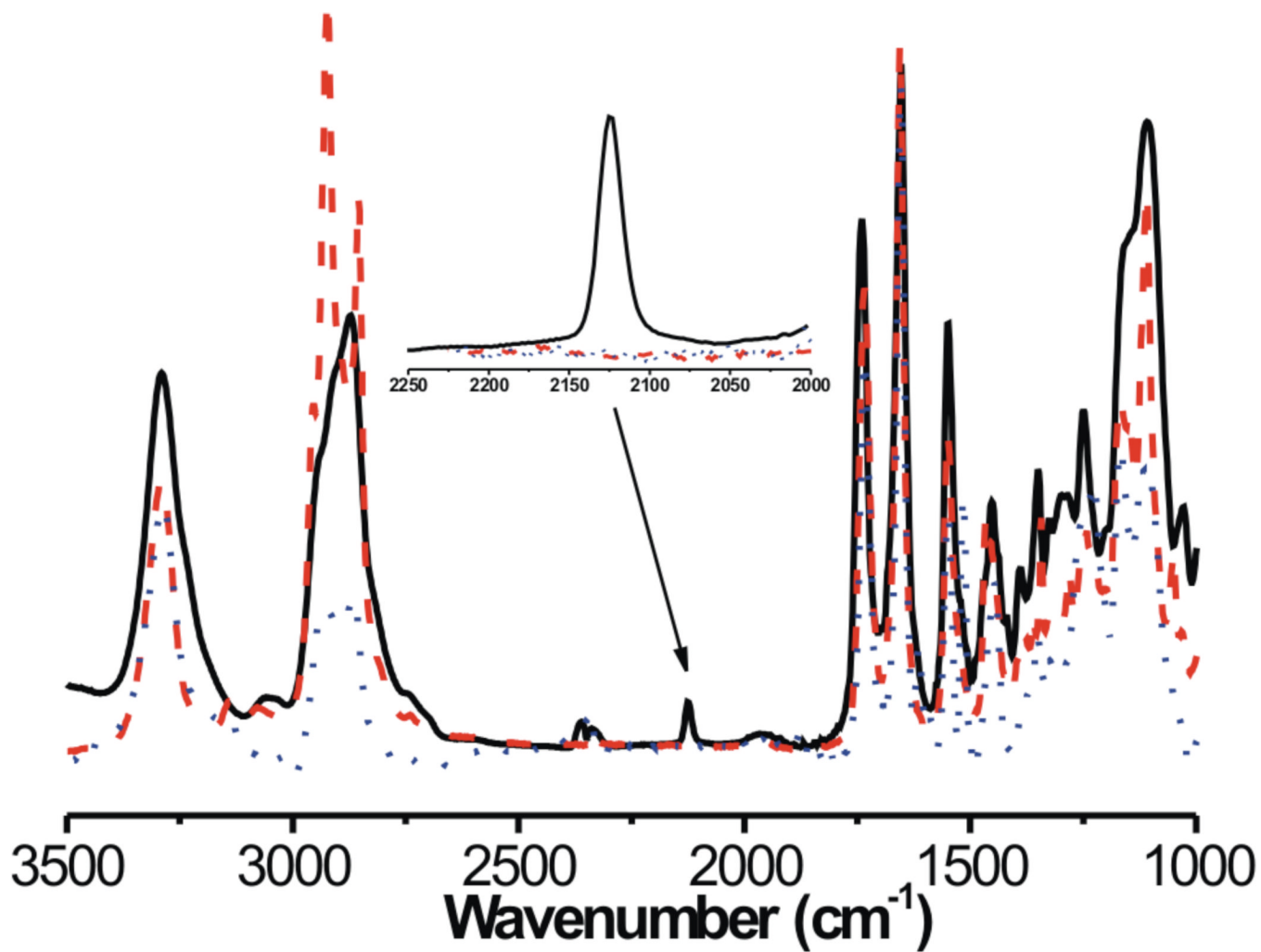


Figure 2. FT-IR of PEG-b-PPLG (solid line), P1 (dashed line) and P6 (dotted line). Inset shows the expanded region between 2250 cm⁻¹ and 2000 cm⁻¹.

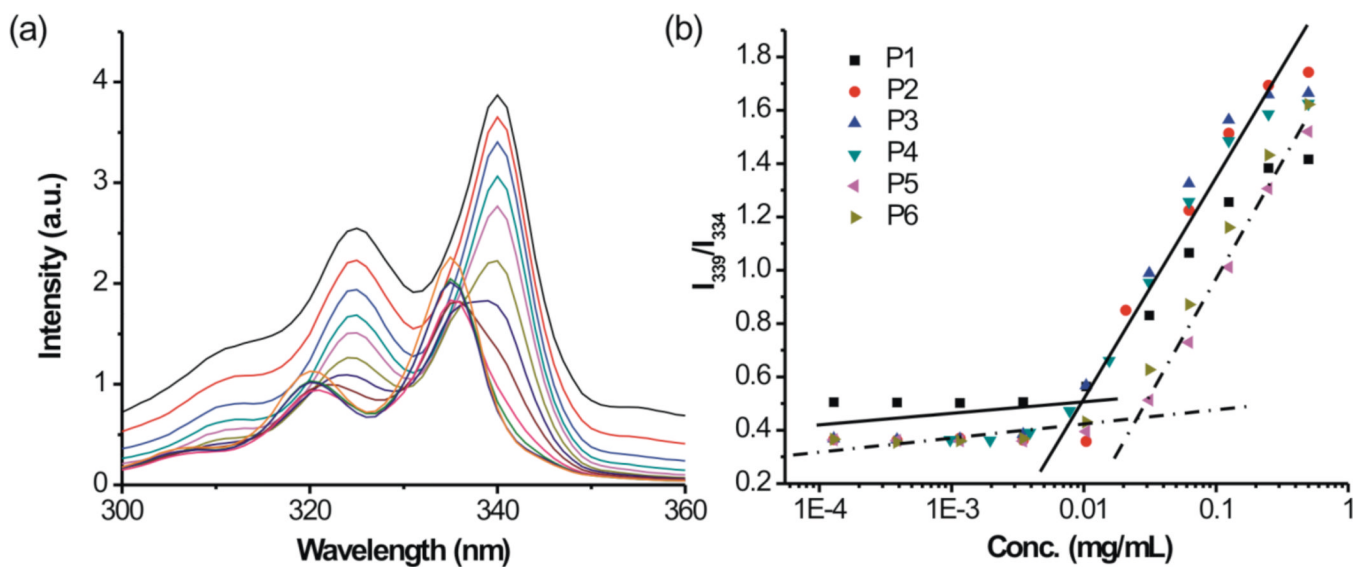


Figure 3. (a) Excitation spectra of pyrene in the presence of P6 in phosphate buffered saline (PBS); (b) Pyrene absorption ratio (I_{339}/I_{334}) as a function of block copolymer concentration.

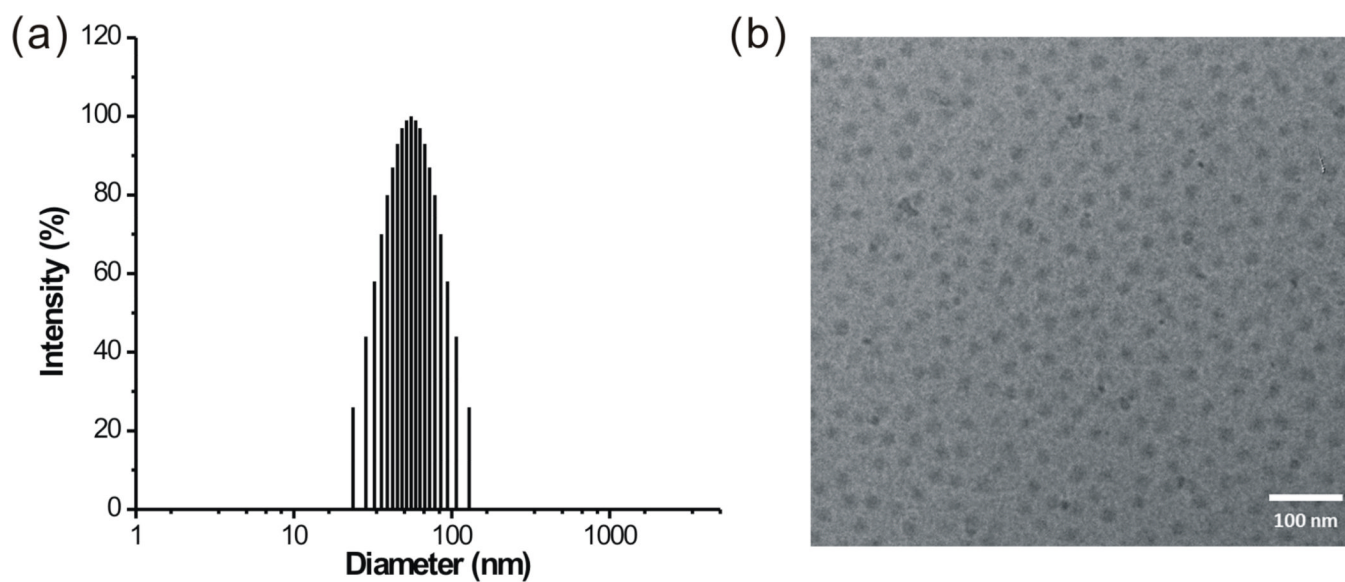


Figure 4.
(a) The size distribution of blank micelles prepared from **P6**. (b) TEM image of blank micelles of **P6**.

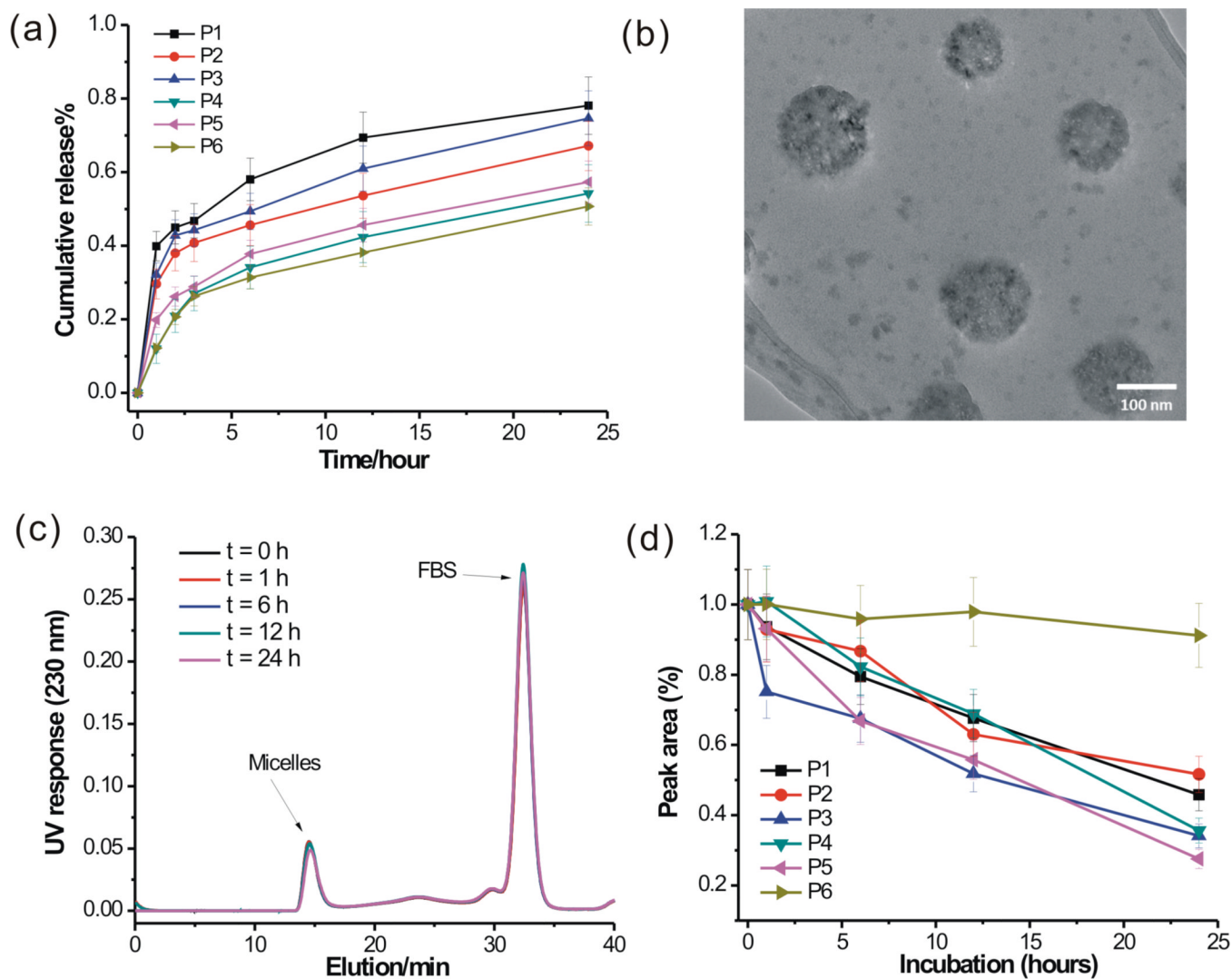


Figure 5. (a) *In vitro* paclitaxel (PTX) release profiles for PTX-loaded micelles in PBS (pH 7.4) at 37 °C. (b) TEM image of PTX-loaded micelles of P6. (c) SEC traces of PTX-loaded micelles of P6 incubated with 20 wt% fetal bovine serum (FBS) for different periods at 37 °C, with very little change in peak area observed for this polymer system; (d) Peak area of different micelles as a function of incubation time. The decrease in peak area with time indicates micelle dissociation in the presence of FBS.

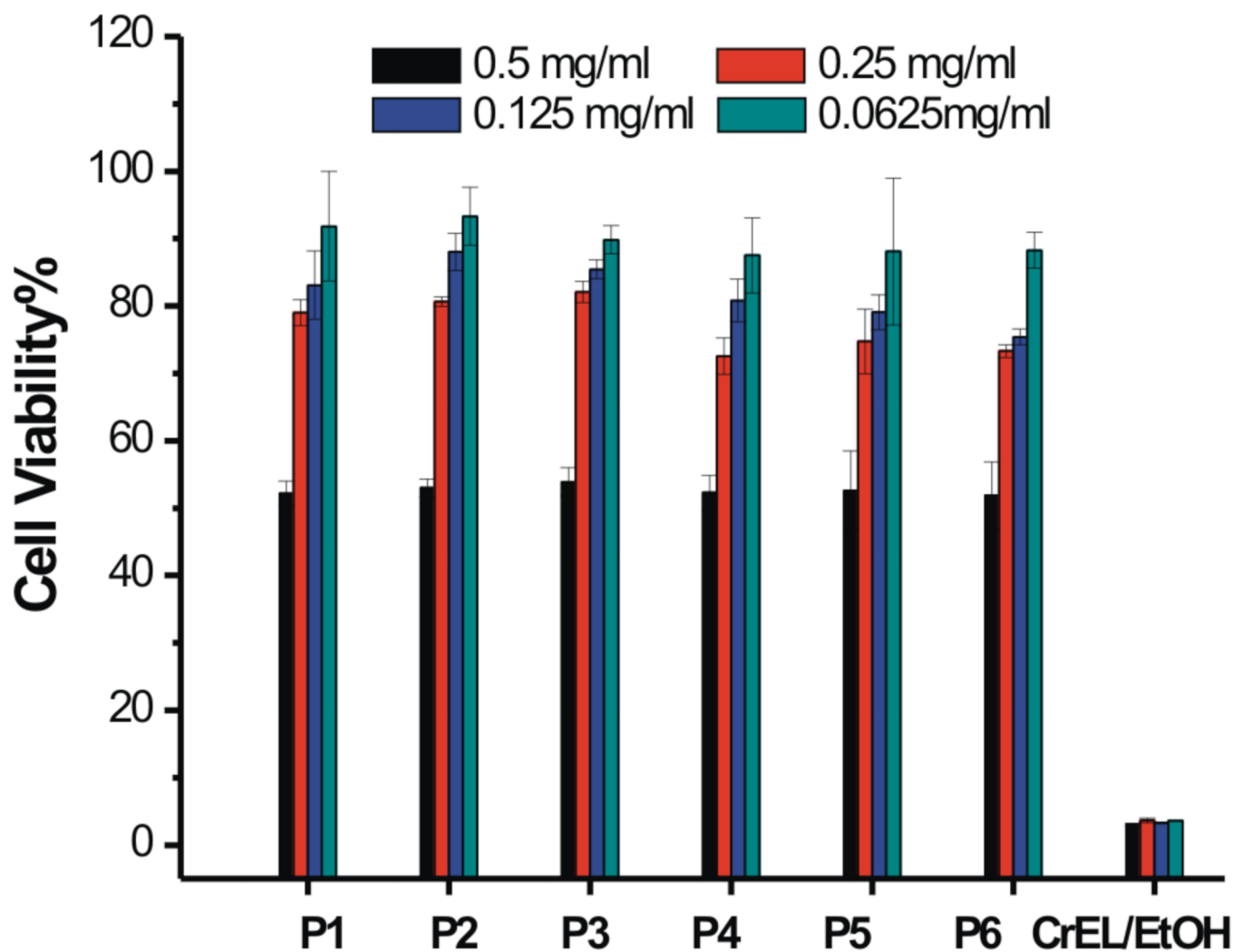


Figure 6. Viability of HeLa cells after incubation with blank micelles of **P1–P6** and CrEL/EtOH (1/1, v/v) in PBS (10 vol%) as a comparison.

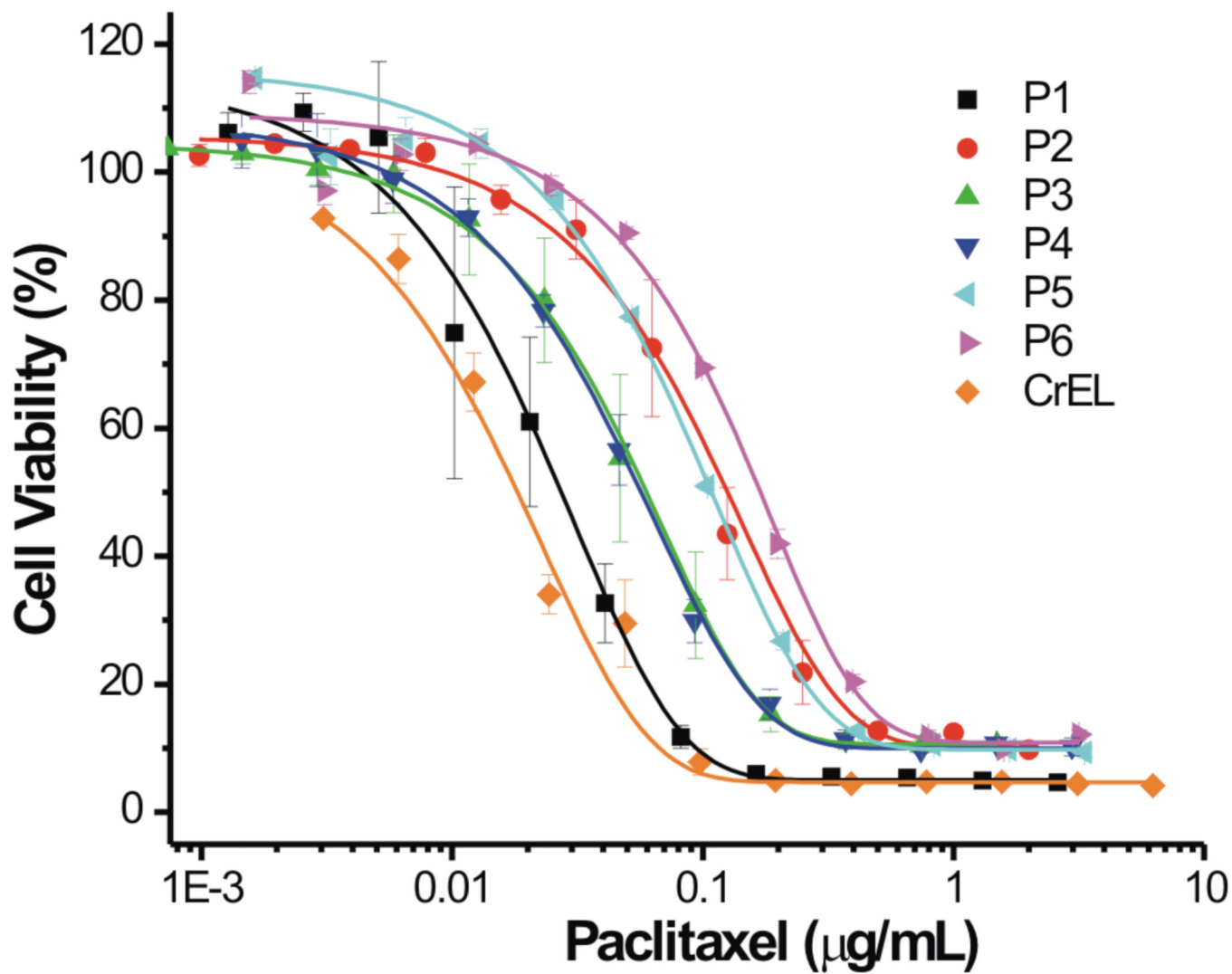
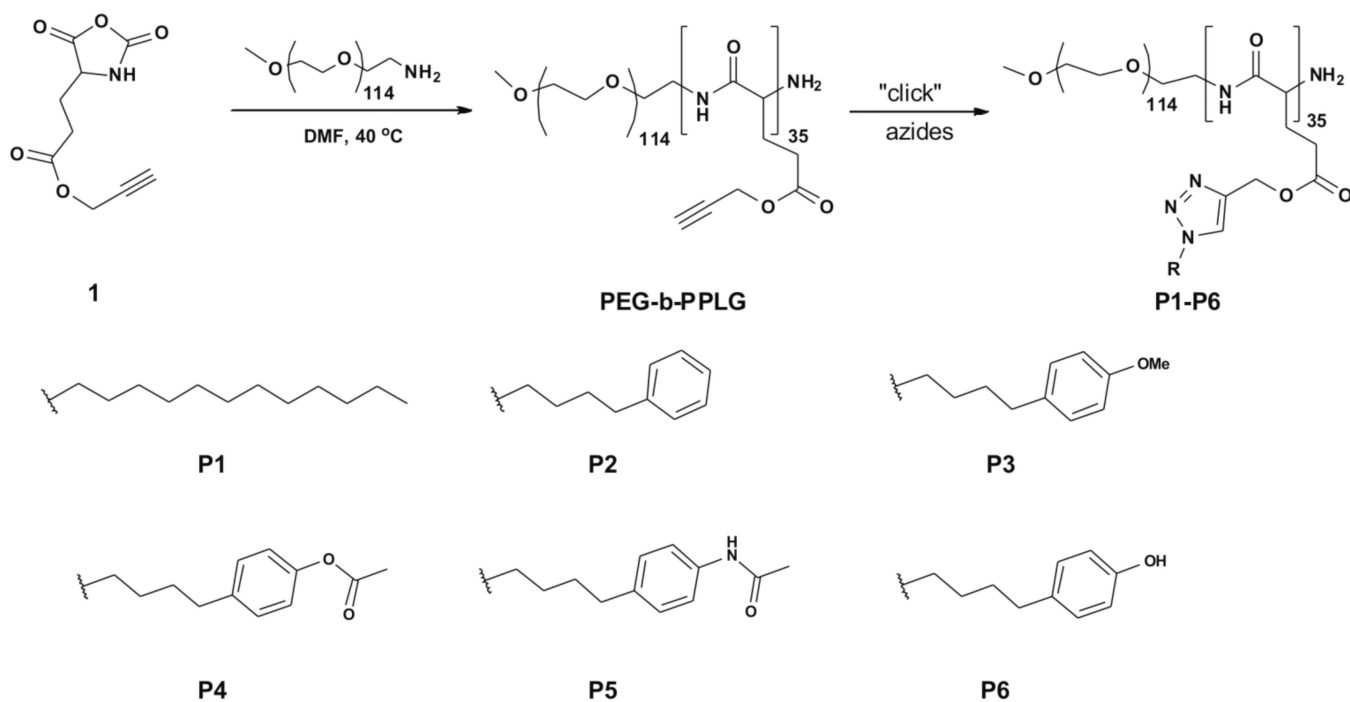


Figure 7. Viability of HeLa cells after being incubated with PTX-loaded micelles and a PTX Cremophor formulation using CrEL/EtOH.



Scheme 1.
 Synthesis of **PEG-b-PPLG** using ROP and subsequent postfunctionalization by CuACC chemistry.

Table 1

Characteristics of **P1–P6**, their blank micelles and PTX-loaded micelles.

	M_n	PDI ^c	DP ^d	CMC (M)	Blank micelles ^e		PTX-loaded Micelles ^e		Drug Loading Content (DLC) wt%(SD)
					d_h	PI	d_h	PI	
P1	18,250 ^a (15,650) ^b	1.2		5.4×10^{-7}	149	0.204	178	0.195	5.7%(0.4%)
P2	16,980 (16,140)	1.1		5.9×10^{-7}	163	0.247	181	0.178	4.9%(0.5%)
P3	18,030 (17,580)	1.1	35	5.5×10^{-7}	109	0.260	195	0.128	4.6%(0.4%)
P4	19,020 (21,430)	1.1		5.3×10^{-7}	136	0.290	160	0.129	7.2%(0.7%)
P5	18,980 (25,010)	1.1		1.6×10^{-6}	59	0.303	189	0.098	6.0%(0.4%)
P6	17,540 (22,610)	1.1		1.7×10^{-6}	43	0.289	157	0.155	6.9%(0.3%)

Note:

^aMolecular weight obtained by ¹H NMR.

^bNumber-averaged molecular weight by GPC in DMF using PMMA standards.

^cPolydispersity index (PDI) by GPC.

^dDegree of polymerization of PPLG block calculated by ¹H NMR.

^eHydrodynamic diameters (d_h) and polydispersity index (PI) of micelles in PBS by dynamic light scattering.