Methotrexate Esters of Poly(Ethylene Oxide)-*Block*-Poly(2-Hydroxyethyl-L-Aspartamide). Part I: Effects of the Level of Methotrexate Conjugation on the Stability of Micelles and on Drug Release

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Received October 29, 1999; accepted February 1, 2000

Purpose. To study the effects of hydrophobicity of the micelle-forming block copolymeric drug conjugate, methotrexate (MTX) esters of poly(ethylene oxide)-*block*-poly(2-hydroxyethyl-L-aspartamide) (MTX esters of PEO-*b*-PHEA), on the stability of micelles and on drug release.
Methods. MTX esters of PEO-*b*-PHEA with three levels of MTX conjugation were synthesized. Size distribution of the micelles was measured by dynamic light scattering (DLS). The critical micelle concentration (CMC) was determined by a light scattering study. Size exclusion high performance liquid chromatography (SEC-HPLC) was used to study the equilibrium between unimers and micelles, and release of MTX at pH 7.4.
Results. MTX esters of PEO-*b*-PHEA with MTX substitution of 7.4%, 22%, and 54% were prepared. The conjugates formed micelles based on DLS. The stability of the micelles correlated with the level of MTX conjugation. The conjugate with 54% MTX had a lower CMC (0.019 mg/mL) than the conjugates with 22% MTX (0.081 mg/mL) or 7.4% MTX (0.14 mg/mL). Micelle dissociation was significantly slower for the conjugate with 54% MTX than that with 22% and 7.4% MTX. Slower release of MTX from the micelles was also observed for the conjugate with the higher MTX attachment.
Conclusions. MTX esters of PEO-*b*-PHEA can be structurally modulated by varying the degree of MTX substitution, which in turn changes the hydrophobicity of the conjugate, thereby modifying micelle stability and controlling drug release.

KEY WORDS: methotrexate; block copolymer; polymeric conjugate; micelles; unimers; drug delivery.

INTRODUCTION

Several different block copolymer micelles have been found potentially useful in the process of drug delivery (1–3). Spherical polymeric micelles have a small core/shell structure and are thermodynamically more stable than surfactants in terms of low critical micelle concentration (CMC) (4). Poly(ethylene oxide) (PEO) is usually oriented on the surface of the micelles after self-assembly of the block copolymers. The hydrophilic PEO shell possesses advantageous surface properties, which determine the high biocompatibility of the micelles (5). Since interactions among the hydrophobic blocks are the driving force in the formation of micelles, the strength of that interaction determines their stability. As a drug carrier, polymeric micelles should not only be stable in circulating blood, but also should transport the drug to its site of action, and release it at an optimum rate (6). Verification of its stability in circulation and its effective release of the drug is, thus, essential in the evaluation of the therapeutic potential of a drug delivery system. Since the structural design of the amphiphilic block copolymer is key in determining the stability of the micelles and their drug-releasing properties, modifying the hydrophobic characteristics of the block copolymer may provide the micelles with high stability and the most desirable drug-release profile.

Studies of drug delivery using polymeric micelles have focused on the characterization of the micelles (7), the solubilization of hydrophobic drugs (8,9), and on the biological activities of drugs and carriers (10). Few studies have addressed the issue of stable and reliable long-term release of the active substance, and the use of polymeric micelles has been limited by a low drug loading capacity and suboptimal drug release. We have described an MTX ester of PEO-*b*-PHEA, a hydrolyzable drug polymer conjugate, which forms stable, approximately 20 nm, micelles, and which releases MTX in a sustained manner (11). We have hypothesized that modifications in the hydrophobic block of the copolymer conjugate by changing the level of MTX conjugation may have an effect on the properties of the micelles, including their thermodynamic stability in terms of CMC, equilibrium between micelles and single polymer molecules (unimers), and release of MTX. Accordingly, experiments were performed to study systematically the relationship between chemical structure and physical and functional properties of the polymer-drug conjugates, MTX esters of PEO-*b*-PHEA. The results show that the degree of hydrophobicity of the block copolymer influences micelle stability and release of MTX.

MATERIALS AND METHODS

Synthesis of MTX Esters of PEO-*b*-PHEA

Poly(ethylene oxide)-*block*-poly(β-benzyl-L-aspartate) (PEO-*b*-PBLA) was graciously provided by Dr. K. Kataoka (University of Tokyo, Japan), and was synthesized as described previously (12). A micelle forming PEO-*b*-PBLA with a PEO block of 12,000 g/mol and 15 units of benzyl-L-aspartate was used for this study. MTX and other chemicals used in the experiments were purchased from Aldrich (Milwaukee, WI). A MTX ester of PEO-*b*-PHEA was synthesized in two steps as described elsewhere (11). First, PEO-*b*-PBLA was converted to PEO-*b*-PHEA by aminolysis in the presence of the catalyst, 2-hydroxypyridine, at ambient temperature. The resulting polymer, PEO-*b*-PHEA, contains hydroxyl groups on the side chains, which serve as the sites of MTX attachment. The conjugation of MTX to PEO-*b*-PHEA was carried out with dicyclohexylcarbodiimide and dimethylaminopyridine as coupling agent and catalyst. MTX esters of PEO-*b*-PHEA were characterized by ^{1}H NMR and ultraviolet (UV) spectroscopy.

The level of MTX substitution was determined by UV analysis after alkaline hydrolysis of the conjugate. MTX esters

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ABBREVIATIONS: CMC, critical micelle concentration; DLS, dynamic light scattering; MTX, methotrexate; PEO, poly(ethylene oxide); PEO-*b*-PHEA, poly(ethylene oxide)-*block*-poly(2-hydroxyethyl-L-aspartamide); SEC-HPLC, size exclusion high performance liquid chromatography; UV, ultraviolet.

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of PEO-*b*-PHEA in methanol (1.0 mg/mL) were hydrolyzed with 1.0 N NaOH. The resulting solution was analyzed on an UV-Vis spectrometer (Pharmacia 4000), and the MTX concentration was measured ($\varepsilon_{304} = 2.35 \times 10^4$ $dm^3mol^{-1}cm^{-1}$). Substitution of MTX on the conjugate expressed as weight was calculated by dividing the weight concentration of MTX by that of the polymeric micelles corrected by a dilution factor. The molar ratio of MTX substitution was subsequently determined.

Critical Micelle Concentration

MTX esters of PEO-*b*-PHEA self-assembled into micelles according to a modified procedure (13). The polymer conjugate (20 mg) was dissolved in N,N-dimethylacetamide (5 mL), and 10–20% (volume ratio) of water was added dropwise to the solution. After dialysis against water with a molecular membrane (molecular weight cut-off = 12,000–14,000 g/mol), the resulting micelle solution was filtered through a nylon membrane (0.45 μm, Fisher, Pittsburgh, PA). A series of concentrations of MTX esters of PEO-*b*-PHEA (0.001 mg/mL −1.5 mg/mL) calibrated by measuring the MTX content was prepared for the measurement of CMC. Light scattering was measured with a fluorometer (Hitachi 3010, Japan), with excitation and emission wavelengths set at 450 nm (14). The average intensity of scattered light from three measurements was plotted against polymer concentration, and CMC was determined from the plot.

Size Distribution of MTX Ester of PEO-*b*-PHEA Micelles

The apparent hydrodynamic diameter and the size distribution of the polymeric micelles were measured by a COULTER® N4 Plus DLS instrument (Beckman) equipped with a 10 mW helium-neon laser source operating at 632.8 nm. Setting the light scattering angle at 90°, the measurements were performed at 25°C. All samples were filtered through a membrane (0.45 μm) before the measurements. The concentration of each sample was in the range of 1.0 to 0.3 mg/ml to obtain optimum light scattering intensity. Particle size distribution was processed with the FORTRAN program "CONTIN".

Stability of the Micelles and Drug Release

Micelles of MTX esters of PEO-*b*-PHEA were lyophilized for storage. The lyophilized form was reconstituted by sonication in 0.10 M phosphate buffer (pH 7.4) containing 0.10% NaN_3. The samples (1.0 mg/mL) were incubated at 37°C in a shaker. Chromatographic analyses of MTX esters of PEO-*b*-PHEA aqueous solution were carried out on a Waters HPLC system consisting of a Waters 501 pump, a WISP 712 autosampler, and a 484 absorbance detector. Aliquots of the micelles were applied onto a SEC column (Shodex, HB 805) equipped with a guard column. The column was calibrated with molecular weight markers (Sigma, 2,000,000 to 500 g/mol). PBS buffer (0.10 M, pH = 7.4) was used as the mobile phase, which was filtered through a 0.45 μm Millipore membrane and degassed prior to use. The flow rate was 1.0 mL/min. The eluent was detected at 302 nm. Each sample was assayed three times. Peak area percentage of released MTX was plotted against time.

RESULTS

Synthesis and Characterization

Figure 1 shows the structure of the MTX esters of PEO-*b*-PHEA. The coupling reaction between PEO-*b*-PHEA and

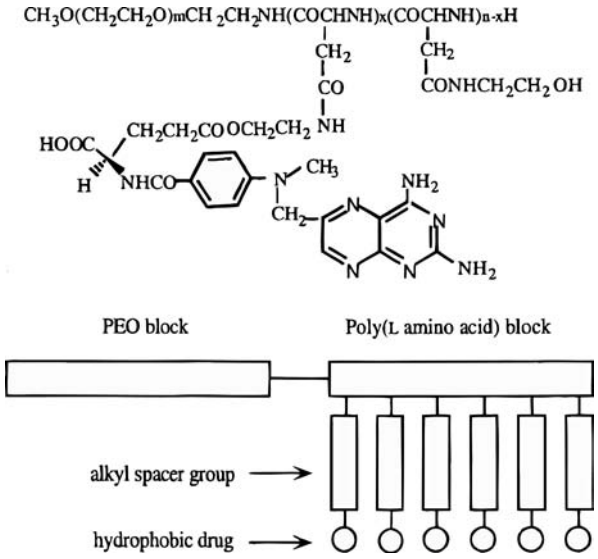

Fig. 1. Chemical structure of MTX esters of PEO-*b*-PHEA.

MTX produced a hydrolyzable drug conjugate. Various levels of MTX conjugation were obtained by altering the reaction conditions, such as concentration of reactants and reaction time. More MTX moieties were conjugated to PEO-*b*-PHEA by increasing the concentration of MTX, prolonging the reaction time, and selecting the proper solvent. We have reported that more drug conjugation can be achieved in dichloromethane than in DMF (11). The conjugate was purified by dialysis against DMSO, followed by precipitation in cold isopropanol. To confirm the purity of the product, the conjugate was analyzed by SEC-HPLC. The column was calibrated with molecular markers consisting of blue dextran (2,000,000 g/mol), proteins (600,000 to 29,000 g/mol), and MTX (454 g/mol). The conjugate unimers with molecular weight of 16,000 g/mol eluted at approximately 10.2 min, and free MTX eluted at 13.1 min. No free MTX was detected in the conjugate solution. The level of MTX substitution for each conjugate was determined after collecting the precipitated powder product. UV analysis was employed to determine the level of MTX substitution. Three levels of MTX substitution were reached for the subsequent studies. The values are summarized in Table I.

Size Distribution

MTX esters of PEO-*b*-PHEA self-assemble into polymeric micelles in aqueous media, and the size distribution of the

Table I. MTX Esters of PEO-*b*-PHEA

MTX Esters of PEO-*b*-PHEA	Molecular weight (g/mol)	Molecular weight of PHEA block	Substitution of MTX[a]	
			Mol %	Weight %
LYD059	15,100	3100	7.4	3.2
LYD043	16,000	4000	22.0	9.0
LYD038	18,100	6100	54.0	19.4

[a] Determined by UV spectroscopy, mol % is based on the residues of aspartic acid; weight % is based on per gram of polymer.

Table II. Micelles of MTX Esters of PEO-*b*-PHEA

Micelles of MTX esters of PEO-*b*-PHEA	HPLC Elution Time (min)		Size[a] (nm)	CMC[b] (mg/mL)
	Unimers	Micelles		
LYD059	10.329 ± 0.007	n.d.	11.7 ± 1.3	0.140
LYD043	10.191 ± 0.007	9.121 ± 0.026	15.9 ± 4.2	0.081
LYD038	10.097 ± 0.019	8.681 ± 0.018	27.1 ± 3.2	0.019

[a] Determined by dynamic light scattering;
[b] Determined by light scattering.

polymeric micelles with various amounts of MTX were examined by DLS. The diameter of the polymeric micelles was dependent on the level of MTX substitution, rising from 11.7 ± 1.3 nm at 7.4%, 15.9 ± 4.2 nm at 22% and 27.1 ± 3.2 nm at 54%, respectively (Table II). The size of the micelles based on MTX esters of PEO-*b*-PHEA was similar to that of PEO-*b*-PBLA micelles formed in aqueous solutions (4).

Critical Micelle Concentration

The CMC of the conjugates was measured by light scattering. When the concentration of the copolymeric conjugate was below the CMC, the solution emitted a low intensity of scattered light. However, when the concentration reached the CMC, the unimers began to assemble into micelles, and a sharp increase in scattered light intensity was observed (Fig. 2). The CMC of MTX esters of PEO-*b*-PHEA was determined by extrapolation of the intensity-concentration curve (Table II). For the conjugate with 54% MTX attachment, the polymeric micelles had a low CMC (0.019 mg/mL), an indication of high stability. In contrast, the conjugate with 7.4% MTX attachment had a relatively higher CMC (0.14 mg/mL), an indication that they were less stable. The CMC of the conjugate with 22% MTX substitution fell between these two boundaries (0.081 mg/mL).

Dissociation of Polymeric Micelles

SEC-HPLC was performed to characterize the unimers and micelles of the MTX esters of PEO-*b*-PHEA. Calibrated with the molecular weight markers, the micelle peak eluted at 9.2 min or earlier, depending on the apparent molecular weight of the micelles. The unimers, however, eluted at 10.0 min or later. The elution time of the conjugates correlated well with the molecular weights derived from the UV analysis. Higher levels of MTX conjugation resulted in higher molecular weights of the unimers, which in turn should correspond to shorter elution times (Table II).

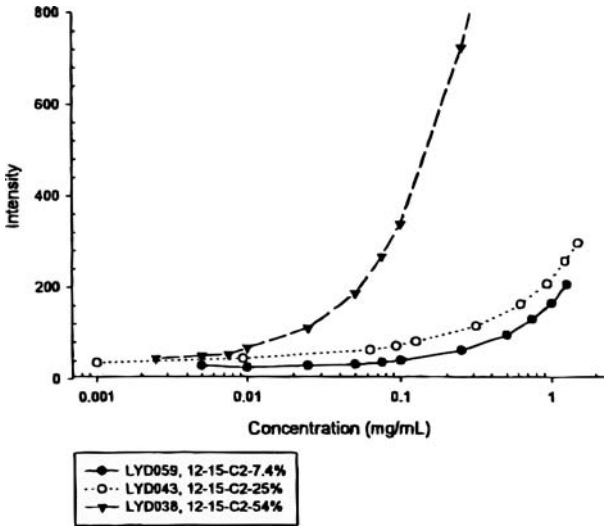

Fig. 2. Light scattering intensity as a function of concentration of MTX esters of PEO-*b*-PHEA (n = 3).

Significant differences in dissociation of the polymeric micelles were observed at different levels of MTX conjugation. Under HPLC conditions, the polymeric micelle solution was diluted with the mobile phase solvent. Therefore, the HPLC system does not provide the exact concentrations of unimers and micelles at equilibrium. Nevertheless, the differences among the different conjugates reflected the relative stability of the micelles. Conjugates with 54% MTX displayed remarkable stability (Fig. 3a). A micelle peak was the main component observed by HPLC, and <20% unimers appeared after 20 days. Conjugates with 22% MTX substitution showed peaks of both unimers and micelles (Fig. 3b). As time progressed, the unimer peak increased while the micelle peak decreased. Finally, the micelle peak disappeared completely, and only unimers remained on the HPLC chart. The conjugates with 7.4% MTX conjugation showed no micelle peak on the HPLC chart, which indicates that the micelles were dissociated due to dilution of the micelle solution by the HPLC eluent (data not shown). These data correlate well with the CMC values. Micelles with lower CMCs were more tolerant of dilution conditions than those with high CMCs. Accordingly, the 7.4% MTX micelles dissociated upon dilution.

Release of MTX from Its Esters of PEO-*b*-PHEA

The release of MTX from its esters of PEO-*b*-PHEA was monitored by SEC-HPLC under physiologic pH. Concentrations of the conjugates for the release study were above their CMCs. All conjugates showed remarkable stability in terms of *in vitro* drug release. However, relatively unstable micelles released drug more freely (Fig. 4). At 20 days, 21% MTX was released from the ester with 7.4% MTX substitution, while only 10% of the drug was released from the ester with 22% MTX substitution and 5% of the MTX from the ester with 54% substitution at the same time point.

DISCUSSION

MTX esters of PEO-*b*-PHEA were synthesized at three levels of MTX to study the relationship between the chemical structure of a block copolymer-drug conjugate and the physical and the functional properties of resulting micelles, particularly

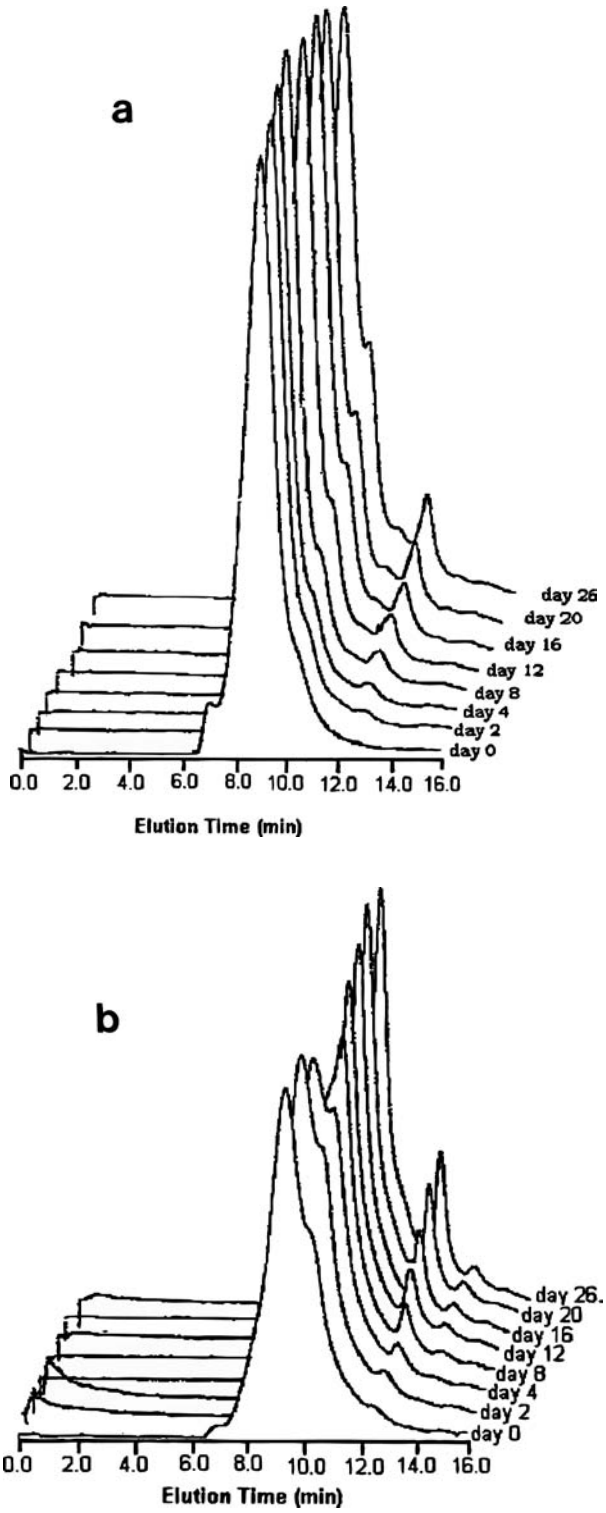

Fig. 3. Size exclusion chromatograms of MTX esters of PEO-*b*-PHEA with (a) 54% MTX substitution; (b) 22% MTX substitution (1.0 mg/mL in 0.10 M phosphate buffer, pH 7.4).

drug release (Fig. 1). Attachment of MTX on the PHEA block by ester bonds altered the size and the hydrophobicity of PEO-*b*-PHEA, and the conjugates self assembled in aqueous solution into micelles. The starting block copolymer, PEO-*b*-PHEA, did not form micelles in aqueous solution, based on transmission electron microscopy and DLS studies (data not shown).

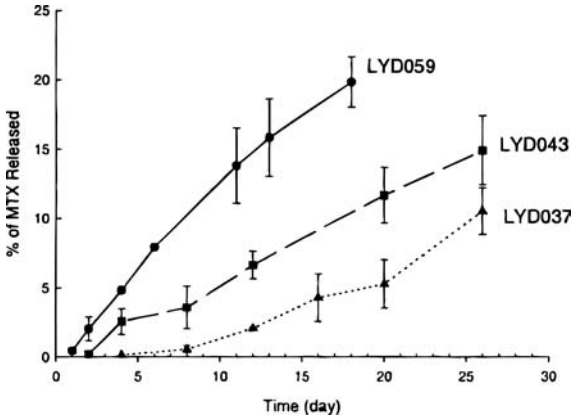

Fig. 4. Release of MTX from the MTX esters of PEO-*b*-PHEA (1.0 mg/mL in 0.10 M phosphate buffer, pH 7.4, n = 3).

Micelles based on MTX esters of PEO-*b*-PHEA had diameters ranging from 10 to 30 nm, depending on the level of drug conjugation. A previous study by transmission electron microscopy revealed spherical micelles of MTX esters of PEO-*b*-PHEA (11). The size and shape is similar to that of serum lipoproteins, biological transport systems for water insoluble lipids. Polymeric micelles may act as synthetic analogs of serum lipoproteins for drug delivery.

Striking effects on the stability of micelles self-assembled from MTX esters of PEO-*b*-PHEA were obtained by adjusting the level of attached MTX. An increase in the level of MTX enhanced hydrophobic interaction responsible for self-assembly, resulting in a more negative free energy of micellization. The CMC, which was determined by light scattering measurements (Fig. 2), decreased from 0.14 to 0.019 mg/mL as the level of attached drug conjugation rose from 7.4 to 54%, a 10-fold change. In addition, an increase in the level of attached MTX resulted in reduced rates of dissociation of micelles into unimers during SEC-HPLC (Fig. 3) The results are consistent with an earlier study by Yokoyama and coworkers, who showed that PEO-*b*-poly(aspartic acid)-doxorubicin conjugates could elute predominately as micelles (7). The unique stability of these polymeric micelles toward dissociation contrasts with low molecular weight surfactants, which reach equilibrium on a time-scale of milliseconds (15).

The release of MTX from the MTX esters of PEO-*b*-PHEA occurred over several weeks and was related to the level

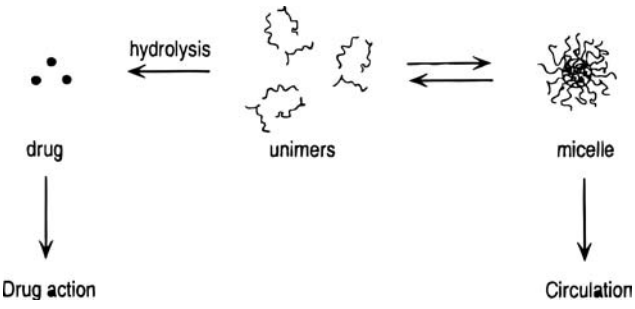

Fig. 5. Model of equilibrium of unimers and micelles and drug release.

of conjugated drug (Fig. 4). For MTX esters of PEO-*b*-PHEA, the hydrolyzable ester bonds were placed in a hydrophobic environment, the core of the micelles. Therefore, the drug is likely released through hydrolysis of the unimers. As illustrated in Fig. 5, at or above the CMC, micelles and unimers are at equilibrium, and the micellar form of the conjugate is available for prolonged circulation. However, the release of the drug is more closely related to the concentration of the unimers. Adjustments in the equilibrium may be critical to obtain the desired drug-release profile. Balancing the association and dissociation of the polymer micelles could be achieved via modulation of the structure of the MTX conjugate, particularly by adjusting the hydrophobicity of the block copolymer. To achieve prolonged circulation in blood, we can increase the level of conjugated MTX, shifting the equilibrium to the micelles (Fig. 5). Lastly, as the release of MTX gradually occurred, polymeric micelles increasingly dissociated into unimers during elution in SEC-HPLC (Fig. 3), and the release of MTX may be faster under nonequilibrium conditions, such as the vascular system.

In conclusion, our data show that MTX esters of PEO-*b*-PHEA can be structurally modulated by varying the level of MTX substitution, which in turn modifies the hydrophobicity of the block copolymer conjugate. A higher percentage of MTX attachment resulted in larger conjugate micelles and lower CMCs, and in slower dissociation and drug release. Therefore, varying the hydrophobicity of the block copolymer enables the controlled release of drug from polymeric micelles used as nanoscopic carriers.

ACKNOWLEDGMENTS

This work was supported by NIH Grant AI43346-01 and the New Investigator Program of the Burroughs Wellcome Fund and the American Association of Colleges of Pharmacy.

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