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P-Glycoprotein Efflux Inhibition by Amphiphilic Diblock Copolymers: Relationship between Copolymer Concentration and Substrate Hydrophobicity

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Abstract: The utilization of surfactants to increase intestinal absorption of drugs is a viable strategy that benefits from increases in drug solubilization and the potential for inhibition of P-glycoprotein (P-gp) mediated efflux. However, the effective concentration range for P-gp inhibition of most surfactants is defined over a narrow concentration range, below the critical micelle concentration (CMC), as a result of significant micelle sequestration of drug. Therefore, the objectives of these studies were to assess if association of P-gp substrates differing in hydrophobicity will impact the effective concentration range for P-gp inhibition by amphiphilic diblock copolymers based on methoxypolyethylene glycol-block-polycaprolatone (MePEG-*b*-PCL). Comparisons between the micelle association and Caco-2 cellular accumulation were evaluated using two structurally homologous P-gp substrates, the relatively hydrophobic R-6G and the hydrophilic R-123, over concentrations above and below the CMC for MePEG-*b*-PCL diblock copolymers. An approximately 3.75-fold enhancement of R-123 accumulation occurred with 2 mM $MePEG_{17}$-*b*-PCL_5, compared to approximately 1.25-fold for R-6G. This decrease in the accumulation enhancement corresponds with the higher R-6G fraction (0.75) associated at 2 mM $MePEG_{17}$-*b*-PCL_5 compared with R-123 (0.25). Interestingly, R-6G accumulation was enhanced over a very broad range of $MePEG_{17}$-*b*-PCL_5 concentrations below the CMC. This was in contrast to R-123, which demonstrated no enhancement below the CMC. A similar concentration dependent accumulation profile was seen with other surfactants such as vitamin E TPGS and Cremophor EL and with two other P-gp substrates differing in hydrophobicity, the relatively hydrophobic paclitaxel and hydrophilic doxorubicin. In conclusion, the effective concentration range for surfactant mediated inhibition of P-gp appears to depend on the P-gp substrate hydrophobicity.

Keywords: P-glycoprotein; diblock copolymer; surfactant; Caco-2

Introduction

The localization and functional expression of the drug efflux transporter, P-glycoprotein (P-gp), on the apical membrane of intestinal epithelia can pose a formidable barrier to the oral bioavailability of many therapeutically important drugs.[1–5] Compounds such as the anticancer drug paclitaxel or the antiretroviral agent saquinavir, among many others, are susceptible to P-gp mediated efflux. Using a mdr1a knockout mouse model (i.e., lacking P-gp), Sparreboom et al. demonstrated an increase in paclitaxel oral bioavailability from 11% in wild type mice to 35% when given to mdr1a(−) mice.[2] Similarly, Huisman et al. demonstrated a significant increase in the plasma AUC of saquinavir when administered orally to mdr1a/1b knockout mice (349 ng/mL) compared to wild type

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(215 ng/mL).[6] The oral bioavailability of paclitaxel in humans increased from 9.3% to 67% when paclitaxel was coadministered with the P-gp inhibitor cyclosporin A.[5] These findings clearly demonstrate that strategies to avoid or inhibit P-gp mediated drug efflux can be an attractive approach to improve drug oral bioavailability.

Although P-gp inhibitors have been developed that reduce P-gp efflux activity, there are numerous studies demonstrating effective inhibition of P-gp using surfactants. Amphiphiles such as polysorbates (Tweens), vitamin E TPGS, Pluronics, Solutols, and Cremophor EL have been shown to enhance cellular accumulation and increase transepithelial flux of P-gp substrates.[7–13] We have previously developed a novel series of low molecular weight amphiphilic diblock copolymers based on methoxy poly(ethylene glycol) (MePEG) and poly(caprolactone) (PCL), MePEG-*b*-PCL, that are capable of increasing the cellular accumulation and decreasing efflux transport of the P-gp substrate, rhodamine 123 (R-123; Figure 1).[14,15] Currently, several potential mechanisms for inhibition of P-gp efflux by surfactants have been proposed including alterations in membrane fluidity, inhibition of P-gp ATPase activity, and depletion of cellular ATP stores.[16–20] However, understanding the mechanisms of action for P-gp inhibition has been hampered by seemingly contradictory results between surfactants. MePEG-*b*-PCL diblock copolymers were shown to stimulate P-gp ATPase activity at concentrations corresponding with enhanced R-123 cellular accumulation.[21] In contrast, Pluronics and vitamin E TPGS decreased P-gp ATPase activity.[16,18] Differences in membrane fluidity changes have also been observed with surfactants, and it has been shown that Cremophor EL, Tween 80, and Pluronic increased membrane fluidity, while vitamin E TPGS and MePEG-*b*-PCL had no effect or decreased membrane fluid-

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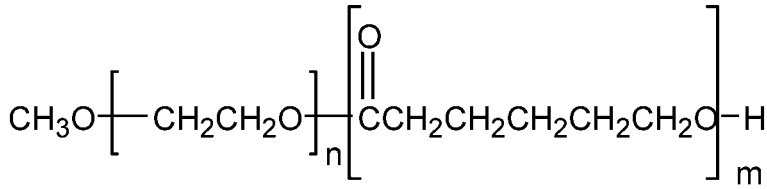
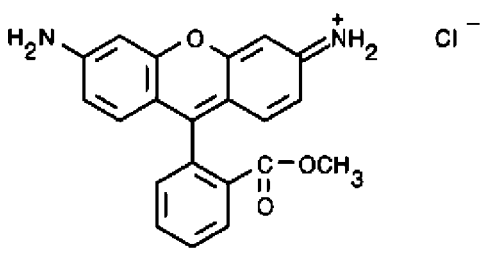
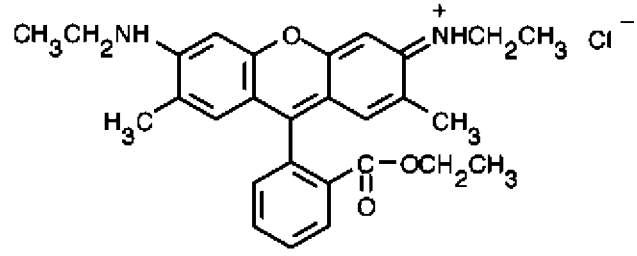


MethoxyPolyethylene Glycol-*block*-Polycaprolactone
($MePEG_n$-b-PCL_m)

n = Degree of Polymerization for MePEG
m = Degree of Polymerization of Caprolactone



Rhodamine 123
(R-123)



Rhodamine 6G
(R-6G)

Figure 1. Chemical structures of methoxypoly(ethylene glycol)-block-poly(ε-caprolactone) (MePEG-*b*-PCL) diblock copolymer, rhodamine 123, and rhodamine 6G.

ity, respectively.[16,18,22] Thus, the suggestion that membrane fluidization and P-gp ATPase inhibition may be a potential mechanism of action for surfactant mediated inhibition of P-gp efflux has not been supported for the wide variety of chemical structures and surfactant architectures that have demonstrated P-gp inhibition.

One advantage in the use of surfactants to inhibit P-gp efflux is the added potential for increasing the solubility of hydrophobic drugs as a result of micellar solubilization. On the other hand, the sequestration of drug within micelles may reduce the fraction of free or unbound drug available for absorption. Surfactant concentrations above the critical micelle concentration (CMC) for Tween 80, Pluronic and Cremophor EL have demonstrated a significant reduction in cellular accumulation of P-gp substrates, corresponding to a reduction in substrate free fraction.[23,24] This was also demonstrated *in vivo* where the oral administration of paclitaxel with Cremophor EL at concentrations greater than the CMC resulted in a 40% decrease in paclitaxel AUC compared to the administration of Cremophor EL below the CMC.[25] In contrast, MePEG-*b*-PCL diblock copolymers increased R-123 cellular accumulation at concentrations 4–100-fold higher than the measured CMC.[15] However, further increases in MePEG-*b*-PCL concentrations resulted in a decrease in the cellular accumulation of R-123 by Caco-2 cells.[15] Thus, the effective concentration range for P-gp inhibition activity of surfactants may be dependent on the partitioning affinity or association of the P-gp substrate into the surfactant micelles. Therefore, the objective of this work was to determine whether differences in partitioning behavior (association) of different P-gp substrates with MePEG-*b*-PCL micelles impacts the effective concentration range of the copolymers for P-gp inhibition. Experimentally, we utilized two structurally homologous P-gp substrates, rhodamine 123 (R-123) and rhodamine 6G (R-6G), which differ in their relative hydrophobicity (Figure 1). The addition of methyl and ethyl groups on R-6G makes this dye more lipophilic with a partition coefficient (K_{part}) of 115.6 compared to R-123 with a K_{part} of 3.4.[26]

Materials and Methods

Materials. Cremophor EL was purchased from Fluka (Oakville, ON). Verapamil, rhodamine 123 (R-123), rhodamine

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6G (R-6G), Triton X-100, and probenecid were obtained from Sigma-Aldrich (Oakville, ON). Paclitaxel was supplied by Hauser (Boulder, CO). Doxorubicin was purchased from Hande Tech (Houston, TX). ^{3}H-Paclitaxel and ^{14}C-doxorubicin were obtained from Amersham Biosciences (Piscataway, NJ) with a specific activity of 66.6 GBq/mmol and 2.15 GBq/mmol, respectively. Vitamin E TPGS 1000 was provided by Eastman Chemical Company (Kingsport, TN).

Cell culture media and supplements, which include Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), L-glutamine, penicillin/streptomycin, trypsin/EDTA, and fetal bovine serum (FBS), were from Invitrogen (Grand Island, NY). Hanks balanced salt solution without phenol red (HBSS), phosphate buffered saline (PBS), and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (hepes) were also from Invitrogen (Grand Island, NY).

Diblock copolymers composed of MePEG with α-caprolactone conjugated to the hydroxyl end of MePEG were synthesized and characterized as previously described.[15,27] These diblock copolymers will be referred to as MePEG$_n$-*b*-PCL$_m$ to denote the degree of polymerization of MePEG (n) and the degree of polymerization of caprolactone (m); for example MePEG$_{17}$-*b*-PCL$_5$. A diblock copolymer consisting of MePEG with a molecular weight of 2000 g/mol and 9 repeat units of D,L-lactide, designated MePEG$_{44}$-*b*-PDLLA$_9$, was synthesized by Angiotech Pharmaceuticals (Vancouver, BC) as previously described.[28]

Cell Culture. Caco-2 cells were obtained from ATCC (Rockville, MD) as passage 17 and cultured as previously described.[15] Cells were used between passages 25–45 and were between 14 and 21 days postconfluency in 48 well plates for cellular accumulation studies. All treatment solutions (diblock copolymers, substrates, and inhibitors) were prepared with HBSS + 10 mM hepes pH = 7.4, which is referred to as "transport buffer" throughout this work. For control purposes, the P-gp inhibitor verapamil (50 μM) and the MRP inhibitor probenecid (100 μM) were utilized to ensure substrate specificity and P-glycoprotein functionality during Caco-2 cell culture.

Caco-2 Cellular Accumulation of Rhodamine Dyes. Caco-2 cellular accumulation of the P-gp substrates R-123 and R-6G was determined with or without various concentrations of MePEG-*b*-PCL diblock copolymers, Vitamin E TPGS 1000, Cremophor EL or MePEG$_{44}$-*b*-PDLLA$_9$ as previously described.[15] Briefly, solutions of 5 μM R-123 or 0.25 μM R-6G in transport buffer with or without diblock copolymers or surfactants were equilibrated at 37 °C for a minimum of 60 min before use. A lower concentration of R-6G compared to R-123 was used in these studies due to R-6G cellular saturation observed at equivalent concentrations of R-123 (data not shown). A concentration of 0.25 μM R-6G was selected to minimize saturation of P-gp activity at high concentrations of R-6G and was based on previous reports utilizing R-6G for P-gp efflux measurements.[29,30] Caco-2 cells grown on 48 well plates were equilibrated with transport buffer for 15 min at 37 °C prior to the addition of copolymer and surfactant samples. Cells were incubated with each treatment group for 90 min at 37 °C, after which the cells were washed using ice cold PBS, then solubilized with 1% Triton X-100. The cellular fluorescence intensity was measured using a CytoFluor 4000 fluorescence microplate reader (PerSeptive Biosystems, Framingham, MA) with λ_{EX} = 485 nm and λ_{EM} = 530 nm for R-123 and λ_{EX} = 508 nm and λ_{EM} = 560 nm for R-6G. Cellular accumulation was normalized with respect to total protein content in each well using the micro BCA protein assay kit (Pierce, Rockford, IL). Results were reported as the fold enhancement in accumulation (accumulation of R-123 or R-6G with diblock or surfactant/accumulation of R-123 or R-6G alone).

Association of Rhodamine Dyes with MePEG-*b*-PCL Micelles. We have previously demonstrated the association of R-123 with MePEG-*b*-PCL diblock copolymers using equilibrium dialysis.[15] Similar methodology was utilized to evaluate the association of R-6G with MePEG-*b*-PCL diblock copolymers. Briefly, an equilibrium dialysis cell from Bel-Art Products (Pequannock, NJ) was used with a Spectra/Por membrane with molecular weight cut off of 1000 g/mol obtained from Spectrum Laboratories (Rancho Dominguez, CA). Increasing concentrations of diblock copolymers with 0.25 μM R-6G in transport buffer were placed in the donor compartment (5 mL) with an equal volume of transport buffer placed in the receiver side (5 mL). The dialysis cell was placed at 37 °C in an Innova 4000 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 100 rpm. R-6G was soluble in transport buffer at 0.25 μM, and control experiments with R-6G in transport buffer demonstrated equilibrium between the donor and receiver chambers within 12 h (data not shown). Therefore, aliquots were taken from both the donor and receiver sides after 24 h to ensure equilibrium, and the fluorescent intensity of R-6G was measured as described above and quantified.

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Above the CMC of a surfactant, there is equilibrium between free and micelle associated solute, and the association coefficient (K_a) of R-123 and R-6G was calculated as follows:

free solute (Sf) + micelles (Saa) $\overset{K_a}{\rightleftharpoons}$ solute associated with micelles (Sm)

$$K_a = \frac{\mathrm{Sm}}{\mathrm{Sf[Saa]}} \quad (1)$$

The equilibrium association coefficient is K_a, and Saa is the concentration of surface-active agent in micelle form (mol/L) and is the total surfactant concentration [Saa_t] minus the CMC (mol/L):

$$[\mathrm{Saa}] = [\mathrm{Saa_t}] - \mathrm{CMC} \quad (2)$$

The micellized or associated fraction of solute (Fb) is given by

$$\mathrm{Fb} = \frac{\mathrm{Sm}}{\mathrm{Sm} + \mathrm{Sf}} \quad (3)$$

Combining eqs 1 and 3 gives

$$\mathrm{Fb} = \frac{K_a[\mathrm{Saa}]}{1 + K_a[\mathrm{Saa}]} \quad (4)$$

Transforming eq 4 to a Scatchard rearrangement gives

$$\frac{\mathrm{Fb}}{\mathrm{Saa}} = K_a - \mathrm{Fb}K_a \quad (5)$$

Caco-2 Cellular Accumulation of Paclitaxel and Doxorubicin. The accumulation of 0.5 μM ^{3}H-paclitaxel or 10 μM ^{14}C-doxorubicin by Caco-2 cells was determined in the presence of increasing concentrations of $MePEG_{17}$-*b*-PCL_5 diblock copolymer. Caco-2 cells, grown on 48 well plates, were exposed to solutions of either paclitaxel or doxorubicin with or without diblock copolymer for 90 min at 37 °C followed by washing and harvesting as described above. Samples were counted using a LS6000TA scintillation counter (Beckman Instruments Inc., Fullerton, CA), and accumulation was normalized to total protein content.

Statistical Analysis. All results are expressed as mean ± standard deviation (SD) with a minimum of $N = 3$ independent experiments. Comparisons between groups were done using a two-tailed two sample *t*-test with a significance level of $p < 0.05$.

Results

Association of R-123 and R-6G with MePEG-*b*-PCL Diblock Copolymers. The fraction of R-123 and R-6G associated with increasing concentrations of $MePEG_{17}$-*b*-PCL_5 diblock copolymer is shown in Figure 2. Above the CMC, both probes accumulated in a concentration dependent manner. A significantly greater degree of association of the relatively hydrophobic R-6G was observed with $MePEG_{17}$-*b*-PCL_5 diblock copolymer compared to R-123 at corresponding concentrations. A similar trend of increased association of R-6G compared to R-123 was found for a series of MePEG-*b*-PCL diblock copolymers varying in the degree of polymerization for each block (data not shown). For each MePEG-*b*-PCL diblock evaluated for R-123 and R-6G association, a Scatchard plot was constructed and the association coefficient (K_a) determined and shown in Table 1. For both R-123 and R-6G, an increase in the PCL block length for the MePEG 750 series resulted in an increase in the K_a. Overall, the K_a value for R-6G was substantially larger than R-123 for each MePEG-*b*-PCL diblock copolymer.

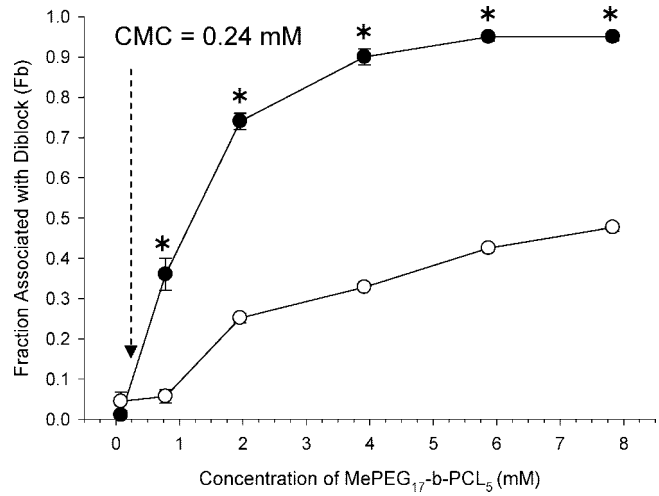

Figure 2. Association of R-123 and R-6G with $MePEG_{17}$-*b*-PCL_5 diblock copolymer. Increasing concentrations of $MePEG_{17}$-*b*-PCL_5 with either (○) 5 μM R-123 or (●) 0.25 μM R-6G were incubated at 37 °C for 24 h in an equilibrium dialysis cell using a 1000 MWCO membrane. Results are expressed as the mean ± SD with $n = 3$ independent experiments. (∗) Statistically significant difference in the fraction associated at corresponding concentrations of diblock copolymer for R-6G and R-123 using a two-tailed two sample *t*-test with $p < 0.05$.

Comparative Caco-2 Cellular Accumulation of R-123 and R-6G with $MePEG_{17}$-*b*-PCL_5. To determine the specificity of R-123 and R-6G for the efflux transporters MRP or P-gp, accumulation experiments were performed with or without the MRP inhibitor probenecid and the P-gp inhibitor verapamil. At 100 μM probenecid, there was no increase in the fold accumulation enhancement of R-123 or R-6G (Figure 3A). However, a 1.5- and 3.0-fold increase in R-123 and R-6G accumulation in Caco-2 cells, respectively, was observed in the presence of the P-gp inhibitor verapamil (Figure 3A). Overall these results indicate that P-gp is the efflux protein involved in limiting R-123 and R-6G cellular accumulation into Caco-2 cells with little or no contribution from MRP isoforms.

The fold enhancement in R-123 and R-6G accumulation by Caco-2 cells with concentrations of $MePEG_{17}$-*b*-PCL_5 below (unimers) and above (micelles) the CMC (240 μM) (represented as a dotted vertical line) is shown in Figure 3B. For R6G, accumulation enhancement occurred at concentrations of copolymer below the CMC followed by a concentration dependent reduction above the CMC. An increase in

Table 1. Association Coefficients and Regression Analysis Obtained from Scatchard Plots for R-123 and R-6G Association Studies with MePEG-*b*-PCL Micelles

			R-123[b]		R-6G[b]	
series	diblock copolymer	CMC[a] (μM)	K_a (M^{-1})	R^2	K_a (M^{-1})	R^2
MePEG 750	$MePEG_{17}$-*b*-PCL_2	2970	10.25	0.771	139.1	139.1
	$MePEG_{17}$-*b*-PCL_5	240	169.6	0.657	874.4	874.4
	$MePEG_{17}$-*b*-PCL_{10}	5.3	295.7	0.943	9273	9273
MePEG 2000	$MePEG_{45}$-*b*-PCL_5	265	207.1	0.909	1994	1994

[a] Obtained from Zastre et al.[15] [b] The association coefficient (K_a) is obtained from the slope of the line from linear regression analysis with a coefficient of variation (R^2).

the R-123 accumulation fold enhancement was observed only with concentrations of $MePEG_{17}$-*b*-PCL_5 greater than the CMC. Caco-2 accumulation of the relatively hydrophobic P-gp substrate R-6G at corresponding $MePEG_{17}$-*b*-PCL_5 concentrations above the CMC resulted in a significantly lower fold enhancement compared to R-123 (Figure 3B). Interestingly, the concentration range of $MePEG_{17}$-*b*-PCL_5 over which accumulation of R-6G increased occurred over a broad range, with concentrations as low as 1 μM resulting in an approximately 2-fold enhancement in R-6G accumulation (Figure 3B). In addition, the fold enhancement of R-6G accumulation with $MePEG_{17}$-*b*-PCL_5 reached a maximum in the vicinity of the measured CMC for the diblock (Figure 3B).

Determination of the MePEG-*b*-PCL Concentration Required for Half-Maximal Fold Enhancement (EC_{50}). It has been previously shown that the reduction in cellular accumulation with concentrations greater than the CMC of surfactant or diblock is due to the partitioning or association of substrate within micelles.[15,23] This micellization of substrates and the resulting decrease in accumulation makes it difficult to quantitatively compare potency between surfactants for enhancement in accumulation. If it is assumed that only the free fraction (Ff) of surfactant or copolymer was able to enter the cells, then at higher concentrations of MePEG-*b*-PCL diblock copolymers, the actual concentration available for accumulation into cells would be proportional to the free fraction. Therefore, the accumulation of R-123 and R-6G with MePEG-*b*-PCL diblock copolymers can be corrected based on the free fraction of R-123 or R-6G at each MePEG-*b*-PCL concentration.

Since the free fraction (Ff) is given by

$$\mathrm{Ff} = \frac{\mathrm{Sf}}{\mathrm{Sm} + \mathrm{Sf}} \tag{6}$$

combining eqs 1 and 6 gives

$$\mathrm{Ff} = \frac{1}{1 + K_a[\mathrm{Saa}]} \tag{7}$$

Since K_a and the CMC are known, at a given concentration of MePEG-*b*-PCL [Saa_t], the accumulation amount of R-123 or R-6G in the cells can be divided by the calculated free fraction (eq 7) to give a so-called "corrected" accumulation level. The resulting dose response curves can then be analyzed using nonlinear regression analysis to establish the half-maximal concentration of diblock required to enhance either R-123 or R-6G accumulation (EC_{50}) for comparisons of the relative potency between diblock copolymers.

Using eq 7, the R-123 and R-6G accumulation profiles for $MePEG_{17}$-*b*-PCL_5 were corrected and shown in Figure 4A and Figure 4B, respectively. For both R-123 and R-6G, the corrected fold enhancement in accumulation appeared to plateau at concentrations of $MePEG_{17}$-*b*-PCL_5 associated with high levels of substrate association. Using the sigmoidal dose response analysis function in SigmaPlot V.9 software, the concentration of diblock producing half-maximal fold enhancement was determined. Table 2 displays the EC_{50} for each diblock copolymer for the accumulation of both R-123 and R-6G. The EC_{50} for R-123 was 2–3 orders of magnitude higher than the EC_{50} for R-6G with all MePEG-*b*-PCL diblock copolymers evaluated (Table 2). At a similar PCL block length of 5 repeat units, increasing the molecular weight of MePEG from 750 to 2000 g/mol appears to have a lower degree of potency for R-123 enhancement. In addition, increasing the PCL block length within the MePEG 750 series from 2 to 5 reduced the EC_{50} approximately 35-fold. Interestingly, all diblocks evaluated demonstrated relatively similar EC_{50} for R-6G enhancement (Table 2).

Fold Enhancement for Paclitaxel and Doxorubicin Accumulation with $MePEG_{17}$-*b*-PCL_5. The accumulation of two structurally dissimilar P-gp substrates, paclitaxel (PTX) and doxorubicin (DOX), with various concentrations of $MePEG_{17}$-*b*-PCL_5 was studied. Similar to the rhodamine dyes evaluated in this work, these P-gp substrates differ in their hydrophobicity, where PTX is relatively hydrophobic ($K_{part} = 99$) compared to DOX ($K_{part} = 0.52$).[31] As observed with both rhodamines, increasing diblock concentration caused an increase in the accumulation of PTX and DOX up to a maximum, followed by a decline in accumulation (Figure 5). Fold enhancement of PTX accumulation reached a maximum in the vicinity of the CMC of $MePEG_{17}$-*b*-PCL_5 with concentrations as low as 1.0 μM demonstrating an approximately 2-fold increase in accumulation (Figure 5). In contrast, a high concentration of approximately 10 mM $MePEG_{17}$-*b*-PCL_5 was required to maximally increase the fold accumulation of DOX (Figure 5). Overall these accumulation profiles are analogous to that of R-123 and R-6G, where maximum fold enhancement for the relatively hydro-

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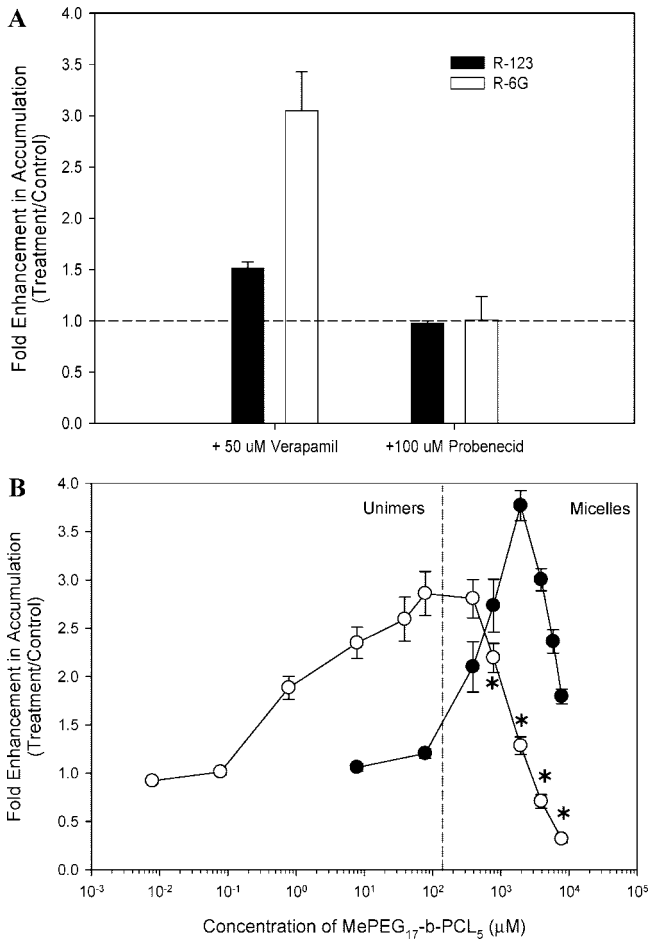

Figure 3. Fold enhancement in R-123 and R-6G accumulation by diblock copolymers in Caco-2 cells. (A) Substrate specificity of R-123 and R-6G for P-gp and MRP isoforms. Cellular accumulation was measured with 5 μM R-123 or 0.25 μM R-6G with or without the P-gp inhibitor verapamil (50 μM) and the MRP inhibitor probenecid (100 μM) for 90 min at 37 °C. (B) Effect of $MePEG_{17}$-*b*-PCL_5 diblock concentration on the fold enhancement of R-123 and R-6G cellular accumulation. Caco-2 cells were incubated with either (●) 5 μM R-123 or (○) 0.25 μM R-6G with or without diblock copolymer at concentrations below (unimers) and above (micelles) the CMC (240 μM; dotted vertical line) for 90 min at 37 °C. Data represents the mean ± SD of $N = 3$ independent experiments. (∗) Statistically significant difference in the fold enhancement between R-6G and R-123 at corresponding concentrations of diblock copolymer using a two-tailed two sample *t*-test with $p < 0.05$.

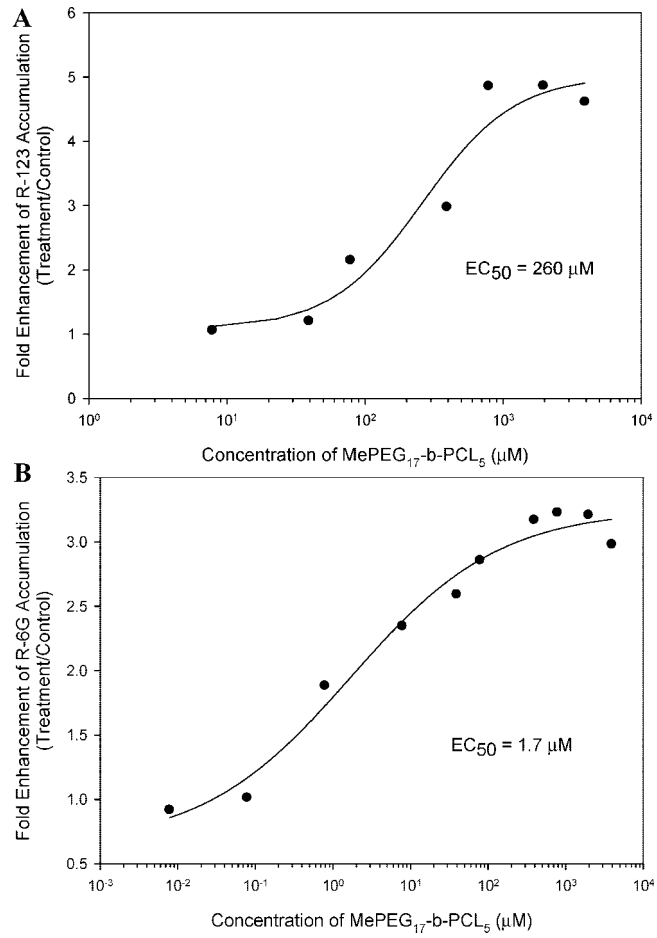

Figure 4. Fold enhancement of (A) R-123 and (B) R-6G accumulation in Caco-2 cells by $MePEG_{17}$-*b*-PCL_5 after correction for free fraction changes. Cellular accumulation was corrected for free fraction changes at concentrations of diblock greater than the CMC using eq 7. Curve represents nonlinear regression analysis using SigmaPlot V.9 software.

Table 2. Concentration of Diblock Copolymer to Achieve 50% of the Maximum Accumulation Enhancement (EC_{50}) for R-123 and R-6G

		EC_{50}[a] (μM)	
series	diblock copolymer	R-123	R-6G
MePEG 750	$MePEG_{17}$-*b*-PCL_2	8980	1.9
	$MePEG_{17}$-*b*-PCL_5	260	1.7
	$MePEG_{17}$-*b*-PCL_{10}	343	0.2
MePEG 2000	$MePEG_{45}$-*b*-PCL_5	1390	0.4

[a] Calculated using the sigmoidal dose response regression analysis from SigmaPlot v.9 software on accumulation data corrected for changes in free fraction of R-123 and R-6G.

phobic substrate was well below the CMC compared to a high concentration above the CMC needed for the relatively hydrophilic substrate (Figure 3B and 5).

Cellular Accumulation of R-123 and R-6G with Other Surfactants. Figure 6 displays the cellular accumulation of R-123 and R-6G in the presence of Cremophor EL (CMC = 30 μM), vitamin E TPGS 1000 (CMC = 132 μM), and a structurally unrelated diblock copolymer $MePEG_{44}$-*b*-$PDLLA_9$ (CMC = 90 μM). Cremophor EL increased the accumulation of both R-123 and R6G. For R-123, a 2-fold increase in accumulation occurred at a concentration approximately 10-fold greater than the reported CMC, with no enhancement below the CMC (Figure 6A). For R6G, the accumulation occurred at concentrations below the CMC (Figure 6A). In contrast, negligible enhancement of R-123 accumulation was found over all concentrations of vitamin E TPGS 1000 and $MePEG_{44}$-*b*-$PDLLA_9$ evaluated (Figure

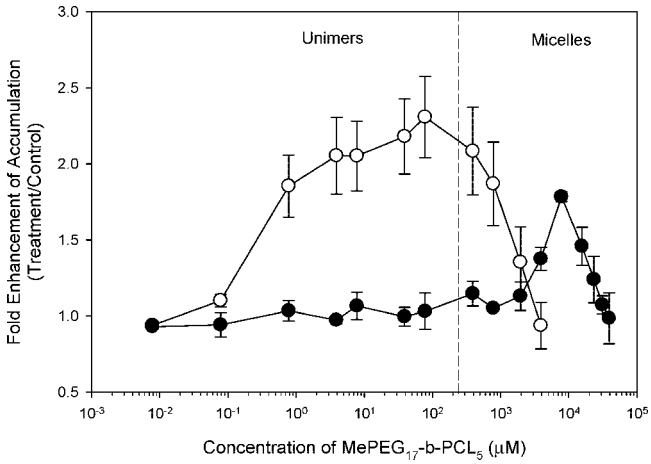

Figure 5. Fold enhancement in the cellular accumulation of paclitaxel and doxorubicin by $MePEG_{17}$-*b*-PCL_5 diblock copolymer in Caco-2 cells. Cellular accumulation of (○) 0.5 μM paclitaxel and (●) 10 μM doxorubicin was determined with or without diblock copolymer at concentrations below (unimers) and above (micelles) the CMC (240 μM; dotted vertical line) for 90 min at 37 °C. Data represents the mean ± SD of $N = 3$ independent experiments.

6B and Figure 6C). However, R-6G accumulation was enhanced approximately 5 fold at vitamin E-TPGS 1000 concentrations below the reported CMC and 2.5 fold at $MePEG_{44}$-*b*-$PDLLA_9$ concentrations as low as 0.2 μM (Figure 6B and 6C). For Cremophor EL, vitamin E TPGS 1000 and $MePEG_{44}$-*b*-$PDLLA_9$, R6G accumulation enhancement dropped in a concentration dependent manner at concentrations above the CMC.

Discussion

Over the years there has been increased interest in developing strategies to overcome the activity of drug efflux transporters such as P-gp, in order to improve drug absorption and distribution to target tissues. The use of surfactants and amphiphilic block copolymers is an attractive approach to overcome P-gp efflux, since the majority of P-gp substrates are hydrophobic drugs with limited solubility. When used above the CMC, surfactants can solubilize hydrophobic drugs within the micelle core.[32,33] Several groups have suggested that sequestration of the substrate within micelles decreased the concentration of substrate available for cellular accumulation, causing a decrease in accumulation and permeability at high surfactant concentrations.[15,23] This potentially may result in formulation and administration restrictions on the use of surfactants to inhibit P-gp activity, since the effective concentration range for the surfactant may be narrowly defined. Too high a concentration of surfactant (above the CMC) may result in a decrease in substrate absorption due to sequestration, and too low a concentration maybe insufficient for P-gp inhibition. This is in contrast to

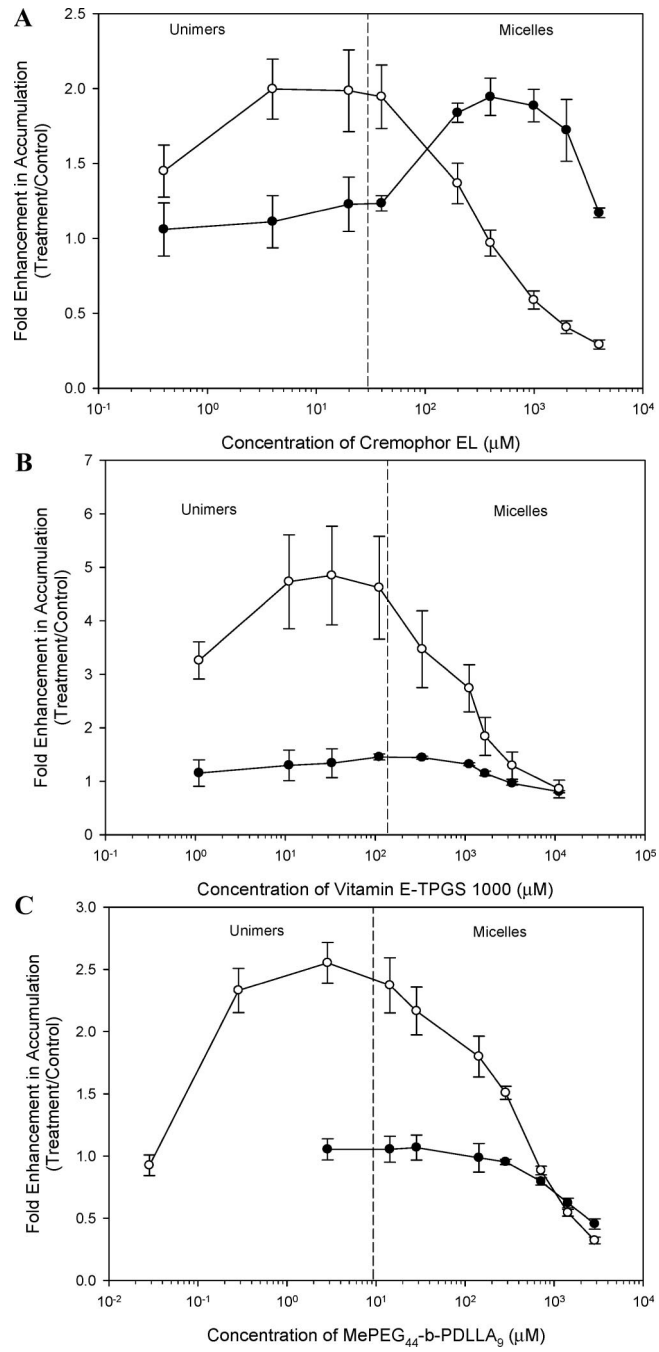

Figure 6. Fold enhancement in R-123 (○) and R-6G (●) accumulation in Caco-2 cells by (A) Cremophor EL, (B) vitamin E TPGS 1000, and (C) $MePEG_{44}$-*b*-$PDLLA_9$. Caco-2 cellular accumulation of either 5 μM R-123 or 0.25 μM R-6G was evaluated with or without increasing concentrations of cremophor EL, vitamin E TPGS 1000 or $MePEG_{44}$-*b*-$PDLLA_9$ diblock copolymer for 90 min at 37 °C. Data represents the mean ± SD of $N = 3$ independent experiments.

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standard P-gp inhibitors such as cyclosporin or verapamil, where the effective concentration range for P-gp inhibition might not be compromised by drug sequestration phenomena.

Both R-6G and R-123 have been utilized extensively as model P-gp substrates, and these studies (Figure 3A) confirm that P-gp mediated efflux limits the cellular accumulation of these two rhodamine dyes in Caco-2 cells.[26,30,34] To determine the impact of micellar association of R-123 and R-6G on the cellular accumulation, increasing concentrations of $MePEG_{17}$-*b*-PCL_5, below and above the CMC (240 μM), were used for cellular accumulation. $MePEG_{17}$-*b*-PCL_5 concentrations up to 8 mM were evaluated since minimal Caco-2 cytotoxicity was previously reported using a lactate dehydrogenase release assay.[15] Concentrations of $MePEG_{17}$-*b*-PCL_5 diblock above the CMC (micelles) resulted in a significant difference in the accumulation enhancement between the relatively hydrophilic R-123 compared with the hydrophobic R-6G (Figure 3B). An approximately 3.75-fold enhancement of R-123 accumulation occurred with 2 mM $MePEG_{17}$-*b*-PCL_5 diblock, compared to approximately 1.25-fold for R-6G. This decrease in the accumulation enhancement corresponds with the higher R-6G fraction (0.75) associated at 2 mM $MePEG_{17}$-*b*-PCL_5 compared with R-123 (0.25) (Figure 2). Since significant accumulation differences occur above the CMC, endocytosis of R-123 or R-6G associated micelles was considered as a possible contributing factor in the cellular accumulation. Several groups have reported endocytosis of polyethylene oxide-polycaprolactone (PEO-*b*-PCL) diblock copolymer micelles as a mechanism for cellular uptake.[35,36] Endocytotic uptake of rhodamine loaded PEO-*b*-PCL block copolymer micelles required long incubation times, upward of 12 h for significant cellular accumulation.[35] Similarly, endocytotic uptake of PEO-PCL micelles containing the fluorescent probe DiI, required greater than 6 h for cellular uptake.[36] These results suggest that cellular uptake of R-123 or R-6G associated with $MePEG_{17}$-*b*-PCL_5 micelles may require longer incubation times greater than the 60 min utilized in this work to facilitate endocytotic uptake of micelles. However, significant endosome distribution of the fluid phase endocytosis marker, horse radish peroxidase was noted within the cytoplasm of Caco-2 cells within 60 min of exposure.[37] In addition, we have previously reported a significant reduction in Caco-2 cellular accumulation of the fluid phase endocytosis marker, Lucifer yellow, after 90 min in the presence of endocytosis inhibitors.[14] Cellular accumulation of R-123 with 2 mM $MePEG_{17}$-*b*-PCL_5 was not significantly reduced in the presence of endocytosis inhibitors after 90 min exposure, suggesting that, at least within this short exposure time, endocytosis does not contribute to the cellular uptake mechanism.[14] Hence, the differences in cellular accumulation (observed at 90 min incubations) between R-123 and R-6G with $MePEG_{17}$-*b*-PCL_5 above the CMC cannot be explained by endocytotic uptake of micelles. Thus, when used at concentrations greater than the CMC, a higher degree of association of a relatively hydrophobic P-gp substrate within MePEG-*b*-PCL micelles appears to result in a lower accumulation enhancement compared with a relatively hydrophilic P-gp substrate.

Interestingly, R-6G accumulation was enhanced over a very broad range of $MePEG_{17}$-*b*-PCL_5 concentrations below the CMC (unimers), with upward of 2-fold enhancement occurring with a diblock concentration of 1 μM (Figure 3B). This is in contrast to when R-123 was used as a substrate with no enhancement of accumulation was observed below the CMC of the diblock. The enhancement profile for R6G was similar to the concentration ranges reported for other surfactants such as Cremophor EL, Tween 80, and Pluronic where enhancement occurs at concentrations primarily below the CMC.[23,24] To quantitatively assess the change in potency of the various diblocks as enhancers of substrate accumulation, the profiles for both R-123 and R-6G with MePEG-*b*-PCL were corrected for changes in free fraction. It was assumed that, at high concentrations of MePEG-*b*-PCL, the decrease in R-123 and R-6G accumulation was the result of a decrease in the free fraction available for cellular uptake and that micelle uptake was negligible as previously reported.[14] Using this approach, Nerurker et al. found that correcting the permeability coefficient for free fraction of solute at high surfactant concentrations resulted in a constant permeability coefficient.[23] Furthermore, the permeability coefficient of amprenavir was found to be constant at high surfactant concentrations when corrected for free fraction.[38] Accordingly, the fold enhancement in R-123 and R-6G accumulation was constant at high $MePEG_{17}$-*b*-PCL_5 concentrations when corrected for free fraction using eq 7 (Figure 4). The EC_{50} values for MePEG-*b*-PCL diblock copolymer-related increases in cellular accumulation were several orders of magnitude higher for the relatively hydrophilic P-gp substrate, R-123, than the hydrophobic R-6G. This suggests that the concentration for P-gp efflux inhibition by MePEG-*b*-PCL for a hydrophobic P-gp substrate may be attained using lower concentrations of diblock copolymer compared to a relatively hydrophilic P-gp substrate.

To assess if compounds differing in hydrophobicity may result in a similar cellular accumulation profile, paclitaxel and doxorubicin were selected since they are well-established

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P-gp substrates with partition coefficients reported to be 99 and 0.52, respectively.[2,31,39] The accumulation enhancement profile for paclitaxel was similar (Figure 5) to the accumulation enhancement observed for R-6G with $MePEG_{17}$-*b*-PCL_5 diblock copolymer (Figure 3B). Maximum accumulation occurred at similar $MePEG_{17}$-*b*-PCL_5 concentrations, with concentrations as low as 1 μM enhancing paclitaxel accumulation by approximately 2-fold (Figure 5). Accumulation enhancement with doxorubicin required a high concentration of 4 mM, well above the CMC of $MePEG_{17}$-*b*-PCL_5 (0.24 mM), complementary to the accumulation enhancement profile observed with the hydrophilic R-123 (Figure 3B).

The differences observed in accumulation enhancement for MePEG-*b*-PCL diblock copolymers with P-gp substrates differing in their relative hydrophobicity are further illustrated by the results in Figure 6, which shows the accumulation of R-123 and R-6G with Cremophor EL, vitamin E TPGS 1000, and $MePEG_{45}$-*b*-$PDLLA_9$. Both Cremophor EL and vitamin E TPGS 1000 have been previously demonstrated to enhance the cellular accumulation of P-gp substrates via a reduction in P-gp mediated efflux.[9,40] Cremophor EL increased R-123 accumulation only at high concentrations above the CMC while enhancement of the hydrophobic R-6G occurred only at concentrations below the CMC (Figure 6A). Vitamin E TPGS 1000 maximally increased R-123 cellular accumulation by 1.4-fold, but enhanced R-6G accumulation by approximately 5-fold at concentrations below the CMC. $MePEG_{45}$-*b*-$PDLLA_9$ is a diblock copolymer with similar MePEG composition to $MePEG_{45}$-*b*-PCL_5, but it possesses poly(D,L-lactide) as the hydrophobic block instead of poly(caprolactone). Accumulation of R-123 was not enhanced over a wide range of $MePEG_{45}$-*b*-$PDLLA_9$ concentrations (Figure 6C). However, $MePEG_{45}$-*b*-$PDLLA_9$ was able to enhance the accumulation of R-6G approximately 2.5-fold at concentrations below the CMC. Taken together, these results demonstrate the importance of considering the physiochemical properties of P-gp substrates on the activity of surfactants to inhibit P-gp mediated efflux activity.

The differences in potency observed for the accumulation enhancement of P-gp substrates differing in hydrophobicity by surfactants may provide insight into the mechanism of action. In general, P-gp substrates are structurally diverse and are hydrophobic in nature. This has led to the currently most accepted model for P-gp mediated efflux proposed by Gottesman et al., called the hydrophobic vacuum cleaner (HVC) model.[41,42] The HVC model postulates that drugs partition into the membrane due to their high partition coefficients, interact with P-gp directly within the lipid bilayer and then are translocated back out to the extracellular milieu. Interaction of substrates with P-gp is considered to involve two stages, substrate partitioning into the membrane and substrate interaction with P-gp binding sites.[43] Substrate partitioning into the cell membrane has been proposed to be the rate-limiting step for the interaction with the P-gp binding domain within the lipid membrane.[44] An increase in the partition coefficients of P-gp substrates has been demonstrated to lead to an increase in P-gp binding.[45] Although a higher degree of lipophilicity of the substrate facilitates membrane penetration and accessibility at the P-gp binding site, the spatial arrangement and type of electron donor or hydrogen bonding acceptor groups within the substrate structure play an important role in determining the strength of the association with P-gp.[46] Increasing the number and type of donor and acceptor groups was associated with a stronger association with P-gp.[46] In comparison, structural features of R-6G and R-123 suggest a similar type and number of donor and acceptor groups capable of interacting with P-gp (Figure 1). However, the additional ethyl and methyl groups on R-6G may result in steric hindrance that could reduce its binding or association with P-gp, resulting in a lower concentration of diblock required to inhibit P-gp mediated efflux compared to the higher concentration of diblock required for R-123.

Recent structural analysis of surfactants by Seelig et al. has demonstrated that surfactants with a high degree of hydrogen-bonding acceptor groups have a greater potential to directly bind with P-gp.[47] However, the mechanism(s) by which detergent-like molecules cause enhanced accumulation of P-gp substrates may depend on numerous factors, including direct binding to P-gp (either at the membrane surface or following membrane penetration), secondary fluidity effect and potentially P-gp-ATPase modulation. For example Triton X-100 is reported to penetrate membranes, induce fluidity

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changes, increase ATPase activity (like Verapamil) and bind to P-gp.[47] Since MePEG-*b*-PCL used in this study has been shown to increase P-gp ATPase activity and penetrate membranes (and presumably directly bind to P-gp), it is likely that these diblock copolymers may also modulate P-gp activity by multiple mechanisms.[21] Considering that the hydrophobic block of MePEG-*b*-PCL is a polyester and was previously demonstrated to associate into cell membranes, it is plausible that MePEG-*b*-PCL diblock copolymers may interact with P-gp and alter the binding affinity of substrates.[21] Therefore, the differences in the potency for enhanced accumulation of P-gp substrates by MePEG-*b*-PCL diblock copolymers may result, in part at least, from the different binding affinities of the substrates for P-gp and the resulting concentration of diblock required to competitively inhibit substrate binding to P-gp.

In conclusion, the effective concentration range for surfactant mediated inhibition of P-gp appears to depend on the P-gp substrate hydrophobicity. As the concentration of the surfactant molecules increases above the CMC, P-gp substrates with a greater degree of association with the micelles will become sequestered and unavailable for accumulation in cells. This effect may explain the reduction in cellular accumulation enhancement activity observed at concentrations above the CMC of the surfactant or diblock. In addition, the different potency for accumulation enhancement by diblock and surfactants with P-gp substrates varying in lipophilicity suggests that surfactants may alter the binding affinities of substrates by directly interacting with P-gp.

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