ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 54 (2002) 299–309

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Enhanced cellular accumulation of a P-glycoprotein substrate, rhodamine-123, by caco-2 cells using low molecular weight methoxypolyethylene glycol-*block*-polycaprolactone diblock copolymers

Jason Zastre[a], John Jackson[a], Mandeep Bajwa[a], Richard Liggins[b], Famida Iqbal[a], Helen Burt[a,*]

[a]*Faculty of Pharmaceutical Sciences, Vancouver, BC, Canada*
[b]*Angiotech Pharmaceuticals Inc., 6660 NW Marine Drive, Vancouver, BC V6T 1Z4, Canada*

Received 1 September 2001; accepted in revised form 1 August 2002

Abstract

A series of diblock copolymers based on methoxypolyethylene glycol-block-poly(caprolactone) (MePEG-*b*-PCL) was synthesized and evaluated for enhancing the cellular accumulation of a P-glycoprotein (P-gp) substrate, rhodamine-123 (R-123), into caco-2 cells. Altering MePEG:caprolactone feed weight ratio allowed diblocks with varying PCL lengths to be synthesized onto MePEG of molecular weight 750 or 2000. The critical micelle concentration (CMC) and the hydrophilic-lipophilic balance all decreased with increasing degree of polymerization of PCL. R-123 accumulation by caco-2 cells increased to a maximum in the presence of increasing concentrations of MePEG-*b*-PCL diblock copolymers (compared to R-123 alone) beginning at concentrations at or above the CMC, with little or no R-123 accumulation enhancement observed below the CMC. Further increases in MePEG-*b*-PCL concentration resulted in a decrease in R-123 uptake back to baseline levels. It is suggested that the higher concentrations of diblock above the CMC were required to serve as a 'depot' for free unimer partitioning into the cell membrane in order to obtain a critical concentration of diblock in the membrane for P-gp modulation. Alternatively, MePEG-*b*-PCL micelles may increase R-123 accumulation via endocytosis of micellized R-123.

Keywords: Diblock copolymers; P-glycoprotein; Critical micelle concentration; Micelles

1. Introduction

The action of intestinal efflux transport proteins, namely P-glycoprotein (P-gp), can serve as an absorption barrier to limit the oral availability of hydrophobic drugs from the gastrointestinal tract [1,2]. Inhibition of P-gp can be achieved using modulators or chemosensitizers that inhibit drug efflux from inside the cell leading to increased drug permeability. A group of compounds found to inhibit P-gp mediated drug efflux and improve intestinal permeation are natural or synthetic fatty acid ester based surfactants. Non-ionic polyethoxylated surfactants such as the polysorbates, solutol, polyethylene glycol – fatty acid diesters, and others have demonstrated reversal of P-gp mediated drug efflux [3–6]. The majority of these compounds are pharmaceutically acceptable components in many parenteral and enteral formulations, utilized as solubilizing or stabilizing excipients. Several groups have demonstrated the potential of using surfactants to block the efflux of drugs susceptible to P-gp in the intestine. Lo et al. demonstrated greater intracellular uptake of epirubicin by caco-2 monolayers in the presence of acacia or polysorbate surfactants compared to either epirubicin alone or with the P-gp inhibitors verapamil and trifluoperizine [7]. Dintaman et al. showed that an amphiphilic derivative of D-α-tocopheryl with polyethylene glycol inhibited P-gp mediated efflux of paclitaxel and the fluorescent probe rhodamine-123 (R-123) from caco-2 cells [8].

Another group of compounds called amphiphilic block copolymers have been increasingly utilized in drug delivery. Typical amphiphilic block copolymers have an A-B diblock or A-B-A/B-A-B triblock structure, where the A is the hydrophilic block such as polyethylene glycol and the B is the hydrophobic block such as poly(D,L-lactide). In an aqueous environment, amphiphilic block copolymers can form micelles with the hydrophobic block forming the core surrounded by a shell of hydrophilic polymer. Polymeric micelles have been utilized for the solubilization and delivery of drugs such as paclitaxel, indomethacin, amphotericin B, adriamycin, and dihydrotestosterone [9–14]. When used for drug delivery, the core forming block is

* Corresponding author. Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: +1-604-822-2440; fax: +1-604-822-3035.
E-mail address: burt@interchange.ubc.ca (H. Burt).

0939-6411/02/$ - see front matter
PII: S0939-6411(02)00119-4

generally composed of biodegradable polymers such as polycaprolactone, poly(β-benzyl L-asparate) and poly(D,L-lactide). Compared to conventional fatty acid ester surfactant based micelles, polymeric surfactant micelles have lower critical micelle concentrations (CMC) and slowly dissociate into unimers when diluted below the CMC [15,16]. This enhanced thermodynamic and kinetic stability of the micelles is a function of the glass transition temperature of the core forming block, the hydrophobic-hydrophilic block ratio, the block architecture, and the presence of solubilizates in the core [17]. Recently A-B-A type amphiphilic block copolymers of poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) also known as Pluronic block copolymers, have been shown to enhance cellular accumulation, membrane permeability, and to modulate multidrug resistance of numerous P-gp substrates [18–24]. Using R-123 as a P-gp substrate, Batrakova et al. demonstrated that Pluronic block copolymers could enhance caco-2 cell accumulation of R-123 at concentrations below the CMC of the Pluronics [20]. In contrast, at concentrations above the CMC, the Pluronic block copolymers were found to cause an increased efflux of R-123 from caco-2 cells [20]. The activity of Pluronics was also shown to be dependent on the hydrophilic-lipophilic balance (HLB), with the most potent Pluronics being those with intermediate hydrophobicity [23]. This limited concentration range (below the CMC) in which Pluronics modulate P-gp efflux may potentially be a disadvantage in terms of formulating and solubilizing hydrophobic drugs.

Several groups have reported the synthesis and characterization of amphiphilic diblock copolymers based on methoxypolyethylene glycol (MePEG) with the polyester, polycaprolactone (PCL) abbreviated MePEG-*b*-PCL [11,12,25,26]. These groups developed diblocks based on high mw MePEG (2000–5000) with large blocks of PCL in the range of 2000–15 000. Larger hydrophobic blocks of PCL form larger micelles with greater drug solubilization capability and slower drug release [11]. To extend this molecular weight (MW) range we have synthesized a series of low molecular weight MePEG-*b*-PCL diblock copolymers. To our knowledge, no studies have been carried out to determine whether amphiphilic diblock copolymers, such as MePEG-*b*-PCL, possess an ability to modulate P-gp mediated efflux of drugs. The objective of this work was to synthesize and characterize a series of low molecular weight MePEG-*b*-PCL diblock copolymers and evaluate their effect on the cellular accumulation of the model P-gp substrate, R-123, in caco-2 cells.

2. Materials and methods

2.1. Materials

MePEG of MW 750 and 2000 and stannous octoate were from Sigma-Aldrich (St. Louis, MO). ϵ-Caprolactone was obtained from Aldrich (Milwaukee, WI). HPLC grade chloroform and dichloromethane (DCM) were from Fisher Scientific (Nepean, ON).

Cell culture media, supplements and Hank's balanced salt solution without phenol red (HBSS) were from Life Technologies (Grand Island, NY). Culture flasks and plates were from Corning-Costar (Cambridge, MA). R-123, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (Hepes), verapamil, cyclosporin A (CSA), 1,6-diphenyl-1,3,5-hexatriene (DPH), and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO). Pluronic P85 was provided by BASF (Parispany, NJ).

2.2. Synthesis of MePEG-b-PCL diblock copolymers

Copolymers of MePEG and PCL were prepared as follows. MePEG with MWs of 750 and 2000 were combined with ϵ-caprolactone in varying weight ratios to control the final MW of the copolymer. The total weight of the two reagents was 50 g. The reagents were placed in a round bottom flask sealed with a ground glass stopper and immersed in a heavy mineral oil bath heated to 140°C. The temperature was controlled using a Dyna-Sense MK-1 controller (Scientific Instruments Inc., Skokie, IL). The reagents were stirred using a teflon coated magnetic stir bar. After the reagents were mixed for 30 min to produce a homogeneous liquid, 0.15 ml of stannous octoate was added to the flask. The polymerization reaction was allowed to proceed for 6 h. Cooling the polymer to room temperature terminated the reaction and no further purification steps were performed on the product since nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) analysis demonstrated no detectable unreacted monomers or NMR homopolymer.

2.3. Characterization of diblock copolymers

Proton NMR spectra of copolymers were obtained using a 10% w/v solution of copolymer in deuterated chloroform from Cambridge Isotope Laboratories (Andover, MD) with a frequency of 200 MHz on a Bruker AC-200 NMR instrument. The spectral data were analyzed in terms of peak position and integrated area using PC-compatible Nuts Lite software (version 19990728).

Copolymer MW was measured by GPC against polyethylene glycol standards in the range of 670–118 400 g/mol. The mobile phase was HPLC grade chloroform flowing at 1 ml/min through two PLgel 10^3 Å columns (Agilent). Sample elution was detected by an HP model 1047 A refractive index detector with a cell temperature of 40°C.

The HLB was calculated based on the Griffin equation for a series of polyethoxylated amphiphilic compounds [25]:

$$\mathrm{HLB} = 20\left[\frac{M_H}{M_L + M_H}\right] \quad (1)$$

Where, M_H = MW of the hydrophilic block (MePEG) and M_L = MW of the lipophilic block (PCL).

The CMC for each diblock copolymer was determined by fluorescence spectroscopy using the hydrophobic probe DPH, as previously described [27]. Increasing diblock copolymer concentrations in HBSS containing 10 mM hepes were incubated at 37°C with 10 μM DPH for 24 h. Aliquots (200 μl) of each sample were then placed into a 96 well flat bottom plate and the fluorescence intensity measured at $\lambda_{EX} = 360/40$ and $\lambda_{EM} = 460/40$ (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA). The CMC was determined to be the intercept from the linear regression of the upper and lower asymptotes of the curve.

2.4. Preparation of diblock copolymer solutions

Thin film hydration was used to prepare diblock copolymer solutions. A known amount of diblock copolymer was placed into a glass test tube and dissolved in 1–2 ml of DCM. The test tube was placed in an evaporation apparatus at 45°C with a N_2 purge for 30 min. The sample was then placed in a vacuum oven operating at 25 inHg vacuum at room temperature overnight to remove residual DCM. The resulting diblock copolymer film was hydrated with prewarmed (37°C) HBSS containing 10 mM hepes (assay buffer). Diblock copolymer solutions were serially diluted and incubated with R-123 for 1 h at 37°C prior to their use in cell culture or fraction bound experiments.

2.5. Caco-2 cell culture

The human colon adenocarcinoma cell line, caco-2, was obtained from ATCC (Rockville, MD) as passage 17. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C and maintained in Dulbecco's modified eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Upon 80–90% confluency, cells were split using 0.25% trypsin plus 1 mM EDTA. Cells were seeded into 48 or 96 well flat bottom plates at a density of 40 000 cells/cm^2 and grown in the plates for a minimum of 14 days and used for experimentation between days 14 and 21. Media was changed every 2nd day and only passages 25–45 were used for experiments.

2.6. Cellular accumulation of rhodamine-123

Caco-2 cells grown in 48 well flat bottom plates were washed with HBSS containing 10 mM hepes (assay buffer) and pre-incubated in assay buffer at 37°C for 15 min. The buffer was then removed and cells exposed to 5.0 μM R-123 in either assay buffer alone or in solutions of diblock copolymers. Standard P-gp inhibitors verapamil and cyclosporin A were used as positive controls and the optimum P-gp inhibition concentrations were found to be 50 and 4.0 μM, respectively, similar to other reports [20,28]. The cells were incubated for various times at 37°C, and the plate then placed on ice. The cells were washed with ice cold phosphate buffered saline followed by the addition of 1% Triton X-100 to solubilize the cells. Cellular debris was removed by centrifugation at high speed for 5 min. Aliquots (50 μl) of the supernatant were used for determining the cellular fluorescent intensity of R-123 using a fluorescence microplate reader (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA) with $\lambda_{EX} = 485/20$ and $\lambda_{EM} = 530/25$. R-123 cellular accumulation was normalized with respect to total protein content using 10 μl aliquots of the supernatant for quantitation of total protein using the BCA method (BCA protein assay kit, Pierce, Rockford, IL).

2.7. LDH release

Lactate dehydrogenase (LDH) release was measured using an LDH assay kit (CytoTox 96, Promega, Madison, WI). Briefly, caco-2 cells grown in flat bottom 96 well plates were washed with assay buffer and pre-incubated for 15 min at 37°C. Serially diluted concentrations of MePEG-*b*-PCL diblock copolymers or Pluronic P85 in assay buffer were added to wells with assay buffer alone used for control and maximum release groups. Plates were incubated at 37°C for 90 min. Triton X-100 was added to provide maximum LDH release. The plate was spun at 1500 rpm at 4°C and an aliquot of supernatant taken to analyze for LDH using reagents and methods provided by the kit manufacturer.

2.8. Association of R-123 with MePEG-b-PCL micelles

In a micellar solution a solute can partition into the hydrophobic core and can equilibrate between the micelle core and the aqueous milieu. The total amount of solute (St) in the system is therefore given by:

$$St = Sf + Sm \tag{2}$$

Where Sf is amount free or unbound and Sm is the amount micellized or bound. The unbound fraction of solute (Fu) is given by:

$$Fu = \frac{Sf}{Sm + Sf} \tag{3}$$

Equilibrium dialysis was performed to evaluate the fraction of R-123 bound within MePEG-*b*-PCL micelles. A 5 ml equilibrium dialysis cell from Bel-Art Products (Pequannock, NJ) was used with a Spectra/Por membrane with MW cut off of 1000 obtained from Spectrum Laboratories (Rancho Dominguez, CA). Initial experimentation evaluated the time to equilibrium using 5.0 μM R-123 in assay buffer placed in the donor compartment with an equal volume of assay buffer placed in receiver side. The dialysis cell was placed at 37°C in an Innova 4000 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 100 rpm. Aliquots where taken from both the donor and receiver side at various time points. The fluorescent intensity of R-123 was measured using a fluorescence microplate reader (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA) with $\lambda_{EX} = 485/20$ and $\lambda_{EM} = 530/25$ and R-123 was

quantified using a standard curve. Once the time to equilibrium was determined for R-123 alone, all sampling for MePEG-*b*-PCL with R-123 was done after this point to ensure equilibrium had been attained. All experimental procedures for 5.0 μM R-123 with diblock copolymer solutions were carried out as described above and the samples were prepared as described in Section 2.4.

3. Results

3.1. Synthesis and characterization of MePEG-b-PCL diblock copolymers

A series of diblock copolymers based on MePEG with MWs of 750 and 2000 were synthesized by ring opening polymerization. The basic structure of the synthesized MePEG-*b*-PCL diblock copolymers and a representative ^{1}H-NMR spectrum with the proton assignments is shown in Fig. 1. The peaks used to establish the degree of polymerization for the PCL block are the peaks at 3.6 ppm (B) from the methylene protons of the oxyethylene group of MePEG and the triplet at 2.2 (C) from the methylene protons of the caprolactone repeat unit. A shift in the cyclic caprolactone monomer peaks to positions C, D, and E was observed by comparing the NMR of the caprolactone monomer to the reacted MePEG-*b*-PCL diblocks supporting polymerization of the caprolactone monomer. The appearance of the B′ peak in the diblock NMR demonstrates that polycaprolactone is attached to the MePEG. The copolymer nomenclature used describes the degree of polymerization (DPN) in subscript behind each block, where $MePEG_{17}$-*b*-PCL_5 has 17 repeat units of ethylene glycol and 5 units of caprolactone. Altering feed weight ratios of MePEG to caprolactone monomer produced diblock copolymers with varying PCL lengths (Table 1) with the monomer to initiator (M/I) ratio determined by NMR agreeing closely with the theoretical M/I ratio.

GPC results demonstrated a single peak for each diblock synthesized which shifted away from the MePEG monomer suggesting an increase in MW. No peaks were seen for caprolactone monomer or homopolymer. The MW determined by GPC was very close to the calculated theoretical MW. Furthermore, the polydispersity index (PDI) was close to 1, signifying a narrow polymer MW distribution. The HLB of the diblocks decreased with increasing PCL length and increased from 12 to 18 by changing MePEG 750 to 2000 with a similar PCL length. Higher HLB values indicate more hydrophilic amphiphiles and lower HLB values indicate more lipophilic amphiphiles. The CMC were deter-

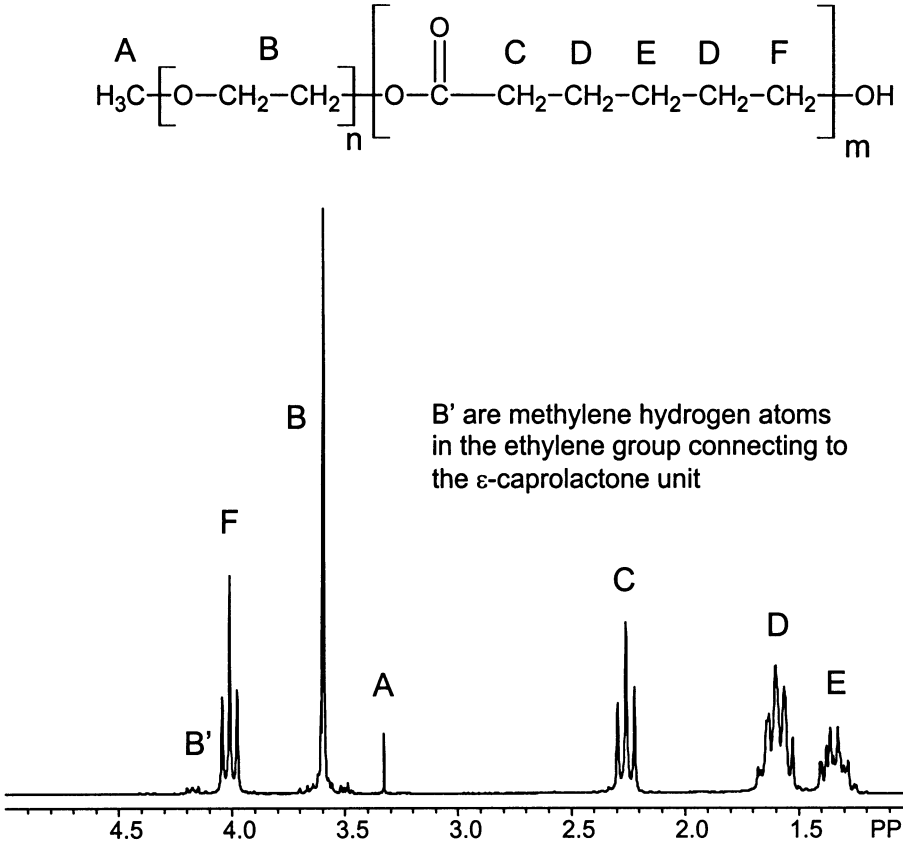

Fig. 1. Structure of methoxypolyethylene glycol-*block*-poly(ϵ-caprolactone) (MePEG-*b*-PCL) diblock copolymers and a representative ^{1}H-NMR spectrum. n = number of ethylene groups, being 17 and 45 for MePEG 750 and 2000, respectively. m = number of caprolactone repeat units.

Table 1
Characterization data for synthesized methoxypolyethylene glycol-*block*-poly(caprolactone) (MePEG-*b*-PCL) diblock copolymers based on MePEG of MW 750 and 2000

Series	Feed ratio[a]	Theo. M/I[b]	NMR M/I[c]	DPN[d]	Theo. MW[e]	MW[f]	PDI[g]	HLB	CMC (% w/v)
MePEG 750	80:20	1.64	1.75	$MePEG_{17}$-*b*-PCL_2	937	951	1.09	16.6	0.29–0.32
	60:40	4.38	4.65	$MePEG_{17}$-*b*-PCL_5	1250	1225	1.10	12.2	0.028–0.034
	40:60	9.86	10.1	$MePEG_{17}$-*b*-PCL_{10}	1875	1887	1.10	8.4	0.0009–0.0011
MePEG 2000	80:20	4.38	4.47	$MePEG_{45}$-*b*-PCL_4	2500	2400	1.11	18.3	0.06–0.072

[a] Feed weight ratio of MePEG:caprolactone.
[b] Theoretical monomer:initiator (M/I) based on feed weight ratio. Where M = mols of caprolactone and I = mols of MePEG.
[c] Monomer:initiator ratio determined by NMR. Calculated by comparing the relative peak areas of caprolactone at 2.2 ppm (equivalent to two protons) to the MePEG peak at 3.6 ppm (equivalent to 68 protons).
[d] DPN of MePEG = MW_{Mepeg}/44, DPN of PCL = rounded off M/I determined by NMR.
[e] Theoretical MW based on the feed weight ratio, e.g. MePEG 750 at a feed ratio of 80:20 will have 80% of the final MW = 750 g/mol. Therefore, 100/80 = X/750 X = 937 g/mol.
[f] Average number MW (Mn) determined by GPC.
[g] Polydispersity index, determined from GPC. PDI = Mw/Mn.

mined by fluorescence intensity using DPH as a probe. The fluorescence intensity of DPH increased rapidly above the CMC of the copolymers (Fig. 2). The CMC values for the MePEG 750 series and MePEG 2000 diblock copolymers are given in Table 1. The CMC of the MePEG 750 series decreased with increasing PCL chain length.

3.2. Effect of diblock copolymer composition and concentration on R-123 accumulation in caco-2 cells

As shown in Figs. 3A–D, for all diblock copolymers, as the concentration of MePEG-*b*-PCL increased, R-123 accumulation increased up to a critical concentration, after which the accumulation began to decrease. Furthermore, the accumulation was greater than or equal to the standard P-gp inhibitors, verapamil and CSA over a wide range of diblock concentrations. All four MePEG-*b*-PCL diblock copolymers showed peak effects at concentrations 4–100 times higher than their respective CMC. Table 2 lists the accumulation enhancement factor (AEF) at the peak effect for the four MePEG-*b*-PCL diblock copolymers. The AEF describes the relative increase in R-123 accumulation and is determined from the ratio of R-123 accumulation with diblock or P-gp inhibitors to R-123 alone. AEF values for

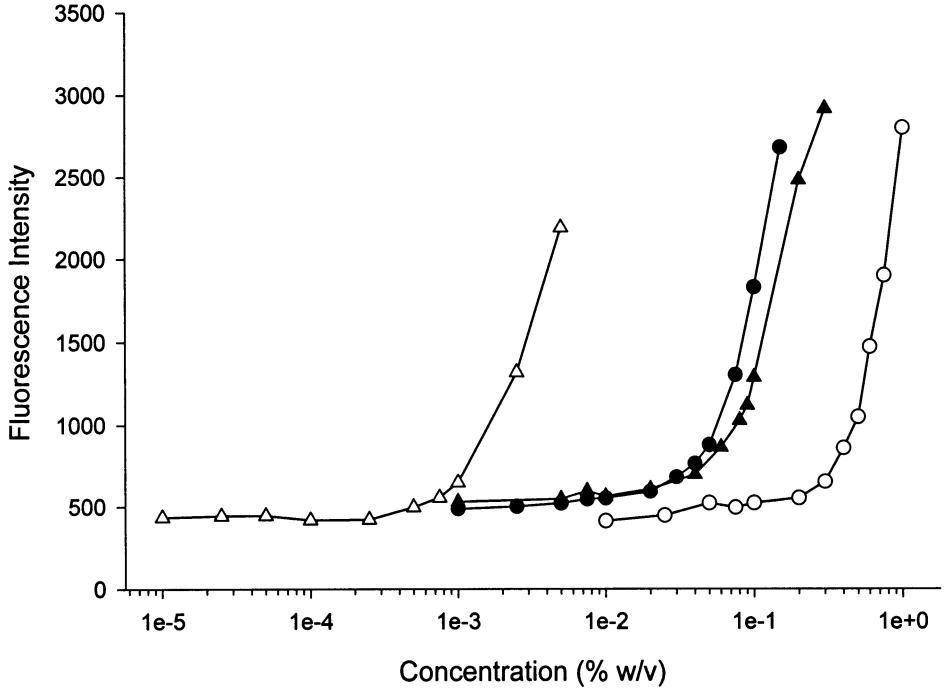

Fig. 2. Determination of critical micelle concentration (CMC) by fluorescence intensity at 37°C after incubating with DPH for 24 h. $MePEG_{17}$-*b*-PCL_2 (○), $MePEG_{17}$-*b*-PCL_5 (●), $MePEG_{17}$-*b*-PCL_{10} (△), and $MePEG_{45}$-*b*-PCL_4 (▲).

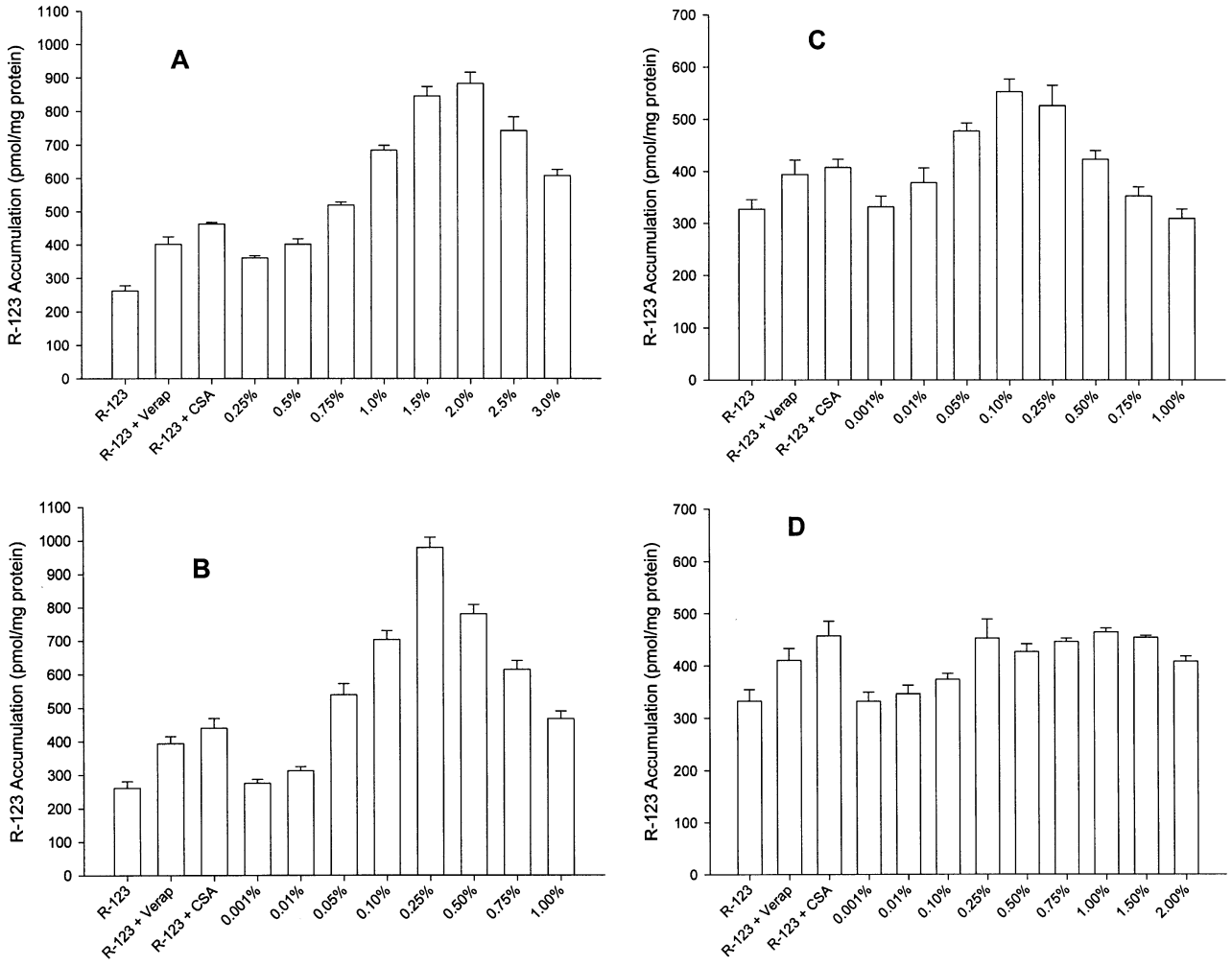

Fig. 3. Effect of MePEG-*b*-PCL composition and concentration on cellular accumulation of R-123 by caco-2 cells at 37^{0}C for 90 min. (A) $MePEG_{17}$-*b*-PCL_2; (B) $MePEG_{17}$-*b*-PCL_5; (C) $MePEG_{17}$-*b*-PCL_{10}; and (D) $MePEG_{45}$-*b*-PCL_4. Data expressed as the mean ± SEM of three independent experiments ($N = 3$).

verapamil and CSA were 1.5 and 1.7, respectively, compared to MePEG-*b*-PCL diblock copolymers, which ranged from 1.7 to 3.8. For the MePEG 750 series, an increase in the PCL chain length from 2 to 5 repeat units had no effect on the AEF (Table 2). But an increase in the length of the PCL block from 5 to 10 markedly reduced the AEF from 3.8 to 1.9. Furthermore, increasing the MW of MePEG from 750 to 2000 (with similar PCL length) resulted in a decrease from 3.8 to 1.7. As a control, R-123 accumulation was evaluated with MePEG 750 to determine whether the observed AEF was a function of MePEG effects alone. MePEG 750 as high as 3% w/v had no effect on R-123 accumulation (data not shown).

Table 2
Accumulation enhancement factor (AEF) of MePEG-*b*-PCL diblock copolymers at concentrations producing maximum R-123 accumulation by caco-2 cells after 90 min incubation at 37°C

Enhancer	Peak Conc. (% w/v)	AEF[a]
Verapamil	N/a	1.5
Cyclosporin A	N/a	1.7
$Mepeg_{17}$-*b*-PCL_2	2.00	3.4
$Mepeg_{17}$-*b*-PCL_5	0.25	3.8
$Mepeg_{17}$-*b*-PCL_{10}	0.10	1.9
$Mepeg_{45}$-*b*-PCL_4	1.00	1.7

[a] AEF determined as the ratio of R-123 accumulation with MePEG-*b*-PCL diblocks or P-gp inhibitors to R-123 alone.

3.3. Time dependent accumulation of R-123

The time dependence of R-123 accumulation was assessed over 3 h with or without diblock copolymers at peak effect concentrations (Fig. 4). For $MePEG_{17}$-b-PCL_2 and $MePEG_{17}$-b-PCL_5 diblock copolymers, there was a large and rapid increase in R-123 accumulation compared to R-123 alone over the first 15 min, which then increased at a slower rate over the next 2–3 h.

3.4. Cytotoxicity of MePEG-b-PCL diblock copolymers

The release of cytoplasmic LDH induced by MePEG-*b*-PCL diblock copolymers and pluronic P85 from caco-2 cells is shown in Fig. 5. Pluronic P85 caused lysis and subsequent

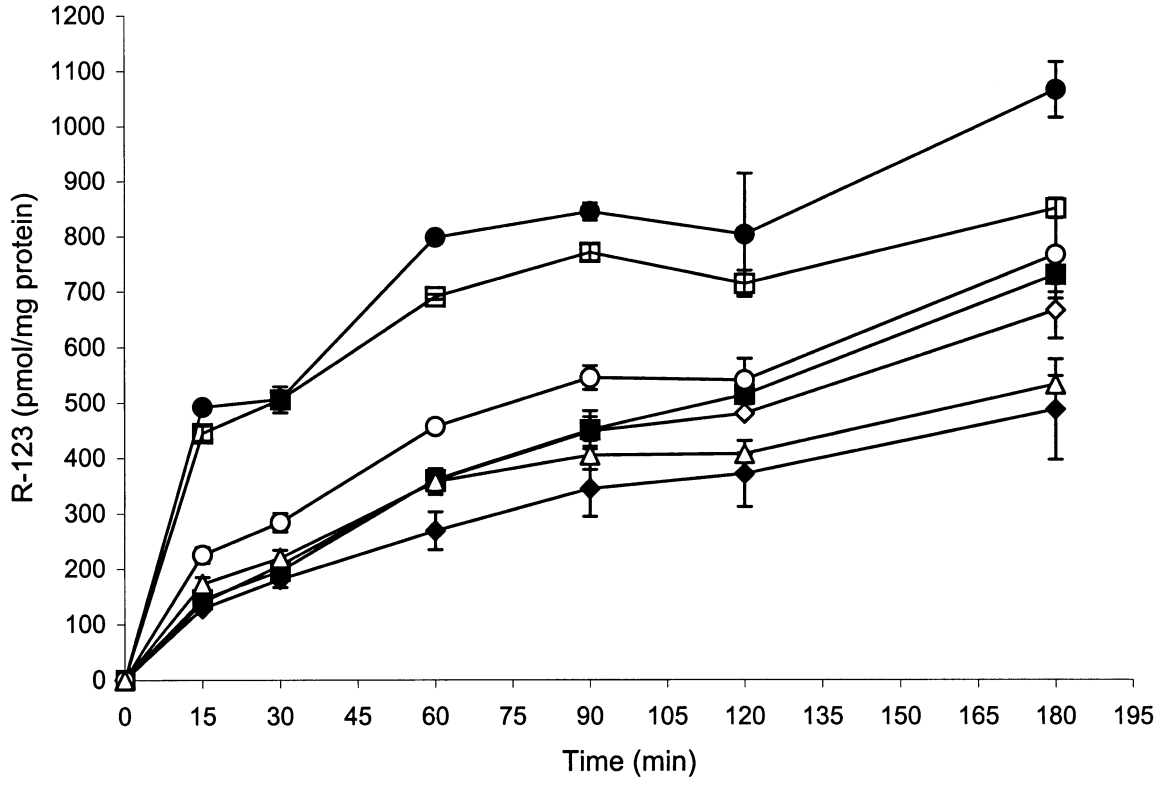

Fig. 4. The time dependent accumulation of R-123 by caco-2 cells. Caco-2 were incubated at 37°C with 5.0 μM R-123 as control (◆), 50 μM verapamil (◇), 4.0 μM CSA (■), 2.0% w/v $MePEG_{17}$-*b*-PCL_2 (□), 0.25% w/v $MePEG_{17}$-*b*-PCL_5 (●), 0.1% w/v $MePEG_{17}$-*b*-PCL_{10} (○), and 1.0% w/v $MePEG_{45}$-*b*-PCL_4 (△). Data expressed as the mean ± SEM of three independent experiments ($N = 3$).

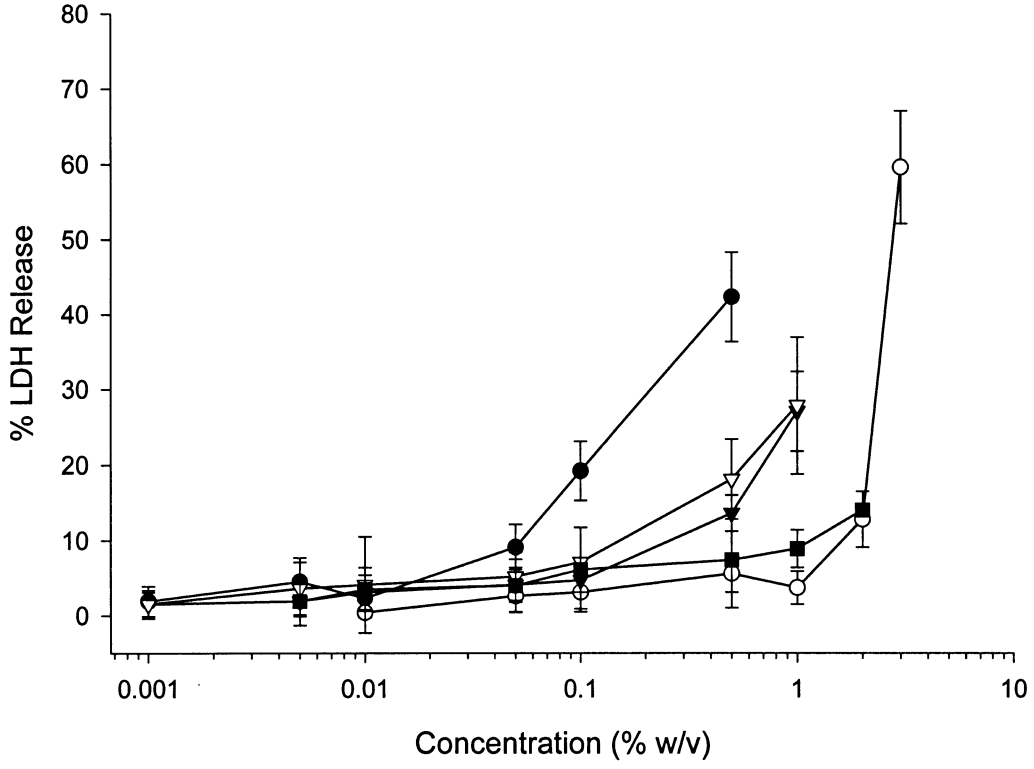

Fig. 5. LDH release from caco-2 cells induced by varying concentrations of pluronic P85 (●), $MePEG_{17}$-*b*-PCL_2 (○), $MePEG_{17}$-*b*-PCL_5 (▼), $MePEG_{17}$-*b*-PCL_{10} (▽), and $MePEG_{45}$-*b*-PCL_4 (■). Incubation was for 1.5 h at 37°C. Data expressed as the mean ± SEM of three independent experiments ($N = 3$).

release of LDH from caco-2 at concentrations above 0.1% w/v. MePEG-*b*-PCL diblock copolymers caused LDH release at concentrations above 0.5% w/v for $MePEG_{17}$-*b*-PCL_5 and $MePEG_{17}$-*b*-PCL_{10}. However, $MePEG_{45}$-*b*-PCL_4 did not induce LDH release up to 2.0% w/v and $MePEG_{17}$-*b*-PCL_2 resulted in less than 10% release at 2.0% w/v, but caused 60% release at 3.0% w/v.

3.5. The fraction of R-123 unbound in MePEG-b-PCL micelle solutions

Using equilibrium dialysis, the unbound R-123 fraction (Fu) with increasing concentrations of MePEG-b-PCL diblock copolymer was determined (Fig. 6). A decrease in the R-123 unbound fraction was observed with increasing diblock concentration for all three diblocks tested. Approximately 80% of the R-123 in solution was not associated with micelles at diblock concentrations of 2.0, 0.25, and 0.1% for $MePEG_{17}$-b-PCL_2, $MePEG_{17}$-b-PCL_5, and $MePEG_{17}$-b-PCL_{10}, respectively which corresponded to the peak in R-123 accumulation concentration (Figs. 3a–c). Further decreases in the unbound fraction were observed as the diblock concentration was increased, correlating with the drop in R-123 accumulation as the diblock concentration was increased beyond the peak activity concentrations.

4. Discussion

MePEG-*b*-PCL diblock copolymers were synthesized by ring opening polymerization of ϵ-caprolactone, initiated by the free hydroxyl on MePEG with stannous octoate used as a catalyst to increase the rate of polymerization. This type of ring opening polymerization to form MePEG-polyester diblock copolymers typically goes almost to completion and gives high yields [9,10]. The DPN of PCL as determined by NMR closely corresponded to the theoretical M/I ratio (Table 1). Increasing PCL length resulted in more hydrophobic amphiphiles as seen with decreasing HLB values for the MePEG 750 series. MePEG-*b*-PCL diblock copolymers could be dissolved in water using the thin film hydration method. $MePEG_{17}$-b-PCL_2 and $MePEG_{45}$-b-PCL_4 were the most hydrophilic copolymers and could be dissolved up to concentrations of 3 and 2% w/v, respectively. $MePEG_{17}$-b-PCL_5 and $MePEG_{17}$-b-PCL_{10} were significantly more hydrophobic and could not be dissolved at concentrations greater than approximately 1% w/v. The CMC for the MePEG 750 series decreased as the DPN of PCL increased and the hydrophobicity of the diblock increased. The CMC inflexion points in Fig. 2 were not well defined since the reported DPN is an average and the copolymer may contain larger or smaller PCL blocks attached to MePEG. Therefore, linear regression was performed with varying points to provide a range of CMC values.

As seen in Fig. 3, MePEG-*b*-PCL diblock copolymers increased R-123 accumulation in caco-2 cells at concentrations above the CMC. For all diblock copolymers, R-123 accumulation enhancement began close to the CMC and increased to a maximum above the CMC, after which accumulation decreased with further increases in copolymer

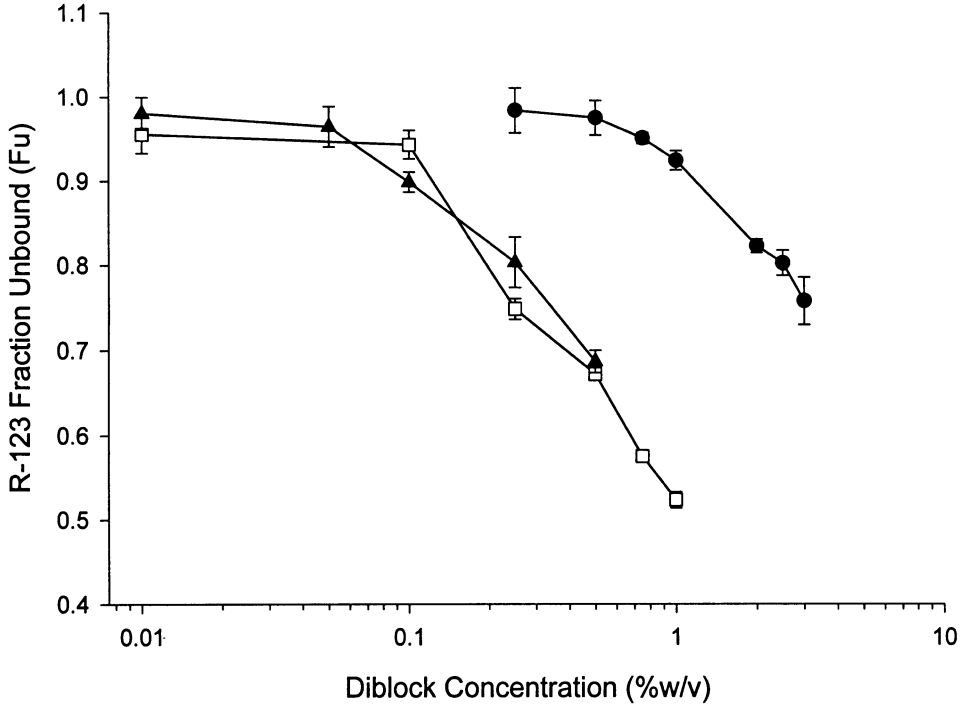

Fig. 6. Unbound fraction of R-123 with increasing concentrations of (●) $MePEG_{17}$-*b*-PCL_2 (□) $MePEG_{17}$-*b*-PCL_5 and (▲) $MePEG_{17}$-*b*-PCL_{10}. Data expressed as the mean ± SD with $N = 3$.

concentration. R-123 accumulation in caco-2 cells in the presence of pluronic triblock copolymers, showed a similar pattern of increasing R-123 uptake up to a maximum followed by a decrease [20]. However, for all pluronics studied, accumulation of R-123 reached maximum levels in the proximity of the CMC and higher pluronic concentrations resulted in a drop in accumulation [20,23]. Similarly, work by Nerurkar et al demonstrated that the apical to basolateral (AP → BL) transport of a model peptide susceptible to P-gp efflux could be enhanced using polysorbate 80 or cremophor EL [6,29]. The AP → BL transport increased at concentrations of polysorbate 80 or cremophor EL below the CMC and reached a maximum in the vicinity of the CMC, after which AP → BL transport decreased with further increases in concentration [6,29]. Nerurkar et al concluded that since the transport increased up to and reached a maximum at the CMC, that the free unimers were responsible for inhibiting P-gp efflux transport [29].

Interestingly, the characteristics of R-123 accumulation in the presence of MePEG-*b*-PCL diblock copolymers in this work differed from the pattern of R-123 accumulation with triblock copolymers, and fatty acid based amphiphiles [20,29]. Maximum accumulation of R-123 for MePEG-*b*-PCL copolymers occurred at concentrations 4–100-fold higher than the corresponding CMC of the diblock copolymers, with almost no R-123 uptake below the CMC. If, as other groups have suggested, the free unimers are responsible for P-gp modulation, then partitioning of unimers into the cell membrane and the concentration of unimers in the membrane will be important parameters governing the modulation of P-gp activity. It is possible that below the CMC the MePEG-*b*-PCL diblock unimers may not have partitioned into the membrane at sufficient concentrations to inhibit P-gp activity. Recent work by Xia and Onyuksel demonstrated that for uptake into, and permeabilization of cell membranes, a surfactant must exhibit a surface pressure greater than 25 dyne/cm and surfactants with surface pressures lower than this threshold may not accumulate into the membrane at sufficient concentrations [30]. Surfactants were shown to achieve this threshold value below the CMC suggesting that micelles were not required to permeate or solubilize membranes [30]. Xia and Onyuksel concluded that penetration enhancement resulted from partitioning of the free surfactant unimers into membranes and that micelles were acting as a 'depot' for free unimer and maintaining the micelle/unimer equilibrium, as unimer is partitioned into the cell membrane [30].

Nerurkar et al. further suggested that the decrease in accumulation seen at high surfactant concentrations was due to a decreasing free drug fraction (bound drug being solubilized within micelles) as the concentration of surfactant increased above the CMC. Hence the amount of available drug for absorption (free fraction) decreased, resulting in a decrease in the cellular accumulation [29]. Since the concentration of R-123 is held constant and assuming that only the free or unbound R-123 fraction is able to enter the cell, then as the concentration of MePEG-b-PCL diblocks are increased, the bound fraction within the micelles will increase and the amount of R-123 available to enter the cell will decrease. Fig. 6 demonstrated that the R-123 unbound fraction decreased at high diblock concentrations along with a decrease in R-123 accumulation (Fig. 3). This suggests that a reduction in the free R-123 fraction available for absorption may be a significant factor in the decrease in cellular accumulation.

Cytotoxicity may further compound the decrease in R-123 accumulation observed after the peak R-123 accumulation concentrations, since all concentrations beyond peak R-123 accumulation concentrations induced LDH release (Fig. 5).

If free amphiphile unimers are responsible for attenuating P-gp drug efflux, how they accomplish this is not clearly understood. Recent work has shown that membrane fluidization by non-ionic surfactants and other fluidizers can reduce P-gp ATPase activity by altering the lipid order around P-gp [31,32]. Furthermore, amphiphiles have been shown to fluidize membranes resulting in enhanced passive diffusion and an increase membrane permeability of P-gp substrates [31,33]. This suggests that amphiphiles act on P-gp in a non-specific manner. Principally, surfactants are commonly used and evaluated as permeation enhancers to improve oral drug absorption by altering membrane integrity and facilitating increased drug transcellular and paracellular diffusion [34,35]. The alteration of P-gp ATPase activity may then be a secondary consequence of the membrane disorder facilitated by the amphiphiles and not the primary mechanism for enhanced permeability. Fig. 4 shows a first order rate of R-123 accumulation into caco-2 cells with or without diblock copolymers. Also, up to 15 min there is a rapid increase in R-123 accumulation using $MePEG_{17}$-*b*-PCL_2 and $MePEG_{17}$-*b*-PCL_5 diblock copolymers. This rapid influx rate may be due to initial membrane permeabilization causing increased R-123 diffusion that continues to follow a first order rate since R-123 concentration gradient is decreasing with time.

Non-P-gp substrate analogs such as Rhodamine 110 (R-110) have been used to assess the role of non-specific permeation enhancement [20]. R-110 accumulation was also evaluated in our model system and no enhancement was seen in the presence of the MePEG-*b*-PCL amphiphiles (data not shown). If increased probe accumulation were due at least in part to enhanced membrane permeability, then R-110 might be expected to result in an increase in accumulation, assuming a permeation enhancement effects of the amphiphiles. Since the structure of R-110 contains a quaternary nitrogen and an ionizable carboxylic acid group, at pH = 7.4, R-110 will carry two charges and has a reported octanol/water partition coefficient of 0.8 [36]. It has been suggested that these charges may hinder the transmembrane diffusion of R-110 compared to R-123 [36], even in the presence of membrane perturbation induced by the amphiphiles.

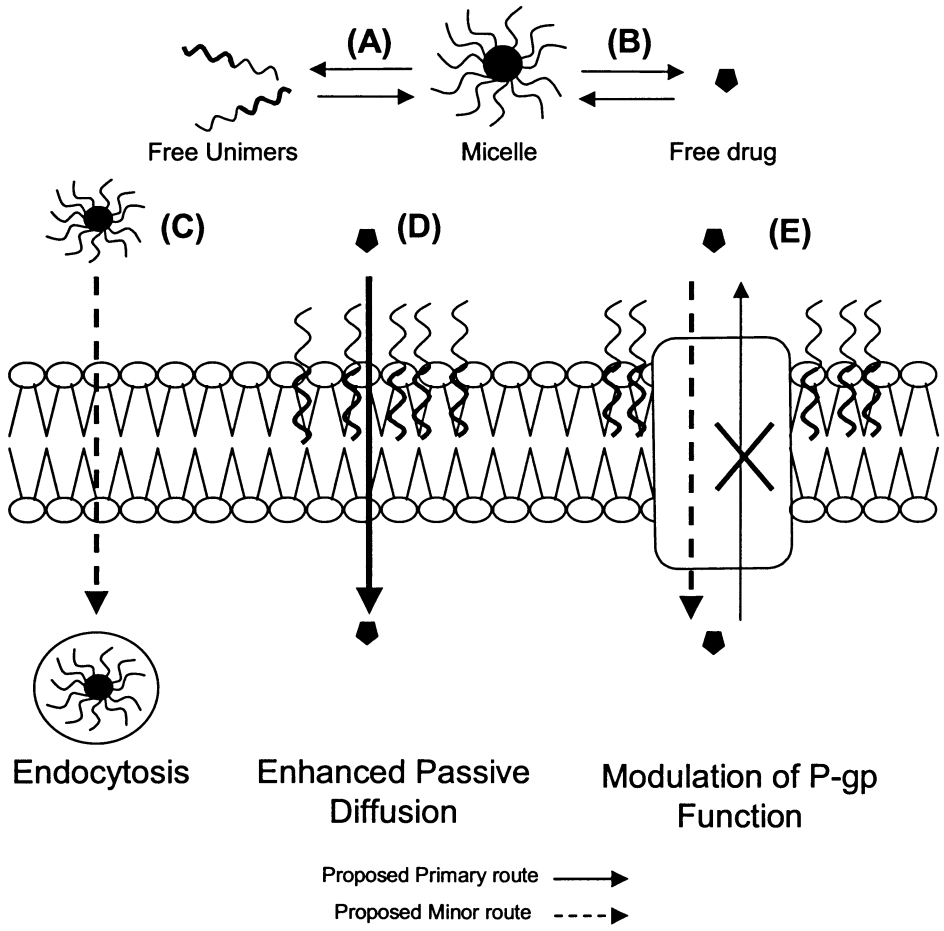

Fig. 7. Schematic representation of enhanced drug permeation of a P-gp substrate mediated by amphiphilic diblock copolymers. Free unimers of MePEG-*b*-PCL are shown with the block in bold face representing the hydrophobic PCL block. (A) Micelle depot for free unimer. (B) Micellization of free drug. (C) Endocytosis of micellized drug. (D) Insertion of free unimers into cell membrane facilitating enhanced passive diffusion of free drug. (E) Insertion of free unimers into cell membrane reducing P-gp ATPase activity resulting in a net accumulation into the cell.

In summary, we have presented the first evidence demonstrating the utility of MePEG-*b*-PCL diblock copolymers to increase the cellular accumulation of a P-gp substrate into caco-2 cells. R-123 accumulation was enhanced at concentrations of MePEG-*b*-PCL diblock copolymers above their CMC with little activity below the CMC and several mechanisms have been proposed and summarized in Fig. 7. Firstly, micelles may be acting as a 'depot' for free unimer partitioning into the membrane, such that a threshold concentration is reached for P-gp modulation (pathway E). Secondly, R-123 loaded micelles maybe be taken up via an endocytic pathway into the cells (pathway C) as demonstrated by Allen et al. [37] and Kabanov and coworkers [22]. Thirdly, fluidization of membranes mediated by free amphiphilic unimer may result in enhanced permeability of a P-gp substrate via enhanced transcellular and paracellular diffusion (pathway D). At high diblock copolymer concentrations R-123 accumulation decreased and is suggested to be most likely due to substantial partitioning of R-123 into micelles reducing free R-123 fraction available for cellular uptake. Cytotoxicity may further reduce R-123 accumulation at high diblock copolymer concentrations. Work is currently underway to assess the contributions of non-specific permeation and endocytosis of MePEG-*b*-PCL diblock copolymer accumulation enhancement of R-123.

Acknowledgements

These studies were financially supported by a Canadian Institutes of Health Research (CIHR) Operating Grant awarded to Helen Burt and by Angiotech Pharmaceuticals Inc. The authors would also like to thank the Science Council of British Columbia for awarding Jason Zastre with the GREAT scholarship.

References

[1] A. Sparreboom, J. van Asperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. van Tellingen, Limited oral bioavailability and active epithelial excretion of pacli-

taxel (Taxol) caused by P-glycoprotein in the intestine, Proc. Natl. Acad. Sci. USA 94 (1997) 2031–2035.
[2] J. Hunter, B.H. Hirst, Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption, Adv. Drug Del. Rev. 25 (1997) 129–157.
[3] L.E. Buckingham, M. Balasubramanian, A.R. Safa, H. Shah, P. Komarov, R.M. Emanuele, J.S. Coon, Reversal of multi-drug resistance in vitro by fatty acid-PEG-fatty acid diesters, Int. J. Cancer 65 (1996) 74–79.
[4] D.M. Woodcock, S. Jefferson, M.E. Linsenmeyer, P.J. Crowther, G.M. Chojnowski, B. Williams, I. Bertoncello, Reversal of the multidrug resistance phenotype with cremophor EL, a common vehicle for water-insoluble vitamins and drugs, Cancer Res. 50 (1990) 4199–4203.
[5] D.M. Woodcock, M.E. Linsenmeyer, G. Chojnowski, A.B. Kriegler, V. Nink, L.K. Webster, W.H. Sawyer, Reversal of multidrug resistance by surfactants, Br. J. Cancer 66 (1992) 62–68.
[6] M.M. Nerurkar, P.S. Burton, R.T. Borchardt, The use of surfactants to enhance the permeability of peptides through caco-2 cells by inhibition of an apically polarized efflux system, Pharm. Res. 13 (1996) 528–534.
[7] Y.L. Lo, C.Y. Hsu, J.D. Huang, Comparison of effects of surfactants with other MDR reversing agents on intracellular uptake of epirubicin in caco-2 cell line, Anticancer Res. 18 (1998) 3005–3009.
[8] J.M. Dintaman, J.A. Silverman, Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), Pharm. Res. 16 (1999) 1550–1556.
[9] X. Zhang, J.K. Jackson, H.M. Burt, Development of amphiphilic diblock copolymers as micellar carriers of taxol, Int. J. Pharm. 132 (1996) 195–206.
[10] H.M. Burt, X. Zhang, P. Toleikis, L. Embree, W.L. Hunter, Development of copolymers of poly(D,L-lactide) and methoxypolyethylene glycol as micellar carriers of paclitaxel, Colloids Surfaces B Biointerfaces 16 (1999) 161–171.
[11] S.Y. Kim, I.G. Shin, Y.M. Lee, C.S. Cho, Y.K. Sung, Methoxy poly(-ethylene glycol) and epsilon-caprolactone amphiphilic block copolymeric micelle containing indomethacin. II. Micelle formation and drug release behaviours, J. Controlled Release 51 (1998) 13–22.
[12] C. Allen, J. Han, Y. Yu, D. Maysinger, A. Eisenberg, Polycaprolactone-b-poly(ethylene oxide) copolymer micelles as a delivery vehicle for dihydrotestosterone, J. Controlled Release. 63 (2000) 275–286.
[13] B.G. Yu, T. Okano, K. Kataoka, G. Kwon, Polymeric micelles for drug delivery: solubilization and haemolytic activity of amphotericin B, J. Controlled Release 53 (1998) 131–136.
[14] M. Yokoyama, S. Fukushima, R. Uehara, K. Okamoto, K. Kataoka, Y. Sakurai, T. Okano, Characterization of physical entrapment and chemical conjugation of adriamycin in polymeric micelles and their design for in vivo delivery to a solid tumor, J. Controlled Release 50 (1998) 79–92.
[15] M. Jones, J. Leroux, Polymeric micelles – a new generation of colloidal drug carriers, Eur. J. Pharm. Biopharm. 48 (1999) 101–111.
[16] G.S. Kwon, T. Okano, Polymeric micelles as new drug carriers, Adv. Drug Del. Rev. 21 (1996) 107–116.
[17] C. Allen, D. Maysinger, A. Eisenberg, Nano-engineering block copolymer aggregates for drug delivery, Colloids Surfaces B Biointerfaces 16 (1999) 3–27.
[18] R. Paradis, C. Noel, M. Page, Use of Pluronic micelles to overcome multidrug resistance, Int. J. Oncol. 5 (1994) 1305–1308.
[19] V.Y. Alakhov, E.Y. Moskaleva, E.V. Batrakova, A.V. Kabanov, Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer, Bioconjug. Chem. 7 (1996) 209–216.
[20] E.V. Batrakova, H.Y. Han, V.Y.u. Alakhov, D.W. Miller, A.V. Kabanov, Effects of pluronic block copolymers on drug absorption in caco-2 cell monolayers, Pharm. Res. 15 (1998) 850–855.
[21] E.V. Batrakova, S. Li, D.W. Miller, A.V. Kabanov, Pluronic P85 increases permeability of a broad spectrum of drugs in polarized BBMEC and caco-2 cell monolayers, Pharm. Res. 16 (1999) 1366–1372.
[22] E.V. Batrakova, H.Y. Han, D.W. Miller, A.V. Kabanov, Effects of pluronic P85 unimers and micelles on drug permeability in polarized BBMEC and caco-2 cells, Pharm. Res. 15 (1998) 1525–1532.
[23] E. Batrakova, S. Lee, S. Li, A. Venne, V. Alakhov, A. Kabanov, Fundamental relationships between the composition of pluronic block copolymers and their hypersensitization effect in MDR cancer cells, Pharm. Res. 16 (1999) 1373–1379.
[24] D.W. Miller, E.V. Batrakova, T.O. Waltner, V.Y.u. Alakhov, A.V. Kabanov, Interactions of pluronic block copolymers with brain microvessel endothelial cells: evidence of two potential pathways for drug absorption, Bioconjug. Chem. 8 (1997) 649–657.
[25] I.G. Shin, S.Y. Kim, Y.M. Lee, C.S. Cho, Y.K. Sung, Methoxy poly(-ethylene glycol)/epsilon-caprolactone amphiphilic block copolymeric micelle containing indomethacin. I. Preparation and characterization, J. Controlled Release 51 (1998) 1–11.
[26] C. Allen, Y. Yu, D. Maysinger, A. Eisenberg, Polycaprolactone-b-poly(ethylene oxide) block copolymer micelles as a novel drug delivery vehicle for neurotrophic agents FK506 and L-685,818, Bioconjug. Chem. 9 (1998) 564–572.
[27] X. Zhang, J.K. Jackson, H.M. Burt, Determination of surfactant critical micelle concentration by a novel fluorescence depolarization technique, J. Biochem. Biophys. Methods 31 (1996) 145–150.
[28] R. Yumoto, T. Murakami, Y. Nakamoto, R. Hasegawa, J. Nagai, M. Takano, Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds, J. Pharmacol. Exp. Ther. 289 (1999) 149–155.
[29] M.M. Nerurkar, N.F. Ho, P.S. Burton, T.J. Vidmar, R.T. Borchardt, Mechanistic roles of neutral surfactants on concurrent polarized and passive membrane transport of a model peptide in caco-2 cells, J. Pharm. Sci. 86 (1997) 813–821.
[30] W.J. Xia, H. Onyuksel, Mechanistic studies on surfactant-induced membrane permeability enhancement, Pharm. Res. 17 (2000) 612–618.
[31] R. Regev, Y.G. Assaraf, G.D. Eytan, Membrane fluidization by ether, other anesthetics, and certain agents abolishes P-glycoprotein ATPase activity and modulates efflux from multidrug-resistant cells, Eur. J. Biochem. 259 (1999) 18–24.
[32] Y. Romsicki, F.J. Sharom, The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter, Biochemistry 38 (1999) 6887–6896.
[33] S. Drori, G.D. Eytan, Y.G. Assaraf, Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability, Eur. J. Biochem. 228 (1995) 1020–1029.
[34] E.S. Swenson, W.J. Curatolo, Means to enhance penetration. 2. Intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity, Adv. Drug Del. Rev. 8 (1992) 39–92.
[35] E.K. Anderberg, P. Artursson, Epithelial transport of drugs in cell culture. VIII: Effects of sodium dodecyl sulfate on cell membrane and tight junction permeability in human intestinal epithelial (caco-2) cells, J. Pharm. Sci. 82 (1993) 392–398.
[36] T.J. Lampidis, C. Castello, A. del Giglio, B.C. Pressman, P. Viallet, K.W. Trevorrow, G.K. Valet, H. Tapiero, N. Savaraj, Relevance of the chemical charge of rhodamine dyes to multiple drug resistance, Biochem. Pharmacol. 38 (1989) 4267–4271.
[37] C. Allen, Y. Yu, A. Eisenberg, D. Maysinger, Cellular internalization of PCL(20)-b-PEO(44) block copolymer micelles, Biochim. Biophys. Acta 1421 (1999) 32–38.