Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 309–319
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration

Jubo Liu, Faquan Zeng, Christine Allen *

Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ont., Canada

Received 29 June 2006; accepted in revised form 1 November 2006
Available online 23 November 2006

Abstract

Methoxy poly(ethylene glycol)-*b*-poly(caprolactone) (MePEG-*b*-PCL) copolymers with varying PEG block lengths and a constant PCL block length were synthesized by cationic ring-opening polymerization and used to form nano-sized micelles. Due to their small size and superior *in vitro* stability, the $MePEG_{5000}$-*b*-PCL_{5000} micelles were selected for further *in vitro* characterization and an *in vivo* evaluation of their fate and stability following intravenous (i.v.) administration. Specifically, $_3$H-labelled $MePEG_{5000}$-*b*-PCL_{5000} micelles were i.v. administered to Balb/C mice at copolymer doses of 250, 2 and 0.2 mg/kg in order to examine the distribution kinetics of (1) copolymer assembled as thermodynamically stable micelles, (2) copolymer assembled as thermodynamically unstable micelles and (3) copolymer unimers, respectively. Overall, it was found that when the copolymer is assembled as thermodynamically stable micelles the material is effectively restricted to the plasma compartment. Interestingly, the copolymer was found to have a relatively long circulation half-life even when administered at a dose that would likely fall to concentrations below the CMC following distribution. Analysis of plasma samples from this group revealed that even 24 h post-administration a significant portion of the copolymer remained assembled as intact micelles. In this way, this study demonstrates that the hydrophobic and semi-crystalline nature of the PCL core imparts a high degree of kinetic stability to this micelle system.

Keywords: Block copolymer micelle; Poly(ethylene glycol)-block-poly(caprolactone); Drug delivery; *In vivo* fate; Kinetic stability; Pharmacokinetics; Biodistribution

1. Introduction

In aqueous media, amphiphilic block copolymers self-assemble to form micelles when the copolymer concentration is at or above the critical micelle concentration (CMC). At concentrations below the CMC the copolymer is present in solution as unimers [1]. Hydrophobic (e.g. paclitaxel) or amphiphilic (e.g. amphotericin B) drugs may be incorporated into the copolymer micelles as a means to formulate or deliver these drugs [2–6]. These formulations have been studied quite extensively with many reports on the *in vivo* fate of the drug following i.v. administration in micelles [3,6–11]. However, only a few studies have examined the *in vivo* fate of the copolymer micelles [6,12–16] and unimers [6,13,15]. Specifically, Burt et al. studied the *in vivo* fate of paclitaxel and MePEG-*b*-poly(D,L-lactide) (MePEG-*b*-PDLLA) copolymers in rats following i.v. administration of a MePEG-*b*-PDLLA micelle formulation of this drug [6]. The *in vivo* pharmacokinetics and biodistribution for micelles prepared from tyrosine (Tyr)-PEG-*b*-PDLLA and tyrosyl-glutamic acid (Tyr-Glu)-PEG-*b*-PDLLA copolymers were also investigated by Kataoka's group [13].

To this point, the effect of the administered copolymer dose on the *in vivo* behavior of micelles as well as the fate of copolymer unimers and micelles remains relatively

* Corresponding author. Department of Pharmaceutical Sciences, University of Toronto, 19 Russell St., Toronto, Ont., Canada M5S 2S2. Tel.: +1 416 946 8594; fax: +1 416 978 8511.
E-mail address: cj.allen@utoronto.ca (C. Allen).

0939-6411/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2006.11.010

unexplored. One of the only studies that has examined the influence of copolymer dose on the distribution kinetics of block copolymers was recently reported by Kabanov's group [15]. Their study evaluated the pharmacokinetics and biodistribution of the Pluronic® triblock copolymer P85 following administration of copolymer concentrations ranging from below to above the CMC of the material after the dilution that occurs upon administration [15].

Additional studies on the *in vivo* behavior and fate of other copolymer unimer and micelle systems are necessary as they will ensure full exploitation of block copolymer micelles as a viable drug formulation strategy. In this study, MePEG-*b*-PCL copolymers of varying PEG block lengths (i.e. $M_n = 20{,}000$, 10,000 and 5000 g/mol) and a constant PCL block length (i.e. $M_n = 5000$ g/mol) were synthesized, characterized and the physico-chemical properties of micelles formed from this series of copolymers were evaluated. The $MePEG_{5000}$-*b*-PCL_{5000} micelle system was selected for an *in vivo* evaluation of copolymer fate and stability following i.v. administration due to its small size and superior *in vitro* stability. Specifically, the pharmacokinetics and biodistribution profiles of the copolymer were evaluated in mice following i.v. administration of three distinct doses of the copolymer.

2. Materials and methods

2.1. Materials

MePEGs ($M_n = 5000$, $M_w/M_n = 1.06$; $M_n = 10{,}000$, $M_w/M_n = 1.10$ and $M_n = 20{,}000$, $M_w/M_n = 1.10$ as determined by size-exclusion chromatography (SEC)) from Sigma–Aldrich (Oakville, ON, Canada) were dried twice by azeodistillation of toluene. The monomer, ε-caprolactone (CL), dichloromethane, and toluene were dried using calcium hydride and distilled prior to use. The tritium (^3H) radiolabelled compound acetyl chloride (CH_3COCl in dichloromethane) was obtained from America RadioLabelled Chemicals Inc. (St. Louis, MO) and used without further purification. All other chemicals were obtained from Sigma–Aldrich (Oakville, ON, Canada) and used as received.

2.2. Synthesis of MePEG-b-PCL copolymers

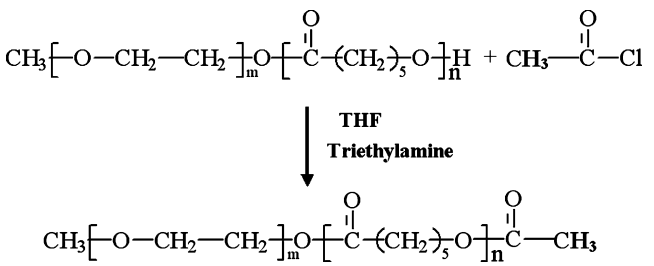
The block copolymer was synthesized using a metal-free cationic method [17]. A typical procedure for the synthesis of the copolymer was as follows: 1.0 g of MePEG (0.2 mmol, $M_n = 5000$, $M_w/M_n = 1.06$) was added to a flame-dried flask and dried twice by toluene azeodistillation. A 10 mL volume of dried dichloromethane and 1.0 g of CL (8.76 mmol, dried and distilled over calcium hydride) were then added to the flask. 0.6 mL (1 M, 0.6 mmol) of the catalyst and hydrogen chloride in diethyl ether was added to the mixture at 25 °C and the reactor was sealed using a rubber septum. The reaction mixture was maintained overnight with vigorous stirring at this temperature and terminated by the addition of 0.1 mL triethylamine. The precipitated triethylamine–HCl salt was removed by filtration and the copolymer was collected by precipitation in ether. The molecular weight of the PCL block was calculated from ^1H NMR and the known molecular weight of the MePEG precursor. ^1H NMR assignments were as follows: signals for the MePEG unit appeared at 3.38 ppm (3H, ***CH***$_3$–O–) and 3.60 ppm (4H, –O***CH***$_2$***CH***$_2$–); while, signals for the PCL block appeared at 1.35 ppm (2H, CO–CH_2–CH_2–***CH***$_2$–CH_2–CH_2–O), 1.55 ppm (4H, CO–CH_2–***CH***$_2$–CH_2–***CH***$_2$–CH_2–O), 2.28 ppm (2H, CO–***CH***$_2$–CH_2–CH_2–CH_2–CH_2–O), and 4.07 ppm (2H, CO–CH_2–CH_2–CH_2–CH_2–***CH***$_2$–O). The composition of the block copolymers was calculated by comparing the relative ratio of the PCL methylene proton signal at 4.07 ppm to the MePEG ethylene proton signal at 3.60 ppm [17].

2.3. Radiolabelling of MePEG-b-PCL copolymer

The radiolabelled block copolymer was synthesized by end-capping the terminus of the PCL block as shown in Scheme 1. Specifically, 10 mg of MePEG-*b*-PCL, that had previously been dried by toluene azeodistillation, was dissolved ($M_n = 10{,}000$ of 5000-*b*-5000 for MePEG-*b*-PCL, Table 1, entry 3) in 1.0 mL of dried THF and charged into a flame-dried glass tube reactor. The triethylamine in THF (1 mg) and 1 mCi of ^3H-labelled acetyl chloride were then added and left overnight at room temperature. The solvent was removed under vacuum. The triethylamine–HCl salt and unreacted acetyl chloride and its derivatives were removed by dialysis (Molecular weight cut-off, MWCO = 1000) against water (Spectrum Laboratories Inc., Dominguez, CA) and the remaining copolymer was dried by lyophilization.

2.4. Characterization of MePEG-b-PCL copolymers

^1H NMR spectra were obtained using a Mercury 400 spectrometer (400 MHz for ^1H, MR Resources Inc., Fitchburg, MA) with $CDCl_3$ as solvent. Chemical shifts were reported in ppm with $CHCl_3$ as the internal standard. SEC measurements were carried out at room temperature using a Waters 590 liquid chromatography system equipped with three Waters Styragel HR 4E columns and a 410 differ-



Scheme 1. The ^3H-labelled $MePEG_{5000}$-*b*-PCL_{5000} copolymer was prepared using an end-capping method. The three tritium atoms in the methyl group are shown in bold.

Table 1
Summary of properties of MePEG-*b*-PCL copolymers and micelles

Exp.		$M_{n,cal}$[a]	$M_{n,SEC}$[b]	$M_{n,NMR}$[c]	M_w/M_n	CMC (±SD) (μM)	CMC (±SD) (mg/L)	Micelle size (±SD) (nm)
1	$MePEG_{20000}$-*b*-PCL_{5000}	25,000	46,500	24,800	1.04	12.0 ± 0.3	299 ± 9	115 ± 13
2	$MePEG_{10000}$-*b*-PCL_{5000}	15,000	27,800	14,600	1.06	7.6 ± 1.4	110 ± 20	124 ± 8
3	$MePEG_{5000}$-*b*-PCL_{5000}	10,000	12,900	9800	1.10	3.2 ± 0.4	38 ± 6	56 ± 6
4	$MePEG_{5000}$-*b*-$AcePCL_{5000}$	10,000	12,900	9800	1.10			49 ± 2

[a] $M_{n,cal} = M_{n,PEG} + M_{n,PCL}$; $M_{n,PCL}$ is the calculated molecular weight of the PCL block based on the feed ratio of caprolactone to MePEG.
[b] $M_{n,SEC}$ is the relative molecular weight as determined by SEC with respect to poly(styrene) standards.
[c] $M_{n,NMR} = M_{n,PEG} + M_{n,PCL}$; $M_{n,PCL}$ was calculated from ^{1}H NMR analysis.

ential refractometer as the detector (Waters Inc., Milford, MA). THF with 1% triethylamine was used as the mobile phase at a flow rate of 1.0 mL/min at 40 °C. Polystyrene standards (Polysciences Inc., Warrington, PA) were used to obtain the calibration curve. The data obtained were recorded and manipulated using the Windows-based Millenium 2.0 software package (Waters Inc., Milford, MA).

2.5. Measurement of critical micelle concentration

The critical micelle concentrations of the MePEG-*b*-PCL copolymers were determined using an established fluorescence-based method [18]. In short, aliquots of MePEG-*b*-PCL stock solutions in chloroform were added to glass vials such that the concentration of copolymer ranged from 0.1 to 200 mg/L. An aliquot of a hydrophobic probe, DPH (1,6-diphenyl-1,3,5-hexatriene) stock solution was then added to each vial such that the concentration of DPH was maintained at 1 mg/L in each solution. The solutions were stirred vigorously for 4 h and the solvent was evaporated under nitrogen. The dried vials were heated to 60 °C and 1 mL of double distilled water was added slowly to each vial. The solutions were equilibrated by stirring overnight at room temperature followed by measurement of the fluorescence emission at 430 nm with excitation at 350 nm (Spectra GeminiXS dual-scanning microplate spectrofluorometer, Molecular Devices, Sunnyvale, CA).

2.6. Preparation of micelles

Aliquots of stock solutions of block copolymers prepared in DMF were added to vials, dried under nitrogen at room temperature and left overnight under vacuum. PBS (0.01 M, pH 7.4) warmed to 60 °C was then added to rehydrate each copolymer film. The solutions were vortexed, stirred for 72 h at room temperature and sonicated for 2 h prior to use.

^{3}H-labelled micelles were prepared from a mixture of the ^{3}H-labelled $MePEG_{5000}$-*b*-PCL_{5000} and the unlabelled $MePEG_{5000}$-*b*-PCL_{5000} copolymer.

2.7. Thermal analysis of micelles

Thermal analysis of each block copolymer micelle system was performed on a 5–10 mg sample of micelles as lyophilized dry powder using a Q100 differential scanning calorimeter (DSC) (TA Instruments, Inc., New Castle, DE). The samples were cooled to −70 °C using a refrigerated cooling system and then heated to 80 °C at a rate of 5 °C/min. The data were analyzed using TA universal software (TA Instruments, Inc., New Castle, DE) and the melting temperature (T_m) was taken to be the peak of the endotherm.

2.8. Characterization of micelle size and morphology

The hydrodynamic diameter of micelles was determined by dynamic light scattering (DLS) at an angle of 90° and temperature of 25 °C (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation; Holtsville, NY). For analysis the samples were diluted to a copolymer concentration of 2 mg/mL using filtered double distilled water. The morphology of the micelles was evaluated by transmission electron microscopy (TEM) with a Hitachi 7000 microscope operating at an acceleration voltage of 75 kV (Schaumburg, IL). The micelle solutions were diluted in PBS buffer and negatively stained using phosphotungstic acid. The sample solutions were deposited on copper grids that had been pre-coated with carbon and negatively charged.

2.9. Evaluation of in vitro stability of micelles

The stability of the block copolymer micelles was evaluated in both the absence and presence of physiologically relevant concentrations of bovine serum albumin (BSA). Specifically, the micelle solutions were mixed with equal volumes of PBS (0.01 M) in the absence and presence of BSA (45 g/L) and incubated at 37 °C. At various time points, 50 μL aliquots of the solutions were removed and analyzed by DLS for size measurement ($n = 3$).

2.10. In vitro evaluation of protein copolymer interactions

The interaction between the $MePEG_{5000}$-*b*-PCL_{5000} copolymer unimers and plasma protein was investigated by incubating copolymer with mouse plasma or PBS at 37 °C for 5 h at copolymer concentrations that were 10-fold below the CMC. The mixtures were then run through a gel filtration chromatography (GFC) column. Specifically 200 μL samples of plasma were run through a Superose 6 gel filtration column (Sigma, Oakville, ON, Canada) with

a flow rate of 0.1 mL/min and 0.01 M PBS (pH 7.4) as the mobile phase. Two hundred microliter fractions were collected from the column and the concentration of copolymer in each fraction was determined by direct scintillation counting (LS 6500 Liquid Scintillation Counter, Mississauga, ON, Canada). The copolymer elution profiles following incubation with plasma or PBS were compared.

2.11. Assessment of in vivo stability of radiolabelled micelles

A method based on GFC was employed in order to evaluate the *in vivo* stability of the $MePEG_{5000}$-*b*-PCL_{5000} micelles in plasma following i.v. administration [13,19,20]. Specifically, the radiolabelled micelle solutions were administered to healthy Balb/C mice (average weight = 20 g) at total copolymer doses of 250 and 2 mg/kg. At specific time points (i.e. 1 h and 24 h following i.v. administration), plasma was collected and immediately run through a GFC column as described in Section 2.10. Samples of block copolymer micelles and unimers in PBS were also run through the column as controls.

2.12. Evaluation of the pharmacokinetics profile and biodistribution pattern of block copolymer unimers and micelles following i.v. administration

The *in vivo* fate of the block copolymer, including the pharmacokinetics profile and biodistribution, was investigated following i.v. administration of three different doses of copolymer. Studies were performed using female Balb/C mice of 8–10 weeks of age with body weights ranging from 16 to 20 g. The mice were housed 4 per cage under standard conditions with access to food and water *ad libitum*. All protocols for these studies were in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and were approved by the Animal Care Committee at the University of Toronto. The block copolymer micelles (^{3}H-labelled) were i.v. injected into the mice via the lateral tail vein. The doses of copolymer administered were 250, 2 and 0.2 mg/kg in order to examine the distribution kinetics of (1) copolymer assembled as thermodynamically stable micelles, (2) copolymer assembled as thermodynamically unstable micelles and (3) copolymer unimers, respectively. At 10 min, 1, 2, 3, 6, 8, 11, 24 and 48 h post-injection the animals were sacrificed ($n = 3$) by cervical dislocation and blood as well as tissue samples (i.e. heart, lung, kidney, liver and spleen) were collected. Blood samples were immediately centrifuged at 1600*g* for 10 min to obtain plasma. The concentration of block copolymer in plasma was measured directly by liquid scintillation counting.

The heart, lung, kidney, liver and spleen samples were washed with PBS to remove excess blood and weighed. The tissue samples were then placed in excess PBS (tissue:PBS = 1:3 (wt:wt)) and homogenized. One hundred and fifty microliter aliquots of the homogenized samples were added to 0.5 mL of Soluene® (Perkin-Elmer Life and Analytical Sciences Inc., Wellesley, MA) and the mixture was incubated at 50 °C overnight. After cooling to room temperature, 50 μL EDTA (200 mM), 200 μL hydrogen peroxide (30%) and 25 μL HCl (10 N) were added. The mixture was then incubated for 1 h at room temperature and 10 mL of scintillation cocktail was added. The samples were analyzed by scintillation counting following 24-h incubation at room temperature. The total area under the plasma concentration–time curve (AUC) and the total area under the first-moment curve (AUMC) for the copolymers were calculated by the linear trapezoidal rule.

2.13. Compartmental modeling and data fitting

Compartmental open models were used for fitting the data from the *in vivo* studies. The computer software Scientist® version 2 (MicroMath Inc., Salt Lake City, UT) was employed for all fitting and simulations. The model selection criterion (MSC) was used as the parameter to determine the optimal model [21].

2.14. Statistical analyses

Statistical analyses of the data were performed using the unpaired, two-tailed, Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Synthesis and characterization of MePEG-b-PCL and radiolabelled MePEG-b-PCL block copolymer

The block copolymers of MePEG-*b*-PCL were synthesized by a metal-free cationic polymerization method with HCl–ether as the catalyst [17]. MePEG was able to initiate CL polymerization in the presence of HCl and as confirmed by SEC analysis little unreacted residual MePEG remained following the procedure. As summarized in Table 1, the copolymers have been found to have the predicted compositions and relatively narrow molecular weight distributions (i.e. $M_w/M_n = 1.04$–1.10). The radiolabelled $MePEG_{5000}$-*b*-PCL_{5000} was synthesized by an end-capping method as shown in Scheme 1. The ^{3}H-labelled acetyl chloride was reacted with the hydroxyl group at the end of the PCL block of the $MePEG_{5000}$-*b*-PCL_{5000} copolymer. Due to the low molecular weight of this radioactive compound as well as the hydroxyl functional group, the labelling of the copolymer does not result in a significant change in the overall chemical composition of the material.

3.2. Determination of critical micelle concentrations of MePEG-b-PCL copolymers

As listed in Table 1, the CMC values for $MePEG_{20000}$-*b*-PCL_{5000}, $MePEG_{10000}$-*b*-PCL_{5000} and $MePEG_{5000}$-*b*-PCL_{5000} copolymers were found to be 299 ± 9 mg/L,

110 ± 20 mg/L and 38 ± 6 mg/L, respectively. The observed trend agrees well with previous findings in that the CMC increases with an increase in the hydrophilic block length of the copolymer, when the length of the hydrophobic block is held constant [22].

3.3. Micelle preparation and characterization

A dry-down method was used to prepare micelles from the three MePEG-*b*-PCL copolymers. Following hydration of the copolymer films in PBS, sonication was employed as a means to reduce the size distribution of the micelles. The size and size distribution of the micelles were measured by DLS and are provided in Table 1. The $MePEG_{5000}$-*b*-PCL_{5000} copolymer was found to form the smallest sized micelles with an effective mean diameter of approximately 56 ± 6 nm ($n = 3$). A significant increase in the viscosity of the solution was observed for the micelle solutions prepared from copolymers having the higher PEG block lengths. Specifically, the $MePEG_{20000}$-*b*-PCL_{5000} micelle solution was gel-like; while, the $MePEG_{10000}$-*b*-PCL_{5000} solution was found to be more viscous than the $MePEG_{5000}$-*b*-PCL_{5000} micelle solution, with all solutions having copolymer concentrations of 50 mg/mL. DLS measurements revealed that all micelle solutions had a monomodal size distribution. The morphology of the particles formed from $MePEG_{5000}$-*b*-PCL_{5000} was also investigated by TEM. As shown in Fig. 1, the electron microscopic analysis revealed a spherical morphology with a relatively uniform size distribution for the micelles.

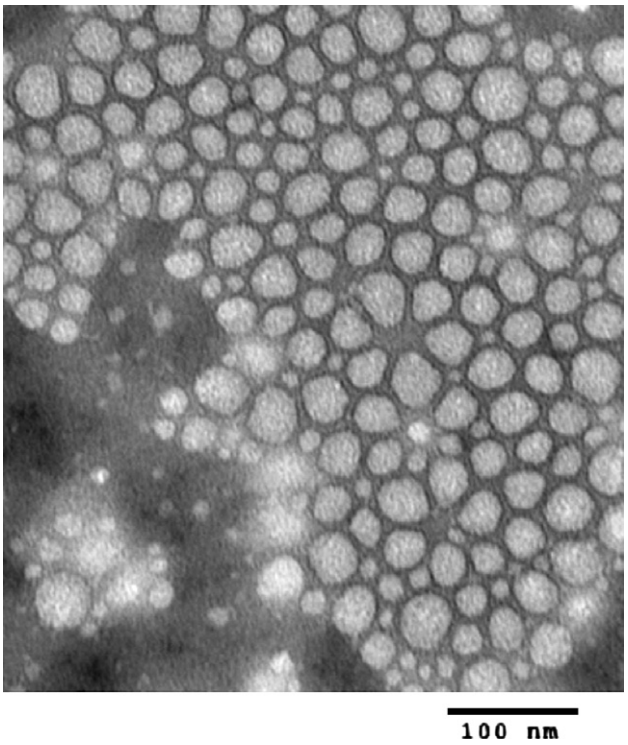


Fig. 1. Transmission electron micrograph of micelles formed from the $MePEG_{5000}$-*b*-PCL_{5000} copolymer.

3.4. Thermal analysis of micelles

DSC measurements were performed to investigate the thermal properties of the MePEG-*b*-PCL micelles (data not shown). The thermograms for all copolymer micelles included a single, symmetrical endotherm that peaks at 61, 58 and 55 °C for $MePEG_{20000}$-*b*-PCL_{5000}, $MePEG_{10000}$-*b*-PCL_{5000} and $MePEG_{5000}$-*b*-PCL_{5000}, respectively. PCL and PEG are both known to be semi-crystalline polymers [1]. The T_m for the MePEG homopolymers were found to be 65, 63 and 62 °C for molecular weights 20,000, 10,000 and 5000, respectively. The T_m for PCL homopolymer of a similar molecular weight has been reported to be between 52 and 60 °C [23]. The single endotherms in the thermograms are the result of the melting of both the PCL and PEG blocks. As expected, as the PEG content within the copolymer is decreased, the T_m for the copolymer approaches the T_m of PCL homopolymer.

3.5. In vitro stability of micelles

The *in vitro* stability of the MePEG-*b*-PCL micelles at 37 °C was investigated in the absence and presence of physiologically relevant concentrations of BSA (45 g/L). The size and size distribution of the micelles in buffer (±BSA) were evaluated via DLS measurements for periods of up to 240 h (i.e. 10 days). The measurements were terminated prior to 240 h if the intensity of a second population increased to more than 40% of the total intensity as this indicates the significant presence of a multi-modal size distribution. As shown in Fig. 2, micelles formed from the $MePEG_{5000}$-*b*-PCL_{5000} copolymer retained their size and a monomodal size distribution over the 240 h incubation period in both the absence (Fig. 2A) and presence (Fig. 2B) of BSA. The values obtained for the hydrodynamic diameters of the $MePEG_{5000}$-*b*-PCL_{5000} copolymer micelles in the absence and presence of BSA were compared by the Student's *t*-test and the differences in the diameters were found to be insignificant ($P > 0.05$) at all time points. However, significant size variations were observed for the micelles formed from the $MePEG_{20000}$-*b*-PCL_{5000} and $MePEG_{10000}$-*b*-PCL_{5000} copolymers, both in the absence and presence of BSA. In addition, the micelle solution formed from the $MePEG_{20000}$-*b*-PCL_{5000} copolymer at a concentration of 50 mg/mL was observed to form a gel upon incubation at 37 °C.

3.6. Interaction of copolymer with plasma protein

The GFC elution profile for copolymer that had been incubated with total plasma was found to be the same as that for copolymer incubated with PBS. The retention time for the copolymer remained unchanged demonstrating that there are no significant interactions between protein and copolymer (data not shown).

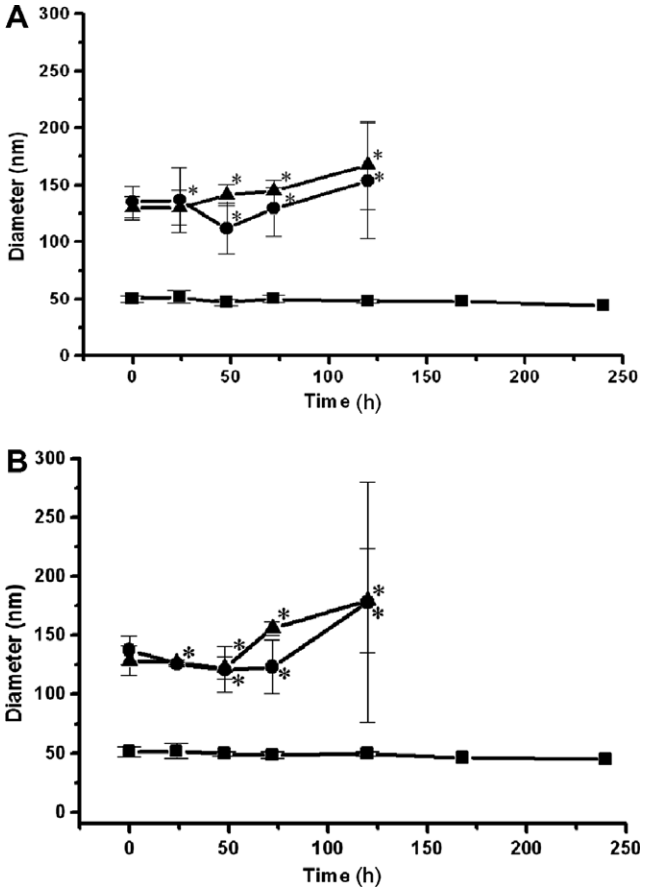
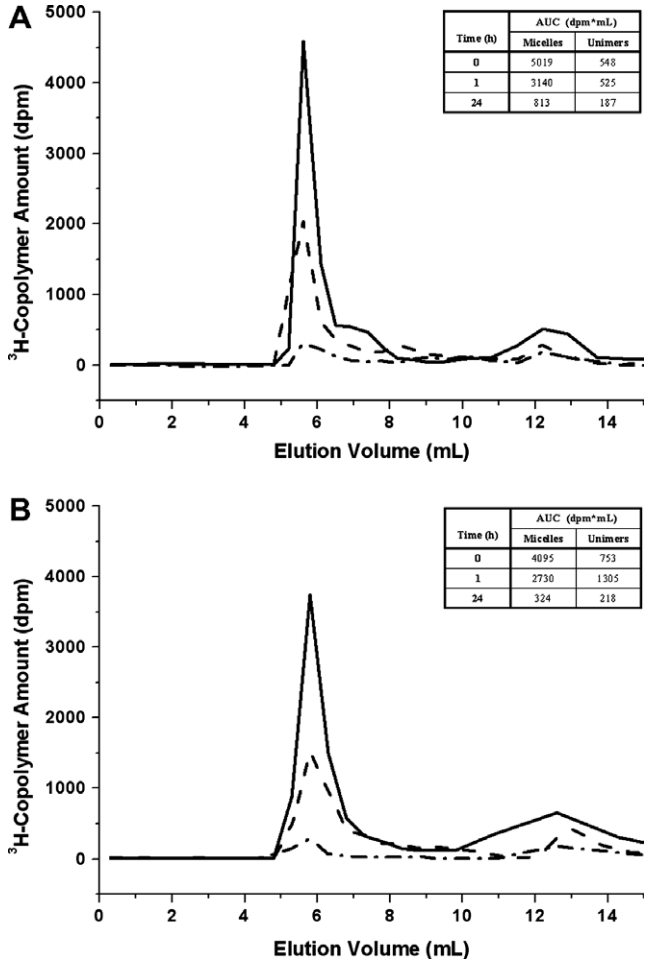


Fig. 2. Mean diameters of micelles formed from MePEG-*b*-PCL copolymers (–●– $MePEG_{20000}$-*b*-PCL_{5000}, –▲– $MePEG_{10000}$-*b*-PCL_{5000}, –■– $MePEG_{5000}$-*b*-PCL_{5000}) in the absence (A) and presence (B) of physiologically relevant concentrations of BSA (45 g/L) (SD shown as error bars). *Signifies that more than one size population has been observed from DLS measurements. Only the mean diameter of the major population is shown on the graph.



Time (h)	AUC (dpm*mL)	
	Micelles	Unimers
0	5019	548
1	3140	525
24	813	187

Time (h)	AUC (dpm*mL)	
	Micelles	Unimers
0	4095	753
1	2730	1305
24	324	218

Fig. 3. GFC chromatograms for plasma samples obtained from mice 1 h (dashed line) and 24 h (dashed-dotted line) following administration of (A) 250 mg/kg or (B) 2 mg/kg copolymer. $MePEG_{5000}$-*b*-PCL_{5000} micelles prior to injection are shown as the solid line. The area under the curve (AUC) for each peak is provided in the inset.

3.7. *In vivo* stability of $MePEG_{5000}$-*b*-PCL_{5000} micelles

The elution volumes for micelles and copolymer unimers had previously been determined to be 5–7 mL and 11–14 mL, respectively. The recovery of total radioactivity from the column for each sample was found to be greater than 92%. This high recovery rate confirms that the cleavage of the ^{3}H label from the copolymer occurs to an insignificant extent in mouse plasma for periods of up to 24 h.

Fig. 3A includes the GFC elution profiles for a plasma sample collected 1 and 24 h post-administration of a copolymer dose of 250 mg/kg. If it is assumed that following administration, the 250 mg/kg copolymer dose is completely diluted in the entire blood volume, then the resulting copolymer concentration would be 70-fold above the CMC of the copolymer (i.e. 38 mg/L). As shown in Fig. 3A, each profile includes a peak eluting at volumes ranging from 5 to 7 mL, confirming the presence of intact micelles in the plasma at both time points. The radioactivity collected in the elution fractions that correspond to the micelle population for both the 1 and 24 h samples was found to be 78% and 74%, respectively, of the total radioactivity administered onto the GFC column. A similar elution profile was obtained for the plasma samples collected from mice administered a 2 mg/kg dose of copolymer (Fig. 3B). In this case, with the same assumption as above, the resulting copolymer concentration would be 2-fold below the CMC of the copolymer. The amount of copolymer collected in the fractions that correspond to intact micelles was 59% and 55% of the total copolymer administered, for the 1 and 24 h samples, respectively.

3.8. Pharmacokinetics profile of block copolymer unimers and micelles following i.v. administration

The plasma clearance profiles for $MePEG_{5000}$-*b*-PCL_{5000} micelles at doses of 250, 2 and 0.2 mg/kg were investigated for periods up to 48 h following i.v. administration. Compartmental fits of the data from both the individual experiments and averaged plasma clearance profiles ($n = 3$) were performed using the Scientist® software. For all doses considered in this study, the fits obtained for individual

experiments were found to provide similar mean values as those obtained from the fits of averaged data (data not shown).

When the copolymer was administered at a dose of 0.2 mg/kg, a very rapid elimination profile from plasma was observed. As shown in Fig. 4, at 10 min, 1 h, 3 h and 6 h, 12.7%, 10.7%, 9.9% and 7.6% of the total injected dose, respectively, remained in the plasma. These values were compared with those from the other two dose groups using the Student's *t*-test and the differences were found to be statistically significant at all time points ($P < 0.01$). The fitting of the plasma clearance data obtained at this dose revealed a one-compartment elimination profile and therefore a one-compartment model was employed to calculate the main pharmacokinetic parameters (MSC = 5.70). The calculated parameters are summarized in Table 2 including an elimination constant of $0.070 \pm 0.003\ h^{-1}$ and volume of distribution of 7.6 ± 0.2 mL.

In contrast, as also shown in Fig. 4, the plasma elimination profiles for the copolymer following i.v. administration of doses of 250 and 2 mg/kg had two-compartment profiles and thus were fit using a two-compartment model. Good fits were obtained using this model as indicated by MSC values of 3.59 and 5.30 for the 250 and 2 mg/kg dose groups, respectively (shown in Fig. 4 as solid lines). The elimination constants for both the central and peripheral compartments (i.e. k_{10}, k_{12}, k_{21} and V_1) for both doses were obtained directly from the fits. Other critical pharmacokinetic parameters were calculated based on these constants and are summarized in Table 2. Overall, the copolymer was found to have a relatively fast distribution phase (0–2 h) followed by a much slower elimination phase (2–48 h). A comparison, using the Student's *t*-test, of the elimination phases for the copolymer when administered at 2 mg/kg versus a dose of 250 mg/kg revealed that the copolymer as thermodynamically unstable micelles is cleared more rapidly than copolymer as thermodynamically stable micelles at all time points ($P < 0.05$). Also, the total clearance rates for the 2 and 250 mg/kg dose groups were found to be 0.14 and 0.06 mL/h, respectively.

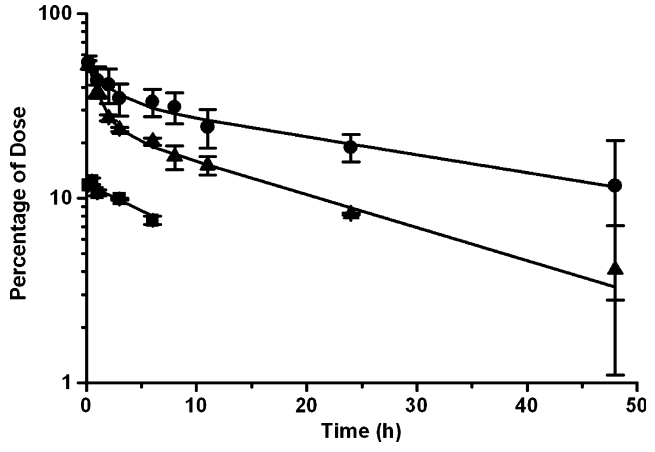


Fig. 4. The plasma clearance of MePEG$_{5000}$-*b*-PCL$_{5000}$ micelles in Balb/C mice ($n = 3$, SD shown as error bars) following intravenous injection at a dose of 250 mg/kg (●, concentration of copolymer above CMC upon dilution following administration) 2 mg/kg (▲, concentration of copolymer above CMC prior to administration but falls below CMC upon dilution) or 0.2 mg/kg (■, copolymer unimers). The plasma concentration data for all groups were fit using compartmental open models by Scientist® software and are shown as solid lines.

3.9. Biodistribution pattern of block copolymer unimers and micelles following i.v. administration

The tissue distribution of copolymer at specific time points following administration is shown in Fig. 5. For all three dose groups, a rapid accumulation of copolymer in the various organs was observed. Among the three dose groups examined, the most significant copolymer accumulation in both the kidney and liver was obtained for the 0.2 mg/kg dose (Fig. 5C). This high accumulation of copolymer in the major elimination organs correlates well with the rapid plasma clearance profile obtained for the copolymer when administered at this dose. On the other hand, the biodistribution profiles for the copolymer at doses of 2 and 250 mg/kg were found to be quite similar at most time points ($P > 0.05$).

As listed in Table 3, the tissue to plasma concentration ratios (K_b) were also calculated for liver, kidney and spleen, which are known to be the main elimination organs for block copolymers [6,13]. For the 0.2 mg/kg dose group, high values for K_b were obtained for all organs (i.e. ranges from 1.3 to 3.8). The volume of distribution of the copolymer in these organs was found to be much larger than their extravascular space as indicated by the high K_b values (i.e. for distribution in extravascular space, liver: $K_b \leqslant 0.3$, spleen: $K_b \leqslant 0.3$) [13]. In contrast, for the 2 mg/kg dose group the values for K_b confirm that most of the copolymer occupies the extravascular space of the liver and vascular space of the spleen (i.e. for distribution in vascular space, liver: $K_b \leqslant 0.1$, spleen: $K_b \leqslant 0.1$) at 1 h following administration [13]. This indicates a low level of cellular uptake of copolymer in these organs. Also, at this dose, the K_b values demonstrate a time-dependent profile with an increase from 0.41 to 1.85 in the liver and from 0.14 to 0.85 in the spleen at 1 and 12 h following i.v. administration. The K_b values for the 250 mg/kg dose group did not vary as significantly with time. The K_b values were 0.27 and 0.41 in the liver and 0.16 and 0.2 in the spleen at 1 and 12 h post-administration, respectively. These small values for K_b also indicate that a significant amount of the injected copolymer, when administered at this dose, remains in the extravascular space of the liver and vascular space in the spleen with only minor uptake by these organs for up to 12 h following i.v. administration.

4. Discussion

In this study a series of MePEG-*b*-PCL copolymers were synthesized and used to form micelles that were characterized *in vitro* as a means to select the most suitable material

Table 2
The main pharmacokinetic parameters for the MePEG$_{5000}$-*b*-PCL$_{5000}$ copolymer following i.v. administration at doses of 250, 2 or 0.2 mg/kg copolymer

Dose of polymer (mg/kg)	K_{10} (±SD) (h^{-1})	K_{12+} (±SD) (h^{-1})	K_{21} (±SD) (h^{-1})	A (mg/L)	B (mg/L)	α (h^{-1})	β (h^{-1})	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	V_1 (±SD) (mL)	V_β (mL)	AUC (mg h L^{-1})	CL_t (mL h^{-1})	MRT (h)	AUMC (mg h^2 L^{-1})
250	0.035 ± 0.004	0.16 ± 0.08	0.30 ± 0.10	1076.0	1775.0	0.47	0.02	1.5	30.8	1.75 ± 0.13	2.74	81246	0.06	43.3	3.5×10^7
2	0.093 ± 0.008	0.42 ± 0.05	0.38 ± 0.06	14.4	10.4	0.85	0.04	0.8	16.7	1.49 ± 0.04	3.30	268	0.14	22.6	6057
0.2	N/A	N/A	N/A	N/A	0.5	N/A	0.07	N/A	10.2	7.60 ± 0.20	N/A	8	0.53	14.4	108

to investigate *in vivo*. Overall the three copolymers were found to be biodegradable, have low CMC values and produce micelles with diameters in the range of 150 nm or less. It should be noted that, in these studies the CMC of the copolymers were measured in aqueous solution in the absence of the vast array of plasma proteins present in blood. Under conditions of thermodynamic equilibrium, protein–copolymer interactions could result in a decrease in the population of unimers present in solution, which could then in turn shift equilibrium towards micelle disassembly. However, it was confirmed in this series of studies that there are no significant interactions between plasma proteins and copolymer unimers. Therefore, it is unlikely that the presence of plasma protein would have a significant influence on the CMC.

Thermal analysis of the copolymer micelles confirmed the semi-crystalline nature of the hydrophobic core which has been shown to enhance the kinetic stability of micelles and result in a slow rate of disassembly once the concentration of the copolymer falls below the CMC [1]. In addition, as listed in Table 1, the polydispersity index (PDI) for each of the three copolymers was found to be relatively low (i.e. PDI < 1.1), which usually leads to a more stable micelle system *in vivo* [13].

As previously reported, the plasma protein adsorption profile and self-aggregation of colloidal carriers are the two key factors that determine their *in vivo* fate [20,24–26]. Therefore, in this study the *in vitro* stability of the micelles in the absence and presence of protein was considered to provide a preliminary indication, although likely an overestimate, of the stability of the formulation *in vivo*. Unlike micelles formed from MePEG$_{20000}$-*b*-PCL$_{5000}$ and MePEG$_{10000}$-*b*-PCL$_{5000}$, the size of the MePEG$_{5000}$-*b*-PCL$_{5000}$ micelles remained constant over the entire 240 h incubation period at 37 °C in the absence and presence of BSA (i.e. 45 g/L). In addition, the values obtained for the hydrodynamic diameters of MePEG$_{5000}$-*b*-PCL$_{5000}$ micelle system in the absence and presence of BSA were compared by the Student's *t*-test and their difference found to be insignificant ($P > 0.05$). This suggests that significant quantities of BSA do not adsorb to this micelle system. These findings are in agreement with our previous report [25]. In this way, the MePEG$_{5000}$-*b*-PCL$_{5000}$ copolymer was determined to be more suitable for *in vivo* use than the other MePEG-*b*-PCL copolymers considered.

The distribution kinetics of the MePEG$_{5000}$-*b*-PCL$_{5000}$ copolymer was examined at three different doses in order to evaluate the behavior and fate of: (1) copolymer assembled as thermodynamically stable micelles (i.e. concentration of copolymer above CMC prior to and upon dilution following i.v. administration), (2) copolymer assembled as thermodynamically unstable micelles (i.e. concentration of copolymer above CMC prior to but not following dilution upon i.v. administration) and (3) copolymer unimers.

The pharmacokinetics profile obtained for the copolymer administered as unimers (i.e. 0.2 mg/kg dose group)

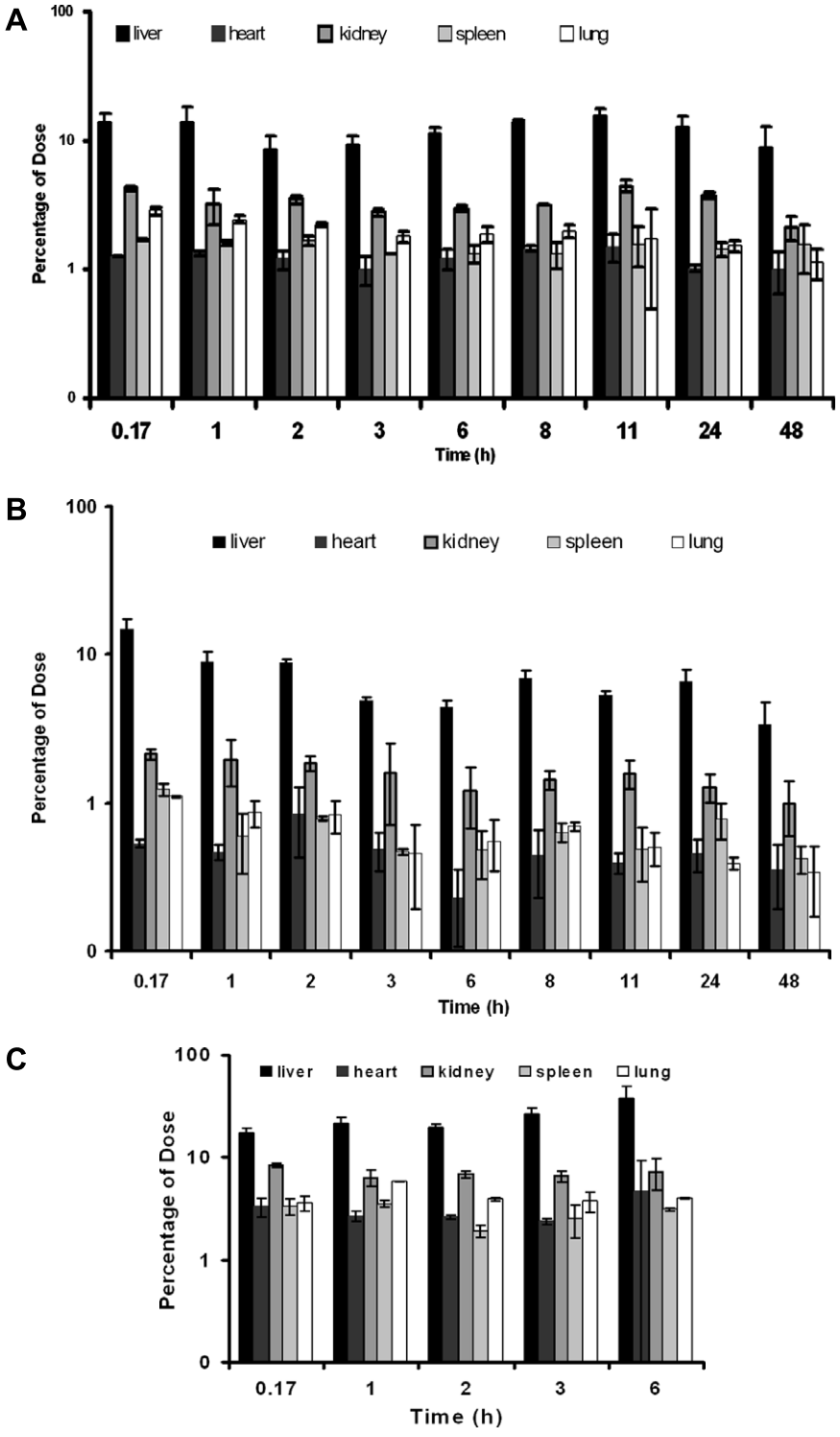


Fig. 5. The tissue distribution profile for the $MePEG_{5000}$-*b*-PCL_{5000} copolymer in mice following i.v. administration of micelles and/or unimers at doses of (A) 250 mg/kg, (B) 2 mg/kg or (C) 0.2 mg/kg. The amount of PEG_{5000}-*b*-PCL_{5000} copolymer is expressed as the percentage of the injected dose versus time. The histograms are representative of the mean of three-independent determinations ($n = 3$, SD shown as error bars).

included a very rapid elimination from the plasma following i.v. administration. The profile for the copolymer was found to be well portrayed by a one-compartment open model with a rapid distribution phase of less than 10 min. The volume of distribution (V_1) was quite large (i.e. $V_1 = 7.6$ mL), in comparison to the total blood volume of a mouse (i.e. 1.75 mL/mouse) [27]. This high value for V_1 confirms a broad tissue distribution for the copolymer to the various tissues and organs within a short period of time. This may be attributed to the relatively low molecular

Table 3
The tissue to plasma concentration ratios for the $MePEG_{5000}$-*b*-PCL_{5000} copolymer following i.v. administration at doses of 250, 2 or 0.2 mg/kg copolymer

Time (h)	K_b (liver)			K_b (spleen)			K_b (kidney)		
	250 mg/kg	2 mg/kg	0.2 mg/kg	250 mg/kg	2 mg/kg	0.2 mg/kg	250 mg/kg	2 mg/kg	0.2 mg/kg
1	0.27	0.41	1.90	0.16	0.14	1.30	0.30	0.47	1.70
6	0.41	0.69	3.80	0.23	0.30	2.30	0.39	0.68	3.10
12	0.41	1.85	N/A	0.20	0.85	N/A	0.43	1.20	N/A

weight (i.e. $M_n = 10{,}000$ g/mol) and amphiphilic nature of the copolymer unimers. In this way, the penetration of the copolymer into tissues may be enhanced, relative to the degree of penetration or entry of intact micelles. As listed in Table 3, the tissue to plasma concentration ratios (K_b) for the copolymer unimers indicate a significant degree of intracellular uptake of the copolymer in these organs [28–30]. The prompt uptake by the liver and spleen leads to a rapid total elimination of the copolymer from the plasma as confirmed by the short half-life (i.e. 10.2 h) and high clearance rate (0.53 mL/h) obtained for the copolymer when administered at this dose.

Following administration of the copolymer as both thermodynamically stable and unstable micelles (i.e. dose groups of 250 and 2 mg/kg, respectively), the presence of intact micelles in plasma both 1 and 24 h post-administration was confirmed by a GFC-based method. The presence of intact micelles in plasma following administration of the thermodynamically unstable micelles (i.e. 2 mg/kg) confirms a high degree of kinetic stability for this system. This may be attributed to both the hydrophobic and semi-crystalline nature of the PCL core as well as the appropriate surface coverage of the PCL core by the MePEG shell [1]. The *in vivo* stability of the $MePEG_{5000}$-*b*-PCL_{5000} micelle system has also been demonstrated in the pharmacokinetics profiles and elimination rates obtained for both the 2 and 250 mg/kg dose groups. Specifically, the plasma clearance profiles for the copolymer at both doses include obvious two-compartment patterns with an initial fast distribution phase in the first 2 h followed by a relatively slow elimination phase. It was found that even the thermodynamically unstable micelles (i.e. 2 mg/kg dose group) had a much longer circulation half-life and slower rate of elimination than the copolymer unimers (i.e. 0.2 mg/kg dose group, $P < 0.05$ at all time points), despite the fact that the copolymer concentration would be expected to reach levels that are approximately 2-fold below the CMC of the copolymer following i.v. injection.

As expected, in comparison to the thermodynamically unstable micelles (i.e. 2 mg/kg dose group) the thermodynamically stable micelles (i.e. 250 mg/kg dose group) were found to have a slower elimination rate from the plasma during the elimination phase (i.e. 2–48 h, $P < 0.05$ for all time points). The concentration ratios of tissue to plasma for the main elimination organs were also found to be lower for the 250 mg/kg dose group: this indicates that the copolymers were more effectively entrapped within the extracellular or vascular space of these elimination organs leading to a lower elimination rate, relative to the 2 mg/kg dose group.

In summary, it has been demonstrated that by optimizing the block lengths of MePEG-*b*-PCL copolymers, a micelle system with a reasonable *in vivo* circulation half-life, that is comparable to that of other well-established long-circulating colloidal particles, may be achieved [12,13,31,32]. The investigation and comparison of the pharmacokinetics and biodistribution profiles for copolymer administered as thermodynamically stable micelles, thermodynamically unstable micelles and copolymer unimers revealed that the aggregation state (i.e. micelles or unimers) of the copolymer plays an important role in determining the *in vivo* fate of the material. These studies also demonstrate that when the copolymer concentration falls below the CMC the kinetic stability of the system, which is largely determined by the nature (i.e. degree of hydrophobicity) and state (i.e. semi-crystalline, amorphous) of the micelle core, can have a significant impact on *in vivo* stability as well as the pharmacokinetics and biodistribution of the material. In this way, the selection of a core-forming block that provides a high degree of kinetic stability may be a strategy for the preparation of micelles that act as true drug carriers and remain intact until reaching the target site.

Acknowledgements

The authors thank NSERC for funding this research. C. Allen is grateful to CIHR/Rx&D for a career award and J. Liu thanks NSERC for a postgraduate scholarship.

References

[1] C. Allen, D. Maysinger, A. Eisenberg, Nano-engineering block copolymer aggregates for drug delivery, Colloids Surf. B Biointerfaces 16 (1999) 3–27.
[2] T.Y. Kim, D.W. Kim, J.Y. Chung, S.G. Shin, S.C. Kim, D.S. Heo, N.K. Kim, Y.J. Bang, Phase I and pharmacokinetics study of Genexol-PM, a cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced maglignacies, Clin. Cancer Res. 10 (2004) 3708–3716.
[3] D. Le Garrec, S. Gori, L. Luo, D. Lessard, D.C. Smith, M.A. Yessine, M. Ranger, J.C. Leroux, Poly(*N*-vinylpyrrolidone)-block-poly(D,L-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: in vitro and in vivo evaluation, J. Control. Release 99 (2004) 83–101.
[4] M.L. Adams, D.R. Andes, G.S. Kwon, Amphotericin B encapsulated in micelles based on poly(ethylene oxide)-block-poly(L-amino acid) derivatives exerts reduced in vitro hemolysis but maintains potent in vivo antifungal activity, Biomacromolecules 4 (2003) 750–757.

[5] R.T. Liggins, H.M. Burt, Polyether–polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations, Adv. Drug Deliv. Rev. 54 (2002) 191–202.
[6] H.M. Burt, X.C. 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 Surf. B Biointerfaces 16 (1999) 161–171.
[7] T. Hamaguchi, Y. Matsumura, M. Suzuki, K. Shimizu, R. Goda, I. Nakamura, I. Nakatomi, M. Yokoyama, K. Kataoka, T. Kakizoe, NK105, a paclitaxel-incorporating micellar nanoparticle formulation, can extend in vivo antitumour activity and reduce the neurotoxicity of paclitaxel, Br. J. Cancer 92 (2005) 1240–1246.
[8] T. Nakanishi, S. Fukushima, K. Okamoto, M. Suzuki, Y. Matsumura, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, Development of the polymer micelle carrier system for doxorubicin, J. Control. Release 74 (2001) 295–302.
[9] S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seo, In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy, J. Control. Release 72 (2001) 191–202.
[10] S.Y. Kim, Y.M. Lee, H.J. Shin, J.S. Kang, Indomethacin-loaded methoxy poly(ethylene glycol)/poly(epsilon-caprolactone) diblock copolymeric nanosphere: pharmacokinetic characteristics of indomethacin in the normal Sprague–Dawley rats, Biomaterials 22 (2001) 2049–2056.
[11] Y. Mizumura, Y. Matsumura, T. Hamaguchi, N. Nishiyama, K. Kataoka, T. Kawaguchi, W.J. Hrushesky, F. Moriyasu, T. Kakizoe, Cisplatin-incorporated polymeric micelles eliminate nephrotoxicity, while maintaining antitumor activity, Jpn. J. Cancer Res. 92 (2001) 328–336.
[12] K. Novakova, M. Laznicek, F. Rypacek, L. Machova, I-125-labeled PLA/PEO block copolymer: biodistribution studies in rats, J. Bioactive Compatible Polymers 17 (2002) 285–296.
[13] Y. Yamamoto, Y. Nagasaki, Y. Kato, Y. Sugiyama, K. Kataoka, Long-circulating poly(ethylene glycol)-poly(D,L-lactide) block copolymer micelles with modulated surface charge, J. Control. Release 77 (2001) 27–38.
[14] Y.J. Park, J.Y. Lee, Y.S. Chang, J.M. Jeong, J.K. Chung, M.C. Lee, K.B. Park, S.J. Lee, Radioisotope carrying polyethylene oxide-polycaprolactone copolymer micelles for targetable bone imaging, Biomaterials 23 (2002) 873–879.
[15] E.V. Batrakova, S. Li, Y. Li, V.Y. Alakhov, W.F. Elmquist, A.V. Kabanov, Distribution kinetics of a micelle-forming block copolymer Pluronic P85, J. Control. Release 100 (2004) 389–397.
[16] S.E. Dunn, A. Brindley, S.S. Davis, M.C. Davies, L. Illum, Polystyrene-poly(ethylene glycol) (PS-PEG2000) particles as model systems for site specific drug delivery. 2. The effect of PEG surface density on the in vitro cell interaction and in vivo biodistribution, Pharm. Res. 11 (1994) 1016–1022.
[17] X.D. Lou, C. Detrembleur, R. Jerome, Living cationic polymerization of delta-valerolactone and synthesis of high molecular weight homopolymer and asymmetric telechelic and block copolymer, Macromolecules 35 (2002) 1190–1195.
[18] 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.
[19] J.E. Diederichs, Plasma protein adsorption patterns on liposomes: establishment of analytical procedure, Electrophoresis 17 (1996) 607–611.
[20] C. Allen, N. Dos Santos, R. Gallagher, G.N.C. Chiu, Y. Shu, W.M. Li, S.A. Johnstone, A.S. Janoff, L.D. Mayer, M.S. Webb, M.B. Bally, Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol), Biosci. Rep. 22 (2002) 225–250.
[21] L. Liu, E. Mak, R.G. Tirona, E. Tan, P.M. Novikoff, P. Wang, A.W. Wolkoff, K.S. Pang, Vascular binding, blood flow, transporter, and enzyme interactions on the processing of digoxin in rat liver, J. Pharmacol. Exp. Ther. 315 (2005) 433–448.
[22] H. Lee, F. Zeng, M. Dunne, C. Allen, Methoxy poly(ethylene glycol)-block-poly(delta-valerolactone) copolymer micelles for formulation of hydrophobic drugs, Biomacromolecules 6 (2005) 3119–3128.
[23] P. Skoglund, A. Fransson, Continuous cooling and isothermal crystallization of polycaprolactone, J. Appl. Polymer Sci. 61 (1996) 2455–2465.
[24] S.C. Semple, A. Chonn, P.R. Cullis, Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo, Adv. Drug Deliv. Rev. 32 (1998) 3–17.
[25] J. Liu, F. Zeng, C. Allen, Influence of serum protein on polycarbonate-based copolymer micelles as a delivery system for a hydrophobic anti-cancer agent, J. Control. Release 103 (2005) 481–497.
[26] J. Liu, H. Lee, M. Huesca, A. Young, C. Allen, Liposome formulation of a novel hydrophobic aryl-imidazole compound for anti-cancer therapy, Cancer Chemother. Pharmacol. (2005) 1–13.
[27] F.G. King, R.L. Dedrick, Physiological pharmacokinetic parameters for *cis*-dichlorodiammineplatinum(II) (DDP) in the mouse, J. Pharmacokinet. Biopharm. 20 (1992) 95–99.
[28] R.K. Jain, Transport of molecules in the tumor interstitium: a review, Cancer Res. 47 (1987) 3039–3051.
[29] R.K. Jain, Determinants of tumor blood flow: a review, Cancer Res. 48 (1988) 2641–2658.
[30] L.T. Baxter, H. Zhu, D.G. Mackensen, R.K. Jain, Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice, Cancer Res. 54 (1994) 1517–1528.
[31] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, et al., Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. USA 88 (1991) 11460–11464.
[32] T.M. Allen, C. Hansen, Pharmacokinetics of stealth versus conventional liposomes: effect of dose, Biochim. Biophys. Acta 1068 (1991) 133–141.