Biomaterials 34 (2013) 3132–3140

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of kinetic stability on biodistribution and anti-tumor efficacy of drug-loaded biodegradable polymeric micelles

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ARTICLE INFO

Article history:
Received 9 November 2012
Accepted 7 January 2013

Keywords:
Kinetic stability
Functional polycarbonates
Micelles
Biodistribution
Anti-tumor activity

ABSTRACT

This study was aimed to investigate the effect of kinetic stability on biodistribution and antitumor efficacy of drug-loaded biodegradable polymeric micelles. Four diblock copolymers of acid- and urea-functionalized polycarbonate (i.e. PAC and PUC) and poly(ethylene glycol) (PEG) with the same polycarbonate length and two different PEG molecular weights (Mn: 5 kDa and 10 kDa), i.e. 5K PEG-PAC, 10K PEG-PAC, 5K PEG-PUC and 10K PEG-PUC, were synthesized *via* organocatalytic living ring-opening polymerization using methoxy PEG as a macroinitiator. These polymers were employed to prepare 5K PEG-PAC/5K PEG-PUC and 10K PEG-PAC/10K PEG-PAC mixed micelles *via* urea-acid hydrogen bonding. An amine group-containing anticancer drug, doxorubicin (DOX) was loaded into the mixed micelles *via* a self-assembly process. DOX-loaded 5K and 10K PEG mixed micelles had particle sizes of 66 and 87 nm respectively with narrow size distribution (polydispersity index: 0.12), and DOX loading levels were 28.9 and 22.8% in weight. DOX-loaded 5K PEG mixed micelles had greater kinetic stability than DOX-loaded 10K PEG mixed micelles due to stronger hydrophobicity of 5K PEG block copolymers. The results of *in vitro* release studies showed that DOX release was sustained without obvious initial burst release. The DOX-loaded mixed micelles effectively suppressed the proliferation of HepG2 and 4T1 cells. The *in vivo* studies conducted in a 4T1 mouse breast cancer model demonstrated that the mixed micelles were preferably transported to the tumor with the 5K PEG mixed micelles accumulating in the tumor more rapidly to a larger extent than 10K PEG mixed micelles, and DOX-loaded 5K PEG mixed micelles with greater kinetic stability inhibited tumor growth more effectively than free DOX and DOX-loaded 10K PEG mixed micelles without causing significant body weight loss or cardiotoxicity. The 5K PEG mixed micelles with sizes below 100 nm and narrow size distribution as well as excellent kinetic stability holds great potential as a delivery carrier for amine group-containing anticancer drugs.

1. Introduction

Most anticancer drugs have limited water solubility and short blood circulation times in the body, leading to frequent administrations [1]. In addition, they cause harmful side-effects due to their non-specificity. To combat these problems, a diverse assortment of biomaterials have been engineered and utilized as vehicles to deliver anticancer drugs in the forms of liposomes, dendrimers, polymeric micelles or peptide assemblies [2]. In recent years, biodegradable polymeric micelles in particular have attracted increasing attention due to the following reasons: 1) their well-defined core–shell nanostructures allow hydrophobic anticancer drugs to be encapsulated in the core while the hydrophilic shell commonly consisting of poly(ethylene glycol) (PEG) allows for increased water solubility, prolonged blood circulation, reduced protein adsorption and recognition by the mononuclear phagocytic system [3]; 2) advanced chemistry is available for making functional copolymers compatible with the drug of choice; 3) their nanosize gives rise to passive accumulation in tumor tissues based on the enhanced permeability and retention (EPR) effect [4]).

There are a number of parameters that should be taken into consideration in the design of polymeric micelles, including drug loading capacity, particle size and size distribution, biocompatibility,

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http://dx.doi.org/10.1016/j.biomaterials.2013.01.042

thermodynamic and kinetic stability. Drug loading capacity is correlated to the amount of polymer required to load a certain amount of drug and drug loading efficiency, which affects the cost of final formulation. Particle size and its distribution govern biodistribution of the micelles in the body. Larger polymeric micelles (>200 nm) tend to be cleared faster in the blood with more particles distributed in the liver, lungs and spleen. Smaller polymeric nanoparticles (<200 nm) with less negative surface charge tend to accumulate mostly in tumors while being cleared from the blood at a slower rate [5]. The critical micelle concentration (CMC) of polymers reflects thermodynamic stability. Micelles made from a polymer with lower CMC have greater thermodynamic stability. The micelles would remain intact even after blood dilution after i.v. injection if the polymer concentration in the blood is above its CMC. On the other hand, the kinetic stability of micelles is another important factor that influences micelle behavior in the blood stream, and it measures how fast the micelles dissociate into individual polymer molecules. The micelles may be still stable in the blood stream for a certain period of time even at polymer concentrations below its CMC. For example, PEG-poly(caprolactone) block copolymer was reported to exhibit longer circulation half-life and slower elimination at a concentration above its CMC [6]. Nonetheless, due to the high kinetic stability of the system, the micellar structure remained unchanged even at a concentration 2-fold below its CMC [6]. The kinetic stability of micelles depends on the interactions between the hydrophobic blocks of the polymer. Non-covalent interactions such as hydrogen-bonding [7,8], stereocomplexation [9] and electrostatic interaction [10] as well as chemical cross-linking [11] were reported to increase drug loading capacity, thermodynamic and kinetic stability, and achieve nanosize. Advanced chemistry such as organocatalytic living ring-opening polymerization (ROP) [12,13] produces polymers with well-defined molecular weight, giving rise to micelles with narrow size distribution [7–9]. In addition, by using this approach, functionality can also be easily introduced into the polymers to realize non-covalent interactions.

In this study, aliphatic polycarbonates were chosen as the building units for copolymers because of their biodegradability and non-toxic degradation products [14,15]. We previously reported the synthesis of urea- and acid-functionalized poly(carbonates) and PEG block copolymers (PEG-PUC and PEG-PAC) with narrow molecular weight distribution *via* ROP of cyclic carbonate monomers. Doxorubicin (DOX, an amine-containing anticancer drug) was loaded into PEG-PAC micelles with extremely high loading levels due to electrostatic interaction between the acid groups in the polymer and the amine group in DOX [7,8]. Blending the acid-functionalized block copolymer with the urea-containing block copolymer formed mixed micelles *via* hydrogen bonding interactions between the acid group and the urea group, significantly improving the kinetic stability of micelles [8]. The present study was aimed to investigate the effect of kinetic stability on biodistribution and antitumor efficacy of DOX-loaded mixed micelles. Block copolymers of acid- or urea-functionalized polycarbonate and PEG with different molecular weights (i.e. 5000 and 10,000 g/mol), i.e. 5K PEG-PAC, 10K PEG-PAC, 5K PEG-PUC and 10K PEG-PUC, were synthesized *via* organocatalytic ROP of acid- or urea-functionalized cyclic carbonate using monomethoxy PEG as a macroinitiator. Mixed micelles were formed from PEG-PAC and PEG-PUC with different PEG lengths, and characterized for CMC, particle size and size distribution, DOX loading capacity, kinetic and serum stability, and drug release profiles. Cytotoxicity of blank and DOX-loaded mixed micelles was investigated in comparison with free DOX against HepG2 human liver carcinoma and 4T1 mouse mammary carcinoma cell lines. The biodistribution of mixed micelles loaded with a near infra-red dye, 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (DiR), was studied in a 4T1 tumor-bearing mouse model *via* live *in vivo* imaging after tail vein injection. The antitumor efficacy of free DOX and DOX-loaded micelles with different PEG lengths was also assessed in the mouse breast tumor model by monitoring tumor size and body weight, and histological analysis.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma–Aldrich and utilized as received unless otherwise indicated. Monomethoxy PEG with Mn 5000 g/mol (PDI 1.04) and Mn 10,000 g/mol (PDI 1.06) obtained from Polymer Source Inc. (Canada) was azeotropically distilled from toluene and dried in vacuum before usage. Sparteine was stirred over CaH_2, distilled in vacuum twice, and then stored in glove box. *N*-(3,5-trifluoromethyl)phenyl-*N*′-cyclohexylthiourea (TU) was prepared according to our previous protocol [16]. TU was dissolved in dry THF, stirred with CaH_2, filtered, and dried *in vacuo*. XenoLight DiR dye was obtained from Caliper Life Sciences Inc. (U.S.A.). HepG2 human liver carcinoma and 4T1 mouse mammary carcinoma cell lines were acquired from ATCC (U.S.A.). Fetal bovine serum was obtained from PAA, Austria.

2.2. Polymer synthesis

2.2.1. Synthesis of urea-functionalized (PEG-PUC) and benzyl-protected acid-functionalized (PEG-P(MTC-OBn)) copolymers (Scheme 1)

The synthesis procedures for preparation of the benzyl-protected acid- (MTC-OBn) and urea-functionalized (MTC-urea) carbonate monomers were reported previously [7,8]. The details of the procedure for organocatalytic ROP of MTC-OBn (or MTC-urea) with 5K PEG are given below as typical examples.

5K PEG-P(MTC-OBn): In a glove box, PEG (0.4 g, 0.08 mmol, Mn 5000 g/mol, PDI 1.04) in CH_2Cl_2 (0.75 mL) was mixed with MTC-OBn (0.2 g, 0.8 mmol) and DBU (12 mg, 0.08 mmol) in CH_2Cl_2 (0.75 mL) and the mixture stirred for 2 h followed by quenching the reaction with benzoic acid (15–20 mg). The reaction mixture was precipitated into diethyl ether (40 mL) with the precipitates collected after centrifugation and dried *in vacuo*. The precipitates were then purified by column chromatography on a Sephadex LH-20 column with THF as the eluent to give 5K PEG-P(MTC-OBn)$_9$ as an off-white solid (0.53 g, 88%). PDI 1.08. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ 7.28 (m, 45H, Ph*H*), 5.11 (s, 18H, PhCH_2-), 4.25 (t, 36H, -CH_2OCOO-), 3.71 (s, 455H, H of MPEG5K), 1.20 (s, 27H, -CH_3).

10K PEG-(MTC-OBn)$_9$: Yield, 82%; PDI 1.13. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ 7.31 (m, 45H, Ph*H*), 5.13 (s, 18H, PhCH_2-), 4.24 (t, 36H, -CH_2OCOO-), 3.70 (s, 909H, H of MPEG10K), 1.24 (s, 27H, -CH_3).

5K PEG-PUC: A solution of thiourea catalyst (TU) (33.3 mg, 0.09 μmol), sparteine (20.3 mg, 90 μmol) and 5K PEG (0.3 g, 0.06 mmol) in CH_2Cl_2 was stirred for 10 min. MTC-urea (0.29 g, 0.9 mmol) in CH_2Cl_2 (1.5 mL) was added to the mixture and stirred for 16 h. Benzoic acid (15 mg) was added to end the reaction and the crude polymer precipitated in 40 mL cold diethyl ether with the precipitates collected after centrifugation and dried *in vacuo*. The precipitates were purified by column chromatography on a Sephadex LH-20 column with THF as the eluent to give 5K PEG-P(MTC-urea)$_9$ (i.e. 5K PEG-PUC) as an off-white solid (0.50 g, 85%). PDI 1.10. ^{1}H NMR (400 MHz, DMSO-d_6, 22 °C): δ 8.60 (s, br, 9H, PhN*H*-), 7.36 (s, 18H, Ph*H*), 7.17 (s, 18H, Ph*H*), 6.85 (s, 9H, Ph*H*), 6.27 (s, br, 9H, -CH_2N*H*-), 4.14 (d, 54H, -CH_2OCOO- and -COOCH_2CH_2-), 3.55 (s, 455H, H of MPEG), 3.31 (s, 18H, -CH_2NHCO-), 1.13 (s, 27H, -CH_3).

5K PEG-P(MTC-urea)$_9$ (i.e. 10K PEG-PUC): Yield, 80%; PDI 1.11. ^{1}H NMR (400 MHz, DMSO-d_6, 22 °C): δ 8.54 (s, 9H, PhN*H*-), 7.36 (s, 18H, Ph*H*), 7.18 (s, 18H, Ph*H*), 6.87 (s, 9H, Ph*H*), 6.22 (s, 9H, -CH_2N*H*-), 4.14 (d, 54H, -CH_2OCOO- and -COOCH_2CH_2-), 3.53 (s, 909H, H of MPEG), 3.33 (s, 18H, -CH_2NHCO-), 1.14 (s, 27H, -CH_3).

2.2.2. Deprotection of benzyl groups in PEG-P(MTC-OBn)

A mixture of PEG-P(MTC-OBn) (0.5 g), THF (7.5 mL), methanol (7.5 mL), and Pd-C (10% w/w, 0.2 g) was swirled under H_2 (7 atm) overnight and filtered through Celite wetted with THF after evacuation of the H_2 atmosphere. Additional washings with THF (15 mL) and methanol (15 mL) were carried out to ensure total transfer. Washings were collected, and the solvents were evaporated. The residue was dialyzed against DMSO using a dialysis membrane with molecular weight cut-off (MWCO) of 1000 Da (Spectra/Por 7, Spectrum Laboratories Inc.) for 48 h, and then against de-ionized (DI) water for an additional two days before being freeze-dried *in vacuo*. Eventually, PEG-PAC was obtained as a white solid (yield: >90%).

2.3. Preparation of DOX-loaded micelles and measurement of DOX loading

DOX encapsulation into various polymeric micelles was carried out through a sonication/membrane dialysis method as described in our previous studies [7,8]. Briefly, 5 mg of DOX was dissolved in 1.5 mL of *N*, *N*-dimethylacetamide (DMAc) and neutralized with 3 mol excess of triethylamine. The DOX solution was mixed with polymer solution (10 mg in 0.5 mL of DMAc) by vortexing, and the mixture was

added dropwise to 10 mL of DI water while being sonicated at 130 W for 2 min by a probe-based sonicator (Vibra Cell VCX 130). The solution was consequently dialyzed against 1 L of DI water utilizing a dialysis bag with MWCO of 1000 Da (Spectra/Por 7, Spectrum Laboratories Inc.). The water was changed at 3, 6 and 24 h. After 48 h, the solution inside the dialysis bag was collected and lyophilized. Each experiment was carried out in triplicates. To measure DOX in the micelles, a known amount of lyophilized DOX-loaded mixed micelles was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and the absorbance of the solution was measured using a UV–Vis spectrophotometer (UV 2501PC Shimadzu, Japan) at 480 nm. A standard line for DOX concentration in the range of 1–100 mg/L was plotted linearly against DOX concentration in DMSO. DOX loading level was determined by using the following formula:

$$\text{Actual loading level(wt\%)} = \frac{\text{mass of DOX loaded in micelles}}{\text{mass of DOX} - \text{loaded micelles}} \times 100\%$$

2.4. Determination of critical micelle concentration (CMC)

The CMC values of the functionalized block copolymers were determined in DI water by a LS50B luminescence spectrometer (Perkin Elmer, United States) and employing pyrene as a probe. Pyrene solution in acetone (10 μL, 6.16×10^{-5} M) was added into each glass vial, following which acetone was evaporated at room temperature. Polymer solution in DI water (1 mL) with various concentrations ranging from 0.01 to 2000 mg/L was added into the glass vials, giving a final pyrene concentration of 6.16×10^{-7} M and the solutions kept overnight. The excitation spectra of the solutions were scanned from 300 to 360 nm with an emission wavelength of 395 nm, and both the excitation and emission bandwidths were set at 2.5 nm. The intensity ratios (I_{337}/I_{334}) were plotted against polymer concentration. The CMC value was given by the intersection of the tangent to the curve at the inflection and the tangent of the points at low polymer concentrations.

2.5. Particle size analysis

Particle size was acquired from freshly prepared micelle (~0.6 mg/mL) and blank micelle solutions (1 mg/mL) using dynamic light scattering (DLS, scattering angle: 90°) using Zetasizer 3000 HAS (Malvern Instrument Ltd., Malvern, UK), which is equipped with a He-Ne laser beam at 658 nm. Large aggregates were removed by centrifugation at 4000 rpm for 10 min. Each sample was measured 3 times and an average particle size was obtained. As the samples might contain individual micelles and aggregates, multimodal analysis was used in order to maximize the resolution and accuracy.

2.6. Stability studies of micelles in the presence of serum

Particle size of DOX-loaded micelles solution (~1 mg/mL) containing 10% fetal bovine serum (FBS) was recorded by DLS using Zetasizer 3000 HAS (Malvern Instrument Ltd., Malvern, UK) over 48 h.

2.7. Kinetic stability studies of blank and DOX-loaded micelles

Kinetic stability of the DOX-loaded micelles in DI water was examined using sodium dodecyl sulfate (SDS) as a destabilizing agent. Briefly, SDS aqueous solution was added to DOX-loaded micelles (1 mg/mL) at a final concentration of 2.23 mg/ml and mixed by pipetting. The change in scattered light intensity was recorded by DLS using Zetasizer 3000 HAS (Malvern Instrument Ltd., Malvern, UK) over 48 h.

2.8. In vitro release studies of DOX-loaded micelles

The lyophilized DOX-loaded micelles were dissolved in PBS buffer (pH 7.4) at a concentration of 1 mg/mL and placed into a dialysis bag with MWCO of 1000 Da (Spectra/Por 7, Spectrum Laboratories Inc.). The bag was submerged into a bottle containing 30 mL of PBS buffer (pH 7.4) inside a 37 °C water bath (Polyscience, Spectra-Teknik (S) Pte Ltd.) while being shaken at 100 rev/min. The solution from the release medium (1 mL) was withdrawn at selected time points and replaced with 1 mL of fresh PBS buffer. The absorbance of DOX in the solution was determined using the UV–Vis spectrophotometer at 480 nm, and DOX content was calculated using the calibration line of DOX in PBS (pH 7.4).

2.9. In vitro cytotoxicity studies

Cytotoxicity of free DOX, blank mixed micelles and DOX-loaded mixed micelles was investigated by MTT assay as described previously [7,8]. HepG2 and 4T1 cells were cultured in DMEM and RPMI-1640 respectively, supplemented with 10% FBS, 5% penicillin-streptomycin, 2 mM L-glutamine and 110 mg/L sodium pyruvate, and incubated at 37 °C, 5% CO_2. D-glucose (4.5 g/L) was additionally supplemented in DMEM. The HepG2 and 4T1 cells were seeded at a density of 10^4 cells per well on 96-well plates and kept overnight. Free DOX, blank mixed micelles, and lyophilized DOX-loaded mixed micelles were dissolved in the corresponding cell culture medium at various concentrations, and the prepared sample solution (100 μL) used to replace the medium in each well. Each sample was tested in eight replicates for each concentration. The plates were incubated at 37 °C, 5% CO_2 for 48 h. Next, the medium with the samples was replaced with fresh growth medium (100 μL), which was mixed with MTT solution (20 μL, 5 mg/mL), and the plates kept at 37 °C, 5% CO_2 for 4 h. To dissolve the purple formazan crystals internalized by live cells, the medium was removed and DMSO (150 μL) was added. The absorbance of formazan crystals in each well was calculated as that at 550 nm deducted by that at 690 nm. Cell viability was formulated as a percentage of absorbance of the non-treated control sample.

2.10. Biodistribution of mixed micelles

Biodistribution of mixed micelles formed from different PEG lengths was investigated through non-invasive bioimaging. A near-infrared fluorophore, DiR, was loaded into the mixed micelles according to the protocol described in Section 2.3. Briefly, polymer (10 mg) and DiR (0.3 mg) were dissolved in DMSO (2 mL) and the mixture pipetted drop-by-drop into 10 ml of DI water with sonication for 10 min. The resulting solution was dialyzed against DI water for 48 h, and the water changed at 3, 6 and 24 h. The loading level of DiR was determined by a method similar to that of DOX (Section 2.3), i.e. dissolving a known amount of lyophilized DiR-loaded mixed micelles in DMSO and measuring its absorbance at 759 nm wavelength.

The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Biological Resource Center, Agency for Science, Technology and Research (A*STAR), Singapore. Female Balb/c mice were injected subcutaneously with 0.5×10^6 4T1 cells each. Two weeks post-injection, when the tumor reached about 5–7 mm in diameter, the mice were treated with 8 mg/kg DiR-loaded mixed micelles *via* tail-vein injection. Whole-body fluorescence images were taken using Xenogen IVIS 100 (Caliper Life Sciences, U.S.A.) with the ICG filter (excitation at 710–760 nm, emission at 810–875 nm) at time points ranging from 30 min to 96 h post-administration. Anesthetized animals ($n = 3$ for each micelle formulation and $n = 2$ for free DiR dye) were placed in one lateral position on the heated plate (37 °C) for image-taking. The exposure time was set to 3s. The tumors and organs were removed from sacrificed mice at 96 h post-administration, and subsequently imaged.

2.11. In vivo therapeutic efficacy and histological analysis

4T1 cells were inoculated subcutaneously at the right flank of 6–8 weeks old BALB/c mice at a dose of 10^6 cells per animal. The mice were randomly divided into 6 groups of ten mice each: PBS control, free DOX, 5K PEG and 10K PEG mixed micelles and their corresponding blank micelles formulations. Ten days post-inoculation, the animals were injected intravenously *via* tail vein at a dose of 5 mg/kg (DOX equivalent) four times at a 4-day interval (Days 0, 4, 8 and 12) with free DOX, DOX-loaded 5K PEG and 10K PEG mixed micelles, the equivalent blank mixed micelles and PBS solution. Tumor volume and body weight were monitored to assess tumor inhibition activity and overall toxicity of each formulation, respectively. The tumor dimensions were measured with a vernier caliper, and the tumor volume was calculated using the formula: Tumor volume = $(\text{width})^2 \times \text{length} \times 1/2$. At the end of the *in vivo* study, the hearts and tumors were excised from sacrificed mice and fixed in 10% formalin solution followed by paraffin embedding and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Apoptotic bodies in the tumor and heart sections were quantified by counting the number of TUNEL-positive nuclei in ten fields of magnification×400 with the highest number of apoptotic bodies and obtaining the mean of the ten fields for each sample. Embedding and staining were done by the Histopathology Unit of Biopolis Shared Facilities, Singapore. Images were acquired and analyzed in the SBIC-Nikon Imaging Center at Biopolis, Singapore using a Nikon AZ 100 microscope.

2.12. Statistical analysis

All data are presented as mean ± SD. The differences in body weight and tumor volume were evaluated using unpaired Student's *t*-test and were considered significant if $p < 0.05$.

3. Results and discussion

3.1. Synthesis of acid/urea-functionalized polycarbonate and PEG diblock copolymers

Organocatalytic ring opening polymerization (OROP) is an attractive approach for synthesis of biomaterials as there is no toxic metal involved and molecular structure of resulting polymers can be well controlled [12,13]. The monomer MTC-OBn was directly derived from ring closure of 2,2-bis(methylol)propionic acid. The benzyl protecting group of MTC-OBn can be removed *via* hydrogenolysis to produce MTC-OH, which was further condensed with phenylureaethanol to give MTC-urea. The diblock copolymers of PEG

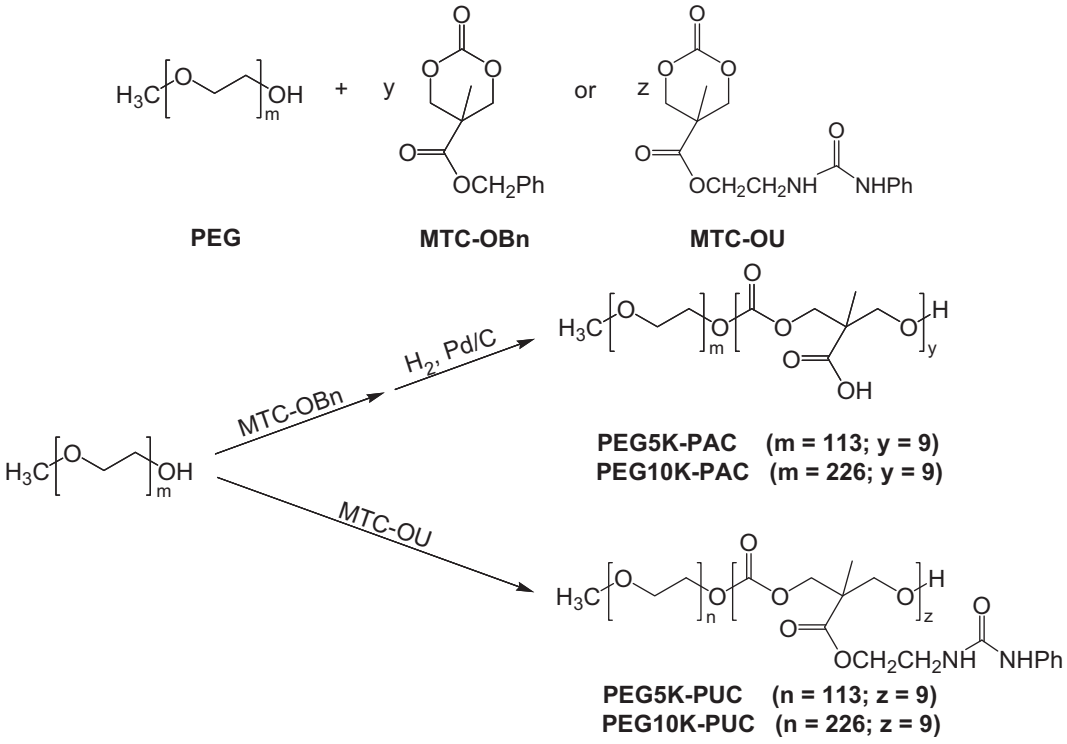
and benzyl-protected acid- or urea-functionalized polycarbonate were synthesized through OROP using monomesthoxy PEG with different molecular weights as macroinitiators (Scheme 1). From their ^{1}H NMR spectra (Fig. S1 in the Supplementary Information), the number of MTC-OBn and MTC-Urea repeat units was 9. The molecular weights of the copolymers estimated from the ^{1}H NMR spectra are congruous with those obtained from GPC analysis (relative to polystyrene standards). The polydispersity indices of molecular weights were 1.08, 1.13, 1.10 and 1.11 for 5K PEG-PAC, 10K PEG-PAC, 5K PEG-PUC and 10K PEG-PUC respectively (Table 1). Benzyl protecting groups were removed by hydrogenolysis (H_2/Pd-C) to free the carboxylic acid groups as evidenced by ^{1}H NMR analysis (Fig. S1), giving rise to the final products.

3.2. Mixed micelles formed from PEG-PAC and PEG-PUC

The formation of mixed micelles from PEG-PAC and PEG-PUC was evidenced by the existence of a single critical micelle concentration (CMC) and a single peak in the dynamic light scattering (DLS) spectra while their individual copolymers exhibited multiple peaks (Fig. S2A and C). The urea-acid hydrogen bonding interactions facilitated the formation of mixed micelles, leading to significantly lower CMC values as compared to their respective acid-functionalized copolymers 5K PEG-PAC and 10K PEG-PAC (17.8 *vs.* 63.1 and 22.4 *vs.* 595.6 mg/L, Table 1). The particle sizes of 5K PEG-PAC and 10K PEG-PAC micelles were 231 and 126 nm with PDI of 0.35 and 0.29, respectively (Table 1). On the other hand, the urea-functionalized 5K PEG-PUC and 10K PEG-PUC copolymers self-assembled into micelles of 41 and 29 nm with relatively lower PDI values (0.19 and 0.18 respectively). Blending PEG-PAC and PEG-PUC before self-assembly in water gave rise to micelles with sizes below 100 nm and narrow size distribution. By simply mixing individual solutions of acid- and urea-functionalized copolymers on the other hand, the resulting solutions exhibited wide size distribution (PDI: 0.38 and 0.52 for 5K PEG and 10K PEG micelles respectively). These findings further suggest that blending acid- and urea-functionalized copolymers before adding water yielded mixed micelles with nanosize and narrow size distribution.

Doxorubicin (DOX) was loaded into micelles *via* a membrane dialysis method. DOX-loaded PEG-PAC or PEG-PUC micelles had sizes close to or above 200 nm with polydisperse distribution (PDI: 0.44–0.78) (Fig. S2B and D, Table 1) due to the formation of aggregates upon dialysis. In contrast, the particle sizes of the drug-loaded mixed micelles formed from 5K PEG-PAC and 5K PEG-PUC or 10K PEG-PAC and 10K PEG-PUC were below 100 nm with monodisperse distribution (66 and 87 nm with PDI of 0.14 for 5K PEG and 10K PEG mixed micelles, respectively) (Table 1, Fig S2B and S2D). The DOX-loaded 5K PEG mixed micelles were further observed under TEM, and spherical nanoparticles were seen (Fig. S3). Particle size is important in the consideration of *in vivo* applications as it would affect the biodistribution of the nanoparticles [17]. Nanoparticles of approximately 10 nm are readily cleared from the blood and excreted *via* glomerular filtration, rendering them unable to accumulate in the tumor effectively [18]. Nanoparticles of more than 200 nm size on the other hand are known to accumulate in the liver and spleen [19] with lower accumulation in the tumor [20]. The mixed micelles reported in this study have sizes of 66 and 87 nm and are thus expected to accumulate in the tumor based on the EPR effect.

Our previous study proved that the presence of the acid groups in micelles significantly increased loading capacity of amine group-containing drugs due to ionic interactions between the acid group in the micelle and amine group in the drug [8]. Similarly, 5K PEG and 10K PEG mixed micelles achieved 28.9 and 22.8% of DOX loading, respectively, which were close to those yielded by the acid-functionalized micelles, but significantly higher than those given by the urea-functionalized micelles (Table 1). PEG10K-PUC led to a lower drug loading level as compared to PEG5K-PUC. Since the overall hydrophobicity of PEG10K-PUC is lower than PEG5K-PUC, PEG10K-PUC micelle formation might be slower and thus more



Scheme 1. Synthesis procedures and structures of acid- or urea-functionalized polycarbonates copolymers using monomethoxy PEG as a macroinitiator.

Table 1
Characteristics of mixed micelles.

Polymers	Mn[a] (Mn/Mw)[b]	CMC (mg/L)	Particle size (nm)		PDI		DOX loading level (wt%)
			Blank	DOX-loaded	Blank	DOX-loaded	
PEG5K-PAC	6440 (1.08)	63.1	231 ± 16	190 ± 17	0.35 ± 0.04	0.78 ± 0.08	33.5 ± 0.5
PEG5K-PUC	7900 (1.10)	15.8	41 ± 1	899 ± 159	0.19 ± 0.01	0.44 ± 0.07	20.6 ± 1.5
PEG5K-PAC/PEG5K-PUC		16.8	66 ± 1	87 ± 1	0.21 ± 0.01	0.14 ± 0.01	28.9 ± 1.3
PEG10K-PAC	11,440 (1.13)	595.6	126 ± 22	274 ± 4	0.29 ± 0.07	0.71 ± 0.05	29.8 ± 0.7
PEG10K-PUC	12,900 (1.11)	17.8	29 ± 1	211 ± 38	0.18 ± 0.01	0.55 ± 0.05	3.2 ± 0.3
PEG10K-PAC/PEG10K-PUC		22.4	55 ± 1	66 ± 3	0.17 ± 0.01	0.14 ± 0.02	22.8 ± 1.7

[a] Determined from ^{1}H NMR spectra.
[b] Acquired from GPC measurement.

DOX might leak out from the dialysis bag, lowering drug loading level. Interestingly, the 5K PEG mixed micelles made from polymers with shorter PEG length exhibited higher drug loading efficiency, which resulted in slightly bigger particle size (87 *vs*. 66 nm). These results show that PEG length exerted an impact on drug loading capacity and particle size.

It is desirable for drug-encapsulated nanoparticles to be stored in a dry form in the long-term as opposed to its aqueous solution because the drug may be released out from the nanoparticles in the latter state. Reconstitution of lyophilized nanoparticles is usually aided with the use of cryoprotectants such as sugars to avoid aggregation and precipitation of nanoparticles. The lyophilized DOX-loaded mixed micelles were able to redisperse in water easily without use of cryoprotectants, and the particle sizes of the micelles after redipersion were close to those measured before lyophilization (86 ± 2 and 74 ± 1 nm for 5K PEG and 10K PEG mixed micelles respectively).

3.3. Stability of DOX-loaded mixed micelles

For *in vivo* application, it is imperative for the micelles to be stable in the presence of serum. As shown in Fig. 1A, the particle size of the DOX-loaded 5K PEG and 10K PEG mixed micelles did not change significantly after 48 h of incubation with 10% serum, and no precipitation or aggregation was observed. This demonstrated the potential of these mixed micelles for *in vivo* application. The kinetic stabilities of the DOX-loaded mixed micelles were further studied by challenging the micelle solutions with a destabilizing agent, SDS, and then monitoring scattered light intensity of the solutions with DLS. The drop in the scattered light intensity reflects the degree of micelle dissociation. As evidenced in Fig. 1B 5K PEG mixed micelles were significantly more stable than 10K PEG mixed micelles. In the two mixed micelle systems, the length of the hydrophobic block of the diblock copolymers was kept constant. Thus, the increase in PEG length increased the overall hydrophilicity of the diblock copolymers and altered the hydrophilicity/hydrophobicity balance, leading to lower kinetic stability. Similarly, Creutz *et al.* showed that by increasing the hydrophobicity of poly((dimethylamino)alkyl methacrylate-block-sodium methacrylate) copolymers, the rate of micelle dissociation was lowered, giving rise to better stability [21]. Additionally, because the cargo loading capacity of the 10K PEG mixed micelles was lower, there was less pi–pi stacking of DOX molecules in the micellar core to maintain the micellar structure, resulting in easier dissociation. These results indicate that the DOX-loaded 5K PEG mixed micelles with greater kinetic stability are more desirable for *in vivo* application as compared to the 10K PEG mixed micelles.

3.4. In vitro drug release and cytotoxicity

Sustained release of drug from nanoparticles represents another important characteristic for a drug delivery system to be applied *in vivo*. The *in vitro* release profiles of DOX from the PEG-PAC/PEG-PUC mixed micelles with different PEG lengths are shown in Fig. 2. The release profiles showed no significant initial burst, and 38.6% and 48.6% of the encapsulated DOX were released in a sustained manner over 8 h for the 5K PEG and 10K PEG mixed micelles, respectively. After 8 h, the release of DOX was too slow to be detected. However, inside cells, DOX release rate may be elevated due to the degradation of the copolymers in the presence of enzymes. DOX release from the

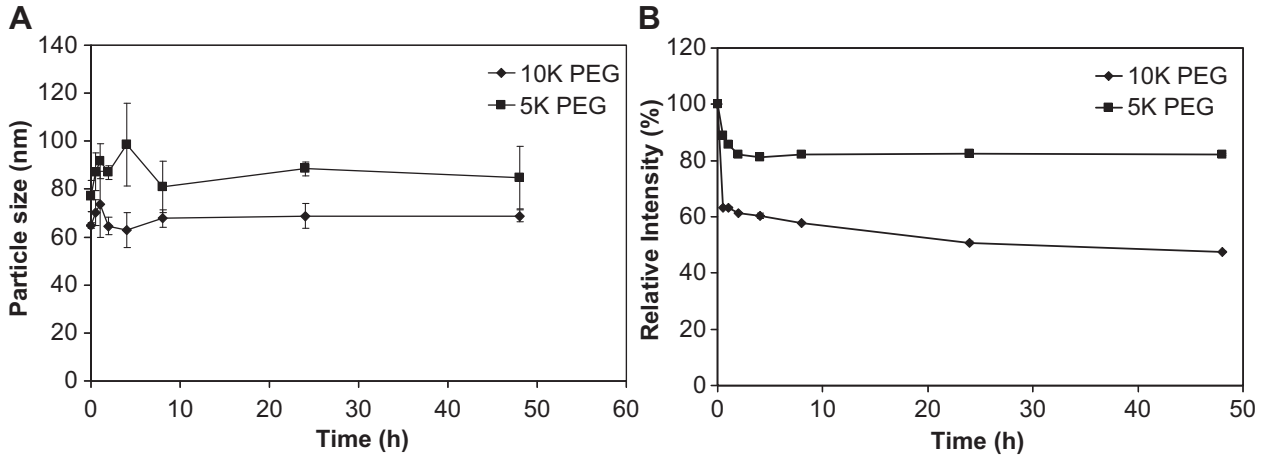


Fig. 1. Micelle stability. (A) Size of DOX-loaded mixed micelles made from the diblock copolymers with different PEG lengths in DI water containing 10% fetal bovine serum changes as a function of time. (B) Scattered light intensity measured at 90° of DOX-loaded mixed micelles against time after being challenged with SDS. Relative intensity (%) is represented as the percentage of the scattered light intensity at time x in relative to the scattered light intensity at time 0.

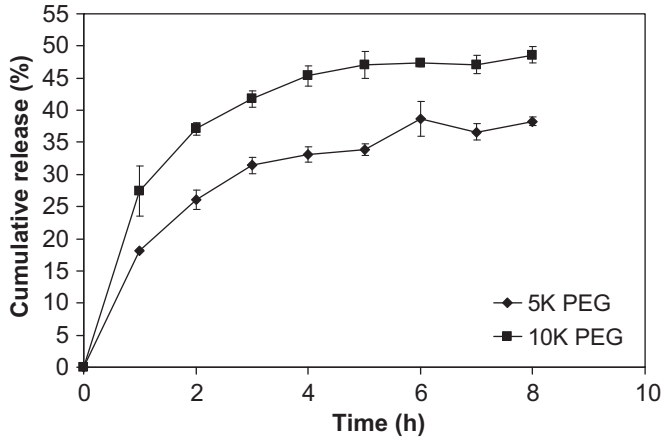


Fig. 2. *In vitro* release profiles of DOX-loaded mixed micelles formed from the diblock copolymers with different PEG lengths in PBS (pH 7.4) at 37 °C.

5K PEG mixed micelles was relatively slower as compared to that from the 10K PEG mixed micelles possibly because DOX took a longer distance to diffuse from the bigger 5K PEG micelles to the medium (Table 1).

The cytotoxicity of DOX-loaded mixed micelles was studied against HepG2 and 4T1 cell lines in comparison with free DOX and blank mixed micelles. From Fig. 3, it can be seen that free DOX and the DOX-loaded mixed micelles of different PEG lengths were able to inhibit cancer cell proliferation efficiently in a concentration-dependent manner. DOX-loaded mixed micelles had relatively higher IC_{50} than free DOX (HepG2: 0.2 mg/L for free DOX and 0.5 mg/L for 5K PEG and 10K PEG mixed micelles; 4T1: 0.7, 1.7 and 2.0 mg/L for free DOX, 5K PEG and 10K PEG mixed micelles respectively) probably due to sustained release of DOX from the mixed micelles. It is interesting to note that DOX-loaded 5K PEG and 10K PEG mixed micelles had comparable IC_{50} values although *in vitro* DOX release from 10K PEG mixed micelles was faster over 48 h (Fig. 2). *In vitro* DOX release from the mixed micelles was most likely due to DOX diffusion as polycarbonate degradation is extremely slow without enzymes [15]. Inside the cells, the presence of enzymes accelerated polycarbonate degradation, and thus DOX release might be dominated by polycarbonate degradation. As both 5K PEG and 10K PEG block copolymers had similar length of polycarbonate block, polycarbonate degradation rate might be similar, leading to similar DOX release rate inside the cells and thus exhibiting similar cytotoxicity. Importantly, blank mixed micelles did not exert any cytotoxicity against either of the cancer cell lines (Fig. S4), suggesting that the cell cytotoxicity of DOX-loaded micelles was solely due to DOX.

3.5. Biodistribution of mixed micelles in tumor-bearing mice

Tumor-targeting ability of the mixed micelles by virtue of the EPR effect was evaluated in BALB/c mice bearing subcutaneous 4T1 tumors through systemic injection. Mixed micelles incorporated with a near infra-red fluorescent (NIRF) dye, DiR, were injected intravenously *via* tail vein, and the biodistribution of the micelles monitored through noninvasive NIRF imaging over 96 h. DiR is a lipophilic dialkylcarbocyanine dye that fluoresces in the NIR band, which prevents any light absorption by tissues, autofluorescence and scattering usually associated with the use of visible light dyes [22]. Whole-body imaging showed no DiR signal-positive area in all mice at 30 min post-administration of the DiR-loaded mixed micelles, suggesting that the micelles had not been accumulated in tissues (Fig. 4A and B). However, DiR signals could be discerned at 2.5 h post-administration of the 5K PEG mixed micelles, and gave DiR signals over a larger area as compared to the 10K PEG mixed micelles. This continued up to 5 h when there was a larger area of DiR signals in mice administered with the 5K PEG mixed micelles. The contrast of DiR signal in the tumor as compared to the rest tissues of the animal was already apparent at 5 h post-administration of the 5K PEG mixed micelles, implying that the micelles started accumulating in the tumor at 5 h (Fig. 4A). However, the contrast between the subcutaneous tumor and the rest of the tissues of the animal was seen only at 24 h onwards for the 10K PEG mixed micelles, and the intensity of DiR signal was weaker in the tumors at each time point as compared to the 5K PEG mixed micelles (Fig. 4). As a control, free DiR dye was also injected at an equivalent dose as that in the mixed micelle formulations. In comparison to the biodistribution images of the mixed micelles, non-specific biodistribution of fluorescent signals was observed with no DiR signal detected in the tumors at any of the time points for the free dye formulation (Fig. S5A). DiR signal was the strongest in the liver and spleen in contrast to the other organs (Fig. S5B), indicating that free DiR could be eliminated by the reticuloendothelial system. These findings demonstrated the ability of the mixed micelles to target the tumors, and the 5K PEG mixed micelles with greater kinetic stability accumulated faster and more in the tumors than the 10K PEG mixed micelles.

The tissue distribution of the DiR-loaded mixed micelles was investigated at 96 h post-administration. The intensity of the DiR

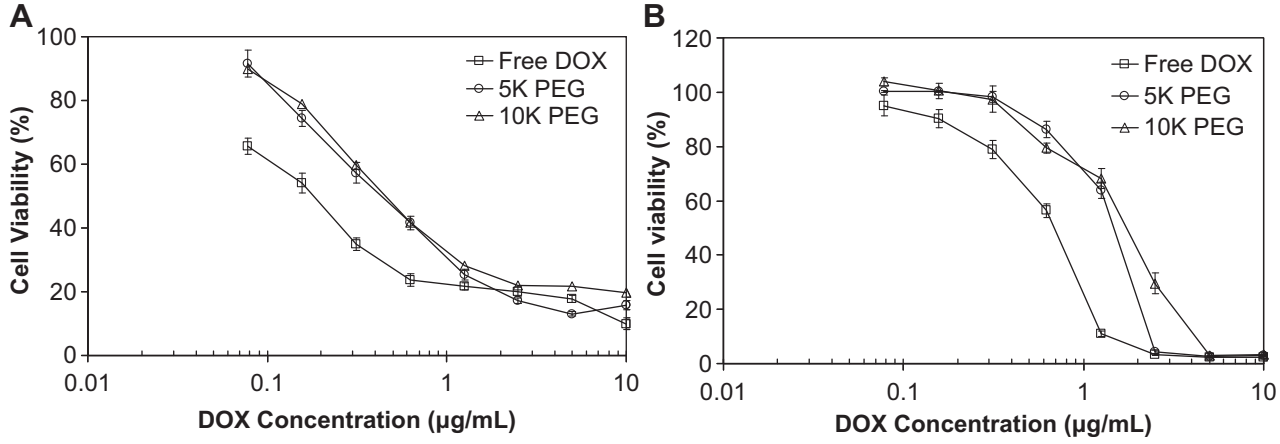


Fig. 3. Viability of (A) HepG2 and (B) 4T1 cells after incubation with free DOX, DOX-loaded 5K PEG and 10K PEG mixed micelles for 48 h at 37 °C.

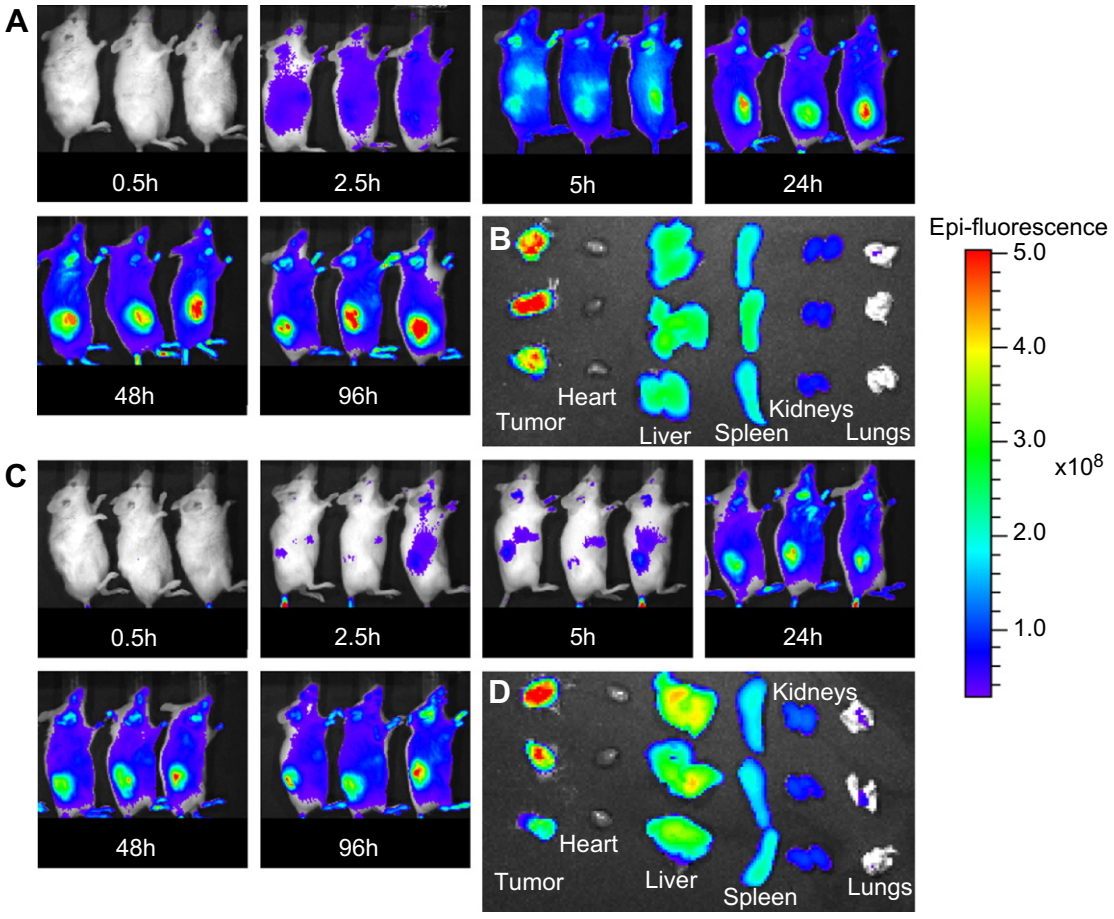


Fig. 4. Whole-body imaging of subcutaneous 4T1 tumor-bearing mice at various time points after tail veil injection of DiR-loaded 5K PEG (A) and 10K PEG (C) mixed micelles, and tissue distribution of DiR-encapsulated 5K PEG (B) and 10K PEG (D) mixed micelles at 96 h post-injection.

signal differed in the various organs with stark contrast seen in the tumors compared to the negligible fluorescence in the heart for both micelle formulations, which is particularly important as DOX can cause cardiotoxicity [23]. In addition, the DiR signal was more pronounced in the tumors for the 5K PEG mixed micelles, while the signal was more intense especially in the liver for the 10K PEG mixed micelles (Fig. 5B and D). This suggests that the 10K PEG mixed micelles dissociated more easily in the blood upon injection

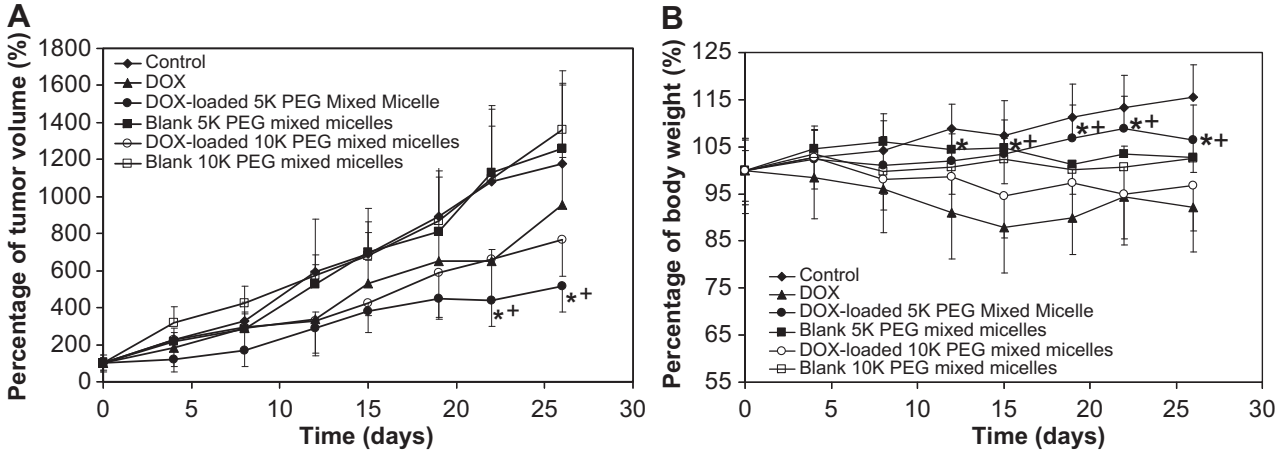


Fig. 5. Evolution of (A) tumor volume and (B) body weight over 26 days for mice bearing 4T1 tumors administered with PBS (control), free DOX, DOX-loaded 5K PEG and 10K PEG mixed micelles and their respective blank micelles. Percentage of tumor volume or body weight was calculated by dividing the tumor volume or weight at a given time point over the respective values at day 0 and being multiplied by 100%. Mice were administered with 5 mg/kg of DOX for free DOX and DOX-loaded mixed micelles and the equivalent weight of blank mixed micelles at days 0, 4, 8 and 12. The symbols * and + indicate significant difference in (A) tumor volume or (B) body weight between DOX-loaded 5K PEG mixed micelles-treated (●) and free DOX-treated (▲) mice and between DOX-loaded 5K PEG mixed micelles-treated (●) and 10K PEG mixed micelles-treated (○) mice respectively ($p < 0.05$).

due to its lower kinetic stability, hence releasing more DiR molecules, and the free DiR molecules would then distribute to the liver, giving rise to pronounced DiR signal in the organ. This finding was in line with what was seen in the control mice that were given free DiR (Fig. S5B). The micelles that remained intact would then accumulate in the tumor. As the 5K PEG mixed micelles were more kinetically stable (Fig. 1B), the micelles accumulated more effectively in the tumor, and are expected to provide higher antitumor efficacy.

3.6. *In vivo* antitumor efficacy

In vivo antitumor efficacy of DOX-loaded mixed micelles was investigated against 4T1 tumor-bearing BALB/c mice *via* tail vein injection, and tumor volume monitored as a function of time. As shown in Fig. 5A, the tumor growth rate was the highest in the negative control groups of mice injected with PBS, blank 5K PEG and 10K PEG mixed micelles, indicating that the mixed micelles themselves had no antitumor effect, and that any effect on tumor growth would be due to DOX. The mice treated with free DOX, DOX-loaded 5K PEG and 10K PEG mixed micelles showed a slower progression of tumor growth in varying extent, and DOX-loaded 5K PEG mixed micelles with greater kinetic stability suppressed tumor growth more effectively than free DOX and DOX-loaded 10K PEG mixed micelles especially at Day 22 and 26. This is due to the EPR effect and accumulation of 5K PEG mixed micelles in the tumor to a higher extent (Fig. 4).

The body weight of mice in the control and treated groups was also monitored as it reflects general toxicity of different delivery formulations. The body weight of the untreated mice increased over time (Fig. 5B), and there was about 15% increase at day 26. As the body weight of the healthy mice without the tumor increased only about 3% from the original weight (Fig. S6), the increase in the body weight of the tumor-bearing mice without treatment might be attributed mainly to tumor growth. Although the tumors in the mice treated with free DOX grew as fast as those in the control group without treatment, the body weight of the free DOX-treated mice decreased by approximately 10% at Day 26. This

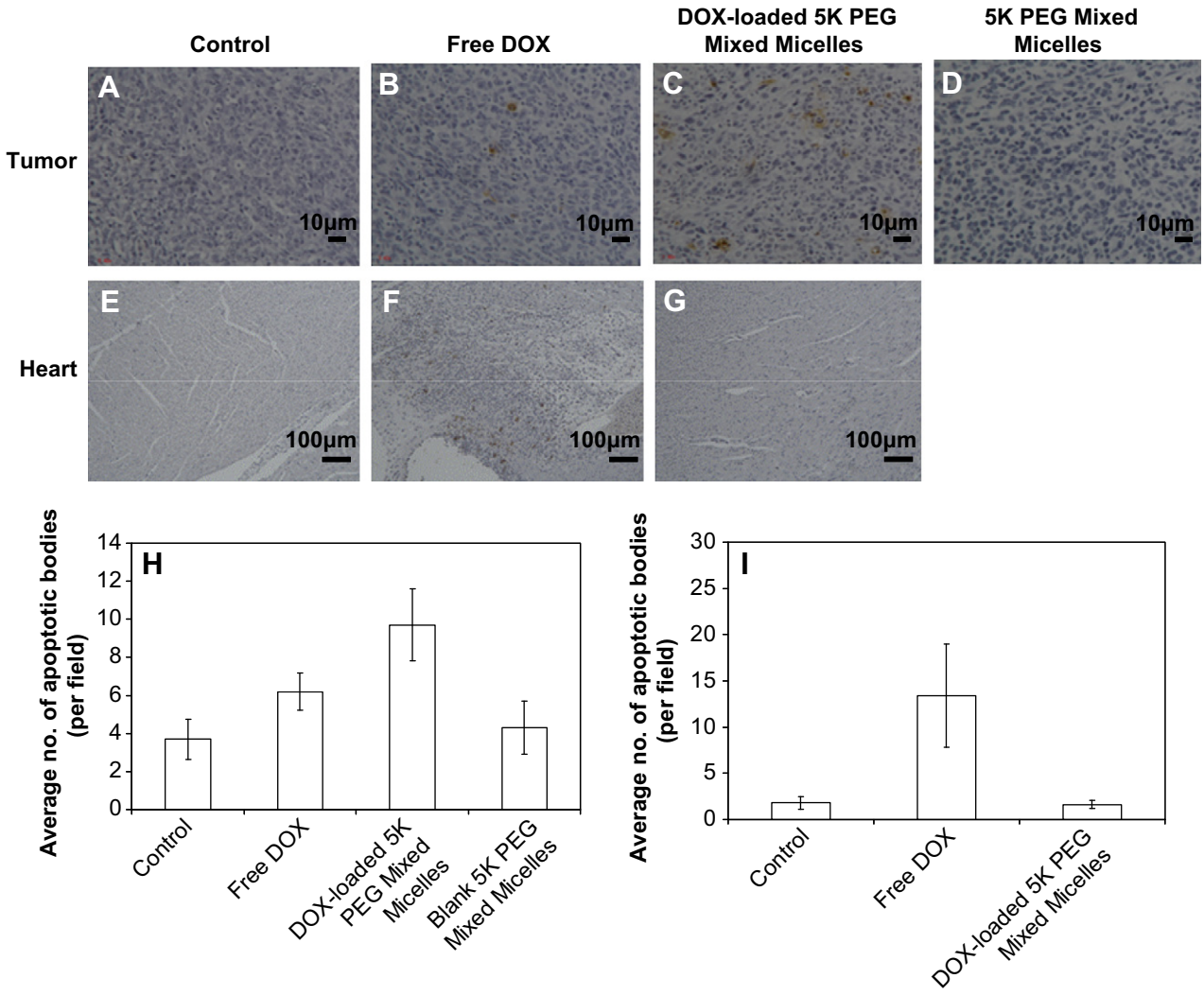


Fig. 6. Histological analysis of 4T1 tumors and hearts at the end of anti-tumor study for TUNEL-positive apoptotic bodies from a representative mouse in each treatment group. Tumor or heart sections from a mouse injected with PBS (A, E); tumor sections from a mouse treated with four doses of 5 mg/kg free DOX (B, F) at days 0, 4, 8 and 12; tumor sections from a mouse treated with four doses of 5 mg/kg DOX-loaded 5K PEG mixed micelles (C, G) and tumor sections from a mouse treated with four equivalent doses of blank 5K PEG mixed micelles (D) at days 0, 4, 8 and 12. Quantitation of mean apoptotic bodies per field (×400) in tumor (H) and heart (I) sections for the ten highest densities of apoptotic bodies was identified.

finding implies DOX toxicity. In addition, there was no significant difference in body weight between the free DOX-treated and DOX-loaded 10K PEG mixed micelles-treated groups. In contrast, the body weight of the mice treated with 5K PEG mixed micelles remained unchanged. These results demonstrated that 5K PEG mixed micelles with greater kinetic stability delivered DOX to the tumor more effectively than free DOX and 10K PEG mixed micelle formulations, hence reducing toxicity caused by non-specificity of DOX.

Since DOX-loaded 5K PEG mixed micelles were more effective and less toxic than DOX-loaded 10K PEG mixed micelles, the histological analysis of tumors and hearts of mice treated with DOX-loaded 5K PEG mixed micelles was conducted. Fig. 6 shows TUNEL-stained histological sections of tumors and hearts. By TUNEL staining, the presence of apoptotic cells in tissues can be visualized by brown regions. The number of apoptotic bodies in the tumors of untreated control mice and mice injected with blank 5K PEG mixed micelles was low (Fig. 6A, D and H, 4 ± 1 per field), which is in agreement with the observation of no therapeutic effect of the blank micelles (Fig. 5A). More apoptotic bodies were seen in the tumor sections of mice treated with free DOX or DOX-loaded 5K PEG mixed micelles. In particular, DOX-loaded mixed micelles induced a higher number of apoptotic cells than free DOX (Fig. 6B, C and H, 6 ± 1 and 10 ± 2 for free DOX and DOX-loaded 5K PEG mixed micelles respectively). These results further demonstrated that DOX-loaded 5K PEG mixed micelles had a higher antitumor activity than free DOX, and the antitumor mechanism of DOX-loaded mixed micelles is based on DOX-induced apoptosis.

It is well known that DOX causes cardiotoxicity in clinical use [23]. As shown in Fig. 6I, the heart sections of mice treated with PBS (Fig. 6E) and DOX-loaded 5K PEG mixed micelles (Fig. 6G) appeared normal with a low number of apoptotic bodies present (2 ± 1), while a significantly greater number of apoptotic bodies were seen in the heart sections of mice treated with free DOX (Fig. 6F, 13 ± 6). This indicates that DOX-loaded 5K PEG mixed micelles would not induce any cardiotoxicity while suppressing tumor growth effectively as the micelles did not amass in the heart, but accumulated mainly in the tumor, thus targeting DOX to the tumor. This reinforces the idea of drug delivery using polymeric micelles that allow for the drug to accumulate in the tumor site with minimal exposure to other organs, reducing or eliminating harmful side-effects caused by non-specificity of the drug.

4. Conclusion

Diblock copolymers with the same length of acid- or urea-functionalized hydrophobic polycarbonate block and different molecular weights of hydrophilic PEG block were successfully synthesized and mixed micelles were formed based on hydrogen bonding between urea and acid groups. DOX was readily encapsulated into mixed micelles with sizes below 100 nm and narrow size distribution as well as high drug loading level due to ionic interaction between the acid in the micelles and amine in the drug. In particular, the DOX-loaded mixed micelles made from diblock copolymers with 5K PEG were more kinetically stable than DOX-loaded mixed micelles made from diblock copolymers with 10K PEG. Although both 5K and 10K PEG mixed micelles preferentially accumulated in the tumor, the former was significantly more effective. Thus, DOX-loaded 5K PEG mixed micelles exerted stronger antitumor activity without inducing significant cardiotoxicity or body weight loss as compared to DOX-loaded 10K PEG mixed micelles. Therefore, 5K PEG mixed micelles can be a promising carrier for targeted delivery of anticancer drugs with amine functional group.

Acknowledgments

This work was supported by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research), Singapore.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.01.042.

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