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PAPER

Folate-conjugated crosslinked biodegradable micelles for receptor-mediated delivery of paclitaxel†

Juan Xiong,[a] Fenghua Meng,[a] Chao Wang,[b] Ru Cheng,[a] Zhuang Liu[b] and Zhiyuan Zhong*[a]

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The poor stability of micellar drug delivery systems *in vivo* due to large volume dilution and interactions with blood pool often leads to premature drug release with low targetability and therapeutic efficacy. Here, we designed folate-conjugated interfacially crosslinked biodegradable micelles consisting of poly (ethylene glycol)-*b*-poly(acryloyl carbonate)-*b*-poly(D,L-lactide) (PEG-PAC-PLA) and folate-PEG-PLA (FA-PEG-PLA) block copolymers for receptor-mediated delivery of paclitaxel (PTX) into KB cells. Micelles with varying amounts of folate ligands were prepared at 0–20 wt.% of FA-PEG-PLA. The resulting micelles, either with or without PTX loading, were readily crosslinked by UV irradiation. The crosslinked micelles had much smaller sizes and better stability as compared to the non-crosslinked controls. Notably, these micelles achieved high drug loading efficiencies of 70–88% at an initial PTX loading content of 10 wt.%. The *in vitro* release studies revealed that crosslinked micelles exhibited significantly inhibited PTX release at low micelle concentrations. MTT assays in KB cells showed that the crosslinked micelles were non-toxic while the toxicity of PTX-loaded micelles, either crosslinked or non-crosslinked, increased with increasing folate contents. Remarkably, at 12 h incubation time folate-decorated PTX-loaded crosslinked micelles composed of 20 wt.% of FA-PEG-PLA displayed markedly higher toxicity to KB cells than free PTX (33% *versus* 50% cell viability), which is most likely due to their much more efficient cellular uptake through FA receptor-mediated endocytosis. Flow cytometry studies showed that folate-decorated FITC-labeled crosslinked micelles were much more efficiently taken up by KB cells than controls without folate ligands. These results indicate that ligand-conjugated interfacially crosslinked PEG-PLA micelles have great potential in targeted cancer therapy.

Introduction

Drug delivery technology is the key to the clinical success of many potent drugs.[1–4] In recent years, polymeric micelles self-assembled from amphiphilic block copolymers have emerged as one of the most promising carrier systems for poorly water soluble drugs including doxorubicin (DOX) and paclitaxel (PTX) in that they offer several advantages such as enhancing the drug's water solubility, prolonged circulation time, passive targeting to the tumor tissues *via* the enhanced permeability and retention (EPR) effect, decreased side effects, and improved drug bioavailability.[1,5–7] In particular, biodegradable micelles based on block copolymers of poly(ethylene glycol) (PEG) and aliphatic biodegradable polyesters such as polylactide (PLA), poly(lactide-*co*-glycolide) (PLGA), and poly(ε-caprolactone) (PCL) are among the most studied systems,[8–13] due to their approved use in medical devices by the US Food and Drug Administration. Notably, a couple of micellar anti-cancer drug formulations, *e.g.* NK911® and Genexol-PM®, have already advanced to clinical trials.[1,5,14,15]

However, one remaining practical challenge for micellar drugs is their low *in vivo* stability since large volume dilution and interactions with blood pool following *i.v.* injection often leads to micelle dissociation or aggregation, premature drug release and/or low drug targetability.[16,17] In the past several years, various crosslinking approaches have been adopted to improve micellar stability.[17–19] The crosslinking of micelles could take place on the hydrophilic shell,[20–22] within the hydrophobic core,[23–27] or at the core-shell interface.[13,28] It should be noted, nevertheless, that there are only a few reports on development of crosslinked biodegradable micelles for anti-cancer drug delivery. Kissel *et al.* reported that core-crosslinked PEG-PCL micelles exhibited significantly enhanced PTX-loading efficiency and thermodynamic stability against dilution.[29] Hennink *et al.* reported that core-crosslinked micelles based on mPEG and N-(2-hydroxyethyl)methacrylamide)-oligolactates (PEG-*b*-p(HPMAm-Lac$_n$)) had prolonged circulation time and much higher accumulation in

[a]*Biomedical Polymers Laboratory, Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, 215123, P. R. China. E-mail: zyzhong@suda.edu.cn; Fax: +86-512-65880098; Tel: +86-512-65880098*
[b]*Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Institute of Functional Nano & Soft Materials (FUNSOM), Soochow University, Suzhou, 215123, P. R. China*
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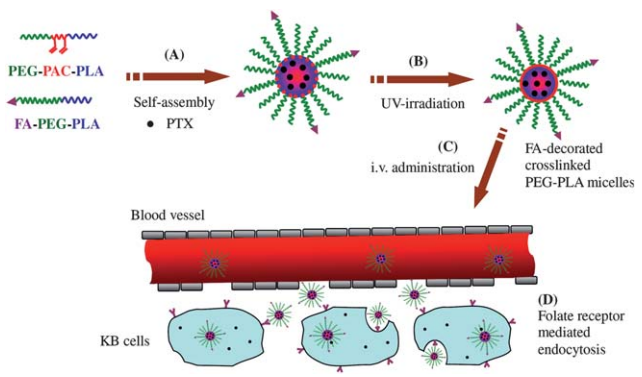
the tumor as compared to the non-crosslinked micelles.[24] More recently, it was shown that pH-activatable DOX-conjugated core-crosslinked PEG-*b*-p(HPMAm-Lac$_n$) micelles led to better anti-tumor activity in B16F10 bearing mice than free DOX.[27] The interfacial crosslinking approach uniquely combining advantages of core and shell crosslinking is particularly interesting.[17,19] On one hand, it allows the crosslinking reaction to take place at high micelle concentrations without inter-micellar crosslinking, and on the other hand, the properties of micellar core will not be altered much by crosslinking. We reported that interfacially crosslinked PEG-PCL micelles retained most drugs even at concentrations close to the CMC, while DOX was released in a rapid and controlled manner under a reductive environment mimicking that of the intracellular compartments.[13]

In this paper, we report on folate-conjugated interfacially crosslinked biodegradable micelles consisting of poly(ethylene glycol)-*b*-poly(acryloyl carbonate)-*b*-poly(D,L-lactide) (PEG-PAC-PLA) and folate-PEG-PLA (FA-PEG-PLA) block copolymers for receptor-mediated delivery of PTX (Scheme 1). FA has been widely exploited for tumor-targeting since FA-receptors are over-expressed on the surfaces of several different types of cancer cells.[30–32] Interestingly, our results revealed that FA-conjugated crosslinked biodegradable micelles have excellent stability with minimal release of PTX under dilute conditions while exhibit apparent targetability and high anti-cancer activity to KB cells. These folate-conjugated interfacially crosslinked biodegradable micelles are highly promising for targeted cancer therapy.

Experimental part

Materials

Methoxy poly(ethylene glycol) (PEG, $M_n = 5000$ g mol^{-1}, Fluka) was dried by azeotropic distillation from anhydrous toluene. D,L-lactide (LA) was recrystallized from dried toluene. Dichloromethane (DCM) were dried under an argon atmosphere by refluxing over CaH_2 and distilled prior to use. Zinc bis[bis[trimethylsilyl]amide] (97%, Aldrich), 1-[4-(2-hydroxy ethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959 or I2959, 98%, Sigma), paclitaxel (PTX, >99%, Beijing Zhongshuo Pharmaceutical Technology Development Co. Ltd.), and fluorescein isothiocyanate (FITC, 98%, Sigma) were used as received. Acryloyl carbonate (AC) monomer was synthesized according to a previous report.[33] Spectra/Pore® dialysis membranes with molecular weight cutoff (MWCO) of 3500 and 12000–14000 were purchased from Spectrum Laboratories Inc., USA.



Scheme 1 Illustration of folate-conjugated interfacially crosslinked biodegradable micelles for targeted delivery of paclitaxel. The micelles are prepared *via* a self assembly process (A) followed by crosslinking with UV irradiation (B). In contrast to non-crosslinked micelles that are prone to dissociation leading to premature drug release after *i.v.* injection, crosslinked micelles are stable in circulation resulting in enhanced accumulation in tumor tissues (C). The folate-conjugated crosslinked micelles are efficiently internalized by the KB cells *via* receptor-mediated endocytosis (D).

Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) as a solvent. The chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatography (GPC) instrument equipped with two linear PLgel columns following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL min^{-1} at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The micelle size was determined using dynamic light scattering (DLS) at 25 °C using Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. The amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase.

Synthesis of PEG-PAC-PLA triblock copolymer

Under a N_2 atmosphere and stirring, a solution of PEG (0.34 g, 0.068 mmol) and AC (0.15 g, 0.75 mmol) in DCM (3 mL) was added with a solution of zinc bis[bis(trimethylsilyl)amide] (0.013 g, 0.034 mmol). The reaction proceeded at room temperature (r.t.) for 3 d. Then, the second monomer, D,L-LA (0.28 g, 1.9 mmol), in 2 mL of DCM was added. The reaction was allowed to proceed at 40 °C for an additional 3 d before termination with acetic acid. The resulting PEG-PAC-PLA copolymer was isolated by precipitation in cold diethyl ether, filtration, and drying *in vacuo* for 2 d.

Synthesis of allyl-PEG-PLA

Allyl-PEG-PLA was synthesized by sequential anionic ring-opening polymerization of EO and D,L-LA in one pot using allyl alcohol/potassium naphthalene as an initiator system. Under a N_2 atmosphere and stirring, EO (1.67 g, 38 mmol) was added to a solution of potassium naphthalene (0.397 g, 2.4 mmol), allyl alcohol (0.018 g, 0.31 mmol) and 18-crown-6 (0.075 g, 0.28 mmol) in THF (5 mL) at 0 °C. The polymerization was conducted at 35 °C for 3 d. Then, one sample was taken for measurements. To the rest of the reaction mixture was added a solution of D,L-LA (1.67 g, 11.6 mmol) in THF (8 mL). The polymerization was allowed to proceed at 40 °C for additional 3 d before termination with acetic acid. The resulting copolymer was isolated by precipitation in hexane, dissolving in DCM and re-precipitation in cold diethyl ether, filtration, washing with diethyl ether, and drying *in vacuo* for 2 d.

Synthesis of amine-PEG-PLA

Under a N_2 atmosphere, to a 25 mL reaction vessel equipped with a magnetic stirrer were introduced allyl-PEG-PLA (0.3g, 0.027 mmol), 2-aminoethanethiol hydrochloride (0.077 g, 0.68 mmol), AIBN (0.0462 g, 0.28 mmol) and dry DMF (4 mL). The mixture was stirred at 60 °C for 24 h. The product, amine-PEG-PLA, was isolated by precipitation in cold diethyl ether, filtration, and drying *in vacuo* for 2 d.

Synthesis of folate-PEG-PLA

The carboxyl groups of folic acid (0.12 g, 3 mmol) was activated in DMSO (13 mL) using DCC (0.062 g, 3.3 mmol) and NHS (0.03 g, 3 mmol) in the dark at r.t. for 12 h. Then, a solution of amine-PEG-PLA (0.658 g, 1 mmol) and TEA (1 mmol) in DMSO (10 mL) was added. The reaction was allowed to proceed at r.t. for 24 h. The product was isolated by filtration to remove 1,3-dicyclohexylurea (DCU), dialysis against DMSO for 48 h and milli Q water for another 48 h (MWCO 3500), and freeze-drying. The degree of folate conjugation was determined by absorbance of folate at 363 nm in the UV-Vis spectrum.

Micelle formation and critical micelle concentration determination

Micelles were prepared by dropwise addition of 4 mL phosphate buffer (PB, 10 mM, pH 7.4) to a DMF solution (1 mL) of PEG-PAC-PLA or PEG-PAC-PLA/FA-PEG-PLA mixture (5 mg mL^{-1}) under stirring at r.t. followed by dialysis against PB. The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The concentration of block copolymer was varied from 9.1×10^{-3} to 45.5 μM and the concentration of pyrene was fixed at 1.0 μM. The fluorescence spectra were recorded using Edinburgh FLS920 fluorometer (Edinburgh Instrument Ltd.) with the excitation wavelength of 330 nm. The emission fluorescence at 373 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{373}/I_{383} at low and high concentration regions.

Crosslinking of micelles

The biocompatible UV initiator, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl -1-propanone (Irgacure 2959 or I2959, Sigma), which has been widely used to prepare photo-crosslinked hydrogels for cell encapsulation and tissue engineering,[39] was employed as a photo-initiator to crosslink the micelles at the interface. 25 μL acetone solution of I2959 was introduced to a micelle solution of PEG-PAC-PLA or PEG-PAC-PLA/FA-PEG-PLA (1 mL, 0.67 mg mL^{-1}), resulting in a final I2959 concentration of 0.025 wt.%. The mixture was ultrasonicated for 1 h to evaporate the acetone. Then, the micelle solution was irradiated under the UV light (Intelli-Ray 400, Uvitron) at an intensity of 100 mW cm^{-2} for 3–15 min to yield crosslinked micelles. The stability of crosslinked micelles against extensive dilution and addition of THF was studied using dynamic light scattering (DLS) as described previously.[13,28]

Preparation of FITC-labeled FA-functionalized micelles

The micelles were prepared as described above from a mixture of PEG-PAC-PLA (0.4 mL, 5 mg mL^{-1}), FA-PEG-PLA (0.16 mL, 5 mg mL^{-1}) and NH_2-PEG-PLA (0.24 mL, 5 mg mL^{-1}). The micelles were crosslinked by UV irradiation. FITC was conjugated to the micelles by treating micelles (4 mL, 0.76 mg mL^{-1}) with FITC (0.12 mg, 0.3 μmol) under the dark at pH 8.5 and r.t. for 5 h. Free FITC was removed by dialysis against PB (10 mM, pH 7.4). The conjugation amount of FITC was quantified by fluorescence using Edinburgh FLS920 fluorometer (Edinburgh Instrument Ltd.).

Loading and release of paclitaxel

Paclitaxel-loaded micelles were prepared by dropwise addition of 4 mL of phosphate buffer (PB, 10 mM, pH 7.4) to a mixture of copolymer (1 mL, 5 mg mL^{-1}) and paclitaxel (50 μL, 10 mg mL^{-1}) in DMF under stirring at r.t followed by ultrasonication for 1 h and dialysis against PB for 12 h at r.t (Spectra/Pore® dialysis membrane, MWCO 3500, Spectrum Laboratories Inc., USA). The micelles were crosslinked as described above by UV irradiation. Drug loading content (DLC) and drug loading efficiency (DLE) were determined as described previously.[34] The drug loaded in the micelles was extracted using acetonitrile, and the amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase. DLC and DLE were calculated according to the following formula:

$$\text{DLC (wt. \%)} = (\text{weight of loaded drug/weight of polymer}) \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug/weight of drug in feed}) \times 100\%$$

The release behaviors of PTX from the micelles were studied in PB (10 mM, pH 7.4) using dialysis tubes at 37 °C at different concentrations. PTX loaded crosslinked or non-crosslinked micelles were divided into two groups: one at a concentration of 0.33 mg mL^{-1} and the other at 0.066 mg mL^{-1}. 1 mL of PTX-loaded micelle dispersions was then transferred to dialysis tubes (Spectra/Pore® dialysis membrane, MWCO 12000–14000, Spectrum Laboratories Inc., USA). The dialysis tubes were immersed in 30 mL of PB. At desired time intervals, 7 mL of release media was taken out for HPLC measurement and replenished with an equal volume of fresh media. The amount of paclitaxel was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase. The release experiments were conducted in triplicate. The results presented are the average data.

MTT assays

Human nasopharyngeal epidermal carcinoma cells (KB cells) were plated in a 96-well plate (5×10^3 cells/well) in folate free RPMI-1640 supplemented with 10% fetal bovine serum (FBS)

and 1% penicillin/streptomycin (Invitrogen) for 24 h. The cytotoxicity of crosslinked PEG-PAC-PLA micelles was evaluated at varying micelle concentrations from 25 to 400 μg mL^{-1} in the presence of 0.05 wt.% photo-initiator I2959. The cells were incubated with micelles for 24 h at 37 °C in an atmosphere containing 5% CO_2. Then, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in 10 μL of PBS were added and incubated for another 4 h. The culture medium was aspirated, the MTT-formazan generated by live cells was dissolved in 100 μL of 10% SDS/0.1 M HCl overnight, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader (Bio-rad, ELX808IU). The cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$).

The cytotoxicity studies for PTX-loaded crosslinked micelles, PTX-loaded uncrosslinked micelles and free PTX (drug dosage 6 μg mL^{-1}) were carried out in a similar way. To evaluate folate-receptor mediated drug delivery, two culture media, *i.e.* folate free RPMI-1640 and folate containing RPMI-1640, were applied. The cells were incubated at 37 °C for 12 or 24 h with PTX-loaded crosslinked micelles, PTX-loaded uncrosslinked micelles and free PTX, respectively, in an atmosphere containing 5% CO_2. Then, 50 μg of MTT in 10 μL of PBS was added and incubated for another 4 h. The culture medium was aspirated, the MTT-formazan generated by live cells was dissolved in 100 μL of 10% SDS/0.1 M HCl overnight, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader (Bio-rad, ELX808IU). The cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$).

Flow cytometry analysis

KB cells were seeded in 24-well plates at 10^5 cells per well (1 mL) for 24 h, incubated with FITC-decorated micelles (either with or without FA ligands) at 37 °C for 12 or 24 h, and digested by 0.25 w/v% trypsin and 0.03 w/v% ethylenediaminetetraacetic acid (EDTA). The suspensions were centrifuged at 1000 × g and 4 °C for 4 min, pelleted in eppendorf tubes and washed twice with cold PBS, and then resuspended in 500 μL of PBS with 2% FBS. Fluorescence histograms were recorded with a BD FACSCalibur (Beckton Dickinson, USA) flow cytometer and analyzed using Cell Quest software. 20,000 gated events were analyzed to generate each histogram. The gate was arbitrary set for the detection of green fluorescence.

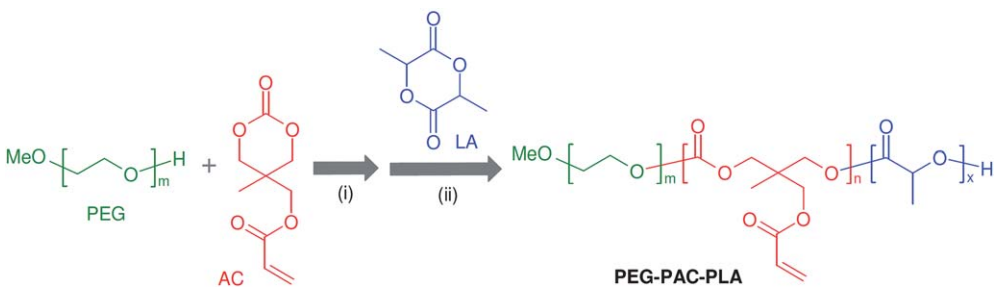
Results and discussion

Synthesis of PEG-PAC-PLA and FA-PEG-PLA block copolymers

The aim of this study was to develop folate-conjugated interfacially crosslinked biodegradable micelles for receptor-mediated delivery of PTX, for which two block copolymers PEG-PAC-PLA and FA-PEG-PLA were prepared. PEG-PAC-PLA was designed to crosslink micelles at the interface by UV irradiation while FA-PEG-PLA was to target at FA-receptor over-expressing cancer cells. Here, both extent of crosslinking and FA ligand density could be readily tuned.

PEG-PAC-PLA triblock copolymer was synthesized by sequential ring-opening polymerization of acryloyl carbonate (AC) and D,L-LA using PEG as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst in CH_2Cl_2 at 30 °C (Scheme 2).[35] As in our previous report, AC could be readily obtained and copolymerized with other cyclic monomers.[33] The ^{1}H NMR spectrum of PEG-PAC-PLA copolymer showed clearly signals at δ 3.38, 3.63, 5.85–6.43, and 5.17 which were attributable to methoxy protons, methylene protons of PEG, acryloyl protons of PAC, and methine proton of PLA, respectively (Fig. 1). The molecular weights of PAC and PLA blocks were determined to be 1.2 and 4.8 kg mol^{-1}, respectively, by comparing integrals of signals at δ 5.85–6.43 and 5.2 to 3.63. GPC revealed that the resulting copolymer had a unimodal distribution with a polydispersity of 1.44 and an M_n of 11.6 kg mol^{-1}, close to the design and that determined by ^{1}H NMR. (Table 1, Entry 1).

FA-PEG-PLA was obtained in three steps, *i.e.* synthesis of allyl-PEG-PLA, conversion of allyl into primary amino function, and conjugation with folate *via* carbodiimide chemistry (Scheme 3). To warrant full exposure of FA at the surface, here PEG was designed to have an M_n of 6.0 kg mol^{-1}, higher than that of PEG in PEG-PAC-PLA triblock copolymer. Allyl-PEG-PLA was obtained by sequential anionic ring-opening polymerization of ethylene oxide (EO) and D,L-LA.[36] ^{1}H NMR spectrum displayed besides signals of PEG (δ 3.63) and PLA (δ 5.17 and 1.57) also resonances of allyl terminal (δ 5.85–6.95 and 5.17) and methine proton next to the hydroxy end (δ 4.35) (Fig. S1A†). ^{1}H NMR end group analysis indicated that PEG and PLA blocks had M_n values of 6.0 and 4.5 kg mol^{-1}, respectively. Notably, GPC



Scheme 2 Synthesis of PEG-PAC-PLA triblock copolymer by sequential ring-opening polymerization of acryloyl carbonate (AC) and D,L-LA (LA) using methoxy PEG as an initiator, zinc bis[bis(trimethylsilyl)amide] as a catalyst, and CH_2Cl_2 as a solvent. Conditions: (i) room temperature, 3 d; and (ii) 40 °C, 3 d.

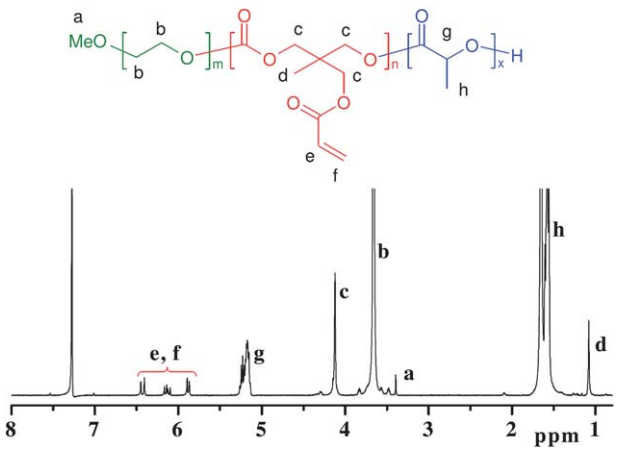


Fig. 1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of PEG-PAC-PLA triblock copolymer.

Table 1 Characteristics of PEG-PAC-PLA and allyl-PEG-PLA copolymers

		M_n (kg mol^{-1})			
Entry	Copolymers	Design	^{1}H NMR[a]	GPC[b]	PDI[b]
1	PEG-PAC-PLA	5.0-2.0-5.0	5.0.-1.2-4.8	11.6	1.44
2	ally-PEG-PLA	6.0-5.0	6.0-4.5	10.6	1.20

[a] Calculated from ^{1}H NMR. [b] Determined by GPC using PS standards.

revealed a low polydispersity of 1.20 and an M_n of 10.6 kg mol^{-1}, which was in line with that determined by ^{1}H NMR (Table 1, Entry 2). The allyl terminal was readily converted into primary amino groups by addition of 2-aminoethanethiol in the presence of AIBN in DMF at 70 °C. ^{1}H NMR showed that signals attributable to the vinyl protons completely disappeared while new peaks assignable to 2-aminoethanethioether moieties (δ 2.9 and 3.2) were detected (Fig. S1B†), indicating quantitative conversion of allyl to amine function. Finally, folic acid was attached to H_2N-PEG-PLA *via* carbodiimide chemistry to yield FA-PEG-PLA. The ^{1}H NMR spectrum showed signals attributable to the FA moieties at δ 6.6–8.6 (Fig. S1C†). UV-Vis measurements indicated nearly 100% conjugation of FA.

Preparation of PTX-loaded interfacially crosslinked micelles

Micelles were readily prepared from PEG-PAC-PLA and FA-PEG-PLA copolymers *via* a solvent exchange method. Dynamic light scattering (DLS) measurements showed average diameters ranging from 80 to 125 nm depending on FA-PEG-PLA contents and polydispersities of 0.07–0.24 (Fig. 2). Using pyrene as a fluorescence probe, the critical micelle concentration (CMC) of PEG-PAC-PLA was determined to be approximately 0.23 μM (*ca.* 2.5 mg L^{-1}).

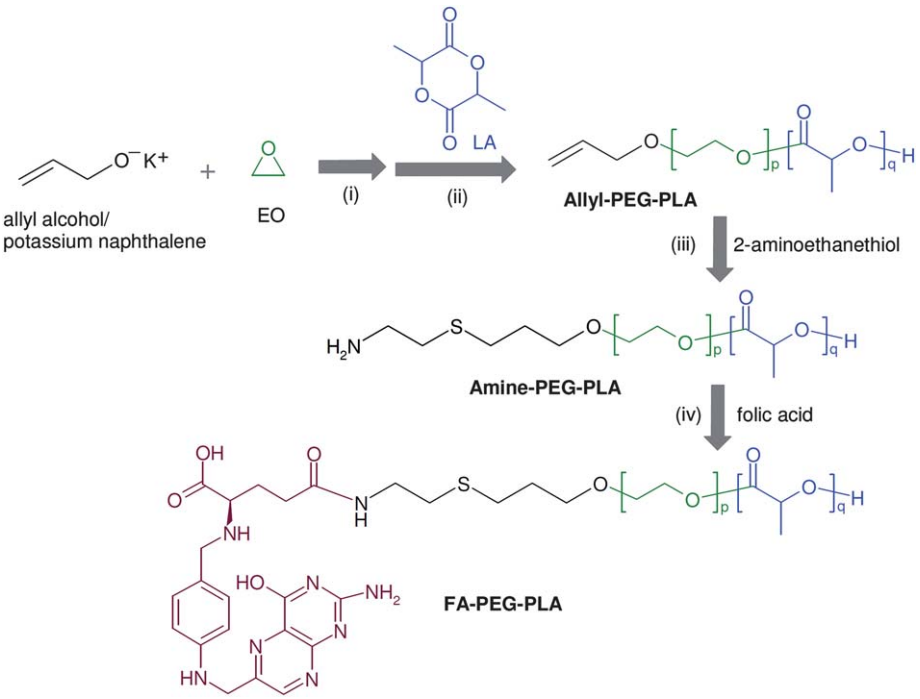
PEG-PAC-PLA/FA-PEG-PLA micelles were conveniently crosslinked by UV irradiation (100 mW cm^{-2}) in the presence of biocompatible photo initiator I2959 (0.025 wt.%) in PB (pH 7.4, 10 mM). DLS measurements showed shrinkage of micelle sizes by 10–15 nm following photo-crosslinking (Fig. 2). UV-vis spectrum of crosslinked micelles (CL micelles) demonstrated complete disappearance of acrylate absorbance at 250 nm (Fig. 3).

PTX, a potent anti-cancer drug, could be efficiently loaded into the micelles. For example, at a theoretical drug loading content of 10 wt.%, high PTX loading efficiencies of 70–88% were obtained (Table 2). PTX loading efficiency decreased with increasing the amount of FA-PEG-PLA, most likely because addition of FA-PEG-PLA decreases micelle crosslinking density leading to more pronounced drug loss during preparation of PTX-loaded micelles. PTX-loaded micelles had average sizes of about 164–172 nm, which were larger than the empty micelles (80–125 nm). Notably, PTX-loaded micelles were readily photo-crosslinked, in which 3 min irradiation yielded robust micelles. In comparison, under otherwise the same conditions, 15 min irradiation was typically needed for crosslinking of empty micelles. The size decrease of PTX-loaded micelles following UV irradiation was a very drastic 50–85 nm (Table 2).

The stability studies using DLS showed that CL micelles maintained similar size distribution even after 5000 times dilution (mimicking *i.v.* injection) (Fig. 4A), while two populations were observed for the non-crosslinked micelles (NCL micelles). In addition, in contrast to complete dissociation into unimers observed for NCL micelles upon addition of 5-fold THF for 3 h, CL micelles swelled to *ca.* 1000 nm with no unimers detected (Fig. 4B), confirming successful crosslinking of micelles. The mixed micelles of PEG-PAC-PLA and FA-PEG-PLA with up to 20 wt.% FA-PEG-PLA after UV crosslinking showed similar thermodynamic stability against dilution and addition of THF to crosslinked PEG-PAC-PLA micelles. These results point out that PTX-loaded interfacially crosslinked micelles can be readily prepared with high drug loading efficiency and superior stability.

In vitro PTX release

The *in vitro* release of PTX from CL and NCL micelles was studied at two concentrations (0.33 and 0.066 mg mL^{-1}) in PB (10 mM, pH 7.4) at 37 °C (Fig. 5). Two types of micelles, one made solely from PEG-PAC-PLA and the other with 20 wt.% FA-PEG-PLA, were used. The results showed that the release of PTX from the CL and NCL micelles at 0.33 mg mL^{-1} were both slow especially for the CL micelles of PEG-PAC-PLA. Interestingly, at a lower micelle concentration (0.066 mg mL^{-1}) the release of PTX from PTX-loaded CL micelles was significantly inhibited, wherein approximately 10% and 30% of drugs were released in 60 h from CL micelles of PEG-PAC-PLA and CL micelles containing 20 wt.% FA-PEG-PLA, respectively (Fig. 5A and B), in agreement with their high stability as shown previously. In contrast, *ca.* 90% and 80% of PTX were released in 60 h from the corresponding NCL counterparts under otherwise the same conditions. PTX-loaded CL mixed micelles containing 20 wt.% FA-PEG-PLA displayed higher release of PTX than the corresponding CL micelles of PEG-PAC-PLA alone (about 30% *versus* 10% release in 60 h), which is likely to be due to their relatively lower crosslinking density (FA-PEG-PLA does not contain crosslinkable acryloyl groups). At first, PTX-loaded CL mixed micelles showed slightly faster release of PTX as compared to the NCL counterparts (about 10% *versus* 5% release in 5 h) at a concentration of 0.33 mg mL^{-1}, which might be because



Scheme 3 Synthesis of FA-PEG-PLA copolymer. Conditions: (i) THF, 35 °C, 3 d; (ii) THF, 40 °C, 3 d; (iii) AIBN, 70 °C, 1 d; and (iv) DCC/NHS, room temperature, 1 d.

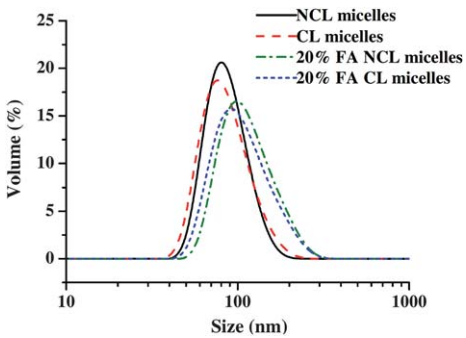


Fig. 2 The size distribution profiles of PEG-PAC-PLA micelles and mixed micelles of PEG-PAC-PLA and FA-PEG-PLA (20 wt.%) before and after UV crosslinking as measured by DLS.

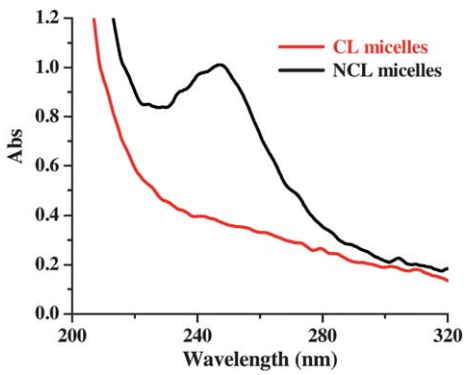


Fig. 3 UV-vis spectra of PEG-PAC-PLA micelle solutions before and after UV crosslinking (UV irradiation time 15 mins).

incorporation of 20 wt.% FA-PEG-PLA alters the properties of micellar core-shell interface and crosslinking pushes drugs towards the periphery of the micellar core. However, after this initial fast release, drug release from PTX-loaded CL mixed micelles was apparently inhibited as compared to the NCL counterparts.

It should be noted that loaded drugs would be released instantaneously from the NCL micelles at a micelle concentration close to or lower than the CMC owing to dissociation of micelles.[13] Here, due to the limit of detection, we were unable to perform the release studies at such low concentration. The current results have, however, indicated that interfacial crosslinking can largely enhance micellar drug stability and may effectively prevent premature drug release following i.v injection (Scheme 1).

Cytotoxicity of folate-decorated PTX-loaded CL micelles in KB cells

The targetability of FA-decorated PTX-loaded CL micelles was evaluated in KB cells, which are known to overexpress folate receptors.[37,38] KB cells were cultured in folate free medium and treated with PTX-loaded micelles for 12 or 24 h. The cell viability was assessed by MTT assays. The results showed clearly that for both NCL and CL micelles, the cytotoxicity of PTX-loaded micelles increased with increasing FA densities (Fig. 6), supporting that PTX is delivered into KB cells *via* a receptor-mediated mechanism. For example, cell viabilities of 65%, 55% and 33% were observed for KB cells following 12 h incubation with CL micelles containing 0, 10 and 20 wt.% of FA-PEG-PLA, respectively (Fig. 6A). Notably, at a short incubation time of 12 h, PTX-loaded CL micelles with 20 wt.% of FA-PEG-PLA

Table 2 Characteristics of PTX-loaded photo-crosslinked micelles[a]

Entry	Content of FA-PEG-PLA (wt.%)	NCL micelles (nm)[b]/PDI	CL micelles (nm)[b]/PDI	PTX loading content (wt.%)[c]	PTX loading efficiency (%)[c]
1	0	171/0.10	139/0.06	8.8	88
2	5	164/0.18	100/0.08	8.4	84
3	10	170/0.24	85/0.14	7.4	74
4	20	172/0.14	122/0.05	7.0	70

[a] Theoretical PTX loading content was 10 wt.%. [b] Determined by DLS. [c] Determined by HPLC.

displayed markedly higher toxicity to KB cells than free PTX (33% *versus* 50% cell viability), which is most likely due to their much more efficient cellular uptake through FA receptor-mediated endocytosis. The inhibition studies using folate-containing culture medium showed that toxicity of FA-decorated PTX-loaded micelles, either crosslinked or non-crosslinked, decreased to a level comparable to that of PTX-loaded PEG-PAC-PLA micelles (without FA), further confirming receptor-mediated uptaking of FA-decorated PTX-loaded micelles. The apparent targetability of FA-decorated micelles could be related to the excellent availability of FA at the micelle surface to interact with FA-receptors in KB cells, resulting from the longer PEG spacer of FA-PEG-PLA than that of PEG-PAC-PLA (6.0 *versus* 5.0 kg mol^{-1}). It should be further noted that there was no significant cytotoxicity difference between PTX-loaded CL and NCL

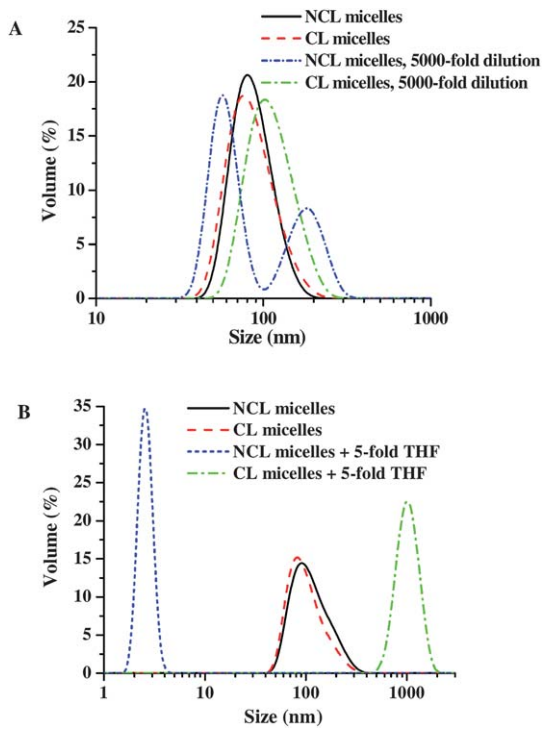


Fig. 4 Stability of NCL and CL micelles of PEG-PAC-PLA against dilution (5000 times) (A) and addition of 5-fold THF (B) as measured by DLS at an angle of 173°. The initial micelle concentration was 0.67 mg mL^{-1}.

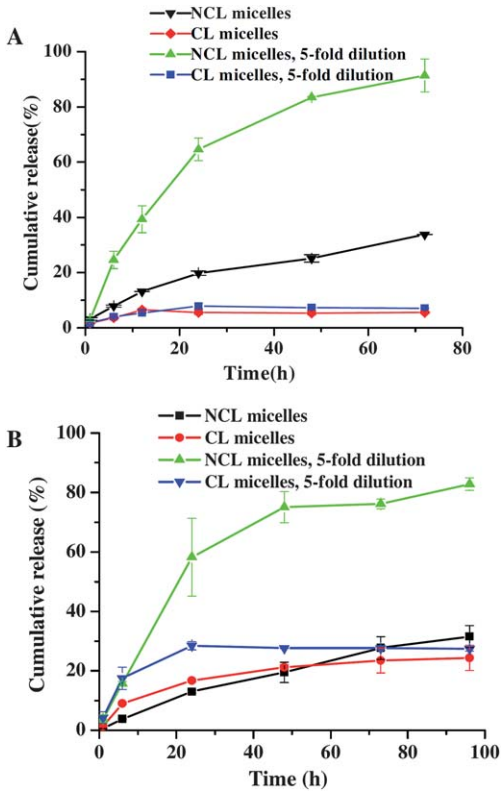


Fig. 5 *In vitro* PTX release from the CL and NCL micelles of PEG-PAC-PLA (A) and those of mixed micelles containing 20 wt.% of FA-PEG-PLA (B) at pH 7.4 and 37 °C. The initial micelle concentration was 0.33 mg mL^{-1}.

micelles at 12 h incubation, especially for FA-decorated micelles containing 20 wt.% FA-PEG-PLA. This result is exceptional and unexpected given the fact that significantly inhibited release of PTX is observed for PTX-loaded CL micelles (Fig. 5). The possible reason is that PTX-loaded CL micelles have much smaller particle sizes as compared to the NCL counterparts (122 nm *versus* 172 nm, Table 2), which has largely facilitated their entry into KB cells *via* the receptor-mediated endocytosis. In other words, the decreased drug release of PTX-loaded CL micelles might be effectively compensated by the active cellular uptake. This result further indicates that the mild photo-cross-linking conditions employed in this study have little influence on the therapeutic activity of PTX drug. The cell viabilities further decreased with increasing incubation time to 24 h, in which 52, 38 and 26% cells were found viable for CL micelles containing 0, 10 and 20 wt.% of FA-PEG-PLA, respectively (Fig. 6B). It should be noted, however, that at this prolonged incubation time (24 h), PTX-loaded CL micelles with 20 wt.% FA-PEG-PLA showed lower toxicity, though not significant, to KB cells than free PTX as well as corresponding PTX-loaded NCL micelles. This observation is in line with our hypothesis that FA-decorated CL micelles are more quickly and efficiently taken up by KB cells as compared to free PTX and the FA-decorated NCL counterparts. At a prolonged incubation time, the difference in cellular uptake between different systems is diminishing and intracellular drug release plays a more significant role in inducing cytostatic effects. It has to be noted, however, that for *in vivo* applications, it is

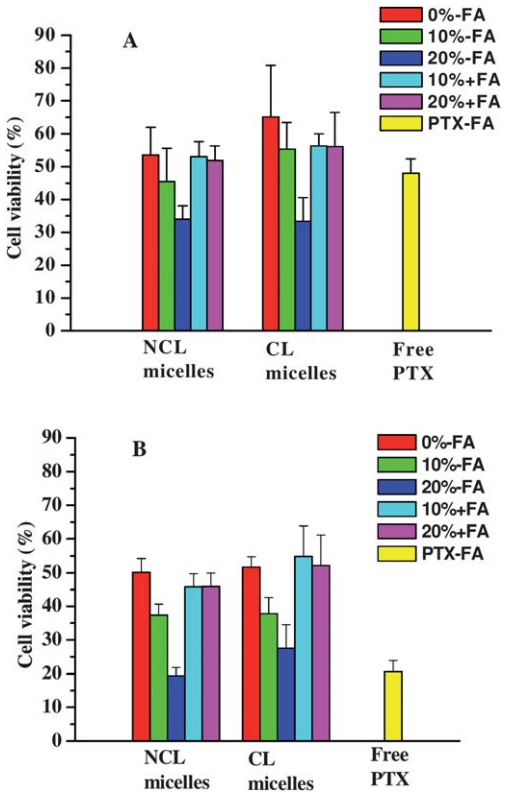


Fig. 6 Toxicity of PTX-loaded CL micelles *versus* PTX-loaded NCL micelles and free PTX in KB cells. PTX dosage was 6 μg mL^{-1}. The cells were incubated with micellar PTX or free PTX for 12 or 24 h in folate free (−FA) or folate containing medium (+FA). Data are presented as the average ± standard deviation ($n = 4$). (A) 12 h incubation; and (B) 24 h incubation.

unlikely that such a high concentration of free PTX or PTX-loaded NCL micelles would be present at the diseased sites for such a long treatment time.

Importantly, MTT assays showed that CL micelles of PEG-PAC-PLA in the presence of 0.05 wt.% photo initiator I2959 at the tested concentrations up to 400 μg mL^{-1} were non-toxic in KB cells after 24 h incubation (Fig. 7). I2959 was previously reported to have good cyto-compatibility.[39] Therefore, FA-decorated PTX-loaded CL biodegradable micelles with markedly enhanced stability over extensive dilution, superior targetability and toxicity to folate receptor-overexpressing cells are excellent candidates for tumor-targeted drug delivery.

Flow cytometry analysis of the cellular uptake of the micelles

The cellular uptake of FA-decorated CL micelles was studied by flow cytometry on KB cells. Flow cytometry has been used for quantitative determination of the cellular uptake of DOX and/or FITC-labeled micelles.[37,38,40,41] FITC-labeled CL micelles were obtained by coupling FITC to the surface of the CL micelles consisting of 30 wt.% NH_2-PEG-PLA and 20 wt.% FA-PEG-PLA. Interestingly, the results showed significantly higher (2.5 fold) cellular uptake of FA-decorated CL micelles than those without FA at 12 h incubation (Fig. S2†). The uptake of FA-decorated CL micelles was, however, drastically inhibited in

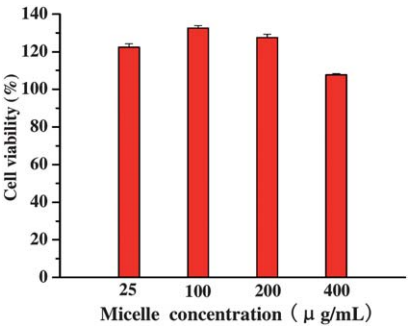


Fig. 7 Toxicity of CL micelles of PEG-PAC-PLA in the presence of 0.05 wt.% photo-initiator I2959 in KB cells. The cells were incubated for 24 h. Data are presented as the average ± standard deviation ($n = 4$).

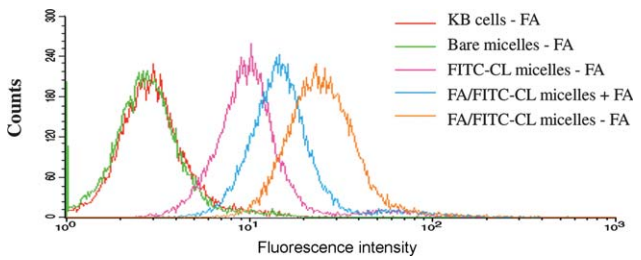


Fig. 8 Flow cytometry measurements on cellular internalization of FITC-labeled CL micelles (FITC-CL micelles) and FITC-labeled FA-decorated CL micelles (FA/FITC-CL micelles) into KB cells in folate free (−FA) or folate containing medium (+FA) following 24 h incubation (micelle concentration 0.2 mg mL^{-1}, cell counts 20000). KB cells and bare micelles (*i.e.* without FITC label) are used as negative control.

folate containing medium. At longer incubation time of 24 h, 3-fold higher cellular uptake was observed for FA-decorated CL micelles as compared to the counterparts without FA (Fig. 8 *versus* Fig. S3†). It is remarkable to note that FA-decorated CL micelles exhibited higher cellular uptake as compared to FA-decorated NCL micelles, which is most probably due to a comparably small size of the crosslinked micelles.

Conclusions

We have demonstrated that folate-conjugated interfacially crosslinked biodegradable micelles based on PEG-PAC-PLA and FA-PEG-PLA block copolymers are able to effectively deliver paclitaxel to KB cells, resulting in high anti-cancer activities. These novel systems present several unique features: (i) they can be readily prepared with varying extents of crosslinking and folate densities, in which FA is designed to be fully exposed to accomplish optimal targeting; (ii) they are highly stable with minimal drug release at low micelle concentrations reflecting the *i.v.* injection; (iii) they have high drug loading levels; and (iv) they are biodegradable and non-toxic. We are currently investigating the *in vivo* biodistribution, tumor-targetability and therapeutic efficacy of PTX-loaded FA-decorated crosslinked micelles of PEG-PLA. In addition, micelles with different types of targeting ligands are being developed. We are convinced that the ligand-conjugated crosslinked biodegradable micelles are highly promising for targeted cancer therapy.

Acknowledgements

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