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PAPER

Cross-linked micelles of graftlike block copolymer bearing biodegradable ε-caprolactone branches: a novel delivery carrier for paclitaxel

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The poor stability of micellar drug delivery system *in vivo* due to large volume dilution often leads to premature drug release with low therapeutic efficacy. In this study, shell cross-linked micelles of graftlike block copolymer bearing biodegradable ε-caprolactone branches (PMAA-*b*-PFM) were prepared to be used as a novel carrier for paclitaxel (PTX). PTX was successfully encapsulated into the hydrophobic cores of the cross-linked micelles using the dialysis method. The resultant PTX-loaded cross-linked micelles were about 99 nm in diameter with spherical shape and high encapsulation efficiency. The PTX-loaded cross-linked micelles had smaller sizes and better stability as compared to the non-cross-linked controls. Fluorescence microscopy and flow cytometry studies showed that PTX-loaded cross-linked micelles had excellent cellular uptake ability by bone marrow derived macrophages and human glioma U87 cells. Cellular uptake of cross-linked micelles was found to be higher than non-cross-linked controls due to smaller size. *In vitro* cytotoxicity studies also revealed that the PTX-loaded cross-linked micelles exhibit high anti-cancer activity to U87 cells. These results suggested that cross-linked PMAA-*b*-PFM micelles could be a potential vehicle for delivering hydrophobic chemotherapeutic drugs to tumors.

1. Introduction

Paclitaxel (PTX), one of the most successful chemotherapeutic drugs, has been confirmed to effectively kill a wide variety of tumor cells including ovarian carcinoma, breast cancer, head and neck cancers and non-small cell lung cancer.[1] However, because of the poor aqueous solubility and low therapeutic index of PTX, the clinical application of PTX is limited. A number of formulations were investigated for solubilization of PTX, including polymeric micelles,[2–5] liposomes,[6] microspheres,[7] nanoparticles,[8] PTX–polymer conjugates[9] and dendritic polymers;[10] among these new delivery vehicles, polymeric micelles have attracted increasing interest.

Polymeric micelles formed by self-assembly of amphiphilic block copolymers in aqueous solution have received widespread attention in the past decade.[11–22] Polymeric micelles have a unique core–shell structure which not only enables the system to incorporate poorly soluble drugs but also protects drugs from inactivation in biological media. Due to their small particle size, these systems exhibit many advantages such as targeting ability, long circulation and easy production of effective delivery systems.[5,23–27] Polymeric micelles are therefore considered to be an excellent delivery system for hydrophobic anti-cancer drugs. However, one remaining practical challenge for micellar drugs is their inferior *in vivo* stability.[28] In the past several years, various cross-linking approaches have been adopted to improve micellar stability.[15,29] The cross-linking of micelles could take place on the hydrophilic shell,[30] within the hydrophobic core,[31,32] or at the core–shell interface.[33] However, there are only a few reports on the development of cross-linked micelles for anticancer drug delivery.[4,34–37]

Poly(methacrylic acid) (PMAA) is one of the most commonly used hydrophilic polymers that possess several specific properties such as bioactive, pH and ionic strength responsiveness. A number of polymeric micelles formed from PMAA-based amphiphilic diblock copolymers have been investigated for various applications.[38–40] Macromonomer FM [CH_2=$C(CH_3)$ $OOCH_2CH_2(OCOCH_2CH_2CH_2CH_2CH_2)_5OH$], bearing long biodegradable hydrophobic ε-caprolactone branches, is commercially available and novel in both structure and properties.[41–43] We have previously reported micelle formation from an amphiphilic graftlike block copolymer of MAA and macromonomer FM (PMAA-*b*-PFM) in aqueous solution.[41,42] Cross-linking of the hydrophilic shell *via* condensation reactions between the carboxylic acid groups and the amino groups ultimately produces cross-linked micelles.[42] In this paper, we report on cross-linked micelles based on PMAA-*b*-PFM for delivery of PTX (Fig. 1). Our results revealed that cross-linked biodegradable micelles have excellent stability with minimal release of PTX under dilute conditions while they exhibit excellent cellular uptake ability and high anti-cancer activity to U87 cells. These

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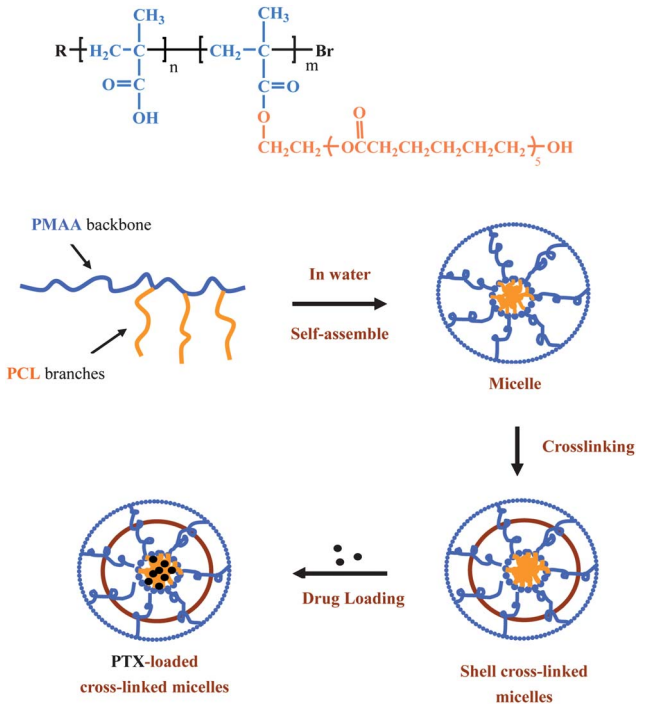

Fig. 1 (A) Illustration on the preparation of PTX-loaded cross-linked micelles of graftlike block copolymer PMAA-*b*-PFM.

cross-linked PMAA-*b*-PFM micelles are highly promising for targeted cancer therapy.

2. Materials & methods

2.1. Materials

Macromonomer FM [CH_2=$C(CH_3)OOCH_2CH_2(OCOCH_2CH_2CH_2CH_2)_5OH$] was purchased from Daicel Chem. Co. Ltd. and used as received. *tert*-Butyl methacrylate (t-BMA) was purchased from Wako, dried over CaH_2, and then distilled under reduced pressure before polymerization. Ethyl-α-bromopropionate (EPN-Br) and PMDETA (99.5%) purchased from Aldrich were used as received. Paclitaxel was purchased from Xi'an Sanjiang Bio-Engineering Co., Ltd. (Xi'an, China). All other reagents from Aldrich were purified following standard procedures prior to use.

2.2. Preparation of PTX-loaded cross-linked PMAA-*b*-PFM micelles

Cross-linked PMAA-*b*-PFM polymeric micelles were prepared as described earlier.[42] Briefly, block copolymer PMAA-*b*-PFM (0.05 g) was dissolved in 100 mL of DMF, followed by filtration of the solution through a 0.45 mm Teflon membrane filter (Chromatographic Specialties, Inc.). The micelles were formed by the addition of the resulting DMF solution into water at a rate of 1 dropper per 60 s with continuous stirring. The micelle solution was stirred for 24 h and then dialyzed in water using a cellulose dialyzer tube. The micellar structure was locked by cross-linking the hydrophilic shell layer through condensation reactions between the carboxylic acid groups of the PMAA block and the amino groups of hexamethylenediamine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide.[44] Briefly, to the micelle solution of PMAA-*b*-PFM was added dropwise an aqueous solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide. The mixture was stirred for about 10 min before the aqueous solution of the cross-linker, 2,2′-(ethylenedioxy)bis(ethylamine), was added dropwise. The reaction mixture was allowed to stir overnight at room temperature and then dialyzed against distilled water for 3–4 days to remove byproducts of the cross-linking reaction. The success of the cross-linking was confirmed by the fact that the resultant micelles maintained their integrity upon switching the medium from water to a solvent mixture containing a large proportion of DMF. DLS studies on the cross-linked particles found that no particle aggregation had taken place, which meant that there had been almost zero interparticle cross-linking.

PTX-loaded cross-linked PMAA-*b*-PFM micelles were prepared by a dialysis technique.[45,46] Briefly, certain amounts of PTX dissolved in methanol were slowly added to cross-linked PMAA-*b*-PFM micelle solutions under stirring, and then the methanol solvent was removed by dialysis (Millipore dialysis tube, molecular weight cutoff 12–14 kDa, USA) to obtain PTX-loaded cross-linked PMAA-*b*-PFM micelles. The dust and impurity in the sample solution were removed by passing through a filter (0.45 μm, Millipore). Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-*glycero*-3-phosphoethanolamine, triethylammonium salt (rDHPE; Invitrogen) was used to label cross-linked PMAA-*b*-PFM micelles and appeared as red fluorescence.

2.3. Characterization of PTX-loaded cross-linked PMAA-*b*-PFM micelles

Average particle size and size distribution of the micelles were measured using a Malvern Zetasizer Naso ZS (Malvern, UK). The shape and surface morphology of the micelles were investigated by atomic force microscopy (AFM): 100 μl of PMAA-*b*-PFM micelle solution was placed on a clean mica surface and then air-dried overnight. The image was obtained with a Benyuan CSPM 4000 AFM system.

2.4. Determination of drug loading capability

Drug loading capability of the cross-linked PMAA-*b*-PFM micelles was determined using high-performance liquid chromatography (HPLC). PTX-loaded micelle solution (50 μl) was transferred into a 10 ml volumetric flask and diluted with methanol to the mark. The solution was centrifuged at 10,000 rpm for 10 min, then 20 μl of supernatant was injected into the chromatographic system. The HPLC system (LC-2010C, Shimadzu, Japan) was equipped with a Lichrospher C18 column (4.6 × 250 mm, 5 μm) with a mobile phase of methanol and water (75 : 25), the flow rate and column temperature were set at 1 ml min^{-1} and 30 °C, respectively. The signals were recorded by a UV detector at 227 nm. A calibration line was conducted to determine the PTX concentration in the range of 0.5–25 mg l^{-1}, and the r^2-value of peak area against PTX concentration was at least 0.999. The encapsulation efficiency (EE, %) and the drug loading coefficient (DL, wt%) were calculated based on the following formulae:

EE (%) = weight of PTX in micelles/weight of the feeding PTX × 100%

DL (wt%) = weight of PTX in micelles/weight of the feeding micelles and PTX × 100%

2.5. PTX releases from cross-linked PMAA-*b*-PFM micelles *in vitro*

PTX release behavior was studied *in vitro* in phosphate buffered saline (PBS) solution. Briefly, the solutions of PTX-loaded cross-linked PMAA-*b*-PFM micelles were respectively placed into visking dialysis tubing (molecular weight cutoff 12–14 kDa, Millipore, USA) and dialyzed against 50 ml PBS with 1% Tween 80 at 37 °C in an air-bath shaker at 50 rpm. Then, 0.5 ml of the release medium was collected and replaced with an equal volume of the fresh release medium at predefined time intervals. The release amounts of PTX were determined by HPLC.

2.6. Cell culture

Mice femur bone marrow was dissociated into single cell suspensions. Bone marrow cells and bone marrow derived macrophages (BMM) were cultured with DMEM with fetal bovine serum, 1% glutamine, 10 mg ml^{-1} ciprofloxacin, and 500 U ml^{-1} macrophage colony stimulating factor (MCSF). Human glioma U87 cells labeled with green fluorescence were obtained from American Type Culture Collection (ATCC). For the MTT assay, cells were plated on 96-well plates to confluence and were allowed to adhere overnight.

2.7. Fluorescence microscopy

The rDHPE-labeled PMAA-*b*-PFM micelles exhibited red fluorescence. Images were captured using a Nikon TE2000-U (Nikon Instruments Inc., Melville, NY) with a swept-field confocal microscope, 488 nm (green) and 568 nm (red) laser excitation, and a 20× objective.

2.8. Flow cytometry

Flow cytometry was used to provide statistics on the uptake of micelles into U87 cells. U87 cells were seeded in six-well culture plates and grown overnight. Then, the micelles dispersed in DMEM culture medium with a concentration of 0.2 mg ml^{-1} were added to different wells, and the cells were incubated at 37 °C for 8 h. After the incubation, samples were prepared for flow cytometry analysis by removing the cell growth medium, rinsing with cold PBS, and treating with trypsin.

2.9. Cell viability assays

U87 and BMM cell viability was determined using the MTT assay. MTT reduction was measured in U87 and BMM with PTX concentration ranging from 0.001 to 1000 μg ml^{-1} for 12 h and then washed out with fresh medium. The assay was based on the ability of active mitochondrial dehydrogenase to convert dissolved MTT to water-insoluble purple formazan crystals. To each BMM or U87 containing well in a 96 well plate, 100 μl of MTT solution [5 mg ml^{-1} solution in 10% fetal bovine serum (FBS) in phosphate buffered saline (PBS)] was added and incubated for 4 h at 37 °C. At the end of the incubation period, the medium was replaced with 100 μl of dimethyl sulfoxide (DMSO) for 15 min at room temperature and absorbance at 490 nm was determined using an ELISA plate reader.

3. Results

3.1. Preparation and characterization of PTX-loaded cross-linked PMAA-*b*-PFM micelles

Our previous work showed that graftlike block copolymer PMAA-*b*-PFM could form nano-sized micelles with a core–shell

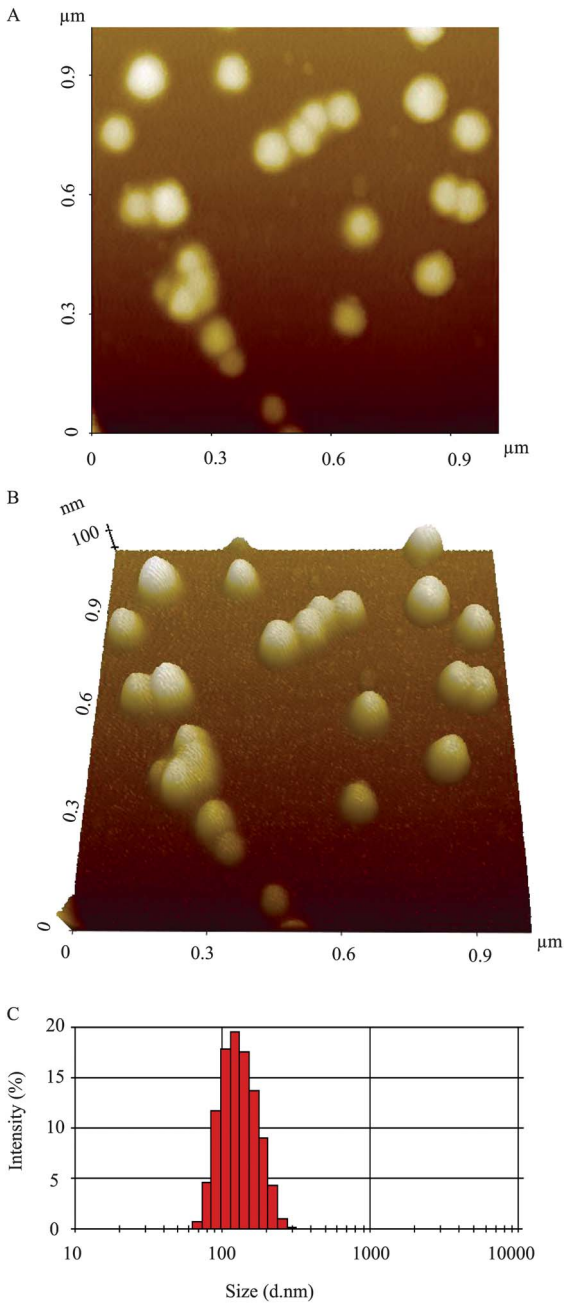

Fig. 2 AFM tapping mode images (A), AFM 3-D images (B) and the size distribution (C) of PTX-loaded cross-linked PMAA-*b*-PFM micelles with a PTX-loading content of 23.1%.

Table 1 Characteristics of the PTX-loaded cross-linked micelles of PMAA-*b*-PFM: particle size, size distribution, drug loading coefficient and encapsulation efficiency

Drug/Carrier[a] (W/W)	Particle Size[b] (nm)	Polydispersity index[b]	Drug Loading Coefficient (%)	Encapsulation Efficiency (%)
1 : 5	83 ± 4.1	0.32 ± 0.06	13.8 ± 1.3	87.8 ± 1.1
1 : 4	96 ± 2.5	0.22 ± 0.05	17.3 ± 0.9	85.3 ± 0.9
1 : 3	99 ± 2.3	0.21 ± 0.03	23.1 ± 1.1	80.1 ± 1.2
1 : 2	125 ± 4.8	0.35 ± 0.04	29.1 ± 1.2	68.1 ± 1.0

[a] The weight ratio of PTX to PMAA-*b*-PFM micelles. [b] The size and size distribution of PTX-loaded cross-linked PMAA-*b*-PFM micelles determined by DLS. Data represent mean ± SE, n = 3.

structure composed of a hydrophobic PCL core and a hydrophilic PMAA shell layer.[42] Cross-linking of the hydrophilic shell *via* condensation reactions between the carboxylic acid groups and the amino groups ultimately produces cross-linked micelles (CL micelles) (Fig. 1). The anticancer drug, paclitaxel (PTX), is known to be highly hydrophobic, so that we hope to use CL micelles of PMAA-*b*-PFM as a carrier for PTX to increase its aqueous solubility. The dialysis method was used to prepare PTX-loaded CL PMAA-*b*-PFM micelles in this study. PTX dissolved in methanol was first added to the micelle solution, and then was dialyzed against aqueous medium. In the dialysis process, methanol was gradually removed, which caused PTX to be spontaneously transferred from the aqueous medium into the hydrophobic cores of the micelles due to the driving force of hydrophobic interaction. AFM was employed to visualize micelle morphology. AFM images (Fig. 2A and 2B) show that PTX-loaded CL micelles of PMAA-*b*-PFM were still spherical in shape. The size distribution of the micelles was unimodal (Fig. 2C), also indicating the formation of the expected monodispersed PTX-loaded micelles.

The physicochemical characterization and drug-loading parameters of PTX-loaded CL micelles of PMAA-*b*-PFM are summarized in Table 1. The amount of PTX (*i.e.*, 10 mg) was kept constant, and the concentration of PMAA-*b*-PFM was varied accordingly (*i.e.*, 20, 30, 50 mg, *etc.*). A formulation of 1/3 (drug/carrier) was found to be appropriate with particle size of 99 ± 2.3 nm, drug loading coefficient of 23.1 ± 1.1% and encapsulation ratio of 80.1 ± 1.2%.

The conventional micelle drug delivery systems based on linear amphiphilic copolymers suffer from instability because micelles tend to disassemble *in vivo*. Maintaining the dimensional stability of micelles is crucial for many applications.[35,47] Cross-linking

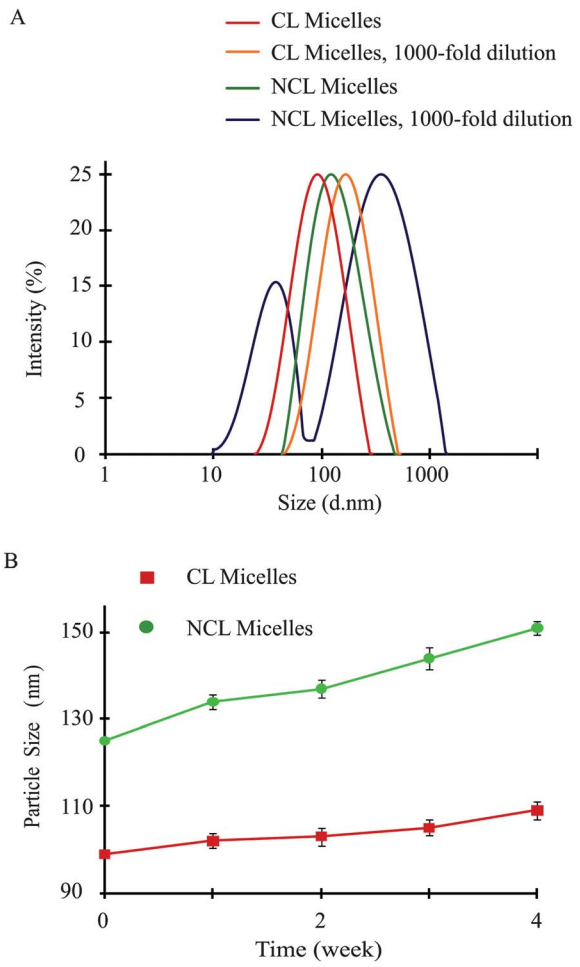

Fig. 3 (A) Stability of PTX-loaded PMAA-*b*-PFM micelles, as measured by DLS. (B) Storage stability of PTX-loaded PMAA-*b*-PFM micelles, micelle size as a function of time. The initial micelle concentration was 0.5 mg mL^{-1}.

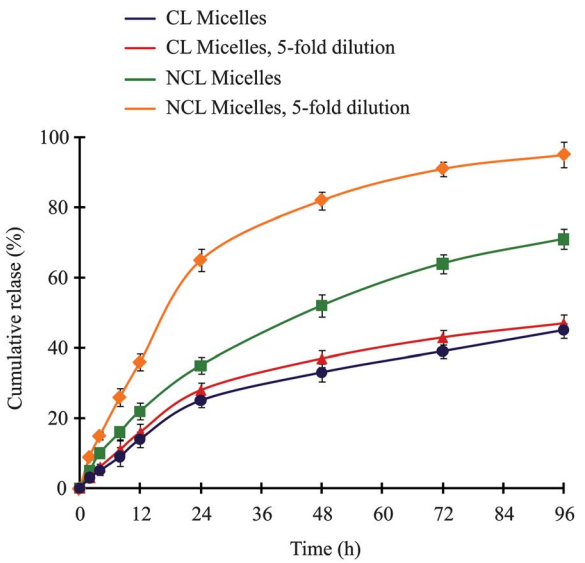

Fig. 4 Release of PTX from CL and NCL PMAA-*b*-PFM micelles in PBS with 1% Tween 80 at 37 °C. The initial micelle concentration was 0.5 mg mL^{-1}.

may improve the stability of the micelles. The stability studies using DLS showed that PTX-loaded CL micelles maintained similar size distribution even after 1000 times dilution (mimicking i.v. injection) (Fig. 3A), while two populations were observed for the non-cross-linked micelles (NCL micelles). Fig. 3B shows the CL and NCL micelle sizes as a function of time during storage. As shown in Fig. 3B, PTX-loaded NCL micelles showed obvious changes in size in 4 weeks, exhibiting poor storage stability, whereas the PTX-loaded CL micelles showed indicate that PTX-loaded shell CL micelles can be readily prepared with high drug loading efficiency and superior stability. This superior stability of the CL micelles was attributed to the cross-linking and the formation of a polymeric network, which restricted the chain segment mobility of the polymer and fixed the structure of the micelles.

3.2. *In vitro* drug release study

PTX release behavior from CL and NCL PMAA-*b*-PFM micelles was studied *in vitro* in PBS with 1% Tween 80 at 37 °C. The cumulative PTX release profiles are shown in Fig. 4. The results showed that the release of PTX from the CL and NCL micelles at 0.5 mg mL^{-1} was slow in both especially for the CL micelles of PMAA-*b*-PFM. Interestingly, at a lower micelle concentration (0.1 mg mL^{-1}) the release of PTX from PTX-loaded CL micelles was significantly inhibited, wherein approximately 40% of drugs were released in 72 h from CL micelles of PMAA-*b*-PFM (Fig. 4), in agreement with their high stability as shown previously. In contrast, *ca.* 90% of PTX was released in 72 h from the corresponding NCL counterparts under otherwise the same conditions. A similar phenomenon was also observed in other cross-linked micelle systems.[4,35] The suppression in release of PTX was also attributed to the cross-linking, which restricted the mobility of PTX, consequently, its release became slower. The current results also indicated that interfacial cross-linking can largely enhance micellar drug stability and may effectively prevent premature drug release following i.v. injection (Fig. 1).

3.3. *In vitro* cellular uptake of micelles

The cellular uptake of CL and NCL PMAA-*b*-PFM micelles was examined to demonstrate the penetration of the micelles into the cells. The micelles were tested for cell uptake at a constant concentration of 0.5 mg mL^{-1}. As shown in Fig. 5 and Fig. 6, representative images reveal increasing intensity of the fluorescence signal of both CL and NCL micelles, demonstrating rapid uptake by BMM and U87 cells. Specifically, CL micelles entered BMM and U87 cells within 1 h of incubation. At 4 h, more than 50% of BMM and U87 cells contained varying levels of micelles and increased to 95% by 8 h. The cellular uptake of CL and NCL micelles was also studied by flow cytometry on U87 cells. Flow cytometry has been used for quantitative determination of the cellular uptake of drug and/or FITC-labeled micelles.[48–51] The results showed significantly higher (2.0 fold) cellular uptake of CL micelles than NCL micelles at 8 h incubation (Fig. 7), which is most probably due to a comparably small size of the cross-linked micelles. Taken together, for PTX-loaded CL micelles of PMAA-*b*-PFM, the smaller size may facilitate the cellular uptake, resulting in higher cellular uptake.

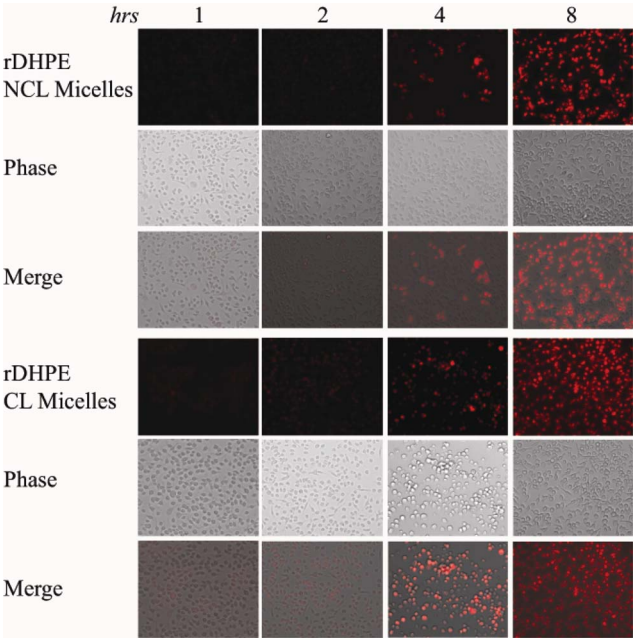

Fig. 5 PTX-loaded CL and NCL PMAA-*b*-PFM micelles uptake by BMM. Fluorescent microscopy images of BMM treated with 0.5 mg mL^{-1} rDHPE-labeled CL and NCL PMAA-*b*-PFM micelles.

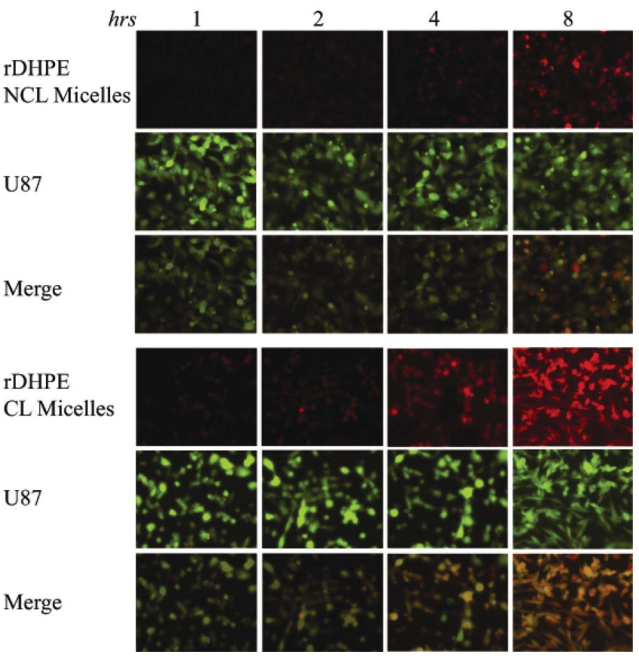

Fig. 6 PTX-loaded CL and NCL PMAA-*b*-PFM micelles uptake by U87 cells. Fluorescent microscopy images of U87 cells treated with 0.5 mg mL^{-1} rDHPE-labeled CL and NCL PMAA-*b*-PFM micelles.

3.4. Toxicity

We investigated PTX-loaded micelle toxicity to macrophages and tumor cells. BMM (Fig. 8A) and U87 (Fig. 8B) were treated with varying concentrations of micelles–blank, micelles–PTX and free PTX and cell viability was measured by MTT assay. We found no significant toxicity to BMM for micelles–blank,

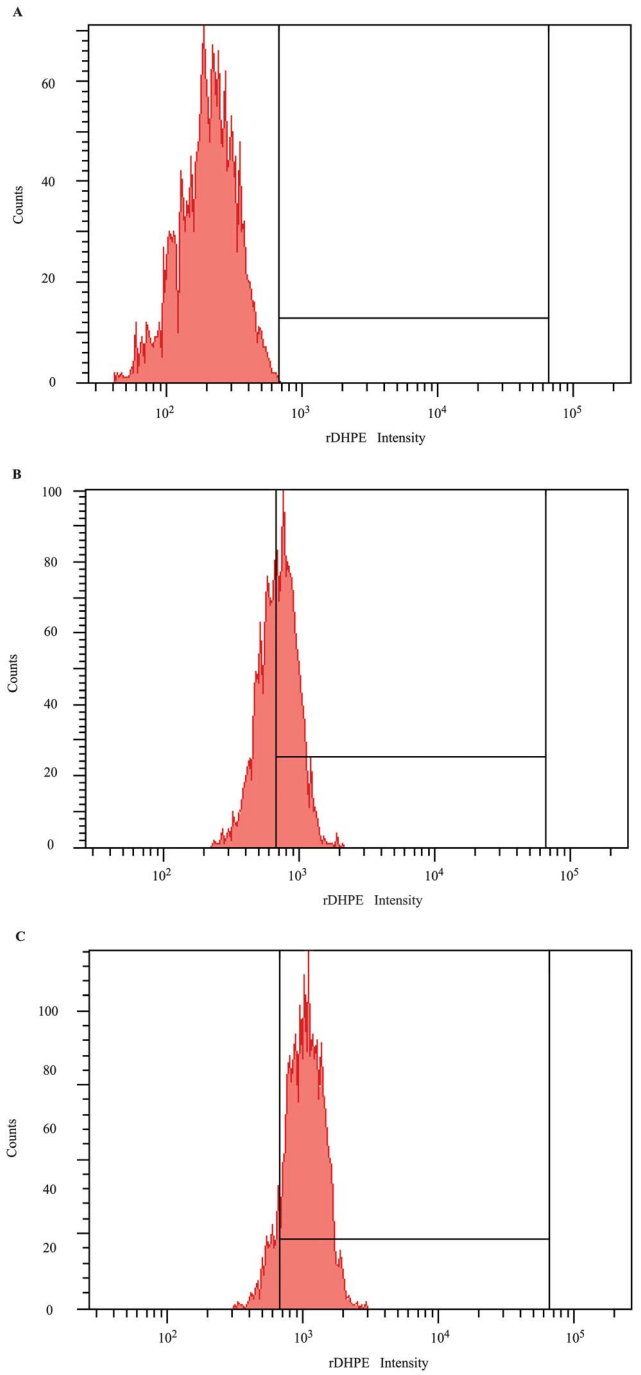

Fig. 7 Flow cytometry measurements on cellular internalization of rDHPE-labeled CL and NCL micelles of PMAA-*b*-PFM into U87 cells following 8 h of incubation (micelle concentration 0.2 mg mL^{-1}). (A) U87 cells are used as negative controls. (B) rDHPE-labeled NCL PMAA-*b*-PFM micelles. (C) rDHPE-labeled CL PMAA-*b*-PFM micelles.

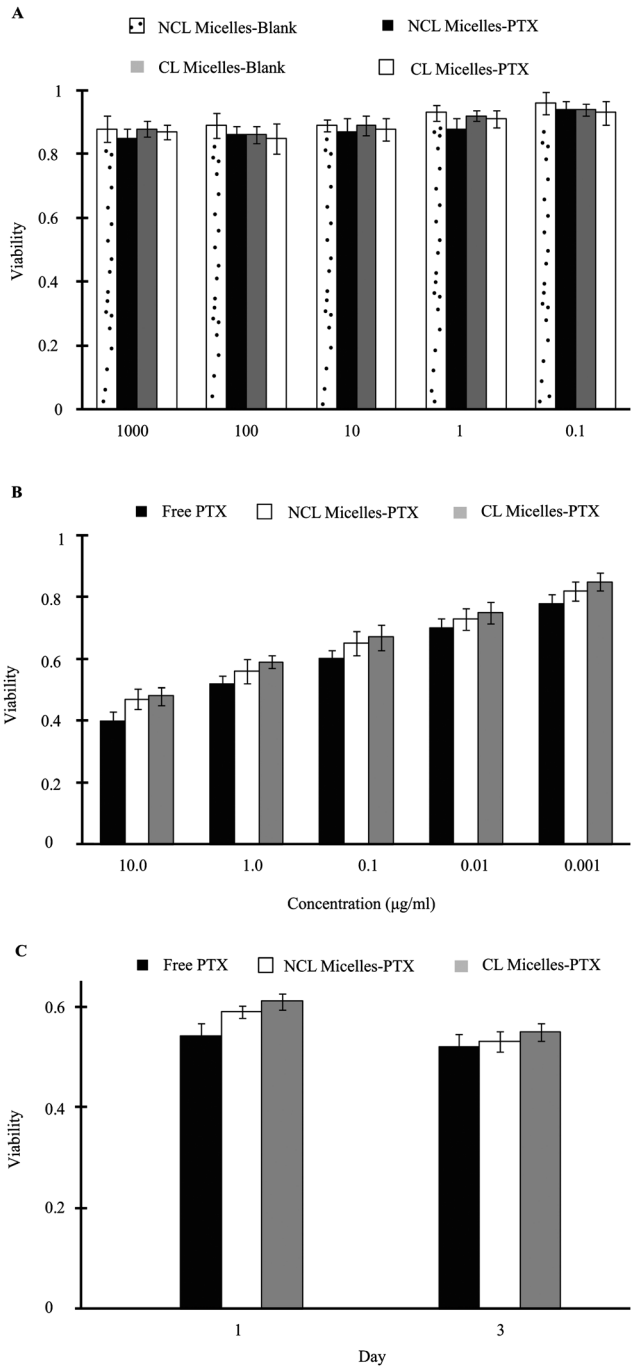

Fig. 8 Cell viability of BMM and U87 incubated with blank and PTX-loaded PMAA-*b*-PFM micelles. (A) BMM cultures were exposed to blank and PTX-loaded PMAA-*b*-PFM micelles for 12 h. MTT analysis of chemosensitivities performed at day 3 post-washout. (B) *In vitro* cytotoxicity of various formulations of PTX against U87 cells, 12 h incubation. (C) U87 cultures were exposed to various formulations of PTX at concentration of 10 μg mL^{-1} for 12 h then washout following fresh medium. MTT analysis of chemosensitivities performed at day 1 and 3.

micelles–PTX under experimental dosages (Fig. 8A). The results also suggest that PMAA-*b*-PFM micelle carrier had limited toxicity to macrophages as well as lower bio-toxicity.

Furthermore, it is shown from Fig. 8B that free PTX and PTX-loaded micelles were specifically toxic to U87 cells at concentrations from 10 to 0.001 μg mL^{-1} at 12 h incubation. For the cancer cells incubated with free PTX at 10, 1, 0.1, 0.01 and 0.001 μg mL^{-1} drug concentration, the cell viability (the survival rate) after 12 h treatment was measured to be 40.0 ± 2.8%, 52.1 ± 2.6%, 59.8 ± 2.9%, 69.7 ± 3.1%, and 78.2 ± 2.9%, respectively. It is straightforward to understand that higher drug concentration

will cause lower cell viability, or equivalently higher mortality of the cells. For the cytotoxicity of the micelle formulations, the same concentrations 10, 1, 0.1, 0.01 and 0.001 μg mL^{-1}, which are encapsulated in the micelles, were applied. After 12 h treatment, the cell viabilities were found to be 47.1 ± 3.3%, 56.0 ± 3.9%, 64.9 ± 3.9%, 73.2 ± 3.5% and 82.3 ± 3.2% for the NCL micelles–PTX, and 47.9 ± 2.8%, 58.8 ± 1.9%, 66.8 ± 4.1%, 74.8 ± 3.4%, and 84.8 ± 2.9% for CL micelles–PTX, respectively. It should be noted that there was no significant cytotoxicity difference between PTX-loaded CL and NCL micelles at 12 h incubation. This result is exceptional and unexpected given the fact that significantly inhibited release of PTX is observed for PTX-loaded CL micelles (Fig. 4). The possible reason is that PTX-loaded CL micelles have much smaller particle sizes as compared to the NCL counterparts (99 nm *versus* 125 nm, Fig. 3), which has largely facilitated their entry into U87 cells. 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 cross-linking conditions employed in this study have little influence on the therapeutic activity of PTX drug.

Next, we compared the timing correlation of chemosensitivity between PTX-loaded CL and NCL micelles. Following a single 12 h treatment of PTX-loaded CL and NCL micelles at a constant PTX concentration of 10 μg mL^{-1}, U87 cells were washed and fresh medium was introduced. The cultures were analyzed by MTT at post-washout day 1 and 3. For the cancer cells incubated with free PTX at 10 μg mL^{-1} drug concentration, the cell viability at day 1 treatment was measured to be 54.6 ± 2.8%. However at day 3 treatment, the cell viability decreased to 51.5 ± 2.6%. For the cytotoxicity of the micelle formulations, at day 1 treatment, the cell viability was found to be 58.9 ± 1.3% for the NCL micelles–PTX, and 61.2 ± 1.5% for CL micelles–PTX. At day 3 treatment, the cell viability decreased to 53.3 ± 2.0% for the NCL micelles–PTX, and 54.9 ± 1.7% for CL micelles–PTX (Fig. 8C). Despite PTX-loaded CL micelles being less toxic to U87 than free PTX and PTX-loaded NCL micelles at day 1 and 3 treatment, the anti-cancer activities differences became smaller between PTX-loaded CL micelles and free PTX, which further proves the controlled and sustained efficacy of the CL micelle formulation.

4. Conclusions

In summary, we successfully prepared cross-linked PMAA-*b*-PFM micelles filled with PTX, and confirmed sustained release of PTX from micelles. Fluorescence microscopy and flow cytometry studies showed that PTX-loaded cross-linked PMAA-*b*-PFM micelles had excellent cellular uptake ability by BMM and U87 cells. Cellular uptake of cross-linked PMAA-*b*-PFM micelles was found to be higher than that of non-cross-linked ones due to their smaller size. *In vitro* cytotoxicity studies also revealed that the PTX-loaded cross-linked micelles exhibit high anti-cancer activity to U87 cells. Taken together, cross-linked PMAA-*b*-PFM micelles seem to be a potential drug delivery system of PTX for cancer chemotherapy.

Acknowledgements

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