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Morphology and in vitro release kinetics of drug-loaded micelles based on well-defined PMPC–b–PBMA copolymer

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ABSTRACT

Well-defined amphiphilic diblock copolymers with different poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) content were successfully synthesized via RAFT polymerization technology. The structure of the copolymers was confirmed using GPC, ^{1}H NMR and FTIR. The polymers have very low critical micelles concentration (6.32×10^{-7} mol/L to 1.01×10^{-6} mol/L), which indicates their high thermodynamic stability needed for intravenous injection. Blank and paclitaxel-loaded polymeric micelles were prepared from the PMPC–b–PBMA copolymers using self-emulsion/evaporation method. TEM analysis revealed a regular spherical shape, small diameter (less than 30 nm) and narrow size distribution of the micelles. The paclitaxel-loaded polymeric micelles had high loading content (above 13%). In vitro release kinetics of paclitaxel from the micelles was also investigated. Less than 30% of the paclitaxel was released within 320 h and the increase of the length of PMPC leads to slower release rate.

1. Introduction

Paclitaxel is a promising anti-cancer drug that can be extracted from Pacific yew tree. It has been proven that paclitaxel is very effective for the treatment of a wide range of cancers like breast cancer, ovarian cancer, and lung cancer (Wani et al., 1971; Rowinsky et al., 1990; Huizing et al., 1995; Wall and Wan, 1995). However, the poor solubility in water of paclitaxel limits its clinical application. Currently, the only commercially available formulation of paclitaxel is based on a 1:1 (v/v) mixture of polyoxyethylated castor oil (Gremophor EL) and ethanol. However, Gremophor EL has been shown to cause some severe side effects such as hypersensitivity reactions, nephrotoxicity, neurotoxicity, cardiotoxicity and so on (Weiss et al., 1990; Onetto et al., 1993; Zuylen et al., 2001; Singla et al., 2002). Therefore, much effort has been made to develop more favorable drug delivery systems for paclitaxel, among which amphiphilic polymeric micelles are most promising (Crosasso et al., 2000; Schmitt-Sody et al., 2003; Dordunoo and Burt, 1996; Constantinides et al., 2000; He et al., 2003; Licciardi et al., 2006).

Usually, polymeric micelles can be obtained by self-assembly of amphiphilic block copolymers which are composed by the hydrophobic blocks and the hydrophilic blocks. A unique feature of polymeric micelles is their core–shell structures. In aqueous environment the hydrophobic blocks of the polymer are segregated from the aqueous exterior to form the inner core that solubilizes lipophilic drugs, and the hydrophilic blocks form the outer shell that make the micelles water-soluble (Kataoka et al., 2001; Xu et al., 2005; Rapoport, 2007). There are many advantages of polymeric micelles for drug delivery use. The nanosize (10–100 nm) and hydrophilic outer shells of polymeric micelles prevent their uptake by reticuloendothelial system (RES), thus can prolong their circulation time in blood (Stolnik et al., 1995; Moghimi et al., 1993). The small size can also lead to passive accumulation of polymeric micelles in solid tumor sites due to the enhanced permeability and retention (EPR) effect of the vascular endothelia at the tumor (Maeda et al., 2000; Jain, 1997; Yuan et al., 1995), which can improve therapy and reduce the side effects of the entrapped drug to healthy tissues.

To date, a variety of amphiphilic block copolymers have been explored for preparing polymeric micelles as drug carrier. Poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), a bioinspired polymer, is an ideal candidate for hydrophilic shell-forming blocks. It is well known that phospholipid is an important component of lipid bilayer which plays a vital role in the biocompatibility of biomembranes. In order to mimic the bilayer surface structure of biomembranes, a great deal of effort has been made to synthesize phospholipid analogues polymers during the past four decades. Among these polymers, 2-methacryloyloxyethyl phosphorylcholine (MPC) is most famous and has been proven that it can inhibit protein absorption and platelet adhesion remarkably (Ishihara, 1997; Lewis, 2000; Nakabayashi and Williams, 2003). Due to its excellent biocompatibility, MPC has been widely used for biomedical applications including drug delivery. Salvage et al. (2005) prepared nanoparticles using PMPC–b–PDPA diblock

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copolymers. In vitro assays showed that these MPC–DPA diblock copolymers had negligible cytotoxicity. Hsiue et al. (2007) prepared PMPC–b–PLA polymeric nanoparticles, low cytotoxicity of the polymer and nanoparticles was confirmed by cytotoxicity assay and growth inhibition assays with HFW (human fibroblasts) cell which indicates the potential use in biomaterials of these nanoparticles. Li et al. (2005) synthesized biocompatible thermo-responsive gels based on PNIPAM–PMPC–PNIPAM triblock copolymer. Cell viability studies indicate that these gels are sufficiently biocompatible to act as a culture medium for V79 cells (hamster lung cells) which suggests their possible applications in drug delivery and tissue engineering. PMPC–b–PBMA amphiphilic block copolymers were synthesized as novel biocompatible polymer micelle reagent by Yusa et al. (2005), whose studies showed that the water solubility of paclitaxel can be enhanced dramatically by use of the aqueous micelles solution. However, the morphology of the micelles and in vitro drug release study were not studied. In this paper, we prepared paclitaxel-loaded PMPC–b–PBMA micelles and investigated the micelles morphology as well as the in vitro drug release kinetics.

Living free radical polymerizations are very useful methods for synthesis of well-defined block copolymers with controlled molecular weight and narrow molecular distribution. In recent years, several living free radical polymerization methods, such as nitroxide-mediated polymerization (NMP) (Georges et al., 1993), atom transfer radical polymerization (ATRP) (Wang and Matyjaszewski, 1995) and reversible addition-fragmentation chain transfer (RAFT) (Chiefari et al., 1998) radical polymerization, have been developed. Among them, reversible addition-fragmentation chain transfer radical polymerization is more versatile compared with other methods since it is metal free (Hu et al., 2007) and can be carried out with a wider range of monomers in various solvents (Donovan et al., 2003; You and Oupicky, 2007; Lin et al., 2007).

In this paper, well-defined PMPC–b–PBMA block copolymers with controlled molecular weight and narrow molecular distribution were synthesized via RAFT technology, followed by preparing nanosized polymeric micelles encapsulated with paclitaxel using the amphiphilic block copolymers by self-emulsion/solvent evaporation method. The micelles morphology, drug loading capacity and in vitro drug release kinetics were also investigated.

2. Experimental

2.1. Materials

MPC was prepared according to the reported method (Ishihara et al., 1990, 1996). n-butyl methacrylate (BMA) was purchased from Shanghai Chemical Agent Company of China and distilled under reduced pressure before use. 4-Cyanopentanoic acid dithibenzoate was synthesized as previously described elsewhere (Mitsukami et al., 2001). 4,4′-Azobis (4-cyanopentanoic acid) was purchased from Aldrich and dried under reduced pressure at room temperature prior to use. Pyrene was purchased from J&K and used as received. Paclitaxel with a purity of 99.5% was obtained from Tianfeng Bioengineering Technology Co., Ltd., Shenyang, China. Other reagents were commercially available and were used as received.

2.2. Measurements

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AVANCE 300-MHZ instrument using $CDCl_3$ or CD_3OD as solvents and TMS as an internal standard.

Fourier transform infrared (FTIR) spectra were obtained on a Nicolet-750 FTIR spectrometer. The samples were pressed into potassium bromide pellets. The scanning range was from 4000 cm^{-1} to 500 cm^{-1}.

Gel permeation chromatography (GPC) was used to determine the molecular weight and its distribution of polymers. GPC analysis was performed at 40 °C on a Waters GPC system equipped with a Waters refractive index detector and Waters Styragel columns. Monodispersed polystyrene standards were used as calibration standards. THF was used as an eluent at a flow rate of 1.0 mL/min.

The critical micelle concentration (CMC) of the block copolymers was determined by fluorescence probe technique using pyrene as a fluorescence probe. Fluorescence spectra were obtained with a fluorescence spectrometer (LS-50B, PERKIN-ELMER). Aliquots of pyrene solution (2×10^{-5} M in acetone, 1 mL) were added to 10-mL empty volumetric flasks. After evaporating the acetone, 10 mL copolymer aqueous solutions with different concentrations (C_p = 0.0001–0.5 mg/mL) were added to these flasks. The solutions were sonicated for 3 h at 60 °C and then kept at room temperature for 24 h to allow the solubilization equilibrium of pyrene. Excitation was carried out at 390 nm, and emission spectra were recorded from 360 nm to 450 nm. The excitation bandwidth was 10 nm and the emission bandwidth was 2.5 nm. The scan speed was set at 300 nm/min. From the pyrene emission spectra, the intensity of the peak at 382 nm (I_{382}) was analyzed as a function of the polymer concentration. CMC value was determined as described else where (Zou et al., 2007).

2.3. Synthesis of PMPC–b–PBMA diblock copolymers

PMPC–b–PBMA diblock copolymers were prepared using RAFT technology. BMA (14.20 g, 100 mmol) was dissolved in 50 mL anhydrous ethanol, and then 4-cyanopentanoic acid dithibenzoate (279 mg, 1.0 mmol) and 4,4′-azobis (4-cyanopentanoic acid) (56 mg, 0.2 mmol) were added to the solution. The mixture was degassed by purging nitrogen for 30 min to remove dissolved oxygen and then heated in an oil bath at 80 °C with vigorous stirring for 12 h at nitrogen atmosphere. PBMA macro-CTA (chain transfer agent) was obtained by precipitation in water/methanol (9:1, v/v) for three times, followed by drying in vacuum for 48 h.

MPC was polymerized using PBMA macro-CTA as chain transfer agent to obtain PMPC–b–PBMA. In a typical procedure, MPC monomer (1.17 g, 60 mmol) was dissolved in 50 mL anhydrous ethanol, then 4,4′-azobis(4-cyanopentanoic acid) (56 mg, 0.2 mmol) and PBMA macro-CTA (11.61 g, 1.0 mmol) were added to the solution. After being purged with nitrogen for 30 min, the solution was placed in an oil bath at 80 °C for 24 h. After the polymerization, the solution was poured into a large excess of n-hexane for precipitation of the polymer, which was then purified by precipitating from ethanol into n-hexane twice. The resulting product PMPA–b–PBMA was vacuum-dried for 48 h.

2.4. Preparation and characterization of micelles

Blank polymeric micelles were prepared using self-emulsion/solvent evaporation method. Typically, 50 mg of the copolymer was dissolved in 5 mL mixture solvent of chloroform and ethanol (3:2, v/v), and then this solution was slowly added to 30 mL distilled water under vigorous stirring to induce the micelle formation. The solution was open to air and stirred overnight to remove the organic solvent by evaporation.

Essentially, drug-loaded polymeric micelles was prepared using the same procedure as described above, except 10 mg paclitaxel was dissolved along with the block copolymers in the chloroform/ethanol. After the evaporation of organic solvent, the micelle solution was centrifuged (2500 rpm) for 30 min followed by filtration using 0.45-μm membrane filters to remove the unloaded drug. Finally, the micelle solution was diluted with distilled water to obtain desired concentrations.

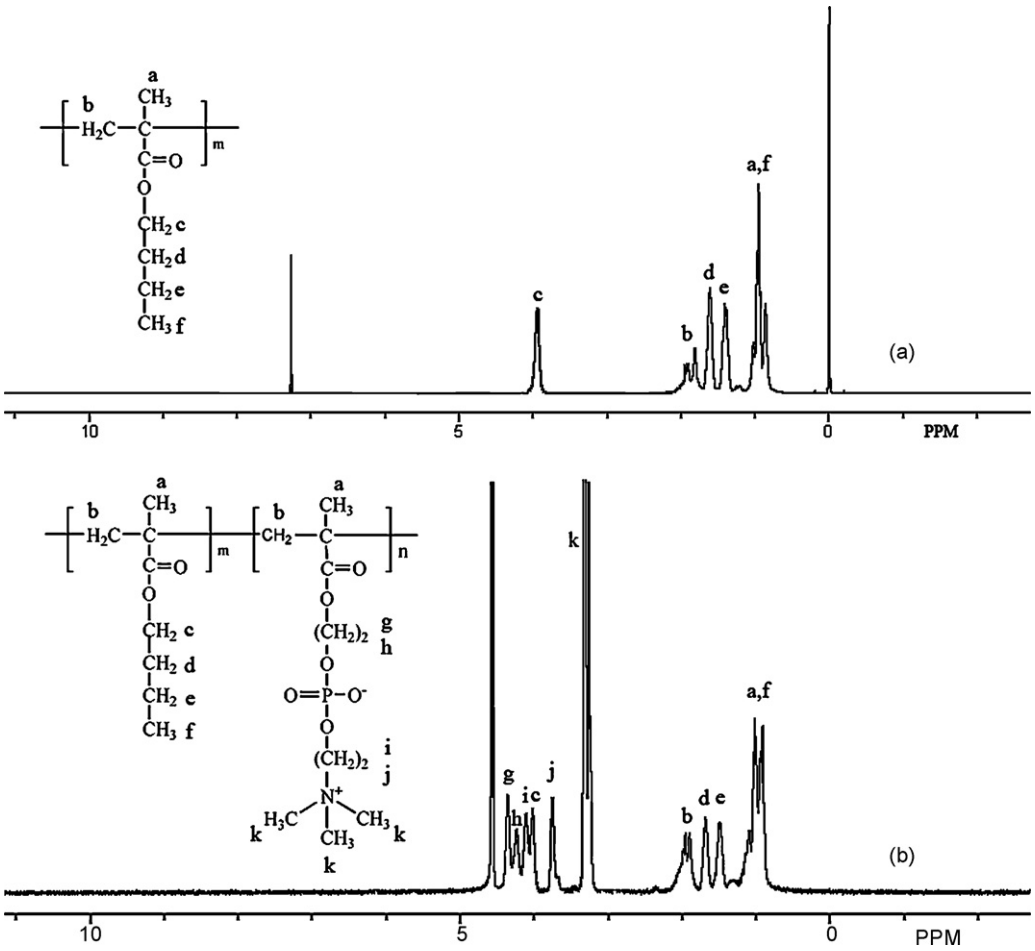

Fig. 1. ^{1}H NMR spectra of (a) the PBMA macro-CTA in $CDCl_3$ and (b) the $PMPC_{107}$–b–$PBMA_{80}$ block copolymer in CD_3OD at 50 °C.

To determine the drug encapsulation efficiency (EE) and drug loading content (LC), the unloaded drug collected by centrifugation and filtration was dissolved in ethanol, then the paclitaxel concentration in the solution was determined by measuring the absorbance at 228 nm on an ultraviolet–visible spectrophotometer (UV-2201, Shimadzu) using a standard calibration curve experimentally obtained with ethanol solutions. The drug encapsulation efficiency was defined as the ratio of the weight of the drug encapsulated in the micelles to the weight of the drug initially used, while the drug loading content was obtained as the weight ratio of the drug encapsulated in the micelles to that of the weight of the polymeric micelles.

The morphology and the size of the blank and drug-loaded micelles were observed by a JEOL JEM-200CX transmission electron microscope operating at an acceleration voltage of 120 kV. To prepare TEM samples, a drop of aqueous micelle solution with a concentration of 0.5 mg/mL was deposited onto copper grids coated with carbon. Several minutes later, excess micelle solution was gently removed using absorbent paper. Samples were then stained by 0.5 wt% phosphortungstic acid aqueous solution, followed by drying at room temperature.

2.5. In vitro release of paclitaxel from the micelles

The in vitro drug release study was conducted by placing a dialysis bag (molecular weight cutoff = 14,000) containing 5 mL of the drug-loaded micelle solution with a concentration of 1.0 mg/mL into 95 mL stirred phosphate buffer solution (PBS, pH 7.4) at 37 °C. At predetermined time intervals, 50 mL PBS solution was taken out and replaced by fresh PBS.

The released content of paclitaxel was determined using a method described in a previous paper (Jackson et al., 2007). The paclitaxel of 50 mL PBS solution was extracted with 10 mL dichloromethane, and then the organic phase was collected and allowed to evaporate. The residue was dissolved in 5 mL mobile phase (a mixture of acetonitrile, methanol and water in the volume ratio of 45:20:35) for HPLC analysis. The samples were injected through a 20-μL sample loop to an Agilent (HP1100) HPLC system equipped with a DAD detector and reverse-phase C-18 column (5 μm, 4.6 mm × 250 mm). The flow rate was 1.0 mL/min and the detection wavelength was set at 227 nm.

3. Results and discussion

3.1. Synthesis and characterization of PMPC–b–PBMA diblock copolymers

PMPC–b–PBMA block copolymers were prepared by RAFT method. Firstly, PBMA was synthesized using 4,4′-azobis(4-cyanopentanoic acid) as initiator and 4-cyanopentanoic acid dithibenzoate as chain transfer agent. Then PMPC–b–PBMA block copolymers were obtained using the same method with different feed ratios of MPC monomer to PBMA macro-CTA. Some characteristics of PMPC–b–PBMA block copolymers were listed in Table 1.

^{1}H NMR was used to confirm the structure of PBMA macro-CTA and PMPC–b–PBMA block copolymers. Fig. 1(a) shows the ^{1}H NMR

Table 1
Compositions, molecular weight and critical micelle concentration of the block copolymers.

Copolymers	DP of BMA[a]	DP of MPC[b]	Mn[c]	CMC (mg/L)[d]
$PMPC_{56}$–b–$PBMA_{80}$	80	56	28,133	17.78
$PMPC_{107}$–b–$PBMA_{80}$	80	107	43,178	43.58

[a] Degree of polymerization of BMA determined by GPC.
[b] Degree of polymerization of MPC estimated by ^{1}H NMR.
[c] Number average molecular weight calculated by the following equation: $M_n = M_{n,\,PBMA\ macro\text{-}CTA} + DP_{MPC} \times M_{MPC}$.
[d] Critical micelle concentration of the block copolymers obtained by fluorescence spectra.

spectra of PBMA macro-CTA in $CDCl_3$. Signals at 0.86–1.05 ppm and 3.95 ppm correspond to the methyl groups of PBMA and the methylene groups neighboring the oxygen atom in the n-butyl moieties respectively. Signals at 1.41 ppm and 1.61 ppm correspond to the methylene groups of the n-butyl moieties and those at 1.81–1.99 ppm correspond to the methylene groups of the main chain. Resonance peaks at 3.33 ppm, 3.75 ppm and 4.12 ppm observed in Fig. 1(b) are attributed to phosphorylcholine moieties of PMPC blocks in the copolymer.

The structure of PBMA macro-CTA and PMPC–b–PBMA copolymers were also characterized by FTIR (Fig. 2). The absorption peaks at 1728 cm^{-1} (C═O), 1244 cm^{-1} (P═O) and 966 cm^{-1} [–$N(CH_3)$] can be observed, which is an evidence of successful polymerization of MPC using PBMA macro-CTA as chain transfer agent.

M_n and polydispersity (PDI) of PBMA were determined by GPC. In Fig. 3, the GPC curve indicated a relatively low PDI (M_w/M_n = 1.24) for PBMA macro-CTA, which can attribute to the use of RAFT technology. However, no suitable eluent could be found for the GPC measurement of PMPC–b–PBMA block copolymers, similar difficulty has been referred for poly(2-acryloyloxyethyl phosphorylcholine)–b–poly(butyl acrylate) (PAPC–b–PBA) by Stenzel et al. (2004). Fortunately, the molar ratio of the MPC to BMA units could also be calculated from the integral intensity ratio of the resonance bands at 3.75 ppm and 1.69 ppm that attributed to the methylene protons neighboring the quaternary ammonium group in phosphorylcholine and n-butyl moiety respectively according to a previous report (Yusa et al., 2005).

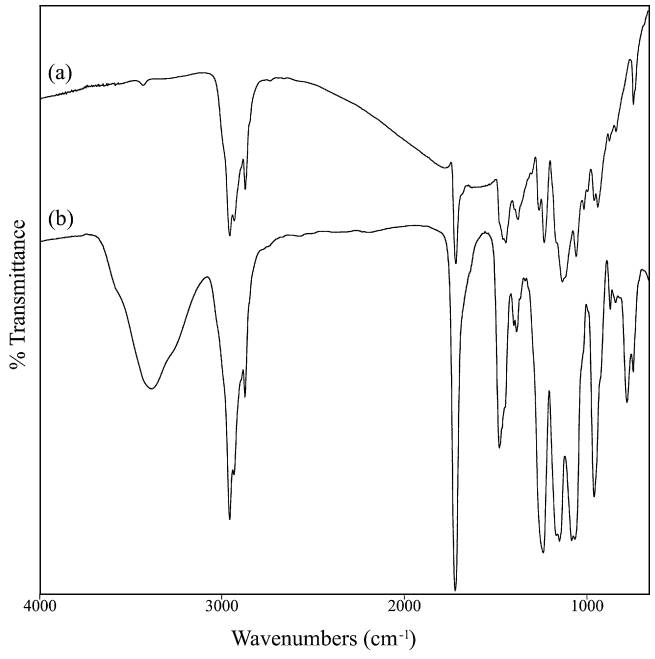

Fig. 2. FTIR spectra of (a) PBMA macro-CTA and (b) $PMPC_{56}$–b–$PBMA_{80}$ block copolymer.

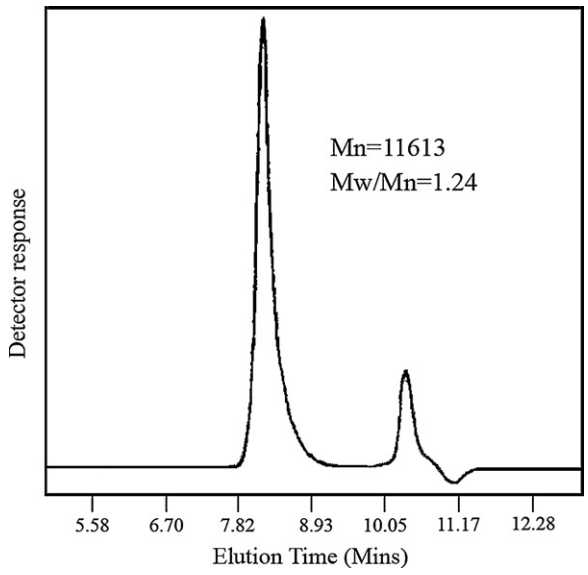

Fig. 3. The GPC trace for PBMA macro-CTA.

3.2. Critical micelle concentration of PMPC–b–PBMA copolymers

The CMC values of the PMPC–b–PBMA copolymers were determined by fluorescence probe technique. Pyrene was used as a hydrophobic probe because of its sensitivity to surrounding polarity. I_{382} was plotted with the concentration of the copolymers, as shown in Fig. 4. From this figure, it was observed that I_{382} nearly kept a constant at low polymer concentrations, but began to increase sharply when the polymer concentration reached a certain value which was defined as the critical micelle concentration. The CMC values of $PMPC_{56}$–b–$PBMA_{80}$ and $PMPC_{107}$–b–$PBMA_{80}$ are 17.78 mg/L (6.32×10^{-7} mol/L) and 43.58 mg/L (1.01×10^{-6} mol/L) respectively. Many researches have shown that CMC values were increased with the length of the hydrophilic blocks when the length of hydrophobic block maintains a constant (Allen et al., 1999), which followed our experimental results. Compared with many low molecular weight surfactants, such a low CMC means high thermodynamic stability and suggests potential use of intravenous injection.

3.3. Preparation and characterization of drug-loaded micelles

Self-emulsion/solvent evaporation method was used to prepare blank and drug-loaded micelles based on PMPC–b–PBMA copolymers. To our knowledge, no single solvent is available for dissolving both PMPC block and PBMA block. Thus, a mixture of chloroform and ethanol (3:2, v/v) was used to dissolve the copolymers and PTX.

Fig. 5 shows the morphology of the blank micelles and drug-loaded micelles prepared from $PMPC_{56}$–b–$PBMA_{80}$ observed by TEM. Obviously in these images, the micelles exhibited a narrow size distribution and relatively regularly spherical shape. The mean diameter of the blank micelles estimated from TEM image was about 22 nm. However, the mean diameter of the drug-loaded micelles reduced to 18 nm. Similar unexpected phenomenon was found by Licciardi et al. (2006) when they used MPC_{30}–b–DPA_{80} for the encapsulation of paclitaxel. The reason might be that the encapsulation of highly hydrophobic paclitaxel could lower the CMC value and consequently decrease the micelle aggregation number, which leads to smaller size.

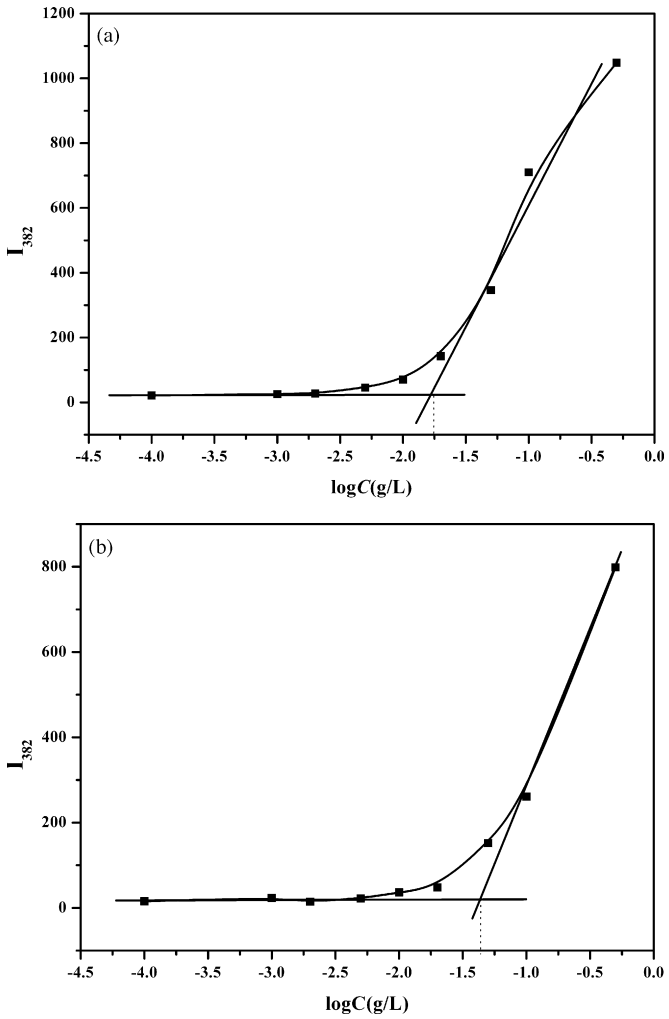

Fig. 4. Determination of CMC value of (a) $PMPC_{56}$–b–$PBMA_{80}$ and (b) $PMPC_{107}$–b–$PBMA_{80}$ using pyrene as fluorescence probe.

Table 2 summarized some properties such as the encapsulation efficiency, drug loading content and the size of the micelles prepared from the copolymers. As shown in Table 2, the drug loading content of PMPC–b–PBMA micelles for PTX is higher than ones of many other micelles systems based on amphiphilic block copolymers (Licciardi et al., 2006). However, there is only a slight difference of the drug loading capacity between micelles systems obtained from $PMPC_{56}$–b–$PBMA_{80}$ and $PMPC_{107}$–b–$PBMA_{80}$. According to previous studies (Rapoport, 2007; Allen et al., 1999), the drug loading capacity of polymeric micelles mainly depends on the length of the core-forming block as well as the compatibility between the drug and the core-forming block, while the length of shell-forming block has effect only to a less extent, which well agrees with the results obtained above. The nanosize (10–100 nm) of polymeric micelles could prevent their uptake by reticuloendothelial system. And the mean diameter of our particles was 18 nm and 28 nm, respectively, which was accorded with nanosize and the difference of their mean diameter was little effect on practical applications.

Table 2
Encapsulation efficiency (EE) and loading content (LC) of paclitaxel in polymeric micelles (feed weight ratio of paclitaxel to block copolymer = 0.2:1).

Copolymers	EE (wt%)	LC (%)	d^a (nm)
$PMPC_{56}$–b–$PBMA_{80}$	69.7	13.8	18
$PMPC_{107}$–b–$PBMA_{80}$	68.8	13.7	28

[a] Mean diameter of paclitaxel-loaded micelles estimated from TEM image.

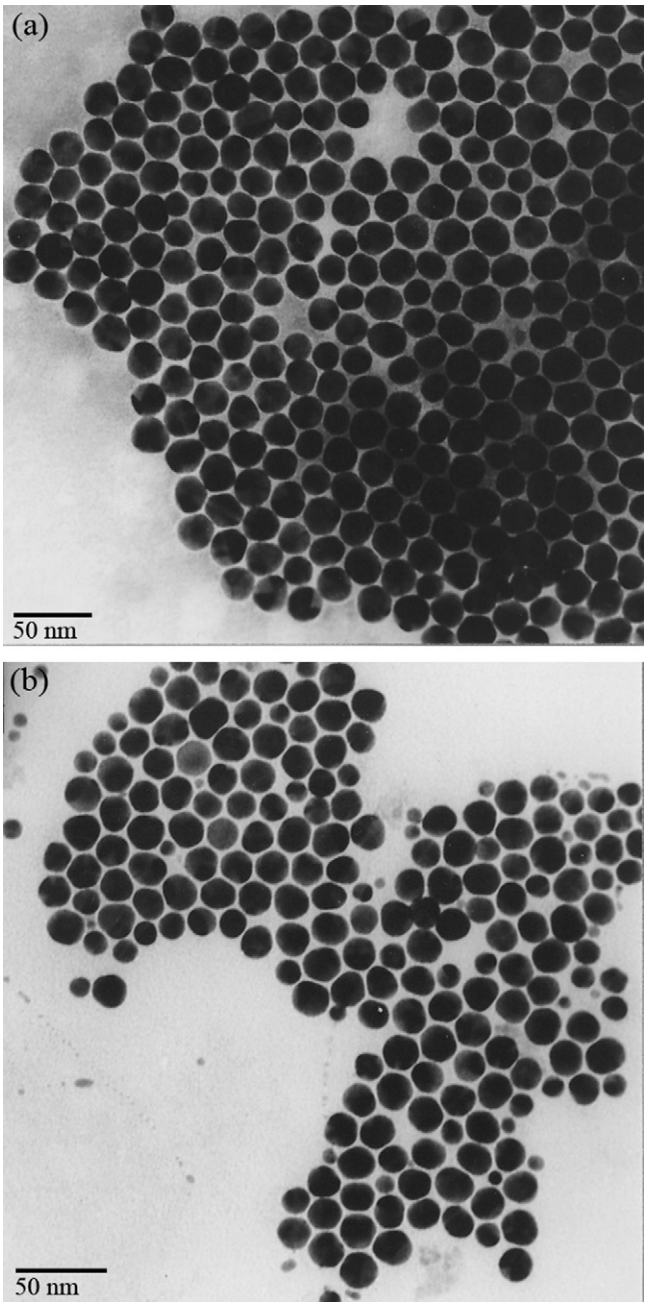

Fig. 5. TEM image of (a) blank micelles and (b) drug-loaded micelles obtained from $PMPC_{56}$–b–$PBMA_{80}$.

3.4. In vitro drug release study

The in vitro drug release study was conducted in phosphate buffer solution (pH 7.4) at 37 °C. The cumulative percentage of paclitaxel released from PMPC–b–PBMA drug-loaded micelles as a function of time is shown in Fig. 6. As can be seen, the release of paclitaxel from both of the micelles based on $PMPC_{56}$–b–$PBMA_{80}$ and $PMPC_{107}$–b–$PBMA_{80}$ is slow and sustained, less than 30% of the incorporated drug was released within 320 h. Besides, the drug release rate from $PMPC_{107}$–b–$PBMA_{80}$ micelles was slightly slower than that from $PMPC_{56}$–b–$PBMA_{80}$ micelles, which can be attributed to the longer hydrophilic block of $PMPC_{107}$–b–$PBMA_{80}$

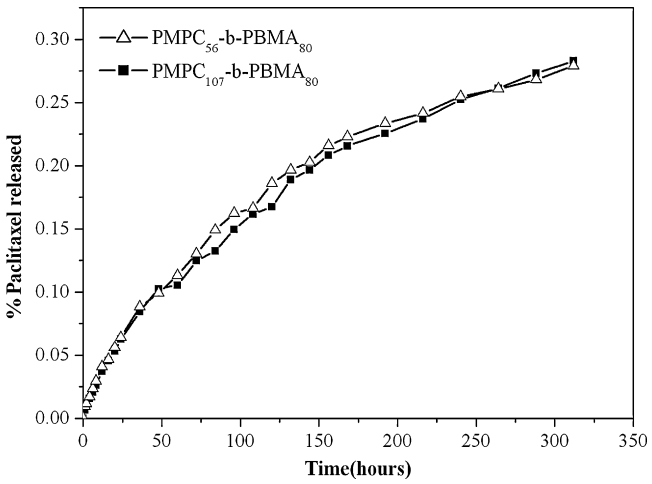

Fig. 6. Paclitaxel released from the PMPC–b–PBMA micelles at 37 °C in PBS with pH value of 7.4.

being disadvantageous to the diffusion of paclitaxel from the micelles core to the external PBS medium.

4. Conclusion

In the present work, well-defined PMPC–b–PBMA block copolymers with different PMPC content were synthesized and used for the encapsulation of paclitaxel. The copolymers had low CMC values (6.32×10^{-7} mol/L to 1.01×10^{-6} mol/L), which suggested their potential using in intravenous injection. High drug loading content (above 13%, w/w) were achieved for both $PMPC_{56}$–b–$PBMA_{80}$ and $PMPC_{107}$–b–$PBMA_{80}$ and no obvious influence of the length of the PMPC block on drug loading capacity was observed. TEM studies indicated small size (less than 30 nm), narrow size distribution and regularly spherical shape of the drug-loaded micelles. In vitro release study showed the release rate of paclitaxel from the polymeric micelles was slow and sustained. At the same time, a longer PMPC block could slightly decrease the release rate. In conclusion, these properties enable this micelles system to be a promising formulation of paclitaxel. Green preparation of paclitaxel-loaded polymeric micelles without using organic solvents is under research.

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