Colloids and Surfaces B: Biointerfaces 86 (2011) 45–57

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Preparation and characterization of thermosensitive pluronic F127-b-poly(ε-caprolactone) mixed micelles

Qi Zhou, Zhao Zhang, Tao Chen, Xing Guo, Shaobing Zhou*

Key Laboratory of Advanced Technologies of Materials, Ministry of Education, School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu 610031, PR China

ARTICLE INFO

Article history:
Received 7 October 2010
Received in revised form 28 February 2011
Accepted 15 March 2011
Available online 23 March 2011

Keywords:
Self-assembly
Micelles
Thermosensitive
Drug delivery system

ABSTRACT

The mixed micelles composed of pluronic F127-b-poly(ε-caprolactone) (F127-CL) and bovine serum albumin (BSA) or polylactic acid (PLA) were fabricated for application as promising drug carriers. F127-CL copolymers were characterized by ^{1}H NMR, FT-IR, GPC, DSC, XRD and POM. They can self-assemble into micelles in water by solvent evaporation method. The thermo-responsivities of the pure and mixed micelles were investigated. The drug release behaviors were investigated in phosphate-buffered solution (PBS) and acetate buffer solution (ABS), respectively, at 37 °C. The hemolysis and coagulation assay and the tumor cell growth inhibition assays were further evaluated. The morphologies of pure micelles underwent from the coexistence of the rods and spheres to the spheres with increasing the lengths of CL. The micelle behaviors were influenced with the addition of BSA and PLA. Both pure and mixed micelles of F127-CL with CL length of 200 show thermo-responsivities from 25 to 45 °C, while form larger aggregations at high temperature. The hemolysis and coagulation assays showed that the micelles possess good blood compatibility. The cytotoxicity results showed that the copolymer was a safe carrier and the encapsulated doxorubicind.HCl remained its potent anti-tumor effect. The in vitro release profiles displayed a sustained release of DOX.HCl from the micelles. The block copolymers can be great potential as a nanocontainer in drug delivery systems.

1. Introduction

Amphiphilic block copolymers used as carriers for drug delivery system (DDS) have gained increasing interest in recent years due to their biodegradability and biocompatibility. They can self-assemble into polymeric micelles in aqueous solution, which typically consist of a hydrophobic inner core that is surrounded by a hydrophilic outer shell [1–5]. Poorly water-soluble drugs can be easily solubilized within the hydrophobic core of the micelle and consequently, polymeric micelles can substantially improve the solubility and bioavailability of various hydrophobic drugs. These micelles with core–shell geometry are widely investigated in drug controlled release system [6–8].

Currently, there has been marked progress in the search for stimulus-responsive nanoparticles [2]. Stimulus-responsive nanoparticles produce physical or chemical changes when subjected to external signals, which include pH [9], temperature [10], magnetic [11], photo [12] and so forth. It would be difficult to meet so many stimulus-responsive requirements for just a single micelle system in DDS [13].

Thereby, the mixed micelle system (MMS) may be a good choice to accommodate the stimulus-responsive behaviors of micelles. The use of mixed micelles obtained from a mixture of two or more block copolymers, with distinctly different properties can be a plausible candidate that is capable of tuning the resulting properties and meeting various requirements for specific application. To date, well studied examples for mixed micelles driven by non-covalent interactions include the mix of thermo-responsive/pH-responsive molecular [14], amphiphilic polymers/thermo-responsive polymers, amphiphilic polymers/pH-responsive molecules [15,16], amphiphilic polymers/diagnosis and targeting polymers [17–20], and so on. Through mixing the polymers with different functions, the mixed micelles have complementary effects in adjusting to external environmental changes, such as in temperature and diluted concentration, target-protein [21]. With these unique characters, the mixed micelles formed from different polymers would exhibit significantly improved stability under various physiological conditions, enhance the target performance of drug loaded carriers, and facilitate the drug release at certain pH.

So far, a large number of block copolymers with different compositions in DDS have been developed [22,23]. Among various copolymers, the amphiphilic diblock copolymers composing of aliphatic polyesters such as poly(ε-caprolactone) (PCL) [24], poly(D,L-lactide) (PLA) [25,26], poly(glycolide) (PGA) and their copolymer poly(lactide-co-glycolide) (PLGA) [27] as hydropho-

* Corresponding author. Tel.: +86 28 87634023; fax: +86 28 87634649.
E-mail addresses: shaobingzhou@swjtu.cn, shaobingzhou@home.swjtu.edu.cn, shaobingzhou@hotmail.com (S. Zhou).

0927-7765/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfb.2011.03.013

bic segments and polyether such as PEG and pluronics [28–30] as hydrophilic segments, have been widely studied. Amphiphilic PEG–PCL block copolymers have been investigated in the morphologies, thermo-responsive hydrogels and drug delivery [31–34].

Pluronic F127, a water-soluble triblock copolymer of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO), are commercially available as a nonionic macromolecular surfactant, which exhibits thermo-responsive properties due to the presence of hydrophobic propylene oxide block, and are also approved by the U.S. Food and Drug Administration (FDA) for use as food additives and pharmaceutical ingredients [35]. PCL is a semi-crystalline, hydrophobic polymer, which has gained many attentions as ideal material for drug delivery and other applications through the decades because of its biodegradability under physiological conditions. A hydrophilic chain such as pluronics was added to the polymer backbone, and could significantly changed the hydrophilicity of the particles, surface roughness, surface charge and water content and could provide a steric barrier to reduce particles aggregation [36].

In the study, the pluronic F127-b-poly(ε-caprolactone) (F127-CLx) copolymers comprised of different ratios of F127 and CL were firstly synthesized through ring-opening polymerization. Later, these amphiphilic copolymers self-assembled into polymeric micelles in a selected solvent to form core–shell architecture by a solvent evaporation method. At the same time, a simple and versatile approach was employed to form mixed micelles through the use of non-covalent interactions with the addition of one-fold hydrophilic (BSA) and hydrophobic (PLA) molecules to the pure micelles to adjust the stimulus-responsive properties. Similarly, Kim et al. [21] have reported mixed micelle system of PNIPAAM–PLLA and PEG–PDLA, in which the hydrophobic core constituting of PDLA and PLLA blocks were driven by strongly non-covalent interactions. BSA is a globular protein because its structure and physicochemical properties are well characterized, capacity, stability, and water solubility, which has a well-established secondary structure, a versatile binding [37]. BSA binds many different types of amphiphilic biological molecules, which are believed to play an important role in determining their physiological function [38]. The properties of micelles were measured by atomic force microscopy (AFM), transmission electron microscopy (TEM) and the dynamic light scattering measurements (DLS). The morphology was also described by altering the ratios between hydrophilic F127 and hydrophobic CL. The thermo responsive behaviors of pure F127-CLx micelles and mixed micelles in aqueous were measured by DLS and the optical method. Finally, the cytotoxicity of the polymeric micelles was evaluated based on osteoblasts and HepG2 tumor cells proliferation studies. The hemolysis assay and coagulation measurements of the copolymers were also studied. These pure and mixed polymeric micelles encapsulated doxorubicin hydrochloride (DOX.HCl) were further used to investigate their functions of suppressing the growth of tumor cells with culturing with HepG2 tumor cells.

2. Materials and methods

2.1. Materials

Pluronic F127 (M_w = 12,600) was obtained from Aldrich, and dried under high vacuum before use. The monomer, ε-caprolactone (Aldrich), was dried over CaH_2 for 2 days and distilled at reduced pressure prior to use. Poly-D,L-lactic acid (PLA, Mn = 600) was synthesized as our previous report [39]. Bovine serum albumin (BSA) with the isoelectric point (IEP) of 4.8 and stannous chloride ($SnCl_2$) were purchased from Shanghai Boao Biotechnology Co., Ltd. (China). Doxorubicin hydrochloride (DOX.HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (China). HepG2 tumor cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). All the other reagents were analytic reagent (AR) grade, and used as received.

2.2. Synthesis of PCL-F127-PCL (F127-CLx) copolymers

F127-CLx was synthesized through ring-opening polymerization (ROP) of ε-caprolactone in bulk using F127 as an initiator and $SnCl_2$ as the catalyst as our previously described [34]. A typical experimental procedure for the synthesis of the copolymers designated as PCL_{100}-F127-PCL_{100} is described as follows. Preweighed ε-CL, F127 and $SnCl_2$ (1 wt%) were quickly added into a 50 mL bottom flask with a stopcock which was preheated to remove the moisture. Later, the reaction system was degassed under vacuum for 30 min with continuous stirring to produce a well-mixed molten phase, and carried out at 140 °C for 6 h. The obtained copolymer was firstly dissolved in dichloromethane, and then precipitated in excess cold aether. Then the mixture was filtered and dried at room temperature under vacuum for 24 h. The F127-PCLx block copolymer used in this study is designated as Fx. In the nomenclature of F200, the numeric 200 corresponds to the degree of CL in theory. The other two copolymers (F127-CL_{100}, F127-CL_{400}) were similar to these processes. The purified copolymers were yellowish, and kept in desiccators before further use.

2.3. Characterization of the copolymers

Fourier transform infrared spectra (FTIR) were obtained using a Nicolet 5700 spectrometer. KBr tablets were prepared by grinding the polymer sample with KBr and compressing the whole into a transparent tablet.

^{1}H NMR spectra were obtained on a Bruker AM 300 apparatus. Tetra-methylsilane (TMS) was used as the internal standard and $CDCl_3$ was used as solvent. Chemical shifts were expressed as parts per million, ppm (δ).

Gel permeation chromatography (GPC) measurements were carried out with a Water 2695 separation module equipped with a Styragel HT4DMF column operated at 40 °C and series 2414 refractive index detector. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by narrow molecular weight distribution polystyrene standards.

The crystallinity of copolymer was characterized using an X-ray diffractometer (XRD, Phlips, X'Pert PRO, The Netherlands) and scanning was done in the 2θ angle range of 5–50°.

Thermal properties of polymers were determined by differential scanning calorimetry (DSC, Netzsch STA 449C, Bavaria, Germany) under a N_2 stream of 20 mL/min. In order to eliminate any unknown thermal history of the samples, heating and cooling were repeated from −50 °C to 100 °C, and then the DSC curves of the second heating and cooling process were obtained. The heating rate was 10 °C/min, and the cooling rate was 5 °C/min.

A polarization optical microscope (POM) (XPN-203, China) at room temperature was used to characterize the isothermal crystallization morphologies of F127, F127-CLx and pure PCL. Firstly, a sample powder of about 5 mg was placed on a glass slide and was heated to melt completely; then, the sample was pressed by another glass slide to obtain a sheet with the thickness of about 20 μm. Afterwards, the sheet was transferred to a room temperature environment, and maintained at this temperature until the crystallization of the sample was finished completely. The crystallization morphologies of the samples were taken images via a digital camera.

2.4. Preparation and characterization of the micelles

The pure and mixed micelle solutions were fabricated by a solvent evaporation method. Briefly, the dry powder of F127-CLx block copolymers was dissolved in 10 mL tetrahydrofuran (THF) as the good solvent, and then the solution was added dropwise using a disposable syringe (21 gauge) under high speed stirring into 10 mL deionized water as the selective solvent. The mixed solution was devolved to a beaker and slowly stirred at room temperature until THF was completely volatile. To prepare the mixed micelles, BSA was pre-added into the water phase, while PLA into the THF phase. The mixed micelles used in this study are designated as addition/polymers. In the nomenclature of BSA/F200, BSA is the addition, and F200 is the polymer.

The DOX.HCl loaded micelles were fabricated as follows. In brief, F200 (200 mg) and DOX.HCl (1.0 mg) were dissolved in a mixture of water (1 mL) and THF (9 mL) to prepare DOX.HCl-loaded polymeric micelles. This solution was added dropwise to 10 mL of deionized water and stirred for 4 h. After the THF had been removed by the evaporation, the transparent dark red solution was centrifugal (Avanti TM J-30I, BECKMAN COULTER, USA) for 8 min at 20,000 rpm. The resultant micelles were rinsed three times with distilled water, and centrifuged three more times to eliminate the unloaded DOX.HCl. Followed this, the slurry of the micelles was lyophilized to leave a dark red powder of DOX.HCl-loaded micelles, which was stored at 4 °C.

The CMC values of F127-CLx micelles were determined by an established fluorescence-based method. A change in the fluorescence excitation spectra of pyrene in the presence of varied concentrations of block copolymers was used to measure the critical micelle concentration (CMC). Pyrene was dissolved in methanol and added to 25 mL volumetric flasks to provide a concentration of 6×10^{-7} M in the final solutions. Methanol was then evaporated and a predetermined volume of copolymer solutions and ultrapurified water was added to the ampules consecutively to get solutions of different micelle concentrations ranging from 5.0×10^{-6} to 1.0 mg mL^{-1}. The excitation spectrum of pyrene for each sample was obtained at room temperature using a Fluoromax spectrometer (F-7000, Hitach, Japan). The emission wavelength and excitation bandwidth were set at 390 and 5 nm, respectively. The intensity ratio of peaks at 339 nm to those at 333 nm was plotted against the logarithm of concentration to measure CMC.

To examine the average size and size distribution of micelles at different temperatures, the pure and mixed micelles were measured with dynamic light scattering (DLS) (ZETA-SIZER, MALVERN Nano-ZS90, Malvern Ltd., U.K.). The experimental temperature range was 25–65 °C. All the samples were immersed in a water bath at each temperature for 15 min to equilibrate. Each measurement was also repeated 3 times and an average value reported.

Micelle morphology was evaluated by TEM with a HITACHI H-700H (Japan) at the acceleration voltage of 150 kV. A droplet of micellar solution was placed on a Formvar coated copper grid and then dried at atmospheric pressure at room temperature. The grids were finally negatively stained by 2 wt% phosphotungstic acid (PTA) aqueous solution, and then dried in air.

The morphologies of the micelles were also tested by tapping-mode atomic force microscopy (AFM) measurements (CSPM5000, Being, China). Some droplets of micellar solution was dripped on clean silicon pellet and then dried at atmospheric pressure at room temperature.

Drug-loading amount was determined from the ratio of the weight of the DOX in micelles to that of the micelles by the UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan).

2.5. Thermo-responsive behaviors

To confirm whether the pure and mixed micelles exhibit a thermal sensitivity as expected, we examined the optical absorbance of F127-CLx micelle aqueous solution as function of the external temperature. The thermosensitive phase transition behaviors were measured with the UV-Vis spectrophotometer. The polymeric pure and mixed micelle solutions were stored at a low temperature place to equilibrate for 30 min. The experimental temperature range was 25–85 °C. At least 10 min was allowed for the temperature equilibration of the sample. The break temperature was determined as the onset point of the abrupt increase in the UV absorption.

2.6. In vitro DOX.HCl release

DOX release profiles from pure and mixed micelles were determined using a previously described method [6]. Briefly, preweighed micelles (0.1 g) were suspended in test tubes containing 10 mL phosphate buffered solution (PBS) at pH 7.4 and acetate buffered solution (ABS) at pH 5.0, respectively. The tubes were kept in a thermostated incubator (Haerbin Dongming Medical Equipment Company, China) which was maintained at 37 °C and 107 cycles per minute. At predetermined intervals, 1.0 mL of supernatant was collected by centrifuge and 1.0 mL of fresh PBS and ABS were added into the test tube. Concentration of DOX.HCl released was determined by UV-visible spectrophotometer at absorbance of 232 nm.

2.7. Hemolysis assay and coagulation measurements

Hemolysis assay was measured based on previous report [40]. In brief, 200 μL human whole blood was added into the micelle with different concentrations in 0.9% NaCl solution. The same volume of human whole blood was added into ultrapure water as the positive control, while into the 0.9% NaCl solutions without the micelles as the negative control. All the samples were incubated in a 37 °C water bath for 60 min. Then, all the samples were centrifuged to obtain the supernatants, and the absorbances of the supernatants were measured in an automated microplate spectrophotometer (ELX800 Biotek, USA) at 540 nm as reference. Percent hemolysis (hemolysis %) was calculated by the formula described as:

$$\text{Hemolysis\%} = \frac{\text{As} - \text{Aneg}}{\text{Apos} - \text{Aneg}} \times 100\%$$

where As, Aneg, Apos are the absorbance of the sample, the negative control and the positive control, respectively.

Coagulation measurements were obtained through the activated partial thromboplastin time (APTT) assay, prothrombin time (PT) assay and thrombin time (TT) assay [41]. Firstly, platelet-poor plasma (PPP) was prepared by centrifuging the whole blood at 3000 rpm for 15 min. Then 0.5 mL PPP was dropped into about 2 mg polymer powder in a 24-well plate. The plate was incubated in a 37 °C water bath for 30 min. The APTT assay was performed by adding 200 μL incubated PPP solutions in a test tube, 100 μL APTT reagent was added into the above tube, followed by adding 100 μL 0.03 M $CaCl_2$ solution after 3 min incubation at 37 °C. The clotting time was measured by a coagulometer (Clot 1A, Innova Co.). The PT assay was performed by adding 200 μL incubated PPP solutions in a test tube, 100 μL PT reagent was added into the above tube after 3 min incubation at 37 °C, and then the clotting time was measured. The TT assay was performed by adding 200 μL incubated PPP solutions in a test tube, 100 μL TT reagent was added into the above tube after 3 min incubation at 37 °C, and then the clotting time was measured.

2.8. In vitro cell cytotoxicity study

The cytotoxicity of the micelles was evaluated using an MTT assay. Osteoblasts from SD rat and HepG2 tumor cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator, respectively. They were seeded in 48-well plates in 1.0 mL medium per well at a density of 6000 cells/well for 6 h. The medium was then replaced with 2.0 mL of medium-containing micelles at the concentrations of 0.25, 0.5, 0.75, 1 and 2 mg/mL, respectively, and incubated for 48 h under 5% CO_2 at 37 °C. After 48 h, the culture medium was removed from cell monolayers cultured in 96-well plates. Each well was reconstituted with 0.2 mL supplemented RPMI 1640 containing 1.0 mg/mL MTT and incubated the cultures for 2–4 h at 37 °C. The supernatants were removed from the wells, and then 0.2 mL dimethyl sulphoxide (DMSO) per well was added in isopropanol and mixed with the content of the wells thoroughly. Finally, the plates were read in an automated microplate spectrophotometer (ELX800 Biotek, USA) at 570 nm as reference.

The HepG2 tumor cells were planted at a density of 2×10^4 cells per well in 1.5 mL medium in 24-well plates and grown for 72 h. The cells were then exposed to a series of DOX.HCl loaded polymeric micelles at different concentrations for 24, 48, 72 h, and DOX.HCl solution in DMSO at the same concentration was used a control. Finally, viability of cells was measured using the MTT method. All data were expressed as the mean ± SD.

3. Results and discussion

3.1. Characterizations of F127-CLx block copolymer

The structure of F127-CLx block copolymers was confirmed by FT-IR, ^{1}H NMR, XRD, POM, DSC and GPC. As seen from FT-IR spectra in Fig. 1A, a typically strong C=O stretching band appeared at 1730 cm^{-1} in F127-CLx, which is attributed to the ester bond because of the chemical binding between F127 and ε-CL, and among ε-CL monomers. The C–H stretching vibrations of PEO segments in F127 and CL are distributed at 2975 and 2888 cm^{-1}. The intensity of peaks at 1117 cm^{-1} is attributed to the characteristic C–O–C stretching vibration in F127. The result implied that the block copolymers were possibly synthesized.

^{1}H NMR spectra is shown in Fig. 1B in order to further confirm the formation of these block polymers. The chemical shifts at δ = 3.62 and 3.55 ppm belong to the hydrogen atoms of individual functional groups on F127. The chemical shifts at 1.66, 2.33, 4.25, 6.97 and 7.99 ppm ascribe to the protons of CL. The peak at 7.25 ppm corresponds to the solvent, deuterated chloroform. The ethylene peak of F127 units at 3.62 ppm (peak a) and the methylene peak of caprolactone units at 2.31 ppm (peak d) in the ^{1}H NMR spectra were used for the determination of molar compositions of the F127-CLx copolymer. The results are summarized in Table 1.

It is very important to investigate the difference in crystalline behavior of the copolymers because it might determine the micelle behaviors in selected solvent. For that, the crystallinity was studied by XRD as shown in Fig. 2A. Two strong F127 crystalline peaks were obviously observed at 2θ = 19.2° and 23.3° in F100 and F200. The crystalline peaks at 2θ = 21.3°, 21.9°, 23.7° belong to CL. As the increase of the length of CL, the peak at 19.2° of F127 becomes smaller and the peaks of CL become sharper. The crystallinity, calculated from XRD curves through a JAD software, increased from 50.21% to 56.26% with the increase of CL, which indicate that the copolymers are semi-crystalline, as shown in Table 1.

It is well known that thermal behavior of copolymer affects the modulus of drug capsule, release behavior and its biodegradation rate. Thus, the melting temperature (T_m) and crystallization temperature (T_c) of the copolymers were investigated using a DSC. Fig. 2B shows one melting transition between 37 and 62 °C in the heating curves (HF100, HF200, and HF400). The melting peaks are overlapped due to that the T_m of F127 is 56 °C and PCL homopolymer about 62 °C. T_m values were 52.4, 52.3, 53.4 °C for F100, F200, F400, respectively, which are less than those of F127 and PCL homopolymers. The reason may be that the crystallinity decreases because of the entwistting of the molecular chains more easily as the increasing of the molecular weight. Compared with H-F400, the thermogram of H-F100 with a smaller PCL molecular weight gave a lower melting transition temperature. In the cooling curves (CF100, CF200 and CF400), wide crystallization peaks at 10–33 °C were observed. As the molecular weight of PCL increased, the crystalline temperature increased from 22.6 °C to 32.4 °C in the cooling curve, indicating that the crystallization peaks at 0–10 and 20–30 °C come from the F127 and PCL, respectively. As the PCL molecular weight increases, the fast crystallizing PCL block freezes the following whole structure and hampers the crystallization process of the F127 [38]. Therefore, the crystallization peak of F127 at 0–10 °C in the cooling curve as well as its corresponding melting peak at 37–62 °C in the heating curve decreased as the molecular weight of PCL increased.

Crystallization behavior of the block copolymers was further visualized by polarization optical microscope (POM) in Fig. 2C. Pure F127 shows a characteristic spherulite structure with the size of about 200 μm and PCL with the size more than 200 μm, which indicates that PCL tends to form larger grains. The spherulite sizes of F127-CL_{100} become obviously smaller, which may be explained that the nucleation density is increasing due to the introduction

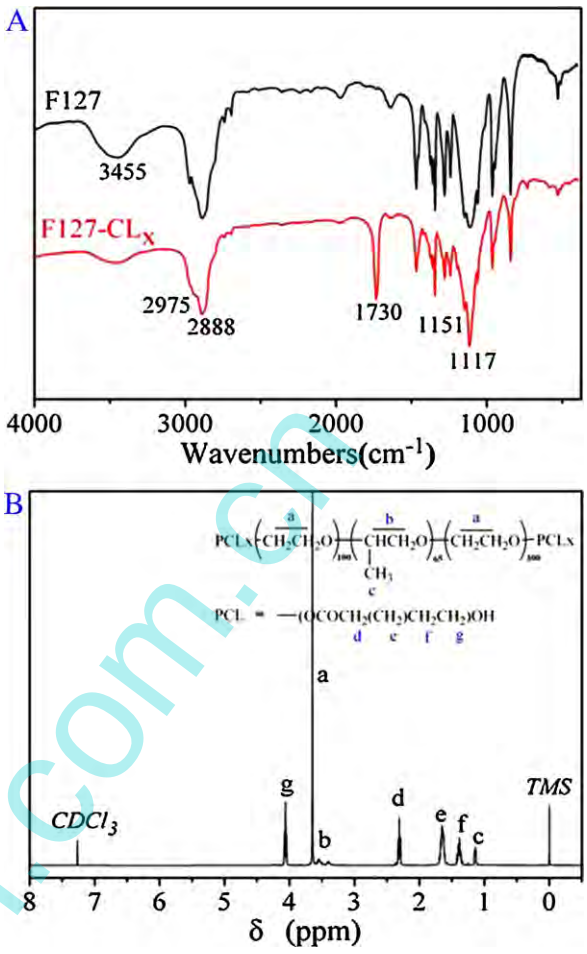

Fig. 1. Characterizations of F127-CLx block copolymer: (A) FITR, (B) ^{1}H NMR.

Table 1
Characterizations of F127-CLx copolymers.

Sample	F127/CL[a]	F127/CL[b]	M_w[c]	PDI[c]	T_m[d]	T_c[d]	Crystallinity (%)[e]
F100	1:100	1:105	14,076	1.38	52.4	22.6	50.21 ± 1.52
F200	1:200	1:185	20,734	1.79	52.3	24.5	51.17 ± 2.01
F400	1:400	1:382	22,787	1.72	53.4	32.4	56.26 ± 0.86

[a] Molar composition in feed.
[b] Molar composition determined by in ^{1}H NMR analysis.
[c] Obtained by GPC.
[d] Determined by DSC.
[e] Calculated using X-ray diffraction through JAD software.

of CL into F127. With increasing the content of CL, the crystals become bigger than the polymers with lower lengths of CL. This is mainly ascribed to the competitive crystal formation between PCL and F127 units [42]. As we know, it is easy to form crystalls for CL and thus F127-CLx can form larger spherulites with increasing CL contents, which is also confirmed by the POM image of pure PCL. From the results from DSC, XRD and POM discussed above, the crystallizing F127 block were restrained because of CL block. Therefore, the increase of nucleation density led to the reducing of the size of the spherulites. The crystallization of CL is predominant along with the increase of CL so that the size becomes bigger [43].

The molecular weight determined by GPC increased with CL segments increasing as shown in Table 1, and the actual ratio of CL and F127 determined by ^{1}H NMR was almost identical with the feed ratio.

3.2. Characterizations of the micelles

The morphologies of the micelles were examined by TEM and AFM. Polymeric micelles are created by a spontaneous self-assembly of individual polymeric molecules (unimers) which are synthetic amphiphilic copolymers comprised of hydrophilic and hydrophobic blocks. The solvent evaporation method offers the advantages of simple and gentle formulation under ambient conditions without the use of chemical additives or harsh formulation processes.

Importantly, the morphology of these nanosized micelles has been shown to influence their performance in applications such as drug delivery. Herein, the effect of the length of CL chains in morphological transitions was studied. In our study, we find that the range of structural characteristic features of these micelles can be

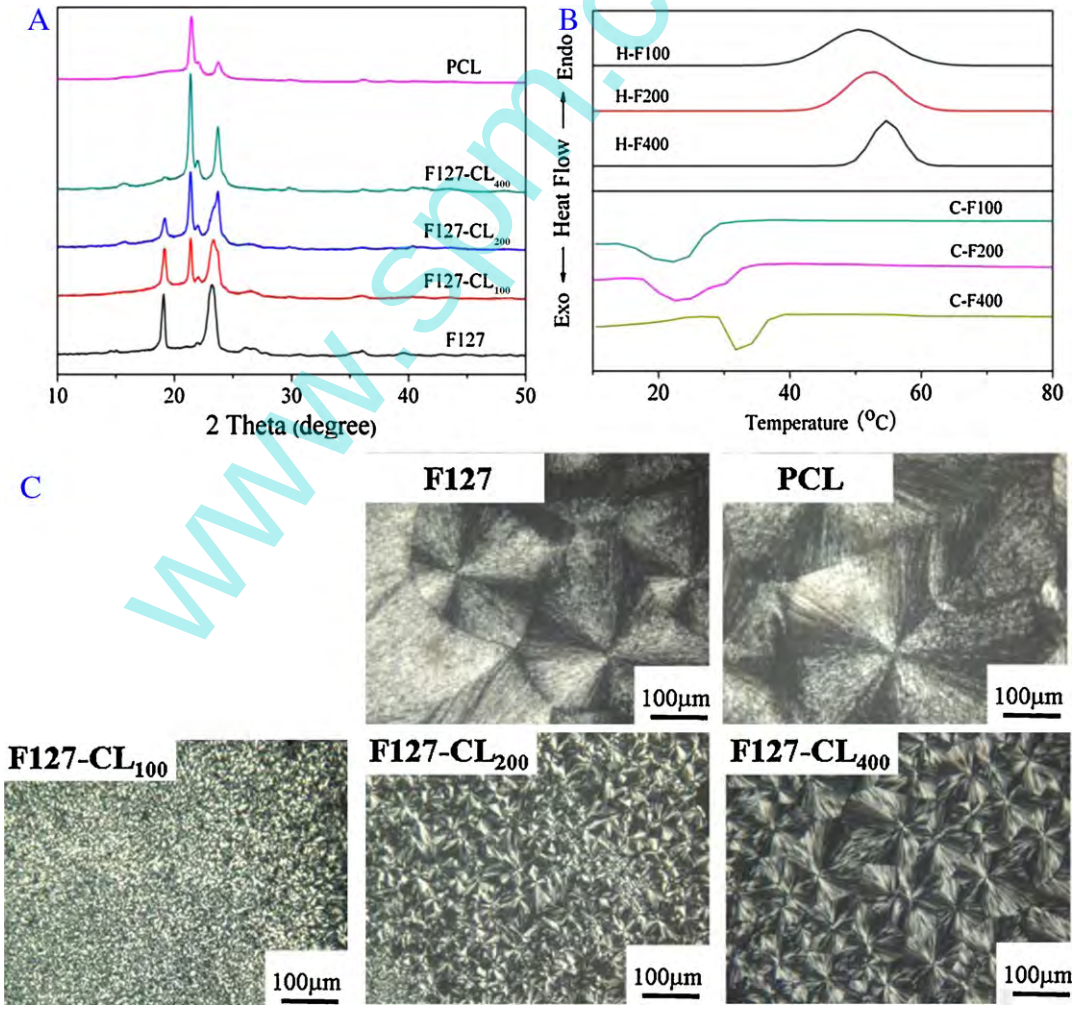

Fig. 2. Characterizations of F127-CLx block copolymer with different lengths of CL: (A) XRD, (B) DSC, (C) POM.

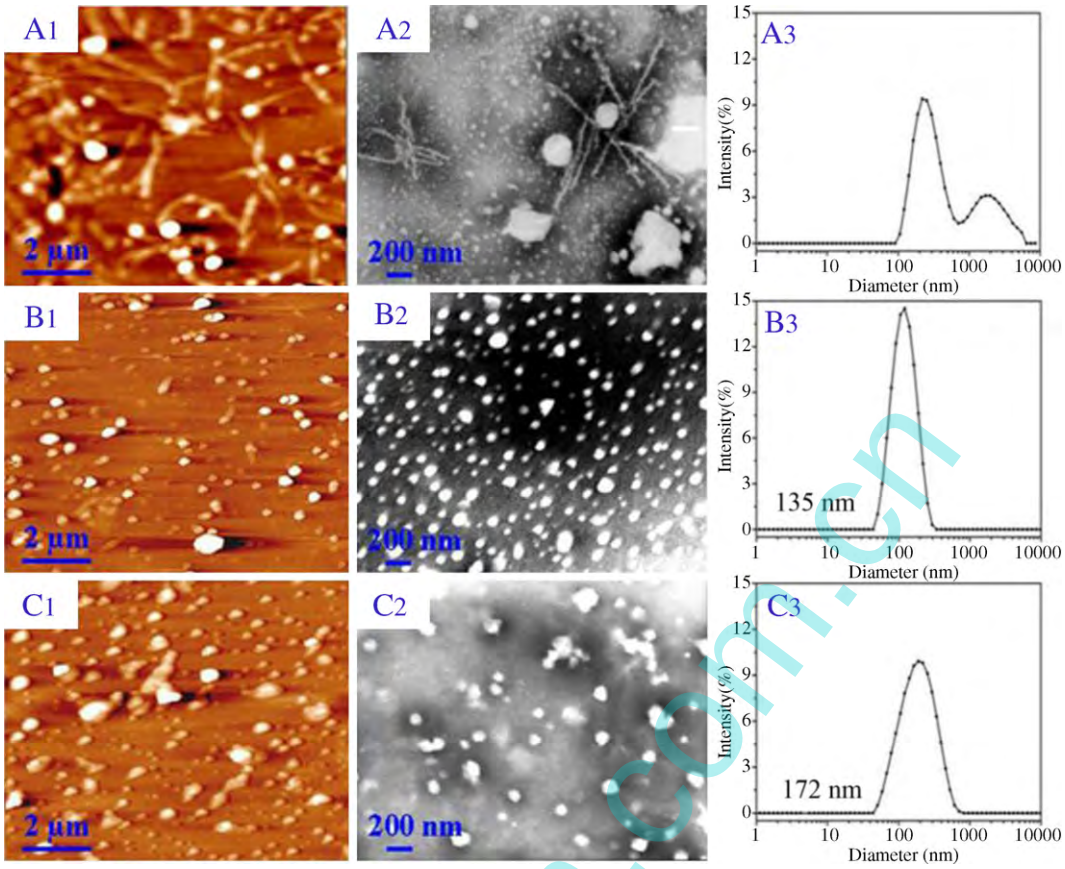

Fig. 3. Characterizations of F127-CLx micelle solutions with different lengths of CL: A1, B1, C1 (AFM images of the micelles at 1 mg/mL); A2, B2, C2 (TEM images of the micelles at 1 mg/mL); A3, B3, C3 (DLS). A1–A3, B1–B3, C1–C3 belong to F100, F200, F400, respectively.

controlled by changing the length of the hydrophobic CL chains. Fig. 3A, B and C shows the AFM, TEM images and size distribution of the micelles of the F127-CLx with different lengths of CL, respectively. The micelle morphologies of F100 were long rod-like mixed with small amount of spherical, which are clearly observed at Fig. 3A1. TEM images (Fig. 3A2) also confirm the coexistence of the two structures. In addition, there also exist large compound micelles (LCM) which comprised of many spherical micelles. Both the LCM and the rods are more stable structure rather than the sphere. For decreasing the free energy of aggregation, the spherical micelles reassemble to the compound vesicle and the rods. At the same time, the surface area of the micelle system is reducing. The tendency that contributes most to the free energy of the micelle system will have the greatest influence on the force balance (the function of all of the impetus) and control the adoption of the lowest energy morphology [20,21].

With the increase of CL length, F200 and F400 micelle morphologies are changed to uniform nanosphere-like as shown in Fig. 3B1, B2 and C1, C2, respectively. The dynamic lighting scanning results are shown in Fig. 3A3, B3 and C3. However, the micelle morphologies are transformed generally from spherical to other morphologies such as vesicles, lamellae, large compound micelles, and tubules with the increase of CL in the previous literatures referred to the block copolymers comprising of PEG and CL [19–21]. The phenomenon mainly contributes to two reasons. On the one hand, it can be explained on the basis of the reduced tethering density. Spherical micelles tend to be formed at a large reduced tethering density, while other structures are formed at a certain value of reduced tethering density. On the other hand, the crystallinity of hydrophobic chain is another important factor. Nikolic and his coworkers [31] have reported CdSe/CdS nanoparticles with bound PEO–N3 chains can self-assemble into spherical, cylindrical, and vesicular structures in dilute solution, with the reduce of polymer/nanoparticle ratios, which results from the increase of crystallinity of the hydrophobic core.

Whereas, the F127-CLx block copolymers undergo the morphologies from the rods and LCM to the sphere. It is different from the previous results, but it is important to point out that pluronic F127 also has the crystallinity because of so large molecular weight up to 12,600 Da, as proved by XRD and POM images discussed above. From the XRD and DSC, we find that the crystallinity of the copolymers are slightly increasing with the increase of CL, while the diffraction peaks of F127 weaken, which might indicate that the crystallizing F127 block were restrained because of CL block. The morphology of F100 are the coexistence of rods, LCM and spheres, which could be explained that the small spheres are joined to a more stable structures to reduce the free energy of aggregation due to the relatively high crystallinity of hydrophilic F127 chain. In other words, the crystallinity of the hydrophilic parts influences the mode of micelle assembly.

CMC, as an important parameter describing the physical properties of the micelles, refers to the thermodynamic stability of the micelle solution. In order to determine the CMC of F127-CLx micelles, fluorescence measurements were carried out using pyrene as a fluorescent probe because its vibrational fine structure is sensitive to polarity and it produces distinct excimer fluorescence under conditions of sufficiently high concentration and mobility. With an increase in F127-CLx block copolymers concentrations, the total fluorescent intensity I increased, and fluorescence spectrum shifted. A negligible change of intensity ratios was observed at a low concentration range, but the intensity ratios show a sharp increase above a certain concentration, which indicates the incorporation of

pyrene into the hydrophobic core region of the micelles. The ratio I_{339}/I_{333} of the pyrene excitation spectra was used to determine CMC of F127-CLx in water. The plot of the intensity ratio I_{339}/I_{333} of the pyrene excitation spectra against the logarithm of the different polymers concentrations is shown in Fig. 4A.

Compared with the micelles with low molecular-weight surfactant, polymeric micelles are generally more stable, exhibiting a remarkably lower CMC. Fig. 4A summarizes the CMC values of the various compositions of F127-CLx block copolymers. The values of F100, F200 and F400 are 1.96×10^{-3}, 7.41×10^{-4} and 3.62×10^{-4} mg/mL, respectively. The hydrophilicity of F127-CLx block copolymers mainly depends on the F127/CL ratio, which further influences the CMC value. The CMC value of F100 is higher than those of F200 and F400. F200 and F400 with longer hydrophobic chains can self-assemble more easily to form micelles, leading to lower CMC values. The lower CMC value indicates a strong tendency toward formation of aggregate. In addition, the CMC values are much lower than those of common low molecular weight surfactants. This result indicates that the block copolymeric system can retain a stable micelle structure even in a diluted condition, which may be useful as drug vehicles.

3.3. Themosensitive behaviors

By the comprehensive comparison to the three ratios of copolymers, F200 was chosen to examine the micelle behavior. The copolymer concentration could influence the morphology of the micelle solution [42]. Herein, diameter changes of F200 aqueous solutions with different concentrations from 1 mg/mL to 10 mg/mL were tested, as shown in Fig. 5A1. The average diameters of F200 with different concentrations distribute between 120 nm and 150 nm. The particle size distributions of the micelles were monodispersed (data not shown). As discussed above, the micelles are spherical. There are almost no morphology transitions with the change of the F200 concentration at the range of 1–10 mg/mL.

Although there is no remarkable difference with various concentrations of F200 micelle solutions, the thermo-responsive behaviors are different. In order to obtain additional informations concerning the temperature-responsive behavior, the micelle size and its distributions for F200 micelles were measured by DLS at various temperatures. Fig. 5B shows the size change of F200 micelle solutions with different concentrations depending on the temperature. Their sizes markedly decrease with increasing the temperature regardless of their concentrations under about 45 °C. Similarly, Kim et al. have reported that the solubility and hydrophilicity of PPO block in F127 markedly decreases with increasing temperature as a result of dehydration, which influence the micelle behaviors [36]. Owing to the increase of inter- and intra-molecular interactions between PPO and PCL as a function of temperature, the shrinkage and the compact structure by the strong chain–chain aggregation could be induced. When the temperature continues to increase, the size of the micelle solution at 2 mg/mL also reduce from 119 nm at 25 °C to 81.9 nm at 65 °C, while the size at 8 mg/mL becomes bigger up to 286 nm at 55 °C, as shown in Fig. 5C and D (the size not tested at 65 °C due to go beyond the measure limit). It can be explained that the solubility and hydrophilicity of PEO block decreases with increasing temperature as a result of dehydration, and then the hydrophobicity of the polymer continue improving, resulting in the micelles congregating and further leading to the increasing of the diameter of micelles with the increase of the temperature.

The solution behaviors of the thermo-responsive block copolymers were also investigated by an optical method. To determine the transition temperature, we firstly observed macroscopically that the solution of the polymeric micelles is almost transparent at room temperature and becomes opaque with the increases of the temperature and then precipitates in Fig. 6A. Later, the optical absorbances

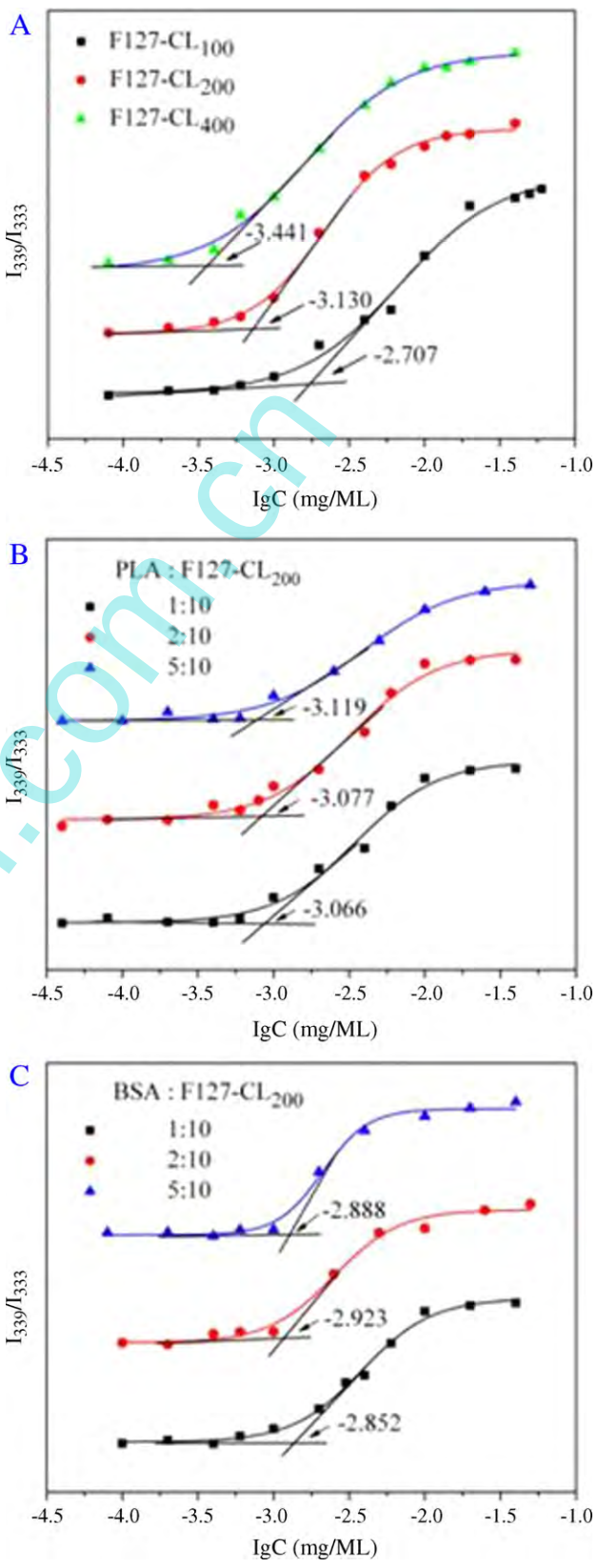

Fig. 4. CMC of F127-CLx with different lengths of CL (A), and CMC dependence of different weight ratios between F127-CL_{200} and the addition of PLA (B), BSA (C). All measurments were at 25 °C.

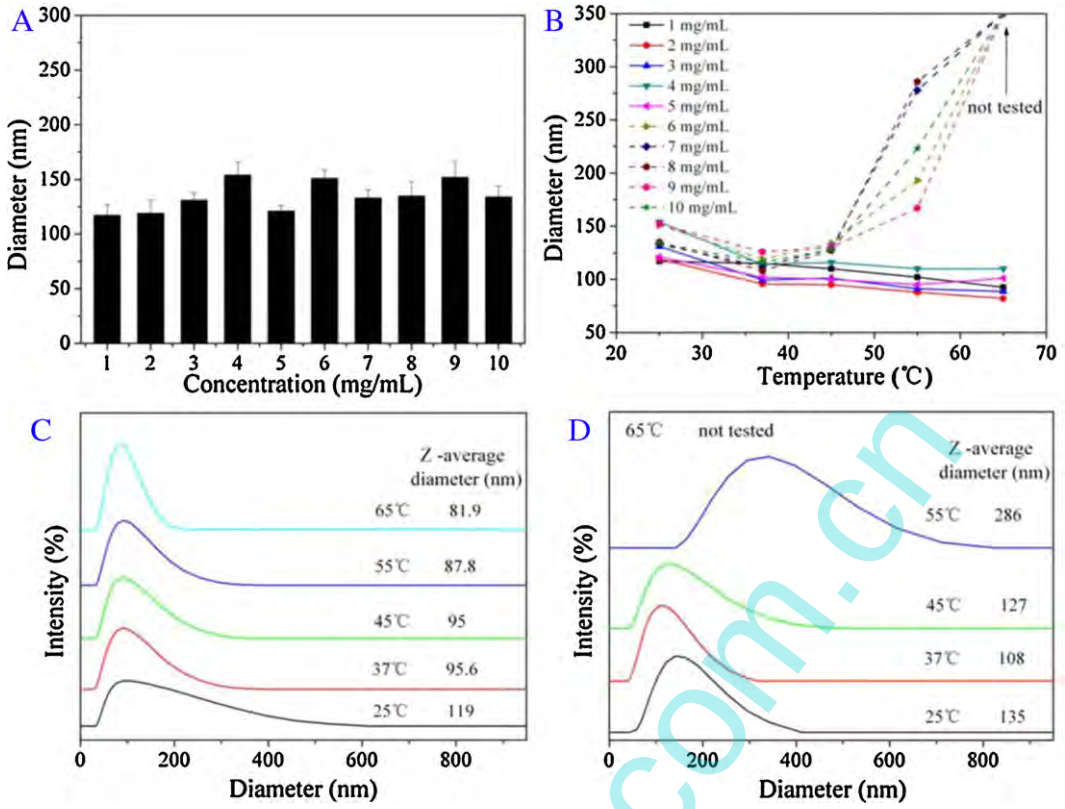

Fig. 5. Diameter changes of F200 aqueous solution with different concentrations from 1 mg/mL to 10 mg/mL (A), and diameter changes as a function of temperature (B), size distributions of F200 aqueous solution at 2 mg/mL (C), 8 mg/mL (D) as a function of temperature.

of polymeric micelle solutions were measured as a function of temperature. Generally, the incorporation of hydrophobic comonomer leads to aggregate for F200 with increasing temperature because the hydrophobic moiety facilitates the chain aggregation. Besides the slightly decrease of the absorbance under 45 °C, there is no obvious change at 2 mg/mL as the temperature increases to 85 °C, which accords with the results that the size does not change as a function of the temperature at a low concentration. But there is a sharply

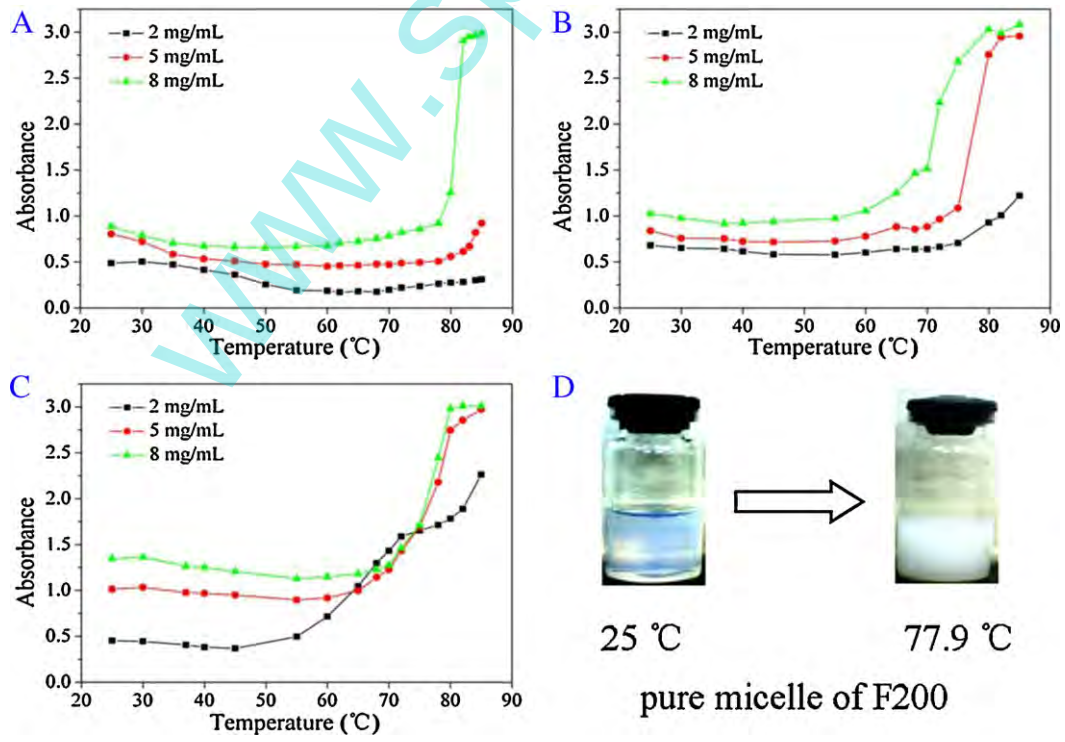

Fig. 6. Absorbance changes of F200 pure micelle solution (A) and mixed micelle solutions with PLA (B) and BSA (C) at different concentrations as a function of temperature. The concentration of PLA remained unchanged at 1 mg/mL while BSA was 0.5 mg/mL. (D) The change of pure micelle solutions at 8 mg/mL from visual observations.

increase of the absorbance at 77.9 °C, when the concentration is up to 8 mg/mL. It contributes that the increase of the concentrations enhances the hydrophobic interaction of inter- and intra-molecular interactions, and then the hydrophilic and hydrophobic chains shrinkage to become more hydrophobic, and further tends to form large aggregates. The thermo-responsive behavior of F200 is influenced by concentration at high temperatures. In other words, the micelle solutions are stable at physiological temperature 37 °C, which is very important to be potential application as drug delivery carriers in nanomedicine.

Mixed micelle solutions were prepared by the method just like preparing the pure micelles. An appropriate ratio between the addition and the surfactant is very important to form stable mixed micelle solutions. Hence, the concentration of the additions such as BSA and PLA were controlled to confirm the mixed micelle solutions stable [44]. Meanwhile, the physicochemical characteristics of mixed micelles with hydrophilic BSA and hydrophobic PLA were investigated. Fig. 4B and C shows the CMC dependence of different weight ratio between the addition of PLA, BSA and F127-CL200. The CMC values of BSA/F200 are 1.41×10^{-3}, 1.19×10^{-3}, 1.29×10^{-3} mg/mL, while the values of PLA/F200 are 8.58×10^{-4}, 8.36×10^{-4}, 7.60×10^{-4} mg/mL, with the weight ratios between the additions and the polymers increasing from 1:10 to 5:10. It can be easily found that the CMC values significantly increase compared to 7.41×10^{-4} mg/mL of pure F200 micelles after adding the hydrophilic molecular BSA. Bovine serum albumins bind many different types of amphiphilic biological molecules, which are believed to play an important role in determining their physiological function [37,44]. Through the results from Kim et al. [44], the hydrophobic chains of surfactants can link to the nonpolar residues of BSA, and the binding of a large number of surfactants may break the intra-chain hydrophobic bonds of BSA, resulting in an extended structure of BSA. Therefore, the extend structures of BSA act as the hydrophilic chains in the mixed micelles, in other words, BSA increases the hydrophilic ratio just like increasing the ratio of hydrophilic F127. Thereby, the CMC values decrease notably. It is worth noting that the values are not influenced regardless of the adding amount of BSA, it might attribute to the assumption that the binding sites of BSA and the surfactant molecular of F200 are limited, it remains further research in future.

In contrast, there is no obviously difference comparing the CMC values with pure F200 micelle when adding hydrophobic PLA. The values slightly decrease, which could be neglected because of experimental error inevitably. It could be believed that the hydrophobic PLA is embedded in the hydrophobic regions of the micelles just as the encapsulation of hydrophobic drugs.

The themosensitive behaviors of mixed micelles were also investigated through UV analysis. Fig. 6B and C shows the absorbance changes of F200 micelle solution mixed with PLA and BSA at different concentrations as a function of temperature. It can be found that all the transition temperatures of the mixed micelles reduce when adding hydrophobic PLA, and the transition temperature reduces to about 70 °C at 8 mg/mL. It is worth noting that while the absorbance begin to increase from 55 °C at 2 mg/mL, because BSA denature at high temperature, while the transition temperature of mixed micelles with BSA over 5 mg/mL do not change obviously at the range of temperature. As we know, the hydrophilicity of copolymer plays a critical role in assembling into stable micelles in aqueous solution. Herein, the introduction of PLA and BSA into pure micelles increased the hydrophobicity and hydrophilicity, separately, which caused the PLA/F200 mixed micelles to being unstable, further resulted in a decrease of the transition temperature, and in the contrary, caused the BSA/F200 micelles to being stable, and then led to the increase of the transition temperature. Fig. 6D shows the change of pure micelle solutions at 8 mg/mL from visual observations.

Considering the results above, the schematic illustration of the pure and mixed micelles can be shown in Fig. 7A. The mixed micelles are also spherical with some small congeries through Fig. 7B,C. As discussed above, both pure and mixed micelles show thermo-sensitive behaviors due to the opening or closing of the F127 shell triggered by the temperature, which plays a significant role in controlling drug loading and release in drug delivery system [28].

3.4. In vitro DOX.HCl release

DOX.HCl was selected as the therapeutic agent, and the loading, in vitro release and cytotoxicity of the nanocarriers were investigated in depth. The diameters of pure and mixed micelles are shown in Table 2. The average diameters after adding BSA and PLA are found to increase, and it was also clear that the size-distribution of mixed micelles became no longer narrow from the polydispersity. The addition to the pure micelle solutions may reduce the stability of micelle system. It is so important that there are so many proteins and other things that can influence the micelle behaviors. Meanwhile, the addition of hydrophobic molecular PLA made the core larger, and hydrophilic BSA enlarged the shell of the micelle, which resulted in the increase of diameters of the mixed micelles. The drug-loading efficiency of BSA/F200 (26.9%) is the lowest among the three micelles. The reason is that the increase of the shell volume resulted from the addition of hydrophilic BSA led the water-soluble DOX.HCl encapsulated into the core to diffusing easily into the solution.

DOX release from pure and mixed micelles were evaluated at pH 5.0 and pH 7.4, respectively. As shown in Fig. 8, both pure micelle and mixed micelles showed much faster DOX release at pH 5.0 than at pH 7.4. At pH 5.0 pure micelles released about 50% of the initially loaded DOX for the original 3 h, and liberated 82% after 48 h. However, at pH 7.4 the micelles released only 28% of DOX for the first 3 h and about 40% after 48 h. Though few differences, BSA/F200, PLA/F200 mixed micelles undergo the same trend that DOX.HCl releases are much faster at pH 5.0 than at pH 7.4. The accelerated DOX.HCl release in acidic pH condition is highly desirable for effective treatment of cancer. It is conceivable that the micelles are passively targeted to the tumor tissue through the enhanced permeation and retention (EPR) effect, thus avoiding the undesirable organ accumulation and toxicity associated with the free drug, with minimized DOX release during circulation in the blood stream. It is likely that some DOX release occurs during rearrangement of the micelles to aggregates while the remainder of the release over a longer time period occurs from the aggregates and is assisted by the increased water solubility of DOX at acidic pH upon protonation of the glycosidic amine. At pH 7.4 the micelles are stable over several days, with no significant changes in the size distribution over this times period [37,44]. Therefore, overall results indicate that the DOX.HCl-loaded pure and mixed micelles have characteristics ideal for the selective release of DOX.HCl in mildly acidic physiological environments.

3.5. Hemolysis assay and coagulation measurements

As shown in Fig. 9B, it can be found that the percent of hemolysis became higher with increasing the concentration of the micelles, which might imply that the micelles with higher concentration can induce hemolysis. Blood coagulation involves a series of proteolytic reactions resulting in the formation of fibrin clot.

Thrombin is formed following a cascade reactions where an inactive factor becomes enzymatically active following surface contact or after proteolytic cleavage by other enzymes; the newly activated enzyme then activates another inactive precursor factor [45]. Fig. 9C shows that the APTT, PT and TT of the block polymers

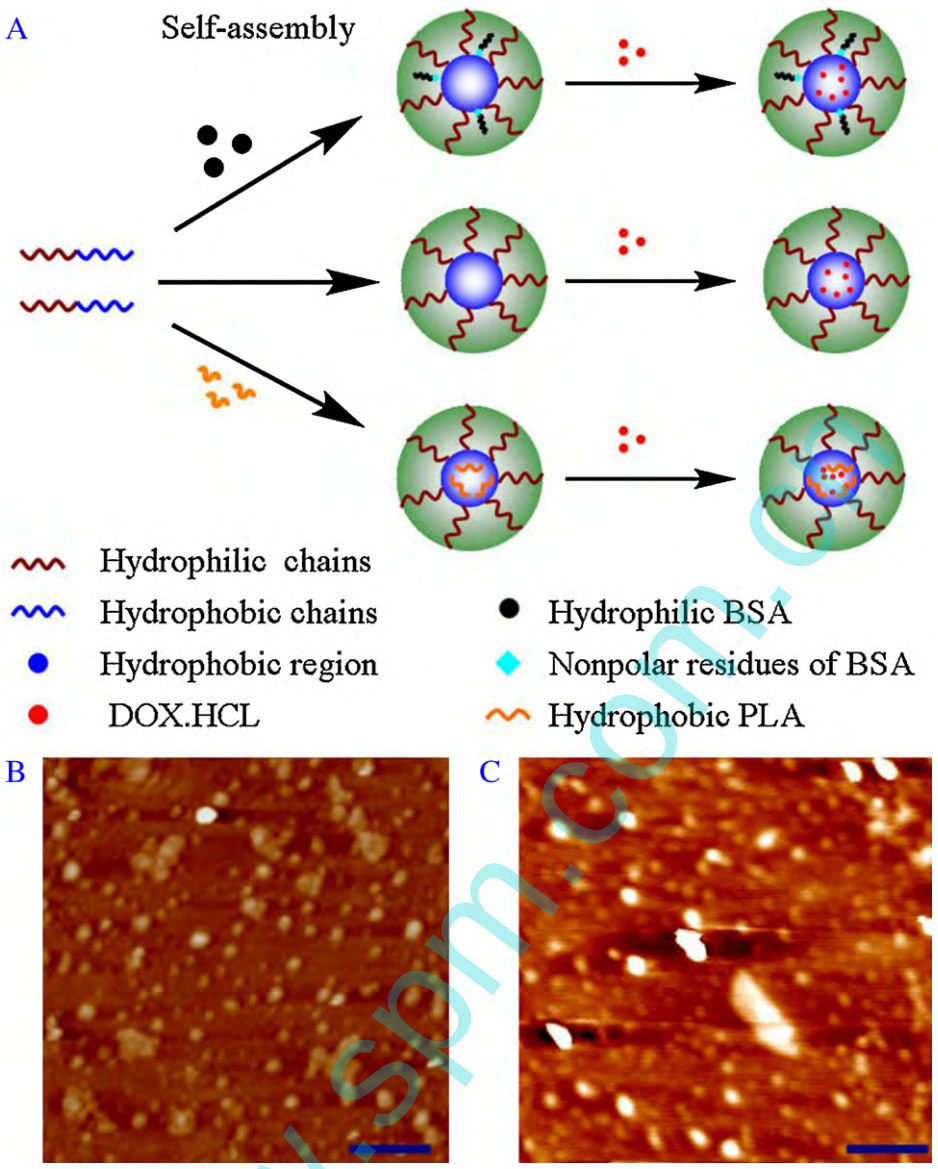

Fig. 7. Schematic illustration of the formation of the pure and mixed micelle solutions with hydrophobic PLA or hydrophilic BSA (A) and AFM images of mixed micelles BSA/F200 (B) and PLA/F200 (C). The scale bar is 2 μm.

are similar to the original plasma, which indicates that the materials have no obvious activation to coagulation factors (FVIII, FIX and FXI) and thrombin generation (TAT). Therefore, the polymeric micelles can be considered that they possess good blood compatibility.

3.6. *In vitro cell cytotoxicity analysis*

One of the major requirements for the block copolymer application in medicine should be non-toxic. Although the F127 and PCL are generally considered as biodegradable and safe polymers, the cytotoxicity of F200 block copolymer micelles is performed by quantitative evaluation of cell viability using osteoblasts and Hep G2 tumor cells. According to the MTT assay results (Fig. 9A), we can see that all of the samples of the micelles with different concentrations performed a degree of cytotoxicity to reduce the cell viability compared to the control group. The cell viability expressed in this study is relative to those at each concentration of the micelles. The cell viability of the micelle at 2.0 mg/mL is about 83.6%, noting that the moderate concentration is a favorable environment for osteoblasts, and at the same time the cell viability of the micelle in tumor cells is similar. This study underlined a fact that the copolymers possess some cytotoxicity as the concentra-

Table 2
Characterizations of F127-CL_{200} pure and mixed DOX-loaded micelle solutions.

Sample	Addition[a]	Blank micelles		DOX-loaded micelles			
		Size ± SD (nm)	PDI	Size ± SD (nm)	PDI	Drug-loading efficiency (%)	Drug-loading content (%)
F200	–	135 ± 5	0.193 ± 0.068	150 ± 8	0.254 ± 0.010	74.1	0.370
PLA/F200	PLA	146 ± 5	0.302 ± 0.113	155 ± 6	0.322 ± 0.028	60.8	0.304
BSA/F200	BSA	144 ± 3	0.388 ± 0.138	186 ± 7	0.446 ± 0.013	26.9	0.135

[a] The weight ratio between the addition and F200 is 2:10.

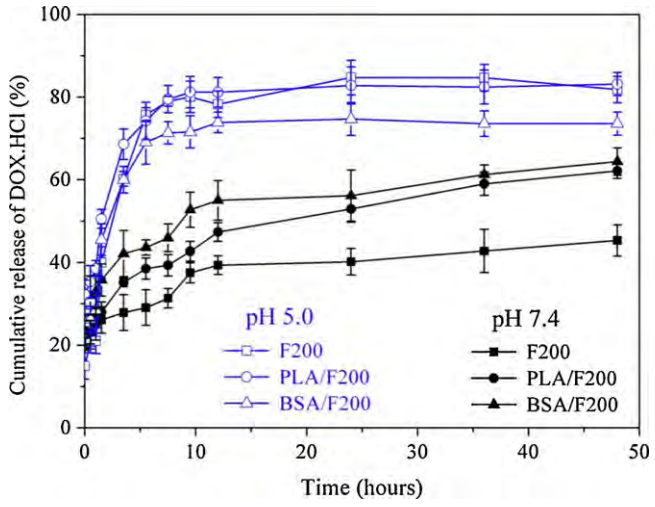

Fig. 8. In vitro release from DOX.HCl-loaded pure micelles and mixed micelles in PBS at pH 7.4, and in ABS at pH 5.0, respectively, at 37 °C. The weight ratios of PLA/F200 and BSA/F200 are 0.2.

tion is above 2 mg/mL, but the micelle with low concentrations is nontoxic. Therefore, these micelles could be regarded as a safe drug delivery carrier.

The MTT assay was performed to evaluate whether micelles influenced the cytotoxicity of DOX.HCl. Fig. 10A displays the effect of drug concentration on HepG2 cell viability. DOX.HCl loaded pure and mixed micelles and free DOX.HCl at various concentrations significantly influenced the growth of the HepG2 cells in the certain dose. The cell viability of all samples decreases slowly during the original 2 days' incubation, whereas the tumor cells are markedly depressed at the third day. With prolonging the culture time, the DOX.HCl loaded micelles exhibit more significant suppress to tumor cell growth compared with free DOX. The result indicates that DOX.HCl-loaded pure and mixed micelles can sustainedly release drug in the culture solution. It is accorded with the release profiles (Fig. 7), which was observed that only 63% DOX.HCl released from these micelles within 48 h. However, BSA/F200 micelles show the cell viability is a bit higher after 3 days due to BSA being a nutrient for most of cells [37,44].

As seen from Fig. 10B, the cell morphology and amount for the samples with low concentrations of DOX.HCl are similar to the control group in the first day, suggesting that the samples have good cell viability. In contrast, almost all the tumor cells are rounded and the numbers of the cells reduced obviously after 3 days. It is more visible to find that the cells are depressed seriously regardless of the blank and mixed micelles. As a greater amount of DOX.HCl could be intra-cellularly delivered into cells in the form of nano-sized micelles by endocytosis, the cells were more vulnerable to the cytotoxic effect of DOX.HCl. DOX.HCl-loaded blank and mixed micelles were equally effective for suppressing the growth of the tumor cells.

As discussed above, the pure micelles show thermosensitive behaviors due to the opening and closing of F127 chains triggered by the temperature, which were confirmed by the DLS and UV optical method. The mixed micelles were prepared to adjust the properties of pure micelles. On one hand, the addition of hydrophobic PLA obviously reduces the transition temperature of the pure micelle and there is few difference in the drug-loading efficiency

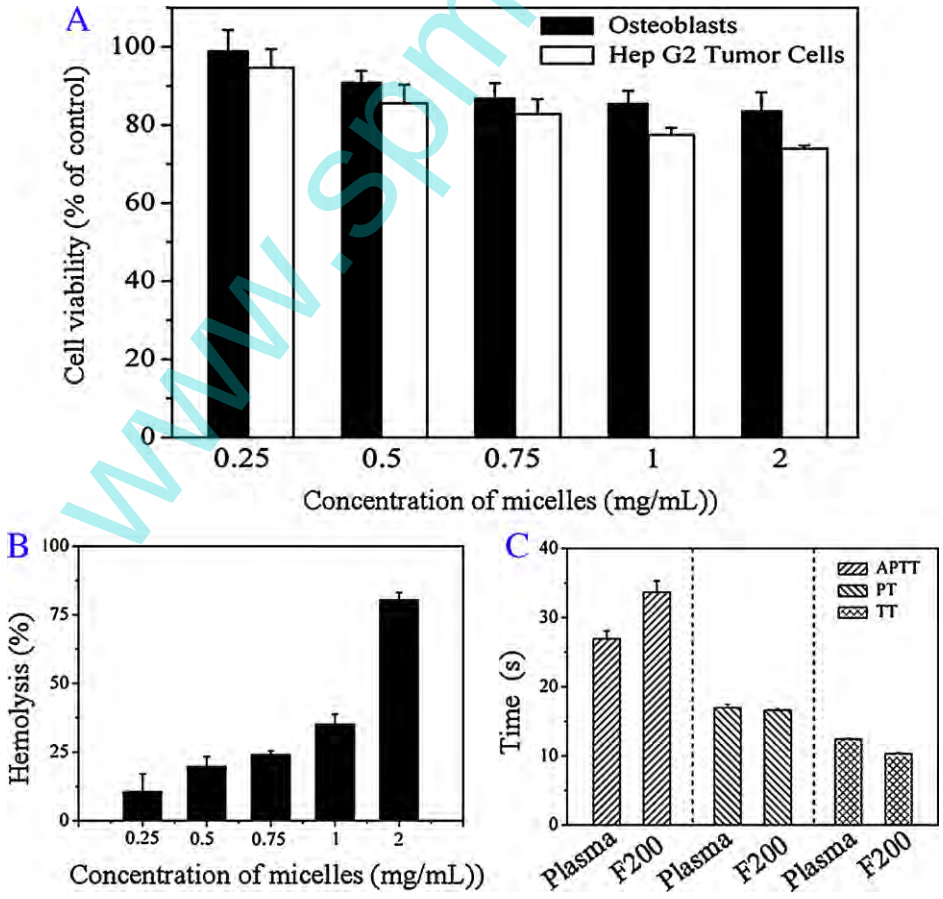

Fig. 9. The cytotoxicity study of the block copolymer: (A) cell viability of osteoblasts and HepG2 tumor cells with different concentrations of micelles after 48 h; (B) hemolysis assay of different concentrations of micelles; (C) the APTT, PT, TT assays of F200.

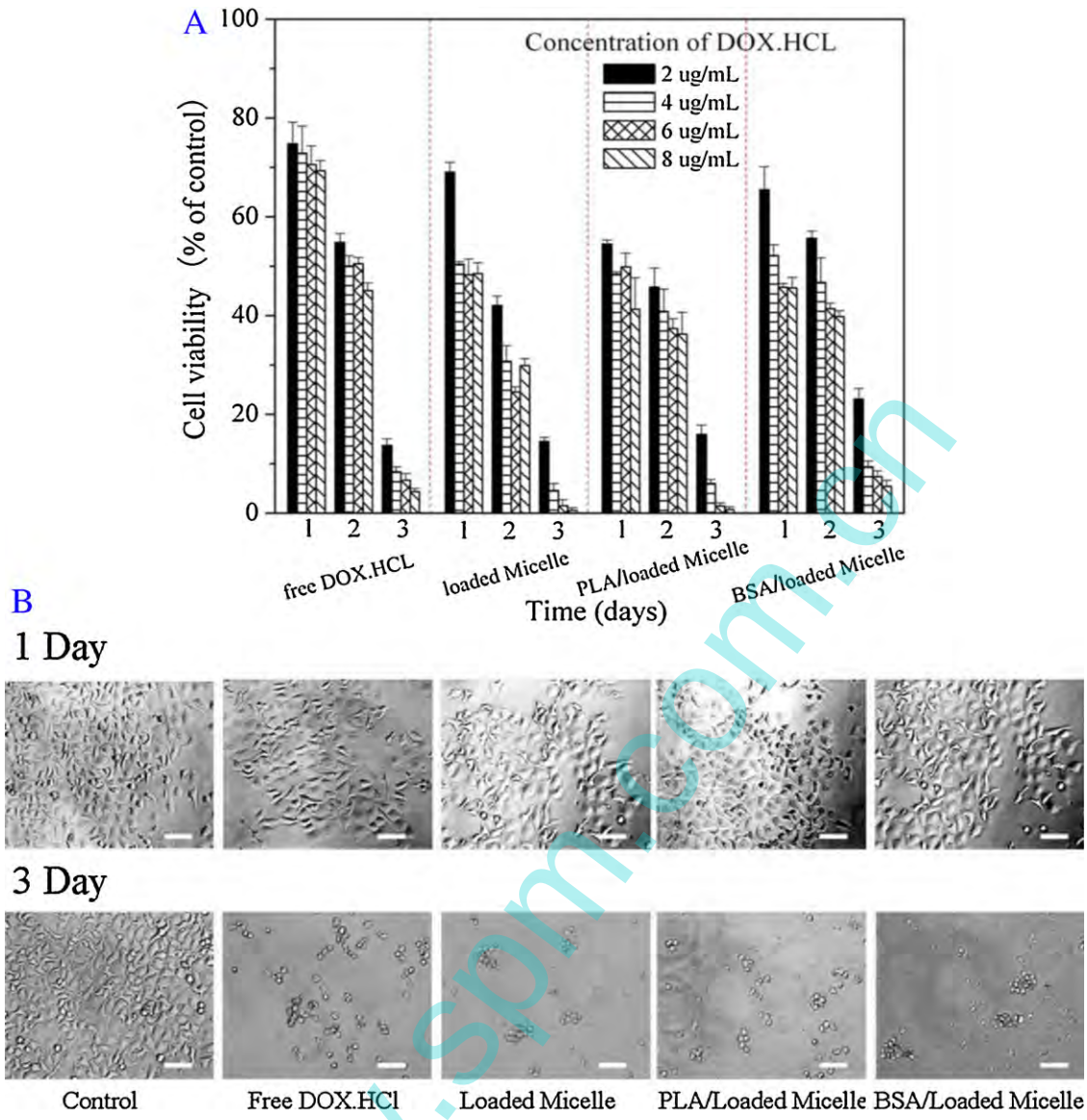

Fig. 10. (A): Cell viability of HepG2 cells. HepG2 cells were exposed to different concentrations of free DOX.HCL or DOX.HCL-loaded pure and mixed micelles for 72 h, respectively. (B) Optical microscopic observation of HepG2 cells incubated at 2 μg/mL DOX.HCl from different samples, and the scale bar is 20 μm.

compared to the pure micelles. On the other hand, the drug-loading efficiency of BSA/F200 mixed micelles is much lower than pure micelles, while the thermosensitive behaviors is almost the same to the pure micelles. Therefore, the addition of the hydrophobic PLA may mainly influence the thermosensitive behaviors of the micelles, while the drug-loading properties may be affected by the addition of hydrophilic BSA in the system. The addition of one-fold hydrophilic and hydrophobic molecules to the pure micelles can adjust their properties such as the stimulus-responsive behaviors. However, either the DOX.HCl-loaded mixed micelles or the pure micelles can suppress the growth of the tumor cells. It may be concluded that the micelles can be altered by mixing either hydrophobic PLA or hydrophilic BSA.

4. Conclusions

We have successfully prepared a kind of thermosensitive mixed micelles from pluronic F127-b-poly(ε-caprolactone) block copolymer by mixing with hydrophilic BSA and hydrophobic PLA. Pure micelles with different lengths of CL undergo morphology transition from the rods to the sphere. The size of F200 at 2 mg/mL becomes smaller with increasing the temperature, while higher concentrations induce aggregations at high temperature. The addition of BSA and PLA can influence the thermosensitive and drug loaded behaviors of the micelles. DOX.HCl-loaded pure and mixed micelles have characteristics ideal for the selective sustained release of DOX.HCl in mildly acidic physiological environments rather than at pH 7.4. As observed from cell cytotoxicity, the block polymers are of excellent biocompatibility and the DOX.HCl-loaded pure and mixed micelles are effective to inhibit the growth of HepG2 tumor cells. Therefore, the properties of the micelles can be adjusted by mixing either hydrophobic or hydrophilic molecules and the thermo-sensitive block polymeric micelles may an attractive vehicle for DOX.HCl delivery.

Acknowledgements

This work was partially supported by National Natural Science Foundation of China (30970723), Programs for New Century Excellent Talents in university, Ministry of Education of China (NCET-07-0719) and Sichuan Prominent Young Talent Program (08ZQ026-040).

References

[1] M.C. Branco, J.P. Schneider, Acta Biomater. 5 (2009) 817–831.
[2] T.Y. Liu, S.H. Hu, D.M. Liu, S.Y. Chen, I.W. Chen, Nano Today 4 (2009) 52–65.
[3] Y.P. Wang, H.P. Xu, X. Zhang, Adv. Mater. 21 (2009) 2849–2864.
[4] A.P. Kumar, D. Depan, N.S. Tomer, R.P. Singh, Prog. Polym. Sci. 34 (2009) 479–515.
[5] S. Kim, J.H. Kim, O. Jeon, I.C. Kwon, K. Park, J. Pharm. Biopharm. 71 (2009) 420–430.
[6] A.J. Zhao, S.B. Zhou, Q. Zhou, T. Chen, Pharm. Res. 27 (2010) 1627–1643.
[7] T.M. Allen, P.R. Cullis, Science 303 (2004) 1818–1822.
[8] X.L. Hu, S. Liu, X.S. Chen, Biomacromolecules 9 (2008) 553–560.
[9] V.K. Garripelli, J.K. Kim, R. Namgung, W.J. Kim, M.A. Repk, S. Jo, Acta. Biomater. 62 (2010) 477–485.
[10] C.Y. Gong, S. Shi, L. Wu, M.L. Gou, Q.Q. Yin, Q.F. Guo, P.W. Dong, F. Zhang, F. Luo, X. Zhao, Y.Q. Wei, Z.Y. Qian, Acta Biomater. 59 (2009) 3358–3370.
[11] D.L. Shi, H.S. Cho, Y. Chen, H. Xu, H.C. Gu, J. Lian, W. Wang, G.K. Liu, C. Huth, L.M. Wang, R.C. Ewing, S. Budko, G.M. Pauletti, Z.Y. Dong, Adv. Mater. 21 (2009) 2170–2173.
[12] B.M. Budhlall, M. Marquez, D. Orlin, Langmuir 24 (2008) 11959–11966.
[13] O.C. Farokhzad, R. Langer, ACS Nano 31 (2009) 16–20.
[14] Y.Y. Li, H. Cheng, J.L. Zhu, L. Yuan, Y. Dai, S.X. Cheng, X.Z. Zhang, R.X. Zhuo, Adv. Mater. 21 (2009) 2402–2406.
[15] D. Kim, E.S. Lee, K.T. Oh, Z.G. Gao, Y.H. Bae, Small 411 (2008) 2043–2050.
[16] C.L. Lo, S.J. Lin, H.C. Tsa, W.H. Chan, C.H. Tsai, C.H.D. Cheng, H.G. Hsiue, Biomaterials 30 (2009) 3961–3970.
[17] T.C. Lu, J. Sun, X.X. Chen, P.B. Zhang, X.B. Jing, Macromol. Biosci. 9 (2009) 1059–1068.
[18] Y.T. Liu, K. Li, J. Pan, B. Liu, S.S. Feng, Biomaterials 31 (2010) 330–338.
[19] H.C. Tsai, W.H. Chang, C.L. Lo, C.H. Tsai, C.H. Chang, T.W. Ou, T.C. Yen, G.H. Hsiu, Biomaterials 31 (2010) 2293–2301.
[20] H.J. Wang, P.Q. Zhao, X.F. Liang, X.Q. Gong, T. Song, R.F. Niu, J. Chang, Biomaterials 3114 (2010) 4129–4138.
[21] S.H. Kim, J.P.K. Tan, F. Nederberg, K. Fukushima, Y.Y. Yang, R.M. Waymouth, J.L. Hedrick, Macromolecules 42 (2009) 25–29.
[22] D. Cohn, A.H. Salomon, Polymer 46 (2005) 2068–2075.
[23] K. Kataoka, G.S. Kwon, M. Yokoyama, T. Okano, Y. Sakurai, J. Control Release 24 (2006) 119–132.
[24] X.W. Wei, C.Y. Gong, M.L. Gou, S.Z. Fu, Q.F. Guo, S. Shi, F. Luo, G. Guo, L.Y. Qiu, Z.Y. Qian, Int. J. Pharm. 369 (2009) 170–175.
[25] M.K. Joo, Y.S. Sohn, B. Jeong, Macromolecules 40 (2007) 5111–5115.
[26] S.B. Zhou, X.Y. Liao, X.H. Li, X.M. Deng, J. Control Release 86 (2003) 195–205.
[27] B. Jeong, Y.H. Bae, S.W. Kim, Macromolecules 32 (2001) 7064–7069.
[28] S.C. Ying, L.C. Guo, J. Wang, J.H. Ma, X.F. Liang, L.R. Yang, H.Z. Liu, Langmuir 23 (2007) 12669–12676.
[29] Y.J. Kim, K. Shibata, H. Uyama, S. Kobayashi, Polymer 49 (2008) 4791–4795.
[30] D. Cohn, H. Sagiv, A. Benyamin, G. Lando, Biomaterials 3019 (2009) 1–8.
[31] M.S. Nikolic, C. Olsson, A. Salcher, A. Kornowski, A. Rank, R. Schubert, A. Frmsdorf, H. Weller, S. Frster, Angew. Chem. Int. Ed. 48 (2009) 2752–2754.
[32] N. Fairley, B. Hoang, C. Allen, Biomacromolecules 9 (2008) 2283–2291.
[33] A. Wittemann, T. Azzam, A. Eisenberg, Langmuir 23 (2007) 2224–2230.
[34] S.B. Zhou, X.M. Deng, Biomaterials 2420 (2003) 3563–3570.
[35] P. Alexandridis, T.A. Hatton, Colloids Surf. A 96 (1995) 1–46.
[36] S.Y. Kim, J.C. Ha, Y.M. Lee, J. Control Release 65 (2000) 345–358.
[37] D. Kelley, D.J. McClements, Food Hydrocolloid. 171 (2003) 73–85.
[38] M.N. Jones, M.N. Chapman, Micelles Monolayers and Biomembranes, Wiley, 1995.
[39] S.B. Zhou, X.M. Deng, W.X. Jia, J. Appl. Polym. Sci. 913 (2004) 1848–1856.
[40] M.J. Gandhi, E. Shapiro, L. Emmert, D.M. Strong, T.H. Price, Transfus. Apher. Sci. 36 (2007) 17–22.
[41] R.X. Hou, L.G. Wu, J. Wang, N. Huang, Appl. Surf. Sci. 256 (2010) 5000–5005.
[42] H.K. Cho, S. Lone, D.D. Kim, J.H. Choi, S.W. Choi, J.H. Cho, J.H. Kim, I.W. Cheong, Polymer 50 (2009) 2357–2364.
[43] W.P. Zhu, W.H. Xie, X.W. Tong, Z.Q. Shen, Eur. Polym. J. 43 (2007) 3522–3530.
[44] S.Y. Kim, K.E. Lee, S.S. Han, B. Jeong, J. Phys. Chem. B 112 (2008) 7420–7423.
[45] Y. Lee, S.Y. Park, H. Mok, T. Gwan, Bioconjugate Chem. 19 (2008) 525–531.