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Novel pH-sensitive polysialic acid based polymeric micelles for triggered intracellular release of hydrophobic drug

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ABSTRACT

Polysialic acid (PSA), a non-immunogenic and biodegradable natural polymer, is prone to hydrolysis under endo-lysosomal pH conditions. Here, we synthesized an intracellular pH-sensitive polysialic acid-ursolic acid conjugate by a condensation reaction. To further test the drug loading capability, we prepared paclitaxel-loaded polysialic acid-based amphiphilic copolymer micelle (PTX-loaded-PSAU) by a nanoprecipitation method. Results showed PTX-loaded-PSAU exhibited well-defined spherical shape and homogeneous distribution. The drug-loading was 4.5% with an entrapment efficiency of 67.5%. PTX released from PTX-loaded-PSAU was 15% and 42% in 72 h under simulated physiological condition (pH 7.4) and mild acidic conditions (pH 5.0), respectively. In addition, *In vitro* cytotoxicity assay showed that PTX-loaded-PSAU retained anti-tumor (SGC-7901) activity with a cell viability of 53.8% following 72 h incubation, indicating PTX-loaded-PSAU could efficiently release PTX into the tumor cells. These results indicated that the pH-responsive biodegradable PTX-loaded-PSAU possess superior extracellular stability and intracellular drug release ability.

1. Introduction

Tremendous efforts have been directed to the development of targeted drug delivery systems because they promised to resolve several key therapeutical issues associated with current clinical practice including low treatment efficacy and significant side effects (Langer, 1998; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001). Until now, various drug delivery systems based on liposomes, nanoparticles, polymeric conjugates and micelles have been intensively explored (Yoo, Lee, & Park, 2002). Polymeric micelles could alter the pharmacokinetic profile of drugs, reduce off-target toxicity and side effects, prolong circulation in the blood owing to its high water-solubility, and enhance the therapeutic efficiency (Cayre, Chagneux, & Biggs, 2011; Kedar, Phutane, Shidhaye, & Kadam, 2010). However, only few drugs could be carried into the intracellular compartments of the cancer cell due to the slow drug release from micelles (Shuai, Ai, Nasongkla, Kim, & Gao, 2004; Sui, Liu, & Shen, 2011). The proton concentration of late endosomes and lysosomes is 100-times lower (pH 5.0) than the physiological condition (pH 7.4), which provide an important stimulus *in vivo* that could be used to trigger intracellular release of hydrophobic drugs (Duncan, 1992; Haag, 2004).

Polysialic acid (PSA), a homopolymer of sialic acid (SA) in either α-2,8 or α-2,9 linkages or a mixture, was first found in *E. coli* (*Escherichia coli*) K-1 and K-235 by Barry (Barry & Goebel, 1957). The 2,8 linked polysialic acid only induced a low level of antibody that could avoid the clearance of modified drugs/carriers from the blood circulation and present long circulation time *in vivo* (Fernandes & Gregoriadis, 1997, 2001). More importantly, it has been proved that PSA is biodegradable and prone to hydrolysis under endo-lysosomal pH conditions (Zhang et al., 2014). Also, the degradation product of PSA, SA, could serve as a targeting ligand for selectin, which is highly expressed in tumor vascular endothelial cells (Greco et al., 2013; Jayant et al., 2007; Zhang et al., 2014). SA could inhibit tumor metastasis by selectin targeting (Zeisig, Stahn, Wenzel, Behrens, & Fichtner, 2004). Obviously, PSA had a great potential to be an effective drug delivery material for targeting cancer disease. Ursolic acid, a natural triterpene, structurally similar to dexamethasone, exhibited antitumor effects in various cell types (Kassi et al., 2007). Research has shown that ursolic acid was relatively non-toxic, and have been used in cosmetics and health products (Liu, 1995).

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In the present work, we reported a novel pH-sensitive degradable micelle for targeted intracellular paclitaxel (PTX) release. Firstly, the amphiphilic copolymer, polysialic acid-ursolic acid conjugate, designed as PSAU was synthesized and characterized. Then, PTX was loaded in PSAU micelle by a nanoprecipitation method. Finally, the particle size, drug entrapment efficiency, drug release behavior *in vitro* and the anti-tumor activity of the PTX-loaded PSAU micelle were investigated.

2. Materials and methods

2.1. Materials

Polysialic acid sodium salt ($M_w = 1.6 \times 10^4$ Da), purchased from Jiangsu Rui Guang Biotechnology Co., Ltd., was further dialyzed against distilled water and lyophilized. Ursolic acid, oxalyl chloride, pyrene, paclitaxel and ethylenediamine were obtained from Aladdin Reagent Int. Bovine serum albumin (BSA) and 3-(4,5-dimethyltiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich Co. All other chemicals and reagents were of analytical grade. The SGC-7901 cell line was a kindly gift from Professor Kan Ding in Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

2.2. Synthesis of the polysialic acid-based amphiphilic copolymers (PSAU)

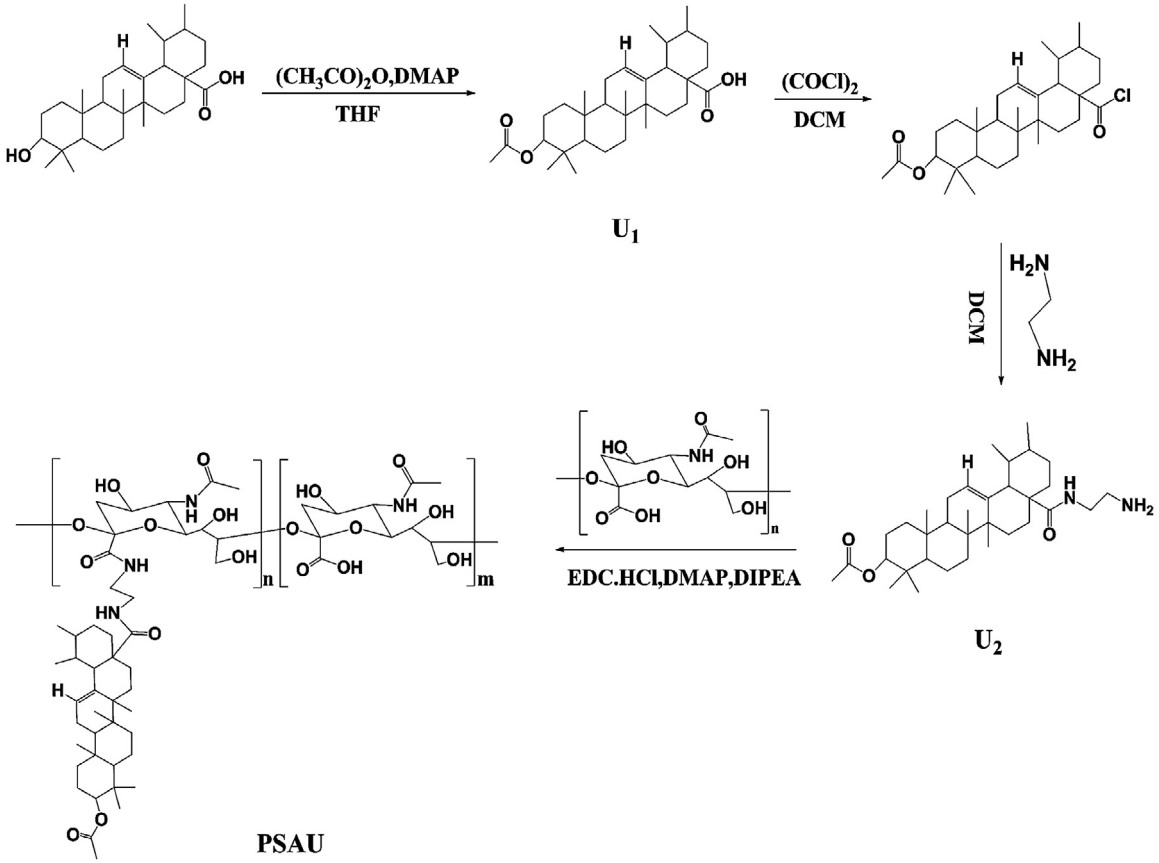
The polysialic acid-based amphiphilic copolymers were synthesized in four reaction steps from PSA, ursolic acid and ethylenediamine (Scheme 1). Specific steps were as follows:

Firstly, 200 mg ursolic acid (0.43 mM) was dissolved in 9 mL anhydrous THF, and 3 mg DMAP (0.025 mM) was added. The resulting solution was cooled by ice bath. Acetic anhydride (600 μL, 6.3 mM) was added dropwise to the solution, and then the mixture was warmed up to room temperature and stirred for another 8 h. The solvent was removed in vacuo and the residual mixture was dissolved with dichloromethane. The solution was washed sequentially with 10% hydrochloric acid, water and brine. Pure U_1 was obtained by silica gel column chromatography.

Secondly, U_1 (0.24 mM) dissolved in 5 mL anhydrous dichloromethane (DCM) was cooled by ice bath. Oxalyl chloride (180 μL, 2.1 mM) was added dropwise. After 30 min, the mixture was warmed up to room temperature and stirred for another 24 h. The solvent was removed in vacuo and the solid was dissolved with anhydrous DCM as a stock solution.

Thirdly, to an ice-cold solution of anhydrous ethylenediamine (12 mM) in 2 mL anhydrous DCM, the stock solution above was added dropwise. After 30 min, the mixture was warmed up to room temperature and stirred for another 12 h. The solution was washed sequentially with 10% hydrochloric acid, water and brine. Aminoethyl U_1 (U_2) was obtained by silica gel column chromatography.

Finally, U_2 (45 mg, 0.083 mM) was dissolved in DMF at 0 °C under N_2, and N,N-diisopropylethylamine (DIPEA, 45 μL, 0.26 mM) was slowly added. The DMF solution was stirred further for 10 min. Polysialic acid (40 mg) was dissolved in 12 mL formamide. DMAP (5.2 mg, 0.043 mM) and EDC·HCl (48 mg, 0.25 mM) were added to the formamide solution within 10 min. The DMF solution and formamide solution were mixed. The mixture was allowed to warm up to room temperature and stirred overnight. The resulting solution was dialyzed against the excess amount of water/methanol (1:3–1:1, v/v) for 1 day and distilled water for another 2 days, respectively. After lyophilization, the polysialic acid-based amphiphilic copolymers (PSAU) were obtained as a white powder.



Scheme 1. Synthetic routes of polysialic acid-based amphiphilic copolymers (PSAU).

2.3. Characterization of PSAU

The chemical structures of UA, U_1, PSA and PSAU conjugate were characterized by ^{1}H NMR (Meng, Song, Yan, & Xia, 2010; Vliegenthart, Dorland, van Halbeek, & Haverkamp, 1982). ^{1}H NMR spectra were recorded on a Bruker Avance III 500 MHz NMR spectrometer. Chemical shifts of UA and U_1 in $CDCl_3$ were referenced to tetramethylsilane (TMS). While HDO (δ = 4.7 ppm) was used as a reference for proton NMR chemical shifts of PSA in D_2O and PSAU in D_2O/CD_3OD.

The degree of substitution (DS), defined as the number of U_2 per 100 sugar residues of PSA polymer, was estimated by elemental analyzer based on the different carbon content.

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. A solution of pyrene in acetone was added to a vial and the solvent was allowed to evaporate to form a thin film at the bottom of the vial. Polymeric micelle solutions at different concentrations were added to the vials and the final pyrene concentration was 5×10^{-7} M in water. The concentrations of polymer micelles varied from 1×10^{-4} mg/mL to 2.0 mg/mL. The solutions were kept on a shaker at room temperature for 12 h to reach equilibrium prior to fluorescence measurement. Fluorescence spectra were recorded on a RF-5301PC fluorescence spectrophotometer at room temperature. The excitation spectra were scanned from 300 to 360 nm at the emission wavelength of 390 nm. Excitation and emission bandwidths were 5 nm and 10 nm, respectively. The fluorescence intensity ratio of I_{338}/I_{333} was analyzed as a function of micelle concentrations.

2.4. Preparation of PTX- loaded PSAU micelles

PTX loaded micelles were prepared by a nanoprecipitation method with minor modification (Li et al., 2008, 2010). Briefly, 12 mg of PSAU and 0.8 mg PTX were dissolved in an aliquot of methanol, which was added dropwise into 8 volumes of distilled water at room temperature. The solution was dialyzed in a dialysis membrane (MWCO 3500–5000 Da, Sigma–Aldrich Co., USA) to remove methanol thoroughly. Solutions of drug-loaded micelles and empty micelles were then lyophilized for further utilization.

2.5. Characterization of PTX-loaded PSAU and drug-free polymeric micelles

The shape and particle size of the micelles were investigated by field emission scanning electron microscopy (FESEM). Micelles solution was placed on a clean silicon chip surface and then air-dried overnight. The image was obtained with a Hitachi S-4800 FESEM system.

2.6. Drug loading content and encapsulation efficiency

Drug loading capability of the PTX-loaded PSAU was determined using high-performance liquid chromatography (HPLC) (Tao, Xu, Chen, Bai, & Liu, 2012). Briefly, 40 μL PTX-loaded micelles solution was diluted with methanol to10 mL. The solution was centrifuged at 10,000 rpm for 8 min, and then 20 μL of supernatant was injected into the chromatographic system. The HPLC system (515 HPLC Pump, waters, USA) was equipped with a Lichrospher C18 column (4.6 mm × 150 mm, 5 m) with a mobile phase of methanol and water (70:30). The flow rate and column temperature were set at 1 mL/min and 30 °C, respectively. The signals were recorded by UV detector at 227 nm. A calibration line was conducted to determine PTX concentrations in the range of 0.5–50 mg/L, and the R^2-value of peak area against PTX concentration was at least 0.998. The following equations were applied to calculate the drug loading content (Eq. (1)) and encapsulation efficiency (Eq. (2)).

$$\text{Drug loading content}\,(\%) = \frac{\text{wt of the PTX in micelles}}{\text{wt of the micelles}\ \times 100\%} \quad (1)$$

$$\text{Encapsulation efficiency}\,(\%) = \frac{\text{wt of the PTX in micelles}}{\text{wt of the feeding PTX}\ \times 100\%} \quad (2)$$

2.7. In vitro *PTX release of the PSAU micelles*

PTX release behavior was studied *in vitro* in phosphate buffered saline (PBS, pH7.4, pH 5.0) solution. Briefly, the solutions of PTX-loaded PSAU micelles were placed into dialysis membrane (MWCO 3500–5000 Da) and dialyzed against 10 mL PBS with 1% tween-80 at 37 °C in an air-bath shaker at 100 rpm. Then, 2 mL release media was collected and replaced with an equal volume fresh PBS at defined time intervals. 2.0 mL DCM was introduced to the release medium to extract PTX. The PTX in DCM solution was separated from the water and kept in a 5.0 mL flask until DCM was volatilized. Finally, 1.0 mL methanol was added to the flask to dissolve PTX. The concentration of PTX in methanol solution was determined by HPLC as above.

2.8. Cell viability assays

The cytotoxicity of drug-free polymeric micelles, PTX-loaded PSAU micelles and free PTX were evaluated by MTT assay (Le Garrec et al., 2004). SGC-7901 cells (5×10^4 cells/well) were plated in a 96-well plate in RPMI-1640 (containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin) and incubated at 37 °C for 4 h. Then polymeric micelles (100 μg/mL), PTX-loaded PSAU micelles (100 μg/mL, containing drug dosage: 4.5 μg/mL) or free PTX (drug dosage: 4.5 μg/mL) were added and incubated for 24 h or 72 h. DMSO was used to solubilize PTX. Then, 20 μL MTT (5.0 mg/mL) was added and incubated for another 4 h at 37 °C. The 96-well plate was centrifuged for 5 min (3500 rpm) and then the medium was aspirated. The MTT-formazan generated by live cells was dissolved in 150 μL DMSO, and the absorbance was measured at 570 nm using a microplatereader (Perlong DNM-9062, China). The cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing cell culture medium alone. Data are presented as mean ± SD (n = 4).

3. Results and discussion

3.1. Synthesis and characterization of PSAU

The synthetic procedure of PSAU was shown in Scheme 1. To obtain compound U_1, ursolic acid was esterified with acetic anhydride. In order to conjugate U_1 to PSA, the carboxyl group of U_1 was first converted to U_2 which possessed a free amine group in the presence of ethylenediamine. PSAU was synthesized through covalently linking amino group with the carboxyl groups on the backbone of PSA. The PSAU structure was identified by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. As showed in Fig. 1, the peaks at 5.30 ppm and 3.25–3.28 ppm were attributed to the protons of the olefinic double bonds and —O—CH— of UA in $CDCl_3$, respectively. Meanwhile, the peaks at 0.82–1.13 ppm and 1.28–2.25 ppm belonged to the protons of the methyl group and —CH— or —CH_2— of UA in $CDCl_3$, respectively. The peaks at 0.82–2.25 ppm were the characteristic resonances of pentacyclic triterpene (Fig. 1a). In comparison with ^{1}H NMR spectrum in Fig. 1a, a new peak at 2.08 ppm (Fig. 1b) appeared, suggesting the successful *O*-acetylation.

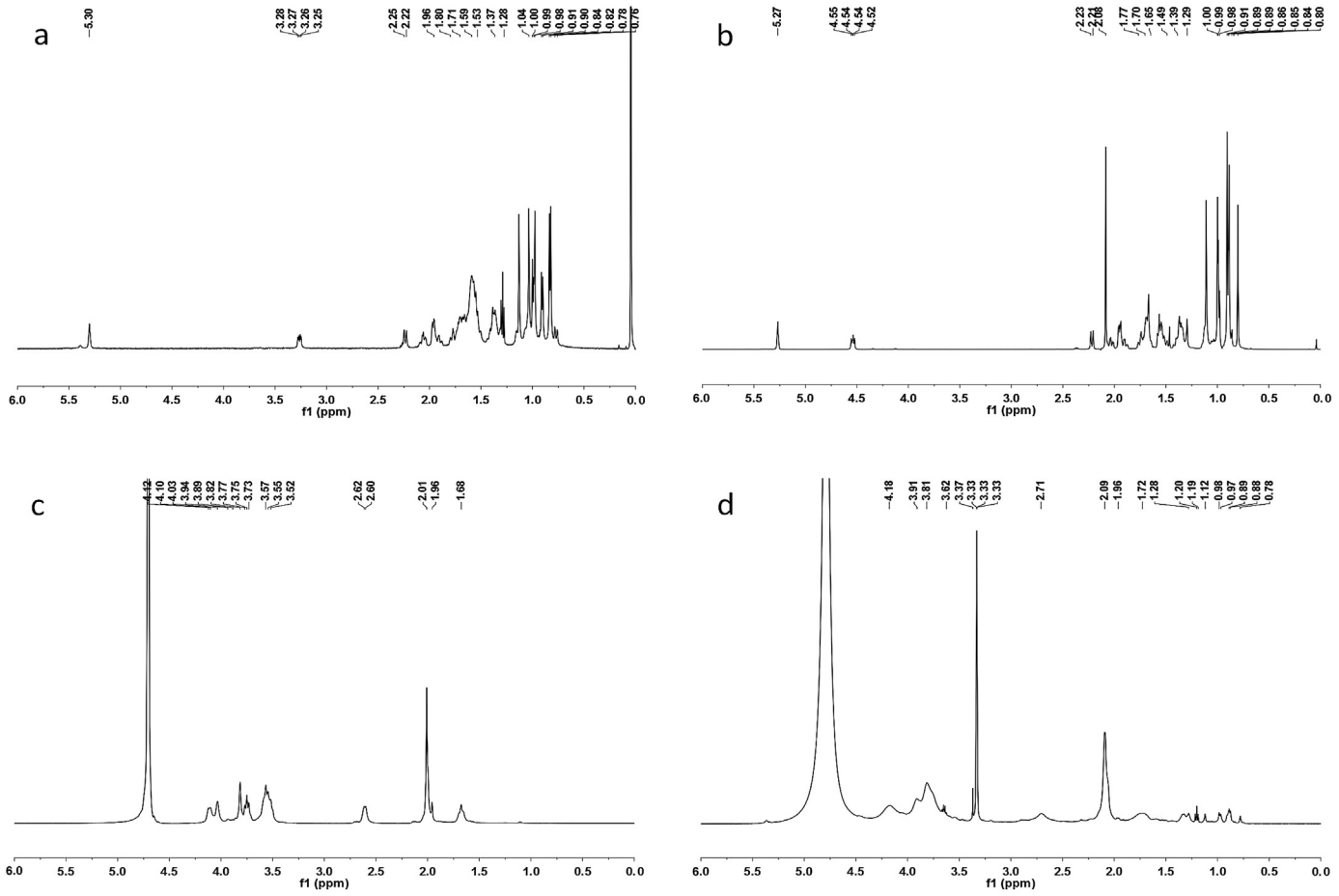


Fig. 1. ^{1}H NMR spectrum of UA in $CDCl_3$(a), U_1 in $CDCl_3$ (b), PSA in D_2O (c) and PSAU conjugates in D_2O and CD_3OD (2:1,v/v) (d).

The peaks at 2.01 ppm were attributed to the protons of the methyl group of PSA. The peaks at 1.68 ppm and 2.60–2.62 ppm belonged to the protons of —CH_2— of saccharide ring of PSA. The peaks at 3.52–4.12 ppm present the other protons of PSA in D_2O (Fig. 1c). ^{1}H NMR spectrum of PSAU in D_2O and CD_3OD (2:1, v/v) showed peaks appearing at 0.7–1.5 ppm due to U_1 moieties (Fig. 1d). These results indicated that ursolic acid was successfully connected to the PSA.

The degree of substitution (DS, defined as the number of U_2 per 100 sugar residues of PSA polymer) was calculated by measuring the content of carbon in U_2, PSA and PSA-U copolymer using elemental analysis. The content of carbon element was shown in Table 1. According to the following equation, the DS of U_2 in PSA-U conjugate was 3.3.

$$\frac{(75.44\% \times W_1 \times n + W_2 \times 36.75\% \times 100)}{(W_1 \times n + W_2 \times 100 - 40 \times n)} = 39.02\%$$

Among them: W_1 is the molar mass of U_2, (540.8 g/mol); W_2 presents the molar mass of PSA unit, (313 g/mol); n is the substitution degree of U_2 in PSA; 40 is the molar mass (NaOH) would be lost when PSA was connected with U_2.

The CMC of amphiphilic PSAU copolymers was determined by a fluorescence spectrometry using pyrene as a probe. Pyrene has been widely used as a hydrophobic fluorescence probe for micelle formation, in which, the ratio I_{338}/I_{333} from excitation spectra, could be used as an index of micelle hydrophobicity (Urbano, Silva, Olea, Fuentes, & Martinez, 2008). As shown in Fig. 2, the intersection of the extrapolated straight line segments yielded the CMC value of 0.11 mg/mL for PSAU copolymers in water at room temperature. The low CMC value suggested that micelle formed from PSAU copolymers would have a good stability in solution.

Table 1
The content of carbon element.

Compound	U_2	PSA	PSA-U
Carbon content (%)	75.44	36.75	39.02

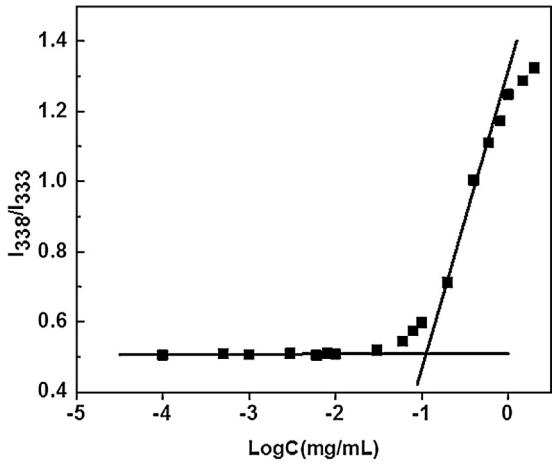
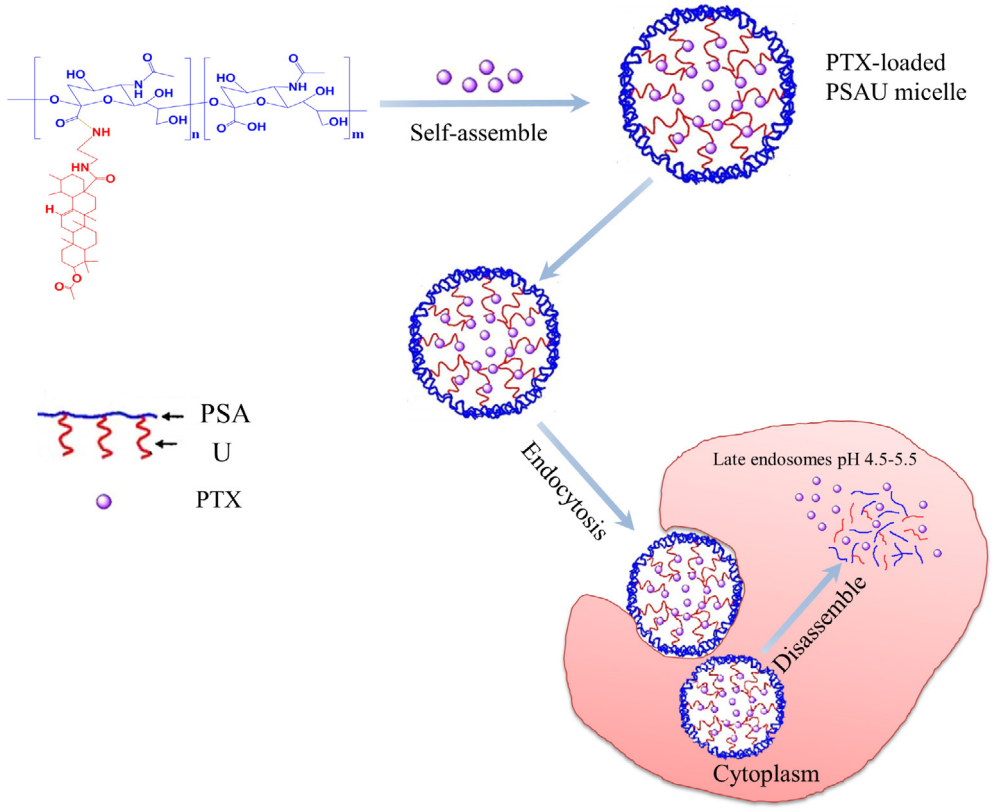


Fig. 2. I_{338}/I_{333} variation of pyrene excitation spectra versus PSA-U_1 concentration in water. The intersection of the extrapolated straight line segments yields the CMC value (0.11 mg/mL).



Scheme 2. Schematic illustration of PTX loading and triggered release from PTX-loaded PSAU micelles in intracellular microenvironment.

3.2. Preparation and characterization of PSAU and PTX-loaded PSAU micelles

The anticancer drug, paclitaxel (PTX), is known to have the highly hydrophobic property (Wang et al., 2008). Therefore, we designed PSAU micelles as a carrier for PTX to increase its aqueous solubility. PTX was physically incorporated into PSAU micelles using a nano-precipitation method at room temperature. The schematic illustration of PTX loading was showed in Scheme 2.

The size and morphology of PSAU and PTX-loaded PSAU micelles were analyzed with Field emission scanning electron microscopy (FESEM). As shown in Fig. 3, the sizes of PSAU micelles ranged from 120 to 150 nm. The particle size increased and ranged from 120 to 180 nm when PTX was incorporated. PSAU micelles showed the nearly spherical shape with increased particle size after drug loading (Fig. 3b). Such small sizes of these PTX-loaded PSAU micelles were considered suitable for passive delivery of PTX to targeted tumors through the EPR effect (Maeda, Wu, Sawa, Matsumura, & Hori, 2000). By calculating the feeding ratio of copolymer and PTX, the drug loading content of PTX into PSAU micelles was detected as 4.5% and the encapsulation efficiency was 67.5%.

3.3. In vitro pH-responsive drug release of PTX-loaded PSAU micelles

In order to investigate the effect of pH on the drug release of PTX-loaded PSAU micelles, we have measured the cumulative release under physiological condition (PBS, pH 7.4) and at pH 5.0 over a period of time.

It could be seen that as the drug-loaded PSAU was immersed in pH 5.0 buffer at 37 °C, paclitaxel was gradually released from PTX-loaded PSAU micelles and the percentage of cumulative release

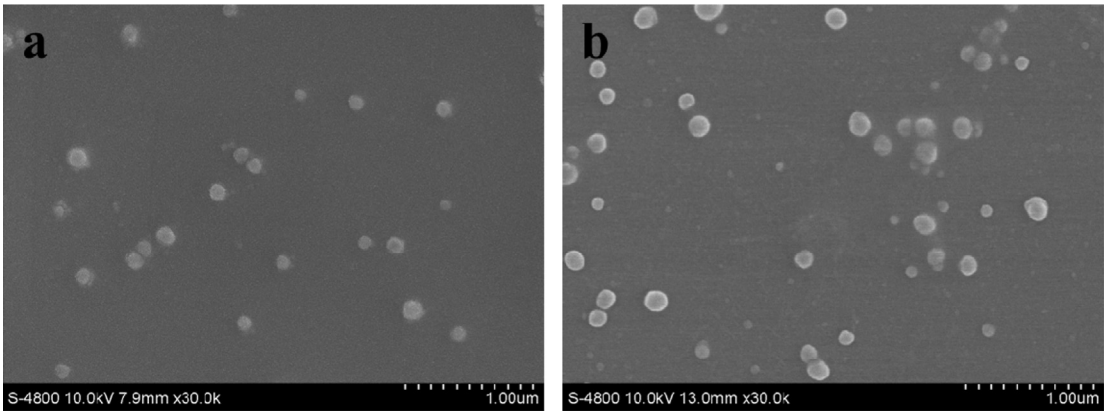


Fig. 3. FESEM photographs of blank PSAU micelles (a) and PTX-loaded PSAU micelles (b).

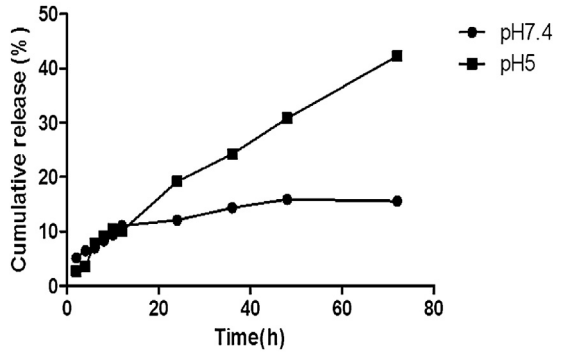


Fig. 4. *In vitro* cumulative PTX release profiles from PTX-loaded micelles in PBS solution with different pH value.

of drug reached to 42% within 72 h (Fig. 4). In the medium of pH 7.4, only 15% PTX was released during 72 h. Hence the drug release depended upon the pH of the media. The initial release might be ascribed to the dissolution and diffusion of drug that was not compactly loaded in the cores of the micelles. It appeared, therefore, that pH sensitive degradable micelles on one hand possessed good stability at physiological pH and on the other hand could release drug at mild acidic pH, mimicking that of endosomal and lysosomal compartments owing to PSA acidic hydrolysis.

3.4. In vitro *cytotoxicity assay*

The cytotoxicity is an important issue for both the drug and the carrier, thus the *in vitro* toxicity of blank micelles, free PTX, PTX-loaded PSAU micelles were evaluated in SGC-7901 cell line for 24 h and 72 h by MTT assay.

The results revealed that empty PSAU micelles had low cytotoxicities (cell viability >80%) at 100 μg/mL during the incubation time (Fig. 5). However, both the free-PTX and the PTX-loaded PSAU micelles showed time-dependent cytotoxicity. After incubation for 72 h, the free-PTX and the PTX-loaded PSAU micelles (PTX dosage: 4.5 μg/mL) showed higher cytotoxicity against SGC-7901 cells (cell viabilities 35.3% versus 53.8%). Compared with the cytotoxicity of free PTX, PTX-loaded PSAU micelles showed a slightly lower cytotoxicity at the concentration tested for 72 h incubation. This may be attributed to the sustained release property of PTX from PSAU micelles and only part of the total encapsulated PTX released from the PSAU micelles during incubation. The result suggested that PTX-loaded PSAU micelles were automatically dissociated inside the tumor cells. The schematic illustration of triggered release of PTX from PTX-loaded PSAU micelles intracellular microenvironment was showed in Scheme 2.

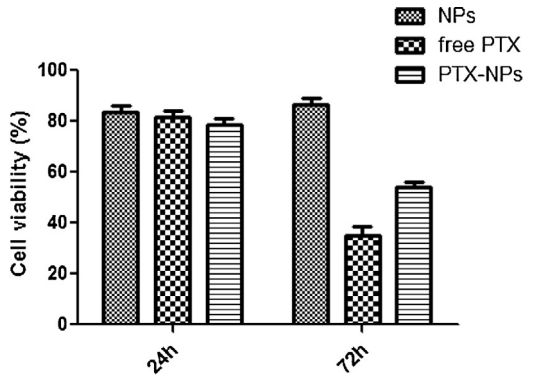


Fig. 5. Cell Viability of the SGC-7901 cells incubated with blank micelles, free PTX, PTX-loaded PSAU micelles for 24 h or 72 h. Blank micelles (100 μg/mL) and free PTX(4.5 μg/mL) were used as controls, PTX-loaded PSAU micelles dosage was 100 μg/mL(4.5 μg/mL of PTX equiv). Data represent mean ± SD, $n = 6$.

Controlled delivery devices that utilize biodegradable polymers have a significant advantage over competing delivery systems in that there is no need for surgical removal of the device (Sahoo, Parida, Rout, & Bindhani, 2012). PSA is a relatively unexplored biodegradable material for the prevention of premature clearance (Bader, Silvers, & Zhang, 2011). The pH-responsive biodegradable PSAU micelles offers a novel and elegant approach to resolve the extracellular stability versus intracellular drug release dilemma of micellar drugs and provide a novel platform for tumor-targeting delivery of anti-cancer drugs.

4. Conclusion

In this study, the amphiphilic polymer, PSAU was synthesized and characterized by ^{1}H NMR and elemental analysis. The particle size of PTX-loaded PSAU micelles ranged from 120 to 180 nm. The PTX-loaded PSAU micelles which possessed superior stability at physiological pH were able to release loaded PTX under a mild acidic condition mimicking that of the endo/lysosomal compartments. *In vitro* cytotoxicity studies revealed that PSAU micelles showed nearly noncytotoxicity against SGC-7901 cells while the PTX-loaded micelles (PTX dosage: 4.5 μg/mL) showed high cytotoxicity against SGC-7901 cells after incubation for 72 h. Taken together, PSAU micelles seemed to be a potential drug delivery system of PTX for cancer chemotherapy.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

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