Drug Delivery

ISSN: 1071-7544 (Print) 1521-0464 (Online) Journal homepage: https://www.tandfonline.com/loi/idrd20

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To cite this article: Wei Xu, Yanan Cui, Peixue Ling & Ling-bing Li (2012) Preparation and evaluation of folate-modified cationic Pluronic micelles for poorly soluble anticancer drug, Drug Delivery, 19:4, 208-219, DOI: 10.3109/10717544.2012.690005

To link to this article: https://doi.org/10.3109/10717544.2012.690005

Published online: 30 May 2012.

Drug Delivery, 2012; 19(4): 208–219

ISSN 1071-7544 print/ISSN 1521-0464 online
DOI: 10.3109/10717544.2012.690005

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ORIGINAL ARTICLE

Preparation and evaluation of folate-modified cationic Pluronic micelles for poorly soluble anticancer drug

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Abstract

The aim of this study was to construct novel targeting polymeric micelles. Folate-Poly (ethylenimine)-Pluronic copolymers were synthesized. A paclitaxel (PTX)-loaded mixed micelles consisting of Folate-Poly (ethylenimine)-Pluronic and Pluronic L121 copolymers have been developed. The mixed micelles showed nano-sized spherical morphology. The solubilization capacity of the mixed micelles was higher than Folate-Poly (ethylenimine)-Pluronic micelles because L121 has high solubilization capacity. MTT colorimetric test revealed that PTX in Folate-Poly (ethylenimine)-Pluronic micelles demonstrated the maximum anticancer activity. Pluronic-poly (ethylenimine) micelles and folate-modified Pluronic-poly(ethylenimine) micelles showed a marked increase of cellular accumulation compared with Pluronic P123 micelles. The biodistribution and retention of intravenously (i.v.) administered micelles to rats were determined. Folate-Poly (ethylenimine)-Pluronic micelles demonstrated enhanced pulmonary retention in rats after injection when compared to Pluronic P123 micelles.

Keywords: Pluronic micelles, poly (ethylenimine), folate modified, biodistribution, tumor targeting

Introduction

Paclitaxel (PTX), the first of a new class of microtubule stabilizing agents, has demonstrated significant antitumor activity in clinical trials against a broad range of solid tumors, including refractory ovarian cancer, metastatic breast cancer, non-small-cell lung cancer, AIDS-related Kaposi's sarcoma, head and neck malignancies and other cancers. However, because of the poor aqueous solubility and low therapeutic index of PTX, the clinical application is extremely limited. It is currently formulated as Taxol, a concentrated solution composed of 50:50 (v/v) mixture of Cremophor EL (polyoxyl 35 castor oil) and dehydrated alcohol. However, intravenous administration of the current Cremophor EL based formulation in a non-aqueous vehicle may lead to serious side effects in some patients such as hypersensitivity, neurotoxicity, nephrotoxicity, and to extraction of plasticizers from intravenous infusion line and to precipitation on aqueous dilution.[1] Thus, there is a need for the development of alternate formulation of PTX to solve these problems.

Among them, employing the polymeric nanoparticulate drug delivery system, especially amphiphilic copolymers, seems to be one of the simplest and most promising ways. Compared with other polymeric carriers, triblock copolymers of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO) (PEO-PPO-PEO), commercially available as Poloxamers or Pluronics, have been widely applied in pharmaceutics. A prominent feature of Pluronic copolymer is that the individual block copolymer molecules are able to self-assemble into spherical micelle structure when the concentration is above critical micelle concentration

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(Received 30 January 2012; accepted 27 April 2012)

(CMC). The micelle structure is comprised of hydrophilic outer shell and hydrophobic inner core in an aqueous media. The hydrophobic core can serve as a microenvironment for incorporating hydrophobic drugs such as anticancer drugs by hydrophobic interaction.[2] The hydrophilic outer shell can serve as a stabilizing interface between the hydrophobic drug and the external medium, which can avoid the micelles being quickly uptaken by the reticuloendothelial system after intravenous administration. Therefore, Pluronic micelles can increase solubility of drug, improve metabolic stability and circulation time for the drug, thus afford a high drug efficacy and reduce side effects.

Micelles constituted from single type of Pluronic copolymer dominated drug delivery efforts until recent years, but lately the binary systems have attracted immense attention because of their advantages. Through the mixing of different polymers to generate mixed micellar systems, many drawbacks of a mono micellar system such as low drug loading, larger particle size and low stability may be compensated.[3] For instance, doxorubicin-loaded mixed micellar system from Pluronics L61 and F127 was the first micellar formulation to get to the clinical trials for cancer chemotherapy.[4] A binary mixing system with Pluronic L121/P123 has been developed to produce a stable carrier for hydrophobic agents. A lamellar-forming L121 was incorporated with spherical-forming P123 to increase thermodynamic stability and enhance drug-loading capacity.[5] Wei et al. have lately reported loading of PTX onto Pluronic P123 and F127 mixed polymeric micelles demonstrating higher loading capacity compared to F127 micelles and enhancement of the antitumor efficacy in MDR human lung tumor cell line A-549.[6] These mixed micelles were further modified via folate-conjugation for selective targeting of cancer cells that enhanced their uptake via a receptor-mediated endocytosis.[7]

In our earlier study, a PTX-loaded multifunctional Pluronic/poly (ethylenimine) nanoparticles were developed by the formation of cross-links in outer shells of Pluronic F127 using macromolecular weight poly(ethylenimine) (MW 25000) and conjugating the folate to the surface of nanoparticles.[8] Nanoparticles modified by folate could be recognized and bound by the receptor expressed on the target cell surface and trigger receptor-mediated endocytosis later, resulting in an increased level of intracellular delivery of the formulation.[9,10] However, the drug loading of nanoparticles was low (about 1%) because of short hydrophobic chains in Pluronic F127 molecule (PEO_{100}–PPO_{69}–PEO_{100}) .

In our present work, folate-modified cationic Pluronic P123/L121 mixed micelles were developed. Firstly, Pluronic-Poly (ethylenimine) copolymer was synthesized with relatively hydrophilic Pluronic P123 (PP123) and poly (ethylenimine) (PEI) (MW 2000). PP123-PEI copolymer was full of amino groups and easy to be protonated. Thus, adding PP123-PEI copolymer into micelle formulation could enhance their uptake by phagocytic cells and change their *in vivo* biodistribution.[11] To further improve the targeting capability of the micelles, folate was connected to the surface of Pluronic-Poly (ethylenimine) copolymer using the reaction between the amino groups of PP123-PEI copolymer and carboxyl group of folate to form Folate-PEI-PP123 copolymer. Then a binary system comprised of hydrophobic and lamellar-forming Pluronic L121 (EO_5–PO_{68}–EO_5) and relatively hydrophilic Folate-PEI-PP123 has been developed. Pluronic P123 (PP123) (EO_{20}–PO_{69}–EO_{20}) and L121 (PL121) (EO_5–PO_{68}–EO_5) were selected because of the long hydrophobic chains in molecules, which could produce a larger core size that allows it for better drug solubilization than Pluronic polymers with short hydrophobic chains in molecule. Poorly water-soluble drug, PTX, was used as model drug and drug-loaded mixed micelles made of Folate-PEI-PP123/PL121 mixture were developed. The characteristics such as particle's size, morphology and *in vitro* release were determined. Cytotoxicity test against HeLa cancer cells was also determined *in vitro*. The tissue distribution *in vivo* for Folate-PEI-PP123/PL121 mixed micelles was studied.

Materials and methods

Materials

PTX was purchased from Yunnan Hande Bio-Engineering Co. Ltd. (Yunnan, China). Pluronic P123 and L121 were purchased from Sigma–Aldrich (St. Louis, MO) and used after additional purification. 1,1-Carbonyldiimidazole(CDI), poly (ethylenimine) (PEI) (MW 2000), folate and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma–Aldrich. All other solvents were of analytical or chromatographic grade.

Synthesis of PP123-PEI copolymers

Preparation of activated Pluronic P123

The CDI-activated Pluronic P123 was prepared using the reported method with modifications.[8] A solution of Pluronic P123 (0.58 g, 0.1 mmol) in anhydrous tetrahydrofuran (THF) (15 mL) was added dropwise (during 2 h) to an excess amount of CDI (0.81 g, 5 mmol) in THF (15 mL) at room temperature under nitrogen atmosphere. After the addition, the mixture was kept stirring for an additional 6 h. The solution was concentrated to a small volume under vacuum and poured into ethyl ether (150 mL), and the precipitate was removed by filtration. This process was repeated three times to remove the unreacted CDI. The filtrate was concentrated to dryness to give the CDI-activated Pluronic P123 as white powder, which was further dried under vacuum at room temperature for 12 h.

Synthesis of PP123-PEI copolymers

The PP123-PEI copolymers were prepared by an emulsification/solvent evaporation method. The activated Pluronic P123 (50 mg) were dissolved in

chloroform (1 mL) and added dropwise to a 10 mL of PEI aqueous solution (5 mg PEI in solution) under stirring. The mixture was sonicated for 3 min and the organic solvent in the emulsion was removed by rotary vacuum evaporation at 50°C for 45 min. The remaining solution was centrifuged at 3000 rpm for 30 min to remove the adhesive fragments. After neutralizing with hydrochloric acid, the solution was dialyzed in a dialysis bag with molecular weight cut-off of 3500 Da against water (pH 4.0). The purified copolymer samples were freeze-dried and confirmed by FT-IR spectrum. The molecular weight was determined by gel permeation chromatography (GPC).

Synthesis of Folate-PEI-PP123 copolymers

To synthesize Folate-PEI-PP123 copolymers, the solution of PP123-PEI copolymers (100 mg) in PBS (10 mL, pH 7.4) was mixed with folate (45 mM in PBS, 0.03 mL). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (9.3 mg) was then added and the mixture was allowed to stand overnight at 25°C. The reaction mixture was dialyzed in a SpectraPor membrane tube with a 3500 Da molecular weight cut-off against diluted aqueous ammonia (0.01%) with two buffer changes for 24 h. The purified copolymer samples were freeze-dried and analyzed by UV absorbance at 363 nm in PBS to measure the folate content. The obtained copolymers were confirmed by proton nuclear magnetic resonance (^{1}H NMR) analysis.

Preparation of drug-loaded micelles

To prepare drug-loaded PP123-PEI/PL121 micelles, the drug (1.5 mg) and 100 mg materials of 80 wt% PP123-PEI and 20 wt% PL121 copolymers were dissolved in chloroform (2 mL) and the organic solvent was subsequently removed by rotary vacuum evaporation. The resulting film was further freeze-dried in vacuum and then hydrated with a suitable amount of 5 mM HEPES-buffered saline to get 5 mM micelle solution. The resulting mixture was filtered through a 0.45 μm Nylon filter. The drug-loaded Folate-PEI-PP123/PL121 micelles were also prepared by the method described above except that 100 mg materials were composed of 30 wt% Folate-PEI-PP123, 50 wt% PP123-PEI and 20 wt% PL121copolymers.

Determination of drug-loading content of micelles

To evaluate the drug-loading (DL) content and loading efficiency (LE) of PTX in micelles, the micelle suspensions were diluted by 200–300-fold by adding DMF (to destroy micelles and release PTX). The exact concentration of PTX in DMF was then determined by high-performance liquid chromatography (HPLC) according to a reported procedure with modifications.[10] The HPLC analysis was carried out using the Agilent 1200 HPLC system consisting of a G1314B UV detector and a G1310A pump, equipped with a reverse phase (RP) column (C18, 5 μm pore size, 250 mm × 4.6 mm, Dikma, China). The mobile phase consisted of acetonitrile and ammonium acetate buffer solution (35 mM, pH 5.0) (50:45, v/v) and was pumped through the column at a flow rate of 1.0 mL/min. The column temperature was maintained at room temperature. The sample injection volume was 20 μL and the detection wavelength was 227 nm. The concentrations of PTX were determined by comparing the peak areas with the standard curve. Drug-loading content (DL %) and loading efficiency (LE %) were calculated by the following equations:

$$\text{DL}\,\% = \frac{\text{weight of drug in micelles}}{\text{weight of micelles}} \times 100$$

$$\text{LE}\,\% = \frac{\text{weight of drug in micelles}}{\text{weight of drug added}} \times 100$$

Appearance and size distribution measurement

The micelle size and size distribution were measured by the dynamic laser light scattering (DLLS) method using a Dawn Heleos, Wyatt QELS, and Optilab DSP instrument (Wyatt Technology Co., Santa Barbara, CA). The incident laser beam (λ = 658.0 nm) was polarized. All measurements were made at a fixed angle of 90° at 25°C.

The morphological features of the micelles were observed by transmission electron microscope (TEM). TEM measurements were carried out on a JEM-100CX electron microscope. The TEM samples were prepared by placing a drop of properly diluted micelle solution on the TEM grid and removing the excess fluid from the edge of the copper disk with a piece of filter paper. The samples were allowed to air dry before they were stained with phosphotungstic acid (2 wt% aqueous solution) for 30 s.

Nuclear magnetic resonance spectroscopy

The proton nuclear magnetic resonance (^{1}H NMR) analysis was performed using ^{1}H NMR spectroscopy (Bruker 300 MHz AMX 300). All spectra were obtained at room temperature from 15% (wt/v) DMSO solutions.

FT-IR analysis

Functional group characterization was carried out by FT-IR analysis using a FT-IR spectrometer (Thermo Electron Scientific Instruments Corp.). The samples were prepared by compressing the samples into pellets with potassium bromide, respectively.

Gel permeation chromatography

Polymer molecular weight was determined by gel permeation chromatography (GPC) (Agilent 1100 Series with RI detector, Santa Clara, CA) equipped with a set of three PLgel 5 μm MIXED-D and -E columns (Agilent). The flow rate was 0.5 mL/min and DMF was used as an eluent. Molecular weights were calculated from obtained chromatograms based on a series of PEG GPC standards (Polysciences Inc., Warrington, PA).

ξ-Potential measurement

Micelle surface charge analysis was performed by ξ-Potential Analyzer instrument (Zetasizer 3000, UK). The concentration of the micelles was 10 mg/mL in a phosphate-buffered saline solution (pH = 7.4), and the temperature was controlled. The measurement was carried out in triplicate.

In vitro release of drug-loaded micelles

The *in vitro* release of PTX from micelles was evaluated by dialysis using a dialysis bag with a molecular weight cut-off of 3000–5000 Da. Sodium salicylate aqueous solution (1 M) was used as the release medium to create a pseudo-sink condition.[12] The PTX-loaded micelle solution (2 mL) was added to the pre-swollen dialysis bag, and the dialysis bag was tied and immersed into the release medium (20 mL) at 37°C with gentle stirring. At each given time interval, an aliquot (2 mL) was withdrawn and fresh release medium (2 mL, 37°C) was added. The PTX concentrations in the aliquots were determined by HPLC.

In vitro cytotoxicity assay

The antitumor activity of the drug-loaded micelles was evaluated by the MTT method.[13] Human epithelial carcinoma cell line, HeLa cells were cultured in the growth medium DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were seeded at a density of 8×10^3 cells per well in 96-well plates and incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C to allow cell attachment. The growth medium was then replaced with 200 μL medium containing increasing concentrations of each of the following substances: free PTX dissolved in DMSO, PTX-loaded PP123/PL121 micelles, PTX-loaded PP123-PEI/PL121 micelles and PTX-loaded Folate-PEI-PP123/PL121 micelles. The cells were incubated for 72 h and washed three times with PBS. Then, the MTT solution (5 mg/mL, 20 μL) was added to each well and the cells were incubated for an additional 4 h at 37°C. Finally, the supernatant was removed and DMSO (200 μL) was added to each well to dissolve the MTT formazan crystals. The absorbance of each well was measured by the enzyme-linked immunosorbent assay reader at 570 nm test wavelength.

Cell uptake test

The rhodanime B (Rh-B)-loaded micelles were prepared by the same method as that of preparation of drug-loaded micelles except that the drug was replaced by Rh-B. Free Rh-B was removed from the Rh-B-containing micelle dispersions via dialysis against DI water for 1 day. The Rh-B-loaded micelles were then filtered using disposable filters (0.22 μm pore size) for sterilization. The different formulations of Rh-B: 5 mM of free Rh-B, PP123/PL121 micelles with Rh-B, PP123-PEI/PL121 micelles with Rh-B, Folate-PEI-PP123/PL121 micelles with Rh-B and Folate-PEI-PP123/PL121 micelles with Rh-B and 100 μM of folic acid were evaluated on HeLa cells, respectively. HeLa cells were seeded in six-well plates at densities of approximately 400,000 per well and incubated using RPMI 1640 medium for 24 h. Next, 1 mL RPMI 1640 medium containing the Rh-B-loaded micelles and the Rh-B solution was added into each well and incubated at 37°C for various time periods. At predetermined time intervals, cells were washed three times with ice-cold PBS, and then the fluorescence images of the samples were observed and obtained by a Nikon Eclipse E400 microscope.

Tissue distribution of polymeric micelles

Tissue distribution of PTX-loaded micelles was studied and all animal procedures were approved by the Shandong University Animal Care and Use Committee. Experimental Kunming strain mice were randomly divided into three groups and administered with solution of PTX-loaded micelles or PTX injection at an equivalent dose of 3 mg/kg PTX versus the body weight via tail vein injection. At predetermined time point (at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h), blood samples were taken from postorbital vein, poured into heparinized tubes and centrifuged to get the corresponding serum. Subsequently, the animals were sacrificed at predetermined time point (at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h) and each tested organ (heart, liver, spleen, lung and kidney) was excised. Organ samples were lightly rinsed, accurately weighed and homogenized with saline. Serum and organ samples were frozen at –20°C till analysis. Liquid-liquid extraction was performed prior to analysis. Briefly, 200 μL samples of plasma were mixed with 1 mL mixed solution of acetonitrile and methanol (50:50, v/v), while 500 μL the supernatants after centrifugation of tissue homogenates were mixed with 3 mL diethyl ether. The samples were extracted on vortex-mixer for 2 min and then centrifuged at 10,000 rpm for 10 min. Then the clear supernatant was transferred to a glass tube and evaporated under a gentle stream of nitrogen. The extraction residue was reconstituted in 100 μL acetonitrile and was filtered using disposable filters (0.22 μm pore size) before HPLC analysis. Mean tissue PTX concentration data were subjected to analyze using DAS 2.0 pharmacokinetic software. Values of AUC and the PTX percentage of the relative distribution of PTX were calculated using the trapezoidal rule.

Results

Preparation of micelles

PP123-PEI copolymers were synthesized adopting emulsifying solvents evaporation method. Activated PP123 by 1, 1′-carbonyldiimidazole (CDI) was conjugated with 1° amino of PEI. The general synthesis of the PP123-PEI copolymers is presented in Figure 1. Figure 2 shows the FT-IR spectra of Pluronic P123, PP123-PEI and Folate-PEI-PP123 copolymers. Compared with the Figure 2A (spectrum of Pluronic P123), Figure 2B clearly demonstrated the

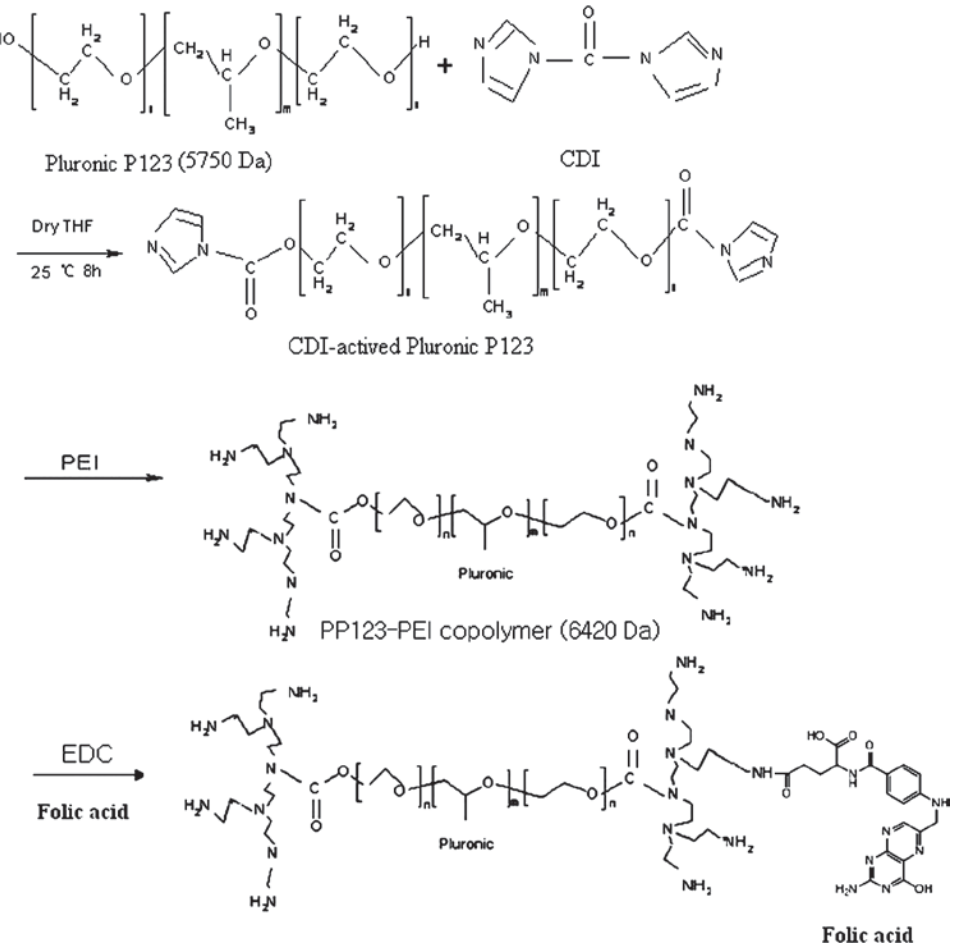

Figure 1. Synthesis and structure of Pluronic PP123-PEI copolymer and Folate-PEI-PP123 copolymer.

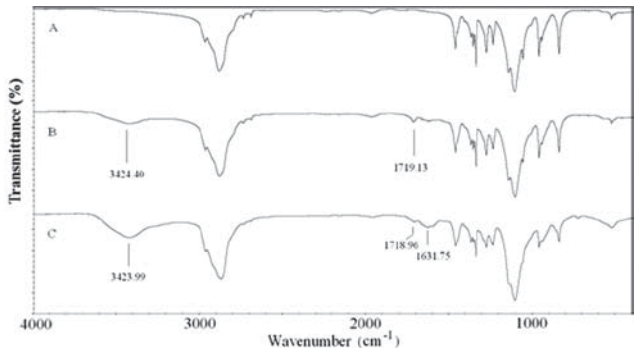

Figure 2. FT-IR spectrum of PP123-PEI and Folate-PEI-PP123 copolymer. (A) Pluronic P123; (B) PP123-PEI; (C) Folate-PEI-PP123.

successful chemical conjugation since a characteristic peak of the amide bond stretching appears at 1719.13 cm^{-1} in PP123-PEI and NH bond stretching appears at 3424.40 cm^{-1}. The molecular weight of PP123-PEI was about 6420 Da based on ^1H NMR and GPC measurements (Table 1).

PP123-PEI copolymer has a significant number of amino groups which could react with folate, generating Folate-PEI-PP123 copolymer. The general synthesis of the Folate-PEI-PP123 copolymer is presented in Figure 1 and the FT-IR spectrum of the Folate-PEI-PP123 copolymer was shown in Figure 2C. In the spectrum of Figure 2C, a peak at 3423.99 cm^{-1} was assigned to the NH group and 1718.96 cm^{-1} and 1631.75 cm^{-1} were assigned to the characteristic peaks of the carbonyl group of Folate-PEI-PP123 copolymer. The FT-IR technique is insufficient to distinguish folate signals in Folate-PEI-PP123. Thus a UV–vis spectrum was applied to determine the content of folate in copolymer and the data were listed in Table 1. The ^1H NMR spectra in DMSO-d_6 was also used to study the chemical structure of Folate-PEI-PP123 copolymer and showed the peaks at δ (ppm) = 1.05 (d, 3H, –CH_3 of PPO), 3.36–3.52 (m, 3H, 4H, –CH_2CHO– of PPO and –CH_2CH_2O– of PEO), 4.48 (C_9-H2 of folic acid), 6.62 (aromatic protons of folic acid), 7.62 (aromatic protons of folic acid), 8.64 (C_7-H of folic acid) (Figure 3). These results indicated that the Folate-PEI-PP123 copolymer was synthesized.

Physicochemical characteristics of drug-loaded micelles

The DL contents in the micelles are shown in Table 1. The DL content was increased from 1.82 ± 0.09% w/w for PP123-PEI micelles and 2.02 ± 0.18% w/w for Folate-PEI-PP123 micelles to 2.38 ± 0.19% w/w for PP123-PEI/PL121 mixed micelles and 2.58 ± 0.24% w/w for Folate-PEI-PP123/PL121 mixed micelles (30% w/w). And this

2.58% w/w DL content was almost kept constant within a broad range of concentrations of micelle-forming materials. Thus, the total quantity of the solubilized PTX per mL of the micelle suspension was increased as the concentration of the Folate-PEI-PP123/PL121 mixture was increased (Figure 4).

The micelle size and the size distribution were measured using dynamic laser light scattering (DLLS) and the results are illustrated in Figure 5. The average diameter of PP123-PEI/PL121 mixed micelles is 69.47 nm, while Folate-PEI-PP123/PL121 mixed micelles were 88.68 nm.

The TEM results (Figure 6) showed that all micelles modified with folate were spherical in shape with a smooth surface and had good dispersibility.

The ξ-potential of micelles was measured. The results showed that modifying the surface of micelles could change their potential (Table 1). PP123-PEI/PL121 mixed micelles displayed positive ξ-potential value (+0.73 ± 0.43 mV) due to the protonization of the amino group of PEI; whereas ξ-potential of Folate-PEI-PP123/PL121 micelles, however, had negative ξ-potential (–2.78 ± 0.35 mV).

The FT-IR spectra over the range of 500–4000 cm^{-1} for free PTX, empty Folate-PEI-PP123 micelles, PTX-loaded Folate-PEI-PP123 micelles and physical mixture of PTX and empty Folate-PEI-PP123 micelles (PTX: 40 wt%) were showed in Figure 7.

In vitro release

The *in vitro* release of PTX from polymer micelles was investigated in an aqueous medium containing 1 M sodium salicylate at 37°C. The sodium salicylate was used because the water solubility of PTX could be increased by 100 times without destroying the micellar structure in sodium salicylate aqueous solution (1 M)[12] and a pseudo-sink condition could be created in this aqueous solution. The results of the cumulative PTX release profile from PP123-PEI/PL121 and Folate-PEI-PP123/PL121 mixed micelles are shown in

Table 1. Characteristics and drug-loading content in the micelles (n = 3).

Sample	PP123-PEI	PP123-PEI/PL121	Folate-PEI-PP123	Folate-PEI-PP123/PL121
DL %	1.82 ± 0.09	2.38 ± 0.19	2.02 ± 0.18	2.58 ± 0.24
LE %	50.2 ± 3.94	64.5 ± 5.53	58.7 ± 4.45	70.23 ± 6.57
MW (Da)	6420			
Folate content (%)			1.3	
ξ-potential (mV)	+0.82 ± 0.45	+0.73 ± 0.43	–4.79 ± 0.55	–2.78 ± 0.35

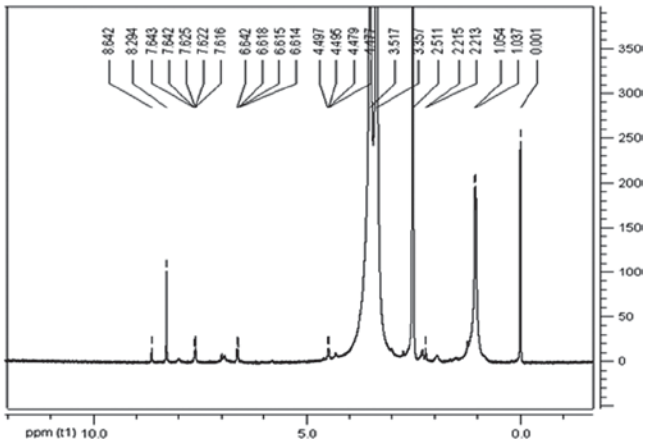

Figure 3. ^{1}H NMR spectrum of Folate-PEI-PP123 copolymer.

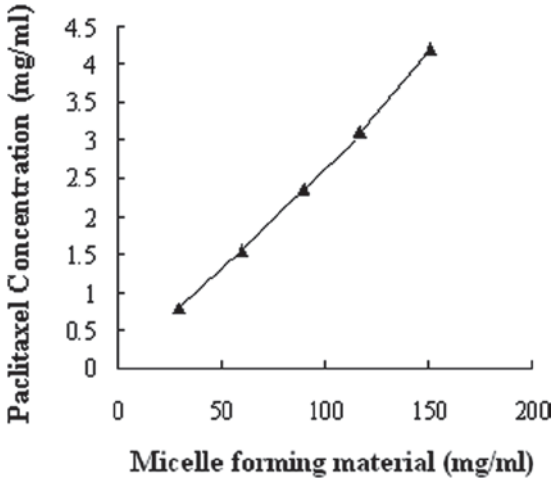

Figure 4. Effect of total micelle-forming material concentration on solubilized PTX concentration in micelle suspension.

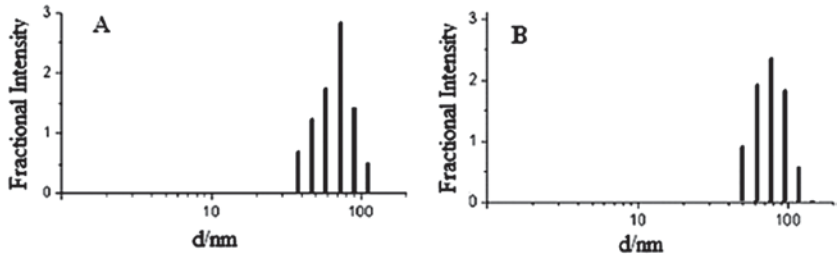

Figure 5. Micelle size and size distribution of PTX-loaded micelles. (A) PTX-loaded PP123-PEI/PL121 micelles; (B) PTX-loaded Folate-PEI-PP123/PL121 micelles.

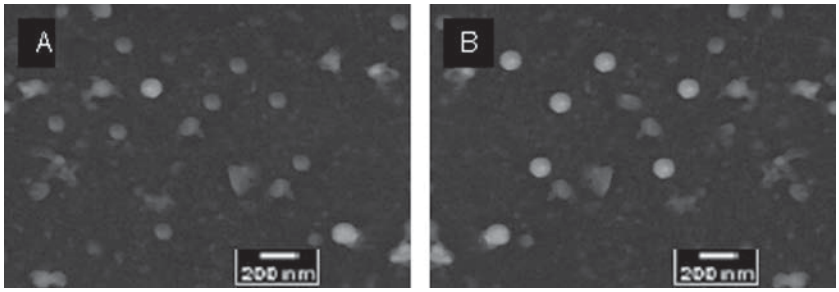

Figure 6. TEM image of PTX-loaded micelles. (A) PTX-loaded PP123-PEI/PL121 micelles; (B) PTX-loaded Folate-PEI-PP123/PL121 micelles.

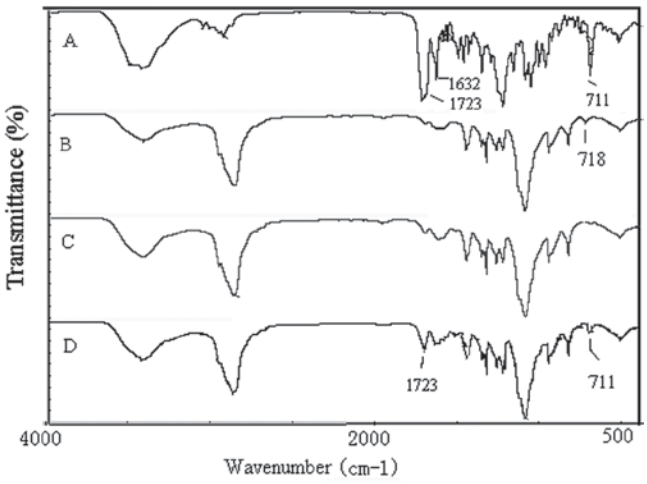

Figure 7. FT-IR spectrum of free PTX and folate-modified micelles. (A) PTX; (B) Empty Folate-PEI-PP123 micelles; (C) PTX-load Folate-PEI-PP123 micelles; (D) Physical mixture of PTX and Folate-PEI-PP123 micelles.

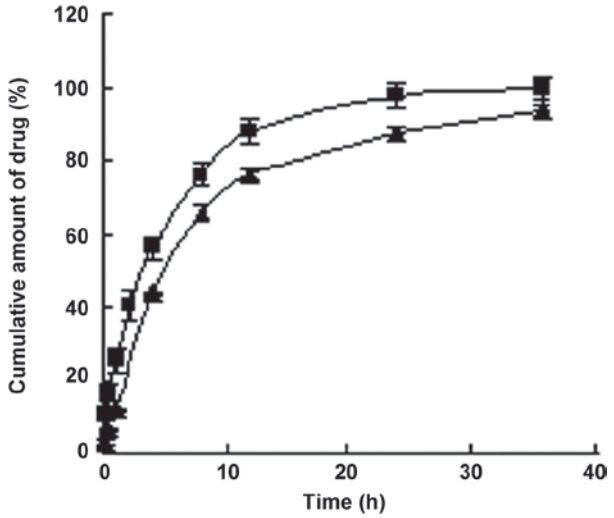

Figure 8. Release profiles of PTX from micelles. from PP123-PEI micelles/PL121 (squares); from Folate-PEI-PP123/PL121 (triangles) ($n = 3$).

Figure 8. According to these results, PTX was initially released rapidly and afterwards steadily from PP123-PEI/PL121 and Folate-PEI-PP123/PL121 mixed micelles, which is consistent with the medication principle of antitumor drugs.

In vitro cytotoxicity

The antitumor activity of the PTX-loaded micelles was evaluated by the MTT method using human epithelial carcinoma cell line; HeLa cells.[14] For comparison, the cytotoxicity of the free PTX were also evaluated (Figure 9). The IC_{50} values for various formulation towards HeLa cells were 29.0 ± 3.3 μg/mL for free PTX, 1.27 ± 0.09 μg/mL for PTX-loaded PP123/PL121 micelles, 0.65 ± 0.05 μg/mL for PTX-loaded PP123-PEI/Pl121 micelles and 0.25 ± 0.02 μg/mL for PTX-loaded Folate-PEI-PP123/PL121 micelles.

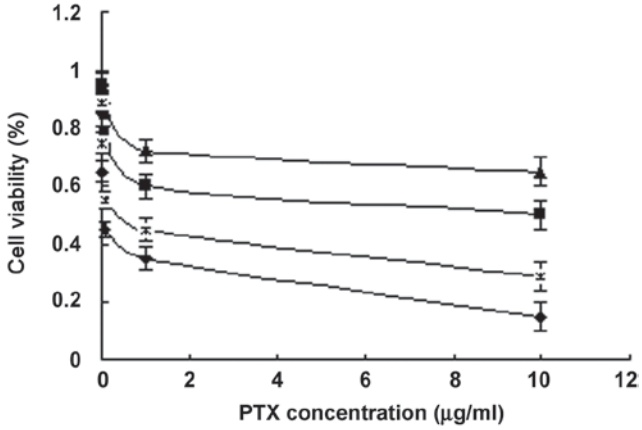

Figure 9. *In vitro* cytotoxicity against HeLa cells. Triangles indicate free PTX solution; Squares indicate PP123/PL121 micelles; *PP123-PEI/PL121 micelles; Diamonds indicate Folate-PEI-PP123/PL121 micelles.

Cell uptake

Results of the fluorescence images of HeLa cells after incubating with free Rh-B and Rh-B-containing micelles at 37°C for distinct durations were shown in Figure 10. As is shown, after exposure of HeLa cells to free rhodamine B, the accumulation of Rh-B in cells was limited throughout the entire course of the study. On the contrary, for the Rh-B-containing PP123/PL121 micelles, PP123-PEI/PL121 micelles and Folate-PEI-PP123/PL121 micelles, the accumulation of Rh-B in HeLa cells was increased compared with that of free Rh-B.

Biodistribution

The *in vivo* behavior of PTX injection and PTX-loaded micelles was investigated after i.v. administration at the

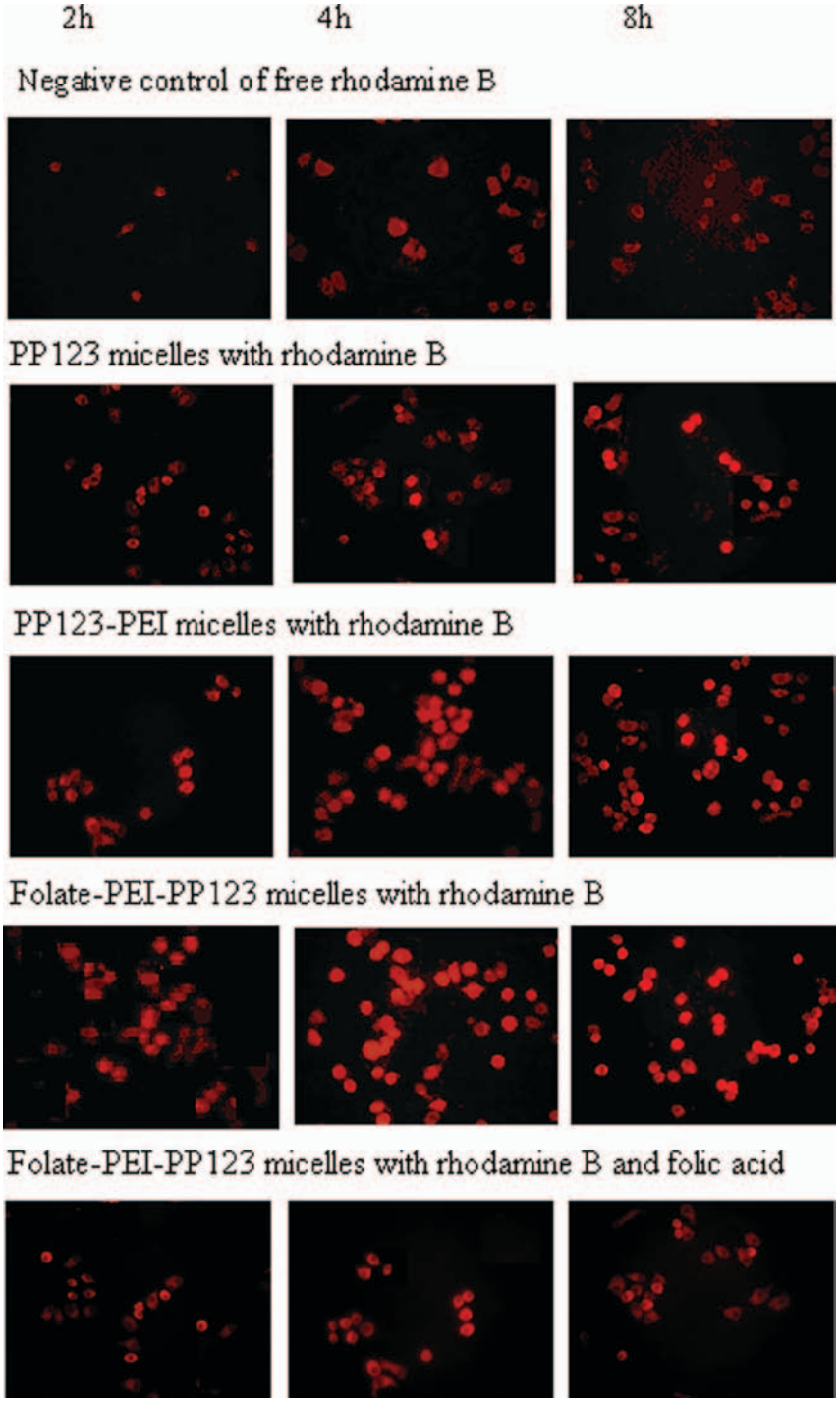

Figure 10. Uptake of rhodamine B-labeled different micelles at different times and effect of free folic acid on uptake of the micelles in HeLa cell lines.

same dose (corresponding to 3 mg/kg of PTX) in mice. Figure 11 presented percentage of different micelles recovered from organs at various time points after i.v. administration. It was found from the results that the PTX-loaded micelles and PTX injection (Taxol) showed similar biodistribution patterns. The concentration of PTX in liver was always higher than that in other organs. PP123-PEI/PL121 and Folate-PEI-PP123/PL121 mixed micelles, however, demonstrated improved pulmonary targeting compared with PTX injection. The highest lung targeting of PP123-PEI/PL121 and Folate-PEI-PP123/PL121 mixed micelles was 31.50% and 46.5%, respectively compared with the PTX injection (15.74%) (Figure 11). The areas under the lung retention percentage time curve (AUCs) of PTX injection and PTX-loaded micelles were also calculated using the trapezoidal rule from 0 to infinite (Table 2). It was found that the areas under the lung retention percentage time curve of PTX-loaded micelles

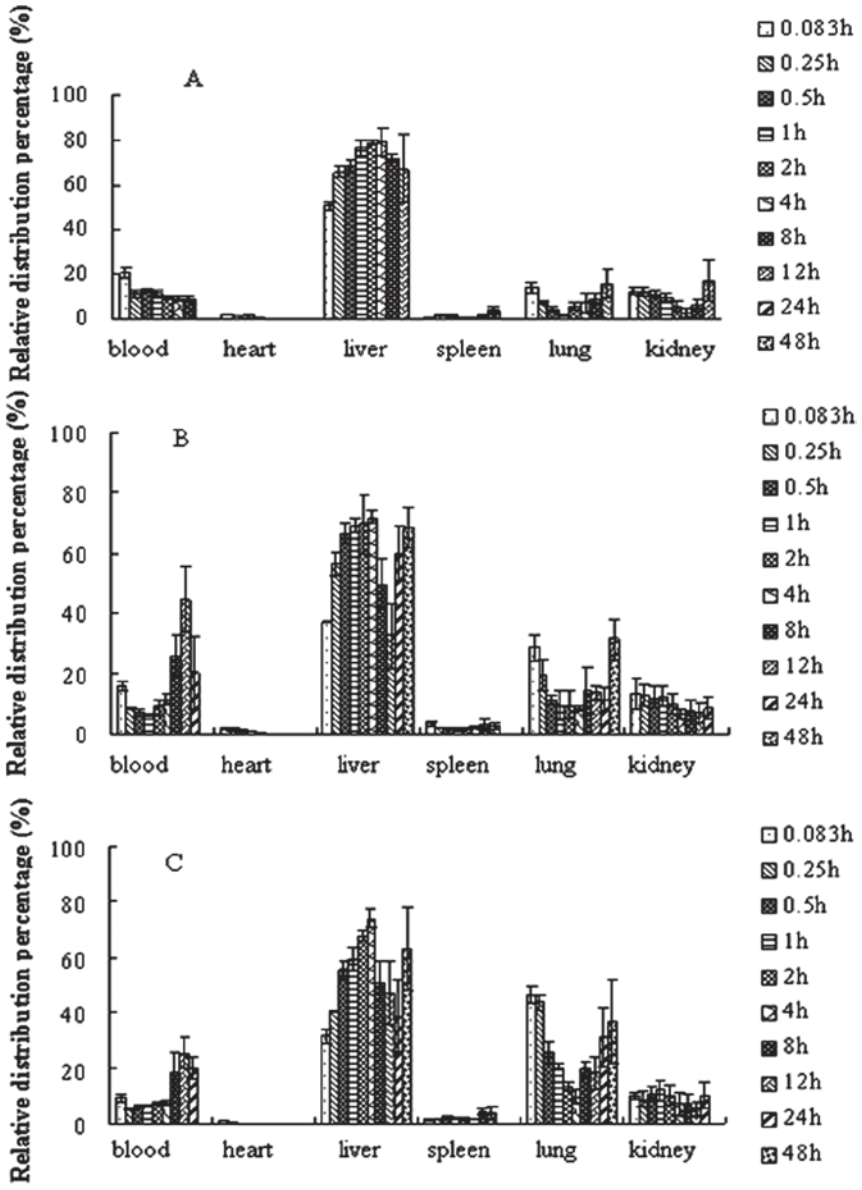

Figure 11. Body distribution of PTX after i.v. administration to mice. (A) PTX injection; (B) PTX-loaded PP123-PEI/PL121 micelles; (C) PTX-loaded Folate-PEI-PP123/PL121 micelles.

Table 2. Average AUC (μg×h/g) values of PTX after i.v. administration of PTX injection and PTX-loaded micelles to mice with dose of 3 mg/kg (n = 3).

	Formulation		
Organ	Taxol	PP123-PEI micelles/PL121	Folate-PEI-PP123 micelles/PL121
Blood	0.663	0.9299	1.8445
Heart	–	–	–
Liver	9.6314	21.3653	15.8020
Spleen	1.3728	3.2533	3.5346
Lung	5.3408	9.4610	14.4609
Kidney	3.6033	5.2630	7.1468

were significantly larger than that of the PTX injection (p < .05). This phenomenon might be due to the ξ-potential effect of PEI and interaction of folate and folate receptor.

Discussions

Pluronic mixed micelles

Pluronic L121 (EO_5–PO_{68}–EO_5) has tremendous potential for solubilization of poorly water-soluble drugs. However, the hydrophilic block PEO of L121 only has 10% of the whole MW, not providing sufficient steric hindrance to form a stable dispersion. Pluronic P123 (EO_{20}–PO_{69}–EO_{20}) has a long PEO chain and similar MW of PPO to L121, and form a stable, nano-sized micelles in aqueous solution. To get a potential drug delivery system, the lamellar-forming Pluronic L121 was incorporated with Pluronic P123 conjutates (Folate-PEI-PP123) to produce nano-sized micelles with high stability due to Folate-PEI-PP123 and high solubilization capacity due to Pluronic L121. The hydrodynamic radius of micelles was determined by dynamic light scattering (DLS) method and results showed that the mean size of PTX-loaded micelles was

smaller than 100 nm. The TEM results (Figure 6) showed that the micelles were spherical in shape with a smooth surface and had good dispersibility, which indicated that stable, nano-sized micelles were formed.

To investigate the drug state in micelles, FT-IR measurements were carried out using a FT-IR spectroscopy (Thermo Electron Scientific Instruments Corp.). Figure 7 showed the FT-IR spectra over the range of 500–4000 cm^{-1} for free PTX, empty Folate-PEI-PP123 micelles, PTX-loaded Folate-PEI-PP123 micelles and physical mixture of PTX and empty Folate-PEI-PP123 micelles (PTX: 40 wt%), respectively. In the FT-IR spectrum of free PTX (Figure 7A), the characteristic bands at 1632 cm^{-1} was attributed to the acylamino group. Furthermore, the peaks at 1723 cm^{-1} and 711 cm^{-1} were assigned to the absorption band of carbonyl group and aromatic ring of PTX respectively. These peaks disappeared in PTX-loaded micelles (Figure 7C). However, the physical mixture of PTX and empty micelles (Figure 7D) had peaks similar to that of free PTX. These results suggested that PTX was molecular dispersed in micelles. More importantly, in the FT-IR spectrum of empty Folate-PEI-PP123 micelles (Figure 7B), the characteristic band at 718 cm^{-1} was attributed to the aromatic ring of folate, which also disappeared in PTX-loaded micelles (Figure 7C). These phenomena implied that hydrophobic interaction between aromatic structure of folate and PTX was caused.

The DL contents of micelles

Pluronic micelles are composed of a hydrophobic core providing a hydrophobic molecule pool and hydrophilic shell that provides aqueous stability. The hydrophobic core can serve as a microenvironment for incorporating hydrophobic drugs such as anticancer drugs by hydrophobic interaction. The solubilization capacity of Pluronic micelles depends on the relative length of hydrophobic blocks. The micelles made of Pluronics with long hydrophobic chains in molecules have higher drug solubilization capacity than Pluronics with short hydrophobic chains in molecule. To increase the DL content, Pluronic L121 (PL121) was added to form the mixed micelles because of the long hydrophobic chains in molecule (EO_5–PO_{68}–EO_5). Insertion of PL121 into micellar system increases the volume of the hydrophobic region of the micelles. This provides a larger space for the hydrophobic drug to be solubilized.[6] The DL content of Folate-PEI-PP123/PL121 mixed micelles (2.58 ± 0.24% w/w) was higher than that of Folate-PEI-PP123 micelles (2.02 ± 0.18% w/w).

More importantly, the results revealed that the total quantity of the solubilized PTX per mL of the micelle suspension linearly depended on the concentration of the Folate-PEI-PP123/PL121 mixture (Figure 4), which corresponds well to a general solubilization pattern of poorly soluble drugs by micelles.[15] Thus, micelle formulations can be prepared containing up to about 5 mg of PTX per mL at still reasonable Folate-PEI-PP123/PL121 concentrations, which is almost the same as the concentration of PTX in injection (Taxol, 6 mg/mL).

In vitro release

The results revealed that PTX was initially released rapidly and afterwards steadily from PP123-PEI/PL121 and Folate-PEI-PP123/PL121 micelles. This result suggested that a fraction PTX was just adsorbed at the micelle surface and could be released rapidly from micelles. Another fraction of PTX was dispersed in the polymer matrix of the micelles and could be released by diffusion from the micelles.

It also demonstrated that Folate-PEI-PP123/PL121 mixed micelles retained the drug even better than PP123-PEI/PL121 mixed micelles, indicating that the presence of folate on the surface of micelles facilitated the stability of its entrapment, probably because of the aromatic structure of folate which may cause stronger hydrophobic interaction between drug and polymers.

In vitro cytotoxicity

The antitumor activity of the PTX-loaded micelles was evaluated by the MTT method using human cervical carcinoma cell line, HeLa cells. The results suggested that PTX-loaded micelles displayed higher cytotoxicity compared with that of the free drug, which could be explained by the enhanced solubility of the poorly soluble PTX in the micelle solution and the firm retention of PTX in the micelles.

In addition, PP123-PEI micelles displayed higher cytotoxicity compared with PP123 micelles, which may be due to the presence of PEI on the surface of PP123-PEI micelles and may cause endosomal break-up through so-called "proton sponge" effect of PEI.[16]

More interestingly, the folate-modified PTX-loaded micelles demonstrated a highest superior cytotoxicity (Figure 9). This result implied that folate-modified PTX-loaded micelles could be taken into HeLa cells better than the no modified PP123-PEI micelles due to the interaction between the folate on the micelle surface and the folate receptors on the HeLa cell surface. This interaction ensured that more drugs were pumped into the tumor cells to give a better anticancer effect. These results also suggested that the antitumor activity of the drug-loaded micelles could be regulated through control of micelle composition, such as incorporating ligand-polymer conjugates into formulation, which provided a useful strategy to build targeted micelles.

Cell uptake

Results of the fluorescence images demonstrated that for the Rh-B-containing micelles, the accumulation of Rh-B in HeLa cells was increased compared with that of free Rh-B, which could be explained by the enhanced solubility of the poorly soluble PTX in the micelle solution and better uptake of PTX-loaded micelles by the cells.

In addition, PP123-PEI micelles have shown a marked increase of cellular accumulation compared with PP123 micelles accumulated in tumor cells, which might be due

to the opposite ξ-potential of PEI. Since cells possess a net negative ξ-potential. Therefore, the electrostatic attraction between the cell membrane and micelle surface with opposite charge causes an increase in the adhesion of micelles to the cells, leading to an increase in phagocytosis.[17]

More importantly, the PTX-loaded Folate-PEI-PP123 micelles showed a marked increase of cellular accumulation compared with non-modified PTX-loaded PP123-PEI micelles (Figure 10). This phenomenon was due to the effective targeting ability provided by folate and folate receptor, whose high affinity contributed to help micelles target tumor cells more than normal cells, which was the reason why folate mediated micelles could decrease side effects at the same time they were able to enhance therapeutic effects.

In order to evaluate the role of folate receptor in the cellular uptake of PTX incorporated into Folate-PEI-PP123 micelles, a competitive binding assay was performed. For these experiments, 100 μM of free folic acid was added to the cell culture wells. As shown in Figure 10, 100 μM of free folic acid significantly reduced the PTX uptake in HeLa cells incubated with PTX-loaded Folate-PEI-PP123 micelles. The observations that free folic acid inhibited uptake of PTX incorporated into Folate-PEI-PP123 micelles suggested that the PTX incorporated into Folate-PEI-PP123 micelles might be endocytosed via the folate receptor.

Body distribution in mice of PTX-loaded micelles

Numerous investigations have shown that the distribution profiles of anticancer drugs can be controlled by their entrapment in nanoparticles. In order to make clear the altered distribution of the PTX-loaded micelles, the *in vivo* behaviors of PTX injection and PTX-loaded micelles were investigated after i.v. administration at the same dose (corresponding to 3 mg/kg of PTX) in mice (Figure 11). The results showed that the concentration of PTX in liver was always higher than that in other organs, which suggested that rapid uptake of micelles was via mononuclear phagocytes in the reticuloendothelial (RES) organs. Since in Taxol injection the PTX was also incorporated into amphiphilic polymer micelles (Cremophor EL micelles), both PTX-loaded micelles and Taxol injection were easily taken up by the macrophages, particularly by the Kupffer cells of the liver. So the highest PTX concentration was observed in liver than in other organs tested.

More interestingly, PP123-PEI/PL121 micelles had significantly better lung targeting and retention (i.e. increased AUC) than PTX injection, which was most likely due to increased cellular interactions once PP123-PEI/PL121 mixed micelles became entrapped in capillaries. Micelles that are more positively charged might interact more with the endothelial cell surface and be hindered in lung. Folate-PEI-PP123/PL121 mixed micelles had also significantly better lung targeting and retention (i.e. increased AUC) than PTX injection, which was most likely due to the ξ-potential effect of PEI and interaction between the folate on the micelle surface and the folate receptors, which is slightly expressed in the lung.

Conclusion

In this study, Pluronic-Poly (ethylenimine) copolymers were synthesized with Pluronic P123 (PP123) and poly (ethylenimine) (PEI). Then folate was connected to the surface of Pluronic-Poly (ethylenimine) copolymers. A PTX-loaded mixed micelles consisting of Folate-PEI-PP123 and Pluronic L121 copolymers have been developed. The mixed micelles showed nano-sized spherical morphology with a high stability in aqueous solution. The solubilization capacity of the mixed micelles was higher than Folate-PEI-PP123 micelles because L121 has high solubilization capacity.

PP123-PEI copolymer was full of amino groups and easy to be protonated. Thus micelles made of PP123-PEI copolymers could enhance their uptake by phagocytic cells and change their *in vivo* biodistribution. The selectivity and targeting property of micelles towards tumor cells expressing more folate receptors could also be improved by modifying folate on the surface of micelles. The cytotoxicity test results confirmed that the *in vitro* anticancer activity of the drugs can be improved when micelles were used as drug transport carriers. More interestingly, Pluronic PP123-PEI micelles exhibited significant antitumor activity compared with the PP123 micelles and folate mediated Pluronic PP123-PEI micelles exhibited highest antitumor activity compared with the no modified micelles and the IC_{50} declined significantly to 0.25 ± 0.02 μg/mL. The biodistribution and retention of intravenously administered micelles to mice were determined. PP123-PEI micelles and Folate-PEI-PP123 micelles demonstrated enhanced pulmonary retention in mice after injection compared to unmodified micelles. These provided theory and test validation for exploring targeting delivery for antitumor drug.

Declaration of interest

This work was supported by Scientific and Technological Project of Shandong Province of China (2008GG10002028) and Scientific Research Foundation for Returned Scholars, Ministry of education of China. This paper has not been published elsewhere and it has not been submitted simultaneously for publication elsewhere. All authors have read and approved it.

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