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A novel targeting drug carrier to deliver chemical bonded and physical entrapped anti-tumor drugs

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ABSTRACT

In this study, we demonstrated a novel targeting drug carrier formed by amphiphilic prodrug based mixed micelle. Along with octadecyl chains, chemical bonded Dox moieties were utilized to entrap free drugs (PTX) and simultaneously acted as therapeutic agents. The formulation of CP-Folate/CPN = Dox/PTX showed spherical micellar structure and possessed high drug loading content, which was up to 22.9%. We examined the cell uptaken capacity and the cytotoxicity of mixed micelle by CLSM and MTT assay. The introducing of folate moiety enhanced intracellular accumulation in HeLa cells and co-delivery of Dox and PTX showed stronger anti-tumor activity even compared with free drugs.

1. Introduction

In the past decades, the basic research on chemotherapy has focused on the designing of all kinds of drug carriers (Batrakova and Kabanov, 2008; Mundargi et al., 2008; Slowing et al., 2008). The application of drug carriers can improve the water solubility of hydrophobic drugs, increase the intracellular drug accumulation and optimize the behavior of drug release (Allen et al., 1999; Kataoka et al., 2001; Moghimi et al., 2001). Conventional drug carriers are utilized to entrap single therapeutic agent, but in practical applications, combination delivery of multi-drugs or any other therapeutic agents is required to deal with complicated cases. For example, cocktails of drugs are also used to treat with HIV infection (Donati et al., 2004), and paclitaxel (PTX) and doxorubicin (DOX) can increase the inhibition of tumor growth (Gehl et al., 1996; Gustafson et al., 2005; Moghimi et al., 2001). Furthermore, combination delivery may hit different targets simultaneously, resulting enhanced therapy efficacy (Harries and Gore, 2002; Moghimi et al., 2001; Reich et al., 1999).

Successful combination delivery of multiply therapeutic agents is not only required a reasonable ratio of each component but also with adequate drug content. Therefore, the first problem in designing multi-drug carriers is to improve the drug loading content. Certain vehicles always involve a considerable amount of inert materials, which show no further functions except as the carrier matrices (Khandare and Minko, 2006). Thus, the percentage of drugs in the carriers is passively decreased. Recently, Shen's group prepared a prodrug based lipsome to encapsulate hydrophilic free drugs and this carrier showed a very high drug loading capacity which was up to 58% (Shen et al., 2010). Li and co-worker have synthesized a targeting prodrug nanoassembly, Biotin-PEG-Dox, to entrap hydrophobic free drugs (Yuan et al., 2013). These works provide us an idea to prepare high drug loading carriers, which is using the drugs as the materials of carrier.

Based on such ways, we present here a novel targeting drug platform to deliver multiply drugs (Scheme 1). Octadecyl-polyethylene glycol$_{100}$-hydrazone-doxorubicin (C_{18}-PEG-hy-Dox) was an amphiphilic prodrug to build up the main structure. A small amount of octadecyl-polyethylene glycol$_{100}$-Folate (C_{18}-PEG-Folate) was added as the targeting component. The moieties, octadecyl chain and Dox, could blend in aqueous solution by the hydrophobic effect to form mixed micelles, which were stabilized by the hydrophilic PEG blocks. Dox processed nearly half percentage of hydrophobic moieties and acted two roles here: the therapeutic agent and the carrier matrix. By utilizing such property, another model hydrophobic drug, PTX was loaded into micelle core. An acid-labile structure was introduced into the system to endow the carrier with pH-sensitive drug release behavior.

2. Experimental part

2.1. Materials

1,1′-Carbonyldiimidazole (CDI), dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from

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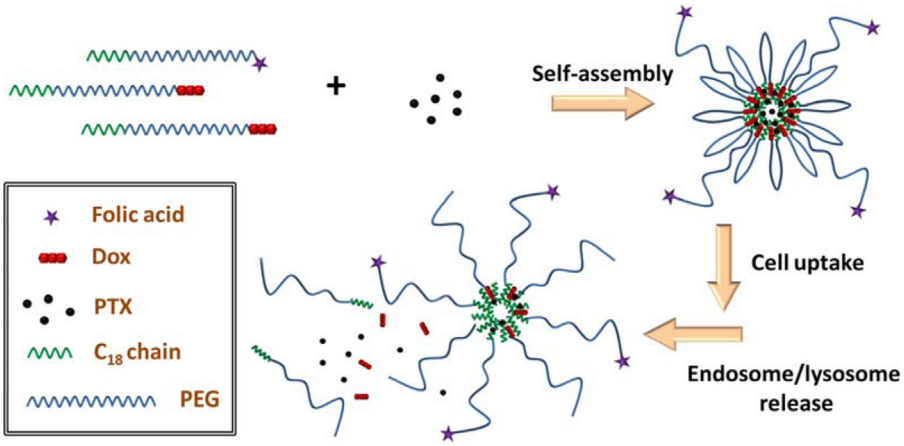


Scheme 1. The schematic illustration of structure of CP-Folate/CPN = Dox/PTX mixed micelle and the process of the targeted drug delivery.

Sinopharm (China) and used as received. Doxorubicin hydrochloride (Dox·HCl) and Brij S 100 (average Mn ~4670) were purchased from Sigma–Aldrich and used as received. Dimethyl sulfoxide (DMSO) was dried over 4 Å molecular sieve and distilled under vacuum. Dichloromethane (DCM) was distilled over CaH_2 before use. Methanol was dried over 3 Å molecular sieve and distilled by rectification. Bovine serum albumin (BSA), Dubelcco's Modified Eagle's Medium (DMEM), penicillin–streptomycin, trypsin, and phosphate-buffered saline (PBS) were purchased from GIBCO Invitrogen Corporation. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hochest 33258 were purchased from Sigma–Aldrich.

2.1.1. C_{18}-PEG-CDI

Brij S 100 (4.67 g, 1.0 mmol) and CDI (1.62 g, 10.0 mmol) were dissolved in DCM (50 mL), and then the solution was allowed at room temperature for 24 h. The most portion of solvent was evaporated and the residue was poured into excess anhydrous diethyl ether. The white precipitate was collected by centrifugation, washed with diethyl ether and dried under vacuum. Yield 4.2 g, (89.9%). ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 8.14 (s, 1H), 7.47 (d, 1H), 7.09 (d, 1H), 4.56 (t, 2H), 3.42–3.88 (m, 400H), 1.57 (m, 2H), 1.25 (m, 30H), 0.88 (t, 3H).

2.1.2. C_{18}-PEG-NH-NH_2

C_{18}-PEG-CDI (476 mg, 0.1 mmol), hydrazine (16 mg, 0.5 mmol) and triethylamine (101 mg, 1 mmol) were dissolved in 10 mL of DCM. The mixture was concentrated by rotary evaporation and the residue was poured into excess anhydrous diethyl ether to form precipitate. The white precipitate was collected by centrifugation, then washed with diethyl ether and dried under vacuum. Yield: 412 mg, (86.6%). ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 4.27 (m, 2H), 3.42–3.88 (m, 400H), 1.57 (m, 2H), 1.25 (m, 30H), 0.88 (t, 3H).

2.1.3. Folate-NH_2

The synthesis of Folate-NH_2 was according to a published procedure (Lee and Low, 1995). Folic acid (441 mg, 1 mmol) was dissolved in 20 mL of DMSO, and then treated with DCC (248 mg, 1.2 mmol) and NHS (230 mg, 2.0 mmol). The mixture was stirring at 50 °C for 6 h. The resulting Folate-NHS was reacted with ethylenediamine (781 mg, 13.0 mmol) and pyridine (500 mg, 6.3 mmol) at room temperature overnight. The mixture was poured into excess acetonitrile, and the precipitate was collected and washed with diethyl ether before drying under vacuum to get yellow powder. Yield: 297 mg, (61.5%). The obtained Folate-NH_2 was directly used in the next step without further purification.

2.1.4. C_{18}-PEG-Folate (CP-Folate)

C_{18}-PEG-CDI (476 mg, 0.1 mmol), Folate-NH_2 (241 mg, 0.5 mmol) and triethylamine (0.21 g, 2.1 mmol) were dissolved in 10 mL of DMSO. The mixture was stirring at room temperature for 48 h, and then dialyzed extensively against with DI water for 72 h (Mw cutoff: 3500 Da). The CP-Folate was obtained by freeze-drying. Yield: 327 mg (63.5%).

2.1.5. C_{18}-PEG-NH-N = Dox (CPN = Dox)

Dox·HCl (87 mg, 0.15 mmol) and C_{18}-PEG-NH-NH_2 (476 mg, 0.1 mmol) were dissolved in 15 mL of anhydrous menthol, and treated with a drop of TFA. The solution was refluxed under dark for 48 and then cooled down to room temperature. The solvent was evaporated under vacuum and the residue was resolved in 10 mL of anhydrous DMSO. The solution was dialyzed against with DMSO for 48 h (Mw cutoff: 3500 Da) and then dialyzed extensively against with DI water to remove the organic solvent and any other impurities. The CPN = Dox was obtained by freeze-drying as dark red solid. Yield: 299 mg (57.2%).

2.2. Methods

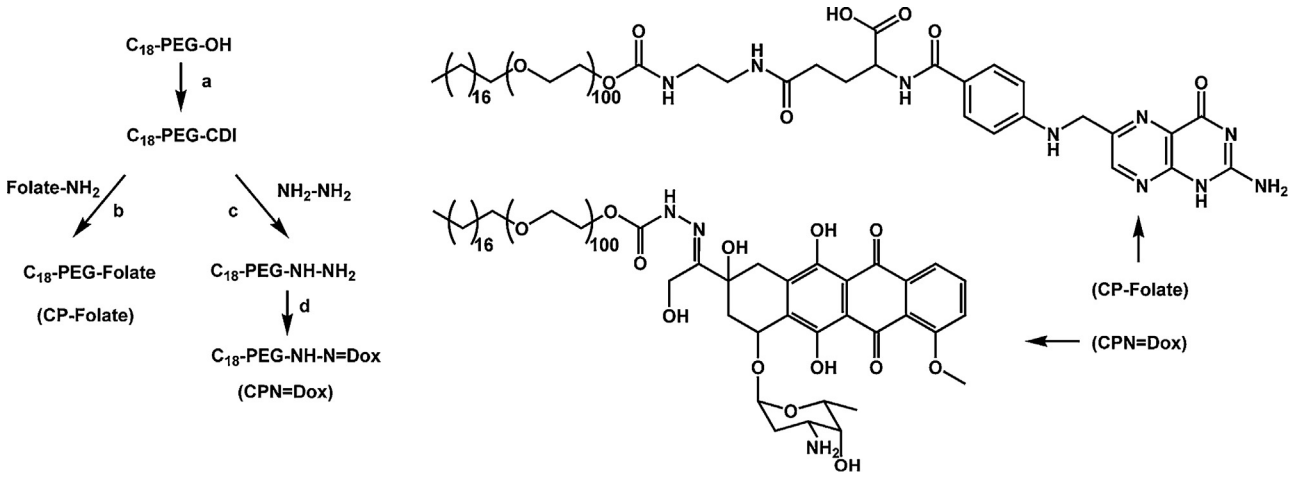
^{1}H NMR spectra were recorded at 300 MHz on a Mercury VX-300 spectrometer by using tetramethylsilane (TMS) as the internal reference. A drop of micelle solution was placed onto a copper grid with carbon film and then stained by phosphotungstic acid. The TEM images were taken by JEM-2100 (HR) transmission electron microscope at an acceleration voltage of 200 keV. Size and distribution of the mixed micelles were measured by Dynamic Light Satter (Zetasizer, Malvern).

2.2.1. Determination of the content of Dox in CPN = Dox

The content of Dox in CPN = Dox was determined using fluorescence spectroscopy. The CPN = Dox was resolved in 1N HCl solution and kept in dark over night at room temperature. The content of Dox was calculated based on the fluorescence intensity at emission wavelength of 560 nm, excitation wavelength of 488 nm, and slit width of 5 nm calibrated by a standard curve of Dox·HCl.

2.2.2. Micelle preparation and drug encapsulation

The mixed micelles (CP-Folate/CPN = Dox or CP-OH/CPN = Dox) were prepared by dialysis. Typically, 1.0 mg of CPN = Dox and 0.2 mg of CP-Folate were stirring in 200 μL of DMSO, and then 1.8 mL of DI water was added dropwise into the above solution. The mixture was further stirred for 2 h and then dialyzed (Mw 3500, cutoff) against with DI water.



Scheme 2. Principle synthesis route and the structure of the polymers. (a) CDI, in DCM, 24 h at r.t.; (b) Folate-NH2 and TEA, in DMSO, 48 h at r.t. (c) Hydrazine and TEA, in DCM, 24 h at r.t. (d) Dox·HCl and TFA, in anhydrous CH3OH, 48 h, reflux.

The loaing of PTX was based on a similar procedure but the mixture was passing through a 0.45 μm filter after dialysis. The amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 6/4 (v/v) mixture of acetonitrile and water as a mobile phase. The content of Dox was calculated based on the UV absorbance at 488 nm calibrated with standard stocks in DI water.

2.2.3. Drug release

The release profiles of DOX and PTX from mixed micelles were studied at 37 °C under two different pH values, (i) acetate buffer, pH 5.4 and (ii) phosphate buffer, pH 7.4. All the concentrations of the release media were set as 0.1 M. Every 2 mL of PTX loaded mixed-micelle solution was sealed into a dialysis tube (Mw 8000, cutoff) and then dialyzed against with 20 mL of the corresponding buffer at 37 °C. At desired time intervals, 2 mL of release medium was collected and added with an equal volume of fresh medium. The amount of released DOX was measured by fluorescence emission spectra. The amount of released PTX was tested by HPLC. All the release experiments were conducted in triplicate and presented as the average value ± SD.

2.2.4. Confocal laser scanning microscopy

Cell internalization was observed on a confocal laser scanning microscopy (CLSM, Nikon C1-si). HeLa cells were seeded in a 35 mm cell culture dish and incubated at 37 °C with 5% CO_2 for 24 h. The mixed micelles prepared in DMEM with 10% fetal bovine serum (FBS) were added to replace the medium. After 30 min of incubation, the sample was removed, and the cells were washed 3 times with PBS. Then the nucleus was stained with Hochest 33258, and the cells were fixed with 4 wt% formaldehyde in PBS for 20 min. The fluorescence was observed on the CLSM with excitation at 405 nm for Hochest 33258 and 488 nm for Dox.

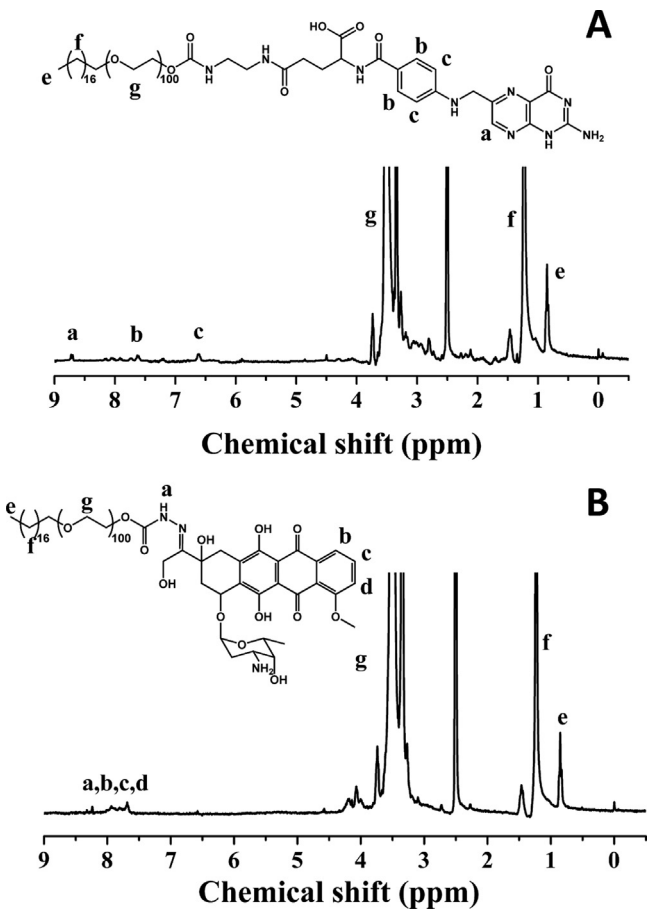


Fig. 1. ^{1}H NMR spectrua of CP-Folate (A) and CPN = Dox (B).

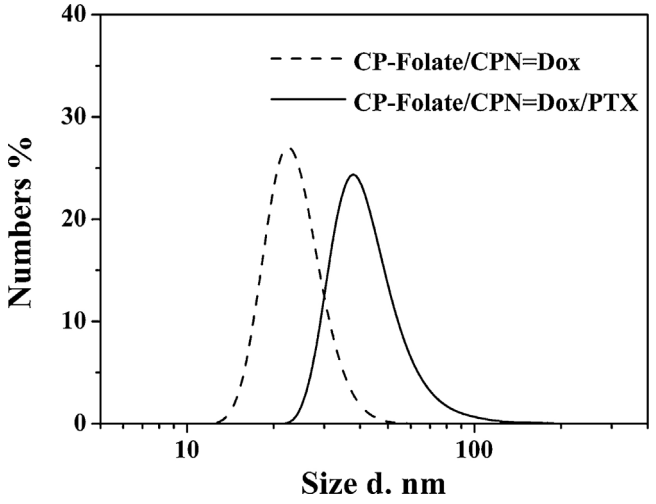


Fig. 2. Size distribution of non-/drug loaded mixed micelles measured by DLS.

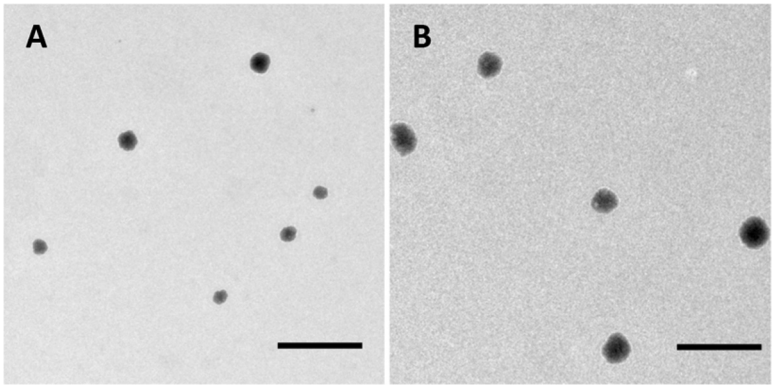


Fig. 3. TEM images of CP-Folate/CPN = Dox and CP-Folate/CPN = Dox/PTX mixed micelles. Scale bar: 100 nm.

2.2.5. MTT assay

In vitro anti-tumor activity was estimated in HeLa cells by using the MTT assay. 100 μL of cell suspension containing 5×10^3 cells was seeded into each well of a 96-well plate. After 24 h of incubation at 37 °C with 5% CO_2, the cells were treated with mixed micelles at various concentrations and carried out a further incubation for 48 h. Finally, the mediums were replaced with 200 μL of flash mediums, and 20 μL of MTT solutioin in PBS (5 mg/mL) was added into each well. The cells were incubated for another 4 h to form violet crystals. The medium in each well was carefully removed and replaced by 100 μL DMSO. When the purple solution was homogeneous, the absorbance at 570 nm was recorded by a microplate reader (Multiskan GO, Thermo Fisher). Cell viability was calculated by

$$\text{Cell viability}\ (\%) = \frac{(A_{\text{treated}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100\%$$

The data are shown as the average value ± standard deviation.

3. Results and discussion

The synthesis route of all the polymers was shown in Scheme 2. Generally, the terminal hydroxyl group of C_{18}-PEG-OH was activated by CDI and then was reacted with Folate-NH_2 to obtain C18-PEG-Folate. The polymeric prodrug C_{18}-PEG-NH-N = Dox (CPN = Dox) was synthesized according the reaction between C9 α-ketol side chain on the Dox molecule and the hydrazine bond on C_{18}-PEG-NH-NH_2. Fig. 1 showed the ^{1}H NMR spectra of C_{18}-PEG-Folate and C18-PEG-NH-N = Dox. In Fig. 1A, peaks at 1.24 and 3.65 ppm were attributed to protons of ethylene on octadecyl chains and polyethylene glycol repeating units, respectively. We also observed the characteristic peaks of Folate moiety at 6.71, 7.63 and 8.73 ppm. This confirmed the structure of C_{18}-PEG-Folate. In Fig. 1B, we observed the characteristic peaks belonged to Dox moiety at 7.72–8.31 ppm. The content of Folate moiety in C18-PEG-Folate and Dox in C_{18}-PEG-NH-N = Dox was determined by the UV absorption at wavelength of 365 nm and 488 nm, respectively. About 82.3% of C_{18}-PEG-OH was successfully modified with Folate —NH_2 while about 91.3 μg of Dox was found in 1.0 mg of C_{18}-PEG-NH-N = Dox.

The mixed micelle formed by CP-Folate and CPN = Dox was prepared based on a dialysis method. It was reported that only small amount of ligands were needed to achieve effective endocytosis (Van Butsele et al., 2009). Therefore, we set the molar ratio of CP-Folate and CPN = Dox as 2/10. As these two polymers

Table 1
Drug loading content and drug loading efficiency for PTX of CP-Folate/CPN = Dox (2/10) mixed micelle (n = 3).

Initial feeding ratio of PTX/CP-folat/CPN = Dox[a]	Content of PTX (wt%)	PTX loading efficiency (%)	Content of Dox (wt%)	Total drug content (wt %)[b]
1/2/10	7.4	87.3	6.8	14.2
2/2/10	13.4	86.4	6.4	19.8
3/2/10	16.7	73.5	6.2	22.9

[a] The initial feeding amount of CPN = Dox was 1.0 mg.
[b] The total drug content was a sum of PTX and Dox.

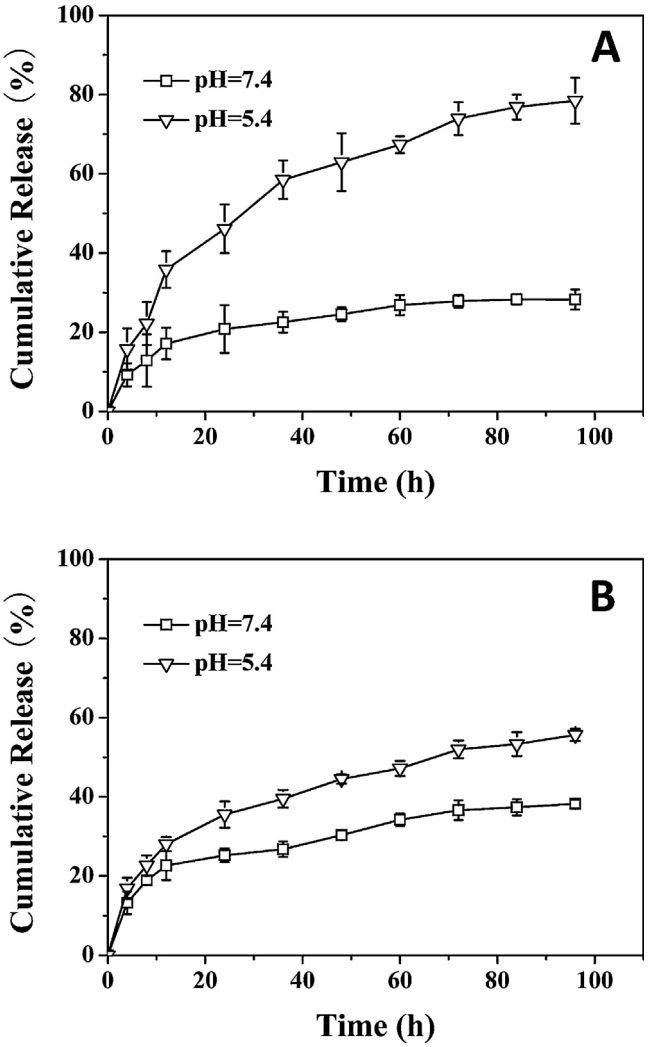


Fig. 4. Dox (A) and PTX (B) release from the mixed micelles at two different pH conditions.

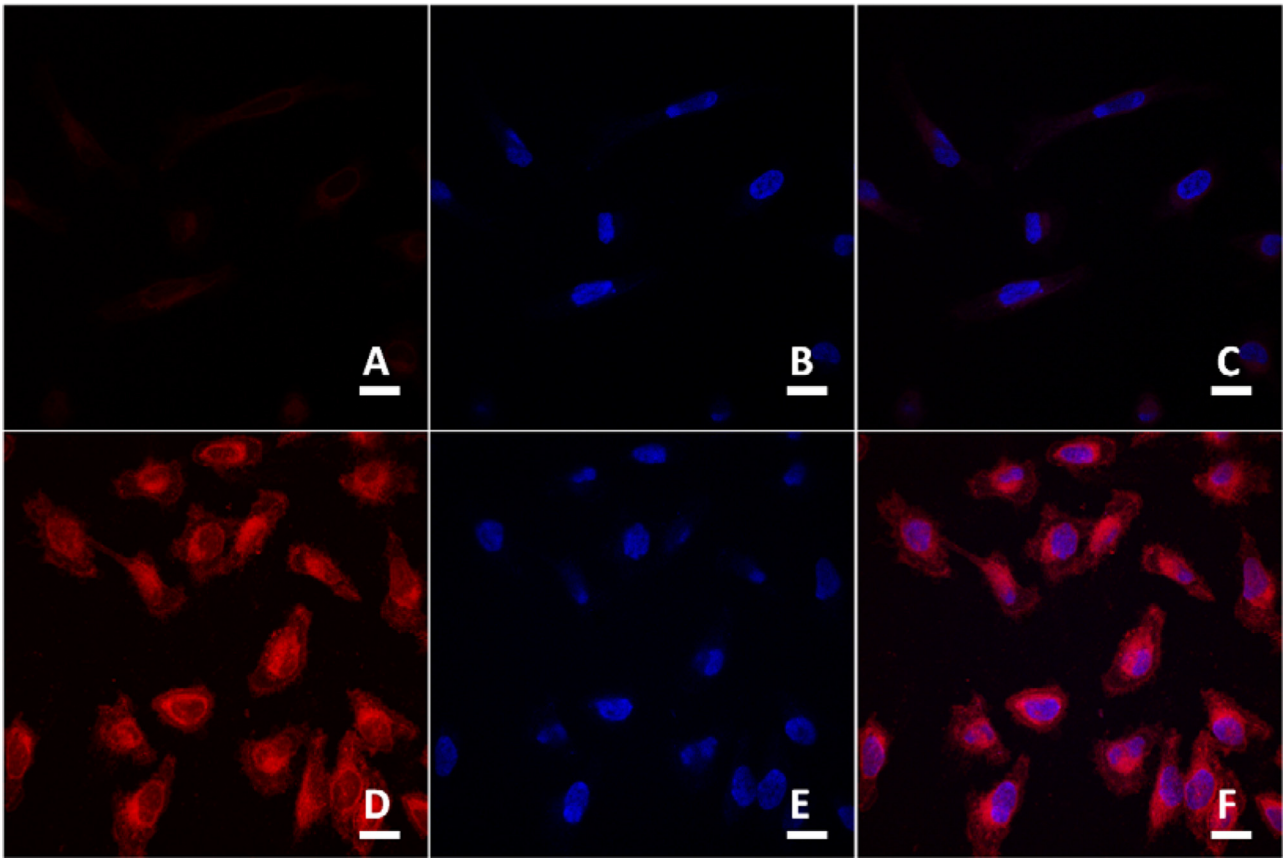


Fig. 5. The CLSM images of HeLa cells incubated with CP-OH/CPN = Dox (A–C) and CP-Folate/CPN=Dox (D–F) for 30 min. (A, D), (B, E) and (C, F) indicated the Dox, Hochest 33258 and merged channels, respectively. The scale bar is 20 μm.

possessed amphiphilic structure and had same hydrophobic components, they could self-assemble into nanoparticles in aqueous solution. We used dynamic light scattering (DLS) and transmission electron microscope (TEM) to estimate the particle size and the morphology of mixed micelle. The size of mixed micelle was about 23.9 ± 0.4 nm (hydrodynamic diameter) and showed spherical micellar structure (Figs. 2 and 3A).

There was a large amount of bonded Dox molecules in the mixed micelle. It not only acted as the therapeutic agent but also supplied extra hydrophobic spaces for stabilizing the micellar structure and encapsulated free drugs. We applied this mixed micelle to load with free PTX, one classic kind of anti-tumor drug. Table 1 summarizes the PTX loading capacities of the mixed micelle CP-Folate/CPN = Dox. This formulation based on polymeric mixed micelle showed good capacity of loading free PTX and the maximum amount of PTX in the mixed micelle was up to 16.7 wt%. The weight percentage of total drugs in this mixed micelle was nearly 22.9 wt%, which was a big improvement compared to traditional micelle based drug carriers (Khandare and Minko, 2006). We estimated the change of particle size after drug encapsulation by DLS. The result showed that there was a significant size increase when the PTX was loaded into the mixed micelles (Fig. 2). The size increase of hydrophobic core led to the enlargement of whole particle size. TEM images also confirmed this phenomenon (Fig. 3B).

As we known, the nanoparticles would face to an acidic condition (pH value range from 6.8 to 4.0) during the process of endocytosis (Bae et al., 2003; Lee et al., 2008; Tannock and Rotin, 1989). In our design, the Dox was conjugated to the amphiphilic polymer by hydrazone bonds, which could hydrolyze at such weak acidic environment. We firstly tested the drug release behavior of the mixed micelle in vitro. In the PBS buffer at pH 7.4, no more than 40% PTX was released from the mixed micelle after 96 h (Fig. 4B). As to the release of Dox, fewer drugs (28.2% after 96 h) were detected in the dialysate due the covalently bonding (Fig. 4A). When the pH was set to 5.4, which was similar to the endosomal acidic conditions, both PTX and Dox were faster released compared to the neutral conditions. It was noted that the release of Dox at pH 5.4 was faster than that of PTX. About 78% of Dox was released in 4 days. The hydrolysis of hydrazone bonds at such pH value led to the rapid release of bonded Dox. Furthermore, the slightly pH sensitivity and hydrophilicity of Dox molecules also contributed to the acid dependented drug release behavior. Enhanced PTX release at pH 5.4 was not so much than Dox, and about 55.6% of PTX was finally released from the system after 4 days. The detachment of hydrazone bonds would increase the ratio of hydrophilic part of mixed micelles but the decrease of internal stress due to the stretch of PEG blocks will counterpoise it. Thus, the structure change of the mixed micelle will be weaken, and the release of PTX at pH 5.4 was slower than that of Dox.

We added small amount of CP-Folate into the mixed micelles, expecting to improve the intracellular drug accumulation. In vitro test was carried out in HeLa cell lines, which was reported to expressing abundant Folic acid receptors (Atkinson et al., 2001; Bae et al., 2003; Nayak et al., 2004). As shown in Fig. 5D, obviously red fluorescences was observed in the HeLa cells that were treated with the CP-Folate/CPN = Dox mixed micelle. CP-OH/CPN = Dox mixed micelle was prepared here as the non-targeting contrast. In Fig. 5A, negligible fluorescence could be detected in the HeLa cells treated with the non-targeting contrast. It proved that the cell

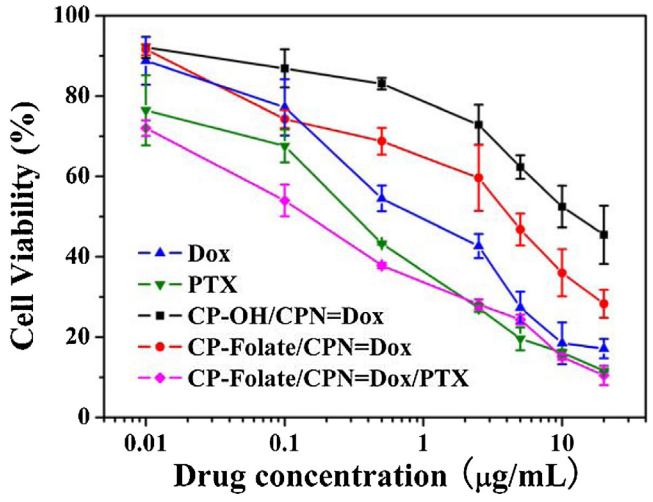


Fig. 6. Cytotoxicity of the mixed micelles in HeLa cells after 48 h of incubation.

uptake of CP-Folate/CPN = Dox mixed micelle was mediated by the FA and its receptors.

The cytotoxicities of the drug loaded mixed micelles were tested by MTT assay in HeLa cells. The data were shown in Fig. 6, CP-OH/CPN = Dox showed the lower cytotoxicity, and the IC_{50} in HeLa cells was about 12.8 μg/mL. However, with the aid of FA moieties, the CP-Folate/CPN = Dox showed more cytotoxicity (IC_{50} value: 4.2 μg/mL) than mPD. It indicated that introducing FA moieties could obviously enhance the intracellular accumulation and endow the drug carrier with tumor selectivity. Co-delivery of Dox and PTX greatly decreased the viability of HeLa cells. The IC_{50} of CP-Folate/CPN = Dox/PTX was about 0.15 μg/mL, which was much lower than that of free Dox (0.92 μg/mL) and free PTX (0.33 μg/mL). Previous literatures reported that the combination delivery of muti-drugs could improve the therapy efficacy as compared to the single therapeutic agent strategy (Shen et al., 2010). Although the reason of this issue has not been entirely understood yet, the co-delivery strategy actually improved the anti-tumor efficacy in vitro.

4. Conclusions

In summary, we demonstrated here a novel targeting drug carrier to simultaneously delivery two anti-tumor drugs, chemical bonded Dox and physical entrapped PTX. CP-Folate/CPN = Dox mixed micelles could effectively encapsulate free PTX, and the drug content was up to 22.9% due to the combined effect of octadecyl chains and chemical bonded Dox moieties. CP-Folate/CPN = Dox/PTX enhanced intracellular accumulation in HeLa cells and showed stronger anti-tumor activity compared to free drugs. We expect our strategy will facilitate to prepare high drug content and multi-drug loading targeting drug delivery systems with the purpose of overcoming multi-drug resistance.

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