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Anticancer drug delivery of PEG based micelles with small lipophilic moieties

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ABSTRACT

Herein, we reported a new type of self-assembly micelles based on amphiphilic polymers of cinnamate and coumarin derivatives modified PEG for drug delivery applications. Lipophilic cinnamic acid (CIN) and 7-carboxyl methoxycoumarin (COU) were immobilized on the terminal groups of poly(ethylene glycol) (PEG) to prepare amphiphiles. The amphiphiles self-assembled into micelles. The amphiphiles and micelles were characterized by ^{1}H NMR, FT-IR, DLS and TEM. Doxorubicin (DOX) was used as a model drug to investigate the lipophilic moieties effects on the drug release behaviors. The DOX loaded micelles were incubated with HepG2 liver cancer cells to study the in vitro anticancer activities. The results showed that DOX could be encapsulated in the micelles efficiently. The mean diameter of the drug loaded micelles was around 100 nm. Drug release profile revealed that the release rate of DOX loaded COU-PEG-COU micelles was significantly slower than that of CIN-PEG-CIN micelles. The DOX loaded micelles could be internalized in HepG2 cells. Both CLSM and flow cytometry results showed that the DOX loaded CIN-PEG-CIN micelles exhibited better anticancer efficacy.

1. Introduction

In the past decades, drug delivery systems have attracted much interest to pharmacists and biomaterials scientist (Kataoka et al., 2001; Kazunori et al., 1993; Spizzirri et al., 2013). As most anticancer drugs are hydrophobic, the poor solubility in aqueous medium is a main problem that chemotherapy faces (Licciardi et al., 2010; Richter et al., 2010; Tian and Mao, 2012). The encapsulation of anticancer drugs in polymeric micelles could not only improve the solubility of drugs but also decreases the risk of multidrug resistance (Li et al., 2010; Tong et al., 2012). More importantly, the size of most drug loaded polymeric micelles is lower than 200 nanometers, which takes the advantages of targeting the tumor tissues due to the enhanced permeability and retention (EPR) effect (Acharya and Sahoo, 2011; Torchilin, 2011). Therefore, amphiphilic copolymers, which could self-assemble into micelles, have been extensively studied as carriers for anticancer drug delivery (Licciardi et al., 2010; Michailova et al., 2010; Zhao et al., 2012). Many polymeric amphiphiles with different architectures including block (Attwood et al., 2007; Boudier et al., 2009), graft (Sun et al., 2010), star-shaped (Rezaei et al., 2012; Gao et al., 2009), dendrimertic (Teow et al., 2013) and hyperbranched (Kolhe et al., 2004; Liu et al., 2010) polymers have been designed and synthesized for loading anticancer drugs.

Among all the amphiphilic copolymers, hydrophilic poly(ethylene glycol) (PEG) is an important even indispensable segment for its advantages in escaping the capture of reticuloendothelial system (RES). These characteristics lead to long circulation of drug loaded micelles during blood vessel transportation (Ambegia et al., 2005; Shi et al., 2006; Basile et al., 2012; Yan et al., 2009). Biodegradable polymers such as poly(lactide acid) (PLA), poly(ε-caprolactone), polycarbonates and their copolymers are usually used as lipophilic segments in polymer amphiphiles (Suksiriworapong et al., 2012; Zhang et al., 2012). PEG–polyester copolymers are traditional and classic amphiphiles for self-assembly micelles. Besides lipophilic polymer chains, small lipophilic molecules such as cholesterol were reported as lipophilic moiety in polymeric micelles (Cai et al., 2011; Yu et al., 2013). However, this micelle was not as stable as block copolymer micelles due to the weak hydrophobic interaction within cholesterol, which acted as the driven force for self-assembly.

Recently, a new series of small lipophilic moieties, which had π–π conjugated structures, were reported to replace biodegradable polymer chains as lipophilic segments in fabricating micelles for anticancer drug delivery (Lai et al., 2012; Liang et al., 2013). Cinnamic acid and 7-carboxyl methoxycoumarin were immobilized on the terminal groups of poly(ethylene glycol) methyl ether (mPEG)

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to simplify the preparation of amphiphiles. The advantages of these amphiphiles were that the encapsulation of anticancer drug in the micelles was not only driven by hydrophobic interaction but also π–π interaction within drugs and carriers. Though the micelles with small π–π conjugated molecules as lipophilic moieties were investigated, the architecture effects of lipophilic moieties on the drug release behaviors and anticancer activity were not reported in details.

In this paper, we aim to study the small lipophilic architecture effects on the drug delivery properties. A facile, one-step strategy for the synthesis of polymeric micelles containing cinnamic acid and 7-carboxyl methoxycoumarin as lipophilic moieties was reported. The amphiphiles and self-assembly micelles were characterized by ^{1}H NMR, FT-IR, DLS and TEM. Anticancer drug doxorubicin (DOX) was loaded in the micelles. The release profiles of DOX loaded micelles with different lipophilic moieties were investigated. The drug loaded micelles were incubated with HepG2 liver cancer cells to study the in vitro anticancer activities.

2. Experimental

2.1. Materials and instruments

7-Hydroxycoumarin was purchased from Alfa Aesar, ethyl-bromoacetate, cinnamic acid were purchased from SinoPharm Chemical Reagent Co., Ltd. 4-Dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC) and poly(ethylene glycol) (PEG, Mw 2000 Da) were purchased from Sigma–Aldrich Co. PEG was dried by an azeotropic distillation in toluene. Doxorubicin hydrochloride (DOX·HCl, Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd., China) was deprotonated by dissolving in water and the pH value was adjusted to 9.6 with NaOH aqueous solution. The obtained precipitation was concentrated and lyophilized to receive doxorubicin (Liu et al., 2011; Pu et al., 2013; Chang et al., 2013). All the solvents were analytical grade and purchased from Kelong Chemical Co. (Chengdu, China). Dichloromethane (CH_2Cl_2) was refluxed with CaH_2 and then distilled prior to use. N,N-dimethylformamide (DMF) was dried over calcium hydride and then purified by vacuum distillation. All other reagents and solvents of analytical grade were used as received unless mentioned.

Dulbecco's modification of eagle's medium (DMEM), fetal bovine serum, and penicillin–streptomycin were purchased from HyClone Inc. The human hepatocellular carcinoma cell line (HepG2) was used for cytotoxicity test.

2.2. Synthesis of 7-carboxyl methoxycoumarin

7-Carboxyl methoxycoumarin (COU-COOH) was synthesized according to a previously reported method. 7-Hydroxycoumarin (1.00 g, 6.17 mmol) and K_2CO_3 (1.25 g, 9.00 mmol) were dissolved in acetone (20 mL) in a round-bottomed flask. The reaction vessel was consecutively evacuated and refilled with nitrogen for three times. Ethyl bromoacetate (1.24 g, 7.42 mmol) was added dropwise under stirring. The reaction flask was lowered into an ice bath for 2 h and then submerged in a 65 °C oil bath for another 3 h. The reaction was terminated and the precipitate was removed. The solution was concentrated under reduced pressure, ethanol (50 ml) was added to the concentrated solution, the mixture was placed in refrigerator at 4 °C for recrystallization. White needle crystal of 7-ethoxy carbonyl methoxycoumarin was obtained after filtration.

NaOH solution was added dropwise to the 1,4-dioxane (40 mL) solution containing 7-ethoxy carbonyl methoxycoumarin (0.70 g, 2.83 mmol) and the solution was stirred at room temperature for 24 h. The pH value was adjusted to 2.0 by the addition of 0.1 N aqueous HCl solution at the end of the reaction. The product was extracted with chloroform/methanol mixed solvent. The solvent was concentrated to 30 mL. After that, it was placed in refrigerator at 4 °C for recrystallization in ethanol. COU-COOH was obtained after filtration.

2.3. Synthesis of COU-PEG-COU and CIN-PEG-CIN

PEG (Mw 2000, 2.00 g, 1.00 mmol), DMAP (0.02 g, 0.18 mmol) and cinnamic acid (0.568 mmol) were dissolved in 20 mL CH_2Cl_2 in an ice bath under nitrogen atmosphere. A solution of DCC (0.59 g, 2.84 mmol) in CH_2Cl_2 (10 mL) was added dropwise in the mixture over 0.5 h. The mixture was stirred at room temperature for 24 hours. The white solid dicyclohexylurea (DCU) precipitate was removed by filtration, the filtrate was concentrated. The crude product was isolated by precipitation in cold ether, filtration and extensive washing. For further purification, the product was dissolved in deionized water and dialyzed in a dialysis membrane tubing (Spectra/Por, MWCO 1000) renewed every 6 h for three days. The product in the membrane was freeze-dried.

The procedure of synthesizing COU-PEG-COU was the same with that of CIN-PEG-CIN, except that the solvent is DMF.

2.4. Preparation of drug loaded micelles

DOX loaded micelles were prepared by a dialysis method. Typically, the freeze-dried amphiphile COU-PEG-COU/CIN-PEG-CIN (20 mg) and DOX (5 mg) were dissolved in 2 mL of DMSO. The solution was added dropwise to 20 mL of double-distilled water under stirring. After stirred at room temperature for 2 h, the solution was transferred to a 1000 Da molecular weight cut-off dialysis tubing and dialyzed for 24 h to remove the organic solvents and free DOX. The solution was further filtered through a 0.45 μm syringe filter to remove the residual DOX aggregates. The solution was freeze-dried to obtain DOX loaded micelles. The whole procedure was performed in dark.

The content of encapsulated DOX was determined by fluorescence measurement with excitation at 480 nm in DMSO using calibration curve obtained from DOX/DMSO solutions with different DOX concentrations. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formulas:

$$\text{DLC}\ \ (\text{wt}\%) = \left[\frac{\text{weight of loaded drug}}{\text{weight of drug loaded micelle}}\right] \times 100$$

$$\text{DLE}\ \ (\%) = \left[\frac{\text{weight of loaded drug}}{\text{weight of drug in feeding}}\right] \times 100$$

2.5. In vitro release of drug loaded micelles

DOX loaded micelles were diluted to 1 mg/mL and then 1.0 mL of solution was transferred into dialysis membrane tubings (Spectra/Por, MWCO 1000). The tubings were immersed into flasks containing 25.0 mL of PBS (0.01 M, pH 7.4). The flasks were put in a shaking bed at 37 °C to acquire sink conditions. 1 mL PBS was withdrawn and replaced by 1 mL of fresh PBS at predetermined time intervals to keep the volume of solution constant. The released DOX was detected by a fluorescence detector with excitation wavelength at 480 nm and the emission wavelength at 550 nm. The release experiments were conducted in triplicate, and the results were presented as the average data with standard deviations.

2.6. Characterization

^{1}H NMR analyses were recorded on a Varian Mercury Plus 400 MHz spectrometer with DMSO-d_6 and $CDCl_3$ as solvents at room temperature. TMS was used as the internal standard. FT-IR spectra were measured using the KBr disk method on Fourier transform infrared spectrometer (FT-IR, Thermo Fisher Nicolet 8700) within the range of 3600–400 cm^{-1}. The DLS measurements were performed with a dynamic light scattering (DLS, Malvern Zetasizer Nano ZS). All samples were measured in aqueous solution with the concentration of 1 mg/mL at room temperature and the scattering angle was 173°. TEM was employed to observe the morphology and particle size of the micelles with a JEOL JEM-100CX-II instrument at a voltage of 400 V. Samples were prepared by dropping micelle solutions onto carbon-coated copper grids and dried overnight at room temperature. To make particle morphology more clear, the samples were stained with phosphotungstic acid (ATP) aqueous solution.

2.7. Cytotoxicity assay

NIH/3T3 fibroblasts and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin. The temperature of the incubator was set at 37 °C and under a humidified atmosphere containing 5% CO_2.

The relative cytotoxicity of COU-PEG-COU and CIN-PEG-CIN micelles was performed by Cell Counting Kit-8 (CCK-8) assay against NIH/3T3 fibroblasts. The cells were seeded onto 96-well plates at a density of 8×10^3 cells per well in 100 μL of medium. After 24 h incubation, the culture medium was removed and replaced with 100 μL medium containing COU-PEG-COU or CIN-PEG-CIN of different concentrations. The cells were incubated for another 48 h. The culture medium was removed and the wells were rinsed with PBS (pH = 7.4) for three times. 100 μL DMEM containing 10 μL CCK-8 solution was added to each well and incubated for another 2 h. The absorbance of each well was measured in at the 450 nm wavelength. Cell viability was calculated by the following equation.

$$\text{Cell viability}\ (\%) = \frac{\text{Ints}}{\text{Int}_{\text{control}}} \times 100$$

where Ints is the absorbance intensity of the cells incubated with the samples and $\text{Int}_{\text{control}}$ is the absorbance intensity of the cells incubated with the culture medium only.

2.8. In vitro anticancer efficacy of drug loaded micelles

The in vitro anticancer efficacy of drug loaded COU-PEG-COU and CIN-PEG-CIN micelles against HepG2 cells was evaluated by CCK-8 assay. Approximate 8×10^3 cells per well were seeded in 96-well plates with 100 μL of DMEM medium for 24 h before the tests. DOX·HCl and DOX loaded micelles with different DOX concentrations (from 0.001 to 200 μg/mL) in DMEM were added to the medium of 96-well plates and incubated for 48 h. The culture medium was removed and the wells were rinsed with PBS (pH = 7.4) for three times. Afterwards CCK-8 assay was conducted as described above. The half maximal inhibitory concentration (IC50) of the micelles and DOX·HCl were calculated using Origin software (n = 4).

2.9. Cellular uptake of drug loaded micelles

Flow cytometry and confocal laser scanning microscopy (CLSM) were employed to evaluate the qualitative and quantitative cellular uptake of the DOX loaded micelles.

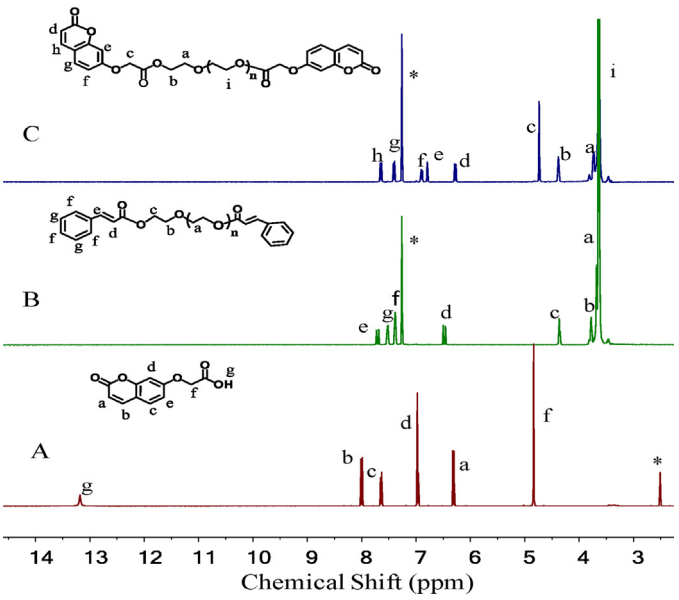


Fig. 1. ^{1}H NMR spectra of COU-COOH (A) in DMSO-d_6, CIN-PEG-CIN (B) and COU-PEG-COU (C) in $CDCl_3$. The asterisks denote the solvent peaks.

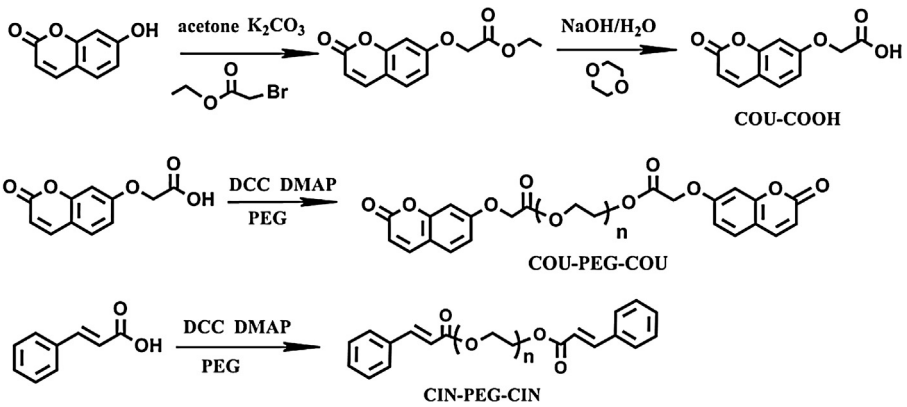
For flow cytometry, HepG2 cells were seeded on six-well plates at a density of 1×10^5 cells per well in 1 mL complete DMEM and cultured for 24 h. The free DOX and DOX loaded COU-PEG-COU and CIN-PEG-CIN micelles were added to different wells with the DOX concentration of 10 μg/mL. Cells without DOX treatment were used as control. After incubated at 37 °C for 0.5 h and 4 h, the culture medium was removed and the cells were washed with PBS for three times. Then the samples were prepared for flow cytometry analysis by removing the culture medium, rinsing with PBS, treating with trypsin and centrifugation. A minimum of 1×10^4 cells were collected for each sample.

For confocal laser scanning microscopy, HepG2 cells were seeded on 35 mm diameter glass dishes at a density of 1×10^5 cells per well. After incubation in DMEM with 10% FBS and 5% CO_2 at 37 °C for 24 h, when the HepG2 cells were at a logarithm phase, 250 μL of DMEM solution of DOX·HCl and DOX loaded micelles were added into the wells with the DOX concentration of 10 μg/mL. The HepG2 cells were incubated at 37 °C for another 0.5 h and 4 h respectively before the culture medium was removed and the dishes were rinsed with PBS (pH = 7.4) for three times. The cell nucleus was stained with DAPI, followed by replacing the culture medium with PBS. The cells were imaged on confocal laser scanning microscopy excited at 485 nm.

3. Results and discussion

3.1. Synthesis and characterization of CIN-PEG-CIN and COU-PEG-COU amphiphiles

The synthetic routes of amphiphiles COU-PEG-COU and CIN-PEG-CIN are illustrated in Scheme 1. These two small lipophilic molecules of cinnamic acid and 7-carboxyl methoxycoumarin were immobilized on the terminal groups of PEG. Fig. 1 provided the ^{1}H NMR spectra of COU-COOH, CIN-PEG-CIN and COU-PEG-COU. The signal at 13.2 ppm in Fig. 1A was assigned to the proton in the carboxyl group of COU-COOH. The proton signals between 6.0 and 8.0 ppm are the characteristic peaks of bifuran ring in coumarin. In addition, as shown in Fig. 1B and C, the peaks locate between 3.4 and 3.6 ppm are attributed to characteristic proton peaks of the repeated unit (CH_2CH_2O) in PEG. After the esterification reaction,



Scheme 1. The synthesis of CIN-PEG-CIN and COU-PEG-COU.

the hydroxy groups in PEG were substituted, which affected the chemical environment of the PEG units connected nearby, thus two single peaks appear at 3.8 and 4.4 ppm, respectively. It is confirmed that the hydroxyl groups of PEG were completely replaced by cinnamic acid and 7-carboxyl methoxycoumarin via calculating the ratio of integrated areas of proton peaks at 3.8 and 4.4 ppm against the proton peaks of cinnamate or coumarin groups. Therefore, ^{1}H NMR spectra in Fig. 1 elucidate the successful synthesis of CIN-PEG-CIN and COU-PEG-COU.

The proposed structure of the amphiphilic polymers has been further verified by the FT-IR technique. Fig. 2 exhibits the representative FT-IR spectra of CIN-PEG-CIN and PEG-PEG-COU samples. In the spectra, the bands at 2947 and 2869 cm^{-1} are attributed to the asymmetric and symmetric stretching vibrations of —CH_2—, respectively. While the —CH_2— asymmetric deformation vibration of COU-PEG-COU/CIN appears at 1464 cm^{-1}. In the spectrum of CIN-PEG-CIN, a sharp peak at 1714 cm^{-1} is attributed to C=O stretching vibration of esters. It is noteworthy that apart from the ester bond linking PEG and coumarin, a lactone structure exists in COU-PEG-COU. The peaks at 1758 cm^{-1} and 1733 cm^{-1} are ascribed to the two carbonyl groups. Meanwhile, a strong C—O—C stretching vibration at 1116 cm^{-1} was also observed. The bands locate between 1460 cm^{-1} and 1650 cm^{-1} are verified to be aromatic ring skeleton vibration. From ^{1}H NMR and FT-IR measurements, it can be concluded that COU and CIN derivates have been successfully bounded onto the hydroxyl terminals of PEG via esterification reaction, affording COU-PEG-COU and CIN-PEG-CIN.

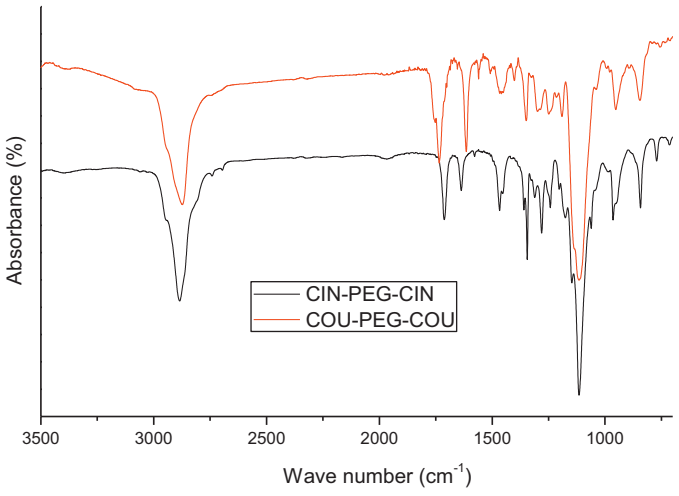


Fig. 2. FTIR spectra of COU-PEG-COU and CIN-PEG-CIN amphiphiles.

3.2. Characterizations of blank and drug loaded micelles

The amphiphiles self-assembled into micelles with coumarin derivatives or cinnamates as the hydrophobic cores and PEG chains as hydrophilic shells were uniformly dispersed in water with appropriate size. DLS measurements verified that the average hydrodynamic diameters were 107 nm and 117 nm for blank COU-PEG-COU and CIN-PEG-CIN micelles, respectively. To further confirm the DLS observation, the TEM measurement was employed to analyze the size and morphology of the COU-PEG-COU and CIN-PEG-CIN micelles. As shown in Figs. 3 and 4, the spherical blank micelles with an average size of ~70 nm were observed and the CIN-PEG-CIN micelles were slightly larger than COU-PEG-COU micelles. The result of TEM images was consistent with the DLS data very well. And it was found that COU-PEG-COU and CIN-PEG-CIN micelles have analogous diameter and morphology whether blank or loaded with DOX because of their substantially similar structure. The smaller size of COU-PEG-COU micelles was attributed to the stronger hydrophobic interaction within the micelles due to the larger size of coumarin backbone comparing to cinnamate.

DOX was used as a model anticancer drug to evaluate the loading and release properties of the two micelles. DOX is widely used to treat various solid malignant tumors by interacting with DNA in the ways of intercalation and inhibition of macromolecular biosynthesis. Fluorescence detection was employed to test the drug loading content (DLC) and drug loading efficiency (DLE) of the micelles (Table 1). The DLC and DLE of COU-PEG-COU micelles were higher than that of CIN-PEG-CIN micelles. The morphologies of blank and drug loaded micelles were shown in Fig. 5. After DOX was loaded, the size of the drug loaded micelles increased significantly, but it still remained around 100 nm, which was favorable for targeting tumor tissues via EPR effect (Maeda et al., 2009; Maeda, 2012).

3.3. In vitro drug release

The drug release of the DOX loaded micelles was investigated at 37 °C in PBS (pH 7.4, 50 mM). As demonstrated in Fig. 6, the typical biphasic pattern including an initial burst release in the first 4 h and following a sustaining release profile for a prolonged time was observed. The drug initial burst release of DOX loaded CIN-PEG-CIN was up to 40% and that of DOX loaded COU-PEG-COU micelles was 20%. This burst release behavior was attributed to the small

Table 1
Drug loading content and drug loading efficiency of the micelles.

Sample	DLC (wt%)	DLE (%)
CIN-PEG-CIN-DOX	11.3	56.7
COU-PEG-COU-DOX	14.2	71.2

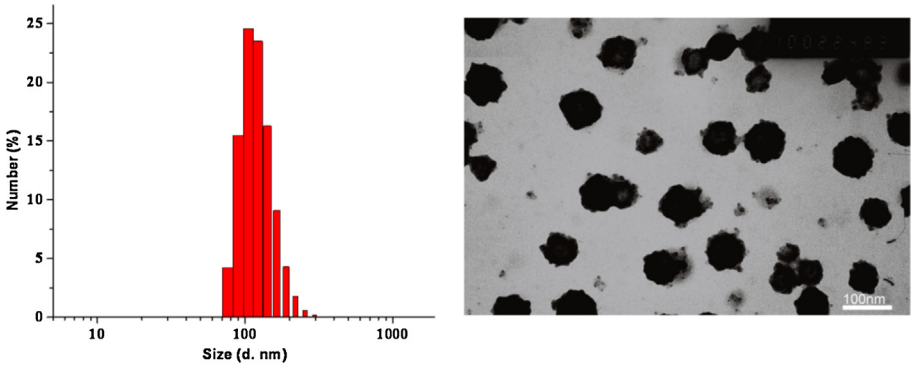


Fig. 3. DLS histogram and TEM photographs of CIN-PEG-CIN micelles.

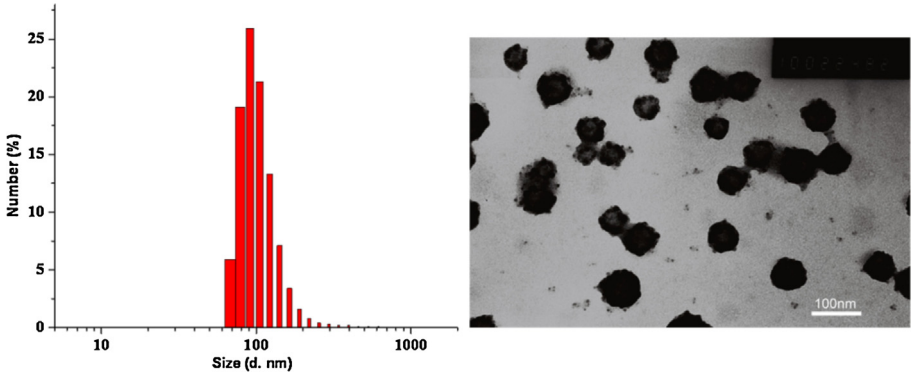


Fig. 4. DLS histogram and TEM photographs of COU-PEG-COU micelles.

proportion of DOX adsorbed to or just beneath the surface of the DOX loaded micelles dissociated from the carriers. This result indicated that most DOX molecules were well encapsulated in the inner core of micelles. After the relative rapid release of DOX in the early stage, the release rate slowed down greatly during the following long period, maintaining a slow and steady release of DOX. By the end of 48 h, the cumulative release of DOX was 33% and 52% for COU-PEG-COU and CIN-PEG-CIN, respectively. The slower release rate of COU-PEG-COU micelles was also attributed to the stronger hydrophilic interaction between DOX and COU lipophilic moieties.

3.4. The biocompatibility of micelles

The relative cytotoxicity of COU-PEG-COU/CIN-PEG-CIN micelles was measured by Cell Counting Kit-8 (CCK-8) assay against NIH/3T3 fibroblasts. The results were presented in Fig. 7. The micelles with different concentrations were incubated with 3T3 fibroblasts for 48 h. The cell viability decreased as the micelles concentration increased. But the relative cells viability was higher than 90% even when the concentration was as high as 2 mg/mL. Thus, it could conclude that the COU-PEG-COU and CIN-PEG-CIN micelles were low toxic to NIH/3T3 fibroblasts.

3.5. In vitro antitumor efficiency

In order to evaluate whether the COU-PEG-COU and CIN-PEG-CIN micelles were efficient carriers for anticancer drug delivery, the in vitro anticancer activity of the drug loaded micelles were measured. The DOX loaded micelles were incubated with HepG2 liver cancer cells. As shown in Fig. 8, the significant proliferation inhibition effect was observed when the cells were treated with DOX loaded micelles. The free hydrophilic DOX·HCl was used as control. The half maximal inhibitory concentration (IC50) was 2.8 μg/mL for DOX loaded CIN-PEG-CIN, and that of DOX loaded COU-PEG-COU was 29.9 μg/mL. The IC50 of drug loaded micelles was much higher than that of free DOX·HCl. It was due to the higher and nonspecific cellular uptake of free DOX·HCl. DOX·HCl is a small molecule and hydrophilic, it is internalized in the HepG2 cells easily and directly via the pinocytosis process (Luo et al., 2002). While the DOX loaded micelles with their diameter around 120 nm were

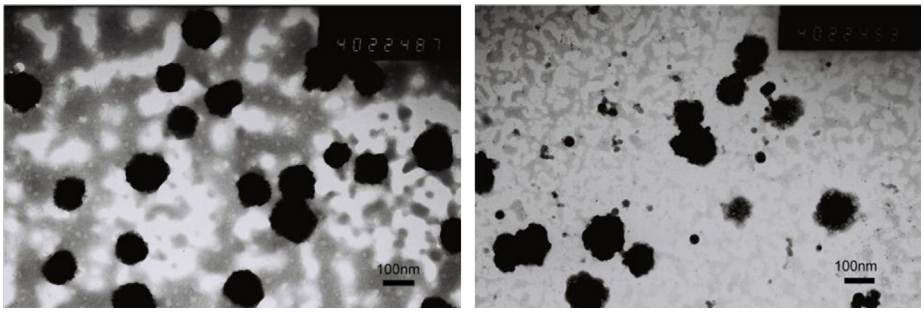


Fig. 5. TEM photographs of DOX loaded COU-PEG-COU (left) and CIN-PEG-CIN (right).

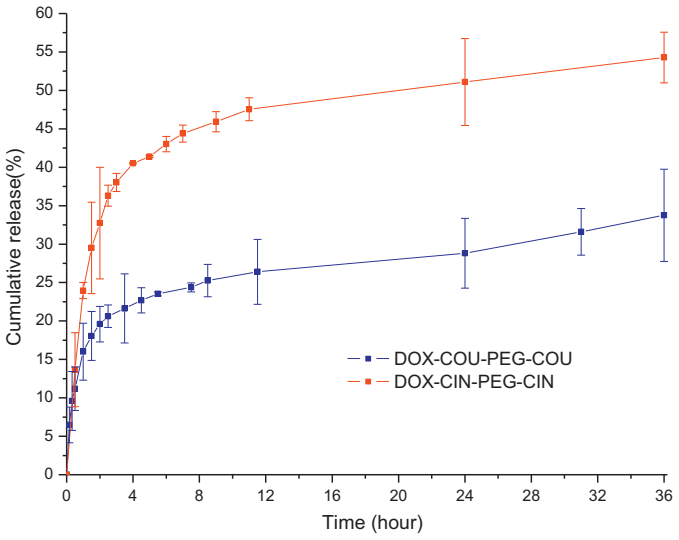


Fig. 6. In vitro release profile of DOX loaded micelles in PBS (0.01 M, pH 7.4) at 37 °C.

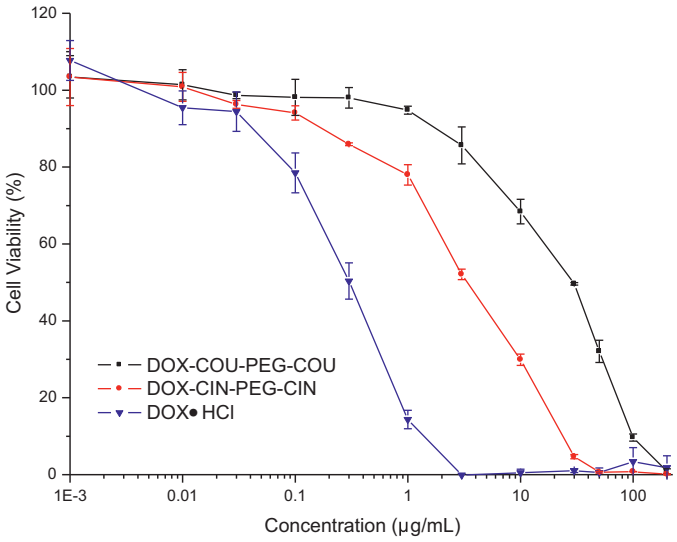


Fig. 8. Cytotoxicity of free DOX·HCl and DOX loaded micelles to HepG2 cells after 48 h incubation ($n = 4$).

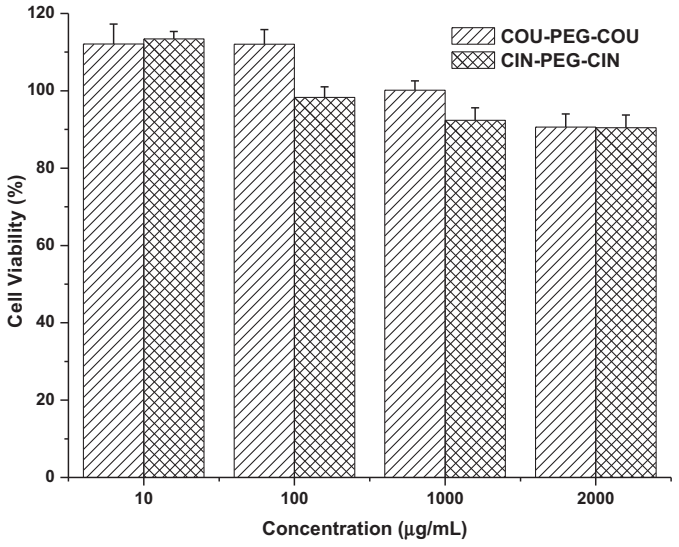


Fig. 7. Viability of NIH/3T3 cells after incubated with DOX-free micelles at different concentrations for 48 h ($n = 4$).

internalized via endocytosis process (Canton and Battaglia, 2012). As the drug release from CIN-PEG-CIN micelles was faster than that from COU-PEG-COU micelles, thus, the cumulated concentration of DOX in cells was higher than that in cells treated with DOX loaded COU-PEG-COU micelles, which resulted more efficient inhibition effect.

3.6. Cellular internalization

Flow cytometry was used to characterize the cellular internalization of DOX loaded micelles. The DOX loaded CIN-PEG-CIN and COU-PEG-COU micelles were incubated with HepG2 cells with equivalent DOX concentration of 10 μg/mL. Fig. 9 showed the histograms of cell-associated DOX fluorescence intensity of HepG2 cells treated with DOX·HCl and the two DOX loaded micelles at the predetermined time intervals of 0.5 h and 2 h. After incubated for 30 min, an evident fluorescence intensity enhancement was observed, indicating the cellular uptake. With a longer incubation time of 4 h, as displayed in Fig. 9, the fluorescence intensity of DOX loaded micelles increased clearly. The relative geometrical mean fluorescence intensities of HepG2 cells treated with DOX loaded COU-PEG-COU micelles, DOX loaded CIN-PEG-CIN micelles

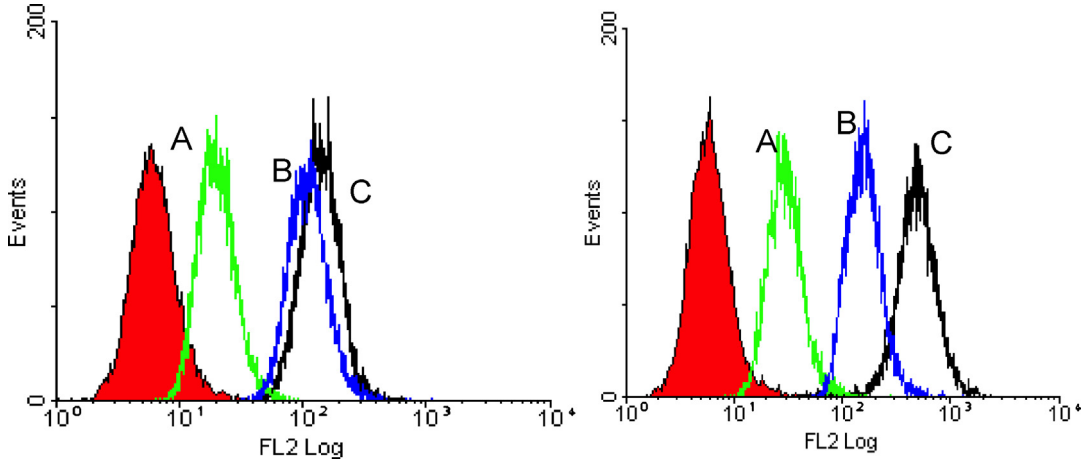


Fig. 9. Flow cytometric profiles of HepG2 cells incubated with DOX loaded COU-PEG-COU (A) micelles, DOX loaded CIN-PEG-CIN (B) micelles and free DOX·HCl (C) for 0.5 h (left) and 4 h (right) at an equivalent drug concentration of 10 μg/mL.

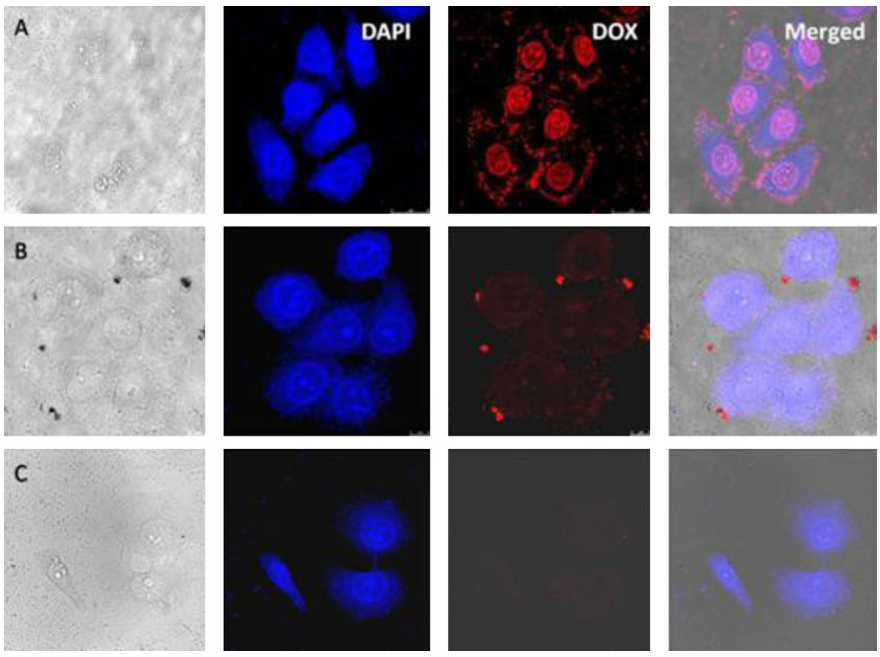


Fig. 10. Confocal microscopy images of HepG2 cells incubated with DOX·HCl (a), DOX loaded CIN-PEG-CIN micelles (b) and DOX loaded COU-PEG-COU micelles (c) for 0.5 h (DOX concentration was 10 μg/mL). Cell nuclei were stained with DAPI.

and DOX·HCl were about 5-fold, 25-fold and 80-fold to the control, which indicated the cellular internalization of drug loaded micelles. The sequence of fluorescence intensity was also closely related to the different cellular uptake mechanisms as discussed before. Free DOX·HCl was internalized via diffusion, and the DOX loaded micelles were internalized via endocytosis process.

CLSM was applied to further investigate the cellular uptake behavior and intracellular distribution of the DOX loaded micelles and free DOX·HCl in HepG2 cells. After predetermined interval of 30 min and 4 h, the nuclei was stained by DAPI and observed by confocal microscopy. The results revealed that the free DOX·HCl largely accumulated in the cell nuclei of HepG2 cells after incubation for 30 min (Fig. 10), while the red fluorescence of DOX was weak in the cells treated with DOX loaded COU-PEG-COU and CIN-PEG-CIN micelles and the red fluorescence mainly located in the cytoplasm. After incubated for 4 h (Fig. 11), an evident red fluorescence of DOX appeared in the cytoplasm and nucleus. The strength of red fluorescence of cells treated with DOX loaded COU-PEG-COU micelles was weaker than that of DOX loaded CIN-PEG-CIN micelles, which was in agreement with the flow

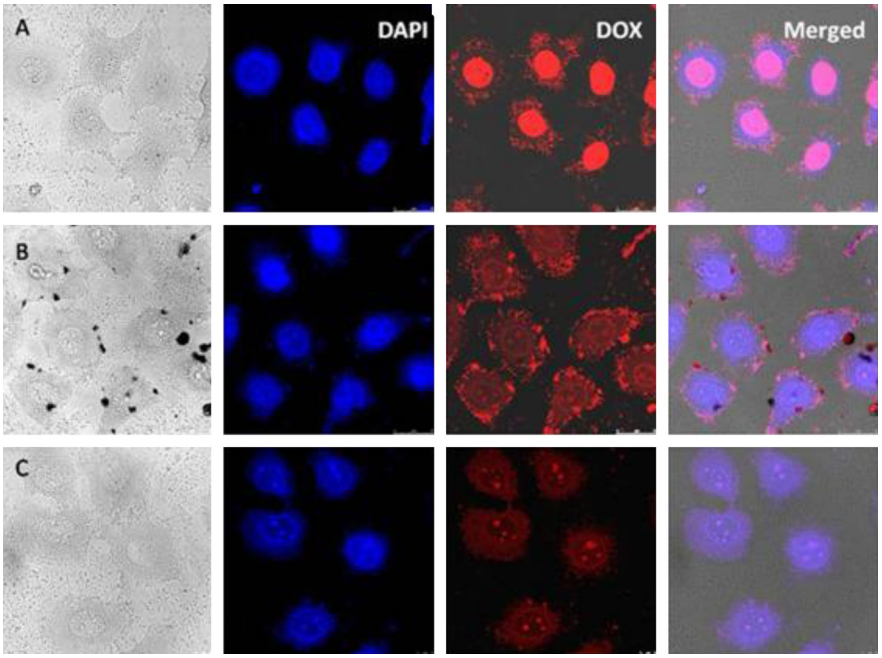


Fig. 11. Confocal microscopy images of HepG2 cells incubated with DOX·HCl (a), DOX loaded CIN-PEG-CIN micelles (b) and DOX loaded COU-PEG-COU micelles (c) for 4 h (DOX concentration was 10 μg/mL). Cell nuclei were stained with DAPI.

cytometry results. Both flow cytometry and CLSM analyses confirmed that the DOX loaded CIN-PEG-CIN and COU-PEG-COU micelles could be internalized in HepG2 cells.

4. Conclusions

In this study, we synthesized COU-PEG-COU and CIN-PEG-CIN amphiphiles with the conjugation of small lipophilic molecules on the terminal groups of PEG chains. The amphiphiles self-assembled into micelles. The mean diameters of blank and drug loaded micelles were less than 100 nm. Doxorubicin was trapped in the micelles. Both the drug loading content and encapsulation efficiency of COU-PEG-COU micelles were higher than those of CIN-PEG-CIN micelles. Drug release from COU-PEG-COU micelles was significantly slower than that of CIN-PEG-CIN micelles. The IC50 was 2.8 μg/mL for DOX loaded CIN-PEG-CIN micelles and 29.9 μg/mL for DOX loaded COU-PEG-COU micelles, respectively. The DOX loaded micelles could be internalized in HepG2 cells. The DOX loaded CIN-PEG-CIN micelles exhibited better anticancer efficacy. The small lipophilic moieties provided a novel strategy for fabricating polymeric micelles for anticancer drug delivery.

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