Department of Pharmaceutical Science[1], Nanfang Hospital, Southern Medical University; School of Pharmaceutical Science[2], Southern Medical University, Guangzhou, China

Preparation, characterization and anticancer activity of norcantharidin-loaded poly(ethylene glycol)-poly(caprolactone) amphiphilic block copolymer micelles

Shui-Fang Chen[1], Wen-Fen Lu[1], Zhi-Yong Wen[1], Qiang Li[2], Jian-Hai Chen[1]

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Prof. Jian-Hai Chen, Department of Pharmaceutical Science, Nanfang Hospital, Southern Medical University
jianhaichen11@yahoo.com.cn

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In this study, a novel amphiphilic block copolymer biomaterial — poly (ethylene glycol)-poly (caprolactone) (PEG-PCL), was used to entrap norcantharidin (NCTD), taking advantage of self-assembly theory. Dialysis and volatilization dialysis were used to prepare copolymer micelles. Drug-loaded micelles were compared with blank micelles in terms of their particle diameter, morphology and IR spectral characteristics. The results revealed that there was no significant difference in respect of morphology and IR spectrum, but particle size differed. Drug-loaded micelles had a smaller particle size than blank micelles. Three important factors influencing particle size, the drug loading content (LC) and the drug entrapment efficiency (EE) of the NCTD-loaded micelles, were studied. The results indicated that the method of preparation and the type of organic solvent had a significant influence on the size of the micelles. LC and EE were greatly affected by the ratio of NCTD to copolymer. *In vitro* release of NCTD from the conjugate micelles showed that its release rate depended on the pH of the phosphate buffer solution (PBS). The amount released was higher at lower pH than under neutral conditions. *In vitro* antitumor activity of the NCTD conjugate against human hepatoma (HepG2) cell line and human lung cancer (A549) cell line was evaluated by the MTT method. Micelles loaded with NCTD demonstrated greater and more satisfactory cell viability inhibition than the free drug. *In vivo* antitumor activity of drug-loaded micelles was investigated in mice bearing S180 mouse sarcoma. NCTD-loaded micelles displayed tumor inhibition effects, better than the free drug. As a new drug delivery system, copolymer micelles present many advantages including easy formulation, good water solubility, low toxicity and high treatment efficacy, and show great potential as carriers of hydrophobic drugs.

1. Introduction

Norcantharidin (NCTD), a demethylation derivative of cantharidin (Fig. 1) which is an active constituent obtained from the dried body of the Chinese blister beetle (*mylabris)* is a new chemotherapy agent that has exhibited very effective activity against cancer and a wide spectrum of tumors, including primary hepatic carcinoma (Chen et al. 2003), colorectal cancer (Chen et al. 2009), oral cancer (Kok et al. 2003), lung cancer, ovarian cancer and breast cancer (Yang et al. 2011). The anti-cancer mechanisms of NCTD have been illuminated by the evidence showing its anti-proliferative, pro-apoptotic and anti-migratory effects on tumor cells. However, the clinical application of NCTD has been limited because of its poor water-solubility and irritation to the urinary system. Thus, development of new drug delivery systems aimed to enhance solubility, decrease side-effects and improve treatment efficacy of NCTD would be a priority. At present, many new formulations of NCTD, such as microspheres (Wang et al. 2006), microemulsions (Zhang et al. 2005a), and nanoparticles (Qin et al. 2010), are being studied to improve the targeted delivery of NCTD so as to reduce the adverse reactions of normal tissue.

Poly (ethylene glycol)-poly (caprolactone), an amphiphilic block copolymer biomaterial, composed of a hydrophilic chain (PEG) and a hydrophobic chain (PCL), has attracted much attention owing to its biocompatibility and degradability. It has been widely investigated as a excellent pharmaceutical carrier in the form of microspheres (Gao et al. 2011), nanoparticles (Li et al. 2009) and niosomes (Discher et al. 1999). In this paper, PEG-PCL was used to encapsulate NCTD by self-assembly in aqueous media into copolymer micelles with a spherical core-shell structure (Fig. 2). Because of this particular core-shell structure, polymer micelles have been considered as important for enhancing drug stability *in vitro* and *in vivo*, increasing drug solubility and improving transport properties of pharmaceutical molecules (Kataoka et al.1993). Polymer micelles also demonstrate other attractive properties as drug delivery systems, such as good biocompatibility, increasing drug bioavailability, minimizing undesirable side-effects and targeting tumor areas, because polymer micelles of small size are able to accumulate spon-

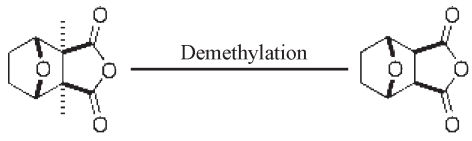

Fig. 1: Chemical structure of cantharidin and norcantharidin

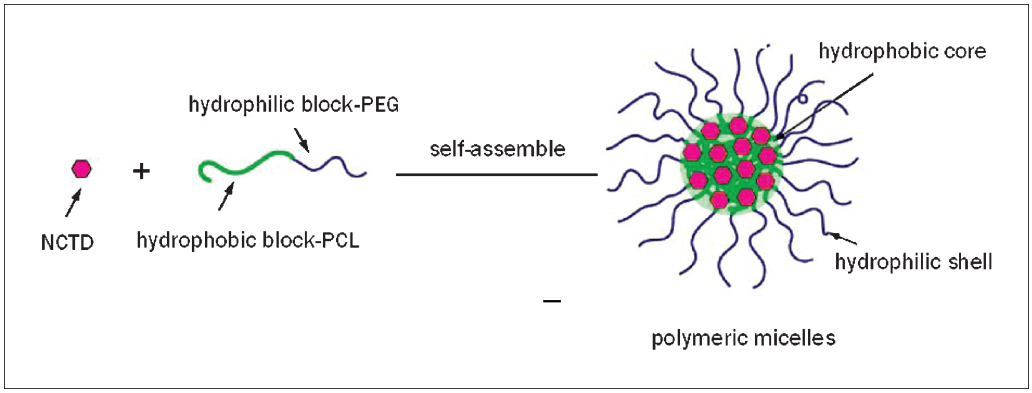

Fig. 2: In aqueous media, amphiphilic block copolymer (PEG-PCL) composed of a hydrophilic domain (PEG) and a hydrophobic domain (PCL) self-assembled into polymeric micelles, containing a hydrophobic core and hydrophilic shell. Because of the special stucture of the micelles, the hydrophobic drug (NCTD) is likely to be loaded in the hydrophobic core

taneously in pathological areas, due to the damaged ("leaky") vasculature of these areas, via enhanced permeability and retention (EPR) effects (Maeda et al. 2003). At the present time, many drugs with poor solubility or high toxicity have been incorporated into micelles, such as paclitaxel (Kim et al. 1999; Kim et al. 2001; Cai et al. 2007), amphotericin B (Yang et al. 2008) and so on.

The aim of this study was to investigate the possibility of producing NCTD-loaded PEG-PCL diblock copolymer micelles. We intended to confirm the formation of micelles by transmission electron microscopy (TEM) and verify NCTD loading in the hydrophobic core by IR. The critical micellization concentration (CMC) of PEG-PCL was determined to elucidate the anti-dilution of micelles. In addition, the anti-cancer effects of polymer micelles were explored both *in vitro* and *in vivo* to illustrate its potential value in clinical applications.

2. Investigations, results and discussion

2.1. Determination of critical micellization concentration (CMC)

CMC, originally defined as the principal thermodynamic parameter for surfactant micelles, is also used as an apparent measurement to characterize the stability of polymeric micelles. Pyrene, often used to determine CMC, can solubilize rapidly into the hydrophobic core of PEG-PCL micelles because of its strongly hydrophobic character, and its fluorescence intensity changes as the concentration of the polymer changes. When the concentration of PEG-PCL was lower than the CMC, the fluorescence intensity was very weak because no micelles were formed, but once polymer concentration was beyond the CMC, pyrene underwent a polarity shift and its fluorescence intensity increased sharply. Thus, a graph having two linear segments with different slopes could be drawn. Fig. 3 shows the ratio of pyrene fluorescence intensity at 338 nm and 335 nm vs. the logarithm of the copolymer concentration. The intersection point of these two segments gave a CMC values of 0.3×10^{-6}mol·L^{-1}. A low CMC is very important for maintaining the stability of micelles both *in vitro* and *in vivo* (Lukyanov et al. 2002). CMC values of the order of 10^{-6} demonstrated that PEG-PCL formed stable micelles that could keep their structure intact upon dilution with body fluids, thus ensuring drug transport within the bloodstream to specific biological target sites. Adequate physical stability is of prime importance to achieve successful drug delivery.

2.2. Size and morphology of copolymer micelles

Figure 4 presents the effective diameters and distribution of drug-loaded micelles and blank micelles. As shown in the figure,

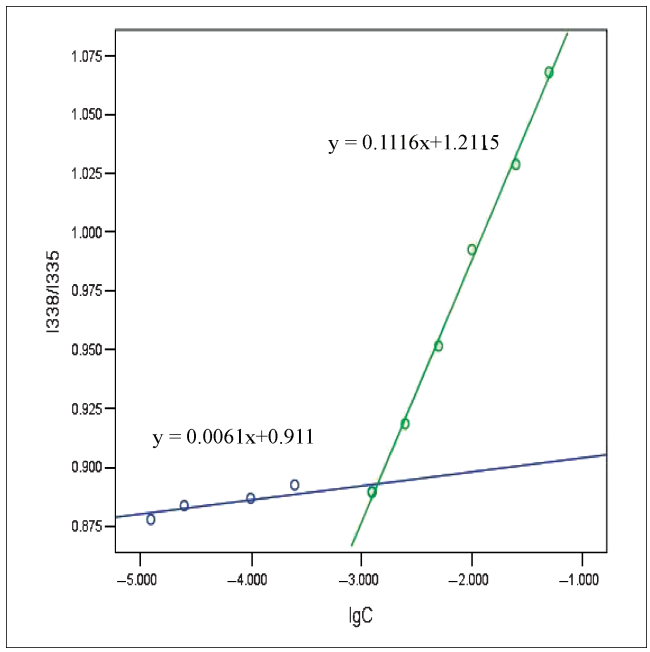

Fig. 3: Plot of ratio of pyrene fluorescence intensity (I338 nm/I335 nm) vs. logarithm of PEG-PCL copolymer concentration

they had a unimodal size distribution and the mean diameters were 87.1 ± 0.2 nm and 126.7 ± 0.5 nm, respectively. We found that drug-loaded micelles had a smaller size than micelles without drug under the same preparation conditions. This conclusion was contrary to results by other researchers that showed drug-loaded micelles to have a slightly increased particle size compared with the blank micelles (Huh et al. 2008; Yang et al. 2009). This phenomenon may be explained by some theories

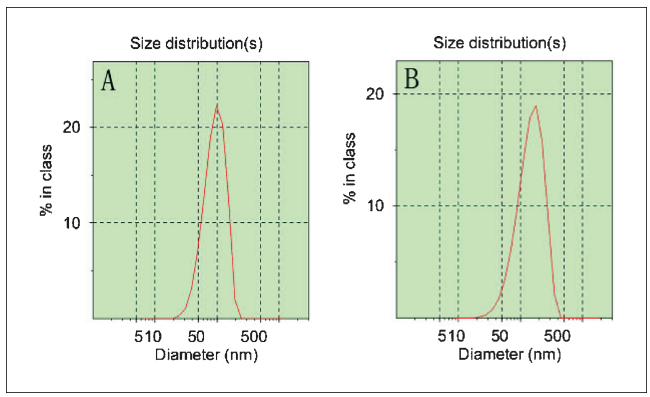

Fig. 4: Particle size and distribution of NCTD-loaded micelles (A) and blank micelles (B) determined by dynamic light scattering (DLS)

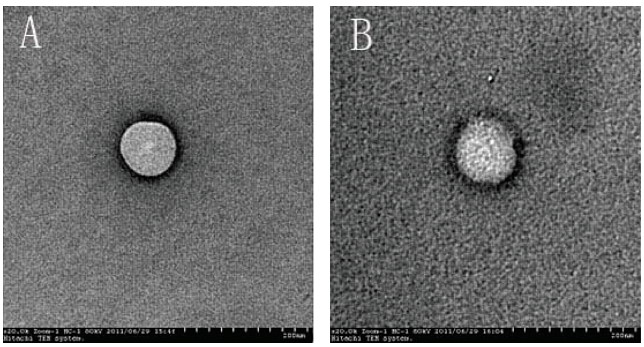

Fig. 5: Transmission electron micrographs of PEG-PCL diblock copolymer micelles loaded with NCTD (A) and without NCTD (B)

of intermolecular forces. Once NCTD entered the hydrophobic core of the micelles, it was covalently combined to polycaprolactone under the action of Van der Waal's force or electrostatic attraction, so that the space in the micelle core was smaller than in blank micelles.

The structures of the micelles were examined using TEM. There were no significant differences between drug-loaded micelles and drug-free micelles except the particle size. TEM microphotographs are presented in Fig. 5, showing the spherical shape and shell-core structure of the micelles. The inner hydrophobic core with a light color is clearly distinguished from the outer hydrophilic dark ring that had absorbed phosphotungstic acid (Wu and Luan 2007). The diameters of micelles estimated from TEM micrographs were approximately 90–100 nm for drug-loaded micelles and 120–130 nm for blank micelles, in good agreement with the results obtained from DLS. The TEM results showed that the core-shell structure had been completely formed during the formation of the micelles.

2.3. *Fourier transform–infrared analysis*

FTIR was utilized to determine the structures of blank micelles, NCTD-loaded micelles and a mixture of NCTD and PEG-PCL. As shown in Fig. 6, NCTD-loaded micelles had different bands and signals compared with the mixture but had the same as the blank micelles. The three had the same characteristic absorption peaks at 2890 to 2950 cm^{-1}, indicating the presence of C-H stretching vibration, at 1724 cm^{-1} from a strong C=O vibration in the repeated -C(O)CH2CH2CH2CH2CH2O- of the polycaprol actone backbone and at 1419 $cm^{-1,}$ attributed to the marked C-O-C in the repeated -OCH2CH2- of polyethylene glycol. All of the above absorptions appertained to the IR spectrum characteristic of PEG-PCL (Liu et al. 2006). The relevant literature (Wang et al. 2009) states that NCTD has remarkably strong carbon-hydrogen bond, carbonyl and epoxy group absorptions; however, in Fig. 6 (C), only carbon-hydrogen bonds at 3484 cm^{-1} could be observed. We conjecture that the characteristic carbonyl and epoxy group absorptions of NCTD were overlapped by those of PEG-PCL that had stronger absorptions in the same groups. What attracted our attention was that none of the characteristic absorptions of NCTD could be seen in Fig. 6(B). From the above facts, the conclusions are clearly that: (1) NCTD was completely incorporated in the micelles so that it could not be detected by IR. (2) IR spectra also confirmed that any free drug not loaded in the micelles had been removed completely. It was confirmed that FTIR was an excellent method to control the purity of polymer micelles.

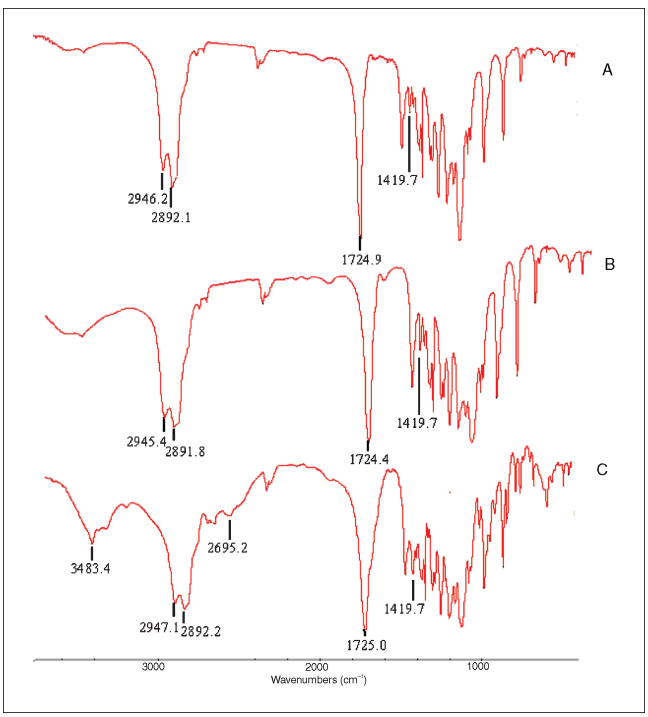

Fig. 6: Fourier transform–infrared spectra of blank micelles (A), NCTD-loaded micelles (B) and mixture of NCTD and PEG-PCL (C).

2.4. *Effects of formulation variables on the size, LC and EE of micelles*

As nanoparticles, the size of micelles plays an important role in determining their *in vitro* properties as well as their fate after administration *in vivo*. Within a certain size range, a smaller particle is more prone to accumulate in the tumor areas due to ERP, which enhances the efficiency and low toxicity of the drug. Drug loading content (LC) and encapsulation efficiency (EE) are also considered to be important parameters for evaluating the loading capability of the drug and the quality of preparation, respectively. LC and EE were calculated as follows:

$$LC = \frac{\text{mass of NCTD encapsulated in micelles}}{\text{mass of PEG-PCL copolymeri c micelles}} \quad (1)$$

$$EE = \frac{\text{mass of NCTD encapsulated in micelles}}{\text{mass of initially added NCTD}} \quad (2)$$

The three factors (preparative method, type of organic solvent and weight ratio of NCTD to polymer in the feed) had a notable influence on the diameter, LC and EE, as summarized in Table 1. Though solvent volatilization has been described as a good method yielding small particle size and an adequate loading efficiency (Blanco et al. 2007), it proved extremely unsatisfactory in this paper. Not only did the product have the largest size (up to 307.7 ± 3.4 nm), but also no advantage was shown in LC and EE. Compared with volatilization dialysis, dialysis was observed to give a marked improvement in NCTD loading, with an LC of 5.5 ± 0.1%, and an EE of 37.6 ± 0.4%, but with a larger diameter of 205.2 ± 3.7 nm. The size of micelles was significantly affected by the organic solvent, showing a great range from 77.3 ± 0.5 to 333.1 ± 0.9 nm. However, there appeared to be little difference in LC and EE with the different organic solvents. When acetone and dehydrated ethanol were used to dissolve the polymer and drug, the micelles showed the smallest and largest sizes, respectively. These facts suggest that the preparation method was not the only determining factor for micelle diameter, the type of organic solvent also having a dramatic influence. It is well accepted that the feed weight ratio of drug and polymer plays a critical role in LC and EE (Dong and Feng 2004; Paul et al. 1998; Govender et al. 1999). It was found that when keeping the mass of polymer constant (320 mg), with an increase in theoretical drug content from 10 to 320 mg, LC increased from 2.1 ± 0.1% to 7.5 ± 0.1%, but the

Table 1: Effects of formulation variables (preparation method, organic solvent and feed weight ratio of NCTD to polymer) on size, LC and EE of NCTD-loaded PEG-PCL polymeric micelles (mean ± SD, n = 3)

Fabrication variables	Size ± SD (nm)	LC ± SD (%)	EE ± SD (%)
Method			
Dialysis	205.2 ± 3.7	5.5 ± 0.1	37.6 ± 0.4
Volatilization dialysis	105.3 ± 1.5	3.4 ± 0.2	29.3 ± 0.7
Solvent volatilization	307.7 ± 3.4	3.1 ± 0.1	26.9 ± 0.7
Organic solvent			
THF	126.6 ± 1.1	3.6 ± 0.1	27.8 ± 0.8
DMF	135.4 ± 0.8	5.8 ± 0.1	26.5 ± 0.5
Acetone	77.3 ± 0.5	5.7 ± 0.1	31.7 ± 0.4
DMSO	268.2 ± 0.8	3.3 ± 0.1	27.7 ± 0.5
Ethanol	333.1 ± 0.9	4.3 ± 0.1	25.8 ± 0.3
Feed weight ratio (drug/polymer)			
1:1	78.1 ± 0.9	7.5 ± 0.1	7.2 ± 0.1
0.5:1	79.5 ± 0.6	6.5 ± 0.1	15.0 ± 0.4
0.25:1	85.9 ± 1.2	5.9 ± 0.1	25.0 ± 0.3
0.125:1	80.7 ± 0.2	5.5 ± 0.1	34.0 ± 0.5
0.0625:1	77.3 ± 0.8	5.6 ± 0.1	48.0 ± 0.5
0.05:1	79.2 ± 0.4	3.3 ± 0.2	52.0 ± 1.3
0.03125:1	76.1 ± 0.6	2.1 ± 0.1	90.0 ± 0.3

EE declined sharply from 90.0 ± 0.3% to 7.2 ± 0.1%. It seemed that the higher the feed weight ratio, the higher was the LC but the lower was the EE. We might explain this phenomenon using the following theory: during the formation of micelles, addition of more drug only increases the denominator, most of the drug being wasted due to the polymer's limited capacity of encapsulating the specific drug, so that EE decreased correspondingly. Despite this, increasing the quantity of drug could slightly facilitate drug loading, so that LC tends to rise. It was concluded that micelle size was significantly influenced by preparation method and organic solvent but there was no significant effect of the ratio of drug to polymer; however, LC and EE were greatly affected by the ratio but only slightly affected by the preparation method and organic solvent.

2.5. *In vitro drug release properties*

The drug release mechanism could involve drug diffusion, polymer matrix swelling, erosion or degradation. Fig. 7 presents the release profiles of NCTD from conjugate micelles at different pH values. Continuous release and similar parabolic release profiles can be observed. Micelles in PBS at pH = 6.5 and 7.0 exhibited a significantly faster release rate than that at pH = 7.4. Furthermore, the percentage of NCTD released from the micelles increased as the pH decreased from 7.4 to 6.5. After oscillation for 72 h, the amounts of NCTD released in buffers of pH 6.5, 7.0 and 7.4 were 83.4 ± 2.5%, 80.0 ± 1.6% and 72.0 ± 1.5%, respectively. It is interesting that *in vitro* drug release did not show initial burst release but strongly depended on the pH of the PBS. This phenomenon could be probably attributed to the pH sensitivity of the cleavage of the ester bonds which link PEG-PCL and NCTD (Xie et al. 2007). It has been reported in the literature (Yamagata et al. 1998) that pH in tumor tissue (pH = 6.5 to 7.0) is less than in normal tissue (pH = 7.4) because more glucose is metabolized to lactic acid in tumor cells. That is to say, the special release properties of the micelles could promote drug release and increase the drug concentration in tumor tissue, thereby enhancing anti-cancer effects and decreasing the side effects with a lower drug concentration in normal tissue.

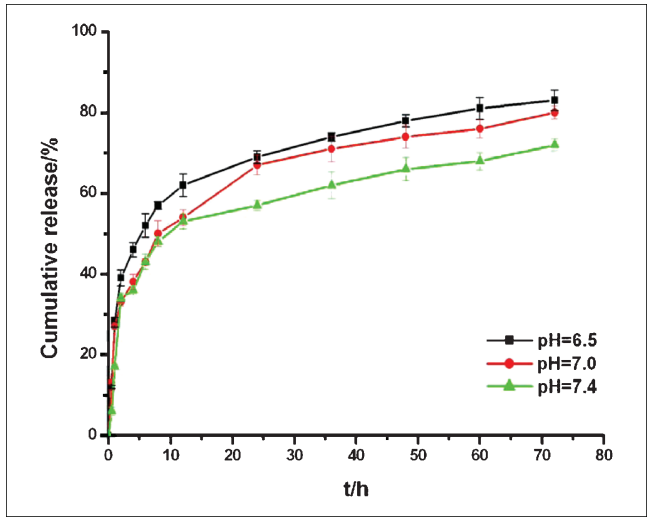

Fig. 7: NCTD release from NCTD-loaded PEG-PCL micelles in 37 °C PBS at different pH values of 6.5, 7.0 and 7.4, respectively. Data are mean ± SD, n = 3

2.6. *In vitro anti-tumor activity*

The cytotoxicity of free NCTD and drug-loaded micelles to both HepG2 cells and A549 cells were compared comprehensively. Figs. 8 and 9 show cell viability at a series of drug concentrations at different incubation times. At the same time, NCTD concentrations resulting in killing 50% of cells (IC_{50}) determined from concentration–dependent cell viability curves are shown in Table 2. As shown in Fig. 8 and Fig. 9, cell growth inhibitory effects depended on drug concentration and incubation time. With increasing drug concentration, a higher cytotoxic activity was found, and an enhancement in cytotoxicity with increasing time of incubation was also observed for both free NCTD and copolymer micelles. Similar conclusions have been reported in previous papers (Zhang et al. 2005b; Fonseca et al. 2002). As expected, with the same incubation time and drug concentration, the copolymer micelles showed stronger inhibition of cell growth than did the free drug, especially for A549 cells. IC_{50} values from Table 2 make the same point. The table shows the IC_{50} of free NCTD and copolymer micelles at different incubation times. After 72 h incubation, IC_{50} values with micelles were 22.13 μg/ml and 11.54 μg/ml for HepG2 cells and A549 cells respectively, which were 1.2- and 2.3-fold lower than those with free NCTD. Polymer micelles could efficiently deliver the drug into the tumor cells by diffusion, membrane fusion or endocytosis, leading to a high drug concentration in the tumor cells. Thus, greater cytotoxicity could be seen with micelles. It is interesting to note that the enhancing effect of micelles was stronger with A549 cells than with HepG2 cells, suggesting that A549 cells were more sensitive to copolymer micelles than HepG2 cells.

Table 2: IC_{50} values (μg/ml) of free NCTD and micelles for HepG2 cells and A549 cells after incubation for 24 h, 48 h, 72 h

Tumor cell line	24 h		48 h		72 h	
	NCTD	Micelles	NCTD	Micelles	NCTD	Micelles
HepG2	359.55	311.27	34.44	26.37	26.00	22.13
A549	291.19	67.69	32.58	14.55	27.27	11.54

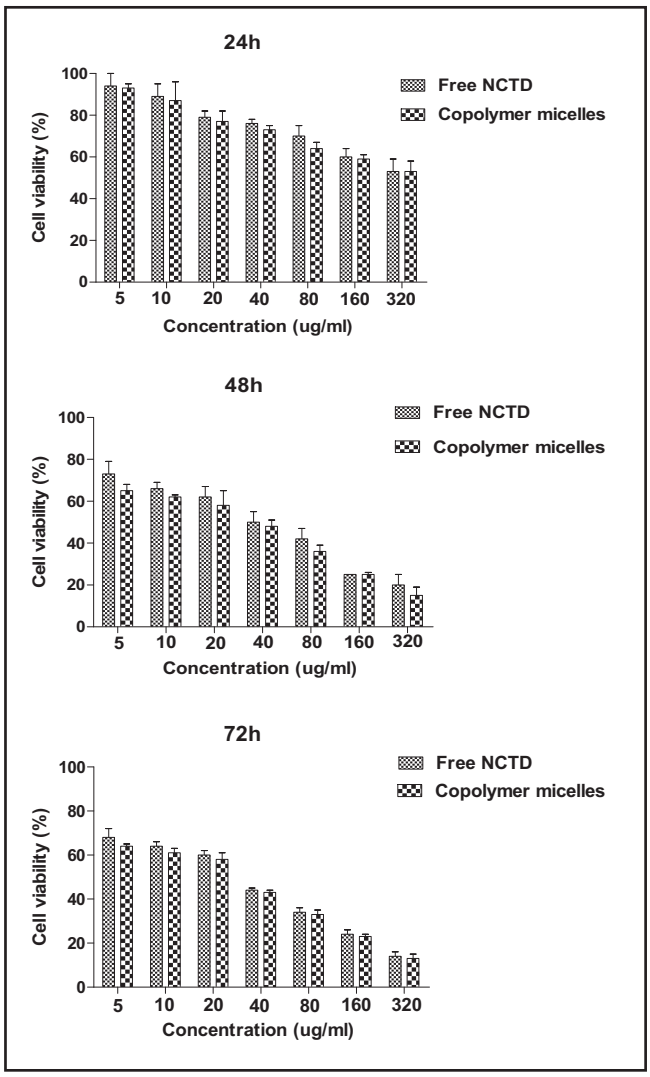

Fig. 8: Cell viability of free NCTD and copolymer micelles to HepG2 cells with different concentrations (5, 10, 20, 40, 80, 160, 320 μg/ml) for 24 h, 48 h, 72 h incubation, respectively, data are mean ± SD, n = 3

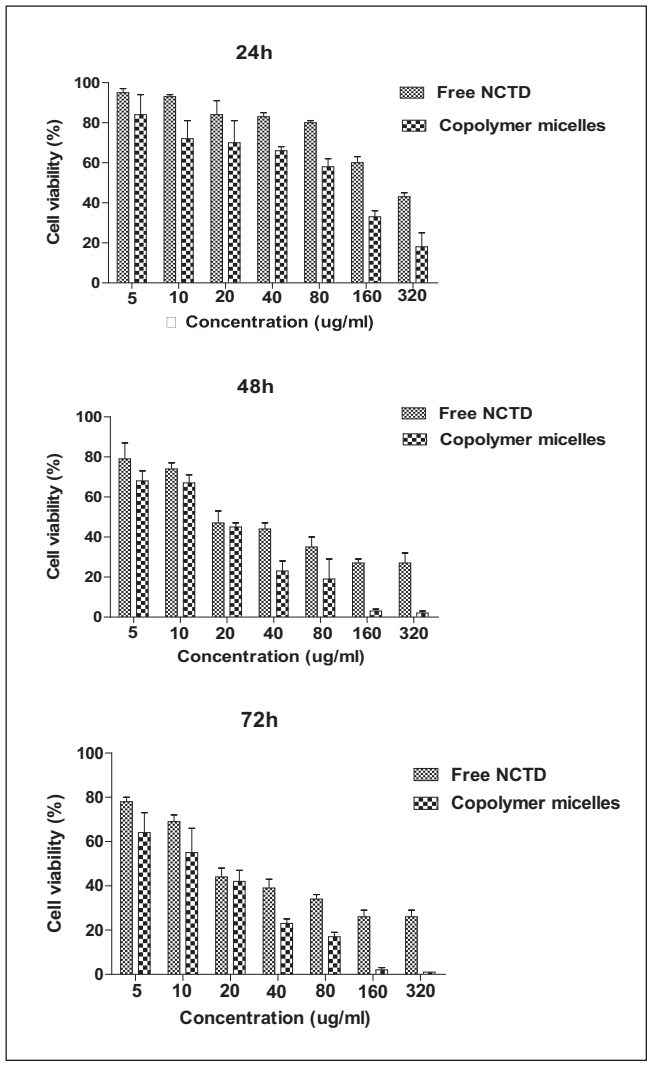

Fig. 9: Cell viability of free NCTD and copolymeric micelles to A549 cells with different concentrations (5, 10, 20, 40, 80, 160, 320 μg/ml) for 24 h, 48 h, 72 h incubation, respectively, data are mean ± SD, n = 3

After only 48 h incubation, the cell viability of A549 cells at 160 μg/ml was close to 0% for the copolymer micelles group, but as much as approximately 40% for the free NCTD group, as can be seen clearly in Fig. 9. Fig. 8 implies that the growth inhibitory effects on HepG2 were unsatisfactory. In spite of 72 h incubation and a high concentration, the cell viability was still approximately 20%.

2.7. In vivo antitumor efficacy

The *in vivo* antitumor efficacy of drug-loaded micelles was evaluated by measuring the growth of tumor volume after administration and the weight of tumor which had been stripped. Fig. 10 shows the trend in tumor growth with one day intervals. It is apparent that there were notable differences among the control group and experimental groups according tumor weight (Table 3). The body weights of mice before administration and after being killed, and the inhibition rates are also given in Table 3. A striking antitumor response was observed in all mice which received the drug. All the experimental material effectively confirmed that free NCTD gave better tumor inhibition than physiological saline, but a worse tumor inhibition effect compared with NCTD-loaded micelles. Micelles with high dose exhibited superior antitumor activity, and the IRw reached 77.63%. Tumor suppression by NCTD-loaded micelles increased in a dose-dependent manner. All the mice gained weight during the experiment, although high doses resulted in less weight gain due to the toxicity of the antitumor drug. We concluded that the delivery of NCTD in micelles could improve its tumor-inhibitory activity *in vivo* because polymer micelles made NCTD accumulate in the tumor tissue through passive targeting strategies and EPR effect. This finding, based on the results above, could have important clinical applications.

3. Experimental

3.1. Animals

Six- to eight-week-old Kunming mice (18–22 g) were supplied by the Experimental Animal Center of Southern Medical University (Guangzhou, China). Mice were kept at 40% humidity at room temperature (25 °C). All the animal experiments were conducted according to the principles of the Committee on Care and Use of Laboratory Animals of Southern Medical University.

3.2. Materials

PEG_{2k}-$PCL_{2.7k}$ was provided by Ji'nan Daigang Inc. (Ji'nan, China). NCTD (100% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). The culture medium used was composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, USA) and 10% fetal bovine serum (FBS, Gibco BRL, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All reagents

Table 3: Body weight, inhibition rate (IRw) on tumor weight in S180 tumor-bearing mice

Groups	Dose (mg/kg)	Body weight (g)		Tumor weight (g)	IR_W (%)
		Start	Sacrifice		
Normal saline	—	20.7 ± 1.3	32.3 ± 3.0*	1.344 ± 0.235	—
NCTD	2.0	20.6 ± 1.0	27.7 ± 2.7*	0.705 ± 0.097	47.50
NCTD-micelles	0.5	20.4 ± 1.3	29.6 ± 2.4*	0.824 ± 0.127	38.69
	2.0	20.5 ± 1.7	27.1 ± 2.4* **	0.520 ± 0.041	61.36
.	4.0	20.6 ± 1.3	24.7 ± 2.7*	0.301 ± 0.053	77.63

* $p < .01$ vs normal saline group
** $p < .01$ vs NCTD group at 2.0 mg·kg^{-1}

for HPLC analysis were of HPLC grade. Other chemicals were of analytical grade.

3.3. Measurement of CMC

The most common method to determine the CMC of a polymer is by using a pyrene fluorescence probe (Kataoka et al. 2001; Sezgin et al. 2006; Zhang et al. 2004). Briefly, a known amount of pyrene in acetone was added to a series of blank micelles with different concentrations. The samples were placed in a water bath at 37 °C to evaporate acetone and then laid out overnight at room temperature. The next day, fluorescence excitation spectra of all samples were measured at an emission wavelength of 393 nm, and excitation wavelengths of 335 nm and 338 nm, respectively. Avoiding light was necessary throughout the process because of the light sensitivity of pyrene.

3.4. Preparation and HPLC analysis of drug-loaded micelles

3.4.1. Preparation of drug-loaded micelles

The dialysis method (Yokoyama et al. 2004) and the volatilization dialysis method were adopted to encapsulate NCTD. In the dialysis method, organic solvent in the dialysis membrane is gradually replaced by fresh water by osmosis, which is a slow and sustained process. In this way, a compact solid micellar core is formed progressively, and the drug is slowly and effectively incorporated into the micelles. Not only is dialysis simple to perform, but it is also a purification process, during which organic solvent and NCTD not incorporated in micelles is removed. In the volatilization dialysis method, a mixed solution of drug and copolymer was dropped at the rate of 10 s/drop into distilled water in a round-bottomed flask with magnetic stirring. After 30 min, stirring was stopped, and the solution was put in a dialysis bag (MW cutoff = 2000 Da), and then dialyzed against distilled water regularly renewed by fresh water for 24 h. Compared with the dialysis method, the steps of dropping and stirring were omitted. Centrifugation was needed to ensure the removal of copolymer which did not form micelles. Finally, a suitable amount of cryoprotectant–trehalose (Di Tommaso et al. 2010) was added before freeze-drying with an FD-1-50 freeze dryer (Beijing, China).

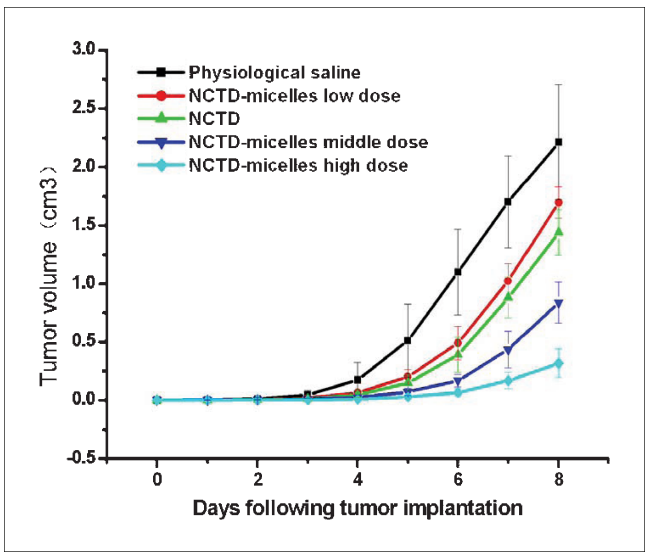

Fig. 10: Tumor growth in S180 tumor-bearing mice at different dosages of physiological saline, NCTD, NCTD-micelles low dose, NCTD-micelles middle dose and NCTD-micelles high dose, data are mean ± SD, n = 6.

3.4.2. HPLC analyses

The quantification of NCTD entrapped in PEG-PCL micelles was with a Shimadzu LC-20A high-performance liquid chromatography (HLPC) system (Shima dzu, Japan) with a reverse-phase C_{18}, 5.0 μm column (4.6 × 150 mm) (ECOSIL, Japan) and a UV detector (SPD-20A, Shimadzu). A 20 μl sample was injected into the HLPC system after the drug had been released from micelles by ultrasonication. The column was eluted with methyl alcohol:potassium dihydrogen phosphate solution (30:70), adjusted to pH = 3.0 using phosphoric acid to separate NCTD from the diblock components at a flow rate of 0.6 ml/min with a detection wavelength of 213 nm. The standard curve was established in the range from 2 to 64 μg/ml with good linear correlation (r = 0.9999).

3.5. Determination of micelle size and observation of morphology

3.5.1. Size determination

The size and size distribution of the micelles were measured by dynamic light scattering (DLS) using a Zetasizer 3000 Hs/IHPL (Malvern Instruments, UK). Analysis was carried out at a scattering angle of 90° and at a temperature of 25 °C. Before manipulation, the sample was appropriately diluted with ultra-purified water and filtered through a 0.45-μm membrane.

3.5.2. Observation of micelle morphology

The shape of the micelles was observed by transmission electron microscopy (TEM) using an H-7650 instrument (Hitachi, Japan). The sample was dripped on a copper grid coated with film and then negatively stained with 0.2% phosphotungstic acid before observation.

3.6. Verification of drug encapsulated in the core of micelles

To confirm that NCTD was actually loaded in the micelles rather than being a physical mixture, infrared spectrometry (IR) using a Thermo Nicolet 6700 FT-IR (Thermo Nicolet, USA) was used to confirm the structures of blank micelles, drug-loaded micelles and a physical mixture. The pre-processing of samples was very important. After the samples were dried thoroughly, KBr was added and the mixture was pressed into thin discs.

3.7. In vitro release of NCTD from micelles

A given solution of micelles in a dialysis bag was placed in a tube containing a fixed amount of phosphate buffer solution (pH 6.5, 7.0, 7.4) with 1% w/v sodium lauryl sulfate (SDS). PBS was used to simulate the internal biological environment, and the use of SDS was to increase the solubility of NCTD in the buffer solution. The tube was placed in an orbital shaker (Spx-100b-D, Shanghai Boxun Holdings Co., Ltd., China) at the rate of 100 r/min in a water bath at 37 °C. At scheduled time intervals, a sample of PBS was taken and replaced by fresh PBS solution. After that, tube was put back in the shaker to continue measurement. NCTD assay was again by HPLC.

3.8. In vitro cytotoxicity tests

In vitro anticancer effects were determined using the MTT ([3-(4,5-dimethyl)-2,5-diphenyl-2*H*-tetrazolium bromide]) assay (Fonseca et al. 2002; Zhang and Feng 2006). Human hepatoma (HepG2) cell line and human lung cancer (A549) cell line (supplied by Clinical Medical Experimental Research Center of Nanfang Hospital, Guangzhou, China) were chosen as target cells to evaluate the antitumor activity of drug-loaded micelles. These two cells were cultured with DMEM cell culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in an

incubator at 37 °C with an atmosphere containing 5% CO_2. When the cells had grown in good condition, the cell density was adjusted to 5×10^4/ml, and 100 μl of cell suspension were added to each well of a 96-well plate (Corning Costar, USA). After culturing in an incubator for 24 h, administration was performed. There were 3 groups: (1) control group: only test cells were added; (2) NCTD group: NCTD was dissolved in DMSO and diluted 100-fold with PBS buffer solution, the final NCTD concentrations being 5, 10, 20, 40, 80, 160 and 320 μg/ml; (3) NCTD-loaded micelles group: the freeze-dried sample was dissolved in PBS, the final NCTD concentrations being 5, 10, 20, 40, 80, 160 and 320 μg/ml. After 24 h, 48 h and 72 h incubation, respectively, 20 μl of MTT solution (5 mg/ml) was added to each well of the plates. The incubation was continued for another 4 h, during which formazan was precipitated (reaction of MTT and living cells). 150 μl DMSO was added to dissolve the formazan precipitate. Finaly, the optical density (OD) of the solution was measured under a microplate reader (SpectraMax M5, USA) at 570 nm. The growth viability was computed as follows:

$$\text{Cell growth viability} = \text{OD}_{\text{test cell}}/\text{OD}_{\text{control cell}} \times 100\% \quad (3)$$

3.9. *In vivo antineoplastic activity*

In vivo antitumor efficacy of NCTD-loaded micelles was investigated in mice bearing S180 mouse sarcoma cells (supplied by Clinical Medical Experimental Research Center of Nanfang Hospital, Guangzhou, China). In order to maintain good viability of the S180 cells, the cells were injected into mice via intraperitoneal implantation for three generations. In the third generation, the ascites were withdrawn and centrifuged. The viable cells at the bottom of the tube were diluted with physiological saline to modulate the cell density to 1×10^7/ml. The tumor cell suspension was injected subcutaneously into the right hind limb of mice (0.1 ml/10 g). After inoculation, the mice were assigned at random to different experimental groups, including a control group (physiological saline) and medication administration groups (NCTD, NCTD-loaded micelles at low dose, NCTD-loaded micelles at moderate dose and NCTD-loaded micelles at high dose). 24 h later, the mice were given an intravenous injection to the tail of the various formulations for 8 days continuously, and tumor size was measured daily with a vernier caliper in two dimensions. Tumor volumes (V) were calculated. by the formula:

$$V = [\text{length} \times (\text{width})^2]/2. \quad (4)$$

On the ninth day, the subcutaneous tumor blocks were stripped and weighed after killing the mice by cervical dislocation. The inhibition rate on tumor weight (IRw) was calculated as follows:

$$IRw = (1 - Wm/Wc) \times 100\%$$

where Wm and Wc are the tumor weight of the drug-administered and control groups, respectively.

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