

Study on docetaxel-loaded nanoparticles with high antitumor efficacy against malignant melanoma

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Docetaxel (Doc) has extraordinary activities against a variety of solid tumors. However, the clinical efficacy of Doc is limited due to its poor solubility, low selective distribution, fast elimination *in vivo*, etc. In the present study, Doc was incorporated into the core-shell structure of nanoparticles prepared based on our previous work. The obtained docetaxel-loaded nanoparticles (DOCNP) were characterized with various biophysical methodologies, and its antitumor efficacy against malignant melanoma was evaluated both *in vitro* and *in vivo*. Our results indicated that Doc could be incorporated into the nanoparticles with high encapsulation efficiency (>90%). The incorporated Doc can be released from DOCNP in a sustained manner. *In vitro* cytotoxicity studies indicated that DOCNP could effectively kill B16 cells and show a dose- and time-dependent efficacy. Furthermore, intratumoral administration revealed that DOCNP has significantly higher antitumor effect and lower toxicity to normal cells and tissues than free Doc. These results suggest that DOCNP may be a promising drug delivery system in therapy for malignant melanoma.

Keywords docetaxel; nanoparticle; melanoma; controlled release

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Introduction

Melanoma, a malignancy of melanocytes mainly found in the skin, causes the greatest number of skin

cancer-related deaths worldwide [1]. Owing to the low response to conventional chemotherapy, the prognosis of metastases melanoma is very poor [2]. Docetaxel (Doc), which is a mitotic spindle poison that accelerates the microtubule assembly from tubulin and blocks the depolymerization of the microtubule [3], has demonstrated extraordinary anticancer effects both *in vitro* and *in vivo* against a variety of tumors, including lung, ovaries, breast, leukemia, malignant melanoma, etc. [4–6]. However, the clinical application of Doc is limited due to several problems. For instance, the poor solubility of Doc in aqueous solution requires a specific solvent system, such as ethanolic solution containing polysorbate 80, to facilitate its clinical use. However, this solvent system may elicit hypersensitivity reactions that necessitate premedication [7]. Another problem is the non-specific distribution throughout the body, which contributes to drug-related side effects, such as neurotoxicity, musculoskeletal toxicity, and neutropenia [8]. Thus, a novel formulation of Doc with less toxicity and better targeting to tumor sites is desirable in clinical application.

Recent developments in drug delivery systems provide promising ways to maximize the localization of the drug toward the tumor while minimizing systemic toxicity [9]. Latest progress in polymer science has greatly improved the efficiency of drug carriers in encapsulating and delivering chemotherapeutics [10,11], among which the amphiphilic block copolymers have attracted the most interest [12,13]. Amphiphilic block copolymers, composed of both a hydrophilic segment and a hydrophobic segment,

are capable to self-assemble into nanoscale spherical structures with a hydrophilic outer shell and a hydrophobic inner core [14,15]. For such a structure, lipophilic drugs can be incorporated into the hydrophobic core without destroying the hydrophilic outer shell which is able to stabilize the system. In most studies, polyethylene glycol (PEG) is usually used as the outer shell due to its hydrophilicity, biocompatibility, low toxicity, negligible antigenicity, and immunogenicity [16,17]. Moreover, the core-shell structure with PEG as an outer shell enables the nanoparticles to escape from the scavenging of the reticuloendothelial systems (RES) effectively after systemic administration [18]. Previous reports have also demonstrated that drug-loaded polymeric nanoparticles preferentially accumulate in tumors due to the enhanced permeability and retention effect (also called passive targeting), and exhibit significantly lower systemic toxicity when compared with conventional drug formulation [19,20]. Therefore, drug delivery systems based on the amphiphilic block copolymers are promising candidates for anticancer drug delivery. However, most previous studies were mainly concentrated on developing nano-delivery systems for paclitaxel, and few published reports were focused on the study of Doc-loaded nanoparticles.

In most previous studies, intravenous administration is the main delivery route in the *in vivo* evaluation of drug-loaded nanoparticles for the reason that intravenous administration is the most commonly used method in clinic. However, melanoma is mostly found in skin. Therefore, it is difficult for systemic delivery of chemotherapeutics to achieve ideal effective concentration in tumor site. To solve this problem, intratumoral administration receives most attraction because of its targeted delivery to tumor. As reported in our previous study, cisplatin was used as a model drug incorporated into nanoparticles formed by amphiphilic copolymers, demonstrating exciting *in vivo* anticancer efficacy against subcutaneous xenografts of murine hepatoma [21]. Compared with free cisplatin, cisplatin-loaded nanoparticles are characterized by the controlled release of incorporated cisplatin, which may overcome the deficiency of cisplatin retention in the tumor and may reach a satisfying outcome in improving antitumor efficacy when combined with intratumoral delivery instead of intravenous route [21].

On the basis of this research, the present paper reported a nano-delivery system for Doc. Polycaprolactone (PCL) was chosen as the hydrophobic segment of the block copolymer because of its good drug encapsulation ability. Doc was used as the model drug. The prepared docetaxel-loaded nanoparticles (DOCNP) were characterized by

dynamic light scattering (DLS), transmission electron microscopy (TEM), and atomic force microscopy (AFM). Coumarin-6 was used as a fluorescent marker to assess the uptake efficiency of nanoparticle by murine malignant melanoma B16 cells. To assess the potential anticancer efficacy of the nanoparticulate system, B16 cell line was used to test its *in vitro* cytotoxicity. Meanwhile, the *in vivo* antitumor efficiency of DOCNP was evaluated by intratumoral delivery in the B16 transplant model. Our results indicated that nanoparticle-based delivery system of Doc greatly enhanced Doc antitumor efficiency both *in vitro* and *in vivo* with relatively lower toxicities to normal tissues.

Materials and Methods

Materials

Doc was kindly provided by Jiangsu Hengrui Pharmaceutical Co. Ltd (Lianyungang, China). Methoxy-polyethyleneglycol (mPEG) was purchased from Sigma (St Louis, MO, USA). All PEG samples were dehydrated by azeotropic distillation with toluene, and then vacuum-dried at 50°C for 12 h before use. ϵ -Caprolactone (ϵ -CL, Sigma) was purified by drying over CaH₂ at room temperature and distillation under reduced pressure. Stannous octoate (Sigma) was used as the catalyst. All other chemicals were of analytical grade and used without further purification. Mouse malignant melanoma B16 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China).

Male 6–8-week-old ICR mice, weighing between 18 and 20 g, were from the Animal Center of Nanjing Medical University. The mice were housed and maintained in the animal facility of the Animal Center of Nanjing Medical University. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Synthesis of mPEG and PCL block copolymers

mPEG-PCL block copolymers were synthesized by a ring opening copolymerization as previously described [21]. Briefly, CL and mPEG at a feeding ratio of 5:1 was added into a polymerization tube with the presence of 0.1% w/w stannous octoate as a catalyst. The tube was then connected to a vacuum system, sealed off, and placed in an oil bath at 130°C for 48 h. At the end of the polymerization, the crude copolymers were dissolved in dichloromethane and precipitated with excessive amount of cold methanol to remove the unreacted monomer and oligomer. The precipitates were then filtered and washed

with water several times before thoroughly dried at reduced pressure.

Preparation of docetaxel-loaded nanoparticles

Doc-loaded nanoparticles were prepared by a nanoprecipitation method as previously described with minor modification [21]. Briefly, 10 mg of mPEG-PCL block copolymers and a predetermined amount of Doc were dissolved in acetone. The obtained organic solution was added dropwise into 10 times volumes of distilled water under gentle stirring at room temperature. The solution was then dialyzed in a dialysis bag (molecular weight cutoff 12 kDa; Sigma) to remove acetone thoroughly. The resulted bluish aqueous solution was filtered through a 0.22 μm filter membrane to remove non-incorporated drugs and copolymer aggregates. Similarly, coumarin-6 and Doc co-loaded nanoparticles were prepared by adding Doc and coumarin-6 together at a weight ratio of 10:1 in the corresponding experimental step. Drug-free nanoparticles were produced in a similar manner without adding drugs. Solutions of drug-loaded nanoparticles and blank nanoparticles were then lyophilized for further characterization and utilization. In all experiments, DOCNP were solved in water.

Characterization of nanoparticles

Size and zeta potential analysis of the nanoparticles

Mean diameter and size distribution of the nanoparticles were measured by DLS with a Brookhaven BI-9000AT instrument (Brookhaven Instruments Corporation, Holtsville, NY, USA). Zeta potential was measured by the laser Doppler anemometry (LDA) on ZetaPlus Zeta Potential Analyzer (Brookhaven Instruments Corporation). All measurements were performed at 25°C. Calculation of the size and polydispersity indices was achieved using the software provided by the manufacturer. The diameters mean value was calculated from the measurements performed at least in triplicates.

Transmission electron microscopy and atomic force microscopy

Morphological examination of the nanoparticles was conducted with JEM-100S TEM (JEOL Ltd, Tokyo, Japan). One drop of nanoparticle suspension was placed on a copper grid covered with nitrocellulose membrane and air-dried before negative staining with phosphotungstic sodium solution (1% w/v). AFM (SPI3800, Seiko Instruments, Tokyo, Japan) was used to study the surface morphology of nanoparticles. One drop of properly diluted nanoparticle suspension was placed on the

surface of a clean silicon wafer and dried under nitrogen flow at room temperature. The AFM observation was performed with a 20 μm scanner in tapping mode.

Drug loading content and encapsulation efficiency

Doc loading content in nanoparticles was determined by HPLC according to the method of Immordino *et al.* [22] with minor modification. The concentration of Doc was assayed on a Shimadzu LC-10AD (Shimadzu, Kyoto, Japan) HPLC system equipped with a Shimadzu UV detector and an Agilent C-18, 5 μm , 200 mm \times 4.6 mm RP-HPLC analytical column. The mobile phase consists acetonitrile (spectral grade, Merck, Darmstadt, Germany)/double-distilled water (50/50, v/v) pumped at a flow rate of 1.0 ml/min with determination wavelength of 227 nm. The concentration of Doc was determined based on the peak area by reference to a calibration curve. The following equations were applied to calculate the drug loading content (DLC) and encapsulation efficiency (EE). $\text{DLC (\%)} = \text{weight of the drug in nanoparticles} / \text{weight of the nanoparticles} \times 100\%$. $\text{Encapsulation efficiency (\%)} = \text{weight of the drug in nanoparticles} / \text{weight of the feeding drugs} \times 100\%$.

In vitro release of Doc-loaded nanoparticles

In vitro release of Doc from the mPEG-PCL nanoparticles was evaluated using a dialysis bag diffusion technique as described in our previous reports with minor modification [21]. First, 10 mg of lyophilized Doc-loaded nanoparticles was suspended in 1 ml of normal saline (NS, 0.9% sterile sodium chloride). The solution was then placed into a pre-swelled dialysis bag with a 12 kDa molecular weight cutoff and immersed into NS, at 37°C with gentle agitation. The incubation medium was sampled at various time points to monitor the Doc releasing rate. After sampling, equal volume of fresh NS was immediately added back to keep the constant volume of the incubation medium. The concentration of Doc released from the nanoparticles was expressed as a percentage of the total Doc in the nanoparticles and plotted as a function of time. The concentration of released Doc was corrected for the dilution due to the addition of fresh NS.

Nanoparticle uptake by tumor cells

About 5×10^5 B16 cells were seeded in a 6-well plate with 2 ml RPMI 1640 supplemented with 10% calf serum and allowed to adhere at 37°C with 5% CO₂ for 24 h prior to the assay. The medium was then replaced with 10 ml fresh RPMI 1640 containing Doc and

coumarin-6 co-loaded nanoparticles. The final concentration of coumarin-6 in the medium is 5 µg/ml. After 2 h incubation, the cell monolayers were rinsed three times with PBS buffer to remove excess nanoparticles. The cells were imaged with a fluorescence microscope.

***In vitro* cytotoxicity studies**

Cytotoxicity of Doc-loaded nanoparticles against B16 cells was assessed by MTT assay [23]. Briefly, cells were seeded in a 96-well plate at a density of ~5000 cells/well and allowed to adhere for 24 h prior to the assay. Cells were exposed to a series of doses of free Doc, blank nanoparticles, or Doc-loaded nanoparticles, respectively, at 37°C. After 24–72 h of incubation, 50 µl of MTT indicator dye (5 mg/ml in PBS, pH 7.4) was added to each well and the cells were incubated for another 2 h at 37°C in the dark. The medium was then removed and 200 µl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) was added to each well and agitated thoroughly to dissolve the formazan crystals. The solution was transferred to a new 96-well plate and immediately read on a microplate reader (Bio-Rad, Hercules, CA, USA). Absorption was measured at the wavelength of 490 nm with 620 nm as a reference wavelength in Microkinetics reader BT2000 (Arbin Instruments, College Station, TX, USA) and the obtained values were expressed as a percentage of the control cells to which no drugs were added. All experiments were repeated three times.

***In vivo* antitumor efficacy**

ICR mice implanted with B16 cell line were used to qualify the relative efficacy of Doc-loaded nanoparticles through intratumoral administration. The mice were raised under specific pathogen-free circumstances and all of the animal experiments were performed in full compliance with guidelines approved by the Animal Care Committee of Nanjing Medical University. The mice were subcutaneously injected at the left axillary space with 0.1 ml of cell suspension containing $4-6 \times 10^6$ B16 cells. Treatments were started after 7–8 days of implantation. The mice with tumor volume of $\sim 100 \text{ mm}^3$ were selected and this day was designated as 'Day 0'.

For intratumoral injections, animals received a single injection volume of ~ 0.2 ml drug solutions with a 21-gauge needle placed in the center of the tumor. The intratumoral injections were infused ~ 10 s, and the needle was allowed to remain in place for an additional 10–15 s and removed through another direction. On Day 0, the

mice were randomly divided into eight groups (each containing 10 mice). The mice were treated intratumorally with free Doc (Commercial Taxotere, provided by Jiangsu Hengrui Pharm. Co. Ltd), Doc-loaded nanoparticles, blank nanoparticles, and saline. Doc solution was administered at doses of 4, 8, and 16 mg/kg. Doc-loaded nanoparticles were administered as a saline solution at the equivalent Doc doses of 4, 8, and 16 mg/kg. All mice were tagged, and tumors were measured every other day with calipers during the period of study. The tumor volume was calculated by the formula $(W^2 \times L)/2$, where W is the tumor measurement at the widest point and L the tumor dimension at the longest point. Each animal was weighed at the time of treatment, so that dosages could be adjusted to achieve the mg/kg amounts reported. Animals also were weighed every other day throughout the experiments.

Statistical analysis

Results were presented as mean \pm SD. Statistical comparisons were made by *t*-test or ANOVA analysis. The accepted level of significance was $P < 0.05$.

Results

Preparation and characterization of DOCNP

Morphology, size distribution, and zeta potential detection

The molecular weight of the mPEG-PCL block copolymers was in a reasonable range as reported in our previous work [21]. The polydispersity of the copolymer (defined as the ratio of weight-average molecular weight to the number-average molecular weight) was narrow, with all < 1.65 . The number-average molecular weight and the weight-average molecular weight of mPEG4 k-PCL20 k were 18,600 and 30,480 Da, respectively.

The solubility of DOCNP in NS is up to 20 mg/ml, which is much better than that of free Doc. **Figure 1** shows that the solution of Doc-loaded nanoparticles is bluish in color, which is caused by the light scattering of the nanoparticles in the solution. The morphology images of the DOCNP obtained from AFM [**Fig. 2(A)**] and TEM [**Fig. 2(B)**] indicate that the nanoparticles, estimated less than ~ 100 nm in size, are in spherical shape with a smooth surface. DLS measurement indicated that the size of DOCNP (88 nm) is slightly larger than that of the blank nanoparticles (~ 73 nm). Meanwhile, LDA measurement of DOCNP showed that the zeta potentials exhibit a negative value (-5 mV), which is much lower than polyester nanoparticles without the PEG coating surface (-35 mV) reported by Zhang *et al.* [12]. This is

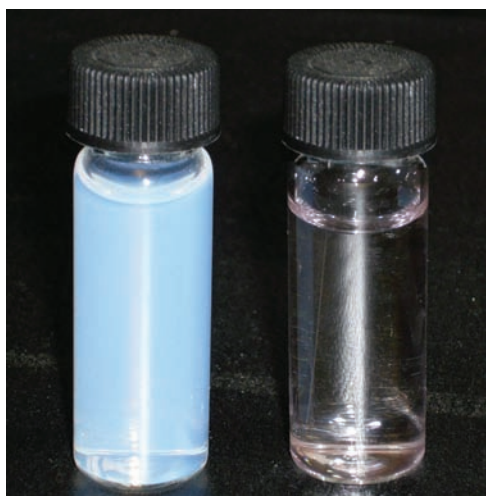


Figure 1 The solution of DOCNP in glass bottle Left: DOCNP solution (5 mg/ml) in water. Right: free water.

because the PEG outer shell is capable to lower the negative surface charge to a certain degree, resulting in a reduction of cell repulsion to the nanoparticles.

Drug loading content and encapsulation efficiency

The data in **Table 1** show the DLC and EE of DOCNP. The highest DLC of Doc in mPEG-PCL nanoparticles was $19.4 \pm 2.4\%$ when the drug/polymer feeding ratio is

at 1% (**Fig. 3**). As shown in **Fig. 3**, EE of DOCNP decreases with the increase of the feeding ratio of Doc and polymer.

In vitro release of DOCNP

Figure 4 shows the sustained release profile of DOCNP. It is obvious that free Doc released much faster than DOCNP. In the first 3 h, an initial burst of more than 70% of free Doc was released from the dialysis bag. Meanwhile, only 30% of Doc in DOCNP was released in the same period. During the next 120 h, no more than 55% of the total Doc was released from the solutions of DOCNP. The difference in release from dialysis bag for both free and loaded Doc is evidently attributed to the prolonged release function of the nanoparticle [21]. This implies that Doc in DOCNP can be released slowly and kept at a constant concentration for long period both *in vitro* and *in vivo*, which is very important for the clinical application.

Cellular uptake of nanoparticles by B16 cells

Figure 5 shows the fluorescent microscopic images of B16 cells incubated with coumarin-6 and Doc co-loaded nanoparticles. In **Fig. 5(B,D)**, the observed coumarin-6 fluorescence was mainly located inside the cells, which indicated that coumarin-6 was mainly transported into

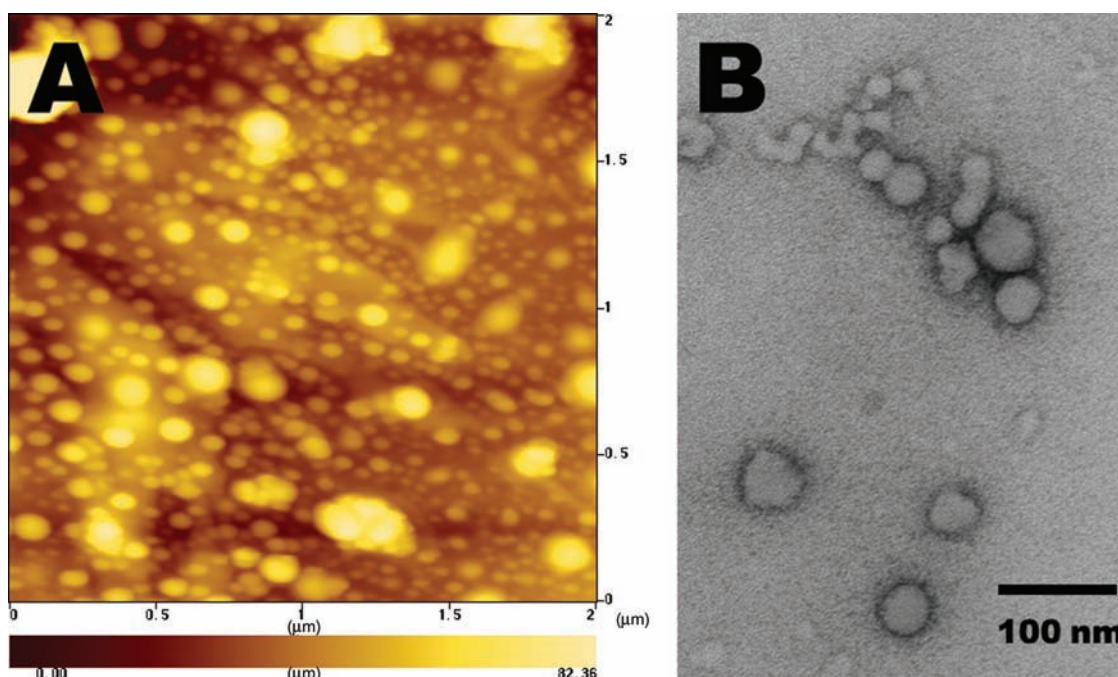


Figure 2 AFM (A) and TEM (B) images of DOCNP (A) AFM image shows that DOCNP is less than ~ 100 nm in size and is in spherical shape with a smooth surface. (B) TEM image confirmed that the diameter of DOCNP is < 100 nm with a spherical morphology as obtained from AFM image.

Table 1 Mean particle size and drug load efficiency of DOCNP

Nanoparticles	Particle size (nm) ^a	Polydispersity	Zeta potential (mV)	DLC (%)	EE (%)
Blank	78.3 ± 7.9	0.14 ± 0.04	-6.5 ± 1.4	/	/
DOCNP	87.5 ± 9.5	0.15 ± 0.03	-5.6 ± 0.7	19.4 ± 2.4	91 ± 4.6

^aThe SD value was for the mean particle size obtained from the three measurements of a single batch. DLC, drug loading content; EE, encapsulation efficiency.

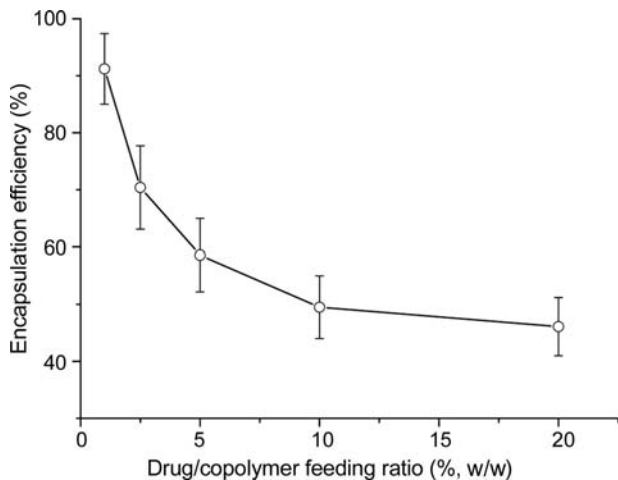


Figure 3 The dependence of DOCNP EE on drug/copolymer feeding ratio From the curve, the EE of DOCNP decreases with the increase of the feeding ratio of Doc and polymer.

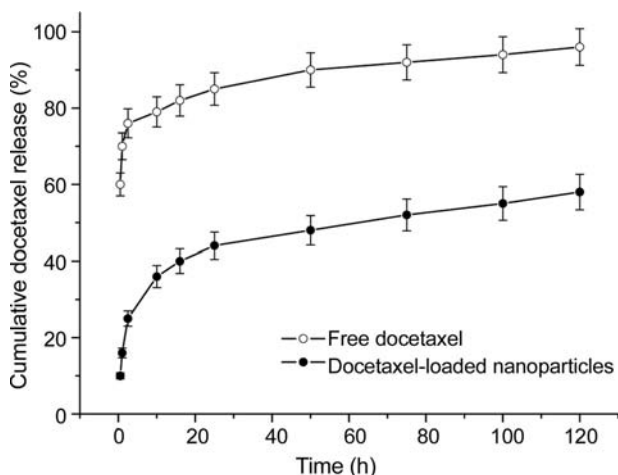


Figure 4 The time course of Doc release from Doc-loaded nanoparticles The open circle curve represents the release of free Doc; the filled circle curve represents the release of Doc from Doc-loaded nanoparticle. The amount of released free and loaded Doc was determined by HPLC as described in Materials and Methods. An initial burst of >70% of free Doc was released whereas only 30% of Doc in DOCNP was released in the same period. This featured a controlled release pattern of DOCNP *in vitro*.

the cells and localized around the nuclei in the cytoplasm. Therefore, it is reasonable to conclude that Doc, encapsulated into the nanoparticles together with coumarin-6, was also transferred into the cells by the nanoparticles as well.

In vitro cytotoxicity of DOCNP against B16 cells

Figure 6 shows the cytotoxicity of DOCNP against murine malignant melanoma cancer cell line B16. Preliminary results of our study showed that blank nanoparticles is nearly non-toxic to B16 cell with the inhibition rate <10% even at a high nanoparticle concentration of 500 µg/ml (data not shown). **Figure 6** clearly indicates that DOCNP show very similar dose- and time-dependent cytotoxicity against B16 cell with free Doc at an equivalent dose from 4 to 128 µg/ml. It can be seen that the survived B16 cells decreased with the incubation time from 24 to 72 h for both free and loaded Doc. Moreover, detailed observation of the cell viability in **Fig. 6** indicated that DOCNP could induce slightly more cell death of B16 than that of free Doc at most corresponding incubation times, implying that DOCNP show higher or, at least, the same cytotoxicity against B16 cell with free Doc at equivalent doses.

In vivo antitumor effect of DOCNP

Figure 7(A) shows changes of tumor volume by intratumoral administration in B16 tumor-bearing mice. It was found that all the tumor volumes treated with different doses of DOCNP were smaller than those treated with the same dose of free Doc ($P < 0.05$), indicating that DOCNP can more effectively inhibit tumor growth. It is also obvious that the inhibition is dose-dependent. For instance, the tumor volume increase at the 9th day was 2.6, 0.7, and 0.4 mm³ corresponding to dose 4, 8, and 16 mg/kg, respectively, for group treated with DOCNP, whereas 3.6, 2.3, and 1.5 mm³ for free Doc treated. It should be noted that the difference in tumor volumes among the groups of Doc-loaded nanoparticles and blank nanoparticles as well as saline was highly significant ($P < 0.01$). More interestingly, DOCNP at a higher

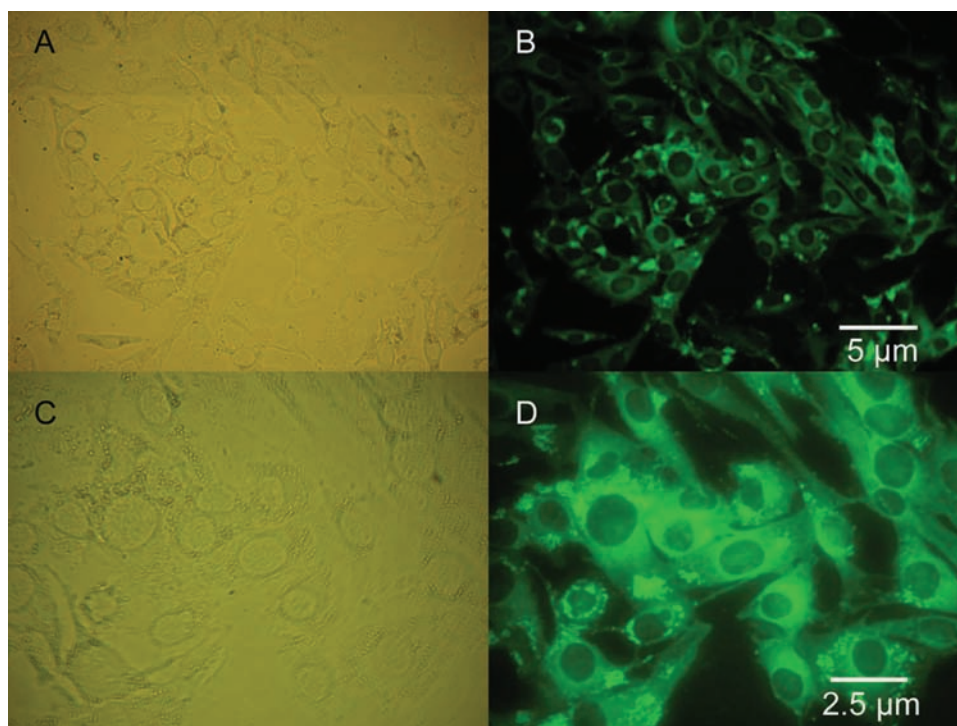


Figure 5 Fluorescent microscopic images of B16 cells incubated with coumarin-6 and Doc co-loaded nanoparticles (A) Lower power bright field of B16 cells incubated with coumarin-6 and Doc co-loaded nanoparticles. (B) The same field of (A) with fluorescence. (C) High-power bright field of B16 cells incubated with coumarin-6 and Doc co-loaded nanoparticle. (D) The same field of (C) with fluorescence.

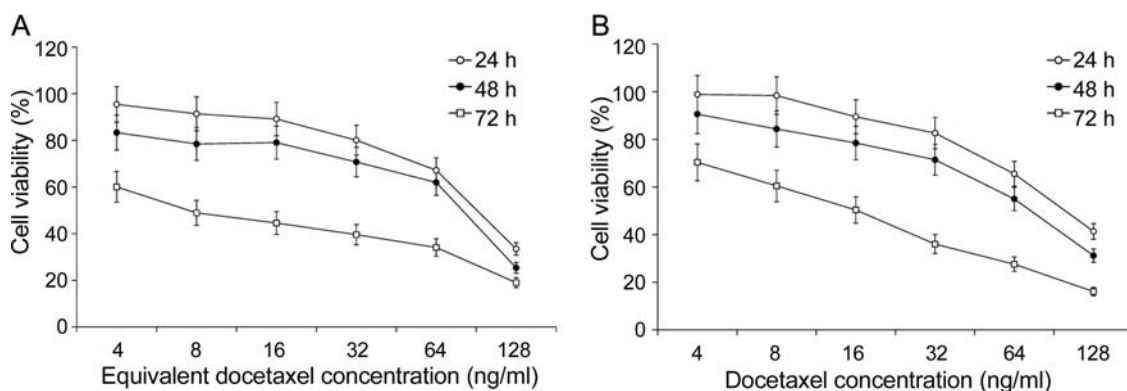


Figure 6 Cytotoxicity of DOCNP to mouse malignant melanoma B16 cells (A) B16 cell viability after 24, 48, and 72 h incubation with different doses of DOCNP. (B) B16 cell viability after 24, 48, and 72 h incubation with different doses of free Doc. All the data are presented as mean \pm SD ($n = 3$).

dose of 16 mg/kg exhibited a super antitumor efficacy than free Doc by delaying tumor growth: no growth of tumor nodules was observed in the first 4–5 days and the tumor mean volumes were no more than 500 mm³ at the end of therapy. Meanwhile, tumor nodules in the groups receiving the same dose of free Doc grew faster with a mean volume more than 2000 mm³ at the same period. **Figure 7(B)** shows the body weight variations of

mice during the intratumoral administration, the body weight of mice treated with lower DOCNP dose (4 mg/kg) had no obvious loss compared with those treated with saline and blank nanoparticles. The decrease in bodyweight was found in the higher doses of 8 and 16 mg/kg of DOCNP, but the extent of weight loss was much smaller than those induced by free Doc. For the latter, the body weight loss was ~20% even at lower

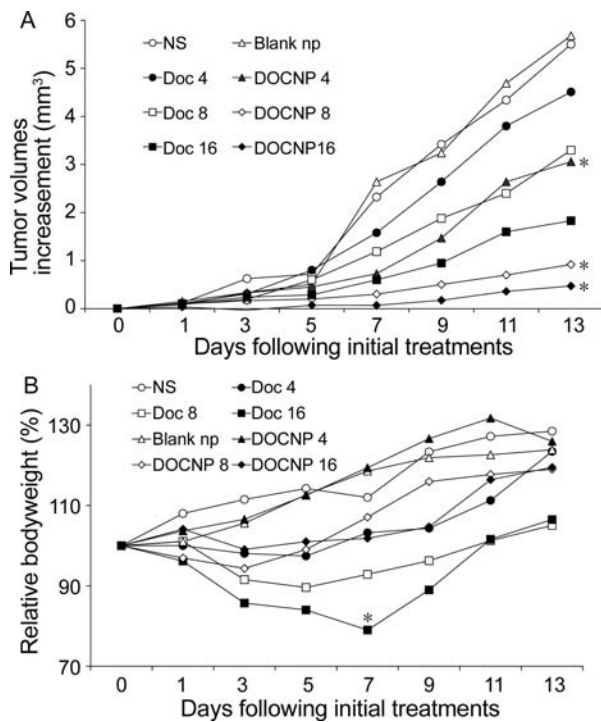


Figure 7 Variation of tumor volume (A) and body weight (B) of ICR mice established B16 xenografts during therapy with different doses of free DOC or DOCNP (A) ICR mice were treated with different protocols as described in Materials and Methods. NS, normal saline (a solution of 0.9% w/v of NaCl); blank NP, solutions of empty nanoparticles in NS; Doc 4, Doc 8, and Doc 16 represent free Doc at doses of 4, 8, and 16 mg/kg, respectively; DOCNP 4, DOCNP 8, and DOCNP 16 represent DOCNP at equivalent Doc doses of 4, 8, and 16 mg/kg, respectively. All the data are presented as mean \pm SD ($n = 10$). (B) Bodyweight change of ICR mice receiving different treatments during therapy. For better readability of the figure data points, the error bar of each point was not shown (the errors of each data point in this figure were $< 15\%$ of the value of the corresponding data point). * $P < 0.05$ versus the group receiving the equivalent dose of free Doc.

dose 4 mg/kg. As to the higher dose of free Doc, the loss was huge. The analysis of body weight variations can be used to define the adverse effects of the different therapy regimens. These results lead to a conclusion that Doc-loaded nanoparticles generate less toxicities to normal organs than free Doc when administered intratumorally. Moreover, we also observed that the mice receiving free Doc were in a weak state in the aspects of movement and spirit, whereas no obvious alteration was observed in the nanoparticle-treated animals.

Discussion

Here we reported for the first time that a spherical core-shell structure DOCNP formed by amphiphilic mPEG-PCL

block copolymers demonstrated superior antitumor efficacy against malignant melanoma both *in vitro* and *in vivo*.

DLC measurement revealed a high loading of Doc. According to a previous research, the strong hydrophobicity of polymers enables a large quantity of Doc to be trapped in the polymeric nanoparticles, which strengthens the stability of the drug delivery system [24]. *In vitro* release study further demonstrated the strong affinity between Doc and PCL. In addition, one thing needs to be clarified that the release of free DOC *in vitro* is actually the diffusion rate of free DOC through the dialysis bag and DOCNP release *in vitro* is the sum of DOC release from nanoparticles and diffusion rate of DOC through the dialysis bag. As the release data showed, Doc was released in a sustained manner from the core-shell structure of polymeric nanoparticles. Thus, the slow dissociation of Doc from the polymeric micelles to the targeted tissues also may provide a more sustained release of the drug. In addition, we demonstrated that the sustained exposure of tumor cells to low concentrations of paclitaxel were more efficacious than shorter periods of exposure to higher Doc concentrations [25]. Therefore, the sustained release of formulated Doc would be less likely to cause systemic toxicity than the continuous systemic administration of conventionally formulated Doc during similar periods of time. It has been demonstrated in other studies that the *in vivo* toxicity in the various drug delivery systems was found to be significantly lower than that of a conventional formulation of paclitaxel [26,27].

In the cytotoxicity test, DOCNP caused a little more death of B16 cells than free Doc. The results are in accordance with previous studies that cytotoxicity of drug-loaded nanoparticles was nearly the same as that of free drugs [12,15,21]. Possible mechanism underlying the slightly enhanced efficacy of DOCNP against B16 cells may include the enhanced intracellular drug accumulation by nanoparticle uptake [28]. Results from cellular uptake experiment also confirmed the high cell affinity of nanoparticles (Fig. 4). Previous reports supported the current findings not only by investigating the cellular uptake efficiency of fluorescence but also by determining the cytotoxicity of drug-loaded nanoparticles at different concentrations [29,30].

Previous studies have reported the satisfied antitumor effect of Doc delivery systems in several cancer models, such as liver cancer and colon cancer [24,31]. However, no report on the efficacy evaluation of nano formulation of Doc against malignant melanoma was identified. Here

in the *in vivo* experiments reported, we compared the *in vivo* efficacy of DOCNP with equivalent dosing with free Doc. As shown in **Fig. 7**, Doc-loaded nanoparticles delayed tumor development significantly than free Doc. Possible mechanisms underlying the superiority of DOCNP against free Doc may include the continuous exposure of tumor mass to released Doc from the nanoparticles. Therefore, the sustained release of Doc is capable to deliver its antitumor efficacy constantly. It has been reported that antitumor activity of chemotherapeutics depends on the dose and exposure time [32]. Moreover, the toxicity of DOCNP is lower than that of free Doc from the observation of mice bodyweight in the *in vivo* evaluation, which will facilitate its future clinical application.

In summary, the current study reported for the first time a spherical core-shell structure Doc-loaded nanoparticle formed by amphiphilic mPEG-PCL block copolymers. In this delivery system, Doc can be well incorporated into mPEG-PCL-based nanoparticles with high EE due to its lipophilicity. The *in vitro* release study showed a sustained and continuous release pattern of Doc-loaded nanoparticles. The *in vitro* studies proved the cytotoxicity of Doc-loaded nanoparticles in a dose- and time-dependent manner against murine malignant melanoma B16 cells. *In vivo* evaluation further demonstrated the superior anticancer efficacy of Doc-loaded nanoparticles with relatively lower side effects compared with free Doc in an established B16 transplanted mice model. It is concluded that nano formulation of Doc delivery is a promising way in countering the spread of malignant melanoma, and continuing research will definitely advance the current study. It is fully understood and appreciated that the development of nanoscale drug formation of chemotherapeutics warrants more intensive research in order to further characterize the detailed mechanism in the uptake of drug-loaded nanoparticles, the interaction between drug release and tumors, and, ultimately, the feasibility and advantages of clinical applications.

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