

RESEARCH ARTICLE

Heat-Treated Emulsions with Cross-Linking Bovine Serum Albumin Interfacial Films and Different Dextran Surfaces: Effect of Paclitaxel Delivery

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Received 7 November 2012; revised 14 January 2013; accepted 17 January 2013

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23468

ABSTRACT: In this study, a type of biocompatible and biodegradable oil-in-water emulsion for hydrophobic drug delivery was evaluated *in vitro* and *in vivo*. Bovine serum albumin (BSA)-dextran conjugates with different dextran molecular weights and different conjugation degrees were used as the emulsifier and stabilizer. Paclitaxel (PTX), a hydrophobic antitumor drug, was effectively loaded inside the oil droplets via high-pressure homogenization. The emulsions were heated at 90°C for 1 h to eliminate the anaphylaxis of BSA. By virtue of the cross-linked BSA films at the oil-water interfaces produced by the heat treatment and the hydrophilic dextran surfaces, the emulsions are stable in blood serum, as well as stable against long-term storage. *In vitro* cytotoxicity study verifies that the unloaded emulsions are biocompatible and the PTX-loaded emulsions have similar antitumor activity as PTX solution. *In vivo* investigation of murine ascites hepatoma H22-tumor-bearing mice demonstrates that the PTX-loaded emulsion with shorter and denser dextran surface has better tumor inhibition and survivability efficacy than the commercial PTX injection. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: albumin; biocompatibility; biodegradable polymers; cancer; drug delivery; emulsion/microemulsion; nanotechnology; polysaccharide; stability; surfactants

INTRODUCTION

About 40% of the new chemical entities discovered by the pharmaceutical industry are hydrophobic compounds, and parenteral delivery of the hydrophobic drugs is a challenging task.^{1,2} For example, paclitaxel (PTX) is an effective antitumor drug and microtubule antiproliferative agent, but its efficacy is greatly limited by its extremely poor solubility in an aqueous solution³ and poor absorption via the oral route.⁴ To enhance its solubility and allow parenteral administration, PTX is currently formulated in a vehicle composed of polyethoxylated castor oil and alcohol.⁵ Another marketed PTX formulation is PTX-human serum albumin (HSA) nanoparticles, produced by oil-in-water emulsion in which HSA acts as an emulsi-

fier, then removing the solvents and redispersion in water.^{6,7} PTX-HSA nanoparticles show a better antitumor activity and less toxicity.

Recently, emulsion has attracted much attention for hydrophobic drug delivery because of the improvement in efficacy of the therapeutic agents.^{8–10} Besides HSA, many other proteins, such as bovine serum albumin (BSA), can also act as emulsifiers for the reason that they have a strong tendency to adsorb at oil-water interface to lower the surface tension.^{2,11,12} BSA has been widely used in drug delivery studies because of its abundance, low cost, ease of purification, and unusual ligand-binding properties.¹³ For example, Han et al.² used BSA as an emulsifier to produce emulsion. By dissolving drug and gelator into the oil phase, the oil droplets have a structure of BSA shell and drug-loaded gel core, which endows the droplets with a temperature-controlled release property. BSA-dextran conjugate, produced via a naturally occurring Maillard reaction between the ϵ -amino group in BSA and the reducing-end

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Journal of Pharmaceutical Sciences

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carbonyl group in dextran, can also be used as an emulsifier,^{14,15} the oil droplets have a structure of BSA film at the oil–water interface and a dextran shell on the droplet surface. The dextran shell can change the surface charges and increase the thickness of the interfacial layers, enhancing the hydrophilicity and steric repulsion of the oil droplets. Furthermore, introducing dextran on the droplet surface can endow the droplets with a “stealth” property^{16,17} that can avoid reticuloendothelial recognition and subsequent elimination of the droplets *in vivo*. These advantages make us think that BSA–dextran emulsion is a good system for the delivery of hydrophobic drugs. Recently, we found that BSA–dextran emulsion can effectively load PTX. Unexpectedly, we also found that this PTX-loaded BSA–dextran emulsion can cause acute death of murine ascites hepatoma H22-tumor-bearing mice after the administration via tail vein. The acute death may be caused by BSA–dextran conjugate, not by PTX, because no acute death was found for the commercial PTX injection. However, in our previous study, we have used BSA–dextran conjugate and doxorubicin hydrochloride to produce doxorubicin-loaded nanoparticles via a pH-adjusting and a heat-treatment process.¹⁸ For the doxorubicin-loaded nanoparticles, no acute death happened during the treatments of H22-tumor-bearing mice. The different results between the emulsion and the heat-treated nanoparticle solution indicate that the acute death is caused by the BSA. As a foreign protein, BSA may cause anaphylaxis in mice, whereas the heat treatment can induce BSA denaturation¹⁹ that may eliminate the anaphylaxis.

In order to demonstrate that the heat treatment is an effective method to eliminate BSA anaphylaxis and BSA–dextran emulsion is a universal vehicle for hydrophobic drugs, in this study, we fabricated thermostable PTX-loaded oil-in-water emulsions, in which BSA–dextran conjugates with different dextran molecular weights and different conjugation degrees were used as the emulsifier and stabilizer. The antitumor effects of the heated emulsions were evaluated *in vitro* and *in vivo*. The results confirm our supposition.

MATERIALS AND METHODS

Materials

BSA (fraction V, 99%) and dextran (molecular weight 10 kDa) were supplied by Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China). Dextran (molecular weight 62 kDa) was from Amersham Biosciences AB (Uppsala, Sweden). Soybean oil for injection was from Jiangxi Golden Crabapple Medicinal Oil Company Ltd. (Yushan, China). PTX was from Jiangsu Yew Pharmaceutical Company Ltd. (Wuxi, China).

Fluorescein isothiocyanate (FITC) was from Tokyo Chemical Industry Company Ltd. (TCI, Shanghai, China). Human oral squamous carcinoma KB cell line was from American Type Culture Collection (ATCC, Manassas, Virginia). Dulbecco's modified Eagle's medium (DMEM) cell culture medium and fetal bovine serum were from GIBCO BRL Life Technologies Inc. (Shanghai, China). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was from Promega Company (Beijing, China). Commercial PTX injection prepared from polyethoxylated castor oil and alcohol was from Harbin Pharmaceutical Group Company Ltd. (5 mL, 30 mg PTX; Harbin, China). Male ICR mice were from Sino-British Sippr/BK Lab. Animal Ltd. (Shanghai, China). All other reagents were of analytical grade. All solutions were prepared by deionized water, which was filtered by 0.45 µm film to remove dust and bacteria.

Preparation of BSA–Dextran Conjugates

BSA–dextran conjugates were prepared by Maillard reaction as reported previously.^{20,21} In this study, 10 and 62 kDa dextran molecules were used to prepare BSA–dextran conjugates. Briefly, BSA and dextran were dissolved together in water to reach molar ratio (MR) of BSA to dextran 1:1–1:7. The solution was adjusted to pH 8.0 with 0.5 mol/L NaOH and then lyophilized. The lyophilized power reacted at 60°C and 79% relative humidity for 48 h. The resultant BSA–dextran conjugates were kept at –20°C before use.

Conjugation Degree Analysis of BSA–Dextran Conjugates

For the conjugates prepared from 10 kDa dextran, the free dextran in the conjugate solution was separated by high-flow ultrafiltration membrane (cutoff molecular weight = 100 kDa; Microcon YM-100; Millipore Corp. Billerica, MA) and was collected in ultrafiltrate. The dextran concentration in the ultrafiltrate was analyzed by gel permeation chromatography (1515 GPC; Waters Corp., Milford, MA) to calculate the conjugation degree. Considering the noncovalent interactions between BSA and dextran as well as the membrane adsorption, a mixture of BSA and dextran with the same MR, the same concentration, and the same ultrafiltration process was used as a standard to calibrate the dextran concentration in the ultrafiltrate.

Preparation of PTX-Loaded Emulsions

BSA–dextran conjugates were used to produce emulsions without purification. For the purpose of simplifying the description, in this paper, only BSA concentrations were presented to denote the conjugate concentrations.

PTX was dissolved/dispersed in soybean oil to reach a concentration of 10–50 mg/mL by ultrasonication. BSA–dextran conjugate aqueous solution of pH 8.0 containing 10 mg/mL BSA (10 mg/mL BSA and additional dextran) was mixed with 20% volume fraction of oil phase. The mixture was pre-emulsified with a homogenizer (FJ200-S; Shanghai Specimen Model Company, Shanghai, China) at 10,000 rpm for 1 min, and then was immediately emulsified by a high-pressure homogenizer (AH100D; ATS Engineering Inc., Shanghai, China) at 800 bar for 4 min. The emulsion was heated at 90°C for 1 h if there is no specific indication. The heated emulsion was stored at 4°C to investigate the long-term stability.

Encapsulation Efficiency Analysis of PTX-Loaded Emulsions

Unloaded PTX in the emulsion was extracted by chloroform and then was analyzed on a high-performance liquid chromatography (HPLC) system (2545 system; Waters Corp., Milford, MA). A standard PTX chloroform solution was used in HPLC analysis to calibrate the extracted PTX concentration. PTX loading efficiency and loading capacity in the emulsion were calculated using following equations:

$$\text{Loading efficiency (\%, w/w)} = \frac{\text{Paclitaxel in feed} - \text{unloaded paclitaxel}}{\text{Paclitaxel in feed}} \times 100\%$$

$$\text{Loading capacity (\%, w/w)} = \frac{\text{Paclitaxel in feed} - \text{unloaded paclitaxel}}{\text{Conjugate in feed}} \times 100\%$$

Characterizations of PTX-Loaded Emulsions

Dynamic light scattering (DLS) measurements were carried out at 25°C on a laser light scattering instrument (Autosizer 4700; Malvern Instruments, Worcs, UK) at 90° scattering angle. The emulsion was diluted to BSA concentration of 0.01 mg/mL for the DLS measurement. Generally, a DLS sample was prepared by taking 4 µL original emulsion from the top layer and then diluting to 3.2 mL with the solution containing the same pH and ionic strength. For fresh produced emulsion, the DLS measurement was performed within the same day or next day, and no special shake was performed. For the emulsion after storage, the sample was shaken and then diluted for the DLS measurement. Every emulsion was measured only once before storage and was measured again after different time of storage. The DLS measurement was performed at the condition of refractive index 1.333 for water and 1.472 for oil, equilibrium time 2 min, duration time 4 min, and slit of 35 nm. The apparent

z-average hydrodynamic diameter (D_h) and polydispersity index (PDI, μ_2 / Γ^2) were obtained by CONTIN mode analysis.^{22,23} At least two batches of the emulsions were produced at the same condition and were measured to assess reproducibility; the average data and deviation were reported.

ζ-Potential measurements were performed at 25°C on a ZetaSizer Nano ZS90 instrument (Malvern Instruments). Electrophoresis mobility was measured and ζ-potential was calculated by the Dispersion Technology Software provided by Malvern according to the Henry equation.²⁴ ζ-Potential sample was prepared by adjusting the emulsion to desired pH value and then diluting the emulsion to BSA concentration of 0.01 mg/mL with the same pH aqueous solution containing 5 mmol/L NaCl. Each sample was measured three times, and the average data were reported.

Transmission electron microscopy (TEM) observations were conducted on a Philips CM 120 electron microscope (FEI Company, Hillsboro, Oregon). TEM sample was prepared by depositing diluted emulsion onto a carbon-coated copper grid and drying in a desiccator containing dried silica gel for 72 h.

Cellular Internalization of FITC-Labeled Droplets

BSA–dextran conjugate was labeled with fluorescence probe FITC as reported in the literature.²⁵ Unreacted FITC was removed by dialysis against 10 mmol/L pH 7.4 phosphate buffer. FITC-labeled emulsion was prepared from 5 mL oil and 20 mL aqueous solution containing FITC-labeled conjugate in which BSA concentration was 10 mg/mL.

To track the internalization, KB cells were seeded in special plates for confocal laser scanning microscopy (CLSM) and incubated overnight at 37°C with complete culture medium (DMEM cell culture medium supplemented with 10 vol % fetal bovine serum and 100 IU/mL penicillin G sodium and 100 µg/mL streptomycin sulfate). Subsequently, the cells were incubated with 1.5 mL/well medium containing FITC-labeled emulsion. After incubation for desired time, the cells were washed with PBS (10 mmol/L pH 7.4 phosphate buffer containing 0.15 mol/L NaCl) for three times and then observed on a CLSM (Leica TSC SP5; Leica Microsystems, Wetzlar, Germany) with fixed laser intensity.

To quantify the cellular uptake of the droplets, the cells were seeded in six-well plates and incubated with 2 mL/well medium containing FITC-labeled emulsion for 1 h. After incubation, the cells were detached and transferred to centrifugal tubes. Subsequently, the cells were centrifugated and resuspended in PBS twice. Finally, the cells were analyzed on a flow cytometer (BD FACS Calibur; BD Biosciences, Franklin Lakes, NJ). Ten thousand cells were counted for each sample.

***In Vitro* Cytotoxicity of PTX-Loaded Emulsions**

Cell viability against free PTX solution and PTX-loaded emulsion was investigated by MTS assay.^{3,26} KB cells were seeded in 24-well plates and incubated with complete culture medium. After 24 h incubation, the cells were treated with 0.5 mL/well of fresh media containing equivalent blank emulsion, PTX-loaded emulsion, and free PTX solution with different PTX concentrations in triplicate. The free PTX stock solution was prepared by dissolving PTX in dimethyl sulfoxide (DMSO) to reach PTX concentration 2 mg/mL. The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration 2 mg/mL. After 72 h incubation, the cell media were replaced by 120 μ L fresh medium containing 16.7 v% MTS and the cells were incubated for another 2 h. Then, the optical density of the plates was read on a BioTek microplate reader.

For the time-dependent cell viability, the cells were treated with the PTX solution and emulsion containing 0.01 μ g/mL PTX for designed time, and then MTS assay was performed.

***In Vivo* Antitumor Effect of PTX-Loaded Emulsions**

All the animal experiments of this report were performed at National Pharmaceutical Engineering Research Center in full compliance with the guidelines approved by Shanghai Administration of Experimental Animals. The commercial PTX injection and the PTX-loaded emulsions were diluted to 1 mg/mL freshly before the administration with sterile physiological saline injection. The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL. Filtration sterilization was performed for the diluted injection and emulsions by 0.22 μ m film.

Survivability

Male ICR mice (18–22 g) were injected in abdomen with 2.4×10^6 murine ascites hepatoma H22 cells. The mice were randomly assigned to various treatment groups with 10 in each group. The treatments started after 4 days of the inoculation. Physiological saline, PTX injection, blank emulsion, and PTX-loaded emulsions were intravenously injected via the tail veins, separately, with PTX doses of 10 and 16 mg/kg every week for 3 weeks. The survivability rate (SR) was calculated according to the following equation: $SR (\%) = (T/C - 1) \times 100\%$, where T is the average survivable time of the PTX-treated group, and C is the average survivable time of the physiological saline-treated group.

Tumor Inhibition

Male ICR mice were subcutaneously injected in right axilla with 3×10^6 H22 cells. The mice were randomly assigned to various treatment groups with 10 in each group. The treatments started after 3 days

of the inoculation. Physiological saline, PTX injection, blank emulsion, and PTX-loaded emulsions were intravenously injected via the tail veins, separately, with PTX doses of 10 and 16 mg/kg every day for 6 days. After 12 days of the inoculation, all mice were sacrificed by cervical dislocation. The solid tumors were taken out and weighed. The tumor inhibition rate (TIR) was calculated according to the following equation: $TIR (\%) = (1 - WT/WC) \times 100\%$, where WT is the average tumor weight of the PTX-treated group, and WC is the average tumor weight of the physiological saline-treated group.

RESULTS AND DISCUSSION

Preparation of BSA–Dextran Emulsions

It was reported that irreversible thermo-aggregation happens for BSA aqueous solution at a concentration of 10 mg/mL when the temperature is higher than 55°C.¹⁹ In this study, the BSA–dextran emulsions were heated at 90°C for 1 h to cause the denaturation and aggregation of the BSA molecules adsorbed at the oil–water interface. The thermo-stability of the emulsions produced from the conjugates with different MR (MR of BSA to dextran) and different molecular weights of dextran (10 and 62 kDa) was investigated by DLS. Table 1 shows that the emulsions produced from the conjugates BD1-62K (62 kDa dextran with MR 1:1), BD6-10K (10 kDa dextran with MR 1:6), and BD7-10K (10 kDa dextran with MR 1:7) do not change their droplet sizes after the heat treatment, whereas the other emulsions with less dextran weight ratio increase their droplet sizes. The unchanged droplet sizes can be explained by the fact that there are enough dextran chains on the droplet surface to prevent the droplets from coalescence and flocculation^{27,28} during the heat treatment. According to the thermo-stability shown in Table 1, BD1-62K and BD6-10K conjugates were used to prepare PTX-loaded emulsions. We chose BD6-10K instead of BD7-10K, which has smaller droplet size, because BD6-10K and BD1-62K have similar weight ratios of BSA to dextran (1:0.90 and 1:0.93, Table 1) that may facilitate the comparison between 10 and 62 kDa dextran. In the following study, all the emulsions were heated at 90°C for 1 h immediately after emulsification.

The stability of the emulsions in physiological condition, pH 7.4 and 0.15 mol/L ionic strength, was investigated by changing the emulsion produced at pH 8.0 to pH 7.4 with 0.5 mol/L HCl and adding 3 mol/L NaCl to reach 0.15 mol/L final concentration. Creaming and whey layers appeared for the emulsion produced from BSA (Table 2). This phenomenon can be explained by the fact that the salt screens the charges of BSA on the droplet surfaces and therefore induces the coalescence and flocculation of the droplets.²⁹

Table 1. DLS Results of the Emulsions Produced from BSA and BSA–Dextran Conjugates with Different MR and Different Molecular Weight (M_w) of Dextran at pH 8.0 Before and After the Heat Treatment at 90°C for 1 h

Sample	Dextran (M_w , kDa)	BSA:Dextran (mol:mol)	BSA:Dextran (w:w)	Before Heat Treatment		After Heat Treatment	
				D_h (nm)	PDI	D_h (nm)	PDI
BSA	—	—	—	346 ± 21	0.17 ± 0.06	377 ± 18*	0.17 ± 0.07
BD1-62K	62	1:1	1:0.93	215 ± 10	0.09 ± 0.01	219 ± 12*	0.10 ± 0.01
BD2-10K	10	1:2	1:0.30	266 ± 17	0.19 ± 0.07	1295 ± 21**	1.0
BD3-10K		1:3	1:0.45	223 ± 6	0.17 ± 0.01	535 ± 106*	0.53 ± 0.16
BD4-10K		1:4	1:0.60	219 ± 3	0.12 ± 0.02	425 ± 239*	0.45 ± 0.25
BD5-10K		1:5	1:0.75	218 ± 8	0.13 ± 0.01	266 ± 44*	0.18 ± 0.01
BD6-10K		1:6	1:0.90	204 ± 6	0.13 ± 0.04	201 ± 5*	0.14 ± 0.02
BD7-10K		1:7	1:1.05	190 ± 1	0.12 ± 0.01	190 ± 6*	0.13 ± 0.01

* $p > 0.05$ compared with the same sample before heat treatment.** $p < 0.0001$ compared with the same sample before heat treatment.

Creaming also happened for the BSA emulsion at pH 5.0, at which the net charges of BSA are close to zero.³⁰ For the BD1-62K and BD6-10K emulsions, the droplet sizes do not change in the media of pH 7.4 with 0.15 mol/L NaCl and pH 5.0. After 3 months of storage at pH 7.4 medium, the BD1-62K and BD6-10K emulsions are homogeneous, BD1-62K emulsion does not change the droplet size, whereas BD6-10K emulsion decreases the droplet size significantly; creaming and whey layers appeared in the BSA emulsion during the storage. The results shown in Table 2 further confirm that the hydrophilic dextran chains on the droplet surfaces provide an effective steric barrier against droplet coalescence and flocculation and thus greatly increase the stability of the emulsions.

For the BD1-62K conjugate, the conjugation degree is 44.6% as reported in our previous study.²⁰ That is to say, about 44.6% of the BSA molecules are conjugated with dextran, in which each BSA molecule has a dextran chain of 62 kDa on average. For the BD6-10K conjugate, about 73.1% of the dextran chains are conjugated to BSA, analyzed by gel permeation chromatography after ultrafiltration separation in this study. That is to say, each BSA molecule has about 4.4 dextran chains of 10 kDa on average. The droplet sizes of the BD1-62K and BD6-10K emulsions shown in Table 1 are 219 and 201 nm, respectively. Compared with the BD1-62K emulsion, much more 10 kDa dextran chains are needed on the droplet surface to stabilize the BD6-10K droplets in an aqueous phase.

In other words, the dextran chains on the BD6-10K droplet surface are much shorter and much denser compared with the dextran chains on the BD1-62K droplet surface.

Encapsulation of PTX in BSA–Dextran Emulsions

Soybean oil was used as a solvent to dissolve/disperse PTX. PTX-loaded emulsions produced from BD1-62K and BD6-10K conjugates were denoted as PTX/BD1-62K and PTX/BD6-10K, respectively. It was reported that PTX is stable and maintains its activity when the heating temperature is lower than 263°C;³¹ therefore, the heat treatment of the emulsions at 90°C cannot cause the loaded PTX to reduce its activity.

The unloaded PTX in the emulsion was extracted by chloroform and analyzed by HPLC. Table 3 shows that more than 95% of the PTX was encapsulated into the droplets when the PTX concentration in the emulsions was in the range of 2–10 mg/mL. The PTX/BD6-10K emulsion increases the droplet size from 191 to 282 nm when the PTX concentration increases from 2 to 10 mg/mL. However, after 2 months of storage, these sizes decrease to 193–199 nm and the PDI values also decrease. Similarly, the BD6-10K emulsion also decreases the droplet size significantly after storage (Table 2). The decrease of the droplet sizes is not caused by the break of the droplets for the reason that the unloaded PTX in the emulsions does not increase after the storage as shown in Table 3. It is possible that some droplets in the top layer of the emulsion

Table 2. DLS Results of the Emulsions After the Changes of the pH and Salt Concentration in the Media as well as Long-Term Storage

Sample	pH 7.4		pH 5.0		pH 7.4 + 0.15 mol/L NaCl		After 3 Months of Storage at pH 7.4	
	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI
BSA	319 ± 21	0.18 ± 0.02			Creaming			
BD1-62K	227 ± 2	0.13 ± 0.01	234 ± 2*	0.14 ± 0.03	228 ± 5*	0.12 ± 0.02	214 ± 11*	0.14 ± 0.01
BD6-10K	203 ± 3	0.13 ± 0.01	209 ± 6*	0.15 ± 0.03	193 ± 1*	0.15 ± 0.05	168 ± 4**	0.14 ± 0.01

The emulsions were produced from BSA, BD1-62K, and BD6-10K at pH 8.0.

* $p > 0.05$ compared with the same sample at pH 7.4.** $p < 0.005$ compared with the same sample at pH 7.4.

Table 3. Results of DLS, Loading Efficiency, and Loading Capacity of PTX-Loaded Emulsions

Sample	PTX Concentration in the Emulsion (mg/mL)	D_h (nm)	PDI	Loading Efficiency (%)	Loading Capacity (%)
PTX/BD1-62K (fresh prepared)	2	211 ± 9	0.11 ± 0.01	100	13.0
	4	203 ± 8*	0.13 ± 0.02		
	6	212 ± 6*	0.12 ± 0.01		
	8	211 ± 10*	0.11 ± 0.01		
	10	203 ± 7*	0.16 ± 0.03		
PTX/BD6-10K (fresh prepared)	2	191 ± 3	0.11 ± 0.01	99.4	13.1
	4	197 ± 16**	0.16 ± 0.03		
	6	223 ± 11**	0.25 ± 0.02		
	8	214 ± 7**	0.24 ± 0.01		
	10	282 ± 45***	0.39 ± 0.05		
PTX/BD6-10K (after 2 months of storage)	2	199 ± 6	0.11 ± 0.02	95.4 ± 2.7	12.6 ± 0.4
	4	193 ± 3****	0.15 ± 0.03		
	6	194 ± 12****	0.15 ± 0.05		
	8	196 ± 17****	0.18 ± 0.04		
	10	198 ± 19****	0.22 ± 0.01		

The emulsions were produced from BD1-62K and BD6-10K at pH 8.0 with different PTX concentrations.

* $p > 0.05$ compared with the fresh PTX/BD1-62K emulsion containing 2 mg/mL PTX.

** $p > 0.05$ compared with the fresh PTX/BD6-10K emulsion containing 2 mg/mL PTX.

*** $p < 0.01$ compared with the fresh PTX/BD6-10K emulsion containing 2 mg/mL PTX.

**** $p > 0.05$ compared with the PTX/BD6-10K emulsion containing 2 mg/mL PTX after 2 months of storage.

flocculated during the heat treatment and the flocculated droplets are dispersible during the storage. For the PTX/BD1-62K emulsion, the droplet size does not increase with the PTX concentration (Table 3) and the BD1-62K emulsion does not decrease the droplet size significantly after 3 months of storage (Table 2). These results suggest that the longer dextran chains on the droplet surface are more effective to prevent the droplets from flocculation.

Considering that the commercial PTX injection contains 6 mg/mL PTX, we further investigated the PTX/BD6-10K emulsion containing 6 mg/mL PTX. The emulsification pH was separately decreased from 8.0 to 7.8, 7.6, 7.4, and 7.2. The resultant emulsions have droplet sizes of 178–197 nm. After 8 months of storage, the droplet size of the emulsion produced at pH 7.6 is 203 ± 4 nm, whereas the droplet sizes of the other emulsions are 230 nm or larger, suggesting pH 7.6 is the optimal emulsification pH for the emulsion containing 6 mg/mL PTX. For the PTX/BD6-10K and PTX/BD1-62K emulsions produced at pH 8.0 containing 2 mg/mL PTX, the droplet sizes are 205 ± 3 and 233 ± 9 nm after 17 months of storage, respectively. Compared with the sizes of 191 ± 3 and 211 ± 9 nm of the freshly prepared PTX/BD6-10K and PTX/BD1-62K emulsions (Table 3), the droplet sizes only change 14 and 22 nm, respectively, after 17 months of storage. Therefore, the PTX/BD6-10K and PTX/BD1-62K emulsions produced at pH 8.0 containing 2 mg/mL PTX were used in the following study. In addition, both PTX/BD6-10K and PTX/BD1-62K emulsions can pass through $0.22 \mu\text{m}$ filter for sterilization.

The stability of PTX-loaded emulsion in blood serum and whole blood was investigated *in vitro*. The PTX/BD1-62K emulsion was separately mixed with fetal bovine serum and mouse blood at a volume ra-

tio 1:1. The mixtures were kept in a shaker at 37°C for 24 h. Then, the released PTX in the mixtures was extracted by chloroform and analyzed by HPLC. The result of no extracted PTX in the mixtures means that the emulsion is stable in blood serum for at least 24 h.

Characterizations of PTX-Loaded Emulsions

Figure 1 shows the ζ -potentials of the emulsions as a function of pH. Both PTX/BD1-62K and PTX/BD6-10K emulsions carry positive charges and negative charges when the pH values are lower and higher than 5.0, respectively. The ζ -potential absolute values of the PTX/BD1-62K and the PTX/BD6-10K emulsions are much smaller than those of the BSA emulsion at the same pH because the droplet surfaces of the emulsions prepared from the conjugates are covered by dextran. The PTX/BD1-62K emulsion has smaller ζ -potential absolute values than the PTX/BD6-10K emulsion at the same pH. Figure 2 shows a TEM image of PTX/BD1-62K droplets. The droplets present polygonal shape because of their soft and deformable nature. It was reported that the real particle size may change in the blood stream because of the deformability of particles and the deformability can facilitate an accumulation of the particles in tumor via the enhanced permeability and retention (EPR) effect.^{32–34} In this study, the soft and deformable property of the droplets may facilitate the accumulation of PTX-loaded droplets in tumor via the EPR effect.

Cellular Internalization of FITC-Labeled Emulsions

The cellular internalization of FITC-labeled emulsions was visualized by CLSM after 3 and 5 h incubation. Figure 3 shows that the droplets, both BD6-10K and BD1-62K, mainly locate in the cytoplasm, and the fluorescence intensities of the BD6-10K are stronger

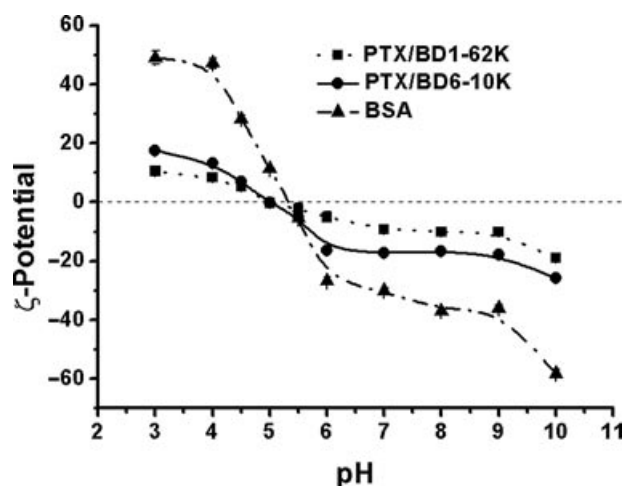


Figure 1. ζ -Potentials of BSA, PTX/BD1-62K, and PTX/BD6-10K emulsions as a function of pH. The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL.

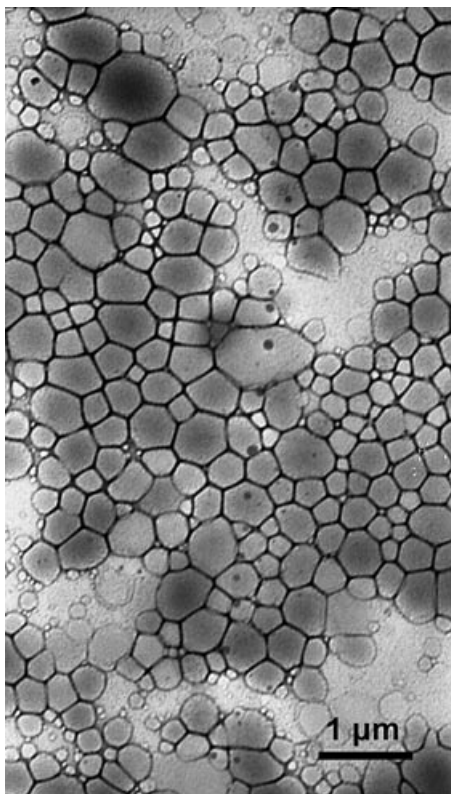


Figure 2. TEM image of PTX/BD1-62K emulsion. The emulsion was produced at pH 8.0 with PTX concentration of 2 mg/mL.

than those of the BD1-62K in the cells. The intracellular fluorescence intensity was quantified by flow cytometry after 1 h incubation. As shown in Figure 4, the fluorescence intensity of the BD6-10K emulsion is significantly stronger than the intensity of the BD1-62K emulsion if the exponential scale of the x -axis is noticed. The results of CLSM and flow cytometry

reveal that the BD6-10K emulsion can enhance the cellular uptake compared with the BD1-62K emulsion. It is possible that the shorter and denser dextran chains on the BD6-10K droplet surface and the smaller droplet size of BD6-10K emulsion (Table 1 and 2) account for the better cellular uptake.

In Vitro Cytotoxicity of PTX-Loaded Emulsions

The cytotoxicity of the unloaded emulsion, PTX-loaded emulsions, and the free PTX solution against KB cell line was investigated and the results are shown in Figure 5. At a PTX concentration of 0.01 μ g/mL, free PTX inhibits more than 90% of the cell growth after 72 h incubation. The PTX/BD6-10K and PTX/BD1-62K emulsions show similar antitumor activity as the free PTX solution, but the PTX/BD6-10K emulsion exhibits better activity than the PTX/BD1-62K emulsion that can be explained by the enhanced cellular uptake of the PTX/BD6-10K emulsion. The unloaded emulsions do not show any cytotoxicity when the concentrations of BSA and dextran are identical to the PTX-loaded emulsions. This result indicates that BSA–dextran emulsions, the carriers of PTX, are biocompatible and nontoxic at the concentrations studied. We further investigated the antitumor activity of the emulsions against incubation time at a fixed PTX concentration of 0.01 μ g/mL. Figure 6 shows that after 48 h incubation, more than 90% of the cells are killed by the PTX solution and PTX-loaded emulsions. The results in Figure 6 further confirm that the PTX/BD6-10K emulsion has better antitumor activity than the PTX/BD1-62K emulsion.

The results of cytotoxicity can be further understood in combination with the result that no PTX was extracted by chloroform in the mixture of PTX/BD1-62K emulsion and mouse blood. In this case, the antitumor activity of the PTX-loaded emulsions shown in Figures 5 and 6 can only be attributed to the PTX that was released inside the cells. The heat treatment of the emulsions can produce cross-linked protein adsorption layer at the oil–water interface because of the formation of disulfide bonds.³⁵ These disulfide bonds can be cleaved in the intracellular compartments in which there is 2–10 mmol/L reducing glutathione.³⁶ The data in Figures 5 and 6 reveal that the PTX-loaded emulsions have similar antitumor activity as the free PTX solution, demonstrating that the loaded PTX can be effectively released from the droplets inside the cells.

In Vivo Antitumor Effect of PTX-Loaded Emulsions

The survivability of H22-tumor-bearing mice treated with the PTX-loaded emulsions was investigated first. The H22 cells were injected in abdomens and grew into ascites; the treatments were performed every week for 3 weeks via the tail veins. The average life-time and SR are listed in Table 4. No acute toxicity

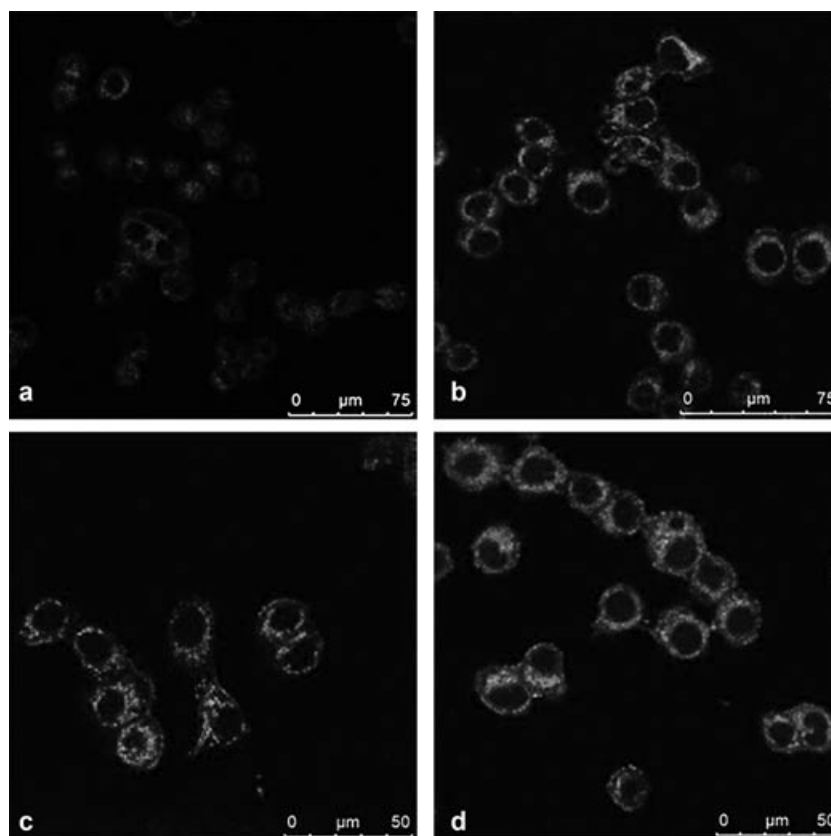


Figure 3. CLSM images of KB cells after incubation with FITC-labeled BD1-62K emulsion for 3 h (a), 5 h (b), and FITC-labeled BD6-10K emulsion for 3 h (c), and 5 h (d).

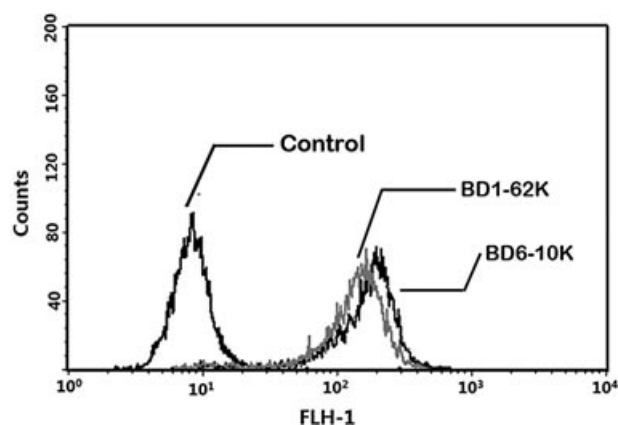


Figure 4. Flow cytometry analysis of the KB cellular uptake of FITC-labeled BD1-62K and BD6-10K emulsions after 1 h incubation.

was observed for the mice injected with the heated emulsions, demonstrating that the heat treatment of the emulsions at 90°C for 1 h can avoid anaphylaxis. This result confirms our supposition that the heat treatment is an effective strategy to eliminate BSA anaphylaxis, although the detailed mechanism is not known at this stage. Possibly, the heat treatment can induce BSA denaturation and aggregation

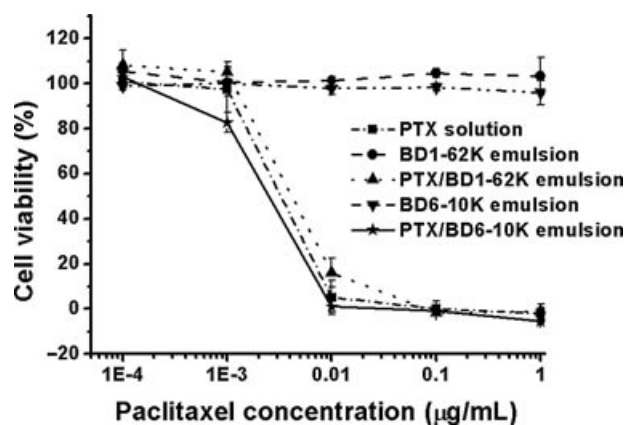


Figure 5. Cell viability against the free PTX solution and the PTX-loaded emulsions as a function of PTX concentration. The unloaded emulsions containing the same BSA and dextran concentrations as the PTX-loaded emulsions were also assessed. The incubation time was 72 h. The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL.

at the oil–water interface that prevent the epitopes from reacting with the antibodies.

Table 4 shows that the unloaded BD6-10K emulsion does not have any effect on the tumor-bearing mice. PTX/BD6-10K emulsion has better

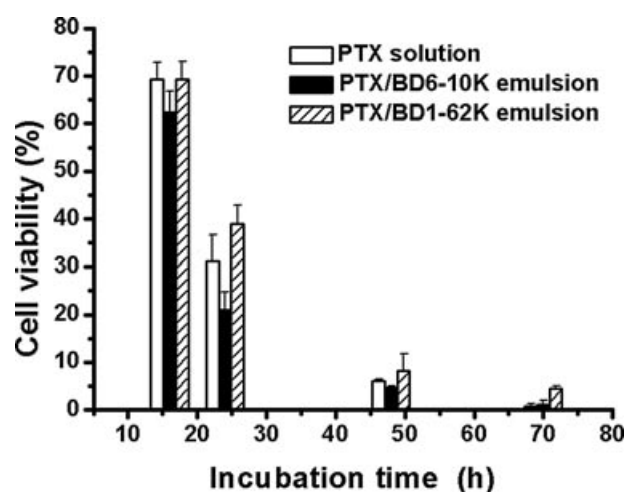


Figure 6. Cell viability against the free PTX solution and the PTX-loaded emulsions after 16, 24, 48, and 70 h incubation at a fixed PTX concentration of 0.01 $\mu\text{g/mL}$. The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL.

Table 4. Survivability of H22-Tumor-Bearing Mice Treated with Unloaded BD6-10K Emulsion, PTX-Loaded PTX/BD6-10K and PTX/BD1-62K Emulsions, and Commercial PTX Injection

PTX Dose (mg/kg)	Group	Average Lifetime (Day)	Survivability Rate (%)
0	Saline	15.4 $\pm$ 2.8	–
	Blank (BD6-10K)	15.3 $\pm$ 2.9	–
10	PTX/BD6-10K	22.8 $\pm$ 1.3*	48.1
	PTX/BD1-62K	21.6 $\pm$ 1.8*	40.3
16	PTX injection	21.9 $\pm$ 2.3*	42.2
	PTX/BD6-10K	23.6 $\pm$ 2.1*	53.2
	PTX/BD1-62K	21.9 $\pm$ 1.5*	42.2
	PTX injection	23.2 $\pm$ 1.8*	50.6

The PTX-loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL.

* $p < 0.01$ compared with the saline group.

survivability effect than PTX/BD1-62K emulsion and commercial PTX injection. At the PTX dose of 10 mg/kg, the SR of the PTX/BD6-10K group is 48.1%, whereas that of the PTX/BD1-62K group is 40.3% and the commercial PTX injection group is 42.2%. Increasing the PTX dose to 16 mg/kg, the SR of the PTX/BD6-10K group increases to 53.2%, which is also higher than 42.2% of the PTX/BD1-62K group and 50.6% of the commercial PTX injection group.

The tumor inhibition effect of the emulsions was also investigated. In the experiments, H22 solid tumors were grown in right axillae and the mice were treated every day for 6 days via tail veins. Table 5 shows that at the PTX dose of 10 mg/kg, the PTX/BD6-10K group achieves 61.1% tumor inhibition rate, whereas the PTX/BD1-62K and the commercial PTX injection groups achieve 35.4% and 41.0% tumor inhibition rate, respectively. It is also noticeable that

the PTX/BD6-10K group increases the average body weight more than the PTX/BD1-62K and commercial PTX injection groups. When increasing the PTX dose to 16 mg/kg, the tumor inhibition rate of the PTX/BD6-10K group is 83.3%, higher than 75.2% of the PTX/BD1-62K group and 72.3% of the commercial PTX injection group. These results demonstrate that PTX/BD6-10K emulsion has better H22 tumor therapy effect. Furthermore, no mice died during the treatment in the two PTX/BD6-10K groups, whereas two or three mice died in the two PTX/BD1-62K groups and one commercial PTX injection group. This further confirms that PTX/BD6-10K emulsion has better survivability effect than PTX/BD1-62K emulsion and the commercial PTX injection.

The results in Tables 4 and 5 demonstrate that PTX/BD6-10K emulsion has better tumor inhibition and survivability effects than PTX/BD1-62K emulsion. The emulsions used in the animal experiments are freshly prepared ones containing 2 mg/mL PTX. The droplet sizes of PTX/BD6-10K and PTX/BD1-62K emulsions are 191 and 211 nm, respectively (Table 3). Possibly, PTX/BD6-10K droplets can enter the tumor tissue more effectively via the EPR effect due to the smaller size. PTX/BD6-10K droplets show better cellular uptake than PTX/BD1-62K droplets (Figures 3 and 4) that may also account for the better tumor inhibition and survivability effects. In addition, it was reported recently that the doxorubicin-loaded poly(alkylcyanoacrylate) nanoparticles with a dense brush dextran surface were less cleared by liver and they could reach highly vascularized organs at higher concentrations compared with the nanoparticles with the dextran chains in a loop conformation.³⁷ In this study, the dextran chains on PTX/BD6-10K droplet surface are much shorter and denser than the dextran chains on PTX/BD1-62K droplet surface that may result in the better tumor inhibition and survivability effects.

The data in Tables 4 and 5 indicate that PTX/BD6-10K emulsion has better antitumor activity and less toxicity than the commercial PTX injection prepared from polyethoxylated castor oil and alcohol. In addition to PTX, other lipophilic compounds can also be encapsulated effectively in BSA-dextran-stabilized oil droplets. Using the same method, we produced camptothecin and fluorescence probe DiR (1,1-dioctadecyl-3,3,3,3-tetramethyl indotricarbocyanine iodide) loaded BD6-10K emulsions whose droplet sizes are 196 and 187 nm, respectively, demonstrating that BD6-10K emulsion is a universal vehicle for hydrophobic compounds. Considering that our fabrication approach is a simple, low cost, green and effective one, the BSA-dextran emulsion is a promising carrier for the delivery of hydrophobic drugs and probes.

Table 5. Tumor Inhibition Effect of H22-Tumor-Bearing Mice Treated with Unloaded BD6-10K Emulsion, PTX-Loaded PTX/BD6-10K and PTX/BD1-62K Emulsions, and Commercial PTX Injection

PTX Dose (mg/kg)	Group	Mice Number (Beginning/End)	Average Body Weight (g, Beginning/End)	Average Tumor Weight (g)	Tumor Inhibition Rate (%)
0	Saline	10/10	18.7/27.8	2.00 ± 0.26	—
	Blank (BD6-10K)	10/10	18.8/27.7	1.92 ± 0.31	—
10	PTX/BD6-10K	10/10	18.6/28.3	0.78 ± 0.45*	61.1
	PTX/BD1-62K	10/8	18.7/23.9	1.29 ± 0.40**	35.4
	PTX injection	10/10	19.2/23.4	1.18 ± 0.44*	41.0
16	PTX/BD6-10K	10/10	18.6/22.3	0.33 ± 0.20*	83.3
	PTX/BD1-62K	10/7	18.7/19.5	0.50 ± 0.28*	75.2
	PTX injection	10/8	18.6/24.0	0.55 ± 0.30*	72.3

The PTX -loaded emulsions were produced at pH 8.0 with PTX concentration of 2 mg/mL.

* $p < 0.001$ compared with the saline group.

** $p < 0.005$ compared with the saline group.

CONCLUSIONS

In this study, we demonstrate that the heat-treated PTX-loaded BSA–dextran emulsions can avoid anaphylaxis. PTX/BD6-10K emulsion has better antitumor activity and less toxicity compared with PTX/BD1-62K emulsion and commercial PTX injection. Furthermore, BSA–dextran emulsion is a universal carrier for hydrophobic compounds including drugs and probes.

ACKNOWLEDGMENTS

The financial supports of Ministry of Science and Technology of China (973 Program 2009CB930402 and 2011CB932503), the innovative team of Ministry of Education of China (IRT0911), National Natural Science Foundation of China (NSFC Project 21274026), and Key Project of the National Twelfth-Five Year Research Program of China (2012ZX09304004) are gratefully acknowledged. We are grateful to Dr. Shaoyong Yu for reviewing the manuscript and offering helpful suggestions.

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