Biodegradable self-assembled nanoparticles of poly (D,L-lactide-co-glycolide)/hyaluronic acid block copolymers for target delivery of docetaxel to breast cancer

Jingbin Huang a,1, He Zhang a,1, Yuan Yu a, Yan Chen a, Dong Wang b, Guoqing Zhang d, Guichen Zhou d, Junjie Liu a, Zhiguo Sun a, Duxin Sun c, Ying Lu *, Yanqiang Zhong **

a Department of Pharmaceutical Science, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China
b Department of Pharmacy, Beidaihe Sanatorium of Beijing Military Area Command, 4 Xihaitan Road, Qinhuangdao, Hebei 066100, China
c Department of Pharmaceutical Sciences, School of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA
d Department of Pharmacy, Eastern Hepatobiliary Surgery Hospital, 225 Changhai Road, Shanghai 200438, China

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A B S T R A C T
To develop biodegradable docetaxel-loaded self-assembled nanoparticles of poly (D,L-lactide-co-glycolide)/hyaluronic acid block copolymers were successfully synthesized. These copolymers could form nanoparticles with small size (<200 nm), an acceptable CMC (~7.9 mg/L), typical core/shell structure and superior stability in one week. DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) showed a biphasic release pattern within 120 h, and exhibited enhanced cytotoxicity toward CD44-overexpressing MDA-MB-231 cells. Cellular uptake study indicated that PLGA 502H-b-HA5.6k nanoparticles (SANPs) were taken up in MDA-MB-231 cells by CD44-mediated endocytosis. Pharmacokinetics study revealed DTX/SANPs could prolong the circulation of DTX in the blood. In vivo studies demonstrated that SANPs exhibited enhanced tumor targeting and antitumor activity with lower systemic toxicity. In conclusion, DTX/SANPs have great potential for targeted chemotherapy for CD44-overexpressing breast cancer.

1. Introduction

Self-assembled nanoparticles composed of amphiphilic block copolymers have received intensive attentions in the field of drug delivery systems [1,2]. Such nanoparticles always have a core–shell structure consisting of a hydrophobic core and a hydrophilic shell [1,3,4]. Typically, the hydrophobic block forms the hydrophobic core of the nanoparticles, while the hydrophilic block forms the outer or hydrophilic shell. The hydrophobic core serves as a compartment for loading hydrophobic drugs, and the hydrophilic shell made of a brush-like corona can stabilize nanoparticles in aqueous solution. Furthermore, hydrophilic shell is expected to help prolong circulation time of self-assembled nanoparticles, due to the steric stabilization which provides protection from opsonization in blood stream. In addition, self-assembled nanoparticles often possess a small size of <200 nm which facilitates the extravasation of nanoparticles at leaky sites of tumors owing to the enhanced permeability and retention (EPR) effect [5].

Although nanoparticles formed from block copolymers have passive targeting to tumors, their therapeutic effect may be limited by insufficient cellular uptake by tumor cells, due to lack of active targeting of unmodified nanoparticles [6,7]. To enhance the active targeting, nanoparticles are generally modified with targeting moieties such as peptide ligands [8–10], nucleotide aptamers [11,12], and antibodies [13–16]. However, the application of these targeting moieties severely suffers from the following disadvantages [17,18]: potential immunogenicity, impaired binding affinity after conjugation and high cost. Hyaluronic acid (HA), a naturally occurring polysaccharide composed of N-acetyl-D-glucosamine and D-glucuronic acid, could specifically bind to its receptors CD44 and RHAMM (receptor for HA-mediating motility) which are well-validated targets in a variety of tumors and be internalized via receptor-mediated endocytosis [19,20]. Thus, HA is regarded as a targeting moiety for tumor targeting. It is noteworthy that,
compared with other types of targeting moieties, HA possesses unique advantages such as non-toxicity, non-immunotoxicity [21], good biocompatibility, biodegradability [22] and modification flexibility [23].

Hyaluronic acid (HA) has been extensively used as a targeting moiety in HA-drug conjugates [24,25], HA nanogels [26] and HA self-assembled nanoparticles [5,20,27,28] for cancer targeting delivery of chemotherapy drugs. In addition to its targeting ability to tumors, HA is a hydrophilic molecule and possesses the ability to form the hydrophilic shell in self-assembled nanoparticles after conjugation with hydrophobic polymers such as poly (ω,ω-lactide-co-glycolide) (PLGA) [29–33]. Generally, the self-assembled nanoparticles composed of HA/PLGA copolymers are categorized into two types: one is nanoparticles composed of PLGA-grafted HA copolymers, in which hydrophobic PLGA chain is grafted onto the backbone of hydrophilic HA [30–33], and the other one is composed of PLGA/HA block copolymers [29]. Both nanoparticles composed of PLGA/HA block copolymers can generally form a core–shell structure, and showed effective cellular targeting and significant cytotoxic effects toward CD44-overexpressing tumor cells [25,30,33]. Notably, compared with the nanoparticles composed of PLGA-grafted HA copolymers, one of the advantages of the nanoparticles composed of PLGA/HA block copolymers is that hydrophobic HA can be freely extended and directed toward aqueous solution while the hydrophobic PLGA incorporate hydrophobic drugs [29]. This unique advantage may contribute to longer circulation and more active targeting toward CD44-overexpressing tumor cells for the nanoparticles composed of PLGA/HA block copolymer. Until now, there have been very few studies about the nanoparticles composed of PLGA/HA block copolymers. Such copolymer was first reported to form typical core–shell nanoparticles in aqueous condition by Dae Hwan Kang’s group, in which the nanoparticles based on PLGA/HA block copolymer had been proven to possess active targeting ability to CD44-overexpressing HCT-116 human colon carcinoma cells [29]. Furthermore, hydrophobic drug (doxorubicin) was successfully loaded into such nanoparticles with high drug loading content, which achieved enhanced cytotoxicity in HCT-116 cells. In addition to its biodegradability and compatibility, this copolymer is a superior material for targeted delivery to CD44-overexpressing cancer. However, the preparation procedure was not optimized, and the effects of molecular weight ratio of HA to PLGA on the characteristics of the nanoparticles such as size and critical micelle concentration (CMC) were not investigated. Furthermore, in vivo tumor targeting, pharmacokinetics and antitumor efficacy of drug loaded nanoparticles based on PLGA block HA should be further investigated. Up to now, there is still no study about docetaxel-loaded PLGA/HA block copolymers based nanoparticles for breast cancer targeting.

In this study, to obtain an optimized type of self-assembled nanoparticles composed of PLGA/HA block copolymers, we synthesized a series of PLGA/HA block copolymers of different molecular weight using an end to end coupling strategy and developed a series of nanoparticles composed of these block copolymers. The size, CMC and zeta potential of these nanoparticles were investigated. Finally, the optimal nanoparticles, PLGA502H-b-HA5.6k nanoparticles (SANPs), with the smallest size and a suitable CMC were developed for further investigation. Characteristics of blank or drug loaded nanoparticles were evaluated and the mechanism of endocytosis against CD44-overexpressed cells was also investigated. Coumarin-6 and DiR were loaded into PLGA502H-b-HA5.6k nanoparticles to track the behavior of the nanoparticles to elucidate the targeting pathway. Pharmacokinetics and in vivo antitumor activity of docetaxel (DTX) loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) were investigated in Sprague–Dawley (SD) rats and tumor-bearing BALB/c nude mice, respectively.

2. Materials and methods

2.1. Materials

Hyaluronic acid with a different molecular weight (Mw) of 5.6, 7.3 or 8.9 kDa was purchased from the Shandong Freda Biopharmaceutical Co., Ltd. (Shandong, China). PLGA (50:50 polymers, Resomer® RG 502 H (MW 13, 600) and RG 503 H (MW 35, 700), were purchased from Boehringer Ingelheim (Ingelheim, Germany). Docetaxel was purchased from Shanghai Biochempartner Co., Ltd. (Shanghai, China). 3-[(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1,4-Diaminobutane, Triton X-100, 4,6-Diamidino-2-phenylindole (DAPI), sodium cyanoborohydride and coumarin-6 were purchased from Sigma–Aldrich Co. Ltd. 1-[(3-Dimethylaminopropyl) -3-ethylcarbodiimide hydrochloride (EDC HCl), N-Hydroxysuccinimide (NHS) and N,N-Diisopropylthelylamine were obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). 1,2-Diododecyl-3,3,3',3'-tretamethyl indocarbocyanine iodide (DiR) was obtained from Biotium (CA). Cell Cycle and Apoptosis Assay Kit and Annexin V-FIT Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Cell culture medium (RPMI 1640 and High Glucose DMEM), trypsin–EDTA, penicillin, streptomycin and fetal bovine serum (FBS) were provided from GIBCO (USA). Two breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from the Cell Culture Center of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). All the other reagents used were of analytical grade and used without further purification.

Sprague–Dawley (SD) rats (male, 200 ± 20 g) and BALB/c nude mice (female, five weeks, ~16 g) were purchased from Shanghai Slac Laboratory Animal Co, Ltd (China, Shanghai) and maintained in laminar flow room under constant temperature and humidity. All animal experiments were performed in accordance with protocols evaluated and approved by the ethics committee of Second Military Medical University.

2.2. Synthesis of PLGA/HA block copolymers (PLGA-b-HA)

PLGA/HA block copolymers (PLGA-b-HA) were synthesized by an end to end coupling strategy as described before [29]. Here, we take one of the PLGA-b-HA copolymers, PLGA502H-b-HA5.6k, as an example to illustrate the procedures of the synthesis of the block copolymers.

(a) Synthesis of amino-functionalized HA

The synthetic strategy was based on a terminal reductive amination reaction [34] between hyaluronic acid (HA) and 1,4-Diaminobutane with sodium cyanoborohydride (NaCNBH3) as a reducing agent (Fig. 1A). In detail, 1 g of HA (5.6 kDa, 0.18 mmol) was dissolved in an acetone buffer (30 ml, pH 5.6, 2% w/v). Then, 1.0 ml of 1,4-Diaminobutane (11.4 mmol) was added into the HA solution under magnetic stirring. Twenty-four hours after stirring at 50 °C, HA reacted with 1,4-Diaminobutane and resulting imine was obtained in the mixture. Subsequently, 0.2 g of sodium cyanoborohydride (3.2 mmol) was added to the mixture each day for three days under stirring. The mixture was purified by dialysis with a dialysis bag (Spectra/Per8, MWCO 3500) against deionized water for 72 h to remove excess 1,4-Diaminobutane and sodium cyanoborohydride. The final product was collected and lyophilized.

(b) Synthesis of N-Hydroxysuccinimide PLGA (PLGA-NHS)

The N-Hydroxysuccinimide PLGA (PLGA-NHS) was synthesized as described before [29]. Briefly, 1 g of PLGA-COOH (RG 502H, 0.083 mmol) was added into methylene chloride (30 ml) in which 48.0 mg of N-Hydroxysuccinimide (NHS, 0.42 mmol) and 80.0 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC HCl, 0.42 mmol) were dissolved. Then, the mixture was stirred for another 24 h at room temperature (Fig. 1B). Following that, the final product PLGA-NHS was precipitated with ethyl ether (5 ml), and washed for three times in an ice-cold mixture of ethyl ether and methanol (50:50, v/v) to remove residual NHS and EDC HCl. Finally, purified PLGA-NHS was dried under vacuum.

(c) Synthesis of PLGA-b-HA

First, 0.5 g of PLGA-NHS (0.042 mmol) was dissolved in dimethyl sulfoxide (DMSO, 20 ml), and 0.35 g of amino-functionalized hyaluronic acid (0.063 mmol) and 20 μl of NHS-Diisopropylthelylamine (0.16 mmol) were added subsequently. The mixture was stirred at 50 °C for 48 h (Fig. 1C). Then, the mixture was purified by dialysis with a dialysis bag (Spectra/Per8, MWCO 12000–14000) against deionized water for 72 h to remove excess amino-functionalized hyaluronic acid. The final product PLGA-b-HA was lyophilized and used to prepare SANPs without further treatment.
2.3 Characterization of PLGA-b-HA copolymers

The chemical structure of PLGA-b-HA was characterized by $^1$H nuclear magnetic resonance ($^1$H NMR) and Fourier transform infrared spectroscopy (FT-IR). For $^1$H NMR analysis, PLGA-b-HA was dissolved in DMSO-$d_6$ and D$_2$O, and analyzed by a NMR Spectrometer (Avance II 600 MHz, Bruker, Switzerland). For FT-IR analysis, samples and potassium bromide (KBr) were mixed homogeneously with a mass ratio of 1:100 to make transparent tablets, and analyzed by a Fourier transform infrared instrument (NEXUS-470, Nicolet, USA).

The critical micelle concentration (CMC) of PLGA-b-HA was determined by a pyrene fluorescence method as described before [19]. In brief, 100 ml of pyrene solution ($6 \times 10^{-5}$ M in acetone) was transferred into a series of volumetric flasks (10.0 ml), and the acetone was evaporated under a gentle nitrogen gas stream for 4 h at room temperature. The PLGA-b-HA solutions with a series of concentrations ranging from $1 \times 10^{-5}$ to 0.1 mg/ml in deionized water were added into each flask, with a final pyrene concentration of $6 \times 10^{-7}$ M. Then, the solutions were allowed to equilibrate for another 12 h at 37 °C in a shaker (SHK6000-8CE, Thermo Scientific, USA). The fluorescence spectrum was recorded using a fluorescent

Fig. 1. The synthetic scheme of PLGA-b-HA. (A) Synthesis of amino-functionalized HA. (B) Synthesis of PLGA-NHS. (C) Synthesis of PLGA-b-HA.
spectrum F-7000 (Hitachi, Japan). The excitation spectrum ranged from 300 to 360 nm with the emission spectrum was fixed at 390 nm, and emission and excitation slit widths were both 2.5 nm and the scanning speed was set at 240 nm/min.

2.4. Preparation and characterization of PLGA-b-HA nanoparticles

PLGA-b-HA nanoparticles were prepared by a modified solvent-dialysis method [36]. In brief, 10 mg of PLGA-b-HA was dissolved in 1 ml of DMSO/dimethyl formamide (DMF) mixed solvent (v/v = 3:1) under stirring for 15 min. Then, deionized water was slowly added, and nanoparticles were developed after stirring for another 15 min. The extra organic solvent was removed by dialysis with a dialysis bag (Spectra/Por®, MWCO 12000–14000) against deionized water for 12 h.

The size and zeta potential were measured by dynamic light scattering using a Malvern Instruments (Zetasizer Nano ZS, Malvern, UK). The morphology of the nanoparticles was observed by a transmission electron microscopy (JEM-2100HT, JEOL, Japan) and an Atomic Force Microscopy (Nanoscope IV, Veeco, USA). For the analysis of TEM, the concentration of the nanoparticles was adjusted to 1.0 mg/ml and stained with 2% (w/v) phosphotungstic acid (PTA) for 1 min, and excess solution was absorbed with a filter paper bar. Then, the samples were air-dried for 10 min for observation. For the analysis of AFM, one drop of the nanoparticles solution (1.0 mg/ml) was dropped on a freshly cleaved mica surface and air-dried at room temperature. Both topography and phase signal images were recorded.

The effects of polymer concentration and pH on the particle size and zeta potential of SANPs were investigated. The stability assay was performed by monitoring the analysis of AFM, one drop of the nanoparticles solution (1.0 mg/ml) was dropped on a freshly cleaved mica surface and air-dried at room temperature. Both topography and phase signal images were recorded.

2.5. Preparation and characterization of DTX-loaded PLGA502H-b-HA5.6k nanoparticles

DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) were prepared by modified solvent-dialysis method (Fig. 2). Briefly, 10 mg of PLGA502H-b-HA5.6k and different doses of DTX (0.2, 0.3, 0.5 mg) was dissolved in 1 ml of DMSO/DNf (v/v = 3:1) mixed solvent under stirring. Then deionized water was slowly added, and DTX/SANPs were developed after stirring for another 15 min. Extra organic solvent was removed by dialysis with a dialysis bag (Spectra/Por®, MWCO 12000–14000) against deionized water for 12 h. Finally, the DTX/SANPs was filtered through a syringe filter (0.45 μm pore size, Millipore, USA) before HPLC analysis.

The status of DTX in PLGA502H-b-HA5.6k nanoparticles was measured by the Powder X-ray diffraction (Bruker, Karlsruhe, Germany) at room temperature, with a scanning rate of 4°/min (the scanning angle (2θ) was set from 5° to 50°).

2.6. In vitro release profile of DTX-loaded PLGA502H-b-HA5.6k nanoparticles

DTX/SANPs (150 μg of DTX) were suspended in 5 ml PBS (pH = 5.5 or 7.4) containing 0.1% (w/v) Tween-80. Then the suspension was transferred into 5.0 ml dialysis tubes (Spectra/Por®, MWCO 8000). The tubes were immersed in 20.0 ml PBS (pH 5.5 or 7.4) containing 0.1% (w/v) Tween-80 and placed in a shaker (SHK6500-8C, Thermo Scientific, USA) with a speed of 100 rpm at 37°C. One milliliter of medium was collected from the tube at predetermined time intervals (1, 2, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96 and 120 h) while an equivalent volume of fresh PBS was added to maintain a sink condition. The collected medium was filtered through a syringe filter (0.45 μm pore size, Millipore, USA) before HPLC analysis.

2.7. Flow-cytometric analysis of CD44 expression

Expression of CD44 receptor level in MCF-7 and MDA-MB-231 cells was analyzed by flow cytometry. When MCF-7 and MDA-MB-231 cells grew at 90% confluence, cells were harvested and rinsed with PBS. Subsequently, about 2 × 10^4 cells were incubated with FITC-CD44 antibody or FITC-IgG2b at 4°C for 30 min in PBS. The samples were washed twice with PBS and re-suspended in 500 μl PBS for flow cytometry analysis.
2.8. In vitro cellular uptake of PLGA502H-b-HA5.6k nanoparticles

2.8.1. Preparation of coumarin-6-loaded PLGA502H-b-HA5.6k nanoparticles

To investigate in vitro uptake of PLGA502H-b-HA5.6k nanoparticles, coumarin-6 was used as a fluorescence probe. Coumarin-6-loaded PLGA502H (C-6/PLGA) and PLGA502H-b-HA5.6k nanoparticles (C-6/SANPs) were prepared in the same way as DTX-loaded nanoparticles, except that DTX was replaced by 50, 100 μg coumarin-6, respectively. Unloaded coumarin-6 was removed by filtering through a syringe filter (0.45 μm pore size, Millipore, USA). And free coumarin-6 was removed by Superdex® 200 10/300GL (USA, Amersham). Finally, coumarin-6-loaded nanoparticles were lyophilized. Drug loading content of coumarin-6 in coumarin-6-loaded nanoparticles was calculated according to a coumarin-6 calibration curve constructed by standard lead coumarin-6 solutions.

2.8.2. Intracellular uptake of coumarin-6-loaded PLGA502H-b-HA5.6k nanoparticles

For in vitro cellular uptake study, MCF-7 and MDA-MB-231 cells were seeded in glass-base dishes at a density of 2.0 × 10^5 per dish and incubated for 12 h at 37 °C. Then, the medium was replaced with 1 ml of fresh medium containing: (1) free coumarin-6 (C-6); (2) coumarin-6-loaded nanoparticles (C-6/PLGA or C-6/SANPs); (3) coumarin-6-loaded nanoparticles with free HA (M_w ¼ 5.6 kDa, 5 mg/ml) which had an equivalent concentration of coumarin-6 (15 ng/ml), the cells were incubated for 1 h at 37 °C. The cells were washed thrice with ice-cold PBS and fixed with 4% (w/w) formaldehyde solution for 15 min. The formaldehyde solution was removed and the cells were further washed thrice with ice-cold PBS. Finally, the cells were treated with Triton X-100 solution (0.1% w/w in deionized water) for DAPI staining and observed using a confocal laser scanning microscopy (TCS-SP5, Leica, Germany). For flow cytometry analysis, cells were rinsed thrice with ice-cold PBS to remove free coumarin-6 or coumarin-6-loaded nanoparticles. Then, the cells were dissociated by 0.25% trypsin–EDTA and collected by centrifugation (250g × 5 min) and resuspended in 0.5 ml PBS before FCM analysis.

2.9. In vitro cytotoxicity

Cytotoxicity of nanoparticles was performed using the methylthiazol tetrazolium (MTT) assay [21,37]. Briefly, MCF-7 and MDA-MB-231 cells (5 × 10^3 cell/well) were seeded in 96-well plates (BD Falcon, USA) and incubated for 12 h. Then the medium was replaced by fresh medium containing a series of concentrations of free DTX, DTX-loaded PLGA502H (DTX/PLGA), DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs), or blank PLGA502H-b-HA5.6k nanoparticles (blank SANPs). Then, the cells were incubated with the blank SANPs at 37 °C for 12 h, 24 h and 48 h, and with free DTX or DTX-loaded nanoparticles for 48 h. After incubation, the cells were washed with PBS thrice to remove the formulations and fresh medium (100 μl per well) was added to the cells. After then, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for additional 4 h. Subsequently, the medium was replaced with 150 μl DMSO per well. The absorbance was measured at 490 nm using a microplate reader (MK-3, Thermo, USA). Cell viability was calculated using the formula: [(A_E - A_B)/(A_C - A_B)] × 100%, where A_E, A_C and A_B were defined as the absorbance of experimental samples, untreated samples and blank controls, respectively. The cell viability was plotted relative to the concentration of docetaxel. The IC50 values were calculated by the formula of logarithmic curves.

Fig. 3. The chemical structure of PLGA-b-HA was characterized by 1H NMR (A, B, C) and FT-IR (D, E, F). 1H NMR spectra of (A) HA5.6k in DMSO-d_6, (B) PLGA502H-b-HA5.6k in DMSO-d_6, (C) PLGA502H-b-HA5.6k in D_2O. FT-IR spectra of (D) PLGA502H, (E) HA5.6k, (F) PLGA502H-b-HA5.6k.
2.10. Cell cycle and apoptosis assays

Cell cycle and apoptosis assays were performed as described below. Briefly, MDA-MB-231 cells were seeded in six-well plates at a density of 5 × 10^4 cells/well. The cells were assigned into four groups (three wells per group): (i) untreated group; (ii) DTX group (free DTX 10 ng/ml); (iii) DTX-loaded PLGA_{502H} nanoparticles (DTX/PLGA, 10 ng/ml); (iv) DTX-loaded PLGA_{502H}-b-HA_{5.6k} nanoparticles (DTX/SANPs, 10 ng/ml). Forty-eight hours after treatment, the cells were cycle analyzed by the Cell Cycle and Apoptosis Analysis Kit (Beyotime, Haimen, China) according to the manufacturer’s protocol, and cell apoptosis was evaluated by the Annexin V–FIT Kit (Beyotime, Haimen, China) according to the manufacturer’s protocol.

2.11. Pharmacokinetics

Eighteen Sprague–Dawley (SD) rats (male, 200 ± 20 g) were randomly assigned to three groups (six mice per group). Free DTX, DTX/PLGA_{502H} nanoparticles (DTX/PLGA) or DTX/PLGA_{502H}-b-HA_{5.6k} nanoparticles (DTX/SANPs) were injected i.v. as a single dose (10 mg/kg DTX) via tail vein. At different time points, 500 ȝl of blood samples were collected from retro-orbital sinus, and immediately centrifuged to collect the plasma fraction. All plasma samples were pre-treated as follows: 200 ȝl of plasma was spiked with 50 ȝl of internal standard (paclitaxel, 1.0 ȝg/ml in methanol), vortexed for 30 s, and extracted by ethyl ether. The supernatant was collected and evaporated under nitrogen flow at room temperature, the residue was dissolved in 100 ȝl methanol for LC/MS analysis. Plasma concentrations of DTX were determined using an Agilent Technologies 1100 series LC/MS system (Agilent, USA) equipped with a Symmetry C-18 column (3.0 × 100 mm, 3.5 μm, waters, USA). The mobile phase was composed of 30:70 (v/v) A/B, where A was 0.1% (v/v) formic acid in water containing 2 mM ammonium acetate, B was methanol, and flow rate was 0.3 ml/min. Quantification was performed using SIM positive mode with DTX ion m/z 829.8 [M + Na]⁺ and paclitaxel m/z 875.8 [M + Na]⁺. The optimal mass parameters were as follows: Drying Gas Flow, 10 L/min; Nebulizer Pressure, 35 psig; Drying Temperature, 35 °C; Capillary Voltage, positive 4000 V. Pharmacokinetics parameters were calculated using the DAS 2.0 software.

2.12. In vivo tumor targeting assays

For the analysis of in vivo tumor targeting of the nanoparticles, the near-infrared dye DiR was loaded into PLGA_{502H} and PLGA_{502H}-b-HA_{5.6k} nanoparticles. The preparation method of DiR loaded nanoparticles was exactly as that of DTX-loaded nanoparticles, except that DTX was replaced by 100 μg DiR. Free DiR was removed by filtration through the Superdex™ 200 10/300 GL (USA, Amersham). The amount of DiR in nanoparticles was determined by a UV–Vis spectrometry method.

BALB/c nude mice (female, five weeks, ~16 g) were inoculated via orthotopic injection on the left side of mammary fat pad with 1 × 10^6 MDA-MB-231 cells. When the volume of tumors reached ~400 mm³, mice were randomly assigned to three groups: (i) PBS (control); (ii) DiR loaded PLGA_{502H} nanoparticles (DiR/PLGA); (iii) DiR loaded PLGA_{502H}-b-HA_{5.6k} nanoparticles (DiR/SANPs). All mice were administrated with the formulations via i.v. injections (tail vein), then anesthetized with 5% of chloral hydrate and individually paced in a stereotaxic apparatus, and the in vivo fluorescence imaging was taken with a MAESTRO in vivo imaging system (Maestro™, Cry, USA) at predetermined time (2 h, 4 h, 8 h, 12 h, 24 h). After 24 h, the mice were sacrificed and tumors as well as organs were excised from the mice. Again, the fluorescence images of the tumors and organs were taken under the same condition mentioned above.

2.13. In vivo antitumor assays

The therapeutic effect of the nanoparticles was performed in a breast cancer xenograft in mice. BALB/c nude mice (female, five weeks, ~16 g) were inoculated via orthotopic injection on the left side of mammary fat pad with 1 × 10^6 MDA-MB-231 cells. When the volume of tumors reached ~50 mm³, mice were randomly assigned to five groups (six mice per group): (i) PBS; (ii) blank PLGA_{502H}-b-HA_{5.6k} nanoparticles (blank SANPs); (iii) DTX; (iv) DTX/PLGA_{502H} nanoparticles (DTX/PLGA); (v) DTX/PLGA_{502H}-b-HA_{5.6k} nanoparticles (DTX/SANPs). All the formulations were given to mice via tail vein every three days for four times at a dose of 10 mg/kg. Mice were weighted, and the volume of tumors reached 400 mm³, mice were randomly assigned to three groups (six mice per group). Free DTX, DTX/PLGA_{502H} nanoparticles (DTX/PLGA) or DTX/PLGA_{502H}-b-HA_{5.6k} nanoparticles (DTX/SANPs) were injected i.v. as a single dose (10 mg/kg DTX) via tail vein. At different time points, 500 ȝl of blood samples were collected from retro-orbital sinus, and immediately centrifuged to collect the plasma fraction. All plasma samples were pre-treated as follows: 200 ȝl of plasma was spiked with 50 ȝl of internal standard (paclitaxel, 1.0 ȝg/ml in methanol), vortexed for 30 s, and extracted by ethyl ether. The supernatant was collected and evaporated under nitrogen flow at room temperature, the residue was dissolved in 100 ȝl methanol for LC/MS analysis. Plasma concentrations of DTX were determined using an Agilent Technologies 1100 series LC/MS system (Agilent, USA) equipped with a Symmetry C-18 column (3.0 × 100 mm, 3.5 μm, waters, USA). The mobile phase was composed of 30:70 (v/v) A/B, where A was 0.1% (v/v) formic acid in water containing 2 mM ammonium acetate, B was methanol, and flow rate was 0.3 ml/min. Quantification was performed using SIM positive mode with DTX ion m/z 829.8 [M + Na]⁺ and paclitaxel m/z 875.8 [M + Na]⁺. The optimal mass parameters were as follows: Drying Gas Flow, 10 L/min; Nebulizer Pressure, 35 psig; Drying Temperature, 35 °C; Capillary Voltage, positive 4000 V. Pharmacokinetics parameters were calculated using the DAS 2.0 software.

3. Results

3.1. Characterization of PLGA-b-HA

The synthetic scheme of PLGA-b-HA was presented in Fig. 1. Take PLGA_{502H}-b-HA_{5.6k} as an example, the chemical structure of PLGA-b-HA was characterized by 1H NMR and FT-IR (Fig. 3). In the analysis of 1H NMR, the specific peaks of methyl (3H, –CH₃), methylene (2H, –CH₂–, –CH₂–C–), –COCH₃ and glucosidic H appearing at 2.85–4.55 ppm, respectively (Fig. 3B1–C). The characteristic peak of N-acetyl group (3H, –COCH₃) in the HA chain was observed at 1.80 ppm (Fig. 3A4–C4), with the peaks of methylene (2H, –CH₂OH) and glucosidic H appearing at 2.85–4.55 ppm (Fig. 3A5 and C5). As expected, the characteristic peaks of PLGA were not observed when the copolymer was introduced into D₂O. The loss of proton signal of PLGA in D₂O reflected that its motion was suppressed, suggesting that PLGA-b-HA was self-assembled to form a core–shell structure in D₂O which shielded the PLGA core. However, when the copolymer was dissolved in DMSO, the chain completely stretched, and both of the peaks of HA and PLGA appeared again (Fig. 3B).

In the analysis of FT-IR, the stretching vibration (3441 cm⁻¹) of the hydroxyl groups in PLGA-b-HA was strengthened after PLGA (3522 cm⁻¹) was conjugated with HA (Fig. 3D–F). The reason is that there is only one hydroxyl group in the terminal of PLGA, whereas there are much more hydroxyl groups in the HA chain. There are

<table>
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<th>Sample</th>
<th>X (μm)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>CMC (mg/L)</th>
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<tr>
<td>PLGA_{502H}-b-HA_{5.6k}</td>
<td>31.8</td>
<td>1116.1 ± 1.7</td>
<td>0.098 ± 0.007</td>
<td>−32.9 ± 1.3</td>
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<td>PLGA_{502H}-b-HA_{5.6k}</td>
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<td>PLGA_{502H}-b-HA_{5.6k}</td>
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<td>192.6 ± 2.3</td>
<td>0.034 ± 0.025</td>
<td>−33.5 ± 1.8</td>
</tr>
</tbody>
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4 X(μm) = HA molecular weight × [μm/mole] × 100.

2.14. Statistical analysis

All the data in this study were analyzed by the statistic package SPSS 16.0 (SPSS Inc., Chicago, IL). Data are expressed as mean ± SD. For values that were normally distributed, direct comparison between two groups was conducted by Independent Sample’s t test. P value of < 0.05 was considered statistically significant.
two types of carbonyl groups in PLGA, and the stretching vibration of these groups was overlapped in 1759 cm$^{-1}$ (Fig. 3D). In PLGA-b-HA, the absorption peak of carbonyl groups in PLGA (1759 cm$^{-1}$) and HA (1745 cm$^{-1}$) was overlapped in 1756 cm$^{-1}$ (Fig. 3F). Another two peaks which belonged to $\equiv$NHC\textsubscript{2}H and $\equiv$COOH in HA appeared in 1556 and 1659 cm$^{-1}$, respectively (Fig. 3E and F). In summary, these results demonstrated that PLGA-b-HA was successfully synthesized.

To clarify the amphiphilic property and self-assembling behavior of PLGA-b-HA, the critical micelle concentration (CMC) of the copolymers was determined using a pyrene fluorescence method [19]. As shown in Fig. 4, a shift of the excitation band at 334 nm could be negligible at low concentrations of copolymers. When the concentration of copolymers increased, a red shift of the excitation band to 336 nm could be clearly detected. The fluorescence intensity ratio ($I_{336}/I_{334}$) was plotted according to the

![Size Distribution by Intensity](image.png)

**Fig. 5.** The size distribution of PLGA\textsubscript{502H}-b-HA\textsubscript{5.6k} nanoparticles measured by DLS. (A) The morphology of PLGA\textsubscript{502H}-b-HA\textsubscript{5.6k} nanoparticles observed by TEM (B: scale bar = 100 nm; C: scale bar = 20 nm) and AFM (D: phase image; E: 3D image).
concentrations of copolymers, and the CMC values of copolymers were defined as the intersection of the lines drawn through the points of flat regions at low concentrations and the drastically increasing regions at high concentrations. The Table 1 showed the CMC values of PLGA-b-HA copolymers, ranging from 2.0 to 19.9 mg/L. With an increase in the molecular weight of HA, the CMC value increased, suggesting that PLGA-b-HA consisting of HA of higher molecular weight tends to have higher CMC values. In particular, PLGA503H-b-HA7.3k had the lowest CMC value (2.0 mg/L), whereas the CMC value of PLGA502H-b-HA8.9k was the highest (19.9 mg/L). Such relatively low CMC is beneficial in maintaining and stabilizing original structure of nanoparticles, thus preventing the quick release of drugs from nanoparticles in vivo before they reach the targeting sites.

3.2. Preparation and characterization of PLGA-b-HA nanoparticles

A modified solvent-dialysis method was used to prepare PLGA-b-HA nanoparticles. With the increase in the molecular weight of the copolymer, the size of PLGA-b-HA nanoparticles increased correspondingly (Table 1). In contrast, the zeta potential of PLGA-b-HA SANPs was not affected by the molecular weight and remained at a relatively steady value of ~30 mV, which may contribute to their stability and long circulation in vivo.

Fig. 5 shows the hydrodynamic size and its size distribution of PLGA502H-b-HA5.6k nanoparticles (SANPs) in aqueous solutions. Such a small size (117 nm) and PDI (0.111) contribute greatly to the stability of the nanoparticles. The morphology of SANPs was observed by TEM and AFM. From the TEM images, a well-defined core–shell structure was observed (Fig. 5B and C). The spherical shape and narrow distribution of the nanoparticles were also validated in the AFM images (Fig. 5C and D). We found that the size of SANPs observed from TEM and AFM (~80 nm) was smaller than that obtained from DLS analysis (~100 nm). The reason is that the nanoparticles measured by DLS were hydrated whereas those measured by TEM and AFM were dehydrated.

The effects of copolymer concentrations and pH on the size and zeta potential of SANPs were also evaluated. Accompanied with increased copolymer concentrations, the particle size increased gradually but the zeta potential was not affected significantly (Fig. 6A). However, both of size and zeta potential of SANPs were significantly affected by pH (Fig. 6B). When the pH dropped from 9 to 2, the particle size (~100 nm) became significantly larger (~180 nm), suggesting that aggregation may take place. As for the zeta potential, it was significantly enhanced by decreased pH and converted to positive charges at pH 2, which was due to the protonation of the carboxyl groups on HA under decreasing pH. It can be safely concluded that aggregation of SANPs would happen when the electrostatic repulsion among nanoparticles was reduced due to pH dropping.

The stability of SANPs was also evaluated for a week at different pH values (pH = 5.5 or 7.4) at 37 °C. The results showed that the particles size and zeta potential did not significantly change within one week, suggesting that SANPs possess an excellent physical stability (Fig. 6C and D).

3.3. Characterization and drug release profile of DTX-loaded PLGA502H-b-HA5.6k nanoparticles

As the same as blank PLGA-b-HA nanoparticles, DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) were prepared by a modified solvent-dialysis method. As shown in Fig. 7A, DTX/SANPs...
had a small size (~160 nm) of a narrow size distribution (PDI < 0.15). Accompanied with the increase in the weight ratio of DTX/polymer, the drug loading of DTX/SANPs increased gradually from 1.67% to 3.11%, although the encapsulation efficiency was reduced (Fig. 7A).

The status of DTX in PLGA502H-b-HA5.6k nanoparticles was evaluated by Powder X-ray diffraction analysis. As shown in Fig. 7B, the physical mixture of DTX and blank PLGA502H-b-HA5.6k copolymer showed similar drug crystal peaks as compared to that of DTX powder. However, no crystal peaks were observed in DTX-loaded PLGA502H-b-HA5.6k nanoparticles and blank PLGA502H-b-HA5.6k copolymer. These results revealed that DTX could exist as the amorphous or molecular state after loaded into PLGA502H-b-HA5.6k nanoparticles.

The in vitro release profile of DTX-loaded PLGA502H-b-HA5.6k nanoparticles was evaluated. As shown in Fig. 7C, a biphasic release pattern could be seen at both pH values. In the first 24 h, a burst release appeared with almost 50% of DTX was released, followed by a sustained release in another 96 h. This biphasic release pattern showed that the burst release could be helpful to suppress the growth of tumor cells in a short time, while the sustained release could provide the possibility to continually fight against cancer cells. The release profiles were similar at pH 5.5 and pH 7.4 in the first 36 h, whereas the release of DTX was a little faster at pH 5.5 than pH 7.4. The faster release of DTX in DTX/SANPs at pH 5.5 than pH 7.4 may be helpful in facilitating drug release in tumors which usually have acidic pH.

3.4. The intracellular uptake of PLGA502H-b-HA5.6k nanoparticles

To evaluate the intracellular uptake of PLGA502H-b-HA5.6k nanoparticles (SANPs), coumarin-6 was loaded into the nanoparticles and the endocytosis kinetics of coumarin-6-loaded nanoparticles was observed by confocal laser scanning microscopy (CLSM) and quantitatively analyzed by flow cytometry analysis (FCM). The CD44 expression of the two breast cancer cell lines (MDA-MB-231 and MCF-7) was evaluated by FCM. MDA-MB-231

<table>
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<tr>
<th>DTX/polymer (mg/mg)</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity</th>
<th>ζ (mV)</th>
<th>Encapsulation efficiency (%) a</th>
<th>Drug loading (%) b</th>
</tr>
</thead>
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<tr>
<td>0:10</td>
<td>156.7±2.7</td>
<td>0.078±0.018</td>
<td>-31.7±3.1</td>
<td>79.56±0.22</td>
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<td>0.2:10</td>
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<td>87.55±0.19</td>
<td>2.71±0.28</td>
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<tr>
<td>0.3:10</td>
<td>164.3±3.3</td>
<td>0.115±0.009</td>
<td>-29.6±1.2</td>
<td>65.26±0.41</td>
<td>2.58±0.21</td>
</tr>
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<td>0.102±0.011</td>
<td>-28.9±1.5</td>
<td>58.38±0.26</td>
<td>3.11±0.14</td>
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<tr>
<td>0.5:10</td>
<td>161.5±2.5</td>
<td>0.095±0.027</td>
<td>-29.7±1.2</td>
<td>55.66±0.68</td>
<td>2.78±0.34</td>
</tr>
</tbody>
</table>

a Encapsulation efficiency (%) = \( \frac{\text{Actual amount of DTX in formulation}}{\text{Input amount of DTX}} \) × 100

b Drug loading (%) = \( \frac{\text{Actual amount of DTX in formulation}}{\text{Amount of DTX-loaded formulation}} \) × 100

Fig. 7. (A) Characteristics of DTX-loaded PLGA502H-b-HA5.6k and PLGA502H nanoparticles. The PLGA502H-b-HA5.6k nanoparticles were prepared at a final polymer concentration of 2.0 mg/ml in deionized water. Data are represented as mean ± SD (n = 3). (B) Powder X-ray diffraction patterns of (a) blank PLGA502H-b-HA5.6k nanoparticles, (b) free DTX, (c) physical mixture of DTX and PLGA502H-b-HA5.6k nanoparticles and (d) DTX-loaded PLGA502H-b-HA5.6k nanoparticles; (C) The in vitro DTX release profile of DTX-loaded PLGA502H-b-HA5.6k nanoparticles in PBS (pH = 5.5 or 7.4) containing 0.1% (w/w) Tween-80 at 37°C (n = 3).
cells are CD44-overexpressed, whereas CD44 is lowly-expressed in MCF-7 cells (Supplementary Fig. 1).

As shown in Fig. 8, the intracellular uptake of various formulations including coumarin-6 (C-6), coumarin-6-loaded PLGA502H nanoparticles (C-6/PLGA), coumarin-6-loaded PLGA502H-b-HA5.6k nanoparticles (C-6/SANPs) and nanoparticles combined with free HA (SANPs + HA) was observed in MCF-7 and MDA-MB-231 cells. In MDA-MB-231 cells, the fluorescence in nanoparticles (C-6/PLGA, C-6/SANPs) treated groups was relative higher than the coumarin-6 treated group. It is noteworthy that the fluorescence in the C-6/SANPs treated group was significantly higher than that of the C-6/PLGA treated groups, whereas in CD44 lowly-expressing MCF-7 cells, the enhanced intracellular uptake of C-6/SANPs than C-6/PLGA was not observed. After competitive treatment with HA, the mean fluorescence was reduced significantly in MDA-MB-231 cells, whereas the reduced fluorescence after competitive treatment with HA was not observed in MCF-7 cells (Fig. 8A). These results strongly suggest that the enhanced uptake of coumarin-6-loaded PLGA502H-b-HA5.6k nanoparticles was dependent on the HA-CD44 specific interaction.

The intracellular uptake of nanoparticles was measured by a quantitative analysis of flow cytometry. As shown in Fig. 8B, the mean fluorescence in the C-6/SANPs treated group was significant higher than that of the C-6/PLGA treated group (P < 0.01) in MDA-MB-231 cells. Moreover, the mean fluorescence of the C-6/SANPs treated group was significantly reduced after competitive treatment with HA (P < 0.001), whereas free HA had no effect on the fluorescence of free coumarin-6 or coumarin-6-loaded PLGA502H nanoparticles (P > 0.05). In MCF-7 cells, no significant difference in mean fluorescence between C-6/PLGA and C-6/SANPs was observed (P > 0.05). In addition, after competitive treatment with HA, the mean fluorescence was not reduced in all the formulations treated groups (P > 0.05). As expected, in CD44 lowly-expressing MCF-7 cells, competitive treatment with HA did not affect the mean fluorescence of free coumarin-6 or nanoparticles (Fig. 8B).

In conclusion, both CLSM and FCM assays firmly demonstrated that coumarin-6-loaded PLGA502H-b-HA5.6k nanoparticles bound to and were internalized in CD44-overexpressing MDA-MB-231 cells in a CD44-dependent way.

3.5. In vitro cytotoxicity

The cell viability was evaluated by the MTT assay. First, the cytotoxic effect of blank PLGA502H-b-HA5.6k nanoparticles was...
evaluated in the two cell lines (Fig. 9A and B). In a wide range of copolymer concentrations (~500 μg/ml), PLGA502H-b-HA5.6k nanoparticles did not show significant cytotoxicity toward the cells at 12, 24 and 48 h, indicating that PLGA502H-b-HA5.6k nanoparticles are nontoxic to tumor cells. DTX, DTX/PLGA502H nanoparticles (DTX/PLGA) and DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) showed a dose-dependent cytotoxicity toward the tumor cells (Fig. 9C and D). As shown in Fig. 9E, the IC50 value of DTX/SANPs (281.5 ± 4.51 ng/ml) was higher than that of DTX (230.44 ± 3.22 ng/ml) in MCF-7 cells (P < 0.05), and no significant difference compared to DTX/PLGA (275.94 ± 8.18 ng/ml) (P > 0.05). However, in MDA-MB-231 cells, the IC50 value of DTX/SANPs (9.68 ± 2.31 ng/ml) was significantly lower than that of DTX/PLGA (14.06 ± 1.46 ng/ml) (P < 0.05). This result suggested that the enhanced cytotoxicity in MDA-MB-231 cells (but not in MCF-7 cells) might be due to the active cellular uptake mediated by HA-CD44 interaction which increased the intracellular concentration of DTX.

3.6. Cell cycle and cell apoptosis assays

To investigate the antitumor mechanism of nanoparticles, we evaluated the cell cycle and cell apoptosis in MDA-MB-231 cells after treatment of equal concentration (10 ng/ml) of DTX, DTX/PLGA and DTX/SANPs for 48 h. As shown in Fig. 10(A1 and A2), the majority of cells were distributed in the G0/G1 and G2/M phases in

![Fig. 9](https://example.com/fig9.png)

**Fig. 9.** In vitro cytotoxicity of blank PLGA502H-b-HA5.6k nanoparticles (SANPs), free DTX, DTX-loaded PLGA502H nanoparticles (DTX/PLGA) and DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) in (A and C) MCF-7 and (B and D) MDA-MB-231 cells at 48 h. Cell viability was evaluated by the MTT assay. Data are represented as mean ± SD (n = 6). (E) IC50 of different formulations calculated by the formula of logarithmic curves from triplicate experiments. Data are represented as mean ± SD (n = 3).
the control group. After treatment of free DTX, the proportion of the cells in the S phase increased significantly, and the majority of cells got arrested in the G0/G1 and S phases. Treatment with DTX/PLGA and DTX/SANPs showed significant arrest at the G2/M phase compared with free DTX treatment. Particularly, the DTX/SANPs treated group showed significant increase in the proportion of cells in the G2/M phase than that of DTX/PLGA treated group ($P < 0.01$).

The apoptosis assay was shown in Fig. 10(B1 and B2). The control group showed negligible apoptotic and necrotic cells (less than 10%). After treatment of DTX, DTX/PLGA and DTX/SANPs, the percentage of early and late apoptotic cells was significantly increased. Specially, the percentage of early and late apoptotic cells or total injured cells in the DTX/SANPs treated group was significantly higher than that of the DTX/PLGA or DTX treated group ($P < 0.01$). The results indicated that DTX/SANPs were more effective in inducing cycle arrest and apoptosis, compared with free DTX and DTX/PLGA.

3.7. Pharmacokinetics

The pharmacokinetic profiles of DTX, DTX/PLGA$_{502H}$ nanoparticles and DTX/SANPs nanoparticles were shown in Fig. 11. Both the DTX/PLGA$_{502H}$ and DTX/SANPs showed some increase in the concentrations of docetaxel in plasma compared with those of DTX (Fig. 11A). The physical pharmacokinetic parameters were calculated using non-compartmental model (Fig. 11B). Plasma half-life ($t_{1/2}$) of DTX/SANPs was significantly more than the DTX/PLGA ($P < 0.05$) and DTX ($P < 0.01$) groups. The clearance of DTX in DTX/SANPs formulation decreased by more than 2.5-fold, 3.9-fold compared with DTX/PLGA and DTX ($P < 0.01$), respectively. The volume of distribution ($V$) was calculated to reflect the theoretical volume over which the DTX is evenly distributed after injection. The calculated $V$ for the DTX/SANPs was only half of those for DTX/PLGA or Free DTX ($P < 0.01$), indicating that active DTX in plasma circulation was of greater retention when loaded into PLGA$_{502H}$-b-HA$_{5.6k}$ nanoparticles. As expected, the AUC for the DTX/SANPs
group was 2.5-fold, 3.8-fold higher than that of the DTX/PLGA and free DTX groups. In conclusion, the DTX/SANPs could prolong the circulation of DTX in the plasma.

3.8. In vivo tumor targeting assays

The real-time images of MDA-MB-231 tumor-bearing mice after i.v. injection of DiR loaded nanoparticles were shown in Fig. 12A. Two hours after intravenously administration of the formulations, obvious DiR signals were observed in the whole body due to the rapid circulation of the nanoparticles in the blood stream. During the time of imaging test, most of the formulations accumulated in the liver and tumors after intravenously administration of both formulations. The fluorescence in the DiR/SANPs treated group was much higher than that of the DiR/PLGA treated group in tumors at the same time points. At 12 h, the fluorescence of nanoparticles achieved the maximized level, and gradually decreased at 24 h. The ex vivo fluorescent images of excised organs and tumors further confirmed that the nanoparticles mainly accumulated in the liver, lung, spleen and tumors. In excised tumors, more fluorescence was observed in the DiR/SANPs treated group than in the DiR/PLGA treated group (Fig. 12B).

As a result, compared with the DiR/PLGA nanoparticles, the SANPs could be more effectively accumulated in CD44-overexpressing tumors.

3.9. In vivo antitumor assays

As shown in Fig. 13A, both PBS and blank SANPs exhibited negligible antitumor activity ($P > 0.05$). The nanoparticles showed enhanced antitumor activity than free DTX ($P < 0.01$). Notably, DTX/SANPs exhibited enhanced antitumor efficacy compared with DTX/PLGA ($P < 0.01$) (Fig. 13B). At the end of the treatment, tumors were excised and shown in Fig. 13C. The DTX/SANPs showed higher tumor inhibitory rate compared with DTX/PLGA ($P < 0.01$) and free DTX ($P < 0.001$) (Fig. 13D and E).

Safety profiles of DTX formulations were evaluated by measuring the changes in body weight as a function of time (Fig. 13F). Body weight did not decrease in the PBS and blank SANPs treated group, suggesting that SANPs had a good biocompatibility. However, there was an appreciable loss in the body weight of tumor-bearing mice after treatment of free DTX, whereas the loss in body weight was significantly decreased in the DTX/SANPs or DTX/PLGA treated groups. At the end of treatment, the body weight decrease rate in the free DTX treated group was much higher than the DTX/SANPs or DTX/PLGA treated groups ($P < 0.001$), whereas no significant difference in body weight decrease rate was observed between the DTX/SANPs and DTX/PLGA treated groups ($P > 0.05$) (Fig. 13G). These results indicated that DTX/SANPs showed significant antitumor activity against MDA-MB-231 xenograft with minimal toxicity.

4. Discussion

Self-assembled nanoparticles consisting of PLGA and HA block copolymers have been received great interest recently, owing to its superior advantages such as good biocompatibility [30], rapid biodegradability and low immunotoxicity [21]. Here we synthesized a series of PLGA-b-HA copolymers and developed
nanoparticles based on these copolymers. One of these copolymers, PLGA502H-b-HA5.6k, which formed nanoparticles with the smallest size and had a suitable CMC, was chosen to fabricate DTX-loaded PLGA502H-b-HA5.6k nanoparticles (DTX/SANPs) for further investigation.

To obtain the optimal PLGA-b-HA copolymer, five PLGA-b-HA copolymers with different hydrophobic and hydrophilic moieties were synthesized by conjugating the aminated HA with PLGA-NHS [29]. It is reported that the hydrophobic and hydrophilic moieties in block polymer had great effects on the important properties such as size and CMC [38,39]. The particle size of all the prepared nanoparticles composed of PLGA-b-HA copolymers in our study increased accompanied with the increase of the molecular weight of the copolymers, but the size of all the nanoparticles was smaller than 200 nm (Table 1). Such a small size of nanoparticles can be helpful in avoiding the clearance of reticuloendothelial system (RES) [40]. The CMC value, which is critical in keeping the core/shell structure of SANPs, is another important parameter in the characteristics of amphiphilic polymers. The CMC values of these copolymers ranged from 2.0 to 19.9 mg/L, which were relatively low CMC values and increased with the increase of the percent of hydrophilic moieties (Table 1). Notably, the zeta potential of all

Fig. 12. In vivo fluorescence images of DiR loaded PLGA502H (DiR/PLGA) and DiR loaded PLGA502H-b-HA5.6k nanoparticles (DiR/SANPs) in MDA-MB-231 tumor-bearing female nude mice after tail vein injection of the formulations. (A) The time-dependent images of whole body showed the distribution of the formulations in tumor-bearing mice. Images were taken at predetermined time intervals. (B) Ex vivo fluorescence images of excised organs and tumors at 24 h post-injection of the formulations.
prepared SANPs was negatively charged (∼30 mV). The negative zeta potential, which is due to the ionized carboxylic group of HA, can be beneficial in promoting the stability of nanoparticles, preventing the nanoparticles from binding with plasma proteins in the blood, and prolonging the in vivo circulation of nanoparticles. We selected one of the copolymers, PLGA502H-b-HA5.6k, which formed the smallest nanoparticles (∼111 nm) in aqueous condition and had a suitable CMC of 7.9 mg/L, in our subsequent experiments. As expected, PLGA502H-b-HA5.6k possessed superior stability, and their size and zeta potential keep relatively unchanged within one week.

Fig. 13. In vivo antitumor activity in MDA-MB-231-bearing female nude mice treated with various formulations. (A) Tumor growth curves evaluated by changes in tumor volume. (B) The enlarged profiles of DTX, DTX/PLGA and DTX/SANPs on the growth of tumors. (C) The images of excised tumor and (D) tumor weight at the end of the test after 18 days observation, scale bar = 1 cm. (E) Tumor inhibitory rate calculated with excised tumor weight. n.s. p > 0.05 vs PBS group; * p < 0.01 vs PBS group; ** p < 0.01 vs DTX/PLGA; *** p < 0.001 vs free DTX (n = 6). (F) Alteration in body weight of MDA-MB-231-bearing female nude mice after incubation of MDA-MB-231 cells. (G) Body weight decrease rate was calculated at the end of the test. "−" represents the body weight was increased. "×" p < 0.001 vs free DTX; n.s. p > 0.05 vs DTX/PLGA (n = 6).

The small size (∼111 nm), typical core/shell structure, a low CMC value (7.9 mg/L) and negative zeta potential (∼32 mV) greatly contribute to the superior stability of PLGA502H-b-HA5.6k nanoparticles. We incorporated DTX as a model drug into PLGA502H-b-HA5.6k nanoparticles. DTX was chosen due to its hydrophobic properties and superior antitumor activity in breast cancer therapy [41]. DTX-loaded PLGA502H-b-HA5.6k nanoparticles were successfully prepared, with a high encapsulating efficiency (58.38–87.55%) and drug loading content (1.67–3.11%), which were significantly higher.
than those of other previous prepared DTX-loaded copolymer nanoparticles [40,42]. The DTX release profile indicated that at low pH 5.5, DTX release in DTX/SANPs was higher than that at pH 7.4. The pH-sensitive property of DTX release in nanoparticles may be beneficial for reducing side effects of nanoparticles in circulation for reduced release at neutral pH and enhancing specific release in tumors which usually possess acidic pH. The cytotoxicity assay showed that although DTX/SANPs did not show significant enhanced cytotoxic effects than free DTX in MCF-7 cells, it showed in MDA-MB-231 cells, demonstrating the specific cytotoxic effect of DTX/SANPs toward CD44-overexpressing tumor cells. The specific targeting of HA-modified nanoparticles to CD44-overexpressing tumor cells was also demonstrated by other independent groups [19,20,29]. Subsequently, the mechanism of uptake was explored extensively. Results of CLSM and FCM analysis consistently showed that SANPs were taken up in MDA-MB-231 cells by CD44-mediated endocytosis via an energy-dependent endocytic pathway. In addition, we used the PLGA nanoparticles as the non-targeted control to elucidate the enhanced cellular uptake of SANPs in CD44 receptor over-expressed cells. On the other hand, the HA competitive assay and examination in two breast cancer cell lines with different CD44 expression provided sufficient evidence to elucidate the enhanced uptake mediated by HA-CD44 interaction.

For nanoparticles, long circulation is an essential desirable property, which can prevent fast elimination of drugs and provide sufficient time for the accumulation of the nanoparticles in target sites. In this study, DTX/SANPs was found to exhibit longer circulation time than that of DTX/PLGA or free DTX, and this result was in accordance with the long circulation time of other nanoparticles coated with HA shell [22].

Due to the permeability and retention effect (EPR), nanoparticles with a small size (<200 nm) can be passively accumulated in tumors. In this study, we examined the in vivo tumor targeting of nanoparticles, using DiR as a fluorescent probe. The results showed that much more DiR/SANPs were accumulated in tumors than DiR/PLGA, despite both nanoparticles also accumulated significantly in the liver and spleen.

The in vivo antitumor study further demonstrated the superior antitumor activity of DTX/SANPs. Although DTX, a potent antitumor reagent toward breast cancer in clinic, would be expected to show superior antitumor activity in vivo, our prepared nanoparticles (DTX/SANPs and DTX/PLGA) exhibited superior antitumor activity than free DTX, indicating the benefits of application of nanoparticles. Further, the superior antitumor activity of DTX/SANPs than DTX/PLGA indicated the necessity of HA shell in DTX/SANPs.

We also evaluated the systemic toxicity of drugs, according to the examination of the change of the body weight during the treatment. Although DTX was not well-tolerated in mice, DTX/SANPs and DTX/PLGA was well-tolerated in mice, indicating that DTX/SANPs and DTX/PLGA is a safe intervention in breast cancer therapy.

5. Conclusion

In this study, a series of PLGA-b-HA copolymers were successfully synthesized by an end to end coupling strategy. These copolymers could form SANPs with small size (<200 nm), typical core/shell structure, low CMC values, and negative zeta potential. The optimized PLGA90GA20-b-PLA35k (SANPs), which formed the smallest nanoparticles (∼111 nm) and had a suitable CMC of 7.9 mg/L was chosen for further studies. Blank SANPs showed good biocompatibility, while DTX/SANPs exhibited enhanced cytotoxicity toward CD44-overexpressing MDA-MB-231 cells. The studies of cellular uptake revealed that SANPs were taken up in MDA-MB-

231 cells by CD44-mediated endocytosis via an energy-dependent pathway. Pharmacokinetics study revealed SANPs could prolong the circulation of DTX in the blood stream. In vivo studies demonstrated that SANPs exhibited enhanced tumor targeting and antitumor activity compared with DTX/PLGA and DTX formulation. In conclusion, PLGA-b-HA nanoparticles can be used as a potential drug carrier to deliver hydrophobic chemotherapeutic drugs to CD44 over-expressed breast cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.09.089.

References


