Reversibly crosslinked hyaluronic acid nanoparticles for active targeting and intelligent delivery of doxorubicin to drug resistant CD44+ human breast tumor xenografts

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A B S T R A C T

The existence of drug resistance poses a major obstacle for the treatment of various malignant human cancers. Here, we report on reduction-sensitive reversibly crosslinked hyaluronic acid (HA) nanoparticles based on HA-lys-LA conjugates (Lys: 1-lysine methyl ester, LA: lipoic acid) for active targeting delivery of doxorubicin (DOX) to CD44+ breast cancers in vitro and in vivo, effectively overcoming drug resistance (ADR). HA-lys-LA nanoparticles formed robust nano-sized nanoparticles (152–219 nm) following auto-crosslinking. DOX-loaded crosslinked nanoparticles revealed inhibited DOX release under physiological conditions while fast drug release in the presence of 10 mM glutathione (GSH). Notably, MTT assays showed that DOX-loaded crosslinked HA-lys-LA10 nanoparticles possessed an apparent targetability and a superior anti-tumor activity toward CD44 receptor overexpressing DOX-resistant MCF-7 human breast cancer cells (MCF-7/ADR). The in vivo pharmacokinetics and biodistribution studies in MCF-7/ADR tumor xenografts in nude mice showed that DOX-loaded crosslinked HA-lys-LA10 nanoparticles had a prolonged circulation time and a remarkably high accumulation in the tumor (12.71% ID/g). Notably, DOX-loaded crosslinked HA-lys-LA10 nanoparticles exhibited effective inhibition of tumor growth while continuous tumor growth was observed for mice treated with free drug. The Kaplan–Meier survival curves showed that in contrast to control groups, all mice treated with DOX-loaded crosslinked HA-lys-LA10 nanoparticles survived over an experimental period of 44 days. Importantly, DOX-loaded crosslinked HA nanoparticles caused low side effects. The reversibly crosslinked hyaluronic acid nanoparticles with excellent biocompatibility, CD44-targetability, and effective reversal of drug resistance have a great potential in cancer therapy.

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1. Introduction

The existence of multidrug resistance (MDR) poses a major obstacle for the clinical treatment of various malignant human cancers [1,2]. In recent years, polymeric nanoparticles, which are of great interest in targeted cancer chemotherapy [3–5], have been reported to provide a potential approach to overcome drug resistance (ADR) in vitro and in vivo [6–8]. As a drug carrier, polymeric nanoparticles should be engineered with high stability therefore achieving long circulation time and minimizing premature drug release following intravenous injection [9,10], specific and efficient tumor cell uptake [11,12], as well as rapid and complete drug release inside the target tumor cells [13–15]. The therapeutic efficacy of nanoparticulate drugs would be markedly decreased if they failed to meet any one of the above requirements.

In the past years, reversibly crosslinked polymeric nanoparticles have been developed to achieve high extracellular stability as well as fast intracellular drug release [13,16]. In particular, disulfide-crosslinked nanoparticles that are prone to rapid de-crosslinking inside the tumor cells have received the most interest in that there exists a high reducing potential in the cytoplasm and cell nucleus [17–19]. Notably, nanoparticles could be crosslinked either with disulfide-containing crosslinkers such as cystamine and 3,3′-dithiobis(sulfosuccinimidyl propionate) [20–24], or via oxidizing free thiol groups in the prepolymer [25–28]. We reported facile preparation of disulfide-crosslinked micelles and nanoparticles from lipoic acid (LA) conjugates [29–32]. The lipoic ring is prone to ring-opening polymerization to form a linear polydisulfide in the presence of a catalytic amount of 1,4-dithio-o-threitol (DTT) under aqueous conditions. The rapid and efficient drug release inside the tumor cells, as triggered by an internal or external stimulus like cytoplasmic reducing potential, endo/lysosomal pH, and near infrared (NIR), would lead to a high intracellular drug concentration, resulting in reversal of drug resistance [33–36]. The anti-tumor effect of nanoparticulate drugs could
further be improved by installing targeting ligands such as peptides, saccharides, antibodies, and aptamers onto the surfaces of polymeric nanoparticles that facilitate efficient and specific cellular uptake via receptor-mediated endocytosis mechanism [37–40]. Notably, there are no reports on design and development of tumor-targeting disulfide-crosslinked nanoparticles for the treatment of drug-resistant cancers.

In this paper, we report on reduction-sensitive reversibly crosslinked hyaluronic acid (HA) nanoparticles based on HA-Lys-LA conjugates (Lys: l-lysine methyl ester, LA: lipoic acid) as a “natural” and multifunctional platform for active CD44-targeting doxorubicin (DOX) delivery and reversal of drug resistance in vitro and in vivo (Scheme 1). HA is a biocompatible and biodegradable natural polysaccharide present in the extracellular matrix and synovial fluids. HA could effectively target to CD44 receptor overexpressing cancer cells including drug-sensitive and resistant human breast adenocarcinoma cells (MDA-MB-231, MCF-7, MCF-7/ADR) and human lung cancer cells (A549, H69, H69/ADR) [41–45]. LA is a natural antioxidant produced by human body and has also been applied for the treatment of varying diseases including Alzheimer’s disease and diabetes [46,47]. Herein, crosslinked HA nanoparticles (denoted as HA X-NPs) were designed with the following features: (i) excellent biocompatibility and stability in blood circulation that minimizes premature drug release and improves in vivo pharmacokinetics; (ii) efficient uptake by DOX-resistant MCF-7 human breast tumor cells (MCF-7/ADR) via the receptor-mediated endocytosis mechanism; and (iii) fast drug release inside the tumor cells owing to reduction-triggered de-crosslinking of nanoparticles. Here, preparation and reduction-triggered disassembly of HA X-NPs, loading and in vitro release of DOX, targetability and antitumor activity toward MCF-7/ADR cells, in vivo pharmacokinetics and biodistribution, as well as therapeutic effects in drug-resistant human breast tumor xenografts in mice were investigated.

2. Materials and methods

2.1. Materials

Sodium hyaluronic acid (HA, molecular weight: 35 kDa, Shandong Freda Biopharm Co., Ltd.), N-tert-butoxycarbonyl-l-lysine methyl ester

Scheme 1. Illustration of disulfide-crosslinked HA nanoparticles (HA X-NPs) based on HA-Lys-LA conjugates as a “natural” and multifunctional platform for active CD44-targeting DOX delivery and reversal of drug resistance (ADR). (i) HA-Lys-LA conjugates are self-assembled into core–shell nanoparticles that allow efficient loading of DOX in water; (ii) DOX-loaded HA nanoparticles (HA NPs) can be crosslinked and stabilized by a catalytic amount of DTT; (iii) DOX-loaded HA X-NPs can be efficiently taken up by CD44-overexpressing drug-resistant cancer cells via a receptor-mediated endocytosis mechanism; and (iv) HA X-NPs will be automatically de-crosslinked inside the cancer cell as triggered by cytoplasmic GSH, leading to nanoparticle swelling, efficient intracellular drug release and reversal of ADR.
hydrochloride (H-Lys[Boc]–OMe·HCl, 98%, GL Biochem (Shanghai) Ltd.), triethylamine (99%, Alfa Aesar), 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (EDC, 98%, Alfa Aesar), N-hydroxysuccinimide (NHS, 98%, J&K), trifluoroacetic acid (TFA, 99%, 9 ding Chemistry (Shanghai) Ltd.), lipic acid (LA, 98%, Acros), 1,3-dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), 4-dimethylaminopyridine (DMAP, 99%, Alfa Aesar), 1,4-dithio-di-o-tolylene disulfide (DTT, 99%, Merck), glutathione (GSH, 99%, Roche), doxorubicin hydrochloride (DOX–HCl, > 99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), anhydrous methanol, and anhydrous formamide were used as received. N,N-dimethyl formamide (DMF) was dried by refluxing over anhydrous magnesium sulphate and distilled under reduced pressure before use. Dichloromethane (DCM) was dried by refluxing over CaH2 and distilled prior to use. Lipic acid anhydride (LAA) was synthesized according to our previous report [29].

2.2. Synthesis of HA-Lys and HA-Lys-LA conjugates

HA-Lys was obtained in two steps. Firstly, triethylamine (85 mg, 0.84 mmol) was added to a stirred solution of H-Lys(Boc)-OMe·HCl (240 mg, 0.80 mmol) in anhydrous methanol (2.0 mL) at room temperature. The mixture was stirred for 1 h. A solution of HA (300 mg, 0.79 mmol carbonyl group), EDC (460 mg, 2.40 mmol) and NHS (140 mg, 1.22 mmol) in D.I. water (5 mL) was added with pH 8.5. The mixture was stirred for 24 h at room temperature. The resulting HA-Lys(Boc) adduct was isolated by extensive dialysis against D.I. water (Spectra/Pore, MWCO 3500) followed by lyophilization. Yield: 94%. Then, the Boc group in HA-Lys(Boc) was removed using TFA/1 M HCl (v/v 1/1). The reaction was allowed to proceed for 6 h. The HA-Lys adduct was isolated by adjusting solution pH to ca. 7.0 using 4 M NaOH, extensive dialysis against D.I. water (Spectra/Pore, MWCO 3500), and lyophilization. Yield: 92%.1HNMR (D2O): HA: δ 1.86–2.01, 3.28–4.02, and 4.21–4.75; Lys: δ 1.13, 1.86–2.01, and 2.86. The degree of substitution (DS) was determined to be 10 (denoted accordingly as HA-Lys10) by comparing the integrals of signals at δ 2.86 (Lys) and 4.21–4.75 (anomic proton in HA). In a similar way, we have also obtained HA-Lys5 and HA-Lys28 by changing H-Lys(Boc)-OMe·HCl/carboxyl group of HA feed ratios to 1:2 and 2:1, respectively.

HA-Lys-LA was prepared in two steps from HA-Lys. Firstly, under a nitrogen atmosphere, to a stirred solution of lipic acid (12 mg, 58 μmol) in DCM (1.0 mL), a solution of DCC (6 mg, 29 μmol) and pyridine (DMAP, 99%, Alfa Aesar), 1,4-dithio-D,L-threitol (DTT, 99%, Merck), glutathione (GSH, 99%, Roche), doxorubicin hydrochloride (DOX–HCl, > 99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), anhydrous methanol, and anhydrous formamide were used as received. N,N-dimethyl formamide (DMF) was dried by refluxing over anhydrous magnesium sulphate and distilled under reduced pressure before use. Dichloromethane (DCM) was dried by refluxing over CaH2 and distilled prior to use. Lipic acid anhydride (LAA) was synthesized according to our previous report [29].

Typically, nanoparticles were prepared by dropwise addition of 4.0 mL of phosphate buffer (PB, pH 7.4, 10 mM) to a formamide solution (1.0 mL) of HA-Lys-LA (5 mg/mL) under stirring at room temperature, followed by extensive dialysis (Spectra/Pore, MWCO 3500) against PB (pH 7.4, 10 mM) for 24 h. The CAC was determined using pyrene as a fluorescence probe. The concentration of the conjugates was varied from 1.0 × 10−5 to 0.1 mg/mL and the concentration of pyrene was fixed at 1.0 μM. The fluorescence spectra were recorded using a FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio I372/I383 at low and high concentration regions. The crosslinking of HA NPs was carried out under a nitrogen atmosphere at room temperature in the presence of 10 mol% DTT relative to the lipoyl units, as reported previously [29]. In brief, DTT (7 μg, 10 mol% relative to the lipoyl units) stock solution in PB (10 mM) was added to 2.0 mL of HA-Lys-LA5 nanoparticle dispersion (0.5 mg/mL). The mixture was stirred at room temperature for 24 h and dialyzed against PB (10 mM, pH 7.4) for 12 h (Spectra/Pore, MWCO 3500). The colloidal stability of X-NPs against large volume dilution as well as high salt concentrations was investigated via DLS.

2.5. Loading and reduction-triggered release of DOX

DOX-loaded nanoparticles were prepared by dropwise addition of 4 mL PB (10 mM, pH 7.4) to a mixture of polymer (1.0 mL, 5 mg/mL) in formamide and DOX (250 μL, 5 mg/mL) in DMSO under stirring at room temperature, followed by dialysis against PB (10 mM, pH 7.4) for 24 h (Spectra/Pore, MWCO 3500). The dialysis medium was changed five times. The whole procedure was performed in the dark. DOX-loaded nanoparticles were crosslinked as described above. The amount of DOX was determined using fluorescence (FLS920) measurement (excitation at 480 nm and emission at 580 nm). For determination of drug loading content (DLC), lyophilized DOX–loaded nanoparticles were dissolved in formamide and analyzed with fluorescence spectroscopy, wherein calibration curve was obtained with DOX/formamide solutions with different DOX concentrations. DLC and drug loading efficiency (DLE) were calculated according to the following formula:

\[
\text{DLC (wt%)} = \left(\frac{\text{weight of loaded drug}}{\text{total weight of drug and polymer}}\right) \times 100\% \\
\text{DLE (wt%)} = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug in feed}}\right) \times 100\%
\]

The drug release from HA-Lys-LA5, HA-Lys-LA10, and HA-Lys-LA28 nanoparticles, either crosslinked or non-crosslinked, was studied using a dialysis tube (Spectra/Pore, MWCO 12000) at 37 °C in two different media, i.e. PB (10 mM, pH 7.4) and PB (10 mM, pH 7.4) containing 10 mM GSH. The release studies were performed at a low nanoparticle concentration of 10 mg/L. In order to acquire sink conditions, drug release studies were performed with 0.6 mL of DOX-loaded nanoparticle dispersion dialysis against 20 mL of the corresponding medium. At desired time intervals, 6.0 mL of release medium was taken out and replenished with an equal volume of fresh medium. The amount of DOX released was determined by using fluorescence measurement (FLS920). The release experiments were conducted in triplicate, and the results presented were the average data with standard deviations.
2.6. In vitro cytotoxicity assays

The cytotoxicity of HA NPs was determined using DOX-resistant MCF-7 human breast cancer cell line (MCF-7/ADR) that expresses a high level of CD44 receptors. MCF-7/ADR cell line was originated from the Cancer Center of the Second Affiliated Hospital of Zhejiang University School of Medicine. For culture of MCF-7/ADR cells, DOX at a concentration of 2–5 μg/mL was added to maintain the drug resistance of the cells. One week before the experiments, the culture medium without DOX was added as replacement. The cells were plated in a 96-well plate (2 × 10^4 cells/well) using RPMI 1640 media supplemented with 10% fetal bovine serum, 1% l-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 24 h, prescribed amounts of bare nanoparticles in 20 μL of PBS were added (final nanoparticle concentrations were fixed at 0.5 and 1.0 mg/mL) and incubated in an atmosphere containing 5% CO_2 for 48 h at 37 °C. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) solution in PBS (20 μL, 5 mg/mL) was added. After incubation for 4 h, the supernatant was carefully aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO for 20 min. The absorbance at a wavelength of 490 nm was measured using a microplate reader (Bio-Tek, ELX808IU). The cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. The experiments were performed in quartets.

The antitumor activity of DOX-loaded HA NPs and free DOX was also studied by MTT assays. Briefly, the cells were plated in a 96-well plate (2 × 10^4 cells/well) using RPMI 1640 media supplemented with 10% fetal bovine serum, 1% l-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 24 h, the prescribed amounts of DOX-loaded HA NPs or free DOX at different DOX concentrations in 20 μL of PBS were added and incubated in an atmosphere containing 5% CO_2 for 4 h at 37 °C. The medium was aspirated and replaced by fresh medium, and the cells were incubated in an atmosphere containing 5% CO_2 for another 48 h at 37 °C. MTT solution in PBS (20 μL, 5 mg/mL) was added. After incubation for 4 h, the supernatant was carefully aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO for 20 min. The cell viabilities were determined by MTT assays as described above. The inhibition experiments were performed by pre-treating MCF-7/ADR cells with free HA (5 mg/mL) for 4 h prior to incubating with DOX-loaded HA-Lys-LA_{10} X-NPs.

2.7. Flow cytometry assays and confocal microscopy measurements

The cellular uptake and intracellular drug release behaviors of DOX-loaded HA-Lys-LA_{10} X-NPs were studied in MCF-7/ADR cells using flow cytometry and confocal laser scanning microscopy (CLSM). The cells were seeded in a 6-well plate (1 × 10^6 cells/well) using RPMI 1640 media containing 10% fetal bovine serum, 1% l-glutamine, and antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 12 h. DOX-loaded HA-Lys-LA_{10} X-NPs, DOX-loaded HA-Lys-LA_{10} NX-NPs, or DOX-HCl in 400 μL of phosphate buffered saline (PBS) was added to each well (5.0 μg DOX/mL). After incubation at 37 °C for 10 h, the cells were digested by 0.25% (w/v) trypsin and 0.03% (w/v) EDTA. The suspensions were centrifuged at 700 × g for 5 min at 4 °C, washed twice with PBS, and then re-suspended in 500 μL of PBS. Fluorescence histograms were then recorded with a BD FACS Calibur flow cytometer (Becton Dickinson, USA) and analyzed using Cell Quest software. We analyzed 20,000 gated events to generate each histogram. The gate was arbitrarily set for the detection of red fluorescence. The inhibition experiments were performed by pre-treating MCF-7/ADR cells with free HA (5 mg/mL) for 4 h prior to incubating with DOX-loaded HA-Lys-LA_{10} X-NPs.

MCF-7/ADR cells were cultured on microscope slides in a 24-well plate (5 × 10^4 cells/well) using RPMI 1640 media containing 10% fetal bovine serum, 1% l-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 24 h, DOX-loaded HA-Lys-LA_{10} X-NPs or free DOX in 100 μL of PBS was added to each well (DOX dosage: 5.0 μg/mL). After 10 h incubation, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were then fixed with 4% paraformaldehyde solution for 20 min and washed with PBS containing 0.1% Triton X-100 for three times. The cytoskeleton was stained with fluorescein isothiocyanate-labeled phalloidin (phalloidin–FITC, green) for 1 h and washed with PBS for three times. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 20 min and washed with PBS for three times. The fluorescence images were obtained using a confocal microscope (TCS SP2). The inhibition experiments were performed by pre-treating MCF-7/ADR cells with free HA (5 mg/mL) for 4 h prior to incubating with DOX-loaded HA-Lys-LA_{10} X-NPs.

2.8. Blood circulation and in vivo imaging

The mice were handled under protocols approved by Soochow University Laboratory Animal Center and the Animal Care and Use Committee of Soochow University. The DOX level in blood was measured by withdrawing ~10 μL of blood from the tail vein of nude mice at different time points post-injection of DOX-loaded HA-Lys-LA_{10} X-NPs, and free DOX (10 mg DOX equiv./kg). Each blood sample was dissolved in 0.3 mL of lysis buffer (1% Triton X-100) with brief sonification. DOX was extracted by incubating blood samples in HCl-IPA at ~20 °C overnight. After centrifugation at 14.8 krpm for 30 min, the DOX level of the supernatant was determined by fluorescence measurement. The blood circulation followed a typical two compartment model. The first phase is distribution phase with usually a rapid decline. The second phase is elimination phase with usually a long period, which is the predominant process for drug clearance. We calculated the half-lives of two phases (t_1 and t_2) according to the following formula:

\[ y = A_1 \times \exp(-x/t_1) + A_2 \times \exp(-x/t_2) + y_0. \]

In order to monitor nanoparticles using fluorescence imaging in vivo, fluorescent molecule Cy7 was loaded into HA-Lys-LA_{10} X-NPs. DOX-resistant human breast tumor xenograft model was established by subcutaneous inoculation of 1 × 10^7 MCF-7/ADR in 50 μL serum-free RPMI 1640 media into the hind flank of each mouse. When the size of tumors reached about 200 mm^3, the tumor-bearing mice were injected with Cy7-loaded HA-Lys-LA_{10} X-NPs via tail vein. The fluorescent scans were performed at various time points (1, 4, 10 and 24 h) post i.v. injection using the Maestro in vivo fluorescence imaging system (CRI Inc.).

2.9. Ex vivo imaging and biodistribution

Taking advantage of the fluorescent nature of DOX, biodistribution studies were also performed with ex vivo fluorescence imaging. A single dose of DOX-loaded HA-Lys-LA_{10} X-NPs, or free DOX in 0.2 mL of PBS was administrated intravenously via the tail vein at a dosage of 10 mg DOX equiv./kg. After 10 h, MCF-7/ADR tumor-bearing mice were sacrificed. The tumor block and several major organs including the heart, liver, spleen, lung, and kidney were collected, washed, and dried. Fluorescence images were acquired with the Maestro in vivo fluorescence imaging system (CRI Inc.).

To quantify the amount of DOX delivered to the tumor and different organs, tumor-bearing mice following 10 h i.v. injection with DOX-loaded HA-Lys-LA_{10} X-NPs or free DOX (10 mg DOX equiv./kg) were sacrificed. The tumor block and organs including the heart, liver, spleen, lung, and kidney were collected, washed, weighed, and then homogenized in 0.5 mL of 1% Triton X-100 with a W6/10 Superfine Homogenizer (Fluko). For DOX measurements, 1.5 mL of the extraction solution (HCl-IPA) was added to tissue lysates, and the samples were incubated at −20 °C overnight. After centrifugation at 14.8 krpm for 30 min, the amount of DOX was quantified by fluorescence measurement.
2.10. In vivo antitumor efficacy

MCF-7/ADR tumor-bearing mice were treated with DOX-loaded HA-Lys-LA10 X-NPs, and free DOX at a dosage of 7.5 mg DOX equiv./kg. The drugs were intravenously injected every six days. PBS was used as a blank control. The tumor sizes were measured by calipers twice a week and volume was calculated according to the formula \( V = 0.5 \times L \times W \times H \), wherein \( L \) is the tumor dimension at the longest point, \( W \) is the tumor dimension at the widest point, and \( H \) is the tumor dimension at the highest point. Relative tumor volumes were calculated as \( V/V_0 \) (\( V_0 \) is the tumor volume when the treatment was initiated). Mice were weighed with the relative body weights normalized to their initial weights. Mice in each cohort were considered to be dead either when the tumor volume increased to 1000 mm\(^3\), or when the mice died during treatment.

2.11. Histological analysis

At the end of the treatment, one mouse of each group was sacrificed, and the tumor, liver, and heart were excised. The tissues were fixed with 4% paraformaldehyde solution and embedded in paraffin. The sliced organ tissues (thickness: 4 mm) mounted on the glass slides were stained by hematoxylin and eosin (H&E) and observed by a digital microscope (Leica QWin).

3. Results and discussion

3.1. Formation of disulfide-crosslinked HA nanoparticles from HA-Lys-LA conjugates

The aim of this study was to develop reduction-sensitive reversibly crosslinked HA nanoparticles for active CD44-targeting DOX delivery, for which “all-natural” HA-Lys-LA (Lys: \( L \)-lysine methyl ester, LA: \( L \)-lipoic acid) conjugates were designed. Lys was used as a biocompatible linker. HA-Lys-LA conjugates were readily obtained in three steps (Scheme S1). Firstly, \( N \)-tert-butoxycarbonyl-L-lysine methyl ester (\( H \)-Lys(Boc)-OMe) was conjugated to HA by carbodiimide chemistry. Then, the Boc group was de-protected using trifluoroacetic acid/1 M HCl (v/v 1/1) to furnish HA-Lys adduct. \(^1\)H NMR spectrum showed clearly signals attributable to Lys moieties (\( \delta \) 1.13, 1.86–2.01, 2.86) and HA backbone (\( \delta \) 1.86–2.01, 3.28–4.02, 4.21–4.75) (Fig. S1). The degree of substitution (DS), which is defined as the number of Lys units per 100 sugar residues of HA polymer, could be determined by comparing the integrals of signals at \( \delta \) 2.86 (methylene protons next to the amine group of Lys) and \( \delta \) 4.21–4.75 (ammonic proton in HA). Here, HA-Lys conjugates with different DS of 5, 10, and 28 were obtained at varying H-Lys(Boc)-OMe/HA carbonyl group molar feed ratios of 1/2, 1/1, and 2/1, respectively. Finally, HA-Lys-LA conjugates were prepared by treating HA-Lys with lipoic acid anhydride (LAA) at an amino group/LAA molar ratio of 1/2. \(^1\)H NMR of HA-Lys-LA displayed besides signals attributable to Lys moieties (\( \delta \) 1.13, 1.86–2.01) and HA (\( \delta \) 1.86–2.01, 3.28–4.02, 4.21–4.75) also resonances due to lipoyl protons at \( \delta \) 1.29–1.68, 2.15–2.47 and 2.88–2.98 (Fig. S2). Notably, a signal at \( \delta \) 2.86 owing to the methylene protons next to the amine group of Lys-LA10, and HA-Lys-LA28 nanoparticles at pH 7.4 and 37 °C at a low drug loading efficiency (DLE) of X-NPs increased with increasing DS, wherein HA-Lys-LA10 X-NPs exhibited excellent colloidal stability (Fig. 1C). Moreover, HA-Lys-LA10 X-NPs showed also clearly better stability against 2 M NaCl than their non-crosslinked counterparts (Fig. S3). However, in the presence of 10 mM GSH, HA-Lys-LA10 X-NPs swelled quickly to over 1000 nm in 12 h and were dissociated into unimers upon extensive dilution (Fig. 1D), indicating that de-crosslinking is fast and complete. The large size increase of X-NPs following treatment with 10 mM GSH is most likely due to increased hydrophilicity of nanoparticle core after converting lipoic groups into dihydroxipic groups (Scheme 1). It is evident, therefore, that HA X-NPs while having superior colloidal stability under physiological conditions are prone to rapid disassembly under a reductive condition mimicking that of the cytoplasm and cell nucleus.

3.2. Loading and reduction-triggered release of DOX

Loading of DOX into HA NPs was performed at a polymer concentration of 1 mg/mL and a theoretical drug loading content (DLC) of 20.0 wt%. Similar to bare nanoparticles, DOX-loaded nanoparticles could be readily crosslinked using a catalytic amount of DTT. DLS showed that DOX-loaded HA-Lys-LA X-NPs had mean diameters of 154–225 nm, which decreased with increasing DS (Table 2). The drug loading efficiency (DLE) of X-NPs increased with increasing DS, wherein HA-Lys-LA10 X-NPs gave a high DLC of 15.1 wt.% and a high DLE of 71.2%. The in vitro drug release studies were carried out using HA-Lys-LA5, HA-Lys-LA10, and HA-Lys-LA28 nanoparticles at pH 7.4 and 37 °C at a low polymer concentration of 10 mg/L close to CAC of HA-Lys-LA conjugates (6.2–8.8 mg/L). The results showed that the release of DOX from HA-NPs under physiological condition (pH 7.4, 37 °C) was largely inhibited by crosslinking (Fig. 2). Taking HA-Lys-LA10 NPs as an example, ca. 24.2 and 80.3% drug was released in 22 h from crosslinked and non-crosslinked NPs, respectively. However, drug release from crosslinked nanoparticles was markedly enhanced in the presence of 10 mM GSH under otherwise the same conditions, wherein ca. 89.4%, 86.5%, and 79.7% drug was released in 22 h from HA-Lys-LA5, HA-Lys-LA10, and HA-Lys-LA28 X-NPs, respectively (Fig. 2). Interestingly, drug release from DOX-loaded HA-Lys-LA X-NPs in the presence of 10 mM GSH was slightly faster than corresponding NX-NPs in the absence of GSH, indicating rapid and complete de-crosslinking. The faster drug

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<th>Polymer</th>
<th>NX-NPs</th>
<th>X-NPs</th>
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<td>PDI ( a )</td>
<td>Zeta ( a ) (mV)</td>
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<td>0.11</td>
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\( a \) Determined by DLS analysis using the Zetasizer Nano-ZS (Malvern Instruments) at 25 °C in water.

\( b \) Determined using pyrene as a fluorescence probe.
release of X-NPs in the presence of GSH is likely due to their enhanced core hydrophilicity (dihydroxylipoyl group) as compared to that in the corresponding NX-NPs (lipoyl group). Similar results have also been observed for crosslinked dextran-LA nanoparticles [29].

3.3. CD44-targeting DOX delivery and antitumor activity

The occurrence of drug-resistance is a major challenge for cancer chemotherapy [2,48]. The antitumor activity of DOX-loaded HA NPs was evaluated by MTT assays in MCF-7/ADR cells, which are known to express high level of CD44 receptors (Fig. S5). Interestingly, MTT assays revealed that all HA-NPs, either crosslinked or non-crosslinked, were nontoxic toward MCF-7/ADR cells up to a tested nanoparticle concentration of 1.0 mg/mL (Fig. 3A), corroborating that "natural" HA-Lys-LA nanoparticles possess excellent biocompatibility. The antitumor activity of DOX-loaded HA-Lys-LA X-NPs to MCF-7/ADR cells was shown to

<table>
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<th>Entry</th>
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<td>HA-Lys-LA10</td>
<td>189</td>
<td>0.18</td>
<td>12.0</td>
<td>54.5</td>
</tr>
<tr>
<td>3</td>
<td>HA-Lys-LA28</td>
<td>154</td>
<td>0.12</td>
<td>15.1</td>
<td>71.2</td>
</tr>
</tbody>
</table>

Table 2: DOX-loaded HA-Lys-LA X-NPs (theoretical drug loading content = 20.0 wt.%).

![Fig. 1. Characteristics of crosslinked and non-crosslinked HA-Lys-LA10 nanoparticles. (A) Size distribution of HA-Lys-LA10 X-NPs and NX-NPs measured by DLS; (B) TEM micrograph of HA-Lys-LA10 X-NPs; (C) colloidal stability of HA-Lys-LA10 X-NPs against extensive dilution; and (D) change of size distribution profiles of HA-Lys-LA10 X-NPs in response to 10 mM GSH in PBS (pH 7.4, 10 mM) at 37 °C.](image)

![Fig. 2. Reduction-triggered release of DOX from HA-Lys-LA5, HA-Lys-LA10, and HA-Lys-LA28 X-NPs at pH 7.4 and 37 °C in PBS buffer. NX-NPs were used as controls. The drug release studies were performed at a low nanoparticle concentration of 10 mg/L. Data are presented as mean ± SD (n = 3).](image)
highly depend on DS of HA in which HA-Lys-LA10 X-NPs exhibited the highest antitumor activity and HA-Lys-LA28 X-NPs the lowest (Fig. 3B). It should be noted that DOX-loaded HA-Lys-LA28 X-NPs had a low half maximal inhibitory concentration (IC50) of ca. 2.8 μg DOX equiv./mL, which was comparable to DOX-loaded non-crosslinked counterparts (2.5 μg DOX equiv./mL) under otherwise the same conditions while free DOX induced no obvious inhibition to MCF-7/ADR cells even at a DOX dosage of 10 μg/mL (Fig. 3B), supporting efficient internalization of DOX-loaded HA-Lys-LA10 X-NPs into MCF-7/ADR cells as well as rapid de-crosslinking and intracellular drug release, leading to effective reversal of drug resistance in MCF-7/ADR cells. To further confirm that MCF-7/ADR cells have a high drug resistance, we have also performed MTT assays in the parental MCF-7 cells (drug sensitive). The results showed that free DOX had a much lower IC50 of 2.9 μg/mL while HA-Lys-LA10 X-NP-DOX displayed a comparable IC50 of 3.4 μg/mL. In contrast, DOX-loaded HA-Lys-LA28 X-NPs displayed a much lower antitumor activity to MCF-7/ADR cells with an IC50 of 13.0 μg DOX equiv./mL, which is likely due to a diminishing targetability caused by over substitution of the carboxylic acid groups in HA. It has been reported that the carboxylic acid groups in HA play a critical role in CD44-targeting [41,49,50]. The antitumor activity of HA-Lys-LA10 X-NPs was significantly reduced by pretreating MCF-7/ADR cells with free HA (Fig. 3B). These results point out that HA-Lys-LA10 X-NPs possessed apparent targetability to CD44-positive cells and can efficiently deliver and release DOX into target cancer cells, inducing high antitumor efficacy and reversal of drug resistance. In the following, HA-Lys-LA28 X-NPs were adopted for further in vitro and in vivo studies. Notably, DOX-loaded HA-Lys-LA10 X-NPs (denoted as X-NP-DOX) following lyophilization could readily be re-dispersed in PBS with little change of nanoparticle sizes and distribution.

The cellular uptake and intracellular drug release behaviors of DOX-loaded HA-Lys-LA10 X-NPs were studied in MCF-7/ADR cells using flow cytometry and confocal laser scanning microscopy (CLSM). As fluorescence of DOX is self-quenched when encapsulated into nanoparticles, DOX fluorescence observed inside cells correlates with the intracellular DOX release [51,52]. As expected, flow cytometry showed that MCF-7/ADR cells incubated with free DOX for 10 h revealed low intracellular DOX fluorescence level (Fig. 4A), likely due to drug efflux or activation of coordinate-ly regulated detoxifying systems. In comparison, MCF-7/ADR cells following 10 h treatment with X-NP-DOX displayed greatly enhanced DOX fluorescence (Fig. 4A). The inhibition experiments showed that DOX fluorescence in MCF-7/ADR cells was markedly reduced by pre-treating the cells with free HA prior to incubating with X-NP-DOX (Fig. 4A), confirming that X-NP-DOX was taken up by MCF-7/ADR cells via a receptor-mediated mechanism. It is evident, therefore, that X-NP-DOX can effectively increase the cellular uptake and the intracellular drug concentration in ADR cancer cells, leading to a potent inhibition effect. CLSM results further showed that strong DOX fluorescence was detected in the nuclei of MCF-7/ADR cells following 10 h incubation with X-NP-DOX while DOX fluorescence was negligible in the cells treated with free DOX and cells pretreated with free HA (Fig. 4B), confirming efficient uptake of X-NP-DOX by MCF-7/ADR cells and fast intracellular drug release.

### 3.4. In vivo pharmacokinetics and imaging studies

In the following, we investigated the in vivo pharmacokinetics of X-NP-DOX in mice. The plasma levels of DOX were determined by fluorescence spectroscopy at different time intervals following a single i.v. injection of X-NP-DOX or free DOX (10 mg DOX equiv./kg) in nude mice. Notably, X-NP-DOX revealed a significantly longer circulation time than free DOX, in which the concentration of DOX in plasma decreased to undetectable levels after 2 h injection of free DOX whereas considerable amount of DOX was observed even at 24 h following administration of X-NP-DOX (Fig. 5A). The distribution phase half-lives were determined to be 0.48 and 0.13 h, while the elimination phase half-lives were 4.83 and 0.42 h, for X-NP-DOX and free DOX, respectively (Fig. 5A). The long circulation time in blood might be attributed to high stability of X-NP-DOX.

To evaluate their in vivo tumor-targetability, Cy7-loaded X-NPs was injected intravenously to mice bearing human MCF-7/ADR tumor xenografts and monitored using a Maestro VX in vivo fluorescence imaging system (CRi, Inc.). The fluorescence images showed significant tumor accumulation of X-NPs at 1 h post injection and Cy7 fluorescence intensity reached maximum in the tumor site at 10 h and was still strong even at 24 h post injection (Fig. 5B), confirming high tumor accumulation and retention of X-NPs.

### 3.5. Ex vivo fluorescence imaging and biodistribution

To examine the distribution of released DOX in tumor-bearing mice, ex vivo fluorescence images of tumor and several major organs following 10 h i.v. injection of X-NP-DOX or free DOX were taken. The
distribution ratios of DOX are summarized in Table 3. These collected data obviously demonstrated that X-NP-DOX could reduce DOX uptake (DOX dosage: 5.0 μg/mL). DOX-loaded HA-Lys-LA10 NX-NPs (NX-NP-DOX) and free DOX were used as controls. The competitive inhibition experiment was performed by pre-treating MCF-7/ADR cells with free HA (5 mg/mL) for 4 h before adding X-NP-DOX, and CLSM images of MCF-7/ADR cells following 10 h incubation with X-NP-DOX (DOX dosage: 5.0 μg/mL) (I). The cells treated with free DOX (II) and cells pre-treated with free HA before adding X-NP-DOX (III) were used as controls. For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), cytoskeleton stained by phalloidin-FITC (green) and overlays of the three images. The scale bars correspond to 25 μm in all the images.

**Fig. 4. Flow cytometry and confocal microscopy studies of DOX-loaded HA-Lys-LA10 X-NPs. (A) Flow cytometry of MCF-7/ADR cells following 10 h incubation with X-NP-DOX (DOX dosage: 5.0 μg/mL). DOX-loaded HA-Lys-LA10 NX-NPs (NX-NP-DOX) and free DOX were used as controls. The competitive inhibition experiment was performed by pre-treating MCF-7/ADR cells with free HA (5 mg/mL) for 4 h before adding X-NP-DOX, and (B) CLSM images of MCF-7/ADR cells following 10 h incubation with X-NP-DOX (DOX dosage: 5.0 μg/mL).**

**3.6. In vivo therapeutic efficacy of X-NP-DOX**

The therapeutic performance of X-NP-DOX was evaluated using MCF-7/ADR tumor-bearing nude mice. When tumors grew up to about 50 mm³ in volume, mice were treated with X-NP-DOX and free DOX (7.5 mg DOX equiv./kg). Mice treated with PBS were used as control. The treatment was repeated every six days. The results showed that tumor growth was effectively inhibited by X-NP-DOX while continuous tumor growth was observed for mice treated with free DOX (Fig. 7A). In the following, the amount of DOX accumulated in major organs such as the heart, liver, spleen, lung and kidney was quantified using fluorescence measurements. It is remarkable to note that the tumor uptake of DOX was 12.71% of injected dose per gram of tissue (% ID/g) for X-NP-DOX, which was about 20-fold as that for free DOX (0.63% ID/g) (Fig. 6B). Moreover, X-NP-DOX displayed reduced accumulation in the heart, spleen, lung, and kidney as compared with free DOX. The tumor-to-normal tissue (T/N) distribution ratios of DOX are summarized in Table 3. These collected data obviously demonstrated that X-NP-DOX could reduce DOX uptake by healthy organs or tissues particularly the heart, spleen, and lung while largely increase DOX accumulation in the human breast tumors.
was observed for mice treated with free DOX. Moreover, the heart tissue of mice treated with free DOX indicated atrophy cardiac myocyte and infiltration of inflammatory cells. It’s interesting to note that liver damage is insignificant for X-NP-DOX, though HA nanoparticles were previously observed with unwanted accumulation in the liver due to cellular uptake of phagocytic cells and/or liver sinusoidal endothelial cells [53]. The “stealth” property of HA nanoparticles could be improved by chemical conjugation with PEG [54]. However, PEGylation will also compromise their CD44 targetability, which is not desired. X-NP-DOX though exhibiting somewhat higher accumulation in the liver than in the other organs such as the heart, kidney, spleen and lung had less liver accumulation than free DOX (Fig. 6B). In contrast, significantly enhanced accumulation in the tumor was observed for X-NP-DOX as compared to free DOX. Therefore, X-NP-DOX possesses excellent CD44 targetability and comparably low accumulation in the healthy organs including liver. Our results point out that X-NP-DOX has not only potent therapeutic efficacy toward drug resistant CD44 positive breast tumor xenografts in mice but also low side effects.

4. Conclusions

We have demonstrated for the first time that reduction-sensitive reversibly crosslinked hyaluronic acid nanoparticles based on HA-Lys-LA conjugates are a robust and integrative platform for active CD44-targeting delivery of doxorubicin, affording potent antitumor effect toward CD44-positive drug-resistant human breast cancers in vitro and in vivo. These multifunctional nanoparticles possess several unique features: (i) they are solely made of “natural” compounds (i.e. hyaluronic acid, lysine and lipoic acid) and are therefore inherently biocompatible and biodegradable; (ii) they have excellent colloidal stability, inhibited drug leakage, and prolonged blood circulation time; (iii) they can actively target to CD44 receptor-overexpressing tumors, giving a remarkably high drug accumulation in the tumor (12.71% ID/g); and (iv) they can be rapidly internalized by CD44-positive tumor cells via a receptor-mediated endocytosis mechanism, and de-crosslinked and destabilized under the cytoplasmic reductive conditions, resulting in superior antitumor effect in CD44-positive drug-resistant human breast tumor xenografts in nude mice. These reversibly crosslinked hyaluronic acid nanoparticles with excellent CD44-targetability, effective reversal of drug resistance, and low side effects have appeared as a novel potent platform for CD44-targeted cancer chemotherapy.

Table 3
Summary of tumor-to-normal tissue (T/N) distribution ratios of DOX at 10 h after i.v. injection. Data are presented as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-NP-DOX</td>
<td>9.39 ± 0.49</td>
<td>7.70 ± 0.19</td>
<td>1.36 ± 0.30</td>
<td>10.40 ± 0.28</td>
<td>2.19 ± 0.31</td>
</tr>
<tr>
<td>Free DOX</td>
<td>0.31 ± 0.12</td>
<td>0.20 ± 0.05</td>
<td>0.05 ± 0.04</td>
<td>0.36 ± 0.17</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

Fig. 6. In vivo biodistribution of X-NP-DOX and free DOX in MCF-7/ADR human breast tumor bearing nude mice at 10 h post intravenous injection. (A) DOX fluorescence images of organs and tumors (1: heart, 2: liver, 3: spleen, 4: lung, 5: kidney, and 6: tumor); and (B) quantification of DOX accumulated in different organs and tumors using fluorescence spectroscopy. DOX uptake is expressed as injected dose per gram of tissue (% ID/g). Data are presented as mean ± SD (n = 3).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.01.012.
Fig. 7. *In vivo* antitumor performance of X-NP-DOX in MCF-7/ADR human breast tumor-bearing nude mice. (A) Tumor volume changes of mice treated with X-NP-DOX, free DOX and PBS, respectively. The drug was given on days 1, 6, 11, and 16 (dosage: 7.5 mg DOX equiv./kg body weight, in 0.2 mL PBS). Data are presented as mean ± SD (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001 (Student's t test); (B) photographs of typical tumor blocks collected from different treatment groups of mice on day 25; (C) body weight changes of mice in different treatment groups within 24 d. Data are presented as mean ± SD (n = 6); and (D) survival rates of mice in different treatment groups within 48 d.

Fig. 8. H&E-stained tumor, liver and heart sections excised from MCF-7/ADR human breast tumor-bearing mice following 25 d treatment with X-NP-DOX. Mice treated with PBS and free DOX were used as controls. The images of tumor and liver were obtained by a Leica microscope at low magnification (100×), and the images of heart were obtained at high magnification (400×).
References


