Dendronized heparin–doxorubicin conjugate based nanoparticle as pH-responsive drug delivery system for cancer therapy

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Abstract

Heparin drug conjugates are currently investigated as excellent candidates for drug delivery vehicles. In this study, we report the preparation and characterization of dendronized heparin–doxorubicin (heparin–DOX) conjugate as pH-sensitive drug delivery vehicle by combination of the features of dendrimer and heparin. Dynamic light scattering (DLS) and transmission electron microscope (TEM) studies demonstrated the dendronized heparin–DOX conjugate self-assembled into compact nanoparticles with negatively charged surface. The nanoparticles with 9.0 wt% (weight percent) of doxorubicin (DOX) showed pH-sensitive property due to the faster drug release rate at pH 5.0 and slow release rate at pH 7.4 aqueous. The nanoparticles were shown to effectively kill cancer cells in vitro. Notably, the nanoparticles resulted in strong antitumor activity, high antiangiogenesis effects and induced apoptosis on the 4T1 breast tumor model due to the evidences from mice weight shifts, tumor weights, tumor growth curves, immunohistochemical assessment and histological analysis. It's also noteworthy that dendronized heparin and its nanoparticle with drug demonstrated no significant toxicity to healthy organs of both tumor-bearing and healthy mice, which was confirmed by histological analysis compared with free drug DOX. The dendronized heparin–DOX conjugate based nanoparticle with high antitumor activity and low side effects may be therefore a potential nanoscale drug delivery vehicle for breast cancer therapy.

1. Introduction

Nanoparticles, as drug delivery vehicles for cancer therapy, are rapidly progressing and are being implemented to overcome the limitations of conventional chemotherapeutic agents such as nonspecific biodistribution and targeting in the body, poor water solubility and low therapeutic indices [1,2]. Nanoscale drug delivery vehicles provide enhanced antitumor efficacy and reduced side effects, owing to their properties such as higher accumulation in tumors via the enhanced permeability and retention (EPR) effect and active cellular uptake [13]. Among the emergent nanoparticles, lipids (liposomes) and polymers (polymeric nanoparticles, micelles and dendrimers) likely have the great potential clinical impact for the foreseeable future [4–7]. However, although those lipids and polymers based nanoparticles demonstrate many advantages as drug delivery systems, there are still many limitations to address such as instability in circulation, inadequate tissue distribution and toxicity.

Dendrimers with nanometer dimensions have provided potential drug delivery vehicles due to their features such as their precise and monodisperse size, low polydispersity, modifiable surface functionality, water solubility and multivalency, resulting in a number of possible advantages, such as pharmacokinetic advantages of typical colloidal or macromolecular delivery systems [8,9]. The fate of injected nanoparticles, including dendrimer based drug delivery, suitable for cancer therapy depends on their sizes and surface characteristics [1,10–14]. However, this kind of drug delivery with ideal antitumor properties in vivo has not been sufficiently achieved, since the reported dendrimers with size less than 10 nm are rapidly cleared from the circulation through extravasation or renal clearance [10]. Increasing generation and surface modification can increase the sizes of the dendrimers, which leads to longer blood circulation and higher antitumor efficiency. However, it’s not easy to prepare higher generation dendrimers due to the steric hindrance to chemical reactions [15]. Simultaneously, the high generation dendrimer leads to side effects due to their slow degradation [15,16].

In order to prepare dendrimer or dendritic polymer based nanoparticles with larger sizes and well-defined molecular objects, dendronization strategy has been utilized to prepare...
nanoscopic objects via connecting dendron to polymer [17,18], in which polymer was used as a multifunctional and polydisperse core, and the new polymers with this architecture were referred to as ‘dendronized polymers’ [19,20]. The dendronized polymers can self-assemble into nanoparticles taking advantage of dendrimer and linear polymer [21,22]. The dendronization and self-assembly can overcome the synthetic challenges associated with preparation of dendrimer based nanoparticles with satisfied sizes. Heparin, a non-cytotoxic, biodegradable, rich in animal tissues and water–soluble natural polysaccharide belonging to the family of glycosaminoglycans [23], has been widely used as an anticoagulant drug owing to its ability to accelerate the rate at which antithrombin inhibits serine proteases in the blood coagulation cascade [24,25], as well as antitumor drug delivery carriers due to its ability to inhibiting tumor growth and metastasis by interacting with tumor related factors such as selectins, heparanases, and growth factors [26–32]. The antitumor drug was conjugated to heparin or encapsulated into the heparin nanoparticles [33–39]. However, the major problems are the unsuitable particle sizes for cancer therapy and poor stability. Based on above observations, our question here was if the dendrimerized heparin–DOX conjugate can self-assemble into nanoparticle and be suitable as nanoscale drug delivery with significant antitumor efficacy as well as good biosafety by combination of the features of dendrimer and heparin. However, currently, few studies on dendronized heparin based nanoparticles as antitumor drug delivery has been reported.

In this study, we described the preparation and characterization of dendrimerized heparin–DOX conjugate as pH-stimuli and nanoscale drug delivery system for breast tumor therapy. Its antitumor efficacy and biosafety were assessed well. The heparin was dendronized with low generation dendron via click reaction; DOX was conjugated to the surface of dendron through pH-sensitive hydrazone bond, resulting in compact nanoparticle via the self-assembly, as shown in Fig. 1. The in vitro and in vivo characteristics of nanoparticle as pH-stimuli drug delivery system, such as size, zeta potential, drug release, antitumor efficacy, antiangiogenic effect and toxicity, were evaluated, which showed the dendrimerized heparin–DOX conjugate based nanoparticles provide a potential drug delivery vehicle for breast cancer therapy.

2. Materials and methods

2.1. Materials and measurements

N,N,N′-tetramethyl-1H-benzotriazole-1-yluronium hexafluorophosphate (HBFU), 1-hydroxybenzotriazole (HOBT), propargylamine, N,N-dioctylpropylenylamine (DIPPA), trifluoroacetic acid (TFA) and doxorubicin hydrochloride were purchased from Sigma–Aldrich and used without further purification. Boc–L-lys (Boc–OH) was purchased from GL Biochem (Shanghai) Ltd. N-Hydroxy sulfo succinimide sodium salt (Sulfo–NHS) and N-[(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) HCl) were obtained from Aladdin. Heparin (sodium salt) with an average molecular weight 6000–20000 Da (activity: 150 u/mg) was obtained from Chem Basel Co. (China). Goat polyclonal against PECAM1 (sc-1505) was obtained from Santa Cruz Biotechnology, Inc. Fluorescein (FITC)–conjugated AffiPure Rabbit Anti–Goat IgG (H + L) was obtained from ZSGB–BIO (China). 4–(N-(tert-butoxycarbonyl–hydrazone)–4–oxo–butyric acid (1.82 g, 7.86 mmol), HOBt (1.06 g, 7.86 mmol) and HBTU (2.98 g, 7.86 mmol) were then added simultaneously as a mixture of solids and the solution was stirred under nitrogen in an ice bath for 30 min. The mixture was allowed to warm to room temperature and stirred for another 24 h. The crude product was then purified by preparative reverse-phase HPLC and the mixture purified by column chromatography (silica, CH2Cl2; MeOH, 10:1) to give the product Dendron 2 in 88.2% yield (1.94 g, 2.31 mmol), 1H NMR (400 MHz, DMSO), δ = 1.11–1.54 (m, 54H, C–CH2), 2.20–2.40 (m, 16H, COCH2CH2CO), 2.97 (s, 6H, CH2NHC(CH2)2HCH2NH), 3.07 (d, 1H, C–CH2), 3.84 (s, 2H, CH2CH2CH2), 4.07–4.22 (m, 3H, COCH2), TOF MS: m/z 1384.3121 [M + Na]+, C75H80N16O12Na.

Dendron 2 (11.1 g, 13.11 mmol) was first dissolved in anhydrous dichloromethane 4 mL, TFA 4 mL was added and the solution was stirred under nitrogen for 30 min. The solvent was removed from the solution by rotary evaporation and the sample was then dried under high vacuum for 1 h. Dithyethyl ether was added and a precipitate appeared. The precipitate was collected by centrifugation and dried. The zeta potential, drug release, antitumor efficacy as well as another 24 h. The solvent was removed by rotary evaporation and the sample was then dried under high vacuum for 1 h. Dithyethyl ether was added and a precipitate appeared. The precipitate was collected by centrifugation and dried. The zeta potential, drug release, antitumor efficacy and toxicity were evaluated, which showed the dendrimerized heparin–DOX conjugate based nanoparticles provide a potential drug delivery vehicle for breast cancer therapy.
determined by measuring the absorbance at 485 nm with UV–vis spectrophotometry, giving 9.0 wt% of DOX.

2.3. Size, shape and zeta potential

Particle size and zeta potential of the nanoparticles were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The dendronized heparin–DOX conjugate and dendronized heparin were diluted to 10 mL with PBS to a final concentration of 100 μg/mL. The TEM samples (nanoparticle concentration of 100 μg/mL) were prepared by dipping a copper grid with formvar film into the freshly prepared nanoparticles solution. A few minutes after the deposition, the aqueous solution was blotted away with a strip of filter paper and then the samples were dried at room temperature. The samples were stained with phosphotungstic acid (ATP) aqueous solution and dried in air.
2.4. In vitro drug release

Dendronized heparin-DOX conjugate based nanoparticles (0.3 mg) was dissolved in 2 mL phosphate buffered saline solution (PBS) with different pH values (pH 5.0 and pH 7.4) and were placed in dialysis bags [molecular weight cut off (MWCO), 3500 Da]. The dialysis bags were then immersed in 30 mL of PBS with different pH values and kept in a horizontal shaker maintained at 37 °C for 170 rpm. 5 mL of the medium was removed at different time points and the same volume of fresh PBS was added. The released DOX was measured by a fluorescence detector with excitation wavelength at 480 nm and emission wavelength at 550 nm.

2.5. In vitro cytotoxicity assays

The cytotoxicity of free DOX and its formulations against 4T1 cells was measured by the MTT assay (CCCK8, Dojindo, Japan). 4T1 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO2. The cells were harvested with 0.02% EDTA and 0.025% trypsin and rinsed. 4T1 cells were cultured on 96–well plates at a density of 5000 cells/well. The cells were incubated at 37 °C, 5% CO2/95% air for 24 h to allow for attachment to the culture vessel. The medium of RPMI 1640 and 1.5 ng/mL to 30 ng/mL DOX equivalents (as DOX, dendronized heparin-DOX conjugate) or medium alone added to each of wells. Cells were incubated for 2 days before assay, and untreated control wells were set at 100% viable.

2.6. In vivo efficacy

Animals were performed in line with national regulations and approved by the animal experiments ethical committee. Female BALB/c mice (20 ± 2 g, 6–8 weeks old) were obtained from West China animal culture center of Sichuan University. 4T1 tumors were induced by subcutaneous injection of 5 × 10^5 injected (in a volume of 50 μL) into the right flank. When tumor reached approximately 250–300 mm³, mice were divided into three groups with each group with 5 tumor–induced ones and marked properly. Mice were administrated with free DOX and dendronized heparin-DOX based nanoparticle (DOX-equivalent, 4 mg/kg DOX) in a final volume of 200 μL via the tail vein every 4 days for 4 times. After 13 days dosage period, surviving mice were allowed to recover and tumor growth was monitored continuously 12 days. In parallel, the body weights of mice were measured every day. The tumor volume and body weight at day 0 were normalized to 100%. Tumor volumes were calculated using the formula V = (a * b^2) / 2, with a being the largest and b being the smallest diameter. All subsequent tumor volumes and body weight were then expressed relative to the tumor volume and body weight determined on the first day of therapy. At the end of the experiment, all of the animals were euthanized and sacrificed, the different organs, such as liver, heart, spleen, lung, kidney and tumor, were separated. At the end of the experiment, the tumors were photographed, weighted and all separated organs were washed with PBS, followed by storage 4 °C formalin and embedded in paraffin, sectioned, and finally stained with hematoxylin and eosin for histological analysis. The tumor tissues were histologically evaluated by anti–CD31 antibody immunofluorescence staining. The tissue slices were observed via inversion fluorescence microscope (Leica DM4000B).

2.7. Assay of in vivo angiogenesis

The microvessel density in the tumor tissues was examined using a CD31 staining method [24,42]. Briefly, the tumor tissues were embedded in paraffin, sectioned. Endothelial cells were immunostained using rat monoclonal anti-mouse CD31 antibody (Santa Cruz Biotechnology, Inc.) followed by Fluorescein isothiocyanate (FITC)–conjugated AffiniPure Rabbit Anti–Goat IgG (H + L) (ZSG8–BIO, China). Slides were then incubated for 5 min with DAPI. The background was stained with DAPI. Immuno–stained slides were scanned at × 200 magnification to identify the areas (hot spot) with the highest number of vessels. Microvessel density was quantified by counting the number of capillaries per microscopic field in 5 random fields in the hot spot per slide by five independent pathologists at × 400 magnification according to the international consensus report. The results are expressed as mean value ± SD.

2.8. In vivo toxicity

Healthy female BABL/c mice (20 ± 2 g) mice, four groups with each group comprising 5 ones, were administered 4 times with 4 mg/kg doxorubicin equivalents of DOX, dendronized heparin–DOX conjugate based nanoparticle and drug–free dendronized heparin via the tail vein in a final volume of 200 μL, and physiological saline was used as control. The body weights of mice were measured, whereas the behaviors of animals were detected. After 13 days dosing period, surviving mice were allowed to recover and weight was monitored continuously 12 days. All animal were sacrificed, the liver, heart, spleen, lung and kidney were separated, washed with PBS and fixed in 3% formaldehyde, embedded in paraffin, sectioned and finally stained with hematoxylin and eosin for histological analysis.

2.9. Statistical analysis

Comparison between groups was analyzed by the two--tailed Student’s t-test was used to address statistical significance. All data are presented as average ± SD, with p values of < 0.05 indicating statistical significance.

3. Results and discussion

3.1. Design and preparation of dendronized heparin–DOX conjugate based nanoparticle

In our previous studies, peptide dendrons/dendrimers have been used as drug/gene delivery vehicles and magnetic resonance imaging probes taking advantage of dendrimer’s multivalency [15,43–49]. However, the dendrimer based drug delivery system can be easily cleaned up form body due to the small size [10]. Despite the high generation dendrimer showed higher size, it resulted in toxicity in vitro and in vivo [15], and it was also associated with synthesis difficulty. The dendronization provided a possibility to preparation of nanoscale materials with dendrimer’s features [50], which can overcome the synthesis problems. Recently, heparin based nanoparticles were utilized as drug delivery carriers by combination of the features of heparin and nanoparticles, which has provide synergistic improvements of drug delivery [23,25,33–37,51]. Thus, here we designed dendronized heparin–DOX conjugate as nanoscale drug delivery vehicle for the therapy of breast tumor.

However, it’s not simple to the conjugating of dendron to heparin, since the high molecular weight of the two compounds lead to relatively larger stereospecific blockade. In an alternative way, the tail of peptide dendron was functionalized with one alkynyl group. The heparin was functionalized with azido group. Further evidence was furnished by the presence of the peak characteristic of azido group (2112 cm⁻¹) in the IR spectra of azido–heparin. The Dendron 2 was covalently attached to heparin via Cu²⁺–catalyzed azide–alkyne cycloaddition (CuAAC) click chemistry, resulting in the final water–soluble dendronized heparin. It should be noted that the product was washed and dialyzed with EDTA solution to fully remove trace amounts of copper, since the chelated copper may cause unwanted toxicity. To further estimate the conjugation efficiency and the number of dendron on heparin, the 1H NMR spectrophotometry analysis was employed to determine the dendron conjugation, indicating approximately one dendron was attached to every five repeat glycosyl units.

The dendronized heparin was easily dissolved in water, so the hydrazone reaction was carried out in NH₄OAc buffer (pH 5.7) [40].

![Fig. 2. In vitro drug releases from dendronized heparin–DOX conjugate based nanoparticle at pH 7.4 and 5.0 (n = 3).](image-url)
Fig. 3. The hydrodynamic size of (a) heparin, (b) dendronized heparin and (c) dendronized heparin–DOX conjugate based nanoparticles via DLS, and the size were 0, about 250 and 90 nm, respectively. The size of (d) drug-free dendronized heparin and (e) dendronized heparin-DOX conjugate nanoparticles via TEM, and the size was around 200 nm and 80 nm, respectively.
The antitumor drug DOX was covalently linked to dendronized heparin. After attachment of DOX, the dendronized heparin-DOX conjugate was nicely purified by dialysis. Small molecular and byproducts were easily removed. UV–vis spectrophotometry analysis was employed to determine the DOX content, resulting in 9.0 wt% (weight percent). In the PBS buffer (pH = 7.4), the dendronized heparin–DOX conjugate based nanoparticle was obtained, where the antitumor drug DOX was conjugated to dendron via an acid–liable hydrazone bond. Thus, unlike other self–assemble nanocarries such as micelles, liposomes and polymersomes, this dendronized heparin conjugate may be stable in PBS or blood circulation because the drug is covalently linked to the polymer [52].

3.2. Release of DOX from nanoparticle

The character of drug release from carriers, especially in intracellular environment, is very important for antitumor efficacy. For a drug delivery system with long circulation and antitumor efficacy, it’s required the high stability in body circular system and fast release in cell. To quantitatively determine release form the nanoparticles, the nanoparticles were in PBS at pH 7.4 (corresponding to the pH of blood) and PBS at pH 5.0 (corresponding to the pH of endosome) at 37 °C, respectively. The amount of released doxorubicin at different predetermined time points was measured by fluorescence detector with excitation wavelength at 480 nm and emission wavelength at 550 nm. The results (Fig. 2) showed that...
the release of DOX from nanoparticle was only 20% at pH 7.4 after 56 h incubation. In contrast, much faster and higher (>80%) drug release was obtained at pH 5.0, since the cleavage of hydrazone linkers accelerated the release of drug at lower pH values. The in vitro release profile suggested the stability of nanoparticle loading drug in circulation system (pH 7.4) and their ability to release the DOX in the acidic endosomes and/or lysosomes where the pH range is 4.0–6.0, indicating the dendronized heparin–DOX conjugate based nanoparticle would be used as pH-stimuli drug delivery system.

3.3. Size and zeta potential of nanoparticle

The size and size distribution of the nanoparticles were measured by dynamic light scattering (DLS) method. The heparin showed no particle diameter due to its good solubility in water (Fig. 3a). Once the Boc groups were removed, the dendron was covalently attached to linear heparin via click chemistry. The drug–free dendronized heparin aggregated to particle with nanoscale size in water (pH = 7.4), giving average hydrodynamic sizes around 250 nm and PDI of 0.310 (Fig. 3b). That’s attributed to the strong H-bonding interaction between the amino groups of dendron and sulfo groups and carboxyl groups of heparin. Once DOX conjugated to dendron via an acid–liable hydrazone bond, as DLS results shown (Fig. 3c), the dendronized heparin–DOX conjugate self-assembled into particle with nanoscale size in water (pH = 7.4), displaying average hydrodynamic sizes around 90 nm and PDI of 0.140. Generally, producing drive force segments are needed to introduce to the drug conjugated heparin for the self–assembly. Fortunately, for our designed drug delivery system, self–assembly behavior was mediated by dendronized heparin–DOX conjugate itself. The primary driving force responsible for the self–assembly behavior is the minimization of the interfacial energy governed by the balance between the hydrophilic interaction of the linear polymer and the hydrophobic interaction of the dendronized heparin–DOX block [53]. Secondly, the driving forces governed self–assembly of our prepared dendronized heparin–DOX, such as π–π stacking, dipole interactions, H-bonding and the preorganized branched architecture should also be considered, since the DOX is composed of multiple domains of different chemical composition, e.g., hydrophobic, aliphatic and aromatic [54]. Meanwhile, the rich sulfo groups and carboxyl groups on the heparin also interacted with DOX and amino groups on dendron via strong H-bonding interaction and electrostatic interaction. It’s noteworthy that the size of drug loading nanoparticles was much smaller than that of drug–free nanoparticles aggregated from dendronized heparin, which also agreed with that the introduced DOX played important role in the formation of nanoparticles.

The TEM was also performed to demonstrate the formation of nanoparticles from dendronized heparin and its drug conjugate, resulting in uniform particles with diameters of about 200 nm and 80 nm, respectively (Fig. 3d,e). The sizes of particles observed by

![Fig. 6. In vivo tumor growth inhibition of nanoparticle. Comparison of the tumor inhibition effect of dendronized heparin–DOX conjugate based nanoparticle with drug (Nanoparticle) versus free drug DOX (DOX) and saline in the breast tumor model (n = 5). The nanoparticle demonstrated significant tumor inhibition (*p < 0.001, compared to saline; $p < 0.001, compared to free drug DOX) (a). During the treatment, the mice administrated nanoparticle showed no significant body lost compared to saline (*p < 0.001, compared to free drug DOX) (b). At the end of this experiment, tumor tissues were collected from each sacrificed animal after 25 days treatment, photographed (c) and weighted (*p < 0.01, compared to saline; $p < 0.05, compared to free drug DOX) (d).]
TEM were smaller (about 10 nm for nanoparticle with drug and about 50–60 nm for drug–free nanoparticle) than those of determined by DLS. This is because the diameter of copolymeric nanoparticles determined by DLS measurement reflected the hydrodynamic diameter of polymeric conjugates, which were swollen in aqueous solution, while that TEM image depicted the actual size at the dried state of sample. Unlike the drug–free nanoparticle with significantly different size between that in aqueous and dried state, the dendronized heparin–DOX conjugate based nanoparticle showed very close size by DLS and TEM, indicating compact nanoparticles. The formation of compact nanoparticle may be due to the strong aggregation of dendronized heparin–DOX conjugate via noncovalent forces mediated by DOX and heparin themselves. Thus, the nanoparticle has a core/shell structure composed of a hydrophobic inner core containing DOX molecules/groups and a hydrophilic heparin shell layer (Fig. 1). It is currently accepted that the diameter of nanoparticles therapeutics should be in the range of 10–100 nm to reach tumor tissues by EPR effect [1]. In this study, our prepared nanoparticles showed just enough diameter (70–100 nm) by both DLS and TEM. The size of nanoparticles as drug delivery systems should be large enough to have accessibility to within disseminated tumors via EPR effect and prevent their rapid leakage into blood capillaries, but also small enough to escape capture by fixed macrophages that are lodged in the reticuloendothelial system, such as the spleen and liver [2], resulting in high antitumor efficacy.

The zeta potential of dendronized heparin and its nanoparticles with drug was −40 and −35 mV, respectively (Fig. 4a,b), which was able to maintain their stability of dispersion in aqueous [55]. The reason for the negatively charged surface should be due to the sulfo groups, along with carboxyl groups on the natural product heparin. Previous studies have showed that the nanoparticles with positively charged surface was cleared rapidly from the circulation after intravenous and intraperitoneal administration [56]. In contrast, the negative charged one can prevent the strong interaction with serum proteins and macrophage uptake in the circulation system and enhance its accumulation into target tissue (tumor) in vivo, leading to higher antitumor efficacy [57]. Thus, in this study, the dendronized heparin–DOX conjugate based nanoparticle with negative charge and diameter of about 90 nm may be as drug delivery system.

3.4. In vitro cytotoxicity

In vitro cytotoxicity of the dendronized heparin–DOX conjugate based nanoparticle was estimated using CCK–8 assay, compared with drug–free dendronized heparin and free DOX. The dendronized heparin–DOX conjugates and doxorubicin were evaluated using mouse breast cancer cell line (4T1) after 48 h incubation and the cellular growth was investigated. As shown in Fig. 5a, the half–maximal inhibitory concentration (IC50) of doxorubicin against 4T1 cells was 27.0 ng/mL, while the dendronized heparin–DOX conjugate based nanoparticle (IC50, 300 ng/mL) was approximately 11 fold of free drug DOX. pH-sensitive nanoparticle showed the possibility of observed lower cytotoxicity produced by DOX may be due to free DOX is an amphipathic and small molecule that can

![Image](https://example.com/image.png)
Histological analysis for different organs of tumor bearing mice administrated control (Saline), free drug DOX (DOX) and dendronized heparin–DOX conjugate based nanoparticle (Nanoparticle). Our analysis showed that nanoparticle demonstrated much higher tumor inhibition (a3: grade IV tumor necrosis with hemorrhage in center area) compared to free drug DOX (a2: grade II necrosis in center area). The free drug DOX resulted in heart toxicity due to the observed necrosis (grade I) with acute inflammatory cells infiltration in epicardium and cardiac myocyte under epicardium (c2). Simultaneously, lobular pneumonia (grade III) and multifocal metastasis (grade I) of tumor was observed in lung tissue (d2). In saline treated group, very small necrosis (grade I) area in center of neoplastic cells was observed (a1). A focal metastasis cancer was observed in liver (b1: grade I), lung tissue (d1: grade III, multifocal metastasis of cancer were observed) and spleen (e1, grade III, multifocal metastasis of cancer).
easily cross the cell membrane. Incubation with equivalent four
highest concentrations of drug-free dendronized heparin (Fig. 5b),
the results showed the cell viabilities for drug-free nanoparticle
were more than 90% after 48 h incubation even at high feed
medium (300 μg/mL), suggesting drug-free dendronized heparin
nanoparticle has non-cytotoxicity and indicating the cytotoxicity of
nanoparticle with drug would not be due to dendronized heparin
block, but the drug DOX. The observed in vitro cytotoxicity of
nanoparticle indicated that DOX can release from the nanoparticle
in the acidic environment of endosomes, and the nanoparticle may
demonstrate in vivo antitumor efficacy once it reach tumor tissue
and been uptaken by cells.

3.5. In vivo efficacy

To evaluate antitumor activity and toxicity, dendronized
heparin–DOX conjugate based nanoparticle and free drug DOX
were intravenously injected mice bearing 4T1 breast tumor model,
while the saline was used as control. The two agents (4 mg equiv-
alent DOX/kg mice) were applied every four days for 13 days. Fig. 6a
shows the tumor growth curves after therapy. The tumors in all
drug–treated groups showed growth retardation compared to the
controls. With respect to antitumor efficacy, DOX only showed
moderate antitumor efficacy. In contrast, the tumors treated with
nanoparticle exhibited a significantly stronger response than the
tumors treated with saline only or free drug DOX, although this
could not be demonstrated statistically within the first 5 days.
Significant difference occurred after second therapy at 5 days
\( p < 0.01 \). Particularly, after 25 days therapy, the statistically
significant was obtained for mice treated with nanoparticles to the
control and DOX treated group due to the much smaller tumor
volume, as shown in the tumor growth curves (Fig. 6a, \( p < 0.001 \)).
This may be due to the lag time required for breast micro
environment and injected breast cancer cells to exhibit influences
and interactions based on different mechanisms by which the local
biochemical and mechanical microenvironment, which is
comprised of various signaling molecules, cell types and the extra-
cellular matrix (ECM), affects the progression of cancerous cells.

At the end of this in vivo experiment, the tumors of all different
groups were removed, photographed and weighted. The tumor
sizes from mice administrated dendronized heparin–DOX conjuga-
ted nanoparticles were obviously smaller than those from
free drug DOX treatment group and controls. (Fig. 6c), which was
proportional to the observed relative tumor volume results
(Fig. 6a). Simultaneously, the tumor weights in mice treated with
nanoparticles were obviously lower compared with the tumors
from free drug DOX treatment group \( p < 0.05 \) and control
\( p < 0.001 \) (Fig. 6d). The high antitumor activity of the nanoparticle
may be attributed to negatively charge surface, longer blood
circulation, potential higher accumulation in tumor via EPR effect
and the accelerated release of DOX from endosomes [1,58]. These
results indicated that inclusion of the nanoscale size and pH-
sensitive characteristics enhanced the antitumor efficacy of
nanoparticles.

Simultaneous monitoring of the body weight of the adminis-
trated animals showed that less body weight shift was observed for
the group administrated nanoparticles with drug compared with the
controls (Fig. 6b), indicating better drug tolerability. Indeed,
examimation of the body weight curves over time of the group
treated with nanoparticles showed a very slight body weight shifts,
suggesting a low degree of systemic toxicity. However, animals
treated with free drug DOX resulted in maximum ~20% body
weight shift. These results demonstrated that nanoparticle, due to
its higher tumor suppression, resulted in much higher in vivo ef-
cacy without significant toxicity.

Angiogenesis, the formation of new vessels, has a major role in
tumor growth, dissemination and metastasis in both solid and
hematological tumors [59]. Immuno histochemical assessment can
be utilized for investigation of tumor vessels in tumor tissue [60–
62]. In this study, to evaluate the antiangiogenic activity of nano-
particles, at the end of experiments, the microvessel density in the
tumors from different groups treated with saline, free drug DOX
and nanoparticles was examined using a CD31 staining method. As
shown in Fig. 7, microvessels were clearly observed by CD31
staining, where the microvessels were labeled with FITC (green
ones). It’s clear that the some microvessels were observed in the
DOX group and controls, and no significant difference was observed
between the two groups (Fig. 7a and b). In contrast, very few
microvessels were observed in the nanoparticles treatment group
(Fig. 7c). Compared with the free drug DOX treatment group and
controls, there was significantly less microvessel density in the
nanoparticles treatment group \( p < 0.01 \) (Fig. 7d). Those results of
the immunohistochemistry study indicated the high anti-
angiogenic effect of dendronized heparin–DOX conjugate based
nanoparticles in tumor, as shown by the microvessel density eval-
uation. Although antiangiogenic effect governed by free drug DOX
was also observed, no statistically significant was obtained
compared with controls. The produced antiangiogenic effects can
block the blood supply and this may be more effective in sup-
pressing tumor growth in vivo. That may be one of reason that the
nanoparticles can provide much higher antitumor activity, as shown
by the tumor growth curves (Fig. 6a) and final tumor weights
(Fig. 6d). The higher antiangiogenic effects produced by dendron-
ized heparin–DOX conjugate based nanoparticles may be due to
the features of nanoparticles, such as higher accumulation in tumor
tissue via EPR effects [1], as well as the features of functionalized
heparin polymers, such as the abilities to effectively inhibit anti-
giogenesis, metastasis and tumor growth [63,64].

To further investigate the antitumor efficacy of dendronized
heparin–DOX conjugate based nanoparticle, the tumor and other
organs of the administrated mice were removed from bodies after
25 days therapy, and histology was used to analyze the produced
toxicity. For the controls, very small necrosis (grade I) area in center

![Fig. 9. Normal animal body weight shifts post-injections of dendronized heparin–DOX conjugate based nanoparticles (Nanoparticle), drug-free dendronized heparin, free drug DOX (DOX) and saline up to day 25 (n = 5).](image-url)
of neoplastic cells in tumor was observed (Fig. 8a1). However, a focal metastasis of cancer in liver (grade I, Fig. 8b1), multifocal metastasis of cancer in lung tissue (grade III, Fig. 8d1) and in spleen (grade III, Fig. 8e1) were observed. Although necrosis of neoplastic cells in center area in tumor was also observed in DOX treated group, the area was only about 10% and the grade of necrosis was II (Fig. 8a2). Simultaneously, the grade I necrosis with acute inflammatory cells infiltration in epicardium and cardiac myocyte under epicardium was observed (Fig. 8c2) and lobular pneumonia (grade III) and multifocal metastasis (grade I) of cancer in serosa membrane of lung were observed (Fig. 8d2). In contrast, in the group treated nanoparticle, the tumor necrosis (grade IV) with hemorrhage in center area was observed. Note that over 50% (area percent) tumor destruction was shown in nanoparticle group and it occurred after 25 days therapy (Fig. 8a3). Meanwhile, all the other organs were normal for the group treated with nanoparticle. It should be noted that animals in all mice were treated with the same amount of DOX. Histology results agreed well with the tumor growth curves. The results suggested that, compared to free drug DOX, the dendronized heparin-DOX conjugate based nanoparticle demonstrated the ability to preventing multifocal metastasis, much better antitumor efficacy and non-observed toxicity. This drug delivery system is unique because it allowed slow elution of doxorubicin into the tumor after administrated due to the features of nanoparticle with better stability in circulation environment (pH 7.4), much longer blood circulation time and higher accumulation in tumor tissue via EPR effect [1,49]. Once the nanoparticles reach to tumor tissues, the DOX is immediately released from the

![Fig. 10. Histological analysis for different organs of normal mice administrated control (Saline), free drug DOX (DOX), drug-free dendronized heparin and dendronized heparin-DOX conjugate based nanoparticle (Nanoparticle) (heart:×200, other tissues:×100). Our analysis showed that the free drug DOX resulted in heart toxicity due to the observed necrosis (grade I) with acute inflammatory cells infiltration in epicardium and cardiac myocyte under epicardium (labeled with blue). In contrast, organs of mice administrated saline, drug-free dendronized heparin and dendronized heparin-DOX conjugate based nanoparticle did not exhibit signs of toxicity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
pH-sensitive drug delivery vehicle followed its entrance into tumor cells, which can enhance the concentration of DOX within the tumor. Finally, the abilities of heparin polymers to inhibiting angiogenesis, metastasis and tumor growth also played important role in this process [63]. Our results again support the advantage of dendronized heparin–DOX conjugate based nanoparticle as its high antitumor activity and low side effects.

3.6. Clinical signs, body weight changes and histology on normal mice

For nanoparticles as drug delivery carriers, one potential problem is their limited biosafety. Thus, in vivo toxicity studies are needed to prove the safety of any nanoparticles. Although a few systematic studies has been recorded to assess the in vivo toxicity of dendrimer/dendron based nanoparticles [15,65–67], the relative toxicity studies on heparin based nano-scale drug delivery systems has not been reported. The fluctuation in body weight and clinical signs, recognized as useful factors for assessing in vivo toxicity of nanoparticles with drug, free drug DOX and drug–free dendronized heparin, was recorded and the physiologic saline as control. For healthy mice, the nanoparticles and free drug DOX (DOX–equivalent) were administrated every four days for 13 days, whereas the dose of DOX was the same with that administrated in tumor mice (4 mg DOX/kg mice). Throughout the study period, the animals administrated saline, nanoparticles and drug–free dendronized heparin showed no apparent signs of dehydration, locomotor impairment, muscle loss, anorexia and other symptoms associated with animal toxicity. In addition, no abnormal physical signs and behaviors were detected. Fig. 9 showed the body weight of mice recorded during 25 days evaluation. Over the whole period, the mice administrated saline did not show any adverse effects on their growth as evident in their normal body weight increased steadily. The two groups injected nanoparticles and drug–free dendronized heparin exhibited slight body increase, which was close to that of the control group. However, the obvious body shifted for DOX administrated mice, resulting in over 15% weight lost. The results may indicate that nanoparticle and drug–free dendronized heparin produced no significant in vivo toxicity compared to drug DOX.

Histological analysis of organs was further used to determine in vivo toxicity of the drug-free dendronized heparin and nanoparticle with drug. As shown in Fig. 10, for mice administrated free drug DOX, the heart toxicity induced by DOX was observed due to the necrosis (grade I) with acute inflammatory cells infiltration at epicardium and cardiac myocyte under epicardium. In contrast, organs of mice administrated with drug–free dendronized heparin and nanoparticle with drug were normal and no visible difference was observed compared to the control, showing no signs of toxicity (Fig. 10). The in vivo toxicity of nanoparticle depends on the formulations, chemical structures, charge, size, exposure duration, biodistribution, location, metabolism as well as the nature of the surface functionality. For polymer based nanoparticles, the toxicity was also correlated with its type, molecular weight and degradability [67]. The non–observed toxicity of drug–free dendronized heparin and its nanoparticle with drug could be attributed to its lower molecular weight and the characteristic of molecular structure, such as the biodegradability of heparin and lysine based dendrimer [23]. The biodegradability of carrier (dendronized heparin) can promote its clearance from organism and thereby enhance the in vivo biosafety. Secondly, for the nanoparticle, the particle may have a high accumulation in tumor tissue but lower accumulation in normal tissue via EPR effects [1,58], which further reduced the side effects to normal organs. In addition, because the drug DOX was covalently linked to dendronized heparin via pH-sensitive hydrazone bond, the drug only can be released from the delivery vehicle in tumor cells with low pH value (pH = 4–6). Thus, the prepared dendronized heparin based nanoscale drug delivery carrier efficiently enhanced the tumor therapeutic index and reduced the side effects.

4. Conclusion

In summary, we have shown an example of amphiphilic dendron and linear polymer conjugate based nanoparticle as drug delivery vehicle that combined the polymer hybrid framework by dendronization and self-assembly. The anticancer drug doxorubicin was conjugated to the dendronized heparin block via an acid–labile hydrazone linkage. The dendronized heparin–DOX conjugate based nanoparticles showed a pH-sensitive drug release property. In aqueous solution, the conjugate can self-assemble into compact nanoparticle with hydrodynamic sizes around 90 nm, where the driving force was mediated by dendronized heparin–DOX itself. The dendronized heparin–DOX conjugate based nanoparticles showed promising biosafety and high tumor inhibition in vivo by combination of the features of dendrimer, polymer hybrid and heparin based nanoparticle, which was confirmed by in vitro IC50 study and in vivo tumor growth curves, immunohistochemical assessment and histological analysis. The overall structural design of dendronized heparin–DOX conjugate based nanoparticle may provide useful design and preparation strategies for soft nanoparticles as safe and efficient drug delivery systems.

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