Integrin-mediated active tumor targeting and tumor microenvironment response dendrimer-gelatin nanoparticles for drug delivery and tumor treatment

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A R T I C L E   I N F O

Article history:
Received 7 October 2015
Received in revised form 8 November 2015
Accepted 13 November 2015
Available online 17 November 2015

Keywords:
Tumor penetration and retention
Breast cancer
Multistage drug delivery system
Matrix metalloproteinase-2
Gelatin nanoparticles

A B S T R A C T

Due to the high morbidity and mortality of cancer, it has become an urgent matter to develop an effective and a safe treatment strategy. Nanoparticles (NP) based drug delivery systems have gained much attention nowadays but they faced a paradoxical issue in delivering drugs into tumors: NP with large size were characterized with weak tumor penetration, meanwhile NP with small size resulted in poor tumor retention. To solve this problem, we proposed a multistage drug delivery system which could intelligently shrink its size from large size to small size in the presence of matrix metalloproteinase-2 (MMP-2) which were highly expressed in tumor tissues, therefore the multistage system could benefit from its large size for better retention effect in tumor and then shrink to small size to contribute to better penetration efficiency. The multistage drug delivery system, RGD–DOX–DGL–GNP, was constructed by 155.4 nm gelatin NP core (the substrate of MMP-2) and surface decorated with doxorubicin (DOX) and RGD peptide conjugated dendritic poly-l-lysine (DGL, 34.3 nm in diameter). In vitro, the size of multistage NP could effectively shrink in the presence of MMP-2. Thus, the RGD–DOX–DGL–GNP could penetrate deep into tumor spheroids. In vivo, this multistage drug delivery system showed higher tumor retention and deeper penetration than both DOX–DGL and DOX–GNP. Consequently, RGD–DOX–DGL–GNP successfully combined the advantages of dendrimers and GNP in vivo, resulting in an outstanding anti-tumor effect. In conclusion, the multistage drug delivery system could intelligently shrink from large size to small size in the tumor microenvironment and displayed better retention and penetration efficiency, making it an impressing system for cancer treatment.

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1 Introduction

Nanoparticles (NP) based drug delivery has gained increasing attention in diagnosis and treatment of various diseases including cancer. Until now, several nanomedicines have been approved (Weissig et al., 2014). Although these approved nanomedicines enhanced the antitumor effect and reduced the drug originated side effect, the outcome is still modest. Commonly, nanomedicines could passively accumulate in tumor because of the enhanced permeability and retention (EPR) effect (Fang et al., 2011). However, tumor is a heterogeneous tissue with unique microenvironment, such as dense tumor extracellular matrix and high interstitial fluid pressure, which extremely restrict the distribution of NP through tumor (Jain, 2001; Junttila & de Sauvage, 2013; Marte, 2013). Therefore, it is important to develop novel NP that could adapt the tumor microenvironment and improve the homogenous distribution of drugs.

For this purpose, several kinds of size changeable NP were constructed to adapt the controversial particle size requirement of tumor penetration and tumor retention because good tumor penetration required small particle size while good tumor retention required large particle size (Li et al., 2015; Ruan et al., 2015a,b; Tong et al., 2012; Wong et al., 2011). When these size changeable NP distributed in tumor according to EPR effect, the large size made them easy to retain in tumor. Then the particle size could considerably reduce, leading to well tumor penetration. Recently, we constructed a matrix metalloproteinase-2 (MMP-2) sensitive NP: DOX–DGL–GNP. The model drug doxorubicin (DOX) was firstly conjugated onto small sized dendritic poly-l-lysine.

http://dx.doi.org/10.1016/j.ijpharm.2015.11.025
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(DGL), then the DOX–DGL was fabricated onto gelatin NP (GNP) to form a large particle (Hu et al., 2015b). The GNP could be degraded by MMP-2, which overexpressed in tumor (Ruan et al., 2015a,b; Zhu et al., 2012), thus the size of DOX–DGL–GNP could reduce and the tumor penetration could be improved. Although some preliminary study showed the DOX–DGL–GNP had better penetration efficiency, rare in vivo study was performed (Hu et al., 2015a,b).

To further improve the tumor targeting effect, active targeting ligand was used in this study (Gao et al., 2013). Neovessel endothelial cells and malignant tumor cells were highly overexpressed integrin receptors, such as αvβ3 (Schottelius et al., 2009), which could specifically bind with arginine-glycine-aspartic acid (RGD) peptide (Ruoslathi, 1996; Ruan et al., 2015c), so RGD was used as a targeting ligand to improve the tumor targeting effect of particles.

In this study, we have proposed a multistage drug delivery system: RGD–DOX–DGL–GNP (Fig. 1). The system could effectively target and retain in tumor which benefited from EPR effect and αvβ3 mediated active targeting. After the degradation by MMP-2 in tumor, the size reduced and RGD–DOX–DGL was released to further penetrate into the core of tumor. To evaluate the tumor targeting and penetration of this system, several experiments were performed.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride was obtained from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Gelatin type A was obtained from MP Biomedicals Co., Ltd. (California, USA). Glutaraldehyde solution (Grade II, 25%) was obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China). DGL–G3 dendrimer was purchased from Colcom (Montpellier Cedex, France). α-Mal- eimide-PEG-succinimidyl carbonate (MW3500), α-mPEG-ω-amine (MW5000) and amine-PEG-carboxyl (MW5000) were obtained from Seebio Biotechnology Co., Ltd. (Shanghai, China). RGDyC was obtained from Phdtpeptides Co., Ltd. (Zhengzhou, China). Aconitic anhydride (CA) was obtained from Alfa Aesar Chemical Co., Ltd. (Tianjin, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy-succinimide (NHS) were obtained from Chengdu Best Reagent Co., Ltd. (Chengdu, China). 6-Diamidino-2-phenylindole (DAPI) was obtained from Beyotime Insitute Biotechnology (Haimen, China). Purified rabbit mAb to CD34 was purchased from Abcam Ltd. (Hong Kong, China). MMP-2 protein (rat) was purchased from Abcam Ltd. (Hong Kong, China). Alexa Fluor® 594-conjugated AffiniPure donkey anti-rabbit was purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA). RPMI1640, 3-(4,5-dimethylthiazol-w)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin and trypsin were purchased from commercial sources (Chengdu, China). Mouse mammary breast tumor cell line (4T1) and human umbilical vein endothelial cells (HUVEC) were purchased from Shanghai Institute of Cell Biology (Shanghai, China). All other reagents and solvents were of analytical or HPLC grade and were used without further purification.

Female BALB/C mice (4-week old, 16–18 g) were purchased from experiment animal center of Sichuan University (Chengdu, China). The mice were maintained under standard housing conditions. All animal experiments were performed in accordance with the principles approved by the experiment animal administrative committee of Sichuan University.

2.2. Synthesis of RGD–DOX–PEG–DGL conjugates

RGD–DOX–DGL–PEG was synthesized according previous procedure (Hu et al., 2015a,b). Aftercis-aconityl doxorubicin (CAD) was synthesized using published procedure (Du et al., 2013), DGL was reacted with NHS-PEG5000-MAL at the ratio 1:8 (molar ratio) in PBS (pH 8.0) for 2 h followed with RGDyC at the ratio of 1:2 (DGL to peptide, molar ratio) in PBS (pH 7.0) for 24 h. After dialyzed in deionized water, RGD–DGL–PEG was freeze-dried and analyzed in a 400 MHz spectrometer. Then DGL–PEG or RGD–DGL–PEG was reacted with CAD in the presence of CDC and NHS in the dark for 12 h. At last, the mixtures were purified by ultrafiltration through a membrane (MWCO10,000), and the amount of DOX conjugation was confirmed by UV–vis spectrophotometry at 480 nm.

2.3. Preparation of RGD–DOX–DGL–GNP

Firstly, GNP were prepared by a two-step desolvation method as previous described with some modification (Wong et al., 2011). Then, EDC (0.8 mg) and NHS (0.8 mg) were added to the GNP solution (pH 6.0, 1 mL) to activate carboxyl groups. After the pH value was adjusted to 8.0, 20 mg of SCM–PEG5000–NH2 was added to the mixture. 2 h later, the pH was adjusted to 6.0 and additional solution of EDC (0.8 mg) and sulfo-NHS (0.8 mg) was added. After stirring for 30 min, the pH value was adjusted to 8.0 again, DOX–DGL–PEG or RGD–DOX–DGL–PEG was added to the resulting mixture. The mixtures were purified by ultrafiltration through a membrane (MWCO100,000) (4500 g × 30 min). After hydrolyzed by 1 N HCl, the doxorubicinone was determined using HPLC as described in Section 2.9 to determine the drug loading capacity (Zhu et al., 2010).

2.4. In vitro release

DOX, DOX–DGL–PEG, RGD–DOX–DGL–PEG, DOX–DGL–GNP and RGD–DOX–DGL–GNP (equivalent to 0.1 mg DOX) were added into dialysis bags secured with clamps respectively, and then placed in plastic tubes containing 40 mL PBS (pH 7.4). The experiment was carried out at 37 °C and a horizontal shaking speed of 50 rpm. At appropriate time intervals (0, 1, 2, 3, 5, 7, 9, 12 and 24 h), 1 mL media was taken away and the corresponding fresh buffer was supplemented. The amount of released DOX was detected by fluorescence spectrophotometer. Each drug release test was performed thrice.

Fig. 1. Schematic illustration to show the size shrinkage of the RGD–DOX–DGL–GNP from large size to small size triggered by MMP-2, a protease highly expressed in tumor extracellular matrix, thus penetrating into deep tumor tissue.
Fig. 2. (A) Synthetic scheme of RGD-DOX-DGL-PEG. (B) $^1$H NMR spectra of RGD–DGL–PEG. (C) Mass spectra of CAD.
2.5. In vitro cytotoxicity assays

The in vitro anti-proliferation activity of various DOX-loaded GNP was evaluated by the MTT assay. 4T1 and HUVEC cells were seeded at a density of $3 \times 10^5$ cells/well into 96-well plates, after 12 h incubation at 37 °C in 5% CO$_2$ atmosphere, these cells were treated with a series of different concentration (from 0.391 mM to 50 mM) of DOX, DOX–DGL–PEG, RGD–DOX–DGL–PEG, DOX–GNP, DOX–DGL–GNP and RGD–DOX–DGL–GNP for 48 h. To assess cell viability, MTT (5 mg/mL, 20 μL) was added into each well and incubated at 37 °C for 4 h. The medium was removed and 150 μL of DMSO was added to each well at 75 rpm for 15 min. After that the percentage of cell viability was detected at 570 nm by fluorescence spectrophotometer.

2.6. Tumor spheroid penetration assay

To obtain tumor spheroid, 4T1 cells were seeded into low melting point agarose precoated 96-well plate at the density of $8 \times 10^3$ cells per well (Gao et al., 2014a,b). Several days later, the uniform spheroids were selected and treated with DOX–DGL–PEG, RGD–DOX–DGL–PEG, DOX–DGL–GNP, RGD–DOX–DGL–GNP, DOX–DGL–GNP (MMP-2) and RGD–DOX–DGL–GNP (MMP-2) at DOX concentration of 12.5 μg/mL for 24 h. After washed with PBS for three times, the spheroids were fixed with 4% paraformaldehyde and then the fluorescence distribution was observed via confocal microscopy.

2.7. Surface plasmon resonance (SPR) analysis

SPR assay was performed at 25 °C using a Biacore T200 instrument (GE, USA) equipped with a GM5 chip. Integrin $\alpha_v\beta_3$ was coupled on the surface of the GM5 chip. Then the diluted samples flow through the surface of chip and the signal was detected. Subsequently, all the samples were eluted with buffer or regenerated reagent.

2.8. In vitro cellular uptake study

The cellular uptake of RGD–DOX–DGL–GNP was evaluated by qualitative and quantitative methods. For flow cytometry experiment, HUVEC were seeded on 6-well plates at $2 \times 10^5$ cells/well and incubated for 24 h. Then, the cells were treated with various samples (12.5 μg/mL DOX equal) under 37 °C for 2 h. After incubation, the cells were washed twice with PBS, trypsinized and resuspended in proper volume of PBS for flow cytometer analysis (Cytomics™ FC 500, Beckman Coulter, Miami, FL, USA).

For qualitative assay, HUVEC were seeded at a density of $1 \times 10^5$ cells/well on microscope slides in 6-well plates. After 24 h incubation, the cells were treated with various samples (12.5 μg/mL DOX equal) for 2 h. After incubation, the microscope plates were washed with cold PBS for three times, fixed with 4% paraformaldehyde for 20 min and then stained with DAPI for 5 min. Finally, slides were imaged by fluorescent microscope (Nikon, Japan).

2.9. In vivo imaging and tumor distribution

At two weeks after 4T1 tumor implantation, DOX, DOX–GNP, DOX–DGL–PEG, RGD–DOX–DGL–PEG, DOX–DGL–GNP and RGD–DOX–DGL–GNP were intravenously injected into the mice at the dose of 5 mg/kg DOX (equal) per mouse (Ruan et al., 2015a). After administration for 24 h, the mice were anesthetized by 4% chloral hydrate and sacrificed, the main organs were taken to observe the accumulation condition of the NP using fluorescent imaging system (IVIS, Caliper, USA).

For quantitative analysis, tissues were weighed and homogenate was prepared. After 1 N HCl was added to the homogenate to hydrolyze conjugates, 20 μL of daunorubicin (200 μg/mL) as internal standard solution was precisely added to 200 μL of tissue homogenates for vortex mixing. Then 1 mL of extracted solution (chloroform:methanol = 4:1) were added for vortex-ultrasonic extraction several times. After centrifugation (12,000 rpm, 10 min, 4 °C), the supernatant was collected and analyzed for DOX concentration with the help of a spectrophotometer (278 nm) and standard curve.
5 min), the organic phase was moved to another centrifuge tube and dried under a stream of nitrogen at 37 °C. The residue was redissolved in 100 μL of mobile phase. After centrifugation (12,000 rpm, 10 min), the supernatant was collected for HPLC analysis. Samples were analyzed by a HPLC method using UV-detection. Agilent liquid chromatographic system (Agilent 1200, USA) was equipped with a UV detector with 479 nm. Apollo C18 (250 × 4.6 mm, 5 μm) was used at 30 °C. The mobile phase was methanol:0.01 M sodium acetate:acetic acid = 70:30:1 (v/v/v), the injection volume was 40 μL and the flow rate was 1 ml/min. The peak area ratio of the sample peak and the internal standard peak were recorded for quantitative analysis. In order to quantitative evaluation of the interior and exterior of the tumors, the tumors were divided into two parts, namely the interior and exterior, each parts were weighted and treated as described above.

For confocal microscopy, the tumors were fixed in 4% paraformaldehyde for 48 h, followed by dehydration with 15% sucrose solution and 30% sucrose solution overnight sequentially until subsidence. Thereafter, the tumors were embedded in OCT (Leica, Germany) at -20 °C, sectioned at 16 μm via freezing microtome section. Finally, Rabbit mAb to CD34 antibody (1:100) was added to sections, then the slices stained with 300 nM DAPI for 5 min and observed via confocal microscopy.

2.10. Degradation of AuNP–GNP in vivo

In order to validate the degradation of this multistage nanocarrier in vivo, AuNP–GNP (200 mg/mL, 0.1 mL) were injected into subcutaneous tumor in situ. The mice were anesthetized after injection for 2 h, then the hearts of mice were perfused with phosphate buffer and paraformaldehyde, thereafter the tumor tissues were sampled from the injection site and the region away from the injection site (about 1 mm³) respectively, and fixed with 25% glutaraldehyde. After a series of dehydration step, the ultrathin sections were stained with osmium for TEM observation.

2.11. Anti-tumor efficacy

BALB/c mice bearing 4T1 tumor were randomly divided into seven groups (n = 5). After the size of tumors reached 100 mm³, mice were given DOX, DOX–DGL–PEG, RGD–DOX–DGL–PEG, DOX–GNP, DOX–DGL–GNP and RGD–DOX–DGL–GNP (at an equal dose of 5 mg/kg) and PBS (the control group) every three days for four times, respectively. The tumor size and animal body weight were measured every three days during the study and the tumor volume was calculated by the formula:

\[
\text{Tumor volume} = \left(\frac{\text{width}^2}{2}\right) / 2
\]

After 27 days, all the mice were sacrificed and the tumors were harvested for imaging, weighing and HE staining.

2.12. Statistical analysis

Data were analyzed using the IBM SPSS 19.0 software. Paired Student’s t test was used for comparison. A probability (p) less than 0.05 was considered statistically difference.

3. Results

3.1. Synthesis and characterization of DGL derivatives

DGL was first decorated with PEG and PEG–RGD (Fig. 2A). In NMR spectra of RGD–DGL–PEG (Fig. 2B), the peak of PEG’s repeat units (–O—CH₂—CH₂—) was presented a sharp peak at 3.4–3.6 ppm (Huang et al., 2013) and the double peak of RGD was found at 6.873 ppm, 6.886 ppm, 7.189 ppm and 7.211 ppm (Miura et al., 2013). Then DOX was conjugated to the residual primary amino groups of RGD–PEG–DGL by cis-aconityl linkage. Mass spectrometry exhibited the mass value of cis-aconityl anhydride-doxorubicin (CAD) was 700.5 (M+H) (Fig. 2C), which was in agreement with previous study (Zhu et al., 2010). The analysis of HPLC also indicated the successive synthesis of CAD. Finally, the activated carboxyl groups of CAD were connected to the amino groups of RGD–DGL–PEG to obtain the final product: RGD–DOX–DGL–PEG. The purified RGD–DOX–DGL–PEG, RGD, and CAD were evaluated by UV–vis absorption spectrophotometer (Fig. 3). The characteristic absorption peaks of RGD and CAD were at 275 nm and 490 nm, respectively, and the above characteristic absorption peaks were observed in the absorption spectrum of the RGD–DOX–DGL–PEG, indicating the successful synthesis of the RGD–DOX–DGL–PEG conjugates (Liu et al., 2012). The calculated numbers of PEG, RGD and DOX per DGL were 7.1, 2.5 and 13.3, respectively.

3.2. Characterization of RGD–DOX–DGL–GNP

The diameters of RGD–DOX–DGL–PEG were 34.4 nm with a PDI of 0.273. After decorated onto GNP, the diameters of RGD–DOX–DGL–GNP were considerably increased to 193.1 nm with a PDI of 0.272. The increase of particle size indicated that small-sized RGD–DOX–DGL–PEG was successfully modified on the surface of GNP.
The DOX loading capacity of RGD–DOX–DGL–PEG was 4.81% ± 0.4%.

3.3. In vitro release of DOX

Compared with the rapid release of free DOX, all particles were showed with sustained release profiles and no burst initial release (Fig. 4). After 24 h incubation, there were lower than 40% of DOX released. The release amounts of DOX–DGL–GNP and RGD–DOX–DGL–GNP were less than 20% at pH 7.4 while the release rates of DOX–DGL–GNP and RGD–DOX–DGL–GNP rapidly increased with the reduction of the pH value of the medium. The phenomenon above mainly attributed to the acid-sensitivity of cis-aconityl bond which was also in accordance with literature (Du et al., 2013). The released profiles suggested this multistage NP would be stable under the normal physiological pH value conditions.

3.4. In vitro cytotoxicity

Cytotoxicity is a primary concern in the development of drug delivery system. The viabilities of mouse mammary breast tumor cells (4T1) (Fig. 5A) and human umbilical vein endothelial cells (HUVEC) (Fig. 5B) were determined after 48 h of incubation with various formulations. All formulations showed dose-related anti-tumor cells activity, and at as high as 50 μM of DOX, the survival of cells treated by all formulations was lower than 40%. Specifically, RGD functionalized particles, RGD–DOX–DGL–PEG and RGD–DOX–DGL–GNP, showed better anti-proliferation effect than the unmodified particles.

3.5. Penetration efficiency of RGD–DOX–DGL–GNP

As shown in Fig. 6A, small sized RGD–DOX–DGL–PEG possessed better penetration efficiency than large sized RGD–DOX–DGL–
GNP, while RGD–DOX–DGL–GNP incubated with MMP-2 could enhance the penetration efficiency, demonstrating smaller sized particles had better penetration ability, which was consistent with our previous reports (Hu et al., 2015a,b; Ruan et al., 2015a,b). In order to better qualitatively compare the penetration efficiency of various NP, the core/surface coefficient was introduced, and the higher the core/surface coefficient was, the stronger the penetration efficiency of NP was. At 100 μm depth, the core/surface coefficient of RGD–DOX–DGL–GNP (MMP-2) was much higher than RGD–DOX–DGL–GNP (Fig. 6B). Besides, the fluorescence intensity profiles of NP at 100 μm depth also indicated that the penetration ability of RGD–DOX–DGL–GNP after degradation was apparently stronger than that before (Fig. 6C). All the results confirmed that this multistage nanocarrier RGD–DOX–DGL–GNP could benefit from RGD active targeting and MMP-2 triggered degradation, which facilitated penetration in tumor.

3.6. SPR analysis

The whole uptake process of NP by cells included in two steps, surface binding on cell membrane and internalization (Guo et al., 2014). SPR was promised to detect the interaction between the sensor surface and samples. Fig. 7 showed representative sensorgrams of the binding of RGD–DOX–DGL–GNP or DOX–DGL–GNP to Integrin αvβ3 on the CM5 chip. The binding proportion of RGD–DOX–DGL–GNP was significantly higher than that of DOX–DGL–GNP (Fig. 7), indicating RGD was successfully modified on the surface of DOX–DGL–GNP and contributed to binding on αvβ3 positive cells.

3.7. In vitro cellular uptake study

Fluorescence microscopy and flow cytometry were used for qualitative and quantitative evaluation of the drug delivery capacity of the RGD–DOX–DGL–GNP into human umbilical vein endothelial cells (HUVEC) in vitro. Both results expressed the cellular uptake of RGD–DOX–DGL–GNP was higher than DOX–DGL–GNP, which meant that RGD–DOX–DGL–GNP possessed the ability of active targeting to tumor angiogenesis (Fig. 7B and C).

3.8. In vivo tumor distribution and penetration

4T1 breast cancer was used as model and ex vivo imaging was utilized to evaluate the tumor distribution and penetration of RGD–DOX–DGL–GNP. At 24 h after tail vein injection, the fluorescent intensity of large-sized NP (DOX–GNP, DOX–DGL–GNP and
Fig. 8. (A) Ex vivo imaging of 4T1-tumor bearing mice at 24h post injection of DOX, DOX-DGL–PEG, RGD–DOX–DGL–PEG, DOX–GNP, DOX–DGL–GNP and RGD–DOX–DGL–GNP at a dose of 5 mg/kg. (B) Semi-quantitative analysis of fluorescent intensity in tumors at 24h post injection (n = 3). (C) Quantitative analysis of DOX in the main organs (n = 3). (D) Quantitative analysis of DOX in the interior and exterior of tumors (n = 3). (E) Fluorescent distribution in slices of 4T1 tumors. Blue represents nuclei, red represents DOX, green represents neovascularure and scale bars represent 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
RGD–DOX–DGL–GNP) in tumor was apparently stronger than that of small-sized NP (DOX, DOX–DGL–PEG and RGD–DOX–DGL–PEG) (Fig. 8A and B), demonstrating large-sized NP possessed better tumor distribution and retention effect. In tumor sites DOX–DGL–GNP exhibited the higher fluorescent intensity than that of DOX–GNP, suggesting the multistage delivery system possessed better tumor targeting efficiency that large sized NP, which might due to not only better tumor retention but also tumor penetration effect. Besides, RGD-modified NP showed stronger fluorescent intensity than non-targeted NP, which due to the active targeting ability of RGD and was consistent with previous studies (Gao et al., 2014a,b; Zhan et al., 2010). Simultaneously, semi-quantitative analysis of the fluorescent intensity in tumor tissues also demonstrated that the intensity of RGD–DOX–DGL–GNP was 1.18, 1.53- and 1.93-fold higher than that of DOX–DGL–GNP, DOX–GNP and RGD–DOX–DGL–PEG, respectively (Fig. 8B), which confirmed this multistage shrinkable RGD–DOX–DGL–GNP could more effectively accumulate in tumor compared with other NP. To further validate this targeted multistage nanocarrier could effectively accumulate in tumor sites, reduce cardiotoxicity of DOX and penetrate to deep area of tumor tissues, the amounts of DOX in the heart, the internal and external tumor sites were measured by HPLC. The distributions of NP in heart were notably decreased compared with free DOX groups (Fig. 8C), which may contribute to the low cardiotoxicity of these NP. Besides, RGD–DOX–DGL–GNP showed highest DOX concentrations in both the internal and outer of tumor (Fig. 8D), confirming the multistage nanocarrier could not only effectively accumulate in the peripheral of tumor but also penetrate into the core of the tumor.

Then vasculature was stained to green and the distribution of DOX (red) in the tumor slices was determined. The fluorescent intensity of fixed-sized NP (DOX, DOX–DGL–PEG, RGD–DOX–DGL–PEG and DOX–GNP) was weaker than that of multistage drug delivery systems (DOX–DGL–GNP and RGD–DOX–DGL–GNP), which was consistent with quantitative analysis by HPLC, indicating the multistage drug delivery systems displayed better tumor targeting and retention effect. In addition, the fluorescence signal of DOX–DGL–GNP and RGD–DOX–DGL–GNP distributed homogeneously in the whole tumor including areas that both rich and lack of neovascularization, which further demonstrated the superiority of this multistage drug delivery system (Fig. 8E).


In order to validate the in vivo shrink effect and tumor penetration of this multistage drug delivery system, gold nanoparticles (AuNP) with 23.2 nm were used to replace DOX–DGL–PEG because of their strong contrast under TEM. Two hours after injection, the mostly integral AuNP–GNP could still be seen in the injection site in situ (Fig. 9A). At the same time, the partial structure of AuNP–GNP could be observed (Fig. 9B), suggesting the GNP based particles could be degraded in tumor due to the highly expressed MMP-2. Furthermore, in the area distanced from the injection site, the released AuNP monomers and oligomers could be clearly observed (Fig. 9C and D), indicating the AuNP with small size could penetrate much deeper than the integral AuNP–GNP with large size. This experiment demonstrated the GNP based multistage delivery system could be successfully degraded in
Fig. 10. In vivo anti-tumor effect. (A) 4T1 tumor growth curves of different groups after treatments (mean ± SD, n = 5). *p < 0.05, **p < 0.01. (B) Body weights of mice after various treatments. (C) Photos of the tumors collected from different groups of mice at the end of treatments (day 27). (D) Average weights of tumors in each treatment group on day 27 (mean ± SD, n = 5). *p < 0.05, **p < 0.01. (E) HE staining of tumor tissue after therapy. The image magnification is 200×. Scale bars represent 100 μm.
tumor and released small-sized NP, leading to penetrating to the deep site of tumor.

3.10. In vivo anti-tumor effect

Finally, the anti-tumor efficacy of this multistage nanocarrier was evaluated in 4T1 tumor bearing mice. By comparison with the control group (PBS), other groups exhibited tumor growth inhibition effect (Fig. 10A, C and D). The multistage drug delivery system, DOX–DGL–GNP, showed more remarkable anti-tumor effect than Free DOX, DOX–DGL–PEG and DOX–GNP, while RGD modification further elevated tumor inhibition from 67.4% (DOX–DGL–GNP) to 78.0% (RGD–DOX–DGL–GNP). In addition, no noticeable change in body weight was observed after administration of both RGD–DOX–DGL–GNP and DOX–DGL–GNP (Fig. 10B), suggesting that they did not have obvious systemic toxicity to mice. However, the weight loss of DOX group was obviously observed in the early administration mainly owing to the severe side effects of DOX (Fig. 10B) (Barry et al., 2007). After HE staining, it is well illustrated that this multistage RGD–DOX–DGL–GNP could effectively induce more apoptosis 4T1 cells compared with other groups (Fig. 10E). Overall, the RGD–DOX–DGL–GNP with shrinkable size achieved our aim of enhancing the tumor accumulation and improving the efficacy of cancer therapy.

4. Discussion

Over the past decades, various NP-based drug delivery systems have obtained a great attention for cancer treatment. However, the application of small-sized NP was greatly restricted by the fast systemic clearance and limited tumor retention, while large-sized NP accumulated in tumor tissue through EPR effect but hardly diffused to deep tumor regions (Sunogrot et al., 2014). As a result, the conventional NP-based drug delivery system of fixed size can’t meet the requirements for both powerful penetration and strong retention in tumors at the same time, so intelligently size-alterable multistage drug delivery systems have been applied for cancer therapy owing to its inherent advantages. Low pH or high concentrations of MMP-2 in tumor microenvironment can be the stimulus of size change of these multistage nanocarriers. For instance, Yu et al. (2014) developed a pH-sensitive micelles which altered its size and surface charge in low pH, increasing the anti-tumor efficacy. In another study, Wong et al. (2011) obtained a multistage quantum dot NP which exhibited powerful tumor penetration in vitro and in vitro degraded by MMP-2.

The overexpression of MMP-2 was found in most cancers including breast cancer, prostate cancer, bladder cancer and brain cancer (Roy et al., 2009) and were highly expressed not only in tumor cells but also in tumor matrix (Lin et al., 2011). Therefore, due to the degradability of gelatin by MMP-2, we constructed a multistage nanocarrier which had a gelatin core and a surface modified with RGD–DOX–DGL–PEG. This multistage nanocarrier, DOX–DGL–GNP, achieved the shrinkable size from large size (177.0 ± 5.4 nm) to small size (34.4 ± 2.2 nm) stimulated by MMP-2 in vitro (Hu et al., 2015a,b). Large NP were key to accumulate in solid tumors by EPR effect and small NP were necessary for deep tumor penetration (Ruan et al., 2015a,b; Wong et al., 2011; Yu et al., 2014). To verify the feasibility of this strategy in vivo, AuNP were used to replace DOX–DGL–PEG due to their small size of 22.3 nm similar to RGD–DOX–DGL–PEG. AuNP–GNP could be degraded by the gelatinase of tumor and then released AuNP; the released AuNP with small size could penetrate deeper than the integral AuNP–GNP with large size. Furthermore, DOX–DGL–GNP incubated with MMP-2 could penetrate deeper into tumor spheroids in vitro by comparison with that without MMP-2. All these results demonstrated the shrinkable property of RGD–DOX–DGL–GNP and the benefit for penetration and retention, which was consistent with other studies (Wong et al., 2011; Yu et al., 2014). In vivo, RGD–DOX–DGL–GNP showed more remarkable tumor growth inhibition effect than other groups, which may ascribe to the shrinkable size of multistage nanocarrier. Specifically, RGD–DOX–DGL–GNP shielded RGD–DOX–DGL–PEG from fast elimination. When RGD–DOX–DGL–GNP accumulated around the leaky regions of tumor blood vessels, high concentration of MMP-2 in tumor environment could degrade GNP core and then released small-sized RGD–DOX–DGL–PEG, and then diffused into deep tumor tissues relaying onself-diffusional ability of small-sized NP, resulting in killing more tumor cells in the hardly accessible tumor areas.

In summary, our multistage nanocarrier RGD–DOX–DGL–GNP possessed the following predominance: (i) enhancing NP retention in tumors, (ii) owning deep tumor penetration and (iii) reducing toxicity of DOX, all of which increased the anti-tumor efficacy of NP.

5. Conclusion

In this study, a multistage nanocarrier, RGD–DOX–DGL–GNP, was constructed and evaluated. Based on the MMP-2 sensitive size shrink, the RGD–DOX–DGL–GNP delivered the DOX to the least access area of solid tumor, the core in tumor tissues, where tumor stem cells usually stayed, thus enhancing therapeutic efficacy. We believe that this study provide a facile strategy towards the design of more intelligent nanocarriers for deep tumor penetration in future.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (81402866, 31571016).

References


