

Studies on Antineoplastic Effect by Adjusting Ratios of Targeted-ligand and Antitumor Drug^{*}

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Abstract A series of drug delivery systems based on a sodium alginate derivative were prepared by mixing glycyrrhetic acid (GA) and doxorubicin (DOX) conjugates at different ratios. GA (a liver-targeting ligand) and DOX (an antitumor drug) were both conjugated to oligomeric glycol monomethyl ether-modified sodium alginate (ALG-mOEG) for prolonged duration of action. These NP-based delivery systems exhibited active cell uptake and cytotoxicity *in vitro* and liver-targeted distribution and anti-tumor activity *in vivo*. In addition, nanoparticles with a 1:1 (*W:W*) ratio of GA-ALG-mOEG and DOX-ALG-mOEG (NPs-3) showed the highest cellular uptake and cytotoxicity *in vitro* and liver-targeted distribution and anti-tumor activity *in vivo*. Specifically, when mixed nanoparticles defined as NPs-3 were injected in mice, liver DOX concentration reached 61.9 $\mu\text{g/g}$ 3 h after injection, and $\text{AUC}_{0-\infty}$ and $t_{1/2}$ of DOX in liver reached 4744.9 $\mu\text{g}\cdot\text{h/g}$ and 49.5 h, respectively. In addition, mice receiving a single injection of NPs-3 exhibited much slower tumor growth (88.37% reduction in tumor weight) 16 days after injection compared with placebo. These results indicate that effective cancer treatment may be developed using mixed NP delivery systems with appropriate ratio of targeted ligand and drug.

Keywords: Sodium alginate derivative; Drug delivery; Targeted-ligand; Antitumor drug; Antineoplastic effect.

INTRODUCTION

Nanometer-sized particles (NPs) display outstanding quantum size effect and have attracted significant attention as delivery systems for hydrophobic antitumor drugs^[1–4]. Tumor-targeting ligands may guide anti-tumor drug-conjugated NPs to tumor tissues, achieving better therapeutic efficacy with reduced drug toxicity^[5–8]. Nonetheless, the targeting effect may not be adequately used if concentration of the targeting ligand is too low^[9]. Similarly, it may be difficult to achieve desired drug efficacy if drug loading of NPs is too low, since the amount of carrier allowed in dosing is usually limited by its system toxicity^[10, 11]. Therefore, an increasing number of research groups have been working on development of new drug delivery systems characterized by high content of targeting ligand or high level of drug loading^[11, 12].

However, these high loading delivery systems might have their own disadvantages. While NPs modified with high density of targeting ligand are more effectively delivered to target cells, they are also more likely to induce immune response and fast clearance by mononuclear phagocytic system (MPS)^[13]. In addition, high loading of hydrophobic drugs in NPs may negatively affect the dispersity^[14]. Thus, we speculated that a balanced loading of drug and targeting ligand in NP-based delivery systems might be critical for optimal therapeutic efficacy with minimal adverse effects.

^{*} This work was financially supported by the National Natural Science Foundation of China (Nos. 51073080 and 51273095), Natural Science Foundation of Tianjin (No. 13JCYBJC25100) and PCSIRT (No. IRT1257).

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Received December 27, 2013; Revised February 11, 2014; Accepted February 17, 2014

doi: 10.1007/s10118-014-1432-y

Although multiple methods have been reported to modify loading of targeting ligands and drugs in NPs^[9, 15–18], optimization of the loading ratio between targeting ligand and antitumor drug to improve therapeutic performance of NPs has rarely been studied. We believe that it is feasible to improve drug efficacy by identifying the appropriate loading ratio of targeting ligand and drug in NPs. Among methods exploited to control loading level in NPs, nanoparticles' blending is a relatively simple, flexible, and effective approach^[19–21]. Lee *et al.*^[22] reported a mixed NP system composed of polymeric micelles that target tumor cells *via* pH-sensitivity and DOX-loaded micelles that are not tumor-targeted. Their results showed that the blended micelles inhibited tumor growth by 80% compared with 40% by free DOX. Kataoka *et al.*^[16] reported a method to control folate concentration on surface of micelles by mixing folate-modified micelles and non-modified micelles at different ratios and demonstrated that cancer treatment efficacy was significantly increased by optimizing the number of folate molecules on surface of mixed micelles. These results demonstrated the feasibility of using blending to create drug delivery systems with desired functions and properties.

Oligomer chain monomethoxy polyethylene glycol (mOEG) modified-ALG (ALG-mOEG) is a water-soluble ALG derivative and has been shown to serve as an efficient carrier with low immunogenicity for hydrophobic chemotherapy drugs such as DOX in our previous study^[14]. Recent studies have also shown that carriers modified with GA (a hepatocyte-targeting ligand) are able to selectively target liver cancer cells^[23, 24], resulting in 2.18-fold higher drug uptake by HepG2 cells than Chang liver cells (a normal liver cell)^[25]. DOX is a very effective chemotherapeutic agent against tumors^[26, 27]. We designed a series of NP delivery systems composed of GA-conjugated ALG-mOEG and DOX-conjugated ALG-mOEG mixed at different ratios, and investigated their biological activities *in vitro* and *in vivo*. First, DOX and GA were conjugated to ALG-mOEG by hydrazone bond to produce DOX-ALG-mOEG and GA-ALG-mOEG, respectively. Then, a series of dual-function NP delivery systems were prepared by mixing DOX-ALG-mOEG and GA-ALG-mOEG at different ratios. The dual-function delivery systems were evaluated in aspects of their cellular uptake and cytotoxicity *in vitro* and biodistribution and anti-tumor effect *in vivo*. The optimal blending ratio of GA-ALG-mOEG and DOX-ALG-mOEG was thereby identified for maximal antitumor effect.

EXPERIMENTAL

Materials

Glycyrrhetic acid (GA, purity > 98% by HPLC) was purchased from Fujie Pharmaceutical Co., Ltd. (Xi'an, China). Sodium alginate (ALG, viscosity is 24 mpa·s, 20 °C, 1% aqueous solution) was supplied by Jing Yan Biotechnology Co., Ltd. (Qingdao, China). Doxorubicin·HCl was obtained from Hua Feng United Technology Co., Ltd. (Beijing, China). 2-Chloro-1-methylpyridinium iodide (CMPI), tert-butyl carbazate and oligomeric glycol monomethyl ether (mOEG) with molecular weight (MW) of 550 was obtained from Alfa Aesar (Tianjin, China). Trifluoroacetic acid (TFA) and tetrabutylammonium (TBA) hydroxide were purchased from Aladdin. DMEM medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and FBS were purchased from Sigma-Aldrich. RIPA Lysis Buffer was purchased from Ding Guo Biotechnology Co., Ltd. (Beijing, China) and the HCl/ethanol (6/94, *V/V*) was prepared by ourselves.

Liver cancer cells (HepG2 cells) and normal cells (Chang liver cells) were maintained under standard cell culture conditions (37 °C, humidified, 5% CO₂). Kunming mice (range, 18–22 g) were purchased from the Laboratory Animal Center, Tianjin Medical University and tested in strict compliance with the “Guide for the Care and Use of Laboratory Animals”.

Synthesis of mOEG-modified ALG (ALG-mOEG)

Tetrabutylammonium-alginate (ALG-TBA) was synthesized as previously reported^[28]. 1.190 mmol ALG-TBA and 0.595 mmol CMPI were added in 50 mL DMF and the solution was kept under nitrogen at 0 °C for 1 h. Then, 1 mL mOEG was added, and the mixture was stirred under nitrogen atmosphere for 24 h. After that the solution was concentrated *in vacuo*, precipitated in absolute dichloroethane, and the precipitate was filtered. The ALG-mOEG was obtained after drying.

Preparation of Nanoparticles (DOX-ALG-mOEG/GA-ALG-mOEG NPs)

Synthesis of DOX-ALG-mOEG

CMPI (0.476 mmol) was added into the DMF with ALG-mOEG (500.0 mg) to activate the carboxyl group of ALG-mOEG. After that, 55.4 mg tert-butyl carbazate was reacted with ALG at room temperature for 1 day and the protective tert-butyl groups in the polymer were removed by 2 mL trifluoroacetic acid (TFA). After repeatedly precipitated and dissolved, the product (NH₂NH-ALG-mOEG) was obtained.

NH₂NH-ALG-mOEG (500 mg) was added into 250 mL DMF, followed by the addition of 200 mg DOX, and a small amount of acetic acid was added into the reaction system as the catalyst. The solution was kept at 60 °C for 48 h. Then, the polymer was purified by precipitation. Finally the precipitate was filtered, dissolved and transferred into a dialysis bag to dialyze against distilled water for 3 days.

Synthesis of GA-ALG-mOEG

The process of synthesis was similar with previous study on minor modifications. In brief, to a solution of ALG-mOEG (1.000 mmol) in 50 mL DMF, 127.7 mg (0.500 mmol) CMPI and 283.3 mg (0.500 mmol) ethanediamine-glycyrrhetic acid was added, the reaction lasted 20 h. Finally, the product was washed for three times by EtOH and transferred into a dialysis bag (MWCO 7000) to dialyze against distilled water for 3 days.

Preparation of DOX-ALG-mOEG/GA-ALG-mOEG NPs

DOX-ALG-mOEG/GA-ALG-mOEG NPs were formed by mixing DOX-ALG-mOEG and GA-ALG-mOEG in solution with the ratios (*W:W*) of the DOX-ALG-mOEG and GA-ALG-mOEG being 4:1, 2:1, 1:1, 1:2 and 1:4 respectively (Fig. 1). Take NPs-1 as the case, DOX-ALG-mOEG and GA-ALG-mOEG with the weight ratio of 4:1 were dissolved in DMF/water (4/1, *V/V*), with ultrasonic treatment at 0 °C for 5 min. Then the mixture was precipitated and washed for three times by EtOH, dialyzed against distilled water for 3 days.

Characterization of the polymers

FTIR spectrometry (Bio-Rad FTs-6000) was performed to provide evidence for successful synthesis of GA-ALG-mOEG and DOX-ALG-mOEG. The ¹H nuclear magnetic resonance (¹H-NMR) spectra of GA-ALG-mOEG and DOX-ALG-mOEG were recorded on a Varian Unity-Plus 400 NMR spectrometer to certify the GA or DOX was incorporated to ALG-mOEG using D₂O as the solvent. The drug load capacity of DOX-ALG-mOEG was analyzed by a UV-Vis spectrophotometer at 480 nm and the amount of GA in the GA-ALG-mOEG was also estimated by the UV-Vis spectrophotometer at 256 nm. Zetasizer Nano ZS90 (Malvern Instruments, UK) was used to measure the hydrodynamic diameter and PDI, the morphology of the NPs was observed by transmission electron microscopy (TEM, Philips TZOST, Philips Tecna Co., NED).

In vitro drug release study

The *in vitro* drug release study of NPs-3 was evaluated as the following procedures. The NPs-3 was dispersed in 2.5 mL of buffer solution (pH = 4.0, 5.0, 6.0, 7.4) and placed in a dialysis bag with molecular weight cut-off of 7 kDa at a density of 2 mg/mL, then it was immersed into another 10.0 mL PBS in the tube. It was incubated at 37 °C with shaking at a constant speed of 100 r/min. At scheduled time intervals, the release medium was removed and replaced by equal volume of fresh medium. The amount of the DOX was evaluated by using a fluorescence spectrometer (excitation = 480 nm, emission = 560 nm). The release studies of NPs-3 was also studied in DMEM containing 1 wt% bovine serum albumin (BSA) and the method was the same as described above. The drug release studies were performed three times for each of the samples.

Biodistribution studies

DOX-loaded nanoparticles with different ratios of DOX and GA (NPs-1, NPs-2, NPs-3, NPs-4, NPs-5 and NPs-6) were injected into the tail vein of female Kunming mice (18–22 g) with a single dose of 7 mg DOX/kg body weight (three rats per group). At appropriate intervals (0.5 h, 3 h, 6 h, 24 h, 72 h, 96 h), the mice were sacrificed and different tissues were removed immediately. After washed with 0.9% NaCl and homogenized in hypotonic phosphate buffer and HCl/ethanol (6/94, *V/V*), the homogenate was centrifugated and the DOX

fluorescence intensity in the clear supernatant was analyzed by fluorescence spectrometer with a fluorescence using excitation and emission wavelengths of 480 nm and 590 nm, respectively. The amounts of DOX in different tissues and $AUC_{0-\infty}$ were calculated.

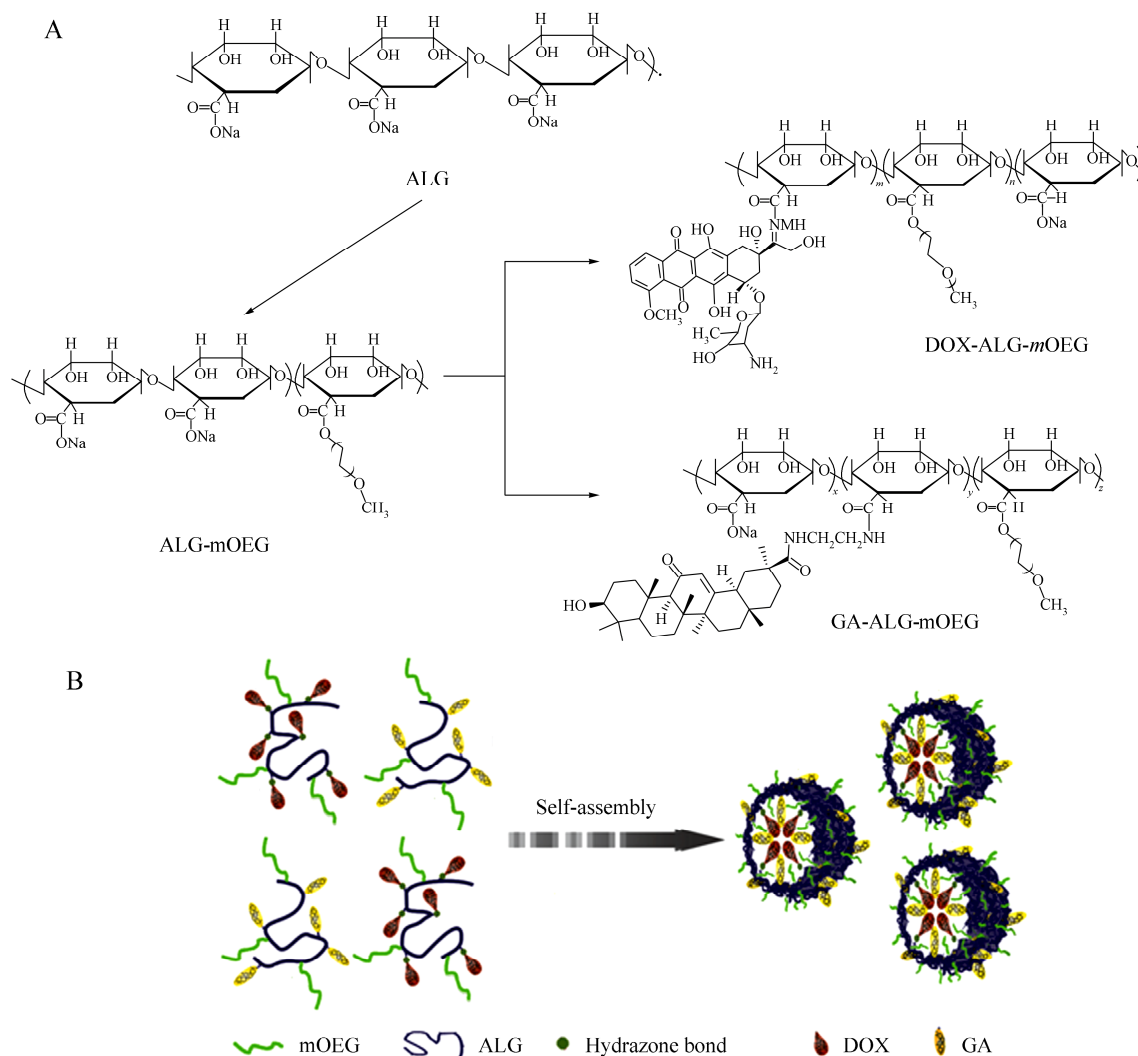


Fig. 1 (A) Synthetic route of ALG-mOEG, DOX-ALG-mOEG and GA-ALG-mOEG and (B) schematic representation of DOX-ALG-mOEG/GA-ALG-mOEG NPs from DOX-ALG-mOEG and GA-ALG-mOEG

In vitro cellular uptake of nanoparticles

The cellular uptake study comparing DOX-ALG-mOEG/GA-ALG-mOEG NPs (NPs-1, NPs-2, NPs-3, NPs-4 and NPs-5) and DOX-ALG-mOEG NPs (NPs-6) was implemented by flow cytometry. HepG2 cells at density of 4×10^4 cells/well were seeded in six-well plates and grown for 24 h. Then, these nanoparticles with a single dose of 10 μ g DOX/mL were respectively added into the six-well plates. After incubated for 4 h, the cells were washed three times with PBS and harvested by trypsinization. The cell suspension was measured with a flow cytofluorometer (BD FACSCalibur).

Cytotoxicity

The antiproliferative effect of DOX-ALG-mOEG/GA-ALG-mOEG NPs and DOX-ALG-mOEG NPs was evaluated by MTT assay on the HepG2 cells and Chang liver cells. HepG2 cells or Chang liver cells were seeded into 96-well plates at a density of 5×10^3 cells/well, and the DOX-loaded nanoparticles (NPs-1, NPs-2, NPs-3,

NPs-4, NPs-5 and NPs-6) were added after incubation for 24 h. Two days later, the culture medium was replaced by MTT solution and incubated for another 4 h. Then, 100 μ L of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured by a microplate reader at dual wavelengths of 570 nm, and cellular growth inhibition was calculated. The cytotoxicity of $\text{NH}_2\text{NH-ALG-mOEG}$ and GA-ALG-mOEG NPs were also evaluated. Cells incubated with culture media was used as control in this study.

In vivo anti-tumor efficacy

The Kunming mice with the H22 tumor in ectopia were chosen as model and the method was referenced by previously reported. The mice were averagely separated into seven groups ($n = 5$). Then the mice were intravenous injected NPs-6, NPs-3, NPs-1, NPs-5, NPs-2, NPs-4 at a dose of 7 mg DOX/kg body weight and saline every 4 days. Sixteen days later, the mice were sacrificed by cervical dislocation, the H22 liver tumors were removed immediately and stored in the formalin, the tumor growth inhibition rate (IR) was calculated.

Statistical analysis

Results are presented as the mean value with standard deviation (mean $\pm$ SD). Pair-sample *t*-Test analysis of variance was used to compare the differences. The value of $*p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of GA-ALG-mOEG NPs, DOX-ALG-mOEG NPs and DOX-ALG-mOEG/GA-ALG-mOEG NPs

Structures of GA-ALG-mOEG and DOX-ALG-mOEG were confirmed by FTIR and $^1\text{H-NMR}$ spectra. In FTIR spectra, peaks at 1616 cm^{-1} and 1421 cm^{-1} were COO^-Na^+ absorption bands in ALG. The peak at 1734 cm^{-1} represented ester bonds, indicating that mOEG moiety was successfully introduced on ALG. In addition, peaks at 1669 cm^{-1} (1665 cm^{-1}) and 1528 cm^{-1} (1531 cm^{-1}) indicated the formation of amide bond (Figs. 2a and 2c). Because of the absorption bands of C=N at 1622 cm^{-1} ^[29], the broad peak from 1616 cm^{-1} to 1669 cm^{-1} indicated that the hydrazone bond formed.

In $^1\text{H-NMR}$ spectra, chemical shifts at 3.65 ($\text{OCH}_2\text{CH}_2\text{O}$) and 3.33 (CH_3O) represented mOEG moiety, and new peaks at 0.6–1.6 (Fig. 3b) indicated formation of GA and ALG-mOEG (ALG) conjugate^[23]; methyl and methylene (0.8–1.4) for DOX of DOX-ALG-mOEG (Fig. 2b) were observed in the $^1\text{H-NMR}$ spectrum which indicated the successful introduction of DOX on ALG-mOEG. Content of GA in GA-ALG-mOEG conjugate was about 11.8% (mol/mol) and drug loading of DOX-ALG-mOEG was about 18% (*W/W*).

Delivery systems of blended DOX-ALG-mOEG/GA-ALG-mOEG NPs were prepared using a simple and convenient method as illustrated in Fig. 1(B). GA-ALG-mOEG was synthesized from ALG-mOEG and ethanediamine-glycyrrhetic acid *via* a conjugation reaction. DOX and ALG-mOEG were covalently linked by hydrazone bond to form DOX-ALG-mOEG. DOX-ALG-mOEG and GA-ALG-mOEG were subsequently mixed at different ratios with ultrasonic treatment under room temperature to produce blended NPs (NPs-1, NPs-2, NPs-3, NPs-4 and NPs-5). NPs-6 was DOX-ALG-mOEG NPs alone.

Hydrodynamic particle sizes of NPs-1, NPs-2, NPs-3, NPs-4, NPs-5, and NPs-6 were determined by Zetasizer Nano ZS90 (DLS) with a scattering angle of 90° (Table 1). These nanoparticles had small PDI with an average diameter of about 260 nm in ultrapure water, while with a smaller hydrodynamic diameter (about 200 nm) at acid environment (pH 4.0). It is inferred that the protonation of COO^- in ALG at pH 4.0 provided stronger hydrophobic interactions to maintain the compactness and formed the smaller sizes. The morphology of the NPs exhibited a regular spherical shape (Fig. 3); the NPs in dry state with a diameters 200 nm was smaller than the diameters measured by DLS because of the swelling of ALG in solution. Zeta potential of these NPs was about -30 mV . The nanoparticles surface in these dual-functional delivery systems is sterically stabilized by mOEG, which is strongly hydrophilic and thus low protein-binding *in vitro* and *in vivo*. In addition, NPs enter cells by the endocytosis pathway. Previous studies have shown that biodistribution and endocytosis of NPs are affected by particle properties such as particle size and zeta potential^[30, 31]. Endocytosis of NPs not only depends on ligand density but also particle size^[32, 33]. Since all NPs prepared in this study had similar size and zeta

potential, experimental results obtained with NPs with a given GA/DOX ratio were reproducible, making it possible to compare and rank performance of particles with different GA/DOX ratios, and thereby identify the optimal ratio for maximal anti-tumor effect.

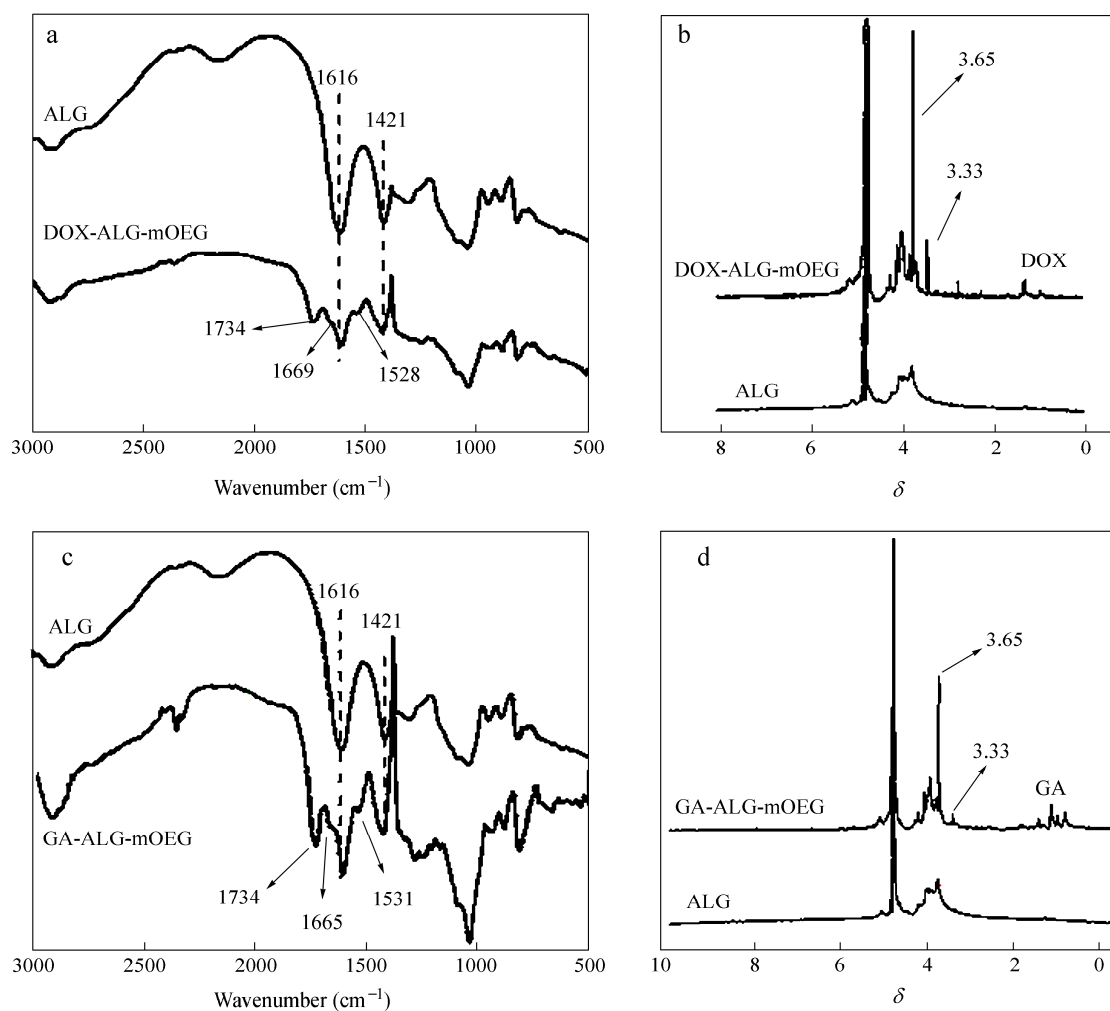


Fig. 2 FTIR spectra (a) and ^1H -NMR spectra (b) of ALG and DOX-ALG-mOEG; and FTIR spectra (c) and ^1H -NMR spectra (d) of ALG and GA-ALG-mOEG

Table 1. The basic characterization of DOX-ALG-mOEG/GA-ALG-mOEG NPs

Sample	The ratio of DOX-ALG-mOEG and GA-ALG-mOEG	Diameter (nm)	PDI
NPs-1 ^a	4:1	272.3 ± 3.22 ^b	0.272
NPs-2	2:1	264.7 ± 7.66	0.282
NPs-3	1:1	254.2 ± 2.55	0.207
NPs-4	1:2	254.7 ± 4.20	0.167
NPs-5	1:4	248.3 ± 3.30	0.213
NPs-6	1:0	274.3 ± 4.11	0.203

^a The mean diameter of nanoparticles in distilled water at 1.0 mg/mL and measured by dynamic light scattering (DLS);

^b The results were presented as mean $\pm$ SD; $n = 3$

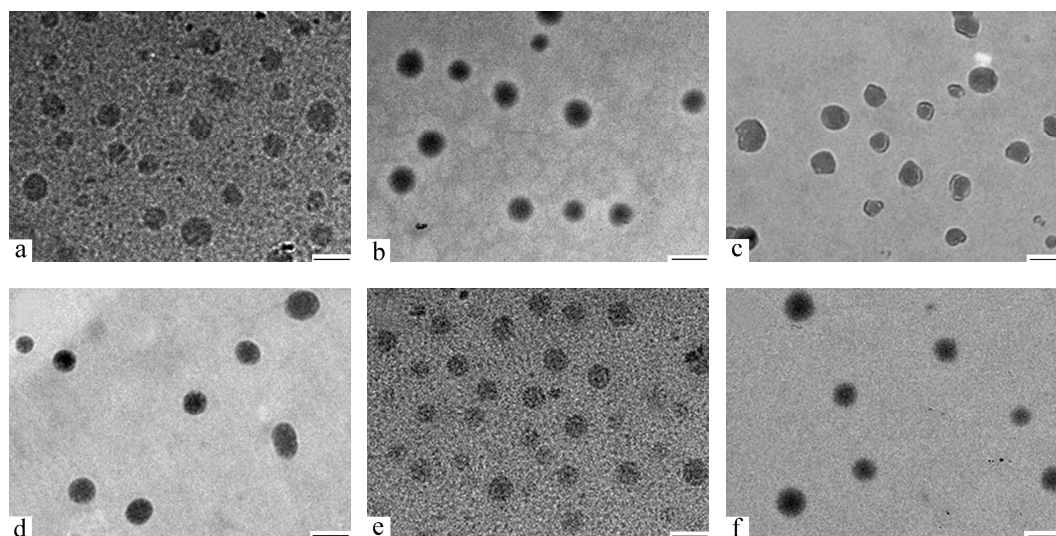


Fig. 3 The TEM images of NPs-1 (a), NPs-2 (b), NPs-3 (c), NPs-4 (d), NPs-5 (e) and NPs-6 (f) (All scale bars are 100 nm.)

In Vitro Drug Release Study

DOX-ALG-mOEG/DOX-ALG-mOEG NPs were speculated to show pH-sensitive DOX release owing to the presence of acid-labile hydrazone bond^[3, 34, 35, 36]. We studied DOX release in NPs under five different buffer conditions including buffer solution at pH 4.0, 5.0, 6.0, and 7.4 and DMEM (pH 7.4) containing 1 wt% bovine serum albumin (BSA). Figure 4 showed DOX release in NPs-3 versus time of incubation under the five conditions. The release profiles showed that the drug release rate of NPs-3 accelerated and the DOX accumulated release amounts increased with the increasing concentration of H^+ . DOX was released at a much faster rate in buffer solution at pH 4.0 (60.9% in 7 days) compared with that in buffer solution at pH 7.4 (10.7% in 7 days), indicating high pH-sensitive hydrolysis of the hydrazone bond (Fig. 4a). Meanwhile, DOX was released at a rate of 13% in 2 days in DMEM containing 1 wt% BSA, a buffer simulating human physiological environment (Fig. 4b). Since DOX was released at similar rates in buffer solution at pH 7.4 and DMEM (pH 7.4), pH was the main property of buffer that affected hydrolysis of the hydrazone bond. Our results were in agreement with previous reports that hydrazone bond was very stable in plasma. In this drug delivery system,

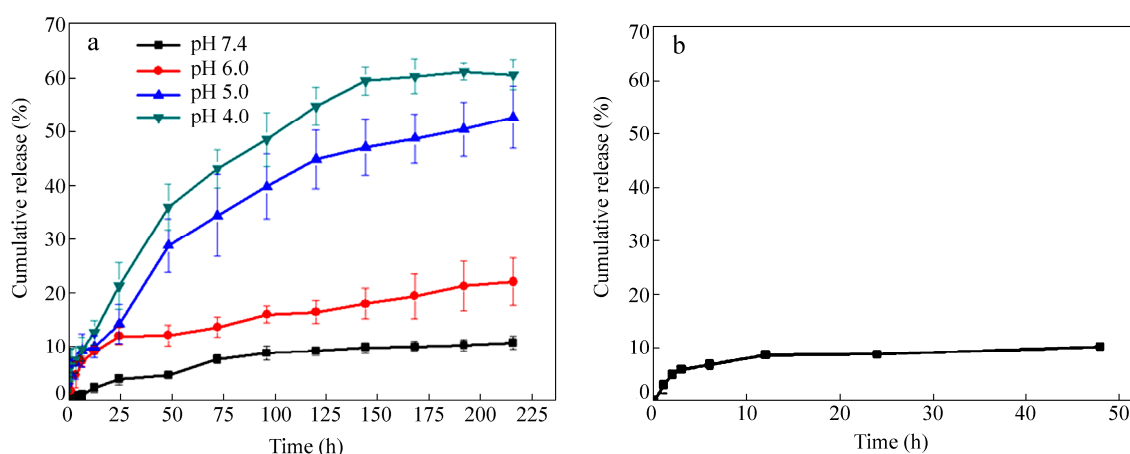


Fig. 4 *In vitro* release of NPs-3 in buffer solution under acidic (pH 4.0, 5.0, 6.0) and neutral (pH 7.4) condition (a) and DMEM under neutral (pH 7.4) condition at 37 °C (b) (Means $\pm$ SD, $n = 3$)

we combined the advantage of the hydrazone bond and GA, so this dual functional drug delivery system can not only reduce drug release during circulation but also specifically enhance intracellular drug concentration because of the GA-mediated role to liver cells. Thus we speculated that a DOX-ALG-mOEG/G-ALG-mOEG NP delivery system with appropriate DOX/GA ratio is suitable for liver-targeted drug delivery.

In Vivo Biodistribution Study

The superior liver-targeting efficiency of GA has been demonstrated in our previous studies^[37]. In the present study, we aimed to develop an optimal drug delivery system by adjusting targeting ligand (GA) and drug (DOX) ratio. First, we examined biodistribution of different NPs (NPs-1, NPs-2, NPs-3, NPs-4, NPs-5, and NPs-6) in mice liver. The six NPs with different GA/DOX ratio showed distinct biodistribution patterns (Fig. 5). Liver DOX concentration in NPs-6 (DOX-ALG-mOEG alone) group was about 22.9 $\mu\text{g/g}$ 3 h after injection, which was higher than that in the DOX·HCl group, attributed to prolonged half-life of the conjugated drug. Liver DOX concentration in NPs-6, however, was the lowest among the six NP groups tested. Addition of GA-ALG-mOEG to DOX-ALG-mOEG increased the liver DOX concentration. Liver DOX concentration in the NPs-1 group reached 50.08 $\mu\text{g/g}$ 3 h after injection. When ratio of GA-ALG-mOEG/DOX-ALG-mOEG increased to 1:1 (NPs-3), liver DOX concentration increased to 61.9 $\mu\text{g/g}$ 3 h after injection. Further increasing the GA/DOX ratio, however, led to decreased liver DOX concentration. Collectively, our results suggested that the liver-targeted ability not only depends on the use of GA as targeting ligand but also the GA/DOX ratio. The highest DOX delivery to liver was achieved at GA/DOX ratio of 1:1 (NPs-3). We found that 23 $\mu\text{g/g}$ of DOX was still detected in liver in NPs-3 group 96 h after injection, while no DOX was detected in DOX·HCl group 48 h after injection. $\text{AUC}_{0-\infty}$ and $t_{1/2}$ of DOX in liver in NPs-3 group were 4744.9 $\mu\text{g}\cdot\text{h/g}$ and 49.5 h, respectively, which were 3.6-fold and 1.4-fold higher than those in NPs-6 group. Therefore, our results demonstrated that more efficient liver-targeted delivery and longer duration of action of DOX were achieved by using blended GA-ALG-mOEG/DOX-ALG-mOEG NPs with appropriate targeted-ligand/drug ratio.

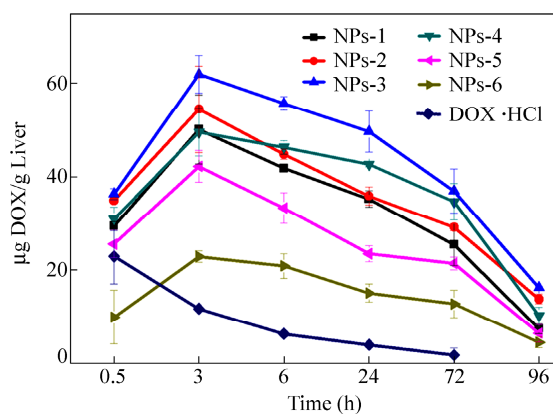


Fig. 5 DOX concentration in the liver after intravenous injection of DOX·HCl, NPs-1, NPs-2, NPs-3, NPs-4, NPs-5 and NPs-6 in Kunming mice at a single dose of 7 mg/kg (The results are given as mean $\pm$ SD with $n = 3$.)

Cellular Uptake Study

We further studied drug delivery performance of these NP-based systems in an *in vitro* cellular uptake assay, in which HepG2 cells treated with different NPs were examined on a flow cytofluorometer to determine cellular DOX uptake. DOX is a fluorophore and served as a fluorescent probe in the uptake assay. Low fluorescence signal was detected with un-treated HepG2 cells and incubation with NPs resulted in increased cellular fluorescence (Fig. 6). We found that cellular uptake of DOX was higher in cells treated with NPs containing GA-ALG-mOEG (NPs-1, NPs-2, NPs-3, NPs-4, NPs-5) compared with NPs of DOX-ALG-mPEG alone (NPs-6). The highest DOX cellular uptake was observed in cells treated with NPs-3, which contained equal amounts of

DOX-ALG-mPEG and GA-ALG-mPEG (1:1). Specifically, DOX uptake in cells treated with NPs-3 was 1.65-fold higher than that in cells treated with NPs-6. Thus, our cellular uptake results demonstrate that the targeting ligand/drug ratio in blended NPs controls the drug delivery efficiency of the system.

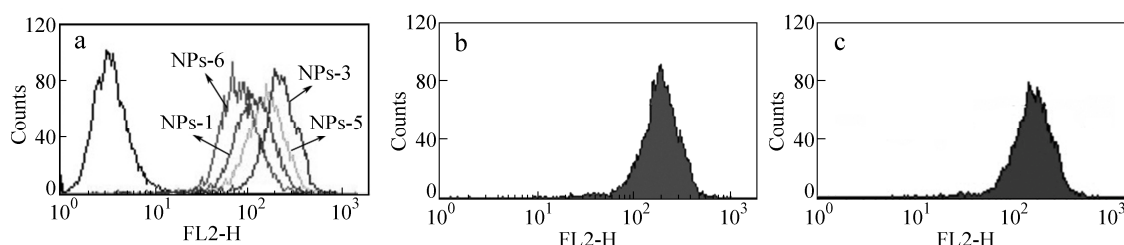


Fig. 6 Flow cytometry histogram profiles of HepG2 cells after the treatment with NPs-1, NPs-3, NPs-5, NPs-6 (a), NPs-4 (b) and NPs-2 (c) for 4 h at 37 °C at DOX concentration of 10 μ g DOX/mL

Cytotoxicity

Cytotoxicity of mixed NPs with different GA/DOX ratios was assessed by MTT assay, in which the viability of HepG2 cells and Chang liver cells were determined after 2 days treatment with NPs. IC_{50} (50% inhibitory concentration) values of different NPs are shown in Fig. 7. We found that all NPs showed cytotoxicity against HepG2 cells and Chang liver cells, indicating that DOX was transported into cells by NPs and released inside cells after endocytosis. In addition, all blended NPs containing GA-ALG-mPEG (NPs-1, NPs-2, NPs-3, NPs-4, NPs-5) showed stronger cytotoxicity than NPs of DOX-ALG-mPEG alone (NPs-6), presumably attributed to more efficient delivery mediated by GA. Cytotoxicity of blended NPs first increased with increasing ratio ($W:W$) of GA-ALG-mPEG to DOX-ALG-mPEG, owing to increased delivery of DOX mediated by higher concentration of GA. However, further increase in GA/DOX ratio led to decreased cytotoxicity, which we believe, mainly resulted from two reasons. First, binding sites for GA were saturated at higher GA levels, therefore GA-mediated transport stopped to increase with concentration of GA-ALG-mPEG beyond a certain point. Secondly, with further increase in GA/DOX ratio, content of DOX-ALG-mPEG in mixed micelles decreased further, resulting in lower drug loading and decreased cellular uptake of drug. Among all NPs tested, NPs-3 showed the highest cytotoxicity ($IC_{50} = 58.1$ ng/mL) in HepG2 cells, in alignment with our cellular uptake data. The similar result was also got in the Chang liver cells group (Fig. 7b), the IC_{50} of the NPs-3 with a value of 106.2 ng/mL was smaller than that of the other NPs which was attributed to the right ratio of liver-targeted ligand and antitumor drug. Thus we demonstrated that preparation of blended NP systems with appropriate targeting ligand/drug ratio is a valid approach to improve drug efficacy *in vitro*.

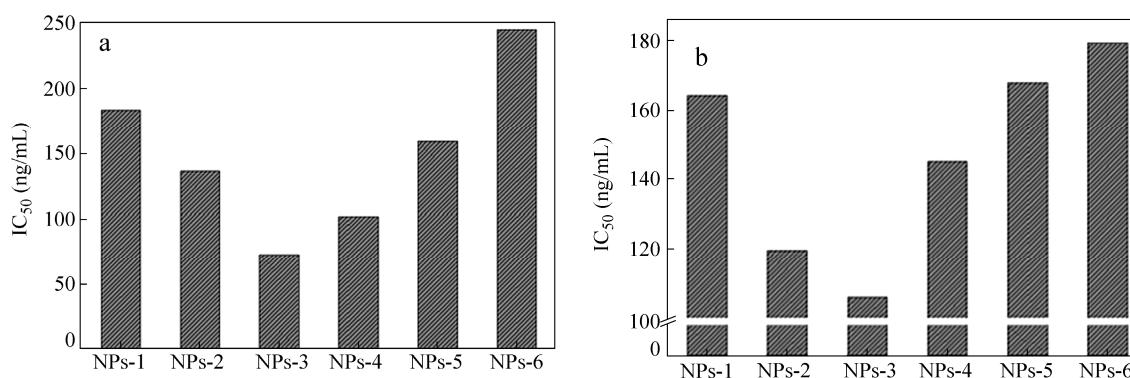


Fig. 7 The IC_{50} of NPs-1, NPs-2, NPs-3, NPs-4, NPs-5 and NPs-6 against HepG2 cells (a) and Chang liver cells (b) after incubation for 2 days

Inhibition of Tumor Growth

To find out whether *in vitro* cytotoxicity and *in vivo* liver-targeted delivery of blended NPs result in improved anti-tumor activity *in vivo*, we tested the effect of NPs on tumor growth in mice bearing H22 liver tumor. Tumor weight was determined 16 days after intravenous injection of NPs (Fig. 8). All NPs inhibited tumor growth in mice. Compared with NPs-6 (DOX-ALG-mPEG alone), NPs-1, NPs-2, and NPs-3 showed better anti-tumor effect attributed to GA-mediated, liver-targeted delivery. NPs-3 with a 1:1 ratio (*W:W*) of GA-ALG-mOEG and DOX-ALG-mOEG exhibited the strongest anti-tumor effect (88.37% inhibition), which was 27.3% higher than that of NPs-2. However, further increase in GA-ALG-mOEG content led to decreased anti-tumor effect, with NPs-5 exhibiting 69.7% inhibition of tumor growth. In addition to factors affecting cytotoxicity *in vitro*, excessive GA levels might induce immune response or drug clearance by the reticulo-endothelial system *in vivo* owing to its hydrophobicity, which might affect DOX delivery and efficacy *in vivo*. Taken together, our findings highlighted the importance of balanced loading of drug and targeting ligand in mixed NP delivery systems for effective tumor suppression.

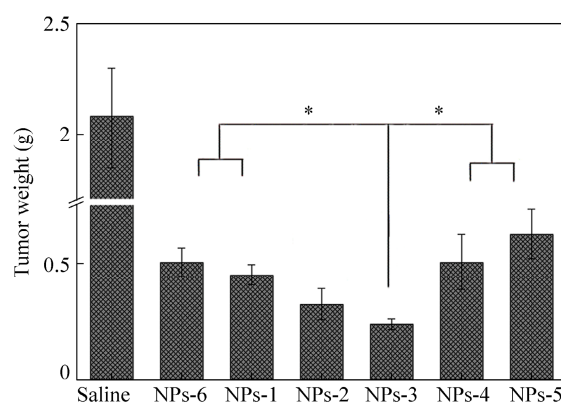


Fig. 8 The ultimate tumor weights of the mice bearing H22 liver tumor after treated with Saline, NPs-1, NPs-2, NPs-3, NPs-4, NPs-5 and NPs-6 (Means $\pm$ SD, * $P < 0.05$.)

CONCLUSIONS

In the present work, we prepared a series of NP-based delivery systems by mixing targeting ligand and anti-tumor drug at different ratios. These NP-based delivery systems showed active cellular uptake and cytotoxicity *in vitro* and targeted distribution and anti-tumor effect *in vivo*. In addition, *in vitro* and *in vivo* distribution and activity of these systems were found to be significantly affected by the ratio of targeting ligand and drug in the delivery system. Our results indicated that effective cancer treatment might be developed using mixed NP delivery systems with appropriate targeting ligand/drug ratio. Our work may help guide further research in this direction.

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