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Doxorubicin-loaded glycyrrhetinic acid-modified alginate nanoparticles for liver tumor chemotherapy

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ABSTRACT

Doxorubicin (DOX)-loaded glycyrrhetinic acid (GA)-modified alginate (ALG) nanoparticles (DOX/GA-ALG NPs) were prepared for targeting therapy of liver cancer. This study focused on the biodistribution of DOX/GA-ALG NPs in Kunming mice as well as their antitumor activity against liver tumors *in situ* and side effects. The biodistribution data showed that the concentration of DOX in the liver reached 67.8 ± 4.9 μg/g after intravenous administration of DOX/GA-ALG NPs, which was 2.8-fold and 4.7-fold higher compared to non-GA-modified nanoparticles (DOX/CHO-ALG NPs) and DOX·HCl, respectively. The concentration of DOX in the heart of mice treated with DOX/GA-ALG NPs at any sampling time was relatively lower than that of mice treated with DOX·HCl. The liver tumor growth inhibition rate (IR) *in situ* was about 52.6% and the mortality was 33% in DOX·HCl group. In contrast, the IR was 76.6% and no mice died in the DOX/GA-ALG NPs group. Histological examination showed tumor necrosis in both experimental groups. Most importantly, the heart cells and the liver cells surrounding the tumor were not affected by administration of DOX/GA-ALG NPs, whereas myocardial necrosis and apparent liver cell swelling were observed after DOX·HCl administration.

1. Introduction

Liver cancer is the fifth most common cancer in the world and the third most common cause of cancer mortality [1]. Surgery and chemotherapy are the main treatments for liver cancer, although the unavailability of liver donors is a major issue when considering surgery. Doxorubicin (DOX) has been shown to be one of the most commonly used chemotherapeutic agents in the treatment of liver cancer, but its clinical application is often limited due to its side effects, in particular cardiotoxicity [2]. Recently, various nano-drug delivery systems have been developed to reduce the side effects of DOX. Dufes et al. found that mice received pronounced weight loss after intravenous injection with DOX solution (10 mg/kg body weight dose level), but weight loss was not observed after administration of DOX-loaded palmitoylated glycol chitosan vesicles [3]. Dong et al. prepared nanoscale complexes (CPX2) composed of cationic gelatin, polyGC-DOX and pH-sensitive pegylated alginate, and found that 20 mg/kg body weight of DOX in the form of CPX2 did not cause death in mice, whereas the same dose of free DOX caused 60% death in 5 days [4].

Targeted drug delivery systems (TDDS) are currently explored for cancer therapeutics. TDDS allow the enhancement of drug concentration in the targeted organ or tissue, and decrease the dosage and side effects, using methods such as passive trapping of nanoparticles by the reticuloendothelium or active targeting based on receptor recognition [5,6]. Active targeting is usually achieved by conjugating a targeting ligand to the nanoparticles, thereby allowing preferential accumulation of nanoparticles in the tumor-bearing organ or in the actual tumor [7,8]. Ligands such as the Arg-Gly-Asp (RGD) [9], transferrin [3], folate [10] and galactosamine [11], modified drug delivery system have been investigated to treat melanoma, epithelial carcinoma, ovarian cancer and liver cancer, respectively. Glycyrrhetinic acid (GA) and glycyrrizin (GL), the main bioactive compounds extracted from licorice, are also common ligands in the TDDS [12]. Recent results show that carrier modified with GA or GL have higher accumulation in the liver and have superior targeting efficiency to hepatocytes, because of the abundant receptors for GA and GL on hepatocyte membranes [13,14]. Tsuji et al. founded that 42.4% of the total GL-modified

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liposomes accumulated in the liver, which was 4-fold more than the control sample [15]. Our previous study indicated that GA-modified chitosan (CTS) nanoparticles (CTS/PEG-GA NPs) had an accumulation about 51.3% in the liver at 3 h post-injection, and the DOX-loaded nanoparticles significantly inhibited tumor growth against subcutaneous tumors [16]. In our opinion, these subcutaneous tumor models do not display the same manifestations of tumor models *in situ*. We are more interested in the antitumor activity of DOX-loaded GA-modified nanoparticles against liver tumors *in situ*. Therefore, the antitumor activity against liver tumors *in situ* of the DOX-loaded GA-modified nanoparticles was estimated in this work. This would be of great importance for the treatment of liver cancer.

Polysaccharides have been proposed for biomedical purposes to a large extent for several years [17,18]. Alginate (ALG) is a nontoxic and biodegradable natural polysaccharide, which has been extensively investigated for pharmaceutical and biomedical application [19,20]. Here, we prepared DOX-loaded GA-modified ALG nanoparticles (DOX/GA-ALG NPs) using equilibrium dialysis method and examined their biodistribution in Kunming mice. In particular, we studied the antitumor activities as well as the side effects of DOX/GA-ALG NPs against Kunming mice bearing H22 liver tumors *in situ*.

2. Materials and methods

2.1. Materials

Sodium alginate (ALG, viscosity: 160 mpa s, 20 °C, 1% aqueous solution) was supplied by Qingdao Crystal Rock Biology Development Co., Ltd. (Qingdao, China). Glycyrrhetinic acid (GA, HPLC purity > 98%) was purchased from Fujie Pharmaceutical Co., Ltd. (Xi'an, China). Doxorubicin hydrochloride (HPLC purity > 98%) was obtained from Alliance Bernstein Technology Co., Ltd. (Beijing, China). Cholesterol (CHO, HPLC purity > 98%) was purchased from Anhui Kebao Bio-engineering Co., Ltd (Anhui, China). Cholesteryl grafted alginate was synthesized and purified according to the method reported by the Yang's group [21]. N-hydroxysuccinimide (NHS), N,N'-dicyclohexyl carbodiimide (DCC) and 1-ethyl-3(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC·HCl) were obtained from GL Biochem Ltd. (Shanghai, China). Pyrene, diamine-poly(ethylene glycol) (PEG-$(NH_2)_2$, Mw = 3400), Hoechst 33342 and tetrazolium salt (MTT) were all purchased from Sigma. Pegylated glycyrrhetinic acid (PEG-GA) was synthesized and purified according to the method reported by our group [16]. Acetonitrile and methanol were of chromatographic grade. All the other chemicals were of analytical grade. Distilled deionized water was used throughout the experiments.

Human hepatocellular carcinoma (HepG2) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Glutamax and 10% fetal bovine serum (FBS) under standard cell culture conditions (37 °C, humidified, 5% CO_2) without antibiotics. Kunming mice (range, 20–30 g) were housed in a ventilated, temperature controlled and standardized sterile animal room. The animal experiment procedures were in strict compliance with the "Guide for the Care and Use of Laboratory Animals".

2.2. Synthesis and characterization of GA-modified ALG

Glycyrrhetinic acid-ethylene diamine (GA-NH_2) and GA-modified ALG (GA-ALG) were synthesized and purified as our previously reported [22]. Briefly, to a solution of ALG (250.0 mg, 1.2 mmol) in 10 mL double distilled water, 25 mL of dimethyl sulfoxide (DMSO) was added dropwise. A certain amount of EDC·HCl was added in the stirring, an appropriate amount of NHS was added [mol_{ALG}: $mol_{EDC\cdot HCl}$: mol_{NHS} = 1:1.2:1.2], and 15 mL of DMSO solution containing GA-NH_2 (631.0 mg, 1.2 mmol) was added. The reaction mixture was stirred at room temperature for an additional 20 h and poured into ethanol. The precipitate was filtered, repeatedly dissoluted and precipitated, and finally freeze-dried to obtain GA-ALG. The chemical structure of GA-ALG was confirmed by ^{1}H NMR spectroscopy (Varian Mercury 400, USA) and IR spectroscopy (Spectrum Instruments Co., Ltd. Brook Germany). Compared with the *IR* spectrum of ALG, GA-ALG showed new peaks at 1658 cm^{-1} and 1542 cm^{-1}, ascribing to the amide bond. In contrast with the ^{1}H NMR spectrum of ALG, the relevant signals (0.75–1.5 ppm) were attributed to the angular methyl protons of GA.

The N content of GA-ALG was determined by elemental analysis (Vanio-EL elemental analyzer, Heraeus, Germany). The substitution degree (SD) of GA was defined as the ratio of reacted sugar unit to the total sugar unit of ALG, which was calculated according to Eq. (1):

$$\mathrm{SD}\ (\%) = \frac{\text{the reacted sugar unit of ALG}}{\text{the total sugar unit of ALG}} \times 100\% \quad (1)$$

2.3. Determination of the critical aggregation concentration (CAC)

The CAC of GA-ALG in an aqueous solution was determined using pyrene as a fluorescence probe. The concentration of GA-ALG (10.0 mL) varied between 1.0×10^{-3} and 1.0 mg/mL, and the final concentration of pyrene was fixed at 6.0×10^{-7} M. The solutions were allowed to stand for 24 h for equilibration before testing. The fluorescence emission spectra were measured on a fluorescence spectrometer (Spectrometer F-7000, HITACHI, Japan) with an excitation wavelength of 335 nm. The intensity ratio of the first band (I_{372}) to the third band (I_{383}) was monitored as a function of GA-ALG concentration. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

2.4. Preparation and characterization of nanoparticles

2.4.1. Preparation of nanoparticles

Doxorubicin hydrochloride was dissolved in N,N-dimethylformamide (DMF) in the presence of triethylamine (1.5 times molar quantity of DOX) with sonication (30 s) to form a DOX-containing solution. GA-ALG (30.0 mg) was dissolved in 30.0 mL of distilled water. The DMF solution of DOX (1.0 mg/mL) was added to the stirred aqueous solution. The suspension was stirred overnight using a magnetic stirrer. Then, the transparent dark red solution was transferred into a dialysis bag (MWCO 7000) to dialyze against distilled water for 48 h. The outer solution was exchanged at appropriate time intervals. The dialysis solution was freeze-dried to obtain DOX/GA-ALG NPs which were stored in a refrigerator until use. For the contrast analysis, DOX-loaded cholesteryl grafted alginate nanoparticles (DOX/CHO-ALG NPs) were prepared as described above.

Blank GA-ALG NPs were prepared by dispersing GA-ALG into distilled water with gentle shaking for 24 h. The GA-ALG NPs suspension was freeze-dried and stored in the refrigerator before use.

2.4.2. X-ray photoelectron spectroscopy analysis

The surface chemical composition of GA-ALG NPs was determined using X-ray photoelectron spectroscopy (XPS, PHI1600ESCA, PERKIN, USA). The binding energy spectrum was recorded from 0 to 1100 eV with a pass energy of 80 eV under the fixed transmission mode.

2.4.3. Transmission electron microscopy, dynamic light scattering and zeta potential

Morphological examination of the nanoparticles was performed using transmission electron microscopy (TEM, Philips TZOST, Philips Tecnai Co., NED). The size of the nanoparticles (diameter, nm), polydispersity index, and surface charge (zeta potential, mV) were determined by dynamic light scattering (DLS) and laser Doppler anemometry using a Zetasizer 3000 (Malvern Instruments, UK) at room temperature.

2.4.4. Drug loading efficiency

The drug loading efficiency was determined by measuring the amount of DOX in the DOX/GA-ALG NPs. The mass of DOX in the DOX/GA-ALG NPs was measured using L6-P6 high performance liquid chromatography (HPLC, Beijing General Instrument Analysis, China) at room temperature as described previously [22]. The chromatographic conditions were as follows: a Dikma Technologies C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) was used. The mobile phase V_{water}:$V_{acetonitrile}$:$V_{methanol}$ = 112 mL:100 mL:12 mL, contained 0.15 mL phosphoric acid and 0.3 g sodium dodecyl sulfate. The flow rate was 1.0 mL/min. The wavelength of the UV detector was 232.8 nm. The injection volume was 20 μL. The drug loading efficiency (DLE) of DOX/GA-ALG NPs was calculated according to Eq. (2):

$$\mathrm{DLE}\ (\%) = \frac{\text{mass of DOX in the nanoparticles}}{\text{mass of DOX/GA} - \text{ALG NPs}} \times 100\% \quad (2)$$

2.5. In vitro drug release

The *in vitro* drug release behavior of DOX/GA-ALG NPs was investigated in phosphate solution at different pH (5.8, and 7.4) using a dialysis-diffusion method. Ten mg freeze-dried DOX/GA-ALG NPs were dispersed in 1.0 mL of phosphate solution, and introduced into a dialysis membrane bag (MWCO: 7000). The release experiment was initiated by placing the end-sealed dialysis bag in 9.0 mL of release medium with different pH values at 37 °C. The release medium was stirred at a speed of 100 rpm. At predetermined time intervals, the whole release medium was removed and replaced by fresh phosphate solution to maintain a sink condition, and the drug concentrations were determined by HPLC as described above.

2.6. Cellular uptake

The cellular uptake of DOX/GA-ALG NPs was evaluated using confocal laser scanning microscopy (CLSM, Leica, Germany) of HepG2 cells in accordance with the protocol of Ma et al. [23]. In brief, HepG2 cells were seeded at a density of 4×10^4 cells per well into a 6-well plate with a clean cover slip placed into each well, in 2.5 mL of DMEM growth medium. After 24 h of incubation at 37 °C, the attached cells