

Functional exosomes modified with chitosan effectively alleviate anthracycline-induced cardiotoxicity

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ABSTRACT

Anthracyclines belong to a class of anti-tumor antibiotics, and their severe cardiotoxicity significantly limits their clinical use. Exosomes play key roles in intercellular communication, characterized by high biocompatibility and specific tissue and organ homing effects. In this study, doxorubicin, an anthracycline anticancer drug widely used in clinical chemotherapy, was selected as a model drug. To address the significant cardiotoxicity associated with doxorubicin, tumor exosomes are utilized as drug carriers. The homing effect of autologous exosomes enhances drug uptake by tumor cells and reduces cardiotoxicity. To enhance the stability of exosomes, improve therapeutic effectiveness, and reduce toxic side effects, chitosan was utilized to modify the surface of exosomes. Chitosan has a specific anti-tumor effect because it can target the CD44 receptor of tumor stem cells and interact with tumor cells through charge adsorption. Through *in vitro* cell experiments, *in vivo* pharmacokinetic experiments, and an *in situ* ectopic nude mouse tumor model, the study demonstrated that chitosan-modified tumor exosomes significantly alleviated the severe cardiotoxicity of doxorubicin, while also showing remarkable anti-tumor efficacy. This study introduces a novel approach to reduce the adverse side effects of anthracycline chemotherapeutic drugs and presents a highly promising nanocarrier delivery system.

1. Introduction

Anthracyclines are glycosylated microbial natural products that are ranked among the most effective chemotherapeutics against cancer. However, their clinical application is restricted due to severe anthracycline-induced cardiotoxicity [1,2]. Doxorubicin (DOX), a potent anthracycline, has been widely used in the clinic for several decades to treat various cancers. Recent studies have demonstrated that DOX causes DNA damage by inhibiting topoisomerase II at various stages of the cell cycle, thereby promoting cell apoptosis. At the same time, it promotes mitochondrial apoptosis and cellular oxidative damage by generating a large amount of reactive oxygen species. Depending on the dosage of DOX and the cellular environment, immune T cells were activated, and cancer cells were induced to undergo senescence, autophagy, ferroptosis, or necrosis [3]. Due to the lack of tumor specificity of DOX, it exhibits serious myocardial toxicity, myelosuppression, and other toxic side effects. To effectively reduce the adverse effects of DOX, pegylated liposomes and non-pegylated liposomes are primarily used for delivering DOX in the market. However, there are still issues with immunogenic reactions, multiple drug resistance, and the emergence of

more severe side effects such as palmar-plantar erythrodysesthesia (PPE), stomatitis, mucositis, and others [4]. Therefore, it is urgent to find a new carrier with low immunogenicity and high biocompatibility to transport DOX for cancer treatment.

Exosomes possess a lipid bilayer membrane structure and are classified as extracellular vesicles with a particle size ranging from 40 to 160 nm. The biogenesis of exosomes involves their origin in endosomes, and subsequent interactions with other intracellular vesicles and organelles generate the final content of the exosomes [5]. The inherent biocompatibility, low immunogenicity, and structural stability within the blood circulation system, imparted by these natural and endogenous sources to membrane-derived vesicles, collectively allow these extracellular vesicles to evade phagocytosis by the reticuloendothelial system [6]. Consequently, they have been widely utilized as drug delivery vehicles in recent years. Exosomes play a significant role in intercellular communication for primary tumors and are implicated in cancer metastasis and immunosuppression [7]. Tumor cells can secrete exosomes in large quantities when deprived of nutrients and oxygen or when stimulated by photoradiation and drugs [8]. The quantity of exosomes secreted by cancer cells, including liver cancer cell lines, is

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significantly higher than that secreted by normal cells [9]. Following the administration of anticancer drugs, the release of extracellular vesicles from hepatocellular carcinoma (HCC) cell lines increased. These extracellular vesicles can be activated by natural killer cells to trigger anti-tumor immunity [10]. At the same time, studies have shown that doxorubicin-loaded tumor-derived exosomes can inhibit metastasis caused by primary tumor exosomes [11]. Autologous exosomes have been proven to be highly effective in delivering drugs for tumor treatment. The extracellular vesicles extracted from A549 cells loaded with DOX and lonidamine exhibited significant anticancer activity in a mouse lung cancer model. *In vivo* studies have shown that smaller extracellular vesicles demonstrate higher anticancer efficacy [12]. The secretion of exosomes from the mouse hepatoma cell line H22 and human hepatoma cell line BEL7402 has been investigated for drug nanoparticle surface modification in cancer treatment. Experiments showed that tumor exosomes have the ability to target tumor cells, enhancing tumor accumulation, penetration, and cellular uptake by tumor cells [13]. Extracellular vesicles secreted by the co-incubated mouse liver cancer cell line H22 and human ovarian cancer cell line A2780, loaded with a series of chemotherapeutic drugs such as adriamycin, methotrexate, hydroxyl camptothecin, or cisplatin, were used to treat the corresponding solid tumors. It was found that tumor cell-derived exosome-encapsulated chemotherapeutic drugs have a higher efficacy in killing tumors compared to traditional chemotherapeutic drugs. Moreover, they could even increase the sensitivity of tumor cells to chemotherapeutic drugs, thus triggering a domino-like or chain reaction that leads to the killing of cancer cells [14]. Therefore, the selection of tumor cells of appropriate origin is a key factor for the successful and effective development of exosome-based targeted drug delivery systems (DDS).

Chitosan is the only natural polysaccharide with a positive charge. As a relatively non-toxic and biocompatible material, it has a variety of chemical applications ($-NH_2$ groups and various molecular weights). The simplicity of chemical modification is a significant advantage of chitosan, which can be optimized to provide suitable biomaterials for therapeutic applications [15]. Chitosan can be administered in various ways, and the degradation rate of chitosan is influenced by the molecular weight and deacetylation degree of the polymer. In the case of nanoparticle formulations, the pharmacokinetic results and bio-distribution are determined by the particle size and charge of nanoparticles. It has been observed that the primary organ for chitosan uptake *via* intravenous injection appears to be the liver. It has been found that the accumulation in the liver increases with the increase in molecular weight [16]. In the study of chitosan-modified curcumin-loaded liposomes, it was found that chitosan modification enhanced high mucoadhesion, storage stability, and encapsulation efficiency [17]. Additionally, the antitumor activity of chitosan is exhibited through its interference with the metabolic growth of tumor cells [18]. The formulation with chitosan coating exhibited high drug loading, sustained-release mode, and enhanced pharmacokinetic parameters. Additionally, it showed improved anticancer activity against human colon cancer cells HT-29 [19]. In addition, the proliferation and metastasis of tumors can be suppressed by low molecular weight chitosan either alone or as a drug carrier. The antitumor activity is associated with the physicochemical properties of chitosan itself [20]. The uptake of tumor cells, antitumor effect, and survival rate in animal models can be enhanced by liposomes combined with low molecular weight chitosan after loading doxorubicin [21]. The CD44 receptor on cancer stem cells in tumor tissues can be bound by chitosan, leading to endocytosis into cells, thereby achieving the goal of targeted elimination and inhibition of tumor metastasis [22]. Cationic liposomes have been shown to selectively accumulate in angiogenic tumor endothelial cells. Therefore, they are expected to serve as drug carriers for delivering drugs to tumor endothelium. The preparation of DOX in cationic liposomes exhibited superior cytotoxicity *in vitro* and antitumor activity *in vivo* [23]. Taking human umbilical vein endothelial cells (HUVEC) as a cell model, *in vitro* experiments have demonstrated that DOX coated

with cationic liposomes possesses excellent tumor vascular targeting capabilities. It has shown a significant effect on inhibiting tumor growth of murine liver cancer cell H22 *in vivo*. In addition, it was also found that there is a positive proportional relationship between the tumor-targeting ability of cationic liposomes and their positive surface charge [24]. The advantages provided by the chitosan coating are diverse, including the stabilization of physicochemical properties, controlled release of drugs and other substances, improved interactions with tissues and cells, and increased efficacy or bioavailability of drugs. The therapeutic effect of doxorubicin-loaded exosomes was superior to that of commercially available doxorubicin-loaded liposomes like Doxil®. The inefficiency of liposomal preparations in suppressing tumor growth has been noted [25]. However, exosomes, which also have a phospholipid bilayer structure and better biocompatibility, have not been discussed in the literature on chitosan modification and drug delivery. Their preparation, characterization, cytotoxicity, uptake, *in vivo*, and *in vitro* research are also lacking.

Therefore, this study aims to isolate exosomes secreted by the HCC cell line HepG2 and subsequently modify them with chitosan. Both autologous exosomes and chitosan can synergistically trigger anti-tumor effects by inhibiting tumor metastasis, enhancing tumor accumulation, promoting tumor penetration, boosting tumor immunity, and improving the antitumor effects of drugs. This synergy can also enhance the tumor-targeting ability of autologous exosomes that already possess tumor-targeting capabilities. In addition, DOX is loaded into chitosan-modified autologous exosomes (CS-EXO@DOX). The efficacy and biological safety of CS-EXO@DOX were comprehensively evaluated through characterization, preparation assessment, cytotoxicity testing, quantitative and qualitative cellular uptake analysis, apoptosis evaluation, *in vivo* pharmacokinetic study, *in vivo* imaging, tissue organ distribution in mice, *in vivo* antitumor study, hematoxylin and eosin (H&E) staining of various tissue sections, and TUNEL fluorescence apoptosis analysis of tumor tissue. To our knowledge, this research represents the first application of chitosan for modifying tumor exosomes and delivering the chemotherapeutic agent DOX to solid tumors in nude mice. Our experimental results demonstrate that chitosan-modified doxorubicin-loaded exosomes are a safe and effective drug delivery system.

2. Materials and methods

2.1. Materials and reagents

Doxorubicin hydrochloride (purity >97 %) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., China. Chitosan with a molecular weight of 1 kDa and a degree of deacetylation of 92.3 %, with a pKa of 6.3, and another chitosan with a molecular weight range of 3–6 kDa and a degree of deacetylation equal to or >90 %, was procured from Hefei Bomei Biotechnology Co., Ltd. in Hefei, China. Ultrafiltration centrifuge tubes with a cutoff molecular weight of 30 kDa/100 kDa were purchased from Merck Millipore in Germany. Dialysis tubing with a cutoff molecular weight of 8–10 kDa was sourced from Shanghai Spectrum Laboratories in China.

Fetal bovine serum (FBS) was purchased from Gibco, USA. Minimum Essential Medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Procell Life Science & Technology Co., Ltd., China. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Taufkirchen, Germany). Phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), FITC-phalloidin, and 4',6-diamino-2-styryl alcohol (DAPI) staining were acquired from Solarbio Science & Technology Co., Ltd., Beijing, China. The Bicinchoninic acid (BCA) kit and radioimmunoprecipitation assay buffer (RIPA) were purchased from Beyotime Bio. Co., Ltd., Shanghai, China. 1,1'-Diocetadecyl-3,3',3',3'-tetramethylindodicarbocyanine perchlorate (DID) was obtained from MedChemExpress in New Jersey, USA. Cell Counting Kit-8 (CCK-8) was purchased from Beijing Labgic Technology Co., Ltd. in Beijing, China. Isoflurane was purchased from Reward Life

Technology Co., Ltd. in Shenzhen, China.

BALB/c nude mice (female and male, 4–6 weeks old, 18–20 g) and Sprague-Dawley (SD) rats (female and male, 6–7 weeks old, 180 ± 20 g) were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd., China. All animal studies were carried out in accordance with the Guidelines for Animal Experimentation of Guilin Medical University and the protocol approved by the Animal Ethics Committee of the institution and complied with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The animal experiments of the project met the requirements of animal welfare ethics and passed the animal welfare ethics investigation by the Institutional Animal Care and Use Committee (IACUC) of Guilin Medical University. The ethics approval reference number is GLMC-IACUC-20241022.

2.2. Cell culture

Human liver cancer cell line HepG2 and H9C2 cells (a rat embryonic ventricle cardiomyocyte cell line) were obtained from the cell bank of the prestigious Chinese Academy of Sciences. The cells were cultured in a complete DMEM medium supplemented with 10 % (v/v) FBS and 1 % antibiotics (100 U/mL penicillin, streptomycin), and maintained in a humidified cell culture incubator (Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at 37 °C with 5 % CO₂. When the cells reached 80–90 % of their capacity, they were transferred for further cell studies.

2.3. Optimization of exosome extraction and isolation

The large-scale cultivation of HepG2 cells was conducted using T75 culture flasks. When the cells reached the logarithmic growth phase, they were rinsed with PBS and replaced with DMEM containing 10 % exosome-free FBS (Gibco, Thermo Fisher Scientific) and 1 % penicillin-streptomycin. The supernatant was extracted after 48 h of culture following UV radiation. A sterile centrifuge tube was used for differential centrifugation purification (1000g for 10 min at 4 °C to remove aggregated and stacked cells, 3000g for 10 min to eliminate dead cells, and 10,000g for 30 min to get rid of cell debris). Subsequently, a 100 kDa MWCO centrifugal filter (Merck Millipore, Darmstadt, Germany) was utilized for concentration. **The concentrated solution was then loaded and separated using a size exclusion chromatography column (SEC, Duolaimi Biotechnology Co., Ltd., Wuhan, China) to obtain relatively pure exosomes.** Exosomes were quantified using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China).

2.4. The preparation and characterization of the formulation

The preparation of EXO@DOX involved adding exosomes and DOX, which were then mixed and sonicated using a SCIENTZ-IIID Ultrasonic Homogenizer (Scientz, China) in an ice bath. Sonication was performed at 150 W for 4 cycles of 2 s on and 3 s off for 5 min, with a 3-minute cooling period between each cycle. The entire process was conducted under light protection. The preparation of CS-EXO@DOX involved slowly adding the chitosan solution dropwise to the EXO@DOX. The mixture was then magnetically stirred for 30 min at room temperature and filtered through a 0.22 µm filter (Millipore, USA). DID-labeled exosomes were prepared in the same manner as doxorubicin-loaded exosomes, with the only difference being the use of DID instead of DOX. The intrinsic fluorescence of DOX was detected at 554 nm using the fluorescence spectrophotometer F-4600 (HITACHI, Tokyo, Japan), with excitation at 465 nm. The quantification of the exosomes loaded with DOX was then performed. The drug encapsulation efficiency (EE) and the drug loading efficiency (DL) of DOX in exosomes were determined using the ultrafiltration-centrifugation method. A specific quantity of drug-loaded exosome solution was added to a centrifuge tube with a 30 kDa molecular weight cut-off. The mixture was centrifuged at 3500 rpm for 10 min. Subsequently, the doxorubicin filtrate was diluted

to the same volume using ultrafiltration. The DOX-exosome solution, without ultrafiltration after ultrasound encapsulation, was measured using fluorescence spectrophotometry. The entire process was conducted in the absence of light. The EE and DL were calculated according to the following formula:

$$EE(\%) = \frac{OD1 - OD2}{OD1} \times 100\%$$

$$DL(\%) = \frac{W}{W_a + W} \times 100\%$$

OD1: The fluorescence value of DOX-exosomes without ultrafiltration after ultrasound encapsulation; OD2: The fluorescence value of free drug in ultrafiltration solution; W_a: The weight of exosomes; W: The weight of the drug contained.

2.5. Nanoparticle tracking analysis (NTA)

For NTA measurements, ZetaView (Particle Metrix, Germany) and the corresponding software ZetaView version 8.05.14 SP7 (Particle Metrix, Germany) were used. The instrument parameters were set as follows: Scatter mode with a sensitivity of 80 and a shutter of 100. For the analysis, a maximum area of 1000, a minimum area of 10, and a minimum brightness of 30 were selected. The binning was set to 5 nm. Samples were diluted with PBS, and measurements were taken at 25 °C.

2.6. Transmission electron microscopy

The microscopic morphology of exosomes, EXO@DOX, and CS-EXO@DOX was observed using a Transmission Electron Microscope (TEM, HT7700, Hitachi, Japan). Briefly, 10 µL of the stock solution of exosomes was taken, appropriately diluted with pure water, and dropped onto the TEM carrier support carbon film. It was blotted dry with filter paper after 10 min. Subsequently, all samples were negatively stained with 2 % uranyl acetate, dried under natural conditions for 10 min, blotted dry with filter paper, and observed and photographed overnight using a transmission electron microscope.

2.7. Western blotting

For biomarker analysis, exosome positive and negative markers were examined by Western blotting using the following antibodies: CD9 (1:5000, 60232-1-Ig, Proteintech Group, Chicago, USA), TSG101 (1:5000, 67381-1-Ig, Proteintech Group, Chicago, USA), CD81 (1:2000, 66866-1-Ig, Proteintech Group, Chicago, USA), and Calnexin (1:5000, 10427-2-AP, Proteintech Group, Chicago, USA). A total of 30 µg of exosome samples were boiled in a 100 °C metal bath for 10 min, separated by 10 % SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). The membranes were blocked with 5 % (w/v) non-fat milk in TBST at room temperature (RT) for 1 h, incubated overnight with the primary antibodies mentioned above, followed by incubation with HRP-conjugated secondary antibodies (1:6000, SA00001-1, SA00001-2, Proteintech Group, Chicago, USA). The bands were visualized using enhanced chemiluminescence (ECL, Vazyme Biotech, Nanjing, China). HepG2 cells were lysed in RIPA lysis buffer and then subjected to Western blot analysis as a control.

2.8. Zeta potential and its serum stability

The zeta potential of the exosomes was determined using a nanoparticle size analyzer (Nan-ZS90, Malvern Instruments Ltd., Malvern, UK). In addition, to evaluate the stability of the preparation *in vivo*, the surface charge stability of EXO, EXO@DOX and CS-EXO@DOX in FBS was determined at specific time points (1, 2, 4, 8, 12, and 24 h). Three samples were taken from each batch, and their mean values were calculated by a machine.

2.9. *In vitro* drug release

In vitro release behavior of doxorubicin-loaded exosomes was determined using the dialysis method [13]. 2 mL of free DOX, EXO@DOX, and CS-EXO@DOX were each placed into separate dialysis tubing with a molecular weight cut-off of 8–10 kDa, and the concentration of DOX in each group was 0.1 mg/mL. The dialysis medium was 50 mL of PBS (pH 7.4), which was placed into a thermostatic shaker for release (shielded from light at 37 °C, with 80 rpm). Samples were taken at the specified time points (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h). Each group was sampled with 1 mL of the release medium at a time, and 1 mL of PBS (pH 7.4) was immediately replenished. The concentration of DOX fluorescence was measured using a fluorescence spectrophotometer (excitation wavelength: 460 nm, emission wavelength: 554 nm). The experiment was repeated three times, and the cumulative drug release percentage (Q) was calculated, with a time curve (t) plotted.

2.10. Cytotoxicity assay

The cytotoxicity was detected using CCK-8. In 96-well plates, 10,000 HepG2 cells were added to each well, incubated overnight, the supernatant was removed after cell adherence, and exosome preparations and materials containing different concentrations were added. The preparations and materials were diluted with DMEM medium. After 24 h of incubation following dosing, 10 µL of CCK-8 was directly added to each well for incubation in the dark for 1 h, and its absorbance was measured at 450 nm with a microplate reader (Bio-Rad 500, Hercules, CA, USA).

Due to the cardiotoxicity of DOX, the efficacy of the formulation in reducing toxicity was tested using H9C2 cells for validation. The MTT assay was used to detect cytotoxicity, where H9C2 cells were added to each well of a 96-well plate and incubated overnight. After the cells had attached, the supernatant was removed, and exosome preparations with materials containing varying concentrations were added. The preparations and materials were diluted and mixed with DMEM medium. After 24 h of culture, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated in a dark incubator for 4 h. The culture medium was then removed, and 100 µL of DMSO was added to each well to dissolve it. The absorbance was measured at 490 nm using a microplate reader.

2.11. Cellular uptake assay

To assess the uptake capacity of HepG2 cells for exosome preparations, sterile coverslips were placed in the wells of a 6-well plate. After rinsing twice with PBS, the coverslips in each well were seeded with a concentration of 3×10^5 HepG2 cells and incubated overnight. DOX, EXO@DOX, and CS-EXO@DOX were added to the cells, respectively. The cells were cultured in MEM (1 % FBS), which is equivalent to 1 µg/mL of DOX, and then incubated at 37 °C for 4 h. The cells were gently rinsed twice with cold PBS (pH 7.4) to stop the uptake process. In the qualitative uptake experiments, 4 % paraformaldehyde and 0.1 % Triton X-100 were added to fix and permeabilize for 10 min, respectively. Subsequently, FITC-phalloidin and DAPI staining were added sequentially to visualize cytoskeletal myofilaments and nuclei. Coverslips containing stained cells were inverted onto glass slides with anti-fluorescence quenching agent applied. The cellular uptake was observed and compared using an inverted fluorescence microscope (IX53, Olympus, Japan). For the quantitative uptake study, after the 6-well plate was incubated for 4 h with the drug, PBS (pH 7.4) was rinsed. RIPA lysate was added to lyse the cells, and the content of DOX in each well was determined using a fluorescent enzyme marker (Infinite M200 Pro NanoQuant, Tecan Co. Ltd., Switzerland) with excitation at 480 nm and emission at 590 nm. The protein content in each well was determined using a BCA kit. The level of DOX uptake in the three preparations was evaluated by the amount of DOX taken up (µg) per unit

of protein (mg).

2.12. Apoptosis assay

In each well of a 12-well plate, 2 mL of HepG2 cell suspension with a concentration of 1.2×10^5 cells/mL was added for incubation for 12 h. After the cells have adhered to the plate, the supernatant was removed, and the cells were washed twice with PBS (pH 7.4). Then, 1 mL of DMEM medium containing DOX, EXO@DOX, and CS-EXO@DOX (with 1 µg/mL DOX) was added to each well for incubation for 16 and 24 h. The procedure was conducted following the guidelines of the Annexin V-FITC/PI apoptosis detection kit (Absin Bioscience, Shanghai, China). Samples stained were preserved in EP tubes in the dark. Eventually, a blank group, a PI staining group, a FITC staining group, and a FITC and PI double staining group were included. All samples were processed within 1 h using a flow cytometer (BD Canto Plus, USA). Data were collected and analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

2.13. Pharmacokinetic studies

SD rats were randomly divided into three groups, and each group was injected with 3 mg/kg of DOX, EXO@DOX, and CS-EXO@DOX through the tail vein. After the rats were anesthetized with isoflurane inhalation, blood was collected from the retroorbital venous plexus of SD rats using a capillary tube at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h, respectively. The blood samples were mixed in a 1.5 mL EP tube containing heparin sodium, and then centrifuged at 3500 g for 10 min. Subsequently, 100 µL of serum was transferred to a new EP tube, and 400 µL of methanol was added. The mixture was then vortexed for 2 min. The sample was then centrifuged at 12000 g for 10 min. 100 µL of supernatant was added to the black 96-well plate, and the fluorescence value of the sample was detected using a fluorescent enzyme marker (Infinite M200 Pro NanoQuant, Tecan Co. Ltd., Switzerland) with excitation at 480 nm and emission at 590 nm. The data were collected for analysis, and the curves of the drug concentration in plasma *versus* time were plotted.

2.14. *In vivo* imaging and tissue fluorescence distribution

In order to investigate whether autologous exosomes have a tumor-homing effect and whether exosomes modified with chitosan have prolonged retention in the liver, EXO@DID and CS-EXO@DID (5 mg/kg DID) were injected into the tail vein of BALB/c mice (5 mice in each group) with an orthotopic liver cancer model at 0.1 mL. Free DID was injected as a blank control. Subsequently, at set times (0, 2, 6, 12, 24 h), mice were anesthetized with isoflurane and then placed into *in vivo* imaging equipment (UVP iBox Scientia, Germany) for observation. Images were captured under 600/640 nm excitation and 668/722 nm emission, and the mice were euthanized at 24 h. The main organs (heart, liver, spleen, lung, and kidney) of the mice were removed, observed on a small animal live imager, photographed, and the data were preserved for *ex vivo* fluorescent intensity analysis.

2.15. *In vivo* antitumor evaluation and biosafety assessment

HepG2 tumor-bearing mice were created by subcutaneously injecting 3×10^6 HepG2 cells per mouse into the flanks of BALB/c mice. When the tumor volume reached approximately 100 mm³, the tumor-bearing mice were randomly divided into four groups, each consisting of five mice. PBS was administered to the control group of mice, while each of the other three groups received a tail vein injection of 5 mg/kg of DOX, EXO@DOX, and CS-EXO@DOX on days 0, 3, 6, 9, 12, 15, and 18. Every three days, body weight and tumor volume were recorded. The tumor volume was estimated using the formula: volume = (length × width²)/2.

Tumors, livers, kidneys, lungs, spleens, and hearts were collected from mice euthanized on day 21. After weighing the tumors, the tissues were preserved in 4 % neutral buffered formalin for 48 h. Subsequently, the morphological characteristics of each organ were monitored to assess the biological safety of the preparation using the Hematoxylin and Eosin (H&E) staining in the usual manner.

2.16. TUNEL apoptosis fluorescence imaging of tumor tissue

A layer of circles was coated around the tissue sections with transparent, colorless, neutral resin initially. Subsequently, 100 μ L of a 20 μ g/mL proteinase K solution was carefully applied to the section, ensuring the operation was conducted in the absence of light, for incubation for 0.5 h to facilitate tissue lysis. After the lysis is complete, the slides soaked in PBS were washed three times for 5 min each. Then, 20 μ L of the prepared TUNEL working solution was added dropwise. After incubating for 1 h, the slides were washed twice with PBS for 2 min each time. Subsequently, a prepared DAPI solution was added for 5 min for nuclear staining. After that, three more PBS washings were conducted for 5 min each time. Finally, the TUNEL staining results were observed at 400 $\times$ magnification under a fluorescence inverted microscope after sealing the fluorescence quenching agent on the slide. The TUNEL-stained tumor tissues were comparatively examined to study tumor cell apoptosis in various treatment groups.

2.17. Statistical analysis

The student's *t*-test was used to compare groups based on the mean $\pm$ standard deviation (SD) data. All statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). In statistics, a *P*-value of <0.05 was considered statistically significant. The significant levels were shown as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

3. Results

3.1. Characterization of exosomes and preparations

A large number of relatively pure exosomes were extracted from the supernatant of a HepG2 culture using an optimized SEC method. The morphology and particle size of tumor exosomes were evaluated using TEM and NTA. The exosomes exhibited a narrow particle size distribution ranging from 40 to 160 nm in the extracellular vesicles. The extracted exosomes exhibited a typical saucer-like bilayer membrane structure in the TEM images (Fig. 1A, a). In nanoparticle tracking analysis, the peak particle size was determined to be 113.3 nm (Fig. 1C, a). Furthermore, Western blotting identified biomarkers such as CD9, CD81, and TSG101. In contrast to the results of lysate detection in HepG2 cells, Calnexin, a protein exclusively present in cells, was not detected in exosomes (Fig. 1D). These fundamental characteristics align with the MISEV2018 guidelines [26].

DOX was loaded using ice bath ultrasound. Under the transmission electron microscopy, certain wrinkles were observed on the surface of exosomes containing drugs (Fig. 1A, b). The particle size measured by NTA after drug loading was 133 nm (Fig. 1C, b). The drug EE and DL were measured as 83 % and 7.54 %, respectively, using a fluorescence spectrophotometer. However, the CS-EXO@DOX formulation, modified through electrostatic adsorption of chitosan, displayed a distinct thin film layer on the surface of the preparation (Fig. 1A, c). The preparation clusters were evenly distributed under the transmission electron microscope (Fig. 1B), and the particle size measured 154.6 nm by NTA (Fig. 1C, c). The particle size of all preparations was <200 nm, which facilitated the accumulation of Enhanced Permeability and Retention (EPR) effects at the tumor site. This led to the selective distribution of the drug in the tumor tissue, thereby enhancing drug efficacy and reducing medication side effects.

The surface potential of exosomes (EXO) was found to be $-18.97 \pm$

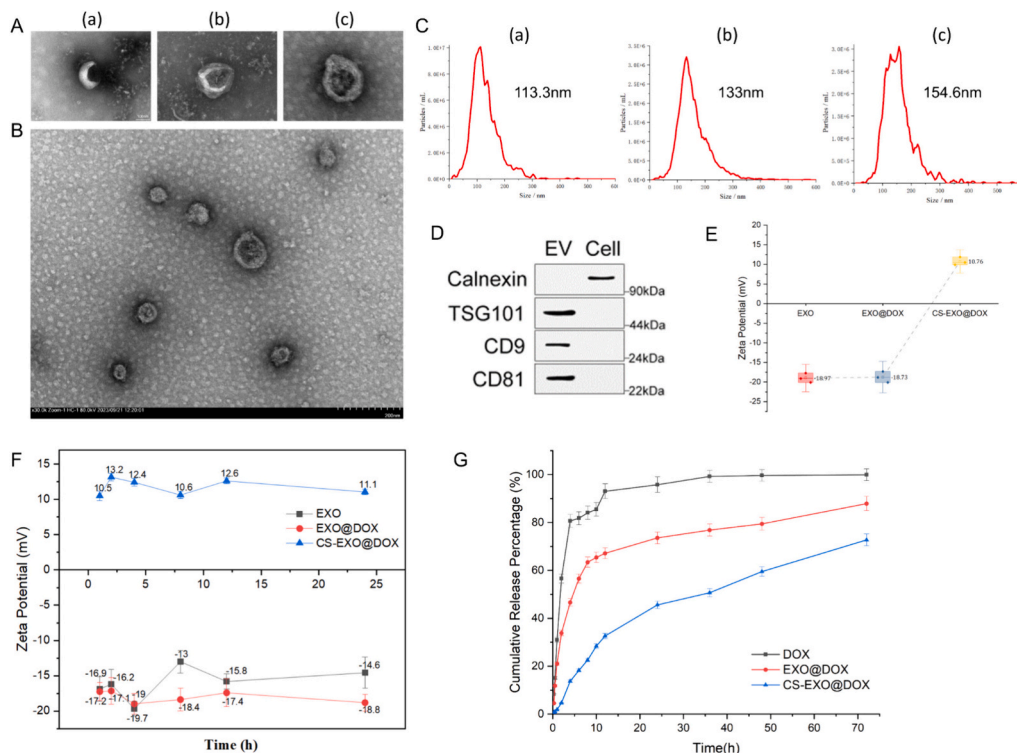


Fig. 1. Characterization of exosomes. (A) The microscopic structures of (a) Exosomes, (b) EXO@DOX, and (c) CS-EXO@DOX were observed using a transmission electron microscope (TEM). Scale bar: 100 nm. (B) Cluster images of CS-EXO@DOX captured using TEM. Scale bar: 200 nm. (C) The NTA detection results of (a) Exosomes, (b) EXO@DOX, and (c) CS-EXO@DOX, respectively. (D) Western blot analysis of the exosome markers. (E) Zeta potential of each preparation. (F) Results of serum stability testing. (G) *In vitro* release behavior of exosomes. The data are expressed as the means $\pm$ SDs (*n* = 3).

1.2 mV, and the surface charge of the doxorubicin-loaded exosomes, EXO@DOX, was determined to be -18.73 ± 1.4 mV. Chitosan, a natural cationic polysaccharide, underwent electrostatic adsorption modification, resulting in a charge reversal of the formulation. The surface potential of CS-EXO@DOX was 10.76 ± 1.0 mV (Fig. 1E), indicating successful modification of chitosan onto the surface of exosomes. In the *in vitro* study of serum stability, it was observed that the surface Zeta potential of CS-EXO@DOX showed minimal fluctuations and remained relatively stable (Fig. 1F). During the experimental process, it was found that the DOX exosomes modified with chitosan settled slowly and remained relatively stable at room temperature. This property can effectively address the issue of DOX settling and precipitating when exposed to light. The drug cumulative release curve indicated that the 72-hour cumulative release amount of EXO@DOX (87.89 ± 2.93 %) was higher than that of CS-EXO@DOX (72.72 ± 2.42 %) (Fig. 1G). *In vitro* release studies confirmed that chitosan-modified drug-loaded exosomes can effectively slow down the release of DOX, thereby achieving the goal of enhancing efficacy and reducing toxicity. The modification of chitosan has an effective protective effect on the biodegradation of exosomes.

3.2. *In vitro* cytotoxicity study

The *in vitro* antitumor effects of free DOX, EXO@DOX, and CS-EXO@DOX on HepG2 cells were evaluated and compared, along with their myocardial protective effects on H9C2 cells (Fig. 2). In all formulations, a dose-response relationship was observed, with higher doses leading to lower cellular viability due to cytotoxicity. In the antitumor *in vitro* study, it has been demonstrated that the vector material (EXO) shows no significant toxicity compared to the modified material (CS) for the sake of experimental rigor. However, the modified material (CS) did exhibit a little inhibitory effect on tumor cells (Fig. 2A). After 24 h of co-incubation of various drugs with HepG2 cells, the cell viability of CS-

EXO@DOX showed the most significant decline. Free DOX exhibited relatively weaker cytotoxicity, while EXO@DOX demonstrated comparable cytotoxicity to CS-EXO@DOX (Fig. 2B). Due to the enhanced delivery efficiency of DOX, EXO@DOX (IC₅₀, 2.1 $\mu\text{g/mL}$) exhibited significantly higher anti-tumor activity than free DOX (IC₅₀, 8.42 $\mu\text{g/mL}$) (Fig. 2C). It is noteworthy that, when compared to EXO@DOX, CS-EXO@DOX (IC₅₀, 0.93 $\mu\text{g/mL}$; 1/8 of free DOX) demonstrated a more potent killing activity, nearly 2.3 times stronger.

Due to the serious myocardial toxicity of DOX, the efficacy of attenuation in formulations was verified. The carrier material (EXO) and the modifying material (CS) showed no significant toxicity to myocardial cells (Fig. 2D). After 24 h of co-incubation of various drug groups with H9C2 cells, cardiomyocytes were found to be most sensitive to free DOX, with only 0.5 $\mu\text{g/mL}$ reaching the median lethal dose. The cytotoxic effects of EXO@DOX and CS-EXO@DOX were relatively weak (Fig. 2E). Significantly, compared with EXO@DOX (IC₅₀, 15.7 $\mu\text{g/mL}$), CS-EXO@DOX (IC₅₀, 73.4 $\mu\text{g/mL}$; 137.6 times the concentration of free DOX) exhibited a more effective myocardial protection function, nearly 4.7 times stronger (Fig. 2F). These results indicate that modifying chitosan could enhance the effectiveness of killing tumor cells and significantly reduce the myocardial toxicity of DOX.

3.3. Cellular uptake and apoptosis *in vitro*

The qualitative cellular uptake was determined by incubating HepG2 cells with doxorubicin-spontaneously fluorescently labeled EXO@DOX and CS-EXO@DOX for 4 h. From the fluorescence images, it was observed that the red fluorescence intensity of cells treated with chitosan-modified CS-EXO@DOX was significantly stronger than that of cells treated with EXO@DOX (Fig. 3A). This suggests that drug-loaded exosomes modified with chitosan could significantly enhance the ability of drug targeting to enter HepG2 cells. The cytoskeleton and nucleus

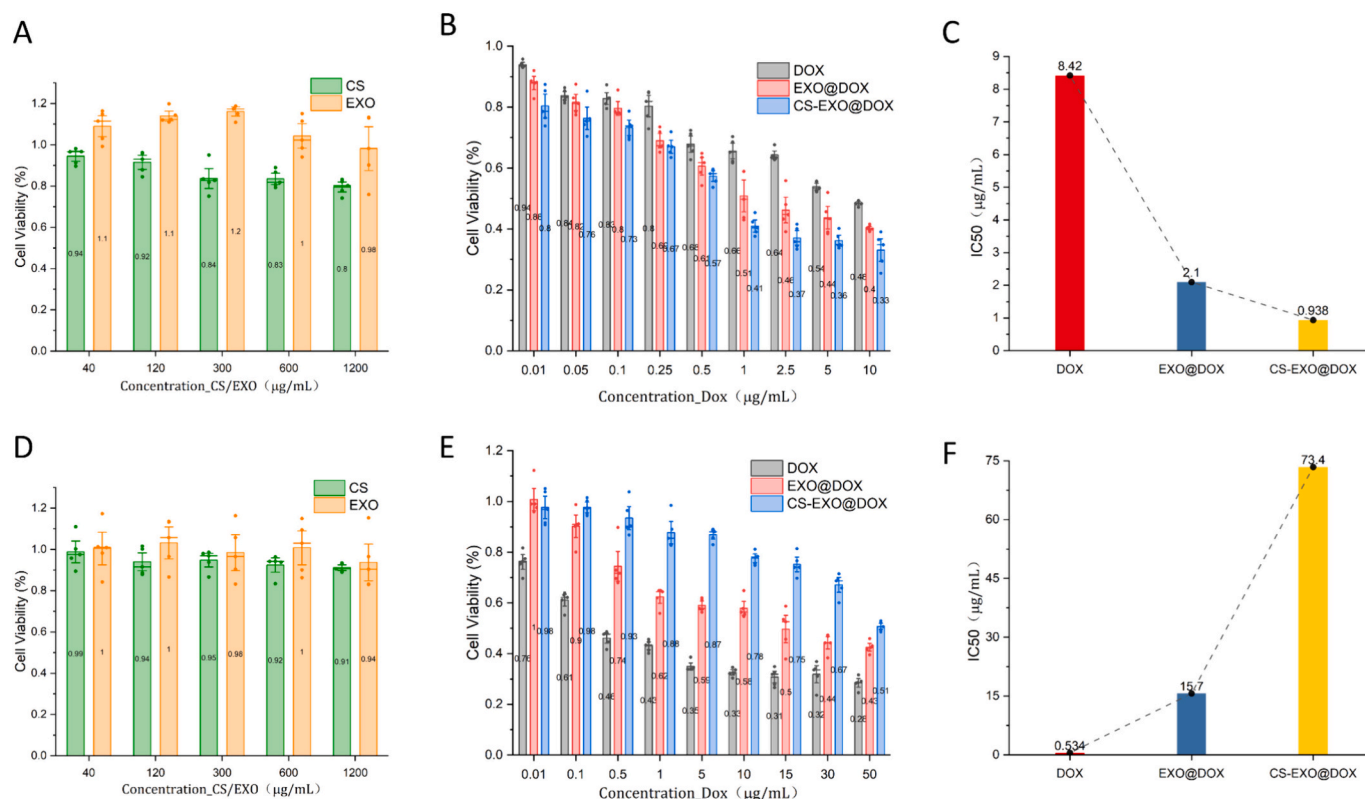


Fig. 2. *In vitro* cellular toxicity of HepG2 and H9C2 cells. (A) Toxic effects of carriers and modified materials on HepG2 cells. (B) Cytotoxicity of doxorubicin-loaded exosomes in HepG2 cells. (C) IC₅₀ of each preparation for HepG2 cells. (D) Toxic effects of carriers and modified materials on H9C2 cells. (E) Cytotoxicity of doxorubicin-loaded exosomes in H9C2 cells. (F) IC₅₀ of each preparation for H9C2 cells. The data are expressed as the means $\pm$ SDs ($n = 5$).

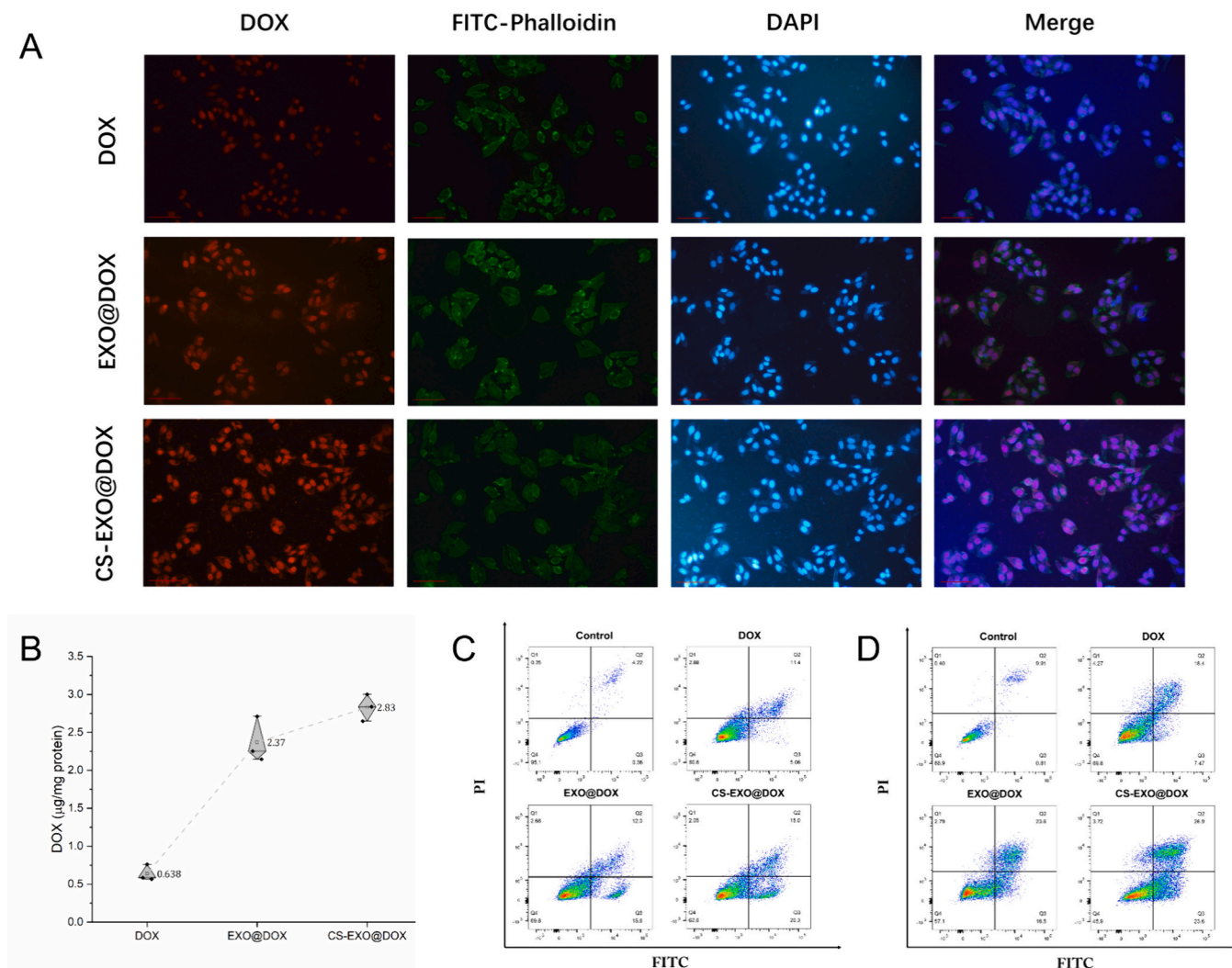


Fig. 3. Qualitative and quantitative cellular uptake and apoptosis of exosomes. (A) Fluorescence uptake images were captured under a fluorescence inverted microscope after HepG2 cells were co-incubated with DOX, EXO@DOX, and CS-EXO@DOX at 37 °C for 4 h. The cytoplasm (microfilament) was stained with FITC-phalloidin, the nucleus was stained with DAPI (blue), and the red fluorescence originated from DOX. Scale bar: 100 μm. (B) Quantitative uptake of DOX, EXO@DOX, and CS-EXO@DOX by HepG2 cells. (C) After a 16-hour co-incubation, a flow cytometry apoptosis assay was performed on doxorubicin-loaded exosomes in HepG2 cells. (D) After a 24-hour co-incubation, a flow cytometry apoptosis assay was performed on doxorubicin-loaded exosomes in HepG2 cells. The data are expressed as the means ± SDs ($n = 3$).

were stained with FITC-phalloidin (green fluorescence) and DAPI (blue fluorescence), respectively. It was obvious that the red fluorescence was only present in the nuclear area, indicating that the cellular uptake of exosomes effectively delivered and released DOX into the nucleus. As shown in Fig. 3B, the quantitative uptake of cells exhibited similar behavioral characteristics to the qualitative uptake. In HepG2 cells, the cellular uptake of EXO@DOX was 2.37 μg/mg, which was 3.7 times greater than that of DOX alone. The cellular uptake of CS-EXO@DOX reached 2.83 μg/mg, which was 4.4 times greater than that of DOX alone. It is worth mentioning that the uptake efficiency increased by 19.4 % compared to EXO@DOX. This further confirms that the chitosan modification effectively enhances the uptake and absorption capacity of HepG2.

Furthermore, the apoptotic effects of free DOX, EXO@DOX, and CS-EXO@DOX on HepG2 cells were analyzed using flow cytometry. After co-incubating drugs with HepG2 cells for 16 h, the apoptosis rates of free DOX, EXO@DOX, and CS-EXO@DOX were determined to be 19.4 %, 30.2 %, and 37.4 %, respectively. Moreover, within 16 h, the EXO@DOX and CS-EXO@DOX groups primarily exhibited early apoptosis (Fig. 3C). With the incubation time increasing from 16 to 24 h, the apoptosis rates

of free DOX, EXO@DOX, and CS-EXO@DOX cells were determined to be 30.2 %, 42.9 %, and 54.1 %, respectively (Fig. 3D). It was observed that the cell apoptosis shifted from early apoptosis to late apoptosis in the EXO@DOX and CS-EXO@DOX groups, with an increase in the number of necrotic cells. Compared to the control group, the percentage of viable cells decreased after administration. The 24-hour cell survival rate was consistent with the cytotoxicity results. The apoptosis rate of the CS-EXO@DOX group was the highest. These results indicate that CS-EXO@DOX could effectively induce apoptosis and necrosis of HepG2 cells. The chemical structure of chitosan may be recognized by the CD44 receptor on cancer stem cells in tumor tissues. This recognition can enhance drug uptake by cancer cells through endocytosis.

3.4. In vivo pharmacokinetic evaluation

An *in vivo* pharmacokinetic evaluation was conducted on Sprague-Dawley rats using EXO@DOX and CS-EXO@DOX, with free DOX serving as the control formulation. The plasma concentration-time curve of DOX in plasma after tail vein injection of free DOX, EXO@DOX, and CS-EXO@DOX at a dose of 3 mg/kg was shown in Fig. 4, and the detailed

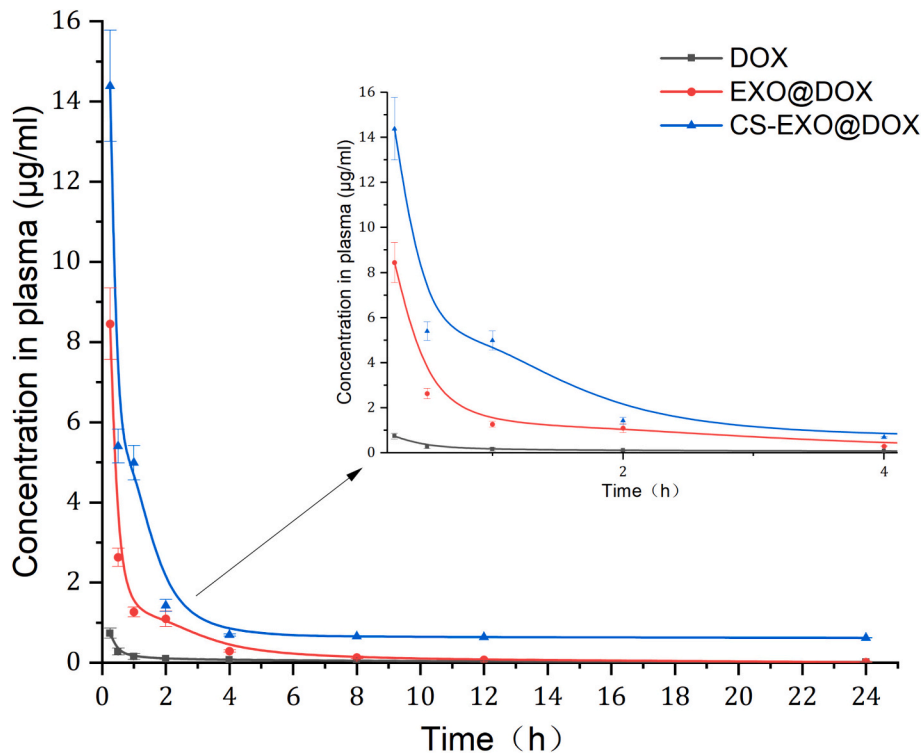


Fig. 4. Pharmacokinetic profiles of DOX, EXO@DOX, and CS-EXO@DOX in rats. The data are expressed as the means $\pm$ SDs ($n = 5$).

pharmacokinetic parameters were calculated and summarized in Table 1. After a single dose administration, it could be observed that free DOX is rapidly cleared from the bloodstream, with almost no detectable presence of DOX in the blood after 4 h. In comparison, both EXO@DOX and CS-EXO@DOX exhibited longer circulation times than free DOX. It is significant to note that the area under the blood concentration-time curve for free DOX, EXO@DOX, and CS-EXO@DOX was determined to be 1.441 ± 0.323 , 9.272 ± 0.947 , and 26.899 ± 1.204 (mg·h/L), respectively. Consequently, EXO@DOX and CS-EXO@DOX exhibited higher bioavailability compared to free DOX. The $T_{1/2\alpha}$ of EXO@DOX and CS-EXO@DOX were found to be 5.4 and 7.4 times longer than that of free DOX, respectively. Furthermore, it was exciting to note that the clearance rate of free DOX was 1.98 ± 0.674 (L/h/kg), whereas the clearance rate of CS-EXO@DOX was significantly reduced to only 0.019 ± 0.015 (L/h/kg). These results indicate that direct encapsulation of exosomes or modification with chitosan could effectively prolong the action time of drugs, thereby enhancing drug efficacy. Within 4 h, the gradual decrease in the blood concentration of CS-EXO@DOX indicated its sustained-release properties, which aligned with the findings of *in vitro* release studies. Importantly, even after 24 h, CS-EXO@DOX continued to release DOX at a consistent and controlled rate, suggesting that CS-EXO@DOX maintained good stability *in vivo*. This indicates that chitosan modification has the potential to improve the pharmacokinetic properties of exosomes.

Table 1
Pharmacokinetic parameters of DOX, EXO@DOX, and CS-EXO@DOX in rats.

Parameters	DOX	EXO@DOX	CS-EXO@DOX
C_{max} (µg/mL)	0.736 ± 0.125	8.451 ± 0.892	14.389 ± 1.389
$AUC_{(0-t)}$ (µg/mL·h)	1.441 ± 0.323	9.272 ± 0.947	26.899 ± 1.204
$VRT(0-t)(h^2)$	41.212 ± 15.478	13.847 ± 2.018	65.218 ± 1.366
$MRT(0-t)$ (h)	5.594 ± 1.559	2.333 ± 0.198	7.062 ± 0.251
$T_{1/2\alpha}(h)$	0.068 ± 0.035	0.094 ± 0.016	0.503 ± 0.035
V_z (L/kg)	21.147 ± 8.07	2.026 ± 0.255	4.065 ± 0.497
CL_z (L/h/kg)	1.98 ± 0.674	0.324 ± 0.036	0.019 ± 0.015

3.5. *In vivo* targeting evaluation and tissue distribution

To investigate the potential of tumor exosomes and exosomes modified with chitosan to specifically deliver DOX to tumor tissues *in vivo*, we established a HepG2 orthotopic liver tumor-bearing nude mice model. Exosomes and chitosan-modified exosomes were labeled with DID dye and injected into nude mice bearing orthotopic liver tumors via the tail vein, while free DID was injected as a blank control. The fluorescence distribution of each group was monitored over 24 h using a small animal *in vivo* imaging equipment (UVP iBox Scientia, Germany) (Fig. 5A). Following the injection of free DID and EXO@DID, the strongest fluorescence in the liver of mice was detected at 6 h post-treatment. The difference was that free DID was significantly metabolized via the liver-kidney pathway, while EXO@DID initially distributed fluorescence throughout the body, then gradually concentrated at the liver tumor site, with notable fluorescence also observed in the lungs. The strongest fluorescence in the liver and spleen was detected at 12 h post-exposure in the CS-EXO@DID group, with a more pronounced concentration of fluorescence observed at the liver tumor site. To analyze the distribution characteristics of drugs more intuitively, the main organs of nude mice in each group were taken out at 24 h for semi-quantitative analysis of fluorescence imaging. The results are shown in Fig. 5B. From top to bottom, the fluorescent distribution images of the tissues and organs are sequentially presented as follows: free DID, EXO@DID, and CS-EXO@DID. In the DID group, significant fluorescence was observed in the liver and kidneys, with minor fluorescence in the lungs. In the EXO@DID group, significant liver and lung fluorescence was observed, with minor accumulation of fluorescence at the liver tumor site. In the CS-EXO@DID group, the fluorescence in the liver and spleen was most pronounced, with stronger fluorescence at the liver tumor site. A semi-quantitative analysis of *in vitro* fluorescence images (Fig. 5C) indicated that DID is primarily metabolized via the liver and kidney pathways. The lung fluorescence intensity was higher in the EXO@DID group, while the liver and spleen fluorescence intensity were highest in the CS-EXO@DID group. The main reason may be related to the positive charge of the preparation. However, this positive charge

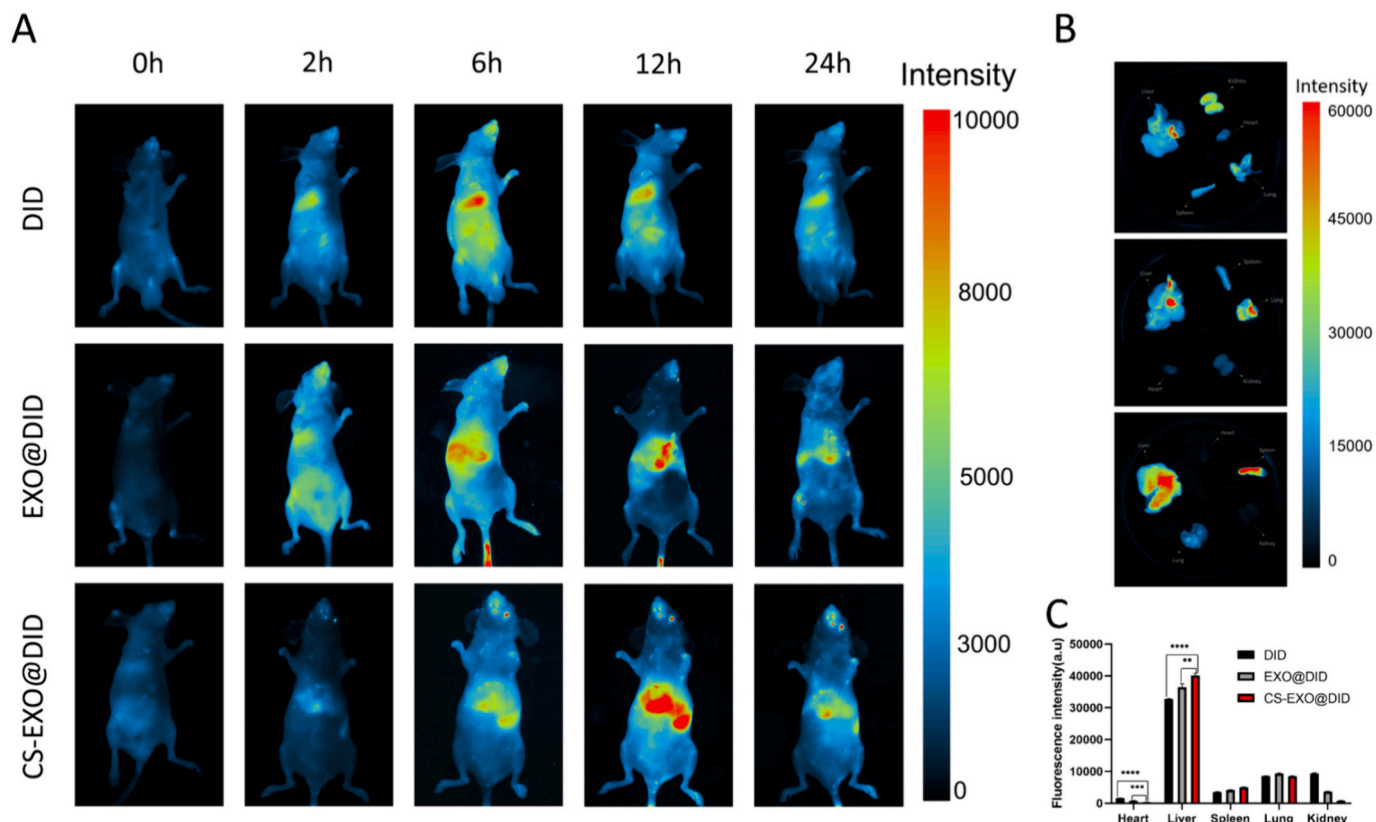


Fig. 5. *Ex vivo* imaging of exosomes in major organs, *in vivo* biodistribution, and *in vitro* fluorescence images of organs. (A) *In vivo* time-dependent whole-body fluorescence imaging of HCC cell line HepG2 orthotopic implantation tumor model mice after intravenous injection of DID, EXO@DID, and CS-EXO@DID. (B) Fluorescence Distribution of Major Organs *In Vitro*: BALB/c mice were intravenously injected with DID, EXO@DID, and CS-EXO@DID, respectively. Organs were harvested 24 h after administration. (C) The statistical graph shows the fluorescence intensity of organs based on a semi-quantitative analysis of *ex vivo* fluorescent images of BALB/c mice 24 h after intravenous injection. Administration with DID, EXO@DID, and CS-EXO@DID. The data are expressed as the means $\pm$ SDs ($n = 3$). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

also enables the preparation to actively target the negatively charged cancer cell membrane, thereby enhancing the penetration, internalization, and retention of the preparation.

These results indicate that exosomes modified by chitosan significantly enhance the retention and accumulation of drugs in the liver, exhibiting specific targeting for liver tumors and the ability to penetrate deep into tumor tissues for drug delivery. Importantly, the fluorescence intensity of the heart in the CS-EXO@DID group was minimal, indicating a negligible impact. This suggests that chitosan-modified exosomal drugs have minimal toxic and side effects on the heart, providing robust myocardial protection, which is consistent with the cytotoxicity results of H9C2 cells.

3.6. *In vivo* antitumor studies

To investigate the *in vivo* therapeutic efficacy of CS-EXO@DOX against tumors, we used HepG2 subcutaneous tumor-bearing nude mice as a model. When the tumor volume reached 100 mm^3 , the mice were randomly divided into groups and treated with free DOX, EXO@DOX, or CS-EXO@DOX (5 mg/kg DOX) via tail vein injection every 3 days. After Day 21, the results are shown in Fig. 6A–D, demonstrating that CS-EXO@DOX exhibited the most effective inhibitory effect. The size, volume, and weight of the tumors were significantly reduced, with the tumor volume decreasing by approximately 3.64 times and the tumor weight decreasing by 80.19 %. Surprisingly, the EXO@DOX group also demonstrated a notable therapeutic efficacy, with the tumor volume reducing by approximately 2.97 times and the tumor weight decreasing by 76.53 %, which may be attributed to the efficient delivery of DOX and the rapid distribution of the drug. It is worth noting that in

Fig. 6B, the body weight of the control group of nude mice has been consistently increasing. In contrast, the body weight of the nude mice in the treatment group initially increased and then gradually started to decrease on day 6. The weight of nude mice in the free DOX group rapidly declined from day 15, and they began to exhibit signs of depression. However, the nude mice in the CS-EXO@DOX group experienced minimal weight loss and maintained good mental and physical vitality. This suggests that the CS-EXO@DOX group effectively alleviated the toxic side effects, such as bone marrow suppression, induced by the chemotherapeutic agent DOX.

The biosafety assessment involved sectioning and H&E staining to analyze the histopathology of the main organs (Fig. 6E). Compared with the control group, the myocardium of nude mice in the free DOX and EXO@DOX groups showed significant damage. Additionally, there was a certain degree of injury and apoptosis in the liver and lung, with more severe damage observed in the free DOX group. However, there were no obvious histopathological abnormalities in the organs of the CS-EXO@DOX group. This finding once again demonstrates that DOX exosomes modified with chitosan could effectively prevent myocardial injury and significantly reduce the toxic and side effects of chemotherapy drugs on the entire body.

In addition, the tumor-suppressing efficacy was confirmed through H&E staining of tumor sections. Clearly defined tumor tissues with distinct blue staining boundaries were prominently observed. It can be observed that in the EXO@DOX group, the tumor tissue was extensively damaged, leading to massive necrosis. In contrast, the CS-EXO@DOX group exhibited concentrated diffusion destruction of tumor cells, with an increase in cell debris. A distinct drug accumulation phenomenon was observed in the sections. In conclusion, these results strongly

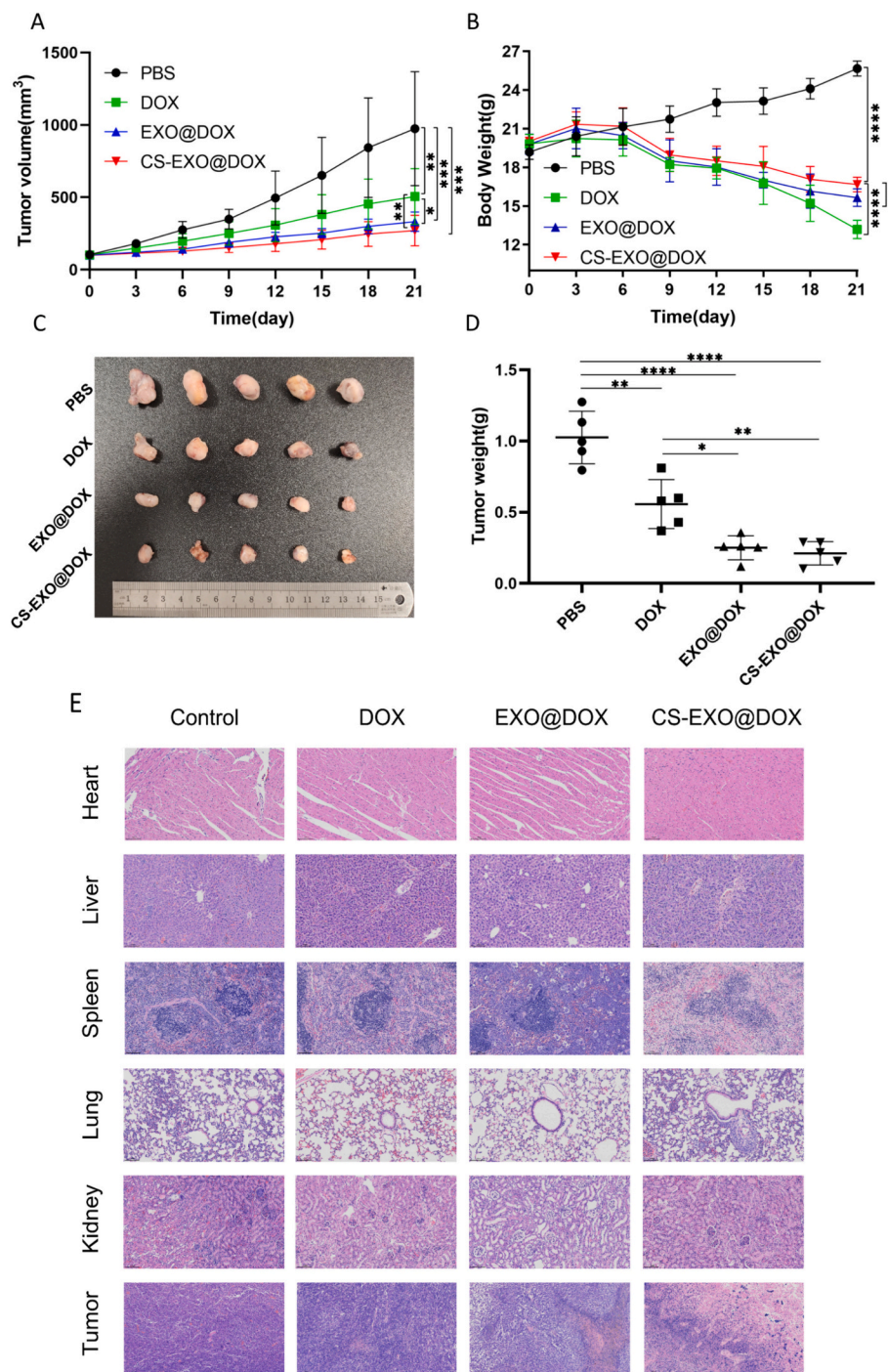


Fig. 6. Antitumor efficacy of CS-EXO@DOX *in vivo*. (A) The relative tumor volume. (B) Body weight changes of the mice in different groups. (C) Images of the tumors on the 21st day post-treatment. (D) Tumor weights after post-treatment in different groups ($n = 5$). (E) H&E staining images of tumors and major organ tissues in mice, which were sacrificed on the 21st day after treatment in different groups. Scale bar: 100 μ m. Data represent the mean $\pm$ standard deviation ($n = 5$). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

demonstrate that both tumor-derived exosomes and their chitosan modifications exhibit excellent anti-tumor effects. However, from a biosafety perspective, it is undoubtedly the best after chitosan modification. All of this data indicates that chitosan-modified exosomes are safe and effective delivery vehicles for tumor chemotherapy drugs.

3.7. Tumor tissue apoptosis evaluation

To further validate the enhanced *in vivo* antitumor activity of free DOX, EXO@DOX, and CS-EXO@DOX compared to the control group,

tumor tissues were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). As shown in Fig. 7, the number of apoptotic cells in the tumor tissues treated with EXO@DOX and CS-EXO@DOX was significantly higher than that in the free DOX group. In the EXO@DOX group, the apoptotic components of the tumors were distributed sporadically, whereas in the CS-EXO@DOX group, the apoptotic components of the tumors exhibited a clustered and diffused distribution. Additionally, a large number of fragmented and necrotic tumor cells were observed in the DAPI-stained cell nuclear images. The aforementioned results indicate that modification with chitosan

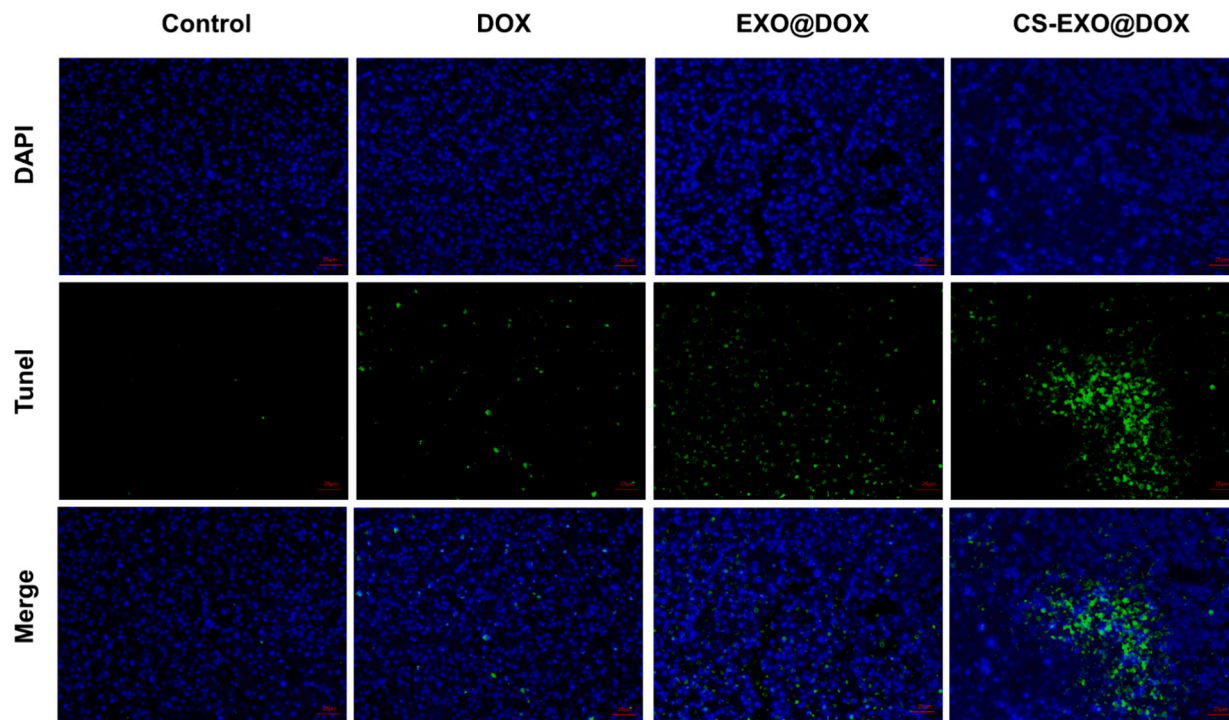


Fig. 7. TUNEL staining images of tumor tissues in mice, which were sacrificed on the 21st day after treatment of various groups. Scale bar: 25 μ m.

facilitates drug accumulation and penetration into tumor tissues, thereby promoting apoptosis and enhancing the antitumor effect *in vivo*.

4. Discussion

Although DOX was widely used as a broad-spectrum antineoplastic agent in clinical therapy, its clinical application was significantly hindered by its cardiotoxicity, which includes cardiomyopathy and congestive heart failure [27]. In the clinic, polyethylene glycol (PEG) was primarily used to modify the surface of liposomes to reduce the toxic and side effects of doxorubicin. However, there were still immunogenic reactions, multiple drug resistance, and even more serious side effects such as palmar-plantar erythrodysesthesia (PPE), stomatitis, and mucositis [4]. However, as carriers with low immunogenicity and high biocompatibility, exosomes could minimize these potential adverse effects [28]. In the study, HepG2 hepatoma cells were used for the mass production of exosomes. Tumor-derived exosomes could express the tetraspanin CD9 on their surface, which facilitates direct membrane fusion with target cells, thereby enhancing the biocompatibility of drug carriers, and inhibiting tumor metastasis [11,29].

In this study, it was found that modifying the surface of exosomes with chitosan was optimal. This modification allowed the original exosomes to retain the expression of their surface features. The modification with chitosan not only enhanced the stability of the exosomes but also increased their affinity for tumor cells. We used chitosan-modified tumor exosomes to deliver the anthracycline drug to solid tumors in nude mice. Chitosan-modified exosomes showed a specific targeting effect on liver tumors and demonstrated the most potent protective effect on the heart [[30–32]]. At the same time, compared with the exosome group, it was found that chitosan-modified exosomes could achieve a longer retention time in the liver. This extended retention time is beneficial for enriching and delivering drugs to the liver. CS-EXO@DOX could effectively enrich and centrally break the ring in the tumor, indicating that chitosan modification on the surface of exosomes plays a crucial role in the delivery of exosomes to the tumor. *In vivo* and *in vitro* experiments have demonstrated that DOX encapsulated in exosomes modified with chitosan exhibits significant synergistic and less

toxic effects compared to the same dose of free DOX [33]. Exosomes modified with chitosan have been shown to exhibit high affinity for HepG2 hepatoma cells both *in vitro* and *in vivo*. The systemic administration of a delivery system modified with chitosan and loaded with DOX, using tumor exosomes as biological carriers, significantly inhibited tumor growth while reducing drug toxicity and side effects. Similar to the findings of previous studies [14,34], it has been suggested that chitosan-modified tumor exosomes are the most promising candidates for the targeted delivery of therapeutic drugs to tumors.

In the study, it was found that chitosan-modified tumor exosomes demonstrated excellent targeting capability. It has four main reasons for CS-EXO@DOX having special targeting properties. Firstly, the particle size of the formulation was controlled to approximately 160 nm, and the EPR effect facilitated the passive targeting of the preparation to tumor tissues. Secondly, after modification with chitosan, the charge of the preparation was reversed. The cancer cell surface became negatively charged, while the normal cell surface remained electrically neutral or slightly positively charged [35]. Consequently, the positively charged CS-EXO@DOX could selectively interact with cancer cells to achieve active targeting. Thirdly, the expression of specific receptor proteins on tumor exosomes enhances the recognition and endocytosis of tumor tissues, thus achieving active targeting, a process known as tumor homing [36]. Lastly, research has confirmed that chitosan could bind to the CD44 receptor on cancer stem cells in tumor tissues, leading to endocytosis into cells, thereby achieving the purpose of targeted elimination and inhibiting tumor metastasis [22]. In the experiment, it was confirmed that the synergistic effects of the aforementioned factors significantly reduced the toxicity of DOX, including myocardial toxicity, bone marrow suppression, and other adverse effects.

Although autologous tumor exosomes have the advantages of the homing ability to target tumor sites and the ability to penetrate deep tumor tissues, there are still concerns about their tumor-activating properties and immune responses when tumor cell-derived exosomes are used as carriers of antitumor drugs [37]. Therefore, the primary focus of further clinical development is the potential impact on the receptor. It is essential to investigate the influence of endogenous nucleic acids and proteins originating from tumors, along with other

components in exosomes, on the immune system of the receptor.

For the clinical application of exosome-based therapies, even the production of exosomes from high-yielding tumor cells struggles to provide the quantities required for clinical use. The large-scale production and comprehensive characterization of exosomes remain challenging. However, with the rise of engineered exosomes, biomimetic exosomes, and exosome hybrid technologies [38], it is believed that the mass production and clinical use of exosomes will be realized soon. Nevertheless, their immunogenicity and safety will still pose significant challenges. Therefore, further clinical development is needed to enhance the technical aspects of chitosan-modified exosomes.

5. Conclusion

In summary, it has been demonstrated that chitosan-modified exosomes serve as a simple and natural nanoscale drug delivery platform. They can efficiently target chemotherapeutic drugs to tumors while significantly reducing the toxic and side effects associated with these drugs. Therefore, this method will be safe and effective for treating human cancer. It is expected that other types of chemotherapeutic drugs can also be specifically transported to tumor tissues through chitosan-modified exosomes. The successful modification of chitosan materials has opened up opportunities for grafting other negatively charged materials onto chitosan. These materials include negatively charged RNA, acidic medium materials, taurine, folic acid, polypeptide proteins, and more. If the high yield and low toxicity of tumor cells can be utilized to produce exosomes, which can then be modified with chitosan or undergo secondary modification with chitosan for efficient drug delivery, these drugs could offer significant potential clinical applications. The limitations of the study include the lack of detection for the expression of oncogenes associated with tumor exosomes. Whether the relevant oncogenic fragments in tumor exosomes can induce tumor metastasis requires further research and validation. Additionally, the impact of tumor exosomes on immune activation and their potential role in regulating the tumor microenvironment also need to be further investigated and substantiated.

CRediT authorship contribution statement

Zhiwei Wen: Writing – original draft, Investigation, Data curation. **Shuiling Qin:** Methodology, Formal analysis. **Huajie Huang:** Methodology. **Xingle Xia:** Data curation. **Wei Zhang:** Project administration. **Wei Wu:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

- [1] M. Tantawy, F.G. Pamittan, S. Singh, Y. Gong, Epigenetic changes associated with anthracycline-induced cardiotoxicity, *J. Clin. Transl. Sci.* 14 (1) (2021) 36–46, <https://doi.org/10.1111/cts.12857>.
- [2] W.C.M. Dempke, R. Zielinski, C. Winkler, S. Silberman, S. Reuther, W. Priebe, Anthracycline-induced cardiotoxicity - are we about to clear this hurdle? *J. Eur J. Cancer.* 185 (2023) 94–104, <https://doi.org/10.1016/j.ejca.2023.02.019>.
- [3] M. Kciuk, A. Gielecińska, S. Mujwar, D. Kolat, Ż. Kaluzińska-Kolat, I. Celik, R. Kontek, Doxorubicin-an agent with multiple mechanisms of anticancer activity, *J. Cells.* 12 (4) (2023) 659, <https://doi.org/10.3390/cells12040659>.
- [4] M. Ibrahim, W.H. Abuwatfa, N.S. Awad, R. Sabouni, G.A. Hussein, Encapsulation, release, and cytotoxicity of doxorubicin loaded in liposomes, micelles, and metal-organic frameworks: a review, *Aust. J. Pharm.* 14 (2) (2022) 254, <https://doi.org/10.3390/pharmaceutics14020254>.
- [5] R. Kalluri, V.S. LeBleu, The biology, function, and biomedical applications of exosomes, *Science* 367 (6478) (2020) eaau6977, <https://doi.org/10.1126/science.aau6977>.
- [6] A. Clayton, C.L. Harris, J. Court, M.D. Mason, B.P. Morgan, Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59, *J. Eur J. Immunol.* 33 (2) (2003) 522–531, <https://doi.org/10.1002/immu.200310028>.
- [7] H. Liu, B. Li, The functional role of exosome in hepatocellular carcinoma, *J. J. Cancer Res Clin Oncol.* 144 (11) (2018) 2085–2095, <https://doi.org/10.1007/s00432-018-2712-7>.
- [8] K.M. McAndrews, R. Kalluri, Mechanisms associated with biogenesis of exosomes in cancer, *J. Mol. Cancer.* 18 (1) (2019) 52, <https://doi.org/10.1186/s12943-019-0963-9>.
- [9] R. Kalluri, The biology and function of exosomes in cancer, *J. J. Clin. Invest.* 126 (4) (2016) 1208–1215, <https://doi.org/10.1172/JCI81135>.
- [10] G. Szabo, F. Momen-Heravi, Extracellular vesicles in liver disease and potential as biomarkers and therapeutic targets, *J. Nat. Rev. Gastroenterol. Hepatol.* 14 (8) (2017) 455–466, <https://doi.org/10.1038/nrgastro.2017.71>.
- [11] X. Xie, S. Lian, Y. Zhou, B. Li, Y. Lu, L. Yeung, L. Jia, Tumor-derived exosomes can specifically prevent cancer metastatic organotropism, *J. J. Control Release.* 331 (2021) 404–415, <https://doi.org/10.1016/j.jconrel.2021.01.030>.
- [12] H. Li, W. Xu, F. Li, R. Zeng, X. Zhang, X. Wang, S. Zhao, J. Weng, Z. Li, L. Sun, Amplification of anticancer efficacy by co-delivery of doxorubicin and lonidamine with extracellular vesicles, *J. Drug Deliv.* 29 (1) (2022) 192–202, <https://doi.org/10.1080/10717544.2021.2023697>.
- [13] T. Yong, X. Zhang, N. Bie, H. Zhang, X. Zhang, F. Li, A. Hakeem, J. Hu, L. Gan, H. A. Santos, X. Yang, Tumor exosome-based nanoparticles are efficient drug carriers for chemotherapy, *J. Nat. Commun.* 10 (1) (2019) 3838, <https://doi.org/10.1038/s41467-019-11718-4>.
- [14] K. Tang, Y. Zhang, H. Zhang, P. Xu, J. Liu, J. Ma, M. Lv, D. Li, F. Katirai, G.X. Shen, G. Zhang, Z.H. Feng, D. Ye, B. Huang, Delivery of chemotherapeutic drugs in tumour cell-derived microparticles, *J. Nat. Commun.* 3 (2012) 1282, <https://doi.org/10.1038/ncomms2282>.
- [15] T. Kean, M. Thanou, Biodegradation, biodistribution and toxicity of chitosan, *J. Adv. Drug Deliv. Rev.* 62 (1) (2010) 3–11, <https://doi.org/10.1016/j.addr.2009.09.004>.
- [16] S.C. Richardson, H.V. Kolbe, R. Duncan, Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA, *J. Int J. Pharm.* 178 (2) (1999) 231–243, [https://doi.org/10.1016/S0378-5173\(98\)00378-0](https://doi.org/10.1016/S0378-5173(98)00378-0).
- [17] G.H. Shin, S.K. Chung, J.T. Kim, H.J. Joong, H.J. Park, Preparation of chitosan-coated nanoliposomes for improving the mucoadhesive property of curcumin using the ethanol injection method, *J. J. Agric. Food Chem.* 61 (46) (2013) 11119–11126, <https://doi.org/10.1021/jf4035404>.
- [18] M.X. Chen, B.K. Li, D.K. Yin, J. Liang, S.S. Li, D.Y. Peng, Layer-by-layer assembly of chitosan stabilized multilayered liposomes for paclitaxel delivery, *J. Carbohydr. Polym.* 111 (2014) 298–304, <https://doi.org/10.1016/j.carbpol.2014.04.038>.
- [19] M.O. Alshraim, S. Sangi, G.I. Harisa, A.H. Alomrani, O. Yusuf, M.M. Badran, Chitosan-coated flexible liposomes magnify the anticancer activity and bioavailability of docetaxel: impact on composition, *J. Molecules.* 24 (2) (2019) 250, <https://doi.org/10.3390/molecules24020250>.
- [20] X. Zhai, C. Li, D. Ren, J. Wang, C. Ma, A.M. Abd El-Aty, The impact of chitooligosaccharides and their derivatives on the in vitro and in vivo antitumor activity: a comprehensive review, *J. Carbohydr. Polym.* 266 (2021) 118132, <https://doi.org/10.1016/j.carbpol.2021.118132>.
- [21] X. Yin, Y. Chi, C. Guo, S. Feng, J. Liu, K. Sun, Z. Wu, Chitooligosaccharides modified reduction-sensitive liposomes: enhanced cytoplasmic drug delivery and osteosarcoma-tumor inhibition in animal models, *J. Pharm. Res.* 34 (10) (2017) 2172–2184, <https://doi.org/10.1007/s11095-017-2225-0>.
- [22] W. Rao, H. Wang, J. Han, S. Zhao, J. Dumbleton, P. Agarwal, W. Zhang, G. Zhao, J. Yu, D.L. Zynger, X. Lu, X. He, Chitosan-decorated doxorubicin-encapsulated nanoparticle targets and eliminates tumor reinitiating cancer stem-like cells, *J. ACS Nano.* 9 (6) (2015) 5725–5740, <https://doi.org/10.1021/nn506928p>.
- [23] J. Wu, A. Lee, Y. Lu, R.J. Lee, Vascular targeting of doxorubicin using cationic liposomes, *J. Int J. Pharm.* 337 (1–2) (2007) 329–335, <https://doi.org/10.1016/j.ijpharm.2007.01.003>.
- [24] X.X. Guo, W. He, X.Q. Wang, X.M. Hu, Preparation and efficacy of tumor vasculature-targeted doxorubicin cationic liposomes coated by N-trimethyl chitosan, *J. Journal of Applied Polymer Science.* 121 (4) (2011) 2149–2156, <https://doi.org/10.1002/app.33944>.
- [25] J. Li, J. Li, Y. Peng, Y. Du, Z. Yang, X. Qi, Dendritic cell derived exosomes loaded neoantigens for personalized cancer immunotherapies, *J. J. Control Release.* 353 (2023) 423–433, <https://doi.org/10.1016/j.jconrel.2022.11.053>.
- [26] C. Théry, K.W. Witwer, E. Aikawa, M.J. Alcaraz, J.D. Anderson, R. Andriantsitohaina, D. Zocco, E.K. Zuba-Surma, et al., Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, *J. Extracell. Vesicles.* 7 (1) (2018) 1535750, <https://doi.org/10.1080/20013078.2018.1535750>.

- [27] V. Bagalkot, O.C. Farokhzad, R. Langer, S. Jon, An aptamer-doxorubicin physical conjugate as a novel targeted drug-delivery platform, *J. Angew Chem Int Ed Engl.* 45 (48) (2006) 8149–8152, <https://doi.org/10.1002/anie.200602251>.
- [28] G. van Niel, G. D'Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, *J. Nat Rev Mol Cell Biol.* 19 (4) (2018) 213–228, <https://doi.org/10.1038/nrm.2017.125>.
- [29] J.G. van den Boorn, M. Schlee, C. Coch, G. Hartmann, SiRNA delivery with exosome nanoparticles, *J. Nat Biotechnol.* 29 (4) (2011) 325–326, <https://doi.org/10.1038/nbt.1830>.
- [30] S.J. Huang, T.H. Wang, Y.H. Chou, H.D. Wang, T.C. Hsu, J.L. Yow, B.S. Tzang, W. H. Chiang, Hybrid PEGylated chitosan/PLGA nanoparticles designed as pH-responsive vehicles to promote intracellular drug delivery and cancer chemotherapy, *J. Int J Biol Macromol.* 210 (2022) 565–578, <https://doi.org/10.1016/j.ijbiomac.2022.04.209>.
- [31] M.U. Akram, N. Abbas, M. Farman, S. Manzoor, M.I. Khan, S.M. Osman, R. Luque, A. Shanableh, Tumor micro-environment sensitive release of doxorubicin through chitosan based polymeric nanoparticles: an in-vitro study, *Chemosphere* 313 (2023) 137332, <https://doi.org/10.1016/j.chemosphere.2022.137332>.
- [32] M. Asariha, S.H. Kiaie, S. Izadi, H. P. F, M. Fouladi, M. Gholamhosseinpour, Extended-release of doxorubicin through green surface modification of gold nanoparticles: in vitro and in ovo assessment, *J. BMC Chem.* 16 (1) (2022) 110, <https://doi.org/10.1186/s13065-022-00895-x>.
- [33] D. Siva, S. Abinaya, D. Rajesh, G. Archunan, P. Padmanabhan, B. Gulyás, S. Achiraman, Mollification of doxorubicin (DOX)-mediated cardiotoxicity using conjugated chitosan nanoparticles with supplementation of propionic acid, *J. Nanomaterials (Basel).* 12 (3) (2022) 502, <https://doi.org/10.3390/nano12030502>.
- [34] H. Al Faruque, E.S. Choi, J.H. Kim, E. Kim, Enhanced effect of autologous EVs delivering paclitaxel in pancreatic cancer, *J. Control Release* 347 (2022) 330–346, <https://doi.org/10.1016/j.jconrel.2022.05.012>.
- [35] B. Chen, W. Le, Y. Wang, Z. Li, D. Wang, L. Ren, L. Lin, S. Cui, J.J. Hu, Y. Hu, P. Yang, R.C. Ewing, D. Shi, Z. Cui, Targeting negative surface charges of cancer cells by multifunctional nanoprobe, *J. Theranostics.* 6 (11) (2016) 1887–1898, <https://doi.org/10.7150/thno.16358>.
- [36] L. Qiao, S. Hu, K. Huang, T. Su, Z. Li, A. Vandergriff, J. Cores, P.U. Dinh, T. Allen, D. Shen, H. Liang, Y. Li, K. Cheng, Tumor cell-derived exosomes home to their cells of origin and can be used as Trojan horses to deliver cancer drugs, *J. Theranostics.* 10 (8) (2020) 3474–3487, <https://doi.org/10.7150/thno.39434>.
- [37] Q.H. Xie, J.Q. Zheng, J.Y. Ding, Y.F. Wu, L. Liu, Z.L. Yu, G. Chen, Exosome-mediated immunosuppression in tumor microenvironments, *J. Cells.* 11 (12) (2022) 1946, <https://doi.org/10.3390/cells11121946>.
- [38] J. Mondal, S. Pillarisetti, V. Junnuthula, M. Saha, S.R. Hwang, I.K. Park, Y.K. Lee, Hybrid exosomes, exosome-like nanovesicles and engineered exosomes for therapeutic applications, *J. J Control Release.* 353 (2023) 1127–1149, <https://doi.org/10.1016/j.jconrel.2022.12.027>.