Research paper

In vivo distribution and antitumor activity of doxorubicin-loaded N-isopropylacrylamide-co-methacrylic acid coated mesoporous silica nanoparticles and safety evaluation

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A R T I C L E   I N F O

Keywords:
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Mesoporous silica nanoparticles
Doxorubicin
Antitumor activity
Safety evaluation
Nanoparticles

A B S T R A C T

The objective of this study was to develop and evaluate the antitumor activity and the safety of a delivery system containing mesoporous silica nanoparticles (MSN) coated with pH-responsive poly (N-isopropylacrylamide-co-methacrylic acid; P NIPAM-co-MAA) for doxorubicin (DOX) delivery (P-MSN-DOX) in vitro and in vivo. We reported that P-MSN-DOX nanoparticles (190 ± 30 nm) offered a DOX-loading coefficient of more than 20%. DOX release from the P-MSN-DOX formulation was pH-dependent with enhanced antitumor effects in vitro compared with traditional MSN-DOX, which was weakly cytotoxic due to negligible drug release at tested pHs. P-MSN-DOX circulated longer, with less cardiac and renal accumulation as shown by pharmacokinetics and biodistribution studies in vivo. Also, the P-MSN-DOX delivery system had greater antitumor activity in mice bearing a murine sarcoma S-180 cell line. This finding was correlated with both in vitro and in vivo. Subacute toxicity tests revealed a low P-MSN-DOX toxicity in vivo, as well. Thus, P-MSN-DOX appears to be an efficacious and safe cancer treatment strategy.

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1. Introduction

Doxorubicin (DOX) is an effective and commonly used anthracycline anticancer drug for treatment of human malignancies. However, its short biological half-life, nonspecific distribution, and significant adverse effects limit its utility [1]. Like most chemotherapeutic agents, DOX toxicity limits the dose that can be used for tumor treatment. Specifically, the clinical effectiveness of DOX is limited by cumulative cardiotoxicity which occurs in a significant percentage of patients who receive DOX in the dosage range of 450–600 mg/m². DOX, as a single chemical entity, has high cytotoxicity, good solubility, and high nuclear affinity [2]. Thus, we attempted to design an improved DOX delivery system to enhance in vivo distribution, enhance tumor site deposition, and reduce cardiotoxicity.

DOX formulations are designed to release drug in response to the tumor microenvironment, so attempts to decrease toxicity in normal tissues have included specifically directing the drug cytotoxicity to tumors. In addition to novel delivery systems, macro-molecular prodrugs have been used to prolong the duration of therapeutic drug activity [3,4]. Several delivery systems developed for DOX were chiefly designed to direct DOX away from the heart toward the tumor, and these formulations did produce modest increases in the therapeutic index of DOX in preclinical and clinical studies [5–7]. Still, a better delivery system is achievable.

Thus, the primary objective of this study was to develop a pH-responsive delivery system that was more efficient in selectively delivering DOX to the tumor and more effective at directing DOX away from the heart to decrease its cardiotoxicity.

Mesoporous silica nanoparticles (MSN) have large surface areas, can be tailored with respect to pore size, particle shape, and particle size, and have high chemical and mechanical stability. Such flexibility makes them especially useful for multiple biomedical applications, and they have been used for such decades [8–11]. However, conventional, pure MSN cannot precisely control drug release. Previously, we developed a cross-linked poly (N-isopropylacrylamide-co-methacrylic acid) coated mesoporous silica nanoparticles (MSN) for doxorubicin (DOX) delivery (P-MSN-DOX) in vitro and in vivo. We reported that P-MSN-DOX nanoparticles (190 ± 30 nm) offered a DOX-loading coefficient of more than 20%. DOX release from the P-MSN-DOX formulation was pH-dependent with enhanced antitumor effects in vitro compared with traditional MSN-DOX, which was weakly cytotoxic due to negligible drug release at tested pHs. P-MSN-DOX circulated longer, with less cardiac and renal accumulation as shown by pharmacokinetics and biodistribution studies in vivo. Also, the P-MSN-DOX delivery system had greater antitumor activity in mice bearing a murine sarcoma S-180 cell line. This finding was correlated with both in vitro and in vivo. Subacute toxicity tests revealed a low P-MSN-DOX toxicity in vivo, as well. Thus, P-MSN-DOX appears to be an efficacious and safe cancer treatment strategy.

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propylacrylamide-co-methacrylic acid) P (NIPAM-co-MAA)-coated magnetic Fe₃O₄ MSN, which had better thermo/pH-controlled release compared to a conventional drug carrier [12]. Surface functionalization of MSN with a stimuli-responsive group prevented premature drug release and drug degradation prior to drug contact with the target tissue. Because pH is critical for appropriate drug release [13], and because most cancers have an enriched blood supply with low extracellular pH compared to normal tissues [14], these features can be exploited for drug design. Specifically, pH-dependent, MSN-based carriers can be created to achieve better drug targeting and treatment efficacy. In this study, we prepared DOX-loaded pH-sensitive MSN/P (NIPAM-co-MAA) without Fe₃O₄, using MSN as a core and a P (NIPAM-co-MAA) polymer for the shell [15].

Few reports describe the behavior of drug-loaded MSN in vivo until now [16,17]. We report an innovative drug design: DOX-loaded MSN. Also, we describe the novel drug formulation’s release kinetics, pharmacokinetics, biodistribution, antitumor efficacy, and safety in vitro and in vivo.

2. Materials and methods

2.1. Materials and animals

MSN and P (NIPAM-co-MAA)-coated MSN (P-MSN) were prepared in our laboratory as previously reported [12]. A hydrochloric acid of DOX was obtained from Beijing Huafeng United Technology Company (Beijing, China). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma–Aldrich (St. Louis, MO). Purified, deionized water was prepared using the Milli-Q plus system (Millipore Co., Billerica, MA). MCF-7, H-460, and S-180 cell lines were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Culture plates and dishes were purchased from Corning Inc. (CITY, NY). The cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate at 37 °C with 5% CO₂ under fully humidified conditions. All experiments were performed on cells in the logarithmic growth phase.

Male Sprague–Dawley (SD) rats (230 ± 20) g and male Implanting Control Region (ICR) mice (20 ± 2) g, supplied by the Department of Experimental Animals, Fudan University (Shanghai, China), were acclimated at 25 °C and 55% of humidity under natural light/dark conditions for 1 week prior to experiments. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University (#2012-7).

2.2. Preparation and characterization of P-MSN-DOX

The preparation of DOX-loaded MSN (MSN-DOX) and DOX-loaded P-MSN (P-MSN-DOX) has been previously described [14]. Briefly, 1.5 mL of DOX solution (1 mg/mL) was dissolved in deionized water and 5 mg of MSN or P-MSN was dissolved in an aqueous solution of sodium hydrate (pH 8.5) with stirring at 25 °C for 12 h. Then, each dispersion was centrifuged to isolate MSN-DOX or P-MSN-DOX, and each isolate was washed with deionized water to remove any remaining external surface adsorption of DOX. The particle size and z-potential of MSN-DOX and P-MSN-DOX were measured using a dynamic light scattering technique with a Zetasiser Nano ZS (Malvern autosizer 4700, United Kingdom).

RP-HPLC analysis of DOX samples was achieved on a C₁₈ Gemini column (5 μm, 150 × 4.6 mm, Phenomenex, CA) and a guard column (Gemini 10 μm C₁₈, 6.6 × 3.0 mm, Phenomenex, CA) with a mobile phase consisting of ammonium acetate buffer solution (10 mM, pH 3.0) and acetonitrile (70: 30, v/v) at a flow rate of 1.0 mL/min. The sample injection volume was 20 μL, and the excitation and emission wave lengths were set at 480 nm and 560 nm on the RF-20A fluorescence detector (Shimadzu, Kyoto, Japan), respectively. The limit of quantification was 1.0 ng/mL, and coefficients of variation were all within 3%.

The drug-loading coefficient (DL%) and encapsulation ratio (ER%) were calculated by the following equations.

\[
DL\% = \frac{\text{weight of the drug in MSNs}}{\text{weight of the feeding polymer and drug}} \times 100\%
\]

\[
ER\% = \frac{\text{weight of the drug in MSNs}}{\text{weight of the feeding drug}} \times 100\%
\]

To measure the release behavior of DOX from MSNs under different pHs, 1 mL of a DOX-loaded MSNs aqueous dispersion (containing 1 mg DOX) was added to 200 mL 0.1 M citrate buffer solution (pH = 5.0 and 7.4) at 37 °C with stirring at 100 rpm for 48 h. At appropriate time intervals, 0.5 mL aliquots were withdrawn and replaced with an equal volume of fresh medium. After centrifugation, the DOX concentration was quantified by HPLC as described above.

2.3. In vitro antitumor activity evaluation

An MTT assay was used to determine the cell viability. Briefly, cells were seeded in 96-well plates (5 × 10³ cells per well). After 24 h of incubation, media were removed and cells were incubated for an additional 72 h in media containing DOX, MSN-DOX, or P-MSN-DOX at various concentrations. After incubation, 5 mg/mL MITT solution (20 μL/well) was added to the cells and cultured for an additional 4 h, and then, the supernatant was discarded after centrifugation at 2000 rpm for 5 min and replaced with dimethylsulfoxide (100 μL/well). The cell suspension was placed on a microvibrator for 5 min, and the absorbance was measured at 570 nm using a microplate reader (Tecan Safire2, Switzerland).

2.4. Pharmacokinetics studies

Fifteen SD rats (230 ± 20 g) were randomly assigned to three groups for pharmacokinetics investigation. Tail veins of rats in each of the three groups were injected with 0.3 mL free DOX, MSN-DOX, or P-MSN-DOX at an equivalent dose of 6 mg/kg DOX. At scheduled time points (0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h) post-injection, 0.2 mL of blood was collected into heparinized polyethylene tubes from the periorbital vein and centrifuged at 1000 rpm for 10 min to obtain plasma [15]. The plasma was stored at -70 °C for further analysis by HPLC. Liquid–liquid extraction was performed prior to HPLC analysis. Briefly, 100 μL plasma samples were mixed with 2 mL chloroform:methanol = 4:1 (v/v) and 20 μL of 2.5 μg/mL daunomycin methanol solution as an internal standard. The samples were extracted on a vortex-mixer for 2 min and then centrifuged at 6000 rpm for 10 min. Then, the underlying organic layer was transferred to a clean tube and evaporated under a gentle stream of nitrogen. The extraction residue was reconstituted in 100 μL methanol, centrifuged at 5000 rpm for 5 min, and supernatant was collected for HPLC analysis. DOX concentrations were measured by HPLC methods described in 2.5. BAPP 2.0 (Bioavailability program package 2.0, 2002, China) was used to analyze pharmacokinetics parameters such as the area under the plasma concentration–time curve (AUC), the apparent volume of distribution (V₂), total body clearance (CL), elimination half-life (t₁/₂b), and the mean residence time (MRT) of DOX for each formulation. The Akaike’s
2.5. Tissue distribution studies

Tumor-bearing mice were prepared by inoculating 0.2 mL PBS containing 1.5 × 10⁶ S-180 cells into the right flank of ICR mice, and the tumor was allowed to grow for approximately 6 days. When the tumor volume reached 1 cm³, mice were divided into three groups at random and injected with 0.1 mL of DOX, MSN-DOX or P-MSN-DOX intravenously via the tail vein (DOX: 4 mg/kg). Then, 0.5, 1, 2, 4, 8, and 12 h after injection, four mice from one group were sacrificed by cervical dislocation after drawing blood (0.2 mL) from the periorbital area. Immediately, blood was treated as described above. Major organs were excised from the animals, washed with cold saline, dried on filter paper, weighed, and stored at -70°C until assessed by HPLC. The tissues were homogenized in saline before extraction.

2.6. In vivo antitumor activity evaluation

The antitumor efficacy of DOX, MSN-DOX, and P-MSN-DOX was evaluated in ICR mice inoculated subcutaneously in the right flank with 0.2 mL PBS containing 1.5 × 10⁶ S-180 cells. The DOX dose schedule started 24 h after tumor cell transplantation to mimic early stage tumor growth. Mice (N = 40) bearing S-180 subcutaneous tumors were randomly assigned to five groups: (1) saline solution group; (2) MSN (25 mg carrier/kg) group; (3) free DOX (5 mg/kg) group; (4) MSN-DOX (5 mg/kg) group; and (5) P-MSN-DOX (5 mg/kg) group. Mice were treated with drug or saline (control) every other day until three administrations were complete. Body weight was measured daily. Finally, 24 h after the last treatment administration, mice were sacrificed by cervical dislocation, and tumors were harvested and weighed.

2.7. Safety evaluation

At the end of the experiment, 24 h of the last treatment administration, blood and serum samples were obtained from tumor-bearing mice to assess hematological and biochemical parameters. First, 0.5 mL blood from each mouse was collected for blood chemistry analysis. paraformaldehyde solution overnight, and then processed into paraffin sections followed by hematoxylin and eosin (H&E) staining for histopathological analysis by an experienced technician who was blinded to the treatment groups.

2.8. Data analysis

Statistical analysis was performed using the Student’s t-test for two groups and one-way ANOVA for multiple groups. Data were expressed as mean ± standard deviation. A probability (p) of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Characterization of P-MSN-DOX and MSN-DOX

MSN and P-MSN particle sizes were (160 ± 10) nm, PDI = 0.09, and (190 ± 30) nm, PDI = 0.05, respectively. DL% of MSN was greater than 20%. In vitro drug release profiles of MSN are shown in Fig. 1, indicating that DOX release from MSN was both time- and pH-dependent. The profile indicated that 85.2% ± 4.8 DOX was released from P-MSN-DOX within 48 h at pH = 5.0 and 12.9% ± 2.2 was released at pH = 7.4. DOX was released slowly from the MSN-DOX formulation: 48.9% ± 3.5 and 27.1% ± 3.0 at pH 5.0 and 7.4, respectively. DOX was released slower, in smaller amounts, and with less pH-dependence in the MSN-DOX formulation, suggesting poor selectivity. The volume phase transition temperature (VPTT) of the P (NIPAM-co-MAA) coat could be changed over various pHs. The cumulative release in vitro revealed a low level of leakage at pH 7.4 below the VPTT, and release was significantly enhanced at pH = 5.0, above the VPTT [12]. As expected, in the P-MSN-DOX formulation, drug release was more responsive to pH compared with conventional, pure MSN. Release profiles of MSN were investigated from pH 5.0 to 7.4, replicating the pH of plasma and tumor tissue [14]. Thus, this experiment revealed that the P-MSN-DOX formulation, in tumors, rapidly released more DOX.

3.2. In vitro antitumor efficacy evaluation

Cytotoxic activity was measured in H-460, MCF-7, and S-180 cell lines incubated with DOX, MSN-DOX, and P-MSN-DOX for 72 h. The results are depicted in Table 1. P-MSN-DOX had similar antitumor effects as free DOX and much higher cytotoxicity than the MSN-DOX formulation in MCF-7, H-460, and S-180 cell lines. Because pure MSNs had no activity over the concentration range used in this study, the increased antitumor activity observed with the P-MSN-DOX formulation could be attributed to more DOX release from the P-MSN-DOX delivery system.

3.3. In vivo pharmacokinetics

At the present time, few papers exist to describe the pharmacokinetics behavior of drug-loaded MSN. We are the first, to our knowledge, to evaluate the pharmacokinetics of DOX-loaded MSN. The plasma concentration–time curves for DOX, MSN-DOX, and P-MSN-DOX were all fitted to the two-compartment model. Data showed that P-MSN-DOX achieved a larger AUC and had long-term antitumor effects as free DOX and much higher cytotoxicity than the MSN-DOX formulation in MCF-7, H-460, and S-180 cell lines. Because pure MSNs had no activity over the concentration range used in this study, the increased antitumor activity observed with the P-MSN-DOX formulation could be attributed to more DOX release from the P-MSN-DOX delivery system.

![Graph showing in vitro release kinetics of drug from P-MSN-DOX and MSN-DOX in citrate buffer solutions at different pHs (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
Pharmacokinetics data showed that DOX-loaded MSN circulated longer and had a slower plasma elimination rate than DOX treatment alone. Such increased circulation time may be attributed to DOX molecules being stabilized within the MSN nanochannels, preventing them from leaking and being metabolized. Such “stealth” behavior of MSN induced by their nanoscopic dimensions and the hydrophilic shell of P (NIPAM-co-MAA) may decrease the rate of mononuclear phagocyte uptake and reduce plasma protein absorption [18]. Therefore, our data illustrate the potential utility of MSN—especially P-MSN—as drug nanocarriers with lasting circulation. Furthermore, we showed that MSN with an efficient surface modification can achieve a long-circulating reservoir for hydrophobic anticancer drugs and preferential tumor targeting [19]. pH-responsive polymer coating evidently influenced the in vivo long-circulating property of DOX to a greater degree than MSN. Similar results have been reported with a self-assembled pH/enzyme-responsive system [20]. The clearance for MSN was significantly lower than that of DOX, implying a longer retention in vivo long-circulating property of DOX to a greater degree than MSN. The DOX AUC0–12h was decreased as follows: spleen > liver > tumor > heart, respectively. The DOX AUC0–12h of MSN-DOX was higher in the liver and spleen and lower in the heart and kidney, compared to DOX. The DOX AUC0–12h of P-MSN-DOX was higher in plasma and lung and lower in the heart and kidney, compared to DOX. According to the literature [21–23], the biodistribution characteristics of nanoparticles were determined by the overall physicochemical properties, such as particle size, surface charge, surface modification, and the hydrophilic/hydrophobic nature of the carrier systems. MSN-DOX notably enhanced liver and spleen accumulation of DOX, and decreased tumor accumulation, decreasing drug efficacy. Thus, a pH-responsive polymer coat was introduced in the system to avoid this decrease in efficacy. After the surface of MSN was covered with P (NIPAM-co-MAA), DOX release was decreased under physiological conditions.

MSN can alter the biodistribution of DOX via nanochannel entrapment [24]. Charge-dependent adsorption of serum proteins greatly facilitates the hepatobiliary excretion of silica nanoparticles. Furthermore, the MSN diameter is smaller than the pore size of the permeable vasculature that is found in many solid tumors, suggesting that MSN should selectively accumulate in solid tumors by an enhanced permeability and retention (EPR) effect. Studies of in vivo biodistribution also revealed that MSN with a 160-nm particle size had a relatively positive surface charge and was mainly targeted to the liver after IV administration [25]. The hydrophilic/hydrophobic nature of the carrier system was suggested to be an important factor in improving nanoparticle biodistribution [26]. P-MSN-DOX with a 190-nm particle size did not significantly increase drug accumulation in the tumor because of weak EPR effect. An EPR is likely to be optimal if nanocarriers can circulate for a sufficient time in vivo, which would then allow accumulation of drug-loaded nanocarriers in tumors. Thus, the ideal particle size of the nanocarrier should be between 10 and 100 nm. Particles charge should be neutral or anionic for efficient escape from the renal elimination. The nanocarriers must be hidden from the reticuloendothelial system (RES), which destroys any foreign particles through opsonization [27]. As depicted in the characterization of P-MSN-DOX delivery system, particle size was ~200 nm, a size that was not-compliant with the defined prerequisites for nanocarriers to achieve an optimal EPR effect. The P (NIPAM-co-MAA) hydrophilic coat of the P-MSN-DOX formulation reduced plasma protein adsorption to MSN, reduced surface charge, increased hydrophilicity, and blocked electrostatic and hydrophobic interactions to allow opsonins to bind to MSN surfaces. These events greatly reduced liver and spleen drug accumulation and RES elimination. Therefore, a pH-sensitive coat did not change hepatic and splenic uptake and tumor accumulation. Additionally, DOX concentrations in the P-MSN-DOX group had greater drug accumulation in the lung than in the DOX and MSN-DOX treatment groups. Reports in the literature suggest that inclusion of a negative charge leads to more drug accumulation in the lung [28]. Coated with hydrophilic P (NIPAM-co-MAA), P-MSN-DOX had a more negative charge which increased the drug distribution in the lung, and this accumulation was greater than the MSN-DOX treatment group. Also, this negative charge greatly reduced cardiac and renal drug accumulation. Therefore, P-MSN-DOX has the potential to be an ideal drug delivery system for DOX in lung cancer treatment, allowing reduced the cardiotoxicity.

3.5. In vivo antitumor efficacy evaluation

Subcutaneous S-180 tumor-bearing mice were selected as animal model because it was one of the classical tumor models for a long time and also introduced in the studies on DOX [20]. The excised tumors were weighed and photographed (Fig. 4). Tumors of negative control group injected with saline solution weighted (0.73 ± 0.40) g. Blank P-MSN had no antitumor effect and tumors

### Table 1

<table>
<thead>
<tr>
<th>Formulations</th>
<th>IC50 (μg/mL)</th>
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<tr>
<td></td>
<td>DOX</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td>H-460</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>S-180</td>
<td>5.90 ± 0.44</td>
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</tbody>
</table>

* P < 0.05, compared with DOX.
** P < 0.01, compared with DOX.

Fig. 2. Plasma concentration–time curves after IV administration of DOX, MSN-DOX, and P-MSN-DOX to SD rats (DOX = 5 mg/kg; n = 5).
While for the DOX, MSN-DOX, and P-MSN-DOX groups, the tumor weighed (0.33 ± 0.21) g, (0.38 ± 0.25) g and (0.26 ± 0.15) g, respectively. These results were in agreement with the antitumor activity of various DOX formulations which showed as arranged tumors. The antitumor efficacy of P-MSN-DOX was greatly superior to that of DOX and MSN-DOX in subcutaneous S-180 tumor model. The antitumor efficacy in vivo for DOX, MSN-DOX, and P-MSN-DOX was consistent well with the cytotoxicity in vitro. These results might be explained by the increased local concentration of DOX in the tumor which was favored by the long circulation time and by EPR effect of MSN. Thus, P-MSN-DOX appeared to retain the desirable effects of passive targeting because the environment in tumor is acid which could trigger a change in the charge on the polymer from negative to a positive, which resulted in the dissociation of the polymer form MSN in the tumor.

3.6. Safety evaluation

3.6.1. Body weight

Animal body weight changes were used as a marker of safety in tumor-bearing animals [29]. Mice treated with DOX lost weight dramatically, losing ≈16% body weight within 24 h after the last drug treatment. This weight loss continued after treatment cessation, too. In contrast, no significant weight loss was observed in groups receiving MSN-DOX or P-MSN-DOX (Fig. 5). Moreover, body weight of mice treated with MSN decreased initially but recovered after drug cessation, suggesting that a slight systemic toxicity occurred in MSN-treated groups. This also indicates less toxicity associated with DOX-loaded MSN than that of free DOX. Controlled release of DOX from MSN apparently was less acutely toxicity as evidenced by no animal loss of body weight.

3.6.2. Hematology

To reveal potential side effects at the cellular level within treated mice, blood biochemistry and hematology were analyzed. We hypothesized that changes observed at this level indicated abnormalities of important systems, such as the immune system [29,30]. DOX significantly decreased WBC and platelets in subcutaneous S-180 tumor model. Mice treated with DOX lost weight dramatically, losing ≈16% body weight within 24 h after the last drug treatment. This weight loss continued after treatment cessation, too. In contrast, no significant weight loss was observed in groups receiving MSN-DOX or P-MSN-DOX (Fig. 5). Moreover, body weight of mice treated with MSN decreased initially but recovered after drug cessation, suggesting that a slight systemic toxicity occurred in MSN-treated groups. This also indicates less toxicity associated with DOX-loaded MSN than that of free DOX. Controlled release of DOX from MSN apparently was less acutely toxicity as evidenced by no animal loss of body weight.

Table 2
Comparative pharmacokinetic parameters of different DOX formulations (n = 5).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2 (a) (h)</td>
<td>DOX</td>
</tr>
<tr>
<td>0.08 ± 0.08</td>
<td>0.71 ± 0.21&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>t1/2 (b) (h)</td>
<td>7.85 ± 5.12</td>
</tr>
<tr>
<td>AU(t0-12h) (μg/L/h)</td>
<td>1493.09 ± 327.68</td>
</tr>
<tr>
<td>AU(t0-∞) (μg/L/h)</td>
<td>1941.98 ± 510.91</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>5.82 ± 4.16</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>3.06 ± 0.74</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>2.33 ± 2.83</td>
</tr>
</tbody>
</table>

<sup>**</sup> P < 0.01, compared with DOX.
<sup>**</sup> P < 0.01, compared with DOX.
neous S-180 tumor-bearing mice compared to control mice ($P < 0.05$) (Table 3). WBC and platelets of MSN-DOX and P-MSN-DOX groups were higher than those of mice receiving free DOX, and the MSN-DOX and P-MSN-DOX groups were not different than the saline group. We presume that MSN ameliorated the lymphocytopenia and reduced hematotoxicity in these animals.

### 3.6.3. Serum biochemistry

Cancer chemotherapy can cause multiple organ toxicity, and DOX specifically targets the heart. DOX triggers the disruption of cardiac myocytes and the release of intracellular CK and AST into the serum [31], indicating muscular damage. Serum CK and AST measurements in experimental animals to evaluate myocardial damage [32] are depicted in Table 4. AST and CK of mice treated with free DOX were significantly higher compared to saline, indicating DOX-induced heart damage. Compared with controls, serum AST level was also increased significantly in the MSN-DOX group ($P < 0.01$). However, there was no statistically significant difference between the P-MSN-DOX group and the control group, suggesting no obvious cardiac toxicity induced by P-MSN-DOX. These results suggest that P-MSN treatment reduced CK and AST caused by the DOX and was superior to MSN alone. P-MSN-DOX-induced less systemic toxicity, probably attributed to less random DOX release into the circulation.

### 3.6.4. Histopathology

Cardiotoxicity is the main drawback of anthracycline use, which ultimately restricts effective drug application in cancer patients. Myocardial degeneration is the most commonly encountered side effect of anthracycline, which can give rise to heart failure and even death [33–35]. Daily injection of DOX caused toxicity, resulting in weight loss and cardiotoxicity [36]. As shown in Fig. 6B, IV administration of free DOX caused noticeable cardiac tissue degeneration, necrosis and heart congestion. However, there was no evidence of acute cardiotoxicity from either MSN-DOX or P-MSN-DOX therapy compared to the control group (Fig. 6A, C, and D). This may mean that the cardiotoxicity of DOX was alleviated when delivered by MSN due to a reduction in DOX accumulation in the heart. Han and colleagues reported that cardiac DOX concentrations with free DOX treatment were higher than with PEG-liposome of DOX (Doxil doxorubicin formulation) [37]. Lu’s laboratory also reported fatty denaturation and fatty degeneration muscle in the free DOX treatment were higher than with PEG-liposome of DOX (Doxil doxorubicin formulation) [37]. Our data suggest a similar efficacy of P-MSN-DOX delivery system for reducing the potential cardiotoxicity of DOX.

### 4. Conclusion

In this study, the antitumor activity and safety of P-MSN-DOX was evaluated in vitro and in vivo. P-MSN-DOX with pH-triggered drug release lead to greater DOX accumulation in cancer cells and produced greater DOX-induced cytotoxicity in vitro compared to MSN-DOX. P-MSN-DOX offered greater therapeutic efficacy in vivo as evidenced by EPR and the pH-responsive property of P-MSN in subcutaneous S-180 tumor-bearing mice. In addition, pharmacokinetics and biodistribution studies show that P-MSN-DOX significantly increased the duration of drug circulation and decreased cardiac accumulation of DOX. Also, P-MSN-DOX appeared to be safe. Therefore, the P-MSN system may be potentially effective for DOX intracellular delivery, and this may enhance chemotherapy efficacy. Modification of P-MSN-DOX with PEG or a target agent may be required in future studies to improve tumor targeting and enhance antitumor efficacy while minimizing side effects.

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**Table 3**

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (10^9/L)</th>
<th>RBC (10^12/L)</th>
<th>Platelet (10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>9.82 ± 0.20</td>
<td>6.80 ± 0.24</td>
<td>1820.0 ± 119.0</td>
</tr>
<tr>
<td>MSN</td>
<td>8.93 ± 1.25</td>
<td>7.15 ± 0.40</td>
<td>1824.8 ± 582.8</td>
</tr>
<tr>
<td>DOX</td>
<td>5.03 ± 0.71</td>
<td>7.85 ± 0.40</td>
<td>1372.7 ± 118.5</td>
</tr>
<tr>
<td>MSN-DOX</td>
<td>12.70 ± 3.00</td>
<td>6.82 ± 0.41</td>
<td>2078.7 ± 498.0</td>
</tr>
<tr>
<td>P-MSN-DOX</td>
<td>9.77 ± 3.73</td>
<td>7.61 ± 0.88</td>
<td>1935.0 ± 376.7</td>
</tr>
</tbody>
</table>

* $P < 0.05$, compared with saline.
* * $P < 0.01$, compared with saline.
* *** $P < 0.001$, compared with saline.

**Table 4**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/L)</th>
<th>CK (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>284.0 ± 86.8</td>
<td>1625.5 ± 582.8</td>
</tr>
<tr>
<td>MSN</td>
<td>302.7 ± 42.1</td>
<td>1727.8 ± 480.0</td>
</tr>
<tr>
<td>DOX</td>
<td>556.5 ± 61.0</td>
<td>3043.7 ± 524.4</td>
</tr>
<tr>
<td>MSN-DOX</td>
<td>280.5 ± 24.9</td>
<td>1253.5 ± 617.0</td>
</tr>
<tr>
<td>P-MSN-DOX</td>
<td>274.3 ± 93.7</td>
<td>1310.7 ± 285.7</td>
</tr>
</tbody>
</table>

* $P < 0.05$, compared with saline.
* * $P < 0.01$, compared with saline.
* *** $P < 0.001$, compared with DOX.

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Fig. 5. Body weight changes in subcutaneous S-180 tumor-bearing mice ($n = 8$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Histopathological analysis of heart sections stained with H&E. Saline (A); DOX (B); MSN-DOX (C); P-MSN-DOX (D). Images were obtained under a Leica microscope (20× objective). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
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References


