Liposome-Coated Arsenic–Manganese Complex for Magnetic Resonance Imaging-Guided Synergistic Therapy Against Carcinoma

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Purpose: A liposome-coated arsenic–manganese complex, denoted as LP@MnAsₓ, was constructed for the targeted delivery of arsenic trioxide (ATO) against carcinoma.

Methods: Arsenite, the prodrug of ATO, was encapsulated within a liposome via electrostatic interaction with the manganese ions. The as-prepared material was characterized with dynamic light scattering and transmission electron microscopy. The entrapment efficiency and drug loading of arsenic in the carrier were measured using inductively coupled plasma spectrometry. The in vitro release of arsenic was evaluated by using the dialysis bag method. Furthermore, the Fenton-like activity and in vitro cytodynamics research of LP@MnAsₓ were monitored in this work. And the cellular uptake study was used to investigate the in vitro entry mechanism. Furthermore, the cytotoxicity, cell apoptosis and cell cycle study were performed to evaluate the tumor-killing efficiency. Also, the pharmacokinetic and antitumor studies were investigated in HepG2 tumor-bearing nude mice.

Results: The as-prepared LP@MnAsₓ possessed a spherical morphology, uniformly distributed hydrodynamic diameter, and excellent drug-loading efficiency. LP@MnAsₓ displayed robust stability and sustained-release profile under physiological environments. LP@MnAsₓ could degrade with high sensitivity to the pH variation in the tumor microenvironment. As such, this could lead to a burst release profile of Mn²⁺ and arsenite to achieve a synergistic therapy of chemodynamic therapy and chemotherapy. When compared to the carrier-free arsenate, in vitro experiments revealed that LP@MnAsₓ exhibited enhanced cellular uptake and tumor-killing efficiency. LP@MnAsₓ also demonstrated significantly enhanced tumor-specific in vivo distribution of arsenic, prolonged systemic circulation lifetime, and increased accumulation at the tumor site.

Conclusion: Based on the experimental results, LP@MnAsₓ is an ideal arsenic-based nanodelivery system, whereby it can improve the non-specific distribution of NaAsO₂ in vivo. Thus, this work can expand the research and application of arsenic trioxide against solid tumors.

Keywords: arsenic–manganese complex liposome, arsenic trioxide, pH-sensitive, hepatocellular carcinoma

Introduction

Primary carcinoma of the liver (PCL) is a type of cancer in the liver cells or intrahepatic bile duct cells, PCL is ranked as the fourth leading cause of cancer-associated deaths worldwide. Among them, hepatocellular carcinoma (HCC) accounts for the vast majority with a high incidence and mortality rate.¹,² Even though various clinically adopted HCC treatment methods have shown to be
significant to ameliorate the disease, the use of chemotherapy is often associated with adverse side effects and limited therapeutic efficacy. Thus, due to the limitation of chemotherapy, it is necessary to explore alternative strategies for the treatment of HCC.

Arsenic trioxide (ATO) is an active ingredient in traditional Chinese medicine and it has been approved by the United States Food and Drug Administration (FDA) as a first-line antitumor drug against acute promyelocytic leukemia. Furthermore, ATO has also demonstrated excellent antitumor effects on various solid tumors, e.g., liver cancer, glioma, and breast cancer, by inhibiting their proliferation and inducing cell apoptosis. Clinically, ATO is administered in the form of the arsinite ion (AsO$_2^-$). Even though ATO can exhibit excellent antitumor effects, the narrow therapeutic window, the lack of specificity in the body distribution, liver toxicity, neurotoxicity, and skin and nail pigmentation have significantly hindered the mass adoption of ATO in the clinical setting. Thus, to utilize ATO as high efficacy antitumor drug, it is necessary to realize targeted ATO delivery to the tumors so that it can achieve high bioavailability and reduced side effects.

Manganese (Mn) is an essential element in the human body and it has been widely applied as a T1 contrast agent for cancer diagnosis due to its excellent biocompatibility. Moreover, Mn$^{2+}$ can form insoluble complexes with multiple anionic groups, and their subsequent biodegradation can be triggered by the acidic environment of the tumor tissue. The released Mn$^{2+}$ can then serve as a T1-weighted magnetic resonance imaging (MRI) contrast agent for real-time detection of the release and in vivo distribution of AsO$_2^-$. Also, Mn$^{2+}$ can catalyze the tumor-abundant hydrogen peroxide (H$_2$O$_2$) to generate toxic hydroxyl radicals (•OH) via Fenton-like reactions (chemodynamic therapy, CDT) to kill the cancer cells. Despite these advantages, arsenic–manganese complex exhibits limited circulation half-life and aggregation tendency, which can tremendously hinder its future application in the clinical setting.

The recent significant advances in nanotechnology have enabled more options in cancer diagnosis and treatment by leveraging the tumor microenvironmennt. Among various drug carriers, liposome, which consists of an amphiphilic lipid bilayer, is of significant importance due to its unique flexible structure and excellent biocompatibility. As such, liposomes have been used for targeted delivery of both hydrophilic and hydrophobic drugs. In particular, hybrid lipid nanoparticle with core-shell nanostructures, which consists of a functional inter-core and an outer lipid bilayer, has demonstrated numerous desirable features. Within this core-shell structure, the lipid layer can enhance the colloidal stability of the inter-core for prolonged circulation time and provide a barrier to prevent the cargo from diffusion. Thus, such a core-shell structure can achieve improved drug-loading yields and controlled release profiles, which can ultimately enhance the therapeutic efficacy of the treatment. Several liposomes have been approved by FDA to utilize as drug carriers, such as liposomal doxorubicin (Doxil). However, the way to directly load ATO into liposomes was limited by low encapsulation efficiency and rapid leakage of ATO, thereby causing systemic toxicity. Recently, metal-arsenic complex has been raised due to its high loading efficiency of ATO, but suffering from its low solubility in water. Previous attempts have shown that actively load ATO into liposome by utilizing metal ions to form a complex in the liposome was a successful strategy to improve the loading efficiency of ATO, enhance the stability of metal-arsenic complex, and prolong the in vivo circulation time in the meantime.

Herein, liposomes were used to encapsulate the arsenic–manganese complex in this work. Based on the results, the liposome-encapsulated arsenic–manganese complex was able to exhibit significantly improved stability and distribution of arsenic in the tumor. The as-prepared liposomal nanoparticle-containing arsenic–manganese complex containing (LP@MnAs$_x$) was able to demonstrate robust stability and sustained-release profile under physiological environments. LP@MnAs$_x$ was able to degrade with high sensitivity towards pH variation in the tumor microenvironment, and this led to a burst release profile of Mn$^{2+}$ and arsenite for synergistic chemodynamic therapy and chemotherapy. Thus, this study on inorganic arsenic-based antitumor drugs may provide a novel approach for the treatment of HCC.

Materials and Methods

Materials

Sodium arsenite (97%) was purchased from West Asia Chemical Industry Co., Ltd. (Shanghai, China). The standard arsenic solution and standard manganese solution were purchased from Beijing Century Audicodes Biological Technology Co., Ltd. (Beijing, China). Manganese acetate (98%) was bought from Alfa Aesar...
(Massachusetts, England). Hydrogenated soybean phospholipids (HSPC, 98%), cholesterol (Chol, 97%) and cholesterol-polyethylene glycol 2000 (Chol-PEG2000, 98%) were bought from Shanghai Dongsheng Biological Technology Co., Ltd. (Shanghai, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Solarbio Co., Ltd (Beijing, China). MTT, penicillin and streptomycin were bought from Gibco (New York, USA). PI kit and apoptosis kit were purchased from BD Biosciences (New Jersey, USA). Sodium azide (NaN₃, 1%) was bought from Amresco (Washington, USA). TUNEL Assay Kit and Ki67 Cell Proliferation Kit were bought from Laval (Quebec, Canada). Nissl dye blue skies was purchased from Shanghai biological technology Co., LTD. (Shanghai, China). LO2 and HepG2 cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Male BALB/c nude mice (6–8 weeks) and male SD rats (weighting 200 ± 15 g) were obtained from Xiamen Medical College. All animal experiments were performed under the protocols approved by the Administrative Committee of Laboratory Animals of Xiamen Medical College (Approval number: 2019008). All animal tests were approved by the Ethics Committee of Xiamen Medical College.

**Preparation of LP@MnAsₓ**

100 mg HSPC, 10 mg Chol, and 5 mg Chol-PEG2000 were dissolved in 10 mL ethanol:chloroform solution (1:1, v/v) in a round-bottom flask. The lipid film was prepared by evaporating the ethanol and chloroform, and then it was hydrated in 30 mg/mL manganese acetate solution (10 mL, pH 7.3) under sonication in a water bath using a probe-type sonicator for 15 min and 10 min, respectively. After which, the liposomes were then extruded through polycarbonate membranes, and then it was ultrafiltrated to remove the excess manganese acetate to obtain LP@Mn. LP@As was prepared using similar procedures, except that sodium arsenite solution (NaAsO₂, 2 mg/mL, 10 mL) was used instead of manganese acetate solution. To prepare LP@MnAsₓ, NaAsO₂ was firstly dissolved in deionized water, and then it was added to LP@Mn. After which, the mixture was reacted at 55°C for 30 min, and then it was poured into an ultrafiltration system. The collected concentrated solution was denoted as LP@MnAsₓ.

**Preparation of Arsenic–Manganese Complex (AMO)**

Chloroform was added to the as-obtained LP@MnAsₓ, and then it was sonicated with using a probe-type sonicator. The upper layer of water evaporated the chloroform, and then the deionized water was ultrasonically dissolved. Subsequently, it was repeatedly washed 10 times. The liquid was then ultrafiltered, centrifuged, and the upper solid was collected and freeze-dried to obtain AMO.

**Characterization**

The hydrodynamic diameter and zeta potential of liposomes were characterized via dynamic light scattering (DLS). The morphologies of the liposomes were observed under transmission electron microscopy (TEM). Inductively coupled plasma spectrometry (ICP, 6300, Thermo Electron Corporation, Waltham, MA, USA) was used to measure the entrapment efficiency (EE%) and drug loading (DL%) of the as-prepared liposomes.

**Fenton-Like Activity of LP@MnAsₓ**

TMB was used to monitor the generated ·OH due to Mn²⁺ of LP@MnAs in the presence of H₂O₂. Briefly, 800 µL LP@MnAsₓ solution of different pH values were mixed with 100 µL H₂O₂ (10 mM) and 100 µL TMB (1 mM). After 10 min, a microplate reader was used to determine the absorbance between 400 to 800 nm.

**In vitro Release Evaluation**

The drug release characteristics of four formulations were investigated by dialysis bag method. The release medium was a phosphate buffer with pH values of 7.4, 6.8, and 5.5. The in vitro release rates of LP@As, LP@MnAsₓ, AMO, and sodium arsenite aqueous solution (NaAsO₂-Sol) were investigated. The released As content was tested using ICP according to formula (1), and the release curve was obtained.

\[ Q_n = \frac{M_n}{M_1} \times 100\% \quad (1) \]

where \( Q_n \) represents the cumulative release at time \( n \), and \( M_1 \) is the content in the sample.

**Cellular Uptake Study**

HepG2 cells were seeded in 6-well plates with a density of 5×10⁵ cells/well, and it was allowed to incubate. The culture medium was replaced with NaAsO₂-Sol, LP@As, and LP@MnAsₓ (all containing about 5 µg/L As) after 24
h of incubation. ICP was used to determine the intracellular drug concentrations after 2, 4, 6, 8, 12, and 24 h. Each experiment was performed in triplicate.

HepG2 cells were seeded in 6-well plates with a density of 1×10^6 cells/well, and then it was incubated for 24 h. It was pretreated with 5 µg/mL sodium azide (NaN₃) without serum for 30 min, suctioned, and washed twice with PBS. The other experimental methods were similar to those used for the cytodynamics research.

**In vitro Cytotoxicity**

Firstly, approximately 8×10^3 HepG2 and LO2 cells were seeded into a 96-well plate. After 24 h of incubation, the medium was replaced with NaAsO₂-Sol, LP@As, and LP@MnAs₃ contained fresh medium with different concentrations. After 24 h of incubation, the medium was replaced with 180 µL fresh medium and 20 µL MTT (0.5 mg/mL). After another 4 h of incubation, 150 µL DMSO was added and incubated for 10 min. The absorbance was subsequently determined by measuring the 490 nm absorption using a microplate reader (Synergy TM2, BIOTEK, Winooski, VT, USA). The cell viability was calculated according to equation (2).

\[
\text{Cell viability} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{contrast}} - A_{\text{blank}}} \tag{2}
\]

**Intracellular Reactive Oxygen Species (ROS) Detection**

The cell-permeable dye DCFH-DA was used to detect ROS production, whereby the ROS could oxidize the non-fluorescent dye to green fluorescent of DCF. In a typical setup, 2×10^5 HepG2 cells were seeded and incubated overnight. Subsequently, it was treated with LP@MnAs₃ for 6 h. Then, the culture medium was then replaced with DCFH-DA (10 µM), and then incubated at 37°C for 30 min. Finally, the sample was observed under a confocal laser scanning microscope (CLSM, Olympus, Tokyo, Japan). And the quantitative analysis of DCF with different treatments was assessed via flow cytometry (Guava EasyCyte, Merck Millipore, Darmstadt, Germany).

**Cell Apoptosis Study**

HepG2 cells were seeded with a density of 5×10^5 cells/well. After 24 h of incubation, each plate was treated with the serum-free medium of NaAsO₂-Sol, LP@As, and LP@MnAs₃. Then, the cells were collected with Annexin V binding buffer for the Annexin V-FITC/PI staining and they were assessed via flow cytometry.

**Cell Cycle Study**

HepG2 cells were seeded with a density of 5×10^5 cells/well, and then it was cultured with medium of NaAsO₂-Sol, LP@As and LP@MnAs₃ for 24 h. After which, the cells were collected and fixed with 70% ethanol for 12 h. Subsequently, it was stained with PI solution and assessed via flow cytometry.

**Pharmacokinetic Study**

Sprague Dawley (SD) rats were randomly divided into three groups (n = 5), and they were intravenously injected with NaAsO₂-Sol, LP@As, and LP@MnAs₃ (As: 0.6 mg/kg). At predetermined time intervals, blood samples were obtained from the orbital vein of the SD rats. The As content in the plasma was tested using inductively coupled plasma mass spectrometer (ICP-MS), and pharmacokinetic parameters were analyzed using the PK Solver pharmacokinetic software.

**Biodistribution**

A total of 5×10^6 HepG2 cells were injected into the back of nude mice. When the tumor volume increased to 50–100 mm³, 15 tumor-bearing nude mice were randomly divided into three groups, ie, NaAsO₂-Sol group, LP@As group, and LP@MnAs₃ group (n = 5). The mice in each group were sacrificed at 24 h after a single injection of drugs. The heart, left lobe of the liver, spleen, left lung, left kidney, brain, abdominal skin, left leg bone, and tumor tissues were collected. ICP was used to determine the drug concentration. The tissue distribution map was plotted with the percentage of tissue per gram of injection volume (% ID/g) as the ordinate.

**In vitro/vivo T1-Weighted MRI Imaging Evaluation**

LP@MnAs₃ with various Mn concentrations, ie, 0, 0.05, 0.1, 0.2, 0.4, and 0.5 mM, were dispersed in buffer solutions with various pH values for 24 h. The MRI images were then obtained via 1.5-T MRI system.

For in vivo MRI imaging, 0.5 mL LP@MnAs₃ was intravenously administrated at Mn concentration of 3 mg/kg. Subsequently, the MRI images were recorded at 1, 2, 4, 8, and 12 h post-injection using the 1.5-T unit MRI system.

**Pharmacodynamics Evaluation**

Twenty tumor-bearing nude mice were randomly divided into four groups, ie, saline, NaAsO₂-Sol, LP@As, and LP@MnAs₃ groups (n = 5). Normal saline or medicine (approximately 0.6 As mg/kg) was injected intravenously...
every day. The tumor volume and body weight of nude mice were recorded every other day. The tumor volume was calculated using Formula (3). On the 15th day, the tumors were collected to weigh.

\[ V = \frac{\text{length} \times \text{width}^2}{2} \]  

(3)

**Pathological Analysis**

To further prove the ROS generation in vivo, 12 tumor-bearing nude mice were intravenously injected with saline, NaAsO₂-Sol, LP@As, and LP@MnAs\textsubscript{x}, respectively (n = 3). At 24 h post injection, the tumor tissues were collected and co-stained with DCFH-DA and DAPI. The generation of ROS within the tumor cells was observed via CLSM.

On Day 15, the nude mice were sacrificed, and the heart, liver, spleen, lung, kidney, hippocampus, and striatum tissue were collected and fixed with 10% formalin to prepare the histological sections. These tissues were then stained with H&E, and histological images were obtained using an optical microscope (AXIO SCOPe.A1, Zeiss, Jena, Germany).

The tumor tissues of nude mice from each group were subjected to TUNEL and Ki67 staining, and the tissue morphology was observed under a microscope.

The brain tissue of nude mice was fixed with 10% paraformaldehyde, stained with Nissl, and then observed under a microscope and imaged.

**Statistical Analysis**

Data were presented as mean or mean ± standard deviation (SD). Statistical significance was evaluated by SPSS 22. \( P < 0.05 \) indicated a statistical difference, while \( P < 0.01 \) was indicated as a significant difference.

**Results and Discussion**

**Characterization of LP@MnAs\textsubscript{x}**

As depicted in Figure 1, the LP@MnAs\textsubscript{x} was designed to entrap arsenic by interacting with Mn\textsuperscript{2+} to realize an MRI-guided synergistic therapy of chemodynamic therapy and chemotherapy. The LP@MnAs\textsubscript{x} was prepared via a two-step method: (1) preparation of manganese acetate-containing liposome using the filming-rehydration method and (2) the formation of the arsenic–manganese complex within the interior hydrophilic space driven by ion gradient. The interactions among the arsenic–manganese complex, including the electrostatic interaction and dipole–dipole interaction, enable the sustained release of the entrapped cargo, and the liposome improves the stability of the as-prepared nanoparticles and it can also facilitate their accumulation at the tumor site for enhanced therapeutic efficacy.\textsuperscript{16}

As shown in the TEM image (Figure 2A), LP@As, LP@Mn, and LP@MnAs\textsubscript{x} possessed spherical morphology and they were uniformly dispersed with average diameters of around 120 nm. Such a result is consistent with the results

![Figure 1](https://doi.org/10.2147/IJN.S313962)  

**Figure 1** Schematic illustration of the synthesis of LP@MnAs\textsubscript{x} and the subsequent cellular uptake and drug release.
obtained by DLS (Figure 2B). When the liposome was removed from LP@MnAsx by subjecting it to chloroform and ultrasonication, the interior core of the nanoparticle was observed by TEM, suggesting the successful formation of AMO within the liposome. According to the ICP results, the As drug-loading rate and encapsulation efficiency in the LP@MnAsx were remarkably high at 4.68 ± 0.82% and 92.96 ± 5.35%, respectively. Furthermore, LP@MnAsx showed excellent colloidal stability over 30 days with negligible changes in the hydrodynamic diameter and zeta potential (Figure 2C), further illustrating the excellent stability of LP@MnAsx.

**Fenton-Like Activity of LP@MnAsx**

To investigate the Fenton-like activity of Mn2+, TMB was selected as a signal for ·OH. As shown in Figure 2D, the absorbance of TMB in LP@Mn and LP@MnAsx reaction system increased significantly as compared to that in LP@As. Such a result indicates the generation of ·OH by the reaction between Mn2+ and H2O2. Especially, a greater extent increase of TMB was induced of LP@Mn and LP@MnAsx under pH 5.5 environment, which demonstrated that the Fenton-like reaction was more evident with the increasing acidity of PBS, displaying the pH-dependent Fenton-like reaction activity. Also, the same results could be clearly observed according to the corresponding digital images of the insert photograph in Figure 2D.

**In vitro Release Evaluation**

The release profiles of the four formulations, ie, NaAsO2-Sol, LP@As, LP@MnAsx, and AMO, were investigated under various pH values, ie, 7.4, 6.8, or 5.5, based on a dialysis method. As shown in Figure 2E-G, more than 95% of As was released from NaAsO2-Sol within 1 h under all pH conditions. Notably, after liposome-coating, there was a slow release of As without pH responsiveness, whereby approximately 80% As was released from LP@As after 24 h under

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**Figure 2** Characterization of LP@MnAsx.

**Notes:** (A) TEM images and (B) hydrodynamic diameter, PDI, and Zeta potential of LP@Mn, LP@As, LP@MnAsx, and AMO. (C) Hydrodynamic diameter and Zeta potential stability of LP@MnAsx. (D) UV-Vis spectra and the color change of aqueous TMB of LP@MnAsx at varying pH values. Cumulative releases of NaAsO2-Sol, LP@As, LP@MnAsx, and AMO at pH 7.4 (E), 6.8 (F), and 5.5 (G).
all pH conditions. However, the LP@MnAs_x demonstrated a sustained-release profile under physiological conditions (pH = 7.4) with a cumulative release of 22.69 ± 0.76% within 24 h. Such a sustained-release profile could be attributed to the synergistic effects of the interactions among the arsenic–manganese complex and the diffusional barrier provided by the liposome (Figure 2E). Upon incubation in a acidic environment, LP@MnAs_x exhibited an obvious pH-dependent drug release behavior, whereby cumulative release amounts of 33.47 ± 3.43% and 63.15 ± 1.90% of As were attained at pH of 6.8 and 5.5, respectively (Figure 2F-G). The biodegradation of arsenic–manganese complex could be triggered by the acidic environment of the tumor tissue,\(^\text{17}\) which could be attributed to the dissociation of the AMO triggered by decreased pH, and the coordination between manganese and arsenic would be weakened in the meantime.

**Cellular Uptake Study**

HepG2 cells were incubated with NaAsO\(_2\), LP@As, or LP@MnAs_x with or without NaN\(_3\) for 24 h. After which, the intracellular As concentration was determined at predetermined time intervals to investigate the internalization dynamics and pathway of various formulations. NaN\(_3\) was used as an endocytosis inhibitor to evaluate the depletion of cellular ATP.\(^\text{35}\) As shown in Figure 3A-C, NaAsO\(_2\) demonstrated a rapid fast entry into HepG2 cells, which was free from the influence of NaN\(_3\) (P > 0.05). LP@As and LP@MnAs_x showed a time-dependent cellular uptake, and obviously enhanced the cellular uptake of As (P < 0.05). While with the addition of NaN\(_3\), the cellular uptake of LP@As and LP@MnAs_x was significantly reduced that by more than a half. This result indicates the energy-consuming endocytosis-mediated internalization pathway.\(^\text{36,37}\) In conclusion, the cellular uptake of LP@As and LP@MnAs_x would be significantly inhibited by the addition of NaN\(_3\), suggesting that their endocytosis way was ATP-dependent (P < 0.05).

**In vitro Cytotoxicity**

The cytotoxicity of these formulations on HepG2 and normal liver cells (LO2) cells was assessed via MTT method. As shown in Figure 3D-E, the viabilities of HepG2 and LO2 cells decreased in a concentration-dependent manner. Based on the IC\(_{50}\) value (Figure 3F), HepG2 cells were more sensitive to each of the formulation as compared to LO2 cells. Thus, the damage to LO2 could be avoided, and the as-prepared formulations could facilitate the subsequent treatment against tumors. Among all samples, LP@MnAs_x exhibited the best tumor-inhibiting effect on HepG2 cells with an IC\(_{50}\) of 1.37 ± 0.47 μg/mL, which was remarkably lower than that of LP@As (3.15 ± 2.29 μg/mL) and NaAsO\(_2\)-Sol (3.83 ± 0.85 μg/mL), indicating the synergistic therapy of chemotherapy by As and CDT by Mn\(^{2+}\).

**Intracellular ROS Detection**

DCFH-DA probes were used to detect intracellular ROS production. As shown in Figure 3G, the green fluorescence of DCF was observed for the groups with LP@Mn and LP@MnAs_x due to the existence of Mn\(^{2+}\) when compared to the control group or LP@As. Moreover, after incubating with LP@MnAs_x, HepG2 cells exhibited morphological alteration, which further illustrated the cytotoxicity of As towards tumor cells. And the results of quantitative analysis via flow cytometry was in line with CLSM (Figure 3H).

**Cell Apoptosis and Cell Cycle Study**

The results of the HepG2 apoptotic rates after being treated with each formulation are shown in Figure 3I-J. Compared with the control group, each treatment exerted significant effects on the apoptosis rate (P < 0.01). The group treated with LP@MnAs_x exhibited the highest apoptotic rate of 42.49 ± 3.36%, which could be attributed to the combined effect of the toxic hydroxyl radicals generated by Mn\(^{2+}\) and the apoptosis-inducing effect mediated by ATO. According to the cell cycle analysis result (Figure 3K-L), the Control, NaAsO\(_2\)-Sol, LP@As, and LP@MnAs_x groups arrested 19.60 ± 1.58%, 46.43 ± 0.94%, 47.73 ± 1.31%, and 69.00 ± 0.90% of HepG2 cells at the 4N phase, respectively. Such results obtained from these groups were significantly different from that obtained from the blank group (P < 0.01). Thus, this can reasonably explain the achievement of apoptosis mainly by shortening the 2N phase and prolonging the 4N phase.

**Pharmacokinetic Study**

A plasma pharmacokinetic study was performed to investigate the pharmacokinetic behavior of various formulations. NaAsO\(_2\)-Sol, LP@As, and LP@MnAs_x were intravenously administered at the As dosage of 0.6 mg/kg, and the blood was collected at pre-determined time intervals for ICP-MS analyses. The As concentration–time curve and main parameters are shown in Figure 4A and Table 1, respectively. It could be observed that all the as-prepared formulations conformed to the two-compartment
Figure 3 The cellular uptake, cytotoxicity, CDT activity, cell apoptosis and cell cycle of LP@MnAsx.

Notes: As concentration in HepG2 cells after treatment with NaAsO₂, LP@As, or LP@MnAsₓ in the absence (A) and presence (B) of NaN₃ pre-treatment. (C) As concentration after treatment with NaAsO₂-Sol, LP@As, or LP@MnAsₓ for 24 h for the normal group and NaN₃ pre-treated groups. Cell viability of LO2 (D) and HepG2 (E) cells and IC₅₀ value of LO2 and HepG2 (F) after treated with NaAsO₂-Sol, LP@As, or LP@MnAsₓ. (G) ROS detection of HepG2 cells after incubating with LP@Mn, LP@As or LP@MnAsₓ. (H) The quantitative analysis by flow cytometry about DCF after different treatments. Cell apoptosis (I, J) and cell cycle (K, L) of HepG2 cells treated with blank medium, NaAsO₂-Sol, LP@As, or LP@MnAsₓ. Data are expressed as means ± SD (n = 3). *p < 0.05; **p < 0.01.

Abbreviations: CDT, chemodynamic therapy.
model. The NaAsO$_2$-Sol was rapidly removed from the body with a short half circulation time of 14.82 ± 2.34 h, and this was shorter than those of LP@As (16.72 ± 6.59 h) and LP@MnAs$_x$ (19.54 ± 4.54 h). When compared to the NaAsO$_2$-Sol, the area under curve (AUC) of LP@As and LP@MnAs$_x$ was increased by 1.69 and 2.06 times, and the mean residence time (MRT) increased by 23.4% and 49.0%, respectively. Such a result indicates that the liposome encapsulation could improve the plasma circulation behavior. When compared to LP@As, LP@MnAs$_x$ further improved the t$_{1/2}$ and AUC, and it decreased the MRT, which could be attributed to the formation of AMO for the sustained drug release. Thus, based on the collective results, LP@MnAs$_x$ displayed superior pharmacokinetic behavior of prolonged circulation time and decreased elimination from the body, benefiting from the liposome-coating.

**Biodistribution**

To evaluate the biodistribution of various formulations, HepG2 xenograft tumor model was constructed. NaAsO$_2$-Sol, LP@As, and LP@MnAs$_x$ were intravenously administered, and the tissues of the heart, liver, spleen, lung, kidney, brain, abdominal skin, bone, and tumor were harvested for ICP-MS examination at 24 h post-administration. As shown in Figure 4B, NaAsO$_2$-Sol was mainly distributed in the liver, spleen, lung, and skin, with only 0.54 ± 0.12%ID/g at the tumor site. After being encapsulated by liposomes, the

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**Figure 4** In vivo distribution and T1-weighted MR imaging of tumor-bearing nude mice.

**Notes:** (A) The concentration-time curve and (B) tissue distribution of As after intravenous injection of NaAsO$_2$-Sol, LP@As, or LP@MnAs$_x$ (n = 5). *P < 0.05. (C) In vitro T1-weighted MR imaging and (D) T1 relaxation rates of LP@MnAs$_x$ at various concentrations after incubating at different pH conditions. (E) In vivo T1-weighted MR imaging of tumor-bearing nude mice after being injected with LP@MnAs$_x$ intravenously. The tumor was marked by red dotted circles.
Table I Main Parameters of the SD Rats After Injected with of NaAsO₂, LP@As, or LP@MnAsₓ Intravenously (n = 5)

| Parameters       | NaAsO₂-Sol      | LP@As          | LP@MnAsₓ       |
|------------------|-----------------|----------------|----------------|
| t₁/₂α (h)        | 0.04 ± 0.01     | 0.03 ± 0.01    | 0.03 ± 0.01    |
| t₁/₂β (h)        | 14.82 ± 2.34    | 16.72 ± 6.59   | 19.54 ± 4.54   |
| CL (L/h)         | 0.35 ± 0.03     | 0.19 ± 0.05*** | 0.13 ± 0.02*** |
| AUC₀₋₂₄ (μg/h/mL)| 2.59 ± 0.25     | 4.38 ± 1.24    | 6.10 ± 1.04*** |
| V₁ (mg/kg/μg/mL)| 6.18 ± 0.72     | 4.33 ± 0.13ª   | 3.55 ± 0.16*** |
| MRT (h)          | 17.57 ± 1.65    | 21.69 ± 4.24   | 26.18 ± 6.49   |

Notes: *p < 0.05 vs NaAsO₂-Sol group, **p < 0.01 vs NaAsO₂-Sol group, ***p < 0.05 vs LP@As group.
Abbreviations: t₁/₂α, absorption half-life; t₁/₂β, elimination half-life; CL, clearance rate; AUC, area under the curve; V₁, volume of distribution; MRT, mean residence time.

Liver, spleen, lung, and skin showed significantly reduced arsenic distribution in the tumor, which indicated an improved arsenic distribution in various tissues. The amount LP@MnAsₓ distribution could be arranged in the following decreasing order: liver > spleen > tumor > lung > heart > skin > bone > brain. Meanwhile, when compared to other groups, the LP@MnAsₓ exhibited the highest accumulation at the tumor site and a decreased distribution in other major organs, which indicated superior tumor targeting ability and improved biodistribution. And the distribution in liver, spleen and lung was due to the reticuloendothelial system (RES) effect.

In vitro/vivo T1-Weighted MRI Imaging Evaluation

Due to the dissociation of LP@MnAsₓ triggered by an acidic environment, the released Mn²⁺ can serve as a contrast agent for T1-weighted MRI contrast agent. With the increased concentration, the T1-weighted MR images of LP@MnAsₓ at pH 5.0 revealed a greater contrasting effect as compared to those at pH 7.4 (Figure 4C). Furthermore, the r₁ relaxivity of LP@MnAsₓ significantly increased from 2.55 mM⁻¹s⁻¹ due to the accelerated release of Mn²⁺ (Figure 4D). Subsequently, T1-weighted MR imaging was monitored at selected times after being intravenously injected with LP@MnAsₓ. An obvious high contrasting effect could be observed at the tumor site, whereby it continuously increased and peaked at 2 h post administration, and then gradually decreased. This result suggests that LP@MnAsₓ was able to respond sensitively to the tumor microenvironment (Figure 4E).

Pharmacodynamics Evaluation

To assess the in vivo therapeutic efficacy, 20 HepG2 tumor-bearing mice were randomly divided into four groups, ie, treated with saline, NaAsO₂-Sol, LP@As, and LP@MnAsₓ. During the 14 days of treatment, tumor volume and body weight of mice were recorded each day. Based on the results, the average tumor volumes of the groups treated with saline, NaAsO₂-Sol, LP@As, and LP@MnAsₓ were 1192.34 ± 482.21 mm³, 648.53 ± 198.62 mm³, 499.67 ± 116.72 mm³, and 316.20 ± 110.45 mm³, respectively, showing that NaAsO₂-Sol, LP@As and LP@MnAsₓ have a difference degrees in the inhibition of the growth of tumor (Figure 5A-C). Compared with the group of NaAsO₂-Sol, LP@As and LP@MnAsₓ could significantly inhibit the tumor, which might be owing to the tumor-targeting ability of liposome and the synergistic therapy of chemotherapy and CDT (P < 0.01). In addition, the body weight of the mice treated with NaAsO₂-Sol group was significantly lower than that of the saline group, indicating systemic cytotoxicity caused by the non-specific distribution of NaAsO₂. In contrast, the body weight of the mice treated with LP@As and LP@MnAsₓ kept increased continuously, which suggests the attenuation of adverse side effects, showing the good biosafety of LP@As and LP@MnAsₓ (Figure 5D).

Pathological Analysis

To further display the in vivo ROS generation, the immuno-histochemical analysis of tumor tissues were investigated by using DCFH-DA as the ROS probe. As shown in Figure 5E, the obvious green fluorescence could be seen in the tumor tissues after LP@MnAsₓ treatment, suggesting a large amount of ROS generation in vivo.

Ki67 and TUNEL staining were used to perform the histological evaluation and statistical analysis so as to determine the tumor cell apoptosis caused by treatment, whereby saline group was used as the control (Figure 5F-G). When compared to other groups, the group treated with LP@MnAsₓ resulted in the largest area of apoptotic cells, and therefore it achieved the best proliferation inhibiting...
effect. When considering the side effects of nerve cytotoxicity caused by arsenic treatment, Nissl staining was used to observe the damage inflicted onto the nerve cells. Based on the results, the number of Nissl bodies in the hippocampus of the LP@As and LP@MnAsₓ groups were significantly less than that in NaAsO₂-Sol groups (P < 0.05). Furthermore, morphological alterations could be detected, which were indicative of severe nerve cytotoxicity.
Meanwhile, LP@MnAs$_x$ showed attenuated damage to the nerve cells with a comparable number of Nissl bodies ($P < 0.01$), and this result is consistent with the biodistribution results. According to the H&E staining result, administrating of NaAsO$_2$-Sol, LP@As, and LP@MnAs$_x$ to the mice did not lead to obvious damage to the major organs relative to the control group (Figure 6).

**Conclusion**

In summary, the LP@MnAs$_x$ was successfully developed for the carcinoma-targeted delivery of the arsenic–manganese complex via magnetic resonance imaging-guided synergistic therapy of chemodynamic therapy and chemotherapy. The enhanced therapeutic efficacy exhibited by the LP@MnAs$_x$ was realized by the pH-sensitive drug release, prolonged circulation time, and improved tumor accumulation. Hence, based on the collective results, the use of the LP@MnAs$_x$ is a promising strategy for carcinoma treatment, overcoming the shortage of ATO, and propelling the clinical translation process of arsenic trioxide based formulation.

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Disclosure
The authors report no conflicts of interest in this work.

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3787
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