1 **RGD conjugated liposome-hollow silica hybrid nanovehicles**
2 **for targeted and controlled delivery of arsenic trioxide**
3 **against hepatic carcinoma**

4 Weidong Fei¹, Yan Zhang¹, Shunping Han¹, Jiaoyang Tao¹, Hongyue Zheng², Yinghui Wei¹,
5 Jiazhen Zhu¹, Fanzhu Li^{1*} and Xuanshen Wang^{3*}

6

7 ¹College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou,
8 310053, China.

9 ²Libraries of Zhejiang Chinese Medical University, Zhejiang Chinese Medical University,
10 Hangzhou, 310053, China.

11 ³Department of Pharmacy, The Second Hospital of Dalian Medical University, Dalian,
12 116027, China.

13

14 *Corresponding Author:

15 Fanzhu Li, College of Pharmaceutical Science, Zhejiang Chinese Medical University,
16 Hangzhou, 310053, China. Tel.: +86-0571-61768130, Fax: +86-0571-86613607, E-mail:
17 lifanzhu@zcmu.edu.cn.

18 Xuanshen Wang, Department of Pharmacy, The Second Hospital of Dalian Medical
19 University, Dalian, 116027, China. Tel.: +86-0571-61768130, E-mail: 542695603@qq.com.

20

21

22

Abstract

The aim of our study was to construct an Arg-Gly-Asp (RGD)-conjugated liposome-hollow silica hybrid nanovehicle for targeted delivery and controlled release of arsenic trioxide (ATO), whose anti-solid tumor effect was hampered by poor pharmacokinetics and dose-limited toxicity. Hydrophobic interactions were used to attach intact lipid membrane to the surface of chlorodimethyloctadecylsilane-modified hollow mesoporous silica nanoparticles. The prepared nanovehicles (RGD-LP-CHMSN) were characterized for uniform structure (silica core of ~140 nm in diameter and liposomal shell of ~6 nm), comparable drug loading efficiency (6.76%), desirable stability and strengthened controlled release. *In vitro*, RGD-LP-CHMSN showed good biocompatibility and low toxicity on HepG2, MCF-7 and LO2 cells. The targeted delivery of ATO by nanocarriers (RGD-LP-CHMSN-ATO) was demonstrated by an enhanced cellular uptake and a reduced half maximal inhibitory concentration (IC₅₀) value. In pharmacokinetic studies, the RGD-LP-CHMSN-ATO group, compared to the free ATO group, prolonged the half time (t_{1/2β}) by 1.7 times and increased the area under curve (AUC) by 2.4 times. In addition, in a H22 tumor-xenograft mouse model, nanovehicles improved the targeting efficiency and anticancer potential of ATO. In conclusion, the strategy of constructing a nanocarrier with targeted delivery and controlled release characteristics is prospective to enhance the antitumor effect of ATO.

Keywords: arsenic trioxide, hollow mesoporous silica nanoparticles, liposomal shell, Arg-Gly-Asp, controlled release, hepatic carcinoma

1. Introduction

For decades, chemotherapy has been a main and effective approach to treat cancer. Most therapeutic agents, including doxorubicin and paclitaxel, kill primary tumor cells effectively, however their effect on metastatic cancer cells or cells with metastatic potential is limited (Minotti et al., 2004; Rowinsky and Donehower, 1995). Since 1970, an active ingredient of traditional Chinese medicine, arsenic trioxide (ATO), has been used to treat cancer (Sweeney et al., 2010). ATO has been approved by FDA in 2000 for treating acute promyelocytic leukemia (APL) (Wang and Chen, 2008). Recent studies have indicated that ATO exerted high toxicity against various cancers by inducing apoptosis (Lu et al., 2007; Miller et al., 2002) in liver, brain, breast, prostate, renal, bladder, and ovarian cancer cells (Dilda and Hogg, 2007; Uslu et al., 2000). In addition, ATO promoted differentiation, reduced invasion and inhibited the migration of surviving cancer cells (Miller et al., 2002). This unique ability allows ATO to inhibit the malignant and metastatic properties of surviving cancer cells, thereby indicating a higher therapeutic effect on solid tumors. Despite its promising pharmacological characteristics, the clinical application of ATO has been severely restricted due to its rapid renal clearance (Swindell et al., 2013), high serum protein binding rate (Shooshtary et al., 2015), low drug concentration around tumor sites and dose-limited adverse reactions, such as liver dysfunction and skin reactions (Song et al., 2014). To address the drawbacks of ATO, various drug delivery systems (DDS) have been designed to encapsulate inorganic ATO into liposomes (Chen et al., 2006; Lee et al., 2011) or polymeric micelles (Zhang et al., 2014a). However, these

nanocarriers of organic nature were incompatible for chemical compositions and resulted in non-stability during storage and unexpected drug release during circulation. Therefore, fabricating a suitable carrier, which efficiently loads the drug and improves its pharmacokinetic (PK) features to enhance antitumor efficacy has drawn great attention.

Regarding the fundamentally inorganic nature of ATO, hollow mesoporous silica nanoparticles (HMSN), a novel inorganic nanomaterial with high stability, excellent biocompatibility, controlled release property, and easy surface modifications (Fang et al., 2012; Fang et al., 2013) are considered promising nanocarriers for ATO. More importantly, the hollow interior of HMSN allows them to efficiently load and encapsulate drugs, and thereby protect agents from enzymatic and/or environmental degradation. However, among the challenges of mesoporous silica carriers were burst release actions (Bhattacharyya et al., 2012; Jalvandi et al., 2015), chronic toxicity, low targeting efficacy and short-term circulation causing by aggregation in the physiological environment (Yang et al., 2012). Lipid-coated DDS decreased self-aggregation, minimized nonspecific binding with proteins (Ashley et al., 2011; Teng et al., 2013), suppressed burst release actions and improved the biocompatibility of nanocarriers. Surface attachment with chlorodimethyloctadecylsilane (Fig. 1A) was to provide the Van-Der-Waals Forces and hydrophobic interactions between hydrocarbon chains on external of CHMSN and liposomal shell, which resulted in increased stability of the core-shell structure in aqueous solutions and within the blood circulation. In addition, one of the key merits of the phospholipid membrane is

its modifiability. Therefore, the phospholipid layer on CHMSN may readily be sensitized with tumor-selective ligands (such as transferrin, RGD or folate), which could enhance accumulation of drugs in proximity of the target (Li et al., 2015; Patil et al., 2016; Wei et al., 2016). To our knowledge, lipid-based hybrid systems combined with tumor-targeting features have not been studied well.

In this study, we reported one novel core-shell hybrid nanocarrier consisting of a hydrocarbon chain modified hollow mesoporous silica core and an RGD-modified liposomal shell for targeted delivery and controlled release of ATO in hepatic carcinoma therapy (Fig. 1). The structure and morphology of nanoparticles were characterized. In addition, the *in vitro* drug release behavior, cellular uptake and cytotoxicity were evaluated. Furthermore, the PK characteristics, distribution into hepatic carcinoma and the therapeutic efficacy of RGD-LP-CHMSN-ATO were investigated.

[Figure 1]

2. Materials and methods

2.1 Materials

Arsenic trioxide (ATO, 90%) was purchased from Alfa Aesar (Shanghai, China). Arsenic standard solution (1 mg/mL) was purchased from Beijing Century Audiocodes Biological Technology Co., Ltd. (Beijing, China). Tetraethyl orthosilicate (TEOS), hexadecyltrimethylammonium bromide (CTAB), ammonia propyl triethoxy silane (APTES), fluorescein isothiocyanate (FITC), Hoechst 33342 and dimethyl

111 sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, USA).
112 Chlorodimethyloctadecylsilane was obtained from Tokyo Chemical Industry Co., Ltd.
113 (Tokyo, Japan). RGD (Arg-Gly-Asp) (purity > 97%) was synthesized by China
114 Peptide Co., Ltd. (Shanghai, China). Distearoyl phosphoethanolamine-PEG
115 (DSPE-PEG), distearoyl phosphatidylcholine (DSPC), distearoyl
116 phosphoethanolamine-PEG-NHS (DSPE-PEG-NHS) and cholesterol were purchased
117 from A.V.T Pharmaceutical Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle
118 medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS),
119 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin G
120 sodium and streptomycin sulfate were obtained from Gibco BRL (Gaithersburg, USA).
121 Human integrin $\alpha_v\beta_3$ PE-conjugated antibody was purchased from R&D Systems
122 China. The propidium iodide (PI) kit was purchased from BD Biosciences (San Diego,
123 USA). Other chemicals and solvents were all of analytical grade, and purified water
124 was produced by a Millipore water purification system.

125 *2.2 Cell culture and animals*

126 Human hepatic carcinoma cells (HepG2), human breast cancer cells (MCF-7)
127 and human liver cells (LO2) were provided by Department of Pharmacology,
128 Zhejiang Chinese Medical University (Hangzhou, China). HepG2 cells were cultured
129 in DMEM supplemented with (10% volume ratio) FBS, 100 IU/mL penicillin G
130 sodium and 100 μ g/mL streptomycin sulfate in 5% CO₂ at 37 °C in a humidified
131 incubator. MCF-7 and LO2 cells were cultured in RPMI 1640 medium supplemented
132 with (15% volume ratio) FBS, 100 IU/mL penicillin G sodium and 100 μ g/mL

streptomycin sulfate in 5% CO₂ at 37 °C in a humidified incubator. Cells for all experiments were in the logarithmic phase of growth.

Sprague-Dawley (SD) rats (220±20 g, both male and female) and ICR mice (20±2 g, male) were supplied by the Laboratory Animal Center of Zhejiang Chinese Medical University. All experiments were performed in accordance with the guidelines for the care and use of animals established by Zhejiang Chinese Medical University.

2.3 Preparation of NH₂-HMSN

Amino-modified hollow mesoporous silica nanoparticles (NH₂-HMSN) were prepared by a “CSASE” strategy as demonstrated by Zheng et al (Fang et al., 2012; Fang et al., 2013) with minor modifications. The entire process consisted of four steps. Firstly, 78 mL of ethanol, 3.1 mL of ammonium aqueous solution (28%) and 10 mL of ultra-pure water were mixed for 30 min, then 6 mL of TEOS was added under stirring for 1 h at 25°C to prepare solid silica nanospheres (sSiO₂). Secondly, 100 mg of sSiO₂ was re-dispersed in 60 mL of an ethanol/water mixture (1:2, v/v), then 15 mL of the mixture (1:2, v/v) containing 150 mg CTAB was added to the nanospheres. After stirring for 30 min, 125 µL of TEOS, 70 µL of APTES and 600 µL of ammonium aqueous solution (28%) were added to the mixture, and incubated for 12 h at 25°C. The products (sSiO₂@CTAB/SiO₂ spheres) were collected by centrifugation and re-dispersed in 20 mL of water. Thirdly, to obtain hollow silica spheres, 450 mg Na₂CO₃ was added to the above mixture and incubated for 12 h at 50°C. Finally, the products were dispersed in 75 mL of NH₄NO₃ ethanol solution (10 mg/mL) for

removing surfactant template. NH₂-HMSN were collected by centrifugation, washed and freeze-dried.

2.4 Preparation of DSPE-PEG-RGD

For the synthesis of RGD-conjugated phospholipid (DSPE-PEG-RGD), DSPE-PEG-NHS (MW: ~2750) were incubated with RGD (MW: 346) (molar ratio 3:1) in DMF for 24 h under a nitrogen flow at 25°C. To remove free RGD and DSPE-PEG-NHS, the products were purified by dialysis (cut off 3.5 kDa MW) for 72 h. Finally, synthetic products were freeze-dried and stored at -80 °C.

2.5 Preparation of CHMSN-ATO, LP-CHMSN-ATO and RGR-LP-CHMSN-ATO

Chlorodimethyloctadecylsilane (0.5 mL) was added to 50 mL of CHCl₃ and 100 mg of NH₂-HMSN and stirred for 12 h. The resulting nanocarriers (CHMSN) were washed with hexane and dried in air. Forty mg of CHMSN was dispersed in 10 mL of ATO solution (ATO-Sol, 0.5 mg/mL) and stirred at 50°C for 6 h. The ATO-loaded CHMSN (CHMSN-ATO) were collected by centrifugation. RGD-conjugated lipid-coated silica nanodrugs (RGD-LP-CHMSN-ATO) were prepared by a combined self-assembly technique and modified thin film hydration method as previously described (Ren et al., 2012; Zhang et al., 2014b). In brief, DSPC, DSPE-PEG, DSPE-PEG-RGD and cholesterol (65:8:2:25, mass ratio) were dissolved in chloroform by using ultrasound at 40°C. The CHMSN-ATO (with a weight ratio of 20% to the lipids) was dispersed by vortexing, followed by evaporating under negative pressure to form a lipid layer surrounding the particles using a rotary evaporator (RE-52C, Shanghai Yaguang Instrument Company, China) at 40°C. **In order to isolate**

RGD-LP-CHMSN-ATO, the hydration dispersion was centrifuged (10,000 rpm, 5 min), the supernatant was discarded and the pellet was re-suspended in 10 ml of PBS. This procedure was repeated five times to remove free lipids (Han et al., 2016; Meng et al., 2015). Lipid-coated silica nanodrugs (LP-CHMSN-ATO) were prepared by a similar approach as described above without the addition of DSPE-PEG-RGD. Nanodrugs were re-dispersed in saline or PBS prior to use. The encapsulation efficiency (EE%) and drug loading efficiency (DL%) were determined by inductively coupled plasma emission spectrum (ICP, 6300, Thermo Electron Corporation, USA) as described previously (Xiao et al., 2016). In addition, DL% was investigated by Thermogravimetric analysis (TGA, Pyris Diamond, Perkin-Elmer Corporation, USA).

2.6 Preparation of FITC labeled nanoparticles

CHMSN (5 mg) were dispersed in 1.0 mL of DMF and incubated in dark with 0.2 mg of FITC (25°C) for 12 h under stirring. The FITC-labeled CHMSN (CHMSN-FITC) were collected by centrifugation and washed with five times with ethanol. The lipid-coating process as mentioned above was repeated to prepare RGD-LP-CHMSN-FITC and LP-CHMSN-FITC.

2.7 Characterization

Transmission electron microscopy (TEM) images of sSiO₂, CHMSN, LP-CHMSN and RGD-LP-CHMSN were obtained by a transmission electron microscope (H-7650, Hitachi, Japan). The surface analysis was performed by nitrogen sorption isotherms at 77 K with micromeritics ASAP-2020 sorptometer. Surface area and pore size distribution were calculated by the Brunauer Emmett Teller (BET)

method and the Barrett Joyner Halenda (BJH) method respectively. Powder X-ray patterns (XRD) were recorded on a Bruker D4 X-ray diffractometer with Ni-filtered Cu K α radiation (40 kV, 40 mA). The size distribution, polydispersity index (PDI) and ζ -potentials of the nanoparticles were measured by Malvern ZEN 3690. Thermogravimetric analysis was performed at a heating rate of 20°C/min in a nitrogen flow from 100 to 800°C. Characterization of DSPE-PEG-RGD was analyzed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS, GCT-Premier, Waters, USA). The infrared features of NH₂-HMSN and CHMSN were measured by FT-IR spectrophotometer (Nicolet 6700, Thermo Electron Corporation, USA). Stability experiments were carried out by dispersing 3 mg/mL FITC-labeled nanoparticles (CHMSN-FITC, LP-CHMSN-FITC and RGD-LP-CHMSN-FITC) in PBS. The particle size was recorded at 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24 and 48 h. Both at the start and at termination of the study, the aggregation of fluorescently-labeled nanoparticles was determined under UV light (365 nm).

2.8 *In vitro* release study

The *in vitro* release of ATO from formulations was performed in PBS (pH 7.4). ATO formulations (ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO or RGD-LP-CHMSN-ATO) were put into a dialysis bag (cut off 7 kDa MW) and immersed into 500 mL of PBS at 37°C. At predetermined sampling time, samples were collected from the media and analyzed by ICP. The removed amount of fluid was instantly replaced with an equal amount of fresh PBS. All experiments were

performed in triplicate.

2.9 Cellular uptake and intracellular disposition

HepG2, MCF-7 and LO2 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight at 37°C . Subsequently, cells were treated with culture medium or nanoparticle-containing culture medium (CHMSN-FITC, LP-CHMSN-FITC or RGD-LP-CHMSN-FITC) at FITC concentrations of $3 \mu\text{mol/L}$. After 4 h, cells were washed three times with PBS, then trypsinized and collected by centrifugation. The fluorescence intensity was determined by Guava Easy Cytometer (Guava Technologies, Millipore, Hayward, CA, USA). To identify intracellular localization of FITC-labeled nanocarriers in cells, confocal laser scanning microscopy (CLSM, Zeiss LSM510, Carl Zeiss, Germany) was applied. HepG2, MCF-7 or LO2 cells were cultured in chambered coverslips at a density of 1×10^5 cells/well and cultured overnight at 37°C . Next, cells were treated for 4 h with nanoparticle-containing culture medium (CHMSN-FITC, LP-CHMSN-FITC or RGD-LP-CHMSN-FITC) at a FITC concentration of $3 \mu\text{mol/L}$. Cells were washed three times with PBS, stained with Hoechst 33342 and fixed with 4% (v/v) paraformaldehyde.

To quantitatively analyze cellular uptake of ATO, HepG2 cells were seeded into 6-well plates at a density of 4×10^5 cells/well for 12 h. Then, cells were treated with 2 mL of culture medium or drug-containing culture medium (ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO or RGD-LP-CHMSN-ATO) ($1 \mu\text{g}$ ATO per mL) for 2, 6, 12, or 24 h. Subsequently, 2×10^6 cells were collected for further analysis. Cells were washed

three times with PBS, extracted by addition of 3 mL of nitric acid combined with 200 μ L of H₂O₂ and transferred to Teflon tubes to be digested in a Microwave Digestion System (Co.Bergho, Germany) followed by the LEAF program. The drug concentration was analyzed by inductively coupled plasma mass spectrometry (ICP-MS, 7500ce, Agilent, USA).

2.10 In vitro cytotoxicity

An MTT assay was used to evaluate the cytotoxicity of drug-free carriers (NH₂-HMSN, CHMSN, LP-CHMSN or RGD-LP-CHMSN) and ATO formulations (1 μ g ATO per mL) on HepG2, MCF-7 and LO2 cells. Briefly, cells were seeded in 96-well plates at a density of 8×10^3 cells/well, and cultured overnight at 37°C. Cells were then treated with culture medium containing drug-free carriers or ATO formulations for 48 h. The cells were incubated with culture medium mixed with MTT (5 mg/mL) for 4 h at 37°C. After discarding the supernatant, precipitates were dissolved in 150 μ L DMSO, and the absorbance of the resulting solution was measured at a wavelength of 570 nm using a microplate reader (Synergy TM2, BIO-TEK Instruments Inc. USA).

2.11 Mechanism of cellular uptake

To verify the internalization mechanism of the nanodrugs, HepG2 cells were pre-incubated for 30 min with medium containing sodium azide (5 μ g/mL), anti- $\alpha_v\beta_3$ (1 mmol/L) or iRGD (1 mmol/L). Next, the medium was discarded. Cells were washed three times with PBS and incubated for 6 h in culture medium containing drug formulations (1 μ g ATO per mL). Then, the process as mentioned in section 2.9

were repeated to detect the drug concentration in HepG2 cells. A blank control was included by adding culture medium alone.

2.12 Cell cycle analysis

HepG2 cells were treated for 48 h with culture medium or drug-containing culture medium at an ATO concentration of 1 µg/mL. For cell cycle analysis, cells were collected after gentle centrifugation at 1000 rpm for 5 min, then fixed with 70% ethanol for 8 h at 4°C. Residual ethanol was removed and the cells were incubated at 37°C for 30 min with 1 mg/mL of DNase-free RNase A. Subsequently, 0.1mL of 0.1% Triton X-100 containing 0.02 mg/mL of PI was added and stained cells were analyzed by Guava Easy Cytometer (Guava Technologies, Millipore, Hayward, CA, USA). All the experiments were performed in triplicate.

2.13 Pharmacokinetic study

SD rats were used for PK studies of the ATO formulations. In brief, SD rats were randomly divided into four groups (n=5), and ATO formulations were delivered by tail vein injection at a dose of 1 mg ATO-equivalent/kg body weight. Blood samples (200 µL) were collected via jugular vein cannula and placed in heparinized tubes by Automated Blood Sampler (INSTECH ABS212, Instech Laboratories, Inc. USA) at the desired times (0.1, 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h). Then, the samples were centrifuged and stored at -80°C for further analysis. In this study, we used ICP-MS to determine the serum concentration of ATO. For ICP-MS determination, 100 µL of serum sample was treated with 40 µL perchloric acid, vortexed for 3 min, and centrifuged for protein precipitation. The supernatant was

transferred and diluted with 2% diluted nitric acid to 5 mL. Working conditions of ICP-MS were as described in our previous study (Xiao et al., 2016).

2.14 *In vivo* biodistribution

In vivo biodistribution analysis was conducted on H22 tumor-xenograft mice, which were established by subcutaneously injecting H22 cells (mouse liver cancer cell line, 1×10^7) into ICR mice. When the tumors reached a size of $\sim 1000 \text{ mm}^3$, the ATO formulations were administered by tail vein injection at a dose of 1 mg ATO-equivalent/kg body weight. The isolated organs of the mice in each group ($n=5$) were excised 24 h after injection and lysed in 2 mL freshly mixed nitric acid and hydrogen peroxide. The samples were heated at 120°C in an oil bath until all liquid was completely evaporated. Remaining residues were re-dissolved in 2% nitric acid and assessed by ICP-MS.

2.15 *In vivo* anti-tumor study

H22 tumor-xenograft mice were randomly divided into five groups ($n=16$). When the tumors reached a diameter of 3-5 mm, ATO formulations at a dose of 1 mg ATO-equivalent/kg body weight (or saline) were intravenously administrated on a daily basis. Body weight and tumor size were recorded every day. Six mice of each group were killed on day 15 and tumor tissues were collected and weighted. The tumor volumes and tumor growth inhibition rate (IR%) were calculated by equation (1) and equation (2) (see below) (Xiao et al., 2016). Tumors were fixed in formalin, embedded in paraffin, and sectioned. To evaluate the apoptosis of tumor cells, sections were stained with hematoxylin and eosin (H&E). The survival of the

remaining mice (n=10) was recorded and analyzed by Kaplan-Meier analysis.

$$V=(a^2 \times b)/2 \quad (1)$$

$$IR\%=(1-W_t/W_c) \times 100\% \quad (2)$$

Regarding the formulas, a, b, W_t and W_c refer to the maximum diameter of the tumor, minimum diameter of the tumor, the mean tumor weight of treated groups and the mean tumor weight of the control group respectively.

2.16 Statistical analysis

All data is presented as the mean $\pm$ SD. Statistical significance was determined by one-way analysis of variance by SPSS software (version 17.0, IBM Inc, Chicago, IL, USA). Survival data is presented using Kaplan-Meier survival curves and was analyzed by the log-rank test. $P < 0.05$ was considered statistically significant, and extreme significance was set at $P < 0.01$.

3 Results and discussion

3.1 Synthesis and characterization of CHMSN, LP-CHMSN and RGD-LP-CHMSN

The sSiO₂ (diameter: 100.67 $\pm$ 1.14 nm and PDI: 0.075 $\pm$ 0.003) were prepared as self-templates. Synthesizing monodispersed NH₂-HMSN with a hydrodynamic diameter of 142.13 $\pm$ 1.54 nm was established by a modified “CSASE” strategy in which all sSiO₂ was etched by Na₂CO₃ to form a hollow structure. Different from other approaches using Fe₃O₄ or phenolic resin particles as a template (Geng et al., 2016; Zhao et al., 2014), the modified HMSN constructing strategy would leave few residual elements, which may cause side reactions. The ζ -potential reversibly

switched from -28.23 ± 0.74 mV for sSiO_2 to 35.00 ± 0.75 mV for $\text{NH}_2\text{-HMSN}$ (Fig. 2E) and the presence of adsorption peak at 1508.09 cm^{-1} in FT-IR spectra resulted from the bending vibration of N-H (Fig. 3A) confirmed the successful modification of amino groups on internal/external surfaces of HMSN. The successful modification of chlorodimethyloctadecylsilane was shown by a C-H stretching absorbance at $2800\text{-}3000\text{ cm}^{-1}$ from $-\text{CH}_3$ in FT-IR spectra of CHMSN (Fig. 3B) and additional weight loss of CHMSN compared to that of $\text{NH}_2\text{-HMSN}$ in TGA study (Fig. 3I). The hollow structure of CHMSN could be identified by a strong contrast between the edge (dark, ~ 30 nm in thickness) and the center (light, ~ 80 nm in diameter) (Fig. 2B). The high ratio of hollow interior/whole diameter (about 4:7) indicated that the CHMSN may be a promising nanovehicle for drug loading and delivery. To better understand the structure of the hollow mesoporous nanocarriers, N_2 adsorption-desorption and XRD analysis were performed. As presented in Fig. 3F, the N_2 adsorption-desorption patterns of $\text{NH}_2\text{-HMSN}$ and CHMSN exhibited a characteristic type IV according to the IUPAC classification with a narrow pore size of 3.15 nm and 2.90 nm (Fig. 3G). The specific surface area and cumulative pore volume of $\text{NH}_2\text{-HMSN}$ and CHMSN were $1106.22\text{ m}^2/\text{g}$ and $1.04\text{ cm}^3/\text{g}$, and $1199.17\text{ m}^2/\text{g}$ and $1.18\text{ cm}^3/\text{g}$, respectively. These values were larger than those of MCM-41 type nanosilica that were reported previously (Xiao et al., 2016). The parallel isotherms (H1 hysteresis loop) in adsorption-desorption patterns and the strong diffraction peak (around 2.30°) between $\text{NH}_2\text{-HMSN}$ and CHMSN further indicated the ordered arrangement of the mesoporous structure (Fig. 3F and Fig. 3H). The hollow structure and ultra-small

pores of HMSN featured this vehicle with a high drug loading capacity and controlled release potential. In addition, all parameters illustrated that the hollow interior and mesoporous structure of HMSN would not be affected by the modification of chlorodimethyloctadecylsilane.

[Figure 2]

To achieve targeted delivery, the RGD peptide was modified on the lipid bilayer. The peak at the m/z of ~ 3083.0 indicated that RGD had been connected to the end of the PEGylated phospholipids (Fig. 3C). The non-specific lipid bilayer was made up of DSPC, DSPE-PEG and cholesterol, whereas the tumor targeting structure was constructed in the lipid bilayer by anchoring DSPE-PEG-RGD. To our knowledge, the principles of coating the phospholipid bilayer on an inorganic/organic carrier can be divided into three types: 1) specific lipid adsorption on the silica surfaces through electrostatic forces (Galanzha et al., 2009; Liu et al., 2009b), 2) Van-Der-Waals Forces and hydrophobic interactions (Liu et al., 2009b; Pereira et al., 2008), and 3) specific interaction between ions of inorganic cores and head groups of lipid (Li et al., 2012). In our studies, chlorodimethyloctadecylsilane was modified on the surfaces of HMSN by silicon-oxygen bond to serve as a support skeleton for supporting the lipid capsule through the Van-Der-Waals Forces and hydrophobic interactions. The coating principle was based on the fact that phospholipids and cholesterol dissolved in chloroform prevail as monomers, then they self-assemble into surface-supported bilayers during hydration (Cauda et al., 2010). The successful assembly of lipid bilayers on CHMSN was demonstrated by the TEM investigation of LP-CHMSN and

RGD-LP-CHMSN with an intact liposomal shell of ~6 nm and the size increased to ~152 nm (Fig. 2C-D and Fig. 2F). It was found that, due to the enhanced permeability and retention (EPR) effect, nanoparticles with a size ranging from 100 nm to 200 nm tended to accumulate more easily in tumor tissues compared to normal tissues (Matsumura and Maeda, 1986). The TGA result (Fig. 3I) showed that weight loss of CHMSN-ATO and LP-CHMSN-ATO was 21.42% and 33.85%, respectively, indicating that the weight percentage of lipids was 12.43%. The reduced specific surface area, cumulative pore volume, and XRD peaks of LP-CHMSN and RGD-LP-CHMSN were due to the pore-blocking effect of the liposomal membrane (Fig. 3F-H).

Considering the adsorption and aggregation of mesoporous materials with high surface energy during the preparation and/or storage, increasing the particle size of nanoparticles was a typical phenomenon, which was not advantageous to drug delivery (Wang et al., 2010). A stability experiment was performed by monitoring the variation of particle size in PBS. As presented in Fig. 3D, the average diameter of the lipid-coated nanoparticles changed slightly in 48 h, whereas the HMSN-FITC and CHMSN-FITC aggregated more intensely. Moreover, a strong green fluorescence at the bottom of vials in both HMSN-FITC and CHMSN-FITC groups indicated the aggregation of nanoparticles (apatite-like precipitate), whereas the suspension of LP-CHMSN-FITC and RGD-LP-CHMSN-FITC remained homogeneous (Fig. 3E). In general, our results not only confirmed the formation of the lipid bilayers on the CHMSN but also demonstrated its contribution as a steric stabilizer, preventing the

aggregation of CHMSN in a salt-contained solution.

[Figure 3]

3.2 Drug loading and release

Drug loading was performed prior to the lipid bilayer coating process. With the presence of hollow interior, CHMSN could accommodate a loading efficiency of 7.75% ATO (TGA analysis) by Van-Der-Waals Forces and electrostatic interactions. The EE% and DL% of CHMSN-ATO as measured by ICP were $67.96 \pm 3.48\%$ and $7.83 \pm 0.37\%$, which was very similar as the TGA results. After encapsulating with the lipid bilayer, the DL% decreased to 6.86% of LP-CHMSN-ATO and 6.76% of RGD-LP-CHMSN-ATO. By monitoring the concentration of ATO in the supernatant during the coating and washing process, we found that the drug loss during these processes was negligible. The reduction in DL% of LP-CHMSN-ATO and RGD-LP-CHMSN-ATO was due to the increased weight after the coverage of lipid bilayer. Even so, the ATO loading capacity of RGD-LP-CHMSN was found to be stronger than that of organic carriers, such as polymeric micelles ($\sim 2.5\%$) and liposomes ($\sim 4.7\%$) (Ahn et al., 2010; Zhang et al., 2014a).

To confirm the controlled release ability of lipid-coated nanoparticles, release experiments of ATO formulations were performed in a physiological environment (Fig 4). Compared to the fast release of ATO-Sol (approximate 95% in 4 h), all ATO-loaded nanoparticles displayed controlled release characteristics. ATO that was released from CHMSN-ATO was about $62.82 \pm 0.53\%$ in 12 h, which was primarily due to the sustained release of cargo by gradual diffusion from inside to outside

controlled by the mesopore. Interestingly, in the initial 2 h, $39.85 \pm 0.84\%$ of ATO was released from CHMSN-ATO. This premature release may be explained by the binding of naked drug around the nanoparticles through weak Van-Der-Waals Forces and may result in off-target toxicity to bystander cells. The release rate from LP-CHMSN-ATO and RGD-LP-CHMSN-ATO within the initial 2 h was remarkably reduced to $19.34 \pm 2.29\%$ and $17.32 \pm 2.10\%$. The reason was that the compact and defect-free lipid membrane has acted as an intact barrier against the escape of encapsulated cargos. This characteristic is key for drug delivery to solid tumors, as it can minimize the amount of premature drug release during the circulation (pH=7.4) and thereby cause drugs to be released in a controlled manner to achieve a sustained anticancer effect.

[Figure 4]

3.3 Cellular uptake and intracellular disposition

In our studies, flow cytometry analysis was carried out to estimate the cellular uptake of FITC-labeled nanocarriers (CHMSN-FITC, LP-CHMSN-FITC and RGD-LP-CHMSN-FITC) by $\alpha_v\beta_3$ -integrin-positive tumor cells (HepG2) or $\alpha_v\beta_3$ -integrin-negative cells (MCF-7 and LO2). As shown in Fig. 5A-D, the mean fluorescence intensity (MFI) of LP-CHMSN-FITC and RGD-LP-CHMSN-FITC groups increased dramatically compared to that of the CHMSN-FITC group ($P < 0.01$). This effect could be ascribed to the easy aggregation of CHMSN-FITC on the cellular surface, thereby resulted in larger structures. Thus, internalization of CHMSN-FITC was more difficult than lipid-coated nanoparticles, which could

diffuse through the membrane (Tada et al., 2014). The MFI of the RGD-LP-CHMSN-FITC group was 2.93-fold higher than that of LP-CHMSN-FITC in HepG2 cells ($P < 0.01$, Fig. 5A and Fig. 5D), whereas the MFI of the two formulations showed a slight change in MCF-7 and LO2 cells (Fig. 5B-D). These evidence indicated that the RGD ligands could specially recognize the target on the surfaces of HepG2 cells and achieve targeted drug delivery.

In order to determine the subcellular localization of nanoparticles, three types of cells were observed by CLSM after incubation for 4 h with medium containing FITC-modified nanocarriers (3 μ M FITC per L). As shown in Fig. 5E, nanoparticles were primarily localized in the cytoplasm. Images of LP-CHMSN-FITC and RGD-LP-CHMSN-FITC clearly showed co-localization of fluorescence signals and the cell membrane, indicating that lipid-coated nanoparticles were internalized by endocytosis. The bright green spots detected in Fig. 5E may be explained by the fact that a large number of FITC-modified nanoparticles are swallowed and gathered in the endosomal/lysosomal compartment after entering into the cells (Rehman et al., 2013). A stronger FITC fluorescence signal could be observed from three cell lines incubated with lipid-encapsulated nanocarriers compared to that of the bare nanocarriers. In HepG2 cells, the fluorescence signal of RGD-LP-CHMSN-FITC was stronger compared to that of LP-CHMSN-FITC, whereas no difference in signal could be observed between RGD-LP-CHMSN-FITC and LP-CHMSN-FITC on MCF-7 and LO2 cells. This finding was inconsistent with the flow cytometry analysis data.

[Figure 5]

3.4 *In vitro* cytotoxicity

To evaluate the biocompatibility of functionalized HMSN, drug-free nanoparticles were incubated with HepG2, MCF-7, and LO2 cells (Fig. 6A-C). More than 85% of all cells remained viable during incubation with the carriers at a concentration below 2.5 $\mu\text{g/mL}$. The results indicated that all drug-free nanocarriers exhibited intrinsically low toxicity and good biocompatibility. By increasing the concentration, NH_2 -HMSN revealed higher toxicity in cells compared to CHMSN, LP-CHMSN, and RGD-LP-CHMSN, which may be attributed to the positive charge- and concentration-dependent hemolytic activity caused by amine-modified nano-materials (Tian et al., 2011). The viability of cells treated with CHMSN was higher than that of cells treated with NH_2 -HMSN, which indicated the modification of organic carbon chain could decrease the cytotoxicity by shielding part of the naked amino group on the surface of NH_2 -HMSN. The lipid-encapsulated formulations further increased the viability of cells since these nanoparticles resembled the “protocell-like” structure and simulated the composition of cell membrane to improve biocompatibility (Ashley et al., 2011).

To evaluate the cytotoxicity of ATO formulations, HepG2, MCF-7, and LO2 cells were exposed to ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO or RGD-LP-CHMSN-ATO for 48 h, respectively. As displayed in Fig. 6D-F, the viability of cells was inhibited with the increase of ATO concentrations. As presented in Fig. 6G, the half maximal inhibitory concentration (IC_{50}) values of ATO-Sol were $18.84 \pm 1.06 \mu\text{mol/L}$, $23.37 \pm 1.30 \mu\text{mol/L}$, and $48.43 \pm 1.34 \mu\text{mol/L}$ in HepG2, MCF-7,

485 and LO2 cells, respectively. This indicated that ATO were more sensitive to tumor
486 cells (HepG2 and MCF-7) than human liver cells (LO2). Notably, LO2 cells
487 maintained a high survival rate (~80%) at an IC_{50} value of HepG2 cells induced by
488 RGD-LP-CHMSN-ATO. Thus, the as-prepared nanodrugs were suitable for treating
489 hepatic carcinoma with limited side effects. The IC_{50} values induced by ATO-Sol
490 were 1.21, 1.16, and 1.24-fold higher compared to those of CHMSN-ATO ($P < 0.05$)
491 in HepG2, MCF-7 and LO2 cells, respectively. The reason for this may be the easy
492 combination between the positively-charged CHMSN-ATO and the
493 negatively-charged cell membrane (Cho et al., 2009) and sustained drug release action.
494 Both the IC_{50} of LP-CHMSN-ATO and RGD-LP-CHMSN-ATO were smaller than
495 that of CHMSN-ATO ($P < 0.05$). The reason for this may be that the lipid bilayer
496 played an important role in increasing the inherent affinity between nanoparticles and
497 the cell membrane, therefore promoting cellular uptake through a mechanism
498 described as contact-facilitated drug delivery (Liu et al., 2009a). Compared with
499 LP-CHMSN-ATO (IC_{50} : $12.61 \pm 1.35 \mu\text{mol/L}$), the higher cytotoxicity of
500 RGD-LP-CHMSN-ATO (IC_{50} : $9.88 \pm 1.21 \mu\text{mol/L}$) on HepG2 cells may result from
501 the higher intracellular concentration of ATO that was improved by receptor mediated
502 endocytosis (RME) ($P < 0.01$). To verify this, the drug concentration in HepG2 cells
503 were assessed after the cell were incubated with ATO formulations for 2, 6, 12, and
504 24 h, respectively. We found that the RGD-LP-CHMSN-ATO group was indeed
505 associated with the highest level of cellular uptake of ATO compared to the other
506 groups ($P < 0.05$) (Fig. 6H).

[Figure 6]

3.5 Mechanism of cellular uptake

To explore the endocytosis pathways of RGD-LP-CHMSN-ATO, a cellular inhibition assay was adopted in integrin-overexpressing HepG2 cells. To determine the impact of energy, sodium azide was employed in our studies. Anti- $\alpha_v\beta_3$ and iRGD were used as either the antibody or as competitive inhibitors to block or competitively inhibit the integrin $\alpha_v\beta_3$. As displayed in Fig. 6I, cellular uptake of CHMSN-FITC, LP-CHMSN-FITC and RGD-LP-CHMSN-FITC was completely inhibited by sodium azide compared to that of the control group ($P < 0.05$), which indicated that endocytosis of nanoparticles was an energy-dependent process. In addition, ligands including iRGD and anti- $\alpha_v\beta_3$ dramatically reduced cellular uptake of RGD-LP-CHMSN-ATO compared to the control group ($P < 0.05$), however no significant changes were found among ATO-Sol, CHMSN-ATO and LP-CHMSN-ATO groups. These results confirmed that the transport of RGD-LP-CHMSN-ATO was primarily dependent on $\alpha_v\beta_3$ integrin-mediated endocytosis.

3.6 Cell cycle analysis

To characterize the inhibitory effect of ATO on cell proliferation, we used flow cytometry analysis to monitor cell cycle progression. HepG2 cells were incubated with culture medium or drug-containing culture medium (1 μ g ATO per mL) for 48 h. As shown in Fig. 7A-F, ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO, and RGD-LP-CHMSN-ATO specifically arrested 26.18 $\pm$ 3.02%, 28.76 $\pm$ 2.13%,

35.51±0.51%, and 39.57±0.62% of HepG2 cells at the G2-M phase of the cell cycle, which was much higher than that of control group (22.30±2.31%). Our results demonstrated all formulations inhibited the proliferation of HepG2 by inducing G2-M transition at different levels with RGD-LP-CHMSN-ATO exhibiting a superior G2-M phase arrest ($P < 0.01$). This may account for the strongest cytotoxicity induced by RGD-LP-CHMSN-ATO in HepG2.

[Figure 7]

3.7 Pharmacokinetic study

The pharmacokinetic properties of ATO have been thoroughly studied (Fujisawa et al., 2007; Hua et al., 2011; Shen et al., 2001), however, few studies have focused on PK characteristics of ATO after loaded by nano-sized DDS. In our study, ATO was initially loaded into hybrid nanoparticles with targeted delivery and controlled release properties and its PK features were studied. The ATO concentration-time profiles are depicted in Fig. 8 and the PK parameters complied with two-compartment model are summarized in Table 1. Compared to the ATO-Sol group, controlled release characteristics of CHMSN-ATO, LP-CHMSN-ATO, and RGD-LP-CHMSN-ATO were evidenced by significantly prolonged $t_{1/2\beta}$ ($P < 0.01$), reduced clearance (CL) ($P < 0.05$), and improved AUC ($P < 0.05$). Remarkably, compared with CHMSN-ATO, a prolonged MRT ($P < 0.05$) and increased bioavailability (AUC) of LP-CHMSN-ATO and RGD-LP-CHMSN-ATO ($P < 0.05$) were attributed to decreased drug leakage and the extended circulation time of lipid-coated nanoparticles. In addition, the PEG chains of DSPE-PEG were able to form mushroom-like clusters

on the surface of the nanoparticles to increase hydration in the aqueous environment, which caused steric hindrance to prevent protein adsorption. Therefore, such nanoparticles shielded the hydrophobic particle surface and thereby reduced opsonization by blood proteins and uptake by macrophages of the reticuloendothelial system (RES) (Hak et al., 2012; Huang and Liu, 2011). The improved PK behavior of ATO by lipid-coated nanodrugs may result in limited side effects and enhanced drug accumulation at the tumor site.

[Figure 8]

[Table 1]

3.8 In vivo biodistribution

We also evaluated the distribution of ATO in H22 tumor-bearing mice, 24 h after intravenous administration of the ATO formulations (Fig. 9). The results showed that the ATO concentration in the tumor treated by ATO-loaded nanoparticles was significantly higher than that of ATO-Sol ($P < 0.05$), which was due to the reduction of the renal clearance rate and the enhanced retention of ATO at the tumor site by EPR effect. The amount of ATO in the tumor induced by RGD-LP-CHMSN-ATO was higher than that of LP-CHMSN-ATO treatment, which further proved the role of the RGD region in specific bond between the designed DDS and integrin receptors of tumor cells. Moreover, we found that ATO-loaded nanoparticles reduced the amount of arsenic distribution in the skin, which may relieve severe skin side reactions caused by arsenic compounds. In addition, the lipid-coated nanodrugs reduced ATO retention by liver and lung capillaries compared to that of CHMSN-ATO treatment.

3.9 *In vivo* anti-tumor efficacy

Based on the promising antitumor effect that we observed *in vitro*, we continued to perform *in vivo* studies using H22 tumor-bearing mice. Fig. 10A displayed the tumor volume with time extension after mice were treated with either saline or ATO formulations (1 mg ATO-equivalent/kg body weight). We found that tumor growth was inhibited in all drug formulations compared to the saline group ($P < 0.01$). On day 15, the mean tumor volume of the RGD-LP-CHMSN-ATO group ($502.67 \pm 92.72 \text{ mm}^3$) was significantly reduced compared to that of the saline group ($2630.06 \pm 427.38 \text{ mm}^3$, $P < 0.01$), ATO-Sol group ($1552.47 \pm 566.45 \text{ mm}^3$, $P < 0.01$), CHMSN-ATO group ($993.81 \pm 263.99 \text{ mm}^3$, $P < 0.01$), and LP-CHMSN-ATO group ($858.69 \pm 149.56 \text{ mm}^3$, $P < 0.05$) (Fig. 10B). And tumor weight showed the same tendency as that of tumor volume in Table 2. The IR% of RGD-LP-CHMSN-ATO group was 82.42%, which was 2.27, 1.58, and 1.27-fold higher than that of the ATO-Sol group, CHMSN-ATO group, and LP-CHMSN-ATO group. Compared with ATO-Sol, the enhanced effect of CHMSN-ATO was primarily caused by two factors: (1) the EPR effect in the tumor and (2) the controlled release action of nanoparticles. The significantly enhanced anti-tumor effect of LP-CHMSN-ATO compared to that of CHMSN-ATO could be attributed to three other facts: (1) the pronounced controlled release feature, (2) the enhanced cellular uptake effect of the supported lipid, and (3) the improved PK characteristics of the drugs. RGD-LP-CHMSN-ATO, which combined the targeted and controlled release effect, showed the best inhibitory effect

on the growth of tumor. At termination of experiments, tumors were removed and sectioned for pathological analysis. H&E staining evaluation of tumor tissues from each group further confirmed the effective destruction of tumor cells by RGD-LP-CHMSN-ATO treatment (Fig. 10E).

[Table 2]

The effects of each treatment on survival of tumor-bearing mice are depicted in Fig. 10C and Table 3. The median survival time of mice that were treated with RGD-LP-CHMSN-ATO was significantly longer compared to that of mice treated with saline ($P < 0.01$), ATO injection ($P < 0.01$), CHMSN-ATO injection ($P < 0.05$), or LP-CHMSN-ATO ($P < 0.05$). These results further demonstrated the promising anti-hepatic carcinoma effect of RGD-LP-CHMSN-ATO treatment.

[Table 3]

For safety evaluation, no noticeable body weight loss was observed in mice, with the exception of the ATO-Sol group from day 13 (Fig. 10D). This may have been caused by the severe toxicity of free ATO to the body. Furthermore, the results suggested that there was no acute or severe toxicity of ATO-loaded nanoparticles at the test dose, because the carriers prevented the exposure of drugs to normal tissue. Moreover, the rapid increase in weight in the first nine days after drug administration was due to the rapidly growing solid tumor. The RGD-LP-CHMSN-ATO group underwent a minimum weight increase during that period, indicated its best antitumor effect.

It is well recognized that targeted delivery and controlled release characteristics

are key in delivering anticancer drugs. This is especially true for drugs with dose-limited toxicity. The present work combined the two strategies to build liposome-hollow silica hybrid nanoparticles. The targeting molecule increased the distribution of ATO to the tumor sites. The prolonged circulation time and enhanced controlled drug release characteristic of lipid-coated nanodrugs resulted in a sustained anticancer effect. Therefore, RGD-LP-CHMSN-ATO had the best treatment effect. From a clinical view point, Trisenox®, an arsenic-containing injection is widely used for APL in humans; whereas its application in solid tumors is hampered by its low therapeutic index and serious side effects. RGD-LP-CHMSN-ATO could be a prospective drug carrier to avoid direct contact between free ATO and normal tissues, and improve drug concentration in a solid tumor. All findings proved this favorable DDS may significantly expand the potential use of arsenic compounds in treating solid tumors.

[Figure 10]

4. Conclusion

Safe core-shell hybrid nanovehicles, in which the features of inorganic mesoporous nanoparticles and functional liposomes are combined, were successfully established as a tumor targeting and on-demand release DDS for enhancing anticancer effects of ATO. Chlorodimethyloctadecylsilane, a non-toxic and pharmacological activity material was innovatively modified on the surface of HMSN for supporting the phospholipid bilayer, which increased the affinity between DDS and the cell

membrane. Results indicated that clear controlled release behavior of ATO was exhibited after it was loaded by CHMSN and pronounced by capping with phospholipids. Improved PK characteristics and significant hepatic tumor-specific distribution of ATO were found in RGD-LP-CHMSN-ATO-treated mice. Additionally, the antitumor efficacy *in vitro* and *in vivo* was remarkably improved by RGD-LP-CHMSN-ATO. In summary, our study encouraged further development of lipid-coated nanosystems for efficient delivery of drugs with poor pharmacokinetics and/or dose-limited toxicity to targets.

Acknowledgment

This study was financially supported by the National Science Foundation of China (NO.81274089, 81473361, 81373982, 81673607 and 81303235), the Natural Science Foundation of Zhejiang Province (LZ13H280001), and the Traditional Chinese Medicine Science and Technology Plan of Zhejiang Province (2016ZA130).

References

- Ahn, R.W., Chen, F., Chen, H., Stern, S.T., Clogston, J.D., Patri, A.K., Raja, M.R., Swindell, E.P., Parimi, V., Cryns, V.L., O'Halloran, T.V., 2010. A novel nanoparticulate formulation of arsenic trioxide with enhanced therapeutic efficacy in a murine model of breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16, 3607-3617.
- Ashley, C.E., Carnes, E.C., Phillips, G.K., Padilla, D., Durfee, P.N., Brown, P.A., Hanna, T.N., Liu, J., Phillips, B., Carter, M.B., Carroll, N.J., Jiang, X., Dunphy, D.R., Willman, C.L., Petsev, D.N., Evans, D.G., Parikh, A.N., Chackerian, B., Wharton, W., Peabody, D.S., Brinker, C.J., 2011. The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. *Nat Mater* 10, 389-397.
- Bhattacharyya, S., Wang, H.S., Ducheyne, P., 2012. Polymer-coated mesoporous silica nanoparticles for the controlled release of macromolecules. *Acta Biomater* 8, 3429-3435.
- Cauda, V., Engelke, H., Sauer, A., Arcizet, D., Brauchle, C., Radler, J., Bein, T., 2010.

Colchicine-loaded lipid bilayer-coated 50 nm mesoporous nanoparticles efficiently induce microtubule depolymerization upon cell uptake. *Nano letters* 10, 2484-2492.

Chen, H., MacDonald, R.C., Li, S., Krett, N.L., Rosen, S.T., O'Halloran, T.V., 2006. Lipid encapsulation of arsenic trioxide attenuates cytotoxicity and allows for controlled anticancer drug release. *J Am Chem Soc* 128, 13348-13349.

Cho, E.C., Xie, J.W., Wurm, P.A., Xia, Y.N., 2009. Understanding the Role of Surface Charges in Cellular Adsorption versus Internalization by Selectively Removing Gold Nanoparticles on the Cell Surface with a I-2/KI Etchant. *Nano letters* 9, 1080-1084.

Dilda, P.J., Hogg, P.J., 2007. Arsenical-based cancer drugs. *Cancer treatment reviews* 33, 542-564.

Fang, W.J., Tang, S.H., Liu, P.X., Fang, X.L., Gong, J.W., Zheng, N.F., 2012. Pd Nanosheet-Covered Hollow Mesoporous Silica Nanoparticles as a Platform for the Chemo-Photothermal Treatment of Cancer Cells. *Small* 8, 3816-3822.

Fang, X.L., Zhao, X.J., Fang, W.J., Chen, C., Zheng, N.F., 2013. Self-templating synthesis of hollow mesoporous silica and their applications in catalysis and drug delivery. *Nanoscale* 5, 2205-2218.

Fujisawa, S., Ohno, R., Shigeno, K., Sahara, N., Nakamura, S., Naito, K., Kobayashi, M., Shinjo, K., Takeshita, A., Suzuki, Y., Hashimoto, H., Kinoshita, K., Shimoya, M., Kaise, T., Ohnishi, K., 2007. Pharmacokinetics of arsenic species in Japanese patients with relapsed or refractory acute promyelocytic leukemia treated with arsenic trioxide. *Cancer Chemother Pharmacol* 59, 485-493.

Galanzha, E.I., Kim, J.W., Zharov, V.P., 2009. Nanotechnology-based molecular photoacoustic and photothermal flow cytometry platform for in-vivo detection and killing of circulating cancer stem cells. *Journal of biophotonics* 2, 725-735.

Geng, H., Zhao, Y., Liu, J., Cui, Y., Wang, Y., Zhao, Q., Wang, S., 2016. Hollow mesoporous silica as a high drug loading carrier for regulation insoluble drug release. *International journal of pharmaceutics* 510, 184-194.

Hak, S., Helgesen, E., Hektoen, H.H., Huuse, E.M., Jarzyna, P.A., Mulder, W.J.M., Haraldseth, O., Davies, C.D., 2012. The Effect of Nanoparticle Polyethylene Glycol Surface Density on Ligand-Directed Tumor Targeting Studied in Vivo by Dual Modality Imaging. *Acs Nano* 6, 5648-5658.

Han, N., Wang, Y., Bai, J.L., Liu, J.J., Wang, Y., Gao, Y.K., Jiang, T.Y., Kang, W.J., Wang, L.L., 2016. Facile synthesis of the lipid bilayer coated mesoporous silica nanocomposites and their application in drug delivery. *Micropor Mesopor Mat* 219, 209-218.

Hua, H.Q., Qin, S.K., Rui, J.Z., Li, J.H., 2011. Pharmacokinetics of Arsenic Trioxide (As₂O₃) in Chinese Primary Hepatocarcinoma Patients. *Asian Pac J Cancer P* 12, 61-65.

Huang, L., Liu, Y., 2011. In Vivo Delivery of RNAi with Lipid-Based Nanoparticles. *Annu Rev Biomed Eng* 13, 507-530.

Jalvandi, J., White, M., Truong, Y.B., Gao, Y., Padhye, R., Kyratzis, I.L., 2015. Release and antimicrobial activity of levofloxacin from composite mats of poly(ϵ -caprolactone) and mesoporous silica nanoparticles fabricated by core-shell electrospinning. *J Mater Sci* 50, 7967-7974.

Lee, S.M., Lee, O.S., O'Halloran, T.V., Schatz, G.C., Nguyen, S.T., 2011. Triggered release of pharmacophores from [Ni(HAsO(3))]-loaded polymer-caged nanobin enhances pro-apoptotic activity: a combined experimental and theoretical study. *Acs Nano* 5, 3961-3969.

Li, J., Yang, Y., Huang, L., 2012. Calcium phosphate nanoparticles with an asymmetric lipid bilayer coating for siRNA delivery to the tumor. *Journal of Controlled Release* 158, 108-114.

Li, Z.Y., Hu, J.J., Xu, Q., Chen, S., Jia, H.Z., Sun, Y.X., Zhuo, R.X., Zhang, X.Z., 2015. A redox-responsive drug delivery system based on RGD containing peptide-capped mesoporous silica

nanoparticles. *Journal of Materials Chemistry B* 3, 39-44.

Liu, J., Stace-Naughton, A., Jiang, X., Brinker, C.J., 2009a. Porous nanoparticle supported lipid bilayers (protocells) as delivery vehicles. *J Am Chem Soc* 131, 1354-1355.

Liu, J.W., Jiang, X.M., Ashley, C., Brinker, C.J., 2009b. Electrostatically Mediated Liposome Fusion and Lipid Exchange with a Nanoparticle-Supported Bilayer for Control of Surface Charge, Drug Containment, and Delivery. *J Am Chem Soc* 131, 7567-7569.

Lu, J., Chew, E.H., Holmgren, A., 2007. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. *Proceedings of the National Academy of Sciences of the United States of America* 104, 12288-12293.

Matsumura, Y., Maeda, H., 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer research* 46, 6387-6392.

Meng, H., Wang, M.Y., Liu, H.Y., Liu, X.S., Situ, A., Wu, B., Ji, Z.X., Chang, C.H., Nel, A.E., 2015. Use of a Lipid-Coated Mesoporous Silica Nanoparticle Platform for Synergistic Gemcitabine and Paclitaxel Delivery to Human Pancreatic Cancer in Mice. *Acs Nano* 9, 3540-3557.

Miller, W.H., Jr., Schipper, H.M., Lee, J.S., Singer, J., Waxman, S., 2002. Mechanisms of action of arsenic trioxide. *Cancer research* 62, 3893-3903.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., Gianni, L., 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacological reviews* 56, 185-229.

Patil, Y., Amitay, Y., Ohana, P., Shmeeda, H., Gabizon, A., 2016. Targeting of pegylated liposomal mitomycin-C prodrug to the folate receptor of cancer cells: Intracellular activation and enhanced cytotoxicity. *Journal of Controlled Release* 225, 87-95.

Pereira, E.M.A., Kosaka, P.M., Rosa, H., Vieira, D.B., Kawano, Y., Petri, D.F.S., Carmona-Ribeiro, A.M., 2008. Hybrid materials from intermolecular associations between cationic lipid and polymers. *J Phys Chem B* 112, 9301-9310.

Rehman, Z.U., Hoekstra, D., Zuhorn, I.S., 2013. Mechanism of Polyplex- and Lipoplex-Mediated Delivery of Nucleic Acids: Real-Time Visualization of Transient Membrane Destabilization without Endosomal Lysis. *Acs Nano* 7, 3767-3777.

Ren, J., Shen, S., Wang, D., Xi, Z., Guo, L., Pang, Z., Qian, Y., Sun, X., Jiang, X., 2012. The targeted delivery of anticancer drugs to brain glioma by PEGylated oxidized multi-walled carbon nanotubes modified with angiopep-2. *Biomaterials* 33, 3324-3333.

Rowinsky, E.K., Donehower, R.C., 1995. Paclitaxel (taxol). *The New England journal of medicine* 332, 1004-1014.

Shen, Y., Shen, Z.X., Yan, H., Chen, J., Zeng, X.Y., Li, J.M., Li, X.S., Wu, W., Xiong, S.M., Zhao, W.L., Tang, W., Wu, F., Liu, Y.F., Niu, C., Wang, Z.Y., Chen, S.J., Chen, Z., 2001. Studies on the clinical efficacy and pharmacokinetics of low-dose arsenic trioxide in the treatment of relapsed acute promyelocytic leukemia: a comparison with conventional dosage. *Leukemia* 15, 735-741.

Shooshtary, S., Behtash, S., Nafisi, S., 2015. Arsenic trioxide binding to serum proteins. *Journal of photochemistry and photobiology. B, Biology* 148, 31-36.

Song, X.L., You, J., Wang, J., Zhu, A.P., Ji, L.J., Guo, R., 2014. Preparation and Investigation of Arsenic Trioxide-loaded Polylactic Acid/Magnetic Hybrid Nanoparticles. *Chem Res Chinese U* 30, 326-332.

Sweeney, C.J., Takimoto, C., Wood, L., Porter, J.M., Tracewell, W.G., Darwish, M., D'Andrea, D.M.,

Remick, S.C., 2010. A pharmacokinetic and safety study of intravenous arsenic trioxide in adult cancer patients with renal impairment. *Cancer Chemoth Pharm* 66, 345-356.

Swindell, E.P., Hankins, P.L., Chen, H., Miodragovic, D.U., O'Halloran, T.V., 2013. Anticancer activity of small-molecule and nanoparticulate arsenic(III) complexes. *Inorganic chemistry* 52, 12292-12304.

Tada, D.B., Suraniti, E., Rossi, L.M., Leite, C.A.P., Oliveira, C.S., Tumolo, T.C., Calemczuk, R., Livache, T., Baptista, M.S., 2014. Effect of Lipid Coating on the Interaction Between Silica Nanoparticles and Membranes. *Journal of Biomedical Nanotechnology* 10, 519-528.

Teng, I.T., Chang, Y.J., Wang, L.S., Lu, H.Y., Wu, L.C., Yang, C.M., Chiu, C.C., Yang, C.H., Hsu, S.L., Ho, J.A., 2013. Phospholipid-functionalized mesoporous silica nanocarriers for selective photodynamic therapy of cancer. *Biomaterials* 34, 7462-7470.

Tian, Y., Alexander, M., Hamidreza, G., 2011. Impact of silica nanoparticle design on cellular toxicity and hemolytic activity. *Acs Nano* 5, 5717-5728.

Uslu, R., Sanli, U.A., Sezgin, C., Karabulut, B., Terzioglu, E., Omay, S.B., Goker, E., 2000. Arsenic trioxide-mediated cytotoxicity and apoptosis in prostate and ovarian carcinoma cell lines. *Clinical cancer research : an official journal of the American Association for Cancer Research* 6, 4957-4964.

Wang, L.S., Wu, L.C., Lu, S.Y., Chang, L.L., Teng, I.T., Yang, C.M., Ho, J.A., 2010. Biofunctionalized phospholipid-capped mesoporous silica nanoshuttles for targeted drug delivery: improved water suspensibility and decreased nonspecific protein binding. *Acs Nano* 4, 4371-4379.

Wang, Z.Y., Chen, Z., 2008. Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood* 111, 2505-2515.

Wei, L., Guo, X.Y., Yang, T., Yu, M.Z., Chen, D.W., Wang, J.C., 2016. Brain tumor-targeted therapy by systemic delivery of siRNA with Transferrin receptor-mediated core-shell nanoparticles. *International journal of pharmaceutics* 510, 394-405.

Xiao, X., Liu, Y., Guo, M., Fei, W., Zheng, H., Zhang, R., Zhang, Y., Wei, Y., Zheng, G., Li, F., 2016. pH-triggered sustained release of arsenic trioxide by polyacrylic acid capped mesoporous silica nanoparticles for solid tumor treatment in vitro and in vivo. *J Biomater Appl* 31, 23-35.

Yang, P., Gai, S., Lin, J., 2012. Functionalized mesoporous silica materials for controlled drug delivery. *Chemical Society reviews* 41, 3679-3698.

Zhang, Q., Vakili, M.R., Li, X.F., Lavasanifar, A., Le, X.C., 2014a. Polymeric micelles for GSH-triggered delivery of arsenic species to cancer cells. *Biomaterials* 35, 7088-7100.

Zhang, X., Li, F., Guo, S., Chen, X., Wang, X., Li, J., Gan, Y., 2014b. Biofunctionalized polymer-lipid supported mesoporous silica nanoparticles for release of chemotherapeutics in multidrug resistant cancer cells. *Biomaterials* 35, 3650-3665.

Zhao, Z., Zhang, H., Chi, X., Li, H., Yin, Z., Huang, D., Wang, X., Gao, J., 2014. Silica nanovehicles endow arsenic trioxide with an ability to effectively treat cancer cells and solid tumors. *Journal of Materials Chemistry B* 2, 6313-6323.

Figure Captions

Fig. 1. Schematic illustration for the synthesis and preparation of RGD-LP-CHMSN-ATO for hepatic carcinoma therapy.

Fig. 2. TEM images of sSiO₂ (A), CHMSN (B), LP-CHMSN (C) and RGD-LP-CHMSN (D). ζ -potential (E) of sSiO₂, NH₂-HMSN, CHMSN, CHMSN-ATO, LP-CHMSN and RGD-LP-CHMSN. Size and PDI (F) of sSiO₂, NH₂-HMSN, CHMSN, LP-CHMSN and RGD-LP-CHMSN.

Fig. 3. FT-IR spectra of NH₂-HMSN (A) and CHMSN (B). MALDI-TOF-MS spectrum (C) of DSPE-PEG-RGD. Stability curve (D) and dispersion state (E) of the HMSN-FITC, CHMSN-FITC, LP-CHMSN-FITC and RGD-LP-CHMSN-FITC in PBS. N₂ adsorption-desorption isotherm (F), pore size distribution (G) and XRD patterns (H) of NH₂-HMSN, CHMSN, LP-CHMSN, RGD-LP-CHMSN. TGA curves (I) of NH₂-HMSN, CHMSN, CHMSN-ATO, LP-CHMSN-ATO and RGD-LP-CHMSN-ATO.

Fig. 4. *In vitro* release profiles of ATO from ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO and RGD-LP-CHMSN-ATO at pH 7.4 during 48 h (n=3).

Fig. 5. Flow cytometry profiles of HepG2 (A), MCF-7 (B) and LO2 (C) cells that were incubated with CHMSN-FITC, LP-CHMSN-FITC or RGD-LP-CHMSN-FITC containing medium (3 μ M FITC per L) for 4 h. Total cellular uptake (D) of FITC-modified nanoparticles by HepG2, MCF-7 and LO2 cells at 37 $^{\circ}$ C, $**P < 0.01$ vs CHMSN-FITC group, $^{##}P < 0.01$ vs LP-CHMSN-FITC group. Confocal images (E) of HepG2, MCF-7 and LO2 cells incubation for 4 h with CHMSN-FITC, LP-CHMSN-FITC or RGD-LP-CHMSN-FITC containing medium. For each group, the cell nucleus were stained with Hoechst 33342 (blue).

Fig. 6. MTT assay of HepG2 (A), MCF-7 (B) and LO2 (C) cells after being treated with medium containing NH₂-HMSN, CHMSN, LP-CHMSN or RGD-LP-CHMSN at concentrations ranging from 0.1 µg/mL to 100 µg/mL for 48 h. The viability of HepG2 (D), MCF-7 (E) and LO2 (F) cells after being treated with ATO formulations during a certain concentration range. IC₅₀ values (G) to HepG2, MCF-7 and LO2 cells induced by ATO formulations, **P* < 0.05, ***P* < 0.01 vs ATO group, #*P* < 0.05, ##*P* < 0.01 vs CHMSN-ATO group, Δ *P* < 0.01 vs LP-CHMSN-ATO group. Quantification of ATO (H) in HepG2 cells incubation with blank medium or ATO formulations for 2, 6, 12 or 24 h, **P* < 0.05, ***P* < 0.01 vs ATO-Sol group, #*P* < 0.05, ##*P* < 0.01 vs CHMSN-ATO group, Δ *P* < 0.05, Δ *P* < 0.01 vs LP-CHMSN-ATO group. Quantification of ATO (I) in HepG2 cells after incubation for 6 h with blank medium (control) or ATO formulations in presence of sodium azide, anti- $\alpha_v\beta_3$ or iRGD, **P* < 0.05, ***P* < 0.01 vs control group.

Fig. 7. HepG2 cell cycle perturbations induced by culture medium (A) or drug-containing medium (ATO-Sol (B), CHMSN-ATO (C), LP-CHMSN-ATO (D) or RGD-LP-CHMSN-ATO (E)) (1 μ g ATO per mL) for 48 h. Distribution of cell cycles (F) after treatment. ** $P < 0.01$ vs RGD-LP-CHMSN-ATO group.

Fig. 8. The profiles of ATO concentration-time in SD rats after intravenous injection (n=5).

Fig. 9. Biodistribution of ATO in mice at 24 h after intravenous injection (n =5). * $P < 0.05$ vs ATO-Sol group.

Fig. 10. Tumor volume curves (A) after intravenous injection of saline, ATO-Sol, CHMSN-ATO, LP-CHMSN-ATO or RGD-LP-CHMSN-ATO (1 mg ATO-equivalent/kg body weight) for 15 days. $**P < 0.01$ vs saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs ATO-Sol group, $^{\Delta}P < 0.05$ vs CHMSN-ATO and LP-CHMSN-ATO group. The tumor photographs (B) on the day 15 (n=6). Kaplan-Meier survival curves (C) of H22-bearing mice treated with saline or ATO formulations (n=10). Mean body weight (D) of the mice in different groups during the treatment (n=6). Tumor H&E histology images (E) of the mice (black arrows indicate the typical necrotic cells in tumors and scale bar is 200 μm).

Graphical abstract

To construct a targeted and controlled arsenic trioxide (ATO, a poor pharmacokinetic drug with dose-limited toxicity) delivery core-shell nanovehicle which combined the features of hollow mesoporous silica nanoparticles (HMSN) and functional liposome (named as RGD-LP-CHMSN-ATO). The chlorodimethyloctadecylsilane was modified on the surface of HMSN for supporting phospholipid bilayer which enhanced the stability of nanoparticles and increased the affinity between nanoparticles and cell membrane. Improved PK features and significant hepatic tumor-specific distribution of ATO were achieved by RGD-LP-CHMSN-ATO. The antitumor efficacy in vitro (HepG2 cell line) and in vivo (H22 xenografts) was remarkably improved by RGD-LP-CHMSN-ATO. All the findings proved this favorable drug delivery system may significantly expand the potential use of arsenic compounds in treating solid tumor.

Table 1

Main parameters of ATO after vein injection in rats (n=5).

Parameters	ATO-Sol	CHMSN-ATO	LP-CHMSN-ATO	RGD-LP-CHMSN-ATO
$t_{1/2\alpha}$ (h)	0.20±0.02	0.31±0.16	0.38±0.05	0.30±0.08
$t_{1/2\beta}$ (h)	14.78±2.34	18.92±6.59 ^{**}	24.86±4.54 ^{**}	24.94±4.68 ^{**}
CL (L/h)	0.23±0.04	0.15±0.05 [*]	0.09±0.02 ^{**}	0.08±0.01 ^{**}
AUC _{0-t} (μg·h/mL)	3.99±0.61	6.10±1.24 [*]	8.63±1.08 ^{**#}	9.44±0.84 ^{**#}
V _{ss} (mg/kg/(μg/mL))	4.65±0.57	3.49±0.13 [#]	3.03±0.16 ^{**}	2.77±0.23 ^{**##}
MRT (h)	20.59±3.34	25.75±4.24 [*]	35.43±6.49 ^{**#}	35.60±6.66 ^{**#}

* $P < 0.05$, ** $P < 0.01$ vs ATO-Sol group, # $P < 0.05$, ## $P < 0.01$ vs CHMSN-ATO group.**Table 2**

Tumor weight and IR% of the H22-bearing mice after treating with ATO formulations (n=6).

Groups	Tumor weight (g)	IR(%)	Compared with			
			Saline	ATO-Sol	CHMSN-ATO	LP-CHMSN-ATO
Saline	1.65±0.13	/	—	—	—	—
ATO-Sol	1.05±0.16	36.36	**	—	—	—
CHMSN-ATO	0.79±0.12	52.12	**	*	—	—
LP-CHMSN-ATO	0.58±0.08	64.85	**	**	*	—
RGD-LP-CHMSN-ATO	0.29±0.07	82.42	**	**	**	**

* $P < 0.05$, ** $P < 0.01$.**Table 3**

Effect of ATO formulations on survival time of the H22-bearing mice (n=10).

Groups	Survival times (days)		Compared with			
	Means	Medians	Saline	ATO-Sol	CHMSN-ATO	LP-CHMSN-ATO
Saline	19.6±2.4	19.0±2.3	—	—	—	—
ATO-Sol	22.4±3.3	22.0±1.5	*	—	—	—
CHMSN-ATO	25.7±4.4	26.0±2.1	*	N.S.	—	—
LP-CHMSN-ATO	29.1±2.4	29.0±3.1	**	**	*	—
RGD-LP-CHMSN-ATO	32.9±4.0	33.0±2.1	**	**	*	*

* $P < 0.05$, ** $P < 0.01$, N.S. $P > 0.05$ by log-rank analysis.