***pH-sensitive mesoporous silica nanoparticles anticancer prodrugs for
sustained release of ursolic acid and the enhanced anti-cancer efficacy
for hepatocellular carcinoma cancer***

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Abstract

Ursolic acid (UA) as a nature product exhibits good anti-cancer activity, low toxicity, and good liver protection features. However, the low-solubility and poor bioavailability restricts its further clinical application. To overcome this problem, a pH-sensitive prodrug delivery system (UA@MSN-UA) incorporated acid-sensitive linkage between drug and silica-based mesoporous nanosphere (MSN) was successfully designed and synthesized. The physicochemical properties of the UA@MSN-UA nanoparticles were investigated for shape, particle size, zeta potential, nitrogen adsorption-desorption and infrared (IR) spectroscopy. The nanoparticles were further evaluated for in vitro cytotoxicity, including proliferation inhibition, cell cycle distribution and apoptotic effects against human hepatocellular carcinoma HepG2 cells. The TEM image showed that the size of synthesized MSN nanoparticle was a near-spherical shape with ~100 nm diameter. In vitro cytotoxicity testing demonstrated that UA@MSN-UA nanoparticles prodrug exhibited higher proliferation inhibition, cell cycle arrest at the G2/M phase and significantly caused the early and late apoptosis in HepG2 cells, which would be contributed to high loading capacity, high cellular uptake and sustained release of UA. Overall, the UA-modified MSN prodrug delivery system can be a promising drug carrier for improving the bioavailability of UA, and further enhance its anti-cancer efficacy.

Key words: Ursolic acid; Mesoporous silica nanoparticles; Amino modification; Cancer therapy; Hepatocellular carcinoma (HCC)

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer globally and HCC is the second leading cause of cancer death in the men worldwide (Bruix et al., 2014; Forner et al., 2012; Jemal et al., 2011). There are 782500 estimated new cases and 745500 estimated deaths in the global during 2012, with China alone accounting for about 50 % of the total number of cases and deaths (Torre et al., 2015) . Current approaches for the treatment of HCC include transarterial embolization (TAE), chemoembolization (TACE), surgical and chemotherapy, among which chemotherapy still the main method for HCC therapy. Chemotherapy drugs for treatment of HCC includes 5-fluorouracil (Liao et al., 2015), docetaxel (Xu et al., 2009), cisplatin (Xu et al., 2012) and cyclophosphamide (Greten et al., 2010) etc. However, traditional chemotherapy often shows poor sensitivity, severe toxic and side effects, as well as drug resistance for the tumor cell. Searching for additional novel therapeutic agents against HCC is thus urgently needed.

Ursolic acid (UA) is a natural resource product and belongs to pentacyclic triterpenoids (Xiang et al., 2015) in the molecular structure. The presence of UA has been confirmed in a variety of plant, including 1) natural medicinal plants, such as *Poterntilla discolor* Bge, *Gardenia jasminoides* Ellis, *Callicarpa macrophylla* Vahl (Chen et al., 2011b; Lee et al., 2009) and other medicinal plants; 2) fruits, such as *Fructus Chaenomelis*, *Eriobotrya japonica* (Thunb.) Lindl, *Malus pumila* Mill, *Crataegus pinnatifida* Bunge and other fruits (Bringe et al., 2006; Yamaguchi et al., 2008); 3) classes of tea plants, such as *Ilex kudingcha* C. J. Tseng, *Ilex paraguariensis*,

etc (Gnoatto et al., 2008); 4) herb plants, such as *Sanguisorba officinalis*, *Rosemarinus officinalis*, *Ocimum species*, *Salvia officinalis* etc (Janicsak et al., 2006; Silva et al., 2008); 5) other plants, such as *Paulownia fortunei* (Seem.) Hemsl (Yu et al., 2008), etc. Ursolic acid possesses various pharmacological effects of anti-tumor (Prasad et al., 2011; Shanmugam et al., 2011) , hepato protective (Saravanan et al., 2006)²⁰, anti-inflammatory (Harmand et al., 2005), anti-viral (Baglin et al., 2003), anti-angiogenesis (Saraswati et al., 2013) activities. Pharmacology research shows that ursolic acid can inhibit the proliferation, invasion and metastasis in human hepatoma carcinoma cells both *in vitro* and *in vivo* (Liu et al., 2014; Shih et al., 2013). Recently, ursolic acid and its derivatives have caused an increasing attention because of its high-efficiency and low-toxicity as a safe and efficient agent for hepatoma cancer metastasis prevention (Dong et al., 2015; Son et al., 2013). However, the poor water solubility and low bioavailability of UA restricts its further clinical application.

Nanoparticles drug delivery system is a vigorous technology which defined as improving the drug bioavailability, controlling drug release, enhancing permeability and retention (EPR) effect in the favour of passive tumor targeting, and has been receiving extensive attention in the field of cancer and biological research (He et al., 2015; Mekaru et al., 2015). Among various nanomaterials for drug delivery applications, mesoporous silica nanoparticles (MSNs) have received great attention since they exhibit security and flexible properties, including prominent biocompatibility and low cytotoxicity, significant chemical inertness, famously mechanical stability, highly specific surface area and large pore volumes, tunable

particle diameter, favorable permeability, and facile surface functionalization (Chen et al., 2011a; Fan et al., 2014; Fu et al., 2013; He and Shi, 2011; Tang et al., 2011; Thomas et al., 2010; Zhao et al., 2010). Various study have shown that MSN can enhance the loading capacity of various pharmaceutical drugs, genes or fluorescent dyes, such as DOX (Luo et al., 2012), CPT (Lu et al., 2012), siRNA (Na et al., 2012), and rhodamine B (Chen et al., 2013), etc. However, there are no reports about using MSN as the delivery systems for the natural product UA till now.

In the present study, we designed and synthesized a silica-based mesoporous nanosphere (MSN) controlled-release prodrug delivery system (UA@MSN-UA) (Fig.1). UA was firstly covalently conjugated to the surface of MSN through an acid-labile amide bond, and then UA was encapsulated inside the MSN by non-covalent interactions. Both covalent coupling and encapsulation by non-covalent interactions could lead to increased solubility and enhanced bioavailability of UA, as well as ensures the active UA to be released from the nanoparticles prodrug at low pH environment within the cells. Moreover, the specific structure of UA conjugated on the surface of MSN could cap the pores of MSNs to prevent entrapped UA quickly release in blood before reaching the tumor tissue. We then conducted the in vitro release experiment to study the release profiles of UA from UA@MSN-UA conjugate. Finally, the UA@MSN-UA prodrug nanoparticle was then subjected to biological evaluation against HepG2 cancer cells by cellular uptake, MTT study, cell cycle, and cell apoptosis analysis.

2. Materials and methods

2.1. Materials

Ursolic acid (UA, >90% pure) was purchased from Sartorius Scientific Instruments Co., Ltd. (Beijing China). Hexadecyltrimethylammonium bromide (CTAB, $\geq 99\%$), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride (EDC), N-Hydroxysuccinimide (NHS), 3-[2- (2-aminoethylamino) ethylamino] propyltrimethoxysilane (AEPTMS, $\geq 98\%$) were obtained from Aladdin Reagents Co., Ltd. (Shanghai, China). Trypsin–EDTA and phosphate buffered solution (PBS) were bought from Gibco-BRL (Burlington, ON, Canada). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies GmbH (Darmstadt, Germany). Tris (2-Hydroxyethyl) Amine (TEA), N, N-Dimethylformamide (DMF) was purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

2.2. Synthesis of mesoporous silica nanoparticles (MSN)

Briefly, Ethanol (0.5 mL, 0.015 mol), H₂O (6.4 mL, 0.36 mol) and CTAB (0.28 g, 0.768 mol) were mixed and stirred at room temperature, and the pH was respectively adjusted to 10 /10.5/11 using Tris (2-Hydroxyethyl) Amine. After stirring at 60°C for 45 min, TEOS (0.73 mL, 3.25 mmol) was added slowly to the solution, then continuously stirred at 60 °C for 2 hours. The products were then centrifuged at 12000 rpm for 15 min, and the supernatant was removed. Then the residue were washed with pure ethanol and deionized water. CTAB was extracted with 28 mL of a solution of EtOH/HCl (5/2) for 2 h at 60°C. After centrifugation, the extraction procedure was repeated twice. The particles were centrifuged and washed with ethanol and water

respectively, and dried with vacuum dryer at -50 °C for 24 h.

2.3. Synthesis of MSN-NH₂ nanoparticles

First, 20 mg MSN was added to 20 mL N, N-Dimethylformamide (DMF), then 10 µL APTES was added rapidly. After stirring at room temperature for 24 h, the mixture were centrifuged at 12000 rpm for 15 min to remove the supernatant, following by washed with pure ethanol and deionized water to remove the unreacted APTES. Finally, the MSN-NH₂ were dried with vacuum dryer at -50 °C for 24 h.

2.4. Synthesis of UA conjugated MSN-NH₂ nanoparticles

5 mg UA was added to 10 mL DMF and then activated with 200 mg EDC and 100 mg NHS. After stirring at room temperature for 30 min, 10 mg MSN-NH₂ was added to the solution and then the mixture were stirred for a further 24 h. The product was collected by centrifugation, washed thoroughly with water, and dried with vacuum dryer at -50 °C for 12 h.

2.5. Characterization of MSN, MSN-NH₂ and MSN-UA nanoparticles

The morphology were characterized by a transmission electron microscope (TEM). The size distribution and zeta potential of the MSN and MSN-UA diluted in deionized solutions were measured by Zetasizer Nano ZS90 (Malver, USA). Nitrogen adsorption–desorption analysis was measured on an adsorption analyzer (V-Sorb 2800P, Beijing, China) Fourier transform infrared (FT-IR) spectra was measured on a FT-IR spectrometer (Bruker IFS 55, Fällanden, Switzerland) using the KBr pellet technique. The surface areas and pore size distribution were calculated according to Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) methods.

2.6. Preparation of UA loaded MSN nanoparticles and in vitro drug release

10 mg of MSN or MSN-NH₂ particles were suspended in 10 mL of 1 mg/mL UA DMF solution, respectively. After shaking for 30 min, the mixtures were stirred at room temperature for 24 h. Following, the nanoparticles were collected through centrifugation and washed with deionized water and then dried in vacuum dryer at -50 °C for 12 h and the resulting product was UA@MSN-NH₂/UA@MSN-UA. To evaluate the UA-loading efficiency, the supernatant and washed solutions were collected and The UA concentration was measured by UV-1750 at a wavelength of 210 nm.

To determine the in vitro release of UA from the UA@MSN-UA, 2 mg free UA, UA @MSN-NH₂ or UA@MSN-UA was resuspended in 1 mL PBS buffer (pH 5.5 or pH 7.4) containing 20% of ethanol at 37 °C and followed by incubation for 96 h. 500 uL particle suspensions were collected and centrifuged. The amount of UA in the supernatant was determined by UV–vis spectroscopy at wavelength of 210 nm at various time points.

2.7. Preparation of FITC labeled MSN-UA

FITC covalently conjugated to APTES by dispersing 3 mg FITC and 20 µL APTES in 3 mL ethanol in a dark condition and stirring for 24 h at room temperature. Then, 200 mg MSN was dispersed in 50 mL ethanol and ultrasonic for 30 min, then mixed with 3 mL FITC/APTES ethanol solution. After stirring under dark conditions for 24 h, the products were then centrifuged and washed with pure ethanol. FITC labeled MSN were reacted with APTES, and then UA was covalently conjugated to

the surface of MSN-NH₂ through an acid-labile amide bond.

2.8. Cell lines and cell culture

HepG2 human hepatocellular carcinoma cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HepG2 Cells were cultured at 37 °C with 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum.

2.9. Cellular uptake

HepG2 cells were seeded in 12-well plates at a density of 1×10^5 per well and incubated overnight. After the plates were washed twice with PBS, the cells were incubated with 100 µg/mL MSN or FITC labeled MSN-UA in 500 µL serum -free DMEM medium for 2 h at 37 °C. Subsequently, the cells in each well were washed with cold PBS for three times, after that, cells were stained with Hoechst 33342 for nuclei. At last, the cells were measured using a Leica confocal microscope (SP-8, Germany).

2.10. *In vitro* cell viability study

HepG2 cells were seeded in a 96-well plate at a density of 10000 cells per well and incubated for 24 h at 37 °C with 5 % CO₂. Then, MSN-NH₂ (5, 25, 50, 100, 150, 200, µg/mL), free UA, UA@MSN-NH₂, MSN-UA or UA@MSN-UA were added to the cells in DMEM medium at different UA concentrations of 1, 5, 10, 20, 30, 40 µg/mL, respectively, and incubated at 37 °C with 5 % CO₂ for 24 h. Subsequently, MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) solution was added to each well and the 96-well plate was incubated for another 4 h at 37 °C.

Then, the DMEM medium were removed and 100 μ L of dimethylsulfoxide (DMSO) was added to the well and shocked for 10 min. The optical density (OD) of each well was measured at 570 nm by TECAN Infinite F200 Microplate Reader.

2.11. Cell cycle analysis

HepG2 cells seeded in a 6-well plate at a density of 5×10^5 cells each well and incubated for 24 h at 37 °C with 5 % CO₂. UA, UA@MSN-NH₂, MSN-UA or UA@MSN-HA with UA concentrations of 10 μ g/mL was added to the well and incubated for 24 h at 37 °C with 5% CO₂. Then, the medium were removed and cells were washed twice with pre-cooling PBS, followed by fixing with 1 mL 70 % ethanol for 24 h at 4 °C. The ethanol was removed and cells were washed twice with pre-cooling of PBS, and then cells were stained with a mixture solution of 1 % Triton X-100, 0.01 % RNase, and 0.05 % PI for 30 min at 37 °C in the dark. Cell cycle was assessed by the FACS Aria III flow cytometer.

2.12. Cell apoptosis analysis

HepG2 cells were seeded in a 6-well plate at a density of cells each well and incubated for 24 h at 37 °C with 5 % CO₂. The cells were treated with UA, UA@MSN-NH₂, MSN-UA or UA@MSN-UA with UA concentration of 10 μ g/mL for 24 h. Then, the treated cells were washed three times with PBS. Subsequently incubated with 5 μ L Annexin V-FITC and 5 μ LPI detection for 10 min. Finally, the samples were analyzed by FACS.

2.13. Statistical ananalysis

All data are presented as meas $\pm$ standard errors. The student t-test and one-way

analysis of variance using least significance difference test (IBM SPSS Statistics 19.0).

3. Results and discussion

3.1. Preparation and characterization of UA@MSN-UA

The synthetic procedures of UA@MSN-UA is summarized in Figure 1. Firstly, MSN nanoparticles were synthesized and the size were optimized, and CTAB was removed from pore of MSN by anhydrous ethanol and concentrated hydrochloric acid. Then, the surface of MSN was modified with an amino group by reacting with APTES. Afterwards, UA was conjugated to the surfaces of MSN-NH₂ by amino and carboxyl condensation. Finally, UA was further loaded in MSN-UA with a physical method.

The size and features of the MSN was characterized by TEM image (Figure 2 A). As shown in Figure 2 A, the synthesized MSN under the pH 10 was approximate spherical nanoparticles with an average diameter of about 90 nm, and a pile of orderly micropores could be distinctly browsed in the MSN. The size diameter (Figure 2 C, D, E) under the condition of different pH of MSN was measured by dynamic light scattering (DLS) (Fig. 3 B). The diameter of MSN was 102.2 ± 6.5 , 141.7 ± 3.7 and 219.9 ± 4.6 nm at the pH of 10, 10.5 or 11, respectively. Tris (2-hydroxyethyl) amine was thought to act as a complexing agent for silicate species and additionally as a growth inhibitor for mesoporous particles, limiting the growth and aggregation of particles (Moller et al., 2007; Wu et al., 2013). Under alkaline conditions, silicates with a high negatively-charged density can only assemble with the cationic

surfactants through strong electrostatic interaction, but a lower pH gave a smaller size of MSN because of the increased condensation rate and thus more nuclei (Lin and Mou, 2002; Wu et al., 2013). The MSN nanoparticles we used in our all section of experiments was synthesized under the condition of pH 10, due to the optimum nanoparticle size (around 100 nm) for the cancer cell uptake and endocytosis. All the MSNs were further characterized by nitrogen adsorption-desorption. Figure 3 A showed a typical type IV isotherm loop, which was the typical characteristic of a porous structure according to the International Union of Pure and Applied Chemistry (IUPAC) classification. The detailed textural characteristics were listed in Figure 3 B. For instance, the surface area, pore diameter and pore volume of MSN under the condition of pH 10 were 1090.9 m²/g, 3.43 nm, and 0.953 cm³/g, respectively. Under potential of hydrogen (pH) 10.5, these parameters were 971.4 m²/g, 3.81 nm, and 0.809 cm³/g, respectively. When pH increase to 11, these parameters become 852.1 m²/g, 3.42 nm, and 2.046 cm³/g, respectively. From the foregoing, we can find that with the value of pH increasing, the particle size of MSN increases, while the surface area decreases gradually and the pore volume increases, however, the pore diameter remained steadily.

The successful grafting of MSN-UA was validated by different methods. As shown in Figure 2 B, the MSN were synthesized under the pH 10, then the particles were centrifuged and washed with ethanol and water, the synthesis of other nanoparticles is not regulated by pH. Zeta potential of the MSN, MSN-NH₂ and MSN-UA diluted in deionized water solutions (neutral pH) were measured. the zeta

potential of MSN was reversed from a negative value of -22.3 mV to a positive one of + 24.5 mV after the formation of MSN-NH₂ due to the amine groups on the silica surface. After grafting UA, the zeta potential was reversed again to a negative one of -34.6 mV, which confirming the successful grating process. A comparison of the FT-IR spectra of of MSN, MSN-NH₂, UA, MSN-UA and UA@MSN-UA is shown in Figure 4. The presence of the Si-OH on MSN was confirmed by the minor peak around 962 cm⁻¹. After MSN reacted with APTES, the appearance of a minor peak around 1539 cm⁻¹ was characteristic of the amino groups, indicating the formation of MSN-NH₂. The surface grafting of MSN-UA onto the MSN-NH₂ surface was validated by the appearance of a N-H stretching band at 1557 cm⁻¹ and C=O stretching band at 1741 cm⁻¹, indicating MSN-UA was synthesized from the reaction between UA and MSN-NH₂ using EDC/NHS between carboxyl group of UA and amino group of MSN-NH₂.

3.2. Drug release of free UA, UA@MSN-NH₂ and UA@MSN-UA in buffer solutions at pH 5.5 and 7.4.

To exploit whether the UA@MSN-UA prodrug nanoparticles can be an effective drug delivery system for sustained release of UA, we performed the drug release experiment under pH 5.5 or pH 7.4 at different time intervals. The encapsulation and drug loading efficiencies for UA loaded with MSN-NH₂ and MSN-UA were > 80 % and > 22 %, respectively. The result was shown in Figure 5. Compared to the rapid release of free UA, both both UA@MSN-NH₂ and UA@MSN-UA exhibited similar sustained release of UA at pH 7.4 and pH 5.5. The release rate of free UA and

UA@MSN-NH₂ at pH 7.4 showed significantly higher than at pH 5.5. But we can see from the profiles that the release rate of UA@MSN-UA at pH 7.4 showed significantly lower than that at pH 5.5, and the UA undergoes rapid appearance followed by a sustained release phase, indicating that under this condition the acid-labile amide bond is quickly hydrolyzed (about 76 % of the UA appears during a 96 h incubation). On the other hand, at pH 7.4, the hydrolysis of the acid-labile amide bond linkage between MSN and UA is slower as compared to the previous case. After 96 h of incubation, free UA is about 53 % of the total initial drug. This result demonstrated that UA@MSN-UA is more able to release UA in acidic environment (like tumor tissue) compared to neutral conditions (like blood).

3.3 Cellular uptake of MSN-UA

To visualize the cellular uptake performance of MSN nanoparticles, FITC labeled MSN-UA were prepared and studied by confocal microscopy. As shown in Figure 6. The images are shown from left to right as follows: Hoechst 33342 channel (blue), FITC channel (green), the merge of the two channels and enlarged view from merge. HepG2 cells in control group only showed the blue color of the nuclei after staining with Hoechst 33342, and the cell nuclei are complete without damage. When the cells were incubated with FITC labeled MSN-UA, strong green fluorescent signals originating from FITC were detected inside the HepG2 cells, indicating that MSN-UA nanoparticles is able to permeate by passive diffusion efficiently and taken up fast by HepG2 cancer cells.

3.3. In vitro cell viability study

The growth inhibition of HepG2 cells was observed with the cells treated with the suspension of MSN-NH₂, Free UA in PBS or in DMSO (5%), UA@MSN-NH₂, MSN-UA and UA@MSN-UA, respectively. As shown in Figure 7, HepG2 cells treated with MSN-NH₂, UA in PBS, UA in DMSO, UA@MSN-NH₂, MSN-UA and UA@MSN-UA showed a UA dosage dependent behavior, respectively. The blank MSN-NH₂ had a negligible reduction in the cell viability with increasing the concentration from 1 to 200 µg/mL, indicating that MSN-NH₂ are excellent biocompatible nano-carrier. Free UA in PBS group exhibited weak cell permeability and negligible cell growth inhibition. The UA@MSN-UA NPs group showed more cytotoxic to HepG2 cells compared to free UA in PBS, UA in DMSO, UA@MSN-NH₂ and MSN-UA groups. Interestingly, the extent of UA@MSN-UA NPs inhibition was comparable to that observed using UA dissolved in DMSO at lower concentrations (1~20 µg/mL). This might be due to UA loaded MSN-UA and UA in DMSO have the similar cellular uptake when UA at the low concentrations. The above results indicated that UA encapsulated MSN-UA NPs (UA@MSN-UA) drug delivery system exhibits improved drug bioavailability and enhanced permeability and retention (EPR) effect in the favour of passive tumor targeting, and the carrier MSN-UA also affects the viability of cells due to UA could release easily from the intracellular prodrugs by cleavage of the amide bond to exert anti-cancer activity.

3.4. Cell cycle distribution

To further analyze the effects of UA@MSN-UA on the cell cycle distribution, the cell cycle study were performed. As shown in Figure 8, free UA, UA@MSN-NH₂,

MSN-UA and UA@MSN-UA were arrested at G0/G1 phase and G2/M phase in HepG2 cells under 10 $\mu\text{g/mL}$ concentration. The cell cycle distribution of UA@MSN-NH₂ showed no obvious difference with control. While cells treated with MSN-UA indicated a little more cells were arrested in G0/G1 phase and G2/M phase compared with control group. 24 h of individual incubation with UA and UA@MSN-UA led to a significant increase of cell population in G0/G1 phase and a decrease of cell population in S phase. Moreover, the percentage of cell population in G2/M phase induced by UA@MSN-UA was larger than that of UA or control group. The inhibition of cell cycle in G0/G1 phase and G2/M phase finally resulted in the down-regulation of captured HepG2 cells.

3.5. Cell apoptosis analysis

The cell apoptosis analysis of free UA, UA@MSN-NH₂, MSN-UA and UA@MSN-UA at UA concentration of 5 $\mu\text{g/mL}$ for 24 h were investigated. It can be observed from Figure 9 that free UA and nano-drug (UA@MSN-NH₂, MSN-UA and UA@MSN-UA) could significantly cause the early and late apoptosis in HepG2 cells, and compared with the free drug, the delivery system (UA@MSN-UA) can be more effective on cell apoptosis under the same drug concentration. Specifically, compared with control (2.74 %, 1.89 % for early apoptosis and late apoptosis, respectively), the early and late apoptosis rates of HepG2 cells treated with UA, UA@MSN-NH₂, MSN-UA and UA@MSN-UA were (4.77 %, 4.72 %), (3.50 %, 2.58 %), (4.86 %, 7.44 %) and (11.7 %, 19.4 %), respectively. And the mechanical damage of HepG2 cells treated with MSN-UA (21.9 %) or UA@MSN-UA (14.2 %) were obviously

higher than that of control (1.14 %), UA (1.78 %) or UA@MSN-NH₂ (3.01 %) group.

This phenomenon may be caused by different drug with electricity efficiencies. The results showed that UA@MSN-UA caused a markedly increased the cellular apoptosis.

4. Conclusion

In this study, a silica-based mesoporous nanosphere (MSN) controlled-release prodrug delivery system (UA@MSN-UA) that both covalent coupling and encapsulation by non-covalent interactions of UA was successfully synthesized. Nanoparticle prodrugs possess a near-spherical shape and small particle diameter (~100 nm). Nanoparticles display suitable specific surface area, pore diameter, pore volume and high drug loading capacity. The in vitro release results indicated that UA@MSN-UA exhibited sustained release profile in the initial 20 h. silica-based mesoporous nanosphere (MSN) controlled-release drug delivery system (UA@MSN-UA) could enhance anticancer efficacy on HepG2 cells by cell cycle arrest, and pro-apoptosis as UA@MSN-UA could be more easily taken in by HepG2 cells. All of the findings suggested that silica-based mesoporous nanosphere (MSN) controlled-release drug delivery system could be a promising drug delivery strategy for improving the anti-cancer efficacy of UA, and more importantly, provide a way for improving the bioavailability and extending the drug release of hydrophobic anti-cancer drug with a carboxylic acid moiety.

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Authors' contributions

JWS and TL designed research, wrote and revised the manuscript. XFC revised the manuscript. JWS and TL developed methodology, collected, analyzed and interpreted data; XFC, LLF, YJL, LQL, YX and SJC performed the experiments. TL acquired figures. JWS reviewed the paper.

Notes

No potential conflicts of interests were disclosed.

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Figure legends

Figure 1. Synthetic procedures of UA@MSN-UA.

Figure 2 Characterization of the nanoparticles: (A) TEM image of synthesized MSN under the pH 10 (scale:50 nm); (B) Zeta potential analysis of MSN, MSN-NH₂ and MSN-UA; (C) DLS analysis of MSN (pH=10); and (D) DLS analysis of MSN (pH=10.5). (E) DLS analysis of MSN (pH=11).

Figure 3 Nitrogen adsorption-desorption isotherms of MSN.

(A) The N₂ adsorption-desorption isotherms of MSNs in different pH value; (B) The structure parameters of MSNs in different pH value.

Figure 4 Fourier-transform IR spectra of MSN, MSN-NH₂, UA, MSN-UA, UA@MSN-UA.

Figure 5 In vitro release of UA. The cumulative UA release rates are compared between free UA, UA@MSN-NH₂ and UA@MSN-UA in PBS (pH 7.4 or pH 5.5) at 37 °C.

Figure 6 Confocal laser scanning microscopy images of MSN-UA taken up by HepG2 cells. HepG2 cells were incubated with FITC labelled MSN-UA (lower panel) or without any treatment as a control (upper panel) for 2 h at 37 °C. Then cells were incubated with hoechst 33342 for another 30 min at 37 °C. (60×, exposure factor was under 10).

Figure 7 Cell cytotoxicity assay. HepG2 cells were incubated with empty MSN-NH₂ or MSN-UA in PBS, free UA in DMSO or PBS, UA loaded MSN-NH₂ or MSN-UA in PBS, and the percentage of viable cells was compared to control wells without treatment. The concentration of MSN is shown on top of each figure, while that of UA

in DMSO, in PBS, or loaded in MSN nanoparticles, are shown under each figure.

Figure 8. Effects of UA, UA@MSN-NH₂, MSN-UA and UA@MSN-UA on the cell cycle distribution. (A) PI staining showed the cell cycle distribution induced by different drug of UA, UA@MSN-NH₂, MSN-UA and UA@MSN-UA. (B) Percentage of cell population in every stage (G0/G1, S, G2/M).

Figure 9. Cell apoptosis analysis. (A) Flow cytometry measurement of HepG2 cell apoptosis treated with free UA, UA@MSN-NH₂, MSN-UA and UA@MSN-UA with UA concentrations of 5 µg/mL. Apoptosis was determined by Annexin-V-FITC/PI labeling. (B) The numbers of viable cells, necrotic cells, early apoptosis cells and late apoptosis cells were expressed as a percentage of the total number.

Figure 1

Figure 2

Figure 3

A

B

pH	Diameter (nm)	Specific surface (m ² /g)	Pore diameter (nm)	Pore volume (cm ³ /g)
10	102.2±6.5	1090.9	3.43	0.953
10.5	141.7±3.7	971.4	3.81	0.809
11	219.9±4.6	852.1	3.42	2.046

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9