

Multi-functionalizations of Hollow Mesoporous Silica Nanospheres and Their HIFU Application

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ABSTRACT

High intensity focused ultrasound (HIFU), as a representative non-invasive therapeutic mode, has attracted extensive attentions among biological and medical researchers. In this paper, we focused on constructing multifunctional hollow mesoporous silica nanospheres (HMSNs) as innovative and efficient theranostic/synergistic agents to explore them in HIFU application. First, Au NPs-coated, PFH-encapsulated and PEGylated mesoporous silica nanocapsules-based nano-platform has been constructed as a contrast-intensified ultrasound (US) imaging agent and simultaneously an intensified US-guided HIFU enhancement agent. Besides, a novel temperature-responsive theranostic system is designed and investigated, using L-menthol as phase-change medium, which enables the substantial co-entrapment of hydrophilic and hydrophobic drugs and their temperature-responsive release, and more importantly, also can be used as both the US contrast agents and HIFU therapeutic enhancement agents.

Keywords: high intensity focused ultrasound, mesoporous, hollow silica, multifunctionalization, ultrasound contrast

1 INTRODUCTION

Recently, the minimally invasive techniques using a range of energy-based methods for *in situ* tumor destruction with largely diminished blood loss and infection, elimination of scar formation and decreased risks of other complications have attracted much focus. Therein, High intensity focused ultrasound (HIFU) is one of interesting noninvasive treatment strategies with low cost and short recovery time compared with other counterparts, whose ultrasound (US) energy passes harmlessly through overlying tissues enroute to a strictly focused target area inside the body with rapid energy deposition and consequently irreversible cell death. However, the unsatisfactory safety and accuracy issues of HIFU have emerged as the primary concerns by both scientists and clinicians. Therefore, highly efficient image-guided HIFU therapy by using image contrast agents (CAs) and HIFU enhancement agents (EAs) are urgently recommended to further enhance the focusing accuracy, therapeutic efficacy and to avoid potential damages to normal tissues. The commercial organic microbubbles or liposomes as US imaging contrast agents, e.g. lipid-coated perfluoropropane microbubble agent, Levovist, Sonovue and Optison et.al,

can be applied to intensify the US contrast and HIFU ablation efficacy because of bubble-induced heat and acoustic effects. Nevertheless, both the limited circulatory life time and low heat-resistance as well as reluctant accumulation in target organs due to their large micrometer size, present great obstacles to the sufficient and accurate responses to the HIFU energy input. Very recently, a mesoporous silica nanocapsule-based inorganic HIFU enhancement agent (MSNC-PFH) [1] and magnetic resonance imaging (MRI)-guided HIFU cancer surgery enhancement agent (Mn-loaded MCNCs) [2] have been developed in our group, which demonstrated effective MR imaging and HIFU therapy due to its high stability, efficient perfluorohexane (PFH, with a favorable phase transition temperature of 56 °C) loading and release, enhanced tumor ablation capability and easy uptake by target tissues.

However, even though these results are encouraging, the concept of ablating tumors noninvasively and sparing surrounding normal tissues simultaneously are still in their infancies. In order to further intensify the ultrasound imaging and enhance the HIFU theranostic effects, herein, we aim to construct an Au NPs-coated, PFH-encapsulated and PEGylated mesoporous silica nanocapsules-based nano-platform (MSNC@Au-PFH-PEG, abb. as MAPP), for use as a contrast-intensified US imaging agent and simultaneously an intensified US-guided HIFU enhancement agent as well. In addition, L-menthol was selected and regarded as an ideal medium to facilitate the execution of multi-functionalizations (temperature responsive and controllable release) (HMSN-LM). These constructed brand-new HMSN-based nano-carriers have several functions: (1) co-entrapment of hydrophobic and hydrophilic drugs, (2) temperature-responsive release behavior, and (3) enhanced ultrasound imaging and HIFU therapeutic efficacy were achieved.

2 EXPERIMENTAL SECTION

All reagents were used without further purification. Deionized water was used in all experiments.

2.1 Synthesis of Au NPs anchored Mesoporous Nanocapsules (MSNC@Au) and Perfluorohexane Encapsulation

Uniform 250 nm mesoporous silica nanocapsules (MSNCs) with a typical 50 nm shell were prepared using a structural difference-based selective etching protocol

developed in our previous reports [3]. In details, 100 mg MSNCs was reacted with 200 μL MPTMS in 100 mL isopropanol and the mixture were refluxed at 80 $^{\circ}\text{C}$ overnight. After centrifugation with ethanol, SH modified MSNCs (MSNC-SH) were dispersed in 30 mL water. 50 mL HAuCl_4 solution (3×10^{-4} M) was prepared and the pH value was adjusted to around 9.5 by 0.01 M NaOH solution. Then, 10 mL of as-obtained MSNC-SH solution was added and reacted with above HAuCl_4 solution. 0.01 M fresh NaBH_4 was added dropwise until the solution turned purple red. Au NPs coated MSNCs (MSNC@Au) formed through in situ reduction of Au^{3+} by NaBH_4 and simultaneously attached to the outer surface of MSNC-SH. After another 10 h stirring, the resulting solution was centrifuged at 10000 rpm for 20 min. The MSNC@Au precipitate was collected and dried under vacuum at room temperature for further use.

In the absence of water, 50 mg MSNC@Au stored in a 5 mL bottle was infused dropwise with 150 μL highly echogenic PFH liquid. Thereafter, the bottle was lidded with scotch tape and capped tightly to prevent the volatilization of PFH. 2 min sonication in ice water was then performed to facilitate the loading process in mesoporous shell and hollow core (MSNC@Au-PFH). Then MSNC@Au-PFH was re-dispersed in 25 mL deionized water under slight magnetic stirring. 5 mL of 10 mg/mL mPEG5000-SH aqueous solution was added dropwise into the above solution and stored for another 2 h at room temperature. After centrifuged at 10000 rpm for 5 min, the as-obtained sample (MSNC@Au-PFH-PEG, MAPP) was dispersed in 8.3 mL PBS solution to cap sensitive PFH and immobilize the enhancement agent system during the transportation and experimentation.

2.2 Loading HMSNs with L-menthol & single or mixed Dyes & CPT-11

Typically, the HMSNs (80 mg) well dispersed in methanol (5 mL) were added to a dye solution in 1-tetradecanol at 80 $^{\circ}\text{C}$. Even after the methanol had been evaporated due to stirring and heating, the HMSNs were still well dispersed in the liquid LM. During this process, the mixture of LM and dye molecules slowly entered the hollow interior of each HMSN by diffusion through the mesopores on the surface and these hollow interiors of HMSN were completely occupied. After 4 h, HMSN-LM-dyes were centrifuged with hot DI water at 13,000 rpm for 15 min to obtain the HMSNs loaded with L-menthol and dyes. The retrieved HMSN-LM-dyes were washed with cold deionized water at least 8 times before the release test.

2.3 HIFU *in vitro* Exposure on Degassed Bovine Livers

A JC HIFU tumor therapy system (Chongqing Haifu Technology, China) was employed for the *in vitro* exposure characterization. In the experiment described below, the

therapeutic and diagnostic frequency was set at 3.5 and 1.1 MHz, respectively. A therapeutic transducer with a diameter of 220 mm and a focal length of 145 mm was fixed at the bottom of a tank filled with degassed water. A diagnostic transducer was localized in the center of the therapeutic transducer; thus, tissues in the path of therapeutic ultrasound waves could be viewed in diagnostic ultrasonic images. Ultrasonography was used to guide HIFU treatment and monitor therapeutic effects in real time. The bovine livers *in vitro* (10 \times 8 \times 5 mm) were prepared and underwent a degas treatment for 60 min. Before exposure, the bovine liver was transferred into a tank filled with degassed water on the HIFU system. MAPP, HMSN-LM (0.3 mL, 7.5 mg $\cdot\text{mL}^{-1}$ in PBS) was injected with an injection depth of 26 mm. By employing different power outputs (70 w for 10 s, 120 w for 5 s), hyperechoes were detected and the pre-and post-gray scale values were recorded using software GrayVal 1.0 (Chongqing Haifu Technology, Chongqing, China).

3 RESULTS & DISCUSSIONS

3.1 Au NPs Intensified Mesoporous Silica Nanocapsules for Ultrasound-guided HIFU Enhancement Agent

Uniform MSNCs with an average diameter of 250 nm and mesoporous shell thickness of 50 nm have been synthesized as shown in Figure 1 (a₁-a₃). The fine and versatile Au NPs conjugated MSNCs (MSNC@Au) were prepared by thiol-Au chemistry with pH=9.5 (Figure 1 b₁-b₃). In detail, the surfaces of MSNCs were functionalized with S-H groups (0.432 mmol/g) before being dispersed in HAuCl_4 solution. Au NPs (5-10 nm and 63.5 $\mu\text{g}/\text{mg}$ of MSNCs) were formed through in situ reduction of Au^{3+} by NaBH_4 and simultaneously attached to the outer surface of MSNCs.

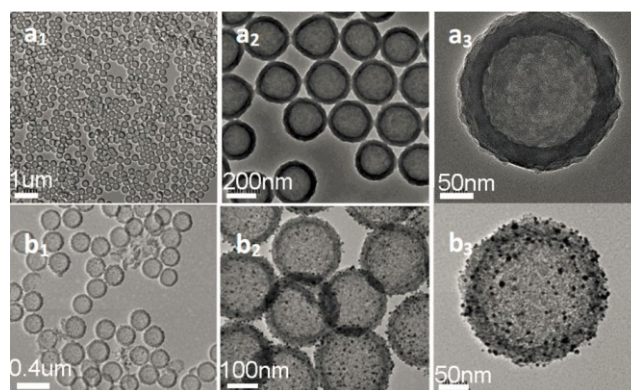


Figure 1: Typical TEM images of MSNCs (a₁-a₃), MSNC@Au (b₁-b₃).

N_2 adsorption/desorption isotherms of MSNCs and MSNC@Au show a typical MCM-41 structure (type IV)

with BET surface area of 449 m²/g, 230 m²/g and pore volume of 0.67 cm³/g, 0.40 cm³/g, respectively. The corresponding pore size distribution curve with average diameter of 4.6 nm and 4.5 nm for MSNCs and MSNC@Au respectively were obtained.

To investigate the contrast-intensified ultrasound imaging of MAPP, PBS control, MSNC@Au-PEG (MAP) and MAPP in PBS solution (both 2 mL at 6 mg/mL) were wrapped up by a plastic layer and immersed in the degassed-water tank for the following ultrasound imaging *in vitro* under different modes (contrast and harmonic) (Figure 2, left). An obvious and significant enhancement in average grey scale for MAPP and MAP, comparing to the PBS control was detected both under contrast mode (with the values of 105 dB, 77 dB to 10 dB) and under harmonic mode (with the values of 176 dB, 146 dB to 6 dB), respectively.

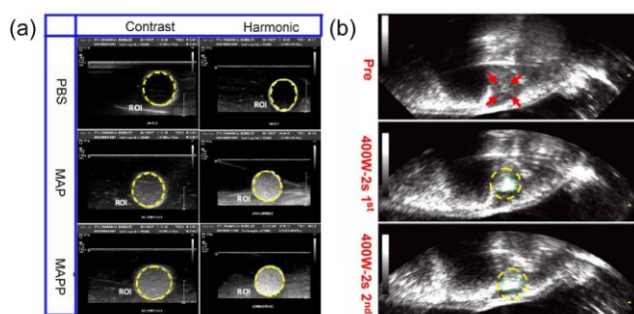


Figure 2: US imaging *in vitro* (a) for PBS control, MSNC@Au-PEG (MAP) and MAPP under different modes (contrast and harmonic), and (b) typical *in vivo* B-mode ultrasonic images before (top) and after HIFU exposure on rabbit liver VX2 tumors using 2 mL of 6 mg/mL MAPP after US irradiation at 400 W for 2 s for once (middle) and twice (bottom). The echogenic changes of tumor before and after ablation were marked by dotted line.

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The evaluation of MAPP to enhance the tumor ablation by HIFU *in vivo* was therefore further carried out on rabbit liver VX2 tumors using 2 mL of 6 mg/mL MAPP at 400 W for 2 s (Figure 2, right). In about 30 min post-injection of MAPP, the tumor profiles could be detected under the diagnostic B-mode ultrasound. The therapeutic results of MAPP were monitored by variation of gray scale on focus

of tumor. Significant enhancement in gray scale of using MAPP by HIFU ablation at 400 W for 2 s one time (139 dB) and twice (188 dB), as compared to nearly 0 dB before ablation, implies an increased necrosis of malignant tumor with this enhancement agent.

3.2 Multifunctional Temperature-responsive Ultrasound imaging Guided-HIFU Theranostic System

The illustration of the temperature-responsive theranostic system was schematically shown in Figure 3a. The prepared HMSNs exhibited well-defined morphology, monodispersity and tunable surface area, particle size and shell thickness by altering the process parameters.

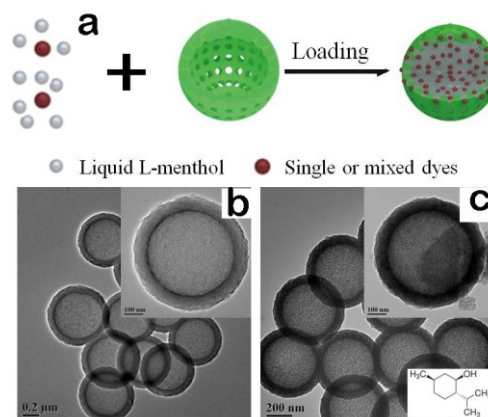


Figure 3: (a) Schematic illustration of loading dyes-doped L-menthol into hollow space of HMSNs. (b, c) Typical TEM images of HMSNs before and after loading with a mixture of L-menthol and dyes (R6G or MB or both), and the inset in c was the molecular formula of L-menthol.

Figure 3b and 3c show the typical TEM images of HMSNs before and after loading of the L-menthol, and distinct contrast differences between HMSN and HMSN-LM could be obtained, indicating that the L-menthol was successfully loaded into the hollow interiors of HMSNs. The FTIR spectrum of HMSN-LM confirms the successful loading of L-menthol. In order to investigate the co-entrapment and temperature-responsive release behaviors of hydrophilic and hydrophobic molecules, rhodamine 6G (R6G) and methylene blue (MB) with different solubility in water were taken as two examples of drug models, because their release behaviors could be easily monitored and quantified by the UV-Vis absorption spectroscopy. Since LM can reversibly change its physical states between solid and liquid phases on environment temperature variation around its melting point, the dyes can be co-loaded with liquid LM into HMSNs at higher than the melting point, and perfectly confined inside the HMSNs by solid LM below the melting point, due to its immiscibility with water. When the local temperature was raised beyond 43 °C, the entrapped dyes would flow out from HMSNs together with

the melted liquid LM. More importantly, during the phase-transition process from solid to liquid state, LM absorbed large amount of heat, that is, it retained large amount of heat energy in liquid state, which is useful for the next-mentioned enhanced HIFU therapeutic efficacy. In short, all these functions in this new system can be achieved merely by remotely manipulating the local milieu temperature.

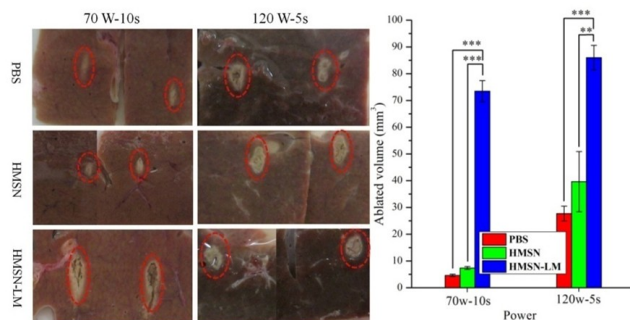


Figure 4: Digital photos of ablated bovine livers at 70 W for 10 s and 120 W for 5 s (left) and the corresponding necrotic volumes (right) after injection of 0.3 mL PBS control, MSNCs, and MSNC-PFH (each column is the average of two data). ** and *** represent significant differences in necrotic volumes found by comparing HMSN-LM with HMSNs or the PBS control at $P \leq 0.005$ and $P \leq 0.0005$, respectively.

To evaluate the enhanced HIFU therapeutic efficacy, the *ex vivo* coagulative necrosis experiments were conducted on degassed bovine livers, and the enhanced ablations of using HMSN-LM as a TEA could be found *via* comparing the gray scale values among control (PBS), HMSNs and HMSN-LM under real-time B-mode ultrasound imaging, as can be found in Figure 4 of the corresponding quantitative necrosis volume. When exposed to HIFU irradiation at a relatively low power output of 70 W for 10 s, HMSN-LM generated the most prominent contrast enhancement and the largest ablated volume (bottom in Figure 4, more than 70 mm³) can be found. Since the transient temperature at HIFU focus at 70 W for 10 s was determined to be around 50 °C, seven degrees above the melting point (43 °C), the encapsulated solid LM in HMSNs could liquidize, and the LM gas bubbles will be generated *via* vaporization of liquid LM under the HIFU irradiation. Similar *ex vivo* results could be obtained in bovine livers under enhanced power input of 120 W for 5 s, as shown in Figure 4. Noticeably, more profound contrast enhancements could be found between pre- and post-HIFU exposures at 120 W for 5 s than that at 70 W for 10 s, which was due to higher HIFU energy and the significantly higher temperature of 60°C reached at 120 W. All ablated volumes were calculated and significant difference (P value) of necrosis volume existed between using HMSN-LM and HMSNs, HMSN-LM and PBS, respectively.

Furthermore, to conveniently and efficiently evaluate *in vivo* HIFU enhanced therapeutic effects of HMSN-LM,

normal liver tissues of New Zealand healthy white rabbits were employed. After exposure to HIFU irradiation at 300 W for 2 s under the monitoring with a B-ultrasonic imaging system, the largest gray scale change of using HMSN-LM was found indicative of the strongest echo-signal.

4 CONCLUSION

In summary, this paper describes two kinds of multifunctional hollow mesoporous silica nanospheres as innovative and efficient theranostic/synergistic agents on HIFU therapy. Such multifunctional theranostic systems demonstrated distinctively enhanced ultrasound imaging and HIFU therapeutic efficacy owing to the remarkable thermal accumulation effects and PFH bubble cavitations, contributed by the capped Au NPs, as well as the controllable and unique phase-transition features of L-menthol under the mediation by HIFU irradiation. Therefore, those systems show great potentials in future cancer diagnosis and therapy, especially in overcoming multi-drug resistance and local controlled drug release in an on-demand fashion.

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