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Surface-engineered nanobubbles with pH-/light-responsive drug release and charge-switchable behaviors for active NIR/MR/US imaging-guided tumor therapy

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Abstract

Active drug-loaded nanocarriers have been widely employed as efficient drug delivery systems for tumor theranostics. Herein, we report folate-mediated “all-in-one” nanobubbles for tumor-targeted NIR/MR/US imaging and combined chemo-photothermal therapy. The surface-engineered nanobubbles are constructed from oleylamine-/IR-780-loaded hollow structures, folate and the Gd^{3+} DTPA-BSA@5-FU complex via electrostatic adsorption and further filled with gas after freeze drying. DLS data show that the nanobubbles have a hydrodynamic diameter of 120.41 ± 18.30 nm. TEM observations show a hollow inner cavity and a shell thickness of approximately 10 nm. The relaxivity (r_1) of the nanobubbles reaches $16.56 \text{ s}^{-1}/\text{mM}$, indicating suitable features for use as a T1-weighted MR contrast agent. Moreover, due to the gas core inside, the nanobubbles are suitable for ultrasound contrast imaging. Interestingly, ξ -potential data and cumulative release measurements demonstrate that the nanobubbles undergo charge-switchable behaviors and pH-/light-sensitive drug-release behaviors after surface engineering, which could facilitate deep tumor penetration and accelerate drug release for efficient killing of cancer cells. In vivo trimodal imaging and chemo-photothermal therapy for MGC-803 tumor-bearing mice reveal selective tumor accumulation, long tumor retention, and enhanced antitumor behaviors. Therefore, the all-in-one nanobubbles could be applied for active tumor-targeting theranostics.

Introduction

To enhance anticancer efficacy and reduce the adverse side effects of anticancer drugs, drug-loaded nanocarriers have been employed as an efficient medium for cancer therapy^{1,2}. There are three main strategies for constructing excellent drug-loaded nanocarriers: (1) endowing nanocarriers with active cancer-targeting properties; (2)

endowing visual tumor-location capacity; and (3) endowing controlled drug-release capacity in response to the tumor microenvironment or external stimulating signals^{3–7}. Therefore, complete drug-loaded nanocarriers with enhanced tumor accumulation, integrated functions for diagnosis and therapy, efficient drug release and deep tumor penetration capacity are required.

Usually, surface modification is required to construct a reasonable and multifunctional drug-loaded nanocarrier. Generally, the surface modification progress leads to improved pharmacokinetics and pharmacodynamics profiles^{8–10}. Based on this strategy, charge-switchable nanoparticles has been developed as a kind of efficient nanocarrier^{11–14}. These nanoparticles can change surface charge from negative to positive in response to external

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stimuli, such as tumor microenvironment pH (6.0–7.0) or endosomal/lysosomal pH (4.0–6.0). The positively charged surface can be utilized for enhanced cellular uptake due to the strengthened interaction between the nanoparticle and the cellular membrane. In addition, during the charge-shifting process, the loaded drugs tend to be released due to the change in environmental pH. These strategies always focus on pH-responsive zwitterionic polymer-based nanoparticles^{15–17}. Yuan et al.¹⁸ reported charge-switchable nanoparticles based on a zwitterionic polymer that showed prolonged circulation time and nonspecific protein absorption properties under physiological conditions; once accumulated in tumor tissue, the zwitterionic polymer-based nanoparticles shifted to positive charge by diminishing the anionic part, which facilitated cellular uptake and therefore enhanced the therapeutic effect. Zhang et al.¹⁹ reported a pH-/thermal-/glutathione-responsive polymer zipper consisting of cell-penetrating poly(disulfide)s and thermosensitive polymers bearing guanidinium/phosphate (Gu⁺/pY[−]) motifs to spatiotemporally tune the surface composition of nanocarriers for precise tumor targeting and efficient drug delivery. However, although those zwitterionic polymers could efficiently enhance cellular uptake and drug release, the required materials are usually difficult to fabricate, which limits their broader application. Therefore, a facile method for fabricating charge-switchable nanoparticles for efficient drug delivery is needed.

Moreover, to improve the visualization of the tumor location and the distribution of drug-loaded nanocarriers, various imaging modalities have been employed for tumor imaging using corresponding contrast agents. Among all imaging methods, fluorescence imaging is the most common method for tracing the distribution of nanocarriers in real time, but has limited tissue penetration depth and low resolution^{20–22}. Magnetic resonance imaging (MRI) is a powerful imaging technique with high spatial resolution and deep tissue penetration but is limited by low sensitivity^{23–25}. Ultrasonic imaging (US) is a safe and convenient imaging tool with high sensitivity and high resolution, especially for structures and boundaries of soft tissue but is prone to interference^{26–28}. Therefore, a nanocarrier with multimodal imaging abilities could offer more precise position of tumor sites for further cancer treatment.

The anticancer drug 5-fluorouracil (5-FU) blocks DNA synthesis. It is widely used in the treatment of a variety of solid tumors such as colorectal cancer, stomach cancer, and breast cancer in the clinic due to relatively strong cell-destruction characteristics²⁹. However, its relatively fast metabolic rate, poor selectivity, high drug resistance, and adverse side effects limit its widespread use. IR-780 iodide is a near infrared (NIR)-absorbing photothermal agent that can be utilized in fluorescence contrast imaging and

photothermal therapy (PTT) with laser irradiation^{30,31}. However, PTT only allows local treatment and limited depth penetration. Therefore, a novel combined therapeutic strategy is required to overcome the disadvantages of these molecules.

In this work, we report folate (FA)-mediated, gadolinium (Gd³⁺)-labeled and IR-780/5-FU-loaded nanobubbles for tumor-targeted NIR/MR/US imaging and combined chemo-photothermal therapy. Oleylamine and IR-780-loaded, poly(lactic-co-glycolic acid) (PLGA)-based hollow structures were first fabricated via the double-emulsion method as the core structure, and then folate and the Gd³⁺DTPA-BSA@5-FU complex were incorporated on the surface of the core structures via electrostatic adsorption. Finally, by freeze-drying, the hollow structures of the nanobubbles were filled with gas for US contrast imaging. Dynamic light scattering (DLS) data indicated that the nanobubbles had a diameter of 120.41 ± 18.30 nm, and TEM images showed that the nanobubbles possessed a hollow inner cavity with a shell thickness of approximately 10 nm. The relaxivity (r_1) of the nanobubbles reached $16.56 \text{ s}^{-1}/\text{mM}$ of Gd³⁺, indicating suitable features for use as a T1-weighted MR contrast agent. Compared with PBS, the gas-filled nanobubbles showed an enhanced ultrasonic signal, demonstrating their capacity for ultrasound contrast imaging. Importantly, ξ -potential data demonstrated that the nanobubbles underwent a charge-switchable progress when the pH value changed from 7.4 to 5.0. Under an acidic pH environment and laser irradiation, the nanobubbles exhibited pH-/light-sensitive drug-release behaviors. Furthermore, when coupled with FA-targeting, the nanobubbles were investigated for use in trimodal imaging (NIR/MR/US imaging) and combined chemo-photothermal therapy, and selective tumor accumulation, long tumor retention times and enhanced antitumor activity were observed. Therefore, these nanobubbles could be employed as excellent nanocarriers for active tumor-targeting theranostics.

Materials and methods

Materials

Bovine serum albumin (BSA), *N,N'*-dicyclohexylcarbodiimide (DCC), oleylamine, *N*-hydroxysuccinimide (NHS), triethylamine (TEA), and anhydrous dimethyl sulfoxide (DMSO) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Folic acid (FA) and gadolinium (III) chloride hexahydrate (GdCl₃·6H₂O) were received from J&K Chemical Ltd. (Shanghai, China). IR-780, 5-FU and diethylenetriaminepentaacetic acid dianhydride (DTPAA) were obtained from Sam Chemical Technology Co., Ltd. (Shanghai, China). Poly(lactic-co-glycolic acid) (PLGA-COOR, Mw 18000) was purchased from Jinan Daigang Biomaterial Co., Ltd. Polyvinyl

alcohol (PVA) was purchased from Sigma-Aldrich (St. Louis, USA). The Annexin V-FITC/PI Apoptosis Detection Kit, Cell Counting Kit (CCK-8) and Calcein-AM/PI Double Stain Kit were purchased from Yeasen Corporation (Shanghai, China). Hoechst 33342 was purchased from Sigma Chemical Corporation (USA). All other chemicals were of reagent grade. Water was purified with a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA).

Synthesis of Gd-DTPA-modified BSA (Gd^{DTPA} -BSA)

Briefly, 1 g of BSA was dissolved in 30 mL of $NaHCO_3$ solution (0.1 M) to form a clear solution, and then 1 g of DTPAA dispersed in 6 mL of anhydrous DMSO was added dropwise. After the pH value was adjusted to 8.5, the solution was stirred for 6 h at room temperature. Subsequently, the solution was dialyzed against 2 L of citrate buffer (0.1 M, pH 6.5), and then 0.5 g $GdCl_3 \cdot 6H_2O$ dissolved in 5 mL of Na-acetate buffer (0.1 M, pH 6.5) was added dropwise to the above dialyzed solution under stirring for 24 h. The resultant Gd^{DTPA} -BSA was purified by successive dialysis against citrate buffer (0.1 M, pH 6.5) and deionized water. The purified Gd^{DTPA} -BSA was obtained by freeze-drying the liquid to a solid. Finally, the Gd^{DTPA} -BSA solid and 5-FU (20:3, w/w) were mixed in deionized water and incubated overnight for further use.

Preparation of IR-780- and 5-FU-loaded Nanobubbles

The nanobubbles were fabricated via a water/oil/water double-emulsion solvent evaporation method. Briefly, 40 mg of PLGA, 5 mg of oleylamine and 1 mg of IR-780 were dissolved in 1.2 mL of chloroform, and 250 μ L of deionized water was added. After cooling to 4 °C, the two-phase mixture was treated with an ultrasonic homogenizer (50 W, 1 min) to form the first emulsion. Subsequently, the first emulsion was added to 5 mL of PVA solution (4%, w/v), and then the mixed solution was treated with a high-speed homogenizer for 1 min followed by treatment with an ultrasonic homogenizer at a power of 75 W for 3 min to form the second emulsion. After removing chloroform by evaporation, 0.5 mg of folate was added to the solution and stirred for 1 h, and the pH value was adjusted to 8.5. Subsequently, 2 mL of aqueous solution containing Gd^{DTPA} -BSA and 5-FU ($[Gd^{DTPA}\text{-BSA}] = 5$ mg/mL, $[5\text{-FU}] = 0.75$ mg/mL) was added and stirred for 1 h. Finally, the purified nanobubbles were obtained by centrifugation at a speed of 18,000 rpm and freeze-dried until further use.

The entrapment efficiency (EE) and drug-loading (DL) were calculated according to the following equations:

$$EE(\%) = ((\text{weight of loaded drug})/(\text{weight of initially added drug})) \times 100\%$$

$$DL(\%) = ((\text{weight of loaded drug})/(\text{total weight of nanobubbles})) \times 100\%$$

Characterization

The morphology of nanobubbles stained with uranyl acetate (2%) was assessed by transmission electron microscopy (Thermo Fisher Scientific Inc., Waltham, MA, USA). The size and ξ -potential of the nanobubbles were measured by dynamic light scattering (DLS) with a NanoBrook Omni Zeta/PLS instrument (Brookhaven instrument Co., Ltd., New York, USA). UV-vis spectra were recorded with a Varian Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA). FL spectra were recorded on a Hitachi FL-4600 spectrofluorometer (Hitachi, Co., Ltd., Tokyo, Japan). The Gd^{3+} content of the nanobubbles was determined by inductively coupled plasma optical emission spectrometry on a Thermo Scientific ICAP 7600 SENES (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Drug release measurement

5-FU has a maximum absorbance at $\lambda = 266$ nm and was quantified in a Varian Cary 50 UV-vis spectrophotometer following construction of a calibration curve. The 5-FU release behavior of the nanobubbles was assessed in different pH solutions (pH 5.0, 6.5, or 7.4) at 37 °C, and the cumulative released amount of 5-FU was quantified at different times. Then, the cumulative released amount of 5-FU (pH 5.0) under 808-nm laser irradiation (1.0 W/cm², 5 min) was quantified at different times.

Photothermal effects of nanobubbles

Dispersed solutions of nanobubbles with different concentrations of IR-780 (12, 6 μ g/mL) in a centrifuge tube were irradiated by laser irradiation for a specified time. Deionized water was irradiated as a control. The temperature changes at different times were monitored by an infrared camera and analyzed using IR Flash thermal imaging analysis software.

In vitro US imaging

US imaging of the nanobubbles in vitro was carried out using a gel mold. Nanobubbles at different concentrations were imaged at a frequency of 40 MHz. Deionized water was imaged as a control, and the samples were scanned using an ultrasonic diagnostic instrument (Prospect 3.0 High Frequency Ultrasound Imaging, S-Sharp) in conventional B mode.

In vitro MRI

To determine the stability of the nanobubbles as T1-weighted contrast agents, the nanobubbles were dialyzed against different environments (pH 5.0, 6.5, 7.4, and 50 °C (pH 5.0)) for 12 h, and the residual Gd^{3+} was determined by inductively coupled plasma optical emission spectro-

metry on a Thermo Scientific ICAP 7600 SENES. In vitro T1-weighted MR images of nanobubbles with different Gd^{3+} concentrations were obtained using an MRI system (MesoMR23-060H-I; Shanghai Niumag Corporation, China). Deionized water was imaged as a control. The measurement parameters were set as follows: repetition time/echo time (TR/TE) = 1150/11.5 ms; matrix acquisition = 256×192 ; number of excitations = 8; field of view (FOV) = 80 mm $\times$ 80 mm; FOV phase of 40 %, thickness = 5.0 mm; 0.5 T, and 32 °C.

In vitro time-dependent fluorescence activity

The MGC-803 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% (v/v) FBS (Gibco) at 37 °C (5% CO₂) for cell studies.

MGC-803 cells (1×10^5 /well) were cultured in 1 mL of culture medium in 12-well plates. Nanobubbles (5 mg/mL of IR-780) in DMEM with a pH of 7.4 or 6.5 were incubated with or without the attached cells for 0.5 h, 1 h and 2 h at 37 °C. Fluorescence images were then captured using a Bruker In Vivo F PRO imaging system (Billerica, MA, USA). The excitation wavelength was 720 nm, and emission was monitored at 790 nm.

Cellular uptake

For the cellular uptake study, MGC-803 cells (1×10^4 /well) were cultured in 24-well plates to adhere and treated with nanobubbles or IR-780 ([IR-780] = 4 μ g/mL) for the indicated times. After washing with PBS twice, the cells were stained with Hoechst 33342 and imaged by a TCS SP8 confocal laser scanning microscope.

To further measure the targeting ability of the nanobubbles, MGC-803 cells and GES-1 cells (0.5×10^3 /well) were cultured in 96-well plates to adhere and treated with nanobubbles ([IR-780] = 4 μ g/mL) for 1 h. After washing with PBS twice, the cells were imaged by a fluorescence microscope.

Cytotoxicity study

The cytotoxicity of the nanobubbles was measured to evaluate the synergetic therapeutic efficacy. MGC-803 cells (5×10^3 /well) were cultured in 96-well plates to adhere and incubated with fresh medium containing nanobubbles, 5-FU, or IR-780 ([IR-780] = 1, 2, 4, 6 μ g/mL, [5-FU] = 1.17, 2.34, 4.68, 7.02 μ g/mL) for 12 h; cells were treated with PBS as a control. After washing with PBS twice and treatment with or without an 808-nm laser (1.0 W/cm², 5 min), the relative cell viabilities were determined by the CCK-8 assay.

To assess the interaction between the nanobubbles and MGC-803 cells at different pH values, MGC-803 cells (1×10^5 /well) were cultured in 12-well plates to adhere and incubated with medium (pH = 7.4 or 6.5) containing

nanobubbles ([5-FU] = 7.02 μ g/mL) for 3, 6, 9, or 12 h. After washing with PBS twice, the relative cell viabilities were determined by the CCK-8 assay.

The Annexin V-FITC/PI Apoptosis Detection Kit was further employed to determine the apoptotic and necrotic cell distributions. In brief, MGC-803 cells were cultured in 12-well plates (1×10^5 /well) to adhere, and the medium was then replaced with fresh medium containing nanobubbles, 5-FU, or IR-780 ([IR-780] = 6 μ g/mL, [5-FU] = 7.02 μ g/mL). After incubation for 12 h, the cells were washed with PBS and treated with or without laser irradiation (1.0 W/cm², 5 min). Subsequently, the cells were washed twice with PBS, trypsinized, and collected for FACS measurement after another 4 h of incubation. The data were analyzed by using FlowJo software 7.6.1.

To confirm the PDT efficacy of the nanobubbles, photodamage of MGC-803 cells by the nanobubbles was visually observed by fluorescence microscopy. MGC-803 cells (1×10^4 /well) were cultured in 24-well plates to adhere, and then the cells were treated with fresh medium containing nanobubbles or IR-780 ([IR-780] = 6 μ g/mL). Cells treated with PBS were used as a control. After incubation for 6 h, the cells were washed with PBS twice and treated with or without irradiation by an 808-nm laser at a power of 1.0 W/cm² for 5 min. Finally, the cells were stained with Calcein-AM (2.0 μ M) and PI (1.5 μ M) and observed by fluorescence microscopy.

Animals and tumor model

All animal experiments were carried out using female BALB/c-nude mice (4–5-weeks old, 18–20 g) purchased from Shanghai Jiesijie Laboratory Animal Co. Ltd. All animals received care in compliance according to the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. MGC-803 tumor-bearing mice were established by injecting 2×10^6 of MGC-803 cells into the right flank subcutaneously. The tumors were allowed to grow 10–14 d to reach a volume of approximately 100–200 mm³ for in vivo or ex vivo experiments.

In vivo tumor-targeted US/MR/NIR imaging

For in vivo monitoring of the nanobubble distribution, fluorescence imaging was carried out by using a Bruker in vivo F PRO imaging system. The nanobubbles were sealed in a 1.5-mL EP tube prior to imaging, and PBS was imaged as a control. Then, 150 μ L of PBS containing nanobubbles, nanobubbles (no folate), or IR-780 (1.0 mg/kg of IR-780 to total mouse body weight) was intravenously injected into each mouse. Fluorescence images were captured (excitation at 720/20 nm; emission at 790/30 nm; exposure time of 60 s) at different times postinjection. The mice were eventually sacrificed by cervical dislocation at 60 h, and the tumor and organs including the heart, liver, spleen, lung, and kidney were imaged with the same

parameters. The average fluorescence intensity of the tumor and organs was quantified by using Bruker Molecular Imaging Software.

For US imaging *in vivo*, 150 μL of PBS containing gas-filled nanobubbles (17.5 mg/kg) was injected via the tail vein. The mice were then placed on a warm platform, and the tumor site was coated with ultrasound gel and imaged using the transducer in B mode with a frequency of 40 MHz. Images were captured at 0.5, 1, and 2 h.

For MR imaging *in vivo*, 150 μL of PBS containing nanobubbles or nanobubbles (no folate) (1.0 mg/kg of Gd^{3+} to total mouse body weight) was injected via the tail vein. T1-weighted MR imaging of the tumor site was performed at different times on a 3.0 T MRI scanner with an animal coil (MAGENTOM, Verio, Siemens Healthcare, Erlangen, Germany). The sequence was set as follows: TR/TE = 1200/12 ms; acquisition matrix = 256×256 ; field of view = 100×100 mm; number of slices = 12; slice thickness = 2 mm; flip angle = 150° .

In vivo combined chemo-photothermal therapy

Mice bearing MGC-803 tumors were intravenously injected with 150 μL of PBS containing 5-FU, IR-780, or nanobubbles (2.0 mg/kg of IR-780 and 2.34 mg/kg of 5-FU to total mouse body weight); 12 h postinjection, the tumor sites of mice treated with IR-780 and nanobubbles were irradiated by an 808-nm laser at a power density of $1.0 \text{ W}/\text{cm}^{-2}$ for 5 min. The temperature and infrared images were recorded in real time using an infrared camera. Tumor volume and body weight were monitored every three days for 18 d. Images of the tumor site were taken using a camera every day to monitor the therapeutic effect on the tumor. The tumor volume was calculated by the following formula (maximum length) $\times$ (minimal width) $^2 \times 1/2$. The mice were sacrificed at 18 d. The heart, liver, spleen, lung, kidney, and brain were excised and further investigated by H&E staining to monitor the morphological features of each organ.

Statistical analysis

All results are reported as the means $\pm$ SD. All statistical analyses were performed with SPSS 22.0 (IBM Corp., USA). Comparisons between groups were performed using Student's *t* test. Levels of $^*P < 0.05$ and $^{**}P < 0.01$ were regarded as significant.

Results and discussion

Preparation and characterization of nanobubbles

Gd^{3+} DTPA-BSA was prepared according to our previously reported method³². The surface-modification process of the nanobubbles is shown in Fig. 1. Oleylamine- and IR-780-loaded nanocapsules were first prepared as the core structures via the double-emulsion evaporation method. Folate was then incorporated on the shell of the core

structures via electrostatic incorporation. The prepared Gd^{3+} DTPA-BSA@ 5-FU complex was then surrounded on the surface to form a stable all-in-one nanoplatform. Finally, after freeze-drying, nanobubbles were formed by compressed air.

The morphology of the nanobubbles was observed by TEM. As shown in Fig. 2a, b, the nanobubbles presented a spherical shape with a distinct hollow structure and a shell thickness of approximately 10 nm. The hydrodynamic size of the nanobubbles was approximately 120.41 ± 18.30 nm (Fig. 2c) after surface modification. When the pH environment changed, as shown in Fig. 2d, the ξ -potential of nanobubbles changed from -11.56 mV (pH 7.4) to 6.23 mV (6.4) and 13.74 mV (pH 5.0). These results indicated that the nanobubbles presented pH-dependent charge-switchable behavior. Considering the acidic environment in tumor tissue, this charge-switching behavior can strengthen the interaction between the nanobubbles and the cellular membrane and further facilitate deep tumor penetration.

To confirm the successful fabrication of the nanobubbles, the UV-vis spectrum changes were recorded during the formation process (Fig. 2e). After loading IR-780 into the nanobubbles, a strong characteristic absorption peak appeared at approximately 792 nm, with a bathochromic shift from 776 nm (free IR-780). After folate was absorbed onto the surface, its characteristic absorption peaks at approximately 302 and 365 nm appeared in the UV-vis spectrum. Similarly, after the Gd^{3+} DTPA-BSA @ 5-FU complex was incorporated into the surface, the characteristic absorption peak of 5-FU appeared at 266 nm. All of these changes in the UV-vis spectrum indicated that the nanobubbles were successfully assembled. In addition, we recorded the photoluminescence (PL) peak of the nanobubbles; compared with free IR-780, the PL peak of the nanobubbles shifted from 790 to 820 nm (Fig. S1), indicating a change in the environment of IR-780.

The entrapment efficiency (EE) and drug loading (DL) were calculated based on the UV calibration curves of IR-780 (778 nm) and 5-FU (266 nm) (Fig. S2). The EE of IR-780 and 5-FU reached 87.6% and 68.3%, and the DL values were 1.97% and 1.71%, respectively. Then, the stimuli (pH or light)-responsive properties were investigated, as shown in Fig. 3a. Within 48 h, the cumulative release of 5-FU from the nanobubbles reached 9.3% (pH 7.4), 27.3% (pH 6.5), and 42.30% (pH 5.0). The nanobubbles presented a pH-dependent drug-release capacity, with greater 5-FU release at lower pH values. This phenomenon is probably due to surface protonation of the nanobubbles at more acidic pH values, which triggered greater 5-FU release to the acidic environment. In addition, upon laser irradiation (808 nm, $1.0 \text{ W}/\text{cm}^2$, 5 min), the cumulative release of 5-FU at pH 5.0 further

Fig. 1 Scheme. Schematic illustration of the preparation of surfaced-engineered nanobubbles with pH-/light-responsive drug-release and charge-switchable behaviors and their use in NIR/MR/US imaging-guided chemo-photothermal therapy in vivo

increased from 42.30 to 59.8% after 12 h, indicating that light-induced heat can dissociate the electrostatic interaction between 5-FU and GdDTPA-BSA . The above results indicate that the nanobubbles exhibited synergetic drug release in response to pH and light, which could contribute to its therapeutic performance in the tumor microenvironment.

IR-780 is an efficient NIR imaging and PTT agent, and the photothermal efficiency of IR-780-loaded nanobubbles was evaluated by monitoring the change in temperature using a thermocouple needle under laser irradiation (808 nm, 1.0 W/cm^2). As shown in Fig. 3b, c, a negligible change was observed in the temperature of water, whereas a dramatic change in the temperature of the nanobubbles was observed in a concentration-dependent manner. The temperature of the nanobubbles increased to 46.1 from 25°C at a concentration of $12 \mu\text{g/mL}$. This temperature increase indicated that the nanobubbles can cause irreversible photothermal damage to tumor cells.

Relaxometry is a key factor for evaluating the capacity of MRI, and the MR relaxometry of the nanobubbles was

measured. As shown in Fig. 3d, the nanobubbles had an MR longitudinal (r_1) value of $16.56 \text{ s}^{-1}/\text{mM}$, similar to that of GdDTPA-BSA ($15.186 \text{ s}^{-1}/\text{mM}$) and approximately fourfold higher than that of the traditional MRI contrast agent (Gd-DTPA). In vitro T1-weighted MR images of nanobubbles were further obtained at different concentrations of Gd^{3+} (Fig. 3e). The signal intensity clearly changed from bright to dark with a decreasing concentration of Gd^{3+} , and the signal of the nanobubbles was much brighter than the signal of GdDTPA at the same concentration of Gd^{3+} . In addition, there was almost no leakage when the pH of the solution was adjusted from 7.4 to 6.5 or the temperature was adjusted to a maximum of 50°C (Fig. S3), mainly due to the strong chelation effect between the Gd ions and DTPA. The above results demonstrated that the nanobubbles can act as stable positive MRI agents.

Ultrasound contrast imaging effects of nanobubbles were also scanned using an ultrasonic diagnostic instrument (Prospect 3.0 High Frequency Ultrasound Imaging, S-Sharp) in conventional B mode. As shown in Fig. 3f,

Fig. 2 Characterization of nanobubbles. TEM imaging of nanobubbles at different magnifications (a) and (b). c Hydrodynamic size of nanobubbles. d ζ -potential of nanobubbles at pH 7.4, 6.5, and 5.0. e UV spectra of free FA, free IR-780, nanobubbles@IR-780, nanobubbles@FA/IR-780, and nanobubbles@FA/IR-780/5-FU

compared with water, the ultrasound signals gradually increased with increasing nanobubble concentration, and these in vitro results demonstrated that the nanobubbles can be used as a contrast agent for efficient ultrasound imaging.

In vitro cellular uptake and intracellular distribution

The effects of the interaction with the nanobubbles on MGC-803 cells at pH 7.4 and 6.5 were evaluated by changes in fluorescence, as shown in Fig. 4a. As the incubation time increased, cells treated with nanobubbles

Fig. 3 Functional characterization of surfaced-engineered nanobubbles. **a** Cumulative release of 5-FU at pH 7.4, 6.5, and 5.0 within 48 h. Laser irradiation induced further cumulative release of 5-FU at 12 h (pH 5.0); the red arrow indicates the change. **b** Temperature changes of the nanobubbles (6 or 12 $\mu\text{g/mL}$ IR-780) in response to laser irradiation (808 nm, 1.0 W/cm^2) within 360 s; water was used as a control. **c** Infrared thermal images of nanobubbles under laser irradiation (808 nm, 1.0 W/cm^2) at an IR-780 concentration of 12 $\mu\text{g/mL}$; water was used as a control. **d** T_1 relaxation rate ($1/T_1$) of the Gd^{3+} -DTPA-BSA complex and nanobubbles. **e** T_1 -weighted MR images of nanobubbles with different Gd^{3+} concentrations. Water (0 mM) was used as a reference. **f** In vitro ultrasound imaging of nanobubbles at concentrations of 10 and 20 mg/mL; water was used as a control

at pH 6.5 exhibited a much stronger red fluorescence signal than at pH 7.4. This result indicated that the induced positive charge (Fig. 2d) created a stronger interaction of the nanobubbles with MGC-803 cells and greater IR-780 release to the intracellular environment from the nanobubbles. By contrast, no changes were observed when the nanobubbles were incubated in culture medium without cells. This sensitive “negative or positive” charge-switchable behavior can facilitate deep tumor penetration and long tumor accumulation.

The in vitro targeting ability of the nanobubbles toward gastric cancer MGC-803 cells and gastric epithelial GES-1 cells was evaluated by confocal laser scanning microscopy. As shown in Fig. 4b, MGC-803 cells presented much a stronger red fluorescence signal than GES-1 cells when incubated with the nanobubbles for a short time, indicating the excellent targeting ability toward MGC-803 cells. With prolonged incubation time, as shown in Fig. 4c, MGC-803 cells treated with nanobubbles emitted an increased red fluorescence signal in the cytoplasm,

Fig. 4 Endocytosis effect of the nanobubbles. **a** FL image of nanobubbles incubated in culture medium with or without MGC-803 cells for different times. **b** Confocal images of MGC-803 cells and GES-1 cells treated with nanobubbles for 1 h. **c** Confocal images of MGC-803 cells incubated with nanobubbles for 0.5, 4, and 12 h. Scale bar, 100 μ m

indicating gradual release of the loaded IR-780 from the nanobubbles after uptake by the cells. By contrast, after incubation for a short time of 1 h, MGC-803 cells treated with IR-780 emitted weaker red fluorescence than cells treated with nanobubbles for the same duration (Fig. S4), further indicating that the nanobubbles were more efficiently endocytosed by MGC-803 cells due to the FA-targeting ability.

In vitro cytotoxicity study

In vitro cytotoxicity was first evaluated by CCK-8 assay. As shown in Fig. 5a, the nanobubbles induced significant lower cell viability compared with free 5-FU, which indicated that the nanobubbles can assist the chemotherapeutic effects of 5-FU. Furthermore, we observed that a lower pH environment induced higher cytotoxicity

with prolonged time (Fig. 5b), probably because the acidic environment induced a positive surface charge and consequently increased cytotoxicity. In addition, the nanobubbles presented much higher phototoxicity than free IR-780 (Fig. 5a) because more of the IR-780 from the nanobubbles was internalized, and thus light induced a greater temperature increase accompanied by accelerated 5-FU release (Fig. 3a). Additionally, the ratio of cell necrosis to apoptosis was detected by flow cytometry (Fig. 5c). The cells treated with nanobubbles exhibited a significant change in the ratio of apoptosis to necrosis from 5.2% (control) to 31.5% in the dark, and the ratio of apoptosis to necrosis was dramatically higher in cells treated with nanobubbles + laser (9.0–62.3%) than in cells treated with IR-780 + laser (9.0–38.3%). Therefore, the nanobubbles exhibited greater therapeutic efficacy

Fig. 5 In vitro cytotoxicity and apoptosis assay. **a** CCK-8 assay: cell viability of MGC-803 cells treated with 5-FU, IR-780, and nanobubbles with or without laser irradiation at different concentrations of IR-780 and 5-FU. **b** Cell viability of MGC-803 cells treated with nanobubbles (equal to 7.02 $\mu\text{g}/\text{mL}$ 5-FU) at different pH values. **c** Flow cytometry: MGC-803 cells were treated with 5-FU, IR-780, and nanobubbles with or without laser irradiation. The red arrow represents PI, and the green arrow represents Annexin V-FITC. **d** Photodamage efficacy of nanobubbles and IR-780 in MGC-803 cells. Each well was treated with and without laser irradiation. The red color represents dead cells (staining with PI), and the green color represents live cells (staining with Calcein-M). All data are presented as the mean $\pm$ SD, * $P < 0.05$ and ** $P < 0.01$

than single 5-FU or IR-780 + laser due to the synergistic effect. Next, photodamage effects were evaluated in vitro. Greater cell death was induced by treatment with nanobubbles + laser than by IR-780 + laser (Fig. 5d). Taken together, these results demonstrated that the nanobubbles have superior therapeutic efficacy and an integrative effect for chemo-photothermal therapy due to good FA-targeting, charge-switchable behavior and stimulus-responsive drug release.

In vivo trimodal tumor-targeted imaging evaluation

The outstanding in vitro performance results encouraged us to evaluate tumor-targeted NIR/MR/US imaging of nanobubbles in MGC-803 tumor-bearing nude mice. FL imaging in vitro, as shown in Fig. S5, showed that the nanobubbles presented a much stronger red fluorescence signal and indicated that the nanobubbles could be used for further FL imaging in vivo. FL imaging in vivo was then performed, as shown in Fig. 6a, b. At 4 h

postinjection, the tumor area present stronger fluorescence than the other sites due to accumulation of the nanobubbles at the tumor site via the blood circulation. A much stronger fluorescence signal was observed in the tumor site between 8 and 24 h. Eventually, the background fluorescence signal decreased over time, but the fluorescence remained clearly visible at the tumor site until 48 h postinjection. However, when folate was absent from the nanobubbles, the nanobubbles exhibited delayed tumor accumulation and a shorter tumor retention time, confirming the targeting ability of FA to MGC-803 tumor cells in vivo. As a control, mice treated with IR-780 (Fig. S6A) did not exhibit a strong contrast red fluorescence signal at the tumor site. Furthermore, ex vivo fluorescence imaging (Fig. S6B and Fig. 6c) indicated that

the nanobubbles accumulate more efficiently at the tumor site.

Moreover, for MRI in vivo, as shown in Fig. 7a, the brightness of the tumor gradually increased when nanobubbles were intravenously injected. The signal of the tumor increased remarkably at 6 h. By 26 h postinjection, the signal of the tumor areas became weaker, probably due to slow release of Gd ions from the tumor site. By contrast, nanobubbles (no folate) showed delayed tumor accumulation due to the lack of specific targeting by folate. These results indicated that folate-mediated nanobubbles are suitable T1-weighted MRI agents in vivo with excellent tumor-targeting ability. Similarly, due to the good ultrasound contrast imaging in vitro, US imaging in vivo was performed. As shown in Fig. 7b, after injection with

Fig. 7 Visualization of tumor location. **a** In vivo T1-weighted MR images post injection of nanobubbles or nanobubbles (no folate). **b** In vivo ultrasound imaging of mice treated with nanobubbles. The red arrow marks the signal changes at the same location

nanobubbles, the tumor site became much lighter compared with preinjection. Notably, the boundary of tumor was constantly legible for 2 h, suggesting potential applications in guiding further tumor therapy.

In vivo combined therapeutic efficacy of nanobubbles

Encouraged by the excellent therapeutic effect in vitro and excellent tumor-targeting ability in vivo, MGC-803 tumor-bearing mice were intravenously injected with free IR-780 or nanobubbles to evaluate the PTT efficacy. The tumor site was irradiated with an 808-nm laser (1.0 W/cm^2) for 5 min at 12 h postinjection. The temperature changes were monitored during laser irradiation, as shown in Fig. 8. The temperature of the tumors in the group treated with free IR-780 reached a maximum of approximately 49.0°C (5 min). By comparison, the mice treated with nanobubbles exhibited a higher maximum of 53.7°C at the tumor site, indicating that the nanobubbles can induce more serious thermal injury to tumor cells than free IR-780 under laser irradiation. As shown in Fig. 9a, b, mice treated with nanobubbles exhibited clear hemorrhagic injury at the tumor site, followed by formation of a solid scar and shedding of the tumor over time. Eventually, as new normal tissue generated, all of the tumors treated with

Fig. 8 In vivo thermal imaging: temperature changes at the tumor site in mice treated with nanobubbles + laser or IR-780 + laser. The images were captured by infrared thermographic maps

nanobubbles + laser disappeared without recurrence or metastasis. However, although the tumor treated with free IR-780 + laser also exhibited a certain degree of

Fig. 9 In vivo chemo-photodynamic therapy. **a** Changes in tumors from mice treated with PBS, 5-FU, IR-780 + laser, nanobubbles or nanobubbles + laser at different times postinjection. **b** Body weight fluctuation curve of MGC-803 tumor-bearing mice treated with PBS, 5-FU, IR-780 + laser, nanobubbles or nanobubbles + laser. **c** Relative tumor volume fluctuation curve of MGC-803 tumor-bearing mice treated with PBS, 5-FU, IR-780 + laser, nanobubbles or nanobubbles + laser. All data are presented as the mean $\pm$ SD ($n = 3$)

hemorrhagic injury, new tumor tissue regenerated and further expanded in volume within 18 d due to insufficient accumulation of IR-780 within the tumor. More, these significant therapeutic effects were not observed in mice treated with PBS + laser, in which the tumor volume increased by 9.26-fold.

To evaluate the nanoprobe's chemotherapeutic efficacy in vivo, MGC-803 tumor-bearing mice were administered 5-FU or nanobubbles, respectively. As shown in Fig. 9a, b, the nanobubble-treated mice without laser irradiation exhibited more efficient inhibition of tumor volume at 18 d. However, the tumor volume in the 5-FU group increased approximately 6.87 times at 18 d, highlighting the excellent tumor-targeting ability and stimuli-responsive drug release of the nanobubbles in the tumor microenvironment. As a control, the tumor volume in the group treated with PBS increased approximately 9.56 times. Taken together with the PTT results in vivo, these results indicated that the nanobubbles exhibited better therapeutic efficacy than free IR-780 or 5-FU. Moreover, the body weights of the mice all

slightly increased within 18 d after administration of the different materials (Fig. 9c), with no obvious side effects of the nanobubbles. In addition, according to H&E staining of organ sections (Fig. 10), the nanobubbles caused no obvious damage to the brain, liver, heart, spleen, kidney, and lung at 18 d, further confirming that the nanobubbles can act as a safe delivery agent.

Conclusion

In summary, we have successfully developed surfaced-engineered nanobubbles that can be used for NIR/MR/US imaging and chemo-photothermal therapy. Interestingly, release of the loaded 5-FU can be triggered by an acidic pH environment and laser irradiation. Moreover, the nanocapsules presented charge-switchable behaviors upon a change in pH, which could facilitate endocytosis and deep tumor penetration. The tumor-targeting and therapeutic capability of the nanobubbles were confirmed both in vitro and in vivo. The combined chemo-photothermal therapy guided by the trimodal imaging

Fig. 10 Histopathological staining analysis. Histopathological examination of H&E-stained organ sections from mice treated with PBS and nanobubbles at 18 d postinjection. Scale bar, 100 μ m

method resulted in remarkably enhanced treatment efficacy, with complete tumor elimination without recurrence. These results indicate that the “all-in-one” nanobubbles could be employed for advanced tumor theranostics.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

- Long, J. T. et al. Anticancer drug-loaded multifunctional nanoparticles to enhance the chemotherapeutic efficacy in lung cancer metastasis. *J. Nanobiotechnol.* **12**, 37 (2014).
- Senapati, S., Mahanta, A. K., Kumar, S. & Maiti, P. Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduct. Target Ther.* **3**, 7–7 (2018).
- Chandran, S. P., Natarajan, S. B., Chandraseharan, S. & Shahimi, M. S. B. M. Nano drug delivery strategy of 5-fluorouracil for the treatment of colorectal cancer. *J. Cancer Res. Pract.* **4**, 45–48 (2017).
- Sun, T. et al. Engineered nanoparticles for drug delivery in cancer therapy. *Angew. Chem. Int. Ed.* **53**, 12320–12364 (2014).
- Wang, H. et al. Precise engineering of prodrug cocktails into single polymeric nanoparticles for combination cancer therapy: extended and sequentially controllable drug release. *ACS Appl. Mater. Interfaces* **9**, 10567–10576 (2017).
- Brigger, I., Dubernet, C. & Couvreur, P. Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev.* **64**, 24–36 (2012).
- Zhang, L. et al. Lipid nanoparticle-mediated efficient delivery of CRISPR/Cas9 for tumor therapy. *NPG Asia Mater.* **9**, e441 (2017).
- Kumari, A., Yadav, S. K. & Yadav, S. C. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids Surf. B Biointerfaces* **75**, 1–18 (2010).
- Zhu, L., Kate, P. & Torchilin, V. P. Matrix metalloproteinase 2-responsive multifunctional liposomal nanocarrier for enhanced tumor targeting. *ACS Nano* **6**, 3491–3498 (2012).
- Liu, Z. et al. Multifunctional nanohybrid based on porous silicon nanoparticles, gold nanoparticles, and acetalated dextran for liver regeneration and acute liver failure theranostics. *Adv. Mater.* **30**, e1703393 (2017).
- Feng, T., Ai, X., An, G., Yang, P. & Zhao, Y. Charge-convertible carbon dots for imaging-guided drug delivery with enhanced in vivo cancer therapeutic efficiency. *ACS Nano* **10**, 4410–4420 (2016).
- Wu, L. et al. Magnetite nanocluster and paclitaxel-loaded charge-switchable nanohybrids for MR imaging and chemotherapy. *J. Mater. Chem. B* **5**, 849–857 (2017).
- Hung, C. C. et al. Active tumor permeation and uptake of surface charge-switchable theranostic nanoparticles for imaging-guided photothermal/chemo combinational therapy. *Theranostics* **6**, 302–317 (2016).
- Chen, S. et al. A surface charge-switchable and folate modified system for co-delivery of proapoptosis peptide and p53 plasmid in cancer therapy. *Biomaterials* **77**, 149–163 (2016).
- Chen, Y. et al. Zwitterionic phosphorylcholine-TPE conjugate for pH-responsive drug delivery and AIE active imaging. *ACS Appl. Mater. Interfaces* **8**, 21185–21192 (2016).
- Ou, H. et al. Surface-adaptive zwitterionic nanoparticles for prolonged blood circulation time and enhanced cellular uptake in tumor cells. *Acta Biomater.* **65**, 339–348 (2018).
- Li, Y., Liu, R., Shi, Y., Zhang, Z. & Zhang, X. Zwitterionic poly (carboxy/betaine)-based cationic liposomes for effective delivery of small interfering RNA therapeutics without accelerated blood clearance phenomenon. *Theranostics* **5**, 583 (2015).
- Yuan, Y. Y. et al. Surface charge switchable nanoparticles based on zwitterionic polymer for enhanced drug delivery to tumor. *Adv. Mater.* **24**, 5476–5480 (2012).
- Zhang, P. et al. Engineering the surface of smart nanocarriers using a pH-/thermal-/GSH-responsive polymer zipper for precise tumor targeting therapy in vivo. *Adv. Mater.* **29**, 1702311 (2017).
- Gao, M., Yu, F., Lv, C., Choo, J. & Chen, L. Fluorescent chemical probes for accurate tumor diagnosis and targeting therapy. *Chem. Soc. Rev.* **46**, 2237–2271 (2017).

21. Kircher, M. F. et al. A brain tumor molecular imaging strategy using a new triple-modality MRI-photoacoustic-Raman nanoparticle. *Nat. Med.* **18**, 829–U235 (2012).
22. Li, C. A targeted approach to cancer imaging and therapy. *Nat. Mater.* **13**, 110–115 (2014).
23. Shin, T. H., Choi, Y., Kim, S. & Cheon, J. Recent advances in magnetic nanoparticle-based multi-modal imaging. *Chem. Soc. Rev.* **44**, 4501–4516 (2015).
24. Liu, C. et al. Magnetic/upconversion fluorescent NaGdF₄:Yb,Er nanoparticle-based dual-modal molecular probes for imaging tiny tumors in vivo. *ACS Nano* **7**, 7227–7240 (2013).
25. Yin, T. et al. In vivo targeted therapy of gastric tumors via the mechanical rotation of a flower-like Fe₃O₄@Au nanoprobe under an alternating magnetic field. *NPG Asia Mater.* **9**, e408 (2017).
26. Du, J. et al. Preparation and imaging investigation of dual-targeted C3F₈-filled PLGA nanobubbles as a novel ultrasound contrast agent for breast cancer. *Sci. Rep.* **8** 3887 (2018).
27. Zhou, D. et al. Superparamagnetic PLGA-iron oxide microspheres as contrast agents for dual-imaging and the enhancement of the effects of high-intensity focused ultrasound ablation on liver tissue. *Rsc Adv.* **5**, 35693–35703 (2015).
28. Ao, M. et al. Gd-DTPA-loaded PLGA microbubbles as both ultrasound contrast agent and MRI contrast agent-A feasibility research. *J. Biomed. Mater. Res Part B* **93B**, 551–556 (2010).
29. Chen, G. & Gong, R. Study on fluorouracil–chitosan nanoparticle preparation and its antitumor effect. *Saudi Pharm. J.* **24**, 250–253 (2016).
30. Yue, C. et al. IR-780 dye loaded tumor targeting theranostic nanoparticles for NIR imaging and photothermal therapy. *Biomaterials* **34**, 6853–6861 (2013).
31. Zhang, W. et al. Synergistic effect of chemo-photothermal therapy using PEGylated graphene oxide. *Biomaterials* **32**, 8555–8561 (2011).
32. Xing, X. et al. An “imaging-biopsy” strategy for colorectal tumor reconfirmation by multipurpose paramagnetic quantum dots. *Biomaterials* **48**, 16–25 (2015).