**pH/Ultrasound Dual-Responsive Gas Generator for Ultrasound Imaging-Guided Therapeutic Inertial Cavitation and Sonodynamic Therapy**

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Herein, a pH-ultrasound dual-responsive gas generator is reported, which is based on mesoporous calcium carbonate (MCC) nanoparticles by loading sonosensitizer (hematoporphyrin monomethyl ether (HMME)) and modifying surface hyaluronic acid (HA). After pinpointing tumor regions with prominent targeting efficiency, HMME/MCC-HA decomposes instantaneously under the cotriggering of tumoral inherent acidic condition and ultrasound (US) irradiation, concurrently accompanying with CO\(_2\) generation and HMME release with spatial/temporal resolution. Afterward, the CO\(_2\) bubbling and bursting effect under US stimulus results in cavitation-mediated irreversible cell necrosis, as well as the blood vessel destruction to further occlude the blood supply, providing a “bystander effect.” Meanwhile, reactive oxygen species generated from HMME can target the apoptotic pathways for effective sonodynamic therapy. Thus, the combination of apoptosis/necrosis with multimechanisms consequently results in a remarkable antitumor therapeutic efficacy, simultaneously minimizing the side effects on major organs. Moreover, the echogenic property of CO\(_2\) make the nanoplatform as a powerful ultrasound contrast agent to identify cancerous lesions. Based on the above findings, such all-in-one drug delivery platform of HMME/MCC-HA is utilized to provide the US imaging guidance for therapeutic inertial cavitation and sonodynamic therapy simultaneously, which highlights possibilities of advancing cancer theranostics in biomedical fields.

1. Introduction

In recent years, sonodynamic therapy (SDT) has been developed as a potential alternative to common therapies for cancer treatment.\(^1\,2\) Different from photodynamic therapy, SDT is an ultrasound-based approach that possesses many inspiring merits, e.g., deeper penetration and minimal invasiveness.\(^3\) Upon activation by ultrasound (US), the sonosensitizers would transfer energy to oxygen molecules, leading to the production of reactive oxygen species (ROS), further causing serious cellular toxicity.\(^4\) Notwithstanding their significant potential, developing multifunctional drug delivery system integrated with coordinated functions of diagnostics and therapy, deserved full credit as an effective means to optimize cancer therapy.

Of various imaging modalities, US imaging was a noninvasive real-time imaging technology for guiding SDT to realize the early diagnosis, real-time therapy monitoring, and the assessment after treatment.\(^5\) Although most ultrasound contrast agents (perfluorochemicals, NaHCO\(_3\), H\(_2\)O\(_2\), etc.) presented strong echo signals, they suffered from inherent drawbacks such as instability and short half-life.\(^6,7\) Currently, such progress has inspired interest in the solid-phase ultrasound contrast agents.\(^8,9\) Among them, the gas generating CaCO\(_3\) nanoparticles could decompose and produce hyperechogenic CO\(_2\) bubbles spontaneously in tumoral lysosome acidic condition (pH 4.3–5.8), displaying their potential as ultrasound contrast agents as well as drug carriers.\(^10\) However, most of the reported solid CaCO\(_3\) nanoparticles were hindered by their low drug loading capacity.\(^11–13\) In addition, their slow inadequate degradation in response to the single stimulus of weak acidic condition led to the insufficiency of gas generation and suboptimal echogenic property.

To resolve these issues, mesoporous calcium carbonate nanoparticles (MCC NPs) were synthesized as the drug delivery carriers in doses sufficient for high therapeutic efficacy in this text, benefiting from their large surface area and great pore volume.\(^6,14,15\) Encouragingly, some emerging exceptional characteristics of MCC NPs were substantiated, including their pH-ultrasound dual-responsive instantaneous decomposition.
behaviors. It might be because the initial small CO$_2$ bubbles generating in tumoral inherent acidic condition would hide in porous channels, but burst timely once encountering ultrasound waves, while the mesoporous structure was not so stable as to withstand the explosive attack which was similar to the cavitation effect. Afterward, the instantaneous disintegration of MCC under the US irradiation triggering was expected in turn, to generate more CO$_2$ bubbles by reacting with acidic substances. It was worthwhile to note that the acidic condition was a prerequisite for the decomposition of MCC NPs, while the mesoporous structure was the pivotal determinant to the ultrasound sensitivity. In this sense, the pH/ultrasound dual-responsive platform based on MCC NPs was well-documented to realize the instantaneous decomposition action, concurrently accompanying with the CO$_2$ generation and “drug dumping” effect with spatial/temporal resolution. Moreover, CO$_2$ bubbling and bursting behaviors of MCC NPs induced a disruptive cavitation bubble effect, which would mechanically destroy cells and induce irreversible cell necrosis.$^{[16]}$ In light of these advantages, we utilized MCC NPs for the first time to integrate US imaging and therapeutic inertial cavitation simultaneously, which exhibited superior advantages in comparison with the conventional solid CaCO$_3$ nanoparticles.

In this study, a pH/ultrasound dual-responsive gas generator was constructed based on MCC NPs loaded with hematoporphyrin monomethyl ether (HMME), a sonosensitizer. The biocompatible hyaluronic acid (HA) as tumor targeting moiety as well as gatekeeper was introduced onto the surface of MCC NPs, rendering the system tumor targeting efficiency and “zero premature release” character.$^{[17,18]}$ As illustrated in Scheme 1, after pinpointing tumor regions via CD44 receptor-mediated endocytosis process, HMME/MCC-HA decomposed instantaneously under the cotriggering of tumoral inherent acidic condition and US irradiation, concurrently accompanying with CO$_2$ generation and HMME release in an on-demand manner. Subsequently, multiple factors including increased osmotic pressure led to the endo/lysosomal disruption and redistribution of nanoplatform from endosomal to cytoplasm for further delivery. On the one hand, the CO$_2$ bubbling and bursting effect under US stimulus resulted in cavitation-mediated irreversible cell necrosis, as well as the blood vessel destruction to further occlude the blood supply, providing a “bystander effect.” On the other hand, US activated ROS generation of HMME targeted the apoptotic pathways for effective SDT effect. Actually, apoptosis and necrosis often shared common initiators and seemed regulated by similar pathways, which could coexist and cooperate in a complementary way to promote cell injury.$^{[19]}$ Reasonably, it was undoubtedly logical that HMME/MCC-HA exploited the merits of synergistic combination of therapeutic inertial cavitation and sonodynamic therapy simultaneously, resulting in antitumor effects with multimechanisms.

More importantly, absences of targeting effect and two local stimulations (acidity and US irradiation) in normal tissues, would significantly avoid the potential side effect. Therefore, such pH/ultrasound dual-responsive gas generator of HMME/MCC-HA was considered as a promising candidate to amplify

antitumor therapeutic efficacy for safe clinical applications. Furthermore, the excellent US imaging contrast performance of MCC-HA at specific tumoral acidic pH provided the opportunity for identifying cancerous lesions, as well as improving the treatment precision, controllability, and biosafety. By this design, such delivery platform of HMME/MCC-HA certainly would provide the US imaging guidance of therapeutic inertial cavitation and sonodynamic therapy for tumors simultaneously, which might offer a valuable direction in multimodality therapeutic applications in cancer treatment.

2. Results and Discussions

The uniform spherical MCC NPs were synthesized via a one-pot approach.[20] The transmission electron microscopy (TEM) image clearly showed the obvious space within the nanoparticles (Figure 1A(a); Figure S1, Supporting Information), depicting the disordered mesoporous structure with a size of ≈250 nm. And the high-angle annular dark-field scanning TEM (HAADF-STEM)-based elemental mapping further confirmed the uniformly distribution of three elements (Ca, C, O) on the MCC NPs. The energy dispersive spectrometer pattern showed that the weight ratio of Ca:C:O in MCC NPs was 39:31:29 (Figure S2, Supporting Information). The weight difference between the synthesized MCC NPs and theoretic CaCO₃ might be due to the existence of the residual reactants such as starch. Moreover, the X-ray diffraction pattern of MCC could be indexed as a product of calcite/vaterite mixture (Figure S3, Supporting Information), which was more soluble than other polymorphs and suitable for degradation in acidic environment.

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**Figure 1.** Characterization of HMME/MCC-HA. A) (a) TEM image (left) and the correspondent HAADF-STEM image (right) of MCC, elemental mapping showed the distribution of Ca (orange), C (green), and O (yellow). b,c) TEM images of HMME/MCC-HA in phosphate buffer saline (PBS) at pH 7.4 (b) or pH 5.8 (c) for 30 min, respectively. d,e) TEM images of HMME/MCC-HA incubated in pH 5.8 PBS for 30 min and then irradiated by the US transducer at a power density of 1 W cm$^{-2}$ for 30 s (d) or 60 s (e), respectively. B) FT-IR spectra. C) UV–vis absorption spectra. D) N$_2$ adsorption–desorption isotherms and pore size distribution plots (insert). E) Leakage rate of HMME from HMME/MCC and HMME/MCC-HA stored in PBS (pH 7.4) containing 10% of FBS at 37 °C. The data points represent mean ± S.D. ($n$ = 3).
Benefiting from their large surface area and great pore volume, MCC NPs could easily serve as the promising drug delivery carriers in doses sufficient for high therapeutic efficacy. In spite of the high drug loading capacity (319 µg mg⁻¹), HMME/MCC without modification might suffer from undesirable drug leakage in circulation. To tackle this issue, HA as the gatekeeper as well as tumor targeting moiety was introduced onto the surface of MCC NPs via amidation reaction. Herein, Fourier transform infrared (FT-IR) results showed that the emerging characteristic −NH−CO− stretching vibration at 1400 cm⁻¹ and the ν(C−N) mode at 1109 and 1026 cm⁻¹ provided direct evidence of the successful HA capping (Figure 1B).

Then the reaction was further supported by the optical property of HMME/MCC-HA, which displayed both the characteristic peak (397 nm) of HMME and the end absorption of HA at 200 nm (Figure 1C). Because of its random-coil structure and high molecular weight, the HA usually act as a “gatekeeper” to prevent free diffusion of drug cargo, which were reported in several research papers.[17,18] It was noteworthy that TEM image of HMME/MCC-HA was in sharp contrast to the pristine sample (Figure 1A(b)), and showed an evident HA coating morphology in the absence of appreciable porosity by wrapping around the nanoparticles. Subsequently, accumulating characterizations of HMME/MCC-HA were measured to attain more insights into the capping efficiency of HA. The N₂ adsorption–desorption isotherm of MCC NPs was assessed and classified as mesoporous characteristic type of IV isotherm (Figure 1D). With a high surface area up to 164.3 m² g⁻¹ and pore size of ≈4.4 nm, MCC NPs allowed small drug molecules to diffuse into their mesoporous channels. Nevertheless, a considerable decrease in these parameters was observed for HMME/MCC and HMME/MCC-HA as listed in Table S1 in the Supporting Information. Notably, it was clear to see that the pore size almost reduced to zero after HA capping, showing an exceptional pore blocking effect. In addition, the leakage experiment depicted that the leakage of HMME/MCC group was 36.4%, while that of HMME/MCC-HA group decreased to 5.8% (Figure 1E). Thus, it was reasonable to infer that HA as gatekeeper played an important role in minimizing premature drug release during the drug delivery in vivo. Meanwhile, with hydrophilic character, HA modification significantly facilitated the stability of nanoparticles without any precipitation over 24 h (Figure S4, Supporting Information), which was of great importance in biomedical application.

According to the dynamic light scattering analysis, the zeta potential of HMME/MCC-HA was measured as −18.1 ± 1.9 mV and average hydrodynamic size was 264.5 ± 4.7 nm (Figure S5, Supporting Information), which was in consistent with the TEM results.

In this system, one key design of HMME/MCC-HA was its pH/ultrasound dual-responsive decomposition behavior. Its morphological evolution was directly observed under TEM. Compared with the compactly assembled original HMME/MCC-HA NPs under pH 7.4 (Figure 1A(b)), they tended to be partially deformed with small particles emerging in the surroundings under pH 5.8 (Figure 1A(c)). And it was undoubtedly logical that the acid-triggered gradual degradation of MCC was supported by the chemical reaction between CaCO₃ with weak acids to produce CO₂ bubbles and Ca²⁺ ions.[21,22] Afterward, when exposed by an external US irradiation for 30 s, the body of HMME/MCC-HA NPs exhibited an appreciable violent contortion and collapse according to the TEM image (Figure 1A(d)). Furthermore, their irregular shape further disintegrated instantaneously, leaving no visible particle structure within as short as 60 s (Figure 1A(e)). The digital photographs of MCC-HA treated with US irradiation also clearly substantiated its disintegration property (Figure S6, Supporting Information). The further ultrasound responsive instantaneous disintegration of HMME/MCC-HA might be contributed to the fact that the initial small CO₂ bubbles hiding in porous channels would burst timely once encountering ultrasound waves, while the mesoporous structure was not so stable as to withstand the explosive attack which was similar to the cavitation effect. Thereafter, the decomposition of MCC was expected in turn, to facilitate more CO₂ bubbles generation by reaction with acidic substances. In this sense, the pH/ultrasound dual-responsive decomposition behavior of HMME/MCC-HA was well-documented, concurrently accompanying with CO₂ generation and HMME release.

The quantitative analysis of CO₂ gas generation was further assessed (Figure 2A(a)). Similar to the HA modified solid calcium carbonate (SCC-HA), MCC-HA generated a negligible amount gas in neutral conditions. By contrast, it was observed that a considerable amount of CO₂ generated under pH 5.8. Of note, a burst release of CO₂ (115 ppm) occurred for MCC-HA (1 mg mL⁻¹) resulting from its ultrasound responsive disintegration. The nanoparticle tracking analysis indicated that the number of particles in a 1 mg mL⁻¹ sample was 3.6 × 10¹¹ particles mL⁻¹. It was calculated that about 3.2 × 10¹⁰ ppm CO₂ was released per particle. However, SCC-HA failed to generate subsequent gas. Meanwhile, the ultrasound-triggered CO₂ bubbling of MCC-HA rather than SCC-HA was also visualized by optical microscopic images (Figure 2A(b)). These comparative observations clearly indicated that the mesoporous structure was a decisive factor to the sensitivity to ultrasound. More importantly, the acidic condition was a prerequisite for the decomposition of CaCO₃. In light of its unique CO₂ bubbling and bursting behaviors, HMME/MCC-HA as the pH/ultrasound dual-responsive gas generator played an important role in US imaging and therapeutic inertial cavitation effect.

Based on above analysis, it was interesting to investigate the echogenic property of the nanoparticles in vitro under neutral and weakly acidic conditions (pH 7.4, pH 5.8), to mimic the environment of normal tissues and tumors, respectively (Figure 2B). The MCC-HA at pH 7.4 showed no significant acoustic reflectivity contrast. Whereas, the brightest contrast contributing to the generation of gas bubbling was observed under pH 5.8 and US irradiation, which was consistent with the CO₂ quantitative production results. Furthermore, the enhanced US imaging of MCC-HA at specific tumoral acidic pH demonstrated it was considered as an excellent ultrasound contrast agent to identify cancerous lesions for guiding cancer therapy.

Encouraged by these desirable properties of HMME/MCC-HA as mentioned above, we moved on to explore its drug release profile under the stimuli of acidic condition and US irradiation (Figure 2C). In this way, it showed that the HMME release increased by about 27.3% at pH 5.8 than that at pH 7.4 due to the acid-degradable property of MCC. Then a remote US irradiation was applied to activate the instantaneous disintegration. As expected, it was not surprised to observe an impulsive,
fast-release profile of HMME with release increasing by even 56.4%, which indicated an ultrasound mediated “drug dumping” effect. Reasonably, such pH/ultrasound dual-responsive platform opened up new possibilities for a more sophisticated on-demand cargo delivery and enhanced antitumor efficacy.

On account that the released HMME could act as the sonosensitizer to generate ROS, especially $^{1}\text{O}_2$, for SDT, the ultrasound activated $^{1}\text{O}_2$ generation from HMME/MCC-HA was then detected by using singlet oxygen sensor green (SOSG) as an indicator, which could emit a green fluorescence at 525 nm after capturing $^{1}\text{O}_2$.[23] As illustrated in Figure 2D, the $^{1}\text{O}_2$ production exhibited HMME concentration and ultrasonic time-dependent features. In addition, before the SOSG assay, the $N, N$-diethylhydroxylamine (DEHA) as the oxygen scavenger was added to reduce the oxygen content of the reaction mixture. It showed that the DEHA pretreatment quenched the SOSG fluorescence intensity significantly (Figure S7, Supporting Information), which further supported the ROS generation from the nanoparticles for SDT. Next, the practicability of the ultrasound activated ROS generation was further examined on MCF-7 human breast cancer cells by using 2’,7’-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe. The results showed that only HMME and HMME/MCC-HA group displayed the strong green fluorescence with US irradiation (Figure 3A). These ultrasound activated ROS generation could be interpreted by the fact that the sonosensitizer would transfer energy to oxygen molecules, leading to the production of ROS.[3] Taken together, HMME/MCC-HA would offer the opportunity for SDT to attain a more effective antitumor nanotherapy.

Prior to explore the antitumor effect of the formulations in vitro, its tumor targeting efficiency should be taken into account. Quantitative flow cytometric analysis demonstrated the far higher uptake of HMME/MCC-HA with a positive cell percentage of 67.2% than that of the nontargeted HMME/MCC with a positive cell percentage of 7.3% in CD44-positive MCF-7 cells (Figure 3B). In addition, the cellular uptake was significantly blocked and reduced to 10.9% by excess free HA (5 mg mL$^{-1}$). Then the targeting ability of HA was further investigated on CD44 negative NIH3T3 fibroblast cells. Quantitative flow cytometric analysis showed the internalization amount of HMME/MCC and HMME/MCC-HA at 4 h was 6.5% and 8.3%, respectively (Figure S8, Supporting Information). No significant enhancement of binding occurred between HA modified nanoparticles and NIH3T3 cells. These findings indicated that HA modified nanoparticles had a high binding specificity with MCF-7 cells via CD44 receptor-mediated endocytosis process and rendered the system prominent tumor targeting efficiency.[17,18] Next, the intracellular distribution of the nanoparticles was further explored (Figure 3C). For the no-US treated group, a frequency of colocalization of the fluorescence of HMME (red) and Lysotracker (green) was visualized by confocal laser scanning microscopy (CLSM), corroborating the endocytic uptake pathway and entrapment within endo/lysosomal compartments. The insignificant HMME intensity in the cells could be explained by the fluorescence quenching effect of metallic materials when HMME was restricted inside MCC NPs.[17] Nevertheless, an enhanced HMME fluorescence evidently separated from lysosomes after US irradiation, indicating the HMME release and endo/lysosomal disruption. Probably, the decomposition of HMME/MCC-HA in response to pH/ultrasound resulted in the Ca$^{2+}$ ions generation, which
was demonstrated by using a calcium-specific dye Rhod-2 (Figure S9, Supporting Information). Meanwhile, the H\(^+\) depletion and chloride influx would also increase the osmotic pressure and cause lysosome swelling.[24] Moreover, ultrasound-activated ROS generation from HMME and inertial cavitation effect of CO\(_2\) bubbles also were responsible for the endo/lysosomal disruption and redistribution of HMME releasing from endosomal to cytoplasm for further delivery, which was supposed to improve the therapeutic efficacy.[14,25]

Keeping in mind the ultrasound activated ROS generation and cavitation effect in cell-free experiments, it was highly desirable to evaluate its feasibility in MCF-7 cells via SEM imaging (Figure 3D). Compared with the control cells, the MCC-HA + US group displayed characteristic features associated with cellular swelling, cytoskeleton collapsing as well as nuclear disintegration, an indication of cell necrosis. HMME treated cells exhibited diverse apoptotic morphology with cytoplasmic enrichment and smaller shape. Indeed, it was clearly to see apoptotic bodies on the cell surface by forming plasma membrane blebs and protuberances. Actually, both necrosis and apoptosis were two major forms of cell death. These comparative cellular morphology induced by MCC-HA and HMME implied the distinct mechanisms of anticancer effect. More specifically, pH/ultrasound dual-responsive CO\(_2\) release system of MCC-HA could generate disruptive cavitation bubble effect, which would mechanically destroy cells and induce irreversible cell necrosis.[16] And it was well established that HMME mediated SDT targeted the apoptotic pathways via ROS generation. Taking advantage of the integration of necrotic and apoptotic cell death simultaneously, the all-in-one HMME/MCC-HA would be bound to show a desirable cytotoxicity. Before that, we had confirmed the safety and biocompatibility of MCC-HA.

Figure 3. In vitro cell experiments. A) Detection of ROS generation by DCFH-DA staining in MCF-7 cells with US irradiation (1 W cm\(^{-2}\), 1 min). B) Flow cytometric assay of cellular uptake with different treatments. C) CLSM of MCF-7 cells incubated with HMME/MCC-HA for 4 h, lysosomes and nuclei were counterstained with LysoTracker (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue), respectively. D) SEM images of MCF-7 cells. Red arrow indicated the apoptotic body. E) Cell viability of MCF-7 cells with different treatments with US irradiation. The data points represent mean ± S.D. (n = 3).

The level of statistical significance is indicated when appropriate (*P < 0.05, **P < 0.01).
on MCF-7 cells and mouse NIH3T3 fibroblast cells without the ultrasound stimulus (Figure S10, Supporting Information). In the case of HMME/MCC-HA, it decreased the cell viability substantially to $14.5 \pm 1.3\%$ with US irradiation, displaying a remarkable therapeutic efficacy as expected (Figure 3E). Assumably, we could infer that the HMME/MCC-HA with tumor targeting effect would release CO$_2$ and HMME under the cotrig-}

tering of tumoral inherent acidic condition and US irradiation, then exhibit the synergistic combination of therapeutic inertial cavitation and sonodynamic therapy, which might promote the development of cancer therapy.

To realize the synchronous imaging diagnosis during cancer treatments, we undertook US imaging performance of nanoparticles in MCF-7 tumor-bearing nude mice (Figure 4A(b)).

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**Figure 4.** In vivo experiments. A) Histogram of US signal intensity profile (a) and US images (b) of tissues (red dotted circle) acquired pre and postinjection of MCC and MCC-HA (10 mg kg$^{-1}$) with US irradiation (1 W cm$^{-2}$, 1 min). $I/I_0$ indicated the ratio of ultrasound intensity to that of preinjection. The data points represent mean $\pm$ S.D. ($n = 3$). B) Ex vivo fluorescence images of tumor region to observe HMME (red) and 1O$_2$ (green) with US irradiation. C) Tumor growth curves after different treatments as a function of time. D) Representative pictures of mice after treatments. E) H&E, CD34 immunohistochemical and TUNEL staining of tumor harvested from mice. Nuclei were stained with DAPI. Note: (a) Control group; (b) Control + US; (c) MCC-HA; (d) MCC-HA + US; (e) HMME; (f) HMME + US; (g) HMME/MCC-HA; (h) HMME/MCC-HA + US. F) The positive index of CD34 with different treatments according to the image in (E). G) Quantitative relative apoptosis deriving from image in (E) with control group as the base. The tumor volumes and pathological analysis for all animals for each different group expressed as mean $\pm$ S.D. ($n = 5$). The level of statistical significance is indicated when appropriate (*$P < 0.05$, **$P < 0.01$).
Compared with that of preinjection, MCC group exhibited bright and strong US signals in tumor site at 3 h postinjection, and particularly after exposure to US stimulus, indicating the pH/ultrasound dual-responsive CO2 bubbling. More importantly, localized spots of enhanced contrast were observed for MCC-HA group, which could preferentially accumulate at the tumor site via CD44 receptor-mediated endocytosis process. Apart from tumors, the ultrasound images were also captured in normal tissues. However, we observed no obvious changes in contrast signal at liver site (Figure 4A(a)), probably due to its neutral pH and the lack of accumulation of MCC-HA. On basis of this proof-of-concept trial, MCC-HA with tumor targeting ability and pH/ultrasound dual-responsive CO2 release features, could be employed as an excellent ultrasound contrast agent at specific tumoral acidic pH to identify cancerous lesions, as well as improving the treatment precision, controllability andbiosafety. And it was plausible that such pH/ultrasound dual-responsive gas generator certainly would provide the guidance of therapeutic inertial cavitation and sonodynamic therapy for tumor simultaneously.

To understand tumor treatment efficacy of various HMME formulations (HMME and HMME/MCC-HA), the biodistribution of HMME in tumor and various main organs was investigated (Figure S11, Supporting Information). The relatively high drug distribution of nanoparticles in liver and lung could be probably interpreted by the sessile macrophages present there.[26] Notably, the HMME level at tumor site of HMME/MCC-HA group was higher than that of HMME group by 4.9 and 6.4-fold at 3 and 12 h postinjection, respectively, which might be attributable to the enhanced permeability and retention effect (EPR effect) and the active tumor targeting ability of HA. Compared to the HMME group, HMME/MCC-HA group could enhance accumulation in tumor, which should be responsible for the antitumor efficiency in vivo. Subsequently, the HMME release behavior in vivo was investigated by injecting HMME/MCC-HA into mice (Figure 4B). An eminent and wide range of red fluorescence in the tumor site via CD44 receptor-mediated endocytosis process. Notably, the HMME level at tumor site of HMME/MCC-HA group was higher than that of HMME group by 4.9 and 6.4-fold at 3 and 12 h postinjection, respectively, which might be attributable to the enhanced permeability and retention effect (EPR effect) and the active tumor targeting ability of HA. Compared to the HMME group, HMME/MCC-HA group could enhance accumulation in tumor, which should be responsible for the antitumor efficiency in vivo. Subsequently, the HMME release behavior in vivo was investigated by injecting HMME/MCC-HA into mice (Figure 4B). An eminent and wide range of red fluorescence in the tumor region indicated that HMME was distributed evenly when exposed to US irradiation. Moreover, the $^1$O$_2$ signal (green) was also detected using SOSG as an indicator. Thus, the ultrasound activated HMME release and ROS generation were confirmed in vivo, which was supposed to improve the SDT effect. Then the antitumor efficacy of HMME/MCC-HA was assessed in vivo. According to the tumor growth curves and photos of MCF-7 tumor-bearing nude mice with different treatments (Figure 4C,D), it was clear that both MCC-HA group and HMME group with US irradiation resulted in a moderate inhibition of tumor growth. HMME/MCC-HA successfully suppressed the tumor volume with the V/Vo of 0.87 $\pm$ 0.13, exhibiting an exceptional therapeutic effect with multimechanisms (Scheme 1B). Actually, after pinpointing tumor regions via CD44 receptor-mediated endocytosis process, MCC based nanoparticles would decompose instantaneously under the cotriggering of tumoral inherent acidic condition and US irradiation, concurrently accompanying with CO2 generation and HMME release in an on-demand manner. On the one hand, the CO2 bubbling and bursting effect under US stimulus resulted in cavitation-mediated irreversible cell necrosis, as well as the blood vessel destruction to further occlude the blood supply, which provided a “bystander effect.” On the other hand, ultrasound activated ROS generation of HMME targeted the apoptotic pathways for effective SDT effect. From this viewpoint, the nanoplatform of HMME/MCC-HA exhibited the synergistic combination of therapeutic inertial cavitation and sonodynamic therapy simultaneously.

Furthermore, to confirm such a therapeutic mechanism, the tumor tissues were removed for H&E, CD34 immuno-histochemical, and terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining. It was clear to observe both dark purple–blue round apoptotic body in H&E staining and TUNEL-positive tumor cells in the group of HMME with US irradiation (Figure 4E,G), which derived from SDT-mediated apoptosis. In comparison, MCC-HA + US group displayed characteristic features associated with substantial tissue cavitation and cell necrosis in H&E staining, as well as lower level of vascular density according to the CD34 immuno-histochemical staining (Figure 4E,F), which convincingly corroborated the cavitation mediated blood vessel destruction. It was noted that apoptotic cell death (TUNEL assay) was also enhanced following MCC-HA treatment, possibly because cellular components excreted from necrotic cells might induce immune responses and lead to the stimulation of the apoptotic pathway.[27] Actually, apoptosis and necrosis often shared common initiators and seemed regulated by similar pathways, which could coexist and cooperate in a complementary way to promote cell injury.[19] Thus, it was not surprised to observe the large-scale cell apoptosis/necrosis in HMME/MCC-HA group, which exploited the merits of synergistic combination of therapeutic inertial cavitation and sonodynamic therapy simultaneously.

Before finding their way into clinical applications, we performed a pilot study to investigate the potential toxicity of the nanoparticles in vivo. Neither abnormalities in body weight nor pathology changes in major organs were found in each group (Figures S12 and S13, Supporting Information), demonstrating its potential safety of HMME/MCC-HA. As a matter of course, the absence of targeting effect and two local stimulations (acidity and US irradiation) in normal tissues, as well as highly solubility and inactivity property of CO2 bubbles, would significantly avoid the potential side effect. In this sense, HMME/MCC-HA was considered a promising candidate to amplify antitumor therapeutic efficacy for safe clinical applications.

### 3. Conclusion

In summary, we have successfully proposed a pH/ultrasound dual-responsive gas generator based on MCC NPs. Herein, HMME/MCC-HA with the specific tumor targeting efficiency would decompose instantaneously under the cotriggering of tumoral inherent acidic condition and US irradiation, concurrently accompanying with CO2 generation and HMME release. Thus, such all-in-one drug delivery platform of HMME/MCC-HA was considered as a promising candidate to provide the US imaging guidance of therapeutic inertial cavitation and sonodynamic therapy for tumor simultaneously, which offered compelling highlights for advancing nanotherapy in biomedical fields. Despite its exceptional therapeutic effect with multimechanisms, future experiments are still required to understand the mechanism of cavitation-mediated cell necrosis in longer terms. Furthermore, other gasotransmitters such as NO, CO,
and H₂S, may also serve as therapeutic agents in the modulation of physiological functions. Based on these advances, we believe these gas generators would provide a new vision on the development of cancer theranostics in the near future.

4. Experimental Section

Synthesis of HMME/MCC-HA: MCC NPs were synthesized via a one-pot approach by using soluble starch as soft template. Briefly, a mixture of CaCl₂ and soluble starch solution was added to a flask and stirred at 30 °C for 30 min. Subsequently, a solution of Na₂CO₃ was quickly added into the above solution until the final mixture was set to a very dilute solution ([CaCl₂] = 2 × 10⁻⁵ M, [Na₂CO₃] = 2 × 10⁻⁵ M, soluble starch (0.25 wt%)). Then the reaction was carried out for another 10 min under vigorous stirring and maintained under static condition for 24 h. As an end, the resultant MCC NPs was collected by centrifugation. To obtain amination derivative of MCC, MCC (20 mg), 3-aminopropyltriethoxy-silane (APTES, 400 µL) and ethanol (2 mL) were mixed and stirred for 24 h. After this, the system was purified by washing with water to obtain MCC-NH₂.

For HMME loading, MCC-NH₂ (5 mg) and HMME (10 mg) were dissolved in 5 mL of ethanol–water mixture (ethanol:water = 1:1). After stirring for 24 h, the retrieved HMME/MCC was collected by centrifugation. Then the free HMME collected from supernatants was quantified spectrophotometrically at 397 nm. Then the reaction was carried out for another 10 min under vigorous stirring and maintained under static condition for 24 h. For HMME pre-loading, MCC-NH₂ (5 mg) and HMME (10 mg) were dissolved in 5 mL of ethanol–water mixture (ethanol:water = 1:1). After stirring for 24 h, the retrieved HMME/MCC was collected by centrifugation. Then the free HMME collected from supernatants was quantified spectrophotometrically at 397 nm. MHMME,loaded = MHMME,prep – MHMME,free. HMME loading capacity (LC) was calculated according to the following formula

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LC = \frac{M_{HMME,loaded}}{M_{HMME,load} + M_{MCC}}
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Before being introduced onto MCC, HA was activated by EDC, HCl, and NHS with molar ratio of 1:1:1 in PBS for 1 h. Then HMME/MCC was added into the mixture and stirred for 24 h. Unreacted HA was removed by centrifugation. Ultimately, the as-prepared HMME/MCC-HA was acquired after freeze-drying.

Characterization: The morphology of samples was estimated by TEM (CM30, Philips). UV–vis spectrophotometry analysis was performed on UV-2550 Shimadzu spectroscope. The FT-IR spectra were recorded on a Nicolet iS10 spectrometer (Thermo, USA). N₂ gas adsorption–desorption isotherms were collected by using an ASAP2020 sorptometer (Micromeritics). The quantification of CO₂ bubbles generated from SCC and MCC was determined by using a quadrupole mass spectrometer (Agilent Technologies, USA). In vitro and in vivo US imaging were carried out by using a Vevo 2100 ultrasound system (Fujifilm VisualSonics, Toronto, Canada) with a 40 MHz transducer.

pH/Ultrasound Dual-Responsive Drug Release: HMME/MCC-HA samples were suspended in 1 mL PBS and sealed in dialysis bags (MWCO of 12 KDa), which were dialyzed in 50 mL of PBS at pH 7.4 and pH 5.8, respectively. The release studies were performed at 37 °C with gentle shaking. For ultrasound responsive drug release study, the sample was repeatedly exposed to an external US irradiation (power intensity: 1 W cm⁻², frequency: 1 MHz, time: 1 min) by using a 2776 Intellect Mobile Ultrasound device (Chattanooga, USA). The released free HMME was determined spectrophotometrically at regular time intervals.

Cellular Experiments: MCF-7 and NIH3T3 cells were incubated with HMME/MCC and HMME/MCC-HA for 4 h, respectively. The cells were then harvested by trypsinization and analyzed by flow cytometry (FACSCalibur) for HMME. For observation by CLSM, the lysosomes and nuclei were counterstained with LysoTracker Green and 4',6-diamidino-2-phenylindole (DAPI), respectively. After washing with PBS, the cells were visualized under CLSM (Leica SP5II).

For cell viability measurements, cells were plated in 96-well plates until adherent, and then treated with different formulations of MCC-HA, HMME, and HMME/MCC-HA (MCC-HA concentration: 20 µg mL⁻¹, HMME concentration: 10 µg mL⁻¹), respectively. sulphorhodamine B (SRB) dye reduction assays were conducted to determine cell viabilities after incubation for 24 h.

Biodistribution Studies: The tumor-bearing mice were i.v. injected with HMME or HMME/MCC-HA ([HMME] = 5 mg kg⁻¹), respectively. After treatment for 0.5, 3, and 12 h, the tissues were collected, weighed, and homogenized in saline. Then the samples were treated and determined by high-performance liquid chromatography (HPLC) with the following conditions: a Symmetry C18 column; mobile phase, methanol/acetonitrile/PBS buffer solution (pH = 6.9); 30/20/50; column temperature: 30 °C; fluorescence detector set: λex = 397 nm and λem = 613 nm.

In Vivo Antitumor Activity: All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals. BALB/c nude mice bearing MCF-7 tumors were randomly assigned to eight groups (n = 5 per group). These groups received i.v. administration of PBS, MCC-HA, HMME, and HMME/MCC-HA ([MCC-HA] = 10 mg kg⁻¹, [HMME] = 5 mg kg⁻¹), respectively, and were then exposed to an external US irradiation (1 W cm⁻², 1 min) at 3 h after injection. The tumor volumes were monitored with a caliper and calculated as tumor width² x tumor length/2. Furthermore, tissues were removed for H&E, CD34 immumohistochemical, and terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining.

Statistical Analysis: Results were expressed as Mean values ± standard deviation (S.D.) from at least three independent measurements. All data sets were analyzed by using GraphPad Prism6 software (GraphPad Software, San Diego, USA). The differences between two groups and multiple groups were analyzed by Student’s t test and one-way ANOVA, respectively. The level of significance was set at probabilities of *P < 0.05, and **P < 0.01.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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