Research Paper

Eradication of solid tumors by chemodynamic theranostics with H$_2$O$_2$-catalyzed hydroxyl radical burst

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Abstract

Activatable theranostics, integrating high diagnostic accuracy and significant therapeutic effect, holds great potential for personalized cancer treatments; however, their chemodynamic modality is rarely exploited. Herein, we report a new in situ activatable chemodynamic theranostics PAsc/Fe@Cy7QB to specifically recognize and eradicate cancer cells with H$_2$O$_2$-catalyzed hydroxyl radical (•OH) burst cascade.

**Methods:** The nanomicelles PAsc/Fe@Cy7QB were constructed by self-assembly of acid-responsive copolymers incorporating ascorbates and acid-sensitive Schiff base-Fe$^{2+}$ complexes as well as H$_2$O$_2$-responsive adjuvant Cy7QB.

**Results:** Upon systematic delivery of PAsc/Fe@Cy7QB into cancer cells, the acidic microenvironment triggered disassembly of the nanomicelles. The released Fe$^{2+}$ catalyzed the oxidation of ascorbate monoanion (AscH$^-$) to efficiently produce H$_2$O$_2$. The released H$_2$O$_2$, together with the endogenous H$_2$O$_2$, could be converted into highly active •OH via the Fenton reaction, resulting in enhanced Fe-mediated T$_1$ magnetic resonance imaging (MRI). The synchronously released Cy7QB was activated by H$_2$O$_2$ to produce a glutathione (GSH)-scavenger quinone methide to boost the •OH yield and recover the Cy7 dye for fluorescence and photoacoustic imaging.

**Conclusion:** The biodegradable PAsc/Fe@Cy7QB designed for tumor-selective multimodal imaging and high therapeutic effect provides an exemplary paradigm for precise chemodynamic theranostic.

Key words: chemodynamic theranostics, activatable, reactive oxygen species, Fenton reaction, multimodal imaging

Introduction

Nanotheranostics, the integration of multimodal diagnostic and therapeutic modalities into a single system, provides opportunities for nanomedicine-based personalized precision treatments [1-6]. In contrast to the conventional nanotheranostics with “always-on” diagnostic signals and pharmacological effects [7-11], the activatable, in particular the stimuli-responsive, materials have attracted great focus for precision medicine [12-19]. These nanomaterials can sense endogenous [20] or exogenous stimuli [21], such as pH changes [22], different enzyme levels [23-25], reactive oxygen species (ROS) [26], laser irradiation [27, 28], or temperature changes [29] and are relatively safe and usually non-toxic. Therefore, enhancement of the diagnostic accuracy towards tumor microenvironments as well as the latent therapeutic effects are essential requirements of these nanomaterials designed for the improved treatment efficacy [30, 31].

Among the tumor-related stimuli, ROS species have been viewed as targets for cancer diagnosis and therapy because of high ROS levels in cancer cells [32-36]. Recently, chemodynamic therapy (CDT) that can generate highly cytotoxic hydroxyl radicals
encapsulating a newly synthesized H2O2-responsive PAsc/Fe@Cy7QB nanomicelles were obtained after [54, 55], such as MnO2/GOX and Fe3O4/GOx [35, 38, observed using inorganic nanotheranostic systems reaction efficiency and accelerating toxic •OH be effective strategies for enhancing the Fenton CDT agents or decreasing GSH concentration should be effective strategies for enhancing the Fenton reaction efficiency and accelerating toxic •OH generation. Despite significant improvement observed using inorganic nanotheranostic systems [54, 55], such as MnO2/GOX and Fe3O4/GOx [35, 38, 47, 49], the single CDT was still not effective against the localized solid tumors [56-58]. In this context, engineered biodegradable chemodynamic theranostics with tumor-selective multimodal imaging and significant in-situ yield of •OH could indisputably enhance the treatment accuracy and efficacy.

In view of the above concerns, herein, we developed theranostic nanomicelles (PAsc/Fe@Cy7QB) with tumor-selective imaging, affording precise and effective eradication of the localized solid tumor via an H2O2-catalyzed •OH burst cascade. Briefly, the polymeric matrix (PAsc-PSFe) was synthesized by copolymerization of ascorbate (polymerized ascorbic acid, PAsc) [59], pH-sensitive segments of Schiff base embedded with Fe2+ (polymerized Schiff base-Fe2+ complex, PSFe), and 2-(diisorpropylamino) ethyl methacrylate (polymerized diisorpropylamino, PDPA). This complex could be packed into a hydrophobic core due to its intrinsic hydrophobicity, and become hydrophilic after acidic environment of endosome/lysosomes could be efficiently converted into highly active •OH via the Fenton reaction. Further, the adjuvant Cy7QB could also be activated by H2O2 to generate the GSH-scavenger quinone methide and enhance •OH yield. The concomitantly generated dye Cy7 could be used as an imaging agent for fluorescence imaging (FLI) and photoacoustic imaging (PAI) to monitor the process. Thus, stimuli-activatable chemodynamic therapeutics are promising alternatives for tumor therapy, as they can significantly improve the bioavailability of therapeutic agents and inhibit tumor growth with high accuracy and minor side effects in vivo.

Materials and Methods

Materials

All chemicals were of reagent grade and used without further purification. Poly(ethylene glycol) methyl ether (mPEG113-OH) and 2-(diisorpropylamino) ethyl methacrylate were purchased from Sigma-Aldrich Corporation (MO, USA). Diethyl ether, ethyl acetate and all other reagents were acquired from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Fetal bovine serum (FBS), penicillin, streptomycin and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Thermo Fisher Scientific Co. Ltd (Beijing, China). Catalase (CAT) and phorbol 12-myristate 13-acetate (PMA) were purchased from Aladdin Reagent, Ltd (Shanghai, China). All aqueous solutions were prepared in ultrapure water, obtained from a Millipore MilliQ water purification system (Millipore, Billerica, MA USA).

Characterizations

The optical characteristics of PAsc/Fe@Cy7QB and Cy7QB were investigated using UV/visible absorption spectroscopy (Lambda-35 UV/visible spectrophotometer, Perkin-Elmer, Waltham, MA, USA). Fluorescence spectra of PAsc/Fe@Cy7QB and Cy7QB were acquired on an LS-55 fluorescence spectrophotometer (Perkin-Elmer). Transmission resonance imaging (MRI) signal was enhanced dramatically along with the release of Fe2+. Notably, the Fe2+ could catalyze the oxidation of AscH- into ascorbate radical (Asc•) to produce H2O2, which, along with the endogenous H2O2, could be efficiently converted into highly active •OH via the Fenton reaction. Therefore, supplementation of additional CDT agents or decreasing GSH concentration should be effective strategies for enhancing the Fenton reaction efficiency and accelerating toxic •OH. As illustrated in Scheme 1, after systematic administration, the PAsc/Fe@Cy7QB nanomicelles accumulated at the tumor site through the enhanced permeability and retention (EPR) effect, then internalized and endocytosed by tumor cells. The acidic environment of endosomes/lysosomes could subsequently induce decomposition of the Schiff base and PDPA protonation, leading to free Fe2+, ascorbate monoanion (AscH) and the adjuvant Cy7QB to the cytoplasm. At this stage, the Fe-mediated T1-magnetic (•OH) from hydrogen peroxide (H2O2) via the Fenton reaction (Fenton-like) by catalyzing ferrous ion (Fe2+) or other Fenton-like ions, has been demonstrated to have potential for cancer treatment [37-44]. However, CDT’s limitations lie in the poor tumor selectivity and low intratumoral •OH yields [45, 46]. Furthermore, the efficacy of CDT is reduced because of the relatively low cellular concentrations of H2O2 and Fe2+, decreasing Fenton reaction efficiency and •OH yield [47-49] as well as clearance of •OH by glutathione (GSH)-abundant antioxidation systems [50-53]. Therefore, supplementation of additional CDT agents or decreasing GSH concentration should be effective strategies for enhancing the Fenton reaction efficiency and accelerating toxic •OH. As illustrated in Scheme 1, after systematic administration, the PAsc/Fe@Cy7QB nanomicelles accumulated at the tumor site through the enhanced permeability and retention (EPR) effect, then internalized and endocytosed by tumor cells. The acidic environment of endosomes/lysosomes could subsequently induce decomposition of the Schiff base and PDPA protonation, leading to free Fe2+, ascorbate monoanion (AscH) and the adjuvant Cy7QB to the cytoplasm. 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electron microscopy (TEM) images were collected under a field emission high-resolution 2100 F transmission electron microscope (JEOL, Japan) operating at an acceleration voltage of 200 kV. The size of the nanomicelles was measured using a ZEN3690 zetasizer (Malvern, USA).

**Synthesis of compound PAsc-PSFe**

FeCl₂ (20.28 mg, 0.16 mmol) in 50 mL of ethanol was added dropwise to a solution of 150 mg PAsc-PS in 50 mL of ethanol, and stirred in a round-bottomed flask. To avoid oxidation of Fe²⁺, a few drops of glacial acetic acid were added. The resulting solution was magnetically stirred for 12 h under nitrogen at 40 °C, and then the solution was evaporated at 25 °C. The precipitated complex was filtered off, washed with ether, recrystallized from ice-cold ethanol, and dried in air. The optimized copolymer PDPA₅₀-b-(PAsc₀.₈₂-PSFe₀.₁₈)₆₅ (named PAsc-PSFe) was analyzed by ¹H NMR in d₆-DMSO. The copolymer PAsc₃₃-PDPA₃₆ (named PAsc-PDPA) with copolymerization of ascorbate and 2-(diisopropylamino) ethyl methacrylate was synthesized according to a similar procedure and analyzed by ¹H NMR in d₆-DMSO. The copolymer PSFe₁₂-PDPA₃₆ (named PSFe-PDPA) with copolymerization of Schiff base-Fe²⁺ and 2-(diisopropylamino) ethyl methacrylate was synthesized using a similar procedure and determined by ¹H NMR in d₆-DMSO.

**Preparation of PAsc/Fe@Cy7QB**

The Cy7QB was synthesized according to a similar protocol previously described and successfully characterized by ¹H NMR [62]. Subsequently, Cy7QB (2 mg) dissolved in CH₂Cl₂ was added to deionized water (2 mL) solution of PAsc-PSFe (10 mg). The mixture was stirred at 25 °C for 4 h. PAsc/Fe@Cy7QB was obtained after dialysis in a cellulose dialysis bag (MWCO 3500 Da) overnight. The resulting solution was freeze-dried for 6 h, and the dry product was re-dispersed in deionized distilled water (pH 7.4) to obtain the PAsc/Fe@Cy7QB solution, which was passed through a 0.22 μm filter to sterilize the sample before use in cultured cells and mice.

**H₂O₂ production in solution**

The H₂O₂ concentration was measured by UV-vis absorbance of oxidized 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of horseradish peroxidase (HRP). Briefly, HRP (150 mU/mL), and TMB (100 μM) were added to Fe²⁺ (0.16 mM)/ascorbate (0, 0.2, 0.4, 0.6, 0.8 mM), or only ascorbate (0.1, 0.2, 0.4, 0.6, 0.8 mM) solutions at pH 7.4 or 5.0, followed by incubation at 37 °C in the dark. Time-dependent UV-vis spectra were recorded. The characteristic absorbance of oxidized TMB at 370 nm was used to quantify the concentration of H₂O₂ based on the standard curve. Copolymers PAsc-PSFe, PAsc-PDPA and PSFe-PDPA were also treated similarly.

**Scheme 1.** Fabrication procedure and scheme of the theranostic mechanism of PAsc/Fe@Cy7QB for MRI/FLI/PAI multi-modal imaging and the H₂O₂-catalyzed •OH burst cascade.

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•OH production in solution

The amount of •OH was determined by the •OH-specific fluorescence turn-on probe benzoic acid (BA). Briefly, BA (10 µM), Fe²⁺ (0.16 mM) and ascorbate (0, 0.2, 0.4, 0.6, 0.8 mM) were added to PBS (pH 7.4 or 5.0) at 37 °C. Subsequently, the fluorescence spectra of the solution were recorded at determined time intervals and the emission intensity at 421 nm was plotted as a function of incubation time. PAsc/Fe@Cy7QB, PAsc-PSFe, PAsc-PDPA and PSFe-PDPA were also treated similarly.

Cell culture and confocal fluorescence imaging

Human hepatoma cells (HepG2) were cultured in DMEM containing 10% FBS (γ-irradiated and sterile-filtered) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% of CO₂. HepG2 tumor cells were seeded in 35 mm confocal culture dishes and allowed to grow for 24 h. Cells were incubated for 2 h, the culture media were removed, and the cells were washed with PBS. Confocal fluorescence imaging analyses were performed using a confocal laser scanning microscope (ZEISS LSM 510 META, Germany). PAsc/Fe@Cy7QB was excited at 680 nm and emission was recorded at 690-730 nm.

Cell uptake

Cell phagocytosis was assessed by confocal laser scanning microscopy (CLSM). HepG2 cells were incubated with PAsc/Fe@Cy7QB (50 µg/mL) for various durations (0, 0.5, 1 and 2 h). The uninternalized nanomicelles were removed by washing several times with PBS.

Cellular ROS detection

The culture medium of HepG2 cells was replaced with fresh DMEM, followed by the addition of PAsc/Fe@Cy7QB, PAsc-PSFe, PSFe-PDPA and PAsc-PDPA (50 µg/mL). After 8 h, the medium was removed and the cells were washed with PBS several times. Subsequently, 10 µM of the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) was added for 30 min to react with ROS and generate the fluorescent product DCF, which could be observed by CLSM.

Cellular GSH levels

A group of HepG2 cells were treated with 0, 12.5, 25, 50 µg/mL of PAsc/Fe@Cy7QB for 2 h. Another group was treated with catalase (CAT) before treatment with PAsc/Fe@Cy7QB (50 µg/mL). The cells were harvested, washed, and then lysed on ice in 40 µL of Triton X-100 lysis buffer. After 20 min, lysates were centrifuged and 10 µL of the supernatant was mixed with 50 µL of Ellman's reagent (0.5 mM DTNB). GSH concentration was determined by measuring the absorbance at 405 nm using a microplate reader. The levels of GSH in the treated cells were compared with the basal GSH levels in untreated cells.

In vitro cell viability assay

The cytotoxicity was studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were seeded in a 96-well plate and cultured in 100 µL DMEM medium for 24 h at 37 °C. Subsequently, the medium was replaced with 200 µL of fresh medium containing different concentrations of the PAsc-PDPA, PSFe-PDPA, PAsc-PSFe or PAsc/Fe@Cy7QB. After incubation for another 24 h, MTT (20 µL, 5 mg/mL) was added and incubated for 4 h to produce purple formazan. The supernatant was then replaced with 150 µL of DMSO. The optical density (OD) was monitored using a microplate reader at 570 nm. Cell viability (%) was determined by comparing the absorbance (570 nm) of the treated cells with that of untreated cells. Five independent experiments were performed for each test.

Tumor mouse model

All animal procedures were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals of South China Normal University, and the experiments were approved by the Animal Ethics Committee of South China Normal University. 4-week-old female BALB/c nude mice were purchased from the Animal Experiment Center, Southern Medical University, and bred in an axenic environment. HepG2 tumor cells (1.0 × 10⁶ cells) in PBS (100 µL) were subcutaneously injected into the flanks of each mouse. The animals were subjected to the treatments when the tumors reached approximately 100 mm³. The tumor volumes were calculated using the following formula: tumor volume = the greatest longitudinal diameter (length) × the greatest transverse diameter (width)² × 0.5.

In vivo pH-responsive MRI

For in vivo T₁-weighed MRI, PAsc/Fe@Cy7QB (100 µL, 50 µg/mL) was intravenously injected into HepG2 tumor-bearing BALB/c nude mice. After 0, 2, 4, 8, 12, and 24 h, the MRI of tumor tissues was recorded by MRI BioSpin ICON 1T (Bruker) with a small animal specific body coil. The relative MRI signal intensity was measured using Image J software.

In vivo FLI and PAI imaging

PAsc/Fe@Cy7QB (100 µL, 50 µg/mL) were injected into the tail veins of the HepG2
tumor-bearing mice, which were imaged and analyzed at 0, 2, 4, 8, 12, and 24 h after injection with an NIR imaging system (Odyssey LI-COR, USA). The fluorescence signals were collected at λ_{em} = 710 nm under excitation with a 680 nm continuous laser. For PAl, the mice were pretreated with saline, catalase (CAT), or phorbol 12-myristate 13-acetate (PMA) for 24 h. PAl was performed by a PA computed tomography system equipped with a 10 MHz, 10 mJ/cm², 384 element ring ultrasound array, and an optical parametric oscillator (OPO) (Surelite II-20, Continuum, Santa Clara, CA, USA) with 4-6 ns pulse duration and 20 Hz pulse repetition rate light source.

**Blood circulation**

The HepG2 tumor-bearing mice were intravenously injected with PAsc/Fe@Cy7QB (100 µL, 50 µg/mL), and approximately 20 µL blood was collected at 0, 2, 4, 8, 12, 24, and 48 h post-injection to which 980 µL of physiological saline containing 10 mM EDTA anticoagulant. Blood concentration of PAsc/Fe@Cy7QB was then determined by measuring the characteristic fluorescence of Cy7. The curve of blood terminal half-life of PAsc/Fe@Cy7QB was fitted based on the single-compartment pharmacokinetic model.

**In vivo biodistribution**

HepG2 tumor-bearing mice were intravenously injected with PAsc/Fe@Cy7QB (100 µL, 50 µg/mL) for biodistribution measurement, and then the mice were sacrificed at various time points post-injection (each group contained three mice). The major organs of mice were collected at 8, 12, 24, 48, and 72 h after the injection and were solubilized by tissue lysate for fluorescence measurement to determine Cy7 content.

**In vivo excretion study**

Mice were intravenously injected with PAsc/Fe@Cy7QB (100 µL, 50 µg/mL). Urine and feces of each mouse were then collected at various time points (12, 24, 48, 72, 96, and 120 h) and solubilized in 10% DMSO in PBS. The absorbance at 710 nm was measured to determine the percentage of injected dose per gram of urine and feces.

**Hemolysis tests**

Whole blood was collected from mice in heparinized-tubes and centrifuged at 3000 rpm for 5 min. The pellet was washed three times with PBS by centrifugation and resuspended in the same buffer. This suspension of red blood cells was always freshly prepared and used within 48 h after collection. Subsequently, 500 mL of 2% erythrocytes (v/v) were mixed with 500 mL of 20, 40, 60, 80, or 100 µg/mL PAsc/Fe@Cy7QB solution and incubated at 37 °C for various times [63-65]. Erythrocytes mixed with pure water were used as 100% hemolysis. The samples were centrifuged and the absorbance of the supernatants at 540 nm was measured. The percentage of hemolysis was calculated using the formula [66]: Hemolysis (%) = (I/I₀) × 100%, where I represents the absorbance of the supernatant for erythrocyte suspension with PAsc/Fe@Cy7QB, and I₀ represents the absorbance of completely hemolyzed erythrocytes in pure water.

**Antitumor activity**

For the evaluation of the antitumor activity against HepG2 tumors, PBS, PAsc-PDPA (200 µL, 50 µg/mL), PSFe-PDPA (200 µL, 50 µg/mL), PAsc-PSFe (200 µL, 50 µg/mL), and PAsc/Fe@Cy7QB (200 µL, 50 µg/mL) were intravenously injected after the tumor volume reached ~100 mm³. Tumor volumes were monitored every two days using a caliper to measure the perpendicular diameter of the tumors. The individual tumor volume (V) was calculated using the following equation: V = X × Y²/2, where X and Y represent the longest and shortest diameters of the tumors. Typical images of HepG2 tumors were obtained using a digital camera. The body weight of each mouse was recorded every two days.

**Histologic examination**

Hematoxylin and eosin (H&E) staining was performed according to the method provided by the vendor (BBC Biochemical). After the experiments were completed, the mice were sacrificed. The major organs (liver, lungs, kidneys, heart, and spleen) and tumor tissues from the mice of the five groups were retrieved, cut into 4-μm-thick sections, fixed in a 10% paraformaldehyde solution for 8 h at room temperature, followed by dehydration with ethanol, and then processed routinely into paraffin. The sliced tissues were stained with H&E and examined using an inverted fluorescence microscope system (Nikon E 200).

**Statistical analysis**

Data analyses were performed using the Prism GraphPad software (Version 7.0, GraphPad Software, San Diego, CA). Statistical analyses were performed according to a t-test. In all cases, * was used for p < 0.05, ** for p < 0.01, and *** for p < 0.001. Values were expressed as the mean ± SD. All experiments were performed at least in triplicates. The samples/animals were randomly allocated to the experimental group.
Results and Discussion

Molecular design principle and preparation of PAsc/Fe@Cy7QB nanomicelles

Ascorbate is recognized as an antioxidant or a pro-oxidant [67]. Generally, the endogenous ascorbate is thought to be an excellent reducing agent by donating one or two electrons to protect biomolecules from oxidative damage [68]. However, ascorbate could also widely exert pro-oxidant effects in the presence of transition metal ions [69-71]. The combination of ascorbate and iron has been used as an oxidizing system for the hydroxylation of alkanes, aromatics, and other oxidants [70, 72]. The mechanism [73, 74] is summarized in Scheme 1 and Figure S1: the oxidation of ascorbate is catalyzed by Fe^{2+} to produce the Asc·- and superoxide radical (O_2·), Reaction (1) + (2); the O_2· in turn results in H_2O_2 and O_2 dismutation, Reaction (3); Fe^{2+} reacts with H_2O_2 to generate Fe^{3+} and •OH in the classic Fenton Reaction (4).

Herein, we designed an in situ activatable theranostic system for H_2O_2-catalyzed •OH burst cascade to selectively enhance CDT’s efficacy against cancers. The all-in-one nanomicelles PAsc/Fe@Cy7QB were constructed from acid-responsive polymersomes incorporating ascorbate and Schiff base-Fe^{2+} complex and Cy7QB in the membranes and inner aqueous cavities, respectively. First, the optimal ratio of ascorbate and Fe^{2+} was determined using BA to monitor the production of •OH via the Fenton reaction (Figure S2) [74]. Subsequently, the optimized copolymer PAsc-PSFe was synthesized via copolymerization of the ascorbate and Schiff base-Fe^{2+} complex from the mPEG_{113}-OH as a micelle matrix for the self-produced H_2O_2 and acid-triggered Fe^{2+} release (Figure S3), and the degrees of polymerization were determined by ^1H NMR (Figure S4). For comparison, copolymer PAsc-PDPA polymerized with ascorbate and copolymer PSFe-PDPA polymerized with Schiff base-Fe^{2+} complex were also synthesized and characterized by ^1H NMR (Figure S5-6). To avoid the clearance of •OH by GSH, the H_2O_2-activatable probe (Cy7QB), a GSH-scavenging adjuvant was synthesized and characterized by incorporating the quinone methide-generating moiety boronate ester into Cy7 [75] (Figure S7-8). The final nanomicelles were then successfully obtained by self-assembly of PAsc-PSFe and Cy7QB; the loading capacity was measured as 84.15% (Figure S9) and the critical micelle concentration (CMC) was 30.12 µg/mL [76, 77] (Figure S10).
Characterization of PAsc/Fe@Cy7QB

The energy dispersive spectrometer (EDS)-elemental mappings and spectrum revealed that PAsc/Fe@Cy7QB consisted of Fe, C, O, and N elements (Figure 1A), confirming the chemical composition of PAsc/Fe@Cy7QB. TEM and dynamic light scattering (DLS) revealed that PAsc/Fe@Cy7QB had well-dispersed spherical morphology under physiological pH (pH 7.4) (Figure 1B-C). Under these conditions, the hydrodynamic size of the nanomicelles was almost unchanged in 48 h, indicating good stability (Figure S11). As a characteristic feature of solid tumors, high glycolytic activity facilitates lactate and H+ secretion, making the tumor microenvironment acidic [78]. Under acidic conditions, the average hydrodynamic diameter of PAsc/Fe@Cy7QB was shortened from 110 ± 2 nm at pH 7.4 to 8 ± 2 at pH 5.0, apparent changes in their morphology were also observed due to the degradation of the acid-sensitive polymer (Figure 1B-C, S12-13). More importantly, T1-weighted MRI became gradually brighter from pH 7.4 to pH 5.0, and the longitudinal relaxivities r₁ at pH 5.0 were 30-fold higher than at pH 7.4 because of the Fe²⁺ release from the Schiff base under acidic conditions (Figure 1D-E). The release of Fe²⁺ from the PAsc-PSFe polymer under acidic conditions was confirmed using the standard potassium ferricyanide and potassium thiocyanate method (Figure S14).

The synchronous release of Cy7QB was also confirmed by UV-vis absorption and emission spectra, consistent with the sensing performance with the free Cy7QB (Figure 1F). Due to the significant absorption responsiveness of PAsc/Fe@Cy7QB towards H₂O₂, the fluorescence intensity gradually increased after the addition of H₂O₂ (Figure S15). The PA signal of PAsc/Fe@Cy7QB also showed a positive linear correlation with H₂O₂ concentration (Figure S16). Altogether, our results indicated that the nanomicelles PAsc/Fe@Cy7QB could disassemble in an acidic microenvironment to release Fe²⁺, Asc⁻⁻, and the molecular adjuvant Cy7QB, accompanied by remarkable enhancement of MR signals and recovery of the H₂O₂-sensitive fluorescent and photoacoustic signals, facilitating the multimodal imaging of the localized tumors.

Generation of H₂O₂ and •OH in solution

After validation of the acid-triggered degradation of PAsc/Fe@Cy7QB, in situ production of H₂O₂ was investigated. We utilized TMB and HRP to quantify the H₂O₂ concentration by measuring the absorbance of oxidized TMB (Figure S17). H₂O₂ concentration reached up to 0.5 mM in the presence of free Fe²⁺ (0.16 mM) and ascorbate (0.8 mM) at pH 5.0 (Figure 2A), and the concentration further increased with the increase in ascorbate (Figure S18). However, H₂O₂ generation was negligible after treatment with ascorbate at both pH 7.4 and 5.0 (Figure S19). These results confirmed that the Fe²⁺ could catalyze the oxidation of ascorbate into Asc⁻⁻ for in situ H₂O₂ production under acidic conditions [74, 79].

Figure 2. Generation of H₂O₂ and •OH in solution. (A-B) Time-dependent H₂O₂ production by Fe²⁺-mediated Fenton reaction in different solutions. Ascorbate (0.8 mM), Fe²⁺ (0.16 mM). (C) Time-dependent increase in fluorescence using BA as the fluorescence probe for the detection of •OH production. The emission of fluorescence by oxidized BA at 426 nm was used to determine the levels of •OH at pH 5.0. (D) Detection of •OH in samples treated with indicated H₂O₂ concentrations with polymer PAsc-PSFe. (E) Fluorescence spectra of BA by •OH generated by different concentrations of GSH-treated with PAsc-PSFe + H₂O₂ and PAsc/Fe@Cy7QB + H₂O₂ at pH 5.0. (F) ESR spectra of PAsc/Fe@Cy7QB + 100 μM H₂O₂ + 10 mM GSH, PAsc-PSFe, PAsc-PDPA, and PSFe-PDPA.
We then investigated the possibility of using PAsc/Fe@Cy7QB to produce H$_2$O$_2$, and PAsc-PDPA without Schiff base-Fe$^{2+}$ and PSFe-PDPA without ascorbate were chosen as controls to accurately quantify H$_2$O$_2$ levels. A similar trend was observed for PAsc-PSFe containing ascorbate and Schiff base-Fe$^{2+}$, suggesting that the ascorbate in PAsc-PSFe could induce H$_2$O$_2$ generation in situ at pH 5.0 (Figure 2B, S20). Subsequently, •OH generation with Fe$^{2+}$ and ascorbate and the depletion effect of GSH on •OH at pH 7.4 or 5.0 were investigated. We found that BA’s fluorescence intensity gradually increased with the addition of ascorbate in the presence of Fe$^{2+}$ at pH 5.0 within 2 h (Figure S21), indicating efficient generation of •OH. However, the levels of •OH remained almost unchanged after the same treatment at pH 7.4 (Figure S22). PAsc-PSFe was also found to efficiently produce •OH under acidic conditions (Figure 2C, S23). The addition of exogenous H$_2$O$_2$ could also boost •OH generation (Figure 2D, S24-25), indicating ability of PAsc-PSFe to produce •OH by utilizing both the produced and endogenous cellular H$_2$O$_2$. However, further investigations showed that BA’s fluorescence intensity in PAsc/Fe@Cy7QB at pH 5.0 was 3-fold stronger than PAsc-PSFe in the presence of 10 mM GSH (Figure S26), ascribed to the removal of GSH by PAsc/Fe@Cy7QB via the in situ generated quinone methide (Figure S27). Overall, acid-degradable PAsc/Fe@Cy7QB, integrating self-catalytic Fenton reactions and the ability to eliminate GSH, could provide a cascade burst of •OH production in the tumor microenvironment for highly efficient and precise eradication of solid tumors (Figure 2E). Furthermore, electron spin resonance (ESR) was utilized to monitor the •OH signals by employing 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin-trapping agent. The results indicated that •OH was effectively generated by PAsc/Fe@Cy7QB that integrated self-catalytic Fenton reaction and the ability to eliminate GSH under acidic conditions (Figure 2F).

**In vitro H$_2$O$_2$-selective bioimaging and intracellular ROS levels**

Given the highly effective *in vitro* generation of •OH by PAsc/Fe@Cy7QB under acidic conditions, we explored their responsiveness to H$_2$O$_2$ and anticancer effects in tumor cells. The fluorescence intensity of HepG2 incubated with PAsc/Fe@Cy7QB increased significantly over time and reached a maximum at 2 h (Figure S28). The appearance of merged yellow dots from green (Lysotracker) and red (PAsc/Fe@Cy7QB) pixels showed that PAsc/Fe@Cy7QB exhibited good colocalization with lysosomes (Figure 3A). The acidic environment of endosomes/lysosomes could induce decomposition and destabilization of the responsive nanomicelles [80], which might explain the disintegration of PAsc/Fe@Cy7QB and release of Fe$^{2+}$, AscH and the adjuvant Cy7QB in a relatively acidic microenvironment. Subsequently, upregulation of H$_2$O$_2$ in HepG2 cells following treatment with PMA (1 mg/mL) led to brighter fluorescence imaging. In contrast, H$_2$O$_2$ depletion following treatment with CAT (10 U/mL) resulted in a decrease in fluorescence intensity, beneficial for monitoring the disintegration and visualizing movement of PAsc/Fe@Cy7QB (Figure 3B, S29).

To understand the intracellular H$_2$O$_2$-catalyzed toxicity of PAsc/Fe@Cy7QB, ROS levels were further monitored by the probe DCFH-DA [81-83], which can emit green fluorescence after oxidation by ROS. As shown in Figure 3C, flow cytometric analysis verified that •OH generation in the PAsc-PSFe group was higher than the control group, which was ascribed to the self-catalytic Fenton reaction. However, •OH generation in the PAsc/Fe@Cy7QB group was higher than the control group due to the synergistic effect of self-catalytic Fenton reaction and GSH depletion. Similar results were observed by CLSM analysis, further verifying a significant H$_2$O$_2$-catalyzed •OH burst cascade by PAsc/Fe@Cy7QB treatment (Figure 3D-E). GSH quantification in HepG2 cells treated with PAsc/Fe@Cy7QB confirmed that nanomicelles could decrease the cellular GSH concentration (Figure S30), consistent with the *in vitro* findings. Also, H$_2$O$_2$ elimination by CAT prevented PAsc/Fe@Cy7QB-induced H$_2$O$_2$ mediated boronate oxidation to generate quinone methide and recover GSH levels in the cells. Thus, PAsc/Fe@Cy7QB were effectively taken up by tumor cells, producing •OH with high efficiency in the acidic tumor microenvironment to induce significant localized toxicity and exert excellent anticancer effects.

**Assessment of PAsc/Fe@Cy7QB-induced cytotoxicity**

Encouraged by the high catalytic performance of PAsc/Fe@Cy7QB, we assessed the cell viability by using the standard MTT cell assay. First, treatment of HepG2 cells with PSFe-PDPA at the same concentration of H$_2$O$_2$ resulted in significant cytotoxicity compared to cells without PSFe-PDPA treatment, demonstrating the stronger cytotoxicity of •OH compared to H$_2$O$_2$ (Figure 4A). The cytotoxicity of PAsc/Fe@Cy7QB was then explored using PAsc-PSFe, PAsc-PDPA and PSFe-PDPA groups as controls. As shown in Figure 4B, the PAsc/Fe@Cy7QB group exerted significant concentration-dependent cytotoxicity, and the percentage of cell survival
following treatment with PAsc/Fe@Cy7QB (26%) was lower than that of PAsc-PDPA (84%) with insufficient intracellular ROS and PAsc-PSFe (49%) with only self-catalytic Fenton reaction. In particular, PSFe-PDPA caused only a slight reduction in cell viability due to the insufficient intracellular H2O2 levels to produce •OH, which was further confirmed by the negligible cytotoxicity of pure Fe²⁺ (Figure S31). The combination index (CI) value of PAsc-PSFe, and PAsc/Fe@Cy7QB was determined to be 0.51 and 0.47, respectively (Table S1), suggesting that the combined effect led to a stronger inhibition compared with the single composition [84]. The high cytotoxicity of PAsc/Fe@Cy7QB was also verified by Calcein AM and propidium iodide (PI) double-staining of liver cells; live cells (green) and dead cells (red) (Figure 4C). Flow cytometric analysis with Annexin V-FITC together with PI as markers provided similar results (Figure 4D). These results confirmed the •OH burst cascade by the self-catalytic Fenton reaction and GSH depletion.

**In vivo multimodal imaging**

Multimodal imaging provides the advantages of various imaging methods, such as high-resolution MRI, highly sensitive FLI and high-depth PAI, to facilitate the screening of early micro-tumors and guide precision therapy [85-89]. The excellent magnetic, fluorescence, and photoacoustic properties of the PAsc/Fe@Cy7QB in vitro prompted us to apply multimodal imaging-guided tumor-targeted in vivo CDT. After intravenous (i.v.) injection of PAsc/Fe@Cy7QB (50 µg/mL, 100 µL) into the HepG2 tumor-bearing mouse, MRI of the tumor region was acquired over time and recorded at 0, 2, 4, 8, 12, and 24 h post-injection (Figure 5A). The T₁-weighted MRI signal was substantially increased from 30% to 75% within 24 h (Figure 5C), confirming efficient tumor accumulation of PAsc/Fe@Cy7QB. Next, NIR fluorescent imaging of HepG2 tumor-bearing mice following PAsc/Fe@Cy7QB injection was performed, as depicted in Figure 5A. The fluorescence in the tumor region was detectable at 2 h post-injection, and its intensity gradually increased reaching a maximum at 12 h post-injection (Figure 5D). After 12 h of i.v injection of PAsc/Fe@Cy7QB, the HepG2 tumor-bearing mice pretreated with saline, CAT, or PMA were monitored by PAI (Figure 5B, E). These results implied that H2O2 could selectively unlock imaging features of the nanomicelles in vivo.

The biodistribution in the main organs and in blood circulation was also measured to determine the in vivo behavior of the nanomicelles. The results showed that the blood level of PAsc/Fe@Cy7QB gradually decreased over time but remained at a relatively effective concentration even at 24 h post-injection (Figure 5F). The eliminating rate constants [38, 90] of PAsc/Fe@Cy7QB were calculated to be -0.2093 µg/mL per hour in the first stage and decreased to -0.01209 µg/mL per hour in the second stage with a shifting time interval of 6.18 h (Figure 5G). The tumor as well as all main organs from

![Figure 3](http://www.thno.org)
HepG2 tumor-bearing mice treated with PAsc/Fe@Cy7QB were harvested after 24 h (Figure 5H). FLI indicated that the theranostic PAsc/Fe@Cy7QB specifically accumulated in the tumor region via the multi-targeting effect of EPR and subsequent pH-triggered decomposition and H2O2-driven deboronation reaction. The rapid decrease in PAsc/Fe@Cy7QB levels within 72 h in various organs after post-injection further revealed its desirable good biodegradation (Figure S32).

Although the relatively high PAsc/Fe@Cy7QB accumulation was found in the liver due to the reticuloendothelial system (RES) [91], the tumor selectivity of the nanomicelles was significantly dominant and could be attributed to the multi-targeting effect. The feces and urine were also collected to study the possible clearance pathway of PAsc/Fe@Cy7QB (Figure S33). High levels of Cy7 were detected in both urine and feces, indicating good metabolic characteristics of PAsc/Fe@Cy7QB that could be excreted through both the kidneys and gastrointestinal system. Very low levels of Cy7-species remained in the mouse body after 120 h. The results were consistent with the relatively high accumulation levels of PAsc/Fe@Cy7QB in the liver, spleen and kidney. Taken together, these results indicated that PAsc/Fe@Cy7QB provided outstanding MR/FL/PA multimodal imaging for defining the tumor region and precise guidance for subsequent CDT.

**In vivo tumor therapeutic effects of PAsc/Fe@Cy7QB**

Based on the H2O2-catalytic substantial production of •OH and remarkable tumor-selective imaging in vitro and in vivo, PAsc/Fe@Cy7QB were injected into HepG2-bearing mice to investigate its anticancer efficacy (Figure 6A). The ROS levels in tumor tissues of treated mice were detected using the DCFH-DA probe. As shown in Figure 6B, bright green fluorescence was observed in the group that was injected with PAsc-PSFe due to the self-catalytic Fenton reaction. However, the group treated with PAsc/Fe@Cy7QB presented brighter imaging due to the combination of self-catalytic Fenton reaction and GSH elimination. To verify the therapeutic efficacy of

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**Figure 4.** Assessment of PAsc/Fe@Cy7QB-induced cytotoxicity. (A) Cytotoxicity of PSFe-PDPA in the presence of H2O2 (50 µM or 150 µM). (B) Cytotoxicity against HepG2 cells at various concentrations. (C) The live/dead assay and (D) Annexin V/PI assay was used to evaluate the cytotoxicity after incubation with PBS, PAsc-PDPA, PSFe-PDPA, PAsc-PSFe, or PAsc/Fe@Cy7QB (50 µg/mL). All images have a scale bar of 20 µm. P values were calculated by two-tailed Student’s t-test (**p < 0.01, or *p < 0.05).
the nanomicelles, mice bearing HepG2 tumors with initial volumes of 100-150 mm$^3$ were chosen and randomly divided into five groups ($n = 3$ per group). One group was injected with saline and used as a control and the other four groups received PAsc-PDPA, PSFe-PDPA, PAsc-PSFe, or PAsc/Fe@Cy7QB i.v. injections and were then subjected to the indicated treatments. Mice in control, PAsc-PDPA and PSFe-PDPA groups exhibited rapid tumor growth, indicating that these agents had almost no therapeutic effect. The PAsc-PSFe group inhibited tumor growth due to the self-catalytic Fenton reaction, whereas the PAsc/Fe@Cy7QB group exhibited the best tumor suppression effect, demonstrating the efficient and superior anticancer effect of the micelles (Figure 6C, S34). H&E staining was subsequently performed to evaluate the treatment effect, and the results demonstrated more necrosis and karyolysis in the PAsc/Fe@Cy7QB group (Figure 6E). No obvious loss in body weight was recorded in any of the experimental groups (Figure 6D), and the hemolysis test reflected the biosafety of PAsc/Fe@Cy7QB even at high concentrations (Figure S35). H&E images of major organs in all treated groups revealed no abnormality or organ toxicity compared to the control group, indicating barely any side effects and good biocompatibility of PAsc/Fe@Cy7QB (Figure S36).

Therefore, the high biodegradability and biocompatibility with the efficient tumor-targeted pharmacological effects suggested great potential of PAsc/Fe@Cy7QB for precise cancer therapy.

Figure 5. In vivo multimodal imaging. (A) In vivo MRI/FLI at 0, 2, 4, 8, 12, and 24 h i.v. post-injection of PAsc/Fe@Cy7QB (50 μg/mL, 100 μL). (B) PAI of each group at 12 h after PAsc/Fe@Cy7QB injection via the tail vein: PAsc/Fe@Cy7QB only; PAsc/Fe@Cy7QB + CAT; PAsc/Fe@Cy7QB + PMA. (C) Analysis of the tumor region by the MRI signals in (A). (D) Fluorescence intensity in mice after PAA/Fe@Cy7QB injection. (E) Analysis of the tumor region by the PAI signals in (B). (F) Blood level curve of PAsc/Fe@Cy7QB in mice fluorescence measurement of Cy7 in blood at different time points post i.v. injection. (G) Eliminating rate curve of injected PAsc/Fe@Cy7QB in blood circulation according to the Ln (concentration)-time relationship. The two stage eliminating rates of PAsc/Fe@Cy7QB are shown. (H) FLI of major organs and tumors of mice after 24 h i.v. injection PAsc/Fe@Cy7QB.
Figure 6. In vivo antitumor efficacy against HepG2 tumors of intravenous injection of PBS, PAsc-PDPA, PSFe-PDPA, PAsc-PSFe, or PAsc/Fe@Cy7QB. (A) Schematic illustration of the establishment of the HepG2 tumor xenograft model and treatment process. (B) DCFH-staining of tumor tissues from different groups at 24 h post-injection. (C) Typical images and the relative tumor weight of HepG2 tumor-bearing mice in various groups after 21 days of treatment. (D) Bodyweight changes of HepG2 tumor-bearing mice in various groups after 21 days of treatment. (E) H&E-stained slices of tumor tissues from different groups collected after 21 days of treatment. Mean ± s.d, n = 3. Scale bar represents 20 μm. P values were calculated by two-tailed Student’s t-test (***p < 0.001, *p < 0.05).

Conclusion

We have successfully developed a novel in situ activatable PAsc/Fe@Cy7QB nanomicelles for the tumor-targeted chemodynamic theranostics to achieve effective eradication of localized solid tumors. The biodegradable nanomicelles featured optimized nanoparticle size, PEG surface, and high stability under physiological conditions and could persist for prolonged periods of times in the blood circulation to improve tumor accumulation. The tumor microenvironmental acidosis facilitated the selective disassembly of PAsc/Fe@Cy7QB, inducing the release of Fe²⁺, AscH⁻ and the adjuvant Cy7QB. The released pharmaceutical components synergized to achieve the tumor-cell recognition with multimodal imaging as well as the burst cascade of •OH to selectively kill tumor cells. Our study provides a new paradigm for Fenton metal-based chemodynamic theranostics, demonstrating great potential for accurate tumor therapies.

Abbreviations

Asc: ascorbate (ascorbic acid); AscH⁻: ascorbate monoanion; Asc·⁻: ascorbate radical; Cy7QB: Cy7 dye incorporated with quinone methide-generating boronate ester; PDPA: poly(2-(diisorpropylamino)ethyl methacrylate); PAsc-PSFe: polymerized ascorbate, Schiff base-Fe²⁺ complex and 2-(diisorpropylamino)ethyl methacrylate; PAsc-PDPA: polymerized ascorbate and 2-(diisorpropylamino)ethyl methacrylate; PSFe-PDPA: polymerized Schiff
base-Fe$^{2+}$ complex and 2-(diisopropylamino) ethyl methacrylate; CDT: chemodynamic therapy; •OH: hydroxyl radicals; $\text{H}_2\text{O}_2$: hydrogen peroxide; GSH: glutathione; EPR: enhanced permeability and retention; EDS: energy dispersive spectrometer; MRI: magnetic resonance imaging; FLI: fluorescence imaging; PAI: photoacoustic imaging; ESR: electron spin resonance; BA: benzoic acid; TEM: transmission electron microscopy; DLS: dynamic light scattering; TMB: 3,3',5,5'-tetramethylbenzidine; HRP: horseradish peroxidase; PMA: phorbol 1,2-myristate 1,3-acetate; CAT: Catalase; DMEM: Dulbecco’s modified Eagle’s medium; CLSM: confocal fluorescence microscope images; DCFH-DA: 2,7-dichlorofluorescein diacetate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (201806010189), Guangdong Province, China. Pearl River Nova Program of Guangzhou Foundation of Guangdong (2019A1515012021), the Guangzhou (2019050001); the Natural Science Foundation of China (21771065); the Natural Science Foundation of Guangdong (2019A1515012021), the Pearl River Nova Program of Guangzhou (201806010189), Guangdong Province, China.

Competing Interests
The authors have declared that no competing interest exists.

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