Multifunctional targeted nanoprobe with high NIR-II PAI/MRI performance for precise theranostics of orthotopic early-stage hepatocellular carcinoma

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1. Experimental Section

1.1 Synthesis of Fe$_3$O$_4$ MNPs

The Fe$_3$O$_4$ MNPs were synthesized according to the previous report. In brief, Fe(NO$_3$)$_3$·9H$_2$O (1.6 g) and sodium oleate (3.65 g) were dissolved in the solvent containing cyclohexane (14 mL), ethanol (8 mL), and distilled water (6 mL). Then the mixture was heated to 70 °C and maintained for 4 hours. Next, the mixture was washed by 50 mL distilled water for 3 times. After removing the cyclohexane in the organic layer, the iron-oleate complex was obtained as a waxy solid. Subsequently, all the iron-oleate complex and oleic acid (355 μL) were dissolved in 14 mL of 1-octadecene, and the mixture was stirred at 120 °C for one hour. After that, the temperature of reaction system increased to 320 °C, which was maintained for 60 min, producing a turbid and brownish black colloidal suspension. Finally, ethanol (300 mL) was added to the reaction mixture, yielding the Fe$_3$O$_4$ MNPs as a black precipitate.

1.2 Synthesis of SP

The synthetic route of SP is presented in Figure S3:

Compound 1 (100 mg, 0.129 mmol), compound 2 (57.65 mg, 0.129 mmol), 2,6-Di-tert-butylphenol (5 mg, 0.024 mmol), and Bis(triphenylphosphine) palladium (II) dichloride (10 mg, 0.0142 mmol) were placed in a 50 mL round-bottom flask and the reaction vessel was degassed and purged with N$_2$. Then toluene (5 mL) was injected into the flask, which was subjected to three freeze-pump-thaw cycles to remove O$_2$. Then toluene (5 mL) was injected into the flask, which was subjected to three freeze-pump-thaw cycles to remove O$_2$. Then the mixture was heated to 100 °C under vigorous stirring. After 5.5 h, the mixture was concentrated and dropped into methanol to precipitate the crude product, which was purified by washing with methanol for three times.

1.3 Fabrication of Targeted nanoprobe

SP (1.0 mg), Fe$_3$O$_4$ MNPs (20 mg), and DSPE-PEG-COOH (30 mg, Ponsure Biological) were dissolved in THF (Tetrahydrofuran) (1.0 mL), and the mixture was rapidly injected into pure water (10 mL) under sonication. Then THF was removed by nitrogen flow blowing, yielding the aqueous solution of non-targeted nanoprobe. Subsequently, EDC (50 mg) and NHS (25 mg) were added to activate the prepared non-targeted nanoprobe for 2 hours at room temperature. Then anti-Glypican 3 (GPC-3) antibody (5 mg, ab207080, Abcam) was added to the mixture, which was subjected to mild stirring for 6 hours at 37 °C. The HCC-targeted nanoparticles were obtained via purifying the
reaction mixture through the ultrafiltration process.

1.4 The Characterization of nanoprobes

The morphology of Fe₃O₄ nanoparticles was captured using transmission electron microscopy (TEM, JEM-1200EX, JEOL). Then the nanoprobes were further observed by TEM after negative staining with uranyl acetate. The Zetasizer Nano ZS (Malvern, USA) was used to measure the dynamic light scattering (DLS) and Zeta potential of nanoprobes. The UV-Vis-NIR absorption spectrum of SP and nanoprobes were recorded using a Shimadzu 1700 spectrophotometer (Shimadzu, Japan).

1.5 The instrument of PAI

1.5.1 The OR-PAM system

The optical-resolution photoacoustic microscopy (OR-PAM) system in this study is built by the Bio-photonics lab of City University of Hong Kong. The brief imaging procedures of OR-PAM system are as follows: (1) The master oscillator power amplifier (MOPA) fiber laser (VPFL-G-20, Spectra-Physics) was used as the 532 nm pulsed laser source (7 nanosecond pulse width, 4 kHz pulse repetition rate); (2) The energy of final output laser beam is adjusted at 75 nJ through the neutral density filter; (3) The 2D scanning is completed by two linear stages (PLS-85, Physik Instrument GmbH & Co. KG); (4) The PA signal were received by an ultrasonic transducer (50 MHz center frequency, 39MHz bandwidth, V214-BC-RM, Olympus); (5) The PA signal is amplified by 48 dB using amplifiers (ZFL-500LN+, Mini-Circuits) and digitized at 500 MHz by a data acquisition card (ATS9360, Alazar Technologies Inc); (6) The collected data is processed into maximum amplitude projection (MAP) images.

1.5.2 The PACT system

The photoacoustic computed tomography (PACT) system used in this study was built by the Bio-photonics lab of City University of Hong Kong. The PA excitation laser source in the system is provided by a tunable optical parametric oscillator (OPO) system (basiScan-M/120/HE, Spectral-Physics, USA), which is pumped by an Nd: YAG laser (Quanta-Ray, INDI-40−20, Spectra-Physics, Santa Clara, California, USA). The detailed specification and data acquisition procedures of the PACT system are as follows: (1) The laser with a pulse width of 6-9 nanoseconds and a repetition rate of 20Hz is emitted by the Nd: YAG laser system; (2) The OPO system was activated and appropriate wavelength (400 to 2000 nm) was selected; (3) The laser energy was set as 10 mJ cm⁻²
in vitro and 14 mJ cm$^{-2}$ in vivo at wavelengths of 1064 nm according to the American National Standard Institute (ANSI) safety limits; (4) The tissue emits PA signals under laser irradiation, then the generated PA signals were received by a linear array transducer (L11-4V, Verasonics, USA) and sampled by an acquisition system (Vantage 256, Verasonics, USA); (5) After data acquisition, the researchers used the special algorithm to reconstruct the final images and analyzed related parameters.

Figure S1 The Schematic illustration of the OR-PAM system.

Figure S2 The Schematic illustration of the PACT system.
Figure S3 The synthetic route of SP.

Figure S4 TEM image of Fe₃O₄ MNPs modified with oleic acid.
Figure S5 Absorption spectrum of SP in THF solution.

Figure S6 Zeta potential of the non-targeted nanoprobe, targeted nanoprobe and the mixture of non-targeted nanoprobe and antibodies.

Figure S7 The Characteristics of nanoprobes. (a) Diameter distribution of the non-targeted nanoprobe. Average diameter of the non-targeted and targeted nanoprobe stored in PBS (pH = 7.4) (b) and in FBS (c) for 30 days.
**Figure S8** (a) Absorption spectra of the targeted nanoprobe with various concentrations ranging from 15.63 to 250 μg mL\(^{-1}\). The normalized absorption values of targeted (b) and non-targeted nanoprobe (c) under laser irradiation for 20 min (\(\lambda_{\text{ex}} = 1064\) nm, 10 mJ cm\(^{-2}\)). The absorbance values were normalized against the first time point (0 min). (d) The PA amplitude fluctuation of targeted nanoprobe under 1064 nm light irradiation (10 mJ cm\(^{-2}\)) with different laser pulses.

**Figure S9** (a) The photothermal performance of non-targeted nanoprobe at different concentrations under continuous 1064 nm laser illumination (1 W cm\(^{-2}\)) for 10 min. (b) Photothermal stability of non-targeted nanoprobe (50 μg mL\(^{-1}\)) upon NIR-II irradiation (1 W cm\(^{-2}\)) for three laser on/off cycles. (c) The temperature evolution of targeted nanoprobe samples (100 μg mL\(^{-1}\)) during one
heating-cooling cycle. The laser was switched off when the temperature reached a plateau at 10 min.
(d) The calculated photothermal conversion efficiency of targeted nanoprobe (100 μg mL⁻¹) according to linear analysis of the cooling process. (e) The temperature evolution of DI water during one heating-cooling cycle. The laser was switched off when the temperature reached a plateau at 10 min. (f) The calculated photothermal conversion efficiency of DI water according to linear analysis of the cooling stage.

Figure S10 The levels of GPC-3 expression in HepG2 and L-O2 cells were detected by western blot assay, and the representative bands were shown in (a). (b) The relative expression levels of GPC-3 in HepG2 and L-O2 cells.

Figure S11 The in vitro biocompatibility and photothermal cytotoxicity of non-targeted nanoprobe. (a) Cell viabilities of HepG2 and L-O2 cells incubated with non-targeted nanoprobe at different concentrations in dark. (b) Cell viabilities of HepG2 cells treated with different concentrations of non-targeted nanoprobe with and without 1064 nm laser irradiation (1W cm⁻²). (c) Live/dead
staining of HepG2 cells after treatments of PBS only, PBS + 1064 nm laser, non-targeted nanoprobe only, and non-targeted nanoprobe + 1064 nm laser. Scale bar: 100 μm.

Figure S12 Normalized PA amplitude of nanoprobe-treated cells under OR-PAM scanning. (i) The HepG2 cells treated with targeted nanoprobe, (ii) The HepG2 cells treated with non-targeted nanoprobe, (iii) The L-O2 cells treated with targeted nanoprobe.

Figure S13 The NIR-II PAI of nanoprobe-labelled cell pellets through PACT. (a) The PA images of HepG2 cell pellets incubated with targeted nanoprobe (i) or non-targeted nanoprobe (ii) at the same concentrations (50 μg mL⁻¹) for 12 h, as well as the pure HepG2 cell pellets (iii) as control (excitation wavelength: 1064 nm, pulse energy: 10 mJ cm⁻²). (b) The quantification results of PA amplitude of the cell pellets in different groups.
Figure S14 The histological examination of hepatocellular carcinoma (a) and liver cirrhosis (b).
Scale bar: 200 μm.

Figure S15 The actual photograph of noninvasive PTT.
Figure S16 Histological examination of the major organs dissected from mice after receiving various treatments. Scale bar: 200 µm.

Table S1 Some recent references about the PCE of some inorganic and organic NIR-II photothermal agents

<table>
<thead>
<tr>
<th>Photothermal agents</th>
<th>Category</th>
<th>Laser wavelength</th>
<th>Laser power</th>
<th>PCE</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Targeted nanoprobe</td>
<td>Inorganic-organic hybrid</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>74.6%</td>
<td>Our work</td>
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<tr>
<td>CuS-Au heterostructures</td>
<td>Inorganic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>36.5%</td>
<td>Chem. Eng. J., 2020, 381, 122613</td>
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<tr>
<td>TeO$_2$/(NH$_4$)$_3$WO$_3$ nanoribbons</td>
<td>Inorganic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>43.6%</td>
<td>Nano Lett., 2019, 19, 1179–1189</td>
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<td>Nb$_2$C nanosheets</td>
<td>Inorganic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>45.65%</td>
<td>J. Am. Chem. Soc. 2017, 139, 16235-16247.</td>
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<tr>
<td>H-SiO$_2$-PEG NPs</td>
<td>Inorganic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>48.6%</td>
<td>Biomaterials 2017, 143, 120-</td>
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<td>Material</td>
<td>Type</td>
<td>Laser Wavelength</td>
<td>Laser Intensity</td>
<td>Conversion Efficiency</td>
<td>Source</td>
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<td>CS-RuO$_2$ NPs</td>
<td>Inorganic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>52.5%</td>
<td>Chem. Commun., 2020, 56, 3019–3022</td>
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<td>OSPA</td>
<td>Organic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>30.5%</td>
<td>Biomaterials, 2020, 232, 119684</td>
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<td>PSQPNs-DBCO</td>
<td>Organic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>33.4%</td>
<td>Biomaterials, 2020, 243, 119934</td>
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<td>DPP-IID-FA NPs</td>
<td>Organic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>49.5%</td>
<td>Chem. Commun., 2018, 54, 13599–13602</td>
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<td>2MPT$^{2+}$-CB NPS</td>
<td>Organic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>54.6%</td>
<td>Angew. Chem., Int. Ed., 2019, 58, 15526–15531</td>
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<td>PPy nanosheets</td>
<td>Organic</td>
<td>1064 nm</td>
<td>1.0 W cm$^{-2}$</td>
<td>64.6%</td>
<td>Nano Lett., 2018, 18, 2217–2225</td>
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References