Electronic Supplementary Information

Enzyme-Responsive Copper Sulphide Nanoparticles for Combined Photoacoustic Imaging, Tumor-Selective Chemotherapy and Photothermal Therapy

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Experimental Details:

Chemicals:

Gelatin type A (from porcine skin, ~ 300 Bloom) was purchased from Sigma-Aldrich in the powder form. Doxorubicin hydrochloride (DOX) was obtained from Beijing Huafeng United Technology Co. Copper(II) chloride (CuCl₂·2H₂O), sodium sulfide (Na₂S·9H₂O), sodium cyanoborohydride (NaCNBH₃) and sodium periodate (NaIO₄) were obtained from Aladdin Chemistry Co. Ltd. Deionized water, with a resistivity of 18.2 MΩ·cm, was obtained from Milli-Q Gradient System (Millipore, Bedford, MA, USA) and used for all the experiments.

The preparation of the gelatin-DOX conjugates:

Briefly, DOX, 10 mg in 4.0 mL 0.01 mol L⁻¹ PBS solution in a 15 mL flask, was mixed with a slight molar excess of 0.1 mol L⁻¹ NaIO₄ (0.2 mL) and incubated for 1 hrs at 40 ºC in the dark. Appropriate amount of glycerol (0.1 mol L⁻¹, 15 μL) was then added to consume excess periodate for another 30 min. The solution of oxidized drug was mixed with 4 mL, 50 mg mL⁻¹ gelatin in carbonate buffer solution (CBS, pH 9.5), and incubated for another 1 hrs at 40 ºC in the dark. NaCNBH₃ (0.5 mL, 0.1 mol L⁻¹) was finally added in the flask, and the reaction was allowed to proceed for 2 hrs in the dark. After the reaction, the whole solution was dialyzed at 40 ºC for one week and
then lyophilized for two days.

**Synthesis of CuS@Gel/DOX NPs:**

Gelatin-DOX conjugates (100 mg) were dissolved in 100 mL DI water at 45 °C, and then CuCl₂·2H₂O (0.0855 g) was added and stirred for 5 min. Thereafter, 1 mL of sodium sulfide solution (Na₂S·9H₂O, 0.12009 g) was added with stirring at 45 °C. After 5 min, the reaction mixture was heated to 90 °C and stirred for 60 min until a dark-green solution was obtained. The mixture was transferred to ice-cold water and uncoated gelatin-DOX was separated by three rounds of centrifugation in centrifugal filter tubes (Millipore, 300 KDa). The CuS@Gel/DOX NPs were obtained and stored at 4 °C for further use. CuS@Gel NPs without chemotherapeutic drug were fabricated with the same procedures.

**Characterization of CuS@Gel/DOX NPs:**

The size distribution of CuS@Gel/DOX NPs was determined using a 90Plus/BI-MAS dynamic light scattering (DLS) analyzer (Brookhaven Instruments Co., USA). Transmission electron microscopic images of CuS@Gel/DOX NPs were acquired by FEI Tecnai G2 Sphera Microscope with a CCD camera operated at 100 kV. The UV-visible-NIR absorption spectra of CuS@Gel/DOX NPs were obtained by a UV-visible-NIR spectrophotometer (Varian Cary 4000) with a quartz cuvette of 1.0 cm optical path length.

**PAT experimental setup and imaging of CuS@Gel/DOX NPs in vitro and in vivo:**

The setup for PAT was made in accordance with previous reports.¹ ² A Q-switched Nd:YAG laser (LS-2137/2, LOTIS TII, Minsk, Belarus) and pumped tunable Ti:sapphire laser (LT-2211A, LOTIS TII, Minsk, Belarus) were employed to excite photoacoustic signals. The laser beam is expanded by a concave lens and homogenized by a ground glass lens and then directed onto the test samples. The incident energy density of the laser beam (808 nm, pulse repetition rate of 10 MHz) was controlled to be less than 10 mJ cm⁻² on the surface of test samples. An unfocused ultrasonic transducer with a central frequency of 2.25 MHz was used to detect the ultrasound signals. The photoacoustic signals detected by the ultrasonic transducer
were amplified and then recorded by a data acquisition card installed in the computer. The photoacoustic images were reconstructed through a modified back-projection algorithm.³

To determine maximum imaging depth, five agar gel cylinders with CuS@Gel/DOX NPs were embedded in agar gel of ~ 2.5 cm in diameter, which was then placed under pieces of chicken breast muscle. Five agar gel cylinders, containing CuS@Gel/DOX NPs of 0.5, 1.0, 1.5, 2.0 and 3.0 mM, were sequentially embedded counterclockwise starting at the twelve o’clock direction. Moreover, two carbon rods were placed in the same plane as the agar objects at the center and eleven o’clock direction, respectively, as references (Fig. S4a). When excited by laser light from the top, the embedded objects emit photoacoustic signals were detected by the circularly scanning transducers and fed into the image reconstruction on the basis of a back-projection algorithm. The tissue’s crosssection containing the embedded objects was imaged when blocks of chicken breast muscle were sequentially stacked to make the embedded objects at the desired depths from the laser-illuminated tissue surface.

For in vivo photoacoustic imaging, Kunming mice (18 ~ 22 g) were used for the imaging experiments. All animal studies (n = 3) were carried out in accordance with institutional guidelines. The mouse was anesthetized and set on a homemade mount so that its head protruded into the water tank filled with water through a hole in the tank’s bottom. The hole was sealed with a piece of polyethylene film, and the head of the mouse was in direct contact with the film but not with water in the tank. One PAT scan was performed before the injection of the contrast agent. Afterwards, CuS@Gel/DOX NPs (100 μL, 5 mM) were injected into the mouse tail vein, then PAT scanning started at predetermined times.

**In vitro drug release from CuS@Gel/DOX NPs:**

CuS@Gel/DOX NPs were incubated in PBS (with or without gelatinase) at 37 °C and the released DOX was determined by fluorescence spectrophotometry using the corresponding standard calibration curve. Briefly, 5 mL aqueous solutions of CuS@Gel/DOX NPs (5 mM) were placed in a dialysis bag, which was immersed in a
vial containing 30 mL PBS (0.01 mol L⁻¹, pH 7.4) and incubated with stirring at 37 °C. After 3 hrs, 0.5 mL of gelatinase (10 mg mL⁻¹, 180 U mg⁻¹) was added. At predetermined time intervals, an aliquot of the release medium (3 mL) was withdrawn from the vial and replaced with an equal volume of fresh PBS at predetermined times. Drug release was determined by fluorescent intensity readings (excitation = 480 nm; emission = 590 nm) using a fluorescent spectrophotometer (Cary Eclipse, Varian).

**Investigation of photothermal effect of CuS@Gel/DOX NPs under NIR irradiation:**

Different concentrations of CuS@Gel/DOX NPs dispersed in RPMI-1640 culture media were suspended in quartz cuvettes (total volume of 3.0 mL), irradiated by continuous-wave diode NIR laser (Xi’an Minghui Optoelectronic Technology, China) with a center wavelength of 808 ± 10 nm and output of 2 W for 10 min. The solution temperature was measured by a digital thermometer with a thermocouple probe every 10 s. RPMI-1640 culture media without CuS@Gel/DOX NPs was irradiated by NIR laser as a control.

Localized photothermal cell toxicity of CuS@Gel/DOX NPs was evaluated on HeLa cells (human cervical carcinoma cell line). HeLa cells were seeded onto a 24-well plate at a density of 5 × 10⁴ cells per well. After overnight incubation, the culture medium was replaced with 400 µL gradient concentrated CuS@Gel/DOX NPs solution at 37 °C. The cells were irradiated with an NIR laser (808 nm, 6 W cm⁻²) for 0 min, 3 min and 5 min, respectively. After laser irradiation, the cells were incubated with fresh RPMI-1640 culture medium containing 10 % fetal bovine serum at 37 °C for 30 min, and then washed with PBS and stained with calcein AM for visualization of live cells.

We further investigated the cell survival efficiency after drug and/or laser treatment with MTT assay. HeLa cells were seeded onto a 96-well plate at a density of 10,000 cells per well. After overnight incubation, the culture medium was replaced with gradient concentrated free DOX, CuS@Gel NPs and CuS@Gel/DOX NPs solution at 37 °C, respectively. Thereafter, the cells were irradiated with an NIR laser (808 nm, 6
W cm⁻²) for 0 min, 3 min and 5 min, respectively. After laser irradiation, the cells were incubated with fresh RPMI-1640 containing 10% fetal bovine serum and incubated at 37 °C for 24 hrs. Cell viability was measured using the MTT assay according to the manufacturer suggested procedures.

Reference
Table S1. Characterization of gelatin-DOX conjugates with different feeding weight ratio of DOX and gelatin.

<table>
<thead>
<tr>
<th>DOX : gelatin (w:w)</th>
<th>C_{conjugated DOX/C_{gelatin-DOX conjugate}}</th>
<th>C_{conjugated DOX/C_{feeding DOX}}</th>
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</thead>
<tbody>
<tr>
<td>1:3</td>
<td>9.73 %</td>
<td>32.33 %</td>
</tr>
<tr>
<td>1:5</td>
<td>5.77 %</td>
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<td>1:10</td>
<td>2.53 %</td>
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<td>1:20</td>
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<tr>
<td>1:30</td>
<td>1.39 %</td>
<td>42.28 %</td>
</tr>
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</table>

Fig. S1 Synthesis of gelatin-DOX conjugates via a standard periodate oxidation method.
**Fig. S2** UV-vis-NIR spectra of 0.05 mg mL\(^{-1}\) gelatin-DOX conjugates aqueous solution.

**Fig. S3** (a) Image of CuS@Gel/DOX NPs aqueous solution; (b) Image of bare CuS aggregates without adding gelatin-DOX conjugates during the synthetic process; (c) Image of bare CuS aggregates which were physically mixed with 1 mg/mL gelatin-DOX aqueous solution.
**Fig. S4** Image of CuS@Gel/DOX NPs dispersed in RPMI-1640 culture medium with different concentrations.

**Fig. S5** Size distribution of CuS@Gel/DOX NPs in DI water (a) and RPMI-1640 culture medium (b) from DLS measurement.
Fig. S6 Agar gels containing CuS@Gel/DOX NPs were embedded in an agar phantom, which was then placed under varying numbers of pieces of chicken breast muscle. Photograph of (a) the cross section of agar phantom with different concentration of CuS@Gel/DOX NPs and two carbon rods, (b) stacked chicken breast muscle blocks.

Fig. S7 The integrated absorption calculated from the in vivo brain images of three mice at different times following the injection of CuS@Gel/DOX NPs. The presented values were normalized to that of the integrated absorption of the image obtained before the injection.
**Fig. S8** Non-invasive PAT imaging of a mouse brain *in vivo* employing CuS@Gel/DOX NPs and NIR light at a wavelength of 808 nm. Photoacoustic image acquired (a) before, (b) 60 min after the intravenous injection of CuS@Gel/DOX NPs. (c) Differential images that were obtained by subtracting the preinjection image from the post-injection images (Image c = Image b – Image a).

**Fig. S9** Photothermal destruction of HeLa cells with CuS@Gel NPs and NIR laser (808 nm, 6 W cm$^{-2}$) treatments (a) 0 min; (b) 3 min; (c) 5 min.