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Electronic Supplementary Material

Near-infrared AIEgen for sulfatase imaging in breast cancer in vivo

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Contents:

1. Experimental Procedures

Materials: Sulfatase was bought from Sigma-Aldrich Co. (Shanghai, China). Hydroxylamine was purchased from Shanghai Macklin Biochemical Co., Ltd. (China). Sodium tert-butoxide and sulfur trioxide trimethylamine complex were purchased from Aldrich Chemistry Co., Ltd. (China). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Jiangsu Keygen Biotech Corp., Ltd (China). All chemical reagents were used with analytical grade without further purification. Ultrapure water obtained from a Millipore Milli-Q water purification system (electric resistance >18.3 MΩ) was used for preparing all solutions.

Instrumentation: Transmission electron micrograph (TEM) images were obtained on a JEM-2100 transmission electron microscope (JEOL, Japan). Fluorescence spectra were obtained on an FL 6500 fluorescence spectrometer (PerkinElmer, Germany). High-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1260 Infinity II Prime system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column, with acetonitrile (0.1% of TFA) and water (0.1% of TFA) as the eluent (Agilent, USA). Mass spectra (MS) were measured with Infini Lab LC/MSD (Agilent, USA). Cell images were obtained on a Ti2-U fluorescence microscope (Nikon, Japan). Animal fluorescence images were captured using an IVIS imaging system (IVIS-CT machine, PerkinElmer).

TEM Sample Preparation

First, 10 μM **QMT-SFA** was incubated with sulfatase (40 U/mL) at 37 °C in PBS (100 mM, pH 7.4, 1% DMSO), 10 μL reaction mixture was dropped on the copper grid. Then, the copper grids dried naturally at room temperature and were immediately observed under TEM.

The Limit of Detection Measurement

The fluorescence intensity of **QMT-SFA** was measured in the presence of sulfatase, and standard deviation of the blank measurement was achieved. The limit of detection (LOD) of **QMT-SFA** for sulfatase was calculated with the following equation:

The Limit of Detection = 3σ/k

Where σ is the standard deviation of the blank measurement, k is the slope between the fluorescence intensity versus various sulfatase concentrations.

Molecular Docking

Firstly, we used the Pymol tool to remove water molecules and unrelated heteroatoms, leaving only the protein. Then, we used the propka3 （https://www.ddl.unimi.it/vegaol/propka.htm） online tool to calculate and assign amino acid pKa values under neutral conditions. proteinsPlus （https://proteins.plus/） was used to predict the best binding sites. Docking structures of receptors and ligands were prepared by Autodock Tools-1.5.7. Molecular docking experiments were

performed using Watvina software with the box size set to a cube with the side length of 30 Å and Spacing step of 0.375. The maximum number of searching conformations was set as 10000, and the genetic algorithm was used for conformation sampling and scoring. The optimal conformation was sorted according to the docking score.

Cell Culture

4T1 cells and L02 cells were cultured at 37 °C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).

Cytotoxicity Assay

The cytotoxicity of **QMT-SFA** to 4T1 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 4T1 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for overnight at 37 °C. Then the medium was replaced with **QMT-SFA** at varying concentration (0 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, 160 μM, and 320 μM). Each concentration was tested in three replicate wells. After incubation for 24 h, cells were rinsed twice with PBS and incubated with 0.5 mg/mL MTT. After incubation for 4 h, the supernatant was discarded and the precipitate was dissolved in DMSO (150 μL) with gentle shaking. The absorbance (OD) of MTT at 570 nm in each well was acquired on the iD3-3914 microplate reader.

Cell Assays

To examine the ability of **QMT-SFA** for imaging sulfatase activity in living cells, we seeded 4T1 cells or L02 cells on 96-well plates at a density of 1.0×10^4 cells/well. Three groups were set: (1) the "4T1" group, in which 4T1 cells were treated with **QMT-SFA** (10 μM) for 1 h; (2) the "L02" group, in which L02 cells were treated with **QMT-SFA** (10 μM) for 1 h; (3) the "4T1+Inh." group, in which 4T1 cells were pretreated with hydroxylamine (1 mM, a sulfatase inhibitor) for 0.5 h, followed by **QMT-SFA** (10 μM) treatment for 1 h. The medium was removed, and carefully washed with PBS (1×) three times. Finally, fluorescence images were captured on a Ti2-U fluorescence microscope (Nikon, Japan).

Animal Protocol

All animal experiments were approved and performed according to the guidelines of the Animal Care and Use Committee of the Southeast University Laboratory Animal Center (No: 20240306009). The 5-week-old female BALB/c nude mice (15 ± 2 g) were purchased from Nanjing Jun Ke Biological Technology Co. Ltd.

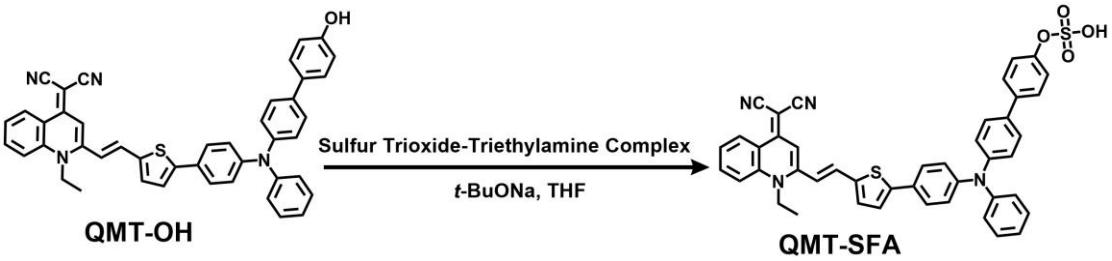
In Vivo Imaging Experiments

To monitor sulfatase activity in tumor-bearing nude mice, all mice were subcutaneously injected with 2×10^6 4T1 cells to establish tumor-bearing nude mice and randomly divided into two groups: "QMT-SFA" and "QMT-SFA+Inh." groups. Then, each mouse was treated with 0.25 mg/kg **QMT-SFA** via intratumoral injection.

For the “QMT-SFA+Inh.” group, 4T1 tumor-bearing mice were pretreated with inhibitor (0.02 mg/kg) for 0.5 h just before **QMT-SFA** injection. After that, the fluorescence signals generated from the tumors in mice were recorded by a small animal imaging system at various time points postinjection (i.e., 0, 0.5, 1, 2, 4, 8, 16 h). During the imaging process, the mice were anesthetized with 2.5% isoflurane gas in an oxygen flow (1.5 L min^{-1}).

2. Syntheses and Characterizations

Scheme S1. The synthetic route for **QMT-SFA**.



*Synthesis of **QMT-SFA***: The synthesis of **QMT-OH** was performed according to our recent publication[1]. Compound **QMT-OH** (6.64 mg, 0.01 mmol) in THF (10 mL) at 22℃ was added a solution of sodium tert-butoxide (0.96 mg, 0.01 mmol) in THF (1 mL) dropwise (10 min). After 15 min, sulfur trioxide trimethylamine complex (1.81 mg, 0.013 mmol) was added as solid. After 3 h, the solvents were evaporated from the reaction mixture. Compound **QMT-SFA** was obtained after HPLC purification using water/acetonitrile mixed solvent (volume ratio from 50:50 to 0:100) added with 0.1% TFA as the eluent. MS: calculated for **QMT-SFA** $[M+H]^+$: 745.2; obsvd. ESI-MS $[M+H]^+$: *m/z* 745.2 (**Figure S1**)

3. Supporting Figures

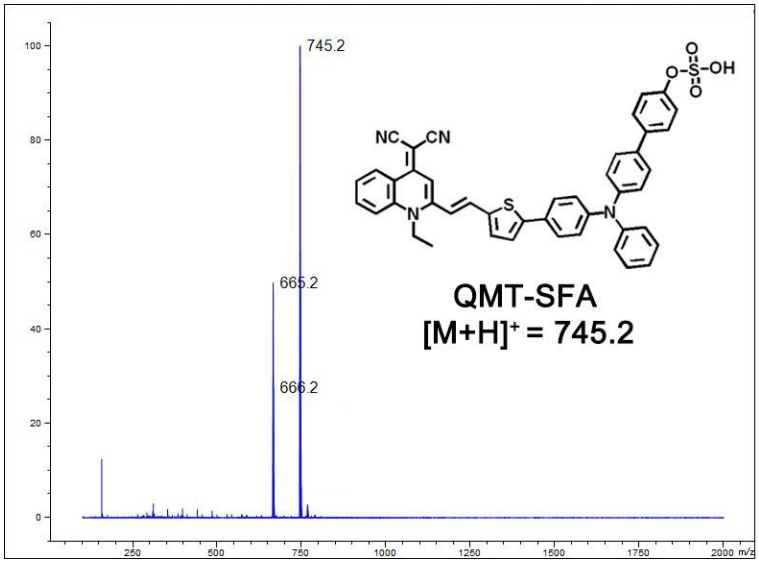


Figure S1. ESI-MS spectrum of **QMT-SFA**.

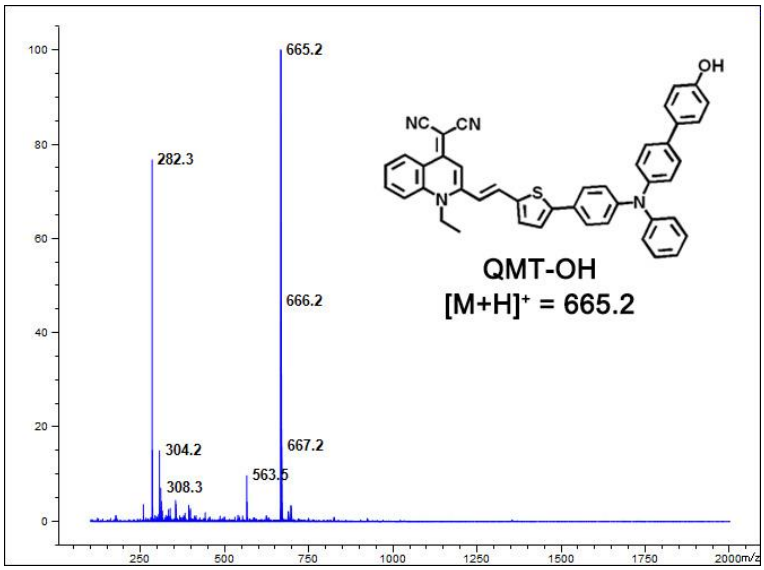


Figure S2. ESI-MS spectrum of the HPLC peak at 18.0 min in Figure 2a.

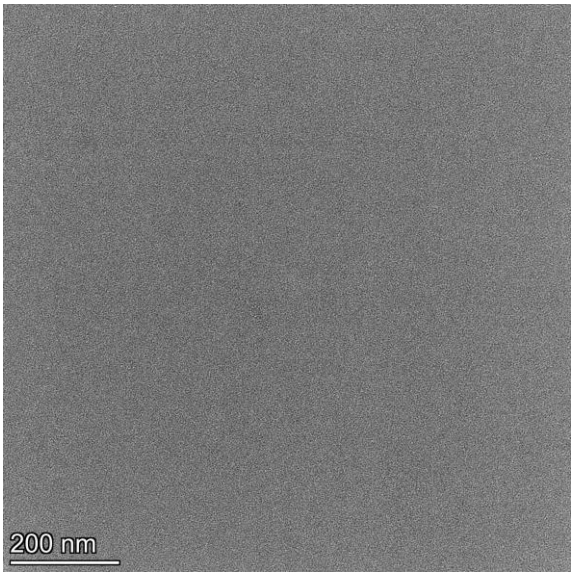


Figure S3. TEM image of 10 μM **QMT-SFA** in PBS (100 mM, pH 7.4, 1% DMSO).

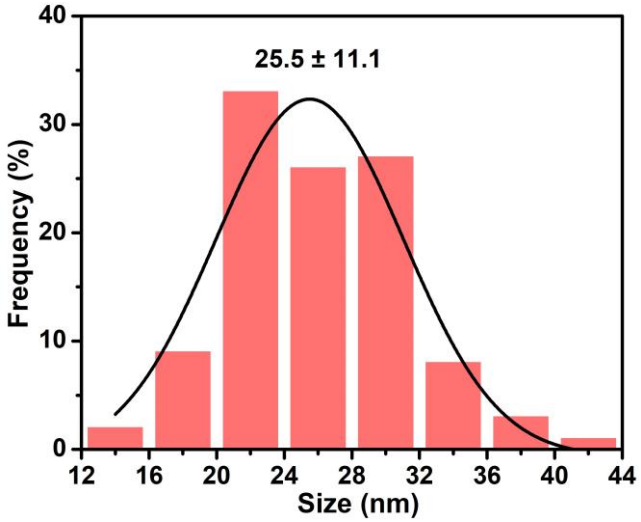


Figure S4. Histogram of diameters of the nanoparticles in the TEM image of Figure 2b.

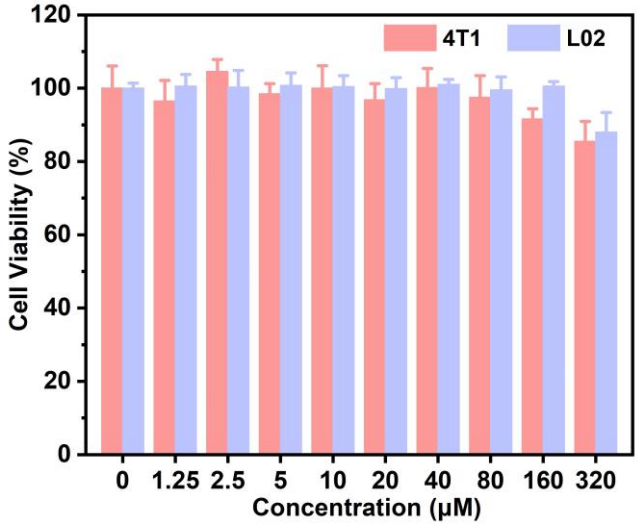


Figure S5. Cell viability of 4T1 or L02 cells incubated with **QMT-SFA** at different concentrations for 24 h.

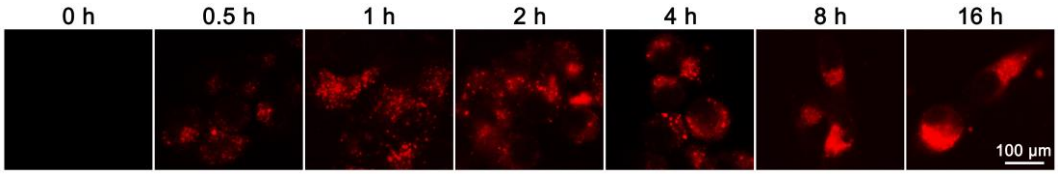


Figure S6. Time-course fluorescence images of 4T1 cells incubated with 10 μM **QMT-SFA** for 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h and 16 h. λ_{ex} = 540-580 nm, λ_{em} = 600-660 nm.

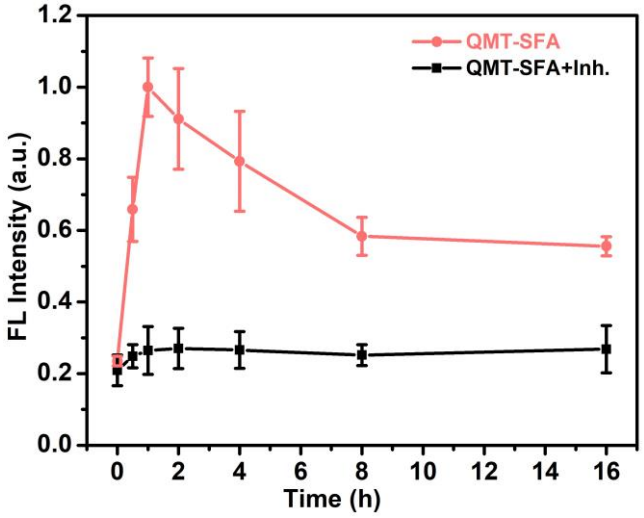


Figure S7. Quantification of fluorescence intensity in Figure 4.

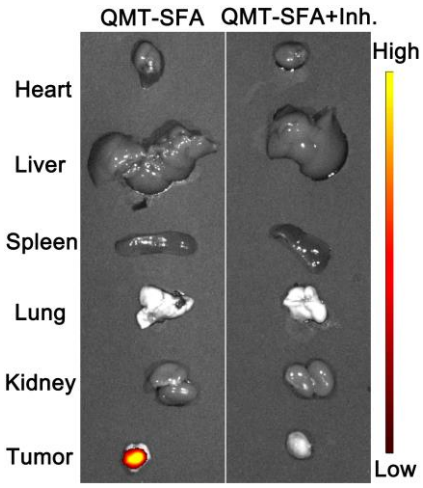


Figure S8. Ex vivo fluorescence images of the main organs and tumors from the mice in Figure 4.

4. Supporting Table

Table S1. Analysis of interaction force at the interface of **QMT-SFA** and sulfatase.

Name	Distance	Category	Type
GLU481:N - LIG:N52	3.04	Hydrogen Bond	Conventional Hydrogen Bond
LIG:H86 - GLY402:O	1.94	Hydrogen Bond	Conventional Hydrogen Bond
SER403:CA - LIG:O46	3.29	Hydrogen Bond	Carbon Hydrogen Bond
SER403:CA - LIG:O50	3.79	Hydrogen Bond	Carbon Hydrogen Bond
GLU481:OE2 - LIG	4.25	Electrostatic	Pi-Anion
VAL91:N - LIG	3.89	Hydrogen Bond	Pi-Donor Hydrogen Bond
TYR379:OH - LIG	3.84	Hydrogen Bond	Pi-Donor Hydrogen Bond
SER403:OG - LIG	3.70	Hydrogen Bond	Pi-Donor Hydrogen Bond
VAL91:CG2 - LIG	3.95	Hydrophobic	Pi-Sigma
VAL91:CG2 - LIG	3.73	Hydrophobic	Pi-Sigma
TYR379 - LIG	5.65	Hydrophobic	Pi-Pi T-shaped
HIS405 - LIG	5.06	Hydrophobic	Pi-Pi T-shaped
HIS405 - LIG	4.43	Hydrophobic	Pi-Pi T-shaped
ALA478 - LIG:C14	4.38	Hydrophobic	Alkyl
LIG:C14 - PRO89	4.66	Hydrophobic	Alkyl
LIG - ALA478	4.27	Hydrophobic	Pi-Alkyl
LIG - ALA478	4.36	Hydrophobic	Pi-Alkyl
LIG - VAL93	4.66	Hydrophobic	Pi-Alkyl
LIG - VAL477	4.72	Hydrophobic	Pi-Alkyl

5. Reference

1 L. Xu, H. Gao, W. Zhan, Y. Deng, X. Liu, Q. Jiang, X. Sun, J.-J. Xu, G. Liang, *J. Am. Chem. Soc.*, 2023, **145**, 27748-27756.