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Dual-modal Molecular Probe for Near-infrared Fluorescence and Photoacoustic Imaging of Peroxynitrite

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ABSTRACT: Peroxynitrite (ONOO⁻), a reactive and short-lived biological oxidant, is closely related with many pathological conditions such as cancer. However, real-time *in vivo* imaging of ONOO⁻ in tumor remains to be challenging. Herein, we develop a near-infrared fluorescence (NIRF) and photoacoustic dual-modal molecular probe (CySO₃CF₃) composed of a water-soluble hemicyanine dye caged with a trifluoromethyl ketone moiety for *in vivo* imaging of ONOO⁻. The trifluoromethyl ketone moiety can undergo a series of ONOO⁻-induced cascade oxidation-elimination reactions, leading to sensitive and specific fluorescence and photoacoustic turn-on responses toward ONOO⁻; while a zwitterionic structure of the hemicyanine component ensures good water-solubility. CySO₃CF₃ thus not only can specifically detect ONOO⁻ in solution and cells with the limit of detection down to 53 nM, but also allows for NIRF and photoacoustic dual-modal imaging of ONOO⁻ in the tumor of living mice.

Reaction oxygen and nitrogen species (RONS) play a vital role in physiological and pathological processes of the living system.¹ Peroxynitrite (ONOO⁻) as a highly reactive RONS can nitrate the biomacromolecules including proteins, nucleic acids and lipids, and disorganize their biological functions,² leading to mitochondria malfunction and cellular apoptosis. In tumor microenvironment, ONOO⁻ is closely related to tumor immunosuppression. Particularly, ONOO⁻ can nitrate p53 protein,³ a critical regulator of tumor suppressor protein that is encoded by gene TP53 (tumor protein p53) in human body, which causes the loss of the control role of p53 protein to cell cycle followed by a series of cytotoxic effects, ultimately accelerating the formation of tumor.⁴ Thereby, real-time imaging of ONOO⁻ in tumor is essential for understanding the pathological functions of ONOO⁻ and developing innovative therapeutic approach to tumor treatment.

Fluorescence imaging has been developed for detection of ONOO⁻,⁵⁻¹¹ but most of fluorescent probes are only responsive to visible light, which is not ideal for *in vivo* imaging due to shallow tissue penetration. In contrast, photoacoustic (PA) imaging is a hybrid imaging modality that combines laser excitation with acoustic detection, offering higher tissue penetration depth (up to ≈ 5 cm) for *in vivo* imaging.¹²⁻¹⁴ Although near-infrared (NIR) molecular dyes,^{15,16} polymer nanoparticles,¹⁷⁻²⁴ fluorescence pro-

teins,^{25,26} porphyrins,^{27,28} upconversion nanoparticles,^{29,30} quantum dots,³¹ gold nanoparticles,³² carbon dots³³ and 2D materials³⁴ have been used for PA imaging, smart activatable probes with specific PA responses toward ONOO⁻ are less exploited.^{35,36} We recently developed organic semiconducting nanoprobes for ratiometric PA imaging of ONOO⁻.³⁷ Although it can be applied for *in vivo* imaging, this nanoprobe was doped with bulky borane in addition to the chromophores to achieve selectivity. Besides, the fluorescence is completely quenched, making it incompatible for fluorescence imaging.

In this study, we report the design and synthesis of a water-soluble small-molecular probe (CySO₃CF₃) for *in vivo* near-infrared fluorescence (NIRF)/PA dual-modal imaging of ONOO⁻. CySO₃CF₃ is composed of trifluoromethyl ketone moiety (TFK),^{6-8,38} an ONOO⁻-responsive unit, caged NIR hemicyanine dye with a zwitterionic structure³⁹ (Scheme 1a). The probe is nonfluorescence because CySO₃OH is in a "caged" state with weak electron-donating ability of the oxygen atom.^{37,40-44} In the presence of ONOO⁻, CySO₃CF₃ is converted into the uncaged molecule (CySO₃OH) through a series of cascade oxidation-elimination reactions. Due to the enhanced electron-donating ability from the oxygen atom in CySO₃OH, such an ONOO⁻-induced conversion results in not only fluorescent turn-on but also red-shifted NIR absorption, enabling NIRF/PA dual-modal imaging.

EXPERIMENTAL SECTION

Chemicals. All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated. Sodium hypochlorite (NaOCl, 5% aqueous solution). Hydrogen peroxide (H₂O₂, 30% aqueous solution), sodium nitrite (NaNO₂) and boron tribromide (BBr₃) were purchased from TCI (Tokyo, Japan). Anhydrous dichloromethane (CH₂Cl₂) were treated with CaH₂ under reflux for 24 h and redistilled before use. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Bedford, U.S.A.). A stock solution (1 mmol/L) of CySO₃CF₃ was prepared by dissolving in dimethyl sulphoxide (DMSO).

Instrumentation. UV-vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence spectra were recorded on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). NMR spectra were measured using a Bruker 300 MHz instruments (Germany). All ¹H chemical shifts (δ) are relative to residual protic solvent (CHCl₃: δ 7.26 ppm). Mass spectra were measured using Thermo LCQ Fleet LC-MS with ESI mode (America). The pH values were measured using a digital pH-meter (Seven-Compact S220, Zurich, Switzerland). High performance liquid chromatography (HPLC) analyses were performed on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with CH₃CN (0.1% of TFA) and water (0.1% of TFA) as the eluent. Fluorescence images of cells were acquired on Laser Scanning Microscope LSM800 (Zeiss) or LX71-inverted fluorescence microscope (Olympus) with Retiga-2000R CCD camera. Fluorescence images of tumor were acquired with IVIS Spectrum imaging system (PerkinElmer, Inc.). *In vivo* PA images were performed with a Multispectral Optoacoustic Tomography scanner (MSOT, iThera medical, Germany) with excitation light of 680–850 nm.

Synthesis

Compound 4: To a solution of 3-(4-methoxyphenyl) propanoic acid (**3**, 2.7 g, 15.0 mmol) in ethanol (20 mL) was added H₂SO₄ (0.7 mL, con.) at room temperature under nitrogen atmosphere. The resulting mixture was refluxed for 4 h. Then ethanol was evaporated under reduced pressure, and the residue was added ethyl acetate (60 mL). The resulting solution was washed with H₂O (3 × 50 mL), saturated sodium bicarbonate (30 mL) and saturated sodium chloride (30 mL), respectively. The organic phase was dried with anhydrous Na₂SO₄, and then concentrated under reduced pressure, which obtained ethyl 3-(4-methoxyphenyl)propanoate **4** (2.99 g, yield 96%) without purification. ¹H NMR (300 MHz, CDCl₃): δ = 7.12 (d, *J* = 8.6 Hz, 2 H), 6.83 (d, *J* = 8.7 Hz, 2 H), 4.12 (q, *J* = 7.1 Hz, 2 H), 3.78 (s, 3 H), 2.89 (d, *J* = 7.8 Hz, 2 H), 2.58 (d, *J* = 7.8 Hz, 2 H), 1.23 (t, *J* = 7.2 Hz, 3 H). MS (ESI): *m/z* = 209.13 [M+H]⁺.

Compound 5: To a solution of ethyl 3-(4-methoxyphenyl) propanoate **4** (2.91 g, 14.0 mmol) and TMSCF₃ (3.1 mL, 21.0 mmol) in tetrahydrofuran (THF, 10 mL) was added CsF (21.2 mg, 0.14 mmol). The mixture was stirred at room temperature under nitrogen atmosphere for 18 h and then CH₂Cl₂ (30 mL) was added to the resulting solution, the mixture was treated with water (3 × 10 mL) and saturated

sodium chloride (10 mL) respectively. The organic phase was dried with anhydrous Na₂SO₄, and then was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 5%) to afford the desired product (2-ethoxy-1,1,1-trifluoro-4-(4-methoxyphenyl) butan-2-yl)trimethylsilane **5**. (4.02 g, yield 86%). ¹H NMR (300 MHz, CDCl₃): δ = 7.14 (d, *J* = 8.2 Hz, 2 H), 6.86 (d, *J* = 8.0 Hz, 2 H), 3.81 (s, 3 H), 3.81–3.56 (m, 2 H), 2.78–2.63 (m, 2 H), 2.18–1.96 (m, 2 H), 1.26 (td, *J* = 7.0, 0.9 Hz, 3 H), 0.24 (d, *J* = 0.9 Hz, 9 H). MS (ESI): *m/z* = 335.19 [M+H]⁺.

Compound 6: To a solution of compound **5** (4.0 g, 12 mmol) in THF (2 mL) was added TBAF (1 M in THF, 13.0 mL) dropwise under nitrogen atmosphere. The mixture was stirred at room temperature for 5 h and then was treated with hydrochloric acid (4.0 M, 4.0 mL) for 3 h. CH₂Cl₂ (30 mL) was added to the resulting solution, which was washed with water and saturated sodium chloride respectively. The organic phase was dried with anhydrous Na₂SO₄, and then was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 5%) to obtain trifluoro-4-(4-methoxyphenyl)butan-2-one **6**. (2.45 g, yield: 88%). ¹H NMR (300 MHz, CDCl₃): δ = 7.11 (d, *J* = 8.7 Hz, 2 H), 6.84 (d, *J* = 8.7 Hz, 2 H), 3.78 (s, 3 H), 3.05–2.98 (m, 2 H), 2.96–2.89 (m, 2 H). MS (ESI): *m/z* = 233.09 [M+H]⁺.

Compound 7: To a solution of 1,1,1-trifluoro-4-(4-methoxyphenyl) butan-2-one **6** (2.4 g, 10.3 mmol) in CH₂Cl₂ (20 mL) was added BBr₃ (5.2 mL, 13.4 mmol) at –78 °C under nitrogen atmosphere. The resulting mixture was then warmed to room temperature and stirred 12 h. Then ice-water was added slowly, and the mixture was added ethyl acetate (50 mL). The resulting solution was washed with H₂O (3 × 20 mL), saturated sodium bicarbonate (30 mL) and saturated sodium chloride (30 mL), respectively. The organic phase was dried with anhydrous Na₂SO₄, and then concentrated under reduced pressure, which was purified by silica gel column chromatography (ethyl acetate/hexane, 25%) to obtain 1,1,1-trifluoro-4-(4-hydroxyphenyl)butan-2-one **7** (1.84 g, yield 82%). ¹H NMR (300 MHz, CDCl₃): δ = 7.06 (d, *J* = 8.6 Hz, 2 H), 6.77 (d, *J* = 8.6 Hz, 2 H), 3.05–2.97 (m, 2 H), 2.95–2.80 (m, 2 H). MS (ESI): *m/z* = 219.16 [M+H]⁺.

CySO₃Cl: A mixture of compound **1** (516 mg, 3.0 mmol), compound **2** (1.76 g, 6.0 mmol) and sodium acetate (246 mg, 3.0 mmol) were dissolved in acetic anhydride (20.0 mL) under nitrogen atmosphere and heated to 90 °C for 1 h with a Dean-Stark condenser. After cooling to room temperature, the resulting green solid was collected, washed with Et₂O (100 mL) and purified by silica gel column chromatography using CH₂Cl₂/ 0–2% methanol as eluent to afford desired product CySO₃Cl as a blue solid. Yield: 1.59 g (73%). ¹H NMR (300 MHz, CDCl₃): δ = 8.43 (d, *J* = 14.2 Hz, 2H), 7.52 (d, *J* = 7.2 Hz, 2H), 7.48–7.32 (m, 4H), 7.28 (d, *J* = 7.0 Hz, 2H), 6.34 (d, *J* = 14.1, 2H), 4.23 (t, *J* = 7.2 Hz, 2H), 2.90 (t, *J* = 6.6 Hz, 2H), 2.75 (t, *J* = 4.2 Hz, 2H), 2.08–1.88 (s, 10H), 1.73 (s, 12H) ppm; ESI-MS, *m/z*: 727.48 [M]⁺.

CySO₃OH: To a stirred solution of resorcinol (220 mg, 2.0 mmol) in acetonitrile (ACN, 15 mL) was added K₂CO₃ (276 mg, 2.0 mmol) at room temperature under nitrogen atmosphere and the resulting mixture was stirred for 20 min.

Then, a solution of CySO_3Cl (727 mg, 1.0 mmol) in DMF (10 mL) was added to the above mixture via a syringe, and the reaction mixture was heated at 50 °C for 4 h. Eventually the solvent was evaporated under reduced pressure, and the crude product was purified by flash column chromatography (petroleum ether/ $\text{CH}_2\text{Cl}_2/\text{MeOH}$ = 25:25:1) on silica gel, affording the desired compound CySO_3OH as a blue-green solid (400 mg, yield 79%). ^1H NMR (400 MHz, CDCl_3): δ = 8.75 (d, J = 14.8 Hz, 1H), 7.63 (d, J = 14.8 Hz, 1H), 7.51-7.57 (m, 2H), 7.45-7.39 (m, 3H), 6.87-6.82 (m, 2H), 6.51 (d, J = 14.8 Hz, 1H), 4.37 (t, J = 7.2 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H), 2.78-2.72 (m, 4H), 2.11-2.05 (m, 2H), 1.99-1.92 (m, 4H), 1.81 (s, 6H) ppm; ESI-MS, m/z : 506.38 $[\text{M}]^+$.

CySO_3CF_3 : To a stirred solution of BTC (17.8 mg, 0.06 mmol) dissolved in dichloromethane (1 mL) and cooled to 0 °C, was added drop-wise a solution of the compound 7 (17.4 mg, 0.08 mmol) in dichloromethane (2.0 mL) and then triethylamine (8.0 mg, 0.08 mol) was added. During addition, the temperature was kept at 0 °C. After the addition was completed, the mixture was allowed slowly to rise to room temperature and stirring was continued for 2 h. Then a solution of CySO_3OH (30 mg, 0.06 mmol) in dichloromethane (2 mL) was added to the above mixture and stirred 30 min. The resulting mixture was concentrated under vacuum, which was purified by silica gel column chromatography (ethyl acetate/hexane, 10%) to obtain CySO_3CF_3 (14.0 mg, yield 31%). ^1H NMR (300 MHz, CD_3OD): δ = 8.64 (d, J = 19.6 Hz, 1H), 7.52-7.39 (m, 5H), 7.42-7.35 (m, 5H), 6.92 (d, J = 7.2 Hz, 1H), 6.89 (s, 1H), 6.43 (d, J = 19.6 Hz, 1H), 4.27 (t, J = 8.8 Hz, 2H), 3.25-3.05 (m, 4H), 2.90 (t, J = 9.2 Hz, 2H), 2.73 (t, J = 8.0 Hz, 2H), 2.68 (t, J = 8.2 Hz, 2H), 2.11-1.85 (m, 6H), 1.77 (s, 6H). ^{19}F NMR (300 M, CD_3OD): δ = -79.28. MS (ESI): m/z = 750.49 $[\text{M}]^+$.

Cell Culture and Cytotoxicity Test. HeLa (Human Cervical Adenocarcinoma Epithelial Cells), RAW 264.7 (Mouse Leukaemic Monocyte Macrophage Cell Line) and NIH 3T3 (Mouse Embryonic Fibroblasts) cells were purchased from the American Type Culture Collection (ATCC). These three kinds of the cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with heat-inactivated fetal bovine serum (10%) in a humidified environment containing 5% CO_2 and 95% air at 37 °C, respectively. Cells were seeded in 96 well plates (1×10^4 cells/well) for 24 h, and then CySO_3CF_3 (final concentration 0, 5, 10, 20, 35, 50, and 80 $\mu\text{mol/L}$) was added to the cell culture medium. The corresponding cells were incubated with or without (control) CySO_3CF_3 for 24 h, followed by the addition of MTS (100 μL , 0.1 mg/mL) for 4 h. The absorbance of MTS at 490 nm was measured by using a microplate reader. The cytotoxic effect (VR) of CySO_3CF_3 were assessed using the following equation: $\text{VR} = A/A_0 \times 100\%$, where A and A_0 are the absorbance of the experimental group and control group, respectively. The assays were performed in six sets for each concentration.

Cell Imaging. After seeded RAW 264.7 cells reached 70–90% confluency, the cells were washed three times using prewarmed PBS buffer (10 mM, pH 7.4). Then the cells were incubated with CySO_3CF_3 in an atmosphere of 5% CO_2 and 95% humidified air at 37 °C for 30 min. After that the cells were stained with 4',6-diamidino-2-phenylindole

(DAPI) (NucBlue Live ReadyProbes Reagent, Thermo Fisher) for the nuclei as protocols. Fluorescence images of the live cells were taken with the wide-field fluorescence microscope Leica DMI8 (Leica, Germany) equipped with scientific CMOS (sCOMS, Hamamatsu, Japan). Excitation/emission wavelengths were 405/490 $\pm$ 20 nm for DAPI, and 680/710 $\pm$ 20 nm for CySO_3CF_3 .

Tumor Mouse Model. All animal experiments were performed under protocols approved by the Laboratory Animal Center of Soochow University. To establish tumors in 6-week-old female nude mice, 2 million 4T1 cells suspended in 1 mL DMEM (containing 10% fetal bovine serum, 1% penstrep, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin) were injected subcutaneously near the armpits of the mouse. Tumors were grown until a single aspect was ~7 mm (approximately 10–15 days) before used for fluorescence and PA imaging.

In Vivo Fluorescence Imaging. Nude mice bearing 4T1 tumors were injected with CySO_3CF_3 (50 μM in 100 μL saline) (n = 3) through the tail vein using a microsyringe. Fluorescence imaging were performed on the IVIS spectrum imaging system at designated time points after CySO_3CF_3 administration (ex: 675 $\pm$ 10 nm, em: 720 $\pm$ 10 nm). Images quantitation was performed using Living Image software for IVIS imaging system.

In Vivo Photoacoustic Imaging. After the nude mice were anesthetized using 2% isoflurane in oxygen, CySO_3CF_3 (50 μM in 100 μL saline) (n = 3) was systematically injected through the tail vein using a microsyringe. Multispectral Optoacoustic Tomography scanner was used to acquire the PA images at 680 nm after systematical administration of CySO_3CF_3 . *In vivo* PA spectra with excitation light from 680 to 850 nm were acquired from the tumor regions after 3 h systemic administration of CySO_3CF_3 .

Data Analysis. The fluorescence data were measured by region of interest (ROI) analysis using Living Image 4.0 Software. *In vivo* and ex vivo PA intensities were quantified with ROI analysis using postprocessing software ViewMSOT. Results were expressed as the mean $\pm$ standard deviations (SD) unless otherwise stated.

RESULTS AND DISCUSSION

Scheme 1b describes the synthesis route of CySO_3CF_3 . (Z)-2-chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde **1** was first reacted with 2,3,3-trimethyl-1-(4-sulfobutyl)-3H-indol-1-ium **2** to afford compound CySO_3Cl , followed by reacted with resorcinol in the existence of K_2CO_3 to obtain CySO_3OH directly for the following reaction. The other component, the esterified ethyl 3-(4-methoxyphenyl) propanoate was firstly attacked by (Trifluoromethyl)trimethylsilane (TMSCF_3) and then eliminated to form compound **6**, which was further treated with BBr_3 to afford compound **7**. Finally, the oxygen atom of compound CySO_3OH was caged with compound **7** using phosgene to afford CySO_3CF_3 . The chemical structure of CySO_3CF_3 was characterized by proton nuclear magnetic resonance (^1H NMR) and electrospray ionization-mass spectrometry (ESI-MS), as shown in the Experimental Section and in the Supporting Information.

To validate ONOO^- -induced oxidation of CySO_3CF_3 , high performance liquid chromatography (HPLC) analysis was conducted (Figure S1, Supporting Information). The data showed that CySO_3CF_3 (HPLC retention time, $T_R = 8.7$ min) was converted to CySO_3OH ($T_R = 4.6$ min). The ESI spectrometry analysis (Figure S2, Supporting Information) of the probe treated with ONOO^- further confirmed the formation of the expected CySO_3OH (m/z 506.38) and the desired spiro-quinone (m/z 234.05). The reaction mechanism was confirmed by the NMR spectra using trifluoro-(4-methoxyphenyl)butan-2-one as a model compound (Figure S3, Supporting Information).

To study the optical response along with ONOO^- -induced cleavage, the spectroscopic properties of CySO_3CF_3 under physiological conditions (1×PBS: phosphate buffer, pH 7.4) were measured before and after addition ONOO^- . CySO_3CF_3 intrinsically had the absorption maximum at 602 nm (Figure 1a); upon addition of ONOO^- , this peak gradually decreased, while a new peak at 686 nm

appeared (Figure S4, Supporting Information). At the saturation point, the absorbance at 686 nm for CySO_3CF_3 was 0.48, which was 9.4-fold higher than that at the initial state (0.051). In addition, the fluorescence at 712 nm dramatically enhanced upon addition of ONOO^- (Figure S5, Supporting Information), which was ~59-fold higher relative to its initial state at the saturation point. Such absorption and fluorescent responses confirmed the ONOO^- -induced conversion as proposed in Scheme 1. Both absorption and fluorescent signals had good linearity with the concentration of ONOO^- (Figure 1b and Figure S6, Supporting Information), showing the limits of detection of 115 and 53 nM, respectively.

The specificity of CySO_3CF_3 towards ONOO^- was tested against other RONS ($\text{O}_2^{\cdot-}$, ClO^- , $^1\text{O}_2$, $^{\cdot}\text{OH}$, H_2O_2 , NO , NO_2^-) and sulphur compounds (S^{2-} , GSH, Cys, Hcy, HSO_3^- , SO_3^{2-}). The results showed that other RONSs and sulphur compounds could not induce any absorption and fluorescent

changes for the probe (Figure 1c and Figure S7, Supporting Information). Thus, CySO_3CF_3 could only be activated by ONOO^- , changing their solution colors from purple to cyan (the inset of Figure 1c). To determine the activation speed of the probe, the reaction kinetics of CySO_3CF_3 with ONOO^- was studied by time-course absorption measurements (Figure 1d and Figure S8, Supporting Information). The corresponding pseudo-first-order rate constant (k) for the present ONOO^- reaction was determined to be $1.49 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. Thus, CySO_3CF_3 should be able to specifically detect ONOO^- in real time.

The PA sensing ability of CySO_3CF_3 was also tested under physiological conditions. After activation by ONOO^- , the PA band ranging from 680 to 750 nm appeared (Figure 1e), consistent with the absorption response. At the saturation point, the PA intensity at 680 nm (PA_{680}) was 5.1-fold higher than that at the initial state (1.73). In contrast, in the present of other ROS ($\text{O}_2^{\cdot-}$, ClO^- , $^1\text{O}_2$, $\cdot\text{OH}$, and H_2O_2), no obvious changes were observed in the PA spectra (Figure S9, Supporting Information). Furthermore, PA_{680} was linearly correlated to the concentration of ONOO^- with the limit of detection of 145 nM, which was closed to that for absorption response. As the concentration of ONOO^- is in the range of 50–100 μM per minute in living systems,⁴⁵ CySO_3CF_3 should be ideal for sensing ONOO^- in living cells and mice.

The low pH (pH 6.8) of tumor tissues was caused by the highly acidic metabolites, e.g. lactic acid produced by anaerobic glycolysis in the absence of oxygen during tumor growth. To validate the feasibility of probe activation in the acidic tumor environment, the absorption responses of CySO_3CF_3 toward ONOO^- was tested at pH ranging from 9.0 to 5.0. The probe presented weakened absorption signals at 686 nm especially when pH decreased from 9.0 to 6.0 (Figure S10, Supporting Information). This could be ascribed to the protonation of the phenoxide product to its conjugate acid at low pH as CySO_3OH has $\text{pK}_a = 7.11$. The maximum absorption signals at 600 nm of the conjugate acid were the same as the initially unactivated state. Note that at pH = 6.8, in the present of ONOO^- , the absorption value at 686 nm was ≈ 0.290 , 3.6-fold higher when compared with its original state (0.081). Hence, despite its pH response, CySO_3CF_3 could be applied to fluorescence and PA imaging of ONOO^- in acidic tumor microenvironments.

To validate the reactivity of CySO_3CF_3 toward ONOO^- in cells, Fluorescence imaging was conducted on murine macrophage (RAW 264.7) cells (Figure 2a). After incubation with CySO_3CF_3 for 30 min, the cells were washed and stained with nuclei indicator, 4',6-diamidino-2-phenylindole (DAPI), which was depicted in blue colors. Images

showed a remarkable red fluorescence in the cytoplasm, which implied that the probe underwent a cascade oxidation-elimination reaction with intracellular ONOO^- . In contrast, the cells pretreated with *N*-acetylcysteine (NAC), an effective RONS scavenger, only showed weak fluorescence signals. Moreover, the cytotoxicity of CySO_3CF_3 was investigated by MTS assay. As shown in Figure 2c and Figure S11 (Supporting Information), the cells including HeLa, RAW 264.7 and NIH 3T3 kept high survival rate upon exposure to CySO_3CF_3 with concentrations below 50 μM . These results illustrated that CySO_3CF_3 could detect endogenous ONOO^- in living cells and thus had a potential for imaging application.

Figure 2. Imaging ONOO^- in cells. a) Confocal microscope fluorescence images of live murine macrophages (RAW 264.7). Cells successively treated with or without NAC (30 min) and then CySO_3CF_3 (30 min), $[\text{CySO}_3\text{CF}_3] = 10 \mu\text{M}$; $[\text{NAC}] = 1 \text{ mM}$. b) Quantification of fluorescence intensities of RAW 264.7 cells incubated with CySO_3CF_3 and other reagents corresponding to Figure 2a. The error bars represent the standard deviation from three separate measurements. c) MTS assay for the survival rate of RAW 264.7 cells treated with various concentrations (0, 5, 10, 25, 35, 50, 80 μM) of CySO_3CF_3 for 24 h. Error bars represent the standard deviations of 6 trials.

Figure 3. *In vivo* NIRF and PA imaging of ONOO⁻ using CySO₃CF₃. **a**) Illustration of the mechanism for NIRF and PA imaging of ONOO⁻ in tumor using CySO₃CF₃. CySO₃CF₃ first accumulates into tumor and then is activated by ONOO⁻ through cascade oxidation-elimination reactions, eventually resulting in enhanced NIRF/PA signals. **b**) Real-time fluorescence imaging of tumor bearing mouse after systemic administration of CySO₃CF₃ (50 μM in 100 μL saline) at 0, 3, and 4 h. **c**) Fluorescence intensity in tumor area as a function of post-injection time for CySO₃CF₃ intravenous injected mice. **d**) Representative PA maximum imaging projection (MIP) of tumor from a systemic administration of a living mouse at 0, 3, and 4 h post-injection of CySO₃CF₃ (50 μM in 100 μL saline). **e**) *In vivo* real-time PA spectra extracted from the tumors in living mice after systemic administration of CySO₃CF₃ via intravenous injection for 3 h. **f**) PA intensity in tumor area as a function of post-injection time for CySO₃CF₃ intravenous injection. Error bars represent standard deviations of three separate measurements ($n = 3$).

The feasibility of using CySO₃CF₃ as a NIRF/PA probe to monitor ONOO⁻ in the subcutaneous 4T1 xenograft tumor of living mice was tested (Figure 3). After systemic administration of CySO₃CF₃ into tumor-bearing living mice through tail vein, both NIRF and PA images were longitudinally recorded and quantified. The NIRF and PA signals gradually enhanced for CySO₃CF₃-treated mice and reached its maxima 3 h post-injection (Figure 3b, 3d). This showed that the probe was accumulated in the tumor, which was also confirmed by *ex vivo* data (Figure S12, Supporting Information). At $t = 3$ h, the PA and fluorescence intensities of CySO₃CF₃-treated tumor were respectively 2.1 and 5.3-fold higher than the background signals of tumor (Figure 3c). The higher signal-to-noise ratio of fluorescence imaging relative to PA imaging has been reported before^{19,46–51}, which should be mainly ascribed to the better sensitivity of fluorescence imaging and higher tissue background for PA background at 680 nm from hemoglobin. The real-time *in vivo* PA spectra extracted from the tumor regions at $t = 3$ h showed a strong signal at 680 nm (Figure 3e); Moreover, the PA spectral profile resembled the solution spectrum of activated CySO₃CF₃. These results thus not only confirmed the selective activation of CySO₃CF₃ by ONOO⁻ in tumor but also highlighted its ability for both NIRF and PA imaging.

CONCLUSIONS

In summary, we synthesized a water-soluble small-molecular probe (CySO₃CF₃) that can be specifically activated by endogenously overexpressed ONOO⁻ to turn on both

NIRF and PA signals. CySO₃CF₃ is different from the most reported RONS PA probes which are nanoparticles.^{32,43,44} CySO₃CF₃ also showed higher NIRF (59-fold) and PA (5.1-fold) enhancement and fast activation kinetics in response to ONOO⁻. With its zwitterionic structure, CySO₃CF₃ has good water-solubility and can be systemically injected into living mice for NIRF and PA dual-modal imaging of ONOO⁻. To the best of our knowledge, this work represents the first smart molecular probe that detects endogenous ONOO⁻ in living system with NIRF and PA dual-modal imaging.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. at DOI: 10.1021/acs.anal-chem.xxxxxxx.

Additional information as noted in the text (Figures S1–S12); Synthesis, characteristic (¹H NMR and MS spectra), UV-Vis absorption, fluorescence and photoacoustic spectra. (PDF)

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