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Supporting Information

A targetable acid-reponsive micellar system for signal activation based high performance surgical resolution of tumors

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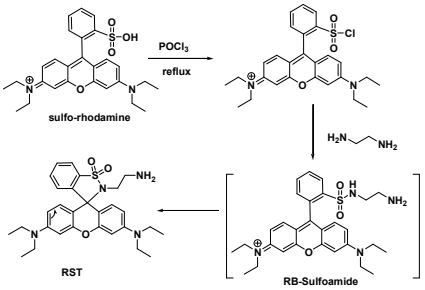
Experimental

Material and methods

LysoTracker Green DND-26 and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were purchased from Invitrogen. Bafilomycin A, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma-aldrich. FBS was inactivated by heating at 60 °C for 30 min. RLT and poly[styrene-*alt*-(maleic anhydride)]$_{40}$ were available from previous study.[1] Sulforhodamine is a gift from Bioluminor. All other chemicals were used as received from Alfa Aesar. Deionized water was used for the preparation of all aqueous solution. QGY-7701 cells and Raw 264.7 cells were obtained from American Type Culture Collection and grown at 37 °C under 5% CO_2 in DMEM.

The fluorescence spectra were recorded on a spectrofluorimeter (Spectramax M5, Molecular Device). Dynamic light scattering and Zeta potential analysis of the micelles were performed on Zetasizer Nano ZS (ZEN3500, Malvern). Confocal microscopic images were obtained on Leica SP5 using the following filters: *λex*@543 nm and *λem*@580-625 nm for RST; *λex*@488 nm and *λem*@500-530 nm for Lysotracker Green; *λex*@405 nm and *λem*@410-480 nm for DAPI. Fluorescence images were merged using Photoshop CS 6.0. Graph by origin 8.5 software. Flow cytometric data were obtained on Beckman Coulter using the fiters: *λex*@488 nm and *λem*@PE channel. Fluorescence quantification of organs of mice were performed on Carestream FX PRO *in vivo* imaging system using an excitation filter of 530 nm and an emission filter of 600 nm. Data were analyzed with Carestream MI SE. ICR mice were gifts from Xiamen University Laboratory Animal Center. H22 hepatocellular carcinoma cells were collected from peritoneal cavity of tunor-bearing mice and then used for transplantation of tumors in ICR mice. All animal experiments were performed in accordance with the guidelines of Xiamen University's Animal Care and Use Committee.

Synthesis of RST



Scheme S1. Synthesis of RST.

Sulforhodamine (5 g) was added to a flask containing phosphorous oxychloride (50 ml), and the resultant solution was heated under reflux for 10 h. The solvent was removed by rotary evaporation, and the residue was dissolved in dichloromethane (20 ml). To the solution was added ethylenediamine (20 ml). The reaction solution was stirred at rt for 2 h and then concentrated by evaporation. The residue was purified by silica gel chromatography using dichloromethane as the eluent to give the desired product as a pale yellow solid (1.2 g, 35%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.89 (m, 1H), 7.49 (m, 2H), 7.03 (dd, 1H, J_1 = 2.28, J_2 = 1.56), 6.84 (d, 2H, J = 9.12), 6.35 (m, 4H), 3.35 (q, 8H, J = 7.08), 2.99 (t, 2H, J = 6.00), 2.55 (m, 2H), 1.18 (t, 12 H, J = 14.08); ^{13}C-NMR (400 MHz, $CDCl_3$): 152.84, 148.93, 145.74, 133.40, 133.21, 129.88, 128.92, 126.47, 120.24, 108.46, 106.63, 97.23, 66.44, 44.34, 44.15, 40.56, 12.59; HRMS ($C_{29}H_{36}N_4O_3S$): calculated ($M+H^+$): 521.2581, found: 521.2579.

Preparation and characterization of the micelles

Poly[(styrene-*alter*-(maleic anhydride)] (2 g, mw: 20, 000) was added into a flask containing anhydrous dimethylformamide (DMF) (10 ml) and triethylamine (1 ml), the solution of RST (250 mg) in DMF (1 ml) was dropwise added. The solution was divided into 4 equal portions to which was respectively added mannosamine (250 mg), glucosamine (250 mg), galactosamine (250 mg) or no addition. The mixtures were stirred at rt for overnight followed by addition of aqueous Na_2CO_3 solution (1 M, 5 ml). The mixtures were first stirred at rt for 1 h and then extensively dialyzed against deionized water using a dialysis tube (MWCO 3500) to remove excess reagents and DMF. The solutions were respectively lyophilized and the resultant solids were dissolved in distilled water and then ultrasonicated for 40 min to afford RST@P-Man, RST@P-Gal, RST@P-Glu and

RST@P. The aqueous solutions of these micelles (1 mg ml^{-1}) were respectively characterized by Zetasizer Nano ZS for their hydrodynamic sizes and zeta potentials. The aqueous solutions of these micelles (2 mg ml^{-1}) were used for the in *vivo* experiment.

pH titration of RST and the micelles

Aliquots of stock solution of RST (10 μl, 0.1 mM in DMF), RLT (10 μl, 0.1 mM in DMF), RST@P (10 μl, 1 mg ml^{-1} in water), RST@P-Glu (10 μl, 1 mg ml^{-1} in water), RST@P-Gal (10 μl, 1 mg ml^{-1} in water), or RST@P-Man (10 μl, 1 mg ml^{-1} in water), were respectively added to sodium phosphate buffers (100 mM, 1 ml) of various pH. The fluorescence emission@ 590 nm of the solutions was recorded as a function of pH using *λex*@560 nm. The titration curves were plotted by fluorescence emission intensities@590 nm versus pH.

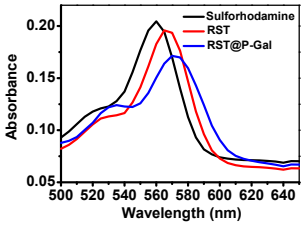


Fig. S1 UV-vis absorbance spectra of sulforhodamine (1 μM), RST (1 μM), and RST@P-Gal (25 μg ml^{-1}) at acidic buffer (100 mM, pH 4.5).

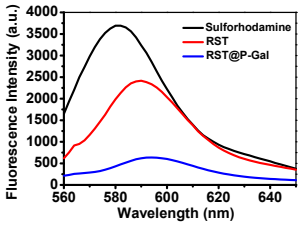


Fig. S2 Fluorescence emission spectra of sulforhodamine (1 μM), RST (1 μM), and RST@P-Gal (5 μg ml^{-1}) at acidic buffer (pH 4.5).

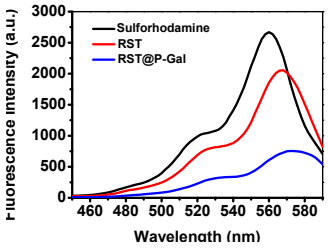


Fig. S3 Fluorescence exitation spectra of sulforhodamine (1 μM), RST (1 μM), and RST@P-Gal (5 μg ml^{-1}) at acidic buffer (pH 4.5).

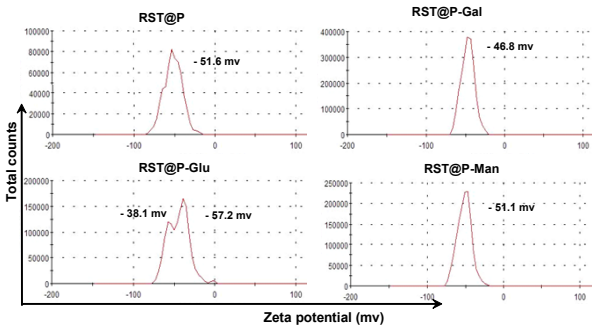


Fig. S4 Zeta potentials of the micelles.

Levels of RST at RST@P-Gal or RST@P-Man

To sodium phosphate buffers (100 mM, pH 4.5, 500 μl) was added RST@P-Gal (25 μg). The solutions were sonicated for 10 min and ananlyzed by UV-vis absorption. The levels of RST at RST@P-Gal was calculated to be 4.3% (w/w). (**D**) RST@P-Man (25 μg) was added to sodium phosphate buffers (100 mM, pH 4.5, 500 μl). The solutions were sonicated for 10 min and ananlyzed by UV-vis absorption. The levels of RST at RST@P-Man was calculated to be 4.4% (w/w).

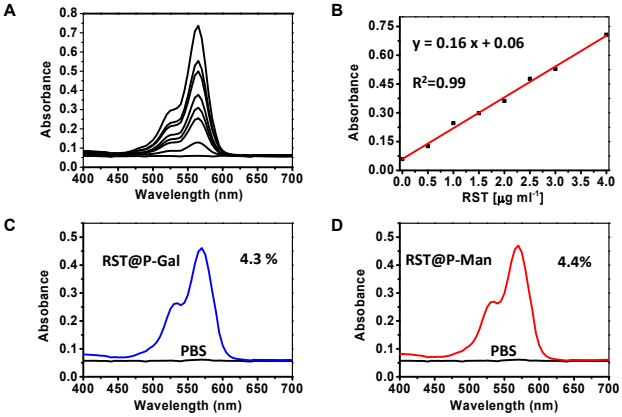


Fig. S5 Levels of RST at RST@P-Gal or RST@P-Man. (A) UV-vis absorption spectra of sodium phosphate buffers (100 mM, pH 4.5, 500 μl) contaning various levels of RST (0-4 μg ml^{-1}). (B) RST standard curve was plotted by absorbance at 560 nm as a function of RST concentrations.

The stability of the micelles at different pH condition

Aliquots of stock solution of RST@P-Gal (50 μl, 10 mg ml^{-1} in water) were respectively added to sodium phosphate buffers (100 mM, 1 ml) of various pH (4-9). The hydrodynamic sizes of RST@P-Gal were recorded as a function of pH.

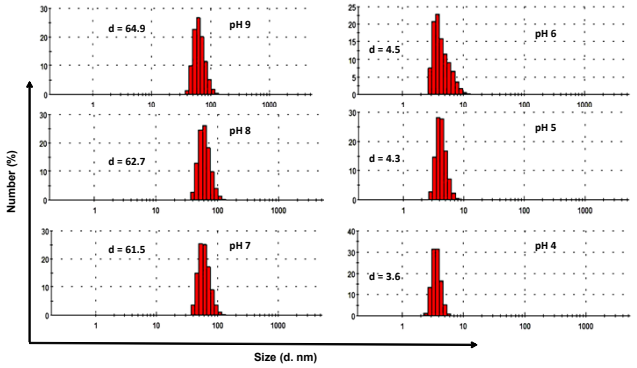


Fig. S6 pH dependent hydrodynamic sizes of RST@P-Gal .

Staining of lysosomes with RST and the micelles

Raw 264.7 cells and QGY-7701 cells were seeded on 35 mm glass-bottom dishes (NEST) and incubated for 24 h in DMEM supplemented with 10% FBS. The cells were stained with DAPI (1 μM) in DMEM for 4 h, and then further cultured for 30 min in DMEM spiked with one of the following species: RST (1 μM), RST@P (10 μg ml^{-1}), RST@P-Glu (10 μg ml^{-1}), RST@P-Gal (10 μg ml^{-1}), or RST@P-Man (10 μg ml^{-1}). The cells were washed with PBS (1 ml) and further incubated with Lysotracker green (1 μM) for 20 min in DMEM. The resultant cells were placed in fresh medium and then analyzed by confocal fluorescence microscopy.

For reversal staining of lysosomes, the cells were pre-incubated for 4 h at 37 °C in the absence or presence of 50 nM BFA, and then cultured for 30 min in DMEM containing one of the following reagents: RST (1 μM), RST@P (10 μg ml^{-1}), RST@P-Glu (10 μg ml^{-1}), RST@P-Gal (1 μg ml^{-1}), or RST@P-Man (1 μg ml^{-1}). The cells were washed with PBS (1 ml), incubated with Lysotracker green (1 μM) in DMEM for 20 min, and then analyzed by confocal fluorescence microscopy.

Flow cytometric analysis of cellular uptake of the micelles

Raw 264.7 cells and QGY-7701 cells were respectively cultured for 1 h in DMEM containing RST@P (10 μg ml^{-1}), RST@P-Glu (10 μg ml^{-1}), RST@P-Gal (10 μg ml^{-1}), RST@P-Man (10 μg ml^{-1}) or no addition. The cells were washed with PBS (1 ml) and then analyzed by flow cytometry.

Lysosomal retention of RST and the micelles

Raw 264.7 cells and QGY-7701 cells were respectively cultured for 2 h in DMEM containing RST@P (10 μg ml^{-1}), RST@P-Glu (10 μg ml^{-1}), RST@P-Gal (10 μg ml^{-1}), or RST@P-Man (10 μg ml^{-1}). The cells were washed with PBS (1 ml) and then cultured in fresh DMEM. At fixed time points (2 h, 24 h, 48 h), the intracellular fluorescence emission within the cells were analyzed by confocal fluorescence microscopy.

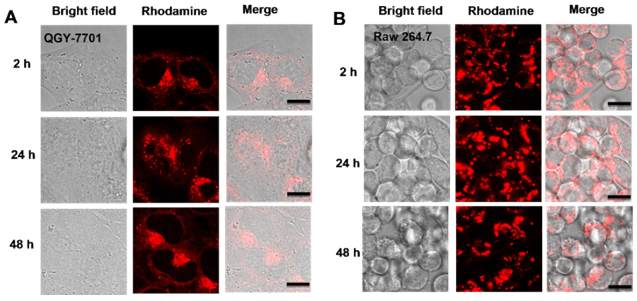


Fig. S7 Lysosomal retention of RST. QGY-7701 cells (A) and Raw 264.7 macrophages (B). Cells were first cultured with each of RST (1 μM) in DMEM for 2 h, and then cultured in fresh DMEM. The intracellular fluorescence emission@580-625 nm were recorded over incubation time (*λex*@543 nm).

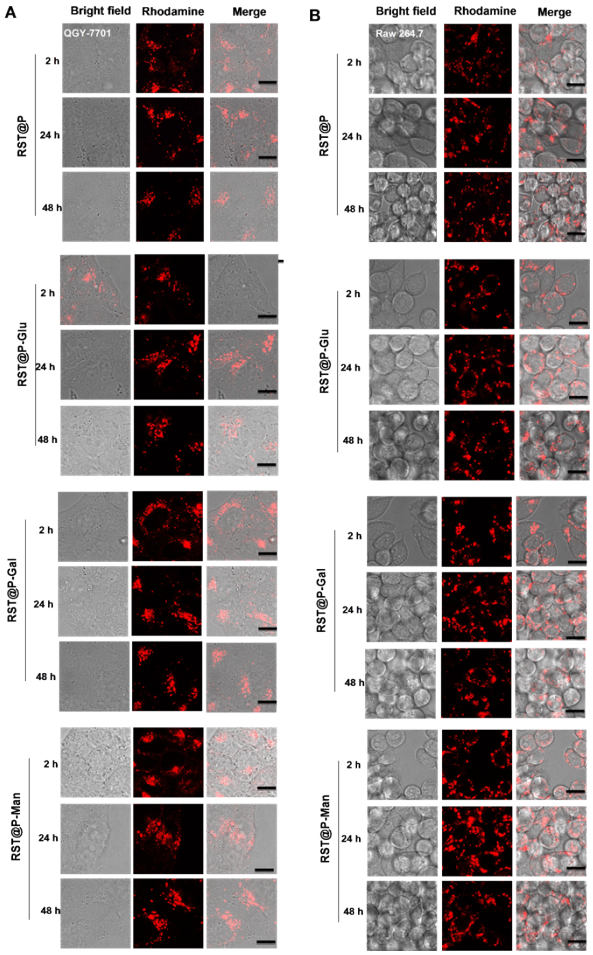


Fig. S8 Lysosomal retention of the micelles in QGY-7701 cells (A) and Raw 264.7 macrophages (B). Cells were first cultured with each of the micelles (10 μg ml^{-1}) in DMEM for 2 h, and then cultured in fresh DMEM. The intracellular fluorescence emission@580-625 nm were recorded over incubation time (*λex*@543 nm).

Time dependent imaging of subcutaneous tumors

ICR mice were xenografted in the flank by subcutaneous injections of H22 cells ($1x10^6$). At 5-10 days after the transplantation, a cohort of tumor-bearing mice were injected intravenously via the tail vein with each of the micelles (10 mg kg^{-1}) or PBS PBS (100 μl). At 4, 10, 17 or 39 h post-injection, the mice were anesthetized, and the tumors and selected organs were excised, washed with PBS and then subjected to *ex vivo* analysis for the fluorescence intensity.

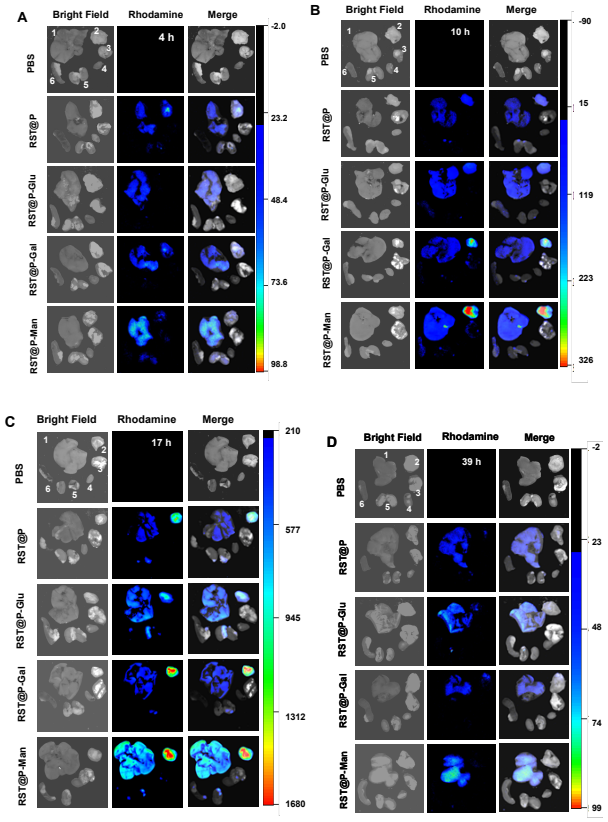


Fig. S9 Time course study on biodistributions of the micelles in tumor-bearing mice. The micelles was respectively injected into mice with subcutaneous tumor (10 mg kg^{-1}). The mice were sacrificed at 4, 10, 17 or 39 h after administration. Representative organs and tumors were excised and analyzed to probe the fluorescence emission (excitation filter: 530 nm, emission filter : 600 nm). The organs were arranged in the following order: liver (1), tumor (2), lung (3) heart (4), kidney (5) and spleen (6).

Detection of liver tumors in mice

ICR mice xenografted in the liver with H22 cells were obtained from the animal center of Xiamen University. At 5-10 days after transplantation, the mice were injected intravenously via the tail vein with each of the micelles (10 mg kg^{-1} in mice) or PBS (100 μl). After 15 h, the mice were anesthetized, and the liver and representative healthy organs were excised, washed with PBS and then subjected to *ex vivo* analysis for the fluorescence intensity.

Cytotoxicity of the micelles

For cell toxicity: Raw 264.7 cells and QGY-7701 cells were respectively cultured in medium containing various levels (0, 25, 50, 100 μg ml^{-1}) of RST@P, RST@P-Glu, RST@P-Gal, or RST@P-Man for 24 h at 37 ℃ with 5% CO_2. The cells were stained with trypan blue. Cell number and cell viability were determined using the trypan blue exclusion test.

For systemic toxicity: Healthy mice were intravenously injected with overdosed RST@P-Gal or RST@P-Man at the doses of 100 mg kg^{-1}. The mice were monitored regularly for 7 days for any adverse effects, and then anesthetized. The representative organs were excised, washed with PBS and then subjected to *ex vivo* fluorescence analysis.

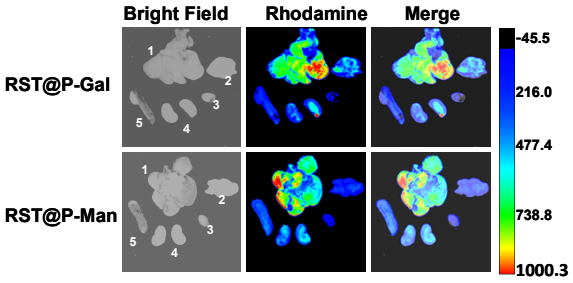


Fig. S10 Biodistribution of over-dosed glyco-micelles in healthy mice. Healthy mice were intravenously injected with RST@P-Gal or RST@P-Man (10, 100 mg Kg^{-1}). The mice were monitored for 7 days and then anesthetized. The representative organs were excised, washed with PBS and analyzed *ex vivo*.