Supplementary Material (ESI) for Chemical Science

Electronic Supplementary Information for

A Dendrimer-Based Platform for Simultaneous Dual Fluorescence Imaging of Hydrogen Peroxide and pH Gradients Produced in Living Cells

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Experimental Section

Synthetic Materials and Methods. SiliaFlash P60 (230 – 400 mesh, Silicycle, Quebec City, Canada) was used for column chromatography. Analytical thin layer chromatography was performed using SiliaPlate TLC (0.25 mm thickness, Silicycle). Dichloro[1,1´-bis(diphenylphosphino) ferrocene]palladium (II), Pd(dppf)Cl_2 was purchased from Strem Chemicals (Newburyport, MA). Bis(pinacolato)diboron was purchased from Boron Molecular (Research Triangle Park, NC). PAMAM-G5 (ethylenediamine core, molecular weight 28824.8, 128 amine end groups, solution 5 wt. % in methanol), phorbol 12-myristate 13 acetate (PMA), diphenylene iodonium (DPI) and concanamycin A were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. Sephadex™ LH-20 was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). The WST-1 cell proliferation reagent was purchased from Roche Applied Science (Indianapolis, IN). Amicon Ultracel-10K centrifugal units (regenerated cellulose 10,000 MWCO) were purchased from Millipore. SpectralPor regenerated cellulose MW3500 was used as a dialysis membrane (Spectrum Laboratories, Inc, Rancho Dominguez, CA). ^{1}H NMR spectra were collected in $CDCl_3$, DMSO-d_6, or $(CD_3)_2CO$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. Mass spectral analyses were carried out using Agilent Technology 1200 series with 6130 Quadrupole LC/MS (Santa Clara, CA). Matrix assisted laser desorption-ionization-time of flight mass spectrometry (MALDI-TOF-MS) was collected on a Voyager DE MALDI-TOF (Perseptive Biosystems, Framingham, MA) equipped with a 337 nm pulsed nitrogen laser. MALDI-TOF spectra were obtained in linear positive mode with the following settings: accelerating voltage of 25,000 V, grid voltage 92 % of accelerating voltage,

extraction delay time of 500 ns. 2,5-Dihydrobenzoic acid (DHB) was used as the matrix. The DHB matrix solution was 20 mg/mL DHB in 1:1 H_2O/DMF. A solution of the analyte (PAMAM ~10 mg/mL) was mixed with the matrix solution at the volume ratio of 1:5, 1:10 and 1:20. The resulting solution was spotted on the MALDI plate (1 μL/spot) and vacuum dried. Dynamic light scattering (DLS) was performed on Zetasizer Nano-ZS (Malvern Instruments, estborough, MA) equipped with 4.0 mW, 633 nm He-Ne laser. DLS measurement was done at 25 °C with buffered solution containing 2 mg/mL dendrimers. All samples was filtered through 0.2 μM PVDF membrane before DLS measurrement.

Isomeric Mixture of 2-(4-(Diethylamino)-2-hydroxybenzoyl)terephthalic Acid (1) and 4-(4-(Diethylamino)-2-hydroxybenzoyl)isophthalic Acid (2). 3-(Diethylamino)phenol (6.6 g, 40 mmol) and 1,2,4-benzenetricarboxylic anhydride (7.7 g, 40 mmol) were added to a 1L round bottom flask filled with 500 mL toluene. The mixture was heated to reflux overnight and allowed to cool down to room temperature. The solid precipitate was collected by vacuum filtration and washed with CH_2Cl_2 to give a yellow-brown solid that can be used in the next step without further purification (8.2 g, 23 mmol, 57% yield). Purification with flash column chromatography (4:1 EtOAc: hexane) gave a yellow solid. ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 8.69 (1H, d, J = 1.6 Hz), 8.31 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz), 8.22 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz), 8.16 (1H, d, J = 8.0 Hz), 7.98 (1H, d, J = 1.6 Hz), 7.55(1H, d, J = 8.0 Hz), 6.91(1H, d, J = 9.2 Hz), 6.87 (1H, d, J = 8.8 Hz), 6.21-6.17 (2H, m), 6.09 (s, 2H), 3.44 (8H, q, J =7.2 Hz), 1.16 (12H, t, J = 7.2Hz). LC-MS: calculated for [MH^+] 358.1, found 358.

Isomeric Mixture of C.SNARF2: 10-(Diethylamino)-3-hydroxy-3'-oxo'3'*H*-spiro[benzo[*c*]xanthenes-7,1'-isobenzofuran]-6'-carboxylic acid (3) and 10-(diethylamino)-3-hydroxy-3'-oxo'3'*H*-spiro[benzo[*c*]xanthenes-7,1'-isobenzofuran]-5'-carboxylic Acid (4). The isomeric mixture of compounds **1** and **2** (170 mg, 0.47 mmol), 1,6-dihydroxynaphthalene

(80 mg, 50 mmol), and methanesulfonic acid (2 mL) were added to a heavy-walled reaction flask. The mixture was sonicated for 3 hours. Dilution of the reaction mixture into ice water (100 mL) gave a purple-colored precipitate, which was collected by vacuum filtration. Purification by flash column chromatography (EtOAc) gave C.SNARF2 as a purple solid (120 mg, 0.25 mmol, 53% yield). ^{1}HNMR ($(CD_3)_2CO$, 400 MHz): δ 8.55 (1H, s), 8.48 (1H, dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz), 8.38 (1H, dd, J_1 = 8.0, J_2 = 1.6 Hz), 8.33 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz), 8.14 (1H, d, J = 8.0 Hz), 7.82 (1H, s), 7.34 (2H, d, J = 8.8 Hz), 7.28 (2H, dd, J_1 = 9.2 Hz, J_2 = 2.0 Hz), 7.21 (2H, t, J = 2.4 Hz), 7.00 (1H, dd, J_1= 8.0 Hz, J_2= 1.6 Hz), 6.78 (2H, t, J = 2.0 Hz), 6.72 (2H, dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz), 6.68 (2H, dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz), 6.67 (2H, dd, J_1 =8.8 Hz, J_2 = 2.0 Hz), 3.45 (8H, q, J = 7.2 Hz, 1.26 (12H, t, J = 7.2 Hz). LC-MS: calculated for [MH^+] 482.1, found 482.

SNARF2-CONHS: 2,5-Dioxopyrrolidin-1-yl 10-(diethylamino)-3-hydroxy-3'-oxo-3'H-spiro[benzo[*c*]xanthenes-7,1'-isobenzofuran]-5'-carboxylate (5). C.SNARF2 (50 mg, 0.10 mmol) and *N*-hydroxysuccinimide (15 mg, 0.13 mmol) and DMF (1 mL) were added to a 4 mL vial. The mixture was sonicated briefly until all solids were dissolved. Then, EDC-HCl (25 mg, 0.12 mmol) was added and the mixture was allowed to stir at room temperature for 3 hours. The product was isolated by flash column chromatography (4:1 EtOAc:Hex) giving isomerically pure SNARF2-CONHS as a purple solid (15 mg, 0.026 mmol, 25% yield). ^{1}HNMR ($(CD_3)_2CO$, 400 MHz): δ 8.62 (1H, s), 8.47 (1H, d, J = 8.0), 8.45 (1H, d, J = 8.8), 7.58 (1H, d, J = 8.0), 7.35 (1H, d, J = 8.8 Hz), 7.29 (1H, dd, J_1 = 8.8 Hz, J_2= 2.4 Hz), 7.21 (1H, d, J = 2.4 Hz), 6.80 (1H, d, J = 8.8 Hz), 6.79 (1H, d, J = 2.4 Hz), 6.76 (1H, d, J = 8.8 Hz), 6.55 (1H, dd, J_1= 8.8 Hz, J_2 = 2.4 Hz), 3.46 (4H, q, J = 7.2 Hz), 2.99 (4H, s), 1.25 (6H, t, J = 7.2 Hz). LC-MS: calculated for [MH^+] 579.1, found 579.

3',6'-Dibromo-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (6). 3-Bromophenol (10.51 g, 50.0 mmol), 1,2,4-benzenetricarboxylic acid (17.30 g, 100.0 mmol), and methanesulfonic acid (50 mL) were added to a 150 mL heavy-walled reaction flask and heated at 135 ºC for 72 h. The reaction was cooled to 25 ºC, poured into 400 mL of an ice/ddH_2O slurry, and stirred vigorously to precipitate a greenish solid. The solid was collected by vacuum filtration and treated with 300 mL of acetic anhydride and 100 mL of pyridine to give a pinkish white solid that was recrystallized three times from a mixture of acetic anhydride and pyridine (2/1). The white solid was treated with 1 M HCl to give isomerically pure carboxylic acid **6** as a bone white powder (6.27 g, 25% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 13.67 (1H, s), 8.26 (1H, d, J = 8.0 Hz), 8.17 (1H, d, J = 8.0 Hz), 7.86 (1H, s), 7.70 (2H, d, J = 2.0 Hz), 7.33 (2H, dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz), 6.90 (2H, d, J = 8.4 Hz). LC-MS: calculated for [M^-] 500.9, found 501.

PF1-COOH: 3-Oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (7). 3',6'-Dibromo-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid **6** (2.01 g, 4.0 mmol), Pd(dppf)Cl_2 (989 mg, 1.2 mmol), potassium acetate (3.94 g, 40.1 mmol), and bis(pinacolato)diboron (4.00 g, 15.8 mmol) were added to a dried 100 mL Schlenk flask under nitrogen. Anhydrous, degassed DMF (50 mL) was added and the solution stirred at room temperature for 5 min. The reaction was heated to 80 ºC for 12 h, cooled to 25 ºC and the solvent removed under reduced pressure, giving a dark brown mass. Purification by flash column chromatography (silica gel, EtOAc) provided a brown viscous oil which was precipitated in minimal Et_2O, washed with Et_2O (3 x 25 mL), and dried *in vacuo* to give **7** as a peach solid (1.47 g, 61% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 13.68 (1H, s), 8.24 (1H, d, J = 8.0 Hz), 8.19 (1H, d, J = 8.0 Hz), 7.67 (1H, s), 7.40 (2H, d, J = 7.6 Hz), 6.95 (2H, d, J = 7.6 Hz), 1.30 (24H, s). LC-MS: calculated for 597.2, found 597.

PF1-CONHS: 2,5-Dioxopyrrolidin-1-yl 3-oxo-3',6'bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (8). PF1-COOH (50 mg, 0.085 mmol), *N*-hydroxysuccinimide (11 mg, 0.086 mmol) and DMF (2 mL) were added to a 4 mL vial. EDC-HCl (17 mg, 0.085 mmol) was then added and the mixture was allowed to stir at room temperature for 3 hours. The product was isolated by flash column chromatography (1:1 EtOAc:Hex) to give PF1-CONHS as a pale yellow solid (30 mg, 0.043 mmol, 51 % yield). ^{1}HvNMR ($CDCl_3$, 400 MHz): δ 8.36 (1H, d, J = 8.0 Hz), 8.16 (1H, d, J = 8.0 Hz), 7.80 (1H, s), 7.77 (2H, s), 7.47 (2H, d, J = 7.6 Hz), 6.83 (2H, d, J = 7.6 Hz), 2.87 (4H, s), 1.36 (24H, s). LC-MS: calculated for [MH^+] 694.2, found 694.

G5-SNARF2. SNARF2-NHS ester (4 mg, 6.9 μmol) in DMSO (1 mL) was added to a solution of PAMAM-G5 in MeOH (40 mg, 1.4 μmol, 5 mg/mL) after which stirring continued for 1 h in the dark at room temperature. This solution of G5-SNARF2 was then ready for further functionalization in the next step. For analysis of the number of SNARF2 dyes on the dendrimer, 200 μL of the reaction mixture was taken into 3 mL H_2O and concentrated with an Amicon 10K centrifugal unit to a final volume of 100 μL. Small organic molecules were removed by size-exclusive gel chromatography using Sephadex LH-20 equilibrated with MeOH. The collected dendrimer fraction was diluted with H_2O to approximately 20 % MeOH and concentrated with an Amicon 10K to a final volume of 50 μL. The solution was kept at 4 °C for analysis with MALDI-TOF

G5-SNARF2-Ac. Triethylamine (20 μL, 143 μmol) followed by acetic anhydride (7 μL, 74 μmol) were added to a solution of G5-SNARF2 (4.4 mL, 0.7 μmol). After stirring for 2 h at room temperature, the reaction mixture was dialyzed against 20% MeOH for 1 h to dilute its DMSO content. The dendrimer solution was concentrated to a final volume of 2 mL with an Amicon 10K centrifugal unit and applied to a Sephadex-LH20 column equilibrated with MeOH.

The collected dendrimer fraction was diluted with H_2O to approximately 20% MeOH and concentrated with an Amicon 10 K to a final volume of 1 mL and then stored in the dark at -20 °C. The concentration of dendrimer solution, determined from the dried mass of a lyophilized sample of known volume, was 11 mg/mL.

G5-SNARF2-PF1. A solution of PF1-NHS ester (5 mg, 7.2 μmol) in DMSO (0.5 mL) was added to a solution of G5-SNARF2 (4.4 mL, 0.7 μmol). After 1 h, 200 μL of reaction mixture was subjected to purification and analysis in a similar procedure as described for G5-SNARF2.

G-SNARF2-PF1-Ac. Acylation of G5-SNARF2-PF1 was achieved by following a similar protocol to the synthesis of G5-SNARF-Ac to give 1 mL of a 12 mg/mL dendrimer solution.

SDS-PAGE In-Gel Fluorescence. G5-SNARF2-PF1-Ac was diluted in to PBS pH 7.4 to the concentration of 200 μg/mL. To a PCR tube was added 15 μL G5-SNARF2-PF1-Ac solution and 15 μL H_2O_2 solution (0, 50, 100, 200, 400, 800 μM in PBS). After 30 minute incubation at 37 °C, 10 μL loading buffer (50% glycerol, 10% SDS, bromophenol blue) was added to the reaction mixture. Samples were loaded into 12% polyacrylamide gel (20 μL/well) in Tris-Glycine running buffer at 200 V gradient. In-gel fluorescence scanning was obtained on a Typhoon 9410 imaging system (GE Helathcare). The dendrimer bands were visualized with Coomassie Blue staining.

Spectroscopic Materials and Methods. Milipore water was used to prepare all aqueous solutions. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter,

switchable 814 photon counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and fluorescence measurements were contained in 1-cm x 1-cm 1.25 mL volume quartz cuvettes (Starna, Atascadero, CA). Spectroscopic properties of G5-SNARF2-Ac at various pH values were measured in 50 mM phosphate buffer. pKa values of G5-SNARF2-Ac were obtained from analysis of the absorption spectra at 597 and 545 nm to fit the following equation,

$$pH = pK_a + c\left[\log \frac{R - R_{min}}{R_{max} - R}\right] + \log \frac{I^a}{I^b}$$

where I^a and I^b are the absorbances in acid and base respectively at the wavelength chosen for the denominator of R; this correction vanishes at the isosbestic wavelength (545 nm). C is the slope. R is the ratio of absorbance at two selected wavelengths, with maximum and minimum values of R_{max} and R_{min}, respectively.

Preparation of Cell Cultures and Cytotoxic Assays. RAW 264.7 macrophage cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose with GlutaMAX™ (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS, Thermo Scientific/Hyclone, Logan, UT). Cells were split 1/30 twice a week. One day before imaging, cells were passaged and plated, and seeded at a density of 3 x 10^5 cells /well, in 4-well Lab-Tek Chamber slides (Thermo Scientific/ Nalge Nunc, Rochester, NY) pre-coated with poly-L-lysine (50μg/mL, Sigma, St. Louis, MO). For cytotoxic assays, RAW 264.7 cells were seeded (50,000 cells/well) in a 96-well cell culture plate and maintained for 24 h. The cells were washed once with Hank's buffered salt solution (HBSS) and incubated with 100 μL of dendrimer probe (500 -1000 ug/mL in DMEM) or in DMEM alone as a control medium. After 3 h, the cells were washed twice with fresh DMEM to remove the dendrimer probe and replaced with 100 μL of WST-1 solution (1:10 dilution) in DMEM. After incubation for 2 h, absorbance was measured at

440 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Five measurements were taken for each concentration of PAMAM dendrimers.

Fluorescence Imaging Experiments. Fluorescence imaging was performed with a Zeiss Axiovert 200M microscope (Zeiss, Jena, Germany) equipped with a mercury light source X-Cite 120 series (Exfo Life Science Divisions, Ontario, Canada) and a 63X/1.4 oil Plan-Apochromat objective lens. During image acquisitions, samples were kept at 37 °C in a 5% CO_2 using a Chemlide TC incubator system, consisting of a chambered cover glass connected to a CU-109 temperature controller, FC-7 gas mixture and a humidifier (Live Cell Instrument, Seoul, Korea). The filter sets used in image acquisition are listed in Table S1 and in the data captions. All imaging was performed with fixed acquisition times of 1000 ms for Cy5, 500 ms for Cy3, 250 ms GFP and automatic mode for DAPI and DIC channel. Stimulation of phagocytosis was achieved by the addition of PMA at 4 μg/mL delivered from a 1 mM stock solution in DMSO. The NADPH oxidase inhibitor DPI was delivered from a stock solution of 5 mM in DMSO. Hoechst 33342 was delivered from a stock solution of 1 mM in water. Concanamycin A was delivered from a 0.1 mM stock solution in DMSO. G5-SNARF2-Ac and G5-SNARF2-PF1-Ac were delivered from stock solutions in water, and imaging was done with 300 μg/mL working concentrations of dendrimer in DMEM. Dendrimers, Hoechst 33342, PMA, DPI and concanamycin A were premixed in DMEM by vortexing. For phagocytosis imaging, the culture medium of RAW 264.7 cells was replaced by the labeling DMEM solution. After 15 min incubation at 37 °C with 5 % CO_2, cells were washed twice with DMEM to remove the dendrimer, and placed in HBSS buffer for imaging of early phagosomes. Cells were further incubated in HBSS for another 30 min for late endosome images. Image analysis was performed on an Axiovision Rel 4.8 (Zeiss). Ch1, Ch2, and Ch3 were adjusted to the same

brightness/contrast setting. Ch4 and DIC were adjusted to the min/max value. Exported TIF color images were cropped by using ImageJ (National Institute of Health).

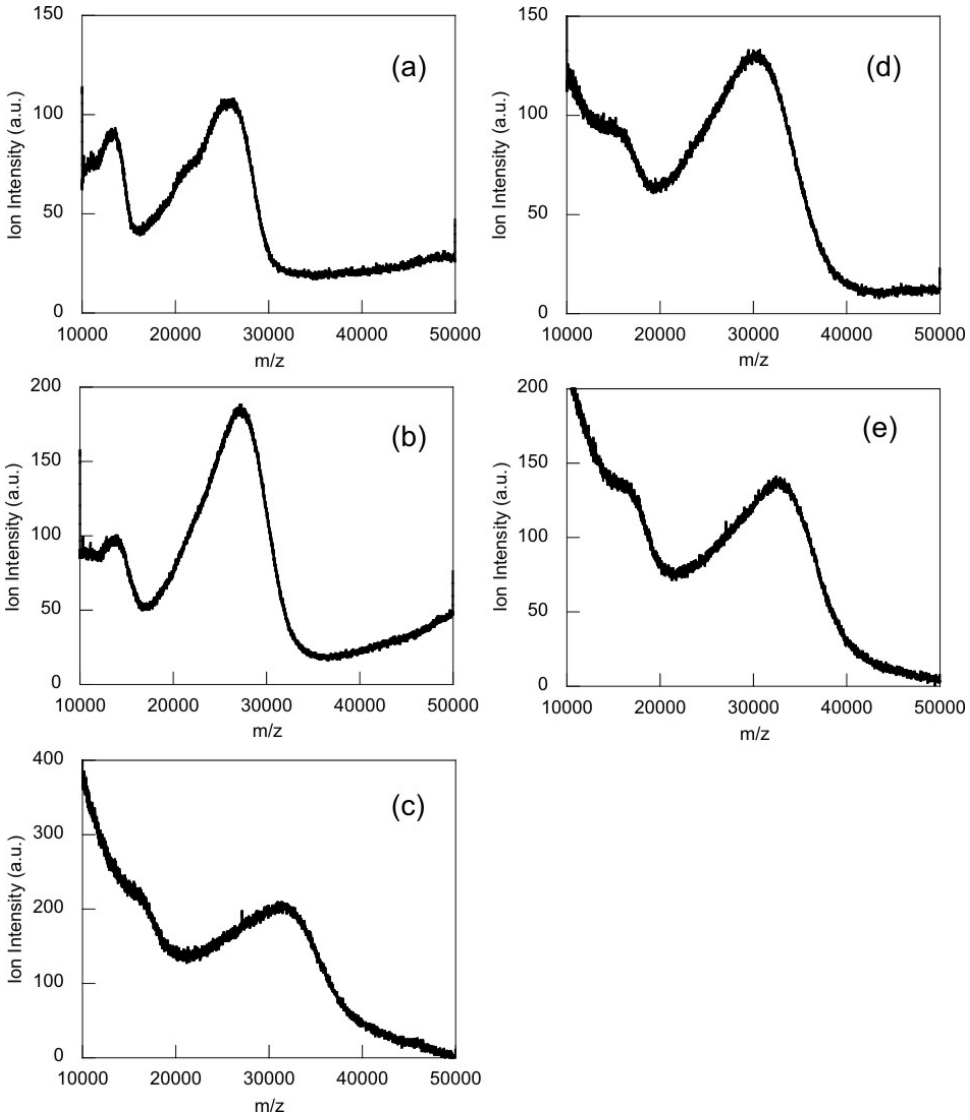

Fig S1. MALDI-TOF spectra of functionalized PAMAM dendrimers. (a) PAMAM-G5 with an average MW of 26,300 Da. (b) G5-SNARF2 with an average MW of 27,200 Da. (c) G5-SNARF2-Ac with an average MW of 31,000 Da. (d) G5-SNARF2-PF with an average MW of 30,660 Da. (e) G5-SNARF2-PF1-Ac with an average MW of 32,500 Da.

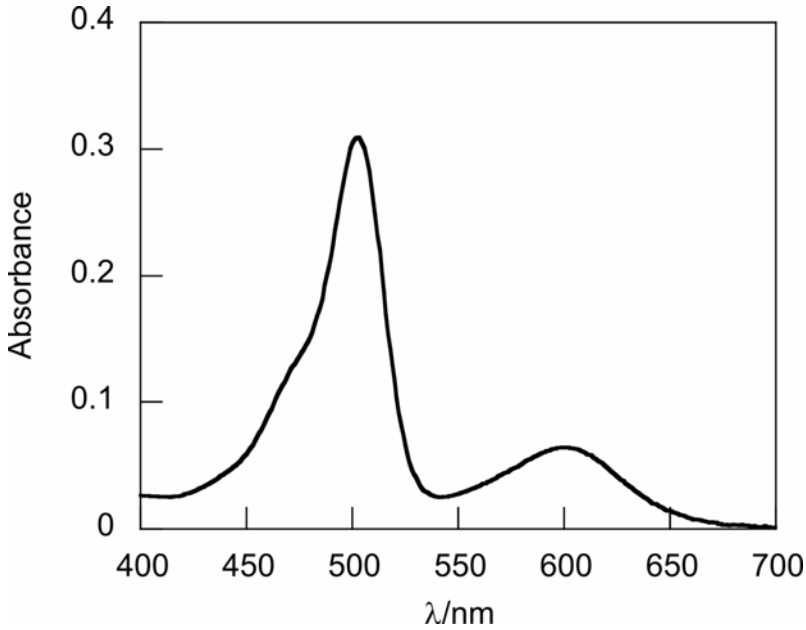

Fig S2. Absorption spectra of G5-SNARF2-PF1-Ac (20μg/mL ~ 0.6 μM) of which PF1 was fully conversed to fluorescein by reaction with 1 mM H_2O_2 for 60 min in PBS pH 7.4. The solution was adjusted to pH 10 with 0.1 M NaOH to give the absorption spectrum of basic SNARF2. Calculation from the known extinction coefficients (dianion fluorescein ε = 76,900 $M^{-1}cm^{-1}$; SNARF2 46,400 $M^{-1}cm^{-1}$) gave six molecules of fluorescein and two molecules of SNARF2 per dendrimer.

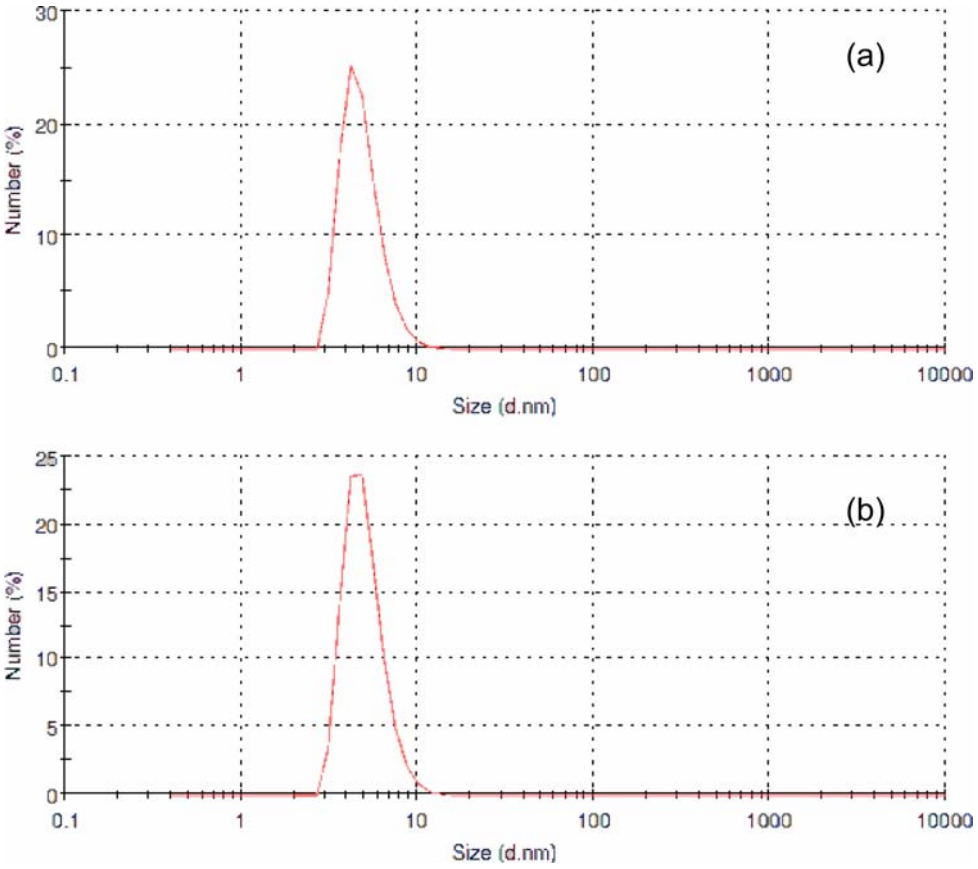

Fig S3. PAMAM-G5 is stable under oxidative conditions. The hydrodynamic diameter of PAMAM-G5 measured by dynamic light scattering (DSL) shows no difference between (a) PAMAM-G5 (average diameter 4.95 nm), and (b) PAMAM-G5 after 2 h with 10 mM H_2O_2 in PBS buffered to pH 7 (average diameter 5.10 nm).

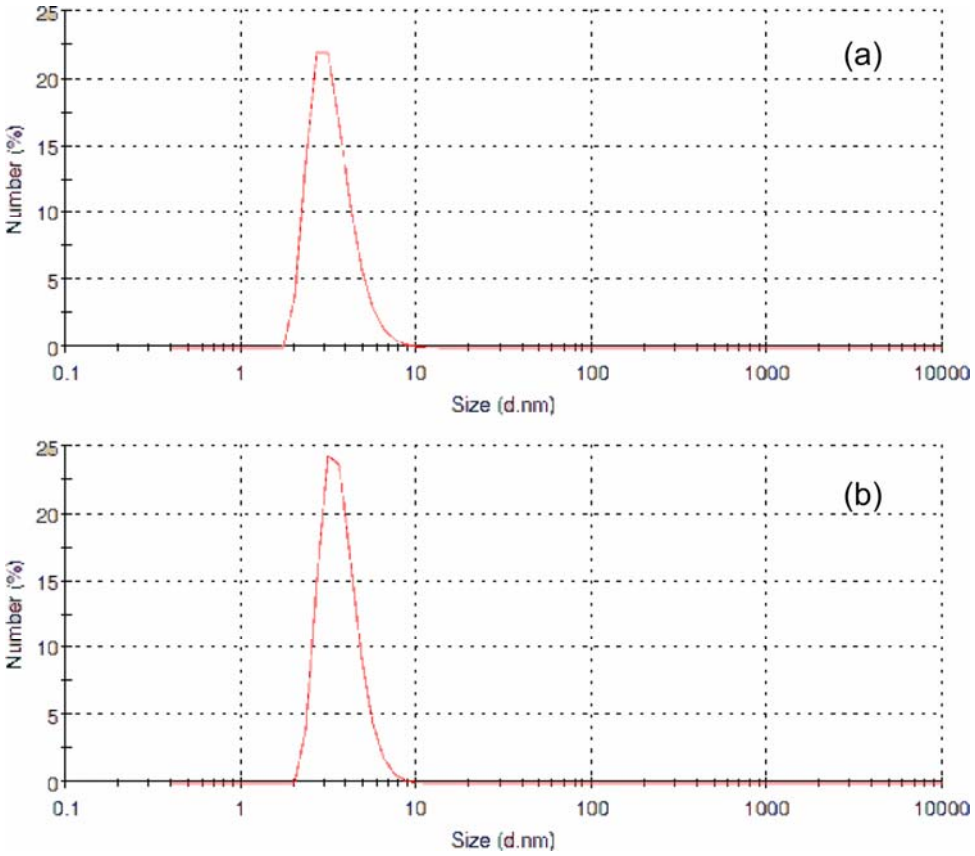

Fig S4. Size distributions of functionalized PAMAM dendrimers as determined by dynamic light scattering (DLS) measurements. The average hydrodynamic diameters of (a) G5-SNARF2-Ac and (b) G5-SNARF2-PF1-Ac are 3.63 and 3.56 nm, respectively. Measurements were done in acidic buffer, 0.1 M citric acid buffered to pH 2.5, in order to avoid 633 nm absorption from basic SNARF2.

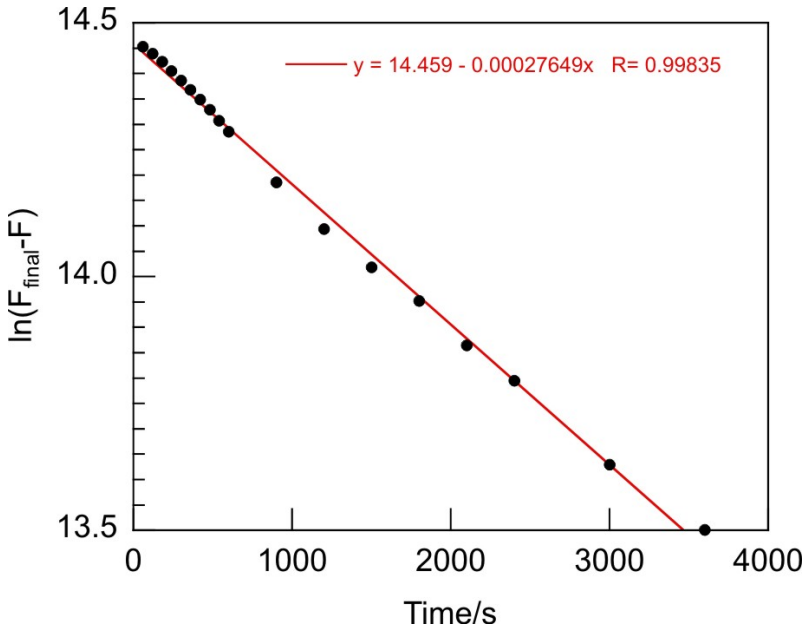

Fig S5. Kinetic plot of the fluorescence emission intensity at 520 nm of the pseudo-first order reaction of G5-SNARF2-PF1-Ac (10 μg/mL, ~1.5 μM PF1) with 1 mM H_2O_2. The slope of the plot corresponds to the observed rate of reaction $k_{obs} = 2.8 \times 10^{-4}$ s^{-1}.

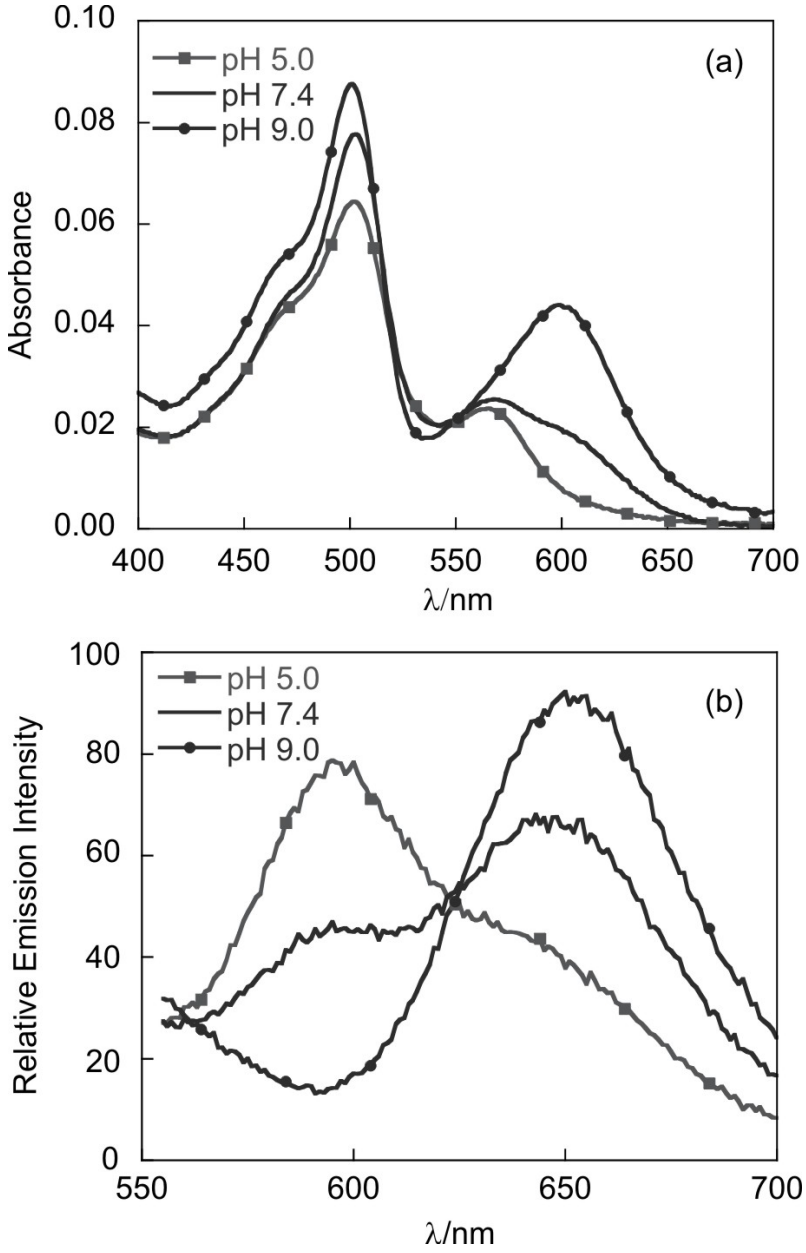

Fig S6. (a) Absorption spectra of G5-SNARF2-PF1-Ac (10 μg/mL) at 30 min after 100 μM H_2O_2 was added. The reaction in phosphate-buffered solution pH 7.4 was adjusted to pH 5.0 and pH 9.0 using 1N HCl and 1M NaOH, respectively. (b) Fluorescence emission spectra of G5-SNARF2-PF1-Ac in (a) using λ_{exc} = 543 nm.

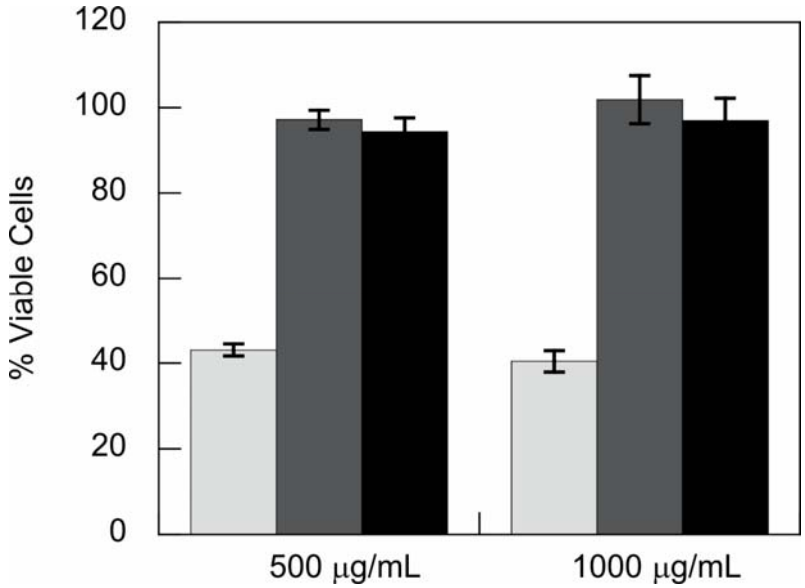

Fig S7. WST-1 cell viability assay of RAW264.7 cells after 3 h incubation with 500-1000 μg/mL dendrimer in DMEM: PAMAM-G5 (light gray), G5-SNARF2-Ac (dark gray), and G5-SNARF2-PF1-Ac (black). Error bars represent standard deviation.

Table S1. Filter sets used in the imaging experiments.

Fluorophore	Channel	Excitation	Beamspliter	Emisssion	Specification
Hoechst 33342	Ch4	G 365	FT 395	BP 445/50	Filter set 49 (488049)
PF1	Ch3	BP 470/40	FT 495	BP 525/50	Filter set 38 HE (489038)
SNARF2 (acid)	Ch2	BP 550/25	FT 570	BP 605/70	Filter set 43 HE (489043)
SNARF2 (base)	Ch1	BP 575-625	FT 645	BP 660-710	Filter set 326 (488026)