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A Cell-Permeable Fluorescent Prochelator Responds to Hydrogen Peroxide and Metal Ions by Decreasing Fluorescence

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Abstract

Described here is the development of two boronic ester-based fluorescent prochelators, FloB (2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-benzylidene-hydrazinocarbonyl]-benzoic acid) and FloB-SI (2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)-benzylidene-hydrazinocarbonyl]-benzoic acid) that show a fluorescence response to a variety of transition metal ions only after reaction with H₂O₂. Both prochelators' boronic ester masks are oxidized by H₂O₂ to reveal a fluorescein-tagged metal chelator, FloS (4(5)-(2-hydroxy-benzylidenehydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid). Chelation of Fe³⁺ or Cu²⁺ elicits a 70% decrease in the emission signal of FloS, while Zn²⁺, Ni²⁺, and Co²⁺ produce a more modest fluorescence decrease. The conversion of FloB to FloS proceeds in organic solvents, but hydrolytic decomposition of its hydrazone backbone is observed in aqueous solution. However, FloB-SI oxidizes cleanly with H₂O₂ within 1 h in aqueous solutions to produce FloS. Fluorescence microscopy studies in HeLa cells with FloB-SI show that the sensor's fluorescence intensity remains unchanged until incubation with exogenous H₂O₂, which results in a decreased fluorescent signal. Incubation with a competitive chelator restores the emission response, thus suggesting that FloB-SI can effectively report on a H₂O₂-induced increase in intracellular labilized metal.

Keywords

fluorescence; metal sensor; oxidative stress; prochelator; iron; copper

1. Introduction

Mounting evidence has drawn attention to the link between metal-mediated oxidative stress and a number of diseases, including Alzheimer's, Parkinson's and macular degeneration [1–3]. Metals such as iron have been shown to exacerbate levels of oxidative stress in these diseases by catalytically producing dangerous reactive oxygen species (ROS). One specific example includes iron's ability to react with naturally occurring H₂O₂ to produce the highly reactive hydroxyl radical, which causes damage to lipids, nucleic acids, and proteins [4, 5].

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Appendix A. Supplementary Material. Supplementary material associated with this article can be found online.

Areas of misregulated iron homeostasis are the most susceptible to this type of oxidative stress, as is found in the brains of neurodegenerative diseased patients [3].

In order to better understand the complicity of iron and H_2O_2 in disease, we seek to develop a fluorescent probe for metal-induced oxidative stress. Recently, a growing interest has emerged in the design of fluorescent sensors for biologically relevant metal cations. However, there are few examples of iron-specific probes [6–12] and an even smaller number that sense redox-active metals within an oxidative environment [13–15]. The current best practice for monitoring intracellular labile iron pools in conjunction with intracellular ROS production is to use a combination of complementary probes, for example calcein as a non-specific metal chelating agent that is quenched by iron and dihydrofluorescein that is oxidized intracellularly by ROS to a fluorescent derivative [15]. We and others have introduced molecules that in principle could act as specific probes of metal-associated ROS [13, 14, 16]. Our previously described FlamB compound is one such example where H_2O_2 activates a fluorescent copper sensor by oxidizing a boronic ester that masks a latent phenolic oxygen donor of a copper chelator [14]. Once unmasked, copper binding triggers a modest turn-on in fluorescence emission. A significant limitation of FlamB, however, is its hydrolytic instability in aqueous solution that results in off-target signal response. By again utilizing our boronic ester prochelator strategy, we sought to create a sensor that would detect iron or copper within areas of dangerously high concentrations of H_2O_2 . Our lab and others have described numerous examples of H_2O_2 -activated prochelators that utilize boronic ester masks [16–20]. These masked prochelators possess little affinity for metal cations until high concentrations of H_2O_2 are available to convert an aryl boronate functionality to a phenolic functionality that provides high-affinity metal coordination. The advantage of this technique is that potentially harmful metals are chelated or sequestered, while biologically essential metals are unperturbed. To expand the scope of our boronic ester prochelators and to overcome the limitations of our first-generation fluorescent prochelator FlamB, we present here two new fluorescein-modified aroyl hydrazone prochelators: FloB and FloB-SI. Both compounds contain boronic ester masks that can be triggered by H_2O_2 to reveal a fluorescein-tagged chelator that responds most significantly to Fe^{3+} and Cu^{2+} by decreasing fluorescence intensity, while Co^{2+} , Ni^{2+} , and Zn^{2+} show less dramatic quenching responses. Notably, we demonstrate that FloB-SI is an effective cellular probe that shows a diminished fluorescence response in cells treated with sublethal amounts of H_2O_2 . Because iron is the most likely metal ion to be released intracellularly upon exposure to H_2O_2 , these preliminary cell microscopy experiments suggest that FloB-SI may be useful for monitoring changes in labile iron upon oxidative insults that involve or generate H_2O_2 .

2. Materials and Methods

2.1. Materials and Instrumentation

All chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise noted; (2-formylphenyl)boronic acid pinacol ester was purchased from Combi-Blocks, Inc and PS-carbodiimide resin was purchased from Biotage. All solvents were reagent grade and all aqueous solutions were prepared from nanopure water. FloS and FloX stock solutions were prepared daily in 10 mM HEPES/100 mM NaCl buffered to a pH of 7.00. FloB stock solutions were prepared daily in MeOH and FloB-SI solutions were prepared in DMSO. Dilutions of FloB were prepared in 10 mM HEPES and absorbance/fluorescence measurements were taken within 5 minutes. 250 μ M [Fe^{3+} (NTA)] stock solutions were prepared by first making a 250 μ M nitrilotriacetic acid (NTA) solution in 10 mM HEPES/100 mM NaCl (pH = 7.00) with excess $FeCl_2$. The solution was allowed to sit overnight and was then filtered to eliminate excess iron.

Liquid chromatography-electrospray mass spectrometry (LC-MS) was performed on an Agilent 1100 Series LC/MSD trap spectrometer with a Daly conversion dynode detector. A Varian Polaris C18 (150 × 1.0 mm) column was used and peaks were detected by UV absorption at 310 nm. For FloB-SI stability studies, a linear gradient from 30% A in B to 80% A in B was run at 40 $\mu\text{L}/\text{min}$ from 2–17 min with a total run time of 25 min where A is MeCN/2% H_2O with 0.3% formic acid and B is $\text{H}_2\text{O}/2\%$ MeCN with 0.3% formic acid. For FloB-SI oxidation studies, a linear gradient from 20% A in B to 30% A in B was run at 40 $\mu\text{L}/\text{min}$ from 2–17 min with a total run time of 22 min where A is MeCN/2% 10 mM ammonium acetate buffer and B is 10 mM ammonium acetate buffer/2% MeCN. High-resolution mass spectra (HR-MS) were recorded on a time-of-flight mass spectrometer (Agilent 6224 TOF). ^1H and ^{13}C NMR spectra were recorded on a Varian Inova 400 or 500 MHz NMR spectrometer; δ values are in ppm and J values are in Hz. UV-Vis spectra were recorded on a Cary 50 UV-Vis spectrophotometer. Emission spectra were recorded on a Jobin-Yvon-Horiba Fluorolog 3 fluorimeter in a 1-cm pathlength quartz cell. Excitation and emission slit widths were 2 nm and emission spectra were collected from 500–600 nm after excitation at 495 nm.

2.2. Synthesis

2.2.1. Preparation of 4(5)-Hydrazino-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid (1)—To a solution of hydrazine monohydrate (0.43 mmol, 21 μL) in 10 mL CH_2Cl_2 and 4 mL of DMF was added 0.2 mL of N-methylmorpholine and the solution was stirred at room temperature. A portion of 5(6)-carboxyfluorescein (0.244 g, 0.645 mmol) was dissolved in 4 mL of DMF and then added to the hydrazine solution. The mixture was stirred for 10 min. PS-carbodiimide resin (0.86 mmol, 1 g) was added directly to the reaction and the solution was stirred for 18 h. The reaction was vacuum filtered to remove the resin and washed with CH_2Cl_2 . The solvent was removed and the resulting orange oil was used without further purification. LC-MS analysis of the solution confirmed that the product was obtained in >90% purity. ESI-MS: m/z 391.0 $[\text{M}+\text{H}]^+$, calcd 390.09 for $\text{C}_{20}\text{H}_{14}\text{N}_2\text{O}_6$.

2.2.2. Preparation of 4(5)-(2-Hydroxy-benzylidene-hydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid (FloS)—A solution of **1** (0.133 mmol) was prepared in 8 mL of MeOH and heated to 60 $^\circ\text{C}$. Salicylaldehyde (0.133 mmol, 14.0 μL) was added dropwise and stirred for 20 min. The flask was cooled over ice to yield an orange precipitate. The product was washed with ether and collected via vacuum filtration (0.0368 g, 56%). ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{SO}$) δ 11.16 (s, 1H), 11.07 (s, 1H), 10.26 (s, 1H), 10.19 (s, 2H), 8.71 (s, 1H), 8.58 (s, 1H), 8.33 (dd, J = 8.11 and 1.18, 1H), 8.27 (dd, J = 8.00 and 0.76, 1H), 8.16 (d, J = 8.02, 1H), 7.81 (s, 1H), 7.66 (dd, J = 7.75 and 1.84, 1H), 7.61 (dd, J = 7.74 and 1.38, 1H), 7.52 (td, J = 4.18, 1.68, 1.68, 1H), 7.47 (d, J = 8.06, 1H), 7.24–7.31 (m, 2H), 6.99 (d, J = 8.39, 1H), 6.96 (dd, J = 6.23 and 2.89, 2H), 6.91 (d, J = 9.32, 1H), 6.71–6.88 (m, 3H), 6.67–6.52 (m, 7H). HR-MS (ESI): m/z 495.1196 $[\text{M} + \text{H}]^+$, calcd 494.1187 for $\text{C}_{28}\text{H}_{18}\text{N}_2\text{O}_7$; UV-Vis (10 mM HEPES/100 mM NaCl, pH 7.0) nm ($\text{M}^{-1}\text{cm}^{-1}$): 495 (42,347).

2.2.3. Preparation of 4(5)-(Benzylidene-hydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid (FloX)—A solution of **1** (0.133 mmol) was prepared in 3 mL of MeOH and heated to 60 $^\circ\text{C}$. Benzaldehyde (0.133 mmol, 13.6 μL) was added dropwise and stirred for 10 min. The flask was cooled over ice and 1 M HCl was added dropwise until an orange precipitate formed. The resulting precipitate was washed with ether and collected by vacuum filtration (0.0222 g, 35%). ^1H NMR (500 MHz, $(\text{CD}_3)_2\text{SO}$) δ ppm 12.20 (s, 1H), 11.99 (s, 1H), 8.57 (s, 1H), 8.53 (s, 1H), 8.40 (s, 1H), 8.33 (d, J = 7.81, 1H), 8.27 (d, J = 7.95, 1H), 8.15 (d, J = 8.02, 1H), 7.80 (s, 1H), 7.77 (d, J = 6.94, 1H), 7.69 (dd, J = 6.37 and 2.73, 1H), 7.46 (m, 7H), 6.69–6.73 (m, 4H), 6.55–6.66 (m,

7H). HR-MS (ESI): m/z 479.1242 $[M + H]^+$, calcd 478.1238 for $C_{28}H_{18}N_2O_6$; UV-Vis (10 mM HEPES/100 mM NaCl, pH 7.0) nm ($M^{-1}cm^{-1}$): 495 (57,859).

2.2.4. Preparation of 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzylidene-hydrazinocarbonyl]-benzoic acid (FloB)—A solution of **1** (0.133 mmol) was prepared in 2 mL of THF and heated to 60 °C. (2-formylphenyl)boronic acid pinacol ester (0.133 mmol, 29.3 μ L) was added dropwise and stirred for 10 minutes. The flask was then cooled over ice and deionized H_2O was added to form a dark brown oil. The oil was washed with ether to produce an orange precipitate, which was collected by vacuum filtration (0.0618 g, 77%). 1H NMR (400 MHz, $(CD_3)_2SO$) δ 12.24 (s, 1H), 12.11 (s, 1H), 8.95 (s, 1H), 8.83 (s, 1H), 8.49 (s, 1H), 8.26 (m, 1H), 8.20 (d, $J = 7.39$ Hz, 1H), 8.12 (d, $J = 7.98$ Hz, 1H), 8.02 (d, $J = 8.03$ Hz, 1H), 7.93 (dd, $J = 15.65$, 8.07 Hz, 1H), 7.69 (m, 2H), 7.52 (m, 2H), 7.40 (m, 3H), 6.66 (m, 4H), 6.55 (m, 7H), 1.32 (s, 12H), 1.27 (s, 12H). HR-MS (ESI): m/z 604.2117 $[M + H]^+$, calcd 603.2126 for $C_{34}H_{29}BN_2O_8$ (^{10}B); UV-Vis (10 mM HEPES/100 mM NaCl, pH 7.0) nm ($M^{-1}cm^{-1}$): 495 (46,054).

2.2.5. Preparation of 2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)benzaldehyde (2)—4-bromomethylphenyl boronic acid, pinacol ester (0.30 g, 1.01 mmol) and salicylaldehyde (106 μ L, 1.01 mmol) were dissolved in 5 mL of anhydrous DMF. Potassium carbonate (0.69 g, 5.05 mmol) was added and the reaction was stirred for 18 h at room temperature. The solvent was removed via rotary evaporation and the resulting solid was taken up in CH_2Cl_2 and extracted with H_2O (3×10 mL). The organic layer was washed with brine (3×10 mL), dried over $MgSO_4$, filtered and solvent removal yielded a crystalline white solid (0.280 g, 82% yield). 1H NMR (400 MHz, $CDCl_3$) δ 10.57 (s, 1H), 7.85 (t, $J = 7.4$, 3H), 7.54–7.49 (m, 1H), 7.44 (d, $J = 7.0$, 2H), 7.03 (dd, $J = 14.6$ and 7.8, 2H), 5.22 (s, 2H), 1.35 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.74, 189.71, 160.98, 139.16, 135.97, 135.21, 128.44, 126.43, 125.15, 121.06, 113.07, 83.94, 70.34, 24.91.

2.2.6. Preparation of 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)-benzylidene-hydrazinocarbonyl]-benzoic acid (FloB-SI)—A solution of **1** (0.5 mmol) was prepared in 6 mL of acetonitrile with a catalytic amount of glacial acetic acid and heated to 80 °C. A solution of **2** (0.182 g, 0.47 mmol) was prepared in 0.5 mL acetonitrile and added dropwise to the heated solution. After stirring for 1 h, the solvent was removed by rotary evaporation and the product was isolated by silica gel column chromatography with 8:2 EtOAc:Hexanes (0.128 g, 38% yield). 1H NMR (400 MHz, $(CD_3)_2SO$) δ 12.16 (s, 1H), 12.00 (s, 1H), 10.18 (s, 4H), 8.87 (s, 1H), 8.72 (d, $J = 6.22$ Hz, 1H), 8.60–8.58 (m, 1H), 8.55 (s, 1H), 8.31 (d, $J = 8.07$ Hz, 1H), 8.25 (d, $J = 8.06$ Hz, 1H), 8.13 (d, $J = 8.08$ Hz, 1H), 7.93 (d, $J = 7.54$ Hz, 1H), 7.86 (d, $J = 7.71$ Hz, 1H), 7.77 (s, 1H), 7.73 (d, $J = 7.23$ Hz, 2H), 7.69 (d, $J = 7.19$ Hz, 1H), 7.53 (d, $J = 7.30$ Hz, 2H), 7.45 (dd, $J = 11.89$, 7.92 Hz, 3H), 7.20 (d, $J = 8.43$ Hz, 1H), 7.12 (d, $J = 8.39$ Hz, 1H), 6.95–7.00 (m, 2H), 6.70 (s, 4H), 6.56–6.60 (m, 7H), 5.26 (s, 2H), 5.21 (s, 2H), 1.29 (s, 24H). HR-MS (ESI): m/z 710.2543 $[M + H]^+$, calcd 709.2545 for $C_{41}H_{35}BN_2O^9$ (^{10}B); UV-Vis (10 mM HEPES/100 mM NaCl, pH 7.0) nm ($M^{-1}cm^{-1}$): 495 (52,756).

2.2.7. Preparation of 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)-benzylidene-hydrazinocarbonyl]-benzoic acid, 3,6-diacetate (AcFloB-SI)—A solution of FloB-SI (0.050 g, 0.070 mmol) and triethylamine (TEA, 49 μ L, 0.35 mmol) was prepared in 6 mL anhydrous CH_2Cl_2 with 0.5 mL anhydrous DMF. The reaction was cooled to 0 °C and

acetyl chloride (11 μ L, 0.154 mmol) was added dropwise. After 10 min, the reaction was warmed to room temperature and stirred for an additional 30 min. The solvent was then removed by rotary evaporation and the remaining solid was dissolved in CH_2Cl_2 , washed with NaHCO_3 (3×10 mL) and brine, and then dried over MgSO_4 . Removal of the solvent yielded a dull yellow powder (36.8 mg, 66 % yield). ^1H NMR (400 MHz, CD_3CN) δ 10.00 (s, 1H), 8.79 (s, 1H), 8.48 (s, 1H), 8.30–8.18 (m, 2H), 8.13 (d, J = 8.11, 1H), 8.06 (d, J = 8.16, 1H), 7.98 (d, J = 8.08, 1H), 7.83 (d, J = 7.58, 4H), 7.56 (d, J = 7.69, 1H), 7.43 (d, J = 7.59, 3H), 7.31 (t, J = 8.01, 8.01, 2H), 7.20–7.24 (m, 1H), 7.17 (d, J = 7.77, 2H), 7.10 (s, 3H), 6.92 (t, J = 7.44, 7.44, 3H), 6.81 (d, J = 7.97, 5H), 6.68–6.62 (m, 1H), 6.54 (d, J = 4.86, 2H), 11.61 (s, 1H), 5.07 (s, 2H), 4.96 (s, 2H), 2.31 (s, 6H), 2.29 (s, 6H), 1.34 (s, 12H), 1.30 (s, 12H). MS (ESI): m/z 795.4 $[\text{M} + \text{H}]^+$, calcd 794.26 for $\text{C}_{45}\text{H}_{39}\text{BN}_2\text{O}_{11}$.

2.2.8. Preparation of 4(5)-(Benzylidene-hydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid, 3,6-diacetate (AcFloX)—A solution of FloX (0.050 g, 0.104 mmol) and TEA (73 μ L, 0.52 mmol) was prepared in 5 mL anhydrous CH_2Cl_2 with 0.5 mL anhydrous DMF. The reaction was cooled to 0 $^\circ\text{C}$ and acetyl chloride (16 μ L, 0.23 mmol) was added dropwise. After 10 min, the reaction was warmed to room temperature and stirred for an additional 20 min. The solvent was then removed by rotary evaporation and the remaining solid was dissolved in CH_2Cl_2 , washed with NaHCO_3 (3×10 mL) and brine, and then dried over MgSO_4 . Removal of the solvent via rotary evaporation revealed a dull yellow colored solid (36.3 mg, 62 % yield). ^1H NMR (400 MHz, CDCl_3) δ ppm 11.56 (s, 1H), 10.65 (s, 1H), 8.56 (s, 1H), 8.35 (d, J = 7.97, 1H), 8.27 (d, J = 7.76, 1H), 8.21 (d, J = 2.83, 1H), 8.05 (d, J = 8.05, 1H), 8.01 (s, 1H), 7.89 (s, 1H), 7.57 (d, J = 6.74, 3H), 7.52 (d, J = 7.11, 1H), 7.35–7.24 (m, 6H), 7.13 (s, 2H), 6.83 (s, 5H), 6.72 (s, 2H), 6.64 (s, 1H), 2.36 (s, 6H), 2.32 (s, 6H). MS (ESI): m/z 563.2 $[\text{M} + \text{H}]^+$, calcd 562.13 for $\text{C}_{32}\text{H}_{22}\text{N}_2\text{O}_8$.

2.3. Quantum Yield Determination

Stock solutions of 40 μM FloS, 100 μM FloX, and 250 μM $[\text{Fe}^{3+}(\text{NTA})]$ were prepared in 10 mM HEPES/100 mM NaCl (pH = 7.0). Stock solutions of 1 mM FloB and 2 mM FloB-SI were prepared in MeOH and DMSO, respectively. Dilutions of FloS, FloX, FloB, and FloB-SI were prepared in HEPES at concentrations such that their absorbance at 495 nm equaled 0.02, 0.04, 0.06, 0.08, and 0.1. Dilutions of the FloS-Fe complex were prepared at 0.5, 1, 1.5, 2, and 2.5 μM in HEPES with 3 equivalents of $[\text{Fe}^{3+}(\text{NTA})]$. Excitation was performed at 495 nm and collected emission was normalized to the HEPES blank and then integrated from 500 to 600 nm. A plot of the integrated fluorescence intensity vs. the absorbance at 495 nm for each concentration was prepared and the positive slope of the linear fit was calculated. The data were compared to a fluorescein standard in 0.1 M NaOH using the following equation, where ϕ_R is the quantum yield of the standard (0.95), Grad is the slope of the absorbance vs. emission line found for each compound, Grad_R is the slope found for the fluorescein standard, η is the refractive index of the sample solutions (1.33) and η_R is the refractive index of the fluorescein solution (1.33):

$$\phi = \phi_R (\text{Grad}/\text{Grad}_R) (\eta^2/\eta_R^2)$$

2.4. Cell Culture

All cell culture reagents including minimal essential medium (MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin (pen-strep), and trypsin-EDTA (0.25%) were purchased from Gibco. HeLa cells were purchased from the Duke University cell culture facility and originally obtained from the American Type Culture Collection. The HeLa cells were cultured in complete growth medium containing

DMEM supplemented with sodium pyruvate, 10% (v/v) FBS and 1% (v/v) pen-strep. The cells were incubated at 37 °C with a fully humidified atmosphere containing 5% CO₂.

2.5. Fluorescence Microscopy Experiments

Live cell microscopy images were collected on a Zeiss Axio Observer widefield fluorescence microscope under 40× magnification using a Plan Neofluar objective lens. A GFP filter set (Zeiss no. 38HE; BP470/40, FT 495, BP525/50) was used to collect fluorescence emission data. Quantification and image processing were performed with MetaMorph. All microscope settings, including transmission density, brightness, contrast and scan speed were held constant.

For each experiment, cells were seeded into a 12-well MatTek glass bottom plate and grown to ~70–80% confluence. To treat the cells with the fluorescent compounds, the growth medium was removed and the cells were washed with 1 mL phosphate buffered saline (PBS) and 1 mL of MEM was added to each well. Aliquots of AcFloB-SI and AcFloX were added directly from DMSO stock solutions to a final concentration of 5 μM. Cells were returned to the incubator at 37 °C. After 30 min, the cells were again washed with 1 mL of PBS and 1 mL of MEM was added to each well. For samples treated with H₂O₂, 5 μL of a 10 mM stock solution prepared in MEM were added directly to the wells and the cells were then incubated at 37 °C for 30 min prior to imaging. For samples treated with the competitive chelator SIH, 10 μL of a 10 mM stock solution prepared in DMSO were added directly to the wells and the cells were incubated at 37 °C for an additional 30 min before imaging.

3. Results

3.1. Synthesis, Nomenclature, and Structure of FloS, FloX, FloB, and FloB-SI

The design of the Flo-series is based on the parent aroyl hydrazone-based BSIH/SIH prochelator/chelator combination, but with an embedded fluorescein moiety [17]. The isonicotinic acid hydrazide used to synthesize BSIH and SIH was replaced by 5(6)-carboxyfluorescein, hydrazide (**1**) and reacted with 2-formylphenyl boronic ester, salicylaldehyde, benzaldehyde or salicylaldehyde equipped with a self-immolative pinacol boronic ester (**2**) to produce FloB, FloS, FloX, or FloB-SI, respectively (Scheme 1, Figure 1). All four compounds were synthesized by Schiff-base condensation reactions carried out in refluxing solutions of MeOH, THF, or MeCN. Each was isolated as a mixture of two structural isomers (positional isomers on the fluorescein phenyl ring at positions 5 and 6). Additionally, FloX and FloB-SI were acetylated with acetyl chloride at the 3 and 6 positions on the xanthene ring to form AcFloX and AcFloB-SI, respectively. These diacetylated versions increase the cell permeability of each compound. The two fluorescent prochelators contain either a pinacol boronic ester, FloB, or a self-immolative pinacol boronic ester, FloB-SI. As we have shown previously, masking a metal chelator directly with a boronic ester renders the prochelator ineffective at binding metal ions until activation with H₂O₂ [17, 19, 21]. Several other groups have employed a self-immolative boronic ester as a protecting group or mask for H₂O₂ responsive small molecules, dendritic chains, and metalloproteinase inhibitors [20, 22–24]. Cleavage of the self-immolative protecting group is initiated by nucleophilic attack on the boronic ester by H₂O₂, followed by hydrolysis of the borate intermediate to yield phenoxide ion. A “head to tail” disassembly through a 1,6-benzyl elimination then releases a phenolic component and quinone methide as a side product that is quickly hydrated to form 4-hydroxybenzyl alcohol [25, 26]. Under the appropriate conditions, FloB or FloB-SI will be converted to the chelator, FloS, a fluorescently tagged derivative of the metal chelator, SIH. The pinacol boronic ester of FloB and the self-immolative boronic ester of FloB-SI mask the phenolic oxygen of FloS. Once unmasked by H₂O₂, iron binding is anticipated to occur at the phenolic oxygen, imine nitrogen, and

carbonyl oxygen and cause a decrease in the fluorescence emission of the fluorescein-tagged FloS. FloX is a non-chelating control compound because it lacks the phenolic oxygen, one of the required donor atoms for iron chelation.

3.2. Absorbance Response of FloS, FloX, FloB, and FloB-SI to Fe³⁺

All four of the compounds have modest solubility in aqueous solutions. Therefore, stock solutions were prepared directly in 10 mM HEPES buffer at pH 7.0 for FloS and FloX; FloB and FloB-SI solutions were prepared by proportioning stock solutions into 10 mM HEPES buffer so that the final MeOH or DMSO content was less than 1%. Due to the fluorescein component, each solution has a green/yellow hue and a strong absorbance peak centered at 495 nm. The extinction coefficient for each compound is greater than 40,000 M⁻¹cm⁻¹, which is expected due to the highly fluorescent nature of fluorescein.

Iron-binding studies were carried out using an iron-NTA solution or ferric ammonium citrate (FAC) as the Fe³⁺ sources. Other iron (III) salts, such as Fe(NO₃)₃ or FeCl₃, undergo fast hydrolysis to form insoluble iron hydroxides in water at neutral pH. However, the tetradentate chelator, NTA, forms a hydrolysis-stable and therefore water-soluble complex with iron. In addition, NTA's affinity for iron in buffered solutions at pH = 7.0 (log K' = 10.84) is weak enough that the FloS chelator is able to remove the iron from its source during the following titration experiments. The weaker affinity citrate ligands also keep iron in a soluble form.

Addition of up to 1 equivalent [Fe³⁺(NTA)] into a 20 μM solution of FloS introduces instantaneous changes to the absorbance spectra and a color change from green/yellow to dark orange. Decreases at 290 and 325 nm and an increase at 380 nm are seen, as shown in Supp. Mat. Figure S1. These data indicate that one equivalent of [Fe³⁺(NTA)] is required to achieve the maximal change in the spectra, which would suggest formation of a 1:1 Fe:FloS complex. However, chelators in the SIH family are known to form 1:2 Fe:chelator complexes, and formation of ternary complexes containing Fe, NTA, and FloS cannot be ruled out. The experiment was therefore repeated with FAC as the Fe³⁺ source. In this case, the spectral changes upon FAC addition were slow and required at least 3 h to equilibrate. The equilibrated spectra shown in Figure 2 show that the absorption changes maximize at 0.5 equivalents of added Fe³⁺, which is consistent with the formation of a bis complex [Fe(FloS)₂]⁺. Further evidence that both 1:1 and 1:2 Fe:FloS complexes can form is provided by ESI-MS analysis, which indeed detects both species (Supp Mat, Figure S6).

In contrast to the changes observed for FloS, addition of [Fe³⁺(NTA)] into solutions of FloB, FloB-SI and FloX produce negligible changes to the absorbance spectra (Supp. Mat. Figures S2–S4), and no visible color changes to the solution occur. These titrations were repeated with FAC to verify that no response is seen when the iron source is coordinated to lower affinity ligands. Both prochelators as well as the control compound lack the phenolic oxygen found in FloS, which suggests that it is required for Fe³⁺ binding, as was seen previously with BSIH [17].

3.3. Fluorescence Response of FloS, FloX, FloB, and FloB-SI to Fe³⁺

All four compounds were shown to be highly fluorescent in aqueous conditions. 1 μM solutions excited at 495 nm show a strong emission peak centered at 520 nm. In addition, high quantum yields were observed for each, ϕ = 0.62, 0.56, 0.59, and 0.63 for FloB, FloS, FloX, and FloB-SI, respectively. Since the fluorescein is an appendage off the chelator, its free carboxylic acid remains open at physiological pH and is unlikely to cyclize into the non-fluorescent lactone version. This feature results in a high fluorescent output for each of the compounds.

Because Fe^{3+} is a paramagnetic metal cation, it was anticipated that the fluorescent signal of FloS would quench upon binding. As expected, titration of Fe^{3+} into a solution of FloS produces a decrease in the emission signal at 520 nm. Adding just 0.5 equivalents of Fe^{3+} yields a dramatic 70% decrease in the emission output (Figure 3a) and lowers the quantum yield to 0.21. This Fe^{3+} -dependent “turn-off” response is reversible with Desferrioxamine (DFO), a hexadentate iron chelator (Figure S5). In contrast, the prochelators, FloB and FloB-SI, and the non-chelating control, FloX, experience no significant changes when Fe^{3+} is added to the solution as either $[\text{Fe}^{3+}(\text{NTA})]$ or FAC. Figure 3b compares the effect of Fe^{3+} on all four compounds.

3.4. Metal Dependent Quenching of FloS

The metal selectivity of the FloS spectral response was examined by titrating excess amounts of other biologically relevant metal cations into the chelator solution. Aqueous solutions of MnCl_2 , NiCl_2 , CoCl_2 , CuCl_2 , ZnCl_2 , NaCl , MgCl_2 and CaCl_2 were titrated into 1 μM solutions of FloS and changes to its fluorescence response were monitored. Mn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} , and Zn^{2+} were studied up to concentrations of 20 μM , while Na^+ , Mg^{2+} and Ca^{2+} were titrated into FloS solutions in concentrations up to 10 mM. Following these titrations, 20 equivalents of $[\text{Fe}^{3+}(\text{NTA})]$ were added to the FloS solution containing the competing metal to interrogate if iron could still induce fluorescence quenching in the presence of the competitor metal.

The bar graph in Figure 4 illustrates the results of the metal competition experiment. Data for each condition are normalized to the fluorescence intensity of the emission band at 520 nm of the 1 μM FloS sample (green bars). Most of the metal cations tested showed little turn-off response between 500–600 nm when excited at 495 nm, as depicted by the striped bars, and the addition of an equal amount of Fe^{3+} showed a greater decrease in the fluorescence emission signal (white bars). Co^{2+} , Ni^{2+} and Zn^{2+} show a modest quenching effect with FloS, but the addition of Fe^{3+} is able to further decrease the emission response of the chelator. However, Cu^{2+} yields a similar turn-off response to Fe^{3+} and the two cannot be differentiated by fluorescence alone. Titration of all four of these metal cations into a solution of FloS showed changes to the absorbance spectra (Supp. Mat. Figures S7–S10).

3.5. Stability of FloB and FloB-SI in Aqueous Solutions and Cellular Media

Based on the instability of the hydrazone backbone seen with the previously reported fluorescent prochelator, FlamB [14], solutions of FloB and FloB-SI were monitored over time by UV-Vis spectroscopy after preparation in buffered solvents and cell culture media. FloB exhibits no spectral changes in either 10 mM HEPES buffer or minimal essential media (MEM) over a 24 h period at 37 °C (Supp. Mat. Figure S11). Likewise, FloB-SI shows minimal spectral changes when monitored in the same solvents (Supp. Mat. Figure S12). Some precipitation was observed for incubations in MEM longer than 6 h, but analysis of a 24 h sample by LC-MS revealed the integrity of FloB-SI (Figure S14). LC-MS analysis reveals that the boronic ester of FloB-SI is susceptible to hydrolysis, as shown by the appearance of its boronic acid version with a $m/z = 629.1$, but no evidence of hydrolysis of the hydrazone linkage is observed (Supp. Mat. Figures S13 and S14). Loss of the pinacol ester is not unexpected for the boronate group due to the equilibrium that exists between the ester and acid species in aqueous solution [27, 28].

3.6. Oxidation of FloB to FloS with H_2O_2 in Organic Solvents

The reaction of FloB with H_2O_2 to produce FloS, as depicted in Scheme 2, was first studied by ^1H -NMR. A 10 μM solution of FloB was prepared in *d*-DMSO and 10 equivalents of H_2O_2 were added. The sample was monitored by ^1H -NMR for 4 h. At 0 h, two singlet peaks are observed for the pinacol methyl protons, at 1.35 and 1.30 ppm. The peaks are attributed

to the two structural isomers of FloB. After 4 h of incubation with H_2O_2 , a shift is seen in the signal for the pinacol methyl protons to 1.15 ppm (Supp. Mat. Figure S15). This signal is consistent with released boronate pinacol ester, as it is shifted from that of the free pinacol diol (1.07 ppm). The oxidized FloB solution was analyzed further by UV-Vis and fluorescence spectroscopy. The absorbance spectrum of the oxidized sample matched that of FloS, displaying peaks at 290, 325 and 495 nm (Supp. Mat. Figure S16). In addition, the fluorescence spectrum of the NMR sample showed quenching upon addition of Fe^{3+} (Supp. Mat. Figure S16) in a manner similar to that of FloS. Finally, analysis of the sample by electrospray ionization mass spectrometry revealed a molecular ion peak of 495.1 m/z confirming the presence of FloS.

3.7. Oxidation of FloB to FloS in Aqueous Solutions

Even though the oxidation of FloB in DMSO proceeded as expected, this result could not be repeated in aqueous conditions. As shown in Figure 5, the addition of 1 mM H_2O_2 to a 20 μM solution of FloB in 10 mM HEPES buffer (100 mM NaCl, pH = 7.00) leads to a decrease in the absorbance band at 290 nm and an increase of the 260 nm band. No spectral changes were observed above 400 nm. However, the final absorbance spectrum does not match that of FloS, as was expected. Upon titration of $[\text{Fe}^{3+}(\text{NTA})]$ into this solution, no changes were observed suggesting that no metal complexation took place (Supp. Mat. Figure S17). Similarly, no fluorescence quenching is seen after addition of H_2O_2 and Fe^{3+} into an aqueous solution of FloB (Supp. Mat.).

3.8. Conversion of FloB-SI to FloS with H_2O_2

The reactivity of the self-immolative boronic ester with H_2O_2 was tested to ensure that the prochelator could efficiently be converted to the chelator. UV-Vis spectroscopy was first utilized to monitor the reaction in a buffered solution. An increase in the absorbance band at 287 nm and a slight shift of the 324 nm band are seen when FloB-SI is incubated with 3 mM H_2O_2 for 1 h (Figure 6a). Although these changes are minor, the resulting spectrum matches that of FloS. Further spectral changes are observed when Fe^{3+} is titrated into the FloB-SI and H_2O_2 solution (Figure 6a), unlike when Fe^{3+} was added to a solution containing only FloB-SI, as previously shown. The reaction was repeated and changes were monitored by fluorescence (Figure 6b). As expected, minimal change to the emission intensity is seen after addition of H_2O_2 into a FloB-SI solution. However, after titration of Fe^{3+} a 50% decrease in emission intensity is observed.

LC-MS was used to further investigate the reaction between FloB-SI and H_2O_2 . Solutions of FloS and FloB-SI were prepared in buffered solvents and analyzed by LC-MS. Their chromatographic traces were compared to that of a buffered FloB-SI solution incubated with H_2O_2 for 1 h. There is only one set of peaks in the reaction's chromatographic trace (Figure 7). Mass spec analysis of these peaks confirm that FloS is the product of the reaction between FloB-SI and H_2O_2 .

3.9. Fluorescence Microscopy of FloB-SI and FloX

To test our fluorescent prochelator concept in cells, cervical carcinoma HeLa cells were treated with the diacetylated versions of FloB-SI and FloX, AcFloB-SI and AcFloX, respectively. Both compounds show a high intracellular fluorescence response when imaged with fluorescence microscopy after 30 min of incubation (Figure 8). After the addition of 50 μM exogenous H_2O_2 , the fluorescence response of cells treated with AcFloB-SI decreases by 67%, but the cells treated with AcFloX exhibit no change in fluorescence response (Figure 8). Cells treated with both AcFloB-SI and H_2O_2 were incubated with 100 μM of the high affinity iron chelator, SIH. Treatment with SIH restored the intracellular fluorescence to 85% of its initial intensity suggesting that the quenching seen with H_2O_2 was due to

intracellular oxidation of FloB-SI and subsequent metal binding. Bright-field images of the cells before and after treatment with the Flo-compounds and H₂O₂ show that the cells remain viable throughout the experiment (Supp. Mat. Figure S18).

Several initial control experiments were performed with the HeLa cells to determine the parameters of the imaging experiment. First, a kill curve experiment was performed in which cells were treated with 50–350 μ M H₂O₂ in MEM (Supp. Mat. Figure S19). Cell death increased with concentration and 50 μ M showed 60% cell death after 24 h, insinuating that this concentration causes intracellular damage, but would not completely kill the cells during the time course of the microscopy experiment.

Additionally, the effect of photobleaching for AcFloB-SI and AcFloX was analyzed by continuously exciting the samples and collecting their emission intensity every 3 seconds. Data were collected for a total of 30–60 seconds and normalized to 100%. The emission signals for both compounds decrease to ~5–10% from the original intensity (Supp. Mat. Figure S20). Although the initial 3 seconds of exposure causes ~20% decrease in emission intensity, only 10–30 milliseconds are needed to take each image. Since the same cells are not imaged more than twice in a given experiment, photobleaching should be negligible within this timeframe.

4. Discussion

4.1. Chelator Design and Metal Induced Spectral Changes

The Flo-series design is modeled from the BSIH prochelator strategy. The major structural difference between the two families of compounds is that a fluorescein is tagged onto the backbones of the Flo-series. By incorporating a fluorophore into the structure, all four compounds, FloS, FloX, FloB, and FloB-SI were found to be highly fluorescent in buffered solutions at a biologically relevant pH.

The design of FloS maintains the binding site of the parent chelator, SIH. The phenolate oxygen, imine nitrogen, and carbonyl oxygen are the three donor atoms. The assumption that the same tridentate design of FloS would facilitate a 2:1 ligand:metal binding ratio with Fe³⁺ seems to hold, as evidenced by the absorption and fluorescence titrations as well as mass spectrometry.

As designed, absorbance and emission spectral changes are seen when FloS is titrated with Fe³⁺ sources. Due to the paramagnetism of Fe³⁺, the changes in absorbance correlate to a 70% decrease in the 520 nm emission band of FloS when excited at 495 nm.

No spectral changes were seen when Fe³⁺ was titrated into the non-chelating control compound, FloX, suggesting that the phenolic oxygen of FloS is a key atom donor. By masking this same phenolic oxygen with a boronic ester, the prochelator, FloB, was shown to prevent iron binding until activation with H₂O₂. FloB showed no absorbance or emission changes upon addition of Fe³⁺. Likewise, the self-immolative boronic ester-protecting group successfully prohibits premature iron binding.

The aroyl hydrazone backbone found in SIH and FloS creates a receptor site capable of binding divalent d-block metal ions in addition to trivalent Fe³⁺ [29, 30]. It is therefore not surprising that spectral changes of FloS were observed for Co²⁺, Ni²⁺, Cu²⁺, and Zn²⁺, all of which had a fluorescence quenching effect. The quenching seen with Zn²⁺ is slightly unexpected due to the fact that Zn²⁺ is a d¹⁰, diamagnetic cation. However, in the cases of Zn²⁺, Ni²⁺ and Co²⁺, addition of Fe³⁺ back into the metal-containing solutions caused the emission signal to further decrease. This result suggests that FloS prefers to bind Fe³⁺ as

opposed to Zn^{2+} or Ni^{2+} . However, Cu^{2+} and Fe^{3+} induce similar amounts of fluorescence quenching and cannot be differentiated based on the fluorescence turn-off response.

4.2. Superior Properties of FloB-SI vs FloB Make it Amenable to for Cell Studies

The basis of the prochelator strategy relies on oxidation of the boronic ester mask and stability of the prochelator and chelator in aqueous solutions. While the oxidative conversion of FloB to FloS by H_2O_2 proceeded as expected in DMSO, attempting the same studies in aqueous solution led to a different observation. Although changes in the absorbance spectrum of FloB were observed with H_2O_2 , the product does not correlate to FloS. Based on the instability seen with FlamB, it is assumed that these changes might correlate to hydrolysis of the imine bond on the hydrazone backbone of FloB. Hydrolysis at this position would lead to formation of the two starting materials, **1** and (2-formylphenyl)boronic pinacol ester. **1** is a highly fluorescent molecule, like FloS; however, it is unable to complex or interact with Fe^{3+} and thus no changes to its absorbance or emission spectra would be observed. After reacting with H_2O_2 , FloB solutions titrated with Fe^{3+} yield no change in their spectra, supporting the conclusion that the hydrolysis product, **1**, has formed. However, unlike FlamB, FloB is stable in aqueous solvents and cell culture media; therefore, the hydrolysis seems to be dependent on the presence of H_2O_2 . In any case, the failure of FloB to convert into a metal chelating agent upon exposure to H_2O_2 prevents further exploration of this particular compound in cellular experiments.

Many of the limitations of FlamB and FloB were overcome by incorporating a boronic ester mask onto FloS via a self-immolative linker to generate FloB-SI as an H_2O_2 -reactive prochelator. FloB-SI is hydrolytically stable in simple aqueous solution and under more demanding conditions of MEM cell culture medium for 24 h at 37 °C. Addition of excess H_2O_2 to an aqueous solution of FloB-SI resulted in a final absorbance spectrum that matched that of the chelator, FloS. In addition, titration of Fe^{3+} into the solution revealed both changes to the absorbance spectrum that indicated iron binding and a decrease in the fluorescence emission at 520 nm. Analysis of a FloB-SI and H_2O_2 solution by LC-MS provided further evidence that FloS is cleanly formed.

4.3. Fluorescence Microscopy Imaging of FloB-SI in HeLa cells

To see if the fluorescent prochelator strategy is capable of responding to intracellular oxidative stress, FloB-SI and FloX were tested in HeLa cells and their fluorescence response to H_2O_2 was monitored with fluorescence microscopy. Acetylating the xanthene ring of each compound renders them non-fluorescent but cell permeable. Once the diacetyl ester caps are cleaved by internal esterases, each shows strong fluorescence intensity. After addition of excess H_2O_2 for 30 min, the intracellular fluorescence signal decreases by ~70% for samples treated with AcFloB-SI. However, samples treated with AcFloX and H_2O_2 show no decrease in fluorescence intensity. These results suggest that the addition of H_2O_2 may have caused intracellular liberation of Cu^{2+} or Fe^{3+} because fluorescence quenching is seen with the prochelator but not with the non-chelating control. To determine if metal binding caused the intracellular response of AcFloB-SI with H_2O_2 , the cells were treated with an excess of SIH as a competitive chelator. After 30 min of treatment with SIH, the signal of AcFloB-SI was restored to 85% of its initial value, suggesting that the quenched emission was due to metal binding. While our fluorescent probe cannot distinguish which quenching metal it actually finds intracellularly, iron is the likely candidate, as it is known that H_2O_2 can deactivate iron-sulfur clusters to release iron [31, 32].

5. Conclusions

By utilizing a prochelator strategy, we were able to develop a fluorescent probe for metal-mediated oxidative stress. The design employs a self-immolative pinacol boronic ester mask to limit the interaction of the fluorescent prochelator, FloB-SI, with metal cations. In the presence of H_2O_2 , the self-immolative boronic ester is oxidized to release a highly fluorescent metal chelator, FloS. Upon complexation with Fe^{3+} or Cu^{2+} , the fluorescence emission signal of the chelator is quenched by greater than 70%, while other d-block metals have less significant quenching effects. The selectivity of this fluorescent probe therefore does not reside in any inherent metal preference, but rather with the reactivity-dependent activation that requires initial exposure to H_2O_2 . Because H_2O_2 is a component of oxidative stress, FloB-SI may be useful for monitoring changes in chelator-accessible pools of metal ions under oxidative stress conditions. While a turn-on fluorescence response [13] is generally more desirable than the turn-off response provided by FloS, we have previously observed that hydrazone-type compounds can produce a turn-on response arising from complications other than direct metal binding [14]. Understanding the intracellular properties of fluorescent probes remains an ongoing challenge. The preliminary cell microscopy experiments presented here are consistent with the *in vitro* fluorescence behavior of FloB-SI exposed to H_2O_2 and Fe^{3+} , but it is important to keep in mind that the complicated milieu of the cell may provide alternative pathways for these fluorescence changes. Continuing efforts to further optimize these compounds for photostability and cellular compatibility are underway.

Highlights

- A series of fluorescein-based metal-binding compounds have been prepared and characterized.
- Two boronate ester prochelators react with H_2O_2 to produce a fluorescent agent capable of chelating a variety of di- and tri-valent d-block metal ions.
- Fe^{3+} and Cu^{2+} elicit a 70% decrease in the chelator's emission signal upon binding, while Ni^{2+} , Co^{2+} and Zn^{2+} induce less significant quenching.
- The fluorescent prochelator termed FloB-SI is cell permeable and responds to exogenously added H_2O_2 by a decrease in fluorescence that is restored upon treatment with a competitive chelator, suggesting that FloB-SI responds to intracellular metal ions in response to H_2O_2 -induced stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BSIH	isonicotinic acid [2-(4,4,5,5-tetramethyl-1,3,2)dioxaborolan-2-yl]benzylidene]-hydrazide
DMEM	Dulbecco's modified eagle medium
EDTA	ethylenediaminetetraacetic acid
FAC	ferric ammonium citrate
FloB	2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4,4,5,5-tetramethyl-1,3,2)dioxaborolan-2-yl)-benzylidene-hydrazinocarbonyl]-benzoic acid

FloB-SI	2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4(5)-[2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)-benzylidene-hydrazinocarbonyl]-benzoic acid
FloS	4(5)-(2-Hydroxy-benzylidene-hydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid
FloX	4(5)-(Benzylidene-hydrazinocarbonyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid
HeLa	immortal cell line derived from cervical cancer cells from Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRMS	high-resolution mass spectrometry
MEM	minimal essential medium
NTA	nitrilotriacetic acid
ROS	reactive oxygen species
SIH	salicylaldehyde isonicotinoyl hydrazone

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References

1. Loh A, Hadziahmetovic M, Dunaief JL. *Biochim. Biophys. Acta*. 2009; 1790:673–649.
2. Lee DW, Andersen JK. *J. Neurochem.* 2010; 112:332–339. [PubMed: 20085612]
3. Altamura S, Muckenthaler MU. *J. Alzheim. Dis.* 2009; 16:879–895.
4. Cadet J, Douki T, Ravanat J-L. *Free Radical Biol. Med.* 2010; 49:9–21. [PubMed: 20363317]
5. Moreira PI, Nunomura A, Nakamura M, Takeda A, Shenk JC, Aliev G, Smith MA, Perry G. *Free Radical Biol. Med.* 2008; 4:1493–1505. [PubMed: 18258207]
6. Xiang Y, Tong A. *Org. Lett.* 2006; 8:1549–1552. [PubMed: 16597107]
7. Lin W, Long L, Yuan L, Cao Z, Feng J. *Anal. Chim. Acta.* 2009; 634:262–266. [PubMed: 19185130]
8. Lee MH, Giap TV, Kim SH, Lee YH, Kang C, Kim JS. *Chem. Commun.* 2010; 46:1407–1409.
9. Chartres JD, Busby M, Riley MJ, Davis JJ, Bernhardt PV. *Inorg. Chem.* 2011; 50:9178–9183. [PubMed: 21812394]
10. Li P, Fang L, Zhou H, Zhang W, Wang X, Li N, Zhong H, Tang B. *Chem. Eur. J.* 2011; 17:10520–10523. [PubMed: 21850713]
11. Breuer W, Epsztejn S, Cabantchik ZI. *FEBS Lett.* 1996; 382:304–308. [PubMed: 8605990]
12. Petrat F, Rauen U, Groot Hd. *Hepatology.* 1999; 29:1171–1179. [PubMed: 10094962]
13. Wei Y, Guo M. *Chem. Commun.* 2009:1413–1415.
14. Hyman LM, Stephenson CJ, Dickens MG, Shimizu KD, Franz KJ. *Dalton Trans.* 2010; 39:568–576. [PubMed: 20023995]
15. Glickstein H, Ben El R, Shvartsman M, Cabantchik ZI. *Blood.* 2005; 106:3242–3250. [PubMed: 16020512]
16. Wei Y, Zhang Y, Guo M. *Chem. Commun.* 2010; 46:4472–4474.
17. Charkoudian LK, Pham DM, Franz KJ. *J. Am. Chem. Soc.* 2006; 128:12424–12425. [PubMed: 16984186]
18. Dickens MG, Franz KJ. *ChemBioChem.* 2010; 11:59–62. [PubMed: 19937900]

19. Leed MGD, Wolkow N, Pham DM, Daniel CL, Dunaief JL, Franz KJ. *J. Inorg. Biochem.* 2011; 105:1161–1172. [PubMed: 21708101]
20. Jourden JLM, Cohen SM. *Angew. Chem. Int. Ed.* 2010; 49:6795–6797.
21. Charkoudian LK, Dentchev T, Lukinova N, Wolkow N, Dunaif JL, Franz KJ. *J. Inorg. Biochem.* 2008; 102:2130–2135. [PubMed: 18835041]
22. Quin C, Robertson L, McQuaker SJ, Price NC, Brand MD, Hartley RC. *Tetrahedron.* 2010; 66:2384–2389. [PubMed: 20418941]
23. Weinstein R, Baran PS, Shabat D. *Bioconjugate Chem.* 2009; 20:1783–1791.
24. Avital-Shmilovici M, Shabat D. *Bioorg. Med. Chem.* 2010; 18:3643–3647. [PubMed: 20231097]
25. Lo L-C, Chu C-Y. *Chem. Commun.* 2003:2728–2729.
26. Jourden JLM, Daniel KB, Cohen SM. *Chem. Commun.* 2011; 47:7968–7970.
27. Springsteen G, Wang B. *Tetrahedron.* 2002; 58:5291–5300.
28. Yan J, Springsteen G, Deeter S, Wang B. *Tetrahedron.* 2004; 60:11205–11209.
29. Koh LL, Koh OL, Loh KW, Long YC, Ranford JD, Tan ALC, Tjan YY. *J. Inorg. Biochem.* 1998; 72:155–162. [PubMed: 10094614]
30. Donia AM, El-Boraey HA. *J. Anal. Appl. Pyrolysis.* 2002; 63:69–84.
31. Jang S, Imlay JA. *J. Biol. Chem.* 2007; 282:929–937. [PubMed: 17102132]
32. Cantu D, Schaack J, Patel M. *PLoS ONE.* 2009; 4:e7095. [PubMed: 19763183]

Figure 1.
Structures of FloS, FloB, FloX, FloB-SI, AcFloB-SI and AcFloX.

Figure 2.

Absorbance spectra of 20 μM FloS in 10 mM HEPES buffer (100 mM NaCl, pH = 7.0) with excess FAC. Solutions were equilibrated for 18 h prior to measurement. Inset shows that the absorbance feature centered at 380 nm plateaus after 0.5 equivalents of Fe^{3+} have been added, suggesting formation of a bis complex. The increases below 325 nm are due to excess FAC.

Figure 3.

(a) Emission spectra of 1 μM FloS in 10 mM HEPES (100 mM NaCl, pH = 7.0) equilibrated for 18 h with increasing concentrations of FAC. Inset shows that maximal fluorescence quenching is achieved by 0.5 equivalents Fe. (b) Bar graph comparing the effect of Fe^{3+} on the emission of each member of the Flo-series. Solutions of 1 μM FloS, FloX, FloB, and FloB-SI were prepared in 10 mM HEPES buffer (100 mM NaCl, pH = 7.0) and the maximum emission at 520 nm was normalized to 1 for each sample. $[\text{Fe}^{3+}(\text{NTA})]$ (3 μM) was added to each and the emission at 520 nm was recorded and normalized to the initial emission intensity. FloS is the only compound that shows significant changes with Fe^{3+} . λ_{ex} = 495 nm.

Figure 4.

Bar graph depicting the metal dependent quenching of FloS to Fe^{3+} over other metal cations. Green solid bars: Normalized fluorescence intensity at 520 nm of 1 μM solutions of FloS in 10 mM HEPES buffer (100 mM NaCl, pH = 7.00). Striped bars: Normalized fluorescence response after the addition of various metal cations. Metal stock solutions were prepared in H_2O from MnCl_2 , NiCl_2 , CoCl_2 , CuSO_4 , and ZnCl_2 and added to a final concentration of 20 μM , or 10 mM for NaCl, MgCl_2 , and CaCl_2 . White bars: Fluorescence response after the addition of 20 μM [$\text{Fe}^{3+}(\text{NTA})$] to the FloS- M^{n+} solutions. $\lambda_{\text{ex}} = 495$ nm.

Figure 5. Absorbance spectra of 20 μM FloB prepared in HEPES buffer (10 mM, pH = 7.00) with 1 mM H_2O_2 . Spectral changes were monitored for 2 h.

Figure 6.

(a) Absorbance spectra of 15 μM FloB-SI (blue line) prepared in 10 mM HEPES (100 mM NaCl, pH = 7.00). The solution was reacted with 3 mM H_2O_2 for 1 h (green line) and 1 equivalent of $[\text{Fe}^{3+}(\text{NTA})]$ was added to the solution (black dotted line). (b) Emission spectra of 1 μM FloB-SI prepared in 10 mM HEPES buffer (100 mM NaCl, pH = 7.00) before and after reaction with 3 mM H_2O_2 . After 1 h, 3 equiv of $[\text{Fe}^{3+}(\text{NTA})]$ was added to produce a 50% decrease in the fluorescence emission. $\lambda_{\text{ex}} = 495 \text{ nm}$.

Figure 7.

Chromatography traces of 200 μ M FloS, FloB-SI, and FloB-SI + 3 mM H₂O₂ prepared in 10 mM HEPES buffer (100 mM NaCl, pH = 7.00). Each trace contains two peaks due to the mixture of structural isomers produced with each compound. Mass spectra corresponding to the LC peaks confirm the identity of FloS (**1** and **1***) and FloB-SI (**2**).

Figure 8.

Fluorescence microscopy images of AcFloB-SI and AcFloX in HeLa cells. Each panel shows an independent view from the same well: (a) cells incubated with 5 μM AcFloB-SI for 30 min; (b) image taken 30 min after addition of 50 μM H_2O_2 to FloB-SI sample; (c) image taken after addition of 100 μM SIH to the FloB-SI/ H_2O_2 -treated sample; (d) cells incubated with 5 μM AcFloX for 30 min; (e) image taken 30 min after addition of with 50 μM H_2O_2 to FloX sample. The bar graph represents the average, background-corrected intensity from 5 randomly selected fields of view collected from each condition.

Scheme 1.
General Synthesis of FloX, FloS, FloB and FloB-SI

Scheme 2.

Oxidation of FloB by H_2O_2 proceeds as depicted in DMSO.