Published in final edited form as:

ACS Chem Biol. 2018 September 21; 13(9): 2595–2602. doi:10.1021/acscchembio.8b00535.

Targeting Fluorescent Sensors to Endoplasmic Reticulum Membranes Enables Detection of Peroxynitrite During Cellular Phagocytosis

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Abstract

Peroxynitrite is a highly reactive oxidant derived from superoxide and nitric oxide. In normal vertebrate physiology, some phagocytes deploy this oxidant as a cytotoxin against foreign pathogens. To provide a new approach for detection of endogenous cellular peroxynitrite, we synthesized fluorescent sensors targeted to membranes of the endoplasmic reticulum (ER). The very high surface area of these membranes, approximately 30 times greater than the cellular plasma membrane, was envisioned as a vast intracellular platform for the display of sensors to transient reactive species. By linking an ER-targeted profluorophore to reactive phenols, sensors were designed to be cleaved by peroxynitrite and release a highly fluorescent ER-associated rhodol. Studies of kinetics in aqueous buffer revealed a linear free energy relationship where electron-donating substituents accelerate this reaction. However, in living cells, the efficiency of detection of endogenous cellular peroxynitrite was directly proportional to association with ER membranes. By incorporating a 2,6-dimethylphenol to accelerate the reaction and enhance this subcellular targeting, endogenous peroxynitrite in living RAW 264.7 macrophage cells could be readily detected after addition of antibody-opsonized tetragel microspheres, without additional stimulation, a process undetectable with other known fluorescent sensors. This approach provides uniquely sensitive tools for studies of transient reactive species in living mammalian cells.

Peroxynitrite (ONOO⁻), an exceptionally strong oxidant, is an inflammatory mediator with important roles in both normal physiology and human pathology.^{1–4} In biological systems, this natural product is derived from the diffusion-limited reaction of nitric oxide free radical (*NO), produced by nitric oxide synthases (NOS), and superoxide radical anion (O₂⁻), generated by NADPH oxidases (NOX), among other pathways. Although protonated peroxynitrous acid (ONOOH, pK_a = 6.8) is highly unstable with a half-life of ~ 1 s at pH 7.4, its conjugate anion ONOO⁻ is relatively stable, and it can be stored at low temperatures. When generated by biological systems, peroxynitrite crosses cell membranes and is thought to diffuse 5–20 microns in its short lifetime.¹ During this time, this oxidant, and its secondary reactive species, can oxidize and damage a wide range of biomolecules, including

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Supporting Information. Additional methods, figures, and synthetic schemes, compound characterization data, and spectroscopic data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

proteins, lipids, and nucleic acids. This process is associated with cardiovascular disease, neurodegenerative disease, host defense, and the antitumor immune response.^{1–5}

To study peroxynitrite in living cells, fluorescence-based methods are of substantial interest. Fluorescent sensors derived from small molecules have been based on cleavable side chains derived from *p*-hydroxyphenol and *p*-hydroxyaniline,⁶ *p*-aminophenol,^{7–9} *p*-aminophenyl-trifluoromethylbutanone,^{10, 11} boronates,¹² indoles,¹³ and other functional groups such as polymethines.¹⁴ In some cases, these sensors have been targeted to intracellular mitochondria^{15–19} and lysosomes.²⁰ Reversible^{16, 21} and genetically-encoded²² fluorescent sensors have also been described. However, detection of low levels of endogenous peroxynitrite, such as those produced during phagocytosis of pathogens by macrophages, remains a challenge. Previously reported fluorescent sensors capable of detecting endogenous peroxynitrite during phagocytosis, such as fluorescein boronate,²³ require additional stimulation of cells by cytokines such as interferon-gamma (IFN- γ) and lipopolysaccharide (LPS). However, some professional phagocytes may kill pathogens²⁴ in the absence of these additional stimulants. To better understand the chemical biology of peroxynitrite, more sensitive sensors are needed.

We report here the synthesis of fluorescent sensors of peroxynitrite that accumulate in the dense tubular membranes of the endoplasmic reticulum (ER).²⁵ We hypothesized that targeting these intracellular membranes might afford highly sensitive sensors because they possess an extensive surface area that is approximately 30 times greater than the plasma membrane. In support of this concept, the ER is a direct target of endogenous peroxynitrite,²⁶ and ER stress resulting from oxidation is thought to contribute to atherosclerotic lesions. Peroxynitrite can also deplete ER-associated calcium and zinc.²⁷ We identified a sensor bearing a 2,6-dimethylphenol side chain that can selectively detect endogenous peroxynitrite in RAW 264.7 macrophage cells upon stimulation with only antibody-coated tetragel beads.

RESULTS AND DISCUSSION

Design and synthesis of fluorescent sensors.

Based on our previous studies of fluorinated rhodol fluorophores that accumulate in membranes of the ER,²⁸ we designed ER-targeted profluorophores bearing *p*-aminophenols (**1–8**) as a site of reactivity with peroxynitrite (Figure 1). These side-chains were designed to be cleaved by this oxidant to release *p*-benzoquinones and a highly fluorescent rhodol product (**9**). Due to its high hydrophobicity (cLogP (**9**) = 4.3, ChemAxon method), this rhodol could remain associated with ER membranes to enhance cellular fluorescence. We investigated phenols bearing both electron donors and acceptors and included a dichloro derivative (**8**) that is similar to a previously reported²⁹ sensor of hypochlorite. These compounds were synthesized from the triflate derivative (**10**) of Pennsylvania Green,³⁰ via Buchwald-Hartwig cross coupling, as shown in Scheme 1. Additional synthetic details are provided in the Supporting Information (Schemes S1–S3).

Photophysical properties of sensors.

Optical spectroscopic properties of sensor **3**, as a representative probe, and the rhodol product **9**, in *n*-octanol as a mimic of ER membranes, are shown in Figure 2. Consistent with prior studies of phenol-linked xanthenes,^{7–9} the fluorescence emission of **1–8** was quenched by up to 1800-fold (for **3**, $\Phi = 0.0004$) compared to **9** ($\Phi = 0.72$). However, the brightness of these sensors, calculated as the product of their measured molar extinction coefficients and quantum yields, varied by up to 30-fold (Supplementary Figure S1).

Reactivity of sensors towards oxidants in vitro.

To examine the reactivity of **1–8** with peroxynitrite in aqueous buffer, we measured kinetic half-times upon treatment with the peroxynitrite generator SIN-1 under pseudo-first-order conditions (Figure 3, panel A). Although all of these compounds reacted rapidly with this oxidant to generate a highly fluorescent product, the 2,6-dimethyl-substituted sensor **3** exhibited the fastest kinetics ($t_{1/2} = 49$ s). The differences in the magnitude of conversion of **1–8** into this fluorescent product may result from side reactions of less stable benzoquinone fragments after cleavage. However, further analysis by HPLC demonstrated that SIN-1 can cleanly convert **3** to **9** (Figure S2). Analysis of the kinetic half-times of the monosubstituted compounds by the Swain-Lupton method³¹ showed a linear free energy relationship ($R^2 = 0.95$, Figure 3, panel B), where electron donating substituents accelerate cleavage of the side-chain. Additional comparison of the reactivity of **3** with pure peroxynitrite, perchlorite, hydroxyl radical, superoxide, peroxides, and nitric oxide, by fluorescence spectroscopy, confirmed that this sensor is highly selective for peroxynitrite under biologically-relevant conditions (Figure 4, panel A). Analysis of the sensitivity of **3** in aqueous buffer revealed that a 2-fold increase in fluorescence intensity, as an approximate limit of detection, could be observed upon treatment with 40 nM of peroxynitrite (Figure 4, panel B).

Detection of peroxynitrite in living cells by flow cytometry.

To detect endogenous peroxynitrite generated during phagocytosis, we treated living RAW 264.7 macrophage cells with amino-tentagel microspheres (10-micron). As shown in Figure 5, these beads were modified with 2,4-dinitrophenyl aminohexanoic acid (DNP) as a ligand of anti-DNP antibodies (IgG). Beads were additionally modified with the coumarin-derived fluorophore Pacific Blue (PB)³² to provide a non-IgG-bound control. We hypothesized that treatment of macrophage cells with tentagel-DNP bound to anti-DNP IgG would lead to recognition of the bead-bound antibodies via Fc receptors, phagocytosis of the beads, and trigger production of cellular peroxynitrite. The proximity of ER-targeted sensors to phagosomal membranes could facilitate conversion to highly fluorescent **9**, and retention of this compound in ER membranes might also enhance sensitivity (Figure 5).

We treated living RAW 264.7 macrophages with sensors **1–8** and quantified cellular fluorescence by flow cytometry (Figure 6). To trigger phagocytosis and production of endogenous peroxynitrite, chemically-modified amino-tentagel beads were also added. These beads were either covalently derivatized with the fluorophore Pacific Blue, to provide a less immunostimulatory control, or covalently conjugated to DNP and non-covalently bound to anti-DNP IgG, to promote bead phagocytosis. To provide a spectrally orthogonal

marker, lysine residues of the antibody were conjugated to PB to allow labeled beads to be distinguished from cells by flow cytometry. As shown in Figure 6 (panel A), sensor **3** exhibited the greatest change in fluorescence (10-fold) upon addition of the immunostimulatory IgG-coated beads, as analyzed by flow cytometry. We further compared ER-targeted with the known non-ER-targeted sensors of peroxynitrite: hydroxyphenyl fluorescein (**HPF**)⁶ and fluorescein boronate (**FI-B**).²³ Because the fluorescence of **HPF** can be affected by serum proteins,³³ cells were treated with this compound in Hank's balanced salt solution (HBSS). As shown in Figure 6 (panel B), both **HPF** and **FI-B** responded to SIN-1 and could increase cellular fluorescence. However, only the ER-targeted sensor **3** could detect peroxynitrite upon mild treatment of cells with antibody-opsonized tetragel beads, demonstrating a major advantage of this approach. Additional co-treatment of cells with **3**, IgG-bound beads, and the peroxynitrite decomposition catalyst FeTMPyP decreased cellular fluorescence by over 50%, further supporting selective detection of this specific oxidant by **3**. Importantly, negligible cytotoxicity of **3** was observed at 10 μ M after 48 h in culture (Figure S5).

Imaging of living cells treated with fluorescent sensors.

The unique ability of ER-targeted sensors to detect endogenous peroxynitrite during phagocytosis was further investigated by confocal laser scanning microscopy. As shown in Figure 7, addition of antibody-opsonized beads to RAW 264.7 macrophages treated with **3** resulted in a dramatic increase in localized cytosolic fluorescence throughout the population of treated cells. This fluorescence enhancement was substantially reduced by co-treatment with the peroxynitrite decomposition catalyst FeTMPyP (Figure S6). The lack of localization of this enhanced fluorescence to specific cells undergoing phagocytosis is likely to be a consequence of rapid exchange of the rhodol **9** between cells in the population. In support of this idea, cellular fluorescence resulting from phagocytosis was only observed in unwashed cells; washing these cells once with complete media resulted in extensive loss of fluorescence within 10–20 min (Figure S6), indicating that efflux of **9** from cellular membranes readily occurs. However, secretion of cytokines by cells undergoing phagocytosis may also stimulate other cells in the population, and diffusion of peroxynitrite to adjacent cells may also contribute to this widespread distribution of cellular fluorescence.

Increased accumulation of sensors in membranes of the ER enhances efficacy of detection of peroxynitrite.

Both the weak intrinsic fluorescence of **3** alone and its highly fluorescent product **9** were confirmed to accumulate in the ER by colocalization with ER tracker blue-white DPX (Figure S3). The high hydrophobicity of these compounds, analogous to ER tracker blue-white DPX (cLogP = 4.2), drives their association with membranes of this organelle, but the molecular basis for this membrane selectivity is not well understood. Analysis of images of cells treated with **2**, **3**, and **5** demonstrated that the cytosolic (ER-associated) fluorescence compared to the nuclear fluorescence of **3** (ratio = 6.7) is substantially higher compared to **2** (ratio = 3.7) and **5** (ratio = 2.6, Figure 8, panel A). These results suggest that the substantially increased efficacy of **3** as a sensor of peroxynitrite compared with the similarly reactive sensor **2** is a consequence of the ability of **3** to more extensively and selectively

accumulate in membranes of the ER. After correcting for differences in brightness, this interpretation was further supported by analysis of cells treated with **1–8** by flow cytometry. Using this method, the concentration of **3** in cells was found to be 4-fold higher than **5** under identical conditions (Figure S4). Comparison of the highly structurally similar sensors **1** (cLogP = 5.9), **2** (cLogP = 6.4), and **3** (cLogP = 6.8), showed a linear correlation between cLogP and cellular fluorescence (Figure 8, panel B), indicating that increased hydrophobicity can provide a driving force for loading of ER membranes. However, the more highly hydrophobic di-*t*-butyl-phenol derivative **4** (cLogP = 9.2), showed lower cellular association than **2** (Figure S4), presumably due to the lower affinity of the branched alkanes of **4** for the straight-chain fatty acids of lipids of ER membranes. Despite its high hydrophobicity, **8** (cLogP = 6.9) showed the lowest cellular association (Figure S4), presumably because the partially-ionized acidic phenol of **8** (calculated pKa = 7.4, ChemAxon method) decreases interactions with ER membranes. These trends offer guidelines for the design of other ER-associated sensors.

The extensive and selective association of **3** with membranes of the ER enables this fluorescent sensor to uniquely detect transient peroxynitrite generated during phagocytosis. Because these convoluted membranes offer a vast intracellular surface area, fluorescent sensors that accumulate in these membranes have potential for highly sensitive detection of a wide variety of transient cellular species.

METHODS

Studies of kinetics of reactivity with SIN-1.

A DMSO stock solution (25 μ M) of each sensor was diluted 1:1000 with PBS to yield a 25 nM solution. This solution was vortexed to mix and 200 μ L was transferred into each of 6 wells of a black fluorescence 96-well plate (Microfluor 1 Flat-Bottom, ThermoFisher Scientific). A freshly-prepared aqueous stock solution of SIN-1 (2 μ L, 100 mM, AdipoGen) was added to 3 wells of each probe to afford a final concentration of 1 mM SIN-1. The fluorescence of the plate was analyzed immediately using a Packard Fusion Universal Microplate Analyzer (Fluorescein 485 excitation filter, Fluorescein 530 emission filter, top fluorescence, light intensity = 1, integration = 0.1 s, high intensity orbital shaking for 10 s before every reading, and 30 s intervals between readings). The efficiency of addition of SIN-1 limited the number of probes analyzed to 3–4 per run. All probes were analyzed on the same day using the same solution of SIN-1. Background-subtracted values were plotted as mean with SEM and analyzed by non-linear regression with a one-phase association model (GraphPad Prism 7) to determine half-times.

Analysis of the selectivity of sensor **3** towards peroxynitrite compared with other oxidants.

A normalized DMSO stock solution of **3** in DMSO (50 μ M) was diluted 1:1000 with PBS (pH 7.4) to yield a 50 nM solution (0.1% DMSO) in triplicate. RNS and ROS were generated as described below and diluted into the solution in volumes that changed the overall volume by 0.5% or less. These solutions were mixed, incubated at room temperature (22 $^{\circ}$ C) for 5 min, and transferred to a quartz cell for analysis by fluorescence spectroscopy. Data was plotted as mean with SD. For these studies, peroxynitrite (ONOO^-) was

synthesized by modification of the procedure of Robinson and Beckman.³⁴ Briefly, an aqueous solution of hydrogen peroxide (0.6 M, 185 μ L) in hydrochloric acid (0.7 M) was added to aqueous sodium nitrite (0.6 M, 200 μ L) at 4 °C. The mixture was made alkaline by rapid addition of aqueous NaOH (3 M, 200 μ L). This mixture was treated with freshly prepared manganese dioxide (ca. 25–50 mg) at 4 °C. After 10–15 min, the resulting suspension was filtered to yield a solution of ONOO⁻ (of up to 48 mM). The concentration of ONOO⁻ was measured by absorbance spectroscopy (ϵ = 1670 M⁻¹cm⁻¹ at 302 nm in aq. NaOH, 0.1 M). To provide stock solutions, ONOO⁻ was diluted with NaOH (aq., 0.1 M). These solutions were diluted 1:1000 into solutions of **3** for analysis. Perchlorate (ClO⁻) was obtained from commercial bleach diluted with DI water to generate stock solutions (50 μ M and 2.5 mM) that were diluted 1:1000 into solutions of **3**. The concentration of ClO⁻ was verified by absorbance spectroscopy (ϵ = 350 M⁻¹cm⁻¹ at 209 nm) in water. Hydroxyl radical ([•]OH) was generated using the Fenton reaction.¹⁵ Briefly, a aqueous stock solution of ammonium iron (III) sulfate hexahydrate (5 mM, Alfa Aesar) and an aqueous stock solution of H₂O₂ (50 mM) were prepared. Each was diluted 1:1000 into the solution of sensor to yield a final concentration of 5 μ M hydroxyl radical. For treatment with hydrogen peroxide (H₂O₂), a concentrated aqueous solution (30%, Fisher) was diluted in DI water to generate a stock solution (5 mM) that was diluted 1:1000 into a solution of **3**. The concentration of H₂O₂ was verified by absorbance spectroscopy (ϵ = 43.6 M⁻¹cm⁻¹ at 240 nm) in water. Superoxide (O₂⁻) was prepared as a saturated DMSO stock solution of potassium superoxide (1 mM, Acros). This solution was diluted 1:200 into the solution of **3** to yield 5 μ M superoxide (0.6% DMSO). *tert*-Butyl hydroperoxide (*t*-BuOOH, 70%, Alfa Aesar) was diluted in DI water to yield a 5 mM stock solution, a source of alkoxy radical (*t*-BuO[•]), that was diluted 1:1000 into the solution of **3**. Nitric oxide (NO) was prepared from a solution of sodium nitroferricyanide (III) dihydrate (5 mM, SNP, Alfa Aesar) in PBS. After incubation at room temperature for 0.5 h, this solution was diluted 1:1000 into a solution of **3**.

Determination of the limit of detection of peroxynitrite by **3**.

A normalized DMSO stock solution of sensor **3** in DMSO (50 μ M) was diluted 1:1000 with PBS (pH 7.4) to yield a 50 nM solution (0.1% DMSO). The concentration of a stock solution of pure ONOO⁻ was measured by absorbance spectroscopy and diluted with aq. NaOH (0.1 M) to provide additional stock solutions. These solutions were diluted 1:1000 into solutions of **3** for analysis in triplicate. These solutions were mixed and incubated at room temperature in the dark for 5 min before being transferred to a quartz cell for analysis by fluorescence spectroscopy. The detection limit of **3** was determined based on a reported method.^{18, 35} The fluorescence emission at 526 nm was normalized between the minimum intensity (0 nM ONOO⁻, $F_{\min}$) and the maximum intensity (500 nM ONOO⁻, $F_{\max}$) using the following equation: $(F - F_{\min}) / (F_{\max} - F_{\min})$. These values were plotted against the concentration of ONOO⁻ (50 nM–400 nM) and analyzed by linear regression (GraphPad Prism 7) to establish the limit of detection as the x-intercept.

Labeling of tentagel beads.

Tentagel M NH₂ microspheres (5 mg, 10 μ m, monosized, 0.2–0.3 mmol/g, Rapp Polymere) were allowed to swell in PBS (1 mL, 1 h) under agitation on a Titer Plate Shaker (speed 7). Based on the specified loading capacity (0.21 mmol/g), the concentration of amines in this

solution was ~ 1 mM. This solution was split into two aliquots (500 μ L each) that were each diluted to 1 mL (~ 0.5 mM amines). To one aliquot, a solution of *N*-succinimidyl *N*-(2,4-dinitrophenyl)-6-aminocaproate (DNP-X-NHS, 3 μ L, 50 mM in DMSO, Sigma Aldrich) was added. To the other, the *N*-succinimidyl ester of Pacific Blue (Pacific Blue-NHS, prepared as previously described³⁶ or commercially available from ThermoFisher Scientific) in DMSO (3 μ L, 20 mM) was added. These solutions were shaken at room temperature (1 h). These beads were pelleted by centrifugation on a personal microcentrifuge (USA Scientific Plastics, ~30 s), and the supernatant removed by pipette. The beads were washed once with EtOH (1 mL) and twice with PBS (pH 7.4, 0.3 mL). The Pacific Blue-labeled beads were diluted in PBS (final volume of 800 μ L) and stored at 4 °C until use. The DNP-labeled beads were incubated with rabbit Anti-DNP IgG (50 μ L, Vector Laboratories) additionally labeled on lysine residues with Pacific Blue-NHS (2 μ M, DOL 4–5) with shaking at room temperature for 1 h. Labeling of this antibody with Pacific Blue on lysines was performed by incubating Anti-DNP (100 μ L, 7 μ M) with Pacific Blue-NHS (0.2 μ L, 20 mM) in a Big Shot III Hybridization Oven for 0.5 h (37 °C). To purify this conjugate, Sephadex G-25 resin (Superfine, Sigma) was suspended in PBS (pH 7.4). The resulting slurry (950 μ L) was added to a minispin column (USA Scientific) and centrifuged (16,000 $\times$ g, 20 s) to remove the buffer and pack the resin. The antibody solution was loaded onto the packed resin and centrifuged (16,000 $\times$ g, 30 s) to separate the protein from unconjugated Pacific Blue. The degree of labeling (DOL) was determined by comparing the absorbance at 280 nm and 425 nm (IgG $\epsilon_{1\%}^{1\text{cm}}$ (10 mg/mL, 280 nm) = 13.7 Lg⁻¹cm⁻¹, Pacific Blue $\epsilon_{425\text{ nm}}$ = 29,500 M⁻¹cm⁻¹), measured with a Nanodrop 1000 Spectrophotometer. The beads were diluted in PBS (final volume = 800 μ L) and stored at 4 °C until needed (used within two days to maximize activity). Beads were counted by diluting 20 μ L of beads with media (180 μ L) in duplicate in a non-treated 96-well plate (USA Scientific). The beads were analyzed by flow cytometry using a Beckman Coulter Cytoflex S flow cytometer (FSC threshold = automatic, flow speed = fast, mixing time = 5 s, backflush time = 5 s, and beads were collected for 20 s). The average of these samples was used to determine to volume of beads to add to cells to trigger production of peroxynitrite.

Optical spectroscopy.

Absorbance spectra were obtained from 190–1100 nm with an Agilent 8453 diode array UV spectrophotometer in a semi-micro quartz cuvette (Sigma Aldrich). Fluorescence spectra were recorded using a Perkin Elmer LS-55 fluorescence spectrometer. Samples were excited at 488 nm and emission was recorded from 510–700 nm (scan speed of 500 nm/min and slit widths of 10 nm) in a quartz SUPRASIL Macro/Semi-micro cell.

Cell culture.

Murine RAW264.7 macrophages (ATCC, TIB-71) were cultured in T75 flasks (37 °C, 5% CO₂) in complete media comprising DMEM (Sigma Aldrich) supplemented with fetal bovine serum (FBS, 10%, HyClone), penicillin (100 units/mL, Sigma Aldrich), and streptomycin (100 μ g/mL, Sigma Aldrich).

Flow cytometry.

Cells were analyzed with a Beckman Coulter Cytoflex S (B2-R0-V2-Y2) flow cytometer. Fluorophores were excited with 405 nm and / or 488 nm diode lasers and emitted photons were collected through 450/45 nm BP (Pacific Blue), 525/40 nm BP (sensors), or 690/50 nm BP (PI) filters (FSC threshold = 500,000, flow speed = fast, mixing time = 5 s, backflush time = 5 s, and cells were collected until 10,000 cells were counted). Background fluorescence from treatment with vehicle alone was subtracted from cellular fluorescence for analysis.

Detection of peroxynitrite resulting from phagocytosis by flow cytometry.

RAW264.7 macrophages were seeded on a non-treated 96-well plate (USA Scientific) in complete media (40,000 cells, 200 μ L per well) 16 h prior to treatment. These cells adhere to treated plastic very strongly, and the use of non-treated plates was required for subsequent release and analysis of cells by flow cytometry. Fluorescent sensors were diluted from DMSO stock solutions into complete media (final concentration of probe = 10 μ M, 0.5% DMSO). Because commercial hydroxyphenyl fluorescein (**HPF**, ThermoFisher Scientific) is supplied in DMF, solutions of this probe contained 0.2% DMF instead of DMSO. Components of complete DMEM can affect the fluorescence of **HPF**, so this sensor, and **3** for direct comparison, was added to Hank's Balanced Salt Solution (HBSS, Corning, pH 7.4) instead. Labeled tentagel beads were added to this complete media or HBSS (200,000 beads/mL). The original media was carefully removed from all wells by aspiration and replaced with the treatment media containing beads and sensors (200 μ L per well) in triplicate. After incubation of treated cells at 37 $^{\circ}$ C for 4 h, aqueous propidium iodide (PI, 20 μ L, 30 μ M, Thermo Fisher Scientific) was added to each well (final concentration of PI = 3 μ M). A p200 multichannel pipette was used to release the cells from the plate via sheer force. For comparison of **3** with **HPF** and **FI-B** (prepared as previously reported),²³ treated HBSS (for **HPF**) or DMEM media (for **FI-B**) was aspirated from the wells and replaced with fresh HBSS or DMEM media containing PI (3 μ M). This reduced the high background fluorescence of **FI-B** and is consistent with previously reported use of this sensor to detect peroxynitrite.²³ Cellular fluorescence was analyzed by flow cytometry. Viability was determined by gating based on staining of cells with compromised membranes with PI. Tentagel beads take up significant amounts of PI, making them brighter than cells at 690/50 nm. Additionally, labeling of tentagel beads with Pacific Blue makes them brighter than cells when excited at 405 nm, and additional excitation at this wavelength was used to assure that all beads were excluded from gating of live cells. Median values of fluorescence of living cells in the FITC-A channel (525/40 nm) were compared and plotted with SD.

Microscopy.

An inverted Leica TCS SPE confocal laser-scanning microscope fitted with a Leica 63x oil-immersion objective was used for imaging. Fluorescent probes were excited with either a 405 nm or 488 nm solid-state laser and emitted photons were collected from 425–500 nm or 500–600 nm. DIC images were obtained with the 488 nm laser line. Unless otherwise noted, laser power and PMT gain settings were identical for all images acquired within a given experiment to allow accurate comparisons of cellular fluorescence.

Analysis of subcellular localization.

RAW264.7 macrophages were scraped to passage and diluted to 300,000 cells/mL in complete medium. These cells were plated in an ibiTreat-coated 8-well μ -Slide (ibidi, 300 μ L of media per well) and incubated at 37 °C overnight. The next day, the wells were washed once with complete media before treatment with fluorescent probes. Fluorescent sensors in DMSO stock solutions were diluted into complete media and added to cells. ER-Tracker Blue-White DPX (ThermoFisher Scientific) was diluted in DMSO to 100 μ M followed by 1:1000 dilution with complete media. Labeled tetragel beads were added to cells at 200,000 beads/mL. Cells were incubated for 4 h at 37 °C followed by imaging by confocal microscopy (without washing). Phagocytosed beads were identified by morphology such as compression of the nucleus around the bead and adjacent fluorescence of ER membranes. Addition of the rabbit anti-DNP IgG was necessary for phagocytosis, and addition of this IgG to cells without beads did not affect the fluorescence of sensors (data not shown). Antibody-opsonized beads but not Pacific Blue-modified beads were observed to be phagocytosed by microscopy. To quantify the ratio of cytosolic to nuclear fluorescence (Figure 8), Leica LAS X 2.0.1 software was used to define regions of interest (ROI) within the cytosol, nucleus, and cell-free regions. Mean fluorescence per pixel was calculated using Leica LAS X 2.0.1 software and plotted with SD (N= 20). ROI from cell-free regions were used to subtract background fluorescence for comparison.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank the NIH (R01-CA211720 and P20-GM103638) and the G. Harold and Leila Y. Mathers Charitable Foundation for financial support. Shared instrumentation was funded by the NIH (P50 GM069663, S10 OD016360, P20 GM103418, S10RR024664). KK thanks the ACS for a MEDI predoctoral fellowship and was supported in part by a NIH Pharmaceutical Aspects of Biotechnology Training Grant (T32-GM008359). We thank Prof. S. Lunte for the RAW 264.7 cell line.

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Figure 1.
 Structures of ER-targeted fluorescent sensors and products of oxidative cleavage by peroxy-nitrite.

Optical properties of sensor **3** and product **9** in *n*-octanol:
Abs. λ_{max} (**3**) = 526 nm; Em. λ_{max} (**3**) = 538 nm; $\Phi = 0.0004$
Abs. λ_{max} (**9**) = 515 nm; Em. λ_{max} (**9**) = 536 nm; $\Phi = 0.72$

Figure 2.
Photophysical properties of sensor **3** and the product of oxidative cleavage of the phenol side-chain (**9**) in *n*-octanol.

Figure 3.

(A) Profiles of reaction of **1–8** (25 nM) with the peroxynitrite generator SIN-1 (1 mM) in phosphate-buffered saline (PBS, pH 7.4, 0.1% DMSO). Pseudo-first-order half-times ($t_{1/2}$, calculated after subtraction of background in the absence of SIN-1) are shown. Dotted lines show fits to a one-phase association model. (B) Analysis of the kinetic half-times of the monosubstituted sensors shown in panel A by the Swain-Lupton method. Electron donating substituents accelerate cleavage of the side-chain.

(A) Selectivity of reaction of **3** (50 nM) with ROS and RNS (B) Limit of detection of ONOO⁻ by **3** (50 nM) in PBS **Figure 4.**

Analysis of the selectivity (A) and sensitivity (B) of reaction of **3** (50 nM) with pure peroxynitrite (A–B) and other oxidants (A). Maximal fluorescence emission was measured by fluorescence spectroscopy after treatment for 5 min at 23 °C (PBS, pH 7.4, 0.1% DMSO).

Figure 5.

Approach for detection of endogenous peroxynitrite in macrophage cells. Receptor-mediated phagocytosis of antibody-bound tentagel beads triggers production of reactive nitrogen species that can be detected by sensors localized in membranes of the endoplasmic reticulum.

Figure 6.

(A–B) Analysis of fluorescence of living RAW 264.7 macrophages by flow cytometry. Cells were treated (4 h) with sensors **1–8** (10 μ M) and 10-micron amino-tentagel beads modified either with Pacific Blue-SE (Beads-PB) or 2,4-DNP-X-SE. To the DNP-modified beads was additionally added rabbit anti-DNP IgG (Beads/IgG), conjugated to Pacific Blue via lysines, to stimulate phagocytosis. In panel B, cells were treated with **3** in DMEM media or HBSS and compared with treatment with hydroxyphenyl fluorescein (**HPF**, 10 μ M in HBSS) and fluorescein boronate (**FI-B**, 50 μ M in DMEM). [SIN-1] = 50 μ M. [FeTMPyP] = 50 μ M.

(A) Treatment of living RAW 264.7 macrophages with **3** (10 μ M)

(B) Treatment of living RAW 264.7 macrophages with **3** (10 μ M) followed by tentagel beads/IgG

Figure 7.

(A–B) DIC (left panels) and confocal laser scanning micrographs (right panels, Ex. 488 nm, Em. 500–600 nm) of living RAW macrophages treated with **3** (4 h). (B) Cells were additionally treated with DNP-modified amino tentagel beads (10 micron) bound to anti-DNP IgG. Cellular green fluorescence is localized to the endoplasmic reticulum. White arrows point at tentagel beads. The bead at the bottom has been phagocytosed, as evidenced by compression of the proximal cell nucleus. Scale bar = 25 μ m.

(A) Image analysis of the subcellular localization of the most reactive sensors (**2**, **3**, and **5**)

(B) Highly structurally similar sensors (**1–3**) show a linear relationship between hydrophobicity and accumulation in membranes of the ER

Figure 8.

(A) Analysis of differences in the subcellular localization of **2**, **3**, and **5** (10 μ M, 4 h) in living RAW 264.7 macrophages by confocal microscopy. Sensor **3** shows the greatest selectivity for localization in the ER as evidenced by the highest ratio of cytosolic to nuclear fluorescence. (B) Linear regression of cellular fluorescence (measured by flow cytometry) / sensor brightness plotted against cLogP for the highly structurally similar sensors **1–3** (10 μ M, 4 h). In this series of compounds, increased hydrophobicity drives accumulation in ER membranes.

Scheme 1.

Synthesis of sensors (**1-8**). R and R' are defined in Figure 1. R''=MOM (for **1-3** and **5-8**) or Boc (for **4**).