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Genetically Encoded Fluorescent Probe for the Selective Detection of Peroxynitrite

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Supporting Information

ABSTRACT: Peroxynitrite is a highly reactive molecule involved in cell signaling and pathological processes. We hereby report a novel genetically encoded probe, pnGFP, which can selectively sense peroxynitrite. A boronic acid moiety was site-specifically introduced into circularly permuted fluorescent proteins. By examining different protein templates followed with site-targeted random mutagenesis, we identified a selective peroxynitrite sensor, which is essentially unresponsive to other common cellular redox signaling molecules. The new probe has been genetically introduced into mammalian cells to image peroxynitrite at physiologically relevant concentrations.

Cellular redox-active molecules play important roles in redox homeostasis and signaling.[1,2] Peroxynitrite ($ONOO^-$) is a redox signaling molecule, which can form in vivo from a diffusion-controlled reaction between $O_2^{\bullet-}$ and $NO^\bullet$ ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$).[3−5] At physiological pH, $ONOO^-$ equilibrates with ONOOH ($pK_a = 6.8$).[3] $ONOO^-$ is a potent oxidant. Early studies focused on its oxidative damage to biomolecules and the resulting cytotoxicity.[6,7] Recently, $ONOO^-$ has also been indicated to modulate cell signal transduction.[8,9] For example, reactive cysteine residues in proteins can be oxidized by $ONOO^-$ and may be further derivatized by other intracellular modifiers, leading to deactivation or activation of the proteins.[10,11] On the other hand, $ONOO^-$ is also an efficient nitration reagent. For example, tyrosine residues can react with $ONOO^-$ to form nitrotyrosine.[12] Proteins may be nitrated for the purposes of signal transduction and immunogenic response.[13,14] Similar to oxidation, misregulated nitration is detrimental and has been linked to Alzheimer's disease, arthritis, cancer, autoimmune and inflammatory diseases, and other disorders.[4,9,12] In this context, it has been of great interest to investigate the dynamics of $ONOO^-$ in cells and how it is regulated to achieve biological functions. Currently, however, there are limited research tools for studying $ONOO^-$.

Due to its very short lifetime (~10 ms) under physiological conditions,[3] it is not possible to directly measure $ONOO^-$ in processed cell or tissue samples. Fluorescent probes can be introduced into cells, and thus represent a promising strategy for detecting this highly reactive molecule. Reduced non-fluorescent leuco dyes such as dihydrorhodamine are frequently used, but, in general, these molecules have little selectivity among various reactive oxidative species (ROS).[15,16] Recent research has generated probes based on other chemical transformations, affording fluorescent dyes for $ONOO^-$ with various degrees of selectivity and sensitivity.[17−23] Prominent examples are a group of boronate organic dyes, which can respond to $ONOO^-$ and hydrogen peroxide (H_2O_2).[24−26] Effort has been invested in the modification of those boronate-derived probes for subcellular localization in mammalian mitochondria and nuclei.[27,28] In addition, an elegant method has been developed to conjugate synthetic boronate probes with proteins of interest through a peptide tag for precise intracellular direction.[29] Despite the progress, no genetically encoded probe is available for direct imaging of intracellular $ONOO^-$. Moreover, it remains a great challenge to distinguish $ONOO^-$ from other ROS (e.g., H_2O_2 and ClO^-)[20,26] and selectively detect $ONOO^-$ in the presence of reducing molecules such as hydrogen sulfide (H_2S) and glutathione (GSH).[22] Herein we present a novel genetically encoded probe for $ONOO^-$ based on a boronic acid-derived circularly permuted green fluorescent protein (cpGFP). A special protein scaffold was identified and further engineered to achieve excellent chemoselectivity and sensitivity, so that $ONOO^-$ at physiological concentrations could be selectively imaged in the presence of a variety of common cell-generated redox-active molecules.

We previously inserted an unnatural amino acid, *p*-azidophenylalanine (*p*AzF), into a cpGFP to create an encoded sensor for H_2S.[30] Along this line, we introduced *p*-boronophenylalanine (*p*BoF) into the same cpGFP by expanding the genetic code of *E. coli*.[31] It is worth noting that Wang et al. also reported the insertion of *p*BoF into GFP in *E. coli* and the response of their protein to H_2O_2.[32] In our previous study, we found that the use of circularly permuted fluorescent proteins could greatly enhance the reactivity of the sensor.[30] So we prepared cpGFP-Tyr66*p*BoF (Supporting Information, Figure S1) from *E. coli*. The yield was quite low, indicating that *p*BoF may negatively impact the folding and solubility of cpGFP. To solve the problem, we performed three cycles of error-prone polymerase chain reaction (EP-PCR)-based direct evolution to improve cpGFP. The enhanced mutant was named cpGFP2, and it showed brighter fluorescence and faster maturation at 37 °C. At the same time, we also directly converted a superfolder GFP, whose folding has been extensively optimized previously,[33] to a circularly permuted topology (designated cpsGFP). Both cpGFP2-Tyr66*p*BoF and cpsGFP-Tyr66*p*BoF were initially nonfluorescent but turned highly fluorescent after reaction with

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H_2O_2 or $ONOO^-$ (Figure S2). The response induced with H_2O_2 is much slower than with $ONOO^-$, but sufficiently dramatic to complicate the selective detection of $ONOO^-$. This observation also corroborates the previous findings that boronate-containing small molecules react with $ONOO^-$ nearly a million times faster than with H_2O_2.[24] To better distinguish $ONOO^-$ from H_2O_2, a site-directed random mutagenesis strategy was undertaken. We fully randomized each of the residues extending from the new N- and C-termini of cpGFP2 and cpsGFP, and the codons for the chromophore tyrosines were remained as TAG (amber codon). The gene libraries were introduced into *E. coli* cells containing an amber suppression plasmid, pEvol-*p*BoF.[34] Bacterial colonies were randomly picked and grown in the media supplemented with *p*BoF. Crude proteins were extracted and tested for $ONOO^-$- or H_2O_2-induced fluorescence changes. After screening a few hundred colonies, we were able to identify a mutant (designated pnGFP) derived from cpsGFP, which responded to $ONOO^-$ (20 μM) but not to H_2O_2 at a 50-fold higher concentration (1 mM).

To further verify the selectivity, we purified the protein and tested its fluorescence changes against a large panel of redox signaling molecules (Figure 1a). Notably, except for $ONOO^-$,

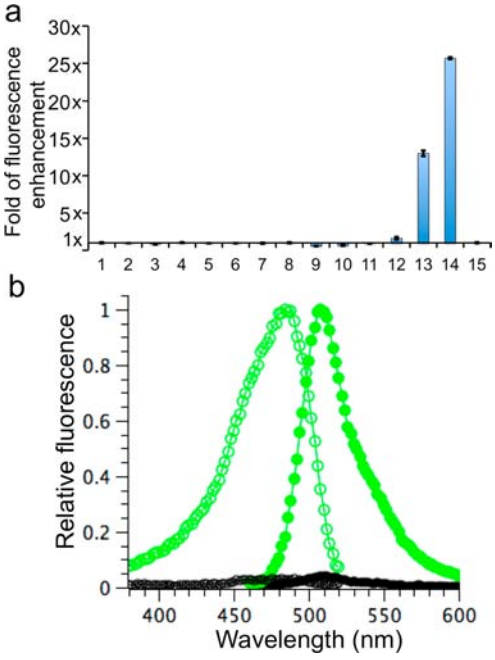

Figure 1. (a) Fluorescence response of pnGFP (0.5 μM) after 20-min incubation with various redox-active chemicals: **1**, 100 μM H_2S; **2**, 1 mM dithiothreitol; **3**,**4**, 5 mM cysteine or glutathione; **5**−**8**, 100 μM HOCl/ClO^-, HOO*t*Bu, $O_2^{\bullet-}$, or NOC-7 ($NO^\bullet$ donor); **9**, $^\bullet$OH (1 mM Fe^{2+} + 100 μM H_2O_2); **10**, $^\bullet$O*t*Bu (1 mM Fe^{2+} + 100 μM HOO*t*Bu); **11**,**12**, 100 μM or 1 mM H_2O_2; **13**,**14**, 20 or 100 μM $ONOO^-$; **15**, PBS. (b) Fluorescence excitation (left) and emission spectra (right) of pnGFP before (black) and after (green) reaction with $ONOO^-$.

all other tested reducing or oxidizing reagents at physiologically relevant (or higher) concentrations triggered no or very limited fluorescence response. Interestingly, glucose and glycerol, which contain boronic acid-interacting *cis*-diols, showed no effect on the sensor's response to $ONOO^-$ (Figure S3). Although the mechanism for the selectivity is not yet elucidated, the N- and C-terminal residues (N−1 and C+1 in Figure S1) of pnGFP were found to be Thr. Presumably, the two Thr residues may interact with the boronic acid group of the chromophore to tune its chemoreactivity (Figure S4). We plan to use X-ray crystallography to further investigate the mechanism.

Next, we examined the spectral changes of pnGFP before and after reaction with $ONOO^-$. Its absorption band shifted from 375 to 480 nm (Figure S5a). Initially, the protein was barely fluorescent, but the product emitted strong fluorescence with excitation maximum at 484 nm and emission maximum at 508 nm (Figure 1b). The spectral profiles are similar to those of pnGFP containing a natural tyrosine-derived chromophore (Figure S5b). These results are aligned with our mass spectrometry (MS) characterization (Figure S6) and previous studies of small boronate molecules,[35] supporting that the main oxidation product induced by $ONOO^-$ has a tyrosine-derived chromophore.

We also investigated the fluorescence response of pnGFP to different concentrations of $ONOO^-$ (Figure S7). The limit of detection (LOD) was determined to be a bolus addition of 553 nM $ONOO^-$ under our experimental conditions. Because $ONOO^-$ itself has a short lifetime in neutral aqueous solution,[3] a slow $ONOO^-$-releasing molecule, 3-morpholinosyndnomine (SIN-1),[36] was utilized to follow fluorescence changes over time (Figure S8). Fluorescence intensity was measured after incubating pnGFP protein with SIN-1 for 1 h. A good linear relationship between the fluorescence intensity and the SIN-1 concentration from 1 to 100 μM was observed (Figure 2), and

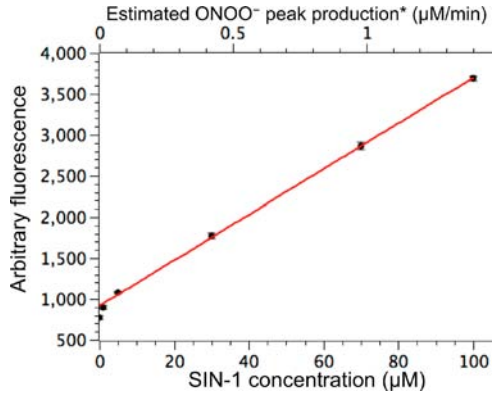

Figure 2. Fluorescence response of pnGFP to SIN-1. The mixtures were incubated at room temperature for 1 h before fluorescence measurement. Linear regression (R^2 = 0.9995) was applied to the data set (1−100 μM SIN-1) to derive the LOD. 1.4% of the SIN concentration was used to estimate the peak production rate of $ONOO^-$.

the LOD was calculated to be 3.38 μM SIN-1. The maximal $ONOO^-$ production rate from SIN-1 was reported to be ~1.4% of the SIN-1 concentration,[36] so the LOD is equivalent to an influx of $ONOO^-$ at the speed of several tens of nM/min. Furthermore, we utilized a previously established method[36] to generate continuous and stable influxes of $ONOO^-$ (0.1−1 μM/min). Gradual but drastic fluorescence enhancement was observed at all conditions (Figure S8c). The steady-state concentration of $ONOO^-$ in cells is estimated in the nanomolar to low micromolar range,[37] and the basal production rate has been estimated to be 0.1−1 μM/min,[3,38] and up to 50−100 μM/min under certain conditions or in confined microdomains (e.g., inflammatory macrophage or cell

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phagosome).[3,4] In biological systems, the production of $NO^{\bullet}$ and $O_2^{\bullet-}$ may result in continuous formation of $ONOO^-$ up to several hours.[3,4] Taken together, our data collectively support that the reaction between pnGFP and $ONOO^-$ is efficient to compete with other $ONOO^-$-consuming pathways, and pnGFP is a promising probe for the detection of biological $ONOO^-$.

To express pnGFP in mammalian cells, it is necessary to identify an orthogonal aminoacyl tRNA and synthetase (tRNA/aaRS) pair for the genetic encoding of *p*BoF in mammalian cells. Previous research only established a *p*BoF-specific tRNA/aaRS pair for *E. coli*.[39] The pair was derived from the archaebacterial *M. jannaschii* tyrosyl tRNA and synthetase, and cannot be used in mammalian cells. We employed a mammalian fluorescent assay to screen existing tRNA/aaRS pairs orthogonal in mammalian cells. These pairs were initially developed for the mammalian encoding of tyrosine analogues such as *p*-azidophenylalanine, *p*-iodophenylalanine, and *p*-methoxyphenylalanine.[40] We constructed a series of mammalian suppression plasmids expressing one synthetase copy and four copies of the corresponding tRNA suppressor.[30,41] These plasmids were used to co-transfect human embryonic kidney (HEK) 293T cells along with another reporter plasmid encoding an EGFP gene with a Tyr39TAG mutation.[42] We found that a tRNA/aaRS pair engineered from the *E. coli* tyrosyl tRNA and synthetase for the genetic encoding of *p*-iodophenylalanine,[43] in the presence of *p*BoF, was able to suppress the TAG codon to synthesize the full-length EGFP protein (Figure S9a). The His-tagged EGFP protein was purified using Ni-NTA agarose beads and subjected to MS characterization, and the observed protein molar mass correlated well with the calculated molar mass of the *p*BoF-containing protein (Figure S9b). The suppression plasmid was named pMAH-POLY (synthetase mutations from *E. coli* tyrosyl synthetase: Y37I/D182S/F183M/D265R) and utilized in our following studies to express pnGFP in mammalian cells.

To validate the use of pnGFP in mammalian cells, we constructed a pcDNA3-pnGFP plasmid containing the pnGFP reporter gene under the control of an enhanced human cytomegalovirus (CMV) promoter. A woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was inserted downstream to the pnGFP reading frame to boost the expression. Both pcDNA3-pnGFP and pMAH-POLY were introduced into HEK 293T cells, which were cultured in media supplemented with *p*BoF. After pnGFP was expressed, the cells were switched into fresh media and treated with SIN-1 (60 μM). The cells were imaged at 90 min after treatment, and prominent fluorescence enhancement was observed (Figure 3). In parallel, cells in the control groups were treated with DMSO, H_2O_2 (1 mM), or ClO^- (100 μM), and no fluorescence increase was detected. These data further support the notion that pnGFP is a selective probe for intracellular $ONOO^-$.

In summary, we have engineered a novel fluorescent probe for $ONOO^-$. The probe combines excellent selectivity and sensitivity, and can detect $ONOO^-$ at biologically relevant concentrations with minimal interference from other cell-generated redox-active molecules. In addition, by exploring the polyspecific properties of the existing orthogonal tRNA/aaRS pairs, we report here, for the first time, the genetic encoding of the unnatural amino acid *p*BoF in mammalian cells. The first genetically encoded $ONOO^-$ probe is expected to enable new studies about the dynamics of $ONOO^-$, leading to a positive impact in a broad area to accelerate our understanding of redox chemistry and biology.

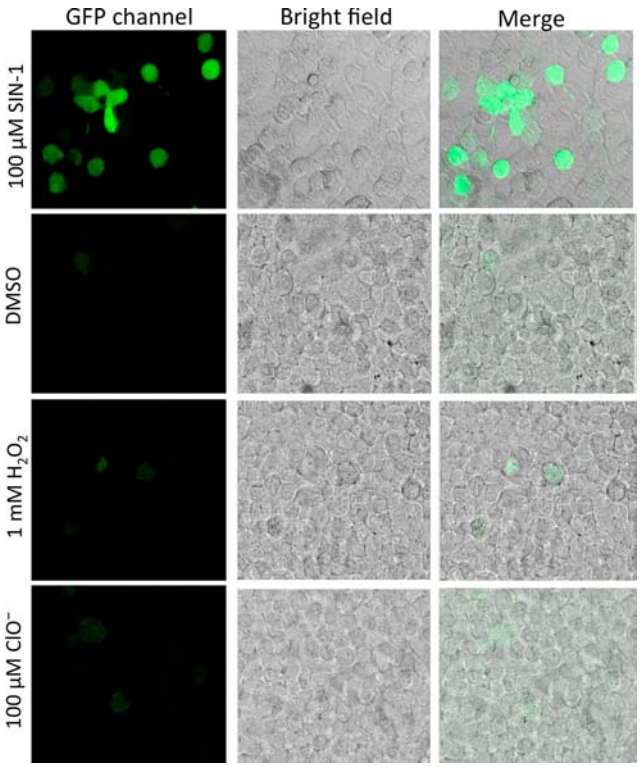

Figure 3. Live-cell imaging of HEK 293T cells transiently transfected to express pnGFP. Cells were treated with the indicated chemicals before imaging.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General methods, protein sequencing alignment, spectroscopic properties, mass spectrometry, determination of detection limits, and confirmation of mammalian encoding of *p*BoF. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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