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Highly Water-Soluble BODIPY-Based Fluorescent Probe for Sensitive and Selective Detection of Nitric Oxide in Living Cells

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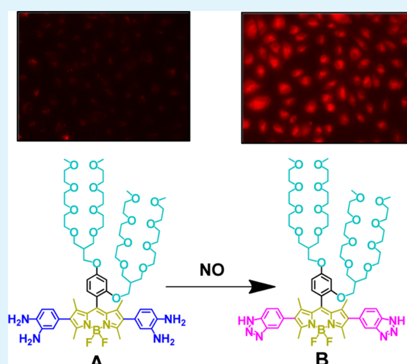
Supporting Information

ABSTRACT: A highly water-soluble BODIPY dye bearing electron-rich *o*-diaminophenyl groups at 2,6-positions was prepared as a highly sensitive and selective fluorescent probe for detection of nitric oxide (NO) in living cells. The fluorescent probe displays an extremely weak fluorescence with fluorescence quantum yield of 0.001 in 10 mM phosphate buffer (pH 7.0) in the absence of NO as two electron-rich *o*-diaminophenyl groups at 2,6-positions significantly quench the fluorescence of the BODIPY dye via photoinduced electron transfer mechanism. The presence of NO in cells enhances the dye fluorescence dramatically. The fluorescent probe demonstrates excellent water solubility, membrane permeability, and compatibility with living cells for sensitive detection of NO.



KEYWORDS: *BODIPY dyes, fluorescent probe, nitric oxide, cell imaging*

1. INTRODUCTION

Nitric oxide (NO) is a highly reactive free-radical gas and a ubiquitous signaling molecule that is responsible for both physiologically positive as well as pathogenic states in many biological systems.[1−7] NO has been shown to have antiapoptotic effects in endothelial cells, lymphoma cells, ovarian follicles, cardiac myocytes, and hepatocytes.[2] In apparent contradiction to this, NO has been shown to possess proapoptotic properties in macrophages, neurons, pancreatic β-cells, thymocytes chondrocytes, and hepatocytes.[3] NO has been shown to have beneficial antiviral, antimicrobial, antiparisital, immunomodulary, and antitumoral effects when induced at the right time and place. Reviews suggest NO in low levels has a protective and proliferative effect on some cells while at high levels induces cell cycle arrest, apoptosis, and senescence.[1,6,7] The pathways that regulate nitric oxide synthase (NOS), the enzyme responsible for endogenous production of NO, differ between cells and species.[8] The regulation of NO production can occur at the transcriptional, post-transcriptional, translational, and post-translational levels. The observed effects of NO are dependent on a number of factors including flux of NO, the timing of NO release, the accumulated dose of NO, and the type of cells exposed to the NO. Clearly defining how much NO is needed and for what duration it is produced to induce the array of activity it has on biological systems is essential to enabling better understanding of the role NO plays in various pathways.

Accurate detection and quantification of NO will provide effective approaches for better understanding of its origins, activities, and biological functions. A variety of methods such as electron paramagnetic resonance spectroscopy,[9] colorimetric,[10] fluorometric,[11−29] electrochemical,[30,31] and chemiluminescence techniques[31] have been developed to detect and monitor endogenous NO for insightful understanding of the complicated functions of NO in living systems. Among these methods, metal-based and *o*-diaminophenyl-based fluorescent probes have been shown very promising to visualize NO *in vitro* and *in vivo* at the cellular level under physiological conditions because of its high sensitivity, selectivity, real-time operation, and simplicity.[11−26] The response mechanism is based on the irreversible reaction of the *o*-diaminophenyl group of the fluorescent probe with byproducts such as the autoxidation of NO to turn on the probe fluorescence via formation of a strongly fluorescent benzotriazole derivative.[11−25] However, some of these fluorescent probes show different undesirable properties such as poor compatibility with living cells, low water solubility, or membrane permeability.

In this paper, we chose BODIPY (4,4′-difluoro-4-bora-3a,4a-diaza-s-indacene) dye as a fluorophore because of its many distinctive and desirable properties such as high absorption coefficients, narrow absorption bands, sharp emissions, high fluorescence quantum yields, and excellent chemical and photostabilities.[32−35] We report highly water-soluble BODIPY-based dye bearing electron-rich *o*-diaminophenyl groups at 2,6-positions as a sensitive and selective fluorescent

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probe for NO by introducing branched oligo(ethylene glycol)methyl ether residues to BODIPY dye at the meso-position and two electron-rich *o*-diaminophenyl groups at 2,6-positions as fluorescent sensing switches. The fluorescent probe was prepared by palladium-catalyzed Suzuki coupling reaction of 4-amino-3-nitrophenylboronic acid pinacol ester with 2,6-diiodo-BODIPY dye in a basic condition, followed by reduction of nitro moieties of 3-amino-4-nitrophenyl groups to amine groups. The fluorescent probe displays sensitive response to NO as the presence of NO significantly enhances the fluorescence intensity of the fluorescent probe by reducing effectiveness of photoinduced electron transfer of electron-rich *o*-diaminophenyl groups at 2,6-positions via irreversible oxidation of the *o*-diaminophenyl groups. The fluorescent probe possesses excellent water solubility, membrane permeability, and compatibility with living cells for sensitive detection of NO in living cells.

2. EXPERIMENTAL SECTION

2.1. Materials. Fluorescent probe **A**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.00 (d, J = 8.4 Hz, 1H), 6.66 (d, J = 8 Hz, 2H), 6.56 (dd, J = 8.4, 2.4 Hz, 1H), 6.51 (d, J = 2 Hz, 1H), 6.48 (d, J = 2 Hz, 2H), 6.45 (dd, J = 8, 1.6 Hz, 2H), 4.02 (d, J = 6 Hz, 2H), 3.95 (d, J = 5.6 Hz, 2H), 3.61−3.43 (m, 50H), 3.38−3.24 (m, 18H) 2.48 (s, 6H), 2.42−2.34 (m, 1H), 2.32−2.22(m, 1H), 1.39(s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.49, 156.80, 153.57, 139.06, 138.37, 134.81, 133.84, 133.54, 131.96, 129.98, 125.74, 122.17, 118.58, 117.21, 116.59, 106.25, 100.38, 72.12, 70.83, 70.82, 70.80, 70.77, 70.73, 70.68, 70.66, 70.50, 69.57, 69.30, 66.60, 66.27, 59.21, 59.17, 40.20, 39.74, 13.58, 12.59. IR (cm^{-1}): 3355, 2870, 1609, 1533, 1463, 1388, 1287, 1203, 1177, 1096, 1004, 849, 767, 707. HRMS (FAB) calcd for $C_{67}H_{103}N_6F_2BO_{18}$ $[M]^+$, 1328.7390; found, 1328.7407.

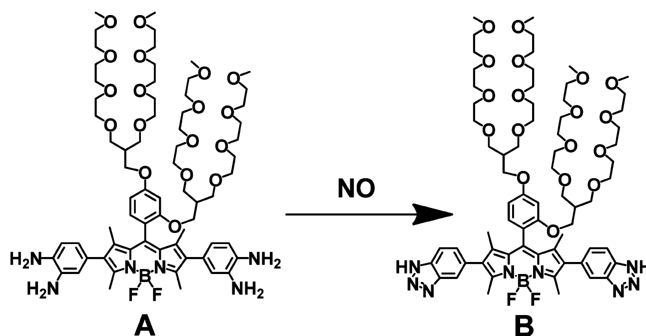
2.2. NOC-7 Treatment and Fluorescence Imaging. Murine macrophages (RAW 264.7) or human retinal pigment epithelial cells (ARPE-19) were washed twice with phosphate buffered saline (PBS) to remove the traces of serum and incubated with 5 μM fluorescent probe **A** in FBS (fetal bovine serum)-free DMEM (Dulbecco's modified Eagle medium) for 4 h at 37 °C. After being repeatedly washed with PBS, cells were treated with 4 μM of NO donor NOC-7 (Santa Cruz Biotechnology Inc., CA) in serum free DMEM for 1−2 h. Untreated cells with NOC-7 are considered as control. Differential interference contrast (DIC) and fluorescence images were acquired using a Zeiss AxioVert 200 M Apo Tome fluorescent microscope with 20× magnification, with an exposure time of 500 ms for murine macrophages and 1 s for human retinal pigment epithelial cells. Emission from the fluorescent probe **A** was detected with the red filter set (with excitation at 540 nm).

2.3. LPS Treatment and Fluorescence Imaging. For studies of endogenously generated NO detection, lipopolysaccharide (LPS) was used to stimulate NO production through iNOS (inducible nitric oxide synthase) for 16 h. After murine macrophage cells were washed with PBS 3 times, 100 ng/mL LPS in FBS-free media was applied to cells. After 16 h incubation with LPS, the cells were washed by PBS for 3 times and 10 μM fluorescent probe **A** dissolved in FBS-free media was applied to cells. A 10 μM portion of 4,5-diaminofluorescein diacetate (DFA) was used as a control for comparison purpose. After 1 h of incubation, cells were washed by PBS 3 times, and the living cell was mounted onto Zeiss AxioVert 200 M inverted microscope for imaging. Images were obtained at an excitation of 500 nm.

3. RESULTS AND DISCUSSION

3.1. Synthetic Approach to Fluorescent Probe for NO. In order to prepare highly water-soluble BODIPY-based fluorescent probe for NO, we introduced branched oligo(ethylene glycol)methyl ether residues to BODIPY dye at the meso positions and electron-rich *o*-diaminophenyl groups at 2,6-positions as fluorescent switches to BODIPY dye (Scheme 2). We demonstrated effective use of branched oligo(ethylene glycol)methyl ether residues to achieve high water solubility of

Scheme 1. BODIPY-Based Fluorescent Probe for NO



BODIPY dyes with emissions ranging from green to near-infrared regions by introducing these residues to BODIPY dyes at all positions such as the meso, 2,6-positions, 3-position, 3,5-positions, 1,7-positions, 1,3,5,7-positions, or 4,4′-positions.[36,37] In order to control effective turn-on switch for sensitive detection of NO, we used an ortho substituent group of branched oligo(ethylene glycol)methyl ether on the meso phenyl ring of BODIPY dye as this approach has been reported to effectively enhance fluorescence quantum yield of BODIPY dye in aqueous solution by preventing dye aggregation resulting from $\pi-\pi$ stacking interactions among BODIPY dyes and free rotation of the meso phenyl ring of BODIPY dye.[37] BODIPY dye bearing branched oligo(ethylene glycol)methyl ether at the meso position (**4**) was prepared according to our reported procedure, starting from 2,4-dihydroxybenzaldehyde (**1**) (Scheme 2).[37] 2,6-Diiodo-BODIPY dye (**5**) was prepared by iodonization of BODIPY dye **4** at 2,6-positions in the presence of iodine and iodic acid in mixed solution of ethanol and water.[37−39] BODIPY dye bearing 4-amino-3-nitrophenyl groups at 2,6-positions (**8**) was prepared by palladium-catalyzed Suzuki coupling reaction of 4-amino-3-nitrophenylboronic acid pinacol ester (**7**) with 2,6-diiodo-BODIPY dye (**5**) in a basic condition under a nitrogen atmosphere (Scheme 2).[39] BODIPY dye bearing electron-rich *o*-diaminophenyl groups at 2,6-positions (fluorescent probe **A**) was prepared by reduction of nitro moieties from 4-amino-3-nitrophenyl groups at 2,6-positions of BODIPY dye (**8**) to amine groups in the presence of hydrazine hydrate and palladium on carbon in ethanol solution.

3.2. Optical Properties of BODIPY Dyes. The absorbance and fluorescence properties and fluorescence lifetimes of BODIPY dyes **4**, **5**, **8**, and fluorescent probe **A** are summarized in Table 1. All BODIPY dyes **4**, **5**, and **A** except **8** are highly soluble not only in water but also in common organic solvents such as tetrahydrofuran, dichloromethane, chloroform, DMSO, DMF, and ethyl acetate. BODIPY dye **4** in PBS solution shows a strong absorption peak at 501 nm due to $S_0 \rightarrow S_1$ ($\pi-\pi^*$) transition and a weaker broad band around 350 nm attributed to the $S_0 \rightarrow S_2$ ($\pi-\pi^*$) transition[32,34,35] and displays a fluorescence peak at 511 nm with high fluorescence quantum yield of 0.32 (Table 1). The high fluorescence quantum yield is attributed to enhanced steric hindrance of the bulky ortho substituent group on the meso phenyl ring of BODIPY dye **4** that can effectively prevent free rotation of the meso phenyl ring and potential $\pi-\pi$ stacking interactions between the BODIPY cores in aqueous solution.[37] 2,6-Diiodo-BODIPY dye (**5**) displays large red shifts (34 and 42 nm) in both the absorption and fluorescence maxima, respectively, compared with BODIPY dye **4** as 2,6-diiodo substituents serve as auxochromes.[39] However, it exhibits low fluorescence quantum yield of 0.009 with short fluorescence lifetime of 0.026 ns in PBS solution because of efficient intersystem crossing

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Scheme 2. Synthetic Route to BODIPY-Based Fluorescent Probe A for NO

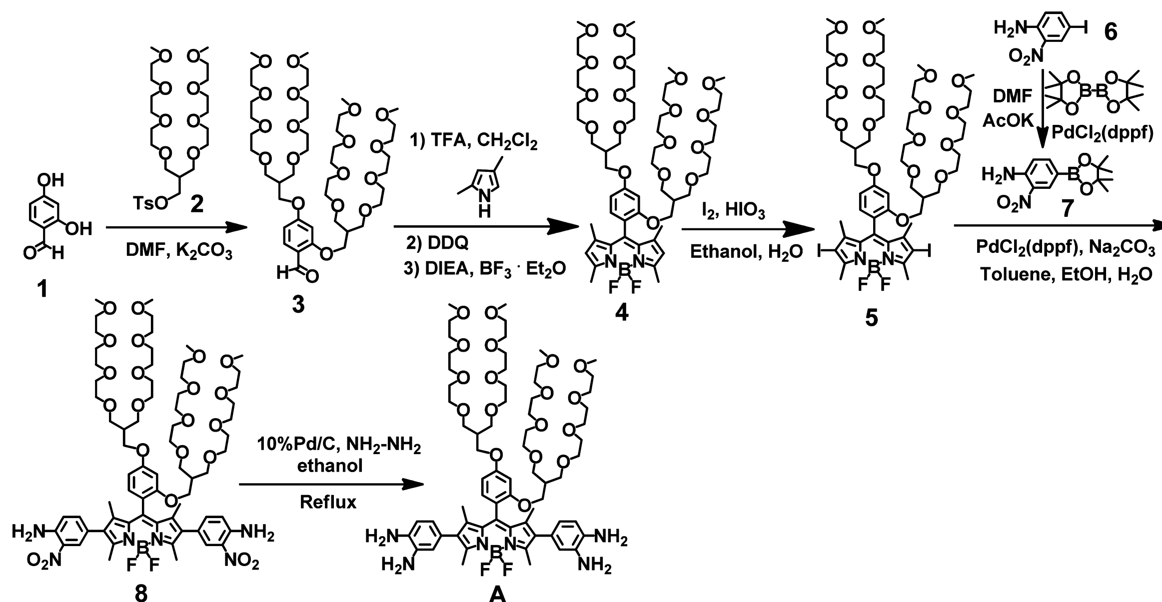

Table 1. Optical Properties, Fluorescence Lifetimes, and Fluorescence Quantum Yields of BODIPY Dyes 4, 5, and A in 10 mM Phosphate Buffer Solution (PBS) (pH 7.0), Acetonitrile, and DMSO and BODIPY Dye 8 in Acetonitrile and DMSO

BODIPY dye	solvent	absorption peak (nm)	emission peak (nm)	fluorescence quantum yield	molar absorption coefficient ($\times 10^4$ M^{-1} cm^{-1})	fluorescence lifetime (ns)
4	PBS	*501*	*511*	*0.32*	*7.54*	2.8
	acetonitrile	501	511	0.74	9.54	5.5
	DMSO	503	514	0.70	8.01	4.9
5	PBS	*535*	*553*	*0.009*	*3.18*	0.026
	acetonitrile	535	553	0.05	5.67	0.076
	DMSO	537	555	0.015	4.48	0.019
8	acetonitrile	*534*	*592*	*0.084*	*6.60*	1.1
	DMSO	538	595	0.052	3.89	0.50
A	PBS	*539*	*570*	*0.001*	*3.52*	2.9
	acetonitrile	539	570	0.006	5.96	4.1
	DMSO	542	574	0.003	4.58	3.7

induced by the heavy atom effect of iodine.[4] BODIPY dye bearing 4-amino-3-nitrophenyl groups at 2,6-positions (**8**) displays poor water solubility in aqueous solution because a hydrophobic feature of 4-amino-3-nitrophenyl groups at 2,6-positions makes the BODIPY core more hydrophobic. It shows absorption and emission peaks at 534 and 592 nm with fluorescence quantum yield of 0.084 in acetonitrile solution. Fluorescent probe **A** displays absorption and emission peaks at 539 and 570 nm, respectively, and shows very weak fluorescence with fluorescence quantum yield of 0.001 in phosphate buffer (pH 7.0) because two electron-rich *o*-diaminophenyl groups at 2,6-positions significantly quench the fluorescence of the BODIPY dye via photoinduced electron transfer mechanism.[11−24] But it can become highly fluorescent after it reacts with NO to generate the electron-deficient product with the triazole rings, which cannot engender photoinduced electron transfer.[11−24]

3.3. Fluorescent Response and Selectivity of the Fluorescent Probe A to NO. We examined the performance of BODIPY-based fluorescent probe to detect NO in aqueous solution by conducting a titration experiment with a NO-saturated stock solution and a fluorescent probe solution (5 μM) in 10 mM phosphate buffer at pH 7.0 (Figure 1). The fluorescence intensity of the fluorescent probe is significantly enhanced upon addition of the NO solution. In addition, the concentration dependent fluorescence

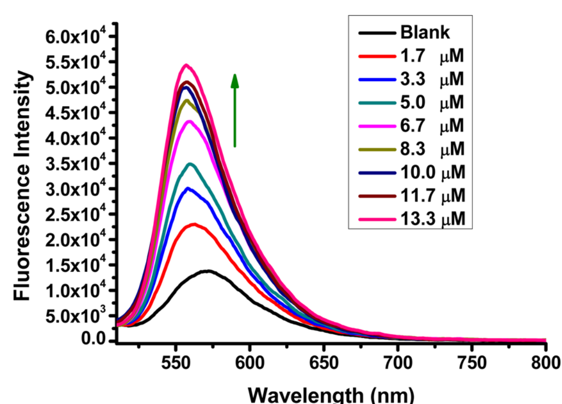

Figure 1. Fluorescence spectra of 5 μM fluorescent probe **A** in the absence and presence of different amounts of NO stock solution in phosphate buffer (10 mM, pH 7.0) at room temperature.

enhancement displays a linearity from 1.7 to 8.3 μM with the detection limit (S/N = 3) of 5.0×10^{-7} M (Figure 2). The sensitivity of fluorescent probe **A** is a little lower than that of 4,5-diamino-fluorescein diacetate in the fluorescent response to NO since the detection limit of 4,5-diaminofluorescein diacetate for NO is 5 nm.[40]

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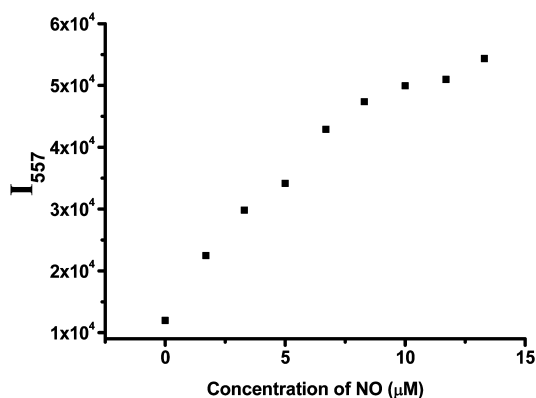

Figure 2. Fluorescent intensity change at 557 nm (I_{557}) of 5 μM fluorescent probe **A** upon titration with NO stock solution (0−13.3 μM).

The detection limit of fluoresceinamine for NO is 44 nm.[41] A detection limit of 6.3 nM in a mixed solution of dichloromethne and ethanol (4:1, v/v) was reported in NO sensor of metal-based conjugated polymer.[42] The increase of the probe fluorescence intensity arises from formation of a strongly fluorescent benzotriazole derivative through reaction of *o*-diaminophenyl groups of the fluorescent probe at 2,6-positions with NO-derivated byproducts such as the autoxidation of NO, which prevents fluorescence quenching via photoinduced electron transfer from *o*-diaminophenyl groups.[11−24] The reaction product, BODIPY dye **B** (Scheme 1), was determined by electrospray mass spectrometry and an ion peak was found at 1349.7 (m/z) $(M - H)^+$, corresponding to the ion that one proton was removed from BODIPY dye **B**.

The selective experiments were conducted by addition of other reactive nitrogen species (RNS) and oxygen species (ROS) to the fluorescent probe **A** in 10 mM phosphate buffer (pH 7.0) (Figures 3 and 4). The fluorescent probe shows high specificity

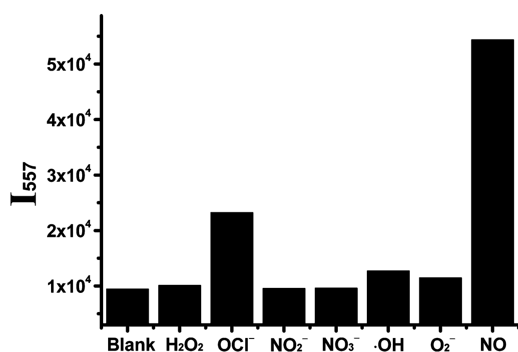

Figure 3. Fluorescent responses of 5 μM fluorescent probe **A** to different ROS and RNS (100 μM, NO: 13.3 μM) in 10 mM phosphate buffer (pH 7.0) at 557 nm.

for NO over other reactive species present in the biological milieu. Except OCl^-, other RNS and ROS such as NO_2^-, O_2^-, NO_3^-, H_2O_2, and ·OH failed to induce significant emission enhancement of the fluorescent probe (Figure 3), that makes this dye a unique tool to measure levels of NO in biological samples and help understand their role in health and disease. This probe showed similar selectivity as 4,5-diaminofluorescein diacetate.[40]

3.4. Use of Fluorescent Probe to Detect Nitric Oxide in Living Cells. NO is a highly reactive free-radical gas that is involved in protein nitration. Studies suggest that NO plays a specific protective and cytotoxic role in cells. Clearly defining

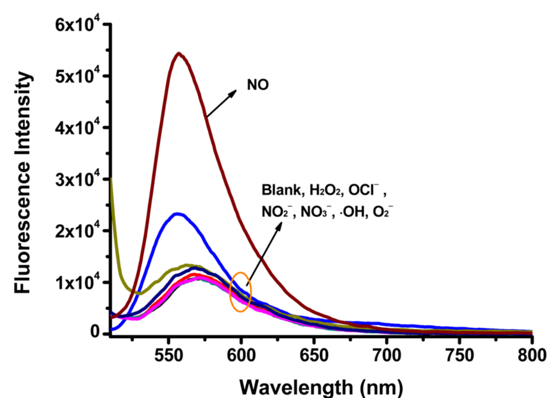

Figure 4. Fluorescent spectra of 5 μM fluorescent probe **A** upon addition of different ROS and RNS (100 μM, NO: 13.3 μM) in phosphate buffer (10 mM, pH 7.0).

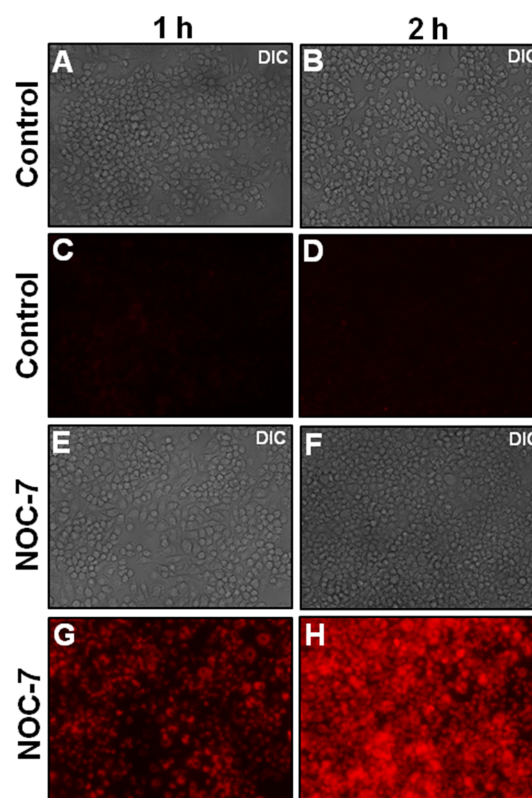

Figure 5. Visualization of NO produced in macrophages (RAW 264.7) using 5 μM fluorescent probe **A** after supply with **NOC-7** (4 μM) for 1 or 2 h: (**A**) bright-field, 1 h, (**B**) bright-field, 2 h, (**C**) fluorescence, 1 h, (**D**) fluorescence, 2 h, (**E**) bright-field, **NOC-7**, 1 h, (**F**) bright-field, **NOC-7**, 2 h, (**G**) fluorescence, **NOC-7**, 1 h, (**H**) fluorescence, **NOC-7**, 2 h.

how much NO is needed and for what duration it is produced to induce protein nitration is critical because excess levels of NO are toxic and will lead to tissue damage and cell death. The fluorescence imaging technique is a very important tool for *in vivo* and *in situ* monitoring of NO.

To evaluate the feasibility of using fluorescent probe **A** to detect NO in cells, we tested fluorescent probe **A** in human-derived retinal pigment epithelium (ARPE-19) and murine macrophages (RAW 264.7). First cells were incubated with 5 μM fluorescent probe **A** in FBS-free DMEM at 37 °C for 4 h and then washed three times with PBS buffer. After that 4 μM NOC-7 (1-hydroxy-2-oxo-3-(*N*-3-methyl-aminopropyl)-3-methyl-1-triazene), an exogeneous NO donor with a

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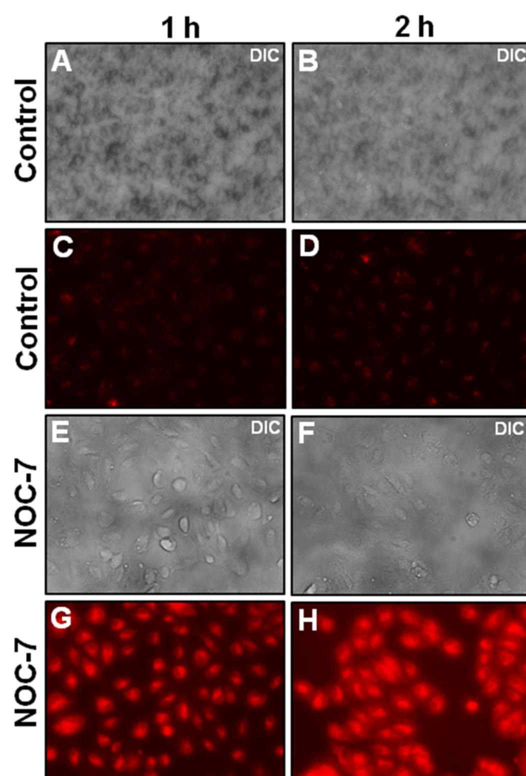

Figure 6. Visualization of NO produced in retinal pigment epithelial cells (ARPE-19) using 5 μM fluorescent probe **A** after supply with **NOC-7** (4 μM) for 1 or 2 h: (**A**) bright-field, 1 h, (**B**) bright-field, 2 h, (**C**) fluorescence, 1 h, (**D**) fluorescence, 2 h, (**E**) bright-field, **NOC-7**, 1 h, (**F**) bright-field, **NOC-7**, 2 h, (**G**) fluorescence, **NOC-7**, 1 h, (**H**) fluorescence, **NOC-7**, 2 h.

half-life time of 5 min at a concentration of 0.1 mM in 0.1 M PBS (pH 7.4),[43] was added and incubated for 1 or 2 h, and cells were washed thrice with PBS. We observed very weak fluorescence of the cells in the absence of **NOC-7** (Figures 5C and D and 6C and D). The purpose of adding the NO donor was to ensure modulation of the NO levels in the cells and that the NO was detected by the probe. This was indeed the case, with an increase in the addition of NOC-7 an increase in fluorescence was observed for both cell types (Figures 5G and 6G). In addition, the fluorescence intensity in both cells increased significantly by longer time (2 h, Figures 5H and 6H). These results confirm that the fluorescent probe **A** is turned on by NO and its fluorescent intensity depends upon the levels of NO in solution. In addition, the fluorescent probe **A** is firmly trapped within a cell without an issue of diffusing out of cells after its initial loading, which was reported in metal-based fluorescent NO probe.[18,44]

We performed fluorescence microscopic imaging of biologically produced NO in RAW 264.7 murine macrophages in order to evaluate the utility of fluorescent probe **A** to detect endogenously generated NO (Figure 7). The RAW 264.7 murine macrophages were stimulated by lipopolysaccharide (LPS; 100 ng/mL) for 16 h and followed by further incubation with 10 μM fluorescent probe **A** for 1 h. In a control experiment, cells were incubated for 16 h with fluorescent probe **A** in the absence of LPS. In addition, we use the commercially available NO fluorescent probe, 4,5-diaminofluorescein diacetate (DAF-2 DA) as a control for the purpose of comparison to probe **A**. A noticeable fluorescence increase in RAW 264.7 murine macrophages was observed at 1 h incubation with fluorescent probe **A** after induction of nitric oxide synthase while it was not observed in the control cells,

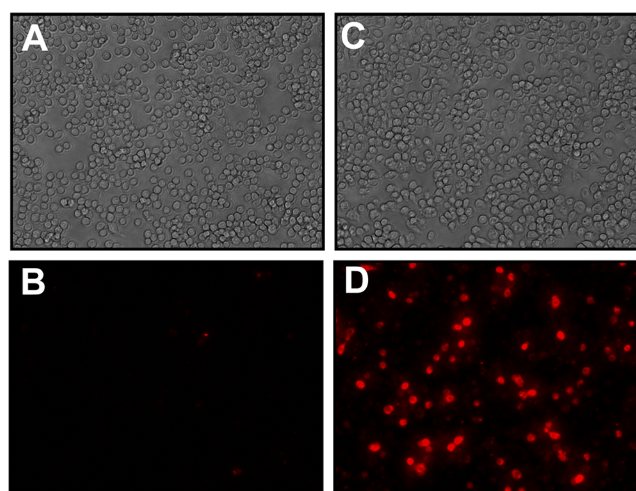

Figure 7. Endogenous NO detection in RAW 264.7 murine macrophages by 10 μM fluorescent probe **A** without and with stimulation of 100 ng/mL lipopolysaccharide: (**A**) bright-field, 1 h, (**B**) fluorescence, 1 h. (**C**) bright-field, LPS stimulation, 1 h, (**D**) fluorescence, LPS stimulation, 1 h.

indicating that fluorescent probe **A** can detect endogenously produced NO. The similar result was observed in control experiments by using DAF-2 DA (Figure S15 in the Supporting Information). The sensitivity of the commercial fluorescent probe (DAF-2 DA) for NO is a little higher than that of this BODIPY-based fluorescent probe in cell imaging. However, the commercial fluorescent probe shows higher fluorescent background in the cells without LPS stimulation under the identical condition (Figure S15 in the Supporting Information).

4. CONCLUSION

We have prepared a highly water-soluble BODIPY-based fluorescent probe for sensitive and selective detection of NO. The fluorescent probe shows differential response to the levels of NO produced endogenously in live murine macrophages and exogenously in human retinal pigment epithelial cells and murine macrophages.

■ ASSOCIATED CONTENT

Supporting Information

Instrumentation; synthesis of compounds **4**, **5**, **7**, **8**, and probe **A**, cell culture, nitric oxide titration, and selectivity measurements; ^{1}H and ^{13}C, absorption, and emission spectra of compounds **4**, **5**, **7**, **8**, and probe **A**, and fluorescence images of murine macrophage RAW264.7 cells using commercial 4,5-diaminofluorescein diacetate. 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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