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Cell-Trappable Fluorescent Probes for Nitric Oxide Visualization in Living Cells

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Abstract

Two new cell-trappable fluorescent probes for nitric oxide (NO) are reported based on either incorporation of hydrolyzable esters or conjugation to aminodextran polymers. Both probes are highly selective for NO over other reactive oxygen and nitrogen species (RONS). The efficacy of these probes for the fluorescence imaging of nitric oxide produced endogenously in Raw 264.7 cells is demonstrated.

The selective detection of nitric oxide (NO) in living systems has attracted much interest since the discovery that NO is the endothelium-derived relaxing factor (EDRF).¹ Nitric oxide is an active signal-inducing molecule in the immune, cardiovascular, and nervous systems, initiating cascades that promote smooth muscle dilation by activating soluble guanylyl cyclase.² More recently, NO has been implicated to modulate synaptic activity in the CNS, where it may play a role in signal transduction in the olfactory bulb.³ Numerous pathological conditions including carcinogenesis, septic shock, inflammation, and neurodegradation have been associated with the misregulation of NO production.⁴

Understanding the multiple biological roles of NO will benefit from the development of tools for its selective detection in vivo. Strategies for monitoring NO have relied on a variety of techniques, including the application of fluorescent probes.⁵ Here we describe fluorescent probes that react directly and specifically with NO that are cell-trappable by means of either ester functionalization or dextran conjugation.

The scaffold of these NO probes is based on that of CuFL1₅ (Scheme 1), previously reported to detect endogenously produced nitric oxide in mammalian and bacterial cells.⁶ The Cu^{II}

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Supporting Information Available Experimental details, spectroscopic data, and diffusion/localization cell imaging information. This information is available free of charge via the Internet at <http://pubs.acs.org>

fluorescein-based NO probe is formed in situ by treating FL1₅ with one equiv of CuCl₂. When non-emissive CuFL1₅ reacts with NO, Cu(II) is reduced to Cu(I) with concomitant *N*-nitrosation of the secondary amine of the ligand to produce the fluorescent species, FL1₅-NO (Scheme 1). CuFL1₅ is minimally cytotoxic, cell-membrane permeable, and selective for NO over other biologically relevant reactive oxygen and nitrogen species (RONS). One limitation of CuFL1₅, however, is that it cannot be trapped within a cell. Under conditions of continual media perfusion, the probe readily diffuses out of cells after initial loading, rendering it ineffective for many biological experiments.

The first strategy that we applied to impart cell trappability to FL1₅ was to incorporate an ester moiety on the quinoline ring, yielding FL1E. The esterified version of this probe is cell membrane permeable until cytosolic esterases cleave the ester to yield a carboxylic acid, FL1A, which is negatively charged at physiological pH and prohibits re-crossing of the cell membrane.⁷ A second strategy was to append the FL1₅ probe to a macromolecule, specifically aminodextran, which because of its high molecular weight can gain entry to cells by endocytotic pathways.⁸ Dextran is hydrophilic polysaccharides with low toxicity and low reactivity that are used as carriers for fluorescent probes.⁹ Their α -1,6-polyglucose linkages cannot be cleaved by most intracellular glycosidases, rendering dextran conjugates stable in cells. We attached the FL1₅ derivative, FL1A, to amino dextran using an acid linker on the quinoline ring for ease of coupling as well as minimal perturbation of the photophysical properties of the fluorophore.

The syntheses of FL1E, FL1A, and FL^{Dex} are outlined in Fig. 1. The Schiff's base formed by condensation of aminoquinoline **1**¹⁰ with fluorescein aldehyde **2**¹¹ in ethanol was reduced with NaBH₄ to afford FL1E. Subsequent hydrolysis under basic conditions yielded the corresponding acid, FL1A. The photophysical properties of FL1A and FL1E are similar to those of FL1₅, as shown in Table 1. The dextran conjugate, FL^{Dex}, was prepared by in situ formation of the NHS-ester of FL1A followed by EDC coupling to 10 kDa aminodextran (2.6 amine/dextran). Optimization of the amide coupling efficiency as well as the fluorescence turn-on with NO of the product conjugate revealed that two equiv of FL1A per primary amine of the dextran provides optimal coupling. Although the coupling was mainly complete, capping excess primary amines with 2-methoxyacetic acid resulted in an enhanced fluorescence turn-on with NO by comparison to the uncapped version. The final conjugate FL^{Dex} was purified by dialysis.

Titration of the various ligands with CuCl₂ revealed that both FL1E and FL1A form 1:1 Cu:ligand complexes, whereas FL^{Dex} binds 1.7 equiv of Cu^{II} per FL1A unit, probably due to non-specific Cu-binding to the dextran backbone. Using the copper stoichiometry determined by titration, exposure of solutions of the probes generated in situ to excess NO under anaerobic conditions led to an immediate fluorescence enhancement with a concomitant bathochromic shift of the emission maxima (Fig. 2 and Table 1). These results are consistent with formation of the *N*-nitrosoamine products.

The relative fluorescence enhancement of CuFL1A when exposed to excess NO was 11.3 ± 0.1 fold, which is similar to that of CuFL1₅ (16 ± 1) (Fig. 2).¹³ Although the fluorescence enhancement of CuFL1E is somewhat diminished (5.4 ± 0.3), it is sufficient to be visualized by spectroscopy and microscopy. Because CuFL1E is not the biologically relevant sensing species, it is therefore not a liability that it is less bright than CuFL1A. CuFL^{Dex} exhibited an intermediate, 6.9 ± 0.8 fold, fluorescence enhancement. This result may be due self-quenching by adjacent fluorophores on the dextran backbone. Treatment of any of the probes with NO under anaerobic conditions in the absence of Cu^{II} did not result in fluorescence turn-on. CuFL1E, CuFL1A and CuFL^{Dex} all turn on with NO and NO donors such as *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), but are only minimally responsive to other physiologically relevant RONS NO₂⁻, NO₃⁻, ClO⁻, H₂O₂, ONOO⁻, HNO and NO₂ (Fig. 3).

Because FL^{Dex} enters cells through endocytosis, we wanted to confirm that the FL1A platform bound Cu^{II} at a pH similar to that encountered in the endosomes.¹⁴ In order to confirm that the CuFL^{Dex} is stable under these conditions and that copper would not dissociate at the low pH of the endosome, FL1A and CuFL1A were used as a model and their UV-vis and fluorescence spectra were monitored as a function of pH. For FL1A, as the pH decreased, the fluorescence maxima blue-shifted; however, for CuFL1A, no shift in maxima was observed from pH 6.5 – 4.0, indicating stability of the copper complex at the acidic pH most likely encountered in the endosome (Fig. 4).

In order to evaluate the efficacy of the probes in live cells, Raw 264.7 murine macrophages, which produce NO from iNOS when induced in response to external activators, were investigated.¹⁵ In these cells, NO production is stimulated by addition of endotoxins, such as lipopolysaccharide (LPS), and cytokines, such as interferon- γ (INF- γ). Raw 264.7 macrophages were therefore treated with LPS and INF- γ , incubating with either CuFL1E or CuFL^{Dex} for 14 h prior to imaging. In both cases, an increase in fluorescence was observed (Fig. 5) confirming turn-on with NO or other biologically relevant NO-transfer agents such as S-nitrosothiols.

Cells that were incubated with the probes without LPS or INF- γ did not display increased fluorescence (Fig. 5). Similarly, stimulation with LPS and INF- γ and treatment with L-N^G-nitroarginine (L-NNA), an iNOS inhibitor (K_i = 4.4 μ M for murine macrophages),¹⁶ showed little fluorescence enhancement. These experiments demonstrate that CuFL1E and CuFL^{Dex} can detect NO produced in vivo. Control experiments demonstrated that CuFL1A is not cell permeable (Fig. S1). In order to test the cell trappability of CuFL1E and CuFL^{Dex}, Raw 264.7 macrophages were stimulated with LPS and IFN- γ and then incubated with the probes for 14 h. The cells were washed and imaged in the microscope incubator, where minimal change in fluorescence intensity was observed over the course of 30 min, confirming cell trappability in contrast to the parent FL1₅ scaffold (Fig. S2–S4). Subcellular localization studies using nuclear and mitochondrial dyes indicated that CuFL1E localizes to the mitochondria whereas CuFL^{Dex} is minimally localized (Fig. S5, S6).

In conclusion, we have prepared two new sensors of the FL1₅ family that impart cell trappability. Both probes maintain sufficient fluorescence to allow for imaging of NO produced endogenously in live Raw 264.7 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Proc Natl Acad Sci U S A 1987;84:9265–9269. [PubMed: 2827174] Palmer RMJ, Ferrige AG, Moncada S. Nature 1987;327:524–526. [PubMed: 3495737]
2. Furchgott RF. Angew Chem, Int Ed 1999;38:1870–1880. Ignarro LJ. Angew Chem, Int Ed 1999;38:1882–1892. Murad F. Angew Chem, Int Ed 1999;38:1857–1868.

3. Kendrick KM, Guevara-Guzman R, Zorrilla J, Hinton MR, Broad KD, Mimmack M, Ohkura S. *Nature* 1997;388:670–674. [PubMed: 9262400] Lowe G, Buerk DG, Ma J, Gelperin A. *Neuroscience* 2008;153:842–850. [PubMed: 18407420]
4. Ignarro, LJ. *Nitric Oxide Biology and Pathobiology*. Academic Press; San Diego: 2000.
5. Nagano T. *J Clin Biochem Nut* 2009;45:111–124. Hetrick EM, Schoenfisch MH. *Ann Rev Anal Chem* 2009;2:409–433. Ye XY, Rubakhin SS, Sweedler JV. *Analyst* 2008;133:423–433. [PubMed: 18365109] Wardman P. *Free Radical Biol Med* 2007;43:995–1022. [PubMed: 17761297] Bedioui F, Villeneuve N. *Electroanalysis* 2003;15:5–18. Kim JH, Heller DA, Jin H, Barone PW, Song C, Zhang J, Trudel LJ, Wogan GN, Tannenbaum SR, Strano MS. *Nat Chem* 2009;1:473–481.
6. Lim MH, Xu D, Lippard SJ. *Nat Chem Biol* 2006;2:375–380. [PubMed: 16732295] Parihar MS, Parihar A, Chen ZH, Nazarewicz R, Ghafourifar P. *Biochim Biophys Acta, BBA* 2008;1780:921–926. Parihar MS, Parihar A, Villamena FA, Vaccaro PS, Ghafourifar P. *Biochem Biophys Res Commun* 2008;367:761–767. [PubMed: 18191636] Gusarov I, Shatalin K, Starodubtseva M, Nudler E. *Science* 2009;325:1380–1384. [PubMed: 19745150] Gusarov I, Starodubtseva M, Wang ZQ, McQuade L, Lippard SJ, Stuehr DJ, Nudler E. *J Biol Chem* 2008;283:13140–13147. [PubMed: 18316370] Patel BA, Moreau M, Widom J, Chen H, Yin LF, Hua YJ, Crane BR. *Proc Natl Acad Sci U S A* 2009;106:18183–18188. [PubMed: 19841256] Shatalin K, Gusarov I, Avetisova E, Shatalina Y, McQuade LE, Lippard SJ, Nudler E. *Proc Natl Acad Sci U S A* 2008;105:1009–1013. [PubMed: 18215992]
7. Tsien RY. *Nature* 1981;290:527–528. [PubMed: 7219539] Izumi S, Urano Y, Hanaoka K, Terai T, Nagano T. *J Am Chem Soc* 2009;131:10189–10200. [PubMed: 19572714]
8. Berthiaume EP, Medina C, Swanson JA. *J Cell Biol* 1995;129:989–998. [PubMed: 7538141]
9. Sonawane ND, Thiagarajah JR, Verkman AS. *J Biol Chem* 2002;277:5506–5513. [PubMed: 11741919] Henry RM, Hoppe AD, Joshi N, Swanson JA. *J Cell Biol* 2004;164:185–194. [PubMed: 14718518] Lencer WI, Weyer P, Verkman AS, Ausiello DA, Brown D. *Am J Physiol* 1990;258:C309–C317. [PubMed: 1689545] Clayton EL, Cousin MA. *J Neurosci Methods* 2009;185:76–81. [PubMed: 19766140]
10. Fahrni CJ, O'Halloran TV. *J Am Chem Soc* 1999;121:11448–11458.
11. Burdette SC, Frederickson CJ, Bu WM, Lippard SJ. *J Am Chem Soc* 2003;125:1778–1787. [PubMed: 12580603] Nolan EM, Burdette SC, Harvey JH, Hilderbrand SA, Lippard SJ. *Inorg Chem* 2004;43:2624–2635. [PubMed: 15074981]
12. Lim MH, Wong BA, Pitcock WH, Mokshagundam D, Baik MH, Lippard SJ. *J Am Chem Soc* 2006;128:14364–14373. [PubMed: 17076510]
13. For comparison with CuFL15, 1300 equiv of NO were used. Addition of 100 equiv of NO yielded similar results; see Table S1.
14. Murphy RF, Powers S, Cantor CR. *J Cell Biol* 1984;98:1757–1762. [PubMed: 6144684] van Weert AWM, Dunn KW, Geuze HJ, Maxfield FR, Stoorvogel W. *J Cell Biol* 1995;130:821–834. [PubMed: 7642700]
15. Bogdan C. *Nat Immunol* 2001;2:907–916. [PubMed: 11577346]
16. Furfine ES, Harmon MF, Paith JE, Garvey EP. *Biochemistry* 1993;32:8512–8517. [PubMed: 7689333]

Figure 1.
Preparation of FL1E, FL1A, and FL^{Dex}.

Figure 2.

Fluorescence turn-on of CuFL1A (1 μ M FL1A, 1 μ M CuCl₂, 1300 equiv NO, 50 mM PIPES, 100 mM KCl, pH 7, 37 $^{\circ}$ C). Inset: Time dependence of fluorescence turn-on.

Figure 3.

Comparison of the selectivity for RONS with CuFL1E, CuFL1A, and CuFL^{Dex}. (50 mM PIPES, 100 mM KCl, pH 7, 37 °C, 60 min, 100 equiv RONS, 1300 equiv NO).

Figure 4.
pH dependence of $\lambda_{max}(\text{emission})$ for FL1A and CuFL1A (5 μM FL, 100 mM KCl, 25 $^{\circ}\text{C}$)

Figure 5. Visualization of NO produced endogenously in Raw 264.7 cells using CuFL1E (left) and CuFL^{Dex} (right) after stimulation with LPS and INF- γ for 14 h. Top: DIC images, Bottom: fluorescence. (a) Probe only, (b) Probe, LPS, INF- γ , and L-NNA (iNOS inhibitor), (c) Probe, LPS, and INF- γ . [Probe] = 4 μ M, [LPS] = (1.25 μ g/mL), [INF- γ] = (625 – 6250 U/mL), [L-NNA] = 10 μ M. Scale bars = 25 μ m.

Scheme 1.
Fluorescence turn-on for the FL1₅ NO probe.

Table 1

Photophysical properties of FL1₅, FL1E, and FL1A.^a

	absorption λ_{max} (nm), $\epsilon \times (10^4 \text{ M}^{-1} \text{ cm}^{-1})$		emission λ_{max} (nm), Φ^b (%)		dynamic range ^e
	unbound	+ Cu(II) ^c	unbound	+ Cu(II) ^c	
FL1 ₅ ¹²	504, 4.2 ± 0.1	499, 4.0 ± 0.1	520, 7.7 ± 0.2	520, nr	16 ± 1
FL1E	506, 2.08 ± 0.05	500, 0.77 ± 0.01	520, 2.57 ± 0.02	520, 3.37 ± 0.08	5.4 ± 0.3
FL1A	504, 6.00 ± 0.07	499, 1.94 ± 0.08	521, 2.37 ± 0.08	520, 2.8 ± 0.2	11.3 ± 0.1

^aSpectroscopic measurements were performed in 50 mM PIPES and 100 mM KCl buffer at pH 7.0.^bQuantum yields are based on fluorescein ($\Phi = 0.95$ in 0.1 N NaOH).^cOne equiv of CuCl₂ was added.^d1300 equiv of NO, 60 min at 37 °C.^eDynamic Range = I_{NO}/I_0 .^fGenerated in situ.