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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 CuII fluorescein-based NO probe is formed in situ by treating FL15 with one equiv of CuCl2. When non-emissive CuFL15 reacts with NO, Cu(I) 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. 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. Dextrans are hydrophilic polysaccharides with low toxicity and low reactivity that are used as carriers for fluorescent probes. 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 FL₅Dex are outlined in Fig. 1. The Schiff’s base formed by condensation of aminoquinoline 1 with fluorescein aldehyde 2 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, FL₅Dex, 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 FL₅Dex was purified by dialysis.

Titrations of the various ligands with CuCl₂ revealed that both FL1E and FL1A form 1:1 Cu:ligand complexes, whereas FL₅Dex binds 1.7 equiv of Cu²⁺ 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 CuFL1E (16 ± 1) (Fig. 2). 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. CuFLDex 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 CuII did not result in fluorescence turn-on. CuFL1E, CuFL1A and CuFLDex 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).

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. 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 CuFLDex 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-NAME, an iNOS inhibitor (Ki = 4.4 μM for murine macrophages), showed little fluorescence enhancement. These experiments demonstrate that CuFL1E and CuFLDex can detect NO.

| Table 1. Photophysical properties of FL1A, FL1E, and FL1A. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | absorption 𝜆max (nm), ε (x 10⁴ M⁻¹ cm⁻¹) | emission 𝜆max (nm), Φ (%) | dynamic range |
|                | unbound + Cu(II) | unbound + Cu(II) | + NOi | + Cu(II) | + Cu(II) |
| FL1A           | 504, 4.2 ± 0.1   | 499, 4.0 ± 0.1  | 520, 7.7 ± 0.2 | 520, nr | 526, 58 ± 2 | 16 ± 1 |
| FL1E           | 506, 2.08 ± 0.05 | 500, 0.77 ± 0.01 | 520, 2.57 ± 0.02 | 520, 3.37 ± 0.08 | 526, 22 ± 1 | 5.4 ± 0.3 |
| FL1A           | 504, 6.00 ± 0.07 | 499, 1.94 ± 0.08 | 521, 2.37 ± 0.08 | 520, 3.2 ± 0.2 | 527, 37 ± 2 | 11.3 ± 0.1 |

*a Spectroscopic measurements were performed in 50 mM PIPES and 100 mM KCl buffer at pH 7.0. b Quantum yields are based on fluorescein (Φ = 0.95 in 0.1 N NaOH). c One equiv of CuCl2 was added. d 1300 equiv of NO, 60 min at 37°C. e Dynamic Range = I₈₅/I₆. f Generated in situ.

Figure 3. Comparison of the selectivity for RONS with CuFL1E, CuFL1A, and CuFLDex. (50 mM PIPES, 100 mM KCl, pH 7, 37°C, 60 min, 100 equiv RONS, 1300 equiv NO).

Figure 4. pH dependence of 𝜆max (emission) for FL1A and CuFL1A (5 μM FL, 100 mM KCl, 25°C)
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\textsubscript{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 FL1\textsubscript{A} scaffold (Fig. S2-S4). Subcellular localization studies using nuclear and mitochondrial dyes indicated that CuFL1E localizes to the mitochondria whereas CuFL\textsubscript{Dex} is minimally localized (Fig. S5, S6).

In conclusion, we have prepared two new sensors of the FL1\textsubscript{A} family that impart cell trappability. Both probes maintain sufficient fluorescence to allow for imaging of NO produced endogenously in live Raw 264.7 cells.

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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

13. For comparison with CuFL1s, 1300 equiv of NO were used. Addition of 100 equiv of NO yielded similar results; see Table S1.