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

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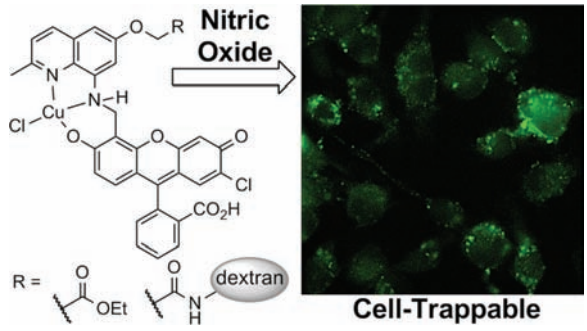
ABSTRACT



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 and that are cell-

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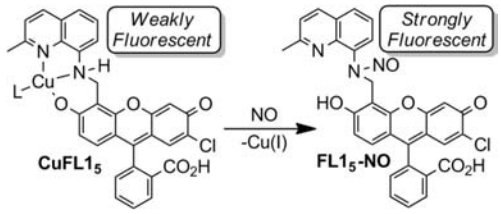
trappable by means of either ester functionalization or dextran conjugation.

The scaffold of these NO probes is based on that of $CuFL1_5$ (Scheme 1), previously reported to detect endogenously produced nitric oxide in mammalian and bacterial cells.[6] The Cu^{II} fluorescein-based NO probe is formed in situ by treating $FL1_5$ with 1 equiv of $CuCl_2$. When nonemissive $CuFL1_5$ 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, $FL1_5$-NO (Scheme 1). $CuFL1_5$ is minimally cytotoxic, cell-membrane permeable, and selective for NO over other biologically relevant reactive oxygen and nitrogen species (RONS). One limitation of $CuFL1_5$, 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.

Scheme 1. Fluorescence Turn-On for the $FL1_5$-NO Probe



The first strategy that we applied to impart cell trappability to $FL1_5$ 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 recrossing of the cell membrane.[7] A second strategy was to append the $FL1_5$ probe to a macromolecule, specifically aminodextran, which because of its high molecular weight can gain entry to cells by endocytotic pathways.[8] Dextrans are 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 $FL1_5$ derivative, FL1A, to aminodextran 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 Figure 1. The Schiff base formed by condensation of amino-

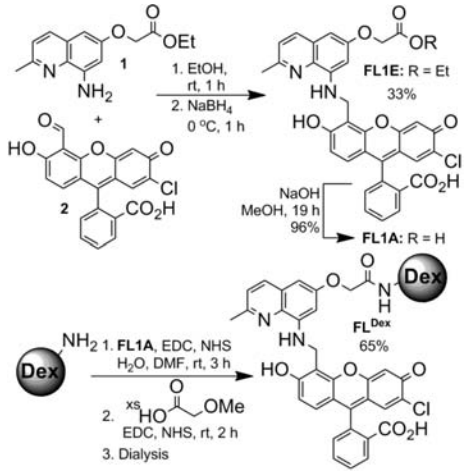


Figure 1. Preparation of FL1E, FL1A, and FL^{Dex}.

quinoline **1**[10] with fluorescein aldehyde **2**[11] in ethanol was reduced with $NaBH_4$ 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 $FL1_5$, 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 2 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.

Titrations of the various ligands with $CuCl_2$ 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 nonspecific 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 (Figure 2 and Table 1). These results are consistent with formation of the *N*-nitrosamine products.

The relative fluorescence enhancement of CuFL1A when exposed to excess NO was 11.3 ± 0.1-fold, which is similar to that of $CuFL1_5$ (16 ± 1) (Figure 2).[13] Although the

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(13) For comparison with $CuFL1_5$, 1300 equiv of NO were used. Addition of 100 equiv of NO yielded similar results; see Table S1 (Supporting Information).

Table 1. Photophysical Properties of $FL1_5$, FL1E, and FL1A[a]

	absorption λ_{max} (nm), $\varepsilon \times (10^4\ M^{-1}\ cm^{-1})$		emission λ_{max} (nm), Φ[b] (%)			
	unbound	+ Cu(II)[c]	unbound	+ Cu(II)[c]	+ NO[d]	dynamic range[e]
$FL1_5$[12]	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[f]	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	527, 37 ± 2[f]	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] 1 equiv of $CuCl_2$ was added. [d] 1300 equiv of NO, 60 min at 37 °C. [e] Dynamic range = I_{NO}/I_0. [f] Generated in situ.

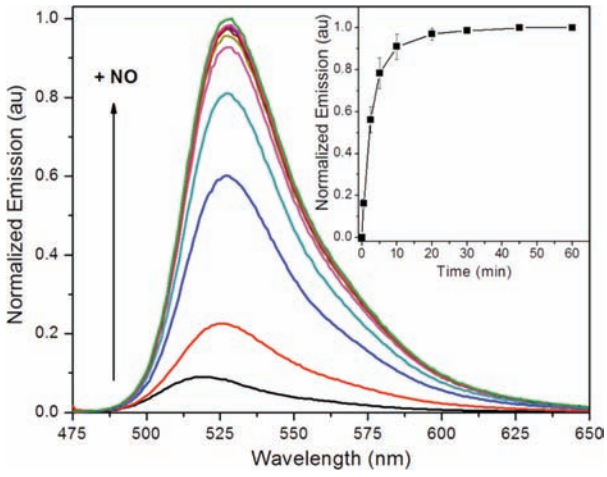

Figure 2. Fluorescence turn-on of CuFL1A (1 μM FL1A, 1 μM $CuCl_2$, 1300 equiv of NO, 50 mM PIPES, 100 mM KCl, pH 7, 37 °C). Inset: Time dependence of fluorescence turn-on.

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, such as NO_2^-, NO_3^-, ClO^-, H_2O_2, $ONOO^-$, HNO, and NO_2 (Figure 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] 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.

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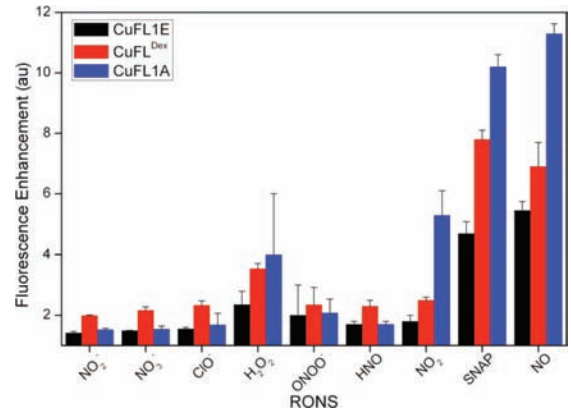

Figure 3. Comparison of the selectivity for RONS with CuFL1E, CuFL1A, and $CuFL^{Dex}$ (50 mM PIPES, 100 mM KCl, pH 7, 37 °C, 60 min, 100 equiv of RONS, 1300 equiv of NO).

For FL1A, as the pH decreased, the fluorescence maxima blue-shifted; however, for CuFL1A, no shift in maxima was observed from pH 6.5 to 4.0, indicating stability of the copper complex at the acidic pH most likely encountered in the endosome (Figure 4).

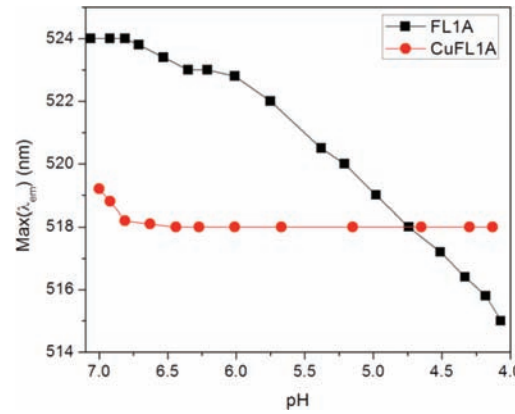

Figure 4. pH dependence of λ_{max}(emission) for FL1A and CuFL1A (5 μM FL, 100 mM KCl, 25 °C).

To evaluate the efficacy of the probes in live cells, Raw 264.7 murine macrophages, which produce NO from iNOS

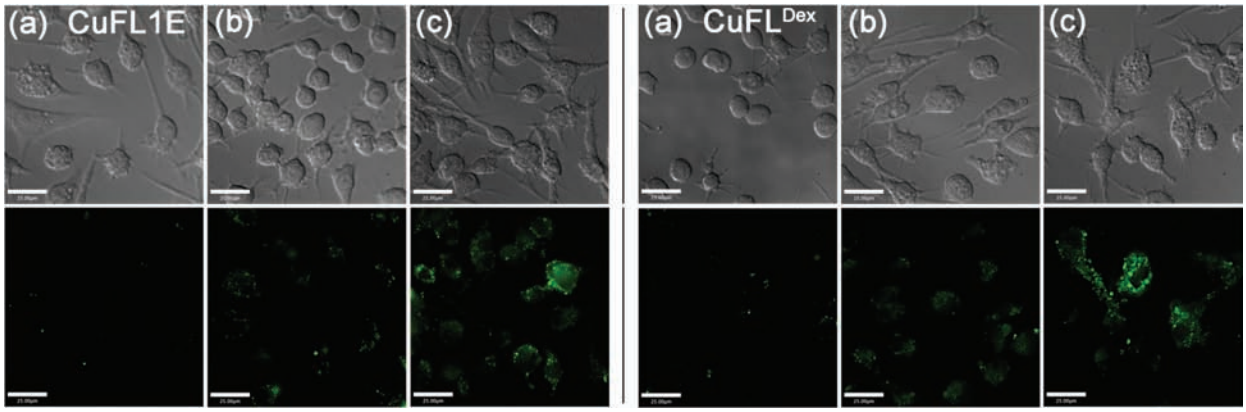

Figure 5. Visualization of NO produced endogenously in Raw 264.7 cells using CuFL1E (left) and CuFL^{Dex} (right) after stimulation with LPS and INF-γ for 14 h. Top: DIC images. Bottom: fluorescence. (a) Probe only. (b) Probe, LPS, INF-γ, and L-NNA (iNOS inhibitor). (c) Probe, LPS, and INF-γ. [Probe]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

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 (Figure 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 (Figure 5). Similarly, stimulation with LPS and INF-γ and treatment with L-N^G-nitroarginine (L-NNA), an iNOS inhibitor (K_i = 4.4 μM for murine macrophages),[16] resulted in 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 (Figure S1, Supporting Information). 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 $FL1_5$ scaffold (Figures S2−S4, Supporting Information). Subcellular localization studies using nuclear and mitochondrial dyes indicated that CuFL1E localizes to the mitochondria, whereas CuFL^{Dex} is minimally localized (Figures S5 and S6, Supporting Information).

In conclusion, we have prepared two new sensors of the $FL1_5$ 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 material is available free of charge via the Internet at http://pubs.acs.org.

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