

Radioactivity-Synchronized Fluorescence Enhancement Using a Radionuclide Fluorescence-Quenched Dye

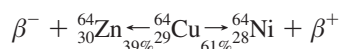
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Metal-sensitive fluorescent probes have been shown to accurately and rapidly report metal complexation through a change in the fluorophore's spectral properties.^{1–3} Previous studies have focused on quasistatic metal–fluorophore complexes, where a single metal of interest complexes with the fluorophore, altering its emission in some discernible fashion. The resultant emission could be further modified by changing the ligands attached to the metal or altering the metal oxidation states. Neither experimental nor theoretical studies have been conducted regarding dynamic metal–fluorophore complexes in which the nature of the bound metal itself is variable. Such changes, for example, may transform a nonfluorescent complex into a fluorescent system if a quencher metal could convert into a nonquencher metal. The difficulty in achieving this transformation arises from the high stability of metals. However, a select group of metals, namely, radionuclides, are ideally suited for this application because spontaneous radioactive decay of an unstable parent metal could transform it to a different, more stable metal.

Herein we propose the first molecular system that demonstrates the feasibility of altering the fluorescence properties of an organic dye as a function of radionuclide decay. Previous studies have shown that ⁶⁴Cu decays to ⁶⁴Zn and ⁶⁴Ni through two pathways, as shown in the following equation:

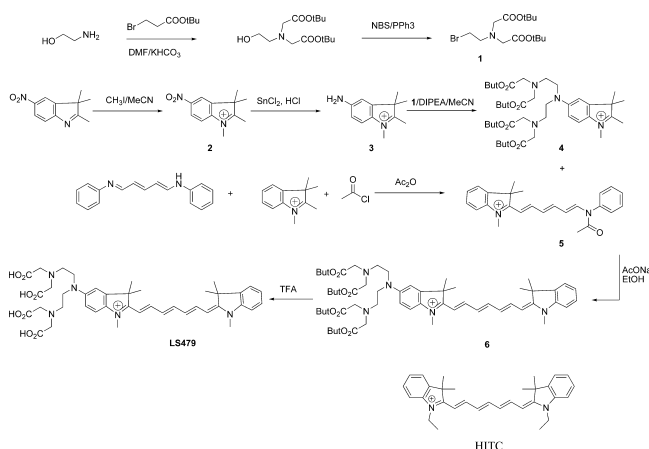


We found a clear differentiation in fluorescence between Cu²⁺ and its decay products, with Cu²⁺ acting as a strong quencher, Zn²⁺ as an enhancer, and Ni²⁺ having no net effect on the dye fluorescence. Correlation of the radionuclide decay with the dye fluorescence enhancement validates the hypothesized metal effect and suggests a realm of possible applications for complementing fluorescence with radioactivity.

To study the influence of intermetal conversion through radioactive decay on the fluorescence properties of organic dyes, we prepared LS479, a novel near-infrared fluorescent dye with a covalently linked metal-chelating group (Scheme 1). LS479 is structurally similar to the known dye 1,1',3,3',3',3'-hexamethylindotricarbocyanine (HITC), which is widely used in analytical chemistry and biological optical-imaging studies.⁴ The chelating group is derived from diethylenetriaminepentaacetic acid (DTPA). The synthesis of LS479 (Scheme 1) involved the methylation of

5-nitro-2,3,3-trimethylindolenine to provide indole analogue **2** in 60% yield. Reduction of the nitro group followed by N-alkylation using *N,N*-bis[(*tert*-butoxycarbonyl) methyl]-2-bromoethylamine (**1**) afforded indole derivative **4** in an overall yield of 65%. Treatment of 1,2,3,3-tetramethylindolinium iodide with glutacetaldehyde dianil, acetyl chloride, and acetic anhydride produced hemicyanine intermediate **5** in 87% yield. Reaction of **5** with **4** under reflux in anhydrous ethanol with anhydrous sodium acetate afforded Boc-protected intermediate **6**, which was isolated in good yield (74.5%). Hydrolysis of **6** to afford LS479 was accomplished with trifluoroacetic acid, and the desired product was purified by preparative HPLC.

Scheme 1. Synthesis of LS479 and Structure of HITC



We used absorption studies to establish that metal complexation would not be lost upon radioactive decay of ⁶⁴Cu²⁺ and that the parent and daughter nuclides remain bound to LS479. A solution of HITC or LS479 in water was titrated with Cu²⁺, Ni²⁺, and Zn²⁺ chlorides, and the absorption and emission spectra were recorded. HITC, lacking the chelating group, did not show any isosbestic points, and the shape of the absorption spectra upon metal titration remained the same (Figures S1–S3 in the Supporting Information), suggesting the absence of metal chelation to the dye molecule. Similarly, we did not observe significant changes in the fluorescence of HITC upon addition of metal salts (Figure S4). In contrast, transformations in the absorption spectra of LS479 (Figures S5–S7) showed that all of the metals remained bound to the dye. In all cases, clear isosbestic points were observed, especially for Zn²⁺ and Ni²⁺.

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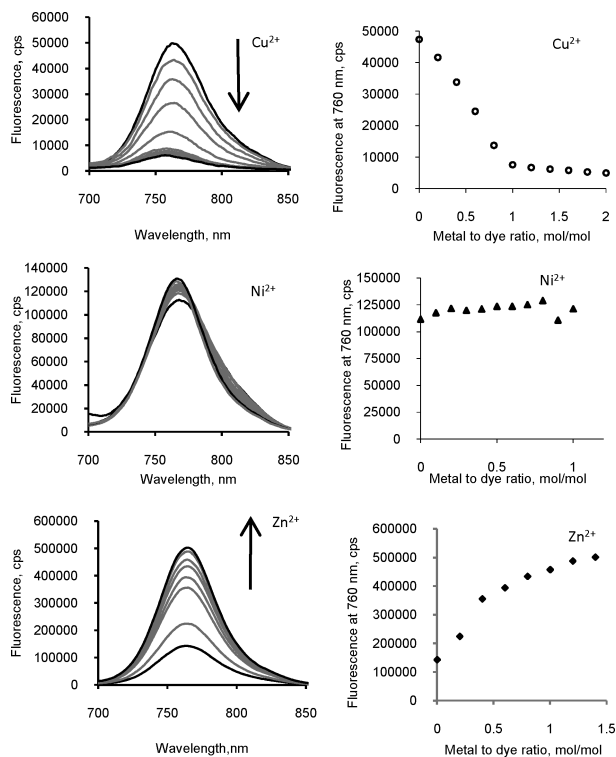


Figure 1. (left) Titration emission spectra of LS479 with (top to bottom) CuCl₂, NiCl₂, and ZnCl₂ in water (excitation at 675 nm). Arrows indicate the direction of increasing metal concentration. Cu²⁺ was shown to quench the fluorescence, Zn²⁺ enhanced it, and Ni²⁺ had no net effect. (right) Fluorescence intensities at the LS479 emission maximum (760 nm) as a function of metal/dye ratio.

Analysis of the fluorescence spectra showed that the three metals displayed different effects on the emission from LS479 (Figure 1). Cu²⁺ had a strong quenching ability, achieving maximum quenching at a 1:1 molar ratio to the dye. The fluorescence intensity of the Ni²⁺–LS479 complex remained the same as that of LS479 at all molar ratios examined. Furthermore, Zn²⁺ significantly enhanced the fluorescence of LS479: at a 1:1 Zn²⁺/LS479 molar ratio, the emission of the Zn²⁺–LS479 complex was ~5 times higher than that of LS479. The metal effects on fluorescence and absorption of LS479 were concentration-dependent. The metal effects on the dye fluorescence were mostly linear until a metal/dye ratio of ~1:1 was established. Obviously, at this point, all the dye molecules were chelated to the metal, and no free dye existed in solution. Further addition of metal resulted in minimal alteration of the spectra.

Having demonstrated that Cu²⁺ was a fluorescence quencher of LS479, Ni²⁺ did not have an effect, and Zn²⁺ significantly enhanced the fluorescence, we hypothesized that a solution of completely decayed ⁶⁴CuCl₂ would possess similar effects on the fluorescence spectra of LS479 as a result of the daughter nuclides Ni²⁺ and Zn²⁺. Since the half-life of ⁶⁴Cu is 12.7 h, we used a completely decayed solution of ⁶⁴CuCl₂ in water (>12 half-lives), which ensured that the solution contained 61% ⁶⁴Ni²⁺ and 39% ⁶⁴Zn²⁺ in addition to any other incipient metals.⁵ The remaining amount of ⁶⁴Cu²⁺ was virtually zero. As expected, the emission spectra were drastically different from the “cold” Cu²⁺ titration spectra (Figure 2). The spectral changes induced by decayed ⁶⁴Cu²⁺ on LS479 were similar to those caused by a model daughter-isotope mixture of ⁶⁴Cu²⁺ decay products (Ni/Zn ratio ≈ 1.5) (see Figures S8 and S9 for the absorption spectra).

The above results using “cold” metal models suggest that “hot” Cu would quench the fluorescence of LS479. Indeed, addition of

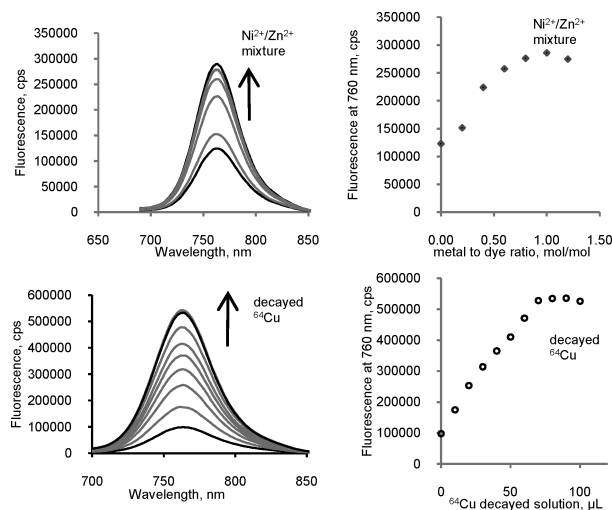


Figure 2. (left) Titration emission spectra of LS479 with (top) a [61% natNi²⁺ + 39% natZn²⁺] mixture and (bottom) decayed ⁶⁴Cu²⁺ (λ_{ex} = 675 nm). (right) Fluorescence intensity at 760 nm as a function of decayed ⁶⁴Cu addition, where the saturation point apparently corresponds to a 1:1 metal/dye ratio. Addition of either solution to LS479 resulted in marked fluorescence enhancement.

⁶⁴Cu²⁺ to a solution of LS479 rapidly quenched the dye fluorescence with a concomitant increase in fluorescence over time (Figure 3). There were no changes in the absorption spectra of LS479, which indicates that the observed fluorescence increases were not diminished or altered by radiolysis. The resultant fluorescence enhancement reflected the transformation of ⁶⁴Cu²⁺ into ⁶⁴Ni²⁺ and ⁶⁴Zn²⁺. As ⁶⁴Cu further decayed to ⁶⁴Ni²⁺ and ⁶⁴Zn²⁺, the quenching effect decreased and the fluorescence increased to 130% relative to the emission of unquenched LS479 for a net enhancement of fluorescence. After 72 h, nearly all of the ⁶⁴Cu decayed into stable ⁶⁴Ni²⁺ and ⁶⁴Zn²⁺ (2% of initial activity remaining). Consequently, there were no changes in fluorescence intensity thereafter.

Fluorescence quenching by Cu²⁺ is a general phenomenon with few exceptions,⁶ spanning the range from organic molecules² to quantum dots⁷ and fluorescent proteins.⁸ However, the mechanism of fluorescence quenching still remains obscure.³ Quenching has been attributed to a spheres-of-action static-quenching mechanism,⁸ the paramagnetic nature of the metal,⁹ a heavy-atom effect,¹⁰ energy transfer from the dye (donor) to Cu²⁺ (acceptor),¹¹ and charge transfer from π^* to d orbitals.¹² We ruled out the first mechanism, spheres-of-action static quenching, because it considers a certain probability of collision quenching,¹³ which is not the case for the Cu²⁺–LS479 complex, where the probability reaches 100% with maximum quenching occurring at 1:1 metal/dye ratio. Paramagnetism is also an inadequate explanation because another paramagnetic complex, octahedral Ni²⁺,¹⁴ did not exhibit any quenching. A heavy-atom effect was discounted on the grounds that the heaviest ion, Zn²⁺, enhanced the fluorescence of LS479 instead of quenching it.

To delineate the contribution of metal chelation to the observed fluorescence properties of LS479 metal chelates, we prepared and compared the absorption spectra of Cu²⁺–, Ni²⁺–, and Zn²⁺–DTPA complexes and the emission spectra of free LS479 (Figure 4). The result showed a large spectral overlap between the dye and the Cu²⁺–DTPA complex and insignificant overlap for the Ni²⁺ and Zn²⁺ complexes, thus supporting the conclusion that an energy-transfer mechanism is responsible for the strong quenching by Cu²⁺ and the neutrality by Ni²⁺ and Zn²⁺. Similarly, quenching could be explained by excited-state charge transfer, where the excited electron travels from the π^* orbital of the dye to

a d orbital of similar energy on Cu^{2+} , where the absorbed energy nonradiatively dissipates. The higher energy of the d orbitals in Ni^{2+} compared with the π^* orbitals precludes this transfer, allowing the fluorescence of LS479 to occur naturally via a $\pi^* \rightarrow \pi$ transition. Indeed, the absorbance maximum in the visible spectra of Cu^{2+} –DTPA was 740 nm, while that for Ni –DTPA was at a shorter wavelength, 610 nm. A similar mechanism of quenching might occur in porphyrin, which in its free form emits at ~ 600 nm but for which both Cu^{2+} and Ni^{2+} cause fluorescence quenching.¹⁵

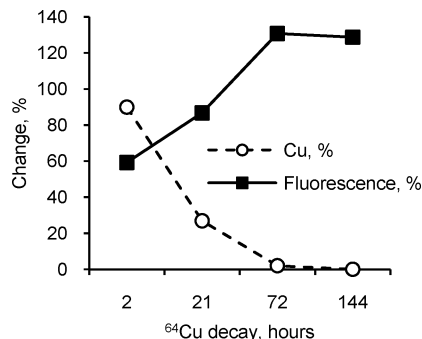


Figure 3. Percent changes in $^{64}\text{Cu}^{2+}$ concentration as a result of decay (calculated from exponential decay) and in relative fluorescence of Cu –LS479 solution as a function of time. Unquenched LS479 (0.5 μM) corresponded to a hypothetical zero-time point for fluorescence. Relative fluorescence was measured as an area under the emission curve after addition of ^{64}Cu solution divided by the area before the addition ($\lambda_{\text{ex}} = 675$ nm). An overall increase in fluorescence intensity was seen as a function of time.

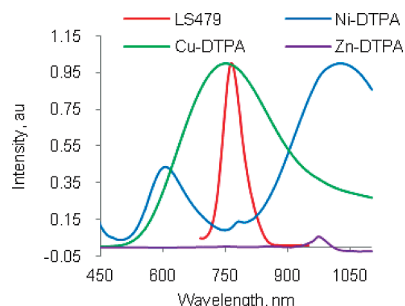


Figure 4. Normalized emission of LS479 ($\lambda_{\text{ex}} = 690$ nm) and absorption spectra of Cu^{2+} –DTPA, and Ni^{2+} –DTPA and Zn^{2+} –DTPA (the absorption maximum for the Zn^{2+} –DTPA complex was <450 nm). Substantial overlap between LS479 and the copper complex suggests that energy transfer is a possible explanation for quenching. The lack of overlap with zinc and nickel should be noted.

The fluorescence enhancement of LS479 observed upon complexation with Zn suggests that the lack of a vacant d orbital precludes ligand-to-metal charge transfer and the absence of absorption bands prevents energy transfer. A distinguishing characteristic of LS479 compared with HITC is the presence of a lone electron pair on the conjugated amine group. Lone electron pairs typically act as highly efficient fluorescence quenchers via an excited-state charge transfer (ESCT) mechanism.¹⁶ Indeed, the quantum yield of LS479 ($\Phi = 0.018$) in unbuffered water was ~ 6 times smaller than that of HITC ($\Phi = 0.11$). In addition, the fluorescence lifetime of LS479 ($\tau = 0.35$ ns) was shorter than that of HITC ($\tau = 0.41$ ns), further indicating the presence of a fluorescence quenching process.

The addition of $^{64}\text{CuCl}_2$ also resulted in fluorescence quenching. The net fluorescence enhancement upon $^{64}\text{Cu}^{2+}$ decay strongly

supports our model of fluorescence enhancement synchronized with radioactive decay. This new method of monitoring fluorescence enhancement through the decay of radionuclides has a variety of potential applications. For example, it may open new possibilities for studying fundamental molecular transformations, such as determining how quickly a molecule readjusts from one state to another after radioactive decay. Furthermore, combining radionuclide decay with fluorescence enhancement could provide a unique complementary signaling mechanism for radionuclear–optical multimodal biological imaging. In fact, the new radiolabeled LS479 represents a paradigm-shifting strategy in multimodal molecular imaging because $^{64}\text{Cu}^{2+}$ and near-infrared fluorescent dyes such as LS479 are routinely used in positron emission tomography¹⁷ and optical imaging¹⁸ of living organisms, respectively. Moreover, the strong correlation between fluorescence quenching and the presence of $^{64}\text{Cu}^{2+}$ chelation provides a dynamic method for studying the in vivo stability of the radionuclide chelates, where fluorescence enhancement reports demetalation of the radionuclide. Finally, the new molecular construct and mechanism could be used to develop visible radioactivity decay devices for environmental and nuclear forensic applications. Future work will focus on these potential applications.

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Supporting Information Available: Synthesis, experimental procedures, calculations, and optical spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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