

# Design, synthesis, and characterization of a calcium-sensitive near infrared dye

April L. Ellis, J. Christian Mason, Hsiau-Wei Lee, Lucjan Strekowski,  
Gabor Patonay, Hoseob Choi<sup>1</sup>, Jenny J. Yang \*

*Department of Chemistry, Center for Drug Design and Advanced Biotechnology, Georgia State University, Atlanta,  
GA 30303, USA*

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## Abstract

Intracellular calcium concentration in biological cells varies from 0.1 to 10  $\mu\text{M}$  depending upon cell signaling and disease states. A direct estimate of calcium concentration in cell tissues within this range is possible with a novel calcium-selective reagent 15C5-774. The molecule of 15C5-774 consists of a near-infrared (NIR) chromophore ( $\lambda_{\text{max}} = 774 \text{ nm}$ ) and a metal complexing moiety of benzo-15-crown-5. The reagent shows a strong calcium binding affinity in a 1:1 ratio and metal selectivity in the order  $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+} \approx \text{K}^+ \approx \text{Na}^+ > \text{Zn}^{2+} > \text{Li}^+$ . The high sensitivity is achieved by conducting absorption measurements in the NIR region where background interference from the biological matrix is low. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Calcium binding; Near infrared; Benzo-15-crown-5; Cyanine dye

## 1. Introduction

As a secondary messenger, calcium regulates many biological processes in various intracellular compartments [1]. Normally the intracellular calcium concentration is  $\sim 0.1\text{--}10 \mu\text{M}$ . Both overloading and depleting the calcium concentration

inside cells is directly related to different disease states in human health, such as Marfan's Syndrome and Osteoporosis [2,3]. In addition to calcium related diseases, many other diseases and conditions are mediated by the improper concentration and availability of other metals. The ability to monitor the concentrations and fluxes of metal ions in the cell would greatly facilitate the treatment of diseases related to an overabundance or insufficiency of those metals. Introduction of a metal binding dye into cells allows for analysis of metal concentrations. Currently, these types of dyes are used to understand certain stimuli that cause a flux of metal ions in the cell and to quantitate the amount of metal ions observed in diseased cells.

*Abbreviations:* Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; 15C5-774, heptamethine cyanine/benzo-15-crown-5 conjugate; MeOH, methanol; NIR, near-infrared.

\* Corresponding author. Tel.: +1-404-651-4620; fax: +1-404-651-2751.

*E-mail address:* chejy@panther.gsu.edu (J.J. Yang).

<sup>1</sup> Present address: Department of Chemistry, Kunsan National University, Kunsan 573-701, Korea.



anhydrous *N,N*-dimethylformamide (10 ml) was heated to 140 °C for 2 h under nitrogen atmosphere. After cooling, the solution was stirred and treated dropwise with aqueous tetrafluoroboric acid (24%, 2 ml), which caused crystallization of the complex 3 (15C5-774)·2HBF<sub>4</sub>. The crystalline precipitate was filtered and washed with water. Crystallization from an acetone/methanol mixture by addition of tetrafluoroboric acid followed by filtration, washing with water and then ethyl ether, and drying at 70 °C/1.0 mmHg gave an analytically pure product: yield 500 mg (63%); mp > 200 °C (decomp.); <sup>1</sup>H NMR (dimethyl sulfoxide-*d*<sub>6</sub>/tetramethylsilane, 400 MHz, 30 °C) δ 1.68 (s, H), 1.88 (t, *J* = 6 Hz, 2H), 2.73 (t, *J* = 6 Hz, 2H), 2.88 (t, *J* = 6 Hz, 2H), 3.37 (s, 6H), 3.65 (m, 6H), 3.85 (m, 4H), 3.90 (m, 4H), 4.20 (m, 4H), 4.28 (m, 4H), 5.69 (d, *J* = 12 Hz, 2H), 7.01 (t, *J* = 7 Hz, 2H), 7.07 (d, *J* = 7 Hz, 2H), 7.27 (t, *J* = 7 Hz, 2H), 7.40 (t, *J* = 7 Hz, 2H), 7.42 (d, *J* = 8 Hz, 2H), 7.78 (d, *J* = 12 Hz, 2H), 8.25 (s, 1H); <sup>13</sup>C NMR (dimethyl sulfoxide-*d*<sub>6</sub>, 100 MHz, 30 °C) δ 20.9, 25.6, 27.3, 28.1, 28.9, 29.6, 31.4, 46.8, 68.2, 68.3, 68.7, 69.4, 70.6, 94.0, 99.9, 101.8, 107.2, 108.2, 111.3, 116.0, 121.0, 121.5, 121.7, 122.3, 125.1, 126.0, 127.9, 128.5, 130.5, 134.4, 134.5, 138.2, 139.5, 140.9, 142.7, 142.8, 143.7, 148.7, 148.9, 153.7, 165.5, 172.6; Vis-NIR (methanol:Tris [20:80 v/v] pH 7.4) λ<sub>max</sub> = 774 nm (ε = 42 600 M<sup>-1</sup> cm<sup>-1</sup>), which varies with pH changes. Analysis, calculated for 3(C<sub>46</sub>H<sub>56</sub>BF<sub>4</sub>N<sub>3</sub>O<sub>5</sub>)·2HBF<sub>4</sub>: C, 63.05; H, 6.52; N, 4.79. Found: C, 63.31; H, 6.28; N, 4.64.

## 2.2. Absorbance analysis

All absorbance measurements were taken on a Shimadzu UV-1601 spectrophotometer interfaced to a PC operating under Windows 98. The data analysis was performed using Microsoft Excel 2000 and KaleidaGraph 3.0. All chemicals used were ACS grade reagents. All metals tested were in the chloride salt form. The titrations were performed with stock solutions of 15C5-774 containing varying concentrations of metal in 20% MeOH and buffered with 10 mM Tris. The Tris buffer was chelexed with Bio-Rad Laboratories Chelex 100 Resin and adjusted to pH 7.4 with

HCl. The pH was measured with a Beckman 360 pH meter by removing 1 ml increments and measuring with a small electrode. A stock solution of 15C5-774 in 100% MeOH was made by dissolving 1.2 mg of dye in 5 ml of MeOH for a concentration of ~ 275 μM.

## 2.3. Metal titrations

First, stock solutions of metal in Tris buffer were made through serial dilutions from 1 M to 1 mM. Then 1 ml dye/metal stock solutions containing a constant 19.52 μM dye concentration (using 275 μM stock dye in MeOH) and various metal concentrations (using stock metal in Tris) were made with the Tris/MeOH buffer. A zero point was made with dye, Tris, and MeOH (no metal) at the same concentration as the 1 ml stocks, and its absorbance was recorded. A certain volume was removed from the cuvette and placed in the waste container. The same volume of one of the dye/metal stock solutions was placed into the cuvette. After mixing well, the absorbance of the dye at that metal concentration was recorded. These steps were repeated until saturation was reached. Depending on the metal being tested, saturation was reached at about 400 μM–60 mM.

## 2.4. Data analysis

The metal concentration at each point was calculated using the Eq. (1),

$$[\text{metal}] = \frac{(V_{\text{LC}} \times M_{\text{C}}) + (V_{\text{A}} \times M_{\text{S}})}{V_{\text{T}}} \quad (1)$$

where  $V_{\text{LC}}$  is the volume left in the cuvette after removal of the exchange volume,  $M_{\text{C}}$  is the molarity of the volume in the cuvette,  $V_{\text{A}}$  is the volume added to the cuvette,  $M_{\text{S}}$  is the molarity of the stock solution of the volume added, and  $V_{\text{T}}$  is the total volume in the cuvette after the volumes are exchanged (normally 1 ml).

The *K*<sub>d</sub> values were calculated by creating a scatter plot of the fractional change versus the metal concentration. Fractional change is given in the Eq. (2),

$$F = \frac{A_p - A_0}{A_{\max} - A_0} \quad (2)$$

where  $A_p$  is the absorbance of that data point,  $A_0$  is the absorbance of the zero point, and  $A_{\max}$  is the absorbance at the most saturated point. The scatter plot was then fit with a curve using the Eq. (3),

$$F = \frac{([D])_T + [M]_T + k_d) - \sqrt{([D])_T + [M]_T + k_d)^2 - 4[D]_T[M]_T}}{2[D]_T} \quad (3)$$

where  $[D]_T$  is the total dye concentration,  $[M]_T$  is the total metal concentration, and  $K_d$  is the dissociation constant of the reaction. When this equation is put into KaleidaGraph, the  $K_d$  is graphically estimated and can be adjusted as necessary to achieve the best result.

### 2.5. pH dependence

For the pH dependence studies, the pH of Tris for the lower pH work was adjusted to 6 with HCl. For the higher pH study, new Tris was made, chelexed, and left at pH 8.7. The stock metal solutions for calcium and magnesium were made with this Tris buffer in 1 ml volumes using serial dilutions. The sample solutions were made with these metal solutions. An additional zero point (without metal) was made to test the initial pH. The pH of the titrated solution was tested after the titrations were complete.

## 3. Results and discussion

### 3.1. Design of 15C5-774

Compound 15C5-774 is a NIR chromophore-modified crown ether. The properties of crown ethers as metal binding compounds have been reported extensively [12–25]. The size of the crown ether ring may be varied to provide a proper cavity for metal binding based on the size of the metal. In particular, the cavity of benzo-15-crown-5 has a diameter of 1.7–2.2 Å, depending on conformation [26]. This cyclic ether can easily accommodate calcium, which has a radius of 0.99 Å [3].

The second functional unit of 15C5-774 is an indolium heptamethine cyanine that absorbs in the NIR region. The crown ether is linked to the central *meso* position of the cyanine. We have shown previously that the wavelength and intensity of absorption of heptamethine cyanine chromophores are greatly affected by a substituent at the *meso* position [27]. More importantly, the equilibrium conformation of an electron donating *meso*-substituent, such as an amino group, greatly influences the absorption by affecting conjugation of the substituent with the electron deficient cationic chromophore. Accordingly, this finding was explored in the design of 15C5-774. It was reasoned that a conformational change of the crown ether moiety upon binding calcium ion would induce absorption changes at the NIR chromophore, thereby providing a basis for the metal determination [28].

### 3.2. Solution properties of 15C5-774

When stored in a solid form at  $-20\text{ }^\circ\text{C}$ , in the dark, and under a dry atmosphere, 15C5-774 is stable indefinitely. Stock solutions in methanol or other organic solvents are stable for at least a month when protected from light and oxygen and stored at  $4\text{ }^\circ\text{C}$ , as monitored by Vis-NIR spectroscopy. In aqueous methanol at  $23\text{ }^\circ\text{C}$  in the presence of oxygen and in the dark, the compound is stable for at least 7 days, as shown by  $^1\text{H}$  NMR spectroscopy. On the other hand, at  $23\text{ }^\circ\text{C}$  in the presence of light and oxygen, the solutions are less stable. A slight decrease in the NIR absorption was observed under these conditions after several hours. Accordingly, freshly made solutions in a methanol:aqueous solvent mixture (20:80 v/v) were used in this work to minimize environmental factors that influence dye degradation.

### 3.3. Metal binding affinities and selectivity of 15C5-774

The Vis-NIR absorption spectra of 15C5-774 in a Tris/MeOH buffer at physiological pH 7.4 in the absence of calcium and as a function of calcium concentration are shown in Fig. 2. The

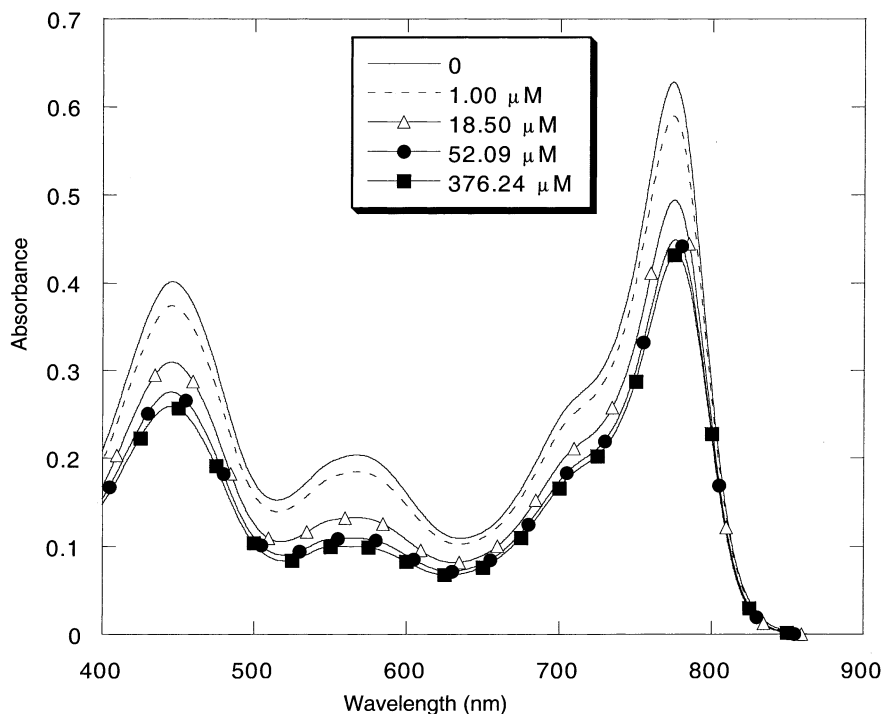


Fig. 2. Absorbance spectra of 19.52  $\mu\text{M}$  15C5-774 with increasing concentrations of  $\text{CaCl}_2$  in 10 mM Tris buffer and 20% MeOH (v/v) pH 7.4.

metal-free compound has a NIR absorption band centered at 774 nm with a Sorens shoulder at about 710 nm and two visible bands at 566 and 445 nm. The visible absorption at 566 nm is unusual in that it is normally not observed for heptamethine cyanines. This phenomenon is discussed in more detail in Section 3.4.

As shown in Fig. 2, the addition of calcium ion results in the decrease of absorbance at all wavelengths. As shown in Fig. 3, the fractional changes of the absorptions at both 774 and 566 nm of the dye can be fitted very well using Eq. (3), suggesting the formation of a 1:1 dye:metal complex [29,30]. Since the calculated  $K_d$  values based on the fractional intensity change at all absorption bands are identical, a summary of the affinities based on the NIR absorption is reported in Table 1.

Calcium is the strongest binding metal of the ions examined. With a  $K_d$  average of 3.17  $\mu\text{M}$  (Table 1), the strong affinity of 15C5-774 for  $\text{Ca}^{2+}$  indicates that this dye could be used for monitoring intracellular calcium concentrations between

the physiological resting and stimulated cell states since intracellular calcium concentrations vary from 0.1 to 10  $\mu\text{M}$ . The strong affinity is consistent with a good molecular fit of calcium ion within a cavity of 15-crown-5. With metal ions of other sizes the binding affinity is not as strong. With a decrease in the ionic radii of metal ions from 0.99 Å for calcium to 0.68 and 0.63 Å, this dye shows a weak binding affinity to zinc and magnesium with  $K_d$  values of 11.4 and 7.2  $\mu\text{M}$ , respectively. These smaller ions may not be able to form bonds efficiently with all of the oxygens in the crown. Strontium has an ionic radius of 1.12 Å, which is larger than that for calcium, and the affinity of this dye for strontium decreases about 4-fold. Its larger size prevents the ion from fitting efficiently into the cavity in the crown. Therefore, 15C5-774 shows metal selectivity for metal ions with ionic size close to the size of a crown cavity (Table 1). In comparison to these divalent metal ions, the size selectivity of 15C5-774 is smaller for monovalent ions.

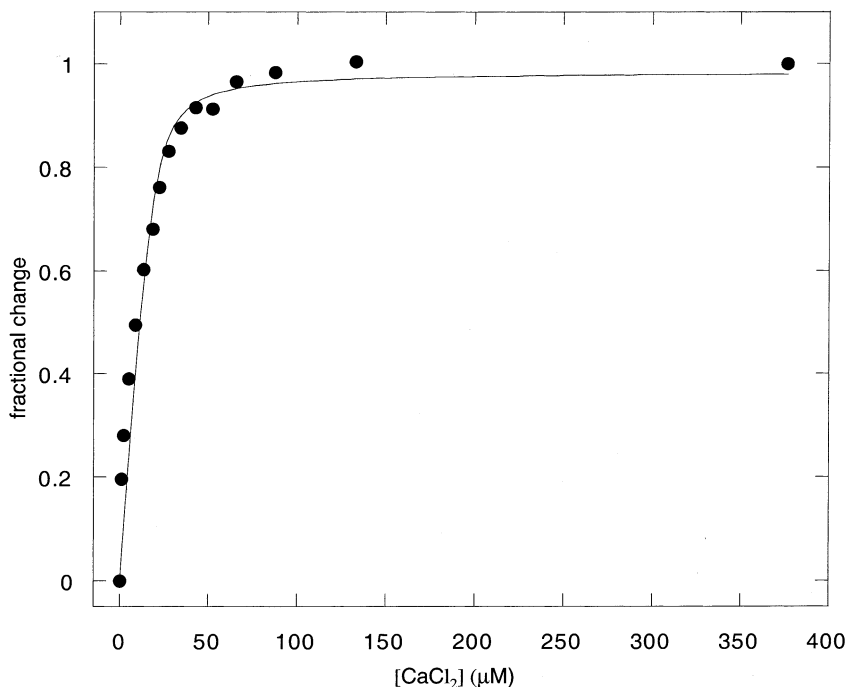


Fig. 3. Fractional change of the absorbance at 774 nm as a function of  $\text{Ca}^{2+}$  concentration in 10 mM Tris and 20% MeOH (v/v) pH 7.4. The solid line was generated by using the fractional change Eq. (3) assuming the formation of a 1:1 metal:dye complex. A virtually identical function was obtained by using the wavelength of 566 nm under otherwise identical conditions.

In summary, the results for both divalent and monovalent metal ions suggest that both the size and charge are the two key determinants contributing to the metal affinity of 15C5-774. As shown in Table 1, for similarly sized ions such as calcium and sodium, the dye binds 2.5 fold stronger to divalent calcium than monovalent sodium. The binding affinity of monovalent lithium is about 3-fold weaker than that of magnesium, despite the similar ionic size.

### 3.4. pH dependence

To test the pH dependence of 15C5-774, the Vis-NIR spectra of the metal-free compound and in the presence of calcium or magnesium were taken in the appropriate Tris buffer at a lower and higher pH than the physiological pH of 7.4. As shown in Fig. 4, the spectra of the dye change with pH. The spectra at pH 8.7 lack absorption at

566 nm and are typical for a heptamethine cyanine chromophore. The unusual absorption at 566 nm appears with decreasing pH, gradually increases in intensity, and becomes dominant at pH 6. These spectral changes are fully reversible with subsequent increases in pH of the acidified solution. On the other hand, the binding affinities of magnesium remain unchanged from pH 6–9 while

Table 1  
The average  $K_d$  values and ionic radii for the metals tested

Metal	$K_d$ ( $\mu\text{M}$ )	Ionic radius ( $\text{\AA}$ )
$\text{Zn}^{2+}$	$11.37 \pm 4.57$	0.63
$\text{Mg}^{2+}$	$7.20 \pm 2.29$	0.66
$\text{Li}^+$	$21.76 \pm 3.11$	0.68
$\text{Na}^+$	$10.69 \pm 0.89$	0.97
$\text{Ca}^{2+}$	$3.17 \pm 2.27$	0.99
$\text{Sr}^{2+}$	$10.14 \pm 0.19$	1.12
$\text{K}^+$	$10.62 \pm 1.11$	1.33

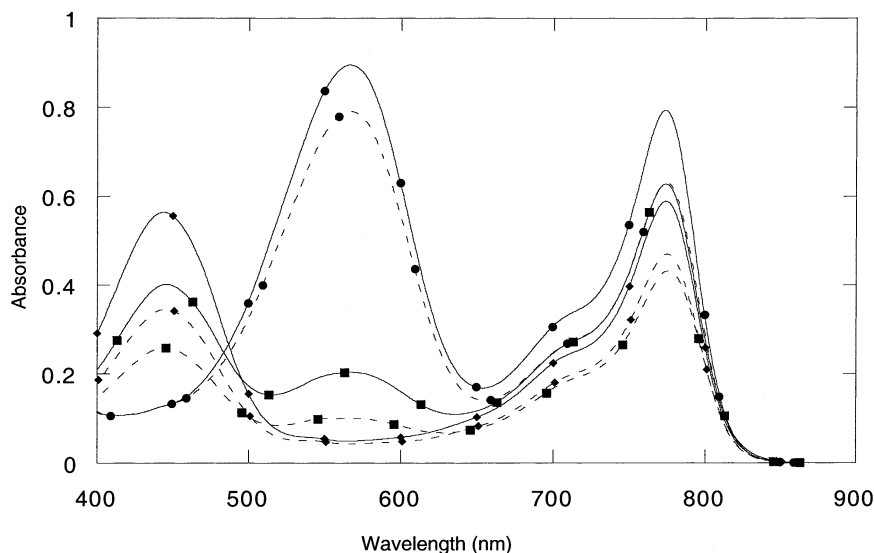


Fig. 4. A comparison of the  $\text{Ca}^{2+}$  free (—) and  $\text{Ca}^{2+}$  loaded (---) spectra of 15C5-774 at pH 6 (●), pH 7.4 (■), and pH 8.7 (▲) in Tris:MeOH (80:20 v/v).

the calcium affinity at pH 6 is identical to that at pH 7, and the affinity at pH 8.7 is decreased only 1.5-fold. These results are highly encouraging for our planned development studies of 15C5-774 as a practical reagent for metal determination *in vivo*. More specifically, variations in physiological pH will have a negligible effect on the calcium determination under different pH conditions *in vivo* that, at present time, are difficult to monitor. Interestingly, in addition to calcium determination, the strong pH dependence of the 566 nm absorption of 15C5-774 may be simultaneously used as the basis for the pH estimate *in vivo*.

Additional studies are needed to understand the presence of the 566 nm absorption in the spectrum of 15C5-774 under low pH conditions. It appears that, in addition to the normal  $s^0 \rightarrow s^1$  transition at 774 nm, a new electronic excitation becomes important for the protonated compound. Most likely, the 566 nm absorption results from excitation of the intramolecular complex between the protonated crown ether and the cyanine chromophore. Consistent with this suggestion are the results of our previous experimental and computational studies that showed the presence of net negative charges on carbons 1' and 7' of the heptamethine chain of cyanines [31] (Fig. 1).

### 3.5. Comparison of 15C5-774 and commercially available dyes

The binding affinity of 15C5-774 for calcium is in the same range as commercially available indicators that are frequently used for intracellular study. Dyes such as FURA-2, INDO-1, and Mag-FURA-2 have nanomolar to micromolar dissociation constants for calcium [6,32,33]. Our dye is well within this range. However, these dyes have maximum absorbance in the ultraviolet to visible regions of the spectrum where background interference is high. Our dye has maximum absorbance in the near infrared region where background interference is nearly nonexistent. Also, illuminating in the near infrared avoids the tissue damage that is seen with ultraviolet irradiation.

Much like FURA-2 and INDO-1, 15C5-774 should be protected from light whenever possible since it will undergo photodegradation. In normal working conditions, however, photodegradation does not severely affect the results since it takes 90 min of constant illumination for a loss of absorbance to be observed whereas INDO has been shown to lose absorbance within 10 min of constant exposure [34]. If the dye is protected from light, it is stable over several days, longer in

organic solvents. Like FURA-2 and INDO-1, 15C5-774 should be stored at  $-20\text{ }^{\circ}\text{C}$ , but, when protected from light, storage in solution at room temperature over several days does not affect the dye's stability. Unlike FURA-2 and INDO-1, the binding affinity of 15C5-774 is not greatly affected by pH.

As a means of transporting the dye into the cell for metal analysis, the dye can be made into ester derivatives in a similar manner as with commercially available dyes. We have reported the synthesis of ester-substituted near-infrared cyanine dyes [28,35]. Also, microinjection, a technique often used, can be used as a means to transport the dye into the cell [9]. Once inside the cell, concentration can be monitored by near UV absorbance ( $\sim 290\text{ nm}$ ), which does not change upon calcium binding. The absorbance at this wavelength is only concentration dependent from the indolium portion of the dye. The development of a dye with a wavelength shift upon calcium binding, by closer coupling of the cyanine chromophore and the metal binding moiety, is currently underway.

#### 4. Conclusions

A novel metal binding near-infrared dye with the advantage of avoiding background interference and possible damage from illumination in the UV-Vis region, 15C5-774, was designed, synthesized, and analyzed for its metal binding properties. The solution properties were examined, and the spectral properties of 15C5-774 were investigated by absorbance and NMR spectroscopy. The solubility of 15C5-774 is comparable to commercially available dyes, and 15C5-774 has been shown to be somewhat more stable. The metal binding affinities of 15C5-774 are in micromolar range for all metals tested and follow the order  $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+} \approx \text{K}^{+} \approx \text{Na}^{+} > \text{Zn}^{2+} > \text{Li}^{+}$ .

The calcium affinity of 15C5-774 is in the same range as FURA and INDO derivatives, indicating that 15C5-774 can be used to monitor intracellular calcium concentrations. The dye could also be used for in vitro work, such as for protein competition assays, since its metal binding affinities are

in the region needed to compare to many calcium binding proteins [36,37]. An additional advantage of 15C5-774 is that, while the binding affinities change only slightly with a pH change, the spectral properties do alter with pH allowing the dye to be used to monitor pH fluxes.

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