

## Ratiometric and Intensity-Based Zinc Sensors Built on Rhodol and Rhodamine Platforms

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A xantheno-forming condensation reaction yields rhodol and rhodamine dyes carrying a zinc-binding ligand that includes the aniline-type nitrogen donor of the fluorophores. Upon zinc coordination in neutral aqueous solution, rhodol RF3 behaves as a ratiometric sensor, and rhodamine RA1 acts as a turn-off intensity-based indicator. Both fluorescent compounds bind the divalent zinc cation with micromolar affinity.

Bioinorganic research continues to unveil the critical and often multifaceted roles of loosely bound metal ions, particularly late elements of the first transition series, in human health.<sup>1–3</sup> Discoveries about the (patho)physiology of these metal ions both stimulate and benefit from research on imaging techniques for biological specimens.<sup>4–7</sup> In this context, fluorescent probes for the spectroscopically silent zinc ion contribute to the study of mobile zinc signals in numerous biological settings,<sup>8–10</sup> including the central nervous<sup>11</sup> and immune systems.<sup>12</sup>

Considerable effort has been devoted in recent years to the development of fluorescent indicators for ratiometric detection of biological zinc.<sup>8,13,14</sup> Probes of this type respond to zinc coordination with a shift of their emission and/or absorption profiles. As such, each ratio of measured inten-

sities at two chosen wavelengths corresponds to a specific analyte concentration, which can be calculated from microscopy data in dual excitation or dual emission experiments. When compared to intensity-based systems, which respond to zinc binding with a change in emission intensity, ratiometric sensors are preferred in bioimaging applications because their detection is less sensitive to dye concentration, sample thickness, and photobleaching.

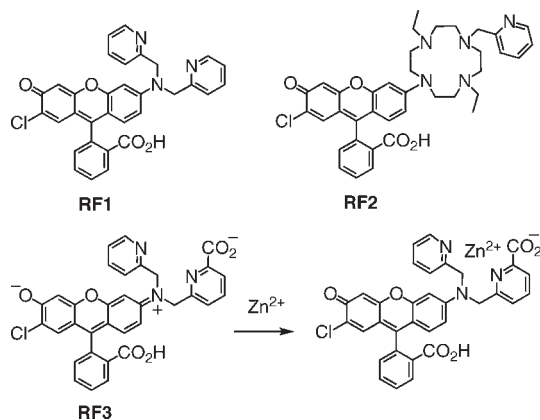
For the construction of a novel ratiometric indicator, we reexamined the asymmetric platform of rhodol (also known as rhodafluor) dyes. These rhodamine–fluorescein hybrid molecules retain several desirable properties of the parent compounds, namely water solubility, brightness, and visible-light excitability.<sup>15</sup> The subject of recent synthetic investigations,<sup>16,17</sup> rhodols have been employed in metal ion sensing. In order to elicit a ratiometric response, the coordinating analyte should interact directly with the  $\pi$ -system of the fluorophore, thereby affecting the energy levels that are responsible for emissive transition(s). The nitrogen donor of rhodol platforms has been incorporated in well-established chelators, such as 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetracetic acid (BAPTA),<sup>18</sup> 1,4,7,10-tetraazacyclododecane (cyclen),<sup>19</sup> and di(2-picolyl)amine (DPA).<sup>19</sup> Whereas the BAPTA systems are ratiometric calcium sensors, the DPA- and cyclen-based sensors (RF1 and RF2, respectively, Chart 1) show little or no affinity for zinc and do not exhibit ratiometric behavior.

In the present study, we modified the structure of RF1 to increase its zinc affinity and to engineer a zinc-induced shift of emission wavelength. A carboxylate moiety, negatively charged in neutral aqueous solution, was introduced on the DPA unit to increase zinc-binding affinity and to influence the charge distribution of the sensor candidate RF3 (Chart 1). Coulombic interactions with the negative charge on the carboxylate group were expected to stabilize a partial positive charge on the nitrogen donor of the rhodol scaffold.

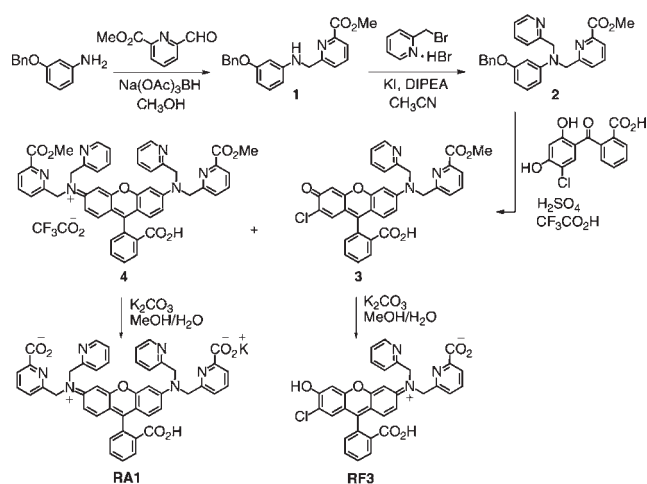
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Chart 1



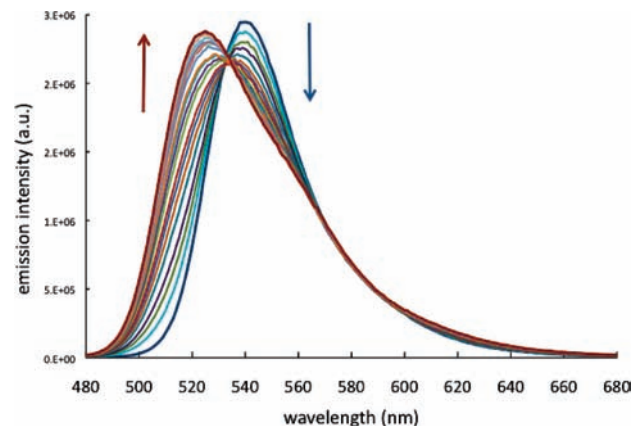
Scheme 1



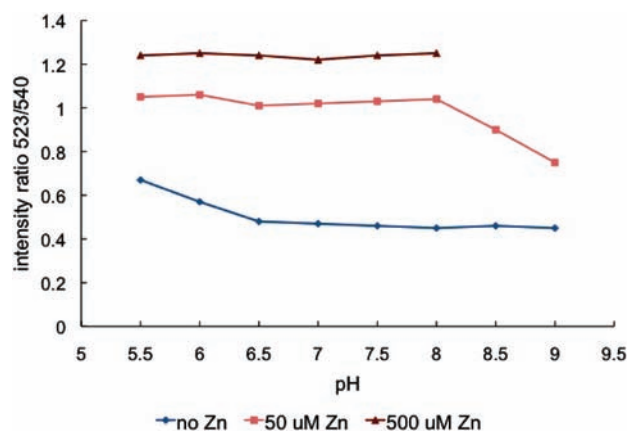
Coordination of the divalent zinc ion would in turn repel the positive charge and modulate the relative populations of the iminophenoxide and aminoquinone mesomers to the bonding description of the fluorophore (Chart 1). Such zinc-induced rearrangement of charge distribution was envisioned to elicit a change in absorption and emission wavelengths.

For the synthesis of rhodol RF3 (Scheme 1), a precursor containing the zinc-binding unit was constructed through reductive amination and subsequent alkylation under standard conditions (Supporting Information). Condensation of compound **2** with 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone afforded ethyl ester rhodol precursor **3** and, among other less abundant side products, compound **4**, an unusual rhodamine carrying two metal-binding units directly installed on the fluorophore. Compounds **3** and **4** were isolated and separately hydrolyzed to give RF3 and RA1, respectively.

Under simulated physiological conditions (50 mM aqueous PIPES buffer, 100 mM KCl, pH 7.0), rhodol RF3 exhibits visible absorption and emission maxima at 514 and 540 nm, respectively. Zinc coordination prompts a hypsochromic shift of the absorption band to 495 nm, with a decrease of the extinction coefficient from  $(9.5 \pm 0.2) \times 10^4$  to  $(4.4 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Supporting Information). In the emission profile (Figure 1), zinc binding is accompanied by a change of emission properties from a quantum yield of  $0.62 \pm 0.01$  at  $\lambda_{\text{max}}$  540 nm to a quantum yield of  $0.52 \pm 0.01$  at  $\lambda_{\text{max}}$  523 nm. As such, rhodol RF3 behaves as a ratiometric zinc



**Figure 1.** Emission intensity changes ( $\lambda_{\text{ex}}$  463 nm) observed upon titration of RF3 (1.0  $\mu\text{M}$ ) with  $\text{ZnCl}_2$  (up to 600 equiv) in neutral aqueous solution (50 mM PIPES buffer, 100 mM KCl, pH 7.0, 25  $^\circ\text{C}$ ).



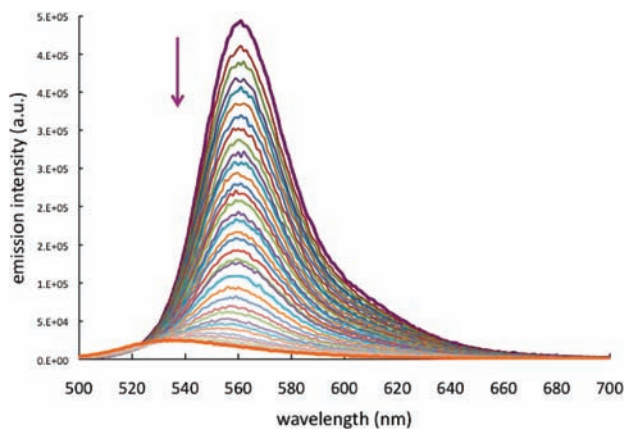
**Figure 2.** Zinc response of RF3 as a function of pH in buffered aqueous solutions (100 mM KCl, 25  $^\circ\text{C}$ ,  $\lambda_{\text{ex}}$  463 nm). Formation of a white precipitate was observed after the second zinc addition (final zinc concentration 500  $\mu\text{M}$ ) at pH 8.5 and 9.0; intensity values under these conditions were therefore not recorded.

sensor and its fluorescence response can be measured by the ratio of emission intensities at 523 and 540 nm ( $F_{523}/F_{540}$ ).

Zinc-induced changes in the emission profile of RF3 were monitored over the course of a fluorometric titration experiment (Figure 1), and Benesi–Hildebrand analysis revealed a dissociation constant ( $K_d$ ) of  $22 \pm 2 \mu\text{M}$  (Supporting Information). Zinc detection was not perturbed by biologically abundant Na(I), Ca(II), and Mg(II) ions. Several divalent transition metals, namely Mn(II), Fe(II), Co(II), Cd(II), and Hg(II), caused little or no interference (Supporting Information); however, Ni(II) and Cu(II) ions quenched the fluorescence emission such that the intensity ratio  $F_{523}/F_{540}$  could not be measured. Because these metal ions are not present in micromolar concentrations in typical biological specimens, they will not limit future applicability of RF3 in a variety of settings. Furthermore, the emission profile and zinc response of RF3 are stable over pH values ranging from 5.5 to 9.0 (Figure 2), and therefore this probe does not suffer from a common weakness of fluorescein-based sensors.

The rhodamine fluorochrome has been employed in numerous fluorometric and colorimetric indicators of metal ions.<sup>20,21</sup>

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**Figure 3.** Emission intensity changes ( $\lambda_{\text{ex}}$  490 nm) observed upon titration of RA1 (1.0  $\mu\text{M}$ ) with  $\text{ZnCl}_2$  (up to 300 equiv) in neutral aqueous solution (50 mM PIPES buffer, 100 mM KCl, pH 7.0, 25  $^\circ\text{C}$ ).

A common sensing mechanism features the analyte-induced opening of a nonfluorescent colorless rhodamine spirolactam to give a fluorescent species. In the present study, the isolated side product RA1 provided the opportunity to investigate a novel rhodamine-based sensor design.

Rhodamine RA1 displays intense absorption ( $\lambda_{\text{max}}$  535 nm) and emission ( $\lambda_{\text{max}}$  561 nm) bands characterized by a molar absorptivity of  $(4.5 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  and a quantum efficiency of  $0.70 \pm 0.01$ . Addition of  $\text{ZnCl}_2$  to a neutral aqueous solution of RA1 causes considerable loss of its visible-light absorption (Supporting Information) and emission features (Figure 3). This behavior is consistent with formation of a nonaromatic spirolactone species, in which the zinc-binding units do not share a positive charge and are available for coordination. According to this proposed reactivity, the sensor maximizes metal coordination capabilities at the expense of xanthene conjugation.

Binding data relative to fluorescence titrations of RA1 with  $\text{ZnCl}_2$  were fit to a 1:2 zinc-binding model by nonlinear least-squares methods. The resulting  $K_{\text{d}}$  values of  $0.34 \pm 0.05$  and  $2.2 \pm 0.4 \mu\text{M}$  are consistent with the moderate zinc-binding affinity of RF3 and, in general, with those of

aniline-fused DPA binding units.<sup>4</sup> The zinc-induced response of RA1 is not affected by millimolar concentrations of Na(I), Ca(II), and Mg(II); however, divalent transition-metal cations interact with the metal-binding units and quench fluorescence emission in part (Mn(II), Fe(II), Co(II), Cd(II), and Hg(II)) or in full (Ni(II) and Cu(II)), depending on their relative affinities and electronic structure (Supporting Information). Conversely, the emission intensity of RA1 is not affected by changes of pH in the 5.5–9.0 range, and the zinc turn-off response is only mildly reduced at pH values below 6.5 (Supporting Information).

In summary, considerations of charge distribution on the rhodol scaffold with respect to metal coordination led to the preparation of sensor RF3, which behaves as a ratiometric probe for zinc ions and indicates a promising direction in sensor design using rhodol platforms. Additionally, standard xanthene-forming condensation procedures afforded the symmetric rhodamine side product RA1, a turn-off intensity-based indicator for several divalent transition-metal cations. Both RF3 and RA1 bind zinc in the micromolar concentration range and are not affected significantly by pH changes in aqueous solution. When used in conjunction with fluorescein-based turn-on zinc indicators, RA1 could provide a useful control signal that is complementary both in color and in zinc response. Owing to their moderate affinity for zinc, RF3 and RA1 should prove valuable for the study of zinc-rich cellular compartments in a variety of organs, including the pancreas, the olfactory bulb, and the hippocampus.

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**Supporting Information Available:** Synthetic procedures and product characterization data, zinc-binding analysis methods, selectivity, and pH dependence charts. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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