Fluorescent dyes of the esculetin and alizarin families respond to zinc ions ratiometrically†

Lu Zhang, Shen Dong and Lei Zhu*

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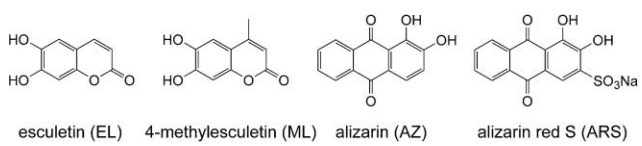
Commercially available dyes of the esculetin and alizarin families are identified as lead structures for constructing ratiometric fluorescent probes for zinc ions.

It is well-known that zinc ions are actively involved in numerous physiological processes.[1] However, the biochemical roles of zinc ions,[2] especially those in neural systems,[3,4] remain unclear. Determination of the spatiotemporal distribution of cellular zinc ion is a key step in unravelling the mechanisms of zinc-involved processes. Fluorescent probes targeting zinc have been developed so that cellular zinc flux can be studied in real time by fluorescence microscopy.[5,6] Among the available probes,[7] a unique group that is capable of ratiometrically determining zinc ion concentration stands out.[8–13] Ratiometric probes are desirable for quantitative analysis in complicated and heterogeneous intracellular media because a ratiometric read-out eliminates environmental influence on the fluorescence intensity.[14] Over the past decade, rapid progress has been made in the development of fluorescent ratiometric probes for zinc.[15] However, the few available structural types that are suitable for constructing them limit the fine-tuning of their photophysical properties and affinities to zinc ion, hence hampering the further development of this technology.

In this communication, we report the study of two fluorescent dye families that respond to zinc ions ratiometrically. The core structures of these dyes—6,7-dihydroxycoumarin and 1,2-dihydroxyanthraquinone—should serve as new lead structures for the development of sensitive and specific fluorescent ratiometric probes for zinc ions.

By surveying the Sigma-Aldrich Handbook of Stains, Dyes and Indicators,[16] we identified four fluorescent dyes (Chart 1) that report $[Zn]_t$ (total zinc concentration) ratiometrically in aqueous media. The apparent 1 : 1 association constants (K_a),[17] and the photophysical data of the dyes and their zinc complexes are compiled in Table 1. The absorption and emission spectra of all the dyes undergo bathochromic shifts upon addition of Zn^{2+}. Two of the dyes—4-methylesculetin (ML) and alizarin red S (ARS)—are discussed in detail. The spectra of the other two dyes are included in the ESI.†



esculetin (EL) 4-methylesculetin (ML) alizarin (AZ) alizarin red S (ARS)

Chart 1

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306-4390, USA. E-mail: lzhu@chem.fsu.edu; Fax: +1 850 644 8281; Tel: +1 850 645 6813

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ML has been used for the spectrophotometric analysis of metal ions, including Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+} and Cu^{2+}.[18,19] Except for Cu^{2+}, which was reported to quench the fluorescence of ML,[20] the fluorescence response of ML to metal ions has not been systematically studied.[21] Upon coordinating Zn^{2+}, both the absorption and fluorescence spectra of ML undergo bathochromic shifts, with an isosbestic point at 362 nm and an isoemissive point at 476 nm, respectively (Fig. 1). ML was known to form 2 : 1[22,23] or 3 : 1[24] coordination complexes with various metal ions. In our case, Job plot analysis indicated a 1 : 1 association between ML and Zn^{2+} at pH 7 (ESI†). The absorption binding isotherm (absorbance *vs.* $[Zn]_t$) of ML at 394 nm was fitted satisfyingly with a 1 : 1 binding equation[25] to afford an association constant (K_a) of $7.2 \times 10^3\ M^{-1}$. The fluorimetric titration[21] reveals a similar K_a of $6.1 \times 10^3\ M^{-1}$.

The fluorescence of ML responds to Zn^{2+} in a typical ratiometric manner. By plotting the ratio of the fluorescence intensity at 520 nm and 452 nm (I_{520}/I_{452}) *vs.* $[Zn]_t$, ML can be used to quantify $[Zn]_t$ over a high micromolar range (Fig. 2A, ◇). We also investigated the metal ion selectivity of ML. Among all the metal ions tested, the fluorescence of ML only responds to Zn^{2+} ratiometrically. ML has very little affinity, and hence little fluorescence response, to divalent metal ions such as Mg^{2+}, Ca^{2+}, Fe^{2+} and even Cd^{2+}, which is a known stereoelectronic isostere of Zn^{2+}. However, it does have substantial affinity for Pb^{2+} (apparent 1 : 1 $K_a \sim 4 \times 10^4\ M^{-1}$) and Cu^{2+} (forming a 2 : 1 complex, ESI†). The interaction with either metal ion results in fluorescence quenching (ESI†). The selectivity of ML toward metal ions of borderline hardness (*e.g.* Zn^{2+}, Cu^{2+} and Pb^{2+}) suggests that the catechol moiety of ML is a borderline base on Pearson's scale. The catechol moiety is fairly polarizable and readily oxidizable due to the involvement of the oxygen atoms in a π system. Therefore, catechol-based bases such as ML are softer than aliphatic alcohols or crown ethers, which selectively associate with hard alkali metal ions.

Literature precedence shows that the internal charge transfer (ICT) type probes, such as the cruciform-type[26,27] and coumarin-based molecules, tend to undergo *hypsochromic shifts* when the electron donor moieties are interacting with metal ions due to destabilization of the charge-separated excited state by metal ion coordination.[28–31] In our case, a *bathochromic shift* of ML was instead observed upon interaction with Zn^{2+} at the electron donor site. The association of ML with Zn^{2+} apparently promotes the

Table 1 Fluorescence response of the probes to Zn^{2+}—coordination and photophysical data in 75% methanol (10 mM HEPES, pH 7.1)

Probe	K_a[a]/M^{-1}	$\lambda_{ex}/\lambda_{em}$ (dye)[b]/nm	$\lambda_{ex}/\lambda_{em}$ (complex)/nm	Φ (dye)[c]	Φ (complex)
EL	3.4×10^3	348/467	400/~520[d]	0.02	0.02
ML	7.2×10^3	346/460	394/520	0.05	0.04
AZ	nd[e]	430/na[f]	530/629	0.001	0.002
ARS	nd[e]	433/566	530/619	0.001	0.005

[a] Apparent 1 : 1 association constant $K_a = [ZnL]_t/([Zn]_t[L]_t)$. $[Zn]_t$ = total Zn(II) concentration, $[L]_t$ = total dye concentration and $[ZnL]_t$ = total complex concentration. [b] λ_{ex} and λ_{em} are excitation and emission wavelengths of maximum intensity, respectively. [c] Φ = Fluorescence quantum yield. [d] Complex emission did not fully develop. [e] nd = not determined. ARS and AZ did not form 1 : 1 complexes with Zn^{2+} under our experimental conditions. [f] na = not applicable. Free AZ has little detectable fluorescence.

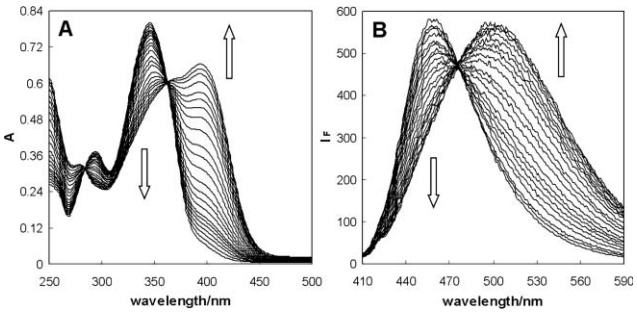

Fig. 1 (A) The absorption spectra of 4-methylesculetin (ML, 68 μM) in 75% methanol (10 mM HEPES at pH 7.1) in the presence of 0–0.3 mM $Zn(ClO_4)_2$. (B) The fluorescence spectra of ML (6.8 μM, λ_{ex} = 394 nm) in the presence of 0–0.3 mM $Zn(ClO_4)_2$. The arrows indicate the spectral change upon addition of Zn^{2+}.

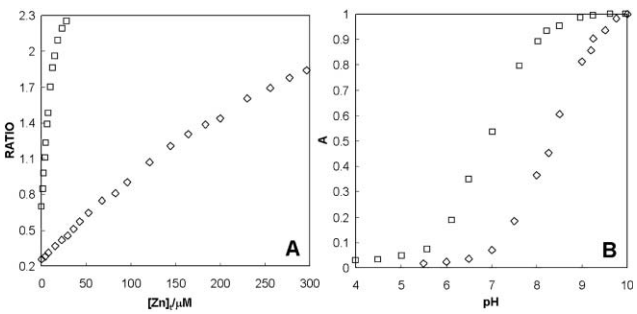

Fig. 2 (A) Fluorescent ratiometric responses of ML (◇, I_{520}/I_{452}) and ARS (□, I_{619}/I_{566}) to Zn^{2+}. (B) Normalized absorbance change of ML (◇, 394 nm) and ARS (□, 530 nm) *vs.* pH value.

deprotonation of ML from a neutral molecule to a coordinated dianion at pH 7 (Fig. 3). The pK_a of ML is estimated to be 8.3, based on the pH profile of the dye (Fig. 2B). At neutral pH, ML is fully protonated. The elevated electron density after Zn^{2+}-coordination-promoted deprotonation decreases the energy gap between the HOMO and LUMO of the $\pi \rightarrow \pi^*$ transition (Fig. 3, see ESI†).[32] Spectroscopically, bathochromic shifts of both absorption and fluorescence spectra were observed.

ARS has been used in the colorimetric analysis of metal ions.[33,34] However, the fluorescence property of ARS was not uncovered until recently for analytical use.[35–37] Kubo *et al.* demonstrated that an ARS–phenylboronic acid ensemble constitutes a fluorescence quenching assay for analyzing Cu^{2+}.[37] In our study, the inherent fluorescence of ARS was found to show a ratiometric response to Zn^{2+}.

At neutral pH, ARS shows two absorption bands (Fig. 4A), presumably from its neutral (440 nm) and monoanionic (530 nm)

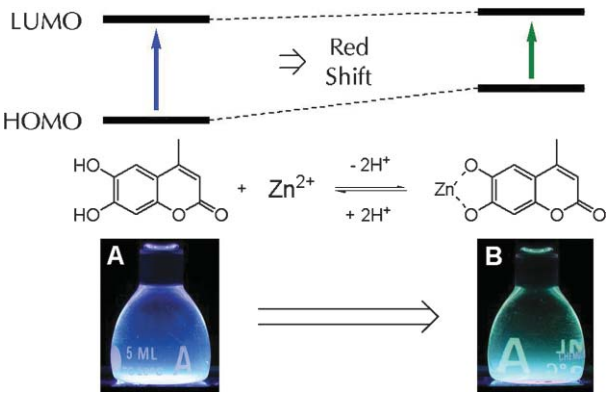

Fig. 3 Metal ion-coordination-promoted deprotonation may be the cause of the bathochromic shift of the spectra of ML. (A) ML (426 μM), (B) ML (426 μM) and $Zn(BF_4)_2$ (426 μM) in HEPES (75% methanol, 10 mM, pH 7).

forms. Upon coordinating Zn^{2+}, the deprotonation of ARS is promoted, and the absorption spectrum undergoes a bathochromic shift. When ARS is excited at 530 nm, where the Zn^{2+} complex absorbs, weak emission was observed. Upon addition of Zn^{2+}, the emission intensity was greatly enhanced (Fig. 4B).[38] The emission enhancement at 619 nm is much more rapid than that at 566 nm, the ratio of the intensities at these two wavelengths can be used to establish a ratiometric correlation with $[Zn]_t$ (Fig. 2A, □) in a sub-micromolar range. To our delight, ARS not only displayed a sensitive ratiometric response to Zn^{2+}, but the fluorescence intensity increased across the spectrum, which also makes it a fluorescence turn-on switch for Zn^{2+}.[10] The Job plot suggested a 2 : 1 (ARS : Zn^{2+}) association (ESI†). A similar 2 : 1

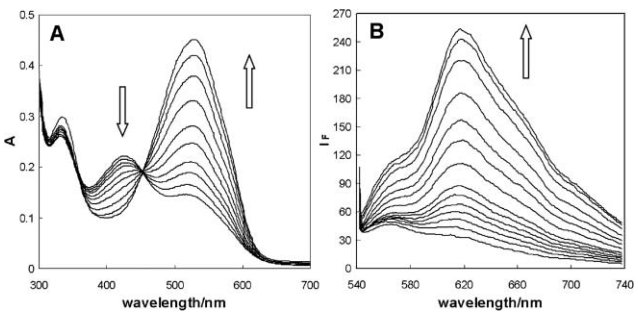

Fig. 4 (A) The absorption spectra of alizarin red S (ARS, 64 μM) in 75% methanol (10 mM HEPES at pH 7.1) in the presence of 0–74 μM $Zn(BF_4)_2$. (B) The fluorescence spectra of ARS (29 μM, λ_{ex} = 530 nm) in the presence of 0–33 μM $Zn(BF_4)_2$. The arrows indicate the spectral change upon addition of Zn^{2+}.

coordination stoichiometry was also observed in the complex of ARS and Cu^{2+}.[36] The fluorescence intensity of ARS ($[ARS]_t$ = 29 μM) reaches a maximum at 30 μM of Zn^{2+} (ESI†), which makes it a much more sensitive probe for Zn^{2+} than ML ($[ML]_t$ = 6.8 μM, not saturated at 300 μM of Zn^{2+}, ESI†) at neutral pH. On the other hand, ARS is not specific to Zn^{2+} as it has been used as an indicator for the EDTA titration of various metal ions.[39] Among the divalent metal ions (Zn^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+} and Pb^{2+}) studied, Zn^{2+} and surprisingly Pb^{2+}, which quenches the fluorescence of ML, gave rise to dramatic ratiometric fluorescence responses with ARS (ESI†).

The different acidities of ML and ARS contribute substantially to the differing affinities to Zn^{2+} at neutral pH. As shown in the pH profiles of ML and ARS (Fig. 2B), ML is a neutral molecule at pH 7. However, one of the phenolic hydroxyl groups of ARS is mostly deprotonated. The lower pK_a of ARS gives rise to its higher affinity to Zn^{2+} than ML. A significantly lower affinity between ARS and Zn^{2+} was observed at pH 5.5, when both phenolic hydroxyl groups are fully protonated. The binding isotherm was fitted to a 1 : 1 binding equation to afford an apparent 1 : 1 association constant of $3 \times 10^4\ M^{-1}$ (ESI†). On the other hand, a titration of ML with Zn^{2+} at pH 8 showed that the absorbance of ML became saturated at a much lower $[Zn]_t$ than observed at pH 7, which indicates a much higher affinity at pH 8. The association stoichiometry also changes with pH. At pH 9, ML forms a 3 : 1 complex with Zn^{2+}, as shown by Job plot analysis (ESI†).

In summary, we have discovered that (1) the fluorescence intensities of 6,7-dihydroxycoumarin- and 1,2-dihydroxyanthraquinone-based dyes respond ratiometrically to Zn^{2+}. The affinity of the latter to Zn^{2+} is much larger than that of the former at neutral pH, due partly to the lower pK_a of dihydroxyanthraquinone. (2) Among the common divalent metal ions, only Zn^{2+} gives a dramatic ratiometric response to ML; however, the fluorescence of ARS changes ratiometrically with either Zn^{2+} or Pb^{2+}. (3) Based on pH profile studies and theoretical calculations using HyperChem (ESI†), we attribute the observed bathochromic shifts of all the dyes upon interacting with Zn^{2+} to coordination-promoted deprotonation of the two hydroxyl groups. 6,7-Dihydroxycoumarin and 1,2-dihydroxyanthraquinone can be used as new lead structures for constructing sensitive and specific fluorescent ratiometric probes targeting zinc ions.

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