

Double discrimination by binding and reactivity in fluorescent metal ion detection

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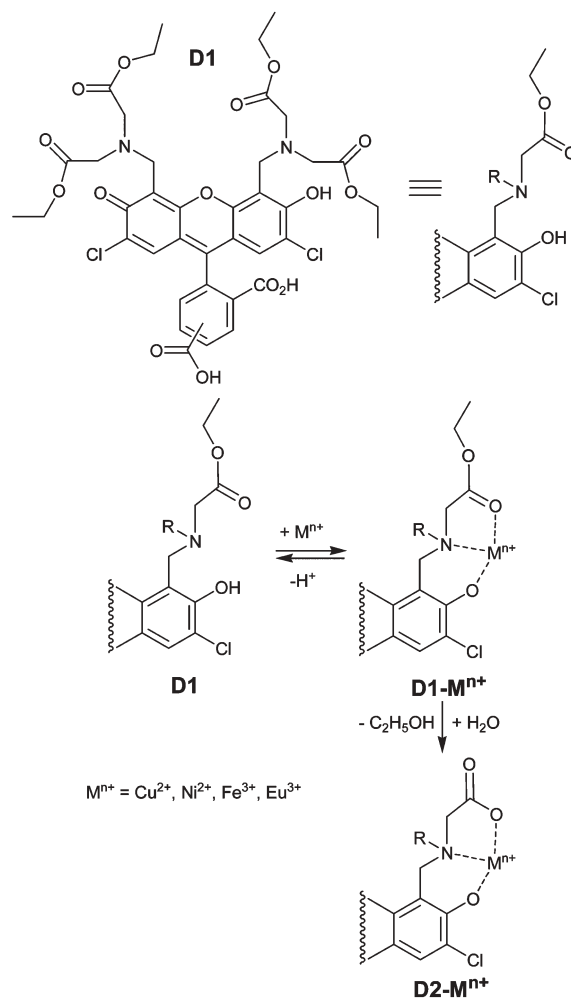
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Metal ion detection using a fluorescent dye containing reactive ester groups allows discrimination on the basis of the hydrolytic reactivities of metal ions, which display the same qualitative fluorescence response on binding only.

Analysis of metal ions (M^{n+}) using fluorescent molecular sensors attracts a lot of attention, because this method is very sensitive, quick, does not require expensive instrumentation and can be performed in a high-throughput format.¹ The sensors usually combine a chelating ligand and a fluorophore, the emission of which is affected by metal ion coordination.² Responses depend on the thermodynamic stabilities of the corresponding M^{n+} complexes, which means that the effect of the metal ions is concentration dependent. For example, fluorescence emission of commercially available metal ion sensors, Phen Green SK, FL,³ Calcein⁴ and Fura-2,⁵ is quenched by Cu^{2+} at $>\mu M$ concentrations. Analogous quenching effects can, however, be achieved by other paramagnetic metal ions, e.g. Ni^{2+} , in more concentrated solutions, which means that samples of unknown composition cannot be unambiguously analyzed.

Here we present a dye, whose fluorescence emission is affected not only by metal ion binding, but additionally by a metal ion promoted reaction. If the former process is faster than the latter one, two parameters can be obtained from every analyte in a single experiment. These are a quick fluorescence change corresponding to the metal ion binding (F/F_0) and a slower change corresponding to the chemical reaction (dF/dt). In comparison, only the parameter F/F_0 is obtained from standard sensors.¹⁻⁴ This increased amount of information should allow discrimination of metal ions, which qualitatively effect the same response of the fluorophore (i.e. either fluorescence quenching or increase). In this paper we present **D1**, the first hybrid sensor⁶ of this type (Scheme 1). It is based on a Calcein framework, the iminodiacetate groups of which are esterified. A carboxylic group introduced at the 5(6)-position increases the solubility of **D1** in water. The dye was synthesized from 2',7'-dichlorofluorescein-3',6'-diacetate-5(6)-carboxylate⁷ and diethyliminodiacetate using the protocol reported for similar derivatives.⁸ After addition of a large excess of Cu^{2+} , Ni^{2+} , Fe^{3+} or Eu^{3+} to **D1** its fluorescence is immediately quenched ($F/F_0 \sim 0$), which indicates that the complexes formed in these solutions are not fluorescent in analogy with the complexes of the parent compound Calcein. In accordance with the structure of **D1**, mono and binuclear complexes can potentially be formed. However, in ESI mass spectra of these solutions no peaks corresponding to binuclear complexes $D1-(M^{n+})_2$ are present,



Scheme 1 Structure of the reactive sensor **D1**. Binding of M^{2+} to **D1** and hydrolysis of the latter.

whereas peaks corresponding to mononuclear $D1-M^{n+}$ complexes can be detected. This suggests that $D1-M^{n+}$ is the only species in these solutions. Stability constants K_d of $D1-M^{n+}$ complexes have been determined by fluorimetry (Table 1). The affinity of **D1** is considerably higher towards the Cu^{2+} ion than the other metal ions (Table 1). However, the metal ion discrimination based solely on the binding event could be masked by increased concentrations of the weaker binding M^{n+} ions. For example, Cu^{2+} (20 μM) or 50 times more concentrated Ni^{2+} , Fe^{3+} or Eu^{3+} produce similar immediate quenching (F/F_0) of the fluorescence of **D1** (Fig. 1, Table 1).

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Table 1 Changes of fluorescence emission intensity of **D1** upon addition of different metal ions and thermodynamic parameters of some **D1**– M^{n+} complexes

| M^{n+} | $[M^{n+}]_{\text{total}}/\text{mM}$ | F/F_0 | $(dF/dt)_0$ | $K_d(\mathbf{D1}-M^{n+})/10^6 \text{ M}^{-1}$ |
|------------------|-------------------------------------|---------|-------------|---|
| Cu^{2+} | 0.02 | 0.42 | -7.12 | 12 ± 3 |
| Ni^{2+} | 0.97 | 0.39 | -0.42 | 601 ± 20 |
| Fe^{3+} | 0.97 | 0.39 | -0.55 | 522 ± 90 |
| Eu^{3+} | 1.2 | 0.40 | -2.54 | 497 ± 550^a |
| Zn^{2+} | 1 | 1.07 | 0.30 | — |
| Ca^{2+} | 1 | 0.94 | ~ 0 | — |
| Mg^{2+} | 1 | 0.98 | ~ 0 | — |
| K^+ | 5 | 0.98 | ~ 0 | — |

^a The high standard deviation of the determined K_d may indicate formation of complexes other than **D1**– M^{n+} in solutions containing excess Eu^{3+} .

The reactive ester group of **D1**– Cu^{2+} is hydrolyzed with formation of the carboxylate complex of **D2** (Scheme 1). It follows from the ESI mass spectral study that only a single ester group of **D1** is hydrolyzed (Fig. 2) even after prolonged reaction time. Since the carboxylic group of **D2** is a better donor than the parent ester group, metal complexes of **D2** are expected to be more stable than those of **D1**. This has been confirmed for Cu^{2+} : $K_d(\mathbf{D1}-\text{Cu}^{2+})/K_d(\mathbf{D2}-\text{Cu}^{2+}) = 3.2$. This effect is reflected in decreasing intensity of the overall fluorescence emission with time upon **D1** hydrolysis in the presence of a metal ion concentration which does not saturate the chelating site (Fig. 1). The initial rate of **D1** hydrolysis is proportional to the linear part of the dependence of fluorescence emission intensity vs. time $(dF/dt)_0$ (Fig. 1, Table 1). The latter values have been used for estimation of activities of metal ions in promoting **D1** hydrolysis, assuming that metal ion affinity to the fluorophore is significantly increased by ester hydrolysis. It is well documented that metal ions have different abilities to activate ester groups toward hydrolysis.⁹ On the basis of $(dF/dt)_0$ values one can clearly distinguish Cu^{2+} (20 μM), Ni^{2+} , Fe^{3+} and Eu^{3+} (about 1 mM), which give very similar immediate fluorescence quenching F/F_0 . Hydrolytic activities of selected metal ions follow the given trend: $\text{Cu}^{2+} > \text{Eu}^{3+} > \text{Fe}^{3+} > \text{Ni}^{2+} > \text{Zn}^{2+} \gg \text{Ca}^{2+}, \text{Mg}^{2+}, \text{K}^+$. In the case of Zn^{2+} the fluorescence of **D1** is

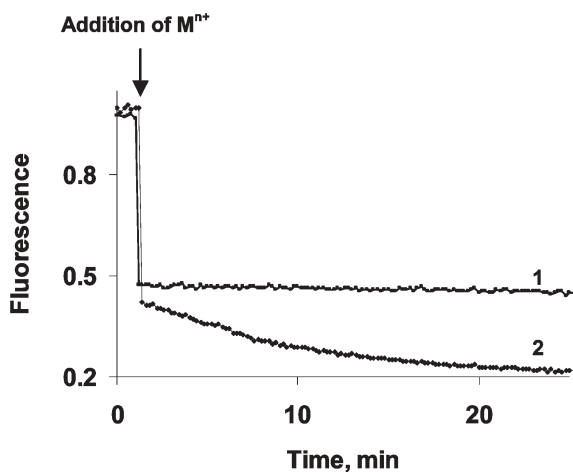


Fig. 1 Dependence of normalized fluorescence (F/F_0) on time after addition of Ni^{2+} (1 mM, trace 1) or Cu^{2+} (20 μM , trace 2). $[\mathbf{D1}] = 1 \mu\text{M}$; MOPS 10 mM, pH 7; NaCl 50 mM; solvent: water with 1% DMSO.

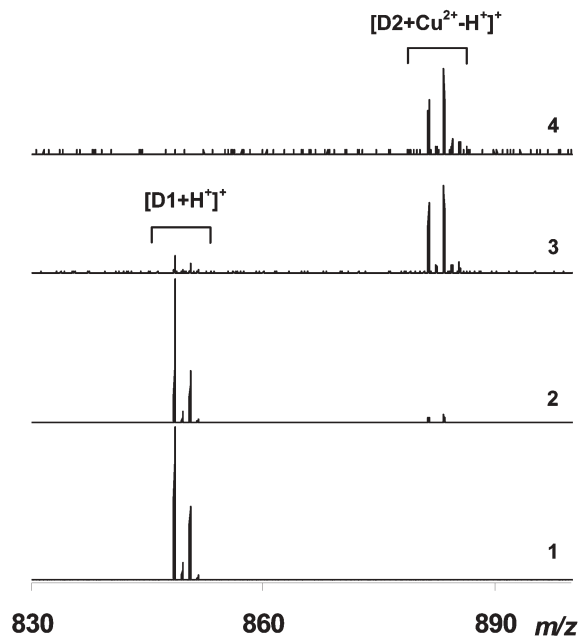


Fig. 2 ESI mass spectra of solution of **D1** (20 μM), CuSO_4 (20 equiv.) in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (1 : 1), titrated with NET_3 to pH 7: (1) immediately after CuSO_4 addition; (2) 25 min after CuSO_4 addition; (3) 60 min after CuSO_4 addition; (4) 90 min after CuSO_4 addition.

enhanced by both binding and ester cleavage by the metal ion, as expected in view of the 1.5 fold increase of Calcein fluorescence at saturation with Zn^{2+} .

Metal ion promoted reactions which affect the fluorescence of a reactant have been used earlier for Cu^{2+} detection by determination of end-point fluorescence intensity (chemodosimetry).¹⁰ Indirect analysis of the same metal ion by kinetic methods has been achieved by replacement/release of a catalytically active Pd^{2+} ion from a macrocyclic complex upon exposure to Cu^{2+} .¹¹ Both methods are Cu^{2+} specific and do not allow a kinetic discrimination of different metal ions.

In conclusion, the fluorescence of a dye which is modified with a “reactive” chelating group is affected by both binding and subsequently in a time-dependent manner by the hydrolytic reactivity of a metal ion. The additional kinetic parameter dF/dt obtained with this sensor type allows to discriminate metal ions (Cu^{2+} , Ni^{2+} , Fe^{3+} , Eu^{3+}) which give the same immediate fluorescence response and are not readily distinguished by a single sensor that relies on metal ion binding only.

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Notes and references

† Synthesis of **D1**: paraformaldehyde (0.48 g, 16 mmol) and diethyliminodiacetate (1.51 g, 8 mmol) were added to acetonitril (50 mL) and the suspension obtained was heated to reflux for 90 min. 2',7'-dichlorofluorescein-3',6'-diacetate-5(6)-carboxylate pyridinium salt (0.98 g, 1.6 mmol) in acetonitril–water (1 : 1, 50 mL) was added and the resulting solution was left stirring for 24 h. After cooling the reaction mixture CH_3CN was removed on a rotary evaporator and the aqueous solution was acidified

with acetic acid to pH 4, cooled down to 4 °C and left standing at this temperature for 2 h. The precipitate formed was filtered, washed with cold water and dried at 0.01 mbar. The product was purified by silica gel chromatography using a CHCl₃-EtOH (9 : 1) mixture containing 0.1% AcOH. The resulting bright orange solid is a mixture of two isomers in ~4 : 1 molar ratio (6 : 5-isomers). Yield 0.62 g, 42%, $R_f = 0.6$ in CHCl₃-EtOH (1 : 5) mixture containing 0.1% AcOH. ¹H NMR (δ , ppm, relative to TMS): 9.59 (broad s), 8.75 (s, 0.3H), 8.42 (dd, 0.3H, $3J = 8.2$ Hz, $4J = 1.4$ Hz), 8.37 (dd, 1H, $3J = 8.0$ Hz, $4J = 1.2$ Hz), 7.90 (s, 1H), 8.13 (dd, 1H, $3J = 8.0$ Hz, $5J = 0.6$ Hz), 7.3 (dd, 0.3H, $3J = 8.0$ Hz, $5J = 0.8$ Hz), 6.62 (s, 0.6H), 6.60 (s, 2H), 4.42 (d, 2H, $2J = 14.3$ Hz), 4.44 (d, 0.6 H, $2J = 14.3$), 4.28 (m, 2.6H), 4.20 and 4.21 (two q, 10.4H, $3J = 7.2$ Hz), 3.60, 3.61 (two s, 10.4H), 2.11 (s, 3.9H), 1.27 (t, 15.6H, $3J = 7.2$ Hz). HR-ESI-MS, positive mode: found 847.1862, calcd. for C₃₉H₄₁Cl₂N₂O₁₅ [M + H]⁺ 847.1883. CHN analysis: found C—52.03%, H—4.91%, N—3.36%, calcd. for C₄₁H₄₈Cl₂N₂O₁₉ ([D1·CH₃CO₂H·(H₂O)₂): C—52.2%, H—5.1%, N—3.0%.

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