

# Discrimination between Hard Metals with Soft Ligand Donor Atoms: An On-Fluorescence Probe for Manganese(II)\*\*

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Manganese is an essential metal in all forms of life.<sup>[1]</sup> It participates as a cofactor in diverse classes of enzymes and the photosynthetic machinery<sup>[2]</sup> and is used widely as a versatile tool for biological studies. For example, high-spin  $\text{Mn}^{2+}$  is an excellent MRI relaxation agent that has been used in clinical diagnosis and is of widespread interest as a tool in neurobiological research.<sup>[3]</sup> However, chronic overexposure can result in movement disorders and mental disturbances and other brain-related toxicities.<sup>[4]</sup> Fluorescent probes would be useful for detection and quantification of  $\text{Mn}^{2+}$ , as this method offers high efficiency, high sensitivity, and easy operation<sup>[5]</sup> among available methods of detection.<sup>[6]</sup> However, development of an effective fluorescent probe for  $\text{Mn}^{2+}$  faces several challenges: 1) Unlike diamagnetic metal ions such as  $\text{Zn}^{2+}$ , paramagnetic  $\text{Mn}^{2+}$  can quench fluorescence. Although chelation-induced fluorescence quenching (CHEQ) is the most commonly used method of paramagnetic metal ion detection,<sup>[7]</sup> “on-fluorescence” probes for  $\text{Mn}^{2+}$  are preferred. 2)  $\text{Mn}^{2+}$  selectivity over abundant cellular metal ions is required, especially  $\text{Ca}^{2+}$  (up to high  $\mu\text{M}$ ).<sup>[8]</sup>  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  share many common properties, underscored by the fact that  $\text{Mn}^{2+}$  can enter cells using some of the same transport systems as  $\text{Ca}^{2+}$ .<sup>[9]</sup> 3)  $\text{Mn}^{2+}$  probes must be compatible with biological environments, including water solubility, biological inertness, long-wavelength excitation and emission profiles to minimize sample damage and native cellular autofluorescence.<sup>[10]</sup> 4) To visualize  $\text{Mn}^{2+}$  in living cells or tissues, membrane permeability is important.<sup>[10]</sup>

Several commercially available chelating dyes produce strong fluorescence enhancement upon binding  $\text{Mn}^{2+}$ .<sup>[11]</sup> However, the fluorescence of available dyes such as calcium green is also enhanced in the presence of  $\text{Ca}^{2+}$ . Bapta (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid) is a known  $\text{Ca}^{2+}$ -selective ligand that serves as the chelating moiety of calcium green.<sup>[12]</sup> We undertook to modify the bapta unit in such a way as to achieve adequate Mn/Ca selectivity.

Optimizing stereoelectronic complementarity between host and guest to achieve efficient complexation is a long-

held principle of supramolecular and coordination chemistry. However, while optimizing a receptor for a substrate leads to strong binding, it may not result in good selectivity over competing substrates. In the case of metal-ion complexation, selection of ligand donor atom can sometimes be used to advantage. For example, choosing soft donor atoms may improve selectivity for relatively soft metal ions in competition with hard ones. Both  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$ , however, are generally considered to be hard and show maximal stability with hard oxygen donors. Thus, superficial considerations would lead away from hard/soft donor atom considerations as a strategy for achieving  $\text{Mn}^{2+}$  selectivity.

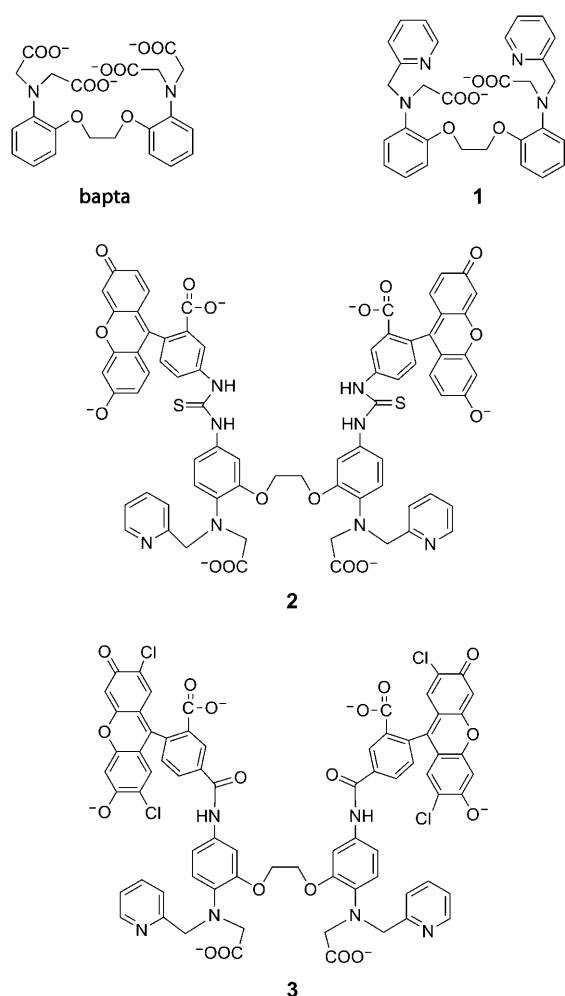
However, although both  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  are classified as “hard” metals and therefore form stronger complexes with oxygen donors,  $\text{Mn}^{2+}$  appears to be more tolerant of softer atom donors than  $\text{Ca}^{2+}$ .<sup>[13,14]</sup> More recently, the relative softness of  $\text{Mn}^{2+}$  compared with  $\text{Mg}^{2+}$  has been debated as the basis for  $\text{Mn}^{2+}$  rescue of activity in dialkylthiophosphate RNAses.<sup>[15–17]</sup> Our hypothesis was thus to replace two or more carboxylate groups in bapta with softer ligating moieties. Since  $\text{Mn}^{2+}$  is a hard metal ion, weaker binding might be expected from such a change, but since  $\text{Ca}^{2+}$  is an even harder metal ion, the effect on  $\text{Ca}^{2+}$  should be more pronounced, resulting in a net increase in selectivity. We chose nitrogen atom donors from pyridine, which is a common binding group in transition metal ion ligands considered to be borderline but softer than oxygen.<sup>[14]</sup> To evaluate the feasibility of our strategy, a prototype ligand **1** was synthesized, which has one carboxylate group of each dicarboxymethylamino moiety of bapta replaced by a pyridine (Scheme 1). The chemical synthesis of ligands **1–3** is included in the Supporting Information.

UV titrations were carried out by addition of  $\text{MnCl}_2$  to MOPS buffered aqueous solution (pH 7.2; MOPS = 3-(*N*-morpholino)propanesulfonic acid) of **1** (Supporting Information). In the absence of  $\text{Mn}^{2+}$ , the spectrum of **1** showed a maximum at 256 nm with a shoulder at 286 nm, similar to bapta.  $\text{Mn}^{2+}$  complexation caused significant hypsochromic shifts towards a limiting spectrum with a small maximum at 278 nm surrounded by shoulders. Absorbance at 256 nm was plotted as a function of  $\text{Mn}^{2+}$  concentration and the minimum level of absorbance was reached upon addition of 1 equivalent of  $\text{Mn}^{2+}$ , suggesting 1:1 metal–ligand complex. The same analysis was applied to determine 1:1 complexation of bapta to  $\text{Mn}^{2+}$ . The binding constants were obtained by titration in pH- and  $\text{Mn}^{2+}$ -buffered aqueous media. The plot of absorbance as a function of free  $\text{Mn}^{2+}$  produced a sigmoidal curve. Nonlinear fitting analysis<sup>[18]</sup> gave association constants ( $\log K$ ) of 8.62 for ligand **1** and 9.14 for bapta (Table 1). These results indicate that substitution of two carboxylate groups of

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**Scheme 1.** Structures of ligands and probes.

bapta with two pyridines impairs binding affinity to  $\text{Mn}^{2+}$  to a surprisingly small extent.

Calcium binding to ligand **1** was investigated in a similar manner. A hypsochromic shift was again observed, but even excess  $\text{Ca}^{2+}$  did not saturate the UV absorption indicating weak affinity. The association constant of **1**– $\text{Ca}^{2+}$  was determined to be  $\log(K_a) = 3.79$ , about 4.83 log units weaker than for  $\text{Mn}^{2+}$ . The  $\log(K_a)$  of bapta– $\text{Ca}^{2+}$  was 6.89,<sup>[11]</sup> only 2.25 log units lower than that of bapta– $\text{Mn}^{2+}$  complex. Therefore, ligand **1** indeed shows much higher selectivity for  $\text{Mn}^{2+}$  over  $\text{Ca}^{2+}$  as compared to bapta. The significant improvement in  $\text{Mn}^{2+}/\text{Ca}^{2+}$  selectivity validates our “selective poisoning” strategy.

To realize the goal of a fluorescent  $\text{Mn}^{2+}$  probe, compound **1** was further functionalized to include a chromophore similar to that present in calcium green. Thus, amino groups installed *para* to the N atoms of both aniline moieties were coupled with fluorescein-5-isothiocyanate in high yield followed by hydrolysis afforded fluorescent probe **2**. Probe **3**, most similar in structure to calcium green-2, was prepared containing chloro substituents and an amide linkage between chelating unit and fluorophore.

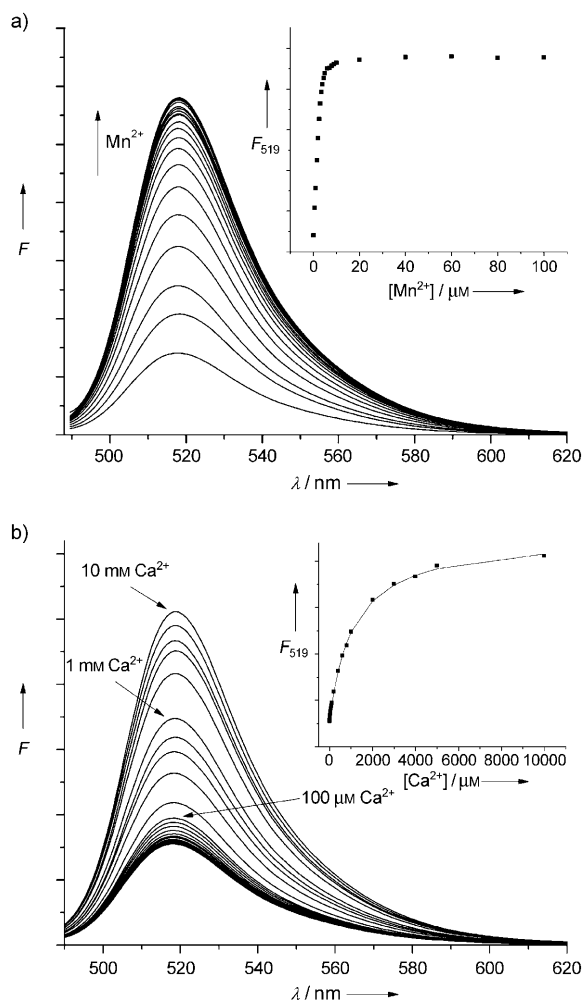
The binding properties of fluorescent probe **2** were first investigated by titration with  $\text{Mn}^{2+}$  (Figure 1 a). When excited at 493 nm, **2** showed an emission maximum at 519 nm. Upon addition of  $\text{Mn}^{2+}$ , an enhanced fluorescence was observed until saturation after 1 equivalent. The quantum yields of the free probe and  $\text{Mn}^{2+}$ -bound complex were determined to be 0.10 and 0.37, respectively. The association constant with  $\text{Mn}^{2+}$  was  $\log K = 7.01$ . Similar to the prototype ligand **1**, only a large excess of  $\text{Ca}^{2+}$  ion (mM) caused fluorescence enhancement indicating weak association of **2** to  $\text{Ca}^{2+}$  ( $\log K = 2.96$ ). In the cellular environment, calcium concentration is generally lower than 100  $\mu\text{M}$ . Therefore, probe **2** has the potential to detect  $\text{Mn}^{2+}$  in the presence of calcium ion interference in biological systems. Screening for selectivity against other metal ions  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{K}^+$  (see Supporting Information) showed no effect on fluorescence intensity of **2**. However, transition metal ions,  $\text{Ni}^{2+}$  and  $\text{Cu}^{2+}$  quench the fluorescence, while  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$  may interfere with  $\text{Mn}^{2+}$  binding. Among these,  $\text{Zn}^{2+}$  (Table 1) is probably the most significant concern for likely applications. Dissociation constants determined for  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Cd}^{2+}$  for bapta (9.13, 10.51, 13.38) and **1** (9.22, 10.23, 13.58) together with data for  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  (Table 1) indicate that the two ligands follow the Irving–Williams series very similarly, and that the discrimination against  $\text{Ca}^{2+}$  is much greater than the effect observed on soft metals.

Compound **3** showed a longer  $\lambda_{\text{exc}}$  (505 nm) and  $\lambda_{\text{em}}$  (530 nm) due to the two incorporated chlorine atoms on the fluorophore (Supporting Information). The  $\text{Mn}^{2+}$  complex showed enhanced fluorescence. Probe **3** maintained high selectivity for  $\text{Mn}^{2+}$  over  $\text{Ca}^{2+}$  with  $\log K$  of 8.00 for  $\text{Mn}^{2+}$  and 3.33 for  $\text{Ca}^{2+}$ . The quantum yield of free probe **3** was 0.13, while that for the  $\text{Mn}^{2+}$  complex was 0.49. Probe **3** shared similar binding properties to probe **2** to other metal ions (Supporting Information). Probe **2** was responsive over physiologically relevant pH 6.8 to 8.2. Interestingly, probe **3** functions well in basic conditions: From pH 8.3 to pH 12.2, the high pH limit of our measurement, the fluorescence showed nearly full response. To our knowledge, probes **2** and

**Table 1:**  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  affinities and spectroscopic properties of ligands.

Ligand	$\text{Mn}^{2+}$	$\log K$ $\text{Ca}^{2+}$	$\log K$ $\text{Mg}^{2+}$	$\log K$ $\text{Zn}^{2+}$	Selectivity $\log(K_{\text{Mn}}/K_{\text{Ca}})$	free ligand	$\lambda_{\text{max}}^{\text{[a]}}$ $\text{Mn}^{2+}$ complex	Ex	Em	free ligand	$\phi_f$ $\text{Mn}^{2+}$ complex
bapta	9.14	6.89	1.69	9.41	2.25	290 (4.8)	278 (4.5)	–	–	–	–
<b>1</b>	8.62	3.79	0.76	9.19	4.83	286 (6.3)	283 (4.1)	–	–	–	–
<b>2</b>	7.01	2.96	0.42	6.16	4.05	493 (84)	493 (100)	493	519	0.10	0.37
<b>3</b>	8.00	3.33	0.93	7.09	4.68	507 (80)	507 (92)	505	530	0.13	0.49

[a] The corresponding molar absorption coefficient  $\epsilon$  [ $10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ ] is given in parentheses.

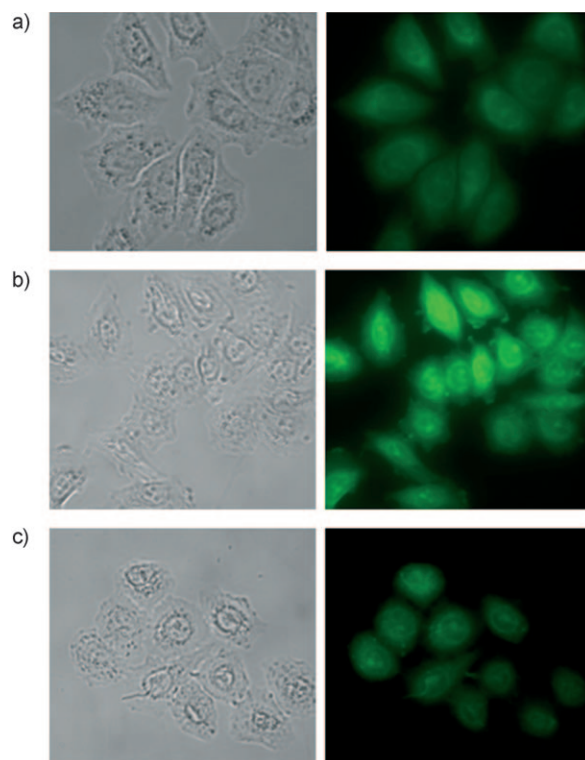


**Figure 1.** Fluorescence titration of **2** (5  $\mu\text{M}$ ,  $\lambda_{\text{exc}} = 493$  nm) with  $\text{MnCl}_2$  and  $\text{CaCl}_2$  (50 mM HEPES, 0.1 M  $\text{KNO}_3$ , pH 7.2): a) 0 to 200  $\mu\text{M}$   $\text{Mn}^{2+}$ ; b) 0 to 10 mM  $\text{Ca}^{2+}$ . Inserts:  $\lambda_{\text{em}} = 519$  nm.

**3** are the first selective “on-fluorescence”  $\text{Mn}^{2+}$  probes reported.

To confirm that the probes would function in a biological environment, intracellular  $\text{Mn}^{2+}$ -sensing was performed with the HeLa cell line. For cell membrane permeability, the ester precursor of **3** (see Supporting Information) was used for in vitro  $\text{Mn}^{2+}$  detection, as it has been reported that permeable ester probes can be hydrolyzed in the intracellular environment.<sup>[19]</sup> HeLa cells were first incubated with  $\text{Mn}^{2+}$ , followed by fluorescent probe treatment. A 2.4-fold enhanced fluorescent signal was observed in the presence of the probe and  $\text{Mn}^{2+}$  (Figure 2), consistent with the measurements performed in solution.

In conclusion, ligand **1** was rationally designed from bapta using a “soft atom poisoning” strategy to differentiate binding affinities to  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$ . Binding preferences were tuned by substitution of carboxylate groups of bapta with pyridines, resulting in much stronger  $\text{Mn}^{2+}$  selectivity over  $\text{Ca}^{2+}$ . Fluorescent probes based on ligand **1** were synthesized. Solution and in vitro properties demonstrated that these sensing compounds have good selectivity towards  $\text{Mn}^{2+}$  with “on” fluorescence response.



**Figure 2.** Detection of  $\text{Mn}^{2+}$  in HeLa cells. a) 5  $\mu\text{M}$  **3** ethyl ester; b) 200  $\mu\text{M}$  added  $\text{MnCl}_2$ ; c)  $\text{Mn}^{2+}$ -supplemented cells treated with 2 mM  $N,N,N',N'$ -tetrakis(2-pyridylmethyl)ethylenediamine (tpen) for 5 min at room temperature.

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