Molecular Recognition

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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.[1] It participates as a cofactor in diverse classes of enzymes and the photosynthetic machinery[2] and is used widely as a versatile tool for biological studies. For example, high-spin 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.[3] However, chronic overexposure can result in movement disorders and mental disturbances and other brain-related toxicities.[4] Fluorescent probes would be useful for detection and quantification of Mn^{2+}, as this method offers high efficiency, high sensitivity, and easy operation[5] among available methods of detection.[6] However, development of an effective fluorescent probe for Mn^{2+} faces several challenges: 1) Unlike diamagnetic metal ions such as Zn^{2+}, paramagnetic Mn^{2+} can quench fluorescence. Although chelation-induced fluorescence quenching (CHEQ) is the most commonly used method of paramagnetic metal ion detection,[7] "on-fluorescence" probes for Mn^{2+} are preferred. 2) Mn^{2+} selectivity over abundant cellular metal ions is required, especially Ca^{2+} (up to high μM).[8] Mn^{2+} and Ca^{2+} share many common properties, underscored by the fact that Mn^{2+} can enter cells using some of the same transport systems as Ca^{2+}.[9] 3) 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.[10] 4) To visualize Mn^{2+} in living cells or tissues, membrane permeability is important.[10]

Several commercially available chelating dyes produce strong fluorescence enhancement upon binding Mn^{2+}.[11] However, the fluorescence of available dyes such as calcium green is also enhanced in the presence of Ca^{2+}. Bapta (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid) is a known Ca^{2+}-selective ligand that serves as the chelating moiety of calcium green.[12] 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 Mn^{2+} and 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 Mn^{2+} selectivity.

However, although both Mn^{2+} and Ca^{2+} are classified as "hard" metals and therefore form stronger complexes with oxygen donors, Mn^{2+} appears to be more tolerant of softer atom donors than Ca^{2+}.[13,14] More recently, the relative softness of Mn^{2+} compared with Mg^{2+} has been debated as the basis for Mn^{2+} rescue of activity in dialkylthiophosphate RNAzymes.[15–17] Our hypothesis was thus to replace two or more carboxylate groups in bapta with softer ligating moieties. Since Mn^{2+} is a hard metal ion, weaker binding might be expected from such a change, but since Ca^{2+} is an even harder metal ion, the effect on 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.[14] 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 $MnCl_2$ to MOPS buffered aqueous solution (pH 7.2; MOPS = 3-(*N*-morpholino)propanesulfonic acid) of **1** (Supporting Information). In the absence of Mn^{2+}, the spectrum of **1** showed a maximum at 256 nm with a shoulder at 286 nm, similar to bapta. 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 Mn^{2+} concentration and the minimum level of absorbance was reached upon addition of 1 equivalent of Mn^{2+}, suggesting 1:1 metal–ligand complex. The same analysis was applied to determine 1:1 complexation of bapta to Mn^{2+}. The binding constants were obtained by titration in pH- and Mn^{2+}- buffered aqueous media. The plot of absorbance as a function of free Mn^{2+} produced a sigmoidal curve. Nonlinear fitting analysis[18] 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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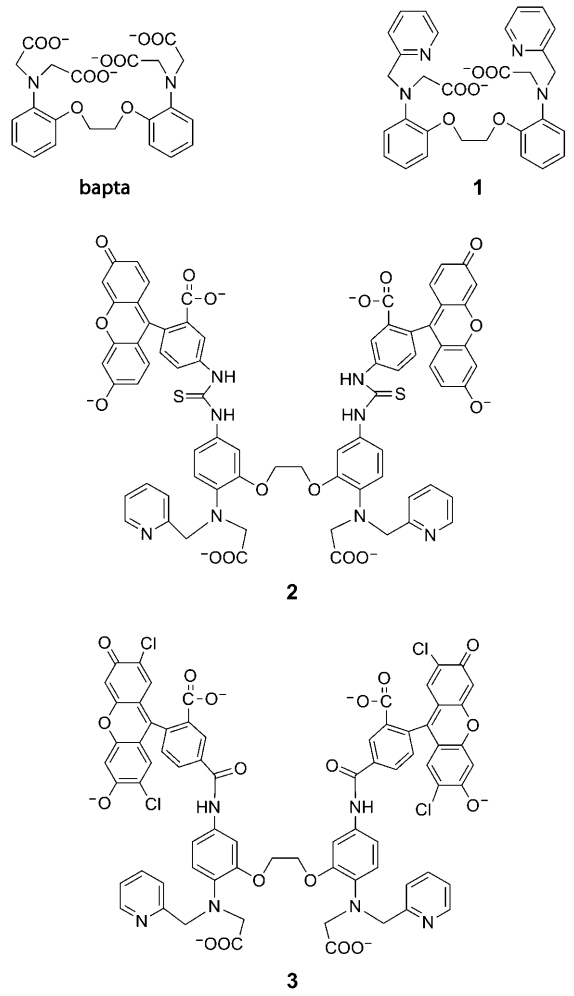

Scheme 1. Structures of ligands and probes.

bapta with two pyridines impairs binding affinity to 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 Ca^{2+} did not saturate the UV absorption indicating weak affinity. The association constant of **1**–Ca^{2+} was determined to be $\log(K_a) = 3.79$, about 4.83 log units weaker than for Mn^{2+}. The $\log(K_a)$ of bapta–Ca^{2+} was 6.89,[11] only 2.25 log units lower than that of bapta–Mn^{2+} complex. Therefore, ligand **1** indeed shows much higher selectivity for Mn^{2+} over Ca^{2+} as compared to bapta. The significant improvement in Mn^{2+}/Ca^{2+} selectivity validates our "selective poisoning" strategy.

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

Compound **3** showed a longer λ_{exc} (505 nm) and λ_{em} (530 nm) due to the two incorporated chlorine atoms on the fluorophore (Supporting Information). The Mn^{2+} complex showed enhanced fluorescence. Probe **3** maintained high selectivity for Mn^{2+} over Ca^{2+} with $\log K$ of 8.00 for Mn^{2+} and 3.33 for Ca^{2+}. The quantum yield of free probe **3** was 0.13, while that for the 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: Mn^{2+} and Ca^{2+} affinities and spectroscopic properties of ligands.

Ligand	log K				Selectivity	λ_{max}[a]		Ex	Em	ϕ_f	
	Mn^{2+}	Ca^{2+}	Mg^{2+}	Zn^{2+}	$\log(K_{Mn}/K_{Ca})$	free ligand	Mn^{2+} complex			free ligand	Mn^{2+} complex
bapta	9.14	6.89	1.69	9.41	2.25	290 (4.8)	278 (4.5)	–	–	–	–
1	8.62	3.79	0.76	9.19	4.83	286 (6.3)	283 (4.1)	–	–	–	–
2	7.01	2.96	0.42	6.16	4.05	493 (84)	493 (100)	493	519	0.10	0.37
3	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 ε [10^3 $L mol^{-1} cm^{-1}$] is given in parentheses.

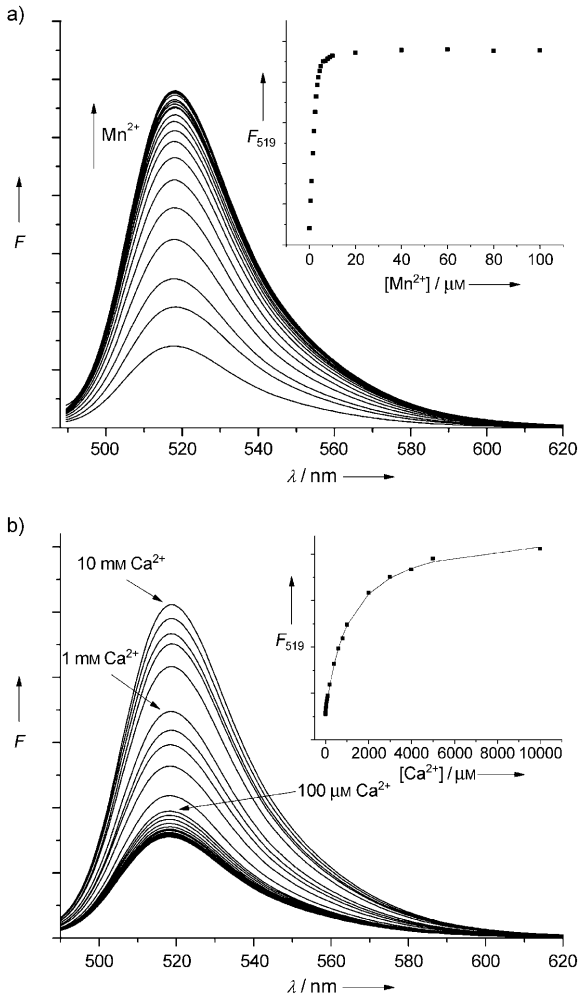

Figure 1. Fluorescence titration of **2** (5 μM, λ_{exc} = 493 nm) with $MnCl_2$ and $CaCl_2$ (50 mM HEPES, 0.1 M KNO_3, pH 7.2): a) 0 to 200 μM Mn^{2+}; b) 0 to 10 mM Ca^{2+}. Inserts: λ_{em} = 519 nm.

3 are the first selective "on-fluorescence" Mn^{2+} probes reported.

To confirm that the probes would function in a biological environment, intracellular 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 Mn^{2+} detection, as it has been reported that permeable ester probes can be hydrolyzed in the intracellular environment.[19] HeLa cells were first incubated with Mn^{2+}, followed by fluorescent probe treatment. A 2.4-fold enhanced fluorescent signal was observed in the presence of the probe and 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 Mn^{2+} and Ca^{2+}. Binding preferences were tuned by substitution of carboxylate groups of bapta with pyridines, resulting in much stronger Mn^{2+} selectivity over Ca^{2+}. Fluorescent probes based on ligand **1** were synthesized. Solution and in vitro properties demonstrated that these sensing compounds have good selectivity towards Mn^{2+} with "on" fluorescence response.

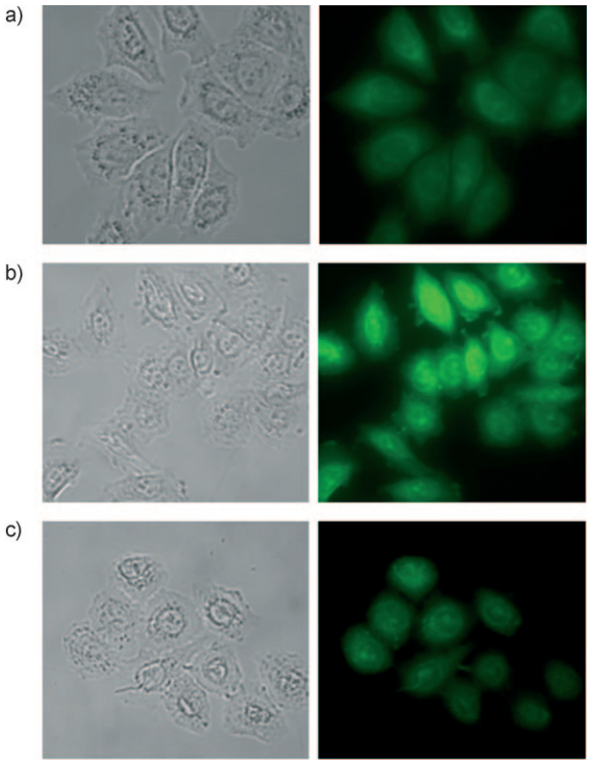

Figure 2. Detection of Mn^{2+} in HeLa cells. a) 5 μM **3** ethyl ester; b) 200 μM added $MnCl_2$; c) Mn^{2+}-supplemented cells treated with 2 mM *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (tpen) for 5 min at room temperature.

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