Fluorescence Sensing of Ionic Analytes in Water: From Transition Metal Ions to Vitamin B13

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Abstract: The fluorescence chemosensor ATMCA has been realised by appending an anthrylmethyl group to an amino nitrogen of TMCA (2,4,6-triamino-1,3,5-trimethoxycyclohexane), a tripodal ligand selective for divalent first-row transition metal ions in water. The ATMCA ligand can act as a versatile sensor for Zn^{II} and Cu^{II} ions. Its sensing ability can be switched by simply tuning the operating conditions. At pH 5, ATMCA detects copper(II) ions in aqueous solutions by the complexation-induced quenching of the anthracene emission. Metal ion concentrations $< 1\,\mu M$ can be readily detected and very little interference is exerted by other metal ions. At pH 7, ATMCA signals the presence of Zn^{II} ions at concentrations $< 1\,\mu M$ by a complexation-induced enhancement of the fluorescence. Again the sensor is selective for Zn^{II} over several divalent metal ions, with the exception of Cu^{II}, Co^{II} and Hg^{II}. Most interestingly, the $[Zn^{II}(atmca)]^{2+}$ complex can act as a fluorescence sensor for specific organic species, notably selected dicarboxylic acids and nucleotides, by the formation of ternary ligand/zinc/substrate complexes. The oxalate anion is detected in concentrations $< 0.1\,mM$; however, no effects on the system's fluorescence is observed in the presence of monocarboxylic acids and long-chain dicarboxylic acids. Among the nucleotides, those containing an imide or amide function are readily detected and an unprecedented high sensitivity for guanine derivatives allows the determination of this nucleotide for $0.05 - 0.5\,mM$ solutions. Moreover, $[Zn^{II}(atmca)]^{2+}$ is a very effective and selective sensor in the case of vitamin B13 (orotic acid) in sub-micromolar concentrations. The operative features of the systems investigated are also clearly suitable for intracellular analyses. The factors at the source of organic substrate recognition, here briefly discussed, are of paramount importance for further developments in the applicability of these sensing systems.

Keywords: chemosensors · fluorescence · molecular recognition · nucleotides · orotic acid · zinc

Introduction

During the last years, fluorescent chemosensors have attracted increasing attention as a result of the great interest in their applications in different fields ranging from environmental analysis to intracellular probing.[1] Much impetus in the search for molecular sensors stems from the new perspectives and novel approaches brought forward by supramolecular chemistry. In fact, these systems combine the recognition abilities of supramolecular receptors with the important sensing advantages of fluorescence techniques to yield analytical tools characterised by high sensitivity, specificity and selectivity.[2] So far, several systems have been successfully realised for the detection of metal ions exploiting the strong interactions with a number of proper ligands.[3] However, many problems stand in the way of a wider range of chemosensors. One general obstacle is that the natural medium for most applications is water and the realisation of water-soluble sensors is still a difficult goal. A second particularly challenging problem is the detection of anions and organic molecules.[4] In this case, most of the interactions that can be exploited for substrate recognition, such as hydrogen bonds or electrostatic attractions, are too weak to give effective binding in water. A well-established strategy, based on the so-called multicomponent or modular approach, suggests the design of anion sensors by linking together a light-emitting fragment

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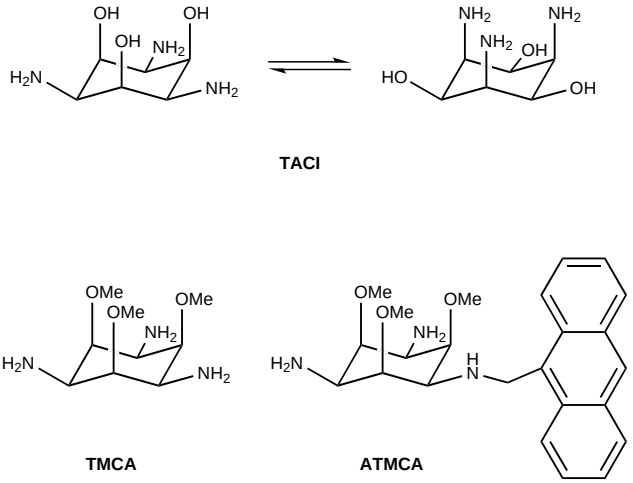
and a metal complex acting as receptor subunit.[5] The recognition of anionic or organic substrates can take place by means of coordinative interactions, which are stronger than electrostatic ones. In this way, the metal ion acts as a structural element that brings a dye-functionalised ligand into contact with the substrate, allowing an intercomponent process which signals the recognition event. The study reported here is an attempt to apply this strategy as well to provide a better definition of its potentiality and limitations.

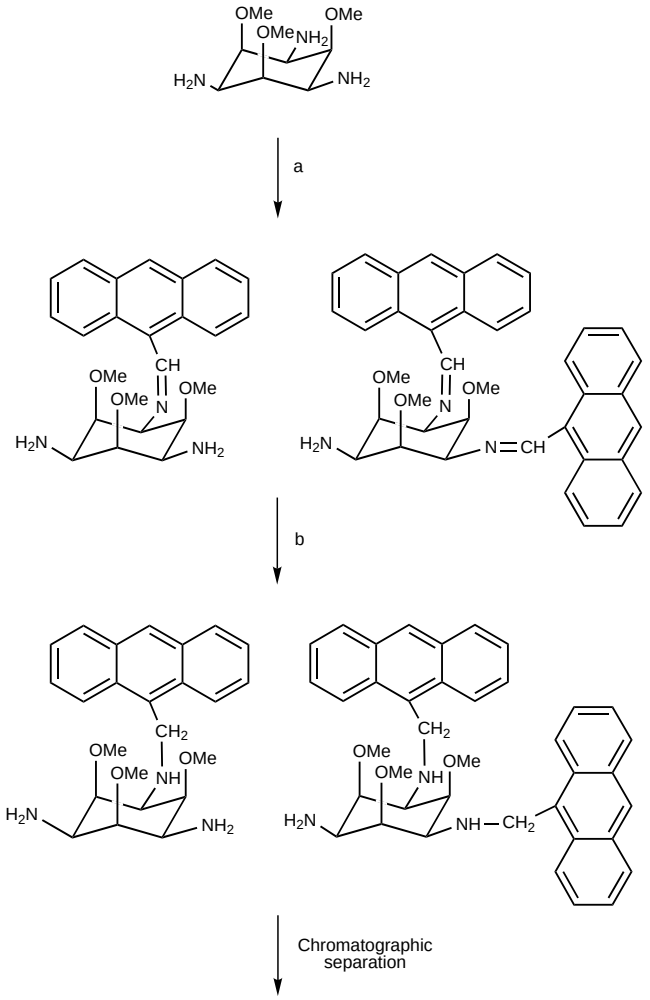
The all-*cis*-2,4,6-triamino-1,3,5-trihydroxycyclohexane (TACI) is a very versatile ligand[6] that is able to form complexes with 30 different metal ions by the use of different



binding sites. It thus appears to be a promising scaffold for the design of fluorescence chemosensors. Among its peculiar features, the following may be highlighted: 1) the selective alkylation of the amino or hydroxy groups allows modulation of the binding properties,[6] 2) the relatively low basicity of the amino groups ensures positive binding even at neutral or slightly acidic pH values,[7] 3) the rigid structure of the ligand leads to relatively high formation constants for metal complexes[6] and 4) the facial coordination mode may leave a large portion of the metal-ion surface available for interactions with other species. On account of this functional flexibility, we considered that an appropriate TACI derivative could act as a fluorescent chemosensor for transition metal ions and, more importantly, for organic molecules and ions. Therefore, we selected the hydroxymethylated derivative, the all-*cis*-2,4,6-triamino-1,3,5-trimethoxycyclohexane (TMCA) as a strong ligand for first-row, divalent, transition metal ions[8] and appended to one of its nitrogen atoms a 9-anthracenyl group as a fluorophore. In accordance with our expectations, a detailed investigation showed that the resulting anthrylamine derivative (ATMCA) is a sensitive and selective sensor for Cu^{II} and Zn^{II} ions, depending on the operative conditions. Moreover, the Zn^{II} ion in the $[Zn^{II}(atmca)]^{2+}$ complex acts as a template for recognition and binding of organic molecules and ions. As a result, the complex was found to act as a fluorescent chemosensor of organic anions and molecules, particularly nucleobase derivatives, with a remarkable selectivity. The features, the mode of action and the scope of this sensor are presented and discussed in the present paper.

Results

Synthesis and properties of ATMCA: ATMCA was prepared according to Scheme 1. Condensation of TMCA with 9-anthraldehyde in refluxing benzene followed by reduction with $NaBH_4$ in ethanol gave a mixture of the mono- and



Scheme 1. Synthesis of ATMCA. a) 9-anthraldehyde, benzene, reflux; b) sodium borohydride, ethanol, RT.

dialkylated derivatives (7:3 ratio). ATMCA was isolated in 62% yield by column chromatography. An attempt to improve the synthesis was carried out by means of amino Z-diprotected or BOC-diprotected TMCA derivatives. The reaction with 9-anthraldehyde in refluxing benzene led to the quantitative formation of the desired imine; however, attempts to perform its reduction following several different methods failed.

ATMCA is soluble up to millimolar concentrations in slightly acidic water or in the presence of metal ions, such as Cu^{II} or Zn^{II}, and up to 1×10^{-4} M in neutral or basic solutions. The features shown in the UV-visible and fluorescence spectra in aqueous solutions are typical for the anthracene chromophore.[9]

The protonation constants of ATMCA and the complex formation constants with Zn^{2+} were determined by potentio-

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metric titrations (see Figure 1 in the Supporting Information). The results obtained are reported in Table 1 and compared to those of TMCA.[8] The pK_2 and pK_3 values for ATMCA (5.8 and 3.7, respectively) are remarkably smaller than the

Table 1. Ligand deprotonation constants (K_n[a]), Zn^{II} complex formation constants ($K(ZnL)$[b]), and deprotonation constants of Zn^{II}-bound H_2O ($K_a(H_2O)$) for TMCA and ATMCA at 25 °C.

	TMCA[c]	ATMCA[d]
pK_1	9.3	9.1
pK_2	6.9	5.8
pK_3	5.2	3.7
$\log K(ZnL)$	10.8	10.2
$pK_a(H_2O)$	8.5[d]	8.3

[a] $K_n = [H_{n-1}L][H_3O^+]/[H_nL]$. [b] $K(ZnL) = [ZnL]/[Zn^{II}][L]$. [c] Data from Ref. [8] (0.1 M KNO_3). [d] This work (0.1 M NaCl).

corresponding values for TMCA. This is probably caused by a less efficient solvation of ATMCA because of the hydrophobic anthracenyl residue.[5d] The other constants are very similar for the two compounds. Interestingly, the attachment of the bulky anthracenyl group to ATMCA, which should disfavour the conformation with the amino groups in the axial position required for the formation of the complex, does not imply a significant decrease in the metal binding affinity.

The pH dependence of the ATMCA fluorescence spectra is reported in Figure 1. The emission intensity at pH > 5 sharply decreases to a plateau value with ≈20% of the initial value. Superimposition of the fluorescence versus pH profile with the distribution diagram for ATMCA clearly shows that the decrease of the emission corresponds to the deprotonation of the second amino group, presumably the anthracenylic one.

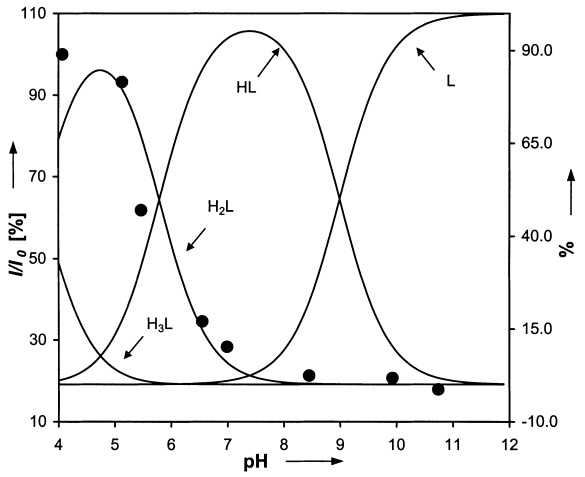

Figure 1. pH-Dependence of the fluorescence intensity (●, $\lambda_{exc} = 368$ nm, $\lambda_{em} = 415$ nm) and distribution diagram for ATMCA (1×10^{-6} M). Conditions: $[CTABr] = 1 \times 10^{-4}$ M, 25 °C.

Sensing of Cu^{II}: Titration of a 1 μM aqueous solution of ATMCA at pH 5.0 (acetate buffer) with $Cu(NO_3)_2$ resulted in a sharp decrease of the emission intensity, reaching a plateau value of ≈10% of the initial intensity after the addition of one equivalent of metal ion (Figure 2). On the other hand, the ATMCA absorption spectra do not appreciably change

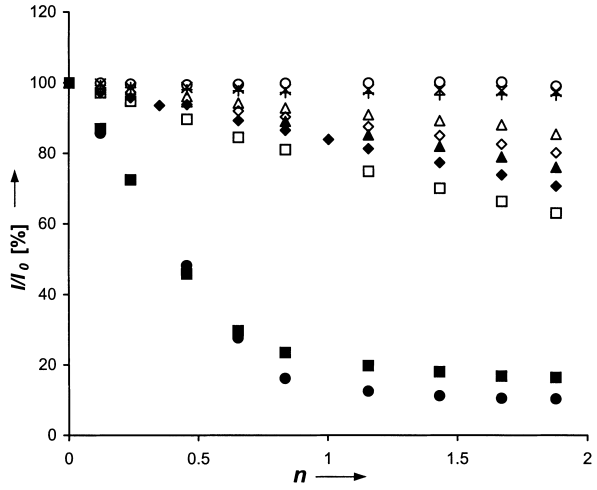

Figure 2. Spectrofluorimetric titrations of ATMCA (1×10^{-6} M) at pH 5.0 with different metal ions: Co^{II} (◆), Ni^{II} (▲), Hg^{II}(□), Zn^{II} (○), Fe^{II} (△), Cd^{II} (×), Mn^{II} (◇), Pb^{II} (+), Cu^{II} (●), Cu^{II} in the presence of all the other metal ions of each 2×10^{-6} M (■). n = number of added equivalents. [Acetate buffer] = 0.01 M, $[CTABr] = 1 \times 10^{-4}$ M, 25 °C.

upon addition of Cu^{II}. Interpolation of the emission intensity versus Cu^{II} concentration, assuming a 1:1 binding model, gives a good fit and allows the estimation of the value of $\log K_{app}$[10] to be 8.0 ± 0.2.

To test the sensor selectivity, we titrated the ATMCA solutions with other divalent metal ions (Figure 2): Zn^{II}, Cd^{II} and Hg^{II} had no effect on the emission intensity of the system, while the addition of Fe^{II}, Mn^{II}, Ni^{II}, Co^{II} and Hg^{II} gave rise to a relatively minor decrease in the emission. Even in the case of Hg^{II}, which shows the largest effect, only a decrease of ≈30% was observed after addition of two equivalents of the metal ion. On the other hand, the most relevant feature of the system is illustrated by the results of the titration with Cu^{II} of a solution containing ATMCA and all the others cations (each 2 μM). The curve thus obtained (defined by ■) is very close to the one obtained in the presence of the Cu^{II} ions alone (defined by ●). This result demonstrates that the presence of an excess of other divalent metal ions does not interfere in the Cu^{II} determination.

Sensing of Zn^{II}: At pH values > 7.0, the fluorescence of ATMCA is low. Titration of a 1 μM aqueous solution of ATMCA at pH 7.0 (HEPES buffer) with $Zn(NO_3)_2$ led to a strong (up to tenfold) increase of the emission intensity (Figure 3). No effects on the absorption spectra were observed. Again the data obtained fit well a 1:1 model to give a $\log K_{app}$ value of 7.0 ± 0.1.

Titrations of the sensor solution with the other metal ions showed no effects on the fluorescence of the system (Figure 3). Only addition of Cd^{II} resulted in an emission increase by 1.3 times. The selectivity in the presence of other metal ions was not so high as in the case of Cu^{II} at pH 5. However, no interference in the Zn^{II} sensing was observed in the presence of Ni^{II}, Fe^{II}, Mn^{II} and Pb^{II} (Figure 2 in the Supporting Information), while the presence of Hg^{II}, Co^{II} and Cu^{II} caused a remarkably lower fluorescence increase after addition of Zn^{II} (Figure 3 in the Supporting Information).

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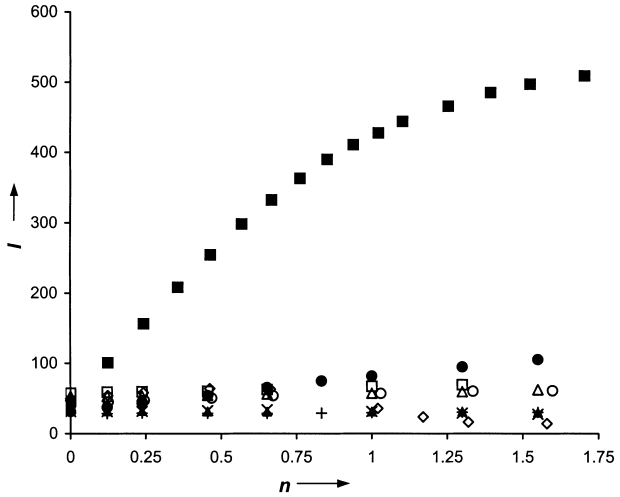
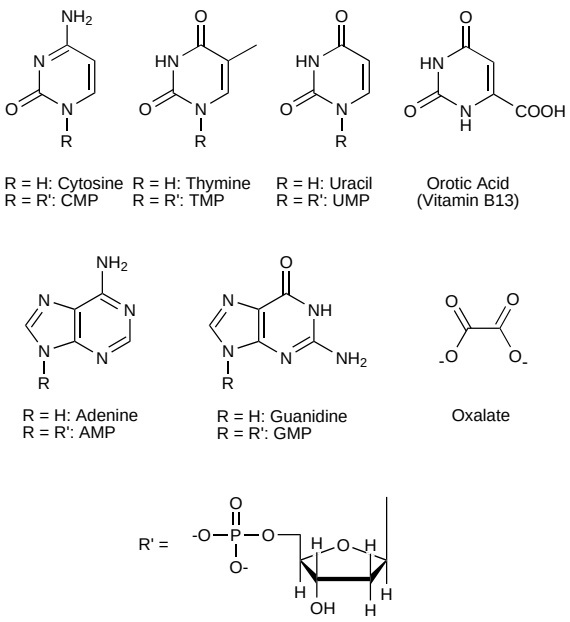


Figure 3. Spectrofluorimetric titrations of ATMCA (1×10^{-6} M) at pH 7.0 with different metal ions: Co^{II} (▲), Ni^{II} (○), Hg^{II}(×), Fe^{II} (+), Cd^{II} (●), Mn^{II} (△), Pb^{II} (□), Cu^{II} (◇), Zn^{II} (■). n = number of added equivalents. [HEPES] = 0.01 M, [CTABr] = 1×10^{-4} M, 25 °C.



Sensing of organic anions and orotic acid (vitamin B13): Several experiments were performed to investigate the ability of the $[Zn^{II}(atmca)]^{2+}$ complex to sense the presence of organic anions or molecules, assuming the possibility that these substrates may form ternary complexes with detectable changes in the fluorescence behaviour. This was studied after the addition of various mono- and dicarboxylic acids, nucleotides, nucleobases and orotic acid (Vitamin B13) to a solution of $[Zn^{II}(atmca)]^{2+}$ (50 μM) at pH 7.2. In each case, when a decrease in the emission intensity was observed, the effects indicated a 1:1 stoichiometry for the binding of the substrates to the Zn^{II} complex. The accessible binding constants ($\log K_{app}$)[11] are reported in Table 2.

A preliminary scrutiny showed that of the carboxylic acids only oxalic acid was detected, whereas no effect was observed in the case of longer chain dicarboxylic acids and monocarboxylic acids. Thus, addition of oxalate dianion to a 50 μM aqueous solution of $[Zn^{II}(atmca)]^{2+}$ led to a 40% decrease of the emission intensity (Figure 4 in the Supporting Information). In this case, fitting of the data, assuming the 1:1 model, gives a ternary complex formation constant ($\log K_{app}$) of 4.3.

Table 2. Apparent complexation constants (K_{app}[a]) and relative fluorescence quenching for different substrates to $[Zn^{II}(atmca)]^{2+}$ at 25 °C and pH 7.2 (HEPES buffer 0.01 M).

Substrate	pK_a	% Quenching[b]	$\log K_{app}$[c]
oxalate		40	4.3 (–[d])
AMP	–	< 5	–[e] (3.5)
CMP	–	14	2.9[e] (3.0)
GMP	9.3[f]	42	4.2 (4.0)
TMP	10.2[g]	20	4.5 (4.3)
UMP	9.5[g]	23	4.0 (4.0)
uracil	9.4[h]	19	3.6 (3.6)
thymine	9.9[h]	20	3.6 (3.7)
cytosine	12.2[h]	< 5	–[e] (–)
orotate	9.45[i]	100	6.6 (6.6)

[a] $K_{app} = [ZnLS]/[ZnL_{tot}][S_{tot}]$, see also Ref. [11]. [b] Fluorescence quenching after the addition of 10 equiv of analyte. [c] As determined by fluorescence and UV/Vis (in parenthesis) titrations. The error in the binding constants is ±0.1 logarithmic units. [d] Not determinable because the spectral variations are too small. [e] No quenching was detected. [f] Data from Ref. [12]. [g] Data from Ref. [13]. [h] Data from Ref. [14]. [i] Data from Ref. [20].

More important are the effects observed by the addition of some nucleotides under the conditions described above (Figure 5 in the Supporting Information). Titrations of a 50 μM aqueous solution of $[Zn^{II}(atmca)]^{2+}$ with guanosine 5′-monophosphate (GMP), thymidine 5′-monophosphate (TMP) and uridine 5′-monophosphate (UMP) result in a 42%, 20% and 23% decrease, respectively, in emission after the addition of ten equivalents of substrate. The fit of the data yields apparent formation constants for the ternary complexes in the range 4.0–4.5 (Table 2).

On the other hand, no effect was observed in the case of adenosine 5′-monophosphate (AMP), while cytidine 5′-monophosphate (CMP) showed a lower emission decrease (14%) and a remarkably lower apparent formation constant for the ternary complexes ($\log K_{app} = 2.9$). Inspection of the nucleotide structures shows that $[Zn^{II}(atmca)]^{2+}$ is particularly effective in the case of nucleotides that feature an acidic amide function. This is supported by the fact that whereas addition of thymine and uracil leads to a decrease in the emission, such an effect is not observed in the case of cytosine, which is a much less acidic amide (Table 2). Unfortunately, we could not test adenine and guanine, since they are virtually insoluble in water at pH 7.2.

In order to gain a better insight into the binding selectivity, we performed UV/Vis titrations with the different nucleotides. In each case, the binding constants determined with these experiments were in agreement with those obtained by fluorescence titrations. In the case of AMP, we observed a different behaviour: this nucleotide did not quench the $[Zn^{II}(atmca)]^{2+}$ fluorescence, but the UV/Vis titration clearly indicated the formation of a ternary complex with a smaller $\log K_{app}$ value (3.5).

The most impressive effect was observed by titration of the $[Zn^{II}(atmca)]^{2+}$ solution (50 μM, pH 7.2, HEPES buffer) with orotic acid (as the orotate anion at the working pH) (Fig-

ure 4). The emission intensity decreased linearly with the substrate concentrations down to total quenching after the addition of one equivalent of orotate. The estimated binding constant ($\log K_{app}$) is 6.6, the same as that obtained from

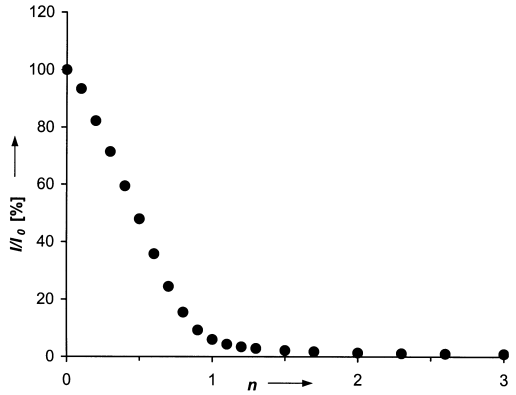


Figure 4. Spectrofluorimetric titrations of $[Zn^{II}(atmca)]^{2+}$ (5×10^{-5} M) in HEPES buffer 0.01 M, pH 7.2 with orotate (●). n = number of added equivalents.

independent UV/Vis titrations. The effect may be partly the result of a concomitant aromatic stacking between the anthracene moiety and the aromatic ring of the substrate. This was indeed the case as shown by the outcome of a UV/Vis titration of the $[Zn^{II}(tmca)]^{2+}$ complex with orotate; from these experiments the resulting binding constant ($\log K_{app}$) was calculated to be 4.4, lower than that estimated for $[Zn^{II}(atmca)]^{2+}$ by 2.2 logarithmic units. Further fluorescence experiments performed with a 1 μM concentration of $[Zn^{II}(atmca)]^{2+}$ complex (not shown) showed that a submicromolar concentration of orotate can be detected. It was important to assess that the above effects were the result of the formation of the $[Zn^{II}(atmca)(orotate)]$ ternary complex and were not caused by other factors. The most worrying among them was the possible removal of Zn^{II} ion by orotate from the $[Zn^{II}(atmca)]^{2+}$ complex which would lead to a decrease of fluorescence intensity to values approaching that of the free ligand. Thus, we performed a ^{1}H NMR titration (Figure 5) on $[Zn^{II}(atmca)]^{2+}$ solutions in D_2O (1 mM, pD 7, 25 °C) in the presence of increasing amounts of orotate. We observed the disappearance of $[Zn^{II}(atmca)]^{2+}$ signals and the appearance of an independent set of peaks. These new signals are different from those of free ATMCA, indicating the formation of the ternary complex. The spectral changes are particularly informative in the aromatic region, in which the signals pertaining to the anthracenyl protons move upfield and undergo a significant broadening. Only the signal of the proton on the C10 atom remains as a sharp singlet. Moreover, the signal of the orotate proton in the ternary complexes is also shifted upfield to $\delta = 5.22$ from $\delta = 6.04$, the chemical shift of the free species. In fact, two distinct signals appear when the experiments are carried out in the presence of an excess of orotate. The upfield shift of both the anthracene and orotate protons can be attributed to the aromatic stacking of the two molecules within the ternary complex,[5d] while the line broadening of most of the anthracene signals is probably the result of hampered rotation of this moiety owing to the formation of the ternary complex.

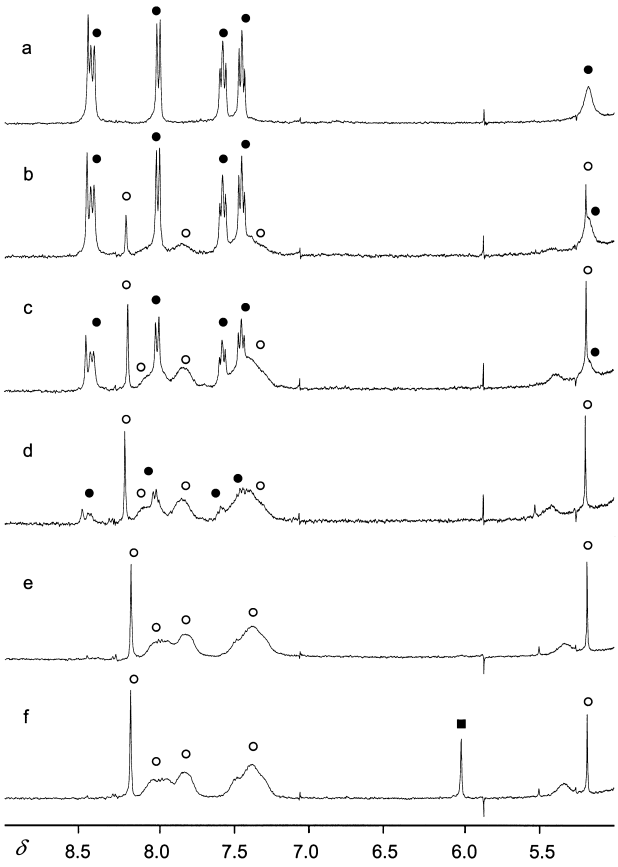


Figure 5. ^{1}H NMR (400 MHz) spectral changes of $[Zn^{II}(atmca)]^{2+}$ (1×10^{-3} M) in D_2O (pD 7.0, phosphate buffer 0.05 M) with increasing concentrations of orotate at 25 °C. The $[Zn^{II}(atmca)]^{2+}$/orotate ratio is: a) 1:0, b) 1:0.25, c) 1:0.5, d) 1:0.75, e) 1:1, f) 1:2. ● indicates the signal of $[Zn^{II}(atmca)]^{2+}$, ○ indicates the signals of ternary complex, ■ indicates free orotate.

Discussion

In this study, ATMCA appears to act as an extremely versatile sensor system that is capable of selectively detecting the presence of Cu^{II}, Zn^{II} and, once complexed with the latter metal ion, some organic species.

The sensitivity of ATMCA fluorescence emission towards the solution pH or the presence of metal ions can be ascribed to the well-described behaviour of polyamines with appended fluorophores.[2a, 3b] The remarkable sensing properties of this class of molecules are known from the pioneering studies carried out by Czarnik[15] which are still attracting a great deal of attention and which will lead to interesting developments. The fluorescence emission of these species can be reversibly switched on and off by different mechanisms on simple modulation of the working conditions. In the free ligand, an electron transfer from the lone pair of the secondary amino group quenches the anthracenyl emission. Its protonation cancels the effect of the amine and allows the restoration of the fluorescence.[3b, 15] Figure 1 clearly shows that this is also the case with ATMCA: there are strong changes in the fluorescence emission following protonation and deprotonation of the benzylic nitrogen ($pK_2 = 5.8$).

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Cu^{II} detection: When Cu^{II} is added (Figure 2) to an ATMCA solution at pH 5.0, the metal ion is complexed by the TMCA subunit by displacement of the protons from the amino groups. This switches off the sensor fluorescence (CHEQ, chelation-enhanced quenching)[1b], usually by an energy- or electron-transfer process.[3b, 3g] The high affinity of the TMCA moiety toward this metal ion[16] ensures a high sensitivity of the system, so that sub-micromolar concentrations of Cu^{II} can be easily detected. Also the complexation of other transition metal ions with partially filled d orbitals, such as Fe^{II} or Ni^{II}, produces an analogous fluorescence quenching. However, at these pH conditions, the affinity of ATMCA toward these metal ions is much lower than that of Cu^{II} and this is at the source of the high selectivity of the sensor.

Zn^{II} detection: At neutral or basic pH values, the complexation of Zn^{II}, which has filled d orbitals, involves the lone pair of the secondary amino nitrogen and thus substantially decreases its reducing properties. This causes a strong enhancement of fluorescence emission. The effect is known as CHEF (chelation-enhanced fluorescence)[1b] and has been exploited in the design of several chemosensors for Zn^{II}, Cd^{II} and alkali metal ions.[1–3, 15] Nevertheless, there is still a great interest in the realisation of CHEF chemosensors for Zn^{II}.[17] Since the zinc ion is an essential cofactor in many biochemical functions,[18] Zn^{II}-specific probes for the study of intracellular regulation of this metal are greatly sought after. Such probes should have high affinity and selectivity, low sensitivity to physiological pH variations, cell permeability and visible light excitation ($\lambda > 350$ nm) and emission ($\lambda > 500$ nm).[1b]

ATMCA positively fits most of these requirements. The relatively low pK_a values of the amino groups, which appeared to be a sort of weakness in the design of the sensor, are in fact a bonus, since this property ensures efficacy at physiological pH and insensitivity to pH variations. ATMCA shows a high affinity and sensitivity to Zn^{II} ions as it can easily detect sub-micromolar concentrations of Zn^{II} and suffers no interference from most metal ions. In addition, interference by Cu^{II}, Co^{II} and Hg^{II}, unavoidable for a polyaminic ligand, should not be of much concern in intracellular applications because of the low in-vivo concentrations of these metal ions.[19] Finally, the good solubility in organic solvents should allow membrane permeability and the need of visible light emission can be easily met by replacing the anthracene group with a more appropriate dye.

Organic anions and orotic acid (Vitamin B13) detection: As emphasised above, the ability of the $[Zn^{II}(atmca)]^{2+}$ complex to detect organic anions and molecules is of particular interest. In this case, the bound metal ion acts as the specific substrate recognition site and the TMCA moiety links the fluorescent dye with the recognition unit. This strategy has been previously explored for the sensing of carboxylic acids (in methanol),[5a,c] amino acids (in methanol),[3b] imidazole (in water)[5b] and thymine (and uracil) derivatives (in water).[5d] The acridinylmethylcyclen–Zn^{II} complex investigated by Kimura can detect the presence of thymidine (and uridine) in aqueous solutions at pH 7.6 ($\log K_{app} = 4.7$) and of guanosine ($\log K_{app} = 3.7$).[5d] The difference in binding constants ensures a good selectivity for imide-containing nucleobase derivatives (thymidine and uridine) and was attributed to a multipoint interaction involving a deprotonated imide–Zn^{II} bond, two hydrogen bonds between the imide carbonyls of the substrate and the cyclen amino hydrogen, and a $\pi-\pi$ stacking interaction between the acridine moiety and the nucleobase.

$[Zn^{II}(atmca)]^{2+}$ shows structural analogies and a similar behaviour. However, this study highlights new intriguing features, illustrated, as discussed below, by the ability to bind all five nucleotides and to selectively detect the presence of the imide- and amide-containing TMP, UMP and GMP. This ability to recognise not only imide-containing nucleotides opens the way to wider applications of such systems and is probably the result of the higher Lewis acidity of the Zn^{II} ion complexed to the tridentate TMCA moiety. Besides the high affinity for the imide-containing TMP ($\log K_{app} = 4.5$ at pH 7.2) and UMP ($\log K_{app} = 4.0$), similar to Kimura's sensor, $[Zn^{II}(atmca)]^{2+}$ has also a high affinity for the amide-containing GMP ($\log K_{app} = 4.2$). The observed fluorescence quenching can be ascribed to an electron-transfer process between the aromatic rings of the bound nucleobase and the anthracene group, as already observed in other fluorescent sensors for organic anions.[5a,c] The extent of quenching is particularly significant with GMP (up to 50%) and allows the determination of this nucleotide in the concentration range 0.05–0.5 mM.

Smaller association constants were determined with CMP ($\log K_{app} = 3.0$) and AMP ($\log K_{app} = 3.5$), which are devoid of an acidic amide function. However, besides the binding abilities of the $[Zn^{II}(atmca)]^{2+}$ complexes, only the imide- or amide-containing nucleotides are efficiently detected by the sensor. The fundamental interactions upon which the selective fluorescence sensing of these nucleotides is based have to be ascribed to the bond between Zn^{II} and the deprotonated amide nitrogen, which reasonably brings the substrate and the anthracene moiety of ATMCA into the correct position for the fluorescence-quenching process. On the other hand, in the case of AMP and CMP, the absence of an amido group on the substrate is likely to induce a different binding mode, which causes a lower affinity and does not assure an optimum communication between the substrate and the signalling subunit.

The ability of $[Zn^{II}(atmca)]^{2+}$ to signal the presence of oxalate (Table 2) indicates the possibility of a second effective recognition mechanism in addition to that involving the interaction of the zinc ion with a strong donor as an imide-deprotonated nitrogen. Carboxylate anions, which are weak ligands for Zn^{II} in water, do not interact effectively with the sensor unless they have the possibility to form a five-membered chelate ring. In fact, among the substrates tested, only oxalate was able to bind the complex and gave a significant (up to 40%) fluorescence quenching ($\log K_{app} = 4.3$ at pH 7.2), while more extended dicarboxylic acids, such as succinic or glutaric acid, did not show any effect.

Orotic acid (vitamin B13), which combines the presence of a relatively acidic amide function ($pK_a = 9.45$) with the ability to form five-membered chelates with metal ions,[20] is the ideal substrate for the $[Zn^{II}(atmca)]^{2+}$ sensor. Orotic acid is an intermediate metabolite in the biosynthesis of pyrimidine

nucleotides.[21] High levels of endogenous orotate are connected to various kinds of disorders, particularly metabolic, and to an increased cancer risk.[22] Moreover, the same molecule which is present in various foodstuffs, such as cow milk, seems to have a positive role in heart protection and in decreasing the cholesterol level.[20, 23] The availability of analytical tools for the detection of orotate in human fluids and food is a goal of great interest[23, 24] and $[Zn^{II}(atmca)]^{2+}$ may prove to be an ideally suitable sensor. The high affinity of the complex for orotate ($\log K_{app} = 6.6$ at pH 7.2) is consequence of the two recognition points, namely, the deprotonated amide and the carboxylate group, and of an aromatic stacking between the aromatic systems of the anthracene moiety and the substrate. The stacking interaction is also suggested by molecular modelling studies: the energy-minimized structure of the ternary complex reported in Figure 6 shows the anthracene subunit and orotate lying in two almost

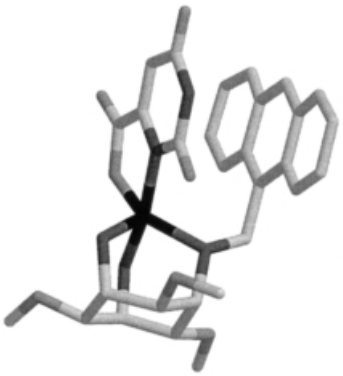

Figure 6. Molecular model for the $[Zn^{II}(atmca)(orotate)]$ ternary complex as obtained by the HyperChem software package (MM+ force field). Hydrogen atoms are omitted for clarity.

parallel planes at a distance ranging from 3.2 to 3.4 Å. NMR experiments confirm the presence of a stacking interaction and, by comparison with $[Zn^{II}(tmca)]^{2+}$ complex data, it is possible to estimate that it contributes 2.2 logarithmic units to the ternary complex formation constant. The high affinity and sensitivity of the complex towards orotate (up to 100% fluorescence quenching) allow it to sense substrate concentrations in the sub-micromolar range. More to the point, the sensor is extremely selective for orotate, even in the presence of potential competitors such as carboxylic acids, nucleotides and nucleobases. It thus appears to be suitable for direct in-situ determination of orotate levels in biological fluids.

Conclusions

The outstanding properties of TACI-derived ligands allowed us to realise the highly effective sensors ATMCA and $[Zn^{II}(atmca)]^{2+}$. The first important feature of these systems is the versatility; in fact, by tuning the reaction conditions (pH or the presence of zinc ions), it is possible to selectively determine the presence of Cu^{II} and Zn^{II} ions as well as relevant organic substrates.

At pH 5, ATMCA can detect Cu^{II} in the sub-micromolar range. Copper is a significant pollutant as a consequence of its widespread use. The high selectivity of the sensor, coupled with its ability to operate in slightly acidic solutions, allows a straightforward Cu^{II} determination in samples which have not been pretreated. At physiological pH values, ATMCA can signal the presence of Zn^{II}, again in the sub-micromolar range, and with a good selectivity under conditions that make it suitable for intracellular Zn^{II} determination. It is emphasised here that, on account of the well-established selectivity of the TMCA moiety toward divalent transition metal ions,[8] also alkaline or alkaline-earth metal ions will not interfere in the detection of Cu^{II} or Zn^{II} ions.

The most important feature of the ATMCA sensing system is the ability of the $[Zn^{II}(atmca)]^{2+}$ complex to detect the presence of important organic species in aqueous solutions. The presence of nucleobases that contain an imide or acidic amide functionality is signalled by the sensor and this occurs with an unprecedented sensitivity in the case of guanine derivatives. Finally, $[Zn^{II}(atmca)]^{2+}$ is a very effective and selective sensor in the case of vitamin B13 (orotic acid). Multiple recognition processes are operative for this important metabolite: the presence on the substrate of a strong binding site such as an acidic amide, the ability to form a five-membered ring by chelation with the bound Zn^{II} ion and aromatic stacking allow the determination of sub-micromolar analyte concentrations. Further investigations are under way to gain a better insight into the factors that are at play in the mode of binding and sensing of organic substrates with $[Zn^{II}(atmca)]^{2+}$.

Experimental Section

General: Solvents were purified by standard methods. All commercially available reagents and substrates were used as received. TLC analyses were performed on Merck 60 F_{254} glass plates precoated with silica gel. Column chromatography was carried out on silica gel 60 (70–230 mesh, Macherey–Nagel). Melting points were determined with a Buchi 510 capillary melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC250F (250 MHz) and a Bruker AM 400 (400 MHz) spectrometer. Chemical shifts are reported relative to internal Me_4Si. Multiplicity is given as usual. Elemental analyses were performed by the Laboratorio di Microanalisi of the Inorganic and Analytic Chemistry Department of the University of Padova (Italy). UV/Vis absorption measurements were performed on a Perkin–Elmer Lambda 16 spectrophotometer equipped with a thermostated cell holder. Fluorescence spectra were recorded on a Perkin–Elmer LS-50B spectrometer equipped with a thermostated cell holder (1 cm quartz cells). Potentiometric titrations were performed with a Metrohom 716 DMS Titrino dynamic titrator. $Cu(NO_3)_2$, $Zn(NO_3)_2$, $Ni(NO_3)_2$, $CoCl_2$, $HgCl_2$, $Fe(NO_3)_2$, $CdCl_2$, $MnSO_4$ and $Pb(NO_3)_2$ were analytical grade products. Metal-ion stock solutions were titrated against ethylenediaminetetraacetic acid (EDTA) following standard procedures.[25] The buffer components were used as supplied by the manufacturers: acetic acid (Aldrich) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma). all-*cis*-1,3,5-Trimethoxy-2,4,6-triaminocyclohexane (TMCA) was prepared as reported.[8]

Synthesis of all-*cis*-1,3,5-trimethoxy-2-(9-anthracenyl)methylamino-4,6-diaminocyclohexane (ATMCA): Anthracene-9-carbaldehyde (0.23 g, 1.1 mmol) was added to a solution of TMCA (0.26 g, 1.2 mmol) in benzene (40 mL). The reaction mixture was stirred at reflux for 4 h, then the solvent was evaporated under reduced pressure. The remaining solid was dissolved in ethanol (35 mL) and $NaBH_4$ (0.20 g, 5.3 mmol) was added to the resulting solution. The reaction mixture was stirred at room temperature for 6 h. Water (20 mL) was added and the aqueous phase extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was dried (Na_2SO_4) and the solvent

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evaporated to yield the crude product as a yellow oil. Purification was performed by gravity column chromatography (silica gel, $CHCl_3$/EtOH/NH_3(aq) 10:2:0.5, $R_f = 0.15$) to yield ATMCA (0.30 g, 61 %) as yellow solid. M.p. (decomp > 200 °C); ^{1}H NMR (250 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 2.96$ (t, $J = 4.1$ Hz, 1H), 3.16 (t, $J = 3.9$ Hz, 2H), 3.44 (s, 3H); 3.50 (s, 6H), 3.67 (t, $J = 4.0$ Hz, 2H), 3.89 (t, $J = 3.8$ Hz, 1H), 4.88 (s, 2H), 7.41–7.56 (m, 4H), 7.99 (d, $J = 7.8$ Hz, 2H), 8.38 (s, 1H), 8.45 (d, $J = 8.8$ Hz, 2H); ^{13}C NMR (63 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 47.78$, 51.46, 56.41, 56.65, 58.78, 78.99, 79.62, 124.65, 124.86, 125.89, 126.95, 128.90, 130.43, 131.53, 132.45; elemental analysis calcd (%) for $C_{24}H_{31}N_3O_3$ (409.5): C 70.39, H 7.63, N 10.16; found: C 70.05, H 7.58, N 10.09.

Potentiometric titrations: Protonation constants and formation constants of the Zn^{II} complex with ATMCA were determined by pH potentiometric titrations (25 °C, 0.10 M NaCl). Solutions of ATMCA · 3 HCl (ca. 1×10^{-3} M) and when necessary $Zn(NO_3)_2$, were titrated with an aqueous NaOH solution (0.1 M). The electrode system was calibrated by titrating an aqueous solution of HCl (0.01 M) so that the pK_w value was 13.78. The data pertaining to the pH and the volume of added NaOH were fitted with the computer program BEST[26] to obtain the desired protonation constants and complex formation constants.

Spectrometric titrations: Small volumes of concentrated substrate solutions were added to a buffered (1×10^{-2} M) solution of the ligand (1×10^{-6} M) or the $[Zn^{II}(atmca)]^{2+}$ complex (5×10^{-5}), and the UV/Vis or fluorescence spectra were recorded. From the spectral changes observed upon addition of the substrate, the K_{app} values were obtained by non-linear regression analyses of the absorbance or fluorescence data (at the selected wavelength) versus metal ion concentrations.

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[10] Defined by the equation: $K_{app} = [ML]/[L_{tot}][M]$, in which M is the metal ion and L the ligand; $[L_{tot}]$ is the total concentration of L in all its protonated forms.

[11] Defined by the equation: $K_{app} = [MLS]/[ML_{tot}][S_{tot}]$, in which ML is the complex $[Zn^{II}(atmca)]^{2+}$ and S the substrate; $[ML_{tot}]$ and $[S_{tot}]$ are the total concentrations of ML and S in all their protonated or deprotonated forms.

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