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Pyrophosphate Detection in Water by Fluorescence Competition Assays: Inducing Selectivity through the Choice of the Indicator**

Luigi Fabbrizzi,* Nathalie Marcotte, Floriana Stomeo, and Angelo Taglietti

Fluorescent sensors have been designed for a variety of analytes during the last decade by following the classical "fluorophore-spacer-receptor" (FSR) approach in which a light-emitting fragment is covalently linked to a receptor subunit.[1] More recently, a different approach has been introduced, the "chemosensing ensemble" (CE) paradigm, which relies on the use of an indicator (**I**) bound to a receptor (**R**) by means of noncovalent interactions.[2] In this approach the highly colored or fluorescent probe **I** is displaced from **R** by the competing analyte (**S**), and this displacement produces a drastic change in the optical properties of released **I**. All the CE systems described until now were designed for anion sensing and most of them were based on hydrogen-bonding interactions. These interactions are relatively weak and in most cases do not compensate for the rather endothermic desolvation of **R** and **S**, which prevents the utilization of such receptors in pure water. We have recently reported the first example of a CE operating through metal–ligand interactions in which the receptor core is a dinuclear Cu^{II} macrobicyclic complex which is able to detect the carbonate ion in water through the revival of visible light emission of the displaced indicator.[3] The availability of optical molecular sensors for anion detection is highly beneficial for the investigation of aqueous media in food science, cell physiology, and environmental chemistry.[4]

Transition metal ions, for example, Cu^{II}, offer substantial advantages when designing CEs for anions. First of all, coordinatively unsaturated Cu^{II} complexes display strong binding tendencies towards anionic substrates because of the d^9 electronic configuration of the metal center, which ensures high ligand field stabilization effects: as a consequence, anionic substrates can be bound and effectively recognized even in the strongly solvating medium water. Moreover, the Cu^{II} ion provides an operative control of the signal as it is able to completely quench the emission of a coordinated indicator, either of an electron-transfer or an energy-transfer nature, by means of very efficient intramolecular processes.[5] We now demonstrate how the judicious choice of the indicator can turn macrocyclic complexes of Cu^{II} ions with well-known recognition tendencies towards anions into smart optical sensing devices, whose spectral and

[*] Prof. L. Fabbrizzi, Dr. N. Marcotte, Dr. F. Stomeo, Dr. A. Taglietti
Dipartimento di Chimica Generale
Università di Pavia
viale Taramelli 12, 27100 Pavia (Italy)
Fax: (+39) 0382-528-544
E-mail: luigi.fabbrizzi@unipv.it

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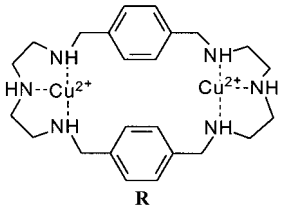
selectivity features can be tuned as required, by simply changing the indicator.

Polyphosphate anions, which participate in several bioenergetic and metabolic processes,[6] represent a relevant target in anion sensing, but very few examples of effective sensors have so far been reported.[7] To the best of our knowledge, the only system able to detect pyrophosphate (PPi) in water at physiological pH values with an off/on fluorescent response was designed following the FSR approach and involved the binding of $H_2P_2O_7^{2-}$ ions by a hexaprotonated anthryloctaamine receptor.[8] However, the fluorescent emission after analyte recognition was only onefold higher than in the absence of analyte, and, most disappointingly, the emission and excitation wavelengths were lower than 400 nm, and thus not suitable for biological applications.[9] Moreover, a receptor comprised of a guanidinium–pyrene conjugate was able to sense PPi through formation of an excimer in MeOH.[10]

We have now chosen as a receptor the dicopper(II) complex of the bisdiene macrocycle 3,6,9,16,19,22-hexaazatricyclo[22.2.2.2(11.14)]triaconta-1(26),11(12),13,24,27, 29-hexaene (**L**). We expected that, in view of the coordinative unsaturation of the two metal centers and the correct $Cu^{II}\cdots Cu^{II}$ distance, the dinuclear complex of **L**, $[Cu^{II}_2(\mathbf{L})]^{4+}$ (**R**), could be a reasonably good candidate for behaving as a fluorescent CE for PPi.[11]

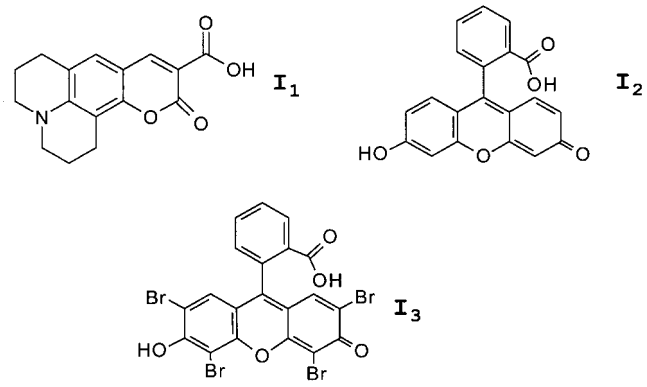


R

Then, we had to find a fluorescent indicator **I** to be coupled to the receptor **R**, whose ideal behavior requires full quenching of **I** when bound to **R** and an appropriate affinity constant (K_I) for the $R + I \rightleftharpoons R{-}I$ equilibrium. In particular, if a substrate **S** displays an affinity constant K_S for **R** at a given pH value, while the interferent **S′** gives its own $K_{S'}$ value, the best situation for discrimination is expressed by the inequality $K_S \gg K_I \gg K_{S'}$.

Under these conditions only the envisaged substrate **S**, and not the interferent **S′**, will be able to displace **I** from **R**, with subsequent revival of indicator fluorescence. In the present case, indicators were chosen on the basis of their spectral features (excitation and emission wavelength higher than 400 nm, high quantum yield) and their capability to interact with the dimetallic receptor: in particular, the indicator should be anionic and should present two donor atoms capable of bridging the two Cu^{II} centers of the receptor. From these perspectives the anion dyes coumarine 343 (**I_1**), fluorescein (**I_2**), and eosine y (**I_3**) containing the carboxylate and phenolate functional groups were chosen.

A complete quenching of the emission was observed on titrating a solution of the indicator (10^{-6} M) buffered at pH 7 (HEPES 0.05 M) with a standard solution of receptor **R**. In all cases, curve fitting of the titration profiles was consistent with



formation of a 1:1 adduct, whose apparent association equilibrium constants (K_I) are reported in Table 1.[12]

Table 1. K_I values for the equilibrium: $\mathbf{R} + \mathbf{I} \rightleftharpoons [\mathbf{RI}]$ at pH 7.

Indicator	Coumarine 343 (**I_1**)	Fluoresceine (**I_2**)	Eosine y (**I_3**)
$\log K_I$	4.5 ± 0.1	5.9 ± 0.1	7.2 ± 0.1

Then, competition assays using CEs of **R/I_1**, **R/I_2**, and **R/I_3** were carried out with several inorganic anions whereby a solution of the chosen **R/I** couple in the spectrofluorimetric cuvette was titrated with a standard solution of the envisaged anion (Figure 1).

The **R/I_1** CE (Figure 1a) is not able to discriminate between phosphates (PPi + orthophosphate (Pi)), but could be used to sense the overall amount of phosphates present in solution through the generation of a substantial enhancement in the probe emission. More interestingly, titrations on the **R/I_2** CE (Figure 1b) showed that only PPi is able to displace the fluorescent probe, and is evident (with a naked-eye-detectable tenfold enhancement of fluorescence) in the 2–20 μM concentration range, even in the presence of any other anions. Competition assays with PPi on the **R/I_2** CE were repeated in the presence of 20 μM nitrate, sulphate, chloride, cyanate, azide, acetate, benzoate, and Pi to confirm the discriminating effects; in all cases, no interference was observed and perfectly superimposable titration profiles were obtained.

Moreover, when using the **R/I_3** couple as the CE, the competition assays displayed discriminating behavior (Figure 1c), with a tenfold fluorescence enhancement even at low concentrations, and allowed detection of PPi at micromolar concentrations. Low detection limits and high sensitivity are related to the K_I value: in particular, the higher the K_I value is, the lower the amount of receptor needed to bind the indicator and to quench its emission almost quantitatively (> 80%), and the lower the concentration of substrate required to displace the equilibrium $[Cu^{II}_2(\mathbf{L})(\mathbf{I})] + \mathbf{S} \rightleftharpoons [Cu^{II}_2(\mathbf{L})(\mathbf{S})] + \mathbf{I}$ to the right. Calibration curves for PPi have been obtained by carrying out a series of reproducible competition assays.

Competition assays of the type described can also be used for determining the association constants for the receptor and the anions. In fact, K_S values for all the investigated substrates[14] at pH 7 can be calculated knowing the relative K_I value.[15] The fitting of the titration profiles with a non-

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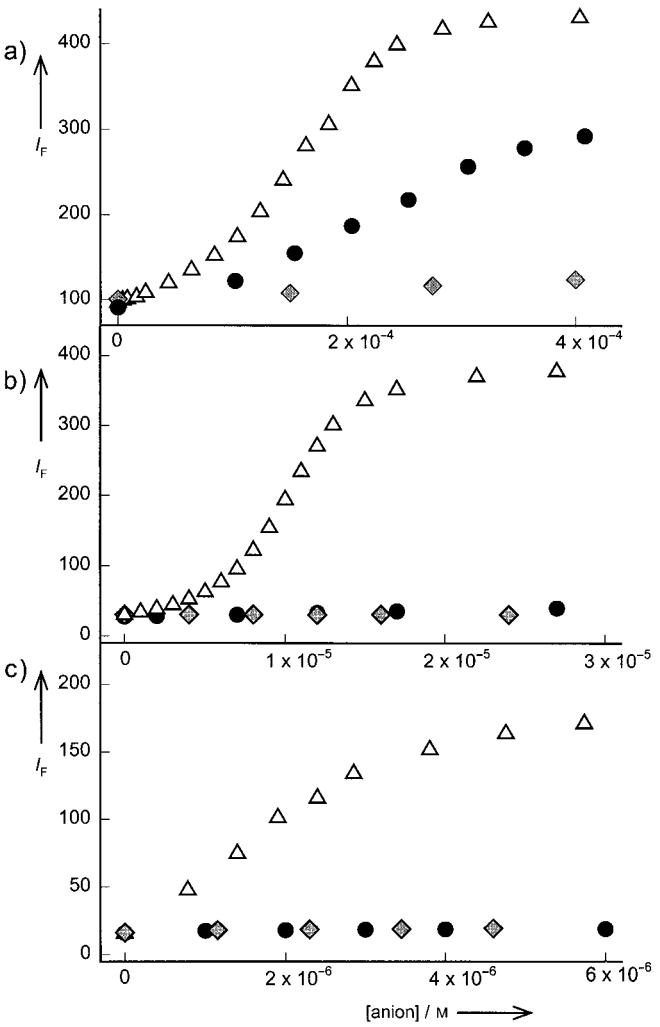

Figure 1. Competitive titrations of aqueous solutions containing chemosensing ensembles {**R**/**I**}, buffered to pH 7, with standard solutions of selected anions: pyrophosphate (△), phosphate (●), chloride, nitrate, sulphate, acetate, and benzoate (◆). a) $[\mathbf{R}] = 2.5 \times 10^{-4}$ M, $[\mathbf{I_1}] = 10^{-6}$ M; b) $[\mathbf{R}] = 1.6 \times 10^{-5}$ M, $[\mathbf{I_2}] = 10^{-6}$ M; c) $[\mathbf{R}] = 2.4 \times 10^{-6}$ M, $[\mathbf{I_3}] = 10^{-6}$ M.

linear least-squares procedure using a model which involves the two competitive equilibria gave the $\log K_S$ values reported in Table 2: the satisfactory agreement of the K_S values obtained for the same anion when using different indicators demonstrates the validity of the approach. The much higher affinity of PPi compared to Pi towards the dimetallic receptor can be ascribed to the capability of the diphosphate ion to coordinate the two Cu^{II} centers by using two oxygen atoms from the two phosphonate groups. On the other hand, Pi is forced to bridge the $Cu^{II}\cdots Cu^{II}$ distance with two oxygen atoms of the same phosphonate group, a strained situation which may induce an endoergonic rearrangement of the polyamine framework. Most interestingly, the observed discrimination effects are confirmed to result from the fulfilment of the inequality $K_S \gg K_I \gg K_{S'}$. For example, the PPi/Pi discrimination observed with the **R**/**I**$_2$ CE is a consequence of $K_S \gg K_I \gg K_{S'}$ being $7.2 > 5.9 > 4.2$. On the other hand, **I**$_1$ forms a poorly stable association complex with the receptor and is also competitively displaced from Pi, and thus fails to provide PPi/Pi discrimination as evident from K_S:K_I:$K_{S'}$ being $7.2 > 4.5 \approx 4.2$. In addition to Pi, other anions that display some competing tendency towards **R** are NCO^- and N_3^-. However, the corresponding association constants (Table 2) are not high enough to allow displacement of **I**$_2$ and **I**$_3$ and cause interference.

In conclusion, the CE approach presents some significant benefits over the FSR paradigm: first, it does not involve any tedious synthetic step for covalently linking together the luminophore and the receptor. Moreover, a further element of selectivity is introduced in addition to that related to the analyte–receptor interaction. This selectivity term is associated with the choice of the indicator, whose affinity towards the receptor determines which analytes can be detected and which cannot. A final point is concerned with the signal transduction mechanism: covalent linking of the fluorophore and receptor in FSR systems does not necessarily imply switching on/off the fluorescence when recognition occurs. In fact, a signal can be generated if the analyte itself is photophysically active and quenches the fluorophore,[16] or it interrupts a pre-existing quenching mechanism specifically designed into the system.[8] Things are much simpler in the metal-containing CE described here, in which efficient quenching of the fluorophore is directly provided by Cu^{II} ions and is suspended when the indicator is released to the solution, with a full revival of the fluorescence detectable by the naked eye. We are currently using the CE approach with transition-metal-containing receptors for the fluorescent detection of biologically relevant substrates which possess an anionic functionality, for example, amino acids.

Table 2. $\log K_S$ values for $\mathbf{R} + \mathbf{S} \rightleftharpoons [\mathbf{RS}]$ equilibria in aqueous solution at pH 7, as obtained from competition measurements with different indicators.

	Fluoresceine (**I**$_2$)	Eosine y (**I**$_3$)	Coumarine 343 (**I**$_1$)
PPi	7.2 ± 0.2	7.2 ± 0.2	> 6.5
Pi	4.4 ± 0.2	4.0 ± 0.2	4.2 ± 0.1
NCO^-	4.7 ± 0.2	4.6 ± 0.2	–
N_3^-	4.0 ± 0.2	–	–
Cl^-, NO_3^-, SO_4^{2-}, N_3^-, acetate, benzoate	< 3	< 3	< 3

Experimental Section

L was prepared as described elsewhere.[17] All other chemicals were purchased from Aldrich/Fluka and used without any further purification. The complex **R** was prepared by dissolving $Cu(NO_3)_2 \cdot 3H_2O$ (1.52 g, 6.28 mmol) in MeOH and adding this to a solution of **L** (1.29 g, 3.14 mmol) in MeOH. The mixture was heated to reflux, and after 30 min the complex $[Cu_2^{II}(\mathbf{L})](NO_3)_4$ (**R**) was collected as a bright blue microcrystalline precipitate by filtration. Yield: 80%. Elemental analysis calcd for $C_{24}H_{38}N_6Cu_2 \cdot (NO_3)_4$: C 36.69, H 4.87, N 17.68; found: C 36.42, H 4.56, N 17.68.

Association constants between **R** and indicators (**I**) were determined in a degassed water solution buffered at pH 7 with 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 10^{-6} M **I**. Aliquotes of a fresh standard solution of **R** were added and the emission spectra of **I** were recorded (**I**$_1$: $\lambda_{exc} = 424$ nm, $\lambda_{em} = 487$ nm; **I**$_2$: $\lambda_{exc} = 489$ nm, $\lambda_{em} = 513$ nm; **I**$_3$: $\lambda_{exc} = 524$ nm, $\lambda_{em} = 540$ nm). The solutions of the chemosensing ensemble for competition assays were prepared by adding **R** to an aqueous solution, buffered at pH 7 with 0.05 M HEPES containing the chosen indicator. The concentration of **R** in the CE

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solution was chosen to quench at least 80% of the emission of the free indicator: 2.5×10^{-4} M for $\mathbf{I_1}$, 1.6×10^{-5} M for $\mathbf{I_2}$, and 2.4×10^{-6} M for $\mathbf{I_3}$. The CE solution was titrated with standard solutions of anions. All spectrofluorimetric titration curves were fitted with the HYPERQUAD program.[18]

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