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Transition-metal-based Chemosensing Ensembles: ATP Sensing in Physiological Conditions

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Sensing of biologically relevant anionic substrates in physiological conditions, employing the strategy of the chemosensing ensembles, is reported. Coordination of a fluorescent indicator to a dicopper(II) polyazamacrocyclic receptor ($[Cu_2(L)]$) results in the collapse of its fluorescence emission. Competitive binding of substrates for the receptor releases the indicator in solution, with full emission recovery. The spectral changes obtained for some indicators and substrates were analysed to determine their respective association constants for the receptor. Discrimination of micromolar ATP quantities from other interferents (small inorganic anions and well-known neurotransmitters) is improved by a judicious choice of the indicator, the resulting ATP sensor promising interesting biological applications.

Keywords: Fluorescent probes; Molecular recognition; ATP; Neurotransmitters; Macrocyclic complexes

The Future of Supramolecular Chemistry

I always liked Supramolecular Chemistry because of its borderline nature, and even if it is very difficult to predict where it can arrive, the road to take is quite clear. Supramolecular Chemistry should be the main route to connect chemistry to other disciplines like life sciences, materials engineering, logics and computing, in order to solve practical problems. Popular examples of close goals for scientists with a multidisciplinary knowledge are functionalisation of surfaces to obtain smart materials for data storage and nanosized electronic components and realization of light-powered molecular level machines. Another classical topic concerns real time monitoring of biologically relevant molecules during their activity *in vitro* or *in vivo* using synthetic sensors: the contribution here presented shows how we can try to answer to a classical biological sensing demand (i.e. revealing the presence of one single neurotransmitter in presence of many other) with a very simple and economic approach.

Angelo Taglietti graduated in Chemistry from the University of Pavia, where he also obtained his PhD in 1996 with a thesis on Electron Transfer Processes in Supramolecular Chemistry, under the supervision of Professor Luigi Fabbrizzi. During postdoc research his main interests were focused on development of transition metal based receptors and sensors for anions. Since 1999 he has worked as a research associate in the University of Pavia, and his current research is centred on design of sensory systems for biologically relevant substrates, kinetic characterization of molecular translocations, design and synthesis of simple molecular level devices.

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INTRODUCTION

Anions play a huge number of key roles in biological processes, and the importance of recognition and sensing of anionic substrates in biological chemistry is of obvious evidence [1–3]. Despite this, there are still few suitable examples of synthetic receptors and sensors working in water solution or in physiological conditions [4,5], the greatest part of papers being devoted to systems that operate in non-aqueous media or solvent mixtures [6–10]. Anions are quite an elusive target: they often have a low density charge, large hydration free energies, and different and complicated shapes, compared with the more easily recognizable metal cations. These are some of the reasons for the arousal of interest in this topic: the problem of anion sensing is nowadays one of the most intriguing challenges for supramolecular chemists.

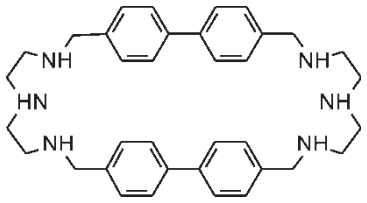
Among existing chemosensors, fluorescent chemosensors are the most well known and utilized. Fluorescence signalling offers clear advantages: high sensitivity, selectivity, possibility to work in real time, non-destructive measurements, low cost of instrumentation [11]. A well-established strategy, based on the so-called multicomponent approach, suggests designing anion sensors by covalently linking a light-emitting fragment to a subunit that displays specific binding tendencies for an analyte [12,13]. An efficient sensor is obtained when the receptor–analyte interaction affects the emission properties of the signalling unit, generally by quenching the fluorescence through a defined mechanism (usually energy or electron transfer—ET and eT) or by enhancing it, by suppressing a pre-existing quenching process. A large number of fluorosensors have been designed following this "fluorophore–spacer–receptor" (FSR) approach [14]. However, most of the interactions that can be used for anionic substrate recognition, such as hydrogen bonds or electrostatic attractions, are not always strong enough to give high affinities in water. The problem can be circumvented by using transition-metal complexes, with at least one free coordination position, as receptor subunits [4,15]. This allows recognition of anionic substrates by means of coordinative interactions, which are usually stronger than electrostatic interactions. In this way, the metal ion should act just like a structural element bringing the fluorophore and substrate close together [16–18]. This role restricts the choice of metal ions to photophysically innocent ones, like Zn^{II}, and rules out any genuine transition metals (such as Cu^{II} and Ni^{II}) that can quench fluorescence by eT or ET processes [19].

More recently, Anslyn introduced a different approach, the "Chemosensing Ensemble" (CE) paradigm (Scheme 1), which relies on the use of an

SCHEME 1

indicator (**I**) bound to a receptor (**R**) by means of non-covalent interactions. In these systems, a highly coloured or fluorescent probe **I** is displaced from **R** by the competing analyte (**S**), this displacement inducing a drastic change in the optical properties of released **I** [20–23].

The use of competition equilibria presents some advantages over classical FSR sensors. First, no time-consuming synthetic work is spent for covalently linking fluorophore and receptor. Moreover, the absence of functionalization of the receptor leaves its coordinating ability unchanged. Last but not least, the indicator can be chosen and changed as required, this choice playing a crucial role in CE features [24,25], as will be shown later. Most of the CE systems described to date are based on hydrogen-bonding interactions, with the aforementioned limitations. This gave us the idea on how to exploit genuine transition metal features of Cu^{II}: strong binding tendencies compared with Zn^{II} (due to high crystal field stabilization energy, CFSE) and quenching properties. We have recently reported two examples of CE based on dinuclear copper complexes able to sense carbonate [26] and pyrophosphate [27], respectively, in water solution at pH 7. In these systems, the two Cu^{II} centres provide well-oriented binding sites able to strongly allocate both bidentate anions [28,29] and anionic indicators. The study reported here shows how to apply this strategy, also in order to better define its potentiality and limitations, to the challenge of triphosphate and ATP detection [30–36]. Not only is ATP the universal fuel in most biological systems, but it is believed to play a crucial extracellular role as a fast neurotransmitter in a variety of tissues [37,38]. Starting from our previous results on the discrimination of pyrophosphate from phosphate and other small inorganic anions [27], we prepared a larger macrocycle **L** (Scheme 2) [39], relying on its dicopper complex



SCHEME 2

ability to selectively bind larger triphosphate anions and thus distinguish ATP from other classical neurotransmitters.

RESULTS AND DISCUSSION

Copper(II) Complexes of Ligand L

The stability of the complexes formed between **L** and Cu^{II} was investigated by potentiometric titrations, using four different ligand-to-metal molar ratios (see Experimental). The potentiometric equilibrium data of the solutions containing a 1:1 and a 1:2 molar ratio of $[\mathbf{L}H_6]^{6+}$ and Cu^{II} are shown in Fig. 1.

The equimolar solution reveals two inflexion points at $a = 4$ and $a = 7$ that appear at $a = 6$ and $a = 8$, respectively, for the solution containing two Cu^{II} equivalents. The inflection point at $a = 6$ undoubtedly corresponds to the completion of the neutralization of the six ammonium ions of the diethylenetriamine moieties, which are easily deprotonated to form dinuclear complexes, as reported for smaller hexaza macrocyclic ligands [28,40,41]. The inflection at $a = 8$ indicates deprotonation of two other titratable protons from $[Cu_2(\mathbf{L})]^{4+}$. The equilibrium constants (Table I) were determined by simultaneously fitting the potentiometric data of the four experiments to avoid any spurious contribution that might be obtained by single-fit calculations. For instance, using a single-fit procedure, the data of the 1:1 molar ratio experiment could be fitted suitably only by taking into account the presence of $[Cu(\mathbf{L})]^{2+}$ species. However, its presence was no longer needed with a global analysis undertaken on the overall data, and, as can be seen from Fig. 1, good fitting results were obtained. The protonation and complexation constants obtained are listed in Table I.

TABLE I Protonation and copper(II) stability constants and stepwise formation for the $\mathbf{L}$–Cu^{II} system ($I = 0.1$ M $NaNO_3$ at 25°C)

Stoichiometry				
L	Cu	H	Log β	Reaction
1	0	2	17.50	$\mathbf{L} + 2H^+ \rightleftarrows [\mathbf{L}H_2]^{2+}$
1	0	3	25.45	$\mathbf{L} + 3H^+ \rightleftarrows [\mathbf{L}H_3]^{3+}$
1	0	5	32.27	$\mathbf{L} + 5H^+ \rightleftarrows [\mathbf{L}H_5]^{5+}$
1	2	0	19.18	$\mathbf{L} + 2Cu^{2+} \rightleftarrows [Cu_2(\mathbf{L})]^{4+}$
1	2	−1	11.59	$\mathbf{L} + 2Cu^{2+} + OH^- \rightleftarrows [Cu_2(\mathbf{L})H_{-1}]^{3+}$
1	2	−2	3.33	$\mathbf{L} + 2Cu^{2+} + 2OH^- \rightleftarrows [Cu_2(\mathbf{L})H_{-2}]^{2+}$
1	1	−1	1.76	$\mathbf{L} + Cu^{2+} + OH^- \rightleftarrows [Cu(\mathbf{L})H_{-1}]^{+}$

Figure 2 displays the species distribution as a function of pH at a total Cu^{II} concentration of 2 mM and a total **L** concentration of 1 mM, at 25°C. The dinuclear complex $[Cu_2(\mathbf{L})]^{4+}$ is the predominant species over the pH range 3.5–7.5 (maximum at pH 5.5, 100%). $[Cu_2(\mathbf{L})H_{-1}]^{3+}$ and $[Cu_2(\mathbf{L})H_{-2}]^{2+}$ are the major complexes in solution at pH >7.5. The monodeprotonated $[Cu_2(\mathbf{L})H_{-1}]^{3+}$ has a favourable pH of 8, above which it is gradually converted to $[Cu_2(\mathbf{L})H_{-2}]^{2+}$. It can be seen in Fig. 2 that at pH 7, the pH at which we performed our absorption and fluorescence studies, nearly 80% of the dinuclear complex $[Cu_2(\mathbf{L})]^{4+}$ is present, the remaining 20% being the monohydroxide dicopper complex. At physiological pH, our candidate $[Cu_2(\mathbf{L})]^{4+}$ receptor, for which each triamine compartment is complexed by one copper ion, can then be considered as major species. In these conditions, with two water molecules expected to be coordinated to each copper ion, the overall +4 charge

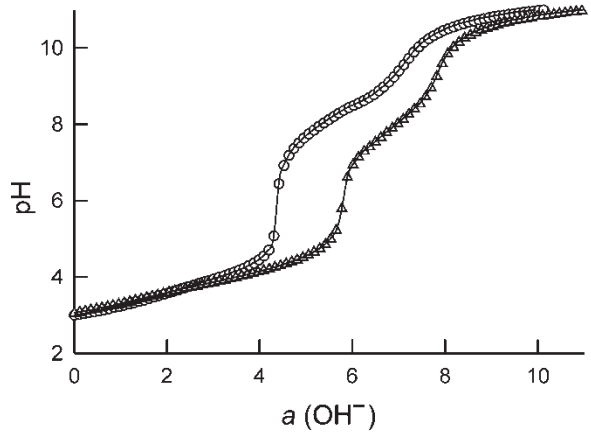


FIGURE 1 Potentiometric titration of the 1:1 (circles) and 1:2 molar (triangles) ratios of $\mathbf{L}$:Cu^{II} in the presence of six-ligand equivalent protons (a = number of moles of NaOH added per mole of ligand, $I = 0.1$ M $NaNO_3$ at 25°C). Symbols represent the experimental data and solid lines the fitting of the data.

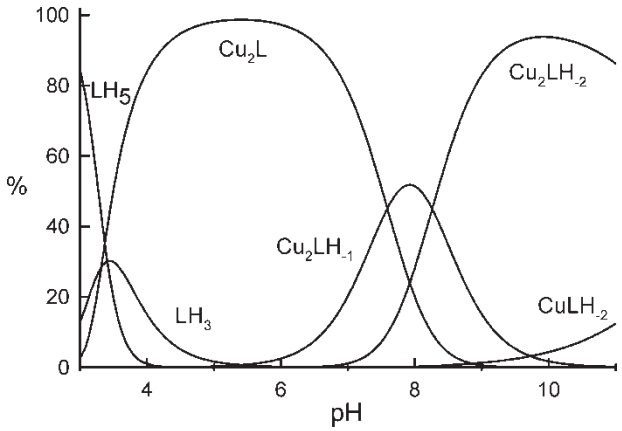
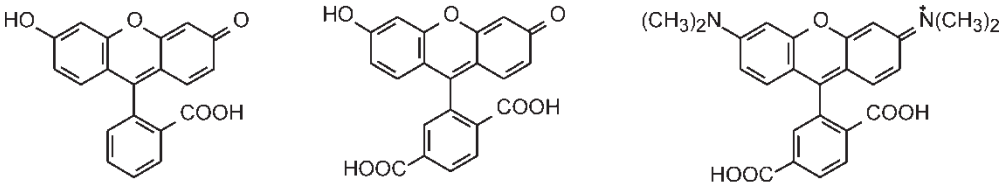


FIGURE 2 Species distribution diagram showing the species formed as a function of pH when 1×10^{-2} M of **L** and 2×10^{-2} of Cu^{II} are equilibrated at 25°C, $I = 0.1$ M $NaNO_3$.



Fluorescein 6-FAM 6-TAMRA

SCHEME 3

should represent an optimal situation for the binding of large anionic substrates.

Force Field Calculations

Geometries optimisation were performed on $[Cu_2(\mathbf{L})]^{4+}$ receptor in which each metal ion is coordinated to two water molecules, by using HYPERCHEM 6 package (MM + method). Its Cu^{II}–Cu^{II} internuclear distance is estimated to be 10.50 Å, a value quite close to that (11.4 Å) obtained from crystallographic data [42]. The same minimizations were repeated in the presence of the bridging triphosphate anion. A slightly shortened distance of 10.25 Å was measured, without any serious conformational change compared with the empty receptor. However, when the calculation was performed in the presence of the bridging pyrophosphate anion, a very distorted conformation was obtained, for which an intermetallic distance of 8.7 Å was calculated. These simple calculations reinforced our expectations on a higher receptor affinity for the larger and longer triphosphate anion.

Indicator to Receptor Complexation Studies

Once the suitable receptor for a given substrate is found, the next step in the realization of a CE is to choose an indicator able to interact with it. In this respect, the binding behaviours of the three dyes presented in Scheme 3 were examined, based on the ability of their anionic and binding functions to interact with the two Cu^{II} centres of the receptor. Note that these well-known indicators absorb light in the visible range of the absorption spectrum (above 400 nm) and are strongly emissive, i.e. they possess some optical properties favourable to measurements in biological conditions [11]. Complexation of indicators was investigated by absorption and fluorescence spectroscopy to determine which indicators might exhibit adequate behaviour to measure triphosphate residue, at especially low-level concentrations.

Absorption Spectroscopy

Absorption spectra of the three indicators are greatly altered by the presence of receptor (Fig. 3). For example, upon addition of $[Cu_2(\mathbf{L})]^{4+}$, the intensity of the main absorption band of 6-TAMRA (2×10^{-5} M, $\lambda_{max} = 548$ nm, Fig. 3a) is first decreased, and then enhanced and shifted 4 nm to the blue. Its shoulder initially peaking at 520 nm shows the reverse trend. The inset of Fig. 3a shows the absorption profiles recorded at 514 and 550 nm, from which the formation of a 1:1 stoichiometry complex is revealed by a plateau beyond 2×10^{-5} M of **L** added. Moreover, the presence of a 2:1 indicator–receptor complex is clearly evidenced by the optical density changes observed at an added ligand concentration of 1×10^{-5} M. The absorption data and receptor concentration were compared to determine the association constants (K_I) between receptor and 6-TAMRA (Table II). The calculated values show an especially high constant for the 1:1 species [$\log(K_{RI}) = 8.1$] with respect to the 2:1 indicator–receptor complex [$\log(K_{RI_2}) = 5.3$]. 6-FAM exhibits an absorption maximum at 492 nm (Fig. 3b), which is slightly blue-shifted to 488 nm and enhanced upon receptor addition. Contrary to 6-TAMRA, the absorption titration profiles (the inset of Fig. 3b shows the 496-nm trace) do not evidence a 2:1 indicator-to-receptor complex. However, the data could be fitted adequately only by taking into account its presence. The absorption maximum of Fluorescein ($\lambda_{max} = 490$ nm) is decreased to the benefit of a new absorption band

TABLE II Log K_I* values for $\mathbf{R} + \mathbf{I} \rightleftarrows \mathbf{RI}$, equilibria in aqueous solution at pH 7, as obtained from direct spectrophotometric and spectrofluorimetric titrations

Indicator	Log K	Absorption data	Emission data
Fluorescein	Log K_{RI}	6.0 ± 0.1	6.2 ± 0.1
6-CF	Log K_{RI}	8.3 ± 0.1	8.4 ± 0.1
	Log K_{RI2}	4.9 ± 0.2	5.4 ± 0.2
6-TAMRA	Log K_{RI}	8.1 ± 0.1	8.1 ± 0.1
	Log K_{RI2}	5.3 ± 0.1	5.7 ± 0.1

*Defined by the equations $K_I = [Cu_2(\mathbf{L})(\mathbf{I})]_{tot}/[Cu_2(\mathbf{L})]_{tot}[\mathbf{I}]_{tot}$, where $[Cu_2(\mathbf{L})(\mathbf{I})]_{tot}$, $[Cu_2(\mathbf{L})]_{tot}$ and $[\mathbf{I}]_{tot}$ are the total concentrations of $Cu_2(\mathbf{L})(\mathbf{I})$, $Cu_2(\mathbf{L})$ and **I**, in all their protonated and deprotonated forms, at a given pH (see also Ref. [5]).

situated at longer wavelengths (502 nm). These absorbance changes (Fig. 3c) lead to a clear isosbestic point at 496 nm, suggesting the existence of a simple equilibrium between the free and complexed fluorophore. The presence of a single 1:1 indicator–receptor complex is supported by our fitting procedure. Association constants of the three fluorophores are gathered in Table II, and results of the fits are shown in the insets of Fig. 3. Although the exact nature of the binding mode is not of crucial importance for our present work, it is interesting to note that the large changes in absorption spectra seem to indicate that the receptor directly interacts with the xanthenic moiety of indicators [25].

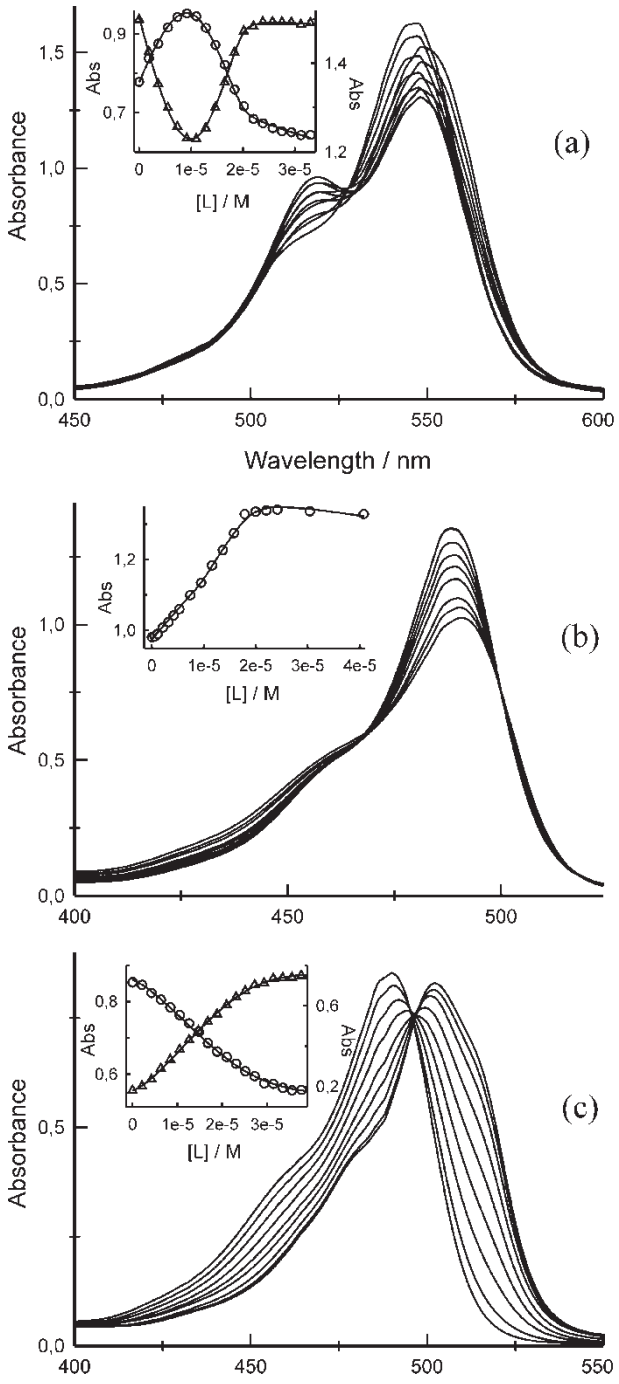


FIGURE 3 Absorption spectra of a solution containing indicators (2×10^{-5} M) and $Cu(NO_3)_2$ (4.5×10^{-4} M) buffered at pH 7 with HEPES (0.05 M) upon titration with ligand **L**. (a) 6-TAMRA, (b) 6-FAM, (c) Fluorescein. Insets: fitting curves of the absorbance changes vs. ligand concentration at 514 (circles) and 550 nm (triangles) for 6-TAMRA, 496 nm for 6-FAM, 490 (circles) and 510 nm (triangles) for Fluorescein.

Fluorescence Spectroscopy

Titrations were repeated by fluorescence spectroscopy, using lower dye concentrations. For all the indicators, a complete quenching of fluorescence was observed upon addition of ligand **L**. As an example, Fig. 4 shows the emission spectra changes obtained in the case of 6-TAMRA.

The optical variations of the fluorescence intensity vs. ligand concentration were processed to assess the interaction between receptor and indicators. The association constants thus calculated (Table II) are in good agreement with those obtained from absorption data. The quality of the fit reached for 6-TAMRA is shown in the inset of Fig. 4. The drastic fluorescence changes observed between the bounded and unbounded fluorophores make them good potential candidates for signalling the recognition of anionic substrates though the CE approach.

Substrates to Receptor Complexation Studies

The complexation ability of receptor $[Cu_2(\mathbf{L})]^{4+}$ towards anions was tested by means of competition

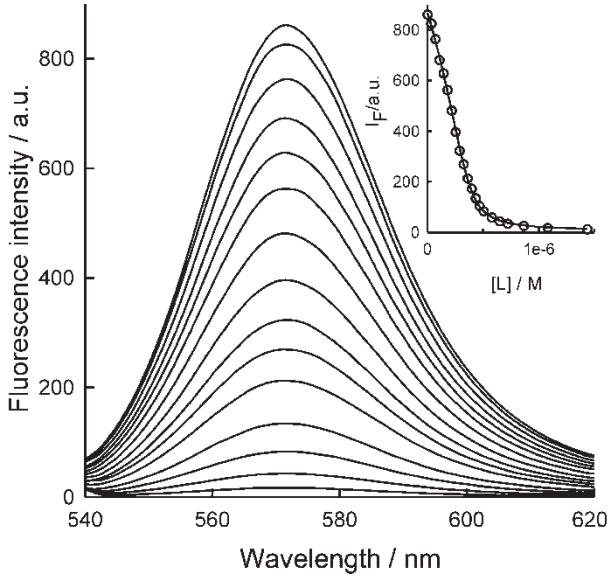


FIGURE 4 Emission spectra of 6-TAMRA (2.5×10^{-7} M) buffered at pH 7 with HEPES (0.05 M) in the presence of $Cu(NO_3)_2$ (8×10^{-6} M) upon titration with ligand **L**, $\lambda_{ex} = 528$ nm. Inset: fitting curve of the emission changes vs. ligand concentration at 572 nm.

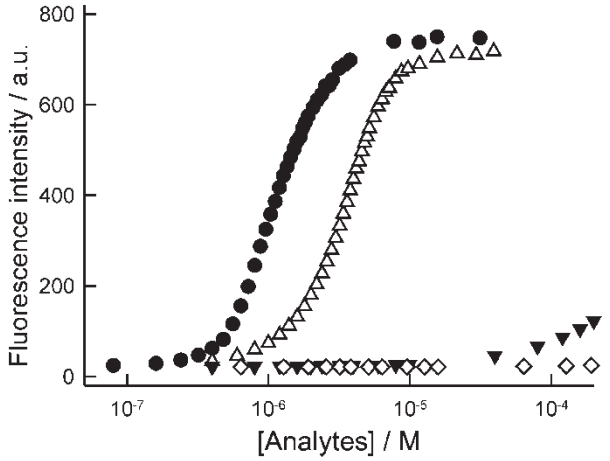


FIGURE 5 Competitive titrations of aqueous solutions containing CEs of 6-TAMRA (2.5×10^{-7} M), $Cu(NO_3)_2$ (8×10^{-6} M), **L** (1.5×10^{-6} M) buffered at pH 7 with HEPES (0.05 M), with standard solutions of selected anions: triphosphate (black circles), pyrophosphate (white triangles), phosphate (black triangles), chloride and nitrate (white diamonds). $\lambda_{ex} = 528$ nm, $\lambda_{em} = 572$ nm.

TABLE III Log K_S* values for $\mathbf{R} + \mathbf{S} \rightleftarrows \mathbf{RS}$ equilibria in aqueous solution at pH 7, as obtained from competition measurements with different indicators

	6-TAMRA	6-CF	Fluorescein
Chloride	$\ll 4$		
Nitrate	$\ll 4$		
Carbonate	$\ll 4$		
Phosphate	4.7 ± 0.2		
Diphosphate	7.3 ± 0.2	7.0 ± 0.2	7.1 ± 0.1
Triphosphate	8.0 ± 0.2	8.1 ± 0.1	8.1 ± 0.1
AMP	5.3 ± 0.2	4.6 ± 0.2	
ADP	7.4 ± 0.2	7.2 ± 0.1	
ATP	7.8 ± 0.2	8.0 ± 0.1	
Aspartic acid	3.9 ± 0.2	4.2 ± 0.1	
Glutamic acid	5.3 ± 0.2	4.9 ± 0.2	
GABA	$\ll 4$		
Acetylcholine	$\ll 4$		
Dopamine	$\ll 4$		
Adenosine	$\ll 4$		

*Defined by the equations $K_S = [Cu_2(\mathbf{L})(\mathbf{S})]_{tot}/[Cu_2(\mathbf{L})]_{tot}[\mathbf{S}]_{tot}$, where $[Cu_2(\mathbf{L})(\mathbf{S})]_{tot}$, $[Cu_2(\mathbf{L})]_{tot}$ and $[\mathbf{S}]_{tot}$ are the total concentrations of $Cu_2(\mathbf{L})(\mathbf{S})$, $Cu_2(\mathbf{L})$ and **S**, in all their protonated and deprotonated forms, at a given pH (see also Ref. [5]).

assays. The CE solutions consisted of a mixture of indicator and receptor, at a concentration that ensured a quantitative complexation. At the ligand concentration used, only the 1:1 indicator to receptor complex was calculated to be present. For each indicator, standard solutions of anions were added to the CE solutions, and the emission spectra were recorded. The titration profiles obtained for several anions using 6-TAMRA indicator are shown in Fig. 5.

When a triphosphate solution is added to the CE, a drastic increase in probe fluorescence is observed, leading to almost complete recovery of the emission, at the micromolar concentration added. This means that triphosphate binds $[Cu_2(\mathbf{L})]^{4+}$ with a high affinity, causing a displacement of 6-TAMRA from the receptor cavity to the solution, and restoring its emission proprieties. The addition of pyrophospate yielded a similar result but with a lower signal enhancement in the same range of concentrations ($< 3\times10^{-6}$ M). This suggests that pyrophosphate has a lower receptor affinity than triphosphate. A higher concentration is then needed to reach the same equilibrium position and to obtain full fluorescence recovery. However, the addition of inorganic anions such orthophosphate (2×10^{-4} M), chloride (2×10^{-2} M) and nitrate (5×10^{-2} M) did not produce any noticeable emission enhancement (less than 10%) in the concentration range investigated. In other words, their affinities are not high enough to kick the indicator off the receptor and restore the fluorescence signal. The use of 6-FAM showed the same discrimination pattern as 6-TAMRA, whereas Fluorescein presented very similar titration profiles for tri- and pyrophosphate, not allowing any discrimination between them.

These different behaviours can be explained by the receptor association constants (K_s) values of the substrates (Table III). The first thing we must underline is that for all the anionic substrates for which K_s calculation was possible, data fitting of the competition assay only accounted for a 1:1 receptor–substrate adduct formation. Also noticeable is the good agreement between the values obtained using different indicators [27].

The best situation for distinguishing between a substrate of interest **S** ($P_3O_{10}^{5-}$ in our case) from an interferent **S′** (like $P_2O_7^{4-}$, HPO_4^{2-}, Cl^-, NO_3^-) is achieved when the association constants of the indicator **I** and the substrate **S** are close, so that an effective competition for binding to the receptor takes place. This situation can be expressed by inequality (1):

$$K_I \approx K_S >> K_{S'} \quad (1)$$

When this condition is achieved, only the substrate **S**, and not the interferent **S′**, is able to displace **I** from **R**, and recovery of indicator emission is expected [24,27].

For our investigations on ATP recognition, assuming that its receptor complexation ability should not be very different from that of triphosphate anion, we decided to focus on the 6-TAMRA indicator. Titrations were repeated with the biologically relevant nucleotides ATP, ADP and AMP. A discrimination pattern nearly identical to that of their corresponding (poly)phosphate anions was observed. Table III reports the calculated stability constants. Once again, in all cases, least-squares

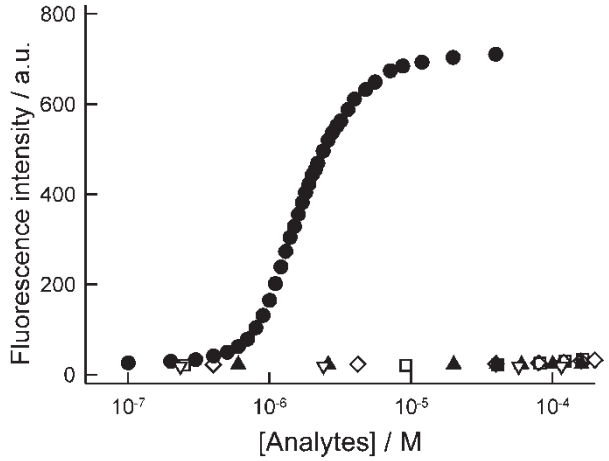


FIGURE 6 Competitive titrations of aqueous solutions containing CEs of 6-TAMRA (2.5×10^{-7} M), $Cu(NO_3)_2$ (8×10^{-6} M), **L** (1.5×10^{-6} M) buffered at pH 7 with HEPES (0.05 M), with standard solutions of selected neurotransmitters: ATP (black circles), dopamine (white triangles), γ-aminobutanoic acid (GABA, black triangles), acetylcholine (white diamonds), glutamic acid (white squares). $\lambda_{ex} = 528$ nm, $\lambda_{em} = 572$ nm.

treatment of the competition assays only gave account for the formation of a 1:1 adduct between receptor and substrate. From the close similarity with their respective inorganic phosphate residues, it can be reasonably hypothesised that nucleotides bind the receptor by some interactions of their anionic tail. However, it cannot be excluded, especially for AMP, that adenosine residue is involved in the binding mode. On the basis of these results, we tested the possibilities of the CE to discriminate ATP from other classical neurotransmitters. The titration profiles of CE $[Cu_2(\mathbf{L})]^{4+}$/6-TAMRA obtained using some well-known neurotransmitters are reported in Fig. 6. They clearly evidence that the CE, able to detect micromolar concentrations of ATP in physiological conditions, can discriminate ATP perfectly from other classical neurotransmitters, even when they are present at a millimolar concentration range. This situation is also illustrated in Fig. 7, which shows emissions of CE solutions (containing the five neurotransmitters at 10^{-5} M) upon excitation with a simple laboratory UV lamp. Tests on the applicability of this sensing system for ATP detection in biological samples are now in progress.

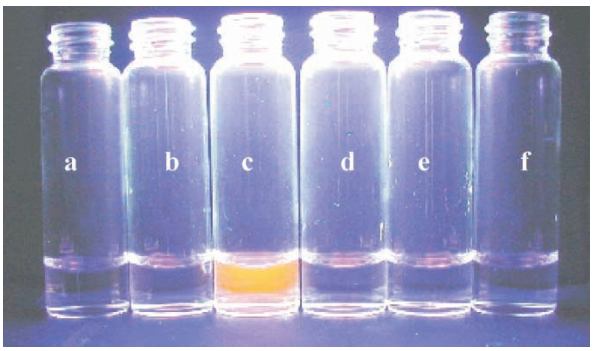


FIGURE 7 Fluorescence emission of the CE solution [6-TAMRA, 2.5×10^{-7} M; $Cu(NO_3)_2$, 8×10^{-6} M; **L**, 1.5×10^{-6} M] in the absence and presence of neurotransmitters: (a) no substrate, (b) dopamine, (c) ATP, d) γ-aminobutanoic acid (GABA), (e) acetylcholine, (f) glutamic acid. (See colour plate 10 at the end of this issue.)

CONCLUSION

The design of a good receptor for a molecule of interest is not always easy, and it can be even more difficult to obtain a useful sensor. In that sense, the use of the CE approach offers a smart way to overcome the problem of time-consuming synthetic work for covalently linking a fluorescent reporter to a host molecule, furthermore leaving its coordinative abilities unchanged. The use of Cu^{II} transition metal ions as a binding core of the receptor allows strong coordinating tendencies for anionic substrates to be established. They also provide a powerful way for controlling the signal with their abilities to quench emission of the fluorescent indicators. Only an analyte of competitive affinity is then able to displace the indicator from the receptor cavity, restoring its emission. The choice of indicator is useful, not only to modulate spectral features of the sensing system, such as emission or excitation wavelengths, but also to tune its discrimination tendencies. The CE reported here was shown to be able to distinguish triphosphate anion from other inorganic anions, and to detect micromolar concentrations of ATP in aqueous solution at pH 7, even in the presence of millimolar quantities of other known neurotransmitters. This particularly interesting feature allows us to envisage its possible use, among different biological applications, as a fluorescent sensory system for a real-time videomicroscopy analysis of ATP release from brain-derived cell cultures. This would offer a new powerful tool to investigate the role played by this neurotransmitter in the complex interplay between neurons and glia cells within the central nervous system.

EXPERIMENTAL

Materials

Spiro(isobenzofuran-1(3*H*),9′-(9*H*)xanthene)-6-carboxylic acid, 3′,6′-dihydroxy-3-oxo-(6-FAM) and xanthylium, 9-(2,5-dicarboxyphenyl)-3,6-bis(dimethylamino)-(6-TAMRA) were obtained from Molecular Probes. All other reagents and substrates were from Aldrich or Fluka and were used as

received, without further purification. All solutions were prepared in deionized water.

Synthesis

The preparation of 7,10,13,24,27,30-hexaazapentacyclo[30.2.2.22,5.215,18.219,22]dotetraconta-2,4,15,17,19,21,32,34,35,39,41-dodecaene (**L**) was adapted from a general procedure previously described [43]. In brief, 4,4′-diformylbiphenyl was obtained by coupling 4-bromobenzaldhehyde (2.44 g, 13.1 mmol) with bis(1,5-cyclooctadiene)nickel (2 g, 7.3 mmol) in dimethylformamide (40 mL) [44]. The resulting dialdehyde (4.7 mmol) was then added dropwise over 1 h to a stirred solution of diethylenetriamine (4.6 mmol) in acetonitrile (140 mL). The reaction mixture was left under agitation at room temperature for 24 h. The pale yellow precipitate (1.1 g) was filtered off and dissolved in 300 mL of methanol, to which 1.25 g of $NaBH_4$ was carefully added. The solution was stirred for 1 h at reflux, the solvent removed, and the residue extracted in CH_2Cl_2:H_2O (80:20). The white powder obtained was recrystallized in methanol. Yield: 65%. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 1.6$ (s, 6H, NH), 2.80 (t, 8H, NCH_2), 2.90 (t, 8H, NCH_2), 3.85 (s, 8H, $ArCH_2$), 7.4 and 7.6 (2 × d, 16H, ArH); FAB: m/z 563 $[MH]^+$. Elemental analysis calcd. for ($C_{36}H_{46}N_6$): C 76.87, H 8.18, N 14.95; found: C 75.75, H 8.30, N 14.55.

Potentiometric Measurements

Potentiometric titrations were carried out with a Titralab Model 90 (Radiometer Analytical) pH meter fitted with glass and calomel electrodes. Experiments were conduced in a jacketed cell thermostated at 25.0 ± 0.1°C kept under an atmosphere of nitrogen. $NaNO_3$ was employed as the supporting electrolyte to keep the ionic strength at 0.1 M. The electrode was calibrated as a hydrogen-ion concentration probe by titration of a known amount of HNO_3 with CO_2-free standardized NaOH solution (0.1 M). The equivalent point was determined by Gran's method, which gave the standard potential, $E°$, and the ionic product of water ($pK_w = 13.68$). Protonation constants of **L** and association constants with $Cu(NO_3)_2{\cdot}3H_2O$ were determined from several potentiometric titrations of solutions containing ligand and Cu^{II} at the molar ratios: 1:1, 1:2, 1:2.5, 1:4. Typical ligand concentrations were approximately $(6–8) \times 10^{-3}$ M, its hexaprotonation being assumed by the addition of HNO_3 (1 M). For each experiment, the titration curves were obtained from about 100 experimental points taken along the pH range 3–11. Equilibrium constants were calculated by simultaneously fitting the titration curves, using the computer program Hyperquad [45].

Determination of Association Constants from Spectroscopic Titrations

The association constants between indicators and receptor (formed by **L**:Cu_2) were determined by steady-state absorption and fluorescence spectroscopy, using the program Hyperquad [45]. Those between substrates and receptor were calculated from fluorescence data [27]. UV-Vis absorption spectra were recorded on a Cary 100 spectrophotometer from Varian, and fluorescence spectra were measured on a Perkin-Elmer LS-50B spectrometer. All experiments were performed in aqueous solution buffered at pH 7 with 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) 0.05 M. For the determination of the indicators constants by absorption measurements, a standard solution of ligand **L** was added to a solution containing the indicator in the presence of an appropriate amount of $Cu(NO_3)_2{\cdot}3H_2O$, assuming a nearly complete and constant formation of the dimetallic complex receptor all along the titration. Fits were performed *simultaneously* on about 20 wavelengths. Fluorescence spectra were recorded by exciting solutions at wavelengths corresponding to isosbestic points (496, 528, 466 nm for Fluorescein, 6-TAMRA and 6-FAM, respectively). The absorbance of indicators (2×10^{-6}, 2.5×10^{-7} and 2×10^{-6} M for Fluorescein, 6-TAMRA and 6-FAM, respectively) was less than 0.05 at the excitation wavelength. Data used for the determination of the indicators constants were obtained by titrating, with a standard solution of ligand **L**, a solution containing indicator and Cu^{II} until an almost complete fluorescence extinction was reached. A known amount of substrates was added to a mixture containing identical concentrations to the latest solution until the maximum fluorescence intensity possible was restored. Emission data thus obtained were used to determine the association constants between receptor and substrates. In the fitting procedure, the protonation constants of **L** and those of (**L**:Cu^{II}) complexes were kept fixed while refining the indicator constants. Their fitted values are then kept fix for the substrates constants determination.

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