A turn-on fluorescent indicator for citrate with micromolar sensitivity†

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Received 20th July 2007, Accepted 2nd August 2007
First published as an Advance Article on the web 21st August 2007
DOI: 10.1039/b711139h

A turn-on fluorescent indicator for citric acid (citrate) has been developed, displaying high emission enhancement (+1500%) and low interference by other carboxylates. The sensor is based on the non-emissive copper(II) complex of a fluorescent amino amide, which, upon addition of citrate decomplexates to yield the emissive ligand. The detection limit estimated for this new chemosensing system is about 0.5 μM. This novel approach to the analysis of citrate constitutes an alternative *ca.* 10^2–10^3 times more sensitive than the standard method based on the enzyme *citrate lyase*.

1. Introduction

Citrate is an organic tricarboxylate playing important roles in the biochemistry of living beings since it is one of the key components of the Krebs cycle.[1] Its concentration has a diagnostic value as defective levels of citrate have been associated to prostate cancer.[2] Citrate has also been used as a molecular marker for the diagnosis of urological diseases, since its concentration in urine can be correlated to some pathological states, like, for instance, nephrolithiasis (kidney stones),[3] or glycogen storage disease.[4] Citrate concentration in this biomedical context is usually determined by the citrate lyase (CL) spectrophotometric test,[5] a well-established methodology.[6] This test consists of a series of enzymatic reactions by citrate lyase, and L-lactate/L-malate dehydrogenases leading finally to the oxidation of NADH to NAD^+, the extent of which can be correlated to the citrate concentration. Several variants of this method have been developed up until now.[7] In contrast to other analytical methods detecting citric acid,[8] fluorescent sensors offer advantages in terms of sensitivity, selectivity and response times.[9] Recently some researchers, pioneered by Anslyn, have focused their attention to the supramolecular recognition and fluorescent sensing of citrate, obtaining very remarkable results.[10] Among those sensors, those developed by Wolfbeis and Parker, based on europium complexes are specially promising for biological imaging.[11] In the biomedical and food sciences the CL method is still the reference procedure to analyze citrate,[2,7,12] and consequently, any new optical methodology to detect this analyte should be compared with it. Here we present a new strategy to sense citrate in aqueous medium, which is between 100 and 1000 times more sensitive than the CL method.

In the course of our research on fluorescent peptidomimetic molecules,[13] we found that the copper(II) complex of amino amide **1** derived from L-phenylalanine (complex depicted in Chart 1 as **2**), is stable in aqueous-methanolic solutions (pH 7.5–8.0) but is very sensitive to traces of citrate. Thus, the non-fluorescent

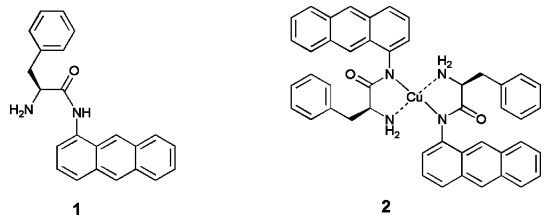

Chart 1 Structures of **1** and its copper(II) complex **2**.

complex **2** leads to a significant emission increase (more than 15-fold) with citrate but it is not so sensitive to the presence of other carboxylates. This positive change takes place within the micromolar range of citrate concentration, whereas the enzymatic CL method is sensitive at millimolar concentrations of this anion. Hence, we believe that **2**, and other related complexes to be developed in the future, could be a competitive alternative for the analysis of citrate (especially for fluorescent microscopy and flow cytometry where high sensitivity is needed).

2. Results and discussion

2.1. Synthesis and characterization

The synthetic route to obtain ligand **1** was adapted from similar syntheses carried out in our group,[14] and is outlined in Chart 2. The N-Cbz protected amino acid L-phenylalanine **3** was activated with *N*-hydroxysuccinimide to yield the activated ester **4**. Coupling with 1-aminoanthracene afforded compound **5**, which

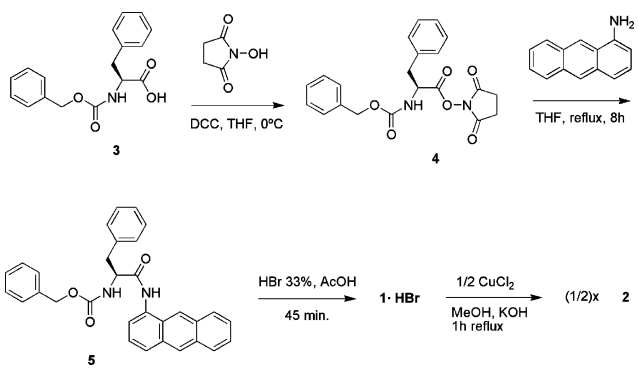

Chart 2 Synthetic route to prepare complex **2**.

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† Electronic supplementary information (ESI) available: Fig. S1–S5. See DOI: 10.1039/b711139h

was deprotected to yield ligand **1** as the bromohydrate salt. Marchelli and co-workers have reported that α-amino amides derived from natural amino acids similar to **1** form stable 2 : 1 (ligand : metal) complexes with Cu(II), yielding in many cases crystals suitable for X-ray analysis.[15] Those structures show a head-to-tail configuration of the ligands around the Cu(II) center with a N_4 coordination sphere. Moreover, Burrows,[16] Polt[17] and Fenton[18] have described the formation of metallic complexes with other organic ligands containing amino amide functionalities. In all the cases, a basic medium for the deprotonation of the amides and complex formation was reported to yield neutral complexes. Hence, **2** was obtained by reaction of two equivalents of **1** and one equivalent of Cu(II) chloride in basic methanol affording a green brilliant solid, in 51% yield. The structure postulated in Chart 1 is presented as tentative according to the analogous complexes described in the literature[15–18] and to the following experimental evidence.

Upon complexation the carbonyl frequency (FT-IR) shifted from 1663 cm^{-1} in **1** to 1591 cm^{-1} in **2** (see ESI†), indicating the participation of the deprotonated amide group in the coordination to the metallic cation and in agreement with related systems.[16,17,18]

The absorption spectrum of **1** (Fig. 1) displays the typically resolved anthracenic band between 350 and 400 nm, with a maximum at 366 nm (6247 M^{-1} cm^{-1}). The absorption spectra of 1-aminoanthracene consists of a broad non-structured band between 340 and 440 nm.[19] Thus, the pair of electrons at the nitrogen attached to the fluorescent group are not delocalized in the anthracene moiety but in the amide group. This fact is supported by the fluorescence spectrum of **1** (Fig. 1) which displays a maximum at 418 nm, whereas that of 1-aminoanthracene occurs at 470 nm.[19] The fluorescence quantum yield of **1** was recorded, with excitation at 356 nm, yielding a value of $\phi = 0.20 \pm 0.02$, both under air and nitrogen atmospheres (using anthracene in degassed ethanol [$\phi = 0.27$] as standard[20]).

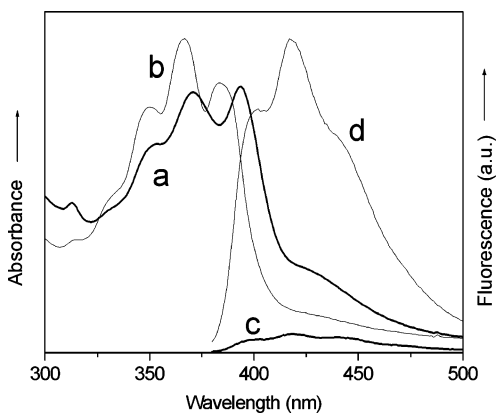

Fig. 1 Comparison between absorption spectra of (a) complex **2** and (b) ligand **1**; and between fluorescence spectra of (c) complex **2** and (d) ligand **1** ($\lambda_{exc} = 370$ nm); water : methanol, 3 : 7, v : v; pH 7.9, NaCl 0.15 M.

The stoichiometry of the complex in solution has been determined to be 2 : 1 (ligand : metal), using the method of continuous variations[21] (Job-Plot) with the absorption and emission spectra, in agreement with structures of related complexes as reported by Marchelli.[15] From the fluorescence Job-Plot (Fig. 2), a sharp variation of emission was recorded at a ligand molar fraction (f)

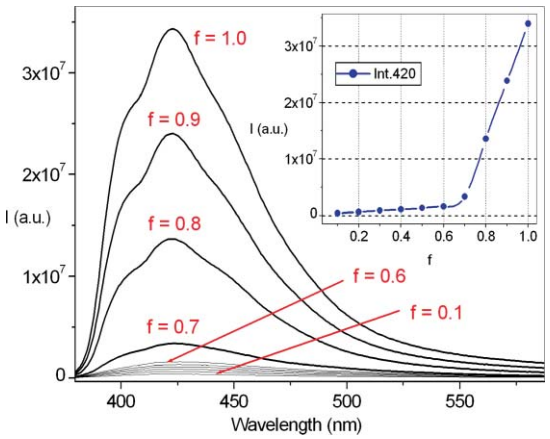

Fig. 2 Determination of the complex stoichiometry by means of a fluorescence Job Plot, where f indicates the molar fraction of ligand in a mixture of ligand and metal, with a total constant concentration of species of 4×10^{-5} M; water : methanol, 3 : 7, v : v; pH 7.9, NaCl 0.15 M. $\lambda_{exc} = 370$ nm.

of 0.7 which indicates the existence of a complex with a 2 : 1 ligand to metal ratio. On the other hand the absorption Job-Plot (Fig. 3) also showed an inflexion point at 0.4. This fact could be associated to the presence in aqueous solution of a minor amount of 1 : 1 complex (theoretical $f = 0.5$). Thus, it is reasonable to assume that complex **2** in aqueous solution is in equilibrium with other species. As reported by Dallavalle,[22] who studied several α-amino amides in the presence of Cu(II), a variety of species can exist in solution. At pH 7.5–8.0, for instance, the prolinamide (ligand L) + Cu(II) system is comprised of CuL_2H_{-2} as the major species in equilibrium with $CuL_2H_{-1}^{+}$, $CuLH_{-1}^{+}$, CuL_2^{2+} and CuL^{2+} as minor components.

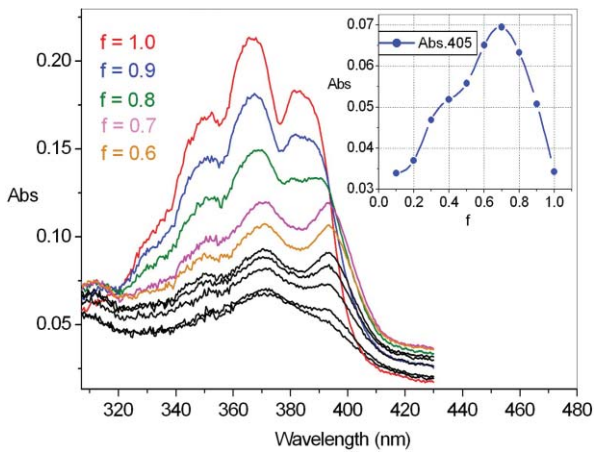

Fig. 3 Determination of the complex stoichiometry by means of an absorption Job Plot, where f indicates the molar fraction of ligand in a mixture of ligand and metal, with a total constant concentration of species of 4×10^{-5} M; water : methanol, 3 : 7, v : v; pH 7.9, NaCl 0.15 M.

Mass spectrometric analysis of **2** using the Fast Atom Bombardment (FAB) technique allowed to record a peak with $m/z = 741.2$, which corresponds to the expected mass $[M]^+$ of the complex (see ESI†). Additional mass spectra were recorded (Electrospray Ionization, ESI) in order to gain further evidence on the structure

of the complex **2**. Thus, in negative mode and in the presence of KCl, the peak $m/z = 776.2$, corresponding to $[M + Cl]^-$ was recorded. In positive mode, the peaks $m/z = 742.4$ $[M + H]^+$ and 780.4 $[M + K]^+$ were detected. No evidence for the 1 : 1 complex formation could be obtained by either of the MS techniques.

In order to further confirm the tetracoordination of the metal center in complex **2** by **1**, with the amides in the deprotonated form, the reactants **1**·HBr and Cu(II) chloride were mixed (0.4 mM and 0.2 mM respectively in MeOH) in a spectrophotometric cell. Aliquots of base (KOH in MeOH) were added stepwise. For the formation of **2**, a double deprotonation of **1**·HBr is needed (one for the HBr and the other one for the amide group). Since there are two ligands in complex **2**, then four equivalents of base, relative to Cu(II), would be required to form the final complex. Fig. 4 and 5 show how the reaction of 2 equivalents of **1**·HBr with 1 equivalent of Cu(II) is complete after the addition of exactly four equivalents of KOH. The same result was obtained using fluorescence spectroscopy (see ESI†).

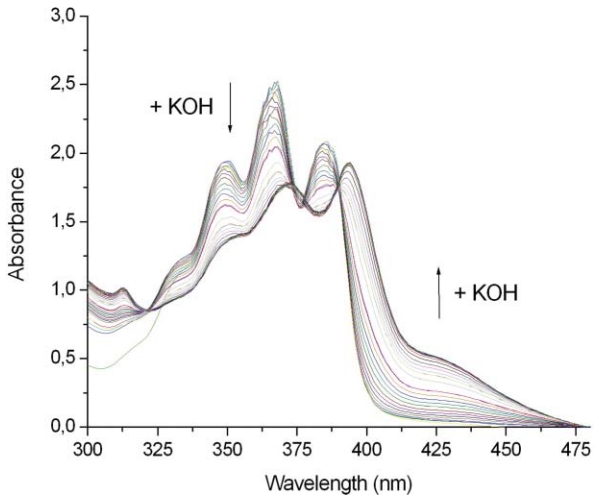

Fig. 4 Change of the absorption spectra of a mixture of **1**·HBr (0.4 mM) and $CuCl_2$ (0.2 mM) upon addition of KOH, in MeOH.

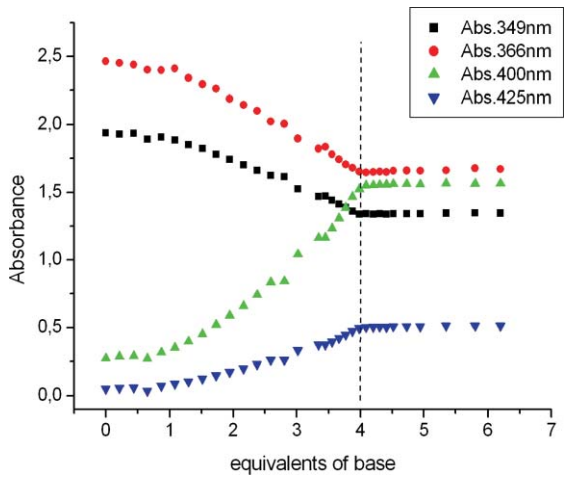

Fig. 5 Change of the absorbance at 349, 366, 400, and 425 nm of a mixture of **1**·HBr (0.4 mM) and $CuCl_2$ (0.2 mM) upon addition of KOH, in MeOH.

2.2. Analytical determinations

Titrations of **2** with citrate were carried out by addition of small aliquots of concentrated analyte (trisodium citrate) to a solution of complex **2** (2×10^{-5} M; water : methanol, 3 : 7, v : v; pH 7.9; NaCl 0.15 M).[23] The non fluorescent solution of **2** became highly emissive as shown in Fig. 6 upon addition of citrate anion. The presence of citrate was detectable even to the naked eye under UV illumination (365 nm) as can be seen in the inset of Fig. 6. This visual feature is especially important for a probe to be useful for microscopy, for which no satisfactory fluorescent probe is commercially available to date. The sensitivity of the complex was evaluated by means of the corresponding titration with citrate over a wide range of concentrations (from 0.1 μM to 100 μM) as can be seen in Fig. 7. For comparison, the CL method was also tested according to the standard commercial protocol[6] (from 0.1 μM to 10 mM) and the results are also plotted in Fig. 7. As is clearly shown, the method using complex **2** is sensitive to concentrations of citrate almost three orders of magnitude lower than the CL methodology.

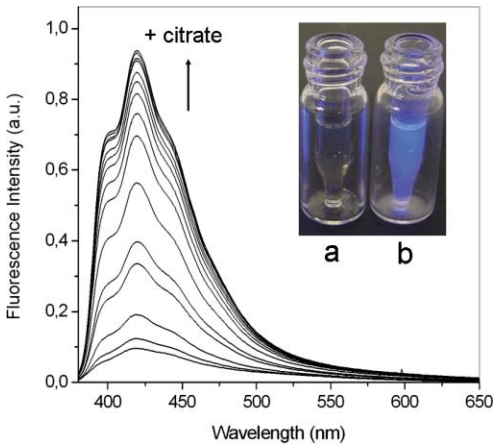

Fig. 6 Fluorescence titration of **2** (2×10^{-5}M; water : methanol, 3 : 7, v : v; pH 7.9; NaCl 0.15 M) with citrate. $\lambda_{exc} = 370$ nm. Upper curve corresponds to a final citrate concentration of 4×10^{-5}M. Inset: (a) initial and (b) final solutions (365 nm exc.).

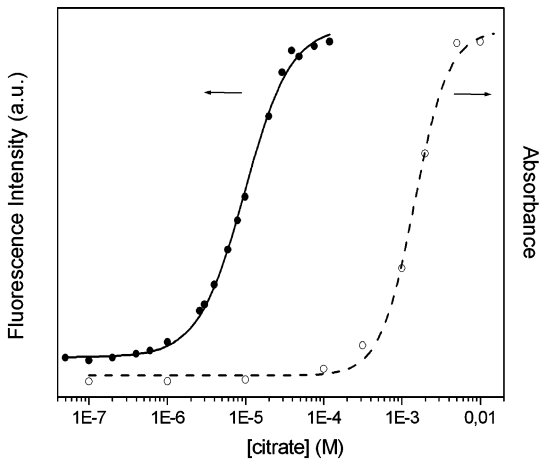

Fig. 7 Comparative sensitivity towards citrate of: CL method (dashed line, absorption measurements) and complex **2** (solid line, fluorescence measurements).

A representation of the lower concentration range revealed linearity up to 10 μM of citrate, with a $R = 0.99764$ (Fig. 8).[24] This calibration allowed the determination of several concentrations of citrate not available directly to the CL test (known concentration of prepared solutions are given in brackets): 3.2 (3.6), 4.3 (4.1), 6.6 (6.1), 9.0 (8.2) μM.

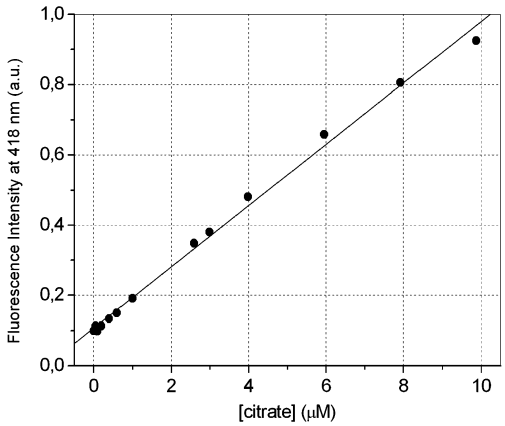

Fig. 8 Calibration curve for **2** (2×10^{-5}M; water : methanol, 3 : 7, v : v; pH 7.9; NaCl 0.15 M). Fluorescence intensity at 418 nm *vs* citrate concentration ($R = 0.99764$). $\lambda_{exc} = 370$ nm.

The selectivity of **2** for citrate determination was evaluated by the corresponding fluorescence titrations with other carboxylates. No significant fluorescence increase took place with succinate, acetate, lactate, glutarate and fumarate (up to 40 μM). With mandelate, tartrate and malate, a small fluorescence enhancement of *ca.* 10–15% occurred (Fig. 9). The selectivity for citrate over malate is specially important for biomedical and food analysis since both species are commonly found in many *real* samples. This causes mutual interferences in analytical protocols, and to avoid this problem new methodologies are continuously under development.[25] The amino acid L-glutamate was also tested provided that it is a potential tri-coordinating species (two carboxylates and one amino group) as citrate (three carboxylates).

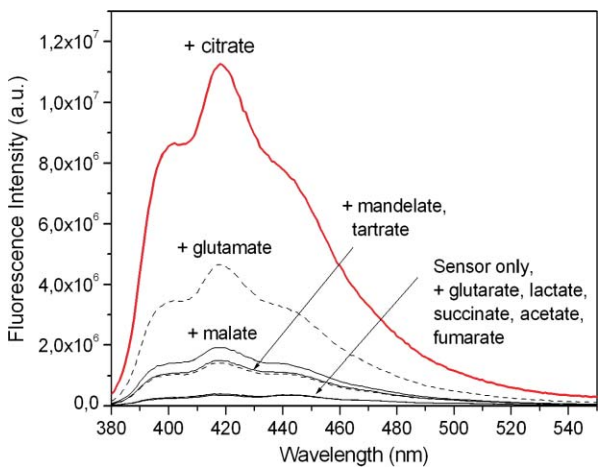

Fig. 9 Fluorescence response of **2** (2×10^{-5}M; water : methanol, 3 : 7, v : v; pH 7.9; NaCl 0.15 M) for citrate and other carboxylates (final concentration: 40 μM).

It was found that the intensity reached about 40% of that recorded with citrate.

From a mechanistic point of view the results can be explained invoking the *decomplexation* of non emissive **2** to yield free fluorescent **1** and copper citrate species (a complex mixture as reported previously[26]). In fact, the fluorescence quantum yield of the final solution, after titration of **2** with citrate, is $\phi = 0.22 \pm 0.02$, which matches, under the experimental error, the value of **1** measured independently ($\phi = 0.20 \pm 0.02$). The absence of emission in **2** is in agreement with the internal fluorescence quenching reported for many emissive ligands when complexed with copper(II) (chelation enhanced quenching (CHEQ) effect).[27] In order to confirm the metal stripping from complex **2**, EDTA (ethylenediaminetetraacetic acid), a stronger chelator than citrate,[28] was added to a solution of **2**. As expected, the non fluorescent solution of **2** became highly emissive upon addition of 40 μM of EDTA. Attempts to analyze mathematically the titration curves of **2** *vs* carboxylates resulted in poor fittings, probably as a result of the complex equilibria of species taking place in aqueous solution. A potentiometric study is probably needed in order to shed some light into the thermodynamics of the complexation–decomplexation process and this is expected to be carried out in future studies. The literature reports an analogous decomplexation-induced fluorescence enhancement, reported by Reymond, to monitor protease activities,[29] in which the stripping of Cu(II) is the basis of the signalling mechanism.

2.3. Combinatorial experiments

Encouraged by the results obtained with the copper(II) complex of amino amide **1**, we decided to test, in a combinatorial fashion, the same amino amide with a series of metals and carboxylates. The purpose was to find, at least qualitatively, some other metallic complexes capable to give fluorogenic sensing of citrate (or other anion). Solutions of the following metallic cations were placed in the B–H rows of a 96-well plate (as chloride salts): Cu(II), Ni(II), Zn(II), Cd(II), Co(II), Cu(I) and Mg(II). The ligand **1** was added to each of the 96 wells. The most appropriate medium for this combinatorial-qualitative experiment was found to be a 2 : 1 (v : v) water : methanol mixture, at pH 7.4 (HEPES 6 mM, NaCl 6×10^{-2} M).[30] Finally the following carboxylates were added to columns 2–12 of the plate : acetate, glutarate, malonate, succinate, tartrate, citrate, lactate, benzoate, ftalate, fumarate and maleate. As can be seen in Fig. 10, the unique metallic cations capable to induce quenching of **1**, at pH 7.4, were Cu(II) and Cu(I) (see column 1 in Fig. 10). Moreover, the unique carboxylate capable of restoring the fluorescence (visually) was citrate. If Cu(II) and Cu(I) were compared, the emission quenching was much more effective with the divalent cation.

The apparent inability of other known quenchers of fluorescence, like nickel(II) for instance, to form a complex with **1** must be attributed to the mild pH conditions (pH 7.4) selected to carry out the experiment. Recording the fluorescence spectra of **1** at different pH and in the presence of Cu(II), Ni(II) or Zn(II) revealed that complexation of **1** by Ni(II) in fact can occur, but at a pH more basic than in the case of Cu(II), in agreement with other amino amide metallic complexes.[31] On the other hand, Zn(II) is not able to quench at any pH the emission of **1**, which is fluorescent over the whole range of pH (see ESI†). We are currently investigating the

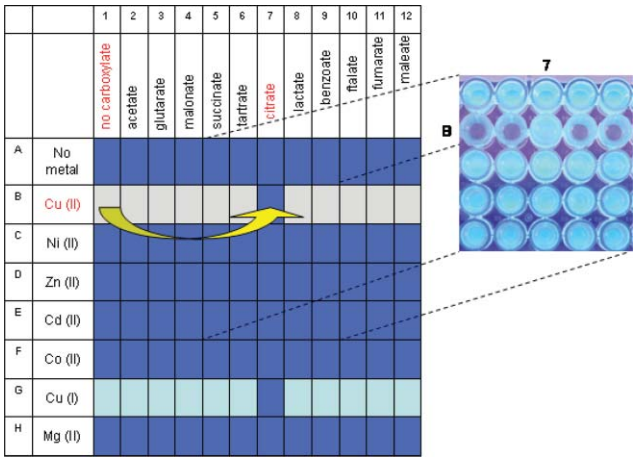

Fig. 10 Schematic representation of the combinatorial experiment carried out with **1**. Final concentrations: ligand **1**: 6×10^{-5} M. Metallic salts: 3×10^{-5} M. Carboxylates: 1.2×10^{-4} M. Medium: water : methanol, 2 : 1 (v : v) pH 7.4 (HEPES 6 mM, NaCl 6×10^{-2} M). Volume in each well: 300 μL. Color code: *blue* indicates strong fluorescence upon illumination (365 nm), *grey* indicates complete quenching, and *cyan* indicates partial quenching. The yellow arrow points to the maximum change of emission from non-emissive complex to released ligand upon addition of citrate.

analytical applicability of new combinations of fluorescent ligands and metallic quenchers.

3. Conclusion

In summary, a new strategy for citrate sensing in aqueous medium with micromolar sensitivity has been described. This methodology for sensing citrate, based on a "*decomplexation-induced fluorescence enhancement*" process (or fluorescence restoration of a latent fluorescent ligand), can constitute a competitive alternative to the *citrate lyase* based methodologies.[32] The concept is not exclusive to Cu(II) amino amidates as described in this paper but could be expanded in the future to other complexes and analytes, just by choosing the appropriate combination of ligand, metal and working pH.

4. Experimental

4.1. Materials and methods

All commercially available reagents (Aldrich or Fluka) were used without further purification. Solvents for reactions were distilled over an adequate drying agent. Water for fluorescence measurements was Millipore® quality. MeOH for fluorescence determinations was spectroscopic grade. The enzymatic analysis of citrate was performed using the citrate lyase kit commercialized by Boehringer Mannheim and according to the protocol described by the manufacturer.

NMR spectra were recorded on a Varian INOVA 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts are reported in ppm using residual undeuterated solvent peaks as internal standards. Mass spectra (ESI) were recorded on a Micromass Quattro LC spectrometer equipped with an electrospray ionisation source and a triple-quadrupole analyzer. FAB-mass spectrum was recorded in a VG Autospec (VG Analytical, Micromass Instruments). Infrared spectra were recorded in a Perkin-Elmer 2000 FT-IR spectrometer. UV-vis absorption spectra were recorded in a Hewlett-Packard 8453 apparatus. Steady-state fluorescence spectra were acquired in a Spex Fluorolog 3–11 equipped with a 450 W xenon lamp. Emission spectra were obtained from air equilibrated samples (otherwise stated) exciting at 370 nm, in right angle mode and using 1 × 1 cm (3 ml) quartz cells. The curves were processed with the appropriate correction files. Excitation spectra were also recorded in order to assure that no impurities were responsible for the recorded emissions. All measurements were performed at 295 K. Emission quantum yields were determined using anthracene in degassed ethanol as standard.[20] All the absorptions were maintained below Abs = 0.10. The samples and the reference were excited at the same wavelength of 356 nm (iso-absorptive) and the emission spectra were corrected to take into account the refractive indices of each medium.

4.2. Synthesis

The synthesis of the *N*-hydroxysuccinimide ester **4** starting from commercially available **3** was made as described previously,[14] with a yield of 86%. Compound **4** (5.0 g, 12.6 mmol) was dissolved in anhydrous THF (40 ml) and 1-aminoanthracene (2.7 g, 12.6 mmol) dissolved in dry THF (10 ml) was added slowly with stirring. The reaction mixture was refluxed for 8 h under an inert atmosphere. The solid formed was filtered off and washed with basic water, neutral water and hot 2-propanol. The product **5** (dark green) was dried under reduced pressure (60–70 °C) for 24 h. Yield: 4.2 g, 70%. mp 236–238 °C. ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 3.04 (dd, 1H, J = 12.7, 9.7 Hz), 3.22 (dd, 1H, J = 13.4, 5.5 Hz), 4.75 (m, 1H), 5.06 (s, 2H), 7.25 (m, 1H), 7.33 (m, 1H), 7.43 (d, 6H, J = 7.1 Hz), 7.50 (t, 2H, J = 7.8 Hz), 7.54 (dd, 2H, J = 6.4, 2.9 Hz), 7.61 (d, 1H, J = 7.0 Hz), 7.80 (d, 1H, J = 8.0 Hz), 7.95 (d, 1H, J = 8.5 Hz), 8.00 (m, 1H), 8.10 (m, 1H), 8.53 (s, 1H), 8.60 (s, 1H), 10.20 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d$_6$) δ 37.6, 56.7, 65.3, 120.6, 121.3, 124.9, 125.6, 125.7, 126.2, 126.3, 127.5, 127.6, 127.7, 128.0, 128.2, 129.3, 130.7, 131.0, 131.7, 133.0, 136.9, 137.7, 156.0, 171.2. FTIR (KBr) 3269, 1684, 1653, 1531 cm^{-1}. Anal. calcd. for $C_{31}H_{26}N_2O_3$: C, 78.5; H, 5.5; N, 5.9; found. C, 78.1; H, 5.8; N, 5.9. ESI-MS m/z = 497.2 [M + H]$^+$, 513.3 [M + Na]$^+$.

Synthesis of **1** [(*S*)-2-amino-*N*-(anthracen-1-yl)-3-phenylpropanamide]: compound **5** (3.0 g, 6.3 mmol) was added to HBr/AcOH (33%) (14 ml) and the mixture was stirred at rt until CO_2 evolution ceased. At this point, diethyl ether was added to the solution, which led to the deposition of a green precipitate. This was filtered off and washed with additional ether. Dark green solid (**1**·HBr). Yield (2.6 g, 97%); mp (dec.) 210 °C; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 3.28 (m, 1H), 3.40 (bs, 1H), 4.55 (s, 1H), 7.33 (m, 1H), 7.40 (m, 4H), 7.51 (t, 1H, J = 7.8 Hz), 7.57 (m, 2H), 7.61 (d, 1H, J = 7.1 Hz), 7.98 (d, 1H, J = 8.5 Hz), 8.00 (d, 1H, J = 8.0 Hz), 8.10 (d, 1H, J = 8.0 Hz), 8.27 (s, 1H), 8.50 (bs, 3H), 8.61 (s, 1H), 10.45 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d$_6$) δ 37.3, 54.1, 120.7, 121.1, 124.9, 126.0, 126.1, 126.2, 126.5, 127.3, 127.9, 128.1, 128.6, 129.7, 130.8, 131.1, 131.6, 131.9, 134.9, 167.5. FTIR (KBr) 3425, 3028, 1663 (carbonyl), 1549 cm^{-1}. Anal. calcd. for $C_{23}H_{21}BrN_2O$: C, 65.6; H, 5.0; N, 6.7; found. C, 65.2; H, 5.4; N, 6.6. ESI-MS m/z = 341.3 [M − Br]$^+$. UV-vis absorption (MeOH), λ (ε, M^{-1} cm^{-1}) 349 nm (4830), 366 nm (6247), 385 nm (5192); Fluorescence emission (MeOH), λ_{max} = 418 nm (exc. at 370 nm).

The synthesis of complex **2** (bis-[(*S*)-2-amino-*N*-(anthracen-1-yl)-3-phenylpropanamidato] copper(II)) was made analogously to similar complexes described in the literature.[15–18] Ligand **1**·HBr (0.22 g, 0.427 mmol) and $CuCl_2{\cdot}2H_2O$ (0.04 g, 0.214 mmol) were dissolved in MeOH (40 ml) (no color change was observed). Then KOH (0.05 g, 0.854 mmol) was added and the solution became darker immediately. The mixture was refluxed for 1 h. The solution was concentrated under reduced pressure and the crude washed exhaustively with hot water. The complex **2** was dried under vacuum (65 °C, 20 h). Brilliant green crystals were obtained (not suitable for X-ray diffraction) soluble in DMSO and partially soluble in alcohols. Yield (0.081 g, 51%). mp 193–195 °C; FTIR (KBr) 3437, 3047, 1591 (carbonyl), 1455 cm^{-1}; UV-vis absorption (MeOH), λ (ε, M^{-1} cm^{-1}) 351 nm (6900), 371 nm (8850), 393 nm (9650), 425 nm (2525). FAB-MS (positive) *m*/*z* 741.20 $[M]^+$, ESI-MS (positive) *m*/*z* 742.4 $[M + H]^+$, 780.4 $[M + K]^+$; ESI-MS (negative) *m*/*z* 776.2 $[M + Cl]^-$. The global yield for the synthesis of **2** starting from **3** was 30%.

Acknowledgements

Financial support from the Spanish Ministerio de Educación y Ciencia (MEC, projects CTQ2006-15672-C05-02/BQU, CTQ2006-26251-E/BQU [Programa Explora]), Generalitat Valenciana (projects GV/2007/277 and ARVIV/2007/079), and Fundació Caixa Castelló-UJI (project P1·1B2004–38) is acknowledged. F. G. thanks the financial support from MEC (Ramón y Cajal Program). L. V. thanks the financial support from MEC (FPU fellowship).

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