Residual and exploitable fluorescence in micellar self-assembled ON–OFF sensors for copper(II)

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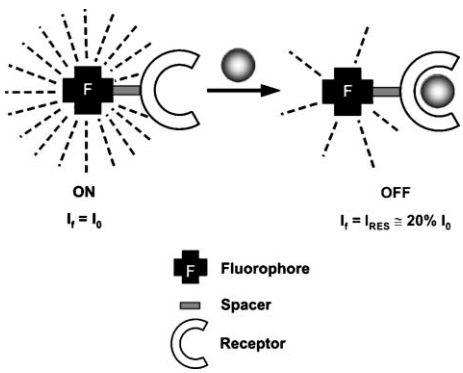
A set of new ligands, **L2**H_2–**L5**H_2, containing the 1,4,8,11-tetraaza-5,7-dione framework has been prepared, The ligands feature lipophilic substituents either on the carbon atom in the 6 position or on the amino groups, or on both. The solution behaviour of the ligands when included in TritonX-100 micelles has been investigated by means of potentiometric titrations and protonation and complexation constants for the Cu^{2+} cation have been determined in micellar medium. Micellar assemblies containing the ligands and pyrene have been prepared, and coupled pH-metric and fluorimetric titrations allowed the determination of the response of the systems as ON–OFF fluorescent sensors for Cu^{2+}. A correlation between the effective lipophilicity of the ligand and the residual fluorescence (*i.e.* the fluorescence of the OFF state) was observed, and with the more lipophilic ligand, **L3**H_2, we obtained a residual fluorescence as low as 8%, with a significant improvement with respect to other published systems. On the other hand, introduction of functionalities on the amino groups of 1,4,8,11-tetraaza-5,7-dione brings the drawback of a small but significant decrease of the exploitable fluorescence, *i.e.* the fluorescence of the system in the absence of added Cu^{2+}, at the pH value suitable for full metal complexation.

Introduction

Fluorescent sensors of the FSR type (FSR = fluorophore–spacer–receptor) are traditional multicomponent molecules that allow the detection of a chemical species thanks to the variation of the fluorescence intensity (I_f) when the target chemical species is bound by the receptor.[1] In particular, ON–OFF fluorescent sensors are said to display full emission (ON state) in the absence of the target species, while the emission is nil (OFF state) when the target species is added.[2] However, this is often an oversimplified view. As a matter of fact, when all the receptors available in the sensing medium (usually a solution) are binding the species to be sensed (*e.g.* in large excess of the target), the read fluorescence is not zero. Calling I_0 the fluorescence of the ON state, and I_{RES} the residual fluorescence intensity in the presence of excess target species, I_{RES} may be as high as 5–20% of I_0.[3] This is pictorially illustrated in Scheme 1. Taking this into account, what is called the OFF state should be better considered a "strongly attenuated" ON state. In FSR sensors this could be due to a not perfect communication between the receptor–target complex and the fluorophore (*e.g.* due to a too long or rigid spacer) or in an only slightly favourable energy balance for the electron-transfer or energy-transfer processes responsible for the quenching mechanism.[4] Moreover, a more subtle consideration should be put forward regarding the ON state, as in many cases, in the conditions in which the receptor can effectively interact with the target species, the F component may not display its full fluorescence. As an example, many sensors of the FSR type contain amino groups in the receptor component and incorporation of the target species (*e.g.* a transition metal cation) into R is complete only at appropriately high pH values, at which some of the amino groups may be unprotonated. Under these conditions partial or significant quenching of the emission of the fluorophore is observed before the target species is added, due to the electron-rich free amino moiety, capable of electron transfer (eT) quenching. Accordingly, it should be remembered that the ON state displays an "exploitable" fluorescence intensity, I_{EXP}, that is lower than I_0, *i.e.* the full emission of the F component in the FSR molecule. As an obvious consequence, the higher I_{RES} and the lower I_{EXP}, the less efficient the sensor response. We recently started the study of micellar fluorescent sensors for transition metal cations and protons,[5] that are obtained as self-assembled multicomponent systems in water.[6] As illustrated in Scheme 2, a fluorophore and several lipophilized ligands are held together inside micelles generating the ON state



Scheme 1 Pictorial view of the transition of a fluorescent sensor from the ON to the OFF state. In many cases, the OFF state must be better considered as "strongly attenuated", *i.e.* fluorescence is low but $\neq 0$.

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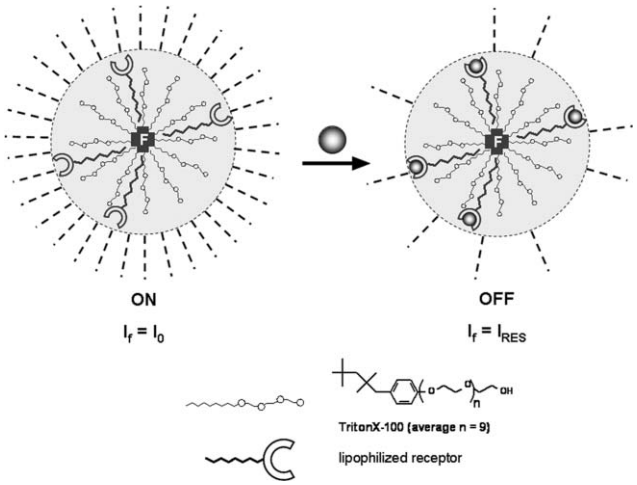
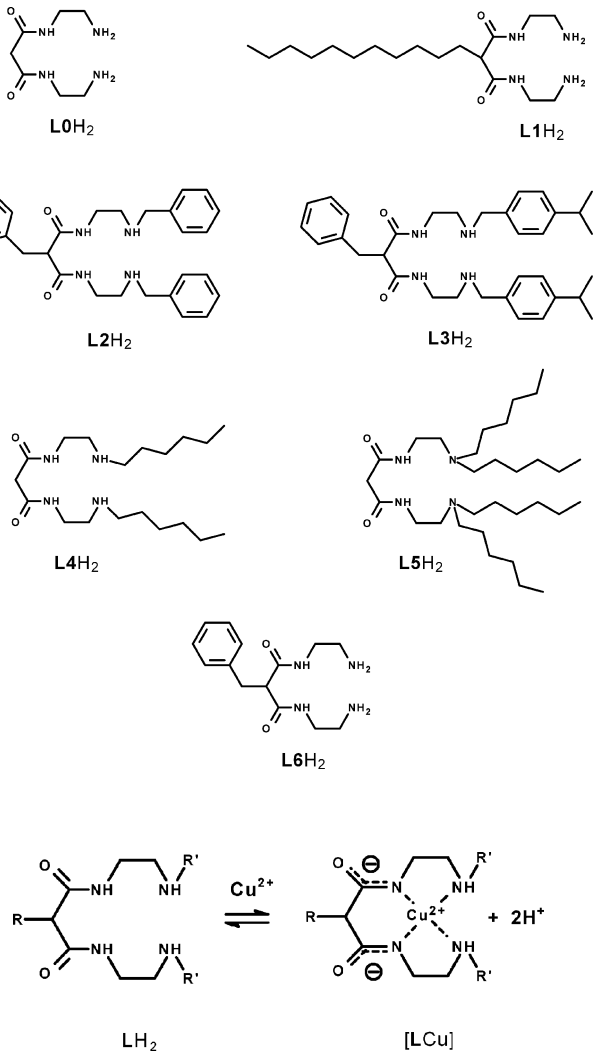


Scheme 2 Pictorial view of the working scheme of micellar multicomponent ON–OFF fluorescent sensors. Also in this case, transition to the OFF state means to obtain a "strongly attenuated" emission.

of the sensing system. Addition of the target cation results in coordination to the ligand and in fluorescence quenching due to intramicellar energy- or electon-transfer processes, between the fluorophore and the metal complex. Residual fluorescence may be particularly significant in this kind of system. In a communication on our first system[5e] we used **L1**H_2 for Cu^{2+} sensing (TritonX-100 as surfactant and pyrene as fluorophore).[5e] In this system, full Cu^{2+} incorporation took place at pH 7.5 with the release of the two amido protons, according to the reaction scheme illustrated in Scheme 3. At pH 7.5 the equilibrium of Scheme 3 is fully displaced to the right and >99% of copper is incorporated inside the bis-deprotonated diamino-diamido framework, with the formation of the neutral [**L1**Cu] complex ($R = C_{12}H_{25}$ and $R' = H$ in Scheme 3). However, the found I_{RES} is 18%. The reason for this high residual value relies on the micellar nature of the sensing system: TritonX-100 micelles are oblate ellipsoids, having 2.7 and 5.2 nm as the minor and major semiaxes,[7] and due to its hydrophobic nature, the [**L1**Cu] quencher adopts a compartmentalized distribution inside the micelle, preferring the less solvated, flattest zones of the ellipsoid. In the working conditions, concentrations were such that every micelle contained one pyrene molecule and ten quenchers. Modeling of the quenching mechanism and interpretation of time resolved fluorescence allowed us to state that only 4 out of the 10 quencher molecules are located in the correct position to be effective in the dynamic quenching of pyrene.[5a,d] On the other hand, in our communication[5e] we also reported an I_{EXP} as high as 97%. At pH 7.5, if no Cu^{2+} is added, 55.5, 42.0 and 2.5% of the diprotonated, monoprotonated and neutral forms of **L1**H_2 are present, respectively, but primary amines are poor quenchers, and they affect fluorescence only marginally.

Positioning inside a micelle of lipophilic molecules containing a metal complex as the quencher may depend on the balance of many factors: quantity and shape of the groups used to lipophilize the ligand backbone, dimensions and hydrophilicity of the complex moiety, modifications of the structure of the micelle induced by the lipophilized complexes. In this full paper we extend the



Scheme 3 Scheme of the [LCu] complex formation for the ligands of the 1,4,8,11-tetraazaundecane-5,7-dione family. The plain ligand (**L0**H_2) has $R = R' = H$.

work on micelles containing **L1**H_2 and pyrene for sensing Cu^{2+}, investigating new ligands to understand which structural factors may be tuned to obtain lower I_{RES}, possibly without affecting I_{EXP}. To this aim, we used micelles made of the same surfactant as that used for **L1**H_2/Cu^{2+} (TritonX-100), we maintained the same binding moiety as in **L1**H_2, and we synthesized three new ligands, **L2**H_2–**L4**H_2, containing the 1,4,8,11-tetraazaundecane-5,7-dione chain with different appended lipophilic groups, and in different positions with respect to **L1**H_2. Primary amines has been changed into secondary ones and, moreover, also the **L5**H_2 ligand, containing tertiary amino groups, has been prepared and studied for comparison. A study in micellar media of the already described **L6**H_2 ligand[8] has also been carried out for comparison. Complete characterization (protonation and complexation constants with Cu^{2+}) is presented for all ligands when included in Triton X-100 micelles. Steady-state fluorescence has been studied in the presence of micellized pyrene as a function of pH, both in the presence and in the absence of Cu^{2+}, and the results correlated with the pertinent distribution diagrams, in order to evaluate the best working pH range and both I_{EXP} and I_{RES}.

Results and discussion

1. Protonation and Cu^{2+} complexation studies on the micelle-included ligands

The acid–base properties of the **L2**H_2–**L6**H_2 molecules have been examined through the determination of ligand protonation constants by means of potentiometric titrations in water containing TritonX-100. Concentration of the surfactant and of the ligands have been chosen identical to those used for the communication on **L1**H_2,[5e] in order to directly compare the obtained data with the published ones. In particular, surfactant water solutions were 0.010 mol L^{-1} in TritonX-100 and the ligands were 0.0010 mol L^{-1}. The average concentration of micelles (C_M) and average number of ligands per micelle (N_{LM}) may be calculated considering the established critical micellar concentration (cmc) and aggregation number (AN) for TritonX-100, that are 2×10^{-4} mol L^{-1} and 100–140 units, respectively.[7,9] C_M is obtained from the relation

$$C_M = \frac{0.01 - \text{cmc}}{\text{AN}}$$

with $C_M \sim 9 \times 10^{-5}$ mol L^{-1} and, accordingly, $N_{LM} \sim 10$. Moreover, in all experiments, 0.05 M $NaNO_3$ was used as the background electrolyte, to buffer ionic strength, and the solutions were thermostatted at 20 °C (see Experimental section for more details). The obtained data are expressed as potential *vs.* volume of added base. They were elaborated with the Hyperquad package,[10] to obtain the protonation constants reported in Table 1. The values determined for the plain 1,4,8,11-tetraaza-5,7-dione ligand **L0**H_2[8] are also listed for comparison, but it has to be remembered that in this case equilibria were studied in water, where **L0**H_2 is soluble also without any added surfactant. It must be stressed that using a standard potentiometric titration apparatus in a micellized water/surfactant medium means measuring a space-averaged value of potential with a macroscopic glass electrode. Accordingly, the calculated protonation constants of the examined molecules are not intrinsic, but are the so-called "observed" or "apparent" protonation constants, *i.e.* they are influenced by the inclusion of the lipophilic bases inside the micelles, where the local water concentration is lower than in the bulk intramicellar solution.[11] Positioning inside the micelle must also be taken into account, as near the core solvation is even less efficient with respect to the more external layers.[5a–d,12] As a consequence, if a protonatable ligand has a preferential distribution inside micelles with respect to water and a deep position inside the micelles due to its lipophilicity, this will result in lowering of the measured (*i.e.* observed) log*K* values. The lower values found for the reference micellized ligand, **L1**H_2, with respect to **L0**H_2, reflects its inclusion in micelle. On examining the new molecules, even lower log*K* values are found for **L2**H_2, **L3**H_2 and **L5**H_2. Although stepping from primary and secondary amines to n-alkyl tertiary amines is generally known to result in a decrease of basicity, the small variations in the protonation constant values reported in the literature for small amines in water[13] suggest that, with our molecules, comparison of what was found in water with **L0**H_2 may be carried out with the values found for **L1**H_2 and **L4**H_2–**L6**H_2. On the other hand, hindering the N atoms with groups such as benzyl gives a more remarkable intrinsic difference[13] so that **L2**H_2 and **L3**H_2 should be considered separately. For our alkyl amines the lowest protonation constants are found for the more lipophilic **L5**H_2 and **L1**H_2. **L6**H_2 displays log*K* values very similar to those of **L0**H_2, and this indicates that it is distributed inside micelles only for a very small percentage. Quite interestingly **L4**H_2 displays protonation constants intermediate between **L0**H_2 in water and **L1**H_2 in TritonX-100 micelles, even if the total number of appended carbons is the same as in **L1**H_2, *i.e.* 12. Although amine secondarization may also play a role, this suggests that the position and distribution of the lipophilizing groups on the same binding unit may play a significant role in obtaining effective lipophilicity, resulting in efficient inclusion and deep positioning inside micelles. As regards the two ligands with benzylamino groups, appending further alkyl groups on the aromatic rings results in a dramatic lowering of log*K* values on stepping from **L2**H_2 to **L3**H_2, as a further indication that the addition of more carbon atoms (six) increases significantly the effective lipophilicity of the ligand.

Formation constants of the copper complexes of ligands **L2**H_2–**L4**H_2 have been determined by means of potentiometric titrations carried out under the same conditions used for determination of the protonation constants, but in the presence of 0.001 M Cu^{2+} (as its triflate salt). The copper cation is thus in 1 : 1 molar ratio with the ligands. The case of **L5**H_2 is different from the other three ligands and is discussed later, while for **L2**H_2–**L4**H_2 the found behaviour parallels what has been already observed for copper complexation with **L0**H_2 and **L1**H_2. Referring to the considered ligand as **L**H_2, we disclosed the formation of three complex species: i) $[LH_2Cu]^{2+}$, due to the interaction of both the amino groups with Cu^{2+}; ii) $[LH_3Cu]^{3+}$, in which one amino group coordinates Cu^{2+} while the second is protonated; iii) [LCu], the neutral complex that forms with the deprotonation of the two amido groups, according to the equilibrium of Scheme 3.

Table 1 Protonation and complexation logarithmic constants for ligands **L0**H_2–**L6**H_2. The values refers to water + TritonX-10 micelles as solvent. Uncertainties are indicated in parentheses. The pertinent equilibria are reported in the column titles, with **L** = **L0–L6**

	$LH_2 + H^+ = LH_3^+$	$LH_3^+ + H^+ = LH_4^{2+}$	$LH_2 + Cu^{2+} = [LH_2Cu]^{2+}$	$LH_2 + Cu^{2+} + H^+ = [LH_3Cu]^{3+}$	$LH_2 + Cu^{2+} = [LCu] + 2H^+$
L0H_2[a]	9.01	8.66	7.20	—	−5.13
L1H_2[b]	8.73	7.62	7.52	12.51	−5.20
L2H_2	8.13 (0.01)	7.20 (0.01)	6.60 (0.01)	12.58 (0.01)	−5.39 (0.01)
L3H_2	7.43 (0.01)	6.41 (0.03)	5.47 (0.03)	—	−6.60 (0.03)
L4H_2	8.90 (0.01)	7.88 (0.02)	9.08 (0.02)	15.39 (0.02)	−4.56 (0.02)
L5H_2	6.17 (0.01)	[c]	—	—	—
L6H_2	8.99 (0.01)	8.34 (0.02)	—	—	−4.94 (0.02)

[a] Values taken from ref. 10 (values obtained in water). [b] Values taken from ref. 5e. [c] Calculation of the second protonation constant for **L5**H_2 was affected by a too high error, so that a reliable value cannot be put forward.

Provided that pH is sufficiently high, the latter is a very stable complex, its geometry is square planar, and it displays a typical pink-violet color corresponding to a d–d absorption with λ_{max} centered usually at 500–520 nm.[3d,5,8] As for **L0**H_2 and **L1**H_2, the distribution diagrams (% of species *vs.* pH) that may be drawn from protonation and complexation constants are dominated by two species, *i.e.* the diprotonated ligand $[\mathbf{L}H_4]^{2+}$ and the complexes of the [**L**Cu] type (see Fig. 1a–c). In particular, it could be noted that [**L2**Cu], [**L3**Cu] and [**L4**Cu] begin to form at pH 5.5, 5.4 and 6.0, and reach 99% at pH 7.1, 7.2 and 7.8, respectively. As typical of the 1,4,8,11-tetraaminoundecane-5,7-dione framework, the $[\mathbf{L}H_2Cu]^{2+}$ and $[\mathbf{L}H_3Cu]^{3+}$ species have a lower (sometimes almost negligible) weight in the species distribution. As a further indication of its almost negligible inclusion in micelles, titration of **L6**H_2/Cu^{2+} in TritonX-100 solution gave complexation constants that are almost identical to those already found in water.[8]

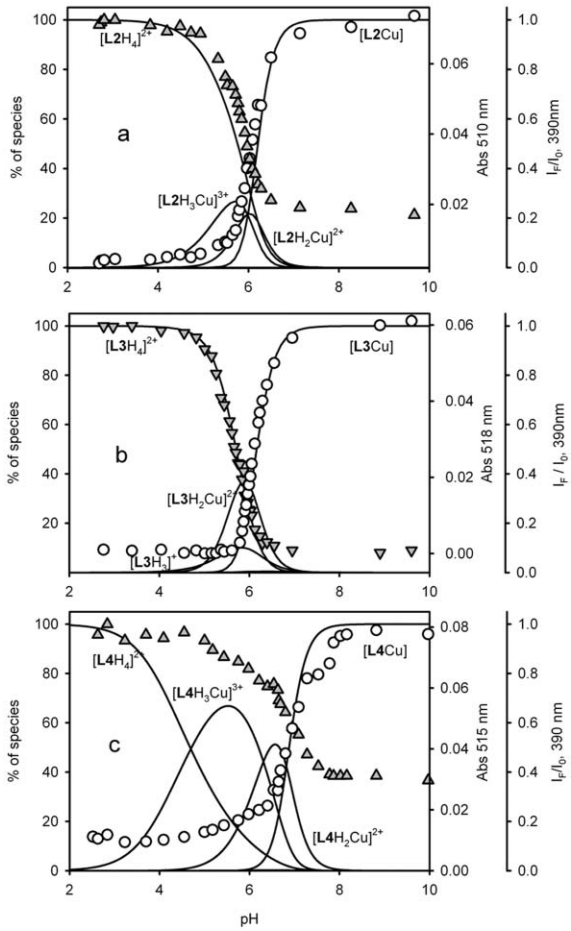


Fig. 1 Distribution diagrams (% of species *vs.* pH) for the systems **L2**H_2/Cu^{2+} (a), **L3**H_2/Cu^{2+} (b) and **L4**H_2/Cu^{2+} (c), calculated for 1 : 1 metal–ligand molar ratio at 0.001 M concentration. The species pertaining to each profile are indicated on the diagrams. White circles report the absorbance measured at the λ_{max} of the [**L**Cu] complexes as a function of pH. Grey triangles report the relative fluorescence intensity (I_f/I_0), measured at the pyrene emission maximum (390 nm), as a function of pH.

The spectrophotometric properties of the copper complexes may be fully individuated for the neutral square planar [**L**Cu] species, as these are the only species existing in solution at pH higher that the appropriate values, as can be seen in Fig. 1. The expected pink-violet colour is observed, with bands displaying λ_{max} at 510 nm ($\varepsilon = 72\ M^{-1}\ cm^{-1}$), 518 nm ($\varepsilon = 60\ M^{-1}\ cm^{-1}$) and 515 nm ($\varepsilon = 78\ M^{-1}\ cm^{-1}$) for [**L2**Cu], [**L3**Cu] and [**L4**Cu], respectively. Coupled spectrophotometric and pH-metric titrations have been also carried out, and plots of the absorbance at the pertinent λ_{max} values *vs.* pH are displayed in the distribution diagrams (white circles in Fig. 1), showing the expected superimposition with the curve relative to the percentage of the [**L**Cu] species. As regards the complexes with the amino groups, $[\mathbf{L}H_2Cu]^{2+}$ and $[\mathbf{L}H_3Cu]^{3+}$, their spectral properties cannot be sharply determined, as there is no pH interval in which they exist as the only species. However, at pH values lower than 5.5, where [**L**Cu] is not yet formed in any case, the solutions display the expected pale blue colour with large absorptions centered at ~670 nm.

Finally, the case of **L5**H_2 should be commented upon. This highly lipophilic ligand displays a very low first protonation constant (Table 1; a value of log*K* around 6, although affect by a large error, can be evaluated also for the second protonation step). When copper is added, in the potentiometric titrations profile no buffer zone is individuated relative to the deprotonation of the two amido groups, *i.e.* the neutral [**L5**Cu] complex does not form. Moreover, pH-spectrophotometric titrations do not show the formation of the typical band of the Cu^{2+} complex of the bis-deprotonated 1,4,8,11-tetraaza-5,7-dione framework: the examined solution becomes pale blue (wide absorption band at λ 650 nm), due to the weak interaction of the tertiary amines with Cu^{2+}. This prompted us also to examine the behaviour of this molecule as monodispersed (*i.e.* not micellized), in order to understand if what we observed was a micelle-induced effect or its intrinsic behaviour. We repeated the coupled pH-spectophotometric titration in a dioxane–water mixture (9 : 1 v/v), and again we did not observe the formation of [**L5**Cu]. These results lead us to the conclusion that when the 1,4,8,11-tetraaza-5,7-dione framework bears tertiary amino groups, it becomes unable to incorporate Cu^{2+}, *i.e.* the equilibrium described in Scheme 3 is not displaced to the right even at very high pH values. It is known that for this ligand framework the release of the two amido protons is very endoergonic, and, accordingly, only Ni^{2+} and Cu^{2+} (the smallest cations of the first transition row) are able to compensate the loss in energy with a favourable crystal field stabilization energy (CFSE) and ligand–metal interactions.[3d] Changing the amines from secondary to tertiary makes the N atoms more hindered and lowers their binding ability, so that the energy spent in the release of the amido protons can not be compensated by Cu^{2+} coordination.

2. Residual fluorescence intensity

The sensing ability towards Cu^{2+} of the **L2**H_2–**L4**H_2 and **L6**H_2 ligands in TritonX-100 micelles has been examined by addition of pyrene to the micellar system, reproducing the situation pictorially described by Scheme 2. We used 10^{-6} M pyrene, so that the average number of fluorophores per micelle is ~0.01. Such a low concentration is chosen both for the very strong fluorescence intensity of pyrene and to avoid the possibility of inclusion of two pyrene molecules in the same micelle and excimer emission. The majority of micelles are thus "empty" as regards the fluorophore, but in the "full" ones only one pyrene molecule is included, and it

is surrounded by an average of 10 molecules of the chosen ligand. It has also to be stressed that these are the same concentration conditions used with the pyrene/TritonX-100/$\mathbf{L1}H_2$ system, so that the results may be directly compared. Coupled pH-metric and fluorimetric titrations allowed us to examine the emission of the system as a function of pH, when Cu^{2+} is added in equimolar ratio with respect to the chosen ligand. Full emission (I_0) is observed at low pH values, at which ligands are protonated and Cu^{2+} is not coordinated, thus not entering the micellar space. Coordination of Cu^{2+} to the secondary amino groups of the micelle-included ligands, *i.e.* formation of the $[\mathbf{L}H_2Cu]^{2+}$ and $[\mathbf{L}H_3Cu]^{3+}$ species, results in an almost negligible lowering of pyrene emission intensity. However, as for $\mathbf{L1}H_2$, entering the bis-deprotonated diamino-diamido ligand, *i.e.* forming [**L**Cu] complexes, results in a sigmoidal descrase of I_f, that reaches its minimum at the pH values at which the [**L**Cu] complexes are >99%. This is the OFF state of the sensors. The process is visualized by plotting the I_f/I_0 ratio (measured at the maximum of pyrene emission, 390 nm) as a function of pH, represented by grey triangles in Fig. 1. It can be seen that the I_f/I_0 ratio reaches a plateau at its minimum value, corresponding to the full formation of [**L**Cu]. From this lowest value I_{RES} may be calculated as

$$I_{RES} = 100 \times I_f/I_0$$

The found I_{RES} values are 18% in the case of $\mathbf{L2}H_2$, 8% for $\mathbf{L3}H_2$ and 38% for $\mathbf{L4}H_2$. I_{RES} in the presence of the [**L6**Cu] complex must also be considered: formation of this complex gives a very small decrease of fluorescence emission, and $I_{RES} = 87\%$. This is a further indication that $\mathbf{L6}H_2$ and its protonated and complexed forms are distributed mainly in bulk water, with only a very small quantity included in micelles. Quite interestingly, considering the comparable set of $\mathbf{L1}H_2$ and $\mathbf{L4}H_2$–$\mathbf{L6}H_2$ ligands, I_{RES} values correlate with $\Delta\log K$, as can be seen in Fig. 2. $\Delta\log K$ is the difference between the first protonation constant of $\mathbf{L0}H_2$ in water and that of the considered ligand in micelles (expressed as their logarithmic values). Based on the considerations made in the section dedicated to the protonation constants, $\Delta\log K$ can be considered a quantity related to the effective lipophilicity of the ligand, due to the combination of two factors: the distribution of the ligand between bulk water and micelles, and the level of its penetration into the micellar core. It is interesting to note that with $\mathbf{L4}H_2$ $I_{RES} = 38\%$, even if the ligand has the same total number of carbon atoms (twelve) appended to the backbone as $\mathbf{L1}H_2$, for which $I_{RES} = 18\%$. As we already pointed out, the different $\Delta\log K$ values indicate how redistribution of the lipophilizing groups may strongly influence the "effective lipophilicity" of the ligands. We may now put forward the hypothesis that it is the effective lipophilicity to tune the observed values of I_{RES}. As a further example to support this consideration, we now find $I_{RES} = 8\%$ with $\mathbf{L3}H_2$, while the less lipophilic $\mathbf{L2}H_2$ displays $I_{RES} = 18\%$. The difference in the first protonation constants for this two ligands is a remarkable 0.7 log units.

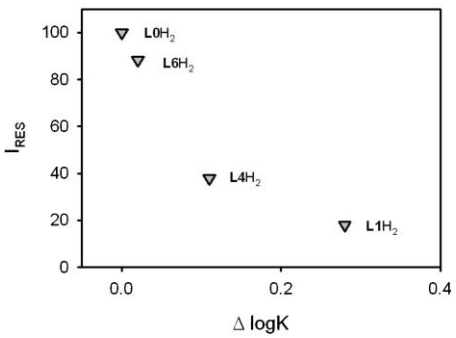


Fig. 2 I_{RES} *vs.* $\Delta\log K$ for the comparable ligand set $\mathbf{L0}H_2$, $\mathbf{L1}H_2$, $\mathbf{L4}H_2$ and $\mathbf{L6}H_2$. $\Delta\log K$ is the difference between the first protonation constant of $\mathbf{L0}H_2$ in water and the first protonation constant of the considered ligand.

3. Exploitable fluorescence

Coupled fluorimetric and pH-metric titrations have been carried out also for solutions containing only micellized ligands and pyrene (*i.e.* with no added Cu^{2+}). Small but significant variations in I_f are found on deprotonating the secondary amines of the ligands, as can be seen in Fig. 3a–c, where grey triangles report the I_f/I_0 values measured at $\lambda = 390$ nm. For a sharper understanding of the reason for the variations of I_f, the fluorescence profiles have

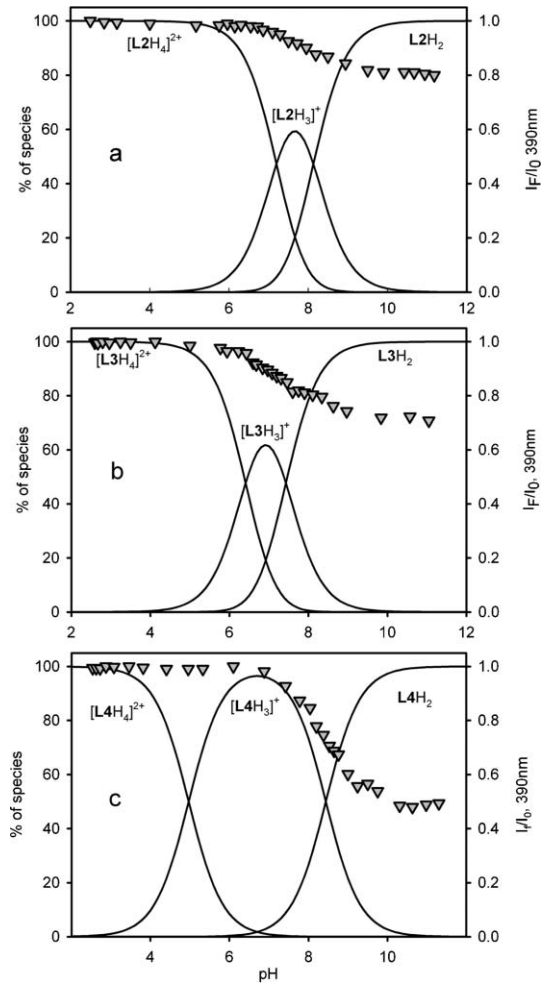


Fig. 3 Distribution diagrams relative to the protonation processes (*i.e.* in the absence of Cu^{2+}) for $\mathbf{L2}H_2$ (a), $\mathbf{L3}H_2$ (b) and $\mathbf{L4}H_2$ (c), expressed as % of species *vs.* pH, calculated at 0.001 M ligand concentration. The species pertinent to each profile are reported in the diagrams. The grey triangles report the relative fluorescence intensity (I_f/I_0), measured at the pyrene emission maximum (390 nm), as a function of pH.

been also superimposed on the distribution diagrams relative to the sole protonation processes: in the cases of **L2**H_2 and **L3**H_2 the first deprotonation step is responsible for the fluorescence variation, while for **L4**H_2 full deprotonation is needed to observe I_f decrease. Charge-dependent and effective-lipophilicity dependent positioning inside the micelle should be taken into account to explain this observation. **L2**H_2 and **L3**H_2 display a high effective lipophilicity, that allows the positioning of the free amine of the monoprotonated form sufficiently near to the micellar core to interact with pyrene. In the case of **L4**H_2, due to its poor effective lipophilicity, the monoprotonated form tends to reside in the more external, hydrated layer of the micelles, and only the neutral ligand is able to penetrate the micellar core allowing static quenching.[5c]

To evaluate the exploitable fluorescence for the sensing systems it is necessary to know the minimum pH at which complexation of Cu^{2+} is complete and to determine the I_f/I_0 percent ratio for sole ligand at the same pH value. From Fig. 1a–c it can be seen that full complexation (*i.e.* [**L**Cu] going at ≥99%) is reached at pH 7.1, 7.2, and 7.8, for [**L2**Cu], [**L3**Cu] and [**L4**Cu], respectively. Using the I_f *vs.* pH points (grey triangles) in Fig. 3a–c, it can be read that at pH 7.1 I_f is 96% of I_0 for **L2**H_2, at pH 7.2 I_f is 87% of I_0 for **L3**H_2 and at pH 7.8 I_f is 86% of I_0 for **L4**H_2. These percentage values are the I_{EXP} values for the three sensing systems. Comparison should be made with **L1**H_2, in which a very high I_{EXP} (~97%) could be evaluated. In that case the ligand contained primary amines, that are not able to efficiently quench pyrene fluorescence.[14] **L2**H_2–**L4**H_2 contain secondary amines, that are more efficient quenchers.

Conclusions

In this work we have demonstrated that when the 1,4,8,11-tetraamino-5,7-dione ligand is used in the assembly of micellar fluorescent sensors for Cu^{2+} the efficiency of the signalling process may be tuned by changing the number and position of its lipophilizing functions. In particular, changing primary amines into secondary ones does not affect the binding ability and allows access to ligands that have a high effective lipophilicity. Increasing the effective lipophilicity of the ligand has the effect of obtaining a low residual fluorescence, when sensing Cu^{2+}. However, secondarization of the amino groups in the 1,4,8,11-tetraaza-5,7-dione framework brings the drawback of a slightly less high exploitable fluorescence. Keeping the amines primary in the ligand framework and increasing its lipophilicity by working on the substituent on the carbon atom in 6 position is a target that we are currently trying to reach. Finally, we have also demonstrated that tertiarization of the amino groups results in ligands that are useless for the assembling of a sensing system, as they are not able to deprotonate their amido groups to form the desired Cu^{2+}complex.

Experimental

Materials

Triton X-100 (*tert*-octylphenoxy polyoxyethylene glycol with and average of 9–10 oxyethylene units) was purchased from Caledon (average molecular weight = 647). **L0**H_2,[15] **L1**H_2[5d] and **L6**H_2[8] were synthesized according to the published procedures. Pyrene (97%) was a Fluka product, used without further purification. Water used for all titrations was distilled twice.

Syntheses

2-Benzyl-*N*,*N*′-bis(2-benzylaminoethyl)malondiamide (L2H_2). 278 mg (1 mmol) of 2-benzyl-*N*,*N*′-bis(aminoethyl)malondiamide (**L6**H_2) and 203 μl (2 mmol) of benzaldehyde were dissolved in 150 ml benzene in a 250 ml flask topped with a Dean–Stark trap equipped with a bulb condenser and heated at reflux with magnetic stirring. After 8 h the solvent was removed with a rotary evaporator and the imino residue was dissolved in 150 ml of absolute ethanol, under an argon atmosphere, and the obtained solution was syringed in a two-necked flask and kept under argon. The solution was heated at reflux and the imino product was reduced with a large excess (1.2 g) of $NaBH_4$, added as a solid in small portions to avoid foaming. After the addition, the mixture was kept at reflux temperature for 8 h, then the solvent was removed on a rotary evaporator obtaining a semisolid residue. This was treated with 100 ml water and extracted with 3 × 100 ml dichloromethane. The organic portions were gathered and dried over Na_2SO_4, then the solvent was removed under vacuum to give a yellowish oil that after treatment with diethyl ether and sonication yielded the product as a white solid. Yield 70%. Mass (ESI): *m*/*z* 459 (**L2**H_2 + H^+). NMR, $(CD_3)_2SO$, δ = 2.46 (t, 4H; CH_2–NH–CH_2–), 3.00 (t, 1H; CO–C*H*–CO), 3.10 (m, 2H; Ph–CH_2–CH), 3.35 (t, 4H; CO–NH–CH_2), 3.6 (m, 4H; Ph–CH_2–NH), 7.2 (m, 15H; aromatic hydrogens), 7.84 (s, 2H; CO–N*H*). Elemental analysis calcd for $C_{28}H_{34}N_4O_2$: C 73.34, H 7.47, N 12.21%; found C 73.32, H 7.49, N 12.20%.

2-Benzyl-*N*,*N*′-bis[2-(4-isopropylbenzylamino)ethyl]malondiamide (L3H_2). 278 mg (1 mmol) of 2-benzyl-*N*,*N*′-bis(aminoethyl)malondiamide (**L6**H_2) and 301 μl (2 mmol) of 4-isopropylxbenzaldehyde were reacted with the same procedure described for **L2**H_2. **L3**H_2 was obtained as a white solid (67% yield). Mass (ESI): *m*/*z* 543 (**L3**H_2 + H^+). NMR, $(CD_3)_2SO$, δ = 1.20 (d, 12H; CH_3–CH), 2.50 (t, 4H; CH_2–NH–CH_2), 2.83 (t, 2H; Ph–C*H*–$(CH_3)_2$), 2.98 (t, 1H; CO–C*H*–CO), 3.10 (m, 2H; Ph–CH_2-CH), 3.33 (t, 4H; CO–NH–CH_2), 3.57 (m, 4H; Ar–CH_2–NH), 7.2 (m, 13H; aromatic hydrogens), 7.8 (s, 2H; CO–N*H*). Elemental analysis calcd for $C_{34}H_{46}N_4O_2{\cdot}0.5(C_2H_5)_2O$: C 74.58, H 8.87, N 9.66%; found C 74.61, H 8.89, N 9.63%.

***N*,*N*′-Bis(2-hexylaminoethyl)malondiamide (L4H_2).** 854 mg (4.54 mmol) of *N*,*N*′-bis(aminoethyl)malondiamide (**L0**H_2) and 648 mg (6.81 mmol) of hexanal were dissolved in 80 ml CH_3OH. To this solution, 427 mg (6.8 mmol) of sodium cyanoborohydride and 637 mg (3.4 mmol) of zinc chloride dissolved in 60 ml CH_3OH were added in one portion, at room temperature. The reaction mixture was stirred under nitrogen for 8 h, after which time it was treated with 20 ml of a 0.1 M solution of NaOH in CH_3OH, and the solvent removed on a rotary evaporator. The semisolid residue was treated with 150 ml of 0.01 M KOH water solution and the obtained mixture extracted with 3 × 150 ml dichloromethane. The organic portions were gathered, dried over Na_2SO_4, and the solvent removed with a rotary evaporator. The oily residue, containing also traces of the mono-, tri- and tetra-hexyl products was separated on a SiO_2 column. The eluent mixture was prepared by shaking in a separatory funnel 50 : 40 : 010 v/v

of n-butanol–water–acetic acid and using the organic phase. **L4**H_2 was the third product obtained from the column, with such an eluent mixture. The fractions containing **L4**H_2 were gathered, the solvent removed under vacuum and 0.01 M aqueous NaOH was added drop by drop until pH 10 was reached. The product precipitated as a deliquescent solid, that was further dissolved in dichloromethane, dried with Na_2SO_4 and, after solvent removal on a rotary evaporator, obtained as a whitish waxy solid. Yield 38%. Mass (ESI): m/z 357 (**L4**H_2 + H^+). NMR, $CDCl_3$: $\delta = 0.9$ (m, 6H; CH_3 of the hexyl chains), 1.3 (m, 16H; CH_2 of the hexyl chains), 2.6 (m, 4H; NH–CH_2– in the hexyl chains), 2.75 (t, 4H; NH–CH_2–CH_2–NHCO), 3.15 (s, 2H; CO–CH_2–CO), 3.35 (t, 4H; CON–CH_2), 7.3 (s, 2H; CO–NH–CH_2). Elemental analysis calcd for $C_{19}H_{40}N_4O_2{\cdot}CH_2Cl_2{\cdot}H_2O$: C 54.17, H 10.00, N 12.63%; found C 54.14, H 9.98, N 12.60%

***N*,*N*′-Bis(2-dihexylaminoethyl)malondiamide (L5H_2).** 187 mg (0.99 mmol) of *N*,*N*′-bis(aminoethyl)malondiamide (**L0**H_2) were dissolved in 40 ml CH_3OH. 285 mg (3 mmol) of hexanal, 157 mg (2.5 mmol) of sodium cyanoborohydride and 204 mg (1.5 mmol) of zinc chloride were dissolved in 40 ml CH_3OH and added to the solution of **L0**H_2 drop by drop, under a nitrogen atmosphere, at room temperature. The reaction mixture was further stirred for 8 h under nitrogen, after which time 20 ml of 0.1 M NaOH in water were added and the solvent removed on a rotary evaporator. The residue was treated with 50 ml 0.1 M NaOH in water and extracted with 3 × 50 ml dichloromethane. The organic solvent was dried on Na_2SO_4 and removed on a rotary evaporator to give a yellow oil containing **L5**H_2 and the mono-, di- and tri-hexyl products as traces. To obtain pure **L5**H_2 the mixture was filtered on a short SiO_2 column, using 1 : 1 v/v n-hexane–ethyl acetate, with a gradient of CH_3COOH from 0.1% to 2%. The fractions filtered with 2% acetic acid were gathered, the solvent removed on a rotary evaporator, the residue was redissolved in 10 ml water and treated drop by drop with 0.1 M NaOH until pH 10 was reached. **L5**H_2 precipitated as a white solid, that was extracted with 20 ml dichloromethane, dried with Na_2SO_4 and obtained as a white pure solid after solvent removal in vacuum. Yield 40%. Mass (ESI): m/z 525 (**L5**H_2 + H^+). NMR, $(CD_3)_2SO$, $\delta = 0.9$ (m, 12H; CH_3– of the hexyl chains), 1.3 (m, 32H; CH_2 in the hexyl chains), 2.6 (m, 8H; NH–CH_2– of the hexyl chains), 2.75 (t, 4H; –HN–CH_2–CH_2–NHCO), 3.15 (s, 2H; CO–CH_2–CO), 3.35 (t, 4H; CONH–CH_2), 7.3 (s, 2H; CO–NH). Elemental analysis calcd for $C_{31}H_{64}N_4O_2{\cdot}0.5\ CH_2Cl_2$: C 66.69, H 11.55, N 9.87%; found C 66.70, H 11.56, N 9.86%.

Titrations

Coupled pH-spectrofluorimetric titrations were carried out on water solutions at 25 °C, made 0.05 M in $NaNO_3$, and containing 6.47 g l^{-1} of TritonX-100, 10^{-6} M pyrene (dissolved by adding aliquots of concentrated pyrene solutions in *tert*-butanol, with a final *tert*-butanol concentration <0.5% v/v), plus the chosen ligand in 10^{-3} M concentration (to observe the effect of protonation on fluorescence) or the chosen ligand and $Cu(CF_3SO_3)_2$ both in 10^{-3} M concentration (to observe the effect of complexation on fluorescence). Solutions of 25 ml were used under a constant flow of nitrogen, they were treated with excess nitric acid and titrated by manual micropipette additions of 10–50 μl aliquots of standard NaOH. A glass electrode for pH measurement was dipped in the bulk solution. At each base addition the pH was recorded and the emission spectra were recorded in the spectrofluorimeter (λ_{exc} = 343 nm). Total NaOH addition, at the end of the titrations, did not exceed 0.8 ml.

Coupled pH-spectrophotometric titrations were also carried out with identical details on solution containing micellized ligand and Cu^{2+}, in the absence of added pyrene.

Protonation equilibria of micellized ligands were studied in water containing 6.47 g l^{-1} of TritonX100, by addition of standard base (KOH) to a 10^{-3} M solution of the chosen ligand containing excess standard nitric acid. Cu^{2+} complexation equilibria of micellized ligands were studied under the same conditions, in the presence of $Cu(CF_3SO_3)_2$ in equimolar quantity with respect to the ligand. Solutions were prepared to contain 0.05 M $NaNO_3$ as the supporting electrolyte, T = 25 °C. Potentiometric measurements were carried out automatically, as already described.[16] The titration curves were fitted and the equilibrium constants were calculated by using the nonlinear fitting program HYPERQUAD.[10] The titrations (both for protonation and complexation constants) were repeated at least twice for each compound. The obtained results were identical within the uncertainty affecting the values.

Instrumentation

Mass spectra were recorded on a Finnigan MAT TSQ 700 instrument, NMR spectra on a Bruker AMX 400. Spectrofluorimetric measurements were performed with a Perkin Elmer LS 50B instrument, absorption spectra were taken on a Hewlett-Packard HP-8453 spectrophotometer. The pH-metric titrations were made with a Radiometer TitraLab 90 titration system.

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