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Fluorescent Sensors for Hg^{2+} in Micelles: A New Approach that Transforms an ON–OFF into an OFF–ON Response as a Function of the Lipophilicity of the Receptor

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Abstract: A new approach to the use of micelles in the fluorescent sensing of metal cations is proposed and applied to the case of Hg^{2+}. We demonstrate how it is possible to transform a system from an ON–OFF to an OFF–ON sensor by changing the length of the chain used to lipophilise a ligand that resides inside TritonX-100 micelles together with pyrene as the fluorophore. Three tetrathia-monoaza macrocyclic ligands have been synthesised with the same ring but functionalised on the nitrogen atom with a methyl (**C1-NS4**), an *n*-butyl (**C4-NS4**) or an *n*-dodecyl (**C12-NS4**) chain. The three ligands have been fully characterised in water containing TritonX-100 micelles by means of potentiometric titrations and their apparent protonation and complexation constants with Hg^{2+} were determined. On the basis of the distribution diagrams obtained, the more lipophilic **C12-NS4** has been developed as an ON–OFF fluorescent sensor for mercury: working at $pH < 4$, in the absence of Hg^{2+} the ligand is inside the micelles, protonated and non-quenching, while on addition of mercury the [**C12-NS4**Hg]$^{2+}$ complex forms which remains inside the micelles and is quenching. On the other hand, the ligand of intermediate chain length, **C4-NS4**, can be used to obtain an OFF–ON sensor at $7.0 < pH < 9.5$. In the absence of added metal at $pH > 7.0$ the ligand is unprotonated, it stays inside the micelles and is quenching, while addition of Hg^{2+} in the 7.0–9.5 pH range results in the formation of [**C4-NS4**Hg]$^{2+}$, which is hydrophilic enough to leave the micelles and to be released into the bulk solution where it is no longer capable of quenching pyrene fluorescence. Additional studies on **C1-NS4**, **C3-NS4** and **C8-NS4** indicate that the optimal chain length to observe this OFF–ON behaviour is C_3–C_4.

Keywords: fluorescence · mercury · micelles · self-assembly · sensors

Introduction

The first examples of systems capable of fluorescent sensing of cations in solution appeared between 1985 and 1988 and dealt with the sensing of H^+ (i.e., pH),[1] alkali-metal cations[2] and Zn^{2+}.[3] These papers were based on a traditional molecular approach, later called FSR (fluorophore–spacer–receptor), in which a fluorophore and a receptor component capable of also acting as a quencher in the absence of the target species were covalently linked by a spacer. These multicomponent molecules thus behaved as sensors of the "OFF–ON" type for the target cation. Later, examples in which a fluorophore was covalently linked to a receptor component capable of acting as a quencher in the presence of the target cation were also introduced, leading to the FSR "ON–OFF" sensors.[4] A huge number of papers reporting on these kinds of molecules continue to be published, showing how fluorescence can be used to conveniently sense a wide range of different metal cations.[5] On the other hand, since the early 1970s, surfactant chemistry has exploited the possibility offered by working in water and confining separate hydrophobic fluorophores and quenchers in the lipophilic core of the same micelle. As an example, the observation of either steady-state or dynamic fluorescence

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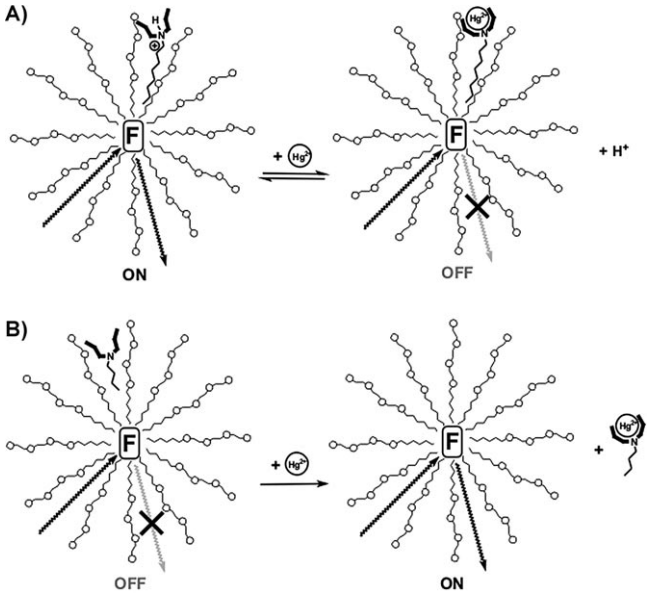
quenching due to intramicellar interactions between a fluorophore and a quencher is a well-established method for calculating the aggregation number (AN).[6] Moreover, kinetic parameters such as the rate constants for the processes involving the fluorophore/quencher systems inside a micelle have also been elucidated by means of fluorescence quenching[7] and sophisticated structural parameters such as the position of a hydrophobic molecule inside a micelle with respect to the water/micelle interface[8] or the shape of a micelle[9] have also been investigated through intramicellar quenching processes between separated components. Very recently, the chemistry of FSR fluorescent sensors and intramicellar quenching processes has been combined to develop the new idea of using micelles as containers in water for assembling supramolecular sensors in which simple, separated hydrophobic fluorophores and receptors/quenchers are kept together and communicate inside the micelle without the need to build a covalent structure.[10,11] Aside, it should also be mentioned that enhanced signal intensity and selectivity have been observed by confining traditional fluorescent sensors inside micelles.[12–14]

Recently, we published our first contribution to this field describing a fluorescent ON–OFF sensing system selective for Ni^{2+} and Cu^{2+} based on a lipophilised diamino-diamido ligand and the fluorescent pyrene molecule, which interacted inside the micelles of the neutral Triton X-100 surfactant.[15] Moreover, we also described a new micellar approach to fluorescent sensors for pH windows, obtained by including in the same micelle one pyrene molecule, a pyridine moiety and a tertiary amine, both made lipophilic by functionalisation with a long alkyl chain.[16] In this paper we turn to the goal of using micelles for the fluorescent signalling of Hg^{2+}, a goal that is particularly interesting as Hg^{2+} is a pollutant arising from both human activities and natural sources[17] that may enter the food chain of human beings through edible fish.[18] A number of papers have reported the fluorescent sensing of Hg^{2+} in water or organic/water mixtures, making use of traditional FSR sensors capable of either an OFF–ON or ON–OFF fluorescent response.[19] In this paper we first show how, by merging some of the possibilities that we have already exploited with the micellar approach, an OFF–ON sensor for Hg^{2+} can be obtained. Considering that 1) lipophilic molecules containing a tertiary amino group give an ON–OFF response with pH if included in micelles containing a fluorophore[16] and 2) the complexation of a heavy-metal cation by a ligand confined in a micelle (e.g. thanks to functionalisation with a long alkyl chain) promotes the quenching of the excited state of a fluorophore included in the same micelle,[15] an ON–OFF sensor can be obtained in micelles containing a separate fluorophore and a tertiary amine-containing ligand (suitable for Hg^{2+}), provided that one can find a pH range in which, in the absence of Hg^{2+}, the ligand is protonated, while in the presence of Hg^{2+} a complex is formed. This approach is represented pictorially in Scheme 1A.

Moreover, a new approach to the use of micelles for fluorescent sensing of Hg^{2+} is also proposed. This approach changes the same system into an OFF–ON sensor thanks to a simple change in the lipophilicity of the ligand. If the ligand is made less lipophilic (e.g., by appending to it a shorter alkyl chain) it will be confined inside the micelle when it is neutral, that is, unprotonated, thus quenching fluorescence (the OFF state). However, when it forms a complex with the Hg^{2+} cation, the charged complex will be released from the micelle, as it seeks better hydration in the bulk water solution: accordingly, as the Hg^{2+} complex will not interact with the micellised fluorophore, the fluorescence will turn ON, and the whole system will work as an OFF–ON sensor. The approach is represented pictorially in Scheme 1B.

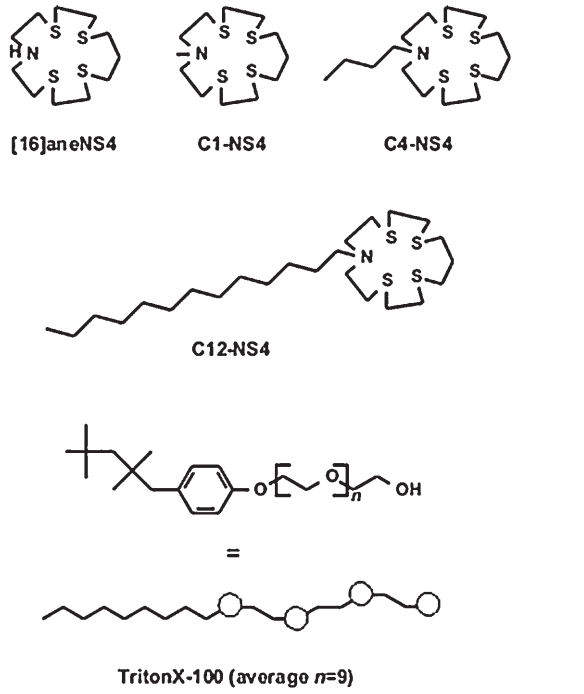
We have chosen to use a ligand, **[16]aneNS4**, that belongs to a category known to be suitable for Hg^{2+} complexation, that is, a polythia-aza macrocycle,[20] and that can be easily synthesised following a procedure described previously.[21] We have functionalised its nitrogen atom with C_1, C_4 and C_{12} *n*-alkyl chains and fully characterised the resulting **C1-**

Abstract in Italian: *In questo lavoro viene proposto, e applicato al caso di Hg^{2+}, un nuovo stile nell'uso delle micelle per il sensing fluorescente dei cationi metallici. Dimostriamo, qui, come sia possibile trasformare un sistema da sensore* ON–OFF *a sensore* OFF–ON *grazie al cambiamento della lunghezza della catena utilizzata per lipofilizzare un legante contenuto in micelle di TritonX-100 insieme a pirene nel ruolo di fluoroforo. Sono stati sintetizzati tre mono-aza tetratia macrocicli dotati dello stesso anello e funzionalizzati sull'atomo di azoto con gruppi metile (**C1-NS4**), n-butile (**C4-NS4**) e n-dodecile (**C12-NS4**). I tre leganti sono stati caratterizzati in acqua, in presenza di micelle di TritonX-100, per mezzo di titolazioni potenziometriche, che hanno consentito di determinarne le costanti apparenti di protonazione e le costanti di complessazione con Hg^{2+}. Sulla base dei diagrammi di distribuzione è stato sviluppato l'uso del legante più lipofilico, **C12-NS4**, per realizzare un sensore fluoresecente* ON–OFF *per il mercurio: lavorando a $pH<4$, in assenza di Hg^{2+} il legante è confinato all'interno delle micelle, è protonato e non è in grado di spegnere la fluorescenza del pirene, mentre per aggiunta di mercurio si forma il complesso $[$**C12-NS4**$Hg]^{2+}$ che altrettanto rimane all'interno delle micelle e spegne la fluorescenza. Per contro, il legante con lunghezza intermedia della catena, **C4-NS4**, può essere usato per ottenere un sensore* OFF–ON *nell'intervallo di pH 7.0–9.5. In assenza di metallo, a $pH>7.0$ il legante non è protonato, rimane all'interno delle micelle e spegne, mentre l'aggiunta di Hg^{2+} nell'intervallo di pH 7.0–9.5 ha come risultato la formazione di $[$**C4-NS4**$Hg]^{2+}$, che è abbastanza idrofilico da abbandonare le micelle e raggiungere il corpo della soluzione, dove non è più in grado di spegnere la fluorescenza del pirene. Studi aggiuntivi sui leganti **C1-NS4**, **C3-NS4** e **C8-NS4** indicano che la lunghezza ottimale della catena per osservare un comportamento* OFF–ON *è C_3–C_4.*



Scheme 1. Pictorial representation of the approaches adopted in this paper to prepare micellar sensors for Hg^{2+} based on fluorescence. The sensor may be transformed from A) ON–OFF to B) OFF–ON, maintaining the same binding unit and changing its lipophilicity and the working pH interval.

NS4, **C4-NS4** and **C12-NS4** ligands (Scheme 2) in water containing micelles (TritonX-100 as surfactant) by means of potentiometric titrations. Determination of the pertinent protonation and complexation constants, and coupled fluorimetric/pH-metric titrations with pyrene as fluorophore, demonstrate that we have obtained systems behaving according to



Scheme 2. Structures of the **C1-NS4**, **C4-NS4** and **C12-NS4** ligands.

our plans. Further studies on NS4 ligands functionalised with C_3 and C_8 *n*-alkyl chains suggest that the best chain length for obtaining an OFF–ON sensor is three to four carbon atoms.

Results and Discussion

Protonation constants of the C*n*-NS4 ligands: The protonation constant of the amino group of the three **C*n*-NS4** ligands was determined in different media, depending on their solubility. In particular, in plain water it was possible to study only the protonation properties of **C1-NS4**, as this is the only ligand among the three that is soluble up to a concentration that allows reliable data elaboration from potentiometric titration experiments ($>5\times10^{-4}$ M). On the other hand, the protonation constant of the tertiary amino group of all the ligands was determined in water/TritonX-100 and in a dioxane/water mixture (9:1 v/v), in which the three ligands are all readily soluble.

The protonation constants in the three media were determined by means of potentiometric titrations using standard methods and instrumentation (see the Experimental Section). The results are summarised in Table 1. While this well-established experimental procedure gives the intrinsic protonation constants for the tertiary amine group in water and in dioxane/water, it has to be remembered that using a standard potentiometric titration apparatus in a micellised water/surfactant medium means measuring a space-averaged value of *E* with a macroscopic glass electrode and that the calculated protonation constants of the examined thia-aza ligands are of course not intrinsic, but are the so-called apparent protonation constants, that is, they are influenced by the fact that the lipophilic bases are included inside the micelles.[22] In this situation, the organic tail is inserted in the hydrophobic micellar core and the more polar amino group presumably lies near the hydrophilic, external layer of the micelle. In the hydrophilic layer of the micelle penetration of the solvent and of any species dissolved in solution is partially allowed, but the local concentration of water and H^+ ions could be significantly lower with respect to the bulk solution.[8] Hence, as has already been observed,[8,16] lower apparent protonation constants are to be expected with increasing ligand lipophilicity. It should also be stressed that the apparent protonation constants depend, in general, on

Table 1. Logarithmic protonation constants of the tertiary amino group of the ligands in different media. The $\log K$ values refer to the equilibrium **C*n*-NS4** + $H^+ \rightleftharpoons$ [**C*n*-NS4**H]$^+$.[a]

	Water	Water/TritonX-100	Dioxane/water (9:1 v/v)
C1-NS4	7.0 (0.1)	6.89 (0.02)	5.73 (0.02)
C4-NS4	–	5.86 (0.02)	5.65 (0.02)
C12-NS4	–	3.81 (0.02)	5.42 (0.02)

[a] Uncertainties are indicated in parentheses.

the concentration of the surfactant. Considering the case of a monobase, low surfactant concentrations may result in distribution of both the protonated and unprotonated forms between the micelles and the bulk water solution, while high surfactant concentrations result in the confinement of both forms inside the micelles (in this case the measured pK_a values should be the highest, that is, the most different from what is found in pure water).[22] Herein, the concentration of the TritonX-100 surfactant was maintained high above the critical micelle concentration (cmc) and at a constant value (0.01 M), and also the concentration of the examined ligands was kept constant (10^{-3} M) in all the experiments. Accordingly, even if a certain degree of distribution occurs between the micelles and the bulk water needs to be taken into consideration, the observed $\log K$ values may be directly compared.

In water/TritonX-100 the $\log K$ value found for **C1-NS4** is 6.89 (Table 1), it drops to 5.86 for **C4-NS4** and on increasing the chain length to **C12-NS4** another dramatic drop in the $\log K$ value is observed (3.81). Even the value for **C1-NS4** is unusual if compared with the protonation of tertiary amines in plain water (triethylamine has a $\log K$ value of 10.68 in water[23]). However, the logarithmic protonation constant for **C1-NS4** measured in water is $\log K = 7.0$, a value very similar to that observed in water/TritonX-100. This leads to two conclusions: 1) this ligand is included only to a negligible extent in the TritonX-100 micelles or it interacts with micelles in the more external, highly hydrated mantle and 2) the nitrogen atom of the tertiary **[16]aneNS4** framework has an intrinsically low basicity, probably related to its particularly hindered nature.[24] Moreover, the protonation data found in the dioxane/water medium (Table 1) are almost identical for the three ligands. This clearly indicates that the huge differences found in the surfactant/water medium are due to micellar inclusion and to the different degree of penetration in the micellar core, but not to any other exotic effect connected to the length of the alkyl chain.

Coordination chemistry of the C12-NS4/pyrene system in micelles: The complexation ability of **C12-NS4** towards Hg^{2+} was examined in the water/TritonX-100 medium by means of potentiometric titrations, carried out under the same conditions as described above and using a ligand/metal 1:1 stoichiometry (both 10^{-3} M). The logarithmic formation constants obtained and the corresponding equilibria are listed in Table 2. Figure 1 (solid lines) displays the distribution diagram that may be drawn from these values for the **C12-NS4**/Hg^{2+} system (1:1 molar ratio) at a concentration of 10^{-3} M.

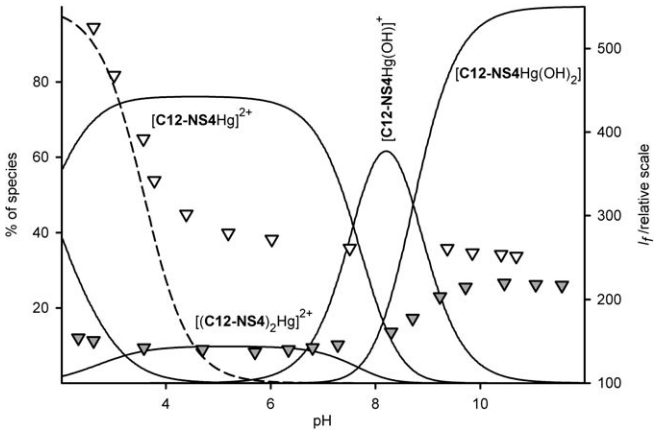


Figure 1. Solid lines: distribution diagram for the **C12-NS4** + Hg^{2+} system (1:1 molar ratio, 10^{-3} M) expressed as a percentage of species (with respect to total ligand) versus pH; the species relating to each curve are indicated in the diagram. Percentage values are to be read on the left-hand axis. Dashed line: percentage of [**C12-NS4**H]$^{+}$ with respect to total ligand versus pH in the absence of added Hg^{2+} (ligand at 10^{-3} M concentration). Percentage values are to be read on the left-hand axis. White triangles: pyrene fluorescence intensity at 386 nm with respect to pH for **C12-NS4** and no added Hg^{2+} (I_f values are to be read on the right-hand axis). Grey triangles: pyrene fluorescence intensity at 386 nm with respect to pH for the **C12-NS4** + Hg^{2+} system (1:1 molar ration, 10^{-3} M; I_f values are to be read on the right-hand axis). All data were obtained in a water/micelle medium, 0.05 M $NaNO_3$, at 25 °C.

The $\log K$ value found for the formation of [**C12-NS4**Hg]$^{2+}$, corresponding to the binding process of the metal cation inside the micellised macrocyclic ring, is particularly low. For the sake of comparison we determined, using the same methods, the formation constants for the complexes formed by **C1-NS4** in water (Table 2), finding a $\log K$ value of 7.98, a value that fits well with what is expected for a Hg^{2+} ion interacting with a polythia-monoaza macrocyclic ligand in water.[20] The low value found in the case of the lipophilic **C12-NS4** in the water/surfactant medium is most probably a consequence of the inclusion of the complex species in the micelle.

In fact, the tendency for the Hg^{2+} ion to interact with further donor atoms once coordinated by the macrocyclic ring in [**C12-NS4**Hg]$^{2+}$ is strongly indicated both by the formation of the two-ligand/one-metal species [(**C12-NS4**)$_2$Hg]$^{2+}$ and by the formation of the hydroxide complexes [**C12-NS4**Hg(OH)]$^{+}$ and [**C12-NS4**Hg(OH)$_2$] at pH > 6. Thus in [**C12-NS4**Hg]$^{2+}$ it is expected that the Hg^{2+} cation is coordinated by water molecules and that the low local concentration of water inside the micelle is probably the main reason for the small value of $\log K$ found. However, as can be seen in Figure 1, the mercury ion is fully complexed by the ligand

Table 2. Logarithmic formation constants for the ligand/mercury complex species.

	C1-NS4[b]	**C4-NS4**[c]	**C12-NS4**[c]
$L + Hg^{2+} \rightleftharpoons [LHg]^{2+}$	7.98 (0.01)	6.34(0.01)	5.60 (0.02)
$2L + Hg^{2+} \rightleftharpoons [(L)_2Hg]^{2+}$	10.23(0.02)	–	9.10 (0.02)
$L + Hg^{2+} + H_2O \rightleftharpoons [LHg(OH)]^{+} + H^{+}$	–	–	−2.00 (0.02)
$L + Hg^{2+} + 2H_2O \rightleftharpoons [(L)Hg(OH)_2] + 2H^{+}$	−13.14(0.02)	−14.25(0.02)	−10.69 (0.03)

[a] Uncertainties are indicated in parentheses. [b] Values obtained in water (0.05 M $NaNO_3$) at 25 °C. [c] Values obtained in water containing micelles (water/TritonX-100, 0.05 M $NaNO_3$) at 25 °C.

(and thus kept in the micelle) at pH 3.0. The formation of a stable [**C12-NS4**Hg]$^{2+}$ species is also confirmed by spectrophotometric titrations (see the Supporting Information). Notably, at pH 3 in the absence of Hg^{2+} the micellised **C12-NS4** ligand is almost fully protonated (Figure 1, dashed line), that is, it exists as [$\mathbf{C_{12}}$-$\mathbf{NS_4}$H]$^+$, while at the same pH addition of Hg^{2+} results in its incorporation in the micelle with the formation of [**C12-NS4**Hg]$^{2+}$ (Figure 1 and Figure S1; concurring with less than 10% of the [(**C12-NS4**)$_2$Hg]$^{2+}$ species).

The C12-NS4/pyrene system in micelles as an ON–OFF sensor for Hg^{2+}: Pyrene was added to the TritonX-100/**C12-NS4** system at a concentration of 10^{-6} M. With such a low concentration of fluorophore the inclusion of two or more pyrene molecules per micelle is ruled out, thus avoiding the possibility of excimer emission due to π–π stacking of two or more pyrene molecules inside the same micelle. As the surfactant and ligand concentrations are kept identical to those discussed in the previous section, some micelles will be "empty" of the fluorophore (average pyrene number per micelle ≅0.01, see also reference [25]). However, in "fluorophore-loaded" micelles, the pyrene molecule will be surrounded by an average of 10 ligand molecules, thus maximising the effect on fluorescence of protonation of the nitrogen atom and of Hg^{2+} complexation. To study the behaviour of pyrene fluorescence a stock solution was prepared containing 10^{-6} M pyrene, 10^{-3} M **C12-NS4** and 0.05 M $NaNO_3$. Two 25 mL portions of this solution were treated with the same quantity of HNO_3 corresponding to full protonation of the nitrogen atom of the ring plus two equivalents excess. To one of the two solutions, [$Hg(CF_3SO_3)_2$] was also added in a 1:1 molar ratio with respect to **C12-NS4**. Then, coupled pH-metric and fluorimetric titrations were carried out on both solutions (λ_{exc} = 343 nm). In the case of the system containing only **C12-NS4**, the expected sigmoidal I_f versus pH profile was obtained, with full emission spectra observed at low pH values and quenching observed above the pK_a value of the tertiary amine, presumably due to an electron-transfer quenching mechanism from the free electron pair on the nitrogen atom to the excited pyrene molecule. In Figure 1, the white triangles show this trend (I_f at 386 nm, the maximum of pyrene emission, versus pH).[26] The dashed line represents the percentage of [**C12-NS4**H]$^+$ versus pH in the absence of added Hg^{2+} (ligand at a concentration of 10^{-3} M) and it superimposes nicely the pertinent I_f versus pH data. On the other hand, fully quenched spectra were observed for the solution containing Hg^{2+} in the whole pH range examined, that is, pH 2–12, owing to the formation of **C12-NS4**/Hg^{2+} complexes in the micelle, as can be seen in Figure 1 (grey triangles) from the plot of $I_{f,386}$ versus pH (a negligible increase in fluorescence intensity is observed at pH > 8, in accord with the formation of the [**C12-NS4**Hg(OH)$_2$] species, as indicated by the superimposition in the distribution diagram). In this case, quenching may be attributed to the "heavy-atom effect"[27] exerted by micellised mercury on the co-micellised pyrene. Comparison of the two I_f versus pH profiles in Figure 1 shows that this micellar system behaves as an ON–OFF sensor for Hg^{2+} if used at pH < 4. Direct experimental evidence was obtained by titrating a solution of micellised pyrene + **C12-NS4** at pH 3.0 with substoichiometric quantities of Hg^{2+} (the pH was maintained at a constant value with an automatic burette working in pH-constant mode).

The emission spectra of pyrene decrease in intensity (Figure 2), reaching a minimum when ~0.5 equivalents of Hg^{2+} had been added, as shown by the $I_{f,386}$ versus Hg^{2+} equivalents profile in the inset of Figure 2. The small

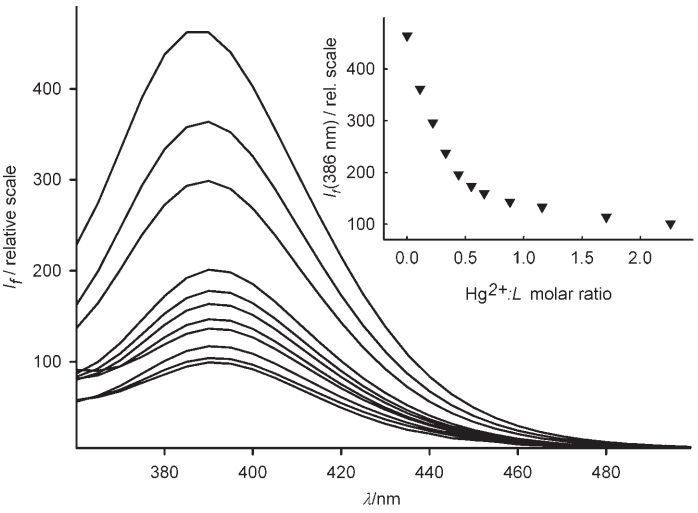


Figure 2. Emission spectra of pyrene in the presence of **C12-NS4** on addition of substoichiometric quantities of [$Hg(CF_3SO_3)_2$] in the water/TritonX-100 medium. The intensities decrease with addition of Hg^{2+}. In the inset, the fluorescence intensity at 386 nm is reported as a function of the equivalents of added mercury (i.e., the Hg^{2+}/ligand molar ratio).

amount (< 1.0 equivalent) of Hg^{2+} needed to achieve the maximum quenching of pyrene fluorescence is related to the high ratio of **C12-NS4** ligands per micelle with respect to pyrene in the "fluorophore-loaded" micelles. Accordingly, complexation of a few of the available lipophilised macrocycles inside each micelle is sufficient for an average quenching to be observed in the bulk solution. We have repeated the same experiments under the same conditions but in the presence of 10^{-2} M NaCl or $NaHCO_3$, to check for any possible effect of Cl^- and HCO_3^- ions (ubiquitous in natural aqueous environments), but without finding significant differences in the sensor response. Selectivity for Hg^{2+} was checked by adding at pH 3.0 up to two equivalents of Ca^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}, which resulted in no significant variation of fluorescence. However, Ag^+ is a serious interferent for Hg^{2+} sensing. We carried out the same kind of titration at pH 3.0 and on addition of $AgNO_3$ in substoichiometric quantities we observed a progressive decrease in I_f, with the emission intensity reaching a minimum after ~0.7 equivalents of $AgNO_3$ had been added. For the sake of comparison, we also carried out a spectrophotometric titration (no pyrene added) at pH 3.0 and compared this with

the same titration carried out with Hg^{2+}. Interpretation of the spectrophotometric data with a simple model consisting of the formation of a 1:1 complex (i.e., the L+M⇌LM equilibrium) allowed us to calculate a conditional $\log K$ value of 6.7 and 5.2, in the case of Hg^{2+} and Ag^{+}, respectively, thus indicating the good degree of selectivity of this system for Hg^{2+} over Ag^{+}. Finally, sensitivity towards Hg^{2+} was checked in the ppm range: we prepared a more dilute sensor solution (10^{-3} M TritonX-100, 5×10^{-5} M **C12-NS4** and 2×10^{-7} M pyrene) obtaining a 10% decrease in I_f by making the same solution 5×10^{-6} M in Hg^{2+}, corresponding to ~1 ppm.

The C4-NS4/pyrene system in micelles as an OFF–ON sensor for Hg^{2+}: We first examined the complexation behaviour of the *n*-butyl-substituted ligand towards mercury in a water/surfactant medium. Potentiometric titrations allowed us to obtain the $\log K$ data for **C4-NS4**/Hg^{2+} complexes reported in Table 2. Interestingly, the logarithmic complexation constant for the formation of [**C4-NS4**Hg]$^{2+}$ is 6.34, intermediate between that found in water for **C1-NS4** and that found in the micelle for **C12-NS4**, a trend also found for the protonation constants (Table 1). A distribution diagram for **C4-NS4**/Hg^{2+} (both 10^{-3} M) is displayed in Figure 3 with, in addition, the profile relative to the percentage of protonated ligand (10^{-3} M) in the absence of added mercury (dashed line). Beside the fact that the neutral ligand is clearly micellised (**C4-NS4** is not soluble in plain water, while it easily dissolves in water/TritonX-100) the question may arise as to whether the short C_4 tail is sufficiently lipophilic

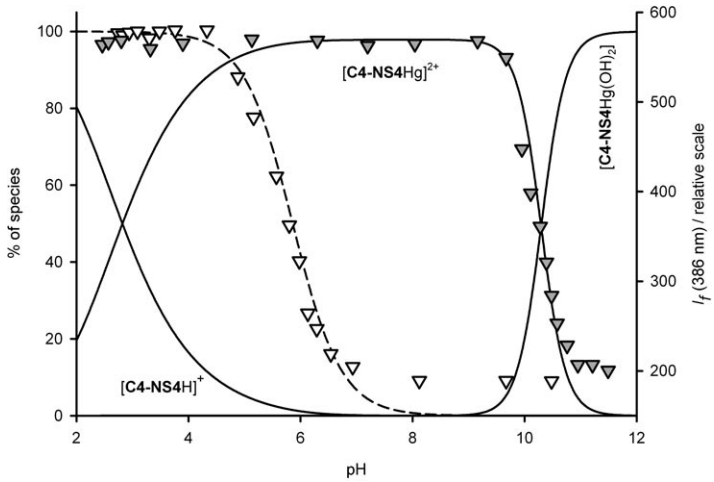


Figure 3. Solid lines: distribution diagram for the **C4-NS4**/Hg^{2+} system in a 1:1 molar ratio (concentration = 10^{-3} M) in a water/TritonX-100 medium, expressed as a percentage of species (with respect to total ligand) versus pH. Dashed line: distribution curve for the [**C4-NS4**H]$^{+}$ species in the absence of added mercury (concentration = 10^{-3} M) in water/TritonX-100 medium, expressed as a percentage of species (with respect to total ligand) versus pH. White triangles: pyrene fluorescence intensity at 386 nm versus pH for the system containing **C4-NS4** and no mercury. Grey triangles: pyrene fluorescence intensity at 386 nm versus pH, for the system containing **C4-NS4** and Hg^{2+} in a 1:1 molar ratio (10^{-3} M).

to keep its protonated form [**C4-NS4**H]$^{+}$ completely inside the micelles. The low value of the protonation constant (Table 1) suggests hydration is hindered owing to its inclusion in the micelle,[8,16] although its location is not as deep inside the micelle as the more lipophilic **C12-NS4**. We prepared a 50-mL solution containing pyrene plus ligand (10^{-6} M pyrene and 10^{-3} M **C4-NS4**), divided it into two portions of 25 mL, added excess acid in the same quantity and on one portion we monitored the effect of the ligand on fluorescence by coupled pH-metric and fluorimetric titrations.

The $I_{f,386}$ versus pH profile obtained is shown in Figure 3 as white triangles and as expected it is superimposed on the dashed line representing the percentage of protonated ligand (in the absence of Hg^{2+}) versus pH profile, that is, showing quenching in accord with the deprotonation of the tertiary amino group. The same coupled pH-metric and spectrophotometric titration was repeated on the second 25-mL portion after addition of an equimolar amount of [Hg$(CF_3SO_3)_2$] with respect to **C4-NS4**. In this case, a completely different profile compared with **C12-NS4** was found: the fluorescence is ON at pH < 9.5 and the intensity decreases in more basic solutions. Superimposition of $I_{f,386}$ (grey triangles, Figure 3) on the distribution diagram for the system containing **C4-NS4** and Hg^{2+} (1:1 molar ratio; Figure 3, solid lines) allows the observed data to be explained: at 5 < pH < 9.5 only [**C4-NS4**Hg]$^{2+}$ exists, while at pH < 5 it exists in equilibrium with the protonated ligand [**C4-NS4**H]$^{+}$. Neither species is able to quench pyrene fluorescence. While this is clear for the protonated ligand (in this regard it does not matter if it remains inside the micelle or leaves it), when [**C4-NS4**Hg]$^{2+}$ forms it is clear that it does not remain inside the micelles, but prefers to be free in the bulk of the solution where it is much better solvated by water. Quenching takes place at high pH with the formation of [**C4-NS4**Hg$(OH)_2$]: this is a neutral complex in which the Hg^{2+} ion is further coordinated to two hydroxide anions and the lipophilicity imparted by the ligand backbone and by the C_4 tail is sufficient to lead it back inside the micelles where it quenches fluorescence, most probably by the heavy-atom effect exerted by mercury.

By examining Figure 3, it can be seen that the system made by micellised **C4-NS4** plus pyrene behaves as an OFF–ON sensor for Hg^{2+}, as pictorially described in Scheme 1B, if the working pH is inside the 7–10 range. Accordingly, we prepared a 25 mL solution containing pyrene, **C4-NS4** and TritonX-100 at pH 8.0, and to this solution we added Hg^{2+} in substoichiometric quantities by means of microadditions of a solution of [Hg$(CF_3SO_3)_2$] made with a micropipette (pH was maintained at a constant value with an automatic burette working in the pH-constant mode). We obtained a series of spectra of increasing intensity, as shown in Figure 4, whose inset also shows I_f at 386 nm versus equivalents of added Hg^{2+}, which reveals that I_f increases with increasing Hg^{2+} concentration with an end point at a ligand/metal ratio of 1:1.

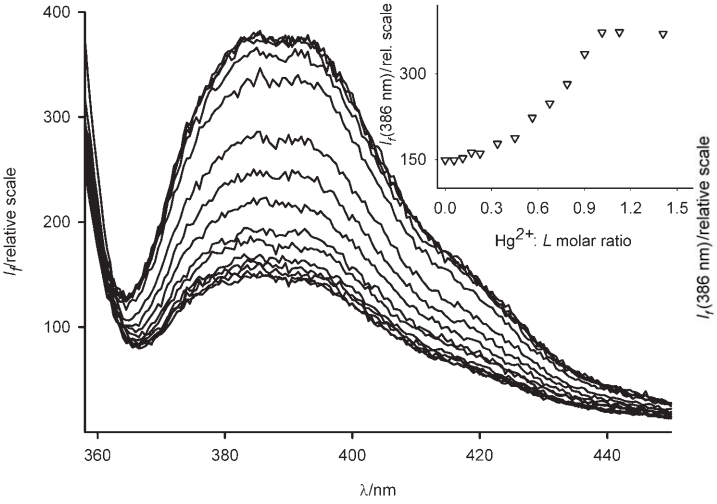


Figure 4. Emission spectra for a solution containing pyrene (10^{-6} M) and **C4-NS4** (10^{-3} M) in water/TritonX-100 at pH 8, obtained by addition of substoichiometric quantities of Hg^{2+}. The spectra increase in intensity with increasing Hg^{2+}. The inset reports $I_{f,386}$ versus number equivalents of added mercury (i.e., Hg^{2+}/ligand molar ratio).

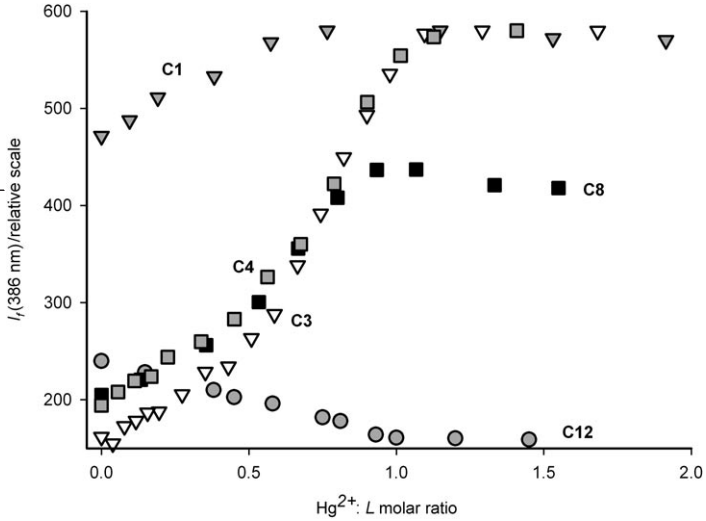


Figure 5. Pyrene fluorescence intensity (at 386 nm) versus equivalents of added Hg^{2+} (i.e., Hg^{2+}/ligand molar ratio) in solutions at pH 8.0–8.2 containing 10^{-3} M ligand. Grey triangles: **C1-NS4**. White triangles: **C3-NS4**. Grey squares: **C4-NS4**. Black squares: **C8-NS4**. Grey circles: **C12-NS4**.

No influence is exerted on the sensing system by Cl^- and HCO_3/CO_3^{2-} ions (identical behaviour is observed under the same conditions but in the presence of 0.01 M NaCl or $NaHCO_3$). The selectivity of this system as an OFF–ON sensor for Hg^{2+} was checked on solutions of micellised **C4-NS4** at pH 8 by addition of one equivalent of Ca^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}, with no significant variation in fluorescence observed. The behaviour of the system with Ag^+ was also examined. Unfortunately, also in this case Ag^+ behaves as an interferent, as its addition to a solution of **C4-NS4** at pH 8 results in an increase in pyrene fluorescence. However, in this case interference is less significant than in the case of the ON–OFF sensor based on **C12-NS4**: revival of fluorescence with mercury at pH 8 is ~350% (as can be seen from Figure 4, inset, or Figure 5), while revival with silver, under the same conditions, is ~180%. Sensitivity for Hg^{2+} was also checked in this case in the ppm range: with a dilute sensor solution (10^{-3} M TritonX-100, 5×10^{-5} M **C4-NS4** and 2×10^{-7} M pyrene) we observed a 15% increase in I_f on addition of 1 ppm Hg^{2+} (final mercury concentration in solution $=5\times10^{-6}$ M).

Tail length and efficiency of the micellised C*n*-NS4/pyrene system as an OFF–ON sensor for Hg^{2+}: To check the role of chain length (and thus of the lipophilicity of the ligand) in the ON–OFF response towards Hg^{2+} of the **C*n*-NS4** ligands in pyrene-containing micelles, we also prepared the *n*-propyl- and *n*-octyl-functionalised NS4 ligands, that is, **C3-NS4** and **C8-NS4**.[28] Unfortunately, we were not able to fully characterise these ligands as regards their protonation and complexation constants: turbidity was observed in the water/TritonX-100 medium at pH > 10 (**C3-NS4**, in the presence of Hg^{2+}) and at pH < 5 (**C8-NS4**, ligand alone) and owing to the formation of two phases we were not able to determine their properties reliably by means of potentiometric titrations. However, they were fully soluble at 5 < pH < 10 (both in the absence and in the presence of Hg^{2+}) and by comparing what we found for the C_1, C_4 and C_{12} analogues we assumed that if no mercury is added at pH ≥ 8.0 they exist entirely in their micellised, unprotonated form in the water/TritonX-100 medium. Accordingly, we prepared a stock solution containing micellised pyrene, we took five 25-mL portions of this solution and prepared five solutions, each one containing one of the five ligands at a concentration of 10^{-3} M. We regulated the pH of each solution in the range 8.0–8.2 by microadditions of 0.1 M NaOH and performed titrations with $[Hg(CF_3CSO_3)_2]$, maintaining the pH at the starting value by means of automatic microadditions of base with a Radiometer ABU901 Autoburette instrument working in the pH-constant mode.

Fluorescence spectra were recorded after each Hg^{2+} addition and the results, expressed as $I_{f,386}$ versus equivalents of added Hg^{2+}, can be compared in Figure 5. It can be seen that while the **C3-NS4** and **C4-NS4** derivatives give a comparable result, with a very significant revival of fluorescence and efficient OFF–ON behaviour, very different profiles are obtained in the other cases. The system containing **C1-NS4** displays an almost fully ON fluorescence even with no Hg^{2+} added: this is in accord with our hypothesis that the methyl-functionalised ligand is not included significantly in the TritonX-100 micelles in its neutral form or that it interacts with them in the more external and more hydrated layer. Accordingly, formation of the hydrophilic $[$**C1-NS4**$Hg]^{2+}$ complex and its total removal from the micellar space result in a very moderate fluorescence revival. The case of **C12-NS4** is the opposite: as shown in Figure 1, at pH ~8 the **C12-NS4**/Hg^{2+} system in a 1:1 molar ratio exists mainly as $[$**C12-NS4**$Hg(OH)]^+$, together with a significant percentage of the $[$**C12-NS4**$Hg]^{2+}$ and $[$**C12-NS4**$Hg(OH)_2]$ forms. Both these complex species and the unprotonated ligand are kept in the

micelle. Moreover, the mercury complexes give a better quenching of fluorescence than the unprotonated ligand. Accordingly, we observed further moderate lowering of the fluorescence intensity on addition of Hg^{2+} to micellised **C12-NS4**. Finally, **C8-NS4** displays an intermediate result: while the neutral ligand gives efficient quenching at pH 8.0, complexation with Hg^{2+} gives only a partial revival of fluorescence. To explain this we propose that the mercury complexes of this ligand have an intermediate lipophilicity that results in a distribution between the micelles and the external bulk water.

Conclusion

In this work we have demonstrated how it is possible to build a fluorescent sensor for Hg^{2+} in water by self-assembling pyrene and a lipophilic ligand suitable for mercury complexation inside TritonX-100 micelles. The ligand is a tetrathia-monoaza macrocycle bearing an alkyl chain of different length on its tertiary amine. We have shown how it is possible to play on pH and on the length of the chain to obtain either an ON–OFF or an OFF–ON fluorescent sensor. When a long chain (C_{12}) is appended to the tertiary amino group of the ligand both the empty, protonated form of the ligand (nonquenching) and the Hg^{2+} complex of the neutral ligand (quenching) are confined in the micellar space: accordingly, the system behaves as an ON–OFF fluorescent sensor. On the other hand, when a "not too long" chain (C_4 or C_3) is appended to the nitrogen atom of the ligand, the neutral free ligand is confined inside the micelle (quenching), while its Hg^{2+} complex is sufficiently hydrophilic to leave the micelle and be released into the bulk solution (nonquenching), so the system behaves as an OFF–ON sensor. This approach, at least in principle, is of general use and suitable for other M^{2+} cations provided that a neutral ligand selective towards the target cation is available and that this ligand contains a tertiary amino group.

Experimental Section

Materials: TritonX-100 (*tert*-octylphenoxypoly(oxyethylene glycol) with and average of 9–10 oxyethylene units) was purchased from Caledon (average molecular weight = 647). **[16]aneNS4** (1,4,10,13-tetrathia-7-azacyclohexadecane) was synthesised according to a published procedure.[21] 1-Bromopropane, 1-bromobutane, 1-bromooctane and 1-bromododecane were purchased from Sigma-Aldrich and used without further purification. Pyrene (97%), was a Fluka product used without further purification. Water used for all titrations was distilled twice prior to use.

Instrumentation: Mass spectra were recorded with a Finnigan MAT TSQ 700 instrument and NMR spectra with a Bruker AMX 400 spectrometer. Spectrofluorimetric and spectrophotometric measurements were performed with Perkin-Elmer LS 50B and Hewlett-Packard HP8453 instruments, respectively. The pH titrations were performed with a Radiometer TitraLab 90 titration system.

Syntheses

7-Methyl-1,4,10,13-tetrathia-7-azacyclohexadecane (C1-NS4): **[16]aneNS4** (129.1 mg, 0.434 mmol) was dissolved in formic acid (0.476 mL, 11.9 mmol) and 37% formaldehyde (0.477 mL, 4.77 mmol). This solution was refluxed for 16 h and after cooling to room temperature treated with 10 drops of concentrated HCl. Then 2 M NaOH (12 mL) was added to the mixture obtained after evaporation of the acid under reduced pressure. The basic solution was extracted with chloroform (3×20 mL). The organic solvent was dried with Na_2SO_4 and evaporated. The product was obtained as a pale yellow oil. Yield: 122 mg, 91%. ^{1}H NMR ($CDCl_3$): δ = 2.80 (br s, 8H; S-CH_2CH_2-S), 2.74–2.66 (m, 12H; N-CH_2CH_2-S and S-CH_2-CH_2-CH_2-S), 2.32 (s, 3H; CH_3-N), 1.93 ppm (q, 2H; S-CH_2-CH_2-CH_2-S); MS (ESI): *m/z*: 312 $[CH_3NS_4+H]^+$.

7-Butyl-1,4,10,13-tetrathia-7-azacyclohexadecane (C4-NS4): **[16]aneNS4** (300 mg, 1.01 mmol), 1-butyl bromide (0.143 mL, 1.575 mmol), potassium iodide (83 mg, 0.50 mmol) and sodium carbonate (0.566 g, 5.24 mmol) were dissolved in dry acetonitrile (26 mL). The mixture was refluxed for 48 h under nitrogen. After cooling, excess base and KI were filtered off and the solvent evaporated under reduced pressure. The product was purified on a basic alumina column using hexane/ethyl acetate (9:1) as eluent and was obtained as a pale yellow oil. Yield: 239 mg, 67%. ^{1}H NMR ($CDCl_3$): δ = 2.89 (br s, 8H; S-CH_2CH_2-S), 2.76–2.65 (m, 12H; N-CH_2CH_2-S and S-CH_2-CH_2-CH_2-S), 2.49 (t, 2H; N-$CH_2CH_2CH_2CH_3$), 1.93 (q, 2H; S-CH_2-CH_2-CH_2-S), 1.44 (m, 2H; CH_2 of the butyl chain), 1.31 (m, 2H; CH_2 of the butyl chain), 0.92 ppm (t, 3H; N-$(CH_2)_3CH_3$); MS (ESI): *m/z*: 354 $[C_4NS_4+H]^+$.

7-Dodecyl-1,4,10,13-tetrathia-7-azacyclohexadecane (C12-NS4): **[16]aneNS4** (1 g, 3.53 mmol) and dodecyl bromide (1.31 mL, 5.30 mmol) were dissolved in acetonitrile (90 mL) with Na_2CO_3 (1.87 g, 17.6 mmol). The obtained mixture was refluxed for 48 h under nitrogen. After cooling to room temperature the inorganic part was filtered off and the solvent evaporated under reduced pressure. The product was purified on a basic alumina column with hexane/ethyl acetate (9:1) as eluent. Pure **C12-NS4** was recovered as a waxy white solid after evaporation of the organic solvent. Yield: 1.13 g, 72%. ^{1}H NMR ($CDCl_3$): δ = 2.89 (br s, 8H; S-CH_2CH_2-S), 2.76–2.65 (m, 12H; N-CH_2CH_2-S and S-CH_2-CH_2-CH_2-S), 2.49 (t, 2H; N-$CH_2(CH_2)_{10}CH_3$), 1.93 (q, 2H; S-CH_2-CH_2-CH_2-S), 1.44–1.28 (m, 20H; N-$CH_2(CH_2)_{10}CH_3$), 0.92 ppm (t, 3H; N-$CH_2(CH_2)_{10}CH_3$); MS (ESI): *m/z*: 466 $[C_{12}NS_4+H]^+$.

Potentiometric titrations: The protonation equilibria of the monodispersed species were studied in solutions of 0.05 M sodium nitrate in water/dioxane (1:9 v/v) at 25 °C by titrating a solution containing the chosen molecule and excess of the nitric acid mixture with a standard base (NaOH). All ligands were studied at a concentration of 10^{-3} M. The behaviour of **C1-NS4** was also investigated in an aqueous solution containing 0.05 M $NaNO_3$ and the ligand at a concentration of 5×10^{-4} M. The protonation equilibria of the micellised species were studied in a 0.05 M $NaNO_3$ aqueous solution containing 6.47 g L^{-1} of Triton X-100 at 25 °C by addition of a standard base (NaOH) to a 10^{-3} M solution of the chosen ligands containing excess standard nitric acid. The complexation equilibria of solutions with the same ligand concentration were studied with $[Hg(CF_3SO_3)_2]$ added in a 1:1 molar ratio with respect to the ligand. The results were obtained as *E* (potential in mV at the glass electrode) versus volume of added base (in mL) and the protonation constants were calculated using the Hyperquad[29] package (which uses nonlinear least-squares regression methods to fit calculated to experimental data), using $E^\ominus$ values for the hydrogen glass electrode determined by the Gran[30] method prior to titration.

Coupled fluorimetric/pH-metric titrations: Coupled pH-spectrofluorimetric titrations were carried out at 25 °C with 0.05 M $NaNO_3$ aqueous solutions containing 6.47 g L^{-1} of Triton X-100, 9×10^{-7} M pyrene (dissolved by adding aliquots of concentrated pyrene solutions in *tert*-butyl alcohol, with a final *tert*-butyl alcohol concentration of < 0.5% v/v), plus the chosen **C*n*-NS4** ligand at a concentration of 10^{-3} M (to observe the effect of protonation on fluorescence) or the chosen ligand and $[Hg(CF_3SO_3)_2]$ both at concentrations of 10^{-3} M (to observe the effect of complexation on fluorescence). Bulk solutions (25 mL, kept under a constant flow of nitrogen) 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 into the bulk solution. For each base addition the pH was recorded and the emission spectra were

recorded with the spectrofluorimeter (λ_{exc}=343 nm). Total NaOH addition at the end of the titrations did not exceed 1.0 mL.

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[24] For the sake of comparison, we measured the protonation constant of **[16]aneNS$_4$** in water (0.05 M $NaNO_3$, 25 °C), finding $\log K = 8.1$ (± 0.2). Diethylamine, in water, has a protonation constant of $\log K = 10.96$ (0.5 M KNO_3; see G. Carlson, J. McReynolds, F. Verhoek, *J. Am. Chem. Soc.* **1945**, *67*, 1334), a further indication of the hindering effect due to the inclusion of an amine group in this thia macrocycle.
[25] It has to be stressed that solubilisation of **C4-NS4** and **C12-NS4** in water/TritonX-100 is a result of their inclusion in micelles. TritonX-100 was used at a concentration of 10^{-2} M, which, based on the cmc (critical micelle concentration) of this surfactant (2×10^{-4} M[31]) and on its AN (100–140[32]), means an average micelle concentration of $\sim 10^{-4}$ M. As the three ligands were studied in this medium at a concentration of 10^{-3} M, an average of ~10 ligand molecules are included in each micelle, at least for **C4-NS4** and **C12-NS4**, which can reasonably be considered as fully micellised. As regards **C1-NS4**, besides its solubility in pure water, its calculated protonation constant indicates that it is not included in the micellar core (see discussion in the same section). The water/surfactant solution contained 0.05 M $NaNO_3$ as the background electrolyte. On the other hand, the dioxane/water mixture used contains sufficient organic solvent to allow solubilisation of the three ligands and sufficient water to allow the measurement of pH with standard pH meters. Mixtures of water with dioxane as the organic component are frequently used when pH-metric or potentiometric titrations are to be carried out on molecular systems that are insoluble in pure water. The choice of dioxane instead of other organic and water-miscible solvents (e.g., acetonitrile or ethanol) derives from the particular stability of dioxane even at the extremes of the pH interval examined (pH 2–12), which allows data from the potentiometric titrations to be collected that are reliable and not affected by noise or errors even at the beginning and end of a titration experiment.
[26] From Figure 1 it can be seen that the residual fluorescence in the presence of unprotonated **C12-NS4** is not negligible, but ~40% of the full emission found in the presence of the protonated ligand. Significant residual fluorescence (20–40%) for TritonX-100/pyrene/quencher systems in the presence of different lipophilic quenching molecules (refs. [15] and [16]) has already been reported by us. To interpret this behaviour, the oblate ellipsoid shape assumed by TritonX-100 micelles has to be considered as well as the consequent selective compartmentalisation of both the fluorophore and the quencher, which results in significant lowering of the effective collisions between them. In this regard, it also has to be mentioned that the choice of pyrene is not casual: we also tested naphthalene, anthracene and dansyl amide as the fluorophore, obtaining, in the presence of **C12-NS4** or other quencher (i.e., free micellised R-NS4 molecules and micellised $[\mathbf{C12\text{-}NS4}Hg]^{2+}$ complexes) a higher residual fluorescence, that is, less intense signal variation in the sensing process.

[27] a) M. Montalti, A. Credi, L. Prodi, M. T. Gandolfi, *Handbook of Photochemistry*, 3rd ed., CRC Taylor & Francis, Boca Raton, **2006**; b) L. Prodi, *New J. Chem.* **2005**, *29*, 20.
[28] **C3-NS4** and **C8-NS4** were synthesised as for **C4-NS4**, using commercial (Sigma-Aldrich) *n*-propyl and *n*-octyl bromide as starting materials, respectively. The products are pale yellow oils. Mass and ^{1}H NMR spectra are consistent with the formulated structures.
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