Detection of trace Cu^{II} by a designed calix[4]arene based fluorescent reagent

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A highly Cu^{2+} selective calix[4]arene based fluorescent reagent, 5,17-bis(4-methylcoumarin-7-azo)-25,26,27,28-tetrahydroxycalix[4]arene, has been designed, synthesized and evaluated. The reagent exhibits excellent selectivity for Cu^{2+} over a wide range of alkali, alkaline earth and other transition metal ions. Quenching of its fluorescence due to a strong Cu^{2+} affinity, induced binding and selective redox reaction is not influenced by the presence of 20- to 10000-fold excesses of Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, NH_4^+, Ni^{2+}, Pb^{2+}, Zn^{2+}, Cl^-, NO_3^-, CO_3^{2-}, SO_4^{2-} or PO_4^{3-}. Furthermore, with this fluorescent reagent a simple, sensitive and highly selective method has been developed for measuring trace Cu^{2+} in real biological fluids. The combination of multiple selective responses presented here may provide a useful design strategy for preparing selective reagents of other species.

Calixarenes are widely used as molecular scaffolds for the preparation of novel acceptors for cations, anions and neutral molecules.[1–3] One of the most intriguing features of calixarenes is the adjustability of their cavity sizes so that a highly selective calixarene-based reagent with a matchable cavity for a given guest size could be readily synthesized. Indeed, great success has been achieved in this regard, especially for some of alkali, alkaline earth and transition metals.[4–10] Besides the matchable size, however, there are many other factors affecting the selectivity of a reagent, such as affinity, thermodynamics and kinetics, and therefore some situations do not follow the widely accepted "size-fit" model.[11] Moreover, the adjustability of the cavity sizes of calixarenes is still limited for the requirements of other guests. So, more and more of the current acceptor design strategy involves extending the calixarene molecules through chemical modifications on their upper or lower rims, which results in a wide variety of calixarene derivatives as efficient and selective reagents for various guests.[1–3] Obviously, in this case the calixarene skeleton no longer plays the so-called cavity role that was assumed before in accommodating guest molecules. Rather, from another viewpoint it could serve as a shielding or covering unit in improving the properties of a reagent, and such an idea has led us to synthesize a highly Ni^{II} selective chromoionophore.[12] Herein, we describe the design of a highly selective fluorescent reagent for Cu^{II}.

Copper is one of the elements studied most extensively by fluorescent detection and measurements. This is not surprising, since it is an essential trace element in biological systems[13–15] and fluorescence signaling offers the advantage of high sensitivity.[16] It is known that excellent fluorescent reagents constitute the basis of Cu^{II} selective detection. However, the major challenge we confront is how to design and synthesize selective and sensitive reagents for Cu^{II}, because Mn^{II}, Fe^{II}, Co^{II}, Ni^{II} and Zn^{II} often coexist with Cu^{II} and all of them have similar reaction behavior with classical fluorescent reagents such as 5-(3-fluo-4-chlorophenylazo)-8-aminoquinoline,[17] 3-carboxy-7-hydroxy-coumarin[18] and 1,1,3-tricyano-2-amino-1-propene.[19] Toward this end, a number of novel fluorescent reagents have been prepared by several research groups.[15b,20–22] For example, Imperiali *et al.*,[15b] Czarnik *et al.*,[20] Tecilla *et al.*[22a] and Marchelli *et al.*[23] have connected fluorescent moieties to different types of selective ligand subunits; Bhattacharya and Thomas[22b] synthesized a thiazole based dipeptide chemosensor. The binding of Cu^{II} ion causes fluorescence quenching of the probes and allows its detection in the micro- or submicromolar range. In most of these systems the quenching is ascribed to a photoinduced metal-to-fluorophore electron transfer mechanism. To find another possible sensing mechanism, the design strategy presented in this paper takes advantage of a combination of a multiple selection processes: besides the shielding action of the calixarene skeleton, response to the target metal ion exploits the high affinity of Cu^{II} for nitrogen atoms and the redox reactivity of the metal ion.

7-Amino-4-methylcoumarin, which contains a diazotizable $-NH_2$, was selected as the fluorescent moiety because of its high fluorescence quantum yield ($\varphi = 0.54$).[24] This moiety was connected to calix[4]arene through a diazo-coupling reaction. The resulting azo group may be expected to serve as not only a chromogen, but also as a strong binding site for Cu^{II} due to the high affinity of Cu^{II} for nitrogen donors.[25] Reversely, this Cu^{II} high affinity would help to induce and reorganize the related binding site to an optimal binding status or geometry. Moreover, calix[4]arene usually has a cone conformation, which is stabilized by the formation of intramolecular hydrogen bonds among the four hydroxyl groups on the lower rim.[26,27] This beaker-like skeleton of calix[4]arene would prohibit other metal ions from approaching the azo group at least in one direction, and could thus make a contribution to the selectivity. To achieve a high selectivity for Cu^{II}, it is not enough to only make use of the high affinity for nitrogen atoms, since many of other transition metals also have a high affinity for them. In basic media, however, Cu^{II} has the highest half-wave potential (*ca.* −0.4 V *vs.* SCE) and is the strongest oxidant among the above mentioned coexisting ions[28] commonly present in biological fluids; therefore, it might oxidize azo compounds, whose half-wave potentials are about –0.7 V (*vs.* SCE).[25,29] This redox reactivity, analogous to the well known Fehling test, would be an important additional factor

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for increasing selectivity; namely, such a reaction would selectively change the fluorescent properties of the reagent, thus eliminating the interferences from many other transition metal ions. Now we report the results of this work.

Experimental

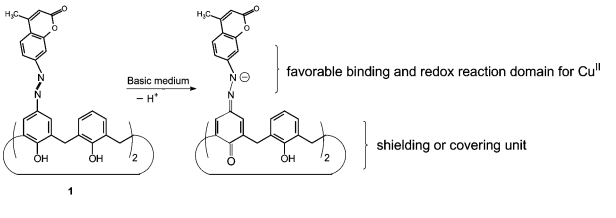
Apparatus and materials

Infrared spectra were taken in KBr disks on a Perkin–Elmer 683 spectrophotometer. ^{1}H nuclear magnetic resonance (NMR) spectra were measured on a Varian Unity 200 spectrometer (200 MHz) in $CDCl_3$ with tetramethylsilane as the internal standard. Negative secondary ion mass (N-SIMS) spectra were recorded with an Apex II (Bruker) Fourier-transform ion cyclotron resonance (FTICR) instrument, and fast atom bombardment (FAB) spectra with a KYKY-ZHP-5 instrument using *m*-nitrobenzyl alcohol (NBA) as a matrix. Elemental analyses were carried out by the analytical laboratory of our institute. Uncorrected melting points were measured on an XT4-100X melting point apparatus (Beijing, China). Fluorescence measurements were performed on a Hitachi F-4500 spectrofluorimeter. Absorption spectra were measured with a Techcomp UV-8500 spectrophotometer (Shanghai, China). A Hitachi 180-70 polarized Zeeman atomic absorption spectrophotometer with an electrothermal atomizer was used for copper measurements and its operating conditions were set as follows: analytical wavelength, 324.8 nm; current of copper hollow cathode lamp, 7.5 mA; slit, 1.3 nm; carrier gas, Ar 200 mL min^{-1} (stop gas flow during atomization); heating procedure of atomizer, dry at 80–120 °C for 50 s, ash at 120–850 °C for 70 s, and atomization at 2700 °C for 7 s. A model 25 pH meter was used for pH measurements.

7-Amino-4-methylcoumarin was purchased from Acros (Belgium). Calix[4]arene was synthesized according to the method available in literature, but the $AlCl_3$ catalyzed de-*tert*-butylation of *p*-*tert*-butylcalix[4]arene was performed at 40 °C for 3 h.[30] A stock solution (0.1 mM) of reagent **1** (Scheme 1) was prepared by dissolving the requisite amount of **1** in 1 mL NaOH (1.0 M) and diluting to 10 mL with distilled-deionized water. Cu^{II} stock solution (1.0 mM) was prepared with cupric sulfate in distilled-deionized water. A working standard solution of Cu^{II} (10 μM) was prepared from this stock solution by serial dilution. A 0.1 M $Na_2B_4O_7$–NaOH buffer solution (pH = 12.0) was employed. All other chemicals were of analytical grade.

Syntheses

5,17-Bis(4-methylcoumarin-7-azo)-25,26,27,28-tetrahydroxycalix[4]arene (1). A solution of 7-amino-4-methylcoumarin (0.175 g, 1 mmol) in 5 mL of 2 M HCl was cooled to 0–5 °C in an ice bath, and to this 2 mL of $NaNO_2$ (0.069 g, 1 mmol) in water was added to produce a diazo salt. Then, the diazo salt solution was added dropwise to a stirred mixture of calix[4]arene (0.106 g, 0.25 mmol) in *N,N*-dimethylformamide (DMF: 10 mL) and pyridine (2 mL) under ice cooling. After stirring for 3 h at pH = 6–8, the red reaction mixture was poured into water. The resulting solution was acidified to pH ~ 1 with HCl, and the dark red precipitate was separated by filtration and washed with water. The pure product **1** was obtained as a reddish brown solid (28% yield) by chromatography ($CHCl_3$–acetone, v/v 5 : 1). **1** is easily soluble in pyridine and DMF, and soluble in NaOH, but not in H_2O and benzene. Mp > 300 °C. IR (KBr): 3400–3100 (br s, OH); 1720 (C=O); 1600, 1450 (C=C, N=N) cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$, 298 K): δ 10.18 (s, 4H, ArOH); 8.02–7.60, 7.20–7.10 (m, 14H, ArH); 6.82–6.76 (m, 2H, ArH); 6.28 (s, 2H, =CHCO); 4.32 (s, 4H, $ArCH_2Ar$); 3.74 (s, 4H, $ArCH_2Ar$); 2.38 (s, 6H, CH_3). FAB-MS: *m*/*z* 796 (M^+). HRMS (*N*-SIMS NBA): *m*/*z* calcd $[C_{48}H_{35}O_8N_4]^-$ 795.246015; found 795.246325. Elem. anal., calcd for $C_{48}H_{36}O_8N_4$: C, 72.35; H, 4.55; N, 7.03%. Found: C, 72.12; H, 4.67; N, 6.20%.



Scheme 1

4-Methyl-7-(3,5-dimethyl-4-hydroxyphenylazo)coumarin (2). Preparation of **2** from 2,6-dimethylphenol was similar to that of **1**. Yield: 56% (from ethanol–water). FAB-MS (*m*/*z*): 308 (M^+). Elem. anal., calcd for $C_{18}H_{16}O_3N_2{\cdot}1.5H_2O$: C, 64.46; H, 5.71; N, 8.35%. Found: C, 64.56; H, 5.75; N, 8.19%.

General procedure for copper detection

To a test solution containing not more than 1.8 μM of Cu^{II}, 2 mL of 0.1 M $Na_2B_4O_7$–NaOH buffer (pH = 12.0) and 0.75 mL of **1** stock solution (0.1 mM) were added, and the final volume adjusted to 5 mL with water. The mixture was heated for 10 min in a boiling water bath. After the solution was cooled to room temperature, the fluorescence intensity was measured with $\lambda_{ex}/\lambda_{em}$ = 362/447 nm. For analysis of trace Cu^{II} in rat brain dialysates, a microdialysis probe (3 mm membrane; 0.22 mm diameter; 18,000 molecular weight cutoff)[31] was inserted into the rat hippocampus. The probe was continuously perfused at 1–1.5 μL min^{-1} with Ringer's solution (145 mM Na^+, 4.0 mM K^+, 1.3 mM Ca^{2+}, pH 7.4). Dialysate was collected every 30 min. Copper concentrations in the dialysates were determined following the same procedure given above. In the meantime, the copper levels in the dialysates were also quantified directly by graphite furnace atomic absorption spectrometry (GFAAS) for comparison purposes. The reported copper levels were not corrected for the probe recovery.

Results and discussion

Based on the above considerations, reagent **1** (Scheme 1) was prepared by diazotizing 7-amino-4-methylcoumarin and then coupling with calix[4]arene. Although some of the diazo coupling reactions on calix[4]arene take place in an autoaccelerative manner due to the specific hydrogen-bonding effect among the calix[4]arene –OH groups,[32] leading to the formation of a tetrasubstituted calix[4]arene as the main product, the present observation is not such a case. Namely, even though an excess of the diazonium salt was used for the coupling, two phenolic units remained unsubstituted, and it was a bis(arylazo) compound instead of the corresponding tetrasubstituted calix[4]arene as main product that was isolated after chromatography. This presumably results from the steric hindrance of the coumarin ring, similar to the literature reports that incompletely *p*-substituted derivatives can be formed.[7,33] The ^{1}H NMR spectral patterns of the methylene hydrogens of the bis(arylazo) compound isolated above appeared as a pair of doublets (4.4–4.2 and 3.75–3.55 ppm) at lower temperature (≤10 °C), but became two broad signals (4.32 and 3.74 ppm) at room temperature (25 °C) and eventually coalesced to a singlet (3.94 ppm) at *ca.* 50 °C. These data suggest that the bis(arylazo) compound is a symmetrical one, the 1,3-bis(arylazo) derivative, and exists in a cone conformation though the

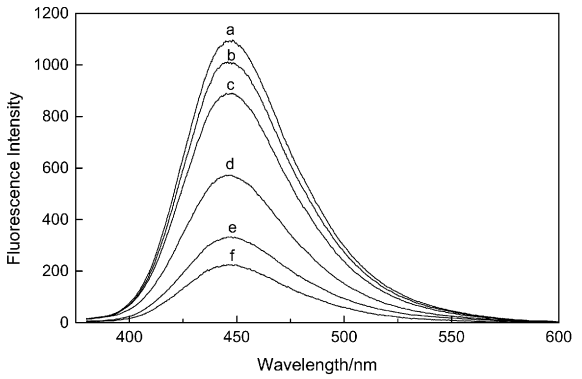


Fig. 1 Fluorescence emission spectra of 10 μM **1** in basic aqueous media (pH 12.0) in the presence of Cu^{II}: (a) 0, (b) 0.15, (c) 0.45, (d) 1.0, (e) 1.5, (f) 1.8 μM. $\lambda_{ex} = 362$ nm, $\lambda_{em} = 447$ nm.

interconversion between mirror image cone conformations is rapid at higher temperature.[1,26,34]

The fluorescence spectrum of reagent **1** in basic aqueous media showed excitation and emission bands at $\lambda_{ex} = 362$ nm and $\lambda_{em} = 447$ nm, respectively. Upon addition of Cu^{II}, the fluorescence intensity of **1** was quenched with no significant change in the positions of the excitation and emission maxima, and the orange color of its solution faded. When the Cu^{II} concentration was increased, the fluorescence intensity diminished accordingly (Fig. 1), accompanying the gradual fading of the reagent solution (Fig. 2). A maximum of ≈80% quenching was observed after 1.8 μM of Cu^{II} was added at pH = 12.

The reversibility of this system was investigated with the chelating agent EDTA. The results showed that, if Cu^{II} was firstly mixed with equimolar EDTA, the subsequent addition of **1** showed no quenching and fading reaction, confirming that the EDTA–Cu^{II} complex is rather stable and that Cu^{II} could not be stripped from the complex by the reagent; in contrast, if Cu^{II} was firstly reacted with **1**, then the addition of EDTA did not restore the fluorescence intensity of **1** and its solution color, demonstrating the irreversible character of this system. Furthermore, the color of **1** mainly originates from the azo group (its reaction would lead to a color change); the quenching and fading phenomenon of the present system therefore support a probable redox reaction of the azo (chromogenic) group. The reaction of **1** with Cu^{II} was also tested in an oxygen-free solution purged with argon but the same quenching and fading result was obtained, indicating that it was not a Cu^{II} catalytic redox reaction between oxygen and **1**. But rather, as expected, **1** is presumably oxidized to an azoxy or arylnitramine compound[35] by Cu^{II} itself, thus leading to the fluorescence quenching of **1** and its solution fading. Moreover,

Table 1 Fluorescence intensity (F) at $\lambda_{em} = 447$ nm (with $\lambda_{ex} = 362$ nm) from different systems[a]

System	F
1. 7-Amino-4-methylcoumarin + calix[4]arene	4239
2. System 1 + Cu^{II}	3546
3. Reagent **1**	1100
4. System 3 + Cu^{II}	831

[a] [Cu^{II}] = 0.5 μM; the concentration of 7-amino-4-methylcoumarin, calix[4]arene and reagent **1** was each 10 μM; pH 12.

in basic media the reagent **1**, being a *p*-hydroxyazo derivative, could lose a proton, and the resulting anion could exist in the form of hydrazone.[35] This hydrazone has a nitrogen atom with a negative charge (see Scheme 1), which facilitates the binding of Cu^{II} and in turn the redox reaction. We tried to separate the formed oxidation product but were unsuccessful due to its complexity or instability in this system.

Under the same conditions, the fluorescence characteristics of systems containing different components were examined (Table 1). System 1 itself had excitation and emission peaks at $\lambda_{ex} = 341$ nm and $\lambda_{em} = 440$ nm, respectively, with a maximum of $F = 4762$. Compared to system 1, reagent **1** (system 3) exhibited longer excitation/emission wavelengths. Although the fluorescence of system 1 was also quenched by Cu^{II}, such quenching had no selectivity, and a number of common ions could produce a similar decrease. For example, 30 μM of Ca^{II} caused a 25% quenching whereas system 3 was not influenced by various common species (*vide infra*). It is understandable that reagent **1** is less fluorescent than 7-amino-4-methylcoumarin, since the diazotization of an amino group in fluorescent molecules usually decreases the fluorescence quantum yield.[36]

To obtain the optimum conditions for the quenching value ΔF of this system ($\Delta F = F_0 - F$, F_0 and F are the fluorescence emission intensities before and after Cu^{II} was added, respectively), three kinds of media, 0.1 M Na_2CO_3–NaOH, 0.1 M $Na_2B_4O_7$–NaOH and 0.1 M Na_2HPO_4–NaOH buffers, were evaluated. The maximum ΔF was achieved in the pH range 11.7–12.3 when a $Na_2B_4O_7$–NaOH buffer was used. Therefore, pH 12.0 may be used and maintained with $Na_2B_4O_7$–NaOH buffer, whose optimal amount was 2 mL for 5 mL of test solution. The effect of the concentration of **1** was also checked. The results indicated that ΔF reached a maximum and constant value with the final concentration ranging from 10 to 30 μM. In this work, 15 μM of **1** was chosen for the following experiments.

The effects of different temperatures and heating times on the reaction of **1** with Cu^{II} are shown in Fig. 3. It can be seen that the reaction at room temperature was very slow. So, a boiling water bath was employed for heating, and the suitable

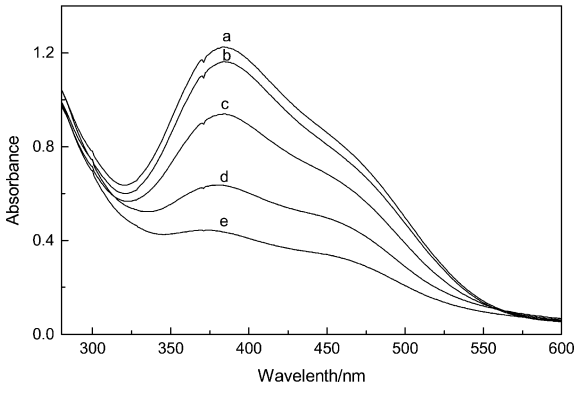


Fig. 2 Absorption spectra of 30 μM **1** in basic aqueous media (pH 12.0) in the presence of Cu^{II}: (a) 0, (b) 2.5, (c) 7.5, (d) 15, (e) 25 μM against water blank.

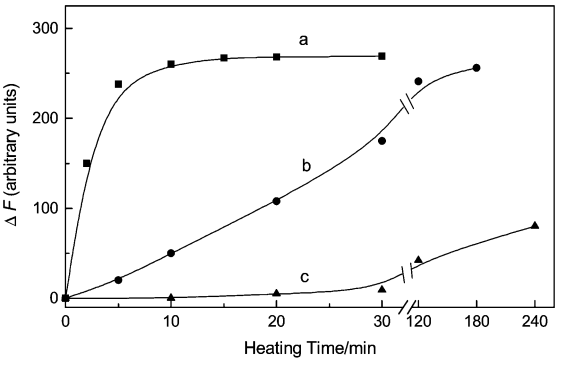


Fig. 3 Effects of heating temperature and time on ΔF. Heating temperature: (a) 100; (b) 50; (c) 25 °C. Conditions: 10 μM of **1**, 0.5 μM of Cu^{II}, pH = 12.0.

Table 2 Effect of various cations on fluorescence intensity of **1** (10 μM)

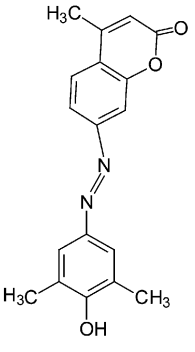
Species	Conc/μM	F^a
None	–	1100
Ag^{I} [b]	5	988
Ca^{II}	100	1070
Co^{II}	50	1044
Cu^{II}	0.5	820
Cu^{II}	1.0	575
Fe^{III} [b]	2	1067
K^{I}	5000	1025
Mg^{II}	500	1050
Ni^{II}	50	1120
Zn^{II}	50	1075

[a] The fluorescence intensity was measured at λ_{ex} and λ_{em} of 362 and 447 nm, respectively. [b] Higher concentration of these ions led to precipitation of metal hydroxides at pH 12.

heating time was found to be 10 min. Under the optimum experimental conditions, ΔF can remain stable for at least 30 h. Furthermore, ΔF is directly proportional to the Cu^{II} concentration in the range of 0.013–1.8 μM. The linear regression equation was determined to be: $\Delta F = 491 \times C$ (μM Cu^{II}) + 5.7, $n = 9$, $r = 0.998$. The detection limit was 6.0 nM ($S/N = 3$). Reproducibility tests ($n = 10$) showed that the relative standard deviation of ΔF was 2.9% for 0.5 μM of Cu^{II}.

To test the selectivity of the reagent, the effects of various species upon the fluorescence intensity of **1** were studied (see Table 2). The fluorescence intensities were not quenched by addition of alkaline or alkaline earth metals at high concentrations. Addition of some other transition metals such as Fe^{III}, Co^{II}, Ni^{II} and Zn^{II}, which are coexistent ions of Cu^{II}, did not influence the fluorescence intensity of **1** either. Although Ag^{I} decreased the fluorescence intensity slightly (this may arise from its high half-wave potential and also possible redox reaction with **1**), its concentration in physiological and environmental samples is much lower than that of Cu^{II}. To further verify the influence of other common species including albumin (the main protein of some body fluids) on the Cu^{II} induced fluorescence quenching, a competitive reaction was used on the determination of Cu^{II} (0.5 μM). The tolerable amounts of these species causing a relative error of no more than ±5% in ΔF were as follows (molar ratio): 10000-fold of K^{I}, Cl^-, NO_3^-, CO_3^{2-}, SO_4^{2-} and PO_4^{3-}; 1000-fold of Mg^{II} and NH_4^{I}; 200-fold of Al^{III} and Ca^{II}; 100-fold of Co^{II}, Ni^{II} and Zn^{II}; 50-fold of Hg^{II}, Mn^{II} and Pb^{II}; 20-fold of Cd^{II} and Cr^{III}; at least 30 μg ml^{-1} of albumin. It may be concluded that **1** exhibits excellent selectivity for Cu^{II} over a wide range of transition, alkali and alkaline earth metal ions, though some other oxidants may cause interferences.

On the other hand, a model compound **2** (Scheme 2) which is a monomer-type of **1**, had excitation and emission maxima



Scheme 2 The structure of model compound **2**.

Table 3 Determination of Cu^{II} in biological samples[a]

Sample	Proposed method Cu^{II} conc/ng mL^{-1}	GFAAS method Cu^{II} conc/ng mL^{-1}
Rat brain microdialysate	60 ± 8	58 ± 7
Human cerebrospinal fluid[b]	78 ± 9	80 ± 9

[a] Analytical results are expressed as the mean of three determinations ± standard deviation. [b] The sample was provided by Xinjing Shihezi Hospital (Xinjiang, China).

at 356 and 443 nm, respectively, and a similar quenching and fading reaction to that of **1** was observed upon addition of Cu^{II}. However, the selectivity of **2** was not as good as **1**, because other transition metal ions such as only 10-fold of Co^{II} and 5-fold of Ni^{II} could also cause a noticeable fluorescence quenching (> 5%). This more selective feature of **1** than **2** for Cu^{II} may be largely attributed to the rational design discussed above, also showing that the beaker-like skeleton of calix[4]arene might play a shielding role in the reagent's recognition behavior. Namely, the high affinity of Cu^{II} for nitrogen donors allows Cu^{II} to occupy the azo groups, and the calix[4]-arene skeleton might prohibit other guests from approaching these reaction sites from some directions, thus contributing to the selectivity.

Following the above general procedure, the proposed method was used to analyze directly trace amounts of Cu^{II} in biological fluids, and owing to the high sensitivity and small volume (at most 0.2 ml) of sample required, no coagulation and precipitation from proteins and other organic components was observed. The obtained results agree very well with the GFAAS method (Table 3).

In conclusion, it has been demonstrated that the fluorescent reagent **1** can be used to detect trace Cu^{II} in real biological fluids, such as cerebrospinal fluid and microdialysates, though the heating required for reaction is its primary shortcoming. The extremely high selectivity of **1** for Cu^{II} results from a multiple selective response to Cu^{II} (besides the shielding action of the calixarene skeleton, response to the target ion depends on the high affinity of Cu^{II} for nitrogen atoms and the redox reactivity of the metal ion) when reacting. Such a design strategy would be useful for preparing reagents for other species.

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