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ORIGINAL PAPER

Rhod-5N as a Fluorescent Molecular Sensor of Cadmium(II) Ion

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Abstract The photophysical and complexing properties of Rhod-5N (commercially available) in MOPS buffer are reported. This fluorescent molecular sensor consists of a BAPTA chelating moiety bound to a rhodamine fluorophore. Its fluorescence quantum yield is low and a drastic enhancement of fluorescence intensity upon cation binding was observed. Special attention was paid to the complexation with Cd^{2+}, a well known toxic metal ion. Possible interference with other metal ions (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Pb^{2+}) was examined. Rhod-5N was found to be highly selective of Cd^{2+} over those interfering cations except Pb^{2+}. The limit of detection is 3.1 μg l^{-1}.

Keywords Fluorescence · Cadmium · Sensing · Fluorescent molecular sensors

Introduction

Cadmium is a heavy metal widely used in industrial processes as electroplating of metals, coloring agents, rechargeable Cd-Ni batteries. Cadmium can then be found in air, in water or in plants as a result of anthropogenic pollution mainly from smelting and refining of nonferrous metals, fossil fuel combustion and municipal waste incineration Unfortunately, cadmium is highly toxic for human being and living things as it accumulates in cells, liver and kidneys with consequent physiological disorders or carcinogenic effects [1]. For this reason, the level of cadmium in drinking water should not exceed 3 μg l^{-1} according to the World Health Organization [2]. The development of fast and reliable methods for sensing traces of cadmium in environment or in biological media is thus of major interest. Along this line, fluorescence techniques offer distinct advantages in terms of sensitivity and selectivity over some other techniques such as UV/vis spectrophotometry, atomic absorption or emission spectroscopies [3–8].

Many papers have reported cadmium sensing studies using fluorescence techniques [9–22], but in most cases, the selectivity is not satisfactory. It should be noted that very few cadmium fluorescent sensors are commercially available and they are not specially designed for cadmium sensing. For example, Phen Green FL, Phen Green 5K, BTC-5N sold by Invitrogen [23] as cadmium indicators are not specific of this metal ion and can also be used to detect other metal ions such as Hg^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} or Zn^{2+} ([23] p. 921 and references therein). Interestingly, we noticed that Rhod-5N based on a 5N-BAPTA ionophore (BAPTA=1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) linked to a rhodamine fluorophore exhibits a stronger affinity for Cd^{2+} than for Ca^{2+} ions ([23] p 915). It has a lower binding affinity for Ca^{2+} than any other BAPTA-based indicator and is suitable for Ca^{2+} measurements from 10 μM to 1 mM. This fact prompted us to study the complexing properties of this ligand with special attention to its

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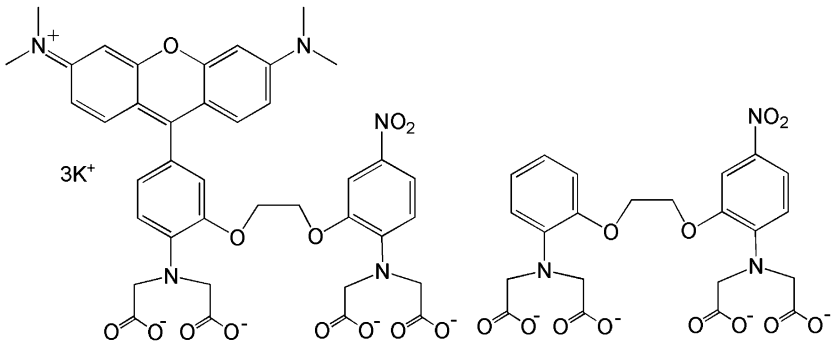
selectivity towards Cd^{2+} over the following interfering ions: Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Pb^{2+}.



Rhod-5N 5N-BAPTA

Experimental

Chemicals

Rhod-5N was purchased from Invitrogen and used without any further purification. Stock solutions of the ligand were freshly prepared. Millipore water (conductivity $< 6\times10^{-8}\ \Omega^{-1}\ cm^{-1}$ at 20 °C) was used for the preparation of the buffered solutions containing 10 mM MOPS (3-(N-morpholino)propanesulfonic acid**).** The pH was adjusted at pH=7.0 by adding KOH (99,99%). Sodium thiocyanate, potassium thiocyanate, calcium perchlorate, zinc perchlorate, lead(II) acetate, magnesium perchlorate and cadmium perchlorate from Aldrich or Alfa Aesar were of the highest quality available. Stock solutions of these salts were prepared at concentrations ranging from 10^{-3} to 0.1 mol l^{-1} in MOPS buffer, except for lead(II) which was prepared in millipore water in order to preclude the formation of insoluble lead(II) hydroxide.

Spectroscopic measurements

Glassware was washed with concentrated nitric acid (70%), then rinsed with millipore water to minimize sorption of cations and their release in the solutions under study. UV/vis absorption spectra were recorded on a UVICON spectrophotometer. Corrected emission spectra were obtained on a Jobin-Yvon Spex Fluoromax spectrofluorometer. The fluorescence quantum yields were determined by using Rhodamine B in water as a standard (Φ_F=0.41) [24]. The complexation constants were determined by global analysis of the evolution of all emission spectra with the Specfit Global Analysis System V3.0 for 32-bit Windows system. This software uses singular value decomposition and nonlinear regression modelling by the Levenberg-Marquardt method [25].

Fluorescence intensity decays were obtained by the single-photon timing method with picosecond laser excitation using a Spectra-Physics set-up composed of a Titanium Sapphire Tsunami laser pumped by an argon ion laser, a pulse detector, and doubling (LBO) and tripling (BBO) crystals. Light pulses were selected by optoaccoustic crystals at a repetition rate of 4 MHz. Fluorescence photons were detected at 576 nm by means of a Hamamatsu MCP R3809U photomultiplier, connected to a constant-fraction discriminator. The time-to-amplitude converter was purchased from Tennelec. Data were analysed by a nonlinear least-squares method using Globals software (Globals Unlimited, University of Illinois at Urbana-Champaign).

Results and discussion

Absorption and steady-state fluorescence measurements

Rhod-5N in aqueous buffer solutions

The absorption spectra of Rhod-5N in 10^{-2} mol l^{-1} MOPS exhibit several bands between 300 to 640 nm as a result of the combination of the absorption spectra of 5N-BAPTA and rhodamine moieties. The emission spectrum (λ_{exc}= 551 nm) of Rhod-5N consists of a single band centered at 576 nm which is typical of rhodamine fluorescence.

The fluorescence intensity of the stock solution is very low, which is generally interpreted as a consequence of the photoinduced electron transfer (PET) occurring from one of the aromatic amine groups of the BAPTA moiety to the rhodamine fluorophore. A drastic enhancement of fluorescence upon cation binding is then due to the reduction of the PET process by the bound cation. Another explanation is the formation of a non-emissive TICT-like (twisted

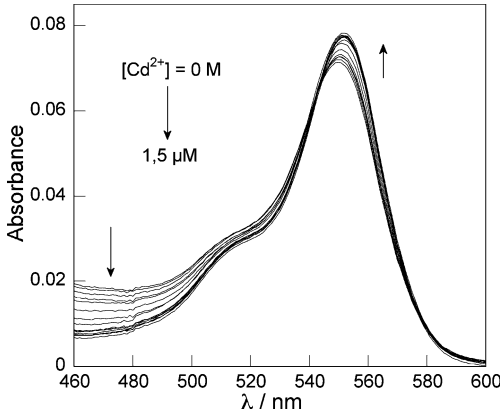


Fig. 1 Evolution of the absorption spectrum of Rhod-5N (1.9×10^{-6} mol l^{-1}) in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0, upon addition of Cd^{2+}

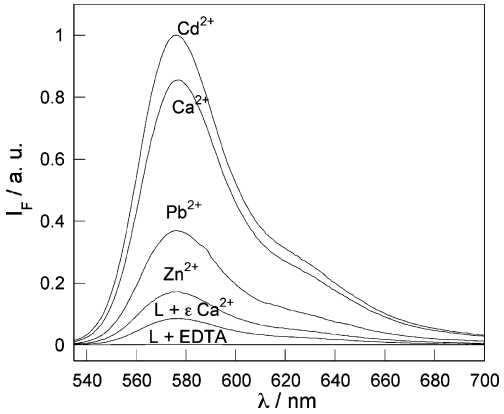


Fig. 3 Corrected emission spectra of Rhod-5N and its complexes with various metal ions in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0. The spectra are normalized according to the relative fluorescence quantum yields

internal charge transfer), as suggested by the experiments on a donor-modified rhodamin, i.e. possessing a dimethylamino group in para position of the phenyl group attached to the heterocyclic part of the molecule [26]. The product state was evidenced by subpicosecond transient and gain spectroscopy. Protonation drastically enhances the fluorescence intensity because the TICT-like state can no longer be formed. Cation binding is expected to cause a similar effect.

We noticed that the fluorescence intensity of the stock solutions can vary from a sample to another because of the presence of residual calcium released from the glassware. This observation was previously reported with Rhod-5N and other BAPTA fluorescent derivatives [27–30]. The concentration of residual calcium can be reduced to less than 10^{-6} mol l^{-1} by washing the cells with concentrated nitric or hydrochloric acid prior to use.

For the determination of the fluorescence quantum yield of the free ligand, we must get rid of residual calcium. Therefore, we added 50 equivalents of dipotassium salt of EDTA ($EDTAK_2$) to an aqueous solution of Rhod-5N (10^{-6} mol l^{-1}) in order to displace the complexation equilibrium of residual calcium with Rhod5N which leads to the free ligand. But the question then arose as to whether $EDTAK_2$ can quench the fluorescence of the rhodamine moiety at this high concentration. To check this point, we added the same amount of $EDTAK_2$ to a rhodamine B aqueous solution, but no quenching was observed. In the presence of $EDTAK_2$, the fluorescence quantum yield of free Rhod-5N was found to be 0.002.

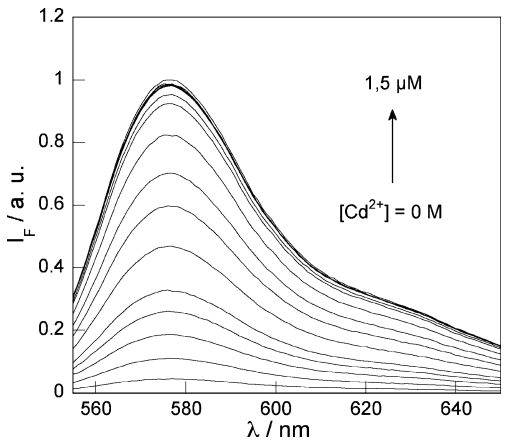


Fig. 2 Evolution of the corrected fluorescence spectrum of Rhod5N (8.6×10^{-7} mol l^{-1}) in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0, upon addition of Cd^{2+} (λ_{exc}=551 nm)

Rhod-5N in the presence of metal ions

Upon addition of metal ions (Fig. 1), the absorption band of the rhodamine moiety is slightly shifted from 549 to 553 nm, and the absorbance is slightly increased. The broad band at 450 nm decreased with increasing metal ion concentration, leading to a broad band centered at 350 nm, as a result of the chelation of metal ion with the 5N-BAPTA moiety. A similar behaviour was reported for the chelation of 5N-BAPTA with calcium ion [31].

The fluorescence response of Rhod-5N in the presence of Cd^{2+}, Ca^{2+}, Zn^{2+}, Pb^{2+}, Mg^{2+}, Na^{+} and K^{+} was then

Table 1 Stability constants of Rhod-5N complexes in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0 determined by the analysis of the evolution of the fluorescence spectra upon addition of metal salts

Cation	log K_{app}	Selectivity K (Cd^{2+})/ K (M^{2+})	ϕ_F^a
Rhod-5N	–	–	0.002
Ca^{2+}	4.20±0.02 3.50[b]/3.85[c]/ 3.20[d]	4.5×10^4	0.30
Zn^{2+}	5.55±0.01	2.0×10^3	0.06
Pb^{2+}	7.95±0.06	8	0.13
Cd^{2+}	8.85±0.05	–	0.35

[a] Error, 5–10%
[b] MOPS 10^{-2} mol l^{-1} buffer at pH=7.2, +0.1 mol l^{-1} KCl, 22 °C [23]
[c] pH=8.05, titrisol [29]
[d] MOPS 10^{-2} mol l^{-1} buffer at pH=6.5, +0.1 mol l^{-1} KCl, 22 °C [27]

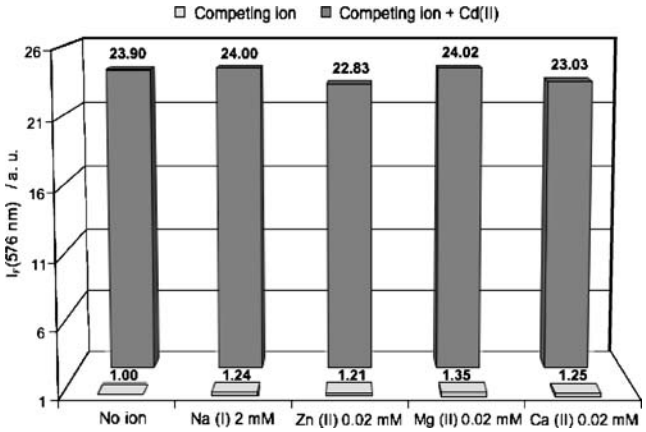


Fig. 4 Fluorescence intensity at 576 nm of Rhod-5N (0.5×10^{-6} mol l^{-1}) in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0, in the presence of Cd^{2+} (0.5×10^{-6} mol l^{-1}) and various interfering ions

investigated. Significant effects on fluorescence were only observed upon addition of Cd^{2+}, Ca^{2+}, Zn^{2+} and Pb^{2+}. It is worth mentioning that potassium ions arising from MOPS buffer have no effect. Cation binding induces an increase in fluorescence intensity (Fig. 2) but no change in the shape of the emission spectra was observed whatever the nature of the cation (Fig. 3).

Analysis of the whole emission spectra at various cation concentrations by means of Specfit software showed that 1:1 complexes are formed in all cases. The fluorescence quantum yields of the complexes (Table 1) are much larger than that of the ligand. The enhancement factors with respect to the free ligand were found to be 150 and 170 for the calcium and cadmium complexes, respectively, and only 30 and 65 for zinc and lead complexes, respectively. The increases in fluorescence quantum yield follow the charge density of the cation whose values are 1.7, 2.02 and 2.06 q $Å^{-1}$ for Pb^{2+}, Ca^{2+} and Cd^{2+}, respectively. Surprisingly, Zn^{2+} induces the smallest increase in fluorescence intensity in spite of its highest charge density.

Table 2 Results of the time-resolved fluorescence measurements on Rhod-5N and its 1:1 complexes in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0

Species	τ_i/ns	α_i^a	f_i^{bb}	χ_R^{2c}
Rhod-5N	1.8±0.1	0.16	0.75	1.27
	0.69±0.32	0.08	0.15	
	$0.05\pm0.01_5$	0.76	0.10	
$Ca^{2+}\subset$ Rhod-5N	1.73±0.03	–	–	1.37
$Cd^{2+}\subset$ Rhod-5N	1.72±0.03	–	–	1.16

[a] Normalized pre-exponential factors
[b] Normalized fractional intensities
[c] Reduced chi-square values

The Specfit software provides the apparent stability constants of the complexes from which the selectivity of Rhod-5N for Cd^{2+} against a given ion, expressed as the ratio of the stability constants, can be calculated (Table 1). The apparent stability constant of the calcium complex (log K_{app}=4.2) was found to be a little bit larger than those previously reported by other authors (log K_{app}=3.20 and 3.50) [27, 29], which can be explained by the different experimental conditions. In fact, these authors added 0.1 mol l^{-1} of KCl to the buffer. At this concentration, the potassium ion may be competitive with the calcium ion and this results in a decrease in the apparent stability of the calcium complex. At pH=8, they found a value of 3.85, but they considered that, depending on the pH, Rhod-5N may exist under two or more species referred as LH_2, LH and L. The fact that we did not take into account the CaLH species may explain the difference with our value. The apparent stability constants of the cadmium complex is very high as compared to those of the calcium and zinc complexes. With the exception of Pb^{2+}, Rhod-5N has a very high selectivity for cadmium over the tested interfering ions.

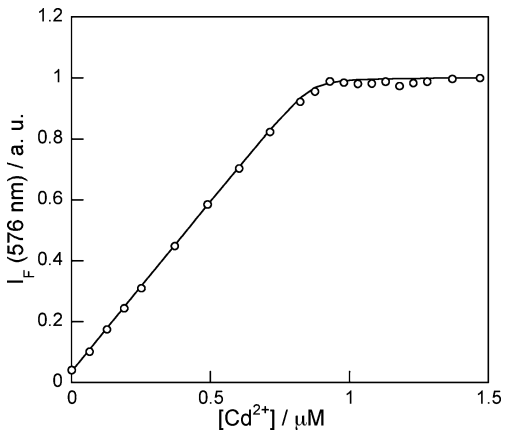


Fig. 5 Calibration curve: variations in fluorescence intensity of Rhod-5N (8.6×10^{-7} mol l^{-1}) as a function of cadmium concentration in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0 (λ_{exc}=551 nm; λ_{em}=576 nm)

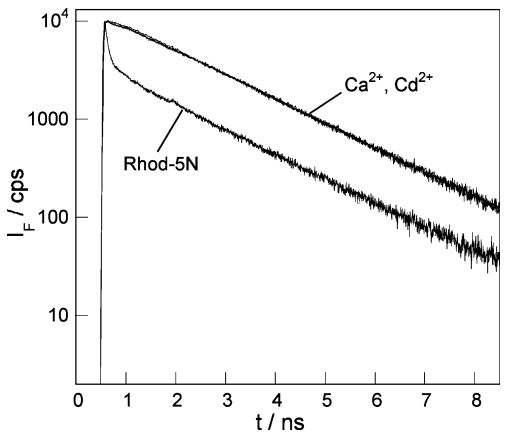


Fig. 6 Normalized fluorescence decays of Rhod-5N and its complexes in MOPS 10^{-2} mol l^{-1} buffer at pH=7.0 (λ_{exc}=495 nm; λ_{em}=576 nm)

We carried out competition experiments between cadmium ion and all the tested interfering ions by adding increasing amounts of metal ion to a solution containing Rhod-5N and Cd^{2+} at 0.5×10^{-6} mol l^{-1}. The results are shown in Fig. 4.

For practical applications, a calibration curve for Cd^{2+} determination is useful (Fig. 5). Under our experimental conditions, the linear part of this curve ranges from 0 to 0.80 μmol l^{-1}. The detection and quantification limits, estimated as three and five times the blank, respectively were found to be 28 and 47 nmol l^{-1}, respectively, i.e. 2.1 and 3.1 μg l^{-1}. The blank was defined as the signal of the ligand in MOPS buffer in the presence of residual calcium.

Time resolved fluorescence measurements

Time resolved fluorescence experiments were first carried out with the ligand in MOPS buffer at pH=7.0. The excitation wavelength was set at 495 nm and the fluorescence was collected at 576 nm. A sum of three exponentials was necessary to get a reasonable χ_R^2 value (Table 2). The shortest time constant (50±15 ps) has the greatest pre-exponential factor (0.78 at pH 7). The other time constants are 0.7±0.3 ns and 1.8±0.1 ns. The latter can be assigned to the lifetime of the complex with the residual calcium. In fact, in the presence of an excess of calcium, the fluorescence decay is a single exponential with a time constant of 1.73±0.03 ns which is the lifetime of the calcium complex. Regarding the time constants of 50 ps and 0.7 ns, it is worth noting that free BAPTA-based Calcium Green-1 exhibits a bi-exponential decay whose time constants were assigned to two conformers which are in equilibrium in the ground state [32, 33]. The presence of conformers of Rhod-5N may also account for the two shortest time constants of the fluorescence decay. But it should be also noted that in the case of a donor-modified rhodamine capable to form a TICT-like state (see above) [26], the decay in decanol or dioxane is a sum of three exponentials. An even larger number of exponentials is necessary to get a satisfactory fit for the decay of calcium ruby [34], a calcium sensor containing a BAPTA-like chelator linked to a rhodamine fluorophore (P. Plaza and MM Martin, personal communication).

The fluorescence decays of the cadmium and calcium complexes are a single exponential with identical lifetimes (Table 2 and Fig. 6).

Conclusion

Rhod-5N shows a much stronger affinity for Cd^{2+} than for Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, but Pb^{2+} appears to be an interfering ion. Therefore, in the absence of Pb^{2+}, Rhod-5N is an outstanding fluorescent molecular sensor for cadmium ion and it is well suited for the detection of this ion in polluted environments (detection limit: 3.1 μg l^{-1}) and for the investigation of its toxic effects in biological media.

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