Coumarin-based ratiometric fluorescent indicators with high specificity for lead ions†

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Total syntheses and spectral properties of fluorescent Pb^{2+} indicators are reported.

Extensive research has shown lead to be a potent neurotoxin, primarily affecting the nervous system during its development, as well as a probable human carcinogen.[1] Moreover, the accumulation of this heavy metal in the environment enhances the chances of lead poisoning. The severe effects that lead ions can cause to environmental and biological systems created a need for the development of selective techniques for its detection.

Fluorimetry stands out as the method of choice of estimation of biological ion concentration.[2] A widespread approach to the matter includes the synthesis of chemical species containing an ionophore group bearing heteroatoms engulfed in a carbon framework. The ionophore group has structural features that assure coordination selectivity for the target ion in the presence of other biological ions. This moiety is connected to a fluorophore group, the latter been designed under the dictation of a host of requirements posed by the practices of fluorescence studies in biological systems.

In recently published works,[3] flavonol- and squaraine-based chemosensors as well as hydroxyflavone derivatives were proposed to bind lead ions. However, their interaction with other heavy metal ions was emphasized in the respective reports. In a study considering cyclooctapeptides as the template in the design of heavy metal indicators,[4] results showed that the binding constants of the systems with Hg^{2+}, Pb^{2+} and Cd^{2+} ions are similar rendering the compounds as non-selective indicators. Calix[4]arene derivatives have also been used in the synthesis of fluorescent lead chemosensors,[5] one of them being highly selective for Pb^{2+} ions.[5b] The properties of these sensors, however, have only been studied in organic solutions.

Selective indicators found to be working in aqueous environments have been reported in the literature,[6] however, limitations related either to the relatively acidic pH required,[6a,b] or to the lack of shift in their spectral maxima upon lead binding (non-ratiometric dyes)[6c,d] narrow the scope of their applications. Moreover, to date only Leadfluor1 (LF1)[6d] seems to be functioning as an indicator capable of detecting Pb^{2+} within living cells.[7]

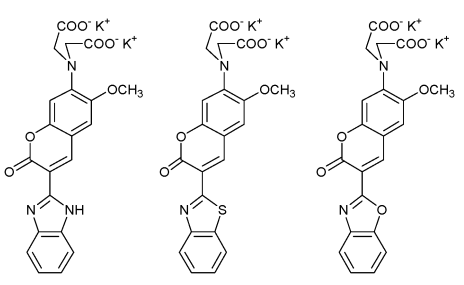
The implication of zinc ions in a host of biological processes[8] and its significant role in the development of numerous neurodegenerative disorders[9] prompted a large number of investigators to study the mechanisms of its involvement in these processes at the cellular level. As a result a host of ion indicators have been synthesized and studied as potential zinc probes, and the respective results have been the subject of recent reviews.[10]

Gee *et al*. published the synthesis of a series of dicarboxylate zinc sensors,[11] namely FuraZin, IndoZin, FluoZin-1, X-RhodZin and FluoZin-2 exhibiting a micromolar affinity for zinc. In common with the corresponding calcium probes,[12] none of the aforementioned compounds may be used as a visible-excited ratiometric probe, a set of highly desirable properties.

In this report we describe the synthesis and fluorescence spectral profile studies of three new ratiometric fluorescent ion probes. Structure-wise all three probes share the *N*-(2-methoxyphenyl)iminodiacetic acid (2-[(carboxymethyl)(2-methoxyphenyl)amino]acetic acid) moiety, as ionophore. According to recent data from our laboratory,[13] these systems were expected to function as visible-excited ratiometric probes and interact with high affinity for zinc ions. However, these dyes proved to be selective for lead ions. Dyes **1**, **2**, and **3** (Scheme 1) were initially studied as putative Zn^{2+} probes. The fluorophores chosen are coumarins, substituted at the 2-position with aromatic heterocycles with the purpose of fine tuning the excitation and emission maxima in their fluorescence spectra and shifting these maxima to the visible region.[14]

All probes were constructed from a common salicylaldehyde intermediate[15] *via* Knoevenagel condensation procedures (see ESI†). Reaction of this intermediate with methyl 2-benzimidazolyl-, 2-benzothiazolyl-, and 2-benzoxazolyl acetate, respectively, gave the dimethyl esters of benzimidazolyl-coumarin (BIC), benzothiazolyl-coumarin (BTC), and benzoxazolyl-coumarin (BXC)-type probes **1**, **2** and **3** which, upon treatment with *t*-BuOK in DMSO, yielded the dipotassium salts of the probes.

The spectral properties of the dyes are presented in Table 1. Dye **1** exhibits an excitation maximum at 469 nm, which undergoes a hypsochromic shift to 401 nm upon zinc ion binding. Dyes **2** and **3** exhibit a very similar profile.



Scheme 1 Chemical structures of indicators **1–3**.

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† Electronic supplementary information (ESI) available: Experimental section, ^{1}H, ^{13}C NMR, ESI-mass and all fluorescence spectra not shown in the text. See DOI: 10.1039/b811569a

Table 1 Spectral properties of fluorescent indicators **1–3** in Zn^{2+} solutions

	λ_{exc}/nm		λ_{emis}/nm	
Indicator	Free	Zn^{2+}-bound	Free and Zn^{2+}-bound	K_d/μM
1	469	401	518	368
2	470	401	526	2404
3	469	400	515	384

Excitation intensity is zinc concentration-dependent for all dyes with an isosbestic point appearing at 427 nm (see ESI†). The emission maxima at 518 nm, 526 nm and 515 nm, respectively, do not undergo any shift in the presence of increasing zinc concentrations. Dissociation constants, calculated according to Tsien's algorithm,[12] range from 368 μM for **1** and 384 μM for **3** to 2404 μM for **2**.

The shifts observed in the excitation maxima of the dyes are expected, since the nitrogen of the iminodiacetate moiety participates in the ion coordination. The dyes, therefore, belong to the general class of photo-induced charge transfer (PCT) probes.[16] In PCT sensors, the fluorophore contains an electron-donating group (such as the 7-amino coumarin substituent) conjugated to an electron-withdrawing group (in this case the coumarin carbonyl), which upon excitation, undergoes internal charge transfer (ICT) from the donor to the acceptor.[17] Coordination of the target ion with the electron-donor moiety destabilizes the system, resulting in a hypsochromic shift in its excitation spectrum.

In an attempt to demonstrate the zinc specificity of the aforementioned coumarin dyes, we performed an ion competition study of a range of metals binding to dye **1** (Fig. 1). To our surprise, lead binding to the sensor appeared to be far stronger than that of zinc. Due to this fact, the dyes were named BICPb, BTCPb and BXCPb, respectively, the prefixes chosen from the well known chomophores in the corresponding calcium indicators.[18]

Spectral studies in solutions of increasing Pb^{2+} concentrations showed that all dyes exhibited similar fluorescent profiles. Their excitation maxima undergo a 70 nm hypsochromic shift upon Pb^{2+} binding, showing distinct isosbestic points. The probes exhibited the same fluorescence emission maxima in their free and lead-bound form, at approximately 520 nm. Excitation and emission spectra of BICPb (compound **1**) are shown in Fig. 2. The fluorescence quantum yields were determined[19] with the highest one (Φ = 0.10) exhibited by BTCPb, a value comparable to that of the calcium indicator BTC (Φ = 0.12).[18b]

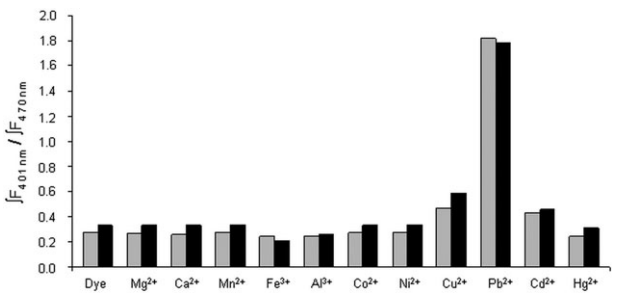

Fig. 1 Ion competition study for dye **1**. First set of measurements: slight increase of fluorescence ratio ($\int F_{401}/\int F_{470}$) in the presence of 50 μM zinc (black bar), as compared to that of the free dye (grey bar). The rest of the measurement sets indicate a fluorescence ratio of the dye in the presence of 50 μM of metal ions (grey bars) *vs.* ratio after addition of 1 equiv. of zinc to the sample (black bars).

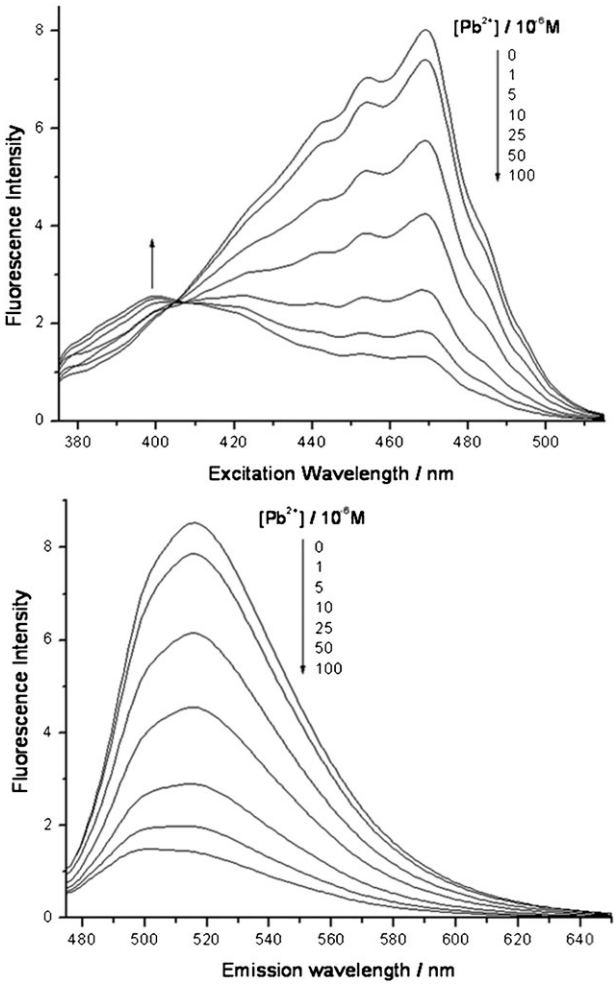

Fig. 2 Excitation and emission spectra of 1 μM BICPb in solutions of increasing Pb^{2+} concentrations in nanopure water. The emission and excitation wavelengths were set at 520 and 470 nm, respectively.

The potential use of compounds **1**, **2** and **3** as selective lead indicators was shown in ion competition studies with a range of metal ions. The study for BICPb is shown in Fig. 3.

Samples were excited at $\lambda_{exc,\ free}$ (470 nm) and $\lambda_{exc,\ bound}$ (401 nm) and the fluorescence ratio was calculated, integrating the emission between 475 and 650 nm. Given that addition of Pb^{2+} increases F_{401} while decreasing F_{470}, an increase in the fluorescence ratio is expected. The presence of other metals (grey bars) appears to have no major effect in lead binding to the sensor, since it does not increase drastically the fluorescence ratio $\int F_{401}/\int F_{470}$ of the free dye (first grey bar), while subsequent addition of Pb^{2+} to these solutions increases the fluorescence ratio of the dye–metal solution to the same extent as in the case of the first measurement (first black bar). It should be noted that iron, aluminium and copper ions, quench the fluorescence of the free dye. Fe^{2+}, K^+, and Na^+ ions in 100 μM concentrations exhibit a similar effect. Respective studies performed with the other two dyes, yielded similar results (see ESI†).

As shown at Table 2, dissociation constant values range from 7.5 μM for BICPb and 8.4 μM for BTCPb to 18.3 μM for

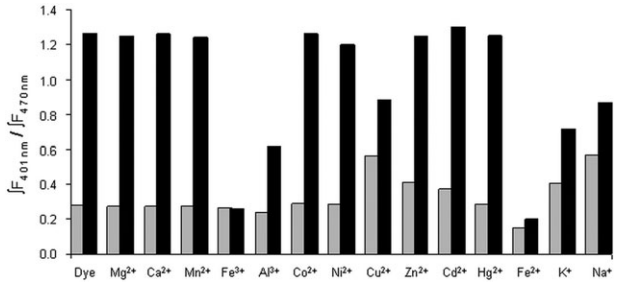

Fig. 3 Ion competition study for dye **1**. First set of measurements: increase of fluorescence ratio ($\int F_{401}/\int F_{470}$) in the presence of 50 μM Pb^{2+} (black bar), as compared to that of the free dye (grey bar). The rest of the measurement sets indicate a fluorescence ratio of the dye in the presence of 50 μM of metal ions (grey bars) *vs.* ratio after addition of 1 equiv. of Pb^{2+} to the sample (black bars). 100 μM solutions were used in the case of Fe^{2+}, K^{+}, and Na^{+}.

Table 2 Spectral properties of fluorescent Pb^{2+} indicators **1–3**

	Free			Pb^{2+}-bound			
Indicator	λ_{exc}/nm	λ_{emis}/nm	Φ	λ_{exc}/nm	λ_{emis}/nm	Φ	K_d/μM
BICPb (**1**)	469	516	0.09	400	516	0.02	7.5
BTCPb (**2**)	470	517	0.10	388	517	0.03	8.4
BXCPb (**3**)	468	514	0.05	398	514	0.02	18.3

BXCPb. It should be noted that the above K_d values are comparable to those reported for LF1 by He *et al.*[6d]

The spectral profile of the free and zinc-bound forms of the dyes was examined over a pH range of 6.5–8.2. No shift in either excitation or emission maxima was observed (see ESI†). Changes in fluorescence intensity were rather small. The most noticeable was that of the Pb^{2+}-bound form of BICPb at pH values 7.8 and 8.2. The observed changes are most likely due to the acidity of the benzimidazole secondary amine.

Electrospray mass spectrometry provided additional evidence for the formation of an [indicator **1** + Pb] complex. This was derived from the resulting mass spectrum in which ions having m/z 630, corresponding to the mass of the protonated molecule of the [indicator **1** + Pb] complex, were observed. [Indicator **1**] represents the anionic $-2K^{+}$ species. The observed isotopic distribution of these ions is in excellent agreement with the theoretical isotopic pattern of the protonated [indicator **1** + Pb] complex. No mass spectral evidence for higher than 1 : 1 indicator-to-Pb complexes was observed (as shown in the relevant spectrum, see ESI†). High intensity mass ions corresponding to $PbCl^{+}$ were also present in the resulting mass spectrum.

Thus dyes **1**, **2** and **3** seem to fulfill most of the criteria required for intracellular lead indicators, as they exhibit high selectivity for Pb^{2+}, they are excited in the visible region shifting their maxima upon binding allowing for ratiometric measurements, and they can be converted into cell permeable derivatives, as we showed in a recent work for those systems,[13] by modifying their carboxylate moieties to the corresponding acetoxymethyl esters.

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