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Synthesis of a Fluorescent Chemosensor Suitable for the Imaging of Zinc(II) in Live Cells

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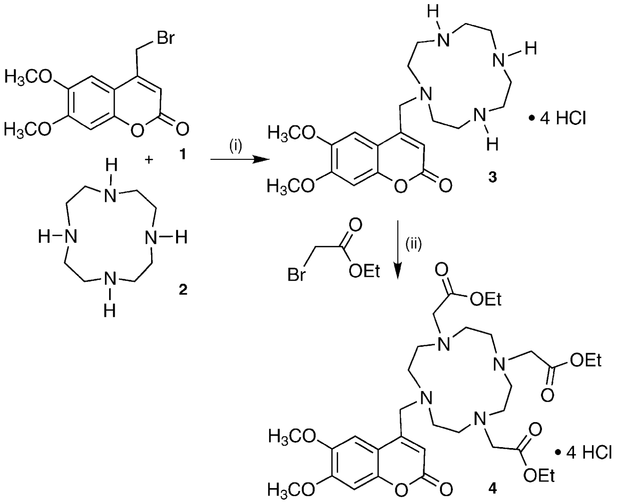
Abstract—The synthesis of a coumarin-cyclen conjugate-based zinc-specific chemosensor and its ability to sense Zn^{2+} in vitro is described. Using fluorescence microscopy, the chemosensor was shown to be capable of imaging Zn^{2+} in live rat pituitary tumour cells.

Zinc is, after iron, the second most abundant transition metal found in the body.[1] Many zinc-dependent enzymes and transcription factors have been described.[1,2] A large body of knowledge exists about the structural chemistry of zinc,[2] however, relatively little is known about the homeostasis of zinc.[3,4] The paucity of knowledge can be attributed to the spectroscopically silent nature of the d^{10} ion Zn^{2+}. Much about the biological function and control of the equally spectroscopically silent Ca^{2+} was learned through the use of chemosensors.[5] One particularly successful chemosensor design principle relies on chelation-enhanced fluorescence (CHEF).[6] Consequently, a number of zinc-specific chemosensors were developed in recent years,[7,8] some of which have shown utility in live cells.[9–12]

No single chemosensor for zinc, however, will allow the probing of all biological systems of interest because each sensor possesses a unique biodistribution profile, affinity and selectivity for Zn^{2+}. Thus, the availability of a number of chemosensors is desirable. Preferably, they are readily synthesized and modified to fit particular biodistribution requirements. In addition, sensors that can be excited and fluoresce at long wavelengths are advantageous for use in biological systems as this minimize light-induced tissue damage. Longer wavelengths also penetrate better and scatter less in optically diffuse systems.

4-Aminomethylcoumarins are known to be susceptible to photoelectron transfer (PET) fluorescence quenching,[13] a prerequisite for the design of a CHEF-type metal ion sensor. Reaction of 4-bromomethylcoumarin **1** with cyclen (**2**) produces chemosensor assembly **3** (Scheme 1). Exhaustive alkylation of **3** with ethyl bromoacetate generates triester **4** in overall modest yields (50%). Both **3** and **4** are isolated and crystallized as their hydrochloride salts.



Scheme 1. Synthesis of chemosensors **3** and **4**. Reaction conditions: (i) CH_2Cl_2, Na_2CO_3, rt 3 days, 60% isolated yield; (ii) CH_2Cl_2, Na_2CO_3, 4 days, 80% isolated yield.

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The alkylation of **3** has multiple effects:[14] (a) The ester or, after hydrolysis in biological media, the carboxyl moiety can assist in the binding of the central metal by means of providing an additional axial oxygen donor; (b) the side chains introduce steric bulk around the macrocycle which may preorganize the ligand toward metal binding; (c) alkylation of the secondary amines alters the pK_b of the ligand; (d) the side chain modulates the solubility (biodistribution) of the sensor. The latter point, in particular, will be demonstrated below. As expected, the aminocoumarins **3** and **4** are only weakly fluorescent (Fig. 1A).[15]

The design of the fluoroionophores **3** and **4** follows the traditional scheme of appending a fluorophore to a polyazamacrocyclic chelator.[8,16] This building block approach is most flexible. A large number of coumarin derivatives are known with excitation wavelengths ranging up to 450 nm,[17] and a variety of reagents can be used to alkylate **3**. Cyclen was chosen as a prototype amine-based chelator, and we expect that reaction of a wide range of amine-based chelates to bromomethylcoumarin **1** is possible. The combination of these options allows the wide adjustment of the optical and biodistribution properties of sensors of type **4**.

Addition of Zn^{2+} causes a 4.4-fold fluorescence enhancement with virtually no concomitant shift of λ_{max} of 448 nm (Fig. 1B). The fluorescence modulation is due to inhibition of the PET-type quenching upon metal coordination to the cyclen amine attached to the fluorophore. Accordingly, protonation of the ring nitrogens also causes a fluorescence increase. A fluorometric pH titration of **4** shows that this increase only becomes significant at a pH below 4, hence the sensor is suitable for use at physiological pH. The degree of Zn^{2+}-induced fluorescence enhancement is as good or better than observed for known Zn^{2+}-specific chemosensors with the exception of zinquin[12].[7,9] The extinction coefficient ε at the wavelength of excitation ($\lambda_{excitation} = 345$ nm) for $Zn^{2+}\cdot$**4** is 9.200 $cm^{-1}M^{-1}$. The fluorescence quantum yield ϕ for the complex is estimated to be better than 0.26.[18] A high ϕ combined with a large ε translates into excellent brightness of the sensor.

A Hill plot demonstrates the expected 1:1 stoichiometry of Zn^{2+}:**4** in the complex. The dissociation constant K_d of this complex was determined from a binding curve to be lower than 1 μM. However, the binding of **4** to Zn^{2+} is very slow (Fig. 1C; 50% of the maximal fluorescence increase is reached after 60 min), complicating the K_d determination. The slow binding kinetics indicate that a considerable reorganization energy is required to make the peripherally substituted and rigidified macrocycle available for metal binding.[14] Slow but strong binding characteristics are frequently observed in polyazamacrocycles.[14]

The integrated fluorescence increase upon exposure of sensor **4** to a 10-fold excess of a variety of metals is shown in Figure 2. Neither the alkaline earth metals, Al^{3+} or the first row transition metals Mn^{2+}, Co^{2+}, Fe^{3+}, or Cu^{2+} elicit any fluorescence increase over the native fluorescence of the free ligand **4**. The observed decrease in fluorescence of the Mn^{2+} and Cu^{2+} complex indicates binding of these paramagnetic and, therefore, fluorescence quenching ions. The soft ions Cd^{2+} and Pb^{2+} and, to a lesser extent, Hg^{2+} show fluorescence increase upon binding. However, the free ion concentration of these metals in any healthy cell is expected to be minimal, reducing the problem of interference with the measurement of Zn^{2+}. The large positive response of Cd^{2+} is a general problem in the design of Zn^{2+}-specific sensors.[10]

Rat pituary tumor cells[4] were incubated with the sensors **3** and **4** and the cells were inspected under a fluorescence microscope (Fig. 3). Only derivative **4** resulted in fluorescent cells. The pendant esters probably turn the chelate into a more lipophilic prodrug. Once passively diffused into the cell, esterases cleave some or all of the esters and the resulting carboxylate may be retained in the cell, leading to better and sustained staining property of the sensor. Such a mechanism was proposed to explain the uptake and retention of zinquin ester.[11] However, other studies show that cleavable ester functionalities are not required for intracellular zinc staining.[12]

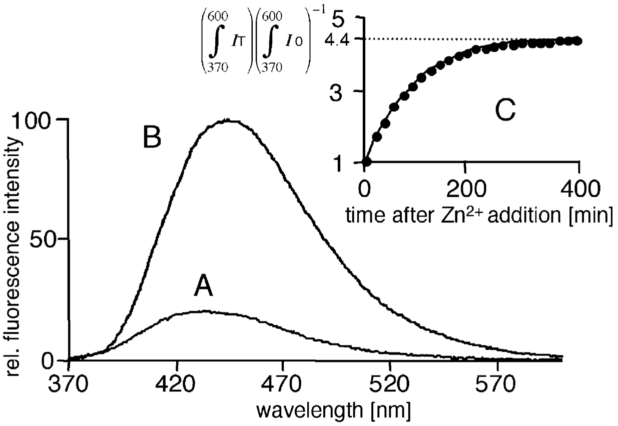

Figure 1. A, Native fluorescence intensity of **4**; B, Addition of 10 equiv. Zn^{2+}, after 12 h; C, Time course of the integrated fluorescence intensity after addition of 10 equiv. $ZnCl_2$. Conditions: $\lambda_{ex} = 345$ nm, 1 mM **4** in 50 mM PIPES buffer, 100 mM KCl, pH 7, 25 °C, aerated solution.

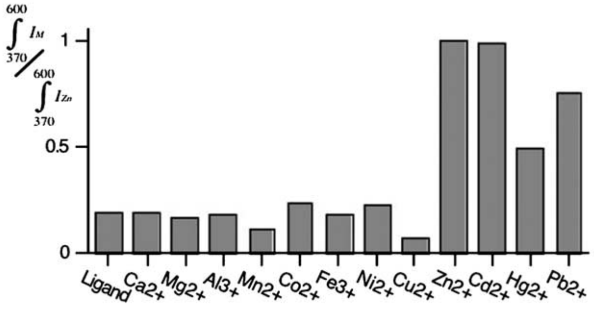

Figure 2. Metal-dependent relative integrated emission intensity ($\lambda_{ex} = 345$ nm) increase ($Zn^{2+} \equiv 1.0$) in the presence of a 20-fold excess of M^{x+} ions (as their chlorides) to **4** (1 μM) in 50 mM HEPES buffer, pH 7, 100 mM KCl, 10 μM EDTA, 30 h at 25 °C, aerated solutions.[10]

The staining of the cells is not homogenous. Instead punctate fluorescence is observed. This vesicle-like staining pattern is similar to that observed before.[12] This may be another indication of the existence of vesicular zinc pools in eukaryotic cells (Fig. 3).

The fluorescence intrinsic to untreated cells is negligible. The fluorescence of treated cells did not diminish over 120 min, an indication that the sensor does not get rapidly excreted or metabolized. Cells treated with the preformed complex Zn^{2+}·**4** did not display any fluorescence. This can be rationalized considering the dicationic charge and, therefore, membrane impermeability of this complex.

Many fluorophores, and coumarins are no exception, show increased fluorescence yields when incorporated into less polar environments such as cell membranes.[1] The following experiments, however, provide compelling evidence that the observed fluorescence in the cells is not due to the uncomplexed sensor **4**. Cells grown in solutions containing 100 μM Zn^{2+} as compared to cells grown in plain basal solutions or containing the zinc chelator diethylenetriaminepentaacetic acid (DTPA) were found to provide subjectively brighter images. The addition of the ionophore pyrithione and Zn^{2+} to the cells incubated with sensor **4** causes an increase in the fluorescence intensity which, however, is partially reversed with exogenously added *N,N,N′,N′*-tetra(2-picolyl)ethylenediamine (TPEN).[10] No fluorescence increase is observed upon addition of exogenous Zn^{2+} in the absence of pyrithione. These experiments provide strong indications that the observed fluorescence is due to intracellular sensor–zinc complex Zn^{2+}·**4** and not due to either Zn^{2+}·**4** adhering to the outside of the cell or to intra- or extracellular **4** which, albeit weakly fluorescing, is not non-fluorescing.

The time scale of the cell staining experiments is $\sim$30 min. The observed intense fluorescence does not increase over the next 120 min. This suggests faster Zn^{2+}-binding kinetics by the sensor in the cell than observed in vitro. The origin of this effect is not clear but may be due to either faster ion binding of the sensor when associated with intracellular membranes or proteins or due to faster ion binding of the (partially) ester-cleaved sensor. Both hypotheses are currently under investigation.

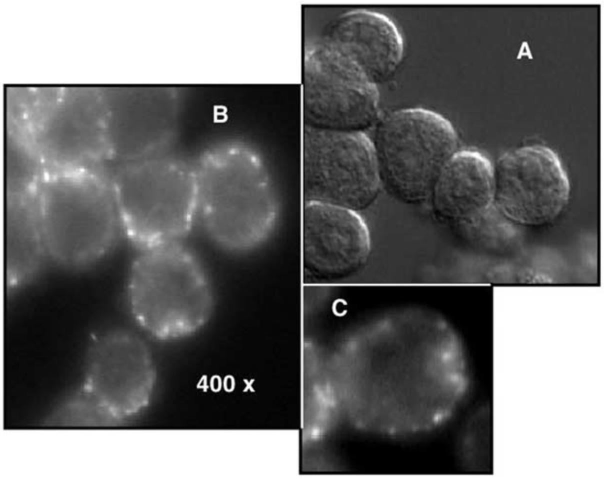

Figure 3. A, Light; B, fluorescence micrograph of cultured GH3 rat pituitary tumor cells incubated with **4**.[4] C, Expansion of single cell from image B. Conditions: incubation for 30 min at 36 °C, 5% CO_2 with 100 mM **4** in 20 mM HEPES, 2 g/L glucose; Zeiss Axiovert 200M with DAPI excitation filter set.

In conclusion, the synthesis of a CHEF-type chemosensor suitable for the Zn^{2+}-specific fluorescence imaging of life cells is presented. The sensor design allows for the synthesis of a family of sensors with varying optical, coordination and biodistribution properties. Studies using these sensors shedding light on the biology of Zn^{2+} can be expected.

Acknowledgements

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