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COMMUNICATION

Modular 'click' sensors for zinc and their application *in vivo*†

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Although the central role that zinc plays in many biological processes and important disease states is now well-established, there remains a pressing need to develop an absolute understanding of the underlying biology of zinc trafficking in terms of its dynamic and quantitative processing in specific organelles. Here we describe the modular synthesis of zinc sensors using a 'click' approach and demonstrate the applicability of our new sensors *in vivo* using a zebrafish model.

It is only relatively recently that zinc has emerged as a key element from the plethora of metal ions utilised in biological systems. Since Berg's manifesto appeared less than fifteen years ago,[1] the importance of zinc in an extraordinary range of biological processes has been revealed, including aspects of brain function and pathology, gene transcription, immune function, and mammalian reproduction. The disruption of zinc homeostasis is now implicated in a host of disease processes, such as type 2 diabetes mellitus (T2DM), Alzheimer's disease, epilepsy, ischemic stroke, infantile diarrhoea and age-related macular degeneration (AMD) to name just a few.[2] However, some of the most basic questions about zinc function remain unanswered and are the subject of vigorous debate. Indeed, it is as yet unclear whether zinc is the prime causal suspect of disease or is in fact employed by biology to prevent its development or progression.[3]

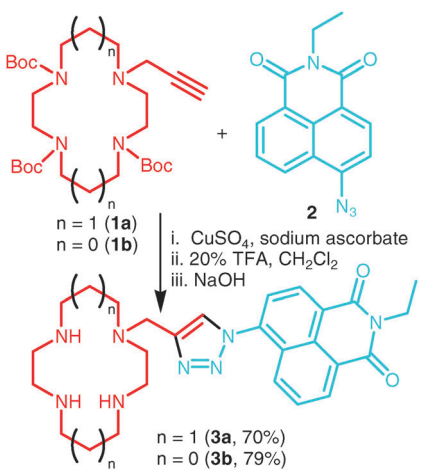
Thus, the development of a fundamental understanding of the role zinc plays in biology and medicine is a contemporary and multi-disciplinary challenge facing chemical-biology. To this end many Zn^{2+} selective fluorescent sensors have now been prepared.[4] Despite these efforts, there remains a pressing need for new constructs,[5] especially those which display: (i) the ability to provide organelle specific zinc sensing and (ii) a ratiometric response to zinc to allow quantification of zinc concentration in a spacially and temporally resolved manner *in vivo*.[6]

We have recently developed highly selective cyclam-based zinc sensors which uphold excellent zinc selectivity over a broad pH range and demonstrated their potential in chemical-biology through the direct observation of increased zinc flux in apoptopic murine thymocytes without the need for the addition of extracellular zinc.[7] A key feature of our approach is the modular nature of the synthesis using 'click' chemistry and we were keen to explore whether this could be applied to the facile development of other Zn^{2+} sensor systems. In particular, we were attracted by the cyclen-based systems of Kimura and co-workers, the utility of which has been comprehensively demonstrated since their seminal report of a dansyl-based sensor in 1996,[8] in the development of a range of related systems.[9]

Herein we report the synthesis and *in vitro* evaluation of a novel cyclen-based sensor which shows excellent selectivity for Zn^{2+} among other biologically relevant metals. Studies towards the *in vivo* detection of zinc were carried out using a live zebrafish model organism.

Sensors **3a**[7a] and **3b** are readily prepared using a Huisgen–Meldal–Sharpless–Fokin [3 + 2] 'click' cycloaddition between azide **2** and alkyne **1a** or **1b** respectively. Simple TFA deprotection and basic work-up furnishes both sensors in good overall yields of 42% and 51% from cyclam and cyclen respectively (see ESI† for full details) (Scheme 1).

We have previously shown compound **3a** to be an excellent switch-on fluorescent sensor for Zn^{2+} under biologically relevant conditions.[7] As expected, sensor **3b** also showed a switch-on response in the presence of Zn^{2+} in HEPES buffer (pH 7) with a significant increase in fluorescence emission at 420 nm



Scheme 1 Synthesis of 'click' zinc sensors **3**.

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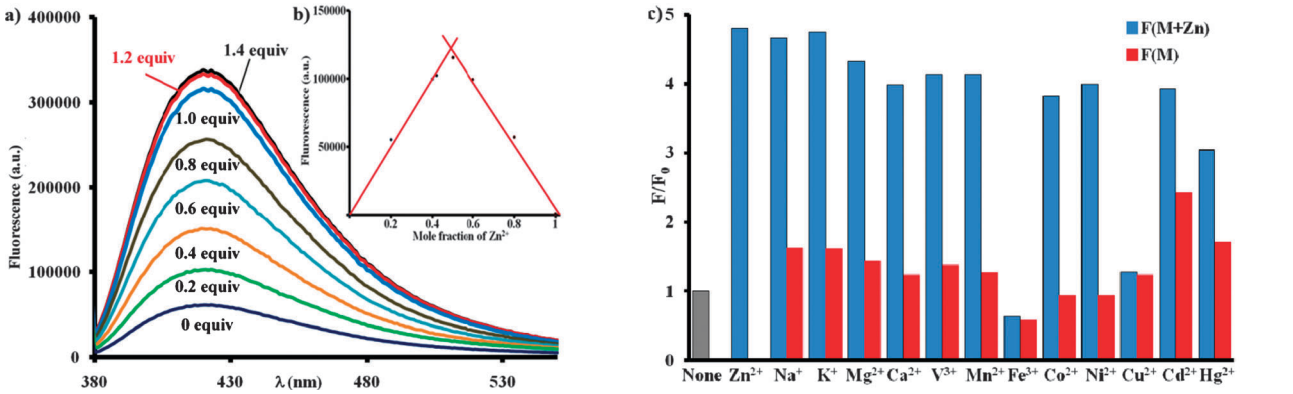

Fig. 1 (a) Emission spectrum of **3b** (0.5 mM) in HEPES buffer (0.1 mM) upon incremental addition of $Zn(ClO_4)_2$ (0.5 mM), (b) Job plot Zn·**3b** binding, (c) competitive binding of **3b** in which the competing metals (1.5 mM) were added to **3b** (0.5 mM) followed by Zn^{2+} (0.5 mM) in HEPES buffer at pH 7.0.

(Φ_{FL} = 0.044) as compared with the free ligand (Φ_{FL} = 0.005) although, intriguingly, the emission maximum of **3b** is slightly red-shifted compared with that of **3a** (λ_{em} = 407 nm). Incremental addition of Zn^{2+} to **3b** gave a steady increase in emission, consistent with the expected 1 : 1 Zn : **3b** complex being formed (Fig. 1a). This binding stoichiometry is corroborated by both a Job plot (Fig. 1b) and by ^{1}H NMR titrations of **3b** with incremental addition of Zn^{2+} (Fig. S1 in the ESI†).

Analysis of zinc binding affinity revealed $K_d = 2.8 \times 10^{-4}\,M^{-1}$ (see the ESI† for details).[10] The response to zinc in HEPES buffer at pH 7 was also upheld over a broad biologically relevant pH range (Fig. S2 in the ESI†). The response of sensor **3b** to the presence of a range of other metal ions (1 equiv.) was also tested (Fig. S3 in the ESI†). No significant switch on response was observed for any of the other metals tested except for Cd^{2+}. This is a common feature of other cyclen-based sensors although in this case the emission for Cd^{2+} was only approximately half that observed for Zn^{2+}. Intriguingly, the response of sensor **3b** for Cd^{2+} was near instantaneous with maximum fluorescence achieved within 5 minutes of the addition of the metal salt, significantly more rapid than that previously observed for sensor **3a**, which required 24 h to achieve its maximum fluorescence with Cd^{2+}.[7a]

An important feature of any putative zinc sensor is the ability to operate in the presence of other biologically relevant metal ions. Therefore to test the efficacy of **3b**, a competing metal ion (3 equiv.) was first added to a solution of **3b** followed by Zn^{2+} (1 equiv.) (Fig. 1c). In the case of Na^+ and K^+ complete fluorescence was recovered, while a significant recovery in fluorescence was observed for Ca^{2+}, Mg^{2+}, V^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and also, rather surprisingly, in the case of Cd^{2+}, and to a lesser extent Hg^{2+}. The partial recovery of fluorescence in the presence of Hg^{2+} is in complete contrast to **3a**, which showed no recovery of fluorescence. As for **3a**, the fluorescent response of **3b** could not be recovered after the addition of Cu^{2+}, but unlike **3a**, Fe^{3+} also resulted in no-recoverable signal. Overall these data reveal that **3b** has excellent switch-on selectivity for zinc, with a significant enhancement in fluorescence in HEPES buffer at physiologically-relevant pH, and with the exception of its behaviour with Hg^{2+} and Fe^{3+}, displays broadly similar behaviour to **3a**.

Slow evaporation of an acetonitrile solution of [Zn(**3b**)]$(ClO_4)_2$ gave crystals suitable for single crystal X-ray analysis (Fig. 2).† The X-ray structure obtained not only corroborates the 1 : 1 binding stoichiometry found through fluorescence and ^{1}H NMR measurements but also confirms the scorpionate binding mode of the proximal triazole nitrogen; the zinc ion adopts a distorted square based pyramidal geometry in which the trizole nitrogen occupies the apical position.

Despite the success of sensors **3** *in vitro* the true test of such devices is their behaviour *in vivo* where factors such as bioavailability and metabolic stability will significantly affect their performance. In recent years *Danio rerio* (zebrafish) have emerged as useful vertebrate models for the assessment of fluorescent sensors both due to the transparent nature of the zebrafish embryos, which allows direct observation of sensor fluorescence, and the high degree of sequence and functional conservation between zebrafish and mammalian genes which renders results obtained in this species of translational relevance.[11] Recently, Guo and co-workers[12] and Xu, Shin and Yoon and co-workers[13] have used a zebrafish model system to image zinc *in vivo*, demonstrating the viability of this approach.

Zebrafish embryos were grown in solutions of sensor **3a** or **3b** at various concentrations[14] and the distribution of fluorescence within the growing embryo monitored (Fig. 3).

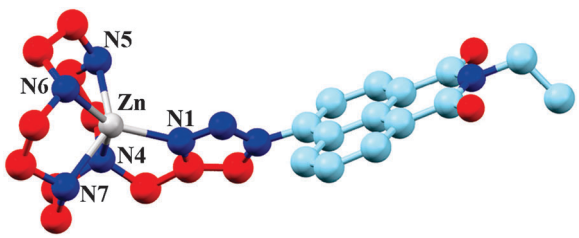

Fig. 2 The single crystal X-ray structure of [Zn(**3b**)]$(ClO_4)_2(CH_3CN)$, with H-atoms, solvent and counter-ions omitted for clarity. Selected bond lengths (Å) and angles (°): Zn(1)–N(1) 2.026(4), Zn(1)–N(4) 2.274(3), Zn(1)–N(5) 2.141(4), Zn(1)–N(6) 2.118(4), Zn(1)–N(7) 2.135(4); N(1)–Zn(1)–N(7) 116.14(15), N(6)–Zn(1)–N(7) 83.19(15), N(1)–Zn(1)–N(5) 101.77(15), N(6)–Zn(1)–N(5) 81.81(15), N(7)–Zn(1)–N(5) 132.04(15), N(1)–Zn(1)–N(4), 78.43(14), N(6)–Zn(1)–N(4) 136.01(14), N(7)–Zn(1)–N(4) 80.08(14), N(5)–Zn(1)–N(4) 79.95(13).

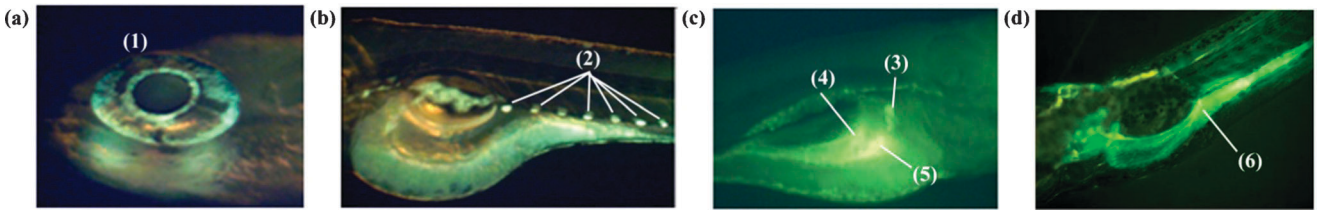

Fig. 3 Fluorescence microscopy images of *Danio rerio* larvae incubated in (a) 50 μM solution of **3a** (5 days), (b) 50 μM solution of **3b** (4 days), (c) 200 μM solution of **3a** (6 days) and (d) 25 μM solution of **3b** (5 days). Fluorescence is observed in the (1) eye, (2) iridophores, (3) gall bladder, (4) pancreas and solitary islet, (5) bile ducts and liver, (6) intestine.

Both sensors showed significant uptake within the fish with clear regions of concentrated fluorescence in the eye (Fig. 3a) and the iridophores (Fig. 3b).[15] Both sensors also clearly visualise the biliary system (gall bladder, liver, pancreas, solitary islet and bile ducts (Fig. 3c). These results are significant as visualisation of the pancreas and solitary islet is potentially of great significance for the understanding of the role of zinc in the onset of T2DM.[16] A significant level of fluorescence was also observed in the intestine, an area known to contain high levels of zinc (Fig. 3d).[17]

The *in vivo* results with sensors **3** are somewhat surprising in light of the previously reported data where zinc was detected using small molecule fluorescent sensors in zebrafish.[12,13] In both previous cases significant fluorescence was mainly observed from ring shaped, bilaterally disposed regions in the head. These areas were not visualised by sensors **3**. The differential localisation observed for sensors **3** may point to serendipitous organ- and system-level targeting or simply the differential cell permeability of sensors **3** compared with those previously reported.

In conclusion we have synthesised and characterised a new cyclen-based zinc sensor **3b** using our recently developed modular 'click' approach. Comparison with our previously reported sensor **3a** reveals that small structural changes in the ligand motif result in subtle changes in metal binding and zinc detection *in vitro*. We have further demonstrated the utility of sensors **3** *in vivo* using a zebrafish model and observed unusual sensor localisation. These preliminary findings suggest that these scorpionate 'click' sensors may have future applications in organ-level imaging of zinc pools *in vivo* in zebrafish animal models. Investigations along these lines are currently underway in our laboratories.

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