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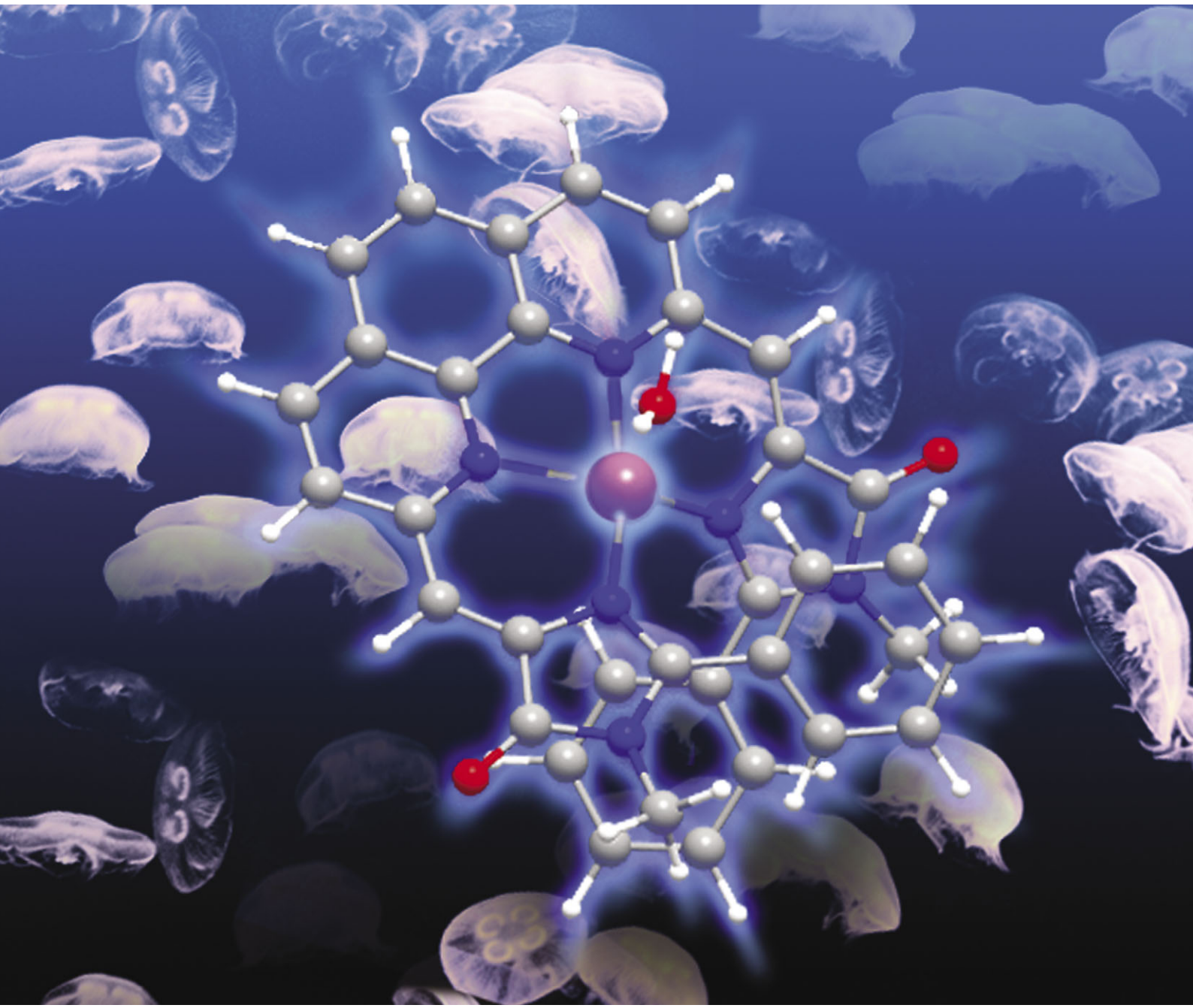

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An OFF–ON fluorescent probe for Zn^{2+} based on a GFP-inspired imidazolone derivative attached to a 1,10-phenanthroline moiety†

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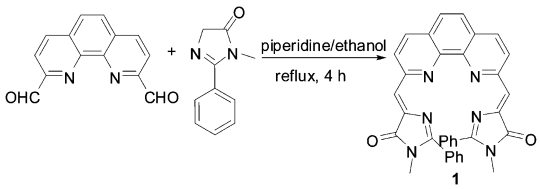
A green fluorescent protein chromophore inspired chemosenor for Zn^{2+} was designed and synthesized. A Zn^{2+} specific fluorescence enhancement was observed due to restricted rotation between the 1,10-phenanthroline and imidazolone moieties.

Green fluorescent protein (GFP) is an indispensable tool for live cell labeling.[1] The GFP chromophore is formed *via* autocatalytic cyclization and dehydration of a Ser-Tyr-Gly tripeptide motif followed by air-oxidization to give an imidazolinone, which produces high fluorescent emission efficiency and a respectable fluorescence lifetime. However, the imidazolinone unit does not fluoresce in solution unless it is anchored covalently and embedded deeply within the hydrogen-bond network of GFP. Seven-membered-ring hydrogen-bonding[2] and complexation by a BF_2 entity[3] have previously been used to restrict rotation about the aryl–alkene bond of the GFP chromophore. With our present work, we set out to design a new sensory system based on restricted rotation about the aryl–alkene bond controlled by metal complexation within the GFP chromophore.[4] Recently, Tolbert *et al.*[4b] have reported the GFP analog 1,2-dimethyl-4-(pyridin-2-ylmethylene)-1*H*-imidiazol-5(4*H*)-one and produced a chromophore which turns on its fluorescence in the presence of Zn^{2+} or Cd^{2+} ions.

The biological significance of Zn^{2+} has led to the development of numerous fluorescent chemosensors.[5] A number of Zn^{2+} chemosensors have been developed using photoinduced electron transfer (PET) and intermolecular charge transfer (ICT) signaling mechanisms.[5b,6] Diverse receptors including di-2-picolylamine,[7] quinoline,[8] and bipyridine[9] *etc.* have exhibited excellent selectivity and sensitivity toward Zn^{2+}. Zn^{2+} induced conformational changes have also been employed to convert the emission of pyrene from monomer to excimer.[10] Other different methodologies for the inhibition of the free rotation have also been reported.[11]

Herein, a GFP analogue **1** was designed and synthesized, where the formation of a complex between **1** and Zn^{2+} results in a remarkable fluorescence enhancement of imidazolinone units, attached to a 1,10-phenanthroline moiety. Our design combines elements of the Mg^{2+} selective porphyrin-like macrocycle[12] and the enhanced metal ion selectivity of the 2,9-di-(pyrid-2-yl)-1,10-phenanthroline unit.[13] We anticipated that the complexation of Zn^{2+} would restrict free rotation of the aryl–alkene bond and result in enhanced fluorescence. The fluorescent spectra changes observed upon addition of various metal ions indicate that **1** is highly selective for Zn^{2+} over other metal ions. We believe this opens the way for the development of many other applications using the GFP chromophore.



Scheme 1 Chemical structure and synthesis of probe **1**.

Compound **1** was prepared according to Scheme 1. Facile condensation of 1,10-phenanthroline-2,9-dicarboxaldehyde[14] with 1-methyl-2-phenyl-1*H*-imidazol-5(4*H*)-one[3] produces probe **1** in a yield of 35% as confirmed by NMR and HRMS analyses.

The spectroscopic properties of **1** were recorded in aqueous buffer (CH_3CN–HEPES, 4 : 1, v/v; HEPES, 50 mM, pH 7.4). An absorption around 400 nm was observed in the absorption spectrum (see Fig. S1, ESI†) and is ascribed to the neutral (A) form[15] similar to that observed for natural GFP. Several metal cations (Na^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Ag^+, Pb^{2+}, Co^{2+}, Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+}) as their perchlorate salts were titrated with probe **1** and as expected a weak fluorescence emission at 506 nm was observed, due to the free rotation of aryl–alkene bonds between the 1,10-phenanthroline and imidazolone units. However, upon the addition of 1 equivalent of Zn^{2+}, the fluorescence emission of **1** shifts from 506 nm to 485 nm indicating a decrease in the electron-donating ability of the nitrogen of the imidazolone moieties, and the intensity increases by more than 10-fold. While with the addition of the other metal ions, no enhancement of emission can be observed in the fluorescence spectra, there is no change with Ca^{2+} and Na^+, and quenching effects by other heavy metal ions (Mg^{2+}, Cd^{2+}, Ag^+, Hg^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}) are observed. The excited state of

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† Electronic supplementary information (ESI) available: Synthesis, characterization, absorption, mass spectra, NMR and DFT data of **1**. See DOI: 10.1039/c1cc10210a

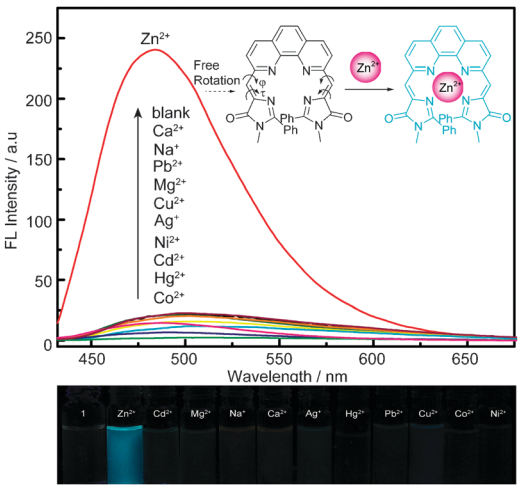

Fig. 1 Top: fluorescence emission of **1** (15 μM) in the presence of 1 equiv. Co^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Ag^{+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Na^{+}, Ca^{2+}, Zn^{2+} (from bottom to top) in CH_3CN–HEPES (4 : 1, v/v; HEPES, 50 mM, pH 7.4). Inset: proposed mechanism for fluorescence enhancement of **1** with Zn^{2+} ion. Bottom: visible fluorescence emission responses of 1.5×10^{-5} M of **1** with 1 equiv. of various metal ions excited using a UV lamp (λ = 365 nm).

chromophore model systems (*e.g.* HBDI) has significant dihedral freedom, which may lead to fluorescence quenching through intersystem crossing.[16] As proposed in the inset of Fig. 1, the bonding of Zn^{2+} with compound **1** may lead to a restriction in the rotational freedom of the chromophore, and result in an increase in fluorescence intensity.

Fig. S2 (ESI†) depicts the pH dependent fluorescence changes obtained for **1**. The plot of the integrated emission intensity reveals that at lower pH values (<6) the fluorescence of **1** is quenched, which is in agreement with Tolbert's work.[4b] The Benesi–Hildebrand analysis[17] of the emission data gives a 1 : 1 stoichiometry for the **1**–Zn^{2+} complex, and the association equilibrium constant (K_{ass}) is 2.44×10^6 M^{-1} (Fig. 2).

The interaction of receptor **1** with zinc ion was further investigated by ^{1}H NMR (see Fig. 3A). After addition of excessive zinc ions, all the hydrogens shift upfield due to complexation with the zinc ions and paramagnetic effect.

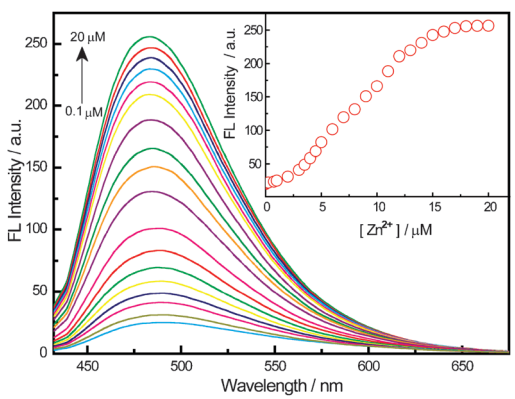

Fig. 2 Fluorescence emission spectra of **1** (1.5×10^{-5} M) upon addition of various amounts of Zn^{2+} in CH_3CN–HEPES (4 : 1, v/v; HEPES, 50 mM, pH 7.4). Excitation at 400 nm. Inset: plot of fluorescence intensity at 485 nm against the concentration of Zn^{2+}.

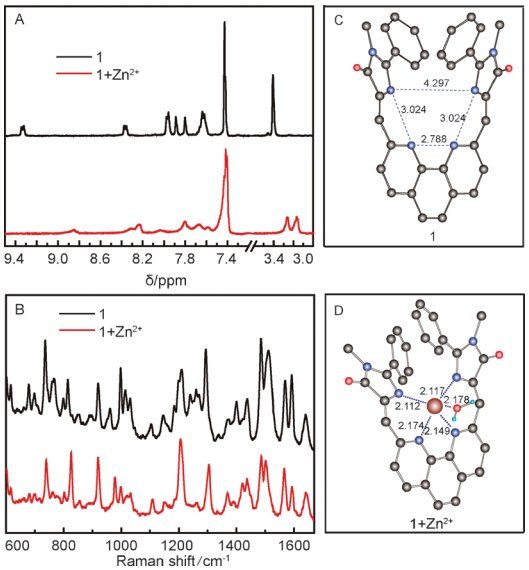

Fig. 3 (A) ^{1}H NMR spectra of **1** in the absence and in the presence of excessive Zn^{2+} in $CDCl_3/CD_3CN$; (B) SERS spectra of 1×10^{-5} M of **1** in the absence and in the presence of excessive Zn^{2+} in $CDCl_3/CD_3CN$; (C) density functional theory (DFT)-optimized geometry of **1**; (D) DFT-optimized geometry of the complexation of **1** with Zn^{2+}. Bond length is in angstrom and unimportant hydrogen atoms are deleted for clarity.

Raman spectroscopy has been used to study the *cis–trans* isomerization of GFP chromophores.[18] Therefore, we decided to probe the effect of Zn^{2+} on a single molecule of **1** using surface enhanced resonance Raman spectroscopy (SERS). On mixing **1** with Zn^{2+} and AgNPs colloid stabilized by citrate, a remarkable difference was observed in SERS spectra (see Fig. 3B). The peaks attributable to modes which are not related to the chromophore do not change, while most of the signals weaken after the addition of Zn^{2+}, which may be due to the enhanced rigidity. Fig. 3B shows the SERS spectra of **1** in the absence and in the presence of Zn^{2+}. Compared to *cis–trans* isomerization of Y66F GFP chromophore (BFPF),[18a] similar changes of the peaks, especially a group of bands around 1100–1300 cm^{-1}, were observed due to the weakening and red-shift of the stretching mode of the C=C bond. This observation gives clear evidence for the conformational change of **1** upon the addition of Zn^{2+} ions.

Density functional theory (DFT) calculations were performed on the proposed Zn^{2+} complexes without (Fig. S3a, ESI†) and with one additional water molecule ligation (Fig. S3b, ESI†), at the B3LYP/lanl2dz level. Without the additional water ligation, the relative energies of the three species in Fig. S4a (ESI†) are 0.0, 9.9, and 13.8 kcal mol^{-1} for the non-oxygen, one-oxygen and two-oxygen ligated conformations, respectively, which means that the non-oxygen-ligated conformation is the most stable structure. With one additional water molecule ligation, the relative sequence is not changed, but, with a smaller energy gap, that is, 0, 3.3 and 7.7 kcal mol^{-1}. The optimized geometries by DFT calculations are presented in Fig. 3C and D, which demonstrate that Zn^{2+} complexation promotes a π–π stacking interaction between the ligands two phenyl groups, and coordination shortens the distance of the four nitrogen atoms. The complex of Zn^{2+} was also analyzed further by high-resolution mass spectroscopy (Fig. S7, ESI†), which shows a peak at 629.1285 assigned to [**1** + Zn + H_2O–H]$^+$.

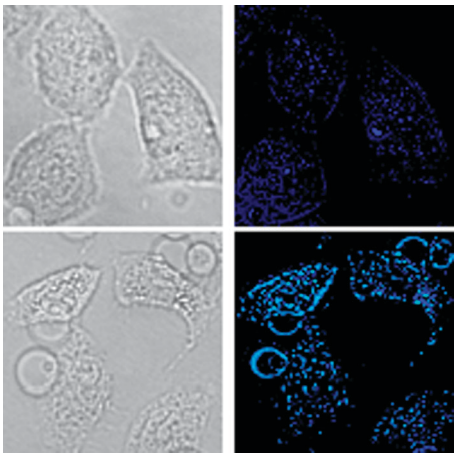

Fig. 4 Phase contrast (left) and fluorescence (right) microscopy images of HeLa cells incubated for 30 min with **1** (40 μM), without (top) and with (bottom) the addition of Zn^{2+} (1 equiv.).

To further explore the selectivity of **1** for Zn^{2+}, we measured the fluorescence intensity of **1** in the presence of Zn^{2+} and other metal ions in aqueous buffer (CH_3CN–HEPES, 4 : 1, v/v; HEPES, 50 mM, pH 7.4) (Fig. S8, ESI†). The emission intensity of Zn^{2+}-bound **1** is unperturbed in the presence of 1 equiv. Na^+, Ca^{2+}, Mg^{2+}, Cd^{2+}, Ag^+, Hg^{2+}, Pb^{2+} and Ni^{2+}, indicating excellent selectivity for Zn^{2+} over these competing cations, whereas the same amounts of Cu^{2+} and Co^{2+} quench the fluorescence, which is due to the displacement of Zn^{2+} by Cu^{2+} or Co^{2+} from the Zn^{2+}–**1** complex.[8d,19] It is important to note that Cd^{2+} does not interfere with the detection of Zn^{2+} by **1**, in stark contrast to the previously reported GFP analogue,[4b] this is due to the porphyrin-like structure of **1** which results in selective complexation of Zn^{2+}.

In vitro Zn^{2+} sensing and imaging was investigated using probe **1** in live HeLa cells (Fig. 4). The cells exhibited strong blue-green fluorescence with the addition of Zn^{2+}. These results indicate that **1** may be used as a possible sensor to detect Zn^{2+} released from stimulated cells.

In conclusion, a GFP-inspired imidazolone derivative containing a 1,10-phenanthroline moiety was designed and synthesized as a Zn^{2+} specific turn-on fluorescent chemosensor. We are currently exploring the use of this design strategy in the construction of other fluorescent chemosensors.

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