A ratiometric fluorescent sensor for phosphates: Zn^{2+}-enhanced ICT and ligand competition†‡

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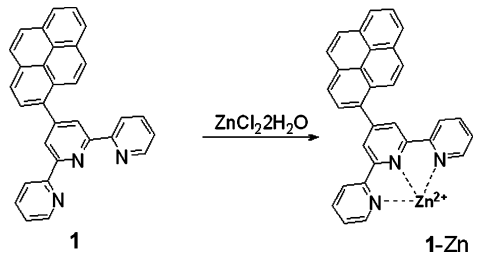
A pyrene–terpyridine–Zn conjugate has been synthesized and characterized, where Zn^{2+} acts as an electron acceptor to enhance molecular ICT with a large emission red-shift (>100 nm). It showed a ratiometric fluorescence change upon addition of phosphate anions in buffered aqueous solution. The selective response to phosphates or pyrophosphates involved ICT and ligand competition processes.

When a fluorophore contains an electron-donating group (often an amino group) conjugated to an electron-withdrawing group, it undergoes intramolecular charge transfer (ICT) from the donor to the acceptor upon excitation by light. The consequence of the ICT is a red shifted emission. Many fluoroionophores have been designed by employing the interaction of cations with the electron-donating group.[1] After being bound to a cation, the electron-donating group loses its donating ability, so the ICT ceases and a blue shift appears, which is often used for the fluorescent ratiometric determination of the cations.[2–6] In contrast to the systems described above, there are only few systems[7–10] in which the binding ions interact with the acceptor part of the charge-transfer sensors. This may result from the limitations of the ion-binding effects on the change of ICT and on the shift of the emission wavelength. Recently Williams has made significant progress and obtained a large red-shifted emission (>110 nm) by binding Zn^{2+} to the acceptor of aniline–terpyridine ICT systems.[11] However, amino groups as the electron-donating groups in ICT systems are subject to interference by protonation when they are used as sensors.[12]

Herein we report a pyrene–terpyridine–Zn system (**1**–**Zn**, Scheme 1) where pyrene, a large π-electron-rich aromatic group (not the traditional amino group), donates the electron, and ICT is enhanced by zinc binding at terpyridine. This new system is not affected by protonation any more. Moreover, the ICT can be modulated by phosphates or pyrophosphates and consequently can be used as an effective ratiometric fluorescent phosphate sensor.

Phosphates and pyrophosphates ($P_2O_7^{4-}$, PPi) are involved in many important biological processes. For example, phosphate anions are present in extracellular fluids at 1–3 mM concentrations, and they play important roles in the formation of extracellular matrix.[13] PPi also plays a role in energy transduction in organisms and could control metabolic processes by participation in enzymatic reactions.[14] As a convenient tool to study biological phosphates and pyrophosphates, fluorescence sensing is particularly attractive due to its simplicity and high sensitivity.[15–20] Recently, Hamachi[21–23] and Akkaya[24] have developed zinc–complex sensors (dpa–Zn^{2+} and bpy–Zn^{2+} respectively, where dpa is dipiconylamine and bpy is 2,2′-bipyridine), which can bind phosphates or pyrophosphates and emit enhanced fluorescence by suppression of PET (photo-induced electron transfer) quenching; Hong *et al.* have obtained a Zn^{2+}–phenolate–dpa sensor which displays 40 nm red-shifted emission when it interacts with pyrophosphates;[25,26] and similarly Yoon's sensor showed a small red fluorescence shift (12 nm) in the binding of pyrophosphate to Zinpyr-1–Zn^{2+} complex.[27] However, up to now, there has been no report of an effective ratiometric fluorescent chemosensor for phosphates or pyrophosphates. Ratiometric fluorescence measurements can increase the selectivity and the sensitivity of detection, because the ratio of the fluorescent intensities at two wavelengths is independent of the concentration of the sensor, the fluctuation of source-light intensity, and the sensitivity of the instrument.



Scheme 1

In contrast to the reported sensors, **1**–**Zn** described herein is based on the suppression of zinc-enhanced ICT, and offers a large blue-shifted emission (>100 nm) in the presence of phosphates or pyrophosphates, which is the first phosphate sensor to be used in the fluorescence ratiometric determinations of the anions in aqueous conditions.

1–**Zn** was synthesized by treatment of **1** with $ZnCl_2$ in ethanol–$CHCl_3$, and recrystallization from nitrobenzene as a yellow powder. Pyrene is known to show a high fluorescence quantum yield and rich electron density. Tpy (2,2′:6′,2″-terpyridine) containing three nitrogen atoms similar to dpa, acts as a receptor for Zn^{2+}.

Compared with the amino-containing ICT systems, the fluorescence of **1** is pH insensitive (Fig. S3†).[28] The fluorescence maxima of **1** undergo slight red shifts (*ca.* 20 nm) with increasing solvent polarity (Fig. S2†). Such behavior is indicative of the intramolecular charge transfer (ICT) character upon excitation.[11] However, after coordination with Zn^{2+}, the fluorescence maximum

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‡ All measurements were carried out in aqueous acetonitrile solution of HEPES buffer (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid).

shifts to *ca.* 550 from 442 nm in CH_3CN–HEPES (9 : 1, v/v; pH = 7.4) (Fig. 1) and at the same time the UV-Vis absorption maxima undergoes 50 nm of red-shift. These phenomena are similar to the results described by Williams.[11] Terpyridines are well-known to show good affinity for Zn^{2+}. The binding of Zn^{2+} to **1** leads to the red-shifts of the spectra due to the complexation-induced ICT process from pyrene to terpyridine.[29] It is unusual that the metal-ion-binding demonstrates such a high capability to act as an acceptor in the charge transfer process that it forces pyrene to donate electrons, as an amino-type electron-donating group does in general ICT systems. It offers a red emission shift up to 100 nm. We define this kind of ICT as "Zn^{2+}-enhanced ICT".

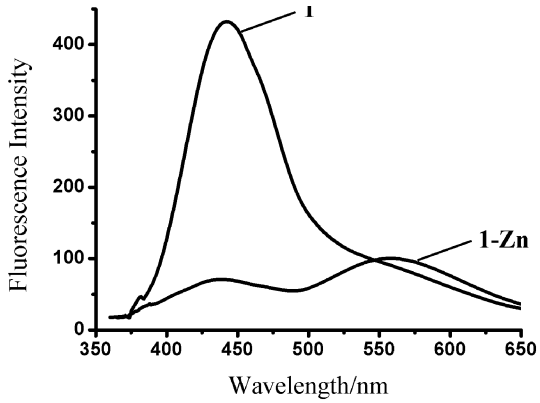

Fig. 1 The fluorescence spectra of ligand **1** (10 μM) and **1**–**Zn** (10 μM) in CH_3CN–HEPES (9 : 1, v/v; pH = 7.4). The spectra were measured with an excitation at 346 nm.

Upon the addition of PPi to the solution of **1**–**Zn** in CH_3CN–H_2O (9 : 1 v/v) at pH 7.4 (HEPES 20 mM), the fluorescence band maximum at 542 nm weakens and at the same time, a new fluorescence band with a maximum at 442 nm develops (Fig. 2). The fluorescence changes from bright yellow to bright blue (Fig. S1†). A similar effective response was observed upon addition of HPO_4^{2-} (Fig. S4†). However, **1**–**Zn** did not show any obvious spectral change upon addition of other anions such as $CH_3CO_2^-$, NO_3^-, F^-, Cl^-, Br^-, I^-, HSO_4^-, SCN^-, *etc.* (Fig. 3, Fig. S5†).

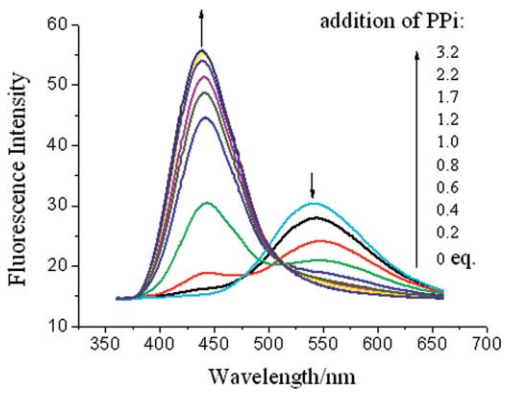

Fig. 2 Change of fluorescence spectra for sensor **1**–**Zn** (100 μM) upon addition of 0–3.2 equivalents of PPi. The spectra were measured with an excitation at 346 nm in CH_3CN–HEPES (9 : 1, v/v; pH = 7.4).

As shown in Fig. 2, the ratio of short-wavelength emission at 442 nm to long-wavelength at 542 nm (I_{442}/I_{542}) increases

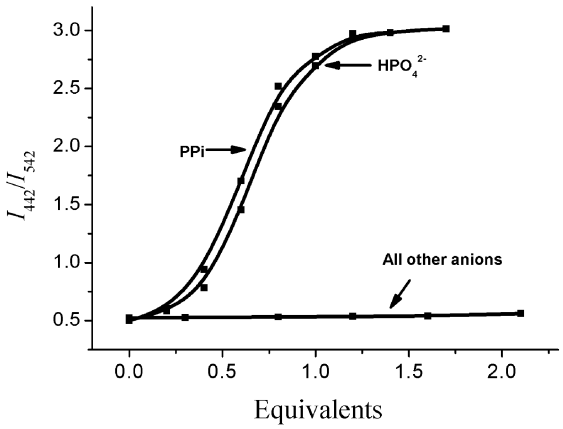

Fig. 3 Plot of the relative fluorescence intensity of I_{422}/I_{542} with equivalents of anions for sensor **1**–**Zn** (100 μM) upon addition of PPi, HPO_4^{2-}, Cl^-, Br^-, I^-, CH_3COO^-, HSO_4^-, SCN^-, NO_3^-. The spectra were measured at an excitation wavelength of 346 nm in CH_3CN–HEPES (9 : 1, v/v; pH = 7.4).

with increasing PPi concentration. When the PPi concentration is 1 equiv., I_{442}/I_{542} is up to 3 (Fig. 3). A similar emission change is also detected upon addition of HPO_4^{2-}. The blue-shifts make the ratiometric measurements possible. The ratio of the fluorescence intensities at two appropriate emission wavelengths provides a more reliable measurement of the concentration.

The dissociation constant (K_d) was calculated to be 7.0×10^{-5} M according to fit plot (Fig. S6†).

Several mechanisms have been proposed to explain the selectivity of Zn^{2+} complexes for phosphates over other anions,[21–26] mostly on the basis of binding or coordination of phosphates to the coordinated Zn^{2+}. Hamachi[21–23] obtained the X-ray crystal structure of the phenyl phosphate–zinc–DPA complex and the fluorescence enhancement induced by the phosphate derivatives was mainly ascribed to the phosphate-assisted coordination of a second Zn^{2+} with a second electron-donating group (DPA) in the PET sensor. Hong *et al.* have obtained the X-ray structure of Zn^{2+}–PPi–dpa-sensor complex[25] and their mechanism was proposed to be that the binding of PPi or ATP weakened the bond between the phenolate oxygen atom and Zn^{2+} and induced a more negative charge characteristic on the phenolate oxygen atom.[26] Akkaya attributed the enhancement of emission of BODIPY–bpy–Zn^{2+} phosphate-sensor to the modulation of oxidative PET.[24]

In the **1**–$\mathbf{Zn}^{2+}$ system, however, we found that the fluorescence enhancement and the blue-shift are just the fluorescence recovery of **1** by a comparison of Fig. 1 and Fig. 2. The UV-Vis spectra of **1**–$\mathbf{Zn}^{2+}$ undergoes a 50 nm blue shift upon addition of PPi and also displays the return of **1**. Separating the blue-shifted fluorescent compound by column chromatography, pure **1** was unexpectedly obtained and identified with TLC, ESI-MS and NMR. It is not surprising when we note the K_{sp} of $Zn_3(PO_4)_2$ is 9.0×10^{-33} (pK_{sp} = 32.04).[30] This means that Zn^{2+} has a great affinity for phosphate ions and a great tendency to deposit insoluble zinc phosphates. Unfortunately, no such K_{sp} of zinc–PPi compounds has been reported, but the strong interaction between Zn^{2+} and PPi or HPO_4^{2-} might be deduced from the above results. As the fluorescence recovery is from the ligands exchange, we call the mechanism "Zn^{2+} ligand competition".

To clarify the mechanism, the fluorescence spectra change of **1**–**Zn** upon addition of EDTA (metal chelator) and TPEN (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (Zn^{2+} chelator) were tested. A similar phenomena to the cases of phosphates and PPi were observed (Fig. S7†).

The ligand competition must compose kinetic steps including the binding of the phosphates on coordinated Zn^{2+}, then the weakening of original coordination and finally the dissociation of new complexes (or insoluble salts) from the big conjugates, accompanied by stereo rigidity adjustments.[31–33] The insoluble zinc salts of phosphates and pyrophosphates may form in mixtures depending on the different pH. $Zn_3(PO_4)_2$, $Zn_2P_2O_7$, $ZnH_2P_2O_7$ and their hydrates are the possible products. As shown in Fig. 3, the molar ratios of **1**–**Zn** to anions are on average 1 : 0.8 ∼ 1 to reach the plateaus. This indicates the formation of on average a 1 : 0.8 ∼ 1 stoichiometric complex. As multiple steps and multi-products are involved in the ligand competition, no isosbestic points in the fluorescence spectra are observed in Fig. 2 and Fig. S4†.

Small wavelength shift recovery[27] and fluorescence intensity recovery[24] also exist in previous reported Zn^{2+}–phosphate/PPi sensors. Besides the reported biding mechanisms, there might be "Zn^{2+} ligand competition" mechanism in these cases to some extent. The difference between the affinities of Zn^{2+} to fluorescence ligands and to phosphate or PPi should be a decisive factor to control the process. The affinities are generally expressed by dissociation constants, K_d, of Zn^{2+}-fluorescence receptor complexes and K_{sp} of zinc salts. The K_d of tpy–Zn^{2+} is much larger than that of dpa (*e.g.* in Zinpyr-1[34a] and BDA[34b]), so the "ligand competition" mechanism dominates the fluorescence recovery of the present case.

This ligand competition mechanism is very similar to the "chemosensing ensemble" developed by Fabrrizzi.[18b,35] In that mechanism, the fluorescent indicator is bound to the receptor through non-covalent interactions (such as coordination), and the fluorescence of the indicator is either quenched or enhanced by the receptor. When the analyte displaces the indicator, the fluorescence recovers. The basis of the chemosensing ensemble method is also on the affinity difference between indicator–receptor and analyte–receptor. Herein Zn^{2+} and **1** are similar to the receptor and the indicator, respectively. But the fluorescence change is the large blue shift *via* Zn^{2+}-induced ICT, and the receptor, Zn^{2+} is much simpler than those polycyclic receptors.[18b,34] Therefore, the "Zn^{2+} ligand competition" should be a special type of "chemosensing resemble" mechanism.

In summary, we have developed a Zn^{2+} enhanced ICT system, pyrene–tpy–Zn conjugate, as the first ratiometric fluorescent sensor to selectively detect phosphates and pyrophosphates in aqueous conditions. In the recognition process, Zn^{2+} is dissociated from the sensor by the action of phosphates or pyrophosphates. A ligand competition mechanism has been proposed.

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