A novel 2,6-diformyl-4-methylphenol based chemosensor for Zn(II) ions by ratiometric displacement of Cd(II) ions and its application for cell imaging on human melanoma cancer cells†

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A new chelating ligand [4-methyl-2,6-bis-(pyridin-2-yl-hydrazonomethyl)-phenol] (1) was prepared by the condensation of 2-hydrazinylpyridine with 2,6-diformyl-p-cresol. Compound 1 exhibits weak fluorescence due to intramolecular photoinduced electron transfer (PET). The sensor (1) demonstrates Zn2+-specific emission enhancement due to the ‘PET off’ process through a 1 : 1 binding mode with the metal ion. The fluorescence quantum yield of chemosensor 1 is only 0.020, and it increases more than 14-fold (0.280) in the presence of one equivalent of the zinc ion. Interestingly, the introduction of other metal ions causes the fluorescence intensity to remain either unchanged or weakened except for Cd2+. The new sensor showed ‘naked-eye’ detection of Zn2+ ions: a color change of the solution from colorless to yellow. Ratiometric displacement of Cd2+ ions from the complex by Zn2+ ions supports the formation of a more stable sensor–Zn2+ complex over the sensor–Cd2+ complex. The experimental findings have been correlated with theoretical results using the B3LYP functional and 6-31G (d, p), LANL2DZ basis set for Cd2+ (2) and Zn2+ (3) complexes, respectively, by the Density Functional Theory (DFT) method. Moreover, the ability of probe 1 to sense Zn2+ within human melanoma cancer cells has been explored, and the Zn2+ -probing process in living cells was found to be reversible with zinc chelator solution of N,N,N,N-tetraakis(2-pyridylmethyl)ethylenediamine (TPEN) or EDTA.

Introduction

The zinc ion is an essential metal in the human body and plays an important role in various fundamental biological processes, such as gene transcription and regulation of metalloenzymes. Disruption of Zn homeostasis has been associated with several pathologies, including ischemia, Alzheimer’s disease and prostate cancer. Ever increasing concerns for ecological issues such as the potential toxicity of zinc pollutants drive the need for more effective detection methods and selective and versatile sensors. A literature survey indicates that many fluorescent probes have been designed which operate on photoinduced electron transfer, Internal Charge Transfer (ICT) or fluorescence resonance energy transfer mechanisms. Although significant progress has been made in relation to the detection of Zn2+ ions, still there is a demand for a fluororeceptor of new architecture with improved fluorescent properties.

Being group 12 elements of the periodic table Zn2+ and Cd2+ show cross-reactivity and record similar spectral changes while coordinated with fluorescent sensors. To date, the common probes for Zn2+ are derivatives of zinquin, 8-aminoquinoline, the Zinpyr family, Zinbo-5, coumarin, and the TQEN family. However, the development of fluorescent chemosensors that can selectively recognize one of the multiple analytes, especially species with similar chemical properties such as Mg2+, Ca2+, Zn2+, Cd2+, etc., has been an active topic of current research because of operational simplicity and high sensitivity. Biological imaging of specific molecules can provide direct information of molecular functions in living systems. The most important breakthrough for this purpose is to create
selective and sensitive tools. Among several approaches made during the last three years, many sensor molecules for detection of chelatable Zn\textsuperscript{2+}-specific sensor molecules have been reported for their importance in monitoring the Zn\textsuperscript{2+} status in living systems. Some of these sensor molecules have become valuable tools for understanding the role of Zn\textsuperscript{2+} in living systems. Zinc ions have been implicated in polymerization and cytotoxicity of sTRAIL in tumor cells. Prostate Zn\textsuperscript{2+} concentrations are among the highest in the body, and a marked decrease in the level of these ions is observed in prostate cancer.\textsuperscript{21} Similarly, the concentration of zinc ions has also some bearing on the expression of pro- and anti-apoptotic factors in high-grade prostate carcinoma cells,\textsuperscript{22} thereby indicating the importance of the zinc ion concentration in cancer studies as well. Fluorescence sensor molecules, which allow visualization of cations or enzymatic activities in living cells, are thus useful tools for providing information on the Zn\textsuperscript{2+} status in living biological systems, particularly because biological functions of Zn\textsuperscript{2+} have been reported for the protein-bound form. In many organs of the human being, disruption in optimum Zn\textsuperscript{2+} concentration can cause severe effects on physiology, as a consequence of modulation of the functions of certain ion channels and receptors.\textsuperscript{23} Our sensor is another addition to the list of efficient sensors that can be used as a tool for detection and determination of the status of Zn\textsuperscript{2+} molecules for various clinical uses where the zinc concentration plays a critical role, as in the case of Alzheimer’s and some other neuro-degenerative diseases, and in certain types of cancer. Recently, a variety of fluorescent Zn\textsuperscript{2+} sensors have been documented but these have certain limitations such as fluorescent towards multiple analytes e.g. Zn\textsuperscript{2+}/Cd\textsuperscript{2+} or Zn\textsuperscript{2+}/Ca\textsuperscript{2+} or Zn\textsuperscript{2+}/Cd\textsuperscript{2+}/Pb\textsuperscript{2+}/Hg\textsuperscript{2+},\textsuperscript{24} expensive synthesis, and cytotoxicity. For these reasons, we have taken care to design a 2,6-diformyl-4-methylphenol-based ligand to overcome these problems.

Herein, we report the facile synthesis, photophysical characterization, and live-cell imaging of a novel chemosensor, 4-methyl-2,6-bis(pyridin-2-yl-hydrazonomethyl)-phenol (1), as a probe for Zn(II) in a (HEPES) buffer [pH = 7.2] at 25 °C by ratiometric displacement of Cd(II) ions (Scheme 1). Sensor 1 is very selective for Zn\textsuperscript{2+} over Cd\textsuperscript{2+}, other biologically relevant alkali and alkaline-earth metal ions, toxic heavy-metal ions, and most of these first-row transition-metal ions. The most interesting feature is that 1 can be successfully applied for sensing Zn\textsuperscript{2+} in DMSO : water = 1 : 9 (v/v), and in human melanoma cancer cells \textit{in vitro}, which makes the sensor useful in detecting the Zn\textsuperscript{2+} ion concentration in certain cells in biological systems that can be cultured \textit{in vitro} without much concern about its cytotoxicity. Sensor 1 is quite selective and also works well in water and even with living cells in DMEM and not just in organic media like >90% of similar reports.\textsuperscript{24–26}

Scheme 1. (a) Cd\textsuperscript{2+} binds with 1, (b) Zn\textsuperscript{2+} replaces Cd\textsuperscript{2+} and (c) EDTA replaces Zn\textsuperscript{2+}. 

\[
\text{EDTA} \quad \text{Cd}^{2+} \quad \text{weak fluorescence} \quad \text{Zn}^{2+} \quad \text{moderate fluorescence} \quad \text{strong fluorescence}
\]
Experimental

Materials and physical methods

Unless otherwise stated, all reagents were purchased from commercial sources and used without further purification. Solvents were dried by standard methods. 2,6-Diformyl-4-methylphenol and 2-hydrazinyl pyridine were prepared by literature methods. All reagents and chemicals were purchased from Sigma and used without further purification. Solvents used for spectroscopic studies were purified and dried by standard procedures before use. Elemental analyses (carbon, hydrogen and nitrogen) were carried out with a Perkin-Elmer CHN analyzer 2400. The UV-Vis spectra of all samples were studied with a Hewlett-Packard UV-Vis spectrophotometer (model 8453). IR spectra (KBr pellet, 400–4000 cm\(^{-1}\)) were recorded on a Perkin-Elmer model 883 infrared spectrophotometer. Fluorescence lifetimes were measured by the method of Time Correlated Single-Photon Counting (TCSPC) using a HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer. The sample was excited using a nanosecond laser diode at 340 nm and the signals were collected at the magic angle of 54.7° to eliminate any considerable contribution from fluorescence anisotropy decay. The typical time resolution of our experimental set-up is ~800 ps. The decays were deconvoluted using DAS-6 decay analysis software. The acceptability of the fits was judged by \(\chi^2\) criteria and visual inspection of the residuals of the fitted function to the data. Mean (average) fluorescence lifetimes were calculated using the following equation:\(^{29}\)

\[
\tau_{av} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}
\]

in which \(\alpha_i\) is the pre-exponential factor corresponding to the \(i^{th}\) decay time constant, \(\tau_i\).

Reagents for cell study

A375, a human melanocarcinoma cell line, was procured from National Center for Cell Science, Pune, India, and used throughout the study. Cells were cultured in DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL), and 1% antibiotic mixture containing PSN (Gibco BRL) at 37 °C in a humidified incubator with 5% CO\(_2\) and cells were grown to 80–90% confluence, harvested with 0.025% trypsin (Gibco BRL) and 0.52 mM EDTA (Gibco BRL) in phosphate-buffered saline (PBS), plated at the desired cell concentration and allowed to reequilibrate for 24 h before any treatment. All experiments were conducted in DMEM containing 10% FBS and 1% PSN antibiotic.

Computational method

Full geometry optimizations of complexes 2 and 3 were carried out using the density functional theory method at the B3LYP level\(^{30}\) with Gaussian03 program package.\(^{31}\) The 6-31G(d) basis set was employed for all the elements except Zn and Cd atoms. For Zn and Cd, we used the LANL2DZ basis set, including Los Alamos Effective Core Potentials.\(^{32}\) The vibrational frequency calculations were performed to ensure that the optimized geometries represent the local minima of potential energy surface and there are only positive eigenvalues. The lowest 40 singlet–singlet vertical electronic excitations based on B3LYP/B3LYP optimized geometries were computed for the time-dependent density functional theory (TD-DFT) formalism\(^{33}\) in methanol using a conductor-like polarizable continuum model (CPCM).\(^{34}\) GaussSum\(^{35}\) was used to calculate the fractional contributions of various groups to each molecular orbital.

Synthesis of the chemosensor (PHMP) (1)

2,6-Diformyl-4-methylphenol was synthesized starting from p-cresol and following a published procedure.\(^{37}\) A methanolic solution (20 mL) of 2-hydrazino pyridine (0.218 g, 2 mmol) was added dropwise to the methanolic solution (15 mL) of 2,6-diformyl-4-methylphenol (0.164 g, 1 mmol) with constant stirring (Scheme 2). The stirring was continued for 30 min and the mixture was refluxed for 5 h at water bath temperature and cooled to room temperature. Excess methanol was removed using a rotary evaporator to obtain a yellow microcrystalline solid. The solid was filtered off, washed thoroughly with cold methanol and dried in a vacuum over fused CaCl\(_2\).

Yield: (0.305 g, 80%). M.P. 245 °C (decomp.). MS (m/z) 346 (M\(^{+}\), 100%), IR/cm\(^{-1}\): \(\nu\text{NH} 3325; \nu\text{COCN} 1661(s); 1597(s); \nu\text{N–N} 1515(s); \nu\text{py} 1023(s).\) Anal. calc. for C\(_{19}\)H\(_{18}\)N\(_6\)O: C, 65.89; H, 5.20; N, 24.27. Found: C, 65.72; H, 5.26; N, 24.21%.

Synthesis of [Cd\(_2\)(L\(_2\))\(_2\)(ClO\(_4\))\(_2\) (2) and [Zn\(_2\)(L\(_2\))\(_2\)(ClO\(_4\))\(_2\) (3)

The ligand PHMP (3.46 g, 10 mmol) was added to a hot solution of Cd(ClO\(_4\))\(_2\)·6H\(_2\)O (4.19 g, 10 mmol) in CH\(_3\)OH–CH\(_2\)CN (30 mL) [1:1 v/v]. The suspension was stirred for 1 h with constant stirring until complete dissolution of the ligand occurred. The resulting yellow solution was filtered to remove any undissolved ligand and left at room temperature. Yellow crystals suitable for
X-ray diffraction were isolated after standing for several days (yield 80%). IR/cm⁻¹: ν_{NaHCO₃} 3401, 3385; ν_{CO/CN} 1625(s), 1539(s); ν_{N-N} 1084(s); ν_{py} 1019(s). UV-Vis (λ max): 233, 322, 371 nm. Anal. calc. for [Cd₂(PHMP)₂(μ₂-O₂)₄](ClO₄)₂ (1): C, 40.86; H, 3.22; N, 30.11. Found: C, 40.87; H, 3.24; N, 30.09%.

Complex 3 was prepared similarly using Zn(ClO₄)₂·6H₂O instead of Cd(ClO₄)₂·6H₂O (yield 75%). IR/cm⁻¹: ν_{NaHCO₃} 3401, 3385; ν_{CO/CN} 1623(s), 1537(s); ν_{N-N} 1082(s); ν_{py} 1019(s). UV-Vis (λ max): 237, 321, 372 nm. Anal. calc. for [Zn₂(PHMP)₂(μ₂-O₂)₄](ClO₄)₂ (1): C, 44.62; H, 3.52; N, 32.87. Found: C, 44.64; H, 3.50; N, 32.84%.

**X-ray data collection and structure determination of 1**

A pale yellow colored crystal of 2 was mounted on a Cryoloop with Paratone-N oil and data were collected at 100 K with a Bruker APEX CCD system using Mo Kα alpha radiation. Data were corrected for absorption with SADABS and structures were solved by direct methods. All non-hydrogen atoms were refined anisotropically by Fourier full matrix least squares on F². Hydrogen atoms on N₂ and N₅ were found from a Fourier difference map and were allowed to refine while all other hydrogen atoms were placed in calculated positions with appropriate riding models.

**Fluorimetric analysis**

Fluorescence quantum yields (Φ) were estimated by integrating the area under the fluorescence curves with the following equation:

\[
Φ_{\text{sample}} = \frac{OD_{\text{sample}}}{OD_{\text{standard}}} \times \frac{A_{\text{sample}}}{A_{\text{standard}}} \times Φ_{\text{standard}}
\]

where A is the area under the fluorescence spectral curve and OD is the optical density of the compound at the excitation wavelength. The standard used for the measurement of the fluorescence quantum yield was anthracene (Φ = 0.32 in ethanol).

**Imaging system**

The imaging system was composed of a fluorescence microscope (Model: LEICA DMLS) with an objective lens of 20× magnification.

**Cell culture**

Cells were rinsed with PBS and incubated with DMEM containing chemosensor 1 making the final concentration up to 20 μM in DMEM [the stock solution (1 mM) was prepared by dissolving chemosensor 1 in the mixed solvent (DMSO : water = 1 : 9 (v/v))] for 10 min at 37 °C. After incubation, bright field and fluorescence images of A375 cells were taken using a fluorescence microscope (Model: LEICA DMLS) with an objective lens of 20× magnification as well as fluorescence images of A375 cells (pre-incubated with 20 μM 1) were taken with the addition of different concentrations (10–50 μM) of zinc nitrate salt at a 10 min interval and consequently fluorescence images were taken after further addition of either EDTA (100 μM) or TPEN (100 μM).

**Cell cytotoxicity assay**

In order to test the cytotoxicity of chemosensor 1, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by the procedure described earlier. After treatment with chemosensor 1 (1, 10, 20, 50 and 100 μM) for 4 h, 10 μL of a MTT solution (10 mg mL⁻¹ PBS) was added into each well of a 96-well culture plate and incubated continuously at 37 °C for 3 h. All media were removed from wells and 100 μL of acidic isopropyl alcohol was added into each well. The intracellular formazan crystals (blue-violet) formed were solubilized with 0.04 N acidic isopropyl alcohol and the absorbance of the solution was measured at a 595 nm wavelength with a microplate reader (Model: THERMO MULTI SCAN EX). The cell viability was expressed as the optical density ratio of the treatment to control. Values are mean ± standard deviation of three independent experiments. The cell cytotoxicity was calculated as % cell cytotoxicity = 100% − % cell viability.

**Results and discussion**

Complexes 2 and 3 were prepared from 2,6-diformyl-4-methylphenol. 2 and 3 were synthesized in a very facile and identical way. It was characterized by X-ray crystallographic analyses, elemental analyses, IR, and UV-Vis spectroscopy.

**X-ray crystal structure of [Cd₂(L)₂](ClO₄)₂ (2)**

A schematic view of the cationic motif in 2 is displayed in Fig. 1; the selected bond lengths and angles are listed in Tables S1 and S2 in the ESI. Complex 2 crystallises in space group C2/c and its unit cell is comprised of four molecules. The structure is built up of dinuclear Cd₂(L)₂⁺ entities in which two cadmium centres are connected through double alkoxo bridges. Within the subunits, the six-coordinate Cd ions are bridged by the deprotonated phenolic oxygen of the ligand at a distance of 3.587 Å. Further donor atoms are the imine and pyridine nitrogens of the ligand side arms. The Cd1–O1–Cd2 angle of 106.08(6)° compares well with the values reported for mono(μ-oxo) dicadmium complexes. There are two perchlorate anions present in each dimeric unit. The coordination of Cd is satisfied....
by the $\text{N}_2\text{O}_2$ chromophore. The equatorial plane consists of $\text{N}1–\text{N}3–\text{N}3_a–\text{O}1$ and $\text{N}4–\text{N}6–\text{N}6_a–\text{O}1_a$ for $\text{Cd}1$ and $\text{Cd}2$ respectively. The axial positions are occupied by $\text{N}1_a$ and $\text{O}1_a$ for $\text{Cd}1$ and $\text{N}4_a$ and $\text{O}1_a$ for $\text{Cd}2$. The equatorial and axial bond distances fall in the ranges 2.2440–2.339 and 2.2440–2.301 for both the $\text{Cd}$ centres.

**Absorption studies**

Fig. 2 depicts spectrophotometric changes upon titrating a fixed concentration of $\text{1} (40 \, \mu\text{M})$ with incremental additions of $\text{Zn(ClO}_4)_2$ (100 μM) in MeOH using HEPES buffer [50 mM, DMSO : water = 1 : 9 (v/v), pH = 7.2]. The absorption spectrum of $\text{1}$ in aqueous methanol ($1 \times 10^{-6}$ M) displayed a broad ILCT absorption band centered at 237 nm, 321 nm and 372 nm (Fig. 2). However, addition of $\text{Zn}^{2+}$ induced dramatic modification both in the maxima and shape of the said bands of $\text{1}$. The band maxima at 237 nm gradually shifted to 246 nm and the band at 321 gradually decreased and three new bands appeared at 295 nm, 326 nm and 343 nm. The band at 372 nm was diminished and a new sharp peak arose at 404 nm and finally the colourless solution turned yellow. This absorption peaks were expected to correspond to coordination of $\text{1}$ with $\text{Zn}^{2+}$. These phenomena illustrated the transformation from free $\text{1}$ to the $\text{Zn}^{2+}$-coordinated species (3). The UV-visible spectral response of $\text{1} (1 \times 10^{-6}$ M) upon introducing perchlorates of biologically significant ions such as $\text{Na}^+$, $\text{Mg}^{2+}$, $\text{K}^+$, $\text{Ca}^{2+}$, $\text{Mn}^{2+}$, $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Co}^{2+}$, $\text{Ni}^{2+}$, $\text{Cu}^{2+}$, $\text{Zn}^{2+}$, $\text{Cd}^{2+}$, $\text{Hg}^{2+}$ and $\text{Pb}^{2+}$ (100 μM each metal ion) in a HEPES buffer [50 μM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C ($\lambda_{\text{exc}} = 350 \, \text{nm}$).

Fig. 3 Emission spectra of $\text{1} (40 \, \mu\text{M})$ in the presence of $\text{Zn}^{2+}$, $\text{Na}^+$, $\text{Mg}^{2+}$, $\text{K}^+$, $\text{Ca}^{2+}$, $\text{Mn}^{2+}$, $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Co}^{2+}$, $\text{Ni}^{2+}$, $\text{Cu}^{2+}$, $\text{Zn}^{2+}$, $\text{Cd}^{2+}$, $\text{Hg}^{2+}$ and $\text{Pb}^{2+}$ (100 μM each metal ion) in a HEPES buffer [50 μM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C ($\lambda_{\text{exc}} = 350 \, \text{nm}$).

Evidently, the changes in the emission spectral profile of $\text{1}$ were significantly more pronounced in the presence of $\text{Zn}^{2+}$ ($\Phi = 0.280$) compared to the other metal ions. It is noteworthy to mention here that unlike many other $\text{Zn}^{2+}$ sensors reported so far where $\text{Cd}^{2+}$, a stereoelectronic isostere of $\text{Zn}^{2+}$, was found to interfere with the sensing but $\text{1}$ showed a negligible fluorescence enhancement with the emission centred at 476 nm in the presence of $\text{Cd}^{2+} (\Phi = 0.130)$, thereby making it advantageous to distinguish between these two ions. The fluorescence intensity of $\text{1} (40 \, \mu\text{M})$ significantly increased when various concentrations of $\text{Zn}^{2+}$ (5–100 μM) were added (Fig. 4), and the fluorescence quantum yield (0.280) increased more than 14-fold. This enhancement is attributed to the introduction of $\text{Zn}^{2+}$ along with the strong complexation occurring with $\text{1}$. Upon complexation, the lone pair of electrons on the $\text{N}$ atom of chemosensor $\text{1}$ is no longer available for PET, leading to fluorescence enhancement. In addition, the enhancement of

**Fluorescence properties and binding behavior**

Fig. 3 shows various metal dependent emission intensities at 476 nm for different metal ions in methanol using HEPES buffer [50 mM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C ($\lambda_{\text{exc}} = 350 \, \text{nm}$).

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**Fig. 2** UV-Vis spectral changes of $\text{1} (40 \, \mu\text{M})$ upon the addition of $\text{Zn}^{2+}$ in a HEPES buffer [50 μM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C, [$\text{Zn}^{2+}$] = 5–100 μM.

**Fig. 4** Emission spectra of $\text{1} (40 \, \mu\text{M})$ in the presence of various concentrations of $\text{Zn}^{2+}$ in a HEPES buffer [50 μM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C ($\lambda_{\text{exc}} = 350 \, \text{nm}$).
the fluorescence intensity was due to the formation of a 1–Zn²⁺ complex, which resulted in the selective CHEF effect. Interestingly, the introduction of other metal ions causes the fluorescence intensity to be either unchanged or weakened. A metal ion selectivity study (Fig. S1 in the ESI†) was then performed for 1 to understand this phenomenon under identical experimental conditions. The fluorescence intensity of 1 (1 μM) was unaffected upon the addition of Na⁺, Mg²⁺, K⁺, Ca²⁺, Mn²⁺, Fe²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, Hg²⁺ and Pb²⁺ (100 μM).

On the basis of non-linear fitting (Fig. S2a in the ESI†), the binding constant was found to be 3.2 × 10⁷ M⁻¹.⁹ The stoichiometry of the coordinating species was examined by the method of continuous variation (Job’s plot) and found to be 1 : 1 with respect to 1 to Zn²⁺ (Fig. S3a in the ESI†). Based on this spectral evidence, mass spectral (Fig. S3b in the ESI†) and CHN analysis, the 1 + Zn²⁺ complex was proposed to have the molecular structure as shown in Fig. 5. The peak at 410.9852 seems to correspond to a peak with a +2 charge based on some of the peak separations (0.5 mass unit: 411.9780, 412.4804, 412.9830). As the pH dependence of fluorescence is generally undesirable in biological applications, the effect of pH on fluorescence was also studied in methanol. It was found that the fluorescence intensity of 1 at 479 nm remained unaffected between pH 5.5 and 9 which makes it suitable for application under physiological conditions (Fig. S4 in the ESI†). These results indicate that 1 can be used as a selective fluorescent probe to recognize and distinguish Zn²⁺ in the presence of various interfering and biologically relevant metal ions. We also carried out a reversibility experiment which proved that Zn²⁺ binding to 1 is reversible. In the presence of EDTA, due to its strong affinity for Zn²⁺, decomplexation of the 1 + Zn²⁺ complex takes place thereby resulting in the quenching of emission at 476 nm (Fig. S5 in the ESI†). A key requirement for an ideal chemosensor function is that guest binding must occur reversibly. As shown in Fig. 8, after the addition of EDTA (100 μM), the emission intensity of the 1 + Zn²⁺ complex was almost completely quenched. The reaction of this Zn²⁺ complex solution with the chelating ligand EDTA ended up with the formation of the original ligand (quantum yield = 0.020). Obviously this gives a tacit support towards the reversible binding of the ligand with formation of Zn²⁺.

Ratiometric displacement of Cd²⁺ by Zn²⁺

The ratiometric displacement of Cd²⁺ ions from the L–Cd²⁺ complex by Zn²⁺ ions was studied from the emission spectral change of the metal sensor titration curve as shown in Fig. 6. For the ratiometric study, 1.5 μM 1 in HEPES buffer was continuously titrated with increasing concentration of Cd²⁺ ions. The spectra show the generation of a blue shifted emission band with maximum at ~476 nm. After the completion of the complexation process with Cd²⁺ ions, the resultant solution was titrated with Zn²⁺ ions. With continuous increase in the Zn²⁺ ion concentration, the concomitant enhancement of the emission band was observed with emission maximum at ~476 nm. The resultant solution was kept for 30 min and then the emission spectrum was recorded. The spectral band position and emission intensity (Φ = 0.280) were found to be intact which suggests that the L–Zn²⁺ complex is more stable than the L–Cd²⁺ complex. The ratiometric displacement is presented in Scheme 1.

Time resolved measurement

A picosecond time-resolved fluorescence technique has been used to examine the excited state behavior of free sensor 1 and its metal complexes in MeOH solvent (Fig. 7). According to the equations, τ⁻¹ = kᵣ + kₙᵣ and kᵣ = Φᵣ/τ, the radiative rate constant kᵣ and the total nonradiative rate constant kₙᵣ of 1 and Zn²⁺-bound species were calculated. The fluorescence decay curves of sensor L and its metal complexes were fitted by bi-exponential functions with acceptable χ² values and all the

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**Fig. 5** Probable cationic structure of the 1 + Zn²⁺ complex (3).
The data suggest that the factor that induces fluorescence enhancement is mainly due to the more than 366 times increase of $k_r$. This enhancement is attributed to the introduction of Zn$^{2+}$ and the strong complexation occurring with 1, as is evident from the large binding constant value. Upon complexation, the lone pair of electrons on the N atom of chemosensor 1 is no longer available for PET, leading to fluorescence enhancement.

**DFT and TDDFT calculations**

The geometries of 2 and 3 are fully optimized by the DFT/B3LYP method using Gaussian03 software. The optimized structural parameters are summarized in Tables S4 and S5 in the ESI for complexes 2 and 3 respectively. The calculated bond parameters are fairly well reproduced the X-ray crystal structure data for complex 3. The optimized geometry of 3 is represented in Fig. 8. The bond distances and bond angles are close to the literature values. Contour plots of some selected molecular orbitals are given in Fig. 9 and 10 for 2 and 3 respectively. The high energy occupied molecular orbitals (HOMOs) and low energy unoccupied molecular orbitals (LUMOs) have been constituted by >90% ligand group of orbitals with a HOMO–LUMO energy gap of 3.58 eV and 3.56 eV for 2 and 3 respectively.

To get detailed insight into the electronic spectra of the complexes, TDDFT/CPCM calculations have been performed in methanol. The calculated singlet–singlet vertical electronic transitions are summarized in Tables S6 and S7 in the ESI for 2 and 3 respectively. The calculated electronic transitions are very close to the experimental electronic bands. All the transitions in the complexes have intra-ligand charge transfer (ILCT) origin.

**Cell studies**

The intracellular Zn$^{2+}$ imaging behavior of 1 was studied on A375, a human melanoma cell line, by fluorescence microscopy. After incubation with chemosensor 1 (20 μM) at 37 °C for 10 min, the cells displayed very faint intracellular fluorescence.
However, the cells exhibited intense fluorescence when exogenous Zn\(^{2+}\) was introduced into the cells via incubation with a zinc nitrate salt solution (Fig. 11C–E). The intensive fluorescence behavior was, however, strongly suppressed when either EDTA or TPEN (100 \(\mu\)M) was also added to the medium. Because of the fact that both EDTA and TPEN have a strong tendency to bind with Zn\(^{2+}\) ions, the sensors with fluorescence property were competitively inhibited to bind with Zn\(^{2+}\) ions; hence the intensive fluorescence disappeared (Fig. 11F and G). Therefore, this renders confirmatory evidence of the sensor to have a specific ability to sense Zn\(^{2+}\) ions. The fluorescence responses of \(\mathbf{1}\) with various concentrations of added Zn\(^{2+}\) are clearly evident from the cellular imaging. Hence, these results indicate that \(\mathbf{1}\) is an efficient candidate for monitoring changes in the intracellular Zn\(^{2+}\) concentration under certain biological conditions; in order to test its cytotoxicity, we performed MTT assay in human melanoma cancer cells treated with various concentrations of chemosensor \(\mathbf{1}\) for up to 4 h. As shown in Fig. 11A, 20 \(\mu\)M \(\mathbf{1}\) did not show significant cytotoxic effects on human melanoma cancer cells for at least up to 4 h of its treatment. This thus suggests that \(\mathbf{1}\) can be readily used for cellular application at the indicated dose and time of incubation without much concern about its cytotoxicity (Fig. 12).

Conclusions

In conclusion, we have successfully developed a new 2,6-diformyl-4-methylphenol based fluorescent chemical probe \(\mathbf{1}\), and it displays high selectivity and sensitivity for Zn\(^{2+}\) in a HEPES buffer [50 mM, DMSO : water = 1 : 9 (v/v), pH = 7.2] at 25 °C. In the presence of Zn\(^{2+}\), significant fluorescence enhancement is achieved, and it is found that the quantum yield was increased more than 14-fold. This is accounted for by the formation of the Zn\(^{2+}\) complex \(\mathbf{3}\) with a high value of the binding constant. By incubation of cultured living cells (A375) with \(\mathbf{1}\), intracellular Zn\(^{2+}\) concentrations could be monitored through selective fluorescence sensing and likewise Zn\(^{2+}\) ions in certain other cells in biological systems that permit in vitro culture could also be monitored with this sensor.

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