Gadolinium(III)—fluorescein complex as a dual modal probe for MRI and fluorescence zinc sensing

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A bimodal imaging Gd–Zpy probe based on magnetic resonance imaging and fluorescence sensing has been synthesized and characterized. Gd–Zpy features a bright green emission and a turn-on fluorescent response manner with high sensitivity for Zn2+ in aqueous solution and is able to luminescent imaging intracellular Zn2+ levels within living cells. It exhibits a 130% increase of the longitudinal relaxation time and a 115% increase of transverse relaxive time upon addition of Zn2+. The results demonstrated that the incorporating of the fluorescein dye having the efficient chelators within a high-spin Gd3+ system was a powerful approach to achieve dual modal probes for MRI and fluorescence sensing.

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1. Introduction

Zinc is a ubiquitous and indispensable element in the human body and the second most abundant d-block metal after iron. Disruptions of zinc homeostasis have been implicated in number of health disorders such as Alzheimer’s disease, diabetes, and certain cancers. Although most forms of biological zinc are tightly bound and serve as essential structural and catalytic components of metalloprotein scaffolds, mobile zinc pools are present in certain mammalian organs. The detailed molecular mechanisms of intracellular Zn2+ accumulation, trafficking, and function are still under debate. While an array of fluorescence sensors for mobile zinc(II) have been devised to help unravel these processes, new noninvasive imaging methods for zinc detection in intact live animals, including humans are therefore in high demand.

Magnetic resonance imaging (MRI) has become increasingly popular in experimental and clinical molecular imaging, because it is noninvasive and capable of producing three-dimensional representations of opaque organisms with high spatial and temporal resolution. While the combination of metal-based MRI contrast agents with selective molecular recognition elements provides a promising new class of chemosensors for molecular imaging of essential s-block and d-block metal ions in biological systems. And the quantitative measurements using MRI as a single modality are still challenging, because the local concentration of contrast agent is unknown, and the sensitivity of MRI technique is relative low compared to other modalities such as optical imaging or PET. In this regard, the attaching of a luminescent imaging to an MRI technique offers a potential powerful approach for the quantitative integration of molecular and cellular information about complex biological signalling networks at a system level, since the fluorescent metal sensors exhibited excellent sensitivity and multiplexed detection capabilities at the cellular level and have been widely exploited in biotechnology and biomedical research fields. The integration is particularly valuable for studying zinc(II), a metal ion involved in numerous molecular process globally, within an intact and complex body, rather than within an isolated in vitro.

High-spin Gd3+, with its seven unpaired electrons, is particularly well-suited to this task, and Gd3+ coordination complexes are currently used in 40–50% of clinical MRI applications. According to Solomon–Bloembergen–Morgan theory, the relaxivity are governed by a variety of factors including the number of bound water molecules (q), the rotational tumbling times (τR) and the mean residence lifetime of Gd3+-bound water molecules (τM).

Inspired by the synthetic strategy of Lippard for the dual-functional molecules for Zn2+ imaging, herein, we reported a water-soluble fluorescein–Gd3+ complex as a dual-function imaging platform for MRI and fluorescence sensing using a fluorescein derivative Zpy, 9-(carboxyphenyl)-2,7-dichloro-4,5-bis[2-pyridylmethyl]aminomethyl]-6-hydroxy-3-xanth enone as chelators. We envisioned that the large molar extinction coefficient and
high emission quantum yield of fluorescein derivatives will lead to an excellent sensitivity and high selectivity for luminescence sensing zinc(II) in aqueous media. And the high-spin \( \text{Gd}^{3+} \) moiety will exhibit larger relaxivity change comparing to the manganese(II) systems in these reported dual-functional zinc(II) probe. Fluorescence experiments established that \( \text{Gd}–\text{Zpy} \) was a bright green emission and a turn-on fluorescent response manner with high sensitivity for \( \text{Zn}^{2+} \) in aqueous solution and was able to luminescent imaging intracellular \( \text{Zn}^{2+} \) levels within living cells. MRI study also showed that \( \text{Gd}–\text{Zpy} \) could respond zinc concentration using the relaxivity change.

### 2. Results and discussion

#### 2.1. Synthesis and structural characterization of \( \text{Gd}–\text{Zpy} \)

Ligand \( \text{Zpy} \) was synthesized according to the literature. Compound \( \text{Gd}–\text{Zpy} \) was prepared by refluxing of \( \text{Zpy} \) and \( \text{Gd(NO}_3\text{)}_3 \cdot \text{6H}_2\text{O} \) in methanol followed by the addition of \( \text{NaClO}_4 \cdot \text{H}_2\text{O} \) to give a perchlorate salt. EA and X-ray powder diffraction evidenced the pure phase of bulk sample. ESI-MS spectra of \( \text{Gd}–\text{Zpy} \) exhibit four intense peaks at \( m/z = 1179.73, 1197.71, 1215.70 \) and \( 1234.01 \) with the isotopic distribution patterns separated by 0.50 Da. Through exact comparison of the experimental peaks with the simulation results obtained on the basis of natural isotopic abundances, these peaks were assigned to the species \([\text{Gd}_2(\text{Zpy})_2(\text{ClO}_4)_4(\text{H}_2\text{O})_2]^{2+}, [\text{Gd}_2(\text{Zpy})_2(\text{ClO}_4)_4(\text{H}_2\text{O})_4]^{4+}, [\text{Gd}_2(\text{Zpy})_2(\text{ClO}_4)_4(\text{H}_2\text{O})_2]^{2+}\) and \([\text{Gd}_2(\text{Zpy})_2(\text{ClO}_4)_4(\text{H}_2\text{O})_4]^{4+}\), respectively. This result suggested the formation of dimeric species in solution with water molecules coordinated to the metal ions. Crystals suitable for X-ray structural analysis were formed by diffusion methanol into a DMF solution for several days. Single crystal structural analysis reveals that a half of the dimeric catic species, two perchlorate anions, two DMF molecules and three lattice water molecules are found within an asymmetric unit. As shown in Fig. 1, the dimeric cationic species is composed of two deprotonated \( \text{Zpy} \) ligands and two \( \text{Gd}^{3+} \) ions. Each \( \text{Gd}^{3+} \) ion is nine coordinated by a hydroxyl moiety, three nitrogen atoms from one DPA moiety from one ligand and a bidentate carboxylate moiety from another ligand. The remaining three sites are occupied by three coordinated water molecules, benefiting the increase of the relaxation rate. The values of the \( \text{Gd}–\text{O} \) bond lengths and the \( \text{Gd}–\text{N} \) bond lengths are in the range of 2.363(3)–2.556(3)\( \text{Å} \) and 2.589(4)–2.643(4)\( \text{Å} \), respectively, comparable well to those reported for other \( \text{Gd}^{3+} \) complex. Each ligand acts as a hexadentate chelating to connect two \( \text{Gd}^{3+} \) ions into a dimeric species.

![Fig. 1. Crystal structure of \( \text{Gd}–\text{Zpy} \) with the atomic-numbering scheme of the metal ions and the coordination donors. Anions, solvents and H atoms are omitted for clarity. Selected bond lengths (\( \text{Å} \)): \( \text{Gd}(1)–\text{O}(1) 2.556(3), \text{Gd}(1)–\text{O}(2) 2.403(3), \text{Gd}(1)–\text{O}(3A) 2.363(3), \text{Gd}(1)–\text{O}(1W) 2.494(4), \text{Gd}(1)–\text{O}(2W) 2.392(4), \text{Gd}(1)–\text{O}(3W) 2.427(3), \text{Gd}(1)–\text{N}(1A) 2.619(4), \text{Gd}(1)–\text{N}(2A) 2.589(4), \text{Gd}(1)–\text{N}(3A) 2.643(4) \). Symmetry code: \( \text{A}–x, 2–y, –z+2 \).](image1)

#### 2.2. \( \text{Gd}–\text{Zpy} \) as zinc fluorescent chemosensor

In a phosphate-buffered saline (PBS) (pH 7.0), the fluorescence of the metal-free probe \( \text{Zpy} \) exhibits a small but significant enhancement upon addition of \( \text{Gd}^{3+} \) gradually. The \( \text{Gd}^{3+} \)-induced fluorescence enhancement is attributed to a mechanism of intramolecular photoinduced electron transfer from a deprotonated benzylc amine resulting in fluorophore quenching and alleviation of such quenching by \( \text{Gd}^{3+} \) chelate formation. The preliminary titration experiments revealed the formation of a 1:1 \( \text{Gd}^{3+} / \text{Zpy} \) complex species in solution. The apparent dissociation constant values for the complex were obtained by linear least-squares fitting of fluorescence titration data to give 1.8 \( \mu \text{M} \). The apparent \( K_d \) value is about 10\(^2\) times greater than those for \( \text{Zn}^{2+} \) in \([\text{ZP1Zn}^2]\), indicating that the \( \text{Gd}^{3+} \) displacement by zinc ions is thermodynamically highly favoured. \( \text{Gd}–\text{Zpy} \) displays an intense absorption band centred at around 510 nm. Upon addition of \( \text{Zn}^{2+} \) to the solution of up to 20 \( \mu \text{M} \) in aqueous solutions, the absorption band shifted to 503 nm with the absorption coefficient increasing a little. When excited at 488 nm, \( \text{Gd}–\text{Zpy} \) (10 \( \mu \text{M} \)) exhibited a strong fluorescein-base emission band with a corresponding emission maximum at 528 nm (\( \Phi = 0.21 \)) in an aqueous solution. Upon addition of \( \text{Zn}^{2+} \), the fluorescence of \( \text{Gd}–\text{Zpy} \) was increasing by ca. fourfold with the quantum yield reached to \( \Phi = 0.8 \) after approximately 20 \( \mu \text{M} \) equivalent of \( \text{Zn}^{2+} \) added (Fig. 2). Job’s plot evaluated from the fluorescence spectra of the titration solution exhibited an inflection point at about 0.66, suggesting the formation of a 1:2 \( \text{Zpy}/\text{Zn}^{2+} \) complex in the aqueous solution. The sigmoidal shape of the titration curve suggested that the \( \text{Gd}^{3+} \) in the \( \text{Gd}–\text{Zpy} \) was substituted by \( \text{Zn}^{2+} \). The \( \text{Zn}^{2+} \)-induced fluorescence response for the displacement reaction was as fast as that of simple \( \text{Zn}^{2+} \) association in the time scale of our steady-

![Fig. 2. Family of fluorescence spectra of \( \text{Gd}–\text{Zpy} \) (10 \( \mu \text{M} \)) probe showing the luminescent enhancement upon addition of \( \text{Zn}^{2+} \) up to 20 \( \mu \text{M} \) in aqueous solutions. Inset picture shows the fluorescence at 528 nm of compound \( \text{Gd}–\text{Zpy} \) (10 \( \mu \text{M} \)) as a function of the zinc concentration. Excitation was at 488 nm.](image2)
state measurements (i.e., typically one titrant addition per minute). Under the optimized conditions (Gd–Zpy was employed at 2 μM), the limit of the detection for Zn" was estimated ca. 1 ppb, and the fluorescence intensity of Gd–Zpy was nearly proportional to the amount of Zn" added.

The selectivity of Gd–Zpy towards the presence of various biologically relevant metal ions and a number of divalent first-row transition metals was investigated (Fig. 3). With the exception of Fe" and Cu", which bind to Gd–Zpy and quench the fluorescence, other metals induce only slight quenching in the fluorescence intensity. Binding by these metal ions appears to be weak since subsequent addition of Zn" to the Gd–Zpy solution enhances fluorescence. The alkaline metals and alkaline-earth metals Na" and Ca" do not diminish the Zn"-induced emission. The results suggested that Gd–Zpy exhibits excellent selectivity for Zn" over these alkali and alkaline earth cations. In contrast, as expected from the Irving–Williams series, Zn"-induced fluorescence turn-on does not occur in the presence of the tightly binding paramagnetic Cu" and Fe" ions.

![Fig. 3. Fluorescence responses of Gd–Zpy (10 μM) to various metal cations in an aqueous solution. The pink bars represent the emission intensities of Gd–Zpy in the presence of 20 μM of Na", K", Ca"", Mg" and Al" and the other cations of interest, respectively. The cyan bars represent the change of the emission that occurs upon the subsequent addition of 20 μM of Zn" to the above solution. The emission was integrated at 528 nm with the excitation at 488 nm.](image)

2.3. Fluorescence monitoring of zinc in living cells

Gd–Zpy exhibited the similar pH-dependent fluorescent behaviour with those dichlororfluorescein-based Zn" sensors. The excellent water solubility and the high sensitivity of Gd–Zpy enables us to investigate the possible applications in monitoring Zn" in living cells. HeLa cells were incubated for 15 min with 10 μM Gd–Zpy at room temperature to allow the probe to permeate into the cells. Under selective excitation with longer-wavelength light at 488 nm, cells gave weak intracellular fluorescence. When the cells stained with solution containing Gd–Zpy were incubated with Zn" (20 μM) in normal saline for another 15 min and washed, a significant green fluorescence intensity in live cells was observed. Bright-field measurement demonstrated that the cells remained viable after treatment with the probes solution and Zn" throughout the imaging experiments (about 2–3 h). These experiments indicate that Gd–Zpy can be used to monitor intracellular Zn" (Fig. 4).

![Fig. 4. Fluorescence images of HeLa cells incubated with Gd–Zpy. (a): Cells treated with Gd–Zpy in the absence of 20 μM of external zinc ions. (b), (d): DIC images. (c): Cells treated with Gd–Zpy in the present of 20 μM of external zinc ions. The excitation was at 488 nm. Micrographs are shown relative to the same intensity scale for direct comparison.](image)

2.4. Gd–Zpy as zinc MRI chemosensor

The most abundant molecular species in biological tissues is water. It is the quantum mechanical ‘spin’ of the water proton nuclei that ultimately gives rise to the signal in all imaging experiments. MRI agents enhance the intrinsic differences in the nuclei that ultimately gives rise to the signal in all imaging experiments. MRI agents affect longitudinal (r1) and transverse (r2) relaxivities to a similar degree and are better suited for T1-weighted imaging. Most of the gadolinium complexes currently used as contrast agents have only one water molecule coordinated to the metal and show low relaxivity (r1=4.5 s⁻¹ mM⁻¹; 20 MHz, 298 K) relative to the theoretically attainable maximum. The initial sensor Gd–Zpy complex having three water molecules coordinated to each of the Gd" ions, thus shows a quite high proton relaxivity (155.14 s⁻¹ mM⁻¹) in the absence of Zn" ions (Fig. 5).

![Fig. 5. T1- and T2-weighted MRIs obtained from aqueous solutions of Gd–Zpy (0.2 mM based on Zpy). (a): T1-weighted MRIs of Gd–Zpy incubated with Zn" (1: 0 mM, 2: 0.1 mM, 3: 0.2 mM). (b): T2-weighted MRIs of Gd–Zpy incubated with Zn" (1: 0 mM, 2: 0.1 mM, 3: 0.2 mM). (c): Relaxation change profile Gd–Zpy upon addition of Zn" up to 1.0 mM.](image)

As shown in Table 1, addition of 0.2 mM equiv of Zn" causes 130% and 115% increase in both T1 and T2, respectively, with longitudinal relaxivity (r1) decreasing to 65.71 s⁻¹ mM⁻¹ from 155.14 s⁻¹ mM⁻¹, and the transverse relaxivity (r2) decreasing to 84.45 s⁻¹ mM⁻¹ from 185.06 s⁻¹ mM⁻¹. Although there are several MRI-based high sensitive Zn" sensors, the responses of Zn"...
through both $T_1$ and $T_2$ are still significant. Given that decreasing in relaxivity need the decrease of number of inner-sphere water molecules in the q-modulation, the significant decrease in the relaxivity observed when bound to Zn$^{2+}$ is likely reflected to the complete changes of the coordination geometry of Gd$^{3+}$. The geometry variation always caused the faster rotational tumbling time ($\tau_\text{R}$), or the decrease in water exchange rates to invoke a relaxivity.\(^{49}\) Relaxivity change profile of Gd–Zpy upon addition of Zn$^{2+}$ was shown in Fig. 5c. With the further addition of Zn$^{2+}$ to 0.4 mM, the longitudinal relaxivity ($r_1$) gradually decreased to ca. 25 s$^{-1}$ mM$^{-1}$. This value is similar to that of free Gd(NO$_3$)$_3$ (relaxivity of 26.12 s$^{-1}$ mM$^{-1}$), confirming the replacement of Gd$^{3+}$ by Zn$^{2+}$.

### 3. Conclusion

In conclusion, we have synthesized a unique paramagnetic Gd–Zpy complex able to serve as a dual modal probe for zinc magnetic resonance imaging and fluorescence sensing. Gd–Zpy features a bright green emission and a turn-off fluorescence response manner with high sensitivity for Zn$^{2+}$ features a bright green emission and a turn-on fluorescent probes.

### 4. Experimental

#### 4.1. Instruments and reagents

Unless otherwise stated, materials were obtained from commercial suppliers and used without further purification. $^1$H NMR spectra were recorded on a VARIAN INOVA–400 spectrometer with chemical shifts reported as parts per million (in CDCl$_3$-spectra were recorded on a VARIAN INOVA-400 spectrometer with internal standard). Mass spectrometric data were obtained on HP1100LC/MSD and LCQ-Toftmass spectrometers. IR spectra were recorded using KBr pellets on a Vector 22 Bruker spectrophotometer in the 4000–400 cm$^{-1}$ regions. Fluorescence emission spectra were obtained using EDINBURGH FS920 luminescence spectrometer. UV–vis spectra were measured on an HP 8453 spectrometer. For all fluorescence measurements, both excitation and emission slit widths were 2 nm. Cell imaging was measured on Nikon eclipse TE2000-5 inverted fluorescence microscopy.

#### 4.2. X-ray crystallographic characterization

Intensity data of Gd–Zpy were collected on Bruker Smart APEX II–CCD single crystal X-ray diffractometer equipped with a graphite-monochromated Mo Kα radiation ($\lambda=0.71073$ Å) using the SMART and SAINT programs.\(^{52}\) Data (45 frames) were collected at 200 K with an oscillation range of 1°/frame and an exposure time of 10 s/frame. Indexing and unit cell refinement were based on all observed reflections from those 72 frames. Crystal data of Gd–Zpy, $\text{C}_{104}\text{H}_{122}\text{Cl}_8\text{Gd}_2\text{N}_{16}\text{O}_{42}$, $M_w=2866.28$, monoclinic, space group $P2_1/n$, $a=15.048(2)$, $b=15.463(2)$, $c=26.837(3)$ Å, $\beta=103.96(1)^\circ$, $V=6059.9(12)$ Å$^3$, $\mu(\text{Mo Kα})=1.352$ mm$^{-1}$, $Z=2$. 35,302 Reflections were collected of which 10,643 reflections were unique ($R_{int}=0.0587$). The final refinement gave $R_1=0.0618$ and $wR_2=0.2017$ for 7238 reflections with $I>2\sigma(I)$. The structures were solved by direct methods and refined on $F^2$ using full matrix least-squares methods using SHELXTL version 5.1.\(^{63}\) Anisotropic thermal parameters were refined for non-hydrogen atoms within the main backbone of the molecules. Except the solvent molecules, hydrogen atoms were localized in their calculated positions and refined using a riding model.

#### 4.3. MRI experiment

MR relaxometry was performed on NMR Analyzing & Imaging system NMI20-Analyzer (Shanghai Niumag Corporation, a 0.5 T magnet, point resolution=156 × 156 μm, section thickness=0.6 mm, TE=11.4 ms, TR=121.4 ms for T$_1$-weighted MR images, TE=78.6 ms, TR=2080 ms for T$_2$-weighted MR images, number of acquisitions=1). The images were fitted using the Levenberg–Marquardt method to calculate $T_1$ values using the Matlab program. We calculated the specific relaxivities ($r_1$ and $r_2$) of the Gd–Zpy from the plot of $T_1$ and $T_2$ versus concentration of contrast agent. The signal intensities of each of the regions of interest in the T$_1$ map and the T$_2$ map were measured for each concentration, which were then used for $r_1$ and $r_2$ calculations, respectively.

#### 4.4. Cell incubation and imaging

HeLa cells were cultured in RPMI-1640 supplemented with 10% FCS (Invitrogen). Cells were seeded in 24-well flat-bottomed plates for Nikon eclipse TE2000-5 inverted fluorescence microscopy. After 12 h, HeLa cells were incubated with 10 μM compound Gd–Zpy (in the culture medium containing 0.5% DMSO) for 30 min at 37 °C under 5% CO$_2$ and then washed with phosphate-buffered saline (PBS) three times before incubating with 20 μM zinc(II) for another 30 min, and cells were rinsed with PBS three times again. The fluorescence imaging of Gd–Zpy in HeLa cells was observed under Nikon eclipse TE2000-5 inverted fluorescence microscopy with a 20× objective lens (excited with green light). For all images, the microscope settings, such as brightness, contrast, and exposure time were held constant to compare the relative intensity of intracellular zinc(II) fluorescence.

#### 4.5. Preparation

Gd–Zpy. Zpy (0.082 g, 0.1 mmol) and Gd(NO$_3$)$_3$·6H$_2$O (0.045 g, 0.1 mmol) were mixed in 20 ml methanol. After refluxing for 1 h and cooling to room temperature, NaClO$_4$·H$_2$O (0.028 g, 0.2 mmol) was added and refluxing was resumed for 1 h. The red precipitate formed was isolated and recrystallized from DMF–methanol (1:4). Yield: 60%. Crystals suitable for X-ray structural analysis were formed by diffusion methanol into a DMF solution containing Gd–Zpy for several days. Anal. Calcd for Gd–Zpy (%): C: 43.54; H: 4.29; N: 7.82; Found (%): C: 43.51; H: 4.27; N: 7.84. IR (solid KBr pellet $\nu$(cm$^{-1}$): 3430 br, 1650 s, 1640 m, 1460 s, 1370 m, 1277 m, 1120 s, 910 w, 766 w, 673 w, 625 m, 484 w.

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### Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2011.10.034.