Phosphorescent Zinc Probe for Reversible Turn-On Detection with Bathochromically Shifted Emission

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ABSTRACT: Phosphorescent molecules are attractive complements to fluorescent compounds for bioimaging. Time-gated acquisition of the long-lived phosphorescence signals provides an effective means to eliminate unwanted background noises due to short-lived autoﬂuorescence. We have previously investigated the molecular principles governing modulation of photoinduced electron transfer in phosphorescence zinc probes that were based on biscyclometalated Ir(III) complexes (Woo, H. et al. J. Am. Chem. Soc. 2013, 135, 4771–4787). The studies established that phosphorescence turn-on responses would be attainable for Ir(III) complexes with high triplet-state energies. This sets an upper limit to an emission wavelength, restricting the development of red- or near-IR-phosphorescence turn-on probes. To address this challenge, we designed and synthesized a new phosphorescent probe having an electron-deﬁcient 2-(2-pyridyl)pyrazine diimine ligand tethering a di(2-picolyl)amine (DPA) zinc receptor. This ligand control led to red phosphorescence emission (λ_{ems} = 596 nm), with an excited-state reduction potential (E_{red}^* ) retained as high as 1.44 V versus standard calomel electrode (SCE). The E_{red}^* value was more positive than the ground-state oxidation potential of DPA (1.05 V vs SCE), permitting an occurrence of photoinduced electron transfer at a rate of 2 × 10^7 s^{-1}. Zinc binding at DPA abolished the electron transfer to produce phosphorescence turn-on signaling. The probe was capable of detecting zinc ions selectively over other competing biological metal ions in aqueous buffer solutions (pH 7.4, 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid)) with the zinc dissociation constant of 109 pM. Finally, bioimaging utility of the probe has been successfully demonstrated by visualizing exogenously supplied zinc ions in live HeLa cells. The research described in this paper demonstrates that judicious ligand control enables retention of turn-on responses in the low-energy phosphorescence region.

I. INTRODUCTION

Phosphorescence modality offers unique advantages in bioimaging, as the long-lived emission facilitates utilization of time domains. Among the various classes of phosphorescent molecules, cyclometalated Ir(III) complexes have been most successful for bioimaging purposes. In particular, a range of phosphorescent sensors that reversibly report the spatiotemporal fluctuations of biometals have been developed. Photoinduced electron transfer (PeT) is utilized to create the metal ion probes, because the nonradiative PeT is several orders faster than phosphorescence transition. Metal ion binding abolishes the PeT, restoring phosphorescence emission. We investigated the effectiveness of PeT for creation of phosphorescence zinc probes, based on biscyclometalated Ir(III) complexes and a di(2-picolyl)amine (DPA) zinc receptor. The studies established that PeT from DPA to the Ir(III) complexes adhered to the Rehm–Weller behavior with −ΔG_{ PeT} = e \cdot [E_{red}^{*} (DPA) - E_{red}^{*} (Ir)] + n \cdot \left( -\Delta G_{Gibbs}^{*} \right) driving force for PeT; e, elementary charge; [DPA] the ground-state oxidation potential, [Ir] the ion-pairing term; E_{red}^{*} = E_{ox} + \Delta E_{T} are the ground-state reduction potential and the triplet-state energy, respectively.) Further mechanistic studies revealed that the PeT was located in the Marcus-normal region of electron transfer. On the basis of these findings, it has been predicted that phosphorescence emission of Ir(III) complexes with small ΔE_T cannot be affected by PeT (Scheme 1a). Indeed, we observed the absence of PeT for Ir(III) complexes exhibiting phosphorescence emission at peak wavelengths longer than 517 nm (i.e., −ΔG_{ PeT} < 0 when ΔE_T < 2.4 eV). This lower bound of ΔE_T is obviously greater than the case for PeT in fluorescence probes. This thermodynamic loss is ascribed to exchange energy (ΔE_{xy}) existing between singlet excited state (S_1) and triplet state (T_1) (Scheme 1b).

There are two potential approaches to address the challenge: One is to increase the E_{red}^* value of an Ir(III) complex, and the other is to decrease the E_{ox} value of a receptor. In the case of the former approach, the modulation of E_{red}^* should rely on control over E_{red} rather than ΔE_T to keep the emission in the long-wavelength regions. We envisioned that such control could be accomplished by incorporating an electron-deﬁcient diimine (N=N) ligand into biscyclometalated Ir(III) complexes.

Supporting Information

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To validate this hypothesis, we chose to employ 2-(2-pyridyl)pyrazine for the N^N ligand. The DPA zinc receptor was introduced to the N^N ligand through an amide linkage. We found that this ligand control indeed enabled red phosphorescence emission with a large \( E^*_{\text{red}} \) value (1.44 V vs standard calomel electrode (SCE)). Herein, we report the design, synthesis, characterizations, and bioimaging applications of a novel phosphorescence zinc probe (ZIrdap in Scheme 2). The sensor produced zinc-induced phosphorescence turn-on responses in the red-emission regions (\( \lambda_{\text{em}} = 596 \text{ nm} \)) in aqueous buffer solutions (pH 7.4, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)). Spectroscopic and electrochemical investigations revealed the occurrence of exoergic PeT (\( -\Delta G_{\text{PeT}} = 0.39 \text{ eV} \)), which was reversibly modulated by zinc binding. Finally, bioimaging utility of the probe was successfully demonstrated by phosphorescence visualization of exogenously supplied zinc ions in live HeLa cells.

II. RESULTS AND DISCUSSION

Synthesis of the Phosphorescence Zinc Sensor. The synthetic route to the phosphorescence zinc sensor (ZIrdap) is depicted in Scheme 2. The Pd(0)-catalyzed Stille reaction between 5-amino-2-bromopyrazine and 2-(tributylstannyl)-pyridine yielded the N^N ligand scaffold. Amidation of the primary amine in the N^N ligand with \( \alpha \)-bromoacetyl bromide, followed by substitution of the \( \alpha \)-bromide with di(2-picolyl)amine, yielded the DPA-appended N^N ligand (dap ligand, hereafter). Finally, chelation of the dap ligand to the bis-cyclometalated Ir(III) core produced the desired complex in an overall 16% yield. Spectroscopic characterization data obtained using multinuclear NMR spectroscopy and high-resolution mass spectrometry were fully consistent with the proposed structure. Reference complexes devoid of DPA and the amido DPA moiety were also synthesized to investigate the origin of the phosphorescence zinc responses (Irp and Irap; see Scheme 2 for the structures). Synthetic details and spectroscopic identification data are summarized in the Experimental Section.

Scheme 2. Synthesis of the Phosphorescent Zinc Sensor (ZIrdap) and Its Reference Compounds (Irp and Irap)
Mechanism for Phosphorescence Responses of the Zinc Sensor. UV–vis absorption spectra of ZIrdap and its reference compounds carry the bands characteristic of bis-cyclometalated Ir(III) complexes (Supporting Information, Figure S1).\textsuperscript{1,33} ZIrdap (10 μM) exhibits orange phosphorescence in acetonitrile solutions with a peak emission wavelength (λ<sub>ems</sub>) at 554 nm under photoexcitation at 345 nm (Figure 1a). Phosphorescence peak wavelengths of the two reference compounds are found in the similar region (555 and 551 nm for Irap and Irp, respectively), while Irp possesses pronounced vibronic structures. Of particular interest is the bathochromic shifts of the phosphorescence spectra of ZIrdap and Irap (λ<sub>ems</sub> = 591 nm) in aqueous buffer solutions (20 mM PIPES, pH 7.4). Such a shift is not observed for Irap. As shown in Figure 1b, the Lippert–Mataga plots of the phosphorescence peak wavelength energies as a function of the solvent polarity parameter demonstrate the strong positive solvatochromism for ZIrdap and Irap.\textsuperscript{34,35} These results suggest that the amidation of the N^N ligand confers significant charge-transfer character in the triplet state. Quantum chemical calculations based on time-dependent density functional theory (TD-DFT; CAM–B3LYP/LANL2DZ:6-311+G(d,p)) on ZIrdap, 10 μM ZIrdap in the absence (black) and presence (red) of 10 μM TPEN (blue). The asterisk (*) indicates a peak resulting from a harmonic of the excitation beam (345 nm).

Figure 1. (a) Phosphorescence spectra (dotted lines, CH₃CN; solid lines, aqueous 20 mM PIPES solution at pH 7.4) of 10 μM zinc sensor (ZIrdap) and its reference compounds (Irap and Irp) in the absence of zinc ion. Photoexcitation wavelength = 345 nm (ZIrdap and Irap) and 363 nm (Irp). The asterisks (*) indicate peaks resulting from the second harmonic of the excitation beams. (b) The Lippert–Mataga plot of the phosphorescence peak wavelengths of 10 μM ZIrdap, 10 μM Irap, and 10 μM Irp as a function of the solvent polarity parameter (f = (ε - 1)/(2ε + 1) = (n² - 1)/(2n² + 1); ε and n are the dielectric constant and refractive index of a solvent, respectively).

Figure 2. Phosphorescence spectra (deaerated acetonitrile solutions) of 10 μM ZIrdap in the absence (black) and presence (red) of 10 equiv of zinc ions and after the subsequent addition of 50 equiv of TPEN (blue). The asterisk (*) indicates a peak resulting from a harmonic of the excitation beam (345 nm).

To estimate −ΔG<sub>PeT</sub>, electrochemical measurements using cyclic and differential pulse voltammetry were performed (Figure 3). The voltammograms of ZIrdap display two anodic peaks at E<sub>ox</sub> = 1.05 and 1.60 V versus SCE, which correspond to oxidation of the dap ligand and the Ir(III/IV) redox process, respectively.\textsuperscript{1,2} The former and the latter waves are also found in the dap ligand and Irap, respectively, confirming the assignments. A reversible wave at E<sub>1/2</sub> = −1.12 V versus SCE due to the one-electron reduction of the N^N ligand is also observed. The E<sub>1/2</sub> value for the phosphorescent moiety in ZIrdap can thus be calculated to be 1.44 V versus SCE, using the ΔE<sub>f</sub> value of 2.56 eV that was determined at the restoration of the original spectrum, demonstrating excellent reversibility. As expected, the reference complexes without the DPA zinc receptors do not show zinc responses. Taken together, the solution behaviors suggest that modulation of PeT is most likely responsible for the phosphorescence turn-on response to zinc ions. Photophysical data for the Ir(III) complexes are compiled in Table 1.

Table 1.

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<th>Compound</th>
<th>μM</th>
<th>E&lt;sub&gt;1/2&lt;/sub&gt; (V)</th>
<th>ΔE&lt;sub&gt;f&lt;/sub&gt; (eV)</th>
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<tr>
<td>ZIrdap</td>
<td>10</td>
<td>−1.12</td>
<td>2.56</td>
</tr>
<tr>
<td>Irap</td>
<td>10</td>
<td>−1.12</td>
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Table 1.
Table 1. Photophysical Data for the Phosphorescent Sensor and Its Reference Compounds

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<th></th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (nm, μ × 10&lt;sup&gt;4&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt; cm&lt;sup&gt;−1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</th>
<th>Φ&lt;sub&gt;fl&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E&lt;sub&gt;vis&lt;/sub&gt; (V vs SCE)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E&lt;sub&gt;red&lt;/sub&gt; (V vs SCE)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;obs&lt;/sub&gt; (μs)</th>
<th>k&lt;sub&gt;φ&lt;/sub&gt; (x 10&lt;sup&gt;13&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>k&lt;sub&gt;nr&lt;/sub&gt; (x 10&lt;sup&gt;13&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>k&lt;sub&gt;PeT&lt;/sub&gt; (x 10&lt;sup&gt;5&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)&lt;sup&gt;f&lt;/sup&gt;</th>
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<td>ZIrdap</td>
<td>360 (1.98)</td>
<td>554&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.016</td>
<td>1.05 (a), 1.16 (qr)</td>
<td>−1.12 (r)</td>
<td>1.44</td>
<td>0.050&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>200</td>
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<tr>
<td>+ Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>354 (2.26)</td>
<td>556&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>N.A.</td>
<td>1.4</td>
<td>6.5&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
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<td>1.63 (r)</td>
<td>−1.16 (r)</td>
<td>1.39</td>
<td>1.2</td>
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<td>1.49 (ir)</td>
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<sup>a</sup>10 μM phosphorescent compound in deaerated acetonitrile; 298 K. <sup>b</sup>Phosphorescence quantum yield relatively determined by employing fluorescein as the standard (0.1 N NaOH; Φ<sub>fl</sub> = 0.79). <sup>c</sup>Determined by cyclic voltammetry. Conditions: scan rate = 100 mV/s; 1.00 mM in Ar-saturated acetonitrile containing 100 mM Bu<sub>4</sub>NPF<sub>6</sub> supporting electrolyte; a Pt wire counter and a Pt disc working electrodes; and a Ag/AgNO<sub>3</sub> couple as the pseudo reference electrode. (r) = reversible wave, (qr) = quasi reversible wave, and (N.A.) = not applicable. <sup>d</sup>Excited-state reduction potential, E<sub>red</sub> = E<sub>vis</sub> + ΔE<sub>T</sub>. <sup>e</sup>Phosphorescence lifetime for 50 μM compound in deaerated acetonitrile observed at λ<sub>em</sub> = 555 nm (λ<sub>em</sub> = 377 nm). <sup>f</sup>Determined by cyclic voltammetry. Conditions: scan rate = 100 mV/s; 1.00 mM in Ar-saturated acetonitrile containing 100 mM Bu<sub>4</sub>NPF<sub>6</sub> supporting electrolyte; a Pt wire counter and a Pt disc working electrodes; and a Ag/AgNO<sub>3</sub> couple as the pseudo reference electrode. <sup>i</sup>Nonradiative rate constant (k<sub>nr</sub>), ΔG<sub>PeT</sub> = 0.39 eV) for PeT in the zinc-free form of ZIrdap. To monitor the PeT process, we acquired phosphorescence decay traces at 555 nm of 50 μM ZIrdap after nanosecond pulsed excitation at 377 nm (Figure 4). In the zinc-free state of ZIrdap, the transient phosphorescence signals follow a biexponential decay model with time constants of 0.031 μs (53) and 0.79 μs (0.054) (values in the parentheses are pre-exponential factors). Corresponding weighted-average lifetime (τ<sub>obs</sub>) is 0.050 μs. This lifetime value is significantly shorter than those of 50 μM Irp (40 μs) and 50 μM Irp (1.2 μs). Addition of 5 equiv of zinc ions elongates the weighted-average lifetime to 6.5 μs, implying that PeT is abolished by the zinc binding. The PeT rate (k<sub>PeT</sub>) is estimated to be 2.0 × 10<sup>7</sup> s<sup>−1</sup> from the relationship k<sub>PeT</sub> = 1/τ<sub>obs</sub> − 1/τ<sub>obs</sub>(Zn), where τ<sub>obs</sub> and τ<sub>obs</sub>(Zn) are the observed phosphorescence lifetimes in the absence and presence of zinc ions, respectively. N.A. indicates not applicable. 10 μM phosphorescent compound in air-equilibrated aqueous buffer solution (20 mM PIPES, pH = 7.4); 298 K. <sup>j</sup>Weighted-average phosphorescence lifetime of biexponential decays: zinc-free ZIrdap, 0.031 μs (53) and 0.79 μs (0.054); zinc-bound ZIrdap, 0.070 μs (0.58) and 7.2 μs (0.46). <sup>k</sup>S equiv of zinc ions.

Figure 3. Cyclic (solid lines) and differential pulse (dotted lines) voltammograms of ZIrdap (blue), the dap ligand (gray), Irp (red), and Irp (black). Conditions: scan rate = 100 mV s<sup>−1</sup> (cyclic voltammetry) and 4.0 mV s<sup>−1</sup> (differential pulse voltammetry); 1.0 mM in Ar-saturated acetonitrile containing 0.10 M Bu<sub>4</sub>NPF<sub>6</sub> supporting electrolyte; a Pt wire counter electrode and a Pt disc working electrode; a Ag/AgNO<sub>3</sub> couple as the pseudo reference electrode.

Figure 4. Photoluminescence decay traces of 50 μM Irp (□), 50 μM Irp (○), and 50 μM ZIrdap (○) in the absence (empty symbols) and presence (filled symbols) of 5 equiv of zinc ions after nanosecond pulsed photoexcitation at 377 nm. Conditions: deaerated CH<sub>3</sub>CN solutions.
10^7 s^-1) within the experimental error. This finding supports the notion that PeT is the dominant quenching process in the zinc-free state of ZIrdap. In addition, the similarity between the k_{on} values of Irap (6.6 × 10^4 s^-1) and the zinc-bound form of ZIrdap (1.5 × 10^5 s^-1) provides evidence for complete suppression of PeT by zinc coordination.

Zinc Detection in the Aqueous Milieu. The phosphorescence intensity in the zinc-free form of 10 μM ZIrdap in air-equilibrated aqueous buffer solutions at pH 7.4 (20 mM PIPES) is higher than the case in acetonitrile. This increased basal intensity can be ascribed to protonation of DPA. Indeed, phosphorescence titration experiments demonstrate that the integrated phosphorescence intensity starts to increase with decreasing pH (Figure 5). The phosphorescence intensity remains relatively large in the range of pH 6–9, and decreases at pH < 6. The lower phosphorescence intensities at acidic pHs may be ascribed to decoordination of the dap ligand, because returning the pH to 7.4 does not restore the original spectrum. Actually, the electrospray ionization mass spectrometry (ESI MS; positive mode) spectra of the zinc-bound form of ZIrdap contained a peak at m/z 1146.0 that corresponded to [Zn(ZIrdap–H^+)(OH)(CH_3CN)_2]^+. This supports this hypothesis (Supporting Information, Figure S7). Furthermore, TD-DFT results for this structure predicted a hypsochromic shift (645 cm^-1) similar to the experimental observation (Supporting Information, Figures S2). Such a hypsochromic shift upon zinc binding is not observed in acetonitrile solutions.

Phosphorescence zinc titration for 10 μM ZIrdap was performed with increasing the total zinc concentration from 0 to 15 μM at a 0.05 μM interval (Figure 7a). The phosphorescence intensity increases in proportion with the added zinc concentration and levels off after 1.0 equiv of ZnCl_2.

Job’s plot analysis supports this 1:1 binding stoichiometry (Supporting Information, Figure S8). Corresponding zinc titration isotherm was fit to the analytical equations of the phosphorescence intensity and the free zinc concentration (eqs 1 and 2 in the Experimental Section). Iterative nonlinear least-squares fit to the equations returns the zinc dissociation constant (K_d) of 109 pM (Figure 7b). This value is 2 orders of magnitude smaller than the K_d value of the previous phosphorescence zinc sensor, indicating tight zinc binding of ZIrdap. The stronger binding capability may originate from participation of the amide group in zinc coordination.31,32

As shown in Figure 8, the phosphorescence response of ZIrdap is selective to zinc ions over other biological metal ions. Presence of 100 mM Na, 100 mM Mg, 100 mM K, 100 mM Ca, 50 μM Cr, 50 μM Mn, and 50 μM Fe(III) ions has no
influence on the phosphorescence zinc (50 μM) response of 10 μM ZIrdap. Weaker responses are observed when 50 μM Fe(II) ions are present, and paramagnetic ions, including Co, Ni, and Cu, abolish the ability of ZIrdap for phosphorescence zinc detection. Similar to other zinc probes,38−54 Cd binding produces turn-on responses. However, the interferences by these metal ions will be minimal due to low concentrations of the chelatable forms in the biological milieu.

Comparison of Zinc and Cadmium Binding Behaviors. Many zinc probes experience interferences by cadmium ions. ZIrdap also displays a phosphorescence turn-on response to Cd ions. However, as shown in Figure 9, the phosphorescence spectrum of the cadmium-bound form differs from those of zinc-free and -bound forms. The peak wavelength of the phosphorescence emission of the cadmium-bound form is 561 nm, which shifts hypsochromically from the zinc-free state by 1200 cm$^{-1}$. The extent of the blue shift is greater than the case for zinc binding (700 cm$^{-1}$). Another notable feature in the phosphorescence spectrum for the cadmium-bound form is the presence of a shoulder peak at 526 nm. This spectral signature is indicative of a Cd-binding mode different from a Zn-binding motif. Indeed, UV−vis absorption titration of ZIrdap (20 mM PIPES, pH 7.4) with increasing either ZnCl$_2$ or CdCl$_2$ concentrations revealed different spectral profiles. As shown in Figure 10, the metal-free ZIrdap exhibits the lowest-energy absorption band at 346 nm. The additions of CdCl$_2$ and ZnCl$_2$ lead to bathochromic shifts of the absorption band with multiple isosbestic points (CdCl$_2$, 358, 318, and 303 nm; ZnCl$_2$, 365, 301, and 273 nm). These red shifts are opposite to the blue shifts in the phosphorescence spectra (Figure 9), suggesting that polar ground states are formed upon metal binding. In the case of the cadmium titration, the absorbance at 337 nm decreases in a monotonic manner and reached a plateau after 1 equiv of Cd ions (Figure 10a,c). Similar linear behaviors are observed in the phosphorescence cadmium titration experiments (Figure 11). In sharp contrast, zinc titration produces two-stage spectral changes (Figure 10b,c). The incremental addition of ZnCl$_2$ up to 0.5 equiv yields absorption changes identical to the spectral signatures of the cadmium titration. Further addition of ZnCl$_2$ evokes the second absorption responses with a hypsochromic shift in the peak wavelength. An isosbestic point at 349 nm is observed, indicating that the second response is not due to instrumental
drifts. The titration isotherm plotting the absorbance at 337 nm versus the added ZnCl₂ concentration reveals the zinc response that is different from the cadmium case (Figure 10c).

To obtain insight into the binding geometries, ¹H NMR spectra were obtained for ZIrdap in the absence and presence of zinc and cadmium ions (Supporting Information, Figure 9). Although the complexity of the NMR spectra hampers complete assignments, the NMR spectra may suggest that the Cd-bound form retains the amido tautomer of the metal-free ZIrdap, whereas zinc binding induces the amido-to-iminolate tautomerization. Several attempts to obtain crystals for the metal-bound forms were not successful. On the basis of the results, it is proposed that ZIrdap adopts a predominant 2:1 (ZIrdap/Zn) binding geometry at low zinc concentrations (i.e., <0.5 equiv), where the amido form of ZIrdap is involved. At high zinc concentrations, a 1:1 complex having the iminolate form becomes the dominant species. This hypothesis is supported by the ESI MS and TD-DFT results for the zinc-bound ZIrdap (Supporting Information, Figures S2 and S7). However, further investigations should be performed to fully elucidate the structure−photophysical response relationship, because the phosphorescence zinc titration exhibits the one-stage (i.e., monotonic) increase in the phosphorescence intensity (Figure 7).

Finally, the cadmium dissociation constant (K_d) was determined using the phosphorescence titration results. Non-linear least-squares fit of the titration data to eq 1 and 2 in the Experimental Section yields a K_d value of 82 pM. This value is comparable to the K_d for zinc binding, implying that ZIrdap binds cadmium ions as strong as zinc ions.

Visualization of Intracellular Zinc. Having established the ability of ZIrdap for phosphorescence zinc detection, we sought to demonstrate bioimaging utility of the zinc probe (Figure 12). HeLa, human cervical cancer cells, were employed for the intracellular zinc imaging experiments. ZIrdap is permeable through the membrane of HeLa cells, as evidenced by phosphorescence halos (λ_ex = 405 nm and λ_em = 441−691 nm) in the cytosol of HeLa cells pretreated with 10 μM ZIrdap (10 min). The spectral profile of the intracellular phosphorescence signals resembles the phosphorescence spectrum of the zinc-bound form of ZIrdap recorded in acetonitrile (Figure 12c), confirming that the ZIrdap is responsible for the photoemission.⁵⁵ The non-negligible phosphorescence intensity might be due to protonation or endogenous zinc levels in HeLa cells comparable to the K_d value of ZIrdap.⁵⁶ Addition of the ionomeric form of zinc ions (50 μM ZnCl₂ + 100 μM sodium pyrithione) leads to a prompt increase in the phosphorescence intensity (Figure 12a). The corresponding
phosphorescence turn-on ratio as quantitated by an average of 11 dishes is 1.5 (Figure 12b), barely enabling zinc imaging. Validity of the phosphorescence turn-on responses was assessed by the fluorescence-activated cell sorting (FACS) analyses (Supporting Information, Figure S11). Subsequent addition of 100 μM TPEN reduces the phosphorescence intensities, confirming zinc responses.

One drawback of using ZIRDap in live cell imaging is noted: ZIRDap is cytotoxic to HeLa cells upon prolonged exposure (>30 min) to the photoexcitation beam. We speculated that this phototoxicity might result from photosensitization of singlet dioxygen (1O2) by ZIRDap. Indeed, the quantum yields for singlet oxygen photogeneration (ΦΔ) of both zinc-free and zinc-bound forms of ZIRDap, determined using methylene blue (ΦΔ = 0.52)58 and 1,3-diphenylisobenzofuran59 as the standard and the 1O2-selective substrate, respectively, approach unity (Supporting Information, Figure S12). The ΦΔ values for IrAP and IrP are also as high as 0.89 and 0.68, respectively, indicating that the cyclometalated Ir(III) complexes are responsible for the 1O2 photosensitization. These results present an additional challenge to the establishment of molecular strategies to attenuate phototoxicity of phosphorescent probes.

III. SUMMARY AND CONCLUSIONS

It has remained as a significant challenge to develop red-phosphorescent probes that can reversibly detect biological zinc ions. This difficulty stems from the thermodynamic forbiddance of PeT in the probes with low-energy phosphorescence emission. We have developed a zinc probe (ZIRDap) that is capable of producing fully reversible turn-on responses in the low-energy phosphorescence regions. The molecular construct for the phosphorescent probe involved the biscyclometalated Ir(III) complex and the DPA-appended 2-(2-pyridyl)pyrazine N–N ligand. An amide linkage was employed for bridging DPA and 2-(2-pyridyl)pyrazine. This ligand structure had two effects on the thermodynamic parameters of PeT: One was increasing E*red of the phosphorescent center, and the other was decreasing Eem of DPA. The net result of these two effects was exothermicity of PeT (−ΔGPeT = 0.39 eV) at a significantly small phosphorescence energy (λem = 596 nm). We monitored the occurrence of the intramolecular PeT and determined the PeT rate to be 2.0 × 107 s−1, by employing transient photoluminescence techniques. This PeT rate was virtually identical to the overall rate of nonradiative transition in the zinc-free form of ZIRDap, indicating the effectiveness of PeT in the phosphorescence modulation. The zinc probe produced turn-on responses, which were fully reversible and selective to zinc ions over other biological metal ions. However, similar turn-on phosphorescence signaling was evoked by cadmium ion, while titration experiment results suggested different metal-binding modes. Finally, the probe was capable of visualizing exogenously supplied zinc ions in live HeLa cells. Despite the advance, however, one challenge to attenuation of 1O2-mediated phototoxicity remains. This calls for future studies to establish molecular strategies for the minimization of 1O2 sensitization.

IV. EXPERIMENTAL SECTION

Materials and Synthesis. Caution! Perchlorate salts of metal complexes are potentially explosive. Only small quantities of material should be handled with care. The chloride-bridged Ir(III) precursor [(dfppy)2Ir(μ-Cl)]2 (dfppy = 2-(2,4-difluorophenyl)pyridinate) was synthesized according to a literature method.60 Commercially available chemicals were used as received. All glassware and magnetic stirring bars were thoroughly dried in a convection oven. Reactions were monitored using thin layer chromatography (TLC). Commercial TLC plates (silica gel 254, Merck Co.) were developed, and the spots were visualized under UV light at 254 or 365 nm. Silica gel column chromatography was performed with silica gel 60 G (particle size...
2-Amino-5-(2-pyridyl)pyrazine. 2-Amino-5-bromopyrazine (2.60 g, 17.0 mmol), 2-(tritylthyllnonylidine (6.19 g, 19.0 mmol), and tetrakis(triphenylphosphine) palladium(0) (1.76 g, 1.70 mmol) were dissolved in toluene (120 mL), and the solution was refluxed for 1 d under an Ar atmosphere. After it cooled to room temperature, the reaction mixture was concentrated and subjected to chromatographic purification on silica gel column (CH2Cl2/CH3OH = 49:1 to 9:1, v/v). Yellow-brown powder (2.30 g, 78%). 1H NMR (CDCl3, 400 MHz): δ 4.71 (s, 2H), 7.23–7.25 (m, 1H), 7.79 (d, J = 7.7 Hz, 1H), 8.03 (d, J = 1.4 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.64 (d, J = 4.8 Hz, 1H), 9.06 (d, J = 1.4 Hz, 1H). 13C NMR (CDCl3, 100 MHz) = 119.73, 122.79, 130.83, 136.83, 140.81, 141.71, 141.97, 154.16, 154.98. HR MS (EI, positive): Calcd for C9H8N4, 172.0749; Found: 172.0746. Anal. Calc for C9H8N4: C, 62.71; H, 4.72; N, 32.21%.

Bromoacetyl bromide (276 μL, 3.20 mmol) was added to a stirred solution of the reaction mixture, which was stirred additionally for 16 h at room temperature. After removal of the solvent, the concentrated mixture was subjected to silica gel column chromatography (CH2Cl2 to CH3Cl/CH2Cl2 = 9:1, v/v) to give a yellow powder (0.460 g, 54%). 1H NMR (CDCl3, 400 MHz): δ 4.80 (s, 2H), 7.33–7.36 (m, 1H), 7.86 (t, J = 7.6 Hz, 1H), 8.36 (d, J = 8.0 Hz, 1H), 8.70 (d, J = 4.8 Hz, 1H), 9.36 (d, J = 1.2 Hz, 1H), 9.53 (d, J = 1.2 Hz, 1H). 13C NMR (deuterated dimethyl sulfoxide (DMSO), 100 MHz) = 31.12, 61.94, 122.22, 125.80, 135.43, 141.58, 148.05, 149.58, 150.95, 165.46, 172.57. HR MS (EI, positive): Calcd for C13H10N4O6Br, 291.9960; found: 291.9957. Anal. Calc for C13H10N4O6Br: C, 45.07; H, 3.09; N, 19.11. Found: C, 44.82; H, 3.06; N, 19.29%.

(2-(Dipicolylamino)acetylamino)-5-(2-pyridyl)pyrazine. An acetonitrile solution (50 mL) containing (bromoacetyl)amino)-5-(2-pyridyl)pyrazine (0.194 g, 0.662 mmol), di(2-picolyl)amine (0.120 g, 0.602 mmol), and K2CO3 (0.128 g, 0.927 mmol) was stirred for 1 d at room temperature. The reaction mixture was filtered to remove residual K2CO3, and the filtrate was concentrated. Chromatographic purification on silica gel column (CH2Cl2 to CH3Cl/CH2Cl2 = 9:1, v/v) gave a brown powder (0.220 g, 88%). 1H NMR (CDCl3, 359 MHz) = 3.59 (s, 2H), 4.04 (s, 4H), 7.17–7.20 (m, 2H), 7.30–7.32 (m, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.66 (s, J = 7.6 Hz, 2H), 7.84 (t, J = 7.6 Hz, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.67 (d, J = 4.8 Hz, 2H), 8.70 (d, J = 4.8 Hz, 1H), 9.40 (d, J = 1.6 Hz, 1H), 9.54 (d, J = 1.2 Hz, 1H). 13C NMR (CDCl3, 100 MHz) = 59.06, 60.80, 120.92, 122.56, 123.15, 123.71, 135.32, 136.77, 137.05, 140.88, 146.42, 148.34, 149.29, 149.48, 154.47, 157.94, 171.18, 207.01. HR MS (EI, positive, m-NBA): Calcd for C20H17N6O4Br: C, 54.78; H, 2.45; N, 11.25%. Found: C, 54.76; H, 2.45; N, 11.25%

Zldap. An anhydrous CH3Cl (40 mL) solution containing the chloride-bridged Ir(III) dimer (dipyrryl)(μ-Cl)2Ir(μ-Cl)2 (0.350 g, 0.290 mmol) and 2-(dipicolylamino)acetylamino)-5-(2-pyridyl)pyrazine (0.200 g, 0.490 mmol) was refluxed for 6 h under an Ar atmosphere. The reaction mixture was cooled to room temperature, and NH4PF6 was added to the solution. After 12 h, the reaction mixture was filtered to remove NH4PF6, and concentrated under vacuum. The crude mixture was subjected to flash column chromatography on silica gel with CH2Cl2 to CH2Cl2/CH3OH = 19:1 (v/v). Further purification by preparative TLC techniques was performed to isolate an orange powder (0.290 g, 44%). 1H NMR (CD3CN, 300 MHz) = 5.30 (d, J = 3.6 Hz, 2H), 6.68–6.76 (m, 2H), 7.07–7.20 (m, 4H), 7.26 (d, J = 7.8 Hz, 2H), 7.51 (m, 1H), 7.62 (m, 3H), 7.73 (m, 1H), 7.90–7.99 (m, 3H), 8.17 (td, J = 7.8, 1.5 Hz, 1H), 8.32 (d, J = 8.7 Hz, 2H), 8.52 (d, J = 8.1 Hz, 1H), 8.57 (m, 2H), 8.85 (s, J = 1.2 Hz, 1H), 9.50 (d, J = 1.2 Hz, 1H). 13C NMR (CD3CN, 100 MHz) δ 59.56, 60.50, 114.04, 122.73, 122.80, 123.83, 124.01, 127.70, 134.98, 136.96, 139.32, 140.43, 144.50, 145.85, 148.84, 149.03, 149.51, 149.82, 151.99, 152.76, 154.22, 157.72, 169.06, 206.97. 19F NMR (CD3CN, 376 MHz) δ = −108.25 (m, 2F), −105.02 (m, 2F), −73.00 (d, J = 771 Hz, 6F). HR MS (FAB, positive, m-NBA): Calcd for C22H16F2IrN6O ([M−PF6]+), 984.2373; found: 984.2376. Spectroscopic Measurements. Milli-Q grade water (18.2 MQ-cm) was used to prepare solutions for spectroscopic measurements. PIPES (≥ 99%) was purchased from Aldrich. A pH 7.4 buffer solution was prepared by dissolving PIPES (20 mM) in milli-Q water and adjusting the pH with standard KOH solution (45 wt %, Aldrich) or concentrated HCl (Aldrich). The buffer solution was further treated with Chelex 100 resin (BIO-RAD) to remove trace metal ions, filtered through a membrane (pore size = 0.45 μm), and its pH was reexamined prior to use. Fresh metal stock solutions (typically, 0.10 or 0.010 M except for CrCl3·6H2O) were prepared in milli-Q water using the corresponding chloride salts: CuCl2 (99.99%, Aldrich), NiCl2 (≥ 99.5%, Aldrich), KCl (puratonic grade, Calbiochem), MgCl2 (99.99%, Aldrich), CaCl2 (99.99%, Aldrich), NaCl (99.99%, Aldrich), AlCl3 (99.99%, Aldrich), FeCl3 (99.99%, Aldrich), CoCl2 (99.99%, Aldrich), NiCl2 (99.99%, Aldrich), CdCl2 (99.99%, Aldrich), ZnCl2 (99.99%, Aldrich), CuCl2·2H2O (99.99%, Aldrich), CaCl2·2H2O (99.99%, Aldrich).
and ZnCl₂ (99.999%, Aldrich). A TPEN solution was prepared by dissolving N,N,N’,N”-tetrakis(2-picolyl)ethylenediamine (299%, Sigma) in DMSO (99.9%, Aldrich). Zn(ClO₄)₂·6H₂O (Aldrich) was dissolved in CH₃CN (spectrophotometric grade, Aldrich) to 1.0, 10, and 100 mM concentrations. The Ir(III) complex solutions were prepared by dissolution in CH₃CN to concentrations of 10 mM, 1.0 mM, 100 μM, and 10 μM. The 10 μM solutions were used for spectroscopic measurements, otherwise stated. For aqueous solutions, 3.0 mL of the PIPES buffer and 3 μL of the Ir(III) complex solution (10 mM in DMSO) were mixed to give a 10 μM solution. A 1 cm x 1 cm fluorimeter cell (Hellma) with a rubber cap was used for the steady-state optical measurements. UV–vis absorption spectra were collected on a Varian Cary 50 spectrophotometer at room temperature.

Photoluminescence spectra were obtained using a Quanta Master 40 scanning spectrorfluorimeter at room temperature. The photoluminescence quantum yields (Φ) were relatively determined according to following standard equation: Φ = Φ₀ref × (I₁ref/I₁) × (A₀ref/A₁) × (n₁/n₀)², where A, I, and n are the absorbance at the excitation wavelength, integrated photoluminescence intensity, and the refractive index of the solvent, respectively. Fluorescein as an aqueous 0.1 N NaOH solution was used as the external reference (Φ₀ref = 0.79). The refractive index of the 0.1 N NaOH solution was assumed to be identical to the value for pure water. The 10 μM solutions were deaerated by bubbling Ar prior to performing the measurements. Ar-saturated 50 μM solution of CH₃CN (CHI Instruments, Inc.) using three-electrode cell working electrodes, respectively. A Ag/AgNO3 couple was used as a reference.

Flow cytometry and Confocal Laser Scanning Microscopy. After the cells were washed with fresh DMEM, a 10 μM ZIrdap in DMSO (biotrace grade, Aldrich) was added to the culture media. The cells were incubated for 10 min at 37 °C. The incubated cells were washed twice with fresh DMEM (serum-free), and photoluminescence micrographs were taken using a Carl Zeiss LSM 510 META confocal laser scanning microscope using a Newport MaiTai eHP DeepSee multiphoton excitation system. An excitation beam (405 nm) was focused onto the dish, and the signals were acquired through 30 emission channels covering the range of 441–691 nm. The cells were imaged after subsequent treatment with 50 μM ZnCl₂/NaPT. Finally, 100 μM TPEN (DMSO) was introduced. ZnPT and TPEN were added directly into the culture media. Exposure to the excitation beam was kept as short as possible (<10 min) to minimize photoinduced cell death. Photoluminescence images and mean intensities were processed using the ZEN and ImageJ software, respectively.

**ASSOCIATED CONTENT**

**Supporting Information**

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


