Development of a Zinc Ion-Selective Luminescent Lanthanide Chemosensor for Biological Applications

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Abstract: Detection of chelatable zinc (Zn^{2+}) in biological studies has attracted much attention recently, because chelatable Zn^{2+} plays important roles in many biological systems. Lanthanide complexes (Eu^{3+}, Tb^{3+}, etc.) have excellent spectroscopic properties for biological applications, such as long luminescence lifetimes of the order of milliseconds, a large Stoke's shift of >200 nm, and high water solubility. Herein, we present the design and synthesis of a novel lanthanide sensor molecule, **[Eu-7]**, for detecting Zn^{2+}. This europium (Eu^{3+}) complex employs a quinolyl ligand as both a chromophore and an acceptor for Zn^{2+}. Upon addition of Zn^{2+} to a solution of **[Eu-7]**, the luminescence of Eu^{3+} is strongly enhanced, with high selectivity for Zn^{2+} over other biologically relevant metal cations. One of the important advantages of **[Eu-7]** is that this complex can be excited with longer excitation wavelengths (around 340 nm) as compared with previously reported Zn^{2+}-sensitive luminescent lamthanide sensors, whose excitation wavelength is at too high an energy level for biological applications. The usefulness of **[Eu-7]** for monitoring Zn^{2+} changes in living HeLa cells was confirmed. This novel Zn^{2+}-selective luminescent lanthanide chemosensor **[Eu-7]** should be an excellent lead compound for the development of a range of novel luminescent lanthanide chemosensors for biological applications.

Introduction

Zinc (Zn^{2+}) is the second most abundant heavy metal ion after iron in the human body, and the total zinc ion concentration in serum is of the order of 10 μM.[1] Zn^{2+} is an essential component of many enzymes (e.g., carbonic anhydrase), and also plays critical roles in maintaining key structural features of gene transcription proteins (e.g., zinc finger proteins).[2] Moreover, chelatable Zn^{2+} is present at especially high concentrations in brain,[3] pancreas,[4] and spermatozoa.[5] Chelatable Zn^{2+} regulates neuronal transmission in excitatory nerve terminals,[3] suppresses apoptosis,[6] contributes to neuronal injury in certain acute conditions,[7] epilepsy,[8] and transient global ischemia,[9] and induces the formation of β-amyloid,[10] which is reported to be related to

the etiology of Alzheimer's disease. Although many reports describe the significance of chelatable Zn^{2+} in biological systems, its mechanisms of action are less well understood than those of other cations, such as Ca^{2+}, Na^+, and K^+. Therefore, there is considerable interest in detecting chelatable Zn^{2+} in biological systems.[11]

So far, several Zn^{2+}-selective fluorescent sensor molecules have been reported.[12] However, novel types of Zn^{2+}-sensitive fluorescent sensor molecules are needed for studies on biological phenomena, and several new types of sensors that are designed for ratiometric measurement,[13] or that are peptide- or protein-based,[14] have recently been introduced. Ratiometric measure-

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ment in particular enables more precise analysis of Zn^{2+} concentrations. The other approach for precise analyses is time-resolved fluorescence (TRF) measurement, which offers a better signal-to-noise ratio. Luminescent lanthanide complexes are suitable for TRF measurement. So, our interest in Zn^{2+}-selective fluorescent sensor molecules was directed toward luminescent lanthanide complexes, in particular complexes of the europium and terbium trivalent ions (Eu^{3+} and Tb^{3+}). These complexes have large Stoke's shifts (>200 nm), long luminescence lifetimes of the order of milliseconds, and high water solubility,[15] whereas the typical organic fluorescent compounds possess small Stoke's shifts (Stoke's shifts of fluorescein and rhodamine are ~25 and ~20 nm, respectively)[12c−e,16] and short fluorescence lifetimes in the nanosecond region. The long-lived luminescence of the lanthanides has the advantage that short-lived background fluorescence and scattered light decay to negligible levels when a pulse of excitation light is applied and the emitted light is collected after an appropriate delay time. For these reasons, sensitization of lanthanide luminescence has been exploited for a number of useful signaling systems for time-resolved assays in the fields of medicine, biotechnology, and biological science.[17] The lanthanide f−f transitions have low absorbance, so the ligand structure requires a sensitizing chromophore for high luminescence.[18] Absorption by the chromophore results in effective population of its triplet level, and efficient intramolecular energy transfer occurs from the excited chromophore to the lanthanide metal, whereby the metal becomes excited to the emission state (Scheme 1).[15a,17g,18b] By means of appropriate chromophore design, it is possible to develop luminescent lanthanide complexes which can be used to sense various biological molecules.

There are only a few reports about lanthanide-based luminescent chemosensors for the detection of Zn^{2+}.[19] Parker and co-workers have developed a luminescent lanthanide agent which binds Zn^{2+} with an apparent dissociation constant K_d of 0.6 μM (295 K, pH 7.3).[19a,c] We employed a different design approach for a ligand and an antenna in a previous report, and

Scheme 1. (a) Schematic View of a Chromophore Incorporated into a Europium Emitter[a] and (b) the General Chromophore-to-Europium Ion Sensitization Process[b]

(a)

(b)
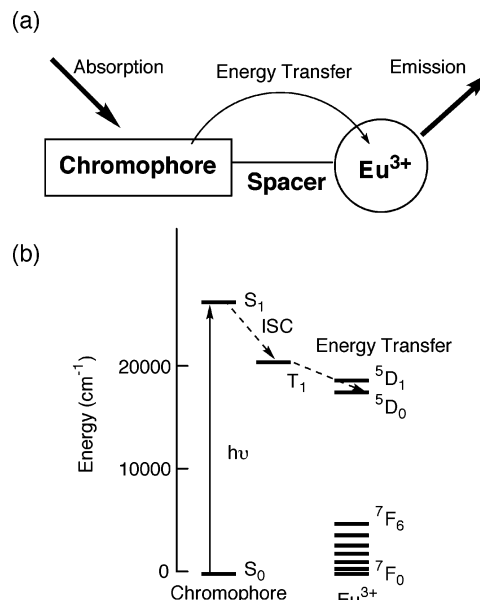
[a] The emission from Eu^{3+} after excitation of the chromophore is shown. [b]Light absorption and lowest-lying singlet excited state (S_1) formation at the sensitizing chromophore are followed by intersystem crossing (ISC), resulting in population of the lowest-lying triplet excited state (T_1). Subsequent chromophore-to-Eu^{3+} energy transfer leads to population of a metal-centered level, which deactivates from Eu^{3+}-emitting states to the relevant ground states.

the novel lanthanide complexes obtained showed a large enhancement of luminescence upon Zn^{2+} addition with an apparent dissociation constant K_d of 2.6 nM (295 K, pH 7.4).[19b] However, these compounds are unsuitable for biological applications, because of their short excitation wavelength, small emission enhancement, inconvenient pH sensitivity, insufficient selectivity for Zn^{2+}, etc. So, it is necessary to develop sensors with a longer excitation wavelength for biological applications without losing the high selectivity and high affinity for Zn^{2+}. Moreover, development of a simple sensor switch for Zn^{2+}, which would serve as both chromophore and Zn^{2+} receptor, would be useful. From this background, we set out to develop a novel lanthanide complex which can detect Zn^{2+} in biological systems, in the relevant concentration range.

Here we report the design and synthesis of the novel Zn^{2+}-sensitive luminescent lanthanide chemosensor **[Eu-7]**; upon complexation with Zn^{2+}, it exhibits strong, long-lived luminescence (of the order of milliseconds), and it also offers a large Stoke's shift (>250 nm), high water-solubility, and high selectivity for Zn^{2+} (Figure 1).

Results and Discussion

Design and Synthesis of [Eu-7] and [Gd-7]. N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine[20] (TPEN) shows high selectivity for Zn^{2+} over other metal ions found under physiological conditions, such as Ca^{2+} and Mg^{2+}. Accordingly, we

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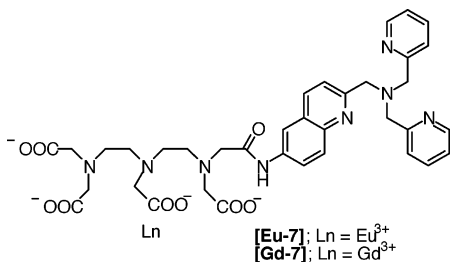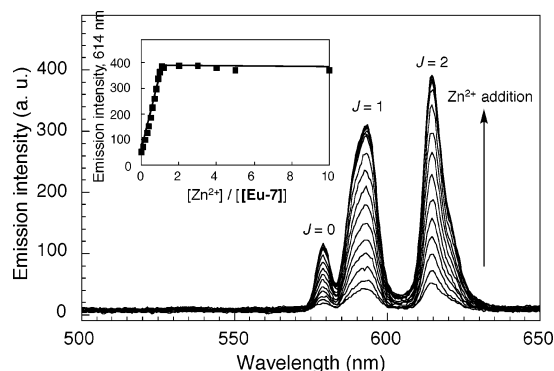
Figure 1. Structures of Eu³⁺ and Gd³⁺ *N*-{*N*-[2-*N*,*N*-bis(2-pyridylmethyl)-aminomethylquinolin-6-yl]carbamoylmethyl}-*N*,*N*′,*N*′′,*N*′′-diethylenetri-aminetetraacetic acid complexes: **[Eu-7]** and **[Gd-7]**.

designed a novel sensitive luminescent sensor for Zn^{2+} **[Eu-7]** by the combination of Eu^{3+}−diethylenetriaminepentaacetic acid (DTPA) complex and a quinoline-containing TPEN-based ligand for Zn^{2+}. The quinoline chromophore was selected as an antenna and a ligand, because quinoline has a longer excitation wavelength (>300 nm)[21] than pyridine and can coordinate to metal ions.

In the structure of **[Eu-7]**, the quinolyl chromophore is fixed close to the Eu^{3+} ion, allowing it to function as an antenna. The synthetic schemes for the lanthanide complexes, **[Eu-7]** and **[Gd-7]**, and details of the chemical characterization of compounds are provided in the Supporting Information.

Spectroscopic Characteristics of a Solution of [Eu-7] upon Addition of Zn^{2+}. The complex **[Eu-7]** in aqueous solution was characterized by a time-delayed luminescence spectrum with a delay time of 0.05 ms. The time-resolved luminescence emission intensity of **[Eu-7]** (50 μM) increased significantly (8.5-fold) upon addition of 1.0 equiv of Zn^{2+}, with a large Stoke's shift of >250 nm. The emission intensity remained at a plateau in the presence of an excess of Zn^{2+} (Figure 2). The luminescence emission displayed three bands at 579, 593, and 614 nm, corresponding to the deactivation from the $^5D_0$ excited state to $^7F_0$, $^7F_1$, and $^7F_2$ ground state, respectively.[18b,21b] The UV−vis absorption spectral change was monitored during Zn^{2+} addition. The absorption spectrum of **[Eu-7]** without Zn^{2+} had $\lambda_{max} = 249$ nm and broad bands at 330 and 318 nm, tailing out to 350 nm (Figure 3). The absorption spectrum of **[Eu-7]** changed upon addition of Zn^{2+} (0−1.0 equiv), with three isosbestic points at 334, 268, and 248 nm, and then remained at a plateau upon further addition of Zn^{2+} (Figure 3 and Figure S4), in accordance with the time-resolved luminescence spectra (Figure 2). The three peaks (330, 318, and 249 nm) of absorption changed to two peaks (320 and 253 nm) upon Zn^{2+} addition. The absorption wavelength changes between 300 and 350 nm were supposed to be due to the photophysical property change of the quinolyl moiety with Zn^{2+} chelation, whereas pyridine−Zn^{2+} coordination resulted in changes below 300 nm.[19b] Thus, 1:1 complex stoichiometry was observed in both the absorption and the luminescence emission spectra of **[Eu-7]**. The Job's plot using the luminescence emission intensity of Eu^{3+} at 614

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Figure 2. Time-delayed emission spectra (excitation at 320 nm) of **[Eu-7]** (50 μM) in the presence of various concentrations of Zn^{2+}: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv of Zn^{2+} with respect to **[Eu-7]**. These spectra were measured at pH 7.4 (100 mM HEPES buffer) and 22 °C using a delay time of 0.05 ms and a gate time of 1.00 ms. The inset shows the changes of the luminescence intensity at $\lambda = 614$ nm. The bands arise from $^5D_0 \rightarrow {}^7F_J$ transitions; the J values of the bands are labeled.
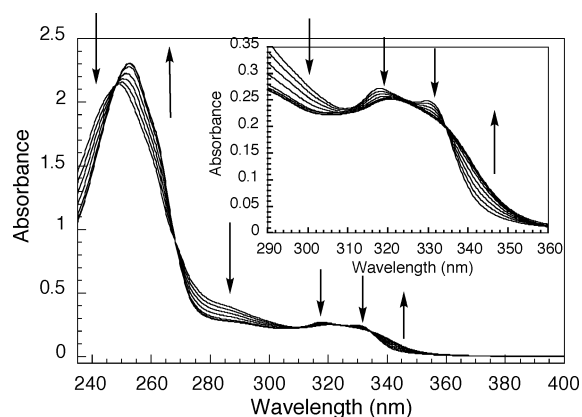
Figure 3. Absorbance spectra of 50 μM aqueous solution (100 mM HEPES buffer; pH 7.4) of **[Eu-7]** at 22 °C upon addition of aliquots of Zn^{2+}, which was added as $ZnSO_4$: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv of Zn^{2+} with respect to **[Eu-7]**. The inset shows the spectra magnified between 290 and 360 nm.
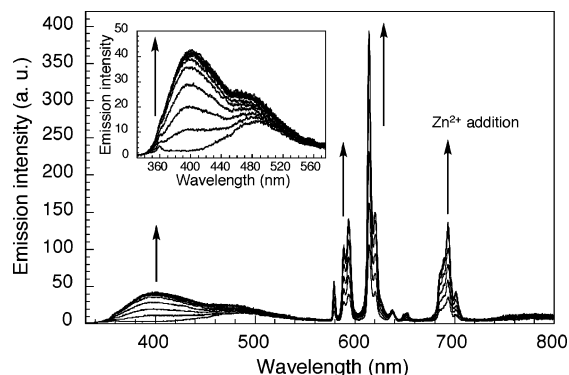
nm for the complex of **[Eu-7]** and Zn^{2+} also indicated the presence of the 1:1 complex (see Supporting Information).[22] For **[Eu-7]** (20 μM), the fluorescence emission spectra were measured without a delay time, with excitation at 320 nm (Figure 4). In the fluorescence emission of **[Eu-7]**, one band with a short lifetime appeared at 397 nm with a concomitant linear fluorescence increase following addition of Zn^{2+} at between 0 and 1.0 equiv to **[Eu-7]**, and it remained at a plateau with further Zn^{2+} addition (Figure S6). The short-lived fluorescence at 397 nm can be ascribed to direct emission from the quinolyl moiety of **[Eu-7]**.[21a,b] All these results can be rationalized in terms of 1:1 complex formation of **[Eu-7]** with Zn^{2+}, via the quinolyl ligand.

Luminescence and Chemical Properties of [Eu-7] and [Gd-7]. The luminescence and chemical properties of **[Eu-7]** and **[Gd-7]** are listed in Table 1. **[Eu-7]** and **[Gd-7]**, even at 10 mM, were highly water-soluble. The phosphorescence spectra of **[Gd-7]** were measured in MeOH:EtOH = 1:1 at 77 K in the absence and in the presence of Zn^{2+}. The triplet energy levels for free and Zn^{2+}-bound **[Gd-7]** were around 20 790 and 20 576

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Figure 4. Fluorescence spectra of **[Eu-7]** (20 μM) in 100 mM HEPES buffer at pH 7.4 without a delay time upon the addition of increasing amounts of Zn²⁺: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv of Zn²⁺ with respect to **[Eu-7]**. Excitation at 320 nm, 22 °C. The inset shows the spectra magnified between 330 and 575 nm.

Table 1. Luminescence and Chemical Properties

	apparent K_d for Zn²⁺ (nM)[a]	ϕ (%)[b] free	ϕ (%)[b] Zn²⁺ complex	τ_{H_2O} (ms)[c] free	τ_{H_2O} (ms)[c] Zn²⁺ complex	τ_{D_2O} (ms)[d] free	τ_{D_2O} (ms)[d] Zn²⁺ complex	q[e] free	q[e] Zn²⁺ complex
[Eu-7]	59	0.9	7.4	0.52	0.58	2.03	2.23	1.42	1.22

	triplet state energy (cm⁻¹)[f] free	triplet state energy (cm⁻¹)[f] Zn²⁺ complex	r_1 (mM⁻¹ s⁻¹)[g] free	r_1 (mM⁻¹ s⁻¹)[g] Zn²⁺ complex
[Gd-7]	20790	20576	6.05	5.81

[a] [Zn²⁺] was controlled by using Zn²⁺/NTA systems below 398 nM free Zn²⁺ and unbuffered Zn²⁺ above 200 μM free Zn²⁺. The buffer contained 100 mM HEPES, pH 7.4, $I = 0.1$ (NaNO₃). [b] Quantum yields were calculated using [Ru(bipy)₃]Cl₂ (bipy = 2,2′-bipyridine; $\phi = 0.028$ in water) as a standard, and measured in 100 mM HEPES buffer at pH 7.4, 25 °C. [c] In H₂O-based buffer (100 mM HEPES buffer; pH 7.4). [d] In D₂O-based buffer (100 mM HEPES buffer; pD 7.4). [e] q values were estimated using the equation $q^{Eu} = 1.2(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.25)$, which allows for the contribution of unbound water molecules.[23] [f] In MeOH:EtOH = 1:1 at 77 K. [g] The R_1 relaxivity as measured at 20 MHz and 25 °C in 100 mM HEPES buffer (pH 7.4).

cm⁻¹, respectively, and both were sufficiently close to the ⁵D₀ level, the excited state, of Eu³⁺ ($E = 17\ 250$ cm⁻¹).[23] The luminescence quantum yield (ϕ) was 0.9% before addition of Zn²⁺ and was increased 8.2-fold to 7.4% by Zn²⁺ addition, under air-equilibrated conditions. This luminescence quantum yield is sufficiently large for luminescence detection. Further, the binding affinity for Zn²⁺ was assessed by using the luminescence intensity. The affinity of **[Eu-7]** for Zn²⁺ ions was measured at pH 7.4, 22 °C, in a high salt background (100 mM HEPES buffer, $I = 0.1$ (NaNO₃)). The apparent dissociation constant K_d for Zn²⁺ was calculated to be 59 nM. This K_d value for Zn²⁺ was larger than that reported for tris(2-pyridylmethyl)-amine (TPA) ($K_d = 0.014$ nM; pH = 7.4, 298 K, $I = 0.1$),[24] probably due to a larger steric repulsion in the case of the quinolyl substituent than the pyridyl substituent, but the value is still sufficiently small for biological applications.[25] Measurements of the decay rate constants of the Eu³⁺ excited state were carried out in both H₂O and D₂O, in the absence and in the

presence of Zn²⁺ (see Supporting Information). The luminescence lifetimes of **[Eu-7]** were found to be 0.52 (without Zn²⁺) and 0.58 (with Zn²⁺) ms in H₂O (τ_{H_2O}), and 2.03 (without Zn²⁺) and 2.23 (with Zn²⁺) ms in D₂O (τ_{D_2O}). These values indicated that the numbers of coordinated water molecules (q values) at the metal center were 1.42 and 1.22, respectively, according to eq 1.[26] Thus, the lanthanide hydration state was hardly affected

no. of water molecules:

$$q^{Eu} = 1.2(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.25)\ (1)$$

by the addition of Zn²⁺. Therefore, it can be considered that the increase of the emission intensity caused by Zn²⁺ addition was not due to a change in the direct interaction of water molecules with Eu³⁺.

Next, the spatial arrangement of **[Eu-7]** was further assessed by measuring the relaxometric properties. The water proton relaxivity R_1 of Gd³⁺ complexes is routinely used as an important parameter for MRI contrast agents.[27] The relaxation enhancement is modulated by the electron−nucleus dipolar interaction, which can be changed by rotation of the complex, by electron spin relaxation of the metal ion, and by the co-ordinated exchange of water molecules.[28] The R_1 relaxivity of **[Gd-7]** at 20 MHz showed similar values in the absence and in the presence of Zn²⁺, i.e., 6.05 and 5.81 mM⁻¹ s⁻¹, respectively. This result indicates that the environment around the Eu³⁺ ion of **[Eu-7]** was hardly changed by Zn²⁺ binding.

Effect of pH and Other Cations on the Long-Lived Luminescence Intensity. The luminescence emission intensity of **[Eu-7]** at 614 nm was examined at various pH values, with excitation at 320 nm. There was almost no effect of H⁺ on the emission spectrum of **[Eu-7]** between pH 3.6 and 8.8 either in the presence or in the absence of Zn²⁺ (see Supporting Information). Thus, the luminescence emission intensity of **[Eu-7]** is stable at around physiological pH. Parker et al. reported a Zn²⁺-sensitive luminescent lanthanide probe based on photo-induced electron transfer (PeT) from the benzylic nitrogen to the chromophore's singlet excited state.[19a,c] This approach has the disadvantage that the pivotal nitrogen for Zn²⁺ binding can also be protonated at lower pH, resulting in strong luminescence owing to inhibition of PeT. However, our compound **[Eu-7]** was not affected by lowering of the pH. The tertiary amine of **[Eu-7]** can be protonated at around pH 3.6, because TPA has pK_a values of 6.10, 4.28, and 2.49.[24] Thus, the finding of insensitivity to lower pH means that the luminescence augmentation was not due to cessation of PeT from tertiary amine. We think that this pH stability of the luminescence is one of the key advantages of **[Eu-7]**.

The effect of adding Na⁺, K⁺, Ca²⁺, Mg²⁺, and various heavy metal ions on the luminescence emission intensity of **[Eu-7]** was also examined. Luminescence emission enhancement of

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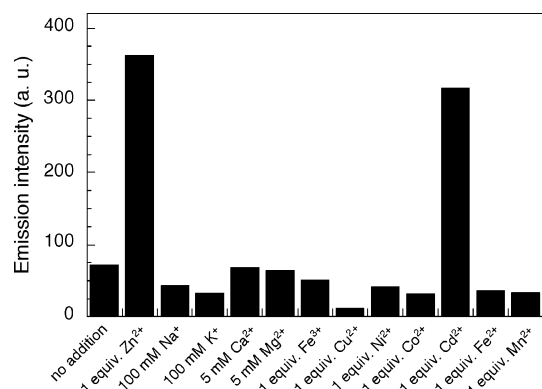
Figure 5. Luminescence intensity change profiles of **[Eu-7]** (50 μM) in the presence of various cations in 100 mM HEPES buffer at pH 7.4, 22 °C (excitation 320 nm, emission 614 nm). Heavy metal ions (1 equiv relative to **[Eu-7]**) were added as $Fe_2(SO_4)_3$, $CuSO_4$, $NiSO_4$, $CoSO_4$, $CdSO_4$, $FeSO_4$, and $MnSO_4$. Other cations were added as $ZnSO_4$ (50 μM), $NaNO_3$ (100 mM), KNO_3 (100 mM), $CaCl_2$ (5 mM), and $MgSO_4$ (5 mM).

[Eu-7] (50 μM) was not observed upon the addition of 100 mM Na^+ or K^+, 5 mM Ca^{2+} or Mg^{2+}, or 50 μM of various heavy metal ions except for Cd^{2+} (Figure 5). Thus, Na^+, K^+, Ca^{2+}, and Mg^{2+}, which exist at high concentrations in biological systems, did not enhance the luminescence intensity of **[Eu-7]**. The luminescence emission intensity was weakened or quenched upon the addition of several cations (particularly Cu^{2+}, Ni^{2+}, and Co^{2+}) together with Zn^{2+}, in comparison with that upon adding Zn^{2+} alone, as shown in Figure S8. However, Zn^{2+} can be distinguished from these heavy metals, since Zn^{2+} chelation selectively enhances the luminescence intensity.

Utility of the Long-Lived Luminescence. To explore further the utility of the long luminescence lifetime of **[Eu-7]**, we tested whether it could be well distinguished from the short-lived background fluorescence and scattered light. Figure 6 presents the emission spectra of **[Eu-7]** solution, with 1 μM rhodamine 6G as an artificial short-lived background, without (Figure 6a) or with (Figure 6b) a time resolution process. There are three fluorescent peaks in Figure 6a due to scattered light (300 nm), the increased fluorescence of the quinoline moiety (around 400 nm), and the fluorescence of rhodamine 6G (around 550 nm). Among them, rhodamine 6G fluorescence can directly interfere with Eu^{3+} luminescence. However, the long-lived luminescence detection of **[Eu-7]** solution was not affected at all by these three peaks (Figure 6b). Thus, the luminescence of **[Eu-7]** should be little influenced by experimental artifacts when measured with the aid of a time-resolution process.

Biological Applications of [Eu-7]. We next examined the application of **[Eu-7]** to cultured living cells (HeLa cells) by fluorescence microscopy (Figure 7). Since **[Eu-7]** can be excited with a relatively long excitation wavelength, this compound is suitable for cellular applications, in contrast to previously reported Zn^{2+}-sensitive luminescent lanthanide sensors. The fluorescence microscope had an optical window centered at 617 $\pm$ 37 nm for the emission due to Eu^{3+}-based luminescence upon excitation at 360 $\pm$ 40 nm (the Stoke's shift is >250 nm). Compound **[Eu-7]** was injected only into the single cultured HeLa cell in the bottom left-hand part of the field of view in Figure 7. We then added Zn^{2+} (150 μM) and a zinc-selective ionophore, pyrithione (2-mercaptopyridine *N*-oxide, 50 μM), to the medium at 3 min, inducing a prompt increase of intracellular luminescence. This luminescence was decreased by extracellular
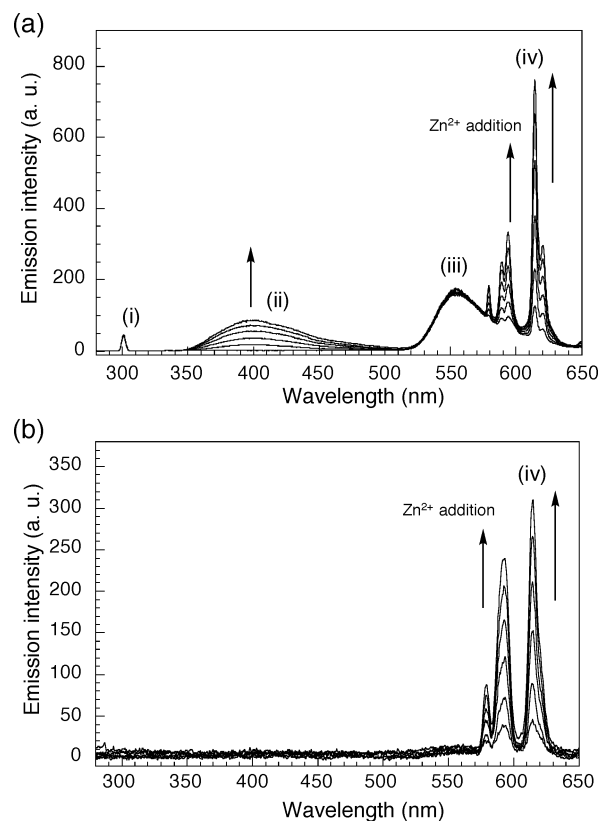

Figure 6. Emission spectra of **[Eu-7]** (50 μM) without (a) and with (b) time resolution in the presence of various concentrations of Zn^{2+}: 0, 0.2, 0.4, 0.6, 0.8, and 1.0 equiv of Zn^{2+} with respect to **[Eu-7]** (excitation at 300 nm, respectively). Both spectra were measured in 100 mM HEPES buffer containing 1 μM rhodamine 6G as a chromophore to provide artificial background fluorescence at pH 7.4 and 22 °C. The emission spectra (a) were measured with a Hitachi F4500, and the time-resolved emission spectra (b) were measured using a delay time of 0.05 ms and a gate time of 1.00 ms with a Perkin-Elmer LS-50B. Bands can be assigned to (i) scattered excitation light, (ii) the fluorescence of the quinolyl chromophore of **[Eu-7]**, (iii) the fluorescence of rhodamine 6G, and (iv) the long-lived luminescence of **[Eu-7]**.

addition of the cell-membrane-permeable chelator TPEN (150 μM) at 7.5 min. Clear images were obtained, because **[Eu-7]** has long-wavelength emission with a large Stoke's shift, which minimizes the influence of the excitation light. These results demonstrate that **[Eu-7]** can be used to monitor changes of intracellular ionic Zn^{2+} reversibly, and has potential for biological applications.

Conclusions

We have designed and synthesized a novel lanthanide-based luminescent sensor molecule for Zn^{2+}, **[Eu-7]**, by using Eu^{3+} as the fluorophore and a quinolyl moiety as the antenna. This compound **[Eu-7]** showed pronounced long-lived luminescence enhancement upon Zn^{2+} addition. Previously reported Zn^{2+}-sensitive lanthanide sensors are not suitable for biological applications, because of their short excitation wavelength, small enhancement of emission, inconvenient pH sensitivity, etc. In contrast, the properties with **[Eu-7]** are favorable for biological applications. It is noteworthy that **[Eu-7]** has a longer excitation wavelength than previously reported Zn^{2+}-sensitive lanthanide sensors, and this permits fluorescence microscopy measurements with **[Eu-7]** to monitor Zn^{2+} concentrations in living cells. We expect that a larger signal-to-noise ratio in cellular imaging
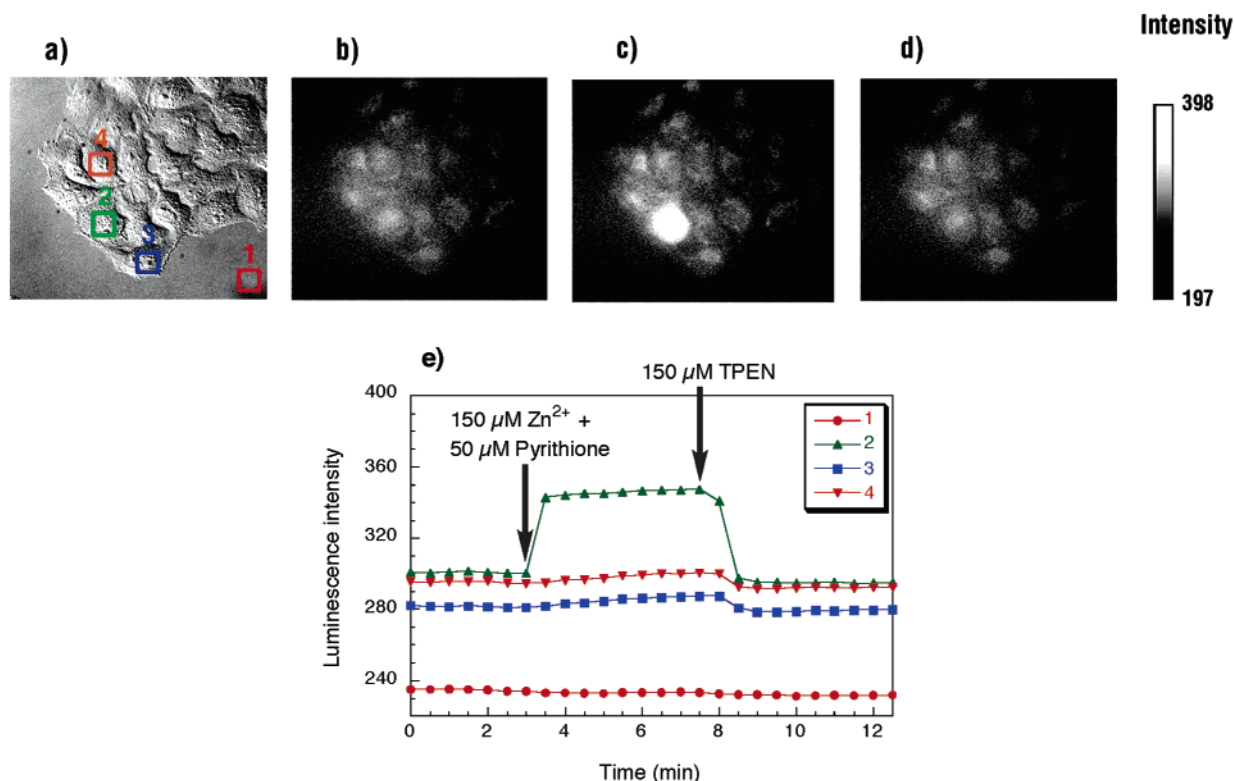
Figure 7. Bright-field transmission and luminescence images of Zn²⁺ in **[Eu-7]**-injected HeLa cells in HBSS buffer. The luminescence at 580−654 nm, excited at 320−400 nm, was measured at 30 s intervals. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO₂/95% air incubator. The cells were then washed with HBSS buffer and injected with **[Eu-7]** solution. (a) Bright-field transmission image (0 min). (b) Luminescence image of (a) (0 min). (c) Luminescence image (5 min) following an addition of 50 μM pyrithione (zinc ionophore) and 150 μM ZnSO₄ to the medium at 3 min. (d) Luminescence image (10 min) following an addition of 150 μM TPEN to the medium at 7.5 min. Luminescence images (b−d) correspond to the luminescence intensity data in (e), which shows the average intensity of the corresponding area or cell area (1, extracellular region; 2, intracellular region of the injected cell; 3, 4, intracellular regions of noninjected cells).

would be achievable by using time-resolved imaging with **[Eu-7]**. Furthermore, we confirmed the advantage of the long-lived luminescence of **[Eu-7]** for eliminating fluorescence background interference. This complex **[Eu-7]** is the first Zn²⁺-sensitive luminescent lanthanide chemosensor that can be used for studies on the biological functions of Zn²⁺, and our design strategy should yield a range of long-lived luminescent lanthanide probes for sensing Zn²⁺ or, after appropriate modification of the acceptor moiety, other molecules of interest in biological applications.

Experimental Section

Materials. DTPA bisanhydride was purchased from Aldrich Chemical Co. Inc. (St. Louis, MO). All other reagents were purchased from either Tokyo Kasei Kogyo Co., Ltd. (Japan) or Wako Pure Chemical Industries, Ltd. (Japan). All solvents were used after distillation. Silica gel column chromatography was performed using BW-300, Chromatorex-NH, and Chromatorex-ODS (all from Fuji Silysia Chemical Ltd.).

Instruments. ¹H and ¹³C NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra were measured with a JEOL JMS-DX 300 mass spectrometer (EI⁺) or a JEOL JMS-700 mass spectrometer (FAB⁺). HPLC purification was performed on a reverse-phase column (GL Sciences (Tokyo, Japan), Inertsil Prep-ODS 30 mm × 250 mm) fitted on a Jasco PU-1587 System. Time-resolved fluorescence spectra were recorded on a Perkin-Elmer LS-50B (Beaconsfield, Buckinghamshire, England). The slit width was 5 nm for both excitation and emission. UV−visible spectra were obtained on a Shimadzu UV-1600 (Tokyo, Japan). Fluorescence spectroscopic studies were performed with a Hitachi F4500 (Tokyo, Japan). The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 700 V.

Measurements of relaxation times T_1 were made using an NMR analyzer operating at 20 MHz (Minispec mq20, Bruker).

Time-Delayed Luminescence Spectral Measurements. The time-delayed luminescence spectra of **[Eu-7]** (50 μM) were measured in 100 mM HEPES buffer at pH 7.4, 22 °C (excitation at 320 nm), with addition of various amounts of Zn²⁺ (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv of Zn²⁺) to **[Eu-7]**. The slit width was 5 nm for both excitation and emission. A delay time of 0.05 ms and a gate time of 1.00 ms were used.

UV−Visible Absorption Spectral Measurements. The absorption spectral changes of **[Eu-7]** (50 μM) upon addition of Zn²⁺ were measured at 22 °C in an aqueous solution buffered to pH 7.4 (100 mM HEPES buffer). Zn²⁺ was added as ZnSO₄ at 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv of Zn²⁺ with respect to **[Eu-7]**.

Fluorescence Emission Spectral Measurements. The fluorescence emission spectra of **[Eu-7]** (20 μM) without a delay time were measured in 100 mM HEPES buffer (pH 7.4) at 22 °C, following excitation at 320 nm. The amounts of added Zn²⁺ were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 equiv with respect to **[Eu-7]**.

Quantum Yield Measurements. The luminescence spectra were measured with a Hitachi F4500 spectrofluorometer. The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. The luminescence spectra of **[Eu-7]** were measured in 100 mM HEPES buffer at pH 7.4, 25 °C, with irradiation at 300 nm. The quantum yields of Eu³⁺ complexes were evaluated using a relative method with reference to a luminescence standard, [Ru(bpy)₃]Cl₂ (ϕ = 0.028 in air-equilibrated water).[29] The quantum yields of Eu³⁺ complexes can be expressed by eq 2,[30] where Φ is the quantum yield (subscript "st" stands for the reference and "x" for the sample), A is

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$$\Phi_x/\Phi_{st} = [A_{st}/A_x] [n_x^2/n_{st}^2] [D_x/D_{st}] \qquad (2)$$

the absorbance at the excitation wavelength, n is the refractive index, and D is the area (on an energy scale) of the luminescence spectra. The samples and the reference were excited at the same wavelength. The sample absorbance at the excitation wavelength was kept as low as possible to avoid fluorescence errors ($A_{exc} < 0.06$).

Luminescence Lifetime Measurements. The luminescence lifetimes of the complexes were recorded on a Perkin-Elmer LS-50B. The data were collected with 10-μs resolution in H_2O (100 mM HEPES buffer at pH 7.4) and D_2O (100 mM HEPES buffer at pD 7.4, based on the equation pD = pH + 0.40[31]), and fitted to a single-exponential curve obeying eq 3, where I_0 and I are the luminescence intensities at the

$$I = I_0 \exp(-t/\tau) \qquad (3)$$

time $t = 0$ and time t, respectively, and τ is the luminescence emission lifetime. Lifetimes were obtained by monitoring the emission intensity at 614 nm ($\lambda_{ex} = 320$ nm).

Relaxation Time Measurements. The relaxation time, T_1, of aqueous solutions of the Gd^{3+} complex **[Gd-7]** was measured in 100 mM HEPES buffer (pH 7.4) at 20 MHz and 25 °C (Minispec mq20, Bruker). The value of T_1 was measured from 10 data points generated by using the standard inversion−recovery procedure. The relaxivity, R_1 (mM^{-1} s^{-1}), of **[Gd-7]** was determined from the slope of the plot of $1/T_1$ vs **[[Gd-7]]** (0, 0.4, 0.6, 0.8, and 1.0 mM). The buffered Gd^{3+} complex (**[Gd-7]**) solution was allowed to equilibrate for at least 10 min after addition of $ZnSO_4$ aqueous stock solution.

Apparent Dissociation Constant (K_d) Measurements. Upon addition of various concentrations of Zn^{2+}, the luminescence intensity and the absorbance of **[Eu-7]** linearly changed up to a 1:1 $[Zn^{2+}]/$ $[[Eu-7]]$ molar ratio, and the luminescence and absorption spectra remained at a plateau with further addition of Zn^{2+}. Furthermore, the Job's plot analysis revealed that maximum luminescence intensity was obtained at a 1:1 ratio. These data suggested that **[Eu-7]** should form a 1:1 complex with Zn^{2+}. So, the apparent dissociation constant, K_d, was determined from the luminescence intensity in 100 mM HEPES buffer (pH 7.4, $I = 0.1$ (NaNO$_3$)) at 22 °C ($\lambda_{exc} = 320$ nm). $[Zn^{2+}]$ was controlled by using 0−9 mM ZnSO$_4$/10 mM NTA (nitrilotriacetic acid) systems[19b,32] below 398 nM free Zn^{2+} and unbuffered Zn^{2+} above 200 μM free Zn^{2+}. The luminescence intensity data were fitted to eq 4, and K_d was calculated, where F is the luminescence intensity, F_{max}

$$F = F_0 + (F_{max} - F_0) ([Zn^{2+}]_f)/(K_d + [Zn^{2+}]_f) \qquad (4)$$

is the maximum luminescence intensity, F_0 is the luminescence intensity with no addition of Zn^{2+}, and $[Zn^{2+}]_f$ is the free Zn^{2+} concentration. The value of K_d was determined from the fittings for the luminescence intensity data shown in the Supporting Information.

Phosphorescence Spectral Measurements. Phosphorescence spectra were obtained with a Hitachi F4500. The phosphorescence spectra of **[Gd-7]** (20 μM) in the absence and in the presence of Zn^{2+} (1 equiv relative to **[Gd-7]**) were measured at 77 K in MeOH:EtOH = 1:1 (excitation at 320 nm) (see Supporting Information). The slit width was 10.0 nm for excitation and 20.0 nm for emission. The photomultiplier voltage was 950 V.

Effect of pH on the Luminescence Intensity. The following buffers were used: 100 mM ClCH$_2$COOH−ClCH$_2$COONa buffer (pH 3.6), 100 mM AcOH−AcONa buffer (pH 4.0−5.5), 100 mM morpholinoethanesulfonic acid (MES) buffer (pH 5.5−6.5), 100 mM HEPES buffer (pH 7.0−8.0), and 100 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 8.5−9.0). The luminescence intensity (excitation

320 nm, emission 614 nm) of each sample of **[Eu-7]** (50 μM) was plotted.

Metal Ion Selectivity Measurements. The luminescence emission enhancement of **[Eu-7]** was measured in 100 mM HEPES buffer (pH 7.4) at 22 °C (excitation 320 nm, emission 614 nm). Heavy metal ions (50 μM) were added as Fe$_2$(SO$_4$)$_3$, CuSO$_4$, NiSO$_4$, CoSO$_4$, CdSO$_4$, FeSO$_4$, and MnSO$_4$. Other cations were added as ZnSO$_4$ (50 μM), NaNO$_3$ (100 mM), KNO$_3$ (100 mM), CaCl$_2$ (5 mM), and MgSO$_4$ (5 mM).

Confirmation of the Utility of the Long Luminescence Lifetime of [Eu-7]. The emission spectra without a time-resolution process were measured with a Hitachi F4500. The time-resolved emission spectra were measured with a Perkin-Elmer LS-50B using a delay time of 0.05 ms and a gate time of 1.00 ms. Spectra of **[Eu-7]** (50 μM) with various concentrations of Zn^{2+} were measured in 100 mM HEPES buffer (pH 7.4) at 22 °C containing 1 μM rhodamine 6G as an artificial provider of short-lived background fluorescence (excitation at 300 nm). The amounts of added Zn^{2+} were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 equiv of Zn^{2+} with respect to **[Eu-7]**.

Preparation of Cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen Corp.), 1% penicillin, and 1% streptomycin (Invitrogen Corp.) at 37 °C in a 5% CO$_2$/95% air incubator. The cells were grown on an uncoated 35-mm-diameter glass-bottomed dish (MatTek, Ashland, MA), and washed twice with Hanks' balanced salt solution (HBSS) buffer (Invitrogen Corp.), and then the medium was replaced with HBSS buffer before imaging. The compound **[Eu-7]** (2 mM) was dissolved in microinjection buffer (HBSS buffer), and injected into the cells with an Eppendorf injection system (Transjector 5246).

Microscopy and Imaging Methods. The imaging system comprised an inverted microscope (IX71; Olympus) and a cooled CCD camera (Cool Snap HQ; Roper Scientific, Tucson, AZ). The microscope was equipped with a xenon lamp (AH2-RX; Olympus), a 40× objective lens (Uapo/340, N.A. 1.35; Olympus), a dichroic mirror (420DCLP; OMEGA), an excitation filter (S360/40×; Chroma), and an emission filter (S617/73m; Chroma). The whole system was controlled using MetaFluor 6.1 software (Universal Imaging, Media, PA). The luminescence images were measured every 30 s. Additions of zinc sulfate (150 μM) with pyrithione (50 μM) or N,N,N',N'-tetra(2-picolyl)-ethylenediamine (TPEN) (150 μM) to cell samples were performed on the microscope stage.

Supporting Information Available: Detailed descriptions of synthetic procedures for **[Eu-7]** and **[Gd-7]**; data on the measurements of the emission lifetime of **[Eu-7]** and on the measurement of the apparent dissociation constant of **[Eu-7]** with Zn^{2+}; phosphorescence spectra of **[Eu-7]**; plot of the absorbance intensity changes detected at 340 nm, shown in Figure 3; Job's plot for complexation between **[Eu-7]** and Zn^{2+}; plot of the changes in the fluorescence intensity detected at 397 and 614 nm, shown in Figure 4; and pH profile of the luminescence intensity of **[Eu-7]**. This material is available free of charge via the Internet at http://pubs.acs.org.

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