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Development of a novel terbium(III) chelate-based luminescent probe for highly sensitive time-resolved luminescence detection of hydroxyl radical

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ABSTRACT

Time-resolved (or time-gated) luminescence detection technique using lanthanide chelates as luminescent probes is a widely used and highly sensitive method for the biological applications. The developments of various functional lanthanide probes that can selectively recognize the biological targets are the essential objective of the technique. In this work, a unique Tb^{3+} chelate-based luminescent probe, *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-(*p*-aminophenoxy)methylene-pyridine] tetrakis(acetate)-Tb^{3+}(BMPTA-Tb^{3+}), has been designed and synthesized for highly selective and sensitive time-resolved luminescence detection of one highly reactive oxygen species (ROS), hydroxyl radical (•OH). The probe is almost non-luminescent, and can selectively react with hydroxyl radical to afford a highly luminescent Tb^{3+} chelate, *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-hydroxymethyl-pyridine] tetrakis(acetate)-Tb^{3+} (BHTA-Tb^{3+}), accompanied by a 49-fold increase in luminescence quantum yield with a long luminescence lifetime (2.76 ms). The luminescence response of the probe to hydroxyl radical is highly selective and insensitive to pH in the physiological pH range. For loading the probe into the living cells, the acetoxymethyl ester of BMPTA-Tb^{3+} was synthesized and used for the HeLa cell loading. Based on this probe, a background-free time-resolved luminescence imaging method for detecting hydroxyl radical in living cells was successfully established.

1. Introduction

In recent years, reactive oxygen species (ROS) have been demonstrated to be involved in many pathogenic processes, and their researches have attracted much attention of the researchers in the chemical, biological, and medical fields. Hydroxyl radical, •OH, is the most reactive member of the diverse class of ROS, and has important functions in biosystems [1–6]. In a normal cellular environment, •OH is an essential species, while in case of overproduction or exhaustion of antioxidants it might become deleterious to life. It has been known that •OH is a second messenger in T cell activation, while it can also damage the DNA bases due to ionizing radiation, and such damage can lead to mutations as well as cell death [7–10]. In order to enable a deeper insight on its biological functions and deleterious effects, there is continuous requirement for new sensitive and specific methods for detecting •OH in biological systems.

To date, although several organic fluorophore-based fluorescent probes that can be used for selectively detecting •OH, including terephthalic acid (TA) [11], N-succinimidyl ester of coumarin-3-carboxylic acid (SECCA) [12], 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (HPF) and [6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) [13], and a fluorescence resonance energy transfer (FRET)-type probe having a DNA skeleton [14], have been reported, some drawbacks of these probes, such as insufficient photostability, broad excitation and emission peaks and small Stokes shift, have limited their effective applications to biological systems [15,16]. Recently, a Tb^{3+} chelate-based probe for the luminescence detection of •OH, relying on the hydroxylation of trimesate by •OH and subsequent chelation by Tb-DO3A (DO3A: 10-(hydroxypropyl)-1,4,7,10-teraazocyclododecane-1,4,7-triacetate), was developed [17]. However, since 2-hydroxytrimesic acid is not an ideal antenna for sensitizing the Tb^{3+} luminescence, only a 11-fold luminescence enhancement was observed after the probe was reacted with •OH.

Herein we describe the design, synthesis and application of a novel Tb^{3+} chelate-based luminescent probe, *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-(*p*-aminophenoxy) methylene-pyridine] tetrakis(acetate)-Tb^{3+} (BMPTA-Tb^{3+}), for specifically detecting •OH both *in vitro* and *in vivo*. This probe is a stable Tb^{3+} chelate with high water solubility, large Stokes shift (>200 nm), and has a high antenna efficiency for sensitizing the Tb^{3+} luminescence [18]. The probe was designed by incorporating an electron-rich aromatic group, *p*-aminophenoxy, into the Tb^{3+} chelate, which could effectively quench the excited state of the

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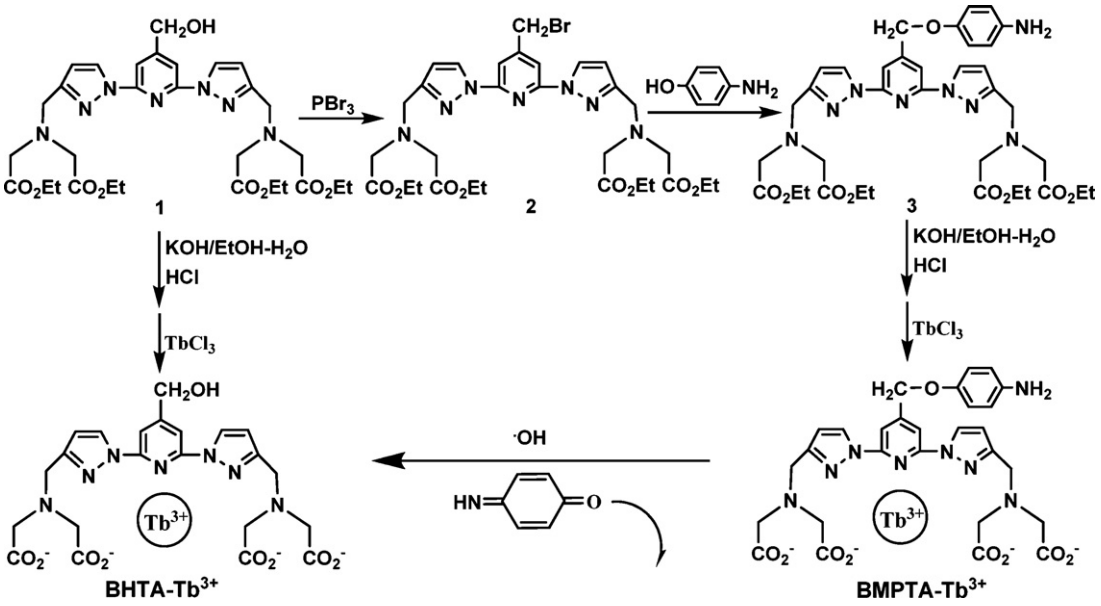

Scheme 1. Synthesis procedures of BMPTA-Tb^{3+} and BHTA-Tb^{3+}.

antenna via a photoinduced electron transfer (PET) process to make the Tb^{3+} luminescence be turned-off [19]. After reacting with •OH, due to the cleavage of the *p*-aminophenyl ether, a highly luminescent Tb^{3+} chelate, *N,N,N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-hydroxymethyl-pyridine] tetrakis(acetate)-Tb^{3+} (BHTA-Tb^{3+}), was generated, which resulted in a 49-fold increase in luminescence quantum yield of the Tb^{3+} chelate (Scheme 1). Moreover, since the *p*-aminophenyl ether in the probe cannot react with other ROS [20], such as H_2O_2, NO, $^1O_2$, $ONOO^-$, and O_2^-, the probe's luminescence can be expected to be selectively turned-on by •OH in aqueous solutions.

2. Experimental

2.1. Materials and physical measurements

Tetraethyl *N,N,N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-hydroxymethyl-pyridine] tetrakis(acetate) (compound 1) was synthesized according to our previous method [21]. 4-Aminophenol was purchased from Acros Organics. Tetrahydrofuran (THF) and acetonitrile were used after appropriate distillation and purification. Acetoxymethyl bromide was purchased from Sigma–Aldrich. HeLa cells were obtained from Dalian Medical University. Hydrogen peroxide (H_2O_2) was diluted immediately from a stabilized 30% solution, and then assayed by using its molar absorption coefficient of 43.6 M^{-1} cm^{-1} at 240 nm [22]. Hydroxyl radical (•OH) was generated by the Fenton reaction from ferrous ammonium sulfate and hydrogen peroxide [22]. Peroxynitrite ($ONOO^-$) was synthesized from sodium nitrite (0.6 M) and H_2O_2 (0.65 M) in a quenched-flow reactor. After the reaction, the solution was treated with MnO_2 to eliminate the excess H_2O_2. The concentration of the $ONOO^-$ stock solution was determined by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M^{-1} cm^{-1} [22]. Singlet oxygen ($^1O_2$) was generated by the reaction of hypochlorite with hydrogen peroxide [23]. Superoxide solution ($O_2^{-\bullet}$) was prepared by adding KO_2 to dry dimethyl sulfoxide and stirring vigorously for 10 min. 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC 13, a NO donor with a half-life of 13.7 min) was synthesized by using a literature method [24] and used as a NO source. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

^{1}H NMR spectra were recorded on a Bruker Avance spectrometer (400 MHz). Mass spectra were measured on a HP1100LC/MSD electrospray ionization mass spectrometry (ESI-MS). Elemental analysis was carried out on a Vario-EL analyser. Time-resolved luminescence spectra and luminescence properties were measured on a Perkin-Elmer LS 50B luminescence spectrometer. The luminescence quantum yields (ϕ) of BMPTA-Tb^{3+} and BHTA-Tb^{3+} were measured in a 0.05 M borate buffer of pH 9.1 and calculated by using the equation $\phi_1 = I_1\varepsilon_2 C_2\phi_2/I_2\varepsilon_1 C_1$ with a standard luminescence quantum yield of $\phi_2 = 0.10$ for the Tb^{3+} chelate of *N,N,N*^{1},*N*^{1}-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl) bis(methylenenitrilo) tetrakis(acetate) (ε_{337nm} = 14,000 cm^{-1} M^{-1})

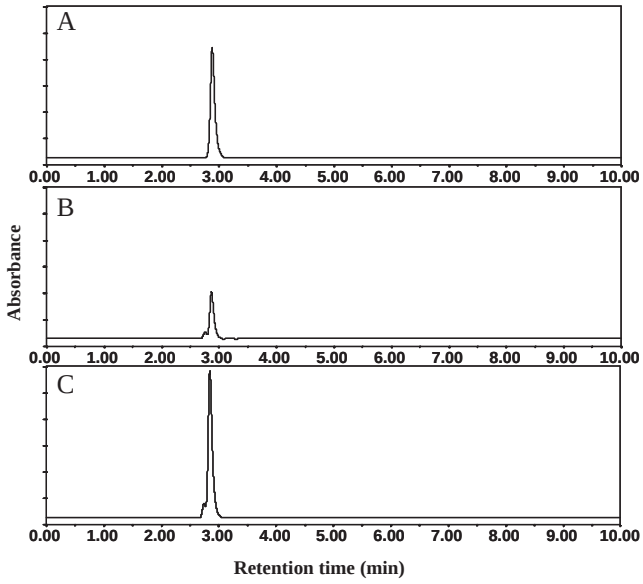

Fig. 1. HPLC analysis of the product of BMPTA-Tb^{3+} reacted with •OH generated by the Fenton reaction (peak retention time, 2.88 min. BMPTA-Tb^{3+} itself showed a broad peak at retention time of ~4.6 min). (A) BHTA-Tb^{3+} (0.25 mM) in 0.05 M HEPES buffer of pH 7.0; (B) the product of BMPTA-Tb^{3+} (0.125 mM) reacted •OH; (C) a mixture of A (0.25 mM) and B (0.125 mM).

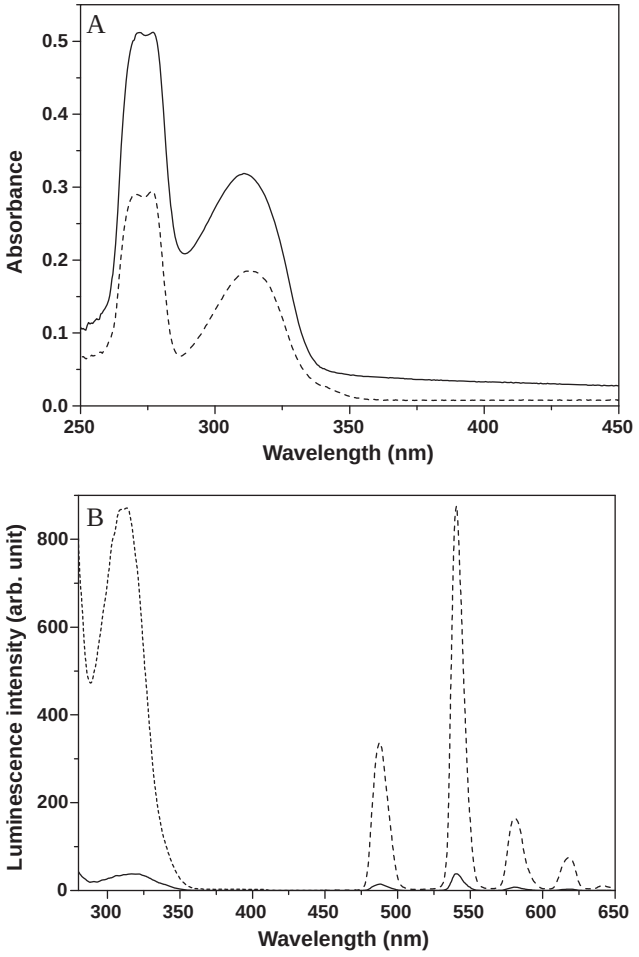

Fig. 2. Absorption (A) and time-resolved luminescence spectra (B) of BMPTA-Tb^{3+} (solid lines) and BHTA-Tb^{3+} (dash lines) in 0.05 M Tris–HCl buffer of pH 7.0. The concentrations of 20 μM and 5.0 μM of BMPTA-Tb^{3+} and BHTA-Tb^{3+} were used for the absorption and luminescence measurements, respectively.

[25]. In the equation, I_1 and I_2, ε_1 and ε_2, C_1 and C_2 are the luminescence intensities, molar extinction coefficients, and concentrations for the measured chelate and the standard chelate, respectively. The time-resolved luminescence measurement for the calibration curve was carried out on a Perkin-Elmer Victor 1420 multilabel counter with an excitation wavelength of 320 nm, emission wavelength of 545 nm, delay time of 0.2 ms, counting time of 0.4 ms, and cycling time of 1.0 ms. HPLC analysis was carried out on a SinoChrom ODS-BP 5 μm (4.6 mm × 250 mm) column using an HPLC system composed of two pumps (P230) and a detector (UV 230+). All bright-field imaging, steady-state luminescence imaging and time-resolved luminescence imaging measurements were carried out on a laboratory-use luminescence microscope [26,27]. The microscope (TE2000-E; Nikon), equipped with a 100 W mercury lamp, a UV-2A filters (Nikon, excitation filter, 330–380 nm; dichroic mirror, 400 nm; emission filter, >420 nm) and a color CCD camera system (RET-2000R-F-CLR-12-C, Qimaging Ltd.), was used for the steady-state luminescence imaging measurement with an exposure time of 20 s. The microscope, equipped with a 30 W xenon flash-lamp (Pulse300, Photonic Research Systems Ltd.), UV-2A filters and a time-resolved digital black-and-white CCD camera system (Photonic Research Systems Ltd.), was used for the time-resolved luminescence imaging measurement with the conditions of delay time, 100 μs; gate time, 1000 μs; lamp pulse width, 6 μs; and exposure time, 60 s.

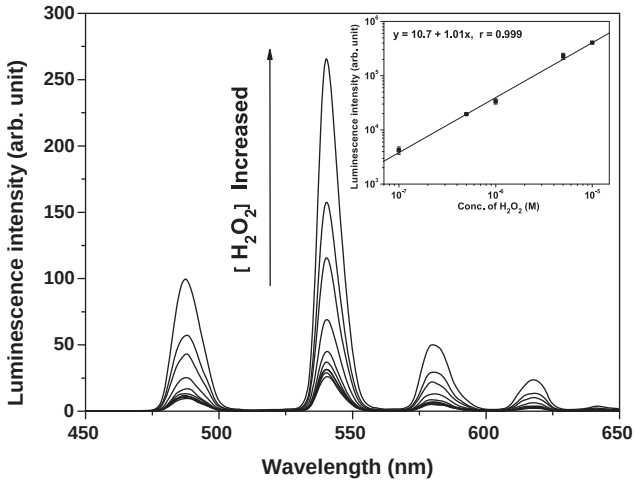

Fig. 3. Time-resolved emission spectra (measured on the Perkin-Elmer LS 50B spectrometer) of BMPTA-Tb^{3+} (5.0 μM) in the presence of ammonium ferrous sulfate (50 μM) and different concentrations of H_2O_2 (0.0, 5.0, 10, 20, 40, 50, 80, 100, 150, 500 μM) for 1 h. The inset is the calibration curve for •OH detection (measured on a Perkin-Elmer Victor 1420 multilabel counter in the H_2O_2 concentration range of 0.1–10 μM).

2.2. Synthesis procedures of BMPTA and BHTA

2.2.1. Synthesis of tetraethyl N,N,N^1,N^1-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-bromomethyl-pyridine] tetrakis(acetate) (compound 2)

To a solution of compound 1 (0.69 g, 1.07 mmol) in 15 mL of dry THF was added dropwise 347 mg of PBr_3 (1.28 mmol). After the solution was stirred at room temperature for 2 h, 150 mL of $CHCl_3$ was added. The solution was washed with 100 mL of water, dried with Na_2SO_4, and then the solvent was evaporated. Purification by silica gel column chromatography with petroleum ether–ethyl acetate (2:1, v/v) as the eluent gave the compound 2 as a white oil (0.56 g, 73.7% yield). ^{1}H NMR ($CDCl_3$): δ = 1.28 (t, 12H), 3.68 (s, 8H), 4.11 (s, 4H), 4.20(m, 8H), 4.47 (s, 2H), 6.60 (d, J = 2.4 Hz, 2H), 7.82 (s, 2H), 8.48 (d, J = 2.4 Hz, 2H).

2.2.2. Synthesis of tetraethyl N,N,N^1,N^1-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-(p-aminophenoxy)methylene-pyridine] tetrakis(acetate) (compound 3)

A mixture of 4-aminophenol (0.125 g, 1.14 mmol) and NaH (27 mg, 1.14 mmol) in 20 mL of dry acetonitrile was stirred at room temperature for 15 min under nitrogen. To the solution was added compound 2 (0.27 g, 0.38 mmol), and the solution was further stirred overnight under nitrogen. After filtration, the solvent was evaporated. Purification by silica gel column chromatography with CH_2Cl_2–CH_3OH (100:4, v/v) as the eluent gave the compound 3 as a yellow oil (150 mg, 53.4% yield). ^{1}H NMR ($CDCl_3$): δ = 0.88 (m, 12H), 3.63 (s, 8H), 4.05 (s, 4H), 4.15 (m, 8H), 5.07 (s, 2H), 6.54 (d, J = 2.4 Hz, 2H), 6.67 (d, J = 8.8 Hz, 2H), 6.84 (d, J = 8.8 Hz, 2H), 7.86 (s, 2H), 8.49 (d, J = 2.4 Hz, 2H).

2.2.3. Synthesis of BMPTA

A mixture of compound 3 (0.15 g, 0.20 mmol), KOH (0.3 g, 4.7 mmol), 0.74 mL H_2O and 8.5 mL ethanol was stirred at room temperature for 20 h. After evaporation, the residue was dissolved in 3 mL water, and pH of the solution was adjusted to ~3 with 3 M HCl. The solution was stirred for 20 h at room temperature and the precipitate was collected by filtration. The dried precipitate was added to 30 mL of dry acetonitrile, and the mixture was refluxed for 30 min. After the precipitate was filtered and dried, BMPTA was obtained as a yellow solid (40 mg, 31.5% yield). ^{1}H NMR (DMSO-

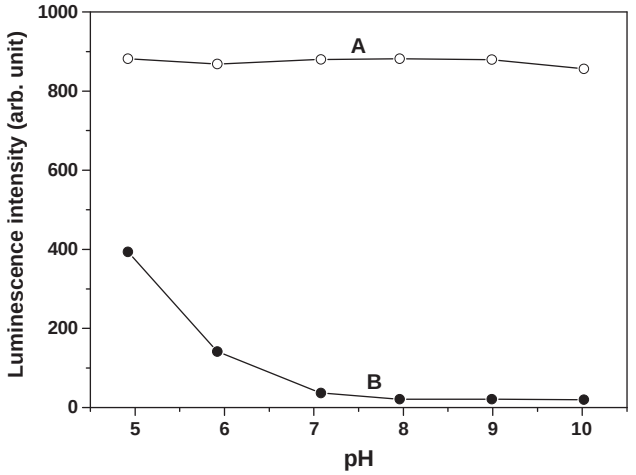

Fig. 4. Effects of pH on the luminescence intensities of BHTA-Tb^{3+} (A, 5.0 μM) and BMPTA-Tb^{3+} (B, 5.0 μM).

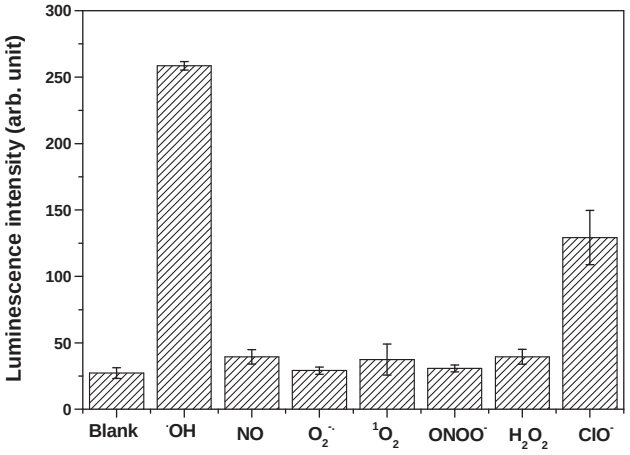

Fig. 5. Time-resolved luminescence intensity responses of BMPTA-Tb^{3+} (5.0 μM) to different ROS (•OH: 50 μM H_2O_2 + 50 μM Fe^{2+}; NO: 50 μM NOC-13; O_2^{-}•: 50 μM KO_2; $^1O_2$: 50 μM NaClO + 50 μM H_2O_2; $ONOO^-$: 50 μM NaONOO; H_2O_2: 50 μM H_2O_2; ClO^-: 50 μM NaClO).

d_6): δ = 3.47 (s, 8H), 3.94 (s, 4H), 5.20 (s, 2H), 6.52 (d, J = 2.4 Hz, 2H), 6.61 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 8.8 Hz, 2H), 7.76 (s, 2H), 8.87 (d, J = 2.4 Hz, 2H). ESI-MS (m/z): $[M]^-$ calcd. for $C_{28}H_{30}N_8O_9$, 621.2; found, 621.3. Anal. calcd. for $C_{28}H_{30}N_8O_9{\cdot}3.5H_2O$: C, 49.05; H, 5.44; N, 16.34; found: C, 49.10; H, 5.17; N, 16.19.

2.2.4. Synthesis of BHTA

A mixture of compound 1 (0.09 g, 0.14 mmol), KOH (0.18 g, 3.3 mmol), 0.54 mL H_2O and 6 mL ethanol was stirred at room temperature for 20 h. After evaporation, the residue was dissolved in 2 mL of water, and pH of the solution was adjusted to ~3 with 3 M HCl. The solution was stirred for 20 h at room temperature and the precipitate was collected by filtration. The dried precipitate was added to 30 mL of dry acetonitrile, and the mixture was refluxed for 30 min. After the precipitate was filtered and dried, BHTA was obtained as a white solid (30 mg, 37.7% yield). ^{1}H NMR (DMSO-d_6): δ = 3.49 (s, 8H), 3.95 (s, 4H), 4.69 (s, 2H), 6.54 (d, J = 2.4 Hz, 2H), 7.71 (s, 2H), 8.85 (d, J = 2.4 Hz, 2H). ESI-MS (m/z): $[M]^-$ calcd. for $C_{22}H_{25}N_7O_9$, 530.2; found, 530.2. Anal. calcd. for $C_{22}H_{25}N_7O_9{\cdot}2.5H_2O$: C, 45.83; H, 5.24; N, 17.00; found: C, 45.84; H, 5.07; N, 16.88.

2.3. HPLC analysis of the product of BMPTA-Tb^{3+} reacted with •OH

The mixture of BMPTA-Tb^{3+} (0.125 mM), H_2O_2 (12.5 mM) and ammonium ferrous sulfate (1.25 mM) in 0.05 M HEPES buffer of pH 7.0 was stirred for 1 h at room temperature, and then subjected to the HPLC analysis with the conditions: eluent, CH_3OH (60%)/HAc (0.1%) aqueous solution; flow rate, 1.0 mL/min; injection volume, 20 μL. The elution was monitored at 315 nm.

2.4. Time-resolved luminescence detection of •OH

To the solutions containing BMPTA-Tb^{3+} (1.0 μM) and various amounts of H_2O_2 in 0.05 mM HEPES buffer of pH 7.0 were added Fe^{2+} (50 μM). The solutions were stirred for 1 h at room temperature. After diluted 10 times by HEPES buffer, the solutions (50 μL) were added to the wells of a 96-well microtiter plate, and then subjected to the time-resolved luminescence measurement on a Perkin Elmer Victor 1420 multilabel.

2.5. Preparation and luminescence imaging of the BMPTA-Tb^{3+}-loaded HeLa cells

2.5.1. Preparation of the acetoxymethyl ester of BMPTA-Tb^{3+}

To a solution of BMPTA (4.79 mg, 0.007 mmol) in 310 μL of anhydrous dimethyl sulfoxide were added 20 μL of triethylamine and 20 μL of acetoxymethyl bromide. After the solution was stirred at room temperature for 20 h, $TbCl_3{\cdot}6H_2O$ (2.61 mg, 0.007 mmol) was added, and then the solution was further stirred for another 0.5 h. The freshly prepared stock solution (20 mM) was used for the cell loading without further purification.

2.5.2. Cell loading and imaging

HeLa cells were cultured in a 25 cm^2 glass culture bottle containing 2 mL of RPMI-1640 medium (Sigma–Aldrich, Inc.), supplemented with 10% fetal bovine serum (Corning Incorporated), 1% penicillin and 1% streptomycin (Gibco). To the culture medium was added the freshly prepared stock solution of the acetoxymethyl ester of BMPTA-Tb^{3+} (25 μL). After the cells were incubated for 2 h at 37 °C in a 5% CO_2–95% air incubator, the cells were washed three times with the isotonic saline solution consisting of 140 mM NaCl, 10 mM glucose, and 3.5 mM KCl, and then incubated with an aqueous solution containing 25 mM of H_2O_2 and 2.5 mM of Fe^{2+} for 1 h at 37 °C in the incubator. The cells were washed five times with the isotonic saline solution, and then used for the steady-state and time-resolved luminescence imaging measurements.

3. Results and discussion

3.1. Synthesis and characterization of the probe

The synthesis procedures of the probe BMPTA-Tb^{3+} and its •OH-reaction product BHTA-Tb^{3+} are shown in Scheme 1. The new Tb^{3+} ligands, BMPTA and BHTA, were well characterized by the NMR, ESI-MS and elementary analysis. In addition, the formation of BHTA-Tb^{3+} by the cleavage reaction between BMPTA-Tb^{3+} and •OH was further confirmed by the reverse-phase HPLC analysis (Fig. 1).

The luminescence properties of BMPTA-Tb^{3+} and BHTA-Tb^{3+} were measured in 0.05 M borate buffer of pH 9.1. The maximum absorption and emission wavelengths of BMPTA-Tb^{3+} and BHTA-Tb^{3+} are 315 nm (ε = 18,700 M^{-1} cm^{-1} for BMPTA-Tb^{3+} and 10,700 M^{-1} cm^{-1} for BHTA-Tb^{3+}) and 541 nm, respectively. As expected, BMPTA-Tb^{3+} itself is almost non-luminescent with a very low luminescence quantum yield (ϕ = 0.16 ± 0.0025%), whereas

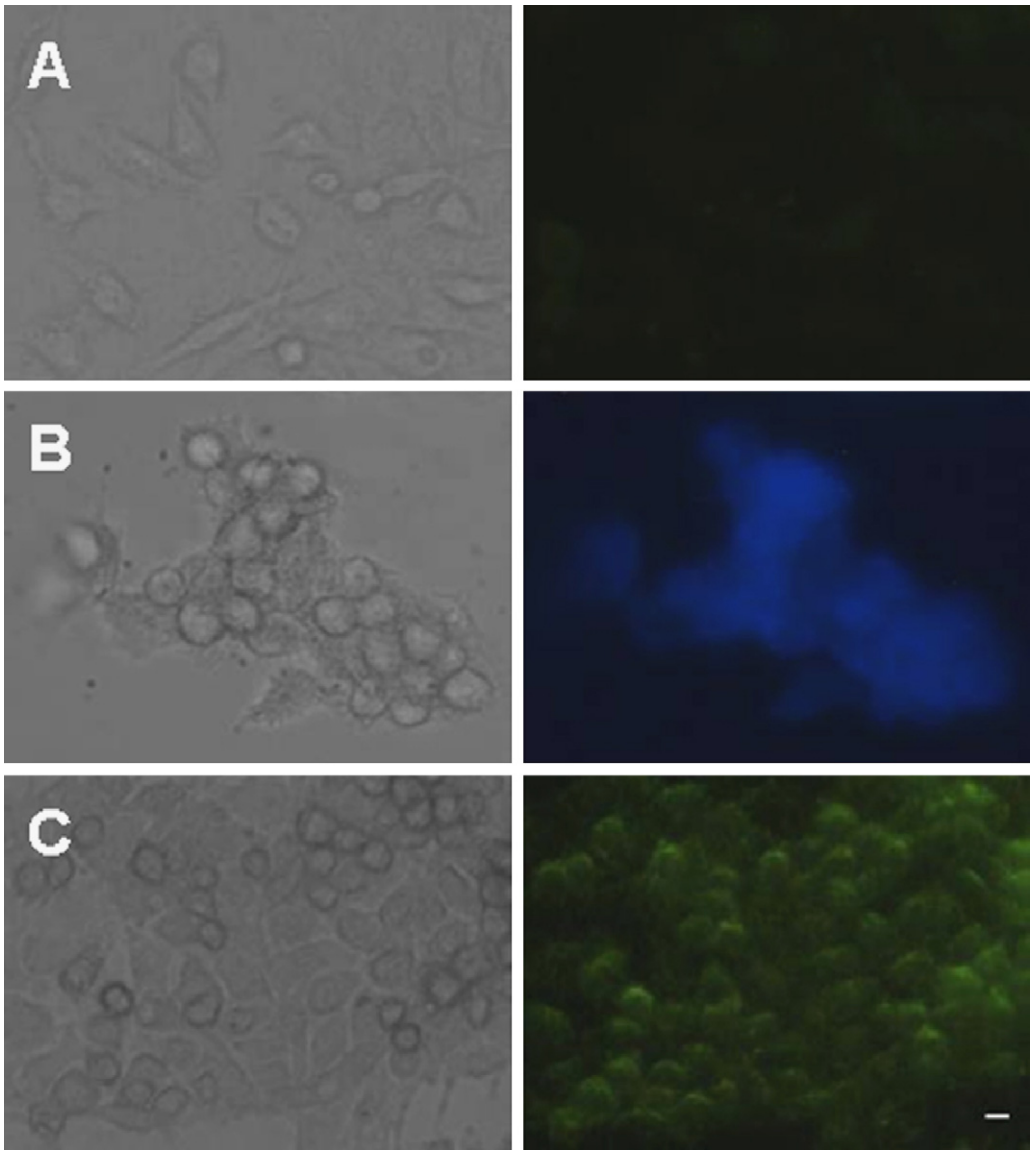

Fig. 6. Luminescence imaging of •OH in the BMPTA-Tb^{3+}-loaded HeLa cells (left: bright-field image; right: luminescence image). (A) Time-resolved luminescence image of the cells incubated with the freshly prepared stock solution of acetoxymethyl ester of BMPTA-Tb^{3+} (0.25 mM) for 2 h. (B) Steady-state luminescence image of the BMPTA-Tb^{3+}-loaded cells treated with H_2O_2 (25 mM) and Fe^{2+} (2.5 mM) for 1 h· (C) Time-resolved luminescence image of the BMPTA-Tb^{3+}-loaded cells treated with H_2O_2 (25 mM) and Fe^{2+} (2.5 mM) for 1 h. Scale bar, 10 μm. The time-resolved luminescence image is shown in pseudo-color (wavelength of 545 nm) treated by a SimplePCI software [22].

BHTA-Tb^{3+} is highly luminescent with a large luminescence quantum yield ($\phi = 7.8 \pm 0.25\%$) and a long luminescence lifetime ($\tau = 2.76$ ms). Fig. 2 shows the absorption and time-resolved excitation and emission spectra of BMPTA-Tb^{3+} and BHTA-Tb^{3+} in 0.05 M Tris–HCl buffer of pH 7.0. The two chelates display the typical Tb^{3+} emission pattern with a main emission peak at 541 nm and several side peaks centred at 488, 581, 618, and 642 nm, respectively.

The solution of BMPTA-Tb^{3+} (5.0 μM) in 0.05 M HEPES buffer of pH 7.0 was used for detecting •OH generated by adding ammonium ferrous sulfate (final 50 μM) and different concentrations of H_2O_2 (Fenton reaction) [28]. Upon addition of H_2O_2 or Fe^{2+} alone, no time-resolved luminescence response was observed. This result indicates that the probe cannot react with H_2O_2 or Fe^{2+}. However, when Fe^{2+} and H_2O_2 were added, the luminescence intensity of the solution was significantly increased, and the luminescence response was proportional to the concentration of H_2O_2 (Fig. 3). The detection limit for H_2O_2, calculated as the concentration corresponding to three standard deviations of the background signal, is 270 nM, which indicates that BMPTA-Tb^{3+} can be used as a luminescent probe for highly sensitive time-resolved luminescence detection of •OH in aqueous solutions. Because the •OH generation by the Fenton reaction is a complicated process [29], and highly reactive •OH can also react with the solvent and buffer molecules in the solution, the exact stoichiometry of the reaction between the probe and hydroxyl radical was not obtained.

3.2. Effects of pH and other ROS

The effects of pH on the luminescence intensities of BMPTA-Tb^{3+} (5.0 μM) and BHTA-Tb^{3+} (5.0 μM) were investigated in 0.05 M Tris–HCl buffers at different pHs ranging from 5.0 to 10.0. As shown in Fig. 4, the luminescence intensity of BHTA-Tb^{3+} is strong and stable in the range of pH 5–10, while that of BMPTA-Tb^{3+} is weak and stable in the range of pH 7–10. The increase of the luminescence of BMPTA-Tb^{3+} at acidic pHs is due to the protonation of the amino group, since which can result in the decrease of the PET efficiency. However, in the physiological pH range, the luminescence intensity change of BMPTA-Tb^{3+} is rather small, which indicates that

BMPTA-Tb^{3+} can be used as an efficient luminescent probe under physiological conditions.

To evaluate the luminescence response specificity of BMPTA-Tb^{3+} to •OH, the reactions of BMPTA-Tb^{3+} with various ROS were examined in 0.05 M HEPES buffer of pH 7.0. As shown in Fig. 5, the luminescence intensity of BMPTA-Tb^{3+} was significantly increased in the presence of •OH generated by the Fenton reaction, whereas almost no luminescence responses of BMPTA-Tb^{3+} to the additions of 10 equiv. of NO, $O_2^{-\bullet}$, $^1O_2$ (singlet oxygen), $ONOO^-$ and H_2O_2 were observed. Although the addition of ClO^- can also induce a ~4.7-fold increase of the luminescence intensity, this luminescence response of the probe is still weaker than that of the probe to •OH. The above result demonstrates that BMPTA-Tb^{3+} is a highly specific luminescent probe for the detection of •OH.

3.3. Time-resolved luminescence imaging of •OH in living cells

For the luminescence imaging detection of •OH in living cells, the acetoxymethyl ester of BMPTA-Tb^{3+} was synthesized according to a previous method [21,30], since which could be easily transferred into the cells with ordinary incubation method, and in the cells, accompanied by the rapid hydrolysis of the acetoxymethyl ester catalyzed by ubiquitous intracellular esterases, the stable BMPTA-Tb^{3+} chelate could be regenerated. After the cultured HeLa cells were incubated with the acetoxymethyl ester of BMPTA-Tb^{3+} (250 μM) for 2 h at 37 °C, and followed by the additions of H_2O_2 (25 mM) and Fe^{2+} (2.5 mM) for another 1.0 h, the cells were imaged with steady-state and time-resolved luminescence modes, respectively. As shown in Fig. 6, in the absence of •OH, no luminescence from the cells could be observed (Fig. 6A). After the cells were incubated in the culture medium containing H_2O_2 and Fe^{2+}, strong pale blue luminescence (mixture of blue and green from cell components and the Tb^{3+} chelate, Fig. 6B) and green luminescence (Fig. 6C) from the cells were observed (the different locations of the cells were used for the imagings to avoid the effect of photoblenching. The morphology changes of the cells can be considered to be attributed to the cytotoxicities of •OH and H_2O_2). Compared to the steady-state luminescence images, highly specific and sensitive time-resolved luminescence images of the cells with strong green luminescence signals were obtained since the autofluorescence from the cell components had been completely suppressed by the time-resolved mode. These imaging results clearly demonstrate that BMPTA-Tb^{3+} is a useful luminescent probe for the background-free time-resolved luminescence imaging detection of •OH in living cells.

4. Conclusions

In conclusion, by incorporating a Tb^{3+} chelate of the polyacid derivative of 2,6-bis(N-pyrazolyl)pyridine into a 4-aminophenoxy moiety, a Tb^{3+} chelate-based luminescent probe that can be used for highly sensitive time-resolved luminescence detection of •OH in aqueous solutions, BMPTA-Tb^{3+}, has been successfully developed. The specific reaction of the probe with •OH in aqueous solutions could afford a highly luminescent Tb^{3+} chelate BHTA-Tb^{3+}, which displayed notable luminescence properties, namely long emission lifetimes (>2.5 ms), large Stokes shift (>200 nm), high stability and good water solubility. These characteristics allowed the probe to be used for the background-free time-resolved luminescence detection of •OH in complicated biological samples. In addition, the results of luminescence imaging by monitoring •OH in living cells demonstrated the utility of the probe for *in vivo* •OH detection. The new luminescence probe, with fine chemical and photophysical properties as well as the time-resolved capacity, provides a useful tool for detecting •OH in complicated biological samples, which could be expected to be used for investigating the pathogenic role of •OH in biological systems.

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