Electronic Supplementary Information for

Artificial luminescent protein as a bioprobe for time-gated luminescence bioimaging

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Experimental details

1. Reagents and materials

Apoferritin was prepared from ferritin by a reductive dissolution procedure.[1] Ferritin was obtained according to the methods described previously.[2,3] All the chemical materials were purchased from commercial sources and used without further purification unless otherwise stated.

2. Physical measurements

The ^{1}H NMR spectra were recorded on a Bruker Avance spectrometer (400 MHz). Elemental analysis was carried out on a Vanio-EL analyzer. The UV-Vis absorption spectra were measured on a Perkin-Elmer Lambda 35 UV-Vis spectrometer. The luminescence lifetime was measured on a Perkin-Elmer LS 50B luminescence spectrometer. The time-gated luminescence spectra were measured on a Perkin-Elmer LS 50B luminescence spectrometer with the conditions of delay time, 0.2 ms; gate time, 0.4 ms; cycle time, 20 ms; excitation slit, 10 nm; and emission slit, 5 nm. The luminescence quantum yields (ϕ) of NTTA-Eu^{3+} were measured in a 0.05 M borate buffer (pH 9.1) with a reported method and calculated by using the equation $\phi_1=I_1\varepsilon_2C_2\phi_2/I_2\varepsilon_1C_1$ with a standard luminescence quantum yield of ϕ_2 = 0.16 for the Eu^{3+} complex of *N,N,N^l,N^l*-(4'-phenyl-2,2':6',2"-terpyridine-6,6"-diyl) bis(methylenenitrilo) tetrakis(acetate) (ε_{335nm} = 14,300 $cm^{-1}M^{-1}$).[4,5] 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 of the measured complex and the standard complex, respectively. The confocal laser scanning microscopy imaging measurement

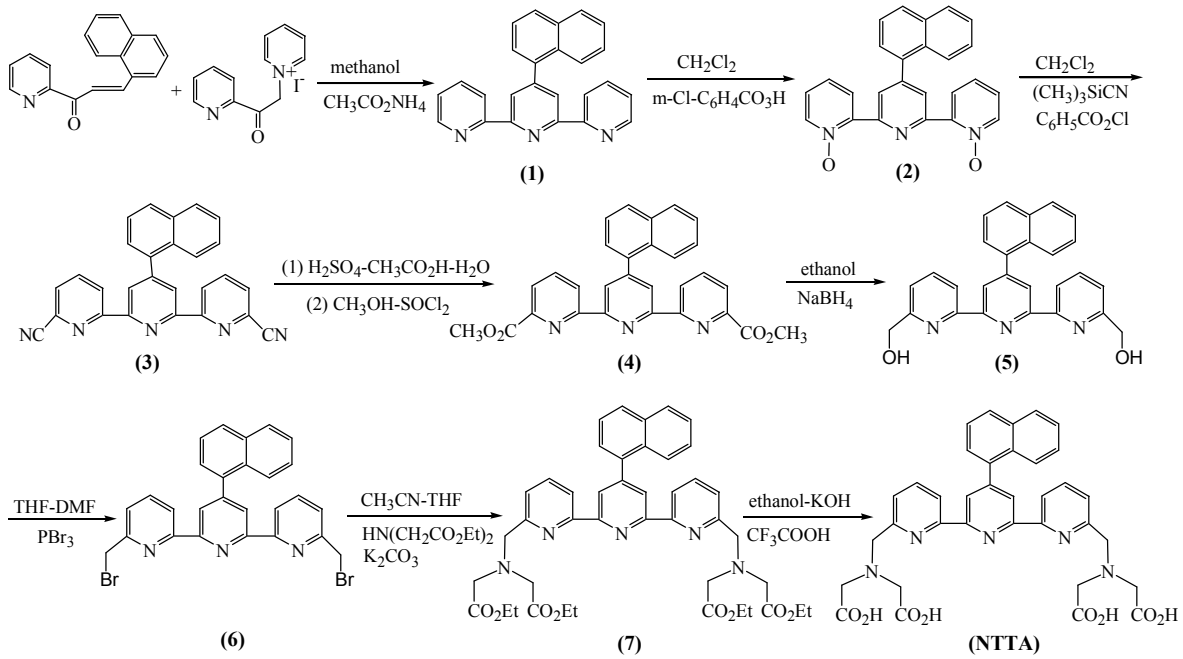
was carried out on a Leica SP5 confocal microscopy. All steady-state luminescence imaging and time-gated luminescence imaging measurements were carried out on a laboratory-use luminescence microscope.[6] The microscope (TE2000-E, Nikon), equipped with a 100 W mercury lamp, a UV-2A filters (Nikon, excitation 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 10 s. The microscope, equipped with a 30 W xenon flash-lamp (Pulse300, Photonic Research Systems Ltd.), UV-2A filters and a time-gated digital black-and-white CCD camera system (Photonic Research Systems Ltd.), was used for the time-gated luminescence imaging measurement with the conditions of delay time, 100 μs; gate time, 1000 μs; lamp pulse width, 6 μs; and exposure time, 300 s. The time-gated luminescence image is shown in pseudo-color (wavelength of 615 nm) treated by a SimplePCI software.[6] All the measurements were carried out at room temperature.

3. Synthesis of the ligand NTTA

The new Eu^{3+} chelating ligand, *N,N,N*^{1},*N*^{1}-[4'-(1-naphthyl)-2,2':6',2"-terpyridine-6,6"-diyl] bis(methylenenitrilo) tetrakis(acetic acid) (NTTA), was synthesized following the eight-step reaction shown in Scheme S1. Because this ligand was synthesized by using the synthesis procedure for a structurally similar Eu^{3+} ligand, *N,N,N*^{1},*N*^{1}-(4'-phenyl-2,2':6',2"-terpyridine-6,6"-diyl) bis(methylenenitrilo) tetrakis (acetic acid),[4,5] and we had synthesized a number of the structurally similar ligands before this work,[6-12] only ^{1}H NMR was used for the structure characterization of the intermediates in the synthesis procedure. However, the target compound NTTA was well-characterized both by ^{1}H NMR and CHN analysis to confirm its structure and purity.

(i) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine (1). The starting materials (E)-3-(1"-naphthyl)-1-(pyrid-2'-yl)prop-2-enone and N-[2-(pyrid-2'-yl)-2-oxoethyl] pyridinium iodide were prepared by the literature methods.[4-12] A mixture of 23.10 g dry $AcONH_4$ (300 mmol), 16.30 g N-[2-(pyrid-2'-yl)-2-oxoethyl] pyridinium iodide (50.0 mmol), 12.97 g (E)-3-(1"-naphthyl)-1-(pyrid-2'-yl)prop-2-enone (50.0 mmol) and 400 mL dry methanol was refluxed for 24 h. After the solvent was evaporated to ~100 mL, the mixture was cooled at -15 °C for 1 h, the precipitate was filtered and washed with cold methanol. After the precipitate was recrystallized from acetonitrile, compound **1** was obtained (7.10 g, 39.51% yield). ^{1}H NMR

($CDCl_3$): δ 8.72 (d, *J* = 8.0 Hz, 2H), 8.68 (d, *J* = 4.8 Hz, 2H), 8.64 (s, 2H), 7.97-7.87 (m, 5H), 7.60-7.45 (m, 4H), 7.33 (t, *J* = 4.8 Hz, 2H).



Scheme S1 Synthesis procedure of the ligand NTTA.

(ii) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine-1,1"-dioxide (2). To 400 mL of CH_2Cl_2 was added 15.70 g of compound **1** (43.7 mmol) and 37.60 g 3-chloroperoxybenzoic acid. The solution was stirred at room temperature for 24 h. After the solvent was evaporated to ~200 mL, the solution was washed with 3 × 300 mL of 10% Na_2CO_3, dried with Na_2SO_4 and evaporated. The product was recrystallized from methanol to yield compound **2** (11.75 g, 68.71% yield). ^{1}H NMR ($CDCl_3$): δ 9.09 (s, 2H), 8.34 (d, *J* = 6.4 Hz, 2H), 8.26 (d, *J* = 7.6 Hz, 2H), 8.13 (d, *J* = 7.6 Hz, 1H), 7.92 (d, *J* = 7.6 Hz, 2H), 7.62 (d, *J* = 6.8 Hz, 1H), 7.57-7.53 (m, 3H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.34 (t, *J* = 6.8 Hz, 2H).

(iii) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine-6,6"-dicarbonitrile (3). To 350 mL of CH_2Cl_2 were added 11.70 g of compound **2** (29.89 mmol) and 23.67 g of $(CH_3)_3SiCN$ (239 mmol) with stirring. After stirring for 20 min, 16.85 g of benzoyl chloride (120 mmol) was added dropwise within 20 min, and the solution was stirred at room temperature for 24 h. The solution was evaporated to ~70 mL, to the solution was added 400 mL of 10% K_2CO_3, and the mixture was further stirred at room temperature for 1 h. The precipitate was filtered and washed with water and cold acetonitrile, and dried. Compound **3** was obtained (9.12 g, 74.50% yield). ^{1}H

NMR (DMSO-d_6): δ 9.05 (d, *J*=7.6 Hz, 2H), 8.54 (s, 2H), 8.33 (m, 2H), 8.18 (d, *J*=7.2 Hz, 2H), 8.12 (m, 2H), 7.86 (d, *J*=7.6 Hz, 1H), 7.69 (m, 2H), 7.61 (m, 2H).

(iv) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine-6,6"-dicarboxylate dimethyl (4). After a mixture of 7.57 g of compound **3** (18.50 mmol), 80 mL of concentrated H_2SO_4, 70 mL of acetic acid and 15 mL of water was stirred at 90 °C for 24 h, the solution was added to 700 mL of ice-water. The precipitate was filtered and washed with water and acetonitrile, and dried. To 450 ml of cooled methanol (ice-water bath) was added dropwise 20 mL of thionyl chloride. After stirring 15 min at room temperature, the half of above precipitate (4.77 g) was added, and the mixture was refluxed for 24 h. After evaporation, the residue was washed with 200 mL of 10% Na_2CO_3, and dried. The product was recrystallized from toluene to yield compound **4** (3.89 g, 73.67% yield). ^{1}H NMR ($CDCl_3$): δ 8.90 (d, *J* = 8.0 Hz, 2H), 8.72 (s, 2H), 8.18 (d, *J* = 8.0 Hz, 2H), 8.05 (t, *J* = 8.0 Hz, 2H), 7.97 (d, *J* = 7.6 Hz, 2H), 7.86 (d, *J* = 8.4 Hz, 1H), 7.59 (d, *J* = 7.6 Hz, 2H), 7.53 (t, *J* = 8.0 Hz, 1H), 7.45 (t, *J* = 8.0 Hz, 1H), 3.97 (s, 6H).

(v) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine-6,6"-dimethanol (5). A mixture of 400 mL dry ethanol, 8.60 g compound **4** (18.10 mmol) and 3.78 g $NaBH_4$ (100 mmol) was stirred at room temperature for 3 h, and further refluxed for 1 h. After the solvent was evaporated, 200 mL of saturated $NaHCO_3$ was added, and the solution was heated to boiling. After cooling, the precipitate was filtered and washed with water and cold acetonitrile. After drying, compound **5** was obtained (7.08 g, 93.28% yield). ^{1}H NMR (DMSO-d_6): δ □□8.60 (d, *J* = 7.6 Hz, 2H), 8.52 (s, 2H), 8.10-8.03 (m, 4H), 7.80 (d, *J* = 8.4 Hz, 1H), 7.68 (t, *J* = 8.0 Hz, 1H), 7.63-7.53 (m, 5H), 5.49 (s, 2H), 4.62 (s, 4H).

(vi) Synthesis of 4'-(1-naphthyl)-2,2':6',2"-terpyridine-6,6"-dibromomethyl (6). To a mixture of 200 mL dry THF, 100 mL dry DMF and 6.71 g of compound **5** (16 mmol) was added 15.10 g of PBr_3 (56 mmol) with stirring. After the solution was refluxed for 4 h, the solvent THF was evaporated. To the solution was added 200 mL of 5% $NaHCO_3$, the precipitate was filtered and washed with water and dried. The residue was purified by silica gel column chromatography using CH_2Cl_2-methanol (99.5:0.5) as eluent. Compound **6** was obtained (5.21 g, 59.75% yield). ^{1}H NMR ($CDCl_3$): δ 8.67 (s, 2H), 8.62 (d, *J* = 7.6 Hz, 2H), 7.97-7.87 (m, 5H), 7.62-7.46 (m ,6H), 4.59 (s, 4H).

(vii) Synthesis of tetraethyl *N,N,N^1,N^1*-[4'-(1-naphthyl)-2,2':6',2"- terpyridine-6,6"-diyl]

bis(methylenenitrilo) tetrakis(acetate) (7). After a mixture of 200 mL dry acetonitrile, 25 mL dry THF, 1.09 g compound **6** (2 mmol), 795 mg diethyl iminodiacetate (4.20 mmol) and 2.80 g K_2CO_3 (20 mmol) was refluxed for 24 h with stirring, the mixture was filtered. After evaporation, the oily residue was washed with hexane and petroleum ether, and purified by silica gel column chromatography using ethyl acetate as eluent. Compound **7** was obtained (1.48 g, 97.11% yield). ^{1}H NMR ($CDCl_3$): δ 8.60 (d, J = 7.6 Hz, 2H), 8.59 (s, 2H), 7.95-7.93 (m, 3H), 7.87 (t, J = 7.6 Hz, 2H), 7.63 (d, J = 7.6 Hz, 2H), 7.58 (d, J = 5.6 Hz, 2H), 7.52 (t, J = 8.4 Hz, 1H), 7.46 (t, J = 7.4 Hz, 1H), 4.10-4.04 (m, 12H), 3.63 (s, 8H), 1.14(t, J = 7.2 Hz, 12H).

(viii) Synthesis of NTTA. A mixture of 100 mL ethanol, 5 g KOH, 7 mL H_2O and 3.05 g compound **7** (4.0 mmol) was stirred at room temperature for 2 h, and further refluxed with stirring for 1 h. After the solvent was evaporated, the residue was dissolved in 100 mL water, and the solution was filtered. To the solution was added dropwise 3 M HCl to adjust the pH to ~2, and the solution was stirred for 10 h at room temperature. The precipitate was collected by filtration and washed with water. After drying, the product was added to 100 mL of acetonitrile, and the mixture was refluxed 10 min with stirring. The precipitate was filtered and dried. NTTA was obtained (2.58 g, 99.2% yield). ^{1}H NMR (DMSO-d_6,): δ 12.37, (s, 4H), 8.61 (d, J = 8.4 Hz, 2H), 8.50 (s, 2H), 8.09 (t, J = 7.6 Hz, 2H), 8.04 (d, J = 7.6 Hz, 2H), 7.88 (d, J = 8.0 Hz, 1H), 7.71-7.56 (m, 6H), 4.03 (s, 4H), 3.51 (s, 8H). Anal. Calcd. for $C_{35}H_{31}N_5O_8 \cdot H_2O$ (NTTA·H_2O): C, 62.96; H, 4.98; N, 10.49. Found: C, 62.93; H, 5.04; N, 10.38. Fig. S1 shows the ^{1}H NMR spectrum of the obtained NTTA in DMSO-d_6, no by-products were found.

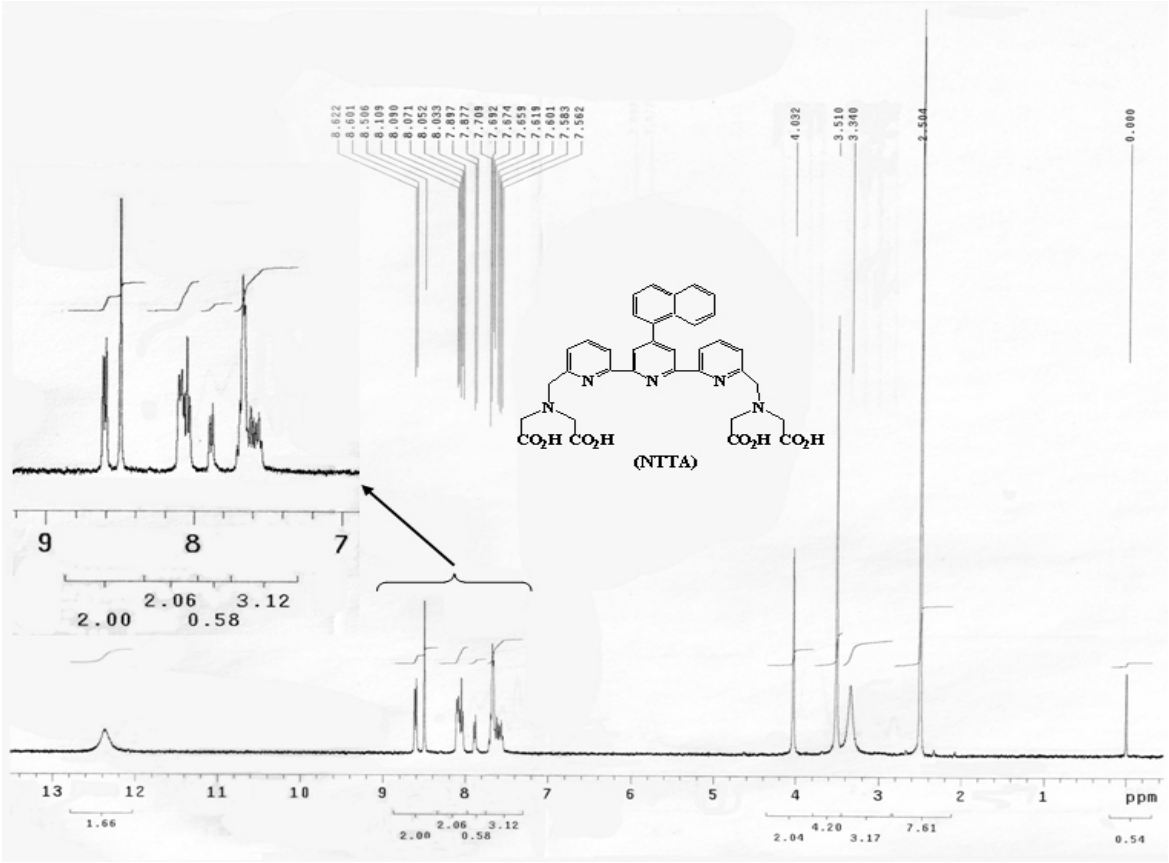


Fig. S1 ^{1}H NMR spectrum of the obtained NTTA in DMSO-d_6.

4. Synthesis and characterization of NTTA-Eu^{3+}

To 0.3 mL DMSO was added 30 μmol NTTA. This solution was then mixed with 0.3 mL aqueous solution containing 30 μmol $EuCl_3$. After mixture, a solution containing 0.05 M of NTTA-Eu^{3+} was obtained as the stock solution.

Fig. S2 shows the luminescence response of NTTA (5.0 μM) to the addition of Eu^{3+} (5.0 μM) in 0.05 M Tris-HCl buffer (pH 5.0). As shown in Fig. S2, upon addition of Eu^{3+} to the NTTA solution, the luminescence intensity of the solution is rapidly increased and reaches to the maximum value, and then keeps at a steady level under the continuous excitation. This result indicates that the complex NTTA-Eu^{3+} can be rapidly formed after NTTA is mixed with Eu^{3+}, which can be considered to be attributed to the strong chelating ability of NTTA to Eu^{3+} due to its nonadentate chelating property.

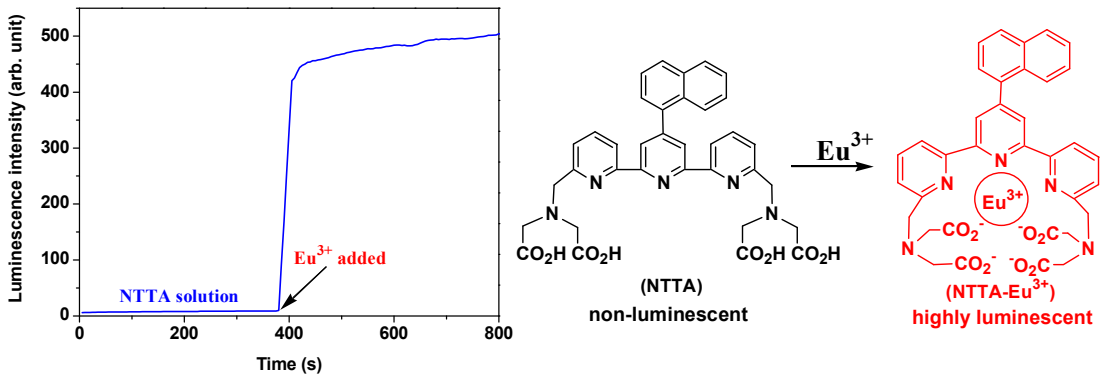


Fig. S2 Time course of the luminescence response of NTTA (5.0 μM) to the addition of Eu^{3+} (5.0 μM) in 0.05 M Tris-HCl buffer of pH 5.0 (left). The right reaction shows the formation of NTTA-Eu^{3+} after adding Eu^{3+} to the NTTA solution.

Fig. S3 shows the effect of pH on the luminescence intensity of NTTA-Eu^{3+}. The luminescence intensity of the NTTA-Eu^{3+} solution has little changes at pH > 3, but remarkably decreases at pH 2 since the complex itself is not stable at pH < 3. When the pH is gradually raised back to neutral, the luminescent intensity of NTTA-Eu^{3+} can be restored accordingly.

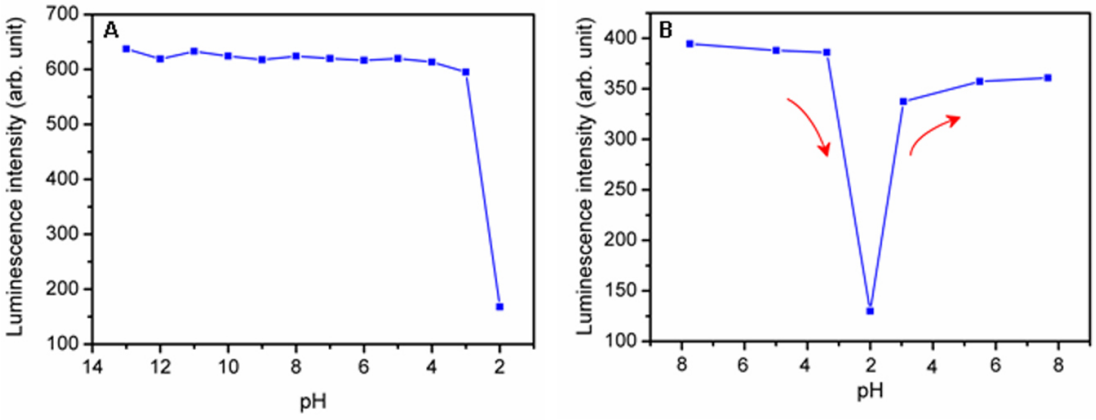


Fig. S3 (A) The effect of pH on the luminescence intensity of NTTA-Eu^{3+} (5.0 μM). After the aqueous solutions of NTTA-Eu^{3+} with different pH values ranging from 2 to 13 were prepared, the luminescence intensities at 611 nm of the solutions were measured with λ_{ex} = 331 nm. (B) The luminescence restorability of an aqueous solution of NTTA-Eu^{3+} (3.0 μM) during the pH adjustment (from 7.7 to 2.0 and then backed to 7.7).

5. Preparation and characterization of NTTA-Eu^{3+}@AFt

To an aqueous solution of apoferritin (4.5 mL, 44 μM) was added dropwise 0.05 M NTTA-Eu^{3+} to a molar ratio of 1:150 (protein/europium), during which, a few drops (~100 μL) of 1.0 M NaOH was added to keep the mixture clear. The pH of the mixture was slowly lowered to 2.0 by adding 1.0 M HCl under stirring, followed by continuously stirring at this pH for 30 min. Thereafter the pH was slowly adjusted to 8.5 by adding 1.0 M NaOH dropwise. The resulting solution was further stirred for 1 h at room temperature, subsequently stood at 4 °C overnight. After centrifugation (12500 g) at 4 °C for 20 min, the supernatant was exhaustively dialyzed against 0.05 M Tris-HCl buffer of pH 7.4, and then purified by Sephadex G-25 (GE Healthcare) column chromatography using the Tris-HCl buffer as eluent. The eluate was monitored on a Perkin-Elmer Lambda 35 spectrometer at 280 nm (for protein) and 324 nm (for NTTA-Eu^{3+}), respectively. Apoferritin-containing fractions were finally collected and concentrated. The protein concentration of as-prepared NTTA-Eu^{3+}@AFt was determined with Bradford method, and the Eu^{3+} concentration was determined by inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 5300DV).

For transmission electron microscopy (TEM) observation, the NTTA-Eu^{3+}@AFt solution was dropped onto a carbon-coated copper grid and dried in air at room temperature, followed by negative staining with phosphotungstic acid. TEM images were obtained using an FEI Tecnai G^2 Spirit microscope operating at an accelerating voltage of 120 kV. Native PAGE analysis of NTTA-Eu^{3+}@AFt, along with apoferritin, was performed on a Bio-Rad electrophoresis system (Mini-PROTEAN 3 Cell) using 4% stacking gel and 6% resolving gel. After electrophoresis, the gel was imaged under a UV lamp irradiation (365 nm), and then stained overnight with 0.25% Coomassie Brilliant Blue R-250, followed by destaining with an aqueous solution containing 7% (v/v) acetic acid and 5% (v/v) methanol.

Fig. S4 shows the time-gated emission spectra of NTTA-Eu^{3+} and NTTA-Eu^{3+}@AFt at different delay times, and the emission intensity changes of NTTA-Eu^{3+} and NTTA-Eu^{3+}@AFt at 611 nm against different delay times. The correlation between the natural logarithm of emission intensity of NTTA-Eu^{3+} and the delay time shows a good linearity with a slope of 0.753, which indicates that the luminescence of NTTA-Eu^{3+} has a single exponential decay with a lifetime of 1.33 ms. Similar to NTTA-Eu^{3+}, the correlation between the natural logarithm of emission intensity of NTTA-Eu^{3+}@AFt and the delay time shows a good linearity with a slope of 0.777,

indicating that the luminescence of NTTA-Eu^{3+}@AFt also has a single exponential decay with a lifetime of 1.29 ms.

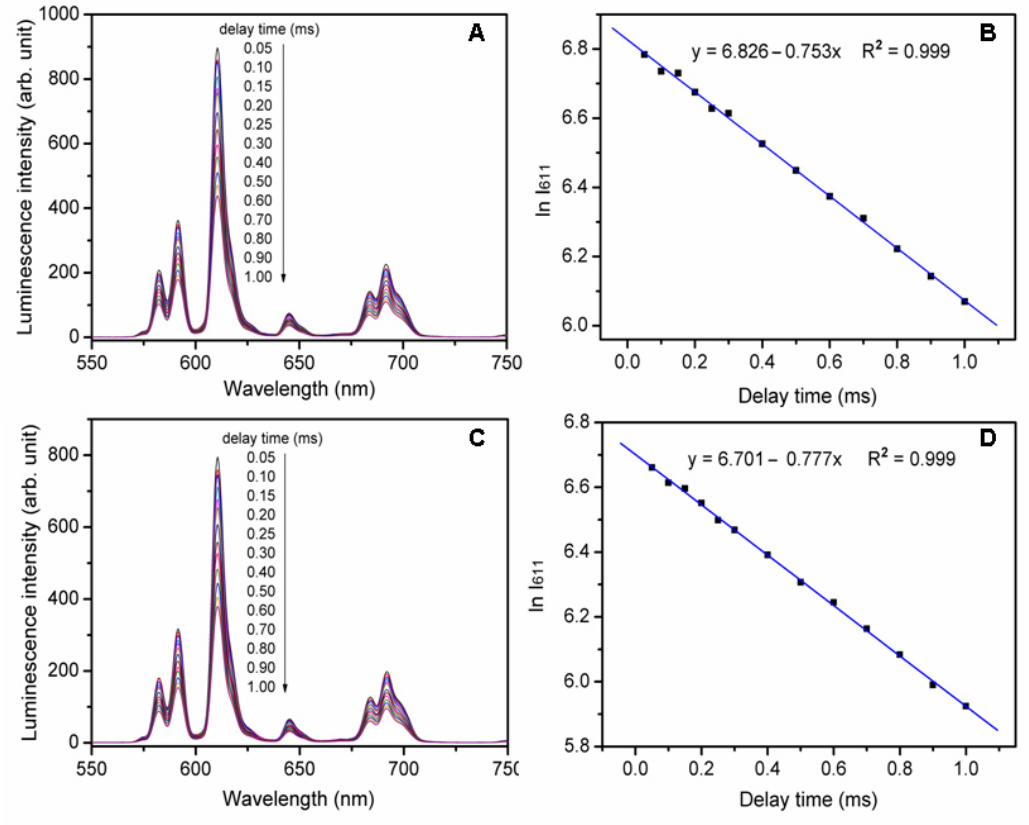


Fig. S4 Time-gated emission spectra of NTTA-Eu^{3+} (A, 5.0 μM) and NTTA-Eu^{3+}@AFt (C, 3.0 μM of Eu^{3+}) in 0.05 M Tris-HCl buffer (pH 7.8) at different delay times, and the emission intensity changes of NTTA-Eu^{3+} (B) and NTTA-Eu^{3+}@AFt (D) at 611 nm against different delay times.

6. Cell imaging experiments

Human hepatoma cell line HepG2 (supplied by ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen) supplemented with 10% (v/v) newborn calf serum (NBCS, Invitrogen) and 1% penicillin-streptomycin (Beyotime, China) in a humidified incubator at 37 °C with 5% CO_2. HepG2 cells were seeded in Petri dishes (Corning, USA) and grown to sub-confluence. The serum-free DMEM containing NTTA-Eu^{3+}@AFt (with a final Eu^{3+} concentration of ~ 6 mM) was subsequently added. After incubation for 5 h, the cells were washed six times with 0.05 M PBS buffer of pH 7.4, and then subjected to the steady-state, time-gated and confocal luminescence imaging detections.

7. References

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