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Supporting Information for

A tumor-targetable probe based on europium(III)/gadolinium(III) complex-conjugated transferrin for bimodal time-gated luminescence and magnetic resonance imaging of cancer cells *in vitro* and *in vivo*

Bo Song,*[a] Jiao Jiang, [a] Huinan Yan, [a] Shengjun Huang[b] and Jingli Yuan*[a]

[a] State Key Laboratory of Fine Chemicals, School of Chemical Engineering, Dalian University of Technology, Dalian 116024, China.

[b] Division of Fossil Energy Conversion, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, Chin

E-mail: bo.song@dlut.edu.cn, jlyuan@dlut.edu.cn;

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1. Experimental section

Materials and physical measurements

N,N-Dimethyl-formamide (DMF), tetrahydrofuran (THF) and acetonitrile were used after appropriate distillation and purification. Cuprous iodide, cyanuric chloride, gadolinium (III) chloride hexahydrate, and europium (III) chloride hexahydrate were supplied by Aladdin. Transferrin (Tf) and 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Regenerated cellulose dialysis bags with a molecular weight cutoff of 10 kD were purchased from Spectrum Laboratories (Rancho Dominguez, CA). MCF-7 cells, LO2 cells, KM mice, and tumor-bearing BALB/c nude mice were obtained from Dalian Medical University. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance spectrometer (400 MHz for ^{1}H and 126 MHz for ^{13}C). Mass spectra were recorded on Thermo TSQ Quantum Ultra LC/MS/MS spectrometer. Elemental analysis was carried out on a Vario-EL analyzer. The contents of Gd and Eu were measured on a PerkinElmer Optima 2000DV inductively coupled plasma-optical emission spectrometer (ICP-OES). Time-gated luminescence (TGL) spectra were measured on a Perkin-Elmer LS 50B luminescence spectrometer with the settings of delay time, 0.2 ms; gate time, 0.4 ms; cycle time, 20 ms; excitation slit, 15 nm; and emission slit, 10 nm. Absorption spectra were measured on a UV-1800 UV-Vis spectrophotometer (Shimadzu Instruments Suzhou Co., Ltd.). Luminescence lifetimes were measured on an Edinburgh FS5 spectrometer. All bright-field, steady-state and TGL imaging measurements were conducted on a laboratory-use luminescence microscope. The measurements of transverse and longitudinal relaxation times were performed on a 0.5 T NM12 MR analyzer (Suzhou Niumag Analytical Instrument Corporation). All the MRI measurements were carried out on an NMI20-030H-I MR imager (Suzhou Niumag Analytical Instrument Corporation).

SDS-PAGE electrophoresis

Samples were mixed with an equal volume of 5×SDS-PAGE loading buffer containing 0.05M

Tris-HCl (pH 6.8), 10% SDS (w/v), 2.5% mercaptoethanol (v/v) and 10% glycerol (w/v). Then, the mixture was boiled for 5 min and cooled before loading on the 12% polyacrylamide gel. The electrophoresis was run at a constant voltage of 80 V for 2 h. After the electrophoresis, gels were stained with Coomassie Blue (0.25%) for 1 min and then destained with distilled water for 15 min. Each set of experiments was repeated three times with the same procedure.

MTT assay

The cytotoxicity of Tf-CNSTTA-Eu^{3+} to MCF-7 cells was determined by MTT assay as previously reported.[1] MCF-7 cells cultured in Dulbecco's modified Eagle medium (DMEM) were washed with isotonic saline (ISS, 140 mM NaCl, 10 mM glucose, 3.5 mM KCl) before use, and then incubated with different concentrations of Tf-CNSTTA-Eu^{3+} probe (0, 1, 2, 3, 4, 6, 8 $g \cdot L^{-1}$) at 37 °C in a 5% CO_2 /95% air incubator for 24 h. After that, the cells were washed with ISS and incubated with ISS containing 5 mg mL^{-1} MTT in an incubator for 4 h. After supernatants were removed, the cells was dissolved in 100 μL DMSO and the absorbance at 490 nm was measured.

TGL imaging of live cells

MCF-7 (human breast adenocarcinoma cell line) and LO2 (human hepatocyte cell line) cells were cultured in a glass bottom culture dishes in DMEM with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37 °C in a 5% CO_2/95% air incubator. For staining cells, the cultured cells were washed three times with ISS, and then incubated with 0.4 g L^{-1} Tf-CNSTTA-Eu^{3+} in culture medium for 4 h. After thoroughly washing with ISS, the cells were subjected to the TGL imaging on the microscope with the conditions of gate time, 1.0 ms; delay time, 10 μs; lamp pulse width, 60 μs; and exposure time, 2 s. For Tf receptors (TfRs) competition experiment, MCF-7 cells were incubated with free Tf solution (5 g L^{-1}) for 1 h. After that, they were loaded with 0.4 g L^{-1} Tf-CNSTTA-Eu^{3+}. After incubation for 4 h, the cells were washed three times with ISS before luminescence microscopic imaging measurements.

In vivo distribution and toxicity evaluation

After three KM mice (female, ~20 g body weight) were anesthetized with 1.5% isoflurane in oxygen, they were injected with Tf-CNSTTA-Gd^{3+} (200 μL, 10 g L^{-1} in physiological saline solution) via tail vein. Then the mice were continuously monitored by sequential T_1-weighted MRI on an NMI20-030H-I Analyzing and Imaging System. In each experiment, the MR intensity analysis of ROIs was performed using the Horos V3.3.1 software for Mac. To qualify the signal enhancement, the signal-to-noise ratio (SNR) was determined using the formula: $SNR = SI_{tumor}/SD_{noise}$, where SI and SD represent signal intensity and s.d, respectively.

To further examine the biocompatibility of the probe Tf-CNSTTA-Eu^{3+}, three KM mice (females, ~20 g body weight) were given Tf-CNSTTA-Eu^{3+} (200 μL, 10 g L^{-1} in physiological saline solution) by intravenous injection. After 24 h, they were sacrificed by dislocating cervical vertebra and the main organs (heart, liver, spleen, lung, and kidney) were surgically dissected. The collected organs were fixed with 4% formaldehyde in PBS and embedded in paraffin. Then the standard hematoxylin and eosin (H&E) staining was carried out for histological analysis.

TGL and MR imaging of tumor-bearing nude mice

To evaluate the performance of Tf-CNSTTA-Ln^{3+} for tumor-targeting TGL/MR imaging *in vivo*, the tumor xenograft models were established by implanting H22 cells (mice hepatoma cell line) in the subcutaneous tissue of BALB/c nude mice (female) with a bodyweight of ~20 g. After the tumor size reached 1.5~2 cm in diameter, six tumor-bearing BALB/c nude mice were randomly divided into two equal groups. For the experimental group, three mice were intravenously administered with 10 g·L^{-1} Tf-CNSTTA-Gd^{3+} (in 200 μL of physiological saline solution). The other three mice in the control group were intravenously administered with 1.0 g L^{-1} free CNSTTA-Gd^{3+} complex (in 200 μL of PPS). After that, the T_1-weighted MR images of the mice were taken at different time points (0, 1, 2, 4, 6, 8 and 24 hour) following the injection. The MRI T_1 signal intensity analyses of the region of interest (ROI) were conducted using the Horos v3.3.1 software for Macintosh.

Furthermore, three tumor-bearing BALB/c nude mice in experimental group were intravenously administered with Tf-CNSTTA-Gd^{3+}/Eu^{3+} (200 μL, 12 g L^{-1} in PSS, Gd^{3+}/Eu^{3+} = 5:1), and the other three tumor-bearing BALB/c nude mice in control group were

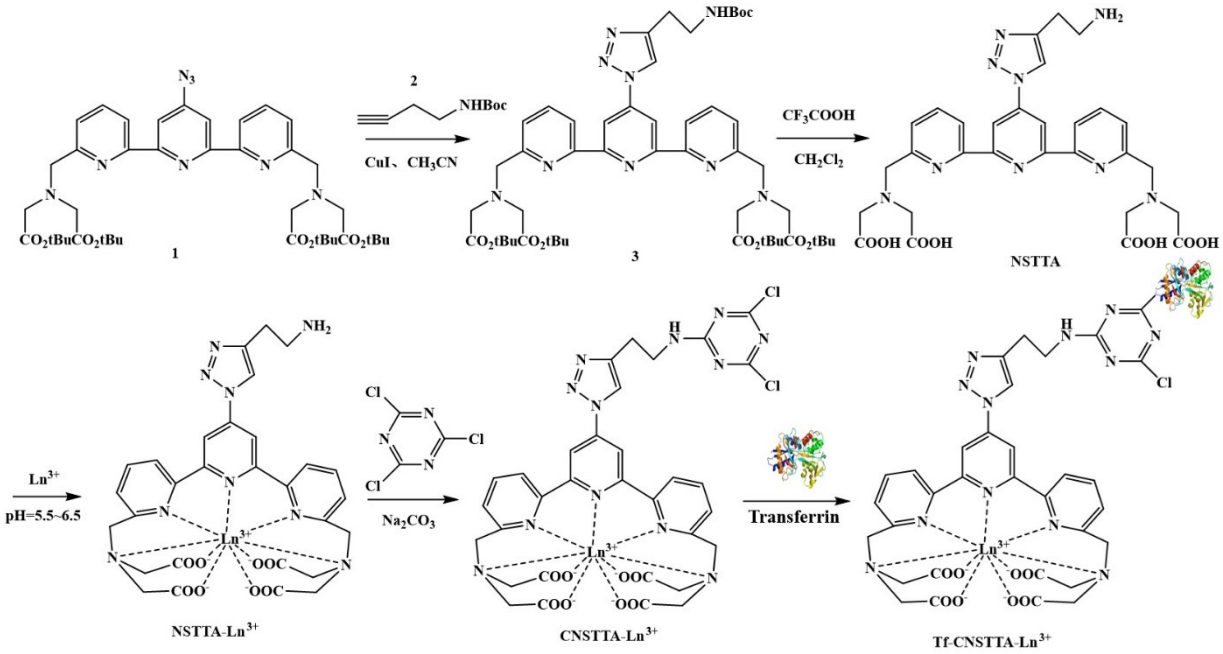
intravenously administered with free CNSTTA-Gd^{3+}/Eu^{3+} (200 μL, 1.2 g·L^{-1} in PSS, Gd^{3+}/Eu^{3+} = 5:1). After that, the mice were subjected to MRI measurement at 4 h postinjection. Then the mice were euthanized, and the tumors were collected and stored at −20 °C for 24 h. The frozen tumor tissues were cryosectioned by a microtome at −20 °C into slices of 20 μm thicknesses for optical imaging measurements on the microscope.

All of above animal studies were conducted in agreement with the guidelines of the Institutional Animal Care (No. 211003700000860) approved by the Animal Ethical and Welfare Committee (AEWC) of Dalian Medical University.

Statistical analysis

All the experiments were performed three times and the values were presented as the mean ± SD. Statistical comparison between the two groups was determined by Student's test. All statistical analyses were conducted with Excel (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). A value of $P < 0.05$ was considered statistically significant.

2. Supplementary scheme S1



Scheme S1. Reaction pathway for the synthesis of the probe.

3. Supplementary Table

Table S1. Photophysical properties of two Eu^{3+} complexes

Complex	$\lambda_{ex,max}$ (nm)	$\lambda_{em,max}$ (nm)	Φ (%)	τH_2O(ms)	τD_2O(ms)	q*
CNSTTA-Eu^{3+}	335	608	14.8	1.29	2.01	0.06
Tf-CNSTTA-Eu^{3+}	335	608	10.8	1.27	2.11	0.05

*The quantum yields (Φ) calculation formula is as follows, $\Phi_x = I_x\ \varepsilon_{ref}\ C_{ref}\ \Phi_{ref}\ / I_{ref}\ \varepsilon_x\ C_x$, where ε is the extinction coefficient of the compound at the excitation wavelength, C is the concentration, and I is the total luminescence intensity. The subscripts x and *ref* refer to compounds with unknown quantum yields and references, respectively. The Eu^{3+} complex *N,N,N′,N′*-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl) bis(methylenenitrilo) tetraacetate-Eu^{3+} (PTTA-Eu^{3+}) was used as the reference (Φ_{ref} = 0.160, ε_{ref} = 14300 $cm^{-1}\ mol^{-1}\ L$). The average number (q) of water molecules in the first coordination sphere of Eu^{3+} was calculated from the equation of $q=1.2(1/\tau_{H2O}-1/\tau_{D2O}-0.25)$. The luminescence lifetimes of CNSTTA-Eu^{3+} and Tf-CNSTTA-Eu^{3+} were measured in H_2O and D_2O buffers, respectively.[2]

4. Characterization of the intermediate compounds and CNSTTA-Ln^{3+}

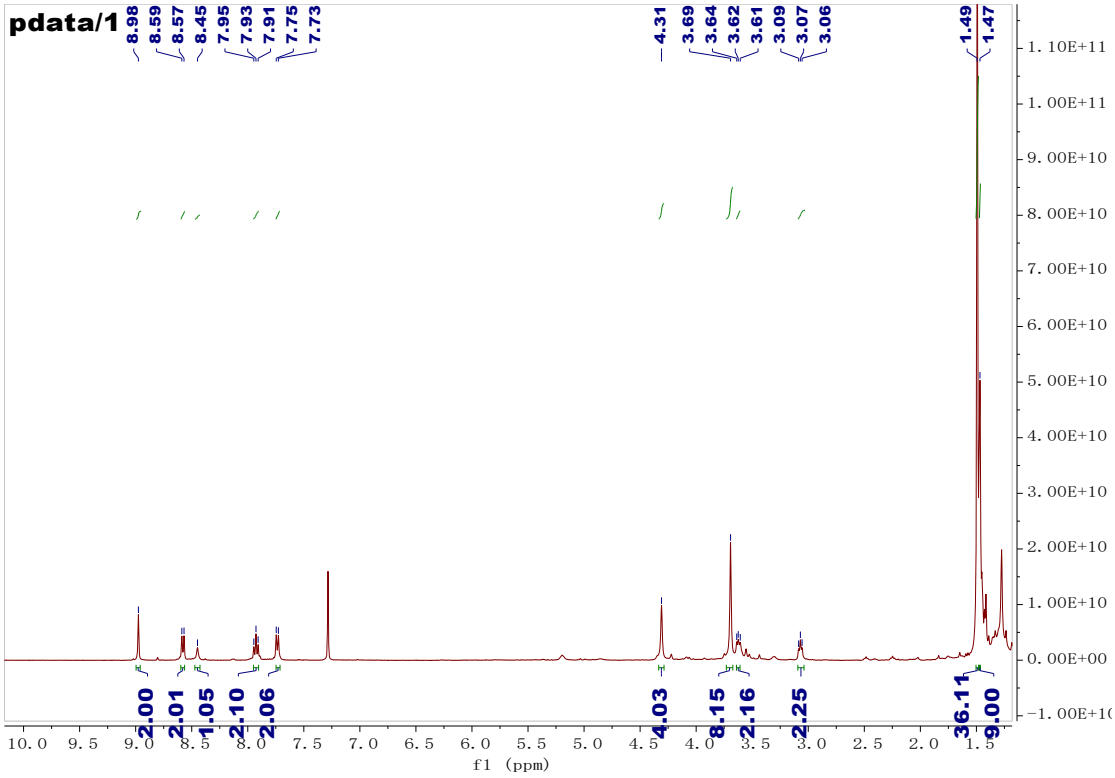


Figure S1. ^{1}H NMR spectrum of Compound 3 (400 MHz, $CDCl_3$).

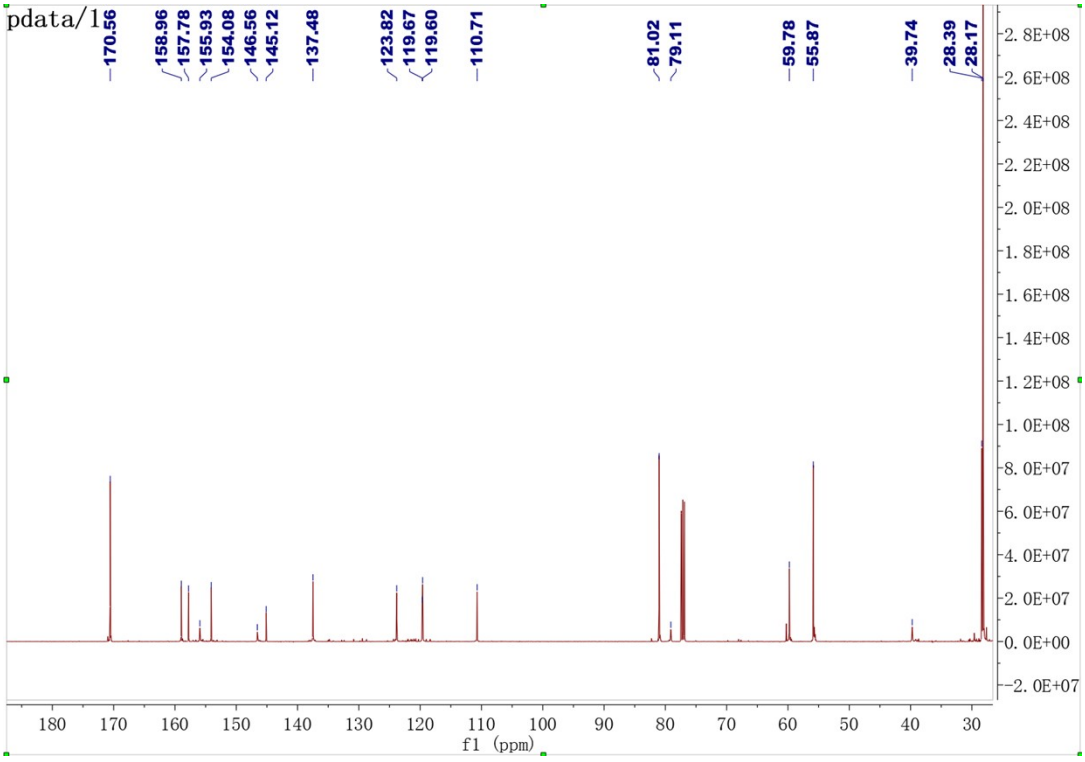


Figure S2. ^{13}C NMR spectrum of Compound 3 (125 MHz, $CDCl_3$).

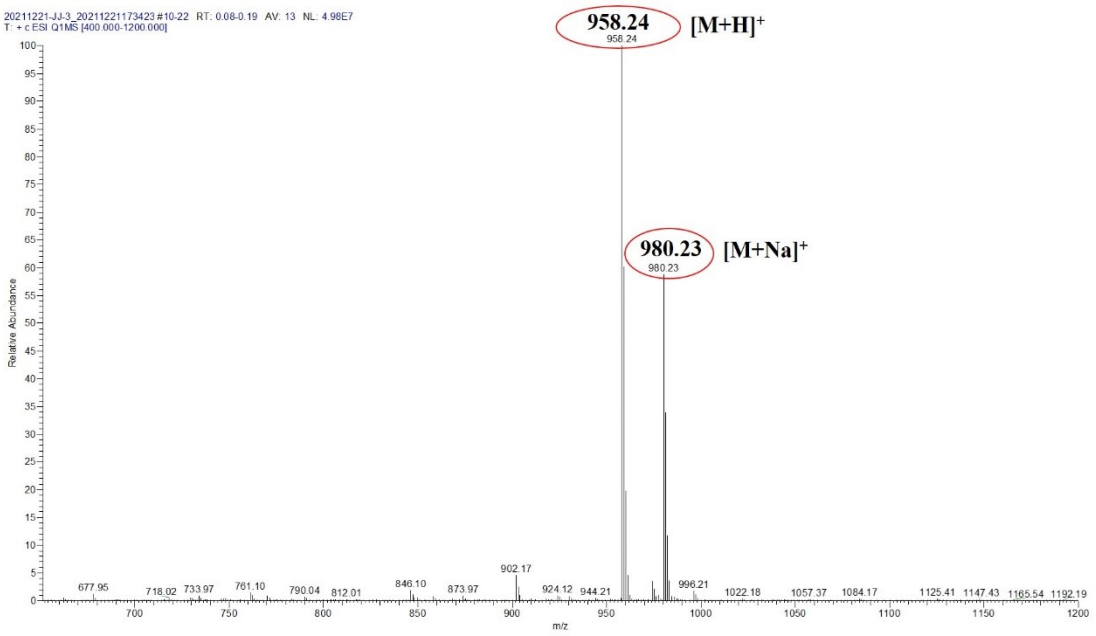


Figure S3. MS spectrum of Compound 3.

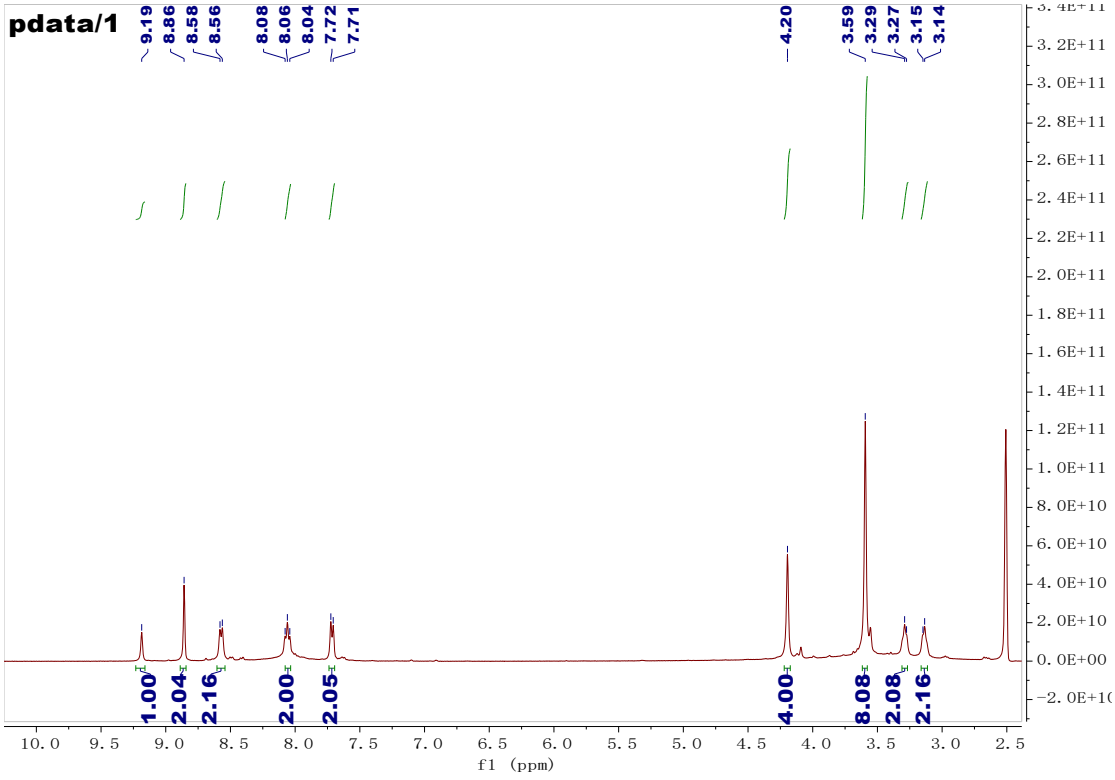


Figure S4. ^{1}H NMR spectrum of NSTTA (400 MHz, DMSO-d_6).

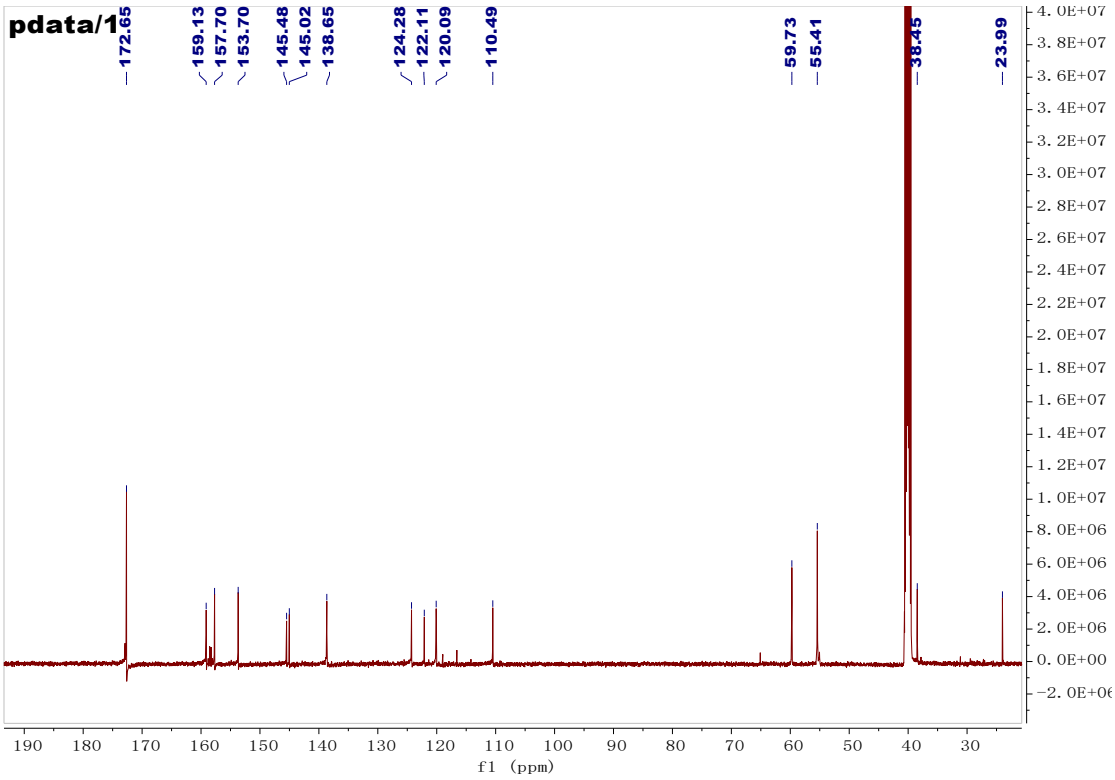


Figure S5. ^{13}C NMR spectrum of NSTTA (125 MHz, DMSO-d_6).

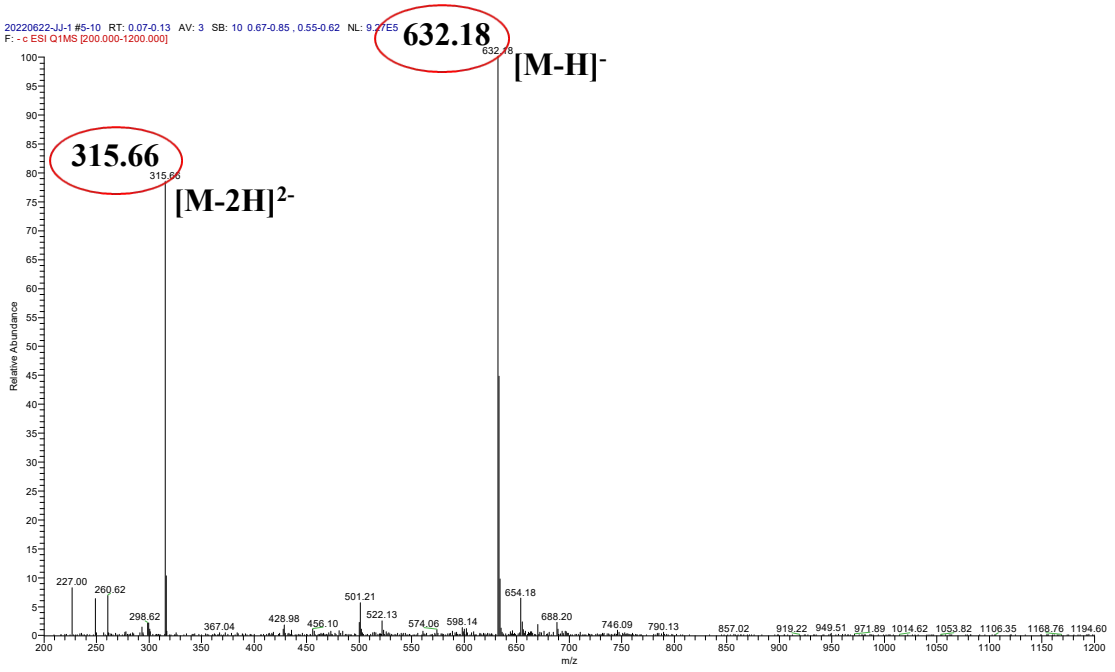


Figure S6. ESI-MS of NSTTA.

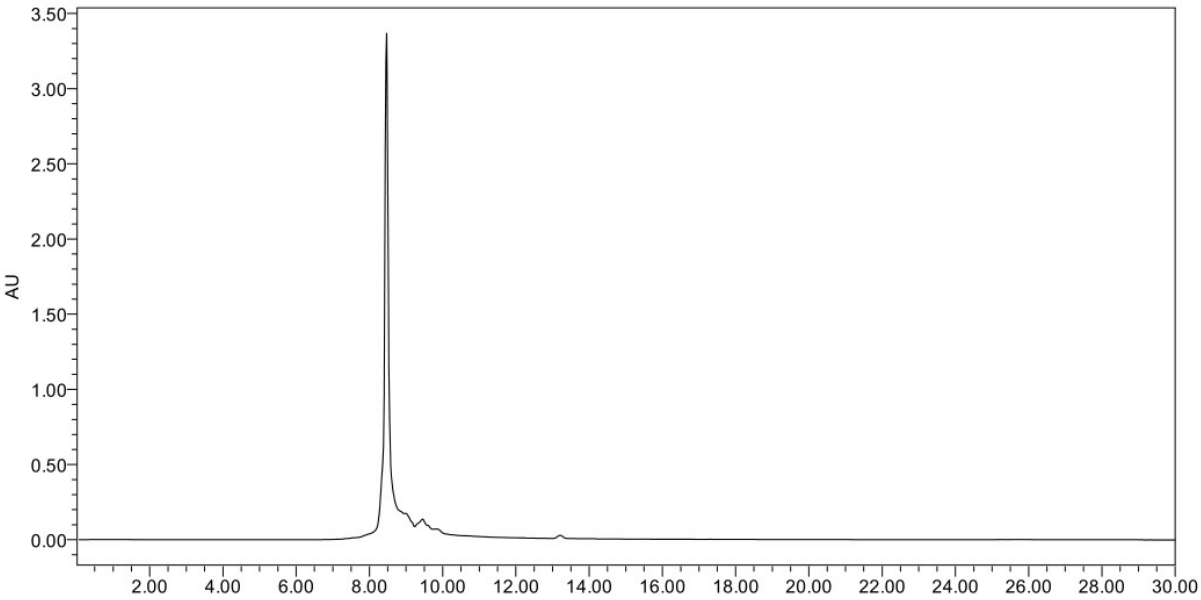


Figure S7. HPLC chromatogram of NSTTA-Eu^{3+}.

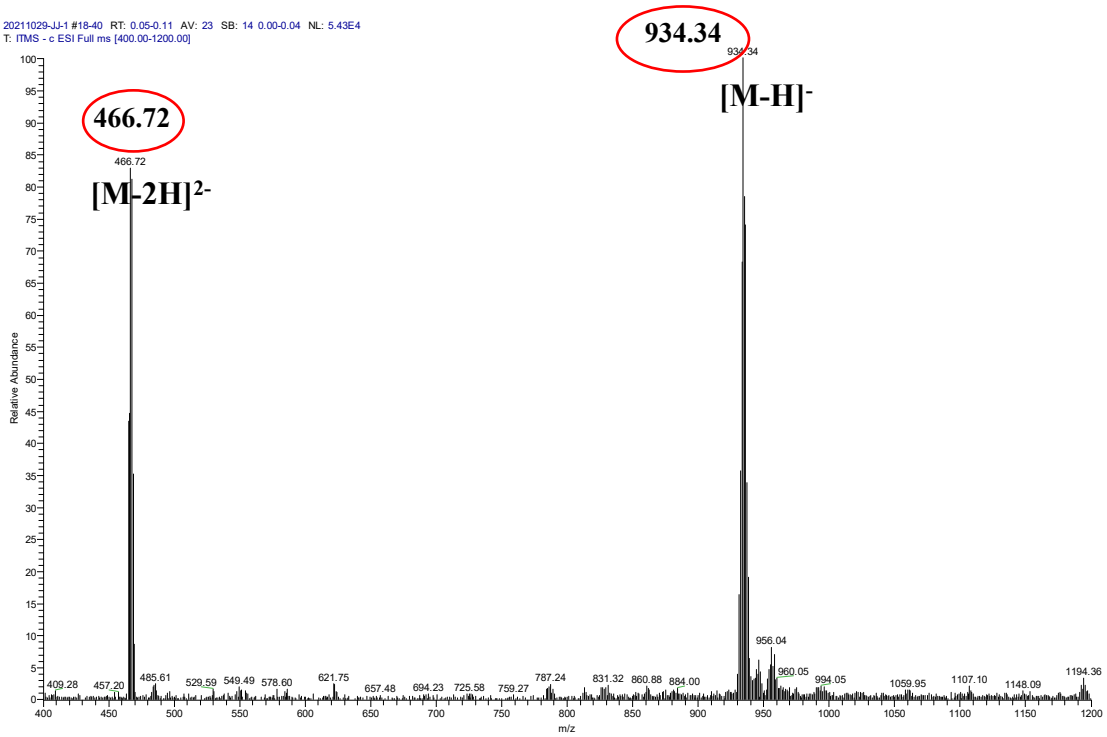


Figure S8. ESI-MS of CNSTTA-Gd^{3+}.

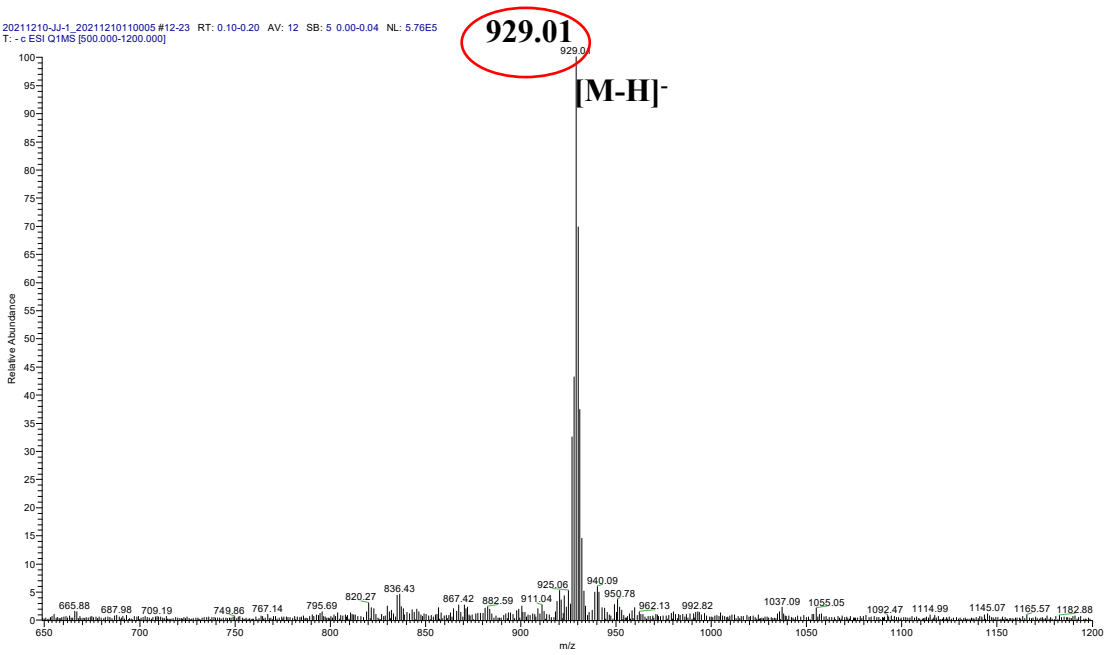


Figure S9. ESI-MS of CNSTTA-Eu^{3+}.

5. Characterization of the probe Tf-CNSTTA-Ln^{3+}

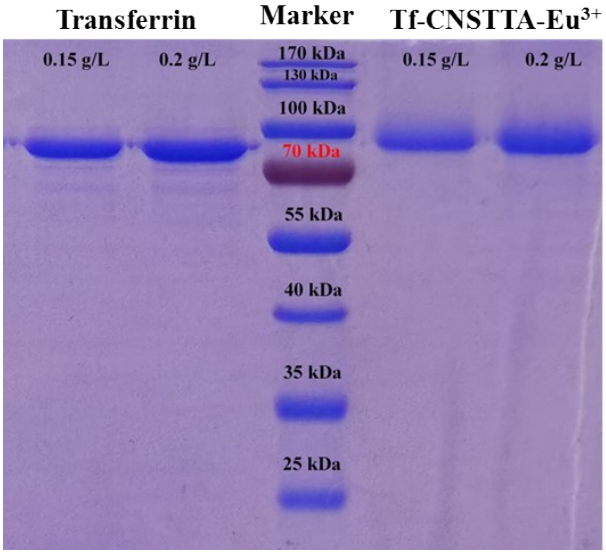


Figure S10. Polyacrylamide gel electrophoresis image of transferrin (0.15, 0.2 g L^{-1}) and Tf-CNSTTA-Eu^{3+} (0.15, 0.2 g L^{-1}) after staining with Coomassie Brilliant Blue R-250.

6. Cytotoxicity and biocompatibility of the probe Tf-CNSTTA-Ln^{3+}

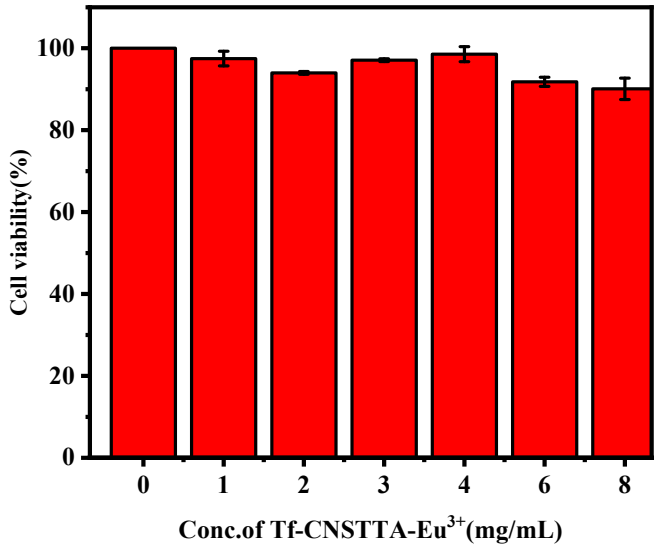


Figure S11. Viabilities of MCF-7 cells after being incubated with different concentrations of Tf-CNSTTA-Eu^{3+} for 24 h.

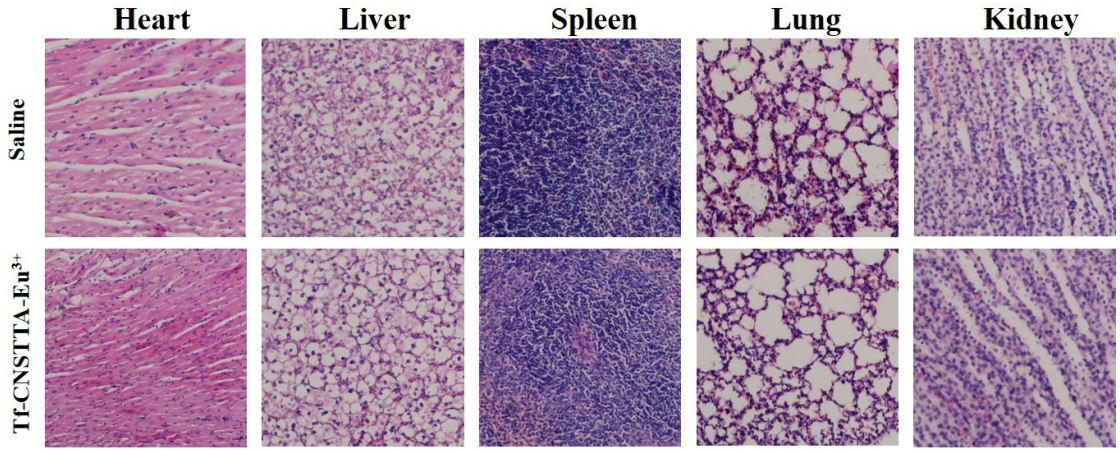


Figure S12. Images of H&E stained main organs of the KM mice after being intravenously injected with physiological saline and Tf-CNSTTA-Eu^{3+} (200 μL, 10 $g \cdot L^{-1}$ in PSS) for 24 h.

7. *In vivo* distribution of Tf-CNSTTA-Ln^{3+}

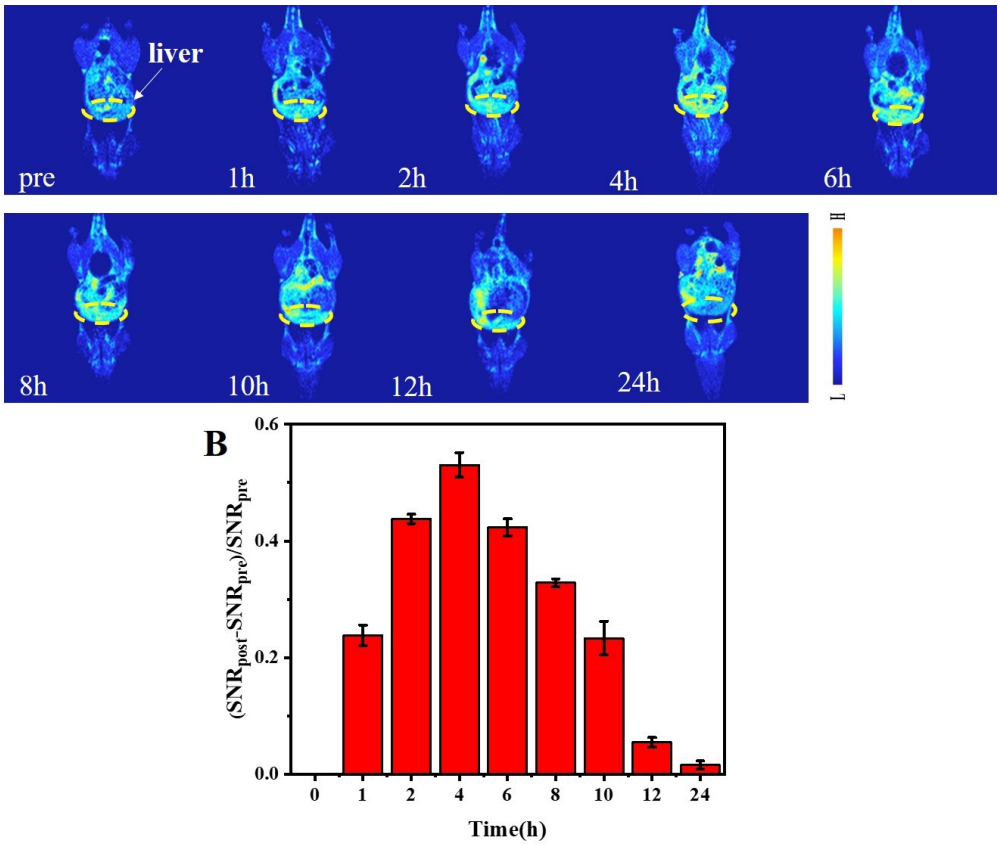


Figure S13. (A) *In vivo* T_1-weighted MR images of KM mice at different time intervals after intravenous injection of the probe Tf-CNSTTA-Gd^{3+} in the longitudinal plane (TR=500, TE=19, recorded at 310 K under 0.5 T magnetic field). (B) Quantification results of liver contrast values in KM mice at different time intervals after injection of Tf-CNSTTA-Gd^{3+}.

8. *In vivo* MRI of tumor-bearing mice loaded with the probe

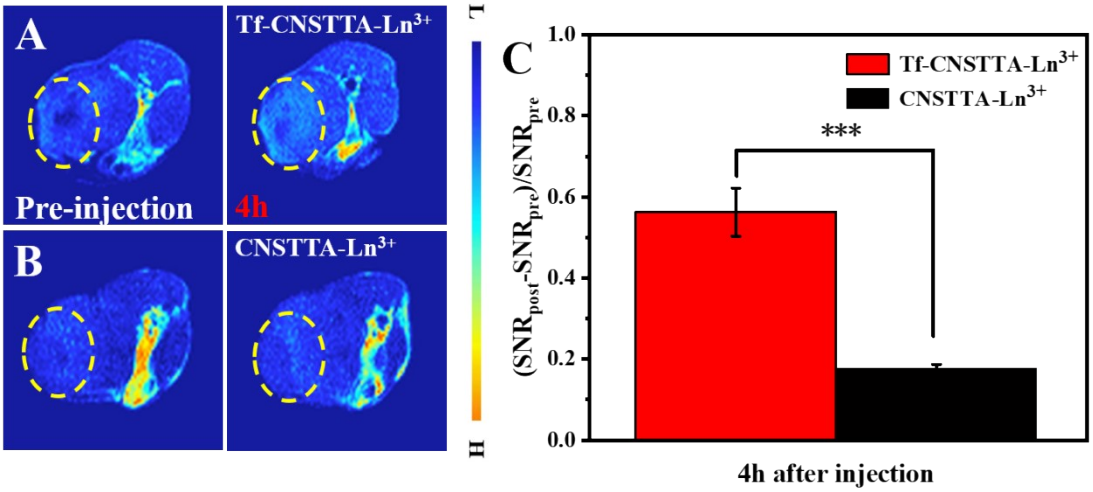


Figure S14. T_1-Weighted MR images of tumor-bearing BALB/c nude mice intravenously injected with Tf-CNSTTA-Gd^{3+}/Eu^{3+} (200 μL, 12 $g \cdot L^{-1}$ in PSS) (A) or CNSTTA-Gd^{3+}/Eu^{3+} (200 μL, 1.2 $g \cdot L^{-1}$ in PSS) (B) in transverse plane areas of tumors (tumors were highlighted with yellow dashed circles, TE=19, TR=500, recorded at 310 K under a 0.5 T magnetic field). (C) The corresponding quantification results of MR contrast values of tumors at 4 h post-injection.

9. References

1 U. Schatzschneider, J. Niesel, I. Ott, R. Gust, H. Alborzinia and S. Wölfl, *ChemMedChem*, 2008, **3**, 1104-1109.

2 B. Song, X. Wen, X. Zhang, Q. Liu, H. Ma, M. Tan and J. Yuan, *J. Mater. Chem. B*, 2021, **9**, 3161-3167.