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Luminescent trimethoprim-polyaminocarboxylate lanthanide complex conjugates for selective protein labeling and time-resolved bioassays

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Abstract

Labeling proteins with long-lifetime emitting lanthanide (III) chelate reporters enables sensitive, time-resolved luminescence bioaffinity assays. Heterodimers of trimethoprim (TMP) covalently linked to various cs124-sensitized, polyaminocarboxylate chelates stably retain lanthanide ions and exhibit quantum yields of europium emission up to 20% in water. A time-resolved, luminescence resonance energy transfer (LRET) assay showed that TMP-polyaminocarboxylates bind to *Escherichia coli* dihydrofolate reductase (eDHFR) fusion proteins with nanomolar affinity in purified solutions and in bacterial lysates. The ability to selectively impart terbium or europium luminescence to fusion proteins in complex physiological mixtures bypasses the need for specific antibodies and simplifies sample preparation.

INTRODUCTION

Ligand-sensitized complexes of lanthanide cations have unique photophysical properties, including large Stokes shifts (>150 nm), multiple, narrow emission bands (<10 nm at half-maximum) and long emission lifetimes (μ s-ms). These properties offer distinct advantages for luminescence-based biological assays and microscopy, most notably high signal-to-background ratios that are obtained by temporally discriminating lanthanide luminescence from short-lifetime (<10 ns) scattering and autofluorescence background.(1–2) Direct conjugation of terbium or europium complexes to oligonucleotides, antibodies and proteins has enabled the development of sensitive luminescence resonance energy transfer (LRET¹, also known as time-resolved fluorescence resonance energy transfer, or TR-FRET) assays of biomolecular interactions in purified biochemical preparations, cellular extracts, and on cell surfaces.(3–5) Recently, we showed that interactions between a terbium complex-labeled protein and a green fluorescent protein (GFP) fusion could be microscopically imaged in living cells by detecting long-lived, terbium-to-GFP LRET in time-resolved mode.(6)

Because lanthanide absorption transitions are parity-forbidden, direct excitation is inefficient, and Tb³⁺ or Eu³⁺ complexes typically incorporate the metal ion into an organic chelating ligand that contains a sensitizing chromophore. When excited with ultraviolet (UV) light in the absorption band, the chromophore undergoes intersystem crossing to its triplet excited state followed by intramolecular energy transfer to the emissive level of the chelated metal.(1) In general, lanthanide complexes used in time-resolved screening or cellular imaging applications must meet several requirements: (i) the molecule must be

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¹Abbreviations: diethylenetriaminepentaacetic acid (DTPA); *Escherichia coli* dihydrofolate reductase (eDHFR); green fluorescent protein (GFP); luminescence resonance energy transfer (LRET); trimethoprim (TMP); triethylenetetraaminehexaacetic acid (TTHA).

kinetically stable; (ii) the molecule must have a high extinction coefficient and quantum yield of emission (i.e., good brightness); (iii) the excitation wavelength should be as long as possible; (iv) the molecule must be resistant to photobleaching; and (v) bioconjugates of the molecule must be easy to prepare. Kinetic stability is particularly important for cellular imaging or screening applications in cell lysates where endogenous chelators could scavenge metal from the complex.⁽²⁾ For microscopy, near-UV (>340 nm) absorption is preferred because typical optics won't transmit below this limit.⁽⁷⁾ While hundreds of luminescent lanthanide chelates have been prepared over the past decades, relatively few meet most or all of the aforementioned criteria.^(1–2)

Polyaminocarboxylates, diethylene triamine pentaacetic acid (DTPA) and triethylene tetraamine hexaacetic acid (TTHA), have proven to be robust scaffolds for developing bright and photostable luminescent chelates of Tb^{3+} and Eu^{3+} . Selvin and coworkers have developed various DTPA and TTHA chelates that utilize 7-aminoquinolinone (carbostyryl 124, or cs124) as the sensitizing chromophore.^(5, 8–13) Conjugable analogs include an amide-linked, amine-reactive or thiol-reactive moiety at one end of the polyaminocarboxylate chain, opposite the sensitizer (Figure 1). The cs124-sensitized chelates have reasonable extinction coefficients ($\epsilon = \sim 10,500 \text{ M}^{-1}\text{cm}^{-1}$ at $\lambda_{\text{max}} = 341 \text{ nm}$) and overall quantum yields of emission up to 40% for Tb^{3+} and up to 28% for Eu^{3+} .^(12, 14) Variants that incorporate 7-amino-4-trifluoromethyl-2-(1H)-quinolinone (cs124CF₃) were shown to be effective Eu^{3+} luminophores, being 2- to 4-fold brighter than their analogous cs124-sensitized DTPA and TTHA analogues with $\sim 15 \text{ nm}$ red-shifted excitation maxima.⁽¹⁵⁾ However, cross-linkable DTPA-cs124 analogues are 8-dentate chelates and therefore suffer reduced brightness and higher kinetic instability because one or more water molecules are coordinated with the metal. This is not the case with TTHA-cs124 conjugates.⁽¹¹⁾ Mustaev and co-workers addressed this issue by developing DTPA-cs124 analogues that could be conjugated to biomolecules via modification of the chromophore moiety (Figure 1).⁽¹⁶⁾ Chromophore-linked DTPA-cs124 conjugates were shown to be highly luminescent and exhibited greater resistance to EDTA challenge than Selvin-type DTPA-cs124 analogues. Analogous thiol-reactive, coumarin-sensitized, DTPA and TTHA chelates were reported by Heyduk and Heyduk, but these molecules only supported Eu^{3+} luminescence and were less bright than cs124-sensitized chelates.⁽¹⁷⁾

In this article, we present results of our continued efforts to develop luminescent lanthanide protein labels for time-resolved LRET microscopy and screening applications. We previously showed that conjugates of the common antibiotic, trimethoprim (TMP) linked to Tb^{3+} -complexed cs124-polyaminocarboxylates or a 2-hydroxyisophthalamide-based macrocycle (Lumi4@-Tb) bound to *Escherichia coli* dihydrofolate reductase (eDHFR) with high affinity, thereby offering an effective means of selectively imparting Tb^{3+} luminescence to fusion proteins.⁽¹⁸⁾ Here, our original goal was to develop a TMP-linked, Eu^{3+} chelate that exhibited good brightness, high kinetic stability and appreciable absorption at 365 nm (for eventual microscopy applications). We prepared conjugates of TMP coupled to 3- and 4-acetic acid derivatives of cs124CF₃ and cs124, respectively (scheme 1), and linked these moieties to DTPA and TTHA (scheme 2). We then assessed the photophysical properties of these chelates as well as our previously reported, Selvin-type, cs124-TTHA-TMP conjugate by measuring the relative quantum yields of Eu^{3+} and Tb^{3+} luminescence using DTPA-cs124 as a reference compound. We assessed comparative kinetic stability by measuring time-dependent decrease in lanthanide luminescence in the presence of a large excess of EDTA.

The previously developed cs124-TTHA-TMP (**10**) and the novel TTHA-cs124CF₃-TMP (**7**) were the most efficient Eu^{3+} complexes with estimated overall quantum yields of $\sim 20\%$, and they exhibited relatively high resistance to EDTA challenge. Affinity for eDHFR ($K_D = \sim 1.5$

nM) was measured directly in purified biochemical preparations and in bacterial lysates by titrating a GFP-eDHFR fusion protein against a fixed concentration (20 nM) of selected, TMP-Tb³⁺ chelates and measuring the protein concentration-dependent increase in Tb³⁺-to-GFP LRET. The ability to selectively impart Tb³⁺ or Eu³⁺ luminescence to fusion proteins and to quantitatively measure binding events in cellular extracts simplifies LRET-based bioassays and eliminates the need for lanthanide chelate-conjugated antibodies. Moreover, the good brightness, kinetic stability and long-wavelength absorption of the Eu³⁺ complex of TTHA-cs124CF₃-TMP (**7**) make it a potentially useful reporter label for live-cell microscopy.

EXPERIMENTAL PROCEDURES

Chemicals and instrumentation

The following chemicals were purchased from Sigma-Aldrich: TTHA, DTPA dianhydride, diamminobenzene, methyl bromoacetate, TMP, hydrobromic acid (HBr, 48%), 1, 8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 2, 2'-(Ethylenedioxy)bis(ethylamine), diethyl-1, 3-acetonedicarboxylate, ethyl bromovalerate, di-*tert*-butyl dicarbonate, dichloromethane, trifluoroacetic acid (TFA), citric acid, O-Benzotriazole-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU), N-Hydroxybenzotriazole (HOBt), benzotriazol-1-yl-oxyltripyrrolidinophosphonium hexafluorophosphate (PyBOP), ethyl acetoacetate, ethyl 4, 4, 4-trifluoroacetoacetate, anhydrous dimethylformamide (DMF, in sure seal bottle), anhydrous dimethyl sulfoxide (DMSO, in sure seal bottle), EuCl₃·6H₂O, TbCl₃·6H₂O, ZnCl₂, diisopropyl ethylamine (DIPEA), pyridine, acetic anhydride, triethylamine (TEA) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH). Glacial acetic acid, NaOH, KOH, NaCl, NaHCO₃, H₂SO₄, anhydrous Na₂SO₄, ethylenediaminetetracetic acid (EDTA) disodium salt dihydrate, acetonitrile, methanol, hexane, ethyl acetate, diethyl ether, BugBuster™, DNase, Halt™ protease inhibitor cocktail, PMSF and imidazole were purchased from Thermo Fisher Scientific. Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) was purchased from Acros Organics. Distilled and deionized (18 MΩ cm⁻¹) water was used throughout. All glassware was washed with a 10% nitric acid solution and thoroughly rinsed with deionized, distilled water. ¹H NMR and ¹³C NMR spectra were recorded at the UIC Research Resources Center (RRC) in the deuterated solvents stated. ¹H and ¹³C chemical shifts were referenced to internal solvent resonances and reported relative to SiMe₄. Low-resolution and high-resolution electrospray (ESI) mass spectra were obtained at the UIC RRC. UV-Visible (UV-Vis) absorption spectra were recorded using a Cary 3000 spectrophotometer (Varian, Inc.). Fluorescence emission spectra were recorded using a Fluoromax 3 fluorimeter (Horiba-Jobin Yvon, Inc.). Time-resolved luminescence intensity was measured using a 96-well plate reader (Perkin Elmer, Victor 3V) with 340 nm excitation (60 nm bandpass) and indicated emission filters (10 nm bandpass).

Purification

Reverse-phase high-performance liquid chromatography (HPLC) was performed using a Beckman System Gold instrument equipped with an analytical scale pump (model 128), a UV-Vis detector (model 168) and a C18 analytical column (GraceVydac, cat. no. 218TP54, 5 μm, 4.6 mm i.d. × 250 mm). A 20 min linear gradient, from 0 to 30%, 3–33% or 5–25% solvent B (solvent A = 0.1M triethylammonium acetate (pH 6.5), solvent B = CH₃CN) was used (Table 1). An initial hold of 3 min was applied for all runs.

Synthesis

Compound 1 (Scheme 1)—Ethyl-4, 4, 4-trifluoroacetoacetate (2.2 mL, 15 mmol) and KOH (0.86 g, 15 mmol) were mixed in 7 mL of DMF and stirred at 40 °C for 20 min. To this mixture, 1.5 mL of methylbromoacetate was added and the solution was stirred

overnight at room temperature under nitrogen. 300 mg (5.2 mmol) of KOH was then added, and stirred at 60 °C for 1 h. This mixture was diluted by the addition of 20 mL of water and was then extracted with chloroform. The organic layer was collected, dried over anhydrous sodium sulfate, and then evaporated *in vacuo*, first at 30 °C, and then at 75 °C for 45 min. The residue obtained was dissolved in 3.5 mL of DMSO, and then 0.76 g (7 mmol) of diaminobenzene was added and the solution stirred at 110 °C for 6 h. This solution was cooled overnight and diluted with 30 mL of 0.05M aqueous NaOH and extracted with ether (2 × 50 mL). The aqueous phase obtained after ether extraction containing product **1**, was acidified by the addition of citric acid to pH 3–3.5; the precipitate was collected by centrifugation, washed with water, dried. Recrystallization from ethyl acetate resulted in the isolation of pure compound **1**. Yield 3%. ESIMS (*m/z*) 287 [M+H]⁺, 309 [M+Na]⁺, 285 [M–H][–]. ¹H NMR (500 MHz, DMF-*d*₇, ppm): δ = 7.65 (d, 1H), 6.87 (d, 2H), 6.36 (s, br, 2H), 4.10 (s, 2H). ¹³C NMR (125 MHz, DMF-*d*₇, ppm): δ = 171.99, 161.81, 152.07, 141.07, 134.82, 126.16, 124.85, 122.00, 112.05, 105.40, 97.18, 31.99.

Compound 2 was prepared as previously reported.(18)

Compound 3 (Scheme 1)—To a stirred solution of compound **1** (11.44 mg, 0.04 mmol) in CH₃CN (7 mL) was added HBTU (18.2 mg, 0.05 mmol), HOBt (6.5 mg, 0.05 mmol) and DIPEA (0.02 mL, 0.12 mmol) and the mixture was stirred for 30 min at room temperature under nitrogen. Then, compound **2** (20 mg, 0.04 mmol) in CH₃CN (3 mL) was added to the reaction mixture and stirred for 3 h. After evaporation of CH₃CN, 15 mL water was added and the mixture was extracted with ethyl acetate (4 × 50 mL). The organic extracts were washed with saturated NaHCO₃ solution, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude reaction mixture was purified on reverse-phase HPLC to obtain the pure compound **3**. ESIMS (*m/z*) 775 [M+H]⁺, 797 [M+Na]⁺, 773 [M–H][–]. HRMS: Pred. *m/z* for C₃₆H₄₅N₈F₃O₈ 775.3385 meas. *m/z* 775.3382 [M+H]⁺.

Compound 4 (Scheme 1)—A suspension of 1.36 g (10 mmol) of ZnCl₂ in 5 mL of DMSO was supplemented with 1.08 g (10 mmol) of diaminobenzene and 2.02 g (10 mmol) of diethyl-1, 3-acetonedicarboxylate. The reaction mixture was stirred at 100 °C for 24 h. This mixture was diluted with 8 mL of ethanol, poured into 150 mL of ice-cold 0.1M citric acid, and left for 3–5 h at 4 °C. The residue was filtered and successively washed with water (4 × 15 mL), and with hot CH₃CN (2 × 10 mL), and then dried *in vacuo*. Yield 53%. From this 200 mg was taken and dissolved in 3 mL dioxane. To this 4 mL of aqueous 2M NaOH was added and stirred at 50 °C for 5 h, and then 15 mL water was added and extracted with ether (2 × 40 mL). The product (**4**) was precipitated from the aqueous phase by the addition of 10% H₂SO₄ to pH 2.5, collected by centrifugation, washed few times with water and dried *in vacuo*. Yield 37%. ESIMS (*m/z*) 219 [M+H]⁺. ¹H NMR (500 MHz, DMSO-*d*₆, ppm): δ = 11.24 (s, 1H), 7.26 (d, 1H), 6.41 (d, 1H), 6.35 (s, 1H), 5.98 (s, 1H), 5.77 (s, br, 2H), 3.65 (s, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆, ppm): δ = 171.97, 162.65, 151.27, 145.40, 141.39, 125.95, 116.60, 111.07, 110.33, 97.42, 38.28.

Compound 5 (Scheme 1)—To a stirred solution of compound **4** (19 mg, 0.09 mmol) in DMF (1 mL) was added PyBop (45.24 mg, 0.09 mmol), and TEA (0.08 mL, 0.58 mmol) and allowed to stir for 30 min at room temperature under nitrogen. Then, compound **2** (15 mg, 0.03 mmol) in DMF (0.5 mL) was added to the reaction mixture and stirred for 12 h. The reaction mixture was purified on reverse-phase HPLC to obtain the pure compound **5**. ESIMS (*m/z*) 707 [M+H]⁺, 705 [M–H][–]. HRMS: Pred. *m/z* for C₃₅H₄₆N₈O₈ 354.1792 meas. *m/z* 354.1797 [M+2H]²⁺.

General procedure for the synthesis of compounds 6, 7, 8 and 9 (Scheme 2)—5 equiv. of DTPA dianhydride/TTHA dianhydride was dissolved in DMF and 20 equiv. of

TEA under nitrogen. 1 equiv. of compound **3**/compound **5** in DMF was added and stirred at room temperature for 8–10 h. A few drops of water were added to quench the reaction. The products were purified by reverse-phase HPLC.

Compound 10 (Scheme 2)—2 equiv. of TTHA dianhydride was dissolved in DMSO and 20 equiv. of TEA under nitrogen. 0.7 equiv. of cs-124 was added and stirred at room temperature for 10 min. 1 equiv. of compound **2** in DMSO was added and stirred for 3–8 h. A few drops of water were added to quench the reaction. The product was purified by reverse-phase HPLC.

Addition of metals

Compound concentration was obtained using measured absorptions and reported extinction coefficients for the fluorophores (cs124, $\epsilon = 10,500 \text{ M}^{-1}\text{cm}^{-1}$ at $\lambda = 341 \text{ nm}$; cs124CF₃, $\epsilon = 21,000 \text{ M}^{-1}\text{cm}^{-1}$ at $\lambda = 341 \text{ nm}$).(12, 15) EuCl₃·6H₂O or TbCl₃·6H₂O was added to the chelate in a 1:1.5 molar ratio at typically >5 μM concentration in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) and allowed to stand for 30 min at room temperature before use.

Quantum yield measurements

The luminescence quantum yield of lanthanide chelates in TBS (pH = 7.6) was determined by standard methods using DTPA-cs124 as a reference from the equation:

$$\Phi_S = \Phi_R \left(\frac{Grad_S}{Grad_R} \right) \left(\frac{\eta_S}{\eta_R} \right) \quad (1)$$

where, Φ_S and Φ_R equal the quantum yields of the sample and reference, respectively. $Grad_S$ and $Grad_R$ equal the slopes of plots of integrated luminescence intensity vs. absorption ($0.02 < \text{Abs} < 0.1$) for either the sample or reference, and η_S or η_R equal the refractive indices of the respective sample or reference solutions.(19) The excitation wavelength was 330 nm in all cases. The results were reported as relative to cs124 (Table 2), so Φ_R was taken to equal 1. The quantum yield of Tb³⁺ emission for DTPA-cs124 was measured using quinine sulfate as a reference ($\Phi = 0.54$ in 0.1M H₂SO₄). Each sample was measured at least twice, and results did not vary by more than $\pm 15\%$.

EDTA challenge assay

In order to measure the stability of the TMP-lanthanide complex conjugates, each metallated compound was diluted to 50 nM in TBS, pH = 7.4 in a 1.5 mL centrifuge tube. EDTA was added from a 0.02 M stock solution (H₂O/NaOH, pH = 8.0) to a final concentration of 10 mM, and the samples were vortexed for 1 min. The samples were then aliquotted (100 μL) into 96-well plates, and the Tb³⁺ or Eu³⁺ emission was measured over time using a time-resolved luminescence plate reader (time delay = 100 μs ; measurement window = 1400 μs ; $\lambda_{\text{ex}} = 340/60 \text{ nm}$; Tb³⁺, $\lambda_{\text{em}} = 545/10 \text{ nm}$; Eu³⁺, $\lambda_{\text{em}} = 615/10 \text{ nm}$). Lanthanide luminescence was normalized to the value measured at the first time point (10 min.) and plotted vs. time using Kaleidagraph (Synergy Software).

Protein expression and purification

GFP-eDHFR was purified from the *E. coli* strain BL21 DE3 (pLysS) transformed with pRSETb-EGFP-eDHFR.(18) A 5mL luria broth (LB) overnight culture was used to inoculate 500 mL of LB (Ampicillin, 100 mg/mL; chloramphenicol, 34 mg/mL). The 500 mL culture was grown at 37 °C, shaking at 200 rpm, to an OD₆₀₀ of ~0.6, at which time

expression of the protein was induced by the addition of IPTG to a final concentration of 1 mM. After growth for another 4 h, the cells were harvested by centrifugation. The pellet was lysed in 30 mL of lysis buffer (1X BugBuster™ Protein Extraction Reagent (Novagen), 10µg/ml DNase, 1mM PMSF, 100mM HEPES, 10mM imidazole, pH 7.5). Samples were placed on an orbital shaker for 30 min., then centrifuged (15000 rpm, 15 min, 4 °C) and applied to a column containing 2.0 mL of HisLink™ Protein Purification Resin (Promega). Following purification, the protein was concentrated (to ~10 µM), dialyzed with phosphate buffer (10mM K₂HPO₄, KH₂PO₄, pH 7.4), and stored at -80 °C.

Cell lysate preparation

Cell lysates containing GFP-eDHFR were obtained from 5.0 mL LB cultures of *E. coli* strain BL21 DE3 (pLysS) transformed with pRSETb-EGFP-eDHFR and treated to induce protein expression as described above. After growth and expression, the cells were harvested by centrifugation. The cell pellets were resuspended in 10mM phosphate buffer (pH 7.4, with Halt™ Protease and Phosphatase Inhibitor, Thermo Scientific, Inc.) to a final concentration of 0.1mg/µL. The cells were then lysed by sonicating for 1 min (2, 15 s on/off cycles) and re-centrifuged (15000 rpm, 15 min, 4 °C). The GFP-eDHFR concentration in the cell supernatant was determined by measuring the absorption of GFP at 484 nm ($\epsilon_{484}=56,000 \text{ M}^{-1}\text{cm}^{-1}$).

Binding affinity assay

The binding affinity of compounds **9** and **10** complexed with Tb³⁺ for eDHFR was determined by measuring Tb³⁺-to-GFP LRET in time-resolved mode. TMP conjugates (20nM), were titrated in 96-well plates with either bacterial cell lysates containing GFP-eDHFR or with purified protein at concentrations ranging from ~0.5 nM to 1000 nM in assay buffer (50 mM K₂HPO₄, KH₂PO₄, 18 mM β-mercaptoethanol, 20 µM β-NADPH, pH 7.2). Titrations were done in triplicate for each sample. Time-resolved luminescence (time delay = 100 µs; measurement window = 1400 µs, $\lambda_{\text{ex}} = 340/60 \text{ nm}$) was measured at $\lambda_{\text{em}} = 520/10 \text{ nm}$ (LRET-sensitized GFP emission signal) and at $\lambda_{\text{em}} = 615/10 \text{ nm}$ (Tb³⁺ donor signal). For each well, the percent change in the donor-normalized LRET signal was calculated from the following equation:

$$\Delta\text{LRET}\% = \left(\frac{\left(\frac{520}{615} \right)_s - \left(\frac{520}{615} \right)_{\text{-ctrl.}}}{\left(\frac{520}{615} \right)_{\text{-ctrl.}}} \right) \times 100 \quad (2)$$

where, $\left(\frac{520}{615} \right)_s$ represents the ratios of the emission signals at the indicated wavelengths for the sample well and $\left(\frac{520}{615} \right)_{\text{-ctrl.}}$ represents the average emission ratio for three wells containing TMP-conjugate (20 nM) and no GFP-eDHFR. Using Kaleidagraph (Synergy Software), $\Delta\text{LRET}\%$ was plotted against protein concentration and dissociation constants were obtained by fitting the data to the following equation:

$$L = L_{\text{min}} - (L_{\text{min}} - L_{\text{max}}) \times \frac{([L]_T + K_D + [P]_T - \sqrt{([L]_T + K_D + [P]_T)^2 - 4[L]_T[P]_T})}{2[L]_T} \quad (3)$$

where L_{min} is the $\Delta\text{LRET}\%$ of the TMP-Tb³⁺ complex with no receptor, L_{max} is the maximum $\Delta\text{LRET}\%$ signal observed at saturating receptor concentration, $[L]_T$ is the total amount of lanthanide complex used, and $[P]_T$ is the total amount of protein used.

RESULTS AND DISCUSSION

Syntheses

The compounds cs124,(20) DTPA-cs124,(12) TTHA dianhydride(21) and an amine-derivatized TMP (**2**)(18) were prepared according to previously reported procedures. Compounds **1** and **4** were obtained by condensing diaminobenzene with ethyl-4, 4, 4-trifluoroacetoacetate and diethyl-1, 3-acetonedicarboxylate, respectively.(16) Compound **3** and **5** were prepared by peptide coupling between compounds **1** or **3** and **2** (Scheme 1). DTPA-based chelates (**6**, **8**) were prepared by reacting an excess of commercially available DTPA dianhydride with **3** or **5** in DMF at room temperature. TTHA dianhydride was similarly reacted with **3** or **5** to form compounds **7** and **9**. Compound **10** was synthesized in a three-component reaction by treating TTHA dianhydride (2 equivalents) with 0.7 equivalents of cs-124, followed by the addition of 1 equivalent of compound **2** in DMSO (Scheme 2). All of the polyaminocarboxylate compounds were purified by reverse-phase HPLC and identified by mass spectrometry (Table 1), UV-Vis absorption spectroscopy, and lanthanide luminescence properties.

Absorption spectra

The absorption spectra of representative TMP conjugates of cs124- and cs124CF₃-polyaminocarboxylates complexed with Eu³⁺ are shown in Figure 2 and compared to DTPA-cs124. The compounds were assumed to retain the molar absorptivities of the sensitizing chromophores (10,500 M⁻¹cm⁻¹ @ 341 nm for cs124, 21,000 M⁻¹cm⁻¹ @ 341 nm for cs124CF₃).^(12, 15) This assumption was supported by the observation that ~1.3 equivalents of Tb³⁺ or Eu³⁺ was sufficient to saturate lanthanide luminescence emission of all the chelates. The absorption spectrum of cs124-TTHA-TMP (**10**) is nearly identical to that of DTPA-cs124, but with a slight red shift of 2 nm. Consistent with previous studies by Mustaev and coworkers, we observed a red shift of 6-nm for the chromophore-linked, cs124 and cs124CF₃ analogs.⁽¹⁶⁾ Thus, TTHA-cs124CF₃-TMP (**7**) has a long-wavelength absorption maximum at 348 nm with a shoulder at 366 nm and DTPA-cs124-TMP (**8**) has a long-wavelength maximum at 348 nm. The analogous compounds DTPA-cs124CF₃-TMP (**6**) and TTHA-cs124-TMP (**9**) exhibited identically shaped spectra to compounds **7** and **8**, respectively (not shown). The two-fold greater absorptivity and longer-wavelength maxima of compounds **6** and **7** allow for efficient excitation with high-power UV LED sources that emit at 365 nm, an important aspect for microscopy.⁽⁷⁾

Emission spectra

All compounds displayed the narrow emission spectra typical of luminescent lanthanide chelates (Figure 3). The emission spectra and relative peak heights of Tb³⁺ emission with all compounds were essentially the same, matching the spectrum shown for **10** (Figure 3D). However, the relative intensities of the different Eu³⁺ emission lines are dependent on the chelate (DTPA or TTHA) but are independent of the sensitizer (cs124 or cs124CF₃).^(12, 15) The Eu³⁺ complexes of the TTHA conjugates, **7** (Figure 3B), **9** (not shown, identical to **7**) and **10** (Figure 3C) have a greater fraction of their luminescence in the sharply spiked peak at 613 nm, whereas the DTPA compounds all have relatively higher emission intensity in the peaks centered around 580 nm and 700 nm, as seen in the emission spectrum for **6** (Figure 3A). The Eu³⁺ emission pattern of **10** varies slightly from that of **7** or **9**, reflecting a different coordination environment resulting from amide bonds at both ends of TTHA. For spectroscopic or microscopic detection of Eu³⁺ luminescence through a narrow-pass emission filter at 613 nm, TTHA chelates are more detectable, as a lesser proportion of the emission intensity is excluded.

Relative quantum yields of emission

We measured overall luminescent quantum yields for both Tb³⁺ and Eu³⁺ complexes of our heterodimeric TMP-linked chelates using appropriately metallated DTPA-cs124 as a reference standard (Table 2). We chose DTPA-cs124 as the reference because its quantum yields for Tb³⁺ and Eu³⁺ luminescence were reported previously (0.32 and 0.10, respectively),(14) and it offered a similar spectral profile and intensity for comparison. We also measured the overall quantum yield of DTPA-cs124(Tb³⁺) using quinine sulfate as a reference and obtained a similar result (0.29) to the reported literature value (0.32).

The quantum yield data revealed some unexpected trends based on prior literature and the molecular architecture of the chelates. For Tb³⁺ luminescence, both chromophore-linked TMP conjugates, **8** and **9**, exhibited quantum yields of only ~20% relative to DTPA-cs124, whereas a similarly structured DTPA-cs124-isothiocyanate was reported by Mustaeu and co-workers to be ~80% as bright as DTPA-cs124.(16) Similarly, for Eu³⁺ luminescence, **8** and **6** had relative quantum yields of 0.77 and 0.56, respectively; substantially lower than the 1.4-fold greater Eu³⁺ luminescence reported for DTPA-cs124-isothiocyanate relative to that of DTPA-cs124.(16) While the previous study did not directly measure quantum yields, the identical absorptivities observed for chromophore-linked vs. conventional cs124 analogs allow for direct comparison. While the aforementioned compounds showed low quantum yields relative to DTPA-cs124 (and thus lower brightness), the Eu³⁺ complexes of **7** and **10** emitted with twice the efficiency of DTPA-cs124 (Table 2). Thus, assuming a ~10% overall Eu³⁺ quantum yield for DTPA-cs124,(14) we estimate a ~20% Eu³⁺ quantum yield for compounds **7** and **10**. The higher quantum yield, greater absorptivity and longer wavelength absorption make compound **7** a potentially useful label for cell-based microscopy.

Relative stability of the lanthanide chelates

Besides photophysical characteristics, stable retention of the metal ion is an important property for studies in living cells or cell extracts where endogenous chelators could scavenge metal from the lanthanide probes. To compare relative stability, we measured Tb³⁺ and Eu³⁺ emission intensity over a period of 3 h in the presence of EDTA for the metal complexes of the TMP-polyaminocarboxylate conjugates and of DTPA-cs124 (Figure 4). In the presence of 10 mM of EDTA, the Eu³⁺ emission intensity for nearly all compounds rapidly decreases and stabilizes after ~75 min suggesting equilibrium was achieved (compound **9** did not appear to equilibrate within 3 h). The Eu³⁺ complex of **10** was the most stable, retaining ~55% of its initial intensity after 3 h, while DTPA-cs124(Eu³⁺) was the least stable (~9% initial intensity). A similar pattern was seen for Tb³⁺ complexes, with TTHA analogues exhibiting greater stability than DTPA chelates, although neither of the TTHA compounds achieved equilibrium with EDTA within the 3 h observation period.

Protein binding affinity measurements

For both *in vitro* and cell-based applications, TMP conjugates must necessarily bind with high affinity to eDHFR fusion proteins. To determine the dissociation constants for binding of select TMP-polyaminocarboxylate Tb³⁺ complex conjugates to eDHFR, we performed a time-resolved LRET assay that detected binding to a GFP-eDHFR fusion protein as Tb³⁺-sensitized, GFP emission. The assay was performed in two formats: 1) purified GFP-eDHFR was titrated against a fixed concentration (20 nM) of either **9** or **10** in assay buffer; and 2) a lysate of *E. coli* expressing GFP-eDHFR was diluted directly into assay buffer without purification and titrated against 20 nM of each compound. Using a time-resolved luminescence plate reader, we measured sensitized, long-lifetime (>100 μs) GFP emission at 520 nm (the LRET signal) and the Tb³⁺ donor emission signal (615 nm). The percent change in the 520 nm/615 nm emission ratio was plotted vs. GFP-eDHFR concentration, revealing characteristic binding isotherms (Figure 5). Nonlinear, least-squares fits of the data

showed that the dissociation constants for binding to GFP-eDHFR equaled ~1.5 nM for both compounds under each assay condition. The ability to quantitatively measure binding events in a complex mixture, coupled with the large dynamic range of the LRET signal (~5,000 % for compound **10** in lysate/buffer) suggests that TMP-lanthanide complex conjugates could be used for high throughput screening or quantification of biomolecular interactions, especially in cases where highly pure biochemical preparations cannot be obtained.

Conclusions

Currently, homogeneous, LRET-based assays of protein-protein or protein-DNA/RNA interactions require direct conjugation of lanthanide reporters to purified proteins or to specific antibodies. However, antibodies are not always available for a particular target, and many proteins cannot be purified. In such cases, and especially for specific protein labeling in living cells, the high-affinity interaction between TMP and eDHFR provides an effective means for imparting lanthanide luminescence to recombinant fusion proteins. Of the TMP-polyaminocarboxylate conjugates described here, the Tb³⁺ and Eu³⁺ complexes of cs124-TTHA-TMP (**10**) and the Eu³⁺ complex of TTHA-cs124CF₃-TMP (**7**) have the requisite brightness and kinetic stability to be effective reporters for time-resolved bioassays. Compound **7** is particularly promising as an Eu³⁺ reporter for cell-based microscopy because of its relatively long-wavelength absorption and high molar extinction coefficient. Paired with a suitable Tb³⁺ complex, **7** (Eu³⁺) could be used for multiplexed lanthanide luminescence imaging.

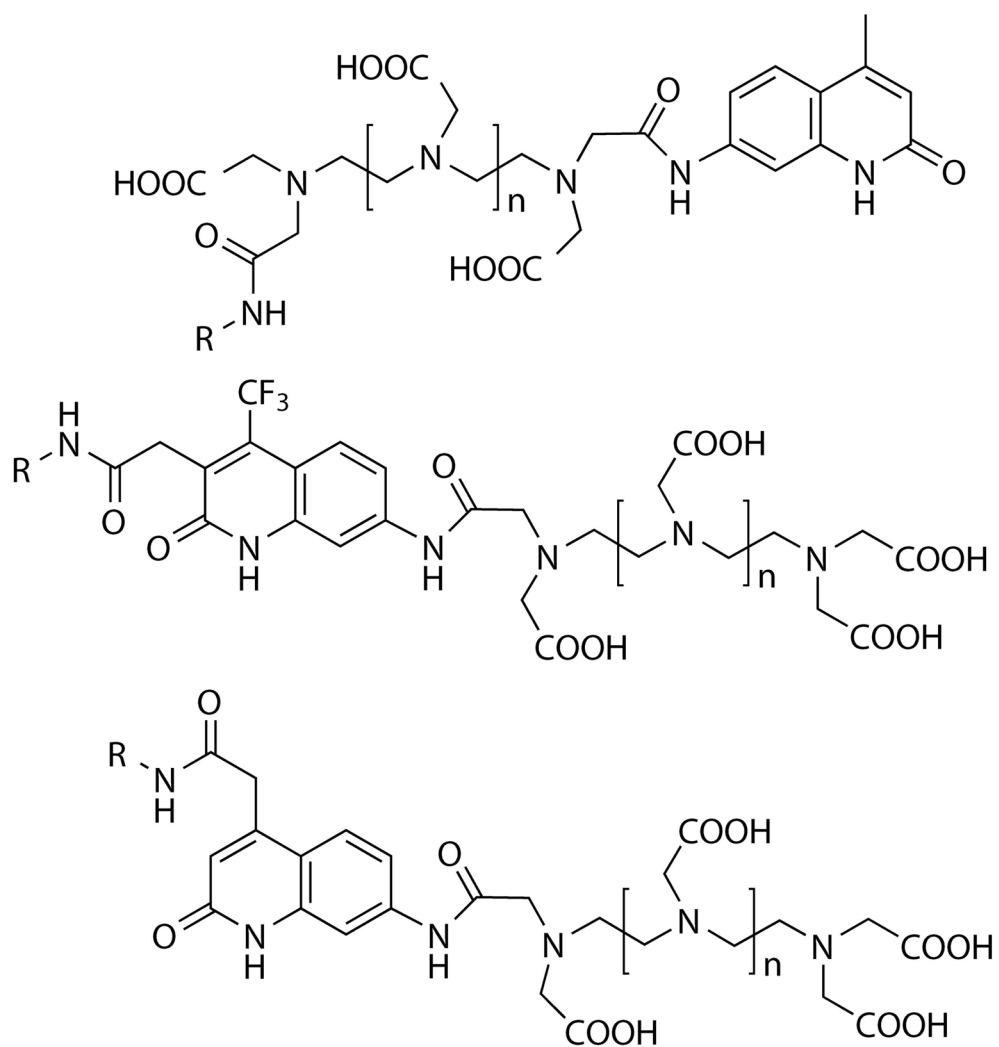
Acknowledgments

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R: Ligand or reactive cross-linker

n = 1: DTPA

n = 2: TTHA

Figure 1. Variants of carbostyryl 124-polyaminocarboxylates and their modes of attachment to biomolecules.

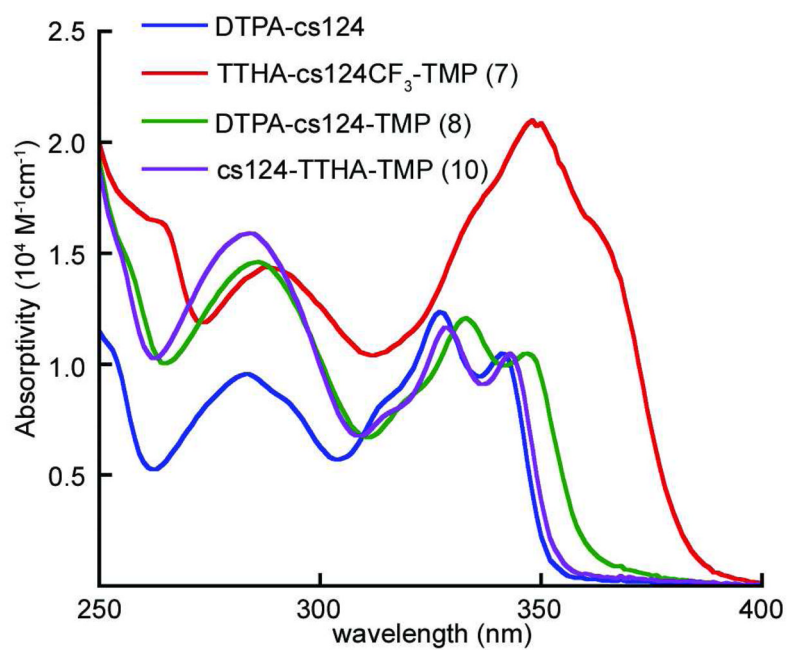


Figure 2. UV absorption spectra of representative trimethoprim-polyaminocarboxylate Eu³⁺ complexes and the reference compound (DTPA-cs124) described in this study.

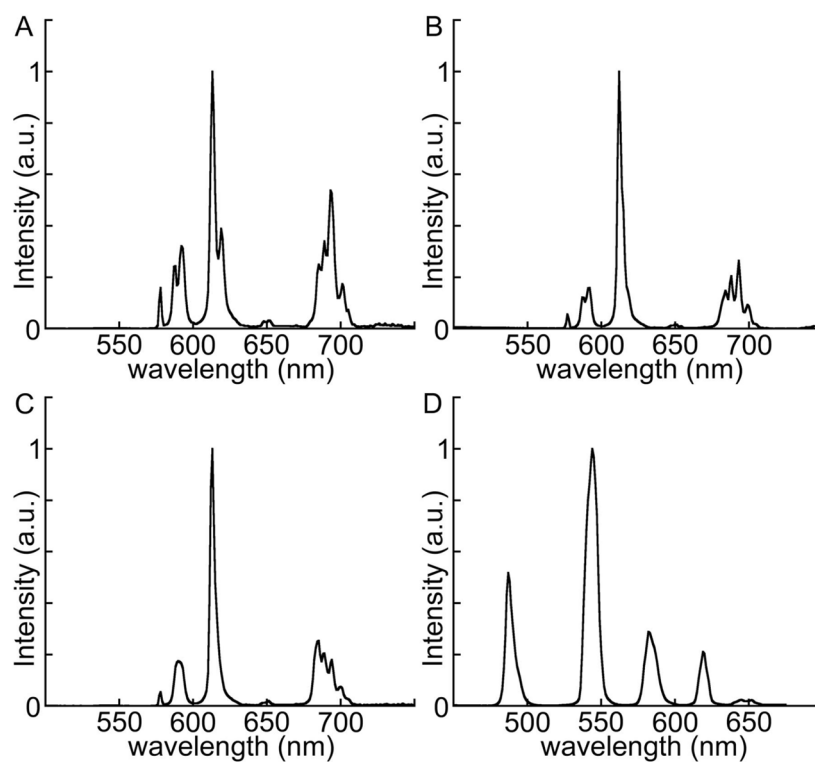


Figure 3. Normalized emission spectra ($\lambda_{\text{ex}} = 330 \text{ nm}$) of Eu^{3+} complexes of (A) DTPA-cs124_{CF3}-TMP (6); (B) TTHA-cs124_{CF3}-TMP (7); (C) cs124-TTHA-TMP (10); and (D) Tb^{3+} complex of 10.

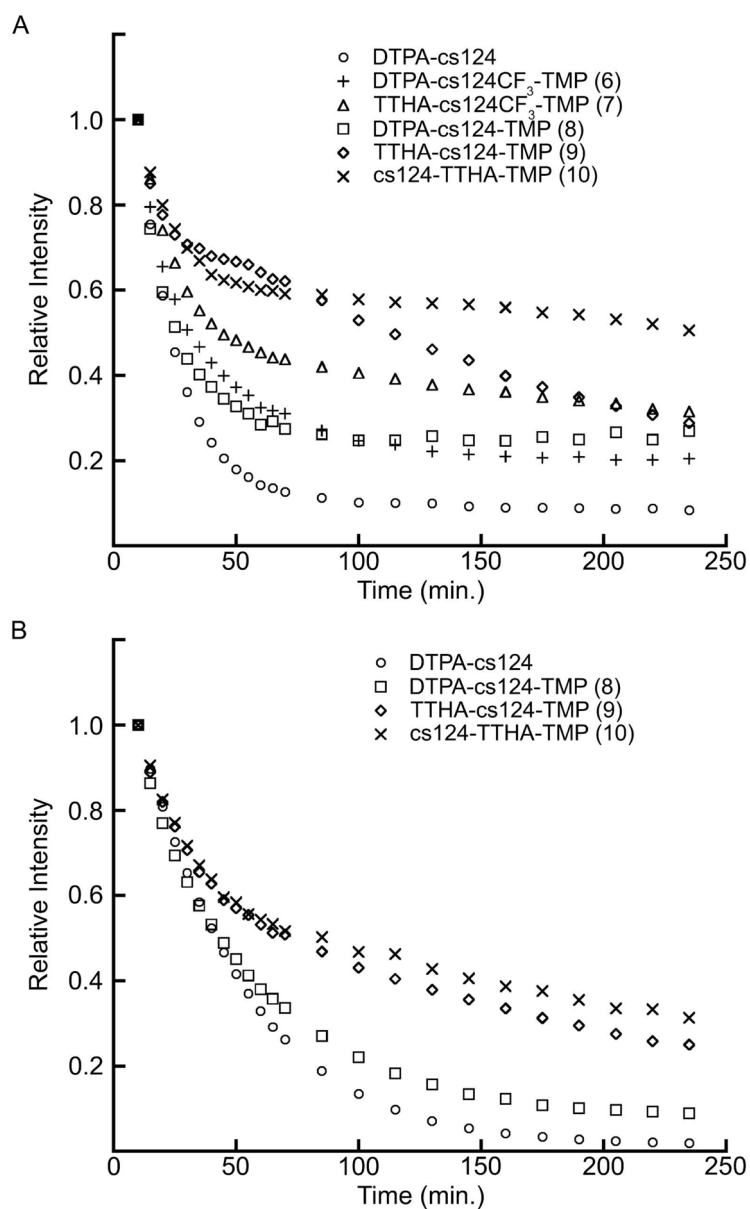


Figure 4. Relative emission intensity over time in the presence of EDTA for trimethoprim-polyaminocarboxylate conjugates and DTPA-cs124. (A) Eu^{3+} complexes. (B) Tb^{3+} complexes.

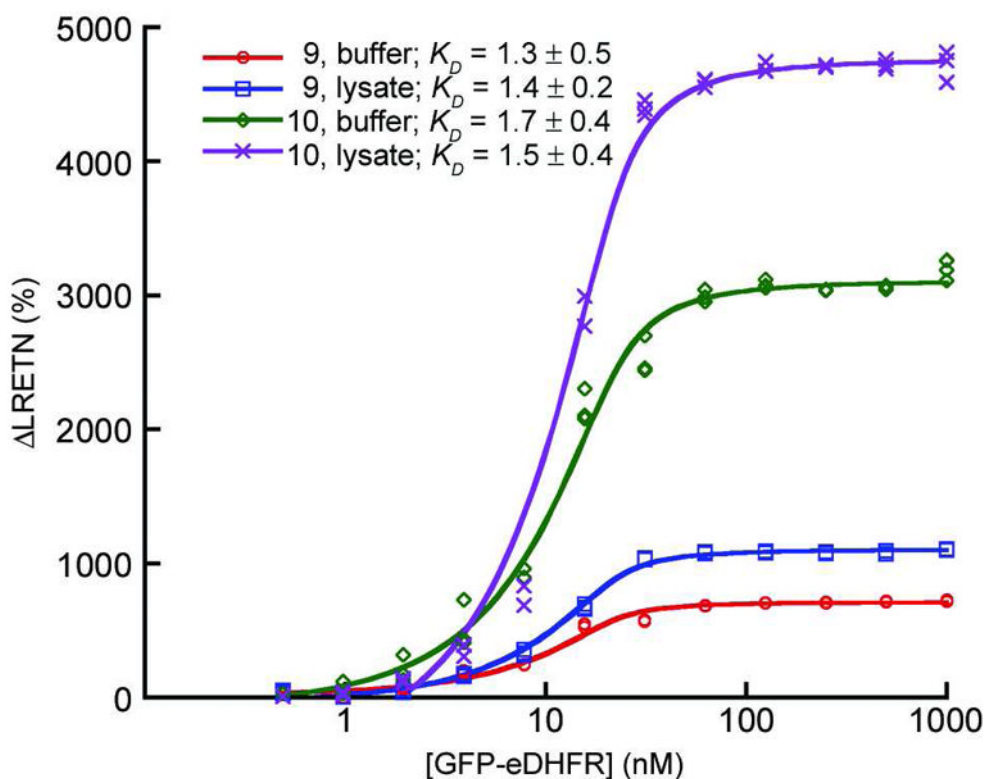
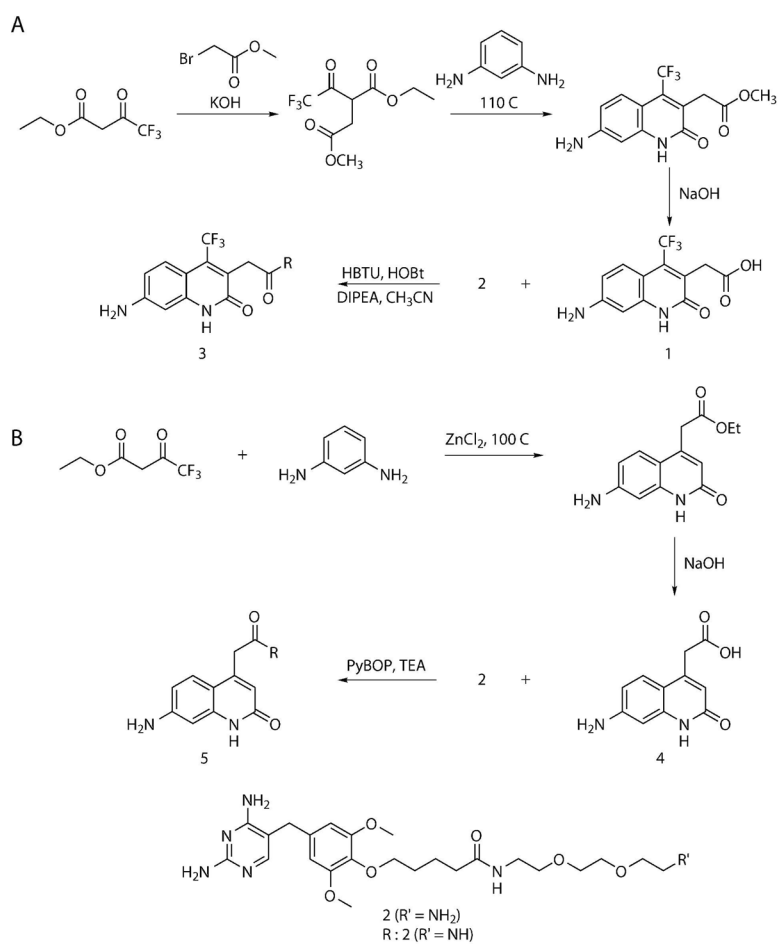
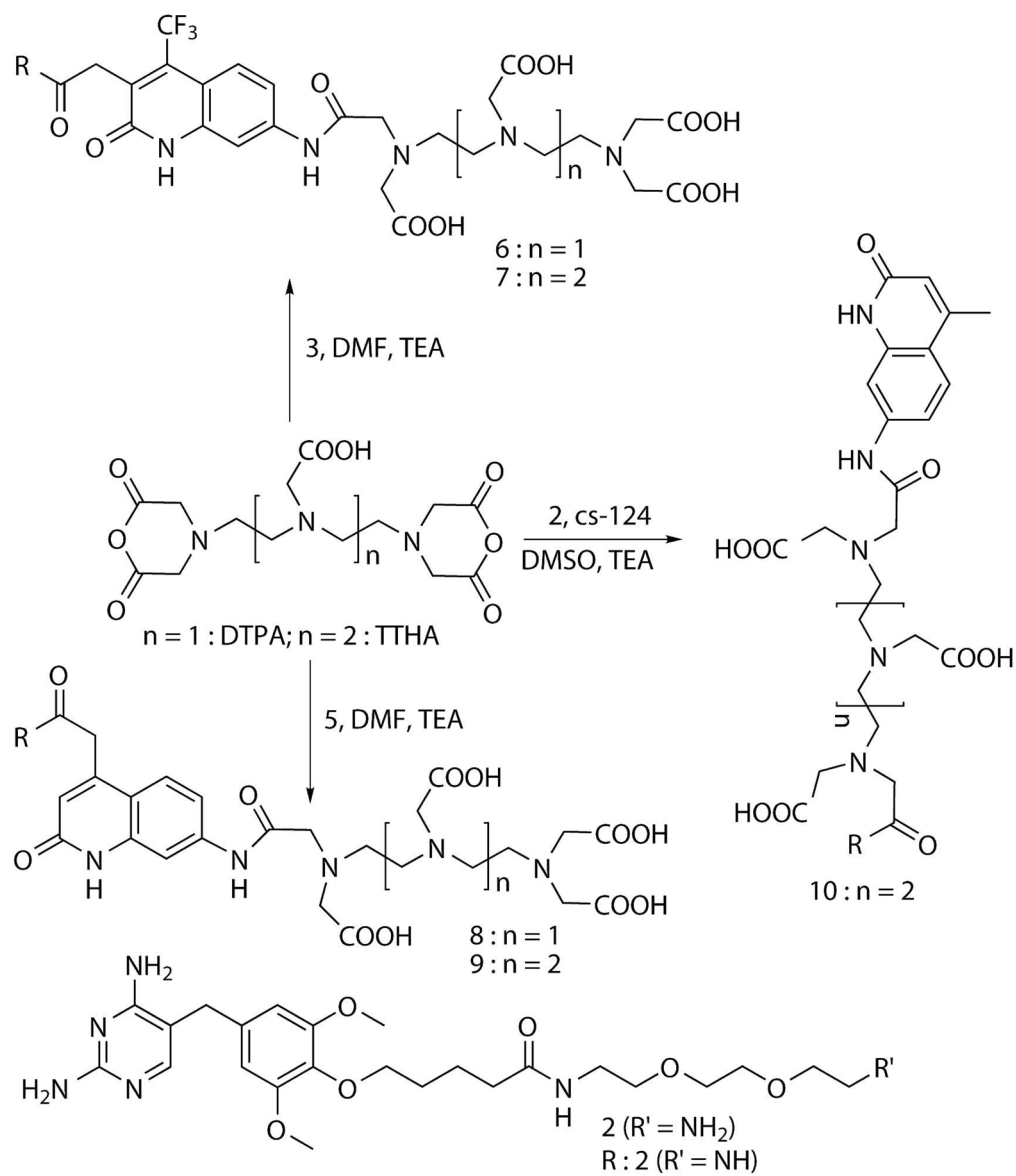


Figure 5.

Intramolecular, luminescence resonance energy transfer (LRET) between eDHFR-bound Tb^{3+} complexes of TTHA-cs124-TMP (9) or cs124-TTHA-TMP (10) and GFP. Increasing concentrations of either purified eDHFR-GFP or a bacterial lysate containing the protein were titrated against a constant concentration (20 nM) of each compound. Sensitized GFP emission (520 nm) and Tb^{3+} emission (615 nm) was detected after a time delay of 100 μs , upon pulsed excitation with near-UV light (~ 340 nm). The y-axis represents the percent change in the 520/615 emission ratio. Lines represent nonlinear least squares fit to the data.



Scheme 1.
Synthesis of TMP conjugated to (A) cs124-CF₃ (**3**) and (B) cs124 (**5**).



Scheme 2.
 Synthesis of sensitized TMP-polyaminocarboxylate conjugates.

Table 1HPLC purification solvents, retention times and detected m/z ratios for chelates prepared in this study.

compound	linear gradient	retention time (min)	ESI MS [M+H] ⁺
cs124CF ₃ -TMP (3)	3–33% CH ₃ CN/TEAA	22	775
cs124-TMP (5)	5–25% CH ₃ CN/TEAA	18	707
DTPA-cs124CF ₃ -TMP (6)	0–30% CH ₃ CN/TEAA	22	1151
TTHA-cs124CF ₃ -TMP (7)	0–30% CH ₃ CN/TEAA	20	1252
DTPA-cs124-TMP (8)	0–30% CH ₃ CN/TEAA	18	1083
TTHA-cs124-TMP (9)	0–30% CH ₃ CN/TEAA	17	1184
cs124-TTHA-TMP (10)	0–30% CH ₃ CN/TEAA	18	1140

Table 2Relative quantum yields of emission for prepared chelates of Tb³⁺ and Eu³⁺.

lanthanide	compound	quantum yield
Tb ³⁺	DTPA-cs-124 ^a	1.00
	DTPA-cs-124-TMP (8)	0.23
	TTHA-cs-124-TMP (9)	0.20
	cs-124-TTHA-TMP (10)	0.90
Eu ³⁺	DTPA-cs-124 ^a	1.00
	DTPA-cs-124-CF ₃ -TMP (6)	0.56
	TTHA-cs-124-CF ₃ -TMP (7)	2.02
	DTPA-cs-124-TMP (8)	0.77
	TTHA-cs-124-TMP (9)	1.56
	cs-124-TTHA-TMP (10)	1.98

^a reference compound