Published in final edited form as:

Bioconjug Chem. 2016 October 19; 27(10): 2540–2548. doi:10.1021/acs.bioconjchem.6b00473.

Brightly Luminescent and Kinetically Inert Lanthanide Bioprobes Based on Linear and Pre-organized Chelators

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Abstract

The synthesis, photophysical properties and kinetic stability of a series of water-soluble, highly emissive Tb(III) and Eu(III) complexes featuring triethylenetetraamine hexaacetic acid (TTHA) and cyclohexyl triethylenetetraamine hexaacetic acid (cyTTHA) chelator scaffolds and carbostyryl sensitizers are reported. The unique and modular design of the chelators gives rise to striking quantum yields of emission in aqueous solutions (up to 54%) as well as the characteristic lanthanides' photophysical properties (long excited state lifetimes, large effective Stokes shifts, and narrow emission peaks). Furthermore, the pre-organized chelators (**L3**, **L4** and **L6**) bind metal within minutes at ambient temperature yet exhibit substantial resistance to transchelation in the presence of a challenge solution (EDTA, 1 mM). Moreover, the Eu(III) complex of **L4** remains stably luminescent in HeLa cells over hours, demonstrating the suitability of these compounds for live cell imaging applications. Representative chelators suitable for derivatization and protein bioconjugation were also prepared that were functionalized with clickable azide and alkyne moieties, biotin and trimethoprim (TMP). With exceptional long-wavelength brightness, enhanced kinetic inertness, and an adaptable synthetic route, the reported lanthanide complexes are promising probes and labels for time-gated bioanalysis, biosensing and optical microscopy.

INTRODUCTION

Luminescent Lanthanide Complexes (LLCs) exhibit unique and useful photophysical properties including large Stokes shifts (>150 nm), long excited state lifetimes (μ s-ms) and multiple, narrow emission bands (<10 nm half-maximal) that span the visible spectrum.¹⁻⁶ These features enable highly sensitive diagnostic assays, high throughput screening methods or optical imaging approaches that employ time-gated detection strategies. Time-gating delivers high signal-to-background ratio by exciting a sample with pulsed light and collecting long-lived emission signals after a brief delay that eliminates ns-scale fluorescence background. Multi-wavelength emission also facilitates multiplexed detection when lanthanide complexes are used as Förster Resonance Energy Transfer (FRET) donors in combination with differently colored, fluorescent acceptors.⁷

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SUPPORTING INFORMATION

Synthetic procedures, NMR spectra, and Supporting Figures.

Structurally, LLCs consist of a chelator and one or more chromophores that serve to sensitize lanthanide ion excitation. In order to be efficiently luminescent, the metal center should be fully encapsulated by the chelating group to minimize solvent quenching effects. Given the hard acid nature of lanthanide trivalent cations, coordinative saturation with chelators that have hard base donor groups is required. In addition, incorporation of a sensitizing moiety is necessary since f-f transitions are formally forbidden by spin and Laporte rules. Upon irradiation in the ligand-centered band, energy transfers from the sensitizer to the chelated metal, usually via the sensitizer's triplet excited state. Generally, ligand-to-metal energy transfer is most efficient when the antenna triplet state energy is about 1500 cm^{-1} greater than that of the metal emissive state energy level, and when the sensitizer is directly coordinated to the metal.^{1-3, 8}

LLCs must meet several requirements in order to be used effectively in luminescent-based biological analysis and time-resolved microscopy. Firstly, the excitation wavelength should be above 340 nm so as to minimize absorbance by other chromophores in biological samples or media (the inner filter effect) and also to allow the use of conventional glass optics (for microscopy applications). Secondly, the complexes should demonstrate high kinetic inertness with respect to metal binding so as to stay intact for extended period in sample media or cells that contain high levels of competing chelators or metal ions. A third requirement is that brightness, which is the product of molar extinction coefficient at the excitation wavelength and overall quantum yield of luminescence, should be as high as possible at physiological pH. Finally, functional groups must be included that permit efficient conjugation to biomolecules. High brightness at relatively long excitation wavelengths ($> 340\text{ nm}$) is especially important when lanthanide complexes are to be used for time-gated, live-cell imaging because signal-to-noise ratio (S/N) scales with the square root of the number of photons detected.⁹ Lanthanide-based cellular microscopy is photon-noise limited because the number of detectable probes and their photon output is low, and imaging times must be kept short so as to capture dynamic events while minimizing photobleaching and phototoxicity. With ms-scale lifetimes, lanthanide photon emission rates are far lower than those of conventional fluorophores, and only the brightest lanthanide luminophores will permit cell imaging under conditions that are comparable to those of conventional fluorescence microscopy.^{4, 9} Probes with relatively long wavelength absorbance are beneficial because they permit the use of conventional microscope optics and are compatible with low-cost, high-power LED excitation sources that emit at 365 nm.^{10, 11}

Many Tb(III) and Eu(III) complexes meet some of the above-mentioned requirements, but only a few have the requisite brightness, long-wavelength absorption and kinetic stability to be used as cellular imaging probes.^{5, 12-21} For example, the octadentate macrocycle of 2-hydroxyisophthalamides, Lumi4-Tb, is bright, stable and has unusually high long wavelength absorptivity for a Tb(III) complex due to the small singlet-triplet energy gap of the 2-hydroxyisophthalamide sensitizing groups.¹⁶ We and others have used Lumi4-Tb(III) to label intracellular and cell-surface proteins for time-gated imaging using either laser (351 nm) or LED (365 nm) excitation.^{5, 6, 20} The 3:2, ligand:metal helicate complexes developed by Bünzli are exceptionally bright and have been validated for live-cell and immunohistochemical imaging.^{12, 13, 21} While most of the helicates have absorption maxima near 320 nm, certain Eu(III) analogs can be excited with visible light.^{21, 21} Parker and co-

workers have introduced the EuroTracker series of Eu(III) complexes based on a triazacyclononane scaffold¹⁷, and analogs have been used for organelle staining²², intracellular pH sensing¹⁸ as well as time-gated FRET detection of a cell surface g-protein coupled receptor.⁵ Cell-permeable EuroTracker analogs with red-shifted absorbance were recently reported.¹⁸

Given the relatively few examples of imaging-compatible LLCs, there remains a clear demand to develop new variants that display the requisite photophysical properties and coordination profile. Herein, we report the modular design and highly efficient synthesis of a new class of lanthanide complexes derived from triethylenetetraamine hexaacetic acid (TTHA) and cyclohexyl triethylenetetraamine hexaacetic acid (cyTTHA) chelators (Chart 1). Selective functionalization of a central carboxylic acid with 7-aminoquinolinone (cs124) or 7-amino-4-trifluoromethyl-2 (1H)-quinolinone (cs124-CF₃) sensitizer chromophores and their reactive analogs, yielded both Tb(III) and Eu(III) complexes with exceptional quantum yields in water (up to 54%). Furthermore, the incorporation of a pre-organized 1S,2S-trans-cyclohexanediamine moiety into the chelator backbone markedly improves the kinetic inertness of the complexes in vitro and in live cells. Moreover, our modular synthesis provides a robust route for preparing multifunctional metal complexes via selective N-alkylation of the chelator backbone.

RESULTS AND DISCUSSION

Chelate design and synthesis

The carbostyryl sensitizers and their corresponding carbamic chloride derivatives, trimethoprim (TMP), and bromoalkylating agent (di-tert-butyl 2,2'-((2-bromoethyl)azanediyl)diacetate) were prepared via previously published methods from our group and others with few modifications (Scheme 1, Scheme S1).²³⁻²⁵ We initiated our probe synthesis by reductive amination of benzaldehyde followed by monoalkylation of the corresponding secondary amine. The subsequent sensitizer incorporation and debenzylation reactions lead to molecule **S8** (Scheme S2). As with previously reported attempts to heterogeneously alkylate TTHA, we observed that formation of piperazin-2-one substantially lowered dialkylation reaction yield^{26, 27} in part because this step requires heat for completion. This side reaction is particularly dominant when preparing the cyclic TTHA analogs. In order to resolve this problem, we designed a modular synthesis that followed a general reaction scheme of N-benzyl protection, N-alkylation with an alkyl halide, benzyl deprotection via hydrogenation, a second alkyl halide N-alkylation, and finally a sensitizer installation via a third alkylation. (Scheme 2) We minimized piperazin-2-one formation by first alkylating with two relatively bulky alkylating agents and then running the subsequent alkylation reaction at room temperature. Following this route, we prepared sensitized chelators in 6 steps with good overall yields (31% for **L1**). Another advantage of our synthetic strategy is it allows the introduction of other chromophores or chelating groups since these groups are added in the last step. We further tested the generality of our synthetic approach by substituting a quinoline chromophore instead of carbostyryl in the central carboxylic arm to yield compound **23**. (Scheme 3)

In order to obtain bioconjugation-ready analogs, we prepared the clickable ligands **L5** and **L6** by modifying the quinolinone sensitizers with azide or alkyne groups, respectively. To demonstrate compatibility of the polyaminocarboxylate ligands with Cu(I)-catalyzed azide alkyne cycloaddition (CuAAC), we first established click reaction conditions by coupling compound **7** to (azidomethyl)benzene in the presence of TTHA (Scheme S3). As with previously reported CuAAC reactions performed in the presence of a strong chelating group, we applied excess Cu(II) sulfate to ensure availability of sufficient copper for catalysis.²⁸ The chelated copper(II) was precipitated out by adding excess sodium sulfide to yield the click product in an excellent yield (Scheme S3). Using this strategy, we prepared the biotinylated compound **25** directly from key intermediate **21** via a one-pot route. Lastly, we prepared a conjugate of TMP with **L1** via anhydride formation to provide terbium probes capable of binding to *Escherichia coli dihydrofolate reductase* (eDHFR) fusion proteins with high affinity.²⁴

Photophysical properties

Selvin and coworkers showed that carbostyryl dyes are effective sensitizers of both Eu(III) and Tb(III) when coupled to the polyaminocarboxylates diethylenetriamine pentaacetic acid (DTPA), triethylenetetraamine hexaacetic acid (TTHA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).²⁹⁻³¹ A common strategy for synthesizing sensitized DTPA or TTHA complexes is to prepare the dianhydride form of the commercially available chelators and react it with the 7-amino group of a carbostyryl or coumarin.^{24, 32, 33} Complexes of cs124-TTHA prepared via the dianhydride route had ca. 50% lower quantum yields than those originally reported.³⁴ It was surmised that the original chelate was a mix of isomers with cs124 appended to either the middle or terminal nitrogens of the TTHA backbone chain, whereas the anhydride-derived form consisted solely of the terminal-functionalized derivative. At the termini, the carbonyl oxygen of the amide linkage between the dye and the backbone must compete with a deprotonated carboxyl for coordination to the metal center. When not coordinated, the antenna is far away from the ion, and energy transfer is less efficient. We hypothesized that TTHA functionalized with cs124 or cs124-CF₃ exclusively at the middle backbone amines would exhibit high emission efficiencies because the antennae would remain coordinated to the metal in solution.

While cs124 effectively sensitizes both Tb(III) and Eu(III), cs124-CF₃ only sensitizes Eu(III).³⁰ Therefore, we prepared Tb(III) complexes of **L1**, **L3** and **L6** and Eu(III) complexes of **L2**, **L4**, and **L5**. The absorption and excitation spectra of the ligands and their metal complexes in TBS buffer at pH 7.4 show the characteristic bands in the near-UV region ($\lambda_{\text{max}} \approx 341$ nm, **L1-L4**, **L6**; 349 nm, **L5**) that are closely matched to those of previously reported carbostyryl sensitized complexes (Table 1, Figure 1, Figure S1).^{24, 30, 31} Characteristically sharp and structured emission spectra of all probes were obtained without the help of augmenting agents with their most intense luminescent transitions centered at 545 nm (⁵D₄ → ⁷F₅) for Tb(III) and 614 nm (⁵D₀ → ⁷F₂) for Eu(III). Quantitative metallation of the complexes was achieved within 15 min at room temperature following addition of LnCl₃, as evidenced by HPLC analysis (Figure S2). Measurement of luminescent lifetimes in H₂O and D₂O and fitting to the Horrocks and Sudnick equation³⁵ showed that water is excluded from the inner coordination sphere (Table 1, Figures S3-S4). The lifetime

data for all of the probes were well fit to mono-exponential decays which confirms the presence of single Tb(III) and Eu(III) emitting species in buffer solutions. The relevant photophysical properties are summarized in Table 1. Most notably, exceptionally high quantum yields of emission (e.g., 0.54, **L3(Tb)**; 0.40, **L4(Eu)**) were observed in aqueous buffer that are among the highest reported values in the literature.^{16-17,22} The efficient emission and lack of bound waters support the hypothesis that inner substitution positions the sensitizer such that it remains coordinated to the metal center. Like our previously developed, 3-substituted cs124-CF₃ analogs²⁴, compound **L5** has an extinction coefficient of ca. 21,000 M⁻¹ cm⁻¹ at 349 nm with a pronounced shoulder at 366 nm. With a brightness of ca. 5500 M⁻¹cm⁻¹ at 365 nm, **L5** is brighter at that wavelength than the Lumi4(Tb) complex (brightness at 365 nm, 5200 M⁻¹cm⁻¹) that we have used successfully for time-gated luminescence microscopy.^{4, 6}

Kinetic Stability in vitro and in living cells

Practical use of lanthanide luminophores in solution phase bioassays or as imaging probes requires stable metal retention at high dilution in the presence of competing chelators or other metals. In general, cryptates or macrocycles are more kinetically inert than relatively flexible, linear chelators. However, they typically have slow metal association kinetics and require metallation at elevated temperatures, which complicates their use as antibody conjugates or with other heat sensitive preparations, although the Lumi4(Tb) cryptate is a notable exception.¹⁶ Introduction of a pre-organized, cyclic moiety into an otherwise linear chelator can induce rigidity and yield more kinetically and/or thermodynamically stable complexes.³⁶ For example, incorporation of trans-1,2 diaminocyclohexane into the backbone of DTPA and TTHA improved the kinetic stability of radiometal complexes.^{37, 38} Ge and Selvin reported enhanced kinetic stability of a 9-dentate polyaminocarboxylate chelate that incorporated 1-oxa-4,7 diazacyclononane.³⁴ The kinetic inertness of **L1(Tb)**, **L2(Eu)**, **L3(Tb)** and **L4(Eu)** was assessed in direct comparison to the benchmark lanthanide complex, cs124-DTPA and the Lumi4-sv(Tb) cryptate (see Figure S5 for structures of comparison chelators).^{16, 31} Luminescence intensity was monitored after dilution to 5 nM in tris-buffered saline (TBS, pH 7.6) that contained the competitive chelator EDTA (1 mM). The intensity of the linear complexes including the reference compound, cs124-DTPA completely diminished within ca. 15 h, whereas their pre-organized counterparts retained > 60% of their initial luminescence after 24 h, approaching the performance of the Lumi4-sv(Tb) cryptate (Figure 2). The competitive challenge assay results clearly show that incorporation of a cyclohexyl moiety into the TTHA backbone imparts considerable solution stability while preserving fast metalation kinetics.

Having achieved the desired solution phase brightness and stability, we next sought to determine that cyTTHA-based complexes retained these properties in living cells. Time-gated microscopy was used to monitor luminescence at 620 nm for a period of 2 h following microinjection of **L4(Eu)** into the cytoplasm of adherent HeLa cells. Emission intensity diminished somewhat during that period, although not much more than that of the Lumi4(Tb) complex that we have previously used for cellular imaging (Figure 3).^{4, 6} In addition to exhibiting good intracellular stability, the anionic complex diffused freely throughout the cytoplasm and nucleus and did not partition to particular organelles. Anionic

probes that diffuse freely throughout cells can be conjugated to cell penetrating peptides and targeting ligands to enable passive cytoplasmic delivery and specific biomolecular or subcellular targeting, as we have previously demonstrated with Lumi4(Tb).^{6, 39}

CONCLUSION

A novel series of Tb(III) and Eu(III) luminescent complexes have been developed on the basis of linear and pre-organized polyaminocarboxylates. Our modular synthesis made it possible to introduce a carbostyryl sensitizing group specifically in the central carboxylic pendant arm of TTHA and cyTTHA chelator scaffolds. The design of the probes yielded exceptional quantum yields of emission in aqueous solution that are among the highest values reported in the literature for both Tb(III) and Eu(III). Incorporation of a pre-organized cyclohexyl ring into the chelator backbone significantly improved kinetic stability in concentrated EDTA solution as well as in live cells while preserving fast metallation rates. Moreover, further derivatization of the probes can be achieved by modifying the sensitizer with clickable azide or alkyne functional groups (e.g., **L5**, **L6**) or reaction of the dianhydride form of the ligand with an amine. We leveraged these conjugation strategies to prepare a biotinylated ligand (**25**) and a TMP- conjugate (**24**) that can bind non-covalently to streptavidin and *Escherichia coli dihydrofolate reductase* (eDHFR) fusion proteins, respectively. The probes reported here are promising candidates for time-gated imaging and bioanalytical applications. Future studies will apply the flexible synthetic route to explore alternative sensitizers or other methods of bioconjugation such as functionalizing the carbon backbone of the chelator.

EXPERIMENTAL PROCEDURES

Chemicals and Instrumentation

All chemicals for synthesis were purchased from Sigma Aldrich Inc. except for trans-1,2-diaminocyclohexane (TCI America) and were used as received. Distilled and deionized (18 M Ω cm⁻¹) water was used for all synthetic and analytical procedures. HeLa cells were purchased from American Type Culture Collection (CCL-2TM). Dulbecco's modified eagle medium (DMEM, 10-014 CV), Dulbecco's phosphate buffer saline (DPBS, 21-030 and 21-031), and 0.25% trypsin/2.21 mM EDTA in HBSS (25-053-CI) were purchased from Corning Cellgro®. MEM non-essential amino acids (11140), DMEM (no phenol red, 21063) and HEPES (15630-080) were purchased from Gibco®. Tris(hydroxymethyl) aminomethane hydrochloride (Tris·HCl) was purchased from Acros Organics. Micropipette Preparation Glass bottom culture dishes (P50G-0-14-F) were purchased from Matek Corporation (Ashlan, MA). XenoworksTM Microinjection System, P-1000 Pipette Puller and Borosilicate glass tubes (BF100-78-10) were used for microinjection (Sutter instruments, Novato, CA). Lumi4-Tb reagents were provided by Lumiphore, Inc. UV-visible (UV-vis) absorption spectra were recorded using a Cary 3000 spectrophotometer (Varian, Inc.). Fluorescence excitation and emission spectra were recorded using a Fluoromax 3 fluorimeter (Horiba-Jobin Yvon, Inc.). Time-resolved luminescence intensity was measured using a micro well plate reader (Perkin-Elmer, Victor 3 V) with 340 nm excitation (60 nm bandpass) and 545 nm emission (10 nm bandpass, for Tb(III) complexes) or 620 nm

emission (10 nm bandpass, for Eu(III) complexes). Preparative HPLC was performed with a LC-20AT Shimadzu on a Luna C18 column (#00G-4252-N0); fractions were detected with a SPD-20A Detector and collected with a Shimadzu fraction collector (#220-91212-71).

Linear gradients of solvents A and B were used (A = HPLC grade acetonitrile containing 0.1 % (v/v) trifluoroacetic; B = de-ionized water containing 0.1 % (v/v) trifluoroacetic acid.

Synthesis

Synthetic procedures and NMR spectra are reported in the Supporting Information.

Preparation of lanthanide complexes

Probes concentration was obtained using measured absorptions and confirmed 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 $\lambda = 341\text{ nm}$). EuCl₃·6H₂O or TbCl₃·6H₂O was added to the chelate in a 1:1.3 molar ratio at typically > 5 μM concentration in TBS buffer (50 mM Tris·HCl, 150 mM NaCl, pH 7.6) and incubated for 15 min at room temperature before use. In all cases, emission intensities saturated within 15 min after incubation. Additionally, HPLC analysis was performed for a representative chelator **L5** to confirm the efficiency of labeling (Figure S2).

Spectroscopy

UV-Vis absorption spectra, excitation spectra and fluorescence emission spectra were obtained in 1 cm cuvettes containing solutions of the ligand:metal complexes (5 μM in TBS, pH 7.6).

Quantum Yield Measurements

The luminescence quantum yields of lanthanide chelates in TBS buffer (pH 7.6) were determined by standard methods using cs124-DTPA(Tb) as a reference from the equation

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

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.⁴⁰ The excitation wavelength used to obtain steady-state fluorescence emission spectra was 330 nm in all cases. The quantum yield of Tb(III) emission for cs124-DTPA was measured using quinine sulfate as a reference ($\Phi = 0.54$ in 0.1M H₂SO₄). Each sample was measured at least three times, and relative standard deviation did not vary by more than $\pm 12\%$.

Luminescent lifetime estimation

Stock solutions of metal complexes were diluted to ~20 nM in TBS buffer (50 mM Tris·HCl, 150 mM NaCl, pH 7.6). Luminescence intensity was measured at 545 nm (for Tb(III) complexes) or 620 nm (for Eu(III) complexes) using a time-resolved fluorescence plate

reader (Perkin Elmer, Victor 3V) following excitation at 340 nm. Intensity values (500 μ s integration) were measured at different time delays (50 μ s increments) from 50 μ s to 900 μ s (for Eu(III)) or 3000 μ s (for Tb(III)). Plots of intensity vs. delay time were fit to a single exponential with KaleidaGraph v4.0, and lifetime was estimated from the equations: 1) $I(t) = A \cdot \exp(-t/\tau)$ (Eu(III) complexes, Figure S3); or $I(t) = A \cdot \exp(-t/\tau) + B$ (Tb(III) complexes, Figure S4). The measurements were repeated 3 times for each compound, and standard deviation was less than 8% of the mean in all cases.

EDTA competition assay

In order to measure the stability of the lanthanide complexes, 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 50 mM stock solution (H₂O/NaOH, pH 8.0) to a final concentration of 1 mM (EDTA) and 5 nM (lanthanide complexes), and the samples were vortexed for 1 min. The samples were then aliquoted (100 μ L) into 96-well plates, and the Tb(III) or Eu(III) emission was measured over time using a time-resolved luminescence plate reader (time delay = 100 μ s; measurement window = 1400 μ s; λ_{ex} = 340/60 nm; Tb(III), λ_{em} = 545/10 nm; Eu(III), λ_{em} = 615/10 nm).

Cell culture

HeLa cells were maintained in DMEM (+) (DMEM supplemented with 10% FBS, 1 $\times$ MEM non-essential amino acids and 15 mM HEPES) at 37 °C and 5% CO₂. The cells were passaged with 0.25% trypsin/2.21 mM EDTA.

Time-gated microscopy and image processing

Time-gated luminescence images were acquired using a previously described, epi-fluorescence microscope (Axiovert 200, Carl Zeiss, Inc.) modified with the following components: 1) a UV LED emitting at 365 nm (UV-LED-365, Prizmatix, Ltd.); 2) a digital delay generator (DG645, Stanford Research Systems, Inc.); 3) a gated image-intensified CCD camera (ICCD), mounted on the side-port of the microscope (Mega-10EX, Stanford Photonics, Inc.); and 4) a computer running Piper Control software (v2.4.05, Stanford Photonics, Inc.). Filter cubes containing the appropriate excitation and emission filters and dichroics allowed for wavelength selection. Images were obtained with a 63X/1.25 N.A. EC Plan Neofluar oil-immersion objective (Carl Zeiss, Inc.). For each time-gated image acquisition, the signal from multiple excitation/emission events was accumulated on the ICCD sensor and read out at the end of the camera frame. The UV LED pulse width and pulse period, the intensifier delay time and on-time, the camera frame length (66.67 ms – 2 s) and the intensifier gain voltage could be varied independently. The source/camera timing parameters were the same for all of the time-resolved images and data presented here: excitation pulse width, 1500 μ s, pulse period, 3000 μ s, delay time, 10 μ s, intensifier on-time, 1480 μ s intensifier on-time. Sensitivity was modulated by either varying the frame length (and thus, the number of integrations) or the intensifier gain voltage. The data reported here was acquired at a gain of 833 V and a frame length of 66.7 ms (22 integrations). The camera control software enabled summation of multiple frames to yield a single composite .TIFF image with a bit depth equal to 1024 multiplied by the number of frames. All images reported here were summations of four frames (bit depth, 4096), and a feature of the camera

control software was enabled that removes large variations in signal resulting from ion-feedback noise of the intensifier. ICCD images (tagged image file format, .TIFF) were captured with Piper control software (v2.4.05, Stanford Photonics, Inc.) Raw, 12-bit images were imported into NIH ImageJ (v1.42q) for all processing operations including cropping and contrast adjustment.

Live-cell microinjection and measurement of intracellular stability

Hela cells were grown to 60-70% confluency in a glass bottom dish. A Sutter Instruments P100 pipette puller was used to prepare micropipettes using the following parameters: heat, ramp-30; pull, 30; velocity, 50; time, 120; and Pressure, 510. Micropipettes were loaded with a solution of **L4(Eu)** (100 μ M) and Lumi4(Tb) (100 μ M) in TBS buffer via capillary action, and the solutions were injected into the cytoplasm with the using the following microinjector parameters: transfer pressure, 33 hPa; injection width, 0.4 sec; injection pressure, 70-100 hPa. Prior to imaging, 100 μ L 1mM patent blue V solution in DMEM (without phenol red) was added to the cells to quench extracellular luminescence from leakage of probes from the microinjection capillary. Mean intensity was measured in a region of interest (ROI) drawn within the cytoplasm of each cell in each image.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R01GM081030). We thank Dr. Darren Magda at Lumiphore, Inc. for providing material and intellectual support.

ABBREVIATIONS

cyTTHA	cyclohexyl triethylenetetraamine hexaacetic acid
TTHA	triethylenetetraaminehexaacetic acid
DTPA	diethylenetriaminepentaacetic acid
FRET	Förster Resonance Energy Transfer
eDHFR	<i>Escherichia coli dihydrofolate reductase</i>
TMP	trimethoprim

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Figure 1. Normalized absorption (dotted lines) and steady-state emission (solid lines) of Tb(III) and Eu(III) complexes ($5 \mu\text{M}$ in TBS buffer at pH 7.6).

Figure 2.

Incorporation of pre-organized cyclohexyl group into TTHA backbone increases lanthanide complex kinetic inertness. Relative luminescence intensity over time in the presence of EDTA (1 mM) for various dilute aqueous solutions (5 nM) of the indicated metal complexes. Each test was repeated 3 times, and relative standard deviation was <4% in all cases.

Figure 3.

Compound **L4(Eu)** is stably luminescent in live HeLa cells. (a) Micrograph: Time-gated luminescence image (λ_{ex} , 365 nm; λ_{em} , 615/10 nm) of HeLa cell injected with a solution containing **L4(Eu)**. (b) Mean, normalized signal intensity in cytoplasmic region of cells injected with either Lumi4(Tb) (3 cells) **L4(Eu)** (4 cells) over time (error bars, sem).

Scheme 1. Synthesis of Tb(III) and Eu(III) sensitizers. **Scheme 1.**

Synthesis of Tb(III) and Eu(III) sensitizers

Reagents and conditions: (a) ethyl 3-oxobutanoate (1.0 equiv) for 3/ ethyl 4,4,4-trifluoro-3-oxobutanoate (1.0 equiv) for 2/ 1-ethyl 4-methyl 2-(2,2,2-trifluoroacetyl)succinate (1.0 equiv.) for 10, DMF, reflux, (b) 2-chloroacetyl chloride (1.1 equiv), NEt₃ (3.0 equiv), DMF, 0 °C- rt. (c) NaH (1.0 equiv), propargyl bromide (1.0 equiv), DMF, rt. (d) 2-(2-(2-azidoethoxy)ethoxy)ethoxyethoxyethanol-1-amine (1.0 equiv), N-(3-Dimethylaminopropyl)O-N'-ethylcarbodiimide hydrochloride (1.0 equiv), DIEA (3.0 equiv), DMF, rt.

Scheme 2.

Synthesis of sensitized lanthanide chelators

Reagents and conditions: (a) PhCHO (2.0 equiv), MeOH, reflux then NaBH₄ (4.3 equiv), 0 °C- rt (b) Na₂CO₃ (3.0 equiv), S2: di-*tert*-butyl 2,2'-((2-bromoethyl)azanediyl)diacetate (2.1 equiv), MeCN, 60 °C. (c) Pd/C (10 wt %), H₂, MeOH, rt. (d) *t*-butyl bromoacetate (0.25 equiv), Na₂CO₃ (1.0 equiv), MeCN, rt. (e) 4 for L2 and L5, 5 for L1 and L3, 12 for L5, 9 for L6 (1.1 equiv), Na₂CO₃ (2.0 equiv), MeCN, 60 °C. (f) TFA/CH₂Cl₂/ iPr₃SiH, 5:5:1.8, v/v, rt.

Scheme 3.

Synthesis of modified probes

Reagents and conditions: (a) 2-(chloromethyl)quinoline (1.0 equiv), Na_2CO_3 (2.0 equiv), MeCN, 60 °C. (b) TFA/ CH_2Cl_2 / $i\text{Pr}_3\text{SiH}$, 5:5:1.8, v/v, rt. (c) acetic anhydride (4.0 equiv), pyridine, 65 °C. (d) S5: N-(2-aminoethyl)-5-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)pentanamide (0.5 equiv), DIEA (10.0 equiv), DMF, rt. (e) 8 (1.1 equiv), Na_2CO_3 (2.0 equiv), MeCN, 60 °C. (g) biotinPEG3azide (0.5 equiv), sodium ascorbate (0.2 equiv), $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (2.1 equiv), 1:1 DMF/ H_2O , rt.

Chart 1.

Chemical structures of TTHA and cyTTHA based chelators capable of sensitizing lanthanide luminescence.

Table 1

Photophysical properties^a and number of bound waters (q) for the reported complexes.

Compounds	$\lambda_{\max}^b$ (nm)	QY (%)	τ /ms (H ₂ O)	τ /ms (D ₂ O)	q
L1(Tb)	341	50	1.72	1.89	0.22
L2(Eu)	341	36	1.11	1.36	0.17
L3(Tb)	341	54	1.73	1.94	0.27
L4(Eu)	341	40	1.07	1.49	0.28
L5(Eu)	349	35	1.11	1.52	0.24
L6(Tb)	341	49	1.65	1.75	0.18

^a Measured in TBS buffer (pH 7.6). Relative standard deviations of quantum yield (QY) and excited state lifetime (τ) values were 12% and 8%, respectively.

^b Peak of longest wavelength absorption band.