New 9- or 10-Dentate Luminescent Lanthanide Chelates

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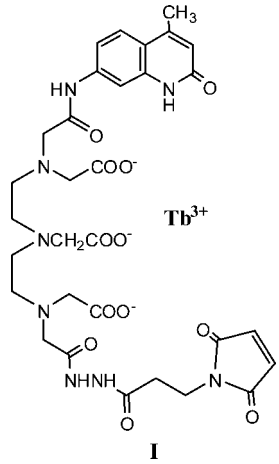
Polyaminocarboxylate-based luminescent lanthanide complexes have unusual emission properties, including millisecond excited-state lifetimes and sharply spiked spectra compared to common organic fluorophores. There are three distinct sections in the structure of the luminescent lanthanide chelates: a polyaminocarboxylate backbone to bind the lanthanide ions tightly, an antenna molecule to sensitize the emission of lanthanide ions, and a reactive group to attach to biomolecules. We have previously reported the modifications on the chelates, on the antenna molecules (commonly cs124), and on the reactive sites. In searching for stronger binding chelates and better protection from solvent hydration, here we report the modification of the coordination number of the chelates. A series of 9- and 10-dentate chelates were synthesized. Among them, the 1-oxa-4,7-diazacyclononane (N_2O)-containing chelate provides the best protection to the lanthanide ions from solvent molecule attack, and forms the most stable lanthanide coordination compounds. The TTHA-based chelate provides moderately good protection to the lanthanide ions.

INTRODUCTION

Luminescence resonance energy transfer (LRET) is a modification of the widely used technique of fluorescence resonance energy transfer (FRET) and can be used to accurately determine the distance between two sites bearing an energy transfer donor and an energy acceptor in a biomolecule (*1, 2*). In LRET, the energy transfer donor is a luminescent lanthanide atom and the acceptor is a conventional (organic) fluorophore. The lanthanide energy transfer donor typically involves a chelate to bind and protect the lanthanide from solvent quenching effects, a covalently attached organic chromophore to act as an antenna to absorb excitation light and transfer the energy to lanthanide ions, and a covalently attached reactive site to attach to biomolecules (see structure **I**, DTPA-cs124-maleimide, as an example).



I

The fundamental advantages of LRET arise because the donor emission is long-lived with millisecond lifetime and is sharply spiked (peaks of a few nanometer widths) (*1*), has a high quantum yield (*3*), and is unpolarized (*4*). This enables temporal and spectral discrimination against the acceptor (a conventional fluorophore), which has nanosecond-lifetime and is broadly spread in wavelength (*1*).

We have published a number of papers on LRET (partially reviewed in *1, 5*), showing its advantages in model systems such as DNA oligomers (*6, 7*) and the ability to measure distance changes of an angstrom reliably even on large protein complexes such as actomyosin (*8, 9*) and in ion channels in living cells (*10–12*). Other researchers have successfully used the technique on DNA−protein complexes (*13–15*), actomyosin (*16*), protein−protein interactions in cells (*17, 18*), and detection of binding of many different biomolecules (*2, 19*).

The current DTPA (diethylenetriaminepentaacetic acid)-based chelates (**I**) works moderately well with both terbium and europium. The disadvantage of such chelates is that they are 8-dentate chelates; therefore, one or more water molecules can bind to lanthanide ions when these chelates coordinate to lanthanide ions (*20*). Because of the hydration on the lanthanide ions, the lifetime is relatively shortened by the solvent quenching effect. Also, the relatively low stability constant or fast dissociation and trans-metalation kinetics limits their application in the physiological environment. On the other hand, here we show that two different chelates, TTHA (triethylenetetraamine-hexanoic acid) and a chelate made from diazacrown ethers, have more than 8 coordination numbers, minimizing the hydration to the lanthanide.

TTHA-based lanthanide chelate is a 10-dentate ligand and has shown no hydration to lanthanide ions when coordinated (*21*). Although TTHA-based lanthanide chelates with coumarin derivatives as sensitizers (which works for Eu^{3+} only) have been reported (*13*), no TTHA-based amine reactive or thiol reactive lanthanide probes with cs124 as sensitizer (which works with both Tb^{3+} and Eu^{3+}) have been synthesized. Here, we report the syntheses of TTHA-based thiol reactive chelates.

The class of macrocycles known as crown ethers has been widely studied since their metal ion-coordinating capabilities were first reported by Pedersen (*22*). Derivations of the crown ether include the replacement of one or more of the ring's oxygen atoms with nitrogen atoms, resulting in azacrown ethers,

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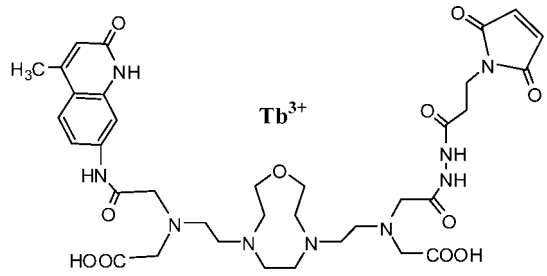
and/or the attachment of one or more side arms to the ring to form a so-called lariat or armed crown ether. There are numerous publications on the metal-complexing properties of diazacrown ethers containing side chains attached to the nitrogen atoms of the macrocycle (*23*). We have synthesized a new type of lanthanide chelate derived from diazacrown ethers. Our chelates contain two ethyliminodiacetic acid side chains and have increased ability to bind lanthanide ions to better protect the lanthanide ions from solvent hydration (see structure **II**) and from other potential chelators such as ATP and EDTA.



II

EXPERIMENTAL METHODS

Chemicals and Materials. The following were purchased from Sigma-Aldrich: triethylenetetraminehexaacetic acid (TTHA), 7-amino-4-methyl-2-(1*H*)-quinolinone (carbostyril 124, cs124), isobutyl chloroformate, anhydrous dimethyl sulfoxide (DMSO, in sure seal bottle), and triethylamine (for reaction). DMSO and triethylamine were dried over activated molecular sieves before use. 1,7-Diaza-12-crown-4 (N_2O_2) was purchased from Acros Organics. Triethylenetetramine hexaacetic acid dianhydride (caTTHA) (*24*), 1-oxa-4,7-diazacyclononane (N_2O) (*25*), and *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine (*26*) were synthesized according to published methods. Glacial acetic acid, methanol, and triethylamine (for making TEAA buffer) were purchased from Fisher Scientific. β-Maleimidopropionic acid hydrazide.TFA (EMPH·TFA) was purchased from Molecular Biosciences (Boulder, CO). (*R*)-2-Amino-2-carboxyethylmethanethiosulfonate (Cys-MTS) was purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Ubiquitin was a gift from Invitrogen Inc. (Madison, WI). Distilled and deionized (18 MΩ cm^{-1}) water was used throughout. All glassware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water. All plastic labware was purchased from Biorad (metal-free). All chemicals were of the purest grade available.

Purification. Most commonly, reverse-phase high-performance liquid chromatography was performed at room temperature on a Waters model 600 system with a Dynamax 60 Å C_{18} column (10 or 25 mm i.d. × 250 mm) using a linear gradient (solvent A = 0.1 M triethylammonium acetate, pH 6.5; solvent B = methanol/or acetonitrile).

Spectroscopy. Time-resolved and gated luminescence measurements were made on a laboratory-built spectrometer described previously, employing a 5 ns excitation pulse at 337 nm followed by time-resolved detection of lanthanide emission (*27*). The number of waters in the primary coordination sphere of the lanthanide was determined via the method of Horrocks and Sudnick (*28*): # of waters = $q(\tau_{H2O}^{-1} - \tau_{D2O}^{-1})$, ($q = 1.05$ for Eu, 4.2 for Tb).

Synthesis (Charts 1, *2*). *N_2O-Tetracarboxylic Acid.* 1-Oxa-4,7-diazacyclononane (N_2O) (100 mg, 0.77 mmol) and *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine (550 mg, 1.56 mmol) were dissolved in 5 mL of CH_3CN. 10 mL of phosphate buffer (2 M, pH 8.0) was added to the mixture. The solution was stirred at room temperature for 36 h. Ethyl acetate (10 mL) was added to the mixture. The aqueous solution was separated and extracted with ethyl acetate 2 × 5 mL. The combined organic layer was washed with water 3 × 5 mL. The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford a colorless oil (458 mg, 89% yield. MS: 673, M + 1, FAB). The product was used for the next step without further purification.

200 mg (0.30 mmol) of the above-prepared material was dissolved in 6 M HCl/MeOH solution (5 mL). The mixture was stirred at room temperature for 2 h. Ether was added to the mixture. White precipitate was formed. The white precipitate was collected by filtration and dried (114 mg, 73% yield. MS: 447, M − 1, ESI).

N_2O_2-tetracarboxylic acid was also synthesized with the same method in similar yield and confirmed by mass spectrometry (MS: 491, M − 1, ESI).

General Procedure for Syntheses of N_2O- and N_2O_2-Based Lanthanide Chelates. 1 equiv of N_2O-tetracarboxylic acid was dissolved in a mixture of DMSO and 7 equiv of Et_3N under N_2. 2.2 equiv of isobutyl chloroformate was added and stirred at room temperature for 1.5 h. 0.7 equiv of cs124 dissolved in DMSO was added slowly to the above solution with vigorous stirring. The reaction was stirred at room temperature for 2 h. The solution was saved for the following steps.

If non-thiol-reactive chelates were to be synthesized, the above solution was quenched by addition of acetic acid and 0.1 M TEAA buffer (pH 6.5). The products were purified by reverse-phase HPLC with a linear gradient (typically 20−40% CH_3CN/TEAA pH 6.5 over 40 min). The yield varies from ∼35% to ∼50% as judged from the HPLC profiles (Table 1).

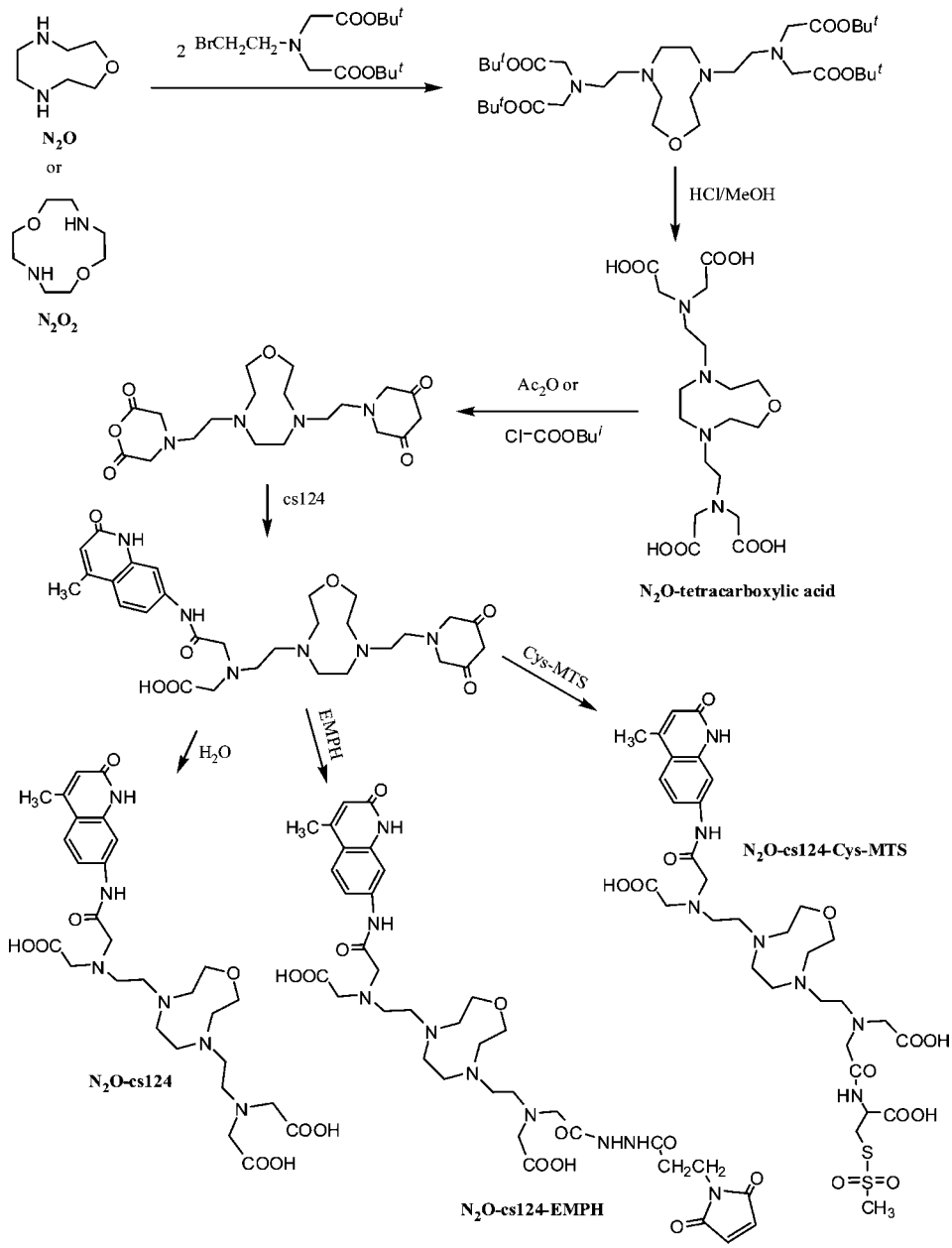
If the thiol-reactive forms of chelates were to be synthesized, the above solution was transferred to a DMSO solution containing 1.3 equiv of EMPH·TFA (or Cys-MTS) rapidly under N_2. The reaction was stirred at room temperature for 1 h. Acetic acid and 0.1 M TEAA (pH 6.5) buffer were added to quench the reaction. The product was purified by reverse-phase HPLC with a linear gradient (typically 20−40% CH_3CN/TEAA pH 6.5 over 40 min). The yield varies from ∼20% to ∼30% as judged from the HPLC profiles (Table 1).

General Procedure for Syntheses of TTHA-Based Lanthanide Chelates. Triethylenetetramine hexaacetic acid dianhydride (caTTHA) (1 equiv) was dissolved in a mixture of DMSO and 5 equiv of Et_3N under N_2. 0.7 equiv of cs124 dissolved in DMSO was added slowly to the above solution with vigorous stirring. The reaction was stirred at room temperature for 2 h. 1.3 equiv of thiol-reactive reagents (EMPH·TFA or Cys-MTS) dissolved in DMSO was added to the above solution. The reaction was stirred for 1 h at room temperature. Acetic acid and 0.1 M TEAA (pH 6.5) were added to quench the reaction. The product was purified by reverse-phase HPLC with a linear gradient (typically 30−50% MeOH/TEAA pH 6.5 over 40 min). The yield varies from ∼20% to 50% as judged from the HPLC profiles (Table 1).

Addition of Metals. $TbCl_3$ or $EuCl_3$ was added to the chelate in a 1:1.2 molar ratio at usually >0.5 mM concentration at pH 6.5−7.5, usually in 0.1 M TEAA, pH 6.5 buffer, and allowed to stand for 30 min at room temperature before use.

Coupling to Biomolecules. Similar labeling conditions for conjugation to muscle proteins (*29, 30*) were employed. The procedure for labeling Shaker K^+ ion channels in *Xenopus* oocytes has also been published (*10, 11*). The thiol-reactive chelates were coupled to Ubiquitin (an 8.9 kDa protein containing a single cysteine) at a typical ratio of 10:1 with a protein concentration of ∼15 μM. The coupling reaction was carried out at 4 °C overnight in 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$. The excess lanthanide chelates were removed either by

Chart 1



passing the reaction mixture through a Sephadex G-50 size exclusion column or by dialyzing the reaction mixture through Pierce Slide-A-Lyzer Dialysis Cassette.

RESULTS AND DISCUSSION

Syntheses. DTPA-based lanthanide chelates can be readily prepared by reacting commercially available DTPA dianhydride (caDTPA) with 1 equiv of cs124 and 1 equiv of thiol-reactive reagents. The same principle can apply to the new chelate syntheses. The synthesis of the dianhydride form of TTHA has been reported (*24*). TTHA dianhydride was reacted with 1 equiv of cs124 first, followed by addition of 1 equiv of thiol-reactive reagents to form TTHA-cs124-EMPH or TTHA-cs124-Cys-MTS in good yield. 1-Oxa-4,7-diazacyclononane (N_2O) and 1,7-diaza-12-crown-4 (N_2O_2) were reacted with 2 equiv of *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine. The resulting tetra-*tert*-butylcarboxylates were then hydrolyzed with HCl in methanol to yield the free acid form of N_2O-tetracarboxylic acid and N_2O_2-tetracarboxylic acid. The two tetracarboxylic acid compounds could react with either acetic anhydride or isobutyl chloroformate to form dianhydride intermediates. These dianhydride intermediates can be either isolated or used for the next step without isolation. Following the sequence by reacting with cs124 first and then thiol-reactive reagents, the lanthanide chelates with N_2O and N_2O_2 in the middle of their backbones were synthesized in satisfy yield.

All of the lanthanide chelates were purified by reverse-phase HPLC and identified by mass spectroscopy, UV−vis, and lanthanide luminescence properties.

Photophysical Properties of the Lanthanide Complexes with the New Chelates. The Tb^{3+} and Eu^{3+} emission spectra are shown in Figure 1. All of the Tb^{3+} and Eu^{3+} complexes exhibit their characteristic sharply spiked asymmetric emission spectra with their most intense luminescent transitions centered at 546 nm ($^5D_4 \rightarrow {}^7F_5$) for Tb^{3+} and 614 nm ($^5D_0 \rightarrow {}^7F_2$). Table 2 lists the excited-state lifetimes of Tb^{3+} and Eu^{3+} measured for their complexes with the new chelates. Table 3 lists the excited-state lifetimes of Tb^{3+} and Eu^{3+} measured for their complexes with the new thiol-reactive chelates labeled to proteins (Ubiquitin, and *Xenopus* oocytes) or glutathione.

Chart 2

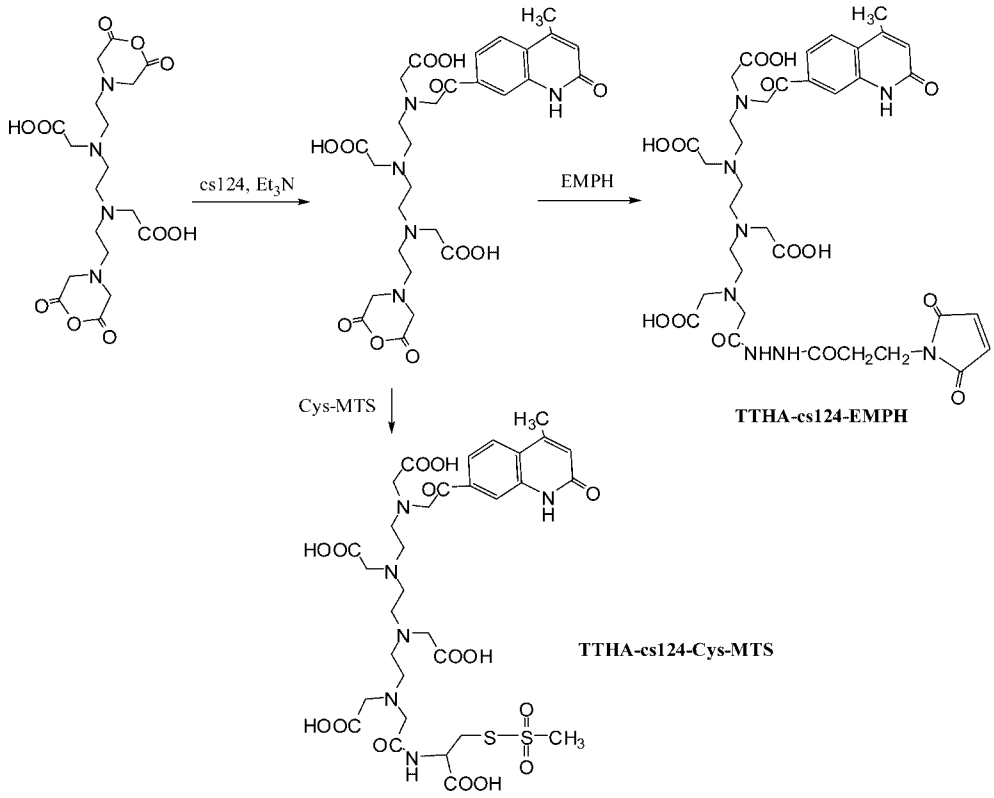


Table 1. Synthesized New Lanthanide Chelates

thiol-reactive chelates	linear gradients	retention time (min)	yield (%)	MS M − 1, ESI⁻
N_2O-cs124	20−40% ACN/TEAA	12	35	603
N_2O_2-cs124	20−40% ACN/TEAA	13	50	647
N_2O-cs124-EMPH	20−40% ACN/TEAA	19	20	768
N_2O-cs124-Cys-MTS	20−40% ACN/TEAA	17	30	784
TTHA-cs124-EMPH	30−50% MeOH/TEAA	22	20	814
TTHA-cs124-Cys-MTS	30−50% MeOH/TEAA	16	30	830

Generally, the Tb^{3+} and Eu^{3+} complexes of these chelates have comparable emission brightness to that of the benchmark chelate, DTPA-cs124. For all (except N_2O_2-cs124) of the non-thiol-reactive chelates, their lanthanide emission intensities are at least 10% higher than that of the reference complex. One apparent reason behind the results is that these chelates are either 9- or 10-dentate chelates. DTPA-cs124 is an 8-dentate chelate. When it coordinates to lanthanide ions, a water molecule is also coordinated to the lanthanide ions in the inner coordination sphere (*20*). The hydration of lanthanide ions is the main source of quenching of lanthanide emission. In contrast, the newly synthesized 9- or 10-dentate chelates can provide enough coordination sites for lanthanide ions that few water molecules can compete to coordinate to lanthanide ions. Therefore, the quenching effect from hydration of lanthanide ions is eliminated. This assumption that little or no water molecules in the lanthanide inner coordination spheres of these new chelate complexes is evident from their corresponding lifetime measurements, as all of these chelates exhibit significantly longer lifetimes than that of DTPA-cs124. Using the Horrocks and Sudnick empirical equation, the number of waters in the primary coordination sphere of Tb^{3+} and Eu^{3+} is found as follows: N_2O-cs124 (0.02, 0.29), N_2O_2-cs124 (0.22, Tb^{3+} only), TTHA-cs124 (0.2, 0.3) (*21*). These data clearly indicate that the new chelates provide better protection to the lanthanide ions from solvent solvation, and limit the accessibility of water molecules to the metal center. Especially for N_2O-cs124, no water molecules coordinate when it binds to Tb^{3+}. Therefore, the new 9- or 10-dentate chelates can shield the lanthanide ions from solvent attack. However, other factors will also contribute to the relative intensity changes. For N_2O_2-cs124, its Tb^{3+} emission intensity is only about 20% that of DTPA-cs124, while its Eu^{3+} emission is undetectable at 1 μM concentration. Yet, its lifetime for Tb^{3+} is significantly longer than that of the reference complex. The calculated number of water molecules coordinated to Tb^{3+} is only 0.22. The facts are that its emission intensity is concentration-dependent and its lifetime is concentration-independent. These observations indicate that the binding stability constant

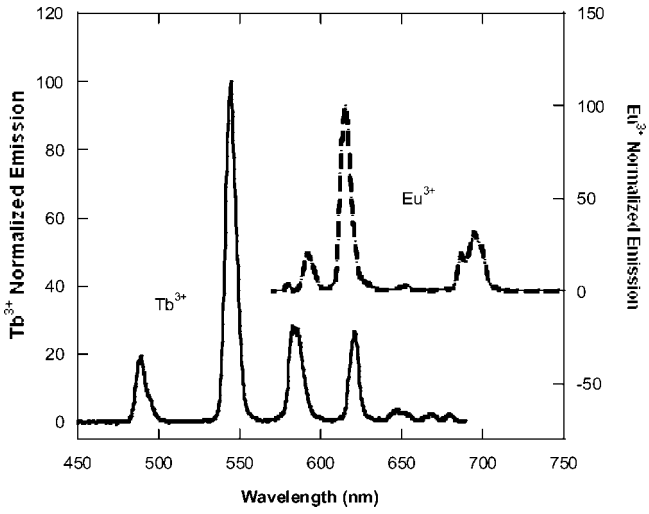


Figure 1. Normalized Tb^{3+} and Eu^{3+} emission spectra of N_2O-cs124 in 0.1 M TEAA pH 6.5 at a concentration of 1 μM. Tb^{3+} and Eu^{3+} display their characteristic sharply spiked spectra. Pulsed excitation was at 40 Hz, 3.5 μJ/pulse for Eu^{3+}, 3.0 μJ/pulse for Tb^{3+}; integration times are 1 s for $Tb^{3+}$and 5 s for Eu^{3+}.

Table 2. Photophysics Data of the New 9- And 10-Dentate Chelates[a]

metal	chelates	τH_2O	τD_2O	$\tau H_2O/\tau D_2O$	no of waters	relative brightness
Tb^{3+}	DTPA-cs124	1.55	2.63	0.59	1.1 (*21*)	1
	N_2O-cs124	1.89	1.91	0.99	0.02	1.3
	N_2O_2-cs124	2.50	2.88	0.87	0.22	0.2
	N_2O-cs124-EMPH	1.93 (81%) 0.72 (19%)				0.4
	N_2O-cs124-Cys-MTS	1.97 (82%) 1.21 (18%)				0.2
	TTHA-cs124	2.10	2.37	0.89	0.2	1.1 (*21*)
	TTHA-cs124	2.10	2.31	0.91	0.19	0.31[b]
	TTHA-cs124-8-me	2.27				0.33
	TTHA-cs124-EMPH	1.97 (31%) 0.81 (69%)				0.73
	TTHA-cs124-Cys-MTS	2.07 (93%) 0.24 (7%)				1.3
Eu^{3+}	DTPA-cs124	0.62	2.42	0.26	1.26 (*21*)	1
	N_2O-cs124	1.0	1.39	0.72	0.29	1.22
	N_2O_2-cs124					N/A[c]
	N_2O-cs124-EMPH	1.03 (41%) 0.60 (22%) 0.04 (37%)				0.3
	TTHA-cs124	1.19	1.79	0.66	0.3 (*21*)	2.7
	TTHA-cs124	1.16				1.45[b]
	TTHA-cs124-8-me	1.15				2.37
	TTHA-cs124-EMPH	1.25				0.89
	TTHA-cs124-Cys-MTS	1.20				0.69

[a] All emissions are measured at 1 μM lanthanide concentration with a slight excess of chelate. [b] From this work. [c] Concentration-dependent. At 1 μM, no emission detected.

Table 3. Lifetimes of Tb^{3+} and Eu^{3+} Complexes of Thiol-Reactive 9-, 10-Dentate Chelates Labeled to Proteins or Glutathione

metal	chelate	protein	lifetimes (ms)
Tb^{3+}	N_2O-cs124-EMPH	Shaker K^+ channels in oocyte cells[a]	1.34 (75%); 0.22 (25%)
		Ubiquitin	1.74 (82%); 0.29 (18%)
	TTHA-cs124-EMPH	Shaker K^+ channels in oocyte cells[a]	1.84 (86%); 0.32 (14%)
		Ubiquitin	1.94 (74%); 0.49 (26%)
		Glutathione	1.89
	TTHA-cs124-Cys-MTS	Ubiquitin	1.92 (63%); 0.33 (37%)
Eu^{3+}	TTHA-cs124-EMPH	Shaker K^+ channels in oocyte cells[a]	0.69 (43%); 0.21 (57%)
		Glutathione	1.04

[a] Details of ion channel expression can be found in [Posson (2005) *Nature 436*, 848−851].

of N_2O_2-cs124 to lanthanide ions is relatively small. There is an equilibrium between lanthanide ion bound and unbound N_2O_2-cs124 in solution. Such results were also observed for TETA-cs124 (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid, TETA) (*21*). After converting to the thiol-reactive form, there is a tendency for the emission intensity to decrease.

The 8-methyl cs124 derivative (cs124-8-me) sensitized TTHA chelate (TTHA-cs124-8-me) was also synthesized. It has a slightly increased lifetime compared to TTHA-cs124 (2.27 ms vs 2.14 ms). This is consistent with the observation of blocked back energy transfer from lanthanide emission to antenna molecule by the 8-methyl group observed for DTPA chelate (*5*). Its Tb^{3+} emission intensity is only about 30% that of the corresponding reference complex, as is the TTHA-cs124 chelate which was made in this work. The hypothesis for this intensity decrease is that Tb^{3+} remains 9-coordinated in TTHA-cs124-8-me and TTHA-cs124. The TTHA chelates provide 10-coordinate sites. The terminal carboxylate arm with sensitizer has to compete with other free carboxylic acid arms to coordinate to the lanthanide center, resulting in an equilibrium between bound and nonbound forms of the carboxylate arms coordinating to the metal center. The case where the cs124 is not bound to the lanthanide probably will not sensitize the emission of terbium because the antenna molecule is far away from metal center. On the basis of the fact that the intensity of TTHA chelates is only about 30% that of the reference complex, it is reasonable to assume that the equilibrium is in favor of the nonbound form, probably due to the spatial hindrance and overall charges of the antenna molecule. At ~pH 7, the side arms in TTHA are deprotonated as carboxylates. When neutral antenna molecule (cs124 or cs124-8-me) attached to form an amide bond, the −1 charge from the carboxylate sidearm will be gone. Hence, the coordination capability of the carbonyl oxygen atom from the amide bond will change. However, we reported that the intensity of TTHA-cs124 is 110% that of the reference complex in our previous work (*21*). We believe that this is because the structures of the two TTHA-cs124 chelates are slightly different. In the present work, we converted TTHA into its dianhydride form first, and then reacted TTHA dianhydride with cs124 and derivatives. By doing this, the antenna molecule is exclusively at the terminals of the TTHA backbone chain. In our previous work, the free acid form of TTHA was activated by reacting with isobutyl chloroformate first, followed by reacting with cs124 in a one-pot reaction. All of the carboxylic acid groups in TTHA could be activated, and hence a mixture is formed with the antenna molecule in both the middle and terminals of the TTHA backbone chain. If the antenna molecule is in the middle of the TTHA backbone chain, it probably will coordinate to the lanthanide center, because after the TTHA backbone chain coordinates to lanthanide, it will bring the carboxylate arm with the antenna molecule very close to the metal center. In contrast, the carboxylate arm with the antenna molecule at the terminals of TTHA backbone chain will have greater freedom to move around.

The Eu^{3+} emission intensity of TTHA-cs124 chelates is significantly increased compared the reference complex. The Eu-TTHA-cs124-8-me emission intensity is 237% that of reference, and the Eu-TTHA-cs124 emission intensity is 145% that of reference. The increased emission intensity is a good indication that the coordination number of Eu^{3+} has increased to 10. It is demonstrated that the $^5D_0 \rightarrow {}^7F_2$ emission intensity increase of Eu^{3+} is linearly proportional to the average coordination number of nitrates to Eu^{3+}, regardless of solvents (*20*). In a 0.047 M Eu^{3+} DMF solution, the intensity increases 1.8× per coordination number increase. Although different coordination chelates were used, the further intensity increase

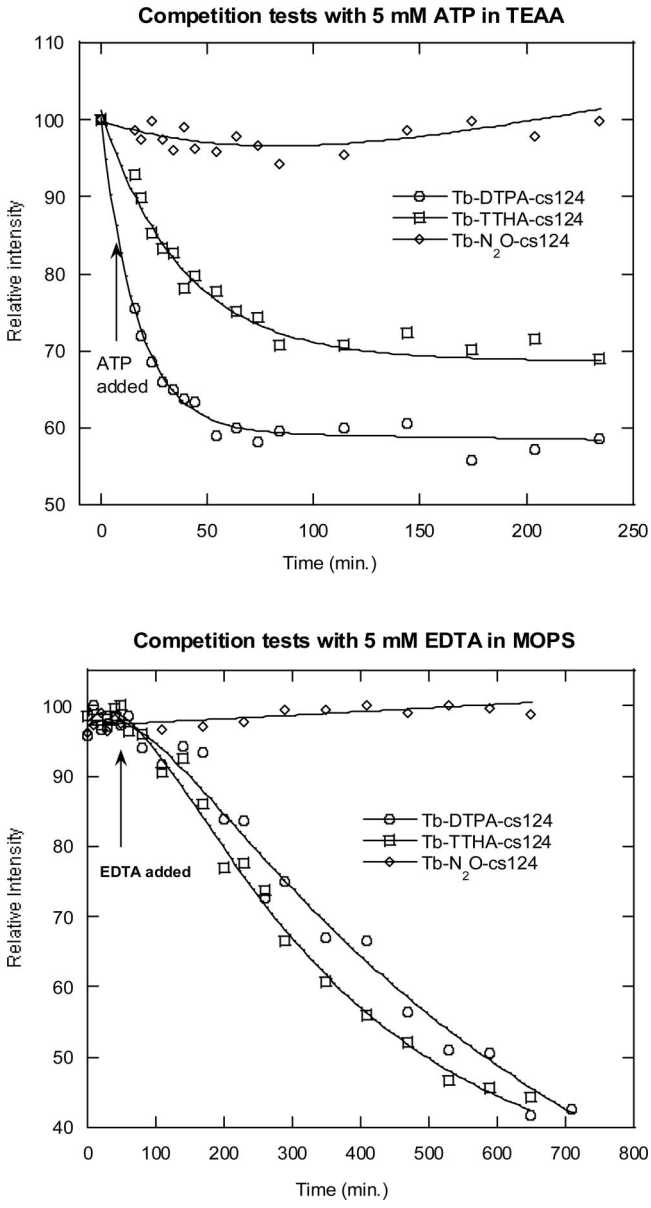


Figure 2. Relative Tb^{3+} emission intensity over time in the presence of EDTA or ATP of chelates of DTPA-cs124, TTHA-cs124, and N_2O-cs124.

of TTHA-cs124-8-me from TTHA-cs124 is probably due to the blocking of energy back transfer effect by the 8-methyl substitute.

To compare the stability of the lanthanide complexes of the new chelates, the Tb^{3+} emission intensity was measured over time in the presence of ATP or EDTA for the nonreactive chelates of DTPA-cs124, TTHA-cs124, and N_2O-cs124 (Figure 2). In the presence of 5 mM of EDTA, the Tb^{3+} emission intensity for both of TTHA-cs124 and DTPA-cs124 decreases to ~50% of the initial intensity in ~8−9 h. In the presence of 5 mM of ATP, the Tb^{3+} emission intensity of DTPA-cs124 decreases to ~60% of its initial intensity in ~50 min after addition of ATP. After this period of time, no further significant intensity decrease was observed, suggesting that equilibrium has been achieved. The Tb^{3+} emission intensity of TTHA-cs124 decreases to ~70% of its initial intensity in ~70 min after addition of ATP, indicating that equilibrium is also achieved at this point. In both cases, the Tb^{3+} emission intensity of N_2O-cs124 virtually does not change. These results suggest that the macrocycle-containing lanthanide chelate, N_2O-cs124, is a much stronger lanthanide binding chelate than the linear DTPA-cs124 and TTHA-cs124 chelates, and its lanthanide complexes are much more stable under physiological conditions.

The thiol-reactive chelates are selectively reacted with Ubiquitin, Shaker potassium ion channel expressed in oocyte, and glutathione. Generally, after labeling to proteins, the luminescent lanthanide emission exhibits a biexponential lifetime decay fit with the majority a long-lifetime component (usually >70%) and the rest a short-lifetime component (<30%). It is interesting to note that, after reacting with glutathione, the originally biexponential thiol-reactive chelates become single-exponential.

CONCLUSIONS

As part of the project to modify the luminescent lanthanide chelate backbone structures to provide stronger and better hydration protection chelates, new 9- and 10-dentate lanthanide chelates have been synthesized. The syntheses of these chelates are straightforward, relatively simple, and in good yields, especially for TTHA-based chelates. The principle for the synthesis is to prepare a backbone precursor similar to DTPA dianhydride. The dianhydride functional groups allow an antenna molecule and a reactive group to attach to the backbone molecule separately. The new chelates provide better protection to lanthanide ions from solvent hydration, as evidenced from their longer lifetimes and relatively higher emission intensity. The 1-oxa-4,7-diazacyclononane (N_2O)-containing chelates provide the best protection to the lanthanide ions from solvent molecule attack. Its Tb^{3+} complex is the most stable complex and does not undergo dissociation in the presence of other competing chelates at relatively high concentration. The TTHA-based chelates provide moderately good protection to the lanthanide ions.

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LITERATURE CITED

(1) Selvin, P. R. (2002) Principles and biophysical applications of luminescent lanthanide probes. *Annu. Rev. Biophys. Biomol. Struct. 31*, 275–302.
(2) Mathis, G. (1993) Rare earth cryptates and homogeneous fluoroimmunoassays with human sera. *Clin. Chem. 39*, 1953–1959.
(3) Xiao, M., and Selvin, P. R. (2001) Quantum yields of luminescent lanthanide chelates and far-red dyes measured by resonance energy transfer. *J. Am. Chem. Soc. 123*, 7067–7073.
(4) Reifenberger, J., Snyder, G. E., Baym, G. A., and Selvin, P. R. (2003) Emission polarization properties of europium and terbium chelates. *J. Phys. Chem B 107*, 12862–12873.
(5) Reifenberger, J. G., Ge, P. and Selvin, P. R. (2005) *Progress in Lanthanides as Luminescent Probes*, Springer, Berlin.
(6) Selvin, P. R., and Hearst, J. E. (1994) Luminescence energy transfer using a terbium chelate: Improvements on fluorescence energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 91*, 10024–10028.
(7) Selvin, P. R., Rana, T. M., and Hearst, J. E. (1994) Luminescence resonance energy transfer. *J. Am. Chem. Soc. 116*, 6029–6030.
(8) Xiao, M., Reifenberger, J. G., Wells, A. L., Baldacchino, C., Chen, L. Q., Ge, P., Sweeney, H. L., and Selvin, P. R. (2003) An actin-dependent conformational change in myosin. *Nat. Struct. Biol. 10*, 402–8.

(9) Xiao, M., Li, H., Snyder, G. E., Cooke, R., G. Yount, R., and Selvin, P. R. (1998) Conformational changes between the active-site and regulatory light chain of myosin as determined by luminescence resonance energy transfer: The effect of nucleotides and actin. *Proc. Natl. Acad. Sci. U.S.A. 95*, 15309–15314.

(10) Cha, A., Snyder, G. E., Selvin, P. R., and Bezanilla, F. (1999) Atomic scale movement of the voltage sensing region in a potassium channel measured via spectroscopy. *Nature 402*, 809–813.

(11) Posson, D. J., Ge, P., Miller, C., Bezanilla, F., and Selvin, P. R. (2005) Small vertical movement of a K^+ channel voltage-sensor measured with luminescence energy transfer. *Nature 436*, 848–851.

(12) Richardson, J., Blunck, R., Ge, P., Selvin, P. R., Bezanilla, F., Papazian, D., and Correa, A. M. (2006) Common topology of prokaryotic voltage-gated ion channels in the lipid bilayer. *Proc. Natl. Acad. Sci. U.S.A. 103*, 15865–15870.

(13) Heyduk, E., Heyduk, T., Claus, P., and Wisniewski, J. R. (1997) Conformational changes of DNA induced by binding of chironomus high mobility group protein 1a (cHMG1a). *J. Biol. Chem. 272*, 19763–19770.

(14) Heyduk, E., and Heyduk, T. (1999) Architecture of a complex between the sigma70 subunit of Escherichia coli RNA polymerase and the nontemplate strand oligonucleotide. Luminescence resonance energy transfer study. *J. Biol. Chem. 274*, 3315–22.

(15) Callaci, S., Heyduk, E., and Heyduk, T. (1999) Core RNA polymerase from *E. coli* induces a major change in the domain arrangement of the sigma 70 subunit. *Mol. Cell 3*, 229–238.

(16) Xu, J., and Root, D. D. (1998) Domain motion between the regulatory light chain and the nucleotide site in skeletal myosin. *J. Struct. Biol. 123*, 150–61.

(17) Root, D. D. (1997) *In situ* molecular association of dystrophin with Actin revealed by sensitized emission immuno-resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 94*, 5685–5690.

(18) Vazquez-Ibar, J. L., Weinglass, A. B., and Kaback, H. R. (2002) Engineering a terbium-binding site into an integral membrane protein for luminescence energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 99*, 3487–3492.

(19) Mathis, G. (1995) Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin. Chem. 41*, 1391–1397.

(20) Bunzli, J.-C. G. (1989) Luminescent Probes, in *Lanthanide Probes in Life, Chemical and Earth Sciences, Theory and Practice* (Bunzli, J.-C. G., and Choppin, G. R., Eds.) pp 219−293, Elsevier, New York.

(21) Li, M., and Selvin, P. R. (1995) Luminescent lanthanide polyaminocarboxylate chelates: the effect of chelate structure. *J. Am. Chem. Soc. 117*, 8132–8138.

(22) Pedersen, C. J. (1967) Cyclic polyethers and their complexes with metal salts. *J. Am. Chem. Soc. 89*, 7017.

(23) Gorden, A. E. V., Xu, J., and Raymond, K. N. (2003) Rational design of sequestering agents for plutonium and other actinides. *Chem. Rev. 103*, 4207–4282.

(24) Zitha-Bovens, E., Muller, R. N., Laurent, S., Elst, L. V., Geraldes, C. F. G. C., van Bekkum, H., and Peters, J. A. (2005) Structure and dynamics of lanthanide complexes of triethylenetetramine-N,N,N′,N″,N‴,N‴-hexaacetic acid (H6ttha) and of diamides H4ttha(NHR) derived from H6ttha as studied by NMR, NMRD, and EPR. *Helv. Chim. Acta 88*, 618–632.

(25) Qian, L., Sun, Z., and Bowman-James, K. (1996) Synthesis of polyaza macrocyclic ligands. *Supermol. Chem. 6*, 313–325.

(26) Williams, M. A., and Rapoport, H. (1993) Synthesis of enantiomerically pure diethylenetriaminepentaacetic acid analogues. L-Phenylalanine as the educt for substitution at the central acetic acid. *J. Org. Chem. 58*, 1151–1158.

(27) Xiao, M., and Selvin, P. R. (1999) An improved instrument for measuring time-resolved lanthanide emission and resonance energy transfer. *Rev. Sci. Instrum. 70*, 3877–3881.

(28) Horrocks, W. D., Jr., and Sudnick, D. R. (1979) Lanthanide ion probes of structure in biology. Laser-induced luminescence decay constants provide a direct measure of the number of metal-coordinated water molecules. *J. Am. Chem. Soc. 101*, 334–350.

(29) Burmeister-Getz, E., Cooke, R., and Selvin, P. R. (1998) Luminescence resonance energy transfer measurements in myosin. *Biophys. J. 75*, 2451–2458.

(30) Chen, J., and Selvin, P. R. (1999) Thiol-reactive luminescent lanthanide chelates. *Bioconjugate Chem. 10*, 311–315.

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