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Luminescent Polyaminocarboxylate Chelates of Terbium and Europium: The Effect of Chelate Structure

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Abstract: We have synthesized and spectrally characterized a series of new luminescent lanthanide complexes based on linear and macrocycle polyaminocarboxylate chelates, covalently joined to an organic sensitizer 7-amino-4-methyl-2(1*H*)-quinolinone (carbostyril 124). These complexes are luminescent with both terbium and europium and have millisecond lifetime, sharply-spiked emission spectra (<10 nm fwhm), large Stokes shifts (>150 nm), excellent solubility, moderate absorption (12 000 M^{-1} cm^{-1} at 327 nm), and high quantum yields for lanthanide emission. These characteristics make them useful alternatives to radioactive probes, to fluorescent dyes, and as donors in energy transfer experiments. A comparison of luminescence, intensity, spectra, lifetime, and number of coordinated waters is made. Net charge varies from −2 to neutral and water coordination number from 0.2 to 1.2. By comparison of lanthanide emission lifetimes with lanthanide chelates without sensitizer, it is shown that the sensitizer does not lead to nonradiative de-excitation and that the quantum yield for lanthanide emission is likely close to unity. Emission of terbium in all chelates is similar. With europium, the macrocycle chelates have enhanced far-red emission (700 nm) and one linear polyaminocarboxylate chelate, triethylenetetraaminehexaacetic acid, has 60% of its emission with europium in a sharply-spiked band around 617 nm with a full width at half-maximum of 3.3 nm. These chelates are the most efficient energy transfer donors yet synthesized. In imaging, they may make possible two-color detection with no spectral overlap and a single excitation wavelength, as well as the ability to discriminate against short-lived autofluorescence background.

Introduction

Luminescent lanthanide (terbium and europium) chelates have many useful applications, including replacements for radioactivity, alternatives to standard fluorescent dyes especially when there is significant autofluorescence, and donors in energy transfer experiments to measure both static and time-varying distances. These applications arise because of the chelates' unusual spectral characteristics, including millisecond lifetime, spiked emission (<10 nm full width at half-maximum), large Stokes shifts (>150 nm), potentially high quantum yields (~1), and excellent solubility. Lanthanide chelates have been used as replacements for radioactive detection,[1,2] and detection limits of 2×10^{-15} M in ethanolic solutions[3] and $\leq 10^{-12}$ M in aqueous solutions[4-6] have been reported. Detection limits of biological macromolecules can be considerably lower because several hundred chelates per macromolecule can be attached with negligible self-quenching and minimal loss of biological specificity.[6-8] As alternatives to fluorescent dyes, Seveus et al. have recently shown a 400-fold contrast improvement in fluorescence imaging of cells by replacing a standard label such as fluorescein, with a europium chelate, and time-resolving to discriminate against short-lived autofluorescence.[5,9] Marriott et al. have shown similar results.[10,11] Furthermore, by using chelates which are luminescent with both terbium and europium, two-color imaging with *no* spectral overlap may be possible. Lanthanide chelates have also been shown to be excellent donors in resonance energy transfer experiments.[12-15] Their spiked spectrum and long-lifetime enable background to be eliminated, and their high quantum yield for lanthanide luminescence yields efficient energy transfer to acceptor molecules: using lanthanides, the characteristic distance at which energy transfer is 50% can exceed 70 Å.

Significant effort has already been expended in designing lanthanide complexes. All complexes contain a chelate which binds the lanthanide and an organic chromophore which absorbs light and transfers energy to the lanthanide.[4,16] The chromophore is necessary to overcome the inherently low absorbance of terbium[17] and europium.[18] Two general synthetic approaches

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have been used: one approach, exemplified by the cryptates,[14] incorporates the chromophore into the structure of the chelate; the other approach, exemplified by a number of polyaminocarboxylate-based chelates,[19-21] contains distinct chelate and chromophore. The latter approach has the possible advantage of optimizing the chelate structure and the sensitizer separately, and the polyaminocarboxylates have excellent solubility and lanthanide-binding properties. Diethylenetriaminepentaacetic acid (DTPA) has been the most widely used polyaminocarboxylate chelate because of its high binding constant for lanthanides,[22] its availability as a dianhydride, its excellent solubility, its relatively high coordination number of 8, and its vibrational spectrum which does not couple away energy from the excited luminescent state of the lanthanide.[23,24] However, one water remains bound to the lanthanide, which quenches luminescence; the emission spectrum with europium is fairly dispersed, and therefore suboptimal for several applications, and greater (or less) net negative charge than DTPA can be advantageous for particular applications.

In this paper we have examined the effect of chelate structure using two linear and two macrocycle polyaminocarboxylate chelates coupled to a 7-amino-4-methyl-2(1*H*)-quinolinone (carbostyril 124) chromophore. The carbostyril moiety has the important attribute that it can sensitize both europium and terbium. The chelates vary in their ability to fill the lanthanide coordination sphere, in net charge, in brightness, and in emission spectra. These chelates are the most efficient energy transfer donors yet synthesized. In imaging applications, they may make possible two-color detection with no spectral overlap and a single excitation wavelength, as well as the ability to discriminate against short-lived autofluorescence background.

Experimental Methods

Chemicals. The following were purchased from Aldrich: diethylenetriaminepentaacetic acid (DTPA) and its dianhydride derivative (caDTPA); triethylenetetraaminehexaacetic acid (TTHA); 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA); 7-amino-4-methyl-2(1*H*)-quinolinone (also called carbostyril 124 or cs124); isobutyl chloroformate; *N,N*-dimethylformamide (anhydrous); triethylamine; europium chloride hexahydrate (99.99%); terbium chloride hexahydrate (99.999%). The sodium salt of 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid (DOTA) was bought from Parish Chemical Co. Distilled and deionized water (18 MΩ cm^{-1}) was used throughout. D_2O (99.9% and 99.96%) was purchased from Cambridge Isotope Laboratory. All glassware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water.[25] All plastic labware was purchased from Bio-Rad (metal-free). All chemicals were the purest grade available.

Synthesis. DTPA−cs124. To a solution of caDTPA (25 mg, 0.069 mmol), 1.5 mL of DMF, and 100 μL of triethylamine was added dropwise cs124 (12 mg, 0.069 mmol) in 200 μL of DMF. The reaction mixture was quenched after 2 h by 5 mL of 1 M triethylammonium acetate (pH 6.5). Product DTPA−cs124 was purified by HPLC. Yield: 65%, estimated by HPLC profile. FAB-MS: *m/e* = 550 ($M + H^+$). ^1H NMR (D_2O): δ 2.70 (3H, s), 3.50 (2H, t), 3.57 (2H, t), 3.63 (2H, t), 3.75 (2H, t), 3.92 (2H, s), 4.02 (2H, s), 4.11 (4H, s), 4.30 (2H, s), 6.70 (1H, s), 7.52 (1H, d), 7.92 (1H, s), 8.01 (1H, d).

TTHA−cs124. TTHA (50 mg, 0.10 mmol) was dissolved in 4 mL of DMF and triethylamine mixture (3:1 v/v) overnight at room temperature. Isobutyl chloroformate (15 μL, 1.1 × 0.10 mmol) in 100 μL of DMF was added dropwise at 0 °C to the mixture. After 15 min, cs124 (17.4 mg, 1.0 × 0.10 mmol) was added, and the reaction was kept at room temperature for 5 h. The reaction mixture was purified by reversed phase HPLC. Yield: 25%, estimated by HPLC profile. FAB-MS: *m/e* = 651 ($M + H^+$).

DOTA−cs124. DOTA sodium salt (15 mg, 0.032 mmol) was dissolved in 5 mL of DMF and triethylamine mixture (3:2 v/v) overnight at room temperature. Isobutyl chloroformate (4.7 μL, 1.1 × 0.032 mmol) in 100 μL of DMF was added dropwise at 0 °C to the mixture. After 15 min, cs124 (6.1 mg, 1.1 × 0.032 mmol) was added, and the reaction was kept at room temperature for 5 h. The reaction mixture was purified by reversed phase HPLC. Yield: 50%, estimated by HPLC profile. FAB-MS: *m/e* = 561 ($M + H^+$).

TETA−cs124. TETA (15 mg, 0.023 mmol) was stirred in 5 mL of DMF and triethylamine mixture (3:2 v/v) overnight at room temperature to give a suspension solution. To this solution was added dropwise isobutyl chloroformate (4.7 μL, 1.5 × 0.023 mmol) in 100 μL of DMF at 0 °C. After 15 min, cs124 (4.0 mg, 1.1 × 0.023 mmol) was added, and the reaction was kept at room temperature for 5 h. The reaction mixture was purified by reversed phase HPLC. Yield: 30%, estimated by HPLC profile. FAB-MS: *m/e* = 589 ($M + H^+$).

Purification. High-performance liquid chromatography was performed at room temperature on a Beckman Model 100 system with a Dynamax 60 Å C_{18} column (10 ID × 250 mm, Rainin). A 30-min linear gradient, from 15% to 60% solvent B (solvent A, 0.1 M triethylammonium acetate (pH 6.5); solvent B, acetonitrile) was used.

Metal Labeling. DTPA−cs124 or TTHA−cs124 at greater than 1 μM was added in 1:1 molar ratio with a solution of $TbCl_3$ or $EuCl_3$ at pH 5−8 (triethylammonium acetate (pH 5), sodium bicarbonate (pH 7.0), or Tris (pH 8.0)). For these linear chelates, the binding was insensitive to pH and was complete within < 30 min. Lanthanide was also bound by adding a slight excess of metal, and excess metal was removed by passing over C-18 Sep-pak cartridge (Waters), eluting with 50/50 MeOH/H_2O, drying, and redissolving in appropriate buffer solution. The results were equivalent.

DOTA−cs124 was bound to terbium or europium using the prelabeling method.[26] Chelate (5 mM) was mixed with equivalent moles of a solution of $TbCl_3$ or $EuCl_3$ in 0.1 M triethylammonium acetate (pH 5) overnight. DTPA (10 mM) in 0.1 M triethylammonium acetate (pH 5) was added to chase any free metal. The mixture was then loaded on a prespin DE-52 anion-exchange column. The eluted fraction contained only the metal complex.

TETA−cs124 was bound to terbium using the prelabeling method at 25 mM chelate with 5× excess terbium in 0.1 M triethylammonium acetate (pH 5) overnight. (pH 7 was also used, with slightly less efficient binding.) The mixture was then loaded on a prespin DE-52 anion-exchange column. The resulting fraction contained metal-bound chelate and free metal. TETA−cs124 at 1.3 mM was also bound to terbium simply by mixing a 1:1 molar ratio of $TbCl_3$ and TETA−cs124 at 0.1 M triethylammonium acetate (pH 5) overnight. The two methods gave the same lifetime results. Attempted binding of europium to TETA−cs124 under the same conditions failed to give any luminescence.

Spectroscopy. All lifetime and wavelength emission spectra were recorded on laboratory-built spectrometer which will be described in detail elsewhere. Briefly, a solution of the lanthanide chelate was placed in a 3 × 3 mm quartz cuvette and excited with a pulsed nitrogen laser (337 nm, 5 ns pulse-width, 40 Hz repetition rate; Laser Photonics) and emission passed through a double-monochrometer (SPEX 1680B, blaze = 500 nm, *f*/4) and was detected by a gallium−arsenide photomultiplier tube operating in photon-counting mode (Hamamatsu R943-02) with associated electronics (MITEQ 500 MHz preamplifier; Ortec 854 gated discriminator; Canberra FMS multichannel scalar with 2 μs time resolution). Measurements were made in D_2O or H_2O with Tris (pH 8.0) used as buffer. When using D_2O, a concentrated solution was dried and resuspended in D_2O at least twice. The cuvette was also washed twice with D_2O. For the europium measurements, the results were confirmed using freshly opened 99.96% D_2O from ampules. For DTPA−cs124, luminescence was also measured from pH 7−9 and no

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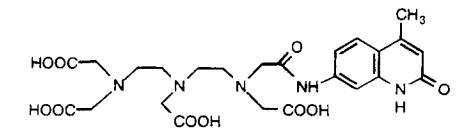
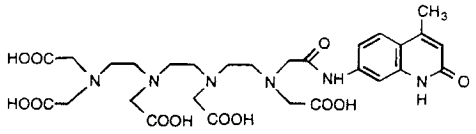


DTPA-cs124



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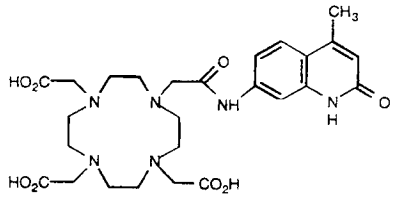
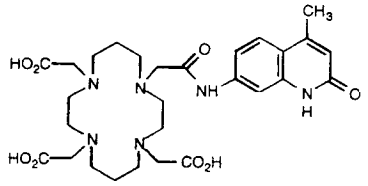


DOTA-cs124



TETA-cs124

Figure 1. Structure of polyaminocarboxylate chelates covalently coupled to carbostyril 124. See text for abbreviations. The position of carbostyril on TTHA is not determined.

difference in lanthanide intensity or lifetime was found. Corrected excitation spectra were taken on a Perkin Elmer LS-5B.

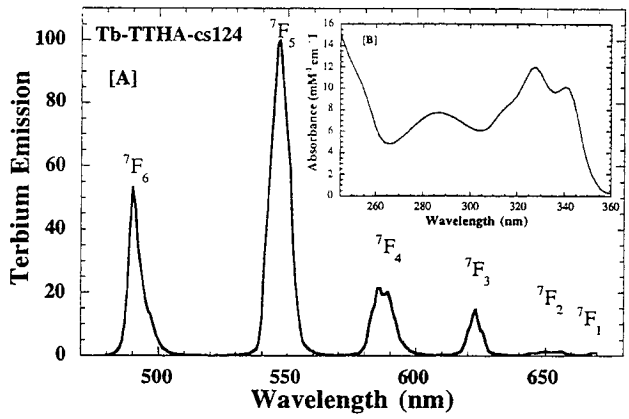
Determination of Extinction Coefficient. Extinction coefficient for cs124 bound to chelate (DTPA) was determined by measuring the absorbance of a solution of DTPA−cs124 and determining its concentration by titrating in a known concentration of terbium chloride and measuring the increase in terbium emission. Emission linearly increased and then reached a plateau.

Results and Discussion

Proof of Structure. Figure 1 shows the structural formulas of the four chelates coupled to carbostyril 124. In addition, DTPA−$(cs124)_2$ was a byproduct of the reaction of the dianhydride of DTPA with carbostyril and was purified. The position of the cs124 in the TTHA complex is not determined since the relative reactivities of the carboxylate groups when combined with isobutyl chloroformate are not known.

Several lines of evidence confirm the synthesis and purity of the compounds shown in Figure 1. Single well-resolved HPLC peaks were found for the products, with starting materials running at different retention rates; mass spectroscopy confirmed the expected mass; products were highly luminescent with terbium and europium, whereas starting materials mixed with lanthanides showed no significant lanthanide luminescence; absorption spectra showed characteristic spectra of carbostyril with the 7-amide (see Figure 2A, inset).

Terbium Chelates. The emission spectrum, absorption spectrum, and excited state lifetime of Tb−TTHA−cs124 in



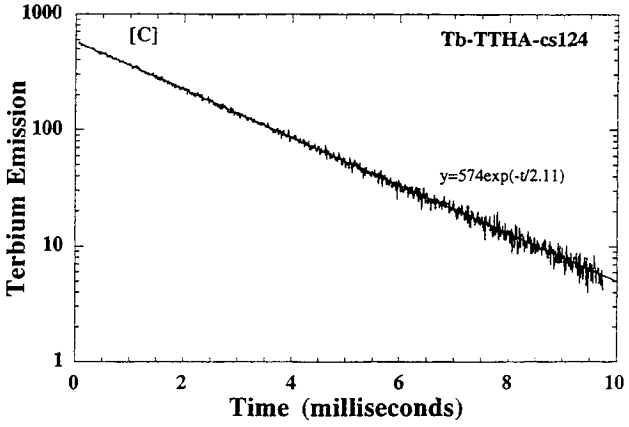


Figure 2. (A) Emission spectrum of Tb−TTHA−cs124. Emission arises from $^5D_4$ excited state to various ^7F_j manifolds. Data collected every 1 nm with a band pass of 1 nm, integrating for 2 s/point, which corresponds to 80 excitation pulses. For each pulse, the signal was collected with a 4.7 ms gate beginning 76 μs after the excitation pulse. Spectral shapes and relative peak heights in D_2O and for other terbium chelates are essentially identical. (B) The inset shows the absorption spectrum of the chelate, which is due to the carbostyril. The excitation spectrum is identical to the absorption spectrum. (C) Lifetimes at 546 nm of Tb−TTHA−cs124. Data collected every 2 μs and digitally binned to 10 μs/point and fit to a single-exponential function (solid line) which showed no residual structure with $r^2 = 0.999$. Lifetimes at all terbium emission wavelengths (see a) were within 5%. Lifetime data for all chelates were of similar quality.

Table 1. Terbium Lifetimes and the Number of Waters Coordinated in the Inner Sphere of Lanthanide and Relative Brightness in H_2O of Various Chelates[a]

Tb complex	τ_{H_2O} (ms)	τ_{D_2O} (ms)	τ_{H_2O}/τ_{D_2O}	no. of waters	relative brightness
DTPA−cs124	1.55	2.63	0.59	1.1	1
TTHA−cs124	2.10	2.37	0.89	0.2	1.1
DOTA−cs124	1.54	2.61	0.59	1.1	1.1
TETA−cs124	2.05	2.30	0.89	0.22	concentration dependent

[a] The relative brightness is the total emission intensity of all wavelengths for the terbium chelates in H_2O-based buffer; total intensity of Tb−DTPA−cs124 is defined to be 1.

H_2O are shown in Figure 2. The emission spectra and relative peak heights of terbium emission with all chelates were essentially identical, although the total intensity and the excited-state lifetime was chelate-dependent (see Table 1). As expected, all emissions arise from the $^5D_4$ state to the various ground-state manifolds ^7F_j, $j = 0-6$. Because of the large degeneracy of excited and ground states, it is not possible to analyze the crystal structure or symmetry of the chelates with terbium. Note that the spectrum is highly spiked: the peak at 546 nm is 1.5

$\times 10^3$ times brighter than the valley at 520 nm; 21.5% of the total emission is in the 490 nm peak (fwhm = 5 nm), and 56.4% is in the 546 nm peak (fwhm = 8 nm).

The absorption (Figure 2B, inset) is due to the carbostyril 124 attached via an amide linkage to the chelate. The absorption spectra of all chelates were very similar and displayed two peaks (ϵ_{327} = 12 000 M^{-1} cm^{-1}; ϵ_{342} = 10 500 M^{-1} cm^{-1}) with ϵ_{337} = 9600 M^{-1} cm^{-1}. The excitation spectra for Eu and Tb bound to DTPA−cs124 were identical to the carbostyril absorption spectra (the other chelates were not tested), indicating that the interaction between the carbostyril and the lanthanide does not significantly perturb the carbostyril wavefunctions. Carbostyril bound to chelate could easily be distinguished from free carbostyril since the absorption spectra of free carbostyril (data not shown) has a maximum at 342 with a shoulder at 330 nm.

The emission lifetimes of Tb−TTHA−cs124 (Figure 2C) and other chelates were all rigorously single exponential. The terbium lifetimes and relative total intensities for the four chelates are shown in Table 1. The number of waters bound to the inner coordination sphere of the lanthanide was determined by the method of Horrocks and Sudnick[18,23,24] where

$$\text{no. of waters} = q(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1})$$

q = 1.05 for Eu, 4.2 for Tb

in which τ is the lifetime of the complex in H_2O or D_2O. The formula is reported to be accurate to ± 0.3 to ± 0.5 water molecules.[18] Fractional water molecules can arise because of this uncertainty, because two species with differing number of water molecules exchange faster than the lanthanide lifetime, or because a water molecule in the outer coordination sphere is bound and has a reduced nonradiative de-excitation efficiency compared to an inner-coordination water molecule.

The presence of H_2O in the lanthanide primary coordination sphere contributes to nonradiative de-excitation and a reduced quantum yield for lanthanide luminescence. If we assume that the quantum yield for terbium emission is 1 in D_2O (that is, once the terbium has received energy, it reemits a photon with unity probability),[27] then the quantum yield in H_2O is just the ratio of lifetimes in D_2O and H_2O (see Table 1). (The quantum yield of the entire chelate can be defined as the efficiency of energy transfer from the sensitizer to the lanthanide, times the lanthanide quantum yield.)

From Table 1 it is clear that the total emission intensities are approximately the same in H_2O-based solutions for the DTPA, DOTA, and TTHA terbium complexes. A standard curve (data not shown) indicates a detection limit of 2 pM (in 100 μL) for Tb−DTPA−cs124. By using more efficient collection optics, this detection limit should significantly improve in the future.

The total intensity is proportional to the efficiency of energy transfer from cs124 to terbium, times the quantum yield of terbium. Since the main determinant of the terbium quantum yield is the number of primary coordinated waters, and since DOTA and DTPA have the same number of waters, we can deduce that the energy transfer from cs124 to terbium is approximately the same in these two complexes. Energy transfer from cs124 to terbium in the TTHA chelate is less efficient—approximately 73% as efficient as the DTPA and DOTA complexes. This is likely because the extra aminocarboxylate group, which excludes water (and increases the terbium quantum yield), prevents the carbostyril from approaching the terbium. For TETA the terbium intensity was concentration-dependent in the micromolar range, with intensity falling off more rapidly than concentration. The terbium excited-state lifetime, however, was independent of concentration. These two results indicate that there is an equilibrium between terbium-bound (luminescent state) and terbium-unbound (nonluminescent) TETA. Although the theoretical stability constant of TETA for Tb is 10^{15} M^{-1},[22] the conversion of one carboxyl to an amide is expected to decrease this number slightly, and more significantly, in solution the stability constant should be expressed as a conditional stability constant.[28] A conditional stability constant of $\leq 10^7$ M^{-1} at pH 8 is consistent with the our data.

The emission lifetime of terbium in DTPA−cs124 can be compared to the terbium emission lifetime of a large number of other Tb−DTPA−sensitizer complexes.[19-21,29] In almost all cases, the lifetimes are very similar. This is indirect but strong evidence that the sensitizer does not contribute to nonradiative deexcitation of the excited-state terbium (via a back-transfer of energy from the excited-state terbium to the sensitizer). This back-transfer process is known to occur with near 100% efficiency with some cryptates.[30] A back-transfer process is dependent on a sensitizer triplet state lying below the $^5D_4$ state of terbium, and the rate is a strong function of the exact energy gap: it is highly unlikely that the equivalent lifetimes of the Tb−DTPA complexes arise because all sensitizers have a low-lying triplet state of almost identical energy; it is much more likely that these sensitizers simply do not have such a state and no back-transfer occurs.

We have also synthesized, purified, and spectrally characterized Tb−DTPA−$(cs124)_2$. The terbium lifetime is biexponential, with a major component (90%) with a lifetime of ≈200 μs and small component with a 1.5 ms lifetime. (The small, long-lifetime component is probably due to trace Tb−DTPA−cs124.) The terbium emission intensity is also significantly (>10-fold) less than the mono Tb−DTPA−cs124 on a per-mole basis. The decrease in the terbium lifetime and intensity in the bis complex is strong indication that terbium emission is being quenched, most likely because the cs124's are interacting and forming a new triplet state which lies slightly below the terbium $^5D_4$ state. This new state is evidently an efficient pathway for nonradiative relaxation in the bis complexes.

Europium Chelates. The emission spectra of the europium chelates are shown in Figure 3a−c, and the lifetime and intensity data are summarized in Table 2. The emission arises from transitions from $^5D_0$ to ^7F_j, j = 0 to 6 (the small peak of j = 6 at about 800 nm is not shown). It is clear that the chelate structure (macrocycle vs linear and symmetric vs assymetric crystal field) significantly affects the strength of the various transitions. The fairly symmetrical macrocycle chelate DOTA has a greater fraction of its luminescence in the peak centered around 700 nm than the assymmetric TTHA and DTPA. We were unable to get efficient binding of europium to the macrocycle TETA complex, although Bryden and Reilley

(27) The quantum yield for lanthanide emission is difficult to measure, and unity quantum yield in D_2O is clearly an upper limit. Nevertheless, the quantum yield in D_2O is probably quite high: D_2O is known to be 100× less efficient at nonradiative deexcitation than H_2O and polyaminocarboxylate chelates are known to have group frequencies which are unable to de-excite lanthanides (see refs 23 and 24), and our carbostyril sensitizer does not contribute to nonradiative de-excitation—see text. Furthermore, in lanthanide-based energy transfer (see refs 12 and 13), this assumption gives results consistent with fluorescence-based energy transfer. For europium, the quantum yield in D_2O (no chelate) has been reported to be 0.78 (see ref 18) and a value of 0.7 has been measured in a clever and probably reliable manner for a europium chelate in H_2O (Drexhage, K. H. *Sci. Am.* **1970**, *222*, 108)

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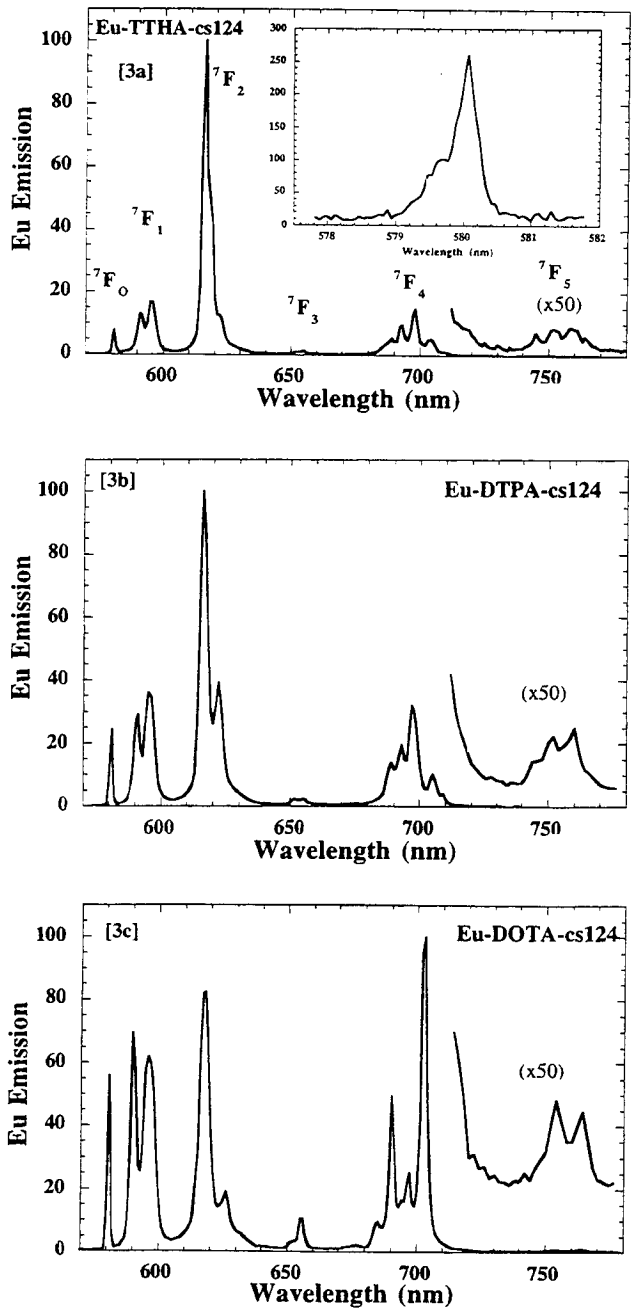

Figure 3. Normalized emission spectra of europium complexes of (a) TTHA−cs124, (b) DTPA−cs124, and (c) DOTA−cs124. Emission arises from the $^5D_0$ excited state, and part a shows the ground-state configuration. The inset to part a shows a high-resolution scan of TTHA which was taken with monochrometer slit bandwidth of approximately 0.05 nm with 0.05 nm step size. All other spectra taken under conditions identical to those of as Figure 2.

showed that, at high concentration, europium bound to TETA (without sensitizer) also displays enhanced far-red emission.[31] The $^5D_0$ to $^7F_1$ magnetic dipole transitions around 585−605 nm are also enhanced in DOTA (and Eu−TETA without sensitizer[31]), presumably because these transitions are even-parity transitions and hence are parity-allowed in a symmetric environment.

The assymmetry of the TTHA complex appears to be greater than any of the other chelates. The magnetic dipole transitions and the emission peaks around 700 nm are highly suppressed in Eu−TTHA−cs124, likely indicating that the crystal field of TTHA is more asymmetric than the other chelates. The shortened lifetime in D_2O of europium in TTHA−cs124, as compared to DTPA−cs124, also indicates a more asymmetric crystal field for TTHA−cs124. (The terbium lifetime in TTHA−cs124 is also shorter than in DTPA−cs124, which may again indicate that the crystal field is more asymmetric in TTHA.) From a practical point of view, the result is that a large fraction of the europium luminescence in the TTHA complex—60%—is in one peak centered around 617 nm, which is also very sharply spiked (fwhm of 3.3 nm). By utilizing 1 nm slits at 617 nm, the emission of Eu in TTHA−cs124 is 3.6 times larger than that of Eu in DTPA−cs124.

In all of the chelate complexes, the $^5D_0$ to $^7F_0$ transition around 580 nm is nondegenerate and cannot be split by the crystal field. The width is intrinsically very narrow, and the width presented in Figure 3 is determined by the instrument slit width. A splitting of this line indicates that there are two different species present. A high-resolution scan shows two structures present in TTHA; a high-resolution scan of DTPA failed to find a splitting, though it is still possible that two or more forms exist but cannot be resolved.

Our europium results can be compared to the results of other workers who have measured europium luminescence bound to DTPA, DOTA, TETA, and TTHA, all without sensitizer.[31−33] In each case the lifetimes and number of water coordinated are the same for the chelate-only and the chelate-sensitizer complex, within experimental error. The emission spectra, with and without sensitizer, for DTPA and for DOTA, are also identical. In addition, the high-resolution scan around the $^5D_0$ to $^7F_0$ transition in the TTHA-only and TTHA-sensitizer complexes are also identical. (The full emission spectra of Eu−TTHA without sensitizer was not reported.[32])

Several conclusions can be reached from these comparisons. (1) Because the europium lifetimes in the chelate-only and in the chelate-sensitizer complexes are the same, this is strong indication that once the europium is excited, nonradiative de-excitation by back transfer to the sensitizer—either vibrationally by phonons or electronically by an excited triplet state of the sensitizer which lies below the $^5D_0$ europium luminescent state—cannot occur. If this were occuring, the europium lifetime in the chelate-sensitizer complex would be shorter than that of the chelate complex. The quantum yield for europium luminescence in the complexes in D_2O is therefore likely to be very high—near unity. This is because D_2O is extremely inefficient at deactivating the europium excited state and the amine and carboxyl ligands of the chelates are also known to be inefficient at deactivating the excited state.[27] (2) Because the numbers of coordinated waters are the same in the chelate and chelate-sensitizer complex, we further conclude that the amide oxygen is able to ligate the lanthanide and that the sensitizer itself is not coordinated to the europium. In a number of DTPA− and DOTA−amide complexes, it has been shown that the amide oxygen is able to ligate the lanthanide.[34−37] If the sensitizer were coordinated to the lanthanide, we would expect a decrease in the number of coordinated waters. (3) The similarity in emission spectra of europium when bound in the chelate-only and in the chelate-sensitizer complexes indicates that the crystal

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Table 2. Lifetime of Europium Complexes in H_2O and D_2O, Their Ratio, the Number of Coordinated Waters in the Lanthanide Primary Sphere, the Total Intensity in H_2O, and the Distribution of the Intensity in Different Emission Peaks

Eu complex	τ_{H_2O} (ms)	τ_{D_2O} (ms)	τ_{H_2O}/τ_{D_2O}	no. of waters	total intensity (570−730 nm) in H_2O	%intensity (617 nm; 700 nm)
DTPA−cs124	0.62	2.42	0.26	1.26	1	48; 25
TTHA−cs124	1.19	1.79	0.66	0.3	2.67	60; 19
DOTA−cs124	0.62	2.25	0.27	1.23	0.66	30; 33
TETA−cs124						

fields seen by the lanthanide are virtually identical in the two complexes. This again indicates that the sensitizer is not bound to the metal. We have recently solved the crystal structure of Eu−DTPA−cs124, and the structure confirms that the amide oxygen at the linkage between DTPA and cs124 is ligated to the metal and that the carbonyl oxygen of the cs124 is not ligated. (4) The high-resolution scan of the TTHA complexes indicates that both chelate-only and chelate-sensitizer have two forms. It is possible that these two forms correspond to a rapidly-exchanging TTHA molecule with europium bound to one and to zero water molecules. Indeed, we find the relative areas under the short- and long-wavelength curves to be 25% and 75%, respectively, which yields an average of 0.25 coordinated waters if the long-wavelength curve corresponds to the zero-water complex. This value is consistent with the value of 0.3 determined by the independent technique of lifetime measurements in H_2O and D_2O (see Table 2).

Table 2 shows that in D_2O the europium lifetime in the DOTA complex is slightly shorter than in the DTPA complex. Since DOTA is more symmetrical than DTPA and because the radiative rate is determined by the assymetry in the crystal field, we would have expected the europium lifetime to be longer in DOTA, assuming the quantum yield is unity in both chelates. Our experimental results indicate that the europium quantum yield in the DOTA complex in D_2O may be slightly less than unity. A europium quantum yield in DOTA of ≤ 0.94 (2.25 ms/2.4 ms) that of in DTPA would account for the lifetime results. H_2O contamination in the D_2O solution of DOTA sample would also account for the shortened lifetime, but we believe this not to be the case.

Table 2 also indicates that the Eu−DOTA−cs124 total intensity is less bright in H_2O-based solution than those of the DTPA and TTHA complexes. Our detection system, however, decreases in sensitivity toward the red, where Eu primarily emits in the DOTA complex, and the emission spectra are not corrected for detector sensitivity.[38] The apparent decrease in europium emission in DOTA as compared to Eu−DTPA may therefore be a measurement artifact. The detector wavelength sensitivity also precludes a quantitative determination of the efficiency of energy transfer from the cs124 to the Eu in the various chelates.

Implications for Chelates as Luminescent Probes and as Energy Transfer Donors. With the exception of TETA, all of the chelates synthesized bind very tightly to the lanthanides.[39] They also emit strong luminescence with both terbium and europium, have excellent solubility, and likely have very high quantum yields for lanthanide luminescence because the chelate and the sensitizer do not allow significant nonradiative deactivation of the lanthanide excited state.

As alternatives to fluorophores in imaging applications, the chelate brightness is the main criteria of goodness, although solubility of the chelate complex and net charge are also important. The solubility becomes important because of potential precipitation problems, especially when labeling a macromolecule with hundreds of chelates, and the net charge is important both for solubility and for avoiding nonspecific labeling of cellular components, which are usually negatively charged. For two-color imaging, spectral overlap between the two labels (terbium and europium, in this case) is also important. For use as energy transfer donors, there are a number of parameters which are important, including the quantum yield for lanthanide luminescence, the fraction of emitted light which arises from electric dipole transitions—magnetic dipole transitions are not capable of transfering energy—and the spectral distribution of lanthanide emission so that overlap with the acceptor's absorption arises. Note that the quantum yield which is relevant to energy transfer is the probability that the lanthanide re-emits light after it has been excited. The total brightness of the chelate is of secondary importance, so long as the chelate is sufficiently bright that photon statistics or detector noise is negligible. For energy transfer in a microscope, this condition may not be met and so the brightness of the chelate is then important.

In both imaging and energy transfer experiments, we expect TTHA−cs124 to be an excellent chelate using both terbium and europium and, in particular, to be better than the more widely used DTPA-based chelates. TTHA−cs124 has the brightest signal, particularly with europium, and the highest lanthanide quantum yield in H_2O. It is also highly negatively charged, which increases solubility and decreases nonspecific interactions with negatively-charged macromolecules.

For two-color imaging, we expect TTHA−cs124 to be useful by utilizing the 546 nm emission of terbium and the 617 nm emission of europium. In particular, the Tb and Eu intensities are approximately equal (Tb intensity at 546 nm = 100; Eu intensity at 617 nm = 72, in arbitrary units), which helps minimize the effect of nonlinearity in the detector and also minimizes the effect of any lanthanide contamination (small amounts of terbium in the europium label, and visa-versa). The narrow-band emission of Eu−TTHA−cs124 (3.3 nm fwhm of 617 nm peak) also enables spectral discrimination against background in imaging applications.

For energy transfer experiments, the sharply spiked spectrum and high lanthanide quantum yield of Eu− (and Tb−)TTHA−cs124 makes this chelate an efficient donor. In addition, in Eu−TTHA−cs124, the magnetic dipole transition around 590 nm is suppressed, which is a transition useless in energy-transfer experiments because it is incapable of transferring energy to an acceptor via standard electric dipole−dipole Förster mech-

(38) We use a GaAs photomultiplier tube which is fairly insensitive to wavelength. Nevertheless, for quantitative measurements, the total throughput of the emission optical path must be measured (including the throughput of lenses and the double-monochrometer), which has not been done.

(39) The log K of each chelate (without sensitizer) for Tb^{3+} and Eu^{3+} is reported to be as follows: DOTA (28.6, 28.2); DTPA (22.71, 22.39); TETA (14.81,15.46); TTHA (23.08, 23.03) [see ref 22 and the following: Hseu, T.-M.; Chang, C.-C.; Lin, Z.-F. *J. Chin. Chem. Soc.* **1987**, *34*, 187]. By comparison the value for EDTA is 17. These data are for the chelate, not bound to a chromophore. We expect the binding constant to be slightly less when the chelate is bound to the chromophore, although this effect is not expected to be large since the amide oxygen can still ligate the lanthanide (see text). These are theoretical binding constants and do not take into account the effect of solvent, which can considerably lower the binding constants. At 2 pM Tb−DTPA−cs124 (pH 8.0) and 10 mM Tris, we find no noticeable dissociation of Tb from DTPA−cs124, indicating that the binding constant under these conditions is $>10^{14}$ M^{-1}.

anism.[13,40] We calculate that energy transfer from Eu−TTHA−cs124 to CY-5[41] has a spectral overlap of 8.43×10^{15} nm^4 cm^{-1} M^{-1} and an R_o of 68.6 Å in H_2O and 73.5 Å in D_2O (assuming the index of refraction equals 1.33, the value for water). This is the largest R_o yet reported in H_2O (see below for D_2O). By comparison, for Eu−DTPA−cs124 and CY-5, we have previously reported a spectral overlap of 6.55×10^{15} nm^4 cm^{-1} M^{-1} and an R_o of 56 Å in H_2O and 70 Å in D_2O. Finally, for energy transfer experiments where the sensitized emission of the acceptor is measured, it is highly advantageous to have little or no donor emission where the acceptor fluoresces.[12−14] With Eu−TTHA−cs124, the $J = 3$ peak is highly suppressed (75× less than the peak at 617 nm) and the europium emission at 668 nm (where CY-5 fluorescence occurs) is 600× less than the peak at 617 nm.

DOTA−cs124 has enhanced far-red europium emission around 700 nm which is expected to be useful in two-color imaging with europium and terbium. By detecting terbium at 492 nm and/or 546 nm and detecting europium around 700 nm, two-color imaging with *no* spectral overlap is possible. The 617 nm line of europium can also be used, but the small terbium emission line at 622 nm causes some overlap. The enhanced far-red emission of DOTA is also useful for energy transfer experiments, since the efficiency of energy transfer depends on λ^4, where λ is the wavelength(s) of spectral overlap between donor and acceptor.[42] The spectral overlap between Eu−DOTA and Cy5.5 is 1.02×10^{16} nm^4 cm^{-1} M^{-1}; the R_o is 76 Å in D_2O assuming the europium quantum yield is 1, the largest R_o yet reported for small molecules. In H_2O the R_o is 62 Å. In addition, sensitized emission of the acceptor can be measured with little or no background because the Eu−DOTA−cs124 emission beyond the 700 nm peaks, where CY5.5 has significant fluorescence, is extremely small: at 735 nm, background is determined by detector noise.

Finally we note that these chelates can be attached to macromolecules in a variety of ways: by activating the carboxylate groups; by reacting the carboxylate groups with diamine-containing compounds which then has one of its amines converted into an isothiocyanate; or by reacting the carboxylate with a heterodimeric cross-linker containing a hydrazide and thiol-reactive functionality. The synthesis of such compounds will be reported shortly.

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