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Luminescence Resonance Energy Transfer in Heterodinuclear Ln^{III} Complexes for Sensing Biologically Relevant Anions

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Dinuclear lanthanide(III) complexes of the macrocycles 1,4-bis[1-4,7,10- tris(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane]-*m*-xylene (**1**) and 1,3-bis[1-4,7,10-tris(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane]-*p*-xylene (**2**) with Ln^{III} = Eu^{III}, Tb^{III} were prepared. Studies using direct excitation ($^7F_0 \rightarrow {}^5D_0$) europium(III) luminescence spectroscopy show that several anions including phosphate, methylphosphate, double-stranded DNA, a DNA hairpin loop, and fluoride bind to both Eu^{III} centers in Eu$_2$(**1**) in solutions at pH 7.0, 0.100 M NaNO$_3$. Recovered luminescence lifetime data is consistent with replacement of water ligands by these anions. There are distinct changes in the relative intensities of Eu^{III} emission peaks for complexes of Eu$_2$(**1**) and Eu$_2$(**2**) with phosphate, fluoride, carbonate, and phosphate ester ligands. This suggests that the Eu^{III} dinuclear complexes show promise as ratiometric sensors for these anions. In contrast, the emission peak ratios for the Tb$_2$(**1**) and Tb$_2$(**2**) complex show a lower response upon binding of each anionic ligand. Luminescence resonance energy transfer (LRET) studies with mixed Eu^{III}/Nd^{III} complexes show that the phosphate complexes of Ln$_2$(**1**) or Ln$_2$(**2**) both have Ln^{III}–Ln^{III} internuclear distances of 7.7 ± 0.9 Å and the methylphosphate complexes of Ln$_2$(**1**) and Ln$_2$(**2**) have internuclear distances of 7.8 ± 0.6 and 8.0 ± 0.7 Å, respectively. LRET experiments also show the two lanthanide ion centers in Ln$_2$(**2**) are 8.4 ± 0.5 Å apart when bound to GC rich duplex DNA. None of the other anions (carbonate, fluoride, DNA hairpins) form complexes that show measurable energy transfer between the lanthanide ions, which is consistent with intranuclear distances >8 Å.

Introduction

The unique photoluminescence properties of Ln^{III} complexes have led to their development as spectroscopic probes.[1–4] One goal of research in this area includes monitoring bioactive ions in aqueous solution for biomedical or environmental applications. Other applications include the use of lanthanide luminescent sensors for cellular imaging of pH and ion concentrations. These Ln^{III} sensors typically contain multidentate ligands to control the number of available coordination sites for interaction with the analyte and to optimize luminescence intensity by minimizing bound water ligands, which efficiently quench lanthanide luminescence. Generally, chromophores are incorporated into the multidentate ligand to act as an antenna for harvesting photons and transferring them to the Ln^{III} ion by resonance energy transfer (RET).[5–10] Many probes based on such sensitized excitation of the Ln^{III} ions have been applied to specific biochemical anions such as those shown in the work by Parker[11–18] and others.[19–27]

The preferred Ln^{III} ions for luminescent probes are Eu^{III} and Tb^{III} because of their favorable luminescence properties, including long excited-state lifetimes in the millisecond range. However, lanthanides such as Yb^{III} and Nd^{III} are being explored for their use as probes because they emit in the near IR (NIR), which is beneficial for biological studies since NIR wavelengths are efficiently absorbed by cells.[28–33] Time-resolved luminescence lifetimes, luminescence intensity,[32] ratiometrics of emission bands,[11,13] excitation spectra, and polarization[34,35] of Ln^{III} photoluminescence are characteristics that have been used to discriminate between anions of biological interest upon interaction with Ln^{III} complexes. Dinuclear Ln^{III} ion complexes are of interest as luminescent sensors partly because their anion binding properties are different than those of their mononuclear analogs.[1,2,22,36,37] For example, we previously proposed that a phosphate monoester bridged two Ln^{III} ion centers in a dinuclear complex and that this interaction resulted in a more stable complex and more effective catalysis for the cleavage of phosphate diesters and RNA.[2] These studies motivated the present investigation to better understand binding of biologically relevant anions to dinuclear complexes by using Eu^{III} excitation spectroscopy and time-resolved luminescence spectroscopy. As an additional tool we investigated the utility of Ln^{III}–Ln^{III} luminescence resonance energy transfer (LRET) to characterize anion binding.

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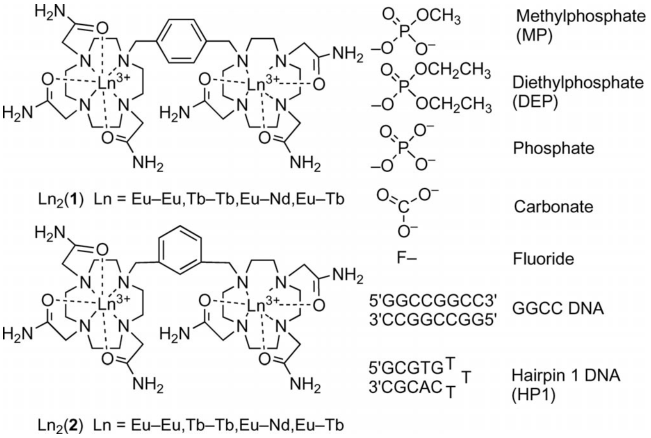
Fluorescence resonance energy transfer (FRET), introduced over 50 years ago by Förster[38,39] describes energy that is transferred from an excited fluorophore through space to another nearby fluorophore by a radiationless dipole–dipole coupled mechanism. In order for this RET to occur, the wavelength of the absorbance band of the acceptor fluorophore must overlap with that of the emission band of the excited fluorophore donor. The analogous process for Ln^{III} ions is LRET.[8]

FRET (or LRET) is typically used as a 'spectroscopic ruler' in biochemical systems to monitor folding, structure,[40] and dynamics of biomolecules.[41] The distance between the donor and acceptor molecules/atoms can be measured by using Equation (1). The relationship between the efficiency of RET and the distance between the donor and acceptor corresponds to $1/r^6$, where r is the calculated distance. The efficiency of energy transfer is E, τ_{DA} is the time-resolved luminescence lifetime of the donor–acceptor pair, τ_D is the lifetime of the donor, and R_0 is the distance for 50% energy transfer to occur or the Förster distance.

$$E = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6} \tag{1}$$

Luminescence resonance energy transfer between Ln^{III} ions in aqueous solution has focused primarily on systems that form dinuclear complexes either through self-assembly of two mononuclear Ln^{III} complexes, usually with bridging hydroxide ligands, or on ligands that bind two Ln^{III} ions.[21,30,42–45] In addition, LRET has been applied to mapping metal ion binding sites of biomolecules.[46] In particular, Ln^{III} are good Ca^{II} surrogates and have been used to study multisite calcium binding proteins such as Troponin-C,[47] parvalbumin,[48,49] and thermolysin.[50] In these studies, mixed lanthanide cations were bound to the proteins and used to estimate distances between metal ion binding sites.[51] The Eu^{III} and Nd^{III} donor–acceptor pair is particularly useful.[48,50,51] Nd^{III} has one of the largest extinction coefficients (~10 $\text{M}^{-1}\,\text{cm}^{-1}$) of all the Ln^{III}, which facilitates LRET over longer distances.[52] Eu^{III} is useful in these photoluminescent experiments because the ion has an excitation transition ($^7F_0 \rightarrow {}^5D_0$), which is not split by ligand fields and therefore allows precise control over which Eu^{III} species is excited. An alternate Ln^{III} pair is Tb^{III}/Eu^{III} although there are fewer examples of direct LRET between Tb^{III} and Eu^{III} in the literature.[53–57] Chrysochoos and coworkers studied Tb^{III} and Eu^{III} donor and acceptor LRET pairs in DMSO[58,59] and aqueous solutions.[60] More recently, energy transfer in dinuclear complexes of Tb^{III} and Eu^{III} have been reported.[61,62] An interesting aspect of this pair is that energy transfer can be observed by exciting specific Tb^{III} transitions and by monitoring Eu^{III} luminescence deriving from energy transfer.[45] LRET between Tb^{III} and Eu^{III} cations is, however, not as favorable as for LRET between Eu^{III}–Nd^{III} donor and acceptor pairs as a result of a smaller spectral overlap between Tb^{III} and Eu^{III}.

Here, we study the Ln^{III} luminescence properties by using time-resolved luminescence and emission/excitation spectroscopy and LRET as tools for the characterization of dinuclear Ln^{III} complexes bound to biologically relevant anions. This work shows that dinuclear Ln^{III} complexes can be used to sense a variety of simple anions in aqueous solution. The macrocyclic complexes and the anions of biological interest studied here are shown in Scheme 1. Dinuclear macrocycles with two different linkers were used to determine whether the LRET properties would vary upon binding of anions that bridge the two Ln^{III} ion centers.



Scheme 1. Macrocyclic complexes and anions used in this study.

Results and Discussion

Eu^{III} Excitation Spectra

The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of Eu$_2$(**1**) in solution at neutral pH shows one major peak at 578.80 nm, which is assigned to the aqua species [Eu$_2$(**1**)(H$_2$O)$_4$] on the basis of the number of bound water ligands as determined by luminescence lifetime studies as described below.[2] A minor peak is observed at 580.5 nm and is assigned to a carbonate species as shown previously.[1,2] To study complexation of the dinuclear complex with biologically relevant anions, changes in the intensity of the Eu^{III} $^7F_0 \rightarrow {}^5D_0$ excitation peak were monitored. Methyl phosphate (MP), fluoride, or phosphate were added to solutions of the Eu$_2$(**1**) complex at pH 7.0 (I = 0.10 M NaNO$_3$) to give the excitation spectra shown in Figure 1 and in Figure S1 in the Supporting Information. In general, both major and minor excitation peaks of the parent dinuclear Eu^{III} complex decrease upon addition of anion with a concomitant increase in intensity of new excitation peaks. The spectra for the complexes of Eu$_2$(**1**) with MP or phosphate show a single new excitation peak. Complexes of Eu$_2$(**1**) with DNA sequences also show a single major peak as reported previously.[1] In contrast, complexes of Eu$_2$(**1**) with fluoride have two major excitation peaks. A peak-fitting analysis of the excitation spectra for each of the fully bound complexes of fluoride, phosphate and methylphosphate is given in Figure S1 in the Supporting Information.

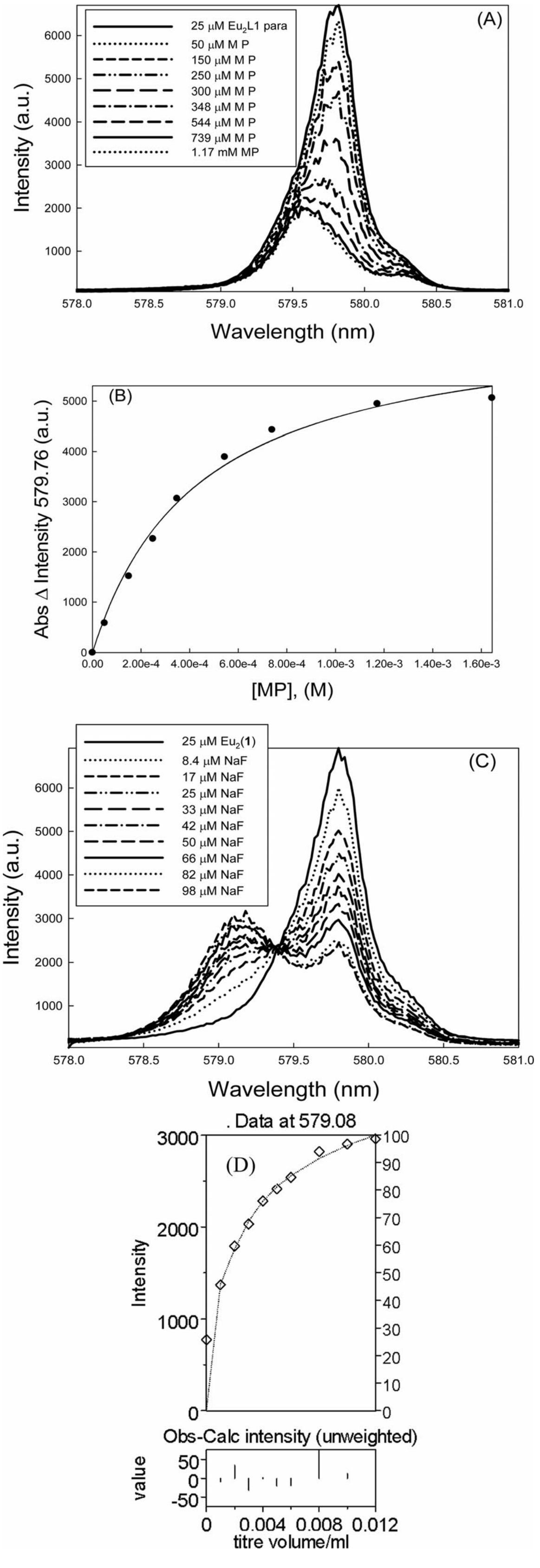


Figure 1. $^7F_0 \rightarrow {}^5D_0$ excitation spectra and binding isotherms for 25 μM Eu_2(**1**) with addition of MP [(A) and (B), respectively] and fluoride (4.09 mM Stock) [(C) and (D)] at I = 0.10 M $NaNO_3$, 20.0 mM HEPES, pH at 7.0. The data is fitted to Equation (5) (MP) and HyperSpec (fluoride).

Changes in the intensity of the major excitation peak of the dinuclear complex Eu_2(**1**) upon binding of anion were used to construct binding isotherms (Figure 1 and Figure S2, Supporting Information). Dissociation constants were calculated by fitting the binding data to Equation (5) (Experimental Section). Binding isotherms for MP and phosphate were fitted as a 1:1 complex of anion to dinuclear Eu^{III} complex, which is consistent with the luminescence lifetime and LRET data given below. The binding isotherm for fluoride and carbonate were fitted to a 2:1 binding model by using HyperSpec (Protonic Software).[83,84] The dissociation constants of all anions used in this study are summarized in Table 1 along with previous data obtained for diethyl phosphate (DEP), methyl phosphate (MP), carbonate, and DNA. More limited data are available for the dissociation constants for the Eu_2(**2**) complexes of the anions.

Table 1. Dissociation constants of anions for Eu_2(**1**) and Eu_2(**2**) at neutral pH. Solutions contained 25 μM Eu_2(**1**), I = 0.10 M $NaNO_3$, 20.0 mM HEPES at pH = 7.0, except where noted.

Anion	Eu_2(**1**) K_d (μM)	Eu_2(**2**) K_d (μM)
CO_3^{2-}	15[a] 0.10 ± 0.01, K_{d1}[b] 95 ± 0.3, K_{d2}[b]	
DEP	1800[c]	430[d]
MP	410 ± 50[e]	60[d] or 25[f]
HP1	2.5 ± 0.4 (n = 8.2 ± 0.5)[a]	
F^-	1.9 ± 0.1, K_{d1}[b] 105 ± 3, K_{d2}[b]	
PO_4^{3-}	45 ± 8[e]	
GGCC	28 ± 2 (n = 6.9 ± 3)[a]	

[a] Determined by luminescence spectroscopy (I = 0.05 M $NaNO_3$) with Equation (6) for DNA (GGCC and HP1), where n as the number of bases per binding site, or with Equation (5) for carbonate (see ref.[1]). [b] Data were fitted with HyperSpec for a two anion binding model (CO_3^{2-} or F^-). [c] I = 0.05 M $NaNO_3$ from ref.[63] [d] Determined by kinetic inhibition (pH = 7.6 and I = 0.05 M $NaNO_3$) from ref.[2] [e] Data were fitted to Equation (5) for a 1:1 anion binding model (PO_4^{3-} and MP). [f] 0.5 mM Eu_2(**1** or **2**) determined by luminescence spectroscopy (pH = 7.6) from ref.[2]

Emission Spectra

Emission spectra were collected by exciting at 465.00 nm, the $^7F_0 \rightarrow {}^5D_2$ transition of Eu^{III}. This transition has a higher quantum yield than the $^7F_0 \rightarrow {}^5D_0$ transition. It also allows for the clear observation of the $^5D_0 \rightarrow {}^7F_0$ and $^5D_0 \rightarrow {}^7F_1$ emission bands by using our laser/MOPO system. The emission spectra of anion complexes with Eu_2(**1**) and Eu_2(**2**) under conditions where the anions were completely bound to the dinuclear Eu^{III} complexes were collected (Figures 2 and S3). For all complexes, the $^5D_0 \rightarrow {}^7F_2$ emission peak (the hypersensitive transition) was the most intense. However, the relative emission peak areas vary for each complex as shown in the comparison of the emission spectra for complexes of Eu_2(**1**) and for Eu_2(**2**) (and Figure S4). Table S1 gives the normalized emission peak areas relative to the $^5D_0 \rightarrow {}^7F_1$ transition for both dinuclear complexes and all of the anions studied. There is clearly a difference in the ratios of the emission peaks of the complexes with bound anions relative to the aqua complexes. This data shows that dinuclear Eu^{III} complexes may be useful for the development of ratiometric sensors for these anions.

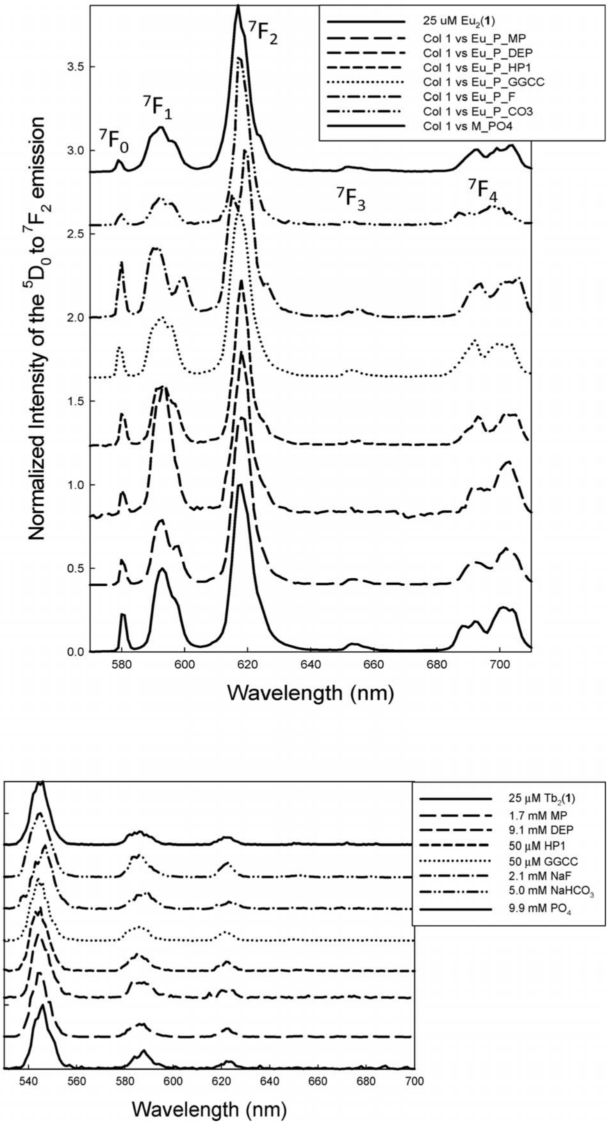


Figure 2. Normalized emission spectra of 25 μM (top) Eu$_2$(**1**) (to the $^5D_0 \rightarrow {}^7F_2$ transition Eu^{III}) and (bottom) Tb$_2$(**1**) (to the $^5D_4 \rightarrow {}^7F_5$ transition Tb^{III}) [with fully bound anions with emission bands $^5D_4 \rightarrow {}^7F_J$ (J = 0,1,2,3,4), I = 0.10 M NaNO$_3$, 20.0 mM HEPES pH at 7.0. Offset for clarity].

The emission spectra of the Tb$_2$(**1**) and Tb$_2$(**2**) complexes were obtained upon excitation of the complexes at 487.00 nm, the $^7F_6 \rightarrow {}^5D_4$ transition. Figure 2 (also Figures S3 and S5) shows the normalized emission spectra of the Tb$_2$(**1**) and Tb$_2$(**2**) complexes bound to the anions. The ratio of the $^5D_4 \rightarrow {}^7F_J$ (J = 5, 4, 3) emission bands are similar for all Tb^{III} complexes with bound anions with the exception of carbonate and fluoride (Table S2). For the carbonate complex of Tb$_2$(**1**), the $^5D_4 \rightarrow {}^7F_5$ intensity is slightly decreased relative to the $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_3$ emission bands, while for fluoride, the $^5D_4 \rightarrow {}^7F_5$ is reduced relative to the other two emission peaks. However, as shown in Table S2, emission band ratios do not vary in the Tb^{III} complexes as greatly as they do in the analogous Eu^{III} complexes, which suggests that the Tb^{III} complexes will be less useful for ratiometric sensing of these anions.

Time-Resolved Luminescence

Eu^{III} luminescence decays were collected for the two homodinuclear complexes of Eu^{III} and for two mixed Eu^{III}/Nd^{III} complexes. For the mixed complexes, a ratio of 1:20 Eu^{III}:Nd^{III} was used to ensure that nearly all bound Eu^{III} had a neighboring Nd^{III} center. All Eu^{III} dinuclear complexes were excited at 579.76 nm, the non-degenerate transition of Eu^{III}. Eu^{III} cations excited at 465.00 nm yielded lifetimes within error of those collected at 579.76 nm (unpublished results).

Luminescence lifetimes (τ_D) were monitored at ca. 618 nm for the Eu$_2$(**1**) and Eu$_2$(**2**) complex with no anion, with MP, DEP, HP1, GGCC, fluoride, CO_3^{2-} and PO_4^{3-} (Table 2) in H_2O and D_2O. This data was used in Equation (2) as refined by Horrocks[64] to determine q, the number of bound waters [A is a constant specific to Eu^{III} (1.1 ms) and k_{H2O} and k_{D2O} are the rate constants for luminescence decay in H_2O and D_2O, respectively]. In this equation, quenching contributions from the three amide groups of the ligand pendent arms are included, $k_{HNC=O}$ (0.075 ms^{-1} for each amide group HNC=O), and α is the total outersphere quenching (0.31 ms^{-1}). Standard deviations of the luminescence lifetimes were propagated to determine the error in the number of water molecules or the q number. The error in the D_2O luminescence lifetimes was assumed to be ≤10%, as shown previously for luminescence lifetimes measured for many different samples on different days.[65]

$$q = A(k_{H20} - k_{D20} - k_{NHC=O} - \alpha) \qquad (2)$$

Absorbance Spectra

UV/Vis absorbance spectra were collected for Nd$_2$(**1**), Nd$_2$(**2**), Eu$_2$(**1**), and Eu$_2$(**2**) complexes in the absence of anions at 1.00 and 10.0 mM concentrations of complex. At the concentrations required for these measurements, the complexes with bound anions were not soluble. Therefore, an assumption is made that the absorbance bands will not be greatly altered in the presence of the anion.

Luminescence Resonance Energy Transfer

The overlap integral, J, was calculated for all complexes and anions with Equation (3) by using the appropriate absorbance spectra (for ligand **1** or **2**) and anion specific emission spectra.

Table 2. All calculated and measured parameters for the determination of inter Ln^{III} distance via LRET for Eu^{III} donor and Nd^{III} acceptor.

Ligand	Anion	τ_D [µs]	τ_{DA} [µs]	Energy transferred	R_0 [Å]	Φ_D	r [Å]	$J \times 10^{18}$ [M^{-1}cm^3]
1	none	347 ± 12	305 ± 11	0.121 ± 0.006	5.7	0.051	7.9 ± 0.4	3.5
2	none	345 ± 14	298 ± 22	0.136 ± 0.011	5.6	0.048	7.6 ± 0.6	3.4
1	$NaHCO_3$	611 ± 13	586 ± 11	0.041 ± 0.001	5.9	0.130	–	1.8
2	$NaHCO_3$	596 ± 39	538 ± 49	0.097 ± 0.011	6.1	0.130	–	2.0
1	DEP	490 ± 17	455 ± 26	0.071 ± 0.005	5.8	0.050	–	4.1
2	DEP	483 ± 6	466 ± 13	0.035 ± 0.001	5.8	0.052	–	4.0
1	MP	520 ± 31	450 ± 27	0.134 ± 0.011	5.9	0.082	8.0 ± 0.7	2.7
2	MP	554 ± 24	455 ± 26	0.179 ± 0.013	6.1	0.092	7.8 ± 0.6	2.9
1	HP1	541 ± 10	515 ± 10	0.049 ± 0.001	6.1	0.078	–	3.5
2	HP1	545 ± 39	481 ± 13	0.119 ± 0.009	6.1	0.083	–	3.3
1	NaF	553 ± 22	523 ± 13	0.054 ± 0.003	6.2	0.084	–	3.8
2	NaF	535 ± 12	515 ± 3	0.037 ± 0.001	6.2	0.079	–	3.9
1	Na_2HPO_4	618 ± 37	498 ± 50	0.194 ± 0.023	6.1	0.109	7.7 ± 0.9	2.5
2	Na_2HPO_4	620 ± 40	507 ± 50	0.183 ± 0.021	6.0	0.128	7.7 ± 0.9	2.0
1	GGCC	515 ± 21	486 ± 8	0.058 ± 0.003	6.0	0.116	–	2.1
2	GGCC	533 ± 11	463 ± 25	0.132 ± 0.008	6.1	0.118	8.4 ± 0.5	2.4

$$J = \int \frac{F(\upsilon)_D\, e(\upsilon)_A \upsilon^4}{F(\upsilon)_D}\, d\upsilon \tag{3}$$

The distance for 50% energy transfer or the Förster distance, term R_0, is calculated with [Equation (4)]. The index of refraction, n, is 1.333 for aqueous solution. Quantum yields, Φ, were calculated for all of the Eu^{III} dinuclear complexes by using a method pioneered by Werts et al. from the collected emission spectra.[66] The orientation factor, κ^2, was taken as 2/3.

$$R_0^6 = 8.79 \times 10^{-25}\, \kappa^2 n^{-4} \Phi J \tag{4}$$

The efficiency of energy transferred from donor to acceptor is E and the distance between the donor and the acceptor is r. For the Eu^{III}–Nd^{III} dinuclear complexes, Eu^{III} is the donor and Nd^{III} is the acceptor. The overlap for this Ln^{III} pair can occur from 570 to 600 nm. All Eu^{III}–Nd^{III} LRET data is summarized in Table 3. Examples of the overlap integral between absorbance spectra and emission spectra are shown for EuNd(**1**) and TbEu(**1**) in Figure 3.

The largest Φ value was calculated for both Eu^{III} carbonate complexes, and the smallest values were for the complexes without anions added. Overlap integrals range from 1.8×10^{-18} to 4.1×10^{-18} M^{-1} cm^3 for all Eu^{III} dinuclear complexes (**1** and **2**) and were calculated from 570 to 610 nm. These overlap integrals are 2–4 times smaller than those reported for Eu^{III}/Nd^{III} pairs bound to proteins or to carboxylate-containing ligands. This leads to R_0 values ranging from 5.7 to 6.2 Å. The error in the distance r was calculated by propagating the standard deviations of the luminescence lifetimes, given that luminescence lifetimes are typically the greatest sources of error in FRET measurements.[67] In Table 3, luminescence lifetime data for the mixed Eu^{III}/Nd^{III} complexes are shown. Overlap integrals and Förster distances are given for the mixed Eu^{III}/Nd^{III} complexes. On the basis of this data, the distance between the two lanthanide centers is estimated by using Equation (1).

Table 3. Luminescence lifetimes in H_2O and D_2O and q numbers for Eu$_2$(**1** and **2**) complexes with and without anions at concentrations for fully bound anion (solutions contained 25 µM Eu$_2$(**1** or **2**), I = 0.10 M $NaNO_3$ and 20.0 mM HEPES pH = 7.0, unless otherwise stated). The q number is per Eu^{III} center.

Eu$_2$(**1**)	λ_{EX} (± 0.02 nm)	τ_{H2O} [µs]	τ_{D2O} [µs]	q
none	579.82	347 ± 12	1700[a]	2.0 ± 0.1
	579.84[a]	300[a]		1.9[a]
MP	579.76	520 ± 31	1700[a]	0.9 ± 0.1
DEP	579.76	490 ± 17	1700[a]	1.0 ± 0.1
HP1	579.76	541 ± 10	1700[c]	0.8 ± 0.1
	579.64[c]	537[c]		0.96[c]
GGCC	579.76	515 ± 21	1600[c]	0.9 ± 0.1
	579.70	490[c]		1.1[c]
Na_2HPO_4	579.56	618 ± 37	1715	0.6 ± 0.1
$NaHCO_3$	580.22	611 ± 13	1333[a]	0.4 ± 0.1
	580.20[c]	587[c]	1330[c]	0.59[c]
	580.45[c]	608[c]	1360[c]	0.50[c]
NaF	579.14	553 ± 22	2021	0.9 ± 0.1
	579.64	374 ± 30	1534	1.7 ± 0.2
Eu$_2$(**2**)	λ_{EX} (± 0.02 nm)	τ_{H2O} [µs]	τ_{D2O} [µs]	q
none	579.82	345 ± 15	1589	1.9 ± 0.1
	579.82[a]	310[a]	1700[a]	1.8[a]
MP	579.76	554 ± 45	1700[a]	0.8 ± 0.1
	579.82[b]	430[b]		0.7[b]
DEP	579.76	483 ± 6	1700[a]	1.1 ± 0.1
	579.82[b]	430[b]		0.7[b]
HP1	579.76	545 ± 39	1881	0.9 ± 0.1
GGCC	579.76	533 ± 11	1650	0.8 ± 0.1
Na_2HPO_4	579.56	620 ± 40	1692	0.5 ± 0.1
	580.22	596 ± 39	1441	0.5 ± 0.1
	579.82[b]	530[b]		0.2[b]
NaF	579.14	535 ± 12	1943	0.9 ± 0.1

[a] 0.5 mM Eu$_2$(**1** or **2**) (I = 0.10 M $NaNO_3$) from ref.[2] [b] 0.5 mM Eu$_2$(**1** or **2**) (I = 0.10 M $NaNO_3$ and 20.0 mM HEPES pH = 7.6), [MP] = 1.0 mM, [DEP] = 20 mM, [$NaHCO_3$] = 2.0 mM, from ref.[2] [c] 0.5 mM Eu$_2$(**1**) (I = 0.05 M $NaNO_3$, [anion] = 20 or 50 µM), errors in $\tau_{H2O} \leq 7\%$ and $\tau_{D2O} \leq 3\%$, from ref.[1] Error in τ is ± 10% if not indicated.

For the Tb^{III}–Eu^{III} dinuclear complex, we studied Tb^{III} as the donor and Eu^{III} as the acceptor. Tb^{III} and mixed Tb^{III}–Eu^{III} dinuclear complexes were excited at 487.00 nm,

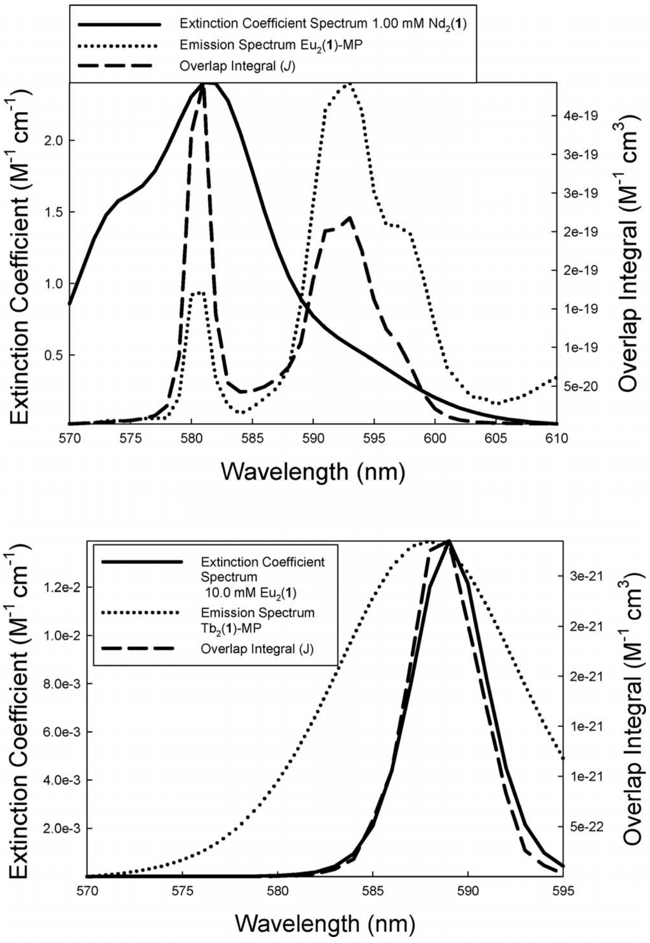


Figure 3. Examples of overlap integrals, absorbance spectra of the Ln^{III} donor, normalized luminescence spectra of the Ln^{III} acceptor to the largest intensity values of each spectrum for clarity (top) EuNd(**1**)-MP $J = 2.7\times10^{-18}\ \mathrm{M}^{-1}\,\mathrm{cm}^3$ and $R_0 = 8.0 \pm 0.7$ Å, (bottom) TbEu(**1**)-MP $J = 1.6\times10^{-20}\ \mathrm{M}^{-1}\,\mathrm{cm}^3$ and $R_0 = 3.0$ Å.

the $^7F_6 \rightarrow {}^5D_4$ excitation transition for Tb^{III}, and luminescence decays were monitored at about 545 nm. In most cases, the Tb^{III} donor luminescence lifetime was within experimental error of that for the Tb^{III}–Eu^{III} complex so that it was not possible to quantitate energy transfer by using luminescence lifetime changes. An added complication was that the luminescence lifetimes for the mixed Eu^{III}–Tb^{III} pairs were less reproducible than those of the homodinuclear Eu^{III} or Tb^{III} complexes for unknown reasons. Other experiments to attempt to observe energy transfer by exciting Tb^{III} to induce Eu^{III} luminescence failed. In these experiments, Tb^{III} excitation at 487 nm did not enhance emission of Eu^{III}; this data is shown in Table S3.

Speciation of Eu^{III} Dinuclear Complexes Bound to Anions

The speciation of dinuclear Eu^{III} complexes with bound anions is followed by monitoring the Eu^{III} $^7F_0 \rightarrow {}^5D_0$ excitation peak. The $^7F_0 \rightarrow {}^5D_0$ transition occurs between two electronic states that are non-degenerate and are thus not split by ligand fields; this gives rise to a single excitation peak for each Eu^{III} complex species, barring the overlap of peaks. Our previous work showed that, in the absence of added anions, Eu_2(**1**) and Eu_2(**2**) each have one major excitation peak assigned to the aqua species on the basis of the number of bound waters (*q* in Table 3) for each Eu^{III} center. In addition, we observed that carbonate binds extremely tightly to Eu_2(**1**) and Eu_2(**2**) so that solutions normally contain carbonate complexes if not specially treated for their removal.[1,2]

Eu_2(**1**) complexes of oligonucleotides, HP1, and GGCC have the most simple excitation spectra with a single major peak at 579.70 nm and a minor peak at 579.47 nm (Figure S1). Assignment of the minor peak to a species is difficult because it is not sufficiently resolved from the major peak to allow for characterization by time-resolved luminescence spectroscopy. One likely assignment is that there are two different diastereomers of the Eu(IIII) macrocyclic complexes in solution, one with a twisted square antiprism (TSAP) geometry and the other with a square antiprism (SAP) geometry. Analogous monomeric Eu^{III} macrocyclic complexes have overlapping excitation peaks, which result from two diastereomers.[36] Excitation at the major excitation peak shows that each Eu^{III} center has lost one water molecule to give hydration (*q*) values close to one. This shows that each Eu^{III} center has innersphere contacts to DNA. Previous studies show that approximately two dinuclear complexes bind to each oligonucleotide. Notably, no carbonate species are observed because of the strong binding of the dinuclear Eu^{III} complexes to DNA.[1]

Excitation spectra for Eu_2(**1**) bound to phosphate and MP are more complicated. A single major peak is observed for both complexes with a small redshifted peak at 580.26 nm. The frequency of the minor peak does not match that of the carbonate complex and remains unassigned. The small minor blueshifted peak is reminiscent of the presence of diastereomeric species (TSAP and SAP) as observed with analogous complexes. Notably, the species that gives rise to the major excitation peak assigned as a MP complex has a single water molecule on each Eu^{III} center, consistent with either a single bridging methylphosphate or with one methylphosphate bound at each Eu^{III} center, as discussed further below. In contrast, the phosphate complexes of both Eu_2(**1**) and Eu_2(**2**) have a *q* number that is less than one. This is similar to the hydration of the carbonate adducts where $q = 0.5$. The lower than expected *q* number may be attributed to a bidentate phosphate or, alternately, to additional outersphere interactions that increase quenching. It is unlikely that there is a complex with two phosphates bound per Eu^{III} center, given that there is only one excitation peak.

The excitation spectrum of Eu_2(**1**) with bound fluoride is the most unusual. A highly blueshifted excitation peak is observed at 579.11 nm in addition to a second major peak at 579.80 nm. Similar large blueshifts in the $^7F_0 \rightarrow {}^5D_0$ excitation peak of the aqueous Eu^{III} ion have been reported.[2] The hydration number of the major new species is close to one, consistent with the replacement of one water ligand on each Eu^{III} center. Given that fluoride is not a ligand that readily forms intramolecular bridges, the complex is best formulated as $Eu_2(\mathbf{1})(F)_2(H_2O)_2$. This formulation is also consistent with the LRET data as discussed below. The product represented by the second major peak (579.8 nm) has a *q* number and excitation peak frequency that is similar to that of the parent dinuclear Eu^{III} complex. One possible assignment for this species is an outersphere fluoride complex. Such outersphere complexes of Eu^{III} complexes have been shown to form with other monoanions such as phosphate diesters through hydrogen bonding to pendent groups and bound waters.[68–70] Formation of an outersphere complex does not perturb the excitation spectrum or change the *q* number.

It is interesting to consider the stoichiometry of anion binding to the dinuclear Ln^{III} complexes. Eu^{III} luminescence lifetime data for dinuclear complexes bound to all anions studied here can be fitted to a single exponential decay upon addition of saturating ligand concentrations. This suggests that either there is one anion bound to each Eu^{III} center (2:1 complex) and each Eu^{III} center acts independently or that a single anion bridges the two centers (1:1 complex). Previous work with $Eu_2(\mathbf{2})$ bound to MP was consistent with the formation of a 1:1 complex. In these earlier studies, the Eu^{III} luminescence intensity was constant after addition of a single equivalent of MP under conditions of tight binding.[63] In addition, the dinuclear Eu^{III} complexes are better catalysts for the cleavage of a phosphate diester than their mononuclear analogs under similar conditions, consistent with cooperative catalysis through two Eu^{III} centers. Thus, a 1:1 stoichiometry was assumed for binding of phosphate and methylphosphate to the two dinuclear complexes in the studies here. Binding of fluoride and carbonate are fitted to a 2:1 model according to our interpretation of the LRET data, which is consistent with the absence of a bridging anion for these complexes.

Comparison of the luminescence lifetimes presented in this work, generally, are in good agreement with our previously reported values (Table 3), especially, if a 10% error is taken into account for all recovered luminescence lifetimes, a common practice in the literature.[65] In addition, some deviation from previous experiments is expected, given that pH, ionic strength, Eu_2 complex concentration, and anion concentration are slightly different and this will influence the coordination environment of the Eu^{III} center.

Binding Strength

There is an increase in the binding affinity of $Eu_2(\mathbf{1})$ following the series DEP < MP < PO_4^{3-}. A stronger binding to the dianionic methyl phosphate relative to that to monoanionic DEP is anticipated on the basis of the higher negative charge of the phosphate monoester. Phosphate is a mixture of monoanionic and dianionic species at neutral pH. The binding constants here are effective binding constants because the protonated form of phosphate bound to Eu^{III} complex is not known. However, a stronger binding to $Eu_2(\mathbf{1})$ is anticipated on the basis of the greater basicity of the phosphate anion (HPO_4^{2-}) relative to those of the phosphate esters, which is consistent with our observations here. A comparison of the binding constants for the two different dinuclear complexes shows that both MP and DEP bind more tightly to $Eu_2(\mathbf{2})$ in comparison to $Eu_2(\mathbf{1})$. These modest differences suggest that the two xylyl linkers modulate the interactions of the Eu^{III} centers with anions.

As shown previously,[1] strong binding interactions are observed for $Eu_2(\mathbf{1})$ and the two DNA oligonucleotides with 1–2 binding sites on each oligonucleotide. Both oligonucleotides displace one water molecule on each Eu^{III} center of complexes of **1** and **2**. However, $Eu_2(\mathbf{1})$ binds the DNA hairpin loop (HP1) 10-fold more strongly than the self-complementary duplex, GGCC. The loop portion of HP1 facilitates phosphates and thymidine bases to orient into a cationic binding pocket.[71] This localization of the phosphate likely explains the larger affinity of the dinuclear complex for HP1 rather than for the canonical GGCC duplex. Thus, there is a modest binding selectivity of the two dinuclear Eu^{III} complexes for different DNA structures.

Fluoride and carbonate bind remarkably strongly to $Eu_2(\mathbf{1})$ with regard to the first anion binding. The second anion binding is weaker (by either one or two orders of magnitude). Carbonate binds tightly to related Eu^{III} complexes that have septadentate macrocyclic ligands and two water ligands. At least one water ligand is replaced by carbonate in these complexes.[11,72] Fluoride displaces a single water molecule on each Eu^{III} center to form a 2:1 complex. Fluoride has been shown to bind strongly to the Eu^{III} aqua ion[68] as the only halide that forms innersphere complexes. Speciation diagrams are shown in Figure S2.

Emission Peak Ratios

Eu^{III} emission peaks are often useful to monitor the binding of anions by using a ratiometric analysis.[13,73] This is because the emission peaks of Eu^{III} arise from alternately either magnetic-dipole- or electric-dipole-allowed transitions and so respond differently to the formation of new complexes by anion addition. The most commonly monitored emission peaks for ratiometric analysis are the $^5D_0\rightarrow{}^7F_1$ and the $^5D_0\rightarrow{}^7F_2$ transitions. A change in the Eu^{III} emission peak ratio upon anion binding is observed here for all of the anions studied (Table S1), which suggests that the Eu^{III} complexes will be useful as ratiometric sensors. In contrast, the ratios of the three prominent emission peaks of Tb^{III}, which are a result of a mixture of magnetic- and electric-dipole-allowed transitions, do not change substantially upon binding of anions.

LRET and Ln^{III}–Ln^{III} Distance

The small R_0 values (6 Å) for the Eu/Nd pairs observed here are attributed to inefficient energy transfer, largely because of the small overlap of donor emission and acceptor absorption in these complexes. This makes it challenging to measure distances in the dinuclear complexes studied here, which have a rather large metal–metal separation. The small overlap integral between donor emission and acceptor absorption gives rise to even smaller R_0 values (3 Å) for the Tb^{III}/Eu^{III} pair. LRET in this Ln/Ln couple is too inefficient for measurement of luminescence lifetime differences in our complexes. Previous LRET studies of either Eu^{III}/Nd^{III} or Tb^{III}/Nd^{III} pairs used complexes with a more favorable spectral overlap.[74,75]

The dinuclear Ln^{III} complexes here display intranuclear distances of 7.6 Å or greater as determined by Eu^{III}/Nd^{III} LRET. The small values of energy transfer derived from small changes in lifetimes are difficult to accurately measure, which leads to large errors in *r*, the intranuclear distance. Luminescence lifetimes generally do decrease for the Eu^{III}–Nd^{III} dinuclear complex relative to the Eu^{III}–Eu^{III} complex, although the decrease, in many cases, is not greater than experimental error. For these cases, we have not calculated intranuclear Ln^{III}–Ln^{III} distances.

Energy transfer is observed for the dinuclear Eu^{III}–Nd^{III} complex in the absence of anions, but the differences in lifetimes are barely larger than experimental error. Our calculations give Ln^{III}–Ln^{III} distances of 7.6 ± 0.6 and 7.9 ± 0.4 Å for Eu$_2$(**1**) and Eu$_2$(**2**), respectively. These are somewhat less than those in the solid-state structure of a dinuclear sodium(I) complex of an analogous ligand, which shows an intranuclear distance of 9 Å.[22]

Luminescence resonance energy transfer experiments for the dinuclear complexes are consistent with two classes of anions. For the first class, a measurable energy transfer between the Eu^{III} and Nd^{III} centers is observed, as indicated by a decrease in the luminescence lifetime for Eu^{III} in mixed Eu^{III}/Nd^{III} complexes, and for the second class, no LRET is seen within experimental error. The phosphate and MP phosphate anions for both dinuclear Ln^{III} complexes are in the first class of anions. The LRET calculations are consistent with a distance of 7.7 to 8.0 Å between the Ln^{III} centers for complexes of MP and phosphate. These intranuclear distances as measured by LRET are larger than those observed in six dinuclear Ln^{III} crystal structures containing a bridging phosphate or phosphate ester (average distance between the dinuclear Ln^{III} centers 6.1 ± 0.2 Å).[76–81] The largest distance is seen for a dinuclear La^{III} bridging phosphate complex (6.2 Å)[78] and the smallest for a dinuclear Eu^{III} bridging complex (5.7 Å).[79] Thus, the LRET data is not entirely consistent with a bridging ligand in either dinuclear Eu^{III} complex although the aqueous complexes are solvated and dynamic, and this may lead to a larger average distance. However, these two anions are clearly unique in that they promote energy transfer, consistent with an interaction of the two Eu^{III} centers mediated through phosphate or MP.

Binding of GGCC DNA to Eu^{III}–Nd^{III}(**2**) leads to an estimated distance of 8.4 Å between the Ln^{III} centers. The LRET data, in conjunction with the luminescence lifetime data, are consistent with the forming of innersphere connections by each Ln^{III} center to distinct sites in DNA. All other DNA complexes have luminescence lifetimes for the homodinuclear Eu^{III} complex (τ_D) that are within experimental error of lifetimes for the mixed Eu^{III}–Nd^{III} complex (τ_{DA}) so that distances cannot be calculated by using LRET methods. Similarly, Ln$_2$(**1**) complexes with other anions including carbonate and fluoride have Ln^{III}–Ln^{III} distances that cannot be measured by using LRET methods. Presumably, these complexes have Ln^{III}–Ln^{III} distances that are even larger than those of the MP or phosphate complexes (>8 Å), which makes it difficult to measure differences in luminescence lifetimes given the small R_0 value for the Eu^{III}–Nd^{III} pair. This data is consistent with the absence of bridging ligands in the Ln$_2$(**1**) and Ln$_2$(**2**) complexes of carbonate and fluoride.

Conclusions

In summary, lanthanide(III) dinuclear macrocyclic complexes that contain aromatic linkers strongly bind to a series of biologically relevant anions. In all cases, water ligands are displaced, and both lanthanide centers interact with the anions. LRET studies are in agreement with a shorter Ln^{III}–Ln^{III} distance for certain anions including phosphate, MP, and GGCC DNA, which is consistent with bridging interactions. Other ligands including DNA hairpin loops, carbonate, and fluoride form dinuclear Ln^{III} complexes with no measurable energy transfer between the Eu^{III}–Nd^{III} pairs. This work suggests that LRET measurements in conjunction with other lanthanide(III) luminescence properties may be useful in distinguishing between different types of biologically relevant anions in solution.

Experimental Section

Materials: Ln(CF$_3$SO$_3$)$_3$ salts were purchased from Aldrich. Synthetic DNAs was purchased from Integrated DNA Technologies Inc. (IDT) in the desalted form. The oligonucleotides were annealed by heating solutions to 90 °C for 5 min and by allowing to cool to room temperature. 1,3-Bis[1-(4,7,10-tris(carbamoylmethyl)-1,4,7,10- tetraazacyclododecane)]-*p*-xylene (**1**) was prepared as described previously.[2] The synthesis of 1,4-bis[1-(4,7,10-tris(carbamoylmethyl)-1,4,7,10- tetraazacyclododecane)]-*m*-xylene (**2**) was either produced as described previously[36] or by using a different synthetic approach.[82] Milli-Q purified or triply distilled water was boiled and bubbled with nitrogen or argon gas for 1 h prior to use in photoluminescence measurements to minimize carbonate concentrations.

General Instrumentation: A 720 SM Titrino (Metrohm, Inc.) equipped with a temperature compensation and Thermo Orion ROSS pH 81152 probes (Thermo Electron, Inc.) were used for all pH measurements. A Beckman Coulter DU® 820 UV/Vis spectrophotometer equipped with a high performance Peltier temperature controller was utilized for all UV/Vis experiments. A 5-cm pathlength cuvette (Starna Cells, Inc.) was used for the UV/Vis absorbance measurements of the Ln$_2$ complexes of **1** and **2**.

Ln^{III} Complexes: The complexes were formed in an aqueous solution by incubation of ligand **1** (or **2**) (1.00 mM, 1 µmol) and Eu^{III}, Nd^{III} or Tb^{III} (2.00 mM) or 1/20 mixtures of Eu/Nd or Tb/Eu(CF$_3$SO$_3$)$_3$ in HEPES (20.0 mM, pH 7.0) over a period of 1 h.

Complex formation was verified by Eu^{III}/Tb^{III} luminescence spectroscopy and by ESI-MS spectroscopy. As previously reported[1] for the removal of Ln^{III}-bound carbonate, $Pb(NO_3)_2$ (25 mM) was added to the Ln_2(**1**) and Ln_2(**2**) solutions to precipitate adventitious carbonate as $Pb(CO_3)$. To remove the excess Pb^{II} were added solutions of NaCl (25 mM) followed by NaOH (carbonate free, 25 mM) to bring the solution back to neutral pH. The solution was vortexed for 30 s and spun in a refrigerated Savant μ-SpeedFuge SFR 13K centrifuge for 60 min at an RCF of 13000×g. The supernatant was removed from the white pellet.

Solutions of complexes for UV/Vis absorbance spectroscopy were prepared in the same manner as the solutions for excitation and emission spectroscopy at complex concentrations of 10.0 mM for Eu^{III} and 1.00 mM for Nd^{III} in HEPES buffer pH at 7.0, $I = 0.10$ M $NaNO_3$.

Photoluminescence Ln^{III} Spectroscopy: Eu^{III} and Tb^{III} steady-state and time-resolved photoluminescence lifetimes were obtained by using a Spectra-Physics Quanta Ray PRO-270–10 Q-switched Nd:YAG pump laser (10 Hz, 60 mJ/pulse) coupled to a MOPO-SL for all luminescence measurements, which is described in detail elsewhere.[65] An alteration was made to the previously described MOPO system: the NIR 5107 Hamamatsu PMT was replaced with a R982 Hamamatsu PMT for emission measurements. The $^7F_0 \rightarrow {}^5D_0$ transition of the Eu^{III} cation was scanned between 578 and 581 nm, while the $^5D_0 \rightarrow {}^7F_2$ emission band was monitored at about 617 nm by using a 628 ± 27 nm bandpass filter (Semrock, Inc.). The Tb^{III} complexes were excited at 487.80 nm, and the emission was monitored at 556 ± 20 nm by using a bandpass filter (Semrock, Inc.). Spectral peaks were fitted by using the program Peak Fit v4.12 (Jandel Software, Inc.).

Steady-State Photoluminescence Emission Spectroscopy: Emission spectra of the dinuclear Ln^{III} complexes were collected at 1-nm increments. The Eu_2(**1** or **2**) and Eu^{III}/Nd^{III}(**1** or **2**) complexes were excited at 465.00 nm, the $^5D_2 \rightarrow {}^7F_0$ transition. Emission spectra were collected from 500 to 720 nm with a resolution of about 1 nm. The emission spectra were recorded for the Tb_2(**1** or **2**) and TbEu(**1** or **2**) complexes that were excited at 487.00 nm, the Tb^{III} $^7F_6 \rightarrow {}^5D_4$ transition. Anions were added at concentrations under which they were fully bound. The emission spectra were blank corrected. Quantum yields were calculated from the area of the Eu^{III} emission peaks[66] by using PeakFit v4.12 (Jandel Software, Inc.); a Voigt function was chosen to fit the peaks in order to determine the peak center maximum, full width at half-maximum (FWHM or peak width), and peak area.

Time-Resolved Photoluminescence: Time-resolved photoluminescence measurements were collected by using a digital Tektronix TDS 3034B oscilloscope. Five data sets per wavelength were fitted to a single exponential decay by using Graphpad Prism 4 (Graphpad Prism Software, Inc.). Lifetimes were averaged from at least three independent samples. Errors in the lifetimes are reported as the standard deviations of the three luminescence lifetimes. In all cases, the best-fit model for the time-resolved intensity decay traces was a single-exponential decay as determined by the linearity of the log intensity vs. time plot and a symmetrical distribution of the residuals about zero. In some cases, the first 20 µs of the decay were not fitted because of asymmetric distributions in the residual plots. This short component is sometimes observed in luminescence decays and ranges from 1 to 30 µs. We have been unable to correlate this short component to the samples and believe it to be an instrumental artefact, which may be related to laser-induced damage of the cuvette. Measurements of the complexes collected in D_2O were at equivalent ionic strength ($I = 0.1$ M $NaNO_3$) and pD = 7.0 by using 20 mM HEPES.

Steady-State Excitation Spectra Titration Experiments: The intensity of the Eu^{III} excitation spectra as a function of anion concentration was fitted by using Equation (5) in Sigma Plot 10 for MP and PO_4^{3-}. Here [LS] is the concentration of the Eu^{III}–anion complex, K_d is the apparent dissociation constant, M is the total concentration of the Eu^{III} complex, A is the anion concentration, n_s is the number of binding sites (fixed at one), X_M is the mol fraction of the Eu^{III} complex, X_{LS} is the mol fraction of the Eu^{III}–anion complex, I_M is the luminescence intensity of free Eu^{III}, and I_{LS} is the intensity of the Eu^{III}–anion complex. The solutions include 25–250 µM Eu^{III}, 20 mM MES buffer pH 6.5, 100 mM NaCl, and 0–5.0 mM anion.

$$[LS] = 0.5\,(K_d+M+n_sA - \sqrt{(K_d+M+n_sA)^2-(4n_sMA)}$$
$$I_{obs} = X_MI_M+X_{LS}I_{LS} \tag{5}$$

The Eu^{III} excitation peak intensity as a function of DNA (HP1 and GGCC) gave binding isotherms that were fitted to Equation (6) for a simple solution system that involves non-competitive tight binding of metal complex to multiple binding sites on DNA. Here I_0 and I are the luminescence intensity in the absence and presence of nucleic acid, R_b is the luminescence intensity of the bound complex, M is the concentration of the complex, K_d is the apparent dissociation constant, n is the number of bases per binding site, A is the nucleic acid concentration, x is the concentration of DNA times the number of bases in the sequence. The solution includes 25 µM complex, 20 mM HEPES buffered at pH 7.0, 0.10 M $NaNO_3$, and 5–50 µM nucleic acid.

$$(I-I_0) = 0.5\,(\frac{R_b}{n})\left[M+n_sA-\sqrt{(\frac{n}{K_d}+nM+Ax)^2-(4nMAx)}\right] \tag{6}$$

For the carbonate and fluoride anions, HyperSpec was used to fit binding isotherms to determine the dissociation constants for the binding of two anions. Conditions for the NaF and $NaHCO_3$ titrations of Eu_2(**1**) were pH 7.0, 20 mM HEPES with $I = 0.10$ or 0.050 M $NaNO_3$, respectively. Two association constants were refined for Eu_2(**1**)(CO_3) and Eu_2(**1**)$(CO_3)_2$. There are potentially other species in solution, but this simple equilibrium modeled the solution chemistry with HyperSpec (Protonic Software).[83,84] The parameter log β for only two species, including Eu_2(**1**)-F and Eu_2(**1**)-F_2, were refined for the titration of Eu_2(**1**) with NaF, and no other equilibria were accounted for during the refinement. HYSS2009 (protonic software) was used to generate speciation diagrams.

Supporting Information (see footnote on the first page of this article): Peak fitting analysis, tables of normalized emission peaks, and binding isotherms are presented.

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