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Luminescent Lanthanide Probes for Inorganic and Organic Phosphates

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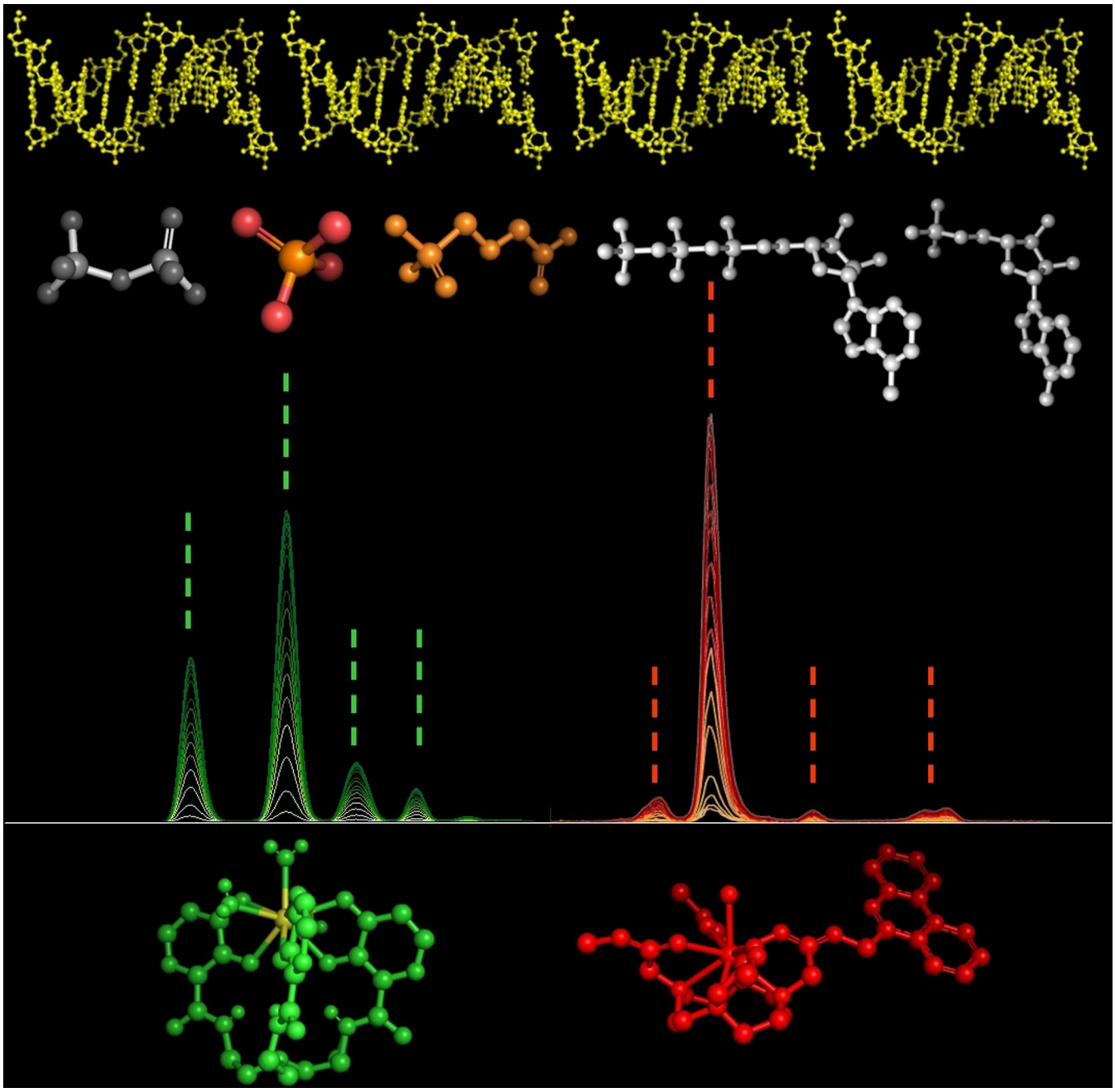

Abstract: Inorganic and organic phosphates-including orthophosphate, nucleotides, and DNA-are some of the most fundamental anions in cellular biology, regulating numerous processes of both medical and environmental significance. The characteristic long lifetimes of emitting lanthanides, including the brighter europium(III) and terbium(III), make them ideally suited for the development of molecular probes for the detection of phosphates directly in complex aqueous media. Moreover, given their high oxophilicity and the exquisite sensitivity of their quantum yields to their hydration number, those luminescent lanthanides are perfect for the detection of phosphates. Herein we discuss the principles that have guided the recent developments of molecular probes selective for inorganic or organic phosphates and how these lanthanide complexes facilitate the study of numerous biological processes.

1. Introduction

Inorganic and organic phosphates are among the most important biological anions. Inorganic phosphates, present as a near 1:1 ratio of $H_2PO_4^-$ and HPO_4^{2-} at neutral pH, buffers intra and extracellular media and is a key component of hard tissues such as bones and teeth. Nucleotides such ATP and GTP are small organic phosphates with crucial roles in energy metabolism, as well as allosteric regulators, physiological mediators, activated intermediates and components of co-enzymes. Nucleotides are also the precursors of nucleic acids, both DNA and RNA, which store and express genetic information. Given their biological significance, numerous small organophosphates have therefore been investigated for both medical and environmental applications.

Understanding and exploiting the intricate roles of both inorganic and organic phosphates, including screening of molecules targeting phosphate-dependent enzymes, is achieved more readily with luminescent probes. The accuracy of these assays require probes that are stable, function in water at neutral pH within the concentration range of the targeted phosphate and with high selectivity over competing anions. Phosphates are, however, highly hydrophilic. The high hydration energy of inorganic phosphate ($\Delta G_{hydration}H_2PO_4^- = -465\ kJ\,mol^{-1}$)[1] positions it among the highest anions in the Hofmeister series. In water, this hydration outcompetes with hydrogen-bonding and electrostatic interactions even if they are predispositioned into tetrahedral symmetry, as is commonly featured in organic receptors. Most organic receptors, therefore, do not function in water.[2]

Lanthanides are well recognized for their strong oxophilic character; a property that, unlike for early transition metals, is not only a result of their hardness but also arises from the low ionization potential of their 4f-valence electrons that contribute to their overall poor electronegativity.[3] Moreover, the ionic nature of lanthanide coordination favours the more basic anions. In the absence of steric hindrance, the affinity of tripodal Gd^{III} complexes for anions was determined to follow directly not with the hardness but with the basicity of the anion.[4] These properties advantageously confer lanthanide complexes with an intrinsic preference for phosphate over bicarbonate and fluoride as well as other anions. Together with their lability that enables rapid coordination of anions, the characteristic coordination chemistry of lanthanides makes them particularly well-suited for the recognition of phosphates in water, the only solvent relevant to biological studies.

It is these characteristics that are increasingly exploited in the design of lanthanide-based luminescent probes for inorganic and organic phosphates that we review herein with a focus on the more recent developments over the last decade. While outside the scope of this contribution, recent progress on lanthanide-based receptors for inorganic phosphate involving luminescent lanthanide MOFs,[5] nanoprobes,[6] and coordination polymers[7] should also be noted.

1.1. Principles of Sensitized Lanthanide Luminescence

The field of luminescent lanthanide probes has grown dynamically over the last several decades and has been the subject of thorough reviews including on their design principles,[8] applications to anion recognition,[9] enzyme activity assays,[10] and DNA probes.[11] The following discussion will therefore focus on the key parameters of lanthanide luminescence as it concerns the recognition and sensing of inorganic and organic phosphates.

Central to the applications of luminescent lanthanide probes for analyte sensing and high throughput screening of enzymes and receptors is the Laporte-forbidden nature of the f→f transitions. Because of this forbidden nature, lanthanide-centered luminescence is characterized by narrow emission bands occurring at fixed wavelength, very large Stokes shifts, and extremely long luminescence lifetimes (Figure 1a). The ms lifetimes of Eu^{III} and Tb^{III} phosphorescence, the most emissive lanthanides, facilitates time-delayed spectroscopy, allowing for the signal to be accurately quantified without interference from the fast-decaying autofluorescence of the sample via the incorporation of a gate time (Figure 1b).

The Laporte forbidden nature of the f→f transitions also dictates that lanthanide ions have a negligible absorption

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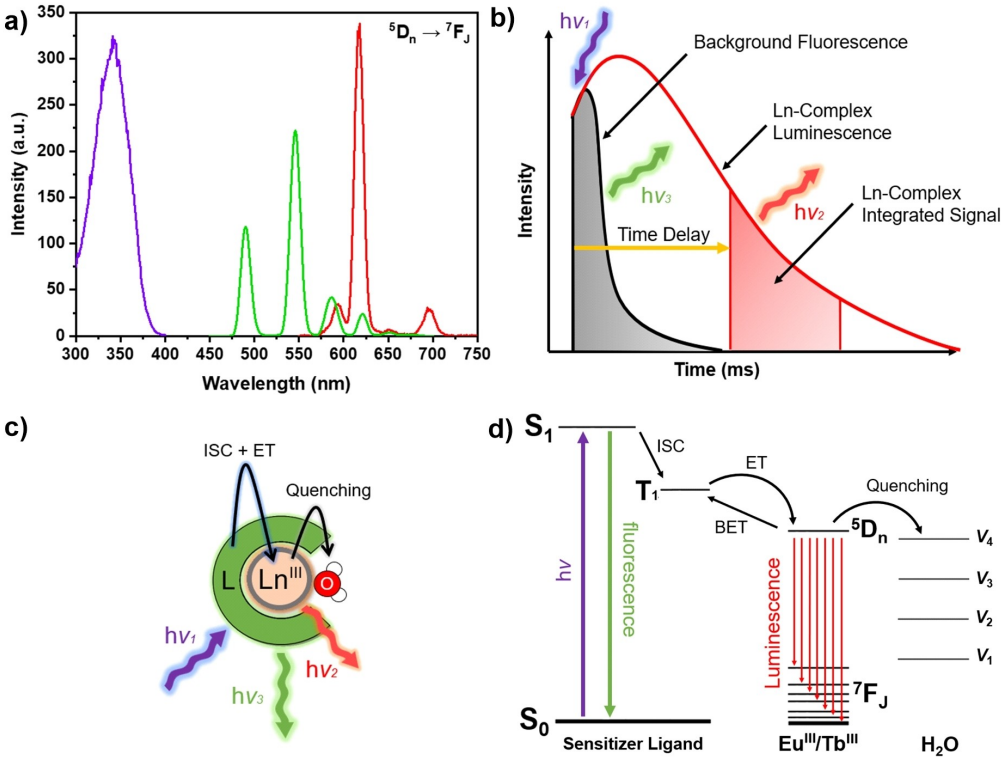

Figure 1. Fundamentals of sensitized lanthanide luminescence: a) Representative absorbance and emission spectra of sensitized Eu^{III} and Tb^{III} demonstrating the characteristic large Stokes shift between absorption of the antenna (blue) and narrow lanthanide-centered emission bands of europium(III) (red) and terbium(III) (green). b) Time-gated spectroscopy exploits the long luminescent lifetimes of lanthanide complexes to remove the interfering autofluorescence of complex biological media via the incorporation of a time delay. c) Excitation pathways within a coordinated lanthanide complex. The antenna absorbs light at hv_1; ISC and ET to the lanthanide enables lanthanide-centered phosphorescence at hv_2 concomitant with ligand-centered fluorescence at hv_3. Coordinated water molecule(s) partially quench lanthanide-centered emission. d) Simplified Jablonski diagram of sensitized luminescence for Eu^{III} and Tb^{III}.

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coefficient. This problem is circumvented by indirect excitation via a sensitizer or "antennae" incorporated in the ligand (Figure 1c and 1d). These chromophores absorb UV-visible radiation and sensitize the lanthanide either via Dexter electron exchange,[12] as is the case for Eu^{III}-1,2-HOPO complexes (Figure 3),[13] or via Förster resonance energy transfer (FRET)[14] mechanisms, as typical for probes where the antennae does not coordinate the lanthanide ion. The quantum yield of the probe (Φ_{Ln}), which defines the efficiency by which photons absorbed by the antennae are emitted by the lanthanide ion, is dependent on, among other parameters, the efficiency of energy transfer (ET) and intersystem crossing (ISC), and the energy difference between the triplet energy state (T_1) and the excited state of the lanthanide ion (^5D_n). If this difference is too large, ET to the lanthanide is inefficient; if it is too small, back energy transfer (BET) to the antennae triplet state occurs, thereby reducing lanthanide emission. Probes that function via a Förster mechanism are highly dependent on the distance separating the antennae from the lanthanide ion, a limitation that has been exploited in the design of responsive probes. Another key parameter influencing the brightness of lanthanide complexes is the fourth overtone of nearby O–H and N–H oscillators, which are slightly lower in energy than the excited ^5D_n states of both Eu^{III} and Tb^{III}. As such, any water molecules directly coordinated to the lanthanide will partially quench lanthanide

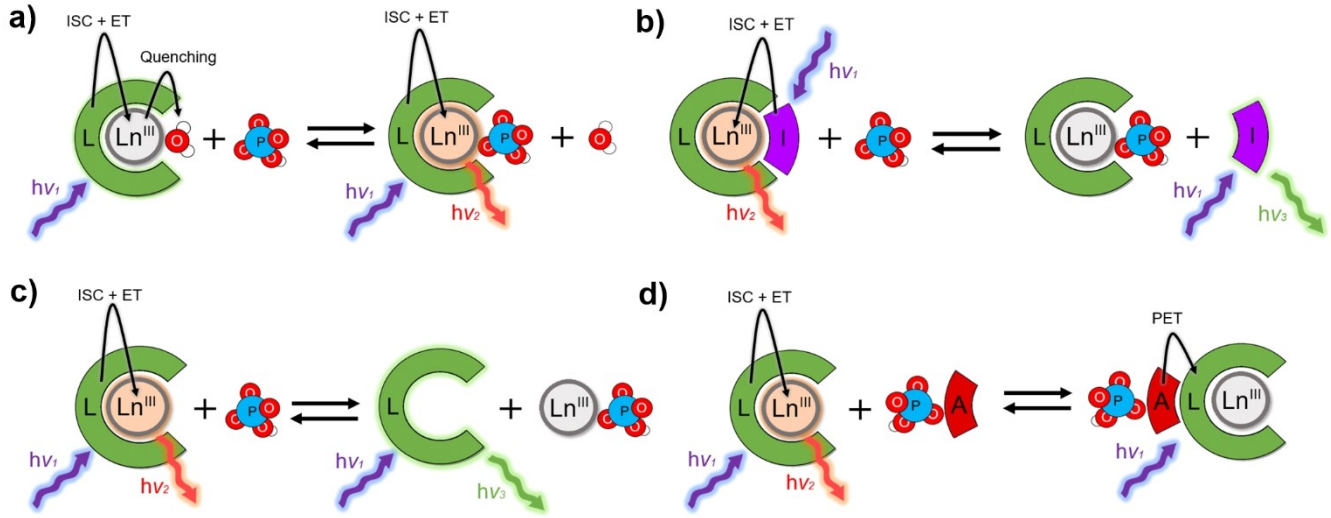


Figure 2. Common design of luminescent lanthanide probes for inorganic and organic phosphates. a) Displacement of water molecules that quench Eu^{III} and Tb^{III}'s phosphorescence by a coordinating phosphate restores the lanthanide-centered luminescence. b) Displacement of a weakly coordinating indicator by a phosphate guest prevents sensitization of the lanthanide by the indicator. c) Weakly coordinating sensitizing ligands undergo ligand exchange with phosphate, resulting in insoluble phosphate salts that are not luminescent. d) Outer-sphere interactions between a phosphate anion and the antenna modulates energy transfer between the antenna and the lanthanide ion.

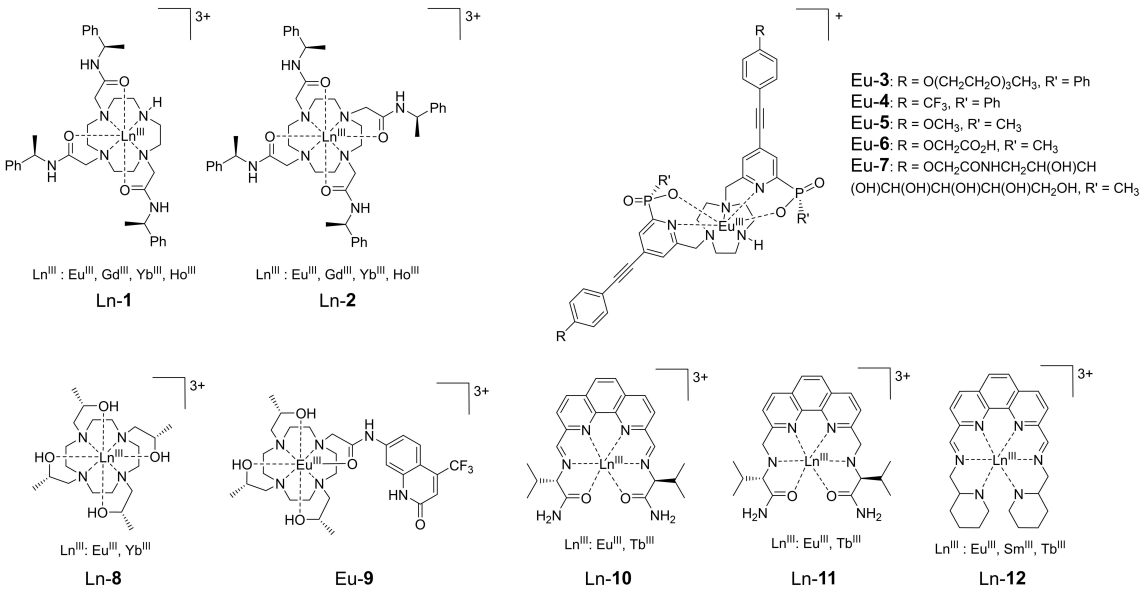


Figure 3. Responsive luminescent lanthanide probes for inorganic phosphate functioning via direct coordination of the anion.

emission and reduce their lifetimes. This dependence is key to the design of numerous probes for phosphates that function by direct coordination to the metal via displacement of water molecules.

Almost all luminescent lanthanide probes employ either Eu^{III}, with a characteristic red emission centered at 615 nm, or Tb^{III}, with a primary emission band at 545 nm, due to the large energy gap between their ground state and their lowest-lying excited state from which luminescence arise (Figure 1a and 1d). Other lanthanides, including Sm^{III} ($^4G_{5/2}\rightarrow{}^6H_J$) and Dy^{III} ($^4F_{9/2}\rightarrow{}^6H_J$), have smaller energy gaps that favor non-radiative decay to peripheral high-energy vibrators, which leads to poor quantum yields and are therefore rarely used.[8a] Although not yet used for the detection of phosphates, fluorescing lanthanides such as Er^{III} ($^4I_{13/2}\rightarrow{}^4I_{15/2}$), Nd^{III} ($^4F_{3/2}\rightarrow{}^4I_J$), and Yb^{III} ($^2F_{5/2}\rightarrow{}^4I_J$) does offer promising characteristics, particularly since their near infra-red (NIR) emission facilitates imaging in biological tissues.[15]

1.2. Design of Receptors for Phosphate

The dependence of lanthanide-based luminescence on the parameters described above opens the door to numerous

approaches for the design of responsive probes that are unique to f-block elements and have been described in prior reviews.[8d] A few of these are particularly well-suited for the detection of inorganic and organic phosphates and will be detailed here.

The most common approach to detecting phosphates exploit the oxophilicity of lanthanide ions which favors coordination of phosphate over water, and the propensity of bound water molecules to quench Eu^{III} and Tb^{III} phosphorescence. In these cases, the presence of one or more inner-sphere water molecules in the "off" state decreases substantially both the quantum yield and the luminescence lifetime of the emitting lanthanide. If the complex $Ln^{III}L$ is stable, phosphate coordination occurs via displacement of the quenching water molecules, resulting in an increase in both Φ and τ (Figure 2a). Since lanthanide ions are labile, this process is reversible, which enables direct monitoring of enzymatic reactions that affect the position of this equilibrium. Advantageously, this mechanism is easily probed by determining the number of inner-sphere water molecules, q, via the isotope effect on the luminescence lifetimes of Eu^{III} or Tb^{III} complexes as follows:[16]

$$q_{Eu} = 1.2[(\ 1/\tau_{H_2O} - \ 1/\tau_{D_2O}\ ) \ - \ 0.25 - 0.0075x] \quad (1)$$

$$q_{Tb} = 5[(\ 1/\tau_{H_2O} - \ 1/\tau_{D_2O}\ ) \ - \ 0.06x] \quad (2)$$

where q is the number of inner-sphere water molecule, τ_{H_2O} and τ_{D_2O} are the luminescence lifetimes of the probe in H_2O and D_2O, respectively, and x is the number of nearby N–H or O–H oscillators arising from the ligand. If coordination of phosphate occurs only via displacement of inner-sphere water molecules, a clear decrease in q will be observed. Of note, similar relationships have been established for NIR fluorescing lanthanides.[17]

Indicator displacement assays are a modification of this mechanism whereby phosphate does not displace inner-sphere water molecules but a weakly-coordinated indicator. If this indicator is also the lanthanide sensitizer, its displacement will results in a decrease in lanthanide-centered emission (Figure 2b). If, on the other hand, the indicator is a quencher, its displacement by phosphate will result in an increase in intensity.

As will be detailed later, these two mechanisms requires stable $Ln^{III}L$ complexes. Unstable ones will undergo ligand exchange where L decomplexes the Ln^{III}, resulting in the formation of insoluble Ln^{III} phosphate salts (Figure 2c). Because of its heterogeneous nature, such equilibriums are poorly controllable and reversible and are not appropriate to the study of complex biological or environmental samples.

Recognition of phosphates does not necessarily require direct coordination to the lanthanide ion. Outer-sphere binding is also an efficient strategy for the recognition and detection of nucleotides and nucleic acids. In these cases, weak interaction between the organic phosphate and the antenna is sufficient to favor photoelectron transfer between the nucleobase and the antenna, which in turn decreases lanthanide sensitization (Figure 2d). This approach is favored for the detection of both nucleotides and nucleic acids.

2. Luminescent Probes for Inorganic Phosphate

Inorganic phosphate (Pi) is present in water at neutral pH as a near-equal ratio of $H_2PO_4^-$ and HPO_4^{2-}. For healthy patients, its concentration in blood ranges from 0.8 – 1.45 mM, while much higher concentrations are present within cells (up to 100 mM). Given the higher concentration of HCO_3^- (23-30 mM) and Cl^- (98-106 mM) in blood, biomedical applications require highly selective probes. Environmental applications have even stringier requirements since the low ppm levels of phosphate present in even polluted surface water (0.1–1 μM) require probes that have a substantially higher affinity for phosphate. Nonetheless, although not all luminescent lanthanide probes fulfill each of these requirements, more recent ones have been developed with sufficient sensitivity, selectivity, and efficacy in water, to be successfully deployed. All function via direct coordination of the oxyanion to the lanthanide ion, although some also employ indicators. Do note that the requirements in term of sensitivity (anion affinity) and selectivity are dictated by the intended application. Luminescent probe for sensing inorganic phosphate in blood or environmental water samples do not need to be selective over nucleotides because there is no nucleotide present in either blood or surface or wastewater samples. For these applications, selectivity over bicarbonate and other endogenous anions are more relevant. The properties and molecular composition of these complexes are summarized in the following Table 1 and Figures 3–6.

2.1. Recognition of Phosphate via Formation of Ternary Complexes

The majority of lanthanide coordination complexes used in biomedical imaging employ polyaminocarboxylate ligands due to their high-water solubility, high stability, and ease of synthesis. In the absence of steric hindrance, most neutrally or positively charged lanthanide complexes of polyaminocarboxylate ligands with one or more open coordination site will bind hard anions such as phosphate. As was first demonstrated by Dickins with Ln-**1** and Ln-**2** (Figure 3), such complexes have nearly no selectivity and also bind bicarbonate, acetate and fluoride with very similar affinity.[18] More recently, Butler developed brighter derivatives employing pyridine-type antennas that permeated cells more effectively due to their hydrophobic nature, distributing primarily in lysosomes.[19] Although brighter than Ln-**1**, the receptors Eu-**3** to Eu-**7** also demonstrated very poor selectivity, binding not only phosphate but also phosphorylated amino acids, bicarbonate, citrate, and lactate.

Some selectivity can be achieved by increasing steric hindrance at the open coordination site. As demonstrated by Morrow, the PARACEST agent Eu^{III}(S-THP) (Eu-**8**) similarly cannot differentiate between organophosphates, inorganic phosphate, carbonate, or carboxylates.[20] Addition of a pendant chromophore near the open coordination site resolves some of the selectivity problem. The derivative Eu^{III}(THPC) (Eu-**9**), for in-

Table 1. Affinity and selectivity in select solvent of lanthanide probes for inorganic phosphate.

Complex	Selectivity	Solvent	Binding Affinity	Ref.
Yb-**1**	P_i	H_2O	n.d.	18
	F		n.d.	
	acetate		n.d.	
	oxalate		n.d.	
	lactate		n.d.	
	HCO_3^-		n.d.	
Eu-**3**	HCO_3^-	H_2O	log K_a 3.47	19
	lactate		log K_a 3.51	
	citrate		log K_a 3.54	
	benzoate		log K_a 3.73	
	P_i		log K_a 3.78	
	O—P-Tyr		log K_a 4.10	
	O—P-Ser		log K_a 3.20	
	O—P-Thr		log K_a 3.30	
Eu-**4**	HCO_3^-	H_2O	log K_a 3.35	19
	lactate		log K_a 3.28	
	citrate		log K_a 4.14	
	Pi		log K_a 3.72	
	O—P-Tyr		log K_a 4.20	
	O—P-Ser		log K_a 3.90	
	O—P-Thr		log K_a 3.70	
Eu-**5**	lactate	H_2O	log K_a 2.70	19
	citrate		log K_a 3.91	
	P_i		log K_a 3.52	
	O—P-Tyr		log K_a 3.37	
	O—P-Ser		log K_a 2.71	
	O—P-Thr		log K_a 2.80	
Eu-**6**	HCO_3^-	H_2O	log K_a 3.33	19
	lactate		log K_a 3.20	
	citrate		log K_a 3.92	
	P_i		log K_a 2.98	
	O—P-Tyr		log K_a 3.71	
	O—P-Ser		log K_a 2.44	
	O—P-Thr		log K_a 2.94	
Eu-**7**	n.d.	H_2O	n.d.	19
Eu-**8**	P_i	H_2O	K^e 0.3, 3.0 mM	20
	acetate		K^e 14 mM	
	lactate		K^e 13 mM	
	citrate		K^e 0.016 mM	
Yb-**8**	HCO_3^-		K^e 33 mM	
	MeP_i		K^e 0.7, 9.8 mM	
	DEP		K_d 1.9 mM	63
	DEP		K_d 2.8 mM	63
Eu-**9**	P_i	H_2O	K^e 4.2 mM	20
	acetate		K^e 0.21 mM	
Eu-**11**	P_i	H_2O	log K_b 5.4	21
	NO_3		log K_b 2.9	
	ATP		n.d.	
	ADP		n.d.	
	AMP		n.d.	
Tb-**11**	P_i	H_2O	log K_b 5.5	21
	NO_3		log K_b 4.6	
	ATP,		n.d.	
	ADP,		n.d.	
	AMP		n.d.	
Eu-**12**	P_i	H_2O	log K_{a1} 4.3	22
			log K_{a2} 3.0	
	HCO_3^-		log K_a 3.1	
	ATP		log K_{sv} 4.0	45
Tb-**12**	P_i		log K_a 4.8	22
	ATP		log K_{sv} 6.3	45
Sm-**12**	P_i		log K_{a1} 4.3	22
			log K_{a2} 3.5	
Gd-**13**	P_i	H_2O	log K_a 17	25, 24
	$HAsO_4^-$		log K_a 7.8	
	HCO_3^-		log K_a 2.7	
	F^-		log K_a 2.0	
Gd-**15**	P_i	H_2O	log K_a 6.1	24
	$HAsO_4^-$		log K_a 11	
	HCO_3^-		log K_a 3.0	
	F^-		log K_a 2.6	
Gd-**16**	P_i	H_2O	log K_a 29	24
	$HAsO_4^-$		log K_a 4.7	
	HCO_3^-		log K_a 3	
	F^-		log K_a 3.1	
Gd-**17**	P_i	H_2O	n.d.	24
	$HAsO_4^-$		log K_a 5.1	
	HCO_3^-		log K_a 3	
	F^-		log K_a 2.9	
Eu-**18**	P_i	H_2O	log K_{a1} 4.9	27
			log K_{a2} 6.5	
Pr-**18**			log $\beta < 9.8$	30
Nd-**18**			log β 9.8	
Sm-**18**			log β 10.4	
Gd-**18**			log β 12.6	
Tb-**18**			log β 13.6	
Dy-**18**			log $\beta > 14$	
Ho/Lu-**18**			log $\beta > 14$	
Eu-**19**	P_i	H_2O	log K_{a1} 3.7	27, 29
			log K_{a2} 5.3	
			log K_{a3} 5.6	
Pr-**19**			log $\beta < 14.5$	30
Sm-**19**			log β 14.7	
Gd-**19**			log β 17.0	
Dy-**19**			log β 15.1	
Ho/Lu-**19**			log $\beta > 17$	
Eu-**23**	P_i	H_2O	log K_{a1} 4.7	29
			log K_{a2} 4.7	
	CN^-		Log β 8.14	28
Eu-**24**	P_i	H_2O	log K_{a1} 5.0	29
			log K_{a2} 5.3	
			log K_{a3} 5.6	
Ln-**27**	P_i	H_2O	n.d.	31
Tb-**29**	P_i	CH_3OH	log $\beta_{1:1}$ 6.6	32
			log $\beta_{1:2}$ 6.8	
	NO_3^-		log $\beta_{1:1}$ 4.7	
Eu-**30**	HSO_4^-	C_2H_5OH	log K_a 3.7	33
	P_i		log K_a 3.8	
	ATP		log K_a 3.42	
	ADP		log K_a 5.99	
	AMP		log K_a 2.64	
Eu-**31**	P_i	H_2O	n.d.	34
	PP_i		n.d.	
	$P_3O_9^{3-}$		n.d.	
Tb-**32**	P_i	H_2O	log K_{sv} 6.8	35
	PP_i		log K_{sv} 7.0	
	ATP		log K_{sv} 7.3	
	ADP		log K_{sv} 6.5	
	AMP		log K_{sv} 4.5	

stance, only binds phosphate and citrate, albeit with a 10-fold lower affinity (Table 1).

Phenanthroline-base complexes such as Ln-**10** and Ln-**11** investigated by Albrecht employ a similar mechanism for staining phospholipids in cells.[21] It should be noted that in the absence of stability studies (vide infra, section 2.3), the quenching of Eu^{III} and Tb^{III} phosphorescence observed upon phosphate coordination could also be attributed to ligand exchange and displacement of the antenna by the phospholipids instead of the formation of a ternary complex postulated by the authors. While it is reasonably selective for most anions other than nitrate, these receptors are unlikely to be selective for inorganic phosphates over their organic counterparts. More recently, Jena reported a similar phenanthroline macrocycle,

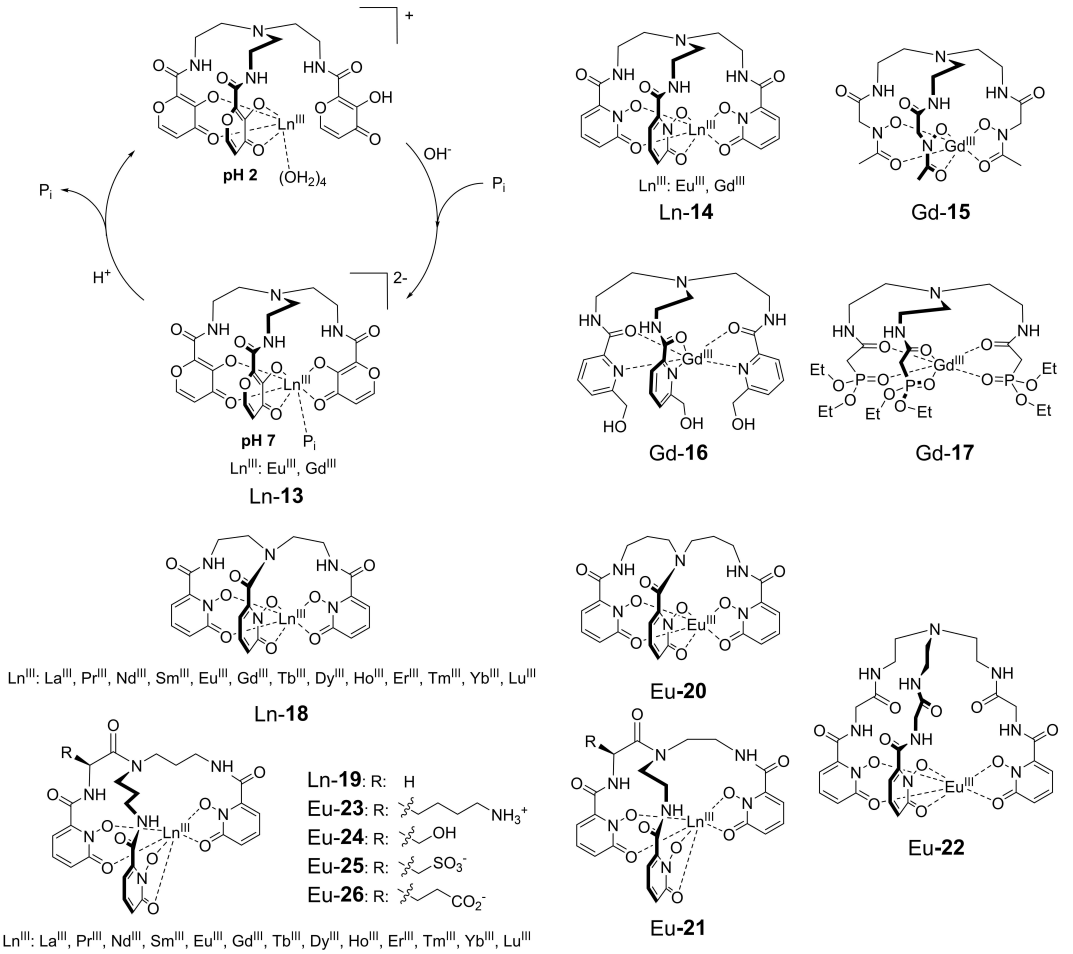


Figure 4. Responsive luminescent lanthanide probes developed by the Pierre group for inorganic phosphate functioning via direct coordination of the anion.

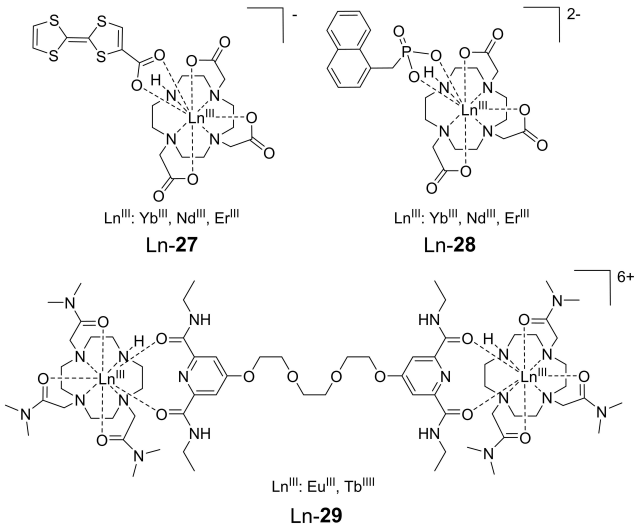
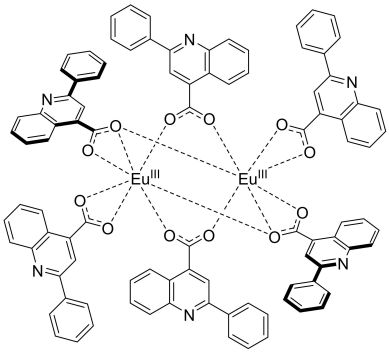
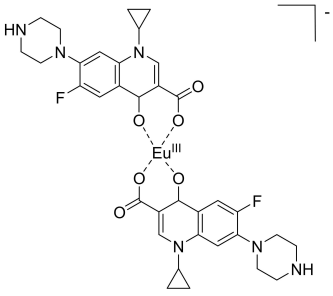


Figure 5. Responsive luminescent lanthanide indicator displacement assays for inorganic phosphate.

replacing the amides by weaker coordinating amines (Ln-**12**), a change that likely destabilizes further the complex.[22] The probe is somewhat selective for phosphate, binding carbonate only weakly. Coordination of a first phosphate anion to Eu-**12** initially results in an increase in luminescence via displacement of an inner-sphere water molecule. Further addition of phosphate quenches the terbium luminescence via a mechanism postulated by the authors to involve photoelectron transfer in a manner similar to that observed by Anslyn for the detection of 2,3-bisphosphoglycerate,[23] although phosphate-based decomplexation has not been ruled out by the authors. It should be noted that "off-on-off" responses to analytes, such as those observed with Ln-**12** have limited practicality.

Importantly, lanthanide receptors can be rationally designed to be highly selective for inorganic phosphate over other anions in water.[24] As we demonstrated with Gd-**13** to Gd-**17** (Figure 4), tripodal lanthanide complexes with 2 inner-sphere water molecules and no steric hindrance at the open coordination sites can bind phosphate and other oxyanions *only* if the complex $Gd^{III}L$ is not too stable, which occurs when the coordinating ligand L is not too basic.[4] In those cases, the selectivity of the selectivity of the Gd^{III} complex always trends in the order phosphate > arsenate > carbonate > fluoride, an order that follows the basicity (not the hardness) of the anion. The more stable the $Gd^{III}L$ complex, the lower its affinity for anion. The more stable, the 2,3-hydroxypyridinonate-based Gd-**14**, has





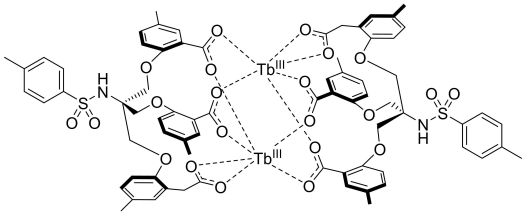


Figure 6. Responsive luminescent lanthanide probes for inorganic phosphate functioning via ligand exchange.

no affinity for any anions whatsoever. Instead of forming ternary complexes, the least stable complexes undergo ligand exchange whereby the anion removes the lanthanide ion, resulting in the formation of insoluble salts. This observation underlies that phosphate "extrusion" of the metal, as opposed to formation of ternary Ln^{III}L·Pi, likely occurs with many poorly stable lanthanide receptors published. Gd^{III}-TREN-MAM (Gd-**13**), which employs a ligand of intermediate basicity, binds phosphate with high affinity in water but not perfect selectivity over bicarbonate.[25] Importantly, this first-generation complex demonstrates the recyclability of this class of receptor and its ability to catch-and-release phosphate upon changes in pH, an important property for applications of phosphate remediation (Figure 4) Indeed, the acidic form of a ligand (i.e. the protonated H_3PO_4) has limited affinity for a metal ion. It is only the basic form (i.e. the deprotonated form) that does. The pH dependency observed for phosphate binding thus follows the general trend of lower stability of coordination complexes at lower pH. Further studies on the mechanism of phosphate recognition demonstrated the highly flexible nature of the receptors, underlining the fact that selective anion recognition can be achieved without rigid predisposition of hydrogen-bonding and electrostatic interactions.[26]

Complete selectivity for phosphate over other oxyanions can be achieved by slightly destabilizing Ln^{III}-TREN-HOPO (Ln-**14**, Figure 3) via slight modification of the ligand cap.[27] The complex Eu^{III}-2,2-Li-HOPO (Eu-**18**) and Eu^{III}-3,3-Gly-HOPO (Eu-**19**) both bind phosphate with very high affinity in water at neutral pH with complete selectivity over bicarbonate, carboxylates, fluorides and other competing anions (Table 1). An important conclusion from this study is that there is *no* relationship between the number of inner-sphere water molecules on the lanthanide ion and the affinity of the complex for phosphate. Some complexes, such as Eu-**14**, Eu-**20**, and Eu-**21**, which also have two inner-sphere water molecules, as well as Eu-**22**, which has a $q=3$, have no affinity for any anions. Only those complexes where the ligand destabilizes one of the 1,2-HOPO chelation bind phosphate. This same destabilization also decreases the quantum yield of the complex. Importantly, selectivity of any lanthanide probe that function via direct coordination of the anion is highly pH dependent. This dependency was demonstrated with Eu-**23**, a probe that is exquisitely selective for phosphate in water at neutral pH, but binds the more basic cyanide at basic pH.[28]

Advantageously, the affinity of these receptors can be tuned at will via the incorporation of charged or hydrogen-bonding moieties in close proximity to the open coordination site.[29] The addition of a neutral hydrogen-bonding alcohol of Eu-**24** increases the affinity of the probe for phosphate by 20-fold, whereas a single ammonium in Eu-**23** increases 200-fold. On the other hand, the presence of a single negatively charged group, such as a sulfonate (Eu-**25**) or a carboxylate (Eu-**26**) is sufficient to block any anion recognition. These observations highlight the stringent dependency of lanthanide-based receptors on nearby electrostatic charges, a dependency that results from the purely ionic nature of lanthanide coordination. The same ionic dependence on anion coordination enables one to tune the affinity of the receptor by changing the lanthanide ion.[30] The later, and smaller, lanthanides have higher affinity for phosphate than the earlier, larger ones. For flexible complexes such as the tripodal Ln-**18** and Ln-**19**, the affinity for phosphate can vary by more than 1,000-fold between La^{III} and Lu^{III}.

2.2. Lanthanide-Based Indicator Displacement Assays

The probes discussed so-far function by the intrinsic effect that displacement of inner-sphere water molecules by coordination anions has on the lanthanide's luminescence. A response can also be induced not by displacing water molecules but by displacing an indicator. An indicator is a small anion that coordinates to the lanthanide more weakly than phosphate such that it can be displaced by the harder oxyanion. If the indicator is also the antenna for Eu^{III} or Tb^{III}, than its displacement results in a decrease of lanthanide-centered emission. This approach was first demonstrated by Faulkner and Pope with

Ln-**27** (Figure 5).[31] The antenna, which coordinates the near-IR emitting lanthanide relatively weakly, is readily displaced by phosphate, resulting in a decrease in the rare earth's fluorescence. An indicator that binds the lanthanide more strongly, such as the phosphonate used in Ln-**28**, is not readily displaced by phosphate and thus does not respond. Gunnlaugsson employed a similar strategy with a dipicolylamide bridging antenna ligand connecting two Tb^{III} metal complexes to make an analogue that responds in the visible region.[32] The probe Tb-**29** is unique in that, in methanol, it also responds to nitrate, an anion generally considered not to coordinate lanthanides ions. The advantage of this approach is that an indicator with a correctly tuned affinity for the lanthanide can bestow the probe with higher selectivity for phosphate over more weakly coordinating anions such as carboxylates or bicarbonates. The disadvantage is that they are likely not reversible in complex media containing several coordinating anions.

2.3. Luminescence Response via Ligand Exchange

A key parameter in the design of the probes discussed so far is the stability of the $Ln^{III}L$ complexes. Phosphate or other anions may coordinate the lanthanide and displace either a water molecule or an indicator, but the polydentate ligand L is strong enough that it is not displaced. These stabilities of the $Ln^{III}L$ probes are crucial to their applications in complex biological or environmental media. In some cases, however, the ligand L is not strong enough to form stable $Ln^{III}L$ complexes. In these cases, addition of phosphate, a strong coordinating anion, does not result in the formation of ternary $Ln^{III}L{\cdot}Pi$ complexes, but in ligand exchange and the formation of insoluble $Ln^{III}Pi$ salts. As discussed previously, such ligand exchange can be observed even with hexadentate ligands of moderate strengths.[4] If the weakly coordinating ligand is also the lanthanide's antenna, its displacement by phosphate will results in a signature complete quenching of the lanthanide's emission. It should be noted that given the low concentrations of probes used in luminescence studies, the lanthanide phosphate salt precipitate is not always observable with the naked eye. For complexes of even moderate stability, complete quenching of lanthanide luminescence is almost always caused by decomplexation of the probe. Quenching by other mechanisms such as PeT cannot be assumed to take place unless the stability of the probe and the intactness of the ternary phosphate adduct have been clearly established by solution thermodynamic studies. Recent examples of this approach includes $Eu(PQC)_6$ (Eu-**30**, Figure 6),[33] the ciprofloxacin (CIP) europium complex (Eu-**31**),[34] and salicylic complex Tb-**32**.[35] More recently, a surfactant micelle probe similar to Eu-**31** utilizing 2-naphthoyltrifluoroacetone sensitizer ligands was also developed.[36] This is an easier approach to detect phosphate in water, albeit one that is not reversible, often poorly selective over other anions, and therefore not as accurate in complex media.

3. Luminescent Probes for Nucleotides

One of the earliest and still most widespread commercial translation of luminescent lanthanide probes is in high throughput screening (HTS) for drug discovery and development. The long luminescent lifetimes of Eu^{III} and Tb^{III} complexes and the facile implementation of time-resolved spectroscopy are ideal for HTS assays since they readily discriminate competing autofluorescence arising from either the enzyme, the substrate(s) or the drug candidate. One of the largest classes of druggable targets with particular significance in oncology are kinases.[37] Kinases are enzymes that catalyzes the transfer of a phosphate group from ATP to a specific substrate with implications in cell signaling, among others. Luminescent lanthanide probes that could thus distinguish between ATP, ADP, AMP, and phosphate thus offers the alluring capability to monitor kinase inhibition and kinetics easily, without the use of antibody or modified substrate. The most relevant probes will be able to differentiate nucleotides *at their cellular concentration*. Healthy cells have ATP concentrations in the range of 1 to 10 mM and a normal ATP:ADP ratio of 1000:1.[38] Hence, kinase studies that are more relevant to cellular conditions require probes that have low affinity for the nucleotides, with K_d between 10^{-2} and 10^{-3}. The properties and molecular composition of these complexes are summarized in the following Table 2 and Figures 7–9.

3.1. Recognition of Nucleotides via Direct Coordination

As discussed in the previous section, the high oxophilicity and hardness of lanthanides favours coordination of hard and highly basic oxoanions such as HPO_4^-. By extension, nucleotides that contain either one, two, or three phosphates (such as AMP, ADP and ATP, respectively) also have a high affinity for lanthanide complexes with open coordination that are not sterically hindered. In most cases, coordination of the phosphoester to the lanthanide ion via displacement of inner-sphere water molecule(s) will also increase the intensity of lanthanide-centered emission by displacing the quenching water molecules (Figure 2a).

Interestingly, the first luminescent lanthanide probe for ATP, developed by Charbonnière and Ziessel, is not a turn-on probe.[39] Instead, coordination of ATP to Eu-**33** (Figure 7) quenches 80% of Eu^{III}'s emission. This significant, but not complete, quenching is not due to transmetallation. Indeed, this is one of the few studies in which formation of the ternary complex was clearly established by a combination of solution studies. The probe is unique in its near-complete selectivity for ATP over ADP and AMP. Unfortunately, the probe cannot differentiate between inorganic phosphate and ATP, an observation that is common to most probes of this class.

In order to avoid transmetallation, most modern probes for nucleotides employ thermodynamically stable and kinetically inert DOTA-type macrocycles. These include the library of probes developed by Butler. The C_2-symmetric cationic complexes Eu-**34** to Eu-**37** can bind reversibly to ATP via displace-

Table 2. Affinity and selectivity in select solvent of lanthanide probes for nucleotides.

Complex	Selectivity	Solvent	Binding Affinity	Ref.
Eu-**33**	P_i	H_2O	log K_a 4.0	38
	ATP		log K_a 4.2	
Tb-**33**	P_i		log K_a 4.5	
	ATP		log K_a 4.6	
Eu-**34**	ATP/GTP	H_2O	log K_a 4.4	39
	ADP/GDP		log K_a 4.6	
	AMP/GMP		log K_a 3.4	
	PPi		log K_a 3.5	
	UTP		log K_a 3.6	
	UDP		log K_a 4.2	
	UMP		log K_a 2.7	
	P_i		log K_a 2.1	
	HCO_3^-		log K_a 3.0	
	ATP–Mg		log K_a 2.82, 3.7	40,41
	ADP–Mg		log K_a 3.37, 4.1	41
	AMP–Mg		log K_a 3.40	
Tb-**34**	ATP		n.d.	41
	ADP		n.d.	
	AMP		n.d.	
Eu-**35**	ATP	H_2O	log K_a 3.0	39
	ADP		log K_a 3.3	
	AMP		log K_a 3.3	
	PPi		log K_a 2.4	
Eu-**36**	ATP	H_2O	log K_a 5.8	39
	ADP		log K_a 5.7	
	AMP		log K_a 4.8	
	PPi		log K_a 4.7	
	GTP		log K_a 5.3	
	GDP		log K_a 5.2	
	GMP		log K_a 3.9	
	UTP		log K_a 4.9	
	UDP		log K_a 5.4	
	UMP		log K_a 4.3	
	P_i		log K_a 2.7	
	HCO_3^-		log K_a 3.0	
	ATP–Mg		n.d.	40
	ADP–Mg		log K_a 4.6	
	AMP-Mg		log K_a 3.8	
Eu-**37**	ATP	H_2O	log K_a 3.8	39
	ADP		log K_a 3.8	
	AMP		log K_a 3.5	
	PPi		log K_a 3.4	
Eu-**38**	ATP	H_2O	n.d.	41
	ADP		n.d.	
	AMP		n.d.	
Eu-**39**	ATP	H_2O	log K_a 3.65	41
	ADP		log K_a 3.34	
	AMP		log K_a 2.77	
	ATP–Mg		log K_a 3.26	
	ADP–Mg		log K_a 2.93	
	AMP-Mg		log K_a 2.85	
Eu-**40**	PAP	H_2O	log K_a 3.6	42
	PAPS		log K_a 4.0	
Eu-**41**	PAP	H_2O	log K_a 3.7	42
	PAPS		log K_a 4.0	
Eu-**42**	PAP	H_2O	log K_a 3.5	42
	PAPS		log K_a 4.0	
	3′-AMP		n.d.	
	5′-AMP		n.d.	
	APS		n.d.	
	CoA		n.d.	
	DeCoA		n.d.	
Eu-**43**	AMP	H_2O	log K_a 3.83	44
Eu-**44**	P_i	H_2O	log K_b 4.15	45
	ATP		log K_b 4.15	
Eu-**45**	P_i	H_2O	log K_b 4.40	45
	ATP		log K_b 4.40	

Table 2. continued

Complex	Selectivity	Solvent	Binding Affinity	Ref.
Eu-**47**	ATP	H_2O	n.d.	46
	ADP		n.d.	
	PP_i		n.d.	
	Citrate		n.d.	
Eu-**48**	ATP	H_2O	n.d.	46
	GTP		n.d.	
	ADP		n.d.	
	GDP		n.d.	
	PP_i		n.d.	
	Citrate		n.d.	
Eu-**49**	ATP	H_2O	n.d.	46
	CTP		n.d.	
	ADP		n.d.	
	PP_i		n.d.	
	Citrate		n.d.	
Eu-**50**	ATP	H_2O	n.d.	46
	CTP		n.d.	
	ADP		n.d.	
	Citrate		n.d.	
Eu-**51**	ATP	H_2O	n.d.	46
	PPi		n.d.	
	ADP		n.d.	
	Citrate		n.d.	
Tb-**52**	ATP	H_2O	K_{sv} 1.28	49
	ADP		K_{sv} 0.85	
	AMP		K_{sv} 0.397	
	GTP		K_{sv} 33.1	50
	GDP		K_{sv} 9.84	
	GMP		K_{sv} 2.84	
	CTP		K_{sv} 0.103	
	CDP		K_{sv} 0.149	
	CMP		K_{sv} 0.139	
	UTP		K_{sv} 0.047	
	UDP		K_{sv} 0.065	
	UMP		K_{sv} 0.070	
Eu-**53**	ATP	H_2O	K_{sv} 0.065	50
	ADP		K_{sv} 0.058	
	AMP		K_{sv} 0.053	
	GTP		K_{sv} 0.099	
	GDP		K_{sv} 0.164	
	GMP		K_{sv} 0.118	
	CTP		K_{sv} 0.006	
	CDP		K_{sv} 0.008	
	CMP		K_{sv} 0.008	
	UTP		K_{sv} 0.001	
	UDP		K_{sv} 0.002	
	UMP		K_{sv} 0.007	
Eu-**54**	ATP	H_2O	log$K_{f2:1}$ 3.89	51
Eu-**55**	AMP	H_2O	log K_a 4.5	53
	ADP		log K_a 4.8	
	ATP		log K_a 4.7	
Eu-**56**	AMP	H_2O	log K_a 4.2	53
	ADP		log K_a 5.5	
	ATP		log K_a 4.6	
Tb-**58**	GMP, 5 μM	H_2O	log K_{a1} 5.4	54
			log K_{a2} 4.1	
	GMP, 20 μM		log K_{a1} 6.1	
			log K_{a2} 5.6	
	GDP, 5 μM		log K_{a1} 6.4	
			log K_{a2} 5.3	
	GDP, 20 μM		log K_{a1} 5.8	
			log K_{a2} 4.9	
	GTP, 5 μM		log K_{a1} 7.1	
			log K_{a2} 5.6	
	GTP, 20 μM		log K_{a1} 5.9	
			log K_{a2} 4.6	
Tb-**59**	GMP	H_2O	n.d.	55
	GDP		n.d.	
	GTP		n.d.	
	(CT)-DNA		n.d.	

Table 2. continued

Complex	Selectivity	Solvent	Binding Affinity	Ref.
Eu-**60**	PP_i	CPB Micellular solution	log β 13.47	57
	ATP		log β 12.26	
	ADP		log β 11.36	
	Citrate		log β 11.42	
	Tartrate		log β 7.32	
	P_i		log β 5.33	
Eu-**61**	PP_i	CPB Micellular solution	log β 13.43	57
	ATP		log β 12.28	
	ADP		log β 11.32	
	Citrate		log β 11.44	
	Tartrate		log β 7.44	
	P_i		log β 5.26	
Eu-**62**	PP_i	CPB Micellular solution	log β 13.49	57
	ATP		log β 12.21	
	ADP		log β 11.37	
	Citrate		log β 11.40	
	Tartrate		log β 7.29	
	P_i		log β 5.42	
Eu-**63**	PP_i	CPB Micellular solution	log β 13.47	57
Eu-**64**	ATP	H_2O	log K_a 6.0	58
	ADP		log K_a 4.7	
	AMP		n.d.	
	P_i		log K_a 4.0	
	PP_i		log K_a 4.9	
Eu-**65**	ATP	H_2O	log K_a 6.6	58
	ADP		log K_a 6.1	
	AMP		log K_a 5.8	
	P_i		log K_a 6.0	
	PP_i		log K_a 6.3	
Eu-**66**	ATP	H_2O	log K_a 6.8	58
	ADP		log K_a 6.6	
	AMP		log $K_a < 5.0$	
	P_i		log K_a 6.5	
	PP_i		log K_a 6.9	

ment of the inner-sphere water molecule concomitant with second-sphere hydrogen-bonding interactions between the quinolone antennas and the nucleobase.[40] Water displacement leads to the telltale increase in both luminescence intensity and the lifetime of europium(III). Key to achieving selectivity for ATP over ADP and AMP is the incorporation of amides on the quinolines of Eu-**34** and Eu-**36** that hydrogen bond to the nucleobase. This study demonstrated that conjugation to a lanthanide ion alone is often insufficient to distinguish between organic phosphates. Although only 3-fold selective for ATP over inorganic phosphate, Eu-**36** can distinguish between ATP, ADP, and AMP at concentrations that are relevant physiologically (1-5 mM ATP) even in the presence of Mg^{II}. The addition of magnesium is indeed crucial to the ability of these probes to successfully monitor kinase kinetics since in cells ATP is stabilized via coordination to Mg^{II}.[41] In more complex assay conditions, coordination of ATP to Eu^{III} therefore requires displacement of Mg^{II}.

In-depth solution studies by the Butler group[42] expanded the functionality of their probes and enabled further study into their mechanism of action. The Tb^{III} analog Tb-**34** is more sensitive to quenching by dissolved oxygen. The cis-configuration of quinolone arms in Eu-**38** favors ligand exchange and decomplexation upon ATP coordination, likely because of steric hindrance. Replacement of amide groups by oxymethyl groups that cannot hydrogen bond to the nucleobase in Eu-**39** decreased affinity for the nucleotides as well as their selectivity. In the presence of Mg^{II}, the apparent association constants decrease by one order of magnitude since the alkali earth competes with the rare-earth for phosphate coordination. Advantageously, principal component analysis (PCA) using all four complexes can distinguish between all nucleotide polyphosphate (NPP) species. Derivative complexes containing distal benzyl groups (Eu-**40** to Eu-**42**) were shown to be able to distinguish between adenosin-3′,5′-diphosphate (PAP) and adenosin-3′-phosphate-5′-phosphosulfate (PAPS) due to unique turn-on intensities, even though they were not particularly selective between NPPs.[43] This unusual selectivity enabled ratiometric screening of sulfotransferase activity. More recently, Butler was able to switch the preference of this class of probes from ATP to AMP, which is uncommon in that the chelate effect usually favors coordination of the triphosphate.[44] This was previously achieved by Albrecht with the lanthanide helicase Eu-**43**.[45] The helicate is indeed perfectly preorganized to bind the phosphate moiety on one end and the purine on the other end, with the length of the helicate favoring AMP (Figure 7). It would be interesting if this approach could be extended to the detection of other nucleotides.

In the absence of second-sphere interactions directed to the nucleobase, almost all lanthanide probes that bind phosphate will also bind ATP with similar affinity. The phenanthroline Ln-**44** and Ln-**45**, which are minor derivatives of the phosphate probes Ln-**10** and Ln-**11**, as well as Ln-**12** also respond to ATP similarly as to phosphate, and likely via the same mechanism.[46] Predictably, the greater the number of open coordination sites on a lanthanide complex, the higher its affinity for phosphate species. Schäferling demonstrated this with the polyaminocarboxylate Eu-**46** to Eu-**51**.[47] All complexes demonstrated higher affinity for ATP and pyrophosphate (PPi) over AMP due to the higher denticity of the former anions. An apparent higher affinity for phosphate species is observed with europium complexes with greater number of open coordination sites. Not surprisingly, the complexes also respond to other coordinating oxyanions such as oxalate and citrate. The mechanism of action, however, is likely not a true formation of a well-defined ternary complex as postulated. Ligands of low denticity are well-known to form poorly stable complexes and are highly susceptible to ligand exchange in the presence of strongly coordinating anions such as phosphates.[4,48] Moreover, often underappreciated, adenosine is a very good antenna for Eu^{III}. Indeed, europium(III) complexes of ATP are quite luminescent.[49] Increase in luminescence intensity is thus not necessarily solely a result of displacement of water molecules and formation of a ternary complex. Determination the nature of the complex formed requires more in-depth solution studies besides luminescence intensity.

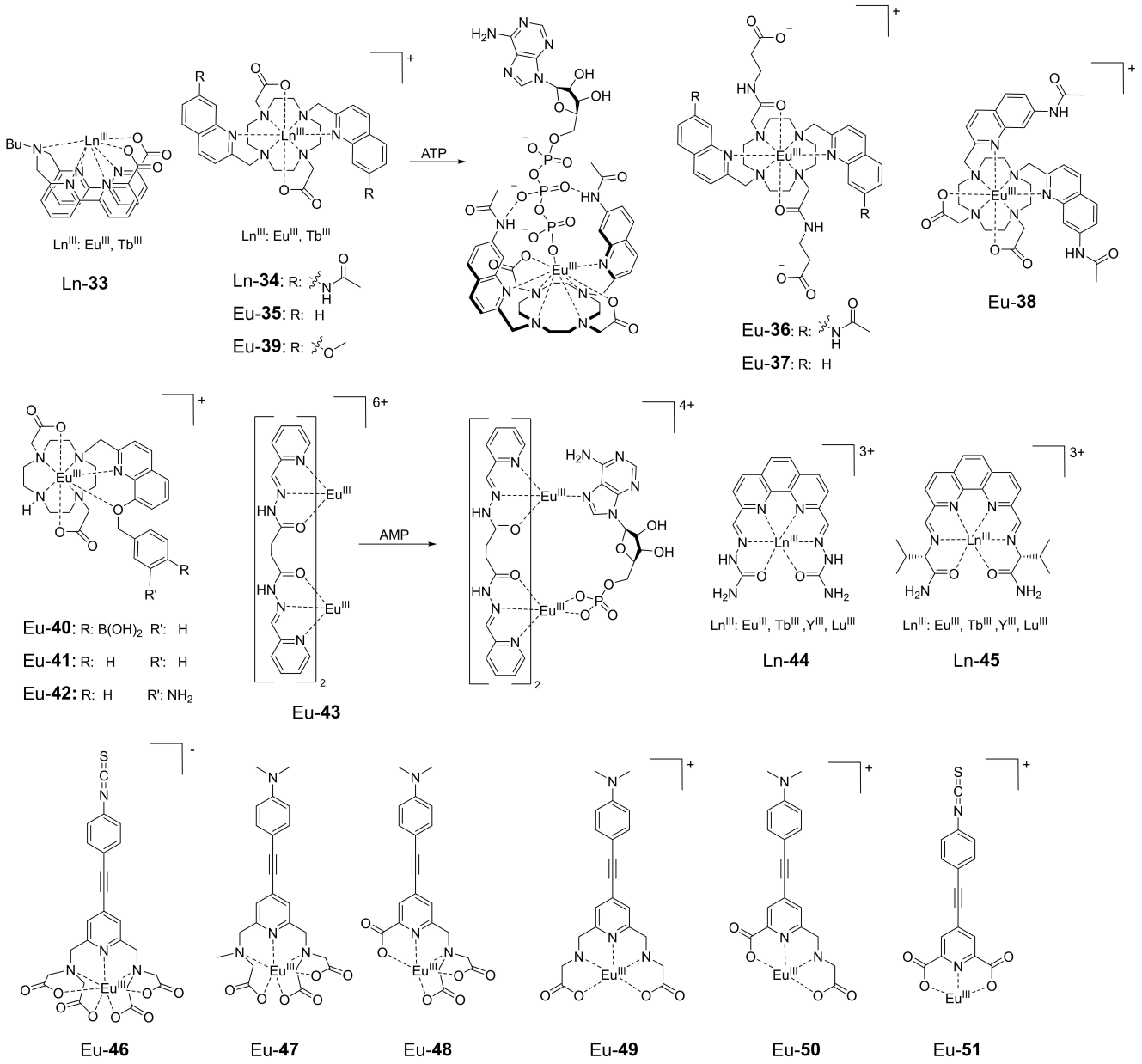


Figure 7. Responsive luminescent lanthanide probes for nucleotides functioning via direct coordination of the anion.

3.2. Recognition of Nucleotides via Second Sphere Interactions

Differentiating nucleotides while minimizing the risks of both ligand exchange and hydrolysis of polyphosphates can be accomplished by preventing coordination of the phosphate moiety to the lanthanide ion, instead relying solely on outer-sphere interactions. This approach enables the use of kinetically inert and thermodynamically stable complexes such as those formed with macrocyclic DOTA-type ligands. Selective recognition of organic phosphate is achieved with the traditional toolbox of organic supramolecular chemistry. Electrostatic and π-stacking interactions are particularly well suited for nucleotides such as ATP and GTP.

This is the approach that we have employed with the ATP-selective receptor Tb-**52** (Figure 8).[50] π-π Interactions between the nucleobase and the phenanthridine enable photoelectron transfer from the purine to the antenna, which prevents sensitization of Tb^{III} resulting in a substantial decrease in the lanthanide's centered emission. Photoelectron transfer and π-stacking is favored for purines over pyrimidines, so the probe is only responsive to adenosine and guanosine nucleotides. Electrostatic interactions with the positively charged Tb-**52** favors the more negatively charged ATP over ADP and AMP. The importance of the electrostatic interactions in water is further supported with studies with the neutral analog Eu-**53**, which does not distinguish between the triphosphate, diphosphate, and monophosphate purine nucleotides.[51] The advant-

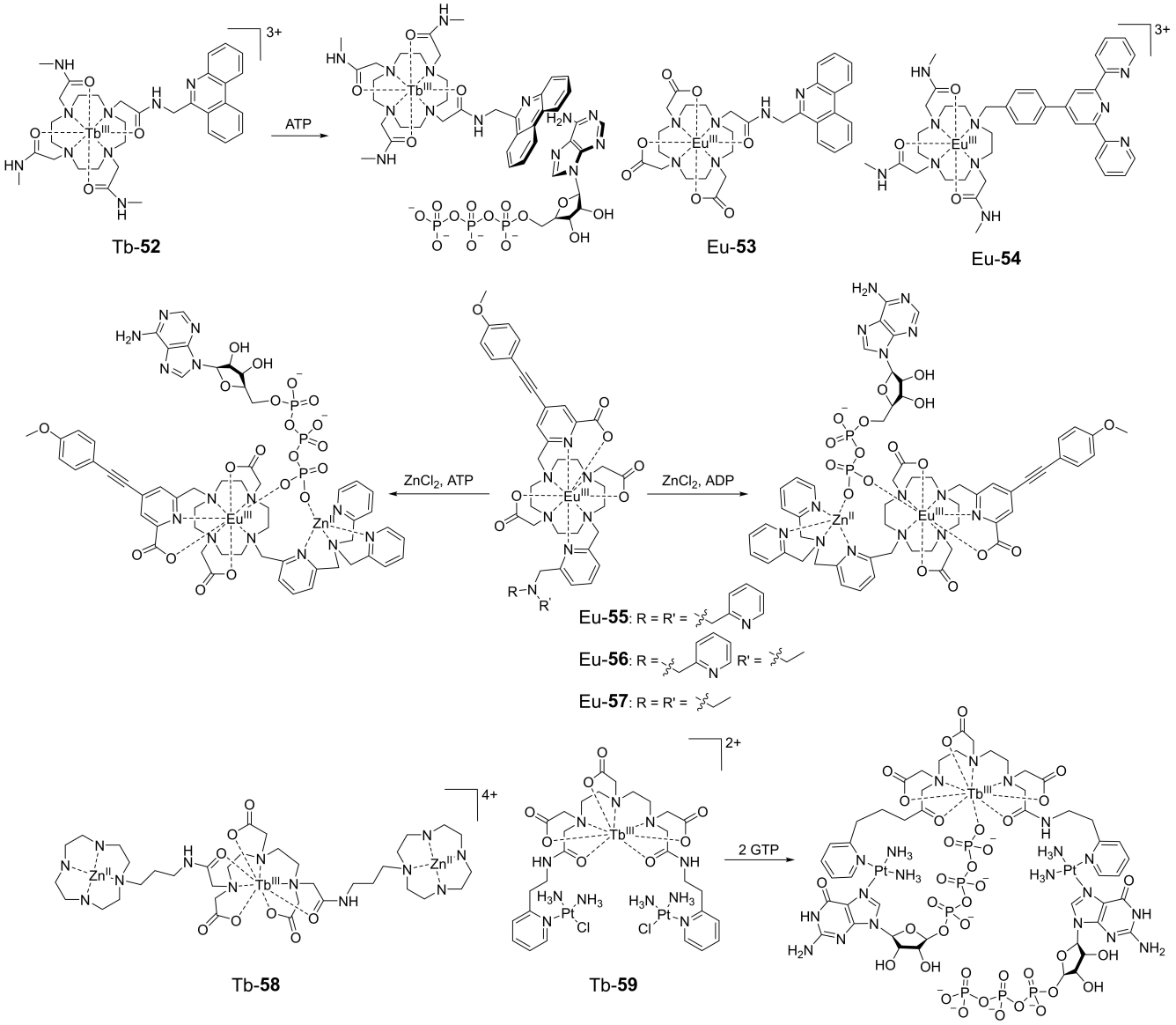


Figure 8. Responsive luminescent lanthanide probes for nucleotides functioning via peripheral interactions.

age of relying on second-sphere interactions is that, since they are weaker, they confer the probe's weak affinity for their substrate. This, in turn, enables measuring nucleotide concentrations in the mM range that are more relevant to cellular conditions. Indeed, concomitant use of the responsive Tb-**52** and unresponsive Eu-**53** probes enabled the first study of kinase kinetics by ratiometric time-gated luminescence spectroscopy. This approach has several advantages over commercial kits: it does not require antibodies, it uses natural substrates that are neither modified nor labeled, and it enables continuous monitoring of the reaction and its inhibition, thereby facilitating kinetic studies of drugs.

Tang subsequently developed the analog Eu-**54** that uses instead a terpyridine antenna and functions similarly.[52] The authors propose that the first equivalent of ATP partially displaces the inner-sphere water molecule causing an increase in luminescence intensity. A second equivalent of ATP causes a decrease in luminescence, likely via PeT. It should be noted that bell-shaped curve responses have limited practicality. More recently, the Patra group reported a similar bis(amido-phenyl-terpyridine) complex with a DTPA backbone and comparable response.[53]

Peripheral or outer-sphere interactions need not be limited to those between organic moieties. The binuclear Tb^{III}–Zn^{II} complexes Eu-**55** to Eu-**57** developed by Parker exploited the ability of the terminal phosphate of both ADP and ATP to bridges the two metal ions to induce a luminescence response.[54] Uniquely, since the two adduct have strong induced circularly polarized emission but of opposite sign, conversion between the two nucleotides can be monitored by measuring the probe's emission dissymmetry factor (Figure 8). The complex Tb-**58** developed by Tuck,[55] also exploits coordi-

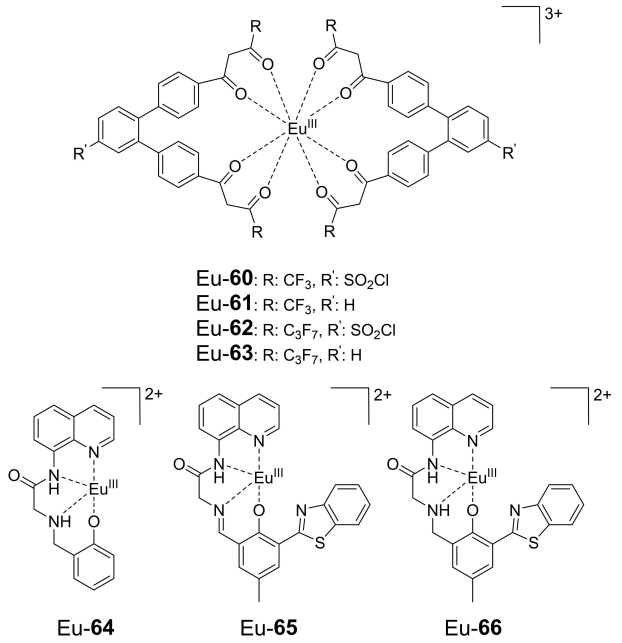


Figure 9. Responsive luminescent lanthanide probes for nucleotides functioning via ligand exchange.

nation to peripheral zinc complexes to distinguish between nucleotides. The trinuclear Tb^{III}-bisZn^{II} is more selective and has higher affinity for GMP via the formation of a 1:1 assembly. Interestingly, this complex is also more selective for acyclic GMP over its cyclic cGMPA derivative, likely due to the higher negative charge of the former. This is a rare example of a molecular probe that can monitor catalytic conversion of cGMP to GMP via a phosphodiesterase enzyme.

Bimetallic complexes, particularly ones employing metals of different hardness, are well suited for distinguishing between nucleotides by enabling coordination to both phosphate and the nucleobase. As discussed throughout this review, lanthanides are highly oxophilic and thus have high affinity for phosphate. On the other hand, softer purines, and in particular guanine, has high affinity for softer metal ions such as platinum(II). The bimetallic Tb^{III}–Pt^{II} developed by Guo, Tb-**59**, is the only example that exploits this duality in metal coordination by nucleotides to favor recognition of GTP.[56] Whereas the phosphate moiety of the nucleotide coordinates Tb^{III} thereby displacing the inner-sphere water molecule, the guanine binds the Pt^{II} by displacing the weaker Cl^- ligand (Figure 8). Unfortunately, coordination of guanosine to Pt^{II} is irreversible, so such bimetallic probes, while sensitive, cannot monitor enzymatic reactions.

3.3. Luminescence Response via Ligand Exchange

As discussed above, weakly coordinating ligands that can also sensitize either Eu^{III} or Tb^{III} are all intrinsically phosphate probes since phosphate, a strong ligand for lanthanides, easily displaces weak ones. Ligand exchange always results in a decrease in lanthanide-centered luminescence intensity. Due to the chelate effect, ATP has higher affinity for lanthanides than ADP, AMP, and orthophosphate, so some selective response can be observed under the right conditions. Schaferling first demonstrated the potential of this approach with the weak Eu^{III}-tetracycline complex.[57] While such metal-exclusion assays are simple, they are poorly selective between phosphate species and toward carboxylates. The formation of insoluble lanthanide phosphate salts, which practically preclude the reversibility of binding, make them ill-suited for monitoring enzymatic reactions. This is demonstrated, for instance, with the β-diketonate europium(III) complexes Eu-**60** to **63** (Figure 9) developed by the Yuan group, which show low selectivity between orthophosphate, pyrophosphate, ATP, and ADP.[58] They are, however, quite sensitive, with limits of detection for pyrophosphate as low as 0.02 μM for Eu-**60**.

More recently, Anzenbacher modified this approach by monitoring the luminescence of the weakly coordinating chromophores as opposed to that of the lanthanide ion.[59] Since europium(III) quenches the luminescence of the carboxamide quinolone, its displacement by phosphate returns the fluorescence of the organic chromophore. Like any displacement assay, the selectivity of Eu-**64** to **66** between phosphate species remains poor.

4. Luminescent Probes for Organic Phosphates

Probes for organophosphates other than nucleotides and nucleic acids are less developed. Yet, the same principles can be applied to their development. Interest in this field focuses primarily on chemical warfare agents and certain pesticides that contain phosphate moieties for which facile detection is the first step in remediating contaminated areas. Markers of biochemical processes, some of which are over expressed in disease tissues, are also of interest. Successful translation hinges on achieving very high selectivity, particularly over orthophosphate. The properties and molecular composition of these complexes are summarized in the following Table 3 and Figures 10–11.

4.1. Recognition of Organophosphates via Direct Coordination

Organophosphates, like nucleotides and inorganic phosphates, have some affinity for many lanthanide complexes with at least one open coordination site. This was first demonstrated by Bretonnière and Parker with the complexes Eu-**67** to **69** (Figure 10), which all bind various phosphorylated species via displacement of the inner-sphere water molecule resulting in the characteristic increase in luminescence intensity.[60] Interestingly, Eu-**68** has higher affinity for phosphorylated tyrosine than phosphorylated serine. The probe is chemoselective. Given a hexapeptide containing both phosphorylated Tyr and Ser residues, it will only bind the phosphorylated tyrosine. Further studies with the analogs Ln-**1** and Ln-**70** demonstrated that the

Table 3. Affinity and selectivity in select solvent of lanthanide probes for organophosphates.

Complex	Selectivity	Solvent	Binding Affinity	Ref.
Eu-**67**	O—P-Ser	H_2O	n.d.	59
	O—P-Tyr		n.d.	
	O—P-Thr		n.d.	
	P_i		n.d.	
	AMP		n.d.	
	Gluc-6-P_i		n.d.	
Eu-**68**	O—P-Ser	H_2O	Log K_a 2.7	59
	O—P-Tyr		Log K_a 4.2	
Eu-**69**	O—P-Ser	H_2O	n.d.	59
	O—P-Tyr		n.d.	
Ln-**70**	O—P-Ser	H_2O	n.d.	59
	O—P-Tyr		n.d.	
	O—P-Thr		n.d.	
	P_i		n.d.	
	AMP		n.d.	
	Gluc-6-P_i		n.d.	
Eu-**72**	Lactate	1 : 1 H_2O/	Log K_a 4.37	60
	P_i	CH_3OH	Log K_a 4.20	
	O—P-Ser		Log K_a 4.80	
	O—P-Tyr		Log K_a 4.80	
	O—P-Thr		Log K_a 4.80	
	LPA	CH_3OH	Log K_a 5.25	
Eu-**74**	Lactate	1 : 1	Log K_a 3.15	60
	P_i	H_2O/CH_3OH	n.d.	
Eu-**75**	Glyphosate	H_2O	Log K_a 5.36	61
	P_i		Log K_a 4.35	
	N-methyl glyphosate		Log K_a 3.93	
	AMPA		Log K_a 3.30	
Eu-**76**	DCP	H_2O	n.d.	62
	DECP		n.d.	
Eu-**77**	GB	H_2O	log K_{sv} 3.56	64
	DMMP		log K_{sv} 3.66	
	DCP		log K_{sv} 3.70	
	TBAF		log K_{sv} 5.0	
	VX		log K_{sv} 5.1	
	VG		log K_{sv} 4.5	
Tb-**77**	GB		log K_{sv} 3.62	
	DMMP		log K_{sv} 3.77	
	DCP		log K_{sv} 3.91	
	TBAF		log K_{sv} 4.7	
	VX		log K_{sv} 5.2	
	VG		log K_{sv} 5.2	
Eu-**78**	R/S pybox	CH_3CN	n.d.	65
	OMA		n.d.	
Eu-**79**	DCP	H_2O	n.d.	66
Eu-**80**	DCP	H_2O	n.d.	66
	DECP		n.d.	

receptor does not bind the protein via its N-terminus, preferring instead monodentate phosphate coordination. This first study demonstrated the role that the ligand can play in selecting for a desired organophosphate.

As demonstrated by Parker with the complexes Ln-**71** to Ln-**74**, chiral *O*-phosphono amino acids can also be probed by circular polarized luminescence (CPL).[61] An ammonium group on the meta position of the benzyl differentiates the two sets of ligands. It is this ammonium that directs coordination of the *O*-phosphono amino acids and model phosphorylated peptide to the lanthanide ion via hydrogen-bonding. The luminescent and water-soluble probe Eu-**72** showed no preference between *O*-phosphono amino acids. However, *O*-P-Ser and *O*-P-Tyr residues could be distinguished by CPL spectral changes. This probe also

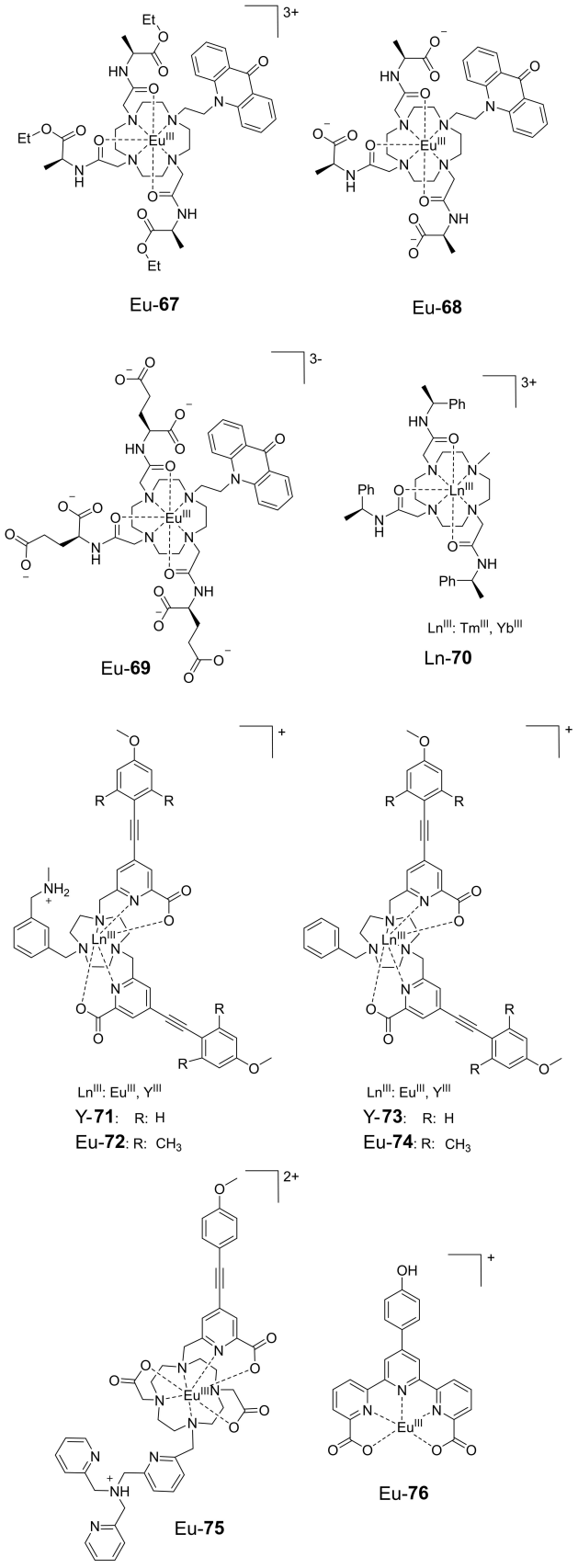


Figure 10. Responsive luminescent lanthanide probes for organophosphates functioning by direct coordination of the anion.

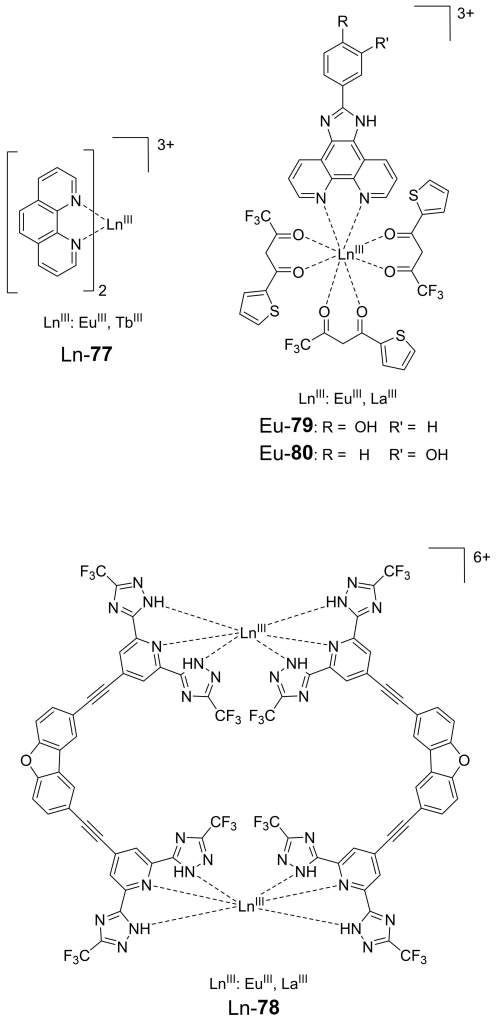


Figure 11. Responsive luminescent lanthanide probes for organophosphates functioning via ligand exchange.

enabled sensitive detection of the ovarian cancer marker oleoyl-L-α-lysophosphatidic acid.

The same motifs that are used for the selective detection of nucleotides can also be employed with organophosphates. Parker, for instance, tuned his bimetallic Eu^{III}-Zn^{II} complexes initially developed for nucleotide sensing for the detection of glyphosate, a problematic herbicide.[62] The zinc(II) ions of Eu-**55** bind the phosphate moiety, thereby assisting its concomitant coordination of the lanthanide ion. In the absence of zinc(II) (Eu-**75**), hydrogen bonding between the phosphate and the pendant tertiary amine substantially increases the affinity for glyphosate, particularly if measurements are done above its pK_a at pH 5.9. Under these conditions, the presence of a hydrogen-bonding motif bestows the probe with selectivitiy of dihydrogenphosphate, aminomethylphosphonate as well as the related herbicide glufosinate.

In a more extreme case, the analyte reacts with the antenna. This is the case with the terpyridine probe Eu-**76** developed by Patra.[63] The G-series nerve agent simulant diethyl chlorophosphate (DCP) not only displaces the three inner-sphere water molecules but also react with the phenol of the antenna, resulting in a characteristic significant increase in europium(III)-centered emission. Phosphorylation of the antenna was monitored by ^{31}P NMR. This probe is highly selective for DCP over most CWA except for diethyl cyanophosphate. Although untested, it is likely to also have high affinity for orthophosphate and pyrophosphate, which would severely limit its translational application.

4.2. Outer-Sphere Recognition of Organophosphates

Organophosphates can also be recognized solely via outer-sphere interactions and without coordination to the lanthanide ion. Morrow followed this approach to detect phosphate diesters with identical ester groups such as diethylphosphate and (bis(4-nitrophenyl phosphate) with the PARACEST (paramagnetic chemical exchange saturation transfer) MRI agent Ln-**8** (Figure 3).[64] Since recognition occurs via outer-sphere hydrogen-bond interactions to the organophosphate rather than by displacing the bound water molecule, the probe is selective for phosphate diesters with two terminal oxygens and two identical ester groups. Similar analytes such as phosphorothioic acid, *O*,*O*-diethylester, ethyl methylphosphonate, *O*-(4-nitrophenylphosphoryl)choline, and cyclic 3,5-adenosine monophosphate do not yield a response. As with our work with nucleotides, Morrow's contribution to anion recognition by lanthanide probes demonstrates that binding does not necessitate coordination to the metal center and that exquisite selectivity can be achieved with the use of outer-sphere supramolecular recognition motifs.

4.3. Luminescence Response via Ligand Exchange

Weak lanthanide complexes readily undergo ligand exchange with strong chelating ligands such as phosphates and phosphonates. Ligand displacement enables sensitive but neither selective nor reversible detection of inorganic phosphate, nucleotides and therefore, by extension, other organophosphates. The phenanthrolines of the very weak Ln-**77** complex (Figure 11), for instance, are readily displaced by V-series organophosphate chemical warfare agents (CWA).[65] The less basic G-series agents have lower affinity for coordinating Eu^{III}. Similarly, omethoate readily displaces the triazole-pyridine ligands of the bis-tridentate assembly of Sun, Eu-**78**.[66] Decomplexation results in complete quenching of the Eu^{III}-centered emission. Likewise, the luminescent but unstable Eu-**79** and Eu-**80** complexes of Patra are displaced by diethyl chlorophosphate, a nerve agent, also resulting in complete loss of phosphorescence.[67] Despite their sensitivity, it is likely that all of these probes also fall apart in the presence of inorganic phosphates or basic organic phosphates, which limits their practicality.

5. Luminescent Probes for Nucleic Acids

While the above discussion has focused on the design of molecular probes that target small molecular weight phosphates, such as orthophosphate and nucleotides, lanthanide complexes have also been designed for macromolecular phosphates. These include dsDNA, aptamers-single-stranded oligonucleotides that selectively recognize targeted analytes due to their unique three-dimensional conformations-and DNAzymes that cleave upon complexation of a lanthanide ion. The molecular composition of these complexes is summarized in the following Figures 12–15.

5.1. Lanthanide Probes for dsDNA

There are two general classes of lanthanide-based molecular probes for dsDNA: metallointercalators and molecular beacons. Metallointercalators intercalate between two base pairs of dsDNA, resulting in a doubling of the rise and, most of the time, a change in the lanthanide-centered luminescence. These form supramolecular assemblies with dsDNA and thus have, for the most part, poor selectivity for a specific sequence, albeit higher sensitivity. Instead, sequence selectivity can be achieved either with molecular beacons or with responsive lanthanide probes covalently conjugated to a complementary strand.

The first lanthanide complexes designed to bind dsDNA were developed by Parker.[68] In most cases, the lanthanide-centered emission is quenched upon interaction with guanines due to the formation of a charge-transfer complex with the excited state of the antenna. Two more recent examples include the complexes Tb-**81** and Tb-**82** (Figure 12), the luminescence of both of which is decreased upon interaction with electron-rich GC base pairs.[69] Of note, in this case, the luminescence of the europium(III) analogs was not affected by guanines. The response of both probes to dsDNA binding was used to monitor changes in nucleic acid in dividing cells.

In the absence of crystal structures of the Ln^{III} complex·DNA adduct, true intercalation can be difficult to prove, although it can be inferred by changes in the excitation spectra of the complex. A recent example of a true intercalator is the heterotrimetallic Eu-**83** developed by Pikramenou.[70] The two square planar terpyridyl platinum(II) moieties intercalate in dsDNA, thereby solidly anchoring the central europium(III) in a hairpin fashion. Unlike other lanthanide-based intercalators, the europium(III) centered luminescence of Eu-**83** increases upon dsDNA binding, likely due to the effect of the electron-rich and hydrophobic environment of the intercalated tpy ligands on the efficacy of ligand-to-ligand charge transfer (LLCT).

In all other cases, intercalation of a lanthanide complex in dsDNA result in significant reduction of the lanthanide-centered emission. This is the case for the two complexes Tb-**52** and Eu-

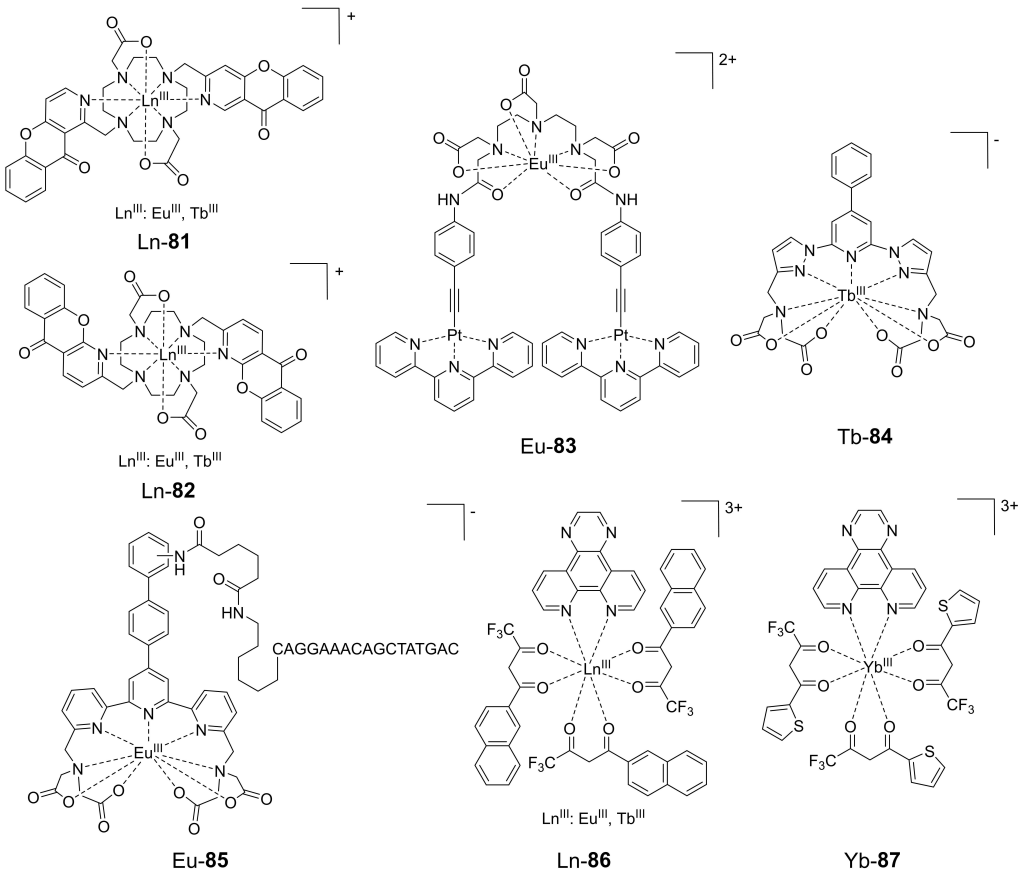


Figure 12. Responsive luminescent lanthanide-based metallointercalators and luminescent probes for dsDNA.

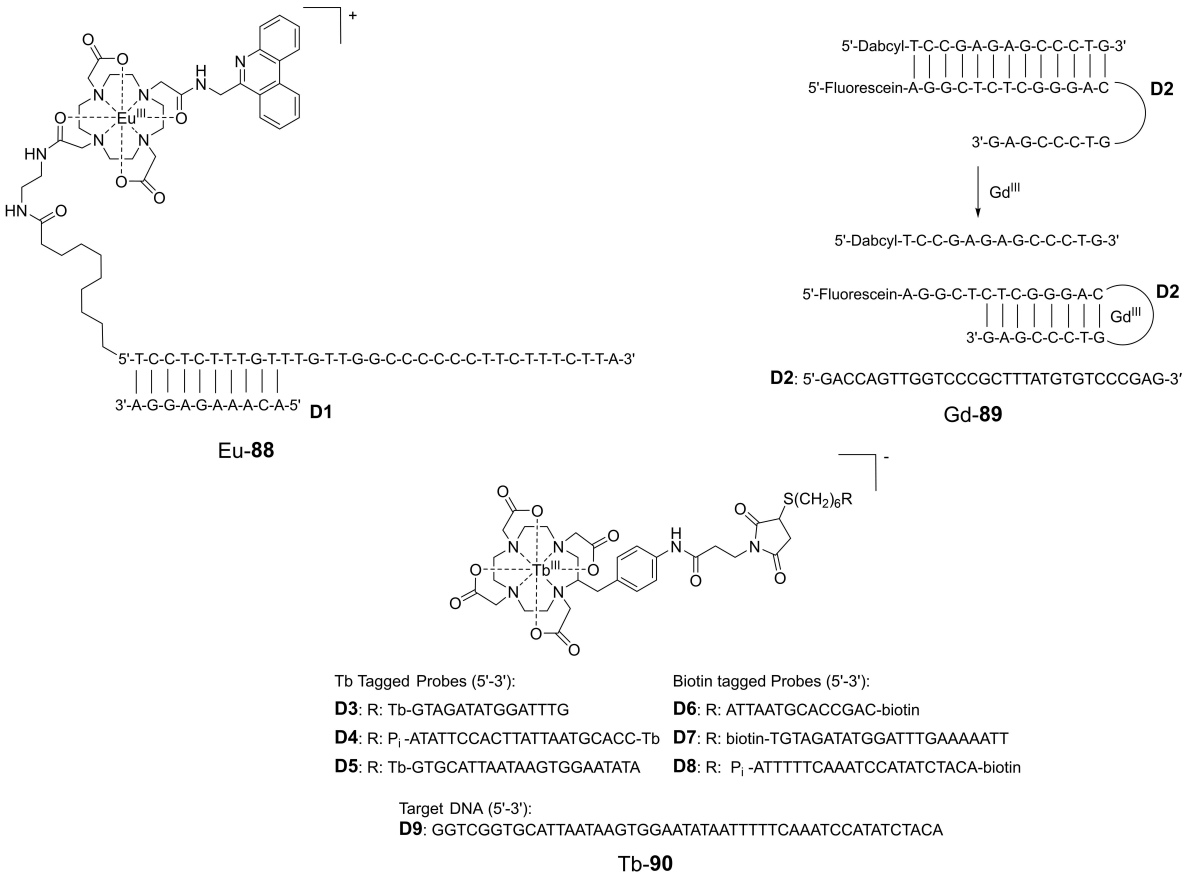


Figure 13. Responsive luminescent lanthanide-based aptamers.

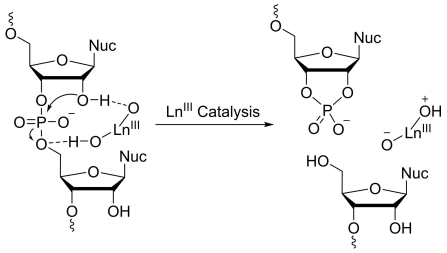


Figure 14. Mechanism of lanthanide ion-catalyzed RNA cleavage in RNA-zymes.

53 previously (Figure 8) reported by our group for nucleotide sensing. These two complexes can intercalate in three-dimensional DNA nanostructure such as Turberfield's tetrahedron[71] with as much as one metallointercalator for every two base pairs.[72] The positively charged Tb-**52** has substantially higher affinity for dsDNA than the neutral Eu-**53**. Advantageously, the supramolecular assembly increases significantly the cell-uptake of the lanthanide complexes in the presence of lipofectamine. Metallointercalators-DNA self-assemblies are thus a unique and efficacious strategy for carrying metalloprobes and drugs that have otherwise poor uptake inside cells.

Supramolecular assemblies can also be achieved by conjugating the terbium(III) complex onto iron oxide nanoparticles via a polyethylene glycol linker.[73] The incorporation of superparamagnetic nanoparticles bestows the construct with a second modality to detect dsDNA. Not only is the Tb^{III} emission quenched, but the formation of three-dimensional nanoparticle-DNA assemblies gives a magnetic response with significant changes in both the longitudinal and transverse relaxivities. Advantageously, since the metallointercalators have poor affinity for denatured DNA, the magnetic assemblies offer a unique strategy for purifying DNA by magnetic catch-and-release.

A metallointercalator can become selective for a specific oligonucleotide sequence if it is covalently conjugated to its complementary strand. One such example is the luminescent lanthanide complexes Tb-**84** and Eu-**85** reported by Matsumoto.[74] The use of a nonadentate ligand ensures that the europium(III) complex is highly stable, and that it has no open coordination site for phosphate coordination. This ensures that the complex intercalates in the π-stack of the dsDNA and prevents coordination by the sugar phosphate backbone, which might result in displacement of the ligand. Intercalation favors energy transfer between the antenna of Eu-**85** and the purines of the dsDNA, and consequently significantly decreases the luminescence intensity of the emitting lanthanides.

In the absence of a strong, octa- or nonadentate ligand, the complex is much less stable in solution. Strongly coordinating phosphates-including the phosphates of the backbone of nucleic acids-can thus displace weak mono- or bidentate

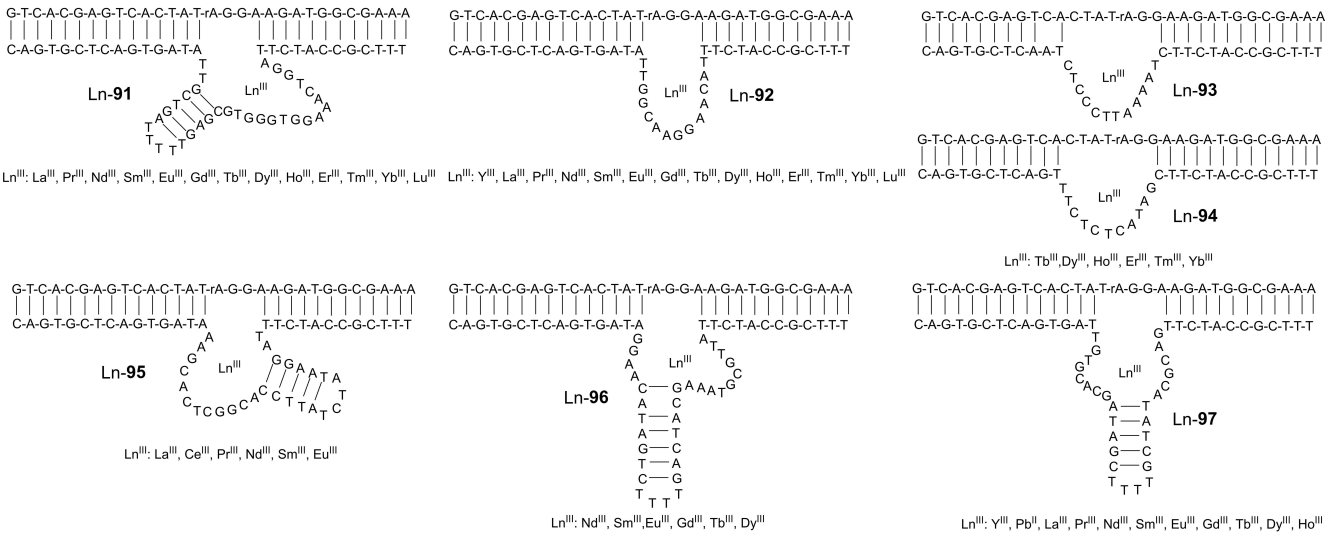


Figure 15. Responsive luminescent RNAzymes.

ligands with much lower basicity. Such complexes will display apparent high affinity for dsDNA, but they will not necessarily stay intact and will behave essentially as indicator displacement assays (section 2c). This is likely the mechanism of action of the probes Ln-**86** and Yb-**87** developed by Patra.[75] Of note, poorly stable probes can still present biological activity. Both Ln-**86** and Yb-**87** cleave DNA hydrolytically, as does a bimetallic complex comprising tolfenamic acid.[76] These studies serve as a reminder not to overestimate the stability of lanthanide complexes and not underestimate their ability to undergo rapid ligand exchange in solution with strong phosphate or even carboxylate ligands. Unless a well-studied, stable and inert ligand structure is used, such as one based on the macrocyclic polyaminocarboxylate DOTA ligand, a mechanism of action should not be asserted without solution studies confirming the stability of the probe and its resistivity to anion-based decomplexation.

5.2. Lanthanide-Based Luminescent Aptamer Probes

Aptamers are short, single-stranded nucleic acids-either RNA or DNA-that bind selectively to a specific target. The target can be small, such as a single ion or small molecule, or large, such as a protein or even a live cell. They are, essentially, nucleic acid versions of antibodies. As such, any luminescent lanthanide probe for nucleic acid can be modified to detect, in principle, any analyte simply by conjugating the lanthanide complex to the corresponding aptamer. (In practice, the number of aptamers reported remain limited.) Indeed, the phenanthridine-based complexes Tb-**52** and Eu-**53** (Figure 8), developed by our group, can monitor enzymatic reactions by monitoring the ratio of ATP/ADP or GTP/GDP;[50–51] the same complexes self-assemble in three-dimensional dsDNA nanostructure and one-dimensional dsDNA as seen in Eu-**88** (Figure 13).[72] Extension of the same principles enable detection of Hg^{II}, a soft-metal, simply by conjugating either complex to an aptamer for Hg^{II}. In the absence of mercury, the phenanthridine intercalates in the dsDNA bound to **D1**, and the europium's luminescence is weak. Coordination of Hg^{II} by the thymines releases the complementary oligonucleotide, which prevents intercalation of the phenanthridine and thus restores the luminescence of the Eu^{III}.[77]

These approaches can be flipped: instead of using a luminescence lanthanide complex to detect an analyte that is not luminescent, one or two organic fluorophores can be used to detect the presence of a lanthanide ion. Edogun demonstrated this methodology with the lanthanide aptamer Gd-**89**.[78] The aptamer strand **D2** was optimized to selectively coordinate gadolinium(III). Lanthanide coordination releases the quenching fluorophore strand resulting in an increase in the luminescence of the fluorescein. This mode of action should not be confounded with chemosensors, in which free metal ions exhibit unique luminescence responses due to sensitization from nucleotide bases.[79]

Lanthanide-labeled aptamers easily enable detection of analytes by luminescence; this can be further coupled with other modalities such as ICP-MS. Many of these modalities, however, suffer from poor sensitivity so that some method of signal amplification must be incorporated. Often, such amplifications are performed by polymerase chain reaction (PCR) which in turn requires heat-stable lanthanide complexes. This is the case with the probe Tb-**90** developed by Linscheid.[80] High sensitivity was achieved using two tagged probes, one tagged with the luminescent terbium(III) complex (**D3-D5**) and the other- the capture probe-labeled with biotin (**D6-D8**). The biotin moiety has high affinity to streptavidin-coated on nanoparticles, which enables amplification of the target sequence **D9** with each PCR cycle. This approach enables detection of the target down to 0.4 fM.

5.3. Lanthanide-Based RNAzymes

RNAzymes, also called RNA enzymes or catalytic RNA, undergo a specific catalytic reaction, behaving essentially as artificial ribozymes. Lanthanide ions, in particular, are good Lewis acids with propensity to cleave the backbones of RNA, and to a lesser extent those of DNA, according to a general mechanism depicted in Figure 14. The lanthanide ion(s) triggers a nucleophilic attack from the alcohol moiety to the phosphate, which cleaves the phosphoester bond in the "rA" (ribo-adenosine) nucleotide. This reactivity enables differentiation of lanthanide ions through cleavage efficiency that can be monitored by luminescence response when a fluorophore is liberated upon release of the targeted rA nucleotide.[81] In a manner similar to that used to obtain aptamers, RNA sequences were conservatively selected for reactivity with desired lanthanide ions, which ultimately enabled the differentiation of rare-earths.

The first generation of lanthanide-selective RNAzymes developed by the Liu group used the lanthanide ion(s) as the cofactor.[82] Although these initial artificial enzymes did not distinguish between all the trivalent lanthanide ions on their own, it was found Ln-**91** (Figure 15) was selective for Ce^{III} over Ce^{IV}. Further studies with Ln-**91** demonstrated that lanthanide coordination was not the rate-limiting step and that guanine had the highest affinity for the lanthanides.[83] Interestingly, this complex had the strongest affinity for Nd^{III} and Ho^{III}, which share three unpaired f electrons. The importance of the size of the lanthanide ion in achieving selectivity was established with Ln-**92**, with which the smaller late lanthanides have the highest reactivity.[84] More RNAzyme families were developed in subsequent studies to enhance the selectivity for heavy lanthanides, including Ln-**93** and Ln-**94**.[85] Ln-**95**, for instance, is more selective for lighter trivalent lanthanide ions and selectively cleaves 2′-5′ RNA.[86] Ln-**96** is selective for lanthanides in the middle of the series.[87] Impressively, when combined together and with Ln-**97** (another middle series selective RNAzyme), the above RNAzymes can differentiate the lanthanide ions with high accuracy, utilizing relative cleaving efficiency.[81] The potential of this unique selective affinity could be functionalized further for lanthanide separation, establishing unique preorganization strategies dependent on conservatively selected sequences.

5.4. An Overlooked Flaw: Phosphodiesterase Activity of Lanthanides

The ability of lanthanide ions to cleave DNA and RNA and function in DNAzymes and RNAzymes arises from their unique properties. They are excellent Lewis acids, with high charge density, large ionic radii, and high oxophilicity favoring phosphate coordination. The combination of these factors also make them excellent catalysts for cleaving phosphoesters. Indeed, several lanthanide complexes with open coordination sites behave as excellent phosphodiesterase mimics. This behavior should be kept in mind when designing probes for polyphosphates such as ATP, since some complexes could be capable of not just binding ATP, but also cleaving it.

The ability of lanthanide complexes to behave as phosphodiesterases was studied primarily by Morrow. The complex Eu-**98** (Figure 16) efficiently cleaves ApUp and oligomers of adenylic acid at 37 °C.[88] Importantly, because it is both thermodynamically stable and kinetically inert, it does not undergo ligand exchange with phosphates. Further studies with macrocyclic complexes established guidelines for predicting lanthanide phosphodiesterase activity.[89] The tetraamide La-**99** and the tetra-hydroxylpropyl Eu-**8** (Figure 3) are both catalytically inactive. On the other hand, the complex Eu-**100**, which also contains four hydroxyl groups, does display good phosphodiesterase activity. Interestingly, it preferentially cleaves α-phosphates on the G residue of the monoribonucleotide m^7GpppG than on the m^7G one, possibly due to electropositive charge of the latter. The complex Eu-**101**, which contains a single amide arm, has similar catalytic activity as its tetrahydroxyethyl analog Eu-**100**. That Eu-**100** displays phosphodiesterase activity whereas its analog Eu-**8** does not is likely due to the increased steric hindrance at the coordination site arising from the four methyl groups of Eu-**8**. Zinc(II), another excellent Lewis acid, enhances the phosphodiesterase activity of lanthanide complexes, likely by facilitating the initial nucleophilic attack.

Phosphodiesterase activity extends to macrocyclic triamide structures similar to many molecular probes developed for nucleotide sensing.[90] Both monoester and diester phosphates coordinate the lanthanide ion of Eu-**102** to **105** via displacement of the inner-sphere water molecule. Phosphate monoester bridges the two lanthanides of the dinuclear Eu-**102** and Eu-**103**. All four complexes promote cleavage of 2-hydroxypropyl-4-nitrophenyl phosphate (HpPNP), an RNA analogue, and uridylyl-3′,5′-uridine (UpU), a dinucleoside, with a simple linear dependence of reactivity on pH. Further mechanistic studies demonstrated that the catalytic activity results from stabilization of the developing negative charge on the phosphorane transition state by the lanthanide ion. Unlike Zn^{II} and Cu^{II}, which hydrolyze at neutral pH to form hydroxo species, the higher pK_a of lanthanide ions favors the formation of an aqua complex that is more reactive.

The phosphodiesterase mimics Eu-**99** to Eu-**105** are quite similar to some of the luminescent lanthanide probes for nucleotide sensing that function by direct coordination to the lanthanide ion. Both classes contain pendant aromatic groups, both have macrocyclic ligands with amide arms, both enable phosphate coordination. This similarity raises the specter that some probes for ATP and GTP could not just bind the nucleotide but also catalyze its hydrolysis. Because such reactivity would undoubtedly affect the ability of the probes to monitor kinase or adipose activity, it should be kept in mind in studying the kinetics of nucleotide binding and determining their mode of action.

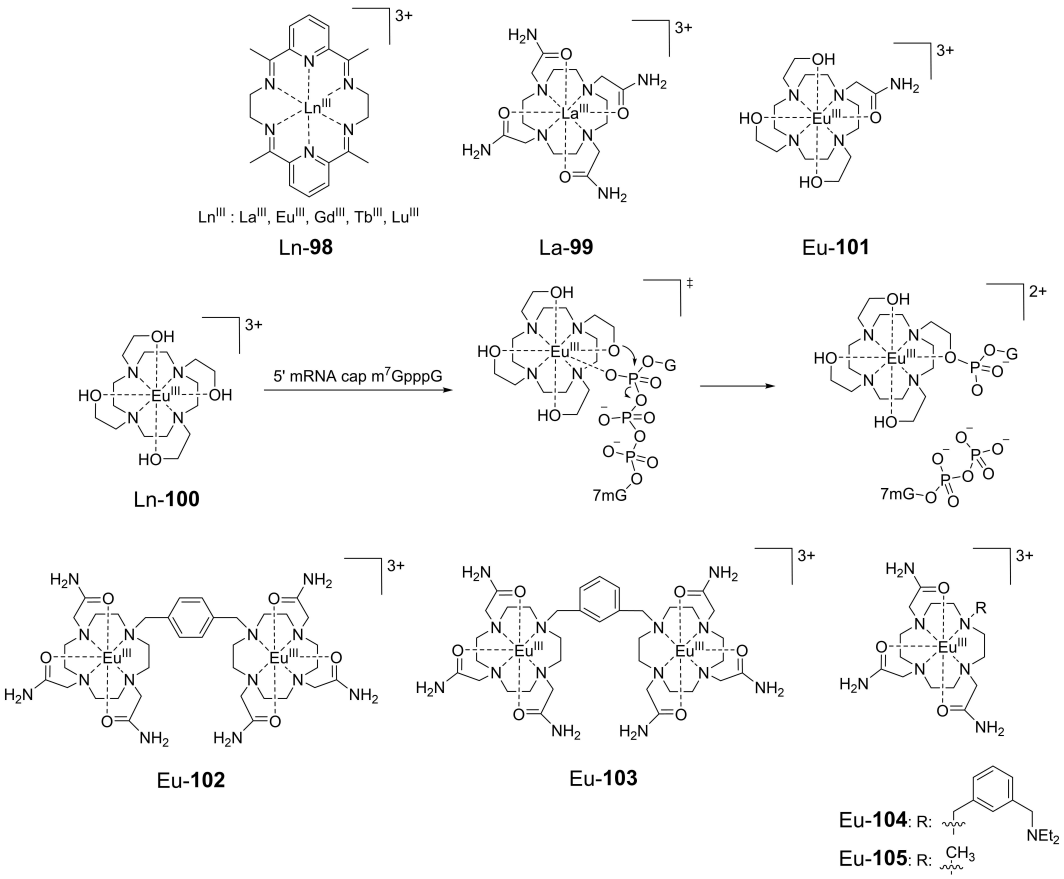


Figure 16. Lanthanide complexes as phosphodiesterase mimics.

6. Conclusion and Outlook

The above discussion has focused on the varied principles that have been exploited successfully over the last decade in the design of lanthanide complexes with unique selectivity for specific inorganic or organic phosphates. The field of lanthanide-based molecular probes for phosphates is now well rounded. Selectivity is almost never complete, but is often sufficient to monitor a variety of enzyme assays or other biological processes with ease. Efficient and bright complexes that are selective for a variety of substrates such as orthophosphate, ATP, or AMP enable their detection directly in water at neutral pH. These should fulfill almost all needs for bioassays in drug discovery, among other applications. Nonetheless, certain areas remain underdeveloped, including the development of sensitive and selective probes for nerve agents and pesticides and sequence-specific lanthanide metallointercalators. Modifications that allow for two-photon excitation and/or near-infrared emission would further empower this class of molecular probes.

The review of the literature has brought up a few points that we advise the reader not to overlook when designing and evaluating lanthanide-based molecular probes. Selectivity toward orthosphosphate should always be determined when evaluating probes targeting organic phosphates and nucleic acids. Selectivity toward other anions should be investigated at relevant conditions; depending on the intended applications, this is often at either the intracellular or blood levels. The mechanism of action, and in particular the nature of the probe·phosphate product, should not be assumed solely based on a change in luminescence intensity. More thorough solution studies, including luminescence lifetime and NMR, are needed to truly assess the formation of the adduct. The luminescent properties of each probe, including quantum yields and extinction coefficient, which determine brightness, are important parameters that also affect the translational potential of probes. Yet, those values are seldom reported. We thus encourage the reader to consider and optimize them in the design of future probes.

Phosphates are excellent chelators for lanthanides, readily capable of displacing a weakly coordinating ligand. As such, the stability of the complex should always be taken into consideration in the design of such probes. Weak ligands of low denticity are ill-advised as they are almost always displaced. On the same note, obtaining a crystal structure of a complex, often from organic solutions, does not necessarily guarantee that the complex will stay intact in solution. Kinetics of phosphate binding are rarely reported, but they offer much insight into the behavior of such probes. We have found, for instance, that phosphate coordination to Eu-**24** can take as long as 15 min to reach equilibrium and form a Eu-**24**·Pi_3 adduct. Those kinetic studies should be performed before titrations so as to ensure that the latter are measured at thermodynamic equilibrium. It is

always preferable to achieve statistical significance by repeating each experiment at least three times. Taking the above points into consideration will help the investigator establish a more accurate picture of the true potential and mode of action of their probes.

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Conflict of Interest

The authors declare no conflict of interest.

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