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ARTICLE

Design and applications of metal-based molecular receptors and probes for inorganic phosphate

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Inorganic phosphate has numerous biomedical functions. Regulated primarily by the kidneys, phosphate reaches abnormally high blood levels in patients with advanced renal diseases. Since phosphate cannot be efficiently removed by dialysis, the resulting hyperphosphatemia leads to increase mortality. Phosphate is also an important component of the environmental chemistry of surface water. Required to secure our food supply, inorganic phosphate is also linked to eutrophication and the spread of algae blooms with an increasing economic and environmental burden. Key to resolving both of these issues is the development of accurate probes and molecular receptors for inorganic phosphate. Yet, quantifying phosphate in complex aqueous media remains challenging, as is the development of supramolecular receptors that have adequate sensitivity and selectivity for use in either blood or surface waters. Metal-based receptors are particularly well-suited for those applications as they can overcome the high hydration enthalpy of phosphate that limit the effectiveness of many organic receptors in water. Three different strategies are most commonly employed with inorganic receptors for anions: metal extrusion assays, responsive molecular receptors, and indicator displacement assays. In this review, the requirements for molecular receptors and probes for environmental applications are outlined. The different strategies deployed to recognize and sense phosphate with metal ions will be detailed, and their advantages and shortfalls will be delineated with key examples from the literature.

1. Relevance of inorganic phosphate in medicine

Inorganic phosphate has numerous biological and medical functions including the synthesis of adenosine triphosphate (ATP), protein phosphorylation and skeletal mineralization.[1] Phosphate is linked to the functions of muscles and nerves and influences calcification. Its level in blood is regulated by the kidneys. Hence, advanced chronic or acute kidney diseases often lead to hyperphosphatemia, a condition defined as an abnormally high serum phosphate concentration of >1.46 mM. This disorder occurs when the reduced glomerular filtration rate that results from impaired kidney function does not sufficiently eliminate excess phosphate and when other homoeostatic mechanisms, including tubular reabsorption regulated by the parathyroid hormone (PTH), do not sufficiently reduce phosphate uptake.[2]

Hyperphosphatemia has long been established as a cause of increased mortality for patients with end-stage renal disease.[3] The inability of dialysis to efficiently remove phosphate from blood further contributes to its progression. Indeed, despite its low molecular weight, phosphate is highly hydrated and possesses a very bulky and tightly coordinated hydration sphere that prevents it from going through the semipermeable membranes used in dialysis. As such, phosphate regulation for patients with advanced kidney diseases instead focuses on the use of phosphate binders: tablets taken orally with intake of food. Phosphate binders are polyamines or weak metal complexes such as calcium acetate and lanthanum acetate that decrease phosphate uptake via adsorption or chelation. They are neither selective nor sufficiently effective to manage the hyperphosphatemia of most patients with advanced kidney disease. More than 70% of patients with advanced chronic

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kidney disease and almost all patients with dialysis-dependent kidney failure suffer from hyperphosphatemia.[4]

In healthy individuals, blood levels of phosphate range between 0.8-1.45 mM.[5] These levels are substantially lower than those of chloride (98-106 mM) and bicarbonate (23-29 mM) and comparable to those of lactate (0.5-1 mM). Of concern, the latter two oxyanions can compete with phosphate for metal coordination in the design of metal-based receptors. Sulfate (0.46-11.9 μM) and citrate (100-150 μM) are usually present at lower concentrations and are thus not a source of concern in the design of receptors. Similarly, organophosphates are typically present at negligible concentrations in serum. For medical applications receptors targeting serum or interstitial phosphate must be effective in water and display modest affinity for the anion but excellent selectivity, particularly over bicarbonate, chloride and lactate.

Intracellular concentrations of inorganic phosphate are higher than those of serum or interstitial fluids, and typically range between 1-5 mM depending on the tissue, subcellular compartmentalization, pH and hormone levels. Levels of organophosphates (phosphocreatine, ATP, ADP, GTP and hexophosphates) in cells can be substantially higher than those of 'free' inorganic phosphate.[5] In muscles for instance, phosphocreatine can reach levels 20 times higher than those of inorganic phosphate. Intracellular quantification of phosphate levels thus requires a probe that has low affinity for phosphate, but high selectivity for phosphate over organophosphates and other intracellular anions such as bicarbonate and chloride. This selectivity can be difficult to achieve.

2. Relevance of inorganic phosphate in aqueous environmental chemistry

Inorganic phosphate is also of concern to the environmental health of surface waters, including lakes, rivers, and coastal waters. Indeed, our society's relationship with phosphorus is a paradoxical one. On the one hand, phosphorus fertilizers are crucial to our food supply. The green revolution was propelled four decades ago in part from the availability of affordable artificial phosphorus-nitrogen fertilizers. Although our consumption of phosphorus has somewhat decreased from its peak of nearly 20 million tons/year in the mid 1980's, ~90% of the phosphorus mined today is still used to support the production of food.[6] Supporting the world growing population depends on the ready availability of inorganic phosphate.

On the other end, the damage caused by the over-use of phosphorus fertilizers to our environment is becoming more immediate. Many inland and coastal waters now suffer from an over-supply of phosphorus due primarily to agriculture run-offs, but also to the processing and transportation of phosphorus.[7,8] This over-supply leads to eutrophication, algal blooms, and, increasingly, to the formation of aquatic dead-zones. Many algal blooms are caused by toxic blue-green alga such as microcystis that produces the toxin microcystin. Such toxins can be deadly to both animals and humans. Algal blooms are now commonly and periodically seen throughout the world, and the economic impact of phosphate accumulation in surface waters increases yearly. In 2014, algal blooms caused the city of Toledo to shut down its drinking water supply to its 400,000 residents for four days.[9,10] The 2017 algae bloom that covered 700 square miles of Lake Erie,[11] and the 2019 algae blooms that forced the state of Mississippi to close all of its beaches highlight the growing economic impact of nutrient runoffs in our water system.[12]

The requirements for sensitivity and selectivity of any assay for a substrate is governed by the sample in which it has to be determined. The phosphate levels of surface waters, even polluted ones, are 1,000 fold lower than those of biological milieus. The guidelines provided by the US Environment Protection Agency (EPA) indicate that the phosphorus level of relatively uncontaminated lake surface waters range from 0.1 – 0.3 μM.[13,14] The recommendation of the EPA is that for prevention of algae bloom, the total phosphates and

phosphorus should not exceed 0.5 μM in any stream at the point where it enters any lake or reservoir, nor 0.25 μM within the lake or reservoir. The desired goal for prevention of plant nuisances in streams or other flowing waters not discharging directly to lakes or impoundments is a total phosphorus concentration below 1 μM.[15] These numbers highlight the different needs for phosphate receptors used for aqueous environmental applications compared to biomedical ones. In particular, efficient environmental phosphate remediation of surface waters requires that the receptors not only function in water but have very high affinity for the inorganic oxyanions.

The media also dictates the requirements for selectivity of a receptor. Anions that can compete with phosphate for a receptor in surface waters differ from those that should be considered for biomedical applications. Bicarbonate and chloride are present in high concentration in both sea water and blood, but unlike serum, surface waters have negligible concentrations of lactate and citrate. Concentrations of nitrates and sulfates, on the other hand, can be sufficient to compete with phosphate. Selectivity over these anions is thus a prerequisite for any aqueous environmental applications of phosphate receptors. Phosphate receptors that are appropriate for biomedical applications are thus rarely appropriate for aqueous environmental applications. The differences between the milieus are such that they preclude the use of a 'one size fits all' approach to the design of receptors for inorganic phosphates.

3. Intermolecular forces at play in phosphate recognition

3.1. Organic and biological receptors

Recognition of inorganic phosphate and phosphorylated molecules in vivo relies primarily on hydrogen-bonding and electrostatic interactions. Examples include the periplasmic phosphate binding class of proteins (PBP).[16] These weak intermolecular forces are rendered more potent when the recognition site is buried within a hydrophobic pocket of a protein receptor. The directionality and geometric arrangement of these interactions are key to tuning the affinity and the selectivity of the receptor for phosphate. Nonetheless, the affinity of those biological receptors for phosphate is low, typically in the mM range, as necessary to regulate the oxyanion that is present in high concentration both intracellularly ('free' phosphate ~ 10 mM) and extracellularly (0.8-1.45 mmol/L mM in blood).

Organic receptors that mimic these biological ones also rely primarily on hydrogen-bonding and electrostatic interactions. Although not a topic of this focused review, excellent reviews on organic receptors for phosphate have been published.[17-25] Hydrogen-bonding moieties in those receptors include amides, ammoniums, imidazoliums, ureas and guanidiniums, the latter reduces the pH dependence of the recognition.[26-29] As a class, many of those probes are characterized by low affinity for phosphate and strong dependence on pH. Discrimination between different oxyanions requires the careful positioning of both the positive charges and the hydrogen-bonding motifs, hence the prevalence of pre-organized receptors with rigid C_{3v} symmetry. In general, the weak intermolecular interactions at play in many of those receptors are insufficient to overcome the high hydration enthalpy of inorganic phosphate.[30,31] As a result, most organic probes for inorganic phosphate do not function in pure water and have thus limited use for aqueous environmental chemistry.

3.2. Inorganic receptors

The low levels of phosphate in surface water, typically in the range of 10-100 μg/L, requires receptors that function not only in water but also with substantially higher affinity for the anion than those displayed by most biological and organic receptors. Achieving such high affinity requires the use of stronger intermolecular interactions that can overcome the high hydration enthalpy of phosphate. Advantageously, phosphate is also an excellent coordinating ligand. Its high Pearson's hardness indicates that one of the most efficient ways to recognize it in water is via coordination to a similarly hard metal ion (Tables 1 and 2), an interaction that although strong, remains highly polar in nature. The advantage of using metals to recognize phosphate in water is that this approach immediately infers strong selectivity over poorly coordinating anions that can be present in high concentration in both blood and surface waters, particularly chloride, nitrate, and sulfate. On the other hand, selectivity over other coordinating hard anions, including bicarbonate, fluoride and arsenate, can be more difficult to tailor than with organic receptors.

Table 1. Gibb's free energy of hydration, radius, absolute hardness (η), and pK_a of conjugate acid of coordinating anions.

	$\Delta G°_{hydration}$ (kJ/mol)	Radius (Å)	η (eV)[a]	pK_a of conjugate acid
HPO_4^{2-}	-465	2.38	2.77	7.2
$H_2PO_4^-$	-	-	3.48	2.1
$HAsO_4^{2-}$	-	2.48	2.68	6.94
$H_2AsO_4^-$	-	-	3.22	2.2
HCO_3^-	-335	1.56	3.52	6.3
F^-	-465	1.26	5.81	3.21

[a] Hardness values calculated at PBE/aug-cc-pVTZ with COSMO solvation.[32]

Table 2. Gibb's free energy of hydration, radius, absolute hardness (η) of metal ions commonly used for phosphate recognition.

	$\Delta G°_{hydration}$ (kJ/mol)	Radius (Å)[a]	η (eV)[b]
Cu^{II}	-2010	0.73	8.27
Zn^{II}	-1955	0.74	10.88
Fe^{III}	-4265	0.64	12.08
Eu^{III}	-3360	1.06	-
Gd^{III}	-3375	1.05	-
Tb^{III}	-3400	1.04	-

[a] radius of transition metals and lanthanides for their CN=6 and 8, respectively, [b] hardness values of those lanthanides are not know but lie between that of La^{III} (15.39 eV) and Lu^{III} (12.12 eV).[33,34]

The properties that affect the affinity and selectivity of inorganic probes for phosphate: Pearson's hardness, lability, steric hindrance at the open coordination site, complex geometry, and chelate basicity, among others, are different than those exploited in organic-based receptors. Many of those parameters are still incompletely understood and are being currently investigated. Advantageously, the strong Lewis acid-base interaction can be used concomitantly with the weak intermolecular interactions used with organic receptors. Careful positioning of hydrogen-bonding and charged moieties can fine-tune the affinity of metal-based receptors as desired. In this regard, recognition of phosphate by any receptor, be it organic or inorganic, depends on the protonation of the oxyanion and thus on the pH. Metal complexes will typically have higher affinity for phosphate at higher pH when phosphate is deprotonated, than at lower ones when the anions is partially or fully protonated. At neutral pH, which is of relevance to both medical and environmental applications, phosphate is present in a near 1:1 ratio of $H_2PO_4^-$ and HPO_4^{2-}.

Metal complexes have been appended to organic receptors to infer a luminescent response or to facilitate the pre-organization of hydrogen-bonding and electrostatic interactions. [35-39] In those complexes, recognition of phosphate is not achieved via coordination chemistry and many such probes suffer from the same limitation as their purely organic precursors. This review focuses instead on phosphate receptors that function via coordination to a metal ion. It also focuses on the recognition of inorganic phosphate; although the principles outlined below are also applicable to organophosphates.

There are three strategies predominantly used to design metal-based receptors and probes for inorganic phosphate: metal extrusion assays, molecular receptors, and indicator displacement assays. They differ primarily on the strength of the auxiliary chelate L of the ML receptors. Metal extrusion assays rely on weak complexes in which the ligand L can readily be displaced by phosphate. They result in the formation of MPi (Pi = $H_2PO_4^-/HPO_4^{2-}/PO_4^{3-}$) salts whose solubility is determined by the metal ion (eqn 1).

$$ML + Pi \leftrightarrows MPi + L \quad (1)$$

Molecular receptors, on the other hand, have stronger chelators L that are not susceptible to trans-chelation, but must leave at least one open coordination site on the metal ion to enable Pi binding. They result in the formation of ternary (n=1) or sometimes quaternary (n=2) complexes (eqn 2).

$$ML + n\ Pi \leftrightarrows MLPi_n \quad (2)$$

Indictor displacement assays combine both strategies with metal complexes that contain two different ligands: one with a strong affinity for the metal, L, that ensures that the metal is always complexed in solution, and one with a weak affinity for the metal, L', that can be displaced by phosphate and is used to give a fluorescent or colorimetric readout (eqn 3).

$$MLL' + Pi \leftrightarrows MLPi + L' \quad (3)$$

The three strategies share several features. They each rely on the coordination of a metal ion by phosphate. Phosphate is a hard Pearson's base and often a mono-dentate ligand. As such, hard metal ions such as Cu^{II}, Zn^{II} and Ln^{III} are more suitable (Table 2). The affinity of the receptors for phosphate, a thermodynamic component of the respective equilibrium, is also strongly influenced by the chelate L. Kinetics is a different parameter that should not be ignored. Metal ions that are labile, that is, according to Taube's definition,[40] exchange a ligand faster than 1 minute, are favored over their inert counterparts for practical reasons. Of note, inert metal complexes are not unreactive; they simply display slow kinetics of ligand exchange from minutes to years. In each case, turning the receptor into a molecular probe, luminescent or otherwise, is a function primarily of the properties of the metal ion M and the chelate L. Regardless of the strategy adopted, one should aim to match the affinity of the receptor to the concentration of phosphate present in the milieu of interest. Probes for intracellular applications should have low affinity for phosphate as appropriate for inorganic phosphate concentration in the 1-5 mM range. Aqueous environmental applications, on the other hand, require a substantially more sensitive probe to measure phosphate in the 100 nM range.

4. Metal extrusion indicators

4.1 Design principles and limitations of metal extrusion indicators and assays

In metal extrusion assays (eqn 1), the chelate L is required to have weaker affinity for the metal M than phosphate so as to be readily displaced by the latter. Note that this does not necessarily require that the receptor ML has an open coordination site since ligand exchange can proceed via a dissociative mechanism. Bidentate and tridentate ligands that have weaker affinity for metal ions than chelates of higher denticity are more appropriate for those assays. The phosphate salts of the metal ions used in such assays such as Zn^{II} ($Zn_3(PO_4)_2$ K_{sp} = 9.0 x 10^{-33}), Cu^{II} ($Cu_3(PO_4)_2$ K_{sp} = 1.40 x 10^{-37}) and lanthanides ($LnPO_4$ K_{sp} = 2 x 10^{-22}) are typically very poorly soluble. This result in pushing equilibrium 1 to the right; hence the high sensitivity of such assays. Do note that those equilibria are pH dependent.

Copper and zinc are commonly used in such assays because these two metal ions can readily convert the receptors into luminescent probes, albeit via different mechanisms. Cu^{II}, which is d^9 and paramagnetic, quenches the luminescence of most of its fluorogenic chelates, usually by a photoinduced metal-to-fluorophore electron transfer (PET) mechanism or an energy-transfer (ET) process.[41] Displacement of Cu^{II} by phosphate removes the quenching metal ions and restores the luminescence of the chelate (Fig. 1a). These extrusion assays are thus 'turn-on'. Zn^{II} and Cu^{I}, isolectronic diamagnetic d^{10} ions but with different geometry and ligand preference, do not quench the fluorescence of their chelate. Instead, they commonly enhance the luminescence of their fluoroionophores typically by blocking the photoinduced electron transfer or

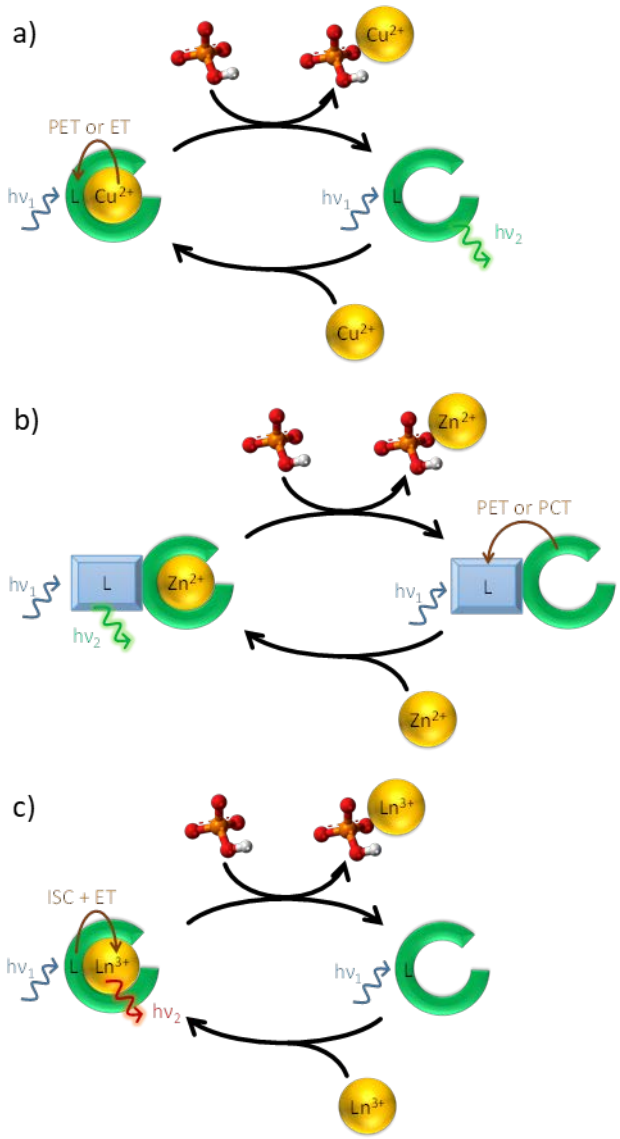


Fig. 1 Metal extrusion assay for inorganic phosphate. (a) Cu^{II} complexes of chromophores are poorly luminescent due to PET or ET from the transition metal to its chelate. Displacement of Cu^{II} by phosphate prevents PET or ET thereby turning on the fluorescence of the probe. (b) Zn^{II} complexes of fluoroionophores behave as turn-off fluorescent metal extrusion indicators (L = ligand). Displacement of the Zn^{II} by phosphate enables PET or PCT from the ionophore to the chromophore, thereby turning off the fluorescence of the probe. (c) Weakly coordinating antennas can sensitize the luminescence of certain lanthanide ions, such as Eu^{III} and Tb^{III}. Their displacement by phosphate results in non-emissive lanthanide phosphate salt precipitates.

photoinduced internal charge transfer (PCT) between the fluorophore and its conjugated ionophore (Fig. 1b).[42] In this case, displacement of the transition metal ion upon binding phosphate restores the electron or charge transfer process and thus 'turns off' the luminescence. Thus, in essence, any molecular luminescent probe for copper or zinc that has weaker affinity for the transition metal ion than does phosphate can also be used as a phosphate probe.

Luminescent lanthanide probes with weak chelates that are particularly apt to being displaced by phosphate function by a similar mechanism (Fig. 1c). Europium and terbium probes that contain an appropriate organic antenna will yield luminescent probes. If tuned correctly, intersystem-crossing followed by energy transfer from the chelate to the metal ion enables lanthanide-centered phosphorescence with its characteristically long emission lifetime uniquely suited for time-gated measurements. Displacement of the lanthanide ion by phosphate prevents this energy transfer resulting in a loss of phosphorescence. These probes typically employ either Eu^{III} (red emission) or Tb^{III} (green emission) due to the higher quantum yields of the luminescent complexes of these two lanthanides.[43,44] Gd^{III} complexes with weak chelates can also function similarly, albeit with a magnetic (relaxometric) readout as opposed to a luminescent one. Other probes with variations on these mechanistic themes have also been reported and will be discussed in greater detail below.

The oft-cited reversibility of those probes can be problematic. How much zinc or copper must be added to return the probe to its initial state depends on how much phosphate was present in the sample initially. Often, those systems result in the formation of phosphate salt precipitates that further muddles real-time monitoring of variations in phosphate levels. Selectivity of these assays is another area of concern. Selectivity versus other strong coordination anions that can also displace the chelate are more difficult to establish than with molecular receptors. In biological and in particular intracellular milieus, selectivity for inorganic phosphate over the large excess of diverse phosphorylated molecules present, in particular phosphocreatine (in muscles), hexose phosphate, ATP, ADP and GTP, if established, is often insufficient. This is unsurprising given the similarly high affinity of these phosphorylated species for metal ions. Selectivity versus other endogenous metal ions, which is rarely mentioned, can potentially be problematic if sufficient concentrations of competing and loosely bound metal ions that can transmetallate with the probe are present in the sample. The requirements for selectivity, just as for sensitivity, is a function of the milieu to be analyzed. The variations between environmental and biological applications preclude a 'one size fits all' approach to the design of fluorescent probe for inorganic phosphate.

4.2. Transition metal-based extrusion indicators

Those metal extrusion indicators are based on the removal of the metal ion by phosphate. As noted above, metal extrusion indicators that use Cu^{II} or similarly hard and paramagnetic metal ions turn-on in the presence of phosphate, whereas those that incorporate diamagnetic Zn^{II} complexes are typically turn-off (Fig. 1). One example of the first mechanism is the Cu^{II} complex Cu-**1** reported by Jang and coworkers (Fig. 2).[45] PET and ICT from Cu^{II} to the benzimidazole-based imine ligand turns off the luminescence of the latter. Addition of PO_4^{3-} to the receptor in HEPES buffered mixed aqueous media (8:2 CH_3CN-H_2O) restores the luminescence of the ligand by displacing the quenching transition metal ion.

This assay is relatively slow, requiring 9.5 min for full displacement to occur, with an estimated limit of detection of 2.8 μM that is not sufficient for environmental applications. Likewise, the Cu^{II} dansyl complex of Bandyopadhyay and coworkers, Cu-**2**, 'turns-on' upon displacement of the

fluorophore by $H_2PO_4^-$ thereby enabling determination of inorganic phosphate levels in biological samples such as human saliva, urine and chicken serum.[46] Chen and coworkers reported a similar 'turn-on' Cu^{II}-based extrusion indicator for $H_2PO_4^-$ using instead the naphthalimide Cu-**3**.[47] This indicator is selective for inorganic phosphate but only works in mixed aqueous media (3:1 MeOH-MOPS buffer). Similarly, the luminescence of deoxycholic acid (steroid)-coumarin based-ligand of Ju and coworkers, Cu-**4**, is quenched upon complexation of Cu^{II} via PET.[48] Careful position of the triazole moieties favors the 2:1 Cu^{II}:ligand stoichiometry. Addition of 2 equivalents of $H_2PO_4^-$ in methanol displaces the Cu^{II} ion and restores the luminescence of the ligand. This complex showed some selectivity over monovalent anions but is affected by the presence of histidines. The Cu^{II} complex Cu-**5**, also by Jang, likewise binds phosphate with log K_a = 3.81 in mixed aqueous media (7:3 DMSO-HEPES buffered H_2O).[49]

Although this class of indicators predominantly use Cu^{II}, any paramagnetic transition metal that is also hard can be employed. Fe^{III}, which has similarly high affinity for phosphate and which can also quench the luminescence of organic ligands either by metal-to-ligand charge transfer (MLCT) or by the para magnetic effect, is also effective. One such example is the Fe^{III} complex Fe-**6** of Meng *et al*. (Fig. 2).[50] Fe-**6** is poorly luminescent due to the effect of the paramagnetic Fe^{III} on fluorescein. Displacement of iron by $H_2PO_4^-$ binding in mixed aqueous media (THF-HEPES buffer) results in a 9.6 fold increase in luminescence. The indicator is surprisingly selective over phosphorylated molecules such as AMP, ADP and ATP and has been used successfully in live breast cancer cells MDA-MB-231 cultures.

Unlike Cu^{II} complexes, weak Zn^{II} complexes instead function as turn-off probes since displacing the Zn^{II} ion returns any PET or PCT quenching of the fluorophore by the ionophore

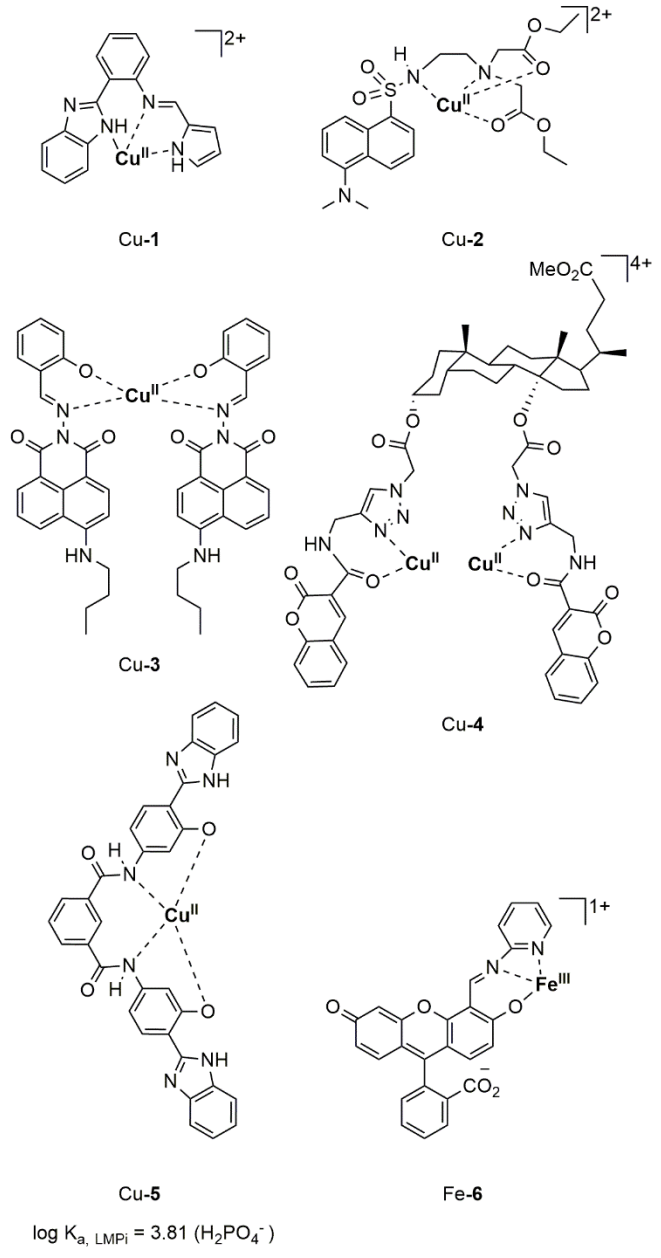


Fig. 2 Turn-on Cu^{II}- and Fe^{III}-based extrusion indicators that function via removal of the metal ion by inorganic phosphate.

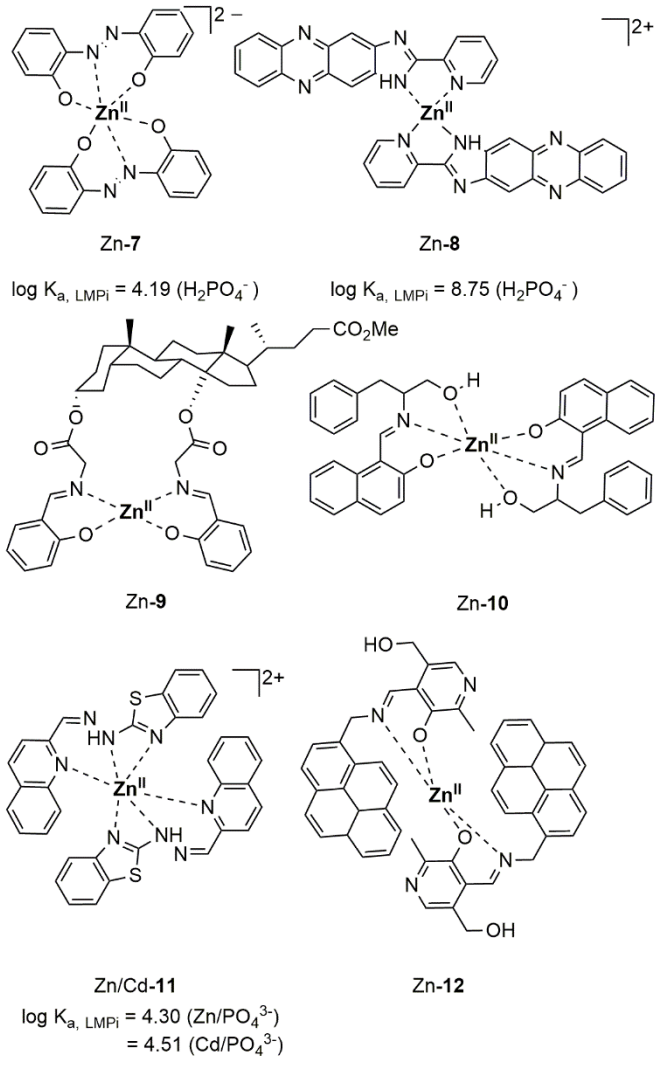


Fig. 3 Zn^{II}-based metal-extrusion indicators that function via removal of the metal ion by inorganic phosphate.

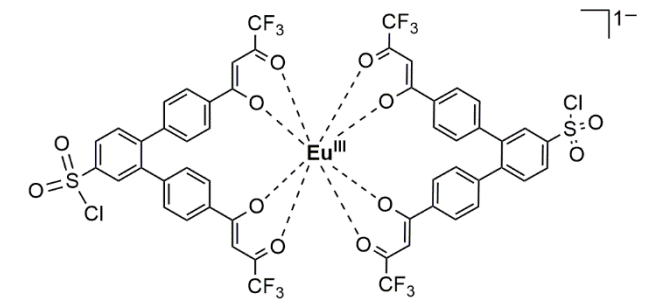
(Fig. 1b). Examples of such indicators are highlighted in Fig. 3. One such example is the 2,2'-dihydroxyazobenzene (DHAB)-Zn^{II} complex of Ha and coworkers.[51] Addition of $H_2PO_4^-$ to the luminescent Zn-**7** in 1:1 H_2O-MeOH mixture results in a decrease in luminescence as the non-fluorescent DHAB receptor is released. This indicator displays a log K_a = 4.19 for $H_2PO_4^-$. Zhang subsequently reported the 2-pyridine-1H-imidazole[4,5-b]phenazine-Zn^{II} complex which behaves similarly. In Zn-**8**, the transition metal blocks the conformation of the ligand thereby preventing the intramolecular charge transfer occurring in its twisted form.[52] Addition of $H_2PO_4^-$ to Zn-**8** in aqueous solution displaces the metal ion, thereby rest oring the ligand to its non-fluorescent twisted conformation. This indicator displays a substantially higher affinity for phosphate (log K_a = 8.75); advantageously, it is one of the few indicators that function in pure aqueous media. Likewise, Ju and coworkers reported on a similar salen-steroid complex, Zn-**9**, for $H_2PO_4^-$.[53] The Zn^{II} complex of the Schiff base 2-hydroxynaphthaldehyde and 2-amino 3-phenyl-1-propanol of Hens, Zn-**10**, also detects $H_2PO_4^-$ and HPO_4^{2-} via the same mechanism.[54] Other examples include the benzothiazole complexes of Zn^{II} and Cd^{II} of Das and coworkers, Zn/Cd-**11**.[55] In the absence of phosphate, metal coordination increases the fluorescence of the ligand by preventing PET from the nitrogen lone pair of the Schiff base to benzothiazole moiety. Phosphate coordination to the metal releases the ligand whose luminescence subsequently decreases. The Cd^{II} complex displays slightly higher affinity for phosphate (log K_a = 4.51) than does the Zn^{II} analog (log K_a = 4.30).

There are a few Zn^{II} displacement indicators for $H_2PO_4^-$ that function via a different mechanism. An example is the pyrene pyridoxal cascade recently published by Sahoo and coworkers.[56] Unlike the typical 'turn-off' based extrusion indicators discussed above, in Zn-**12**, Zn^{II} complexation enhances the fluorescence at 485 nm via the formation of static pyrene excimers (Fig. 3). Displacement of Zn^{II} by $H_2PO_4^-$ releases free monomeric pyrene that has weaker fluorescence. This receptor is not selective for phosphate over thiol containing biomolecules such as cysteine.

4.3. Lanthanide-based extrusion indicators

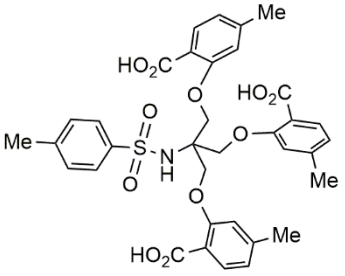
Lanthanide-based metal extrusion indicators typically use either Eu^{III} or Tb^{III}. The ability to sensitize the emission of those two lanthanide ions with coordinating organic antennas render them particularly attractive because such indicators will inherently be luminescent probes. One such example is the tetradentate β-diketonate Eu-**13** (Fig. 4) reported by Yang.[57] Since pyrophosphate has higher affinity for the lanthanide ion than the β-diketonate ligand, Eu-**13** dissociates in the presence of PPi to form a europium pyrophosphate precipitate. Whereas the ligand **13** can sensitize Eu^{III} emission, PPI cannot. Hence, ligand exchange (metal displacement) results in a loss of sensitized Eu^{III} emission and an 'on-off' probe for pyrophosphate. In such an assay, weaker coordinating anions cannot displace the β-diketonate ligand, and hence do not affect Eu^{III}'s luminescence.

A similar approach was employed by Peng and coworkers in their dimetallic Tb^{III} complex of the tripodal substituted salicylate **14** (Fig. 4).[58] Phosphate, a stronger chelating ligand, displaces the sensitizing salicylate ligand **14**, resulting in a turn-off response. The Eu^{III} and Tb^{III} complexes of the chiral phenanthroline-based ligand Ln-**15** behave similarly.[59,60] This complex, which is poorly stable, responds to HPO_4^{2-} as well as to the phosphorylated nucleotides ATP, ADP and AMP in buffered aqueous solutions at pH 7.4. [59,60] It is a rare example of a metal extrusion indicator that has been used successfully to image phosphate levels with cells, in this case green microalga.[59,60] Eu-**16**, another weak Eu^{III} complex, also exhibit the characteristic turn-off luminescence response when



Eu-**13**

log K_a = 5.42
affected by: PPi>ATP>ADP>Citrate>Tartrate



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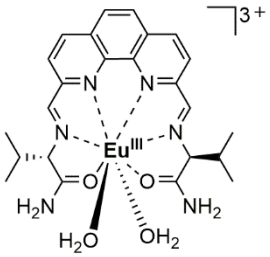
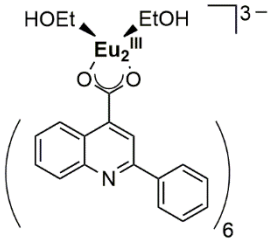
K_{sv} (M^{-1}) = 6.58 x 10^6 ($H_2PO_4^-$)
= 7.04 x 10^6 ($H_2PO_4^{2-}$)
affected by: other phosphorylated molecules



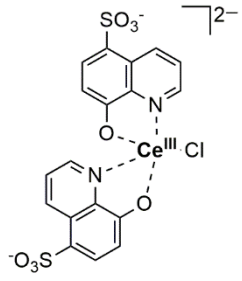
Ln-**15** (Eu/Tb)

log K_b = 5.4 ~ 5.5
affected by: NO_2^-



Eu-**16**

log K_a = 3.81 ($H_2PO_4^-$)
affected by: HSO_4^-



Ce-**17**

log K_a = 6.47 (HPO_4^{2-})

Fig. 4 Lanthanide-based extrusion indicators that function via removal of the metal ion by inorganic phosphate.

phosphate displaces the 2-phenyl-4-quinoline carboxylate ligand.[61] Ce-**17** uses a different lanthanide ion, cerium(III), to modify this mechanism of action so that it instead yields a turn-on luminescence response.[62] In this case, Ce^{III} quenches the ligand-centered fluorescence emission. Coordination of Ce^{III} by phosphate concomitant with release of the bis(8-hydroxy quinoline-5-sulphonate) ligand restores the fluorescence of the latter. The kinetics of transmetallation of lanthanide-based extrusion indicators have not been reported in the corresponding literature. Nonetheless, lanthanide ions are well-known to be kinetically labile. We expect, based on the lanthanide-based molecular receptors (Section 5.3) that those equilibria take no longer than a few minutes to be reached.

5. Molecular receptors and the formation of ternary complexes

5.1. Design principles for molecular receptors and probes

In inorganic molecular receptors for phosphate, the oxyanion does not displace the metal from its chelate. Instead, it occupies an open coordination site, initially filled by water or solvent molecules, resulting in the formation of either ternary MLPi co mplexes or, more rarely, quaternary MLPi2 complexes (eqn 2). Inorganic molecular receptors thus differ primarily from their metal extrusion assay counterparts by the stability of the ML complex. Whereas metal extrusion assays require weak chelates that result in less stable ML complexes that can readily exchange ligand; molecular receptors require strong chelates that form very stable ML complexes. Molecular receptors must, however, have at least one open coordination site, initially filled with water or another weak ligand, relatively unobstructed so as to enable coordination of the rather bulky oxyanion.

Turning molecular receptors for phosphate into probes, luminescent or otherwise, does not typically follow the same mechanism as metal extrusion assays. Lanthanides are particularly well-adapted for this class of receptors since their physical properties, luminescent or magnetic, are largely a function of the presence of any water molecule in their inner coordination sphere. Phosphate, which can often (but not always) displace this labile water, can thus also alter the luminescent properties of Tb^{III}, Eu^{III}, Tm^{III}, Dy^{III}, and Sm^{III} as well as the relaxivity of Gd^{III} complexes (Fig. 5).

There are a number of advantages to molecular receptors over metal extrusion assays, in terms of sensitivity, selectivity, and reversibility. The sensitivity of this class of probes can be more readily fine-tuned by the nature of the metal ion M, the donor strength or basicity of the ligand L, steric hindrance at the open coordination site, and the addition of peripheral hydrogen-bonding and charged motifs, thereby combining the strength of the organic receptors with their inorganic counterparts. Cumulative binding constants, β, of inorganic receptors for phosphate range over 8 orders of magnitude. Selectivity over endogeneous metal ions is usually less of a worry given the high stability of the ML complex. Selectivity over competing anions can also be optimized, again, by the nature of the metal ion M and its chelate L, and by the addition of well-positioned and oriented peripheral groups. These receptors thus offer the possibility to be fine-tuned as desired for any application. Reversibility is a key advantage of this class of probes. Since only one simple binding equilibrium is involved, real-time monitoring of changes in phosphate concentration in a sample is feasible.

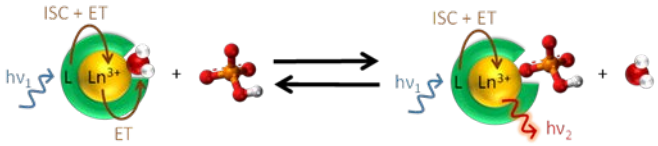


Fig. 5 Lanthanide-based assay for inorganic phosphate. In the absence of phosphate, excitation of the ligand enables sensitization of the lanthanide, whose emission is subsequently quenched by the inner-sphere water molecule. Coordination of phosphate in the open coordination site, concomitant with displacement of the water molecule, eliminates this quenching mechanism resulting in an increase in luminescence.

5.2. Molecular receptors based on transition metals

The design of selective receptors for phosphate and phosphorylated molecules was initially inspired by the reversible coordination of phosphates to one or more Zn^{II} ions in the active binding sites of metalloenzymes. Over two decades ago, Kimura and co-workers demonstrated for the first time that macrocyclic Zn^{II} complexes can have high affinity for phosphate and phosphorylated species with a series of tris (Zn^{II}-cyclen) derivatives and multi-Zn^{II} centered macrocyclic complexes.[63,64] X-ray crystal structure analysis of the phosphate adduct of Zn- **18** (Fig. 6) established that three of the phosphate oxygens of PO_4^{3-} can each coordinate to a Zn^{II} ion as a monodentate apical donor, resulting in a C_3-symmetic assembly. [65]

Similar Zn^{II} complexes can also be used to sense pyrophosphates. In early 2000's, Himachi and coworkers demonstrated that tridentate dipicolyamine (DPA) receptors with three nitrogen donors have good affinity and selectivity for Zn^{II} over other biological relevant metal ions. The open coordination site of the metal complexes enables phosphate or pyrophosphate coordination.[66-68] Since then, a diverse library of DPA analogues for pyrophosphate sensing have been reported by several groups. These are reviewed elsewhere. [69-71]

Recognition of an anion in water can sometimes be enhanced by simultaneously recognizing a counter cation. One such example for phosphate is Gunning's ditopic receptor Zn-**19**. It contains both a Zn(II) aza macrocycle, which is responsible for phosphate binding, and a conjugated crown ether, that recognizes Na^+ or K^+ (Fig. 6).[72] In neutral HEPES buffer, Zn-**19** has higher affinity for potassium phosphate (log K_a = 4.96) than sodium phosphate (log K_a = 4.69), which highlights the role of the counter cation in enhancing the affinity of the receptor for phosphate. Control experiments with $NaClO_4$ and $KClO_4$ supported the proposed diptotic binding nature of the complex. An analogous heteroditopic receptor Zn-**20**, later reported by Gunning, demonstrated the importance of cooperativity in achieving higher binding affinity for phosphate as higher affinity for the anion were observed when the cation matched the size of the receptor's crown ether.[73]

The difficulty in designing copper and zinc molecular probes for phosphate based on those receptors is to modify them in such a way that phosphate binding induces a luminescent response. One such example is the tetranuclear penta-coordinated Zn^{II} receptor reported by Chen *et al*, Zn-**21**.[74] In methanol, coordination of $H_2PO_4^-$ and formation of a phosphate

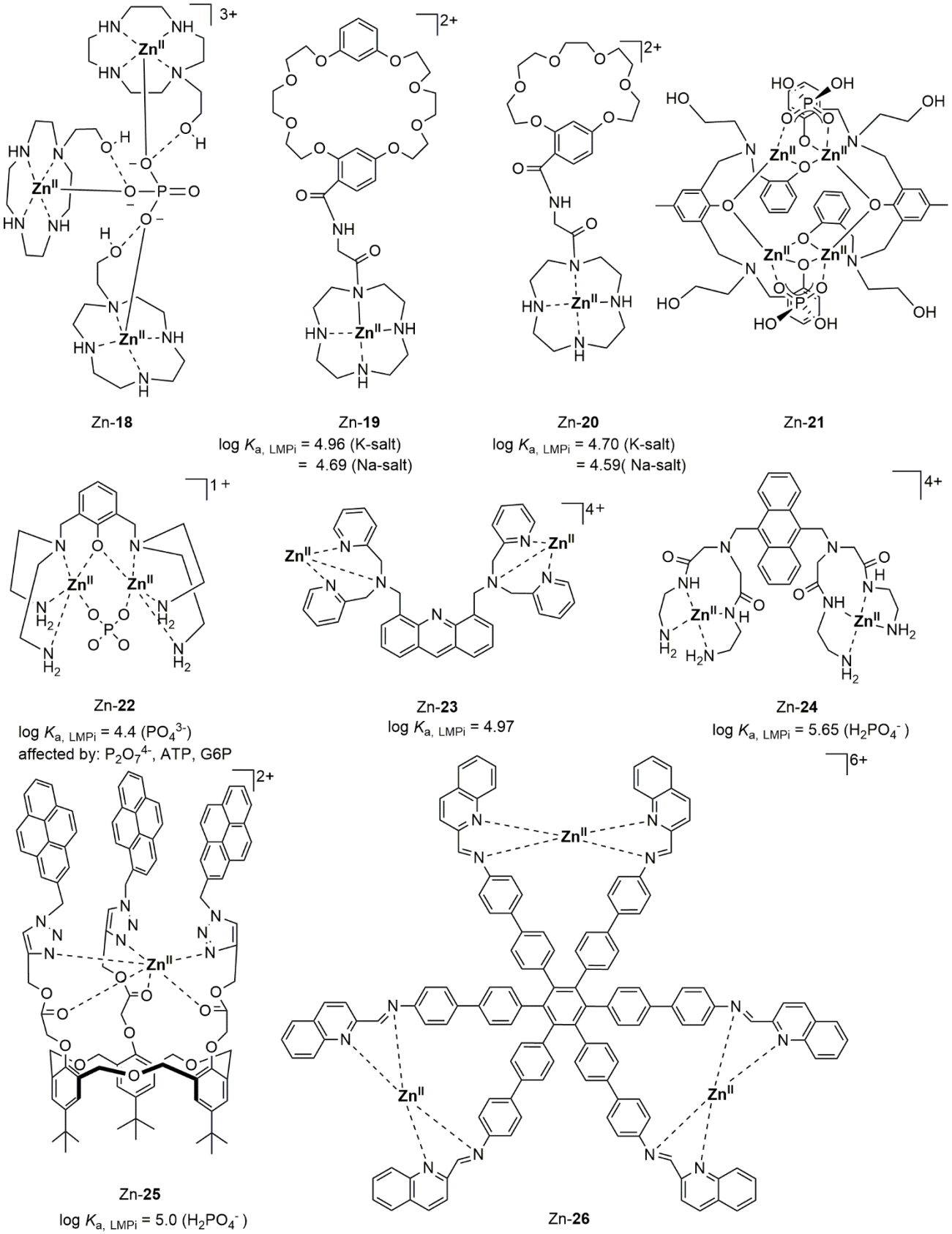


Fig. 6 Molecular receptors for phosphate based on Zn^{II}.

bridge between two Zn^{II} ions necessitates dissociation of the hydroxyl groups of the ligand, which in turn decreases the fluorescence of the receptor. A similar fluorescence quenching effect was reported by Ambrosi *et al*. [75] with a polyamino-phenol complex Zn-**22** that binds PO_4^{3-} with log K ~ 4.4. Interestingly, the dipicolyamine-acridine-Zn^{II} complex Zn-**23** reported by Yoon and co-workers binds both inorganic phosphate and pyrophosphate, albeit differently and with different fluorescence response: phosphate coordination increases the fluorescence of Zn-**23**, whereas pyrophosphate decreases it.[76] The large chelation enhanced fluorescence (CHEF) effect upon phosphate coordination to Zn^{II} was attributed to cooperative hydrogen-bonding interactions with the acridone nitrogen. Pyrophosphate, on the other hand, induces PET from the benzylic amine to the acridine moiety which decreases the latter's fluorescence.

Other mechanisms of CHEF have been reported for Zn^{II}-based receptors for phosphate. Huang et al. reported an anthracene-dipocolyamine-based Zn^{II} ratiometric fluorescent sensor Zn-**24** for $H_2PO_4^-$ that functions in aqueous media (0.01M HEPES at pH 7.4).[77] The formation of dimers between two anthracene moieties induced upon $H_2PO_4^-$ coordination to the Zn^{II} binding sites enhances the excimer fluorescence at 490 nm and decreases the monomer emission at 432 nm, thereby yielding a ratiometric response. The affinity of Zn-**24** for phosphate (log K_a = 5.65) is comparable to many of its class. A similar ratiometric fluorescent response for $H_2PO_4^-$ was reported by Ni *et al*. with a pyrene-linked triazole modified homooxacalix[3]arene based C_3-symmetric receptor Zn^{II} receptor, Zn-**25** (Fig. 6).[78] In mixed aqueous media, addition of $H_2PO_4^-$ increases the excimer emission of the complex and decreases that of its monomer. The affinity of Zn-**25** for phosphate (log K_a = 5.0) is comparable to that of Zn-**24** (Fig. 6).

A PET mechanism is also at play in the fluorescence response of the quinoline moiety appended hexaphenylbenzene Zn-**26** reported by Bhalla and co-workers.[79] This unique propeller-shaped Zn^{II} ensemble recognizes both $H_2PO_4^-$ and AMP in organic solvents (3:1 ethanol-THF) albeit, again, with different responses. Since Zn^{II} suppresses PET from the imino nitrogen to the photoexcited hexaphenylbenzene moiety, the metallic ensemble Zn-**26** (Fig. 6) is brightly fluorescent. Addition of 6 equivalents of $H_2PO_4^-$ results in a hypsochromic shift of the fluorescence emission band, with a significant fluorescence decrease at 438 nm and an increase at 366 nm. This observation indicates a weakening of the Zn^{II}-ligand bonds upon formation of the Zn-**26**:$H_2PO_4^-$ ternary complex. AMP, on the other hand, causes a marginal hypsochromic shift from 438 nm to 431 nm concomitant with a fluorescence increase, indicative of a weaker effect of AMP on the coordination of Zn^{II} by the ligand **26**.

Martell and coworkers later reported that Cu^{II} complexes could also recognize phosphate via direct coordination to the transition metal ion. The 1:2 Cu^{II} complex of the polyamine macrocyclic ligand O-BISDIEN (Cu-**27**, Fig. 7) recognizes HPO_4^{2-} with an affinity that spans over 5 orders of magnitude between pH 2 and 11 (log K_a =1.5-7.0), demonstrating early on the strong pH-dependence of metal-based receptors for coordinating oxyanions.[80] In 2003, Ren and co-workers evaluated the phosphate binding properties of a similar dimetallic small azamacrocyle (16aneN6/Cu_2) Cu-**28**. Interestingly, the binding affinity of this receptor for phosphate was *ca* 1 order magnitude less in HEPES buffer than in un-buffered aqueous solution (log K_{rel} = 4.38 and 3.62 in unbuffered and buffered solutions, respectively).[81] This observation highlights the sometimes substantial influence that buffers, many of which have weak affinities for both metal ions and phosphate, can have on the behavior of metal-based receptors.

That same year, Anslyn and coworkers reported the first mononuclear Cu^{II} complexes with high affinity for inorganic phosphate and high selectivity over other anions, except for arsenate.[82] Selectivity for phosphate over arsenate is, as a general rule, very difficult to establish with this class of receptors due to the similar geometry, hardness and coordination ability of the two anions. Of note, the affinity of Cu-**29** and Cu-**30** (Fig. 7) for phosphate in neutral aqueous mixture (98:2, H_2O-MeOH) is high (log K_a = 4.17), due to the addition of electrostatic and hydrogen-bonding components.

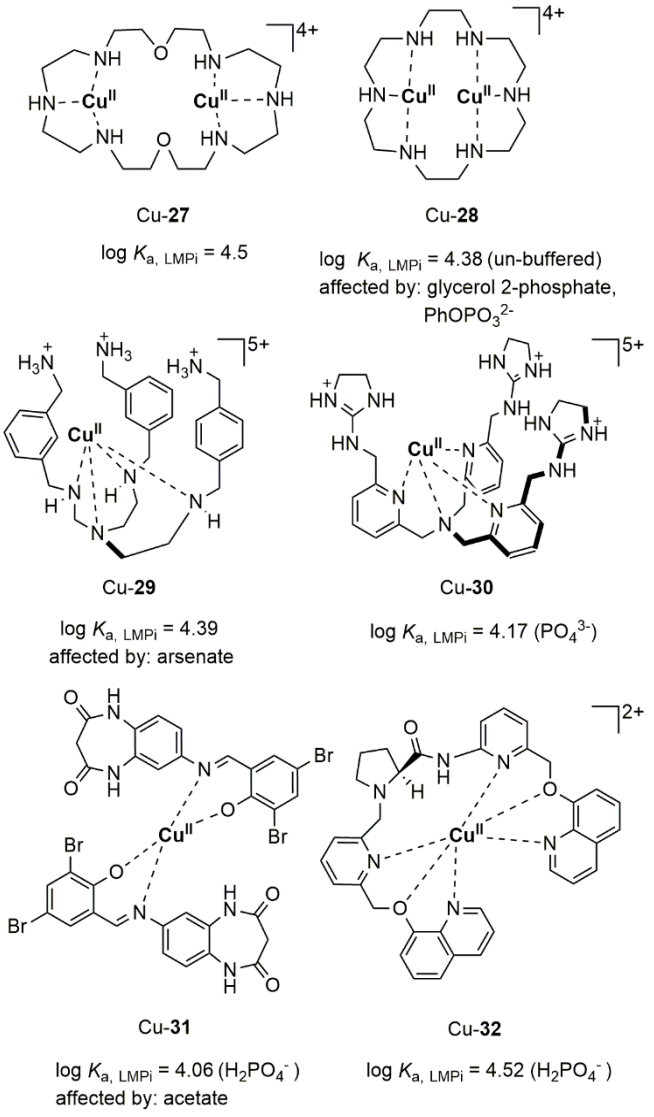


Fig. 7 Molecular receptors for phosphate based on Cu^{II}.

Interestingly, further studies indicated that the two copper complexes do not display the same energetics of phosphate binding. Whereas in both cases metal ligation contributed significantly to the high affinity for phosphate, phosphate-recognition by the ammonium-based receptor Cu-**29** is mostly entropy driven, whereas that of the guanidium derivative is primarily enthalpy-driven.[83] A small change in the structure of the ligand can thus have significant consequences on the nature of the guest binding. Cu-**31** is a simpler 2:1 Cu^{II} complex:phenolic receptor reported by Lin and co-workers that function via a similar mechanism.[84] Phosphate coordination to the Cu^{II} center and formation of a ternary complex is enhanced by cooperative hydrogen bonding interactions with the diamide. The metallic receptor has comparable affinity for phosphate in DMSO (log K_a = 4.2).

Copper-based molecular receptors can also be rendered responsive. The fluorescence of the conformationally restricted chiral Cu^{II} receptor Cu-**32** (Fig. 7) reported by Goswami *et al*. increases substantially upon addition of phosphate.[85] This receptor shows good selectivity over common competing anions although it functions in organic solvents such as CH_3CN and with an affinity that remains insufficient for environmental applications (log K_a = 4.52). Uniquely, the turn-on fluorescence response of this receptor was ascribed not to the extrusion of the Cu^{II} ion, but to the formation of a Cu-**32**:$H_2PO_4^-$ ternary complex whose increased rigidity decreases non-radiative decay of the excited state.

5.3. Molecular receptors based on lanthanides

Gadolinium(III) complexes with open coordination sites have been used as contrast agents for Magnetic Resonance Imaging (MRI) for over three decades.[86] Gd^{III}, like all lanthanide ions, are hard oxophilic metal ions with high affinity for hard anions such as phosphate, carbonates, and carboxylates.[25,43,44,87-90] Therefore, not surprisingly, all clinical MRI contrast agents display some affinity for phosphate in water at neutral pH, albeit to varying degree. The affinity of macrocyclic polyaminocarboxylate complexes with one inner-sphere water molecule such as Gd-DOTA (Gd-**33**, Fig. 8, log *K*a = 2.16) is slightly higher than that of its linear analogue Gd-DTPA (Gd-**34**, log K_a = 2.06) or Gd-DTPA-BMA (Gd-**35**, log K_a = 2.0). In general, increasing the number of open coordination sites increases the affinity of polyaminocarboxylate-based ligands for phosphate, as is apparent from the ca. 3 orders of magnitude higher affinity of Gd-DO3A for phosphate (Gd-**36**, log K_a = 4.8).[91,92] Of note, those complexes show poor to no selectivity over another hard anion: bicarbonate. This impacts their utility as probes for phosphate. Selectivity of lanthanide-based receptors for phosphate over competing coordinating anions, or vice-versa, remains difficult to predict and achieve. Importantly, these observations cannot be generalized to other non-polyaminocarboxylate-based lanthanide complexes.

Most Gd^{III} complexes evaluated as contrast agents for MRI have some affinity for phosphate and bicarbonate, and sometimes, also to fluoride and/or carboxylates. Nonetheless, a subtle structural modification can have a significant impact on the ability of lanthanide complexes to bind anions and to transform them into efficient anion receptors. For instance, the picolinate-based complex Gd-**37** (Fig. 8) reported by Tóth and

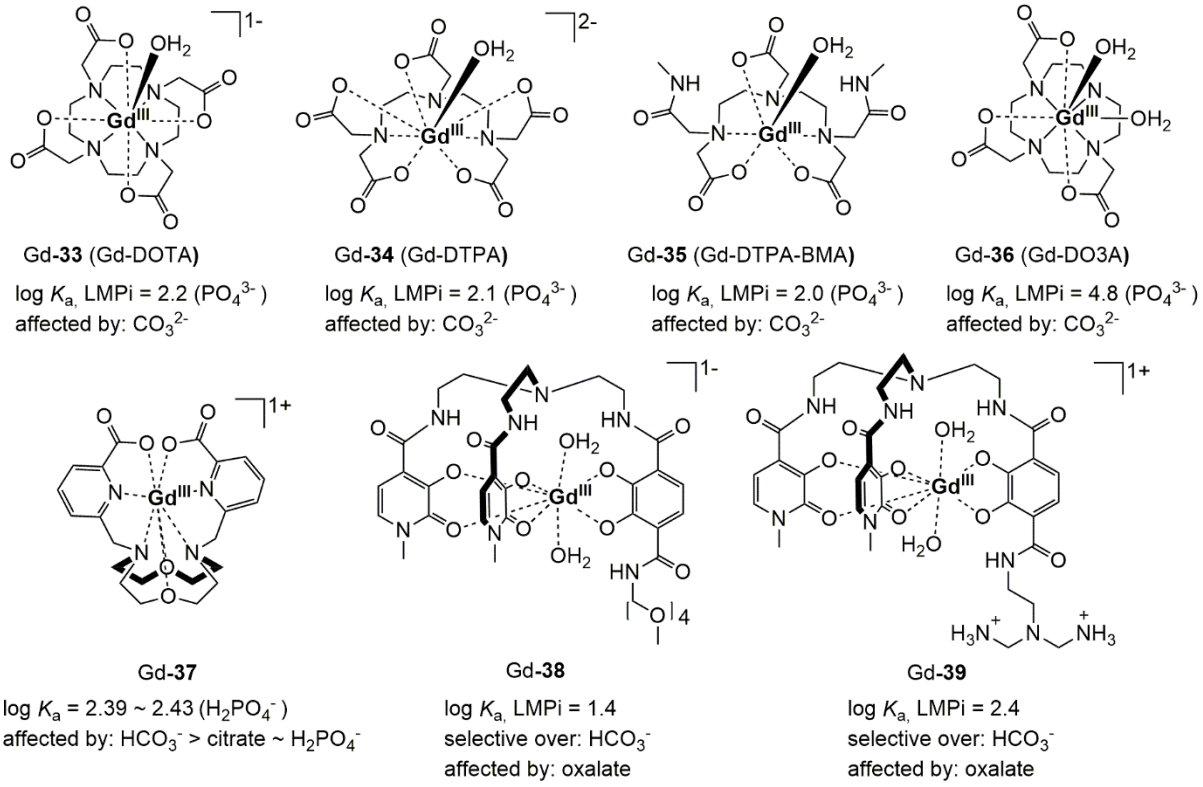


Fig. 8 Molecular receptors for phosphate based on Gd^{III}.

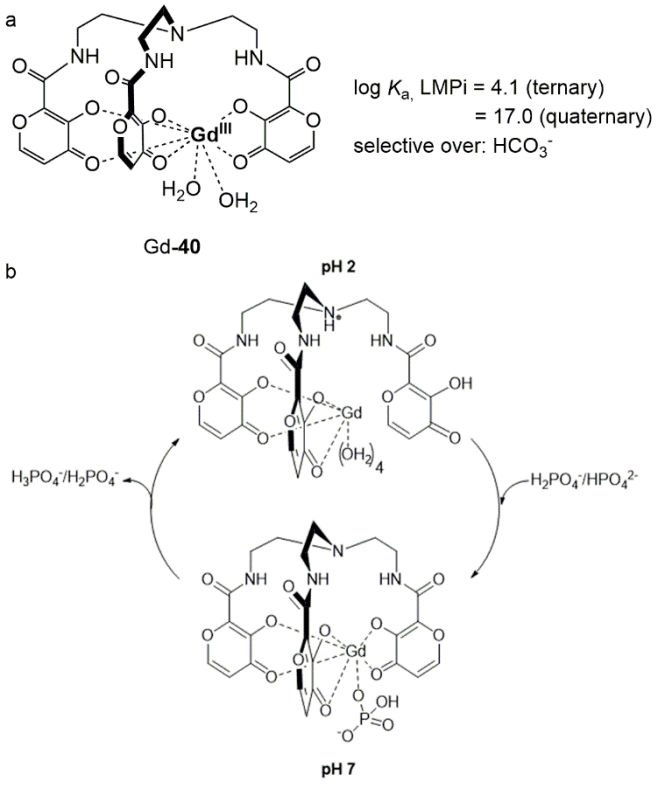


Fig. 9 a) Chemical structure of molecular receptor Gd-**40** and b) its pH dependent phosphate sequestration.

coworkers has high affinity for $H_2PO_4^-$ but also for citrate and HCO_3^-.[93] The lower affinity of this complex for phosphate (log K_a = 2.4) compared to bicarbonate (log K_a = 2.8) was ascribed to the larger steric demand required for phosphate binding that necessitates distortion of the coordination environment of the metal center.

Our group investigated the affinity of lanthanide complexes with tripodal, tris-bidentate architecture using different chelating moieties towards anions using either longitudinal relaxometry (for the Gd^{III} complexes) or luminescence (for the Eu^{III} and Tb^{III} complexes). The first two complexes of this series, Gd-**38** and Gd-**39** (Fig. 8), both have two open coordination sites, initially filled with water in aqueous solutions. These Gd^{III} complexes differ from the polyaminocarboxylate-based ones in that they bind $H_2PO_4^-$ in water at neutral pH with very high selectivity over HCO_3^-.[94] Surprisingly, despite their two open coordination sites, Gd-**38** and Gd-**39** have comparable affinity for $H_2PO_4^-$ than polyaminocarboxylate-based Gd^{III} complexes with one inner-sphere water molecule (Log K_a, LMPi = 1.4 and 2.4 for Gd-**38** and Gd-**39**, respectively). The higher affinity of Gd-**39** for phosphate compared to Gd-**38** arises from the positive charge and the presence of a hydrogen-bonding solubilizing moiety in Gd-**38**. As for transition metal-based molecular receptors, electrostatic and hydrogen-bonding components can, if positioned correctly, increase the affinity of lanthanide receptors for phosphate without affecting their selectivity.

The low affinity of the HOPO-based Gd^{III} complexes Gd-**38** and Gd-**39** for phosphate was resolved with a tripodal Gd-TREN-MAM complex, Gd-**40** (Fig. 9a). Gd-**40** retains the outstanding selectivity for phosphate over bicarbonate and other anions of its predecessors but has substantially higher affinity for phosphate at neutral pH (log K_a = 4. 1 and 17.0 for ternary and quaternary complexes, respectively).[95,96] Indeed, the affinity of Gd-**40** for phosphate in water remains one of the highest one for a gadolinium receptor that forms a quaternary $GdLPi_2$ complex (equilibrium 2). Attempts to further increase the affinity of Gd^{III} complexes for phosphate via the use of more basic podands resulted in too unstable complexes that would instead exchange ligand with phosphate as depicted in equilibrium 1.[96]

Gd-**40** presents another feature: its affinity for phosphate is strongly pH dependent. Whereas Gd-**40** has very high affinity for phosphate and can catch the anion at pH 7, it has negligible one and releases phosphoric acid below pH 2. This enables the complex to also function as a reversible receptor for catch-and-release of phosphate directly from aqueous media (Fig. 9b). This class of complex thus has potential for environmental and biomedical applications requiring phosphate sequestration. These examples show that Ln^{III} complexes with open coordination sites can, when designed properly, function as effective receptors for phosphate.

Gd^{III} complexes are powerful relaxation agents. Since their relaxivity is largely determined by whether the open coordination site is filled by water or phosphate, every Gd^{III}-based receptor for phosphate that functions via direct coordination of the anion to the metal center also acts as a relaxometric probe. Advantageously, every lanthanide ion throughout the series has similar coordination properties. As a result, lanthanide ions can be swapped according to the physical properties one wants to measure with only minor effects on the selectivity and affinity of the lanthanide receptor for phosphate. Whereas Gd^{III} complexes function as powerful relaxometric probes, Tb^{III} and Eu^{III} are instead phosphorescent ones with characteristic long luminescence lifetime in the ms range, ideally suited for time-gated measurements in complex aqueous media.[97] Favorably, both the quantum yield and the luminescent lifetime of Tb^{III} and Eu^{III} can be quenched by the 4th overtone of the O-H vibration of water. As such, the luminescence of Tb^{III} and Eu^{III} is also dependent on the number of inner-sphere water molecules. Tb^{III} and Eu^{III} receptors that function via direct coordination of the anion to the metal center also act as luminescent probes. A number of luminescent Tb^{III} and Eu^{III} probes for phosphate following this principle have thus been reported (Fig. 10).

The first in-depth studies on how the structure of the ligand affects the affinities of luminescent Eu^{III} and Tb^{III} complexes for anions were performed by Parker and coworkers with the chiral complexes Ln-**41**, where Ln = Eu^{III} or Tb^{III} (Fig. 10).[98,99] The complex Ln-**42** where R=Me has higher affinity for anions than Ln-**41** where R=H. Both of those complexes showed marginally higher affinity for phosphate than for acetate, bicarbonate, and lactate. Although those receptors do function in water, the binding affinity of Ln-**42** for phosphate (log K = 4.15) is comparable to that of copper and zinc-based receptors and remains too low for environmental applications. Interestingly, in each case, the Tb^{III} complex has higher affinity for anions than

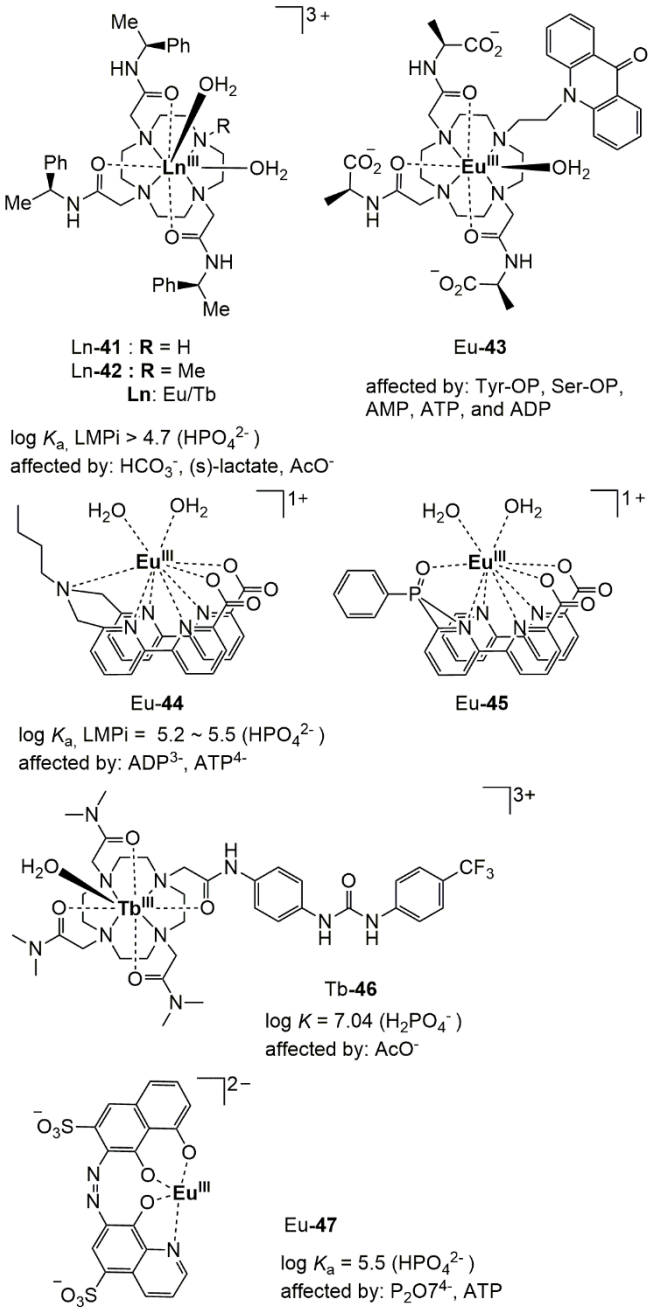


Fig. 10 Molecular receptors for phosphate based on Eu^{III} and Tb^{III}.

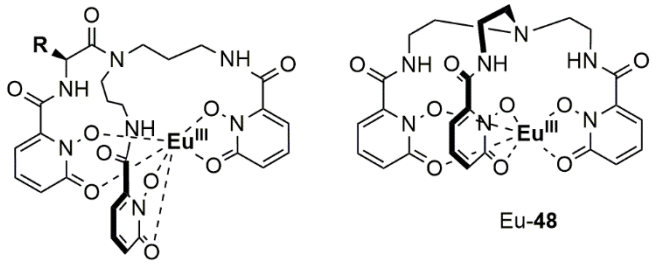
the Eu^{III} one, likely due the more acidic character of Tb^{III} compared to Eu^{III}. Interestingly, the negatively-charged acridone analogue Eu-**43** shows only weak affinity for phosphate but stronger one for phospho-tyrosine and phospho-serine.[100] The distinct emission spectra of the phospho-tyrosine adduct highlights the unique dependency of Eu^{III} emission on its coordinating environment, a dependency that Tb^{III} does not have.

The Eu^{III} and Tb^{III} complexes of receptor Eu-**44** that uses a bypyridylcarboxylate ligand reported by Ziessel and co-workers bind both HPO_4^{2-} and ATP with high affinity in buffered aqueous solution at pH 7.0.[101] Characterization of both adducts indicated that only one of the two inner-sphere water molecules was displaced by either ATP or HPO_4^{2-}, leading to a ternary LnLPi or LnL(ATP) complex. Further studies indicated that the strongly coordinating anions could displace the ligand's coordinating nitrogen. The phenyl phosphine ligand **45** was thus designed in order to stabilize the lanthanide complex with the hope of favoring the formation of the ternary complexes.[102] The affinity of the more stable Eu-**45** for HPO_4^{2-}, ATP and ADP is indeed ca. 20-fold higher than the original Eu-**44**, although the selectivity of the molecular receptor for phosphate over phosphorylated nucleotide was not improved. This highlights one of the disadvantages of this approach.

Other luminescent Eu^{III} and Tb^{III} molecular receptors for phosphate have been reported. In acetonitrile, the diaryl urea-based Tb^{III} complex Tb-**46** (Fig. 10) displays a characteristic increase in metal-centered time-delayed luminescence upon coordination of phosphate due to the displacement of the inner-sphere water molecule.[103] Further characterization indicates that acetate also coordinates the lanthanide, but that $H_2PO_4^-$, unlike acetate, binds to both the metal center and the urea antenna.

Uniquely, Eu-**47** does not yield a luminescent but a colorimetric response to phosphate that is visible to the naked eye.[104] The pink complex turns blue upon coordination of phosphate, pyrophosphate or phosphorylated nucleotides such as ATP, but not to other inorganic anions. Eu-**47** binds phosphate in water with a 2:1 stoichiometry with an association constant log K_a = 5.5. Interestingly, it binds pyrophosphate with a 1:1 stoichiometry and with a substantially lower affinity (log K_a = 3.74).

Following our initial success in developing Gd^{III}-based receptors for phosphate, our group has expanded our strategy to the design of luminescent Eu^{III}-based ones. These complexes use 1,2-hydroxypyridinone (1,2-HOPO) as both the lanthanide coordinating moiety and its sensitizers since previous report by the Raymond group indicated that 1,2-HOPO-based Eu^{III}



Eu-**49** : **R** = NH_3^+

log β = 11.34, log K_{a1} = 5.66, log K_{a2} = 5.67

Eu-**50** : **R** = OH

log β = 15.88, log K_{a1} = 4.99, log K_{a2} = 4.95, log K_{a3} = 5.61

Eu-**51** : **R** = H

log β = 14.50, log K_{a1} = 3.67, log K_{a2} = 5.27, log K_{a3} = 5.55

Eu-**52** : **R** = CO_2^-

Eu-**53** : **R** = SO_3^-

} no binding to phosphate and other competing anions

Fig. 11. Luminescent Eu^{III}-based probes for inorganic phosphate.

complexes displayed some of the highest quantum yields in water.[105] Interestingly, the parent Eu-TREN-1,2-HOPO (Eu-**48**, Fig. 11) has no affinity for phosphate or any other anion in water despite its two open coordination sites.[106] As is apparent from this observation, increasing the number of open coordination sites on a metal complex does not necessarily increase its affinity for anions. We postulated, however, that opening up the geometry of the complex with an extended ligand cap would favour the 9 coordinate state, which would, in turn, favour anion coordination.

Indeed, in water at neutral pH, Eu^{III}-Lys-HOPO (Eu-**49**) has high affinity for phosphate and very high selectivity for phosphate over bicarbonate and other competing anions. Uniquely, increasing the pH to 10 decreases selectivity for phosphate and enables direct coordination of CN^-.[107] This observation highlights the dependency of the selectivity of metal-based receptors for anions on the pH of the media, in accordance with the differing pKa's of competing anions. In water at neutral pH, Eu-**49** forms a $MLPi_2$ complex with the highest affinity of any metal receptor for phosphate (log β= 11.34). Unlike the Cu-**28** complex of Ren,[81] neither the affinity nor the selectivity of the complex is affected by the presence of HEPES buffer. The high affinity of this receptor for phosphate is due to a combination of factors: the high affinity of the lanthanide for the coordinating anion, and the presence of a primary ammonium group near the open coordination site that increases affinity for phosphate via both hydrogen-bonding and electrostatic interactions.[108]

Indeed, Eu-**50**, an analog that bears instead an alcohol group that is neutrally charged but can still hydrogen-bond, has an affinity for phosphate that is one order of magnitude lower than the positively charged Eu-**51** (Fig. 11). Removing the alcohol functionality in Eu-**50** further decreases the affinity of the receptor for phosphate by another order of magnitude. The negatively charged Eu-**52** and Eu-**53** have no affinity for phosphate or any other anion whatsoever. As is apparent from these examples, the affinity of metal-based molecular receptors for coordinating anion can be fined-tuned with the addition of appropriate pendant moieties. Advantageously, tuning the affinity can be achieved without affecting the high selectivity of these receptors.[108]

6. Metal-based indicator displacement assays

6.1. Design principles for metal-based indicator displacement receptors and assays

The main difference between the metal extrusion assays reported in section 4 and the metal-based molecular receptors reported in section 5 lies in the stability of the metal complex ML. Metal complexes used in extrusion assays have low stability, such that the stronger coordinating phosphate can readily displace the weak ligand L resulting in the formation of metal phosphate precipitates. Metal complexes used in molecular receptors, on the other hand, are highly stable. The incoming phosphate does not displace the ligand L, but rather occupies an open coordination site to form a ternary MLPi complex. The advantage of metal extrusion assays is that they can readily be designed to yield a fluorescent response. Molecular receptors, on the other hand, do not lead to the formation of phosphate salt precipitate, are more reversible, and can be more readily amended so as to achieve the desired affinity for phosphate in water and higher selectivity for inorganic phosphate over other competing anions. It is however, more difficult to design a fluorescence response from them, especially in the case of transition-metal based ones.

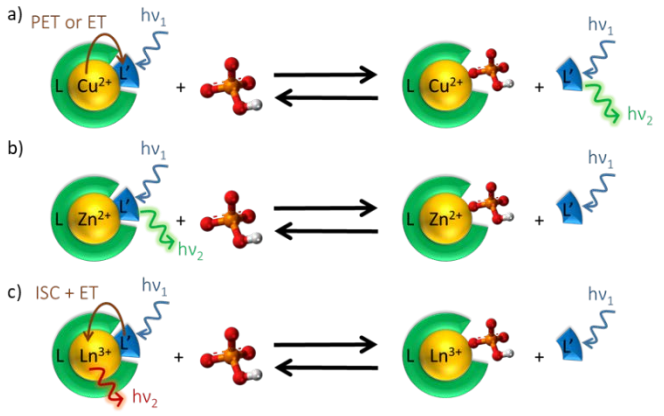


Fig. 12 Metal-based indicator displacement assay for inorganic phosphate. a) Cu^{II} quenches the luminescence of the weakly coordinated indicator ligand L'. Displacement of L' by phosphate reverts the luminescence of the indicator. b) Zn^{II}, on the other hand, enhances the luminescence of the weakly coordinated indicator ligand L'. Displacement of L' by phosphate results in a decrease in the fluorescence of the indicator L'. c) Lanthanide-based indicator displacement assays rely on the sensitizing capability of the antenna. Displacement of the latter by phosphate quenches the luminescence of the lanthanide.

The two strategies can, advantageously, be combined so as to exploit the advantages of each of them. In indicator displacement assays, the metal M is coordinated by two different ligands: a strong one, L, that keeps the receptor and its phosphate adduct in solution and that can be used to tune the affinity of the receptor for phosphate, and a weak one, L', that is used to give a luminescence response. Phosphate can only compete with the weak ligand L' but does not affect coordination of M by L. Addition of HPO_4^{2-} to MLL' thus results in the formation of a ternary complex MLPi upon displacement of the ligand L' (eqn 3). The fluorescence response of such receptors arises from the effect of the metal M on the fluorescence of the ligand L'. The design of their response thus mirrors that of the metal extrusion assays discussed in section 4 (Fig. 12a). Paramagnetic Cu^{II} typically quenches the fluorescence of any coordinated chromophore. If that chromophore is also a weakly coordinating ligand, it can be displaced by phosphate. Freed from Cu^{II}, the chromophore L' can fluoresce. In this case, the resulting ternary complex MLPi is not fluorescent. Zn^{II} and Cu^{I} function as opposites (Fig. 12b). As described above, Zn^{II} instead enhances the fluorescence of certain coordinating chromophores. When HPO_4^{2-} displaces the ligand L' from Zn^{II}, the fluorescence of L' decreases. One should keep in mind that complex media can affect such assays, especially if they contain other metal ions or receptors that can efficiently compete for L'.

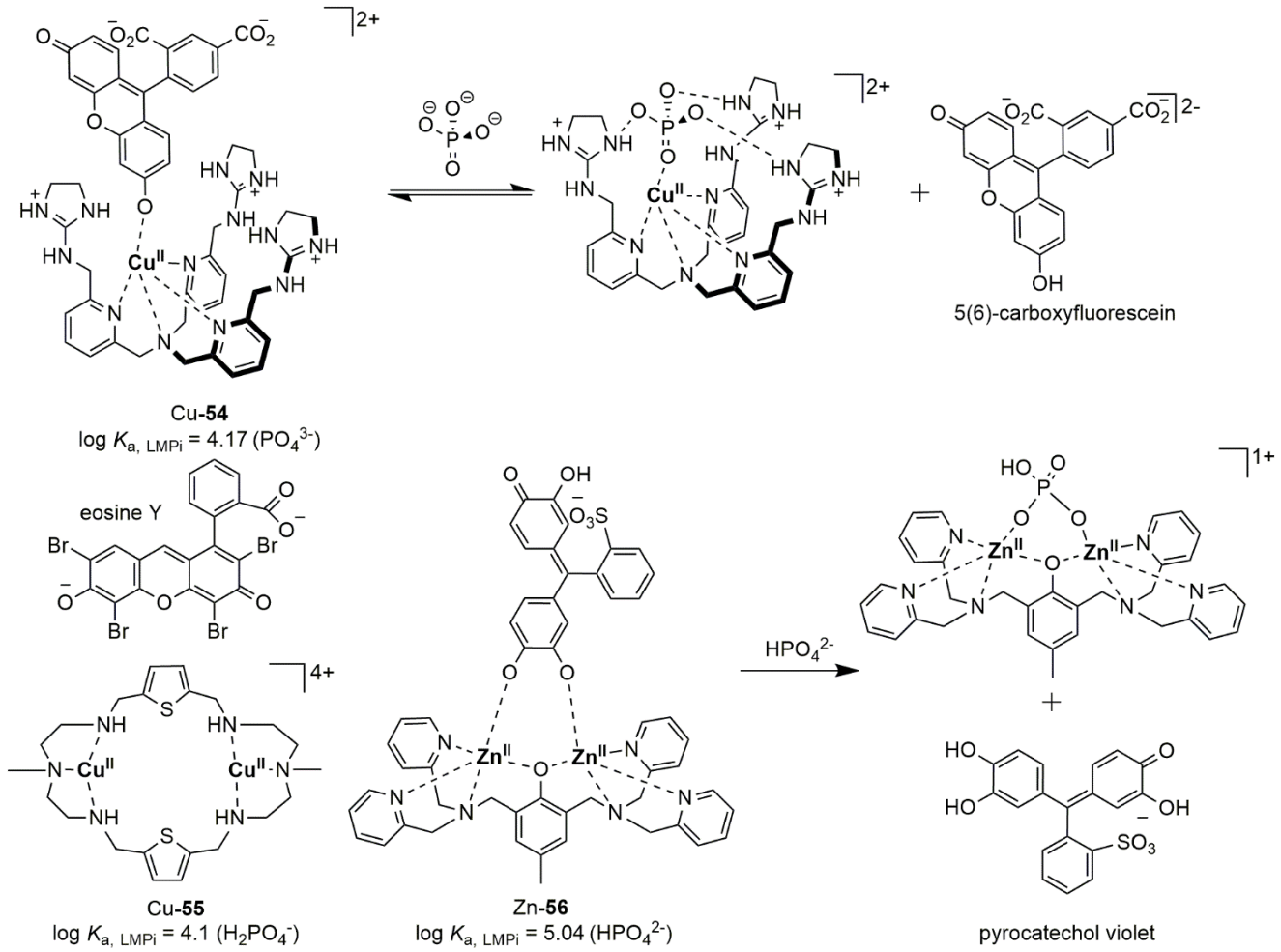


Fig. 13 Transition Metal-Based Indicator Displacement Receptors.

Lanthanides can also be used in indicator displacement assays, although the origin of their luminescent response is slightly different than that of transition-metal based ones. In transition metal-based indicator displacement assays it is the indicator that luminesces, whereas in lanthanide-based ones, it is the metal ion (Fig. 12c). While copper or zinc modulates the luminescence the indicator, luminescent lanthanide-based assays rely instead on the antenna effect and the ability of the indicator to sensitize lanthanide luminescence. Displacement of the antenna prevents sensitization of the lanthanide and as such results in near complete quenching of the metal-centered luminescence. The disadvantage of lanthanide in such assays is that they are always turn-off. Their advantage, is that their long luminescence lifetime readily enable time-gating of the residual auto-fluorescence of the media.

6.2. Transition metal-based indicator displacement receptors and assays

Anslyn and his group, who pioneered indicator displacement assays, have successfully used this approach with a C_{3v} symmetric Cu^{II} complex, Cu-**54** (Fig. 13**)**, which gives a colorimetric readout.[109] The weak ligand 5(6)-carboxyfluorescein is yellow in mixed aqueous solution (1:1 CH_3OH-H_2O) at pH 7.4, but orange when coordinating Cu^{II}. Phosphate has much higher affinity for CuL (log K_a = 4.17) than does the 5(6)-carboxyfluorescein L' ligand, and thus displaces the latter from the Cu^{II} receptor, resulting in an orange to yellow color change. This displacement indicator is selective for phosphate. Its weak affinity for the anion enabled its use to determine phosphate concentration in human saliva and horse serum, concentrations that are typically in the low mM range.

Hossain and coworkers used a similar approach with a macrocyclic dinuclear copper(II) complex, Cu-**55**, to detect phosphate in water.[110] The receptor Cu-**55** adapts a boat-shaped conformation in which both metal ions have a square-pyramidal geometry. The receptor is thus predisposed to bind the weak fluorescent ligand eosine Y. As with other Cu^{II}-based probes, coordination to the transition metal quenches the fluorescence of the indicator. Phosphate is a stronger ligand for Cu-**55** than eosine, and thus displaces and restores the fluorescence of the latter. The weak affinity of Cu-**55** for phosphate (log K = 4.1) and its high selectivity over other tested anions renders it a promising candidate for determination of phosphate levels in biological media.

The first Zn^{II}-based indicator displacement assay for phosphate was reported by Kim and Han two years after Anslyn.[111] The dimetallic receptor Zn-**56** (Fig. 13) uses

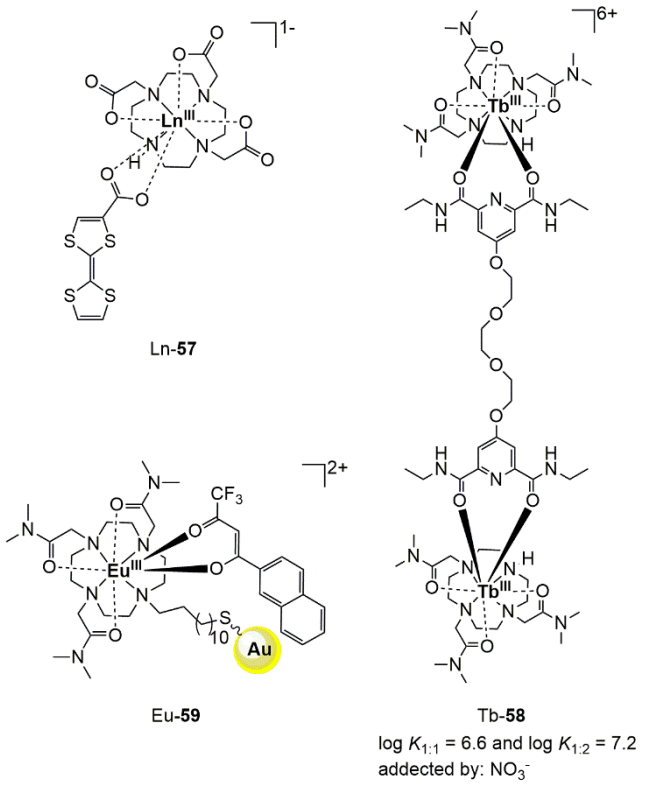


Fig. 14 Lanthanide-Based Indicator Displacement Receptors.

pyrocatechol violet as a colorimetric indicator that, in the absence of phosphate, bridges the two Zn^{II} ions. Phosphate, which has higher affinity for Zn^{II} than the catecholate, displaces the indicator, resulting in a colorimetric change at neutral pH in water that is visible to the naked eye. The affinity of Zn-**56** for phosphate (log K_a = 5.04) is ca. 10 fold higher than that of the di-copper system of Hossain. Smith and coworkers later confirmed that the affinity of the receptor for phosphate, its colorimetric response, and its selectivity over competing anions were strongly dependent on the nature of the bridging weak indicator that is displaced by phosphate.[112] Of the 11 indicators tested, the affinity of the corresponding indicator displacement assays for phosphate spanned over two orders of magnitude (log K_a = 4.4 to 6.4).

6.3. Lanthanide-Based Indicator Displacement Receptors and Assays

Although the majority of indicator displacement assays are based on transition metals, lanthanides are also uniquely suited for such applications. Importantly, because phosphates are strong competing anions for lanthanides, such assays require the use of thermodynamically highly stable, and preferentially kinetically highly inert, LnL complexes. Macrocyclic, DOTA-type ligands are thus better suited for the design of such assays than their linear DTPA-like analogues. The first such displacement indicator for phosphate was reported in 2006 by Faulkner and Pope.[113] The assay makes use of lanthanide complexes of the heptadentate macrocyclic ligand DO3A in which the last coordination site is occupied by a carboxylate-based antenna (Ln-**57**, Fig. 14). In methanol, displacement of this antenna by phosphate prevents sensitization of Nd^{III} by the antenna and consequential decrease in lanthanide-centered emission. As predicted, whereas phosphate can displace a weak carboxylate-based antenna, it cannot displace a stronger phosphonate-based one. A ternary complex using a phosphonate-based antenna is thus not responsive to phosphate.

A similar approach was followed by Gunnlaugsson in 2014 with a self-assembled indicator whereby the dipicolylamide antenna bridges two Tb^{III} complexes Tb-**58** (Fig. 14). In methanol, phosphate displaces the sensitizing bridge, breaking apart the assembly, resulting in quenching of Tb^{III}-centered emission at 545 nm.[114] Interestingly, this is a rare case of a lanthanide-based receptors that is also responsive to nitrate. The Gunnlaugsson group further demonstrated that lanthanide-based indicator displacement assays that employ this mechanism of action can also function when the lanthanide complex is functionalized onto a nanoparticle such as a gold one.[115] The indicator Eu-**59**, which makes use of a similar lanthanide complex with a β-diketonate-antenna functions in water at neutral pH, albeit with no selectivity for inorganic phosphate over phosphorylated biomolecules.

Conclusions and Outlook

The design of responsive probes, luminescent or otherwise, for inorganic phosphate strongly benefits from the inclusion of metal ions, notably Cu^{II}, Zn^{II} and Ln^{III}. The high affinity of these hard metal ions for the hard oxyanions enable the receptors to overcome the high hydration energy of phosphate. As a result, metal-based receptors typically have higher affinity for inorganic phosphate in polar protic solvents than their purely organic counterparts. Three different strategies have been employed in the design of metal-based molecular probes for inorganic phosphate. Metal extrusion assays rely on weak ML complexes where the ligand L is readily displaced by phosphate. These assays can readily be rendered fluorescent or colorimetric, although the heterogenous nature of their response due to the formation of metal phosphate salt hinders their reversibility. The two other strategies are therefore receiving increased attention. Molecular receptors in which phosphate coordinates in the open coordination sites of the metal complex offer a purely homogenous assay, and the ability to modulate the affinity of the receptor for phosphate via the inclusion of peripheral electrostatic or hydrogen-bonding components. This strategy is uniquely well suited for lanthanide ions since their luminescence (Eu^{III} and Tb^{III}) or relaxometric properties (Gd^{III}) are strongly dependent on whether the open coordination site is filled with water or a coordinating anion. Transition metals, on the other hand, can be rendered responsive more easily with indicator displacement assays. Such assays require the use of both a strong (L) and a weak (L') ligand. In principle, the affinity of such receptors for phosphate can also be tuned via the inclusion of electrostatic or hydrogen bonding on the coordinating ligand L. When designed properly, molecular receptors and indicator displacement receptors can bind HPO_4^{2-} directly in water at neutral pH with some selectivity over most common coordinating anions. Selectivity remains problematic, in particular for inorganic phosphate over

arsenate and other phosphorylated biomolecules such as ATP. Despite their strength, of the many probes and receptors reported, few function in water. With few exceptions, those that do still typically have low affinity for phosphate, many in the mM range. While such affinity may be sufficient for biological or medical applications given the high concentration of phosphate in serum and cells, they remain far too weak for environmental application. The low concentration of inorganic phosphate in surface water, typically in the 0.1-1 µM range, still requires the development of new and improved metal-based molecular receptors and probes for environmental applications.

Conflicts of interest

There are no conflicts to declare.

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We discuss and review the strategies of metal-based receptors targeting phosphate.