Reaching selective fluorescence phosphate detection over pyrophosphate with a cerium-based chemosensor in aqueous media

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Abstract

Phosphate ions are important molecules used in critical activities, such as those in agriculture or medicine. However, because of their chemical makeup, sensing them using a chemosensor is difficult. In the past, chemists attempted to solve this issue by creating sophisticated chemical structures as receptors, but with mixed results. Here, using just chemicals that are readily accessible on the market, we establish a straightforward metal extrusion-based fluorescence indicator displacement assay (MEFID) for phosphate detection. Using a fluorophore and cerium ammonium nitrate (CAN), phosphate ions in aqueous solutions are probed. With an average concentration higher than copper and being the most prevalent rare earth on the planet (48 p.p.m.), cerium is an intriguing metal for the identification of analytes. We demonstrate, that the inorganic complex detects phosphate ions in low micromolar concentrations, either spectrophotometrically, by ^{31}P NMR, or with the naked-eye, with high selectivity over common competing anions, including halides, acetate, carbonate, and, remarkably, pyrophosphate. In addition, perturbation of the sensor with phosphorylated molecules such as ATP is minimal. The development of receptors that endures a precipitation offers systems for the purification of surface waters as presented by the probing of environmental samples.

Significant efforts have been devoted to the design of chemosensors that are able to recognize important anions such as phosphorylated molecules e.g pyrophosphate.[1–8] In contrast, chemosensors for phosphates are less common.[2,3,9–11] The difficulty of host-guest recognition of this anion relies on its large size, its hydrophilicity[12] (molar Gibbs energy of hydration of $PO_4{}^{3-}$ $\Delta_{hyd}G^* = 2765$ kJ/mol), its multiple protonation states, and the Hofmeister series, especially at physiological pHs.[2–4,9] However, the development of such phosphate receptors is fundamental because of the importance of this anion in medicine or agriculture for ex-

ample.[1–4,10] In this context, colorimetric or fluorimetric chemosensors are ideal candidates. The gold standard for colorimetric detection of phosphate is the malachite green test relying on molybdenum, yet it presents several drawbacks: 1) The test works only under acidic pH, which limits the detection of phosphate around neutral pH. 2) Excess of phosphate generates a precipitation of molybdenum which has to be compensated with additives for improved solubility. 3) Molybdenum exists in many oxidation states, and could therefore interfere with redox reactants, generating parasitic reactions.[13,14] To offset these problems, we reported

in a previous paper on a dinuclear cerium based sensor for the selective naked-eye detection of phosphate under physiological conditions.[2] However, some challenges still needed to be addressed: this sensor is colorimetric, it does not allow quantitative phosphate determination, it is not commercially available, and it does not work in highly complexed mixtures. Therefore, research still need to be carried out to build selective and sensitive sensors which are able to accurately and practically determine phosphate concentrations in various media.[15] Very recent work has divided displacement assay for the recognition of analytes in three methodologies; 1) Metal based indicator displacement assays 2) Ternary complex molecular receptors 3) Metal extrusion indicator.[16] The metal extrusion indicator presents the advantage of sensitivity due to the low solubility of the resulting product of the reaction. Coupled to a fluorescent indicator and lanthanides, it may offer remarkable spectrophotometric properties for the recognition of phosphate derivatives. Such an example is given by the tetradendate β-diketonate europium complex **Eu-1** (see Figure 1a) reported by Yang and coworkers.[17] The complex dissociates in the presence of pyrophosphate to form a precipitate. A dinuclear based approach was used by Peng and coworkers (see Figure 1b) where a bimetallic Tb(III) tripodal substituted salicylate complex **Tb-2** was used.[18] Phosphate displaces the labile ligand and forms a terbium phosphate precipitate. Similar are the complexes of Tb(III) and Eu(III) (see Figure 1c) based on a phenanthrolin ligand **Eu-3**[19] for the detection of phosphorylated molecule such as: HPO_4^{2-}, ATP, ADP and AMP. This complex was able to detect phosphate levels in cells, in this case a green microalga.[19,20] However, the complexe suffers from poor stability. An additional example is the weak europium complex **Eu-4** (see Figure 1d) able to turn-off luminescence response when phosphate displaces the 2-phenyl-4-quinoline carboxylate ligand. A cerium(III) based chemosensor **Ce-5** was developed by Ganjali and coworkers[21] (see Figure 1e). The cerium(III) complex quenches the ligand-centered fluorescence emission. Co-

ordination of phosphate to the complex restores the fluorescence, leading to a transmetallation. However, no precipitation of the phosphate product was reported.
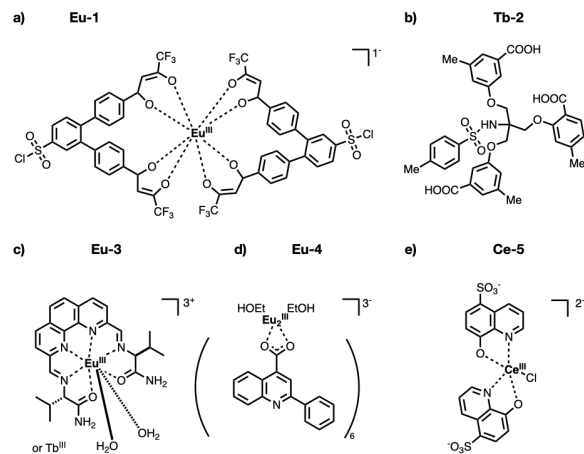

Figure 1: Some metal extrusion based receptors for the recognition of phosphorylated molecules in water.

Herein, we present a simple, cheap, and easy to assemble chemosensor for phosphate, based on cerium (IV) indicator displacement assay.[4,22–25] Cerium (IV) is rarely reported in the literature for sensing.[2,3,26–28] Due to the very low solubility of cerium phosphate, we concentrated our effort toward building a metal extrusion indicator displacement assay, thus providing good sensitivity.[29] Here cerium ammonium nitrate (CAN) is combined with calcein blue (CB), forming $[Ce(CB)]^{2+}$ able to detect selectively phosphate anions with a MEFID in aqueous media. Our host has high sensitivity due to metal extrusion, fluorescent properties and excellent selectivity for phosphate ions over other anions, allowing quantitative determination of phosphate. Additionally, its recognition mechanism is elucidated. Finally, we reveal, as proof of principle, that our complex can be used to sense phosphate ions in complex samples environmental samples.

Dissolving calcein blue (CB, 250 μM) and cerium ammonium nitrate (CAN, 250 μM) in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, 10 mM, pH=7.4) affords $[Ce(CB)]^{2+}$ (250 μM), which is water-soluble and pink at working

micromolar concentrations (see Figure 2 and Figure 3c, d and e).[30] Screening at various pH (5 to 7) allowed us to conclude that CB in insoluble, and therefore no complex formation was observable with CAN. Additionally, we were unable to form the complex $[Ce(CB)]^{2+}$ in pure water or in saline solution only as a result of the low solubility of CB below pH=7. We therefore selected pH=7.4 for the continuation of our study as CB is soluble at that pH as described in the literature for other applications.[31] In the next step, we hypothesized that the $[Ce(CB)]^{2+}$ complex could be used to assemble a receptor for phosphate derivatives, exploiting metal-ligand interactions for selective target recognition.[9] To the best of our knowledge, this mononuclear complex has never been described before in the literature as a selective MEFID-chemosensor for phosphate derivatives. We chose the reported calcein blue (CB), a fluorescent dye, as the fluorigenic indicator for the sensor, due to its coordination ability to metal complexes, especially to lanthanides.[30] Finally, in order to test the ligand exchange abilities of our sensor, which is known to occur with lanthanides, we exposed the complex $[Ce(CB)]^{2+}$ to various metal cations (1 eq.) such as: Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+} and Mg^{2+}, Ca^{2+}, Fe^{3+} all known to form inorganic complexes with CB. Only one of them interacted clearly (at a 1:1 stoichiometry) with $[Ce(CB)]^{2+}$: Zn^{2+}, which is remarkable (see supporting information, Figure 6S and 7S). These results suggest that certain cations might interfere in real-world applications with $[Ce(CB)]^{2+}$, though in aqueous solutions for example, the concentration of them would be far lower than our Ce(IV) complex. In addition, it may finally be beneficial since the extrusion of the sensing metal Ce(IV) would be favored by the formation of parasitic metal complexes, thus allowing better interactions of the liberated Ce(IV) with analytes.

The metal-based sensor $[Ce(CB)]^{2+}$ was prepared at pH=7.4 by simply mixing CAN and CB in a 1:1 molar ratio in an aqueous solution (see experimental part). The natural fluorescent of CB at pH=7.4 is quenched when coordinated to metals (see Figure 2).[30] Therefore, the
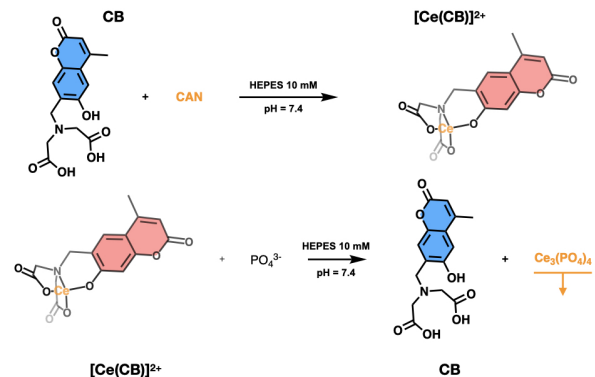

Figure 2: The proposed multi-step reaction mechanism presenting the fluorescent indicator displacement assay based on CB added to cerium ammonium nitrate (CAN) forming $[Ce(CB)]^{2+}$ at 250 μM in HEPES pH=7.4 for the detection of phosphate in 10 mM HEPES pH=7.4.

displacement of the receptor-bound calcein blue by phosphate anions is visually observed under the UV lamp (254-400 nm), as well as being readily measured spectrophotometrically via fluorescence (see Figure 3). The competition assay developed here is illustrated schematically in Figure 2. The sensing ensemble was prepared by simply mixing CAN and CB in a 1:1 molar ratio in an aqueous solution of 10 mM HEPES buffer pH=7.4, resulting in a non-fluorescent complex.

In the next step, we titrated CAN with CB (visible as fluorescent blue) at λ_{ex}=350 nm and λ_{em}=450 nm in order to determine the affinity constant of CAN for CB. The calculated affinity constant[32,33] (see supporting information, Figure 1S) is 3.98 x 10^4 M^{-1}, which is in good agreements with other similar described systems.[2,30] We then performed a Job Plot titration (see supporting information, Figure 9) in order to determine the stoichiometry of our complex $[Ce(CB)]^{2+}$. Out of the absorbance titration, we concluded that the bell-shape curve has a maximum at 1 equivalent and λ=450 nm of CAN versus CB and confirms the formation of a defined 1:1 non-fluorescent complex. The addition of phosphate anions to
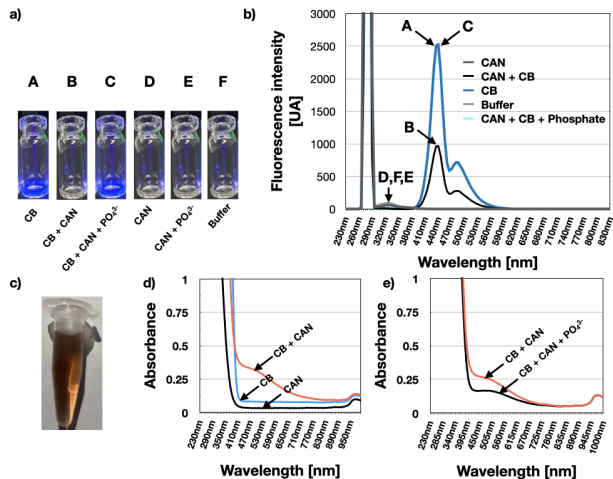
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Figure 3: a) Addition of calcein blue (**A**, CB, 250 μM, HEPES pH=7.4, (λ_{ex} =400 nm) to cerium ammonium nitrate (**B**, CAN, 250 μM, HEPES 10 mM pH=7.4 allows quenching of the fluorescence. Addition of phosphate to **B** (250 μM, HEPES 10 mM pH=7.4) allows a MEFID (**C**, 250 μM, HEPES 10 mM pH=7.4). **D**, **E** and **F** are negative controls (all chemicals at 250 μM, HEPES 10 mM pH=7.4) b) Addition of phosphate (250 μM) to $[Ce(CB)]^{2+}$ allows a MEFID (λ_{ex}=350 nm and λ_{em}=450 nm) c) $[Ce(CB)]^{2+}$ at 125 μM in HEPES 10 mM pH=7.4 d) Absorbance spectras of $[Ce(CB)]^{2+}$, CB and CAN (125 μM in HEPES pH=7.4) e) Absorbance spectras of $[Ce(CB)]^{2+}$ at 83 μM and in the presence of phosphate (2 eq.) in HEPES 10 mM pH=7.4.

the aqueous solution of this purple ensemble (see Figure 3) resulted in the appearance of fluorescence and in the decrease in absorbance (see Figure 3d).

The present sensor exhibits excellent selectivity towards phosphate ions over other anions, including pyrophosphate and sulfate ions (see Figure 4). To our knowledge, $[Ce(CB)]^{2+}$ is the first fluorometric sensor based on cerium(IV) to date that can detect phosphate ions with high selectivity in an aqueous media. Sensors that can detect analytes by fluorescence are of particular interest due to their high sensitivity. The use of the present sensor for such a purpose is demonstrated in Figure 4: the fluorescence is recovered only when phosphate ions are added to the aqueous solution, while

all other anions tested failed to cause this remarkable fluorescent change; even pyrophosphate and carbonate have not reacted, which is very difficult to achieve (see Figure 4).[34] Additionally, we investigated the perturbation of an anion mix (all anions together except phosphate) on the $[Ce(CB)]^{2+}$ (see supporting information, Figure 11s). We found that at a 1:1 stoichiometry, the mix perturb the complex (see supporting information, Figure 12s) but it decreases quickly to have no influence at 0.01 eq. corresponding to a 2.5 μM solution of mixed anion. This situation is more reminiscent of a utilization of the sensor in real conditions. Finally, as a negative control, halides such as chloride, bromide and iodide did not interfere (quenching) with the fluorescence of calcein blue (see supporting information, Figure 8S).
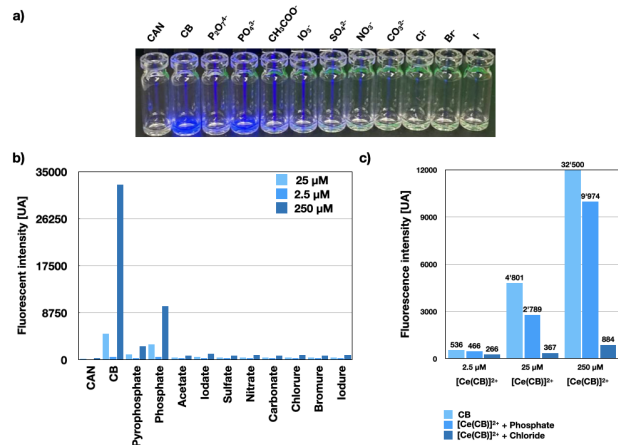

Figure 4: a) Screening of $[Ce(CB)]^{2+}$(250 μM in HEPES 10 mM at pH=7.4, aqueous solution) in the presence of various anions (1.25 mM, 5 equivalents) and exposed to a UV lamp at 400 nm b) Fluorescence measurement of the mixture of $[Ce(CB)(anions)]^{2+}$(250 μM): from left to right exposed to various anions; CAN, CB, $Na_2P_2O_7$, Na_3PO_4, CH_3COONa, $NaIO_3$, $NaSO_4$, $NaNO_3$, Na_2CO_3, NaCl, NaBr, NaI (λ_{ex}=350 nm and emission scan optimum: λ_{em}=450 nm) c) Detection limit of $[Ce(CB)]^{2+}$

To test the selectivity abilities of our sensor against phosphorylated molecules, we screened a collection of phosphorylated analytes, namely: ATP, ADP, AMP, β-
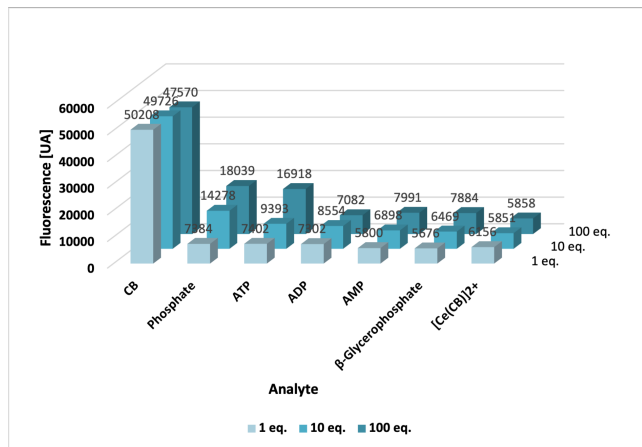
Figure 5: a) Screening of $[Ce(CB)]^{2+}$ (250 μM in HEPES 10 mM at pH=7.4, aqueous solution) in the presence of various phosphorylated analytes (1 to 100 equivalents).

Glycerophosphate. Of all the tested analytes, only ATP disturbed the sensor at high concentration (100 eq.) versus sensor. Whereas, at lower concentrations, only phosphate was detectable. It suggests the good selectivity of our chemosensor even in the presence of phosphorylated molecules.

In order to determine the stoichiometry of phosphate for $[Ce(CB)]^{2+}$, we carried out a Job Plot titration (see supporting information, Figure 9s). A maximum of fluorescence is reached when a 1 eq. of $[Ce(CB)]^{2+}$ is mixed with 0.8 eq. of CB.

Table 1: Affinity constant of CAN for CB

System	Affinity constant $[M^{-1}]$
$[Ce(CB)]^{2+}$	$K_a=3.98 \cdot 10^4$ M^{-1}

Investigation of the detection limit of $[Ce(CB)]^{2+}$ allowed us to observe that our sensor detects phosphate at a concentration of 25 μM (see Figure 4c). However, the measured signal at a concentration of 2.5 μM is still acceptable (signal-to-noise of 1.75 times) and allows to conclude that our sensor has a detection limit in the low micromolar range. This ranks it at the top of the systems described so far for the detection of phosphate by metal based DA such as reaching the abilities of the malachite green assay (see Table 2).[3] Finally, few papers report explicitly the detection limit

of metal based chemosensors for phosphate which renders a classification and a comparison very difficult to obtain or even relevant.

Table 2: Comparison of different detection limits for various phosphorylated compounds in ethanol and buffered water

	Systems	Described detection limit
1[2]	$[Ce_2(HXTA)(PO_4^{3-})]$	1 μM
2[11]	Fe(III)-HOPO-fluo (in ethanol)	3.5 μM
2[35]	$[Zn_2(H$-$bpmp)(H_2PO^{4-})]$	20 μM
3[36]	bis(Zn^{II}-DPA) for phosphorylated compounds	20 μM
4[37]	$[Cu_2(La)(PPi)]$	20 μM
5[38]	$[Cu^{II}_2$-PV] for glyphosate	4.8 μM
6[39]	Malachite green	Useful range: 0.02 to 40 μM
7[thiswork]	$[Ce_3(PO_4^{3-})_4]$	up to 2.5 μM

In order to understand the disassembly of $[Ce(CB)]^{2+}$, we hypothesized that the low solubility (S=2,915·10^{-34} M) of cerium phosphate might generate a precipitation. We therefore prepared a solution of CAN in water (0.1 M) and a solution of sodium phosphate (0.1 M) in water. The mix of the two solutions in a 1:1 ratio generated a precipitate that we interpreted as $Ce_3(PO_4)_4$ (see Figure 6), suggesting the high affinity of cerium for phosphate. Additionally, to understand at micromolar concentration the behaviour of phosphate to $[Ce(CB)]^{2+}$, we exposed CAN and $[Ce(CB)]^{2+}$ (83.3 μM, HEPES in D_2O pH=7.4) with phosphate (1 eq.) and recorded ^{31}P NMRs. The complete disappearance of the signal at -110.38 ppm suggests, that even at that concentration, the phosphate precipitates in the presence of cerium (IV) (see supporting information).

Next, we decided to investigate the detection in environmental samples. We spiked distilled water, tap water and commercial water (Roucous®) with 5 eq. of phosphate versus
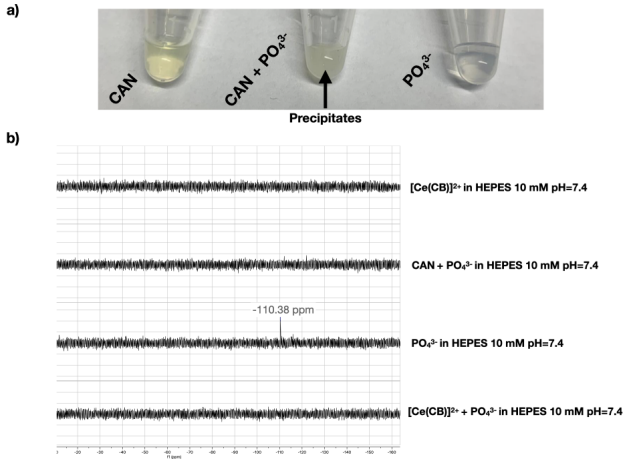
Figure 6: a) Precipitation of cerium phosphate when CAN is dissolved in 0.1 M in water and is mixed with a 0.1 M solution of sodium phosphate in water b) Phosphorus NMR spectra of $[Ce(CB)]^{2+}$ (83.3 μM in 10 mM HEPES pH=7.4) in the presence of phosphate (1 eq.). For both $[Ce(CB)]^{2+}$ and CAN a precipitation occurs in the presence of phosphate, since no phosphorus signal is observable.

Table 3: Exposition of a 250 μM solution of $[Ce(CB)]^{2+}$ to phosphate (5 eq.).

Systems	Fluorescence [UA] (λ_{ex}=350 nm and λ_{em}=450 nm)
$[Ce(CB)]^{2+}$ + Phosphate (5 eq.)	3267 ± 9
CB	8881 ± 25
$[Ce(CB)]^{2+}$	901 ± 60

$[Ce(CB)]^{2+}$ in HEPES 10 mM pH=7.4. Addition of the samples to the sensor allowed a MEFID response in all cases (see supporting information, Figure 12S) with little disturbance by the water composition. It is especially true for the commercial water Roucous® (see Table 4). It suggests that our system can be used in real environmental application for sensing but also for removing phosphate out of water in complexed conditions.

In conclusion, we have described a simple assay for the detection of phosphate ions in an aqueous media. The assay is based on com-

Table 4: Measurements of various water types spiked with 5 eq. phosphate

Sytem	Fluorescence [UA] (λ_{ex}=350 nm and λ_{em}=450 nm)
$[Ce(CB)]^{2+}$	105 ± 1.5
$[Ce(CB)]^{2+}$ + Distilled water spiked with 5 eq. phosphate	1653 ± 83
$[Ce(CB)]^{2+}$ + Tap water spiked with 5 eq. phosphate	2331 ± 140 (70% accuracy)
$[Ce(CB)]^{2+}$ + Roucous® water spiked with 5 eq. phosphate	1853 ± 54 (89% accuracy)

mercially available chemicals (cerium salts and a fluorescent indicator) and allows quantifying phosphate at low micromolar concentration range by fluorescence measurement. The interference of common anions is remarkably low, especially for phosphate over pyrophosphate. With a detection limit of around 2.5 μM, our system is, to the best of our knowledge, the most sensitive metal-based sensor for phosphate described so far. Moreover, the assay is very easy to perform and requires no prior synthesis. First evidence of the utility of our phosphate assay was provided by the analysis of phosphate in environmental samples.

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