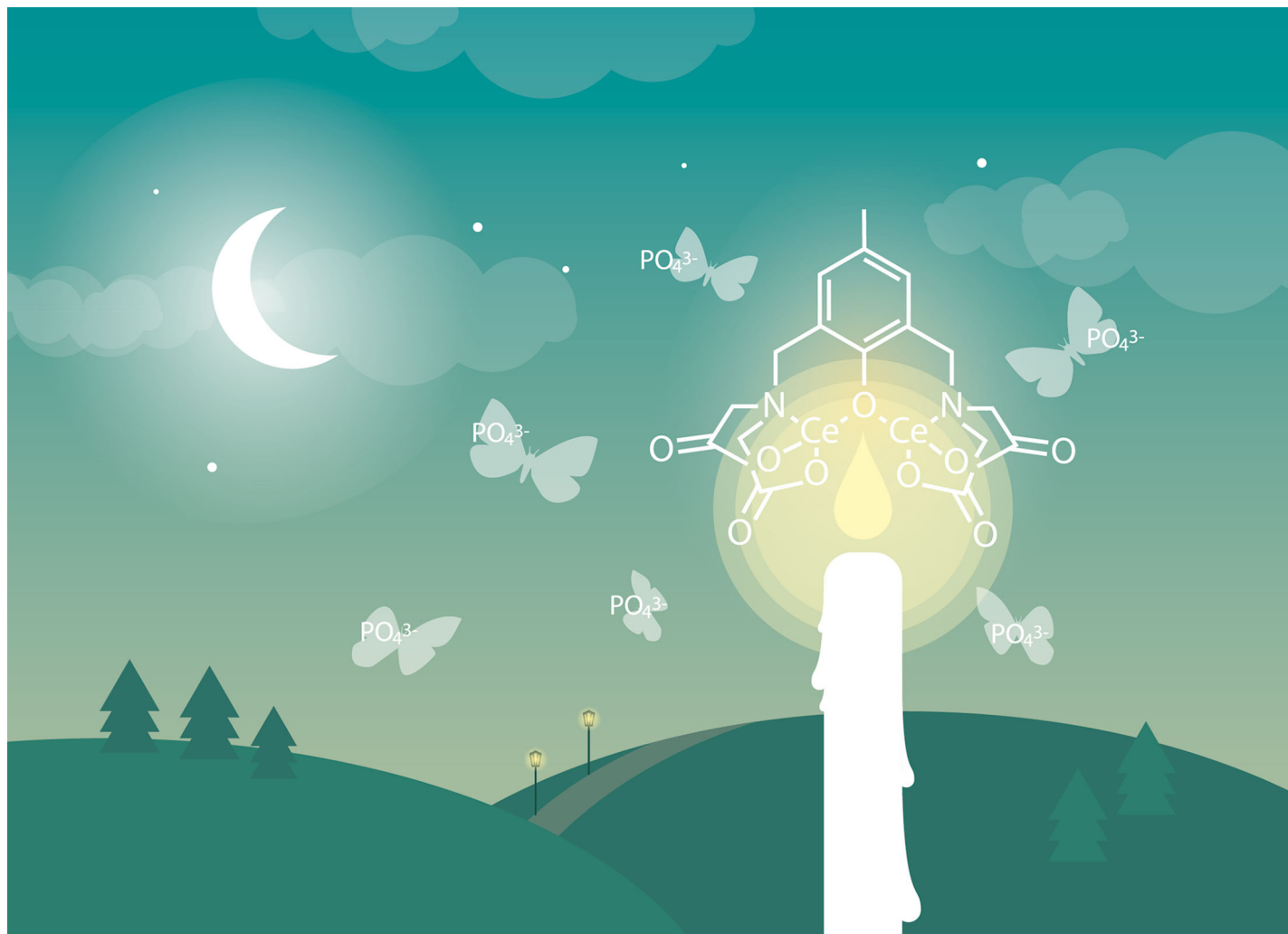




Showcasing research from Rossel's group at Gymnase français de Bienne in Switzerland. Image designed and illustrated by Muriel Hediger.

A biomimetic cerium-based biosensor for the direct visual detection of phosphate under physiological conditions

An indicator displacement assay was used to probe phosphate ions in a neutral aqueous medium using a dinuclear cerium based complex. The homoleptic complex can be used to detect phosphate ions with nanomolar affinity either spectrophotometrically or with the naked-eye.

As featured in:



See Thibaud Rossel and Marc Creus, *Chem. Commun.*, 2019, **55**, 14894.



ROYAL SOCIETY
OF CHEMISTRY

Celebrating
IYPT 2019

rsc.li/chemcomm

Registered charity number: 207890



Cite this: *Chem. Commun.*, 2019, 55, 14894

Received 25th June 2019,
Accepted 31st October 2019

DOI: 10.1039/c9cc04840e

rsc.li/chemcomm

A biomimetic cerium-based biosensor for the direct visual detection of phosphate under physiological conditions†

Thibaud Rossel^{‡*} and Marc Creus^{‡b}

An indicator displacement assay (IDA) was used to probe phosphate ions in an aqueous medium at neutral pH using a dinuclear cerium based complex $[\text{Ce}_2(\text{HXTA})]^{3+}$. The homoleptic complex can be used to detect phosphate ions with nanomolar affinity either spectrophotometrically or with the naked-eye. To our knowledge, this is the first dinuclear cerium biomimetic IDA detection system with the highest affinity known to date for selective, naked-eye based phosphate recognition under physiological conditions (pH = 5–7) and even in pure water and complex samples such as sea water.

The development of selective and sensitive assays for the detection of analytes in biological samples is a challenging and important field of research.^{1–4} In particular, there is a need to develop inexpensive and easy-to-use sensors against a range of important analytes, such as phosphorylated compounds.^{3,5–7} Phosphates are fundamental molecules in biology and play key roles in living systems. Phosphates are also privileged in various cellular mechanisms including (1) energy storage systems, such as through the well-known ADP-ATP⁸ tandem-reaction catalyzed by ATP-synthase;⁹ (2) key constituents of DNA;¹⁰ (3) components of membrane bilayers, for instance, in the form of phospholipids;¹¹ and (4) signal transducers through enzymatic processes involving kinases and phosphatases.¹² Selective binding and recognition of phosphate derivatives by proteins is a crucial highly regulated event in living systems and of great interest for medicinal or analytical purposes.¹³ A relevant example of an important phosphate-containing signaling molecule is sphingosine-1-phosphate (S1P), a naturally occurring bioactive lipid.¹⁴ The FAB fragment of LT1009 (sonpeizumab), a humanized monoclonal antibody, was developed for binding S1P with high affinity (pi-comolar)

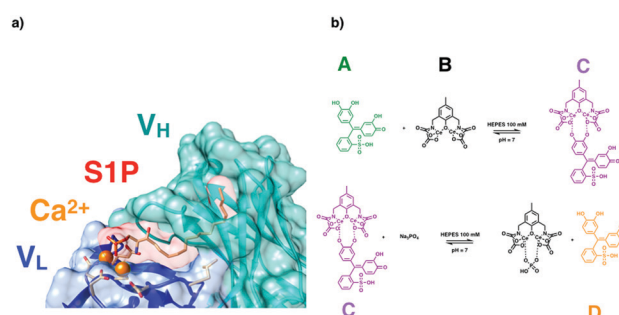


Fig. 1 Crystal structure of S1P in conjugation with LT1009 (PDB 3I9G); in blue the monomer V_L, in green the monomer V_H, in red the phospholipid S1P, in orange the dinuclear calcium based binding site¹⁵ (a). The multistep reaction mechanism presenting the indicator displacement assay based on the description of the cerium complex by L. Que *et al.*²³ (b).

and selectivity used for therapeutic purposes (see Fig. 1a).¹⁵ The exquisite selectivity is due to (1) the first coordination sphere interactions between the dinuclear calcium based binding site and the phosphate part of the sphingolipid (see Fig. 1a) and (2) the second coordination sphere interactions between the protein and the tail of the sphingolipid (see Fig. 1a). Inspired by the mode of binding to the phosphate moiety of the S1P antibody,¹⁵ we sought to design a biomimetic binder of phosphate based on a bi-metallic bioinorganic complex.¹⁶ Furthermore, we sought to exploit this binder of phosphate to develop an easy and inexpensive assay for its detection through the development of an indicator displacement assay (IDA). The construction of phosphate receptors is a daunting task due to the large size of the anion, its hydrophilicity, its multiple protonation states and the Hofmeister series, especially at physiological pH.³ Here we report a novel IDA for phosphate based on the first di-nuclear cerium complex $[\text{Ce}_2(\text{HXTA})]^{3+}$ (C see Fig. 1) that mimics the first coordination sphere of the metal-mediated binding of an anti-S1P-antibody with an exquisite nanomolar affinity for phosphate (see Fig. 1b).^{6,17–20}

Dissolving HXTA-H5 (2,2',2'',2'''-(((2-hydroxy-5-methyl-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetic acid) and

^a Gymnase français de Bienne, Prê Jean-Meunier 1, Moutier, 2740, Switzerland.
E-mail: thibaud.rossel@emsp.gfbienne.ch; Tel: +41 32 494 52 80

^b University of Basel, Klingelbergstrasse 50/70, Basel, 4056, Switzerland.
E-mail: marc.creus@unibas.ch; Tel: +41 61 207 20 92

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cc04840e

‡ Dr Thibaud Rossel conducted the research and wrote the manuscript. Dr Marc Creus revised the research and wrote the manuscript.

cerium ammonium nitrate (CAN) in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, 100 mM, pH = 7) formed $[\text{Ce}_2(\text{HXTA})]^{3+}$ (**B** see Fig. 1), which is water-soluble and colourless at working micromolar concentrations (Fig. 2). By analogy with other reported dinuclear complexes,¹ we hypothesized that the $[\text{Ce}_2(\text{HXTA})]^{3+}$ (**B** see Fig. 1, $\epsilon_{(595\text{nm})} = 736 \text{ L mol}^{-1} \text{ cm}^{-1}$) complex could be used to assemble a receptor for phosphate derivatives, exploiting metal-ligand interactions for selective target recognition. To the best of our knowledge, this dinuclear complex has never before been described in the literature as a selective IDA-biosensor for phosphate derivatives. We chose the reported pyrocatechol violet (**A**, PCV, see Fig. 1, $\epsilon_{(595\text{nm})} = 4587 \text{ L mol}^{-1} \text{ cm}^{-1}$), a catechol-type pH-sensitive dye,²¹ as the chromogenic indicator for the sensor, due to its phenoxo-bridged described coordination to dinuclear metal complexes.²²

In addition to being a pH-sensitive chromophore, pyrocatechol violet (**A** see Fig. 1) is yellow in colour at a neutral pH ($\epsilon_{(595\text{nm})} = 4587 \text{ L mol}^{-1} \text{ cm}^{-1}$) and the reported peak is at $\lambda_{\text{max}} = 444 \text{ nm}$; it changes to blue (reported $\lambda_{\text{max}} = 624 \text{ nm}$) when coordinated to a metal. Therefore, the displacement of the receptor-bound pyrocatechol violet by a phosphate anion is communicated visually and readily measured spectrophotometrically (**D** see Fig. 1 and 2). Investigation of various pHs showed the best discrimination at pH = 7 with the naked-eye. However, the detection works under buffered conditions from pH = 5 to pH = 7 and even in pure water and sea water highlighting the outstanding versatility of the sensor for the detection of phosphate, in particular in complex solutions (see ESI†). The competition assay developed is illustrated schematically in Fig. 1b. The sensing ensemble was prepared by simply mixing CAN, HXTA-H5, and PCV in a 2:1:1 molar ratio in an aqueous solution of 100 mM HEPES buffer pH = 7, which results in a violet complex (**C** see Fig. 1, peak with $\lambda_{\text{max}} = 580 \text{ nm}$, $\epsilon_{(595\text{nm})} = 2700 \text{ L mol}^{-1} \text{ cm}^{-1}$). The absorbance of the construct depends on the concentration according to the

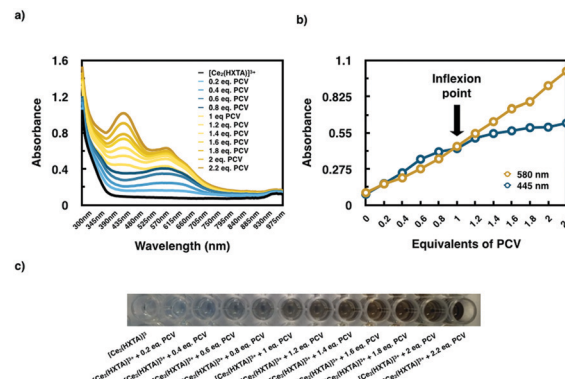


Fig. 3 (a) Titration of $[\text{Ce}_2(\text{HXTA})]^{3+}$ using 0 eq. to 2.2 eq. of PCV and showing an increase in the absorbance band at 580 nm; (b) absorbance plot versus wavelength (the experiment has been done in duplicate with a maximal standard deviation of ± 0.031); (c) visual detection of the titration of $[\text{Ce}_2(\text{HXTA})]^{3+}$ with the addition of 0 eq. to 2.2 eq. of PCV.

Beer-Lambert law ($[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+ = 250 \mu\text{M}$ (**C** see Fig. 1) with an absorbance of 0.43 at $\lambda_{\text{max}} = 580 \text{ nm}$).

Additionally, the titration of the complex $[\text{Ce}_2(\text{HXTA})]^{3+}$ (**B** see Fig. 1) with PCV (**A** see Fig. 1) leads to the appearance of an absorbance band at 580 nm, visible as violet, a colour that is not visible in either precursor alone. The ratio of the 445 nm band, which is present in the PCV precursor, to the 580 nm band, is maintained up to a 2:1:1 mixture of precursors to form $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$; upon further addition of PCV, beyond this stoichiometry, the increase in absorption at 445 nm is more marked than at 580 nm (Fig. 3). Hence, we conclude that the inflection point at 1 equivalent of PCV to $[\text{Ce}_2(\text{HXTA})]^{3+}$ confirms the formation of a defined $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ (**C** see Fig. 1) complex of purple colour.

For comparison, other reported systems reported a decrease in absorbance at 444 nm and an increase in absorbance at 624 nm.^{1,16} In order to determine the stoichiometry and the affinity of the complex formation, a Job plot and a titration (see ESI† and Table 1) was carried out (see Fig. 4). The Job-plot confirms that a maximum of absorbance is reached for various wavelengths (445, 580 and 660 nm) at a 2:1:1 stoichiometry, ascribed to the formation of the complex $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ (Fig. 4). The calculated affinity constant^{16,24} (see ESI† and Table 1) is $K_a = 4.48 \times 10^4 \text{ M}^{-1}$, which is in good agreement with other described systems.^{1,16} The addition of phosphate anions to the aqueous solution of this violet ensemble resulted in a change of colour to pale yellow, and led to changes in the UV/Vis absorption spectra (**D** see Fig. 1 and 2). The present sensor exhibits excellent selectivity towards phosphate ions over other anions, including pyrophosphate and sulfate ions (Fig. 5). Selective phosphate detection is much more challenging compared to pyrophosphate due to charges and co-ordination differences in favor of pyrophosphate.³ In addition, to our knowledge, $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ is the first colorimetric sensor based on a dinuclear lanthanide complex to date that can detect phosphate ions with high selectivity in a buffered aqueous solution. Sensors that can detect analytes by the naked eye, without resorting to any spectrometer, are of particular interest

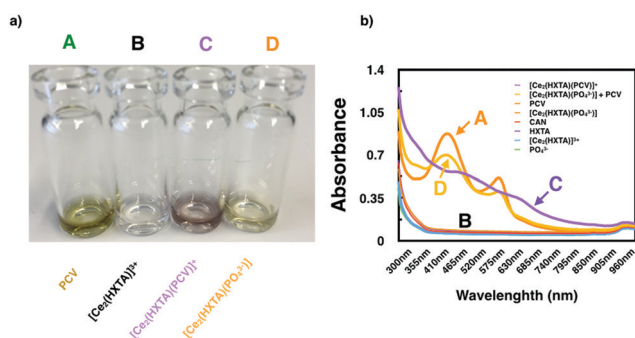
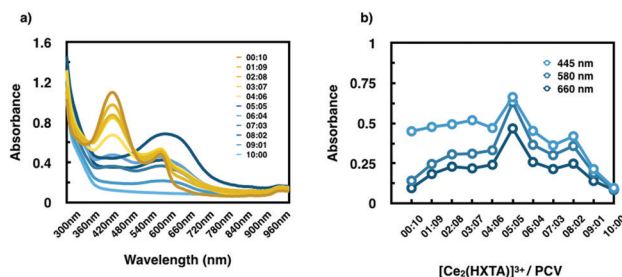
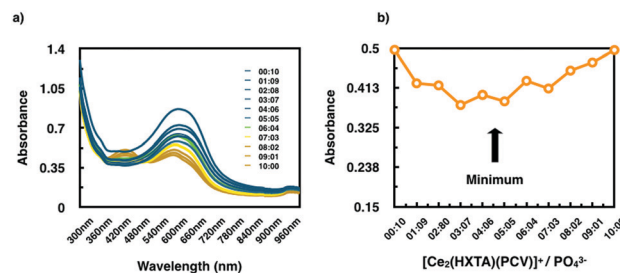
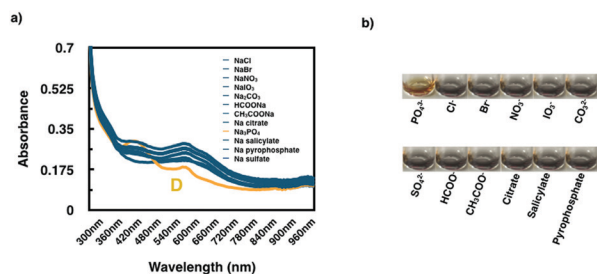


Fig. 2 (a) Naked-eye or (b) spectrometric detection of PO_4^{3-} . The assay is based on the violet coloured $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ (**C**), which upon addition of PO_4^{3-} , changes colour to yellow (**A**). (**B**) is a $250 \mu\text{M}$ solution of $[\text{Ce}_2(\text{HXTA})]^{3+}$ in an aqueous buffer (HEPES 100 mM, pH = 7) solution. The colour of the pyrocatechol violet (**A**) changes from yellow ($\lambda_{\text{max}} = 445 \text{ nm}$) to violet (**C**) ($\lambda_{\text{max}} = 580 \text{ nm}$) upon the formation of $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$. The addition of the phosphate anion ($250 \mu\text{M}$) displaces pyrocatechol violet $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ to form a pale yellow solution with no absorbance peak between 400 and 800 nm (**D**).

Table 1 Comparison of different affinity constants and detection limits based on IDAs for various described dinuclear complexes in buffered water and malachite green

Entry	Systems	Affinity constant (K_a)	Described detection limit
1 ^{thiswork}	$[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$	$4.48 \times 10^4 \text{ M}^{-1}$	10 μM
2 ¹⁶	$[\text{Cu}_2(\text{La})(\text{PCV})]^+$	$3.54 \times 10^5 \text{ M}^{-1}$	20 μM
3 ¹	$[\text{Zn}_2(\text{H-bpmp})(\text{PCV})]^+$	$5.30 \times 10^5 \text{ M}^{-1}$	50 μM
4 ^{thiswork}	$[\text{Ce}_2(\text{HXTA})(\text{PO}_4^{3-})]$	$1.44 \times 10^7 \text{ M}^{-1}$	10 μM
5 ¹⁶	$[\text{Cu}_2(\text{La})(\text{PPI})]$	$1.9 \times 10^4 \text{ M}^{-1}$	20 μM
6 ¹	$[\text{Zn}_2(\text{H-bpmp})(\text{H}_2\text{PO}_4^-)]$	$1.20 \times 10^4 \text{ M}^{-1}$	50 μM
7 ^{25,26}	Malachite green	None	Useful range: 0.02 to 40 μM

**Fig. 4** (a) Job plot of $[\text{Ce}_2(\text{HXTA})]^{3+}$ with PCV with the following ratio of $[\text{Ce}_2(\text{HXTA})]^{3+}$ compared to PCV: 00:10/01:09/02:08/03:07/04:06/05:05/06:04/07:03/08:02/09:01/10:00; (b) absorbance plot at three different wavelengths: 445 nm, 580 nm and 660 nm.**Fig. 6** (a) Job plot of $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ with the following ratio of $[\text{Ce}_2(\text{HXTA})]^{3+}$ compared to PO_4^{3-} : 00:10/01:09/02:08/03:07/04:06/05:05/06:04/07:03/08:02/09:01/10:00; (b) absorbance plot versus wavelength at 445 nm.**Fig. 5** (a) Absorbance spectra of $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ (10 μM in HEPES 100 mM at pH = 7, aqueous solution) in the presence of various anions (10 μM); (b) the coloured mixture of $[\text{Ce}_2(\text{HXTA})]^{3+}$ (250 μM) in the presence of anions (2500 μM) from left to right; Na_3PO_4 , NaCl, NaBr, NaNO_3 , NaIO_3 , Na_2CO_3 , Na_2SO_4 , HCOONa , CH_3COONa , Na Citrate, Na salicylate, Na pyrophosphate and Na sulfate.

because of their convenience. The use of the present ensemble for such a purpose is demonstrated in Fig. 5a: the color change from blue-violet to yellow occurred only when the phosphate ion was added to the aqueous solution of the ensemble, whereas all other anions tested failed to cause this remarkable color change (Fig. 5b).

In addition, we investigated the direct evidence of phosphate coordination to the dinuclear cerium complex $[\text{Ce}_2(\text{HXTA})]^{3+}$. At a concentration of 3.33 mM, $[\text{Ce}_2(\text{HXTA})]^{3+}$ is pink as described in the literature and is well investigated for its chemical structure (dinuclear complex).²³ Direct addition of phosphate to the complex allows a shift in color from pink to violet to be seen leading to the formation of an heteroleptic complex supporting our proposed mechanism (see Fig. 2 and ESI†). In addition, here we present a new methodology for directly proving the detection of analytes in addition to the IDA

described in our investigation without using any indirect methodology such as IDAs.² To our knowledge, this has never been described in the literature for the detection of analytes.

In order to determine the affinity constant of phosphate and the stoichiometry for $[\text{Ce}_2(\text{HXTA})(\text{PO}_4^{3-})]$ ($\epsilon_{(595\text{nm})} = 2148 \text{ L mol}^{-1} \text{ cm}^{-1}$), we carried out a titration (see ESI†) and a Job plot titration (see Fig. 6a). At a 2:1:1:1 mixture a minimum of absorbance was reached at 425 nm and the calculated affinity constant^{16,24} is $K_a = 1.44 \times 10^7 \text{ M}^{-1}$, which is at least 1000 times higher than similar described systems for the detection of phosphate and derivatives (see Table 1 and Fig. 6). To our knowledge, this is to date the best system for selective naked-eye based phosphate recognition with a dinuclear complex based on an IDA.

Comparison with other non-IDA photometric based systems for the detection of phosphates allows it to be concluded that $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ (C see Fig. 1) is an excellent assembly for the detection of phosphate (see Table 1). In addition, our cerium biosensor has the best affinity constant in the nanomolar range (100 nM) compared with similar systems (see Table 1). It is also remarkable to mention that there are very few descriptions of effective dinuclear metal complexes for the recognition of phosphates compared to pyrophosphate.³

Finally, $[\text{Ce}_2(\text{HXTA})(\text{PCV})]^+$ rivals the corresponding gold standard malachite green assay based on molybdenum, phosphate and malachite green assembly^{26,27} that doesn't rely on an indicator displacement assay (0.02–40 μM of phosphate as routine detection, see Table 1²⁶ entry 7). Hence, the malachite green assay has limitations³ compared to our cerium system: (1) it works only under an acidic pH which limits the detection of phosphate under physiological conditions;²⁷ (2) excess of

phosphate with the assay generates a precipitation of the formed molybdenum complex that has to be compensated with for example polyvinyl alcohol for improved solubility;²⁷ (3) as a transition metal, molybdenum exists in many oxidation states and in the assay could interact with any redox reactant to generate parasitic and catalytic redox reactions that perturb the accuracy of phosphate detection.²⁶ Other best strategies for phosphate detection rely on the improvement of the malachite green protocol or on biological entities such as enzymes.³ These strategies could remain however toxic, not working in physiological conditions and or in a complex medium and may need some special handling in particular for enzymes that could be, in addition, expensive.³

In conclusion, our strategy is quick, non-toxic and useful at physiological pH, in pure water or in complex samples such as sea water. The detection of phosphate can be done using an indicator displacement assay or with the direct detection of phosphate. Our system doesn't require special handling, doesn't suffer with poor stability and is very cheap. It is the first description of a selective cerium based sensor for the naked eye detection of phosphate and our system has the best affinity constant of similar systems described to date. Finally, it also offers a biomimetic platform with great potential for the further development of selective binders, which may be able to target phosphorylated compounds of important analytical and therapeutic interest. Future work will focus on the engineering of further selectivity by exploiting interactions with the second coordination sphere such as incorporating the complex into a protein.

We thank Prof. Thomas R. Ward for the loan of a spectrophotometer. Furthermore, we are grateful to Prof. Reinhard Neier for his good advice on this research. We thank the analytical service (NPAC) of the University of Neuchâtel. Additionally, we thank the students that participated in the reproduction of these experiments, in particular Bruno Cabete, Liam MacGillivray, Anthony Racine and Giuseppe Giordano, as well as the Gymnase français de Bienne for providing lab equipment. We thank Cecile Hediger for the affinity constant calculations. Finally, we dedicate this research in honor of Dr Yvonne Wilson and Colette Rossel.

Conflicts of interest

The authors declare no competing interests.

References

- 1 M. S. Han and D. H. Kim, *Angew. Chem.*, 2002, **114**, 3963–3965.
- 2 B. T. Nguyen and E. V. Anslyn, *Coord. Chem. Rev.*, 2006, **250**, 3118–3127.
- 3 A. E. Hargrove, S. Nieto, T. Zhang, J. L. Sessler and E. V. Anslyn, *Chem. Rev.*, 2011, **111**, 6603–6782.
- 4 T. Rossel and M. Creus, *CHIMIA International Journal for Chemistry*, 2019, **73**, 599–603.
- 5 S. Aoki and E. Kimura, *Rev. Mol. Biotechnol.*, 2002, **90**, 129–155.
- 6 S. Goswami, S. Paul and A. Manna, *RSC Adv.*, 2013, **3**, 10639–10643.
- 7 W. Liu, Z. Du, Y. Qian and F. Li, *Sens. Actuators, B*, 2013, **176**, 927–931.
- 8 D. Stock, A. G. Leslie and J. E. Walker, *Science*, 1999, **286**, 1700–1705.
- 9 P. D. Boyer, *Annu. Rev. Biochem.*, 1997, **66**, 717–749.
- 10 J. D. Watson and F. H. Crick, *Cold Spring Harbor Symp. Quant. Biol.*, 1953, 123–131.
- 11 E. D. Korn, *Science*, 1966, **153**, 1491–1498.
- 12 Y. Nishizuka, *Nature*, 1984, **308**, 693–698.
- 13 P. Blume-Jensen and T. Hunter, *Nature*, 2001, **411**, 355.
- 14 S. Spiegel and S. Milstien, *Nat. Rev. Mol. Cell Biol.*, 2003, **4**, 397.
- 15 J. M. Wojciak, N. Zhu, K. T. Schuerenberg, K. Moreno, W. S. Shestowsky, M. Hiraiwa, R. Sabbadini and T. Huxford, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 17717–17722.
- 16 W. Yu, J. Qiang, J. Yin, S. Kambam, F. Wang, Y. Wang and X. Chen, *Org. Lett.*, 2014, **16**, 2220–2223.
- 17 X. Liu, H. T. Ngo, Z. Ge, S. J. Butler and K. A. Jolliffe, *Chem. Sci.*, 2013, **4**, 1680–1686.
- 18 M. E. Branum, A. K. Tipton, S. Zhu and L. Que, *J. Am. Chem. Soc.*, 2001, **123**, 1898–1904.
- 19 H. Miyaji, W. Sato and J. L. Sessler, *Angew. Chem.*, 2000, **112**, 1847–1850.
- 20 F. Zaubitzer, A. Buryak and K. Severin, *Chem. – Eur. J.*, 2006, **12**, 3928–3934.
- 21 V. M. Ivanov and G. A. Kochelayeva, *Russ. Chem. Rev.*, 2006, **75**, 255.
- 22 K. D. Karlin, Y. Gultneh, T. Nicholson and J. Zubieta, *Inorg. Chem.*, 1985, **24**, 3725–3727.
- 23 M. E. Branum, A. K. Tipton, S. Zhu and L. Que, *J. Am. Chem. Soc.*, 2001, **123**, 1898–1904.
- 24 P. Thordarson, *Chem. Soc. Rev.*, 2011, **40**, 1305–1323.
- 25 T. L. Abdulazeez and S. B. Adeloju, *Talanta*, 2013, **114**, 191–203.
- 26 P. V. Veldhoven and G. P. Mannaerts, *Anal. Biochem.*, 1987, **161**, 45–48.
- 27 P. A. Lanzetta, L. J. Alvarez, P. S. Reinach and O. A. Candia, *Anal. Biochem.*, 1979, **100**, 95–97.