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Fluorescent ratiometric supramolecular tandem assays for phosphatase and phytase enzymes†

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Ratiometric fluorescent assays have a built-in correction factor which enhances assay accuracy and reliability. We have developed fluorescent ratiometric supramolecular tandem assays for phosphatase and phytase enzymes using a mixture of three molecular components. One of the molecules is a tetra-cationic fluorescence quencher called **CalixPyr** which can bind and quench the polyanionic pyrene fluorophore, **CMP**, that emits at 430 nm. Polyphosphates can disrupt the **CMP**/**CalixPyr** complex and alter the fluorescence intensity (responsive signal). **CalixPyr** has no effect on the fluorescence emission of cationic pentamethine cyanine fluorophore, **cCy5**, which emits at 665 nm and acts as a non-responsive reference signal. The continuous ratiometric fluorescent assay for alkaline phosphatase monitored hydrolytic consumption of adenosine triphosphate (ATP). The continuous ratiometric fluorescent assay for phytase activity monitored hydrolytic consumption of phytate. With further development this latter assay may be useful for high throughput assessment of phytase activity in individual batches of fortified animal feed. It is likely that the three-molecule mixture (**CMP**, **CalixPyr**, **cCy5**) can become a general assay platform for other enzymes that catalyse addition/removal of phosphate groups from appropriate molecular substrates.

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Introduction

One of the major technical achievements of supramolecular chemistry over the last twenty years is the development of indicator displacement assays.[1] Fluorescent versions of these assays employ a paired combination of fluorophore and host molecule that have the capacity to reversibly associate and form a non-covalent complex with modified fluorescence signal. In the presence of a competing analyte the mole fraction of fluorophore/host complex is altered and so is the corresponding fluorescence signal. A popular manifestation of the fluorophore displacement strategy is the supramolecular tandem assay (STA) which is used to monitor enzymatic reactions that modulate the concentration of a competing analyte over time.[2–4] Typically, these assays track the change in fluorescence intensity at a single wavelength (intensiometric detection); however, it is well-established that a more reliable assay design tracks a responsive signal relative to a non-responsive reference signal at a separate wavelength (ratiometric detection).[5,6] Thus, there is a need for generalizable methods of converting an intensiometric fluorescent STA into a ratiometric STA. The simplest idea is to add a second reference fluorophore to the responsive fluorophore/host mixture. The reference fluorophore must possess a specific set of properties including: (a) an emission wavelength that can be detected separately from the wavelength of the responsive fluorophore, and (b) negligible affinity for the other molecules in the assay mixture. Here, we describe a new supramolecular strategy for converting an intensiometric STA into a ratiometric STA.

The host molecule in this study is calixpyridinium (**CalixPyr**), a tetra-cationic molecule that was invented by Tsukube and co-workers in 1998 (Scheme 1a).[7] Over the intervening years, **CalixPyr** has been incorporated into various supramolecular assembly paradigms.[8–19] A notable property of **CalixPyr** is its capacity to complex and quench the fluorescence of polysulfonated pyrene fluorophores such as **HTPS** and **PyTs**. Moreover, the quenching efficiency can be lowered by the presence of competing polyphosphates or polycarboxylates that have affinity for the tetra-cationic **CalixPyr**.[20–22] This has led to the exploitation of fluorophore/**CalixPyr** pairs within intensiometric enzyme assays that track ATP hydrolysis by phosphatase enzymes.[23] While the commercial availability of **HTPS** and **PyTs** is an attraction, the pH sensitivity of **HTPS**,[24] and the low excitation wavelength of **PyTs** (340 nm which is below the common diode laser wavelength range) are drawbacks that limit the scope of enzyme assays that can be developed. We reasoned that a ratiometric and pH-insensitive fluorescent STA could be achieved by combining **CalixPyr** in a mixture with two new fluorophores that have a unique set of

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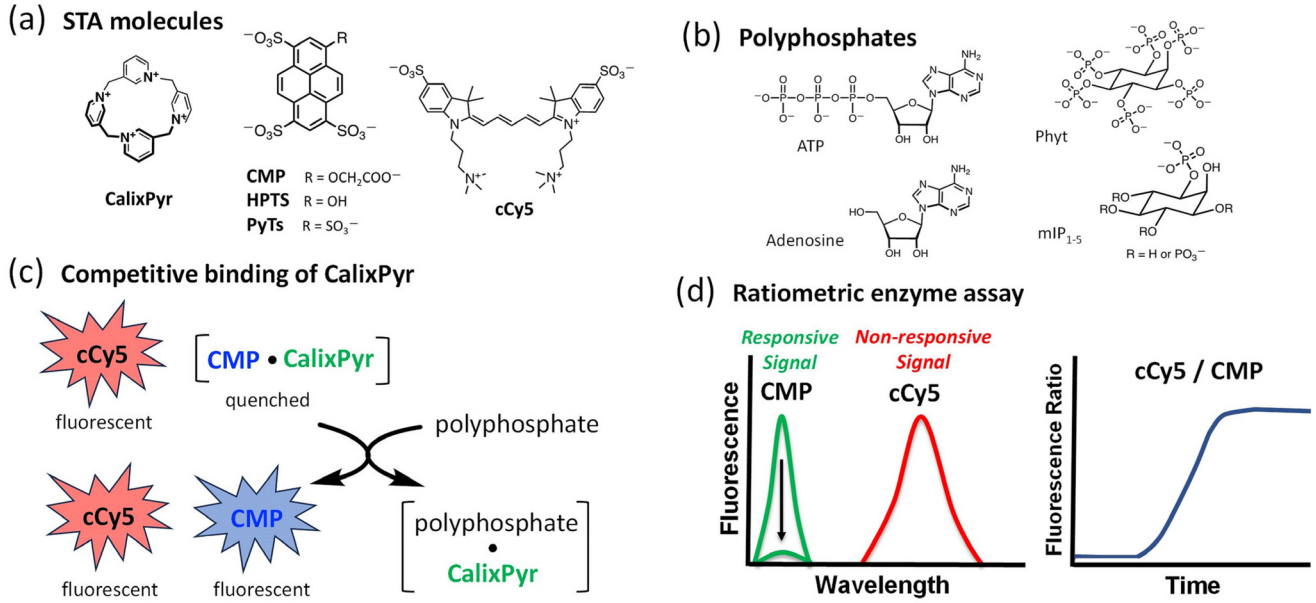

Scheme 1 (a) Chemical structures of the STA molecules used in this study. (b) Polyphosphates used in this study. (c) Selective binding and quenching of **CMP** fluorescence by **CalixPyr** is modulated by the presence of polyphosphate. (d) Ratiometric assay reports time-dependent change in fluorescence signals for non-responsive reference **cCy5** and responsive **CMP** which can be plotted as the **cCy5/CalixPyr** fluorescence ratio.

spectral and supramolecular properties. The first new fluorophore is the polyanionic pyrene derivative **CMP** which exhibits a pH-insensitive emission at 430 nm.[25] We hypothesized that **CalixPyr** would bind to polyanionic **CMP** and quench its fluorescence, and if so **CMP** would be a superior replacement for **HTPS** or **PyTs** as a **CalixPyr**-responsive fluorophore within an STA. The second new fluorophore is the non-responsive reference fluorophore **cCy5** with 665 nm emission. The chemical structure of **cCy5** has a spatially wide distribution of opposite charges and a net positive charge, thus we hypothesized that it would exhibit negligible affinity for **CalixPyr**. We have confirmed both hypotheses by conducting fluorescence and NMR titration studies that show **CalixPyr** can selectively complex and quench **CMP** in the presence of **cCy5**. Moreover, we find that competitive polyphosphate binding to **CalixPyr** (Scheme 1c) selectively modulates the **CMP** fluorescence signal and enables us to exploit this three-molecule mixture (**CMP**, **CalixPyr**, **cCy5**) as a fluorescent ratiometric STA (Scheme 1d) for two separate enzymes that remove phosphate groups from polyphosphate molecules, specifically the enzymatic conversion of ATP to adenosine, and the enzymatic conversion of phytate (Phyt) to *myo*-inositol-phosphate$_{1-5}$ (mIP$_{1-5}$) (Scheme 1b).

Results and discussion

Molecular design and compound synthesis

CMP, **CalixPyr**, and **cCy5** were synthesized by adapting literature procedures as outlined in the ESI.† It is worth emphasizing that **CalixPyr** is prepared in one step from commercially available 2-bromomethyl pyridine and that it exhibits very low cell toxicity.[26] The fluorophore **CMP** is a member of the Cascade Blue family of pyrene fluorophores which are known to be very bright and photostable.[27] **CMP** is tetra-anionic at neutral pH, it can be excited by a common 405 nm diode laser and its fluorescence spectrum with 430 nm maxima is invariant above pH 4.[25] **cCy5** is a deep-red pentamethine cyanine dye with absorbance and emission maxima peaks at 644 nm and 665 nm respectively.

CalixPyr affinity and selectivity for **CMP** was measured by conducting a series of fluorescence titration experiments that added aliquots of **CalixPyr** to separate solutions of **CMP** and monitoring the decrease in **CMP** fluorescence intensity at 430 nm. Each titration isotherm fitted nicely to a 1 : 1 binding model (Fig. S1–S5†) and the derived association constants (K_a) are listed in Table 1. The K_a of 1.3×10^5 M^{-1} for the formation of **CalixPyr·CMP** in water, pH 6.8 is comparable to the reported K_a values for **CalixPyr** binding to **HTPS** or **PyTs** in water.[20–22] The measured K_a values were somewhat higher in biological buffers, (Table 1) but there was a substantial decrease, due to electrostatic screening, when a high concentration of potassium phosphate was used as the buffer.[28]

Table 1 Association constants for formation of **CalixPyr·CMP** in different buffers

Buffer	Association constant (K_a)	pH
Water	$(1.3 \pm 0.1) \times 10^5$ M^{-1}	6.8
10 mM NaOAc	$(1.3 \pm 0.2) \times 10^6$ M^{-1}	7.2
5 mM TES	$(3.6 \pm 0.8) \times 10^6$ M^{-1}	7.2
10 mM HEPES	$(3.0 \pm 1.0) \times 10^6$ M^{-1}	7.0
100 mM K-Phos	$(3.1 \pm 1.8) \times 10^3$ M^{-1}	7.0

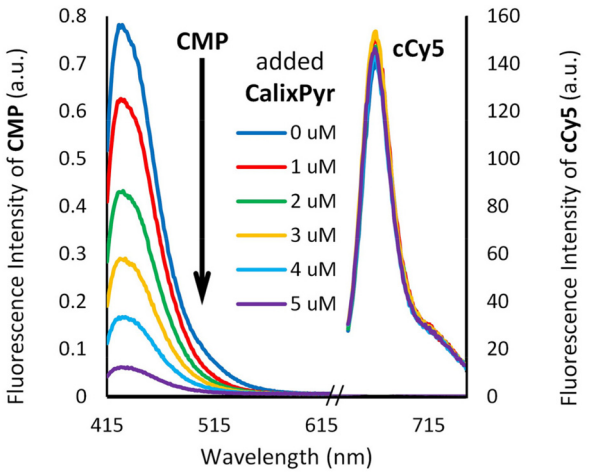

Fig. 1 Fluorescence spectra for a single solution containing a binary mixture of **CMP** (5 μM) and **cCy5** (5 μM) in water (pH 6.8) and titrated with **CalixPyr**. λ_{ex} = 401 nm (**CMP**) and 630 nm (**cCy5**), slit width = 1 nm.

The capacity of **CalixPyr** to selectively quench **CMP** in the presence of **cCy5** was demonstrated by titration experiments that added aliquots of **CalixPyr** to a solution containing a binary mixture of **CMP** and **cCy5**. As shown by the fluorescence spectra in Fig. 1 the titration produced selective quenching of **CMP** fluorescence intensity at 430 nm and negligible change in **cCy5** fluorescence at 665 nm.

^{1}H NMR titration

^{1}H NMR spectroscopy provided confirmation of **CalixPyr** binding selectivity for **CMP** over **cCy5**. Shown in Fig. 2 are comparisons of NMR spectra for binary mixtures of **CalixPyr** with **CMP** or with **cCy5**. The spectra in Fig. 2a show that mixing **CalixPyr** with **cCy5** at a 1 : 1 ratio produces no change in chemical shift for any of the peaks, strongly indicating no measurable interaction. In contrast, the spectra in Fig. 2b clearly show that mixing **CalixPyr** with **CMP** at a 0.5 : 1 ratio produces large up field changes in chemical shift for both species, indicating formation of a **CalixPyr·CMP** complex that is in rapid exchange with the free molecular species (see expanded NMR spectra in Fig. S7 and S8†). When the **CalixPyr·CMP** ratio was increased to 1 : 1 (1 mM each compound) there was substantial broadening of the peaks and formation of yellow aggregates (Fig. S6†) seen by the naked eye. Complex precipitation is not surprising since a 1 : 1 complex of tetra-cationic **CalixPyr** and tetra-cationic **CMP** has a net neutral charge and a moderately hydrophobic aromatic surface.

CMP/CalixPyr fluorescence selectivity for ATP

The association constant between **CMP** and **CalixPyr** was determined to be 1.3×10^6 M^{-1} at pH 7.2 in 10 mM NaOAc solution (Table 1). Previous studies using the same buffer have found that tetra-cationic **CalixPyr** has affinity for adenine nucleotides in the order ATP (5.0×10^4 M^{-1}) > ADP (1.4×10^4 M^{-1}) > AMP (4.8×10^2 M^{-1}) which reflects the differences in nucleotide anionic charge. This trend suggested to us that the complementary pair of **CMP** and **CalixPyr** can be used to create an ATP-selective indicator displacement assay.[23] Experimental confirmation was gained by conducting titration experiments that added ATP, ADP, or AMP to separate solutions containing **CMP** (1 μM), **cCy5** (1 μM), and **CalixPyr** (4 μM) in 10 mM NaOAc solution at pH 7.2. The titration isotherms in Fig. 3 show that the order of fluorescence response (*i.e.*, increase in **CMP** fluorescence intensity at 430 nm) was ATP > ADP > AMP.

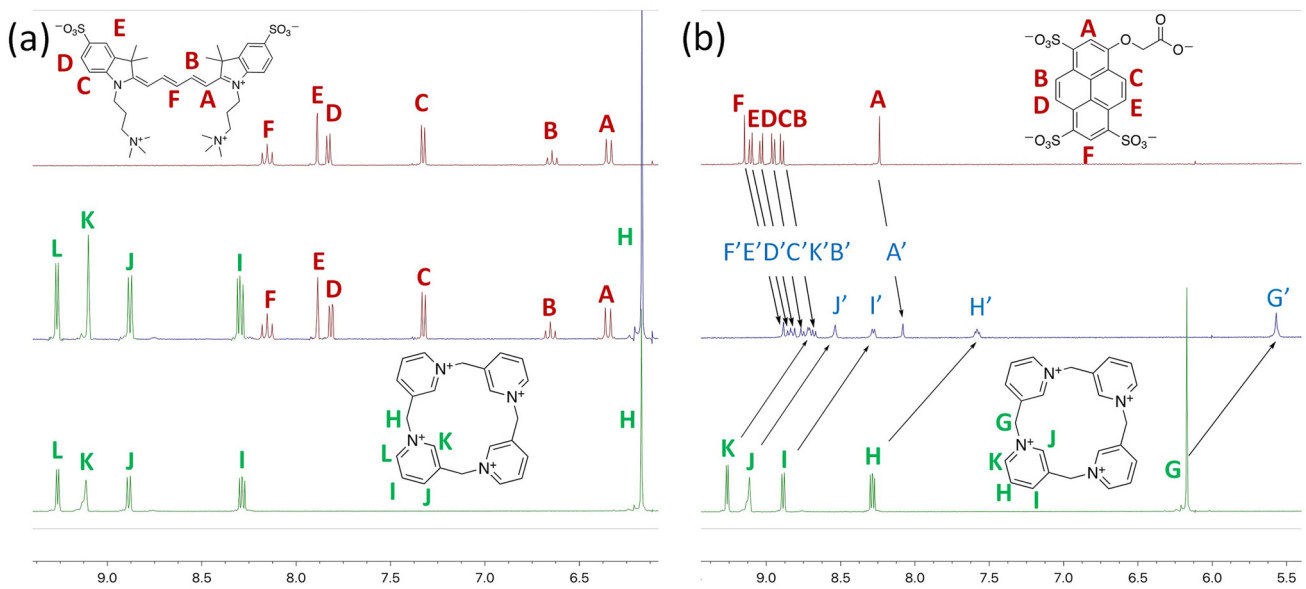

Fig. 2 (a) Partial ^{1}H NMR spectrum (500 MHz, D_2O, pD 6.63, 25 °C) of **cCy5** [1 mM] (top), **CalixPyr** [1 mM] + **cCy5** [1 mM] (middle), and **CalixPyr** [1 mM] (bottom). (b) Partial ^{1}H NMR spectrum (500 MHz, D_2O, pD 6.63, 25 °C) of **CMP** [1 mM] (top), **CalixPyr** [0.5 mM] + **CMP** [1 mM] (middle), and **CalixPyr** [1 mM] (bottom).

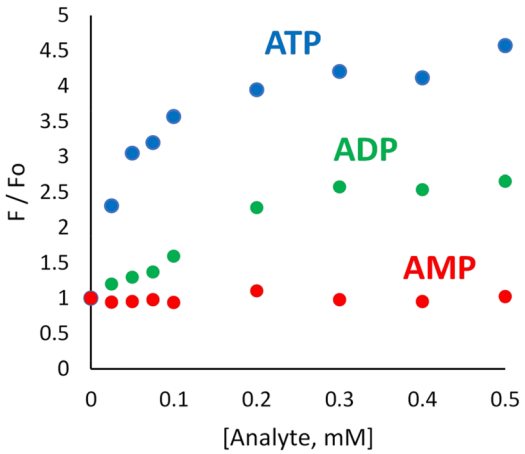

Fig. 3 Change in fluorescence intensity (F/F_0) for **CMP** (λ_{ex} = 401 nm, λ_{em} = 430 nm) upon incremental addition of ATP, ADP, or AMP to separate solutions containing **CMP** (1 μM), **cCy5** (1 μM) and **CalixPyr** (4 μM) in 10 mM NaOAc solution, pH 7.2, room temperature.

Moreover, the titrations induced no change in the **cCy5** fluorescence intensity (Fig. S12†) indicating that the ratio of the two fluorescence signals (*i.e.*, **cCy5**/**CMP**) could be monitored as a ratiometric response. In the following sections, we describe how this three-molecule mixture (**CMP**, **CalixPyr**, **cCy5**) was developed as a fluorescent ratiometric STA for two different phosphate hydrolase enzymes (Scheme 1d).

Alkaline phosphatase assay

We first used the three-molecule mixture (**CMP**, **CalixPyr**, **cCy5**) to create an STA for alkaline phosphatase (AP), a ubiquitous enzyme that can hydrolyse ATP.[23] High levels of alkaline phosphatase in clinical fluids such as serum are indicators of health problems such as Pegat's disease, breast and prostate cancer, vitamin D deficiency, or liver damage.[29] Robust high throughput assays of AP activity are also needed in drug discovery programs that aim to identify and characterize AP inhibitors.[30] The literature contains many fluorescence assays that track consumption of ATP but only a small fraction of them produce a ratiometric response.[4,31–37] In Fig. 4a is a schematic summary of our continuous ratiometric AP assay. At time zero, **CalixPyr** is bound by polyanionic ATP and thus cannot bind and quench the fluorescence of **CMP**. Addition of AP catalyses sequential removal of phosphate groups from the ATP to eventually produce adenosine. Consumption of ATP enables the **CalixPyr** to selectively bind **CMP** and quench its fluorescence signal at 430 nm and not the **cCy5** signal at 665 nm (Fig. 4b and S13†). The data is replotted in Fig. 4c as a time-dependent change in the ratio of fluorescence intensities, F_{cCy5}/F_{CMP}.

Phytase assay

The AP assay described above was conducted in a buffer of 10 mM NaOAc solution at pH 7.2. Most likely at this pH, the assay could have successfully employed the literature fluo-

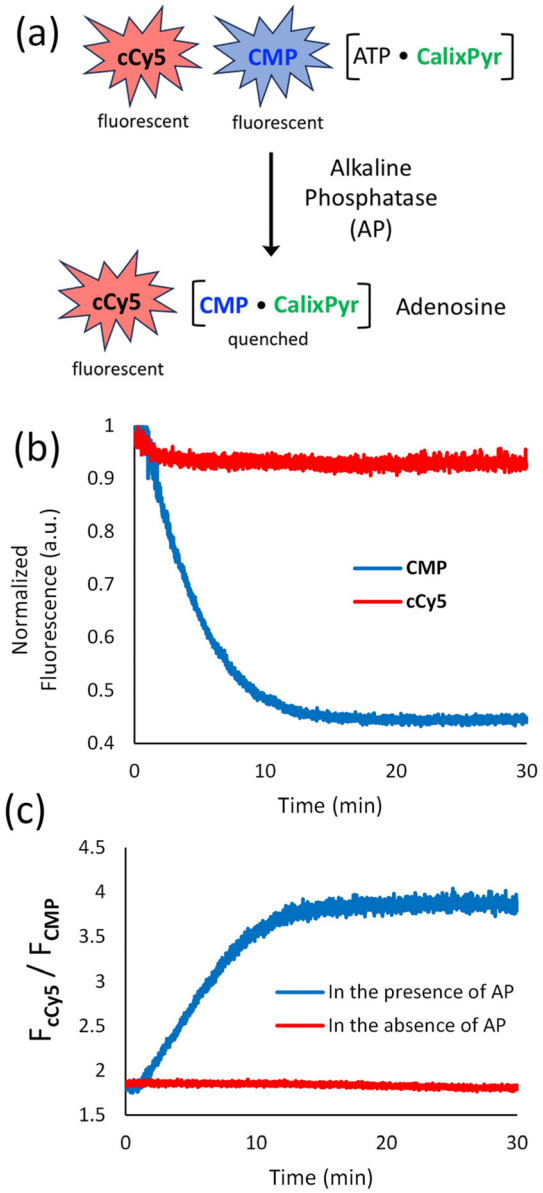

Fig. 4 (a) Summary of continuous ratiometric fluorescent assay that monitors consumption of ATP catalysed by AP. (b) Typical data set showing change in normalized **CMP** and **cCy5** fluorescence after addition of AP (1.5 U mL^{-1}) at 1 min, also in the assay mixture was **CalixPyr** (4 μM), **CMP** (1 μM), **cCy5** (1 μM), ATP (60 μM), 10 mM NaOAc, pH 7.2, 37 °C. (c) Same assay data set plotted as the ratio of fluorescence intensities, F_{cCy5}/F_{CMP}, in the presence and absence of AP, (**CMP**, λ_{ex} = 401 nm, λ_{em} 430 nm; **cCy5**, λ_{ex} = 630 nm, λ_{em} 665 nm).

rescent dye **HTPS** instead of **CMP**.[20] But the pH sensitivity of **HTPS** (pK_a ***~ 7.3) prevents its use in the second STA we developed, which measures the activity of the enzyme phytase in weak acid.

Phytate (Phyt, Scheme 1b) is the conjugate base of phytic acid, and it is stored within plant seeds as salts with the phosphate groups strongly chelating biologically important minerals such as calcium, iron, or zinc. While Phyt has high nutritional value, it is not readily digested by humans or monogas-

tric livestock such as pigs and poultry.[38] Not only is there the problem of untapped nutritional potential, but the excreted Phyt leads to elevated phosphorus concentrations in manure and contributes to surface water eutrophication.[39] Phytases are a group of phosphate hydrolase enzymes that remove phosphate groups from Phyt, and they are widely employed as feed supplements to enhance Phyt nutrition and to reduce phosphate discharge into the environment.[39–41] Phytase catalyses sequential hydrolytic removal of phosphate groups from Phyt leading to the formation of lower *myo*-inositol-phosphates$_{1–5}$ (mIP$_{1–5}$, Scheme 1b) which reduces metal-binding affinity and enhances mineral bioavailability. The nutritional and environmental benefits of phytase in animal feed and agricultural produce provides substantial motivation to develop rapid and accurate analytical methods to quantify phytase content in individual batches of whole grain and legume feedstocks.[42–44] The most commonly employed analytical methods measure the amount of released inorganic phosphate but sometimes this does not produce a sufficiently accurate assessment of actual phytase activity. Recently, discontinuous methods that measure loss of Phyt have been developed based on ion chromatography or lateral flow strips, but they are resource intensive and likely to be costly on a large scale.[45,46] There are surprisingly few fluorescent sensing systems that report Phyt levels,[47–50] and there appears to be no published example of a ratiometric fluorescent assay for phytase activity.[51–53]

To demonstrate proof of concept we developed the continuous ratiometric phytase assay that is summarized in Fig. 5a. A series of titration experiments proved that Phyt could bind to **CalixPyr** under acidic conditions (pH 5.1, 5 mM HEPES buffer) and prevent quenching of **CMP** (Fig. S9†). A competitive titration experiment (Fig. S10 and S11†) determined the association constant between Phyt and **CalixPyr** to be 2.0×10^6 M^{-1} at pH 5.1 and an additional fluorescence experiment revealed that Phyt has no effect on **cCy5** fluorescence intensity. This suggested that the STA in Fig. 5a was feasible. At assay time zero, **CalixPyr** is bound by polyanionic Phyt and cannot bind and quench the fluorescence of **CMP**. Addition of phytase induces sequential removal of phosphate groups from the Phyt and production of mIP$_{1–5}$. The consumption of Phyt allows the **CalixPyr** to bind and selectively quench the **CMP** signal at 430 nm and not the **cCy5** signal at 665 nm (Fig. 5b). The profiles in Fig. 5c show change in the ratio of fluorescence intensities, $F_{\mathbf{cCy5}}/F_{\mathbf{CMP}}$ over time. An assay sample containing 0.005 units per mL of phytase produced an observable change in ratiometric signal after ten minutes but there was no measurable response when the phytase activity was 0.0025 units per mL, thus reflecting the assay's limit of detection. An unusual feature of the assay response profile is the initial lag phase, and this kinetic feature is attributed to the multistep chemical and supramolecular process. When the assay begins, all the inositol molecules in the sample are Phyt which has a −6 charge and binds to the tetra-cationic **CalixPyr**. As the Phyt phosphate groups are removed by phytase catalysed hydrolysis there is sequential conversion of highly anionic mIP$_{1–5}$ species to less anionic mIP$_{1–5}$ species that have reduced affinity for

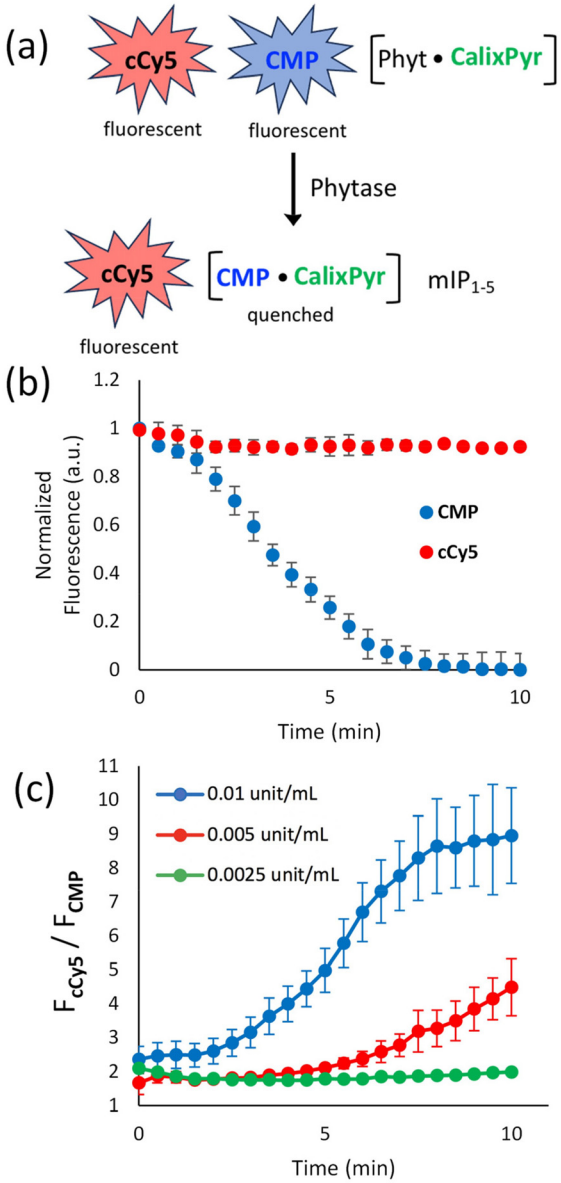

Fig. 5 (a) Summary of continuous ratiometric fluorescent assay that monitors consumption of Phyt catalysed by phytase. (b) Typical data set showing change in normalized **CMP** and **cCy5** fluorescence after addition of phytase (0.01 U mL^{-1}) at time zero the assay mixture contained **CalixPyr** (4 μM), **CMP** (1 μM), **cCy5** (1 μM), and phytate (100 μM) in 5 mM HEPES buffer, pH 5.1 at 45 °C. (c) Ratio of fluorescence intensities, $F_{\mathbf{cCy5}}/F_{\mathbf{CMP}}$, over time for samples containing different of phytase levels, (**CMP**, λ_{ex} = 401 nm, λ_{em} 430 nm; **cCy5**, λ_{ex} = 630 nm, λ_{em} 665 nm).

CalixPyr.‡ After about two minutes (when phytase is 0.01 unit per mL) **CalixPyr** binding of tetra-anionic **CMP** becomes favourable and there is an increasing amount of **CMP** fluorescence quenching.

‡The affinity of **CalixPyr** for the different mIP$_{1–5}$ species is not known, and it is not clear how quickly affinity decreases as the mIP$_{1–5}$ species become less anionic. Most likely, the assay lag time could be reduced by optimizing the concentration of assay components.

Conclusions

Fluorescent ratiometric supramolecular tandem assays for phosphatase and phytase enzymes were developed using the three-molecule mixture (**CMP**, **CalixPyr**, **cCy5**). The common supramolecular features of each assay are: (a) tetra-cationic **CalixPyr** can bind and quench the polyanionic pyrene derivative **CMP** which exhibits a pH-insensitive emission at 430 nm. (b) Polyphosphates can disrupt the **CMP**/**CalixPyr** interaction and alter the fluorescence intensity (responsive signal). (c) **CalixPyr** has no effect on the fluorescence emission of cationic **cCy5** at 665 nm and so it can be used as a non-responsive reference signal. Demonstration of utility was first gained by developing a continuous ratiometric fluorescent assay for alkaline phosphatase (AP) catalysed hydrolysis of ATP. A more innovative and impactful result is development of a continuous ratiometric fluorescent assay that monitors consumption of Phyt catalysed by phytase. It is likely that this three-molecule mixture can be deployed as a ratiometric supramolecular tandem assay for other enzymes that catalyse addition/removal of phosphate groups from appropriate molecular substrates.[4,31–37]

Experimental

Polyphosphates, reagents and solvents were purchased from Sigma-Aldrich, BioChemika, VWR, Oakwood, Thermo Fisher, Ambeed or TCI and used without further purification unless stated otherwise. Calf intestinal alkaline phosphatase was purchased from Invitrogen, catalogue number: 18009019. Phytase from wheat was purchased from Sigma-Aldrich, catalogue number: P1259. The reagent solutions were freshly prepared daily. The pH and pD values were verified on a Mettler Toledo Benchtop F20 pH mV^{-1} Standard Kit pH meter calibrated with three standard buffer solutions. The pH readings were converted to pD by adding 0.4 units.

Synthesis and characterization

All synthetic procedures and compound characterization are provided in the ESI.†

Titration methods

Direct titrations measured the association of **CMP** and **CalixPyr** in different aqueous solutions. A solution of **CMP** (1 mL) was placed in a cuvette and aliquots of **CalixPyr** solution were added to the **CMP** solution, keeping the [**CMP**] constant. Spectral changes were recorded after each aliquot addition. A plot of fluorescence intensity for **CMP** at 430 nm was fitted to a 1 : 1 binding model, as described in the ESI.†

The association of Phyt and **CalixPyr** was measured by a competitive titration method. A solution of **CMP** and **CalixPyr** (1 : 4 ratio, 1 mL) was placed in a cuvette and aliquots of Phyt solution were added, keeping the [**CMP**/**CalixPyr**] constant. A plot of **CMP** fluorescence intensity at 430 nm was fitted to a 1 : 1 competitive binding model, as described in the ESI.†

Enzyme cuvette studies

Separate stock solutions of **CMP**, **CalixPyr**, **cCy5**, Phyt, ATP, were prepared in ultrapure deionized water or the designated buffer. Aliquots of **CMP**, **CalixPyr**, **cCy5**, Phyt, ATP were added to cuvettes containing the designated buffer and were warmed to the appropriate temperature. AP or phytase was added and the solutions (2.0 mL) were mixed by stir bar while changes in the fluorescence spectra were measured using a spectrometer in time-scan mode. Iterative sequential time scan cycles scanned **CMP** and then **cCy5** for a total acquisition time of 2 seconds, followed by 28 seconds interval before the next data point. Enzyme assays were repeated in triplicate. These assays employed a spectrometer, but they should be amenable to a plate reader.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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References

1 A. C. Sedgwick, J. T. Brewster, T. Wu, X. Feng, S. D. Bull, X. Qian, J. L. Sessler, T. D. James, E. V. Anslyn and X. Sun, *Chem. Soc. Rev.*, 2021, **50**(1), 9–38.
2 R. N. Dsouza, A. Hennig and W. M. Nau, *Chem. – Eur. J.*, 2012, **18**(12), 3444–3459.
3 M. Nilam and A. Hennig, *RSC Adv.*, 2022, **12**(17), 10725–10748.
4 Y. C. Liu, S. Peng, L. Angelova, W. M. Nau and A. Hennig, *ChemistryOpen*, 2019, **8**(11), 1350–1354.
5 Y. L. Qi, Y. Z. Li, M. J. Tan, F. F. Yuan, N. Murthy, Y. T. Duan, H. L. Zhu and S. Y. Yang, *Coord. Chem. Rev.*, 2023, **486**, 215130.
6 M. H. Lee, J. S. Kim and J. L. Sessler, *Chem. Soc. Rev.*, 2015, **44**(13), 4185–4191.
7 S. Shinoda, M. Tadokoro, H. Tsukube, S. Shinoda and R. Arakawa, *Chem. Commun.*, 1998, **2**, 181–182.
8 K. Wang, H. X. Dou, M. M. Wang, Y. Wu, Z. H. Zhang, S. Y. Xing, B. L. Zhu and Y. X. Feng, *Chem. Commun.*, 2019, **55**(81), 12235–12238.
9 K. Wang, G. J. Gao and X. Y. Wang, *J. Mol. Liq.*, 2021, **343**, 117654.
10 K. Wang, G. J. Gao, X. Y. Wang, M. M. Wang and H. X. Dou, *Langmuir*, 2021, **37**(38), 11422–11428.
11 K. Wang, X. W. Ren, X. Y. Wang, S. Y. Xing, B. L. Zhu and C. Liu, *Langmuir*, 2019, **35**(32), 10505–10511.
12 K. Wang, M. N. Wang, Q. Q. Wang, Y. X. Feng, Y. Wu, S. Y. Xing, B. L. Zhu and Z. H. Zhang, *Tetrahedron Lett.*, 2019, **60**(52), 151357.

13 K. Wang, M. N. Wang, Q. Q. Wang, C. Liu, Y. H. Du, S. Xing and B. Zhu, *Langmuir*, 2020, **36**(37), 11161–11168.
14 K. Wang, Q. Q. Wang, M. N. Wang, Y. J. Xie, X. Qiao, S. Xing and B. Zhu, *Dyes Pigm.*, 2021, **188**, 109203.
15 K. Wang, Q. Q. Wang, M. N. Wang, S. Xing, B. Zhu and Z. H. Zhang, *Langmuir*, 2019, **35**(27), 9020–9028.
16 K. Wang, X. Y. Wang, G. J. Gao, X. W. Ren, X. Y. Cai, Q. K. Yu, S. Xing and B. Zhu, *Tetrahedron*, 2020, **76**(46), 131620.
17 K. Wang, X. J. Zhang and X. Meng, *ACS Appl. Nano Mater.*, 2022, **5**(9), 13208–13217.
18 K. Wang, S. C. Liu, G. J. Gao, S. Xing and B. Zhu, *ACS Appl. Nano Mater.*, 2023, **6**, 12248–12257.
19 K. Wang, X. Y. Wang, G. J. Gao, M. N. Wang, Y. Y. Yu, S. Xing and B. Zhu, *Langmuir*, 2022, **38**, 2145–2152.
20 S. Atilgan and E. U. Akkaya, *Tetrahedron Lett.*, 2004, **45**(50), 9269–9271.
21 K. Wang, J. H. Cui, S. Y. Xing and X. W. Ren, *Asian J. Org. Chem.*, 2017, **6**(10), 1385–1389.
22 M. H. Luo, H. X. Dou, K. Wang, Y. X. Feng, S. Y. Xing, B. L. Zhu and Y. Wu, *ChemistrySelect*, 2019, **4**(18), 5228–5234.
23 K. Wang, J. H. Cui, S. Y. Xing and H. X. Dou, *Org. Biomol. Chem.*, 2016, **14**(9), 2684–2690.
24 Y. Avnir and Y. Barenholz, *Anal. Biochem.*, 2005, **347**(1), 34–41.
25 E. A. Legenzov, N. D. A. Dirda, B. M. Hagen and J. P. Y. Kao, *PLoS One*, 2015, **10**(7), 1–18.
26 X. Yu, X. Ren, M. Wang, K. Wang and D. Zhang, *J. Inclusion Phenom. Macrocyclic Chem.*, 2021, **99**(1–2), 109–115.
27 J. E. Whitaker, R. P. Haugland, P. L. Moore, P. C. Hewitt, M. Reese and R. P. Haugland, *Anal. Biochem.*, 1991, **198**(1), 119–130.
28 J. H. Jordan, H. S. Ashbaugh, J. T. Mague and B. C. Gibb, *J. Am. Chem. Soc.*, 2021, **143**(44), 18605–18616.
29 U. Sharma, D. Pal and R. Prasad, *Indian J. Clin. Biochem.*, 2014, **29**(3), 269–278.
30 E. A. Sergienko and J. L. Millán, *Nat. Protoc.*, 2010, **5**(8), 1431–1439.
31 J. Kim, J. Oh and M. S. Han, *RSC Adv.*, 2021, **11**(17), 10375–10380.
32 S. H. Hewitt, J. Parris, R. Mailhot and S. J. Butler, *Chem. Commun.*, 2017, **53**(94), 12626–12629.
33 A. Nag and S. Das, *Isr. J. Chem.*, 2021, **61**(3–4), 169–184.
34 J. Hu and G. Li, *Chem. – Asian J.*, 2022, **17**(14), 1–11.
35 S. Wheeler, C. Breen, Y. Li, S. H. Hewitt, E. Robertson, E. A. Yates, I. L. Barsukov, D. G. Fernig and S. J. Butler, *Org. Biomol. Chem.*, 2022, **20**(3), 596–605.
36 M. L. Shipton, F. A. Jamion, S. Wheeler, A. M. Riley, F. Plasser, B. V. L. Potte and and S. J. Butler, *J. Chem. Sci.*, 2023, **14**(19), 4979.
37 L. X. Huang, Y. B. He, J. Kim, A. Sharma, Q. Y. Cao and J. S. Kim, *Chem. Commun.*, 2021, **57**(99), 13530–13533.
38 U. Schlemmer, W. Frølich, R. M. Prieto and F. Grases, *Mol. Nutr. Food Res.*, 2009, **53**, S330–S375.
39 E. Humer, C. Schwarz and K. Schedle, *J. Anim. Physiol. Anim. Nutr.*, 2015, **99**(4), 605–625.
40 C. K. Madsen and H. Brinch-Pedersen, *Int. J. Mol. Sci.*, 2020, **21**(20), 1–19.
41 X. G. Lei and J. M. Porres, *Biotechnol. Lett.*, 2003, **25**(21), 1787–1794.
42 L. Qvirist, N. G. Carlsson and T. Andlid, *J. Biol. Methods*, 2015, **2**(1), e16.
43 T. Sun, Y. Zhang, S. Wang, B. Guo, Q. Yang and H. Zhao, *Int. J. Food Sci. Technol.*, 2022, **57**, 6435–6444.
44 G. Marolt and M. Kolar, *Molecules*, 2021, **26**(1), 174.
45 A. Verma, S. Singh, L. K. Thawait, M. K. Mahatma and A. L. Singh, *J. Food Sci. Technol.*, 2022, **59**(11), 4479–4486.
46 E. C. D. Willard, A. Sundquist, V. Glitsoe, J. O. Sorbara, P. Tamez-Hidalgo, C. Heine, T. Ricker, M. Lehmann, A. Bergman, S. Etheve and C. V. Chatelle, *ACS Omega*, 2022, **7**(6), 5292–5299.
47 T. Minami and Y. Kubo, *Chem. – Asian J.*, 2010, **5**(3), 605–611.
48 Y. Xing, C. Guo, X. Guo, Y. Liu, K. Wei, M. Kang, X. Yang, M. Pei and G. Zhang, *J. Photochem. Photobiol., A*, 2023, **442**, 114797.
49 M. Lee, J. H. Moon, E. J. Jun, G. Kim, Y. U. Kwon, J. Y. Lee and J. Yoon, *Chem. Commun.*, 2014, **50**(44), 5851–5853.
50 N. Dey, *Analyst*, 2020, **145**(14), 4937–4941.
51 S. M. Farahani, M. Dadmehr, M. K. Ali, B. Korouzhdehi, M. K. Amin and M. Rajabian, *Food Chem.*, 2022, **396**, 133711.
52 D. F. Berry and K. Harich, *J. Environ. Qual.*, 2013, **42**(1), 56–64.
53 C. Dussouy, E. Dubreucq, P. Chemardin, V. Perrier, J. Abadie, H. Quiquampoix, C. Plassard and J. B. Behr, *Bioorg. Chem.*, 2021, **110**, 104810.