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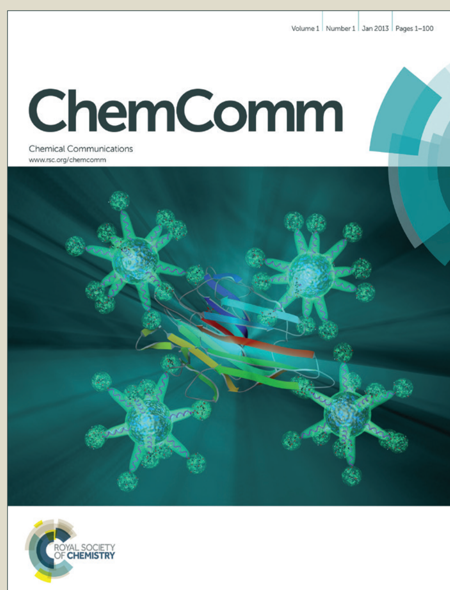

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An off-the-shelf sensing system for physiological phosphates

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An off-the-shelf supramolecular sensing system was designed to discriminate biologically relevant phosphates in neutral water using multivariate data analysis. The system is based on an indicator displacement assay comprising only two unmodified commercially available components: a dendritic poly-electrolyte and a common fluorescent dye. Effective discrimination of nucleotide diphosphates and inorganic diphosphate was achieved through principal component analysis (PCA).

Advanced data analysis methods have long been available, but much of the data collected for analytical purposes is still univariate, even though the simultaneous collection of several variables to yield multivariate data is well within the capabilities of most modern instrumentation. Multivariate analysis methods are invariably more powerful than univariate ones, thanks to the additional information contained in variable correlations which is not captured in univariate data sets.[1] Nonetheless, such techniques have been slow to percolate to the general chemical audience.

To showcase the power of multivariate techniques applied to supramolecular sensing systems, we built an extremely simple sensing system comprised of only two components: a host and a colorimetric indicator. We show here that any univariate response from the system is insufficient for analytical discrimination; however, a multivariate data set collected from the same system captures extra information that allows for discrimination.

Phosphates were chosen as our analytical target due to their significance in biological events such as DNA replication, genetic information transfer, bioenergetics and metabolism.[2] Accordingly, molecular recognition systems for the detection of phosphates have been widely sought after in past and recent years,[3] and a number of selective fluorimetric or colorimetric chemosensors have been developed for the detection of some of these anionic species.[4] However, if more than one analyte is of interest, multiple highly specialized receptors are required, whose syntheses impose a significant burden on the prospective user. An alternative method is the construction of a cross-reactive sensor array, such as those proposed by Anslyn,[5] Anzenbacher,[6] and others,[7] but the systems reported so far also rely on targeted molecular receptors; albeit simpler in structure, these receptors still represent a challenge to the method's adoption.

We present here a sensing system for physiologically relevant phosphates based on an indicator displacement assay made of just two off-the-shelf chemical components: a dendrimer and a fluorescent dye. To the best of our knowledge, this is the first reported sensing system composed of only one commercially available receptor and one indicator that can detect multiple physiologically relevant phosphates in aqueous media.

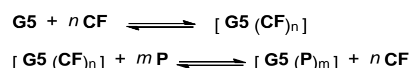

Fig. 1 Indicator displacement assay for the detection of physiologically relevant phosphates. **P** = phosphate analyte

Our system uses an indicator displacement assay based on the displacement of a dye from a host-dye complex for detection of phosphates (Fig. 1). An amine-terminated poly(amidoamine) (PAMAM) dendrimer of generation 5 with 1,2-diaminoethane core was used as the host (**G5**, see structure in ESI). These polycationic polymers are known to uptake dye molecules,[8] and phosphates.[9] In particular, a generation 5 dendrimer was the best compromise between high affinity and affordability: lower generation dendrimers have too few surface charges, and therefore too low apparent affinities for use at micromolar concentration; larger dendrimers display higher affinity for the phosphate analytes but, crucially, their selectivity is not improved over G5, and their high cost would be a significant drawback for the practicality of the proposed method. In addition, we chose 5(6)-carboxyfluorescein (**CF**, Fig. 2) as the indicator. Although the stoichiometry of the complexes involved in the assay can only be roughly estimated from the binding data (see ESI), it is worth noting that this is not a key factor in the application; for the same reason, we did not attempt to determine thermodynamic association constants.

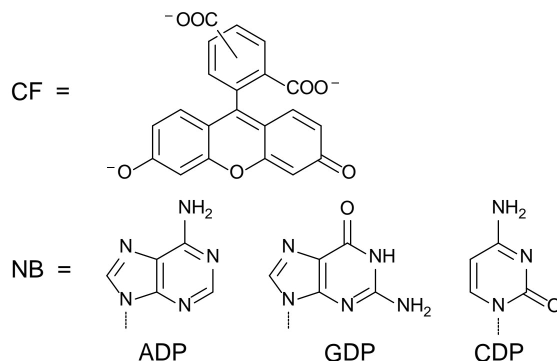

Fig. 2 Structures of the **CF** dye and nucleobases in the target phosphates.

ADP, CDP, GDP (Fig. 2) and diphosphate (PPi) were selected as targets because of their small differences in structure and similar charge state in neutral aqueous solution, making the problem of

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their differentiation particularly challenging.[10]

The fluorescence of **CF** is first quenched upon binding to **G5** to form the sensing ensemble [**G5**(**CF**)$_n$]. The response of the sensing ensemble to these anions was first verified by fluorescence spectroscopy. Representative data for PPi is shown in Fig. 3: the fluorescence is dramatically revived by the addition of aliquots of PPi to a solution of the [**G5**(**CF**)$_n$] sensing ensemble, until the emission typical of free **CF** is reached, indicating that PPi has fully displaced the indicator from the dendrimer-indicator complex. All binding events involved in the sensing process were confirmed to be fast, reversible equilibria (details on these experiments are reported in the ESI). Similar results were obtained for ADP, GDP and CDP. Relative affinities of the ensemble for the phosphate analytes can be estimated from the titration profiles in Fig 4.

Further studies were conducted on a multimode microwell plate reader that allows faster data acquisition. On a single multiwell plate, we monitored absorbance at 6 wavelengths, fluorescence intensity with 5 filter combinations, and fluorescence anisotropy measurements with 2 filter combinations, for a total of 13 collected variables, affording a large multivariate data set (details in ESI).

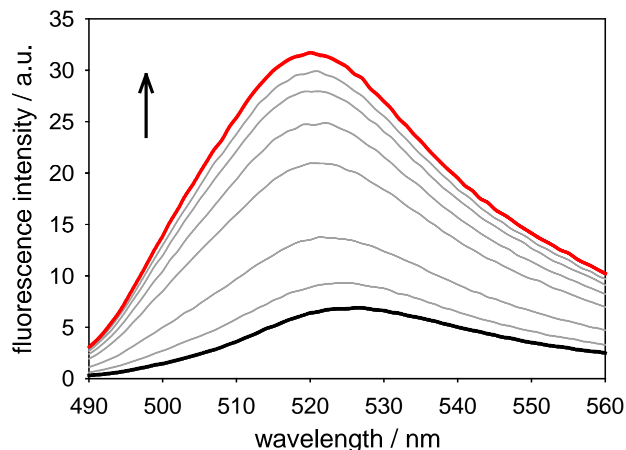

Fig. 3 Family of fluorescence spectra obtained upon addition of aliquots of pyrophosphate to the [**G5**(**CF**)$_n$] system in H_2O buffered to pH 7.4 with 50 mM HEPES. λ_{ex} = 480 nm. [**CF**] = 2.0 x 10^{-6} M, [**G5**] = 4.6 x 10^{-7} M.

All four target phosphates displaced the dye from the dendrimer-dye complex, as indicated by the fluorescence recovery behaviour in Fig. 4; the signal trends in absorbance and anisotropy also confirmed dye displacement induced by phosphates. However, the signal differences were small overall; for instance, the similar displacement behaviour displayed by PPi and ADP in the single-wavelength fluorescence intensity signal would prevent direct differentiation of these two phosphates (Fig. 4). In this system, discrimination is not possible on the basis of the univariate data.

In order to overcome this limitation, we analysed the multivariate data set through principal component analysis (PCA).[11] We used PCA to reorganize the high-dimensionality data set representing all the instrumental responses we collected. The algorithm creates a new set of variables, called *principal components* (PC); the first principal component (PC) is guaranteed to contain the largest possible fraction of the information from the original data set that can be represented as a linear combination of the original variables; higher order components carry less and less. Therefore, retaining the first few PCs only and discarding the rest greatly decreases the complexity of the dataset with minimal loss of information.

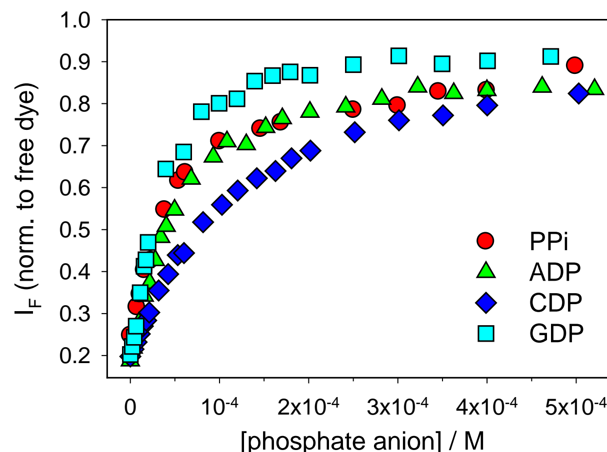

Fig. 4 Dye displacement from the [**G5**(**CF**)$_n$] sensing ensemble as a function of the molar concentration of phosphate anion (ADP, CDP, GDP and PPi). [**CF**] = 2.0 x 10^{-6} M, [**G5**] = 4.6 x 10^{-7} M in H_2O buffered to pH 7.4 with 50 mM HEPES.

In the present case, we retained the first two principal components out of the 13 present in the system (see ESI): together, these two components account for 96% of all the information available, and yet that information can be conveniently represented as a two-dimensional plot (i.e. the PCA score plot). The score plot (Fig. 5) shows clear clustering of the data; sample replicates from each of the phosphates under study were grouped close together in well-separated clusters.

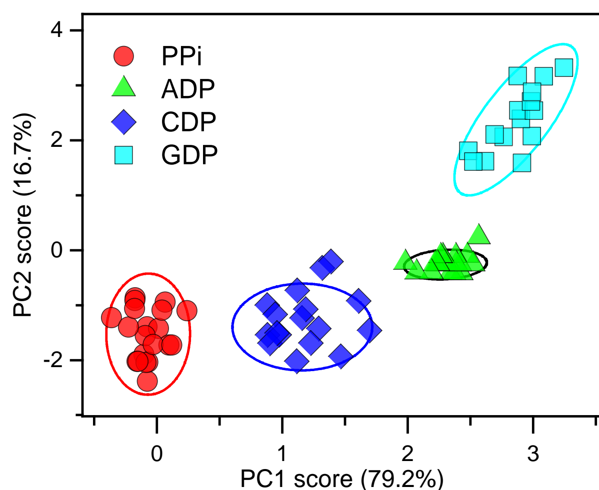

Fig. 5 Two-dimensional PCA score plot for the analysis of four phosphates (confidence ellipsoids at 99% probability). Note the tight clustering of analyte replicates, together with good inter-cluster separation.

In short, the PCA multivariate analysis achieves what the univariate approach could not, in that it is able to discriminate the four phosphates. Crucially, good cluster dispersion was achieved, giving remarkable discrimination capacity that compares well with successful pattern sensing systems reported in the literature for other applications,[12] and validating our multivariate approach.

Further features of the score plot are worth mentioning. ADP and PPi were easily discriminated by the multivariate approach, whereas they were indistinguishable on the basis of the single-variable data, as shown for instance by their overlapping

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titration profiles in Fig. 4. The relative positions of the clusters also make good "chemical sense"; for instance, the ADP and GDP replicates cluster closest together in the PCA results since both are purine-based nucleotides, a similarity that was not captured by the univariate approach.

The PCA loading plot (Fig. 6) reports on the relative contribution of the original variables (i.e. each absorbance or fluorescence "channel") to each of the two principal components that we selected.[13] This provides insight into the origin of the discriminatory power of this system. Consider, for instance, the absorbance measurements at 500-520 nm, a region of the spectrum in which the dye absorbs when bound to G5; and the anisotropy measurements, which are also sensitive to the extent of dye binding. These variables both contribute to the first principal component. Conversely, absorbance values typical of the free dye (460-470 nm) contribute to the second principal component. In other words, instrumental responses linked to the same underlying chemical process, namely the interaction of the dye with **G5**, were found to contribute to the same principal component.

Finally, we were intrigued to find that absorbance values measured at 600 nm also contributed to the discrimination, even though no significant absorption was expected at that wavelength. Upon closer inspection of the full spectra, however, we realized that scattering phenomena influence the absorbance in solution. In fact, anion binding to these dendrimers causes some aggregation because of reduced electrostatic repulsion. This effect manifests itself as a rising background throughout the absorbance spectrum, but it is particularly prominent in the measurement at 600 nm, of all the wavelengths we monitored, since no other change is expected there. Each phosphate anion induces this effect to a different extent, thus providing one more clue towards their discrimination. Such a behaviour was not at all apparent to us when we first inspected the data, nor would it have been captured in a univariate data set; in fact, it is quite unlikely that it would have even been recorded at all!

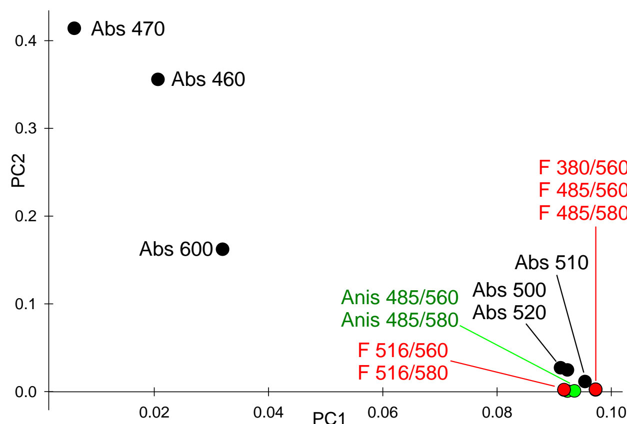

Fig. 6 PCA loading plot: relative contributions of each measured physical property to the first two principal components from the PCA analysis.

In summary, we have demonstrated an utterly simple off-the-shelf sensing ensemble, composed of one dendrimer and one indicator. Our system requires no covalent synthesis: it can be prepared by mixing two commercially available compounds of reasonably low cost and wide availability. With the help of multivariate detection, this system can discriminate multiple physiologically relevant phosphates in neutral aqueous solution, a feat typically achieved only by much more complex synthetic sensor arrays.

We are working towards the quantitative determination of phosphates, and the tackling of analyte mixtures. We also intend to extend the scope of our system to a wider gamut of physiologically relevant nucleotide phosphates (tri- and monophosphates; other nucleobases), thanks to the ease with which different hosts and indicators can be swapped in and out of the sensing ensemble.

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Notes and references

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† Electronic Supplementary Information (ESI) available: structure of PAMAM dendrimers; titration conditions; chemometrics procedures, stoichiometry, reversibility studies. See DOI: 10.1039/b000000x/

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