Electronic Supplementary Material (ESI) for Chemical Communications

An off-the-shelf sensing system for physiologically relevant phosphates

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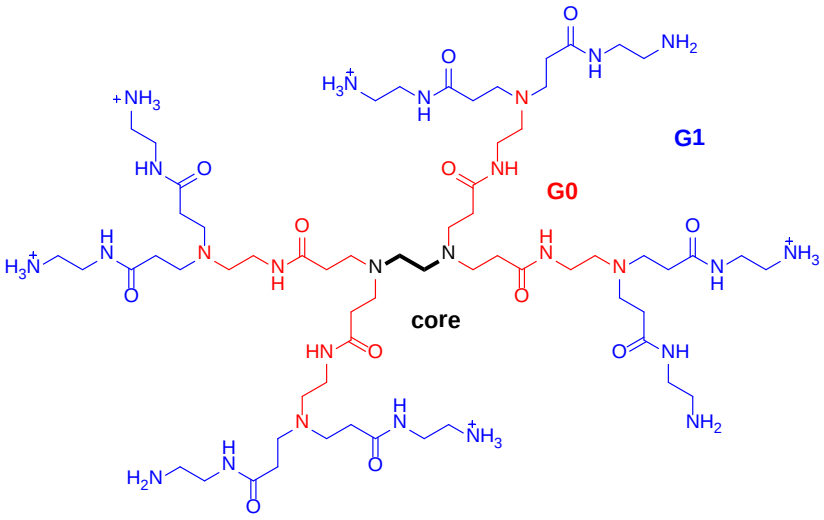
1. Materials:

Solvents, HEPES buffer (free acid), and sodium pyrophosphate were purchased from VWR. Amine-terminated PAMAM dendrimers with ethylenediamine core of generation 5 was purchased directly from the manufacturer, Dendritech Inc. and received as MeOH solution. Nucleotide phosphates (sodium salts) and 5(6)-carboxyfluorescein were purchased from Sigma. Materials were used as received without further purification.

2. Structure of PAMAM dendrimers:

In this study we used an amine-terminated poly(amidoamine) (PAMAM) dendrimer with an ethylenediamine core. The structure of a small, first generation dendrimer (G1) belonging to this family is shown to the right, with each component generation highlighted in color for clarity. A dendrimer of generation 5 would be too cumbersome to draw in its entirety, but its structure can be inferred from the one shown. A G5 PAMAM dendrimer has a total of 128 amine groups on its surface. The terminal amines in these structures have been shown to be ca. 50% protonated in water at neutral pH,[1] thus providing a number of cationic binding sites with which the **CF** dye and the phosphates can interact.



3. Instrumentation:

All the spectroscopic data presented in this paper, with the exception of the displacement titration with pyrophosphate, was acquired on a BioTek Synergy II multimode microwell plate reader, capable of measuring absorbance spectra (through a monochromator), and steady-state fluorescence intensity and polarization (through bandpass filter sets and plastic sheet polarizers). The sample compartment in this instrument is electrically thermostatted.

Experiments were laid out by hand using Eppendorf Research multichannel pipettors and disposable plastic tips into microwell plates with clear bottom for UV and fluorescence (Greiner BioOne), in 96-well configuration. The plates were made of non-treated (medium binding) polystyrene with black walls (to minimize scattered light) and clear flat bottoms. Each well invariably contained 300 µL of solution.

Displacement titrations, such as the one with pyrophosphate reported in the manuscript, were also carried out on benchtop instruments for comparison: a HP 8452a diode array UV-Vis spectrophotometer, and an ISS PC1 spectrofluorimeter equipped, monochromators for wavelength selection and calibrated manual slits for resolution control. The excitation and emission channels on this instrument are also equipped with removable computer-controlled high-aperture Glan-Thompson calcite polarizers. Excitation is provided by a 300 W high-pressure xenon arc lamp. Excitation correction is carried out through a rhodamine B quantum counter with a dedicated detector. The sample compartments are thermostatted by an external circulating water bath.

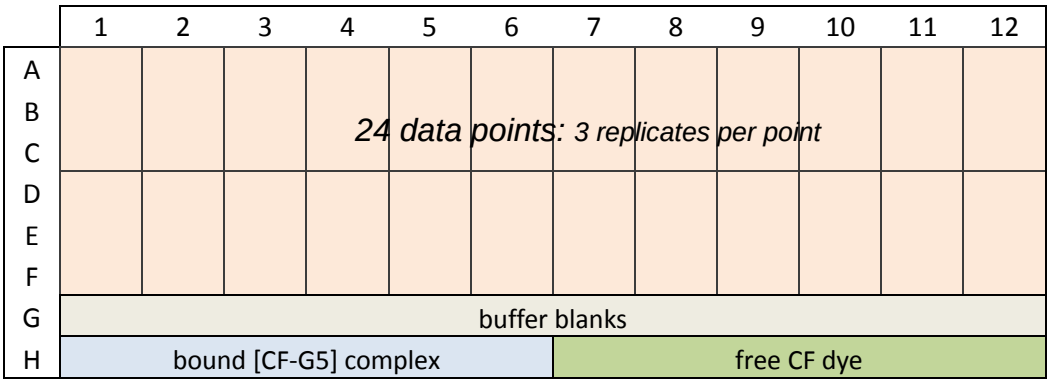
4. Experimental conditions:

All dye solutions were prepared from the same concentrated stock (6.88×10^{-4} M) across all experiments described here. The stability of this stock was checked from time to time by taking its absorbance spectrum. No degradation was observed throughout the course of the data acquisition period.

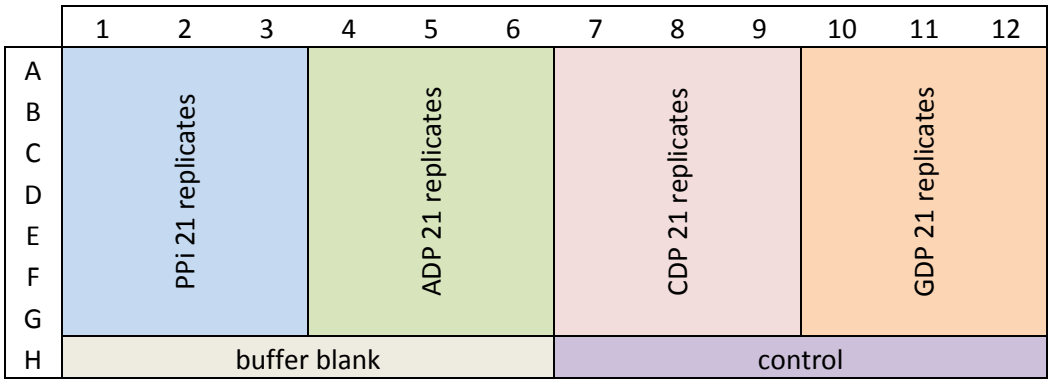
All experiments were carried out in aqueous solutions buffered to pH 7.4 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 50 mM). Experimental temperature was controlled by external circulating water bath on benchtop instruments; the plate reader was thermostatted internally to 24°C.

A new microwell plate was used for each experiment. Plates were read in a multimode plate reader immediately after preparation. Manual dispensing of solutions into each plate generally required less than half an hour; reading time typically required 30-45 minutes per plate. In that time, we did not observe any significant evaporation, so we could afford not to seal the plates, which might have impacted the sensitivity of the measurement.

For a typical displacement experiment, a 96-well plate was laid out to contain the following: 24 data points, each one in triplicate; 12 replicates of buffer (used for blanking), 6 replicates of free dye; 6 replicates of dye-dendrimer complex. Replicates were averaged after blanking. Each data point corresponded to a titration aliquot addition. The fluorophore concentration was kept constant at 2.0×10^{-6} M. The **G5** dendrimer concentration was 4.56×10^{-7} M: this concentration was chosen on the basis of our prior knowledge of the binding properties of the **CF-G5** system to provide a degree of binding around 85%. In our experience, those conditions ensure the best dynamic range for the displacement. The concentration of each phosphate analyte was varied across the plate up to a maximum concentration ensuring complete displacement.



For a typical multivariate discrimination plate, a 96-well plate was used. A full experiment was laid out on a single plate. The 96-well plates were typically laid out to contain blank replicates and dye-dendrimer complex replicates for reference; the rest of the plate was used for the four analytes of interest (PPi, ADP, CDP, GDP). Each analyte solution contained 5(6)-carboxyfluorescein dye (2.0×10^{-6} M), **G5** dendrimer (4.56×10^{-7} M), and phosphate analyte (1.8×10^{-4} M). Replicates were blanked, but not averaged, and fed directly into the data analysis routines.



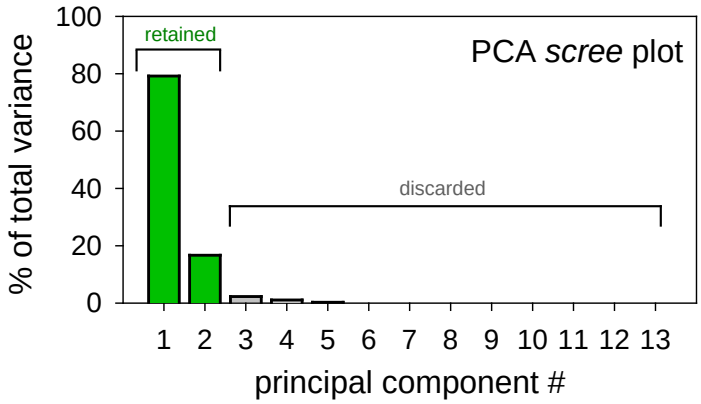
Single-wavelength absorbance readings, non-polarized fluorescence emission, and fluorescence polarization data were acquired. Steady state fluorescence intensity readings were obtained through a 485/20 nm and a 560/40 nm filter set for excitation and emission respectively. In the case of fluorescence, automatic detector gain adjustment was used, so that the highest reading from each plate reached 85% of the instrument full scale. Plastic sheet polarizers were installed in the machine for the determination of emission polarization. Both absorbance and fluorescence emission raw readings were blanked by subtracting the corresponding average reading for the wells containing buffer.

5. Multivariate analysis:

In order to evaluate the discrimination ability of the **CF-G5** sensing ensemble for phosphates, we used principal component analysis (PCA)[2] as a multivariate analysis method to evaluate the datasets. All multivariate analyses were performed as implemented in the commercial MINITAB® program (release 16 for Windows). PCA is a statistical treatment that is used for reinterpretation of a multidimensional data set into a dimensionally reduced space which encloses the most significant characteristics of the original data (*i.e.* the variance).[3]

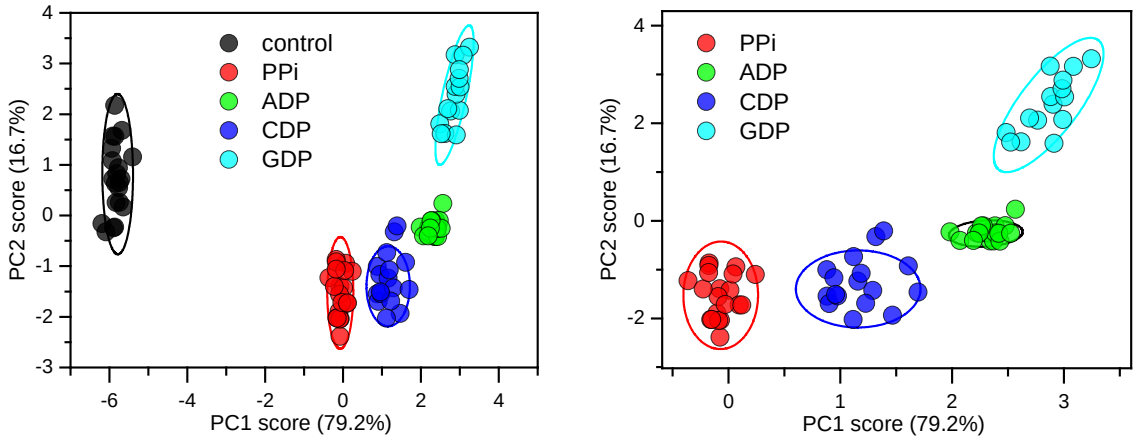
The dataset we used for multivariate analysis contains 13 instrumental variables: fluorescence intensity was collected in the following channels ($\lambda_{ex}/\lambda_{em}$): 380/560 nm, 485/580 nm, 485/560 nm, 516/560 nm, and 516/580 nm; absorbance was collected at the following wavelengths: 460, 470, 500, 510, 520 and 600 nm; fluorescence anisotropy was collected in the following channels ($\lambda_{ex}/\lambda_{em}$): 485/560 nm, 485/580 nm.

The multivariate data treatment was carried out with the help of principal component analysis (PCA). PCA is a mathematical transformation of the original data into a new coordinate system by construction of information-rich principal components from appropriate linear combinations of the original variables. The information that is represented by each principal component is displayed below in the form of a so-called PCA ***scree plot***. Information content in such a plot is reported as a percentage of the total variance present in the original data set that is represented by each principal component. Principal components are constructed in such a way that they are ordered by decreasing information content.



Inspection of the scree plot above shows that the first two components alone capture a total of 94.3% of the total variance present in the original data set. We therefore chose to retain only those two and disregard the other 11 components; in so doing, we significantly reduced the size of the dataset, from 13 to 2 dimensions, without any significant loss of information content. Conveniently, the reduced data set is now representable as a two-dimensional plot, a PCA ***score plot*** (Fig. 5 in the manuscript), in which each data point is plotted within the new coordinate system spanned by the principal components, the “scores” of each data point being its coordinates in this new system.

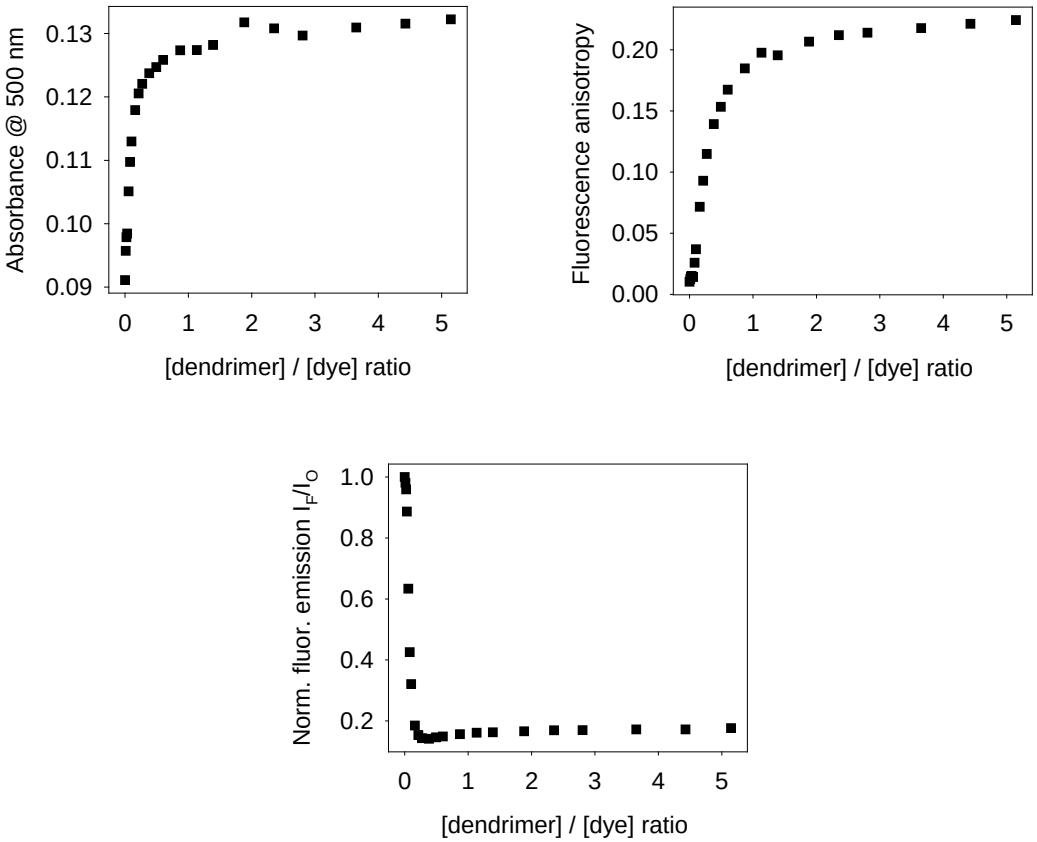
Reported below is the score plot obtained from the PCA analysis. The plot on the left includes the control data points (i.e. pure **CF-G5**, no anion present); the plot on the right excludes the control data points for the sake of clarity. PCA easily and starkly differentiates the analytes from the control.



6. Binding of the **CF** dye – an estimate of stoichiometry:

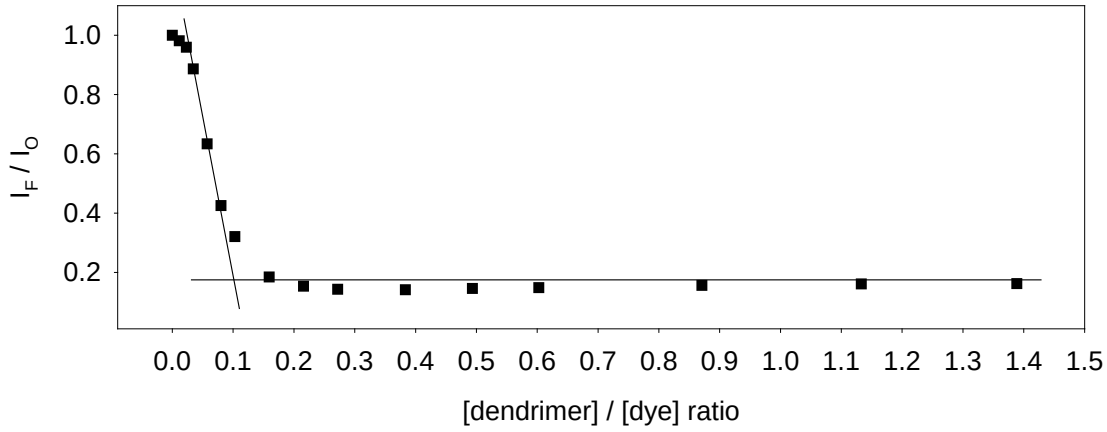
In the main text of this communication we mentioned the fact that stoichiometry determination was not a primary concern in the presented work becauase it does not impact the functionality of the system proposed. Indeed, we have not attempted a full elucidation of the complexes involved. At the micromolar working concentration used here, stoichiometry is very difficult to ascertain. In particular, the binding curves presented below do not report directly on the stoichiometry of binding. We have attempted to use mass spectrometry with little success because of complexities induced by the polycationic host and the possibility of forming multiple complexes, each with multiple charge states.

We present below a series of titration profiles obtained from the titration of a solution containing a constant concentration of **CF** dye (2.0×10^{-6} M) with aliquots of **G5** PAMAM dendrimer until signal saturation is achieved. The binding titration was carried out in a microwell plate; all three instrumental parameters were measured on the same solution.



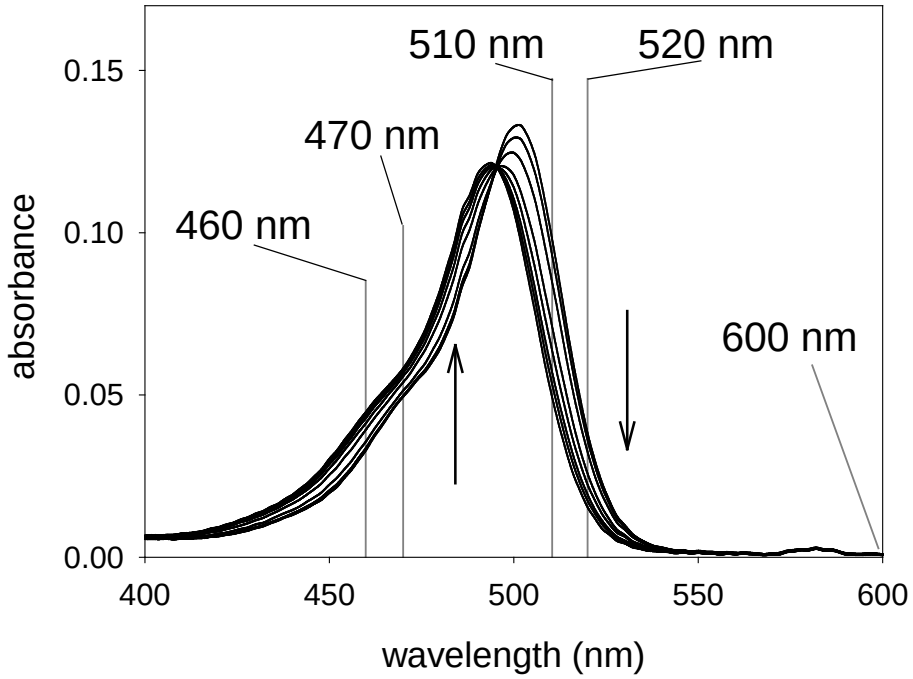
Our best estimation of the *maximum* stoichiometry of the dye-dendrimer interaction stems from the tell-tale shape of the fluorescence intensity binding titration profile shown above (see also expansion below). In fact, that profile presents a trough corresponding to the formation of the highest stoichiometry dye-dendrimer complex because of dye self-quenching through resonance energy transfer, as we reported previously.[4]

From the [dendrimer]/[dye] ratio corresponding to that point, a binding ratio close to 10 molecules of **CF** dye per **G5** dendrimer host can be estimated. Of course, however, extracting stoichiometry from such information is approximate at best.



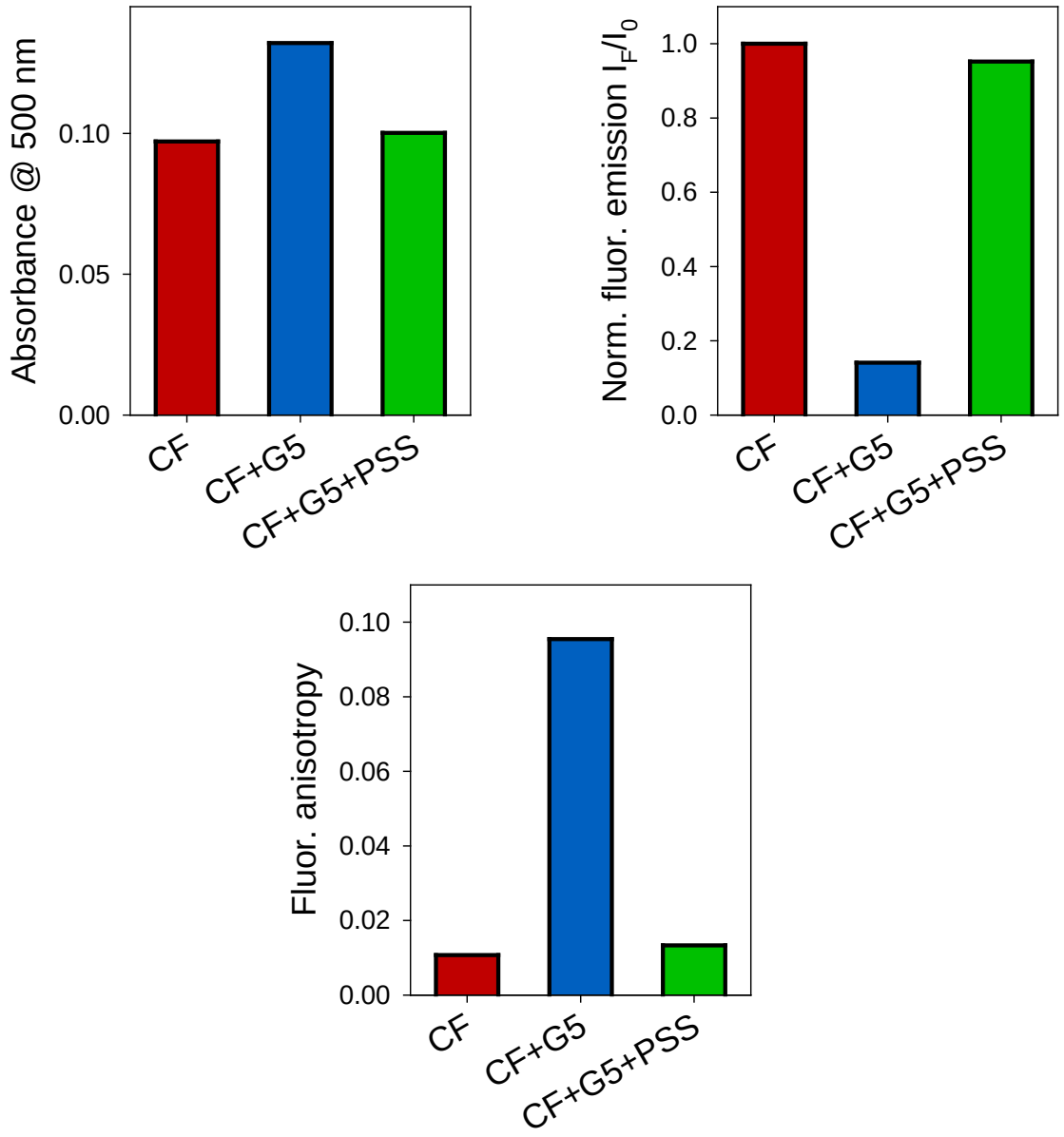
7. Spectral regions in absorbance: displacement of **CF** from **G5** with PPi

Following is a family of spectra obtained during the displacement titration of **CF** from the **CF**-**G5** complex using aliquots of a solution of pyrophosphate anion as the displacer. The wavelengths at which absorbance was monitored are indicated on the spectrum. As can be readily seen, no major component of this system absorbs significantly at 600 nm.



8. Reversibility of the dye-dendrimer binding interactions:

The water-soluble polyanionic poly(styrene sulfonate) (**PSS**) forms insoluble complexes with the cationic PAMAM dendrimer used in this study. Addition of an excess of **PSS** to a solution of the dendrimer causes its precipitation and complete removal from the solution. We took advantage of this property to demonstrate the reversibility of the dendrimer-dye binding. We first prepared a solution of dye alone and measured its optical response (data points indicated as "CF" in the plots below). We then added enough **G5** to that solution to form the sensing ensemble [**CF•G5**] (data labeled "CF+G5" below). Finally, we added an excess of **PSS** to the latter solution, causing the precipitation and removal of **G5** ("CF+G5+PSS" below). Upon removal of **G5**, the **CF** dye clearly returns to its free state in solution, thus demonstrating the reversibility of its binding to the dendrimer.

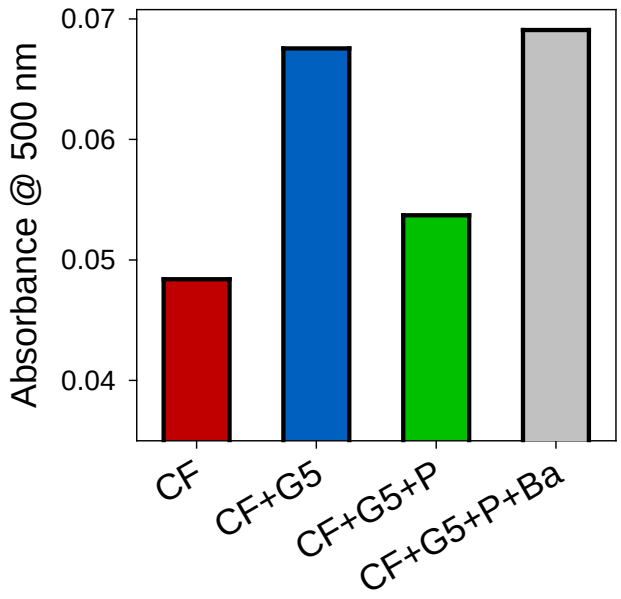


9. Reversibility of the phosphate-dendrimer binding interactions:

We used a similar approach to display the reversibility of the anionic phosphate – dendrimer interaction. We concentrated in particular on inorganic diphosphate as a representative example, monitored by absorbance measurements. In this case, we took advantage of the extremely low solubility of the barium(II) salt of this anion in neutral water (8.820 mg / 100 g H_2O, K_{sp} = 7.6 x 10^{-12} M^{-3}).[5]

We first prepared a solution of dye alone and measured its optical response (data points indicated as "CF" in the plot below). We then added enough **G5** to that solution to form the sensing ensemble [**CF•G5**] (data labeled "CF+G5" below). To this solution we then added enough pyrophosphate (80x [**CF**]) to cause the complete displacement of **CF** from its dendrimer complex (data labeled "CF+G5+P" below).

Finally, we added to the latter solution a large excess of $BaCl_2$ (1800x [pyrophosphate]) as an aqueous solution, causing the quantitative precipitation of $Ba_2P_2O_7$ and the complete removal of inorganic diphosphate from that solution ("CF+G5+P+Ba" below). Upon removal of pyrophosphate, the **CF** dye clearly returns to its fully bound state in solution, thus demonstrating the reversibility of the overall sensing system.



10. References

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[2] (a) J. W. Gardner and P. N. Bartlett, *Electronic Noses: Principles and Applications*; Oxford University Press, New York, 1999.
(b) K. J. Albert, N. S. Lewis, C. L. Schauer, G. A. Sotzing, S. E. Stitzel, T. P. Vaid and D. R. Walt, *Chem. Rev*., 2000, **100**, 2595−2626.

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[4] M. Bonizzoni, S. R. Long, C. Rainwater and E. V. Anslyn, *J. Org. Chem.*, 2012, **77**, 1258–1266.

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