VIP Analytical Methods

DOI: 10.1002/anie.200702611

Rational Design of a Fluorescence-Turn-On Sensor Array for Phosphates in Blood Serum**

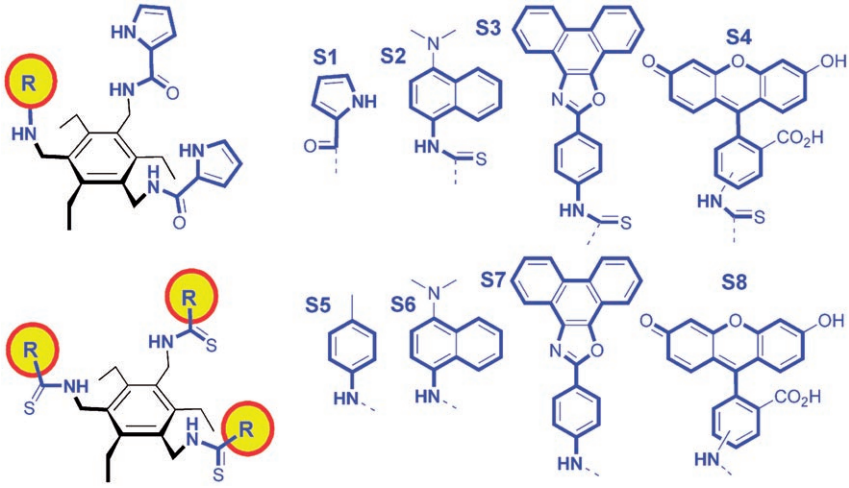
*Grigory V. Zyryanov, Manuel A. Palacios, and Pavel Anzenbacher, Jr.**

In memory of Dmitry M. Rudkevich

Phosphate anions, both inorganic and organic, hold a unique position in nature, as they take part in almost all metabolic processes. Nucleotide phosphates such as AMP or ATP are important for their role in bioenergetics, metabolism, and transfer of genetic information.[1] Phosphates in biological liquids are often present in high concentrations and may be utilized in the diagnosis of certain diseases.[2] Human serum contains 0.80–1.45 mM phosphate;[2b] higher phosphate levels are directly connected to cardiovascular disease and acute renal failure.[2,3] Similarly, ATP is present in resting muscle at a concentration of 4 mM, and in erythrocytes at a concentration of just under 2 mM.[4]

Numerous materials have been developed to sense phosphate ions in water at biological pH values.[5] The sensors based on anion-induced enhancement of intrinsic fluorescence (turn-on) are attractive as they offer the potential for high sensitivity[6,7] and, unlike the displacement assays,[8] are instantly reversible. Alas, few fluorescence-turn-on sensors exist for phosphate that function in electrolyte solutions.[9] To the best of our knowledge, fluorescence-turn-on sensors capable of sensing phosphate ions in complex biological milieu, such as blood serum, have not yet been developed.

Recently, we demonstrated that simple anion sensors based on hydrogen-bonding interactions can bind and sense anions in hydrophilic polymer matrices, despite their inability to bind anions in water.[10,11] This method combined with the sensitivity of the fluorescence-turn-on signaling could allow for the sensing of phosphate ions in aqueous environments, including biological milieu such as blood serum.[12] Here, we present tripodal[13] sensor molecules **S1**–**S8** (Scheme 1) based on 1,3,5-triaminomethyl-2,4,6-triethylbenzene[14] which can form arrays of six hydrogen-bond donors.



Scheme 1. The sensor molecules **S1**–**S8** utilize six hydrogen-bond donors to form the sensor–anion complex.

The binding of anions by **S1**–**S8** was studied in solution by ^{1}H NMR and fluorescence titrations, as well as in the solid state. The ^{1}H NMR titrations at 22 °C and −80 °C showed a concerted downfield shift of the NH resonances, which suggests the formation of a 1:1 complex with C_3 symmetry, in which all three receptor arms are equally involved in the formation of the complex. These results confirm that **S1**–**S8** were not deprotonated by the anions and that the observed increase in fluorescence is due to anion–receptor association. The fluorescence amplification suggests that the binding of the anions to the thiourea moieties results in restriction of their conformational freedom, thus improving the electronic coupling between the chromophore and the hydrogen donors while limiting the rotational and vibrational modes, which would otherwise result in nonradiative decay without strong fluorescence output.[6] These structural features result in an up to 200-fold increase in the fluorescence intensity for phosphate, pyrophosphate, and acetate ions (Figure 1). The anion-binding affinities of **S2**–**S4** and **S6**–**S8** follow the general order: $H_2P^- > HPP^{3-} > AcO^- \gg Cl^- > Br^-$, which also corresponds to the relative degree of amplification of

[*] G. V. Zyryanov, M. A. Palacios, Prof. Dr. P. Anzenbacher, Jr.
Department of Chemistry and
Center for Photochemical Sciences
Bowling Green State University
Bowling Green, OH 43403 (USA)
Fax: (+1) 419-372-9809
E-mail: pavel@bgsu.edu

[**] P.A. acknowledges support from the Alfred P. Sloan Foundation, BGSU (Technology Innovations Enhancement grant), and the NSF (SENSOR no. 0330267). M.A.P. acknowledges support from the McMaster Endowment. We would like to thank Prof. Franz P. Schmidtchen for helpful discussions during the preparation of this manuscript.

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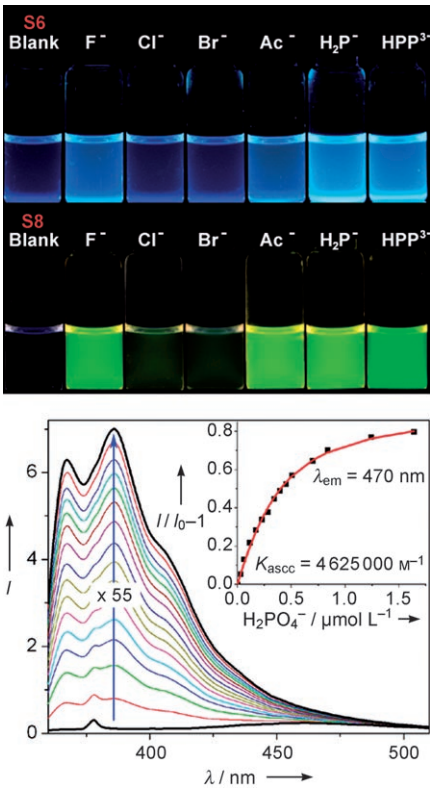

Figure 1. Top: Response of sensor molecules **S6** and **S8** to anions in solution (DMSO/H_2O 95:5). Bottom: Fluorescence amplification of **S6** (0.4 μM) in the presence of tetrabutylammonium hydrogen pyrophosphate ($TBA^+H_2PO_4^-$).

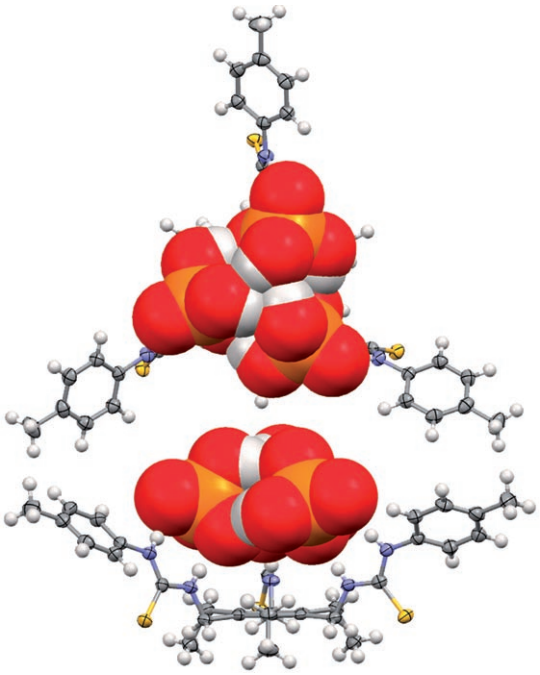

Figure 2. Top and side view of **S5**·3 TBA·3 H_2PO_4 determined by X-ray crystallography. **S5** is shown as thermal ellipsoids at the 50% probability level, while dihydrogen phosphate anions are in a space-filling form. The TBA cations have been omitted. The complex shows C_3 symmetry.

the fluorescence signal (I/I_o). Anion affinities described by association constants (M^{-1}) range between $3.8–5.0\times10^6$ for F^-, 10–100 for Cl^-, $1.0–5.0\times10^6$ for AcO^-, $2.3–5\times10^6$ for dihydrogen phosphate (H_2P^-), and $1.0–5.0\times10^6$ for hydrogen pyrophosphate ($HP_2O_7^{3-}$, HPP^{3-}) in DMSO.

The strong fluorescence-turn-on signal combined with the relatively low selectivity of **S2**–**S4** and **S6**–**S8** for any specific phosphate anion suggest that these materials may be excellent candidates for differential sensing in arrays where the individual sensors are not highly selective toward specific analytes, and where the specificity comes from recognition of the response pattern unique for the specific analyte.[15]

We presume that the binding preference for phosphates is due to the binding of three phosphate oxygen atoms by the C_3-symmetrical receptor. To investigate the **S1**–**S8** structure and anion association we performed several X-ray analyses of the receptors. Interestingly, crystals of **S5** grown from a concentrated phosphate solution revealed a C_3-symmetrical complex comprising an all-up receptor conformation together with three tightly bound phosphate anions (Figure 2). This finding suggests that these simple tripodal receptors have the unique capability to bind up to three phosphate anions, and opens up the possibility of using these sensor molecules for biologically important phosphates, including ATP.

The differences in the propensity of **S1**–**S8** to bind one phosphate anion in solution as opposed to more anions in the solid state may be due to entropic effects which prevent effective desolvation and organization of the multiple anions inside the shallow cavity of the receptor. Conversely, in the solid phase, where the receptors may be more preorganized and enthalpic contributions to binding are generally stronger, the binding of anion clusters can be observed.[16]

We realized that we could detect biological phosphate ions in an arrangement capable of emulating the solid state. We decided to construct arrays utilizing **S2**–**S4** and **S6**–**S8** embedded in hydrophilic polyurethane matrices to circumvent the fact that **S2**–**S4** and **S6**–**S8** are not soluble in water. The polymer/sensor solutions were cast onto a microtiter plate[17] (well radius: 500 ± 25 μm, depth: 230 ± 10 μm). The anions used for this purpose were fluoride, chloride, acetate, benzoate (BzO^-), phosphate, pyrophosphate, AMP, and ATP. The tests were performed in two ways: First, with the anions dissolved in water, and second with 1.0 mM anions in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) at pH 7.4. The first arrangement allows for playing out the intrinsic pH value of the salts, while in the second experiment the effect of the pH values is mitigated by the buffer. In both experiments, the films doped with **S2**–**S4** and **S6**–**S8** responded to the presence of anions by a dramatic increase in the fluorescence intensity, the degree of which is specific to a particular analyte (Figure 3). The changes in fluorescence are unique to each anion, thus enabling the identification of these anions.

The integration of the nonzero pixels in the blue and green channels (Figure 3, bottom) is compared to that before application of the sample. Multivariate analysis of the data

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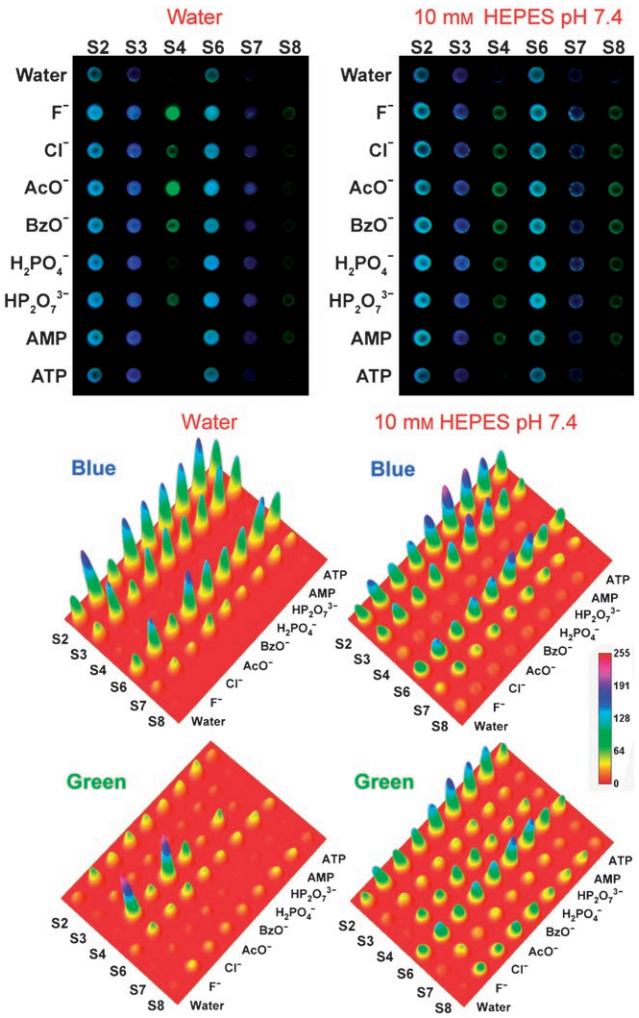

Figure 3. Top: Changes in the fluorescence intensity of the sensor–polyurethane films upon addition of aqueous solutions of anions. The anions (400 nL, 1 mM, except ATP and AMP, which were 5 mM) were added in water (left) and in 10 mM HEPES pH 7.4 (right). Bottom: The gray pixels in the blue (380–500 nm) and green (480–600 nm) channels were integrated to quantify the changes in the fluorescence in the array.

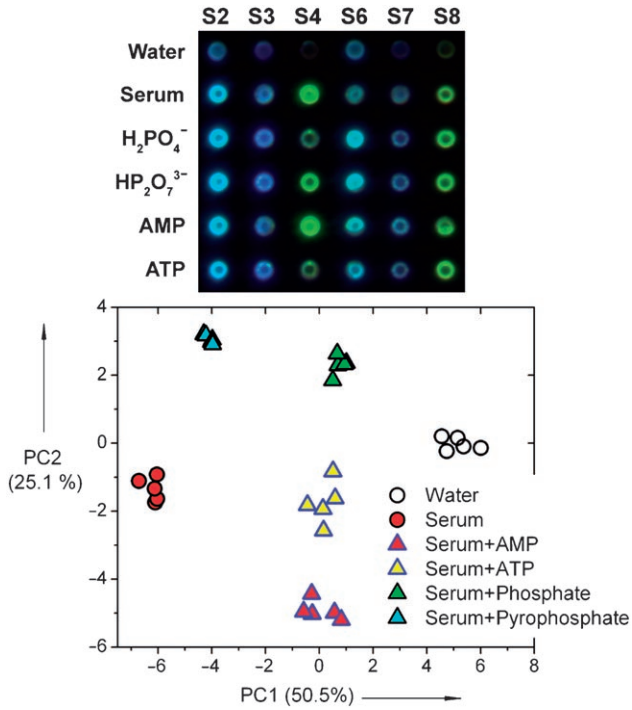

Figure 4. Top: Qualitative changes in the fluorescence of the sensor–polyurethane films after addition of human blood serum and serum with added anions (to increase the concentration to 5 mM). Bottom: PCA score plot:[18] Proximity in space on the plot directly correlates to similarities in the array responses induced by the anions added to the blood serum.

then allows for identification of the anions (Supporting information).[18]

Blood serum, with its protein and electrolyte content, will behave as a unique buffer which also contains phosphate and various carboxylate ions,[2] and is therefore likely to give a unique response. In the next array (Figure 4) we applied water (control), serum, and serum samples with added anions (phosphate, pyrophosphate, AMP, or ATP). As expected, because of its intrinsic anion content, the serum itself turned-on the fluorescence of the array, thereby creating a unique fluorescence response, which was further modulated by the added anions. Principal component analysis (PCA)[18] shows that films of **S2–S4** and **S6–S8** allow phosphate, pyrophosphate, AMP, and ATP to be distinguished. Interestingly, in human serum analysis, the nucleotide (adenine) in AMP and ATP plays an important role in generating a unique response for these anions over inorganic phosphates.

In summary, the effective sensing of anions, including biologically important phosphates, has been successfully achieved by using simple sensors embedded in polyurethane. We have shown that even simple receptors utilizing six hydrogen bonds provide turn-on sensors capable of sensing anions in aqueous media. These simple tripodal sensors are capable of accommodating various phosphate ions including AMP and ATP. Preliminary experiments suggest that sensors in polyurethane films may be used for the fabrication of turn-on arrays that will allow analysis of phosphate-type anions even in complex biological milieu, such as blood serum. The successful recognition of various biologically important phosphate ions in human blood serum suggests the potential for the development of a point-of-care method for monitoring phosphate homeostasis.

Received: June 14, 2007
Published online: August 15, 2007

Keywords: analytical methods · anions · host–guest systems · sensors · supramolecular chemistry

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