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Detecting Biologically Relevant Phosphates with Locked Salicylaldehyde Probes in Water

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This paper describes a disassembly based approach for detecting biologically relevant di- and triphosphates in water using locked fluorescent salicylaldehyde probes.

Supramolecular analytical chemistry strives for developing straightforward approaches for analyte detection in biologically relevant media such as water. Over the years, important steps forward, from purely fundamental research towards real-world application, have been observed in this relatively young research area. In fact, a plethora of indicator systems for detecting anions of biological, medicinal and environmental concern (e.g. F, CN, H$_2$PO$_4$ / HPO$_4^{2-}$) have been developed and subsequently improved in terms of sensitivity, selectivity, water compatibility and response time.

Most of these systems follow one of the three most common approaches: (i) chemosensor, (ii) chemodosimeter or (iii) indicator displacement approach (IDA). The working principle of an IDA is shown in Scheme 1. The displacement of a bound indicator from a receptor-indicator complex by a stronger coordinating analyte leads to the displacement of the indicator concomitant with a detectable signal.

Inspired by this versatile strategy (Scheme 1 top), we present herein an extended version: the disassembly approach (Scheme 1 bottom). In this method, an analyte displaces selectively a metal ion from a metal-chelate containing receptor and the demetallated probe disassembles subsequently into its molecular entities (Scheme 2; ‘Disassembly’). One of these molecular building blocks represents the signaling unit of the reaction and hence, ‘unlocking’ of the reagent leads to a detectable change of signal (Scheme 1 bottom and scheme 2).

Scheme 1. Displacement (top) and disassembly based approach (bottom).

Scheme 2. Molecular design of the Zn$^{2+}$-salen reagent (1, 2) and its disassembly to green fluorescent salicylaldehyde derivatives (5, 6) upon addition of di- and triphosphates (ATP, ADP and PPI) in aqueous media ([TRIS] = 10 mM, pH 7.4). ['The ('unlocked') free-base salen (3, 4) is detected only in organic medium (DMSO)].
fluorometric detection of biologically relevant di-, and triphosphates in water. Our reagent, a zinc salen complex, is composed of Zn\textsuperscript{II}, ethylenediamine and two salicylaldehyde subunits. Zn\textsuperscript{II} chelation stabilizes the imine functionalities of the reagent against hydrolysis in water (Scheme 2). Analyte induced metal ion displacement (‘Unlocking’; Scheme 2) is then activating the reagent for fast hydrolysis into its subunits (‘Disassembly’; Scheme 2). The ‘unlocked’ salicylaldehyde subunits produce a ‘turn-on’ green fluorescence at physiological pH and hence act as signaling units in our approach. Indeed, the probes were designed to take advantage of both the affinity of Zn\textsuperscript{II} towards phosphate ions and the instability of the unlocked, free-base salen ligand in water.

The probes (1, 2) were synthesized as described elsewhere. The probes were first tested for the detection of various phosphates and other commonly occurring anions in water at physiological pH 7.4 ([TRIS] = 10 mM). Compound 1 with two sulfonate groups was used for all the studies in water. The addition of ATP and PPI (pyrophosphate) to 1 led to a remarkable red shift of 57 nm in the absorption spectrum (Fig. 1). Some small changes were also detected upon addition of ADP. The other phosphates (e.g. H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}, AMP) did not induce any change in absorbance (Fig. 1). None of the other commonly occurring anions (e.g. F\textsuperscript{-}, Cl\textsuperscript{-}, Br\textsuperscript{-}, I\textsuperscript{-}, OAc\textsuperscript{-}, SCN\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}) showed any effect or interferences (Fig. S1 and S2, ESI). Titrations of 1 were therefore performed with both ATP and PPI showing concomitant increases in absorbance at 380 nm with a gradual decrease at 337 nm (Fig. S3, ESI).

Sensing of ATP and PPI could also be followed with fluorescence spectroscopy. The addition of ATP or PPI to 1 shifted the fluorescence emission band of 1 from 467 nm (blue) to 490 nm (green) (Fig. 1 and S4, ESI). A small change was also observed with ADP. None of the other anions showed any shift in the emission spectra of 1 (Fig. S4, ESI).

To understand the observed optical changes, absorption and emission spectra of reaction mixtures of 1 and ATP (or PPI) were compared with spectra of the free-base salen compound 3 and the salicylaldehyde derivative 5 (Fig. S5, ESI). Indeed, similarities were observed with the absorption spectrum of derivative 5, but not with compound 3 (SSa, ESI). Similarly, the emission maximum of 5 was identical with that of a mixture of 1 and ATP (or PPI) suggesting the formation of the salicylaldehyde derivative 5 and ethylenediamine during detection (SSb, ESI).

This reaction behaviour was further supported by mass spectral analysis. The spectra of the reaction mixtures displayed an ion at m/z 200.986 that is consistent with the molecular formula of the compound 5 [M-H\textsubscript{2}O]. All of these experiments indicate that ATP and PPI displace Zn\textsuperscript{II} from probe 1 generating free-base salen 3 that hydrolyses rapidly in water into its molecular entities, the salicylaldehyde derivative 5 and ethylenediamine. Job plot analysis indicated 1:1 stoichiometries for the displacement of Zn\textsuperscript{II} from 1 with PPI, ATP and ADP in water (pH 7.4; Fig S8, ESI). The experiments suggest that only one equivalent of PPI, ATP or ADP is required to strip Zn\textsuperscript{II} from its salen complex. Equilibrium constants for the displacement of Zn\textsuperscript{II} from 1 with PPI, ATP and ADP were calculated for 1:1 interaction using non-linear regression analysis (Fig S9, ESI). The equilibrium constant values (logK, 4.54±0.04 (PPI), 3.81±0.07 (ATP) and 3.29±0.1 (ADP) indicate stronger interactions between 1 and PPI or ATP compared to ADP at physiological pH.

Further insights into the mode of the reaction were obtained with \textsuperscript{1}H and \textsuperscript{31}P NMR studies. The addition of ATP to 1 in D\textsubscript{2}O showed the emergence of new peaks in the \textsuperscript{1}H NMR spectra concomitant with the gradual decrease in intensity of existing peaks (b-e) (Fig. 2).

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2 | J. Name., 2012, 00, 1-3

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With increasing addition of ATP, all the proton peaks of 1 disappeared and the new peaks (a'-e') became prevalent. A distinct signal appeared at ~10.01 ppm. This chemical shift is indicative for the proton of an aldehyde moiety (b'), and hence, supports the formation of salicylaldehyde derivative 5 (Fig. 2, line 5). The appearance of new signals at ~3.3 ppm was also observed. They were assigned to the protons of ‘unmasked’ ethylenediamine, the other product of the disassembling process.

Titrations were also performed with PPI, ADP and AMP (Fig. S10–12, ESI). Reactions with the former two anions are comparable to that with ATP. The addition of AMP to 1 did not lead to any changes in the 1H-NMR spectrum. This behaviour indicates that at least two adjacent phosphate subunits are required for demetallation of the reagent. This was further supported by 31P NMR studies of reactions between 1 and ATP, ADP and PPI (Fig. 3 and S13, ESI). In this series of reactions, maximal shifts were observed for the β- and γ-phosphorous atoms (β-P, γ-P) of ATP (~4.0 and 2.5 ppm; Fig. 3), whereas the α-P was almost not affected (~0.5 ppm). This behaviour suggests coordination of the two outer P-atoms (β- and γ-positions) to the released ZnII ion. The observation is in line with significant shifts of the α- and β-phosphorous atoms of ADP and PPI (~3.7 and 1.2 ppm; Fig. 3 and Fig. S13, ESI). No changes were observed with AMP.

All of these results strongly suggest that a bidentate chelating phosphate moiety is required for demetallating the reagent and explain nicely the observed selectivity of ATP, ADP and PPI over Pi and AMP.10 We attribute the selectivity pattern to mainly two factors: the chelating effect of di-and triphosphates as well as their higher anionic charge density of the participating O-P moieties compared to the monophosphates (Pi and AMP). These effects are also expressed in the trend of the equilibria constants of the displacement reaction (PPI>ATP>ADP).

All of these studies have unambiguously proven the disassembly of 1 upon addition of ATP, ADP and PPI into 5 and ethylenediamine in aqueous medium. However, the proposed intermediate of the reaction, the free-base salen ligand 3 was not observed under the reaction conditions. We attribute this behaviour to the fast hydrolysis of the imine functionalities of 3 in water and decided therefore to test the reaction in organic media. All of these studies were performed in organic medium (dimethyl sulfoxide, DMSO) with probe 2, a ZnII salen complex lacking the sulfonate functionalities. The absorption spectra of 2 were recorded in the presence of different phosphate oxanions and other potentially interfering anions (e.g. F-, Cl-, Br-, I-, OAc-, SCN-, and NO3-) in DMSO. In agreement with the reaction in water, immediate changes in absorption were only observed with ATP, ADP and PPI (Fig. S14, ESI). Minor changes were also observed for H3PO4 and AMP, but not for the other tested anions (Fig. S14, ESI). This time the reaction of 2 with ATP, ADP or PPI resulted into a blue shift (~40 nm) from 337 nm to 317 nm. This reaction was assigned to the formation of free-base salen 4 by comparing its UV-vis spectra to that of the pure compound (Fig S15, ESI). The displacement reaction of 2 with ATP (PPI) in DMSO was also followed by mass spectrometry suggesting the formation of the (demetallated) free-base salen compound 4 (Fig. S16-17, ESI).

Further evidence for the displacement of ZnII was given by 1H NMR studies. The titration of 2 with ATP in DMSO resulted into downfield shifts for Hz (0.2 ppm), Hα (0.8 ppm) and Hγ (0.7 ppm), whereas Hz was shifted upfield (~0.3 ppm; Fig. 4). Selective demetallation of 2 (‘unlocking’ Scheme 2) with ATP in DMSO-d6 was then unambiguously confirmed by comparing the 1H NMR spectra of a mixture of 2 and ATP and free-base salen 4 (Fig 4 and S18, ESI). Again, the two NMR spectra are almost identical. 1H NMR titrations of 2 with PPI and ADP gave similar results (Fig. S19-20, ESI). This confirms that the ZnII-containing reagent acts as an indicator for biologically relevant phosphate ions (ATP, ADP and PPI) in DMSO following a displacement approach.
is first demetallated with di-and triphosphates (ATP, ADP and PPI) to the free-base salen ligand that is then rapidly hydrolysed into its molecular subunits, salicylaldehyde and ethylenediamine.

![Scheme 3. Proposed mechanism for the displacement reaction with PPI.](image)

The new detection method convinces also with its rapid detection. Indeed, quantitative optical changes were observed instantaneously after adding ATP or PPI (10 eq.) to the reagent (1; 20 μM) (Fig. S22, ESI). Nevertheless, the current reagent still has some limitations due to its relatively low stability in buffered medium (Fig. S22, ESI). Future improvements by structural modifications of the complexes are therefore required. Metal-sal(ophen) complexes offer ample opportunities for this purpose and may also lead to new probes with altered selectivity.

The sensing of biologically relevant phosphate anions with metal complexes is common. However, only two studies reported so far on Zn-sal(ophen)-based receptors for the detection of nucleoside phosphates. These compounds combined elegantly Zn-phosphate coordination with π-π stacking or hydrogen bonding interactions for molecular recognition of adenosine di and triphosphate. To the best of our knowledge, a Zn-based reagent for detecting biologically relevant phosphates following a displacement strategy has not been reported so far.

In summary, we describe herein the disassembly approach as novel strategy for analyte detection. In particular, we introduced as proof-of-concept, a Zn-salen complex as locked fluorescent salicylaldehyde probe for ‘turn-on’ detection of biologically relevant di-and triphosphates under physiological conditions. It is anticipated that this strategy is not limited to the current examples and will find other applications in the near future.

A post-doctoral fellowship to N.K. by the ‘Forschungskredit (grant number FK-14-107)’ of the University of Zurich is gratefully acknowledged. Prof. H. J. Jessen is kindly acknowledged for a generous gift of tetrabutylammonium salts of ATP, ADP and AMP. Mr. U. Stalder and Dr. T. Fox are kindly acknowledged for their help with MS and NMR studies.

Notes and references