A fluorescent sensor for pyrophosphate based on a Pd(II) complex†

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A mixture of the Pd(II) complex [Pd(NO$_3$)$_2$(bipy)] (bipy = 2,2′-bipyridine) and the fluorescent dye Methylcalcein blue (MCB) constitutes a chemosensing ensemble which can be used for the detection of pyrophosphate (PPi) by fluorescence spectroscopy. The sensor operates in buffered aqueous solution at neutral pH, and allows sensing PPi at low to mid micromolar concentrations with very good selectivity over other anions such as phosphate, acetate, nitrate, sulfate, chloride, and bicarbonate.
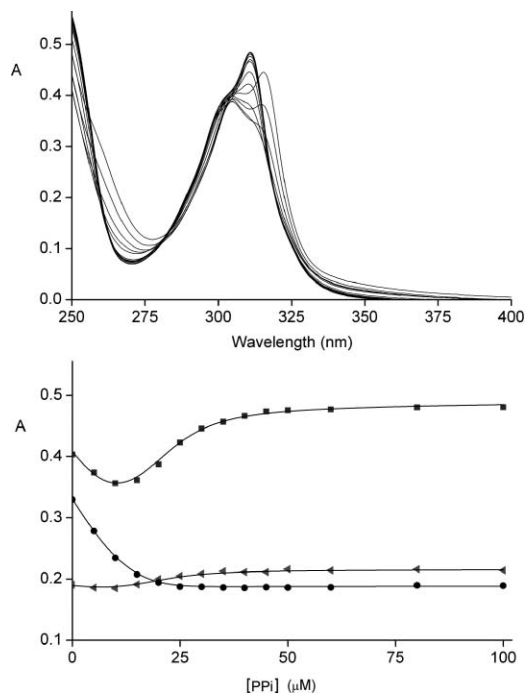
Introduction

Pyrophosphate is a biologically important anion which is involved in numerous cellular processes.[1] It is also used as an additive in food (E450), toothpaste, baking powder, and detergents.[2] Not surprisingly, considerable efforts have been made to develop chemosensors for the optical detection of PPi.[3] Most often, these sensors contain metal complexes as the recognition units. Zn(II) complexes are frequently used in this context,[4] but other metal ions such as Cu(II),[5] Cd(II),[6] Mn(II),[7] Eu(III),[8] and Tb(III)[9] have been employed as well. Some of these sensors display remarkable sensitivity and selectivity.[3] However, their preparation generally requires considerable synthetic efforts. Below we describe a Pd(II)-based chemosensor, which can be obtained by dissolving easily available compounds in buffered aqueous solution. The sensor allows the selective detection of PPi by fluorescence spectroscopy.

Results and discussion

Recently, we have reported that Pd(II) complexes can be employed as versatile recognition units in sensor arrays for peptides[10] or oligodeoxynucleotides.[11] During the course of these studies we observed that [Pd(NO$_3$)$_2$(bipy)] (**1**) displays a high affinity for PPi. This prompted us to investigate whether complex **1** could be used to create a chemosensor for this anion.

In a first set of experiments we investigated the complexation of PPi to the Pd complex in more detail. A buffered aqueous solution (25 mM HEPES buffer, pH 7.0) of complex **1** (25 μM) displays absorptions in the UV region of the spectrum with maxima at $\lambda =$ 307 and 316 nm. In the presence of increasing amounts of Na$_4$P$_2$O$_7$ (0–100 μM), the absorption at $\lambda =$ 320 nm constantly decreases, whereas the band at $\lambda =$ 310 nm first decreases and then increases (Fig. 1).

The absorption at $\lambda =$ 310 nm shows a minimum for a Pd/PPi ratio of ~ 2 : 1. This result suggests that a dinuclear complex is formed at low PPi concentrations, which is then converted into

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Fig. 1 Top: absorption spectra of solutions containing the palladium complex **1** (25 μM) and different amounts of PPi (0–100 μM). The spectra were recorded in aqueous HEPES buffer solution (25 mM, pH 7.0) after equilibration. Bottom: variation in absorbance at $\lambda =$ 290 (◄), 310 (■) and 320 nm (●). The lines represent the best fit to the model discussed in the main text.
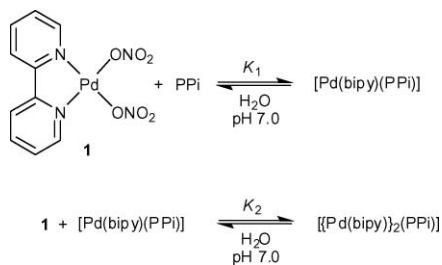
a mononuclear adduct at higher PPi concentrations (Scheme 1). Accordingly, it is possible to fit the absorption data to a model that involves 2 : 1 and 1 : 1 complexes (Fig. 1). The following binding constants were derived from the fitting procedure: $K_1 =$ 3.4(±0.3)·10^6 M^{-1} and $K_2 =$ 4.2(±0.4)·10^4 M^{-1}. Additional evidence for the formation of a dinuclear Pd–PPi complex was obtained from a Job plot (Fig. 2). The absorbance at $\lambda =$ 320 nm was recorded for solutions with different [Pd]/([Pd]+[PPi]) ratios and a maximum was found for [Pd]/([Pd]+[PPi]) ~ 0.66 (Fig. 2).

For the 1 : 1 complex, it is likely that PPi acts as a bidentate chelate ligand, replacing the two weakly bound ligands opposite to the bipy N-donor ligand.[12] For the 2 : 1 complex, single crystals were obtained by slow evaporation of an aqueous solution and its structure was unambiguously determined by X-ray diffraction
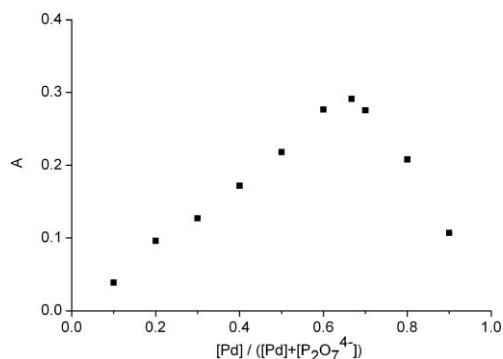
Scheme 1 Complexation of PPi to the Pd complex **1**. The formulas given for the PPi complexes indicate only the stoichiometry; charges, solvation and counter ions are neglected.

Fig. 2 Job plot for the binding between complex [Pd(NO₃)₂(bipy)] and PPi. The data were obtained by recording the absorbance at 320 nm of solutions (25 mM HEPES buffer, pH 7.0) containing different ratios of Pd complex and PPi ([Pd + P₂O₇⁴⁻]ₜₒₜₐₗ = 100 μM).

analysis. The solid-state structure of the complex is shown in Fig. 3. The structure features two Pd(bipy) units connected by a bridging μ-O,O',O'',O'''-PPi chelate ligand, similar to that observed for the dinuclear Pt(II) complex [{Pt(dach)}₂(P₂O₇)] (dach = *trans*-1,2-diaminocyclohexane).[13] The two bipy ligands are parallel and slightly off-set from one another, with a mean plane-to-plane distance of *c.a.* 3.7 Å, typical of π–π stacking interactions. Similar interactions propagate throughout the crystal, with adjacent molecules forming one-dimensional columns along the crystallographic *c*-axis. The asymmetric unit also features six water molecules, which form a network of O–H$_{water}$···O$_{PPi}$ hydrogen bonds linking adjacent columns.
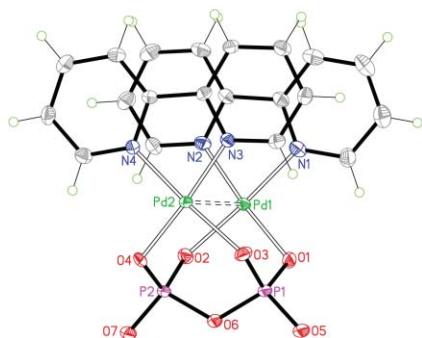

Fig. 3 Solid-state structure of [{Pd(bipy)}₂(PPi)]. Thermal ellipsoids are shown at 50% probability level. Selected bond lengths: Pd1–Pd2 3.0511(3) Å; Pd1–N1 1.997(2) Å; Pd1–N2 1.990(2) Å; Pd1–O1 2.0339(17) Å; Pd1–O2 2.0243(16) Å; Pd2–N3 1.9930(19) Å; Pd2–N4 1.9948(19) Å; Pd2–O3 2.0346(17) Å; Pd2–O4 2.0202(15) Å.

Having established that complex **1** displays high affinity for PPi in neutral aqueous solution, we were looking for a means to convert the binding of PPi into an optical signal. *A priori*, two strategies could be envisioned: (1) the covalent attachment of a chromophore or fluorophore to the bipyridyl ligand, or (2) the utilization of an indicator displacement assay (IDA).[14] The latter strategy appeared more appealing because it is typically very easy to implement (the sensor is generated *in situ* by mixing the receptor with the indicator).

For the creation of an IDA for PPi we needed an indicator which binds to complex **1** with a similar affinity as PPi. Furthermore, the bound and the free indicator should have different optical properties. We found that Methylcalcein blue (MCB) can be employed for this purpose. MCB is a commonly available and water-soluble fluorescent dye which is known to bind metal cations such as Cu(II) and Ca(II).[15]

Titration experiments with a fixed concentration of MCB (20 μM) and variable amounts of complex **1** (0–360 μM) resulted in almost complete quenching for a Pd/MCB ratio of ~ 18 : 1. A Stern–Volmer plot showing the initial (I_0) to observed (I_F) fluorescence intensity ratio as a function of [**1**]$_{tot}$ is given in Fig. 4. The relative fluorescence intensity of MCB decreases linearly up to concentrations of [**1**]$_{tot}$ = 75 μM and the data can be satisfyingly fitted to a model which takes into account static quenching according to $I_0/I_F = (1 + K_s \cdot [\mathbf{1}]_{tot})$, where K_s is the static quenching constant. Best fits gave $K_s = 2.07(\pm 0.5) \cdot 10^4$ M⁻¹, indicating that the fluorophore complexes to **1** with a binding constant of log K = log $K_s \approx 4.3$. For [**1**]$_{tot}$ > 100 μM a deviation from linearity emerges, indicating the on-set of possible dynamic quenching processes. The implied ground-state complex, however, was evidenced by UV/vis spectroscopy (data not shown); spectral changes observed on titrating **1** into a solution of MCB (20 μM)
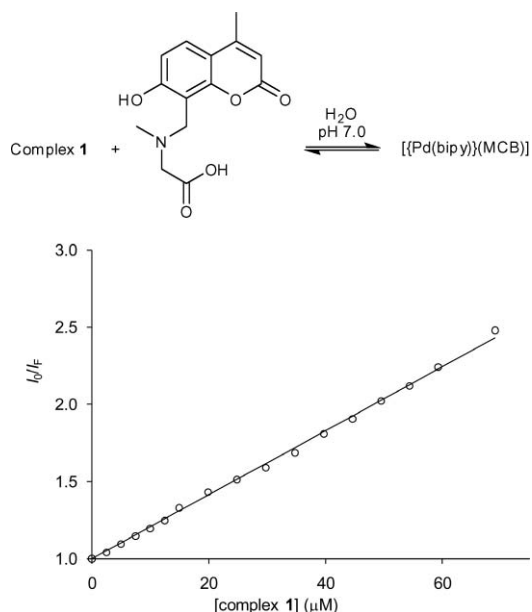
Fig. 4 Stern–Volmer plot showing fluorescence quenching of MCB (20 μM) by complex **1** (0–360 μM, λ_{ex} = 410 nm, λ_{em} = 440 nm). The spectra were recorded in aqueous HEPES buffer solution (25 mM, pH 7.0). The line represents the best fit to the model discussed in the main text. The formulas indicate only the stoichiometry; charges, solvation and counter ions are neglected.

could be modelled by taking into account three absorbing species and least-squares fitting of the data to a 1:1 binding isotherm gave log K_1 = 4.35(5), which compares favorably with the Stern–Volmer quenching studies. Taken together, these data clearly show that MCB binds to the Pd complex in water, with substantial attenuation of its fluorescence emission.

Next, we examined whether a mixture of complex **1** and MCB could be used as a chemosensing ensemble for the detection of PPi. A buffered aqueous solution of MCB (50 μM) and complex **1** (500 μM) showed only weak fluorescence when excited at λ = 410 nm. The use of a 10-fold excess of complex **1** ensures that the fluorophore is in its fully bound and non-emissive (*vide supra*) state. In the presence of PPi (400 μM), however, the original fluorescence of MCB was fully recovered and a strong emission at λ = 440 nm was observed (Fig. 5). The sensing ensemble displays a very good selectivity for PPi over other simple anions (Fig. 5). This was demonstrated by recording the fluorescence spectra of solutions containing a large excess of fluoride, chloride, bromide, dihydrogenphosphate, chlorate, sulfate, acetate, bicarbonate, nitrate and salicylate (1.0 mM, added in the form of their sodium salts). Only the addition of NaH_2PO_4 and $NaClO_4$ resulted in a minor increase in fluorescence (Fig. 5).
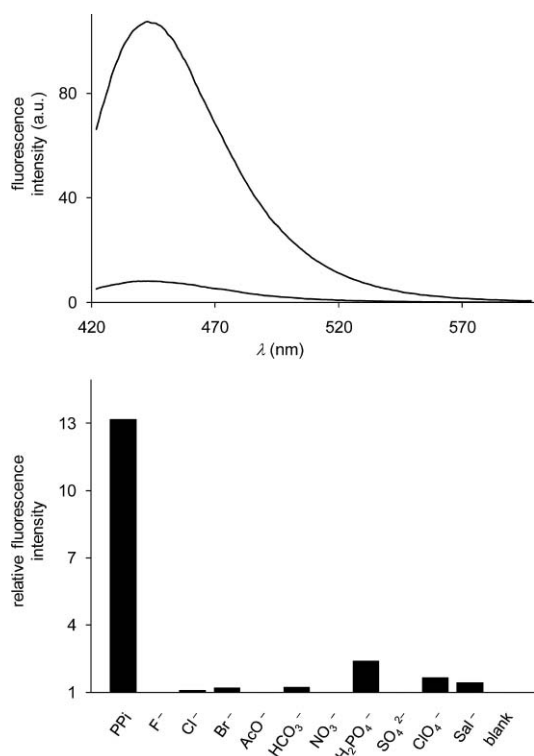
Fig. 5 Top: fluorescence emission spectra of sensing ensemble comprised of complex **1** (500 μM) and MCB (50 μM) in the absence and in the presence of $Na_4P_2O_7$ (400 μM). Bottom: relative fluorescence emission at 440 nm (λ_{ex} = 410 nm) in the presence of $Na_4P_2O_7$ (400 μM) or NaF, NaCl, NaBr, NaOAc, $NaHCO_3$, $NaNO_3$, NaH_2PO_4, Na_2SO_4, $NaClO_4$, and sodium salicylate (1.0 mM each). The spectra were recorded in aqueous HEPES buffer solution (25 mM, pH 7.0) after equilibration.

We have also measured the fluorescence of samples containing a variable PPi concentration of 0 to 600 μM (Fig. 6). The sigmoidal shaped curve reflects the fact that both 1:1 and 2:1 complexes
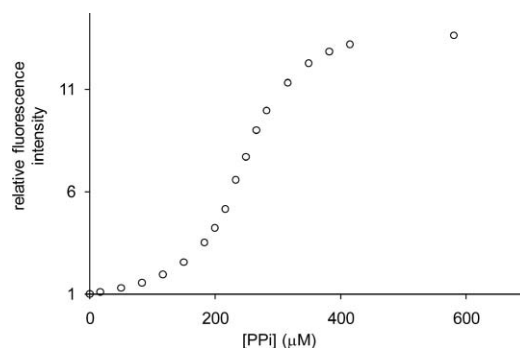
Fig. 6 Relative fluorescence emission at 440 nm (λ_{ex} = 410 nm) of the sensing ensemble comprised of complex **1** (500 μM) and MCB (50 μM) in the presence of variable amounts of $Na_4P_2O_7$ (0–600 μM). The spectra were recorded in aqueous HEPES buffer solution (25 mM, pH 7.0) after equilibration.

of **1**–PPi are formed during the titration. The resulting data can be used as a calibration curve for the sensing of PPi at low to mid micromolar concentrations, with a dynamic range of approximately 50–450 μM and an estimated detection limit of *c.a.* 50 μM for quantitative determination.

Conclusions

We have described a chemosensing ensemble comprised of the Pd complex **1** and the fluorescent dye MCB. The addition of PPi gives a strong turn-on fluorescence signal, which allows the detection of PPi in the low micromolar concentration range. The selectivity for PPi over inorganic anions such as chloride, phosphate, or bicarbonate is very good. Contrary to some of the Zn-based sensors described previously,[4] our system cannot be used to detect PPi in a biological environment, because the Pd complex will bind to peptides[10] and oligonucleotides.[11] For the detection of PPi in an abiotic environment, however, our sensor should be well suited. An important advantage from a practical point of view is the fact that the sensor can be obtained *in situ* by mixing easily accessible compounds.

Experimental

General

Methylcalcein blue (MCB) (Sigma-Aldrich), 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES) (Sigma-Aldrich), and sodium pyrophosphate decahydrate (Acros) were purchased and used as received. The palladium complex [Pd(NO$_3$)$_2$(bipy)] (**1**) was prepared as described in the literature.[16] HEPES buffer solution (25 mM, pH 7.0) was prepared with bidistilled water. Stock solutions of all compounds were prepared in HEPES buffer (25 mM, pH 7.0) and stored in the refrigerator. UV/Vis spectra were recorded at room temperature with a Perkin Elmer Lambda 40 spectrometer. Fluorescence measurements were recorded at 25 °C on a Varian Cary Eclipse spectrophotometer equipped with a thermostatted cell holder.

Spectrophotometric titration of complex 1 and PPi

Stock solutions of complex **1** and PPi in HEPES buffer (25 mM, pH 7.0) were used to prepare a series of solutions with a constant Pd concentration of 25 μM and a variable concentration of PPi. The final anion concentrations were 0–100 μM. After equilibration at room temperature in the dark, the absorption spectra were recorded in the range 250–400 nm (a kinetic study with 25 μM PPi and 25 μM of complex **1** showed that the equilibrium is reached within 5 min). The resulting data were fitted to a model involving 1 : 1 and 2 : 1 complexes of Pd and PPi. The calculations were performed with an in-house built routine in MATLAB®, which implements evolving factor analysis and a Newton–Gauss multi-non-linear least squares fitting algorithm.[17,18] The standard deviations for the fitted parameters (K) were estimated using the sum-of-squared residuals and inverted curvature matrices as obtained from the iterative fitting processes. To take into account pipetting and/or concentration errors in the stock solutions, the minimum error was set at 10%.

Fluorimetric and spectrophotometric titrations of complex 1 and MCB

Stock solutions of complex **1** and MCB in HEPES buffer (25 mM, pH 7.0) were used to prepare a series of solutions with a constant MCB concentration (20 μM) and a variable concentration of complex **1** (0–400 μM). The fluorescence ($\lambda_{ex} = 410$ nm, $\lambda_{em} = 440$ nm) and UV/Vis ($325 < \lambda < 500$ nm) spectra were measured after equilibration at room temperature in the dark (a kinetic study showed that the equilibrium is reached within 5 min). Data fitting was carried out as described above, or in the main text.

Sensing of PPi

Concentrated solutions of PPi were added in 1 μL increments to a solution (3 mL) containing complex **1** (500 μM) and MCB (50 μM) in HEPES buffer (25 mM, pH 7.0). The fluorescence spectra ($\lambda_{ex} = 410$ nm, $\lambda_{em} = 440$ nm) were measured after equilibration for 5 min at room temperature in the dark. Any changes in concentration of **1** and MCB were sufficiently small as to be negligible (< 1%). Control experiments with other anions were performed with NaCl, NaBr, NaF, NaH_2PO_4, $NaClO_4$, Na_2SO_4, NaOAc, $NaHCO_3$, $NaNO_3$ and sodium salicylate (final conc.: 1.0 mM).

Crystallography

Diffraction intensities were measured at 100(2) K on a Bruker APEX II diffractometer equipped with a graphite monochromated Mo-Kα radiation source. Data were reduced using EvalCCD[19] and corrected for absorption using the semi-empirical method.[20] Structure solution, refinement and geometric calculations were performed with SHELX.[21] Crystallographic data for [{Pd(bipy)}$_2$(PPi)]·6H$_2$O: $M = 807.20$, Monoclinic Cc, $a = 16.583(3)$, $b = 12.6047(15)$, $c = 13.4967(6)$ Å, $b = 105.850(8)°$, $V = 2713.8(5)$ Å^3, $Z = 4$; $\rho_c = 1.976$ Mg m^{-3}, $F(000) = 1608$; crystal dimensions $0.47 \times 0.27 \times 0.24$ mm^3; μ(Mo-Kα) $= 1.516$ mm^{-1}, $T = 100$ K. A total of 21969 reflections were measured in the range $3.23 \leq \theta \leq 27.50°$ (hkl range indices: $-21 \leq h \leq 21$, $-16 \leq k \leq 16$, $-17 \leq l \leq 17$), 5829 unique reflections ($R_{int} = 0.0265$).

The structure was refined on F^2 to $R_w = 0.0164$, $R = 0.0160$ (5764 reflections with $I > 2\sigma(I)$) and GOF $= 1.081$ on F^2 for 407 refined parameters and 51 restraints. The absolute structure (Flack) parameter converged to 0.001(11). Largest peak and hole 0.329 and −0.494 eÅ^{-3}. CCDC 776083.

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