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Tuning colourimetric indicator displacement assays for naked-eye sensing of pyrophosphate in aqueous media†

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A library of anion receptors comprising cyclic peptide scaffolds bearing two (Zn^{II} -DPA) anion binding sites have been synthesised and their anion binding abilities evaluated using colourimetric indicator displacement assays with three different indicators. The resulting chemosensing ensembles provided excellent discrimination between polyphosphate ions, with several of the receptor : indicator combinations providing naked-eye sensing of pyrophosphate ions in aqueous solutions containing more than 100 fold excess of ATP.

Introduction

The design of receptors capable of the selective recognition and sensing of biologically relevant anionic species is an area of intense recent interest.^{1–7} Anions such as pyrophosphate ($P_2O_7^{4-}$, PPi) and adenosine triphosphate (ATP) play important roles in metabolic and bioenergetic processes. For example, in the DNA polymerization reaction, the hydrolysis of ATP occurs with concomitant release of PPi, and thus sensors capable of discrimination between these two phosphate oxoanions have the potential for real-time monitoring of such a reaction.⁸ However, the selective recognition of phosphate oxoanions in aqueous media under physiological conditions remains a significant challenge. Although recent advances in receptor development have identified several sensors capable of selectively recognizing PPi in water, selectivity for the binding of PPi over other polyphosphate oxoanions such as ATP and ADP remains modest and in general is unsuitable for practical use.^{9–21} Furthermore, very few PPi sensors have been shown to operate in biologically relevant media, where high salt concentrations and the presence of a range of competing anions and cations are expected to affect both the binding affinity and selectivity.²²

Indicator displacement assays (IDAs) have been widely used for the sensing of anionic species.^{2,23} The ability to readily alter the indicator in such assays provides an additional means by which selectivity of a receptor can be increased.^{17,24,25} However, careful tuning of the interactions between receptor, indicator,

target analyte and other interfering species in solution is required to achieve the desired selectivity and affinity. We recently reported the use of a family of receptors based on the bis(Zn^{II}) complexes of backbone modified cyclic peptide scaffolds with pendant dipicolylamino (DPA) arms, capable of the selective recognition of PPi under physiological conditions using a fluorescent IDA.^{19,26} We have also demonstrated that these cyclic peptide based chemosensing ensembles exhibit enhanced selectivity for PPi over ATP in complex, biologically relevant media such as Krebs buffer (commonly used for cell and tissue culture and approximately isotonic with blood serum²⁷).²² We envisaged that by screening libraries of both receptors and indicators, chemosensing ensembles with improved selectivity for PPi over other polyphosphates might be obtained. We report here our initial studies towards this goal using small libraries of both anion receptors and colourimetric indicators, and demonstrate that with careful selection of the indicator together with a suitable scaffold, near complete selectivity for PPi over other anions including ATP and ADP in both aqueous buffer and Krebs buffer can be achieved at biologically relevant concentrations. In addition, we have found that the stoichiometry of the receptor : indicator complex is an additional factor that can be used to tune selectivity in IDAs.

Results and discussion

Guided by our previous work which indicated that backbone modified cyclic peptide receptors bearing two (Zn^{II} -DPA) functionalised sidechains provide good selectivity towards polyphosphate ions and that smaller ‘non-binding’ sidechains, *e.g.* alanine derivatives, favour binding of PPi over ATP and ADP,^{19,22,26} we prepared a new family of bis(Zn^{II} -DPA) complexes **1·Zn₂–8·Zn₂** using our previously reported solid phase peptide synthesis approach²⁶ with slight modifications (see ESI for details†). Compounds **4·Zn₂–8·Zn₂** contain shortened

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sidechains to alter the distance between the cyclic peptide scaffold and the anion binding sites since we have previously found that cyclic peptides of this type can contribute to anion binding through hydrogen bonding interactions with the amide protons and that reducing the distance between the peptidic scaffold and side chain binding sites results in increased anion binding affinity.^{28–30} For comparison we also prepared receptor **9·Zn₂**³¹ which has previously been used in IDAs for sensing of PPi,^{16,32} but was not able to distinguish between PPi and ATP in IDAs with a pyrocatechol violet (PV) indicator.³²

Despite their lower sensitivity, an advantage of colourimetric over fluorescent IDAs is that they allow an assessment of the presence of target analytes by the naked eye, providing the ability to visually screen samples, while also allowing ratiometric assays to be developed using spectrometric techniques. We therefore visually screened a number of commercially available colourimetric indicators to identify those suitable for use in IDAs with the bis(Zn^{II}-DPA) complexes. Of these, the three catechol-type indicators pyrocatechol violet (PV), bromopyrogallol red (BPR) and pyrogallol red (PR) (Fig. 1) were found to exhibit distinctive color changes upon mixing with 1 equiv. of receptors **1·Zn₂**–**9·Zn₂** in aqueous HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at room temperature (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), attributable to binding of a bridging catecholate moiety to the two metal ions,³³ and a reversal in this colour change was observed upon addition of less than five

equivalents of PPi in all cases. Subsequently, UV-visible titration experiments were carried out to quantify the association constants between receptors **1·Zn₂**–**9·Zn₂** and the three indicators in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at 25 °C. The absorption spectrum of PV (20 μM) changes upon incremental additions of each receptor. The absorption band at 640 nm increases with a concomitant decrease in the band at 444 nm (Fig. 2a). The change quickly reaches saturation as approx. 1 equiv. of each receptor is added, suggesting the formation of strong 1 : 1 complexes. The apparent association constants (Table 1) were estimated *via* non-linear least squares fitting of absorption data to a 1 : 1 binding model using the Hyperquad® package,³⁴ and stoichiometry was confirmed by Job plots. The apparent association constant determined for the complex between **9·Zn₂** and PV ($\log K_a = 8.8$) is in agreement with previous reports^{32,35} after consideration of the higher ionic strength used in the present study. Similar titrations were carried out for BPR and PR. Surprisingly, despite their similar structures to PV, while both of these indicators were observed to bind strongly to all nine receptors, the binding data for PR to **1·Zn₂**–**9·Zn₂** could not be fit to a suitable binding model, and the data for BPR was best fitted to a stepwise 1 : 1 to 2 : 1 receptor : indicator model, with 2 : 1 stoichiometry confirmed by Job plot analysis. Job plot analysis of the complexes of **1·Zn₂**–**9·Zn₂** with PR did not provide clear evidence for compound stoichiometry, suggesting the presence of multiple equilibria in solution.

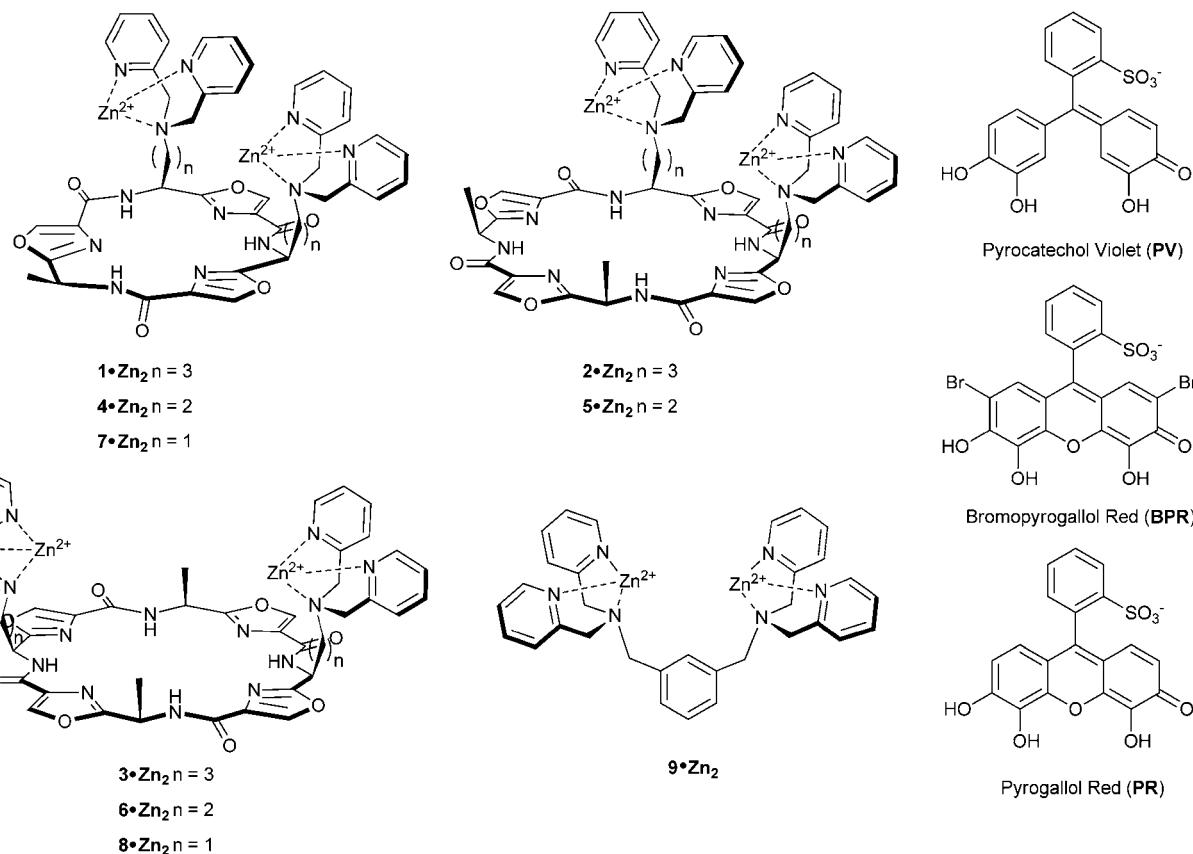


Fig. 1 Structures of receptors **1·Zn₂**–**9·Zn₂** and indicators PV, BPR and PR.



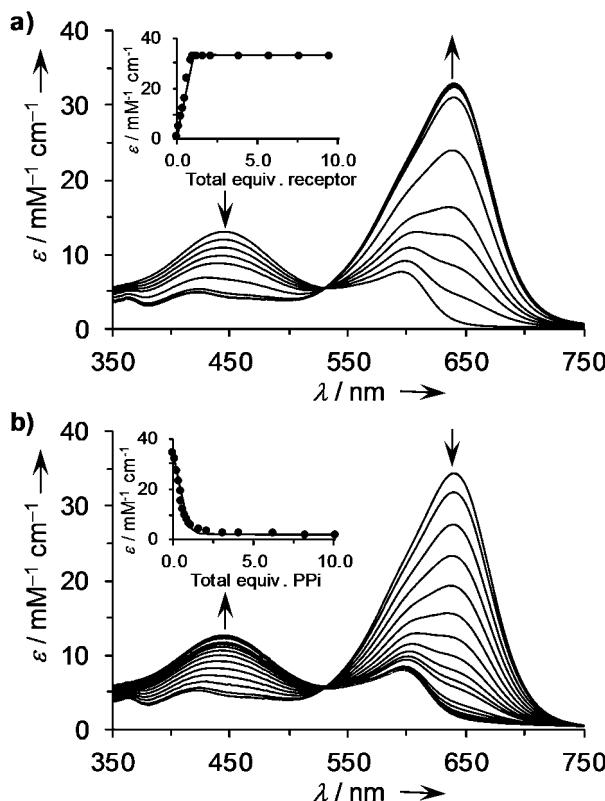


Fig. 2 (a) Changes in absorbance of PV solution (20 μ M) upon addition of **3**·Zn₂, $[3\text{-Zn}_2] = 0, 2, 4, 6, 8, 12, 16, 20, 24, 29, 39, 71, 102, 130$ and 156μ M. (b) Changes in absorbance for 1 : 1 mixtures of receptor **3**·Zn₂ : PV (20 μ M each) upon addition of PPI (sodium salt), $[\text{PPI}] = 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 60, 79, 116, 152, 183 \mu$ M. All spectra were measured in aqueous HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at 25 °C. Insets: fitting curves at 640 nm.

Despite the differences observed in stoichiometry between complexes of **1**·Zn₂–**9**·Zn₂ and the three indicators, we employed 1 : 1 receptor : indicator mixtures in subsequent IDAs with a range of anions in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl),^{23,36} to investigate whether we could obtain a chemosensor for PPi with high selectivity over other anions. We initially focussed on use of PV as an indicator because the 1 : 1 binding stoichiometry observed above allowed ready quantification of the apparent stability constants of a range of anions with receptors **1**·Zn₂–**9**·Zn₂ using the standard equilibria previously described for competition assays.³⁷ Addition of five equivalents of a range of anions (nitrate, acetate,

hydrogensulfate, iodide, phosphate, phenylphosphate, phosphotyrosine, phosphothreonine, phosphoserine, AMP, ADP, ATP, PPi and citrate) as their sodium salts to 1 : 1 mixtures of the receptors and PV (20 μ M each) indicated that only PPi, ATP, ADP and, in some cases, citrate were able to displace PV from the cyclic peptide derived receptor : indicator complexes, as indicated by a colour change from blue to yellow or green (Fig. 3). Titrations of 1 : 1 solutions of receptors **1**·Zn₂–**9**·Zn₂ and PV with PPi, ATP, ADP and citrate under the same conditions and curve fitting of the absorption data based on the equilibria for competition assays³⁷ using the Hyperquad® program afforded the apparent receptor–anion binding constants in Table 1 (see Fig. 4a for an example of titration data). Under these conditions all cyclic peptide receptors (**1**·Zn₂–**8**·Zn₂) showed modest selectivity for PPi against ATP and ADP, while the simple *m*-xylene-based receptor (**9**·Zn₂) did not discriminate between these anions,³⁸ indicating that selectivity for PPi is enhanced when using cyclic peptide derived chemosensing ensembles. In three cases (receptors **5**·Zn₂, **6**·Zn₂ and **8**·Zn₂), only ATP and PPi displaced the indicator to any extent indicating that shortening the side chains between the DPA groups and the cyclic peptide improves the ability of these receptors to discriminate between polyphosphate anions and suggesting that the optimum scaffolds for PPi selectivity are those derived from the larger tetraoxazole rather than trioxazole scaffolds.

We next screened receptor : indicator complexes using PR and BPR as the indicators to investigate whether a change of indicator could be used to enhance selectivity for PPi over ATP, ADP and citrate. While it has previously been established that optimum selectivity in IDAs is obtained when $K_{\text{guest}} \gg K_{\text{ind}} \gg K_{\text{other}}$,^{24,39} in the current case the differences in indicator : receptor stoichiometry (and in the case of PR the ill-defined



Fig. 3 The colours of the 1 : 1 mixtures of **2**·Zn₂ : PV (20 μ M each) with and without anions (sodium salts) from left to right: no anion, PPi, ATP, ADP, AMP, c-AMP, phosphothreonine, phosphoserine, phosphotyrosine, HPO₄²⁻ and citrate (5 equiv. each).

Table 1 Apparent association constants ($\log K_a$) for **1**·Zn₂–**9**·Zn₂ with anionic species in HEPES buffer^a

	1 ·Zn ₂	2 ·Zn ₂	3 ·Zn ₂	4 ·Zn ₂	5 ·Zn ₂	6 ·Zn ₂	7 ·Zn ₂	8 ·Zn ₂	9 ·Zn ₂
PV	8.4	9.0	8.8	8.5	9.0	8.9	7.4	6.6	8.8
PPi	>9	>9	>9	>9	>9	>9	8.4	7.1	>9
ATP	7.2	7.9	7.2	8.8	6.7	4.5	5.4	4.7	>9
ADP	7.4	7.0	4.6	7.2	— ^b	— ^b	4.6	— ^b	>9
Citrate	5.9	6.2	4.4	6.0	— ^b	— ^b	— ^b	— ^b	5.4

^a Titrations were performed at 25 °C in aqueous solutions buffered at pH 7.4 with 5 mM HEPES in the presence of 145 mM NaCl. [PV] and [receptor] were 20 μ M each for competition assays. Estimated errors in $\log K_a < 0.2$. ^b Changes too small to fit.

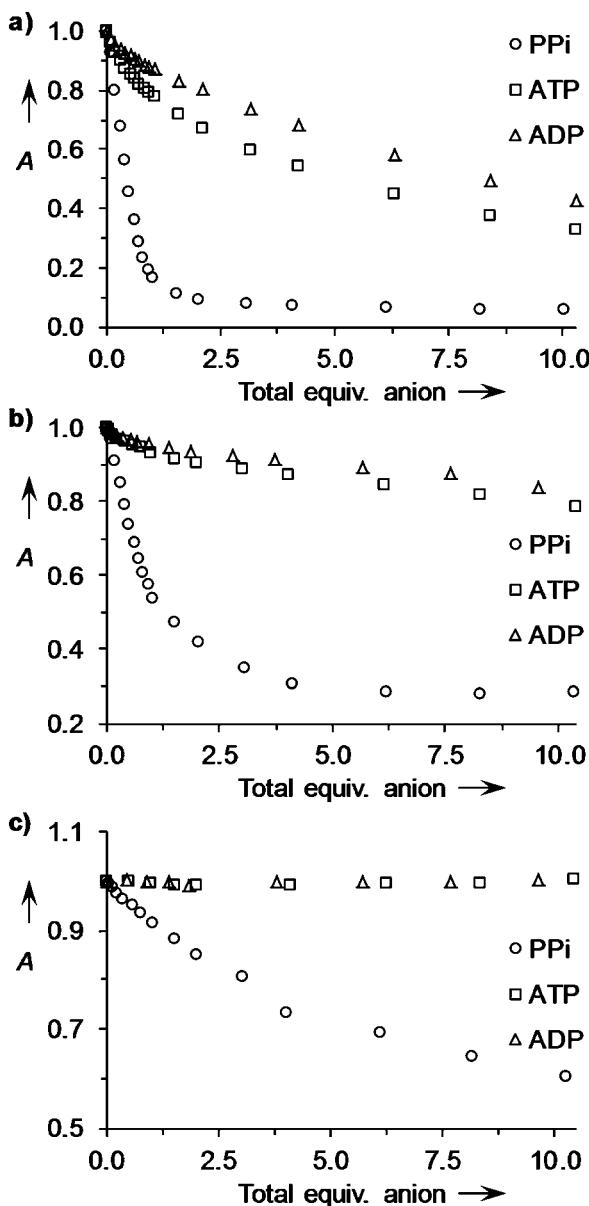


Fig. 4 Changes in absorbance for 1 : 1 mixtures of (a) **3·Zn₂** : PV (20 μ M each) at 640 nm; (b) **3·Zn₂** : PR (20 μ M each) at 600 nm and (c) **3·Zn₂** : BPR (10 μ M each) at 610 nm; upon addition of up to 10 equiv. of anions (sodium salts) in aqueous solutions of HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at 25 °C. A = relative absorbance (arbitrary units).

nature of this complex) make it difficult to predict how selectivity will be affected upon changing the indicator. Gratifyingly, in many cases use of PR and BPR indicators resulted in an improvement in the ability of the chemosensing ensembles to discriminate between polyphosphate and polycarboxylate ions. Using PR as the indicator, receptors **1·Zn₂**, **2·Zn₂**, **7·Zn₂** and **8·Zn₂** exhibited excellent selectivity for PPI with no indicator displacement by ATP, ADP or citrate to any significant extent (<15% change in absorbance at 600 nm after addition of 10 equiv. of anion). Of the cyclic peptide derivatives examined with PR as the indicator, **3·Zn₂** showed the lowest level of discrimination between PPI, ATP and ADP (Fig. 4b). This was

significantly improved upon use of BPR as the indicator (Fig. 4c). In this case addition of at least 10 equiv. of ATP or ADP did not result in displacement of the indicator from any of the receptors examined (**1·Zn₂**–**3·Zn₂** and **9·Zn₂**), notably including the xylene derivative **9·Zn₂**, which has previously shown poor discrimination between polyphosphate ions in IDAs with PV.³² These results indicate that the selectivity of IDAs can be tuned through choice of indicator, in agreement with previous observations^{17,24} and suggests that stoichiometries of >1 : 1 for the receptor : indicator complex provide improved selectivity for these chemosensing ensembles.

Given that our previous studies have indicated that in a fluorescent IDA employing receptor **3·Zn₂**, selectivity for PPI over ATP and ADP improved in Krebs saline (composition: 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, 0.4 mM KH₂PO₄, 0.3 mM NaH₂PO₄, 10 mM glucose, 10 mM Tris-base buffered at pH 7.4), compared to 145 mM aq. sodium chloride solution, we were intrigued to see how chemosensing ensembles comprising **1·Zn₂**–**9·Zn₂** and a colourimetric indicator function in media with compositions matching those in biological systems such as extracellular fluid or urine.²² This was of particular interest since the effect of relatively high Mg²⁺ and Ca²⁺ concentrations on the colourimetric indicators used in the current assays has not previously been established and might be expected to be significant given the use of these compounds as complexometric indicators.⁴⁰ IDAs were carried out with receptors **1·Zn₂**–**7·Zn₂** and **9·Zn₂** in Krebs buffer using indicator PV to allow quantification of the apparent stability constants between the receptors and PPI, ATP, ADP and citrate for direct comparison with those obtained in saline solution (Table 2). In all cases, the colour of a PV solution changed from yellow to blue on addition of the receptors, indicating that the other anions and cations present in Krebs buffer did not interfere substantially with receptor–indicator binding.

While the apparent stability constants of all receptors with PV were lower in Krebs saline in comparison to those observed in HEPES buffer, those for the smaller scaffolds (**1·Zn₂**, **4·Zn₂**, **7·Zn₂** and **9·Zn₂**) were more strongly affected by the change in media composition (dropping by approximately two orders of magnitude) than those of the tetraoxazole based receptors. This is most likely a result of increased competition with phosphate ions, which have previously been shown to bind to **9·Zn₂** (ref. 16 and 41). Notably, for the **3·Zn₂** : PV complex, there was only a

Table 2 Apparent association constants ($\log K_a$) for **1·Zn₂**–**7·Zn₂** and **9·Zn₂** with anionic species in Krebs saline^a

	1·Zn₂	2·Zn₂	3·Zn₂	4·Zn₂	5·Zn₂	6·Zn₂	7·Zn₂	9·Zn₂
PV	6.1	8.2	8.5	6.8	8.3	7.8	6.6	6.3
PPI	6.3	8.4	8.7	8.8	>9	8.2	7.0	9.0
ATP	4.1	— ^b	— ^b	5.3	— ^b	— ^b	— ^b	6.8
ADP	— ^b	6.6						
Citrate	— ^b	5.4						

^a Titrations were performed at 25 °C in Krebs saline solutions buffered at pH 7.4. [PV] and [receptor] were 20 μ M each for competition assays. Estimated errors in $\log K_a$ < 0.2. ^b Changes too small to fit.



very small change in the affinity constant on moving from HEPES buffer to Krebs saline. In all cases, selectivity for PPi over ATP, ADP and citrate was enhanced in Krebs saline as previously observed for fluorescent IDAs.²² While for **9·Zn₂** and **4·Zn₂**, ATP and ADP still exhibited significant indicator displacement under these conditions, receptors **2·Zn₂**, **3·Zn₂**, **5·Zn₂**, **6·Zn₂** and **7·Zn₂** exhibited almost complete selectivity for PPi. As illustrated in Fig. 5a, the binding of 4 equiv. of PPi to a 1 : 1 mixture of **5·Zn₂** and PV led to ~90% restoration of PV's absorption, whereas 10 equiv. of either ATP or ADP only resulted in minor absorption changes (<20%). This level of selectivity represents a remarkable improvement compared to that observed in HEPES buffer (Fig. 4a). This can be attributed to two predominant factors: the numerous competitive equilibrium processes occurring in the complex Krebs mixture as a result of strong binding of Ca²⁺ and Mg²⁺ to PPi, ATP and ADP (log *K*'s ~ 3–4);^{42–44} and the higher affinity for PPi by receptors **2·Zn₂**, **3·Zn₂**, **5·Zn₂**, **6·Zn₂** and **7·Zn₂** compared to ATP or ADP that enables this ion to displace the strongly bound PV. Similarly, addition of 6 equiv. of PPi to a 1 : 1 mixture of **5·Zn₂** and PR led to ~70% restoration of PR's absorption while addition of 10 equiv. of either ATP or ADP gave only minor changes (<8%, Fig. 5b).

Given the excellent levels of selectivity for PPi observed for the cyclic peptide receptors **2·Zn₂**, **3·Zn₂**, **5·Zn₂**, **6·Zn₂** and

7·Zn₂ in Krebs saline, it was of interest to examine whether these receptors would be useful for PPi sensing applications. Using the **3·Zn₂** : PV chemosensing ensemble (20 μ M each), we carried out calibration experiments for PPi detection in Krebs buffer. The first calibration was obtained over the range of [PPi] between 1 and 36 μ M without any ATP present, and the second calibration was carried out the same way but in the presence of 0.25 mM ATP. In both cases, a strong absorption response to PPi was observed as PV was displaced from the sensing ensemble and the presence of a large excess of ATP and other competing anions and cations did not appear to have a significant impact on the displacement capability of PPi (see ESI†). Ratiometric absorbances at 444 nm and 640 nm afforded linear calibration curves over the measured range of PPi concentrations in both experiments with similar slopes and *R*² values being obtained (*R*² = 0.999 for the calibration plot without ATP – see ESI†; *R*² = 0.998 for the calibration plot with ATP – Fig. 6a). The results indicate that less than 2 μ M of PPi can be detected even in the presence of >125 times excess of ATP in the same solution. Similar results were obtained with **2·Zn₂** : PR chemosensing ensemble in HEPES buffer (Fig. 6b), indicating that these IDAs are useful for sensing applications where the detection of low

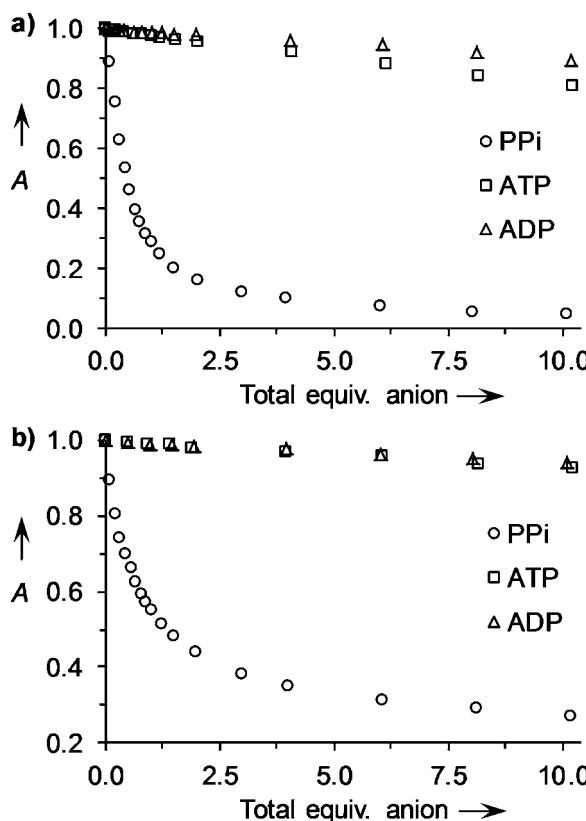


Fig. 5 (a) Changes in absorbance at 640 nm for 1 : 1 mixture of **5·Zn₂** : PV (20 μ M each) upon addition of up to 10 equiv. of anions (sodium salts); (b) changes in absorbance at 600 nm for 1 : 1 mixture of **5·Zn₂** : PR (20 μ M each) upon addition of up to 10 equiv. of anions (sodium salts). Measurements were carried out in Krebs saline solutions (pH 7.4) at 25 °C. A = relative absorbance (arbitrary units).

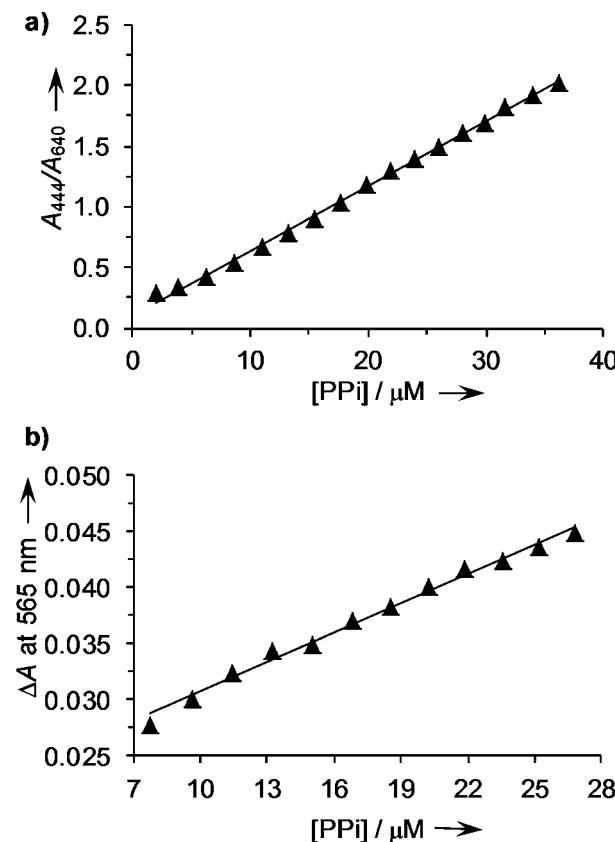


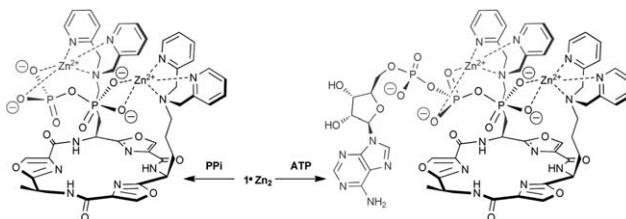
Fig. 6 (a) Calibration plot for PPi obtained from ratiometric absorbances at 444 nm and 640 nm using the **3·Zn₂** : PV chemosensing ensemble (20 μ M each) in the presence of ATP (250 μ M). UV-vis measurements were carried out in Krebs saline solutions (pH 7.4) at 25 °C. *R*² = 0.998. (b) Calibration plot for PPi obtained from absorbance at 565 nm using the **2·Zn₂** : PR chemosensing ensemble (20 μ M each) in the presence of ATP (250 μ M). UV-vis measurements were carried out in HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at 25 °C. *R*² = 0.991.



amounts of PPi is required in the presence of a large excess of ATP, such as the monitoring of DNA polymerase reactions.

To investigate the reason for the remarkable selectivity observed for PPi with these receptors, we investigated the mode of binding between the cyclic peptide-based bis(Zn^{II}-DPA) complexes and polyphosphate anions. ³¹P NMR experiments were carried out in D₂O for receptor **1**·Zn₂ with PPi and ATP. A change in chemical shift from -6.5 to -3.9 ppm for the ³¹P (202 MHz) signal for PPi was observed upon addition of 1 equiv. **1**·Zn₂ (see ESI†), indicating strong binding and that all four negatively charged O-P oxygen atoms bind to the two Zn²⁺ centers in the symmetrical fashion. On the other hand, the three ³¹P signals of ATP behave differently upon binding to **1**·Zn₂ (Fig. 7). While the signals attributable to P^β and P^γ gradually moved downfield (from -22.7 and -10.5 ppm to -21.1 and -8.5 ppm for P^β and P^γ, respectively at 2 equiv. **1**·Zn₂), that of P^α shifted slightly upfield from -11.1 to -12.3 ppm. This indicated that only the two outer phosphate groups play a significant role in binding to the Zn²⁺ ions. These results suggest the binding modes illustrated for PPi and ATP by **1**·Zn₂ in Scheme 1. The selectivity of the cyclic peptide derived receptors for PPi over ATP can be attributed in part to the difference in the total anionic charge density involved in the binding, as proposed by Lee *et al.*¹⁰ for other bis(Zn^{II}-DPA) receptors. The relatively smaller total negative charge of the four O-P oxygen atoms of ATP complexed to the Zn²⁺ ions compared to those of PPi results in reduced binding affinity.

However, there is an additional factor that contributes to the enhanced selectivity for PPi by the cyclic peptide receptors compared to the *m*-xylene based receptor **9**·Zn₂, which is related



Scheme 1 Possible binding modes for **1**·Zn₂ with PPi and ATP suggested by ³¹P NMR data.

to the size and geometry of the receptors themselves. The high affinity of **9**·Zn₂ for all three anions (PPi, ATP and ADP) can be attributed to a tighter fit due to the shorter and more rigid side-arms of the Zn^{II}-DPA binding sides compared to the cyclic peptide derived receptors. The increased selectivity for PPi observed for **1**–**8**·Zn₂ may be attributed to the presence of the additional side chains on the cyclic peptide-based receptors, which can interfere with ATP binding through steric blocking. Amongst the cyclic peptide receptors, those based on the larger tetraoxazole scaffolds generally exhibit higher binding affinities and selectivities than the smaller trioxazole based receptors. This difference in selectivity can be explained by additional steric interference with the larger ATP and ADP ions due to the second Ala residue on the tetraoxazole scaffolds. For the tetraoxazole scaffolds, where the two Zn^{II}-DPA binding sites are positioned either adjacent to each other or on opposite sides of the macrocycle, there is no significant difference on PPi binding affinity or selectivity. This suggests that the relatively flexible bis(Zn^{II}-DPA) side-arms can readily accommodate PPi in an induced-fit binding manner.

Conclusions

The synthesis of a library of cyclic peptide receptors has enabled the identification of chemosensing ensembles with excellent selectivity for PPi over other polyphosphate anions. Chemosensing ensembles comprising the cyclic peptide derived receptors **1**–**8**·Zn₂ and pyrocatechol violet show enhanced discrimination between PPi and other polyphosphate ions (ATP, ADP) in comparison to those containing the simple bis(Zn^{II}-DPA) complex **9**·Zn₂. The increased selectivity can be attributed to steric blocking of the larger anions by additional side chains on the cyclic peptide scaffold. The best discrimination between anions was observed for receptors **5**·Zn₂ and **6**·Zn₂ in which the (Zn^{II}-DPA) binding sites are appended to a tetraoxazole scaffold, with spacers of two methylene units between the scaffold and the anion binding sites. These structures provide the best compromise between the flexibility required for induced-fit binding of the PPi guest and the steric blocking of other anions by the scaffold. Notably, the selectivity of the chemosensing ensembles can be further tuned by changing the indicator, with pyrogallol red and bromopyrogallol red both providing enhanced discrimination between polyphosphate ions when compared to pyrocatechol violet, despite binding with different stoichiometries to the cyclic peptide receptors. Ensembles of all

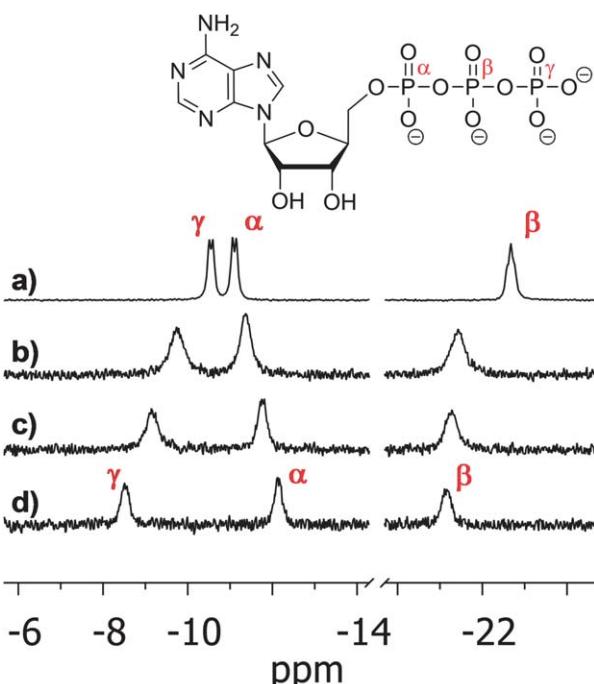


Fig. 7 ³¹P NMR spectra (202 MHz) of ATP (10 mM) in the absence and presence of receptor **1**·Zn₂ in D₂O at 300 K. (a) [1·Zn₂] = 0. (b) [1·Zn₂] = 0.5 equiv. (c) [1·Zn₂] = 1 equiv. (d) [1·Zn₂] = 2 equiv.



three indicators with the cyclic peptides **1–8·Zn₂** provide colour changes visible to the naked eye. Importantly, we have found that such chemosensing ensembles are effective in Krebs saline solution and enable the ratiometric detection of PPi in the presence of a large excess of ATP. This will be applied in the monitoring of enzymatic reactions and detection of PPi levels in biological samples in due course.

Acknowledgements

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