Pyrophosphate recognition and sensing in water using bis[zinc(II)dipicolylamino]-functionalized peptides

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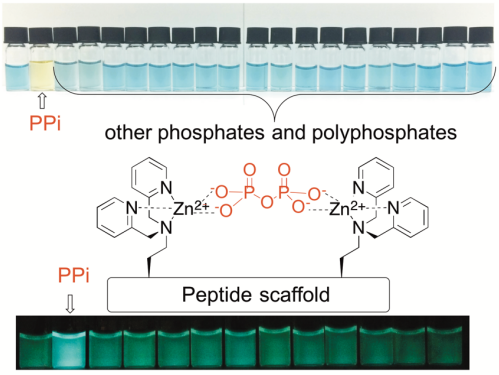
Conspectus

Phosphate oxoanions and phosphorylated biomolecules (such as nucleotides, lipids and proteins) play key roles in a wide range of biological processes. The ability to selectively detect these ions in the presence of each other has numerous applications in biochemistry and biomedicine. However, receptors and sensors that can discriminate between polyphosphate species with high selectivity and in biologically relevant conditions are rare.

In this Account, we show how peptides (both cyclic and linear) can be used to position two zinc(II)dipicolylamine [Zn(II)DPA] binding sites in an appropriate arrangement to provide selective binding of pyrophosphate (PPi) in the presence of other polyphosphate species, including ATP, and in complex media such as cell growth buffer.



The use of peptide scaffolds to position the Zn(II)DPA anion binding sites allowed the synthesis of small receptor libraries in which the arrangement of the two binding sites could be subtly altered to evaluate the factors affecting both binding selectivity and affinity for PPi. We altered a number of structural elements including peptide length, cyclic peptide ring size, amino acid composition, the positioning of the binding sites with respect to one another and the relative stereochemistry of the peptides. Backbone modified cyclic peptides based on the

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Lissoclinum class of natural products were initially employed to provide an added degree of preorganization to the receptors, although it was subsequently found that short, flexible bis[Zn(II)DPA]-functionalized linear peptides are also effective scaffolds for selective pyrophosphate recognition.

The peptidic receptors were successfully employed for the detection of PPi in aqueous media by indicator displacement assays using both colorimetric and fluorescent indicators, with the best compounds able to bind to PPi selectively in both cell growth media and artificial urine and also allow the accurate determination of PPi concentrations in physiologically relevant ranges (micromolar concentrations) in these complex media. Improved pyrophosphate selectivity was observed upon increasing the complexity of the media from HEPES buffer to cell growth media (Krebs saline).

Pyrophosphate sensors in which a fluorescent indicator was covalently attached to either a linear or cyclic peptide scaffold through a flexible linker were then constructed. When the Zn(II)DPA binding sites and the indicator were suitably placed with respect to one another on the peptide scaffold, these 'intramolecular indicator displacement assays' showed improved selectivity for PPi over other polyphosphate anions, such as ATP, when compared to the intermolecular indicator displacement assays. This observation provides the basis for the design and application of future PPi sensors in biochemistry and biomedicine.

1. Introduction

Anions are critical to the maintenance of life, with almost every biochemical process involving the recognition, transport or transformation of anions. This has led to growing interest in the design of artificial anion receptors for sensing applications in biochemistry and

biomedicine.[1,2] Phosphate oxoanions are of particular interest because they play crucial roles in a variety of fundamental biological processes, including genetic information storage, energy transduction, signal processing and membrane transport. For example, pyrophosphate ($P_2O_7^{4-}$, PPi) is released during DNA/RNA polymerase reactions, in the biosynthesis of secondary messengers such as cAMP and is a hydrolysis product of ATP.[3] PPi is also found at elevated levels in synovial fluid or urine in patients with a variety of diseases, including osteoarthritis[4,5] and urolithiasis,[6] and abnormal intracellular PPi levels have been suggested as a tool for cancer diagnosis.[7] Therefore, the development of receptors that can distinguish between the large number of biologically occurring phosphate anions [(poly)phosphates and phosphorylated biomolecules] to bind selectively to a target ion (e.g. PPi) in physiological conditions has numerous potential bioanalytical applications.

Anions can interact with receptors through electrostatic interactions, hydrogen bonds or coordination bonds with metal ions. However, in water, anions such as PPi are highly solvated,[8] so the development of selective receptors typically requires the use of strong electrostatic interactions or coordination bonds. One of the most successful approaches to date for the recognition of phosphate oxoanions in water is the use of Zn(II)dipicolylamine (Zn(II)DPA) binding motifs, which have been found to be particularly selective towards phosphate derivatives over other anions.[9,10] Receptors capable of discrimination between phosphate oxoanions can be prepared through the preorganisation of multiple Zn(II)DPA binding sites using an appropriate molecular scaffold. For example, receptors bearing two appropriately positioned Zn(II)DPA binding sites have been shown to bind selectively to poly-phosphorylated peptides via a two-point binding interaction,[11] whereas the *meta*-xylyl derivative **1**[12] is able to discriminate between PPi and Pi. For further examples the reader is directed to a recent review.[9] The distance between the two Zn(II) centres in these complexes is

key to controlling their selectivity, since phosphate oxoanions bind to the two metal ions in a bridging mode, with one or two oxygen atoms bound to each Zn(II) centre.

While a large range of different molecular scaffolds have been employed to preorganize the Zn(II)DPA binding sites,[9] the design of receptors able to discriminate between PPi and other di- and tri-phosphate derivatives (e.g. ATP) remains a challenge.[13] We show here how our work on the use of peptidic scaffolds to position two Zn(II)DPA binding sites for anion recognition has led to the development of selective receptors for PPi that are able to function in complex media (e.g. cell growth buffers) and selectively sense PPi in the presence of high concentrations of other phosphate derivatives, including ATP.

2. Scaffold design

The key requirements for developing selective receptors that can bind to phosphate oxoanions in relevant conditions (aqueous solution, presence of interferants) are: 1) that the receptors complement the large size and shape of these species; 2) that binding sites are suitably positioned to discriminate between structurally similar anions and 3) that the binding sites provide sufficiently strong interactions with the target analyte for binding to occur. This mandates the use of suitably large scaffolds that position the binding elements in a complementary arrangement to the geometry of a particular guest. The use of peptides for this purpose provides a means of readily varying the size and shape of the scaffold through alteration of amino acid composition, relative stereochemistry and peptide length. One of the key advantages of peptidic scaffolds is the ability to generate libraries of receptors with subtle structural modifications to tease out the factors providing selectivity for specific anionic guests. To circumvent the lack of defined geometry (and therefore preorganization) displayed by short, linear peptides, scaffold rigidification can be achieved through cyclization[14,15] and additional

preorganization can be achieved through the incorporation of peptide backbone modifications such as heterocycles.[16]

The *Lissoclinum* family of cyclic peptides contains cyclic hexa-, hepta- and octa-peptides characterised by the presence of azole heterocycles alternating with proteinogenic amino acid residues.[17] A network of bifurcated hydrogen bonds between the azole nitrogen atoms and the amide protons reduces the flexibility of these compounds, providing a relatively flat macrocycle from which all amino acid side-chains project onto one face of the scaffold (if they are of the same absolute configuration) (Figure 1).[18] These modified cyclic peptides, and analogues thereof, have been used as molecular scaffolds in molecular recognition, as molecular switches and in combinatorial chemistry.[15,16,19] They appeared an ideal starting point from which to develop selective receptors for PPi, as two Zn(II)DPA units could be positioned in a convergent manner on the peptide side chains and the peptide structure could be readily varied to introduce additional binding sites or change the positions of the Zn(II)DPA units with respect to one another.

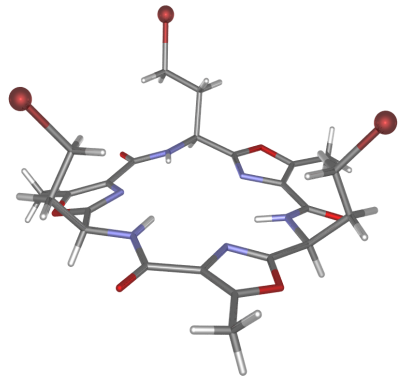


Figure 1. X-ray structure of a representative *Lissoclinum* cyclic hexapeptide (sidechains = CH_2CH_2Br), illustrating the projection of all three side chains from one face of the scaffold. Disorder and solvent removed for clarity.

These cyclic peptide scaffolds are readily prepared via either cyclooligomerisation of an oxazole amino acid monomer[20-22] or dimer,[23] or alternatively through solution[24] or solid-phase[23] peptide synthesis of a linear peptide containing the preformed azole heterocycles, followed by macrocyclization. Side chain amine groups can be deprotected and functionalized through reductive amination with picolylaldehyde, followed by complexation with Zn(II) to install the Zn(II)DPA anion binding motifs.

3. Anion recognition using indicator displacement assays

In order to provide a ready method for sensing anion binding events, without the need for additional synthetic effort to incorporate a chromophore or fluorophore, we initially employed indicator displacement assays (IDAs)[25] to investigate anion binding with these Zn(II)DPA-functionalized peptides. Our first receptor, **2** (Figure 2), was based on a cyclic octapeptide scaffold bearing two Zn(II)DPA groups at an appropriate distance to complement the size and geometry of PPi.[26] The binding affinity of **2** for indicator **3** was first determined by fluorescence titration and subsequent non-linear curve fitting of the emission data to a standard 1 : 1 binding model. The apparent association constants of **2** for anions were then determined using IDAs, using a non-linear least squares fitting procedure based on the equilibria described for competition assays.[27] In IDAs in combination with **3**, **2** exhibited high affinity for PPi anions (log K_a = 8.0) under mimicked physiological conditions (pH 7.2, 5 mM HEPES buffer, 145 mM NaCl). The **2·3** chemosensing ensemble was found to display complete selectivity for PPi over monophosphate derivatives, including AMP and HPO_4^{2-}, which were unable to displace the indicator even upon addition of a large excess of anion. Significant selectivity for PPi over ATP (log K_a = 5.9) and ADP (log K_a = 5.6) was also observed. In comparison, the use of the smaller diketopiperazine scaffold **4**[30] resulted in reduced binding affinity and selectivity for PPi

(log K_a = 6.0 for PPi and 5.3 for ATP), suggesting that the larger scaffold is required for appropriate placement of the two Zn(II)DPA groups to develop an effective PPi sensor.

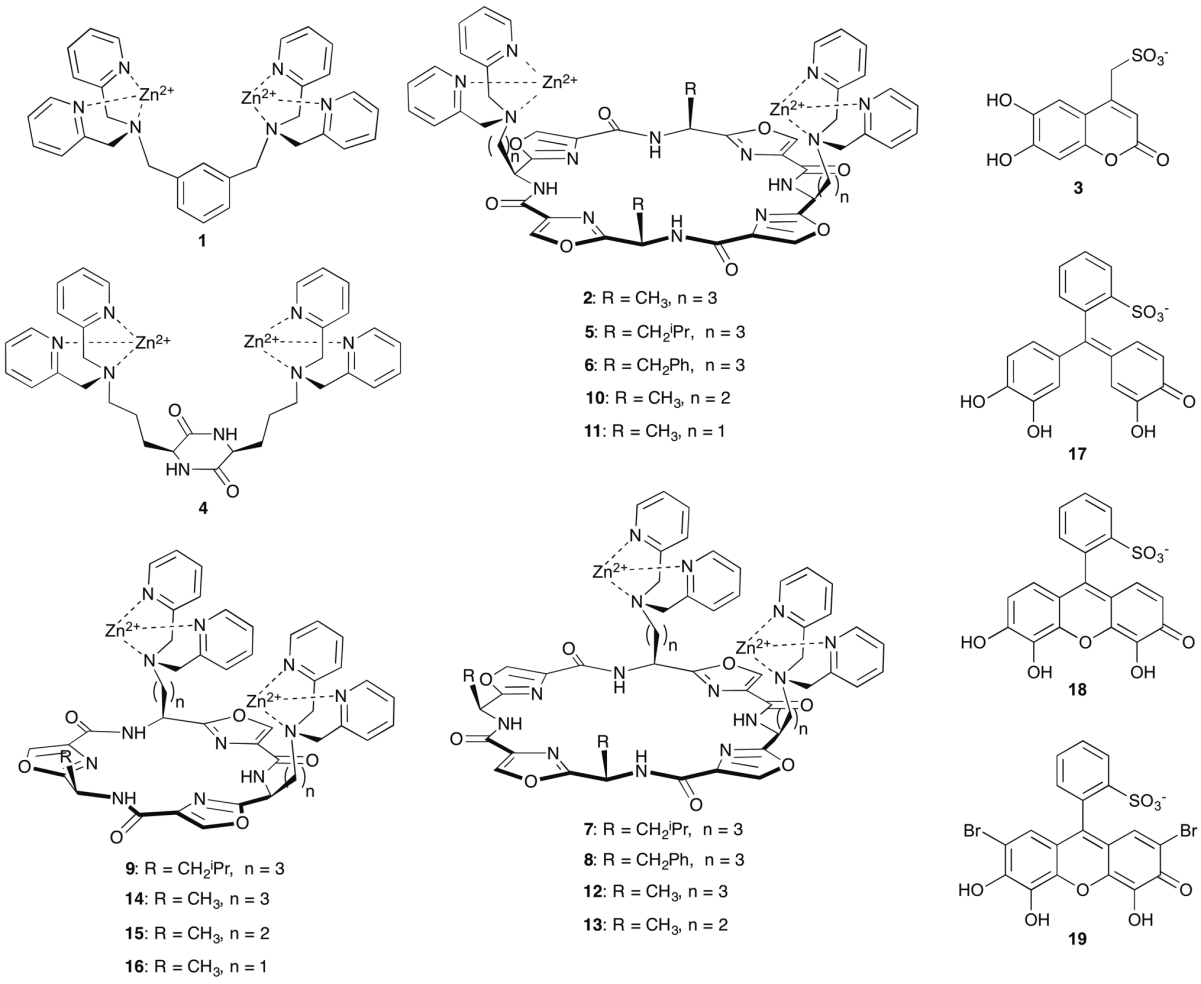


Figure 2. Structures of the Zn(II)DPA receptors and indicators used in IDAs. All complexes were prepared as the nitrate salts, except **4** (acetate).

Since the discrimination between PPi and ATP observed for the **2·3** chemosensing ensemble was not high enough to detect small quantities of PPi in the presence of excess ATP, we subsequently synthesised a small library of analogues (**5-9**) to investigate how selectivity for PPi could be improved by modifying the scaffold size, relative position of the two Zn(II)DPA groups and the steric bulk of the additional 'non-binding' side chains.[23,29,30] Leucine (Leu) and

phenylalanine (Phe) were incorporated to increase the steric bulk of the non-binding side chains, as it was expected that this might improve selectivity for PPi over the bulkier ATP and ADP. In IDAs, using the same indicator (**3**) as above but under slightly different conditions (pH 7.4, 5 mM HEPES buffer), we observed differences in binding affinity for PPi of over two orders of magnitude for this library, with **9** exhibiting the highest PPi affinity (log K_a = 9.8), while **5** had the lowest affinity (log K_a = 7.2).[29] Significant differences in the ability of these receptors to discriminate between PPi and ATP were also observed. In contrast to our expectations, for the series **2**, **5**, **6** in which the steric bulk of the non-binding side chains was varied, it was found that the smaller methyl side chains of the alanine (Ala) derivative **2** provided better selectivity for PPi over ATP and ADP than the more bulky and hydrophobic Leu (**5**) and Phe (**6**) analogues. In the case of the Phe derivative **6**, slightly higher affinity for ATP (log K_a = 8.6) than PPi (log K_a = 8.4) was observed, with molecular modelling suggesting that the increased affinity for ATP results from a secondary π–π stacking interaction of one of the Phe side chains with the adenine moiety (Figure 3), whereas the Leu derivative **5** was unable to discriminate between PPi, ATP and ADP (log K_a = 7.4 – 7.6 for all three anions). Moving the Zn(II)DPA binding sites next to each other on the peptide scaffold (**7**, **8**) also produced improved selectivity for PPi over ATP and ADP. Reduction of the size of the peptide scaffold to a hexapeptide (**9**) resulted in higher binding affinities for both PPi and ATP than the analogous octapeptide **7** (with similar discrimination between these two anions), even though the Zn(II)DPA binding sites are positioned the same distance apart in these two systems. Similar trends in the binding affinities were observed upon increasing the ionic strength of the media to better mimic physiological conditions (pH 7.4, 5 mM HEPES buffer, 145 mM NaCl) (Table 1). These results suggested that both the size and positioning of the non-binding side chains could be used to tune the properties of these receptors to provide improved discrimination of PPi from ATP and other similar polyphosphate derivatives.

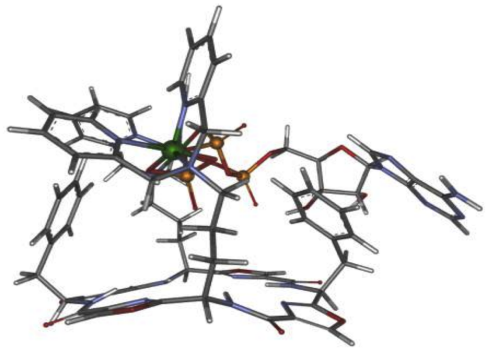


Figure 3. Molecular structure of the complex between receptor **6** and ATP illustrating the proposed π–π interaction with the phenylalanine side chain, modelled using SPARTAN 06 (MMFF94). Reprinted with permission from ref 29. Copyright 2012 John Wiley & Sons.

We also observed that in more complex media (Krebs buffer: 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 2.8 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 10 mM Tris base, 10 mM glucose, buffered to pH 7.4) these receptors displayed improved selectivity for PPi over ATP and ADP (Table 2). For example, for chemosensing ensemble **9·3**, only PPi was able to fully displace the indicator to provide a turn-on of fluorescence, with addition of ATP or ADP providing only minor fluorescence enhancements in this medium (Figure 4). This enabled the **9·3** chemosensing ensemble to detect small amounts of PPi in the presence of high concentrations of ATP, ADP and other di-and tri-phosphate derivatives, opening applications for these compounds in the monitoring of enzymatic reactions. These results illustrated the importance of the composition of the medium in indicator displacement assays, suggesting that the choice of both the indicator and media could be used to further tune the selectivity of the receptors in IDAs.

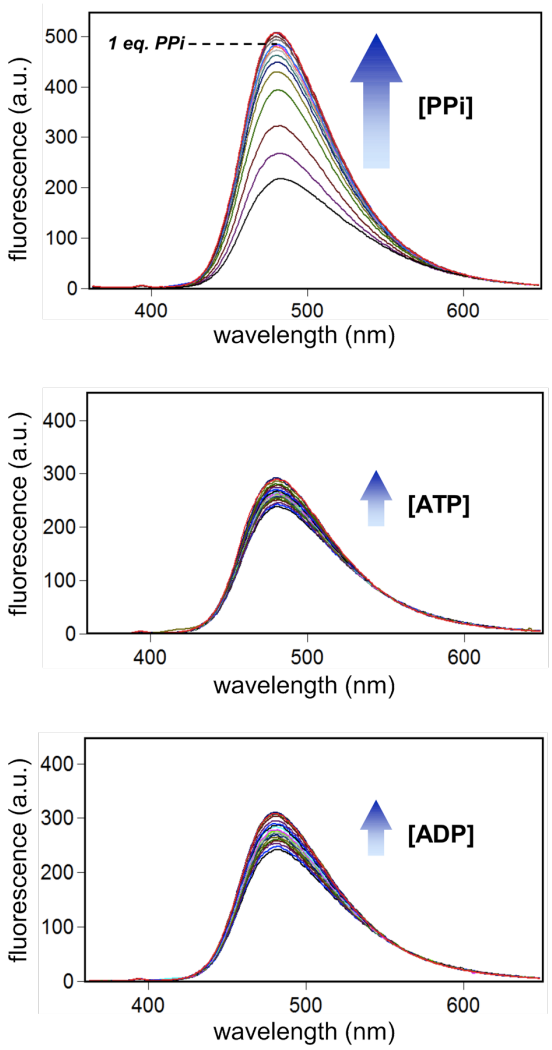


Figure 4. Representative change in fluorescence emission of the complex **9·3** (10 μM) in Krebs buffer upon the addition of 10 equivalents of: PPi (top); ATP (middle) and; ADP (bottom). Measurement conditions: aqueous solution of Kreb's buffer (pH 7.4), λ_{ex} = 347 nm, 25 °C. Reprinted with permission from ref 29. Copyright 2012 John Wiley & Sons.

We subsequently evaluated the effect of changing the indicator in the IDA on binding selectivity, using a series of colorimetric indicators and a modified library of receptors (**2**, **10-16**) in which the distance between the Zn(II)DPA groups and the cyclic peptide scaffold was varied, with Ala 'non-binding' side chains incorporated to favour PPi binding as determined above.[30] We had previously observed that decreasing the distance between the scaffold and

anion binding sites significantly improved anion recognition for hydrogen-bonding receptors based on similar scaffolds,[31] and reasoned that bringing the Zn(II)DPA sites closer to the peptide scaffold might provide additional hydrogen-bonding interactions between the anion and the peptide backbone amides. We found that receptors with shorter side chains (**10**, **11** and **13**) exhibited improved selectivity for PPi and that the cyclic tetraoxazoles provided better PPi selectivity than the smaller cyclic trioxazole scaffold.

The use of colorimetric indicators (pyrocatechol violet **17**, pyrogallol red **18** and bromopyrogallol red **19**) rather than the fluorescent indicator **3** allowed the development of ratiometric assays for detection of PPi and enabled 'naked-eye' detection of binding events (Figure 5). Excellent selectivity for PPi over ATP and ADP was observed with this receptor library in IDAs with **17-19** (Table 1), with the best selectivity observed when **19** was employed as the indicator (Figure 6). As observed previously, selectivity for PPi over other species was significantly improved in Krebs buffer (Table 2) as compared to HEPES buffer, allowing the accurate detection of PPi concentrations from 1-36 μM in the presence of 250 μM ATP (Fig. 7). In contrast, while receptor **1** also exhibits high affinity for PPi in an IDA using **17** in Krebs saline, the ability of the **1**·**17** ensemble to discriminate between PPi, ATP and ADP is considerably lower than that of the cyclic peptide derivatives, illustrating the importance of the peptide scaffold in obtaining selective binding.

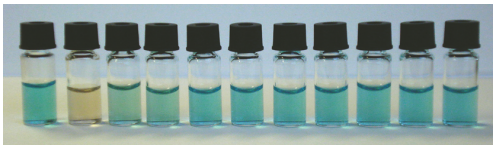


Figure 5. The colours of the 1:1 mixtures of **12**·**17** (20 μM) with and without anions (sodium salts) from left to right: no anion, PPi, ATP, ADP, AMP, cAMP, phosphothreonine, phosphoserine, phosphotyrosine, HPO_4^{2-} and citrate (5 equiv. each). Reprinted from ref. 30 with permission from the Royal Society of Chemistry.

Table 1. Apparent association constants (log K_a) determined using IDAs with **17** as indicator (pH 7.4, 5 mM HEPES buffer, 145 mM NaCl at 25 °C)[a,b]

Receptor	Indicator **17**	PPi	ATP	ADP
1[c]	8.8	>9	>9	>9
2[c]	8.8	>9	7.2	4.6
10[c]	8.9	>9	4.5	-[e]
11[c]	6.6	7.1	4.7	-[e]
12[c]	9.0	>9	7.2	4.6
13[c]	9.0	>9	6.7	-[e]
14[c]	8.4	>9	7.2	7.4
15[c]	8.5	>9	8.8	7.2
16[c]	7.4	8.4	5.4	4.6
20[d]	6.6	8.7	7.2	5.8
21[d]	6.6	>9	6.6	5.3
22[d]	5.4	7.8	5.4	4.2
23[d]	8.3	>9	7.1	5.3
24[d]	8.3	>9	8.2	5.5
25[d]	6.9	>9	6.7	5.0
26[d]	6.8	>9	6.5	4.9
27[d]	8.2	>9	7.8	5.3
28[d]	7.0	>9	6.4	4.6
29[d]	6.1	>9	5.9	4.7

[a]Estimated errors in log K_a ±10%; [b][receptor] and [**17**] were each 20 μM for the IDAs; [c]Data taken from ref 30; [d]Data taken from ref 32; [e]Indicator displacement too weak to fit to a binding model.

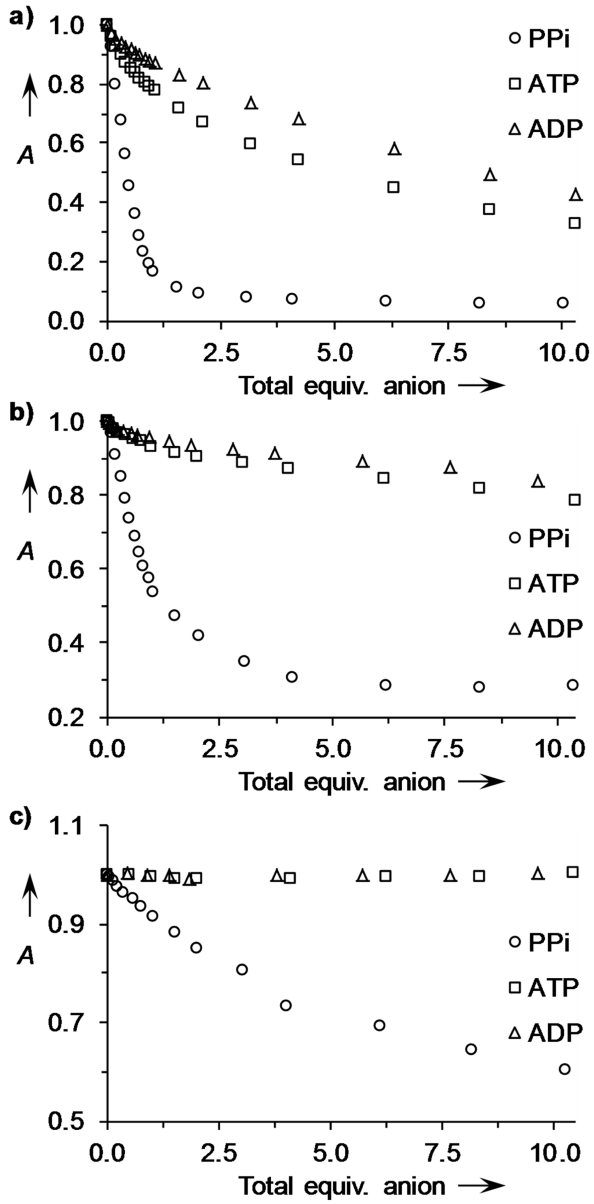


Figure 6. Changes in absorbance for 1:1 mixtures of a) **2·17** (20 μM each) at 640 nm; b) **2·18** (20 μM each) at 600 nm and c) **2·19** (10 μM each) at 610 nm; upon addition of up to 10 equiv. of anions (sodium salts) in aqueous solutions of HEPES buffer (5 mM, pH 7.4, 145 mM NaCl) at 25 °C. A = relative absorbance (arbitrary units). Reprinted from ref 30 with permission from the Royal Society of Chemistry.

Table 2. Apparent association constants (log K_a) determined using IDAs with **17** as indicator in Krebs saline at 25 °C, pH 7.4[a,b]

Receptor	Indicator **17**	PPi	ATP	ADP
1[c]	6.3	9.0	6.8	6.6
2[c]	8.5	8.7	-[f]	-[f]
10[c]	7.8	8.2	-[f]	-[f]
12[c]	8.2	8.4	-[f]	-[f]
13[c]	8.3	>9	-[f]	-[f]
14[c]	6.1	6.3	4.4	-[f]
15[c]	6.8	8.8	5.3	-[f]
16[c]	6.6	7.0	-[f]	-[f]
30[d]	7.2	7.7	4.5	4.2
31[d]	7.0	7.6	4.4	4.3
32[d]	6.5	6.9	4.3	4.0
33[d]	6.3	6.9	3.8	3.5
34[e]	6.9	7.0	4.5	4.0
35[e]	6.8	7.3	4.0	3.9
36[e]	6.8	7.5	4.1	4.0
37[e]	6.2	5.0	3.8	3.9
38[e]	6.8	5.9	4.1	4.1

[a]Estimated errors in log K_a ±10%; [b][receptor] and [**17**] were each 20 μM for the IDAs; [c]Data taken from ref 30; [d]Data taken from ref 32; [e]Data taken from ref 33; [f]Indicator displacement too weak to fit to a binding model.

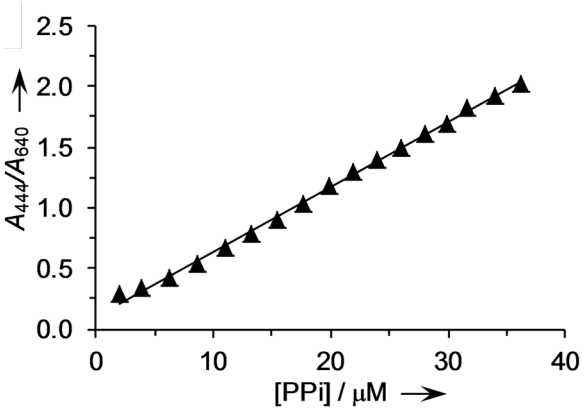


Figure 7. Calibration plot for PPi obtained from ratiometric absorbances at 444 nm and 640 nm using the **2**·**17** chemosensing ensemble (20 μM each) in the presence of ATP (250 μM). UV–vis measurements were carried out in Krebs saline solutions (pH 7.4) at 25 °C. R^2 = 0.998. Reprinted from ref 30 with permission from the Royal Society of Chemistry.

In contemporaneous studies, we evaluated the ability of linear peptides bearing two Zn(II)DPA binding sites adjacent to each other or with a spacing 'non-binding' amino acid (**20-29**) (Figure 8) to selectively discriminate between phosphate oxoanions in IDAs with **17**.[32] While these short linear peptides lack the preorganisation of the cyclic derivatives described above, their synthesis can be achieved entirely on solid phase, greatly simplifying the production of receptor libraries. We prepared a library of receptors incorporating a variety of side chains on the spacer amino acid. Despite their lack of preorganization, linear peptides bearing two Zn(II)DPA binding sites on the side chains were found to display high affinities and a distinct selectivity for di- and tri-phosphate derivatives (e.g. PPi, ATP, ADP) over monophosphates (e.g. AMP, HPO_4^{2-}, phosphorylated amino acids) in IDAs with indicator **17** (pH 7.4, 5 mM HEPES buffer, 145 mM NaCl) (Table 1). In all cases PPi was observed to bind with higher affinity than ATP or ADP and the introduction of non–binding side chains had observable effects on both selectivity and affinity, with the presence of hydrophobic residues providing enhanced affinity for both PPi and ATP. Receptors **28** and **29** with aromatic groups (e.g. Trp, Nal) on the spacer amino acid side chain were found to have increased selectivity for PPi over ATP, presumably due to steric clashes with the larger ATP anion.

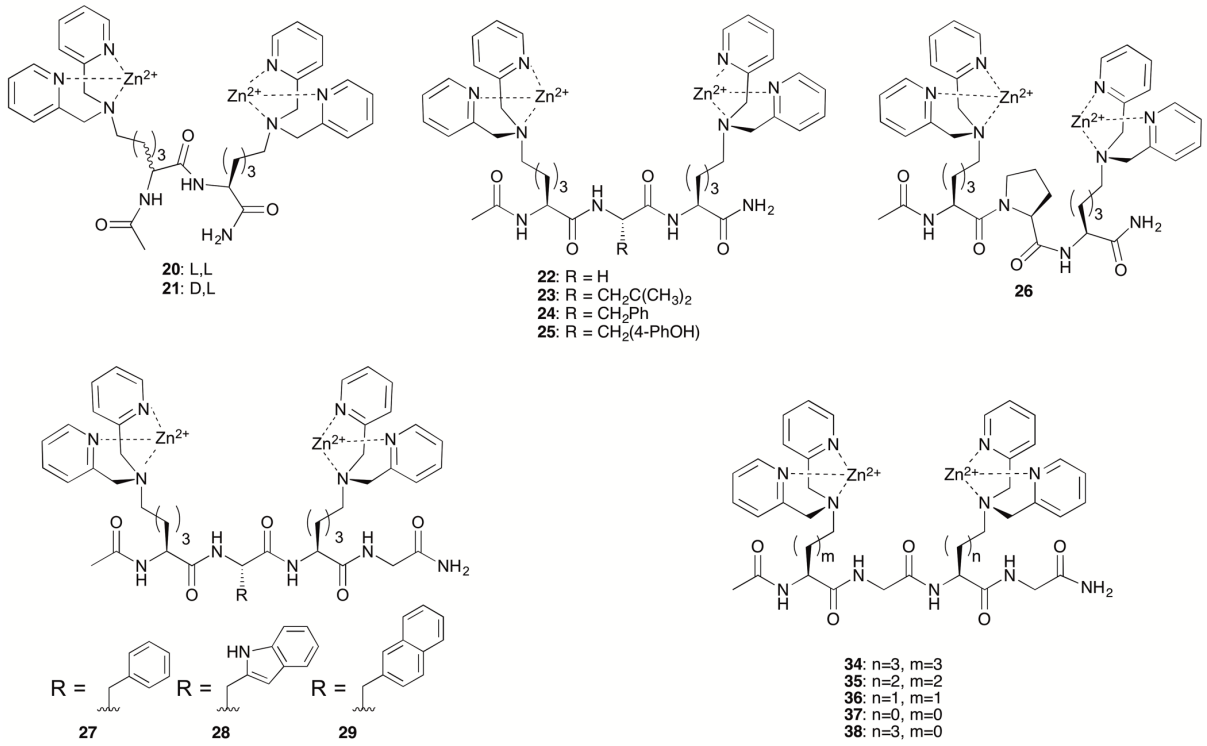


Figure 8. Structures of the bis(Zn(II)DPA) linear peptides used in IDAs. All complexes were prepared as the nitrate salts.

Alteration of the Zn(II) DPA binding sites on short peptide scaffolds was also evaluated, with the introduction of a triazole moiety to provide an additional coordination site for each Zn(II) centre (**30**, **31**) (Figure 9).[33] These compounds bound to **17** in a similar manner to the 'standard' Zn(II)DPA complexes (**32**, **33**) and were employed in IDAs to evaluate anion binding. In Krebs saline, similar anion binding trends were observed for compounds with and without the additional triazole unit, although slightly higher affinities and selectivities for PPi were found for compounds containing the triazole ligands (**30**, **31**) compared to those without (**32**, **33**), attributed to an increase in receptor binding site preorganization due to the extra coordination bond to the triazole unit.

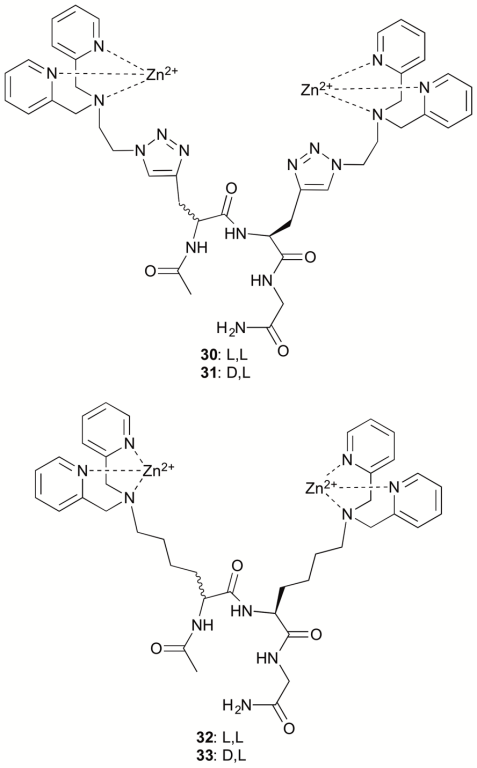


Figure 9. Structures of receptors **30-33**. All complexes were prepared as the nitrate salts.

A further series of linear peptides (**34-38**) was then evaluated in Krebs saline (Figure 8).[34] As previously observed for the cyclic peptide receptors, reducing the length of the side chains from four (Lys, **34**) to two (Dab, **36**) methylene units resulted in increased affinity for PPi and enhanced selectivity for PPi over ATP (Table 2). NMR studies suggested that the increased affinity for PPi was a result of the presence of secondary hydrogen bonding interactions from the peptide backbone to the anion in addition to the coordination interactions between the anion and the Zn(II) centres (Figure 10). Remarkably, in Krebs buffer, compound **36** binds to PPi with comparable affinity to the more preorganized cyclic peptide **15** (log K_a = 7.5 for **36**; 8.8 for **15**), and exhibits similar selectivity for PPi over ATP (log K_a for ATP = 4.1 for **36**; 5.3 for **15**), allowing ratiometric sensing of PPi in the presence of a large range of other anions. For example, in the presence of 200 μM ATP, chemosensing ensemble **36·17** can detect PPi

concentrations as low as 2 μM, with a linear calibration plot across a [PPi] range of 2-38 μM (Fig. 11).

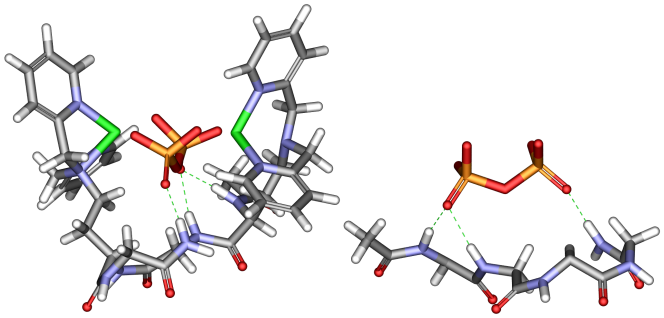


Figure 10. DFT-optimised molecular structures of: (left) the **36·PPi** complex and (right) the **36·PPi** complex upon rotation about the z-axis where the Zn(II)-DPA side arms have been omitted for clarity; hydrogen bonds are represented by the green dashed lines. Reprinted from ref 34 with permission from the Royal Society of Chemistry.

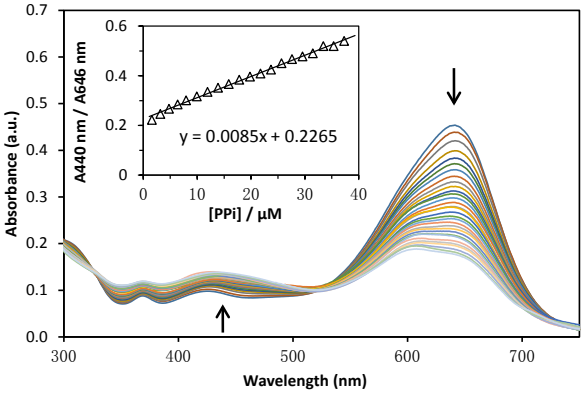


Figure 11. UV-Vis spectrum showing the changes in absorbance of a solution of **36·17** (20 μM each) in Krebs buffer (25 °C, pH 7.4) upon the addition of PPi (0.0–3.0 equiv.) in the presence of ATP (200 μM). Inset: Corresponding calibration plot for [PPi], A440 nm/A646 nm = f([PPi]). Reprinted from ref 34 with permission from the Royal Society of Chemistry.

Given the high PPi selectivity and affinity displayed by the **36·17** chemosensing ensemble, we evaluated its use in binding to PPi in artificial urine samples, since it has been suggested that

the determination of PPi concentration in urine might allow the diagnosis or monitoring of kidney related diseases.[35] Even in these very competitive conditions (1.1 mM lactic acid, 2.1 mM citric acid, 25 mM $NaHCO_3$, 166 mM urea, 0.2 mM uric acid, 7.1 mM creatinine, 2.5 mM $CaCl_2$, 89 mM NaCl, 0.004 mM $FeSO_4$, 2.0 mM $MgSO_4$, 9.9. mM Na_2SO_4, 7.0 mM KH_2PO_4, 6.9 mM K_2HPO_4, 24 mM NHCl, 1 g L^{-1} peptone L37, 0.01 g L^{-1} yeast extract powder) the **36·17** chemosensing ensemble bound PPi with log K_a = 6.8 (Figure 12) and allowed determination of [PPi] across a range of 2-22 μM in this simulated physiological fluid, illustrating the remarkable selectivity of these receptors for PPi.

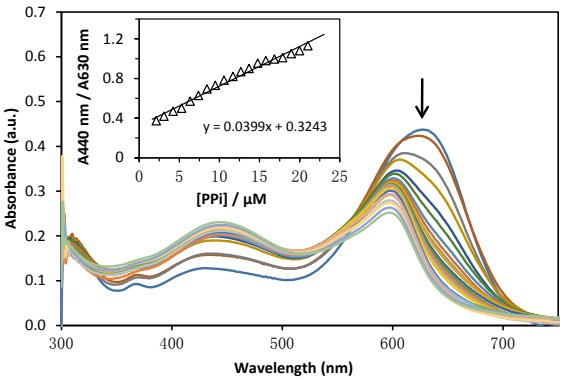


Figure 12. UV-Vis spectrum showing the changes in absorbance of a solution of **36·17** (20 mM each) in artificial urine (25 C, pH 7.4) upon the addition of PPi (0.0–2.0 equiv.); Inset: Corresponding calibration plot for [PPi], A440 nm/A630 nm = f([PPi]). Reprinted from ref 34 with permission from the Royal Society of Chemistry.

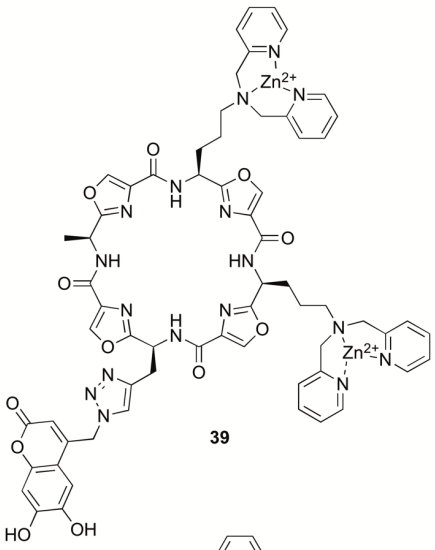
Overall, we have found that in IDAs the cyclic peptide based receptors provide slightly better selectivity and affinity for PPi than the linear receptors (Table 2). For both receptor classes, moving the Zn(II)DPA binding sites closer to the peptide scaffold provides improved selectivity for PPi over ATP and ADP. The effects of non-binding side chains are not as significant for the linear peptides as they are for the cyclic peptides. We attribute this to the

differences in structure between the two receptor classes; the non-binding sidechains are held in close proximity to the Zn(II)DPA binding sites in the cyclic peptides whereas the linear peptides are more flexible and can adopt conformations in which the non-binding side chain does not interfere with the anion binding sites.

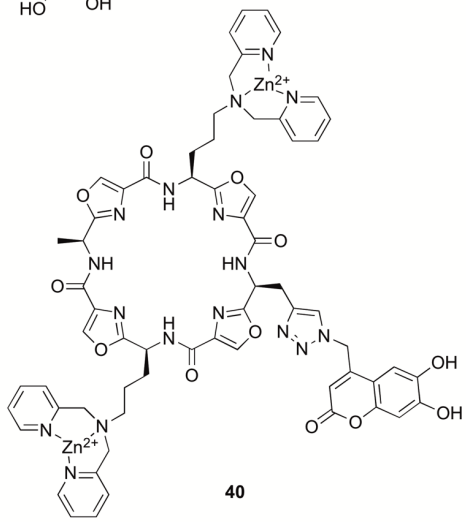
4. Anion Recognition with covalently attached indicators

While our peptidic Zn(II)DPA receptors displayed excellent selectivity and affinity for PPi using IDAs in physiological media, including cell growth buffer and artificial urine, there are some applications for PPi sensing for which IDAs are unlikely to be viable (e.g. determination of intracellular [PPi]). We envisaged that an intramolecular IDA,[36] in which the indicator is covalently attached to the receptor *via* a flexible linker that allows either binding to the Zn(II) centres or displacement, would provide a novel means by which to create PPi sensors that could be used in these applications. Therefore, we covalently attached a coumarin fluorophore to two cyclic Zn(II)DPA peptide scaffolds, to provide **39** and **40** for comparison with the analogous **2·3** and **12·3** chemosensing ensembles. We also prepared a linear peptide analogue **41** with the same covalently attached indicator (Figure 13).[37] Receptors **39** and **41** exhibited a large fluorescence enhancement upon addition of PPi (pH 7.4, 5 mM HEPES buffer, 145 mM NaCl, 25 ^{o}C) (Figure 14) and only minimal changes in emission upon addition of ATP or ADP, whereas receptor **40** exhibited a much smaller increase in fluorescence intensity upon addition of PPi and similar responses to ATP and ADP, indicating that **40** is both less sensitive and selective for PPi than **39** or **41**. This difference in response was attributed to the relative positioning of the indicator and the two Zn(II)DPA units on the peptide backbone. Whereas in **40**, the indicator is able to bridge both Zn(II) centres, making it difficult to displace, in **39** and **41** it is likely that the indicator binds to only the closest of the metal centres, making it easier

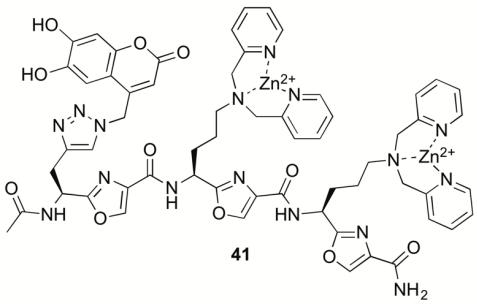
to displace than in **40**. When compared to the chemosensing ensembles **2·3** and **12·3** it was found that covalent indicator attachment in **39** and **41** provides a significant improvement in selectivity for PPi over ATP and ADP, although the sensitivity is lower as a result of the intra- versus inter-molecular indicator displacement process.



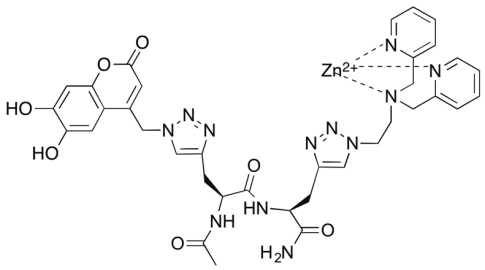
39



40



41



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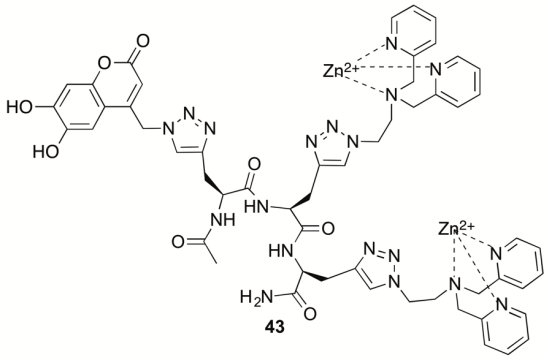

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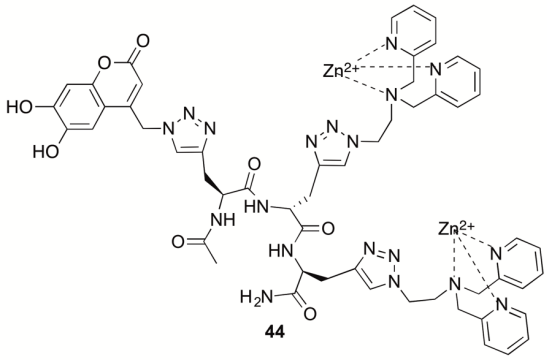

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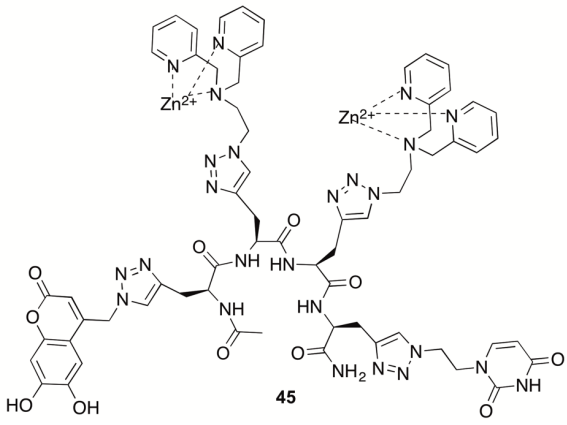

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Figure 13. Structures of Zn(II)DPA peptides with covalently attached indicators. All complexes were prepared as the nitrate salts.

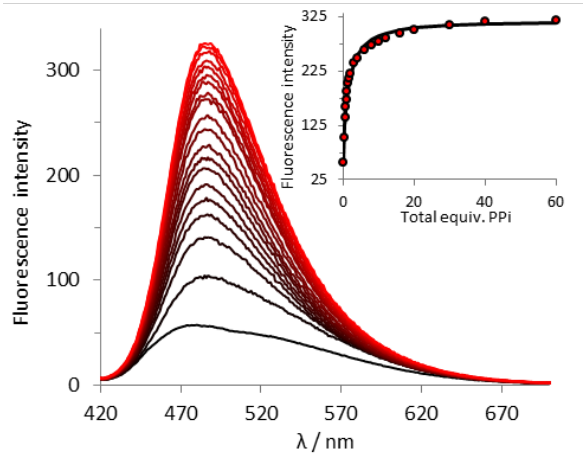

Figure 14. Fluorescence emission spectra of **39** (5 μM) in response to PPi (0-300 μM) in HEPES (5 mM, 145 mM NaCl, pH 7.4) at 25 °C. λ_{ex} = 390 nm, slits = 5 nm/5 nm. Inset: fitting curve for the titration data at 485 nm to a 1:1 binding model. Reprinted from ref 37 with permission from the Royal Society of Chemistry.

We subsequently employed **39** and **41** in the real-time monitoring of the activity of pyrophosphatase, an enzyme that catalyses hydrolysis of PPi to produce two phosphate ions. In these assays, PPi the decrease in emission of **39** and **41** at 485 nm was monitored as PPi was consumed in the enzymatic reaction, with larger quantities of the enzyme resulting in a more rapid decrease in fluorescence, consistent with a more rapid reduction in PPi concentration in the presence of more enzyme (Figure 15). However, the solubility of these receptors in Krebs

saline was too low to allow studies in this more competitive medium or use in determination of intracellular PPi concentrations.

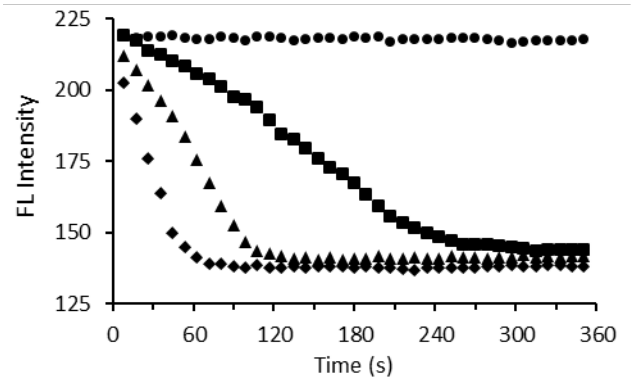

Figure 15. Real-time assay of PPi (80 μM) hydrolysis catalysed by different amounts of pyrophosphatase (● = 0 units, ■ = 0.5 units, ▲ = 1.0 units, ♦ = 2.0 units) in the presence of **41** (15 μM) in Tris buffer (10 mM, 10 mM $MgCl_2$, pH 7.5) at 30 °C, λ_{ex} = 390 nm, slits = 5 nm/5 nm. Reprinted from ref 37 with permission from the Royal Society of Chemistry.

Given the improved selectivity observed using the intramolecular IDA approach, together with the increased PPi affinities observed for linear peptides bearing the triazole-modified Zn(II)DPA units, we combined these two structural features to provide a new series of compounds **42-45** (Figure 13) and investigated their anion recognition properties.[38] Under our standard assay conditions (pH 7.4, 5 mM HEPES buffer, 145 mM NaCl, 25 ^{o}C) upon addition of a range of anions [PPi, ATP, ADP, AMP, cAMP, GTP, GDP, phosphothreonine, phosphoserine, citrate, sulfate, phosphate], only the addition of PPi resulted in a significant enhancement in fluorescence by **43-45** (Figure 16). In contrast, **42**, which only contains a single Zn(II)DPA unit, exhibited a weak fluorescence turn-on response upon addition of several anions, including PPi, ATP and ADP, indicating that two Zn(II)DPA units are required to

provide strong and selective binding to PPi. Emission data for titrations of **43-45** with PPi was fitted to a 1:1 binding model to give log K_a = 6.2, 5.8 and 5.6 for receptors **43**, **44** and **45**, respectively, indicating that these simple receptors bind to PPi with similar affinity and selectivity as the more structurally complex cyclic peptide **39**.

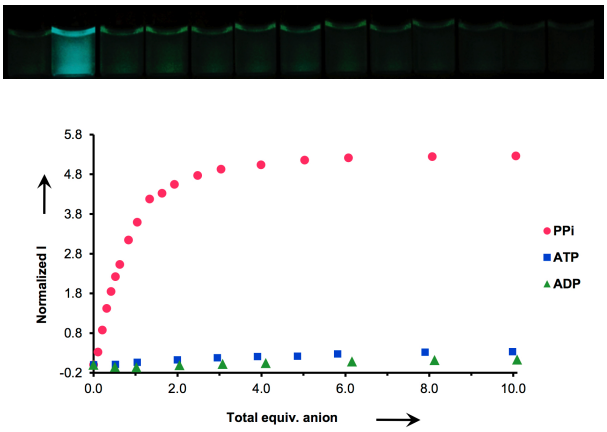

Figure 16. Top: Fluorescence changes of **43** (20 μM) in the presence of 5 equiv. of different anions (sodium salts) in aqueous solutions of HEPES buffer (5 mM, 145 mM NaCl, pH 7.4). From left to right: no anion, PPi, ATP, ADP, AMP, cAMP, GTP, GDP, phosphothreonine, phosphoserine, citrate, sulphate, phosphate. Bottom: Changes in fluorescence intensity of **43** (5 μM) at 480 nm upon addition of up to 10 equiv. of anions (sodium salts) in aqueous solutions of HEPES buffer (5 mM, 145 mM NaCl, pH 7.4) at 25 °C, λ_{ex} = 390 nm. Normalized I = normalized fluorescence intensity.

5. Conclusions

We have found that the use of peptidic scaffolds to position two Zn(II)DPA binding sites provides an effective method for the construction of receptors that show a remarkable ability to discriminate PPi from other polyphosphate derivatives, including nucleoside triphosphates. Both IDAs and receptors with covalently attached indicators can be employed for the selective detection of PPi in complex media such as cell growth buffer and artificial urine. However, it

remains to be seen whether these compounds will be capable of accurately sensing PPi concentrations in more complex biological samples e.g. in synovial fluid or in cells. The design of similar receptors capable of selective recognition of other polyphosphate ions, e.g. ATP, in the presence of PPi, to provide a suite of selective receptors for different phosphate species, also presents a future challenge.

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Biographical Information

Katrina (Kate) A. Jolliffe received her BSc (Honours 1) in 1993 and PhD in 1997 from the University of New South Wales. She then held positions at Twente University, The Netherlands; the University of Nottingham, UK and the Australian National University before taking up an Australian Research Council QEII research fellowship at The University of Sydney in 2002. In 2007 she became a Senior lecturer at the same institution and was promoted to Associate Professor in 2008. Since 2009 she has been Professor of Chemistry at The University of Sydney. A major focus of her research is the use of peptides as scaffolds in supramolecular chemistry.

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