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The Potential of Ion Mobility Mass Spectrometry for Tuning Synthetic Host Guest Systems: A Case Study Using Novel Zinc(II)dipicolylamine Anion Sensors

Chris Nortcliffe^a, Lukasz G. Migas^a, Xuejian Liu^b, Huy Tien Ngo^b, Katrina A. Jolliffe^b,
Perdita E. Barran^a

^a *The Michael Barber Centre for Collaborative Mass Spectrometry, The School of Chemistry,
Manchester Institute of Biotechnology, The University of Manchester,
perdita.barran@manchester.ac.uk*

^b *School of Chemistry (F11), The University of Sydney, NSW 2006, Australia,
kate.jolliffe@sydney.edu.au; Fax: +61 2 9351 3329; Tel: +61 2 9351 2297*

Abstract

Synthetic approaches to produce scaffolds that will act as efficient biosensors have been the subject of considerable research efforts in recent years. The preparation of host complexes which will specifically bind a given guest necessitates structural analysis of the potential complexes formed, as well as an assessment of the strength of the binding interaction. A challenge to the down-stream analytical scientist is to develop a rapid method that can probe the strength of binding, and the architecture of the complex. In this work we apply nano-ESI MS and Drift Tube ion mobility mass spectrometry (DT IM-MS) to examine host guest interactions. We present data on 5 cyclic peptide bis (Zn^{II}-dipicolylamines) which have been systematically designed as potential molecular biosensors for pyrophosphate (PPi). The competition of the macrocycles for PPi compared with other small anions is investigated. Mass spectrometry provides the stoichiometry of the complex, and the proportion of complex preserved into the mass spectrometer correlates with solution phase data. We find the macrocycles have a higher affinity for PPi than for ATP or pyrocatechol violet (PV). The

absolute scale of affinity from mass spectrometry is less ($4-5 \text{ Log } K_a$) than that found in solution ($4-9 \text{ Log } K_a$), but within the series of macrocycles trend in relative affinities are comparable. DT IM-MS shows that the collision cross sections ($^{DT}\text{CCS}_{\text{He}}$) of the macrocycles are unchanged on binding PPI, indicating that the anion is within the designed binding pocket. We also find that binding has tightened the conformation of the macrocycle, as revealed from analysis of the collision cross section distributions ($^{DT}\text{CCSD}_{\text{He}}$), where for 4/5 of the macrocycles there is a significant decrease in FWHM. Molecular mechanics (MMFF94) and semi empirical methods (ONIOM PM6:B3LYP/aug-cc-pvDZ) were used to generate candidate geometries for **4** to support our assertions on the nature of the binding pocket in the uncomplexed host, and to provide a route for improvement in the design.

Keywords

Ion Mobility-MS

Binding Studies

Non-covalent complexes

Pyrophosphate

Anion Sensing

1. Introduction

1.1. Mass Spectrometry

Mass spectrometry (MS) is widely used for studying many different molecular classes and with the development of soft ionisation, it is now possible to routinely analyse non-

covalently bound complexes in the gas phase[1,2]. As well as providing mass and hence stoichiometric information on the formed complex, mass spectrometry can also be used to measure the strength of binding interactions, yielding dissociation constants within minutes using only ng amounts of sample [3]. Furthermore using the more recently developed hybrid technique of Ion Mobility Mass Spectrometry (IM-MS), rotationally averaged collision cross sections can be measured yielding information on conformational changes occurring during complex formation [4–7].

1.2. Anion Sensing

Low molecular weight anions (<500 Da.) have many key biological functions[8–10]. They are often found in metabolomic[11] and cell signalling processes[12,13], and are also critical to enzyme turnover[14,15]. Cellular phosphate is an example: kinase enzymes add and remove phosphates to control protein activity and folding[16,17]; phosphates are also a major component in the DNA and RNA backbones of cells.[18–20] Pyrophosphate (PPi $\text{P}_2\text{O}_7^{4-}$), is a by-product of many enzymatic reactions, such as those carried out by DNA transferases and polymerases,[21] so the ability to directly detect PPi concentrations would allow real-time analysis of biochemical reactions such as DNA amplification[22]. Furthermore since the elevation of PPi is implicated in several illnesses (osteoarthritis, uremia, and acromegaly) a developed assay for PPi could be applied for medical benefit [23–25].

The first selective inorganic phosphate sensor was designed over 100 years ago, with the development of a molybdenum(IV) assay by Taylor and Miller in 1914[26]. Although this method has been improved over the years[27–32] it still has several flaws including the generation of toxic metal waste; it is also highly time consuming and suffers from interference from other anions which can lead to false positives in the results. Other methods

have involved chemical addition of chromophores to molecular receptors however these require special handling, high cost, and complicated synthetic procedures[33].

There has been a great deal of interest in the generation of selective anion sensors for use in aqueous environments [33–49], or which could be used directly upon biological samples; however this is a challenge due to the high hydration energies of the ions. The large size of the phosphate anion along with its high hydrophilicity, place it near the bottom of the Hofmeister selectivity series [50]. Further complications are caused by the acid-base properties of phosphate anions, and the large concentration of other biological anions such as chloride, carboxylates, and hydroxide.

Dipicolylamine (DPA) complexes have been proposed as a bio-compatible tool for anion sensing providing a tridentate ligand specific to Zn^{2+} as opposed to other common biological cations. Previous work has generated a series of macrocyclic peptides with two Zn(II)-DPA substituted side arms for the purpose of binding pyrophosphate[51–53] (Fig. 1). The receptors possess similar structural features with an oxazole-modified cyclic peptide of varying sizes with two DPA arms attached by varying length of linkers- this is shown in Fig. 1 as full chemical structures and simplified cartoons. Using indicator displacement assays (IDAs), previous work has revealed that inclusion of small methyl sidechains on the opposing side of the macrocycles to the linking arms favours the specificity of the binding pocket for PPi over ATP. However, differences in peptide binding affinity to the indicators (predominantly pyrocatechol violet) were also observed and the indicator displacement approach does not allow a direct measurement of binding affinity to the anions, providing only apparent K_a values. Synthesis and design of these molecules have been detailed in previously published work[51]. The five molecules (**1**, **3-5**, and **7** Fig. 1) can be separated into two categories: those with a three (**1**, **4**, **7**) or four (**3**, **5**) oxazole cyclic scaffold. Within each series differences include modifications of side arm length and position. Solution phase

studies of all 5 of the molecules shown in Fig. 1 showed full indicator displacement upon addition of 1 equivalent of PPI, suggesting a 1:1 complex and it was found that PPI bound more tightly than all other anions examined. The hypothesized binding mode for PPI-macrocycle, (supported through ^{31}P NMR experiments) involves PPI bridging between the two metal ions[54].

1.3. IM-MS

Ion mobility mass spectrometry (IM-MS) is a hybrid technique that separates ions based on mass, charge and conformation. Ion mobility allows the elucidation of structural isomers and ligand interactions by adding an extra dimension to a standard mass spectrometry experiment. Interesting structural features such as different binding modes can be tested against experimental data through the use of molecular modelling strategies to determine which model best fit with observed results. Many supramolecular systems have been studied using IM-MS as this technique allows assembly mechanisms and intermediate structures to be investigated [55–60].

In this work we use Drift Tube IM-MS (DT IM-MS). In a typical DT IM-MS ions are stored and pulsed into a cell of known length filled with inert buffer gas across which is a weak (5-40 Vcm^{-1}) electric field. The velocity at which the ion drift through the cell is proportional to the electric field applied, with the constant of proportionality being the mobility of the ion (K) and is dependent on the nature of the drift gas ion interaction and on the temperature of the drift gas. It is common to report K in term of the reduced mobility K_0 which is normalised for drift gas temperature and pressure. K_0 is inversely proportional to the ions rotationally averaged collisional cross-section (CCS) (Ω) as described by equation 1:

$$K_0 = \frac{3ze}{16N} \left(\frac{2\pi}{\mu k_B T} \right)^{1/2} \frac{1}{\Omega} \quad (1)$$

Where K_0 is the reduced mobility (measured mobility corrected to temperature and pressure) z is the ion charge state, e is the elementary charge state, N is the number gas density, μ is the reduced mass of the ion-pair, k_B is the Boltzmann constant and T is the buffer gas temperature. It is possible to generate theoretical CCS from atomistically resolved structures obtained *via* crystallography, NMR or from calculations or other sources[4,61–63]. In order to do this we rely on a number of algorithms which evaluate the CCS for a given set of molecular coordinates. This synergistic use of experiment and theory to obtain CCS values for comparison can be very powerful. It can be used to suggest candidate geometries to fit experimental findings and hence to understand the relation between structure, function and specificity[64–68].

MS and DT IM-MS are here employed along with computation to examine five macrocyclic peptides and their binding to pyrocatechol violet, PPi and ATP.

2. Experimental Details

2.1. Materials

All the macrocycles analysed here were synthesized as described previously [51–53,69]. Aqueous stock solutions of each macrocycle at 500 μ M were made up from lyophilised powder with 5mM ammonium acetate (Fisher Scientific, UK AR grade), and diluted to 50 μ M, 5mM ammonium acetate with 5% propan-2-ol (Fisher Scientific,) and the pH adjusted to 6.8. It was found that in solution conditions more commonly used for small molecule analysis (50:50 MeOH: H₂O 0.1% Formic Acid) the Zn²⁺ ions bound to the dipicolylamines dissociate and the cyclic peptides were mainly observed with the Zn²⁺ ions unbound (Data not shown). This was not the case in the aqueous ammonium acetate conditions, which also are more akin to those used for the solution phase assays[51], where we could retain the bound zinc ions, although it was necessary to use propan-2-ol to improve

the spray quality, especially with solutions containing the phosphate anion. Ligands were prepared at stock concentrations of 1 mM in 5 mM ammonium acetate and then serially diluted to values between 0-400 μ M. Ligands used were pyrocatechol violet (Sigma Aldrich, UK), tetra-sodium pyrophosphate (Sigma Aldrich, UK) & adenosine 5'-triphosphate disodium salt hydrate (Sigma Aldrich, UK). Each ligand was made up to a concentration of 50 μ M and was incubated with a macrocycle at an equivalent concentration for a stoichiometry of 1:1, for 20 minutes. No increase in binding was observed with longer incubation. For the K_d assay (see below) experiments were repeated at a range of anion concentrations (0-400 μ M).

Nano-electrospray tips were made using a Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA, USA) using 0.5mm walled glass capillaries. Ionisation potentials were applied through platinum wire (0.125mm Goodfellow) inserted into the pulled glass tips.

2.2. nESI-Mass Spectrometry

A Waters Micromass Q-ToF-2 quadrupole time-of-flight mass spectrometer (Manchester, UK) with an orthogonal nano-electrospray source was used to obtain all mass spectra. Instrument parameters in the source region were optimised to retain non-covalent complexes and maintained across experiments. Particular care was taken to minimise the source cone voltage since this correlated with optimum successful transfer of species that were complexes. Typical instrument source operating settings involved: capillary voltage ~1.4 kV, cone voltage ~25V, source temperature 80°C. Experiments were performed with three technical repeats; values reported are mean with standard deviation.

2.3. Determination of dissociation constants from nESI-MS data

Measurement of K_d from the mass spectra was performed based upon the technique shown by Sannes-Lowery, Griffey and Hofstader[3] with titrations of the peptide against solutions containing one ligand, along with two-ligand competitive binding experiments. nESI-MS allows the relative stoichiometries and abundances of both the holo and apo complexes simultaneously *via* their monoisotopic peak intensities. The intensities of each species including adduct peaks was summed and normalised to total macrocycle concentration. This is shown in equation (2) where R is the free macrocycle concentration, L is the free ligand, and RL is the 1:1 macrocycle- ligand complex.

$$K_d = \frac{[R][L]}{[RL]} \quad (2)$$

The amount of bound and unbound species was then compared and converted into the values of concentration of free ligand and ratio of molar concentration of bound ligand to total molar concentration of macrocycle. The total peaks owing to the macrocycle should be equivalent in intensity to the total concentration of macrocycle added. K_d 's were obtained by plotting these values against each other as $[RL]/[R]$ vs $[RL]$ yields a slope of $1/K_d$.

Several assumptions were made in the fitting of the K_d measurements which may have had an effect on the values obtained. Firstly it was assumed that all the species behaved similarly in the source of the instrument with respect to ionisation and retention of bound complexes. Next creating the baseline of the unbound species, all species owing to the macrocycle were utilised including those that did not have Zn^{2+} and those which had Na^+ and K^+ bound, however the absence or presence of these metal ions will have had a significant effect on the affinity of the macrocycle for the ligands, altering the results. Peak intensity was measured using the monoisotopic peak rather than the integration of peak area however previous work has shown this has little effect on K_d measurements when the mass of the complex and the uncomplexed ions are relatively similar [70,71]. Finally the starting

concentrations were created from stock solutions and are therefore subject to errors present in balances and pipettes which may have altered final solutions. K_d measurements are in μM and K_a are in M . Data is reported in K_a when we are making comparisons to previous work[51] (where this was the form of the equilibrium constant used). When comparing within the work presented here, K_d is used for clearer comparison of trends within data

2.4. Anion Competition Assay

nESI MS was used to determine the competition for binding of PPI and PV to selected macrocycles (**3** and **5**) because they both showed strong binding to each anion. Macrocycles were incubated with PV ($100\ \mu\text{M}$) for 20 minutes leading to the macrocycle: PV complex accounting for $\sim 100\%$ of the spectra. PPI was thereafter added to solutions in increasing concentrations and spectra were obtained measuring the appearance of the macrocycle: PPI complex.

2.5. Ion mobility Mass Spectrometry

IM-MS experiments were performed on an in-house modified Waters Micromass Q-ToF quadrupole time-of-flight mass spectrometer that includes a 5.1 cm drift cell (pressurised to ~ 4 Torr with He) located before the quadrupole as described previously[7]. The voltage across the drift cell was altered from $\sim 60 - 20\text{V}$ in seven discrete steps whilst temperature and pressure were recorded at each step and averaged over the acquisition time. Data was acquired for 5 minutes at each voltage and summed with all arrival-time distributions recorded synchronously with each injection of ions through the cell. Rotationally averaged collision cross-sections ($^{DT}\text{CCS}_{\text{He}}$) were obtained by plotting experimental arrival times

against P/V for each drift voltage (V) as described previously[7,72,73]. Experiments were performed with three technical repeats; values reported are mean with standard deviation.

2.6. Calculations

Molecular structures were built using ChemDraw [74] with previous ^{31}P NMR restraints in place[51]. The structures were pre-optimised using AVOGADRO and the MMFF94 level of theory. This was followed by ONIOM PM6:B3LYP/aug-cc-pvDZ calculations in Gaussian09[75]. In previous studies, ^{31}P NMR shifts have shown that PPI binds to Zn^{2+} via all four negatively charged O-P oxygen atoms in a symmetrical fashion. This binding mode was taken into account when generating potential structures. The structures were then used for collision cross section calculations using trajectory method in MOBCAL[76,77] and Projection Superposition Approximation (PSA)[78–81]; the calculated values were then compared to drift-time ion mobility measurements[82]. Full details are provided in Supplementary Information.

3. Results

3.1. nESI MS to provide anion affinity

Fig.2 shows nESI spectra in 5mM ammonium acetate for **3** with each of the anions examined here. In the full spectra (Supporting Information Fig. S1-5) all five macrocycles present mainly in two charge states with the majority for each as a $[\text{M}-2\text{H}\cdot 2\text{Zn}]^{2+}$ ion which is shown for **3** in an expanded region of the mass spectrum in Fig. 2(a-d). The assignment of the charge and elemental composition of each ion is supported by fitting theoretical isotopic distributions to the abundant ions (see inserts in Fig.2 (a-d)), and by the excellent mass agreement; for example **3** +2 species a measured mass of 564.145 versus a predicted of 564.142. Though the $[\text{M}-\text{H}\cdot 2\text{Zn}]^{3+}$ ion is observed at low abundance there is no evidence of

the $[M \cdot 2Zn]^{4+}$ species. For each macrocycle the +2 ion is most abundant and represents the macrocycle with the loss of two H^+ ions with two Zn^{2+} ions resulting in an overall charge of +2 (see Fig. 2 and supporting Fig. S1-5). For the bare macrocycle (Fig. 2(a) and S1-4(a)) some ions are observed due to the loss of one or both Zn^{2+} ions, this effect is most pronounced for **7**, which indicates that the binding of zinc ions is not as favoured for **7** as for the other macrocycles, a point which will be discussed later. The side chains are shortest on **7** affording it less conformational flexibility and possibly some steric hindrance for binding of the Zn(II) ions. Some binding of acetate was also observed in the unbound species, likely to the highly positively charged Zn^{2+} ions, and highlighting the softness of our nESI source.

Ligand bound species are observed at both charge states, with the ratio between bound and unbound being highly comparable for each charge state. In addition upon ligand binding there is no shift in the relative intensities of the unbound forms (Fig. 2B-D and Fig. S1-4B-D). It is interesting to note that upon ligand binding, all macrocycle: anion complex peaks are always observed with two Zn^{2+} ions bound, and no zinc loss species; this strongly suggests that in each case the ligand stabilises the dipicolylamine-Zn interactions in the gas phase and that the ligand binds preferentially to the di-zinc form of the macrocycle, as has previously been suggested by Hamachi and co-workers for other bis(ZnII)dipicolylamino complexes[83]. **7** exhibits the lowest affinity for PPI (Fig. S4(b)) which can be attributed to steric hindrance resulting from the short linker arms between the scaffold and dipicolylamine group. The two macrocycles with the larger scaffolds **3** and **5** display similar ratios between the intensities of the bound and unbound species despite the linker arms being located at different positions on the macrocycle, implying that there is sufficient flexibility between them that the mode of binding is unaffected by this orientation.

To quantify the effects of anion binding, the intensities of the monoisotopic peaks from each species were used to determine binding affinities for each ligand following a

method adapted from that previously described [3]. Table 1 reports the K_d values obtained from the nESI-MS titration experiments, which can be compared with those obtained previously (Table S1). There is generally good agreement between the nESI titration data and the colorimetric/UV binding assay; with both approaches the macrocycles showed higher affinity for PPi than PV, however we observed a greater affinity for ATP than PV for both **3** and **5** which differs from solution findings (Table 1 *cf.* Table S1). With the addition of a 1:1 equivalent of PPi to macrocycle, the unbound peak had almost totally disappeared in favour of the bound peak. The K_d values are displayed in Fig. 3, illustrating the relative affinity across macrocycles and ligands. All the trends match well with solution phase data with **4** showing the tightest binding to PPi and **7** showing the greatest discrimination between ligands. Lower dissociation constants are observed using MS than in the previous IDAs which can be attributed to several factors including: loss of anion during ionisation, the limit to μM concentrations causing inaccuracy for strong binding, and related issues with the dynamic range of the mass spectrometer as a detector. Solution phase data was measured in $\log K_a$ therefore we will compare the data in this format. Looking specifically at **5** the PPi binding constants were >9 in solution and 4.8 through mass spectrometry whilst ATP binding constants were 6.7 and 4.7 respectively. Mass spectrometry consistently shows weaker binding than found by UV. The strongest binding observed through MS is for **4** at 5.5 $\log K_a$ which is several orders of magnitude lower than solution. As shown in the original work differing buffers lead to different binding strengths, therefore it is safe to assume that the ammonium acetate values will differ from HEPES and Krebs buffers, however the retention of the trends points to the value of the techniques employed.

3.2. Competition Assay

Experiments were carried out to measure how easily PPi could displace PV from a macrocycle: PV complex. This experiment was designed to accurately recreate the solution

work and to show the effect of PV displacement on PPI dissociation constant. It was found that PPI easily displaced PV with a similar ratio of bound vs. unbound PPI observed (see Fig. 4 for data at stoichiometric ratios). This supports the solution phase results and the design rationale whereby the macrocycle bound to the dye is easily displaced by PPI leading to a visual colour change that can be measured.

3.3. IM-MS

Ion Mobility experiments were carried out on each apo macrocycle along with each of the three phosphate ligands. Collisional cross sections were obtained through measurement of arrival time distributions (ATDs) across a range of drift voltages which were converted to CCS through the use of eqn (1). This data is presented in Table 2 and Fig.S 5 and allows us to correlate binding and structural information.

We compare similar macrocycles to obtain trends from the ion mobility data. For **1,4** & **7** with each further reduction in linker arm length we see a corresponding reduction in cross section in the apo form of the complex from 219 Å² to 201 Å². However when binding ligands between **1** & **4** there is a reduction in ^{DT}CCS_{He} as expected from 218 Å² to 207 Å², however **7** shows a larger ^{DT}CCS_{He} for all Holo complexes than expected (216 Å²). **7** has the shortest linker arms of any macrocycle which may prohibit anions binding as tightly as in the other systems. Macrocycles **3** & **5** have equal length linker arms located at different positions on the ring. **5** shows a smaller cross section than **3** for the all complexes though the differences are more pronounced for the apo and PPI: holo complex. Complexes **1,4**, and **5** all show a reduction in ^{DT}CCS_{He} upon binding of PPI which indicates conformational tightening from a hollow complex to a filled one.[81] This tightening is best realised by a consideration of the widths of the collision cross section distributions (^{DT}CCSD_{He}) where it can be clearly observed that the PPI: Holo Complex is narrower than the Apo macrocycles (Fig.5). **7** shows

an increased $^{DT}CCS_{He}$ upon PPI binding however the width of the $^{DT}CCSD_{He}$ is reduced. **3** holo:PPI presents a wide $^{DT}CCSD_{He}$ (Fig. (5b)) with two major conformations observable in some ATD (data not shown). The $^{DT}CCSD_{He}$ ranges from a form that is smaller and more compact than the **3** Apo form, towards a more extended conformers. The Apo form of **3** already has the broadest distribution from $\sim 175 \text{ \AA}^2$ up to $\sim 350 \text{ \AA}^2$. These broad distributions are likely due to the alternate placement of the linker arms.

3.4. Calculations Results

In vacuo and solvated molecular dynamics calculations were carried out on **4**; the MD trajectories were analysed for the radius of gyration to determine the compactness of the modelled structures as well as root mean square deviation (RMSD) to determine the similarity of the model to the lowest energy structure (Fig.S6). Approx. 50 structures were extracted from each trajectory and their CCS_{He} were calculated in MOBCAL[76,77] and PSA[78–81].

The structures fell broadly into two distributions; one more compact and one extended (Supplementary Data Figures S6 and S7). *In vacuo* the compact structures range between ~ 210 to $\sim 225 \text{ \AA}^2$, whilst the extended group range from ~ 230 to $\sim 270 \text{ \AA}^2$. Structures as extended as this, which were not observed during the experiment, have a common feature of the linker arms spreading out from the macrocycle forming more flattened structures where Zn^{2+} ions are as separated as possible. The compact cluster retains a similar structure to that of the starting model with the linker arms extending above the macrocyclic ring. Fig. 6 shows the experimental arrival time distribution for the unbound macrocycle, which has been converted to a CCS_{He} and superimposed upon this is an example low energy structure for each of the compact, extended geometries observed. A lower populated geometry is classified ‘intermediate’ and is also shown.

Discussion

We have used nESI mass spectrometry to examine the structure of complexes formed between a series of peptide based macrocycles and biologically relevant anions, and to measure directly the strength of the binding interaction. We have followed the same principles that are common in the field of native mass spectrometry, where the synthetic complex is incubated in an aqueous salty solution and directly infused with nano-ESI conditions that are kept as gentle as possible. All macrocycles have affinities of 15 μ M or less for PPi, and each complex is readily observed, which supports the design criteria and the direct infusion approach taken. Macrocycles **1** & **4** have the highest affinity for PPi indicating that the optimum linker arm length for this anion is 2-3 carbon atoms. When the linker arm was reduced to a single carbon as in **7** the affinity for PPi is reduced, and the discrimination between PPi and PV was increased. For **3** and **5** where the arm location has been altered, the binding is also weaker than for **1** and **4**, implying that both arm length and location are critical for binding. The mass spectrometry method indicates less discrimination between the macrocycles for each anion (Table 1 *cf.* Table S1) although values found in the solution assays were taken with higher buffer strengths and were buffer dependent suggesting some competing effects that we have not examined here with the low salt concentration (5 mM) of the volatile ammonium acetate.

Collision cross sections of PPi and four of the macrocycle complexes (**1**, **3**, **4**, and **5**) change little on binding PPi suggesting the pocket is pre-configured for binding this anion—again indication of a good design. This is strongly highlighted in Fig. 5 whereby a narrowing of the arrival time distribution is observed indicating a reduction of flexibility. This reduction in width of the ATD is in the order of ~10 to ~50% (FWHM) between macrocycles. Given the mass increase on binding PPi, the equivalence of the $^{DT}CCS_{He}$ values for the bound and unbound forms implies conformational tightening in the complex. By manually docking the

anion into our low energy structure and using the Trajectory Method [66,67] we obtained a pseudo holo CCS_{He} of $\sim 250 \text{ \AA}^2$ which is larger than the apo form by $42 \text{ \AA}^2$. This highlights how well the anion fits into the designed binding pocket to provide a complex with the experimental $^{\text{DT}}\text{CCS}_{\text{He}}$ of $207 \text{ \AA}^2$. By contrast the **7**:PPi complex is 7% larger than apo **7**, suggesting a less well configured binding site. For each macrocycle the complexes formed with PV and ATP are larger than found for the apo and PPi bound forms, with the PV complexes consistently the largest (Table 2 and Fig. 5); this combined with reduced affinity measured (Table 1) suggests that the binding pocket is not as well tuned for PV. It can also be seen in Fig.5 (c) of the **4**:PV Holo complex that there is a broadening of the distribution further providing evidence that the binding pocket is not configured for this ligand. The larger complexes observed for ATP and PV may be due to steric hindrance from the non-binding side chains on the cyclic scaffold. PPi binds completely between the two Zn ions and therefore does not come in contact with these methyl groups. This evidence of conformational tightening only in the presence of the targeted anion, supports the receptor design process and viability.

Given that these macrocycles have been designed to bind PPi, it is interesting to consider PPi binding and $^{\text{DT}}\text{CCS}_{\text{He}}$ trends as a function of decreasing arm length (**1**, **4**, **7**) along with increasing the ring size (**4** *cf.* **5**). Between **1** and **4** there is a small decrease in cross section as would be expected but no great change in pyrophosphate binding, there is less specificity for PPi over PV. This could be due to an optimum flexibility in the arms that facilitates the most pre-organised binding site; the further reduction of arm length in **7** has reduced affinity for both anions as well an increase in cross section upon ligand binding. This is likely due to the shorter arms making ligand access more restricted forcing a greater conformational change. When comparing PPi complexes for macrocycles **4** and **5**, where the

arm length is the same and the ring size and position of the steric groups alter, the $^{DT}CCS_{He}$ values are very close and unaltered from the apo forms.

3 has the ring size of **5** and the arm length of **4** however with a differing placement of arms. From the cross section data we can see that the difference in $^{DT}CCS_{He}$ from **4** to **3** is greater than from **4** to **5**. This increase from the trend may be attributed to the parallel arm placement resulting in a comparatively larger sized complex for **3**. The fact that we have similar affinities for each ligand for **3** *cf* **5** indicates that with the optimum arm length (here 2 carbons) the placement of the arms does not alter the affinity any further. The multiple conformations that the PPI bound form presents in may be due to differing binding modes whereby the PPI may bind across the complex with each Phosphorus binding to both Zn^{2+} atoms rather than one each.

Candidate structures were generated for **4** using molecular dynamics and found to have their CCS_{He} ranging from ~ 211 - $\sim 270 \text{ \AA}^2$. The larger structures were found to belong to an extended conformation in which the two linking arms flex away from the macrocycle. The extended conformer is observed in both solution and gas phase calculations but not well sampled in the IM-MS experiments, suggesting that it collapses on the time scale of our experiment.

4. Conclusions

This paper has analysed the binding of a series of novel Zinc(II) dipicolylamine sensors for anionic ligands using a variety of mass spectrometry methods *borrowed* from those more commonly taken with protein ligand complexes. Anion receptors were shown to have greater affinity for PPI over the dye molecule PV thought due to the alignment of four negatively charged O-P bonds with the Zn^{2+} atoms. The reduced specificity to ATP could be attributed to only 3 O-P bonds being involved in binding; the lower charge density on each

oxygen atom involved in binding or the steric effect of the nucleoside base upon with the non-binding sidechains. Ion mobility has provided insights as to how macrocycle structure influences strength of ligand binding. We have shown how IM-MS can provide K_d values as well as insights to the binding mode which can be used to infer a mechanism. This highlights the role for ion mobility as a tool in structural drug design, with greater versatility than solution phase methods.

The high specificity of PPI over other anionic ligands results in a tight complex (**5** shows ~50% tighter cross section (FWHM)) that is smaller than the unbound macrocycle, testament to the selectivity of these anion receptors and to the success of their design.

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Figure Captions

Fig.1 Chemical structures of five macrocyclic peptides designed to bind pyrophosphates that have been investigated here. Molecules **1**, **4**, & **7** vary through the length of the hydrocarbon linker between the oxazole ring and the dipicolylamine groups. For **1** it is $(\text{CH}_2)_3$, **4** is $(\text{CH}_2)_2$ and **7** is (CH_2) . **3** & **5** include a larger ring with differing placement of the linker arms around the ring these species have the same length of linker arm which is $(\text{CH}_2)_2$. Simplified cartoons are presented next to each macrocycle to visualise trends.

Fig. 2 nESI spectra of the 2+ charge state of **3** for (A) the Apo form, and with: (B) PPi^{4-} , (C) PV^{1-} , (D) ATP. Insets show theoretical isotope fitting of labelled species superimposed on experimental isotope distribution. Conditions 50 μM **3**, 5mM ammonium acetate, 5% propan-2-ol, pH 6.8. (B) 200 μM sodium pyrophosphate (C) 200 μM pyrocatechol violet (D) 100 μM Adenosine 5'-triphosphate (ATP) disodium salt hydrate. Impurity peaks are observed and annotated as sodium (*) and potassium (**) adducts.

Fig.3 K_d (μM) Measurements for all anions and macrocycle molecules displayed with error bars to observe trends. Simplified cartoons of structures are shown beneath

Fig. 4 nESI spectra of the 2+ charge state for (A) Competition experiment between **3** with 50 μM PV and 50 μM PPi. (B) Competition experiment between **5** with 50 μM PV and 50 μM PPi Impurity peaks are observed and annotated as sodium (*) and potassium (**) adducts.

Fig. 5 Arrival time distributions (Converted to Collision Cross Section) ($^{DT}\text{CCS}_{\text{He}}$) For Apo and PPi: Holo complex of all macrocycles [A **1**, B **3**, C **4**, D **5**, E **7**]. All the macrocycles show evidence of conformational tightening in the presence of the ligand (except **3**). **4** has been shown with the ATD of the PPi: Holo complex to show that upon PV binding there is a large shift in CCS and an expansion of the structure.

Fig. 6 Experimental ($^{DT}ATD_{He}$) arrival time distribution for **4**. Annotations denote computationally generated structures with CCS predicted using the Trajectory Method.

Table Captions

Table 1. K_d values with associated errors for macrocycle binding experiments with a series of anions

Table 2 Rotationally averaged collisional cross-sections ($\text{\AA}^2$) ($^{DT}CCS_{He}$) for all macrocycles and ligands

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Table 1

	PPi	PV	ATP
1 · Zn ₂	4.9 ± 0.7	16.2 ± 1.4	
3 · Zn ₂	12.3 ± 2	25.8 ± 1.8	16 ± 0.5
4 · Zn ₂	3.5 ± 0.3	5.5 ± 0.5	
5 · Zn ₂	13.7 ± 2.3	18 ± 1.9	17.9 ± 2.6
7 · Zn ₂	14.9 ± 1.7	40.5 ± 8.9	

Errors quoted are associated with standard deviation of three biological repeats and accuracy of fitting.

Table 2

	Unbound	PPi	PV	ATP
1 · Zn ₂	218.9±1.8	218.6±0.61	275.5±5.4	256.52±3.8
3 · Zn ₂	240.7±3.7		289.7±3.9	276.7±3.2
		264.2±2.26/		
		282.9±3.4		
4 · Zn ₂	207.9±3.2	206.7±2.1	261.1±6.4	245.6±5.2
5 · Zn ₂	217.4±1.1	217.9±2.9	277.5±1.9	265.9±2.5
7 · Zn ₂	201.6±2.3	215.8±5.2	263±3.5	252.8±9.4

Estimated errors Standard deviation over three repeats