Conformational Study of an Artificial Metal-Dependent Regulation Site for Use in Designer Proteins

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Abstract. This report describes the dimerisation of glutathione, and by extension, other cysteine-containing peptides or protein fragments, with a 5,5′-disubstituted-2,2′-bipyridine or 6,6′-disubstituted-2,2′-6′-2″-terpyridine unit. The resulting bipy-GS₂ and terpy-GS₂ were investigated as potential metal ion dependent switches in aqueous solution, and were found to predominantly adopt the transoid conformation at physiological pH. Metal complexation with CuII and ZnII at this pH has been studied by UV/Vis, CD, NMR and ion-mobility mass spectrometry. ZnII titrations are consistent with the formation of a 1:1 ZnII:terpy-GS₂ complex at pH 7.4, but bipy-GS₂ was shown to form both 1:1 and 1:2 complexes with the former being predominant under dilute micromolar conditions. Formation constants for the resulting 1:1 complexes were determined to be log K_M 6.86 (bipy-GS₂) and 6.22 (terpy-GS₂), consistent with a higher affinity for the unconstrained bipyridine, compared to the strained terpyridine. CuII coordination involves the initial formation of 1:1 complexes, followed by 1.5Cu:bipy-GS₂ and 2Cu:1terpy-GS₂ complexes at micromolar concentrations. Binding constants for formation of the 1:1 complexes (log K_M 12.5 (bipy-GS₂); 8.04 and 7.14 (terpy-GS₂)) indicate a higher affinity for CuII than ZnII. Finally, ion-mobility MS studies detected the free ligands in their protonated form, and were consistent with the formation of two different Cu adducts with different conformations in the gas-phase. We illustrate that the bipyridine and terpyridine dimerisation units can behave like conformational switches in response to Cu/ Zn complexation, and propose that in future these can be employed in synthetic biology with larger peptide or protein fragments, to control large scale folding and related biological function.

Introduction

The primary, secondary, tertiary and quaternary structure of proteins, are all crucial in controlling biomolecular recognition events and biological function. In some cases small local structural changes can greatly impact the secondary, tertiary and even quaternary structure of a whole domain, and as a result can influence the biological activity or function. Regulation processes can arise from contact with other biomolecules, and can themselves sometimes be triggered in response to an external stimulus such as pH.[1] Substructure stabilisation or destabilisation, in response to an external stimulus or binding of an effector molecule, allows the protein to communicate molecular events over long distances, through a large variety of signal-response pathways.[2] Allostery is the generic term used to describe a regulation site which is distinct from the active site in a biomolecule.

Chemists have invested significant effort into trying to model or engineer allosteric sites.[3] One attractive application is to introduce these into artificial biomolecular constructs for use in synthetic biology.[4] Metal complexation by bipyridine (bipy) and terpyridine (terpy) have previously been exploited as allosteric switches in organic solvents,[5,6] and have been inserted into a range of macromolecules including polymers[7] and DNA.[8] A number of reports describe their insertion into peptide sequences,[9–11] however, the majority focus on using them to achieve dimerisation on metal ion complexation. Only a small fraction takes advantage of a cisoid-transoid conformational transition on metal ion complexation, in an effort to achieve allosteric regulation. Kelly and co-workers prepared several short peptides with a bipy unit introduced directly into the peptide backbone. The peptide sequence and the pH were found to influence the conformational state of the bipy, as a result the free ligand was only able to adopt the transoid conformation under alkaline conditions. At pH = 9.5 they demonstrated that CuII coordination led to a structural reorganisation of the bipy linker to the cisoid conformation, resulting in a secondary structure transition from random coil to β-sheet. However, the bpy linker could not be used as a switch at a physiologically relevant pH.[12] This highlights the need for a better understanding of the factors (c.a. protonation of pyridine, substituent nature and positioning) which govern the conformational state of bipyridine and related polypyridine...
units, if they are to be exploited as potential artificial allosteric regulation sites.

The aim of this work was therefore to study the potential for using these polypyridine units as conformational metal ion dependent switches in designed artificial protein architectures. We therefore prepared and studied two small model compounds comprising a polypyridine switchable unit substituted at either end with a short peptide (rather than a complex protein fragment). It was proposed that metal ion coordination would constitute the triggering event, resulting in a conformational change from transoid to cisoid and subsequent realignment of the two peptide moieties. Ultimately our goal is to extend this work to the alignment of larger peptides and small protein fragments, however, the preparation of model compounds containing short peptides (3 amino acids) will allow the conformational change of the polypyridyl linker to be studied in greater detail.

Results

Design and Synthesis

Various coupling techniques have been reported in the literature, however, in order for this approach to be widely applicable and for these polypyridyl units to be incorporated into structures based on natural protein motifs, we felt it was necessary to utilise native functionality for coupling. We therefore chose to take advantage of selective coupling to the sulphhydryl group of cysteine, the most nucleophilic naturally occurring amino acid. The model compounds reported here consist of polypyridyl linkers coupled through the Cys side chain of the naturally occurring tripeptide, glutathione (GSH).

Two different polypyridine units, differing in substitution pattern, were investigated. The conformation of the first, a 5,5'-disubstituted bipyridine (bipy) model, was proposed to be largely unaffected by the presence of metal ions, as upon transoid to cisoid rearrangement, the substituent at position 5 of the 2,2'-bipyridine experiences a 180° rotation around the 2,2'-inter ring linkage, relative to the substituent at position 5. As these two substituents are located along the rotation axis the distance separating them remains unchanged (see Figure 1A). In our design, one needs to further account for the length and the flexibility of the dimethyl sulfide bridge linking the polypyridyl unit and the peptide backbone. The dimethyl sulfide bridge can freely rotate (see Figure 1C), and is therefore able to effectively compensate for the rotation of the 2-pyridinyl. For these reasons 5,5'-disubstituted-2,2'-bipyridine units can be considered allosteric ineffective.[13]

The second model compound contains a 6,6'-disubstituted-2,2';6',2''-terpyridine (terpy) as the linker unit. In contrast to the bipy linker, a transoid to cisoid rearrangement of the terpy linker would alter the relative distance between the two peptide substituents. Methyl-methyl distances for cisoid and transoid conformations of both model compounds, were estimated based on reported structural information for analogous compounds (see Figure 1).[6,14–17]

![Figure 1. Schematic illustrating the impact of conformational transition in A) bipy-GS2 and B) terpy-GS2 on the inter-substituent distance and relative orientation. C) Illustration of the flexibility of the thioether linkage of (2-polypyrindine) substituted at position 5. Distances displayed are estimated based on reported structures for analogous compounds.[6,14–17] (DOI: 10.1002/zaac.201300274)](Image 307x565 to 547x734)

Circular Dichroism (CD)

The CD spectra of 350 μM solutions of bipy-GS2 and terpy-GS2, recorded from 400 to 200 nm, did not display any notable signal. However, the addition of increasing concentrations of ZnCl2 to the solution of bipy-GS2 at pH 7.4 led to the appearance of new positive transitions centred at 220, 241, 310 and 320 nm, as well as a negative transition at 266 nm, with isosbestic points at 251 and 285 nm (Figure 2A). In contrast, addition of ZnCl2 to terpy-GS2 resulted in negative transitions at 228, 329, and 340 nm and positive transitions at 283 and 290 nm, with isosbestic points at 270 and 298 nm (Figure 2B). A plot of molar ellipticity as a function of ZnCl2 concentration reaches a plateau at ca. 0.9 (bipy-GS2) and 1.0 equivalents (terpy-GS2) of ZnII per model switch (Figure 2).

![Image 453x739 to 548x757]
clear isosbestic points at 265 and 292 nm. Further addition of CuCl$_2$ resulted in a gradual shift of the positive transitions toward 251 and 310 nm, and the negative transition toward 282 nm. All signals decreased in intensity, reaching a minimum on addition of 1.5 equivalents of CuCl$_2$. No further spectral changes occur on addition of up to 3.0 equivalents CuCl$_2$ (see Figure 3A) The titration of increasing concentrations of CuCl$_2$ into a 350 μM solution of terpy-GS$_2$ at pH 7.4 resulted in the appearance of a negative transition centred at 215 nm. Two overlapping negative transitions at 335 and 347 nm appear on addition of more than 1.0 equivalent of CuCl$_2$. A plot of molar ellipticity as a function of CuCl$_2$ concentration reaches a plateau for all transitions at ca. 2.0 equivalents of Cu$^{II}$ per terpy-GS$_2$ (see Figure 3B).

In all four cases, addition of excess EDTA (20 equiv. with respect to metal) resulted in CD spectra which were in good agreement with those of bipy-GS$_2$ and terpy-GS$_2$ recorded in the absence of metal ions.

**UV/Vis Spectroscopy**

Similar to the UV/Vis spectra of unsubstituted 2,2’-bipyridine and 2,2’:6,2’-terpyridine,[19] the absorbance of bipy-GS$_2$ and terpy-GS$_2$ in aqueous solution is sensitive to the pH. The UV/Vis spectra of a 5 μM solution of bipy-GS$_2$ recorded between pH 6 and 10 display two transitions with $\lambda_{\text{max}}$ at 295 ($\varepsilon_{295}$ nm 19,600 m$^{-1}$ cm$^{-1}$) and 245 nm ($\varepsilon_{245}$ nm 15,200 m$^{-1}$ cm$^{-1}$), assigned as $\pi \rightarrow \pi^*$ transitions. The analogous terpy-GS$_2$ spectra display a peak with $\lambda_{\text{max}}$ 297 nm ($\varepsilon_{297}$ nm 20,300 m$^{-1}$ cm$^{-1}$) attributed to $\pi \rightarrow \pi^*$, however, the $\pi \rightarrow \pi^*$ transition which occurs around 221 nm, overlaps with that for the peptide bond (Figure 4 and S2). Upon acidification by addition of concentrated HCl, these bands decrease in intensity whilst new bands appear at lower energy. A plot of absorbance as a function of pH allows for an approximation of the associated pK$_a$ values (see Figure S2).

A similar red-shift of the $\pi \rightarrow \pi^*$ bands are observed upon Zn$^{II}$ and Cu$^{II}$ complexation, allowing an apparent binding constant to be determined[10] taking into account the competitive metal ion binding of the phosphate buffer employed in these experiments.[21] Aliquots of a stock solution of ZnCl$_2$ were titrated into a 5 μM solution of either bipy-GS$_2$ or terpy-GS$_2$ in 20 mM phosphate buffer pH 7.4. This resulted in the steady decrease in the absorbance at 295 (bipy-GS$_2$) and 297 nm (terpy-GS$_2$), and an increase in the absorbance at 308 and 340 nm (bipy-GS$_2$), and 330 and 340 nm (terpy-GS$_2$), respectively (see Figure 4A and B). A plot of the absorbance as a function of Zn$^{II}$ concentration indicates the formation of a 1:1 complex between Zn$^{II}$ and both the model ligands (see Figure 4E). The observation of an isosbestic point at 303 (bipy-GS$_2$) and 313
Figure 4. UV spectra for the metal titration of model switches. (A and B) ZnCl₂ or (C and D) CuCl₂ was added to solutions containing 5 μM of either bipy-GS₂ (A and C) or terpy-GS₂ (B and D) in 20 mM phosphate buffer pH 7.4. (black line) 0 equivalent metal added, (dark grey line) 1 equivalent CuCl₂ added, (light grey line) 1 equivalent ZnCl₂ added, (dark grey dotted line) or (light grey dotted line) between 0 and 1 equivalent of CuCl₂ or ZnCl₂ added, (dark grey half dotted line) or (light grey half dotted line) more than 1 equivalent added. For (C) buffer concentration was 100 mM and 20 mM glycine was added as competitor. (E) Plot of absorbance (monitored at 320 (H17039), 328 nm (H17040) for bipy-GS₂ and 340 (H17033), 335 nm (H17034) for terpy-GS₂) vs. the equivalence of metal ion. Line represents best fit for a 1:1 metal:model switch binding ratio.

Table 1. Summary of data obtained for CuII and ZnII coordination to model switches.

| Metal | λ/nm | ε_ML/ m⁻¹ cm⁻¹ | K_app | K_M | R² |
|-------|------|----------------|-------|-----|----|
| CuⅡ  | bipy-GS₂ | 328 | 1.95 ± 0.01 E +04 | 6.73 ± 0.27 E +05 | 3.39 ± 0.14 E +12 | 0.9987 |
|       | terpy-GS₂ | 335 | 1.34 ± 0.00 E +04 | 3.33 ± 0.10 E +06 | 1.09 ± 0.03 E +08 | 0.9997 |
|       |       | 347 | 1.53 ± 0.02 E +04 | 4.21 ± 0.19 E +05 | 1.38 ± 0.06 E +07 | 0.9988 |
| ZnⅡ  | bipy-GS₂ | 320 | 2.27 ± 0.02 E +04 | 1.21 ± 0.10 E +06 | 7.30 ± 0.61 E +06 | 0.9965 |
|       | terpy-GS₂ | 340 | 1.99 ± 0.03 E +04 | 2.77 ± 0.15 E +05 | 1.67 ± 0.09 E +06 | 0.9983 |

a) Titration performed with glycine (20 mM) as competitor in addition to phosphate buffer.

nm (terpy-GS₂) is consistent with the clean formation of the ZnⅡ complex. The extinction coefficient at 320 nm for bipy-GS₂ and [Zn(bipy-GS₂)Xₙ]⁺ were determined to be 1,180 m⁻¹ cm⁻¹ and 23,000 m⁻¹ cm⁻¹, respectively. The extinction coefficient at 340 nm for terpy-GS₂ and [Zn(terpy-GS₂)Xₙ]⁺ were estimated to be 800 m⁻¹ cm⁻¹ and 19,850 m⁻¹ cm⁻¹, respectively. Formation constants, log K_M, were calculated to be 6.86 ± 0.04 for [Zn(bipy-GS₂)Xₙ]⁺ and 6.22 ± 0.03 for [Zn(terpy-GS₂)Xₙ]⁺, see Table 1.

The analogous titration performed with CuCl₂, resulted in a decrease in the absorbance at 295 nm (bipy-GS₂) and 297 nm (terpy-GS₂), accompanied by an increase in the absorbance at 317 and 328 nm (bipy-GS₂) and at 335 and 347 nm (terpy-GS₂) assigned to the formation of [Cu(bipy-GS₂)Xₙ]⁺ and [Cu(terpy-GS₂)Xₙ]⁺, respectively. Monitoring the absorbance at 328 nm (bipy-GS₂) and 335 nm (terpy-GS₂) and plotting this as a function of CuCl₂ equivalence, is also consistent with the formation of a 1:1 complex with extinction coefficients estimated to be ε₃17 19,500 m⁻¹ cm⁻¹ ([Cu(bipy-GS₂)Xₙ]⁺) and ε₃35 13,400 m⁻¹ cm⁻¹, ε₃47 15,300 m⁻¹ cm⁻¹ ([Cu(terpy-GS₂)Xₙ]⁺). The isosbestic points at 305 (bipy-GS₂) and 315 nm (terpy-GS₂) are again consistent with a single equilibrium (see Figure 4C and 4D), Formation constants, log K_M, were estimated to be 12.5 ± 0.1 for
Figure 5. UV spectra for the CuII titration of model switches. CuCl2 was added to solutions containing either (A) 350 μM bipy-GS2 or (B) 100 μM terpy-GS2 buffered at pH 7.4. Plot of absorbance monitored at (C) 622 (■) and 440 nm (○) for bipy-GS2 and (D) 670 (■) and 500 nm (○) for terpy-GS2, vs. the equivalence of CuII.

[Cu(bipy-GS2)Xn]m+ and two slightly different values of 8.04 ± 0.01 (A335) and 7.14 ± 0.02 (A347) were obtained for [Cu(terpy-GS2)Xn]m+, depending on the wavelength monitored, see Table 1.

The addition of CuCl2 to solutions of bipy-GS2 and terpy-GS2 buffered at pH 7.4 was also accompanied by transitions between 400–900 nm. Aliquots of a stock solution of CuCl2 titrated into a more concentrated 350 μM solution of bipy-GS2, resulted in an increase in the absorbance at 622 nm up to 1.5 equivalents of CuII. Further addition of CuCl2 led to only a small increase in the absorbance at 622 nm and an increase at 440 nm, consistent with addition of CuCl2 to the blank buffered solution (see Figure 5A). An analogous titration of CuCl2 into a 100 μM solution of terpy-GS2 at pH 7.4, resulted in an increase in the absorbance at 675 nm up to 2.0 equivalents of CuII, see Figure 5B, which blue-shifted slightly to 670 nm on addition of between 1.0 and 1.5 equivalents of CuII. No further changes were observed upon addition of between 2.0 and 3.0 equivalents of CuCl2.

1H NMR Spectroscopy

The 1H NMR spectrum of a 5 mM solution of bipy-GS2 in D2O at pD 1, displays two singlets at δ = 8.83 and 8.42 ppm in the aromatic region, integrating to two and four protons, respectively. In contrast, one singlet at δ = 8.59 ppm and two overlapping doublets at 8.04 and 8.00 (AB pattern), each integrating to 2 protons, are observed on raising the pD to 7.4 (see Figure S3). A titration of ZnCl2 into a 5 mM solution of bipy-GS2 in D2O at pH 7.4, results in the broadening and decrease in intensity of the peaks at δ = 8.59, 8.04 and 8.00 ppm, and the appearance of new peaks at 8.66 (broadened, H6), 8.42 (doublet, H3) and 8.20 (doublet, H4) ppm on addition of 1.0 equivalent of ZnCl2 (see Figure 6A). A plot of peak integration of the overlapping doublets (8.04 and 8.00 ppm) as a function of equivalence of ZnCl2 indicates a 1:2 Zn:bipy-GS2 ratio. Similarly a plot of peak integration for the resulting doublet for the Zn-bipy-GS2 adduct at δ = 8.20 ppm, also plateaus at 0.5 equivalence ZnCl2 consistent with a 1:2 Zn:bipy-GS2 ratio (see Figure 6C). After 0.5 equivalents of ZnCl2 have been added the peaks at δ = 8.59, 8.04 and 8.00 ppm appear to have been replaced with broad new peaks at δ = 8.42 and 8.20 ppm. Upon addition of between 0.5 and 1.0 equiv. ZnCl2 these peaks sharpen into doublets and a broad peak attributed to H6 appears at higher frequency (δ = 8.66 ppm). Only very small changes are observed on addition of between 1.0 and 2.0 equivalents of ZnCl2 (see Figure 6A).

At acidic pD (ca. 1) the 1H NMR spectrum of terpy-GS2 recorded in D2O displays 5 aromatic signals (all doublets of doublets) at 8.68 (2H3a,b), 8.67 (2H4a,b), 8.59 (2H3), 8.47 (1H4) and 8.17 ppm (2H5a,b), which were assigned using COSY and NOESY NMR (see Figures S4 and S5). The COSY spectrum displays cross-peaks between H3–H1–H2 and H4–H1–H5a (n.b. H3a and H4a are too close, so the cross-coupling overlaps with the diagonal peaks), see Figure S5A. In contrast, NOESY NMR recorded under similar conditions, displays an additional H3a–H5a inter-ring coupling (see Figure S5B).

On raising the pD to 7.4, three aromatic resonances are observed at 8.08 (1 H4, 2 H3, 2 H3a), 7.96 (2H4) and 7.55 ppm (2H5a). A titration of ZnCl2 into a 5 mM solution of terpy-GS2 in D2O buffered at pH 7.4, resulted in the decrease in intensity of the peaks at δ = 8.08, 7.96 and 7.55 ppm, and the appearance of new broad peaks at δ = 8.31, 8.13 and 7.65 ppm.
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Figure 6. $^1$H NMR Zn$^{II}$ titration of bipy-GS$_2$ and terpy-GS$_2$ in solution buffered at pH 7.4 for (A) bipy-GS$_2$ and (B) terpy-GS$_2$. Plot of change in percentage peak integration as a function of equivalence of ZnCl$_2$ for (C) 8.04–8.00 ppm resonances for H$_3$ and H$_4$ of the free model switch, bipy-GS$_2$ (●), as well as for the new peak at $\delta$ = 8.20 ppm for H$_4$ of the Zn-bipy-GS$_2$ adduct (X), and (D) the terpy-GS$_2$ methyl singlet (♦) at $\delta$ = 3.99 ppm. The peak at $\delta$ = 3.96 ppm labelled with *, was attributed to the $^{13}$C satellite relative to the -N-CH$_2$-COOH signal of EDTA centred at $\delta$ = 3.72 ppm.

This is accompanied by a decrease in the intensity of the singlet at $\delta$ = 3.99 ppm assigned to the CH$_2$-pyridinyl group. A plot of the peak integration for the singlet at $\delta$ = 3.99 ppm, as a function of ZnCl$_2$ concentration (Figure 6B and D), is consistent with formation of a 1:1 complex between Zn$^{II}$ and terpy-GS$_2$.

In both cases, addition of excess EDTA (20 equiv. with respect to ZnCl$_2$) resulted in $^1$H NMR spectra which are in good agreement with those of bipy-GS$_2$ and terpy-GS$_2$ recorded in the absence of ZnCl$_2$ (see Figure 6A and 6B).

Ion Mobility Spectrometry (IMS) Mass Spectrometry (MS)

Ion mobility spectrometry (IMS) coupled to electrospray ionisation (ESI) mass spectrometry (MS), has been used to examine the model switches, bipy-GS$_2$ and terpy-GS$_2$, in the absence and presence of CuCl$_2$ and ZnSO$_4$. A single species is detected for the terpy-GS$_2$ model switch with a drift time (DT) of 6.72 ms, which is consistent with [M + H]$^+$ and for which a collision cross-section (CCS) of 193.6 Å$^2$ has been calculated (see Figure 7). When terpy-GS$_2$ is combined with 1.0 equivalent of CuCl$_2$, it forms a split peak, indicating the presence of 2 species, in a 60:40 ratio, separated by 1 Da. Extracting these and looking more closely at their isotopic distributions, it appears that these are consistent with [M+Cu]$^+$ and [M – H+Cu]$^+$ (see Figure 7). The [M+Cu]$^+$ species displays a near identical DT and CCS when compared to terpy-GS$_2$ in the absence of CuCl$_2$. However, the [M – H+Cu]$^+$ species with a DT of 7.06 ms, shows a small change in the calculated CCS (200.0 Å$^2$).

A single species is also detected for the bipy-GS$_2$ model switch in the absence of metal ions, with a DT of 6.02 ms, consistent with [M + H]$^+$ and a CCS of 179.3 Å$^2$ (see Figure S6). Similar results are obtained when bipy-GS$_2$ is combined with 1.0 equivalent of CuCl$_2$, i.e. 2 species separated by 1 Da and consistent with [M+Cu]$^+$ and [M – H+Cu]$^+$. The first species, [M+Cu]$^+$, displays a similar DT and CCS to bipy-GS$_2$, and the second species, [M – H+Cu]$^+$, displays a longer DT
Discussion

ZnII Coordination of Model Switches

Titration of ZnII into a 5 mM solution of terpy-GS2 monitored by 1H NMR, is consistent with a 1:1 binding ratio (see Figure 6B). Similar CD and UV/Vis titrations performed at micromolar concentrations, were also consistent with the formation of a 1:1 [Zn(terpy-GS2)Xn]m+ complex (where X can be an exogenous ligand such as a water molecule, hydroxide or chloride).

In contrast, the titration of ZnII into a 5 mM solution of bipy-GS2 indicates the formation of a 2bipy-GS2:1ZnII complex. This is in stark contrast to the analogous titrations (CD, UV) recorded under more dilute (14 times and 1000 times respectively) and biologically relevant conditions, suggesting that the formation of a 2:1 complex with bipy-GS2 only occurs at high concentrations, and that the 1:1
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That the lower energy transition for [Zn(terpy-GS2)Xn]m+ species dominates under more dilute conditions.

The relatively featureless CD spectra of the model switches, are altered dramatically upon coordination of ZnII. Titrations of bipy-GS2 and terpy-GS2, display chiral induced CD signals relative to the bipy and terpy π→π* bands (250–400 nm range). The cotton effect induced upon ZnII addition is opposite for terpy-GS2 and bipy-GS2 (see Figure 2). This indicates that the lower energy transition for [Zn(terpy-GS2)Xn]m+ might arise from π→π* electronic absorptions involving molecular orbitals composed mainly from atomic orbitals from atoms composing the central pyridine ring, as previously suggested. In contrast, the second lower energy transition (centred at 287 nm) for [Zn(bipy-GS2)Xn]m+ could involve orbitals comprising contributions mainly from the external pyridines, and is therefore more similar to the lower energetic absorption band for [Zn(bipy-GS2)Xn]m+ (see Figure 2). These observations are consistent with formation of a 1:1 complex with the ZnII coordinated to the intended polypyridine chelate of the model switches. Glutathione units might also contribute to the coordination sphere, but this currently remains unclear.

CuII Coordination of Model Switches

The shift in the bipy-GS2 and terpy-GS2 π→π* band (200–400 nm range) in the UV/Vis spectra upon addition of either CuII or ZnII, is consistent with a ca. 1:1 ratio in all cases. However, CD spectra suggest that the complexation of CuII is more complicated, and involves the formation of two different CuII complexes with differing contributions to the metal-bound π→π* bands. This is not obvious in the UV/Vis spectra in this range, but is observed for CuII complexation to both bipy-GS2 and terpy-GS2 by CD, due to exciton effects (see Figure 3). The CD titration of CuII into bipy-GS2 results in chiral induced signals relative to the bipy π→π* band (250–400 nm range) up to one equivalent consistent with the formation of a 1:1 complex involving coordination through the pyridine units. However, the CD titration indicates that this is followed by the formation of a 1.5Cu:bipy-GS2 complex, as a result of a reduction in these induced CD signals. In contrast, the analogous CD titration with terpy-GS2 did not result in the formation of induced CD signals relative to the terpy π→π* band up to one equivalent of CuII. However, it is consistent with the initial formation of a 1:1 complex, followed by a 2Cu:terpy-GS2 complex (see Figure 3B).

Similarly, monitoring of the metal-to-ligand charge-transfer (MLCT) band by UV/Vis spectroscopy (400–900 nm range), which appears upon coordination of CuII, indicates the final formation of a 1.5Cu: bipy-GS2 and 2Cu:terpy-GS2 complex (see Figure 5C and 5D). The MLCT for CuII coordination to the model switches in a 1Cu: bipy-GS2 and 1Cu:terpy-GS2 ratio, (bipy-GS2: λmax = 620 nm, ε620 = 51 M⁻¹ cm⁻¹; terpy-GS2: λmax = 675 nm, ε675 = 131 M⁻¹ cm⁻¹) is consistent with previous reports for the formation of [Cu(bipy-GS2)2(OH2)] and [Cu(terpy-GS2)2(H2O)]2+, as only a small difference in λmax is expected for substitution with chloride. Small MLCT shifts are observed upon addition of between 1–1.5 equiv. CuII (bipy-GS2) or 1–2 equiv. CuII (terpy-GS2), consistent with only minor changes to the Cu ion coordination environment. Similarly the lack of contribution from the π→π* on formation of the 1.5Cu:1 bipy-GS2 and 2Cu:1terpy-GS2 complexes, is consistent with no significant change to the polypyridine coordination chemistry with respect to the analogous 1:1 complexes.

We hypothesise that on formation of the initial 1:1 complexes, the CuII coordinates to the bipyridine or terpyridine ligand, and either exogenous water (or hydroxide) molecules or groups from the glutathione units (for example the N- or C- termini, amino acid side chains or amide bonds). However, the formation of the 1.5Cu:1 bipy-GS2 complex could involve the formation of a new intermolecular CuII coordination site between two Cu(bipy-GS2) complexes. Whereas on formation of the 2Cu:1terpy-GS2 complex, the second CuII could be coordinated exclusively by the glutathione units.

Binding Constants

The shift of the π→π* band was used to estimate the CuII and ZnII binding constants to the model switches. Titrations were performed at low micromolar concentrations, and where necessary a competitor was introduced. Plots of the lower energy absorbance maxima for the resulting complexes π→π* transition vs. metal ion concentration, were fit to a 1:1 model, as this shift corresponds to formation of the 1:1 polypyridine:metal complex (see Figure 4E). The binding constants reported in Table 1 are in good agreement with those reported previously for related ligands using similar methods.

Our model compounds, bipy-GS2 and terpy-GS2, display higher affinity for CuII than for ZnII, consistent with previous reports. Our model compounds, bipy-GS2 and terpy-GS2, display higher affinity for CuII than for ZnII, consistent with previous reports and bipy-GS2 displays a higher affinity for both CuII and ZnII than terpy-GS2. The latter observation is consistent with lowering of the binding constant for terpy-GS2 resulting from the strain introduced by substitutions at position 6- and 6”-, as previously reported for polypyridine amino-acid conjugates. In the case of terpy-GS2, fitting the data for the two π→π* transitions as a function of CuII concentration lead to different affinities, related by a factor of 10 (see Table 1). We postulate that these are due to the different CuII coordination environments, where the two species contribute differently to the absorbance at 335 and 347 nm (fitted to obtain the formation constants).

Conformational Study of the Model Switches

UV/Vis Spectroscopy

Nakamato first studied the pH dependence of 2,2-bipyridine and 2,2’-6’,2”-terpyridine in water, and demonstrated that at low pH, free bipy and terpy display similar absorption profiles to those of the metal complexes, consistent with a cisoid-conformation. However, the π→π* bands shift to higher energy on increasing the pH, and resembles those recorded in organic solvents, consistent with the transoid-conformation.
was hypothesised that deprotonation of the pyridyl ring on increasing the pH, resulted in a conformational transition of the bipy and terpy from cisoid- to transoid-, and has been supported more recently by theoretical studies. The equilibria are characterised by clear isosbestic points, and pKₐ values of 4.44 (bipy), 2.59 and 4.16 (terpy) were reported. Similar pH titrations performed on our model compounds, bipy-GS₂ and terpy-GS₂, are consistent with the reported protonation constants (Figure S2) and the presence of a predominantly transoid-conformation at physiological pH. These results importantly illustrate that introduction of short peptides into the design, does not alter the pH dependent behaviour of these poly(pyridine) switching units as monitored in solution under dilute micromolar conditions by UV/Vis spectroscopy. Therefore, unlike Kelly and co-workers' model switches adopt the transoid conformation at neutral and physiologically relevant pH. This could be due to coupling through an amino acid side chain, rather than introduction into the peptide backbone. Notably the addition of Cu²⁺ and Zn²⁺ to our bipy-GS₂ and terpy-GS₂ model compounds at pH 7.4, lead to a shift of π→π* bands toward higher energy, consistent with a transoid-to cisoid-conformational transition. Importantly these results indicate that metal ions can be used to control our model switches under biologically relevant conditions (under dilute conditions in aqueous solution and at a physiologically relevant pH). 

**1H NMR Spectroscopy**

The 1H NMR spectrum of bipy-GS₂ recorded under acidic conditions is very different from that recorded at neutral pH. A single resonance, attributed to H₃ and H₄ of bipy-GS₂, is observed at pH 1, however, an AB pattern where the two overlapping doublets are located at a lower chemical shift, is observed at pH 7.4. Addition of ZnCl₂ to a 5 mM solution of bipy-GS₂ at pH 7.4 results in new broad peaks which indicate a species in slow/intermediary exchange on the NMR time-scale. A plot of peak integration as a function of Zn²⁺ equivalence is consistent with the formation of a 2bipy-GS₂:1Zn complex. This spectrum at 0.5 equivalents Zn²⁺ does not display any signal assigned to H₆, most likely due to signal broadening as a result of the clash between the two bipyridine in the binary complex. However, a broad resonance assigned to H₆ reappears on addition of more ZnCl₂ (between 0.5 and 1 equivalent) and sharpens in the presence of excess Zn²⁺ (5 and 10 equivalents), which may be consistent with conversion of the 2bipy-GS₂:1Zn complex into a 1bipy-GS₂:1Zn complex. Theoretical studies suggest that even though monoprotonated bipyridine and bidentate metal complexes of bipyridine have energy minima with similar conformations (cisoid), flexibility around the axial bond of bipyridine is much higher in the monoprotonated bipyridine compared to the metal complexes. In fact, the difference in potential energy separating cisoid and transoid conformations has been reported to be comparable for the monoprotonated bipyridine and the free bipyridine. This could account for the similarity of resonances assigned as H₃ and H₄ in spectra of bipy-GS₂ recorded at acidic and neutral pD, which in turn differ from those recorded for the Zn²⁺ complex (see Figure S3). NMR spectra of bipyridine or derivatives where H₃ and H₄ resonances overlap have previously been recorded in aqueous solution at both acidic and physiological pH, however, this is not exclusively the case. Interpretation of bipyridine conformation based on NMR chemical shift can therefore lead to contradictory results. For example, theoretical studies suggest that H₃ are deshielded in the transoid (cation free) bipyridine due to the close proximity with the nitrogen on the second ring. Cation binding to bipyridine is expected to result in deshielding of aromatic resonances. In contrast, H₃ can display only a moderate deshielding or shielding upon transoid to cisoid conformational transition, as deshielding effects on H₃ from the proximal nitrogen are lost. Therefore an attractive method by which to assign the bipy-GS₂ conformation is by monitoring intra-ring coupling by NOESY NMR, however, this is only possible for an asymmetric bipyridine for which H₃ peaks are inequivalent, and so cannot be applied to bipy-GS₂.

In contrast, NOESY can be applied to terpy-GS₂. At pH 1, the cross-peak observed between H₃ and H₃a in the NOESY spectrum is consistent with at least half of the terpyridine adopting a cisoid conformation (see Figure S5B). The 1H NMR spectrum of terpy-GS₂ recorded at pH 7.4 is different from that recorded at acidic pH, and is not suitable for determination of intra-ring coupling as the resonances for H₃ and H₃a overlap. Upon raising the pH from 1 to 7.4, all aromatic signals move to lower frequency, and proton signals relative to external pyridine (H₃a, H₄a and H₅a) experience higher shielding than their counterpart (H₃ and H₄), consistent with a transition from mixed (cis-trans) conformation to a transoid (trans-trans) conformation. Addition of ZnCl₂ lead to formation of broad resonances for a 1terpy-GS₂:1Zn complex in slow/intermediary exchange on the NMR timescale. Signals are generally broad and difficult to assign, but integration of the three signals indicates that the spectrum is different from that recorded for terpy-GS₂ both at acidic and neutral pH, and could be consistent with the cisoid conformation of a terpyridine metal complex.

**Ion Mobility Spectrometry (IMS) Mass Spectrometry (MS)**

The transition between cisoid- and transoid- poly(pyridine) conformers is not the only conformational transition involved in our peptide conjugates of bipy or terpy. The thioether linkage and the peptide backbone will contribute to the overall global structure of the molecule and is likely to dominate when considering the molecules collisional cross-section (CCS). In an attempt to monitor the overall conformation of the model switches in the gas-phase, we have employed ion mobility spectrometry (IMS) couple to ESI-MS to study bipy-GS₂ and terpy-GS₂ in the absence and presence of Cu²⁺ and Zn²⁺. In the absence of any metal ions these measurements found that both bipy-GS₂ and terpy-GS₂ were detected in the protonated form in the gas-phase (samples prepared at pH 6.7), which is associated with the cisoid (bpy) and a mixed cis-trans confor-
Conformational Study of an Artificial Metal-Dependent Regulation Site for Use in Designer Proteins

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Conformation (terpy) in solution. Therefore, it is possible that any changes observed in the CCS can be attributed to changes in the orientation of the glutathione units, rather than a transoid-cisoid conformational transition.

As expected bipy-GS$_2$ traverses the mobility T-Wave with a short drift time (DT) and is consistent with a smaller CCS when compared with the terpy-GS$_2$ model. Coordination of Zn$^{II}$ to bipy-GS$_2$ or terpy-GS$_2$ leads to the formation of a species consistent with [M – H+Zn]$^+$ with a longer drift time but similar CCS to the model switch in the absence of Zn$^{II}$. The ion-mobility spectrum recorded for bipy-GS$_2$ in the presence of Zn$^{II}$ indicates the formation of at least three different species. We have not been able to assign the remaining two, but they may involve partial decomposition of the complex and cluster formation in the gas-phase.

The addition of Cu$^{II}$ resulted in the detection of two different species by IMS. One of these Cu adducts could be consistent with Cu$^+$ replacing the proton and has a near identical drift time and CCS to the model switch in the absence of metal ions. The second Cu adduct has been assigned as [M – H+Cu]$^+$ (consistent with Cu in the +2 oxidation state), is detected after a longer drift time and has a slightly larger CCS than the model switch in the absence of metal ions. Comparison of the CCS measurements suggests that there is potentially some structural difference, which we propose is due to complexation of the bipyridine/terpyridine and subsequent repositioning and potential coordination of the glutathione units. Formation of Cu$^{II}$ complexes in the gas-phase involving a mixture of bipyridine and peptide ligands, have previously been described.[43] Both the Cu adducts are formed in nearly equal amounts on coordinating to the terpy-GS$_2$ model switch (ca. 60:40), however, the second Cu adduct, [M – H+Cu]$^+$, is almost exclusively formed on coordination to bipy-GS$_2$ (ca. 5:95). This would be consistent with formation of polybipyridine-peptide complexes with different stability constants in the gas-phase,[43] similar to the formation of a more stable bipy-GS$_2$ complex with Cu$^{II}$ in aqueous solution. The different CCS for the various species formed (Cu/Zn) could be due to the different preferred metal ion coordination geometries. Similar metal ion conformational dependence in the gas-phase was described previously in an IMS study of the Gramicidin peptide.[44]

Conclusions

In conclusion, two model switches for the metal dependent spatial alignment of protein fragments in synthetic biology, have been prepared and studied in aqueous solution by UV/Vis, CD, NMR and IMS-MS. The model switches, bipy-GS$_2$ and terpy-GS$_2$, contain a polybipyridine linker (either based on 5,5'-disubstituted-2,2'-bipyridine or 6,6'-disubstituted-2,2':6',2''-terpyridine) coupled through the native Cys side chain of the tripeptide glutathione. This approach can be exploited to align these fragments in order to achieve enhanced biomolecular recognition.

Experimental Section

NaHCO$_3$, Na$_2$SO$_4$, NaNO$_2$, NaOH, KOH, mono- and dihydrogen potassium salts, Tris Base, ethylene diamine tetra acetic acid (EDTA), chloroform (CHC$_3$), methanol (CH$_3$OH), ethanol, tetrahydrofuran (THF), toluene, dichloromethane (CH$_2$Cl$_2$), acetonitrile, dioxane, water (HPLC grade), hydrobromic acid 48%, HC$_1$ 32%, ammonia 35%, bromine, were all obtained from Fisher Scientific. 5,5'-Di-(methyl-2,2'-bipyridine) used in the reaction was obtained from Sigma-Aldrich. 2,6-dibromomethyl-2,2'-bipyridine, tetrakis-(triphenylphosphine) palladium, trifluoroacetic acid (TFA), tributyltin chloride, zinc chloride (ZnCl$_2$) were obtained from BOC. N-Bromosuccinimide was obtained from Alfa-Aesar, and l-glutathione from Fluka (> 97 % pure by HPLC, as a sum of enantiomers). Deuterated solvents (CD$_3$Cl and D$_2$O) were obtained from Cambridge Isotope Laboratory Inc.

HRES and ES-TOF MS were recorded with a Micromass LCT TOF spectrometer equipped with a 3000 V capillary voltage, and a cone voltage of 35 V. GC–MS were recorded with a Waters GCT Premier Micromass equipped with an Electronic Impact (EI) probe.

Synthesis of 5,5’-Dibromomethyl-2,2’-bipyridine

5,5’-Dimethyl-2,2’-bipyridine (0.389 g, 2.11 mmol), N-bromosuccinimide (0.756 g, 4.23 mmol) and azobisobutyronitrile (10 mg, 0.06 mmol) were dissolved in dichloromethane (20 mL), refluxed using a 500 W halogen lamp, and the reaction progress monitored by TLC (SiO$_2$, eluent: CH$_2$Cl$_2$/CH$_3$OH (9/1)). After 4 hours, more N-bromosuccinimide (0.375 g, 2.10 mmol) was added and the reaction was refluxed for a further 2 hours. The mixture was allowed to cool to room temperature and the solution extracted with 0.1 M aqueous NaHCO$_3$ (5 × 20 mL). The organic layer was dried with Na$_2$SO$_4$, filtered, and concentrated in vacuo. The solid was re-dissolved in 15 mL of a 50/50 mix CHCl$_3$/CH$_3$OH, and the resulting solution stored...
in the freezer overnight. A white solid precipitate was collected by filtration, air-dried, and recrystallized from CHCl₃ to afford white crystals (0.219 g, 30%). ¹H NMR (300 MHz, CDCl₃): 8.69 (d, J₁H₆₋₅₁ = 2.1 Hz, 2 H, H₁), 8.41 (d, J₁H₅₋₄₁ = 8.2 Hz, 2 H, H₂), 7.86 (dd, J₁H₄₋₅₁ = 2.3, J₁H₅₋₆₁ = 8.2 Hz, 2 H, H₃), 4.54 (s, 4 H, H₄). ¹³C NMR ¹H decoupled (100 MHz, CDCl₃): 155.4 (C₂), 149.4 (C₆), 137.9 (C₄), 134.1 (C₁), 121.4 (C₉), 29.6 (C₇). HRES-TOF (CH₃Cl₂): calculated mass for C₂₆H₂₆Br₂Na = 362.9108; measured = 362.9126 ([M + Na⁺], 100%).

**Synthesis of 2-Bromo-6-methylpyridine**

2-Bromopyridine (10.8 g, 115 mmol) in hydrobromic acid (40 mL, 48%) was cooled to −20 °C in an ethanol bath. Bromine (14.4 mL, 280 mmol) was added dropwise, and the solution was stirred for 90 min at −20 °C. An aqueous solution of NaNO₂ (30 mL, 9.090 g, 50%). ¹H NMR (300 MHz, CDCl₃): 7.43 (t, J₁H₄₋₅₁ = J₁H₅₋₄₁ = 7.7 Hz, 1 H, H₁), 7.29 (d, J₁H₅₋₄₁ = 7.9 Hz, 1 H, H₂), 7.10 (d, J₁H₄₋₅₁ = 7.4, 1 H, H₃), 2.54 (s, 3 H, H₄). ¹³C NMR ¹H decoupled (100 MHz, CDCl₃): 160.2 (C₁), 141.5 (C₆), 138.7 (C₄), 125.2 (C₃), 122.2 (C₂), 24.3 (C₇). HRES-TOF (CH₃Cl₂): calculated exact mass for C₁₂H₆Br = 170.9684; measured = 170.9688: ES-TOF m/z = 171.0 ([M⁺]+, 50%), 92.0 ([M-Br]+, 100%), 65.0 ([M-C₆H₅]+, 75%).

**Synthesis of 6,6'-Dimethyl-bipyridine**

A solution of 2,6-dibromopyridine (1.39 g, 5.9 mmol), 2-tributylstannyl-6-methyl-pyridine (5.02 g, 13.1 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.38 g, 0.33 mmol) was refluxed in degassed toluene (40 mL) for 5 days under a nitrogen atmosphere. Extra tetrakis(triphenylphosphine)palladium(0) (0.38 mg, 0.33 mmol) was added and the reaction was refluxed for a further day under a nitrogen atmosphere. The crude mixture was then concentrated in vacuo, and dichloromethane (50 mL) and 6 M hydrochloric acid (10 mL) added to form a dark brown slurry. The aqueous layer was washed with dichloromethane (2 × 50 mL) and the combined organic layers were washed with 6 M hydrochloric acid (3 × 10 mL). The combined aqueous layers were filtered and cooled in ice. Ammonia was slowly added until a light brown solid precipitated out. The resulting solid was filtered, air dried, redissolved in dichloromethane, dried with Na₂SO₄ and concentrated in vacuo. The crude product was purified over chromatography column (Al₂O₃, hexane/DCM gradient) to afford the pure 6,6’-dimethyl-2,2'-6,2''-terpyridine as a white solid (0.63 g, 41%). ¹H NMR (300 MHz, CDCl₃): 8.46 (d, J₁H₅₋₄₁ = 7.8 Hz, 2 H, H₁), 8.41 (d, J₁H₄₋₅₁ = 7.8 Hz, 2 H, H₂), 7.93 (t, J₁H₄₋₅₁ = 7.8 Hz, 1 H, H₃), 7.73 (t, J₁H₅₋₄₁ ~ J₁H₆₋₅₁ = 7.7 Hz, 2 H, H₄), 7.19 (d, J₁H₆₋₅₁ = 7.6 Hz, 2 H, H₅), 2.65 (s, 6 H, H₆). ¹³C NMR ¹H decoupled (100 MHz, CDCl₃): 158.0 (C₆), 155.9 (C₅), 155.8 (C₄), 137.8 (C₇), 137.1 (C₈), 123.3 (C₉), 121.0 (C₁), 118.3 (C₂), 24.8 (C₃). HRES-TOF (CH₃Cl₂): calculated mass for C₁₈H₁₈N₆Sn = 384.1164; measured = 384.1152: ES-TOF m/z = 284.0 ([M + Na⁺], 100%).

**Synthesis of 6,6''-Dimethyl-2,2':6',2''-terpyridine**

A solution of 6,6'-dimethyl-2,2':6',2''-terpyridine (0.44 g, 1.7 mmol), N-bromosuccinimide (0.77 g, 4.3 mmol) and azobisisobutyronitrile (10 mg, 0.06 mmol) in dichloromethane (30 mL) was refluxed using a 500 W halogen lamp. Progress of the reaction was monitored by TLC (SiO₂, eluent: CH₂Cl₂/CH₃OH (9/1)). After 15 hours, further N-bromosuccinimide (0.3 g, 1.7 mmol) was added and the reaction refluxed for a further 17 hours. The mixture was extracted with 0.1 M NaHCO₃ (5 × 50 mL), the organic layers dried with Na₂SO₄ and concentrated in vacuo. Addition of a 50/50 mix CHCl₃/CH₃OH (15 mL) and storage at −20 °C overnight resulted in the formation of a white precipitate. This was collected by filtration, air-dried, and recrystallized from CHCl₃ to afford white crystals (0.117 g, 58%). ¹H NMR (300 MHz, CDCl₃): 8.53 (d, J₁H₅₋₄₁ = 7.8 Hz, 2 H, H₁), 8.52 (d, J₁H₄₋₅₁ = 7.8 Hz, 2 H, H₂), 7.96 (t, J₁H₄₋₅₁ = 7.8 Hz, 1 H, H₃), 7.86 (t, J₁H₅₋₄₁ ~ J₁H₆₋₅₁ = 7.8 Hz, 2 H, H₄), 7.50 (d, J₁H₆₋₅₁ = 7.7 Hz, 2 H, H₅), 4.66 (s, 4 H, H₆). ¹³C NMR ¹H decoupled (100 MHz, CDCl₃): 160.4 (C₅), 151.6 (C₄), 155.1 (C₆), 138.0 (C₈), 137.0 (C₉), 123.6 (C₇), 121.6 (C₈), 120.4 (C₆), 34.3 (C₃). HRES-TOF (CH₃Cl₂): calculated exact mass for C₁₇H₁₇N₅Br₃Na = 441.9353; measured = 441.9355 ([M + Na⁺]).

**Synthesis of bipy-GS₂: 5,5'-Bis(methyl-S-glutathionyl)-2,2'-bipyridine**

A solution of L-glutathione (30.3 mM, 5 mL, 0.152 mmol) in 100 mM Tris·HCl buffer pH 8.0 was added to a solution of 5,5'-bis(bromomethyl)-2,2'-bipyridine (15.3 mM, 5 mL, 0.152 mmol) in acetonitrile. The resulting suspension was degassed with N₂ for 10 minutes and then stirred for 11 hours at room temperature. The solvent was evaporated in vacuo at 50 °C to yield a pink gel. Deionised water (ca. 5 mL) and few drops of HCl (35%) were added to the gel resulting in complete solubilisation. The pH was neutralised on addition of NaOH (1 M solution). The product was purified by preparative RP-HPLC (C18 Phenomenex, monitoring absorbance at 210 and 290 nm) using 0 to 15% gradient acetonitrile in water (containing 0.05% TFA) over 30 min. The solvent was evaporated in vacuo to yield pure 5,5'-bis(methyl-S-phine).
glutathionyl)-2,2′-bipyridine as a pink solid (42 mg, 68 %). $^1$H NMR
(300 MHz, D$_2$O, pH ~ 1): 8.68 (dd, J$_{H9-H10}$ = 8.1, J$_{H8a-H9}$ = 6.2 Hz, 2 H, H$^8$), 8.67 (dd, J$_{H13-H12}$ = 8.1, J$_{H12-H11}$ = 2.7 Hz, 2 H, H$^{11}$), 8.59 (dd, J$_{H11-H10}$ = 7.5 Hz, 1 H, H$^9$), 8.47 (dd, J$_{H9-H8}$ = 7.1, J$_{H8a-H8b}$ = 8.8 Hz, 1 H, H$^8$), 8.17 (dd, J$_{H12-H11a}$ = 6.2, J$_{H11a-H11b}$ = 2.5 Hz, 2 H, H$^{11a}$), 4.56 (dd, J$_{H9a-H9b}$ = 8.2, J$_{H9b-H10a}$ = 5.5 Hz, 2 H, H$^{10a}$), 4.38 (s, 4 H, H$^{10a}$), 4.01 (t, J$_{H9a-H9b}$ = 7.5 Hz, 2 H, H$^{9b}$), 3.89 (s, 4 H, H$^{9b}$), 3.15 (dd, J$_{H11-H10a}$ = 14.2, J$_{H10a-H10b}$ = 5.6 Hz, 2 H, H$^{10b}$), 2.94 (dd, J$_{H8a-H8b}$ = 14.2, J$_{H8b-H8a}$ = 8.3 Hz, 2 H, H$^{8b}$), 2.51 (dd, J$_{H11-H11a}$ = 7.0, J$_{H11a-H11b}$ = 7.9 Hz, 4 H, H$^{11b}$), 2.14 (m, 4 H, H$^{11b}$), $^{13}$C NMR $^1$H decoupled (125 MHz, D$_2$O): 174.8 (C$^1$), 173.3 (C$^2$), 172.7 (C$^3$), 172.1 (C$^4$), 155.6 (C$^5$), 148.4 (C$^{5a}$), 148.1 (C$^{5b}$), 147.7 (C$^6$), 142.5 (C$^7$), 128.6 (C$^8$), 126.2 (C$^9$), 124.3 (C$^9$), 53.3 (C$^9$), 52.8 (C$^9$), 41.6 (C$^{10}$), 33.9 (C$^{10}$), 33.6 (C$^{10}$), 31.5 (C$^{10}$), 26.0 (C$^{10}$); HRES-TOF (water): calculated exact mass for C$_{37}$H$_{45}$N$_9$O$_{12}$S$_2$Na = 894.2527; measured = 894.2542; ES-TOF: m/z = 795.5 ([M + H]$^+$), 100 %, 817.2 ([M + Na]$^+$), 45 %.

**Synthesis of terpy-GS$_2$ ·6,6″-Bis(methyl-S-glutathionyl)-2,2′:6,2″-terpyridine**

To a suspension of 6,6″-bis(bromomethyl)-2,2′:6,2″-terpyridine (2.52 mM, 25.2 μmol, 10 mL) in acetonitrile, was added 100 mM aqueous Tris.HCl buffer pH 8.0 (5 mL) and a solution of t-glutathione (20.1 mM, 5 mL, 0.100 mmol) in the same buffer. The suspension was degassed with N$_2$(g) for 10 min, heated to 45 °C and stirred at this temperature for 8 hours. The solvent was evaporated in vacuo at 50 °C to yield a colourless gel. Deionised water (ca. 5 mL) and few drops of HCl (35 % w) were added to the gel resulting in complete solubilisation. The solution was allowed to equilibrate for 10 min prior to recording the pH on a Jenway 3510 pH meter, and recording a UV/Vis spectrum.

For the metal titrations, aliquots of an aqueous 0.75 mM stock solution of either CuCl$_2$ or ZnCl$_2$, were titrated into 3 mL of a 5 μM solution of model switches in 20 mM potassium phosphate buffer pH 7.4, and the spectrum recorded after 3 min equilibration. Non-linear fitting were performed with Kaleidagraphe software version 4.0. $K_{app}$ values were calculated by fitting data for the absorbance maximum of the metal complexes as a function of Cu$^{2+}$/Zn$^{2+}$ concentration, to Equation (1) and (2): $A = \frac{[\text{Ligand}][\text{M}]}{K_{app} + [\text{Ligand}][\text{M}]} + \text{Abs}_{blank}$

(1)

$[\text{Ligand}][\text{M}] = \frac{[\text{M}] + K_{app} + \frac{[\text{Ligand}]}{K_{app}}}{2} - \frac{[\text{Ligand}][\text{M}]}{K_{app}}$

(2)

The constant b corresponds to the cuvette pathlength, [complex] corresponds to the concentration of 1:1 complex, [Ligand] the total bipy-GS$_2$ or terpy-GS$_2$ and [M] the total CuCl$_2$/ZnCl$_2$ concentration at each point.

In order to ensure an accurate estimation, measurements were performed at concentrations close to the apparent dissociation constants, such that:

$\frac{[\text{Ligand}]}{[\text{M}]} < \frac{1}{K_{app}} < [\text{Ligand}]$

The $K_{app}$ values were corrected by accounting for the contribution from phosphate metal ion binding, based on values reported in the literature,\textsuperscript{21} see Equation (3):

$K_{app} = K_m \ast (1 - \frac{K_{MPM}[P]}{1 + (K_{MPM}[P])})$

(3)

This is based on the assumption that the amount of free phosphate is equal to the total amount of phosphate in solution. $K_m$ and $K_{MPM}$ correspond to the estimated binding constant of the metal ion to the ligand of interest and the phosphate anion, respectively. When glycine was present, $K_m$ was estimated using Equation (4), based on the reported Cu$^{2+}$ binding constant for glycine.\textsuperscript{47} The contribution from the phosphate anion was regarded as negligible, when 20 mM glycine was present.

$K_{app} = K_m \ast (1 - \frac{K_{GM}[Gly]}{1 + (K_{GM}[Gly])})$

(4)

**NMR Spectroscopy**

All $^1$H and $^{13}$C NMR spectra were collected with either Bruker DRX500 (500 MHz $^1$H and 125 MHz $^{13}$C, T = 300 K), AVIII400
(400 MHz ¹H and 100 MHz ¹³C, T = 298 K) or AVIII300 (300 MHz ¹H, T = 293 K) spectrometer equipped with a 5 mm probe. Titrations were performed by addition of aliquots of a 0.1 M stock solution of ZnCl₂ in D₂O to a 5 mM solution of polyppyridyl-conjugate in 50 mM phosphate buffer pH 7.4 in D₂O, and either 4 mM dioxane (δ = 3.75 ppm) or 1 mM acetone (δ = 2.22 ppm) was used as an internal reference. Addition of two equivalents ZnCl₂ resulted in no more than a 10% increase to the total volume. 0.8 mL of a 0.25 M solution of ethylenediaminetetraacetic acid (EDTA) in D₂O (pH adjusted to 8), and 100 μL D₂O, were then added (20 equivalents EDTA vs. Zn) to the NMR sample, resulting in a dilution of the sample by two. Changes in peak integrations (see Figure 6C and 6D) are reported relative to the internal dioxane reference.

2D NMR of terpy-GS₂ in D₂O at acidic pD (dioxane internal reference), were first recorded on AVIII400 (400 MHz, T = 298 K): DQF-COSY and Gradient NOESY spectra (400 ms mixing time). Additionally, Gradient NOESY (States-TPPI, 450 ms mixing time) and Phase cycle ROESY (States-TPPI, 450 ms mixing time, 10 KHz spin lock field and an offset of 10 KHz in spin lock period to minimise HOHAHA effects) experiments were recorded on DRX500 (500 MHz, T = 300 K, partial presaturation of water signal), both displaying the previously mentioned inter-ring coupling.

Chemical shifts (δ) are given in parts per million (ppm) to higher frequency compared to the methyl signal of the sodium salt of 3-(trimethylsilyl)propanesulfonic acid at 0 ppm.[48] Data were processed using Bruker Topspin version 2.1 (300 and 400 MHz) or 1.3 (500 MHz) and Mestrenova Lite version 5.2.5.

Ion Mobility Spectrometry (IMS) Mass Spectrometry (MS)

Samples of bipy-GS₂/terpy-GS₂ in methanol:water (1:1) were diluted to a final concentration of 5 μM and the pH adjusted to 6.7, prior to infusion. Similar solutions were prepared with 1.0 equivalent of either CuCl₂ or ZnSO₄ and 1.4 (bipy-GS₂) or 1.9 equivalent (terpy-GS₂) of CuCl₂, the pH adjusted and the samples allowed to equilibrate for 10 minutes prior to subsequent dilution and infusion. IMS was used to separate ions in the gas phase based on their mobility in the nitrogen carrier gas, and subsequently identify them by ESI-MS. IMS-MS experiments were performed on a Waters Synapt G2 based on a previously reported procedure.[49] The T-Wave ion mobility device was calibrated with a species of known collision cross-section (CCS) determined using standard drift tube instruments. Polyalanine was employed as a calibrant to measure against and include into our collision cross-section (CCS) calculation.

Supporting Information (see footnote on the first page of this article): Analytical HPLC (Figure S1), UV/Vis pH titrations (Figure S2), ¹H NMR spectra (Figures S3 – S5) and IMS-MS spectra for bipy-GS₂ in the absence and presence of Cu/Zn (Figure S6) are reported.

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