Separation of stereoisomers of dinuclear metal complexes by binding affinity chromatography using non-duplex DNA†

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Affinity chromatography – using non-duplex DNA as the affinity ligand – has been used as a highly efficient means of separating stereoisomers of dinuclear polypyridyl ruthenium(II) complexes.

Affinity chromatography – involving "biologically specific" interactions as a means of separation – has long been used in the purification and isolation of biomolecules.[1] Common applications of the technique include the purifications of antibodies and antigens,[2] oligosaccharides and glycoproteins,[3] enzymes,[4] and hormones.[5] Often, immobilised nucleic acids are employed in the role of the affinity ligand, with the efficient isolation of target molecules being achieved by means of their specific or non-specific interactions.[6,7] This technique was first reported by Litman in 1968 for the purification of DNA polymerase,[8] but has since been applied to numerous other DNA-binding proteins[7,9] as well as the purification of other nucleic acids *via* DNA—DNA and DNA—RNA interactions.[10]

There has been considerable interest in DNA binding by inert metal complexes, and the factors influencing that binding – which include intercalation, chiral selectivity, van der Waals interactions, hydrogen bonding, covalent interactions, and so on. Chiral selectivity[11] has received limited attention with regard to its applicability to the chromatographic resolution of enantiomeric mixtures of metal complexes. Baker *et al.* have reported the enantiomeric resolution of racemic mixtures of the complexes $[Ru(phen)_3]^{2+}$ and $[Ru(bpy)_2(ppz)]^{2+}$ by means of elution through a DNA–hydroxylapatite column.[12] In each instance, the Λ isomer was found to elute prior to the Δ isomer, consistent with the stronger DNA-binding affinity observed for the Δ form.[13] Similar mononuclear DNA-intercalating complexes have been resolved by Aldrich-Wright and co-workers using a HPLC technique featuring a covalently-bound DNA stationary phase.[14] They also developed a technique in which DNA-impregnated cellulose paper was used to compare retention times of DNA-binding complexes and relate them to the intercalative ability of complex ligands.[15] A similar paper chromatographic technique was reported by Hannon *et al.* to resolve the enantiomeric forms of metallo-supramolecular triple-helicates (although the DNA-free cellulose was itself quite efficient at resolving the enantiomers).[16] Chiral separations have also been accomplished using capillary electrophoresis with a selection of chiral buffer additives that includes calf-thymus DNA.[17]

Our laboratory has developed a cation-exchange chromatographic technique using SP Sephadex C-25 which we have used extensively for the separation of stereoisomers (diastereoisomers/geometric isomers and enantiomers) of mono-, di- and oligonuclear species. For example, the resolution of the dinuclear complex *rac*-$[\{Ru(pp)_2\}_2(\mu\text{-BL})]^{4+}$ {where pp = polypyridyl terminal ligands such as 1,10-phenanthroline (phen) or 2,2′-bipyridine (bpy) and BL = rigid polypyridyl bridging ligands such as 2,2′-bipyrimidine (bpm) or 1,4,5,8,9,12-hexaazatriphenylene (HAT)} was achieved using aqueous disodium (−)-*O*,*O*′-dibenzoyl-L-tartrate solution as eluent.[18,19] Resolution of analogous dinuclear complexes featuring more flexible bridging ligands, such as 2,3-bis(2-pyridyl)pyrazine (2,3-dpp), proved much more difficult: effective column lengths in excess of 30 m have failed to clearly resolve a racemic mixture of the ΔΔ and ΛΛ enantiomers. Stereoisomer separations such as these have been routinely undertaken for the purpose of acquiring stereoisomerically-pure complexes for DNA-binding studies. We therefore considered that DNA-based columns might yield more efficient resolutions, while at the same time providing a general indication of relative DNA-binding affinities. To this end we have developed a new column-based affinity chromatography technique for the separation of the stereoisomeric forms of polypyridyl metal complexes.

The ability of immobilised streptavidin to strongly bind biotinylated substances has been exploited in numerous affinity chromatography applications.[20] Accordingly, we chose to utilise this interaction in the immobilisation of selected non-duplex oligonucleotides to a stationary phase for use in DNA-affinity chromatography of metal complexes. HiTrap Streptavidin HP columns (1 mL) – purchased from Amersham Biosciences – contain a medium consisting of streptavidin immobilised on Sepharose. The column (0.7 × 2.5 cm) was attached to a Gilson Minipuls 3 peristaltic pump and equilibrated with approximately ten column volumes (10 mL) of 10 mM sodium phosphate/0.075 M sodium chloride/pH 7.5 buffer solution at a rate of 1 mL min^{-1} (all solutions being filtered through a 0.45 μm filter before being applied to the column). Approximately 300 nmol of a selected biotinylated oligonucleotide (GeneWorks) was then loaded on the column in 1–2 mL of the same buffer solution at a rate of 0.1–0.5 mL min^{-1}, and the column subsequently washed with another 10 mL of the buffer (at 1 mL min^{-1}). The absorbance of the column wash was checked at 260 nm to ensure no DNA was present (if DNA was present, the wash was recycled onto the column until it was DNA-free). Once the DNA was immobilised and washed, approximately 100 nmol of the target metal complex

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mixture was loaded on the column and eluted with the same buffer solution at about 0.5 mL min^{-1}. Elution times/volumes varied (depending on the oligonucleotides and complexes used); however separations of complexes, diastereoisomers, and enantiomers could clearly be observed on the white column due to the highly coloured nature of the complexes. The identity of the eluted species was confirmed by examination of the UV/visible and circular dichroism (CD) spectra of the eluate solutions.[21]

The first test of this method involved the separation of the equivalent diastereoisomer of two different complexes – *meso*-$[\{Ru(Me_2bpy)_2\}_2(\mu\text{-bpm})]^{4+}$ [18] (Me_2bpy = 4,4′-dimethyl-2,2′-bipyridine) and *meso*-$[\{Ru(phen)_2\}_2(\mu\text{-HAT})]^{4+}$ [22] – on an immobilised tridecanucleotide possessing an unpaired adenine base (or "bulge"), d(CCG**A**GAATTCCGG)$_2$. This particular oligonucleotide was selected as it has been used in several prior DNA-binding studies by our group.[23,24] A more concentrated eluent was used for this first separation (20 mM sodium phosphate/0.15 M sodium chloride/pH 7.5 buffer solution) and a clear separation of the two complexes was observed over the 2.5 cm length of the column. Collection of a green band corresponding to the bpm-bridged complex took place after 3–8 mL of eluent had passed through the column, while the trailing purple band of the HAT-bridged species was collected after the passage of 130–200 mL of eluent. The elution order of these complexes reflects the relative binding affinities of the two complexes with the oligonucleotide as observed in fluorescent intercalator displacement (FID) assays:[25] *meso*-$[\{Ru(Me_2bpy)_2\}_2(\mu\text{-bpm})]^{4+}$ was found to decrease the fluorescence of d(CCG**A**GAATTCCGG)$_2$-bound ethidium bromide (EthBr) by some 33%, while *meso*-$[\{Ru(phen)_2\}_2(\mu\text{-HAT})]^{4+}$ caused a 62% decrease (FID data are included in Fig. S1, ESI†). The procedure was repeated on a column to which an icosamer featuring a 6-base CT hairpin loop, d(CACTGGT**CTCTCT**ACCAGTG), had been bound and the same separation was observed. In order to ascertain that the DNA (rather than the Sepharose medium) was responsible for the separation, the same mixture of complexes was loaded on a column to which no DNA had been bound. No separation was observed.

The second system attempted was the separation of two diastereoisomers (*meso*- and ΛΛ) of the dinuclear complex $[\{Ru(bpy)_2\}_2(\mu\text{-HAT})]^{4+}$.[22] Using a column containing the immobilised bulge sequence d(CCG**A**GAATTCCGG)$_2$ with an eluent of half the previous concentration (*i.e.* 10 mM sodium phosphate/0.075 M sodium chloride), a clear separation of two purple bands was achieved. The first band was collected after 25–30 mL of eluate, the second after 35–50 mL. CD spectra revealed that the first band was the ΛΛ isomer,[21] while the second band was the *meso* isomer (see Fig. 1). The greater apparent affinity of the *meso* isomer for the bulge sequence is in concordance with FID assay results that showed that *meso*-$[\{Ru(bpy)_2\}_2(\mu\text{-HAT})]^{4+}$ form induced a 47% decrease in fluorescence whereas addition of the ΛΛ form results in a decrease of only 15% (Fig. S1, ESI†).

For the third example, we attempted the resolution of a racemic mixture of $[\{Ru(Me_2bpy)_2\}_2(\mu\text{-bpm})]^{4+}$. FID assays conducted on this complex showed no difference between the binding of the ΔΔ and ΛΛ enantiomers to the bulge sequence (fluorescence decreases of 29% in each case; Fig. S1, ESI†), however NMR studies have demonstrated total enantioselectivity in the binding of these complexes to the bulge site.[24] Resolution of a racemic mixture of

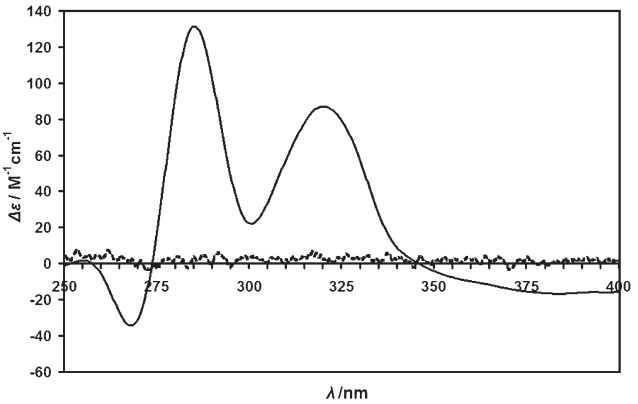

Fig. 1 CD spectra of the first (solid line) and second (dashed line) bands collected from a mixture of ΛΛ- and *meso*-$[\{Ru(bpy)_2\}_2(\mu\text{-HAT})]^{4+}$ separated on a DNA-affinity column loaded with the bulge oligonucleotide.

the complex was again accomplished with 10 mM sodium phosphate/0.075 M sodium chloride eluent at a flow rate of about 0.5 mL min^{-1}. Two green bands eluted from the column, with CD spectra identifying the first band as the ΛΛ isomer and the second band as the ΔΔ isomer (see Fig. 2).[21] This elution order is in agreement with the enantioselectivity observed in NMR experiments, although the ease of separation does not reflect the FID data.

The final example was the resolution of a racemic mixture of a 2,3-dpp bridged species, $[\{Ru(phen)_2\}_2(\mu\text{-2,3-dpp})]^{4+}$, which had proven extremely difficult by our routine cation-exchange procedure. Initial attempts at this particular resolution using the bulge column were unsuccessful, although at an eluent concentration of 10 mM sodium phosphate/0.075 M sodium chloride some elongation of the single purple-pink complex band was observed. Consequently, the resolution was re-attempted but with a second HiTrap column bearing the hairpin loop attached in serial with the bulge column. Using a lower concentration of the eluent (5 mM sodium phosphate/0.0375 M sodium chloride) and a lower flow rate (~0.1 mL min^{-1}) due to the increased back-pressure, the elution proceeded quite slowly. However, the complex eventually resolved into two purple-pink bands which eluted close together

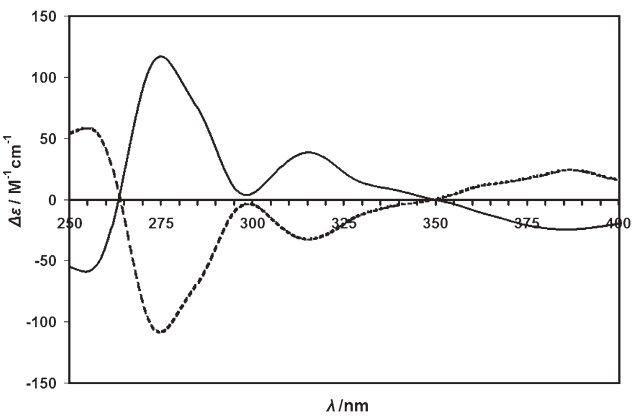

Fig. 2 CD spectra of the first (solid line) and second (dashed line) bands collected from a racemic mixture of ΛΛ- and ΔΔ-$[\{Ru(Me_2bpy)_2\}_2(\mu\text{-bpm})]^{4+}$ separated on a DNA-affinity column loaded with the bulge oligonucleotide.

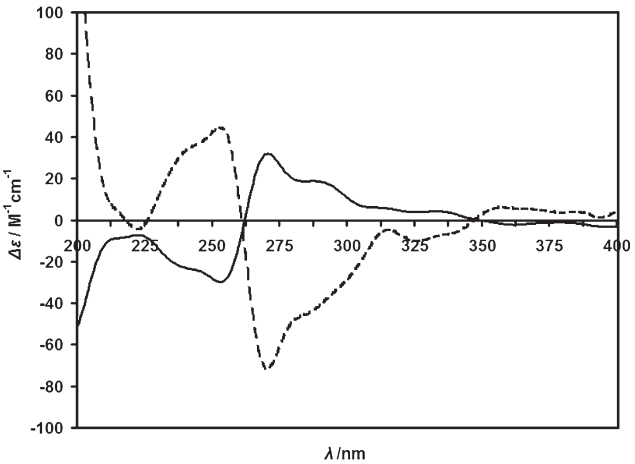

Fig. 3 CD spectra of the first (solid line) and second (dashed line) bands collected from a racemic mixture of ΛΛ- and ΔΔ-[{Ru(phen)$_2$}$_2$(μ-2,3-dpp)]$^{4+}$ enriched on two DNA-affinity columns in serial.

after 1.0–1.2 L of eluent had passed through the columns. CD spectra taken of the leading edge of band 1 and the tailing edge of band 2 again suggested that the bands were enriched in the ΛΛ and ΔΔ enantiomers, respectively (see Fig. 3).[21] In contrast to the [{Ru(Me$_2$bpy)$_2$}$_2$(μ-bpm)]$^{4+}$ resolution, the elution order of the [{Ru(phen)$_2$}$_2$(μ-2,3-dpp)]$^{4+}$ enantiomers was representative of the relative binding affinities observed in FID assays (ΔΔ > ΛΛ with regards to binding affinity for both oligonucleotides). Upon addition to the bulge sequence, decreases of 70 and 49% were observed for the ΔΔ and ΛΛ enantiomers, respectively; addition to the hairpin sequence yielded decreases of 73 and 59% for those same enantiomers (Fig. S1, ESI†).

We have demonstrated that DNA-assisted binding affinity chromatography is an efficient means of separating stereoisomers of polypyridyl transition metal complexes. On the scale described herein, this technique provides a robust means of establishing the relative binding affinities of metal complexes to a particular oligonucleotide, however extension to preparative scales is also feasible. We are currently using the technique to conduct a more comprehensive survey into the interactions between an array of different metal complex/oligonucleotide systems and trying to relate those results to binding affinities assessed by other means, such as NMR spectroscopy and FID assays. Of particular interest is the discrepancy between the apparent binding affinities of the [{Ru(Me$_2$bpy)$_2$}$_2$(μ-bpm)]$^{4+}$ enantiomers as seen in the FID and chromatographic/NMR experiments. These results suggest that the relationship between the DNA-binding affinities of these metal complexes and their ability to induce a fluorescence decrease in DNA-bound EthBr is less straightforward than previously imagined. There are clearly effects in operation beyond the simple displacement of the fluorescing intercalator, and we are investigating the true relationship between FID results and the actual DNA-binding affinities of metal complexes.

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