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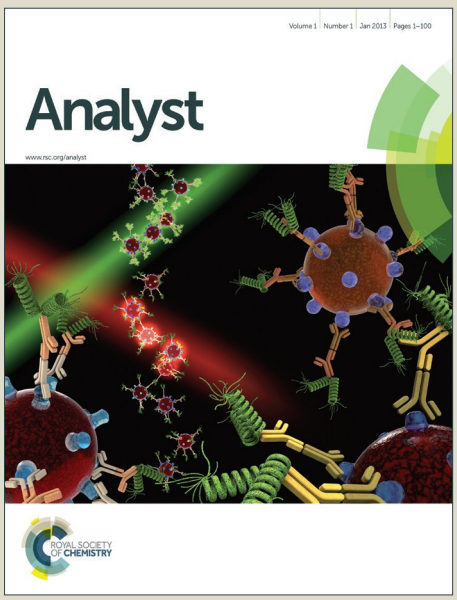

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Improved DNA Equilibrium Binding Affinity Determinations of Platinum(II) Complexes using Synchrotron Radiation Circular Dichroism

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Abstract

The binding affinity of a series of square planar platinum(II) compounds of the type $[Pt(A_L)(I_L)]^{2+}$, where A_L is 1,2-diaminoethane and I_L are 1,10-phenanthroline (phen), 4-methyl-1,10-phenanthroline (4Mephen), 5-methyl-1,10-phenanthroline (5Mephen), 4,7-dimethyl-1,10-phenanthroline ($47Me_2$phen), 5,6-dimethyl-1,10-phenanthroline ($56Me_2$phen) or 3,4,7,8-tetramethyl-1,10-phenanthroline ($3478Me_4$phen) has been reinvestigated using Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy. The additional peaks exhibited considerably greater intensity than those observed between 200 and 400 nm affording additional binding affinity determinations. In addition, the authors have reviewed the various mathematical approaches used to estimate equilibrium binding constants and thereby demonstrate that their mathematical approach, implemented with Wolfram *Mathematica*, has merit over other methods.

Introduction

Circular Dichroism (CD) spectroscopy is a well-established method used for biophysical examination of chemical and biological structures, however, in conventional CD instruments low flux of the light source in the far UV and vacuum UV wavelengths can limit the acquisition of information. These limitations may be overcome using Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy.[1, 2] The application of synchrotron radiation to CD was first reported over 20 years ago, the intense light source increasing effectiveness particularly as a spectroscopic technique for both structural and functional genomics.[3, 4] An additional advantage of SRCD includes the ability to obtain data at shorter wavelengths encroaching into the vacuum UV region, and as a result, superior structural information.[3]

A comparison of the SRCD and conventional CD spectra of ct-DNA (Figure 1), illustrates the extended wavelength range of SRCD. Note that the data is scaled to facilitate this comparison. These intensely absorbing bands in the vacuum and far UV region are seen for many compounds, including proteins and other biomolecules.[3, 4] Chiral metal complexes (MCs), such as [(5,6-dimethyl-1,10-phenanthroline) (1*S*,2*S*-diamino cyclohexane) platinum(II)] dichloride (56MESS) and [(5,6-dimethyl-1,10-phenanthroline) (1*R*,2*R*-diaminocyclohexane) platinum(II)] dichloride (56MERR), also show additional intense absorption bands well into the far UV (Figure 2).

Equilibrium binding experiments are extensively used to investigate interactions between small molecules and biomacromolecules, such as proteins and DNA.[5-14] These interactions may involve intercalation, H-bonding, van der Waals groove binding or π stacking. To evaluate the binding affinity of related small molecules for a particular

biomacromolecule under specific experimental conditions the binding constant K_b is determined; often using methods that poorly estimate this value.

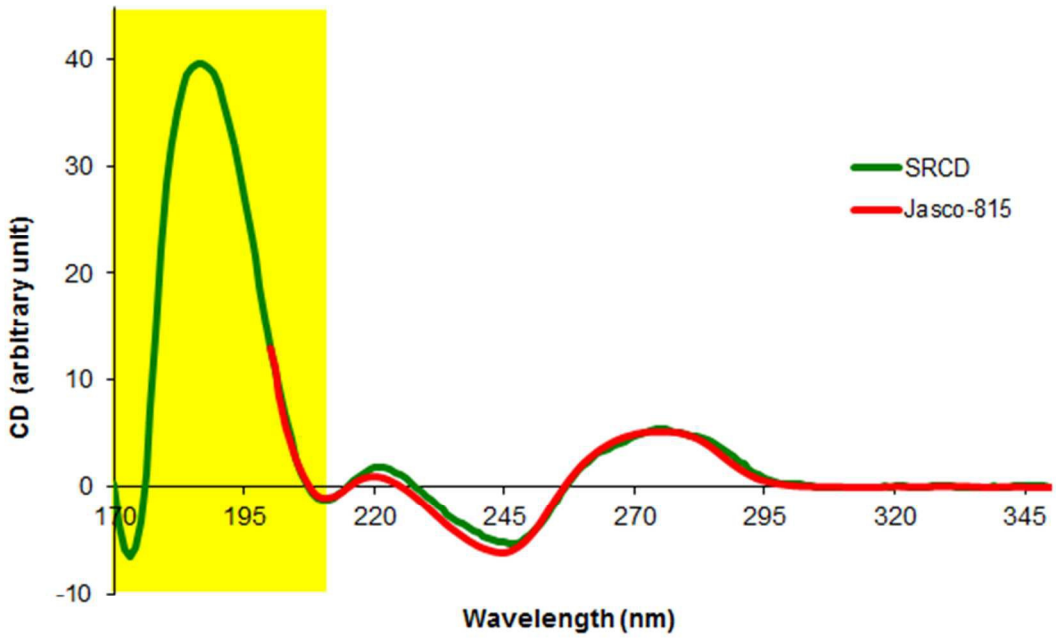


Figure 1 The spectra of ct-DNA as measured on a conventional CD (red) and SRCD (green) spectrophotometer, where the spectra have been scaled to allow direct comparison. CD spectrum was recorded between 200–350 nm, 35 μM, 1.0 cm path length, ambient temperature and 10 accumulations. SRCD spectrum was recorded between 170–350 nm, 1.1 mM, 0.01 cm path length, 25 ^{o}C and 3 accumulations.

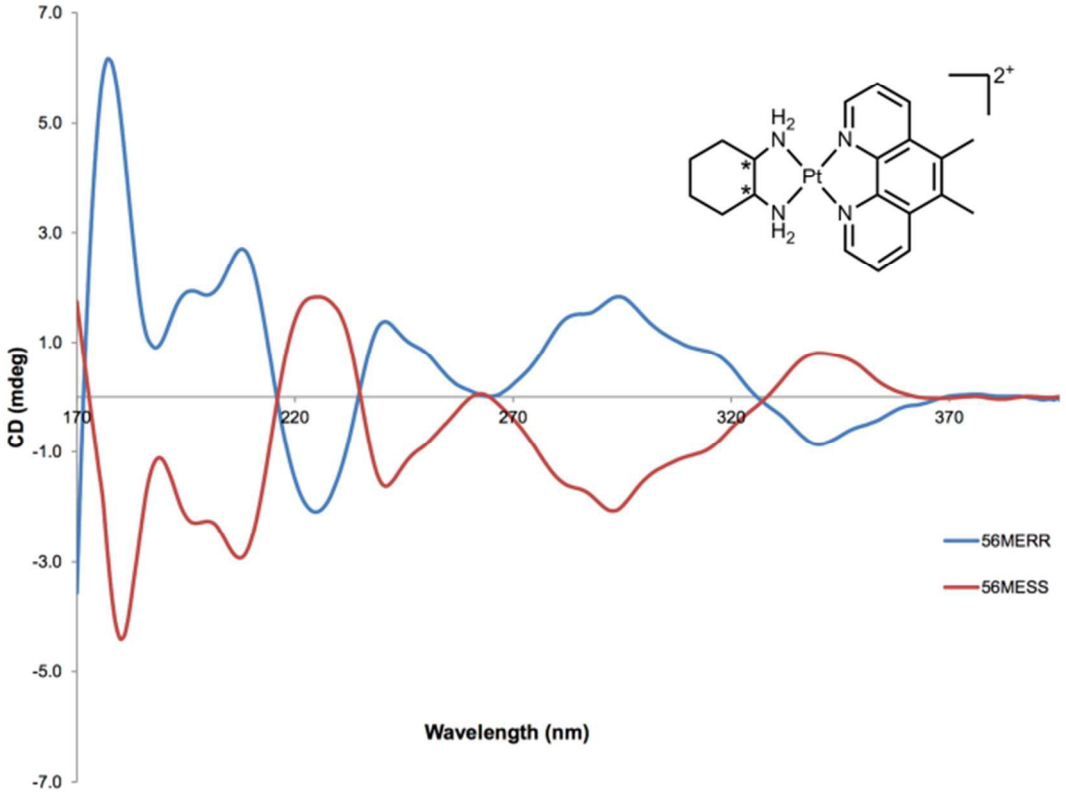


Figure 2 The SRCD spectra of 56MESS and 56MERR. Wavelength = 170–400 nm, concentration = 4 mM, path length = 0.01 cm. Inset: the structure of 56MESS/56MERR, * denotes the chiral centres.

Determining an Equilibrium Binding Constant

The equilibrium binding constant (K) and the number of bases per binding site (n) have heretofore been calculated using a variety of methods as developed by Scatchard,[15] Schmechel and Crothers,[16] McGhee and von Hippel,[17] Nordén,[18, 19] Wolfe et al.,[20] Rodger,[7, 21-23] Rodger and Nordén,[24] and Kumar and Asuncion,[25-27] among others. These approaches all share a common assumption that artificially determines the binding constant at very low bound-ligand concentrations. Our method,[28] developed from first principles using the relation $L_F + S_F \underset{K}{\leftrightarrow} L_B$ (where L_F and S_F are free ligand and free substrate

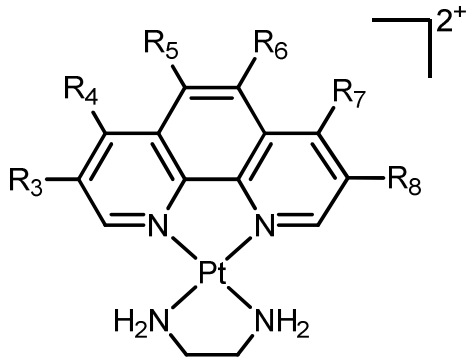
concentrations respectively and L_B is the bound ligand concentration) and represented in Eq. 1, obtains a value of K by accounting for the entire concentration range.

$$L_B = 0.5R\left(\frac{1}{K} + L_T + S_T - \sqrt{\left(\frac{1}{K} + L_T + S_T\right)^2 - 4S_T L_T}\right) \qquad (1)$$

The fitting of Eq. (1) to experimental data has been accomplished with a script (called a 'notebook') in *Mathematica* and is given in the SI. Of note is that Eq. (1) has a mathematical point of inflexion which is indicated on the fitted curve as a red dot if a valid fit is achieved.

In this work we initially use this method in *Mathematica* for the fitting of Eq. (1) to reprocess previously obtained CD binding data for [(1,10-phenanthroline)(1,2-diaminoethane)platinum(II)] dichloride (PHENEN, 1) with ct-DNA to assure that the results are consistent. We also demonstrate that the method can also be used to determine the binding constant for UV data. Finally, we interrogate recently obtained SRCD binding data for ct-DNA and the platinum(II) complexes PHENEN, **(1)**, [(4-methyl-1,10-phenanthroline)(1,2-diaminoethane)platinum(II)] dichloride (4MEEN, 2), [(5-methyl-1,10-phenanthroline)(1,2-diaminoethane) platinum(II)] dichloride (5MEEN, 3), [(4,7-dimethyl-1,10-phenanthroline)(1,2-diaminoethane)platinum(II)] dichloride (47MEEN, 4), [(5,6-dimethyl-1,10-phenanthroline)(1,2-diaminoethane)platinum(II)] dichloride (56MEEN, 5) and [(3,4,7,8-tetramethyl-1,10-phenanthroline)(1,2-diaminoethane)platinum(II)] dichloride (3478MEEN, 6) (Figure 3) by using this improved method to observe what effect the increased beam intensity and shorter wavelengths contribute to the experimental results. Here we present a method,

implemented with Wolfram *Mathematica*, for the quick and effective determination of the binding constant.



1 = PHENEN, all R = H
2 = 4MEEN, R4 = CH_3
3 = 5MEEN, R5 = CH_3
4 = 4,7MEEN, R4,7 = CH_3
5 = 5,6MEEN, R5,6 = CH_3
6 = 3478MEEN, R3,4,7,8 = CH_3

Figure 3 The general structure of the platinum(II) complexes investigated.

Experimental

Materials

All platinum(II) complexes were synthesised and characterised as previously reported.[5] Ct-DNA, disodium hydrogen phosphate, sodium dihydrogen phosphate sodium fluoride and *D*-10-camphoursulfonic acid (CSA) were all purchased from Aldrich Chemical Company. Aqueous solutions were prepared with Milli-Q water (Millipore, MA). All other reagents and solvents were of analytical grade.

Instrumentation

Conventional CD were recorded as previously reported.[5] In summary: a JASCO J-810 CD spectropolarimeter, at the ambient temperature in the wavelength range of 200–350 nm, at ct-DNA concentrations of 35 μM (2600 μL) and MC solution of 10 mM, using a path length of 1 cm, a data pitch of 0.5 nm with 10 accumulations. Aliquots of MC (1 μL)

were titrated into a cuvette containing the ct-DNA and the titration continued until no further change was observed in the CD spectrum, indicating that the saturation point of the DNA had been reached. Baseline subtraction was achieved by subtraction of the ct-DNA/buffer spectrum.

SRCD spectra were recorded at the ASTRID synchrotron at ISA, Aarhus University, Denmark (beamline CD1) operating at 25 °C. The instrument was calibrated for magnitude and polarisation daily, using D-10-camphorsulfonic acid (CSA, 7.4 mg/mL). SRCD spectra were measured using a quartz cell of type QS124 (Hellma GmBH, Germany) with a path length of 0.01 cm. Spectra were measured over the wavelength range 170–350 nm, using a slit width of 0.25 nm, a data pitch of 1 nm, and an average dwell time of 2.0 s. Spectra were averaged from 3 accumulations and smoothed with the CD data processing software CDTool.[29]

A Thermo Scientific Evolution 300 UV-Vis spectrophotometer, with a 1 cm quartz cell at room temperature scanning over a 200–420 nm wavelength range, was used to determine the concentration of CSA (ε_{285} nm = 34.6 $M^{-1}cm^{-1}$)[30] and ct-DNA (ε_{260} nm = 13200 $M^{-1}cm^{-1}$).[31] For all experiments the ratio of the 192.5:290 nm peaks ranged between 2.04 and 2.06.

Binding experiments

Solution preparation

Some experimental changes to the published methods were made in order to reinvestigate the binding affinity of these platinum complexes with ct-DNA using SRCD.[5] Individual solutions (11 samples per experiment, 2 experiments per MC) were prepared for measurement to determine affinity constants for the MCs with ct-DNA. The DNA

solution (1.1 mM) in phosphate buffer (3.6 mM) with NaF (36 mM) at pH 7.1 and MC solutions (2 mM) were prepared. Maintaining a constant volume of ct-DNA (220 µL), different volumes MC solutions (2 mM) were prepared off-site prior to the binding study (details in Table 1) and transported to the Synchrotron. An aliquot from the titration mixture (usually 25 µL) was loaded onto the base of the demountable cell (Figure 4) by micropipette. The cover of the cell was then placed over the solution with care to avoid bubble formation. All samples were degassed before they were loaded into the cells. The spectra were recorded over the range 170–350 nm with 3 accumulations.

Table 1 Prepared solutions for SRCD DNA binding study.

Tube No	Buffer µL	DNA µL	MC µL	Buffer µL	total µL	DNA mM	MC mM	Ratio
0	300	0	0	0	300	0	0.00	0
1	-	220	0	80	300	1.1	0.00	0
2	-	220	1	79	300	1.1	0.07	0.06
3	-	220	1.5	78.5	300	1.1	0.10	0.09
4	-	220	2	78	300	1.1	0.13	0.12
5	-	220	2.5	77.5	300	1.1	0.17	0.15
6	-	220	3	77	300	1.1	0.20	0.18
7	-	220	3.5	76.5	300	1.1	0.23	0.21
8	-	220	4	76	300	1.1	0.27	0.24
9	-	220	5	75	300	1.1	0.33	0.30
10	-	220	6	74	300	1.1	0.40	0.36
11	-	220	12	68	300	1.1	0.80	0.72

Figure 4 Assembly of demountable 0.01 cm quartz cell of type QS124 (Hellma GmBH, Germany).

Processing of data

Processing of the experimental data was performed by fitting the data to Eq. (1) across all wavelengths. This was implemented using Wolfram *Mathematica* version 9.†

Results and Discussion

Model Verification

As a quality assurance test of the implementation, the previously published CD ct-DNA titration spectra[5, 28] of **1** was re-analysed (Figure 5). The *Mathematica* notebook produces an interactive dialogue allowing the entire wavelength range to be analysed and outputs this data in tabulated form. The results for **1** are listed in Table 2. Selection of an appropriate wavelength is evident when Eq. (1) fits the curve, a red dot is drawn at the point of inflexion, as shown in Figure 5, thus providing an unbiased evaluation of the data. Previously, the binding constant was determined to be $2.7 \pm 0.2 \times 10^5$ at 211 nm using the least squares method. Employing the method presented here the binding constant was calculated to be $2.5 \pm 0.1 \times 10^5$ at 212 nm which is in close agreement with the previously published value.[5]

It is noteworthy that this equation does not always achieve a valid fit of the experimental data. When the conditions are met the observation that the calculated equilibrium binding constant varies with wavelength is apparent and that not all wavelengths produce a mathematically sound fit, highlighting an important feature in our interpretation of the binding model with Eq. (1). This demonstrates an important progression as it removes any influence of subjectivity in the selection, calculation and reporting of binding constants. As shown in the derivation of Eq. (1) (SI), previous

methods make assumptions to simplify the calculations and as such can only make approximations of the equilibrium binding constant at a given wavelength. As SRCD spectra is the results from all the combined couplings of transitions of all bases, we attribute the differences in *K* observed with changing wavelength to be a consequence of the interactions at specific bases. Although this interaction will change the overall spectra it may do more so in the region where the resulting couplings of the transitions from this interaction dominate. This suggests that the data may suffers from experimental noise or that the binding may be more complex than this model is capable of describing as the definition is that *K* is a constant across all wavelengths.

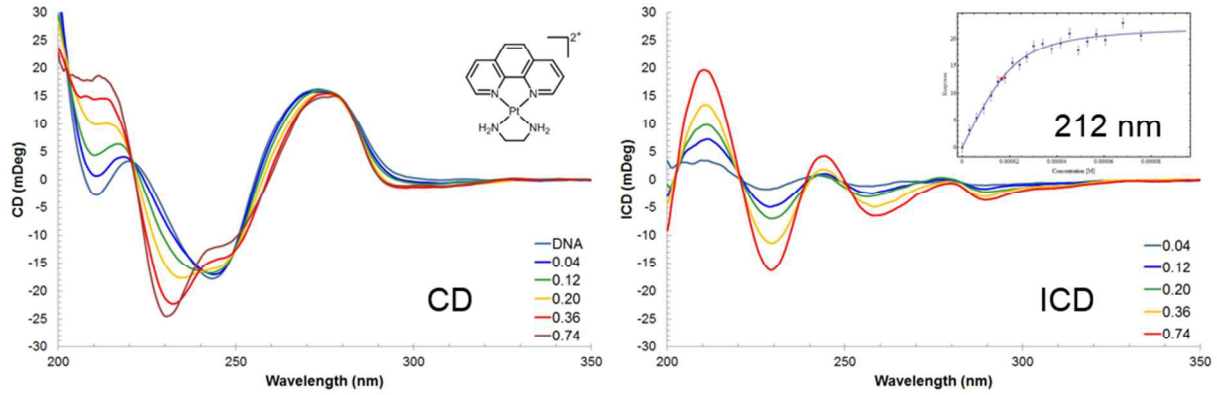


Figure 5 CD (left) and ICD spectra (right) at varying ratios of [MC]/[DNA] (listed) $[Pt(en)(phen)]Cl_2.2H_2O$ into ct-DNA, PS buffer. Binding curve at 212 nm (inset). The solid line is a fit of equation (2), $K = 2.5 \pm 0.1 \times 10^5$ M. The red dot, at the point of inflexion, indicates a good fit of Eq. (1).

The data collected from the conventional CD also includes UV spectrum data which was likewise examined using the *Mathematica* notebook producing the UV spectra illustrated in Figure 6. The inset depicts the response curves at peak maxima 227 and 274 nm which show that this data does not fit Eq. (1). In fact for this binding interaction there

was no wavelength for which a point of inflexion was obtained, i.e. the UV data does fit Eq. (1).

Table 2 Summary of successful fits of previously reported CD spectra[28] of **1** titrated into ct-DNA, as determined by *Mathematica* notebook implementation of Eq. (1), with associated error values and bases per binding site.

Wavelength (nm)	K ($\times 10^5$)	*n* per complex
206.5	2.6 ± 0.1	3.8 ± 0.1
208.0	6.3 ± 0.4	3.2 ± 0.1
212.0	2.5 ± 0.1	3.7 ± 0.1
213.0	2.2 ± 0.1	3.8 ± 0.1
214.5	1.1 ± 0.1	5.0 ± 0.2
249.0	7.8 ± 0.1	1.1 ± 0.1
267.5	5.3 ± 0.2	2.6 ± 0.3
269.0	1.3 ± 0.1	2.8 ± 0.3
269.5	0.8 ± 0.1	2.8 ± 0.1

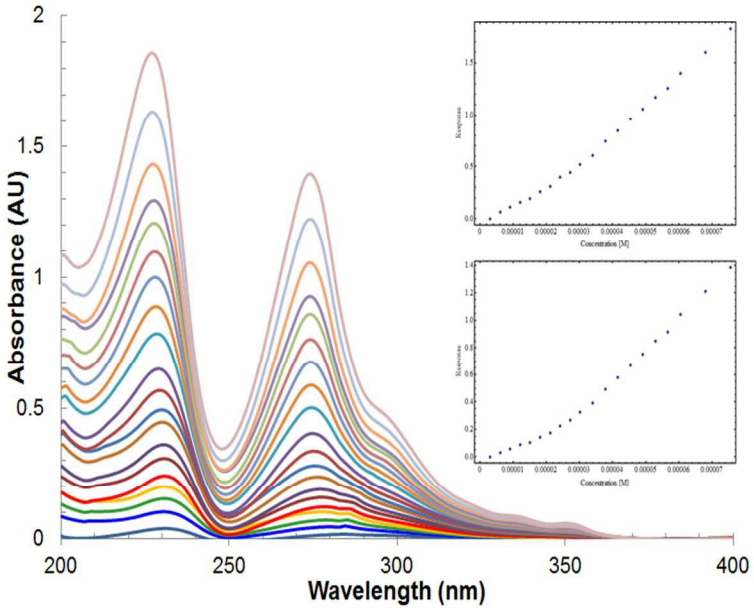


Figure 6 UV spectra at varying ratios of [MC]/[DNA]. **1** into ct-DNA in PS buffer. Titration curves at 227 and 274 nm (inset). The lack of a curve indicates poor fit of Eq. (1).

SRCD Experiments

The SRCD spectra of **1**–**6** each with ct-DNA were obtained in duplicate. The SRCD and induced SRCD (ISRCD) titration spectra for **1** are shown in Figure 7. For each MC, a previously unseen and significantly more intense peak at ~185 nm was evident. The *Mathematica* notebook was used to interrogate the spectra of each MC; the red dot on the binding curve indicating a true fit of Eq. (1). An example is illustrated in Figure 7 (inset) with the data provided in Table 3 (Figures and Tables for the duplicate experiment for **1** and for **2**–**6** are provided in the SI). It is evident from this analysis that the resulting SRCD data did not cover a comprehensive concentration range; the samples were pre-prepared and transported to the synchrotron for measurement with no facility for further preparation. Despite this, the binding constant obtained at 211 nm was determined to be $1.6 \pm 0.2 \times 10^5$ M with the average $K = 2.2 \pm 0.3 \times 10^5$ M and $n = 3.0 \pm 0.1$ across repeats. Though slightly lower, this is comparable to the previously reported CD value for **1**.

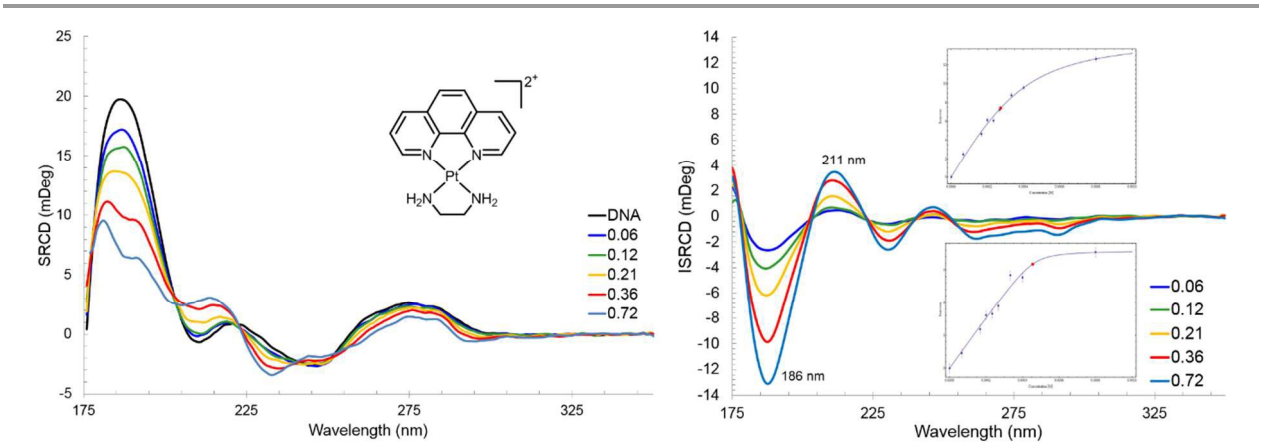


Figure 7 Experiment A showing the SRCD and ISRCD spectra of varying concentrations of **1** with ct-DNA (1.1 mM) in 5 mM phosphate buffer, 50 mM NaF at 7.5

pH. Binding curve determined at 186 and 211 nm (inset). The solid line is a fit of equation (2); the red dot indicates a good fit.

Table 3 Complex 1 binding data from SRCD experiment.

Wavelength (nm)	K $\times 10^5$	n per complex
186	0.11 ± 0.0*	3.0 ± 0.1
189	0.1 ± 0.0*	2.8 ± 0.1
192	0.1 ± 0.0*	3.4 ± 0.1
211	1.6 ± 0.4	2.4 ± 0.0*
228	0.6 ± 0.1	2.0 ± 0.0*
231	1.6 ± 0.3	2.2 ± 0.0*
257	2.7 ± 0.5	2.8 ± 0.1
264	0.3 ± 0.0*	2.1 ± 0.0*

* Error less than 0.03

The binding results from the SRCD (Table 4) are typically an order of magnitude lower compared with those determined from the CD binding data using the least squares method. Whereas previously determined CD binding constants were reported at a given wavelength (230 nm), a range of SRCD data values satisfied the mathematical fit of Eq. (1), thus the average across all fitted wavelengths is reported. Despite the limitations of the titration concentrations the program was able to assess the binding affinity and number of binding sites for **1**–**6** is comparable to previously reported values.

Table 4 Summary of DNA binding affinities of the Pt(II) complexes determined by conventional CD (as previously reported)[5] and SRCD (using Eq. (1)).

		CD[a]			SRCD[b]	
	Compound	Nm	$K \times 10^6$	N	$K \times 10^5$	n
1	$[Pt(en)(phen)]Cl_2.2H_2O$	230	2.2 ± 0.5	2.0 ± 0.3	0.9 ± 1.0	2.6 ± 0.5*
2	$[Pt(en)(4\text{-Mephen})]Cl_2$	230	1.2 ± 0.4	5.8 ± 1.1	1.8 ± 0.8	2.7 ± 0.6
3	$[Pt(en)(5\text{-Mephen})]Cl_2$	230	0.7 ± 0.2	3.8 ± 0.7	2.3 ± 1.0	3.6 ± 2.5
4	$[Pt(en)(4,7\text{-}Me_2phen)]Cl_2$	230	1.3 ± 0.4	4.6 ± 0.1	0.3 ± 0.1	2.3 ± 0.3
5	$[Pt(en)(5,6\text{-}Me_2phen)]Cl_2$	230	1.5 ± 1.1	4.9 ± 0.9	0.6 ± 0.4	3.0 ± 0.8
6	$[Pt(en)(3,4,7,8\text{-}Me_4phen)]Cl_2$	230	0.7 ± 0.3	6.0 ± 0.4	1.0 ± 0.9	6.1 ± 2.2

[a]The intrinsic approach and Scatchard model[24] was used to determine K and n from the ct-DNA titration data,
[b]Average of values across wavelengths determined using the *Mathematica* notebook implementation.

The small shift in wavelength at peak maxima, which is apparent across all SRCD experiments, is attributable to DNA conformational changes induced by the MCs. The intercalating nature of the phenanthroline ligand causes elongation of the DNA and is especially evident with **1**–**5**. In previous methods these subtle shifts are disregarded and a wavelength value is chosen intuitively; this is avoided in our implementation as the fit may be at any wavelength provided that it is a mathematically sound. This is symptomatic of the difficulty with determining binding constants and is not always apparent by using other methods.

The shoulder appearing at higher concentrations, seen in Figure 7 at ~180 nm, is evident in all but the spectra of **6**. This suggests an additional mode of binding other than intercalation. The binding of **6** may be by groove binding alone, the tetramethylated phenanthroline being too large to insert between the strands. This is also evidenced by the overall disparity in the titration spectra between **6** and the other MCs.

Obtaining ideal SRCD Spectra

Collecting data on CD instruments below 190 nm is limited by a number of experimental conditions. The power of the synchrotron beam can be several orders of magnitude brighter than a Xenon lamp and does not experience a comparable flux decrease across the UV and vacuum UV wavelength ranges. Any reduction in light source intensity is amplified by absorptive losses within the quartz optics, atmospheric oxygen in the light path (despite nitrogen purging) as well as sample and buffer absorption.[32] These factors combine to limit the collection of meaningful data at wavelengths below 190 nm. Oxygen is excluded from the light path of SRCD as it is under vacuum, excepting the sample chamber which is constantly purged with dry nitrogen. Measurements in aqueous

solutions can be extended down to 168 nm by short path length quartz sample cells (typically 0.01 cm) and the judicious choice of buffer.[32, 33] The reduced sample volumes, accommodated by the improved signal-to-noise, allow samples to be measured even in absorbing buffers, lipids and detergents.

The shortest wavelength at which a spectrum can accurately be measured (cut-off wavelength) is influenced by salts, particularly chloride, and buffer composition (Table 5). If the components of the solution (i.e. buffers and salts) absorb strongly in the region of measurement then it should be substituted with a non-absorbing equivalent. The worst informal offender is chloride anion, so sodium chloride should be replaced where possible by either sodium fluoride or sodium sulfate. Buffers such as tris(hydroxymethyl)aminomethane (Tris) can be acidified with phosphoric or sulfuric acid in place of hydrochloric acid. Low pH buffers with carbonyl groups (citrate, acetate and glycine) and neutral buffers such as HEPES and tricine all absorb strongly, however low concentrations and short path length cells can minimise these effects on spectra.[32, 34] Phosphate buffer (3.6 mM) replaced the Tris buffer that had been used in the previous experiment. Concentrations of up to 100 mM in path lengths of less than 50 μm can be used for a phosphate buffer.

While the concentrations of DNA and MC used in SRCD experiments is higher than comparable CD experiments, they are still relatively low (< ~1 mM) and the impact is minimal as the obtained spectra are equivalent. This is somewhat expected as the relative concentrations are equivalent and the viscosity of the solutions still close to that of the solvent.

Table 5 Absorption properties of selected buffer and salts in the far UV.

Component/s	Light intensity cut-off	Absorbance (10 mM solution, 0.1 cm path length cell)			
	nm	180 nm	190 nm	200 nm	210 nm
NaF	170	> 0.01	>0.01	>0.01	>0.01
KF	170	> 0.01	>0.01	>0.01	>0.01
$NaClO_4$	170	> 0.01	>0.01	>0.01	>0.01
Boric acid	180	> 0.01	>0.01	>0.01	>0.01
NaH_2PO_4	195	0.15	0.01	>0.01	>0.01
Na borate (pH 9.1)	200	0.3	0.09	>0.01	>0.01
NaCl	205	>0.5	>0.5	0.02	>0.01
Na_2HPO_4	210	>0.5	0.3	0.05	>0.01
Cacodylate (pH 6.0)	210	>0.5	0.22	0.20	0.01
Na acetate	220	>0.5	>0.5	0.17	0.03
Tris/H_2SO_4 (pH 8.0)	220	>0.5	0.24	0.13	0.02
HEPES/Na^+ (pH 7.5)	230	>0.5	>0.5	0.5	0.37
MES/Na^+ (pH 6.0)	230	>0.5	0.29	0.29	0.07
NaOH	230	>2	>2	>2	0.5
MOPS (pH 7.0)	230	>0.5	0.28	0.34	0.10
Tricine (pH 8.5)	230	>0.5	>0.5	0.44	0.22
EDTA*	230	>0.5	>0.5	0.42	0.20

Data are adapted from Kelly,[32] Schmid.[35] Additional data can be found in Rosenheck and Doty[36] and Buck et al.[37] * Data from this work.

Conclusions

The DNA binding affinity of a range of metal complexes was reinvestigated using SRCD and a new method was implemented for determining the binding constants. The implementation accommodates quick and effective determination of the binding constant across an entire spectrum, and importantly the ability to observe the potential for error in determining binding constants. The results compared favourably to previous methods but also highlight the potential shortcoming of reporting binding constants at particular wavelengths. Binding constants cannot, by definition, be wavelength dependent and should be constant. We have provided the means to investigate the entire spectrum and in doing so observed that the choice of wavelength makes a difference. Ct-DNA has many binding sites and when one is used up others will be occupied with different induced CD

signals. It is only when the shape of the induced CD spectrum is the same at the mixing ratios that the data can used to determining binding constants. It is likely that the spectrum is complicated by signals from other binding interactions.

The *Mathematica* notebook implementation allows immediate processing and feedback of experimental data with the inclusion of an interactive spectrum navigator function and tabulated results. Implemented into an experimental technique this automated process has the advantage of avoiding insufficient or excessive data collection as well as identification of which wavelengths the binding constant can be determined with sufficient accuracy. Future refinements to the data processing procedure are planned including the ability to titrate DNA into a solution of MC and using the integral of the curve. The notebook is available upon request.

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Notes and References

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† The *Mathematica* binding notebook is provided in the SI. It can also be obtained by contacting the corresponding author.

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