

A porphyrin-DNA chiroptical molecular ruler with base pair resolution

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Abstract

DNA-based molecular rulers enable scientists to determine important parameters across biology, from the measurement of protein binding interactions, to the study of membrane dynamics in cells. However existing rulers can suffer from poor nanometre resolution due to the flexible nature of linkers used to tether to the DNA framework. We aimed to overcome this problem using zinc and free-base porphyrin chromophores attached via short and rigid acetylene linkers. This connectivity enables the distance and angle between the porphyrins to be fine-tuned along the DNA scaffold. The porphyrins undergo favourable energy transfer and chiral exciton coupling interactions to act as highly sensitive molecular ruler probes. To validate the system, we monitored the detection of small changes in DNA structure upon intercalation of ethidium bromide. CD spectroscopy showed the porphyrins undergo highly sensitive changes in excitation coupling to facilitate base pair resolution of the novel system.

1. Introduction

Förster resonance energy transfer (FRET) is routinely used in the determination of conformational changes or intermolecular interactions in biomolecules, where it gives insight into function and activity. However, most FRET systems have chromophores attached through long and flexible linkers which can limit their sensitivity.¹⁻³ Since the interaction of chromophores, in view of energy transfer, is highly dependent on their alignment, and FRET has an inverse sixth order distance (R) dependency, this does not allow for a precise distance measurement with sub-nanometre resolution due to fluctuations in position of the FRET pair,³ which can be overcome to some extent using time resolved FRET (trFRET).⁴ In addition, when the FRET pair distance is comparable to the linker length, contact quenching can occur. Yet the detection of small structural changes in DNA is highly desirable as it can give information on the DNA topology, e.g. upon binding of proteins,⁵ in intercalator-DNA interactions,⁶ or in base pair mismatches.⁷ To this end, tailor made DNA multi-chromophore systems are now well established.^{8, 9} Specific systems include, for example, Cy3 and Cy5 dyes which are typically attached through short alkynyl linkers to the nucleobase and can provide useful FRET analysis at short distances, though this was not used for analysis of conformational changes.¹⁰ Analogously, fluorescent base analogues which were incorporated within the base stacking region of the DNA and thus are inherently held rigidly in place, resulted in a high control of the orientation factor (κ) and hence

very distinct FRET changes as the number of bases separating the base analogues were varied.¹¹ The combination of Cy5 / Cy3, however, is suitable to detect global structural changes in hairpin ribozymes,¹² and in bivalent peptide complexes,¹³ even when having flexible linkers. Gating of the Cy5 dye with a green laser enabled FRET values at much shorter distances to be obtained than in conventional systems.¹² Stilbene chromophores, which were attached to both ends of a DNA hairpin, showed exciton-coupled circular dichroism (EC-CD) which varied strongly along a helical turn and could serve as a molecular ruler; however, the CD signatures are very complex and not straight-forward to interpret.¹⁴ The use of pyrenes has shown strong fluorescence enhancement when attached to DNA with short linkers and can serve as a structure-sensitive probe for DNA.¹⁵ Ultrafast Energy Transfer in pyrene dimers yielded information on structural dynamics of DNA, but the system is complicated by the presence of two electronic coupling pathways, involving the base pair of the DNA.¹⁶

On a single molecule level, total internal reflection fluorescence (TIRF) microscopy, combined with single molecule FRET (TIRF-smFRET) enables single base pair resolution with 100 ms temporal resolution, though this requires sophisticated equipment setup.¹⁷ DNA origami tiles were used as an elegant breadboard for measuring distances using single molecule FRET systems,^{18, 19} but at much larger distances than single base pair resolution. Gold nanoparticles (AuNPs) have also been investigated and held at specific distances using dsDNA spacers; the plasmon coupling of 80 nm AuNPs was shown to allow distance measurements between 1 and 80 nm with a time resolution <50 ms and with absolute distance errors ranging from <1 nm to around 20 nm.²⁰ Plasmon rulers could certainly be an alternative to FRET for *in vitro* single-molecule experiments,²¹ in particular because sub-nanometre resolution can be achieved.²² Methods other than FRET or plasmon resonance in using DNA as a molecular ruler that have been used include pulsed electron paramagnetic resonance (PELDOR) spectroscopy,²³ X-ray scattering²⁴ and EPR spectroscopy.²⁵

Despite the advances made in determining the impact of sequence and external factors on the structure of DNA, including in a time dependent manner, a system with a simple optical readout that gives unambiguous information on local changes down to the single base pair level is still missing. We have studied the characteristics of porphyrin modified DNA extensively.²⁶ Notably a mixed free-base and zinc porphyrin zipper-array allowed for reversible formation of photonic wires.²⁷ In particular CD spectroscopy has proven to be an invaluable tool in analysing interactions in porphyrin-DNA due to the strong exciton coupling between the porphyrins, which shows strong dependence on the type of linker between porphyrin and DNA used, as well as on the underlying sequence of the DNA.^{28, 29} Thus this system seemed perfectly well suited to investigate its ability to monitor the local changes in DNA structure.

2. Experimental

The synthesis of the porphyrin-dU building block and its incorporation into DNA were performed as described previously.²⁶ Phosphoramidite chemistry and solid support DNA synthesis followed standard protocols for the natural nucleotides. For the incorporation of the porphyrin-dU, the phosphoramidite was dissolved in MeCN-DCM 10:1, and an extended coupling time of 5 minutes was used. The DNA strands were purified and analysed using RP-HPLC. Post-synthetic metalation of the porphyrin strand was done in an aqueous solution using Zn(OAc)₂ as published earlier.²⁹

UV-Vis spectroscopy was conducted using a Varian Cary 300 Bio spectrometer with quartz cells of 1 cm path length; scans were carried out at 25 °C covering 200 – 800 nm. Fluorescence spectroscopy was conducted using a Varian Cary Eclipse spectrometer with quartz cells of 1 cm path length; scans were carried out at 25 °C with excitation wavelength at $\lambda = 425$ nm. CD spectra were recorded at

beamline B23 (Module B) at Diamond Light Source, equipped with an Olis DSM20 Monochromator and a photo multiplier tube detector. CD titration with ethidium bromide (EtBr) was performed using a 1.5 mM stock solution to give a total volume 500 μ L (4 μ M DNA), and a concentration of EtBr equal to 40, 80, 200, 400 and 800 μ M. Concentrations and buffers are given in the figure legends. The theoretical predictions of FRET were performed as described previously, using a custom build MATLAB based program FRETmatrix described elsewhere.³⁰ The geometry of the porphyrin-DNA duplexes was modelled using Schrödinger's software MacroModel.³¹

The data for the titration were analysed using a non-linear fit of the Scatchard's plot of r/C_f vs r using the following equation for n binding sites:^{32, 33}

$$r/C_f = K(1 - nr) \left[\frac{1 - nr}{1 - (n-1)r} \right]^{n-1} \quad (1)$$

where $r = C_b/C_p$, $C_b = [\text{EtBr}]_{\text{bound}}$, $C_b = C_0 - C_f$, $C_0 = [\text{EtBr}]_{\text{total}}$, $C_f = [\text{EtBr}]_{\text{unbound}}$, $C_p = [\text{phosphate groups}]$.

For **Y1**, the $\Delta\theta$ at 415 nm was used, and for **Y2** the $\Delta\theta$ at 420 nm was used. The values of $|\theta_{\text{max}} - \theta_{\text{min}}|$ were fitted to obtain the theoretical value of maximum change (highest possible intercalation. From this, the fraction of bound EtBr was calculated, assuming that the intercalation on a global scale leads to a linear response of $\Delta\theta$.

3. Results and Discussion

3.1. Synthesis and stability of the FRET system

In this study, we used an electronically coupled system based on zinc porphyrin as donor (**Zn-P**) and free-base porphyrin as acceptor (**2H-P**) which has previously shown efficient energy transfer within a DNA supramolecular assembly.^{27, 29, 34} We built an array of DNA duplexes to study the energy transfer properties for this two porphyrin system (see Fig. 1, **Z1** to **Z7**, and ESI for sequences). The position of the Zn-P was fixed throughout, whilst the 2H-P was varied. The corresponding base pair separation between the porphyrins change both the distance and dipole-dipole alignment between them. The porphyrin-nucleoside building block was synthesised according to previously published procedures and used for solid phase synthesis of the porphyrin-DNA.^{29, 34} The thermal UV-denaturing analysis shows a T_m of $\sim 47^\circ\text{C}$ for all porphyrin containing duplexes, with $\Delta T_m = \sim -3^\circ\text{C}$ compared to the native DNA, which is in the expected range. Both porphyrins display the characteristic absorption and emission spectra when measured either as single strand or as duplex with the unmodified complementary strands (Fig. 2). The ground state absorbances in the mixed porphyrin duplexes **Z1** to **Z7** are largely unperturbed and can be described as a superposition of the absorbance of DNA duplexes which contain either **Zn-P** (**Z8**) or **2H-P** (**Z9**).

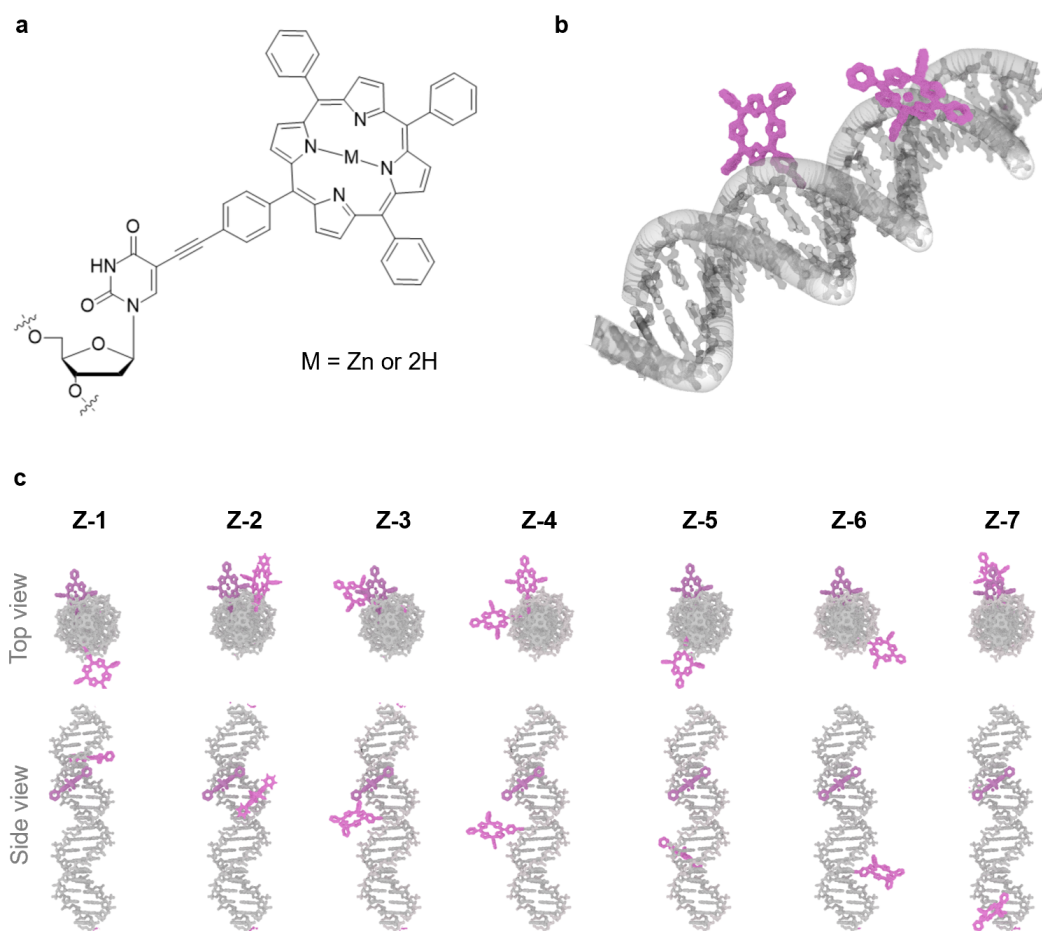


Figure 1. a) Chemical structure of porphyrin DNA modification connected *via* a rigid acetylene linker, the central cavity contains either a Zn or free-base (2H). b) 3D rendering of duplex DNA (grey) modified with two porphyrin molecules (purple). c) Top and side view of porphyrin-DNA duplexes assayed containing Zn-porphyrin (donor) and 2H-porphyrins (acceptor) (purple), the Zn-porphyrin position is held constant, whilst the 2H-porphyrin is varied, giving rise to seven different porphyrin-porphyrin distances and angles.

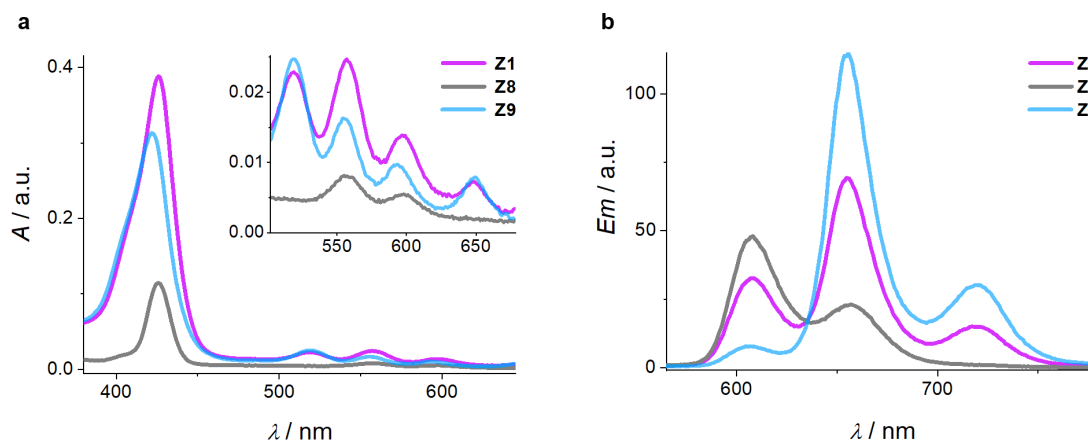


Figure 2. Representative absorbance (a) and emission (b) spectra of the Zn and 2H-porphyrin FRET pair (Z1), Zn-porphyrin (Z8) and 2H-porphyrin (Z9). [DNA] = 2 μM, 100 mM NaCl, 100 mM Na₂HPO₄ buffer pH 7.0.

3.2. Determining FRET efficiency

The quantum yield and Förster distance of the **Zn-P-2H-P** pair were measured previously to be $\Phi = 0.12$ and $R_0 = 28.4$ Å, respectively.³⁵ The distances and angles of the chromophores in the DNA duplexes used here were obtained from energy minimised structures, using the effective transition moment along the 5,15-axis through the acetylene linker as determined by *Berova et al.*³⁶⁻³⁸ The calculated E_{FRET} values (see electronic supporting information [ESI] for details)³⁰ show deviation from the idealised model ($\kappa^2 = 2/3$) (Fig. 3a); for the calculation of the E_{FRET} efficiencies κ^2 was taken into account and calculated for each pair as described previously. The experimentally determined E_{FRET} values from the donor emission peak at 605 nm show a steady decrease in FRET efficiency with increasing donor-acceptor distance. As expected, **Z2** gives the highest E_{FRET} value due to the smallest porphyrin-porphyrin spacing. Conversely, **Z3** and **Z4** exhibit higher E_{FRET} values than **Z1**, even though they have the same, or are separated by one extra base pair, respectively. These differences arise from the attachment points on the complementary strands which positions the porphyrins in **Z1** on opposite sides of the duplex, whereas in **Z2** and **Z3** the porphyrins are located in the same hemisphere of the duplex (see Fig. 1 for DNA models).

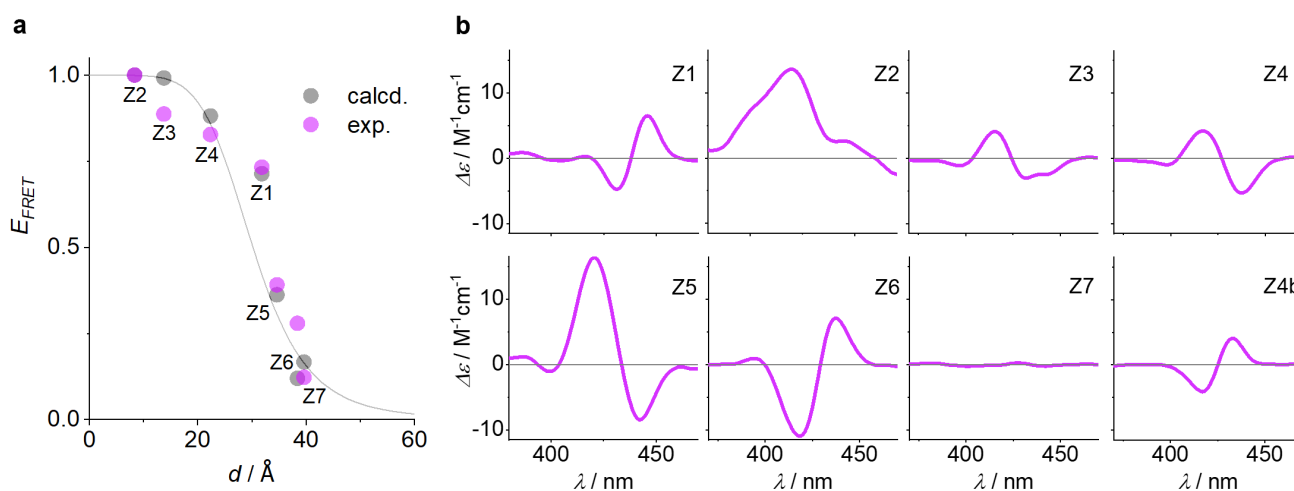


Figure 3. a) E_{FRET} as function of **Zn-P** distance (d), comparing calculated³⁰ and measured FRET efficiencies (the black line represents idealised behaviour with $\kappa^2 = 2/3$). b) CD spectra of the **Zn-P** duplexes (porphyrin region), including the CD spectrum of **Z4b** where the angle of the porphyrin dipole moment has opposite sign to **Z4**. Conditions as in Fig. 2.

The influence of the angle between the chromophores is clearly visible in the CD spectra. The excitonic coupling between the porphyrins display both positive and negative Cotton effects as a function of angle between the dipole moments (Fig. 3b), which is defined as the projection angle of the dipole moments and is either (+) or (−) (viewing down the helical axis of the DNA). Generally, a positive Cotton effect at longer wavelengths and negative one at shorter wavelengths determines a positive exciton couplet and arises from a positive angle (positive exciton chirality).³⁹ This theory has successfully been used to determine the chirality in systems having identical chromophores, but not with mixed chromophore systems. Here, the angles match perfectly well with the CD chirality (Table 1), but only when assuming both porphyrins as equal. Thus, the porphyrins are close to being degenerate: the small ground state differences from zinc to free-base porphyrin are not discriminated by the exciton chirality method, and the system reports two identical chromophores. This is in contrast to the direct energy transfer as seen in FRET. To demonstrate this, we have synthesised the duplex **Z4b** where the relative position of the two porphyrins is inverted with respect to **Z4**, meaning that in **Z4b**

the zinc porphyrin is now downstream of the free-base porphyrin. While the base pair separation and through-space distance of the porphyrins is maintained, the repositioning of the porphyrins results in an inverted exciton chirality, and the CD spectra are near mirror images of each other (Fig. 3b; the E_{FRET} values were not determined for this system).

Table 1. Structural parameters, FRET efficiencies and CD data of the donor-acceptor systems.

DNA duplex	Base pair separation	Porphyrin distance [Å] and dipole angle [deg]	E_{FRET} , calcd. and exp. values	κ^2 value	Exciton couplet $A_{\text{CD}}^{[a]}$ and exciton chirality ^[b]
Z1	2	30.2 / +158	0.71 / 0.73	2.704	+11.3 / (+)
Z2	0	11.0 / +22	1 / 1	1.001	+16.2 / (+)
Z3	2	13.7 / -60	0.99 / 0.89	0.759	-7.1 / (-)
Z4	3	22.0 / -92	0.88 / 0.83	0.963	-9.5 / (-)
Z4b	3	21.6 / +90	n.d. ^[c]	n.d. ^[c]	+8.2 / (+)
Z5	5	34.7 / -167	0.36 / 0.39	1.012	-24.8 / (-)
Z6	7	39.8 / +121	0.12 / 0.28	0.448	+18.0 / (+)
Z7	11	41.1 / +23	0.17 / 0.12	0.806	+0.4 / (+)

[a] The difference between the CD extrema at longer wavelengths and at shorter wavelengths (in $\Delta\epsilon$) determines the amplitude A_{CD} of the exciton couplet.³⁹ [b] The exciton chirality corresponds to the sign of the dipole angle. [c] not determined.

3.3. Detecting ethidium bromide intercalation at single base pair level

We developed an intercalation assay to test the sensitivity of the porphyrin system. Given that the energy transfer method to measure distance changes is still ambiguous due to relatively small expected effects (e.g. see difference from **Z1** to **Z4**), the CD spectra seem to respond much more sensitively to the relative arrangement of the porphyrins. We therefore tested the response of the porphyrin arrays to the intercalation of ethidium bromide (EtBr). EtBr is a well-known intercalator by stacking inside the DNA duplex (sliding in-between base pairs) and is commonly used as a nucleic acid stain in gel electrophoresis to visualise the DNA bands. The binding of EtBr to DNA has been studied extensively, with global binding constants (K_D) in the range of $3.4 \times 10^3 \text{ M}^{-1}$ to $1.3 \times 10^6 \text{ M}^{-1}$, depending mainly on salt concentration.^{32, 33, 40-45} Also, the binding mode indicates that EtBr intercalates at approximately every two to three base pairs with $n \sim 2.5$,⁴⁴ though n -values ranging from 1.6⁴⁵ up to 9^{32, 33} have been reported. In addition, EtBr can show multiple binding interactions, including intercalation, semi-intercalation and electrostatic binding.^{32, 33} We set out to test if our system would be suitable to directly detect the binding of an intercalator on both a large range and single base pair system.

We synthesised two additional porphyrin-DNA strands **Y1** and **Y2** (Fig. 4), with two porphyrins being either at five base pairs apart, or on adjacent base pairs, respectively. This DNA system is shorter and simpler, providing only A-T base pairs in-between the porphyrin sites, and flanking G-C base pairs to maintain duplex stability. Since the metallation state does not influence the exciton coupling, both reporter porphyrins were used as 2H-porphyrins which also simplifies the synthesis by avoiding post-synthetic metalation step. To probe the system, we titrated in EtBr to **Y1** and observed a corresponding change in the exciton coupling of the porphyrins (Fig. 4a and ESI). The large reduction in signal intensity can be attributed to a change in helicity of the DNA upon EtBr intercalation, and with it a

change in chromophore distance and angle. This is, however, based on the assumption that interactions outside of the porphyrin modification sites will not greatly affect the local structure around the porphyrins, which may not exactly be true. The use of an excess of EtBr (> 500 equivalents) did not indicate any significant changes in CD signal after addition of 20 equivalents, and up to that point the EtBr did not itself produce a significant signal in the porphyrin region that would interfere with the analysis. The binding constants were calculated from a non-linear fitting of the Scatchard plot of r/C_f vs r (see experimental section); the apparent global binding constant in our case is $K_D = 1.43 \pm 0.2 \times 10^5 \text{ M}^{-1}$, with $n = 2.6$ binding sites. This tends towards the upper limit of binding constants reported. The analysis is based under the assumption that any outside binding, e.g. electrostatic interactions with the phosphate groups or groove binding, does not give rise to a change in porphyrin coupling as it would not lead to large structural changes. Also, we have not found any indication that EtBr would interact strongly with the porphyrins and lead to a change in CD signature (see ESI). Therefore, we can assume that this apparent K_D represents a value for the intercalation under our experimental conditions. The number of binding sites also compares well to reported values.

More interesting is the response of the system when the porphyrins are at adjacent base pairs as in **Y2**. The order of the porphyrins was switched to give opposite exciton chirality which distinguishes it clearly from the system **Y1**. The intercalation can also be monitored from a large reduction in the exciton coupling of the porphyrins. Here, it is assumed that any EtBr that would bind at random parts of the DNA (internally or externally) will not be detected, and only the single binding site occupation observed. The data points were again fitted according to equation (1) and yielded a K_D of $3.54 \pm 0.4 \times 10^5 \text{ M}^{-1}$, with $n = 2.6$. The K_D is higher than the global binding constant, but the weaker binding is a reflection of the competing sites outside of the porphyrin region which would lead to a lower effective molarity of the available EtBr. This is also shown by the same n -value for both systems. This result shows that the intercalation of a molecule can clearly be detected on the single base pair level using the strong exciton coupling of porphyrins, which is very sensitive down to the nanometer scale.

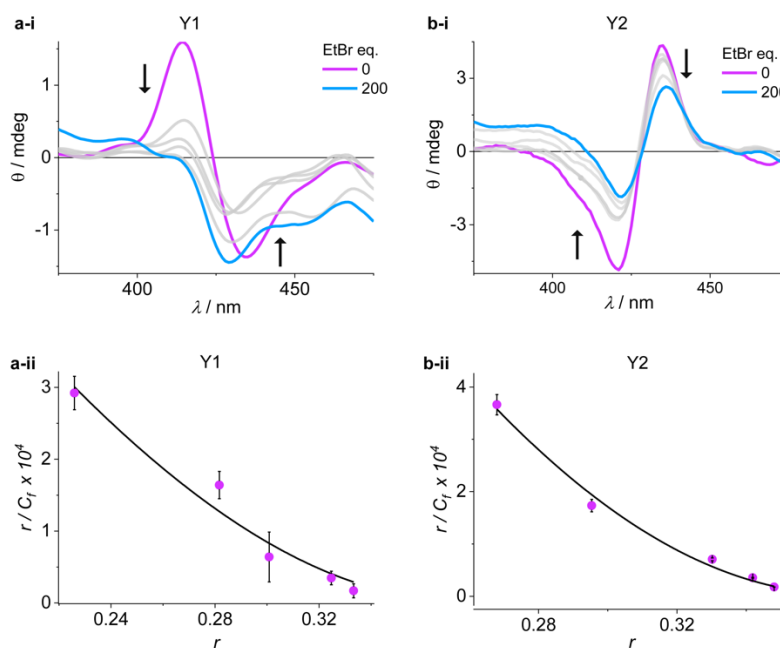


Figure 4. Example CD spectra of the titration experiments of a-i) **Y1** and b-i) **Y2**, the arrows indicate the peak shift upon addition of EtBr, and corresponding Scatchard plots shown in a-ii) and b-ii), respectively, together with the fitted curves according to eq (1).

4. Conclusions

Based on our previously established porphyrin-DNA system, we have demonstrated that varying the position of two porphyrins along a DNA helix can be used to create a molecular ruler, that responds well to changes in distance and structure. Using a well-established FRET pair based on zinc metallated and free-base porphyrins, the FRET efficiency can be correlated to the position of the porphyrins. However, the helicity can lead to ambiguity when FRET is used to determine the distance of the chromophores. This is mainly attributed to the helical structure and arrangement. Circular Dichroism, on the other hand, has revealed that the exciton coupling is independent on the metallation state, most likely because the absorbances of the two porphyrins (Zn, 2H) are very close. This is visible from the exciton couplet which follows the helical geometry of the porphyrin system, not the donor-acceptor energy order. Furthermore, the CD response is shown to be highly dependent on the relative orientation and distance of the porphyrins. This can be explored to monitor structural changes such as those that are induced by intercalation. While the system certainly needs to be evaluated in greater detail, the obtained binding constants for EtBr as a model system compare well with literature data. In this respect, changes can be detected down to the single base-pair level, which gives nanometer resolution in DNA analysis.

5. Data Availability Statement

All datasets generated for this study are included in the article / Supplementary Material.

6. Supplementary Material

Supplementary material is available: details on the FRET matrix determination, full CD titration spectra and modelling of the porphyrin-DNA strands.

7. Funding

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8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Author Contributions

JB designed the ruler system, JB and JW synthesised the porphyrin-DNA strands and performed the experiments, JB, JW and ES analysed the data, ES supervised the project, ES and JB wrote the manuscript.

10. Data Availability Statement

The datasets analysed for this study can be found in the University of Southampton Institutional Research Repository, ePrints Soton: DOI: <https://doi.org/10.5258/SOTON/D1227>.

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