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An Estrogen–Platinum Terpyridine Conjugate: DNA and Protein Binding and Cellular Delivery

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Abstract: A platinum metal complex in which terpyridine joins estradiol (via an ethynyl link) to a platinum with a labile ligand (chloride) has been designed, synthesised and its X-ray crystal structure determined. The aim of this work was to link a targeting motif (in this case estrogen) to a metal-based biomolecule recognition unit (the platinum moiety). The target molecule: 17α-[4′-ethynyl-2,2′:6′,2′-terpyridine]-17β-estradiol platinum(II) chloride (PtEEtpy) has been shown to bind to both human and bovine serum albumin (SA) and to DNA. FTICR mass spectrometry shows that the bimolecular units are in each case linked through coordination to the platinum with displacement of the chloride ligand. Circular dichroism indicates that a termolecular entity involving PtEEtpy, SA and DNA is formed. A range of electrospray mass spectrometry experiments showed that the PtEEtpy complex breaks and forms coordination bonds relatively easily. A whole cell estrogen receptor assay in an estrogen receptor positive cell (MCF-7) confirms binding of both EEtpy and PtEEtpy to the estrogen receptor in cells. The work demonstrates the concept of linking a targeting moiety (in this case estrogen) to a DNA binding agent.

Keywords: bioinorganic chemistry · DNA recognition · steroid conjugates · ternary complex · terpyridines

Introduction

Methods to take drugs to specific cells and to transport them into and localize them within those cells are an important goal in biomedical chemistry since they offer the potential to simultaneously reduce both drug dosage and side effects. Estrogenic steroids are attractive vectors for cellular targeting and delivery as it is known that they are transported through the blood (bound to steroid transport proteins) and across the cellular membrane[1] and that, by binding to the estrogen receptor (ER) in the cytoplasm, they are finally transported into the cell nucleus. Moreover, steroids localise in specific tissues (e.g. breast) which affords a level of targeting.[2] We have been interested in using estrogenic steroids to deliver metal centres into cells and report herein a steroidal metal complex in which an estrogen is used to deliver a platinum-based DNA-binding unit.

Nuclear DNA is believed to be the major biological target of many platinum-based agents,[3–5] generally due to their ability to undergo covalent coordination[6,7] or intercalative binding[8–14] with the DNA bases. Although the clinical anticancer agent cisplatin[5,15–20] is able to react with a wide range of biomolecules, its mechanism of action is largely attributed to the irreversible coordination of its platinum(II) centre (via the two chloro leaving groups) to the N^7 atoms of two guanine bases (and to a lesser extent, a guanine and an adenine base) of DNA, commonly in the form of 1,2-in-

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trastrand crosslinks.[5,16,21] Biologically active platinum(II) complexes which intercalate between DNA base pairs have also been developed.[22–25] Their intercalative ability results from an aromatic moiety of sufficient surface area either coordinated directly or linked indirectly to the platinum(II) centre which can π-stack between two adjacent DNA base pairs. Much of the early research concerning platinum(II) metallointercalators was conducted by Lippard and colleagues using complexes containing 2,2′:6′,2″-terpyridine (tpy).[13,26] Although the terpyridine-based platinum(II) complexes were shown to bind intercalatively to DNA, the complex $[PtCl(tpy)]^+$ was also observed to slowly form a metal coordination with DNA under certain ionic conditions. Replacement of the chloride by thiols[27] or an alkyl[28] gives inert complexes which exclusively intercalate, similarly replacement of the chloride with pyridine leads to a dicationic intercalating agent with higher DNA binding affinity[24] (although over time the pyridine can be displaced by nucleobases leading to the platination binding mode). Attempts to further improve the biological effectiveness of such terpyridine–platinum(II) complexes against tumour cells have led to the development of similar mono- and bifunctional compounds, including a number of drugs synthesized by Lowe et al.[29–32] containing 4′-substituted terpyridines and tpy-linked bispyridine ligands to create bis-intercalators with rigidly separated binding sites to explore DNA topology.[33] Pikramenou has recently linked two platinum–terpyridyl units to a dithiol substituted lanthanide complex to form very elegant visible and near-IR luminescent hairpin bis-intercalators[34] and the unit has also been attached to a thiol-substituted carborane.[35]

In addition to their DNA-binding, platinum(II)–terpyridyl $[Pt(tpy)X]^{n+}$ units are also known to bind to proteins, particularly at cysteine residues. They have been shown: to possess activity against ovarian carcinomas;[31] to possess antiprotozoanal activity[32] by being active against the parasites;[30,36] and to inhibit human thioredoxin reductase[29] and the renal sodium pump.[37]

Most platinum-based drugs are nonspecific in the types of cells they target. Thus, several attempts have been made to make them more specific for targeting tumour cells of particular organs.[5,15,38] One approach for treating hormone-dependent tumours is to link a cytotoxic species to a hormonal agent such as estrogen. As a result, the receptor of the natural hormone binds to the hormonal-like portion of the drug and selectively guides the normally non-selective cytotoxic segment of the drug to the hormone's target tissue, for example, the mammary tissue.[39–41] Early studies on estrogenic metal complexes focused primarily on cisplatin derivatives linked to estrogens through one of the two steroid hydroxy groups.[38,42–47] These two hydroxy groups are very important in receptor recognition[48] and, probably in consequence, there was little gain from such conjugation. Jaouen[49] explored conjugation of steroids to organometallic carbonyl compounds (for application in carbonylmetalloassays) and demonstrated that organometallics could be attached at the 17α-position of estradiol with retention of some steroid receptor binding ability. Katzenellenbogen explored attaching organometallics to the 7α-position with similar success.[50] Inspired by Jaouen's work we have explored the use of estradiol steroids as cellular and nuclear delivery vectors to deliver a variety of metal centres into cells. We have been able to demonstrate that, despite bearing cationic charge, 17α-functionalised steroidal metal complexes of a variety of metals (including palladium(II) and platinum(II)) are both delivered into the cell and retained some receptor binding (3–12% RBA).[51] Osella has more recently described an agent bearing a platinum–malonato unit attached through the 17α-position but which shows negligible receptor binding,[43] while Bérubé has prepared mixtures of estrogens with dichloroplatinum units attached at the 16α- and 16β-positions which show more promise.[52–54]

Lippard has demonstrated that steroid hormones can sensitise cancer cells to platinum drugs (through induction of HMG over-expression)[55] and has attempted to harness this affect through design of an estrogenic platinum(IV) agent intended to hydrolyse and reduce, thus forming cisplatin and an estrogen after entering the cell.[56]

The purpose of this work was to develop a molecule which combined: i) a steroid binding motif to facilitate cellular delivery to cells with estrogen receptors, and ii) a DNA binding motif designed for interaction with DNA. As steroids are transported through the blood by proteins such as human serum albumin, HSA, binding to HSA was also investigated. In this work we report the design, synthesis and biomolecule binding of 17α-[4′-ethynyl-2,2′:6′,2″-terpyridine]-17β-estradiol platinum(II) chloride (PtEEtpy), in which a terpyridine group links the steroid to the metal (Figure 1).

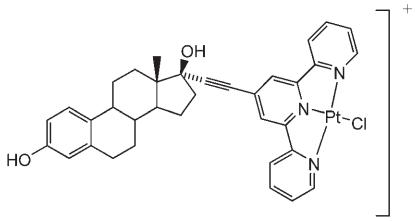

Figure 1. 17α-[4′-Ethynyl-2,2′:6′,2″- terpyridine]-17β-estradiol platinum(II) chloride, $[Pt(EEtpy)Cl]^+$.

Results and Discussion

Molecular design: Our design focused on attaching the DNA-binding unit to the 17α-position of the steroid. Previous work[51,66] has shown that this position locates the unit on the underside of the steroid away from the top recognition face and the hydroxy recognition units. Moreover, our previous work has shown that steroidal metal complexes conjugated through this point are transported into cells and retain some receptor binding.[66] Linking the steroid and metal complex via an alkyne linker is attractive due to its

synthetic feasibility and because, as a spacer unit, it introduces distance without introducing steric bulk. The platinum terpyridyl DNA-binding unit is attractive because its DNA binding is well studied and it has interesting biological activity. In addition, and as detailed below, Sonogashira couplings provide a powerful synthetic route to introduce an aromatic unit, making attachment of this unit to a steroid synthetically practical. We anticipated that the introduction of the large steroid group at the 4′-position of the terpyridine unit would prevent substantial intercalative insertion and thus drive the DNA-binding unit to adopt a covalent platination binding mode, thereby simplifying the binding.

Synthesis and characterization of [Pt(EEtpy)Cl]$^+$: The terpyridine derivatised estrogen ligand, EEtpy, was synthesized in good yields from ethynylestradiol and 4′-[{(trifluoromethyl)sulfonyl}oxy]-2,2′:6′,2″-terpyridine in a palladium-catalysed Sonogashira coupling. Purification (primarily removal of phosphine contaminants from the catalyst) was achieved by column chromatography on alumina. Mass spectral data reveal a parent ion EEtpy peak and NMR data are consistent with the coupled product. In particular, in the NMR spectrum the appropriate ratios of the integrals for the three aromatic estradiol protons with respect to the pyridyl protons are observed and the ethynyl proton (δ 2.8 ppm) from the starting steroid is absent. Recrystallisation of the ligand from a methanol/propan-2-ol mixture afforded crystals suitable for X-ray diffraction and the structure of this steroidal ligand has been determined.

The structure of the ligand (Figure 2) confirms that attachment of the terpyridyl unit through the 17α-position does indeed ensure the chelating unit lies below the steroid away from the two hydroxy groups and the upper face of the steroid which are important in receptor binding. The rings of the terpyridyl unit are approximately coplanar (torsion angles in the range 10–16°) and the nitrogens adopt the normal transoid arrangement observed in other crystal structures of free oligopyridines and which minimises lone pair-lone pair and dipole–dipole repulsions while maximising conjugation.[67–74] The planar terpyridyl ligand is oriented approximately parallel to the long axis of the steroid ligand; this orientation presumably permits the most efficient packing of the molecule. The presence of the terpyridyl unit does not interfere with the capacity of the steroid hydroxy groups to engage in hydrogen bonding with the phenolic and the tertiary hydroxy groups and methanol solvent molecules. Each steroid hydroxy group acts as an hydrogen-bond donor to one solvent molecule and an hydrogen-bond acceptor to another. The solvent-bridged hydrogen bonds link the steroids giving rise to steroid tube structures as shown in Figure 3. There are also a number of (very offset) π-stacking interactions and these and further packing representations are shown in the Supporting Information.

While coordinating a tridentate ligand to a square-planar metal might be expected to be facile, the synthesis of platinum(II) complexes of terpyridyl ligands can be challenging and indeed there have been studies devoted solely to this topic.[75,76] The synthesis is complicated by a tendency to form insoluble tetrachloroplatinate salts[75] and further complicated by different solid-state packing motifs that are believed to be responsible for dramatic differences in colour (yellow through red) and solubility. Moreover, no one synthetic route has been identified which is suitable for all differently substituted terpyridines. We first explored platinum cyclooctadiene dichloride as a starting material, following routes outlined by Annibale[75] and by Lowe.[76] Our first attempt afforded a clean pale yellow product of good solubility. However, we were unable to reproduce this synthesis, obtaining materials of varying colour and very poor solubility. Exploring variations on the approach, we were able to obtain complexes (evidenced by parent ion [Pt(EEtpy)Cl]$^+$ peaks in FAB MS) from both methanol or acetone solution and with and without pre-treatment with a silver salt to remove one or both chlorides. However, none of these routes gave products of good solubility (indeed most products obtained were insufficiently soluble for the NMR to be recorded). Finally we explored platinum bisbenzonitrile dichloride as a starting material. It was pre-treated with a silver(I) tetrafluoroborate or hexafluorophosphate salt to remove one of the coordinated chlorides. The reaction in dichloromethane led to a poorly soluble product, but by using

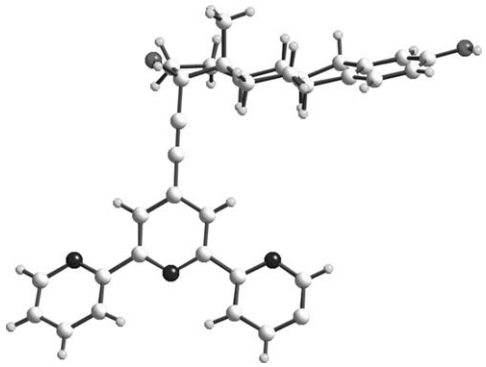

Figure 2. Crystal and molecular structure of EEtpy.

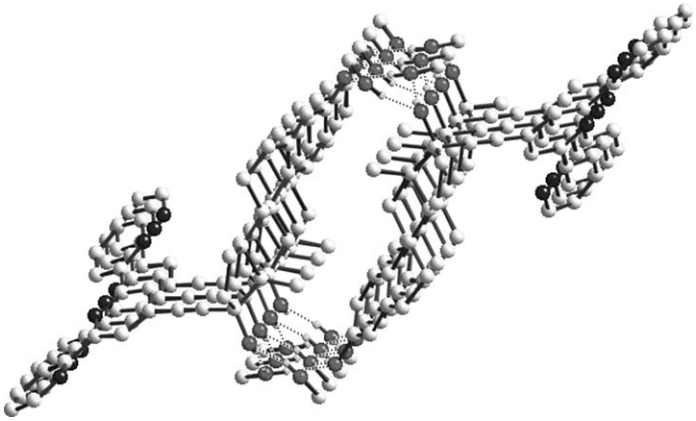

Figure 3. Hydrogen bonding of EEtpy with methanol solvent giving rise to steroid tube structures. For clarity, hydrogens not involved in the hydrogen bonds are omitted.

acetonitrile as reaction solvent a soluble orange tetrafluoroborate salt could be obtained. Anion metathesis was used to obtain the analogous chloride salt which has better solubility in alcohols and was used for the DNA binding studies. In their FAB and ESI mass spectra both the tetrafluoroborate and chloride salts show a peak at *m*/*z* 757 (with the correct isotopic distribution) corresponding to $[Pt(EEtpy)Cl]^+$. Partial microanalytical data are consistent with the formulations $[Pt(EEtpy)Cl]BF_4 \cdot H_2O$ and $[Pt(EEtpy)Cl]Cl \cdot 3H_2O$ and the high resolution FTICR mass spectrum (Figure 4) of the chloride salt confirms the identity of the cation with a monoisotopic mass of 756.1882 Da in agreement with the theoretical value of 756.1880 Da. The proton NMR spectra of the complexes show shifts in the pyridyl resonances consistent with coordination, and platinum satellites can be distinguished from the baseline for proton H_6. The UV/Visible spectrum of [Pt(EEtpy)Cl]Cl shows an absorbance centred around 415 nm ($\varepsilon = 2400\ mol^{-1}\,m^3\,cm^{-1}$) consistent with that for $[Pt(tpy)Cl]^+$ (400 nm), arising from a metal ligand charge transfer transition and again confirming platinum complexation.

Crystals of the tetrafluoroborate salt were grown from acetonitrile solution by the slow diffusion of benzene. The structure of the cation (Figure 5) reveals the anticipated *pseudo*-square-planar platinum(II) centre coordinated to a tridentate terpyridyl unit and a monodentate chloride. The terpyridine unit constrains the geometry at the metal and the Pt–N bond length to the central ring is consequently shorter than that to the outer rings (Table 1). The bond lengths and angles are similar to those observed in other platinum(II)–terpyridyl complexes.[77–80] The metal complex hangs below the estrogenic steroid, as in the free ligand, with the tpy unit again lying along the approximate direction of the long axis of the steroid. The complexes are stacked to form chains of platinum(II)–terpyridyl units, decorated with estrogenic steroids (Figure 6). There are no short Pt···Pt interactions (shortest contact > 4.3 Å) but rather the shortest contacts to the Pt centres are contacts to pyridyl rings above and below the square-plane of their coordination sphere. In each case this is to the C and N atoms of the central pyridyl ring, with the contact being shortest to the C (Pt···C 3.40 Å) for one such interaction and for the other to the centroid of the C–N bond (Pt···centroid 3.37 Å). All the pyridine rings are fully engaged in face to face π-stacking interactions and each tpy unit forms three stacking interactions to the tpy above and three to the tpy below (typical offset stacked interactions of coplanar rings with interplanar distances of the order of 3.4 Å and with centroid–centroid distances in the range 3.74–3.95 Å). This stacking motif is somewhat different from that usually observed: planar platinum–terpyridyl complexes do usually form extended stacks, however, within this stack it is usual to see platinum-platinum contacts, most usually Pt···Pt dimers[77–80] or polymers.[79] While previously reported Pt–terpyridyl stacks also contain some π-stacking interactions, not all rings have been fully engaged in that stacking. In this $[Pt(EEtpy)Cl]^+$ structure, the loss of potential Pt···Pt interactions (usually assumed to

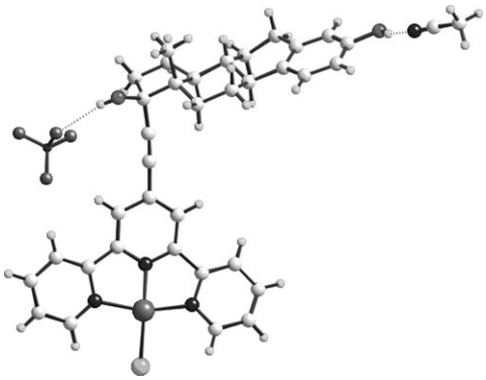

Figure 5. Crystal and molecular structure of $[Pt(EEtpy)Cl][BF_4]$.

Table 1. Selected bond lengths [Å] and angles [°] for $[Pt(EEtpy)Cl]BF_4$.

Pt(1)–N(1)	2.033(6)
Pt(1)–N(8)	1.901(6)
Pt(1)–N(14)	2.011(6)
Pt(1)–Cl(1)	2.295(2)
N(8)-Pt(1)-N(14)	81.1(2)
N(8)-Pt(1)-N(1)	81.1(2)
N(14)-Pt(1)-N(1)	162.1(2)
N(8)-Pt(1)-Cl(1)	178.6(3)
N(14)-Pt(1)-Cl(1)	99.78(19)
N(1)-Pt(1)-Cl(1)	98.06(19)

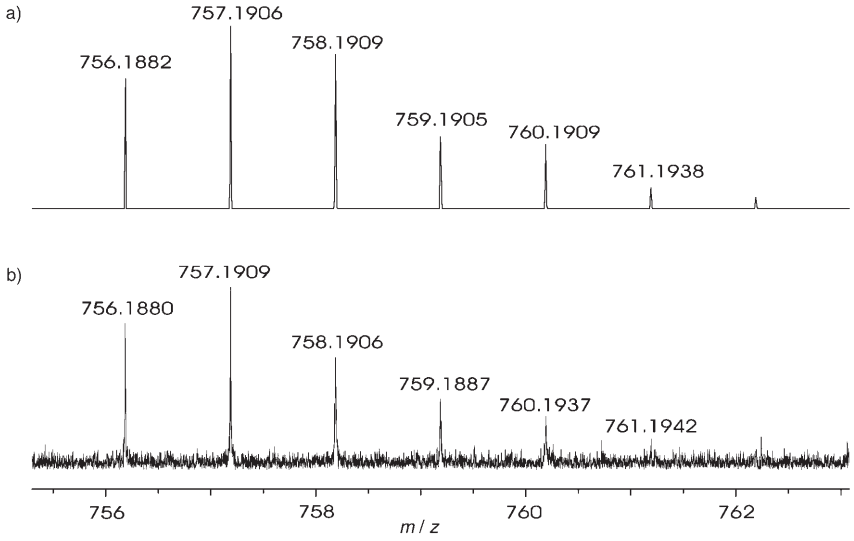

Figure 4. a) Theoretical and b) experimental FTICR mass spectrum of PtEEtpy chloride salt dissolved in water/acetonitrile/formic acid (49:49:2 v/v).

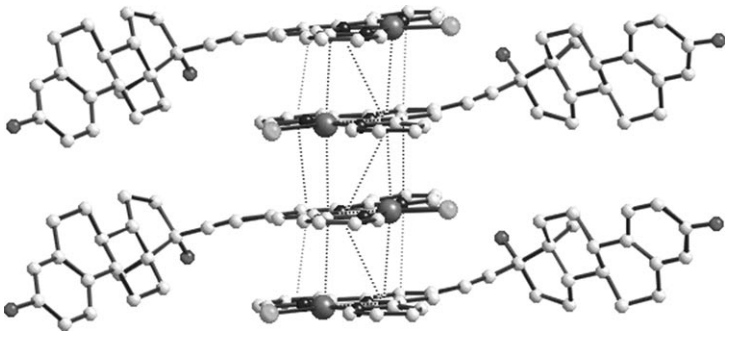

Figure 6. Chains of [Pt(EEtpy)Cl]+ cations formed through π–π and Pt-π interactions. Hydrogens are omitted for clarity.

be strong interactions)[79] appear to have been balanced by an increase in the π-stacking. This balance between multiple competing interactions is reminiscent of the "frustration" approach that we have recently explored as a means of supramolecular library design.[81,82] Indeed we have observed how such competition can lead to a variety of spiral or stacked structures in silver(I) terpyridyl compounds. While platinum–terpyridyl stacks are traditionally ascribed to Pt···Pt and π–π interactions, the contacts from the Pt centres to the rings above and below are particularly intriguing. They are very reminiscent of the η^2-type π-interactions typically seen for Ag···π interactions.[81,83–86] Moreover examination of previous platinum–terpyridyl structures reveals quite similar contacts at the points in the stacks where Pt···Pt contacts are absent. It is unclear whether these might represent additional weak bonding interactions or whether these contacts arise simply as a consequence of the aromatic stacking.

The steroids from adjacent chains are packed together to form an extended solid-state structure (see Supporting Information). As in the free ligand, both alcohol groups of the steroid engage in hydrogen bonding interactions in the crystal lattice, with the phenolic hydroxy hydrogen bonding with an acetonitrile solvent molecule (O···N 2.82 Å; OH···N 2.00 Å) and the tertiary alcohol with a tetrafluoroborate anion (O···F 2.93 Å; OH···F 2.15 Å). From the structure it is clear that the steroid would prevent intercalation into DNA via the central ring of the tpy unit, the initial binding mode observed for platinum complexes of unsubstituted tpy ligands and used so elegantly by Lowe[24,25,79] and by Pikramenou.[34] Some partial intercalation through a terminal pyridine ring might be possible but would not be extensive. By contrast the estrogen should have no steric effect on the alternative DNA-binding mode in which chloride is replaced by a DNA base (most usually N^7 of a guanine residue). The metal complex is buried beneath the steroid and does not interfere with the top face of the steroid nor with the hydroxy groups which, as the structure demonstrates, retain their ability to form hydrogen bonds.

Biomacromolecule binding: The molecular design concept of PtEEtpy was to use the steroid as a transport and localisation motif to deliver a DNA-binding platinum unit. Thus it was important to show that the complex bound to estrogen receptors in cells. The other key molecules of interest are human serum albumin (the main steroid transporter protein) and DNA.

Estrogen receptor whole cell binding assays: The relative binding affinities of the steroidal ligand and metal complex for ERs in viable MCF-7 cells were determined by a competitive radiometric binding assay. The assay involves competition of the steroidal conjugates with 16α-[^{125}I]-estradiol during incubation of viable cells in a medium containing a fixed concentration of 16α-[^{125}I]-estradiol and various concentrations of the competing steroidal ligand or metal complex. The bound 16α-[^{125}I]-estradiol is then extracted from the cells and measured using a gamma counter. From these data the concentration of competing chelate or metal complex required to displace half of the 16α-[^{125}I]-estradiol that would be bound to the ER in the cell in the absence of the test compound is calculated. This is denoted by the IC_{50} displacement measurement for the compound. Low IC_{50} values thus correlate with high binding affinities. This whole cell estrogen receptor assay depends on: i) the ability of the conjugate to be transported into the cell, and ii) the ability of the conjugate to bind to the estrogen receptor in the presence of other biomolecules in the cell which interferes with its binding to the ER (e.g. random metallation of protein amino-acid residues). In each experiment diethylstilbestrol was used as a control since it is known to have a high affinity for the ER (the concentration of DES at which 50% of the ^{125}I is displaced: IC_{50} = 0.6 nM in each experiment) and its use enables comparison of the receptor affinity to be made between assays.

The IC_{50} values (mean data from a minimum of two experiments) for the two molecules are: EEtpy: 80 nM and PtEEtpy: 500 nM. From these results it may be concluded that upon substituting the estrogen at the 17α-position with the tpy derivatives: receptor binding is retained (albeit at a lower relative binding affinity relative to estradiol, approximately 2% for the free ligand and 0.3% for the metal complex);[51] cellular delivery is achieved; and cationic charge (due to the Pt^{2+}) is not greatly detrimental to either delivery or binding. The magnitude of the receptor binding is weaker than the slightly more flexible complexes we have previously reported[51] where the platinum is chelated to a ligand with a single aromatic ring (to which the steroid is attached) and two flexible arms, as well as a single chloride ligand. The IC_{50} values for those complexes are ~15 nm. This may indicate that the planar terpyridine ligand is a little too large for the pocket adjacent to the receptor binding site though the assay does also probe other factors such as binding to other biomacromolecules and cellular transport. In

support of the latter option, a lysed cell-fraction ER assay gave a value of 6 nM for the IC_{50} of PtEEtpy (DES IC_{50} = 0.1 nM). The key conclusion is that receptor binding is retained upon conjugation of the steroid to the quite large terpyridine unit.

Spectroscopic studies of biomacromolecules binding: Circular dichroism (CD) is a measure of the chirality of a system and any changes in the observed signal when two molecules are mixed together is a sensitive probe of changes in the chiral environment of both chiral and achiral molecules. All the molecules of interest to this work are chiral, so, to use CD to probe change, it is essential that the intrinsic CD spectrum of each component (at the appropriate concentrations) is subtracted from the spectrum of the mixture. If the result is non-zero then there is an interaction. Linear dichroism (LD) is also a differential spectroscopy technique; in this case the light used is linearly polarized parallel and perpendicular to a sample orientation axis. In our experiments the DNA is oriented by flowing it (in a Couette cell).[62,63] DNA is the only molecule studied in this work which can be flow oriented, thus the LD signal is a useful indicator of whether anything is bound to the DNA, since for example, PtEEtpy will only give an LD signal if it is bound to the DNA. The magnitude of the DNA LD signal (when the ligands are not significantly contributing at 260 nm) is also a good indicator of whether the ligand bends[87] or stiffens the DNA.[63]

To probe whether PtEEtpy binds to either DNA or proteins the CD spectra of DNA, HSA, PtEEtpy in isolation and mixed together were measured. The ICD spectra of Figure 7 show that PtEEtpy binds both to DNA and to HSA. The perturbation to the PtEEtpy (P) spectrum (the region above 300 nm) by DNA (D) and HSA (H) are different (DP–D–P and HP–H–P in Figure 7) and that of the three-component mixture is distinct from that of any of the two-component mixtures or any simple combination of them (the simple average of the DNA–PtEEtpy and HSA–PtEEtpy ICD is illustrated in Figure 7). The ICD above 350 nm is largely (though not completely) due to the loss of the Pt–Cl ligand to metal charge transfer band (following loss of the Cl, see below). The terpyridine in-ligand band centred at ~325 nm is perturbed differently in all the mixtures. The spectra of the three-component mixtures are more similar, but not identical to an average of that induced by the HSA and DNA suggesting that the Cl is lost upon binding and the tpy is perturbed by both biomacromolecules. If the DNA is added first the ICD resembles that of the DNA–PtEEtpy ICD more closely than if the HSA is added first. This suggests that in each case there is a population of molecules adopting the preferred DNA binding and a population adopting the preferred HSA binding mode with the order of addition affecting this distribution. Thus it may be concluded that there is formation of some kind of heterobiomolecular complex of the three components: HSA–PtEEtpy–DNA. The complex thus formed is probably not a unique species.

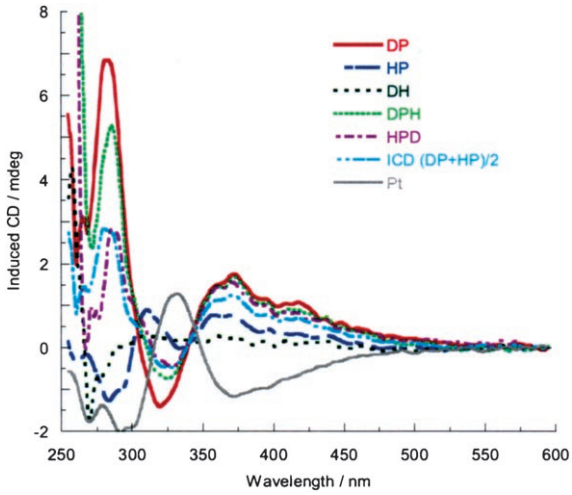

Figure 7. Induced CD spectrum for various mixtures of chiral components. In each case the spectrum is calculated by taking the baseline corrected spectrum of the mixture and subtracting the baseline corrected CD spectra of the relevant chiral components (PtEEtpy, HSA or DNA). DNA+PtEEtpy (denoted DP), HSA+PtEEtpy (denoted HP) and HSA+PtEEtpy+DNA (HPD). The notation denotes the order of mixing, e.g. DPH indicated the components were added in the order DNA then PtEEtpy then HSA. All spectra were collected in a 1 cm pathlength cuvette and data were smoothed over ±5 nm intervals. Concentrations are: PtEEtpy 30 μM, HSA was 16 μM, and ct-DNA 300 μM in 10 mM sodium cacodylate (pH 7) and 40 mM NaCl. The theoretical average of the DNA + PtEEtpy and HSA + PtEEtpy (denoted (DP+HP)/2) ICD spectra is also shown.

The LD spectra corresponding to the CD spectra of Figure 7 (see Figure S5, Supporting Information) confirm the simultaneous interaction of all three species. The LD of PtEEtpy+DNA shows a positive maximum at 470 nm and a negative maximum at 410 nm. Below 400 nm there are three negative bands with the signal at 258 nm being larger in magnitude than that for the DNA alone. The 330 nm tpy in-ligand band is small and negative which could be consistent with the tpy lying parallel to the DNA base pairs if the binding constant is relatively small—as would be expected either for intercalation or for covalent binding of the Pt to guanine N^7. The LD for the three component mixture (HSA + PtEEtpy + DNA) is smaller in magnitude than the PtEEtpy + DNA complex but similar in shape. This is further support for the formation of a heterobiomolecular array, and suggestive of the PtEEtpy adopting the same orientation on the DNA as in the absence of the HSA. The loss of LD magnitude in the ternary complex is due to a decrease in the orientation of the system, in contrast to the effect of HSA on DNA in the absence of PtEEtpy. The decrease could be caused by a "bending" or "kinking" of the DNA in the tri-molecular complex. The bending or kinking is indicative of a non-intercalative binding mode for PtEEtpy though it might be partially inserted into the edge of a base pocket.

ESI Mass spectrometry: The spectroscopic studies show both bi- and trimolecular complexation. Whether these com-

plexes are covalent or noncovalent cannot be determined from these experiments, though the data are suggestive of loss of the Cl ion from the Pt^{II} atom. If data can be collected, the high resolution of FTICR MS with a 9.4 T magnet provides the possibility of distinguishing covalent and noncovalent binding modes. Detailed experiments with a Qtof2 and non-self-complementary DNA sequences were also employed to further probe the complexation.

FTICR Mass spectrometry: BSA was used for these experiments as an HSA sample of sufficient purity to get good mass spectral data could not be obtained. The BSA spectrum of Figure 8 displays a charge state distribution ranging from $[M+28H]^{28+}$ to $[M+40H]^{40+}$. For each of the charge states shown, a degree of isotopic fine structure was observed, which allowed the average mass of BSA to be assigned as 66397 ± 1.4 Da, which is in good agreement with the theoretical average mass of 66397 Da calculated from the complete amino acid sequence.[88] Addition of PtEEtpy (30 μM) to BSA (30 μM) resulted in the formation of a BSA–PtEEtpy complex as shown in Figure 8 with a concomitant shift in the charge state distribution to lower charge states: the highest observed charge state in the un-complexed BSA was $[M+40H]^{40+}$ whereas the highest observed charge state in the complexed BSA was $[M+34H]^{34+}$. This shift in the charge state distribution upon the addition of PtEEtpy could reflect the BSA adopting either a more compact or a shielded conformation as a result of PtEEtpy binding. It was only possible to obtain the mass spectrum for the BSA–PtEEtpy complex in 8 K format as opposed to 512 K used for the free BSA and isotopic resolution for the complex was not achieved. However, the average mass of the observed complex, corresponded to BSA plus 721 Da, indicating that the complex contained only one PtEEtpy molecule without the chlorine attached. The theoretical ESI mass spectrum derived from the theoretical mass of BSA plus the mass of EEtpy minus chlorine was compared to the experimental spectrum and found to be in excellent agreement. Thus we conclude that the PtEEtpy has covalently bound to the BSA through the platinum rather than non-covalently through the estrogen as we had supposed (since BSA is a steroid transporter protein). This is not altogether surprising since the ratio of steroid binding sites to cysteine or methionine residues is ~1:15 for BSA.

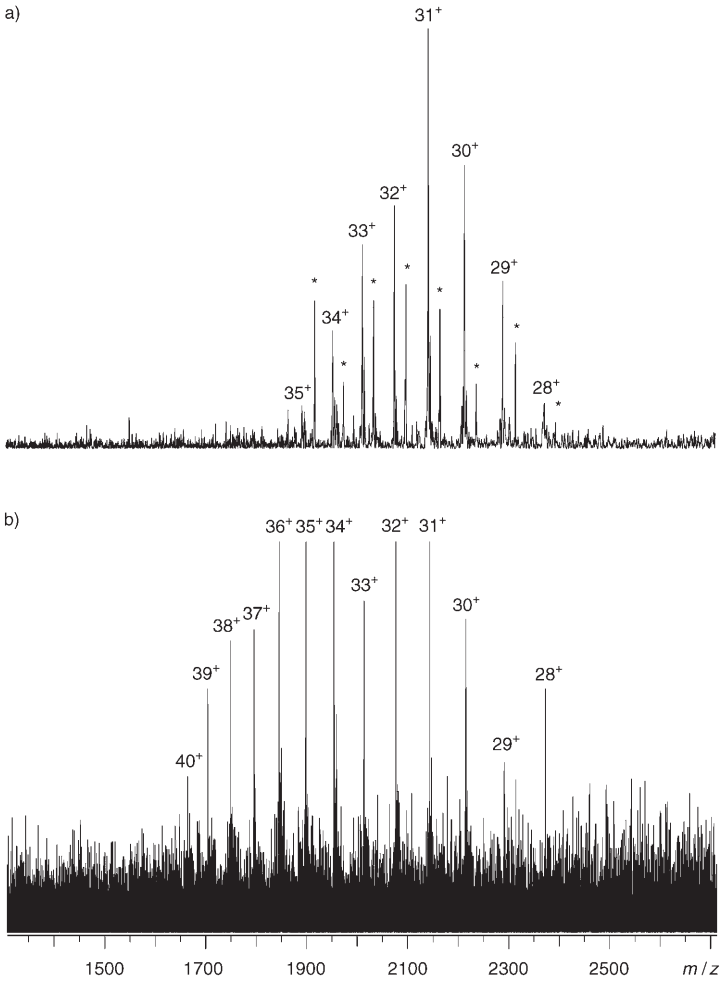

Figure 8. a) ESI mass spectrum of BSA (30 μM) + EPT (30 μM) and b) BSA (30 μM) in water/acetonitrile/formic acid (49:49:2 v/v/v). * Denotes peaks arising from EPT–BSA complex.

The proposed mode of action for PtEEtpy involves its ability to bind to DNA as well as serum albumin. The high resolution of the FTICR data means that ss and ds self-complementary oligonucleotides can be distinguished so the classical Dickerson–Drew dodecamer, (DD) 12-mer (5′-CGCGAATTCGCG-3′), was chosen to probe the DNA binding. The ion derived from this DNA with no sodium ions attached and without any protonation at the phosphodiester groups is denoted herein as $[DD]^{11-}$; the ion derived from the double-stranded Dickerson–Drew DNA (DD_2) would therefore, be assigned as $[DD_2]^{22-}$. DNA commonly retains sodium ions and frequently these sodium ions exchange with protons in solution resulting in various mixtures of cation combinations and charge states. In this work the concern was with maximizing the transfer of intact double stranded DNA from solution into the gas phase, so that the mode of PtEEtpy binding to DNA could be studied. The solvent adopted to maximize DD_2 and stability of the spray was water/acetonitrile/formic acid (49:49:2 v/v/v). CD was used to confirm that these conditions did not alter the DNA structure.

The negative ion mass spectrum of the DNA (200 μM oligonucleotide) obtained by nanospray ionisation shows (Figure 9) the presence of both DD and DD_2. There is a distribution envelope of sodium ions attached to the ss DD in the DD^{2-} and to the ds DD_2^{4-} charge states. Mass accuracies of less than 2 ppm were achieved for all the FTICR DNA spectra presented in this paper. Addition of PtEEtpy (50 μM) to the DNA (200 μM) resulted in several significant changes in the DNA spectrum compared to that of the free DNA (Figure 10). Firstly there was a shift in the

charge-state envelope for free DD_2 from low-charge states (3^-) to higher charge or more negative states (5^- and 6^-). Secondly, there was the appearance of peaks, which corresponded to a PtEEtpy–DD_2 complex in the M^{5-} and M^{6-} charge states. Thirdly, the single stranded DNA [DD], which was observed in the free DNA spectrum was no longer present. Finally, there was increase in the number of Na^+ ions attached to the free double stranded DNA (DD_2). Prior to the addition of PtEEtpy the charge state distribution for $[DD_2]^{4-}$ centred around the attachment of 12 Na^+ ions, upon the addition of PtEEtpy this increased to 16 Na^+ ions for free $[DD_2]^{4-}$. The poor signal intensities and broad isotopic distributions of the 5^- and 6^- PtEEtpy–DNA complex ions made assignment of the exact mass of the complex difficult despite repeated attempts. The average mass of the complex corresponded to DD_2 plus 721 mass units, indicating that the PtEEtpy+DNA complex contained only one PtEEtpy molecule without the chlorine attached, suggesting that the EEtpy covalently binds to the DNA through the platinum as in the case of BSA and as we anticipated in the molecular design. Thus we may conclude that it binds monofunctionally to the DNA, presumably to the N^7 of guanine,[12,13] that is, in the major groove.

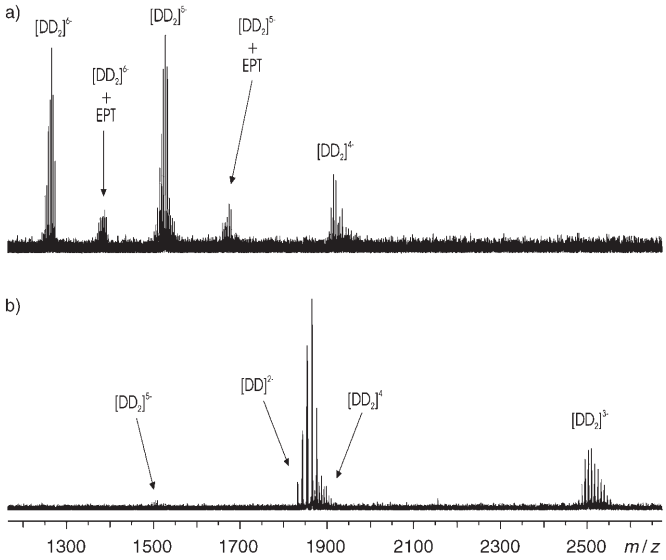

Figure 10. Negative ion nanoflow ionisation mass spectrum of a) Dickerson–Drew 12-mer (200 μM) + PtEEtpy (50 μM) in water:acetonitrile (1:1, v/v) and b) Dickerson–Drew 12-mer (200 μM). EPT denotes PtEEtpy.

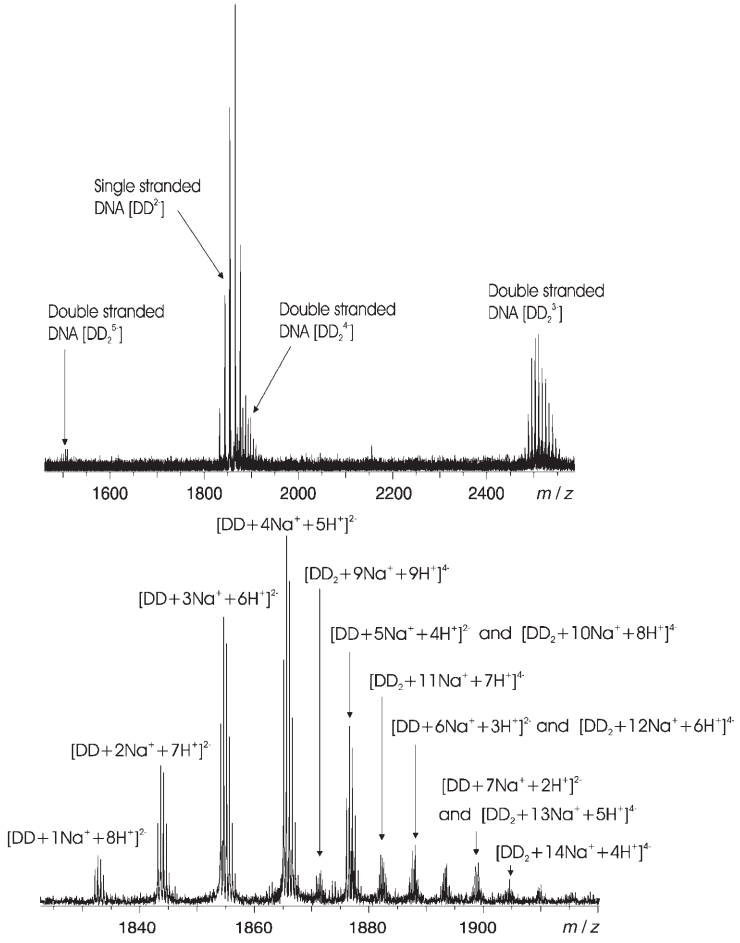

Figure 9. Top: Negative-ion nanospray ionisation FTICR mass spectrum of a Dickerson–Drew 12-mer (200 μM) in water/acetonitrile (1:3 v/v); bottom: expansion of the *m*/*z* 1830–1920 region of the spectrum. The Na^+ distribution centres around the attachment of 4 Na^+ for $[DD]^{2-}$ and 11 for $[DD]^{4-}$.

Binding to (5′-CCCATGCACACC-3′)·(5′-GGTGTGCATGGG-3′): The FTICR MS experiments produce data with isotopic resolution and enable identification of species present in solution, including differentiation of single- and double-stranded DNA and covalent or non-covalent binding. However, the experiments are non-trivial and attempts to observe the heterobiomolecular BSA–PtEEtpy–DNA complex in either positive ion or negative ion mode were unsuccessful. One of the issues appeared to be the small amounts of the PtEEtpy–DNA complex present in solution. Experiments were therefore undertaken to try to shift the reaction mixture towards the formation of the complexes following protocols developed for other coordinate bond forming DNA ligands.[65] A Qtof2 mass spectrometer was used to monitor the production of complexes. PtEEtpy was treated with SJW1 and the resulting complex was separated from unreacted ssDNA using HPLC.

Comparison of the −3 and −4 charge states of the SJW1–1PtEEtpy complex at *m*/*z* 1418 and 1063, respectively with −3 and −4 charge states of unreacted single stranded SJW1 at *m*/*z* 1177 and 889 (Figure 11) shows the effect of time and pH on the binding of PtEEtpy to SJW1. For example, at pH 5.0 (data not shown) the relative intensity of the 4^- complex ion becomes strongest after 6 h and after 122 h, the intensity of complex signals decreased significantly suggesting that the complex is not stable over long periods of time. At pH 7.0 the reaction is somewhat faster and the product more stable. At pH 8.5 the reaction is even faster though the amount of product is smaller. The pH 5 yields of the 1:1 complex are the greatest, but at this pH there are also noticeable amounts of 2:1 (*m*/*z* 1244 and 1659, 4^- and 3^- ions, respectively) and 3:1 PtEEtpy–DNA complex produced. Complexes of SJW1 with 2 PtEEtpy molecules (*m*/*z* 1244 and 1659, 4^- and 3^- ions, respectively) were also evident at the higher mixing ratio.

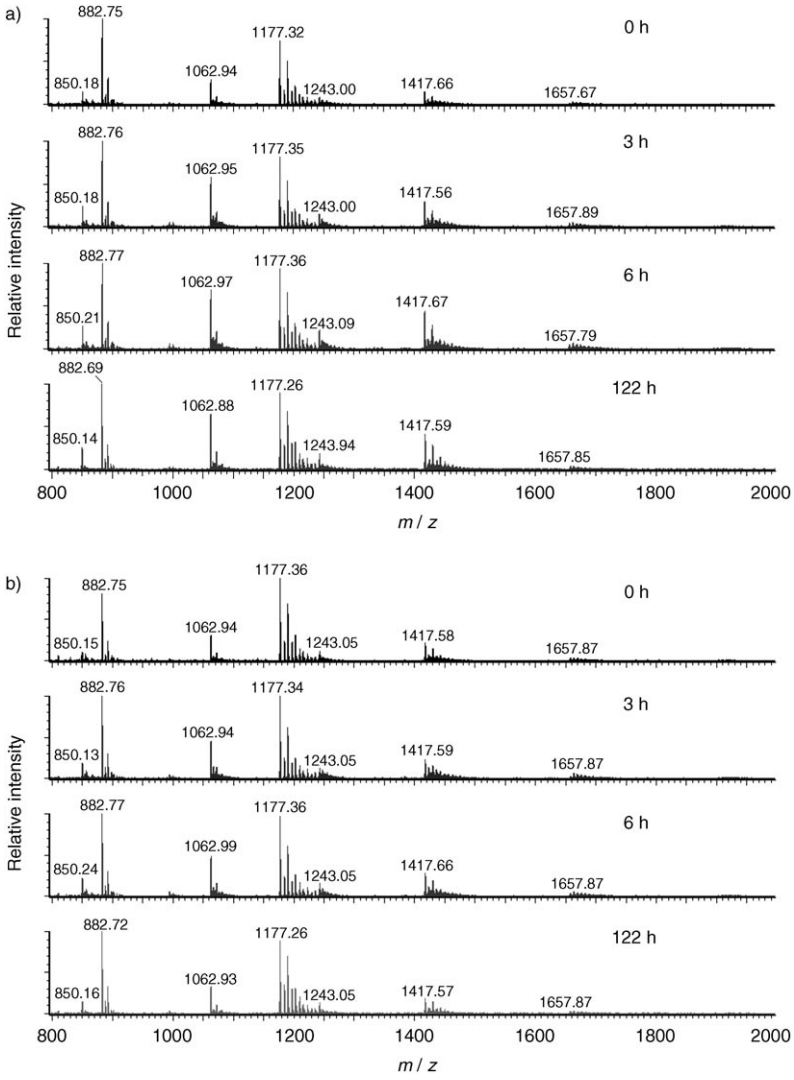

Figure 11. ESI-MS spectra acquired for SJW1–PtEEtpy at a ratio of 1:3 at a) pH 7.0 and b) 8.5 after various time increments as indicated in the figure.

The conditions for maximizing the 1:1 SJW1+PtEEtpy complex formation without significant amounts of 1:2 or 1:3 were therefore concluded to be: pH 7.0 with a ratio of 1:3 for 3 h. As judged by ESI-MS, these conditions seemed to produce an approximately equal amount of SJW1+1PtEEtpy complex and free SJW1 with small amounts SJW1+2PtEEtpy complexes. HPLC purification (Figure 12a) of the reaction mixture resulted in four major fractions (F1–F4). The mass spectrometry indicated fraction 1 comprised solely of the single-stranded SJW1, fraction 2 contained a mixture of SJW1 and the SJW1+PtEEtpy complex with an increased percentage of the complex being observed compared with the unpurified sample (Figure 12b). It is most likely that this fraction was eluted from the column as SJW1+PtEEtpy complex, however, by the time its mass spectrum was measured the solution had reached an equilibrium of bound and free ssDNA. Fraction 3 and 4 did not contain any DNA or complex (data not shown).

Fraction 2 (which contained ssDNA–PtEEtpy) was annealed with the complementary oligonucleotide SJW2. The ESI-MS spectrum of the resulting sample is shown in Figure 13. In this spectrum the high intensity of the 5^- ion of dsSJW1–SJW2 (*m*/*z* 1457) indicates it is present in very high amounts compared to the ds-SJW1–SJW2–PtEEtpy seen at *m*/*z* 1601. The relatively small amount of the dsDNA–PtEEtpy observed suggests that the annealing process (including the high temperature) has facilitated the dissociation of the SJW1–PtEEtpy complex.

Conclusion

We have synthesised a metallo–estrogen derivative that binds to serum albumin (a steroid transport protein), to DNA and to both protein and DNA simultaneously, showing that steroidal metal complexes can be used as bifunctional agents to assemble heterobiomolecular arrays. The X-ray diffraction crystal structure of PtEEtpy shows the shape of the molecule to be such that it is extremely unlikely to intercalate into the DNA but that the steroid would not prevent

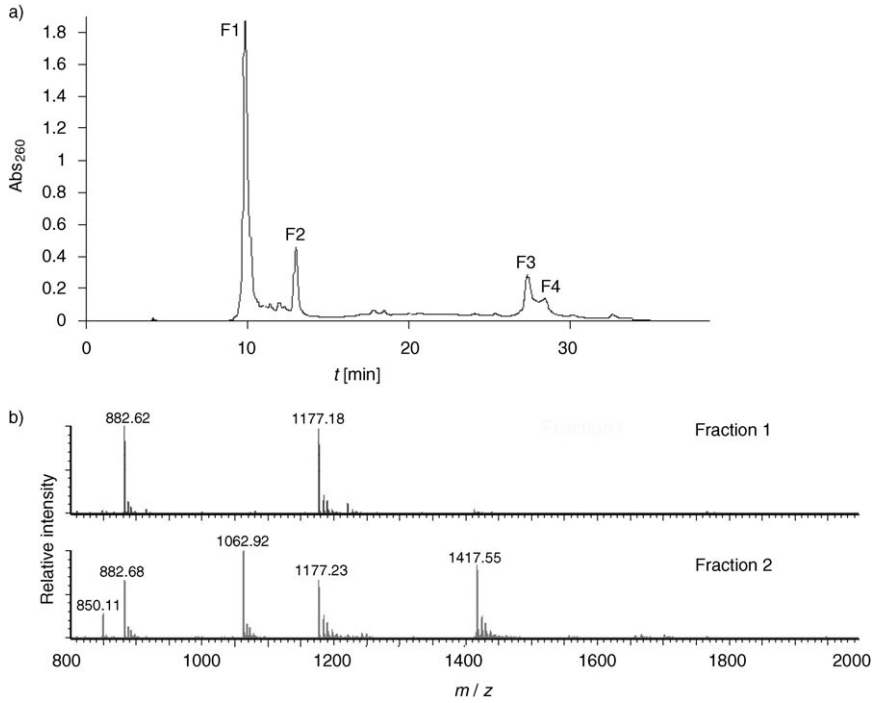

Figure 12. a) HPLC trace obtained from the purification of products from the reaction of SJW1 with PtEEtpy after reacting for 3 h at a ratio of 1:3 and pH 7.0; b) ESI-MS analysis of fractions 1 and 2.

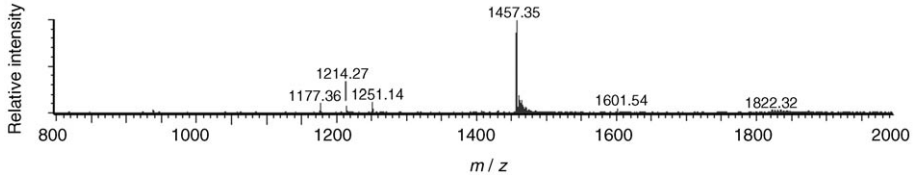

Figure 13. ESI mass spectrum of fraction 2 obtained from a HPLC separation of SJW1 reactions with PtEEtpy after annealing with SJW2.

it binding through metal coordination to the bases in the major groove of the DNA. The intermolecular interactions in the crystal are dominated by π–π stacking interactions and show no Pt–Pt interactions.

The FTICR mass spectra indicated that the bimolecular complexes of PtEEtpy with both DNA and BSA were metal coordination complexes formed by displacing the Cl^- ion from the metal. However, DNA and BSA cannot both bind in this manner in the trimolecular complex. The lability of the complex shown in the Qtof2 mass spectrometry experiments enables us to rationalize the fact that the CD spectroscopy showed a trimolecular array whose structure was in part dependent on order of addition of components: on the timescale of preparing and measuring the CD spectrum the complex could rearrange to a more stable form. The terpyridine is a very strong σ-donating π-accepting ligand so makes the position *trans* to it more labile than one might expect for a Pt–Cl bond. The shapes of the LD spectra suggest that the coordination bond to DNA is the one that is retained and that the protein effects a net bending of the DNA when it binds in or near the same site. Thus this monofunctional platinum agent with protein binding unit attached seems to have the ability to show a similar ultimate effect on DNA (kinking) as bifunctional cisplatin-like agents. The biological significance of the molecular design is shown by the in vitro whole-cell assay which showed that both the neutral metal-free ligand and the cationic platinum complex were transported and delivered to the cell, thus showing that the steroid is an effective delivery vehicle for cellular transport of metal centres. The work demonstrates that estrogenic steroids are effective delivery vehicles for DNA-binding/platinum agents and further investigations with other platinum units are ongoing.

Experimental Section

Synthesis

General: Starting materials were purchased from Aldrich, Avocado and Lancaster and used without further purification. ^{1}H NMR spectra were recorded on a Bruker DPX 300 MHz instrument. EI, CI and FAB mass spectrometry were carried out by the Warwick Mass Spectrometry Service using a matrix of 3-nitrobenzyl alcohol on Micromass Autospec spectrometers. ESI mass spectrometry was carried out by the EPSRC Mass Spectrometry Service Centre, Swansea on a Micromass Quatro II (low resolution triple quadrupole mass spectrometer. Infra Red spectra were recorded on a Bruker Vector 220 instrument fitted with ATR golden gate. UV/Visible spectra were recorded on a Jasco V-550 UV/Vis absorption spectrometer. Microanalyses were carried out by the Warwick Analytical Service on a Leeman labs CE44 CHN analyser. 4′-[{(Trifluoromethyl)sulfonyl}oxy]-2,2′:6′,2″-terpyridine was prepared in a three-step synthesis following the method of Potts.[57]

(17α-[4′-Ethynyl-2,2′:6′,2″-terpyridine])-17β-estradiol (EEtpy): 4′-[{(Trifluoromethyl)sulfonyl}oxy]-2,2′:6′,2″-terpyridine (33 mg, 8.66×10^{-4} mol), 17α-ethynyl estradiol (260 mg, 8.77×10^{-4} mol), palladium(II)bis(triphenylphosphine)dichloride (60 mg, 8.55×10^{-5} mol) and copper(I) iodide (9 mg, 4.73×10^{-5} mol) were mixed in degassed diisopropylamine (20 mL) under nitrogen, in the dark and stirred for 24 h. The solvent was removed in vacuo from the resulting orange solution and the solid was redissolved in diethyl ether (40 mL). The insoluble portion was removed by filtration and the filtrate removed to dryness yielding a light orange solid, which was dried over P_2O_5. Purification of the product was achieved by column chromatography using grade I alumina activated with 1% water and eluted with 1% methanol/chloroform giving a white solid (240 mg, 4.6×10^{-4} mol, 53%) which was dried over P_2O_5 under vacuum. Crystals suitable for X-ray diffraction were obtained by slow evaporation of an ethanol/propan-2-ol solution. ^{1}H NMR ($CDCl_3$): δ = 8.69 (br d, J = 4.7 Hz, 2H, H_6), 8.59 (br d, J = 7.9 Hz, 2H, H_3), 8.47 (s, 2H, $H_{3'}$), 7.86 (td, 2H,

$J=7.8$ Hz, H_4), 7.35 (ddd, 2H, $J=7.8$, 4.7 Hz, H_5), 7.14 (d, 1H, $J=8.3$ Hz, H_a), 6.63 (dd, 1H, $J=8.5$, 2.4 Hz, H_b), 6.54 (d, 1H, $J=1.9$ Hz, H_c), 2.79–1.23 (m, 21H, estradiol non-aromatic protons); IR: $\tilde{\nu}$ = 2930s, 2868s, 2812w, 1603w, 1584s, 1566m, 1547m, 1503m, 1469s, 1392s, 1286w, 1255m, 1119w, 1003w, 788s, 734m, 616w cm^{-1}; UV/Vis (CH_3OH): λ_{max} (ε): 317 (4000), 276 (14000), 248 nm (18000 $mol^{-1}\,m^3\,cm^{-1}$); EI+ MS: m/z: 527 $[M]^+$; CI+ MS: m/z: 528 $[M+H]^+$.

{(17α-[4′-Ethynyl-2,2′:6′,2″-terpyridine])-17β-estradiol}-chloroplatinum(II) tetrafluoroborate, [Pt(EEtpy)Cl]BF_4: Bis(benzonitrile)platinum(II) chloride (17 mg, 3.60×10^{-5} mol) in acetonitrile (5 mL) was added dropwise to a solution of silver tetrafluoroborate (9.7 mg, 5.0×10^{-5} mol) in acetonitrile (5 mL) and the resulting solution was heated at reflux for 16 h. Silver chloride, which had precipitated, was filtered off through Celite and to the filtrate was added EEtpy (19 mg, 3.61×10^{-5} mol). This solution was heated at reflux for a further 24 h after which the silver chloride which had precipitated was filtered off. The solution was reduced in volume by approximately two thirds and cooled in ice resulting in the precipitation of an orange solid (6.2 mg, 7.35×10^{-6} mol, 20%). Crystals suitable for X-ray crystallography were obtained by from acetonitrile solution by slow vapour diffusion of benzene. ^{1}H NMR (CD_3CN): δ = 8.89 (br d, $J=6.7$ Hz, H_6), 8.23 (t, $J=9.0$ Hz, H_4), 8.12 (br d, $J=7.4$ Hz, H_3), 8.10 (s, $H_{3'}$), 7.70 (br t, $J=7.4$ Hz, H_5), 7.00 (d, $J=7.9$ Hz, H_a), 6.42 (d, $J=10.0$ Hz, H_b), 6.37 (br s, H_c), 2.46–1.25 (m, 21H, estradiol non-aromatic protons); +ve FAB MS: m/z: 758 $[M-BF_4]^+$; elemental analysis calcd (%) for $Pt_1C_{35}H_{33}N_3O_2Cl_1B_1F_4{\cdot}1H_2O$: C 48.7, H 4.1, N 4.9; found: C 48.8, H 4.1, N 4.8.

{(17α-[4′-Ethynyl-2,2′:6′,2′-terpyridine])-17β-estradiol}-chloroplatinum(II) chloride, [Pt(EEtpy)Cl]Cl: Bis(benzonitrile)platinum(II) chloride (27 mg, 5.72×10^{-5} mol) in acetonitrile (5 mL) was added dropwise to a solution of silver hexafluorophosphate (14 mg, 5.54×10^{-5} mol) in acetonitrile (5 mL) and the resulting solution was heated at reflux for 16 h. Silver chloride, which had precipitated, was filtered off through Celite and to the filtrate was added EEtpy (31 mg, 5.89×10^{-5} mol). This solution was heated at reflux for a further 24 h after which additional silver chloride, which had precipitated out, was filtered off. Counter ion exchange was carried out by addition of *tert*-butyl ammonium chloride in acetonitrile (2 mL) resulting in the precipitation of an orange solid (31 mg, 3.96×10^{-5} mol, 69%), which was collected on a sinter and washed with cold acetonitrile (5 mL) and cold diethyl ether (5 mL). ^{1}H NMR (CD_3OD): δ = 9.10 (d, 2H, $J=6.8$ Hz, H_6), 8.55 (d, 2H, $J=7.8$ Hz, H_3), 8.52 (s, 2H, $H_{3'}$), 8.44 (t, 2H, $J=7.8$ Hz, H_4), 7.92 (v2H, $J=6.0$ Hz, H_5), 7.13 (d, 1H, $J=8.6$ Hz, H_a), 6.57 (d, 1H, $J=8.6$ Hz, H_b), 6.52 (s, 1H, H_c), 2.52–1.28 (m, 21H, estradiol non-aromatic protons); IR: $\tilde{\nu}$ = 3350s, 3067s, 2358w, 1713m, 1606s, 1556w, 1478w, 1418w, 1372w, 1292m, 1243m, 1097w, 1033m, 784m cm^{-1}; ESI-MS: m/z: 758 [Pt(EEtpy)Cl]$^+$; FAB+ MS: m/z: 758 [Pt(EEtpy)Cl]$^+$; UV/Vis (2% CH_3OH/H_2O): λ_{max} (ε) = 415 (2400), 334 (8000), 287 (13000), 259 (16000), 224 nm (18000 $mol^{-1}\,m^3\,cm^{-1}$); elemental analysis calcd (%) for $Pt_1C_{35}H_{33}N_3O_2Cl_2{\cdot}3H_2O$: C 49.6, H 4.6, N 5.0; found: C 49.5, H 4.5, N 4.9.

X-ray crystallography

Data were measured at 180 K with a Siemens-SMART-CCD diffractometer[58] equipped with an Oxford Cryosystem Cryostream Cooler.[59] Refinement used SHELXL96.[60]

Crystal data for EEtpy: $C_{37}H_{41}N_3O_4$, $M=591.73$, monoclinic, space group $C2$, $a=29.05630(10)$, $b=7.4338(2)$, $c=15.3049(3)$ Å, $\beta=99.128(2)°$, $V=3263.97(11)$ Å^3 (by least squares refinement on 3476 reflection positions), $T=180(2)$ K, $Z=4$, $\rho_{calcd}=1.204$ $Mg\,m^{-3}$, $F(000)=1264$; μ ($Mo_{K\alpha}$) = 0.078 mm^{-1}; crystal character: colourless plates; crystal dimensions $0.5\times0.4\times0.2$ mm; θ_{max} = 28.60°; *hkl* ranges were −38/30, −9/9, −5/20; 6189 reflections measured, 5559 unique [s(int) = 0.0194]; absorption correction: semi-empirical from equivalents; minimum and maximum transmission factors: 0.63, 1.0; no crystal decay.

Structure analysis and refinement: Systematic absences indicated space group $C2$, Cm or $C2m^{-1}$. The first was chosen because of the known chirality of the system and shown to be correct by successful refinement. The structure was solved by direct methods using SHELXS[61] with additional light atoms found by Fourier methods, including two lattice methanol molecules; their protons were located directly from difference maps, but were refined as restrained atoms. Hydrogen atoms were added at calculated positions and refined using a riding model with freely rotating methyl groups. Anisotropic displacement parameters were used for all non-H atoms; H-atoms were given isotropic displacement parameters equal to 1.2 (or 1.5 for methyl hydrogen atoms) times the equivalent isotropic displacement parameter of the atom to which the H-atom is attached. The absolute structure was determined from the known chirality of the molecule. Refinement of a delta-f" multiplier did not give any discrimination. Floating origin constraints were generated automatically. The weighting scheme was calculated $w=1/[\sigma^2(F_o^2)+(0.0551P)^2]$ where $P=(F_o^2+2F_c^2)/3$. Goodness-of-fit on F^2 was 0.842, $R1$[for 3703 reflections with $I>2\sigma(I)$] = 0.0411, $wR2=0.1009$; data/restraints/parameters 5559/1/404; largest difference Fourier peak and hole 0.113 and −0.145 e Å^{-3}.

Crystal data for [Pt(EEtpy)Cl]BF_4: $C_{43}H_{42}BClF_4N_4O_2Pt$, $M=964.16$, orthorhombic, space group $C222_1$, $a=6.7686(3)$, $b=25.2199(9)$, $c=47.0667(16)$ Å, $V=8034.4(5)$ Å^3 (by least squares refinement on 7971 reflection positions), $Z=8$, $\rho_{calcd}=1.594$ $Mg\,m^{-3}$, $F(000)=3840$; μ($Mo_{K\alpha}$) = 3.620 mm^{-1}; crystal character: red-brown plates; crystal dimensions $0.58\times0.18\times0.03$ mm; θ_{max} = 28.53°; *hkl* ranges were −5/9, −33/33, −60/55; 24648 reflections measured, 9361 unique [R(int) = 0.0802]; absorption correction: semi-empirical from equivalents; minimum and maximum transmission factors: 0.41, 0.93; no crystal decay.

Structure analysis and refinement: Systematic absences indicated space group $C222_1$. The structure was solved by direct methods using SHELXS[61] additional light atoms found by Fourier methods, including solvent benzene and acetonitrile molecules. Hydrogen atoms were added at calculated positions and refined using a riding model with freely rotating methyl and OH groups. Anisotropic displacement parameters were used for all non-H atoms; H-atoms were given isotropic displacement parameters equal to 1.2 (or 1.5 for methyl hydrogen atoms) times the equivalent isotropic displacement parameter of the atom to which the H-atom is attached. The known absolute structure was confirmed by refinement of a delta-f" multiplier. Absolute structure parameter $x=0.002(11)$. The weighting scheme was calculated $w=1/[\sigma^2(F_o^2)+(0.0400P)^2]$ where $P=(F_o^2+2F_c^2)/3$; goodness-of-fit on F^2 was 0.982, $R1$ [for 6395 reflections with $I>2\sigma(I)$] = 0.0634, $wR2=0.1105$; data/restraints/parameters 9361/0/504; largest difference Fourier peak and hole 2.883 and −3.759 e Å^{-3}; all the largest difference peaks are close to Pt; a number of relatively large discrepancies between observed and calculated structure factors are attributed to the length of the *c*-axis causing some overlap between peaks (but not enough to hinder structure solution or refinement).

Receptor binding and delivery

The binding to ERs in viable MCF-7 cells was investigated by a competitive radiometric binding assays against 16α-[^{125}I]-estradiol, denoted ^{125}I. The concentration of competing agent required to displace half the ^{125}I bound to the ER is presented as an IC_{50} displacement measurement. Low IC_{50} displacement values thus correlate to high binding affinities. EEtpy and PtEEtpy (10 mM) were dissolved in methanol to give a stock solution that was subsequently further diluted with assay buffer. Solutions were sonicated to ensure dissolution. A 2 mM stock solution of diethylstilbestrol (DES) was prepared in ethanol.

Cell growth conditions and receptor preparation: The estrogen receptor positive MCF-7 (human breast adenocarcinoma) cells (obtained from the American Type Culture Collection) were routinely grown at 37°C in 5% carbon dioxide/95% air and maintained by weekly passage in Minimum Essential Medium (MEM) (Gibco) supplemented with 2 mM L-glutamine, 50 IU per 50 μg mL^{-1} penicillin/streptomycin, 1% non-essential amino acids and 10% foetal bovine serum.

For radioligand binding assays MCF-7 cells were seeded in growth media at a concentration of 1×10^4 cells per well (1 mL) into 24 well culture plates. After a period of 24 h, media was removed from the wells and the cell monolayer was washed with PBS to remove residual media and serum. Estrogen free medium (1 mL) which consisted of MEM without phenol red (Gibco) supplemented with 2 mM L-glutamine, 50 IU per 50 μg mL^{-1} penicillin/streptomycin, 1% non-essential amino acids and controlled process serum replacement-1 (CPSR-1, Sigma) was added to

each well. Incubation was continued for a further three days after which time cells were used in radioligand binding studies.

Radioligand binding assays: Binding affinities were determined from a minimum of two independent assays in each case.

Viable cells assay: The medium was removed from a 24 well plate and monolayer washed with PBS (1 mL). The medium was replaced with 200 μL assay buffer (MEM without phenol red containing 0.1% BSA) containing 0.1 nM 16α-[^{125}I]-iodo-3,17β-estradiol plus or minus competing compound at desired concentration was added to the wells. The cells were incubated at 37 °C for 1 hour after which time assay reagents were removed and replaced with 500 μL equilibration buffer (5 mM sodium phosphate, 0.25 M sucrose, 0.5% BSA, 10% glycerol, pH 7.5). Cells were further incubated at 37 °C for 30 min. The buffer was removed and cells were washed rapidly with ice-cold equilibration buffer. Ethanol (1 mL) was added to each well to extract the radioactive component. After 30 min at room temperature, the ethanol was transferred to counting vials and radioactivity counted by a Wallac Wizard gamma counter.

Spectroscopy

Normal absorption spectra were collected on a Cary 1E or Jasco V-550 spectrometer in a 1 cm pathlength cuvette. Circular dichroism in a 1 cm or 1 mm pathlength cuvette and linear dichroism (LD), in a 1 mm path length Couette flow cell[62,63] spectra were collected on a Jasco J-715 spectropolarimeter adapted for LD measurements. All spectra were recorded at room temperature and were baseline corrected for any intrinsic signal in the cuvette or instrument. Induced CD (ICD) spectra were obtained by subtracting the baseline corrected spectrum of all chiral components of any mixture from the baseline corrected spectrum of the mixture of the components.

HSA was purchased from Sigma. Stock solutions (8 μM and 32 μM) were prepared by accurately weighing the appropriate mass of HSA (M_W 66437) and dissolving it in sodium cacodylate buffer (10 mM) and NaCl (40 mM), pH 7. These solutions were used without further purification. PtEEtpy stock solutions were prepared (by accurately weighing the solid) in 20:80 methanol (HPLC grade BDH):water (Elga Maxima 18.2 MΩ) v/v. DNA base molar concentrations were determined using $\varepsilon_{258\,nm} = 6600\ cm^{-1}m^3mol^{-1}$ for calf thymus DNA (highly lyophilized from Sigma).[64] The synthetic oligonucleotide (from Oswell, Southampton UK) and serum albumin (from Calbiochem, UK) concentrations were determined by weight assuming pure samples.

Mass spectrometry

Dickerson Drew DNA and serum albumin mass spectrometry experiments: Fourier transform ion cyclotron (FTICR) mass spectrometric experiments were undertaken with a 9.4 T Bruker Apex II instrument. Source parameters were carefully controlled during the experiments to minimise unwanted dissociation of the analyte and its complexes. Nanospray needles for the DNA experiments (Protana, USA) were coated in palladium and gold with a 50 μm internal diameter. The needles, which were tapered closed at the spray end, were opened by gently touching the needle against the end plate of the instrument. The exit of the tapered needle was measured under a microscope and found to be approximately 10–20 μm; a pressure of ~10 psi was applied to the proximal end of the nanospray needle. A capillary voltage of 300–500 V was typically used. Attempts to use HSA in the mass spectrometry experiments, even after desalting using a PD-10 column (Sephadex G25M, Amersham, Pharmacia Biotech) were unsuccessful. BSA was therefore used as it displays a very high sequence homology with HSA (76%), the same characteristic disulfide bond pattern and a very similar biological function. BSA and BSA–PtEEtpy 1:1 complexes were analysed by positive ion mode ESI. CO_2 was used as a drying gas in the source at a temperature of approximately 50 °C. The analyte was directly infused into the source at a flow rate of 1 μL min^{-1}. DNA and DNA:EPT (1:1) complexes were analysed using negative ion nano-flow ionisation. Due to its poor water solubility, a stock solution (200 μM) of PtEEtpy was prepared in 20% aqueous acetonitrile. For mass analysis the stock solution was diluted to 30 μM in water/acetonitrile/formic acid (49:49:2 v/v). For mass analysis the BSA was prepared to a concentration of 2 mg mL^{-1} (30 μM) in water/acetonitrile/formic acid (49:49:2 v/v). A synthetic self-complementary Dickerson–Drew 12 mer (5′-CGCGAATTCGCG-3′) was purchased from Oswell (Southampton, UK) and used without any further purification. For mass analysis the DNA was prepared to a concentration of 200 μM in water/acetonitrile (1:3 v/v). The DNA–PtEEtpy complex was analysed at a ratio of four oligonucleotides per one PtEEtpy.

Preparation and ESI-MS analysis of ssDNA-PtEEtpy: A 1 mM stock solution of PtEEtpy was prepared in 100% methanol. Oligonucleotide 5′-CCCATGCACACC-3′ (SJW1) was dissolved in 10 mM NH_4OAc adjusted to pH 5.0, 7.0 or 8.5 with dilute acetic acid or ammonia solutions. Appropriate volumes of PtEEtpy stock solution were taken and mixed with single-stranded (ss) DNA (SJW1) giving ratios of PtEEtpy–ssDNA of 1:1, 3:1 and 6:1. The final concentration of ssDNA was 50 μM. Aliquots of these reaction mixtures were taken immediately and at 3, 6, 24 and 122 h after the time of mixing, diluted 50-fold in 50% CH_3CN in 5 mM NH_4OAc and placed on ice prior to electrospray ionization (ESI) mass spectrometry (MS) analysis. Samples were kept in the dark for all steps. Mass spectrometry of these samples was carried out using a Micromass Qtof2 mass spectrometer with a capillary voltage of 2600 V, cone voltage of 40 V, source block temperature 80 °C and desolvation temperature 120 °C. Typically 30 acquisitions were summed to obtain representative spectra.

Purification of ssDNA–PtEEtpy and annealing with complementary DNA: The double-stranded (ds) DNA–PtEEtpy samples were prepared as follows: PtEEtpy was mixed with SJW1 in 10 mM NH_4OAc pH of 7.0 at a ratio of 3:1. The final concentration of SJW1 was 0.4 mM, and the volume of the reaction mixture was 60 μL. After 3 h this solution was injected on to a Symmetry 300 C18 column (Waters) that had been equilibrated with 10 mM NH_4OAc. A linear gradient (0–65% CH_3CN in 10 mM NH_4OAc) over 30 min was used. Fractions were collected based on UV absorbance detection, dried using a vacuum centrifuge, and then redissolved in 0.1 M NH_4OAc, pH 8.5. Fractions were analysed by ESI-MS as above. This procedure was repeated a number of times to obtain concentrations of ssDNA-PtEEtpy suitable for annealing to SJW2 (5′-GGTGTGCATGGG-3′). An equimolar amount of SJW2 in 0.1 M NH_4OAc (pH 8.5) was then added to the SJW1 containing fractions. This produced ~60 μL of ~200 μM dsDNA. These solutions were held at 60 °C for ~15 min, and then allowed to cool slowly overnight. Samples were diluted in 0.1 M ammonium acetate, pH 8.5 prior to negative ion ESI-MS using a Micromass Qtof2 mass spectrometer as previously described.[65]

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