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Electrochemical Biosensor Assay of the Interaction between $[PtCl_n(NH_3)_{4-n}]^{(2-n)}$ (n = 0–4) Complexes and ds-DNA[‡]

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We report on the results of electrochemical DNA biosensor assay of the interaction between double-stranded DNA (ds-DNA) and a series of six neutral, anionic or cationic Pt complexes of the general formula $[PtCl_n(NH_3)_{4-n}]^{(2-n)}$ [n = 4, **1**; n = 3, **2**; n = 2, isomers cisplatin (**3**) and transplatin (**4**); n = 1, **5**; n = 0, **6**]. The ability of the electrophilic Pt^{II} agents generated in solution to interact with DNA, and hence to form Pt–DNA adducts, was measured as a function of the decrease in the guanine oxidation signal recorded on a screen-printed electrode by using square wave voltammetry. Hydrolysis of the platinum complexes was studied by using time-resolved RP-HPLC and conductivity measurements to determine the aquation rate, which modulates the formation of the electrophilic agent prone to quickly interact with DNA. Our findings indicate that, if time is allowed for sufficient hydrolysis to occur, the interaction of these Pt^{II} complexes with ds-DNA follows the order **2** > **1** > **3** ≈ **4** > **5** >> **6**.

Introduction

The formation of Pt–DNA adducts is believed to be the main determinant of the cytotoxicity of platinum complexes.[1] Cisplatin, the most successful anticancer metallodrug, coordinates to DNA mainly through the N7 atoms of adjacent guanines (G) on individual strands of DNA (1,2-intrastrand cross-linking adducts). To bind to these nucleophiles on DNA, cisplatin needs to be converted by aquation into its active electrophilic forms, namely $[PtCl(H_2O)(NH_3)_2]^+$ and $[Pt(H_2O)_2(NH_3)_2]^{2+}$.[2]

Because DNA–drug interaction and potential antitumour effects are so closely related, the molecular recognition of nucleic acids by low-molecular-weight compounds is a key area of pharmacological study. A variety of chemical and biological techniques must be combined when characterizing DNA adducts in order to obtain qualitative and quantitative information about the extent and nature of the binding. Electrochemical tools, particularly electrochemical biosensors (a specific type of chemical sensor comprising a biological recognition element and an electrochemical signal transducer) provide advantages over other combined biological and chemical assays. Mascini et al.[3] developed biosensors involving DNA, the biological sensing element, immobilized on the surface of screen-printed electrodes (SPEs), the signal transducer, to rapidly detect and measure the oxidation peak of G. Since guanine has the highest electron density and thus the lowest oxidation potential among the DNA bases, its oxidation peak is the easiest parameter to observe.[4] Any compound directly interacting with G and causing a decrease in the electron density on the nucleobase will also decrease the intensity of its oxidation peak. By calculating S%, that is, the ratio between the height of the guanine oxidation peak following interaction with the drug and the height obtained from DNA alone, the amount of guanine sites in DNA modified by the interaction can be roughly estimated.

This is admittedly rather a crude approach, since the experimental conditions are quite different from those in real cells: there are no membrane barriers, the double-stranded DNA (ds-DNA) is not condensed around histones and hence protected, and, finally, in solution there are no competing platinophiles such as S-proteins. Nonetheless, the technique has been employed successfully in the comparative study of the interaction of a series of antitumour metallodrugs with DNA.[5–10] It has also been used in deriving complete, albeit semiquantitative, descriptions of (1) the hydrolysis timescale of the metal complex to afford an electrophilic agent, (2) the possible mass effect of anions acting as leaving ligands (i.e., chlorides), (3) the intrinsic affinity of the generated electrophilic agent to DNA and (4) the strength of the perturbation in the DNA chain caused by such metallodrugs.

In this paper we describe the interaction between DNA and a series of six neutral, anionic or cationic platinum complexes of the general formula $[PtCl_n(NH_3)_{4-n}]^{(2-n)}$ (n = 4, **1**; n = 3, **2**; n = 2, isomers *cis*-**3** and *trans*-**4**; n = 1, **5**; n = 0, **6**) (Figure 1).

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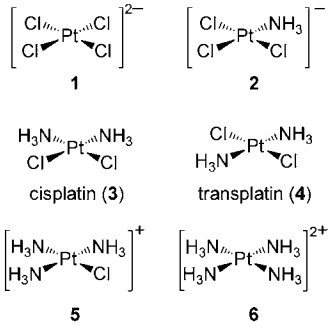

Figure 1. Sketch of the platinum complexes under investigation belonging to the series $[PtCl_n(NH_3)_{4-n}]^{(2-n)}$ (n = 4, **1**; n = 3, **2**; n = 2, isomers *cis*-**3** and *trans*-**4**; n = 1, **5**; n = 0, **6**).

Results and Discussion

Interaction between Platinum Complexes and DNA Biosensors

The DNA biosensor protocol consists of three steps: (1) electrochemical conditioning of the SPE surface, (2) electrochemical deposition of platinated ds-DNA onto the SPE and (3) interrogation of the electrode surface by dipping the electrode in a suitable buffer, and performing a square wave voltammetry (SWV) scan (Figure 2).

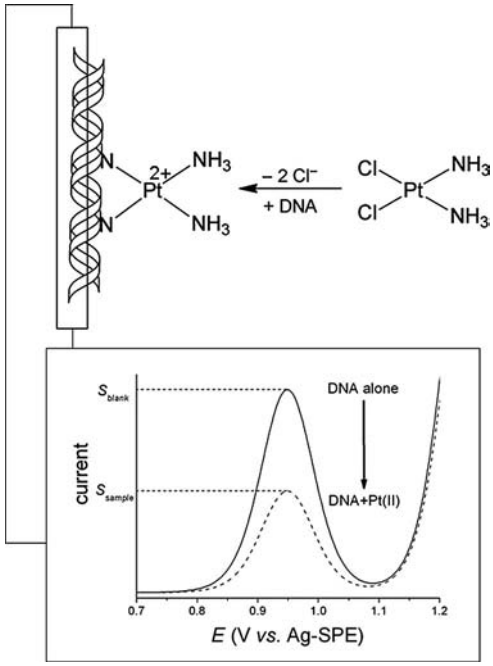

Figure 2. Sketch of the DNA biosensor experiment.

In step (2) the platinum complexes were pre-incubated in phosphate buffer (PB) solutions with ds-DNA, and, at fixed times, a freshly activated SPE was dipped into the solution to allow electrodeposition of platinated DNA on the electrode surface. The SWV scan (step 3) was then performed.

The interaction between the complexes and DNA was evaluated as the decrease (S%) in the height of the guanine oxidation signal, that is, the ratio between the height of the guanine peak of the platinated DNA adsorbed onto the SPE at a given reaction time (S_{sample}) and the peak height of the DNA in the buffer solution without the drug (S_{blank}), $S\% = (S_{sample}/S_{blank}) \times 100$.

Figure 3 shows the S% values vs. incubation time obtained after incubation of 0.25 mM concentrations of Pt complexes and ds-DNA ([Pt]/[NP] = 2.2) in 10 mM PB/5 mM NaCl (pH = 7.4) solutions, 25 °C.

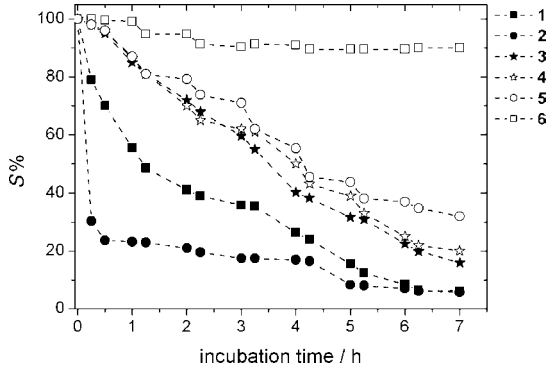

Figure 3. S% vs. incubation time for 0.25 mM Pt complexes and ds-DNA ([Pt]/[NP] = 2.2) in 10 mM PB/5 mM NaCl (pH = 7.4) solutions. Each time point is the mean of three independent experiments (standard deviation ≤ 10%). Electrochemical conditions: electrode pretreatment +1.6 V (vs. Ag-SPE) for 120 s and +1.8 V (vs. Ag-SPE) for 60 s in 0.25 M acetate buffer containing 10 mM KCl (pH 4.75); ds-DNA immobilization at +0.5 V vs. Ag-SPE pseudoreference for 300 s by dipping the strip in the test solution containing ds-DNA and the platinum drug (sample test) or ds-DNA alone (blank test); final SWV measurement scan in 0.25 M acetate buffer containing 10 mM KCl (pH 4.75) from +0.2 V to +1.40 V vs. Ag-SPE with E_{step} 15 mV, $E_{amplitude}$ 40 mV and frequency 200 Hz.

Compounds **1** and **2** react very quickly with DNA: after 2 h the guanine peaks dropped to about 40% and 20%, respectively, the signals fading almost completely at the end of 7 h. The reaction of complexes **3**–**5** with DNA is less marked. In contrast, compound **6** undergoes no significant interaction with DNA, as revealed by the relatively minor decrease in the guanine peak over the 7 h period. This is true notwithstanding the fact that complex **6** is a dication, and therefore, in principle, prone to electrostatic interaction with the negative, external, phosphate backbone of ds-DNA. The present findings confirm that a simple electrostatic interaction, as opposed to coordination bonding, is incapable of altering the oxidation peak of G, as previously reported for free cations, such as Cu^{2+} or Zn^{2+}.[7]

These results are in line with those obtained by Macquet and Theophanides:[11,12] the interaction of platinum with extracted DNA, quantified as the number of platinum atoms bound per nucleotide, was found to be 0.22 in the case of **5**, 0.44 in the case of **3** and **4**, 1.5 in the case of **1** and **2**, while **6**, not having any labile site, was not able to platinate DNA.

It should be noted that, although complex **3** is potentially bidentate, the response of its interaction with DNA was not much greater than that of complexes **4** and **5**, which only produce monodentate adducts. This can be attributed

to the inability of the DNA biosensor to quantitatively distinguish between the electrochemical response of Pt–G and Pt–G_2 adducts.

Solution Behaviour

The importance of the ligand exchange reaction in the platination of DNA by cisplatin **3** has been stated by Lippard and Reedijk.[13,14] The overall reaction is controlled kinetically, rather than thermodynamically, the rate-determining step being the hydrolysis of the first chloride ion. The pseudo-first-order rate constant for the disappearance of cisplatin in the presence of DNA was found to be very close to that of the first hydrolysis of cisplatin. Moreover, the half-lives for the formation of monofunctional Pt–DNA adducts for the two isomers, **3** and **4**, calculated from a kinetic analysis of the time-resolved ^{195}Pt NMR spectra, are quite similar ($t_{1/2}$ is 1.9 h for **3** and 2.0 h for **4** in 1 mM PB/3 mM NaCl),[13] corroborating the behaviour observed with the DNA biosensor.

To shed light on the differences in the timescale reactivity of the compounds with DNA, the RP-HPLC technique was used to evaluate the solution behaviour of the complexes (0.5 mM) under abiological conditions (1 mM PB/5 mM NaCl, pH 7.4, 25 °C) similar to those used for the measurements with the electrochemical biosensor.

The hydrolysis of the six complexes was monitored for 7 h. Changes in the initial peaks cannot be quantified with accuracy because of a partial overlap of HPLC signals belonging to the buffer and the complexes. However, from a qualitative point of view, RP-HPLC confirmed the trends observed with the biosensor: complexes **1** and **2** showed the fastest hydrolysis; complexes **3** and **4** hydrolyzed at similar rates (the initial chromatographic peak decreasing about 50% over 7 h), while the chromatographic peak of compound **5** maintained approximately 60% of its initial value in the same time interval. As expected, the peak of complex **6** showed no change, since the Pt–N bond is more stable than the Pt–Cl bond and undergoes no significant hydrolysis.

The solution behaviour of **3** is well known,[15] and the hydrolysis of some of the other complexes in the series has previously been studied in different halide solutions and with different techniques.[16–22] The behaviours observed are consistent with data from the literature. The formation of mono- and diaqua species from complexes **1** and **2** has been reported[16,17] with different half-times of the first aquation; for instance, $t_{1/2}$ was reported to be about 4.9 h and 5.4 h for complexes **1** and **2**, respectively (in Na_2SO_4 solution, ionic strength: 0.318 M, concentration of the complexes: 16.6 mM, 25 °C, with spectrophotometric and potentiometric titration techniques).[20–22]

Cisplatin (**3**) and transplatin (**4**) share a similar first aquation rate constant,[15] and both monoaquated species are able to interact with guanine before hydrolysis of the second chloride. For compound **5**, the formation of the monoaqua species has been reported ($t_{1/2}$ about 8.8 h at 25 °C and in the absence of chlorides).[23]

To confirm and quantify the results obtained from the RP-HPLC analysis, measurements of the conductivity (Λ) of solutions of **1**–**6** vs. hydrolysis time were performed (experimental conditions: unbuffered, neutral 0.5 mM solutions of **1**–**6** containing 2% DMSO to increase the solubility of the Pt complexes; 25 °C). Conductivity measurements have traditionally been used to confirm the stoichiometry of metal complexes and to evaluate their long-time stability in solution. While this technique may not give detailed information about the nature of the species produced, it provides fast, continuous and quantitative measurements of the changes occurring in solution.

The results are shown in Figure 4. The Λ data are reported as $\Delta\Lambda = \Lambda_{measured} - \Lambda_{t=0}$, so the behaviour of complexes bearing different charges can be compared. By this procedure, the initial Λ values for all complexes were reset: all the ions present in solution (i.e., chlorides and solvolyzed complexes) will contribute similarly to the increase in conductivity values. This normalization produces a kinetic profile that is independent of the original starting point (i.e., it is independent of the sign and the amount of charge brought by the parent complex).

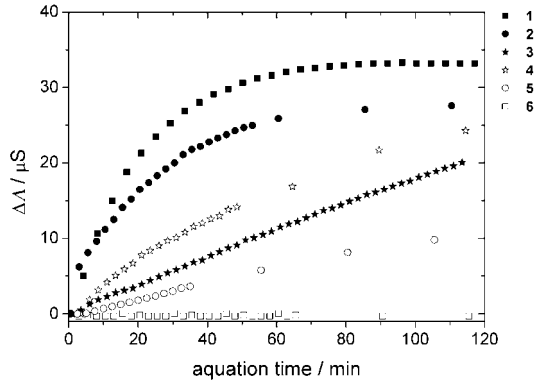

Figure 4. Normalized conductivity values $\Delta\Lambda$, where $\Delta\Lambda = \Lambda_{measured} - \Lambda_{t=0}$, vs. time of 0.5 mM solutions of **1**–**6** (unbuffered, neutral water + 2% DMSO, 25 °C).

In all cases but one (complex **6**), the value of $\Delta\Lambda$ increases with time, reaching values compatible with the release of one chloride ion, albeit with different speeds. When the timescale is limited to two hours, the time period in which the main differences in S% values are observed (Figure 4), it is possible to split the six complexes into three categories: (1) "fast" **1** and **2**, (2) "slow" **3**–**5** and (3) "unreacted" **6**. This rough classification is similar to that obtained by RP-HPLC experiments and in line with the results obtained by means of DNA biosensor.

Thus, despite the crudeness of the technique employed (i.e., the electrochemical biosensor) the Pt–DNA interaction may be explained by means of the kinetics of solvolysis, which produces active, electrophilic Pt metabolites.

Conclusions

The interaction of a series of six neutral, anionic or cationic Pt complexes of general formula $[PtCl_n(NH_3)_{4-n}]^{(2-n)}$

(n = 0–4) with ds-DNA in PB has been evaluated by using an electrochemical DNA biosensor. The results indicate that the interaction of the Pt^{II} complexes of the series with ds-DNA follows the order **2** > **1** > **3** ≈ **4** > **5** >> **6**, in line with other results obtained by using different techniques.[11,12] The overall behaviour is strictly related to the solvolysis of the complexes, as corroborated by RP-HPLC and conductivity measurements.

These results cannot immediately be used to predict the overall cytotoxicity. Providing that DNA is the major target for all Pt^{II} antitumor drugs, the discrepancy between metal–DNA interaction and overall biological activity depends on the type of cell line employed and on the experimental conditions, which modulate the intracellular accumulation of the drugs.[2,25,26] For instance, the complexes under study were previously tested for mutagenicity on Chinese hamster ovary (CHO) cells,[24] revealing a different order: **3** > **2** > **5** > **1**, **4**, **6**.

However, the DNA biosensor may be of interest in studying the possible reaction of a metal complex in solution and hence the formation of DNA-active or -inactive species by reaction with water or other molecules acting as ligands. For the above reasons, the DNA biosensor could give useful and quick information when integrated in a panel of biological and chemical tests in order to quickly evaluate the affinity of low-molecular-weight molecules with DNA.

Experimental Section

Milli-Q grade water (resistivity 18 MΩcm at 25°C) was used to prepare all the solutions. The inorganic salts were of analytical grade (Aldrich) and used without further purification. Complexes **1**, **3** and **4** were purchased from Alfa Aesar; complex **2**,[27–29] **5**[30] and **6**[31,32] were synthesized according to published procedures. Calf thymus double-stranded DNA (ds-DNA) type XV was purchased from Sigma. The concentration of nucleotide phosphate ([NP]) was determined by UV absorbance at 260 nm; the extinction coefficient, ε_{260}, was taken as 6600 $M^{-1}cm^{-1}$ per nucleotide phosphate.[33]

Electrochemical measurements on the screen-printed electrodes (SPEs) were made with an Autolab PGSTAT12 electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) interfaced to a personal computer running GPES 4.9 electrochemical software. The planar, screen-printed electrochemical cell consisted of a graphite working electrode, a graphite counterelectrode, and a silver pseudoreference electrode. SPEs were printed as previously reported;[9] each electrode was disposable.[34] The electrode surface was pretreated by applying a potential of +1.6 V for 2 min and +1.8 V for 1 min in acetate buffer (0.25 M, pH 4.75) containing KCl (10 mM); this procedure is necessary to activate the electrode surface and make it conducive to the immobilization of DNA. After pretreatment of the electrode, the ds-DNA was immobilized at a fixed potential (+0.5 V vs. Ag pseudoreference electrode for 300 s) onto the SPE surface by dipping the strip into the test solution containing ds-DNA and the platinum complex (sample test) or only ds-DNA (blank test). Then, the sensor was immersed in acetate buffer, and a square wave voltammetry (SWV) scan was carried out to measure the oxidation peak height of *unplatinated* guanine residues (at +0.95 V vs. Ag pseudoreference electrode) on the DNA attached to the electrode surface. Interaction of the platinum drugs with DNA was evaluated by changes in the magnitude of the oxidation peak for guanine. The DNA modification was evaluated by the value of the percentage decrease (S%) in the signal, which is the ratio between the guanine peak height of the platinated DNA adsorbed onto the SPE at different reaction times (S_{sample}) and the guanine peak height of the DNA in the buffer solution without any drug (S_{blank}), $S\% = (S_{sample}/S_{blank}) \times 100$. Analyte solutions of the platinum complexes (0.25 mM) were prepared in a phosphate buffer (10 mM, pH = 7.4), containing NaCl (5 mM). After adding the ds-DNA to the solutions ([Pt]/[NP] = 2.2), the resulting mixtures were gently stirred at 25 °C, and the electrochemical activity of the G residues on DNA were sampled at various times with the sensor. The SWV parameters were: frequency, 200 Hz; step potential, 15 mV; amplitude, 40 mV; potential range 0.2–1.4 V vs. Ag pseudoreference electrode. All experiments were done in triplicate, and the mean value was calculated. The relative standard deviation is not reported on the graphs for the sake of clarity; nevertheless, it was below ±10% in all analyses.

The solution behaviour of the complexes was studied by RP-HPLC using a Waters 2695 Separations Module equipped with a 2487 Dual λ Absorbance Detector set at 210 and 254 nm. Separation of the analytes was performed on a silica-based C18 column Phenomenex Gemini 250 × 3.00 mM, 5 μm as the stationary phase and a 70:30 mixture of an aqueous solution of HCOOH (15 mM) and MeOH (flow 0.5 mL min^{-1}, isocratic elution) as eluent. The complexes (0.5 mM) were dissolved in PB (1 mM)/NaCl (5 mM, pH = 7.4) and studied at 25 °C.

Conductivity measurements were performed with an AMEL (Milan, Italy) model 160 conductivity meter. The analyses were carried out on freshly prepared solutions of the complexes in DMSO diluted with water to a final 2% organic content and 0.5 mM concentration of the Pt complex. A thermostatic circulating bath was used to maintain a constant temperature of 25 ± 0.1 °C, and the conductivity was measured at regular time intervals over 8 h.

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