

Electrochemical biosensor evaluation of the interaction between DNA and metallo-drugs[§]

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Abstract

Electrochemical techniques were used to study the interaction between a panel of antiproliferative metallo-drugs and double-stranded DNA immobilized on screen-printed electrodes as a model of the analogous interaction occurring in solution. The propensity of a given metal drug to interact with DNA was measured as a function of the decrease of guanine oxidation signal, which was detected by square wave voltammetry. Estimates of variations in experimental parameters, such as the concentration of complexes, time following dissolution (ageing time) and the presence of chloride, are provided.

Introduction

Electrochemical biosensor techniques are being adopted more and more frequently for the investigation of drug–biomolecule interactions because of their advantages over other kinds of chemical or biological assays: they are relatively simple, reliable, and minimize the sample volume required. Researchers have begun to explore a new class of affinity biosensor consisting of nucleic acid layers combined with electrochemical transducers. Such DNA biosensors have already been applied in testing water, food, soil, and plant samples for potential mutagenic pollutants (Erdem and Ozsoz, 2002). Mascini *et al.* have developed biosensors from double-stranded (ds)

or single-stranded (ss) DNA immobilized on the surface of screen printed electrodes (SPEs), and exploit quick methods of 'interrogation', such as chronopotentiometry or square wave voltammetry (SWV), to measure the oxidation peak of guanine (2-amino-6-hydroxypurine, G) (Chiti *et al.*, 2001). It is important to recall that between the four nucleobases, G has the highest electron density and lowest oxidation potential, making it the preferred target (Steenken and Jovanovic, 1997). Any agent causing a decrease in the electron density on G also leads to a reduction in the oxidation process.

This procedure was developed mainly as a rapid means of environmental risk assessment (ERA) and for swift screening of polluted areas (Lucarelli *et al.*, 2002b).

In addition to its useful role in environmental applications for detecting genotoxic substances, it

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seems straightforward to employ such electrodes in drug studies, especially in cancer chemotherapy where DNA is the main target. With this in mind, this paper presents our findings for a number of compounds, which we hope, prove useful.

Briefly, screen-printing machines and commercially available inks were used to prepare SPEs (details in Materials and methods). The biosensor test protocol included four steps: (i) electrochemical conditioning of the electrode surface, (ii) ds-DNA immobilization, (iii) interaction of the analyte with the DNA, and (iv) interrogation of the electrode surface to measure G oxidation, by dipping the electrode in a suitable buffer and performing a SWV scan. Interaction of DNA with

the panel of compounds under study (Figure 1) was detected and estimated on the basis of decrease in the electrochemical signal of guanine, S% (see Materials and methods).

Materials and methods

Milli-Q grade water (18 M Ω) was used in the preparation of all solutions. Inorganic salts and titanocene dichloride were obtained from Aldrich. The following complexes were prepared according to published procedures: *cis*-diamminedichloro platinum(II) [Pt(NH₃)₂Cl₂] (*cis*platin) (Dhara,

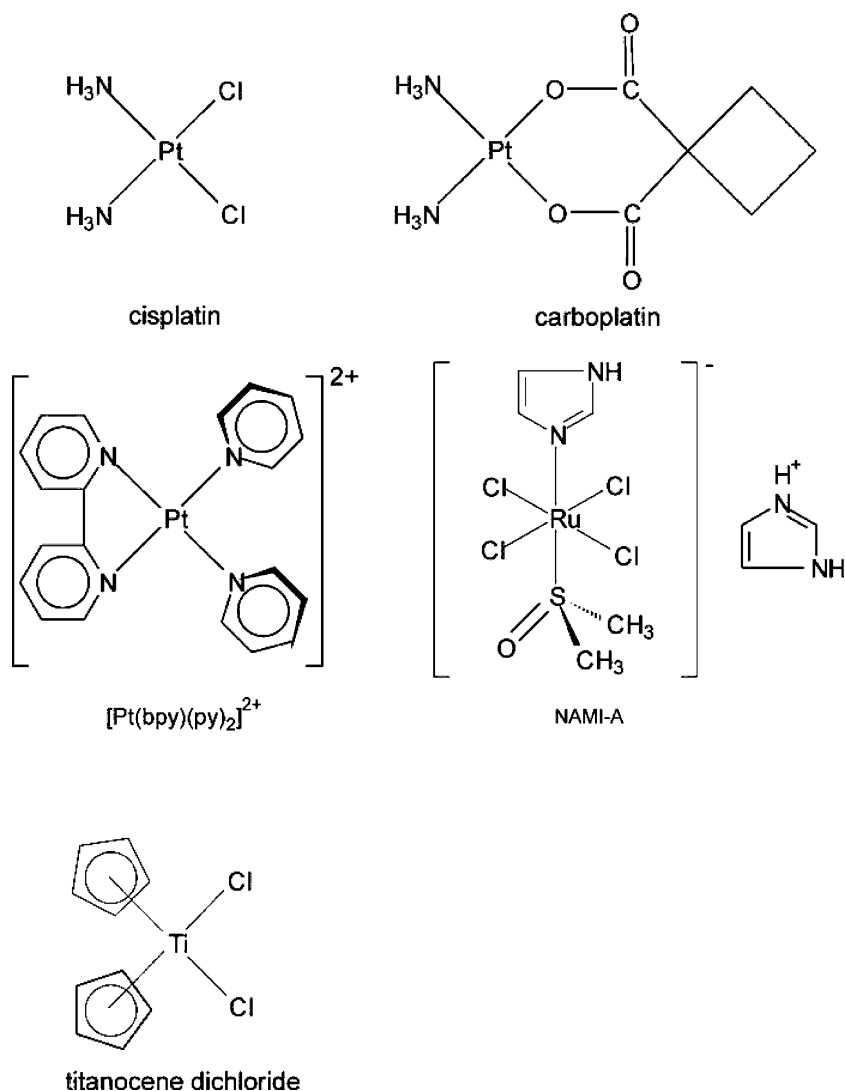


Figure 1. Sketch of the complexes under investigation.

1970), *cis*-diammine-(1,1-cyclobutanedicarboxylato) platinum(II) [Pt(NH₃)₂(CBDCA)] (carboplatin, CBDCA = 1,1-cyclobutane dicarboxylate) (Harrison *et al.*, 1980), 2,2'-bipyridylbis(pyridine)platinum(II) bis(hexafluorophosphate) [Pt(bpy)(py)₂][PF₆]₂ (Cusumano *et al.*, 1996) and *trans*-tetrachlorodimethylsulfoxideimidazoleruthenate(III) [ImH][RuCl₄(DMSO)(Im)] (NAMI-A) (DMSO = dimethylsulfoxide, Im = imidazole) (Mestroni *et al.*, 1998). Calf thymus double-strand (ds-DNA, type XV activated, lyophilized powder) and single-strand (lyophilized powder) DNA were purchased from Sigma.

Electrochemistry

Electrochemical measurements on screen-printed electrodes (SPEs) were taken using a μ Autolab type II electrochemical analysis system running GPES 4.9 software (Metrohm). The planar, screen-printed electrochemical cell consisted of graphite working electrode (with a 3 mm diameter surface), a graphite counter electrode and a silver pseudo-reference electrode. Each electrode was disposable (Cagnini *et al.*, 1995). SPEs were printed using a Model 248 screen printer, obtained from DEK (Weimouth, UK). Three types of ink were used: graphite-based (Electrodag 423 SS, Acheson Italiana, Milan, Italy), silver (Electrodag 410 PF, Acheson Italiana, Milan, Italy) and insulating (Vinyl fast 36-100, Argon, Lodi, Milan, Italy). The substrate was a polyester flexible film (Autostat HTS) obtained from Autotype Italia (Milan, Italy). The electrode surface was pre-treated by applying a potential of +1.6 V for 2 min and +1.8 V for 1 min in 0.25 M acetic buffer (pH 4.75) containing 10 mM KCl under stirred conditions; this procedure is required in order to activate the electrode surface and to sensitize it to the following DNA immobilization step. The biosensor was developed by immobilizing ds-DNA at a fixed potential (+0.5 V vs. Ag pseudo-reference electrode for 300 s) on the SPE surfaces. During the immobilization step, the strip was immersed in acetate buffer solution containing 50 ppm ds-DNA or ss-DNA. The following cleaning step involved dipping the biosensor in a fresh acetate buffer solution, in open-circuit conditions. The incubation step was performed by sinking the electrode in a solution containing the analyte under stirred conditions for 2 min. Obviously, the decrease of G peak height is

proportional to the time of interaction between solution and SPE. The chosen time (2 min) represents the best compromise between sensibility and promptness of the method. The sensor was then washed, immersed in acetate buffer, and analyzed by square wave voltammetry (SWV) to evaluate the oxidation of G residues on the electrode surface. The height of the peak of G oxidation (at +0.95 V vs. Ag pseudo-reference electrode) was measured. The interaction of the compounds under study with DNA was evaluated as the percentage of G peak height (S%) calculated as below:

$$S\% = [(GPH_s/GPH_b)] \times 100$$

where GPH_s is the G peak height after interaction with the sample and GPH_b is the G peak height in buffer solution alone.

The analyte solutions were prepared in buffered (phosphate buffer, pH 7.4) or unbuffered (5 mM or 100 mM NaCl or NaClO₄) solutions. The SWV parameters were frequency, 200 Hz; step potential, 15 mV; amplitude, 40 mV; and potential range 0.2–1.4 V vs. Ag pseudo-reference electrode.

Results and discussion

Cisplatin

Figure 2 shows the SWV signal of the ds-DNA modified SPE before and after interaction with cisplatin. The redox behavior of original ds-DNA–SPE exhibits two oxidation processes of guanine

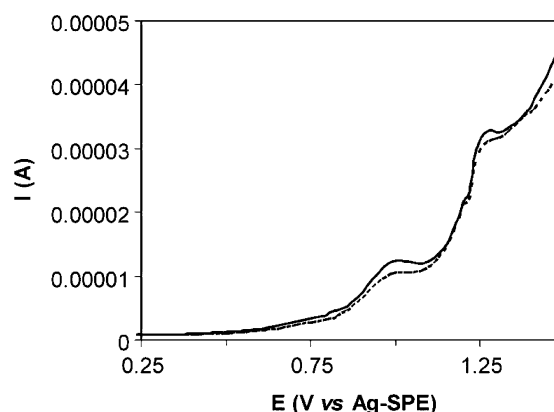


Figure 2. SWV signal of the ds-DNA modified SPE before and after interaction with a 0.1 mM solution of cisplatin.

(G, *ca.* +0.95 V vs. Ag pseudo-reference electrode) and adenine (A, *ca.* +1.30 V vs. Ag pseudo-reference electrode). Both nucleobases are known to interact with cisplatin, but only the peak assigned to G gives a significant variation, while the

A oxidation is partially obscured by solvent discharge.

In particular, the bonding between Pt and G diminishes the electrochemical response of the immobilized DNA in a dose-dependent fashion. In

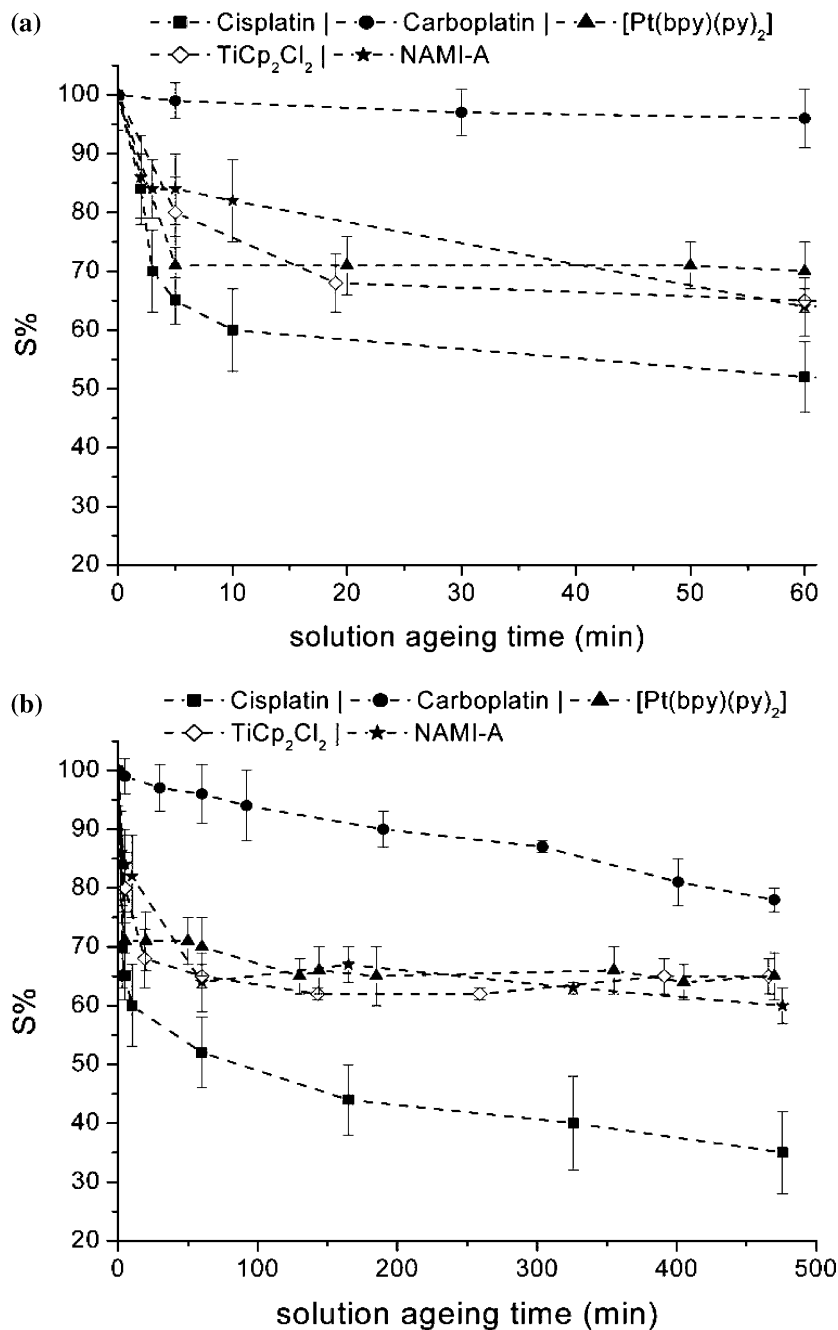


Figure 3. Guanine oxidation signal S% vs. solution ageing time for 0.5 mM solution of the metal complexes in 0.25 M phosphate buffer (pH = 7.4) and 5 mM NaCl: (a) 0–60 min time range, (b) 0–500 min time range.

fact, by increasing the concentration of the metal complexes in solution (in the range 0–1.0 mM), a gradual decrease of the G oxidation signal S% is observed (Figure 3). No oxidation signal for cisplatin was obtained with the bare SPE at the potential range between 0.2–1.4 V vs. Ag pseudo-reference electrode. Thus, the change in G oxidation signals arises from the interaction of metal complex at the DNA-modified SPE surface. Each experiment was repeated at least three times on different electrodes and resulted in reproducible results, including a relative standard deviation of less than 10%. Since each experiment was irreversible, a new electrode was employed for each measurement; therefore all the results should be referred to a non-equilibrium system.

Cisplatin, like several other metallo-pharmaceuticals, acts as a pro-drug. Indeed, a loss of chloride groups is required before cisplatin will interact with DNA, (Miller and House, 1991). The high chloride concentration in extra-cellular fluids (100 mM) suppresses the formation of the active mono- and di-aqua *cis*-Pt(II) aqua-species. In cytosol, the chloride concentration is about 5 mM, so hydrolysis of cisplatin occurs efficiently. In this case, water molecules replace one or both chloride leaving groups, thus allowing the formation of species that are reactive (coordinating/electrophilic)

towards the target biomolecules (Miller and House, 1991). We have already observed on SPE that the behavior of cisplatin is strictly dependent on the concentration of NaCl (Ravera *et al.*, 2004): high concentrations of chlorides (100 mM) inhibit the aquation of cisplatin and, hence, its interaction with DNA.

For this reason, we employed a 0.25 M phosphate buffer (pH=7.4), containing NaCl (5 mM) for cisplatin and all the other metal drugs to be tested, in order to maintain constant pH and ionic strength.

The hydrolysis of metallo-drugs is often the key step in their activation, but the timescale is extremely variable, ranging from tenths of a second for titanocene dichloride, to hours for cisplatin, to days for carboplatin. We found on SPE that the interaction of cisplatin with DNA increases with the solution ageing time, especially at low concentrations of chlorides (Ravera *et al.*, 2004). Figure 3a and b illustrate a comparison of the behavior of the five metallo-drugs in identical experimental conditions when the solution ageing time is varied. As expected, a stronger effect of solution ageing time on S% is observed for cisplatin. In the case of TiCp_2Cl_2 , the effect is not as evident, due to the extremely fast hydrolysis rate, while in the case of NAMI-A and $[\text{Pt}(\text{bpy})(\text{py})_2]^{2+}$,

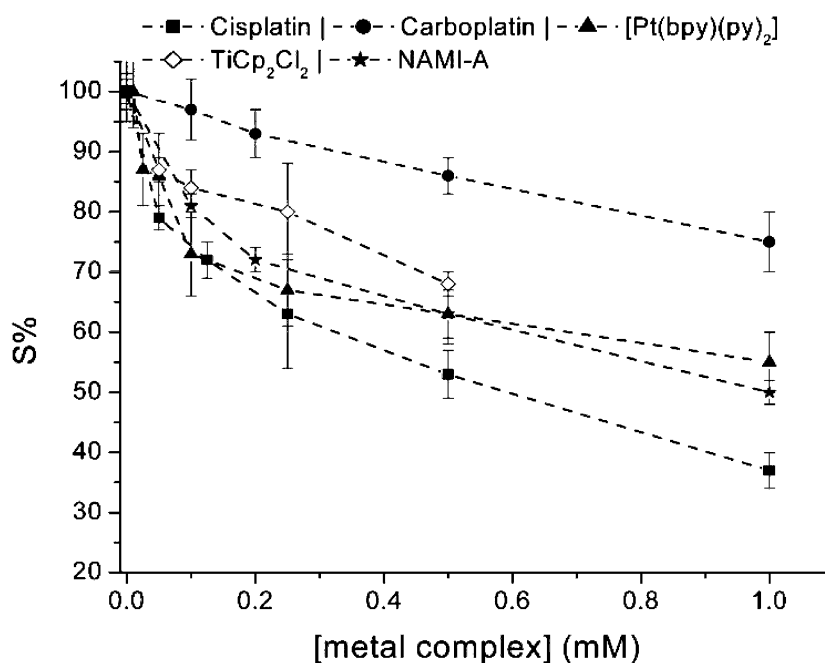


Figure 4. Guanine oxidation signal S% resulting from increasing concentrations of metal complexes in 0.25 M phosphate buffer (pH = 7.4) and 5 mM NaCl.

hydrolysis proved not to be the limiting process (Ravera *et al.*, 2004) or it is not required, respectively. The behavior of carboplatin will be discussed in a separate section. Due to these variations, we had to select a sort of 'ripening time' for all the solutions. Each complex was therefore pre-activated for 60 min before testing interaction with DNA, in order to permit the transformation into the active aqua species, for the complexes needing this incubation time, and to compare the behavior at the same solution ageing time, for the complexes with a different rate of hydrolysis.

Figure 4 shows overall comparison of the behavior of the five metallo-drugs in identical experimental conditions when the concentration is varied. For all complexes, the interaction between metal–drugs and the immobilized DNA decreases S% in a dose-dependent fashion.

Blank experiments

Since the Pt and Ti complexes become, after hydrolysis, doubly positive charged species, we have checked whether a simple electrostatic interaction in lieu of a real coordination to DNA is able to affect the oxidation signal of G. For this purpose, two solutions, the former containing Zn(II) (0.07 mM), the latter containing Cu(II) (0.04 mM), were prepared in 0.25 M acetic buffer (pH 4.75) and tested with the biosensors. No variation in the G signal was observed (S% = 100%) and no residual electroactivity was found by analyzing the bare electrode in the range of potential of interest.

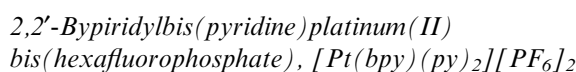
Carboplatin

Carboplatin and cisplatin produce the same electrophilic agent upon hydrolysis, namely $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})_2]^{2+}$, with the same affinity for DNA. Figures 3 and 4 reveal that the interaction of carboplatin is much lower than that of cisplatin even after 500 min (i.e. S% = 77), and this must be due to the different rate of hydrolysis.

The dicarboxylato ring opening producing aquated Pt(II) is indeed much slower than the ready aquation of cisplatin. In fact $t_{1/2}$ in chloride-free phosphate (37 °C) is about 450 h (Frey *et al.*, 1993) compared with 2 h for cisplatin (Johnson *et al.*, 1980; Bancroft *et al.*, 1990; Orton *et al.*, 1993). *In vivo* the difference in activity is not so dramatic: enzymatic cleavage (Cleare *et al.*, 1978),

the presence of oxygen free radicals in the cell (Tonetti *et al.*, 1993), and differences in pharmacokinetics (Elferink *et al.*, 1987) have been hypothesized as the cause of more efficient activation of carboplatin *in vivo* than *in vitro*.

The presence of chloride ions causes a slight but evident decrease in the G oxidation signal. This can be explained by an exchange between the 1,1-cyclobutanedicarboxylato and chlorides producing cisplatin that in turn undergoes activation by aquation (Heudi *et al.*, 1999) (Figure 6). A similar exchange is more difficult for perchlorates, which are weaker Lewis bases (results in Figure 5).



This complex lacks appropriate leaving groups and is therefore unable to produce an activated electrophile (in other words, $[\text{Pt}(\text{bpy})(\text{py})_2]^{2+}$ is devoid of any alkylating properties). Indeed $[\text{Pt}(\text{bpy})(\text{py})_2]^{2+}$ is able to intercalate DNA as unambiguously shown by Cusumano *et al.* (1996). We do, however, observe a significant decrease in S%, albeit lower than that caused by cisplatin. Difficulty in the oxidation of G may be caused by depletion of electron density on the nitrogen atom in 7-position of G (N7-G) due to the efficient and extensive delocalization throughout the DNA chain as clearly demonstrated by Barton and co-workers (Holmlin *et al.*, 1997). This electronic communication renders the G oxidation sensitive to the double positive charge localized on the

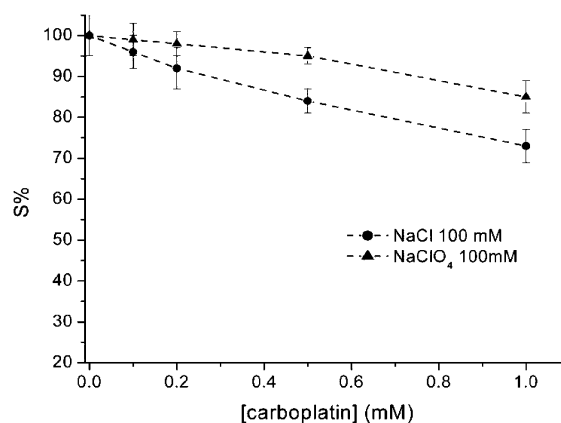


Figure 5. Guanine oxidation signal S% obtained with different concentrations of carboplatin in 100 mM NaCl or 100 mM NaClO₄ respectively.

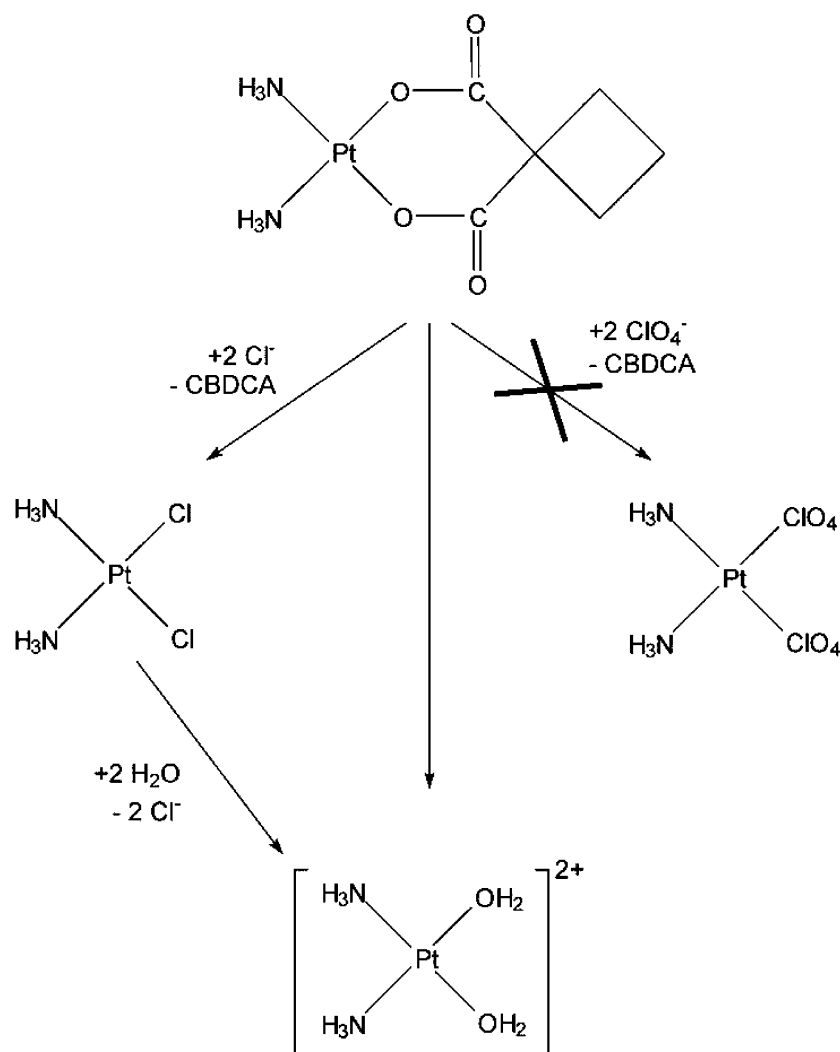


Figure 6. Reaction pathway for the formation of Pt aqua-species from cisplatin and carboplatin in different media.

Pt(II) centre. Interestingly, the $S\%$ value was practically time-independent since the complex does not need dissociation of inner-sphere ligands to be activated for the intercalation.

In order to compare its intercalating properties, a 0.5 mM solution of $[\text{Pt}(\text{bpy})(\text{py})_2]^{2+}$ was tested on both ds- and ss-DNA. An $S\%$ value of 92 ± 3 was obtained after interaction of the Pt complex with ss-DNA vs. an $S\% = 65 \pm 3$ obtained in the case of ds-DNA. As expected, $[\text{Pt}(\text{bpy})(\text{py})_2]^{2+}$ is devoid of any significant ability to interact with single-stranded DNA, as indicated by the higher residual value of $S\%$. This result validates the electrochemical test proposed in this paper as a means of qualitative evaluation of interaction between

metallo-drugs and DNA even if the compound act as an intercalator in lieu of an alkylating agent.

Titanocene dichloride TiCp_2Cl_2 ($\text{Cp} = \eta^5\text{-cyclopentadienyl}$)

TiCp_2Cl_2 unambiguously shows a lower degree of interaction with SPE adsorbed ds-DNA than cisplatin. Because of its poor solubility in water, we employed a water/ethanol solution 90/10 v/v; nevertheless, the maximum concentration of complex achieved was about 0.5 mM. The trend of $S\%$ with solution ageing time (after the initial 60 min) proved almost constant. The hydrolysis of TiCp_2Cl_2 proceeds much faster than cisplatin

(Kuo *et al.*, 1996): the half-life of the first aquation of chloride ligand is too fast to be measured and the second aquation step has a $t_{1/2} \approx 50$ min. We therefore expect that both the active species $[\text{TiCp}_2(\text{H}_2\text{O})\text{Cl}]^+$ and $[\text{TiCp}_2(\text{H}_2\text{O})_2]^{2+}$ were present in solution during the experiment. Slow hydrolysis of the Cp rings produced uncharacterized polymers that were biologically inactive (Kopf-Maier *et al.*, 1980). This is confirmed by our experiments over a longer time-scale (Figure 3): after an initial decrease of S% in the first 240 min, the formation of inactive species results in an increase of S% and, hence, loss of interaction. Keeping in mind that each measurement is performed at a fresh electrode containing fresh DNA, it is clear that our experimental conditions vastly differ from the situation in the cell, where transferrin (a specific Fe(III) carrier) provides a transport mechanism for Ti(IV) (Guo and Sadler, 1999; Guo *et al.*, 2000). Initial studies suggested a correlation between DNA binding and antitumor activity in that complexation of the DNA nucleotides occurs with the metallocene (Kopf-Maier, 1989; McLaughlin *et al.*, 1990; Murray and Harding 1994). Later studies indicate that metallocene dihalides do not bind strongly to DNA at neutral pH, so it is unlikely that their antitumor activity involves nucleic acids (Kuo *et al.*, 1996). Furthermore, interaction studies carried out using UV-Vis and fluorescence spectroscopy indicated that the binding is dominated by coordination to the oxygen atoms of phosphate (Yang and Guo, 1999).

Obviously, the ionic interaction between the Ti cation and the external phosphate backbone has less of an effect on oxidation of G than the direct coordination of N7.

NAMI-A, trans-tetrachlorodimethylsulfoxideimidazoleruthenate(III)
 $[\text{ImH}][\text{RuCl}_4(\text{DMSO})(\text{Im})]$
 (DMSO = dimethylsulfoxide, Im = imidazole)

In a previous paper, we reported that for NAMI-A the S% value decreases as concentration increases (Ravera *et al.*, 2004). We have inserted these data in Figure 3 for the sake of comparison. It is immediately evident that the interaction between NAMI-A and immobilized DNA is definitely lower than that of cisplatin. This confirms the results reported by Gallori *et al.* who showed that

NAMI-A interacts with DNA at concentrations significantly higher than those at which cisplatin produces similar effects (Gallori *et al.*, 2000).

Instead, the chloride concentration played a minor role. Figure 4 shows that NAMI-A does not require ageing time for activation. This was quite surprising since the activation of NAMI-A has been supposed to involve chloride dissociation, followed by aquation reactions. Very recently, it has been reported that the *trans*- $[\text{Ru}^{(\text{III})}\text{Cl}_4(\text{DM-SO-S})_2]^-$ complex reacted with protonated G base, coordinating it in the axial direction without detaching the four equatorial chlorides and producing *trans*- $[\text{Ru}^{(\text{III})}\text{Cl}_4(\text{GH})(\text{DMSO-S})]^-$ (GH = protonated guanine, Figure 7) (Turel *et al.*, 2004). Since for steric reasons the nucleobase is coordinated through a single N atom (in this particular case N9, with N7 being protonated), the resulting interaction is certainly weaker than that the analogous case involved in bifunctional *intra*-strand cross-linking.

Conclusions

While disposable electrochemical DNA-based biosensors represent a simple device for rapid screening of genotoxic compounds whose applicability to river and wastewater samples has been

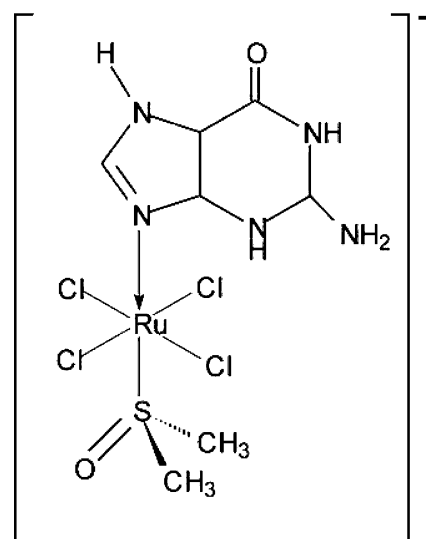


Figure 7. Sketch of the structure of *trans*- $[\text{Ru}^{(\text{III})}\text{Cl}_4(\text{GH})(\text{DMSO-S})]^-$ (GH = protonated guanine) (Turel *et al.*, 2004).

amply demonstrated (Chiti *et al.* 2001; Lucarelli *et al.*, 2002a, b), their use in pharmacokinetics deserves some caution. First of all, the study of the interaction between immobilized DNA and coordinating/electrophilic (often called alkylating) agents is carried out in a cell-free system. This implies that there are no membrane barriers and that DNA is not highly condensed around histone proteins as in the nuclei.

The cytotoxic activity of cisplatin is certainly correlated with the amount of Pt bound to DNA (Zwelling *et al.*, 1979; Knox *et al.*, 1986; Lindauer and Holler, 1996), but many factors control the DNA platination. These include drug uptake (which varies among different cells), the rate of Pt adduct formation and repair, and the concentration of cellular thiols (glutathione and metallothioneins) able to preferentially bind and then intercept the cisplatin. All these limiting factors are not present in the experiments with DNA-based biosensors. Pera *et al.* measured the amount of DNA platination in many cell lines and compared this to the degree of cytotoxicity (Pera *et al.*, 1981). They concluded that cytotoxicity occurs when there are around 2–10 nmoles of Pt/g DNA, which reflects about 1 Pt atom/100,000–500,000 nucleotides. In order to understand at which Pt/DNA ratio the biosensor really works, we measured the amount of DNA immobilized onto the electrode according to Steel *et al.* (1998). The determination revealed that about 3×10^{-9} g of DNA coated the biosensor. Taking into account this quantity of immobilized DNA, only 1 attomole (10^{-18} mole !) of Pt is required to reach the cytotoxic ratio. Thus, at millimolar concentration, numerous metal compounds could interact with G, even without being active antitumour drugs.

Nevertheless, this study of antiproliferative metallo-drugs offers some interesting, albeit qualitative, information concerning (i) the reactivity of the metal complex when acting as a pro-drug being activated by aquation, (ii) the possible mass effect of anions acting as a ligand (i.e. chlorides), (iii) the intrinsic affinity of the electrophilic agent to each nucleophilic component of the DNA, and (iv) the strength of perturbation caused directly to the DNA chain by such metallo-drugs on the electron density of N7-G that is quantifiable in such a measurements.

It is important to recall that simple metal aquaions (i.e. Zn^{2+} and Cu^{2+}) with the same

charge and at similar concentration of the active Pt or Ti species were found to unaffected the G signal. This means that simple electrostatic interactions are not sufficient *per se* to decrease S%. Only the presence of covalent interactions involving the electronic density of N7-G (i.e. on its lone pair of electrons) causes a decrease in its oxidation peak as well. Therefore, it is possible to roughly determine whether or not DNA is a possible target for different metallo drugs.

Acknowledgements

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