A Suggestion of Electrochemical Biosensor for Study of Platinum(II)-DNA Interactions

Sona Krizkova, a,b Vojtech Adam, a,b Jiitka Petrlova, a Ondrej Zitka, a,c Karel Stejskal, a,c Josef Zehnalek, a Bernd Sures, d Libuse Trnkova, e Miroslava Beklova, f Rene Kizek * a

a Laboratory of Molecular Biochemistry and Bioelectrochemistry, Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
b Department of Genetics and Molecular Biology, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic
c Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic
d Universitat Karlsruhe, Zoologisches Institut I, Ökologie-Parasitologie, D-76128 Karlsruhe, Germany
e Department of Theoretical and Physical Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic
f Department of Veterinary Ecology and Environmental Protection, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-612 42 Brno, Czech Republic

Received: July 21, 2006
Accepted: September 19, 2006

Abstract

As a consequence of the employment of platinum based cytostatic drugs in tumor diseases treatment, it became necessary not only to detect them in biological samples but also to determine and study the Pt-DNA adducts, which is highly important during investigation of resistance on antitumor treatment. Thus, the main objective of this work was to study the interactions between DNA and cisplatin. Primarily, we studied changes in electrochemical behavior of cisplatin in the presence of different concentrations of sodium chloride using differential pulse voltammetry. We found out that the highest signal of cisplatin was measured in 0.75 M NaCl with detection limit of 100 pM. The current accepted opinion about platinum based cytostatics mechanism of action is that the drugs induce their cytotoxic properties through binding to the nuclear DNA. That is why we followed by investigation of DNA interactions with cisplatin. For these purposes we have suggested a new biosensor, EDTA metallothionein modified hanging mercury drop electrode (EDTA MT biosensor) and used cyclic voltammetry as a comparative technique. The average concentration of the drug bound to DNA was estimated as 8.1 ng of cisplatin per 500 ng of DNA by EDTA MT biosensor. It follows from the results obtained that the suggested EDTA MT biosensor could be a new useful tool for investigation of interaction of DNA with cisplatin, because give a response only in the present of Pt(II)-DNA adduct.

Keywords: Cisplatin, DNA, Electrochemistry, Cyclic voltammetry, Differential pulse voltammetry, Adsorptive transfer stripping techniques, Biosensors, Pt(II)-DNA adducts

DOI: 10.1002/elan.200603737

1. Introduction

Platinum complexes play an important role in the chemotherapy of various malignancies [1–5]. The biological activity of the first platinum based cytostatic drug – cisplatin (cis-diaminedichloroplatinum(II)), which is still one of the most frequently used cytotoxic agent, was discovered in 1965 by Rosenborg during his studies on the effects of an electric current on bacterial growth [6]. Since then hundreds of platinum(II) and platinum(IV) complexes have been synthesized and evaluated as anticancer agents over past 40 years. To be specific, the “second generation” Pt drug carboplatin [cis-diammine(1,1-cyclobutane-dicarboxylato)platinum(II)] was develop in the 1980s as a less toxic alternative to cisplatin, providing less severe side effects [7–9]. Oxaliplatin [oxalato-1,2-diaminocyclohexane platinum(II)] was developed in the 1990s as a novel (third generation) Pt drug to overcome cisplatin and carboplatin resistance [1, 10, 11]. Besides these commonly used anticancer drugs, many research groups around the world still develop new ones such as LA-12 [(OC-6–43)-bis-(acetato)(1-adamantylamine)amminedichloroplatinum(IV)] [12–14] and analogues of clinically ineffective transplatin [15, 16]. In addition, behavior of these platinum compounds in an organism with respect of kinetics of different complex formation has not been well described. Based on in vitro experiments influencing of platinum complexes stability by pH, concentration of ions and biologically active compounds in blood has been described [17, 18]. Particularly, concentration of chlorides anions in blood (about 100 mM) is different markedly from its concentration in cell cytoplasm (about 4 mM). Due to this difference structural changing of a platinum based cytostatics passing through cytoplasmic membrane appear (according to mathematical
modeling). The changes of cisplatin structure under these conditions based on distribution diagrams are shown in Figure 1 [17, 19]. It clearly follows from the distribution diagrams that the predominant complex among others is [Pt(NH₃)Cl₂]. Total content of this complex is about 80% in comparison with other ones where their content does not exceed 1–3%. On the other hand, distribution of the complex in cell cytoplasm differs markedly (Fig. 1). Here predominates [Pt(NH₃)OH₂] complex, whereas content of other forms of cisplatin is higher in comparison with complexes in blood. It is supposable that these complexes can coordinate to DNA [20–22] and Fig. 1.

Chaney et al. [22–24] and others [21, 25] have found that cisplatin, carboplatin and oxaliplatin form the same types of adducts at the same sites on the DNA. Particularly, a platinum based cytostatic drug can coordinate to N7 of two neighboring guanine and/or adenine bases, as this nitrogen does not form H bonds with other bases, in the same or in opposite DNA strands (approximately 60–65% intrastrand GG, 25–30% intrastrand AG, 5–10% intrastrand GNG, and 1–3% interstrand GG diadducts, see in Fig. 1) [21, 22].

As a consequence of the employment of platinum based cytostatic drugs in tumor diseases treatment, it became necessary not only to detect them in biological samples but also to determine and study the Pt-DNA adducts, which is highly important during investigation of resistance on antitumor treatment [26]. There are many techniques, which have been used for the determination of platinum based cytostatic drugs such as HPLC coupled to different kinds of detectors [27–29] and/or electrochemical methods [30–32]. On the other hand a few techniques have been employed for the direct detection of Pt-DNA adducts [33–37]. In addition the Pt-DNA adducts biosensor have not been suggested yet.

The main of this work was to study the interactions between DNA and cisplatin. For these purposes we suggested a new biosensor, EDTA metallothionein modified hanging mercury drop electrode (EDTA MT biosensor), for detection of Pt(II)-DNA adducts. Cyclic voltammetry has been used as a comparative technique.

2. Experimental

2.1. Chemicals

Rabbit liver MT (MW 7143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) is produced by Molecular Probes (Evgen, Oregon, USA). Sodium chloride and other chemicals used were purchased from Sigma Aldrich. Stock standard solutions of MT with 10 μg mL⁻¹ were prepared by ACS water (Sigma-Aldrich, USA) and stored in the dark at the temperature of –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH of prepared solution was measured using WTW InoLab Level 3 (Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Electrochemical Measurements

Electrochemical measurements were performed with the AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with the drop area of 0.4 mm². The reference electrode was the Ag/AgCl/3 M KCl electrode and the auxiliary electrode was the graphite electrode. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. All experiments were carried out at room temperature. For smoothing and

![Fig. 1. Scheme of the equilibrium constant and chloride concentration influencing the distribution between the hydrolysis products of cisplatin that can react with nucleophilic sites on DNA to form Pt-DNA adducts.](image-url)
baseline correction, the software GPES 4.4 supplied by EcoChemie was employed.

2.2.1. Differential Pulse Voltammetry of Cisplatin

The electrochemical determination of cisplatin was done according to Vrana et al. [38] with modifications. The supporting electrolyte (sodium chloride (0.05–2 M, pH 5.1) was purchased from Sigma Aldrich in ACS purity. DPV parameters were as follows: the initial potential of −1.0 V, the end potential −1.75 V, the modulation time 0.057 s, the time interval 0.2 s, the step potential 1.05 mV/s, the modulation amplitude of 25 mV. The pH of solution analyzed was measured using pX module in connection with pH-electrode (SenTix-H, pH 0–14/3 M KCl).

2.2.2. Cyclic Voltammetry of DNA

DNA has been analyzed by adsorptive transfer stripping (AdTS) in connection with cyclic voltammetry (CV). The supporting electrolyte (0.3 M ammonium formate, pH 6.5) was used. CV parameters were as follows: the initial potential of 0 V, the vertex potential −1.8 V and the end potential 0 V, the step potential of 2 mV/s, the scan rate 1 V, the deposition potential −1.7 V and \( \tau_A = 3 \text{s} \), purging 200 s.

2.2.3. Suggestion of EDTA MT Biosensor

A detailed description of the metallothionein modification method has been previously published [39]. Briefly, scheme of adsorptive transfer stripping technique used for suggestion of heavy metals biosensor; (1) renewing of the hanging mercury drop electrode (HMDE) surface; (2) adsorbing of MT in a drop solution onto the HMDE surface at open circuit (240 s); (3) washing electrode in sodium chloride (0.5 M, pH 6.4); (4) interaction of Pt(II)-DNA adducts in a drop solution with the protein modified HMDE surface at open circuit (this parameter was optimised, see Results and discussion section); (5) washing electrode in sodium chloride (0.5 M, pH 6.4); (6) measurement of MT by DPV in 0.5 M sodium chloride, pH 6.4. The samples of the MT were reduced by 1 mM tris(2-carboxyethyl)phosphine (TCEP) according to [40] and treated by 100 mM ethylenediamine-N, N, N', N'-tetraacetic acid according to [41] before each measurement. The supporting electrolyte (sodium chloride: 0.5 M NaCl, pH 6.4) was purchased from Sigma Aldrich in ACS purity. DPV parameters were as follows: the initial potential of −1.2 V, the end potential −0.3 V, the modulation time 0.057 s, the interval 0.2 s, the step potential 1.05 mV/s, the modulation amplitude 25 mV.

2.3. Real Samples

2.3.1. Preparation of Cisplatin

The chemotherapeutic drug cisplatin was synthesized and provided by Pliva-Lachema (Brno, Czech Republic) [42]. Stock standard solutions of cisplatin at 0.5 mg mL\(^{-1}\) were prepared by sodium chloride solution (0.5 M, pH 6.4) and stored in the dark at the temperature of −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions.

2.3.2. Preparation of DNA Adduct with Cisplatin
dsDNA obtained from chicken erythrocytes (100 μg/mL) was modified in the presence of 10 mM NaClO\(_4\) by cisplatin (10, 25, 50, 100, 250, 350 and 450 μM). The experiments were conducted for 24 hours at the temperature of 25 °C in the dark of a thermostatic box (Model TER-5/1, Chirana, Brno, Czech Republic).

2.3.3. Purification of DNA Adduct

The obtained DNA adduct was purified for the period of 24 hours by ultrafiltration (Microcon YM-30, Millipore). The DNA adduct was laid on Microcon YM-30 membrane and centrifuged (Eppendorf, 14000 g) for 10 min at 20 °C. Each of the low-molecular compounds, such as free cisplatin and fragments of DNA present in the solution came through the nitrocellulose membrane. Consequently, the sample reservoir was turned round and centrifuged (14000 g, 10 min, 20 °C). Afterwards, the modified DNA was obtained.

2.4. MEDUSA Program

Make Equilibrium Diagrams Using Sophisticated Algorithms (MEDUSA program) was used for construction of distribution diagram of different cisplatin under specific conditions. Basic parameters including equilibrium constants that are necessary for construction of distribution diagrams are in program database. The program author is Ignasi Puigdomenech from Inorganic Chemistry of Royal Institute of Technology, Stockholm, Sweden. MEDUSA program is free ware available on http://www.kemi.kth.se/medusa [43, 44].

2.5. Statistical Analysis

STATGRAPHICS (Statistical Graphics Corp, USA) was used for statistical analyses. Results are expressed as mean ± S. D. unless noted otherwise. Value of \( p < 0.05 \) was considered significant.

3. Results and Discussion

3.1. Electrochemical Analysis of Cisplatin

Methods used for monitoring of platinum based cytostatics directly in body liquids have been suggested [28, 30, 45, 46], whereas suggesting of new ones could be very helpful to detect therapeutic concentration of the drug and, thereby, to
improve a treatment of tumor diseases [47–52]. Electro-analytical techniques belong to preferred techniques in this field of study due to their low operating costs and very low detection limits of platinum group metals [30, 38, 53–56]. Typical DP voltammogram of cisplatin (0.25 and 0.5 μM) measured in the presence of sodium chloride (0.75 M) is shown in inset in Fig. 2A. The signal corresponding to cisplatin can be observed at potential of $-1.452 \pm 0.009$ V ($n = 10$). Electrochemical process occurred during analysis of cisplatin is probably same as for electrochemical detection of osmium [57, 58]. Supporting electrolytes as solutions of salts with higher ionic strength have been utilized for determination of cisplatin most frequently due to higher stability of platinum drugs in these electrolytes [38, 55, 56, 59]. Here, we investigated changes of cisplatin signal with increasing concentration of sodium chloride (0.05–2 M). pH of the solutions measured has been monitored during the analysis and have changed about $5.04 \pm 0.04$. $[\text{Pt(NH}_3\text{)Cl}_2]$ could be considered as predominating platinum complex among others within the range of NaCl concentrations used (Fig. 1). It clearly follows from the results obtained that height of cisplatin signal increased with increasing concentration of NaCl up to 0.75 M, afterwards decreased very slightly. The decrease could be associated with changing of predominating cisplatin complex, and thereby, with different behavior of them on the surface of working electrode (Fig. 1). In addition, the cisplatin signal shifted to more positive potentials with increasing concentration of NaCl (Fig. 2A).

Choosing of sodium chloride (0.75 M) for following experiments resulted from the results obtained and described above. We construed dependence of height, area and potential of cisplatin signal on its concentration in the range from 2 to 1000 nM (Fig. 2B). The height of the signal of interest was proportional to cisplatin concentration, whereas we obtained strictly linear dependence in the range from 2 to 125 nM (inset in Fig. 2B). Detection limit ($3 \text{S/N}$) were estimated as 100 pM of cisplatin with $\text{RSD} \ 1.5–2.5\%$. The suggested method could be utilized for determination of cisplatin not only in drugs but also in biological samples.

3.2. Investigation of DNA Interactions with Cisplatin by Means of Cyclic Voltammetry

Although platinum based cytostatic drugs have been successfully used in the chemotherapy of cancer for more than 30 years, its biochemical mechanism of action is still unclear [15, 26, 60]. The current accepted opinion about their mechanism of action is that the drugs induce their cytotoxic properties through binding to the nuclear DNA and subsequent interference with normal transcription, and/or DNA replication mechanisms [3, 61]. As we mentioned above, therapeutic effect of cisplatin probably relates to conformational changes in DNA structure due to their coordinating to N7 guanine. A several models of possible Pt(II) complex with DNA have been suggested [62]. The interactions can be studied electrochemically as a decrease...

![Fig. 2. Analysis of cisplatin. Influence of different concentrations of sodium chloride (0.05–1.75 M) on height and potential of cisplatin signal (A). Inset: typical DP voltammogram of cisplatin (0.25 and 0.5 μM) measured in the presence of sodium chloride (0.75 M). Dependences of height, area and potential of cisplatin signal on its concentration in the range from 2 to 1000 nM (B) and from 2 to 125 nM (inset). DPV parameters were as follows: the initial potential of $-1.0$ V, the end potential $-1.75$ V, the modulation time 0.057 s, the time interval 0.2 s, the step potential 1.05 mV/s, the modulation amplitude of 25 mV.](image)
of guanine signal [63–70]. DNA (100 μg/mL) gives well developed signals, particularly the reduction signal of adenine and cytosine (peak CA) [71–73] and the oxidation signal of reduction guanine product (peak G) [74–76], using AdTS CV (time of accumulation – 120 s, Fig. 3A). Subsequently, the DNA was modified by different concentrations of cisplatin in the presence of 10 mM NaClO4 at 37 °C for 60 min. according to [32]. Changes in cyclic voltammograms of G peak with increasing concentration of cisplatin are shown in Fig. 3B, where decrease of G peak height is well detectable. Dependence of G peak height on different cisplatin concentration is shown in Fig. 3C. Particularly, the G peak height decreased markedly up to the cisplatin concentration of 50 μM, which could be characterized by the equation followed $y = -1.1442x + 119.34 (R^2 = 0.9999)$. The break point of dependence appears between 50 to 100 μg/mL of cisplatin. Then, the decrease of guanine signal was more gradual, which could be characterized by the equation followed $y = -0.0208x + 60.948 (R^2 = 0.9944)$. In addition, the G peak decreased with increasing interaction time and after more than 150 min the signal decreased by about 35% in comparison with the highest obtained response after 5 min of interaction (Fig. 3D). After 24 hours of interaction it gets to decrease of the G peak height by about 60% (not shown). The decrease of the G peak height relates to the sequential formation of complex of Pt(II) and guanine contained in the DNA molecule.

3.3. Analysis of Pt(II)-DNA Adducts by EDTA MT Biosensor

If we have conducted the above mentioned experiments, we would be able to observe course of DNA interaction with the drug. Nevertheless, we are not able to distinguish unmodified DNA molecules from modified ones. Thus, we suggested procedure how to purify and detect only DNA molecules modified by cisplatin (Fig. 4). High molecular compounds have been separated from low molecular ones (below 1.000 g/mol) by ultrafiltration, but the fraction obtained contained both modified (Pt(II)-DNA adducts) and unmodified DNA. If we will use AdTS CV to analyze the fraction, we detect total content of guanines and cannot recognize Pt(II)-DNA adducts. Thus, we suggested unique EDTA MT biosensor to detect platinum bound in DNA (Fig. 4). Recently, we suggested easy-to-use, rapid and sensitive heavy metals biosensors [39, 77, 78]. Here, we optimized MT biosensor by EDTA treating, which removes effectively all bound metals in MT. DP voltammogram of EDTA MT biosensor is shown in Figure 5A. If we attempted

![Detection of G peak by AdTS CV](image)

Fig. 3. Cyclic voltammetry of DNA. Typical cyclic voltammogram of unmodified 100 μg/mL DNA (A). The changes in guanine peaks with increasing cisplatin concentration after the baseline correction (a – 0, b – 10, c – 25, and d – 50 μM; B). Dependences of the guanine peak height on different concentration of cisplatin (0, 10, 25, 50, 100, 250, 350, and 450 μM; C); interaction time (0, 10, 20, 30, 60, and 150 min; D). CV parameters were as follows: initial potential of 0 V, vertex potential – 1.8 V; end potential 0 V, step potential of 2 mV/s, scan rate 1 V, deposition potential – 1.7 V and $t_d = 3 s$, purging 200 s.
to detect DNA (100 µg/mL), we did not observe any changes in CdT signal (Fig. 5B). On the other hand if we did the same experiments and detected Pt(II)-DNA adduct instead of unmodified DNA, we observed decrease in CdT signal (Fig. 5C). The decrease has been more detectable, higher time of interaction has been used (Fig. 5C-D). CdT signal

Fig. 4. Scheme of preparation and followed detection of the Pt(II)-DNA adducts.

Fig. 5. Analysis of Pt(II)-DNA adducts. DP voltammogram of 10 µM MT + 100 mM EDTA – EDTA MT biosensor (A), EDTA MT biosensor + 100 µg/mL DNA (B) and EDTA MT biosensor + 100 µg/mL DNA modified by cisplatin at different interaction times (s); inset: PtMT signal, other details see in [80]. Dependences of the CdT peak height on different interaction times (D) and CdT and G peak heights on concentration of Pt(II)DNA adduct (E). DPV parameters: time of accumulation 240 s, time of interaction 400 s. For other details see Section 2.

decreased with time of interaction higher than 20 min., but it is not suitable for practical purposes. Besides CdT signal, we detected signal called PtMT at potential of −1.11 V during analysis Pt(II)-DNA adducts (inset in Fig. 5C). The same signal has been detected during analysis of cisplatin by MT biosensor [79]. We used CdT signal for determination of Pt(II)-DNA adducts, because is more sensitive to Pt(II)-DNA adducts in comparison with PtMT. The results obtained were compared with cyclic voltammetry (Fig. 5E). It clearly follows from the figure that CdT signal decreased linearly with increasing concentration of Pt(II)-DNA adducts (y = 0.0371x + 0.3481; R² = 0.994). The average concentration of the drug bound to DNA was estimated as 8.1 ng of cisplatin per 500 ng of DNA, which is in good agreement with results obtained by Vrana et al. [38]. It follows from the results obtained that the suggested EDTA MT biosensor could be a new useful tool for investigation of interaction of DNA with cisplatin, because give a response only in the present of Pt(II)-DNA adduct.

4. Conclusions

The mechanism of antitumor activity of all platinum-based drugs is of continuing interest because understanding it may help in designing new platinum drugs with better or alternative therapeutic properties. The suggested EDTA MT biosensor could be a new tool how to study the interaction between platinum based cytostatics and DNA, which would cast the light on cytotoxicity of these drugs.

5. Acknowledgements

The results of this work were presented on 11th International Conference on Electroanalysis, Bordeaux, France. The authors wish to thanks to Dr. Premysl Lubal for discussions. We gratefully acknowledge by following grant agencies for financial supporting of this work: GACR 525/04/P132, INCEMBIOL 0021622412, GA AV CR A100040602 and MSMT 6215712402.

6. References
