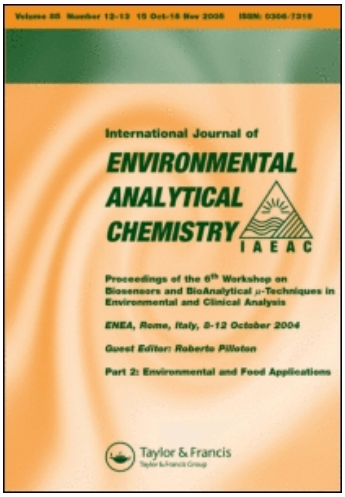

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Adsorptive transfer stripping voltammetry applied to the study of the interaction between DNA and actinomycin D

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ADSORPTIVE TRANSFER STRIPPING VOLTAMMETRY APPLIED TO THE STUDY OF THE INTERACTION BETWEEN DNA AND ACTINOMYCIN D

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Actinomycin D is an antitumor antibiotic which intercalates within DNA. The interaction of actinomycin D with double-stranded and thermally denatured (single-stranded) calf thymus DNA was studied in solution as well as at the electrode surface using differential pulse transfer voltammetry with carbon paste electrode in 0.2 M acetate buffer, pH 5.0.

Keywords: Actinomycin D; Adsorptive transfer stripping voltammetry; DNA; CPE; Intercalator; Anti-cancer drugs

INTRODUCTION

Actinomycin D (ACTD) is one of the most widely studied biologically active small molecules. The widespread interest in ACTD is due to the drug's activity as an antibiotic and as an antitumor agent [1,2]

The ability of actinomycins to bind with double-helical DNA is responsible for their biological activity and cytotoxicity. X-ray studies of a crystalline complex between ACTD and deoxyguanosine permitted formulation of a model that appears to explain the binding of the drug to DNA. The planar phenoxazone ring of ACTD intercalates between adjacent guanine–cytosine base pairs of DNA, where the guanine moieties are on opposite strands of the DNA, while the polypeptide chains extend along the minor groove of the helix. The summation of these interactions provides great stability to the actinomycin D–DNA complex, and, as a result of the binding of ACTD, the transcription of DNA by RNA polymerase is blocked. The DNA-dependent RNA

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polymerases are much more sensitive to the effects of actinomycin D than are the DNA polymerases. In addition, ACTD causes single-strand breaks in DNA, possibly through a free-radical intermediate or as a result of the action of topoisomerase II [3–5]

The interactions between DNA and ligands have so far been studied by various analytical techniques, such as gel or capillary electrophoresis, high-performance liquid chromatography, X-ray crystallography and surface plasmon resonance [6,8]. The electroactivity of nucleic acids has allowed the development of new more rapid and more sensitive electrochemical techniques, based on the differences in the electrochemical behavior of DNA-targeted molecules and DNA [9–19].

DNA biosensors, based on nucleic acid recognition processes, are rapidly being developed towards the goal of rapid, simple and inexpensive testing of genetic and infectious diseases, and for the detection of DNA damage and interactions useful to drug design applications. Unlike enzyme or antibodies, nucleic acid recognition layers can be readily synthesized and regenerated for multiple use [20]. A number of these compounds belonging to known carcinogens (polycyclic aromatic hydrocarbons, aromatic amines) or antitumor antibiotics (daunomycin, doxorubicin, epirubicin, echinomycin, bleomycins, etc.) have been studied to explore the mechanism of their interaction with DNA using electrochemical methods [21].

In previous papers the interaction of acridine orange and ethidium bromide with DNA have been studied [22,23]. In this article, electrochemical DNA-sensors are applied in order to study the interactions of ACTD with DNA on a carbon paste electrode (CPE). Furthermore, the aim is to compare the different behavior of the drug after interaction with DNA in solution or after immobilization of DNA on the electrode surface. The results suggest a new approach for the detection of toxic and potentially toxic compounds, along with a better understanding of the intercalation phenomenon.

EXPERIMENTAL

Reagents

Double-stranded calf thymus DNA (dsDNA) (Catalog No.D-1501, highly polymerized) and Actinomycin D (16,053-9), 95% p.a., were purchased from Sigma. The supporting electrolyte for differential pulse voltammetric experiments was acetate buffer solution 0.2 M (pH 5.0). Trichloroacetic acid, 6 M in water (TCA), was purchased from Fluka.

Thermally denatured (single-stranded) DNA (ssDNA) was prepared by boiling a solution of double-stranded DNA (1 g/L) for 15 min and keeping it at 4°C for 10 min. The stock solution of dsDNA (1 g/L) was prepared with a solution of 10 mM Tris-HCl and 1 mM EDTA at pH 8.0. Stock solutions of ACTD (4 mM) were prepared with water, while dilute solutions were prepared by successive dilutions just before use. The water used was doubly distilled and sterilized.

Apparatus

Differential pulse voltammetric measurements were performed with a Metrohm 647 VA-Stand controlled by a 646 VA-Processor. The working electrode was a CPE (6 mm diameter), the reference electrode was a saturated Ag/AgCl/3M KCl and the

counter electrode was a platinum wire. The carbon paste was prepared in the usual way by hand-mixing graphite powder and nujol oil. The ratio of graphite powder (Fluka) to nujol oil was 75 : 25. The resulting paste was packed tightly into a Teflon sleeve. Electrical contact was established with a stainless steel screw. The surface was polished to a smooth finish before use. The electrode was pretreated by applying a potential at +1.7 V for 1 min without stirring prior to the accumulation step. The electrochemical pretreatment produces a more hydrophilic surface state and a concomitant removal of organic layers.

All water and pipette tips were sterilized by autoclaving for 20 min. The electrochemical cells were cleaned with diluted nitric acid, rinsed with water and sterilized for 20 min. Ultrapure nitrogen was used to deaerate the solutions for 5 min before each experiment.

Procedures

Interaction of Solution-phase DNA with ACTD

The analysis of solution-phase DNA with ACTD consisted of mixing the two components, followed by accumulation and transduction by transfer voltammetry in the differential pulse mode. The electrode was rinsed with water for 5 s prior to each medium exchange. Stock DNA (1 g/L) and ACTD solutions were added to 0.2 M acetate buffer to produce the required concentrations and the mixture was left to stand for 20 min. A freshly polished CPE was immersed in the mixture solution and was pretreated as described above. Accumulation of the mixture was performed by applying a potential of +0.5 V for 5 min. The transduction was carried out in the blank acetate buffer solution, with an initial potential of +0.1 V and a scan rate of 50 mV/s.

Interaction of Surface-confined DNA with ACTD

The procedure consists of DNA immobilization, interaction of ACTD with immobilized DNA and transduction by transfer voltammetry in the differential pulse mode. Prior to each medium exchange, the electrode was rinsed carefully with water for 5 s. After pretreatment of the electrode surface, the nucleic acid was subsequently immobilized onto the electrode surface by adsorptive accumulation for 5 min at +0.5 V. The dsDNA-coated electrode was transferred to the stirred sample solution (analyte plus 0.2 M acetate buffer solution, pH 5.0) for 120 s, while maintaining a potential of +0.2 V. The transduction was performed in the blank acetate buffer solution. The same procedure was followed for the immobilization of ssDNA and the study of the ssDNA-sensor interaction with ACTD.

Sample Preparation

1.5 mL of TCA was added to 1 mL of bovine serum and the mixture was subsequently centrifuged for 5 min at 3000 rpm. 40 μL of the supernatant were transferred into the electrochemical cell, which contained 10 mL of 0.2 M acetate buffer solution, pH 5.0. The solution was deaerated for 5 min before each measurement.

RESULTS AND DISCUSSION

Transfer Voltammetry of ACTD and DNA

In our studies we used 0.2 M sodium acetate (pH 5.0) for the background electrolyte as we found it more suitable than the 0.01 M acetate buffer at pH 4.8 that we also examined. Native double-stranded (ds) DNA yielded a positive peak at +1.02 V, which was more intense in the first background electrolyte. Thermally denatured (single-stranded) DNA yielded a higher peak at +1.02 V due to the oxidation of adenine residues and another feeble one at +0.86 V due to the oxidation of guanine residues.

The accumulation potential and the accumulation time have a profound effect upon the response. The effect of accumulation potential on the oxidation signal of dsDNA for an accumulation time of 120 s is shown in Table I. The peak current is slightly affected by increasing the potential in the range 0.0 to +0.6 V, while it decreases rapidly at higher potentials.

Actinomycin D produces a well-developed peak at +0.985 V with a preconcentration step at +0.2 V for 120 s. Linearity is observed in the range $0–50 \times 10^{-6}$ M (sensitivity 7.227 nA/μM, $r = 0.78$), while saturation of the electrode surface is observed at higher concentrations.

Interactions of Surface-Confined DNA with ACTD in Solution

The DNA-modified electrode was prepared by immersing the CPE in a solution of dsDNA at a concentration of 0.1 g/L in 0.2 M acetate buffer (pH 5.0) for 5 min at +0.5 V. The electrode was pretreated as previously described and subsequently immersed in ACTD solutions of different concentrations ranging from 0 to 1.417×10^{-10} M (in 0.2 M sodium acetate, pH 5.0, for 2 min at +0.2 V). Differential pulse voltammograms were taken after the transfer of the electrode into a blank background electrolyte. The optimal interaction time was selected to be 2 min as shown in Table II, while the interaction potential was selected to be +0.2 V according to the optimal preconcentration step for ACTD. By increasing the drug's concentration, a decrease of the DNA peak was observed, while the peak potential of DNA shifted to more negative values. Figure 1 shows the current changes of the characteristic guanine peak of dsDNA in relation to the drug's concentration added to the solution along with the changes induced in the oxidation potential.

TABLE I The effect of accumulation potential for dsDNA (0.1 g/L) immobilization on the CPE surface for a constant accumulation time of 120 s

Accumulation potential (mV)	*I* (nA)[a]
200	350.27
400	330.12
500	380.43
600	318.13
800	150.08
900	81.09

[a]dsDNA peak at + 0.995 V.

TABLE II The effect of interaction time between the immobilised dsDNA (0.1 g/L) and a constant concentration of ACTD (31.4×10^{-8} M) in the solution at a potential of + 0.2 V at pH 5.0

Interaction time (s)	*I* (nA)[a]
0	430.25
60	43.27
120	51.39
240	48.68
360	36.12
480	50.59
600	34.17

[a]dsDNA peak at + 0.995 V.

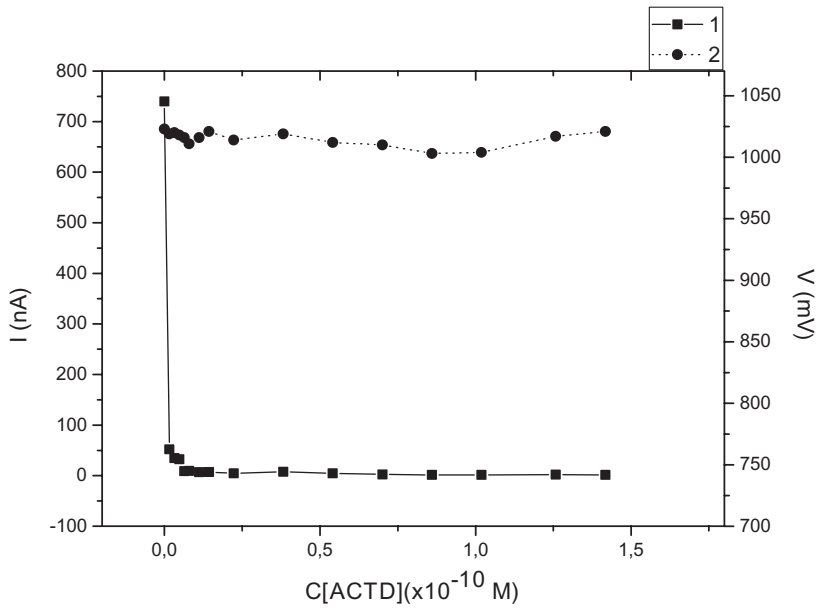

FIGURE 1 (1) Dependence of peak oxidation current for guanine residues in dsDNA immobilized on the electrode surface on increasing concentrations of ACTD. The CPE was pretreated at +1.7 V for 1 min followed by adsorptive accumulation of dsDNA at +0.5 V for 5 min and was immersed in ACTD solutions of different concentration. The incubation time prior to each scan was 2 min at +0.2 V (left scale). (2) Dependence of the oxidation potential of guanine residues on increasing concentrations of the drug under the above conditions (right scale).

In the case of the interaction between the immobilized thermally denatured (single-stranded) ssDNA and ACTD in solution, the characteristic oxidation peak of ssDNA at +1.02 V decreased, while the other one at +0.86 V was gradually increased by increasing concentrations of the drug. Figure 2 shows the current increase of the characteristic guanine peak and the decrease of the adenine peak in relation to the actinomycin concentration. The oxidation potentials of both peaks are shifted to more negative values.

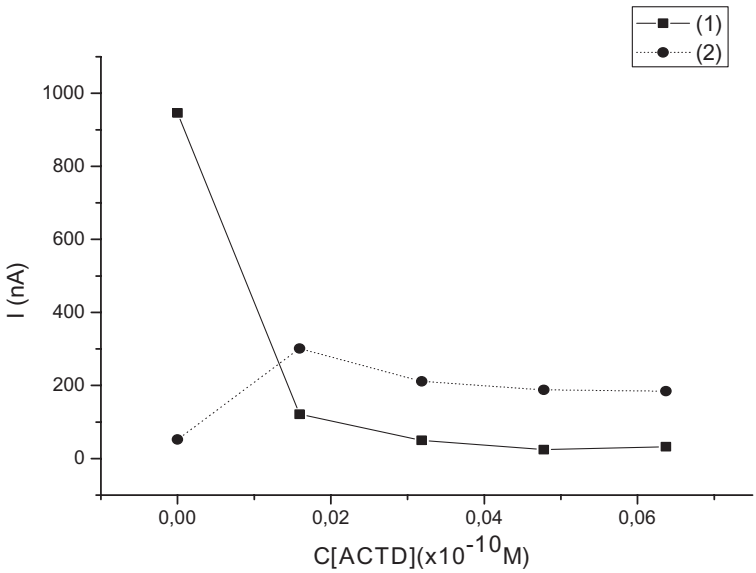

FIGURE 2 (1) Dependence of peak oxidation current for adenine residues in ssDNA immobilized on the electrode surface on increasing concentrations of ACTD. The CPE was pretreated at +1.7 V for 1 min followed by adsorptive accumulation of ssDNA at +0.5 V for 5 min and was immersed in ACTD solutions of different concentration. The incubation time prior to each scan was 2 min at +0.2 V. (2) Dependence of peak oxidation current for guanine residues in ssDNA immobilized as described above on increasing concentrations of ACTD. Experimental conditions are as above.

Interactions of ACTD and DNA in Solution

The incubation time of the two components is a very important factor affecting the response. Figure 3 shows the effect of the incubation time of ACTD at a constant concentration of 7.96×10^{-7} M and dsDNA (0.1 g/L) in solution, namely that the current response increases as the incubation time is increased. The time selected was 20 min, since no dramatic change in the peak current occurs after this point.

A solution of 0.1 g/L double-stranded DNA was left to react for 20 min with different concentrations of ACTD ranging from 0 to 7.36×10^{-6} M. The characteristic oxidation peak of dsDNA is increased by increasing concentrations of the drug, while a new peak appears at +1.134 V, probably due to the excess of ACTD when its concentration equals 2.66×10^{-6} M. Figure 4 presents the dependence of the characteristic peak of dsDNA on increasing amounts of ACTD after incubation in solution. The oxidation potential of the guanine residues is shifted to more negative values.

A similar procedure was followed using ssDNA. Figure 5 shows an increase in both peak currents of ssDNA due to the oxidation of guanine residues and adenine residues with increasing amounts of ACTD. When the concentration of the drug reaches 9.4×10^{-6} M a peak at +1.16 V appears indicating that the drug is in excess.

Application

The two electrodes, carbon paste and dsDNA-modified, were applied in the determination of ACTD in bovine serum samples. The CPE was first pretreated as described previously and then immersed in the buffer solution containing the sample,

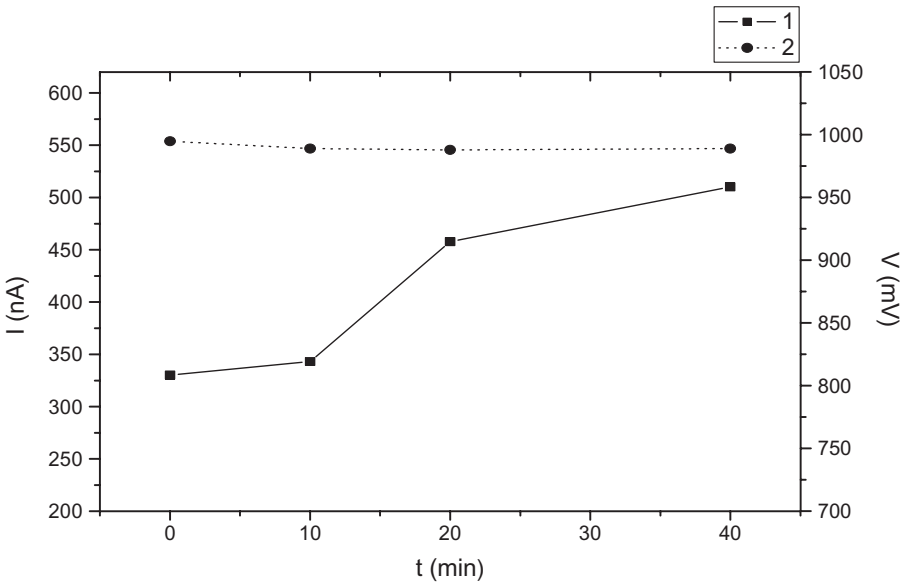

FIGURE 3 (1) Dependence of peak oxidation current for guanine residues in dsDNA in solution with a constant concentration of ACTD (7.96×10^{-7} M) on the incubation time in relation to the potential (left scale). (2) Dependence of the oxidation potential on the incubation time under the above conditions (right scale).

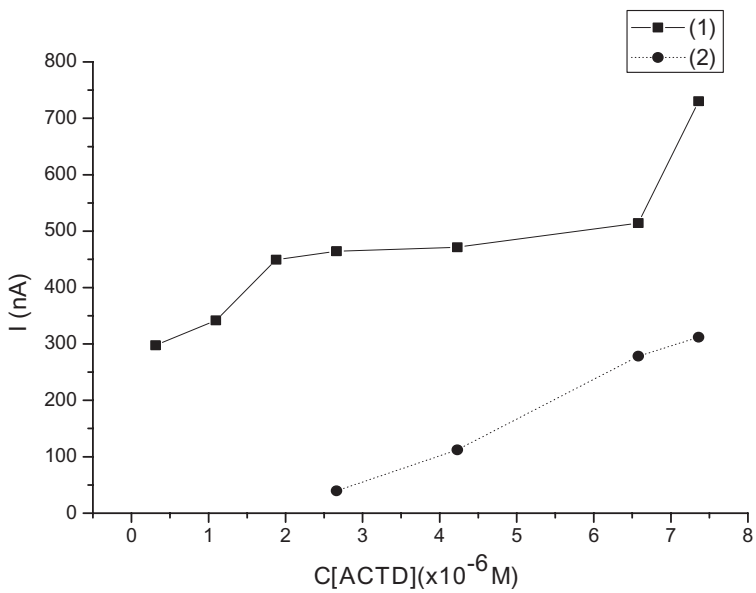

FIGURE 4 (1) Dependence of peak oxidation current for guanine residues in dsDNA on increasing concentrations of ACTD after incubation of stock dsDNA with the drug in 0.2 M acetate buffer for 20 min. The CPE was immersed in the mixture solution, the accumulation was performed at +1.7 V for 1 min followed by application of a potential of +0.5 V for 5 min. (2) Dependence of the characteristic peak current on increasing drug concentrations under the above experimental conditions. See text for a possible explanation.

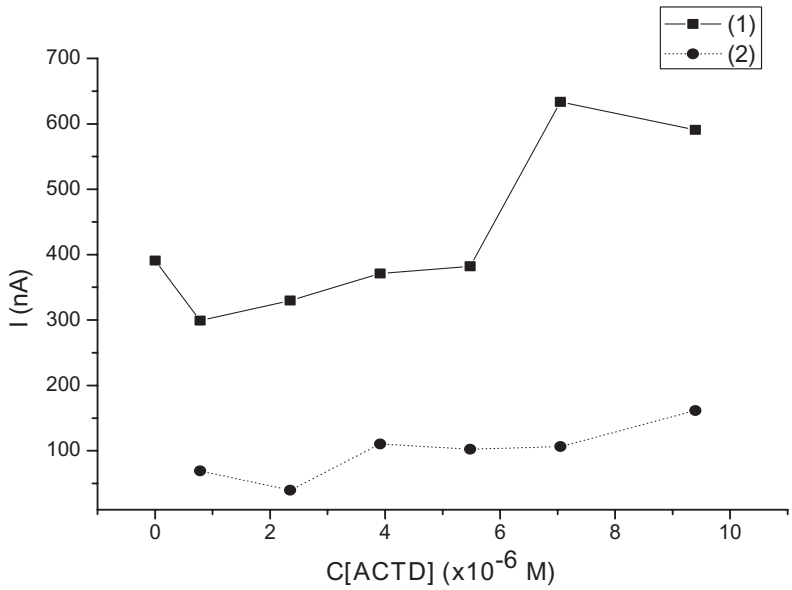

FIGURE 5 (1) Dependence of peak oxidation current for adenine residues in ssDNA on increasing concentrations of ACTD after incubation of stock ssDNA solution with ACTD in 0.2 M acetate buffer, pH 5, for 20 min. The CPE was immersed in the mixture solution, the accumulation was performed by applying a potential at +1.7 V for 1 min followed by application of a potential at +0.5 V for 5 min. (2) Dependence of peak current of oxidation of guanine residues in ssDNA after incubation with increasing concentrations of ACTD as described above. Experimental conditions are as above.

and differential pulse voltammetry was performed. Linearity was observed in the range 5–25 μM ACTD (sens. 0.076 nA/μM, $r = 0.913$).

A similar procedure was followed with the dsDNA-modified electrode. Linearity was observed in the range 39.8–915.4 nM ACTD (sens. 122.4 nA/μM, $r = 0.904$).

Comparing the sensitivity values, the DNA-modified electrode proves to be more sensitive and thus suitable for the determination of lower concentrations of ACTD.

CONCLUSIONS

In this article we have shown that intercalation of ACTD within DNA, immobilized at the CPE surface, can be monitored by differential pulse adsorptive transfer stripping voltammetry.

The interaction of ACTD with DNA in solution is completely different from the interaction of surface-confined DNA with ACTD in solution. In the first case the characteristic oxidation peak of guanine residues in dsDNA is increased by increasing concentrations of the drug, while the oxidation potential is shifted to more negative values. In the second case, the characteristic oxidation peak decreases by increasing the drug's concentration, while the oxidation potential is again shifted to more negative values. According to previous studies [24] positive shifts in the peak potentials of intercalators is a result of their intercalation in dsDNA. In contrast to intercalation

involving hydrophobic interactions in the DNA double helix, electrostatic interactions at the surface of the DNA molecule may result in a negative shift in the peak potential (E_p) of the interacting compound.

In addition, in the first case, the increase of the characteristic oxidation peak of dsDNA with increasing concentrations of the drug in the bulk solution could be attributed to a bending of the DNA molecule and its ability to adhere to the rough CPE surface [17]. In the case of the interaction of ACTD with dsDNA immobilized on the CPE, the drug binds preferentially to the sites of the duplex oriented towards the solution. We have to take into account that DNA is electrostatically adsorbed on the CPE surface through the negatively charged sugar–phosphate backbone by forming a flat layer on the electrode surface and that the side of the duplex that is in intimate contact with the electrode surface is not easily accessible. By increasing the drug's concentration the characteristic guanine peak of DNA is decreased probably due to conformational changes at the DNA backbone, since ACTD is bound preferentially to regions rich in guanine.

We have also studied the interaction with single-stranded DNA. After immobilization of ssDNA on the CPE surface, the oxidation peak of ssDNA at +1.02 V decreased, while the one at +0.86 V remained almost constant as the concentration of the drug in the solution increased. We could use the differences in the characteristic oxidation peak of guanine residues occurring with dsDNA and ssDNA as a criterion for intercalation. The conformational changes of ssDNA due to the interaction with the drug favour an increase of the oxidation signal of adenine residues.

In the case of the incubation of ssDNA and the drug and subsequent immobilization at the CPE surface, both of the characteristic peaks of ssDNA increase and then level off probably due to easier accessibility of guanine and adenine residues at the CPE surface. It is obvious that we cannot speak about an intercalative complex formed as a result of the interaction between ssDNA and ACTD, but other modes of DNA binding should be taken into account, such as outer-sphere electrostatic binding. The different results of the interaction at the CPE surface or in the solution will be further investigated.

In conclusion, we applied a dsDNA and a ssDNA-modified electrode which can be used in the detection of ACTD. The changes of the characteristic peak of guanine can be useful in the determination of very low concentrations of the drug. The study of these changes and the different mode of action with dsDNA or ssDNA could highlight the mechanism of the interaction between different chemical compounds. Finally, we have shown that adsorptive transfer stripping voltammetry in the differential pulse mode is a useful method for the study of the differences in the interaction of the antitumor drug ACTD with dsDNA and ssDNA and for exploration of the mechanism of DNA intercalation.

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