THE REDUCTION OF DOXORUBICIN AT A MERCURY ELECTRODE AND MONITORING ITS INTERACTION WITH DNA USING CONSTANT CURRENT CHRONOPOTENTIOMETRY

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Received October 1, 2009
Accepted November 16, 2009

Dedicated to the memory of Professor Jaroslav Heyrovský on the occasion of the 50th anniversary of the Nobel Prize for polarography.

In this report, voltammetry with linear scan and chronopotentiometric stripping (CPS) with constant current were used for the analysis of doxorubicin (DOX) at a hanging mercury drop electrode (HMDE). CPS was used for the study of DOX *in situ* electrochemical reduction in adsorbed state and for *ex situ* (adsorptive transfer) analysis of the drug. For the first time, CPS was used to study the reversible reduction of the DOX quinine moiety at –0.45 V (vs Ag|AgCl|3 M KCl) as well as electrode processes giving rise to an irreversible signal around –1.45 V at the HMDE in 0.2 M acetate or Britton–Robinson buffers at different pH values. The dependence of the latter signal on pH revealed involvement of protonation equilibria; however, neither CV nor CPS data confirmed the catalytic character of the electrode reaction previously suggested by other authors. The CPS method was also applied to monitor the DOX interaction with double- (ds) and single-stranded (ss) DNA. In the presence of dsDNA, more pronounced changes in DOX signal intensity were observed, in agreement with a strong intercalation of the DOX redox centre into the DNA double helix.

Keywords: Antibiotics; Anthracyclins; Doxorubicin; Adsorption; Reduction; Plasmid DNA; Mercury electrode; Voltammetry; Chronopotentiometry; Electrochemistry.

Doxorubicin (DOX) is a cytotoxic anthracycline antibiotic originally isolated from *Streptomyces peucetius* (for the structural formula of the drug, see Fig. 1). It has been used in the treatment of several human malignancies[1].

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Collect. Czech. Chem. Commun. 2009, Vol. 74, Nos. 11–12, pp. 1727–1738

doi:10.1135/cccc2009512

The pharmacological effects of the drug have been ascribed to interaction with DNA (primarily via intercalation of the anthracycline moiety into the DNA double helix) which causes inhibition of replication and transcription of DNA in the cancer cells[2]. The development of sensitive analytical tools for the determination of DOX and related substances, as well as for studying their interactions with DNA, is essential for both basic research and clinical applications. In addition to UV-Vis spectrophotometry, fluorimetry, Raman spectroscopy, mass spectrometry, viscosimetry, and radioimmunoanalysis[3–6], electrochemical methods[7] also proved useful for sensitive determination of DOX which has been shown by many authors in recent times.

The polarographic behavior of DOX at the dropping mercury electrode has already been studied[8,9]. It was shown that DOX is an electroactive substance[9] which is irreversibly adsorbed at different electrode surfaces (such as mercury[10] or carbon[11]). The electrochemical properties of DOX at a hanging mercury drop electrode (HMDE) and pyrolytic graphite electrode (PGE) were studied[10–13] using alternating current voltammetry (ACV) and voltammetry with linear scan (LSV). Other authors have demonstrated analytical applications of differential pulse and cyclic voltammetry (CV) at a carbon paste electrode (CPE) for the DOX determination[14,15]. In addition, square wave voltammetry (SWV) with HMDE and paraffin-impregnated graphite electrode was used for the DOX study[16]. Recently, *ex situ* SWV and constant current chronopotentiometric stripping (CPS) at DOX-modified electrodes[17,18], have been used for drug analysis based on faradaic and electrocatalytic currents[18,19].

Both studies of DOX electrochemical behavior and its analytical determination and monitoring of drug interactions with DNA using the above-mentioned methods have previously been published (reviewed in refs[7,20]). CV in combination with HMDE was used for the detection of fish sperm

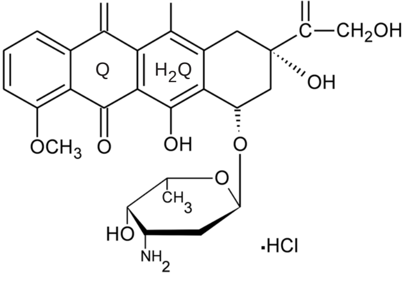

FIG. 1
Chemical formula of DOX with both quinone (Q) and hydroquinone (H_2Q) redox centres

DNA complex with DOX[21]. The interaction was indicated by the decrease of the DOX reduction peak in a weakly acidic medium. Similarly, SWV at HMDE was applied for analysis of the interaction of DOX with DNA in solution and in isolates from neuroblastoma cells[17]. Measurement of the quinone reduction peaks at HMDE was used for the monitoring of interactions of selected anthracyclines with calf thymus DNA[22]. A constant concentration of the drug was titrated by different concentrations of dsDNA and the results indicated the following order of affinities of the anthracyclines to DNA: daunomycin < 4-deoxyadriamycin < DOX < nogalamycin. Oxidative DNA damage upon electrochemical reduction of the drug in the presence of oxygen was also detected using voltammetric methods at a glassy carbon electrode[23]. Moreover, CPS at CPE was applied to detect the interaction of another anthracycline antibiotic daunomycin with calf thymus DNA in the solution and at the electrode surface using oxidation peaks of the drug and of the DNA guanine residues[24].

This paper focuses on CV and CPS studies of DOX adsorbed at the HMDE, with the emphasis on processes related to electrochemical reduction of the DOX quinone group. In addition, CPS technique was applied in the *ex situ* (adsorptive transfer stripping) mode for the monitoring of drug interactions with DNA.

EXPERIMENTAL

DOX hydrochloride salt (Adriamycin), buffer components and other chemicals were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Calf thymus ssDNA and plasmid supercoiled DNA [pBSK$^{(-)}$] were prepared as described[25,26]. All solutions were prepared using deionized water (Millipore, Milli-Q water system, USA).

DOX was analyzed using adsorptive stripping (*in situ*, with the working electrolyte dipped in the analyte solution) electrochemical measurement procedure with the HMDE (0.4 mm^2) if not stated otherwise. In the case of DOX *ex situ* (adsorptive transfer stripping)[27] analysis, HMDE was dipped into 5-μl aliquots of the sample. After an accumulation period, the electrode was subsequently washed by deionized water and placed in an electrochemical cell. All electrochemical measurements were performed at room temperature with an Autolab analyzer (EcoChemie, Utrecht, The Netherlands) connected to VA-Stand 663 (Metrohm, Herisau, Switzerland) in three-electrode setup (Ag|AgCl|3 м KCl) electrode as a reference and platinum wire as an auxiliary electrode). Settings for LSV and CPS, as well DOX concentrations and accumulation times are given in figure legends. Argon was used to remove oxygen from the supporting electrolyte.

RESULTS AND DISCUSSION

The molecule of DOX contains a quinone (Q) and a hydroquinone (H_2Q) redox centre (Fig. 1). These moieties have been shown to undergo reduction or oxidation at carbon electrodes[18]. At the mercury electrodes it is possible to detect, by polarography[8,9] or voltammetry[10,11], only reduction of the Q centre at potentials around –0.5 V (oxidation of the H_2Q group occurs at potentials too positive to be attainable with the mercury electrodes). Figure 2 shows the reversible electrochemical reduction of the Q centre in 0.2 M acetate buffer (pH 5), giving rise to the pair of peaks I_C/I_A at a potential around –0.45 V (see CV in Fig. 2a). A well developed peak due to the Q reduction was also observed using CPS (peak I_C at –0.475 V, Fig. 2b). Besides the reversible reduction of Q, in the CV, we also observed two irreversible peaks (denominated as peaks A and B, Fig. 2a) at more negative potentials (–1.25 and –1.4 V, respectively). When CPS was used instead of CV, only one peak (A/B) at –1.42 V was observed under conditions otherwise the same (compare Figs 2a and 2b).

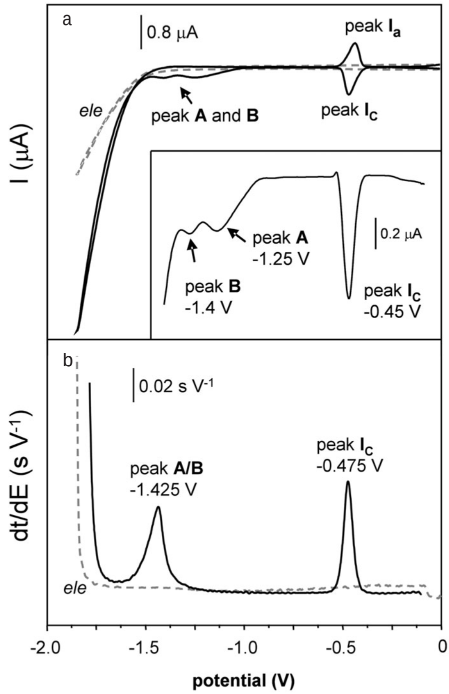

FIG. 2

Electrochemical reduction of DOX at HMDE: (a) CV and (b) CPS with constant current. DOX (5 μM) was adsorbed (t_A = 60 s) at HMDE surface directly in 0.2 M acetate buffer (pH 5) at E_A = 0 V (the inset represents zoom of the cathodic part of voltammogram in Fig. 2a). The scan rate (for CV) and stripping current (for CPS) was 1 V s^{-1} and –10 μA, respectively

DOX is very strongly adsorbed at metal as well as nonmetal surfaces due to the planar structure of its anthracycline moiety. Thus, adsorptive accumulation of DOX at the electrode surfaces can easily be attained. Owing to this efficient accumulation, it has proved possible to analyze nano- and picomolar concentrations of DOX[28]. In the majority of experiments conducted in this work, we adsorbed DOX at a positively charged surface of HMDE, using accumulation potential $E_A = 0$ V and accumulation time $t_A = 60$ s. From the dependences of the LSV peaks I_C and A, one can see that the surface of HMDE was saturated after a 60-s accumulation for DOX concentration ≥ 5 μM (Fig. 3a). The electrode process corresponding to the peak I_C is two-electron reduction of the Q moiety according to reaction $Q + 2\,H^+ + 2\,e^- \rightarrow H_2Q$ (ref.[16]). This reaction, which runs through a semiquinone

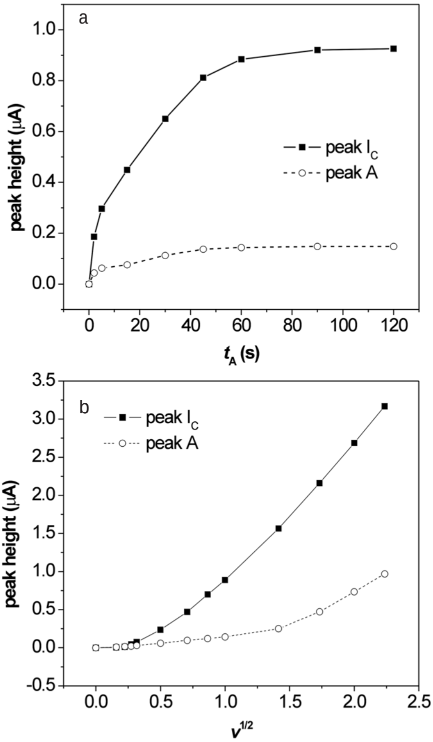

FIG. 3

Dependence of the height of DOX reduction peaks on the (a) time of accumulation and (b) scan rate (as $v^{1/2}$) using LSV measurement. DOX (5 μM) was adsorbed (for (b), $t_A = 60$ s) at HMDE surface directly in 0.2 M acetate buffer (pH 5) at $E_A = 0$ V. LSV parameters: scan rate 1 V s^{-1} for (a), $E_{init} = 0$ V (semiQ) intermediate[16,29], is accompanied by reductive elimination of the glycosidic part (daunosamine). The reduction process in the more negative potential region (LSV peaks A and B or CPS peak A/B) was irreversible contrary to that giving rise to the peak I_C. Previously Kano et al.[10] observed an analogous signal with DOX and its aglycone form (doxorubicinon). The authors ascribed this signal to a kinetic process that occurs at the mercury electrode in the presence of the anthracycline moiety at the negative potentials. However, our results do not allow us to reach such a conclusion. The current of the peak A increased with the square root of the scan rate, following a similar course to the peak current of the faradayic peak I_C (Fig. 3b). The nonlinear (supralinear) shape of this dependence corresponded to the reduction processes of DOX (or product of its reduction at –0.45 V) adsorbed at the HMDE surface[30]. Such behavior did not suggest the involvement of a chemical catalytic reaction (in acetate buffer pH 5.0) proposed in ref.[10]

Besides the shape of the dependence of the peak current heights on the square root of the scan rate in LSV (expected to be decreasing for a kinetic electrode process[30]), the dependence of chronopotentiometric peak A/B height on stripping current (Fig. 4) did not suggest the catalytic nature of peak A. Significant shifts of the potentials of both CPS signals, peak I_C and peak A/B, to more positive potentials (Fig. 4a) and increasing peaks heights (Fig. 4b) were observed with decreasing value of the negative stripping current. Again, both signals followed similar trends. In the case of a coupled catalytic reaction, one could expect that application of low stripping currents will lead to a more pronounced increase of the peak A/B height, compared to a faradayic signal (such as the peak I_C)[18,31,32]. This was not the case in the peak A/B (Fig. 4b).

In subsequent experiments we studied conditions influencing the peak A/B. It was found that this peak was markedly influenced by the potential of accumulation (E_A). DOX was adsorbed at the electrode surface in E_A range from 0.1 to –1.1 V. The height of peak A/B was almost constant for $E_A > -1$ V, but for $E_A \leq -1$ V the peak A/B decreased steeply with the E_A shifting to more negative values. This behavior suggests that during electrolysis at a constant potential ≤ –1 V, the electroactive centre of the adsorbed DOX, giving rise to the peak A/B, was depleted and the peak was diminished. On the other hand, the peak I_C due to the reversible Q/H_2Q process did not decrease upon DOX accumulation at the negative E_A values between –1.0 and –1.4 V, and only a partial decrease of peak I_C was observed around –1.5 V (when hydrogen evolution from the background electrolyte started to occur and induce desorption of the DOX reduction products[33]).

Desorption of the reaction product due to the voltage scanning through potentials < –1 V was indicated also by ACV and SWV measurements (not shown).

In addition to effects connected with accumulation of DOX at HMDE surface, we studied the influence of pH (in the range 2–11) of the background electrolyte on the heights (Fig. 5a) and potentials (Fig. 5b) of the DOX reduction peaks. DOX yielded well developed electrochemical responses in both acetate buffer (pH 3.6–5.6) and the Britton–Robinson buffer (pH 2.0–7.0). The height of the peak I_C was practically unchanged in the pH range from 2 to 7, while at pH values ≥ 8 this peak gradually decreased with increasing pH. Such effect reflected the mechanism of electrochemical reduction of the Q moiety, involving the consumption of two protons. With

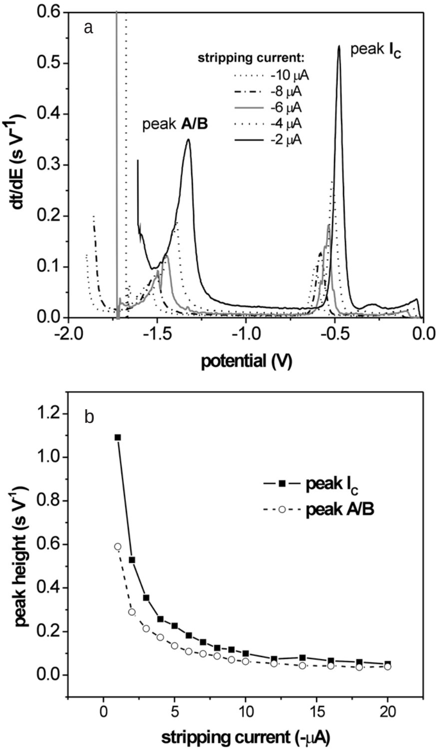

FIG. 4
CPS measurement of 5 μM DOX at HMDE. (a) CPS records and (b) peak height of DOX reduction peaks at different stripping currents. DOX was adsorbed (t_A = 60 s) at HMDE surface directly in 0.2 M acetate buffer (pH 5) at E_A = 0 V

an increase in the pH of the background electrolyte, the potential of peak I_C was shifted to more negative values, with average slope of 45 mV per pH unit. This value was in a good agreement with results obtained by Kano[13] by means of the CV. The pH dependence of peak A/B exhibited a maximum of around pH 7, suggesting interplay between more than one acidobasic equilibrium (possibly involving hydroxyl groups of the drug and the amino group of the sugar residue). Deprotonation of DOX was observable visually via a change in the color of its solution (from orange to blue-violet at pH values around 8). Deprotonation of hydroxyl groups of H_2Q centre was observed by various authors in pH values ≥ 8.15 (reviewed in ref.[34]). This fact accords with the dependence of the peak I_C height on pH (Fig. 5a). The potential of the peak A/B was first slightly shifted to more negative values with increasing pH, but starting from pH ~ 7 it was shifted to more positive potentials. From the previous literature[35], it has been established that DOX

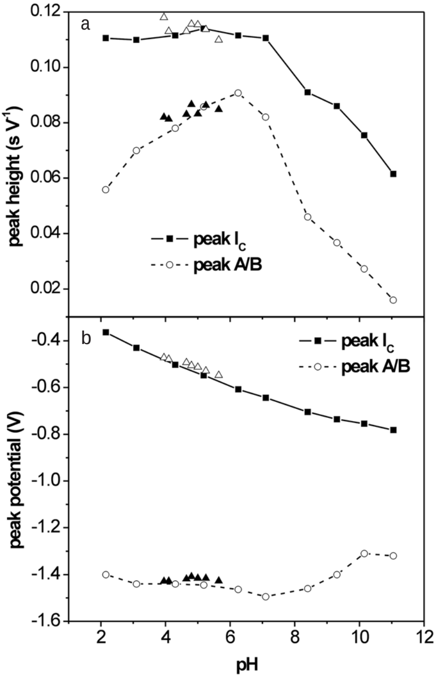

FIG. 5
Effect of the pH of Britton–Robinson (■ and ○) and acetate (△ and ▲) buffers on the (a) height and (b) potential of DOX CPS reduction peaks. DOX (5 μM) was adsorbed (t_A = 60 s) at HMDE surface directly in acetate buffer (pH 5) at E_A = 0 V; stripping current –10 μA

is stable at pH 3–7. At pH > 7, the deprotonized DOX can undergo hydrolysis, which may affect its electrochemical behavior at alkaline pH values.

Finally, we used CPS at the HMDE to monitor DOX interaction with DNA. Basically, different approaches can be used to follow electrochemically the interactions of a drug with DNA [36,37]. The interaction can be detected via changes in the electrochemical behavior of DNA (using intrinsic DNA electrochemical signals at mercury[38] or carbon[24] electrodes) in the presence of the drug. Alternatively, electrochemical responses of drugs, which are often altered due to its interaction with DNA, can be measured[20]. Considering the electrochemical activity of DOX, we chose the latter approach and measured peak I_C, the potential of which is well separated from both tensammetric and reduction responses of the DNA (occurring at potentials ≤ –1.2 V, thus overlapping with the DOX peak A/B). We used *ex situ* chronopotentiometric measurements, allowing the analysis of microliter samples. Adsorption of DOX was conducted at open current circuit from 5 μl of sample (see Experimental). Dependence of the peak I_C heights on the DOX concentration in the absence or presence of DNA is shown in Fig. 6. In the absence of DNA, the signal intensity increased steeply up to ca. 4 μM and the electrode surface was saturated at concentrations ≥ 10 μM. In the presence of scDNA (supercoiled plasmid DNA, 25 μg ml^{-1}), the dependence of peak I_C height was S-shaped (△ in Fig. 6). This behavior reflected the

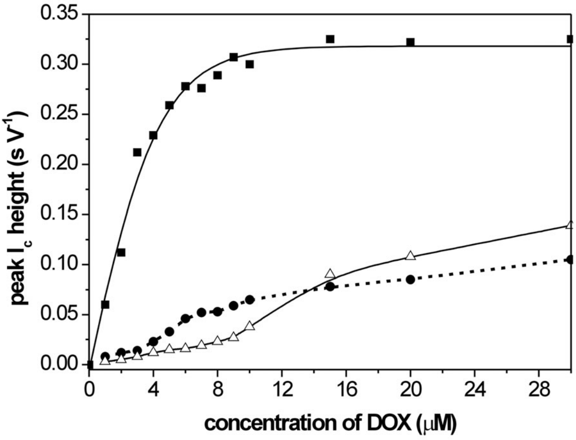

FIG. 6

Ex situ CPS of DOX and its interaction with DNA. Dependence of peak I_C intensity on the concentration of DOX in absence of DNA (■), and in the presence of ssDNA (●) and scDNA (△). DOX at the given concentration was preincubated with ssDNA and/or scDNA (25 μg ml^{-1}) in water at 37 °C for 40 min; t_A = 60 s, stripping current –5 μA strong intercalative interaction of the drug with dsDNA which, at lower DOX concentrations (< 8 μM), resulted in depletion of free DOX from the solution. The Q redox centers of the intercalated DOX were not available for reduction at the HMDE, resulting in the apparent inactivity of the drug at its low concentrations. After saturation of dsDNA by DOX molecules, the peak I_C increased relatively steeply (within the inflexion region) due to availability of free (DNA-unbound) DOX molecules in the solution. From the shape of the concentration dependence in the presence of dsDNA, one can estimate the intercalation of a DOX molecule per ca. 10 base pairs of dsDNA, which is close to the value obtained previously from measurements with calf thymus DNA at carbon electrodes[18]. In the presence of denatured ssDNA, the S-shape of the concentration dependence was less well pronounced and between 4 and 10 μM DOX, the intensities of peak I_C were remarkably higher than in the presence of the same concentration of dsDNA. Considerably lower intensities of the peak I_C observed in the presence of both ssDNA and dsDNA at higher DOX concentrations (such as 20 μM), compared to peak heights obtained in the absence of DNA, were caused by concurrent adsorption of DNA, competing with the drug for the electrode surface. As expected, this effect was more pronounced in the ssDNA exhibiting higher affinity to the mercury surface, owing to accessibility of hydrophobic nucleobase residues, compared to dsDNA which had the nucleobases hidden within the double helix interior.

CONCLUSION

DOX and related anthracycline drugs possess four condensed cycles containing two redox active centers. The pronounced electrochemical activity of these moieties makes the electrochemical methods suitable for a sensitive analysis of these substances as well as for studying their interactions with DNA. In this paper, voltammetric and CPS techniques have been applied to study the electrochemical properties of DOX at the HMDE. CPS was used for the first time to follow a reversible reduction of DOX quinone at the HMDE at ca. –0.45 V and to detect electrode processes giving rise to an irreversible signal at potentials around –1.45 V. Although pH dependence of the latter signal revealed the involvement of protonation equilibria, neither CV nor CPS data confirmed the catalytic character of the corresponding electrode process previously suggested by other authors. Using a reversible signal at –0.45 V we studied interactions of the drug with dsDNA and ssDNA. In the presence of dsDNA, a more pronounced decrease of the

DOX signal was observed, in agreement with a strong intercalation of the DOX redox centre into the DNA double helix.

This work was supported by the Czech Science Foundation (203/07/1195), by the Grant Agency of the Academy of Sciences of the Czech Republic (IAA 400040903), by the Ministry of Education, Youth and Sports of the Czech Republic (LC06035), by the Academy of Sciences of the Czech Republic (1QS500040581), MSM 6198959216 and by institutional research plans No. AV0Z50040507 and No. AV0Z50040702. The authors are indebted to Dr. S. P. Hardy (Department of English and American Studies, Masaryk University, Czech Republic) for language correction.

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