Talanta 80 (2010) 1347–1355

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Voltammetric studies on the potent carcinogen, 7,12-dimethylbenz[*a*]anthracene: Adsorptive stripping voltammetric determination in bulk aqueous forms and human urine samples and detection of DNA interaction on pencil graphite electrode

Y. Yardım[a], E. Keskin[a], A. Levent[a], M. Özsöz[b], Z. Şentürk[a,*]

[a] *Yüzüncü Yıl University, Faculty of Science and Letter, Department of Analytical Chemistry, Campus Zeve, 65080 Van, Turkey*
[b] *Ege University, Faculty of Pharmacy, Department of Analytical Chemistry, 35100 Izmir, Turkey*

ARTICLE INFO

Article history:
Received 18 July 2009
Received in revised form 7 September 2009
Accepted 18 September 2009
Available online 24 September 2009

Keywords:
7,12-Dimethylbenz[*a*]anthracene (DMBA)
Cyclic voltammetry
Adsorptive stripping square-wave voltammetry
Non-aqueous and aqueous media
Urine
DNA interaction
Glassy carbon and pencil graphite electrodes

ABSTRACT

7,12-Dimethylbenz[*a*]anthracene (DMBA), is a widely studied polycyclic aromatic hydrocarbon that has long been recognized as a very potent carcinogen. Initially, the electrochemical oxidation of DMBA at the glassy carbon and pencil graphite electrodes in non-aqueous media (dimethylsulphoxide with lithium perchlorate) was studied by cyclic voltammetry. DMBA was irreversibly oxidized in two steps at high positive potentials, resulting in the ill-resolved formation of a couple with a reduction and re-oxidation wave at much lower potentials. Special attention was given to the use of adsorptive stripping voltammetry together with a medium exchange procedure on disposable pencil graphite electrode in aqueous solutions over the pH range of 3.0–9.0. The response was characterized with respect to pH of the supporting electrolyte, pre-concentration time and accumulation potential. Using square-wave stripping mode, the compound yielded a well-defined voltammetric response in acetate buffer, pH 4.8 at +1.15 V (vs. Ag/AgCl) (a pre-concentration step being carried out at a fixed potential of +0.60 V for 360 s). The process could be used to determine DMBA concentrations in the range 2–10 nM, with an extremely low detection limit of 0.194 nM (49.7 ng L^{-1}). The applicability to assay of spiked human urine samples was also illustrated. Finally, the interaction of DMBA with fish sperm double-stranded DNA based on decreasing of the oxidation signal of adenine base was studied electrochemically by using differential pulse voltammetry with a pencil graphite electrode at the surface and also in solution. The favorable signal-to-noise characteristics of biosensor resulted in low detection limit (*ca.* 46 nM) following a 300-s interaction. These results displayed that the electrochemical DNA-based biosensor could be used for the sensitive, rapid, simple and cost effective detection of DMBA–DNA interaction.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings. They are widespread environmental contaminants resulting from incomplete combustion of organic materials during both natural events and human activities, such as forest fires and volcanic eruptions, and burning of fossil fuels and petroleum products during industrial production, food processing, of machinery operation, including automobiles, airplanes and ships [1]. In view of the large amount of environment pollution studies being carried out on PAHs because some of these compounds are highly carcinogenic and mutagenic, the need has arisen for developing suitable quantitative methods for their determination and monitoring in various types of matrices. PAHs themselves are relatively non-reactive chemicals toward biological macromolecules under physiological conditions. In mammalian system they are metabolized principally in the liver to yield both detoxification products, which are more polar and excretal, and bioactivation products, which are more reactive and genotoxic. Their biological effects are actually attributed to the oxidation process that occurs during biotransformation of the mother compounds. PAH metabolites, including bay-region diol epoxides and free radical cation intermediates formed by monooxygenation and one-electron oxidation, respectively, are molecules that may bind to cellular DNA forming covalent DNA adducts responsible for carcinogenic process [2–5]. Some PAHs are activated exclusively by one of these mechanisms, and others are activated by a combination of both. Recent studies have demonstrated that activation of PAHs can also be achieved by light irradiation [2]. When the skin concomitantly exposed to environmental contaminants and

* Corresponding author. Tel.: +90 432 2251661; fax: +90 432 2251663.
E-mail address: zuhresenturk@hotmail.com (Z. Şentürk).

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doi:10.1016/j.talanta.2009.09.035

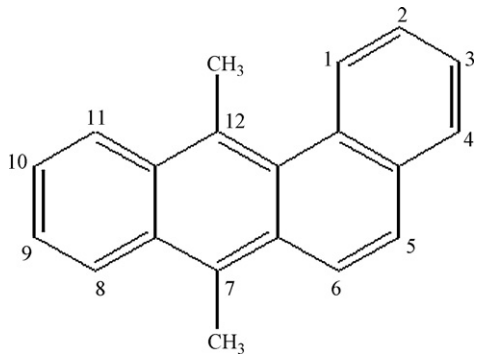

Fig. 1. Chemical structure of DMBA.

sunlight, these chemicals may be activated by photo-irradiation to cause cellular damages and exert toxicity including carcinogenicity.

DNA adducts generated by carcinogenic chemicals or their metabolites are directly related to tumor formation. Therefore, the investigation of DNA interactions is of great importance in order to understand the action mechanisms of some DNA-damaging agents and the design of preventive strategies [3,6–8]. Furthermore, this interaction could be used for rapid detection and quantitative determination of compounds hazardous for all living organisms in the environment, food and clinical samples [9–13].

7,12-Dimethylbenz[*a*] anthracene (DMBA) (its structure is shown in Fig. 1) is one of the PAHs used extensively in experimental studies as a presumptive prototype mutagen, a signal transduction modulator and a known breast and skin carcinogen [14]. The CH_3 groups at C-7 and-12 play an important role in the carcinogenic activation of DMBA [15]. The relationship between the interaction of DMBA with DNA and its carcinogenic potency has been recognized for over 40 years [16]. This compound has been investigated more intensively than any other PAH in terms of its biological activities and biotransformation.

For the above reasons, mostly analytical methods have been employed to characterize and determine DMBA–DNA adducts, and to understand the mechanism of metabolic activation of PAHs in living organisms. So far, chromatographic methods such as GC and HPLC [4,17–24], fluorescence, NMR and mass spectroscopic studies [4,18,25–28], ^{32}P-postlabeling methods [29–32], immunoassay and immunohistochemistry [33–35] are used for these purposes. However, since experimental complications and tediousness or high-cost, most of these methods are not affordable for most laboratories. Although electrochemical techniques were introduced in DNA biosensor technology with a certain delay, as compared to other sophisticated methods, there is a growing interest for design of electrochemical genosensors for biomedical, environmental and forensic applications during the last years. In addition to their useful role in environmental applications, it seems straightforward to employ such electrodes in cancer chemotherapy where DNA is main target. This is mainly due to the capability of fast and sensitive detection by using a relatively low-cost, low-power and pocket-size portable instrumentation. Nonetheless, electrochemical biosensors that detect toxins, pollutants and carcinogenic or mutagenic agents, in particular PAHs [36–41], based on the changes in the oxidation peak of the bases caused by the interaction DNA-analyte, have been scarce so far.

A survey of the literature indicates that very little attention has been paid to the redox behavior of DMBA. In those early reports, electrochemical properties of DMBA was not well documented, only the oxidation potentials of DMBA and related polycyclic carcinogenic hydrocarbons have been studied in non-aqueous medium by cyclic voltammetry using platinum or glassy carbon electrodes [42,43]. Furthermore, there have not yet been any literature reports about the electrochemical detection of the interaction between DMBA and DNA. Therefore, this study is the first attempt describing the detailed voltammetric assay of DMBA and electrochemical investigation of its interaction with DNA.

The current paper is broadly divided into three sections. The first part involves cyclic voltammetric studies of DMBA in non-aqueous solvent at surface of glassy carbon and pencil graphite electrodes. Secondly, special attention will be given to the use of adsorptive stripping voltammetry on disposable pencil graphite electrode in aqueous solutions for the quantification of DMBA in bulk forms and spiked human urine samples. Finally the detection of DMBA–DNA interaction based on the changes of adenine signal using differential pulse voltammetry on pencil graphite electrode will be discussed.

2. Experimental

2.1. Caution

DMBA is a hazardous chemical and should be handled with extreme caution using the guidelines for carcinogenic materials developed by National Cancer Institute [44].

2.2. Apparatus

Cyclic voltammetry (CV) on the glassy carbon electrode (GCE, φ: 3 mm) was performed with BAS 100B/W electrochemical analyzer (Bioanalytical System, USA). Voltammetric measurements with the pencil graphite electrode (PGE) were carried out using electrochemical system μAutolab type III (EcoChemie, The Netherlands) driven by a GPES software version 4.9 (EcoChemie) in connection with a personal computer. The raw voltammograms of differential pulse (DPV) and square-wave (SWV) voltammetry were treated by using Savitzky and Golay filter (level 2) of the GPES software, followed by the moving average baseline correction with a peak width of 0.01 V.

In all measurements, the reference electrode was an Ag/AgCl (3 M NaCl) (Model RE-1, BAS, USA) and the auxiliary electrode was a platinum wire. The measurements with the GCE and PGE were carried out in a standard 10 mL and homemade 5 mL glass one-compartment voltammetric cell, respectively, at a laboratory temperature (20 °C). For stripping analysis, two cells were used, a pre-concentration cell containing the analyte solution and measurement cells containing a blank background electrolyte. Before use, glass cells were soaked into 3 M HNO_3, and rinsed several times with water and working buffer. If no stated otherwise, the convective transport was provided with a magnetic stirring of 400 rpm.

2.3. Chemicals

DMBA standard and native double-stranded fish sperm DNA (ds-DNA) were kindly provided by Merck and Sigma, respectively. All other reagents were of analytical grade quality. Stock solutions of 4×10^{-3} M (corresponding to *ca.* 1 mg mL^{-1}) DMBA were prepared daily by dissolving appropriate amounts of the compound in dimethylsulphoxide (DMSO). When it was necessary, more dilution of working solutions was prepared with the use of same solvent. CV experiments were recorded in DMSO and ionic strength was maintained at 0.1 M with lithium perchlorate ($LiClO_4$) as the supporting electrolyte. In case of stripping analysis in aqueous solutions, three different supporting electrolytes, namely acetate (0.1 M, pH 4.8), phosphate (0.1 M, pH 3, 7.4 and 9) and Britton–Robinson (0.1 M, pH 6) buffer solutions containing 0.02 M NaCl were used. The supporting electrolyte under voltammetric investigation of the DMBA–DNA interaction was a phosphate buffer solution (0.05 M, pH 7.4) containing 0.02 M NaCl, which served as physiological

medium. The stock solution of the ds-DNA (1 mg mL^{-1}) was prepared in TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.00).

Solutions at all stages of the study were made with deionized water (Milli-Q quality), and were kept in the dark and in refrigerator.

2.4. Procedures

2.4.1. Pretreatment of glassy carbon electrode

Before each measurement, GCE was thoroughly polished with aqueous slurry of alumina powder (φ: 0.01 μm) on a damp smooth polishing cloth (BAS velvet polishing pad), and then was subjected to an electrochemical treatment by keeping it for 60 s at +1.6 V in DMSO/0.1 M $LiClO_4$ mixture.

2.4.2. Fabrication and pretreatment of pencil graphite electrode

The preparation of PGE was carried out as described earlier [45]. In brief, a mechanical pencil Model T 0.5 (Rotring, Germany), was used as a holder for pencil lead (Tombo, Japan), which were purchased from a local bookstore. All leads had a total length of 60 mm and a diameter of 0.5 mm. Electrical contact to the lead was achieved by wrapping a metallic wire around the metallic part of the pencil. A total of 10 mm of lead was immersed in solution per measurement. Such length corresponds to an active electrode area of 15.9 mm^2. Each measurement was performed using a new pencil surface.

To obtain a more sensitive and stable analytical signal, PGE surface was pretreated by applying a potential of +1.4 V for 60 s (in case of cyclic voltammetric studies, +1.6 V for 90 s) in the selected supporting electrolyte without stirring.

2.4.3. Adsorptive stripping voltammetric procedure

For stripping studies, the pre-concentration/medium exchange/voltammetry scheme was adopted. Required aliquot of the DMBA working solutions were placed in a pre-concentration cell containing a buffer solution. The pretreated bare PGE (unless otherwise indicated) was kept in the cell, and the solution was stirred at a chosen accumulation potential throughout the selected the accumulation period. The electrode was then placed in the measurement cell containing the same buffer solution, previously washed with water and with the blank background electrolyte. Subsequently, an anodic sweep was carried out towards more positive potentials using the selected voltammetric technique. The measurements were made at room temperature. The common instrumental parameters for SWV (unless otherwise indicated) were as follows: pulse amplitude, 25 mV; frequency, 50 Hz; step potential, 16 mV; rest period, 10 s; stirring rate, 400 rpm.

2.4.4. Determination in spiked urine samples

Blank urine samples were obtained from a healthy and non-smoking donor (male, age 30 years). An aliquot volume of sample was fortified with DMBA dissolved in DMSO to achieve final concentration of 4×10^{-5} M, and treated with 4.95 mL acetonitrile then the volume was completed to 10 mL with the same urine sample. The samples tubes were vortexed for 1 min and then centrifuged for 10 min at 5000 rpm for removing of unknown endogenous chemicals (an unspiked sample of the same urine was kept as a blank). Appropriate volumes of the supernatant were mixed with the acetate buffer (0.1 M, pH 4.8)/0.02 M NaCl in the voltammetric cell. The pretreated bare PGE was immersed in the solution for 360 s at a fixed potential of +0.60 V using 400 rpm stirring rate (pre-concentration step). The electrode was then washed with water and with the blank acetate buffer (pH 4.8), and transferred into the measurement cell containing the acetate buffer at pH 4.8 (with 0.02 M NaCl). Subsequently, the square-wave voltammogram was recorded from +0.40 to +1.40 V in steps of 16 mV at 50 Hz with a pulse amplitude of 25 mV. Successive measurements were carried out by repeating the above assay protocol on a new PGE surface using a new pre-concentration solution.

2.4.5. Electrode preparation for DMBA-DNA interaction studies

DNA immobilization: The ds-DNA was immobilized onto the pretreated PGE surface by adsorptive accumulation for 300 s at +0.5 V in supporting electrolyte containing 10 μg mL^{-1} of DNA under stirred conditions. The electrode was then gently rinsed with a clean supporting electrolyte for 5 s for removal of the unbound ds-DNA at the electrode surface.

Interaction of surface-confined DNA with DMBA: After the DNA immobilization step described above, the ds-DNA-coated PGE was transferred to the stirred supporting electrolyte containing DMBA concentration of 1 μg mL^{-1} (unless otherwise indicated). The adsorption, at open circuit conditions, was allowed to proceed for 300 s (unless otherwise indicated). The biosensor was then rinsed for 5 s in a clean supporting electrolyte.

Interaction of solution-phase DNA with DMBA: DMBA in the concentration level of 1 μg mL^{-1} was added to supporting electrolyte containing DNA at a concentration of 10 μg mL^{-1}. A PGE was first pretreated as described above and subsequently immersed into the mixture solution. The accumulation of the mixture was performed for 300 s while holding the potential at +0.5 V under stirred conditions. The electrode was then washed with a clean supporting electrolyte for 5 s.

Signal transduction: The oxidation signals of DMBA and adenine were measured by using DPV (amplitude: 50 mV; step potential: 8 mV; scan rate: 16 mV s^{-1}) between +0.45 and +1.40 V in a fresh supporting electrolyte.

Successive measurements were carried out by repeating the above assay protocol on a new PGE surface using a new pre-concentration solution.

3. Results and discussion

3.1. Cyclic voltammetry on the glassy carbon and pencil graphite electrodes in non-aqueous solution

The voltammetric oxidation of 8×10^{-4} M DMBA solution on the GCE and PGE was investigated in DMSO without prior accumulation time. DMBA exhibited one distinct and well-defined oxidation peak (peak I) with a maximum at about +1.15 V and one broad peak (peak II) at more positive potential (*ca.* +1.33 V) at scan rate of 100 mV s^{-1} for both electrodes as depicted by cyclic voltammograms given in Fig. 2A and B. It seems to be relatively high background current of PGE arising from clays and polymers present in pencil graphite. However, its usage was proved to be much more sensitive, yielding current densities (obtained by subtracting the background currents from the recorded currents) *ca.* two times higher than those obtained by using GCE.

The initial oxidation step (peak I) could be due to the one-electron transfer which corresponds to the formation of DMBA radical cation intermediate, benzylic radical. As reported on the literature data [15], the CV of DMBA in dimethylformamide under reversible conditions revealed that the oxidation of this compound involves only one electron. The point concerning the broad peak (peak II), which follows the initial oxidation of DMBA, remained obscure. Bearing in mind the anodic voltammetric behavior of benzo[*a*]pyrene, unsubstituted PAH compound, in non-aqueous solvents which has been previously discussed [46], we suggest that peak II could be attributed to either the oxidation of the product from solvent interaction with the DMBA cation radical or the formation of DMBA dication. No corresponding reduction peaks were observed on scan reversal, indicating irreversibility of

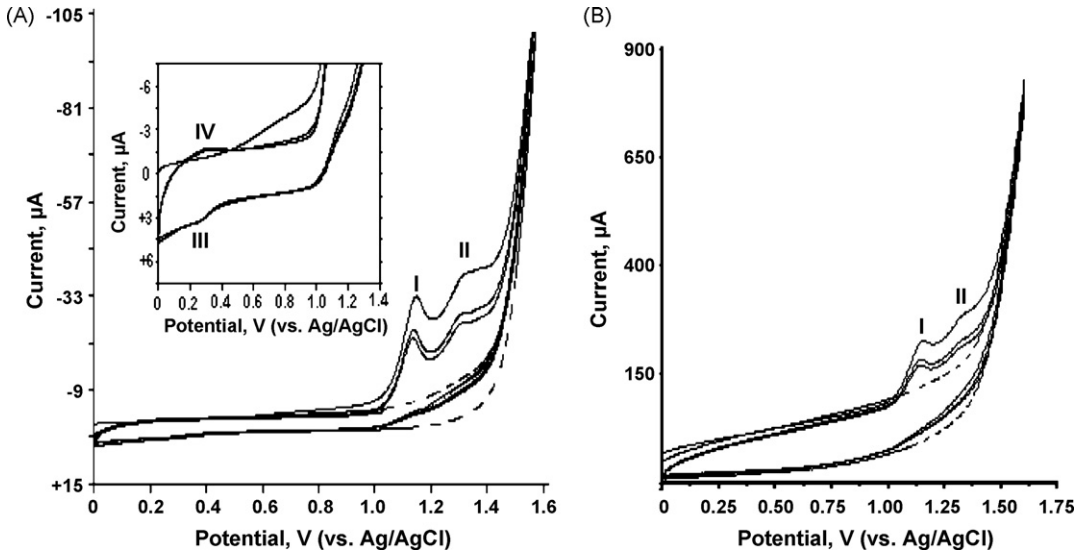

Fig. 2. Repetitive cyclic voltammograms of 8×10^{-4} M DMBA solutions in DMSO for GCE (A) and for PGE (B). Scan rate, 100 mV s^{-1}. Inset: enlargement of reversible redox couple for A. Dashed lines represent background current.

the processes. At lower concentrations of DMBA, these two processes became ill-defined waves, their shape changing from peak to sigmoidal. For the reverse scan and in subsequent scans, a new reversible redox couple producing a reduction (wave III) and re-oxidation (wave IV) wave, appeared in considerably lower positive potential region (Fig. 2A, see inset: enlargement of reversible redox couple). However, this couple was poorly defined in DMSO, where the product solubility may be low. The appearance of these closely spaced waves supported that a chemical reaction subsequent to the electron step occurs, giving rise to species more readily oxidized than DMBA. If successive scans are made, it can be observed that the oxidation process at more positive potentials decreases as the number of scans increase, while the intensity of this couple increases slightly, which confirms this couple arises from the product of follow-up chemical reaction of the DMBA oxidation. Such behavior was also observed and became more pronounced for the oxidation of benzo[*a*]pyrene in the same solvent (our unpublished results will be reported elsewhere soon).

Since the initial oxidation step was more intense, it was selected for analysis purposes. When the scan rate was varied from 5 to 1000 mV s^{-1} at GCE, a 160-mV positive shift in the peak potential was observed as expected for an irreversible oxidation with the simultaneous increase in the peak current. Plotting the logarithm of peak current versus logarithm of scan rate gave up a straight line (correlation coefficient 0.997) with a slope of 0.51. The slope means that process is predominantly controlled by diffusion, although partially coupled to weak adsorption in the whole scan rate range studied. Furthermore, a linear dependence of the peak current upon the square root of the scan rate (correlation coefficient 0.994) was found, demonstrating a diffusional behavior.

3.2. Stripping analysis on the pencil graphite electrode in aqueous solution

Under the voltammetric investigation leading to analytical methodologies, we planned to study in aqueous samples, not only to alleviate the need for a high volume of organic solvents but in order to make possible the application of proposed method in PAHs-contaminated aquatic environments. For this reason the diluted aqueous solutions of DMBA ($\leq 4 \times 10^{-5}$ M) were used due to its low solubility in water. In order to confirm the adsorption of DMBA on working electrodes, preliminary medium exchange experiences were then performed. The PGE electrode was kept in contact with a quiescent solution of assayed concentration of DMBA in background electrolyte for a fixed accumulation time under open circuit. Then it was transferred to another cell containing only the clean electrolyte and the voltammogram was recorded. On the contrary, when the experiment was repeated maintaining the electrode in a stirred solution of DMBA, the voltammogram recorded presented higher peak current. These facts indicate that DMBA undergoes an adsorptive pre-concentration on the electrode surface. Adsorptive stripping voltammetric (AdSV) behavior of DMBA was studied by CV, DPV and SWV techniques after medium exchange.

At first, basic voltammetric behavior of DMBA in aqueous solutions was investigated using CV measurements. Before the measurements, 4×10^{-5} M DMBA in stirred various types of electrolyte solutions (all in 0.1 M containing 0.02 M NaCl), including acetate, phosphate and Britton–Robinson buffers over the pH range of 3–9 was accumulated on the working electrodes (PGE or GCE) using a 300-s open circuit accumulation. Cyclic voltammograms were recorded in the potential range of 0 V to +1.6 V at scan rate of 100 mV s^{-1}. In all of the investigated pH values, two anodic peaks observed in concentrated non-aqueous solutions merged into a single one (Fig. 3A). No peaks were observed in the cathodic scan which indicated that the process was irreversible. Since the DMBA concentration was lower, redox couple could not also be detected. Cyclic voltammogram obtained using the GCE exhibited a similar behavior at comparable potentials; however the peak separation from the background was not better, giving rise to inaccurate measurements of the currents. Furthermore, the GCE severed surface contamination, and the single-use PGE was much superior. For this reason, further electroanalytical experiments were realized using PGE.

The attention was then turned to the effect of solution condition such as pH, which is a critical factor affecting both the rate and equilibrium state of the accumulation process and the rate of the electrode reaction. Due to the poorly resolved stripping signals obtained by CV, the effect of solution acidity on oxidation process was studied using DPV and SWV. Voltammograms were carried out in stirred 4×10^{-5} M DMBA solution with an open circuit mode at 300 s. In application of both DPV and SWV, the appearance of two distinct anodic peaks, together with the improvement of their shapes could be seen between pH 3–7.4. Fig. 3B obtained by applying square-wave scan is representative. At pH 9.0, the second peak

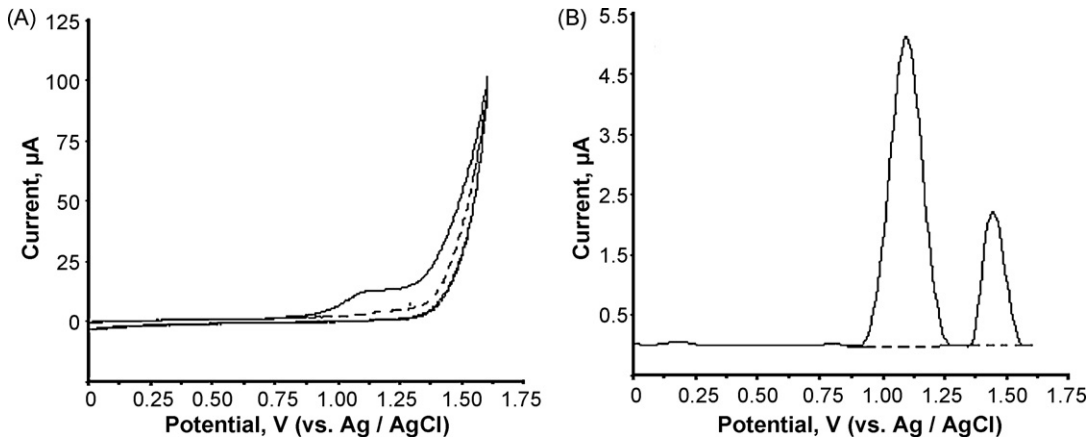

Fig. 3. Cyclic (A) and square-wave (B) voltammograms of 4×10^{-5} M DMBA solution in acetate buffer pH 4.80. CV conditions: scan rate, 100 mV s^{-1}; SWV conditions: pulse amplitude, 20 mV, frequency, 25 Hz, step potential, 8 mV. Dashed lines represent background current.

was not extracted from the background discharge that occurred at less positive potential. The pH did not indicate a significant change in the peak potential over the range studied. For the first peak, a 55- and 70-mV negative shift was only observed upon increasing the pH from 3 to 9 using DPV and SWV, respectively. For the first oxidation step, the peak intensities reached a maximum in acetate buffer pH 4.8. Moreover, the application of square-wave mode for the stripping of adsorbed DMBA was proved to be much more sensitive, yielding signals *ca.* 2.5 times higher than those obtained by applying differential pulse scan. The second oxidation step exhibited a similar behavior however the peak currents were smaller than those of the first one. For remainder of the work, SWV technique was applied in acetate buffer at pH 4.8 by considering the primary oxidation peak.

Further optimization of the analytical signal focused on studying the effect of pre-concentration/stripping conditions, such as accumulation potential and time. The influence of the accumulation potential either at open circuit potential or at a potential range from +0.1 V to +0.8 V was studied with accumulation time of 300 s in stirred 4×10^{-5} M DMBA solution. The value for the stripping current obtained at +0.1 V was nearly equal to the value obtained at open circuit voltage. The maximum peak current was obtained at an accumulation potential of +0.6 V because of an increased accumulation rate, due to more favorable alignment of the molecule by the electric field at the electrode–solution interface. After fixing the accumulation potential at this value, the deposition time was varied between 30 and 480 s. The increasing the peak currents were obtained at a fast rate up to about 360 s and starting to level off for longer times above this value. The peak potentials undergo a small displacement towards more negative potentials; this indicates the need of a larger energy to attain the stripping of the adsorbate from the electrode. For further analytical studies, the deposition stage was carried out at +0.6 V for a pre-concentration time of 360 s.

Square-wave stripping voltammograms at different concentrations of DMBA were recorded under the maximum current experimental conditions. The use of DMBA oxidation at about +1.15 V as the first analytical peak allowed quantification in the range of 2 nM to 10 nM with a regression equation of i_p (μA) = 0.217 C (nM) − 0.134 (correlation coefficient, 0.992). A detection limit of 0.194 nM was estimated based on signal-to-noise characteristics of the data for S/N = 3. The repeatability of the stripping signal was realized in terms of relative standard deviation for ten identical measurements carried out at a concentration level of 6 nM DMBA of a new PGE surface using a new solution in the cell and found to be 5.1%. A variation of the DMBA concentration was found to have a slight influence on the peak potential. The peak potential shifted towards more negative values when the concentration was increased.

The selectivity of such determination could be limited by interactions of structurally similar compounds with DMBA. However, this is not a case of samples with one class of compounds or one analyte present.

Since DMBA solutions undergo photooxidation in air and light, they were freshly prepared and kept in dark-colored, tightly closed containers. However, to check its stability, the voltammograms of stock solutions in DMSO stored in the dark and in refrigerator were recorded after two-week periods. When the voltammograms were compared with those of samples freshly prepared they did not show any appreciable change, demonstrating that DMBA solutions were stable for this period. Taking into account that the stability of aqueous solutions containing PAHs is a major problem due to their tendency to be absorbed on surfaces of polyethylene containers [47], all solutions were stored in glass vials. In daily laboratory practice, no significant decrease was evident among the calibration samples throughout the study period.

In view of the sensitivity (extremely low detection limit) of the proposed method, PGE electrode was applied to the determination of DMBA in simulated human urine.

The presence of proteins, major components of the sample matrix which could be adsorbed on the electrode surface together with analyte is an important drawback of adsorptive voltammetric techniques. In case of our study, when the voltammograms were recorded in 1:100 blank urine dilutions smooth curves were not obtained in the potential range from +0.4 to +1.4 V comparing to those of the background electrolyte. To obviate this problem, the extraction with acetonitrile followed by centrifugation protocol, that was quick and easy, was applied together with a medium exchange procedure. After medium exchange technique (analogous to solid phase extraction methods), none of the potentially interfering electroactive urine components contributed to the response and DMBA could easily be measured as shown in Fig. 4.

In order to check if the linearity persists, a new calibration plot was carried out in urine samples. In this case, the voltammetric response measured in urine was linearly related to the concentration of DMBA within the range of 0.2–0.8 μM according to the equation: i_p (μA) = 2.39 C (μM) + 0.025 (correlation coefficient, 0.999) with a detection limit of 0.04 μM. Under these conditions a decrease in the signal was observed comparing to that obtained in aqueous solution without urine. The matrix of the sample did not reduced the reproducibility of the signal as demonstrated by recording three voltammograms for each of the urine samples containing DMBA in the concentration range mentioned above. In

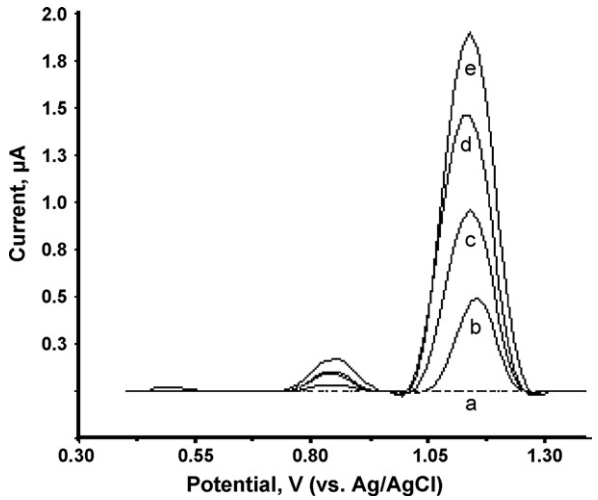

Fig. 4. Square-wave voltammograms obtained for the determination of DMBA in spiked urine. Blank urine (a), urine spiked at a DMBA level of 0.2 μM (b), 0.4 μM (c), 0.6 μM (d), 0.8 μM (e). For operating conditions, see Section 2.4.4.

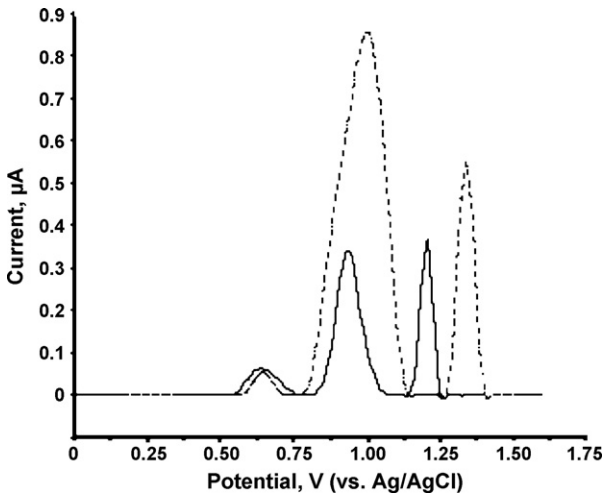

Fig. 5. Differential pulse voltammograms for 4×10^{-5} M (10.3 μg mL^{-1}) DMBA (dashed line) and 10 μg mL^{-1} DNA (solid line). Supporting electrolyte, phosphate buffer pH 7.40.

urine, results were characterized by higher precision comparing to those obtained in aqueous solutions, probably due to the fact that concentration in urine samples was relatively higher. An average relative standard deviation was found to be 2.9%. There was no degradation of the DMBA in solution during the experiments.

3.3. Interaction with DNA on the pencil graphite electrode

Before interaction differential pulse voltammograms for DNA and DMBA in phosphate buffer (pH 7.40) are shown in Fig. 5. Under the conditions of the experiment, the redox behavior of original ds-DNA immobilized PGE exhibited two oxidation processes of guanine (G, *ca.* +0.93 V) and adenine (A, *ca.* +1.20 V) residues. DMBA produced two oxidation peaks at around +1.05 and +1.40 V with a pre-concentration step on PGE at open circuit condition for 300 s in a stirred supporting electrolyte (explained above). Guanine and DMBA (first peak) oxidation peaks coincided with each other, whereas separation of adenine and DMBA (first and second peaks) oxidation peaks was possible even if at high concentration of DMBA. That is why we could compare only adenine oxidation peak changes.

Fig. 6 displays the DPV signals, which were monitored in blank supporting electrolyte, obtained before/after interaction of 1 μg mL^{-1} DMBA with 10 μg mL^{-1} ds-DNA both at the electrode surface and in solution. In the case of both interaction protocols (following the association with DMBA), a considerable diminution in peak current was observed.

It is known that planar condensed aromatic ring systems play a major role in their intercalative DNA binding, primarily involving stacking interactions. Binding of the intercalators to DNA results in a significant change in DNA conformation, and can be followed by electrochemical methods [48,49]. On the other hand, it is reported the synthesis of two detectable DNA adducts by the electrochemical oxidation of DMBA in the presence of dA. A covalent bond is formed specifically at the 12-CH_3 and 7-CH_3 group of DMBA for the N-7 and N-3 position of adenine, respectively [15]. Taking into account these findings, the decrease in the adenine signal could be attributed to either the penetration into the DNA double-helix between adjacent base pairs (non-covalent interaction) or forming adducts through covalently binding to sites in DNA.

The reproducibility of DNA biosensor protocol was also evaluated from three repetitive measurements for the concentration 10 μg mL^{-1} DNA and 1 μg mL^{-1} DMBA. In the absence of DMBA, measurements yielded a reproducible result with a mean peak height of 374 nA and a relative standard deviation of 3.74%, indicating that the preparation of working electrode was satisfactory. After interaction of DMBA at electrode surface or in solution, the series resulted in mean peak heights of 166 and 159 nA and relative standard deviations of 4.86 and 13.46%, respectively. Following a 5 min accumulation, the detection limits of DMBA, which were esti-

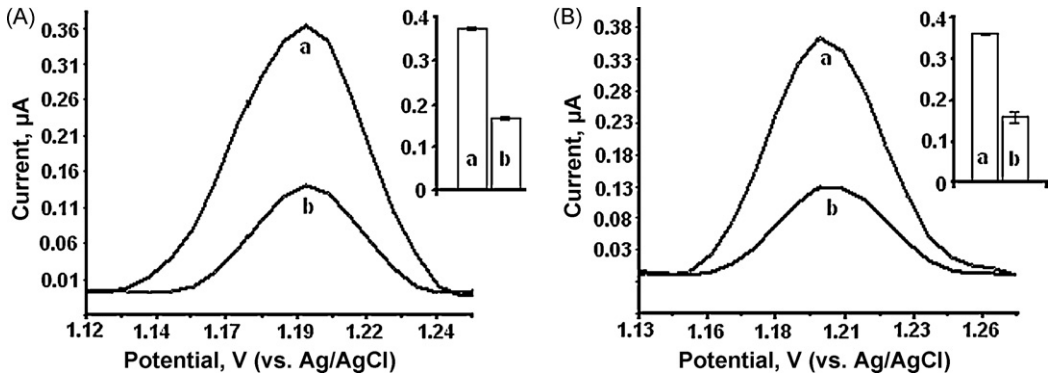

Fig. 6. Differential pulse voltammograms for 0 (a) and 1 μg mL^{-1} (b) DMBA after the interaction of surface-confined DNA (A) and solution-phase DNA (B) at a 5-min accumulation. Inset: the results were also demonstrated with bar charts. For operating conditions, see Section 2.4.5.

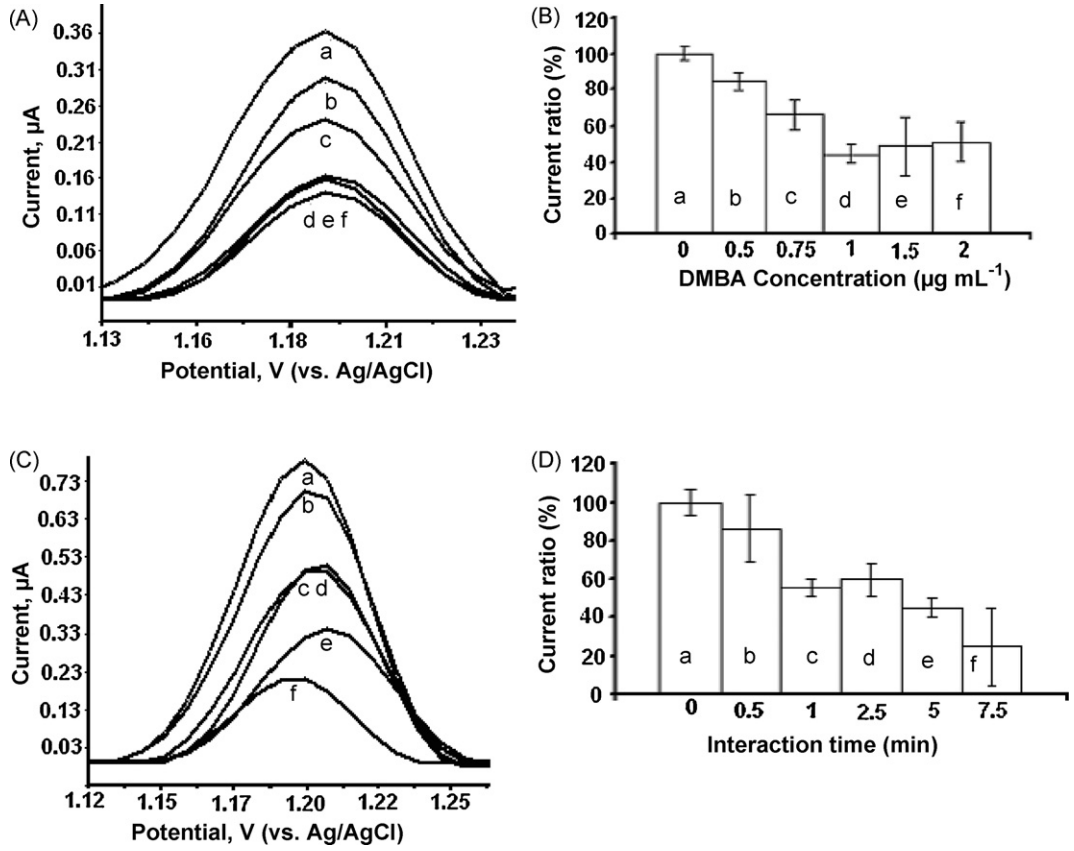

Fig. 7. The effect of DMBA concentration (A) and accumulation time (C) upon the DPV response of adenine after interaction of surface-confined DNA for 5 min (A) and with 1 μg mL^{-1} DMBA (C). The results were also shown as the peak current ratio (B and D). For operating conditions, see Section 2.4.5.

mated from the signal-to-noise characteristics of the data (S/N = 3), were found as 11.8 and 11.9 ng mL^{-1} (corresponding to *ca.* 46 nM) for interaction of surface-confined and solution-phase ds-DNA, respectively. Bearing in mind that the size (ring number), and conformation of the molecule play a major role in its DNA binding [40,50], four-ring structure of DMBA may account for its higher sensitivity. Such detection limits are low enough for assays of polluted environmental samples. The results obtained by the interaction in solution phase were found to be less suitable for this study because of the relatively poor reproducibility. Thus, the assay protocol at the electrode surface was chosen for remaining parts.

The dependence of DMBA concentration (in the range 0–2 μg mL^{-1}) on the adenine signal was shown in Fig. 7A and B. In the presence of DMBA, adenine oxidation peaks decreased linearly with concentration up to 1 μg mL^{-1} according to the equation: i_p (μA) = −0.294 C (μg mL^{-1}) + 0.465 (correlation coefficient, 0.999), and almost leveled off for more concentrated solutions. In repetitive measurements, the relative standard deviation values of DMBA measurements did not exceed 8% in the range 0.5–1 μg mL^{-1}, whereas the higher values (above 10%) occurred at the concentration above the linearity of calibration curve. Since the linear range looks shorter, the improvement in application of assay protocol would be resulting in relatively wide range of DMBA concentration.

Fig. 7C and D displays the influence of DMBA interaction time (in the range 0–7.5 min) at ds-DNA immobilized PGE surface upon the changes in the adenine signal. There was a decrease on adenine signal till 1 min. The response remained nearly constant from 1 min to 2.5 min then decreased again. The time-dependent profile which differs from compound to compound is due to the binding kinetics of the analyte-DNA interaction [49]. The optimum time for interaction of DMBA with ds-DNA was found as 5 min, since the changes at the adenine peak before/after the interaction with DMBA could clearly be observed following this accumulation time.

4. Conclusion

In the present study, the DMBA molecule was chosen for two specific reasons. First, it is a highly interesting and valuable tool in investigations of bioactivation processes as they relate to the etiology of several important pathological conditions. Second, it is a synthetic model PAH compound of controlled laboratory environment. Thus, the present observations dealing with voltammetric studies on DMBA may be similar to the finding of other large hydrocarbon systems.

We note that elucidating the mechanism of DMBA electrochemical oxidation is not an easy task and is beyond the scope of the present work. It only shows how the compound is oxidized in aqueous and non-aqueous media at carbon-based electrodes. Although the sufficient data are not available to determine the exact oxidation mechanism, it is hoped that knowledge of the voltammetric oxidation behavior of DMBA would help with a better understanding of the carcinogenic and oxidative pathways of PAHs in their metabolism and provide a more comprehensive picture.

The anodic voltammetry of aromatic hydrocarbons has been studied in great detail in acetonitrile, dimethylformamide, methylene chloride, propylene carbonate and nitrobenzene. The stability of the intermediates formed is affected markedly by the solvent, undergoing a series of chemical and additional electrochemical steps. In case of our study, DMSO was selected as the solvent. For medicine purposes, DMSO has proved a popular choice due to its suitability as fatty emulsions for the intravenous administration of

DMBA, maintaining the stability [51]. This encouraged its use in a clinical measurement sensor for the animal assays in future.

GCE is the most common carbon-based electrode in current use. However, anodic voltammetry at GCE is more frequently used for research purposes than for routine analytical determinations because of its passivation which can decrease of the sensitivity. Although disposable PGE is relatively new type of carbon electrode, it has become of interest in the field of sensitive and simple monitoring of toxic compounds including carcinogenic compounds by virtue of its high electrochemical reactivity, good mechanical rigidity, low-cost, low technology and ease of modification. In the environmental sense, disposable PGE in combination with field-based experiments can be much more attractive than the electrodes commonly used in the laboratory, i.e. GCE, because of the avoiding the need for extensive time delays, and considerable risk of contamination or loss of analyte of interest [52]. In this study, the PGE combined with more highly sensitive and accurate AdSV could allowed to an attractive trace analysis of DMBA from the aqueous solutions with the detection limit that reached the level as low as 0.194 nM (49.7 ng L^{-1}). This value is comparable to those obtained by commonly used chromatographic techniques for environmental carcinogens [53]. Therefore this technique could not only be used for monitoring the efficiency of destruction of DMBA in laboratory wastes, but serve as a model for the determination of trace risk chemicals in contaminated aquatic environments [54].

Following PAHs exposure, most of the absorbed compounds via different exposure pathways, i.e. inhalation, dermal contact, and ingestion of contaminated food are metabolized and eliminated in urine as phenols [55] while only a smaller amount is excreted unchanged in urine [56]. However, measurement of unmetabolized PAHs, instead of metabolites, in urine can be very useful for several reasons: their elimination is directly associated to previous exposure; they are less susceptible to intra-individual variability; they are characterized by a high degree of specificity [57]. With this aim, the possibility of measuring the DMBA in simulated human urine was also demonstrated. Owing to the complexity of the biological samples in which chemical carcinogens are sought, conventional biomonitoring methods are often time-consuming, labor intensive and expensive. They often incorporate a preliminary separation, involving extraction, filtration, fractionation, drying of extracts, redissolution and other steps. Using proposed technique, no sample pretreatment was required, other than precipitation in the presence of *ca.* 50% acetonitrile and dilution steps. Solvent addition is expected to increase DMBA solubility and reduce its absorption on container walls as well as to eliminate the influence of proteins. The performance of the stripping step in clean electrolyte was very convenient from the point of eliminating possible interferences with similar redox potential (not or slightly adsorbed) in urine. However, its detection limit found urine samples (0.04 μM corresponding to 10.3 μg L^{-1}) was not low enough to reach the levels expected in urine after the exposure to PAHs through especially multiple media [55]. We plan to undertake the investigations to improve the sensitivity together with the selectivity by combining HPLC with proposed adsorptive stripping technique at PGE.

In this study it was also presented for the first time a new screening tool for DMBA. Such use of DNA interaction as a basis for electrochemical biosensor results in a simple, cost effective, rapid, reliable and sensitive detection of DMBA. This promising approach might be a key step not only to detect very low concentrations of DMBA, but also to clarify its reaction mechanism concerning its role in the tumor formation.

Due to the fact that PAHs are very hydrophobic, they tend to be stored in fat tissue, liver and kidney (common target organs) [51]. Considering this knowledge, the preliminary investigation (experiments in vitro) was performed in these tissues of the rats which were first treated with DMBA dissolved in olive oil as orally, and then followed for tumor production. The use of proposed method represented satisfactory results giving well-defined voltammetric responses in the tissue extracts. Studies on laboratory animals are continuing in the development of these measurements.

Acknowledgements

This work was supported by the Yüzüncü Yıl University Scientific Research Foundation (Project no. 2007-FED-B44). It is produced from PhD thesis of Yavuz Yardım and Ertuğrul Keskin (Yüzüncü Yıl University, Institute of Natural and Applied Sciences). The authors thank to Gökhan Oto, PhD (Yüzüncü Yıl University Faculty of Medicine) for his kind help on partially supplying DMBA standard sample.

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