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Sequential-injection stripping analysis of nifuroxime using DNA-modified glassy carbon electrodes

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Abstract

The voltammetric behavior of nifuroxime was investigated comparing stationary voltammetric methods with the recently proposed sequential-injection stripping analysis (SISA), by using cyclic voltammetry (CV) and differential-pulse voltammetry at bare and DNA-modified glassy carbon (GC) electrodes. In cyclic voltammetry, reduction of nifuroxime at DNA-modified electrodes gives rise to a well-defined peak, and in contrast to bare GC surfaces, a re-oxidation peak could be observed. Optimization of the pre-concentration process at the DNA-modified surface led to a significant enhancement of the voltammetric current response, a better defined peak shape and an improved dynamic range. Based on this optimized voltammetric procedure, SISA has been evaluated for the determination of nifuroxime. The flow-system significantly facilitates the regeneration of the DNA-modified electrode surface, hence diminishing problems related to accumulation and memory effects. The linear detection range could be extended to 65 μM with a detection limit (3 s) of 0.68 μM, which corresponds to an absolute amount of 21 ng nifuroxime.

Keywords: Nifuroxime; Modified electrode; Sequential-injection stripping analysis; DNA-modified electrode

1. Introduction

In recent years, there has been a considerable interest in developing electrochemical sensors for detection of DNA, and in using DNA as surface-modification element in electrochemical biosensors [1,2]. DNA-based sensors have a variety of possible applications, e.g. for the detection of the presence of genes associated with human diseases [3], or as electrode surface modifiers for the detection of the intercalation properties of transition-metal complexes [4,5]. The possibly most important application of DNA biosensors is related to the determination of a variety of analytes (e.g. carcinogens, drugs, pollutants, etc.) interacting with the structure of DNA thus leading to a modulation of the sensor properties. If the analyte compound itself is electroactive, it can be directly detected using voltammetric methods [6,7]. However, even electrochemically inert compounds may be detected via changes in the electrochemical signal related to the DNA's redox reactions [8,9].

Nifuroxime (5-nitro-2-furancarboxaldehyde oxime) belongs to a group of nitrofurans, which is used as antimicrobial and as cellular-sensitizing drug in therapeutics [10,11]. Different analytical methods have been applied for the determination of nifuroxime in its pharmaceutical preparations. These methods include high performance liquid chromatography (HPLC) [12], thin layer chromatography [13], colorimetry [14], spectrophotometric methods [15], or electron spin resonance (ESR) [16].

The electrochemical properties of nifuroxime have been reported [17] and it is known that the biological activity of nitrofurans is derived from the reductive metabolism of the nitro group, a process catalyzed by a variety of nitroreductase activities [18]. Although the mechanism of the reaction between the enzyme-generated reduction product and DNA is not completely elucidated, it has been proposed that the nitro group is primarily reduced to a hydroxylamine intermediate which can then either bind to DNA nucleotides forming guanine or cytosine adducts [19], or interact with DNA causing a lesion characterized by destabilization and damage of the double helix [20].

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Modification of the electrode surface with an immobilized layer of DNA was successfully used for the accumulation of drugs [7], therefore in this communication, we aim at the elucidation of the voltammetric behavior of nifuroxime in aqueous solution at bare glassy carbon (GC) and DNA-modified glassy carbon electrodes by using stationary stripping voltammetry on the one hand, and the automation of the determination of nifuroxime using sequential-injection stripping analysis (SISA) on the other hand. SISA was recently introduced for determination of traces of mercury in water samples [21]. It is expected that SISA will be employed for the facilitated and highly sensitive determination of nifuroxime.

2. Experimental

2.1. Chemicals

Nifuroxime was purchased from Aldrich Chemical (Milwaukee, USA). Calf thymus DNA (sodium salt, type 1) was obtained from Sigma-Aldrich Chemie (Steinheim, Germany) and was used as received without further purification. Acetate buffer (0.2 M) pH 4.5 was used to prepare and condition the DNA-modified electrode, 0.1 M phosphate buffer solution (PBS) pH 7.2 was used as electrolyte in all experiments.

Single-stranded DNA (ssDNA) solution was prepared by dissolving 4 mg of double-stranded DNA (dsDNA) in 0.5 ml of 70% perchloric acid. The acid was neutralized with 0.5 ml of 9 M sodium hydroxide solution followed by an addition of 9 ml of acetate buffer pH 4.5 [8]. All solutions were prepared using analytical grade reagents and triply distilled water from a quartz distill.

2.2. Electrode modification

Glassy carbon (GC) electrodes (0.07 cm^2) were polished with 3-, 1-, and 0.3-μm alumina suspension to obtain a mirror-like, shiny electrode surface, washed with water and sonicated in an ultrasonic bath for 3 min to remove residual alumina particles. The DNA-modified GC electrode was prepared following a procedure described by Brett et al. [8] with minor variations; a thinner DNA layer was formed on the electrode surface, using a 50% decreased amount of DNA as reported before [8]. In short, 3 mg of dsDNA were dissolved in 160 μl of acetate buffer solution. 80 μl of this solution was placed onto the GC electrode surfaces using a pipette and allowed to dry. The electrode was then integrated into a three-electrode electrochemical cell using acetate buffer as electrolyte. The electrode potential was scanned following a differential pulse voltammetry profile from -0.9 to $+1.4$ V at a scan rate of 10 mV s^{-1} and a pulse height of 50 mV. After this, the electrode was poised to a constant potential of $+1.4$ V for 5 min. It was then transferred to a freshly prepared solution of ssDNA for conditioning, where differential pulse voltammograms were recorded in the potential range from 0 to $+1.4$ V followed by keeping a constant potential of $+1.4$ V for 2 min under constant stirring. The conditioning was stopped after stabilization of the peak currents corresponding to the adenine and the guanine oxidation reaction. The electrode was then transferred into acetate buffer solution for 2 min, then removed and left to dry. This procedure led to the formation of a relatively thick DNA layer with good conductivity on the electrode surface [22].

2.3. Apparatus and electrochemical measurements

Electrochemical measurements were performed with a Polarographic Analyzer/Stripping Voltammeter Model 264 A from EG&G Princeton Applied Research (Bad Wildbad, Germany) connected to an X–Y recorder BD 90 (Kipp and Zonen, Netherlands).

All stationary voltammetric measurements and electrode conditioning were performed in a one compartment cell containing the GC or DNA-modified GC electrode as working electrode, a Pt wire auxiliary electrode, and a Ag/AgCl/(3 M KCl) reference electrode. Cyclic voltammetry (CV) was performed in 0.1 M phosphate buffer pH 7.2, scan rate 100 mV s^{-1}. Differential pulse voltammetry was performed at a scan rate 10 mV s^{-1} and a pulse height of 50 mV.

For sequential-injection stripping analysis (SISA) measurements, we used the On-Line General Analyzer (OLGA) [23] developed in our laboratory. The system consists of a high precision micro pump (Tecuria, Chur) that has the ability to pump liquid segments down to a volume of 1 μl but also to pump continuously forward and backward with flow rates up to 50 μl s^{-1}; a polyacryl injection manifold with six equivalent inlet/outlet channels connected to a central channel (IBA, Göttingen); magnetic snap valves (Sirai, Hannover) which can be individually closed or opened; and a flow-through electrochemical cell specifically designed for the integration of stick-type GC electrodes. The pump and the snap valves are controlled by a modular software package developed using VisualBasic 3.0 (Microsoft, Redmond). Bare GC or DNA-modified GC electrodes were used as working electrodes, Ag/AgCl/(3 M KCl) as reference electrode and Pt wire as counter electrode.

3. Results and discussion

3.1. Behavior of nifuroxime at bare GC and DNA-modified GC electrodes

The voltammetric behavior of the nitrofurans has been described taking into account the rather complex mechanism of their electrochemical reduction due to the theoretical capability of the nitro group to accepting up to six electrons under formation of the corresponding amine [17]. Generally,

two reduction waves were observed in aqueous acidic media. The first wave, corresponding to the reduction of the nitro group to form the intermediate hydroxylamine involves four electrons, and the second wave corresponding to the reduction of the hydroxylamine to the related amine involves two electrons. For some nitrofurans however, only one wave was observed in neutral to alkaline media corresponding to a one-step six-electron transfer process.

Fig. 1 shows the cyclic voltammograms of 0.3 mM nifuroxime in PBS pH 7.2 at unmodified and DNA-modified GC electrodes. Although a better peak definition can be obtained at more acidic pH-values PBS at pH 7.2 was chosen as a model for physiological media. Only one reduction wave was observed for nifuroxime which corresponds with previous observations that nofuroxime and nifuroxazide [17,24], both members of the nitrofuran family, show only one reduction wave which is most probably due to the reduction of the nitro group to the corresponding hydroxylamine under transfer of four electrons as shown in Eq. (1).

$$RNO_2 \xrightarrow[-H_2O]{4e^- + 4H^+} RHNOH. \quad (1)$$

As compared to the voltammogram obtained at a bare GC electrode (curve B), the DNA-modified GC electrode (curve A) showed a shift of the nitro group reduction wave to more negative potentials (about 40 mV). However, the observed current response was significantly higher and the shape of the peak was much better defined. The increased current is ascribed to the interaction of nifuroxime with the immobilized DNA on the electrode surface. In addition, during the positive half-scan, an oxidation peak appeared which was also seen in similar applications of DNA-modified GC for the analysis of the nitroimidazoles [6]. This wave was ascribed to the interaction of the reduction products of the nitroimidazoles with the DNA backbone on the electrode surface. Thus, it can be assumed that also the formed nifuroxime reduction product interacts with the DNA backbone on the electrode.

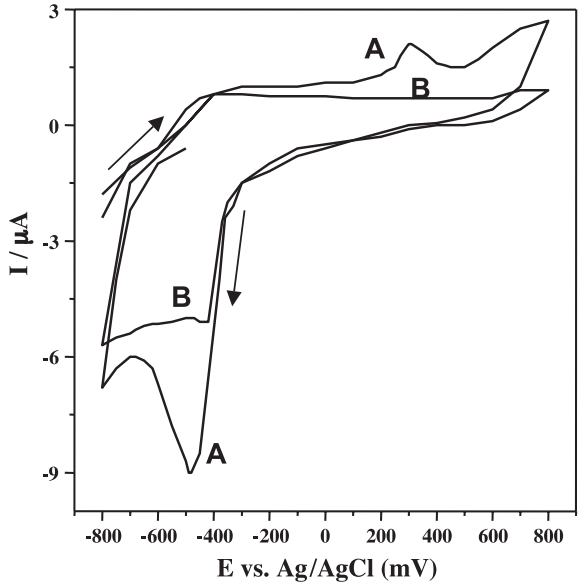

Fig. 1. Cyclic voltammograms of 0.3 mM nifuroxime in 0.1 M PBS (pH 7.2) at: (A) DNA-modified GC electrode and (B) bare GC electrode (scan rate 100 mV s^{-1}).

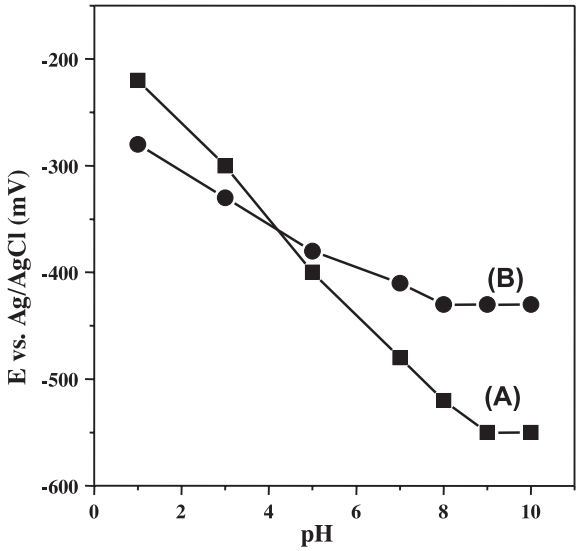

Fig. 2. Plot of the nifuroxime reduction potential E vs. pH for 0.3 mM of nifuroxime solution at: (A) DNA-modified GC electrode and (B) bare GC electrode.

3.2. Effect of pH on the reduction-wave potential of nifuroxime

Like the other nitro-aromatic compounds, the reduction-wave potential for the nitro group of nifuroxime depends on the pH of the buffer solution. The shift in reduction potential with pH was observed when both bare GC and DNA-modified GC were used as working electrodes. Plots of the reduction-wave potential against the pH-value for nifuroxime at both the DNA-modified GC and bare GC are shown in Fig. 2. A shift of the reduction potential to more negative values was observed as the pH-value increases, this shift has been attributed to an acid–base pre-equilibrium followed by the first electron transfer to form the nitro radical [25,26]. Also, Fig. 2 shows that in alkaline media the reduction wave of nifuroxime (bare GC at pH> 8, and for DNA-modified GC at pH> 9) is independent of pH, which agreed with the previous studies using mercury and GC electrodes for the reduction of nitro groups of metronidazole [7,27].

3.3. Reduction of nifuroxime using differential pulse voltammetry

Differential pulse voltammetry was used to investigate the reduction of nifuroxime in more detail. The interaction between the DNA backbone on the modified electrode and nifuroxime allows for the pre-concentration of nifuroxime or

its immediate reduction products on the electrode surface, which subsequently leads to a much lower detection limit than the one obtained at the unmodified electrode. The pre-concentration time was optimized with respect to maximum sensitivity and a pre-concentration time of 1 min was found to be optimal. No further improvement was achieved with shorter and longer pre-concentration times. However, using long pre-concentration times caused problems due to the memory effect at the DNA-modified electrode that occurs from the accumulation of nifuroxime and/or its intermediate at the electrode surface during the adsorptive stripping method. Therefore, washing of the electrode at anodic potentials can remove the un-reacted adsorbed analyte. The unpredictable adsorption of reaction product on the DNA-modified GC surface also implies the necessity to regenerate the electrode after each measurement. The regeneration was achieved by intensive washing of the electrode with buffer solution. It is well known that DNA can strongly and irreversibly adsorb on GC electrodes if the modified surface was allowed to get dry. Under these circumstances, the adsorbed DNA layer on GC electrodes is stable even in the presence of 1 M HCl solution [28]. The success of the regeneration process was checked by means of cyclic voltammetry in buffer solution. In order to minimize the impact of this memory effect on the sensor response and concomitantly to reduce the time needed for the sensor regeneration, we have chosen only 30 s as pre-concentration time instead of the optimal 1 min in all further measurements.

Apart from the ability of the modified electrode to pre-concentrate nifuroxime, a better peak shape was obtained at the DNA-modified GC electrode as compared to the unmodified GC electrode as shown in Fig. 3. The problems of

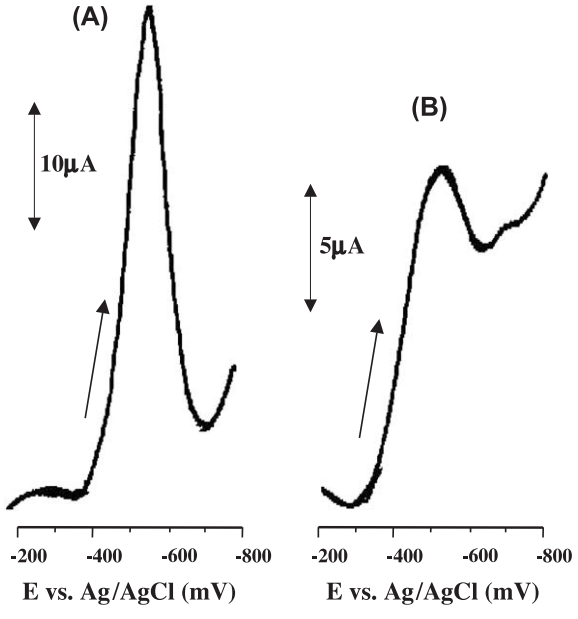

Fig. 3. Differential pulse voltammograms for the reduction of 25 μM of nifuroxime at: (A) DNA-modified GC electrode and (B) bare GC electrode in 0.1 M PBS (pH 7.2) (scan rate: 10 mV s^{-1}; pulse amplitude: 50 mV).

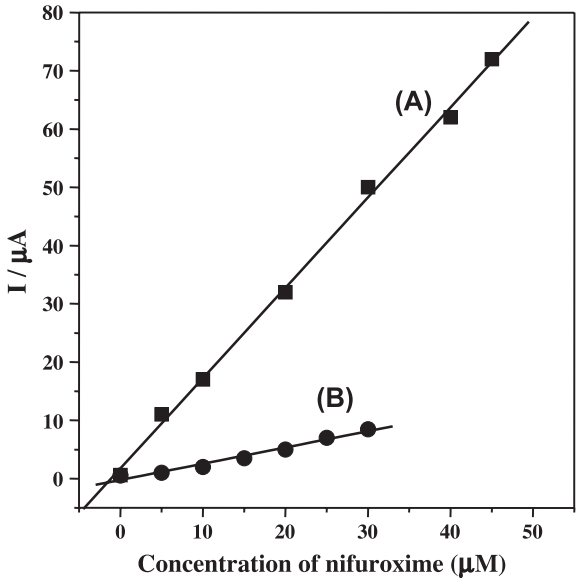

Fig. 4. Calibration curves for nifuroxime reduction at: (A) a DNA-modified GC electrode with 0.5 min pre-concentration time and (B) a bare GC electrode with a 1-min pre-concentration time in 0.1 M PBS (pH 7.2).

accumulation and memory effects were eliminated by using sequential-injection stripping analysis (SISA), which proofed to be a more easy and more practical technique to regenerate the electrode as will be shown in the next section.

Calibration curves were derived from the reduction currents of nifuroxime at the DNA-modified GC and at bare GC (Fig. 4). The plots clearly indicate that the DNA-modified GC electrodes exhibit a better sensitivity and longer dynamic range than the unmodified GC electrode. The detection limit obtained at the DNA-modified electrode was 8.6×10^{-7} mol l^{-1} in contrast to a detection limit of 7.5×10^{-6} mol l^{-1} obtained at the unmodified electrode in the differential pulse voltammetry mode as calculated from three times the noise level of the background electrolyte.

3.4. Oxidation of nifuroxime at DNA-modified GC electrodes

In addition to the reduction, also the anodic wave (curve A in Fig. 1) observed in the cyclic voltammogram of nifuroxime at DNA-modified electrodes could be used to obtain quantitative information about nifuroxime concentrations as seen in Table 1. As a matter of fact, this is only possible with the DNA-modified electrode because of the interaction of the reduction product of nifuroxime with the DNA backbone immobilized on the electrode. The pre-concentration potential (E_p) also has a significant effect on the current response of the anodic wave as shown in Fig. 5. The highest current response was obtained using a pre-concentration potential $E_p = -0.5$ V. This potential is slightly more negative than the one observed for the reduction of nifuroxime at the DNA-modified GC electrode. This shows that it is the reduced form of nifuroxime (most probably the corresponding hydroxyl-

Table 1
Comparison of the results obtained for the reduction and oxidation of nifuroxime at DNA-modified and bare GC electrodes by means of stationary voltammetry and SISA

Electrode	Prec. time	Linear range (μm)	D.L. (μm)	(Δ*I*/Δ*C*) (μA/μM)	*r*
Statistics[a]					
GC[b]	1 min	2–30	7.5	0.2670	0.9874 (*n*=7)
DNA-GC[b]	0.5 min	1–45	0.86	1.6009	0.9988 (*n*=7)
DNA-GC[c]	0.5 min	20–120	10	0.1286	0.9981 (*n*=7)
SISA					
GC[b]	60 s	2–50	3.8 (118 ng)	0.4136	0.9938 (*n*=6)
DNA-GC[b]	20 s	0–65	0.68 (21 ng)	1.777	0.9989 (*n*=9)

[a] At constant stirring.
[b] Reduction.
[c] Oxidation at −0.5 V.

amine) that is pre-concentrated on the DNA-modified electrode surface rather than nifuroxime itself.

Hence, the observed oxidation current is due to the oxidation of the accumulated hydroxylamine to the nitroso derivative (curve A in Fig. 1) as shown in Eq. (2).

$$\mathrm{RHNOH} \overset{2e^- + 2H^+}{\rightleftharpoons} \mathrm{RNO} \qquad (2)$$

The optimized pre-concentration time found for the oxidation of nifuroxime at the modified electrode is 1 min. Using the optimized pre-concentration time and potential, a good linear relationship between the peak current and the concentration of nifuroxime in solution was obtained (not shown).

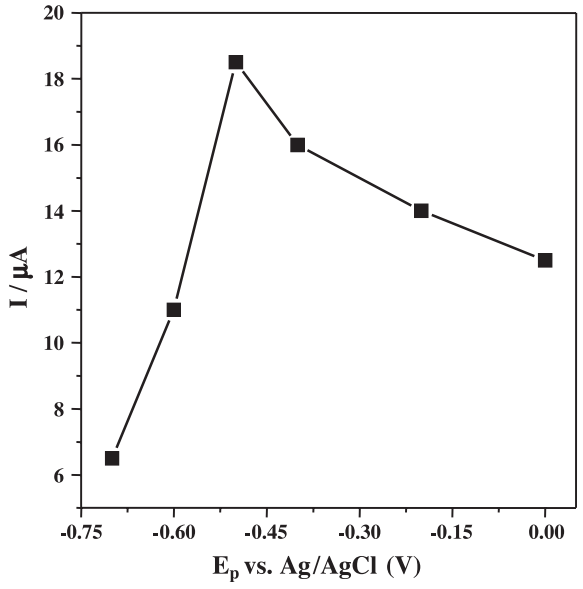

Fig. 5. Dependence of the nifuroxime oxidation current on the pre-concentration potential for the oxidation of 0.2 mM nifuroxime at a DNA-modified GC electrode with a 0.5-min pre-concentration time under constant stirring in 0.1 M PBS (pH 7.2).

3.5. Sequential-injection stripping analysis (SISA) of nifuroxime

The use of biosensors as quantitative detectors in flow-injection analysis is primarily due to their excellent selectivity, high sensitivity and good reproducibility [29]. The combination of the highly sensitive electrochemical stripping voltammetry with flow-injection analysis (FIA) lead to the development of SISA [21]. This technique is on-line, gives improved quantitative results and overcomes problems related to structural changes at the electrode [30], electrode degradation, or the leaching of active component from the electrode commonly associated with conventional flow-injection analysis. The schematic setup of SISA is shown in Fig. 6 based on the previously reported On-Line General Analyzer (OLGA) [23].

Another feature that distinguishes SISA from conventional FIA is that the sample solution reaches the measuring cell only during the real measurements. In situations where the electrode surface needs conditioning or regeneration, pure buffer solution can be pumped through the electrochemical flow-through cell at a low flow rate (10 μl s^{-1} for 3 min).

3.5.1. Optimization of stripping analysis parameters for the reduction of nifuroxime

In preliminary experiments, it became obvious that both the flow rate and the pre-concentration time mainly affect the stripping-injection analysis. Hence, these parameters were studied and optimized. Fig. 7 shows the correlation between pre-concentration time and the peak current for the reduction of 2×10^{-5} mol l^{-1} nifuroxime in PBS using stripping analysis. An optimal pre-concentration time of 20 s was found for the DNA-modified GC electrode, and it could be demonstrated that with longer accumulation times, no further increase in the peak current was obtained. This

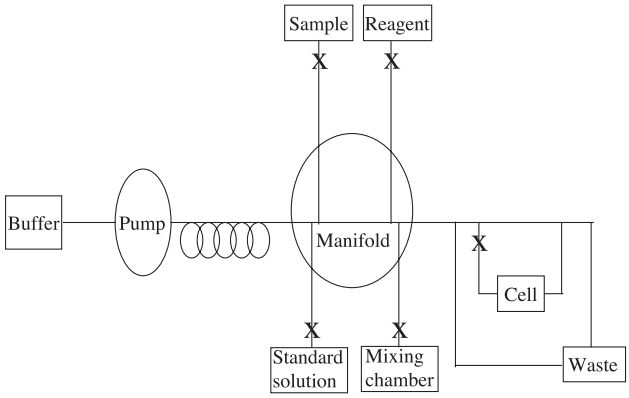

Fig. 6. Schematic diagram of the sequential-injection analyzer used for SISA measurements.

indicates clearly that with a pre-concentration time of 20 s, the modified electrode surface is primarily saturated with the analyte molecules. In contrast, for the unmodified GC electrode, changing the pre-concentration time did not have significant effects on the current response. A flow rate of 10 μl s^{-1} was found to be optimal for stripping injection analysis.

Using the optimized conditions for SISA, nifuroxime-containing solutions were analyzed concomitantly avoiding the problems of accumulation and memory effects by cleaning the electrode in buffer at a flow rate of 10 μl s^{-1}. Although the nature of interaction between nifuroxime and DNA remains unclear, obviously, at least the reduced form of the analyte can be successfully accumulated at the DNA-modified electrode and the obtained stripping current is significantly larger than for a comparable but non-modified GCE. However, there is no possibility to differentiate between an adsorption process probably facilitated by charge interactions between the negatively charged DNA backbone and the reduced analyte or an intercalation of the nifuroxime within the double helix. Due to the relatively mild regeneration process, it can be assumed that the accumulation is most probably due to adsorption. After cleaning, the electrode was checked using CV in pure buffer solution to assure that the cleaning process was successful and no memory effects from previous measurements could be seen. The method yielded very reproducible results as shown in Fig. 8. Each curve in Fig. 8 represents the current response obtained for 40 μM nifuroxime at a flow rate of 10 μl s^{-1} and 20-s pre-concentration time for five consecutive measurements. No significant change in the magnitude of the current response was observed (RSD = 1.7%, $n = 6$).

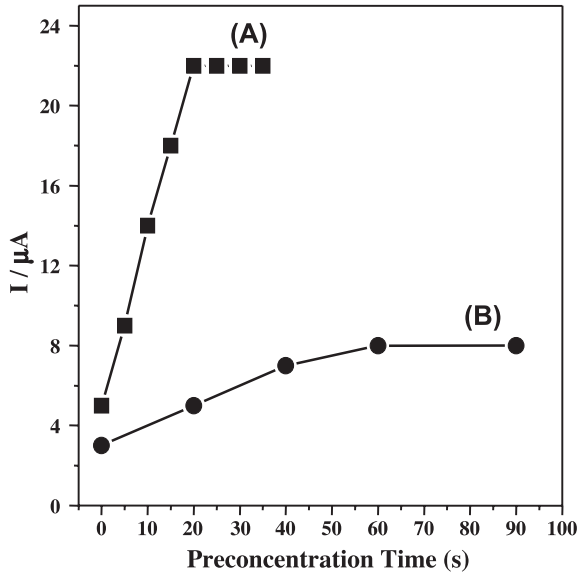

Fig. 7. Effect of the pre-concentration time on the stripping voltammograms for the reduction of 20 μM nifuroxime in 0.1 M PBS (pH 7.2) using SISA for (A) a DNA-modified GC electrode and (B) a bare GC electrodes.

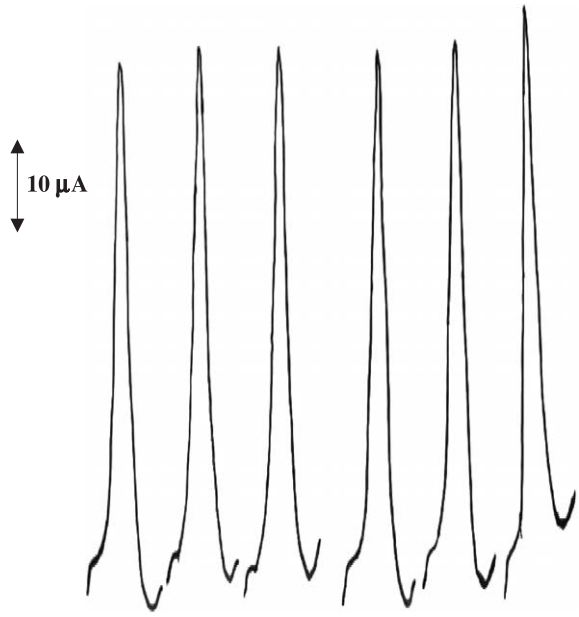

Fig. 8. Subsequent differential pulse voltammograms of the reduction of 40 μM nifuroxime in 0.1 M PBS (pH 7.2) using SISA demonstrating the reproducibility of the analysis.

The stability of the DNA-modified electrode was evaluated. It was found that there was no significant change of the current response measured with the same electrode after 1 week of storage in air. With daily measurements, the electrode was found to be very reproducible with a relative standard deviation of 1.9% ($n = 7$).

A summary of the results obtained from stationary voltammetry and SISA is presented in Table 1. It can be seen that a wider linear range and a lower detection limit was obtained using SISA as compared with individual stationary measurements.

4. Conclusion

DNA-modified GC electrodes have been used for the determination of nifuroxime. The modified electrode was capable of accumulating nifuroxime on the surface due to the interaction between the DNA backbone on the electrode surface and nifuroxime in solution. This led to an increase in sensitivity using the DNA-modified electrode towards the reduction of nifuroxime as compared with the unmodified electrode. Both, the oxidation and reduction wave of nifuroxime can be used for its quantification at the DNA-modified electrode; whereas only the reduction wave can be observed at the unmodified electrode. Electrochemical techniques are often not used in the analysis of pharmaceuticals because of the surface passivation that occurs after the electrochemical event. However, using SISA, the electrode surface can be easily regenerated during the overall analytical cycle and hence a low detection limit of 0.68 μM corresponding to an absolute value of 21 ng of nifuroxime could be obtained.

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