

Amperometric DNA-Peroxidase Sensor for the Detection of Pharmaceutical Preparations

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Abstract: Novel DNA-sensor with enzymatic amplification of the signal has been developed on the base of glassy carbon electrode modified with ds-DNA and horseradish peroxidase (HRP). Phenothiazine dyes Methylene Blue and Methylene Green were used as electrochemical markers for the detection of sulfonamide and anthracycline preparations able to interact with DNA. The biosensor signal related to HRP oxidation of the markers depends on the relation between their bonded and readily oxidized forms which depends on the nature and concentration of pharmaceuticals. Sulfonamides diminish surface concentration of MB accessible for HRP reaction whereas anthracyclines release intercalated marker and increase the signal. The DNA-HRP sensor developed makes it possible to detect down to 0.002 nmol L⁻¹ of sulfamethoxazole, 0.1 nmol L⁻¹ of sulfadiazine, 0.01 nmol L⁻¹ of sulfamethazine, 0.1 nmol L⁻¹ of sulfaguanine, 0.05 μmol L⁻¹ of rubomycin and 0.08 μmol L⁻¹ of doxorubicin.

Key words: DNA sensor, affinity biosensor, horseradish peroxidase, sulfonamide determination, anthracycline determination

1. Introduction

A growing interest to DNA-sensors has emerged recently due to the perspectives they possess in clinical diagnostics [1-3]. Double-stranded (ds-) and single-stranded (ss-) DNAs show remarkable advantages over traditionally used enzymes or antibodies due to their higher stability and variety of interactions with biological targets, i.e. metabolites, pharmaceuticals, microbial and viral DNAs. The DNA probes can be easily isolated from living beings or synthesized *ex vivo*, selected against appropriate analytes and then used for their detection and quantification with various optical or electrochemical measurement devices. Main attention is paid to the application of ss-oligonucleotides for detecting microbial and viral pathogens [4, 5] and gene mutations [6]. Meanwhile, ds-DNAs are reported to detect specific DNA binding proteins (e.g. anti-DNA antibodies [7]), DNA-targeted drugs [8-11] and mutagenic pollutants [12-14]. In comparison with optical or piezoelectric devices, electrochemical DNA-sensors seem attractive for further commercialization due to simple and inexpensive design and compatibility with conventional electrochemical analyzers.

The generation of the signal in electrochemical DNA-sensors is mainly achieved by two approaches. The interaction of DNA probe with target analyte can affect the electrochemical oxidation of guanine residues [15,16]. This provides detecting the hybridization events, DNA cleavage and determining compounds that coordinate near guanine residues in DNA duplex. Guanine signals are often recorded together with oxidation peaks of intercalators, e.g. daunomycin [17] and other anthracycline drugs [8, 10] which intercalate ds-DNA and lose their ability to direct oxidation on the electrode. This confirms the results of hybridization detection and improves sensitivity of detection of DNA point mutation and sequence mismatches.

The second approach assumes introduction of labels that could be covalently bonded to the DNA probe or addition of diffusion-free markers. The enzymes [7, 18-20] and ferrocene labels [21, 22] have been described for electrochemical DNA-sensor design. Thus, the hybridization of target oligonucleotide with ss-DNA immobilized on Au electrode resulted in the electrostatic accumulation of cytochrome *c* followed by the enhancement of electron transduction between cytochrome *c* and the electrode [18]. Alkaline phosphatase and galactosidase were used for differentiation of two DNA targets related to breast cancer gene in a single measurement [19]. The products of enzymatic reactions, i.e. phenol and α -naphthol, produced two well resolved peaks measured in chronopotentiometric mode. Streptavidine - alkaline phosphatase conjugate was used for enzyme amplification of the hybridization response on the Au electrode covered with thiol monolayer containing synthetic oligonucleotide probe [20]. For the detection of anti-DNA antibodies, ds-DNA was co-immobilized with cholinesterase on a glassy carbon electrode [7]. The formation of specific DNA-protein complex limited the access of the enzyme substrate and decreased the biosensor response in accordance with kinetics of apparent competitive inhibition. The DNA-cholinesterase sensor was suggested to test antibody level in the blood samples of bronchial asthma patients.

Diffusion-free markers change their electrochemical characteristics in reaction with DNA placed on the electrode surface. In addition to anthracyclines mentioned, phenothiazine dyes [5, 23-25] and transition metal complexes [17, 25-30] have been tested for this purpose. Most of them are coordinated

near guanine residues of DNA and hence are accumulated on the electrode surface. The effect is much more pronounced with ss-DNA and decreased after its reaction with complementary DNA sequence. Phenothiazine dye Methylene Blue (MB) was reported to be effective electrochemical marker of DNA interactions due to its high affinity to ss- and ds-DNAs and easy electrochemical detection. Thus, nanomolar amounts of target oligonucleotides relative to Hepatitis B [5] and bloom-forming cyanobacteria [25] were detected with this marker of hybridization events.

The further improvement of the sensitivity of amperometric DNA-sensors based on this marker can be achieved by coupling DNA interaction with peroxidase (HRP) oxidation of MB within mutual surface layer. Phenothiazines are known as efficient mediators of electron transfer on HRP [31, 32]. The rate of their enzymatic oxidation can be easily determined by the cathodic current of the reduction of the products measured at -50...- 400 mV vs. Ag/AgCl. The involvement of the phenothiazines in the HRP reaction is affected by their specific interaction with ds-DNA on the electrode surface. This makes it possible to determine low-molecular compounds that compete with phenothiazines for DNA binding. In this work, the DNA-HRP sensor was developed for the determination of pharmaceutical preparations, i.e. sulfonamides and anthracyclines.

2. Experimental

2.1. Apparatus

Voltammetric measurements were carried out in a standard three-electrode electrochemical cell thermostated at 25 ± 1 °C with electrochemical analyzer CV-50W (BAS, W.Lafayette, IN, USA). Peak currents referring to the reduction of phenothiazines oxidized in HRP reaction were measured at -140 mV for MB and at -250 mV vs. Ag/AgCl reference electrode for Methylene Green (MG). Square-wave and direct current voltammetry were used for the signal measurement. Glassy carbon rod tightly pressed into the PTFE tube with the outer diameter of 9 mm was used as transducer for the DNA-HRP sensor design. The geometric surface area of the electrode was about 2.5 mm². Pt wire auxiliary electrode and Ag/AgCl reference electrode (RE-5B, BAS) were used in all the measurements.

2.2. Reagents

All the stock solutions were prepared in distilled water. The 500 bp ds-DNA from chicken erythrocytes was purchased from Reanal (Budapest, Hungary), HRP (EC 1.11.1.7), specific activity 250 U mg⁻¹ solid, RZ 3.4, Methylene Blue (MB), Methylene Green (MG), sulfamethoxazole (SMX), sulfamethazine (SMZ), sulfadiazine (SDZ), sulfaguanidine (SG) were purchased from "Sigma-Aldrich Chemie GmbH" (Steinheim, Germany), rubomycin hydrochloride (daunorubicin) and doxorubicin hydrochloride (adriamycin) from "Ferane" (Moscow, Russia), glutaraldehyde, 25% aqueous solution, from "Lancaster" (Eastgate, England). All the other reagents used were of analytical grade ("Reakhim", Russia, and "Fluka", Neu-Ulm, Switzerland). Stock DNA solution was prepared by dispensing the preparation in citrate buffer solution (0.15 mol L⁻¹ NaCl + 0.015 mol L⁻¹ sodium citrate, pH 7.0). All

the electrochemical measurements were performed in 2.0 mmol L⁻¹ phosphate buffer solution containing 0.1 mol L⁻¹ Na₂SO₄, pH 7.0.

2.3. DNA-sensor development

Glassy carbon electrode was first polished with alumina, then electrochemically cleaned by repeating scanning the potential between -600 and +800 mV, washed with phosphate buffer solution, dried and fixed upside-down. After that, 3 μL of ds-DNA solution were spread onto the working area, dried to form wet film and covered with 5 μL of HRP solution containing 0.01% gelatin. The final loading of the components corresponded to 0.01-0.1 mg cm⁻² of ds-DNA and 5 U cm⁻² of HRP. The content of the surface layer corresponded to the optimal immobilization conditions established earlier [7] for the detection of DNA-protein interactions. The DNA-HRP sensor was left at room temperature for 15 min. Before its complete drying, 5 μL of 1 % glutaraldehyde were added to bind the proteins and prevent DNA loss. DNA-HRP sensors can be stored at 4°C for at least ten days in dry conditions. DNA- and HRP-sensors were prepared in a similar way. The conditions for glutaraldehyde cross-binding were the same for all the biosensors developed.

2.4. Procedures

2.4.1. Signal measurement

DNA-HRP- or HRP-sensor, Pt auxiliary and Ag/AgCl reference electrodes were immersed in 5 mL of 2×10⁻³ mol L⁻¹ phosphate buffer solution containing 0.1 mol L⁻¹ Na₂SO₄, pH 7.0. After that, 50 μL of H₂O₂ were added to its final concentration of 2.0 mmol L⁻¹ and the background current was recorded. The signal was measured in 5 min after pipetting 10 μL of MB or MG at -140 mV and -250 mV, respectively. The standard deviation of the response for six successive measurements performed with the same sensor did not exceed 5 %.

2.4.2. Pharmaceuticals determination

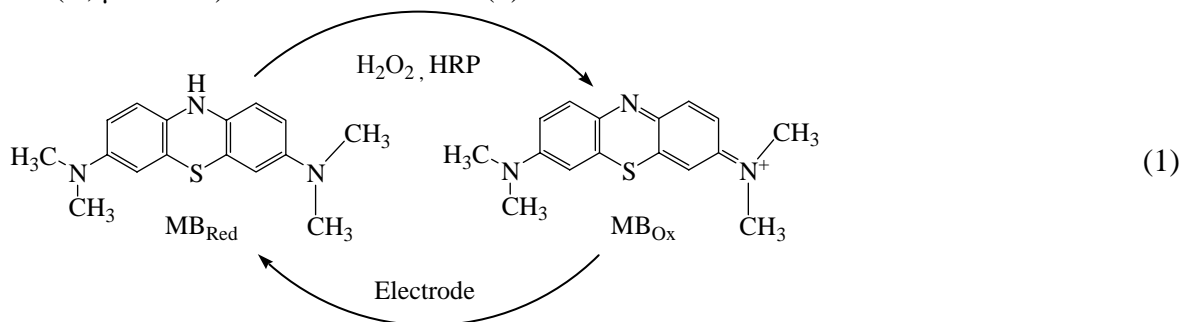
DNA-HRP sensor was incubated in the solution of pharmaceutical preparation for 10 min, whereupon phenothiazine marker was added and the response measured as described above. The changes in the signal against that in blank experiment were calculated and used for quantification of pharmaceutical preparations.

3. Results and Discussion

3.1. Investigation of the marker signal

Both phenothiazine markers investigated, i.e. MB and MG, are active in electrochemical and enzymatic conversion on glassy carbon electrode and biosensor containing HRP in the surface layer. In the latter case, the peak referred to the reduction of an oxidation product appears on cathodic leg of cyclic voltammogram after H₂O₂ addition in accordance with the reaction (1) given for MB as an example. The efficiency of HRP oxidation of MG was found much lower than that of MB. This results

in the lower signal and its sensitivity toward MG concentration. In square-wave voltammetry, the symmetrical cathodic peak was recorded with the maximum (i , μA) proportional to the phenothiazine concentration (C , $\mu\text{mol L}^{-1}$) in accordance with (2).



$$\begin{aligned} \text{MB: } i, \mu\text{A} &= (0.083 \pm 0.003) + (1.5 \pm 0.1) \times (C, \mu\text{mol L}^{-1}), \quad R=0.9981 \\ \text{MG: } i, \mu\text{A} &= (0.27 \pm 0.06) + (0.063 \pm 0.002)(C, \mu\text{mol L}^{-1}), \quad R = 0.9956 \end{aligned} \quad (2)$$

The intercept values of calibration curves obtained for the HRP sensor were significantly different from zero. Probably they reflect the contribution of the sorption to the surface concentration of the markers. The most reproducible response was obtained with $2.0 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$. The mean current obtained with $1.0 \mu\text{mol L}^{-1}$ of MB was $1.8 \mu\text{A}$ with R.S.D. of 4.2 % for six consecutive measurements performed with the same HRP-sensor. Although the following increase in H_2O_2 concentration increases the peak current, the reproducibility of the signal diminishes to about 6% at 5.0 mmol L^{-1} of H_2O_2 . At higher concentration, the peak current becomes lower probably to the non-enzymatic oxidation of MB in bulk solution and decrease of its surface concentration. The maximum signal in phosphate buffer solution was obtained at pH 6.9-7.0. This corresponds well to the pH maximum of free HRP activity in the same measurement conditions.

The introduction of ds-DNA from chicken erythrocytes into the surface layer was performed by physical sorption from the phosphate buffer solution followed by its entrapment in the protein film formed by cross-binding with glutaraldehyde. When the $0.001\text{-}0.04 \text{ mg mL}^{-1}$ DNA solution was taken for immobilization, an increase of the signal toward MB was observed in comparison with that on HRP-sensor (Fig.1). Hereafter, the DNA concentration refers to the solution used for the sorption of DNA on the electrode surface.

The following increase in ds-DNA loading diminishes its influence. This corresponds well to the mechanism of MB interaction with ds-DNA. As was mentioned, MB both intercalates ds-DNA near G-C pairs and forms complexes at minor grooves near A-T pairs [33]. Thus DNA placed on the electrode provides accumulation of MB in the form accessible for HRP oxidation. A high efficiency of electron transduction in the system ds-DNA-MB-HRP was confirmed by improving sensitivity of H_2O_2 determination with the sensor contained poly(allylamine) as immobilization matrix [34]. For MG, the modification of the electrode with ds-DNA does not increase the current of MG reduction and accuracy of the response measurement. Meanwhile, the slope of appropriate calibration curve as well as the range concentrations to be determined did not significantly differ from those obtained with

HRP-sensor. The appropriate calibration plots obtained for 0.015 mg mL^{-1} DNA taken for immobilization are presented with Eq.(3).

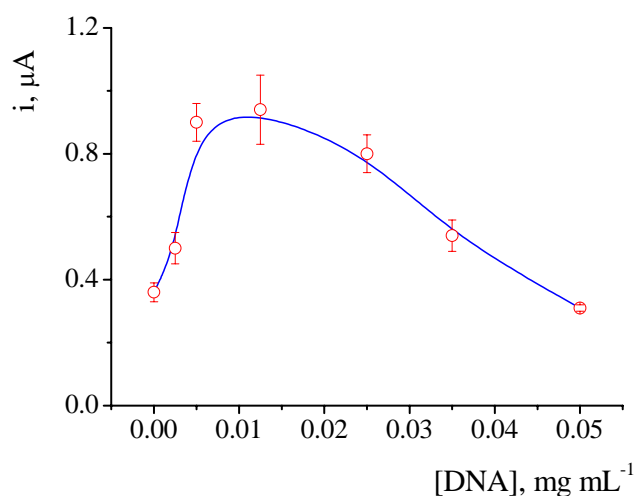


Figure 1. The dependence of the signal of the DNA-HRP sensor on the concentration of solution used for DNA immobilization, $0.4 \text{ } \mu\text{mol L}^{-1}$ MB, 2.0 mmol L^{-1} H_2O_2

$$\begin{aligned} \text{MB: } i, \mu\text{A}, &= (-0.13 \pm 0.04) + (2.30 \pm 0.06)(C, \mu\text{mol L}^{-1}), R = 0.9870 \\ \text{MG: } i, \mu\text{A}, &= (0.14 \pm 0.02) + (0.054 \pm 0.001)(C, \mu\text{mol L}^{-1}), R = 0.9996 \end{aligned} \quad (3)$$

The supposed mechanism of ds-DNA-MB and ds-DNA-MG interactions was proved by investigation of ds-DNA melting with microcalorimetric measurements (Fig.2). The effect of MB on the DNA melting curve was found to be different from that of ethidium bromide, a typical DNA intercalator. In the latter case, intercalation results in the shift of the maximum of the melting curve toward higher temperature accompanied by an increase of the peak area and appropriate changes in the starting and ending points. In the presence of MB the shift of distinctive points was much lower and in the opposite direction. The difference in the DNA thermostability observed in the presence of ethidium bromide and MB makes it possible to assume that MB is preferably coordinated at minor grooves of DNA helix. MG did not affect DNA melting curve in the concentration range investigated. Probably, the nitro group at aromatic ring of MG weakens the interaction of the dye with ds-DNA due to hindering optimal coordination of the reactants.

3.2. Determination of sulfonamides

The sensitivity of the signal of enzymatic oxidation of MB in the presence of ds-DNA on the electrode makes it possible to use DNA-HRP sensor for the detection of low-molecular compounds interacting with DNA and competing with the marker for appropriate DNA binding sites. Previously we have established that sulfonamides, i.e. SMX and ST, successfully replace anti-DNA antibodies in

the DNA complexes. The coordination of sulfonamides near DNA minor grooves was confirmed by microcalorimetric experiments [7].

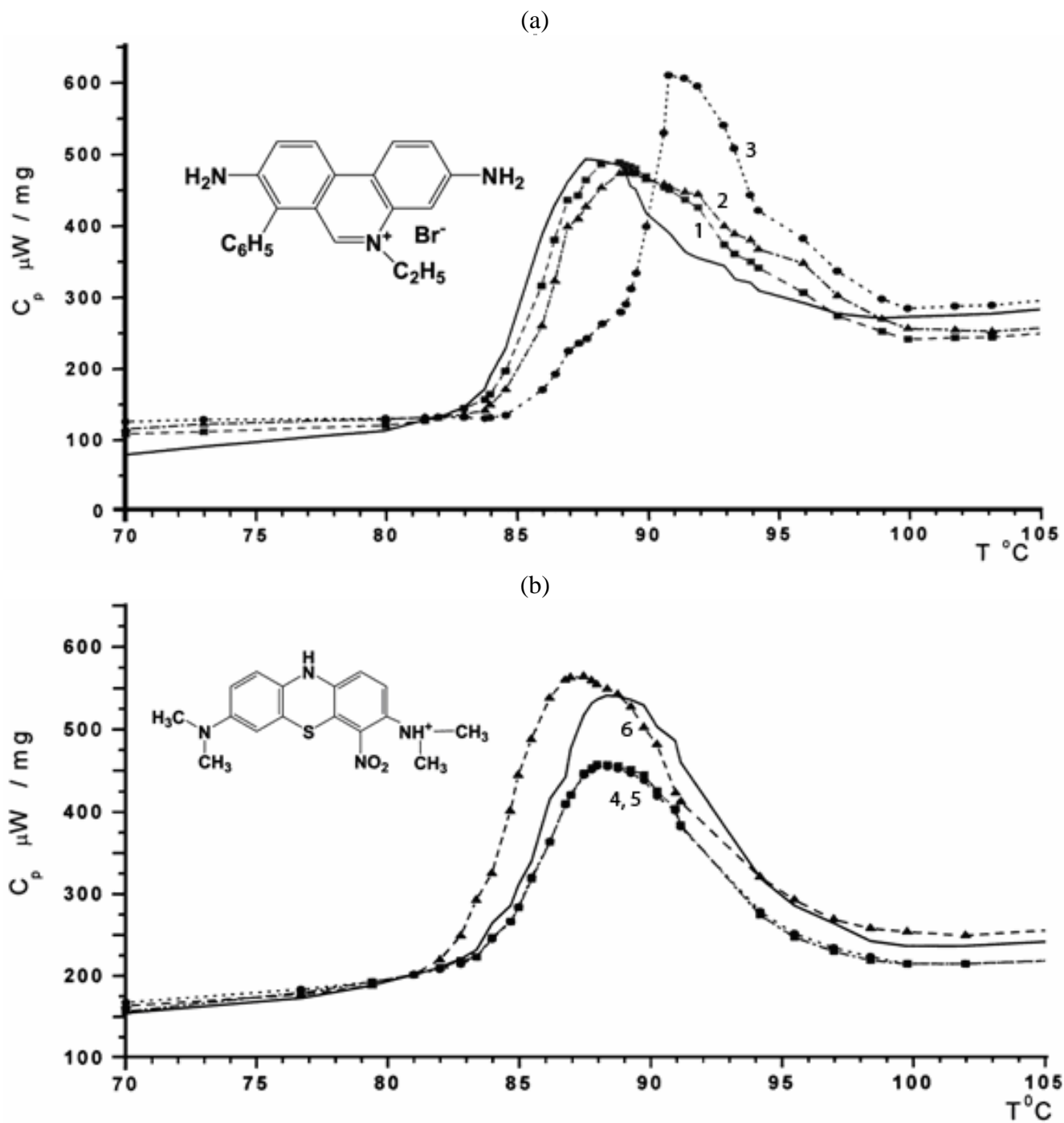


Figure 2. Melting curves of ds-DNA (solid curves) and of its mixtures (dashed curves) with ethidium bromide (a, (1) - 10, (2) - 12 and (3) - 15 $\mu\text{g mL}^{-1}$) and MB (b, (4) - 5, (5) - 10 and (6) - 15 $\mu\text{g mL}^{-1}$). C_p – specific heat capacity

Indeed, the addition of various sulfonamide preparations to MB solution prior to its contact with the DNA-HRP sensor resulted in remarkable decrease of the signal recorded (Fig.3). It should be mentioned that sulfonamides investigated did not alter the activity of free HRP so that all the changes in the MB oxidation are related to the DNA present. The decay of the signal increases with incubation period with the maximum reached to 10 min contact. After that, current changes became irregular and less reproducible.

The limits of detection of sulfonamides investigated as well as other characteristics of the response are summarized in the Table 1. The limit of detection (LOD) corresponds to the concentration of the substrates with the signal equal to the triplicate error of the current measurement ($S/N=3$). Concentration range refers to the linear piece of the curve used for sensitivity calculation.

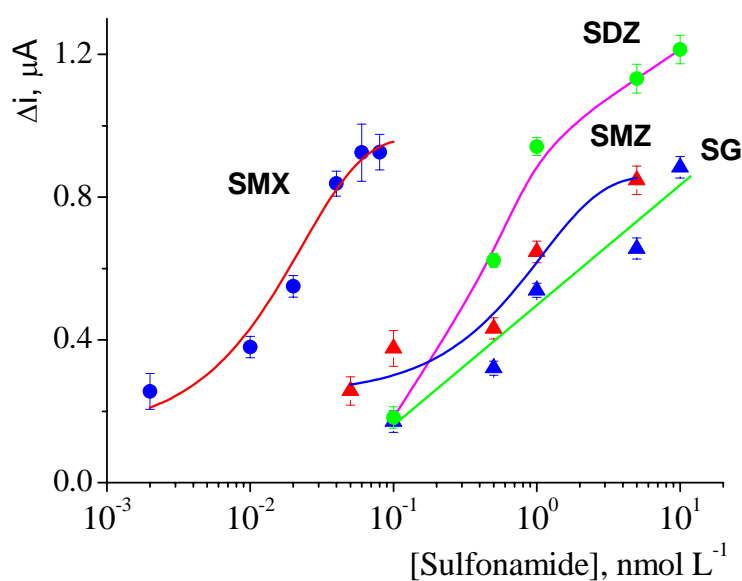


Figure 3. The influence of the sulfonamide concentration on the decay of the DNA-HRP sensor signal. $0.4 \mu\text{mol L}^{-1}$ MB, incubation 10 min.

Table 1. Determination of sulfonamide preparations with DNA-HRP sensor.

MB $0.4 \mu\text{mol L}^{-1}$, H_2O_2 2 mmol L^{-1} , incubation 10 min, phosphate buffer solution, pH 7.0

Sulfonamide	LOD, nmol L^{-1}	Sensitivity, $\Delta i / \Delta \log(C, \text{nmol L}^{-1})$	Concentration range, nmol L^{-1}
SMX	0.002	0.73	0.005-0.2
SDZ	0.1	0.80	0.1-1.0
SMZ	0.01	0.30	0.02-2
SG	0.1	0.33	0.1-10

The maximum shift of the current remains much less than the difference of the response of DNA-HRP and HRP sensor to the same MB concentration. This suggests that only part of MB bonded to the ds-DNA can be replaced with sulfonamides. The saturation level depends on the pharmaceutical preparation used.

The MG signal depends on the sulfonamides investigated to a much smaller extent. Only SMX in micromolar range of concentration decreased the signal of DNA-HRP signal. Other sulfonamides either increase the signal by 10-15 % or leave it as is. Probably the changes in MG signal can be related to non-specific sorption of sulfonamides due to their electrostatic interaction with negatively charged phosphate skeleton of ds-DNA. In all the cases the relative shift of the signal did not exceed 30% of that recorded with MB as a marker.

The difference in the MB and MG behavior on DNA-HRP sensor can prove the determination of sulfonamide determination. HRP activity can be affected by various species, i.e. metabolites or pharmaceuticals that belong to enzyme substrates or effectors. In the presence of these species, the activity of HRP and hence the signal of MG and MB oxidation will alter in a similar manner. However, sulfonamide that effect the availability of phenothiazine markers due to DNA interaction will decrease only MB signal whereas MG current will remain about the same.

3.3. Anthracyclines determination

Contrary to sulfonamides, anthracyclines refer to DNA intercalators. Their interaction with dsDNA results in decrease of their electrochemical activity and of the oxidation current recorded by pulse or differential voltammetry at high anodic potentials [8, 10]. The anthracyclines react preferably with G-C pairs. Changes in their oxidation current correlate with appropriate changes in oxidation peaks of guanine residues which could be observed on the same voltammograms. In the presence of MB as electrochemical marker, the addition of rubomycin and doxorubicin resulted in an increase of the signal (Fig.4). The LOD was found to be $0.05 \mu\text{mol L}^{-1}$ of rubomycin and $0.08 \mu\text{mol L}^{-1}$ of doxorubicin for 10 min incubation and $1.0 \mu\text{mol L}^{-1}$ of MB.

Probably, anthracyclines replace intercalated MB from the DNA helix and increase surface concentration of the form accessible for HRP oxidation. In accordance with relative changes of the MB current recorded with HRP and DNA-HRP sensors in the presence of sulfonamides and anthracyclines it could be concluded that about 60-70% of MB intercalate ds-DNA can be replaced with other intercalators. Residuary 30-40% of MB are bonded to ds-DNA weaker and released in the presence of the compounds coordinated out of DNA helix, preferably at minor grooves. This suggestion is confirmed by similarity of appropriate curves and saturation levels observed for both preparations investigated (see Fig.4).

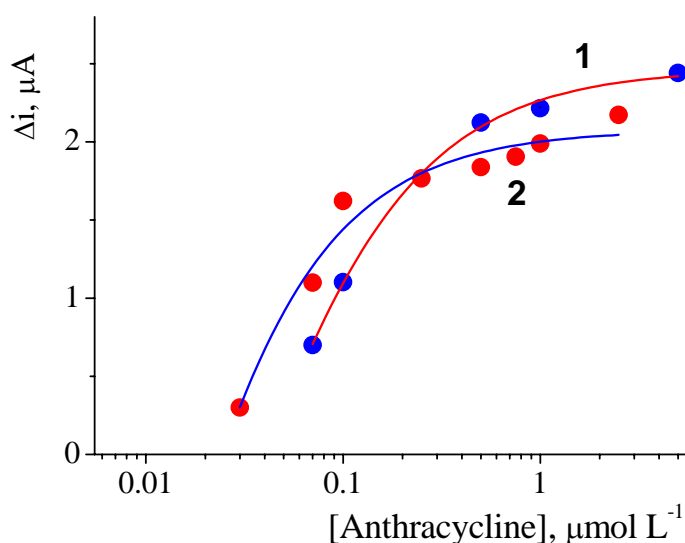


Figure 4. The influence of doxorubicin (1) and rubomycin (2) on the MB signal recorded with DNA-HRP sensor. 1.0 μmol L⁻¹ MB, incubation 10 min

Conclusion

The use of two competitive reactions of phenothiazine markers, i.e. their binding with ds-DNA and HRP oxidation coupled with electrochemical detection provides the possibility for a fast and sensitive detection of affinity interactions with low-molecular compounds of medical significance. The response of the DNA-HRP sensor based on biochemical amplification depends on the marker used and measurement conditions. This allows changing the sensitivity and selectivity of detection of sulfonamides and anthracyclines in accordance with the particular aim of analysis. Moreover, simultaneous consideration of MG and MB response makes it possible to avoid mistakes related to the presence of HRP substrates and effectors in the sample tested. As was shown, changes in the MB signal depend on how the marker is bonded with ds-DNA. Anthracyclines release firmly bonded marker and increase the signal recorded whereas sulfonamides replace weakly bonded MB and hence decrease the concentration of readily oxidized part of the marker and its signal. This provides not only separate detection of both groups of analytes but also investigation of the mechanisms of DNA interactions with various low-molecular compounds of medical significance. Even though the target analyte-DNA interaction is less specific than that in the case of immunosensors the selectivity toward various groups of medicines makes it possible to use DNA-HRP sensor for the estimation of the total amounts of pharmaceuticals of known origin. This could be important for monitoring patient conditions under intensive therapy or for testing efficiency of anti-cancer drug treatment.

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