Electrochemical DNA sensors on the basis of electropolymerized thionine and Azure B with addition of pillar[5]arene as an electron transfer mediator

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A DNA sensor was developed on the basis of glassy carbon electrode coated with polymeric forms of thionine and Azure B. Introduction of carbon black and pillar[5]arene into the electrode composition increases the efficiency of polymerization and the oxidation peak currents of dyes due to the mediating effect of the macrocycle. The addition of DNA onto the sensor surface and into the reaction mixture differently influences the electrochemical activity of poly(Azure B) and polythionine. The control of changes in current-voltage characteristics allowed us to identify the heat denaturation of DNA and its oxidation by reactive oxygen species generated upon the reaction of hydrogen peroxide and copper(II) salt. The DNA sensors can find application in the diagnosis of DNA damage on exposure to carcinogens and in screening of cytotoxic anticancer drugs.

Key words: electropolymerization, biosensor, pillar[5]arene, polythionine, DNA damage.

Electrochemical DNA sensors find wide application in bioanalysis and electroanalytical chemistry due to the capability of recognizing complex biomolecules, such as nucleic acid fragments, regulatory proteins, anticancer agents, and intercalating agents.[1—3] They are in demand as agents for disease diagnostics,[4] monitoring of carcinogenic pollutants in environment[5] and food products,[6] and in screening of cytostatic agents.[7] DNA sensors are applied in pure researches in the field of molecular biology and biochemistry.[8]

Despite great advances in the design of electrochemical DNA sensors, the improvement of methods for the measurement of their signals remains topical, especially upon detection of low-molecular-weight compounds or when nonspecific DNA damage (heat denaturation or oxidative damage on exposure to reactive oxygen species) should be determined.[9] The direct oxidation of DNA on an electrode requires high anode potentials to be applied, which is undesirable due to the effect of oxidizable components of a sample on the signal. In addition, this requires relatively high concentrations of nucleic acids and, consequently, the sensitivity of recording their damage will be low. For this reason, new approaches contemplating the use of covalently bound labels or the inclusion of DNA into the composition of surface complexes involving electrochemically active components are being extensively developed at the present time.

Among electrochemically active components, a great attention is focused on polymer materials obtained on electrodes from monomer solutions in electropolymerization reactions.[10] Polymerization is typically initiated by the oxidation of a monomer on an electrode followed by condensation to deposit oligomers on the electrode. It is convenient to monitor the course of the reaction by current-voltage characteristics varying upon a change in the amount of the product on the electrode. When polymerization is performed in the presence of DNA, the biopolymer is trapped in a growing film; the negative charge of phosphate residues in the DNA helix favors polymerization due to accumulation of intermediate and final cationic products. The effect of DNA charge on polymerization and electrochemical properties of products is called template effect. It is manifested to the maximum extent in the case of polymerization of aniline. Polyaniline exhibits conductive properties and electrochemical activity as an electron transfer mediator predominantly in the acidic medium being in the semioxidized form of emeraldine.[11] Deoxyribonucleic acid as a polymeric counter-ion stabilizes this state favoring the retention of electrochemical activity in a weakly acidic or neutral regions, which are more suitable for recording biochemical interactions.[12—14] DNA has similar, but less pronounced effect on polymerization of pyrrole.[15] At the same time, the study of poly-

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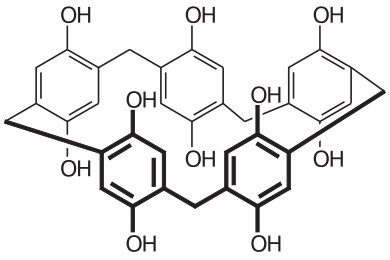
merization of other electron transfer mediators, such as thiazine and phenothiazine dyes, remains virtually unstudied. For example, the effect of DNA on the electrochemical behavior of polymeric forms of methylene blue, methylene green, and neutral red obtained in the presence of DNA and the possibility of recording the oxidative damage of DNA by a change in the parameters of redox peaks of polymers were described.[16] Poly(neutral red)[17] and polythionine[18—20] have found application as electron transfer mediators upon recording the hybridization of complementary DNA sequences. Single-stranded DNA was used as a template for the preparation of polythionine as a part of the electrochemical sensor for hydrogen peroxide.[21] In contrast to polyaniline, electropolymerization of the above-mentioned dyes in the presence of DNA proceeds slightly slower than that in its absence due to a partial decrease in the electrode surface area upon adsorption of a nonconductive biopolymer. The insertion of additional electron transfer mediators can increase both the efficiency of coprecipitation of polymer and DNA and the sensitivity of recording the changes associated with specific reactions of DNA. Earlier, we have shown that decahydroxylated pillar[5]arene reversibly oxidizing on the carbon black-coated electrode is sensitive to the presence of DNA and, at the same time, shows a capacity for efficient electron transfer.[22] In the present work, conditions for the preparation of a coating made of the polymeric forms of thionine and Azure B in the presence of DNA and pillar[5]arene were studied and the possibility to discriminate biosensor signals depending on the thermal and chemical damages of native DNA molecules was shown.



Pillar[5]arene

Experimental

4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), low-molecular-weight DNA from fish testes, thionine acetate, Azure B (Sigma Aldrich), and carbon black (IMERYS, Belgium) were used in the work. Other reagents were of analytical grade. Pillar[5]arene was synthesized according to a known procedure[23] and its structure was confirmed by IR and ^{1}H NMR spectroscopy, as well as by elemental analysis.

Working solutions were prepared using Millipore Q® deionized water. A solution of DNA was prepared in HEPES buffer (0.1 *M* HEPES containing 0.03 *M* NaCl, pH 7.0). Voltammetric measurements were performed in the same solution. DNA was oxidized mixing its solution with an oxidizing mixture containing 0.15 *M* NaCl (15 vol.%), water (75 vol.%), and 0.04 *M* $CuSO_4$ (10 vol.%). Immediately prior to addition of DNA, 30% aqueous H_2O_2 (1.4 μL) was added to the oxidizing mixture. Heat denaturation of DNA was performed immediately prior to its use as a part of the biosensor by heating a solution of DNA for 30 min at 95 °C followed by its cooling in an ice bath for 5 min. A suspension of carbon black for modification of the electrode was prepared dispersing the substance (1 mg) in a 0.375% solution of chitosan in 0.05 *M* HCl for 2 min under ultrasonication.

Electrochemical measurements were made using a CHI 440B electrochemical workstation (CH Instruments, USA). The working electrodes were SU2000 glassy carbon rods (NIIGraphit, Moscow) embedded in a polytetrafluoroethylene body and equipped with a copper collector. The working surface area was 1.77 mm^2. The counter electrode was a platinum wire and the reference electrode was silver chloride (Ag/AgCl/(3.0 *M* KCl)) electrode (CH Instruments, USA). All measurements were carried out in a 5-mL three-electrode cell.

The glassy carbon electrode was modified after mechanical and electrochemical purification placing a carbon black suspension (2 μL) with a concentration of 1 mg mL^{-1} and 1.0 *M* NaOH (1.5 μL) on the electrode surface followed by drying at 50 °C until a dense uniform layer formed. The electrode was washed from the excess of alkali using deionized water. A 10 m*M* solution of pillar[5]arene (2 μL) in acetone was placed on the electrode and dried in air. For electropolymerization, the electrode was fixed in a working cell containing a 0.1 m*M* solution of thionine or Azure B in a working HEPES buffer. The electrode potential was scanned repeatedly in a potential range from −0.5 to 1.1 V at a scan rate of 50 mV s^{-1}.

The true surface area of the modified electrode was determined by the Randles—Sevcik equation[24]

$$I_p = 2.69 \cdot 10^5 n^{3/2} A D^{1/2} \nu^{1/2} c,$$

where I_p (A) is the cathode current peak of ferricyanide ion $[Fe(CN)_6]^{3-}$; n is the number of electrons transferred in the redox event (usually $n = 1$); A (cm^2) is the electrode surface area; D is the diffusion coefficient ($D = 7.6 \cdot 10^{-6}$ cm^2 s^{-1}); ν (V s^{-1}) is the potential scan rate; and c (mol L^{-1}) is the concentration of ferricyanide ion.

Results and Discussion

Electropolymerization of thionine dyes. Thionine and Azure B (*N*,*N*,*N*′-trimethylthionine chloride) are oxidized to form one quasireversible pair of peaks in a region of −0.27 V and 0.17 V corresponding to oxidation-reduction of the phenothiazine core of their molecules. The study was performed in the presence of carbon black, which increases the recorded currents and the stability of precipitating polymerization products. The presence of carbon black on the glassy carbon electrode did not change the morphology of voltammetric curves compared to pure glassy carbon, but increased the recorded currents due to a larger working surface area. The required amount of deposited carbon black was determined by the reach of complete surface filling and the stability of recorded

voltammetric curves. When the amount of the modifier exceeded 2 μg per electrode, a portion of the coating delaminated during measurement and was released from the electrode surface. The true surface area was determined from the Randles—Sevcik equation by the reduction current of ferricyanide ion $[Fe(CN)_6]^{3-}$. The roughness factor showing how much the true surface area of electrode exceeds the geometric one was 2.3 under specified modification conditions.

For the polymeric product to be deposited on the electrode, the maximum scan potential should correspond to the oxidation of the starting dye into a dication initiating polymerization.[25] If scanning does not cover the potential region of 1.0—1.2 V, no polymerization of the dyes is observed. As an example, Fig. 1, *a* shows cyclic voltammograms (CV) obtained in a solution of thionine on the carbon black-modified electrode. Similar curves were obtained by using a solution of Azure B.

The nature of the polymerization products of thionine and Azure B have not been established earlier. As assumed in Refs 26 and 27, the reaction proceeds by the mechanism of head-to-tail addition to form a C—N bond between the aromatic ring and the amino group of monomer and oligomers. The cathode reduction peaks on voltammograms are slightly higher than the anode ones and have a shape typical of surface electrode reactions, which can be attributed to sorption accumulation of oxidized (positively charged) dye molecules on negative carbon black particles.

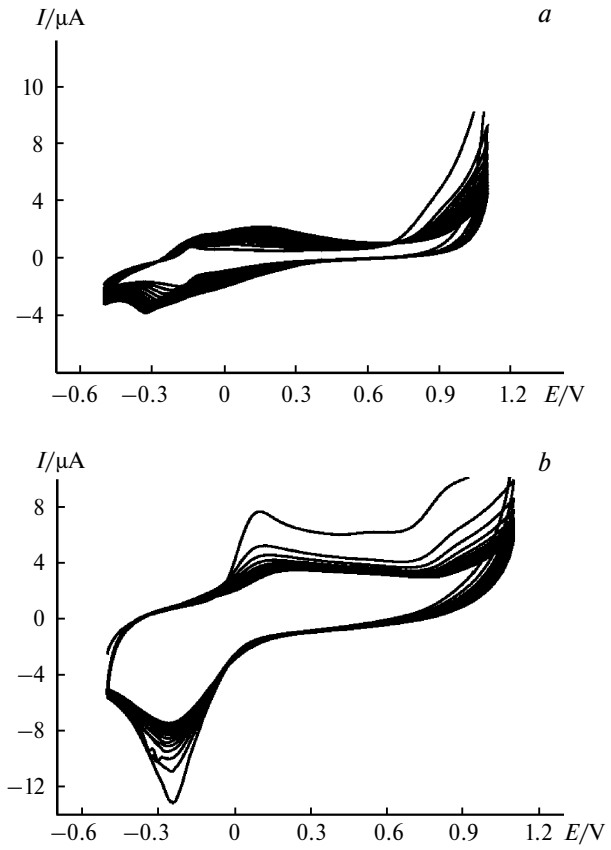


Fig. 1. Electropolymerization of thionine on the glassy carbon electrode modified with carbon black (2 μg per electrode) in the absence (*a*) and in the presence (*b*) of pillar[5]arene (20 nmol) in the surface layer. The measurements were carried out using a HEPES buffer (pH 7.0) at a scan rate of 50 mV s^{-1}.

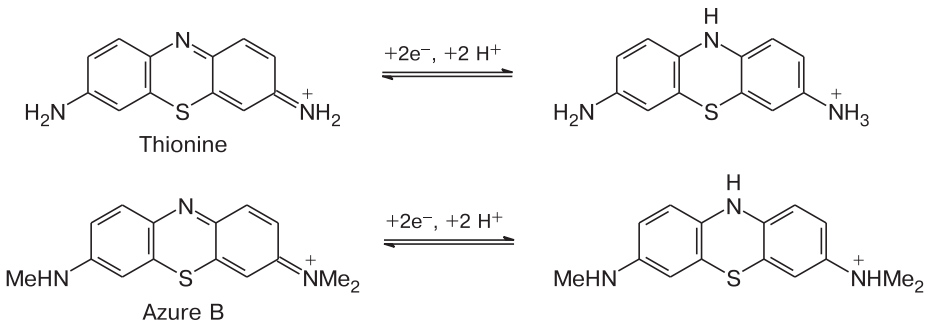
The redox capacity of the polymer slightly differs from that of the corresponding monomers. Therefore, no new peak pair emerges during polymerization; instead of this, voltammetric peaks initially assigned to the monomeric forms of dyes become broader. As far as polymerization products accumulate, the difference between the cathode and anode current peaks decreases. When the modified electrode was transferred after polymerization into a buffer solution free of monomeric dyes, the peaks of polythionine and poly(Azure B) became more symmetric, although a great difference of potentials remained.

The measurement of formal redox potential (the half-sum of oxidation and reduction peak potentials) as a function of pH of a buffer solution showed the equivalent numbers of hydrogen ions and electrons transferred at the rate-limiting step for both dyes (54 mV per pH unit in a range of pH 4—7 for thionine and 47 mV per pH unit for Azure B). A slight decrease in the curve slope relative to the theoretical value of 59 mV per pH unit can be explained by intrinsic buffer properties of the polymer exhibited due to the presence of amino groups therein involving in acid-base interactions along with the carboxylate groups of the carbon species. The plot of the cathode peak current as a function of the scan rate formally corresponds to the diffusion nature of the process (linear dependence of the cathode peak current on the square root of scan rate). Since no diffusion-free dye molecules were present in the system, this can be explained by a shuttle electron transfer between oxidized and reduced moieties of the polymer chain.

Thus, the electrochemical behavior of polymer corresponded to Scheme 1 and was in line with reversible oxidation-reduction of the phenothiazine core of the monomers.

Introduction of pillar[5]arene into the composition of the carbon black surface layer increased the oxidation-reduction peaks of the dyes by 2.5—4-fold (see Fig. 1, *b*). During potential scan, the current values decreased due to limited conductivity of the polymer layer and the peaks became broader. Changes were especially noticeable after the first scan. Since the electrochemical activity regions of the macrocycle and the dyes almost coincide,[28] the contributions of individual reactions could not be distinguished. The oxidation-reduction peaks of polymer dyes in the presence of macrocycle were more pronounced and the cathode peaks had a narrower symmetric shape than those in the absence of pillar[5]arene, which is typical of surface electron transfer reactions. The sensitivity of signals to the pH change was almost independent of whether the

Scheme 1



macrocycle was introduced into the surface layer or not. The stability of resulting coatings is a significant advantage of polymerization in the presence of the macrocycle. When the electrode after polymerization in the absence of macrocycle was transferred into a buffer solution free of dyes, the signals on CV curves changed during five-ten measurements due to leaking of low-molecular-weight oligomers and monomers of the dyes from the film. The peak currents decreased compared to the starting values by 25—30% and peaks themselves became less pronounced and transformed into a wave. Upon electropolymerization in the presence of pillar[5]arene, the signals for the polymer layer stabilized even after the second potential scan and kept stable characteristics (current and potential) for at least two weeks of measurements.

Effect of DNA on the electrochemical characteristics of the coatings. The addition of DNA to the reaction system decreased the recorded currents upon polymerization of Azure B and increased them in the case of thionine. The effect of the biopolymer depended on the sequence of addition and polymerization. The highest effect in the case of thionine was observed upon dropwise dispension of a DNA solution onto the electrode prior to polymerization, while the deposition of DNA over the polythionine layer changed the CV curves similarly to addition of DNA into a solution of thionine at the polymerization step.

In the case of Azure B, the signal decreased; highest changes occurred upon addition of DNA to a polymerization solution, while modification of the electrode prior to its contact with the monomer and over the poly(Azure B) layer had virtually identical and considerably weaker effect on CV curves. The corresponding plots are shown in Fig. 2. Preliminary heat treatment of DNA and its oxidation with peroxide radicals generated by the reaction of hydrogen peroxide with copper(II) ions (the mechanism of generation of peroxide radicals was considered in Ref. 29) decreased the effect of native DNA on the signal for Azure B. The current-voltage curves obtained in the presence of heat-denaturated DNA held an intermediate position. This is likely due to the fact that, in the chosen denaturation method, a sufficient portion of the biopolymer preserves a structure close to the native one.

In the case of polythionine, the signal for the polymer layer in the presence of DNA increases; with regard to a decrease in the level of effect the oxidized and heat denatured DNAs are after the native DNA. Addition of the biopolymer to the electropolymerization mixture considerably increases the effect of DNA on the CV curves of polythionine compared to its deposition under or over the polymer layer. The polythionine coatings in the presence of DNA demonstrate more symmetric peaks with a lower contribution of sorption on the CV curves, whereas, in the case of Azure B, the peaks keep asymmetry with a slightly higher cathode peak and a sloping anode peak.

The effect of DNA on the CV curves of polymer dyes depends on the amount of the added biopolymer (Fig. 3). Figure 3 shows relative changes in the cathode peak current of the polymer dye (I) compared to the signal obtained under the same conditions in the absence of DNA (I_0). The effect of DNA becomes apparent at concentrations of 0.05—1.0 mg mL^{-1} in a solution for polymerization of the dye and then comes to the ultimate value. In the case of thionine, the native DNA increases the signal more than 6-fold , which cannot be explained only by the template effect, especially since the current values vary 2—2.5-fold. One can assume that DNA has an effect not only on the amount of polythionine accumulated on the electrode, but also on the efficiency of pillar[5]arene as an electron transfer transmitter mediating reactions of the dye. Indeed, it has been shown earlier[22] that in the absence of polymer dye films adsorption of DNA can increase the oxidation-reduction peak currents of pillar[5]arene itself, although not so much as in the present work. This can be explained by disaggregation of molecular DNA complexes on the electrode where the macrocycle molecules are combined into supramolecular polymer particles through hydrogen bonding between the hydroxy groups of the rim. The formation of these particles was confirmed by scanning

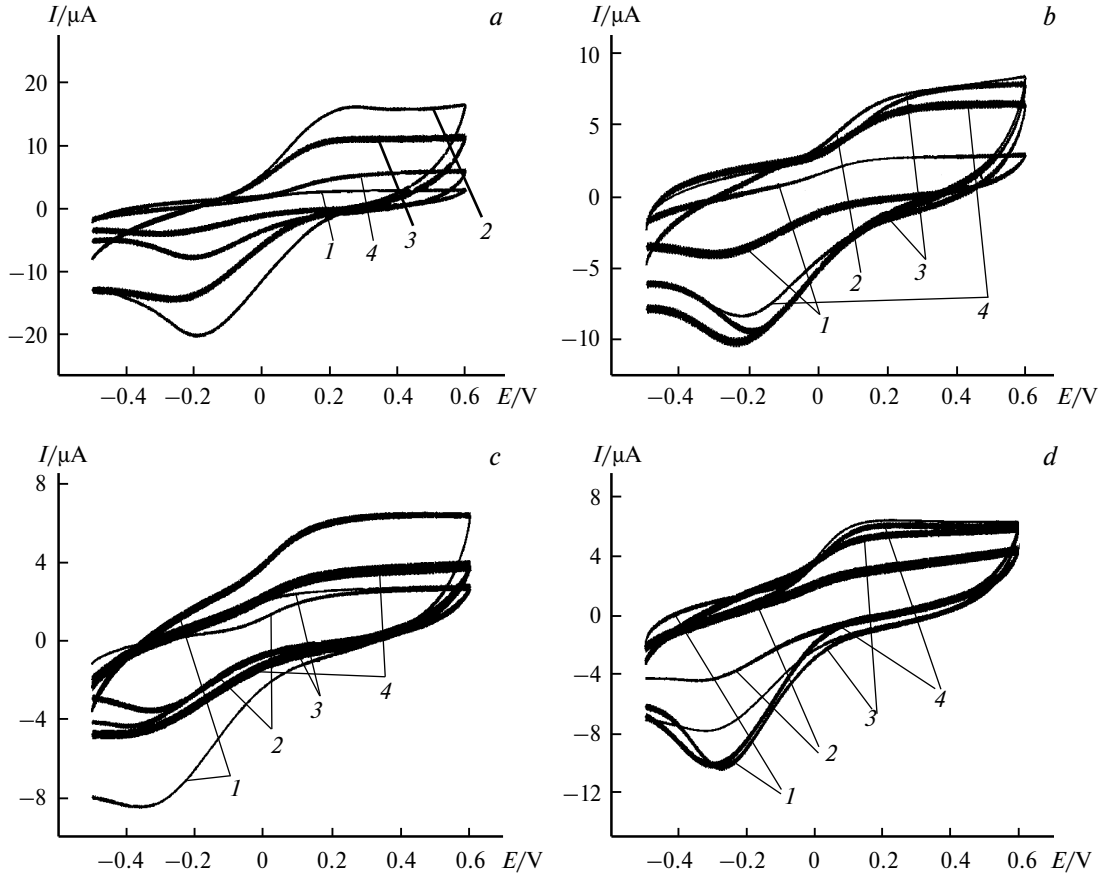


Fig. 2. CV curves for polythionine (*a*, *b*) and poly(Azure B) (*c*, *d*) obtained on the glassy carbon electrode modified with carbon black and pillar[5]arene in the presence of DNA added to a polymerization solution at a concentration of 1 mg mL^{-1} (*a*, *c*) or deposited onto the electrode in an amount of 2 μg prior to polymerization (*b*, *d*). *1* is the control experiment, *2* is native DNA, *3* is oxidized DNA, and *4* is heat-denaturated DNA.

electron microscopy. Also, associates were detected in the study of the electrochemical activity of pillar[5]arene in organic solvents.

It appears that DNA present in solution accumulates thionine molecules favoring their oxidation and polymerization on the electrode. Denaturation of DNA, either

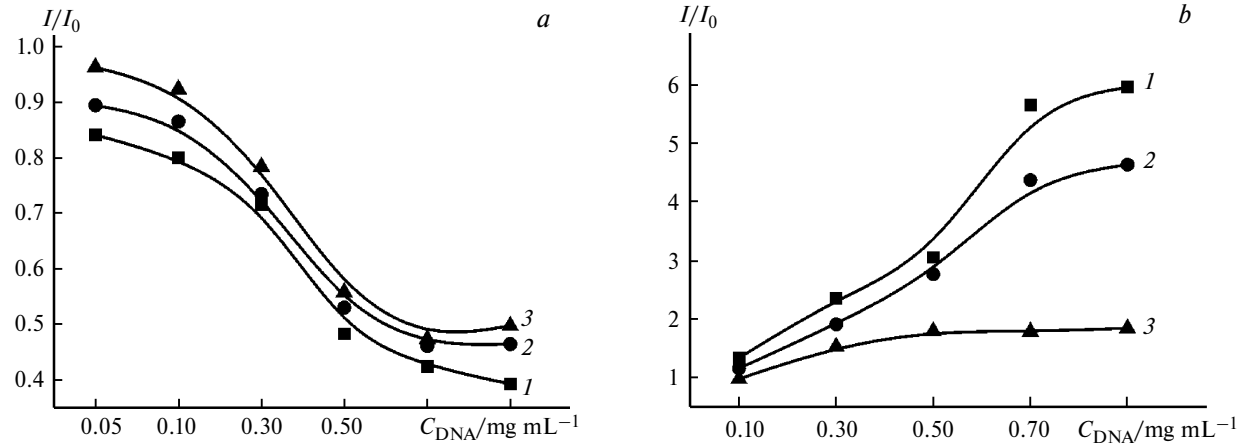


Fig. 3. Relative change in the cathode peak current of poly(Azure B) (*a*) and polythionine (*b*) as a function of the concentration of DNA added to the polymerization mixture. *1* is native DNA, *2* is chemically oxidized DNA, and *3* is heat-denaturated DNA. *I* and I_0 are the peak currents of polymer dyes in the presence and in the absence of DNA, respectively.

heat or oxidative, impairs the structural linearity and structural regularity of the template; therefore, the DNA effect decreases. On the sensor surface, pillar[5]arene associates separate under the action of negative phosphate residues of the DNA backbone, which multiply increases the number of functional groups involved in the electron transfer.

Azure A differs from thionine by the presence of three methyl substituents at the amino groups of the phenothiazine moiety. They separate positive and negative centers of the dye oligomers, DNA, and carbon black. As a result, there occurs mechanical blocking of a portion of the electrode surface rather than the template effect and separation of pillar[5]arene molecules and, as a consequence, currents on the CV curves decrease.

Thus, DNA exerted an opposite effect on the electrochemical characteristics of electropolymerized dyes due to combination of two factors: restriction of accumulation of Azure B sterically more hindered than thionine and additional effect of pillar[5]arene on associates acting under these conditions as an electron transfer mediator between the phenothiazine centers of dyes and the electrode.

Although effective concentrations of DNA are quite high, aliquots taken upon its application onto the electrode do not exceed 2 μL, which allows one to use microgram amounts of DNA. Despite the fact that the developed DNA sensors are of single use (since a damaged DNA cannot be regenerated in the layer), the procedure for electrode modification is very simple and can be reproduced immediately prior to measurement, which facilitates its standardization. Also, one can expect that any other biochemical interactions, including intercalation of native DNA with anticancer cytostatic drugs, can have an effect on interactions between components of the layer. Looking into the future, this will allow one to develop biosensors for screening of anticancer drugs, whose action is based on similar principles of signal generation.

Thus, in the present work we established that polymerization of two structurally related phenothiazine dyes on the electrode modified with pillar[5]arene and carbon black retains the mechanism of reversible two-electron oxidation-reduction of the phenothiazine moiety. The presence of the macrocycle by its inclusion into the reversible electron transfer chain increases the recorded currents and reversibility of redox reactions of the dyes. Upon addition of DNA to the system, the signal for polythionine increases and the peaks for poly(Azure B) decrease as a result of combination of two following factors: template effect being manifested in accumulation of the dyes on the DNA molecules due to electrostatic interactions and blocking of a portion of the electrode surface with inactive and nonconductive biopolymer molecules. An unusually high (more than 5-fold) increase in the oxidation-reduction currents of polythionine upon addition of the native DNA to the reaction mixture is caused by further increase in the efficiency of the mediating effect of the macrocycle due to disconnection of its associates linked through intermolecular hydrogen bonding. An imperfection in the native DNA structure resulting from exposure to heat or peroxide radicals decreases the effect of DNA on the electrochemical parameters of polymer films. Discrimination of the native, heat-denaturated, and chemically oxidized DNAs can be confirmed further by their different effects on the polymer forms of thionine and Azure B.

The developed sensors combining electropolymerization and electrostatic inclusion of DNA into the resulting modifying coatings can find application for monitoring physical and chemical factors, which cause DNA damage.

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