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Echinomycin and cobalt-phenanthroline as redox indicators of DNA hybridization at gold electrodes

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1. ABSTRACT

A bis-intercalator echinomycin (ECHI) and a simple intercalator $[Co(phen)_3]^{3+}$ were used as a novel electrochemical redox indicators to detect DNA hybridization at gold electrodes (AuE). In order to minimize the nonspecific adsorption of oligonucleotides (ODN), the thiol-derivatized oligonucleotides were immobilized onto AuE in the first step, and the exposition of AuE to 6-mercapto-1-hexanol (MCH) followed in the second step of this procedure. In this arrangement good reproducibility and discrimination between single-stranded (ss) probe and double-stranded (ds) hybrid DNA were obtained. While both redox indicators showed a good ability to discriminate between the ss probe and ds hybrid DNA, the signals of ECHI were by an order of maginitude higher than those of $[Co(phen)_3]^{3+}$ in a good agreement with stronger DNA binding by the bis-intercalator as compared to the simple intercalator. In addition, DNA single-base mismatch (DNA point mutation) was easily detected by means of ECHI.

2. INTRODUCTION

Recently there has been considerable interest in developing DNA hybridization biosensors (genosensors) for rapid genetic analysis (1-3). Electrochemical DNA biosensor has an enormous potential for the detection of the presence of genes or mutant genes associated with inherited human diseases providing a simple, rapid and low cost point-of-care detection of specific nucleic acid sequences (4-7).

Many electrochemical studies are dealing with the interactions of DNA with redox indicators, which are used in determination of the DNA nucleotide sequence (4,5,8-25). These indicators are usually represented by small electroactive substances such as organic dyes, such as methylene blue (11-14), anthracycline antibiotics such as daunomycin (15,16) or cationic metal complex compounds with aromatic ligands, e.g. $[Co(phen)_3]^{3+}$ or $[Co(bipy)_3]^{3+}$ (5,8,17,18), etc. The DNA indicators interact in a different way with single-stranded (ss) and double-stranded (ds)

Table 1. Oligonucleotides

Thiol-capped probe	
P1	5' – SH- TAC GAG ACG GGG GGC TT — 3'
P2	5'– SH- TTT TTT TTT CGA TCG AG — 3'
Complementary	
T1p	5' – AAG CCC CCC GTC TCG TA — 3'
T2p	5' – CTC GAT CGA AAA AAA AA — 3'
Noncomplementary	
NC	5' – TGA AAC GAT TAT GAT AC — 3'
T2m	5' – CTC GAT CGA AGA AAA AA — 3'

DNA providing different electrochemical responses for ds and ssDNA. The performance of such redox indicators frequently depends on the nature of the electrode used as a transducer in the sensor.

In this report we compared electrochemical behavior of ECHI and $[Co(phen)_3]^{3+}$ in detection of DNA hybridization by self-assembled monolayer (SAM) immobilization onto gold electrodes. SAM technique was frequently used in many electrochemical studies dealing with DNA hybridization (26). SAM is formed on the gold surface as a result of spontaneous adsorption of molecules possessing high affinity towards the surface, in our case molecules oligodeoxynucleotides (ODN) containing –SH group. There are many strategies how to prepare DNA SAM on a gold surface (19,27,28).

Millan and coworkers (17,20) reported earlier electrochemical detection of DNA hybridization in the presence of electroactive indicator, $[Co(phen)_3]^{3+}$ that interacts preferentially with dsDNA. Carter et al. (21) showed that the homogenous DNA-binding properties of tris (1,10-phenanthroline) cobalt (III) could be determined electrochemically, since the peak currents depend on diffusion coefficients and the DNA-bound complexes diffuse much more slowly than the free complexes. Recently this indicator was used to detect calf thymus DNA at gold nanoparticles (10).

In our previous papers we studied electrochemical behavior of echinomycin (ECHI) and its interaction with ss and dsDNA (22-25) at hanging mercury drop electrode (HMDE) or mercury film electrode. We found that ECHI is electroactive yielding several voltammetric or chronopotentiometric signals. We confirmed a strong binding (intercalation) of ECHI to dsDNA, which was in good agreement with results obtained by other authors (29-31). Electrochemical studies concerning interaction of ECHI with DNA were recently summarized in several reviews (1,2,32-34). Quite recently the interaction of ECHI with guanine was investigated either by cyclic voltammetry at guanine modified glassy carbon electrode or by spectroscopic techniques (ultraviolet/visible adsorption spectroscopy and Fourier-transform infrared spectroscopy) in solution. Spectral data confirmed formation of adduct between alanine contained in ECHI molecule and guanine residues within DNA (35).

In this study a bis-intercalator ECHI, as a novel electrochemical indicator for DNA hybridization, and the well known indicator $[Co(phen)_3]^{3+}$ were used for the first time in order to detect DNA hybridization at gold electrode (AuE) surface. $[Co(phen)_3]^{3+}$ is known to associate with the DNA double helix by intercalative and minor-groove binding (36). To minimize the nonspecific adsorption of oligonucleotides, the following protocol was exploited: (1) the thiol-derivatized oligonucleotides were immobilized on AuE and (2) exposed to 6-mercapto-1-hexanol (37) in the second step of this procedure. The results obtained with $[Co(phen)_3]^{3+}$ and ECHI were compared.

3. MATERIALS AND METHODS

3.1. Apparatus

Voltammetric signals were collected with an AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.8 software package (Eco Chemie, The Netherlands). The three electrode system consisted of the gold working electode (AuE) (Bioanalytical Systems-BAS, USA), the Ag/AgCl/KCl reference electrode and a platinum wire as the auxiliary electrode. The convective transport was provided by a magnetic stirrer.

3.2.Chemicals

Echinomycin (ECHI) and MCH (6-mercapto-1-hexanol) were purchased from Sigma – Aldrich Chemical Company (Germany). Tris (1,10-phenanthroline) cobalt (III) prechlorate, $[Co(phen)_3 (ClO_4)_3]$ was synthesized as reported by B. Cetinkaya following the literature Dollimore and Gilliard et al. (38) using reagent grade cobalt (II) chloride and bromine (Sigma), perchloric acid (Sigma) and ligand 1,10-phenanthroline (Sigma).

The 17–mer thiol capped probe, complementary and noncomplementary DNA oligonucleotides (ODN), were purchased (as lyophilized powder) from Thermo Electron Corp. (Germany). Their base sequences are shown in Table 1.

All ODN stock solutions were prepared with ultrapure tri-distilled water and kept frozen. More dilute solutions of ODN were prepared with either 0.50 M acetate buffer (pH 4.80) or 20 mM Tris-HCl buffer (pH 7.00), according to the hybridization protocol. Other chemicals were of analytical reagent grade. The in-house distilled and deionized water was used in all solutions.

3.3. Electrode pretreatment

The AuE surface was prepared for modification by polishing with a 0.1 μm alumina/water slurry on a polishing cloth for 5 min and it was sonicated during 5 min. Then electrode was then cleaned by using cyclic voltammetry procedure in potential range – 0.9 and + 0.9 V in 0.05 M H_2SO_4 solution at a scan rate of 150 mV/sec until reproducible curves were recorded. The electrode was rinsed with distilled water for 5 sec before probe immobilization.

The AuE SAM modification and the detection of hybridization were performed as shown in Figure 1A.

3.4. Thiol-linked probe immobilization onto the surface of gold electrode

Probe immobilization onto the AuE preatreated surface was performed as follows: The AuE was inverted

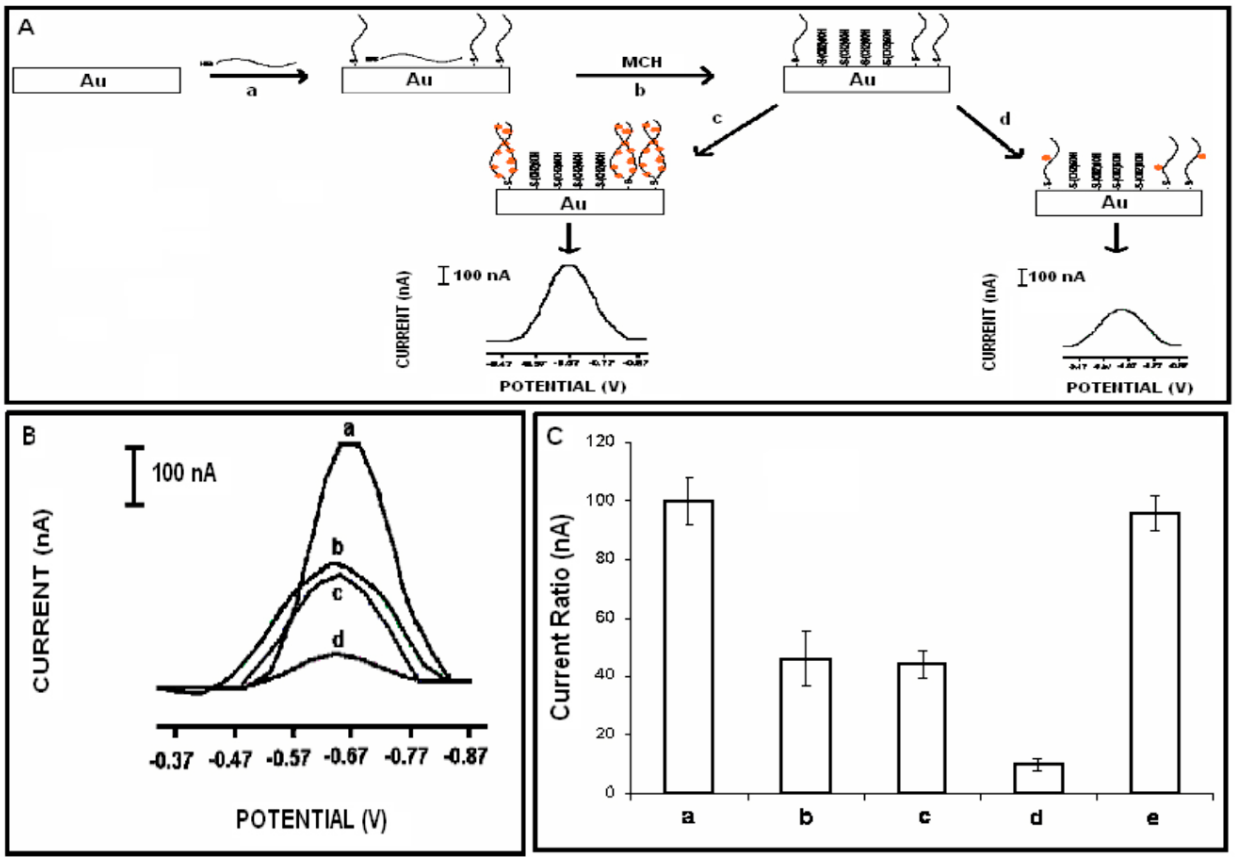

Figure 1. A. Scheme representing electrochemical detection of DNA hybridization with a bis-intercalator ECHI. (a) SH-linked probe immobilization onto gold electrode (AuE) surface, (b) addition of 0.1 mM MCH in order to provide a better surface, (c) electrochemical measurement based on ECHI signal, after binding of ECHI to DNA hybrid (between probe and complementary), (d) electrochemical measurement based on ECHI signal, after binding of ECHI to DNA probe. B. Differential pulse voltammograms of ECHI: (a) hybridization of the probe P1 with complementary strand T1, (b) probe P1 alone, (c) hybridization of the probe P1 and noncomplementary strand (d) bare electrode. C. Histograms for the relative reduction signals of ECHI obtained: (a) after hybridization of the probe P1 with complementary strand T1, (b) probe P1 alone, (c) hybridization of the probe P1 and noncomplementary strand (d) bare electrode, (e) hybridization between probe and complementary T1 in the presence of noncomplementary in ratio (1:1).

and 20 µL of 10 µg/ml thiol-linked probe in 0.5 M acetate buffer solution, pH 4.80 (ABS), was pipetted onto the surface of AuE. The droplet was air-dried for 3 h. It was then rinsed with ABS for 10 s to remove unbound ODN material.

SAM was prepared by the immersion of the probe modified AuE in freshly prepared 75 : 25 (v/v) ethanol : water solution containing 0.1mM MCH. AuE was incubated in this ethanolic solution for 1 h. The AuE / SAM was rinsed with 75 : 25 (v/v) ethanol : water.

3.5. Hybridization

For detection of hybridization, after the immobilization of thiol-linked probe, the probe modified AuE was inverted and 20 µL of 15 µg/ml complementary or noncomplementary or mismatched DNAs in 20 mM Tris buffer solution pH 7.0 (TBS) was pipetted onto the surface. The target droplet was air-dried for 1 h. It was then rinsed with TBS for 10 s to remove unbound ODN material.

3.6. Label binding to DNA

After immersing the electrode into the stirred 20 mM TBS containing either 2.5 µM ECHI or 0.2 mM $[Co(phen)_3]^{3+}$ with 20 mM NaCl, label was accumulated onto the surface during 5 min without applying any potential. Then, the electrode was rinsed with 20 mM TBS for 5 sec.

3.7. Voltammetric transduction

The reduction signal of the ECHI or $[Co(phen)_3]^{3+}$ was measured in the 20 mM phosphate buffer, pH 7.4, by using differential pulse voltammetry (DPV) in potential range between +0.6 V and –0.9 V at scan rate 50 mV/s and pulse amplitude 50 mV. The raw data were treated using the Savitzky and Golay filter (level

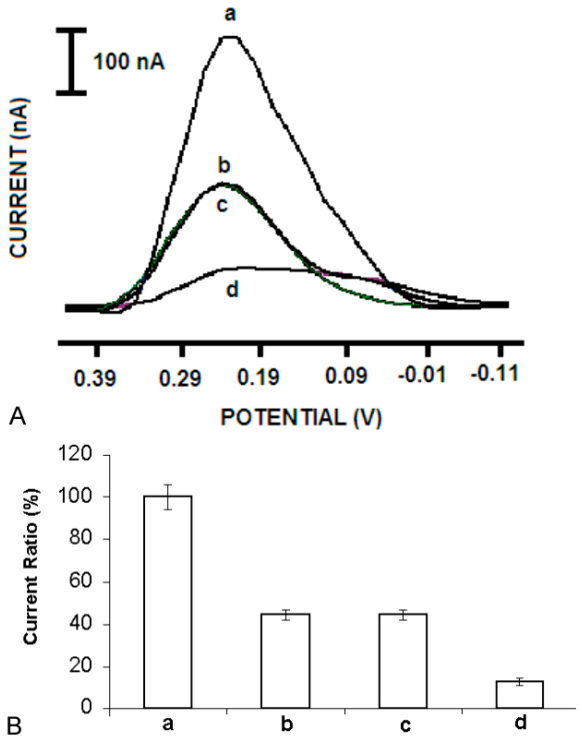

Figure 2. A. Differential pulse voltammograms of $[Co(phen)_3]^{3+}$: (a) hybridization of the probe P1 with complementary strand T1, (b) probe P1 alone, (c) hybridization of the probe P1 and noncomplementary strand (d) bare electrode. B. Histograms for the relative reduction signal of $[Co(phen)_3]^{3+}$ obtained: (a) after hybridization between probe and complementary, (b) probe P1 alone, (c) interaction between probe and noncomplementary, (d) bare electrode.

2) of the GPES software, followed by the moving average baseline correction with a "peak width" of 0.03.

Repetitive measurements were carried out by renewing the surface and repeating the above assay format.

4. RESULTS AND DISCUSSION

The electrochemical transduction of the ODN hybridization between the thiol-linked probe and complementary sequences was performed. The detection of hybridization was accomplished via the reduction of ECHI at -0.65 V, where the electroactivity of this label served for detection of the DNA duplex formation (hybridization) (Figure 1A). This increase in ECHI differential pulse voltammetric signal upon hybridization is shown in Figure 1B. The signal of ECHI observed with probe alone was significantly lower (Figure 1B-b). After hybridization with non-complementary (NC) (Figure 1B-c), ECHI signal was almost the same as that obtained with the probe alone. ECHI at bare electrode produced only a very small signal (Figure 1B-d).

Figure 1C shows ECHI signals after hybridization between probe and either complementary or NC ODN or the mixture of complementary and NC ODN in 1:1 ratio. The signal obtained with the hybrid was higher than the one with probe signal. The hybridization of the probe and NC ODN was also investigated (Figure 1C-c). Even in the presence of equimolar concentrations of NC ODN and complementary ODN, the DNA hybridization (with the complementary strand) was selectively detected.

To obtain the optimum concentration of complementary ODN, DNA hybridization was performed by using different concentration levels of complementary ODN between 10 µg/mL and 20 µg/mL. The lowest hybridization signal was obtained at 10 µg/mL concentration level. When the probe was exposed to complementary ODN at concentration of 15 µg/mL and 20 µg/mL, almost constant ECHI signal was obtained. Thus, 15 µg/mL was chosen as the optimum concentration of the complementary strand.

The detection of DNA hybridization was also investigated using signal of the $[Co(phen)_3]^{3+}$ well-known as an redox indicator of the DNA hybridization at carbon electrodes (21,22). In Figure 2A, the voltammograms represent the hybridization in the presence of complementary (Figure 2A-a) and NC ODN (Figure 2A-c). In agreement with previously reported works using carbon paste electrode (CPE) (4,5) and glassy carbon electrode (GCE) (17), the highest signal of $[Co(phen)_3]^{3+}$ at AuE was obtained with duplex DNA after the hybridization.

Figure 2B-a presents also the histogram upon hybridization with complementary ODN, showing a dramatic increase in current signal of $[Co(phen)_3]^{3+}$. After hybridization with NC ODN a signal similar to that observed with the probe alone was obtained (Figure 2B-c). The signal of $[Co(phen)_3]^{3+}$ at bare electrode is shown in Figure 2B-d. Our results thus show little difference in the ability to discriminate ssDNA from hybridizated dsDNA with $[Co(phen)_3]^{3+}$ or ECHI.

Three subsequent experiments using 15 µg/mL concentration of target ODN gave reproducible results as a mean ECHI signal of 290 nA and a RSD value of 10.6 % (n=3) by using AuE and 1 hour hybridization time. The detection limit (DL) estimated from S/N=3, corresponds to 26 fmole/mL target concentration. There has been no report yet regarding to detection of DNA hybridization using ECHI and $[Co(phen)_3]^{3+}$ at AuE surface. In comparison to the earlier reports of the detection of the DNA hybridization at carbon electrodes with $[Co(phen)_3]^{3+}$ redox indicator (4,5), here the reproducibility of the experiments was much better and DL was much lower.

Our results show that both ECHI and $[Co(phen)_3]^{3+}$ gave good results in DNA hybridization detection at gold electrodes covered with MCH as a second SAM. Without such a SAM the reproducibility and discrimination between ss and ds DNAs were rather poor (not shown).

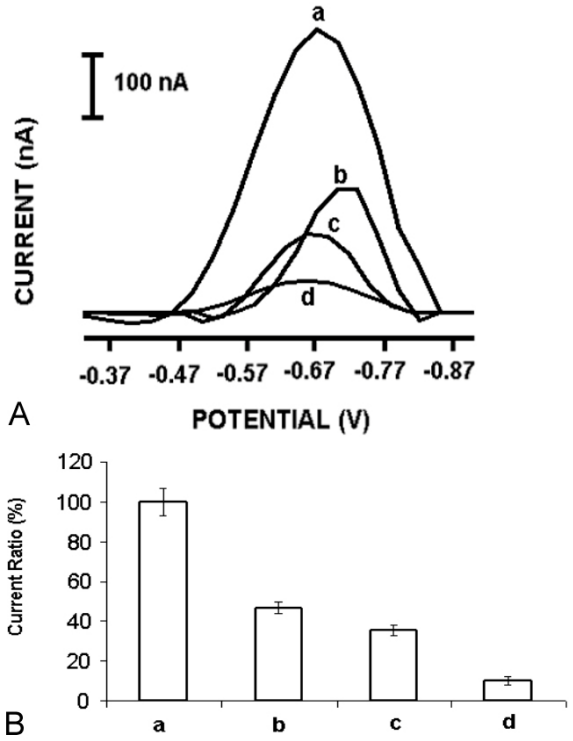

Figure 3. A. Differential pulse voltammograms of ECHI: (a) hybridization of the probe P2 with complementary T2p, (b) interaction of P2 and mismatched T2m, (c) probe P2 alone (d) bare electrode. B. Histograms for the relative reduction signal of ECHI obtained: (a) after hybridization of the probe P2 with complementary T2p, (b) interaction of P2 and mismatched T2m, (c) probe P2 alone (d) bare electrode.

4.1. ECHI sensitively reflects DNA point mutation

In another set of experiments we tested the ability of ECHI to detect a single base mismatch within the duplex (point mutation in target DNA). The sequence of DNA probe was optimized for ECHI bis-interacalation (sequence P2, Table I) and the target DNA was represented either by perfectly matching sequence (T2p, Table I) or by almost the same sequence in which only one adenine was replaced by guanine (T2m, Table I). Similarly to the previous measurements (Figure 1B), the reduction signal of ECHI produced by the perfectly matched duplex (P2/Tp2) was substantially higher than the signal of the probe (Figure 3A,B-a,c). On the other hand the single-base mismatch-containing hybrid (P2/T2m) produced much smaller signal (Figure 3A,B-b) than the perfectly matched P2/T2p (Figure 3A,B-a) but still significantly higher than the signal of the single-stranded probe (Figure 3A,B-c).

Both ECHI and $[Co(phen)_3]^{3+}$ gave good results in DNA hybridization detection at gold electrodes covered with MCH as a second SAM. Concerning to use MCH as a second SAM layer, our results show also a very important advantage compared to the reproducibility and discrimination between ss and dsDNAs, which were rather poor without such a SAM layer on gold surface.

Typical intercalators are planar aromatic cations, which insert their aromatic ring system between DNA base pairs. Bis-intercalators have two potential intercalating ring systems connected with linkers, which can vary in rigidity and length (39,40). The interaction of the ring systems with DNA can be controlled to a great extent by the characteristics of the linker. Crystal structure of echinomycin complex with d(CGTACG) was determined (41); in such a structure the quinoxaline rings was bis-intercalated with the C-G sequence sandwiched between the intercalated rings in agreement with solution studies.

To test two different redox indicators in the first part of this paper (Figs. 1 and 2) we chose a random probe sequence (P1) without considering the binding properties of the bis-intercalator ECHI. Under the given conditions ECHI showed properties of a good redox indicator but its ability to discriminate between ss and dsDNAs did not significantly differ from that of $[Co(phen)_3]^{3+}$ (Figs. 1 and 2). On the other hand the current values (obtained with the perfectly matched DNA) produced by $[Co(phen)_3]^{3+}$ (Figure 2) were almost ten times smaller than those yielded by ECHI (Figure 2A). Using another probe sequence (P2), which was designed to produce one optimum binding site for ECHI bis-intercalation and only two binding sites for $[Co(phen)_3]^{3+}$, the ratio of current values of ECHI/$[Co(phen)_3]^{3+}$ corresponded almost to 20, i.e., it was almost two-fold higher than that obtained with sequence P1.

Considering that signals of these redox indicators obtained at bare AuE (without DNA or MCH) did not significantly differ (Figs. 1 and 2), we may conclude that ECHI bound more strongly to the DNA duplex, in agreement with its bis-intercalation mode. On the other hand the ability of $[Co(phen)_3]^{3+}$ to discriminate between single-stranded and double-stranded DNA was not substatially different from that of ECHI. Excellent ability of ECHI to recognize the single base mismatch at AuE (Figure 3-b) makes this redox indicator worth of further investigations. Our results and particularly the ability of recognizing the single-base mismatch resemble the results of J. Barton et al. (42) based on the charge transfer through the base stack of the DNA duplex. On the other hand it is rather strange that in our experiments the single-stranded probe DNA produced appreciable signals (both with ECHI and $[Co(phen)_3]^{3+}$ redox indicators, Figs. 1B and 2A). The electrode was modified by MCH and efficiently screened against direct contact of the free redox indicator as documented by a negligible signals of unbound redox indicators (Figs. 1B and 2A). If the signals of perfectly matched duplexes are due only to the electron transfer through the duplex base stack the question would arise what is the reason for the signal of single-stranded DNA? Does it also conduct electrons or is there any other mechanism in operation?

At this stage, our results do not allow us, to make any definite conclusions about the possible charge transfer through the dsDNA base stack (9), because the orientation of the probe ODN molecules (upright position or laying flatly on the surface) was not controlled in our experiments.

For deeper study of the charge transfer in DNA duplexes a strategy described by Barton et al. (42,43) should be used. Such experiments are under way in our laboratory. ECHI showed excellent discrimination between ss and ds DNAs at the mercury electrodes (22-25). We obtained similar results at gold electrodes but in addition we showed that a single-base mismatch can be detected by this redox indicator at AuE.

5. CONCLUSION

Our results show that both ECHI and $[Co(phen)_3]^{3+}$ enable selective monitoring of DNA hybridization at AuE surface. The signals of DNA-bound ECHI are by an order of magnitude higher than those of DNA-bound $[Co(phen)_3]^{3+}$. Moreover, using ECHI as a redox indicator we were able to sensitively detect single-base mismatch in the DNA duplex. Many diseases are associated with point mutation at specific locations of the genome. Electrochemical detection of such single-base mutation represents a promise for faster and less expensive technology for decentralized diagnostics. First successful electrochemical detection of point mutation was achieved in 1996 by using PNA probe instead of DNA in combination with a $[Co(phen)_3]^{3+}$redox indicator at carbon electrodes (44). While with PNA probe satisfactory results were obtained at these electrodes, this indicator failed in detection of the point mutation with a DNA probe. Since that time a number of other methods of electrochemical detection of point mutations have been developed (reviewed in (45,46)) many of them being based on different stabilities of perfectly matched and single-base mismatched duplexes. In contrast to the latter methods, which usually require measurements at elevated temperature, the proposed analysis with ECHI indicator is simple and can be done at room temperature. We may conclude that detection of point mutation with ECHI at AuE represents an interesting extension of the available methods. This detection method will require further research to find optimum experimental conditions and to understand better the processes at the electrodes, which are behind the ability of the system to identify the DNA point mutations.

6. ACKNOWLEDGEMENTS

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Abbreviations: ECHI - echinomycin, dsDNA - double-stranded DNA, ssDNA - single-stranded DNA, ODN - oligodeoxynucleotide, HMDE - hanging mercury drop electrode, DPV - differential pulse voltammetry, MCH - mercaptohexanol, AuE - gold electrode, ABS - acetate buffer, TBS - TRIS-HCl buffer, NC – noncomplementary

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