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Enzyme-Amplified Amperometric Sandwich Test for RNA and DNA

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A one-step enzyme-amplified amperometric sandwich hybridization test for RNA and DNA is described. The test utilizes a carbon electrode, modified with a film of co-electrodeposited avidin and redox polymer; the redox polymer electrically "wiring" horseradish peroxidase (HRP) reaction centers upon contact. The film is made specific for the particular RNA or DNA sequence tested by conjugating its avidin with a biotinylated oligonucleotide, complementary to the assayed sequence. This oligonucleotide-modified redox polymer film, prepared prior to the test, forms the base of the sandwich. The center layer of the sandwich, added in the test, is the analyte RNA or DNA; its top is a second complemetary oligonucleotide, which is HRP-labeled, and is cohybridized in the test. The test consists of mixing the analyte DNA or RNA solution, the HRP-labeled oligonucleotide solution, and a hydrogen peroxide solution, immersing the base-layer carrying electrode applying a potential of 0 V versus Ag/AgCl, and measuring the H_2O_2 electroreduction current. Completion of the sandwich brings the HRP label into electrical contact with the redox polymer, converting the nonelectrocatalytic base layer into an electrocatalyst for the electroreduction of H_2O_2 to water. Flow of H_2O_2 electroreduction current when the electrode is poised near Ag/AgCl potential indicates the presence of the analyte RNA or DNA. The current density for the maximally sandwich-covered electrode was 250 μA cm^{-2}, exceeding more than a 100-fold the current density flowing upon nonspecific binding of the HRP-labeled oligonucleotide. High concentrations of irrelevant DNA and diluted serum did not interfere with the assay. When the electrodes were rotated in order to make the solution-phase mass transport rapid, the test was completed in ∼30 min. The test was applied in probing for the presence of a 60-base *E. coli* mRNA sequence.

There are two traditional ways of detecting DNA and RNA. The first involves immobilization of the target DNA, followed by its detection with a labeled probe.[1] The second involves double hybridization in a "sandwich", where the analyte RNA or DNA (the "target") is hybridized to a particle or surface-bound oligonucleotide and then hybridized with a marker-labeled probe.[2] The disadvantage of the apparently simpler first approach is that, unlike sandwich hybridization, it requires modification of the surface with an immobilization-enabling function. In either approach, radioactive, [3,4] luminescent, [5−8] and enzyme labels [6,9,10] have been used. The assays require amplifying the analyte DNA by PCR.[12] Here we "wire" the reaction centers of the probe-labeling enzyme of the sandwich to an electron-conducting redox polymer-modified electrode and detect the hybridization of the probe to the surface-bound RNA or DNA "target" or analyte as an electrical current. The current flows as a result of the electrocatalysis of the electroreduction of hydrogen peroxide to water by the redox polymer "wired" peroxidase. The physical platform on which the assay is built is an electrodeposited film of the electron-conducting, enzyme "wiring" redox polymer, containing co-electrodeposited avidin.

Electron-conducting, enzyme 'wiring" redox hydrogels have been employed earlier in electrodes assaying substrates of the "wired" enzymes,[13−16] the enzymes,[17] antibodies,[18,19] and oligonucleotides in non-sandwich-type DNA assays.[20−22] Electroanalytical methods not involving enzymes, sensitively detecting DNA

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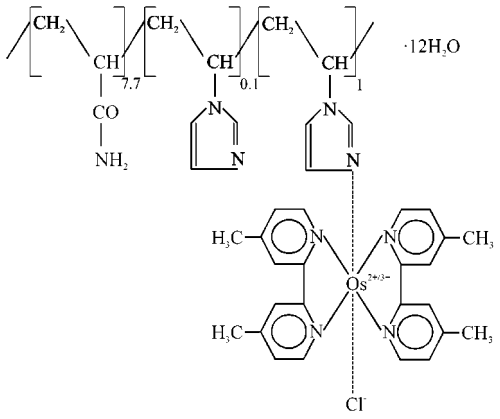

Figure 1. Structure of hydrazine-treated copolymer of poly(acrylamide) and poly(*N*-vinylimidazole) complexed with [osmium(4,4′-dimethyl-2,2,′-bipyridine)$_2$ Cl]$^{+/2+}$.

and RNA, have been reported. These were based on conductive polymers,[23] nucleic acid-modified electrodes,[24,25] bonding of oligonucleotides to electrode surfaces,[26−29] and the use of redox-active probes that bind with nucleic acids.[25,30,31]

The redox polymer used in this study is a water-soluble copolymer of acrylamide and vinylimidazole, complexed with osmium (4,4′-dimethyl-2,2′-bipyridine) chloride (PAA−PVI−Os, Figure 1).[32] Polyacrylamide-based gels are advantageous, because they do not bind nonspecifically proteins or nucleic acids.[21,32] Incorporation of avidin in the electrodeposited base layer provides a generic platform to which any commercially available biotinylated oligonucleotide can be promptly and simply attached.

EXPERIMENTAL SECTION

Materials. The glassware was washed (with detergent), sprayed with RNaseOUT (Gene Technology Inc.), and then rinsed with deionized water. Sodium periodate (Catalog No. 31,144-8) was obtained from Aldrich. Avidin (Catalog No. A9390), salmon testes DNA (Catalog No. D9156), and biotinamidocaproyl-labeled peroxidase (biotinylated HRP or B-HRP) (Catalog No. P9568) were obtained from Sigma. All buffer salts and other inorganic materials were obtained from Sigma or Aldrich unless otherwise stated. Phosphate-buffered saline (PBS) was 8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4 (BupH-modified Dulbecco's phosphate-buffered saline pack, Pierce). The hybridization buffer (HB) consisted of 10 mM *N*-(2-hdroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 1 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0. Tris/EDTA (TE) buffer consisted of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris), 1 mM EDTA pH 7.75. Goat serum (Catalog No. 005-000-121) was purchased from Jackson Immuno Research Laboratories (West Grove, PA.).

The electron-conducting redox polymer (PAA−PVI−Os), a 7:1 copolymer of acrylamide and 1-vinylimidazole, the imidazole function being complexed with [Os(4,4′-dimethyl-2,2′-bipyridine)$_2$Cl]$^{2+/3+}$, was prepared using a modified version of an earlier procedure.[22] Briefly, 24 g of acrylamide, 7.0 mL of *N*-vinylimdazole, 0.69 mL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 0.6 g of ammonium persulfate were reacted in 350 mL of water in a tightly closed vessel. The resulting polymer was purified by twice precipitating in methanol and then vacuum-dried overnight. Os(dmebpy)$_2$Cl$_2$ (dmebpy = 4,4′dimethyl-2,2′-bipyridine) was prepared as reported.[33] The osmium-derivatized polymer was prepared by refluxing Os(dmebpy)$_2$Cl$_2$ (624 mg) with the copolymer of acrylamide and *N*-vinylimidazole (430 mg) in 50 mL of anhydrous ethylene glycol under argon for 24 h. The polymer was then precipitated in ether−acetone (800 mL/200 mL) and dried under vacuum.

The oligonucleotide sequences were synthesized by Synthetic Genetics (San Diego, CA.). The capture probe, which we termed EPRO, was a 52-mer with a 12-T spacer and biotin label at the 3′-end and was complementary to positions 1−40 at the 5′-end of the *Escherichia coli* 16S rRNA. The oligonucleotide sequences are shown in Table 1. The detection probe, which we termed ETAR1, was a bacterial domain-specific 30-mer probe with a 12-T spacer and HRP label at the 5′-end. It was complementary to positions 338−355 in the *E. coli* 16S rRNA. EMID was a 75-mer complementary to EPRO and ETAR1 at their 5′- and 3′-ends, respectively, and was designed to serve as a test DNA for hybridization. Complementary to EPRO, ETAR2 was also a 52-mer with a 12-T spacer and an HRP label at the 3′-end. It was designed to serve as a test oligonucleotide for verification of hybridization and connection of EPRO to the redox polymer. *E. coli* 16S + 23S rRNA (Catalog No. 206 938) was obtained from Boehringer Mannheim.

Equipment. Electrochemical measurements were performed with a computer-controlled CH Instruments (Austin, TX) model 832 bipotentiostat. All tests were carried out in a Faraday cage using a water-jacketed, three-electrode electrochemical cell consisting of a disk rotating electrode, an Ag/AgCl reference electrode, and a platinum counter electrode. The working electrode was rotated using a Pine Instruments AFMSRX rotator and an MSRX speed controller. The rotating disk elecctrodes were prepared by embedding a carbon rod (3-mm diameter, Atomergic V10) in a Teflon shroud using epoxy (Polysciences, Catalog No. 01916). Miniature reference electrodes (Catalog Nos. EE008 and EE009) were made by Cypress Systems. Prior to use, the electrodes were polished with 0.3-μm alumina paste, sonicated in deionized water, and then washed again in deionized water.

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Table 1. Oligonucleotide Sequences

name	sequence (5′-3′)
EPRO	GCC AGC GTT CAA TCT GAG CCA TGA TCA AAC TCT TCA ATT TTT TTT TTT TTT T
ETAR1	TTT TTT TTT TTT GCT GCC TCC CGT AGG AGT
EMID	AAA TTG AAG AGT TTG ATC ATG GCT CAG ATT GAA CGC TGG CTT TTT TTT TTT TTT TTT ACT CCT ACG GGA GGC AGC
ETAR2	AAA TTG AAG AGT TTG ATC ATG GCT CAG ATT CGC TGG CTT TTT TTT TTT T

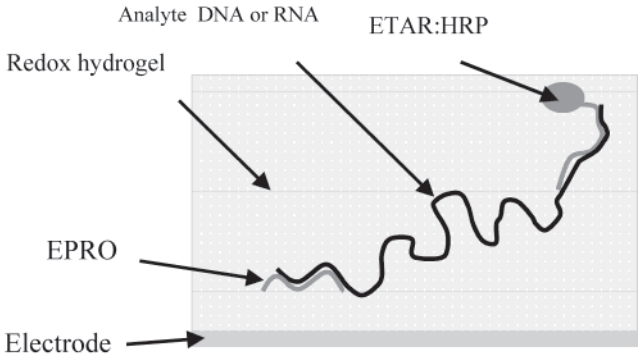

Figure 2. Scheme of the DNA/RNA sandwich hybridization assay.

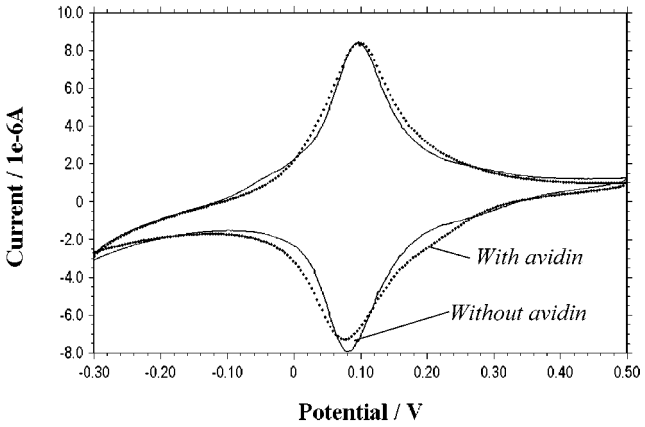

Figure 3. Cyclic voltammograms of the electrodeposited films: 3-mm glassy carbon electrode; scan rate 50 mV/s; pH 7.4 PBS.

Electrodeposition and Electrochemical Measurements. The avidin-containing redox polymer was electrodeposited in a 15-mm-diameter, 1-mL machined acrylic cell fitted with a miniature Ag/AgCl reference electrode. A 100-μm-thick platinum foil served as counter electrode and as the base of the cell. A 30-μL aliquot of the PAA−PVI−Os polymer solution (10 mg/mL in water), 600 μL of deionized water, and 15 μL of avidin (1 mg/mL in PBS) were mixed together in an Eppendorf plastic vial. Immediately prior to deposition 25 μL of $NaIO_4$ (42.8 mg/mL in water) was added to the solution, mixed well, and placed in the cell. The electrode was lowered to within 1−2 mm of the platinum foil and a potential of −600 mV versus Ag/AgCl was applied for 300 s while the electrode was rotated at 500 rpm. After deposition the electrode was washed with deionized water.

Oligonucleotide Attachment and Amperometric Detection of Hybridization. The scheme for detecting either DNA or RNA through sandwich hybridization is shown in Figure 2, which also explains the terms used. Prior to the test, the carbon electrode is coated with the avidin-containing electron-conducting redox hydrogel, to which the biotinylated analyte-selective capture sequence, termed EPRO, is bound. In the field test, the electrode is exposed to the tested solution, which contains the analyte DNA or RNA, the detection sequence ETAR:HRP, and H_2O_2. Hybridization of the mid-sandwich, now HRP-labeled, DNA or RNA with the base sequence results in electrical contact between the redox hydrogel and the HRP label. Upon contact the redox hydrogel, which by itself does not catalyze the electroreduction of hydrogen peroxide to water, becomes an electrocatalyst for the reduction. Flow of the electroreduction current thus indicates the presence of the analyte DNA or RNA in the solution.

The electrochemical measurements were performed in 2 mL of hybridization buffer (10 mM HEPES, 1 M NaCl, 1 mM EDTA, pH 7) at 37 °C with the electrode rotating at 1000 rpm and an applied potential of 0.0 V versus Ag/AgCl. The attachment of the oligonucleotides and the hybridizations were carried out in a simple one-step process. The disk electrode was connected to the potentiostat, polarized at 0.00 V versus Ag/AgCl, and rotated at 1000 rpm while the change in current with time was measured. To the cell was added 1×10^{-10} mol of EPRO and ETAR1, bringing the concentrations to 5×10^{-8} M. Immediately afterward, the test DNA, the analyte DNA, termed EMID, or the *E. coli* 16S rRNA, the analyte RNA, was added to the buffer and the solution left to hybridize for 20 or 30 min, respectively. When detecting rRNA, the rRNA was denaturated prior to introduction by heating at 80 °C for 3 min using a block heater (Digi-Block Jr). After the hybridization period, H_2O_2 was added to the solution bringing the concentration to 1 mM and the change in current due to the catalytic electroreduction of the H_2O_2 was monitored.

RESULTS

Electrodeposition of Avidin/Redox Polymer Films. The steady-state cyclic voltammograms of an electrodeposited film of PAA−PVI−Os only and PAA−PVI−Os with co-electrodeposited avidin are shown in Figure 3. The voltammograms of electrodes with co-deposited avidin had slightly larger peak areas and their peaks were more separated, indicating increased capacitance and slower electron transport. The peak currents increased linearly with scan rate up to 500 mV/s, as expected for a surface-immobilized reversibly oxidized/reduced redox couple.[34] The reproducibility of the electrodeposited coatings was tested by repolishing and recoating the same electrode 50 times, using a fresh solution for each deposition. The peak heights of the cyclic voltammograms of the films were identical, with a standard deviation of less than ±5%. When different 3-mm glassy carbon disk electrodes were used, or when the coatings were deposited

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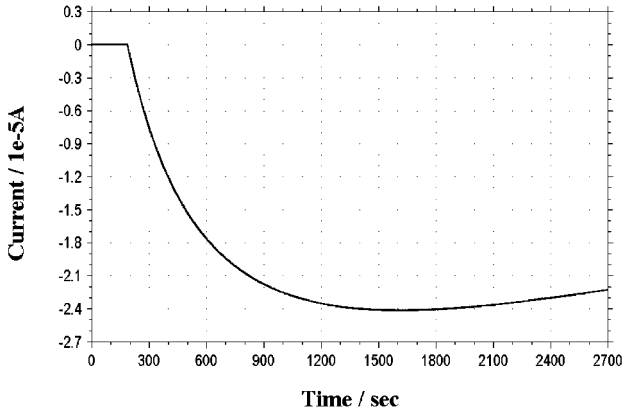

Figure 4. Current change upon adding B:HRP. The B:HRP and the H_2O_2 were added at $t = 200$ s. B:HRP concentration 5×10^{-8} M; 1 mM H_2O_2; hybridization buffer; 37 °C.

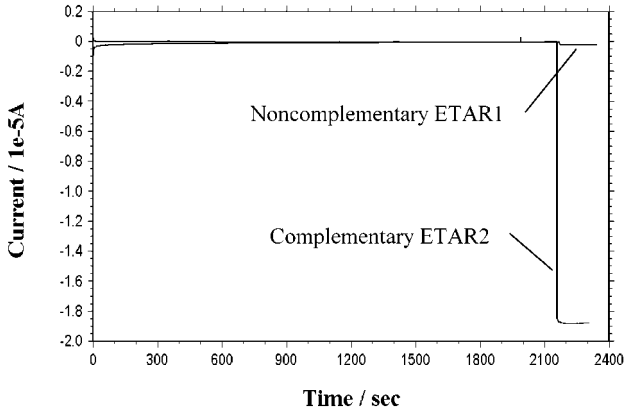

Figure 5. Current change upon hybridization of EPRO and ETAR2 and EPRO and ETAR1. Oligomer concentrations 5×10^{-8} M; after 20-min hybridization in hybridization buffer at 37 °C; 1 mM H_2O_2 .

in different electrochemical setups, the voltammograms remained the same. The electrodeposited films could be thoroughly air-dried and rehydrated with no observable change in their peak current or in their other electrochemical properties.

Incorporation of avidin in the films was confirmed by measuring the amount of conjugated biotinylated horseradish peroxidase (B:HRP). Because binding of HRP converts the film from non-electrocatalytic to electrocatalytic, an H_2O_2 electroreduction current appears upon adding B:HRP to the cell (Figure 4). The time required for maximum binding of the biotinylated-HRP was less than 15 min. In a test buffer containing 1 mM H_2O_2 and 5×10^{-8} M B:HRP, the current was ~ 25 μA (current density $\sim$350 μA/cm^2). In comparison, in an earlier study, electrodes made with a much thicker drop-deposited layer of an osmium polymer and avidin reached electroreduction current densities of only 20−30 μA/cm^2. Apparently, the electrodeposited films were richer in B:HRP-accessible avidin.[35]

The electrodeposition step was optimized with respect to the amount of electroactive polymer deposited (tracked by cyclic voltammetry) and accessible avidin content (tracked by measuring the B:HRP-produced H_2O_2 electroreduction current). Fortuitously, the optimal periodate concentrations and deposition potentials were the same for both. Rotation of the electrodes was necessary for significant deposition of polymer or polymer−avidin.

Detection of DNA−DNA Hybridization. Figure 5 shows the change in current upon hybridization of the complementary HRP-labeled ETAR2 to the electrode-bound probe EPRO. When 5×10^{-8} M concentrations of both the biotinylated EPRO and its complementary HRP-labeled ETAR2 were allowed to hybridize for 20 min in HB at 37 °C, a sharp increase in the reduction current was observed upon adding H_2O_2 (1 mM). When the noncomplementary HRP-labeled ETAR1 was added instead of ETAR2, the change in the reduction current, upon adding H_2O_2, was negligible. For the complementary HRP-labeled ETAR2 the current was 19 μA, while for the noncomplementary HRP-labeled ETAR1 the current density was $\sim$15 nA, the signal-to-noise ratio exceeding 100. The current was also higher than that observed in a nonsandwich hybridization assay, where the probe oligonucleotide was covalently bound to the electrodeposited redox polymer layer on a microelectrode through carbodiimide activation of its terminal phosphate.[19] An additional advantage of using the biotinylated oligonucleotides instead of the carbodiimide-activated ones was their absence of reaction with water, expected to translate to a better shelf life.

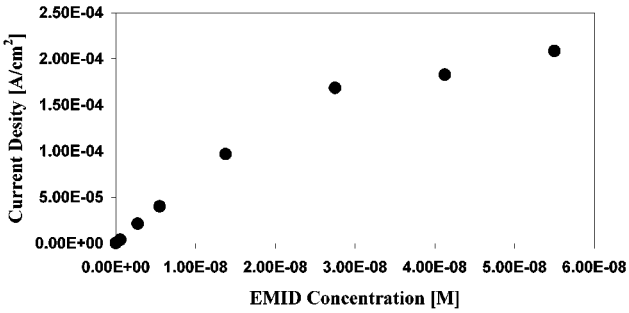

Figure 6. Dependence of the change in current on the concentration of EMID. EPRO and ETAR concentrations 5×10^{-8} M; after 20-min hybridization in hybridization buffer at 37 °C; 1 mM H_2O_2 .

Sandwich Hybridization. Figure 6 shows the current change when 5×10^{-8} M concentrations of both the biotinylated EPRO and the HRP-labeled ETAR1 were allowed to hybridize with the test oligonucleotide EMID at 0, 0.55, 2.75, 5.5, 13.8, 27.5, 41.3, and 55 nM concentrations for 20 min in hybridization buffer at 37 °C and adding H_2O_2. The increase in the reduction current was as sharp as that seen upon the direct hybridization of the oligonucleotide probe with the complementary HRP-oligonucleotide. The dependence of the EMID "wiring" signal on the hybridization time is seen in Figure 7. When 5×10^{-8} M aliquots EPRO, ETAR1, and EMID were allowed to hybridize for various times (0, 2, 5, 10, 15, 30, 60 min) in hybridization buffer at 37 °C, the current increased with hybridization time, reaching a plateau at $\sim$15 min.

In previous electrochemical DNA and RNA assays in biological fluids, the effects of interferences were minimized by washings, chromatographic separation, or extraction. These added to the time, complexity, and cost of the assays. To show that the hybridization and detection can be carried out in unseparated biological solutions, the tests were repeated in a solution containing an excess (0.2 mg/mL) of irrelevant salmon testes DNA and an excess (50 vol %) of goat serum. Figure 8 shows the current response for hybridizations of 5×10^{-8} M EPRO, ETAR1, and EMID at 37 °C for 20 min in goat serum (nonbuffered solution, with low salt concentration) and in 0.2 mg/mL ss-DNA from

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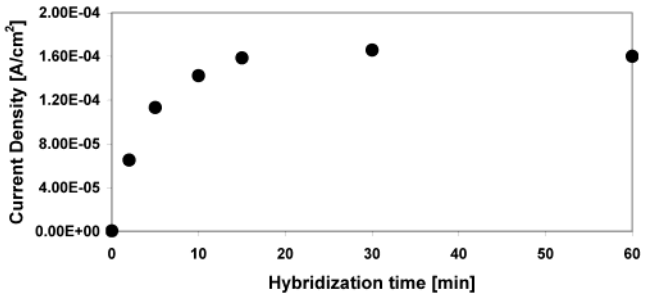

Figure 7. Dependence of the current change on the hybridization time. Oligomer concentrations 5×10^{-8} M; hybridization buffer at 37 °C; 1 mM H_2O_2.

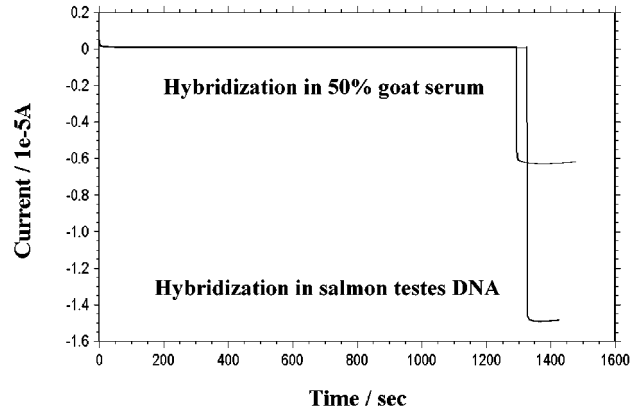

Figure 8. Current change upon hybridization of EPRO, ETAR, and EMID in 50 vol % goat serum (nonbuffered solution with low salt concentration) and in hybridization buffer containing 0.2 mg/mL ss-DNA from salmon testes. EPRO, EMID, and ETAR concentrations 5×10^{-8} M; after 20-min hybridization; 37 °C; 1 mM H_2O_2.

salmon testes (in hybridization buffer). The test showed that the irrelevant DNA did not change the current response, while the goat serum reduced the current to only to half of its value in absence of serum.

Detection of *E. coli* 16S rRNA. The overall objective being to determine whether a microorganism can be rapidly and easily detected by the simple enzyme-amplified amperometric sandwich assay, the assay was applied to the detection of the available mixture of *E. coli* 16S + 32S rRNA. The 16S rRNA strand was detected. The current change after 30-min hybridization in the solution containing the denaturated *E. coli* 16S + 32S rRNA (hybridization buffer at 37 °C, 5×10^{-8} M concentrations of both EPRO ETAR1) was 5 times that observed in absence of the *E. coli* rRNA (Figure 9).

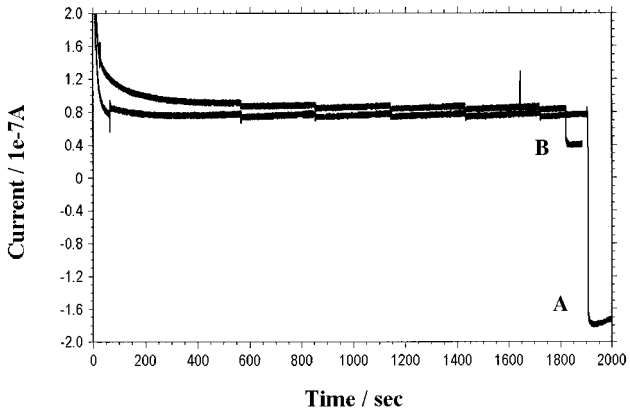

Figure 9. Trace A: current change upon hybridization of EPRO, ETAR1, and *E. coli* 16S + 32S rRNA. Trace B: current change when the *E. coli* rRNA is omitted. Conditions: EPRO and ETAR concentrations 5×10^{-8} M; 30-min hybridization; 37 °C; 1 mM H_2O_2 .

CONCLUSION

An electron-conducting enzyme-wiring redox polymer and periodate-oxidized avidin were readily coelectrodeposited on vitreous carbon to form a convenient and reproducible 30-min DNA/RNA sandwich-type detection platform. Even in the absence of a blocking buffer, the test could be performed in the presence of extraneous DNA and serum.

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