Anal. Chem. **2002,** *74,* 4370−4377

Screen Printing of Nucleic Acid Detecting Carbon Electrodes

Murielle Dequaire and Adam Heller*

Department of Chemical Engineering and Texas Materials Institute, The University of Texas at Austin, Austin, Texas 78712

A large fraction of the presently mass-manufactured (> 10^8 units/year) electrochemical biosensors, used mostly by diabetic people to monitor their blood glucose levels, have screen-printed carbon working electrodes. An earlier study (Campbell, C. N., et al. *Anal. Chem.* 2002, *74,* 158−162) showed that nucleic acids can be assayed at 1 nM concentrations by a sandwich-type amperometric method. The assay was performed with vitreous carbon working electrodes on which an electron-conducting polycationic redox polymer and avidin were coelectrodeposited. Because the rate of the electrodeposition increases with the surface density of the polycationic redox polymer, its practicality depends on pretreatment of the surface, which adds anionic functions. (Gao, Z., et al. *Angew. Chem. Int. Ed.* 2002, *41,* 810−813). Here it is shown that the required conducting redox polymer films can be electrodeposited on potentially mass manufacturable electrodes made by screen-printing hydrophilic carbon inks on polyester sheets. The modified electrodes are made in two steps. First a polycationic electron-conducting redox polymer is cross-linked and electrodeposited by applying a negative potential. Next, an amine-terminated 20-base single-stranded oligonucleotide is electrodeposited by ligand-exchange. Both steps involve exchange of a labile inner sphere chloride ligand of the polymer-bound osmium-complex: Cross-linking and electrodeposition of the redox polymer result when inner-sphere chloride anions of the osmium complexes are exchanged by imidazole functions of neighboring chains. Incorporation of the oligonucleotide in the redox polymer results in the formation of a coordinative bond between the terminal amine (attached through a spacer to the oligonucleotide) and the osmium complex. In testing for the presence of a 38-base oligonucleotide, the analyte, in a 15- or 25-μL droplet of hybridization solution, is hybridized with and captured by the 20-base electrode-bound sequence; then it is hybridized with an 18-base horseradish peroxidase labeled sequence. When the HRP label electrically contacts the redox polymer, the film becomes an electrocatalyst for the reduction of H_2O_2 to water at 0.10 V (Ag/AgCl). Flow of the H_2O_2-reduction current indicates the presence of the assayed sequence.

Electrochemical assays of nucleic acids based on the intercalation of redox couples such as $Co(bpy)_3^{3+}$ in DNA, were introduced by Mikkelsen and co-workers.[1] Barton and her colleagues probed the integrity of alkanethiol-terminated DNA monolayers on gold with nonintercalating $[Fe(CN)_6^{3-/4-}]$, then monitored intercalation of redox-active methylene blue under anaerobic conditions.[2] Thorp and co-workers analyzed fractions of PCR-amplified product from the HER-2 gene by $Ru(bpy)_3^{2+}$-mediated electrooxidation of their G nucleotides.[3] Kayyem and co-workers applied ferrocene-labeled detection probes and gold-electrode microarrays coated with monolayers containing capture probes in electrochemical sandwich hybridization assays, detecting the C282Y single-nucleotide polymorphism associated with hereditary hemochromatosis.[4]

Accurate reproduction of the selectively hybridizing dots in arrays is central to their usefulness, because in most analyses, it is the difference between signals from different dots that is measured. Better reproduction improves the ability to detect small differences and improves thereby the sensitivity. In electrochemical assays, the reproducible electrodeposition of single-stranded DNA on electrodes with identical dimensions is of particular importance. Korri-Youssoufi et al.[5] attempted, without success, to electropolymerize pyrrole-bearing oligonucleotides on electrodes. They succeeded, however, in electrodepositing polypyrroles functionalized with carboxylic acids and phthalimides, which they converted to *N*-hydroxysuccinimide esters then condensed with amine-functionalized oliginucleotides. Wang et al. electropolymerized pyrrole in oligonucleotide solutions, obtaining oligonucleotide-doped polypyrrole films.[6]

A sandwich-type enzyme-amplified amperometric assay of nanomolar concentrations of nucleic acids was reported recently by Campbell et al.[7] Such solutions are produced by PCR amplifications. The assay of PCR-amplified DNA is carried out now by fluorescence detectors that are larger, more expensive, and much more complex than the electrochemical systems that are now widely used in glucose assays performed by diabetic people. The sandwich-type assay was based on electrodeposited, electron

(1) (a) Millan, K. M.; Mikkelsen, S. R. *Anal. Chem.* **1993**, *65*, 2317−2323. (b) Millan, K. M.; Saraullo, A.; Mikkelsen, S. R. *Anal. Chem.* **1994**, *66*, 2943−2948.
(2) Boon, E. M.; Ceres, D. M.; Drummond, T. G.; Hill, M. G.; Barton, J. K. *Nat. Biotechnol.* **2000**, *18*, 1096−1102.
(3) Armistead, P. M.; Thorp, H. H. *Anal. Chem.* **2000**, *72*, 3764−3770.
(4) Umek, R. M.; Lin, S. W.; Vielmetter, Jr; Terbrueggen, R. H.; Irvine, B.; Yu, C. J.; Kayyem, J. F.; Yowanto, H.; Blackburn, G. F.; Farkas, D. H.; Chen, Y.-P. *J. Mol. Diagn.* **2001**, *3*, 74−84.
(5) Korri-Youssoufi, H.; Garnier, F.; Srivastava, P.; Godillot, P.; Yassar, A. *J. Am. Chem. Soc.* **1997**, *119*, 7388−7389.
(6) Wang, J.; Jiang, M.; Fortes, A.; Mukherjee, B. *Anal. Chim. Acta* **1999**, *402*, 7−12.
(7) Campbell, C. N.; Gal, D.; Cristler, N.; Banditrat, C.; Heller, A. *Anal. Chem.* **2002**, *74*, 158−162.

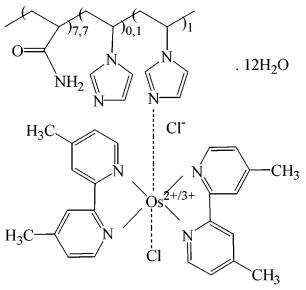
10.1021/ac025541g CCC: $22.00
Published on Web 07/20/2002

Table 1. Oligonucleotide Sequences for Capture Probe (Surface-Immobilized) Target and Detection Probe (labeled with HRP) DNA[a]

symbol	sequence (5′ → 3′)	% T
C_1	TTT TTT TTT TTT GGG GGG GGG GGG GAG CAA AGG TAT TAA CTT TAC TCC C	38.8
D_1	TTT TTT TTT TTG GGA GTA AAG TTA ATA CCT TTG CTC CCC CCC CCC CCC	
C_2	TTT TTT TTT TTT CAC TTC ACT TTC TTT CCA AGA G	58.8
T_2	AGG CAT AGG ACC CGT GTC CTC TTG GAA AGA AAG TGA AG	
T_2'	AGG CAT AGG ACC CGT GTC CTC TTG GAA **T**GA AAG TGA AG	
T_2''	AGG CAT AGG ACC CGT GTC CTC T**C**G GAA AGA AAG **A**GA AG	
D_2	GAC ACG GGT CCT ATG CCT	

[a] Capture probes ($C_{i=1,2}$) are modified with 5′-amine-terminated 6-carbon spacers. A 12-T spacer is appended on the 5′ end of each capture probe to allow for better hybridization. Detection probes D_1 and D_2 are 5′ and 3′-labeled, respectively, with the HRP. A 6-carbon spacer is used between the oligo and the enzyme. Targets for the model sandwich hybridization T_2, $T_{2'}$, and $T_{2''}$ are designed with no, one, and two mismatched-bases, respectively. The desalted target sequence, the capture probe, and the horseradish peroxidase-labeled detection probe were custom-prepared by Synthetic Genetics, San Diego, CA.

conducting films of redox polymers which, by themselves, were not electrocatalysts, but after hybridizing oligonucleotide-labeling peroxidases, they catalyze the electroreduction of H_2O_2 to water.[7] As reported by Gao et al.,[8] the electrodeposition of the redox polymer films proceeds by coordinative cross-linking of adsorbed redox polymer films and is rapid only when the surface coverage is high. Earlier, amperometric detection of the hybridization of a horseradish peroxidase labeled oligonucleotide was reported by de Lumley et al.[9] Using tips of 7-μm-diameter carbon fiber, Caruana and Heller[10] detected amperometrically a single base mismatch in an 18-base sequence. They prepared the microelectrodes by reactively electrodepositing a redox polymer and the oligonucleotide on the carbon fiber tips. Their polymer was derived of PAA−PVI−Os, by converting part of the amides to hydrazides. These functions condensed with *N*-hydroxysuccinimide (phosphate) esters of the polyanionic single-stranded oligonucleotides. Their complementary detection-probe sequences



PAA-PVI-Os

were labeled with thermostable soybean peroxidase so as to allow hybridization with the perfectly complementary 18-base strand in a solution heated to a temperature just below the melting temperature of the hybrid, at which point the hybrid with a single mismatched base pair was already denatured.

The cited studies established the feasibility of electrochemical assay of nucleic acids at concentrations readily produced by PCR amplification. The nature of the electrode surfaces was, however, a critical variable, defining in the sandwich-type assay of Campbell et al.[7] whether the essential redox polymer can be electrodeposited. The deposition required preoxidation of the surface of the vitreous carbon electrode to increase the density of acidic functions and increase thereby the coverage by the cross-linkable polycationic redox polymer film.[7,8] Although numerous DNA hybridization assays have been routinely used in diagnostic laboratories, there is interest in screen-printed DNA-hybridization sensors, because these can be mass-produced by existing manufacturing processes at low cost.[11−13] Here, we affirm the feasibility of performing sandwich-type amperometric assays of oligonucleotides with mass-manufacturable carbon electrodes screen-printed on polyester sheets.

EXPERIMENTAL SECTION

Chemicals and Reagents. The salmon testes DNA, Tween 20, *o*-phenylenediamine, and the inorganic chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The phosphate-buffered saline solution (PBS: 8 mM $NaPO_4$, 2 mM KPO_4, 140 mM NaCl, 10 mM KCl) was from Pierce (Rockford, IL); the hybridization buffer (4.3 mM NaH_2PO_4, 15.1 Na_2HPO_4, 500 mM NaCl, 100 μg mL^{-1} salmon DNA), washing buffer (4.3 mM NaH_2PO_4, 15.1 Na_2HPO_4, 500 mM NaCl, and 0.5% Tween 20), TE buffer (10 mM TRIS; pH 7.7, 1 mM EDTA), and all other solutions were prepared using deionized water (Barnstead, Nanopure II, Van Nuys, CA). The electron-conducting redox polymer, a 7:1 copolymer of acrylamide and 1-vinylimidazole, its imidazole functions complexed with [Os(4,4′-dimethyl-2,2′-pyridine)$_2$Cl]$^{+/2+}$, termed PAA−PVI−Os, was synthesized as previously described.[14] The desalted analyte−oligonucleotide sequence (Table 1), the capture probes having 5′-amine-terminated 12-T spacers, and the horseradish peroxidase labeled detection probes were custom prepared by Synthetic Genetics, San Diego, CA. In-house *o*-phenylenediamine-based colorimetric assay of the HRP label of the detection probes showed that on a molar basis,

(8) Gao, Z.; Binyamin, G.; Kim, H, H.; Calabrese Barton, S.; Zhang, Y.; Heller, A. *Angew. Chem., Int. Ed.* **2002**, *41*, 810−813.

(9) de Lumley-Woodyear, T.; Campbell, C, N.; Caruana, D. J.; Heller, A. *Anal. Chem*. **1999**, *71*, 394−398.

(10) Caruana, D. J.; Heller, A. *J. Am. Chem. Soc.* **1999**, *121*, 769−774.

(11) Lucarelli, F.; Palchetti, I.; Marrazza, G.; Mascini, M. *Talanta* **2002**, *56*, 949−957. Mascini, M.; Palchetti, I.; Marrazza, G. *Fresenius' J. Anal. Chem*. **2001**, *369*, 15−22. Marrazza, G.; Chiti, G.; Mascini, M.; Anichini, M. *Clin. Chem*. **2000**, *46*, 31−37.

(12) Azek, F.; Grossiord, C.; Joannes, M.; Limoges, B.; Brossier, P. *Anal. Biochem*. **2000**, *284*, 107−113. Authier, L.; Grossiord, C.; Brossier, P.; Limoges, B. *Anal. Chem*. **2001**, *73*, 4450−56.

(13) Wang, J.; Cai, X.; Rivas, G.; Shiraishi, H.; Dontha, N. *Biosens. Bioelectron*. **1997**, *12*, 587−599.

(14) de Lumley-Woodyear, T.; Rocca, P.; Lindsay, J.; Dror, Y.; Freeman, A.; Heller, A. *Anal. Chem*. **1995**, *67*, 1332−1338.

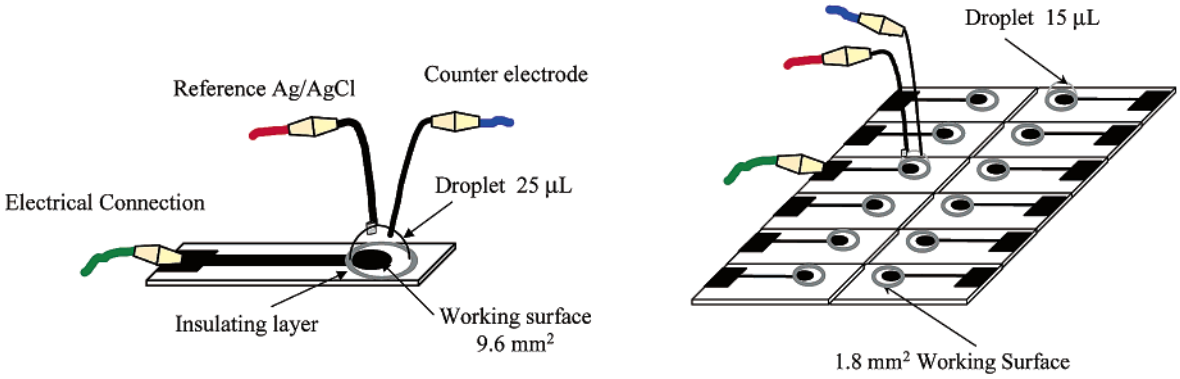


Figure 1. Scheme of the SPE droplet-based cell and array.

the activity of the probe-bound HRP did not differ from that of the pure enzyme. The in-house assay confirmed the assay of the supplier, reporting a specific activity of 10^4 units mg^{-1}. The ds-DNA was prepared by heating ss-DNA with a 2-fold excess of complementary DNA in TE buffer to 90 °C and cooling over 2 h to room temperature.

Equipment and Screen-Printed Electrodes. The hybridizations were performed on a DIGI−BLOCK JR, Laboratory Devices block heater, purchased from Sigma-Aldrich. The electrochemical measurements were performed with a CH Instruments model 832A electrochemical detector (Austin, TX) interfaced to a computer (Dell OptiPlex Gxi, Austin, TX). The experiments were performed first with 3.6-mm-diameter screen-printed carbon electrodes (SPEs) printed on a flexible polyester film with a homemade polystyrene-based ink, a 2:3 mixture of polystyrene foam and graphite particles (2−15 μm, Alfa Aesar, Wardhill, MA) in mesitylene.[15] The SPEs (Figure 1) were made hydrophilic by exposure to a 0.5 Torr air plasma for 90 s.

The homemade ink was substituted in later experiments by the commercial carbon ink Electrodag 423SS (Acheson, Port Huron, MI). This ink was used to screen-print the 12 SPE arrays of Figure 1 (right). To avoid the spreading of the 10−35-μL droplets beyond the 3.6- or 1.5-mm-diameter working electrodes, a hydrophobic circle was drawn around each SPE with a felt-tip pen containing a hydrophobic ink (DAKO Pen, S 2002, DAKO Corporation, Carpinteria, CA). The electrochemical cell formed in the confined droplet had a screen-printed carbon working electrode, a 0.9-mm-diameter mechanical pencil carbon lead (hardness HB) or a 0.5-mm-diameter platinum wire counter electrode, and a Ag/AgCl microreference electrode (3 M KCl saturated with AgCl) (Cypress, Lawrence, KS), *to which all potentials are referenced.*

Electrodeposition of the ds-Oligonucleotide-Containing Redox Polymer-Films. Redox polymer films containing ds-oligonucleotide were electrodeposited from solutions of 0.62 mg mL^{-1} PAA−PVI−Os and 8−80 μg mL^{-1} ds-oligonucleotide by one of two procedures: (a) applying for 2 min a steady reducing potential, usually −1.4 V; or (b) potential cycling, in which a train of 60 pairs of 2-s-long square waves of +0.8 and −0.6 V was applied. After rinsing with water, the SPEs were scanned at 50 mV s^{-1} between − 0.15 and 0.5 V to measure the amount of electroactive polymer on the electrode. The ds-oligonucleotide was then denatured by dipping the SPE in boiling water for 1 min. The unbound ss-oligonucleotide was removed by rinsing the SPE with DI water.

Electrodeposition of ss-Oligonucleotide Containing Redox Polymer Films. The films were deposited from solutions made by mixing at 1:7.5 weight ratio 0.133 mg mL^{-1} ss-oligonucleotide in PBS with 1 mg mL^{-1} of PAA−PVI−Os, also in PBS. A 15-μL aliquot of the mixed solution was pipetted onto the SPE, and the polymer-oligonucleotide film was electrodeposited by holding a fixed reducing potential as described above. When the ss-oligonucleotide had a particularly high mol-fraction of T, the films were prepared in two-steps: First, the redox polymer was electrodeposited by applying 60 pairs of 2-s square waves of +0.8 and −0.3 V; then the amine-terminated oligonucleotide was incorporated by similar potential cycling.

Storage of the electrodes at 4 °C for two weeks did not change their activity.

Confirmation of the Electrodeposition. The presence of the ss-oligonucleotide in the electrodeposited films was confirmed by hybridization with the complementary HRP-labeled oligonucleotide, which converted films to H_2O_2 electroreduction catalysts. The H_2O_2 electroreduction current was measured with the electrodes by cyclic voltammetry at 0.1 V. The hybridization with 0.75−7.5 pmol of HRP labeled oligonucleotide in 25-μL droplets of hybridization buffer took 40 min at 37 °C. After hybridization, the electrode was immersed for 10 min in 5 mL of rinsing buffer agitated by a rotary mixer so as to remove any nonhybridized HRP-labeled oligonucleotide, then washed in PBS for 5 min. A 30-μL droplet of PBS was then applied to the SPE, the presence of the redox polymer was confirmed by cyclic voltammetry (5 mV s^{-1}scan rate), 5 μL of 1 mM H_2O_2 was added, and the increase in the steady-state H_2O_2 electroreduction current was measured.

Sandwich-Type Assay. A 25-μL portion of the analyte-containing hybridization buffer-solution was pipetted onto the SPE and held at 53 °C for 15 min. After cooling and holding at room temperature for 10 min, the SPE was rinsed sequentially in 5 mL of washing buffer (10 min) and hybridization buffer (5 min). Next, it was incubated with 25 μL of a 50 nM solution of the detection probe in hybridization buffer at 37 °C for 40 min, allowed to cool, and held at room temperature for 10 min. After rinsing, the H_2O_2 electroreduction current was measured as described above.

RESULTS

Electrodeposition of the Redox Polymer Film without Oligonucleotide. When the positive charge of the redox polymer

(15) Bagel, O.; Limoges, B.; Schöllhorn, B.; Degrand, C. *Anal. Chem.* **1997**, *69*, 4688−4694.

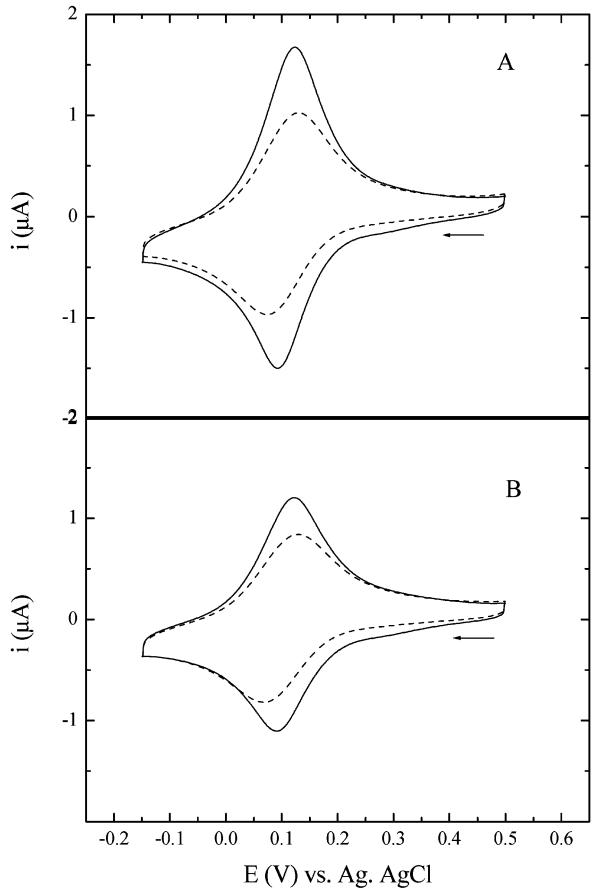


Figure 2. Sixth cycle voltammograms of the films electrodeposited by the first procedure. Solid line: PAA−PVI−Os polymer only, deposited from a 0.62 mg mL^{-1} solution; A, as deposited; B, after dipping in boiling water for 1 min and rinsing. Dashed line: coelectrodeposited (dissolved polymer to C_1C_1* weight ratio 7.5) redox polymer and double stranded C_1C_1* oligonucleotide; A, as deposited; B, after dipping for 1 min in boiling water to denature the double-stranded oligonucleotide and produce the capture probe (C_1)-modified redox polymer film. Scan rate, 50 mV s^{-1}; PBS; 3.6-mm-diameter SPE.

was about balanced by the negative charge of the oligonucleotide, their electrostatic adduct was least soluble. To avoid precipitation of the adduct during the coelectrodeposition of the redox polymer and the capture probe, the solution-phase redox polymer/oligonucleotide weight ratio was maintained at ∼8:1. When a lesser ratio was desired, the redox polymer and the capture probe were deposited separately from their respective solutions, the redox polymer being deposited first. A typical voltammogram of the redox polymer, electrodeposited on a 3.6-mm-diameter SPE by poising it at − 1.4 V for 2 min, is shown in Figure 2A (solid line). The redox potential of the polymer was + 107 ± 2 mV; at 50 mV s^{-1} scan rate, the peaks of the voltammetric waves were separated by $\Delta E_p = 35 \pm 2$ mV. Integration of the voltammogram yielded a faradaic charge of 1.4 ± 0.1 μC for the reduction wave and 1.6 ± 0.2 μC for the oxidation wave, corresponding to coverage by (1.6 ± 0.1) × 10^{-10} moles cm^{-2} of electroactive $Os^{2+/3+}$. The standard deviation for 20 electrodes from 5 random batches was ± 9.0%. The $Os^{2+/3+}$ coverage of the 1.5-mm-diameter SPEs (hydrophilic ink, no plasma treatment) was (1.3 ± 0.1) × 10^{-10} mol cm^{-2}; the standard deviation in a batch of 16 electrodes was ± 11%. Whether the films were deposited by poising the electrodes at a fixed potential or by square-wave cycles, their electroactive $Os^{2+/3+}$ coverage was the same. The redox polymer films deposited by holding the potential at −0.2, −1.025, or −1.4 V for 2 min were indistinguishable in their electroactive $Os^{2+/3+}$ content.

Table 2. Surface Density of Electroactive $Os^{2+/3+}$ [a]

	2-min reduction at −1.4 V	potential cycling	2-min reduction at −0.3 V	2-min adsorption
screen-printed carbon ink	19.2	18.1	19.2	16.9
vitreous carbon	10.1	17.7	8.8	4.6

[a] Units, μC cm^{-2}.

Comparison of the amount of the passively adsorbed electroactive $Os^{2+/3+}$ on the O_2 plasma treated (5 min, 1 Torr) hydrophilic vitreous carbon electrodes with that on the SPEs showed that the latter adsorbed 4 times more redox polymer (Table 2). Following electrodeposition by square-wave cycling, the vitreous carbon electrodes and the SPEs had identical electroactive $Os^{2+/3+}$ coverages. Their coverages differed, however, when the films were deposited by poising the electrodes at a fixed reducing potential: When the films were deposited in 2 min at −1.4 V, the vitreous carbon electrodes had 40% less electroactive $Os^{2+/3+}$ than the SPEs. When deposited at −0.3 V, the coverage of the vitreous carbon electrodes was less than one-half of the coverage of the SPEs. The reproducibility of the coverages depended foremost on the hydrophilicity of the carbons, which also affected the actual coverage. Unless the ink was intrinsically hydrophilic or the electrodes were O_2 plasma treated, the $Os^{2+/3}$ coverage was less reproducible and 20−40% lower. The presence of 1−10 mM H_2O_2 did not affect the coverage upon electrodeposition at a steady − 0.3 V potential.

Figure 2A (solid line) shows a typical voltammogram of an electrode with an electrodeposited redox polymer film, and Figure 2B (solid line) shows the voltammogram of the same electrode after it was dipped in boiling water for 1 min and rinsed. Dipping in boiling water decreased the peak heights by ∼30% and increased the separation of the peaks from ∼35 to ∼60 mV.

Electrodeposition of ds-Oligonucleotide-Containing Redox Polymer Films and Their Dehybridization/Hybridization. The 5′-amine terminus of oligonucleotide C_1 allowed the coordinative binding of the C_1C_1* hybrid to the redox polymer through exchanging the inner-sphere chloride of the polymer's osmium complex by the C_1 amine. After denaturing the hybrid and washing off the C_1* chain, the C_1 probe remained polymer-bound. The voltammogram of the polymer with the coordinatively bound hybrid is shown in Figure 2A (dashed line). The voltammetric peak heights of the hybrid-containing films were ∼30% lower than those of films without the ds-oligonucleotide, and their separation was ∼60 mV versus the ∼35 mV separation of the films without oligonucleotide. Dipping in boiling water for 1 min to denature the hybrid caused a further 30% decrease in the heights of the voltammetric peaks. (Figure 2B, dashed line).

Mass transport was enhanced in the droplet during hybridization by heating the block on which the SPE was placed to 37 °C, the temperature gradient between the heated bottom of the droplet and its evaporatively cooled surface, producing a convective flow

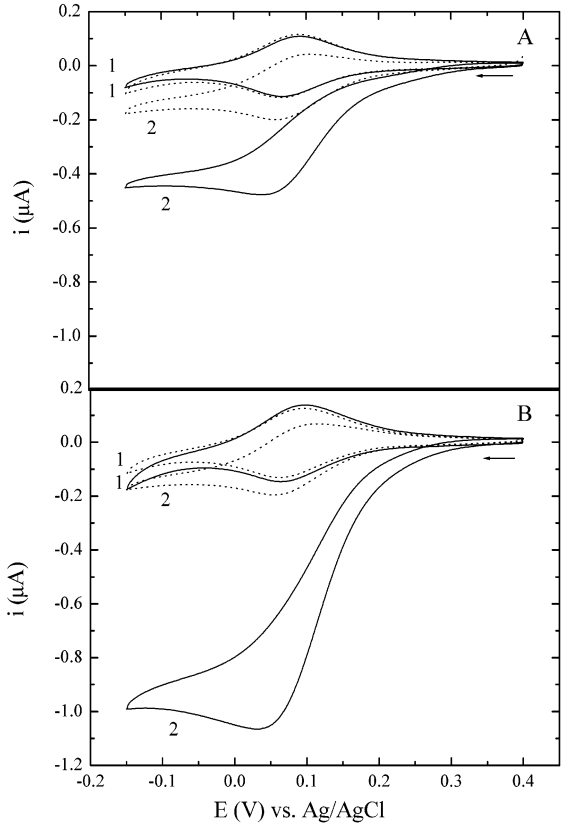


Figure 3. (A) Cyclic voltammograms of the electrode with the single-stranded capture probe (C_1) made by denaturing the coelectrodeposited redox polymer without (dotted line) and with (solid line) C_1C_1* oligonucleotide (solid line) after its hybridization with the HRP-labeled detection sequence D_1. The deposition solution contained 0.62 mg mL^{-1} redox polymer and 0.08 mg mL^{-1} C_1C_1*. (B) Cyclic voltammograms of the electrode made by coelectrodepositing the redox polymer without (dotted line) and with (solid line) the single-stranded capture probe (C_1), then hybridizing with the HRP-labeled detection sequence D_1. The deposition solution contained 0.62 mg mL^{-1} redox polymer and 0.04 mg mL^{-1} C_1. The voltammograms before adding H_2O_2 are labeled with the digit 1 and after adding H_2O_2 (0.2 mM), with digit 2.

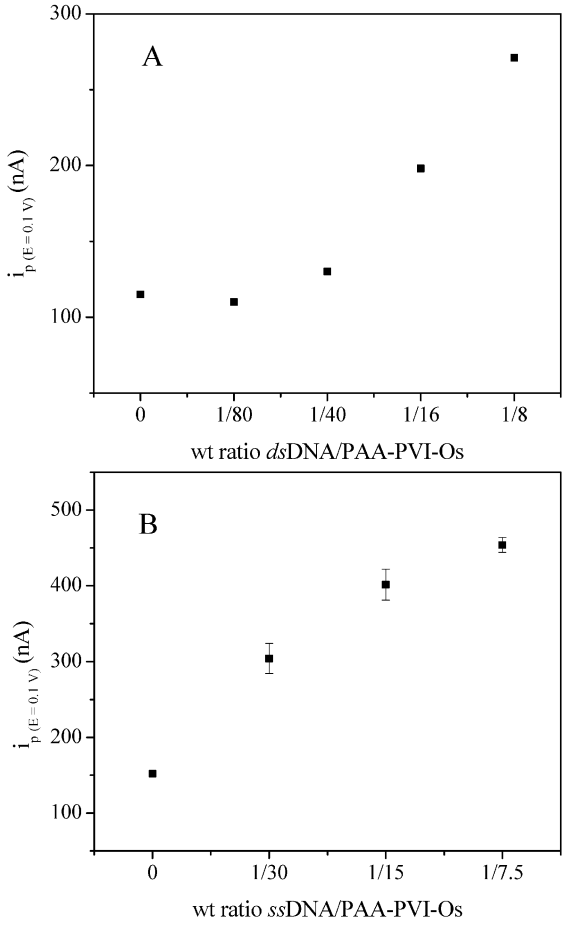


Figure 4. Dependence of the signal on the oligonucleotide/polymer weight ratio. (A) Coelectrodeposition of redox polymer films in the presence of ds-oligonucleotide C_1C_1* checked with the hybridization of 330 nM D_1. (B) Coelectrodeposition of redox polymer films in the presence of ss-oligonucleotide C_1 checked with the hybridization of 35 nM D_1. Scan rate, 5 mV s^{-1}; 0.2 mM H_2O_2 in PBS.

loop.[16−20] Though mass transport increased with the temperature gradient, it was not possible to raise the block temperature above 37 °C, because at the ∼70−80% relative humidity and the 25 °C temperature of the ambient air, the droplet evaporated too rapidly.

After hybridizing (40 min, 25-μL droplet) the coordinatively polymer-bound capture probe C_1 on the electrode with 330 nM of the HRP-labeled complementary probe D_1 (Table 1), the film catalyzed the electroreduction of H_2O_2 water at +0.1V (Figure 3A). The magnitude of the electrocatalytic current (i_p) at + 0.1 V was chosen as the analytical response. At 0.05 V, where the H_2O_2 electroreduction reached its plateau on the catalytic electrode, a minuscule, though still measurable, current was observed with PAA−PVI−Os−C_1-coated SPEs covered by a drop containing H_2O_2. In the absence of hybridization with the HRP-labeled target, at 0.1V, this current was no longer measurable. In the PAA−PVI−Os−C_1C_1* coated electrodes prepared by depositing films at varying solution-phase polymer/oligonucleotide ratios between 8 and 80, the signal increased with the weight fraction of the oligonucleotide in the solution. (Figure 4A).

Electrodeposition and Hybridization of ss-Oligonucleotide-Containing Redox Polymer Films. Figure 3B shows voltammograms of electrodes made by coelectrodepositing the single stranded oligonucleotide C_1 with the redox polymer before and after hybridization with their HRP-labeled complement D_1. When the solution-phase polymer/ss-oligonucleotide ratio was raised stepwise from 7.5 to 30, the signal increased with the ss-oligonucleotide content. The highest signal was observed at a 1:5 ss-oligonucleotide/polymer solution-phase weight ratio (Figure 4B). The HRP-labeled D_1 was detected at 5 nM (125 fmol of D_1 in the 25-μL droplet) at a signal-to-noise ratio of 6 on the 3.6-mm-diameter electrodes, on which C_1/PAA−PVI−Os was coelectrodeposited from a 1/15 weight ratio solution.

Arrays of 12 Electrodes of 1.5-mm-Diameter with 15-μL droplets. The redox polymer films had (1.21 ± 0.13) × 10^{-5} C cm^{-2} of electroactive $Os^{2+/3+}$ when deposited in 2 min from 15 μL of the 1 mg mL^{-1} PAA−PVI−Os solution on the 1.5-mm-

(16) Tio, K. K.; Shadal, S. S. *J. Heat Tranfer* **1992**, *114*, 220−226.
(17) Rizza, J. J. *J. Heat Transfer* **1981**, *103*, 501−507.
(18) Maruyama, S.; Kurashige, T.; Matsunmoto, S.; Yamagushi, Y.; Kimura, T. *Microscale Thermophys. Eng.* **1998**, *2*, 49−62.
(19) Chandra, S.; Di Marzo, M.; Qiao, Y.; Tartarini, P. *Fire Saf. J.* **1996**, *27*, 141−158.
(20) Tartarini, P.; Lorenzini, G.; Randi, M. R. *Heat Mass Transfer* **1999**, *34*, 437−447.

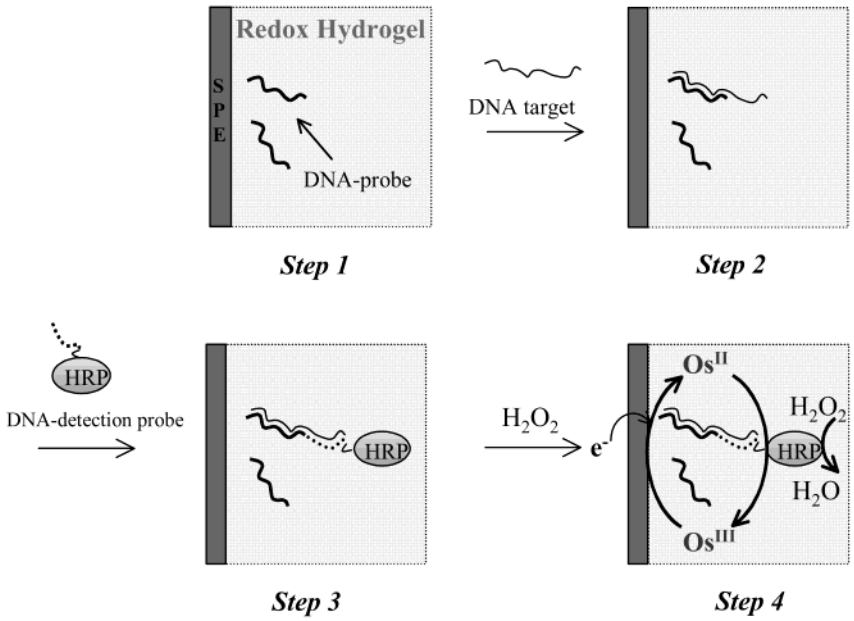


Figure 5. Steps of the sandwich-type assay: (1) The redox polymer and the oligonucleotide probe are electrodeposited on the screen-printed electrode (SPE); (2) the capture probe and the target are hybridized; (3) the electrode-bound target and the HRP-labeled oligonucleotide are hybridized, the HRP labels are in electrical contact with the redox polymer; and (4) the electrocatalytic reduction current of H_2O_2 to water is measured.

diameter electrodes of the 12 SPE arrays shown in Figure 1 (right).

Coelectrodeposition of single-stranded C_1 and hybridization with the HRP-labeled detection probe D_1 allowed the detection of D_1 at 3 nM concentration, with 75 fmol of the HRP-labeled oligonucleotide in the 25-μL droplet at a signal-to-noise ratio of 5.

Sandwich Hybridization of a 38-Base Sequence and Detection of a Single Base Mismatch. The assay sketched in Figure 5 was carried out with the capture probe C_2 (Table 1) having an amine-terminated 6-carbon spacer arm, 12 nonhybridizing T bases, and a 20-base-long specifically hybridizing sequence. The negative charge on an oligonucleotide increased with the mole fraction of T, the only base without an amine. As a result, the electrostatic adduct of C_2 and PAA−PVI−Os precipitated even at a polymer/oligonucleotide weight ratio of 30:1. For this reason, the C_2-containing redox polymer films were prepared by first electrodepositing the PAA−PVI−Os film, then incorporating C_2 in the film by electrochemically induced ligand exchange. The incorporation resulted in the exchange of inner-sphere chlorides of the Os complex of the redox polymer by the terminal amine of the spacer arm of C_2. Each of these steps required 2 min. The accumulation of C_2, which decreased the voltammetric peak heights and increased their separation (see above), was monitored by cyclic voltammetry. Hybridization of the PAA−PVI−Os−C_2 films with their HRP-labeled complement D_2 yielded electrodes on which H_2O_2 was electroreduced at a current density identical with that seen in the PAA−PVI−Os−C_1–modified, D_1-hybridized electrodes.

In the sandwich-type assay, the PAA−PVI−Os−C_2, 20-hybridizing-base capture probe was hybridized with the 38-base target T_2 in a 25-μL droplet (15 min at 53 °C), then the HRP-labeled 18-base probe D_2 was hybridized with the target. The optimal concentration of the HRP-labeled detection probe D_2 was 50 nM

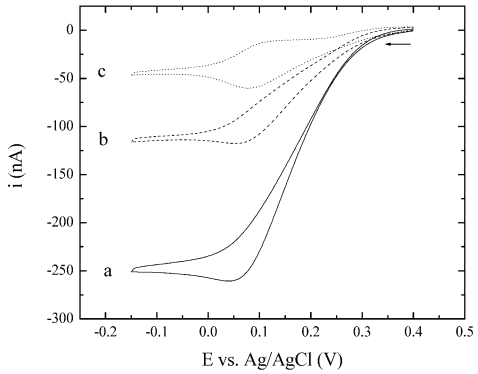


Figure 6. Cyclic voltammograms of the sandwich hybrids with 0, 1, and 2 mismatched base pairs. The electrodeposited PAA−PVI−Os films were reacted with capture probe C_2; hybridized (a) with the perfectly matched target T_2, (b) with the target with a single mismatched base T_2', or (c) with the target with two mismatched bases T_2''. (PBS, 10 nM target, 16 mM H_2O_2, scan rate v = 5 mV s^{-1}).

when the concentration of the target T_2 was 10 nM. The voltammograms of the electrodes modified with the perfectly matched target T_2, or with the single-base-mismatched target T_2', or with the two-base-mismatched target T_2'' of Table 1 are shown in Figure 6. The signal was halved when a single base pair was mismatched and decreased further when two base pairs were mismatched. The target T_2 was detected at 200 pM concentration, at which concentration the 25-μL droplet contained 5 fmol of T_2.

DISCUSSION

Characteristics of the Assay. Screen-printed electrodes on plastic sheets are produced at low cost. The potentially low-cost system detects DNA above 200 pM concentration, making it useful and highly cost-competitive with fluorescence-based methods in

confirming the PCR amplification of a specific oligonucleotide. The number of D_1 copies producing the observed current was estimated from the turnover rate of the HRP label after its attachment to the oligonucleotide, 756 s^{-1} at 25 °C. Because two electrons are transferred per turnover, the current per active label is 2.4×10^{-16} A. The observed current, 70 nA after hybridization of 0.125 pmol (5 nM in 25 μL) of D_1, results therefore from 3×10^8 copies of active and wired HRP hybrids. This number represents ~1% of the number of copies of the DNA contained in the assayed droplet. The corresponding surface capture-probe coverage on the 3.6-mm-diameter SPE is 5×10^{-15} mol cm^{-2}, in the midrange of the theoretically estimated packing of a 49-base-pair oligonucleotide on a solid surface.[21] Assuming that the electrodeposition of the 1:15 C_1/PAA−PVI−Os weight ratio solution produces ss-DNA redox polymer films with the same 1:15 weight ratio, 90 fmol of the capture probe are electrodeposited on the 3.6-mm-diameter SPEs. Thus, ~6% of the surface-bound C_1 capture-probes were actually hybridized.

Electrostatic Adducts and Their Precipitation. In absence of attractive interaction between the redox polymer and the capture probe DNA, the two would phase-separate (as do all macromolecules because of the low entropy of mixing). Phase separation would preclude the essential electrical contact between the detection-probe-bound HRP and the redox polymer (Figure 5). The formation of the electrostatic adduct, however, adversely affects the electronic conductivity of the redox polymer films and limits the useful range of redox polymer/capture probe ratio. Although the response improves when the weight fraction of DNA is increased in the deposition solution (Figure 4), phase separation of the electrostatic adduct limits the composition. Oligonucleotides rich in A, G, and C have a smaller net negative charge than T-rich oligonucleotides, because amines of A, G, and C balance in part their anionic phosphate charge. Thus, poly-T-rich sequences precipitated more readily, and the preparation of their films required a two-step process in which the redox polymer was electrodeposited first from a solution without DNA, following by coordinative binding of the amine terminus of the capture probe's DNA to the deposited redox polymer. Because the single-stranded oligonucleotide had only one-half of the negative charge of the hybrid, its concentration limit in the deposition solution was twice that of the double-stranded capture probe. The signal obtained with films deposited from the single-stranded capture probe solution was, correspondingly, twice that obtained with films deposited from solutions of the double-stranded then denatured probe solutions (Figure 3).

Incorporation of the DNA polyanions in the redox polymer increased the density of the ionic bridges, reduced the mobility of the redox polymer's segments, reduced the segmental collisions leading to electron exchange between reduced and oxidized centers of the redox polymer, and as a result, reduced the conduction of electrons in the films.[22] Furthermore, immersion in boiling water annealed the film, its polyanions and polycations assuming the most stable, best electrostatically bound, most rigid, and therefore, most resistive structure (Figure 2B).

Electrodeposition of the Redox Polymer and Coordinative Attachment of Amine-Terminated DNA to the Redox Polymer. The electrodeposition of redox polymers through their coordinative cross-linking has been described elsewhere in detail.[8] The process involves the exchange of labile, inner-sphere chloride ligands of $Os^{2+/3+}$ complexes of one chain with more strongly coordinated nitrogen ligands of a second chain. Because Os^{3+} binds the chloride anion more strongly than Os^{2+}, the exchange proceeds only when the chloride-comprising Os complex is reduced. Amine-terminated ds- and ss-oligonucleotide capture probes are similarly incorporated into the electrodeposited redox polymer films as the amines also replace inner sphere chloride of the Os^{2+} complexes. (Figure 2A, dashed line). The rate of electrodeposition of the redox polymer on the carbon electrodes is defined by the density of the exchanged ligands and the exchanging ligands on the carbon surface. When a polymer like PAA−PVI−Os, having both exchangeable chloride and chloride-exchanging ligands, is electrodeposited, the rate of its cross-linking upon electroreduction of its Os^{3+} centers to Os^{2+} increases with the square of the surface density of the polymer molecules. Thus, the rate is slow on carbon surfaces that do not strongly adsorb the redox polymer and is fast on those that do. Of the graphite surfaces, the most strongly adsorbing ones are those perpendicular to the van der Waals plane. On vitreous carbon surfaces, the density of adsorbed polycationic PAA−PVI−Os polymer is high only after the surface is enriched in anionic oxygenated functions either by exposure to an oxygen plasma or by electrooxidation.[23] Oxidation is not needed, however, on small graphite particles, where the reactive carbon radicals in surfaces perpendicular to the van der Waals plane are spontaneously oxidized in air. As a result, the redox polymer is electrodeposited on the small graphite particle-based carbon inks even when the electrodes are not cycled through oxidation (surface preparation)−reduction (polymer deposition) cycles. Furthermore, it is sufficient to poise the electrodes at a mildly reducing potential (−0.3 V), where the $Os^{2+/3+}$ is electroreduced, for the deposition to be rapid (Table 2).

Absence of Oxidative Damage to the Oligonucleotide upon Its Coelectrodeposition. Because reactive oxidizing species (such as hydrogen peroxide, ·OOH radicals, and singlet oxygen) might be produced in the faradaic processes at electrodes in the air-saturated droplets during the electrodeposition process, and because these species may oxidize the oligonucleotides, the initially chosen coelectrodeposited sequences were designed so as to shift part of the oxidative damage from the recognition sequence to remote poly-G sequences that were nonessential for the assay.[24−27] Thus, the 49-base C_1C_1* hybrid (Table 1) had sacrificially oxidizable G-rich 5′ sequences, that would have been preferentially oxidized if the oligonucleotide were oxidatively attacked. The hybrid oligonucleotide (of the C_1* hybrid capture sequence) was initially believed to be better protected against oxidation than the single-stranded one by sacrificially oxidizable G sequences. For this reason, the hybrid was initially deposited,

(21) Chan, V.; Graves, D. J.; McKenzie, S. E. *Biophys. J.* **1995**, *69*, 2243−2255.
(22) de Lumley-Woodyear, T.; Rocca, P.; Lindsay, J.; Dror, Y.; Freeman, A.; Heller, A. *Anal. Chem.* **1995**, *67*, 1332−1338.
(23) Anderson, C. W.; Lung, K. R.; Paul, M. *J. Electrochem. Soc.* **1982**, *129*, 2505−2508.
(24) Kan, Y.; Schuster, G. L. *J. Am. Chem. Soc.* **1999**, *121*, 10857−10864.
(25) Sanii, L.; Schuster, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 11545−11546.
(26) Sistare, M. F.; Codden, S. J.; Heimlich, G.; Thorp, H. H. *J. Am. Chem. Soc.* **2000**, *122*, 4742−4749.
(27) Heller, A. *Faraday Discussions* **2000**, *116*, 1−13.

then denatured in boiling water. The protection against oxidation proved, however, to be unnecessary, and as the process evolved, it was focused on electrodepositing single-stranded capture sequences.

Assay Time: Convection in the Heated Droplet. To avoid slow mass transport that would have extended the time required for the assay, convective flow was maintained in the droplets by producing a temperature gradient. The temperature gradient between the heated bottom of the droplet and its evaporatively cooled surface produced a convective flow loop.[16−20] In the absence of HRP, the heated surface of the electrode was maintained at 53 °C, 20 °C below the melting temperature of the hybrid, because at 53 °C, the hybridization rate was at its theoretical maximum.[28] In the presence of the HRP-labeled detection probe, which lowered the surface tension and thereby caused the spreading of the droplet, the droplets evaporated excessively rapidly at 53 °C (in 15−20-min). For this reason, the hybridization temperature was maintained at 37 °C.[16,20] With the hybridization performed in the enclosed chamber of a strip similar to that of electrochemically glucose assaying strips, evaporation would be a lesser problem, and a higher temperature at which the convection and evaporation rates are optimal could be maintained for assays performed with a thermostable enzyme label, such as thermostable soybean peroxidase.[29]

(28) Anderson, M. L. M. In *Gene Probes 2, A Practical Approach*; Hames, B. D., Higgins, S. J., Eds.; Oxford University Press, Inc.: New York, 1995; pp 1−29.
(29) Vreeke, M. S.; Yong, K. T.; Heller, A. *Anal. Chem*. **1995**, *67*, 4247−4249.

ACKNOWLEDGMENT

The research reported in this document/presentation was performed in connection with Contract/Cooperative Agreement number DAAD17-01-D-0001 with the U.S. Army Research Laboratory. The views and conclusions contained in this document/presentation are those of the authors and should not be interpreted as presenting the official policies or position, either expressed or implied, of the U.S. Army Research Laboratory or the U.S. Government unless so designated by other authorized documents. Citation of manufacturer's or trade names does not constitute an official endorsement or approval of the use thereof.

Received for review January 24, 2002. Accepted June 24, 2002.

AC025541G