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Amperometric Detection of DNA by Electroreduction of O_2 in an Enzyme-Amplified Two-Component Assay

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Abstract : The two-component type enzyme amplified amperometric DNA assay is described to use an ambient O_2 of the substrate of the DNA labeling enzyme. Although the assay detects DNA only at >0.5 nM concentration, a concentration $\sim 10^6$ fold higher than the sandwich-type enzyme amplified amperometric DNA assay, it can be run with an always available substrate. The assay utilizes screen-printed carbon electrodes (SPEs) which were pre-coated by a co-electrodeposited film of an electron conducting redox hydrogel and a 37-base long single-stranded DNA sequence. The DNA in the electron conducting film hybridizes and captures, when present, the 37-base long detection-DNA, which is labeled with bilirubin oxidase (BOD), an enzyme catalyzing the four-electron reduction of O_2 to water. Because the redox hydrogel electrically connects the BOD reaction centers to the electrode, completion of the sandwich converts the film from non-electrocatalytic to electrocatalytic for the reduction of O_2 to water when the electrode is poised at 200 mV vs. Ag/AgCl. The advantage of the assay over the earlier reported sandwich type enzyme amplified amperometric DNA assay, in which the amplifying enzyme was horseradish peroxidase, is that it utilizes ambient O_2 instead of the less stable and naturally unavailable H_2O_2 .

Key words : DNA Detection, Electrochemistry, Amperometry, Enzyme-Amplification, Screen-printed carbon electrode, Bilirubin oxidase

1. Introduction

In two component-type DNA assays (a) a capture sequence is immobilized on a surface; (b) the modified surface is exposed to the analyte solution so that, if present, the single-stranded detection DNA with an enzyme-labeled is captured.[1-5] The enzyme-labeled surface-bound duplex is detected through an enzyme-catalyzed reaction. Because redox enzymes can be conveniently "wired" through redox hydrogels to electrodes, the presence of the enzyme-labeled duplex can be detected amperometrically through electrooxidation or electroreduction of the substrate of the enzyme. In earlier studies by Heller group the labeling enzyme was a peroxidase, such as horseradish peroxidase (HRP), or thermostable soybean peroxidase.[6,7] With screen printed carbon macroelectrodes (SPEs) the lowest DNA concentration detected by the HRP-involving H_2O_2 requiring assay was 20 pM, though with microelectrodes DNA was detected already at 0.5 fM concentration.[8-10] At this concentration the analyzed 10 μL droplets contained only 3000 copies of DNA.[10]

To "wire" the labeling peroxidase and to cause the electrodes to selectively capture the single stranded target DNA an electron conducting redox hydrogel was electrodeposited on the electrodes, then the capture sequence was co-electrodeposited.[8] In the electrodeposition, Os^{3+} complexes with inner-sphere chloride, bound to the backbone of a water-soluble vinyl-imidazole co-polymer, were electroreduced to Os^{2+}.[11] The Os^{2+}, unlike Os^{3+}, exchanges its inner-sphere chloride by nitrogen, whether of an amine or heterocycle. Thus, exchange of the inner sphere chloride of an Os^{2+} center of one chain, by an imidazole of a neighboring polymer chain, causes linking of the two chains and multiple crosslinking leads to the precipitation of the polymer on the electrode. The DNA sequence selectively capturing the analyte-DNA carries a spacer-arm, with a terminal primary amine. When this amine exchanges an inner-sphere chloride of the Os^{2+}, the capture sequence is bound to the film. Thus, both the redox polymer and the capture sequence are co-electrodeposited at a reducing potential.

Because the earlier the detection-sequence labeling enzyme was HRP, the assay required hydrogen peroxide. For an assay at home or in a field it is much more convenient to use atmospheric O_2 as the substrate. Here we show that DNA can be detected amperometrically through a two-component type assay by four electron electroreduction of O_2 to water, with bilirubin oxidase (BOD) serving as the label of the duplex. Unlike laccase, which also catalyzes the electroreduction of O_2 to water, but looses most of its activity in chloride-containing solutions,[12-17] BOD retains its activity and could be used as a label in assays in plasma or serum.[18-23]

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2. Experimental

2.1 Chemicals

Bilirubin oxidase (BOD) (EC 1.3.3.5) from *Myrothecium verrucaria* was purchased from Sigma, St. Louis, MO. The desalted analyte ("target") sequence, the capture sequence with a 5′-amine-terminated 12-T spacer and the detection sequence with a 5′-amine-terminated 7-C and 11-T spacer (Table 1) were custom prepared by Synthetic Genetics, San Diego, CA. The buffering salts and other chemicals were purchased from Sigma (St. Louis, MO) and from Aldrich (Milwaukee, WI) and were used as received.

The phosphate buffered saline solution (PBS: 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, 140 mM NaCl), the hybridization buffer (4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, 500 mM NaCl, and 10 mM EDTA), the washing buffer (4.3 mM NaH_2PO_4, 15.1 Na_2HPO_4, 500 mM NaCl and 0.5% Tween 20®), and all other solutions were prepared using deionized water (Barnstead, Nanopure II, Van Nuys, CA).

The electron-conducting redox polymer, PAA-PVP-$[Os(bpy)_2Cl]^{+/2+}$, a co-polymer of polyacrylamide (PAA) and poly (N-vinylpyridine) (PVP) complexed with $[Os(2,2'\text{-bipyridine})_2Cl_2]^{2+/3+}$, was synthesized as previously described (Fig. 1).[9]

2.2 Instrumentation and 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 the commercial carbon ink Electrodag® 423SS (Acheson, Port Huron, MI).[8] To avoid the spreading of the 25 μL droplets beyond the 3.5 mm-diameter working electrodes, a hydrophobic circle was drawn around each SPE with a felt-tip pen containing 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 0.5 mm diameter platinum wire counter electrode; and a Ag/AgCl micro-reference electrode (3 M KCl saturated with AgCl) (Cypress, Lawrence, KS), to *which all potentials are referenced.*

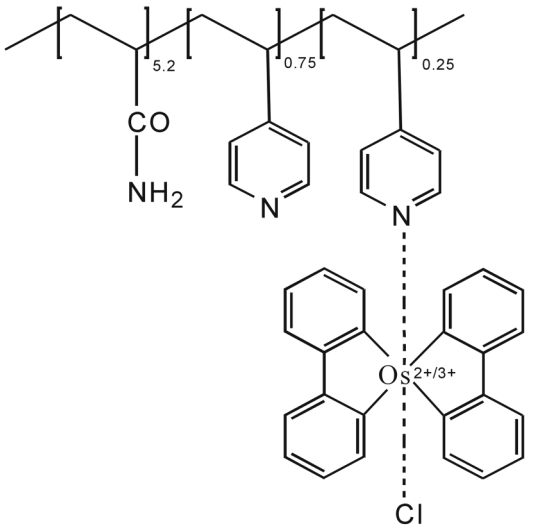

Fig. 1. Structure of PAA-PVP-$[Os(bpy)_2Cl]^{+/2+}$.

2.3 Electrodeposition of the redox polymer PAA-PVP-$[Os(bpy)_2Cl]^{+/2+}$

The earlier described electron-conducting redox polymer PAA-PVP-$[Os(bpy)_2Cl]^{+/2+}$, a copolymer of polyacrylamide and poly-N-vinylimidazole, the imidazoles complexed with $[Os(bpy)_2Cl]^{+/2+}$ (2,2-bipyridine), was electrodeposited as follows:[8,9] 25 μL drops of the 1 mg/mL polymer in saline PBS solution were pipetted onto the SPEs. The SPEs were poised at -1.4 V vs. Ag/AgCl for 2 minutes, rinsed with deionized water and with PBS, and then scanned between 100 mV and 500 mV vs. Ag/AgCl, to confirm the electrodeposition of the redox polymer. The preparation of the electrodes was completed by pipetting onto them 25 μL drops of the 2 μM 5′-amine terminated-capture sequence *(C1)* solution in PBS, co-depositing the sequence by poising the electrodes at -1.4 V vs. Ag/AgCl for 2 minutes and rinsing.

The assay was comprised of the hybridization of the 5′-amine-terminated detection probe (*D1*). The electrode was placed on the block heater block (Digi-Block JR, Aldrich). The 30 μL of the 5′-amine terminated 37-base detection, of which a 37-base part was complementary to the capture sequence, in hybridization buffer was pipetted onto the SPEs (0.5 nM~10 nM, 40 min, 50°C). The electrode was rinsed thoroughly with PBS and with the hybridization buffer. The resulting amine-functionalized film was reacted first with 30 μL drop of a 1.0% glutaldehyde solution in PBS, 20 min, 25ºC, then rinsed with deionized water and with PBS, then pipetted onto the SPEs with 30 μL of the bilirubin oxidase solution (1 mg/mL in PBS), then finally rinsed thoroughly with PBS. The O_2 electroreduction current was then measured with the electrode poised at 200 mV vs. Ag/AgCl in 30 μL of PBS solution at 25ºC (Fig. 2.).

3. Results and Discussion

The steady-state cyclic voltammograms of an electrodeposited film PAA-PVP-$[Os(bpy)_2Cl]^{+/2}$ with co-electrodeposited C1,

Table 1. Oligonucleotide Sequences for Capture Probe (Surface Immobilized), NH_2-labeled Detection probe DNA.

	Sequence (5′→ 3′)
Capture(*C1*)	NH_2 6C-spacer- TTT TTT TTT TTT *GGG GGG GGG GGG GAG CAA AGG TAT TAA CTT TAC TCC C*
Detection(*D1*)	NH_2 7C-spacer- TTT TTT TTT TT*G GGA GTA AAG TTA ATA CCTTTG CTC CCC CCC CCC CCC*

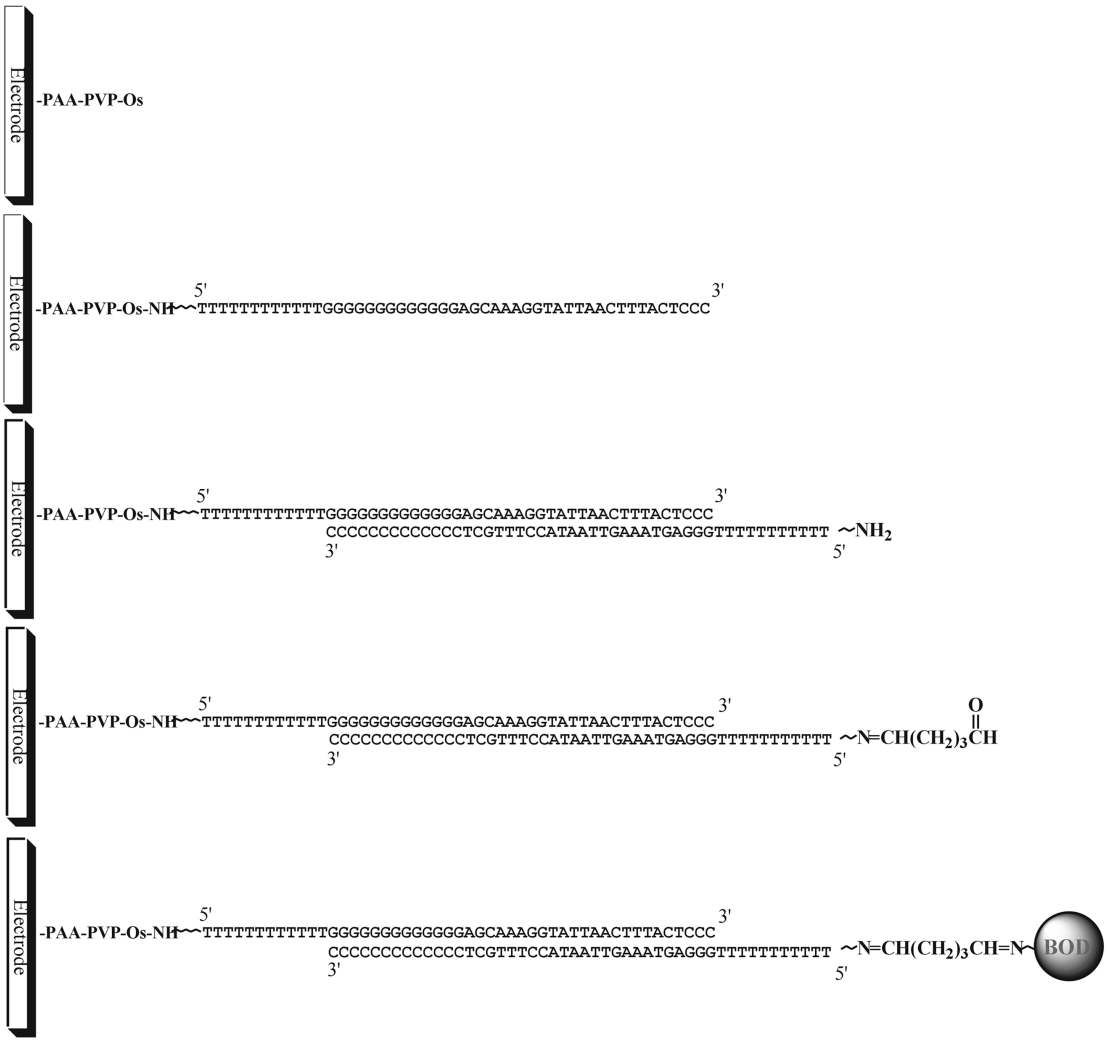

Fig. 2. Schematic diagram of the enzyme-amplified amperometric detection. (1) Electrodeposition of the PAA-PVP-Os redox polymer (2) Co-electrodeposition of the 5′-amine terminated-capture probe to the redox polymer (3) Hybridization and surface immobilization of the 5′-amine terminated-detection probe (4) Reaction of the primary amine of the detection probe with glutaldehyde (5) Binding of bilirubin oxidase (BOD) to the aldehyde.

PAA-PVP-Os with co-electrodeposited oligonucleotides (C_1) and hybridizing detection probe (D_1) and with/without BOD are shown in Fig. 3. The votammograms of electrodes with deposited D_1 had slightly smaller peak areas and their peaks were more closely, indicating lost Os polymers and faster electron transfer. After hybridizing (40 min, 50°C) the coordinatively polymer-bound C_1 on the electrode with 25 µl droplets of 50 nM BOD-labeled complementary prode D_1 (Table 1). The catalytic electroreduction of O_2 starts at +400 mV versus Ag/AgCl and the catalytic current plateau was reached at +200 mV versus Ag/AgCl.

The five steps of the process leading to the electrical "wiring" of BOD when the analyte sequence is present in the test solution are shown in Figure 1. Fig. 4 shows the electron transfer steps underlying the catalysis of O_2 electroreduction to water when BOD is electrically contacted with the $Os^{2+/3}$ complex comprising redox polymer.

As seen in Fig. 5, the O_2 electroreduction current increased linearly with the concentration of the analyte DNA up to 5.0

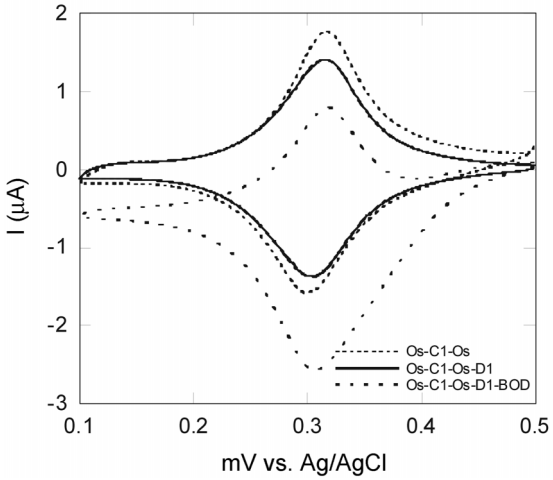

Fig. 3. Cyclic voltammograms of electrodeposited PAA-PVP-Os films with *C1* and *D1* with/without BOD under air; 3.5 mm-diameter screen-printed carbon electrode; scan rate 5 mV/s; pH 7.1 PBS with 0.14 M NaCl.

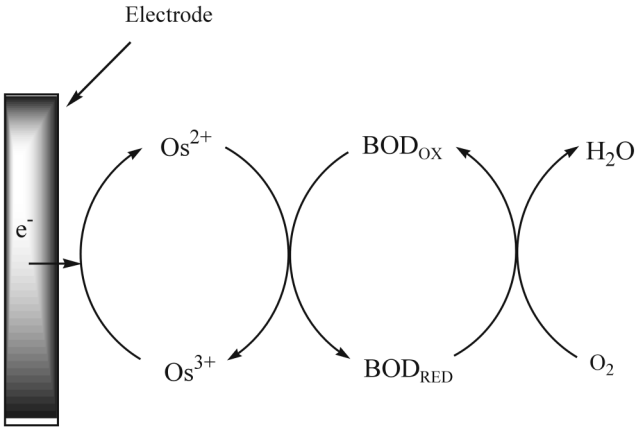

Fig. 4. Steps of electron transfer in the electroreduction of O_2 to H_2O.

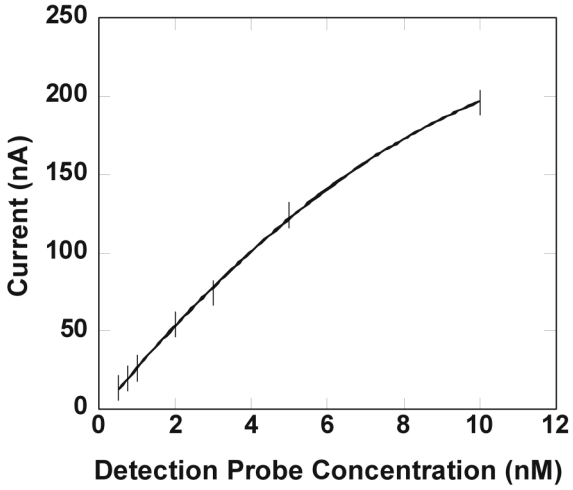

Fig. 5. Dependence of the O_2 electroreduction current on the concentration of the 38-base analyte DNA (*D1*). The currents were measured with the 3.5 mm-diameter SPEs poised at 0.2 V (Ag/AgCl), pH 7.1 PBS buffer with 0.14 M NaCl, 25ºC in air.

nM concentration. The lowest concentration detected was 0.5 nM. The detection limit, 0.5 nM, was about six orders of magnitude higher than the 0.5 fM limit for the detection with H_2O_2 as substrate and a microelectrode instead of the SPE.[8-10)]

Figure 6 shows the current change when 5 nM of the complementary HRP-labeled D_1 at 10, 20, 30, 40 and 50°C were allowed to directly hybridize in hybridization buffer. This result shows that mass transport was enhanced in the droplet during hybridization by heating the block on which the SPEs were placed, the temperature gradient between the heated bottom of the droplet and its evaporatively cooled surface producing a convective flow loop.

The dependence of the complementary *D1* current signal on the direct hybridization time is seen Fig. 7. When 5 nM aliquots *D1* hybridize for various times (10, 20, 30, 40, 50 minute) in hybridization buffer at 40°C, the current increased with hybridization time, reaching a plateau at 40 minute.

Finally, the detection limit, 0.5 nM, was about six orders

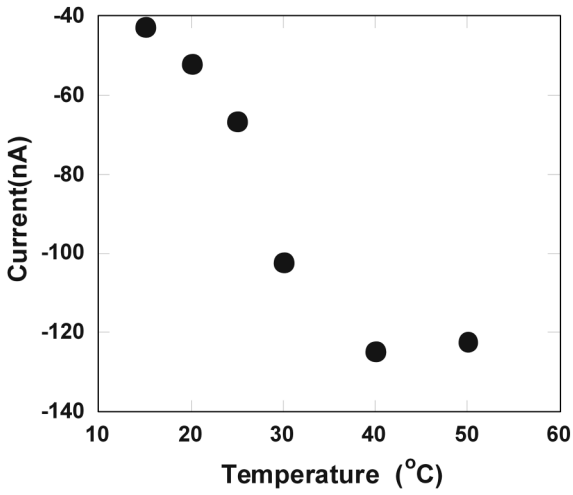

Fig. 6. Dependence of the current change on the different hybridization temperature with 5 nM of *D1*. The currents were measured with the 3.5 mm-diameter SPEs poised at 0.2 V (Ag/AgCl), pH 7.1 PBS buffer with 0.14 M NaCl, 25ºC in air.

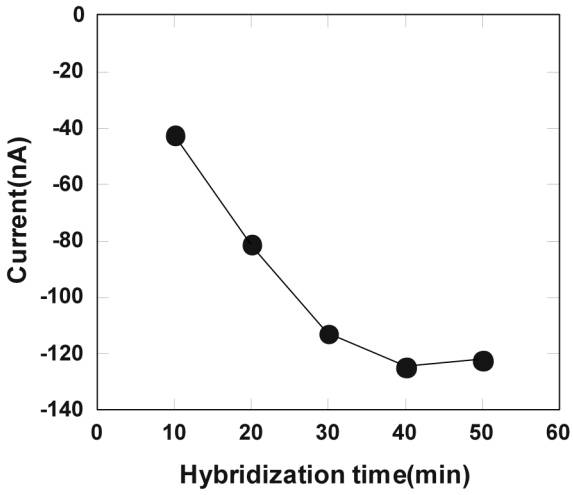

Fig. 7. Dependence of the current change on the different hybridization time with 5 nM of *D1*. The currents were measured with the 3.5 mm-diameter SPEs poised at 0.2 V (Ag/AgCl), pH 7.1 PBS buffer with 0.14 M NaCl, 25ºC in air.

of magnitude higher than the previous results using H_2O_2 as substrate of a microelectrode instead of the screen printed carbon electrodes (SPEs). The detection was also more complex, because it required not only hybridization, but also the formation of an additional reagent, Bilirubin Oxidase (BOD). Nevertheless it established that enzyme-amplified amperometric detection of DNA with ambient O_2 as the substrate is very feasible.

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