Direct Enzyme-Amplified Electrical Recognition of a 30-Base Model Oligonucleotide

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Electrochemical sensors recognizing hybridization of DNA strands based on electrogenerated chemiluminescence[1−5] and cyclic voltammetry of intercalated ruthenium and cobalt complexes[6,7] and organic dyes[8,9] and of redox functions covalently bound to single DNA strands[10] have been reported. Also a direct enzyme-amplified amperometric affinity assay for the avidin−biotin conjugate has been described.[11] In this system, bonding of biotinylated horseradish peroxidase to avidin covalently attached to an electron-conducting redox hydrogel resulted in the "wiring" of the enzyme, i.e. its electrical connection to the electrode. Such connection produced an H_2O_2 electroreduction current proportional to the number of conjugates. Here we show that the hybridization of a model oligonucleotide can be directly measured as an electrical current. The current flows as a result of continuous electroreduction of H_2O_2, electrocatalyzed by the horseradish peroxidase (HRP) label of an oligonucleotide strand when the complementary strand is covalently bound to a hydrogel that electrically "wires" the HRP (Figure 1). In such a hydrogel, electrons diffuse via self-exchange of electrons between colliding segments of the redox polymer network, which become mobile when hydrated.[12]

A strand of poly(deoxythymidine)-5′-phosphate ($pd(T)_{25-30}$), oligonucleotide **I**, was attached to a film of a polyacrylamide-based, electron-conducting redox hydrogel on a vitreous carbon electrode.[13] The complementary strand, poly(deoxyadenosine)-5′-phosphate ($pd(A)_{25-30}$), oligonucleotide **II**, was labeled with HRP. Upon hybridization the enzyme was electrically wired to the electrode, and H_2O_2 was electrocatalytically reduced. The resulting current density was 1.4 ± 0.2 $\mu A/cm^2$ on an electrode poised at 0.0 V versus Ag/AgCl. The current density resulting from nonspecific binding of HRP bound to a noncomplementary oligonucleotide, such as $pd(T)_{25-30}$ or poly(deoxyguanosine)-5′-phosphate ($pd(G)_{12-18}$), oligonucleotide **III**, was only 0.07 ± 0.02 $\mu A/cm^2$.

The electron-conducting redox hydrogel was formed by cross-linking on a vitreous carbon electrode the copolymer PAA−PVI−Os (Figure 2a) with polyacrylamide−hydrazide (Sigma, Cat# P-9905) (Figure 2b) using poly(ethylene glycol diglycidyl ether) (Polysciences Cat# 08211) as a cross-linker. The respective weight fractions of the three polymers were 61:37:2, and the total polymer loading was 0.4 mg cm^{-2}. Oligonucleotide **I** was covalently bound by carbodiimide coupling

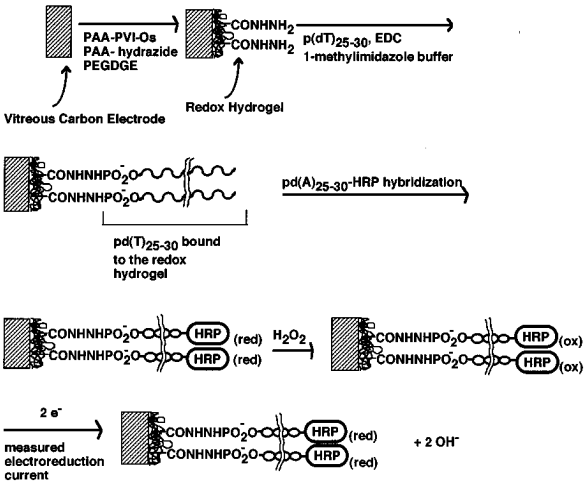

Figure 1. Design of the oligonucleotide probe. The redox film was formed of aqueous solutions of 5 g/L PAA−PVI−Os, 3.3 g/L PAH, and 0.21 g/L PEGDGE mixed in a 1:1:1 v/v ratio by loading 10^{-6} L of the mixture on a 3 mm vitreous carbon electrode. For binding the 5′-phosphate esters of the oligonucleotide to hydrazides of the hydrogel by carbodiimide coupling, 1.85×10^{-4} g of pd(T) (5 units) was dissolved in 2.47×10^{-4} L of water to which 2.7×10^{-5} L of (0.1 M) 1-methylimidazole buffer was added, and the $pd(T)_{25-30}$ solution was mixed in a 5.5:1 ratio with a 0.5 M solution of EDC. The 5.5:1 mixture (6×10^{-6} L) was syringed on the redox hydrogel coated surface. The electrodes were kept for 16 h at room temperature in a water-saturated atmosphere to prevent the 6×10^{-6} L drop from drying. When $pd(T)_{25-30}$ was hybridized with HRP-labeled $pd(A)_{25-30}$, the enzyme was wired and an H_2O_2 electroreduction current was measured. The hybridization solution contained 5×10^{-2} M Tris HCl, 0.2 M NaCl, 4×10^{-7} M $pd(A)_{25-30}$ labeled with HRP, and the excess HRP (4×10^{-6} M) that was not consumed in the labeling of $pd(A)_{25-30}$.

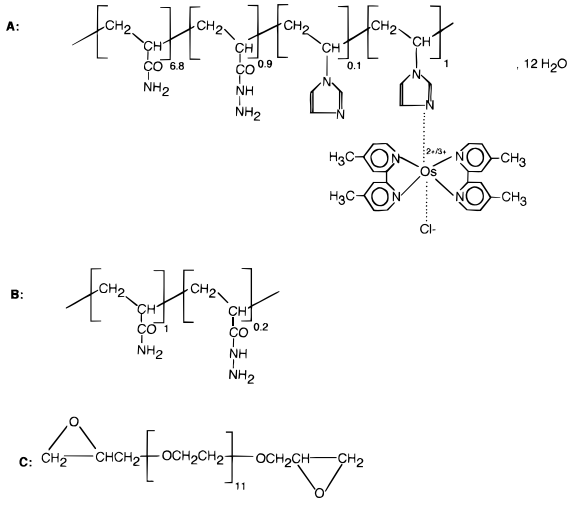

Figure 2. (A) Structure of the water soluble copolymer of acrylamide and vinylimidazole, modified with hydrazine and $[Os(dmebpy)_2Cl]^{+/2+}$ (dmebpy = 4,4′-dimethyl-2,2′-bipyridine functions), PAA−PVI−Os (MW 1502.9). (B) Structure of polyacrylamide hydrazide (PAH), with 9×10^{-4} mol/g hydrazide. (C) Structure of the cross-linking agent, poly(ethylene glycol 600 diglycidyl ether) (PEGDGE).

to hydrazide functions of the hydrogel by a reported process.[14] Oligonucleotide **II** was HRP labeled, as shown in Figure 3; 1.85×10^{-4} g of $pd(A)_{25-30}$ was treated with 3×10^{-3} L of a 0.1 M imidazole, 0.15 M EDC, 2.5×10^{-3} M hydrazine monohydrate solution at 23 °C for 16 h.[15] To remove the excess hydrazine, the oligonucleotide was precipitated in ethanol,

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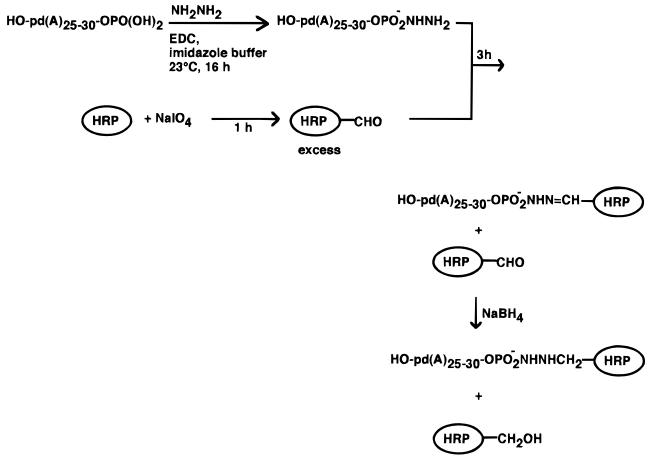

Figure 3. Hydrazine-modified 5′-phosphorylated oligonucleotides $pd(A)_{25-30}$ labeled with HRP, part of the oligosaccharide which had been previously oxidized to aldehydes.

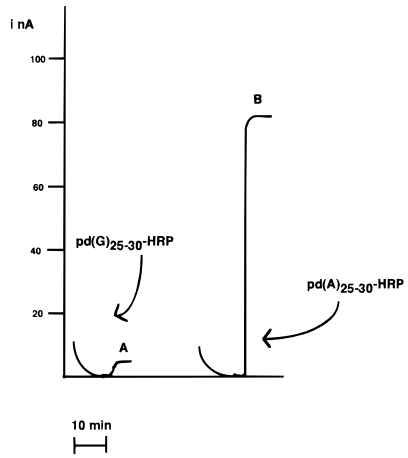

Figure 4. Increase in the electroreduction current upon addition of 10^{-4} M H_2O_2. (A) The coated electrode was incubated in a pretreatment solution of 3% nonfat dry milk in PBS for 10 min, hybridized in 5×10^{-2} M Tris HCl, 1 M NaCl, 4×10^{-7} M $pd(T)_{25-30}$ labeled with HRP, 4×10^{-6} M HRP, 0.2% Tween 20 for 20 min, and rinsed in 5×10^{-2} M Tris HCl, 1 M NaCl, 0.2% Tween 20 for 20 min. (B) As in A, except that 4×10^{-7} M $pd(T)_{25-30}$−HRP was replaced by 4×10^{-7} M $pd(A)_{25-30}$ labeled with HRP.

recovered by centrifugation, and redissolved in 10^{-3} L of 0.1 M sodium bicarbonate buffer. HRP (20 g/L) and $NaIO_4$ (12 g/L) were mixed in a 1:2 ratio. The mixture was allowed to react 1 h in the dark at room temperature. After oxidation of the enzyme oligosaccharides, the excess sodium periodate was removed by dialysis using an Amicon Centriprep 30. HRP solution (10^{-3} L) was collected and reacted with 10^{-3} L of the $pd(A)_{25-30}$ solution for 3 h at room temperature. A 10-fold excess of HRP was used to avoid the binding of multiple $pd(A)_{25-30}$ units to the same HRP molecule. The Schiff's bases formed between aldehydes of the HRP and the hydrazides of the 5′ end of the oligonucleotides were then reduced with $NaBH_4$. The 10-fold excess of free HRP alcohol, which was not separated, would have interfered with the recognition of hybridization if its nonspecific binding to the redox hydrogel (in which oligonucleotide **I** was immobilized) would have been, contrary to our findings, significant.

In order to prevent nonspecific binding of the codissolved HRP, the electrode coated with the redox hydrogel with bound oligonucleotide **I** was treated with a blocking buffer (3 wt % nonfat dry milk in phosphate-buffered saline). The electrode was then hybridized in a 4×10^{-7} M solution of HRP-labeled oligonucleotide while rotating at 1000 rpm. The hybridization solution contained 5×10^{-2} M Tris HCl, 1 M NaCl, 0.2% Tween 20, and either 4×10^{-7} M $pd(A)_{25-30}$−HRP or 4×10^{-7} M oligonucleotide **III**−HRP and also 4×10^{-6} M unbound HRP residue from the labeling process of the oligonucleotide. For the amperometry a three-electrode cell with a Ag/AgCl reference and a platinum counter electrode was used. The rise in H_2O_2 electrocatalytic reduction current (eq 1) was measured

$$H_2O_2 + 2\ e- \rightarrow 2\ OH- \qquad (1)$$

for pairs of identical electrodes. One electrode of each pair was incubated for 20 min in a hybridizing solution containing the HRP-labeled oligonucleotide **II**, while the second electrode was incubated in a hybridizing solution containing either plain unbound HRP or HRP bound to oligonucleotide **III**. In the presence of the complementary HRP-labeled oligonucleotide **II**, the current density increased upon injection of H_2O_2 from 0.03 ± 0.02 to 1.4 ± 0.1 $\mu A/cm^2$. When the complementary oligonucleotide was absent, but a noncomplementary HRP-labeled oligonucleotide was present, the current did not increase significantly, reaching only 0.07 ± 0.02 $\mu A/cm^2$. Results identical to those above were obtained when, instead of testing pairs of electrodes in oligonucleotide **III**−HRP or oligonucleotide **II**−HRP containing solutions, only a single electrode was tested, first in 4×10^{-7} M oligonucleotide **III**−HRP and then in oligonuclotide **II**−HRP (Figure 4).

In another set of control experiments the electron-conducting hydrogel on the electrode was identically prepared and tested, except that EDC was not added in the step where oligonucleotide **I** was bound to the hydrazide functions of the hydrogel. Consequently any oligonucleotide **I** incorporation into the gel would have resulted from noncovalent, e.g. electrostatic, binding. In the absence of covalent binding of oligonucleotide **I** to the redox hydrogel, there was no significant increase in current in this case. Incubation with an HRP-labeled, noncomplementary oligonucleotide led to a current increase of 0.10 ± 0.02 $\mu A/cm^2$, and incubation with HRP-labeled oligonucleotide **II** led to a current increase to 0.30 ± 0.02 $\mu A/cm^2$ under hybridization conditions. Apparently, the positively charged PAA−PVI−Os surface did electrostatically bind some oligonucleotide **I**. However, covalent binding of oligonucleotide **I** to the gel improved the specific binding with oligonucleotide **II** and reduced the nonspecific electrostatic interaction between the positively charged redox gel and oligonucleotide **III**−HRP.

Currents of $\approx 10^{-10}$ A are measurable with conventional electrochemical systems. With systems used for scanning electrochemical microscopy, 10^{-13} A currents are measured. An HRP molecule turns over about 1800 times per second; i.e. about 3600 electrons per second pass through one molecule. The corresponding current per enzyme molecule is 6×10^{-16} A. Thus, using conventional electrochemical equipment, a current of 2×10^5 hybrids would be measurable, and with a state-of-the-art system a current of about 20 hybrids would be measurable. At the observed current density of 1.4 $\mu A/cm^2$ the diameter of the smallest electrode required to deliver a measurable current is about 70 μm with a conventional system and about 2 μm for an advanced one. These estimates are conservative, as current densities in microelectrodes, where electron diffusion through the HRP-wiring hydrogel is radial, are at least an order of magnitude higher than in the macroelectrodes of this study.[16,17]

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