Enzyme-Amplified Amperometric Detection of Hybridization and of a Single Base Pair Mutation in an 18-Base Oligonucleotide on a 7-μm-Diameter Microelectrode

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Abstract: A single base pair mismatch in an 18-base oligonucleotide was detected amperometrically with a 7-μm-diameter carbon microelectrode. The hybridization was followed directly and in real time by steady-state amperometry. The microelectrode was coated with a hybridization-sensing layer in a two-step electrophoretic process, which yielded microelectrodes with reproducible dimensions. In the first step, a thin film of an electron-conducting redox polymer was deposited electrophoretically at constant potential in a low ionic strength solution. In the second step, a carbodiimide-activated single-stranded probe was reactively electrophoretically deposited and covalently attached to the redox polymer film. The labeling enzyme, thermostable soybean peroxidase (SBP), was covalently bound to the 5′-end of the target single-stranded oligonucleotide. When the redox polymer and the enzyme were brought to close proximity by hybridization of the target and probe oligonucleotides, the film on the electrode switched from being a noncatalyst to a catalyst for H_2O_2 electroreduction at −0.06 V vs Ag/AgCl. The current observed corresponded to that generated by ∼40 000 surface-bound and electrically connected SBP molecules.

Introduction

Measures of the relative advantages of different oligonucleotide-sensing systems include size, the number of copies detected, the selectivity, assessed by the ability to sense mutations, and the cost of the system.[1] There is no accepted figure of merit of the combination of these four measures. In some applications, particularly those relevant to sensing in combinatorial arrays where the samples are small, the combination of the size of the sensing element, the number of copies detected, and the ability to differentiate between long oligonucleotide sequences differing in a single base are particularly relevant. The size of the detecting elements defines their surface density in the array; the number of copies detected defines the number of PCR cycles needed to detect a particular sequence, the error rate increasing with the number of cycles; and the ability to differentiate between oligonucleotides of increasing length, differing only in a single base, defines the balance between the ability to locate mutants and the specificity. [2,3]

Millan et al.,[4] Singahal *et al.*,[5] and Napier *et al.*[6] have shown that electrochemical techniques are well suited for measuring hybridization events. A desired yet unapproached goal is to accurately detect a single base mutation in a single copy of a gene, using a sensing element the dimensions of which are not different from that of the smallest gate in an integrated circuit. The use of microelectrodes has proven successful in numerous applications where miniaturization was important.[7−9] Frequently, particularly in arrays where signals of different elements were compared, it was, however, not the electrode size that prevented the use of micrometer-sized electrodes but the reproducibility of their specificity-providing coatings. Routes to selectively coated electrodes have been described.[8,9] Korri-Youssoufi et al. electrochemically copolymerized oligonucleotide-modified monomers of pyrrole[10] and then constructed an array of 48 microelectrodes of 50 μm × 50 μm, each derivatized with a different oligonucleotide sequence.[11] With these they determined the hybridization through measuring the fluorescence of a hybrid-bound molecule.

In this paper, we describe results of a study aimed at the simultaneous reduction of electrode size and the number of copies required for detecting a single base mismatch in an 18-base oligonucleotide. We show that 7-μm-diameter oligonucleotide-detecting microelectrodes with reproducible electrochemical characteristics can be prepared and that these detect a single base mutation in an 18-base sequence, through measuring the current generated by 40 000 copies.

Experimental Section

Materials. All glassware was washed by soaking in Aquet (VWR) overnight then rinsed with deionized water. Sodium periodate (Catalog

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Table 1. The Oligonucleotide Sequences of This Work[a]

	sequence	base pairs	mp (°C)
probe	TTTTTTTTTTTTAAATATCATTGGTGTTTC	30	
complementary target	GAAACACCAATGATATTT	18	59.5
single-base-mismatched target	GAAACACCAGTGATATTT	18	54.5−52.5
four-base-mismatched target	GAAACACCAAAGATGATA	18	37−40

[a] The melting points were calculated using the equation of Bolton and McCarthy.[26]

No. 31,144-8) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Catalog No. 16,146-2) were purchased from Aldrich. Imidazole (Catalog No. I-20-2) and soybean peroxidase (SBP) (Catalog No. P-1432) were purchased from Sigma. All buffer salts and other inorganic chemicals were obtained from Sigma or Aldrich. The electron-conducting redox polymer, (PAA-PVI-Os), a 7:1 copolymer of acrylamide, acrylhydrazide, and 1-vinylimidazole, the imidazole functions complexed with $[Os(4,4'\text{-dimethyl-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$, was synthesized as previously described.[12] The probe and target oligonucleotides used were purchased from Genosys and are listed in Table 1. The 18-base probe was purchased with a 12-T spacer, and the 18-base targets were purchased with 12-carbon spacers between their oligonucleotide and terminal primary amine functions. The primary amines were used to form Schiff bases with aldehyde functions of periodate-oxidized oligosaccharides of SBP, which were subsequently $NaBH_4$ reduced to secondary amines.

Equipment. The current−time traces were recorded with a *Y*−*t* recorder (Kipp & Zonen). Electrochemical measurements were carried out using a low-noise CH Instruments model 832 electrochemical detector in conjunction with a Pentium computer. The water-jacketed cell was placed in a grounded faraday cage. Unless otherwise stated, the three-electrode system used consisted of the microelectrode, a Ag/AgCl reference electrode, and a large-area platinum flag or wire. For measurements above 25 °C, an agar salt bridge was used to maintain the reference electrode at a constant temperature of 25 °C. Microelectrodes were built in-house by sealing individual 7-μm-diameter carbon fibers (Goodfellow) in soft glass tubes (Kimble Products) in a butane flame. The electrode surface was exposed by fracturing the glass and polishing, first with sand paper and then with aluminas of decreasing particle size to 0.3 μm. The electrodes were tested by cyclic voltammetry in ferrocenemethanol and in pH 7.0 phosphate buffer solution for the absence of leaks and for perfection of the glass seal of the carbon fiber. The repolished microelectrodes were stored in deionized water.

Polymer Deposition. In the miniature cell that was designed for the electrophoretic deposition, the counter electrode was a 100-μm-thick platinum foil serving as the base of the cell. A silver wire served as a pseudoreference electrode. A total of 100 μL of a 0.085 mg mL^{-1} PAA-PVI-Os redox polymer solution in deionized water was placed in the cell and was used for up to 25 depositions before being replaced. After the microelectrode was connected to the potentiostat, it was lowered into the redox polymer solution, using a micromanipulator, until the tip of the microelectrode was 1 mm from the counter electrode at the base of the cell. The redox polymer was electrophoretically deposited by poising the potential of the microelectrode at −1.025 V (Ag/AgCl) for 2 min, after which the electrode was washed with deionized water. The deposition was then confirmed by cyclic voltammetry in buffer solution. It was essential that neither the working electrode nor its contact nor any part of the cell be touched, as the buildup of static electricity could change the electrochemical characteristics of the redox polymer coating of the microelectrode.

Probe Oligonucleotide Attachment. A solution of 450−550 μg of the probe oligonucleotide in 50 μL of pH 7 20 mM methylimidazole buffer was added to 50 μL of 0.2 M EDC in the same buffer. This mixture was kept overnight at 4 °C. The solution was then diluted with 450 μL of water, and the volume was reduced to 50 μL using a Microcon tube (Amicon) with a 3000-Da cutoff membrane. This procedure was repeated twice to remove the buffer salts from the solution. The solution was reduced to 50 μL, then transferred to an electrochemical cell similar to the one described above for the redox polymer deposition, and used for the electrophoretic deposition of the probe oligonucleotide. Because the oligonucleotide was EDC-activated, it reacted with hydrazide functions of the redox polymer on the microelectrode. The deposition conditions of the EDC-activated oligonucleotide were similar to those of the deposition of the redox polymer except that the applied potential was +0.9 V (Ag/AgCl) and the duration was 5 min, unless otherwise stated. After the oligonucleotide was deposited, a cyclic voltammogram was recorded in buffer solution containing 1 M NaCl.

Labeling of Oligonucleotides with SBP. The three 18-base target oligonucleotides of Table 1, one perfectly complementary to the probe, one with a single base mismatch, and one with four mismatched bases, were purchased with 5′-amine-terminated 12-carbon spacers. They were labeled with SBP as follows: 10 mg of SBP was dissolved in 0.25 mL of pH 7, 0.1 M phosphate buffer, and 0.25 mL of freshly dissolved sodium periodate in water was added. The solution was left to stand for 1 h in the dark and then passed through a standard G-25 gel filtration column (60 cm long 1.5-cm diameter). The concentration of the resulting oxidized enzyme was determined by the Bio-Rad protein assay (protein assay kit II). A 10-fold molar excess of enzyme was added to between 200 and 300 μg of oligonucleotide, bringing the volume to 0.5 mL. The solution was allowed to react for 3 h before 0.5 mL of 0.4 M $NaBH_4$ was added and then left for 13 h at 4 °C. The resulting oligonucleotide concentration was between 35 and 50 mM.

The activities of the SBP labels of the oligonucleotides were derived from the measured protein concentration and the rate at which the SBP catalyzed H_2O_2 oxidation of the leuco dye 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfuric acid) (ABTS) to the dye. The initial rate of dye formation was measured spectrophotometrically, using a Hewlett-Packard diode array UV/visible spectrophotometer (model 8452A). The procedure involved adding 2.9 mL of 5 mM ABTS to 50 μL of peroxidase solution followed by 50 μL of 60 mM H_2O_2 to initiate the reaction. The change in absorbance at 404 nm was recorded for 60 s.

The specific activity of the native SBP was, as reported**,** 45% of the specific activity of native horseradish peroxidase at 25 °C.[13] After attachment of the SBP to the oligonucleotide, 57% of the activity was conserved. The loss of activity was caused by the periodate oxidation step, not the $NaBH_4$ reduction step. The isoelectric points were measured by isoelectric focusing electrophoresis (Phastgel System, Pharmacia). The isoelectric points were 9.1 for the native enzyme, 4.5 for the periodate-oxidized enzyme, and 8.0 for the periodate oxidized and then $NaBH_4$-reduced enzyme.

Amperometric Detection of Hybridization. Hybridization was carried out in 1 mL of pH 7 *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer containing 1 M NaCl, 1 mM H_2O_2, and 1 mM ethylenediaminetetraacetic acid (EDTA). The buffer solution was thermostated at the stated temperature and was stirred using a rotating glass paddle powered by an air-powered motor. The electrode was connected to the potentiostat and polarized at −0.06 V vs Ag/AgCl. After 20 s, the current stabilized between −5 and −15 pA and 10 μL of the oligo-SBP conjugate was injected into the 1-mL cell to bring the concentration to 0.4 nM. The change in the current with time was recorded, and the observed hybridization transients were fitted to a diffusion-limited Langmuir equation using Sigma Plot graphing software (SPSS).

To determine the current reached when the redox polymer-precoated electrodes were densely coated with SBP, the periodate-oxidized enzyme was allowed to self-cross-link on the redox polymer films of the microelectrodes. The cross-linking resulted from the condensation of aldehyde functions of the oxidized oligosaccharide of the enzyme with its surface (lysine and arginine) amines. Because in these

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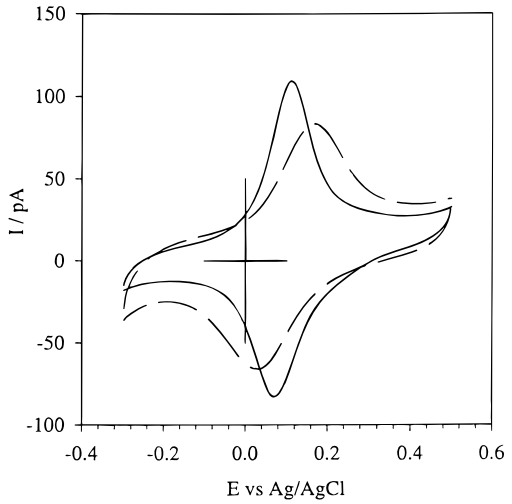

Figure 1. The fifth cyclic voltammograms of the redox polymer (solid line) and of the redox polymer reacted with the probe (dashed line): 7-μm-diameter carbon microelectrode; scan rate 50 mV s^{-1}; pH 7 HEPES buffer containing 1 M NaCl and 1.0 mM EDTA.

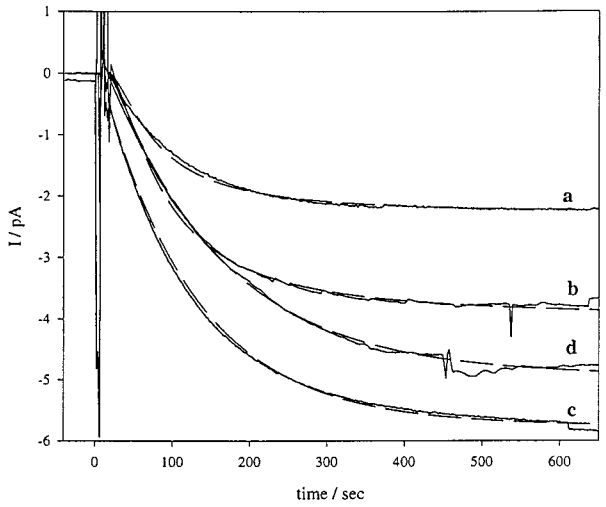

Figure 2. Increase in the catalytic current in microelectrodes loaded with different amounts of the probe oligonucleotide after adding the SBP-labeled target. The probe was deposited for (a) 1, (b) 2.5, (c) 5, and (d) 10 min. Stirred, 1 mL of pH 7 HEPES/1.0 M NaCl buffer with 1.0 mM EDTA/1.0 mM H_2O_2; −0.06 V (Ag/AgCl). A 10-μL sample of the 40 nM SBP-labeled target solution was added at 0 s.

experiments the redox polymer did not have hydrazide functions, covalent linking of the redox polymer and the oxidized enzyme was not the cause of SBP immobilization. In a second experiment the electrode was precoated with the hydrazide-functionalized redox polymer. The EDC-activated probe oligonucleotide was bound to the redox polymer through reactive electrophoretic deposition. The periodate-oxidized SBP was then allowed to bind and self-cross-link on the electrode. These experiments were carried out in the hybridization buffer containing 0.4 nM periodate-oxidized SBP.

Results

When the periodate-oxidized SBP was allowed to self-cross-link on the electrode coated with the redox polymer without reactive hydrazide functions, the H_2O_2 electroreduction current at 25 °C increased from nil to −45 pA and then leveled off. The current leveled off at −30 pA when the electrode was coated with the hydrazide-functionalized redox polymer and then reacted with the electrophoretically deposited EDC-activated probe. No catalytic current was detected after the oxidized SBP was reduced with $NaBH_4$, with or without the oligonucleotide attached, and also if not initially reacted with periodate, showing that nonspecific adsorption of SBP to the redox polymer was not significant.

The solid curve of Figure 1 is a typical voltammogram of the electrophoretically deposited redox polymer on the 7-μm microelectrode. The permeability of the redox polymer film to small molecules was probed using ferrocenemethanol. The permeability was so high that the diffusion-limited current at 0.5 V was only 2−5% below that of the uncoated electrode. To test the reproducibility of the coatings formed by electrophoretic deposition, the same electrode was repolished and recoated using the same redox polymer solution 25 times. The peak heights of the cyclic voltammograms of the 25 films were identical, the standard deviation in their heights being ±5%.

The deposition of the EDC-activated probe oligonucleotide onto the redox polymer resulted in the coupling of the reactive 5′-phosphate with hydrazide functions of the redox polymer. The reactive electrophoretic deposition of the probe increased the separation of the anodic and cathodic peaks (Figure 1, dashed line). The integrals of the anodic and cathodic waves did not change, however, indicating that no redox polymer was lost as a result of the oligonucleotide deposition.

The effect of the amount, i.e., loading, of the EDC-activated probe oligonucleotide on the rate of target hybridization and on the resulting H_2O_2 electroreduction current was studied. As seen in Figure 2 the optimal probe loading was reached when the duration of its electrophoretic deposition was 5 min. When the probe deposition was continued for 10 min, the rise in current, following introduction of the SBP-labeled target, was slowed and the maximum current was reduced. For deposition times shorter than 5 min, the rise in current was not faster but the maximum current was reduced.

When redox polymer without reactive hydrazide functions on the backbone was deposited in the same way, the current observed after 5 min of hybridization of the SBP-labeled target oligonucleotide with the film, which was coated by 5 min of electrophoresis of the probe oligonucleotide, was only 0.35−0.75 pA at 25 °C.

Figure 3 shows the current evolution at 25, 45, and 57 °C for the perfect, single-base-mismatched and four-base-mismatched SBP target oligonucleotides listed in Table 1. At 25 °C, H_2O_2 was electroreduced on electrodes with any of the three SBP-labeled targets. At 45 °C, H_2O_2 was electroreduced only when the target was perfectly matched or when only one of its bases was mismatched, but not when four of the bases were mismatched. At 57 °C, H_2O_2 was electroreduced efficiently (45 pA current) only when the target was perfectly matched. The current dropped to 12 pA when a single base was mismatched and to 6 pA when four-base pairs were mismatched. The currents for the perfectly matched hybrid at 25, 45, and 57 °C were, respectively, 6, 28, and 45 pA. [14]

Figure 4 shows an experiment at 45 °C where at $t = 550$ s the SBP-labeled target with four mismatched bases was introduced, followed by the fully complementary SBP-labeled target at *t* (EDTA)1450 s. The respective attained currents were 2.3 and 25 pA.

Discussion

The scheme for detecting the probe is shown in Figure 5A. A redox polymer film was electrophoretically deposited on the

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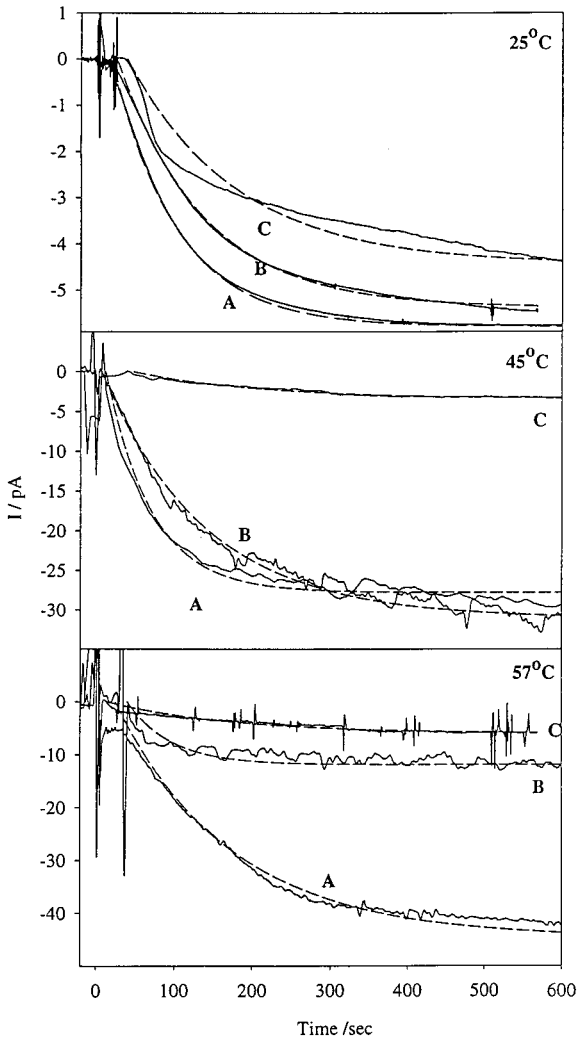

Figure 3. Increase in the catalytic current of microelectrodes at 25, 45, and 57 °C after adding the SBP-labeled target fully complementary or partially mismatched targets: (a) perfectly matched target; (b) target with a single mismatched base; (c) target with four mismatched bases. A 10-μL sample of the 40 nM SBP-labeled target solution was added at 0 s. Stirred 1 mL of pH 7 HEPES buffer/1 M NaCl with 10 mM H_2O_2; −0.06 V (Ag/AgCl). The dashed line represent the best fit of the data to eq 4.

electrode. This film was reacted in a second electrophoretic deposition step with the 5′-activated poly-T spacer of the probe. Upon hybridization with the SBP-labeled target, electrons were flowing from the electrode through the redox polymer to heme centers of the label, reducing these. SBP was preferred over the more active horseradish peroxidase for labeling the target because of its superior thermal stability.[15]

Because electrophoretic deposition was restricted to the conductive area of the glass-embedded carbon fibers, redox polymer films of reproducible dimensions and characteristics were deposited when the electrode and solution were also reproducible. This is important because, if the microelectrodes are to be used in gene-detecting arrays, then differences between their currents will be measured and the significance of small differences in current will depend on the reproducibility of the coatings. The dimensions of the electrodes are likely to be reproduced when made by the processes used in the manufacture of microelectronic circuits, within $\pm$ 0.05 μm. In 25 successively deposited films the voltammetric peaks showed a normal

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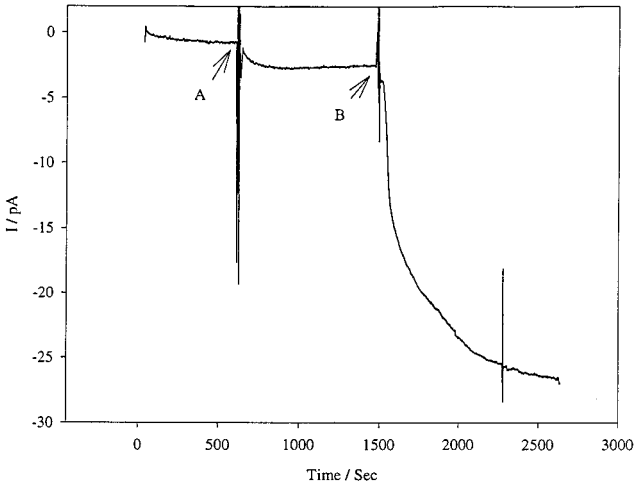

Figure 4. Current−time plot of the catalytic current of a microelectrode coated with the probe-bearing redox polymer. A 10-μL sample of 40 nM SBP of the SBP-labeled target with four mismatched bases was introduced at 550 s, followed by 10 μL of 40 nM SBP of the SBP-labeled perfectly matching target at 1450 s. Stirred 1 mL of pH 7 HEPES buffer/1 M NaCl. with 1.0 mM H_2O_2; −0.06 V (Ag/AgCl) thermostated at 45 °C; stirred.

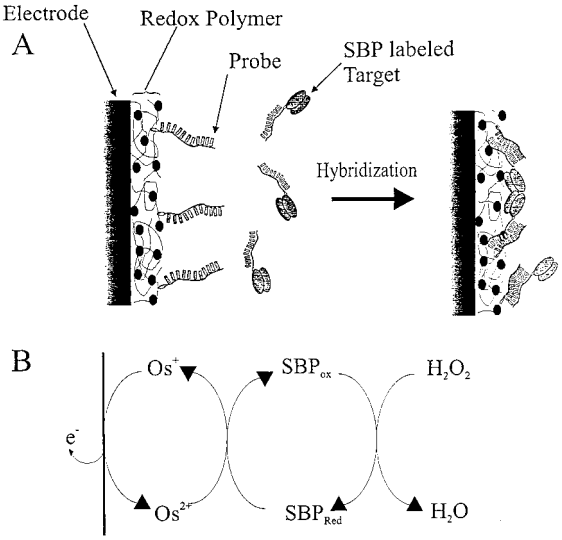

Figure 5. (A) Schematic diagram of the detection system. The 18-base probe oligonucleotides were covalently bound to the electron-conducting redox polymer on the microelectrode through 12-base poly-T spacer arms. The SBP labels were covalently bound to the target oligonucleotides through 12-carbon spacer arms. Upon hybridization, electrical contact was established between the SBP heme centers and the electrode via the redox polymer. This contact enabled the electrocatalytic reduction of H_2O_2 to water through the cycle shown in (B). Hybridization was thereby translated to current of H_2O_2 electroreduction.

distribution, with the standard deviation, σ, being $\pm$5%. Typically, the faradaic charge was 5.6×10^{-11} C, corresponding to 5.8×10^{-16} mol of osmium complex on the electrode.

The purpose of precoating the microelectrodes with a thin layer of redox polymer was to make the electrical contact, between the reaction centers of SBP and the electrode independent of the orientation of the target-labeling SBP. In the absence of a redox polymer film, electrical contact is established only with those SBP heme centers that are near the electrode surface. In the case of horsradish peroxidase (HRP) on vitreous carbon, a small fraction of the HRP molecules is always electrically connected to the carbon surface because some of the molecules are so oreinted that their heme redox centers are

close enough to the surface of the electrode for electrons to be transferred. This fraction is usually ~1%.[16] In contrast, when the peroxidase is embedded in an electron-conducting redox polymer, electrons are transferred from the redox polymer to all the heme centers of the enzyme irrespective of their orientation. As a result, the current density is ~100-fold higher. The electrons are now transferred through collisions of heme centers with randomly moving segments of the cross-linked redox polymer film. The mobility of these tethered segments increases when the redox polymer is hydrated. Electrons diffuse through the redox polymer by the related process of collisional self-exchange between approaching redox function-carrying segments of the polymer network.[17] Figure 5B shows the scheme of electron transport between the electrodes and the SBP labels of the targets. The transport results in the catalysis of the electroreduction of H_2O_2 to water (eq 1) at −0.06 V (Ag/AgCl).

$$H_2O_2 + 2\,e^- + 2H^+ \rightarrow 2H_2O \qquad (1)$$

At this potential, H_2O_2 is not electroreduced on carbon in the absence of the catalyst.

Because of the fast electron exchange and because the redox polymer is well adsorbed on the electrode, the peaks of the anodic and cathodic waves of the voltammogram of the redox polymer-coated microelectrodes are separated only by ~20 mV (Figure 1, solid curve). The peak separation is increased to 180 mV after the probe oligonucleotide is bound to the redox polymer (Figure 1, dashed curve), indicative of much slower electron transport.[18,19] The apparent cause of the sluggish transport is the formation of ion bridges between the polyanionic probe and the polycationic redox polymer, which restricts the segmental mobility of the redox polymer and thereby the frequency of electron-transferring collisions.

Figure 2 shows that overloading of the redox polymer film with the probe oligonucleotide reduced the current after the SBP-labeled hybrid was formed. The current increases initially (Figure 2, curves a−c) as the amount of probe is built up and more of the SBP-labeled target is captured. However, when the amount of probe is excessively increased, the current decreases and the rate of hybridization, evidenced by the rate of change in current, also slows (Figure 2, curve d). The apparent cause of the reduction in current is again the restriction of the movement of segments of the redox polymer upon excessive ion bridging, compounded by the dilution of the electron-exchanging redox centers. Consistently, the current is reduced by 30% upon incorporation of probe oligonucleotide in the redox polymer film not only when the current was flowing through the SBP label of the hybrid but also in the absence of hybridization, when periodate-oxidized SBP is allowed to self-cross-link on the film. The sluggish increase in current when the redox polymer film is overloaded with the probe is also attributed to the formation of ion bridges. These make the film rigid, slowing the access of the SBP-labeled target to probe oligonucleotides.

The optimal duration of the deposition process is 5 min. The time dependence of the current, which represents the rate of enzyme binding through hybridization, e, is well described (R^2 ≅ 0.99) by the diffusion-limited Langmuir equation (eq 2).[20]

$$e = e_{max}[1 - \exp(-k_D t^{1/2})] \qquad (2)$$

In the equation k_D is the surface binding rate (here the rate of hybridization of the SBP-labeled target to the electrode-bound probe), which is related to the rate of diffusional mass transport, and e_{max} is the maximal surface concentration of enzyme, reached after hybridization of all possible probes that can hybridize. For a thin redox polymer film and neglecting diffusion of the substrate (H_2O_2 in the present case) the saturation current is given by eq 3. k being the rate constant of the reaction between

$$I_{cat} = 2k[Os^{2+}][S]FAe \qquad (3)$$

the reduced redox center and the enzyme, $[Os^{2+}]$ the concentration of the reduced redox centers, and $[S]$ the substrate (H_2O_2) concentration. Because in the experiments the substrate concentration is high, the current is limited either by electron diffusion in the film or by the enzyme's turnover rate. Through combining eq 2 with the simplified eq 3, eq 4 is derived, where

$$I_{cat} = b[1 - \exp(-k_D t^{1/2})] \qquad (4)$$

$b = 2k[Os^{2+}][S]FAe_{max}$. This equation fits the experimental curves of Figures 2 and 3.

The best-fit parameters for the hybridization transients of Figure 2 are summarized in Table 2. While the k_D values for films a−c that are not overloaded with the probe are similar, k_D of the overloaded film is significantly lower, suggesting that the hybridization-causing diffusive step was restricted in the matrix with the excessive amount of oligonucleotide. The time dependence of the current fits the diffusion-limited Langmuir equation also when the hybridization is carried out at different temperatures and with mismatched bases in the oligonucleotides. However, as the temperature is increased, the noise increases and the value of R^2 is reduced.

The best-fit parameters to the experimental hybridization transients for the one fully and the two imperfectly matched oligonucleotide sequences are listed in Table 3. The dashed curves in Figure 3 represent the equation fitted with the constants listed in Table 3. The diffusion-limited Langmuir equation fits these transients particularly well, especially in the case of the complementary oligonucleotide at 25 °C shown in Figure 2.[21] In some cases the fitting is made difficult by the noise and in one case, that of the four-base-pair mismatch at 25 °C, the fit is poor.

The increase in the currents with temperature in the case of the perfectly matched hybrid (25 °C, 6pA; 45 °C, 28pA; 57 °C, 45pA) yields an activation energy of 60 kJ mol^{-1}, similar to the activation energy reported for a related redox polymer.[22]

Table 2. Best-Fit Parameters to Eq 4 for Microelectrodes with Different Probe Loadings[a]

oligonucleotide deposition time (min)	b (pA)	$k_D/(s^{-1})$	R^2
1	−2.26	0.089	0.99
2.5	−3.86	0.093	0.99
5	−5.79	0.095	0.99
10	−4.6	0.077	0.99

[a] The calculated and the measured (Figure 2) currents fitted eq 4 with a mean difference of 2% or less in any of the experiments.

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Table 3. The Best-Fit Parameters to Equation 4 for the Three Targets at 25, 45, and 57 °C Shown If Figure 3

temp (°C)	target oligonucleotides	*b* (pA)	k_D/s^{-1}	R^2
25	complementary	−5.74	0.096	0.99
	single base mismatched	−5.48	0.098	0.95
	four bases mismatched	−4.46	0.084	0.76
45	complementary	−27.7	0.117	0.99
	single base mismatched	−30.2	0.086	0.89
	four bases mismatched	−3.44	0.077	0.97
57	complementary	−44.6	0.082	0.91
	single base mismatched	−11.8	0.133	0.56
	four bases mismatched	−5.9	0.079	0.66

[a] The calculated and the measured (Figure 2) currents fitted eq 4 with a mean difference of 2% or less in the experiments at 25 °C, 4% at 45 °C, and 10% at 57 °C.

The similarity in the activation energies for electron transfer through the redox polymer and the catalytic H_2O_2 electroreductioon current suggests that the current is limited by transport of electrons through the redox polymer.

Because the SBP label contacts the redox polymer electrically only below the melting temperature of the hybrid, and the activation energy for electron transport is high, the currents at 25, 45, and 57 °C differ significantly when the hybrids were perfectly matched, had a single base-pair mismatch, or contained four mismatched base pairs. The theoretically estimated melting temperature of the 18-base hybrid with a single mismatched base pair is approximately 5−7 °C below that of the perfect hybrid when the mismatch is in the middle of the oligonucleotide and the mismatched base pair is GC.[23] For the four-base-pair mismatch, the theoretically estimated melting temperature is 20−23 °C below that of the perfectly matched hybrid. The actual melting points of the 18-base-pair hybrids when perfectly matched, mismatched in a single base pair, and mismatched in four base pairs are 59.5, 54, and 37−40 °C, respectively. Consequently, a current should flow in the case of the perfectly matched hybrid at any of the three temperatures, 25, 45, or 57 °C. In the case of the hybrid with a single mismatched base pair a current should flow at 25 °C and at 45 °C, but not at 57 °C; and in the case of the hybrid with four mismatched base pairs, a current should flow only at 25 °C, not at 45 °C or at 57 °C. That this is indeed the case is seen in Figure 3. For example, at 57 °C, the current for the perfectly matched hybrid is 45 pA, while the current for the hybrid with a single mismatch is 11 pA. Figure 4 shows the result of an experiment carried out at 45 °C, where first the SBP-labeled target with four mismatched bases was added, hybridizing below 37−40 °C, followed by the complementary SBP-labeled target, hybridizing at temperatures up to 59.5 °C. The experiment shows that the presence of an extraneous oligonucleotide with a partially matching sequence does not interfere with the hybridization of the matched target, nor does it affect the magnitude of the current (∼30 pA) reached upon hybridization (Figures 3B and 4).

The number of copies producing the current was estimated from the turnover rate of the SBP label. The rate of turnover of the SBP label is 460 s^{-1} at 25 °C. With two electrons being transferred per turnover, this turnover rate corresponds to a current of 1.5×10^{-16} A per label. At 25 °C, the saturating current measured upon complete hybridization was 5 pA, the output of the "wired" and active labels of 34 000 copies. For the 7-μm-diameter electrode, the corresponding surface coverage was 1.4×10^{-13} mol cm^{-2}, which agrees well with the theoretically calculated surface density range of $(0.03-3.8) \times 10^{-13}$ mol cm^{-2} for a probe with 18 base pairs on a solid surface.[24]

Conclusion

The study shows that a single base mismatch in an 18-base oligonucleotide can be amperometrically sensed with, and amplified by, a redox polymer-coated microelectrode. The detected current is generated by ∼40 000 active and "wired" copies of the thermostable SBP-labeled hybrid. In future studies, a part of a single-stranded gene, consisting of a few hundred bases, will be hybridized to the ≥18-base peptide oligonucleotide or deoxyribose oligonucleotide[25,26] and bound thereby to the redox polymer. The presence of the bound gene part will be then queried with an SBP-labeled ≥18-base sequence, hybridized to a different region of the gene.

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