Unlabeled Hairpin DNA Probe for Electrochemical Detection of Single-Nucleotide Mismatches Based on MutS−DNA Interactions

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The paper described a label-free assay for the detection of single-nucleotide mismatches in which an unlabeled hairpin DNA probe and a MutS protein conjugate (His6-MutS-linker peptide-streptavidin binding peptide (HMLS)) are exploited for the detection of mismatches by electrochemical impedance spectroscopy (EIS). We demonstrate this method for eight single-nucleotide mismatches. Upon hybridization of the target strand with the hairpin DNA probe, the stem-loop structure is opened forming a duplex DNA. In duplexes containing a single nucleotide mismatch, the mismatch is present at the solvent exposed side, enabling more effective HMLS recognition and binding. The binding event is evaluated by EIS and analyzed with the help of Randles' equivalent circuits. The differences in the charge transfer resistance ΔR_{CT} before and after protein binding to the duplex DNA allows the unequivocal detection of all eight single-nucleotide mismatches. ΔR_{CT} allows the discrimination of a C-A mismatch with the concentration of the target strand as low as 100 pM.

One of the major focal points of current genomics research is the detection of single-nucleotide polymorphisms (SNPs) within known gene sequences, as well as in the genome as a whole.[1] Point mutations, which can be a deleterious type of SNP, are the hallmarks of several known diseases (e.g., Tay Sachs,[2] cystic fibrosis,[3] thalassaemia[4]), and their detection is, and in fact genotyping is in general, of fundamental importance for the early identification and diagnosis of such diseases and may potentially allow for a more personalized approach to medicine. Current optical assays for mismatch detection are based on the hybridization of a labeled DNA target strand to a complementary capture strand.[5−8] In order to enhance the signal output, polymerase chain reaction (PCR) is often used to amplify the target DNA.[9,10]

Structured DNA probes, such as hairpin DNA, have been used to recognize specific sequences[11−16] but have relied in many cases on the presence of a fluorophore and a quencher. In an effort to develop "label-free" optical biosensors, hairpin DNA was functionalized by a fluorophore, and the immobilized substrate[17] or nucleotide base[18] served as a quenching agent. While the resulting sensor systems are in many cases highly sensitive and allow even single nucleotide mismatch detection,[15,16] the systems still require specific labeling with probes, such as fluorescent dyes.

Electrochemical detection is an interesting and convenient alternative that has allowed one to simplify the detection and increase the sensitivity of the measurements.[19−27] Currently, electrochemical measurements of surface supported hairpin DNA

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rely on the presence of redox labels, including ferrocene,[28,29] methylene blue,[30–32] and others,[33] which are attached to the 5′ or 3′ terminal of the DNA strand. For the most part, these assays focus on the detection of hybridization events and, to some degree, on the detection of single nucleotide mismatches. Recently, we reported an unlabeled hairpin DNA as a probe for the detection of all eight possible single-nucleotide mismatches in detail by electrochemical impedance spectroscopy (EIS) exploiting $[Fe(CN)_6]^{3-/4-}$ as a solution based redox probe.[34] Mismatch evaluation is based on evaluating the difference in the charge transfer resistance between a film of duplex DNA after the hybridization of unlabeled hairpin DNA with the target strand in the presence and absence of Zn^{2+} at pH ≥ 8.7. In addition, two reports about hybridization measurements also appeared involving an unlabeled hairpin DNA.[35,36] MutS is a component of the *Escherichia coli* DNA mismatch repair system. It was shown that purified MutS binds to ds-DNA containing mismatched and/or unpaired nucleobases, which is a promising approach for mismatch detection.[37–44] Previous work indicates that the flanking regions adjacent to the mismatch appear to play only a minor role and appear not to influence MutS−DNA interactions to a large extent.[40]

In this report, we build on our earlier work on hairpin DNA and, in combination with a MutS conjugate (His6-MutS-linker peptide-streptavidin binding peptide (HMLS)), we are able to distinguish all eight mismatches by EIS. This assay has a number of advantages over previous work: (a) it avoids the diluting step to achieve the HMLS interaction with ds-DNA compared to the previous impedance assay,[38] (b) it does not require labeling of the target strand and, thus, is a label-free approach for mismatch detection involving a MutS construct, (c) it does not require the

addition of metal ions such as Zn^{2+}, and (d) interactions of the bulky HMLS construct are expected to cause significant changes in the electric film properties that can be probed by EIS. In the case of a C-A mismatch, this assay allows the discrimination of the mismatch at target strand concentrations as low as 100 pM.

EXPERIMENTAL SECTION

Materials. Ten DNA sequences were synthesized by standard solid phase techniques using a fully automated DNA synthesizer in Shanghai (Shanghai Sangon Biological Engineering Technology & Service Co. Ltd.).

5′-HO-(CH₂)₆-SS-(CH₂)₆-GCA-CGC-GTC-ACG-ATG-GCC-CAG-TAG-TTG-CGT-GC-3′ (**1**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-CG-5′ (**2**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-C*C*-5′(**3**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-C*A*-5′ (**4**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GC*C*-CG-5′ (**5**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-*A*G-5′ (**6**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-*G*G-5′ (**7**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GCA-*T*G-5′ (**8**)

3′-CAG-TGC-TAC-CGG-GTC-ATC-AAC-GC*T*-CG-5′ (**9**)

3′-CAG-TGC-TAC-CGG-GTC-A*A*C-AAC-GCA-CG-5′ (**10**)

One complementary strand and eight single-nucleotide mismatched strands (mismatch in italic) are listed above. The resulting duplexes and mismatches are shown in Table 1.

NaClO₄, K₃[Fe(CN)₆], K₄[Fe(CN)₆], Tris (Tris-(hydroxymethyl)-aminomethane)), and 6-mercaptohexanol were purchased from Aldrich and used without further purification. His₆-MutS-linker peptide-streptavidin binding peptide (HMLS) without dithiothreitol (DTT) was purified as reported before (see Supporting Information).[41] Deionized water (18.2 MΩ·cm resistivity) from a Millipore Milli-Q system was used throughout this work. The working gold electrodes, 99.99% (w/w) polycrystalline with a diameter of 2 mm, were purchased from CH Instrument Inc. and cleaned prior to use.[38]

FILM PREPARATION

The freshly cleaned gold electrodes (2.0 mm diameter) were incubated in a solution of 0.01 mM hairpin-structured strand **1** in 50 mM Tris−ClO₄ buffer (pH = 7.6) for 5 days. Then, the electrodes were washed with the buffer solution and subsequently incubated in 1 mM 6-mercaptohexanol for 2 h. The electrodes were washed with Tris−ClO₄ buffer and incubated in the target strand solution for 10 h to form the fully matched ds-DNA (**1** + **2**) and films containing a single-nucleotide mismatch (see Table 1). After washing with Tris−ClO₄ buffer, the electrode was mounted into an electrochemical cell at 0 °C and EIS measurements were carried out. Subsequently, binding of HMLS with ds-DNA was performed by incubating

Table 1. Matched and Mismatched Duplex DNA Used for the Detection of Mismatches

1 + 2 matched	1 + 3 C-C	1 + 4 C-A	1 + 5 T-C	1 + 6 G-A	1 + 7 G-G	1 + 8 G-T	1 + 9 T-T	1 + 10 A-A

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Scheme 1. Schematic View of Hairpin-DNA Probe (1) for Hybridization and the Detection of Single-Nucleotide Mismatches[a]
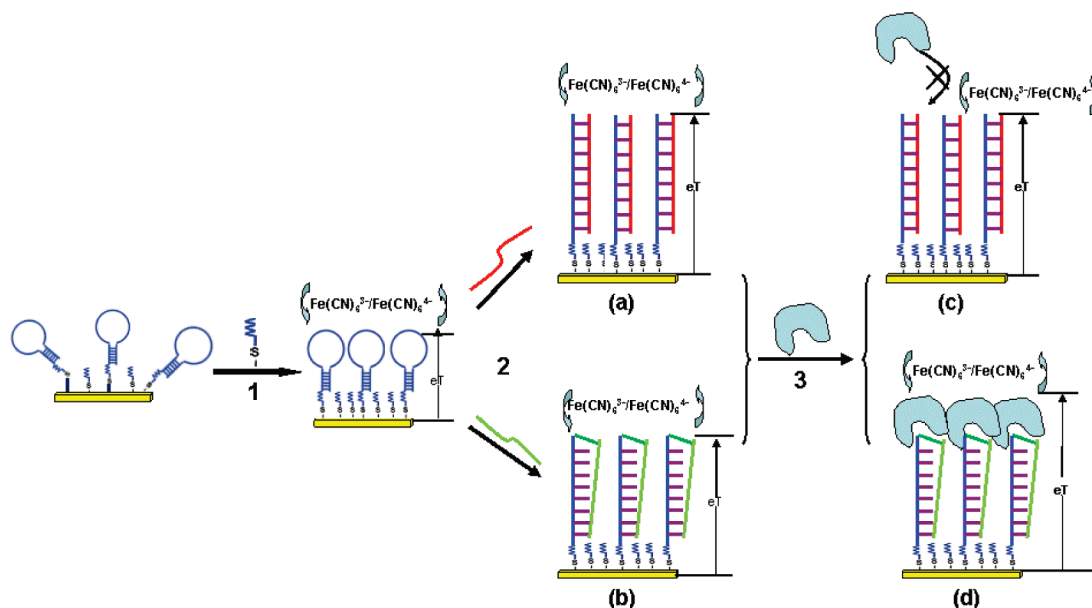
[a] (1) 6-Mercaptohexanol is applied to occupy the possible pinholes on the electrode modified with thiolated hairpin DNA and force the immobilized DNA to align reproducibly in an orientation extending away from the electrode surface. (2) Hairpin DNA opens the stem-loop structures to hybridize with target strand to form the matched (a) and mismatched (b) ds-DNA. (3) (c) and (d) represent the binding properties of HMLS with matched (a) and mismatched (b) ds-DNA.

the ds-DNA modified electrode in HMLS solution (20 mM Tris−ClO$_4$, pH 7.6, 150 mM NaClO$_4$, 1 mM ATP, 5 mM MgCl$_2$) at 0 °C for 30 min, and EIS of the films were recorded.

ELECTROCHEMICAL MEASUREMENTS

A conventional three-electrode system was used, and all the measurements were carried out at 0 °C in an enclosed and grounded Faraday cage. The reference electrode was constructed by sealing a Ag/AgCl wire into a glass tube with a solution of 3 M KCl that was capped with a Vycor tip. The counter electrode was a platinum wire. Impedance spectra were measured using a potentiostat/frequency analyzer (EG&G 2273). The ac voltage amplitude was 5 mV, and the voltage frequencies used for EIS measurements ranged from 100 kHz to 100 mHz. The applied potential was 250 mV vs Ag/AgCl (formal potential of the redox probe [Fe(CN)$_6$]$^{3-/4-}$ in the buffer solution). All measurements were repeated for a minimum of five times with a separate electrode to obtain statistically meaningful results.

RESULTS AND DISCUSSION

Loosely packed films of hairpin DNA (1) were prepared by incubating freshly cleaned gold electrodes in solutions of 0.01 mM 32-mer strand 1, followed by backfilling potential pinholes and defects by soaking the film in 1 mM 6-mercaptohexanol in 50 mM Tris−ClO$_4$. This procedure prevents the molecules from laying flat on the surface and aligns the immobilized DNA hairpins in an upright orientation (see Scheme 1).[45] During hybridization to the 26-mer target strands 2−10, the hairpin DNA opened the stem-loop structure and ds-DNA was formed (see Supporting Information).[34] The combination of strands 1 and 2 results in the formation of matched ds-DNA, whereas the combination of 1 with

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strands 3−10 results in ds-DNA films containing a single-nucleotide mismatch at the top of the duplex, as shown in Table 1. Subsequently, the modified gold electrodes are incubated with HMLS in the binding buffer (shown in Scheme 1). EIS is applied to evaluate the binding results. The representative Nyquist plots for films of 1 + 2 and 1 + 3 containing a C-C mismatch are shown in Figure 1. The impedance spectra for all systems were analyzed with the help of a modified Randles' equivalent circuit, relating specific properties to resistive and capacitive components (see inset, Figure 1). Individual fitting results for all films are listed in Table 2.

The solution resistance, R_s, is the resistance between the reference electrode and the DNA-modified gold electrode. For each measurement, the position of the two electrodes is kept the same. All measurements were carried out under identical conditions of electrolyte concentration (20 mM Tris−ClO$_4$) and at 0 °C to minimize variations in R_s, which ranged from 0.3 to 0.4 kΩ.

$C_{monolayer}$ accounts for the capacitance of the DNA films on the gold electrodes. There are two observations from the evaluation of $C_{monolayer}$: (i) $C_{monolayer}$ is slightly smaller for the film having matched ds-DNA compared to the films containing a single-nucleotide mismatch at the top of the duplex. The possible interpretation is the film thickness variations due to the fraying of the strands containing a single-nucleotide mismatch.[34] For the films of 1 + 3 containing a C-C mismatch and 1 + 7 containing a G-G mismatch, $C_{monolayer}$ is slightly smaller, presumably due to a better interaction between nonpaired base pairs and the solvent, resulting in a thicker film. (ii) As expected, MutS binding will increase the film thickness and this in turn will increase the film capacitance $C_{monolayer}$, which is probably caused by an increased dielectric constant after binding with MutS. The combination of R_x and the
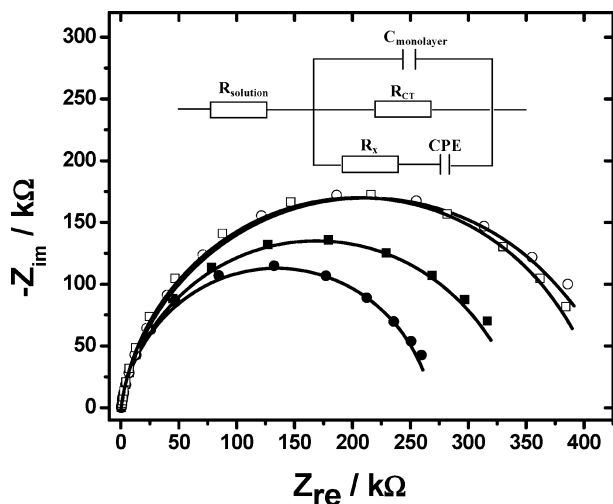
Figure 1. Representative Nyquist plots ($-Z_{im}$ vs Z_{re}) of the films prepared from a 0.01 M solution of 32-base hairpin DNA **1** hybridization with 0.01 M 26-base strand **2** to form matched DNA **1** + **2** before (○) and after (□) incubating with HMLS, and with strand **3** to form a C-C mismatched DNA **1** + **3** before (●) and after (■) incubating with HMLS. Measured data are shown as symbols with calculated fit to the equivalent circuit as solid lines. Inset: the measured data are fit to the equivalent circuit; R_s, solution resistance; $C_{monolayer}$, capacitance of the DNA monolayer; R_{CT}, charge transfer resistance of DNA monolayer; R_x and CPE, resistance and nonlinear capacitor accounting for 6-mercaptohexanol film.

constant phase element (CPE) accounts for the behavior of the 6-mercaptohexanol-diluted films on the electrode surfaces. CPE acts as a nonlinear capacitor accounting for the inhomogeneity of the films on the electrode surface with the exponential modifier $n = 0.8$.[46] Diffusion of the redox probe from the solution to the DNA film is not important in this system, as is apparent from the absence of any Warburg impedance, as shown in Figure 1.

The charge transfer resistance, R_{CT}, is the result of the resistance to charge transfer from the solution based redox probe $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. For any given film, R_{CT} for a matched DNA film is larger than that for a mismatched DNA film. Since a single-nucleotide mismatch will introduce disorder into the film, the redox probe may penetrate the film to a larger extent, giving rise to a lower R_{CT}. On the other hand, R_{CT} is increased after binding with HMLS. As shown in Figure 1, the Nyquist plot clearly showed that a film of matched DNA **1** + **2** has a higher R_{CT}, as compared to the film with a C-C mismatch (**1** + **3**). Binding of the MutS construct to films containing a mismatch increases the charge transfer resistance R_{CT}. The values for R_{CT} are listed in Table 2. As reported, purified MutS protein binds DNA containing mispaired bases but does not bind equally well to DNA without mismatches.[41] As for the matched DNA **1** + **2**, R_{CT} is slightly increased, presumably due to nonspecific absorption of HMLS on the film, and partly hampered the charge transfer through the DNA films.[47] Though HMLS can bind with a C-C mismatched DNA **1** + **3**, nonspecific absorption of HMLS on the

film may be unavoidable.[47] As a result, for the film of the matched DNA **1** + **2**, the value R_{CT} for ds-DNA is 412(11) kΩ and increased to 423(18) kΩ after the addition of the protein. The difference in R_{CT} (ΔR_{CT} 11(1.2) kΩ) is small compared to the interaction in the presence of single nucleotide mismatches. Typical results for films of **1** + **3** containing C-C mismatch show a value of ΔR_{CT} of 71(6.1) kΩ. The values for ΔR_{CT} for other mismatched DNA duplexes are listed in Table 2 and range from 186(14.1) kΩ for a C-A mismatch, 175(12) kΩ for a G-T mismatch, 65(5.3) kΩ for a G-A mismatch, 62(3.3) kΩ for a C-T mismatch, 49(2.1) kΩ for a T-T mismatch, 38(3.1) kΩ for a G-G mismatch to 34(3.8) kΩ for A-A mismatch. On the basis of this approach, we were able to detect all eight mismatches using hairpin DNA and HMLS without the need for prior labeling.

Next, we explored the detection limit of a mismatch using the C-A mismatch as an example. The concentration of target strands **2** and **4** were gradually decreased from 10^{-5} to 10^{-12} M; then, the strands were hybridized with hairpin-DNA films of strand **1** on the gold electrodes to form matched and C-A mismatched DNA. The impedance spectra for the combinations of **1** + **2** and **1** + **4** were recorded in the presence and absence of the MutS construct, and the value of ΔR_{CT}, obtained after fitting to the equivalent circuit (Figure 1), was plotted as a function of added concentration of single strand (**2** or **4**). As shown in Figure 2, the value of ΔR_{CT} of a fully matched DNA film decreases with decreasing concentrations of single strand **2**, while for the C-A mismatched film **1** + **4**, the value of ΔR_{CT} as a function of concentration of single strand **4** added remains fairly constant. At a concentration of 100 pM of single stranded DNA added, the value of ΔR_{CT} for the matched and unmatched films are indistinguishable.

The possible reason for changes is related to the formation of film (from hairpin to duplex).[27,36,48] Given a sufficiently high binding affinity of the target strand, at higher concentrations of the target strand DNA, a large number of hairpin DNA on the electrodes will open the stem-loop structure and hybridize with the target strand to from the duplex, followed by the interaction with the MutS construct. At lower concentration, less and less stem-loops will open and, thus, less MutS construct will bind to the electrode, resulting in decreasing changes in charge transfer resistance. Ultimately, at about 100 pM matched and mismatched systems are indistinguishable.

CONCLUSION

In this paper, a label-free approach for the detection of single-nucleotide mismatches exploiting ds-DNA−protein interactions is presented. We chose films prepared from hairpin DNA, which enable facile monitoring of the hybridization to complementary strands by electrochemical impedance spectroscopy. In the presence of a single nucleotide mismatch at the top of the ds-DNA, MutS will be able to bind the ds-DNA, resulting in reproducible changes in the charger transfer resistance R_{CT}. Eight different single-nucleotide mismatches are detected by evaluating the difference in charge transfer resistance (ΔR_{CT}) of ds-DNA film before and after protein binding. Generally, the value

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Table 2. Equivalent Circuit Element Values for Matched and Eight Mismatched DNA Duplex Films before and after Binding with HMLS[a]

		circuit elements						
		R_s (kΩ)	$C_{monolayer}$ (µF)	R_{CT} (kΩ)	R_x (kΩ)	CPE (µF)	n	ΔR_{CT} (kΩ)
match	DNA	0.4(0.01)	0.15(0.01)	412(11)	0.20(0.03)	0.48(0.02)	0.8(0.03)	11(1.2)
	DNA + MutS	0.4(0.01)	0.17(0.01)	423(18)	0.33(0.03)	0.51(0.02)	0.8(0.02)	
C-C	DNA	0.4(0.01)	0.13(0.01)	268(23)	0.32(0.02)	0.58(0.03)	0.8(0.02)	71(6.1)
	DNA + MutS	0.4(0.01)	0.23(0.01)	339(21)	0.43(0.03)	0.57(0.04)	0.8(0.02)	
C-A	DNA	0.4(0.01)	0.17(0.01)	222(16)	0.21(0.02)	0.39(0.03)	0.8(0.02)	186(14.1)
	DNA + MutS	0.4(0.01)	0.19(0.01)	408(27)	0.32(0.03)	0.42(0.04)	0.8(0.01)	
C-T	DNA	0.4(0.01)	0.20(0.01)	132(11)	0.25(0.02)	0.33(0.03)	0.8(0.03)	62(3.3)
	DNA + MutS	0.4(0.01)	0.21(0.01)	194(12)	0.28(0.02)	0.35(0.03)	0.8(0.02)	
G-A	DNA	0.3(0.01)	0.15(0.01)	286(13)	0.22(0.01)	0.48(0.03)	0.8(0.02)	65(5.3)
	DNA + MutS	0.4(0.01)	0.16(0.01)	351(21)	0.21(0.01)	0.50(0.03)	0.8(0.01)	
G-G	DNA	0.4(0.01)	0.14(0.01)	201(9)	0.30(0.02)	0.51(0.04)	0.8(0.03)	38(3.1)
	DNA + MutS	0.4(0.01)	0.16(0.01)	239(10)	0.29(0.02)	0.54(0.04)	0.8(0.02)	
G-T	DNA	0.4(0.01)	0.18(0.01)	88(11)	0.26(0.02)	0.29(0.02)	0.8(0.03)	175(12)
	DNA + MutS	0.4(0.01)	0.24(0.01)	263(13)	0.28(0.02)	0.33(0.03)	0.8(0.02)	
T-T	DNA	0.4(0.01)	0.19(0.01)	392(28)	0.33(0.02)	0.41(0.04)	0.8(0.01)	49(2.1)
	DNA + MutS	0.4(0.01)	0.20(0.01)	441(13)	0.48(0.02)	0.48(0.03)	0.8(0.03)	
A-A	DNA	0.4(0.01)	0.19(0.01)	208(16)	0.34(0.02)	0.50(0.03)	0.8(0.01)	34(3.8)
	DNA + MutS	0.4(0.01)	0.18(0.01)	242(26)	0.35(0.03)	0.52(0.04)	0.8(0.02)	

[a] The values in parentheses represent the standard deviations from at least five electrode measurements.
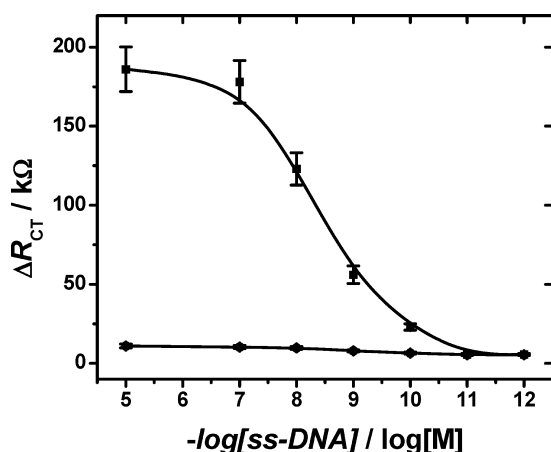

Figure 2. Relationship between ΔR_{CT} and the concentration of the target strand of **2** (■) and mismatch containing target strand **4** (●). The gold electrode was modified with hairpin DNA **1** and diluted with 6-mercaptohexanol. Error bars are derived from a minimum of five electrodes.

of ΔR_{CT} for mismatched films exceeds that of a matched film significantly. Importantly, the presence of a C-A mismatch is detectable down to concentrations of 100 pM of target strand. At present, we are in the process of improving the performance of this assay and are exploring microelectrodes as transducer surfaces, including microelectrode arrays, and we are working on more realistic life conditions, including serum and cell lysates.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.