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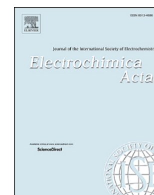
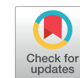


Amplified detection of single base mismatches with the competing-strand assay reveals complex kinetic and thermodynamic behavior of strand displacement at the electrode surface



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Detection of single-base mismatches with respect to a probe strand has been a predominant pursuit in electrochemical biosensor efforts, due to links found between single nucleotide polymorphisms (SNPs) and the predisposition to various diseases. We report an osmium tetroxide bipyridine-based, thermally-controlled, competitive-strand electrochemical assay to allow amplified detection of single-base mismatches. Optimally designed competitive-strand displacement and hybridization temperature allows us to distinguish the single-mismatched-target from the fully complementary sequence with unambiguous, highly reproducible, robust signal differences of over 90%. Furthermore, we find a complex interplay between the position of the redox label, variations in strand displacement kinetics due to mismatches incorporated into the competitive strand, and alterations in the melting temperature of DNA duplexes tethered on the gold surface, when probed by square-wave voltammetry. These insights will apply to any surface-tethered DNA-based electrochemical biosensor, and can help with understanding complex phenomena involved in these types of assays.

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1. Introduction

The presence of single nucleotide polymorphisms (SNPs) in the genome is considered to be a powerful diagnostic indicator for predicting both disease predisposition and drug effectiveness [1], and is also important in forensic analysis of degraded DNA samples [2,3], allowing for investigations with amplicon lengths of only 50 bases [4]. SNP characterization can also be suitable for human identification [5] and ancestry determination [6,7]; thus a reliable, efficient, and rapid technique for SNP detection is desirable. Electrochemical methods have been proposed as promising tools for analyzing SNPs, owing to their high-sensitivity, portability, efficiency, and low-cost compared with optical methods. While hybridization-based assays are popular in electrochemical genetic detection, fundamentally small differences in duplex stability between the fully complementary strand and one containing a single mismatch require the use of advanced strategies to increase sensitivity, such as addition of chemical agents or the use of peptides and proteins, specific probe designs, and manipulation of temperature [8,9]. Thermally stringent conditions are used in PCR, on Southern blots and in microarrays. Temperature has also been

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determined to be a crucial parameter in various electrochemical hybridization assays for DNA [10–12] and RNA [13] detection (reviewed in Ref. [14]). Excessively elevated temperatures may affect a hybridization sensor's stability [15], but thiol linkers immobilizing DNA strands onto gold are stable up to 65 °C [16]. Temperature influences sequence-specific pairing reactions in the DNA-duplex: in the presence of mismatches, base-pairing bonds become weaker, which is reflected in decreased melting temperature (T_m) of the double-stranded DNA (dsDNA). Thus, temperature can be used to provide stringency for discrimination of SNPs.

Multiple thermal electrochemical DNA-hybridization studies show that duplex melting behaves differently at the immobilized surface than in solution. It is important to note that the definition of melting temperature may be different for these electrochemical solid-state studies and the solution-state UV setting, as the resulting curves report on different phenomena: in the UV setting, the effects of hypochromism have been described rigorously and the inflection point in the absorbance curve correlates to a 50% population of unpaired bases [17], furthermore the total concentration of nucleic acids in the solution is known; for electrochemical analyses it is unclear what population of unpaired bases are reflected in the point where the signal begins to decrease, and the surface density of the tethered duplex is not exactly determinable. Using osmium tetroxide [18], change points in electrochemical temperature curves were found to be significantly lower at the electrode surface than in solution, and varied depending on whether the bulk solution was heated, or the electrode itself was heated via electrical resistance. Ferrocene redox-label-based studies [19] also showed that surface-immobilized DNA can have T_m reduced as low as 20 °C as compared to solution measurements. Using fluorescent labels [20], it was found that surface probe density can also affect surface melting temperature of DNA duplexes, with an apparent increase in Tm for increasing density. Shen and coworkers used a microscale platform with a built-in Pt heater to distinguish full-match, single- and double-mismatch by means of square wave voltammetry [21]. Inspection of the results and sequences used in this paper reveals that this localized heating also showed lower melting temperatures than expected in solution, although this was not emphasized in the study. Yang and coworkers employed a real-time melting curve analysis of surface-immobilized hybridization probes using a heated microfluidic device and alternating-current voltammetry, where temperature-dependent changes in redox current revealed differences in T_m for full-matched and mismatched targets [22]. Here as well, the reported T_m values seem to be much lower than solution calculations would predict.

Recent studies using a thermostated surface plasmon resonance cell also showed that on gold, immobilized DNA-DNA and DNA-RNA duplexes exhibited melting temperatures decreased by up to 20 °C as compared to the respective solution-state conditions for the same sequences [23]. Furthermore, it was found that DNA-RNA hybrids showed a T_m that was up to 15 °C lower than the respective DNA-DNA version, further decreasing the T_m to around room temperature, which obviously creates an unfavorable situation for RNA-hybridization-based genetic sensors employed at standard conditions.

In addition to temperature, the number of complementary bases and their position within the duplex play a role in thermal stability of dsDNA, not only in solution, but also on surfaces [24]. In the presence of mismatches, a minimal number of seven consecutive matching base pairs seems to improve thermal stability of dsDNA, compared to six or fewer, when followed by a mismatch. Mismatches more central in the sequence destabilize more than near-terminal or terminal mismatches [25]. Another consideration important to the behavior of the electrochemical hybridization assay is the position of the redox label. In 2008, Hartwich et al. used covalent osmium(II/III)-complex labels for capture probe strands and ferrocene as a covalent label at the target strands; these experiments confirmed that distance from the electrode surface influences redox kinetics at well-ordered DNA probe-target layers [26], while suggesting that SAMs of single-stranded DNA are not well ordered. The interaction of the redox labels used for biosensors with the electrode surface may also be important. Various polycyclic substances, such as methylene blue and ferrocene, which are used as redox labels, can adsorb strongly onto metal and graphene surfaces [27]. This property, attributed to the interaction of delocalized π electrons, has been utilized in efforts to remove contaminants from the environment, in the case of methylene blue [28], and create novel surfaces, for instance with ferrocene [29], and with methylene blue for multi-layered immunosensors [30]. In a recent study we discovered and characterized the strong adsorption of the osmium tetroxide bipyridine label onto gold, and subsequent increase in the strength of adsorption of DNA onto the gold electrode when labeled with this chemical [31]. Furthermore, we were able to unambiguously distinguish the positions of the square-wave voltammetric peaks for the pure redox label, versus the adsorbed, labeled DNA, and versus labeled thiolated DNA that was bound with a covalent attachment at the thiol linker. Due to these effects, redox labels designed to lie closer to the electrode may result in different hybridization behavior than those designed to be at a more distal region of the target.

A popular hybridization-based electrochemical assay uses single-stranded probes which hybridize to redox-labeled DNA targets. Another strategy is a hairpin molecular-beacon (MB) system, which employs terminally redox-labeled probes composed of a self-complementary stem region and a target-complementary loop region [32]. Here, formation of linear duplexes upon hybridization results in increased distance between the redox label and the electrode surface, which leads to signal reduction. The MB assay can provide an improvement over the simple linear-hybridization method for single-base mismatch detection, because hybridization of DNA target to the hairpin requires breakage of hydrogen bonds in the stem duplex, which is less thermodynamically favorable than a simple standard hybridization reaction. Thus, in the presence of a mismatched target, the force which opens the hairpin's structure and allows its hybridization to the target will be diminished, which can lead to an improvement in specificity [33]. However, the electrochemical MB assay also suffers from its own difficulties, because it relies on detection of the target via a sometimes-small reduction in signal (the signal-off method), which can be difficult to distinguish and can be caused by alternate phenomena [34]. The MB is an example of a competitive assay which increases sensitivity to a mismatch using thermodynamics.

Osmium tetroxide 2,2′-bipyridine ($[OsO_4(bpy)]$) is a redox reagent that reacts with thymine bases in single-stranded DNA in a click-reaction [35,36], forming a labeled nucleic acid which is then electrochemically detectable [37,38]. The labeling can be performed in-house [39] and so does not require purchase of commercially-modified DNA. The reagent does not readily react with thymines in double-stranded regions, and thus selective labeling of specific thymines can be achieved using “protective-strands.” These protective oligos are hybridized to a given strand before labeling, and preserve the recognition region for later use. The protective strand leaves all other thymines in a sample sequence exposed, so that labeling of multiple thymines outside of the sequence in question occurs. The osmium labels do not prevent the hybridization of the protected recognition region to the immobilized probe, which occurs when exposed to the modified electrode in a highly specific manner [37,38].

Here we analyze a linear competitive strand-displacement assay

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