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A universal and label-free impedimetric biosensing platform for discrimination of single nucleotide substitutions in long nucleic acid strands

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ABSTRACT

We report a label-free universal biosensing platform for highly selective detection of long nucleic acid strands. The sensor consists of an electrode-immobilized universal stem-loop (USL) probe and two adaptor strands that form a 4J structure in the presence of a specific DNA/RNA analyte. The sensor was characterized by electrochemical impedance spectroscopy (EIS) using $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox couple in solution. An increase in charge transfer resistance (R_{CT}) was observed upon 4J structure formation, the value of which depends on the analyte length. Cyclic voltammetry (CV) was used to further characterize the sensor and monitor the electrochemical reaction in conjunction with thickness measurements of the mixed DNA monolayer obtained using spectroscopic ellipsometry. In addition, the electron transfer was calculated at the electrode/electrolyte interface using a rotating disk electrode. Limits of detection in the femtomolar range were achieved for nucleic acid targets of different lengths (22 nt, 60 nt, 200 nt). The sensor produced only a background signal in the presence of single base mismatched analytes, even in hundred times excess in concentration. This label-free and highly selective biosensing platform is versatile and can be used for universal detection of nucleic acids of varied lengths which could revolutionize point of care diagnostics for applications such as bacterial or cancer screening.

1. Introduction

A great number of hybridization-based sensors have been explored for the detection of specific DNA or RNA sequences in clinical molecular diagnostics of human diseases, environmental monitoring and food safety (Gerasimova et al., 2014; Sun et al., 2012; Tosar et al., 2010; Zhao et al., 2015). Among various strategies, electrochemical transduction is routinely adopted for nucleic acid detection because of the simplicity, high sensitivity, low cost and portability (Farjami et al., 2011; Liu et al., 2015; Ricci et al., 2007; Wu et al., 2014). First reported by Fan et al. (2003), a class of electrochemical DNA sensors (E-DNA) which included an immobilized stem-loop (SL) probe with a covalently bound redox marker were developed. This class of E-DNA sensors undergo a conformational change upon target binding and the change in current is measured (Lubin et al., 2006.) Other electrochemical constructs of these sensing platforms such as a double-stem (pseudoknot) DNA probe (Cash et al., 2009; Xiao et al., 2007) and triple-stem DNA probe (Xiao et al., 2009) have been developed to address limitations in differentiating single nucleotide substitutions (SNS). Although E-DNA sensors offer good sensitivity, they still fall short when detecting SNS using folded probes despite the improvements made using the double and triple stem DNA probes.

The selectivity of SL folded probes has been improved by the development of multicomponent probes for optical reporters (Gerasimova et al., 2010; Gerasimova et al., 2013). This approach utilizes adaptor strands along with the conventional molecular beacon (MB) probe, a hairpin-folded DNA strand conjugated with a fluorophore and quencher on opposite ends (Tyagi and Kramer, 1996; Kolpashchikov, 2012). The multicomponent approach exhibits improved selectivity in a broad range of temperatures (5–40 °C) compared to the MB or SL probe due to the design of the short analyte-binding arm of one adaptor strand (Gerasimova et al., 2010; Kolpashchikov, 2006; Stancescu et al., 2016). The short analyte binding arm will not bind at the SNS site and will in turn destabilize the 4J structure, resulting in the SL probe to be thermodynamically favored in its hairpin conformation rather than the 4J structure.

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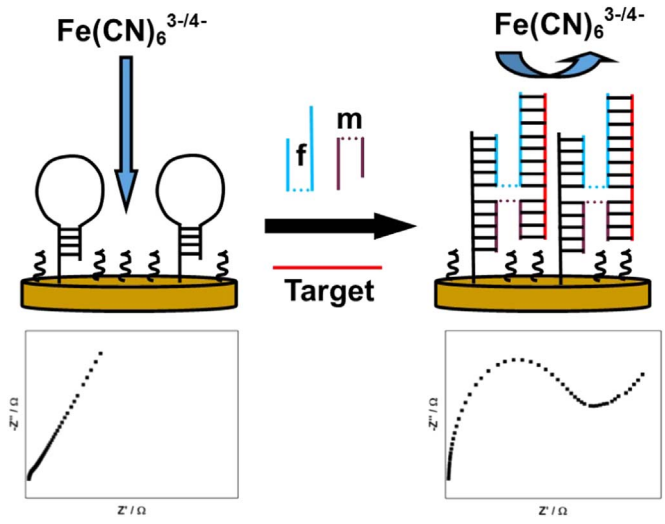
We have used this multicomponent approach to detect DNA and RNA targets (~ 22 bases) with the ability to discriminate SNS and have characterized the sensor using voltammetry techniques (Mills et al., 2017). Other electrochemical 4J sensor designs were explored for the analysis of microRNAs (~ 22 bases), but the previous designs involved the use of multiple covalently bound redox labels or enzyme assisted amplification strategies to reach low limits of detections (Labib et al., 2013, 2015). Although many techniques have been developed to overcome challenges with trace analyte detection, the user-friendly aspect has been eliminated as more steps and costs are incorporated to achieve lower limits of detection (Li et al., 2012; Lin et al., 2014). Therefore, electrochemical impedance spectroscopy (EIS) was also investigated for detection of nucleic acids to achieve low limits of detection (fM-aM) without use of additional amplification steps or expensive bound redox markers (Su et al., 2017). EIS is a powerful technique that monitors interfacial changes upon surface modifications (Lee and Shim, 2001; Li et al., 2002; Zhang et al., 2008). A few studies have explored EIS detection of varied analyte lengths, e.g. 17, 21, and 27 base DNA analytes reported by Wang et al. (2013) or PCR products of 90 bases reported by Minaei et al. (2016), but did not focus on how the length of the analyte affected sensor performance. On the other hand, the size of the analyzed nucleic acid should affect sensitivity of EIS since larger analytes bound to the electrode surface should prevent access of redox couple and thus increase the signal in greater extent than smaller analytes.

In this work, we registered EIS signals produced by analytes of varied lengths (22 bases, 60 bases, 200 bases) sensed by electrochemical 4 J platform to explore the performance and the impact of target length on discrimination of SNS, which has not yet been explored. The sensor includes an immobilized universal stem-loop (USL) probe attached to a gold substrate via a thiol bond and two adaptor strands (m and f) as seen in Scheme 1. The adaptor strands have a SL-binding arm and target-binding arm complementary to the target. In the presence of the target, the USL probe changes its conformation to form a bulky 4J structure that serves as a barrier to the redox couple in solution, resulting in a large charge transfer resistance.

The change in electron transfer resistance was used to monitor the hybridization for nucleic acid targets of varied lengths. An equivalent circuit model was used to analyze the performance of the sensor upon fabrication and hybridization with the nucleic acid analytes. The developed multicomponent sensor could be used for an inexpensive, selective and label-free detection of potentially any RNA or DNA analyte of varied length for point of care clinical diagnostics.



Scheme 1. A schematic of the design of the label-free impedimetric electrochemical 4J sensor.

2. Materials and methods

2.1. Reagents and materials

Tris(2-carboxyethyl phosphine hydrochloride (TCEP), Trizma hydrochloride (Tris-HCl), 6-Mercapto-1-hexanol (MCH) and magnesium chloride ($MgCl_2$) were purchased from Sigma Aldrich (St. Louis, USA). Sodium hydroxide (NaOH), sodium chloride (NaCl), potassium ferrocyanide ($K_4[Fe(CN)_6]$ $3H_2O$), potassium ferricyanide ($K_3[Fe(CN)_6]$), ethanol, hydrogen peroxide (H_2O_2) and sulfuric acid (H_2SO_4) were purchased from Fisher Scientific (Pittsburg, USA). Solutions were prepared with deionized water (18 MΩ cm resistivity) using a Milli-Q Integral Water Purification System from EMD Millipore (Massachusetts, USA). The oligonucleotides used in this study were purchased from Integrated DNA Technologies (Coralville, USA) and used as received (Table 1). An immobilization buffer (IB) was prepared with 50 mM Tris-HCl, 250 mM NaCl. A hybridization buffer (HB) was prepared with 50 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$. A hybridization buffer (HB) containing bovine serum was prepared with 50 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$. The buffers were adjusted to a pH of 7.4 using 1 M NaOH.

2.2. Instrumentation

A CHI660D Electrochemical Workstation (CH Instruments, Austin, USA) was used to perform EIS, Cyclic Voltammetry (CV), and Linear Sweep Voltammetry (LSV). Gold screen printed electrodes (DROPSENS, Spain) served as the working electrode (WE) in a three-electrode system which included an external Ag/AgCl (3 M KCl) reference electrode (RE) and platinum counter electrode (CE) (CH Instruments, Austin, USA). EIS, CV, and LSV measurements were recorded in HB containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, this par redox was used since it is very well stablished, characterized and widely used. EIS measurements were taken over a frequency range of 10 kHz to 10 Hz at an AC potential of 0.15 V in a faraday cage to reduce electrical noise. The experimental data is presented as Nyquist plots which were fitted by an equivalent circuit using instrumental software. CV measurements were obtained at a scan rate of 100 mV/s. At least three electrodes were used in each experiment to acquire statistically significant data.

Kinetic studies were performed to calculate the electron transfer rate constant (k_f) at the clean gold electrode, upon USL probe and MCH immobilization, and upon hybridization with the three different target lengths (T-22, T-60 and T-200). Experiments were executed using a gold rotating disk electrode (Au-RDE) of a 5-mm diameter. The electrode was coupled to a modulated speed rotator (MSR) system from PINE Research Instrument, Inc. (Durham, US). Speed rotations were performed from 100 to 3600 rpm.

2.3. Electrode preparation

The WE was activated in 0.5 M H_2SO_4 using CV in the range from 1.6 to − 0.1 V at a scan rate of 100 mV/s. The real surface area of the gold screen printed electrode was calculated from the CVs in sulfuric acid solution by integrating the reduction charge of gold oxide monolayer as described in the literature (Carvalhal et al., 2005; Trasatti et. al, 1991). The WE was then rinsed with DI water and dried with nitrogen prior to use.

2.4. Immobilization and hybridization

The immobilization of the USL probe was achieved using a gold-thiol bond. First, 1 mM TCEP was added to the USL probe and was vortexed for 1 h to reduce the disulfide bonds. This solution was diluted to 0.1 μM in IB and 15 μL of the solution was drop casted and incubated on the electrode for 30 min at room temperature (Mills et al., 2017). Then, the electrodes were rinsed using IB and dried with nitrogen. Next,