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Rapid detection of different DNA analytes using a single electrochemical sensor

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ABSTRACT

A unique platform for the detection of two distinct DNA sequences by using one single electrochemical sensor was developed. The sensor consists of a universal stem loop probe attached to a solid surface, and two analyte-specific adaptor strands. These adaptor strands hybridize to a nucleic acid analyte and provide both highly specific recognition and high binding affinity. The universal probe can be regenerated by a simple and quick rinse with urea and water. As a proof of concept, we differentiated long target sequences of Zika (141 nt.) and Dengue (114 nt.) viruses that contain secondary structures. The proposed sensor provides a platform for rapid and cost-efficient detection of potentially any DNA or RNA sequence using a single electrochemical sensor.

1. Introduction

Traditional sensors for nucleic acid analysis are tailored for detection of DNA or RNA targets by utilizing a target-specific probe coupled to a transducer. When a target nucleic acid hybridizes with the probe, a change in signal is observed. A number of detection approaches have been explored for selective analysis of nucleic acids, e.g. optical, piezoelectric and electrochemical sensors [1]. Electrochemical transducers present advantages such as efficient analysis due to their low cost, portability and high sensitivity. The electrochemical signal output is measured upon hybridization of the target-specific probe, which triggers a change in current based on the placement of a redox marker [2]. However, present electrochemical sensors targeting nucleic acids still face challenges such as: i) for each different analyte, a specific probe needs to be designed, making it impossible to detect multiple analytes using the same immobilized probe, hindering mass production for future commercialization; and ii) the inability to detect long and stable DNA/RNA secondary structures at ambient temperature. Here, we propose a universal sensor for analysis of potentially any ssDNA or RNA target by implementing an original detection scheme. Furthermore, this platform allows for sensor regeneration, thus creating a basis for applying the same sensor in analyses of multiple, different targets. In addition, we demonstrate the detection of nucleic acids with long sequences that contain stable secondary structures, which traditionally present a great challenge for nucleic acid analysis. Thus, this proposed technology offers a platform for a broad range of biomedical, environmental and forensic applications.

Zika (ZIKV) and Dengue (DENV) viruses were chosen as model analytes for this study. These flavivirus infections are transmitted by mosquitos and have quickly spread throughout the Americas causing major health concerns [3–6]. ZIKV and DENV typically cause mild influenza-like symptoms such as fever, rash and headaches. However, ZIKV can present serious complications such as Guillain-Barré syndrome and fetal microcephaly, while DENV can be fatal due to dengue hemorrhagic fever, which causes hemorrhage and plasma leakage [7–12]. Around 80% of ZIKV infections are asymptomatic and the other 20% have non-specific symptoms that mimic those of other viruses, such as DENV and Chikungunya, which make its clinical diagnosis challenging [13]. ZIKV and DENV are commonly detected by enzyme-linked immunosorbent assays (ELISAs) [14,15]. However, due to the difficulty of differentiating flaviviruses (e.g. ZIKV versus DENV) using ELISAs, significant interest and research efforts are being explored to develop molecular diagnostic tests [10,21–23]. The standard method reverse-transcription polymerase chain reaction (RT-PCR) can provide accurate results in hours [16–20], however it can be expensive, involve large instrumentation that may not be portable, and require skilled personnel which may limit these testing methods in remote locations. In addition, more specifically, there is a need for a rapid point-of-care

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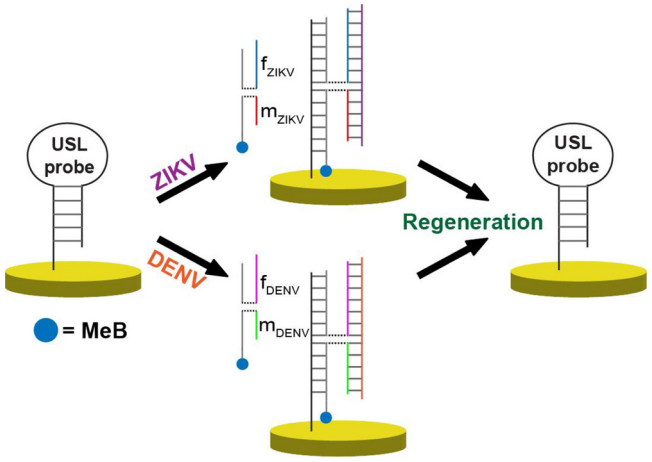

Scheme 1. Design of a USL probe used for the detection of the ZIKV or DENV. A simple consecutive rinse with 7 M urea and water will regenerate the USL probe. Design details and sequences of the DNA/RNA components are included in the SI.

(POC) test as diagnosis and treatment are delayed with the current clinical protocols [24]. Several technologies for the detection of viruses have been proposed, such as a molecular detection platform combined with nucleic acid sequence-based amplification (NASBA) and a RNA probe-based sensor, reported by Pardee et al. [25]. However, this technology would require two different sensors one is ZIKV and the other is DENV specific.

Here, we developed a universal electrochemical sensing platform which can detect both ZIKV and DENV with high selectivity. This platform is composed of a universal stem loop (USL) probe and two adaptor strands (m and f), that hybridize to a long target sequence to form a four-way junction (4J) structure (Scheme 1). The m-strand is labelled with a methylene blue (MeB) redox marker for electrochemical detection. This strand provides high selectivity for the sensor, forming a stable complex only in the presence of a target with fully complementary sequence due to its relatively short binding region. The other adaptor strand (f) has a longer target-binding arm to aid in unwinding the secondary structures of nucleic acids and hybridize with the target [26,27]. Therefore, it is suitable for detection of long nucleic acid sequences (over 100 nt.), such as RNA fragments from Zika and Dengue viruses. We have previously shown the benefits of this platform's sequence-specific analysis for short nucleic acid sequences (microRNAs) and selective detection of nucleic acids through impedance measurements [28,29]. Now here, we demonstrate the power of this technique for detection of long nucleic acid sequences containing secondary structures. Moreover, we demonstrate for the first time that a single sensor can be used for the detection of ZIKV sequence, regenerated rapidly through washing with urea and deionized water, and then used for the detection of DENV sequence.

2. Experimental

Gold disc electrodes (GDE) were used as working electrodes (WE). This WE was cleaned before modification by immersion in a piranha solution (1:3 ratio of H_2O_2:H_2SO_4) and then manually polished on a microcloth with a set of alumina slurries (1.0 µm, 0.3 µm and 0.05 µm; Buehler, Lake Bluff, USA). Residual alumina particles on the electrode surface were removed upon sonication of the WE in water and in ethanol, both for 2 min. The area of the WE was determined by performing cyclic voltammetry (CV) in 0.5 M sulfuric acid from 1.6 to –0.1 V at a scan rate of 100 mV/s [30].

Next, the disulfide bond of the USL probe was reduced using 1 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) while shaking for 1 h. Then, the solution was diluted with Immobilization Buffer (IB, see SI for buffer components) to a final concentration of 0.1 µM. Subsequently, 15 µL of the solution was drop casted and allowed to incubate on the electrode for 30 min at room temperature. Then, the electrodes were rinsed using IB and dried with nitrogen. 15 µL of 2 mM 6-mercapto-1-hexanol (MCH) was drop casted and incubated on the electrode for 30 min to reduce nonspecific adsorption, then rinsed with IB and dried with nitrogen. The target solutions (ZIKV or DENV) were diluted in Hybridization Buffer (HB) to appropriate concentrations and mixed with 0.25 µM m-strand and 0.5 µM f-strand. These concentrations of adaptor strands were previously optimized as reported by Mills et al. [28]. Finally, 15 µL of this solution was drop casted on the electrode and incubated for variable times as specified in each section. As mentioned before, Scheme 1 displays a representation of the mechanism of the universal electrochemical platform for detection of ZIKV and DENV, including the ability of the USL probe to be regenerated. The electrode was regenerated by subsequent rinsing with 7 M urea and DI water to disrupt the 4J structure.

3. Results and discussion

3.1. Sensor optimization

USL probe was immobilized on the solid surface via a gold-thiol bond. The probe sequence was earlier optimized for the 4J sensor in solution [28]. The target fragment for ZIKV was selected based on a publication by Pardee et al [25], who found that a specific set of primers produce a nucleic acid sequence-based amplification (NASBA) product with high efficiency starting from RNA of Zika Virus MR 766 strain. The target fragment for DENV was chosen based on the work by Waggoner et al. [31], who selected a fragment of DENV gene than can serve as a good marker for differentiation of Dengue from Zika and Chikungunya virus.

Adaptor strands m and f were designed to the chosen analyte fragments according to the strategies described previously [32]. To enable highest current signal output (Figure S1), different lengths of the oligothymine (T) spacer connecting the USL to the GDE were studied (1T, 5T, 10T and 15T). It was observed that the USL spaced with ten thymines (10T) resulted in the highest average current density, likely due to a compromise between the successful formation of the 4J structure, which is expected to be highest for longer spacers, and efficiency of the electron transfer from the redox marker to the electrode surface, which should be highest for shorter spacers. Additionally, the influence of length and sequence of adaptor strands for DENV (sequences shown in Table S1) were also optimized. Results (Figure S2 and Table S2) and discussion of this optimization can be found in the supporting information.

3.2. Sensor response and cross-reactivity

ZIKV and DENV share a significant similarity in their RNA-genome sequence and can interfere via cross-reactivity during detection [33,34]. However, here fragments from different regions with no overlap were investigated to improve specificity by using a single sensor. Thus, Fig. 1 shows the response of the Zika sensor to the presence of either Zika (T-ZIKV) or Dengue target (T-DENV). In a similar way, the response of the Dengue sensor was evaluated for T-DENV and T-ZIKV. The cross-reactivity study of the sensors is shown in Fig. 1, where the same USL probe was used to detect both targets, with adaptor strand sequences corresponding to each target.

As observed in Fig. 1, the Zika sensor produced a high electrochemical signal (current density) in the presence of T-ZIKV and corresponding adaptor strands m-ZIKV and f-ZIKV (Fig. 1A, blue line). Upon sensor regeneration by rinsing with 7 M urea solution and water (Fig. 1B, red line), this sensor (containing m-ZIKV and f-ZIKV adaptor strands) was evaluated for T-DENV resulting in a negligible electrochemical signal (Fig. 1B, blue line). This demonstrates high selectivity

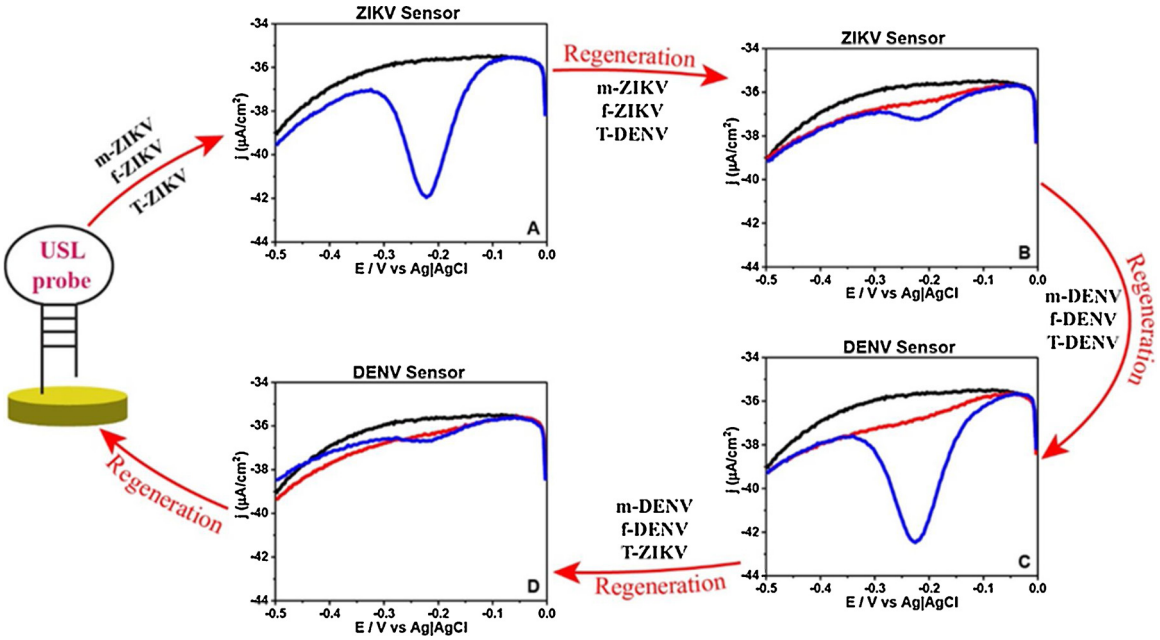

Fig. 1. Regeneration of the USL probe for detection of the ZIKV and DENV. Square wave voltammetry (SWV) response after immobilization of the USL probe and backfilling with MCH and hybridization with (A) m-ZIKV, f-ZIKV and 50 nM T-ZIKV, (B) m-ZIKV, f-ZIKV and 50 nM T-DENV, (C) m-DENV, f-DENV and 50 nM T-DENV and (D) m-DENV, f-DENV and 50 nM T-ZIKV. Black lines indicate baseline signal, blue lines indicate signal after hybridization with the target, 10 min for ZIKV and 30 min for DENV, and red lines indicate signal after regeneration of the USL probe. Between each new hybridization, the electrode was regenerated using 7 M urea for 5 s and water for 30 s (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of the sensor. Upon a second regeneration of the sensor (Fig. 1C, red line), the Dengue sensor was interrogated for T-DENV in the presence of corresponding adaptor strands m-DENV and f-DENV and a large current density was displayed (Fig. 1C, blue line). Finally, upon a third regeneration (Fig. 1D, red line), the Dengue sensor (containing m-DENV and f-DENV adaptor strands) was evaluated for T-ZIKV, and a negligible signal was observed (Fig. 1D, blue line). The quick dehybridization of the probe-analyte complex in 7 M urea and water can be attributed to the reduced stability of the 4J structure, especially at low [Mg^{2+}] [35]. These results demonstrate that the same electrochemical sensor can be used for the detection of both ZIKV and DENV by just switching the adaptor strands before hybridization. This discrimination of DENV and ZIKV is very important to facilitate appropriate clinical diagnosis and treatment. To further evaluate the selectivity, the sensors were interrogated in samples containing a mixture of ZIKV and DENV targets. The response obtained with Zika sensors in the mixture of 50 nM ZIKV + 50 nM DENV RNA (Figure S4, B) is the same as the response obtained for 50 nM ZIKV RNA alone (Figure S4, A). Similar results were obtained for the Dengue sensor where the response in the mixture of 50 nM ZIKV + 50 nM DENV sample (Figure S4, C) is similar to the response obtained for 50 nM DENV sample (Figure S4, D). Values for current density can be found in Table S3 (SI). These results confirm the high selectivity of the sensors and suggested that no interference should be expected when both targets are present in a sample.

3.3. Sensor hybridization time

The sensor response is dependent on the hybridization time of each target (ZIKV and DENV). Thus, various hybridization times were tested to obtain the highest electrochemical signal output. Fig. 2A displays the response for the Zika sensor, where it shows an increase in current density as the hybridization time for T-ZIKV increased from 1 to 90 min. Although 90 min provided the highest signal, subsequent analysis was performed with 10 min hybridization time to reduce the overall assay time. This analysis time was chosen as the corresponding current density can be easily differentiated against the blank. For the Dengue sensor, as the hybridization time increased from 1 to 30 min, an increase in current density was observed (Fig. 2B). However, the current density remained relatively constant from 30 to 90 min. For subsequent analysis, 30 min hybridization time was used for this sensor. This time was chosen since the signal intensity was close to that obtained for the Zika sensor after 10 min of hybridization. The difference in optimized hybridization times are likely due to the variance in stability of the secondary structures for the ZIKV and DENV target sequences chosen (Figure S3) as well as the sensor design. Further studies are needed to investigate this difference in the response to two different analytes. However, the hybridization time achieved is adequate for the selective detection of each analyte in 10 min assay. A rapid analysis time would be beneficial for POC clinical diagnostics to determine the presence or absence of each virus, rather than its concentration, allowing this sensor to be used in a qualitative diagnosis capacity to determine a course of treatment.

3.4. Sensor calibration curve

The response of the Zika sensor increased linearly as the concentration of T-ZIKV increased from 1 to 75 nM (Fig. 3A). The limit of detection (LOD) obtained was 0.98 nM, which was calculated as three times the standard deviation of the blank divided by the slope from the calibration curve. The sequence of the target-binding arm on the adaptor strands was easily tailored for the detection of T- DENV using the same USL probe. The response of the Dengue sensor increased linearly as the concentration of T-DENV increased from 1 to 75 nM (Fig. 3B), with a LOD of 1.04 nM. The LOD of both sensors was in the nM range which is adequate for detection of RNA samples amplified from body fluids using NASBA protocol. NASBA produces RNA fragments in the range of nM to μM concentrations depending on operational conditions. Thus, the sensors demonstrate promising results for detection of nucleic acid fragments, which may be useful for developing future POC tests targeting unique sequences of different viruses or other RNA analytes. In that respect, ZIKV was spiked in diluted serum samples to investigate possible matrix effects. As displayed in Figure S5 (SI)

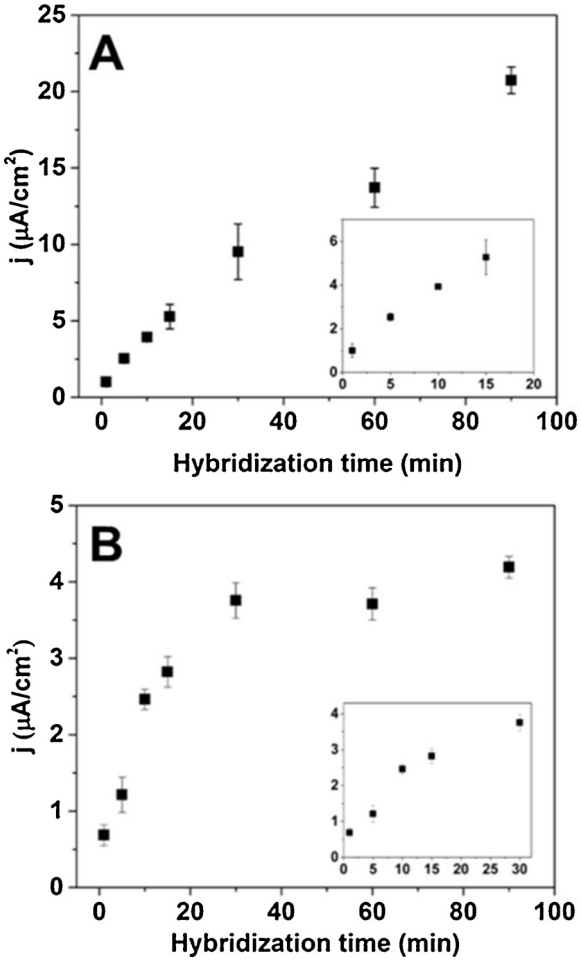

Fig. 2. Hybridization time affects the signal of the ZIKV and DENV sensors. (A) ZIKV Sensor response (current density) with m-ZIKV, f-ZIKV and 50 nM T-ZIKV at varied hybridization times. (B) DENV Sensor response (current density) with m-DENV-11, f-DENV-19 and 50 nM T-DENV at varied hybridization times. The insets display the current density versus hybridization timing from 1 to 15 min for ZIKV and 1 to 30 min for DENV.

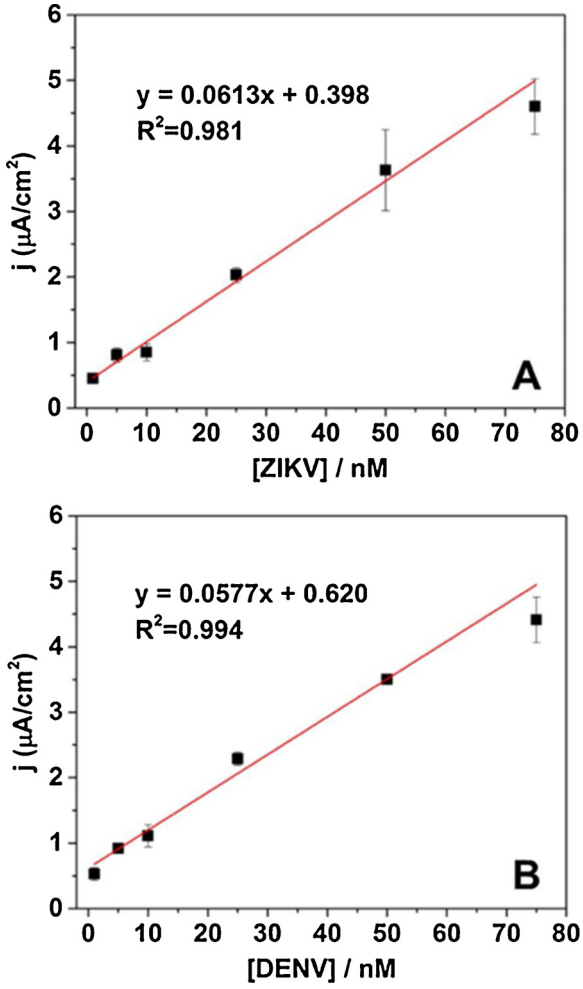

Fig. 3. Calibration curves for ZIKV and DENV sensors. (A) ZIKV sensor response (current density) upon 10 min of hybridization with varied concentrations of T-ZIKV along with m-ZIKV and f-ZIKV. (B) DENV sensor response upon 30 min of hybridization with varied concentrations of T-DENV along with m-DENV-11 and f-DENV-19.

negligible matrix effect was observed for serum:buffer dilutions of 1:20 and 1:50.

4. Conclusions

The USL sensor demonstrated the capability to discriminate two different long targets with secondary structures at room temperature, which is an extremely important attribute since conventional probes lack this capability. Furthermore, the potential of using and reusing one sensor by a quick and simple rinse with urea and water before hybridization of a new target reduces the time and cost when different DNA/RNA sequences may need to be analyzed. For example, in practice, the sensor could be used to test the presence of multiple viruses for a single patient following by simple amplification procedures, such as NASBA. This technology is potentially automatable: a simple device can be designed for continuous delivery samples premixed with adaptor strands to the universal sensor and provides quick urea/water washes between each sample. The proposed methodology showed high selectivity and the possibility of being used for detection and discrimination of sequences with high similarity, such as ZIKV and DENV. Zika analysis was also demonstrated in diluted serum samples. Thus, the same USL probe can rapidly detect potentially any RNA sequence of other flaviviruses in a simple and cost-effective manner. Therefore, this points toward the development of single universal electrochemical sensor that can be used for the analysis of several different analytes with high selectivity under ambient conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.04.149.

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