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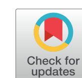
Short communication

Electrochemical voltammetric behaviors of synthetic dengue virus RNAs at ITO sensing electrode



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ABSTRACT

An electroconductive indium tin oxide (ITO) electrode was assembled and used as a sensing platform for direct detection of Dengue virus (DENV) RNAs in the phosphate buffer solution at pH 7.4. The voltammetric behaviors of DENV RNAs were examined at different volume ratio with matched and mismatched RNAs. The stability of the DENV RNAs hybrid duplexes and the microenvironment on the electrode surface was found to affect their voltammetric profiles. In this work, the detection limit was estimated to be 2 amol. No labeling, reverse transcription and/or polymerase chain reaction is required for this RNA detection technology. This simple and efficient ITO sensing electrode may be a promising platform in Dengue diagnosis.

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1. Introduction

Dengue is a serious disease worldwide transmitted by mosquito [1], as Dengue virus (DENV) can cause hemorrhagic fever that is usually fatal [2]. No antivirals or vaccines are available for DENV [3]. DENV is a positive single-strand RNA virus, belongs to the *Flaviviridae* family, and consists of 4 related serotypes (DENV1-4). Dual or multiple infections of viruses can cause antibody-dependent enhancement (ADE) for Dengue [4], leading to severe consequences, such as hemorrhagic fever. Although antibody detection is a sensitive tool for Dengue diagnosis [5], it is unfeasible for rapid and early Dengue diagnosis because it lacks serotype specificity and is time-consuming [6]. Additionally, although dual or multiple infections of viruses can be detected using reverse transcription polymerase chain reaction (RT-PCR) method, the method is limited in serotyping the four Dengue viruses and requiring extensive work to identify them [7], and the specificity and repeatability of detection need to be further improved. Therefore, it is highly desired to develop a simple, low-cost and reliable diagnosis method that can rapidly identify the serotypes in the early phase.

Note that as the hemorrhagic fever is asymptomatic in the early stage, it is difficult to achieve identification and prevention. Therefore, early detection of DENV is particularly important. Electrochemistry has been an effective tool since the genome can be sensitively and specifically detected at the early stage of the symptoms, without labeling, reverse transcription and/or polymerase chain reaction [8,9]. Various electrochemical immunosensors have indeed been reported for early diagnosis of Dengue virus [10,11], based on the detection of proteins in Dengue virus. In this work, we demonstrate an electrochemical detection method based on the RNAs in the Dengue Virus. It is well-known that detection of RNAs is more specific and direct than those with protein biomarkers, thus showing great advantages in disease diagnosis. However, RNAs typically exhibit high oxidation potential, and produce only poor electrochemical signals due to the oxygen evolution reaction. In addition, the sensitivity of conventional cyclic voltammetric technique is generally too low to detect the RNAs in aqueous solution, while detection by electrochemical impedance spectroscopy lacks specificity. By contrast, square-wave voltammetry (SWV) can be exploited as a highly sensitive and specific tool [12]. Additionally, electroconductive indium tin oxide (ITO) electrode can provide a stable and sensitive electrochemical sensing platform for the detection of biomolecules [13]. Therefore, in this study, we assembled an ITO/DENV RNAs/nafion electrode for the electrochemical detection of DENV RNAs, where high sensitivity and specificity was achieved in SWV measurements. To our best knowledge, detection of DENV RNAs via ITO sensing electrode has not been reported previously.

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2. Materials and methods

2.1. Reagents

The target Dengue RNAs and hybrid probes were designed from the RNA genome with the inclusion of a general probe that was identical in all four serotypes. The target Dengue RNAs were synthesized and purified by IDT (https://www.idtdna.com/site). The probes were synthesized in the Huang lab by using solid phase phosphoramidites chemistry and thereafter purified using Glen Pak column. ITO slides (7–10 Ohm/sq) were purchased from Vin Karola Instruments.

2.2. Preparation of modified electrodes

Different volume ratios of target DENV RNAs and hybrid probes were mixed. Hybridizing of RNAs and probes was carried out in water at 90 °C for 2 min. The solutions were drop-cast onto the ITO chip, which was then covered by 5 μL of nafion.

2.3. Electrochemistry

Electrochemical measurements were performed in a three-electrode configuration using a CHI 1030 electrochemical workstation. The ITO slide was used as the working electrode, which was covered with epoxy resin leaving an open area of 2 mm in diameter. A Pt wire acted as the auxiliary electrode. All the potentials were referred to a Ag/AgCl (sat'd KCl) reference electrode. A 0.1 M phosphate buffer solution (PBS, pH 7.4) was used as the electrolyte in all experiments.

3. Results and discussion

3.1. Specific detection of various DENV RNAs

In this work, the DENV probes were designed using different regions of viral RNA genomes. All the probes were modified with an amino group which affords immobilization on the ITO glass chip surface. The probes have two regions, an RNA region and a DNA region. The RNA region is 2′-*O*-methylyted, which stabilizes the RNA region and protects it against RNases. DENV Probes and DENV RNAs were listed in Table S1 (see ESI† for details). DENV probes (5 pM) and DENV RNAs (5 pM) at different volume ratios were mixed. A microchip was assembled by drop-casting the above hybridizing solution onto an ITO chip. ITO is an n-type semiconductor. At the interface between ITO and solution, the ITO region is positively charged and the solution negatively charged. When the potential is swept positively, the holes are driven toward the solution to electrooxidize RNA by the electric field. The response can be analyzed electrochemically.

As shown in Fig. 1, when DENV probes and target DENV RNAs were mixed at 1:1 molar ratio, only featureless voltammetric response was observed (blue curves in Fig. 1), suggesting remarkable stability of DENV RNA hybridization. At other mixing ratios, when the potential was swept positively, an oxidation peak appeared at ca. +2.00 V. This can be exploited as a sensing mechanism for DENV RNA hybridization. As well-known, a hybrid duplex is formed between two complementary RNA strands, which are more stable than single-strand RNA and cannot be electro-oxidized easily. The peaks currents decrease in linearity with content of hybridizing RNA-RNA increasing (Fig. S1). When DENV

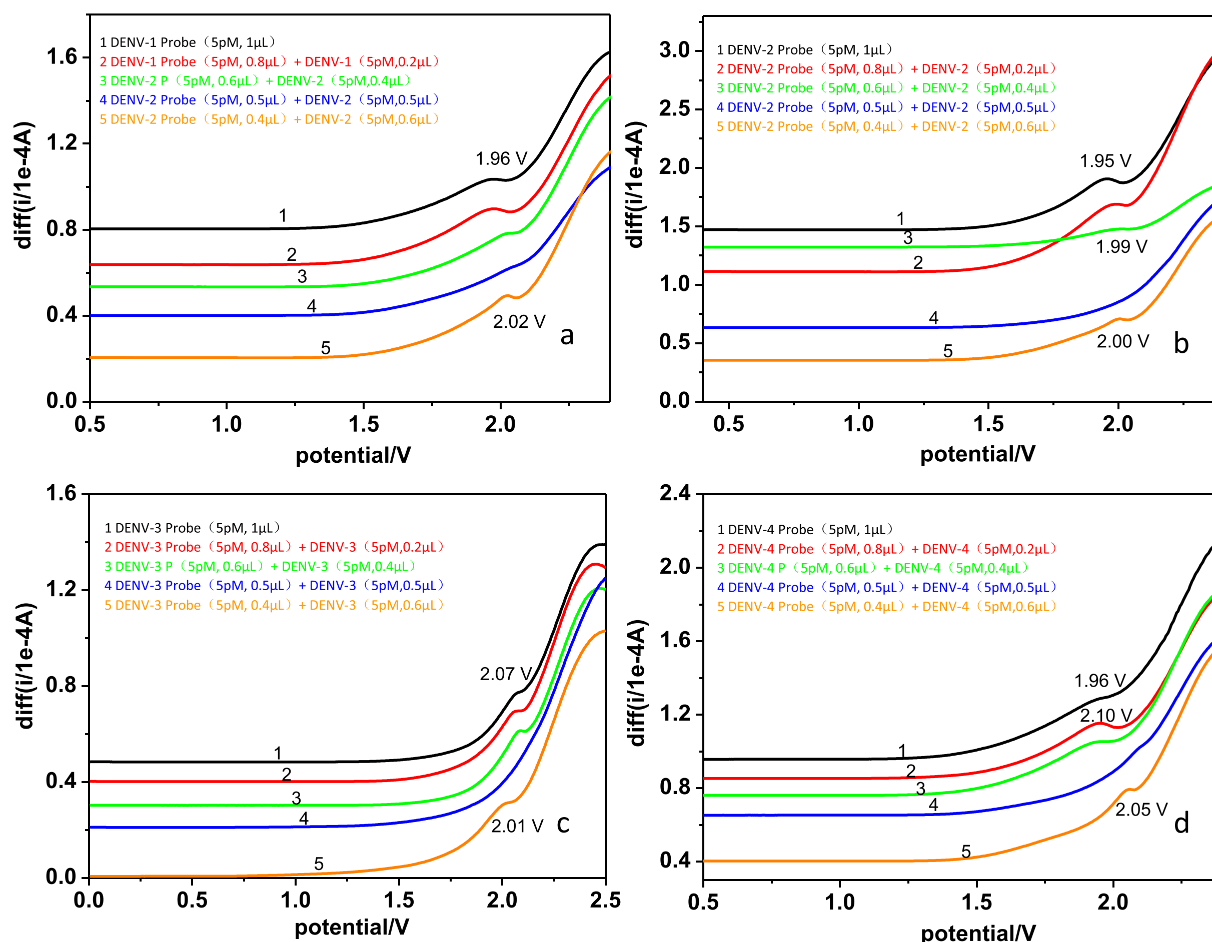


Fig. 1. Electrochemical superstability of DENV RNAs hybridized with the same molar ratio. SWV curves for the hybridizing solutions of DENV Probes and DENV RNAs with different volume ratios: (a) DENV-1 probe + DENV-1 RNA; (b) DENV-2 probe + DENV-2 RNA; (c) DENV-3 probe + DENV-3 RNA; (d) DENV-4 probe + DENV-4 RNA.

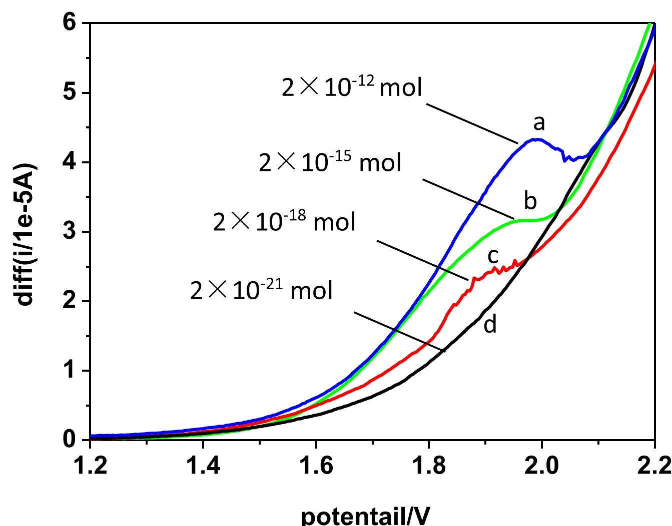


Fig. 2. SWV curves acquired at different Dengue-2 probe concentrations: (a) 2×10^{-12} mol; (b) 2×10^{-15} mol; (c) 2×10^{-18} mol; (d) 2×10^{-21} mol.

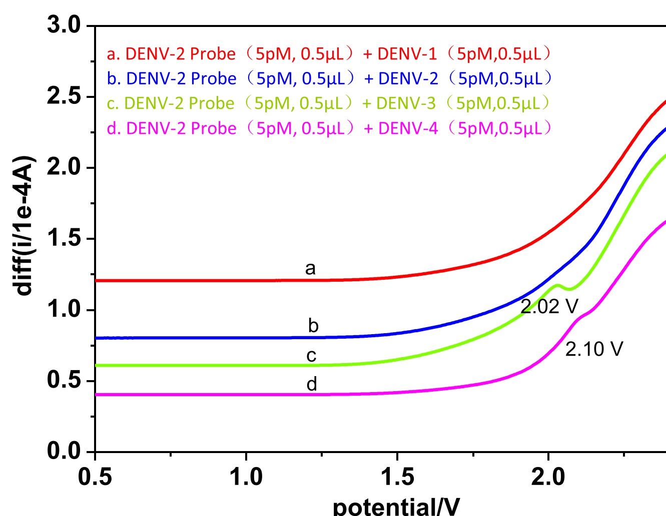


Fig. 3. The stability of the hybrid duplexes affects the electrochemical behavior of Dengue virus RNAs. SWV curves for the hybridizing solutions of Dengue 2 probe and DENV 1-4 RNAs with the same mole ratio: (a) DENV-2 probe + DENV-1 RNA; (b) DENV-2 probe + DENV-2 RNA; (c) DENV-2 probe + DENV-3 RNA; (d) DENV-2 probe + DENV-4 RNA.

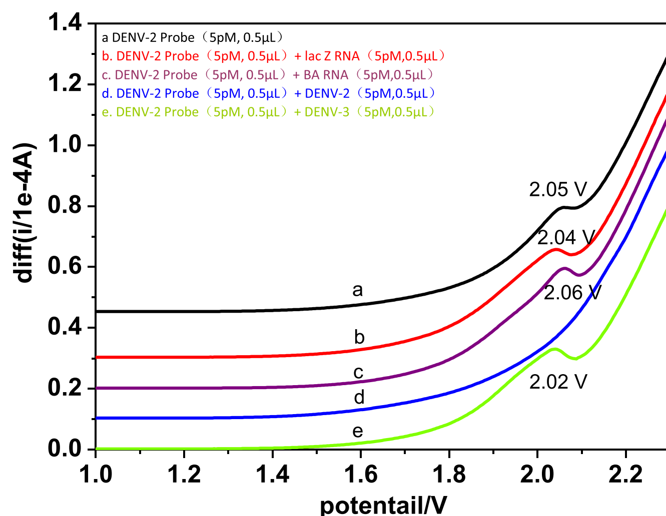


Fig. 4. Further confirming the relationship between the stability of the duplexes and electrochemical behavior. SWV curves for the hybridizing solutions of DENV-2 probe and DENV2-3 RNAs (or lac Z RNA, or BA RNA) mixed with the same mole ratio: (a) DENV-2 probe; (b) DENV-2 probe + lac Z RNA; (c) DENV-2 probe + BA RNA; (d) DENV-2 probe + DENV-2 RNA; (e) DENV-2 probe + DENV-3 RNA. The oligonucleotide sequences of *E. coli lacZ* RNA (*lacZ*) and *Bacillus anthracis* RNA (BA) were listed Table S2 in ESI†.

probes and target DENV RNAs were hybridized at 1:1 molar number, DENV probes and target DENV RNAs were perfectly matched, producing only stable hybrid duplexes. Therefore, no oxidation peak was observed in electrochemical measurements (blue curves in Fig. 1). The same electrochemical behavior was also observed in DENV-5 RNA hybrid solution, as shown in Fig. S2 (see ESI† for details). From these measurements, taking Dengue-2 probe as the example (Fig. 2), the detection limit can be estimated to be 2×10^{-18} mol (2 amol), which is higher than those obtained from electrochemical immunosensing detection of DENV reported recently in the literature [14].

DENV-2 is becoming the most widely spreading viral disease worldwide. DENV-2 has multiple antigen epitopes-related antibody-dependent enhancement (ADE) [15], and vaccine is only 33.6% effective against DENV-2 in patients aged over 9 years old [16]. As DENV-2 is more likely to cause hemorrhagic fever than other types of viruses [17], this study will focus on DENV-2 RNA detection. DENV-2 probe (5 pM) and DENV RNAs (5 pM) were mixed at 1:1 volume ratio. The electrochemical results were shown in Fig. 3. From Fig. 3, with DENV-2 probe, the electrochemical results are the same for both DENV-1 RNA and DENV-2 RNA. No oxidation peak was observed (Fig. 3a–b). That is, DENV-1 and DENV-2 cannot be differentiated. By contrast, others show an oxidation peak at 2.02 V ~ 2.10 V (Fig. 3c–e), obviously, DENV-2, DENV-3, and DENV-4 could be differentiated easily. This can be explained by their hybridization competition with the DENV-2 probe. There is only one different single nucleotide between DENV-2 RNA and DENV-1 RNA, while other DENV RNAs have 2 different nucleotides (Table 1). The hybridization will afford G:G mispairing between DENV-2 probe and DENV-1. While DENV-3 will afford C:A and U:G mispairing with DENV-2 probe, DENV-4 will afford A:C and G:T mispairing with DENV-2 probe. The mismatched nucleotides increase in the following sequence of DENV-2 < DENV-1 < DENV-3~4, which reduces the stability of the hybrid duplexes and enhances their

Table 1
DENV probes and DENV RNAs [7].

DENV-2 Probe-2: 5′-TACGCCAT-[2′-Me-r(*GCGUACAGCUUCC*)]-NH_2-3′

DENV-2 RNA (Acc.: U87411.1; 10470-10490 nt): 5′-GGAAGCUGUACGC-AUGGCGUA-3′

DENV-1 RNA (Acc. no.: U88536.1; 10484-10504 nt): 5′-GGAAGCUGUACGC-AUGGGGUA-3′

DENV-3 RNA (Acc. no.: AY099336.1; 10457-10477 nt): 5′-GGAAGCUGUACGC-ACGGUGUA-3′

DENV-4 RNA (Acc. no.: AF326825.1; 10393-10413 nt): 5′-GGAAGCUGUACGC-GUGGCAUA-3′

Note: Underlined sequences are viral RNAs complementary to italicized 2′-*O*-Me-RNAs of RNA probes, and mismatched nucleobases are marked in red.

electrochemical activity. As shown in Fig. 3, when DENV-2 probe and DENV-2 RNA or DENV-1 RNA was mixed to form hybrid duplexes, the hybrid duplexes could not be oxidized (Fig. 3a–b), whereas others were oxidized at ca. 2.00 V or so (Fig. 3c–e). That is, SWV measurements can specifically differentiate between closely related DENV species, and the stability of the DENV RNAs hybrid duplexes will affect their electrochemical response, which was consistent with results obtained from the above analysis (Fig. 1).

To further confirm the relationship between the stability of the duplexes and electrochemical behavior, we investigated the electrochemical profiles of *E. coli lacZ* RNA and *Bacillus anthracis* RNA (BA) using a Dengue-2 probe. The results as shown in Fig. 4. In all samples, only target Dengue-2 RNA was able to perfectly match Dengue-2 probe in the hybridizing solution, while Dengue-3 RNA (Fig. 4e), *E. coli lacZ* RNA (Fig. 4b) and *Bacillus anthracis* RNA (BA) (Fig. 4c) did not match Dengue-2 probe. In particular, the latter two are completely mismatched, and the hybrid duplexes formed were not stable, as manifested by the electro-oxidization peak at ca. 2.00 V. Moreover, reproducible SWV responses were obtained by using different ITO electrodes prepared in the same manner.

3.2. Oxidation peaks shifting with scan times

Interestingly, the oxidation peaks of DENV RNAs exhibit a positive shift in electrochemical scanning. DENV-2 RNA hybrid duplexes are taken as an example (Fig. 5). Three SWV measurements were carried out consecutively; then the fourth scan was acquired 1 h later. Fig. 5 shows the SWV curves in these different sweeps. At the 4:1 volume ratio of DENV-2 probes (5 pM) to DENV-2 RNA (5 pM), DENV-2 RNA hybrid duplexes (curve 1 in Fig. 5) exhibited a positive shift of the oxidation peak at +1.88 V, +1.99 V, and + 2.24 V during the first three scans. After 1 h, the system was scanned again and the oxidation peak now shifted negatively to +2.10 V (Fig. 5d). Similar phenomenon was observed at the volume ratio of 1:4 (curve 2 in Fig. 5).

Such a shift of the oxidation peak is likely due to a change of the microenvironment on the electrode surface (Scheme 1). The samples (about 1 μL) were covered with nafion, which formed a stable microenvironment on the electrode surface [18]. Generally, the electrochemical system will involve oxygen reaction in the aqueous solution [19,20]. Anodic reactions such as oxygen evolution will take place at the electrode (Reaction 1) [21,22], which can result in a change of pH on the electrode surface. At longer scans, the concentration of hydrogen ion increases in the micro-environment, decreasing the pH and increasing the electrochemical potential of nucleic acids [23]. Therefore, the oxidation peaks of DENV-2 RNA hybrid duplexes shifted positively as the scanning times increased.

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \quad (1)$$

In addition, the nafion membrane has excellent proton conductivity [18]. When the system was kept for 1 h, hydrogen ions in the microenvironment would diffuse through the nafion membrane into the bulk solution, thus restoring the microenvironment of the electrode surface and the potential of DENV-2 RNA hybrid duplexes to a less positive value (Fig. 5d).

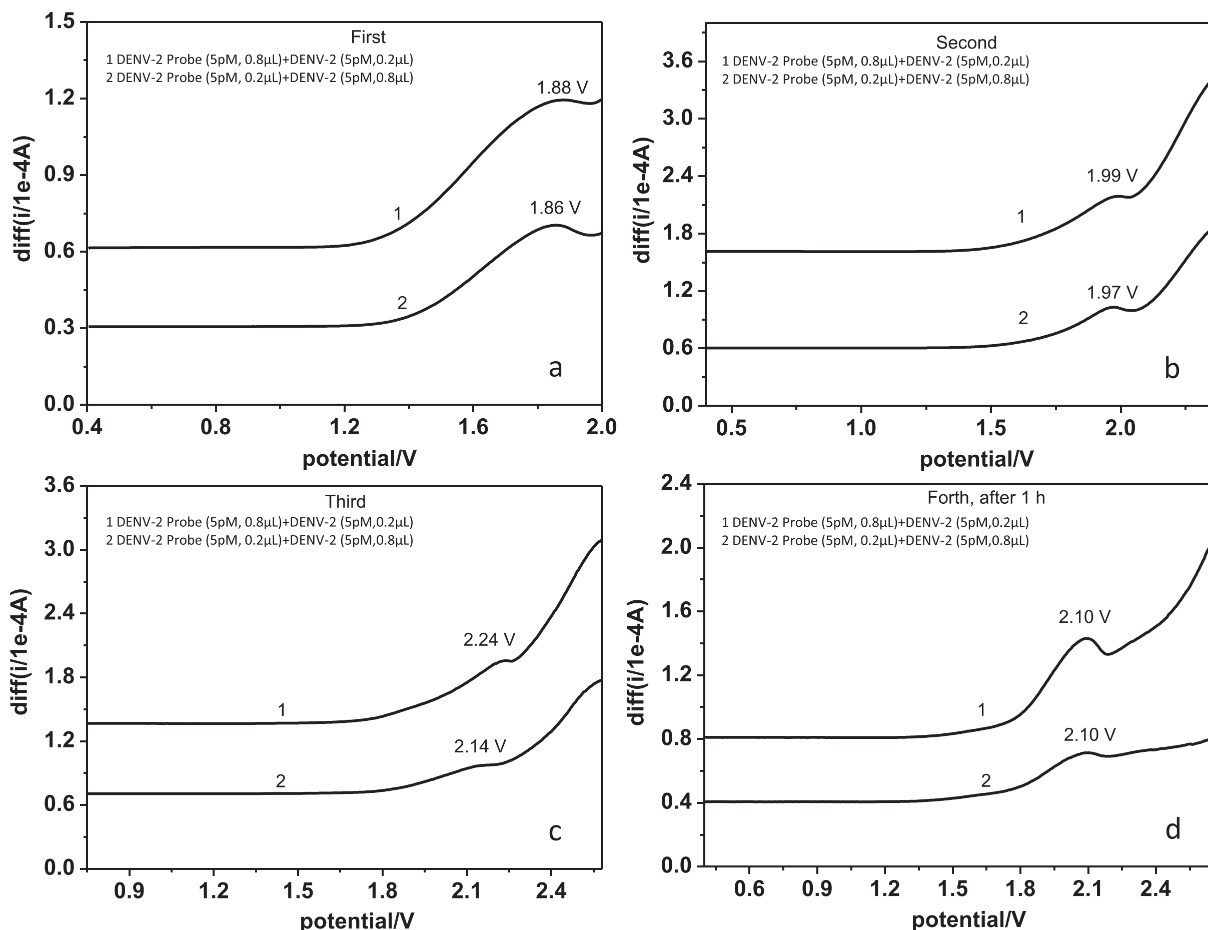
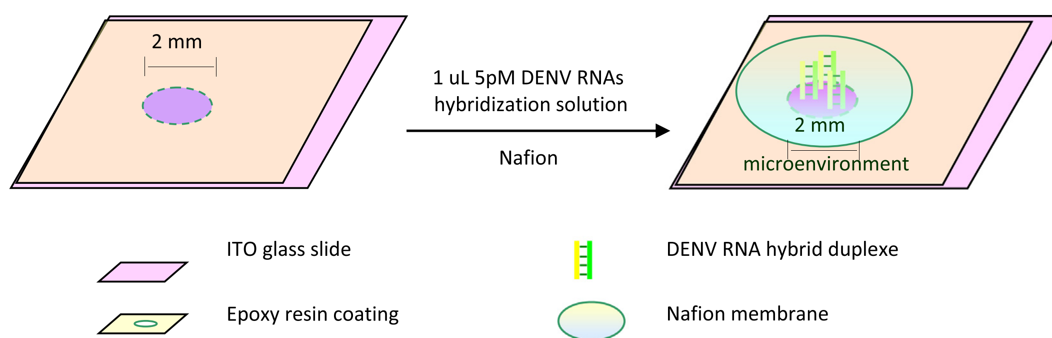


Fig. 5. Shift of oxidation peaks with scanning times. SWV curves at different scanning times: (a) First; (b) Second; (c) Third; (d) Fourth. The hybridizing solutions of DENV-2 probe and DENV-2 RNA with different volume ratios: (1) DENV-2 probe (5 pM, 0.8 μL) + DENV-2 RNA (5 pM, 0.2 μL); (2) DENV-2 probe (5 pM, 0.2 μL) + DENV-2 RNA (5 pM, 0.8 μL).



Scheme 1. Illustration for the microenvironment of the electrode surface.

4. Conclusions

In this work, SWV was used as a sensitive tool to investigate the electrochemical behaviors of DENV RNAs in PBS with a conductive ITO electrode, which could differentiate closely related DENV species with high sensitivity. The results showed that the stability of DENV RNA hybrid duplexes and the micro-environment on an electrode surface could impact the electrochemical response. Results from this study suggests that the ITO sensing electrode may be used as a promising platform for rapid and specific sensing of DENV RNAs that will be of significance in early Dengue diagnosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jelechem.2019.113463.

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