An ultrasensitive label-free electrochemical impedimetric DNA biosensing chip integrated with a DC-biased AC electroosmotic vortex

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A label-free electrochemical impedance spectroscopy (EIS)-based DNA biosensing chip is integrated with DC-biased AC electroosmotic (ACEO) stirring using concentric double ring-single disk electrodes to greatly promote the hybridization efficiency of 20-base target DNA (tDNA) fragments. The +0.7 V-biased ACEO flow of 3 V m and 380 Hz applied at the outer ring electrode (ORE) and inner ring electrode (IRE) can drive the tDNA-containing 1 mM Tris solution from the ORE to the probe’s DNA-modified disk electrode (DE) to achieve 90% saturation hybridization within 141 s. The DNA biosensor used Pd-deposited ORE as the pseudo-reference electrode and IRE as the counter electrode for on-chip-type three-electrode EIS measurement, and presented good linearity and repeatability in the range of 1 aM–10 pM and exhibited an ultrasensitive detection limit of 0.5 aM. The label-free EIS-based DNA sensing chips with integrated DC-biased ACEO vortex can achieve rapid hybridization, high selectivity and ultrasensitive detection for different tDNA samples.

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1. Introduction

The determination of specific nucleic acid sequences originating from organisms has attracted widespread interest for use in diagnostic applications, including the detection of food contamination, the identification of pathogenic species and the recognition of genetic diseases. DNA biosensors based on different principles have been developed to estimate the degree of hybridization between complementary target DNA (tDNA) fragments and probe DNA (pDNA) fragments. In particular, label-free methods, such as quartz crystal microbalance [1], surface plasmon resonance spectroscopy [2], mass-sensitive cantilever [3] and electrochemical impedance spectroscopy (EIS) [4–7], promise easy, fast and cost-effective DNA detection. Among the label-free methods, EIS-based biosensors exhibit several advantages, including good compatibility with electrical instruments and ease of large-scale production, making them well-suited for use in miniature diagnostic systems [8]. EIS biosensors can use either impedimetric or capacitive methods, which respectively estimate the change in the resistive and capacitive properties of electrode/electrolyte interfaces during DNA hybridization [9]. Compared with two-electrode impedimetric and capacitive detectors, three-electrode EIS-based biosensors can provide further analysis of the electrochemical properties of ionic diffusion and electron transfer across electrode/electrolyte interfaces by using a redox couple as the probe [8,9]. In EIS detection, electron transfer resistance (Rct) can be used as an indicator to quantify tDNA concentrations and to distinguish single-base mismatched hybridization [7,10–14].

Although label-free DNA biosensors can significantly simplify DNA detecting procedures, the hybridization process still takes tens of minutes to reach a plateau in stationary solutions [12,13]. The long hybridization time is mainly due to the Brownian motion of nanometer-scale tDNA, moving stochastically toward the pDNA immobilized on the sensor surface. Furthermore, the lower the tDNA concentration is, the smaller the diffusive flux of tDNA toward the immobilized pDNA becomes, thus resulting in longer hybridization times and lower binding densities when detecting lower tDNA concentrations.

To date, direct current (DC) and alternating current (AC) electrokinetic techniques such as electrophoresis, dielectrophoresis (DEP), electrothermal flow and electroosmotic flow [15], have been used to manipulate bioparticles and fluids in microanalysis systems without the use of external pumps or valves. Sosnowski et al. demonstrated that a DC field can regulate the transportation, concentration and hybridization of DNA fragments [16]. As compared with a DEP force proportional to particle volume, AC electroosmotic

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(ACEO) flow combined with an electrophoretic force is more effective at concentrating 20-base tDNA of a nanometer-scale size [17]. Moreover, the AC electrophoretic flow has been used to improve the efficiency of affinity interaction with a shorter hybridization time and a larger sensing response [7,18,19]. Therefore, the integration of the AC electrophoretic flow and the EIS-based sensor can completely fulfill the requirements for lab-on-a-chip with transportation, hybridization and in situ detection functions combined on the same substrate.

Generally, the ACEO flow is more effective than the AC electrothermal flow for obtaining larger velocities in excess of 100 μm/s using lower voltages (typically <5 Vpp) [20,21]. The lower driving potential can reduce the distortion of the thin-film electrodes and the pDNA-modifying layer, and prevent the formation of bubbles. Our previous study showed that the background electric properties of the pDNA-sensing layer exhibited significant change when an ACEO potential greater than 1.5 Vpp was applied in the DNA biosensing chip adopting the same electrodes for EIS detection and ACEO stirring [7]. Therefore, the electrode design integrating the EIS detector and the ACEO stirrer determines the detecting properties of the biosensing chip and the efficiency of DNA hybridization.

This article presents an advanced evolution to integrate a DC-biased ACEO vortex into a label-free three-electrode EIS-based DNA biosensing chip by using an on-chip-type concentric double ring-single disk electrode array. The potential of driving biased ACEO flows was applied at the palladium (Pd)-deposited outer ring electrode (ORE) and inner ring electrode (IRE) to produce a flow from the ORE to the IRE and the central disk electrode (DE) via the fluidic inertia for fastening DNA hybridization. When performing EIS measurement, the pDNA-modified DE, palladium (Pd)-deposited IRE and ORE can be, respectively, used as the working electrode (WE), counter electrode (CE) and reference electrode (ReE). The effects of ACEO stirring on the sensing characteristics of the EIS-based chips were evaluated in detail.

2. Experimental

2.1. Reagents

Specific sequences of 5′-GAGCTCGAGCTCGATTAG-3′ derived from the highly conserved AC1 gene of Squash leaf curl Philippines virus were used as the detected tDNA [22], designated as dtDNA. 20-base pDNA modified with the SH-(CH₂)₃ group at the 5′-end with the sequences of 5′-SH-(CH₂)₃-CCTAATCCGCTCGACCCTG-3′ was perfectly complementary to the dtDNA. Moreover, single base mismatched sequences of 5′-GAGCTCGAGATCGATTAG-3′, designated as smDNA, were used to estimate sensor selectivity. All DNA strands were synthesized by Bio Basic Inc. (Markham, Canada) and purified by high performance liquid chromatography. Sodium phosphate dibasic, sodium phosphate monobasic dihydrate, Tris(hydroxymethyl)aminomethane (Tris), N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 6-mercapto-1-hexanol (MCH), potassium hexacyanoferrate(III), hexacyanoferat(II) trihydrate, and fluorescence-labeled carboxylate-modified latex beads (with a mean diameter of 1.0 μm, an excitation wavelength of 470 nm, and an emission wavelength of 505 nm) were purchased from Sigma. All chemicals were of reagent grade and were used without further purification. All solutions were prepared with water purified through a Milli-Q system.

2.2. Electrode fabrication

50-nm Ti and 200-nm Au thin films were patterned as a 4 × 4 array of double ring-single disk electrodes on a cleaned glass substrate by lift-off microfabrication techniques. The widths of ORE and IRE were, respectively, 70 μm and 140 μm. The diameter of the central DE was 200 μm and the interelectrode gap between DE, IRE and ORE was 25 μm. Moreover, the working area of all patterned electrodes was designed by a 7-μm-thick negative photoresist, SU8-3010 (MicroChem, Newton, MA). In addition, the ORE and IRE were electrodeposited using Pd particles to, respectively, act as the pseudo-RefE and CE for EIS measurement and to supply the stable potential control of Faradaic charging for biased ACEO stirring. First, Pd particles were nucleated using the sweeping potential ranging from +0.6 V to 0.0 V at the scan rate of 50 mV/s for 5 times in the 0.1 M H₂SO₄ solution (pH 1.1) containing 1 mM K₂PdCl₄. Then, the constant potential of +0.43 V was performed for 90 s to form the Pd film.

2.3. Quantification of ACEO velocity

Our previous study covered the method of quantifying the ACEO velocity in detail [7]. Fluorescence-labeled latex beads suspended in 1 mM Tris solution (6.1 μS/cm, pH 9.3) with a concentration of 10⁶ particles/mL were used as markers to probe the ACEO velocities by measuring the particle trajectories from a set of continuous images. A function generator (33220A, Agilent, Santa Clara, CA) was connected to the ORE and the IRE for bias-varied ACEO driving, setting the positive bias voltage at the ORE versus the IRE used as the ground. Simultaneously, time-lapse fluorescent images of particles were recorded via a CCD camera (600D, Canon, Taiwan) equipped on an upright fluorescent microscope stage (BA310LED, Motic, Hong Kong) to analyze the ACEO velocities occurring above the electrode surface. According to the equivalent circuit model of the electrolyte resistor and electric double layer (EDL) capacitor in series [23], the theoretical frequency, defined as fₘw, of the maximum ACEO velocity (fₘ) can be numerically calculated from the angular frequency (ω₀ = 2πfₘw). Here, ω₀ = 2πfₑ/πɛₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κₑ·κ水肿合
measurement was carried out in the frequency range of 1 Hz to 100 kHz at 0 V potential added with a 5 mV amplitude sine wave versus versus the ReF of Pd-deposited ORE and the CE of Pd-deposited IRE. The IM-6/THALES software package was used for the acquisition and analysis of impedance spectra, and the simulation of equivalent circuits.

3. Results and discussion

3.1. Bias-varied ACEO

Theoretically, electrolytes with lower ionic strength can increase the EDL thickness to produce a larger ACEO velocity [23], but the lower cationic concentration of electrolytes may increase the difficulty of DNA hybridization due to the stronger repulsive force between pDNA and tDNA [11]. Our previous work showed that 1 mM Tris (pH 9.3) solution provides a compromise between ACEO driving and DNA hybridization [7]. The ACEO applied at the asymmetric ORE/IRE electrodes expectedly produces a unidirectional flow from the narrower ORE to the wider IRE [24]. The real $f_{mv}$ was explored by observing the trajectories of moving latex beads. Fig. S1 (see Supporting information) shows the time-lapse bead position and the calculated velocity of beads as a function of position when performing the ACEO of 3 Vpp at 350, 380 and 410 Hz. The velocities of beads at 350, 380 and 410 Hz, calculated at 50-μm far position from the edge of IRE, were respectively 52.2, 61.2 and 46.1 μm/s. The result indicates that the frequency of 380 Hz is the closest to the real $f_{mv}$ above the theoretical $f_{mv}$. 347 Hz. Moreover, while the frequencies fluctuate above and below 380 Hz, the corresponding velocities are smaller than that induced at 380 Hz. This phenomenon is attributed to the fact that, at frequencies higher than $f_{mv}$, the applied potential drops increasingly across the electrolyte so that the induced surface charges in EDL tend to zero. On the other hand, as the frequencies gradually fall below $f_{mv}$, the applied potential drops increasingly across EDL so that the tangential field in EDL approaches zero [20].

Fig. 1(a) shows a typical fluorescent image of beads, collected on the electrode surface with ACEO stirring of 3 Vpp and 380 Hz for 30 s applied at the IRE and ORE. The image shows that partial beads are attracted at the inner side of IRE and ORE edges due to the positive DEP force, and most beads moving along the IRE surface are collected at a ring-shaped fixed position, called the stagnation point, attributed to the changes in tangential electric fields [21,25]. The theoretical stagnation points, calculated at the $1/2.5$ width of IRE [25], are depicted as the yellow dotted lines of Fig. 1. The result shows that the experimental stagnation points under ACEO

![Image](https://example.com/image1.png)

**Fig. 1.** Fluorescent images of beads collected on the electrodes after performing bias-varied ACEO stirring for 30 s at 3 Vpp and 380 Hz in 1 mM Tris solution. (a), (b), (c) and (d), respectively, show the application of an extra DC bias of 0, +0.1, +0.5 and +0.7 V at ORE during ACEO driving. Red dashed, blue dashed, and yellow dotted lines, respectively, show the edges of the electrodes and insulator, and the stagnation point (S). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
stirring are very consistent with the theoretical points. In addition, the beads suspended above the IRE surface can move toward the DE via the flow inertia and flow out of the insulator–shielded area of the DE wire. The same situation occurred at +0.1 V- and +0.5 V-biased ACEO stirring, as shown in Fig. 1(b and c). The number of beads collected at stagnation points increased with the DC bias and the bead position gradually closes to the DE as the bias increases, attributed to the larger velocity of bias-increasing ACEO flow. It is worth noting that, as shown in Fig. 1(d), the Faradaic charging is sufficiently strong to produce a fast unidirectional flow from ORE to DE, thus the +0.7 V-biased ACEO flow drives the beads at speeds too fast for beads to accumulate at the stagnation point [25]. The continuous images of particles driven by +0.1 V- and +0.7 V-biased ACEO flow are, respectively, shown in the files “Movie-01V-biased ACEOF” and “Movie-07V-biased ACEOF” (see video data). However, the application of +1.1 V bias would immediately induce water electrolysis to produce bubbles at ORE, which is adverse for biased ACEO stirring and EIS measurement.

As shown in Fig. 2, driven by the bias-varied ACEO flow, the velocity of beads was a function of bias voltage, obtained from continuous images to calculate the bead position from the DE edge every 0.25 s and thus the corresponding velocity. Bead velocities increased with the DC voltage of biased ACEO stirring, resulting from the increasing Faradaic charging at ORE. Furthermore, the fluctuation of bead velocities increased with the DC voltage of biased ACEO stirring in the 0.1–0.5 V range. The larger fluctuation possibly results from the unstable yield of the Faradaic reaction, resulting in the increased flow change. In contrast, the fluctuation of +0.7 V-biased ACEO velocity was lower than that of +0.5 V-biased ACEO flow, and the average velocity was 4.5 times faster than that of ACEO flow. The results suggest that the stronger Faradaic charging of +0.7 V-biased ACEO flow can effectively produce stable bulk flow from the outer ORE to the central DE. To consider the stability of fluidic velocity, the hybridization efficiency was only compared with ACEO stirring of 0 V and +0.7 V bias.

3.2. Properties of the on-chip-type three-electrode EIS system

Most related studies have adopted an external ReFe in the three-electrode system to perform CV and EIS for evaluating the characteristics of electrochemical sensors followed by modification and hybridization [4–7,10–14,26,27]. This process makes the development of a miniature and disposable EIS-based biosensing chip difficult. In this study, the double ring-single disk electrodes were designed for not only ACEO stirring but also in situ EIS measurement. The ORE and IRE were electrodeposited by a Pd film to provide stable surface potential for EIS measurement. Figs. 2S and 3S (Supporting information), respectively, show the surface morphology of Pd-deposited ORE and the corresponding open circuit potential (OCP) versus an external Ag/AgCl electrode before and after the pDNA/MCH modification. The Pd-deposited film made the ORE surface rougher and allowed the Fe(CN)₆³⁻/⁴⁻ redox couple to exhibit a stable Nernstian potential at about +187.5 mV with only 0.2 mV drift within 30 min before the pDNA/MCH modification. Although the adsorption of thiolated molecules on Pd is a less-well-characterized system of self-assembled monolayers [28], the Pd-deposited ORE showed little change in terms of OCP value after modifying the thiolated pDNA/MCH layer. This result demonstrates that the Pd-deposited ORE in the Fe(CN)₆³⁻/⁴⁻-containing solution can serve as a competent pseudo-ReFe for EIS measurement.

Fig. 3(a and c), respectively, shows the cyclic voltammograms of Fe(CN)₆³⁻/⁴⁻ measured on the bare DE versus the Ag/AgCl ReFe and the Pd-deposited ORE ReFe followed by pDNA immobilization, MCH blocking, and dtDNA hybridization. A well-defined redox wave of Fe(CN)₆³⁻/⁴⁻ can be observed on the bare DE using both the Ag/AgCl and the Pd-deposited ORE ReFe. The peak-potential separation (∆Eₚ) versus the two kinds of ReFEs exceeded 60 mV, implying quasi-reversible behavior. Moreover, no matter which ReFe the CV measurement used, the current response exhibited a similar change after pDNA/MCH modification and dtDNA hybridization. After pDNA immobilization, the redox current decreased significantly and the redox current peaks became unobservable. This result is attributed to the electrosstatic repulsion [29] and the steric hindrance of the phosphate backbone of nucleotides to the negatively charged Fe(CN)₆³⁻/⁴⁻ mediators due to the surface blocking of the nonspecific adsorption of pDNA on the gold surface through the nitrogen-containing nucleotide bases [30]. Subsequently, the current measured on the pDNA/MCH-modified electrode was significantly larger than that of the pDNA-modified electrode. These results indicate that the modification of MCH can eliminate the nonspecific adsorption of pDNA due to its hydroxyl-terminated group [30]. After hybridization, the current of kinetic control on the dtDNA–pDNA/MCH-modified DE became smaller than that on the pDNA/MCH-modified DE due to the increase of structural hindrance and the stronger electrosstatic repulsion of the double strand DNA (dsDNA) to Fe(CN)₆³⁻/⁴⁻.

The behavior of electron transfer on the modified electrodes was further quantified by EIS. Fig. 3(b and d) shows the Nyquist plots, respectively, measured versus the Ag/AgCl ReFe and Pd-deposited ORE ReFe at the +0.19 V and 0 V potential added with a 5 mV amplitude sine wave. The curves (a) shown in Fig. 3(b and d) are the Nyquist plots obtained on the bare DE, which include a linear correlation of Z_R and Z_M at lower frequencies, indicating a diffusion-controlled reaction, and a semicircle region at higher frequencies, indicating a kinetic reaction of electron transfer. This EIS behavior can be explained by the Randles equivalent circuit [31].

After immobilizing pDNA, the diffusion-controlled part of the impedimetric spectrum disappeared entirely, and the diameter of semicircle increased drastically, implying the increase of the R_M. Following MCH blocking, the diameter of the semicircle decreased significantly due to the elimination of nonspecific adsorption of pDNA fragments. Afterward, the diameter of the semicircle of the dtDNA-hybridized electrode became larger than that of the pDNA/MCH-modified electrode. When the impedimetric spectrum only contains the kinetics-controlled part, the EIS behavior can be explained using the 1R/C model, consisting of one resistor (solution resistance (R_s)) in series with one parallel circuit comprising a resistor (R_M) and a capacitor (constant phase element (CPE)) [31]. The impedance of CPE can be expressed by Z_CPE(ω) = Z_0(ω)^n, where Z_0
Fig. 3. Cyclic voltammograms (a and c) and Nyquist plots (b and d) measured with an external Ag/AgCl reference electrode (a and b) and the Pd-deposited ORE (c and d) in 10 mM TES containing 5 mM equimolar Fe(CN)$_6^{3−/4+}$ on the same (1) cleaned bare DE, followed by (2) immobilization of 1 μM pDNA for 2 h, (3) blocking of 1 mM MCH for 1 h, and (4) hybridization of 1 nM dtDNA. The scanning rate is 20 mV/s.

is a constant, $j$ is the imaginary number, $\omega$ the angular frequency, and $0 < \alpha < 1$. When $\alpha$ is closer to 1, CPE becomes more capacitive.

Table 1 compares the $R_{ct}$, CPE and $\alpha$ values of the computer fitting results for the modified electrodes from the EIS measurement using the Ag/AgCl and the Pd-deposited ORE RefE. Most notably, after modification and hybridization, the fitting values of the $R_{ct}$ and CPE elements obtained against the Ag/AgCl RefE and the Pd-deposited ORE RefE presented a statistically insignificant difference with a $p$-value larger than 0.05 estimated by the Student’s $t$-test. These results demonstrate that the Pd-deposited ORE can be used as a pseudo-RefE like the Ag/AgCl electrode for the on-chip-type three-electrode EIS measurement.

The bare DE had the smallest $R_{ct}$ and the largest CPE values, respectively attributed to the lowest resistance of the electrode surface and the thinnest EDL. After modifying the pDNA, the electrostatic repulsion of pDNA layer caused the $R_{ct}$ value to increase significantly, and the adsorbed pDNA fragments caused the decrease of the CPE value due to the larger thickness and smaller permittivity of the pDNA layer relative to that of EDL of the bare DE. Furthermore, the pDNA/MCH-modified DE had smaller $R_{ct}$ and CPE values than those of the pDNA-modified DE, attributed to the pDNA desorption from the gold surface via the MCH modification. After 1 nM dtDNA hybridization, the $R_{ct}$ and CPE values, respectively, increased to 1.56 times and 1.30 times that of pDNA/MCH-modified DE. The results indicate that the $R_{ct}$ value has a larger response to the modifying and hybridizing processes than does the CPE value. Therefore, the $R_{ct}$ value was used to estimate the dtDNA–pDNA interaction on the three-electrode EIS-based DNA chip.

3.3. ACEO effect on hybridization

The convective flow produced by external pressure [32,33] or ACEO driving [7] has been demonstrated to increase the efficiency of DNA hybridization. Moreover, the hybridization efficiency depends on the velocity and range of fluidic flow. Fig. 4 shows the effect of bias-varied ACEO stirring on the hybridization efficiency estimated by the time-lapse $\Delta R_{ct}$, dtDNA (= $R_{ct}$, dtDNA – $R_{ct}$, pDNA/MCH) value. After performing biased ACEO stirring in 1 mM Tris solution containing 1 nM dtDNA for 30 s, the Tris solution was manually replaced by the 10 mM TES solution containing 5 mM equimolar Fe(CN)$_6^{3−/4+}$ to measure the $R_{ct}$, dtDNA. The procedure was repeated until the hybridization curve was completed. The hybridization for 1 nM dtDNA with +0.7 V-biased ACEO and ACEO stirring, respectively, took 150 s and 270 s to reach a plateau and obtained respective saturated $\Delta R_{ct}$, dtDNA responses of 168.6 ± 3.9 kΩ and 145.2 ± 1.6 kΩ. These phenomena indicate that the +0.7 V-biased ACEO stirring can produce shorter hybridization times and greater hybridization density than ACEO stirring due to the larger
The change in $R_\text{et}$ value as a function of time before and after the pDNA/MCH-modified DE performs the hybridization in (a) 1 nM dtDNA (circle)- or 1 nM smDNA (triangle)-containing and (b) blank 1 mM Tris (pH 9.3) solution with ACEO (dashed line) and +0.7 V (solid line)-biased ACEO stirring of 380 Hz and 3.0 $V_\text{pp}$. Each measurement was performed with at least three repetitions.

### Table 1

Values of the circuit elements were individually fitted using the Randles’ equivalent circuit for the bare electrodes and by the 1R/C model for pDNA-, pDNA/MCH-, and dtDNA-pDNA/MCH-modified electrodes, using the external Ag/AgCl electrode and Pd-deposited ORE (Pd/ORE) as the Re/E. The experimental parameters are the same as in Fig. 3 (b) and (d). The statistical values of the mean ± standard deviation were calculated from three repetitions.

<table>
<thead>
<tr>
<th>Electrodes</th>
<th>$R_\text{u}$ (kΩ)</th>
<th>CPE (nF)</th>
<th>$\sigma$ (×1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ag/AgCl</td>
<td>Pd/ORE</td>
<td>Ag/AgCl</td>
</tr>
<tr>
<td>Bare</td>
<td>6.2 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>91.6 ± 0.7</td>
</tr>
<tr>
<td>pDNA</td>
<td>324.5 ± 1.6</td>
<td>323.8 ± 0.6</td>
<td>65.9 ± 0.7</td>
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<tr>
<td>pDNA/MCH</td>
<td>214.1 ± 0.8</td>
<td>213.6 ± 0.5</td>
<td>27.5 ± 0.3</td>
</tr>
<tr>
<td>dtDNA-pDNA/MCH</td>
<td>333.7 ± 0.7</td>
<td>333.8 ± 0.3</td>
<td>36.6 ± 0.4</td>
</tr>
</tbody>
</table>

**Fig. 4.** The change in $R_\text{u}$ value as a function of time before and after the pDNA/MCH-modified DE performs the hybridization in (a) 1 nM dtDNA (circle)- or 1 nM smDNA (triangle)-containing and (b) blank 1 mM Tris (pH 9.3) solution with ACEO (dashed line) and +0.7 V (solid line)-biased ACEO stirring of 380 Hz and 3.0 $V_\text{pp}$. Each measurement was performed with at least three repetitions.

velocity and wider range of fluidic flow. Furthermore, the saturated $\Delta R_\text{et-dtDNA}$ values obtained in this study was significantly larger than that (21.4 kΩ) obtained by using the single ring-disc electrodes (RE/DE) with ACEO stirring to hybridize the same dtDNA concentration in our previous paper [7], although both of these studies adopted the same 200 µm diameter sensing area. The result implies that the ORE/IRE design of a wider flow range can increase hybridization density so as to obtain the larger $\Delta R_\text{et-dtDNA}$ response. In contrast, the hybridization curve of 1 nM smDNA with the +0.7 V-biased ACEO stirring shows time-independent $\Delta R_\text{et-dtDNA}$ values. After applying +0.7 V-biased ACEO stirring for 150 s, the $\Delta R_\text{et-dtDNA}$ ratio of smDNA to dtDNA was about 1.4%, implying that the EIS-based DNA sensor has excellent selectivity. This result is attributed to the shear force of +0.7 V-biased ACEO flow exceeding the binding strength between pDNA and smDNA due to the reduced hydrogen bonds and the larger bulge area of the smDNA. Future work could explore the shear force needed to determine mismatched hybridization to promote the selectivity of DNA sensors.

**Fig. 4(b) shows the effect of bias-varied ACEO velocity on the stability of pDNA/MCH-modified DE within a 300 s interval in blank Tris solution estimated by the change in $R_\text{u}$ ($\Delta R_\text{u}$) value. The averaged $\Delta R_\text{u}$ values with the ACEO and +0.7 V-biased ACEO driving were, respectively, 0.5 ± 0.1 kΩ and 1.1 ± 0.1 kΩ. The result indicates that the larger ACEO velocity may change the structure of the pDNA-modified layer to cause a greater $\Delta R_\text{u}$. Furthermore, these $\Delta R_\text{u}$ values were smaller than those obtained at the EIS/ACEO chip of mutual WE with ACEO stirring of 1.5–3.0 $V_\text{pp}$ [7], attributed to the lack of an ACEO electric field to affect the pDNA/MCH-modified layer. The smaller background $\Delta R_\text{u}$ values allow the EIS-based DNA sensors to exhibit better stability.

The 90% response times (RT$_{90\%}$) of the saturated hybridization of 1 nM dtDNA with the ACEO and +0.7 V-biased ACEO driving were, respectively, 231 s and 141 s (Fig. 4(a)). Although the RT$_{90\%}$ with +0.7 V-biased ACEO is longer than that (119 s) of our former RE/DE design [7], increasing the AC driving voltage will reduce the RT$_{90\%}$ because the ACEO velocity is proportional to the square of AC voltage [34]. As compared to the RE/DE design [7], the ORE/IRE/DE chip allows the application of a larger AC voltage to produce a stronger biased ACEO velocity and has less influence on the stability of pDNA/MCH-modified layer. Experimentally, ACEO stirring for 270 s and the +0.7 V-biased ACEO stirring for 150 s were used on the pDNA/MCH-modified electrodes for each concentration-varied dtDNA sample to explore the effect of ACEO flow on the sensing characteristics.

### 3.4. Sensing characteristics

Following the fitting of the 1R/C model, Fig. 5 plots the $\Delta R_\text{et-dtDNA}$ values versus the sequential hybridization of the 1 nM–10 pM dtDNA samples. To further define the linearity over the 1 nM–10 pM concentration, linear regression analysis of $\Delta R_\text{et-dtDNA}$ values against the dtDNA concentration with ACEO and +0.7 V-biased ACEO stirring was, respectively, expressed as $\Delta R_\text{et-dtDNA}$ (kΩ) = 19.34 log(dtDNA)+ 354.91 with $R^2$ = 0.9945 and $\Delta R_\text{et-dtDNA}$ (kΩ) = 22.73 log(dtDNA)+ 418.44 with $R^2$ = 0.9979. Both present high correlation coefficients, implying a good linearity between the $\Delta R_\text{et-dtDNA}$ values and the dtDNA concentration. Moreover, in the linear range the maximal relative standard deviation of $\Delta R_\text{et-dtDNA}$ response with +0.7 V-biased ACEO stirring did not exceed 4%, meaning that the EIS-based biosensor presents good repeatability. The sensitivity of the biosensor with +0.7 V-biased ACEO stirring was larger than that with ACEO stirring, attributed to the wider range of the biased ACEO flow. Moreover, the sensitivity values in this study
were much larger than that (2.46 kΩ/log[dtDNA]) in our previous RE/DE chip [7] when measuring the same dtDNA concentration with the WE of the same sensing area. This indicates that the design of the ORE/IRE/DE array can obtain better hybridization with TDNA under biased ACEO stirring.

After incubation with 1 µM dtDNA with ACEO and +0.7 V-biased ACEO stirring, the mean ΔRΔt-dtDNA value was, respectively, 3.3 kΩ and 11.4 kΩ. The background noise of the ΔRΔt of pDNA/MCH-modified DE before dtDNA hybridization with ACEO and +0.7 V-biased ACEO stirring was, respectively, 0.5 kΩ and 1.1 kΩ (Fig. 4(b)). Therefore, the corresponding calculated LOD was, respectively, 0.5 µM and 0.5 µM, based on the IUPAC recommendation of the signal response being greater than three times the background noise. In contrast to the previous label-free impedimetric DNA sensors for hybridization of tens of base tDNAs in unstirred conditions [11,13,14] and with the assistance of pressure-driven [32,33] and ACEO-stirred [7] flows, the LOD of 0.5 µM obtained in this study was much lower than those found in previous studies.

4. Conclusions

This study develops a label-free three-electrode EIS-based DNA sensing chip integrated with biased ACEO stirring using a concentric double ring-singl disk array. The ACEO driving voltage was only applied on the ORE and IRE to eliminate the effect of the electric field on the stability of the pDNA/MCH-modified DE. The +0.7 V-biased ACEO flow of 3.0 Vpp and 380 Hz can effectively move the fluid from OR to DE at velocity 4.5 times that of ACEO flow. Moreover, the Pd-deposited ORE can act as a stable pseudo-RefE to construct the on-chip-type three-electrode EIS system. The +0.7 V-biased ACEO-stirred sensing chips presented a larger ΔRΔt-dtDNA response and a shorter RT90% (141 s) for dtDNA hybridization than the ACEO-stirred chips. The calculated LOD of the biosensor can be as low as 0.5 µM and exhibited good linearity and repeatability over the 1 µM–10 µM concentration range. The EIS-based DNA biosensors with +0.7 V-biased ACEO stirring can perform rapid saturated hybridization along with ultrasensitive detections to specifically measure TDNA concentrations and have a promising potential to construct a miniature DNA diagnostic platform.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.11.078.

References


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