An impedimetric bioaffinity sensing chip integrated with the long-range DC-biased AC electrokinetic centripetal vortex produced in a high conductivity solution

Ming-Jie Lin,¹ Yen-Fu Liu,¹ and Ching-Chou Wu^{1,2,a)}

¹Department of Bio-industrial Mechatronics Engineering, National Chung Hsing University, No. 145, Xingda Rd., South Dist., Taichung City 402, Taiwan

²Innovation and Development Center of Sustainable Agriculture, National Chung Hsing University, No. 145, Xingda Rd., South Dist., Taichung City 402, Taiwan

(Received 15 May 2018; accepted 25 June 2018; published online 6 July 2018)

Immunoreaction of specific antibodies to antigens is widely used in numerous immunoanalysis applications. However, diffusion-dominated transport in stationary solutions limits the rate and binding density of immunoreaction. This research describes the construction of chip-type concentric multi-double ring electrodes and single central disk electrode. A +1 V-biased 6 V_{pp} voltage was applied to the multi-double ring electrodes to induce a long-range DC-biased AC electrokinetic flow (ACEKF). The immunoreaction was quantified by electrochemical impedance spectrum (EIS). Fluorescence-labeled secondary antibody (FLSA) and protein A were exemplified as an immunoreacting model to demonstrate the effect of ACEKF on immunoreaction efficiency. The results showed that FLSA binding can reach a plateau in 8 min with the DC-biased ACEKF vortex, and the increment of electron transfer resistance is 2.26 times larger than that obtained in the unstirred solution. The sensitivity of the calibration curves obtained by EIS detection with the aid of DC-biased ACEKF vortex is 1.51 times larger than that obtained in an unstirred solution. The label-free EIS-based sensing chip integrated with the long-range DC-biased ACEKF vortex promises to facilitate immunoreaction efficiency, which is beneficial for the development of a miniature and fast-detection *in vitro* diagnostic device. Published by AIP Publishing. <https://doi.org/10.1063/1.5040231>

I. INTRODUCTION

Immunoassay relies on the use of specific antibodies to detect antigenic targets for various applications,^{1,2} including clinical diagnostics,³ food safety,⁴ and the detection of environmental pollutants.⁵ Label-free techniques, such as surface plasmon resonance,⁶ quartz crystal microbalance,⁷ mass-sensitive microcantilever,⁸ and electrochemical impedance spectroscopy (EIS)-based immunosensors,^{9–11} have attracted increasing attention due to the reduced number of immunoassay steps, improved time efficiency, and reduced reagent cost. In particular, the EIS-based immunosensor presents high sensitivity, high cost-effectiveness, and ease of mass production and miniaturization.^{10,12,13}

In general, immunoreactions require tens of minutes to reach the hybridization plateau in a stationary solution,¹⁴ thus limiting its applicability for *in vitro* diagnostics. Moreover, the lower the analyte concentration in a stationary solution, the longer the immunosensor's saturated immunoreaction due to the lower diffusive flux from bulk solution to the electrode surface. Several studies have demonstrated the use of microfluidics to promote the sensing properties of immunosensors.^{13,15–18} Compared with pressure-driven microfluidics, electrokinetics-controlled techniques have more promising potential for use in portable and miniaturized devices. Yan *et al.* used a

^{a)} Author to whom correspondence should be addressed: ccwu@dragon.nchu.edu.tw, Tel.: +886-4-2285-1268, Fax: +886-4-2287-9351.

high direct current (DC) field to produce an electrophoretic force to reduce the immunoreaction incubation time to 2 min.¹⁹ Furthermore, as types of AC electrokinetics (ACEK), the alternating current electrothermal flow (ACETF), and AC electroosmotic flow (ACEOF), both present effective strategies to transport suspended antigens to the antibody (Ab)-modified electrodes via ACEK flow (ACEKF).^{13,17}

Although the ACEOF can achieve a velocity exceeding $100\text{ }\mu\text{m/s}$ at a lower voltage,^{20,21} the ACEOF is insignificant in high conductivity solutions. In contrast, ACETF is an effective method for producing fluidic flow in high conductivity biofluids, such as blood ($4\text{--}8\text{ mS/cm}$) or urine ($11\text{--}25\text{ mS/cm}$),²² due to the gradient of conductivity or dielectric coefficient induced by Joule heat.^{18,23} Furthermore, a Faradaic reaction induced by a DC bias can easily produce a conductivity gradient via the production of protons or hydroxide ions on the electrode surface. Therefore, a DC bias is imposed on an AC field to control the velocity and direction of fluidic flow, called DC-biased ACEKF.²⁴ Ng *et al.* demonstrated that the velocity of DC-biased ACEKF performed in a 0.1 mM KCl solution with the low conductivity of 0.01 mS/cm can reach $700\text{ }\mu\text{m/s}$ by applying the $+2\text{ V}$ -biased 20 V_{pp} voltage of 500 kHz working frequency.²⁴ Generally, the lower the solution conductivity is, the less the electrolysis degree of solution is, which is benefit for the application of large DC bias and AC voltage to produce fast ACEKF. However, lowering the conductivity of biofluids implies that the high dilution procedure is necessary. Consequently, the concentration of analyte is simultaneously reduced during the dilution process. It is adverse to the analyte detection. Presently, few studies explore the effect of high conductivity solution on the properties of DC-biased ACEKF. Furthermore, the electrode pattern determines the distribution of electric field, affecting the magnitude, direction, and range of ACEKF. Several types of electrode patterns have been designed for ACETF and DC-biased ACEKF, such as asymmetric planar interdigitated electrode pairs,²⁵ three-dimension electrode array,²⁶ and single concentric ring-disk electrodes (DEs),²⁷ to produce unidirectional flow or centripetal flow. However, few studies have attempted to integrate DC-biased ACEK vortex and EIS-based immunosensors on the same substrate as an *in vitro* diagnostic device.

This study presents the design of concentric multi-double ring electrodes and disk electrode (DE) and produces a long-range DC-biased ACEK centripetal vortex in a high conductivity solution, driving the antigen to the Ab-immobilized central DE, improving immunoreaction efficiency. Moreover, the double ring electrodes can be used as a reference electrode and counter electrode, when the DE is used as the working electrode during EIS measurement. The recombinant protein A (PA) and the immunoglobulin G (IgG) are exemplified as a model of immunoreaction due to their high affinity between PA and the Fc region of IgG.²⁸ The effect of DC bias-varied ACEKF on the velocity of centripetal flow and the sensing properties of the EIS-based immunosensors is estimated in detail.

II. MATERIALS AND METHODS

A. Reagents

Recombinant PA was purchased from BioVision (Cat. 6500B). Highly specific fluorescence-labeled secondary antibody (FLSA), a type of goat polyclonal anti-mouse Ab, and bovine serum albumin (BSA) were obtained from Sigma (SAB4600387). Potassium hexacyanoferrate(III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$), potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), and sodium chloride (NaCl) were purchased from Showa. Sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic dehydrate (NaH_2PO_4), 3-mercaptopropionic acid (MPA), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES), potassium chloride (KCl), and fluorescence-labeled carboxylate-modified latex beads (with a mean diameter of $1.0\text{ }\mu\text{m}$, an excitation wavelength of 470 nm , and an emission wavelength of 505 nm) were purchased from Sigma-Aldrich. The 10 mM MPA solution was prepared in double distilled water. All chemicals were of reagent grade and were used without further purification. All solutions were prepared with water purified through a Milli-Q system.

B. Electrode fabrication

The chip-type electrodes consist of seven pairs of concentric double ring electrodes and one central DE, patterned on a cleaned glass substrate by lift-off microfabrication and sputtered with 20 nm Ti and 200 nm Au films. Figure 1 shows the scheme of electrode layout. The width of all outer ring electrodes (OREs) and inner ring electrodes (IREs) was, respectively, $40\ \mu\text{m}$ and $120\ \mu\text{m}$. The diameter of the central DE was $200\ \mu\text{m}$. The interelectrode gap (G) between the ORE and IRE was $40\ \mu\text{m}$, and the distance (D) between the two pairs of double-ring electrodes was $200\ \mu\text{m}$. The width ratio of IRE to ORE and D to G is, respectively, 3 and 5, which refers to the suggestion of Hong's study, to obtain the larger average velocity of ACEKF.²⁵ The interelectrode gap between the first pair IRE, called IRE_{1st}, and DE was $40\ \mu\text{m}$. The distance from the outermost ORE to the center of the DE was $2700\ \mu\text{m}$. A $7\text{-}\mu\text{m}$ -thick SU8-3010 (MicroChem, Newton, MA) layer was used to define the working area of all electrodes. In addition, all IREs and OREs were electrodeposited by Pt particles to hinder MPA absorption on the electrode surface and thus helps to stabilize the surface potential of the electrode during the control of DC-biased vortex and the EIS measurement.²¹ Pt particles were nucleated using the sweeping potential from $+0.8\ \text{V}$ to $0.0\ \text{V}$ at a scan rate of $50\ \text{mV/s}$ for 9 cycles in the $0.1\ \text{M}$ H_2SO_4 solution containing $1\ \text{mM}$ K_2PtCl_6 . The Pt film was then formed by applying $+0.43\ \text{V}$ potential for 200 s.

C. Production and quantification of ACEK vortex

The method used to quantify the ACEK velocity is described in the previous study.²¹ Fluorescence-labeled latex beads of 1×10^6 particles/ml concentration suspended in $1\ \text{mM}$ phosphate buffer solution (PBS, pH 7.4) containing $14\ \text{mM}$ NaCl (PBS(NaCl)) with a high conductivity of $2.06\ \text{mS/cm}$, simulating the conductivity of diluted serum samples, were used to measure the velocities of DC-biased ACEKF based on the particle trajectories from a set of continuous images. A function generator (33220A, Agilent, Santa Clara, CA) was connected to a high voltage power amplifier (HA805, Pintek, Taiwan), with two output terminals linked to all OREs and IREs to drive DC-biased ACEKF. Moreover, the DC bias was imposed on all OREs versus all IREs used as a ground electrode. The center DE was not applied with voltage during the performance of DC-biased ACEK vortex. Simultaneously, the chip was placed on an upright fluorescent microscope stage (BA310LED, Motic, Hong Kong) equipped with a CCD camera (600D, Canon, Taiwan) to obtain time-lapse fluorescent images of particles. The particle trajectories were analyzed as markers to quantify the ACEKF velocities from observations taken at an interval of $0.25\ \text{s}$.²¹

FIG. 1. (a) Concentric multi-double ring electrodes and one central DE and (b) the dimensions of central DE, IRE, and ORE.

D. Immunosensor preparation and measurement

A polydimethylsiloxane slab was drilled to form a circular hole with the diameter of 5.8 mm. The center of hole was aligned to the central DE and the hole-containing slab was fixed on the sensing chip to form a container by using a home-made clamp. 10 μ l aliquot of 10 mM MPA was dripped on the cleaned chip-type electrodes at 30 °C for 1 h to form a self-assembled monolayer, which was used as a link for the covalent immobilization of PA. The MPA-modified electrodes were soaked overnight in 20 mM MES solution (pH 4.6) containing 30 mM EDC and 2 mM NHS to activate the carboxyl group of MPA. Subsequently, 10 μ l aliquot of 0.2 mg/ml PA prepared in 10 mM PBS was placed on the EDC/NHS-activated MPA-modified electrodes for 1 h at 30 °C and then blocked with 1% BSA. The modified electrodes were alternately dipped in PBS containing 0.05% Tween 20 and PBS to remove the unbound PA and BSA. The PA-modified electrodes can be reacted with FLSA in the PBS(NaCl) solution under DC-biased ACEK stirring. 30 μ l aliquot of FLSA-containing solution was dripped in the container for the immunoreaction. Optical images of the FLSA-immunoreacted chips were analyzed using Image J software to obtain the fluorescent intensity of FLSA.

The 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ was added in the 10 mM PBS with 100 mM NaCl as a mediator to evaluate the electrochemical characteristics of the electrode/solution interface in each modification step and to quantify the immunoreaction. Cyclic voltammetry in a range of -0.2 V to $+0.2$ V was performed with a scanning rate of 50 mV/s. EIS measurement of DE was performed in a frequency range of 1 Hz to 100 kHz at $+0$ V potential added with a 5 mV amplitude sine wave versus the reference electrode of Pt-deposited $\text{ORE}_{1\text{st}}$ and the counter electrode of Pt-deposited $\text{IRE}_{1\text{st}}$. Measurement of the impedance spectra and simulation of equivalent circuits were performed using the IM-6/THALES software package.

III. RESULTS AND DISCUSSION

A. ACEK velocity

Theoretically, when the frequency of driving ACET is lower than the relaxation frequency (f_{re}) of the bulk electrolyte, the fluid can be driven from a high conductivity area to a low conductivity area due to the application of Coulomb force.²⁴ The strength of the Coulomb force is about 10 times larger than that of dielectric force produced at frequencies higher than f_{re} .²⁹ In this study, the f_{re} , calculated from $\sigma/2\pi\epsilon$, where σ and ϵ are, respectively, the conductivity (2.06 mS/cm) and permittivity (7.08×10^{-10} F/m) of the PBS(NaCl) solution, is 46.2 MHz. Therefore, a frequency of 100 kHz was chosen as the working frequency for the asymmetric ORE/IRE electrodes. Moreover, varied DC bias was applied to all OREs and superimposed on the AC voltage for the DC-biased ACEKF. The trajectories of particles driven by 6 V_{pp} and $+1$ V-biased 6 V_{pp} were observed on the electrode surface and are, respectively, shown in movie s1 and movie s2. The two movies show that the ACEKF of 6 V_{pp} and $+1$ V-biased 6 V_{pp} can produce a long-range centripetal flow to drive most beads across the ORE/IRE gap and the interval between two neighbor ORE/IRE pairs. Furthermore, the bead motion was observed not only on the electrode surface but also at a plane 40 μ m above the electrode surface. After calculating bead velocity as a function of position from the outer edge of $\text{ORE}_{2\text{nd}}$, the corresponding velocities of beads are shown in Fig. 2. The velocities increased with the amplitude of AC voltage due to the increasing strength of the electric field. Moreover, the bead velocity obtained in the 40 μ m high plane and driven by a DC-biased ACEKF was significantly faster than that driven by the ACETF at the same AC voltage. This phenomenon is attributed to the fact that the DC-biased AC voltage produces a conductivity gradient greater than the same AC voltage on an electrode surface due to Faradaic reactions.²⁴ However, when the bias exceeded $+2.0$ V, the water electrolysis immediately occurred to form bubbles at all OREs, thus hindering the flow. The results of Fig. 2 also show that the bead velocity in the 40 μ m high plane driven by the ACETF is faster than that on the electrode surface. Similarly, Ng *et al.* observed that the fastest flow occurred in a plane at 40% of the total microchannel height when applying DC-biased ACEKF at two symmetric electrodes.²⁴ Similar results were also found by Hong *et al.*,

FIG. 2. Bead velocity as a function of positions calculated from the outer edge of ORE_{2nd} with the different DC-biased ACEKF, observed in the 40 μm high plane above the chip surface (a) and on the chip surface (b) ($n = 3$).

using COMSOL software to simulate the velocity contours of the ACET pump driven at two asymmetric electrodes with the 5 μm interelectrode gap in a closed microchannel.²⁵ The fastest flow velocity occurred in the 5 μm high plane, with a flow direction from the narrow electrode to the wide electrode. Furthermore, it is worth noting that the bead velocity on the chip surface induced by +1 V-biased 6 V_{pp} was faster than that induced by 8 V_{pp} [Fig. 2(b)]. This is attributed to the conductivity gradient produced by the +1 V-biased 6 V_{pp} being larger than that produced by the 8 V_{pp} .

B. ACEK effect on affinity efficiency

The effect of ACEKF on affinity efficiency was estimated by fluorescence and EIS methods. Figure S1(a) (supplementary material) shows a fluorescent image of the bare chip surface. The electrode area is black, indicating that there is no native fluorescence on the electrode surface. In a binary image produced using Image J software, the electrode area and non-electrode area are, respectively, presented in gray and white [Fig. S1(b), supplementary material]. Figure S2 (see in supplementary material) shows the Image J-processed images of PA-modified immunosensors after immunoreaction with 10 $\mu\text{g/ml}$ FLSA without and with ACETF (4 and 6 V_{pp}) vortex for 10 min. The red color indicates the FLSA-immunoreacted positions. The surface of the IRE_{1st} and ORE_{1st} had the FLSA binding position and increased with the AC voltage of ACETF. Figure 3 shows the time-lapse fluorescent intensity of FLSA counted in the DE area by Image J software with and without the voltage-varied ACEK vortex. The inset of Fig. 3 shows that the FLSA/PA immunoreaction takes about 90 min to plateau in a stationary solution. Reaching 95% saturated immunoreaction, respectively, took about 8, 10, and 12 min for +1V-biased 6 V_{pp} , 6 V_{pp} , and 4 V_{pp} , suggesting that the immunoreaction time is negatively correlated to the ACEKF velocity, referring to the results of Fig. 2. The faster flow velocity can allow the immunoreaction to plateau faster. Moreover, the plateau intensity obtained by the +1 V-biased 6 V_{pp} ACEKF was, respectively, 1.09, 1.58, and 3.17 times larger than that obtained by 6 V_{pp} , 4 V_{pp} ACETF and without ACEKF. The results indicate that the convective transport of DC-biased ACEKF can significantly improve the binding efficiency. Moreover, the stronger fluorescent intensity on the DE surface implies that more FLSAs were brought to the DE surface due to the wider flow range.

C. Effect of Pt electrodeposition

As shown in Figs. S1(c) and S2 (supplementary material), many FLSAs were adsorbed on the PA-modified OREs and IREs. This behavior may reduce the magnitude of FLSA binding to

FIG. 3. FLSA intensity on the surface of the PA-modified DE as a function of different voltage of ACEK for 10 $\mu\text{g/ml}$ FLSA ($n = 3$). The IREs and OREs have no Pt film electrodeposition film.

the PA-modified DE. To avoid FLSA absorption on the OREs and IREs, all OREs and IREs were electrodeposited by Pt particles to prevent MPA modification from the surface of OREs and IREs. After EDC/NHS activation, little PA was immobilized on the Pt-deposited OREs and IREs. Figure S1(d) (supplementary material) shows the J-processed image after performing the +1 V-biased 6 V_{pp} ACEK vortex for 12 min. The red area of the bound FLSA on the DE is more significant than that on the IRE_{1st}. The calculated fluorescence intensity on the Pt-deposited IRE_{1st} was 0.58 times smaller than that on the Au IRE_{1st} [Fig. S1(c), supplementary material]. Moreover, the fluorescence intensity obtained on the DE surface of the Pt-deposited chip was 1.2 times larger than that on the Pt-unmodified chip. These results suggest that the Pt deposition can prevent the FLSA binding on the surface of OREs and IREs due to the low PA immobilization, thus increasing the magnitude of FLSA binding on the DE via the ACEK vortex.

D. EIS-based sensor

Most of the previous studies used an external reference electrode and a counter electrode for EIS-based immunosensors which is not conducive to the development of portable and miniaturized immunosensors.^{10,12,13} In this study, the Pt-deposited ORE_{1st} and IRE_{1st} electrodes were used not only to drive the ACEK vortex but also, respectively, as the reference electrode and the counter electrode for EIS measurement. Wu's study showed that the Pd-deposited electrode provides a stable equilibrium potential of $\text{Fe}(\text{CN})_6^{3-/4-}$ for EIS measurement following the modification of thiolated molecules.²¹ The electron transfer behavior on the modified electrodes was evaluated by cyclic voltammetry and EIS. Figure 4(a) shows the cyclic voltammograms measured at the cleaned DE following surface modification and FLSA affinity binding. A well-defined redox wave of $\text{Fe}(\text{CN})_6^{3-/4-}$ can be observed on the cleaned DE using the Pt-deposited ORE_{1st} as a reference electrode. The peak-potential separation (ΔE_p) was about 88 mV, implying a quasi-reversible behavior. Moreover, the equilibrium potential ($= (E_{pa} + E_{pc})/2$) of the redox peak was calculated at about 0 mV, suggesting that the bare DE and the Pt-deposited ORE_{1st} have almost the same surface potential in the $\text{Fe}(\text{CN})_6^{3-/4-}$ containing PBS. Following MPA modification, the redox peak current was slightly smaller than that of the cleaned DE due to the electrostatic repulsion between the carboxylic acid of MPA and the negatively charged $\text{Fe}(\text{CN})_6^{3-/4-}$.⁹ Following PA immobilization and FLSA binding, the peak current decreased and the ΔE_p increased, due to the steric hindrance of PA and FLSA binding.

Figure 4(b) shows the corresponding Nyquist plots. The curve (i) of Fig. 4(b) obtained on the clean DE exhibits a dominant linear region at lower frequencies, implying the redox

FIG. 4. (a) Cyclic voltammograms and (b) Nyquist plots obtained in a 10 mM PBS solution containing 100 mM KCl and 2.5 mM equimolar $\text{Fe}(\text{CN})_6^{3-/4-}$ at the bare Au (i) followed by modification of 10 mM MPA for 60 min (ii), immobilization of 0.2 mg/ml PA for 60 min (iii) and affinity of 10 $\mu\text{g}/\text{ml}$ FLSA for 100 min in the stationary solution (iv).

reaction of the $\text{Fe}(\text{CN})_6^{3-/4-}$ mediator as a diffusion-controlled behavior, and a small semicircular region at higher frequencies, implying a fast kinetic rate of electron transfer.¹³ Furthermore, the radius of the semicircle increased with the progression of MPA modification, PA immobilization, and the FLSA affinity. These results indicate that the impedance of the solution/electrode interface increased with the modification steps. These Nyquist plots can be fitted using the Randle's equivalent circuit to explain EIS behavior.¹³ The Randle's equivalent circuit includes the resistance (R_s) of the electrolyte solution, the Warburg impedance (Z_w) resulting from the analyte diffusion, the electric double layer (EDL) (C_{dl}), and the electron transfer resistance (R_{et}).^{10,12} The constant phase element (CPE) is used instead of C_{dl} to represent the non-ideal capacitive response of the electrode surface.¹² The CPE component is presented by $Z_{CPE}(\Omega) = Z_0(j\Omega)^{-\alpha}$, where Z_0 is a constant, j is an imaginary number, Ω is the angular frequency, and α is between 0 and 1. When α is closer to 1, CPE becomes more capacitive. Table I shows the R_{et} , CPE , and α values of the fitting results for the modification steps using IM-6/THALES software. The bare DE had the smallest R_{et} and the largest CPE values, respectively, attributed to the fastest electron transfer rate and the thinnest electric double layer (EDL). After the MPA modification, the R_{et} value increased due to the carboxylic acid of the MPA layer, and the CPE value decreased due to the MPA monolayer being thicker than the EDL. Afterwards, the R_{et} value significantly increased with PA immobilization and the FLSA binding, suggesting that the R_{et} value can be used as the indicator to probe the immunoreaction.

E. Effect of ACEKF on sensing properties

In principle, the convective flow can promote the immunoreacting efficiency.^{13,30} Figure 5 shows the effect of ACEK stirring on immunoreaction efficiency. The time-lapse

TABLE I. Values of the circuit elements fitted using the Randle's equivalent circuit for the bare electrodes followed by MPA modification, PA immobilization, and FLSA attachment. The experimental parameters are the same as in Fig. 4(b).

Electrode	R_{et} (k Ω)	CPE (μF)	α ($\times 1000$)
Bare	63.53 ± 3.82	9.89 ± 0.24	839.8 ± 1.7
MPA	117.67 ± 9.61	7.92 ± 0.43	860.5 ± 3.7
PA/MPA	143.50 ± 0.71	4.74 ± 0.08	898.7 ± 4.6
FLSA/PA/MPA	183.83 ± 12.82	4.97 ± 0.05	894.3 ± 2.1

FIG. 5. The ΔR_{et} value as a function of immunoreaction time measured at the PA-modified DE with +1 V-biased 6 V_{pp} ACEK vortex of 100 kHz for 10 $\mu\text{g/ml}$ FLSA ($n = 3$). The inset shows the time-lapse immunoreaction in an unstirred solution ($n = 3$).

ΔR_{et} ($=R_{et-FLSA}-R_{et-PA}$) values were obtained at the PA-modified DE after performing the +1 V-biased 6 V_{pp} ACEK vortex or dipping in the unstirred PBS (NaCl) solution containing 10 $\mu\text{g/ml}$ FLSA. The results show that the ACEK vortex causes the immunoreaction curve to plateau in 8 min with the ΔR_{et} value of 87.0 ± 1.18 k Ω . The calculated 90% response time was 7.3 min. In contrast, the immunoreaction in the unstirred solution takes at least 60 min to reach saturation with a ΔR_{et} value of 38.53 ± 0.37 k Ω . The calculated 90% response time was 55 min. This indicates that the +1 V-biased 6 V_{pp} ACEK vortex can reduce the time needed to achieve a saturated immunoreaction to 0.13 times of that with the unstirred immunoreaction and increases the magnitude of saturated FLSA binding to 2.26 times ΔR_{et} value of that obtained in the unstirred solution. The result indicates that the long-range centripetal ACEK vortex moves more FLSA to the central DE in a shorter time.

Figure 6 shows the calibration curve of the chip-type immunosensors with +1 V-biased 6 V_{pp} ACEK vortex for 8 min for each concentration-varied FLSA sample. The linear regression

FIG. 6. Calibration curve of the PA-modified DE for the concentration-varied FLSA, respectively, obtained in an unstirred solution for 60 min immunoreaction (triangle) and with +1 V-biased 6.0 V_{pp} ACEK vortex for 8 min immunoreaction (square) ($n = 3$).

equation of $\Delta R_{et} \text{ (k}\Omega\text{)} = 13.89 \log [\text{FLSA}] + 22.90$ with a correlation coefficient (R) of 0.996 in the range of 1 ng/ml to 10 $\mu\text{g/ml}$, implying good linearity. The R_{et} value of the PA-modified DE was $143.50 \pm 0.71 \text{ k}\Omega$, implying that the background noise was 0.71 k Ω . The calculated limit of detection (LOD) was 32.0 pg/ml, based on the IUPAC recommendation ($S/N > 3$).²¹ In contrast, the calibration curve obtained in the unstirred solution for 60 min for each FLSA concentration shows a linear regression equation of $\Delta R_{et} \text{ (k}\Omega\text{)} = 9.19 \log [\text{FLSA}] + 2.68$ with $R = 0.994$, suggesting good linearity. The calculated LOD was 871.3 pg/ml. The sensitivity of the immunosensors with +1 V-biased 6 V_{pp} ACEK vortex was 1.51 times larger than that obtained in an unstirred solution, implying that the +1 V-biased 6 V_{pp} ACEK vortex can significantly increase the magnitude of FLSA binding and reduce the LOD. The calibration curves were obtained from three individual chip-type immunosensors. The relative standard deviation for the different FLSA concentrations obtained with the DC-biased ACEK vortex and the unstirred immunoreaction was, respectively, smaller than 4.51% and 4.11%, indicating that the chip-type immunosensors have good reproducibility.

Table II shows the LOD of different immunosensors for the detection of protein analytes with or without the ACEKF. The LOD obtained by the EIS-based detection with the long-range DC-biased ACEK centripetal vortex is obviously lower than that obtained in an unstirring solution.^{31–33} Moreover, the saturated immunoreactive time, 8 min, of this study is shorter than that of several tens of minutes required in an unstirring solution.^{31–33} Although the velocity of ACEOF is faster than that of DC-biased ACEKF, the conductivity of solution needed for the ACEOF is lower than 150 $\mu\text{S/cm}$,^{13,34–37} implying that the high dilution procedure is required for high conductivity biofluids. Furthermore, it is worth noting that the pattern of concentric multi-double ring electrodes and single DE has not been developed for driving ACEKF in the previous studies. The electrode pattern could affect the LOD. Li *et al.* used interdigitated electrodes to produce ACETF and dielectrophoresis (DEP) in the 15 mM NaCl-containing 1 mM PBS of high conductivity;³⁰ the LOD obtained by the capacitive detection is much higher than that obtained in this study. The low LOD of this study attributed to the electrode design can produce the long-range centripetal vortex, transporting more FLSA from bulk solution to the PA-modified DE.

TABLE II. Comparison of LOD obtained by different immunosensors in unstirring solutions and with ACEKF stirring for the detection of proteins.

Electrokinetics/ detection method	Electrode geometry	Affinity reaction	LOD (pg/ml)	References
-/EIS	Pt electrode	MBP/anti-MBP ^a	150	31
-/EIS	ITO electrode	Cry1Ab/anti-Cry1Ab ^b	370	32
-/EIS	SPCE electrode	Mb/anti-Mb ^c	80	33
ACEO + DEP ^d /EIS	Single ring-disk Au electrode	Ab/PA	200	13
ACEO + DEP/optical	Spiral Au electrode	Bacterial collection	...	34
ACEO + DEP/QCM	Interdigitated Au electrodes	FLSA/Ab	...	35
ACEO/optical	Microtip asymmetric Au microelectrode pairs	HER2/anti-HER2 ^e	0.001	36
ACET + DEP / capacitance	Interdigitated Au electrode arrays	IgG/Ab	10 000	30
DC-biased ACEK/EIS	Concentric multi-double ring electrodes and single Au DE	FLSA/PA	32	This study

^aMBP: Myelin basic protein.

^bCry1Ab: Pesticidal crystal protein.

^cMb: Myoglobin.

^dDEP: Dielectrophoresis.

^eHER2: Epidermal growth factor receptor 2.

IV. CONCLUSIONS

This study constructs chip-type concentric multi-double ring electrodes and one central DE to produce a long-range DC-biased ACEK centripetal vortex to improve immunoreaction efficiency. Moreover, the immunoreaction magnitude can be quantified by *in situ* EIS-based detection using Pt-deposited IRE_{1st} and ORE_{1st}. The +1 V-biased 6 V_{pp} ACEK vortex can effectively reduce the saturation immunoreaction time to 8 min, as opposed to 60 min for the unstirred immunoreaction. Moreover, the ΔR_{et} value of the FLSA/PA immunoreaction is 2.26 times larger than that obtained in the unstirred solution. The calculated LOD obtained using the +1 V-biased 6 V_{pp} ACEK vortex is as low as 32.0 pg/ml. The EIS-based immunosensors integrated with the long-range ACEK vortex show good promise for the development fast and miniaturized *in vitro* diagnostic devices.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for fluorescent images of the bare chip surface and image J-processed images of PA-modified immunosensors after immunoreaction with FLSA with the voltage-varied ACETF. Movie s1 and movie s2 show the trajectories of particles driven by 6 V_{pp} and 6 V_{pp} + 1 V_{DC} and observed on the electrode surface, respectively.

ACKNOWLEDGMENTS

This work was financially supported by the Ministry of Science and Technology of Taiwan under the Grant Nos. MOST104-2313-B-005-036-MY3 and MOST104-2622-B-005-007-CC2, the collaborative grant from TCVGH-NCHU1057605, and the “Innovation and Development Center of Sustainable Agriculture” from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

- ¹I. A. Grimaldi, G. Testa, G. Persichetti, F. Loffredo, F. Villani, and R. Bernini, *Biosens. Bioelectron.* **86**, 827–833 (2016).
- ²M. Konecna, K. Novotny, S. Krizkova, I. Blazkova, P. Kopel, J. Kaiser, P. Hodek, R. Kizek, and V. Adam, *Spectrochim. Acta, Part B* **101**, 220–225 (2014).
- ³D. Y. Chen, Y. M. Chen, W. C. Tsai, J. C. Tseng, Y. H. Chen, C. W. Hsieh, W. T. Hung, and J. L. Lan, *Ann. Rheum. Dis.* **74**, e16 (2015).
- ⁴F. A. Esteve-Turrillas, J. V. Mercader, C. Agulló, A. Abad-Somovilla, and A. Abad-Fuentes, *Food Control* **50**, 393–400 (2015).
- ⁵M. A. González-Martínez, R. Puchades, and A. Maquieira, *Trends Anal. Chem.* **18**, 204–218 (1999).
- ⁶X. Liu, Y. Hu, S. Zheng, Y. Liu, Z. He, and F. Luo, *Sens. Actuators, B* **230**, 191–198 (2016).
- ⁷C. March, J. V. Garcia, A. Sanchez, A. Arnau, Y. Jimenez, P. Garcia, J. J. Manclus, and A. Montoya, *Biosens. Bioelectron.* **65**, 1–8 (2015).
- ⁸C. Xue, H. Zhao, H. Liu, Y. Chen, B. Wang, Q. Zhang, and X. Wu, *Sens. Actuators, B* **156**, 863–866 (2011).
- ⁹C.-H. Lin, C.-C. Wu, and Y.-F. Kuo, *J. Electroanal. Chem.* **768**, 27–33 (2016).
- ¹⁰C. S. Chen, K. N. Chang, Y. H. Chen, C. K. Lee, B. Y. Lee, and A. S. Lee, *Biosens. Bioelectron.* **26**, 3072–3076 (2011).
- ¹¹P. Kongsuphol, H. H. Ng, J. P. Pursey, S. K. Arya, C. C. Wong, E. Stulz, and M. K. Park, *Biosens. Bioelectron.* **61**, 274–279 (2014).
- ¹²M. I. Prodromidis, *Electrochim. Acta* **55**, 4227–4233 (2010).
- ¹³I. F. Cheng, H. L. Yang, C. C. Chung, and H. C. Chang, *Analyst* **138**, 4656–4662 (2013).
- ¹⁴S. Boujday, A. Bantegnie, E. Briand, P. G. Marnet, M. Salmay, and C. M. Pradier, *J. Phys. Chem. B* **112**, 6708–6715 (2008).
- ¹⁵A. Bange, H. B. Halsall, and W. R. Heineman, *Biosens. Bioelectron.* **20**, 2488–2503 (2005).
- ¹⁶M. Selmi, R. Khemiri, F. Echouchene, and H. Belmabrouk, *Appl. Therm. Eng.* **105**, 77–84 (2016).
- ¹⁷X. Liu, C. Cheng, J. Wu, S. Eda, and Y. Guo, *Biosens. Bioelectron.* **90**, 83–90 (2017).
- ¹⁸A. Salari and M. Thompson, *Sens. Actuators, B* **255**, 3601–3615 (2017).
- ¹⁹F. Yan, J. Wu, F. Tan, Y. Yan, and H. Ju, *Anal. Chim. Acta* **644**, 36–41 (2009).
- ²⁰A. Ramos, H. Morgan, N. G. Green, and A. Castellanos, *J. Colloid Interface Sci.* **217**, 420–422 (1999).
- ²¹C.-C. Wu, W.-C. Huang, and C.-C. Hu, *Sens. Actuators, B* **209**, 61–68 (2015).
- ²²Y. Lu, T. Liu, A. C. Lamanda, M. L. Sin, V. Gau, J. C. Liao, and P. K. Wong, *J. Lab. Autom.* **20**, 611–620 (2015).
- ²³J. Wu, M. Lian, and K. Yang, *Appl. Phys. Lett.* **90**, 234103 (2007).
- ²⁴W. Y. Ng, A. Ramos, Y. C. Lam, I. P. Wijaya, and I. Rodriguez, *Lab Chip* **11**, 4241–4247 (2011).
- ²⁵F. J. Hong, J. Cao, and P. Cheng, *Int. Commun. Heat Mass Transfer* **38**, 275–279 (2011).
- ²⁶E. Du and S. Manoochchhri, *Appl. Phys. Lett.* **96**, 034102 (2010).
- ²⁷T. Liu, M. L. Sin, J. D. Pyne, V. Gau, J. C. Liao, and P. K. Wong, *Nanomedicine* **10**, 159–166 (2014).
- ²⁸A. Makaraviciute and A. Ramanaviciene, *Biosens. Bioelectron.* **50**, 460–471 (2013).
- ²⁹A. Ramos, H. Morgan, N. G. Green, and A. Castellanos, *J. Phys. D: Appl. Phys.* **31**, 2338–2353 (1998).
- ³⁰S. Li, H. Cui, Q. Yuan, J. Wu, A. Wadhwa, S. Eda, and H. Jiang, *Biosens. Bioelectron.* **51**, 437–443 (2014).

- ³¹B. Derkus, E. Emregul, C. Yucesan, and K. C. Emregul, [Biosens. Bioelectron.](#) **46**, 53–60 (2013).
- ³²M. Freitas, W. Correr, J. Cancino-Bernardi, M. F. Barroso, C. Delerue-Matos, and V. Zucolotto, [Sens. Actuators, B](#) **237**, 702–709 (2016).
- ³³R. Khan, M. Pal, A. V. Kuzikov, T. Bulko, E. V. Suprun, and V. V. Shumyantseva, [Mater. Sci. Eng., C](#) **68**, 52–58 (2016).
- ³⁴D. Hou, S. Maheshwari, and H. C. Chang, [Biomicrofluidics](#) **1**, 14106 (2007).
- ³⁵R. Hart, E. Ergezen, R. Lec, and H. M. Noh, [Biosens. Bioelectron.](#) **26**, 3391–3397 (2011).
- ³⁶M. J. Shiddiky, R. Vaidyanathan, S. Rauf, Z. Tay, and M. Trau, [Sci. Rep.](#) **4**, 3716 (2014).
- ³⁷R. Vaidyanathan, S. Dey, L. G. Carrascosa, M. J. Shiddiky, and M. Trau, [Biomicrofluidics](#) **9**, 061501 (2015).