Development of Integrated Electrochemical–Quartz Crystal Microbalance Biosensor Arrays: Towards Ultrasensitive, Multiplexed and Rapid Point-of-Care Dengue Detection

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Abstract: Dengue is an infectious mosquito-borne viral disease that affects approximately 50 million people annually worldwide and is prevalent mostly in the tropics. Severe cases of dengue can be fatal, making early detection and fast diagnosis crucial towards improving patient care and survival rates. Currently, early detection can be achieved through detection of NS1 protein, using ELISA technique. Unfortunately, ELISA is an expensive method, making it unsuitable as a screening technique, especially in low-resource settings. In this work, we present a prototype device and its early validation studies, of an integrated electrochemical and mass-sensor for dengue NS1 antigen. The sensor is connected to open source mass-sensing software and hardware, OpenQCM which makes it easily portable. Having dual-measurement capabilities (mass and impedance) increases the sensitivity of the sensor. Preliminary studies suggest that the prototype could achieve ultralow limit of detection as low as 10 ng mL^{-1}, dual-sensing cross-validation capability, portable size, sample-to-analysis time of less than 30 minutes, and parallelization of multiple assays. This work could lead to early and accurate dengue detection in routine point-of-care settings.

1 INTRODUCTION

Dengue is an infectious tropical disease transmitted by Aedes mosquitoes, which pose serious health threats, especially in tropical and subtropical regions. Dengue virus has four serotypes (DENV 1–4), which produces different symptoms starting from mild fever to the more severe forms, which may lead to fatal illnesses such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Malaysia is among the seriously affected countries with a rapid increase in the number of dengue fever cases from 43,346 with 92 deaths in 2013, to 101,357 cases including 237 deaths in 2016 (Ahmad et al., 2018; Shafie et al., 2017). In Indonesia, there was a reported average of 94 564 cases and between 472 and 1446 deaths per year in between year of 2001 and 2011 (Wahyono et al., 2017). On the other hand, three serotypes of dengue virus (1, 2 and 3) detection are circulating in Saudi Arabia where 1790 confirmed dengue cases between 2005 and 2016 with highest outbreaks in 2016 (555 cases) (Mohammed et al., 2018). In Africa, outbreaks of DF are increasing in size and frequency but are not being consistently reported to the WHO.

NS1 (non-structural) dengue protein antigen capture immunoassays, have been widely used as a dengue fever biomarker for the early stage detection (up to 7 days) in dengue diagnosis (Cecchetto et al., 2015). The NS1 protein is conserved among all four dengue serotypes, and is expressed or secreted by infected host cells (Masrinoul et al., 2011). A practical diagnostic titre in serum sample of human bodies can range from 40 ng mL^{-1} to 2000 ng mL^{-1} (Dias et al., 2013). The combination of the NS1 antigen and specific antibody tests such as IgM and IgG are popularly discussed to enhance the diagnostic efficiency for early diagnosis of dengue infection

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(Casenghi et al., 2014; Kassim et al., 2011; Omar and Fen, 2018). It was also previously shown that NS1 also presents itself in asymptomatic patients (Ashshi, 2017).

Recently, lab-on-chip (LoC) devices have been increasingly exploited for biosensor applications due to its ability to execute comprehensive laboratory protocols on a single integrated miniature MEMS device, with lower time and reagent consumption than a normal laboratory protocol. Biosensing techniques such as electrochemistry (Electrochemical Impedance Spectroscopy, EIS) and mass sensing (Quartz Crystal Microbalance, QCM) measurement have been extensively used due to their high sensitivity and are easy to miniaturize for use in point-of-care (PoC) applications (Pal et al., 2015). Among emerging applications of PoC biosensors include dengue virus detection for clinical screening and diagnosis. Many of these biosensors rely on immunoaffinity principles, similar to conventional enzyme-linked immunosorbent assay (ELISA), where antigen-specific antibody is immobilized on a solid sensor surface. In typical clinical practice, ELISA is used for dengue diagnosis; complemented by virus isolation, RT-PCR and serology methods. These methods however are time-consuming, and require tedious steps, expensive instrumentations and trained personnel (Darwish et al., 2015).

QCM measures minuscule changes in mass on the surface of its sensor through the shifts in oscillation and resonant frequency due to the damping effects that occurs when analytes are deposited onto sensor surface, as alternating current (AC) is subjected through the piezoelectric quartz substrate (Liu et al., 2013a). QCM is favoured in applications requiring high sensitivities to microscale perturbations. The typical configuration of QCMs is as single element sensors, but a multichannel QCM array (MQCMA) on the same crystal has been recently proposed (Liu et al., 2013a; Zhang et al., 2017).

Electrochemical biosensors measure perturbations in current or impedance on electrode surface due to biological redox reaction or surface interactions (Wang, 2006). One of the earliest commercial examples of electrochemical biosensor is the glucometer, which was introduced by Clark et al. in 1962, in which screen-printed electrodes are modified with glucose oxidase enzyme as a bio-receptor (Silva et al., 2017). Electrochemical biosensors are often preferred in development of point-of-care applications due to its relatively low cost, high sensitivity, and multiplexing capabilities (Cecchetto et al., 2015).

Integrated sensors employing QCM and electrochemical measurements are known as electrochemical quartz crystal microbalance sensors (EQCMs). These sensors are the combination of detection methods which includes simultaneous monitoring of mass change performed in parallel with cyclic voltammetry (CV) and/or electrochemical impedance spectroscopy (EIS) measurements on common electrodes. In EQCM, the electrodes serve both as QCM excitation electrodes, as well as the electrochemical working electrode. The counter and reference electrodes are added on one side of the quartz surface (Xiao and Zeng, 2013). Several works have been demonstrated for EQCM biosensors, highlighting capabilities for higher sensitivity from the QCM, higher selectivity and linear range from electrochemistry, and cross-validation for accuracy (Ma et al., 2015). More development is required towards practical applications of EQCM in point-of-care settings, however it offers a promising avenue for new clinical insights.

In this position paper, we present a preliminary work towards an ultrasensitive and rapid point-of-care (PoC) device for early detection of dengue, based on integrated EQCM. In section 2, the working principles that form the basis of the EQCM sensor are elaborated. We present in section 3 the development of a multichannel EQCM array that serve as an early functional prototype for a future integrated EQCM (IEQCM) PoC device. We also show that it can perform basic biosensing tests for dengue virus antigens. In section 4, we analyze the potential scientific and socio-economic impact of this innovation, should it be successfully realized and brought into clinical practices.

2 PRINCIPLES

2.1 Device Operation

To determine the optimal design of the integrated biosensor, the principle of Quartz Crystal Microbalance (QCM) and the electrochemistry system is used here. Figure 1 depicts the geometrical design and cross section of integrated biosensor.

The AC signal is applied to the top-bottom electrode of AT-quartz to generate acoustic wave energy (resonance frequency). Concurrently, for electrochemistry detection typically consisting of three metal electrodes: working, counter and reference (Regiart et al., 2016). AC/DC signal is excited between the two working and counter electrodes; in which, the electric field will be induced

on these electrodes by the electric potential when a probe marker or electrolyte is introduced into the system (Mansor and Ibrahim, 2016). Thus, integration of on-chip QCM with the electrochemistry technique is realized by fabricating a semicircular counter electrode next to top electrode on the same side of the quartz crystal. Variables of *d* and *w* indicate the diameter of working and width of counter electrode respectively. The gap, *g*, is the distance size between working and counter electrodes.

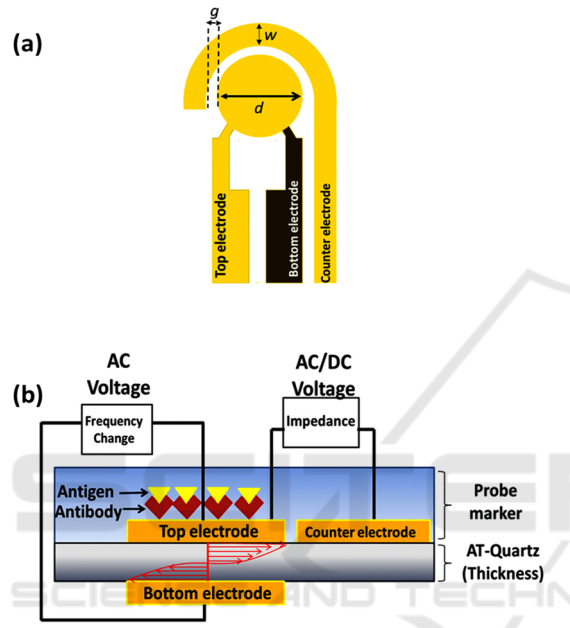

Figure 1: (a) Design of integrated biosensor. (b) Cross-section view of integrated biosensor. It indicates the frequency and impedance changes due to the binding of bio-molecules (antigens-antibodies) to the surface.

2.2 Resonance Frequency

The resonant frequency of the QCM is determined by the thickness of quartz (Shi et al., 2016). The highest resonance frequency is needed to produce the best sensitivity of this integrated biosensor. This resonance frequency is induced when acoustic waves propagate between these two electrodes through the quartz substrate, as AC signal is applied between the top and bottom electrodes. The resonant frequency of the QCM is theoretically given as Eq. (1) (Liu et al., 2013b):

$$f_0 = \frac{n}{2h_q}\sqrt{\frac{\mu_q}{\rho_q}} \tag{1}$$

Specifically, biosensing in liquids contributes significant properties of density-viscosity and acoustic impedance effects to the frequency changes as follows in equation (2) (Kankare, 2002):

$$\frac{\Delta f}{f_0} = -\frac{\Delta m}{\rho_q h_q}\left[1 - \frac{\rho_l}{\rho_f}\mathrm{Re}\left(\frac{\mu_l}{\mu_f}\right)\right] \tag{2}$$

Where f_0 is the resonant frequency of the quartz of the resonator. Δf is its frequency change due to the layer on the surface of the resonator. Δm is the change of mass which is proportional to the change of frequency and h_q is the thickness of the resonator. ρ, η, and μ is the density, viscosity and shear modulus with indices l, f, and q indicating the liquid, film (electrode), and quartz respectively. The material properties and simulation work used in this work are detailed in (Zainuddin et al., 2018, 2016).

2.3 Capacitance

Electrochemical biosensors measure current or impedance changes in electrode surface due to redox reaction or surface reactions (Wang, 2006). Generally, these sensors use three-electrode setup for *in situ* electrochemistry systems. Firstly, working electrode is the main active electrode on which the nano-range changes of chemical reactions occur (Koyun et al., 2012). Secondly, counter electrode is used to apply the electric current. Finally, reference electrode is utilized to provide the reference voltage for electrochemical tests. During the electro-migration of ions mobility process, the change of resistance and diffusion current will be affected on working electrode surface due to the successful capture of specific biological targets (Rezaei et al., 2016). Higher current density is a crucial design consideration as it corresponds to higher measurement sensitivity. Apart from that, increasing the total capacitance or constant phase element (CPE) in the system level is needed to maximize sensor sensitivity. All the theoretical studies regarding on this concept is detailed in (Zainuddin et al., 2016). The total capacitance or CPE, at electrode-electrolyte interface denoted as in equation (4) (Nevill and Malleo, 2012):

$$CPE = \varepsilon_r.\varepsilon_0\frac{A_{GEO}}{g^2} \tag{3}$$

Where ε_0 is the permittivity of free space and has the value 8.854 x 10^{-12} Fm^{-1}. ε_r is the relative dielectric constant of the medium between two electrodes. A_{GEO} is the electrode active geometry surface area. g is the gap between electrodes. From this equation, reducing the gap g will increase total capacitance in this system.

3 PRELIMINARY RESULTS

3.1 COMSOL Simulation

The mechanical (resonance frequency) and electrochemical operation in the IEQCM array were simulated using COMSOL 5.3. The detail of simulations were discussed previously (Zainuddin et al., 2017).

3.1.1 Resonance Frequency

Figure 2 indicates the measurements of resonance frequency for the working electrode diameter, from 200 µm to 4000 µm. From the results, the highest total-displacement value (170 pm) with the resonance frequency of 9.826 MHz (i.e. natural frequency of 168 µm quartz wafer) was produced at diameter of 4000 µm. Consequently, d = 4000 µm was selected since it showed the highest total displacement compared to other diameter based on simulation results.

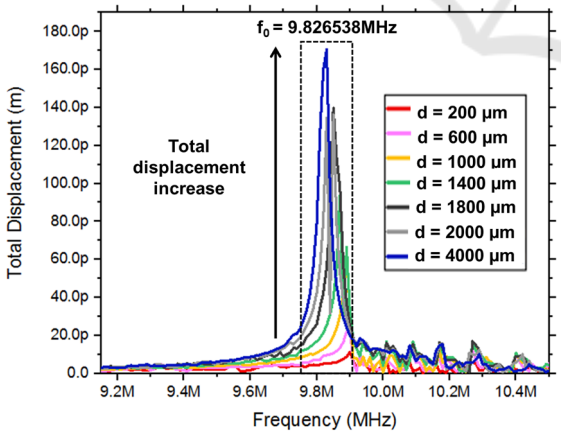

Figure 2: Total-displacement versus resonance frequency when electrode of working diameter, d = 200 µm, 600 µm, 1000 µm, 1400 µm, 1800 µm, 2000 µm, and 4000 µm. The dotted line box represents the total displacement for all diameter values around quartz resonance frequency of 9.826 MHz.

3.1.2 Frequency Interference

The optimal centre-to-centre distance of the adjacent electrodes, (s) was chosen based on the lower resonance frequency interference. It also produced the same response of resonance frequency for all mass sensors. Figure 3 shows the resonance frequency interference simulation of the centre to centre distance, (s). From the results, the resonance frequency interference decreased with the enlargement of the distance. From the results, s = 4 mm (Figure 3(a)) indicated only two peaks were observed at the close resonance frequency values. For s = 6 mm (Figure 3(b)), all QCM sensors showed the same response resonance frequency of f_0 = 9.8420 MHz. Finally, for s = 8 mm (Figure 3(c)), it was observed that, all QCM sensors showed the same response of resonance frequencies, however the resonance peaks were not uniform due to electrodes position near to the edge of quartz. Therefore, s = 6 mm was indicated as the optimal value to allow minimization of the signal interference between other channels.

3.1.3 Determination of Gap Separation

The optimal gap between working and counter electrodes was selected based on the higher magnitude of total capacitance. Figure 4 shows that the gap of 70 µm was indicated as the optimal result to allow maximization of total capacitance in this simulation as compared to others. Apart from that, it was observed that widening the counter electrode width will increase the total capacitance in this design. Therefore, the gap of (d = 70 µm) and the counter width of 1000 µm were chosen since these values showed the highest total capacitance as compared to other parameters studied in this work.

3.1.4 Fabricated Final Design

Diameter 4000 µm was selected for our biosensor since it showed an optimal size with a high mechanical displacement and low interference while also allowing dimensional fit into a radial array of 3 identical sensors on a single quartz crystal substrate of diameter, d_q = 14 mm as shown in Figure 5. Sensor was fabricated using standard photolithography process. Fabrication work was performed in XLIM Research Institute, University of Limoges, France.

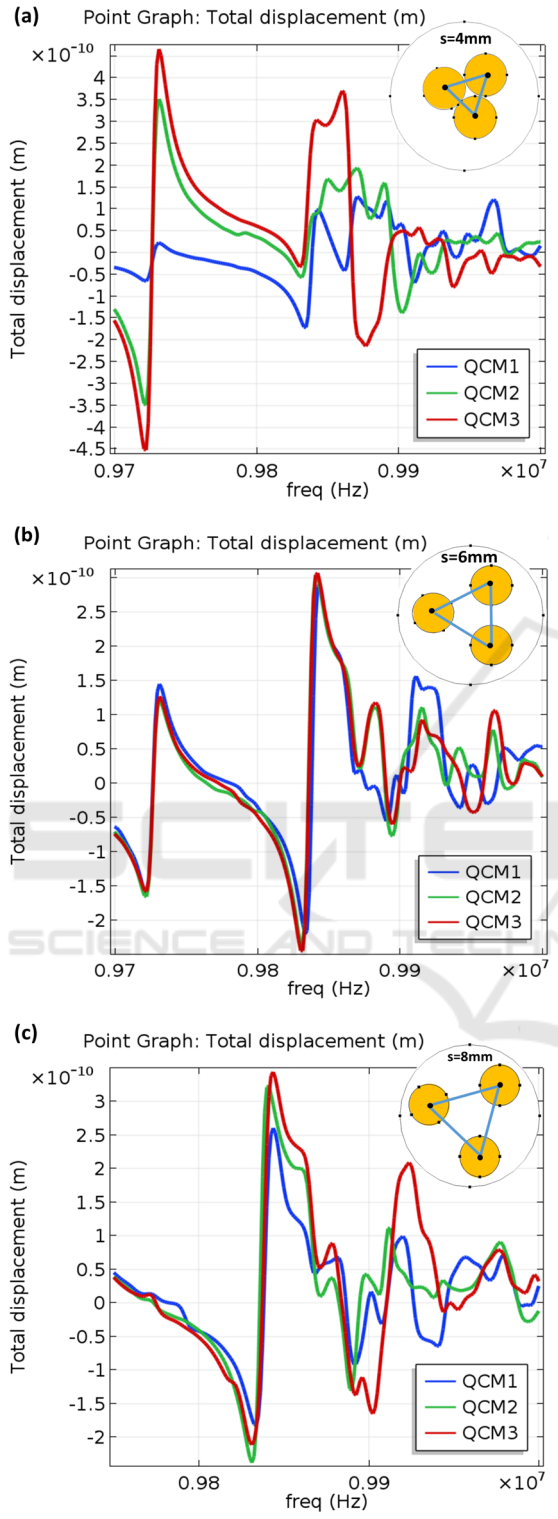

Figure 3: Simulation of frequency interference between the 3 QCM sensors placed symmetrically in the centre of quartz. (a) s = 4 mm, (b) s = 6 mm, (c) s = 8 mm.

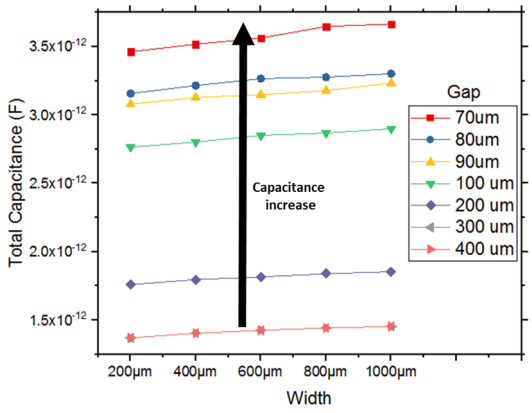

Figure 4: Simulation of total capacitance for gap of 70 µm to 400 µm and counter width of 200 µm to 1000 µm.

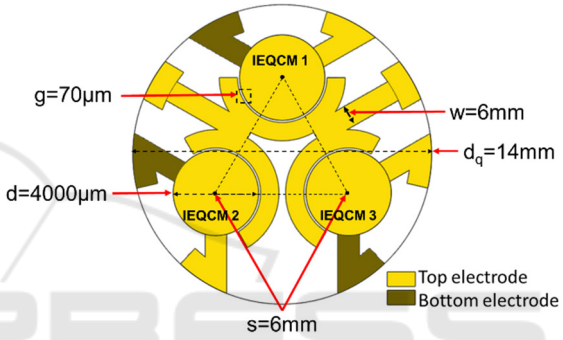

Figure 5: Proposed design and dimension of integrated electrochemical quartz crystal microbalance in array (IEQCM) biosensor in a single chip. This sensor has an array of 3 identical integrated biosensors (IEQCM1, IEQCM2, IEQCM3) equipped with dual-function detection system.

3.2 Detection of NS1 Dengue based on Antigen and Antibody Interactions

In this work, the dengue detection was based on antigen and antibody binding. Several concentrations of NS1 antigens (10 ng mL^{-1}, 100 ng mL^{-1}, 1000 ng mL^{-1}) were measured for demonstration of the functionality of the IEQCM using a custom enclosure. Electrochemical impedance spectroscopy (EIS) and QCM were used to investigate the binding interactions. Prior to NS1 antigen injection, the working electrode was coated with self-assembly monolayer to immobilize the anti-NS1 IgG antibodies. It was followed by the incubation of glycine in PBS to block the nonspecific interaction. Figure 6 shows the measurement results of EIS and frequency changes before and after incubation with anti-NS1 reflect the concentrations of NS1. The electrochemical signals were recorded in the presence of a redox probe ($[Fe(CN)_6]^{3-/4-}$) via potentiostat to

monitor changes in charge-transfer resistance associated with target binding. The frequency changes were monitored using OpenQCM software by injecting 100 µl of phosphate-buffered saline (PBS) onto the sensor. The frequency change measurement was started after a stable baseline was formed as shown in Figure 6(b). Using this new measuring system, we reliably detected concentrations of dengue NS1 down to 10 ng mL^{-1}, well below clinical diagnostic range. Early measurements of three common human serum proteins shows that EIS and QCM signals from nonspecific proteins are no higher than 3 standard deviations of blank measurements, suggesting the sensor is highly NS1-selective, however further investigation is required. Based on these preliminary results, further validation of the IEQCM will be performed to allow calibration and to specify the limit of detection and sensitivity. These validation steps will support further development of the technology, and translation of the IEQCM into clinical applications in the future.

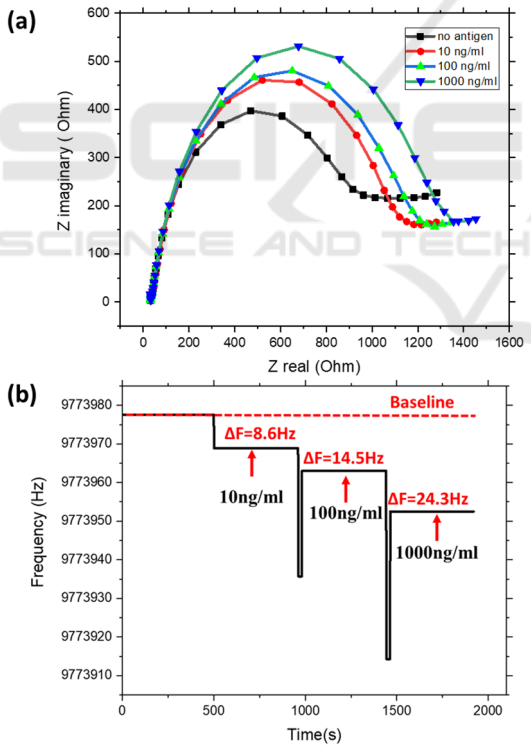

Figure 6: NS1 dengue detection: (a) Nyquist plots from EIS, (b) frequency change from QCM.

4 SCIENTIFIC AND SOCIO-ECONOMIC IMPACTS

From a scientific innovation perspective, we present the first multichannel array IEQCM biosensor ever reported. This biosensor brings together biotechnology, semiconductor and electronics on a single chip in providing rapid detection of dengue, noted as the fastest-growing mosquito-borne viral infection. The array of 3 identical integrated biosensors equipped with dual-function detection system offers high-throughput functionalities in detecting a wide range of dengue antigen concentrations. Additionally, the ability to multiplex and parallelize biosensing on a single platform will allow development of integrated multi-disease detection panels, such as Aedes-related infections (dengue, Zika, chikungunya, yellow fever). A portable, integrated instrument is currently under active development. Further developments will enable the device to be operated conveniently by clinical laboratory personnel or semi-trained staffs, as multiplexed measurement automation and pre-loaded protocols are developed in the future. These approaches have the potential to become PoC applications that improve standard of care, particularly in resource-limited settings.

Socio-economically, dengue is a disease that impacts an estimate of 50 million persons annually across the globe, heavily concentrated in the tropical and sub-tropical regions (Special Programme for Research and Training in Tropical Diseases and World Health Organization, 2009). By enabling deployment of an ultrasensitive rapid PoC diagnostic, a better healthcare delivery for dengue can be achieved. The portability of the device allows for use in smaller clinics that often have wider reach into communities as compared to hospitals with full laboratory capacity. The prototype is estimated to be able to run sample analyses in less than 30 minutes. This rapid analysis allows same-day sample-to-results cycle, which facilitates faster decisions on treatment plans for febrile patients suspected of dengue infection. Perhaps the most novel merit of this device is the ability to directly detect dengue virus in solution at very low concentrations, well below typical clinical diagnostic range of dengue fever, which promises potential integration into screening programs for early, pre-viremia detection of dengue infection. It also could potentially enable screening for non-asymptomatic carriers for epidemiological study purposes.

5 CONCLUSIONS

In this position paper, we demonstrate the early development of an integrated EQCM device for dengue biosensing. Our working hypothesis is that using dual-function sensors could not only increase the sensitivity to very low limits of detection and increase dynamic range of disease quantification, but also provide higher diagnostic accuracy through cross-validation of parallel measurement techniques. This presents potential capability for new clinical insights, including early detection of dengue infection, and identification of asymptomatic carriers. Furthermore, to the best of our knowledge, this is the first time a multichannel IEQCM array has been described. To a larger context, we propose that it is a worthwhile endeavour to explore more parallel dual-measurement techniques and their integration strategies to enhance sensitivity and accuracy of diagnostic tests.

When completed, this technology could potentially improve dengue patient care significantly, as dengue could be detected at an earlier stage, and enables a faster and higher accuracy of diagnosis at the point-of-care. As this technology utilizes a generic immune-affinity sensing scheme, this technique could also be expanded into other diseases.

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