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A non-optical multiplexed PCR diagnostic platform for serotype-specific detection of dengue virus

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ABSTRACT

Viable point-of-care multi-target diagnostics requires single-sample multiplexed detection in a single PCR reactor with non-optical instruments. We report an integrated multiplexed PCR chip platform with a novel ion-selective membrane sensor array that eliminates the key selectivity and sensitivity issues for multiplexed PCR, such as primer dimerization and nonspecific amplification (mis-priming). The depletion action of the ion-selective membrane renders the sensor insensitive to pH and ionic strength variation or non-specific binding, which plague the current electrochemical sensor arrays. We validate the platform with robust, sensitive and selective detection of each of the four dengue virus (DENV) serotypes, even in the presence of misprimed non-target products, with plasma samples spiked with heterogeneous RNA populations that include all the serotypes. The assay time is ~ 90 min with a detection sensitivity of DENV RNA template down to 100 copies per mL. By replacing the optical sensing technology of RT-PCR with the membrane sensor, the platform is scalable and can allow simultaneous screening for multiple viral infections in a single sample within a single PCR reactor chip for point-of-care applications.

1. Introduction

In a laboratory, reverse-transcription PCR (rt-qPCR) based on optical detection can be done on a single sample in a 96-well format. With different primer sets in each well, large number of targets can be screened with one single sample. Such large-library multiplexed PCR cannot be applied to point-of-care (POC) applications, because of the bulkiness of well-loading robotics and optical instruments. One potential solution is to do multi-target detection in a single or a small number of PCR reactors with multiple primer sets. However, with the large DNA and primer population in an untreated heterogeneous POC sample, interfering reactions like primer dimerization and mispriming will significantly reduce the sensitivity and selectivity of an optically based single-PCR reactor platform. Both reactions compete with the target-amplification reaction to reduce the latter's yield (sensitivity). Molecular beacon type optical reporters cannot differentiate between the target amplicons and the products of these interfering reactions (selectivity). Even if these selectivity/sensitivity issues are overcome, it is challenging to select proper fluorophores with appropriate spectral bandwidth to avoid spectral emission overlap and output signal interference. This limits optics-based PCR platforms to the detection of no more than five targets simultaneously for POC applications [1].

Recently, electrochemical sensor arrays, particularly ion-sensitive field-effect transistor (ISFET) arrays, have been suggested as a replacement to the optical sensor to allow multiplexed PCR in a single PCR reactor [2–4]. By functionalizing oligo probes complementary to the target, it is hoped that only the amplified targets will be detected, after they hybridize with the probes to form duplexes, even in the presence of interfering reactions. However, electrochemical sensors that detect charge or electron transfer reactions to or from ions have their own sensitivity and selectivity issues. Due to Debye screening of the target charge and current, its signal is sensitive to the pH, the ionic strength and the multitude of factors that control the length of the hybridized duplexes: the duplex conformation, the pairing sequence location on the target and the lengths of the dangling tails [3,5,6]. Such factors are difficult to control for POC samples without extensive pretreatment. Given that PCR reactions change the pH significantly, the pairing sequence location can be arbitrary and non-specific

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hybridization are quite ubiquitous, electrochemical sensor arrays have not enabled multiplexed PCR. A most recent attempt is to carry out multiplexed PCR in low ionic strength such that the Debye length is sufficiently large to accommodate the hybridized target, regardless of the duplex conformation, the pairing sequence location and length of the dangling tails that bracket the pairing sequence [7]. However, screening of electrostatic repulsion between annealing nucleic acids becomes weak at low ionic strengths very low PCR yield results. The yield is so low that it cannot reach the necessary sensitivity for early screening of pathogens from bio-samples.

Clearly, a viable POC multiplexed PCR platform has not been reported. Instead of the electrochemical sensor array, we use an array of ion-selective membrane (IEM) sensors [8]. We demonstrate, for the first time, the ion-depletion action of the ion-selective membrane promotes the selectivity and sensitivity of the sensor without sacrificing the yield of the PCR reaction, which is still carried out in the usual 50 mM ionic strength of the PCR buffer. Ours is the first POC multiplexed PCR platform that can identify the serotype of a Dengue virus in a heterogeneous blood sample filled with the RNAs of other vector-borne viruses.

We validate our POC multiplexed PCR platform with an important POC application: sensitive and selective detection of specific dengue serotype in blood with viral RNAs from other vector-borne diseases, including those from the other serotypes with similar sequences. Dengue virus (DENV) is one of the rapidly spreading global health problems. It is a common tropical infection in more than 120 counties, including Southeast Asia, Africa, Central and South America, and the Western Pacific [9]. Some 2.5 billion people or one third of world population are at a risk from dengue infection at any given time [10]. World Health Organization (WHO) currently estimated over 50–100 million infections worldwide every year [11,12]. Dengue is caused by a virus of the Flaviviridae family and exists as four genetically closely related serotypes DENV (1–4) [13,14]. DENV is transmitted by infected mosquito vectors, mainly Aedes aegypti, and can cause symptoms such as high fever for 4–7 days with headache and joint pain [15,16]. However, infections due to other arboviruses such as Zika virus (ZIKV), Yellow Fever virus (YFV) and Chikungunya virus (CHIKV) also show similar symptoms, making it very difficult to identify DENV based on symptoms [17–21]. Despite the absence of an effective DENV treatment, an early diagnosis can improve clinical outcomes and is crucial for monitoring disease outbreaks. Specifically, as the severity of the disease varies by serotype and history of past infection, early identification of DENV serotype is clinically important.

Immunoassay techniques, although rapid and accurate, typically can be used only after the body has begun to mount an immunological response to the foreign pathogen through antibody production and are hence also ineffective for rapid epidemic control [22,23]. Moreover, antibody-based assays suffer from cross-reactivity, especially in identifying species within different flavivirus genus, such as ZIKV or different DENV serotypes.18 Multiplexed PCR in a single reactor that is sufficiently sensitive and selective remains the most viable solution.

In our new IEM sensor array-based platform, serotype-specific oligoprobe is attached to each sensor of the multiplex IEM sensor module for selective capture and identification of the amplified DENV serotype products. When an electric field is applied across an IEM, external concentration polarization occurs across the membrane. The depletion side is almost ion-free and hence controls the ion current through the membrane. A limiting current regime develops at a sufficiently high voltage such that the differential resistance is almost infinite. At higher voltages, an overlimiting current is observed when an electroconvective instability sets in [8,24]. When IEMs are functionalized with oligo probes on the depletion side, the duplexes formed after hybridization with the oligos cannot be depleted. Consequently, the hybridized targets produce a large shift in the non-equilibrium ionic current that results a shift in current-voltage curve (CVC) in the overlimiting current of the IEM [8,25]. Moreover, the vortices of the electroconvective instability in the over-limiting region also serves to shear off non-specifically bound targets whose bonds with the probes are weaker. We further enhance the selectivity with a hydrodynamic shear on our IEM biochip.

Importantly, the overlimiting current only occurs after ions have been depleted from the membrane surface, thus significantly increases the Debye length to enhance both the sensitivity and selectivity of the sensor, much like the recent work on low-ionic strength electrochemical sensing [7]. The sensitivity is enhanced because the presence of a small number of charged targets (and their counterions) in the ion-depleted region can produce a large change in the current. Selective because the Debye thickness is large (> 30 nm) in the ion-depleted region so the current signature is not sensitive to the length, conformation of the duplex and the location of the pairing sequence. The hybridized target molecules hence do not protrude beyond the Debye layer. The platform also uses hydrodynamic shear to shear off non-specifically adsorbed non-targets from the membrane surface, thus further enhancing the detection selectivity of the platform and minimizing any false positive detection. Additionally, the platform does not require extensive sample pretreatment and expensive lab equipment. It uses a simple off-chip chitosan modified filter paper-based dip and rinse technique to remove PCR inhibitors and isolates the negatively charged target DENV RNAs from the lysing buffer (TRIzol™ reagent) treated RNA spiked plasma sample for downstream amplification of different DENV serotypes. Hence, the POC platform has the potential to be used as epidemiologic surveillance tool by identifying the presence of DENV serotypes from a large population of potentially infected people in a low-resource setting. The multiplex target-specific probes allow the platform to be scaled up so that a large number of targets can be identified with a one-step procedure from a single sample, although we will only demonstrate its potential with 4 targets—the 4 serotypes of Dengue.

In this paper, we present a low-cost non-optical integrated on-chip PCR-based diagnostic platform coupled with a multiplexed ion exchange membrane (IEM) sensor for analysis of the PCR amplified products for the detection of four DENV (1–4) serotypes. A serotype-specific oligoprobe is attached to each sensor of the multiplex sensor module for selective capture and identification of the amplified DENV serotype products. When IEM captures and hybridizes with the target, it produces a change in non-equilibrium ionic current that results a shift in current-voltage curve. Without any sample pretreatment, this platform uses a simple off-chip chitosan modified filter paper based dip and rinse technique to remove PCR inhibitors and isolates the negatively charged target DENV RNAs from the lysing buffer. Yet, it demonstrates higher selectivity and sensitivity against all other PCR-based technologies. Hence, the platform has the potential to be used as epidemiologic surveillance tool by identifying the presence of DENV serotypes from a large population of potentially infected people in a low-resource setting.

2. Materials and methods

2.1. Sample preparation

All DENV serotypes were obtained from Naval Medical Research Center (NMRC) and the following strains are used: WP-74 (DENV-1); OBS8041 (DENV-2); CH53489 (DENV-3) and H241 (DENV-4). The isolated ZIKV RNA was received from Emory University and PRVABC59 strain is used. The extracted DENV and ZIKV RNA were then spiked into the human blood plasma sample (ZenBio Inc., NC, USA). All oligonucleotides including primers and probes were obtained from Integrated DNA Technologies, Inc.

2.2. Chitosan modification of Fusion 5 filter paper

Chitosan (medium molecular weight), MES (2-(N-morpholino)-ethanesulfonic acid), sodium hydroxide, sodium dodecyl sulfonate

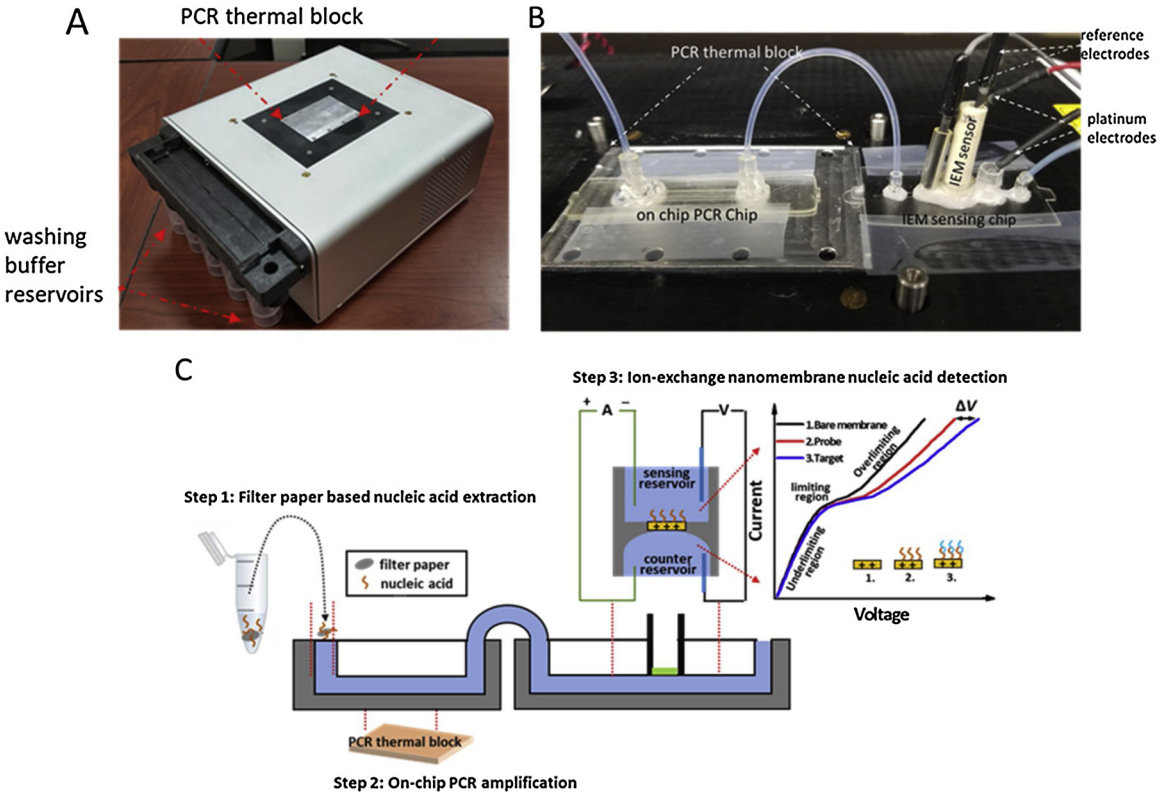

Fig. 1. (A) In house box for pumping washing buffers. (B) polycarbonate based microfluidic sensing chip. (C) Schematic of the integrated microfluidic platform for the detection of DENV (1–4) serotypes. The platform consisting of a filter paper-based sample pretreatment unit capable of capturing nucleic acid molecules for downstream PCR amplification, an on-chip PCR unit to amplify the target DENV RNA serotypes, an IEM based sensor to detect amplified products.

(SDS), benzophenone-3,3,4,4-tetracarboxylic acid, N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fusion 5 filter paper was obtained from Whatman (GE Healthcare, Pittsburgh, PA) and modified with chitosan according to literature.27 Briefly, a 5 cm diameter size Fusion 5 filter paper was first treated by oxygen plasma for 2 min and then incubated in chitosan solution (0.05 % (w/v) in 0.1 % acetic acid, pH 6.0) for overnight and then washed three times with DI water. Finally, the sample was vacuum dried. The dried 5 cm diameter piece of filter paper was cut into 1 mm diameter discs and stored at room temperature for experimental use.

2.3. RNA extraction from human plasma sample

Different concentrations of DENV and ZIKV RNA were obtained by spiking 10 µL extracted DENV and ZIKV RNA into 1 mL human plasma with serial dilution. Then, a piece of 1 mm diameter chitosan-modified filter paper disc was immersed into the spiked sample and incubated for 5 min. The filter disc was carefully removed followed by rinsing with 1 % SDS and DI water. Finally, the filter disc was mixed with RT-PCR mixture for on-chip RT-PCR amplification.

In order to demonstrate the performance of chitosan modified filter paper-based RNA extraction from plasma sample with DENV, 10 µL extracted DENV RNA (100 copies total) was first spiked into 0.2 mL human plasma sample, human whole blood sample and DI water, respectively. TRIzol™ was added and shake vigorously for 30 s followed by 0.2 ml chloroform and shake again for 60 s to separate out the aqueous phase. The supernatant was then collected and a small piece (1 mm diameter) chitosan-modified filter paper and incubated for 5 min. The filter paper was then carefully removed followed by rinsing with 1 % SDS and DI water. Finally, the filter disc was mixed with PCR mixture for on-chip single step RT-PCR amplification.

2.4. On-chip PCR amplification

On-chip PCR amplification was performed following RNA extraction using chitosan-modified filter paper disc from 1 mL of human blood plasma samples in the microchip. The targeted sequences of DENV RNA were reverse transcribed into a respective complimentary DNA (cDNA) and PCR amplified using the DENV (1–4) RT-PCR assay protocol published elsewhere [26]. The assay was run with each DENV serotype in a separate chip (for initial optimization study) or in multiplex (the four DENV serotypes were amplified in the same reaction using four primer sets). All RT-PCR reactions were performed using iTaq™ Universal SYBR® Green One-Step Kit (Bio-rad, cat. no. 1725150). To assemble a multiplex RT-PCR reaction, a 20 µL PCR mixture comprised of 12.5 µL of PCR reaction mixture, 0.5 µL of forward and reverse primers for DENV-1 and DENV-3, 0.25 µL of forward and reverse primers for DENV-2 and DENV-4, 0.5 µL of iScript reverse transcriptase and 5 µL of nuclease-free H_2O was made first, then the filter disc with extracted RNA was put into the PCR mixture. After the PCR mixture was loaded into the microfluidic chip, the inlets and outlets were sealed and placed over a lab-developed thermocouple plate. A calibrated thermal recipe was used for PCR amplification, including reverse transcription at 50 °C for 30 min, RT inactivation at 95 °C for 2 min followed by 35 cycles of 95 °C for 15 s and annealing at 55 °C for 1 min. The amplicon products were collected from the reaction chamber to confirm amplification with gel electrophoresis or heated at 95 °C for 5 min in the same chamber to get ssDNA for the downstream detection.

2.5. Nanomembrane sensor

The nanomembrane sensor is an anion-exchange nano porous membrane (AME), produced using polystyrene–divinylbenzene fine particles with strong basic quaternary ammonium groups supported by polyethylene as a binder and polyamide/polyester textile fiber (Mega

a.s., Czech Republic). For each nanomembrane sensor one or 4 pieces of $0.9 \times 0.3 \times 0.3\,mm^3$ (l/w/t) was cut and framed by a resin mold. To perform the DENV detection, AME sensor was first functionalized with oligoprobe using EDC protocol as previously described [8].

2.6. Fabrication of integrated microfluidic chip

The integrated microfluidic chip was designed using Adobe Illustrator with dimension of $10 \times 4 \times 0.1\ cm^3$ (l/w/t) and fabricated using three layers of 0.3 mm polycarbonate thermo softening plastic by cutting plotter Graphtec Pro FC 7000MK2-60 and sealed together via thermal/pressure bonding. It consisted of a 25 μL on-chip PCR chip and an AEM sensing chip with input and output channels for performing on-chip PCR amplification and AEM -based sample detection together. The sketch of the microfluidic chip is depicted in Fig. 1B and C.

2.7. Recording unit

After successful attachment of oligoprobes on the nanomembrane sensor, the functionalized nanomembrane sensor was mounted on AEM sensing chip so that sample was in direct contact with sensing region. An in-house constructed box of dimension $102 \times 240 \times 305\ mm^3$ (l/w/t), consisting of a fluidic pump and a thermocouple heater was used to perform on-chip PCR and deliver different buffers (Fig. 1A). The current voltage characteristics (CVC) of the membrane sensor was established using a Gamry 500 potentiostat (Gamry Instruments, Warminster, PA, USA) connected to a PC for data acquisition and further analysis using Gamry Framework software. Two reference electrodes (Ag-AgCl) were used to measure the potential across the nanomembrane sensor whereas two platinum electrodes were used to apply the current load from 0 to 40 μA at a step rate of 1 μA/s.

3. Results and discussion

3.1. Platform layout

The visual representation of the integrated microfluidic platform is shown in Fig. 1. The assay protocol involves three steps and takes ~90 min to detect four DENV serotypes from 1 mL of DENV RNA spiked in plasma sample. Step 1, the DENV RNA is extracted upstream from TRIzol™-chloroform treated RNA spiked plasma sample using a chitosan-modified filter paper-based dip and rinse extraction technique (10 min). Step 2, the extracted RNA filter paper is then mixed with PCR reagents and introduce in a microfluidic chamber for simultaneous denature and create ssDNA. The solution is then passed through the microfluidic chip to interact with multiplexed IEM sensor. Finally, the sensors are washed with 4x PBS and current-voltage signal is measured sequentially in 0.1x PBS for simultaneous detection of the target serotypes (20 min).

We used a simple dip-and-rinse filter paper-based pretreatment process to remove PCR inhibitors and capture the target DENV RNA serotypes from the aqueous phase of TRIzol™-chloroform treated RNA spiked plasma sample. Several reports revealed that Fusion 5 filter paper can be successfully used to extract nucleic acids from a number of different samples due to the physical entanglement and electrostatic adsorption of long-chain nucleic acid fragments on the fiber matrix [27–29]. In 2009, Jangam et al. reported isolation method for HIV proviral DNA extraction from whole blood using a Fusion 5 filter paper [29]. Furthermore, Liu et al. developed a microfluidic device that integrated a chitosan modified filter disc to extract DNA from various raw samples for PCR amplification [28]. They demonstrated a very high recovery rate of ~98 % and 95 % for K562 human genomic DNA and bacteriophage λ-DNA, respectively [28]. Hence, we selected the chitosan modified filter. paper to rapidly capture nucleic acid and remove PCR inhibits from human plasma or whole blood and to get a good quality RNA for downstream PCR amplification of DENV serotypes. Due to the higher affinity of chitosan polymers, RNA molecules are trapped on the filter fiber matrix, allowing us to wash off the inhibitors from filter surface.

The external ion concentration polarization signature of IEM is used to detect the PCR amplified product. IEMs are ion-selective membrane having a fixed positive (anion-exchange) or negative (cation-exchange) surface charge that only allows the transport of counter ions through the membrane under an applied electric field. This asymmetric migration of ion reduces the ionic strength on one side of the membrane (known as the depletion zone) and increases the ionic strength on the other side (known as the enrichment zone) [8,25]. The depletion zone is formed beyond a critical voltage. Its low ionic strength significantly increases the overall ionic current resistance, with a differential resistance that can be orders of magnitude higher than the membrane resistance. Consequently, onset of depletion produces a signature limiting current regime in the second critical voltage. For detection of target amplified product, the depletion side of an AEM is used where the hybridization of target with specific oligoprobes are attached to the AEM [30]. The charge on the oligoprobes suppresses the depletion action and the electro-convective instability [30]. As a result, the overlimiting current in the CVC (current-voltage curve) shifts by several volts. An additional voltage shift occurs upon the hybridization of target as additional charge is introduced to the surface to further reduce the resistance of the depletion region. As the signals are due to large change in the ionic strength, the voltage signals are 10–100 times larger than those from electrochemical sensors due to electron transfer reactions and offers sensitive detection [8,30].

3.2. RNA extraction from plasma sample and on-chip RT-PCR amplification

We used chitosan modified Fusion 5 filter paper-based dip and rinse technique to isolate DENV RNA template from plasma sample. First, a series of 10 μL DENV RNA samples containing 50, 10^2 and 10^4 copies of DENV-1 were spiked into 1 mL human plasma and then a 1 mm size chitosan modified filter paper disc was immersed into each sample respectively. After incubating for 5 min, the filter disc was carefully removed followed by rinsing and washing. Finally, the filter disc was introduced into an on-chip PCR chamber along with the RT-PCR reagent for amplification. DENV-1 RNA first reverse transcribed to single stranded cDNA and then amplified with DENV-1 specific primers in a single step process. The amplified product was then identified using gel electrophoresis (Fig.2). The overall extraction process takes about 10 min, including 5 min incubation with plasma sample and 5 min for rinsing and washing. As shown in Fig. 2A, the 110 bp PCR amplicons for DENV-1 were successfully amplified with each filter disc containing different copies of DENV-1 RNA templates. Fig. 2A further demonstrates that the isolation of paper-based dip and rinse technique can go down to 50 copies of RNA per mL However, the PCR amplification efficiency is very low when no extraction was done. This demonstrates the effectiveness of the filter paper extraction process in removing the PCR inhibitors from the human plasma and capable to isolating enough good quality RNA templates for downstream PCR amplification.

After successful optimizing the extraction process with DENV-1 RNA spiked in plasma sample, we validated the process with other DENV (2–4) serotypes. We used two different strategies for this validation study. The RT-PCR reaction was performed with one primer set and with corresponding target spiked in plasma sample and four primer sets but with only one target spiked in plasma sample. Fig. 2B and C show the successful on-chip RT-PCR amplification of RNA samples extracted from human plasma containing one or four sets of primers respectively. As expected, the amplicon sizes of DENV-1, DENV-2, DENV-3 and DENV-4 are 110 bp, 98 bp, 78 bp and 83 bp, respectively. The negative control experiment without any DENV RNA template (NT) in both cases did not show any detectable amplification. However, with four primer set in Fig. 2C, we observed some spurious bands in the gel image,

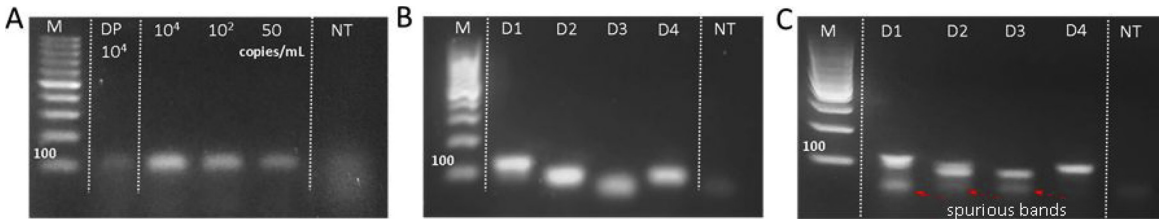

Fig. 2. Showing the successful on-chip PCR amplification with the RNA extracted sample using chitosan-modified filter paper technique. (A) Comparison of PCR amplification efficiency of DENV-1 RNA from human plasma by using different initial copy numbers of DENV-1 RNA with/without filter paper extraction. (Lane M: DL 1000 DNA marker; DP: direct amplification without filter paper extraction; NT: no RNA template) (B) On-chip RT-PCR amplification for DENV (1–4) RNA serotypes with one primer set and the corresponding DENV RNA spiked in plasma sample (D1: DENV-1; D2: DENV-2; D3: DENV-3; D4: DENV-4) (C) On-chip RT-PCR amplification for DENV (1–4) RNA serotypes with four primer sets and with only one DENV RNA spiked in plasma sample.

indicating false positive amplification or primer dimer cross amplification. This suggests that with multiple primer set, the RT-qPCR could show false positive result as the detection specificity depends on primer and probe design. However, with the probe-functionalized sensors of our platform, such false-positive non-specific PCR products do not contaminate our CVC electrical signals, as described in the next section.

3.3. Specific detection of DENV serotypes using nanomembrane sensor

The amplicons from on-chip PCR amplification (Fig. 2B) were then detected using nanomembrane sensor functionalized with the corresponding target specific oligoprobes. The specificity of each nanomembrane sensor was then evaluated against other DENV RNA serotypes and ZIKV RNA amplified products. Zika virus is also a mosquito-borne flavivirus that shows very similar symptoms to DENV [31]. After incubating each denatured PCR amplified product for 15 min, the sensor was washed with 4xPBS to remove all non-targets. The CVC of IEM was then measured in 0.1xPBS. As seen in Fig. 3 (B–E), a large voltage shift is only observed when the sensor is exposed to the DENV sample that is complementary to the oligoprobes attached to the sensor surface, otherwise the shift is within noise level for NT, Zika RNA and other DENV serotypes samples, despite the presence of non-specific amplification products. A shift of 0.55 V, 0.61 V, 0.67 V and 0.53 V were observed when DENV-1, DENV-2, DENV-3 and DENV-4 PCR amplicons were exposed respectively with their corresponding target-specific oligoprobe sensor. These results clearly reveal that platform is very specific and can easily discriminate non-target PCR amplification products.

Reproducibility of the sensor is then investigated by comparing the voltage shift from four different sensors hybridized with each DENV serotype. As shown in Fig. 4, significant CVC shifts were observed only due to specific hybridization between probes and ssDNA target sequence from corresponding DENV serotypes. All the CVC shifts from target group are larger than 0.4 V. While there were no obvious CVC shifts from control group PCR samples that are in the absence of viral RNA or presence of other non-specific viruses (the remaining three DENV serotypes, ZIKV), all the CVC shifts from control groups were less than 0.2 V (which might be due to the attachment of non-specific molecules on the nanomembrane surface). As seen from Fig. 4, the detection cutoff threshold can be set as 0.2 V, which is much lower than the average shifts observed with each detected DENV RNA serotype sample (~0.4 V). The CVC shifts clearly demonstrate no overlap between the target and control group for all DENV serotypes samples, indicating the excellent selectivity and reproducibility of the membrane sensors.

To bench mark the limit-of-detection of our current technology, we compared it against other reported DENV detection technologies for human blood sample in Table 1. As is evident, our LOD outperforms all other PCR detection technologies by at least a factor of 3, despite the fact that our platform requires minimum pretreatment. This superior performance over optical detection platforms is most likely because the latter suffers from noise from primer dimerization or non-specific amplification (mis-priming). As our platform uses target specific oligoprobe on the sensor surface, we can easily remove any non-specifically amplified product by hydrodynamic shear (flow), thus making our sensor selective with very low background noise. Hence, the amplified product from a small number of targets is sufficient for our platform to produce a detectable change in the current-voltage signal.

3.4. Simultaneous detection of two DENV serotypes

After successful demonstrating selective detection of each DENV serotype, we extended our strategy to simultaneously detect two DENV serotypes using 2-membrane sensor in a single sensor module. Infection with any DENV serotypes can provide lifelong immunity against the infected serotype. A secondary infection with any of other three DENV serotypes could lead to a more severe infection and clinical manifestations [38]. For example, severe dengue infections usually occur with the primary infection of DENV-1, followed by secondary infection of DENV-2 or DENV-3 [23,39]. Hence, simultaneous and rapid identification of different DENV serotypes could provide more detailed information of the infection status to reduce the risk of severe DENV complications. Toward this objective, we designed two addressable membrane sensors to selectively identify two DENV serotypes simultaneously. Similar to one-membrane sensor, we attached target specific oligoprobes in each sensor. In one set of sensors, we attached DENV-1 and DENV-3 serotype specific oligoprobes and in other set, we attached DENV-2 and DENV-4 serotype specific oligoprobes. We used the ZIKV amplified product to check the detection selectivity of the 2-membrane sensor. We then sequentially exposed the sensor with DENV-1 and DENV-3 serotype denatured PCR amplified product. Followed by sample incubation and appropriate buffer washing, the CVCs were subsequently measured by two identical sets of electrodes in each of the reservoirs (Fig. 5A). As shown in Fig. 5B, only DENV-1 and DENV-3 serotype PCR amplified product shows significant shifts in CVC (0.47 V for DENV-1, 0.54 V for DENV-3) at 40 μA while ZIKV and the other DENV serotype show no detectable shift at both sensors. The other set of sensors also shows a similar selective detection (Fig. 5C) with DENV-2 and DENV-4 serotype PCR amplified products (a shift of 0.51 V for DENV-2 and 0.43 V for DENV-4 in CVC were observed). This clearly demonstrations the 2-membrane sensor can selectively detect the presence of the two DENV serotypes simultaneously. Importantly, the DENV specific probe functionalized sensor only produced significant CVC shift (larger than 0.4 V) in the presence of target group, and there was no overlap between the target group and non-specific ZIKV samples (Fig. 5D).

3.5. Detection of four DENV serotypes using 4-membrane sensor

We further expanded the multiplex detection efficacy of the membrane sensor to differentiate simultaneously four DENV serotypes. The four DENV serotypes can be differentiated by RT-qPCR, though this remains challenging with other laboratory methods [40,41]. Further,

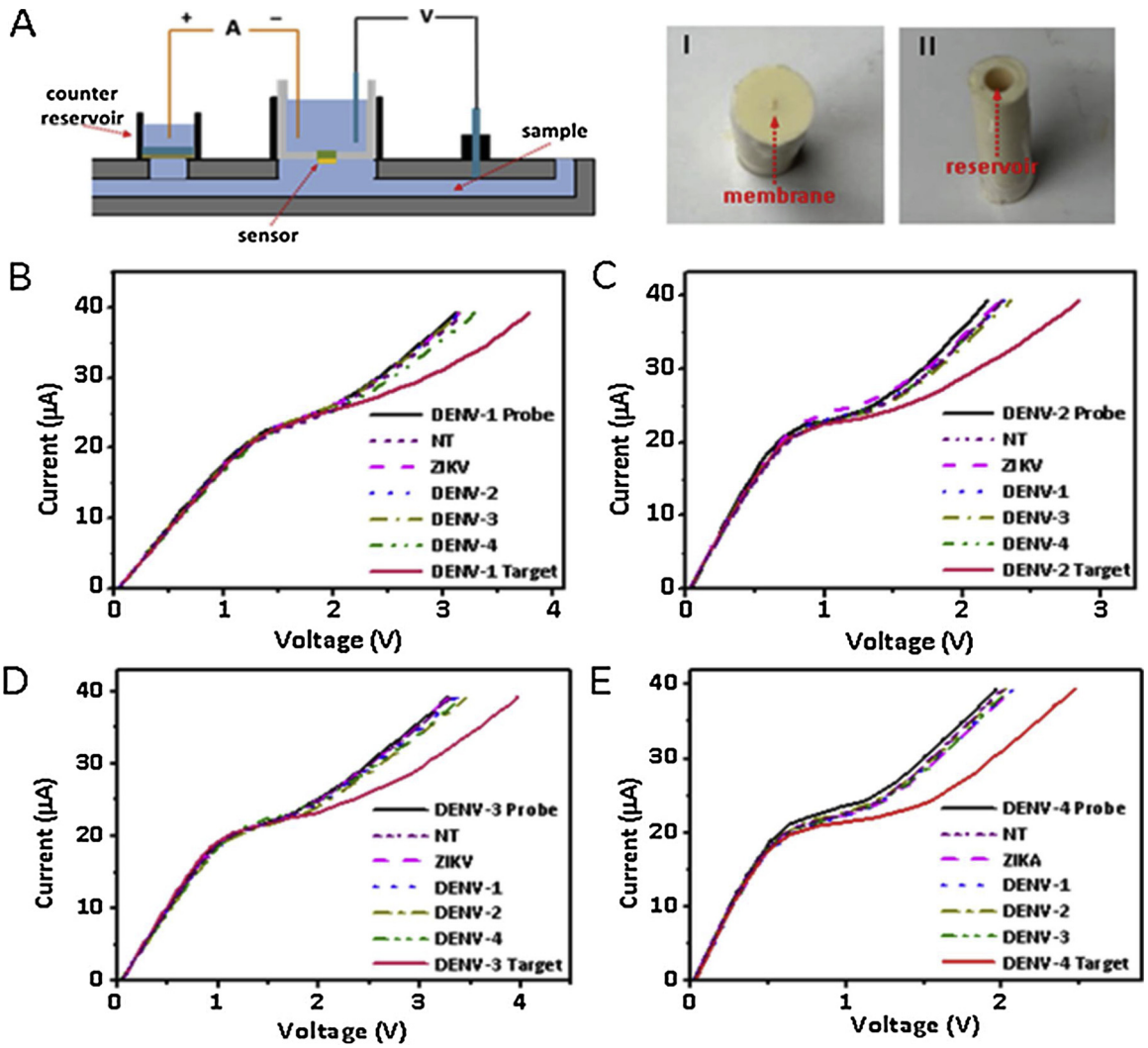

Fig. 3. Specific detection of each DENV serotypes using a membrane sensor. (A) Schematic and pictures of the sensor and reservoir. Specific detection of (B) DENV-1; (C) DENV-2; (D) DENV-3; (E) DENV-4. Serotype-specific detection of DENV indicating the sensor's capability to specifically detect denatured ssDNA target sequence from DENV PCR products.

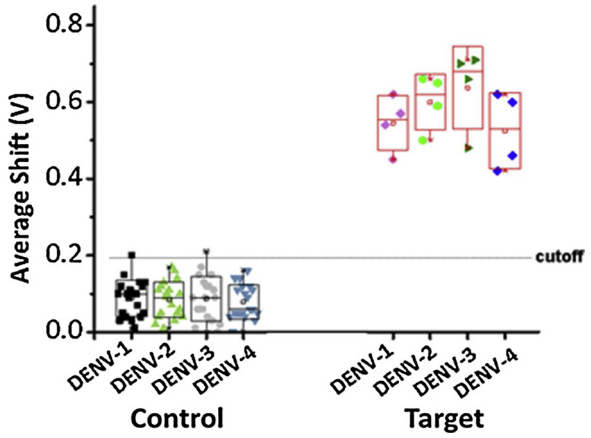

Fig. 4. Specificity and reproducibility of the nanomembrane sensor. Four different sensors for each of the DENV serotypes were tested by incubating the sensors with ssDNA sequences from different DENV serotypes. No overlap was observed between the target and control group for all DENV serotypes.

Table 1
Developments in dengue virus detection limit.

Sensing Technology	Limit of Detection	Reference
PCR	350 copies/mL	[32]
CDC RT-PCR	1×10^3 GCE/mL	[33]
ELISA	600 copies/mL	[34]
Optical Surface Plasmon Resonance	2×10^4 particles/mL	[35]
FCCS	9×10^9 particles/mL	[36]
Nanoporous Alumina Membrane	16×10^8 particles/mL	[37]
IEM Sensor	100 copies/mL	Current Technology

Abbreviations: PCRpolymerase chain reaction, CDC RT-PCRcenters for disease control and prevention real-time PCR, GCEgenome copy equivalents, ELISAEnzyme-linked immunosorbent assay, FCCSfluorescence cross-correlation spectroscopy, IEMion exchange membrane.

an extensive sample pretreatment step is necessary so that the isolated sample is pure enough for successful amplification of RT-qPCR, which limits its use for point-of-care application. Similar to the design of 2-membrane sensor, the 4-membrane sensor consists of four isolated reservoirs, as shown in Fig. 6A. By functionalizing individual probes on the membrane surface at different reservoirs, each of the membrane can

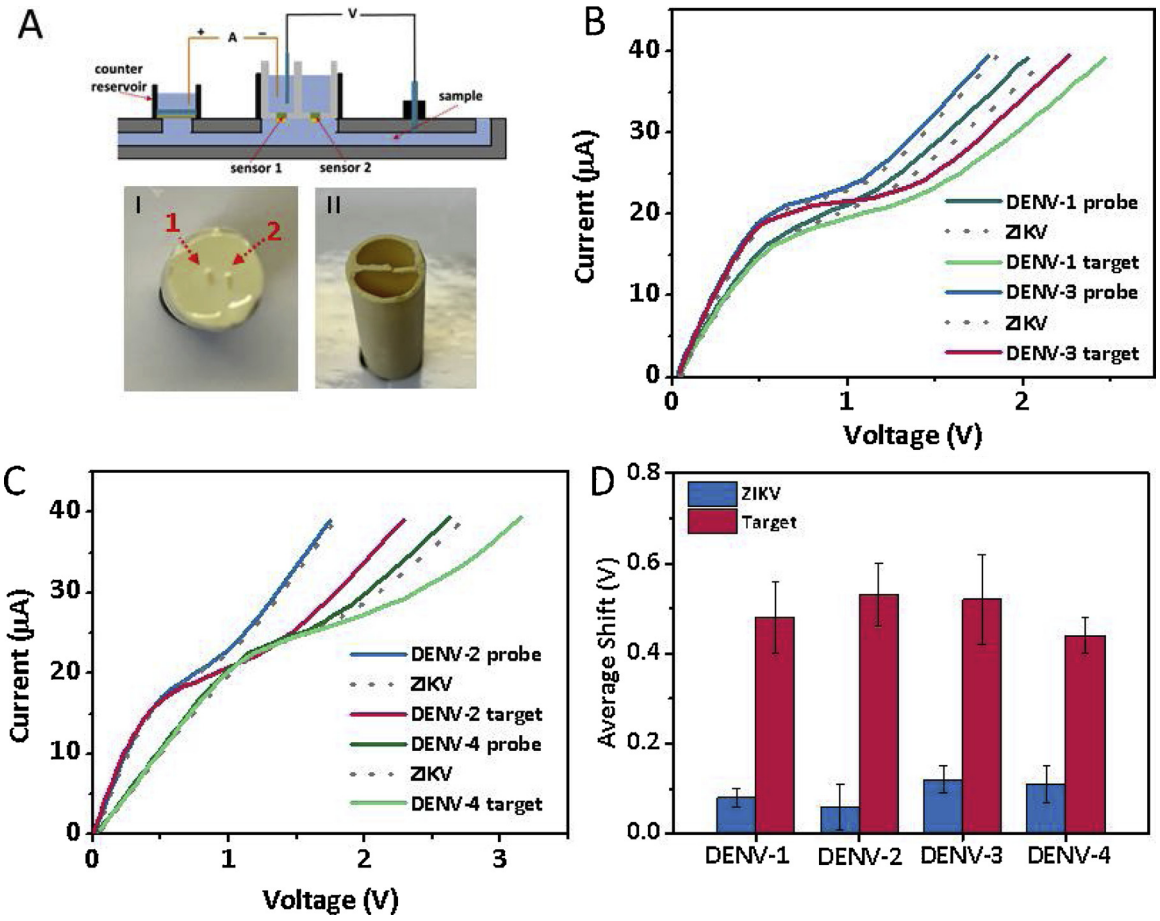

Fig. 5. Simultaneous detection of DENV serotypes using a 2-membrane sensor. (A) Schematic and pictures of the 2-membrane sensor and reservoir. (B) Simultaneous detection of DENV-1 and DENV-3. (C) Simultaneous detection of DENV-2 and DENV-4. (D) Average voltage shifts from probe level for four DENV serotypes (red column) and ZIKV (blue column).

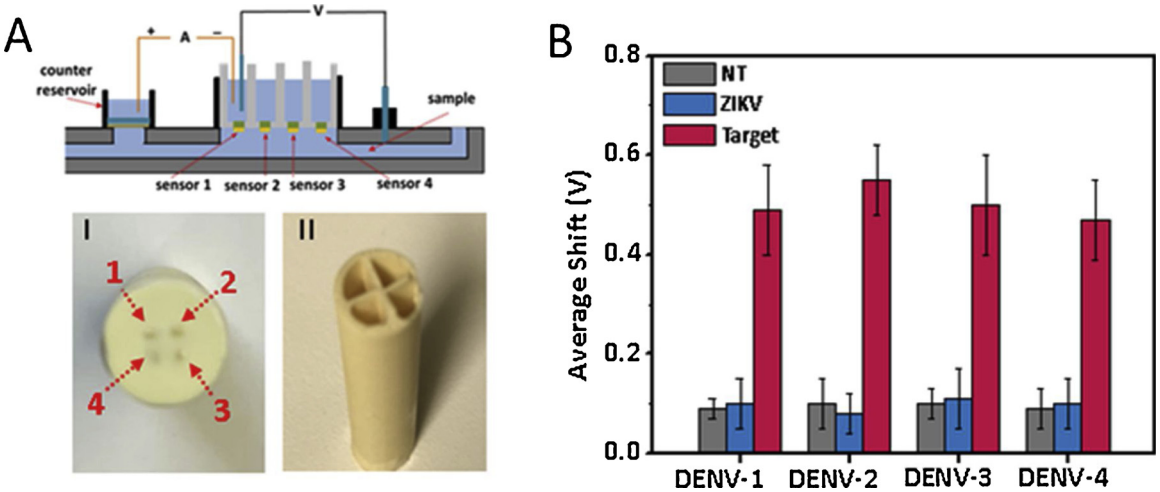

Fig. 6. Selective detection of four DENV serotypes with 4-membrane sensor. (A) Schematic and pictures of 4-membrane sensor and four isolated reservoirs. (B) CVC shifts from probe level for PCR amplicons of no DENV RNA template (grey column), ZIKV (blue column) and different DENV serotypes (red column) at a current of 40 μA.

serve as an independent sensor to differentiate different DENV serotypes from single run. Fig. 6B shows the result of CVC shifts from probe level for different PCR amplified product using the 4-membrane sensor. For this study, PCR amplified product with no template and ZIKV were used as a negative control. As expected, there were no significant CVC shifts observed from control groups, while each of the probe-functionalized membrane sensors showed significant CVC shift after incubating the sensor with the corresponding PCR amplified target product. More importantly, no overlap between the target and control group were observed. This confirms that the 4-membrane sensor is capable of differentiating four DENV serotypes with high detection selectivity.

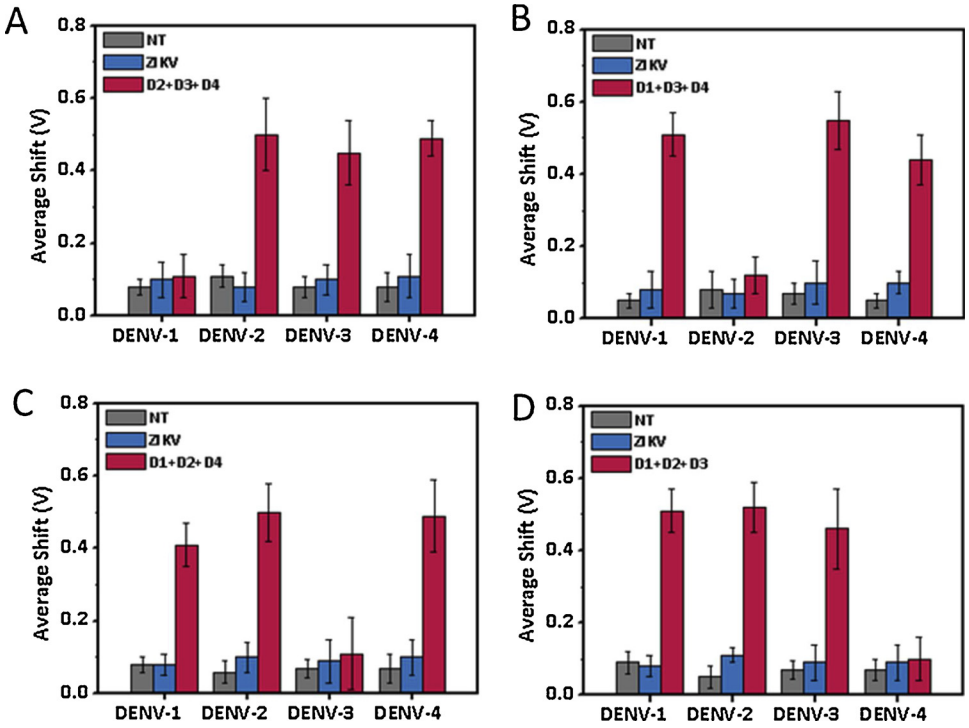

Fig. 7. Selective detection with 4-membrane sensor to differentiate four DENV (1–4) serotypes. Average voltage shifts from probe baseline is observed after incubating with a heterogeneous sample consisting of any three DENV serotypes indicate the differentiation of (A) DENV-1; (B) DENV-2; (C) DENV-3; (D) DENV-4.

In order to further evaluate the selectivity of the 4-membrane sensor and its capability of differentiating four DENV serotypes, we designed an experiment mimicking a practical sample consisting of three DENV serotypes amplified products. We functionalized the 4-membrane sensor surface with specific DENV-serotype oligoprobes of DENV-1, DENV-2, DENV-3 and DENV-4 respectively. Then a lab-created heterogeneous sample consisting of equal proportions (10 μL each) of the PCR amplicons of DENV-2, DENV-3, and DENV-4 was exposed with the 4-membrane sensor. Significant shifts in the CVC were observed for 0.51 V for DENV-2, 0.43 V for DENV-3 and 0.47 V for DENV-4 while no detectable shift was observed for DENV-1 probe sensor. This clearly confirms the 4-membrane sensor's ability to capture the specific target ssDNA sequence from the heterogeneous mixture (Fig. 7A). Three other sensors were tested with lab-constructed heterogeneous samples without the presence of ssDNA of DENV-2, DENV-3 or DENV-4, respectively. As expected, the sensors did not show any significant shift for the sample that does not contain the target DENV serotype (Fig. 7B, C and D). This further demonstrates the selectivity of the 4-membrane sensor in the presence of other non-specific arthropod-borne virus sequences, including three other DENV serotype sequences.

As DENV RNA in plasma is predominantly encapsulated within virions and hence, a lysing step is necessary to release the internal RNAs for their downstream analysis. In order to validate the performance of our dip and rinse RNA extraction technique with chemically lysed sample, we designed an experiment where DENV RNA spiked plasma sample is first treated with TRIzol™ lysing buffer and then dip and rinse technique is used to isolate the RNAs from the aqueous phase of TRIzol™-chloroform emulsion. This aqueous phase in addition to DENV RNAs also contains a higher amount of PCR inhibitor, Guanidinium thiocyanate, a chaotropic agent that is generally present in the TRIzol™ lysing buffer to denature proteins and stabilize RNAs. As seen Fig. 8A clearly shows the successful amplification of the DENV RNA (1–4) from the isolated samples, thus indicating that the dip and rinse technique is capable of isolating RNAs and removing PCR-inhibitors from the TRIzol™ treated sample. The chitosan modified positively charged filter paper preferentially captures the negatively charged RNAs and provides good quality sample for downstream PCR amplification. The presence of multiple bands in the gel image also suggests that the filter paper probably captured other nucleic acids from the plasma sample (could be due to use of lysing buffer TRIzol™ reagent, otherwise unexpected amplified products are mostly absent (see Fig. 2C). This non-specific amplification could be an issue if the targets were identified by RT-qPCR. However, since our nanomembrane sensor is functionalized with specific DENV oligoprobes, it allows to differentiate any primer dimerization and nonspecific amplification of non-targets by selectively capturing the DENV target amplified products. Fig. 8B clearly demonstrates the successful capture of the PCR amplified targets by the specific probe attached on membrane surface and provides an alternative strategy of identifying the correct targets even though the RT-PCR amplification is not very selective.

4. Conclusion

We have successfully developed a multiplexed and turn-key integrated on-chip PCR microfluidic platform for rapid extraction of DENV RNA from human plasma for selective detection of all four DENV serotypes in a single chip with a single PCR reactor that contains all the primer sets. A chitosan-modified filter paper was used to extract nucleic acids of different DENV serotypes. The dip and rinse technique can isolate template DENV RNAs from as low as 100 copies per mL of RNA spiked human plasma. DENV specific oligoprobes functionalized on 4-membrane sensors allow selective detection of four DENV serotypes against other non-specific arthropod-borne virus sequences with high detection specificity and sensitivity in a heterogeneous plasma sample. The selectivity of the sensor against primer dimerization and non-specific amplification also allows amplification of all four targets within a single PCR chip. Unlike optical sensors in RT-PCR, which is limited to less than 5 targets due to spectral overlap, the current membrane sensor design can be scaled up indefinitely to allow massively multiplex pathogen detection from a single test, adding an important tool for the diagnosis and monitoring of infectious diseases in a low-resource setting. Further, the use of electrical signal to identify PCR amplified

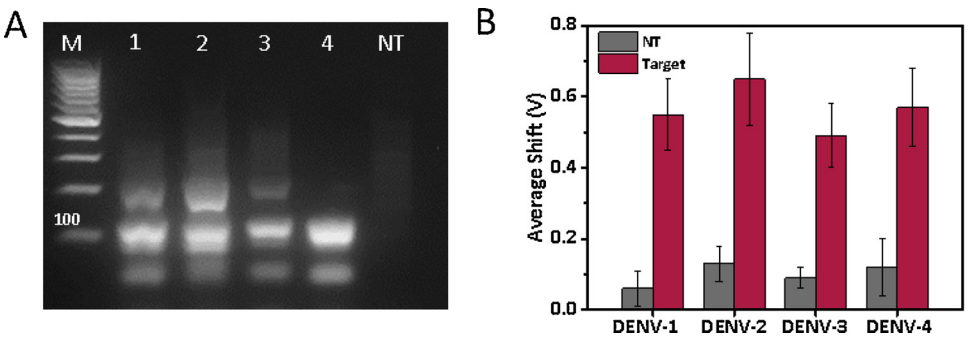

Fig. 8. Multiplex detection of four DENV serotypes from TRIzol™ treated DENV RNA spiked plasma samples with 4-membrane sensor. (A) On-chip RT-PCR amplification for all DENV (1–4) RNA serotypes with four primer sets after isolating DENV RNA (1–4) from aqueous phase of TRIzol™-chloroform treated plasma sample using chitosan-modified filter paper extraction process (Lane M: DL 1000 DNA marker; 1: DENV (1–4) RNA spiked in plasma; 2: DENV (1–4) RNA spiked in human whole blood; 3: DENV (1–4) RNA spiked in DI water; 4: pure DENV (1–4) RNA without TRIzol™ treatment; NT: no DENV RNA template. (B) CVC shifts from probe level for PCR amplicons of no DENV RNA template (grey column) and a heterogeneous sample consisting of four DENV serotypes extracted from plasma blood (red column) at a current of 40 μA.

products provide an option to run the whole assay on a battery powered system, making the platform suitable for point-of-care settings. While the use of DENV RNAs spiked plasma sample somewhat diluted impact of this study, we have nevertheless validated the effectiveness of our nanomembrane technology for detecting multiple targets from a single sample by one-step PCR. Further, our capability of successful RT-PCR amplification of the targets from the aqueous phase of TRIzol™-chloroform treated RNA spiked plasma sample clearly shows the potential of the platform to analyze plasma or whole blood samples infected with DENV. The multiplex target-specific probes allow the platform to be scaled up so that a large number of targets can be identified with a one-step procedure from a single sample. Our platform allows us to easily detect more than four-targets by placing multiple 4-membrane sensors in series. Our next step is to test the clinical samples and we are currently working towards this goal.

Declaration of Competing Interest

The authors declare no competing nonfinancial interests but do declare the following competing financial interests. Both S. Senapati and H.-C. Chang hold some stock in AgenDx Biosciences Inc., a startup biotechnology company that has licensed some parts of the proposed sensing platform.

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