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A heparin-functionalized carbon nanotube-based affinity biosensor for dengue virus☆

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ABSTRACT

Dengue virus is an arthropod-borne virus transmitted primarily by *Aedes* mosquitos and is major cause of disease in tropical and subtropical regions. Colloquially known as Dengue Fever, infection can cause hemorrhagic disorders and death in humans and non-human primates. We report a novel electronic biosensor based on a single-walled carbon nanotube network chemiresistive transducer that is functionalized with heparin for low-cost, label-free, ultra-sensitive, and rapid detection of whole dengue virus (DENV). Heparin, an analog of the heparan sulfate proteoglycans that are receptors for dengue virus during infection of Vero cells and hepatocytes, was used for the first time in a biosensor as a biorecognition element instead of traditional antibody. Detection of DENV in viral culture supernatant has similar sensitivity as the corresponding viral titer in phosphate buffer despite the presence of growth media and Vero cell lysate. The biosensor demonstrated sensitivity within the clinically relevant range for humans and infected *Aedes aegypti*. It has potential application in clinical diagnosis and can improve point-of-care diagnostics of dengue infection.

1. Introduction

Dengue virus (DENV) is a positive-sense, single-stranded RNA virus of the genus *Flavivirus* (family *Flaviviridae*) that is primarily transmitted by the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. Since the 1970s, it has spread to urban and semi-urban areas in over 100 countries and the incidence of dengue infections throughout the world has increased 30-fold (Bhatt et al., 2013). More than 3.6 billion people are at risk and an estimated 390 million infections occur annually. With no vaccine or specific treatment, early detection plays a significant role in decreasing fatality rates (Gubler, 2011; Sharp et al., 2015).

The World Health Organization has separated detection methods for diagnosing a dengue infection into two categories: indirect and direct. While the indirect methods can be rapid and accessible, the targeted human anti-dengue IgG and IgM are not readily detectable until approximately 7 days after the onset of fever during a primary infection (Guzman and Harris, 2015; World Health Organization, 2009). Thus, their use is limited to late-stage diagnosis; increasing patient mortality and further delaying implementation of outbreak control measures (Welch et al., 2014). Additionally, recent evidence indicates that sylvatic DENV amplifications can introduce new serotypes and correlate with outbreaks in nearby urban environments (Cardosa et al., 2009; Mustafa et al., 2015). Human IgG and IgM are not found in all potential clinical samples (e.g., *Aedes* mosquitos) and thus cannot be used to monitor the sylvatic cycle of the virus.

Direct detection methods are most useful when the virus is readily detectable in the serum, 0–7 days post infection (Blacksell, 2012), making them ideal for early diagnosis of DENV. These methods isolate and identify live virus by cell culture or detect components of the virus such as the viral genome by PCR or viral nonstructural proteins (NS1) by ELISA. However, PCR sensitivity varies between serotypes and neither PCR nor ELISA provide additional infectious viruses for future analysis and characterization (Kao et al., 2001). Isolation of viruses in cell culture is preferable and considered a gold standard detection method due its reliability, ability to propagate additional infectious virus for further study, high compatibility with a wide variety of sample sources, and its demonstrated ability to isolate DENV from mosquitos in field conditions (Philip Samuel and Tyagi, 2006; Race et al., 1978; Salje et al., 2014). Commonly, 6–10 days post infection, when samples containing virus concentrations as low as 1–10 pfu/ml have propagated to (Kao et al., 2005) detectable levels, the cell culture supernatant is screened for infection with immunofluorescent antibodies (IFA) (Gubler et al., 1984; World Health Organization et al., 2009). This

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delays detection and limits diagnostic capabilities. Additionally, IFA, ELISA, and PCR require temperature-controlled storage of samples and diagnostic devices/reagents, sophisticated equipment, and highly trained personnel.

Clearly, there is a need to develop low cost diagnostic devices capable of decreasing the time, equipment, and personnel training needed to detect DENV during the 0–7 days post infection window. Electronic biosensors are analytical devices that combine a bioreceptor with an electronic interface to convert a binding event of the target analyte into a measurable signal. They offer a portable, sensitive, reliable, and inexpensive diagnostic method with rapid detection capabilities. Biosensors using single-walled carbon nanotubes (SWNT) have demonstrated superior performance due to their excellent electrical properties and size similarity to the target of interest (Allen et al., 2007).

Biosensors commonly use antibody-based approaches to target analytes with high selectivity/specificity (Lee et al., 2011; Ramnani et al., 2015). However, antibodies are less than ideal for biosensing in field conditions as they are sensitive to denaturation by sunlight, changes in pH or temperature, as well as degradation by enzymes, metals, and other agents in a sample matrix (Vlasak and Ionescu, 2011; Wang et al., 2007). Heparin is a close structural homologue of heparan sulfate, a receptor for the four serotypes of DENV. Heparin has been shown to inhibit entry of DENV 1–4 into host cells and can bind tightly to DENV-2. Additionally, heparin is stable during storage in adverse conditions. Thus, it has excellent potential to act as the bioreceptor for DENV during biosensing (Chen et al., 1997; Hidari et al., 2013).

In this study, we report a highly sensitive, antibody-free, chemiresistive biosensor for the detection of the dengue virus. The biosensor consists of an SWNT network chemiresistor functionalized with heparin, instead of antibody, as the biorecognition molecule that detects the virus. SWNT network chemiresistor transducer was prepared using a previously reported self-assembly approach on lithographically patterned interdigitated gold electrodes (Ramnani et al., 2013). Heparin was side-on attached on SWNT network using a technique modified from (Pieper et al., 2000; Wissink et al., 2001) in which the carboxyl groups of heparin were cross-linked to primary amine groups on the pyrene-linker, 1-pyrenemethylamine, previously adsorbed onto the SWNTs. The performance of this biosensor was evaluated for detection of dengue type 1 virus in phosphate buffer and we demonstrate compatibility with DENV isolation in cell culture. Influenza virus H1N1 was used as a negative control to evaluate the biosensor selectivity.

2. Materials and methods

2.1. Virus propagation and titration

Vero cells (ATCC, CCL 81) were cultured at 37 °C and 5% CO_2 in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, buffered with sodium bicarbonate, 100 U/ml of penicillin, and 100 mg/ml streptomycin.

Vero cells were infected with dengue type 1 virus, TH-Sman (ATCC, VR 1586), using MEM with 2% fetal bovine serum (MEM-2). The supernatant was harvested at day 5 post-infection, clarified by low g-force centrifugation (10 min, 4,000g, 4 °C) and the cell debris pellet was discarded. The viral culture supernatant containing DENV was inactivated with 0.05% formalin (22 °C, 48 h) and stored at −80 °C until used for low titer laboratory viral culture detection measurements. Several DENV stocks were further concentrated to create high titer samples by ultracentrifugation in a 20% sucrose cushion in dH_2O (3.5 h, 100,715×*g*, 4 °C) to pellet the virus. Pellet was resuspended in 10 mM phosphate buffer, pH 7.4 (PB), aliquoted and stored at −80 °C. Viral titer was approximately 5.5×10^3 $TCID_{50}$/mL in low titer laboratory viral culture stocks and 8.4×10^5 $TCID_{50}$/mL in high titer stocks, as determined by $TCID_{50}$ with Vero cells in a 96-well plate.

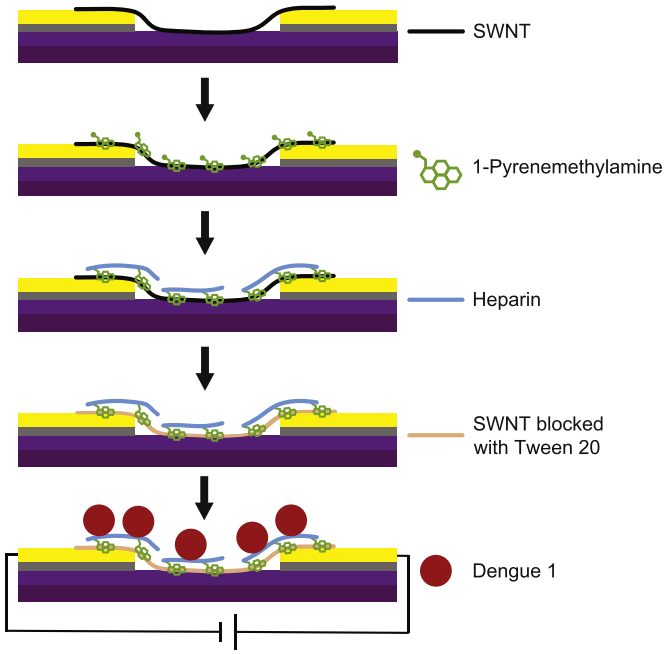

Fig. 1. Functionalization schematic representation of the fabrication of the chemiresistor used for detection of Dengue virus.

In order to mimic any potential variables from the DENV culturing and harvesting process, monolayers of Vero cells were washed with PBS and MEM-2 was added. The cells were freeze/thawed to simulate cytopathic effects and similarly clarified with low g-force centrifugation. The cell culture supernatant containing MEM-2 and Vero cell lysate was collected and stored in aliquots at −80 °C until used for control measurements or to create high titer viral culture stocks in which ultracentrifuged viral pellets were resuspended into aliquoted freeze-thawed Vero cell culture lysate.

2.2. Fabrication of SWNT biosensors

The microelectrodes and SWNT networks were fabricated according to a previously reported method (Ramnani et al., 2013) and subsequently functionalized with heparin following the protocol depicted in Fig. 1. Briefly, SWNT networks were first functionalized by non-covalent modification with 1-Pyrenemethylamine (Pyr-NH_2) (Sigma-Aldrich, USA) by incubating with 10 µL of 6 mM Pyr-NH_2 in dimethylformamide (DMF) for 1 h under high humidity conditions, followed by thoroughly washing with PB. Next, in a separate reaction tube ethyl-3-dimethylaminopropyl carbodiimide (EDC), N-hydroxy succinimide (NHS), and heparin were mixed in 2-morpholinoethane sulfonic acid buffer (pH 5.5) and incubated for 10 min prior to exposure to the previously modified SWNT for 2 h at room temperature. Devices were washed with PB then incubated with 0.1% Tween 20 (*BIO-RAD*) for 1 h to block any non-specific adsorption to the SWNT, followed by thorough rinsing with PB.

2.3. Detection of the virus

10 µL of sample was pipetted onto each biosensor to simultaneously cover each of the five microelectrode sensing regions, incubated for 10 min, and then gently washed with PB. 10 µL PB was then pipetted to cover the sensing region for resistance measurement. Measurements were taken in the dark at room temperature with the humidity controlled using a petri dish containing DI water. The resistance of each electrode was measured using linear sweep voltammetry with an electrochemical analyzer (Model 1202 A, CH Instruments, Inc., TX, USA). The voltage was swept from −200 to

200 mV and the current recorded. Linear regression analysis was used to calculate the slope of the current/voltage curves between −100 and 100 mV and inversed to calculate resistance. Relative resistance changes were calculated using the formula $\frac{R_1 - R_0}{R_0} * 100$, where R_1 is the resistance of the channel after each exposure and R_0 is the resistance of the PB control; in the case of the MEM-2, R_0 is the resistance of previous incubation.

3. Results and discussion

3.1. Heparin

Heparan sulfate is a variably sulfated glycosaminoglycan attached to some cell surface protein-core molecules and is utilized by DENV during invasion of host cells (Artpradit et al., 2013). Heparin, an analog of heparan sulfate, can inhibit DENV infection of Vero cells by incubation of the virus with heparin for 5 min prior to exposure to the cells. Furthermore, heparin has been shown to have high conformational flexibility and can significantly inhibit DENV infection at very low concentrations (50% inhibiting dose=0.3 μg/mL). Previous studies have shown an excellent binding affinity of DENV-2 to heparin conjugated albumin (K_d=56 nM) (Chen et al., 1997; Marks et al., 2001).

Fabrication of the biosensor was monitored with resistance measurements (Fig. 2). Consistent with the literature and our previously reported devices, the measurements show an increase in resistance after each step of functionalization; Pyr-NH_2, heparin, and Tween 20. This is attributed to π–π stacking interaction of the pyrene moiety of Pyr-NH_2 with the walls of the SWNT as well as electron donation to the SWNT and/or scattering potential generated by the immobilization of fabrication components (Gruner, 2005; Ramnani et al., 2013).

Heparin is highly sulfated; as a result, it has the highest negative charge density of any known biological macromolecule (Capila and Linhardt, 2002). DENV envelope protein has positively charged β-strands located within a well-conserved binding pocket for electrostatic interaction with receptor sulfate groups (Thullier et al., 2001; Zhang et al., 2013) but the net surface charge is negative (Kostyuchenko et al., 2013; Pereira et al., 2010). Thus, both the attachment of heparin and binding of DENV to the heparin receptor is expected to cause an increase in the resistance on p-type SWNTs. Since DENV is between 40 and 60 nm in diameter, only charges within the Debye length are expected to contribute to changes in the resistance (Stefansson et al., 2012).

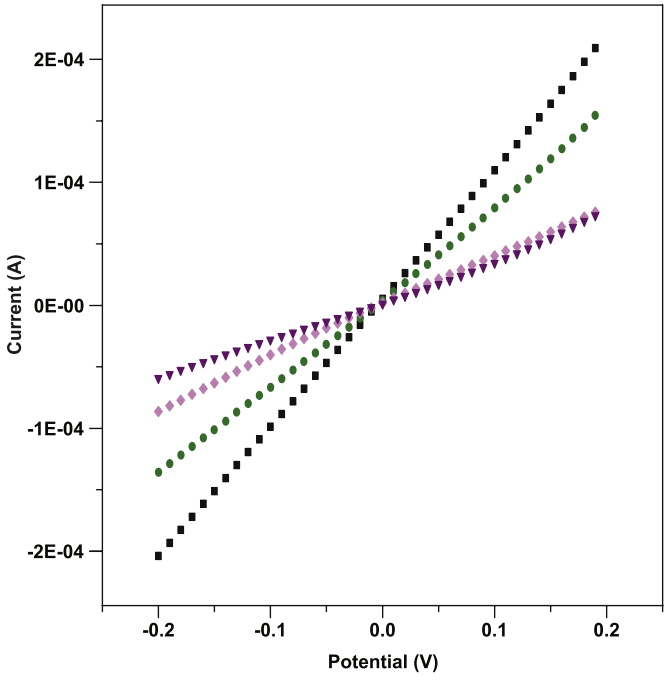

Fig. 2. I-V Curve of bare SWNTs in PB (■), after functionalization with 1-Pyrenemethylamine (●), immobilization of the heparin glycosaminoglycan (♦), and blocking of unoccupied sites with Tween 20 (▼).

3.2. Chemiresistor performance characterization

The heparin biosensor was first characterized in PB containing minimal potential inhibiting agents. Sensors were incubated with 10 μL PB to check for background signal, microelectrodes with ± 5%

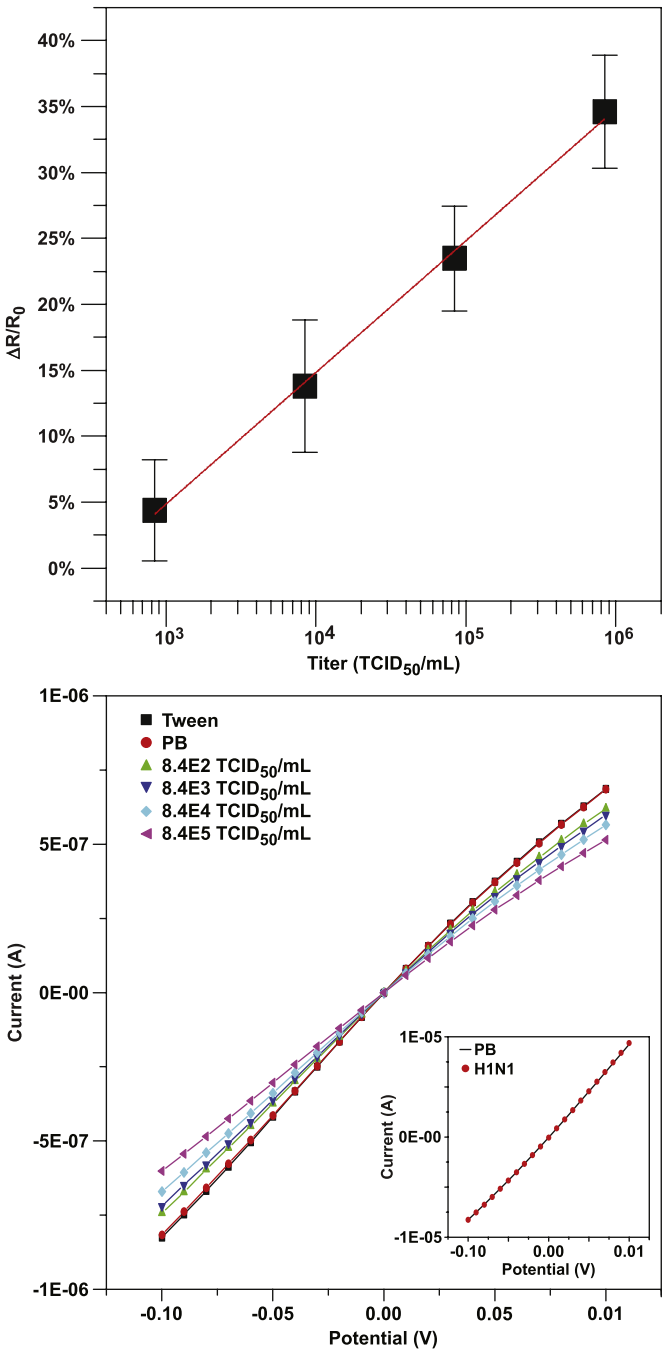

Fig. 3. Chemiresistor calibration plot and I-V curve. Upper: Resistance increase in response to incubation with dengue in phosphate buffer. The data points are the mean of measurements from 11 independent biosensors, and error bars represent ± 1 standard deviation (y=0.10x −0.25, R^2=0.998). Lower: Current-voltage characteristics of a single electrode to the titers of DENV. Insert: Current-voltage characteristics of a single electrode to H1N1.

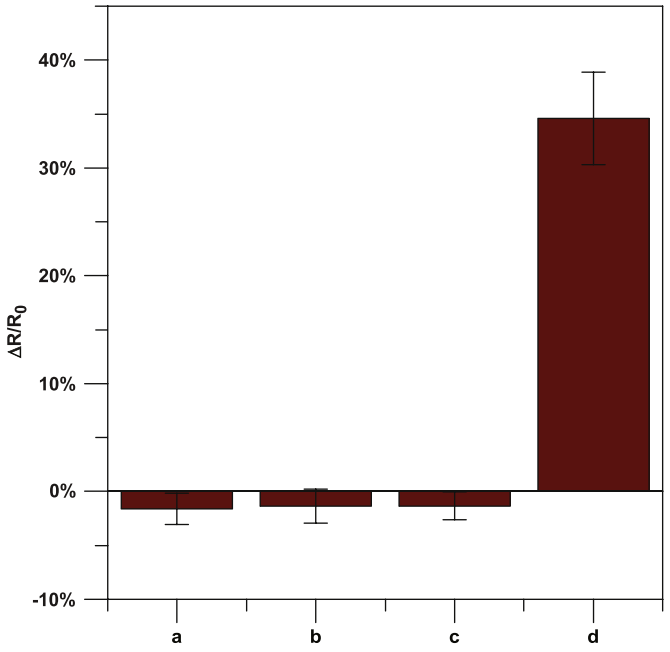

Fig. 4. Chemiresistor selectivity: (a) Response of Tween 20 functionalized chemiresistor to 8.4×10^5 $TCID_{50}$/mL dengue virus in phosphate buffer [n=7]. Response of heparin functionalized chemiresistor to: (b) Influenza A H1N1, (c) phosphate buffer, (d) 8.4×10^5 $TCID_{50}$/mL dengue in buffer. Unless noted, the data points are the mean of measurements from 11 independent biosensors, and error bars represent ± 1 standard deviation.

background noise were not used for virus sensing. As shown in Fig. 3, the response was a linear function of the logarithm of the viral concentrations between 8.4×10^2 $TCID_{50}$/mL and 8.4×10^5 $TCID_{50}$/mL ($y=0.10x-0.25$, $R^2=0.998$) with a sensitivity of 10% per log $TCID_{50}$/mL. The lowest concentration detected was 8.4×10^2 $TCID_{50}$/mL, which equates to approximately 8 $TCID_{50}$/chip. Sample titers were within the clinically relevant range for humans and infected *Aedes aegypti*: $1–10^9$ pfu/mL and $9\times10^3–10^4$ pfu/mL, respectively (Salazar et al., 2007; Xi et al., 2008).

Two control experiments were performed to confirm that the observed resistance increase was the result of specific heparin/DENV interaction. First, heparin functionalized biosensors were incubated with PB and second, SWNTs blocked by Tween 20 but without heparin receptors were incubated with DENV suspended in PB (Fig. 4). The negligible resistance changes of $-1.34\pm1.31\%$ and $-1.62\pm1.44\%$, respectively, were significantly different (n=11; $p<0.01$ and n=7; $p<0.01$, respectively) from the response to lowest viral titer detected, confirming the DENV did not interact with the biosensor in the absence of the heparin receptor.

To investigate biosensor selectivity, dialyzed H1N1 influenza virus (A/Puerto Rico/8/34) suspended in PB was incubated on heparin-functionalized sensor. H1N1 utilizes the negatively-charged sialic acid as the receptor for viral invasion of host cells. Incubation of H1N1 resulted in a negligible change in resistance of $-1.37\pm1.57\%$, which was not significantly different from the PB controls (n=11; $p>0.05$) (Fig. 4b). These results further confirm that the increase in resistance was due to attachment of DENV to the heparin receptor and there was no increase due to non-specific interaction by charged viral particles.

3.3. Detection of DENV after isolation from Vero cells

As a real-world application of the developed biosensor, we evaluated the detection of viruses in the presence of potential interferents that may be present in the viral culture process. For this application, the biosensor should be able to detect DENV in the presence of proteins, amino acids, and other compounds produced during the lytic cycle. Protocols for DENV isolation typically use low g-force centrifugation to clarify the virus from the cells used in viral culture. Low g-force centrifugation removes large cell organelles leaving virus particles, soluble proteins, and other cell lysates in the supernatant. Thus, any samples resulting from the viral culture supernatant are a complex mixture of DENV, MEM-2, and Vero cell lysates (ML+D). Control solutions taken from freeze-thawed Vero cell culture supernatant are a complex mixture of MEM-2 and Vero cell lysates (ML-).

3.3.1. Identification of matrix effects

In our initial experiments, we used the heparin-based biosensor to detect the virus in the ML+D with no pretreatment of the biosensor, i.e., without blocking. The result was a resistance increase much greater than when detecting the same DENV concentration in phosphate buffer (data not shown); indicating interference from chemicals in MEM-2 and/or compounds produced during the lytic cycle. To identify the source(s) of interference, a systematic analysis was performed using heparin-functionalized and Tween 20 blocked biosensors against MEM-2 alone and ML-.

Results of this investigation identified MEM-2 and ML- to cause major interference. Heparin-binding epidermal growth factor-like growth factor is a membrane-bound protein found in Vero cells (Goishi et al., 1995; Wang et al., 2006) and is expected to be present in Vero cell lysate. MEM-2 is a complex mixture of 21 amino acids, inorganic salts, and vitamins that have varying binding affinities for physisorption onto gold (Hoefling et al., 2010). Binding of compounds and nonspecific interaction with gold caused an increase in resistance due to electrostatic gating and/or modulation of the Schottky barrier formed in the SWNT-gold contact region; the response was consistent with p-type semiconductors reported in literature (Byon and Choi, 2006). It has been reported that modulation of the Schottky barrier by adsorption to gold near the SWNT-gold interface may contribute more to the biosensor response than targeted analyte-SWNT interaction (Heller et al., 2007). Thus, we and other researchers have blocked non-specific binding of chemicals in sample matrix by passivating gold with 6-mercaptohexanol. However, while the initial challenge of MEM-2 and ML- may have resulted in a large increase in resistance, a repeat challenge demonstrated minimal interaction with biosensors, both with or without heparin, even after multiple washings. This observation led us to investigate using MEM-2 for passivating the gold electrodes. The response to a second challenge of MEM-2 and ML- were not significantly different ($p>0.05$) but indicate that MEM-2 has a small matrix effect on the biosensors causing a background signal of $4.26\pm2.65\%$ increase in resistance (see supplementary information Fig. S1).

3.3.2. DENV detection

Viral detection was performed by sequential 10-min incubations in MEM-2, ML-, and ML+D on a heparin functionalized biosensor. ML+D was tested at two DENV concentrations; a low titer sample (5.5×10^3 $TCID_{50}$/mL) taken directly from viral culture and a simulated high titer (8.4×10^5 $TCID_{50}$/mL) sample created by spiking ML- with ultracentrifuged DENV harvested from the same viral passage as the PB spiked samples discussed in Section 3.2. The resistance increase after incubating was $18.39\pm3.91\%$ (n=8) for low titer ML+D and $39.07\pm6.06\%$ (n=8) for high titer ML+D. Using min-max normalization, the resistance change for these two DENV titers in the MEM-2/lysate matrix was similar to the increase in resistance for their respective titers in PB (Fig. 5) (low titer ML+D: p=0.61, high titer ML+D p=0.78). Additionally, a direct comparison of biosensor response after the incubation in high titer ML+D shows that the increase in resistance was $4.33\pm2.57\%$ higher than that of the equivalent titer of DENV spiked PB. This is not significantly different from the matrix effect identified with independent controls in section 3.3.1 (p=0.94). Thus, the difference in biosensor response to incubation in viral culture media and PB can be diminished further by removing the mean ± SD of the matrix effect. The mean ± SD of the difference is $14.14\pm4.72\%$ and $34.81\pm6.61\%$ for the low titer ML+D and high titer ML+D, respec-

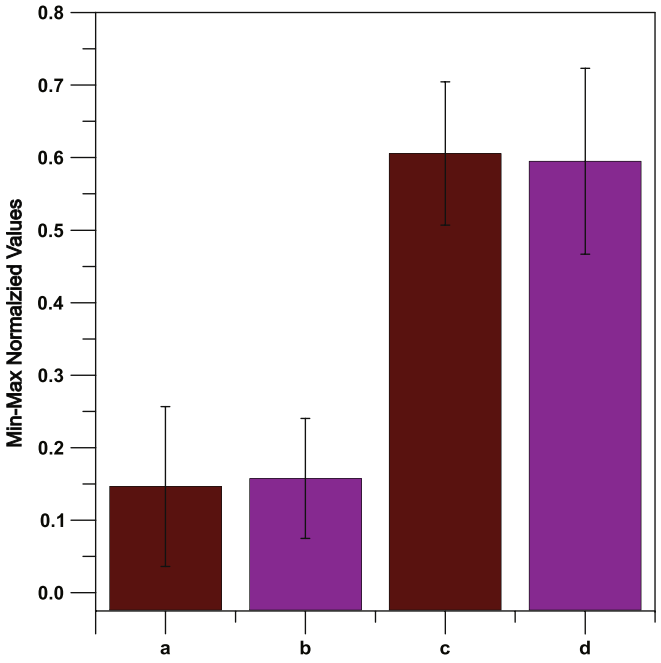

Fig. 5. Comparison of DENV spiked PB and MEM-2: Comparison of Min-Max normalized chemiresistor response to dengue in phosphate buffer [n=11] and to DENV suspended in MEM-2 containing Vero cell lysate (ML+D) [n=8]. (a) Response to 8.4×10^3 $TCID_{50}$/mL DENV in PB, (b) 5.5×10^3 $TCID_{50}$/mL ML+D, (c) 8.4×10^5 $TCID_{50}$/mL DENV in PB, (d) 8.4×10^5 $TCID_{50}$/mL ML+D. The data points are the mean of measurements from independent biosensors, and error bars represent ± 1 standard deviation.

tively, which is not significantly different from equivalent titers in PB (low titer ML+D: p=0.72, high titer ML+D p=0.69). Incubation of ML+D on biosensors without heparin resulted in an increase of resistance that was no different than the matrix effect (see supplementary information).

The lowest concentration detected was 8.4×10^2 $TCID_{50}$/mL (~8 DENV/chip) with only a 10-min incubation. This was not only more rapid but was also more sensitive than similar SWNT-based whole virus immunosensors reported in literature for herpes simplex virus type 1 (Bhattacharya et al., 2011) and influenza H1N1 (Lee et al., 2011). These immunosensors use antibodies that are 5–9 nm in length, which can prevent or decrease the portion of the target analyte from binding within the Debye length of the biosensor resulting in a decreased electrostatic gating effect and decreased sensitivity. When using side-on attachment, the distance between binding sites on heparin and the SWNT surface is ~1 nm. Thus, larger portions of virus are within the detectable region of the heparin-based biosensor, increasing sensitivity through the electrostatic gating effect. Recently, biosensors for DENV monitoring have been described. Loureiro et al., (2016) reported a surface plasmon resonance (SPR)-based immunosensor integrated with a microfluidic cell (for transport of reagents and sample) for a label-free detection of DENV with a limit of detection of 2×10^4 viral particles/mL, a concentration relevant to acute infection phase. However, antibodies are subject to denaturing and aggregation after only a few weeks of room temperature storage (Thiagarajan et al., 2016; Wang et al., 2007). Navakul et al. (2016) reported a molecularly imprinted polymer (MIP)-graphene based electrochemical sensor for DENV and anti-DENV antibodies detection. The sensor was reported to detect DENV ranging from 1 to 2000 pfu/mL and between different DENV serotypes and H5N1. While an MIP as a recognition element has the advantage of eliminating antibody transducers, it lacks the specificity and binding affinity of antibodies (Kryscio and Peppas, 2012; Navakul et al., 2016). Previous researchers have tested heparin for heat stability and it maintained integrity for 250 h at 100 °C. Heparin is inexpensive and stable for years at room temperature and in direct sunlight, both as dry powder and sterile solution, as long as the pH of the solution does not become acidic (Akeel et al., 2013; Fu et al., 2014; Jandik et al., 1996; Pritchard, 1964). Cheng et al., (2015) developed an impedimetric biosensor utilizing a poly-L-lysine modified screen-printed carbon electrode to monitor BHK-21 cells for cytopathic effects (CPE) of DENV propagation in real-time. The biosensor similarly relies on *in vitro* propagation of DENV; giving the advantage of compatibility with clinical samples. However, while the sensitivity to CPE is high, reliance on CPE dictates that other methods must be used to determine if observed CPE is caused by the propagation of DENV.

Viral isolation and plaque assays are gold standards for detection and diagnosis of a viral infection. As such, they are widely used for isolation of DENV from a variety of clinical samples; such as rhesus macaques, *Aedes* mosquitos, and human fluid and tissue samples (Martina et al., 2009). Incorporation of viral culture into sample analysis expands the biosensor's capabilities to cover many potential sample sources and can allow amplification of the viral titer from a low pfu/mL into detectable limits. However, DENV isolated from these sources can result in a range of titers depending on host cell line, day harvested, serotype, and subtype (Putnak et al., 1996). Thus, we have demonstrated that our biosensor is viral culture compatible and capable of detecting DENV in a range of titers from a 10-μL sample in 10 min without antibodies. Small samples can be easily withdrawn from a viral culture and analyzed much sooner than IFA, without disrupting viral propagation and reducing the need for a large numbers of cultures. Thus enabling facile and affordable detection within the 0–7 days post-infection period and monitoring the disease in the sylvatic cycle using the same chemiresistive biosensor. With further study, direct analysis of these samples may be possible with heparin-based biosensors.

4. Conclusion

This study demonstrates the successful application of a carbon nanotube-based chemiresistor functionalized with heparin for sensitive detection of DENV. The electrical resistance increased during each step of functionalization and upon the binding of DENV to the heparin receptor. With only a 10-min incubation of a 10 μL sample, a limit of detection of 8.4×10^2 $TCID_{50}$/mL (~8 DENV/chip) was achieved for detection of DENV suspended in PB. The selectivity tests also indicated that a functionalized sensor was responsive to the presence of DENV but not Influenza H1N1. Additionally, the biosensor was successfully applied for the detection of DENV titers in Vero cell culture and stock after only a simple low g-force centrifugation to remove large Vero cell organelles. Ultimately, the SWNT-heparin chemiresistor can be used as a low-cost, label-free, ultra-sensitive, and electric detection of DENV. SWNT-based electronic biosensors are an important step towards achieving a point-of-care detection as well as enhancing the capabilities of existing surveillance programs for monitoring pathogens with sylvatic cycles. As monitoring systems improve and the resolution of the data increases, modelling and predicting the outbreak of zoonotic diseases becomes increasingly viable.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://doi:10.1016/j.bios.2017.01.017.

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