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Development of Carbon Nanotube-Based Biosensors to Detect Dengue Virus

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ABSTRACT OF THE DISSERTATION

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by

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Since 1970, human activities have permitted the spread of Dengue virus (DENV) and the primary mosquito vectors *Aedes* spp. to virtually every continent. Infection rates have increased more than 30-fold and it has become the most prevalent arboviral disease in the world. Every year, 3.6 billion people are at risk of infection and there are 390 million new infections, mostly among children. With no vaccine or specific treatment, early detection plays a significant role in decreasing fatality rates. Dengue infection has no pathognomonic clinical features, thus diagnostic tools are essential for diagnosis.

In addition to a human transmission cycle, a variety of forest-dwelling non-human primates are hosts for DENV in a sylvatic cycle. Unfortunately, sylvatic cross-over events occur regularly and have resulted in disease outbreaks within humans. Vector surveillance plays a critical role in dengue detection and outbreak prevention. Current laboratory methods for detection and diagnosis of DENV require highly trained personnel and costly equipment that are impractical for regular surveillance and diagnostic use.

Thus, new technologies to facilitate and enhance diagnostic and surveillance capabilities within each transmission cycle are urgently needed. This research describes the development of two novel biosensors using single-walled carbon nanotube transducers functionalized for the detection of whole DENV or DENV Non-Structural Protein 1 (NS1). Heparin, an analog of the heparan sulfate proteoglycans that are receptors for DENV, was used as a bioreceptor for detection of whole DENV virions within viral culture. This permits detection of DENV virions from a variety of viral culture-compatible samples; such as fluid or tissue samples from monkeys, vector mosquitos, and humans. Anti-dengue NS1 monoclonal antibodies were used to detect DENV NS1, a clinically accepted biomarker for DENV infection. This biosensor will allow early detection and diagnosis of the disease in *Aedes* mosquitos and human saliva. Both biosensors were selective and sensitive for their target analyte in a 10-μL sample over the clinically relevant concentration range with detection occurring in only 10-20 min. Each was constructed to be a portable, rapid, and inexpensive diagnostic tool suitable for use by minimally-trained personnel in the field, laboratory, or point-of-care location.

Table of Contents

List of Figures

List of Tables

Abbreviations

1D: One-Dimensional
APTES: 3-Aminopropyltriethoxysilane
BSA: Bovine Serum Album
C: Dengue Capsid Protein
CPE: Cytopathic Effects
DENV1-5: Dengue virus
DHF: Dengue Hemorrhagic Fever
DMF: Dimethylformamide
DSS: Dengue Shock Syndrome
E: Dengue Envelope Protein
EDC: Ethyl-3-dimethylaminopropyl Carbodiimide
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: Fetal Bovine Serum
GAC-ELISA: IgG Antibody-Capture Enzyme-Linked Immunosorbent Assay
GAG: Glycosaminoglycans
H1N1: H1N1 Influenza Virus
HI: Hemagglutination-Inhibition Assay
HRP: Horse Radish Peroxidase
HS: Heparan Sulfate
IFA: Immunofluorescence Antibody Assay
IgG: Immunoglobulin G
IgM: Immunoglobulin M
LFIA: Lateral-Flow Immunoassay
LOD: Limit of Detection
MAC-ELISA: IgM Antibody-Capture Enzyme-Linked Immunosorbent Assay
MEM: Minimal Essential Medium with 10% Fetal Bovine Serum
MEM-2: Minimal Essential Medium with 2% Fetal Bovine Serum
MES: 2-Morpholinoethane Sulfonic Acid Buffer
MIP: Molecularly Imprinted Polymer
ML-: A mixture of MEM-2 and Vero cell lysates without DENV
ML+D: A mixture of MEM-2 and Vero cell lysates with DENV
NHS: N-hydroxy succinimide
NS1: Dengue Virus Non-structural Protein 1
NS1-5: Dengue Non-Structural Protein
PB: Phosphate Buffer
PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction
PLL: Poly-L-Lysine
prM/M: Dengue PreMembrane/Membrane Protein
Pyr-COOH: 1-Pyrene Butanoic Acid
Pyr-NH2: 1-Pyrenemethylamine
Pyr-NHS: 1-Pyrenebutyric Acid N-hydroxysuccinimide Ester
QCM: Quartz Crystal Microbalance
RDT: Rapid Diagnostic Test
RNA: Ribonucleic Acid
RT-PCR: Reverse-Transcription Polymerase Chain Reaction
SERS: Surface Enhanced Raman Spectroscopy
SPR: Surface Plasmon Resonance
SWNT: Single Walled Carbon Nanotube
$TCID_{50}$: 50% Tissue Culture Infectious Dose
Vero: Cell line derived from the African green monkey kidney
WHO: World Health Organization

Chapter 1: Introduction

1.1 Dengue

Dengue virus (DENV) is a member of the genus *Flavivirus* (family *Flaviviridae*) and is primarily transmitted by the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. There are four confirmed serotypes of DENV, dengue-1, 2, 3, and 4. While each shares around 65% of its genome with the other serotypes, they most likely emerged independently from the sylvatic cycle (Holmes and Twiddy, 2003). A possible fifth serotype (DENV-5) was isolated from a patient in Malaysia in 2013. DENV-5 is genetically similar to the other serotypes and is theorized to have recently emerged into the human cycle from rhesus macaque monkeys (Mustafa et al., 2015). Each serotype can be further categorized into genotypic categories that have yet to be formally named but are classified as "a group of dengue viruses having no more than 6% sequence divergence" (Holmes and Twiddy, 2003). As an RNA virus, DENV is highly variable and even within a serotype, the genotype of a virus can be highly divergent (Pyke et al., 2016).

DENV infection is a major cause of disease in tropical and subtropical regions. Considered a re-emerging disease, transmission among humans has greatly intensified. More than 3.9 billion people are at risk and an estimated 390 million infections occur annually, making dengue the most important viral disease transmitted to humans by mosquitoes (Simmons et al., 2012). While the majority of infections are asymptomatic or subclinical, roughly one-fourth of those infected experience dengue fever with mild to severe arthralgia and myalgia 4 to 9 days post-infection (Bhatt et al., 2013). The severe

pain associated with infection gives the disease its popular name, “Break-bone Fever”. A minority of those infected progress to “severe dengue”, previously known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Severe dengue is characterized by plasma leakage and hemorrhagic tendencies that often result in death if not properly diagnosed and treated (Gubler et al., 2014). Unfortunately, the broad range of symptoms makes clinical diagnosis by pathognomonic features extremely difficult and clinicians cannot predict which patients will progress to severe dengue. Thus, laboratory confirmation is highly recommended (World Health Organization, 2009). To further complicate treatment of a DENV infection, patients infected with one serotype develop a robust and long-term immunity to the homologous serotype but not to the other three. The antibodies developed during the primary infection can cross-react with the other serotypes; and upon a secondary infection by another serotype, there is an increased risk of developing severe dengue (Libraty et al., 2002a). Fluid replacement therapy is the only recognized treatment. Early diagnosis and intervention can reduce mortality from 20% to below 1% (Guzmán and Harris, 2015).

1.2 Dengue Structure

DENV is a spherical, enveloped, positive-sense, single-stranded RNA virus approximately 40-50nm in diameter (Kuhn et al., 2002). The genome is encapsulated by capsid (C) proteins and contains approximately 11,000 bases and encodes for a large, self-cleaving polyprotein. This polyprotein contains three structural proteins and seven nonstructural proteins within a single open reading frame along with 5' and 3' untranslated

regions. While DENV is an enveloped virus, the lipopolysaccharide envelope is sandwiched between the C proteins and an outer layer of premembrane/membrane (prM/M) and envelope (E) proteins. The outer protein layer has a jagged appearance on immature virions; the prM and E proteins form protruding trimeric spikes. Upon maturation of the virus, pr is released and the M and E protein fold to lie flat against the envelope resulting in a smooth surface. The E protein consists of 3 domains that occupy the majority of the viral surface. Domain I of the E protein provides structural flexibility, domain II contains the hydrophobic viral fusion peptide for cellular entry, and domain III has protein motifs for binding to cellular receptors in the mammalian and mosquito hosts. Neutralizing antibodies developed during the human immune response commonly target the E protein. The DENV virions do not contain nonstructural proteins (NS); instead they are produced by host cell machinery during the initial translation of the viral genome. The NS proteins are largely responsible for viral replication by acting as a RNA-dependent RNA polymerase, hijacking cellular machinery, and subverting host defenses (Modis et al., 2005; Zhang et al., 2013).

1.3 The origin and emergence of dengue virus

The most widely accepted theory for the origination of *Flavivirus* is based on phylogenetic studies that suggest it originated approximately 10,000 years ago as a non-vectored mammalian virus in Asia or Africa (Gould et al., 2003). However, more recent analyses performed with a relaxed molecular clock and Bayesian methods on the genome

of tick-borne flaviviruses suggest a much older origination, perhaps as many as 158,900 years ago (Heinze et al., 2012; Pettersson and Fiz-Palacios, 2014).

Ancestral human-cycle DENV is thought to be the result of a cross-species transmission from monkeys in Asia approximately 1000 years ago (Holmes and Twiddy, 2003; Twiddy and Holmes, 2003). The oldest description of dengue fever-like illness comes from 265-420 A.D. in China. DENV emerged into the "New World" approximately 300 years ago via the African slave trade. Consequently, the first DENV infection to be well-documented was recorded in Philadelphia, Pennsylvania, USA by Benjamin Rush in 1780 (Gubler, 1998; Vasilakis et al., 2011).

Prior to World War II, DENV epidemics were large but infrequent, primarily centered around hubs for mass transit. The majority of affected regions with autochthonous circulation had one or two serotypes in co-circulation. The outbreaks were so infrequent that DENV was not considered a major public health problem (Gubler, 2011b; Beaumier et al., 2014). During the war, thousands of DENV infected soldiers moved rapidly between cities and countries, introducing new serotypes and creating the beginnings of the global DENV pandemic (Sabin, 1952). Following the war, unplanned urbanization, housing with inadequate protection against the mosquito vector, and the deterioration of sewer and water management systems provided ideal breeding grounds for both DENV and *Aedes* spp. in Southeast Asia. It is in these conditions that a new disease emerged: the first established epidemic of DHF occurred in 1954 in Manila, Philippines. By the 1980s, the leading cause of hospitalization and death for children in Southeast Asia was DHF. By 1994, air travel

had introduced multiple strains of each DENV and the Americas became hyperendemic (Gubler, 2011b; Gubler, 2011c; Gubler, 2011d; Simmons et al., 2012).

More recently, global warming, population growth, and other human activities have further resulted in habitat expansion for the vector mosquitoes (Bai et al., 2013; Ramasamy and Surendran, 2016). In 2013, Europe reported autochthonous transmission of DENV in Croatia and France, the first autochthonous cases since 1928 (Bonizzoni et al., 2013; Succo et al., 2016). Furthermore, habitat modeling predicts further geographic expansion of the *Aedes* spp. habitat range. Failure to control the spread of *Aedes* spp. has resulted in the DENV transmission range to be similarly expanded (Gubler, 2011a, d; Bonizzoni et al., 2013).

1.4 Transmission Cycles

1.4.1 Sylvatic Transmission

Each of the four DENV serotypes is maintained in two ecologically and evolutionarily distinct transmission cycles: a sylvatic cycle and a human cycle (also referred to as the urban cycle). Phylogenetic research has identified strains of sylvatic DENV that are distinct from strains found in the human cycle (Rico-Hesse, 1990). Despite this, the strains from each cycle are remarkably similar. Both zoonotic and anthroponotic transmission can occur without major genetic and phenotypic changes. Studies have shown that DENV from the human cycle can readily infect monkeys, and sylvatic DENV has been identified in human patient samples on a number of occasions (Vasilakis et al., 2008; Franco et al., 2011; Durbin et al., 2013; Hanley et al., 2014; Mustafa et al., 2015).

Sylvatic DENV was first identified with serosurveys of arboreal monkeys in Penang, Malaya. Smith (1956) noted that while canopy-dwelling monkeys tested seropositive, very few ground-dwelling animals tested seropositive, suggesting DENV circulation primarily within canopy. Similarly, Rudnick (1965) was performing a serosurvey in Malaysian forest reserves and found DENV-neutralizing antibodies in wild arboreal monkeys, implicating a sylvatic transmission cycle. Direct evidence of a sylvatic cycle was finally obtained in 1975 by isolation of sylvatic DENV strains from sentinel monkeys placed in the forest canopy (Rudnick et al., 1986). Within the same year, direct evidence of the link between canopy-dwelling and ground-dwelling mammals was found when DENV4 was isolated from *Aedes niveus,* a mosquito that preferentially feeds within the canopy but will opportunistically feed on ground-dwelling mammals (Rudnick, 1986).

Sylvatic DENV strains circulate between non-human mammals and various *Aedes* spp. within a given region. A massive mosquito surveillance program was initiated in the savannah and forest galleries located in the southeastern part of Senegal in Kedougou. Diallo et al. (2003) isolated DENV2 in 4 species of mosquitoes in the forest gallery. Surprisingly, the biting activity of *Ae. aegypti* mosquito (a primary vector in the human cycle) was limited and *Ae. furcifer* was found to bite humans at the highest rate, especially in villages. Thus developed the first model for sylvatic transmission of DENV, cross-over events, and evidence that sylvatic transmission of DENV has separate epizoonotic amplification events that do not always result in a spill over into humans. While the mammal and mosquito species differ from region to region, the transmission cycle follows a similar pattern. (Figure 1.1) A few mosquito species preferentially lay their eggs and feed

on non-human mammals within the forest environment. Some mosquito species preferentially feed peridomestically, feeding both within the forest and within human settlements that border the forest. These species act as a zoonotic vector into the human cycle and anthroponotic vector into the sylvatic cycle. *Ae. furcifer* and *Ae. luteocephalus*, two species known to occupy this role, are highly susceptible to DENV from both cycles. Finally, certain mosquito species (*Ae. aegypti* and *Ae. albopictus*) feed preferentially within urban environments and are almost exclusively vectors in the human transmission cycle (Diallo et al., 2003; Diallo et al., 2005; Hanley et al., 2013).

Contribution of the sylvatic cycle of dengue to the overall human disease burden is controversial and poorly understood. It has been long thought that the sylvatic cycle is maintained sufficiently away from human contact that no major epidemics have been associated with sylvatic DENV (Rico-Hesse, 1990; Vasilakis et al., 2008; Vasilakis et al., 2011). However, mounting evidence indicates that sylvatic DENV amplifications can introduce new subtypes and correlate with outbreaks in nearby urban environments (Diallo et al., 2003; Cardosa et al., 2009; Althouse et al., 2012; Durbin et al., 2013; Mustafa et al., 2015).

1.4.2 Human Transmission

Ae. aegypti mosquito has been highly domesticated. It prefers to rest indoors, feed on humans, and lay its eggs above the water line in artificial containers that are likely flood to such as tires, trash, and flower pots. The adult mosquitos are predominantly found in urban environments and the eggs are very tolerant to high humidity and high temperatures.

Thus, it is considered to be one of the primary vectors in the human transmission cycle, especially in warmer climates. (Figure 1.2) The female *Ae. aegypti* mosquito has been described as a "nervous feeder": the slightest sign of movement or danger will cause the mosquito to flee. Consequently, during a single blood meal the mosquito may feed or probe without taking blood on several individuals (Putnam and Scott, 1995; Platt et al., 1997). This may result in the infection of multiple individuals from a single infected mosquito. Once bitten by an infected mosquito, it takes 4 to 9 days before DENV can be detected in peripheral blood. If a mosquito feeds on this individual, in 8 to 12 days, the mosquito itself may become infectious and able to transmit the virus.

The second major mosquito vector, *Ae. albopictus*, prefers the peridomestic environment such as rural, suburban, and vegetated urban areas. It is an opportunistic feeder, feeding on any organism it can locate such as birds, reptiles, and amphibians, although preferring mammals and especially humans (Delatte et al., 2010). Thus, it is considered secondary to *Ae. aegypti*. However, *Ae. albopictus* mosquito eggs can survive in much colder temperatures by the use of diapause, a survival strategy in which the eggs go dormant during cold weather. (Figure 1.2) Thus, *Ae. albopictus* is able to survive in subfreezing and dry climates (Braks et al., 2003). When the *Ae. aegypti* mosquito is rare or absent, *Ae. albopictus* can be the primary vector in the human cycle; such as the autochthonous European outbreaks discussed previously (Hawley et al., 1987; Effler et al., 2005; Benedict et al., 2007; Gjenero-Margan et al., 2011).

Both *Ae. aegypti* and *Ae. albopictus* have shown the ability to vertically transmit DENV to their offspring. At this time, the mechanism of vertical transmission is poorly understood but the most widely accepted is transovarial transmission in which the developing eggs are infected by DENV. However, infection of the mature egg could occur at the time of oviposition. Vertical transmission has been demonstrated in both laboratory and wildtype populations and grants DENV the ability to survive weather conditions lethal to adult mosquitoes by overwintering within the eggs (Joshi et al., 2002; Martins et al., 2012). A vertically transmitted DENV infection persists into adulthood can be transmitted during feeding behaviors (Kyle and Harris, 2008).

1.5 A Brief Overview of Dengue Replication

When an infected mosquito feeds on a human, it injects DENV as well as a number of salivary proteins into the blood and surrounding tissues to aid in the acquisition of a blood meal. These proteins act as an anti-inflammatory, anti-coagulant, vasodilatory, and most importantly to DENV, an immunomodulatory substance (Schneider and Higgs, 2008; Sim et al., 2012). Immunomodulating proteins such as Interleukin-10 and -4 can suppress T-cell activation and interferon expression to diminish host immune response, consequently promoting DENV infection of the host (Ejrnaes et al., 2006).

Once in the blood stream, the first step in DENV infection of the human host is to bind to a cellular receptor. A large number of potential DENV receptors have been identified on a wide variety of human cells (Cruz-Oliveira et al., 2015) including but not limited to, the heparan sulfate glycosaminoglycan on hepatic cells, (Chen et al., 1997) DC-

SIGN, an adhesion molecule for dendritic cells, (Navarro-Sanchez et al., 2003) and the mannose receptor on macrophages (Miller et al., 2008). The recognition of a large number of binding sites is likely a consequence of the evolutionary pressures associated with infecting both mosquitos and a variety of mammals.

Following attachment to a viral receptor, the virus is taken into the cell via endocytosis. The cell acidifies the endosome which triggers rearrangement of the E proteins to expose domain II. The viral membrane is then fused with the endosome membrane and the viral nucleocapsid is released into the cytosol (Rodenhuis-Zybert et al., 2010). The positive-sense RNA becomes associated with the rough endoplasmic reticulum where a ribosome translates the large single open reading frame into the polyprotein (C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). Host signal peptidase and the NS2B serine protease cleave the polyprotein into the individual proteins. NS1 is secreted from infected cells to serum concentrations as high as 50 μg/ml (Alcon et al., 2002) but is also located intracellularly and can become associated with the cell membrane. Intracellular NS1 is involved with RNA replication and inhibition of the complement system. The role of secreted and membrane-associated NS1 is discussed in section 1.5.1. NS2A inhibits interferon signaling as well as combining with NS1, NS3, NS4A/B, and NS5 for RNA replication. Additionally, NS4A/B can block interferon signaling and NS5 acts as the RNA-dependent RNA polymerase (Alcaraz-Estrada et al., 2010; Akey et al., 2014).

During replication, newly synthesized viral RNA is released directly into virions budding from the endoplasmic reticulum where the RNA becomes associated with the C

protein. The immature viral particle is transported through the secretory pathway during which the prM protein prevents E protein rearrangement into a mature particle, which in turn prevents premature membrane fusion. Upon exposure to the neutral pH of the extracellular space pr is released from M, marking the complete maturation of the DENV virion. In 2-4 days, the range of DENV titer in human blood can be as high as 10^9 pfu/mL (Simmons et al., 2015).

1.5.1 Role of Secreted and Membrane-Associated Dengue NS1

A portion of dengue NS1 is secreted into the extracellular space as a hexamer and has a multi-faceted role that enhances the virus's ability to infect the host. In both primary and secondary DENV infections, the immune response generates high concentrations of anti-NS1 IgG antibodies against both secreted and membrane-associated-NS1. Unfortunately, these anti-NS1 antibodies cross-react with a number of host proteins such as human blood clotting factors (platelets, fibrinogen, and fibronectin) and integrin/adhesion proteins. This autoimmune response prevents proper protein function and causes tissue damage that ultimately increases leakage of the vascular system (Falconar, 1997; Chang et al., 2002; Lin et al., 2002; Lin et al., 2003). Additional cellular damage occurs when secreted NS1 directly activates the complement pathway; stimulating the inflammatory cytokines and soluble membrane attack complexes (Kurosu et al., 2007). However, not all of the anti-NS1 antibodies are damaging. Anti-NS1 monoclonal antibodies have been protective in mice when challenged with a lethal concentration of DENV2. Additionally, patient recovery does not coincide with a decrease in circulating

anti-NS1 antibody (Henchal et al., 1988; Amorim et al., 2012; Amorim et al., 2014). Thus, while NS1 plays some part of the pathogenesis of DENV, its role is not fully understood.

1.5.2 Infection of the *Aedes aegypti*

When an *Ae. aegypti* mosquito feeds on an infected organism and ingests a large titer of DENV, the virus passes through the tracheal system and into the midgut. While the virus can infect the tracheal system, it has been demonstrated that this does not result in a systemic infection of the mosquito. Upon arrival into the midgut, a network of crisscrossing proteins is erected around the blood to form the midgut infection barrier to aid in digestion and prevent the spread of infectious agents (Anderson and Rico-Hesse, 2006). The strength of this barrier along with a second midgut escape barrier, play a key role in determining vector competence by preventing or delaying systemic DENV infection (Bennett et al., 2002; Black et al., 2002). Each serotype has demonstrated the ability to bind to the putative R67 and R80 receptors on the midgut epithelial cells (Mercado-Curiel et al., 2006). About 2 days post-ingestion, the virus will have bypassed both barriers and will have begun to infect the midgut epithelial cells followed by dissemination of mature virions to infect other organs. By 7-10 days, the average DENV titer is 9×10^3-10^4 pfu/ml and the virus can be detected in salivary glands indicating that the mosquito is infectious (Salazar et al., 2007; Xi et al., 2008).

1.6 Dengue Clinical Manifestations

In 2006, the classic dengue classification scheme was called into question as patients suffering with DHF/DSS did not meet the official criteria and were improperly

diagnosed. Therefore in 2010, the World Health Organization officially updated the classification scheme to a two category scheme; dengue and severe dengue (Deen et al., 2006; World Health Organization, 2009; Simmons et al., 2015). Despite this, many works published after the scheme change still use the previous dengue classification scheme.

1.6.1 Dengue Illness

The majority of primary DENV infections in humans are asymptomatic or mildly symptomatic. Patients with secondary infections are more likely to suffer from more intense symptoms and may be more likely to have severe dengue (Dejnirattisai et al., 2010; Wahala and de Silva, 2011). Those who suffer more intense systems tend to follow three phases: febrile, critical, and recovery. The febrile phase marks the beginning of symptoms, with the sudden onset of fever 2 to 7 days post infection. (Figure 1.3) Additional symptoms include headache, generalized myalgias and arthralgias, abdominal pain, a variety of rashes, nausea, and an enlarged liver. At this time, a DENV infection must be diagnosed and fluid therapy needs to begin. Severe and non-severe dengue symptoms are indistinguishable during the febrile phase (World Health Organization, 2009).

The critical phase generally occurs on days 3 through 7 of illness. Symptoms of an intense non-severe dengue are still indistinguishable from those of severe dengue. The start of this phase is marked by patient defervescence, an increased capillary permeability, and a decrease in platelet and white blood cell count. During this phase, patients with non-severe dengue will begin to recover but those with severe dengue will continue to worsen. Within hours, the severe dengue patient's condition may rapidly deteriorate with

hemorrhagic manifestations. Plasma leakage may increase to the point of shock with pleural effusion due to the significant loss of plasma volume followed by liver failure. Cardiorespiratory collapse may occur within minutes of dengue-induced shock. Prolonged high titers of DENV and NS1 in the blood are potential indicators of severe dengue but no significant clinical trials have been performed. If the patient survives the critical phase, s/he enters the recovery phase. The extravascular fluid is gradually reabsorbed, the generalized myalgias and arthralgias dissipate and white blood cells and platelet counts will begin to rise (Vaughn et al., 2000; Libraty et al., 2002b; Lum et al., 2002; World Health Organization, 2009).

1.7 Current Dengue Detection Methods

Diagnosis of a DENV infection early in the illness is critical for proper patient management. Thus, many diagnostic tools for DENV have been developed to aid in the detection, confirmation, and surveillance of DENV. Determining which diagnostic tool to use is largely dependent on the disease phase, available laboratory facilities and equipment, expertise of the personnel, and diagnostic time to results. (Figure 1.3) Generally, tests with a high diagnostic accuracy are more complex and require specialized laboratories with costly equipment and highly trained personnel. Tests that are relatively cheaper, rapid, and accessible compromise sensitivity for ease of use.

1.7.1 Virus Isolation

Isolation of DENV in viral culture is the oldest of the golden-standard dengue diagnostic techniques (Lanciotti et al., 1992; Alcon et al., 2002; World Health

Organization, 2009). There are currently two common protocols for viral isolation of DENV: intrathoracic inoculation of live adult mosquitoes and isolation in mosquito or mammalian cell lines. Intracerebral inoculation of suckling mice was popular early in DENV research but the technique is no longer recommended (Guzmán and Kourí, 2004; Philip Samuel and Tyagi, 2006). Of these, while less sensitive, isolation in a cell line is the most common due to ease of use, lower level biosafety protocols, and relatively low cost.

Isolation of DENV in mosquito cell lines most commonly use the C6/36 line derived from the larvae of *Ae. Albopicutus*. However, AP-61 from *A. pseudoscutellaris*, and TRA-284 from *Toxorhynchites amboinensis* are popular cell lines as well. Of the three, C6/36 is most compatible with immunofluorescent assays, most susceptible to DENV, and most commonly used for routine surveillance (Igarashi, 1978; Gubler et al., 1984; Kuno et al., 1985; Philip Samuel and Tyagi, 2006). Isolation in mammalian cell lines most commonly use Vero cells derived from kidney epithelial cells of an African green monkey, LLC-MK_2 derived from kidney epithelial cells of a rhesus monkey, or BHK-21 derived from kidney fibroblasts of a baby Syrian golden hamster. Typically, blood or tissue samples are introduced into a T-75 flask containing a monolayer of the chosen cell line, given time to infect the cells, and the samples removed from the flask. The cells are incubated for approximately 6-10 days until cytopathic effects (cell rounding, refraction of light, and detachment) are observed. Most commonly, viral titer is then determined by an immunofluorescence assay (IFA), adding an additional 2-5 days for results (Bhat et al., 2015). Determining viral titer via plaque assays is not recommended; not all DENV strains form plaques, not all cell types permit plaque formation, and strains that do form plaques

are typically cell culture adapted (Philip Samuel and Tyagi, 2006). Isolation in cell culture can detect low titers of DENV (1-10 pfu/ml) from a sample and is a viable detection method in the first 0-7 days of the febrile stage. (Figure 1.3) Additionally, it amplifies viral titer from the initial sample and provides additional viruses for further virological analysis. However, the technique requires temperature-controlled storage of the cells and reagents, sophisticated equipment, and highly trained personnel. Thus, viral isolation in cell culture is used more often in academic and surveillance laboratories. (Gubler et al., 1984; World Health Organization, 2009; Peeling et al., 2010).

Intrathoracic inoculation of live adult mosquitoes is the most sensitive DENV isolation technique. Thus, it is most commonly used for experimental investigations when isolation of the virus, rather than diagnosis, is the priority (Shu and Huang, 2004). The technique is much too costly and labor intensive for routine diagnostics as it requires a large amount of mosquitos from an insectary. The *Toxorhynchites splendens* mosquito is preferred over *Ae. aegypti*. First, the *Toxorhynchites splendens* mosquito does not bite humans and does not feed on blood, greatly reducing the danger. Second, DENV recovery from the heads, thorax, and abdomen is approximately 5 times greater than *Ae. aegypti*. Viral detection occurs 10-12 days post-inoculation using IFA on the salivary glands and brain tissue (Philip Samuel and Tyagi, 2006).

1.7.2 Nucleic Acid Detection

Recently, nucleic acid detection with reverse-transcription polymerase chain reaction (RT-PCR) was added to the list of gold-standard diagnostic techniques. DENV

RNA can be rapidly detected and serotyped in field-caught mosquitoes, tissues, whole blood, or sera of infected organisms in the 0-5 days of infection (Chow et al., 1998; Philip Samuel and Tyagi, 2006). In some cases, nucleic acid detection has shown a similar sensitivity but more rapid detection capability than isolation via cell culture. A number of variations of RT-PCR techniques have been developed, including quantitative, multiplex, real-time, and single-step assays. General detection can be achieved by targeting highly conserved regions of the DENV genome such as the prM gene sequence. Serotyping can be achieved by analyzing amplicon size, melting curve analysis, or by using serotype specific primers that target a gene sequence unique to each serotype such as the 3'-noncoding region of the genome. Detection directly from a tissue or serum sample can be achieved with titers as low as 20 pfu/mL in as little as 3 hours (Lanciotti et al., 1992; Houng et al., 2001; Shu et al., 2003; Yong et al., 2007; Conceição et al., 2010; Waggoner et al., 2013). RT-PCR is also compatible with isolation via cell culture and can shorten the time to results to 1-5 days depending on inoculation titer (Shu and Huang, 2004). Despite the advantages, RT-PCR has a number of drawbacks that prevent it from being used at the point-of-care and in resource lacking countries for routine diagnostics and surveillance. It requires high technical skills, a number of expensive sample treatments and reagents for nucleic acid extraction and amplification, and is highly susceptible to contamination.

1.7.3 Serological tests

Serological tests have the longest history of being the most rapid and accessible diagnostic testing methods. Even the recently developed, commercially available, rapid diagnostic tests (RDTs) predominantly use serological detection for DENV diagnosis.

These tests target and detect the human IgM and IgG antibodies that are developed by the patient's immune system to fight a DENV infection. During a primary infection, IgM antibodies are the first to appear in the serum and are detectable 3-10 days after the onset of symptoms. IgG antibodies are detectable 7 or more days after the onset of symptoms and may be detectable for life (Guzmán and Harris, 2015; Parkash and Shueb, 2015; Shamala, 2015). During a secondary infection, IgG antibodies are rapidly detectable while IgM antibody concentrations remain low or are undetectable. The IgM/IgG ratio can be used to aid in determining primary vs. secondary DENV infections and thus can aid in determining clinical management (Shu and Huang, 2004; World Health Organization, 2009; Blacksell, 2012; Bhat et al., 2015).

For many years, the hemagglutination-inhibition (HI) was the gold standard of rapid and accessible diagnostic testing. The assay, while easy to perform, is laborious and requires at least 3 days and easy access to goose blood to perform. It has since been replaced by the enzyme-linked immunosorbent assay (ELISA) for laboratory diagnostics and RDTs for point-of-care diagnostics (Peeling et al., 2010; Shamala, 2015). ELISA-based methods such as IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) and IgG antibody-capture enzyme-linked immunosorbent assay (GAC-ELISA) are now accepted as gold-standard diagnostic techniques. Modern serological RDTs largely rely on the same detection methodologies as the ELISA-based methods. However, RDTs have been designed to be used by untrained personnel at the point-of-care. While serological RDTs are cheap, rapid, commercially available, and the most accessible detection method, they suffer from the same drawbacks as ELISA and the other serological detection

methods. First, IgM and IgG antibodies are not produced until approximately day 5 into a primary infection and therefore are not effective for an early diagnosis. Second, time of sampling, DENV serotype, and primary/secondary infections can greatly affect the results; low sensitivity (30-95%) and specificity (46-100%) have been reported. Third, human IgM/G are broadly cross-reactive across serotypes and other flaviviruses, resulting in false positives. Fourth, a false-positive can be triggered by a previous flavivirus infection. Fifth, serological-based methods were developed for human diagnosis; they cannot be used for vector surveillance (mosquitos don't produce antibodies) or field surveillance unless special target organism anti-antibodies are substituted. (Schilling et al., 2004; Blacksell et al., 2006; Blacksell et al., 2007; Vazquez et al., 2007; Blacksell et al., 2008; World Health Organization, 2009; Huhtamo et al., 2010; Wang and Sekaran, 2010; Blacksell et al., 2011; Blacksell et al., 2012; Hunsperger et al., 2014; Welch et al., 2014; Bhat et al., 2015)

1.7.4 NS1 Antigen Detection

Similar to nucleic acid detection methods, circulating DENV NS1 can be detected in sera as early as one day after onset of symptoms using ELISA or RDTs. NS1 is detectable up to 18 days during a primary infection (Young et al., 2000; Alcon et al., 2002). During a secondary infection, detection of the NS1 protein is limited by host antibody-NS1 interactions but can still be detected within the first 8 days of symptoms (Winkler et al., 1989; Huang et al., 2013). Additionally, unlike serological tests, diagnostic tests for the NS1 protein are not cross-reactive with other flaviviruses. Development of commercially available RDTs for the detection of DENV NS1 have significantly improved point-of-care diagnostic capabilities but rely on visual (colorimetric) detection. The devices have

sacrificed reliability for a portable, rapid, and easy to use diagnostic tool. Reports on NS1 detection vary widely, sensitivities generally range from 55-95% and specificity from 89-100%. However, sensitivities under 50% have been reported during the first two days of illness when NS1 concentrations are lower (Shu et al., 2009; Lima et al., 2010; Osorio et al., 2010; Singh et al., 2010; Blacksell et al., 2012; Shamala, 2015).

1.8 Advanced Dengue Detection Methods

While the burden of dengue has grown to global proportions, DENV still predominantly effects developing countries. Clearly, there is a need to develop low cost DENV diagnostic devices that combine high sensitivity and specificity with portability, rapidity, and accessibility. Biosensors are biodiagnostic devices that combine biological recognition elements (bioreceptor) with a signal transducer to convert a binding event of the target analyte with the bioreceptor into a measureable and user-friendly signal. The bioreceptor typically consists of enzymes, antibodies, nucleic acids, or other proteins that can be immobilized onto a particular transducer to specifically bind to the desired target analyte. A number of biosensor transducer technologies have been developed for signal generation and amplification; such as piezoelectric, nanomaterials, electrochemical, quartz crystal microbalances, or surface plasmon resonance.

1.8.1 Quartz Crystal Microbalance

Quartz crystal microbalance (QCM) is a piezoelectric transducer that can measure small changes in micro-mass on the surface of a resonating quartz crystal. As mass attaches or detaches from the surface of the crystal, there is a corresponding frequency change that

allows for label-free, real-time biosensing. Pirich et al. (2017) developed a QCM biosensor for the DENV NS1 protein that utilizes cellulose nanocrystals as the receptive surface for monoclonal anti-NS1 antibodies. The biosensor was able to detect NS1 in PBS from 0.03 µg/mL; a comparable sensitivity to ELISA. However, once NS1 was suspended in serum, a complex sample matrix with interfering chemicals and proteins, sensitivity decreased to 0.32 µg/mL. In a different approach, Sz-Hau et al. (2009) utilized two oligonucleotide-functionalized gold nanoparticles to detect DENV RNA; one to increase the mass of viral RNA and one to increase the specificity of the assay. This biosensor was able to detect the viral RNA at an equivalent of 2 pfu/mL in serum. QCM devices are low-cost and easy to use. However, the sensitivity of the QCM assay is negatively affected by vibrations from the external environment, which limits the portability of QCM-based devices (Sz-Hau et al., 2009; Parkash and Shueb, 2015; Pirich et al., 2017).

1.8.2 Electrochemical Detection

Electrochemical biosensors monitor the current of an electrode before and after an electrochemical oxidation/reduction reaction. Electrochemical impedance spectroscopy or cyclic voltammetry are used to measure the change generated by the electrochemical reaction. The impedance change is proportional to the concentration of the target analyte within the sample matrix. Dias et al. (2013) captured DENV NS1 from a serum sample with unlabeled monoclonal anti-NS1 antibodies immobilized onto a carbon nanotube-ink printed electrode. Next, anti-NS1 antibodies conjugated with horse-radish peroxidase were permitted to bind to the captured DENV NS1. The horse-radish peroxidase was reacted with hydrogen peroxide and the amperometrical response generated by the reaction was

monitored with cyclic voltammetry. This multi-step assay achieved a limit of detection (LOD) of 12 ng/mL. Similarly, Silva et al. (2015) achieved an LOD of 15 ng/mL using a poly(allylamine)/carbon nanotube layered immunoelectrode to monitor the perioxidase-H_2O_2 reaction. While high sensitivity can be achieved through electrochemical reactions, these biosensors use a tedious multi-step and multi-reagent method for indirect detection of DENV NS1.

1.8.3 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a label-free optical method that measures the change in refractive index when the target analyte binds to the bioreceptor immobilized onto a reflective surface. The intensity and angle of the light reflected off of the surface is proportional to the concentration of the target analyte. Using long-range surface plasmon waveguides, Wong et al. (2016) detected DENV NS1 in plasma with a LOD of 5.73 pg/mm^2. While SPR can detect low concentrations of the target analyte, the cost of the optics and electronics is extremely high, the machines are bulky, and the assays are not user-friendly (Breault-Turcot and Masson, 2012).

1.9 Chemiresistors

While many of these biosensors are sensitive, they require extensive training to use and many of the technologies involved are not readily portable, making point-of-care and vector surveillance difficult. The need for high sensitivity, selectivity, and portability has driven research towards nanoscale devices. Due to their pseudo-one-dimensional structure, single-walled carbon nanotubes (SWNT) have unique physical and chemical properties

that can improve biosensing technologies. Electric biosensors that utilize SWNT as a transducer element have demonstrated increased performance due to the SWNT electrocatalytic activity and size similarity to the target of interest (Allen et al., 2007). Additionally, electric biosensors have demonstrated many desirable traits, especially when considering the needs of DENV diagnosis; offering a portable, sensitive, reliable, and inexpensive diagnostic method with rapid detection capabilities.

1.9.1 Carbon Nanotubes

Unbeknownst to those utilizing them, humanity has been synthesizing and capitalizing on the carbon nanotube's mechanical properties for hundreds of years. Swords and daggers were strengthened by rolling soot into metals to produce stronger materials such as Damascus steel (Reibold et al., 2006; Reibold et al., 2009). In 1952, Radushkevich and Lukyanovich published what are thought to be the first images of multi-walled carbon nanotubes then-named, "hollow carbon fibers." Oberlin et al. (1976) published the first images of SWNT as "Filamentous growth of carbon" (Oberlin et al., 1976; Grobert, 2007; Kumar and Ando, 2010). However, it wasn't until 1991, with the publication of 'Helical microtubules of graphitic carbon' that carbon nanotubes were recognized by the scientific community. Since then, scientific interest in carbon nanotubes and SWNT biosensors has increased exponentially (Iijima, 1991; Iijima and Ichihashi, 1993).

SWNT are an allotrope of carbon in which a honeycomb-like structure with a one-atom-thick layer of carbon atoms form a hollow tube about 1nm in diameter. All carbon atoms are on the surface resulting in a quasi-one-dimensional (1D) nanostructure with an

ultra-high surface area-to-volume ratio. The helicity of the SWNT determines the electrical properties; armchair nanotubes are conducting, while zigzag and chiral nanotubes can be either conducting or semiconducting (Yang et al., 2015). Additionally, SWNT exhibit superior electrical conductivity, mechanical strength, and chemical inertness. These properties make them extremely sensitive to minute changes in surface charge density due to electrostatic interactions with the target analyte (Gruner, 2006; Grobert, 2007; Reich et al., 2008).

Unlike two-dimensional graphene sheets, the 1D SWNT restricts the electrical current flow along the length of the nanotube. As the electric field from the target analyte penetrates into the bulk of the SWNT, it creates regions of electron accumulation or depletion within the SWNT. Electrical transport through these regions results in detectable changes in the conductivity/resistivity of the SWNT microelectrodes resulting in a label-free detection of the target. Devices that operate using these principles are known as chemiresistors (Balasubramanian and Burghard, 2006; Allen et al., 2007; García-Aljaro et al., 2010).

1.9.2 Functionalization of SWNT

While chemiresistive sensors without receptors have been reported, the sensors have virtually no selectivity (Collins et al., 2000; Ramnani et al., 2016). Choice of functionalization method and bioreceptor can significantly alter the performance of the biosensor. There are two functionalization methods that are commonly used; the first method is to oxidize the SWNT, breaking carbon-carbon bonds in order to introduce a

coupling/linker molecule into the sidewalls. The chemical groups introduced using this method are readily available for covalent bonding to a bioreceptor. Typically, carboxyl or epoxide groups are introduced followed by reaction with an amine group of the desired bioreceptor. While this method produces robust biosensors that survive well in harsh environments, the breakage of the carbon-carbon bonds on the SWNT sidewall causes disruptions in the π-system, significantly increasing electrical resistance and often results in greatly diminished sensitivity (Wang and Swager, 2011). The second method utilizes aromatic pyrene-linkers to non-covalently functionalize the side walls of the SWNT. The aromatic pyrene moieties form π-π interactions with the sp^2-bonded carbon in the sidewalls without affecting conductivity. Additionally, the functional group linked to the pyrene moiety can be chosen based on the desired linker-bioreceptor chemistry. Thus, even bioreceptors without amine groups can easily be functionalized to the SWNT (Balasubramanian and Burghard, 2006; Chen et al., 2013).

1.10 Objectives

Dengue virus is the currently the most important arbovirus in the world. As such, a number of diagnostic techniques and technologies have been produced. Virtually all of the laboratory and commercial DENV diagnostic methods require a painful blood draw for sample analysis, which can be dangerous and decreases patient willingness. The diagnostic tools were designed for the diagnosis of DENV in humans or are too expensive and complex for sustainable mosquito vector surveillance. Additionally, none of these have managed to combine sensitivity and selectivity in a portable, rapid, and inexpensive

diagnostic tool suitable for use by minimally-trained personnel in the field, laboratory, or a point-of-care location. As such, there is a need for a sensitive and selective, non-invasive, user-friendly, portable, low-cost diagnostic device that is compatible with all potential DENV hosts.

In this project, two novel biosensors using SWNT transducers functionalized for the detection of whole DENV or DENV Non-Structural Protein 1 (NS1) were developed. The first utilizes heparin, an analog of the heparan sulfate proteoglycans that are receptors for DENV, as a bioreceptor for detection of whole DENV virions. Heparin has demonstrated the ability to maintain its structure and reactivity despite prolonged periods of direct sunlight and high temperatures; creating a robust and sensitive biosensor. The second utilizes anti-dengue NS1 monoclonal antibodies to detect DENV NS1, a clinically accepted biomarker for DENV infection. NS1 is a highly conserved 46-kDa protein secreted at high concentrations during viral replication in virtually every host organism and can be detected in human saliva. Both biosensors offer a facile, affordable, and label-free method for direct detection of the target analyte.

1.11 Figures

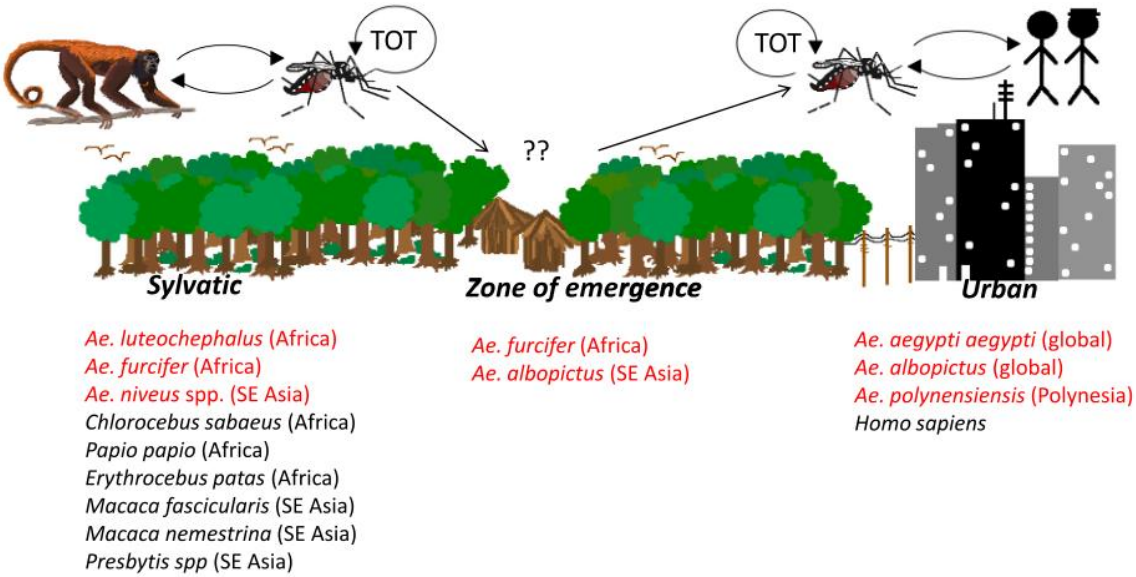

Figure 1.1 Comparison of the major mosquito vectors (in red text) and primate hosts (in black text) involved in sylvatic transmission, spillover and urban transmission of dengue virus. Adapted with permission from Hanley et al. (2013)

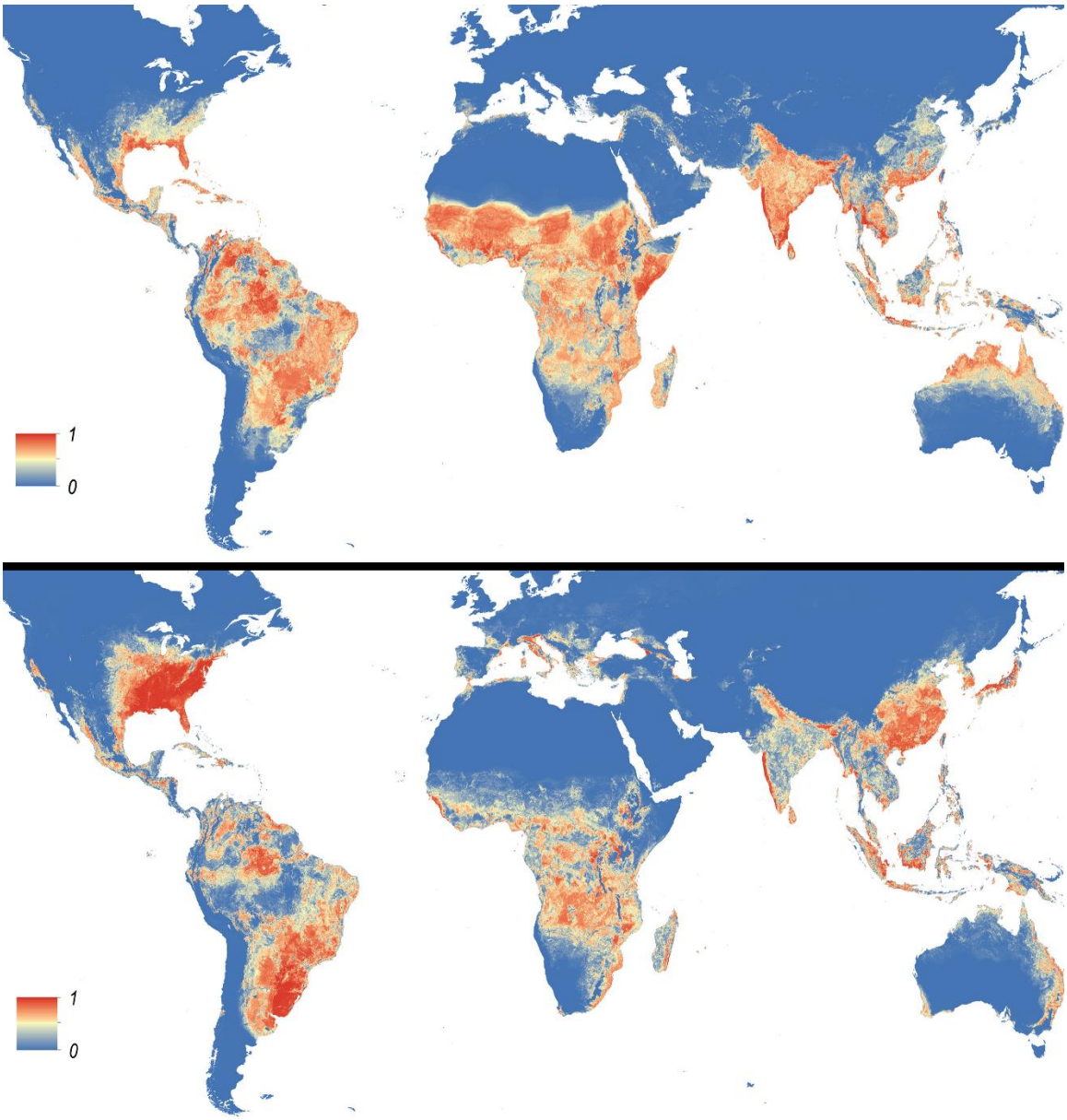

Figure 1.2 Global map of the predicted distribution of *Ae. aegypti* (top) and *Ae. albopictus* (bottom). The map depicts the probability of occurrence (from 0 blue to 1 red) at a spatial resolution of 5 km × 5 km. Adapted with permission from Kraemer et al. (2015)

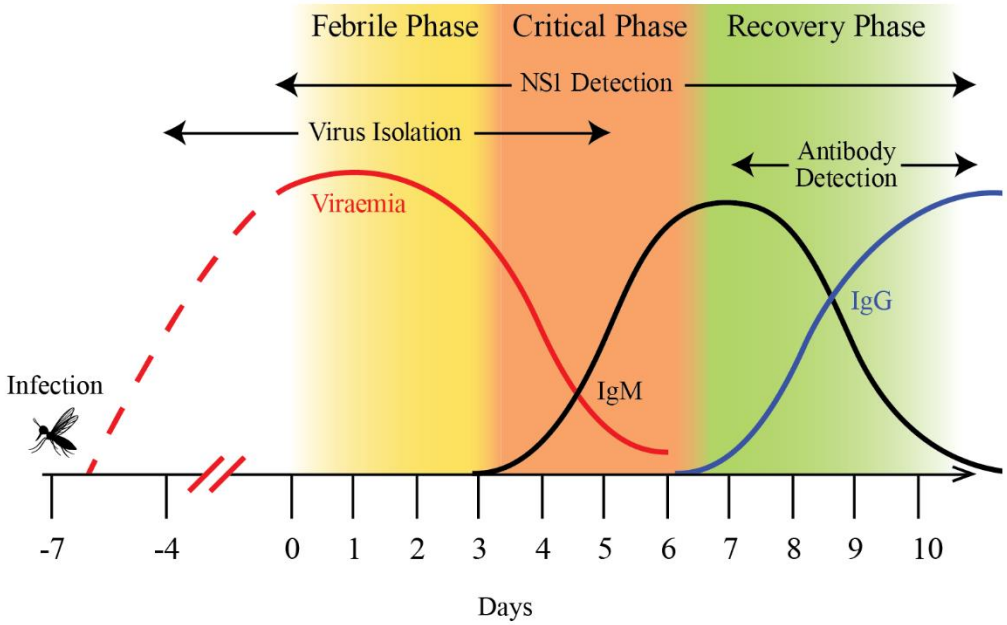

Figure 1.3 Timeline of a primary dengue infection and diagnostics.

1.12 References

Akey, D. L., Brown, W. C., Dutta, S., Konwerski, J., Jose, J., Jurkiw, T. J., DelProposto, J., Ogata, C. M., Skiniotis, G., Kuhn, R. J. and Smith, J. L. (2014). "Flavivirus NS1 Structures Reveal Surfaces for Associations with Membranes and the Immune System." Science **343** (6173): 881-885.

Alcaraz-Estrada, S. L., Yocupicio-Monroy, M. and del Angel, R. M. (2010). "Insights into dengue virus genome replication." Future Virology **5** (5): 575-592.

Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V. and Flamand, M. (2002). "Enzyme-Linked Immunosorbent Assay Specific to Dengue Virus Type 1 Nonstructural Protein NS1 Reveals Circulation of the Antigen in the Blood during the Acute Phase of Disease in Patients Experiencing Primary or Secondary Infections." Journal of Clinical Microbiology **40** (2): 376-381.

Allen, B. L., Kichambare, P. D. and Star, A. (2007). "Carbon Nanotube Field-Effect-Transistor-Based Biosensors." Advanced Materials **19** (11): 1439-1451.

Althouse, B. M., Lessler, J., Sall, A. A., Diallo, M., Hanley, K. A., Watts, D. M., Weaver, S. C. and Cummings, D. A. (2012). "Synchrony of Sylvatic Dengue Isolations: a Multi-Host, Multi-Vector SIR Model of Dengue Virus Transmission in Senegal." PLoS Negl Trop Dis **6** (11): e1928.

Amorim, J. H., Alves, R. P., Boscardin, S. B. and Ferreira, L. C. (2014). "The dengue virus non-structural 1 protein: risks and benefits." Virus Res **181**: 53-60.

Amorim, J. H., Diniz, M. O., Cariri, F. A., Rodrigues, J. F., Bizerra, R. S., Gonçalves, A. J., de Barcelos Alves, A. M. and de Souza Ferreira, L. C. (2012). "Protective immunity to DENV2 after immunization with a recombinant NS1 protein using a genetically detoxified heat-labile toxin as an adjuvant." Vaccine **30** (5): 837-845.

Anderson, J. R. and Rico-Hesse, R. (2006). "*Aedes aegypti* Vectorial Capacity is Determined by the Infecting Genotype of Dengue Virus." Am J Trop Med Hyg **75** (5): 886-892.

Bai, L., Morton, L. C. and Liu, Q. (2013). "Climate change and mosquito-borne diseases in China: a review." Global Health **9** (1): 10.

Balasubramanian, K. and Burghard, M. (2006). "Biosensors based on carbon nanotubes." Anal Bioanal Chem **385** (3): 452-468.

Beaumier, C., Garcia, M. N. and Murray, K. O. (2014). "The History of Dengue in the United States and its Recent Emergence." Current Tropical Medicine Reports **1** (1): 32-35.

Benedict, M. Q., Levine, R. S., Hawley, W. A. and Lounibos, L. P. (2007). "Spread of the Tiger: Global Risk of Invasion by the Mosquito *Aedes albopictus*." Vector Borne Zoonotic Dis **7** (1): 76-85.

Bennett, K. E., Olson, K. E., Muñoz, M. D., Fernandez-Salas, I., Farfan-Ale, J. A., Higgs, S., Black, W. C. and Beaty, B. J. (2002). "Variation in Vector Competence for Dengue 2 Virus Among 24 Collections of *Aedes aegypti* from Mexico and the United States." Am J Trop Med Hyg **67** (1): 85-92.

Bhat, V. G., Chavan, P., Ojha, S. and Nair, P. K. (2015). "Challenges in the Laboratory Diagnosis and Management of Dengue Infections." Open Microbiol J **9**: 33-37.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J. and Hay, S. I. (2013). "The global distribution and burden of dengue." Nature **496** (7446): 504-507.

Black, W. C. t., Bennett, K. E., Gorrochótegui-Escalante, N., Barillas-Mury, C. V., Fernández-Salas, I., de Lourdes Muñoz, M., Farfán-Ale, J. A., Olson, K. E. and Beaty, B. J. (2002). "Flavivirus susceptibility in *Aedes aegypti*." Arch Med Res **33** (4): 379-388.

Blacksell, S. D. (2012). "Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs?" J Biomed Biotechnol **2012**: 12.

Blacksell, S. D., Bell, D., Kelley, J., Mammen, M. P., Jr., Gibbons, R. V., Jarman, R. G., Vaughn, D. W., Jenjaroen, K., Nisalak, A., Thongpaseuth, S., Vongsouvath, M., Davong, V., Phouminh, P., Phetsouvanh, R., Day, N. P. and Newton, P. N. (2007). "Prospective Study To Determine Accuracy of Rapid Serological Assays for Diagnosis of Acute Dengue Virus Infection in Laos." Clin Vaccine Immunol **14** (11): 1458-1464.

Blacksell, S. D., Jarman, R. G., Bailey, M. S., Tanganuchitcharnchai, A., Jenjaroen, K., Gibbons, R. V., Paris, D. H., Premaratna, R., de Silva, H. J., Lalloo, D. G. and Day, N. P. (2011). "Evaluation of Six Commercial Point-of-Care Tests for Diagnosis of Acute Dengue Infections: the Need for Combining NS1 Antigen and IgM/IgG Antibody Detection to Achieve Acceptable Levels of Accuracy." Clin Vaccine Immunol **18** (12): 2095-2101.

Blacksell, S. D., Jarman, R. G., Gibbons, R. V., Tanganuchitcharnchai, A., Mammen, M. P., Jr., Nisalak, A., Kalayanarooj, S., Bailey, M. S., Premaratna, R., de Silva, H. J., Day, N. P. and Lalloo, D. G. (2012). "Comparison of Seven Commercial Antigen

and Antibody Enzyme-Linked Immunosorbent Assays for Detection of Acute Dengue Infection." Clin Vaccine Immunol **19** (5): 804-810.

Blacksell, S. D., Mammen, M. P., Jr., Thongpaseuth, S., Gibbons, R. V., Jarman, R. G., Jenjaroen, K., Nisalak, A., Phetsouvanh, R., Newton, P. N. and Day, N. P. (2008). "Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos." Diagn Microbiol Infect Dis **60** (1): 43-49.

Blacksell, S. D., Newton, P. N., Bell, D., Kelley, J., Mammen, M. P., Jr., Vaughn, D. W., Wuthiekanun, V., Sungkakum, A., Nisalak, A. and Day, N. P. (2006). "The Comparative Accuracy of 8 Commercial Rapid Immunochromatographic Assays for the Diagnosis of Acute Dengue Virus Infection." Clin Infect Dis **42** (8): 1127-1134.

Bonizzoni, M., Gasperi, G., Chen, X. and James, A. A. (2013). "The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives." Trends Parasitol **29** (9): 460-468.

Braks, M. A., Honório, N. A., Lourenço-de-Oliveira, R., Juliano, S. A. and Lounibos, L. P. (2003). "Convergent Habitat Segregation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Southeastern Brazil and Florida." J Med Entomol **40** (6): 785-794.

Breault-Turcot, J. and Masson, J. F. (2012). "Nanostructured substrates for portable and miniature SPR biosensors." Anal Bioanal Chem **403** (6): 1477-1484.

Cardosa, J., Ooi, M. H., Tio, P. H., Perera, D., Holmes, E. C., Bibi, K. and Abdul Manap, Z. (2009). "Dengue Virus Serotype 2 from a Sylvatic Lineage Isolated from a Patient with Dengue Hemorrhagic Fever." PLoS Negl Trop Dis **3** (4): e423.

Chang, H.-H., Shyu, H.-F., Wang, Y.-M., Sun, D.-S., Shyu, R.-H., Tang, S.-S. and Huang, Y.-S. (2002). "Facilitation of Cell Adhesion by Immobilized Dengue Viral Nonstructural Protein 1 (NS1): Arginine-Glycine-Aspartic Acid Structural Mimicry within the Dengue Viral NS1 Antigen." J Infect Dis **186** (6): 743-751.

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J. and Marks, R. M. (1997). "Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate." Nat Med **3** (8): 866-871.

Chen, Y., Star, A. and Vidal, S. (2013). "Sweet carbon nanostructures: carbohydrate conjugates with carbon nanotubes and graphene and their applications." Chem Soc Rev **42** (11): 4532-4542.

Chow, V. T., Chan, Y. C., Yong, R., Lee, K. M., Lim, L. K., Chung, Y. K., Lam-Phua, S. G. and Tan, B. T. (1998). "Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing." Am J Trop Med Hyg **58** (5): 578-586.

Collins, P. G., Bradley, K., Ishigami, M. and Zettl, A. (2000). "Extreme Oxygen Sensitivity of Electronic Properties of Carbon Nanotubes." Science **287** (5459): 1801-1804.

Conceição, T. M., Da Poian, A. T. and Sorgine, M. H. (2010). "A real-time PCR procedure for detection of dengue virus serotypes 1, 2, and 3, and their quantitation in clinical and laboratory samples." J Virol Methods **163** (1): 1-9.

Cruz-Oliveira, C., Freire, J. M., Conceição, T. M., Higa, L. M., Castanho, M. A. and Da Poian, A. T. (2015). "Receptors and routes of dengue virus entry into the host cells." FEMS Microbiol Rev **39** (2): 155-170.

Deen, J. L., Harris, E., Wills, B., Balmaseda, A., Hammond, S. N., Rocha, C., Dung, N. M., Hung, N. T., Hien, T. T. and Farrar, J. J. (2006). "The WHO dengue classification and case definitions: time for a reassessment." Lancet **368** (9530): 170-173.

Dejnirattisai, W., Jumnainsong, A., Onsirisakul, N., Fitton, P., Vasanawathana, S., Limpitikul, W., Puttikhunt, C., Edwards, C., Duangchinda, T., Supasa, S., Chawansuntati, K., Malasit, P., Mongkolsapaya, J. and Screaton, G. (2010). "Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans." Science **328** (5979): 745-748.

Delatte, H., Desvars, A., Bouétard, A., Bord, S., Gimonneau, G., Vourc'h, G. and Fontenille, D. (2010). "Blood-feeding behavior of *Aedes albopictus*, a vector of Chikungunya on La Reunion." Vector Borne Zoonotic Dis **10** (3): 249-258.

Diallo, M., Ba, Y., Sall, A. A., Diop, O. M., Ndione, J. A., Mondo, M., Girault, L. and Mathiot, C. (2003). "Amplification of the Sylvatic Cycle of Dengue Virus Type 2, Senegal, 1999-2000: Entomologic Findings and Epidemiologic Considerations." Emerg Infect Dis **9** (3): 362-367.

Diallo, M., Sall, A. A., Moncayo, A. C., Ba, Y., Fernandez, Z., Ortiz, D., Coffey, L. L., Mathiot, C., Tesh, R. B. and Weaver, S. C. (2005). "Potential Role of Sylvatic and Domestic African Mosquito Species in Dengue Emergence." Am J Trop Med Hyg **73** (2): 445-449.

Dias, A. C., Gomes-Filho, S. L., Silva, M. M. and Dutra, R. F. (2013). "A sensor tip based on carbon nanotube-ink printed electrode for the dengue virus NS1 protein." Biosensors and Bioelectronics **44**: 216-221.

Durbin, A. P., Mayer, S. V., Rossi, S. L., Amaya-Larios, I. Y., Ramos-Castaneda, J., Eong Ooi, E., Jane Cardosa, M., Munoz-Jordan, J. L., Tesh, R. B., Messer, W. B., Weaver, S. C. and Vasilakis, N. (2013). "Emergence potential of sylvatic dengue virus type 4 in the urban transmission cycle is restrained by vaccination and homotypic immunity." Virology **439** (1): 34-41.

Effler, P. V., Pang, L., Kitsutani, P., Vorndam, V., Nakata, M., Ayers, T., Elm, J., Tom, T., Reiter, P., Rigau-Perez, J. G., Hayes, J. M., Mills, K., Napier, M., Clark, G. G., Gubler, D. J. and Hawaii Dengue Outbreak Investigation, T. (2005). "Dengue Fever, Hawaii, 2001-2002." Emerg Infect Dis **11** (5): 742-749.

Ejrnaes, M., Filippi, C. M., Martinic, M. M., Ling, E. M., Togher, L. M., Crotty, S. and von Herrath, M. G. (2006). "Resolution of a chronic viral infection after interleukin-10 receptor blockade." J Exp Med **203** (11): 2461-2472.

Falconar, A. K. I. (1997). "The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis." Arch Virol **142** (5): 897-916.

Franco, L., Palacios, G., Martinez, J. A., Vázquez, A., Savji, N., De Ory, F., Sanchez-Seco, M. P., Martin, D., Lipkin, W. I. and Tenorio, A. (2011). "First Report of Sylvatic DENV-2-Associated Dengue Hemorrhagic Fever in West Africa." PLoS Negl Trop Dis **5** (8): e1251.

García-Aljaro, C., Cella, L. N., Shirale, D. J., Park, M., Muñoz, F. J., Yates, M. V. and Mulchandani, A. (2010). "Carbon nanotubes-based chemiresistive biosensors for detection of microorganisms." Biosensors and Bioelectronics **26** (4): 1437-1441.

Gjenero-Margan, I., Aleraj, B., Krajcar, D., Lesnikar, V., Klobučar, A., Pem-Novosel, I., Kurečić-Filipović, S., Komparak, S., Martić, R., Duričić, S., Betica-Radić, L., Okmadžić, J., Vilibić-Čavlek, T., Babić-Erceg, A., Turković, B., Avšić-Županc, T., Radić, I., Ljubić, M., Sarac, K., Benić, N. and Mlinarić-Galinović, G. (2011). "Autochthonous dengue fever in Croatia, August-September 2010." Euro Surveill **16** (9).

Gould, E. A., de Lamballerie, X., Zanotto, P. M. d. A. and Holmes, E. C. (2003). Origins, evolution, and vector/host coadaptations within the Genus *Flavivirus*. Advances in Virus Research, Academic Press. **Volume 59:** 277-314.

Grobert, N. (2007). "Carbon nanotubes - becoming clean." Materials Today **10** (1-2): 28-35.

Gruner, G. (2006). "Carbon nanotube transistors for biosensing applications." Anal Bioanal Chem **384** (2): 322-335.

Gubler, D. J. (1998). "Dengue and Dengue Hemorrhagic Fever." Clin Microbiol Rev **11** (3): 480-496.

Gubler, D. J. (2011a). "Dengue, Urbanization and Globalization: The Unholy Trinity of the 21st Century." Tropical Medicine and Health **39** (4 Suppl): 3-11.

Gubler, D. J. (2011b). "Dengue, Urbanization and Globalization: The Unholy Trinity of the 21st Century." Trop Med Health **39** (4 Suppl): 3-11.

Gubler, D. J. (2011c). "Dengue, Urbanization and Globalization: The Unholy Trinity of the 21st Century." Tropical Medicine and Health **39** (4SUPPLEMENT): S3-S11.

Gubler, D. J. (2011d). "Prevention and Control of *Aedes aegypti*-borne Diseases: Lesson Learned from Past Successes and Failures " Asia-Pacific Journal of Molecular Biology and Biotechnology **3** (19): 111-114.

Gubler, D. J., Kuno, G., Sather, G. E., Velez, M. and Oliver, A. (1984). "Mosquito Cell Cultures and Specific Monoclonal Antibodies in Surveillance for Dengue Viruses." Am J Trop Med Hyg **33** (1): 158-165.

Gubler, D. J., Ooi, E. E., Vasudevan, S. and Farrar, J. (2014). Dengue and Dengue Hemorrhagic Fever, CABI.

Guzmán, M. G. and Harris, E. (2015). "Dengue." The Lancet **385** (9966): 453-465.

Guzmán, M. G. and Kourí, G. (2004). "Dengue diagnosis, advances and challenges." Int J Infect Dis **8** (2): 69-80.

Hanley, K. A., Guerbois, M., Kautz, T. F., Brown, M., Whitehead, S. S., Weaver, S. C., Vasilakis, N. and Marx, P. A. (2014). "Infection Dynamics of Sylvatic Dengue Virus in a Natural Primate Host, the African Green Monkey." Am J Trop Med Hyg **91** (4): 672-676.

Hanley, K. A., Monath, T. P., Weaver, S. C., Rossi, S. L., Richman, R. L. and Vasilakis, N. (2013). "Fever versus fever: the role of host and vector susceptibility and interspecific competition in shaping the current and future distributions of the sylvatic cycles of dengue virus and yellow fever virus." Infect Genet Evol **19**: 292-311.

Hawley, W. A., Reiter, P., Copeland, R. S., Pumpuni, C. B. and Craig, G. B. (1987). "*Aedes albopictus* in North-America: Probable Introduction in Used Tires from Northern Asia." Science **236** (4805): 1114-1116.

Heinze, D. M., Gould, E. A. and Forrester, N. L. (2012). "Revisiting the Clinal Concept of Evolution and Dispersal for the Tick-Borne Flaviviruses by Using Phylogenetic and Biogeographic Analyses." J Virol **86** (16): 8663-8671.

Henchal, E. A., Henchal, L. S. and Schlesinger, J. J. (1988). "Synergistic Interactions of Anti-NS1 Monoclonal Antibodies Protect Passively Immunized Mice from Lethal Challenge with Dengue 2 Virus." J Gen Virol **69 (Pt 8)** (8): 2101-2107.

Holmes, E. C. and Twiddy, S. S. (2003). "The origin, emergence and evolutionary genetics of dengue virus." Infect Genet Evol **3** (1): 19-28.

Houng, H.-S. H., Chung-Ming Chen, R., Vaughn, D. W. and Kanesa-thasan, N. (2001). "Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1–4 using conserved and serotype-specific 3′ noncoding sequences." J Virol Methods **95** (1-2): 19-32.

Huang, C.-H., Kuo, L.-L., Yang, K. D., Lin, P.-S., Lu, P.-L., Lin, C.-C., Chang, K., Chen, T.-C., Lin, W.-R., Lin, C.-Y., Chen, Y.-H. and Wu, H.-S. (2013). "Laboratory diagnostics of dengue fever: an emphasis on the role of commercial dengue virus nonstructural protein 1 antigen rapid test." J Microbiol Immunol Infect **46** (5): 358-365.

Huhtamo, E., Hasu, E., Uzcátegui, N. Y., Erra, E., Nikkari, S., Kantele, A., Vapalahti, O. and Piiparinen, H. (2010). "Early diagnosis of dengue in travelers: Comparison of a novel real-time RT-PCR, NS1 antigen detection and serology." Journal of Clinical Virology **47** (1): 49-53.

Hunsperger, E. A., Yoksan, S., Buchy, P., Nguyen, V. C., Sekaran, S. D., Enria, D. A., Vazquez, S., Cartozian, E., Pelegrino, J. L., Artsob, H., Guzmán, M. G., Olliaro, P., Zwang, J., Guillerm, M., Kliks, S., Halstead, S., Peeling, R. W. and Margolis, H. S. (2014). "Evaluation of Commercially Available Diagnostic Tests for the Detection of Dengue Virus NS1 Antigen and Anti-Dengue Virus IgM Antibody." PLoS Negl Trop Dis **8** (10): e3171.

Igarashi, A. (1978). "Isolation of a Singh's *Aedes albopictus* Cell Clone Sensitive to Dengue and Chikungunya Viruses." J Gen Virol **40** (3): 531-544.

Iijima, S. (1991). "Helical microtubules of graphitic carbon." Nature **354** (6348): 56-58.

Iijima, S. and Ichihashi, T. (1993). "Single-shell carbon nanotubes of 1-nm diameter." Nature **363** (6430): 603-605.

Joshi, V., Mourya, D. T. and Sharma, R. C. (2002). "Persistence of Dengue-3 Virus Through Transovarial Transmission Passage in Successive Generations of *Aedes*

aegypti Mosquitoes." American Journal of Tropical Medicine and Hygiene **67** (2): 158-161.

Kraemer, M. U., Sinka, M. E., Duda, K. A., Mylne, A., Shearer, F. M., Brady, O. J., Messina, J. P., Barker, C. M., Moore, C. G., Carvalho, R. G., Coelho, G. E., Van Bortel, W., Hendrickx, G., Schaffner, F., Wint, G. R., Elyazar, I. R., Teng, H.-J. and Hay, S. I. (2015). "The global compendium of *Aedes aegypti* and *Ae. albopictus* occurrence." Sci Data **2**: 150035.

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S. and Strauss, J. H. (2002). "Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion." Cell **108** (5): 717-725.

Kumar, M. and Ando, Y. (2010). "Chemical Vapor Deposition of Carbon Nanotubes: A Review on Growth Mechanism and Mass Production." J. Nanosci. Nanotechnol. **10** (6): 3739-3758.

Kuno, G., Gubler, D. J., Vélez, M. and Oliver, A. (1985). "Comparative sensitivity of three mosquito cell lines for isolation of dengue viruses." Bulletin of the World Health Organization **63** (2): 279-286.

Kurosu, T., Chaichana, P., Yamate, M., Anantapreecha, S. and Ikuta, K. (2007). "Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1." Biochem Biophys Res Commun **362** (4): 1051-1056.

Kyle, J. L. and Harris, E. (2008). "Global Spread and Persistence of Dengue." Annu Rev Microbiol **62** (1): 71-92.

Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J. and Vorndam, A. V. (1992). "Rapid Detection and Typing of Dengue Viruses from Clinical-Samples by Using Reverse Transcriptase-Polymerase Chain-Reaction." Journal of Clinical Microbiology **30** (3): 545-551.

Libraty, D. H., Endy, T. P., Houng, H. S., Green, S., Kalayanarooj, S., Suntayakorn, S., Chansiriwongs, W., Vaughn, D. W., Nisalak, A., Ennis, F. A. and Rothman, A. L. (2002a). "Differing Influences of Virus Burden and Immune Activation on Disease Severity in Secondary Dengue-3 Virus Infections." J Infect Dis **185** (9): 1213-1221.

Libraty, D. H., Young, P. R., Pickering, D., Endy, T. P., Kalayanarooj, S., Green, S., Vaughn, D. W., Nisalak, A., Ennis, F. A. and Rothman, A. L. (2002b). "High Circulating Levels of the Dengue Virus Nonstructural Protein NS1 Early in Dengue Illness Correlate with the Development of Dengue Hemorrhagic Fever." J Infect Dis **186** (8): 1165-1168.

Lima, M. d. R., Nogueira, R. M., Schatzmayr, H. G. and dos Santos, F. B. (2010). "Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil." PLoS Negl Trop Dis **4** (7): e738.

Lin, C.-F., Lei, H.-Y., Shiau, A.-L., Liu, C.-C., Liu, H.-S., Yeh, T.-M., Chen, S.-H. and Lin, Y.-S. (2003). "Antibodies From Dengue Patient Sera Cross-React With Endothelial Cells and Induce Damage." J Med Virol **69** (1): 82-90.

Lin, C.-F., Lei, H.-Y., Shiau, A.-L., Liu, H.-S., Yeh, T.-M., Chen, S.-H., Liu, C.-C., Chiu, S.-C. and Lin, Y.-S. (2002). "Endothelial Cell Apoptosis Induced by Antibodies Against Dengue Virus Nonstructural Protein 1 Via Production of Nitric Oxide." J Immunol **169** (2): 657-664.

Lum, L. C., Goh, A. Y., Chan, P. W., El-Amin, A.-L. and Lam, S. K. (2002). "Risk factors for hemorrhage in severe dengue infections." J Pediatr **140** (5): 629-631.

Martins, V. E., Alencar, C. H., Kamimura, M. T., de Carvalho Araújo, F. M., De Simone, S. G., Dutra, R. F. and Guedes, M. I. (2012). "Occurrence of Natural Vertical Transmission of Dengue-2 and Dengue-3 Viruses in *Aedes aegypti* and *Aedes albopictus* in Fortaleza, Ceara, Brazil." PLoS ONE **7** (7): e41386.

Mercado-Curiel, R. F., Esquinca-Avilés, H. A., Tovar, R., Díaz-Badillo, Á., Camacho-Nuez, M. and Muñoz, M. d. L. (2006). "The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells." BMC Microbiol **6** (1): 85.

Miller, J. L., de Wet, B. J., Martinez-Pomares, L., Radcliffe, C. M., Dwek, R. A., Rudd, P. M. and Gordon, S. (2008). "The Mannose Receptor Mediates Dengue Virus Infection of Macrophages." PLoS Pathog **4** (2): e17.

Modis, Y., Ogata, S., Clements, D. and Harrison, S. C. (2005). "Variable Surface Epitopes in the Crystal Structure of Dengue Virus Type 3 Envelope Glycoprotein." J Virol **79** (2): 1223-1231.

Mustafa, M. S., Rasotgi, V., Jain, S. and Gupta, V. (2015). "Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control." Med J Armed Forces India **71** (1): 67-70.

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J.-L., Arenzana-Seisdedos, F. and Desprès, P. (2003). "Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses." EMBO Rep **4** (7): 723-728.

Oberlin, A., Endo, M. and Koyama, T. (1976). "Filamentous Growth of Carbon through Benzene Decomposition." Journal of Crystal Growth **32** (3): 335-349.

Osorio, L., Ramirez, M., Bonelo, A., Villar, L. A. and Parra, B. (2010). "Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection." Virol J **7** (1): 361.

Parkash, O. and Shueb, R. H. (2015). "Diagnosis of Dengue Infection Using Conventional and Biosensor Based Techniques." Viruses **7** (10): 5410-5427.

Peeling, R. W., Artsob, H., Pelegrino, J. L., Buchy, P., Cardosa, M. J., Devi, S., Enria, D. A., Farrar, J., Gubler, D. J., Guzman, M. G., Halstead, S. B., Hunsperger, E., Kliks, S., Margolis, H. S., Nathanson, C. M., Nguyen, V. C., Rizzo, N., Vázquez, S. and Yoksan, S. (2010). "Evaluation of diagnostic tests: dengue." Nat Rev Microbiol **8** (12 Suppl): S30-38.

Pettersson, J. H. and Fiz-Palacios, O. (2014). "Dating the origin of the genus *Flavivirus* in the light of Beringian biogeography." J Gen Virol **95** (Pt 9): 1969-1982.

Philip Samuel, P. and Tyagi, B. K. (2006). "Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes." Indian J Med Res **123** (5): 615-628.

Pirich, C. L., de Freitas, R. A., Torresi, R. M., Picheth, G. F. and Sierakowski, M. R. (2017). "Piezoelectric immunochip coated with thin films of bacterial cellulose nanocrystals for dengue detection." Biosensors and Bioelectronics **92**: 47-53.

Platt, K. B., Linthicum, K. J., Myint, K. S., Innis, B. L., Lerdthusnee, K. and Vaughn, D. W. (1997). "Impact of Dengue Virus Infection on Feeding Behavior of *Aedes aegypti*." Am J Trop Med Hyg **57** (2): 119-125.

Putnam, J. L. and Scott, T. W. (1995). "Blood-Feeding Behavior of Dengue-2 Virus-Infected *Aedes aegypti*." Am J Trop Med Hyg **52** (3): 225-227.

Pyke, A. T., Moore, P. R., Taylor, C. T., Hall-Mendelin, S., Cameron, J. N., Hewitson, G. R., Pukallus, D. S., Huang, B., Warrilow, D. and van den Hurk, A. F. (2016). "Highly divergent dengue virus type 1 genotype sets a new distance record." Scientific Reports **6**: 22356.

Ramasamy, R. and Surendran, S. N. (2016). "Mosquito vectors developing in atypical anthropogenic habitats: Global overview of recent observations, mechanisms and impact on disease transmission." Journal of vector borne diseases **53** (2): 91-98.

Ramnani, P., Saucedo, N. M. and Mulchandani, A. (2016). "Carbon nanomaterial-based electrochemical biosensors for label-free sensing of environmental pollutants." Chemosphere **143**: 85-98.

Reibold, M., Paufler, P., Levin, A. A., Kochmann, W., Pätzke, N. and Meyer, D. C. (2006). "Materials: carbon nanotubes in an ancient Damascus sabre." Nature **444** (7117): 286.

Reibold, M., Paufler, P., Levin, A. A., Kochmann, W., Pätzke, N. and Meyer, D. C. (2009). Discovery of Nanotubes in Ancient Damascus Steel. Physics and Engineering of New Materials. Cat, D. T., Pucci, A. and Wandelt, K. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 305-310.

Reich, S., Thomsen, C. and Maultzsch, J. (2008). Carbon Nanotubes: Basic Concepts and Physical Properties, John Wiley & Sons.

Rico-Hesse, R. (1990). "Molecular Evolution and Distribution of Dengue Viruses Type 1 and 2 in Nature." Virology **174** (2): 479-493.

Rodenhuis-Zybert, I. A., Wilschut, J. and Smit, J. M. (2010). "Dengue virus life cycle: viral and host factors modulating infectivity." Cell Mol Life Sci **67** (16): 2773-2786.

Rudnick, A. (1965). "Studies of the ecology of dengue in Malaysia: a preliminary report." J Med Entomol **2** (2): 203-208.

Rudnick, A. (1986). "Dengue virus ecology in Malaysia." Inst. Med. Res. Malays. Bull **23**: 51-152.

Rudnick, A., Lim, T. W. and Ireland, J. (1986). Dengue fever studies in Malaysia, Institute for Medical Research.

Sabin, A. B. (1952). "Research on Dengue during World War II." The American Journal of Tropical Medicine and Hygiene **1** (1): 30-50.

Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E. and Beaty, B. J. (2007). "Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes." BMC Microbiol **7**: 9.

Schilling, S., Ludolfs, D., Van An, L. and Schmitz, H. (2004). "Laboratory diagnosis of primary and secondary dengue infection." J Clin Virol **31** (3): 179-184.

Schneider, B. S. and Higgs, S. (2008). "The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response." Trans R Soc Trop Med Hyg **102** (5): 400-408.

Shamala, D. S. (2015). "Laboratory Diagnosis of Dengue: A Review." International Medical Journal Malaysia **14** (1): 17-28.

Shu, P.-Y., Chang, S.-F., Kuo, Y.-C., Yueh, Y.-Y., Chien, L.-J., Sue, C.-L., Lin, T.-H. and Huang, J.-H. (2003). "Development of Group- and Serotype-Specific One-Step

SYBR Green I-Based Real-Time Reverse Transcription-PCR Assay for Dengue Virus." J Clin Microbiol **41** (6): 2408-2416.

Shu, P.-Y. and Huang, J.-H. (2004). "Current Advances in Dengue Diagnosis." Clin Diagn Lab Immunol **11** (4): 642-650.

Shu, P.-Y., Yang, C.-F., Kao, J.-F., Su, C.-L., Chang, S.-F., Lin, C.-C., Yang, W.-C., Shih, H., Yang, S.-Y., Wu, P.-F., Wu, H.-S. and Huang, J.-H. (2009). "Application of the Dengue Virus NS1 Antigen Rapid Test for On-Site Detection of Imported Dengue Cases at Airports." Clin Vaccine Immunol **16** (4): 589-591.

Silva, M. M. S., Dias, A. C. M. S., Silva, B. V. M., Gomes-Filho, S. L. R., Kubota, L. T., Goulart, M. O. F. and Dutra, R. F. (2015). "Electrochemical detection of dengue virus NS1 protein with a poly(allylamine)/carbon nanotube layered immunoelectrode." J Chem Technol Biot **90** (1): 194-200.

Sim, S., Ramirez, J. L. and Dimopoulos, G. (2012). "Dengue Virus Infection of the *Aedes aegypti* Salivary Gland and Chemosensory Apparatus Induces Genes that Modulate Infection and Blood-Feeding Behavior." PLoS Pathog **8** (3): e1002631.

Simmons, C. P., Farrar, J. J., Nguyen v, V. and Wills, B. (2012). "Dengue." The New England journal of medicine **366** (15): 1423-1432.

Simmons, C. P., McPherson, K., Van Vinh Chau, N., Hoai Tam, D. T., Young, P., Mackenzie, J. and Wills, B. (2015). "Recent advances in dengue pathogenesis and clinical management." Vaccine **33** (50): 7061-7068.

Singh, M. P., Majumdar, M., Singh, G., Goyal, K., Preet, K., Sarwal, A., Mishra, B. and Ratho, R. K. (2010). "NS1 antigen as an early diagnostic marker in dengue: report from India." Diagn Microbiol Infect Dis **68** (1): 50-54.

Smith, C. E. G. (1956). "The history of dengue in tropical Asia and its probable relationship to the mosquito *Aedes aegypti*." J Trop Med Hyg **59** (10): 243-251.

Succo, T., Leparc-Goffart, I., Ferré, J. B., Roiz, D., Broche, B., Maquart, M., Noel, H., Catelinois, O., Entezam, F., Caire, D., Jourdain, F., Esteve-Moussion, I., Cochet, A., Paupy, C., Rousseau, C., Paty, M. C. and Golliot, F. (2016). "Autochthonous dengue outbreak in Nimes, South of France, July to September 2015." Euro Surveill **21** (21).

Sz-Hau, C., Yao-Chen, C., Yi-Chen, L., Hsiu-Chao, L., Yun-Liang, Y. and Chih-Sheng, L. (2009). "A method of layer-by-layer gold nanoparticle hybridization in a quartz crystal microbalance DNA sensing system used to detect dengue virus." Journal of Nanotechnology **20** (21): 215501.

Twiddy, S. S. and Holmes, E. C. (2003). "The extent of homologous recombination in members of the genus *Flavivirus*." J Gen Virol **84** (Pt 2): 429-440.

Vasilakis, N., Cardosa, J., Hanley, K. A., Holmes, E. C. and Weaver, S. C. (2011). "Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health." Nat Rev Microbiol **9** (7): 532-541.

Vasilakis, N., Tesh, R. B. and Weaver, S. C. (2008). "Sylvatic Dengue Virus Type 2 Activity in Humans, Nigeria, 1966." Emerg Infect Dis **14** (3): 502-504.

Vaughn, D. W., Green, S., Kalayanarooj, S., Innis, B. L., Nimmannitya, S., Suntayakorn, S., Endy, T. P., Raengsakulrach, B., Rothman, A. L., Ennis, F. A. and Nisalak, A. (2000). "Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity." J Infect Dis **181** (1): 2-9.

Vazquez, S., Hafner, G., Ruiz, D., Calzada, N. and Guzman, M. G. (2007). "Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic dengue infections." J Clin Virol **39** (3): 194-198.

Waggoner, J. J., Abeynayake, J., Sahoo, M. K., Gresh, L., Tellez, Y., Gonzalez, K., Ballesteros, G., Pierro, A. M., Gaibani, P., Guo, F. P., Sambri, V., Balmaseda, A., Karunaratne, K., Harris, E. and Pinsky, B. A. (2013). "Single-Reaction, Multiplex, Real-Time RT-PCR for the Detection, Quantitation, and Serotyping of Dengue Viruses." PLoS Negl Trop Dis **7** (4): e2116.

Wahala, W. M. and de Silva, A. M. (2011). "The Human Antibody Response to Dengue Virus Infection." Viruses **3** (12): 2374-2395.

Wang, F. and Swager, T. M. (2011). "Diverse Chemiresistors Based upon Covalently Modified Multiwalled Carbon Nanotubes." J Am Chem Soc **133** (29): 11181-11193.

Wang, S. M. and Sekaran, S. D. (2010). "Early Diagnosis of Dengue Infection Using a Commercial Dengue Duo Rapid Test Kit for the Detection of NS1, IGM, and IGG." Am J Trop Med Hyg **83** (3): 690-695.

Welch, R. J., Chang, G.-J. and Litwin, C. M. (2014). "Comparison of a commercial dengue IgM capture ELISA with dengue antigen focus reduction microneutralization test and the centers for disease control dengue IgM capture-ELISA." J Virol Methods **195**: 247-249.

Winkler, G., Maxwell, S. E., Ruemmler, C. and Stollar, V. (1989). "Newly Synthesized Dengue-2 Virus Nonstructural Protein NS1 is a Soluble Protein but Becomes Partially Hydrophobic and Membrane-Associated after Dimerization." Virology **171** (1): 302-305.

Wong, W. R., Sekaran, S. D., Adikan, F. R. and Berini, P. (2016). "Detection of dengue NS1 antigen using long-range surface plasmon waveguides." Biosensors and Bioelectronics **78**: 132-139.

World Health Organization (2009). Dengue: Guidelines for Diagnosis Treatment Prevention and Control: New Edition. Geneva, World Health Organization.

Xi, Z., Ramirez, J. L. and Dimopoulos, G. (2008). "The *Aedes aegypti* Toll Pathway Controls Dengue Virus Infection." PLoS Pathog **4** (7): e1000098.

Yang, N., Chen, X. P., Ren, T. L., Zhang, P. and Yang, D. G. (2015). "Carbon nanotube based biosensors." Sensors and Actuators B: Chemical **207**: 690-715.

Yong, Y. K., Thayan, R., Chong, H. T., Tan, C. T. and Sekaran, S. D. (2007). "Rapid detection and serotyping of dengue virus by multiplex RT-PCR and real-time SYBR green RT-PCR." Singapore Med J **48** (7): 662-668.

Young, P. R., Hilditch, P. A., Bletchly, C. and Halloran, W. (2000). "An Antigen Capture Enzyme-Linked Immunosorbent Assay Reveals High Levels of the Dengue Virus Protein NS1 in the Sera of Infected Patients." J Clin Microbiol **38** (3): 1053-1057.

Zhang, X. K., Ge, P., Yu, X. K., Brannan, J. M., Bi, G. Q., Zhang, Q. F., Schein, S. and Zhou, Z. H. (2013). "Cryo-EM structure of the mature dengue virus at 3.5-Å resolution." Nat Struct Mol Biol **20** (1): 105-110.

Chapter 2: A Heparin-Functionalized Carbon Nanotube-Based Affinity Biosensor for Dengue Virus

2.1 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.

2.2 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 (World Health Organization, 2009; Guzmán and Harris, 2015). 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 (Race et al., 1978; Philip Samuel and Tyagi, 2006; Salje et al., 2014). Commonly, 6 to 10 days post infection, when samples as low as 1-10 pfu/ml have propagated into (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, 2009). This 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., 2016). 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 (Wang et al., 2007; Vlasak and Ionescu, 2011). 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 has 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.

2.3 Materials and Methods

2.3.1 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, 4,000 × g, 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 x 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 $5.5x10^3$ $TCID_{50}$/mL in low titer laboratory viral culture stocks and $8.4x10^5$ $TCID_{50}$/mL in high titer stocks, as determined by $TCID_{50}$ with Vero cells in a 96-well plate.

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.

2.3.2 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 Figure 2.1. 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 hour to block any non-specific adsorption to the SWNT, followed by thorough rinsing with PB.

2.3.3 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 a semiconductor parameter analyzer (Model 1202A, CH Instruments, Inc., TX, USA). The voltage was swept from −200 mV to 200 mV and the current recorded. Linear regression analysis was used to calculate the slope of the current/voltage curves between −100 mV to 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.

2.4 Results and Discussion

2.4.1 Heparin

Heparan sulfate is a variably sulfated glycosaminoglycan (GAG) attached to some cell surface protein-core molecules and is utilized by DENV during invasion of host cells (Artpradit et al., 2013). Heparin, an analogue 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 (Figure 2.2). 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, 2006; 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 (Pereira et al., 2010; Kostyuchenko et al., 2013). 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 40-60 nm in diameter, only charges within the Debye length are expected to contribute to changes in the resistance (Stefansson et al., 2012).

2.4.2 Reduction of matrix effects of MEM-2 and ML-

After the biosensors were incubated in MEM-2 alone for 10-min, the increase in resistance was 33.99±6.15% (n=8). However, when incubated a second time for an additional 10-min, the increase in resistance was minimal: 4.49±2.48%. When a complex mixture of MEM-2 and Vero cell lysates (ML-) was similarly tested, the first incubation resulted in a 40.60±6.81% increase in resistance. This was greater than MEM-2 alone (n=8; p=0.04) and indicates that Vero cell lysate may interact with heparin. However, when the biosensor was challenged with a second incubation in ML-, the increase in resistance

(4.29±3.52%) was not significantly different from the second incubation in MEM-2 alone (n=8; p>0.05)**. (**Figure 2.3**)**

To elucidate the interactions of Vero cell lysate on the biosensor, the biosensors were first blocked with MEM-2 alone for 10-min followed by a 10-min incubation in ML-, resulting in an increase of 32.89±5.86% and 11.65±4.68%, respectively. The presence of Vero cell lysate in the second incubation resulted in a larger increase in resistance than the second incubation in MEM-2 alone; (p>0.05) indicating binding of Vero cell lysate to the heparin receptor. Propagation of virus in cultured Vero cells requires the use of MEM-2 to maintain viability of the cell line and support infection. As viral infection progresses through the lytic phase, a number of biologically-active proteins and other cellular components are released into the culture media. Heparin-binding epidermal growth factor-like growth factor is a membrane-anchored protein found in Vero cells (Goishi et al., 1995). Thus, cell lysate interaction with heparin was not unexpected.

Negative control experiments were performed with biosensors functionalized by Tween 20 without the heparin receptor. When incubated in MEM-2 alone, there was a large increase in resistance: 34.26±7.41%. However, subsequent challenge with the complex mixture of DENV, MEM-2, and Vero cell lysates (ML+D) resulted an increase of only 3.97±1.98% (n=7). With the SWNTs blocked by Tween 20 to prevent non-specific binding to the SWNT, the response to incubation MEM-2 and Vero cell lysate continued to be minimal. The increase in resistance of the second challenge was no greater than those on

functionalized devices ($p>0.05$); despite presence of DENV and Vero cell lysate. These results confirm Vero cell lysate and DENV interaction with Heparin. (Figure 2.3)

The increase in resistance upon first incubation with MEM-2 was not significantly different between any experiments utilizing MEM-2 ($p>0.05$). When biosensors were challenged with a second incubation, the response to MEM-2, ML-, and the negative control 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±2.65% increase in resistance. Heparin functionalization did not affect background signal or interaction of MEM-2 with the sensor. Upon blocking the gold electrodes by incubation with ML-, the biosensors had minimal response to incubation in either MEM-2 or ML-, even after multiple washings. The heparin-functionalized SWNT biosensor can be used to detect DENV in the supernatant of Vero cell culture without further treatment. (Figure 2.3)

2.4.3 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% background noise were not used for virus sensing. As shown in Figure 2.4, the response was a linear function of the logarithm of the viral concentrations between $8.4x10^2$ $TCID_{50}$/mL and $8.4x10^5$ $TCID_{50}$/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 $8.4x10^2$ $TCID_{50}$/mL, which equates to approximately 8 $TCID_{50}$/chip. (Figure 2.5) Sample titers

were within the clinically relevant range for humans and infected *Aedes aegypti*: 1-10^9 pfu/mL and 9×10^3-10^4 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 (Figure 2.6). The negligible resistance changes of -1.34 ± 1.31% and -1.62 ± 1.44%, 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 ± 1.57%, which was not significantly different from the PB controls ($n=11$; $p>.05$) (Figure 2.6b). 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.

2.4.4 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-).

2.4.5 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., 2008). 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 ($p>.05$) but indicate that MEM-2 has a small matrix effect on the biosensors causing a background signal of $4.26 \pm 2.65\%$ increase in resistance. (Figure 2.3)

2.4.6 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 ($5.5x10^3$ $TCID_{50}$/mL) taken directly from viral culture and a simulated high titer ($8.4x10^5$ $TCID_{50}$/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 $18.39 \pm 3.91\%$ (n=8)

for low titer ML+D and 39.07 ± 6.06% (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 (Figure 2.7) (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 ± 2.57% 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 ± 4.72% and 34.81 ± 6.61% for the low titer ML+D and high titer ML+D, respectively, 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 $8.4x10^2$ $TCID_{50}$/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. (2017) 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 $2x10^4$ viral particles/mL, a concentration relevant to acute infection phase. Additionally, antibodies are subject to denaturing and aggregation after only a few weeks of room temperature storage (Wang et al., 2007; Thiagarajan et al., 2016). 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 (Pritchard, 1964; Jandik et al., 1996; Akeel et al., 2013; Fu et al., 2014). 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 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 minutes 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.

2.5 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 10μL sample, a limit of detection of $8.4x10^2$ $TCID_{50}$/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.

2.1 Figures

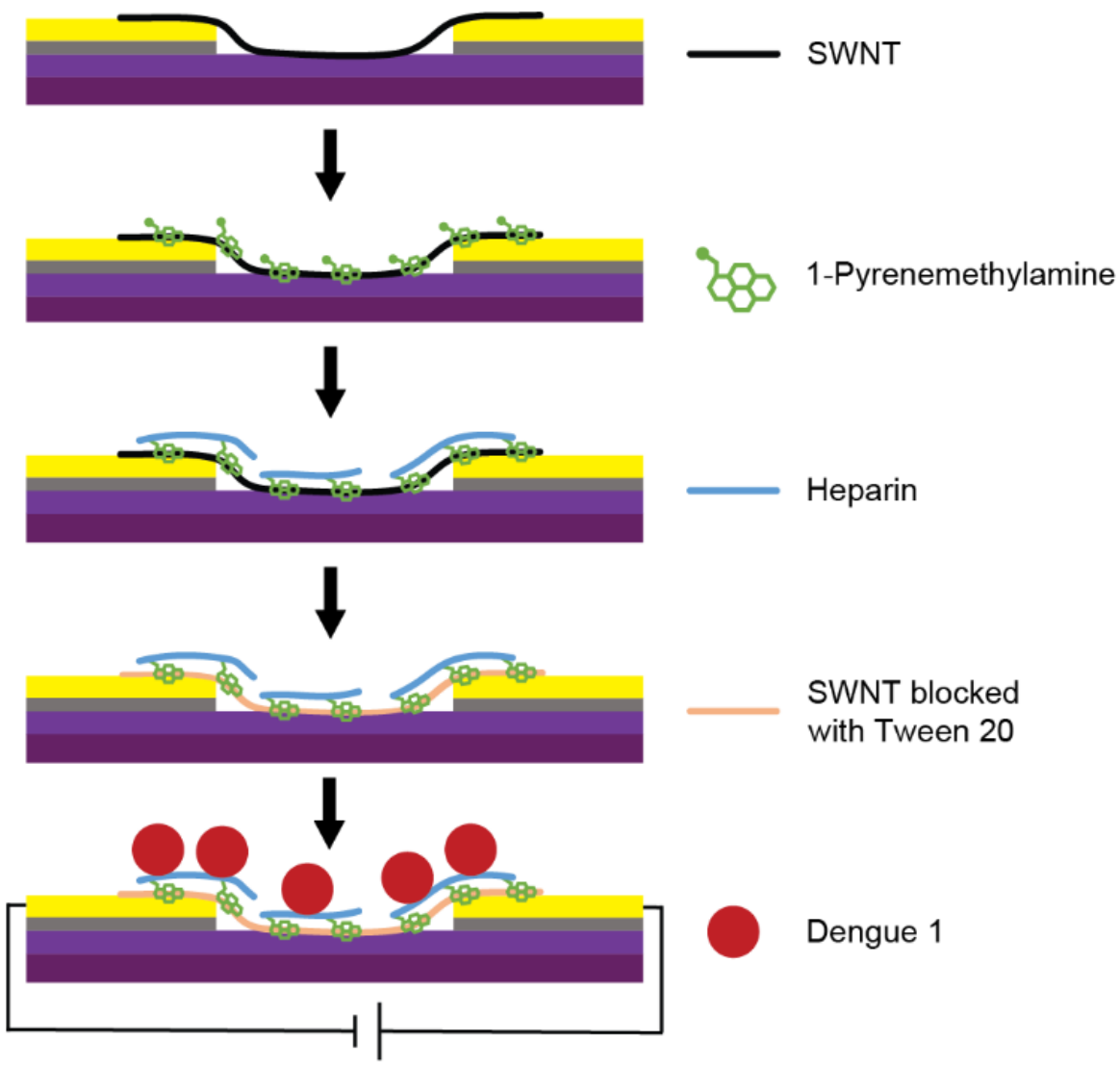

Figure 2.1 Functionalization schematic representation of the fabrication of the chemiresistor used for detection of dengue virus.

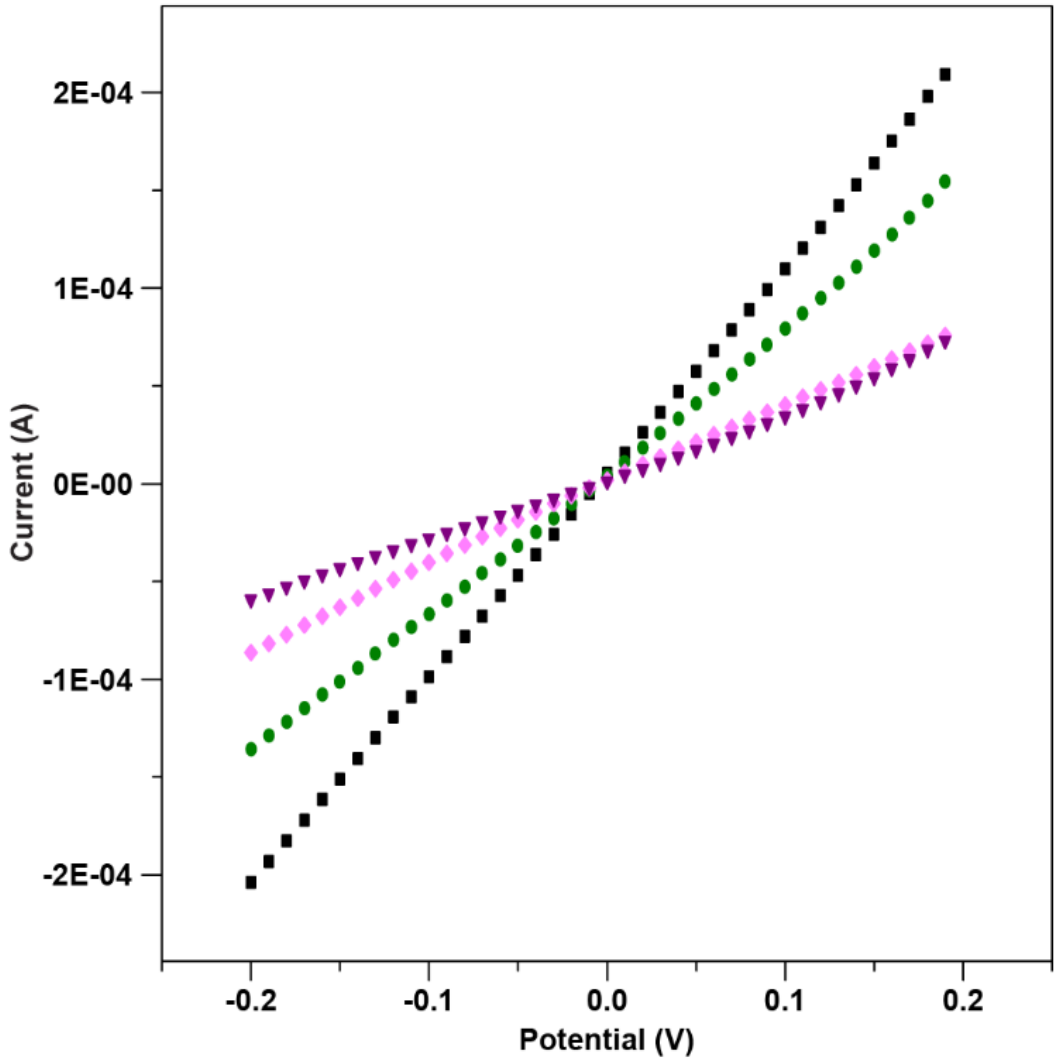

Figure 2.2 IV curve of bare SWNTs in PB (■), after functionalization with 1-Pyrenemethylamine (●), immobilization of the heparin glycosaminoglycan (♦), and blocking of unoccupied sites with Tween 20 (▼).

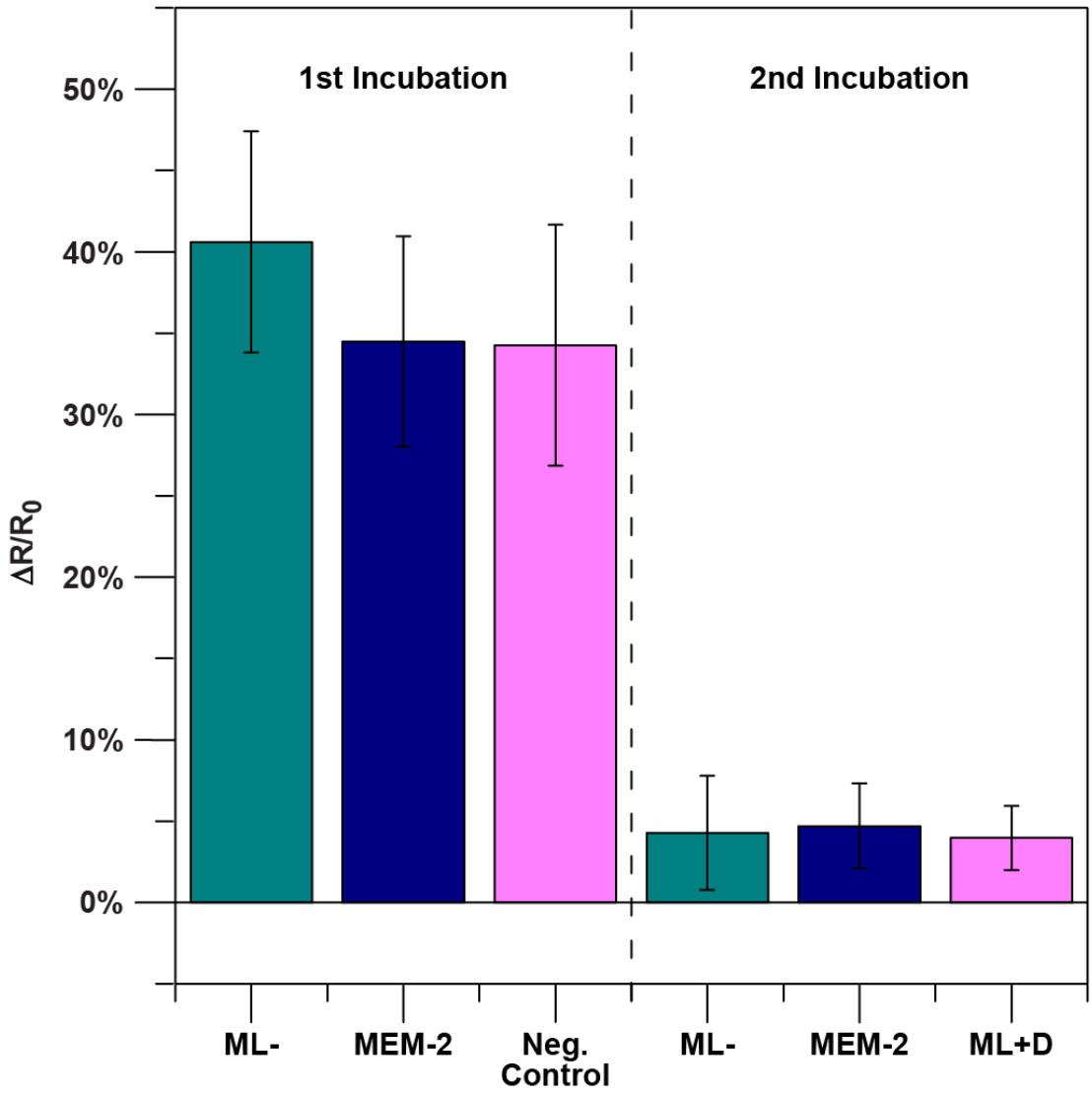

Figure 2.3 Blanking controls of chemiresistor response during control measurements. No significant difference between the 1st incubation of MEM-2 alone (blue) on heparin functionalized biosensors and biosensors only functionalized with Tween 20 (pink). First incubation of MEM-2 containing Vero cell lysate (ML-, teal) on functional device resulted in a larger increase in resistance [n=8; p=0.04]. DENV suspended in MEM-2 containing cell lysate (ML+D) incubated on biosensors only functionalized with Tween 20 (second incubation; pink) was not significantly different from the second incubations of ML- or MEM-2 alone on heparin functionalized devices [n=7, p>0.05]. The data points are the mean of measurements from independent biosensors, and error bars represent ±1 standard deviation.

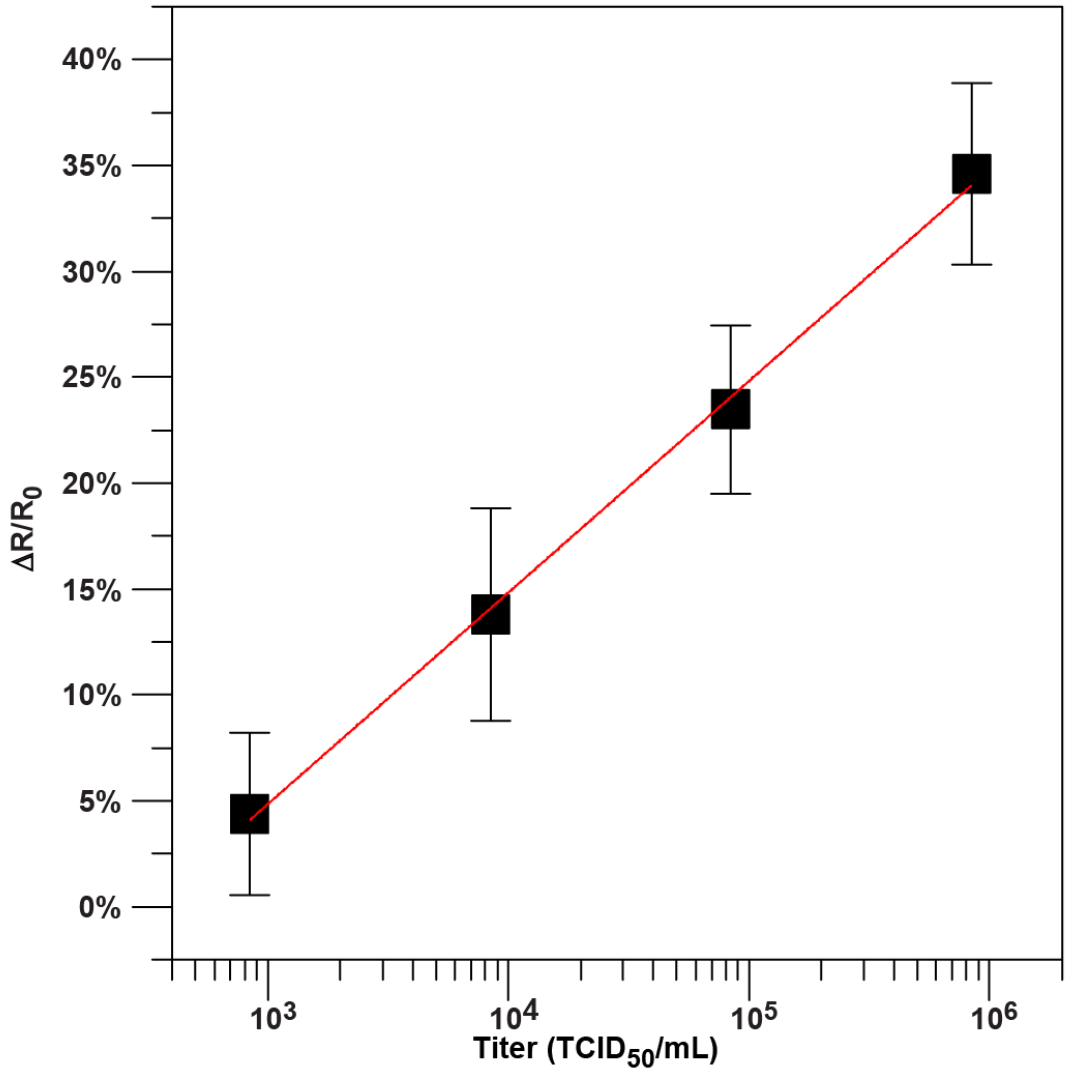

Figure 2.4 Chemiresistor response to incubation with dengue in phosphate buffer. The data points are the mean of measurements from 11 independent biosensors, and error bars represent ±1 standard deviation ($y = 0.10x - 0.25$, $R^2=0.998$).

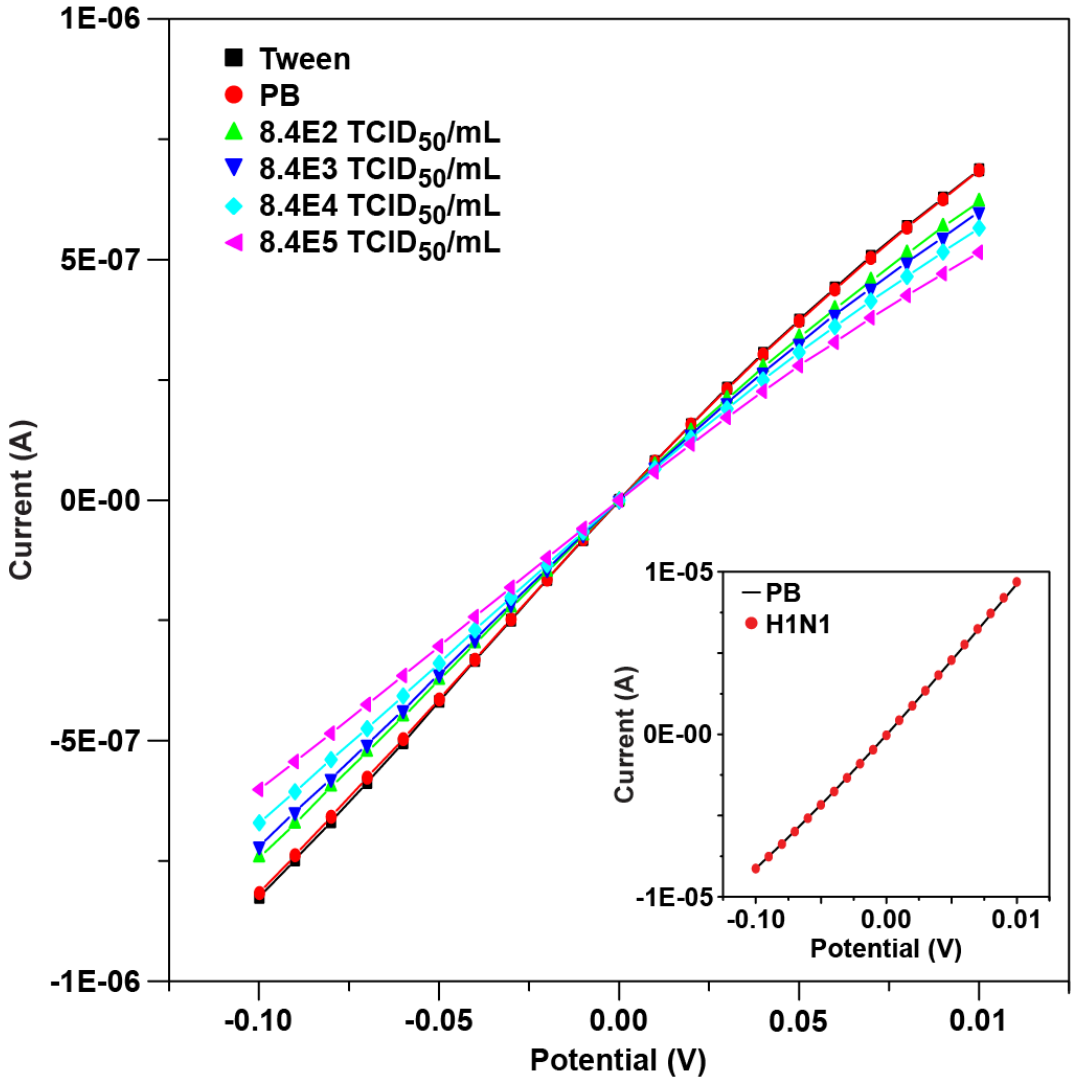

Figure 2.5 IV curve of a single electrode to the titers of DENV. Insert: Current-voltage characteristics of a single electrode to H1N1.

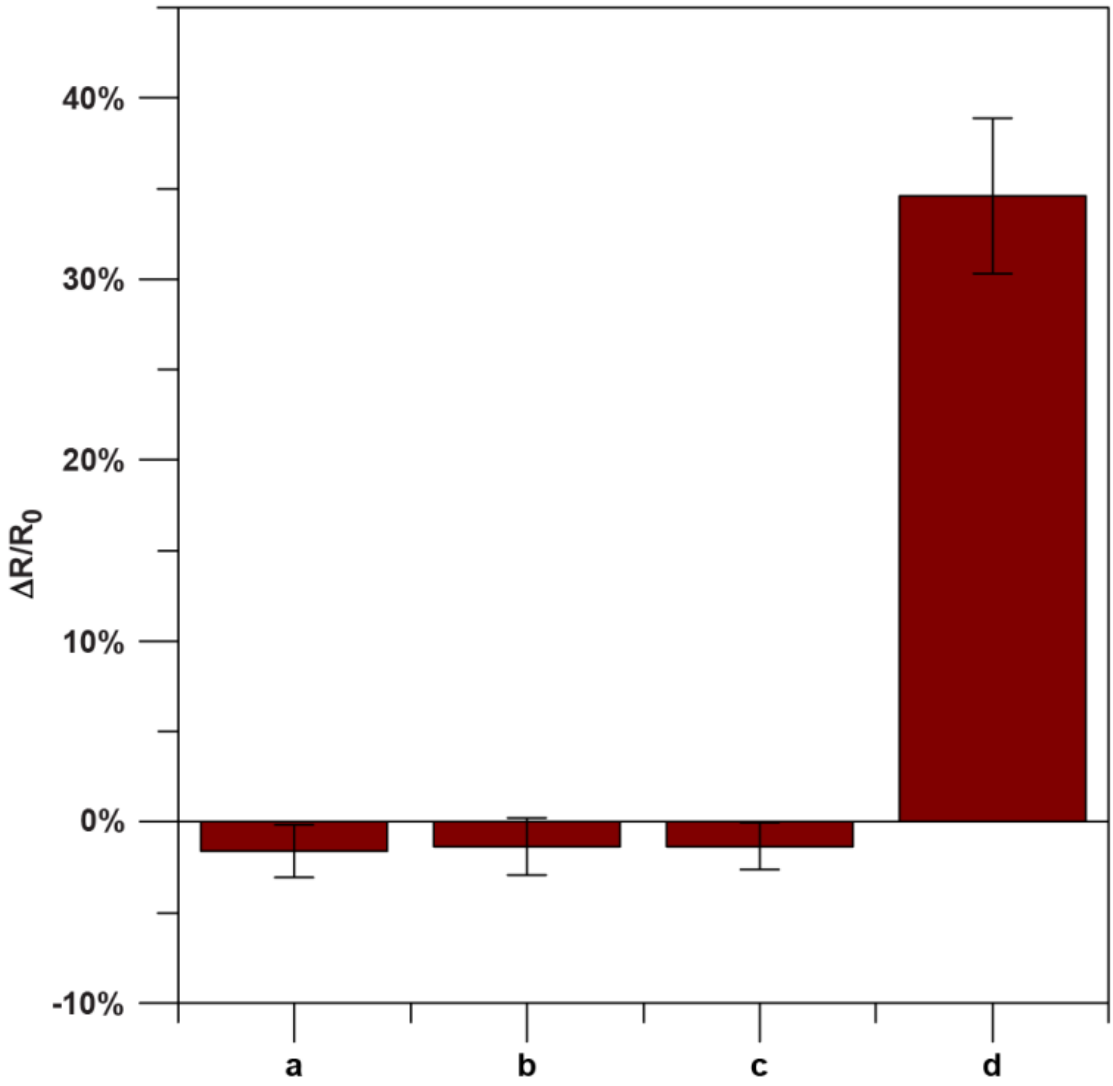

Figure 2.6 Comparison of the (a) response of Tween-20 functionalized chemiresistor to $8.4x10^5$ $TCID_{50}$/mL dengue virus in phosphate buffer [n=7] and the response of heparin functionalized chemiresistor to (b) Influenza A H1N1 (c) phosphate buffer (d) $8.4x10^5$ $TCID_{50}$/mL dengue in buffer. Unless noted, the data points are the mean of measurements from 11 independent biosensors, and error bars represent ±1 standard deviation.

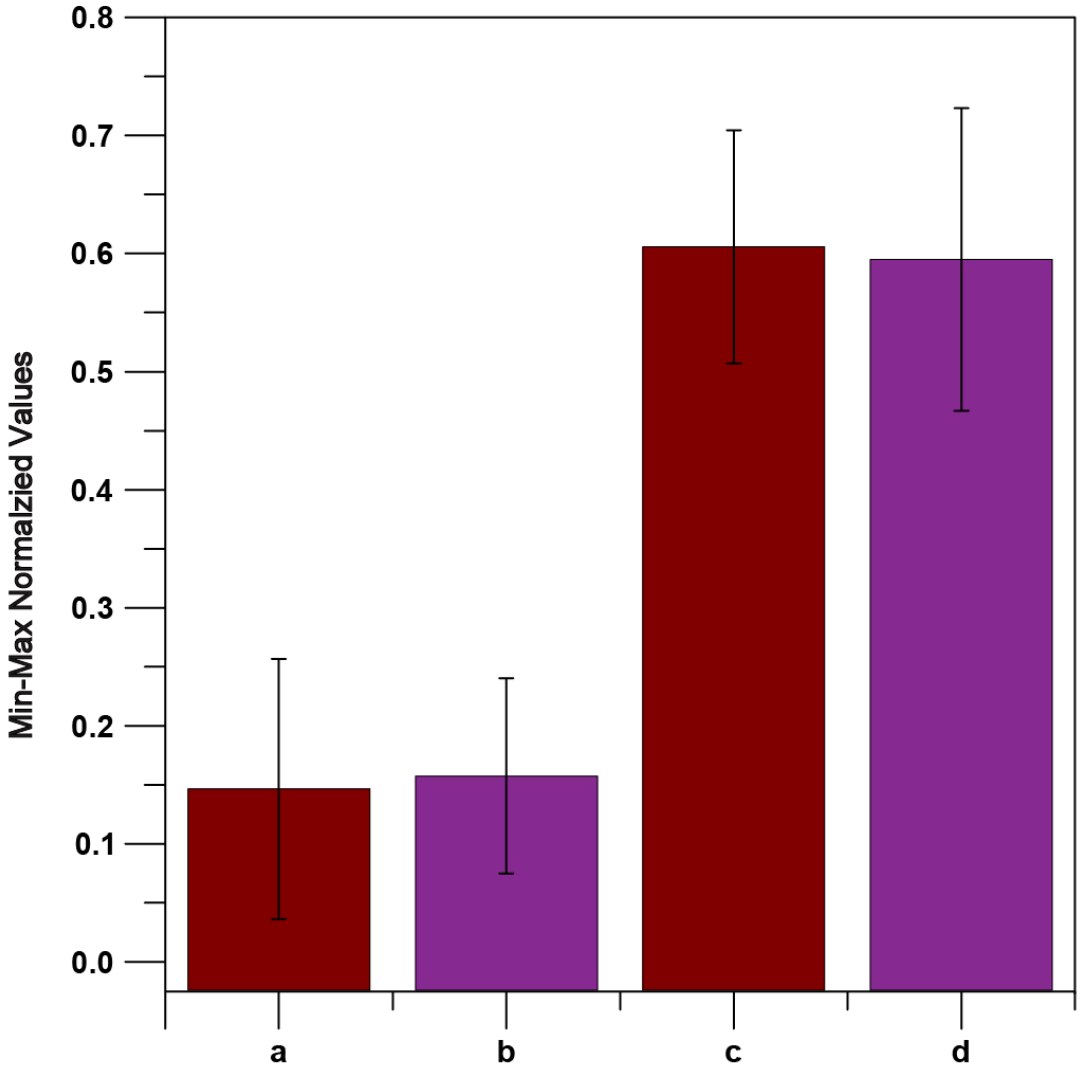

Figure 2.7 Comparison of Min-Max normalized chemiresistor response to dengue in phosphate buffer [n=11] and to DENV suspended in MEM-2 containing Vero cell lysate (ML+D). [n=8] (a) Response to $8.4x10^3$ $TCID_{50}$/mL DENV in PB (b) $5.5x10^3$ $TCID_{50}$/mL ML+D(c) $8.4x10^5$ $TCID_{50}$/mL DENV in PB (d) $8.4x10^5$ $TCID_{50}$/mL ML+D. The data points are the mean of measurements from independent biosensors, and error bars represent ±1 standard deviation.

2.1 References

Akeel, A., Sibanda, S., Martin, S. W., Paterson, A. W. and Parsons, B. J. (2013). "Chlorination and oxidation of heparin and hyaluronan by hypochlorous acid and hypochlorite anions: effect of sulfate groups on reaction pathways and kinetics." Free Radic Biol Med **56**: 72-88.

Allen, B. L., Kichambare, P. D. and Star, A. (2007). "Carbon Nanotube Field-Effect-Transistor-Based Biosensors." Advanced Materials **19** (11): 1439-1451.

Artpradit, C., Robinson, L. N., Gavrilov, B. K., Rurak, T. T., Ruchirawat, M. and Sasisekharan, R. (2013). "Recognition of heparan sulfate by clinical strains of dengue virus serotype 1 using recombinant subviral particles." Virus Res **176** (1–2): 69-77.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J. and Hay, S. I. (2013). "The global distribution and burden of dengue." Nature **496** (7446): 504-507.

Bhattacharya, M., Hong, S., Lee, D., Cui, T. and Goyal, S. M. (2011). "Carbon nanotube based sensors for the detection of viruses." Sensors and Actuators B: Chemical **155** (1): 67-74.

Blacksell, S. D. (2012). "Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs?" J Biomed Biotechnol **2012**: 12.

Byon, H. R. and Choi, H. C. (2006). "Network Single-Walled Carbon Nanotube-Field Effect Transistors (SWNT-FETs) with Increased Schottky Contact Area for Highly Sensitive Biosensor Applications." J Am Chem Soc **128** (7): 2188-2189.

Capila, I. and Linhardt, R. J. (2002). "Heparin-Protein Interactions." Angewandte Chemie International Edition **41** (3): 391-412.

Cardosa, J., Ooi, M. H., Tio, P. H., Perera, D., Holmes, E. C., Bibi, K. and Abdul Manap, Z. (2009). "Dengue Virus Serotype 2 from a Sylvatic Lineage Isolated from a Patient with Dengue Hemorrhagic Fever." PLoS Negl Trop Dis **3** (4): e423.

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J. and Marks, R. M. (1997). "Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate." Nat Med **3** (8): 866-871.

Cheng, M. S., Lau, S. H., Chan, K. P., Toh, C.-S. and Chow, V. T. (2015). "Impedimetric cell-based biosensor for real-time monitoring of cytopathic effects induced by dengue viruses." Biosensors and Bioelectronics **70**: 74-80.

Fu, L., Li, L., Cai, C., Li, G., Zhang, F. and Linhardt, R. J. (2014). "Heparin stability by determining unsubstituted amino groups using hydrophilic interaction chromatography mass spectrometry." Anal Biochem **461**: 46-48.

Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995). "Phorbol Ester Induces the Rapid Processing of Cell Surface Heparin-Binding EGF-like Growth Gactor: Conversion from Juxtacrine to Paracrine Growth Factor Activity." Mol Biol Cell **6** (8): 967-980.

Gruner, G. (2006). "Carbon nanotube transistors for biosensing applications." Anal Bioanal Chem **384** (2): 322-335.

Gubler, D. J. (2011). "Dengue, Urbanization and Globalization: The Unholy Trinity of the 21(st) Century." Trop Med Health **39** (4 Suppl): 3-11.

Gubler, D. J., Kuno, G., Sather, G. E., Velez, M. and Oliver, A. (1984). "Mosquito Cell Cultures and Specific Monoclonal Antibodies in Surveillance for Dengue Viruses." Am J Trop Med Hyg **33** (1): 158-165.

Guzmán, M. G. and Harris, E. (2015). "Dengue." The Lancet **385** (9966): 453-465.

Heller, I., Janssens, A. M., Männik, J., Minot, E. D., Lemay, S. G. and Dekker, C. (2008). "Identifying the Mechanism of Biosensing with Carbon Nanotube Transistors." Nano Letters **8** (2): 591-595.

Hidari, K. I., Abe, T. and Suzuki, T. (2013). "Carbohydrate-Related Inhibitors of Dengue Virus Entry." Viruses **5** (2): 605-618.

Hoefling, M., Iori, F., Corni, S. and Gottschalk, K.-E. (2010). "Interaction of Amino Acids with the Au(111) Surface: Adsorption Free Energies from Molecular Dynamics Simulations." Langmuir **26** (11): 8347-8351.

Jandik, K. A., Kruep, D., Cartier, M. and Linhardt, R. J. (1996). "Accelerated Stability Studies of Heparin." J Pharm Sci **85** (1): 45-51.

Kao, C.-L., King, C.-C., Chao, D.-Y., Wu, H.-L. and Chang, G.-J. (2005). "Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health." J Microbiol Immunol Infect **38** (1): 5-16.

Kao, C.-L., Wu, M.-C., Chiu, Y.-H., Lin, J.-L., Wu, Y.-C., Yueh, Y.-Y., Chen, L.-K., Shaio, M.-F. and King, C.-C. (2001). "Flow Cytometry Compared with Indirect

Immunofluorescence for Rapid Detection of Dengue Virus Type 1 after Amplification in Tissue Culture." J Clin Microbiol **39** (10): 3672-3677.

Kostyuchenko, V. A., Zhang, Q., Tan, J. L., Ng, T.-S. and Lok, S.-M. (2013). "Immature and Mature Dengue Serotype 1 Virus Structures Provide Insight into the Maturation Process." J Virol **87** (13): 7700-7707.

Kryscio, D. R. and Peppas, N. A. (2012). "Critical review and perspective of macromolecularly imprinted polymers." Acta Biomater **8** (2): 461-473.

Lee, D., Chander, Y., Goyal, S. M. and Cui, T. (2011). "Carbon nanotube electric immunoassay for the detection of swine influenza virus H1N1." Biosensors and Bioelectronics **26** (8): 3482-3487.

Loureiro, F. C. C. L., Neff, H., Melcher, E. U. K., Roque, R. A., de Figueiredo, R. M. P., Thirstrup, C., Borre, M. B. and Lima, A. M. N. (2017). "Simplified immunoassay for rapid Dengue serotype diagnosis, revealing insensitivity to non-specific binding interference." Sensing and Bio-Sensing Research **13**: 96-103.

Marks, R. M., Lu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernáiz, M. J. and Linhardt, R. J. (2001). "Probing the Interaction of Dengue Virus Envelope Protein with Heparin: Assessment of Glycosaminoglycan-Derived Inhibitors." J Med Chem **44** (13): 2178-2187.

Martina, B. E., Koraka, P. and Osterhaus, A. D. (2009). "Dengue Virus Pathogenesis: an Integrated View." Clin Microbiol Rev **22** (4): 564-581.

Mustafa, M. S., Rasotgi, V., Jain, S. and Gupta, V. (2015). "Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control." Med J Armed Forces India **71** (1): 67-70.

Navakul, K., Warakulwit, C., Yenchitsomanus, P.-T., Panya, A., Lieberzeit, P. A. and Sangma, C. (2016). "A novel method for dengue virus detection and antibody screening using a graphene-polymer based electrochemical biosensor." Nanomedicine **13** (2): 549-557.

Pereira, E. M., Dario, A. F., França, R. F., Fonseca, B. A. and Petri, D. F. (2010). "Binding of Dengue Virus Particles and Dengue Proteins onto Solid Surfaces." ACS Appl Mater Interfaces **2** (9): 2602-2610.

Philip Samuel, P. and Tyagi, B. K. (2006). "Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes." Indian J Med Res **123** (5): 615-628.

Pieper, J. S., Hafmans, T., Veerkamp, J. H. and van Kuppevelt, T. H. (2000). "Development of tailor-made collagen-glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects." Biomaterials **21** (6): 581-593.

Pritchard, J. (1964). "Stability of heparin solutions." J Pharm Pharmacol **16** (7).

Putnak, R., Barvir, D. A., Burrous, J. M., Dubois, D. R., D'Andrea, V. M., Hoke, C. H., Sadoff, J. C. and Eckels, K. H. (1996). "Development of a Purified, Inactivated, Dengue-2 Virus Vaccine Prototype in Vero Cells: Immunogenicity and Protection in Mice and Rhesus Monkeys." J Infect Dis **174** (6): 1176-1184.

Race, M. W., Fortune, R. A., Agostini, C. and Varma, M. G. (1978). "Isolation of Dengue Viruses in Mosquito Cell Cultures under Field Conditions." Lancet **1** (8054): 48-49.

Ramnani, P., Gao, Y., Ozsoz, M. and Mulchandani, A. (2013). "Electronic Detection of MicroRNA at Attomolar Level with High Specificity." Anal Chem **85** (17): 8061-8064.

Ramnani, P., Saucedo, N. M. and Mulchandani, A. (2016). "Carbon nanomaterial-based electrochemical biosensors for label-free sensing of environmental pollutants." Chemosphere **143**: 85-98.

Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E. and Beaty, B. J. (2007). "Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes." BMC Microbiol **7**: 9.

Salje, H., Rodriguez-Barraquer, I., Rainwater-Lovett, K., Nisalak, A., Thaisomboonsuk, B., Thomas, S. J., Fernandez, S., Jarman, R. G., Yoon, I.-K. and Cummings, D. A. (2014). "Variability in Dengue Titer Estimates from Plaque Reduction Neutralization Tests Poses a Challenge to Epidemiological Dtudies and Vaccine Development." PLoS Negl Trop Dis **8** (6): e2952.

Sharp, T. M., Moreira, R., Soares, M. J., Miguel da Costa, L., Mann, J., DeLorey, M., Hunsperger, E., Muñoz-Jordán, J. L., Colón, C., Margolis, H. S., de Caravalho, A. and Tomashek, K. M. (2015). "Underrecognition of Dengue during 2013 Epidemic in Luanda, Angola." Emerg Infect Dis **21** (8): 1311-1316.

Stefansson, S., Kwon, H. H. and Ahn, S. N. (2012). "Targeting Antibodies to Carbon Nanotube Field Effect Transistors by Pyrene Hydrazide Modification of Heavy Chain Carbohydrates." J. Nanotechnol. **2012**: 1-8.

Thiagarajan, G., Semple, A., James, J. K., Cheung, J. K. and Shameem, M. (2016). "A comparison of biophysical characterization techniques in predicting monoclonal antibody stability." mAbs **8** (6): 1088-1097.

Thullier, P., Demangel, C., Bedouelle, H., Mégret, F., Jouan, A., Deubel, V., Mazié, J.-C. and Lafaye, P. (2001). "Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism." Journal of General Virology **82** (8): 1885-1892.

Vlasak, J. and Ionescu, R. (2011). "Fragmentation of monoclonal antibodies." mAbs **3** (3): 253-263.

Wang, W., Singh, S., Zeng, D. L., King, K. and Nema, S. (2007). "Antibody Structure, Instability, and Formulation." J Pharm Sci **96** (1): 1-26.

Wang, X., Mizushima, H., Adachi, S., Ohishi, M., Iwamoto, R. and Mekada, E. (2006). "Cytoplasmic Domain Phosphorylation of Heparin-Binding EGF-like Growth Factor." Cell Struct Funct **31** (1): 15-27.

Welch, R. J., Chang, G.-J. and Litwin, C. M. (2014). "Comparison of a commercial dengue IgM capture ELISA with dengue antigen focus reduction microneutralization test and the centers for disease control dengue IgM capture-ELISA." J Virol Methods **195**: 247-249.

Wissink, M. J., Beernink, R., Pieper, J. S., Poot, A. A., Engbers, G. H., Beugeling, T., van Aken, W. G. and Feijen, J. (2001). "Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation." Biomaterials **22** (2): 151-163.

World Health Organization (2009). Dengue: Guidelines for Diagnosis Treatment Prevention and Control: New Edition. Geneva, World Health Organization.

Xi, Z., Ramirez, J. L. and Dimopoulos, G. (2008). "The *Aedes aegypti* Toll Pathway Controls Dengue Virus Infection." PLoS Pathog **4** (7): e1000098.

Zhang, X. K., Ge, P., Yu, X. K., Brannan, J. M., Bi, G. Q., Zhang, Q. F., Schein, S. and Zhou, Z. H. (2013). "Cryo-EM structure of the mature dengue virus at 3.5-Å resolution." Nat Struct Mol Biol **20** (1): 105-110.

Chapter 3: Point-of-use nanobiosensor for detection of dengue virus NS1 antigen in adult *Aedes aegypti*: A potential tool for improved dengue surveillance

3.1 Abstract

Dengue virus (DENV) and its primary mosquito vectors *Aedes* spp., have spread to every continent except Antarctica, causing outbreaks and autochthonous transmission in previously disease-free regions. Recently, the spread of other arboviruses carried by invasive *Aedes* spp., such as Chikungunya and Zika, seem to be following similar trends as DENV and have renewed interest in monitoring and modelling arboviruses within mosquito vectors. Unfortunately, current commercially available detection methods are designed for the diagnosis of DENV in humans or are too expensive and complex for sustainable monitoring. We report a novel electronic nanobiosensor utilizing a single-walled carbon nanotube networks chemiresistor transducer functionalized with anti-dengue NS1 monoclonal antibodies for rapid detection of the dengue nonstructural protein 1 (NS1). NS1 is a highly conserved protein secreted at high concentrations during viral replication and is a biomarker for DENV infection. NS1 was successfully detected in spiked adult *Aedes aegypti* homogenate over a broad dynamic range with high sensitivity and selectivity. The biosensor is compatible with "gold-standard" adult mosquito field-collection protocols and generates electronic data that can be readily stored or wirelessly transmitted. Thus it has potential for remote and real-time monitoring of wild mosquito populations.

3.2 Introduction

Dengue virus (DENV) has one of the most severe global health burdens of any arthropod-borne disease and is a leading cause of death and disease in endemic nations, especially in poor communities and among children. DENV has long been associated with tropical and subtropical regions. However, global warming, population growth, and other human activities have resulted in habitat expansion for the primary arboviral vectors, *Aedes aegypti* and *Aedes albopictus*, causing a 30-fold increase in the incidence of dengue infections since 1970 (Gubler, 2011a; Simmons et al., 2012; Bai et al., 2013; Ramasamy and Surendran, 2016). Unfortunately, habitat modeling predicts further expansion of the habitat range (Anker, 2004; Gubler, 2011b; Bonizzoni et al., 2013). Similarly, West Nile, Chikungunya, and Zika viruses have emerged as global threats and have spread to every inhabited continent causing explosive and unprecedented outbreaks (Diallo et al., 2012; Petersen et al., 2013; Berthet et al., 2014; Weaver and Lecuit, 2015). Importantly, these viruses have similar epidemiology, transmission cycle, and diffusion patterns as DENV (Musso et al., 2015). Understanding the transmission cycle and sylvatic maintenance of DENV will be key in understanding transmission of these diseases.

Sylvatic DENV amplifications can result in the introduction of a sylvatic strain into the human cycle and outbreaks in urban environments (Althouse et al., 2012; Durbin et al., 2013). With large populations of asymptomatic humans, relying on hospitalized patients underestimates the incidence and prevalence in the human cycle. Thus, monitoring the diffusion of DENV in mosquito populations is an effective and efficient method for simultaneous monitoring of the disease in both cycles. Early detection of an arbovirus

amplification event gives time for medical personnel and government agencies to prepare for a potential outbreak and implement disease prevention and control protocols (Centers for Disease and Prevention, 2001; Sharp et al., 2015).

Despite its importance, relatively few detection methods are being used to monitor the spread of the disease through vector populations. Commonly, DENV surveillance is performed by field collection of adult mosquitoes with aspirators or box traps followed by detection of DENV with costly and complex laboratory-based methods, such as culturing and PCR (Frentiu et al., 2014; Ritchie et al., 2014). Unfortunately, these detection techniques are impractical for large-scale vector surveillance, especially in resource-limited countries. Researchers have proposed using cheaper rapid diagnostic tests (RDT) that target the dengue nonstructural protein 1 (NS1) as a biomarker for infection for vector surveillance (Tan et al., 2011; Voge et al., 2013; Sylvestre et al., 2014). NS1 is a highly conserved 46-kDa protein secreted at high concentrations during viral replication in virtually every host organism (Winkler et al., 1989). However, commercial RDTs have limitations in field surveillance because these devices rely on the user for optical detection, thus compromising the sensitivity and limit of detection.

Thus, new sensing technologies that can assist in rapid assessment of transmission risk and facilitate vector surveillance of DENV infected mosquitoes are urgently needed. Electronic immunosensors with single-walled carbon nanotube (SWNT) transducers are rapidly emerging as facile, field-deployable, and cost-effective analytical devices with exquisite sensitivity for transducing antibody-analyte binding events into a measurable

electric signal without any label. The SWNT are pseudo-1D cylindrical fullerenes with a large surface-to-volume ratio for binding a large fraction of analyte molecules. The analyte-binding events penetrate into the 'bulk" of the nanometer diameter to modulate the charge carriers and produce a large electrical resistance/conductance change. Thus, even low concentrations of analyte can generate significant changes in conductance to produce electronic signal/response that can then be stored and/or wirelessly transmitted. The flexibility of the SWNT platform allows for the use of different bioreceptor elements for the detection of other target analytes, including arboviruses (Wasik et al., 2017). Additionally, they offer a reliable, rapid, inexpensive, and sensitive diagnostic method that is easily accessible by untrained personnel (García-Aljaro et al., 2010; Ramnani et al., 2016).

Here we report the development of a novel label-free chemiresistor immunosensor for the detection and quantification of dengue NS1 in adult mosquito homogenate that is compatible with "gold-standard" field-collection techniques such the BG-Sentinel and CDC Backpack Aspirator. A network of SWNTs was self-assembled onto lithographically patterned interdigitated gold electrodes and functionalized with anti-dengue NS1 monoclonal antibodies. The developed immunosensor had high sensitivity and specificity in both phosphate buffer and mosquito homogenate.

3.3 Materials and Methods

3.3.1 Adult *Aedes aegypti* Homogenate

Uninfected adult female *Aedes aegypti* were homogenized using a modified technique common to DENV/NS1 detection (Armstrong et al., 2011; Muller et al., 2012; Voge et al., 2013; Ritchie et al., 2014; Duong et al., 2015). A single adult female *A. aegypti* mosquito was placed into a 2-mL microcentrifuge tube containing 1 mL of 10 mM phosphate buffer, pH 7.2 (PB) and two 5-mm glass beads. The microcentrifuge tube was vortex mixed until homogenization was achieved and care was used to avoid extracting visible debris during aliquot extraction.

3.3.2 Preparation of Anti-Dengue Virus NS1 Glycoprotein Antibody and NS1 Glycoprotein

Mouse monoclonal anti-dengue virus NS1 glycoprotein antibodies (ab138696, Abcam) were buffer exchanged into PB with Micro Bio-Spin P-6 Gel Columns in 10 mM Tris–HCl buffer, pH 7.4 (732-6222, Bio-Rad) according to the manufacturer's instructions. The final antibody concentration after buffer exchange was 240 μg/mL. The full-length DENV NS1 glycoprotein (ab64456, Abcam) was similarly buffer exchanged to a final concentration of 0.1 mg/mL.

3.3.3 Microelectrode Fabrication

Using photolithography, microelectrodes were patterned onto a 300-nm SiO_2 layer on a silicon wafer. E-beam evaporation was then used to deposit a 20-nm Ti adhesion layer and a 180-nm Au gold contact layer. The microelectrode pattern was an interdigitated

fingers sensing region with 10 pairs of fingers, 5 μm wide and separated by a 3-μm gap. Each chip/assay had 5 of these regions in close proximity for simultaneous sensing.

Microelectrodes were cleaned in a 30% ammonium hydroxide (Fisher Scientific, Inc., A669-212) bath for 30 min and rinsed with deionized water (DI). Next, 3-aminopropyltriethoxysilane (APTES) (Acros Organics, 99%) was applied to the sensing regions of the chips for 30 min followed by washing in DI. A dense, uniform SWNT network was then formed by dropcasting of 95% semiconductive SWNT (Nanointegris Inc., CA) to bridge the 3-μm gap of the interdigitated sensing region. Finally, chips were annealed for 1 hour at 250°C in an open-ended quartz tube.

3.3.4 Immunosensor Fabrication

Microelectrode fabrication and self-assembly of SWNT networks were performed according to a previously reported method (See Supplementary). A 3:1 molar ratio of 1-Pyrenebutyric acid (Sigma Aldrich, USA) and 1-Pyrenebutyric acid N-hydroxysuccinimide ester (Sigma-Aldrich, USA) was mixed into 1 mL of dimethylformamide (DMF). This mixture was incubated on the SWNT networks for 1 h under high humidity for the non-covalent attachment of the pyrene moieties to the SWNT by π-π stacking while leaving the N-hydroxysuccinimide ester available to form an amide bond with anti-NS1 antibody. (Kim et al., 2009; Stefansson et al., 2012) Devices were washed with DMF and dried with N_2 gas. Further incubation steps occurred in the dark at 4 °C, in high humidity environment. Ten μL of buffer-exchanged antibody was incubated on the device for 2 h followed by gentle washing with PB. Next, the devices were incubated

successively with 0.1 mM ethanolamine (Sigma-Aldrich, USA) for 15 m to neutralize unreacted N-hydroxysuccinimide ester, 0.1% Tween 20 (BIO-RAD, USA) for 1 h to block any naked/bare sites on SWCNTs to prevent any non-specific adsorption, and 1% bovine serum albumin (BSA) for 1 h, with gentle washing with PB after each incubation step.

3.3.5 Detection of the NS1 Protein

Biosensor current was monitored over time to measure response to different concentrations of NS1 protein in aqueous solutions. Measurements were performed at a fixed source-drain potential (V_{DS}) of 0.1 V, in the dark at room temperature, with humidity modulated by petri dish containing DI water. Current from each electrode were collected approximately every 3 s with a Keithley 2636 digital multimeter controlled by Arduino Mega 2560 with a LabVIEW interface. The five sensing regions of the microelectrode were covered by a 20-μL drop of PB and allowed to equilibrate. As a control, 10 μL PB was then gently added and microelectrodes with ≥ ±5% response to this PB addition were excluded from sensing. Next, 10 μL of increasing concentrations of NS1-spiked PB were added and the current was allowed to equilibrate between each addition. Analysis of mosquito homogenate (MH) followed this protocol with a slight modification. After the initial 10 μL PB control, two additions of 10 μL MH without NS1 were added to the PB with equilibration between each addition. To normalize the measurements, they were averaged over 30-s intervals and the percent resistance changes were calculated using the equation $[(R_1 – R_0)*100/R_0]$ where, R_1 is the equilibrated resistance of the microelectrode after each addition and R_0 is the equilibrated resistance of the initial 10-μL PB addition.

3.4 Results and Discussion

As shown in Figure 3.2, a decrease in current occurs after each step of functionalization with anti-dengue NS1 antibodies, Tween 20, and BSA. The decrease is consistent with literature and attributed to immobilization of fabrication components onto semiconducting p-type SWNT; π−π stacking of the pyrene moieties onto SWNT and/or electron donation and scattering potential of the components (Gruner, 2006; Ramnani et al., 2013; Wasik et al., 2017).

Figure 3.3 shows a representative dynamic/real-time response trace of a single electrode to increasing concentrations of NS1-spiked PB. The sensor produced an observable response (normalized resistance change) upon each addition that reached a plateau, which were used to generate a calibration curve. Following the final NS1 detection, 10 μL of PB was added into the sample, resulting in a negligible response (-0.66±0.40%). The positive control experiment, in which a biosensor with anti-NS1 antibody was exposed to BSA spiked PB (Figure 3.4) exhibited minimal response to even high concentrations of BSA protein confirming the decrease in resistance was due specific antibody-NS1 interaction and not due to non-specific interaction by charged proteins. Additionally, the negative control experiment, in which the sensor without anti-NS1 antibody was exposed to NS1 spiked PB (Figure 3.4), had a minimal response that was not significantly different from the positive control ($n=8$, $p>0.05$), confirming NS1 is captured by the antibody Figure 3.4 illustrates the calibration plot of the averaged plateau responses of the immunosensors as a function of the logarithm of NS1 concentration. The immunosensor exhibited a sigmoidal calibration plot that was linear between 0.03 ng/mL

and $1.39x10^2$ ng/mL ($y = -7.47x - 59.19$, $R^2=0.999$; n=12) with a sensitivity (slope) of -7.47±0.14% per log ng/mL. The limit of detection of the immunosensor for NS1 in PB based on a signal to noise (S/N) of 3 was estimated to be 0.09 ng/mL.

In order to simulate field collection, uninfected *A. aegypti* mosquitos were reared in the Insectary and Quarantine Facility at the University of California, Riverside where they were trapped via aspirator, frozen at -20 °C, and sexed. For analysis of mosquito homogenate (MH), multiple 10-µL MH aliquots were first added to the 20-µL drop of PB placed on the sensor. It was determined that two additions were required to blank the immunosensor; further additions of MH resulted in an insignificant response with an average normalized resistance change of -0.22±0.21% per addition, further demonstrating that biosensor response is not due to nonspecific protein interactions (data not shown).

Similar to NS1 spiked PB, the real-time response trace (Figure 3.5) of the immunosensor sensor produced an observable response upon each addition of NS1 spiked MH. When an immunosensor without antibody was substituted (negative control), the normalized resistance changed on average by -0.6±0.07% per addition of NS1 spiked MH (Figure 3.6). The normalized resistance change of the MH calibration plot was significantly different from MH controls at each concentration (Figure 3.6, n=12, $p<0.01$) and was a function of the logarithm of NS1 concentration, which was linear between 0.02 ng/mL and 1.23×10^2 ng/mL with a sensitivity (slope) of -7.71±0.2% per log ng/mL ($y = -7.71x - 63.96$, $R^2=0.998$). The limit of detection of the sensor for NS1 in MH for an S/N=3 was estimated to be 0.04 ng/mL. The analytical characteristics of the immunosensor response

to NS1 were nearly identical in sensitivity and linear dynamic range in both PB and MH with an insignificant difference ($n=12$; $p>0.05$) at all NS1 concentrations when min-max normalized (Figure 3.7). Within the working range, inter-chip (4 assays per chip) assays had a %RSD from 2.85 to 13.29% across both PB and MH assays; indicating excellent inter-chip reproducibility for simultaneously fabricated assays. The intra-chip (3 chips with 4 assays per chip) %RSD was 16.88% across the working range of the PB and MH assays. These results indicate an excellent inter-chip and good intra-chip reproducibility, especially for handmade devices. Both inter- and intra-chip assays had highest %RSD at the lowest NS1 concentration.

Compared to the presented SWNT immunosensor, established detection methods such as ELISA and various commercially available RDTs, which are currently preferred for use in the field, have a higher limit of detection (~10 ng NS1/mL) and take longer to obtain results (Young et al., 2000; Allonso et al., 2014). Furthermore, research on various biosensor technologies for DENV NS1 detection has largely been performed in buffer(Tai et al., 2005; Cavalcanti et al., 2012; Figueiredo et al., 2015; Sinawang et al., 2016) and/or human serum samples, (Dias et al., 2013; Antunes et al., 2015; Cecchetto et al., 2015; Silva et al., 2015) not mosquito homogenate. However, when compared to these reported biosensors for NS1 detection in PB or PBS, the 0.03 ng/mL limit of detection of this SWNT immunosensor is one of the lowest (Table 3.1 Comparison of Dengue NS1 detection methods). Additionally, many of the biosensors use two antibodies, an enzyme label (such as horseradish peroxidase) for signal generation, and multiple incubation/washing steps;

making these protocols too tedious/labor intensive and complex for field deployment by untrained personnel.

3.5 Conclusion

Vector surveillance plays a critical role in dengue detection and prevention by acting as an early warning system. Early detection of a sylvatic amplification event coupled with a successful outbreak prevention program is much less costly than eradication or control programs established after an outbreak has occurred. Few, if any, biosensors have been reported for detection of NS1 in the vector mosquitoes. Our immunosensor offers a facile, affordable, and label-free method for direct detection of the NS1 protein using electrical detection with a high potential for enhancing existing vector surveillance programs and point-of-use diagnostics. The extremely low detection limit, high sensitivity, and 4-orders of magnitude concentration dynamic range of our biosensor makes it well-suited for detection in field-collected mosquitoes (Bennett et al., 2002; Anderson and Rico-Hesse, 2006). The SWNT platform's flexibility allows for the use of different bioreceptor elements for detection of other target analytes such as whole DENV,(Wasik et al., 2017) other arboviruses, and potentially multiplexing biosensors.

3.6 Acknowledgements

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3.7 Figures

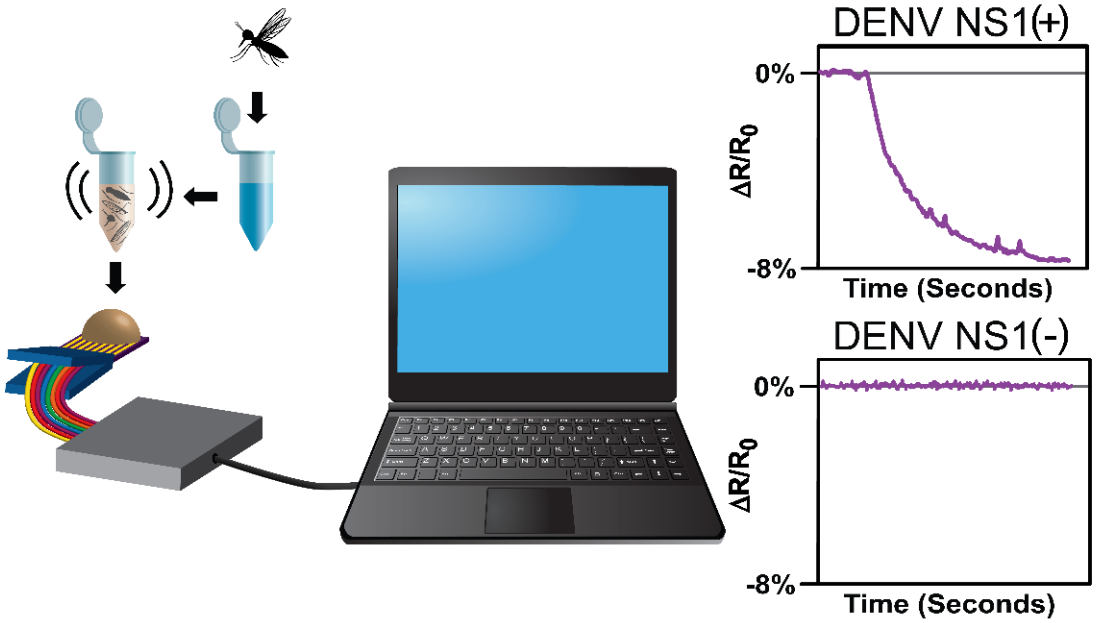

Figure 3.1 Diagram of experimental procedure.

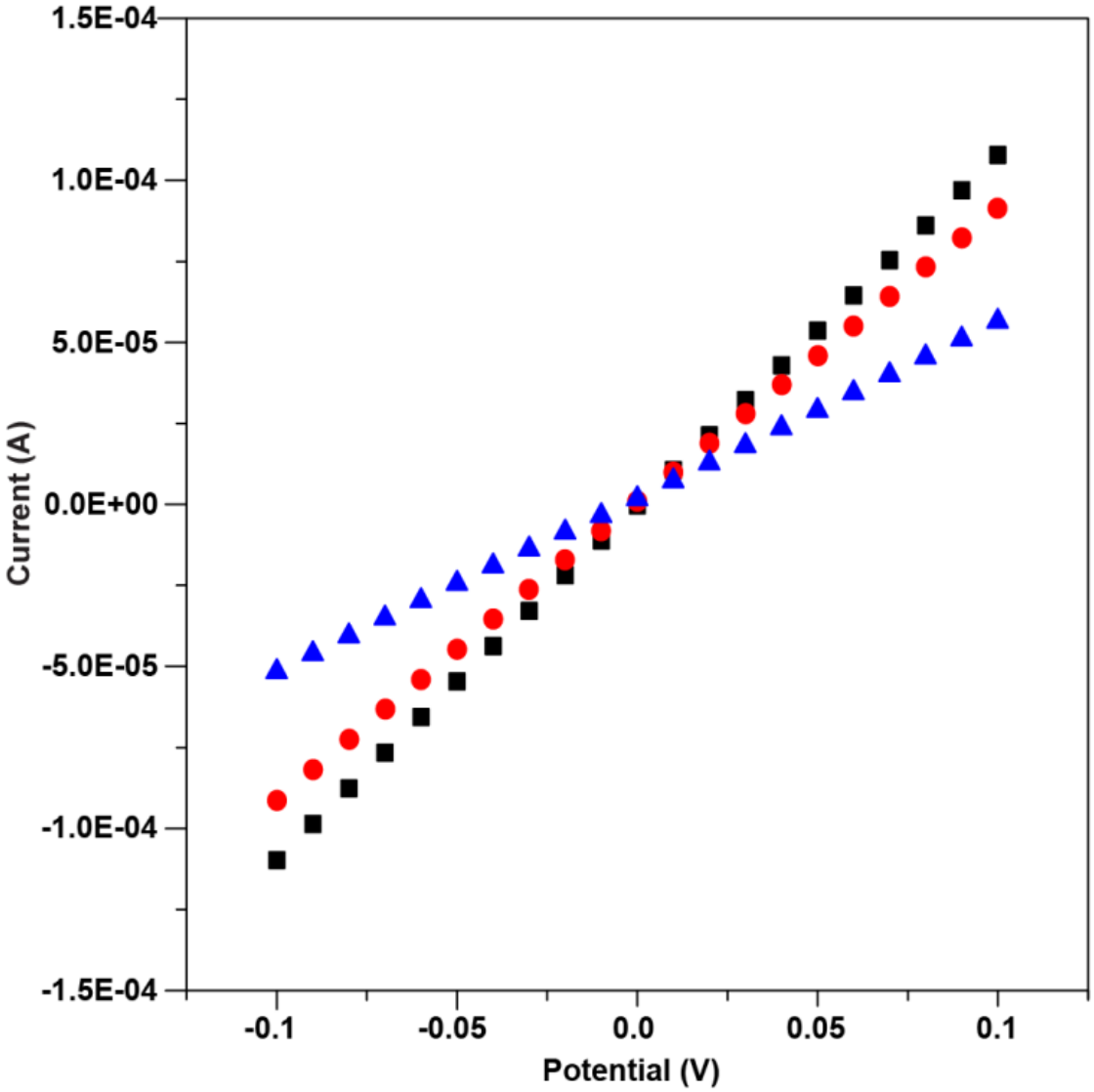

Figure 3.2 I-V Curve of bare SWNTs in PB (■), after functionalization with a 3:1 molar ratio of 1-Pyrenebutyric and 1-Pyrenebutyric acid N-hydroxysuccinimide ester (●), after immobilization of the antibody and blocking with ethanolamine, Tween-20, and BSA (▲).

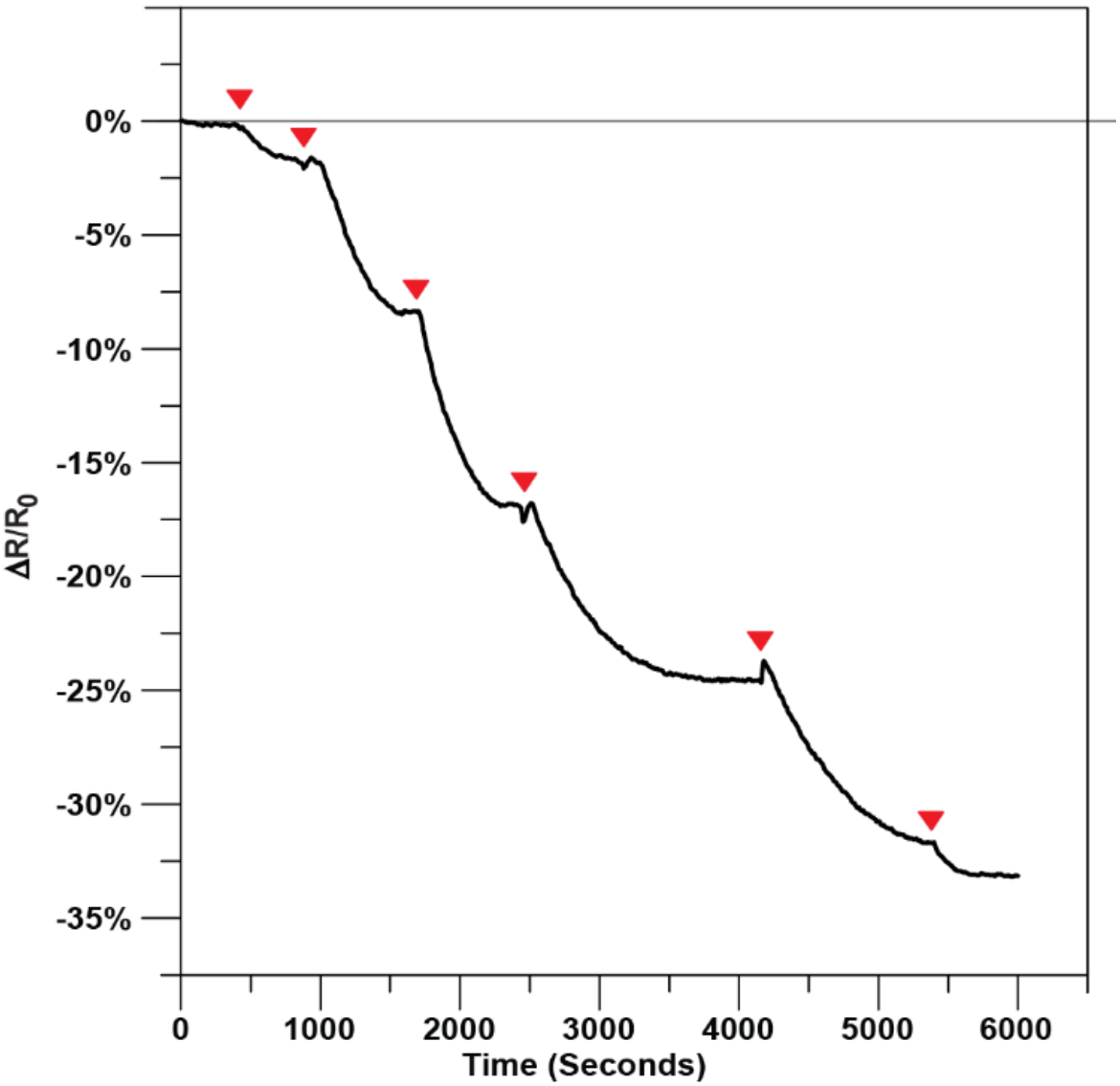

Figure 3.3 Normalized response of a single microelectrode to the addition (▼) of increasing concentration of NS1 protein in PB.

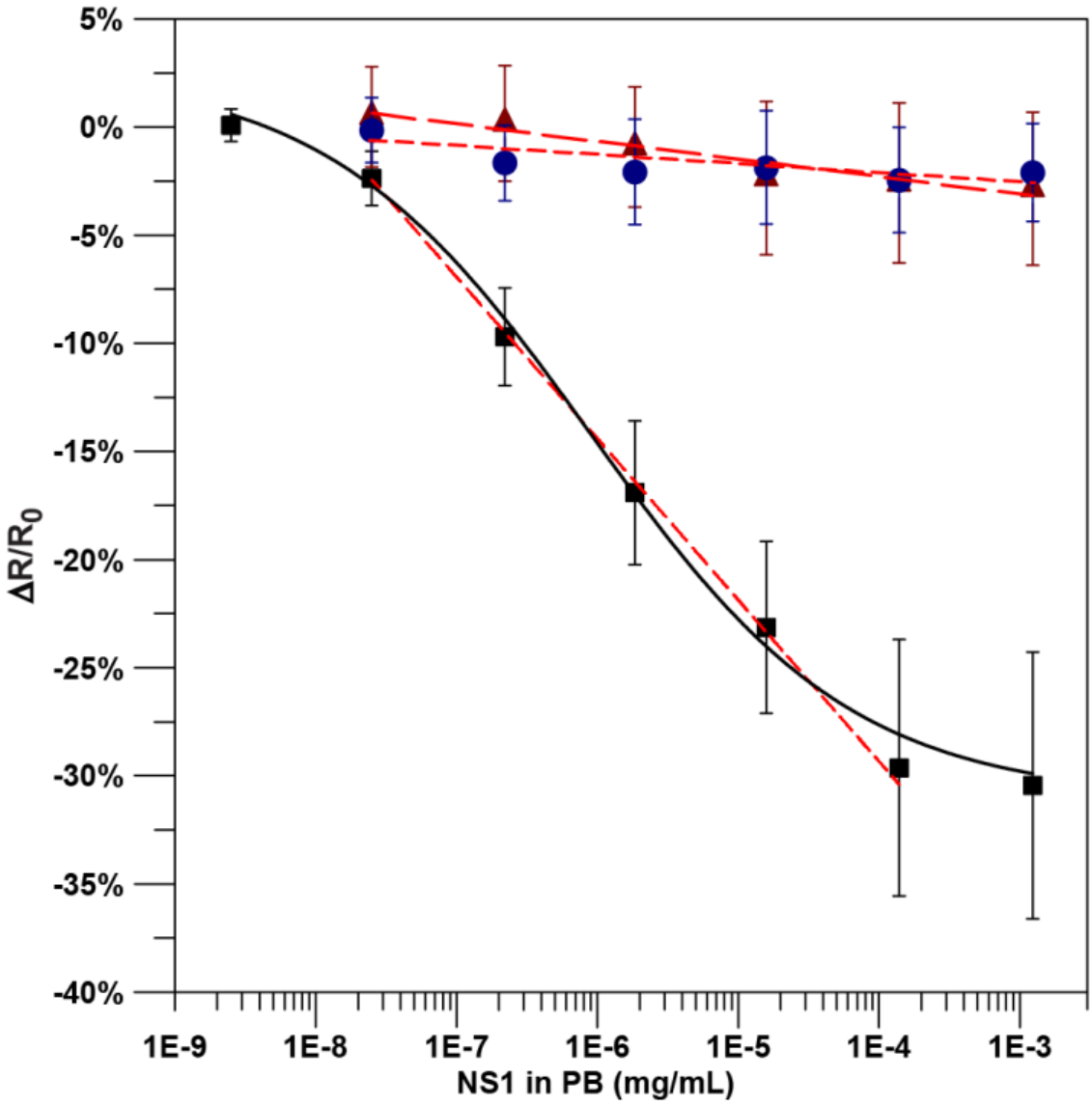

Figure 3.4 Calibration plot of the normalized response of the immunosensors functionalized with BSA, Tween-20, anti-NS1 antibody to the addition of NS1 protein in PB [n=12; y = -7.47x −59.19, R^2=0.999] (■) and to BSA protein in PB. [n=9; y = -0.81x −5.57, R^2=0.94] (▲). Normalized response of an immunosensor functionalized with BSA and Tween-20 but without antibody to the addition of NS1 protein in PB [n=8; y = -0.43x −3.89, R^2=0.66] (●).The data points are the mean of measurements from independent immunosensors, and error bars represent ±1 standard deviation.

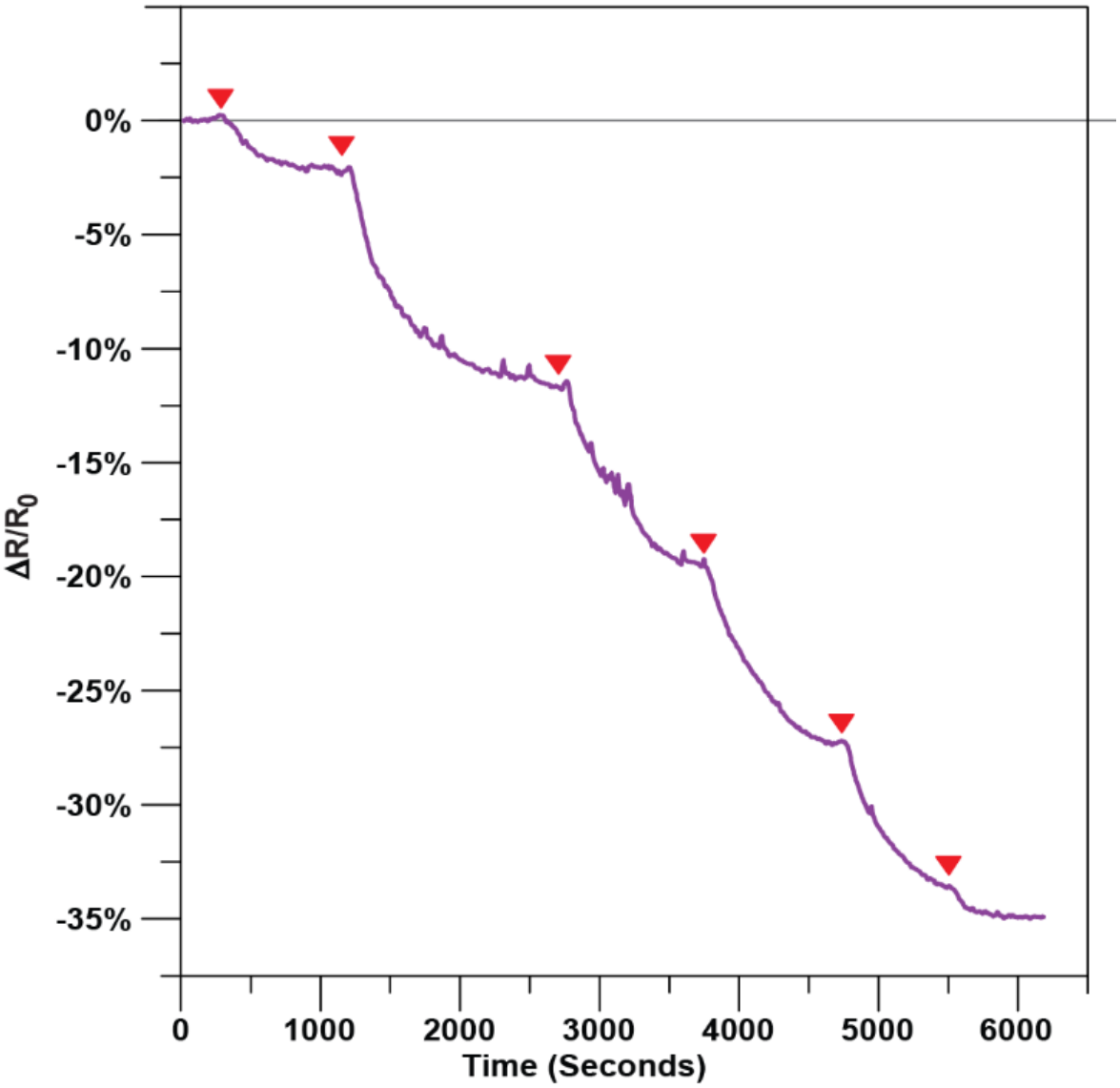

Figure 3.5 Normalized response of a single microelectrode to the addition (▼) of increasing concentration of NS1 protein in spiked adult Aedes aegypti homogenate.

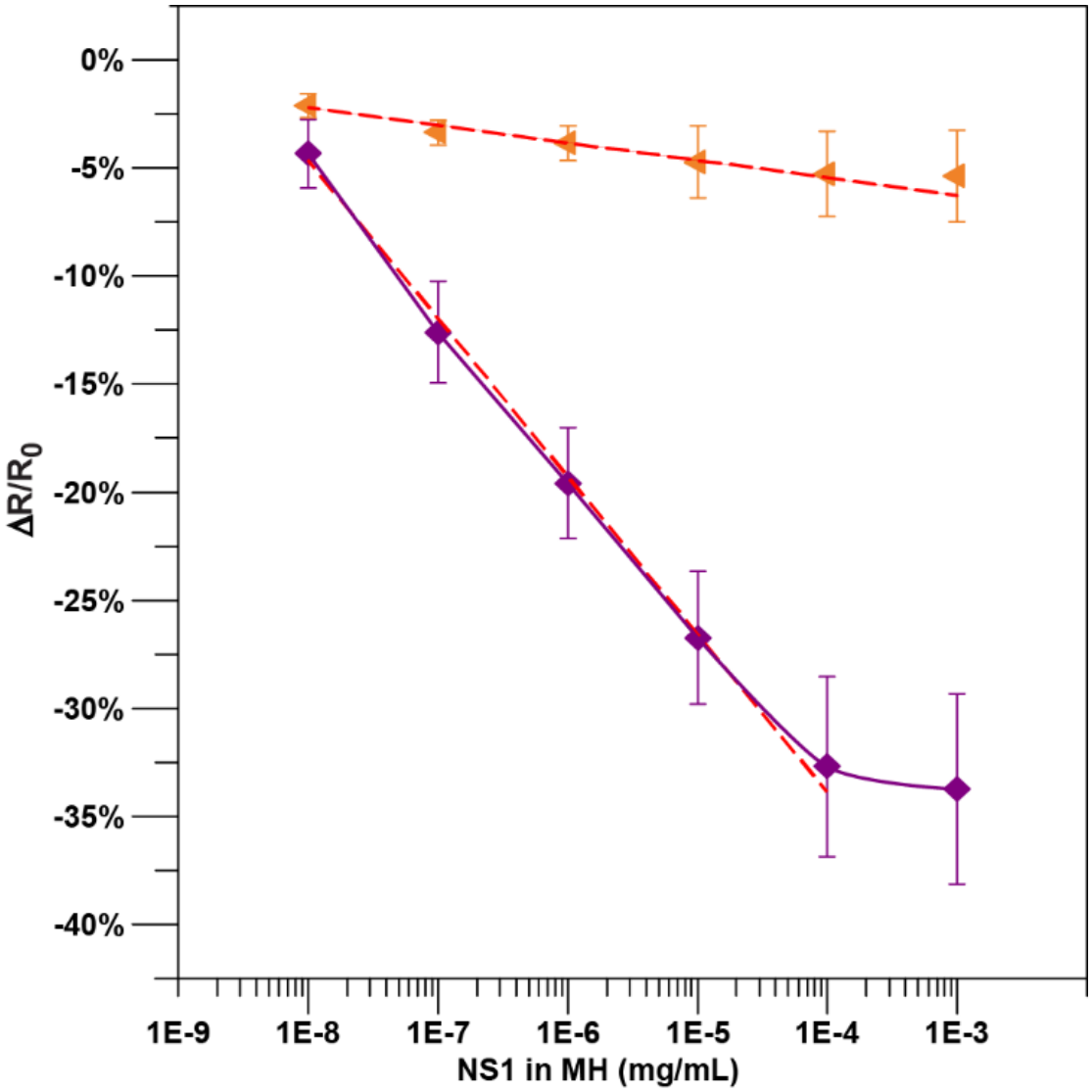

Figure 3.6 Calibration plot of the normalized response of the immunosensors functionalized with BSA, Tween-20, anti-NS1 antibody to the addition of NS1 protein in adult Aedes aegypti homogenate [n=12; y = -7.71x −63.96, R^2=0.998] (♦). Normalized response of an immunosensor functionalized with BSA and Tween-20 but without antibody to the addition of NS1 protein in adult Aedes aegypti homogenate [n=5; y = -0.43x –3.89, R^2=0.94] (◄). The data points are the mean of measurements from independent immunosensors, and error bars represent ±1 standard deviation.

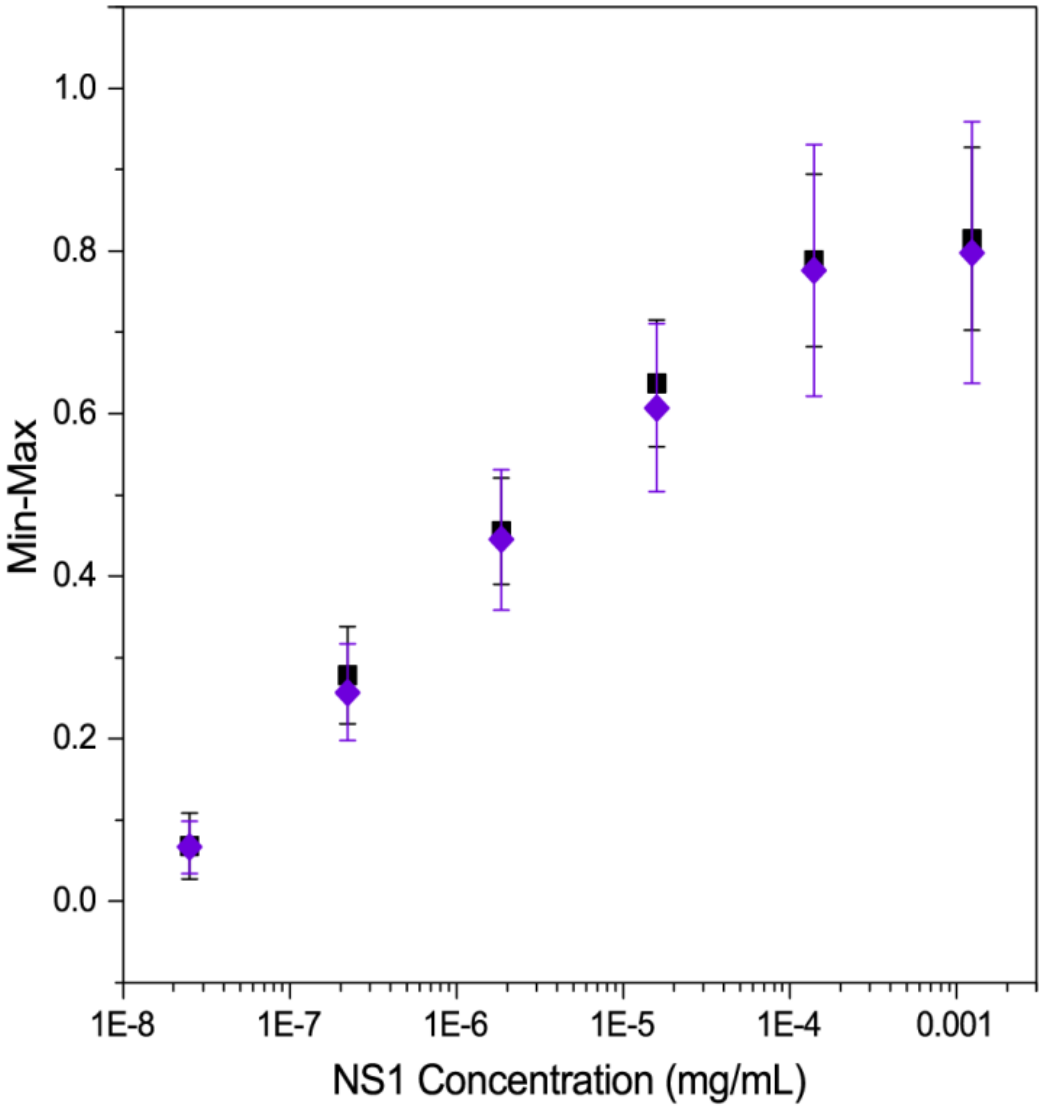

Figure 3.7 Comparison of Min-Max normalized immunosensor response to NS1 spiked PB (■) [n=12], and to NS1 spiked adult *Aedes aegypti* homogenate (♦) [n=12]. The data points are the mean of measurements from independent biosensors, and error bars represent ±1 standard deviation.

Table 3.1 Comparison of Dengue NS1 detection methods

detection	method	range	sample vol.	time (min)	LOD	sensing element	source
Primary antibody	Chemiresistor Immunosensor	0.03-1200 ng/mL	10 µL	10-20	0.03 ng/mL	Single-walled carbon nanotubes	
Primary antibody	Long range surface plasmon polariton	n/a	20 µL	30-40	5.73 pg/mm^2	Gold stripe embedded in Cytop claddings with etched micro-fluidic channel	Wong et al. (2016)
Immobilized anti-NS1 antibodies	Differential pulse voltammetry	1-100 ng/mL	n/a	n/a	0.33 ng/mL	Recordable compact disk chip	Cavalcanti et al. (2012)
HRP conjugated secondary antibodies	Electrochemical / amperometric	40-2000 ng/ml	10 µL	30	12 ng/ml	Carbon nanotube-screen printed electrodes	Dias et al. (2013)
HRP conjugated secondary antibodies	Electrochemical / amperometric	500-2000 ng/mL	n/a	120	30 ng/mL	Screen printed carbon electrodes	Parkash et al. (2014)
Immobilized anti-NS1 antibodies	QCM & energy dissipation monitoring	0.01-10 µg/mL	100 µL	10-30	100 ng/ml	Bacterial cellulose nanocrystals	Pirich et al. (2017)
ELISA	ELISA (in serum)	7-284 ng/mL	n/a	3-5 h	7.3 ng/mL	Enzyme immunoassay	Allonso et al. (2014)
RDT	Platelia Rapid Diagnostic Assay	n/a	100 µL	140	11.94 ng/mL	One-step sandwich microplate enzyme immunoassay	Pal et al. (2014)
RDT	Biorad NS1 Ag Strip (in mosquitoes)	n/a	50 µL	15-30	10^6 RNA copies	Immunochromato-graphic test	Tan et al. (2011)
Immobilized anti-NS1 antibodies	Optomagnetic readout	<20 µg/mL	6 µL	8	25 ng/mL	Magnetic nanoparticles	Antunes et al. (2015)
immunoelectroactive nanobeads	Electrochemical lateral flow immunosensor	1–25 ng/mL	50 µL	35	0.5 ng/mL	One-step sandwich microplate enzyme immunoassay	Sinawang et al. (2016)
Immobilized anti-NS1 antibodies	Electrochemical impedance spectroscopy	0.01-2.00 µg/mL	50 µL	30	3 ng/ml	Au electrode	Cecchetto et al. (2015)
Immobilized IgY anti-NS1 antibodies	Electrochemical impedance spectroscopy	0.1-10 µg/mL	300 µL	5-20	0.09 µg/mL	Disposable Au electrode	Figueiredo et al. (2015)
Molecularly imprinted polymers	Quartz crystal microbalance	5-50000 ng/ml	100 µL	5+	n/a	Molecularly imprinted polymers	Tai et al. (2005)
HRP conjugated secondary antibodies	Electrochemical / amperometric	0.1-2.5 µg/mL	10 µL	30	0.035 µg/mL	Multi-walled carbon nanotubes	Silva et al. (2015)
Fluorescent dyes conjugated to antibody coated polystyrene beads	Spectrofluorometric / ELISA	2-500 ng/mL	4 µL	45-60	5.2 ng/mL	Sandwich fluorescent immunolinked sorbent assay	Linares et al. (2013)
FITC-tagged secondary antibodies	Fluorescent microscopy	0.05-1000 ng/mL	5 µL	n/a	50 pg/ml	Micro-Spot with integrated pillars	Gunda et al. (2013)
Immobilized anti-NS1 antibodies	Localized surface plasmon resonance	n/a	n/a	30-60	0.074 µg/ml	AuNPs deposited on the endface of a standard multimode fiber	Camara et al. (2013)

3.8 References

Allonso, D., Meneses, M. D., Fernandes, C. A., Ferreira, D. F. and Mohana-Borges, R. (2014). "Assessing Positivity and Circulating Levels of NS1 in Samples from a 2012 Dengue Outbreak in Rio de Janeiro, Brazil." PLoS ONE **9** (11): e113634.

Althouse, B. M., Lessler, J., Sall, A. A., Diallo, M., Hanley, K. A., Watts, D. M., Weaver, S. C. and Cummings, D. A. (2012). "Synchrony of Sylvatic Dengue Isolations: a Multi-Host, Multi-Vector SIR Model of Dengue Virus Transmission in Senegal." PLoS Negl Trop Dis **6** (11): e1928.

Anderson, J. R. and Rico-Hesse, R. (2006). "*Aedes aegypti* Vectorial Capacity is Determined by the Infecting Genotype of Dengue Virus." Am J Trop Med Hyg **75** (5): 886-892.

Anker, M. (2004). Using Climate to Predict Infectious Disease Outbreaks: A Review. Corval C(Eds):. Geneva, World Health Organization.

Antunes, P., Watterson, D., Parmvi, M., Burger, R., Boisen, A., Young, P., Cooper, M. A., Hansen, M. F., Ranzoni, A. and Donolato, M. (2015). "Quantification of NS1 dengue biomarker in serum via optomagnetic nanocluster detection." Scientific Reports **5**: 16145.

Armstrong, P. M., Andreadis, T. G., Finan, S. L., Shepard, J. J. and Thomas, M. C. (2011). "Detection of Infectious Virus from Field-collected Mosquitoes by Vero Cell Culture Assay." J Vis Exp (52): 2889.

Bai, L., Morton, L. C. and Liu, Q. (2013). "Climate change and mosquito-borne diseases in China: a review." Global Health **9** (1): 10.

Bennett, K. E., Olson, K. E., Muñoz, M. D., Fernandez-Salas, I., Farfan-Ale, J. A., Higgs, S., Black, W. C. and Beaty, B. J. (2002). "Variation in Vector Competence for Dengue 2 Virus Among 24 Collections of *Aedes aegypti* from Mexico and the United States." Am J Trop Med Hyg **67** (1): 85-92.

Berthet, N., Nakouné, E., Kamgang, B., Selekon, B., Descorps-Declère, S., Gessain, A., Manuguerra, J.-C. and Kazanji, M. (2014). "Molecular Characterization of Three Zika Flaviviruses Obtained from Sylvatic Mosquitoes in the Central African Republic." Vector Borne Zoonotic Dis **14** (12): 862-865.

Bonizzoni, M., Gasperi, G., Chen, X. and James, A. A. (2013). "The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives." Trends Parasitol **29** (9): 460-468.

Camara, A. R., Gouvêa, P. M., Dias, A. C., Braga, A. M., Dutra, R. F., de Araujo, R. E. and Carvalho, I. C. (2013). "Dengue immunoassay with an LSPR fiber optic sensor." Opt Express **21** (22): 27023-27031.

Cavalcanti, I. T., Guedes, M. I. F., Sotomayor, M. D. P. T., Yamanaka, H. and Dutra, R. F. (2012). "A label-free immunosensor based on recordable compact disk chip for early diagnostic of the dengue virus infection." Biochem Eng J **67**: 225-230.

Cecchetto, J., Carvalho, F. C., Santos, A., Fernandes, F. C. B. and Bueno, P. R. (2015). "An impedimetric biosensor to test neat serum for dengue diagnosis." Sensors and Actuators B-Chemical **213** (0): 150-154.

Centers for Disease, C. and Prevention (2001). "Underdiagnosis of dengue--Laredo, Texas, 1999." MMWR. Morbidity and mortality weekly report **50** (4): 57-59.

Diallo, D., Sall, A. A., Buenemann, M., Chen, R., Faye, O., Diagne, C. T., Faye, O., Ba, Y., Dia, I., Watts, D., Weaver, S. C., Hanley, K. A. and Diallo, M. (2012). "Landscape Ecology of Sylvatic Chikungunya Virus and Mosquito Vectors in Southeastern Senegal." PLoS Negl Trop Dis **6** (6): e1649.

Dias, A. C., Gomes-Filho, S. L., Silva, M. M. and Dutra, R. F. (2013). "A sensor tip based on carbon nanotube-ink printed electrode for the dengue virus NS1 protein." Biosensors and Bioelectronics **44**: 216-221.

Duong, V., Lambrechts, L., Paul, R. E., Ly, S., Lay, R. S., Long, K. C., Huy, R., Tarantola, A., Scott, T. W., Sakuntabhai, A. and Buchy, P. (2015). "Asymptomatic humans transmit dengue virus to mosquitoes." Proc Natl Acad Sci U S A **112** (47): 14688-14693.

Durbin, A. P., Mayer, S. V., Rossi, S. L., Amaya-Larios, I. Y., Ramos-Castaneda, J., Eong Ooi, E., Jane Cardosa, M., Munoz-Jordan, J. L., Tesh, R. B., Messer, W. B., Weaver, S. C. and Vasilakis, N. (2013). "Emergence potential of sylvatic dengue virus type 4 in the urban transmission cycle is restrained by vaccination and homotypic immunity." Virology **439** (1): 34-41.

Figueiredo, A., Vieira, N. C., dos Santos, J. F., Janegitz, B. C., Aoki, S. M., Junior, P. P., Lovato, R. L., Nogueira, M. L., Zucolotto, V. and Guimarães, F. E. (2015). "Electrical Detection of Dengue Biomarker Using Egg Yolk Immunoglobulin as the Biological Recognition Element." Scientific Reports **5**: 7865.

Frentiu, F. D., Zakir, T., Walker, T., Popovici, J., Pyke, A. T., van den Hurk, A., McGraw, E. A. and O'Neill, S. L. (2014). "Limited Dengue Virus Replication in Field-Collected *Aedes aegypti* Mosquitoes Infected with Wolbachia." PLoS Negl Trop Dis **8** (2): e2688.

García-Aljaro, C., Cella, L. N., Shirale, D. J., Park, M., Muñoz, F. J., Yates, M. V. and Mulchandani, A. (2010). "Carbon nanotubes-based chemiresistive biosensors for detection of microorganisms." Biosensors and Bioelectronics **26** (4): 1437-1441.

Gruner, G. (2006). "Carbon nanotube transistors for biosensing applications." Anal Bioanal Chem **384** (2): 322-335.

Gubler, D. J. (2011a). "Dengue, Urbanization and Globalization: The Unholy Trinity of the 21(st) Century." Trop Med Health **39** (4 Suppl): 3-11.

Gubler, D. J. (2011b). "Prevention and Control of *Aedes aegypti*-borne Diseases: Lesson Learned from Past Successes and Failures " Asia-Pacific Journal of Molecular Biology and Biotechnology **3** (19): 111-114.

Gunda, N. S., Singh, M., Purwar, Y., Shah, S. L., Kaur, K. and Mitra, S. K. (2013). "Micro-spot with integrated pillars (MSIP) for detection of dengue virus NS1." Biomedical Microdevices **15** (6): 959-971.

Kim, J. P., Lee, B. Y., Lee, J., Hong, S. and Sim, S. J. (2009). "Enhancement of sensitivity and specificity by surface modification of carbon nanotubes in diagnosis of prostate cancer based on carbon nanotube field effect transistors." Biosensors and Bioelectronics **24** (11): 3372-3378.

Linares, E. M., Pannuti, C. S., Kubota, L. T. and Thalhammer, S. (2013). "Immunospot assay based on fluorescent nanoparticles for dengue fever detection." Biosensors and Bioelectronics **41**: 180-185.

Muller, D. A., Frentiu, F. D., Rojas, A., Moreira, L. A., O'Neill, S. L. and Young, P. R. (2012). "A portable approach for the surveillance of dengue virus-infected mosquitoes." J Virol Methods **183** (1): 90-93.

Musso, D., Cao-Lormeau, V. M. and Gubler, D. J. (2015). "Zika virus: following the path of dengue and chikungunya?" Lancet **386** (9990): 243-244.

Pal, S., Dauner, A. L., Mitra, I., Forshey, B. M., Garcia, P., Morrison, A. C., Halsey, E. S., Kochel, T. J. and Wu, S.-J. (2014). "Evaluation of Dengue NS1 Antigen Rapid Tests and ELISA Kits Using Clinical Samples." PLoS ONE **9** (11): e113411.

Parkash, O., Yean, C. Y. and Shueb, R. H. (2014). "Screen Printed Carbon Electrode Based Electrochemical Immunosensor for the Detection of Dengue NS1 Antigen." Diagnostics (Basel) **4** (4): 165-180.

Petersen, L. R., Brault, A. C. and Nasci, R. S. (2013). "West Nile Virus: Review of the Literature." JAMA **310** (3): 308-315.

Pirich, C. L., de Freitas, R. A., Torresi, R. M., Picheth, G. F. and Sierakowski, M. R. (2017). "Piezoelectric immunochip coated with thin films of bacterial cellulose nanocrystals for dengue detection." Biosensors and Bioelectronics **92**: 47-53.

Ramasamy, R. and Surendran, S. N. (2016). "Mosquito vectors developing in atypical anthropogenic habitats: Global overview of recent observations, mechanisms and impact on disease transmission." Journal of vector borne diseases **53** (2): 91-98.

Ramnani, P., Gao, Y., Ozsoz, M. and Mulchandani, A. (2013). "Electronic Detection of MicroRNA at Attomolar Level with High Specificity." Anal Chem **85** (17): 8061-8064.

Ramnani, P., Saucedo, N. M. and Mulchandani, A. (2016). "Carbon nanomaterial-based electrochemical biosensors for label-free sensing of environmental pollutants." Chemosphere **143**: 85-98.

Ritchie, S. A., Buhagiar, T. S., Townsend, M., Hoffmann, A., Van Den Hurk, A. F., McMahon, J. L. and Eiras, A. E. (2014). "Field Validation of the Gravid *Aedes* Trap (GAT) for Collection of *Aedes aegypti* (Diptera: Culicidae)." J Med Entomol **51** (1): 210-219.

Sharp, T. M., Moreira, R., Soares, M. J., Miguel da Costa, L., Mann, J., DeLorey, M., Hunsperger, E., Muñoz-Jordán, J. L., Colón, C., Margolis, H. S., de Caravalho, A. and Tomashek, K. M. (2015). "Underrecognition of Dengue during 2013 Epidemic in Luanda, Angola." Emerg Infect Dis **21** (8): 1311-1316.

Silva, M. M. S., Dias, A. C. M. S., Cordeiro, M. T., Marques, E., Jr., Goulart, M. O. and Dutra, R. F. (2014). "A thiophene-modified screen printed electrode for detection of dengue virus NS1 protein." Talanta **128**: 505-510.

Silva, M. M. S., Dias, A. C. M. S., Silva, B. V. M., Gomes-Filho, S. L. R., Kubota, L. T., Goulart, M. O. F. and Dutra, R. F. (2015). "Electrochemical detection of dengue virus NS1 protein with a poly(allylamine)/carbon nanotube layered immunoelectrode." J Chem Technol Biot **90** (1): 194-200.

Simmons, C. P., Farrar, J. J., Nguyen v, V. and Wills, B. (2012). "Dengue." The New England journal of medicine **366** (15): 1423-1432.

Sinawang, P. D., Rai, V., Ionescu, R. E. and Marks, R. S. (2016). "Electrochemical lateral flow immunosensor for detection and quantification of dengue NS1 protein." Biosensors and Bioelectronics **77**: 400-408.

Stefansson, S., Kwon, H. H. and Ahn, S. N. (2012). "Targeting Antibodies to Carbon Nanotube Field Effect Transistors by Pyrene Hydrazide Modification of Heavy Chain Carbohydrates." J. Nanotechnol. **2012**: 1-8.

Sylvestre, G., Gandini, M., de Araújo, J. M., Kubelka, C. F., Lourenço-de-Oliveira, R. and Maciel-de-Freitas, R. (2014). "Preliminary evaluation on the efficiency of the kit Platelia Dengue NS1 Ag-ELISA to detect dengue virus in dried *Aedes aegypti*: a potential tool to improve dengue surveillance." Parasit Vectors **7** (1): 155.

Tai, D.-F., Lin, C.-Y., Wu, T.-Z. and Chen, L.-K. (2005). "Recognition of Dengue Virus Protein Using Epitope-Mediated Molecularly Imprinted Film." Anal Chem **77** (16): 5140-5143.

Tan, C.-H., Wong, P.-S., Li, M.-Z., Vythilingam, I. and Ng, L.-C. (2011). "Evaluation of the Dengue NS1 Ag Strip(R) for detection of dengue virus antigen in *Aedes aegypti* (Diptera: Culicidae)." Vector Borne Zoonotic Dis **11** (6): 789-792.

Voge, N. V., Sánchez-Vargas, I., Blair, C. D., Eisen, L. and Beaty, B. J. (2013). "Detection of Dengue Virus NS1 Antigen in Infected *Aedes aegypti* Using a Commercially Available Kit." Am J Trop Med Hyg **88** (2): 260-266.

Wasik, D., Mulchandani, A. and Yates, M. V. (2017). "A Heparin-Functionalized Carbon Nanotube-Based Affinity Biosensor for Dengue Virus." Biosensors and Bioelectronics **91**: 811-816.

Weaver, S. C. and Lecuit, M. (2015). "Chikungunya Virus and the Global Spread of a Mosquito-Borne Disease." The New England journal of medicine **372** (13): 1231-1239.

Winkler, G., Maxwell, S. E., Ruemmler, C. and Stollar, V. (1989). "Newly Synthesized Dengue-2 Virus Nonstructural Protein NS1 is a Soluble Protein but Becomes Partially Hydrophobic and Membrane-Associated after Dimerization." Virology **171** (1): 302-305.

Wong, W. R., Sekaran, S. D., Adikan, F. R. and Berini, P. (2016). "Detection of dengue NS1 antigen using long-range surface plasmon waveguides." Biosensors and Bioelectronics **78**: 132-139.

Young, P. R., Hilditch, P. A., Bletchly, C. and Halloran, W. (2000). "An Antigen Capture Enzyme-Linked Immunosorbent Assay Reveals High Levels of the Dengue Virus Protein NS1 in the Sera of Infected Patients." J Clin Microbiol **38** (3): 1053-1057.

Chapter 4: Salivary detection of dengue virus NS1 protein with a label-free immunosensor for early dengue diagnosis.

4.1 Abstract

Dengue virus (DENV) is an arthropod-borne virus transmitted primarily by the bites of *Aedes* mosquitoes. Annually, 3.6 billion people are at risk of infection with 390 million new infections, mostly among children. Early diagnosis of a dengue infection is critical for clinical management. Detection of the DENV Nonstructural Protein 1 (NS1) is a clinically accepted as a biomarker of DENV infection and will allow early diagnosis of the disease. Unfortunately, virtually all of the laboratory and commercial DENV diagnostic methods require a blood draw for sample analysis which limit point-of-care diagnostics and decreases patient willingness. Alternatively, saliva collection is relatively non-invasive, painless, inexpensive, and simple to collect; even by minimally trained personnel. In this study, we present a label-free chemiresistive immunosensor for detection of the DENV NS1 protein utilizing a single walled carbon nanotube network functionalized with anti-dengue NS1 monoclonal antibodies. NS1 was successfully detected in spiked artificial human saliva over the range of clinically relevant concentrations with high sensitivity and selectivity. It has potential application in clinical diagnosis and the ease of collection allows for self-testing, even within the home.

4.2 Introduction

Dengue virus (DENV) is an arthropod-borne *flavivirus* primarily transmitted by the mosquito vectors *Aedes aegypti* and *Aedes albopictus* (Guzmán and Harris, 2015). Prior to

World War II, the mosquito vectors and therefore the virus, was mostly limited to tropic and sub-tropic regions. However since the 1950s, increased air travel, global commerce, unplanned urbanization, and global warming have permitted the mosquito vectors to proliferate in previously uninhabited regions (Weaver and Lecuit, 2015). Autochthonous infections have now been reported as far north as France, Japan, and the US indicating that DENV is a growing global threat (Teets et al., 2014; Kutsuna et al., 2015; Succo et al., 2016).

Dengue fever, the most frequent result of a DENV infection, has the highest incident rate among humans of any of the arboviral diseases. It has recently been estimated that 390 million new infections occur annually (Bhatt et al., 2013) and 3.6 billion people are at risk of infection (Simmons et al., 2012) with the highest rates of infection occurring among children who are 15 years of age or younger (World Health Organization, 2009). The clinical manifestations of dengue fever vary from asymptomatic to severe arthralgia and myalgia; with typical infections manifesting as a nonspecific febrile disease. In some cases, the infection causes severe dengue, which can result in failure of the circulatory system, the liver, and death if not adequately managed (Shamala, 2015).

There are five antigenically distinct dengue viruses, DENV1-DENV5, each are capable of causing dengue fever and severe dengue (Mustafa et al., 2015). There is no specific treatment for a DENV infection, however, early intervention with fluid replacement therapy can reduce mortality from 20% to below 1% (Guzmán and Harris, 2015). Therefore, diagnosis of a dengue infection is critical for clinical management,

especially when late or inadequate treatment can be lethal. Unfortunately, dengue and severe dengue have no pathognomonic clinical features and can manifest differently in adults and children; making a clinical diagnosis and differentiation of dengue or severe dengue by clinical features alone extremely difficult (Souza et al., 2013; Shamala, 2015).

A number of diagnostic tests have been produced to aid in clinical diagnosis by detecting the virions, nucleic acids, serologic, or antigenic components of a DENV infection. Viral isolation by cell culture and nucleic acid detection using polymerase chain reaction-based techniques require a dedicated laboratory, expensive equipment, and highly trained personnel which are impractical for routine clinical diagnostics (Parkash and Shueb, 2015). Current commercially available rapid diagnostic tests (RDTs) are relatively inexpensive, easily accessible by untrained personnel, and they employ various techniques for serological or antigenic detection. Unfortunately, the IgG and IgM are not highly specific to DENV and thus serological assays are known to be cross-reactive against other flaviviruses. Additionally, it can take up to 7 days post-infection for antibody concentrations to reach detectable limits (World Health Organization, 2009; Guzmán and Harris, 2015) and the antibodies from any flavivirus infection stay in the blood for months, triggering future false positives (Parkash and Shueb, 2015; Shamala, 2015). Antigenic RDTs typically use a lateral flow or ELISA-based methods for detection of the highly conserved DENV Non-Structural Protein 1 (NS1). NS1 is a 46-kDa protein secreted by infected cells, has a clinical range from 0.04 - 2 µg/mL in human serum, and can be detected within the first 18 days of a primary infection (Winkler et al., 1989). NS1 is an ideal target for early detection of a DENV infection and high NS1 concentrations and/or a rapid decline

of soluble NS1 may be an indicator of a severe DENV infection (Paranavitane et al., 2014; Thomas, 2015). However, RDTs cannot quantify NS1 and sensitivities under 50% have been reported during the first two days of illness, when serum NS1 concentrations are low (Shu et al., 2009; Blacksell et al., 2012). Importantly, each of these diagnostic assays require a blood draw to analyze whole blood, serum, or plasma samples. Venous blood collection requires expertise and equipment typically found in a clinical setting but can be a limiting factor for in-home testing or for use by workers with limited training Additionally, the blood draw also exposes personnel to bloodborne pathogens, needle-sticks injuries, and are sources of pain for both adults and children, leading to an unwillingness to agree to such procedures (Inal and Kelleci, 2012; Tarigan et al., 2015; Lee et al., 2016; Rahim, 2017).

Alternatively, saliva collection is relatively non-invasive, painless, inexpensive, and simple to collect; even by minimally trained personnel. Whole saliva is produced by a continuous flow of water, mucins, and enzymes generated by salivary glands as well as serumnal components transported from the blood into saliva (Dawes et al., 2015; Khanna and Walt, 2015). While the relationship between NS1 concentrations in saliva and blood is not well known; a few small studies have identified NS1 detection in saliva as a diagnostic possibility and have shown that saliva NS1 concentrations correlate with DENV-RNA serum levels (Radzol et al., 2013; Korhonen et al., 2014; Radzol et al., 2014; Andries et al., 2015). Andries et al. (2015) reported that in a cohort of 267 patients, aged 3-16, NS1 in saliva was detectable up to two weeks after the onset of fever. NS1 concentrations in saliva samples obtained by direct spitting were an average of 3.8 ng/ml and ranged between

0.5 ng/ml and 41.5 ng/ml; too low for reliable diagnosis with commercially available RDTs (Shu et al., 2009; Blacksell, 2012).

Electronic biosensors fabricated with single-walled carbon nanotubes (SWNT) transducer elements make ideal detection tools when ultra-low protein concentrations are expected (Tlili et al., 2010; Tlili et al., 2011; Tan et al., 2015). SWNT have high surface-to-volume ratio for functionalization with antibody bioreceptors (immunosensors) that enable selective and specific detection of target analytes. The pseudo-1D structure of the SWNT allow small perturbations of antibody-analyte binding events to be converted into a measurable electric signal, even at low analyte concentrations. Additionally, immunosensors are portable, rapid, and inexpensive diagnostic tools suitable for in-home and point-of-care use.

In this study, we have fabricated a chemiresistive immunosensor for the detection and quantification of dengue NS1 in saliva. Anti-dengue NS1 monoclonal antibodies were functionalized onto a dense network of self-assembled SWNT on lithographically patterned interdigitated gold electrodes. The immunosensor performance was assessed in phosphate buffer (PB) and artificial human saliva where it demonstrated quantification of DENV NS1 protein concentrations with high sensitivity and specificity.

4.3 Materials and Methods

4.3.1 Fabrication of the SWNT Immunosensor

Photolithography and e-beam evaporation was used to form the microelectrodes by depositing a 20-nm Ti adhesion layer and a 180-nm gold contact layer onto the 300-nm SiO_2 layer of a silicon wafer (Ramnani et al., 2013). Each sensing region of the microelectrode has 10 pairs of 5 μm wide interdigitated Au fingers separated by a 3-μm gap. A single chip/assay had 5 microelectrodes with the sensing regions in close proximity for simultaneous sensing. Each chip was bathed in 30% ammonium hydroxide (Fisher Scientific, Inc., A669-212) for 30 min and rinsed with deionized water (DI). Next, the sensing regions were covered by 3-aminopropyltriethoxysilane (APTES) (Acros Organics, 99%) for 30 min followed by washing in DI. 95% semiconductive SWNT (Nanointegris Inc., CA) were dropcasted onto the sensing region for 1 hour followed by annealing for 1 hour at 250°C in an open-ended quartz tube. Annealed sensors were stored under vacuum until use.

4.3.2 Preparation of Anti-Dengue Virus NS1 Glycoprotein Antibody and NS1 Glycoprotein

Micro Bio-Spin P-6 Gel Columns in 10 mM Tris–HCl buffer, pH 7.4 (732-6222, Bio-Rad) were used to buffer exchange mouse monoclonal anti-dengue virus NS1 glycoprotein antibodies (ab138696, Abcam) into phosphate buffer according to the manufacturer's instructions. After buffer exchange, antibody concentration was 240 μg/mL. Similarly, full-length dengue virus NS1 glycoprotein (ab64456, Abcam) was buffer exchanged into PB for a final concentration of 0.1 mg/mL.

4.3.3 Preparation of spiked PB and artificial human saliva samples

Artificial saliva was prepared as previously described in Tlili et al. (2010) with 0.6 g/L Na_2HPO_4, 0.6 g/L anhydrous $CaCl_2$, 0.4 g/L KCl, 0.4 g/L NaCl, 4 g/L mucin, and 4 g/L urea dissolved in DI. The mixture was adjusted to pH 7.2 and then sterilized by autoclaving and kept at 4 °C until use. Buffer exchanged DENV NS1 protein was spiked into an aliquot of artificial saliva and then serially diluted into other saliva aliquots. NS1 spiked saliva samples were immediately clarified by centrifugation at 14,000 × *g* for 5 min at 4°C. The supernatant was extracted and kept on ice for immediate analysis; the pellet was discarded. NS1 spiked PB samples were similarly prepared.

4.3.4 Functionalization of SWNT Networks with Anti-Dengue Virus NS1 Glycoprotein Antibody

1-Pyrenebutyric acid (Sigma Aldrich, USA) and 1-Pyrenebutyric acid N-hydroxysuccinimide ester (Sigma Aldrich, USA) was mixed at a 3:1 molar ratio into 1 mL of dimethylformamide (DMF) and incubated on the SWNT for 1 h with humidity controlled by a petri dish containing DI (Kim et al., 2009; Stefansson et al., 2012). Biosensors were washed with DMF, followed by drying with N_2 gas. Each of the following incubation steps were in the dark at 4 °C with humidity controlled by petri dish containing DI. The entire surface of the chip was covered by 2mM 6-mercaptohexanol in DMF for 30 min followed by washing with DMF. Next, a 10 μL drop of buffer-exchanged antibody was pipetted onto the sensing region and incubated for 2 h. In order to block non-specific interactions with the SWNT or antibodies, the senor was incubated with 0.1% Tween 20

(*BIO-RAD*) for 1 h, gently washed with PB, and then 1% bovine serum albumin (BSA) for 1 h followed by gentle washing with PB.

Functionalization was monitored by measuring resistance of each electrode voltage using the linear sweep voltammetry function of the Keithley 2636 digital multimeter controlled by Arduino Mega 2560 with a LabVIEW interface. The current was recorded as the voltage was swept from −200 mV to 200 mV. The IV curve was inversed and linear regression analysis was used to calculate the slope (resistance) between −100 mV to 100 mV.

4.3.5 Detection of the NS1 Protein

Measurements were performed in the dark with humidity controlled by a petri dish containing DI. The current of each electrode was continuously monitored at 0.1 V by a Keithley 2636 digital multimeter controlled by Arduino Mega 2560 with a LabVIEW interface which took measurements approximately every 3 seconds. Furthermore, sample analysis consisted of repeated cycles of two steps with very gentle washing between each transition. The first step (incubation phase) is a 10 min incubation of 10uL of sample blanks or sample containing NS1 on the sensing region. The sample blank consists of saliva without NS1 for analysis of NS1 spiked saliva and PB without NS1 for analysis of NS1 spiked PB. During the second step (detection phase), the five microelectrode sensing region is covered by a 20μL drop of PB, and the chronocurrent curve permitted to equalize. As a control, two cycles of samples blanks are analyzed prior to sample analysis, microelectrodes with ≥±5% change in normalized resistance were excluded. Next, starting

at lowest concentration, NS1 spiked samples were cycled through for analysis. The concentration of NS1 was increased 10-fold every cycle and the temperature of each serial dilution was permitted to warm to room temperature immediately prior to incubation. Following the cycle of highest NS1 concentration, a PB blank cycle was performed. The chronocurrent data were averaged over 30s intervals and the percent resistance changes were calculated using the equation $[(R_1 - R_0)*100/R_0]$ where, R_1 is the equilibrated resistance of the microelectrode for the analysis cycle and R_0 is the equilibrated resistance taken during the detection phase of the second control cycle.

4.4 Results and Discussion

As expected, the functionalization of the immunosensor with anti-dengue NS1 antibodies, Tween 20, and BSA resulted in an increase in resistance which matches previously reported biosensors using p-type semiconducting SWNT. (data not shown) The increase in resistance is likely due to the π−π stacking of the pyrene moieties and/or the electron scattering/donation potential of the functionalization components (Gruner, 2006; Ramnani et al., 2013; Wasik et al., 2017).

The dynamic response trace shown in Figure 4.1 is a single electrode cycling through the increasing concentrations of NS1 spiked into PB. As shown in the figure, the normalized resistance of the immunosensor increased during the incubation phase and plateaued at a lower normalized resistance during the detection phase. Incubation with increasing NS1 concentrations results in a detection phase plateau with a lower normalized resistance than the previous cycle. In Figure 4.2, the combined average response of the

immunosensors resulted in a sigmoidal response curve to increasing concentrations of NS1. Relative to the previous detection phase, the sensor had minimal response to 0.1 ng/ml of NS1 in PB (0.95±1.3%) but upon the incubation of 1.0 ng/mL NS1, the average normalized resistance changed by (-5.89±1.6%) resulting in a limit of detection of ~1ng/mL NS1. The immunosensor calibration plot for NS1 in PB was linear between 1.0 ng/mL and 1000.0 ng/mL ($y = -4.92x - 35.41$, $R^2=0.99$; n=7) with a sensitivity (slope) of -4.92±0.07% per log ng/mL. Incubation with 10,000 ng/mL NS1 only resulted in a change of -1.02±0.40% relative to the previous detection phase, which gives an upper limit of quantification of ~1000 ng/mL. A positive control in which cycles of NS1 spiked PB were performed on biosensor without anti-NS1 antibody and a negative control in which devices with antibody were cycled with PB alone resulted in minimal change in normalized resistance per cycle, an average -0.34±0.05% and -0.36±0.06%, respectively. When an immunosensor with anti-NS1 antibody was incubated with PB spiked with BSA, the average normalized change in resistance was -0.24±0.13% per cycle. The three controls were not significantly different from each other (n=23, $p>0.05$) but the device response to the presence of NS1 was significantly greater than the controls at concentrations ≥ 1ng/mL NS1. (n=7, $p<0.01$)

Without centrifugation of the spiked artificial saliva, the mucus left behind a visible residue that was not easily removed. Even after washing, the residue caused the resistance of the immunosensor to remain relatively high during the detection phase which prevented NS1 detection. (data not shown) On the other hand, centrifuged saliva samples did not leave behind any visible residue, even without washing. The dynamic/real-time response trace of a single electrode of the immunosensor (Figure 4.3) shows that, unlike NS1 in PB,

the spiked and centrifuged saliva samples resulted in relatively large increases of normalized resistance during the incubation phase. However, after washing and application of PB during the detection phase, the resistance of the immunosensor returned to a lower resistance in which NS1 detection and quantification was possible.

As shown in Figure 4.4, the average response of the immunosensors exhibits a sigmoidal curve to increasing concentrations of NS1 spiked into artificial saliva. When saliva spiked with the lowest concentration of NS1 (0.1 ng/ml) was cycled onto the immunosensors, a -0.06±1.20% normalized resistance change occurred which was no different than any of the controls. (n=17, $p>0.05$) However, when the concentration was increased to 1 ng/ml of NS1 in saliva, the immunosensor responded with a normalized -5.85±1.67% change in resistance. At the highest NS1 concentration, 10,000 ng/ml, the normalized resistance only changed by -1.15±1.34% relative to the previous detection phase. Similar to NS1 spiked PB, ~1000 ng/ml NS1 is the upper limit of quantification. Through the quantification range, the resistance decreased linearly ($y = -4.79x – 34.3$, $R^2=0.997$; n=17) as a function of the logarithm of NS1 concentration. The immunosensor had a sensitivity of -4.79±0.13% per log ng/mL and an average of ±0.76% at 95% confidence interval across 17 independent microelectrodes indicating high reproducibility. The terminal PB blank cycle resulted in a minimal 0.08±1.3% average change of resistance across the same immunosensors. Fully-functionalized sensors exhibited a minimal average responses to each cycle of BSA spiked saliva (-0.14±0.09%) and each cycle of saliva alone (-0.27±0.05%). (Figure 4.4) Additionally, when sensors without antibody were incubated with NS1 spiked saliva, the normalized sensor response was -0.52±0.06% per cycle which

was not significantly different than the other controls at any concentration. ($n=5$, $p>0.05$) All three controls were not significantly different from each other; indicating that response of the functionalized immunosensor is due capture of DENV NS1 by the anti-NS1 antibody. ($n=18$, $p>0.05$)

Radzol et al. (2015a) reported detection of NS1 in saliva using Surface Enhanced Raman Spectroscopy (SERS) at concentrations as low as 10ng/mL (Radzol et al., 2013; Radzol et al., 2015b). However, SERS requires expensive equipment and expertise not readily available in the point-of-use environment. Andries et al. (2016) found that a "prototype immunochromatographic kit" developed by Standard Diagnostics for detection of NS1 in whole saliva lacked sensitivity (33.9%) and specificity ($n=59$). Similar to the experiment presented here, the viscous mucin and other protein substances present in saliva interfered with NS1 detection and caused the "prototype immunochromatographic kit" to have a lower sensitivity (Zhang et al., 2015; Andries et al., 2016). However, unlike the prototype kit, with centrifugation of saliva the immunosensor presented here was able to detect NS1 with a 10 min incubation of a 10μL sample of saliva.

Additionally, NS1 concentration in a saliva sample may be affected by collection technique. In a separate study, Andries et al. (2015) used saliva samples obtained by direct spitting and was able to detect NS1 in whole saliva down to 0.5ng/mL using a relatively simpler one-step ELISA. However, it required an incubation of 2 hours and large sample volumes to be collected. However, Anders et al. (2012) used the Bio-Rad NS1 Platelia ELISA, with a limit of detection of 11.94 ng/mL (Allonso et al., 2014), to detect NS1 in

eluates from oral swabs in 64.7% patients (n=85). More studies are needed to understand interferents present and to establish a proper saliva collection and treatment protocol.

Currently, there are no pathognomonic clinical features to distinguish dengue from severe dengue. While the association between NS1 concentration and severe dengue has yet to be elucidated; (Casenghi et al., 2014) several studies have demonstrated that high concentration or a sudden decrease in concentration may be an indicator of severe dengue. Unlike RDTs, the presented biosensor can quantify NS1 concentration and thus may be useful for distinguishing the diseases (Young et al., 2000; Casenghi et al., 2014; Nguyen et al., 2017).

4.5 Conclusion

Treatment of a DENV infection is inexpensive and very effective but early diagnosis is critical for proper clinical management. However, clinical diagnosis based on symptoms alone is unreliable. Thus, clinicians rely on laboratory confirmation by analysis of drawn blood samples; a method that is risky to the medical personnel as well as painful and unattractive to patients, especially children. A non-invasive sampling technique, such as saliva collection is, less dangerous, much more likely to be accepted by patients, and can be collected by untrained personnel. With a quantification range of ~1ng/mL to 1,000ng/mL of NS1 in saliva, the proposed biosensor capable of quantifying DENV NS1 in a clinically relevant salivary concentration range with a 10 min incubation of 10μLof saliva. As such, the immunosensor will improve clinical utility and diagnostics in the point-of-care setting, especially when a blood draw is unavailable and may distinguish dengue

from severe dengue. The ease of collection even allows repeated self-testing by a potentially infected individual within their home.

4.6 Figures

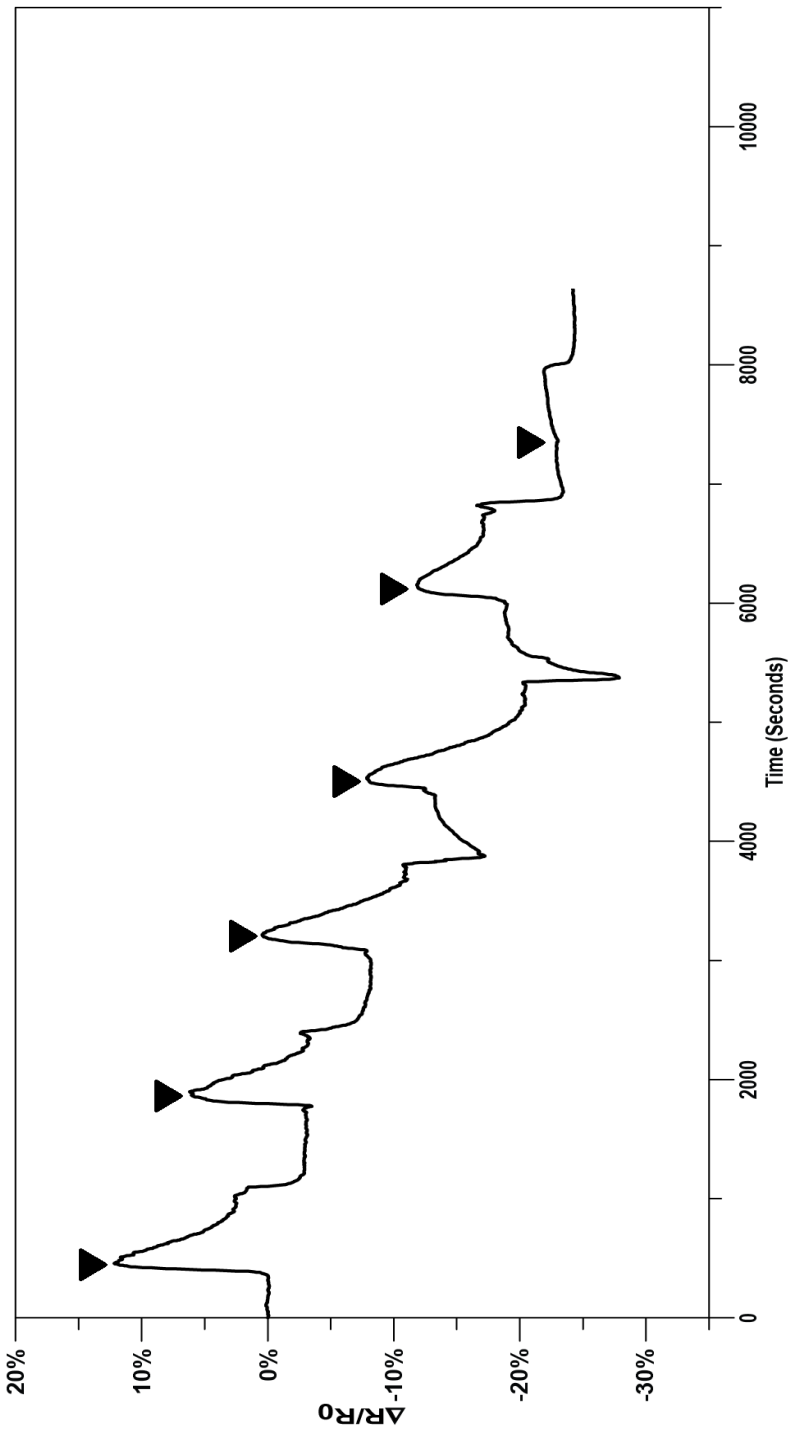

Figure 4.1 Normalized response of a single microelectrode to the incubation (▼), washing, and detection of NS1 protein in PB.

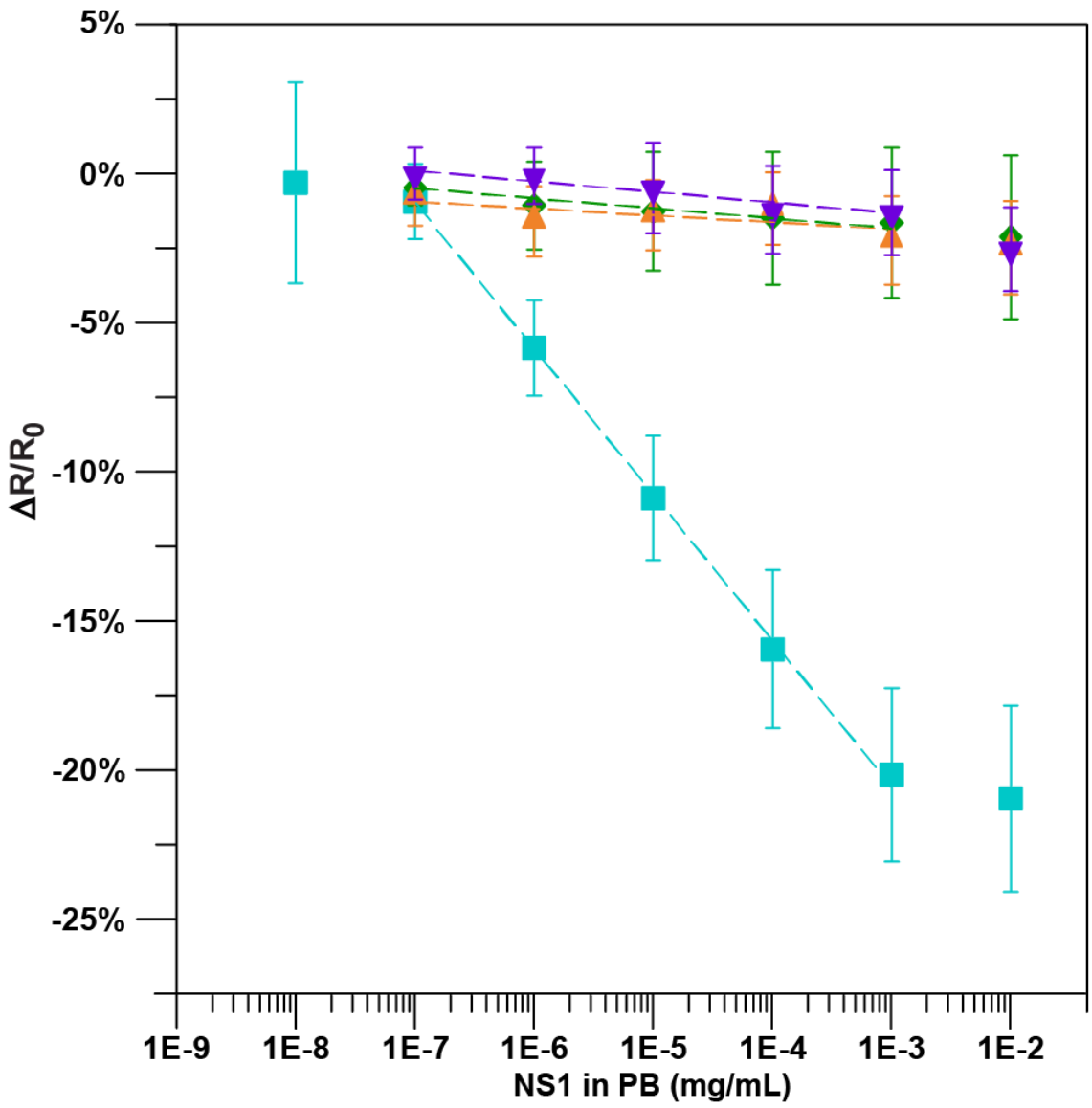

Figure 4.2 Calibration plot of the normalized response of the immunosensors functionalized with BSA, Tween-20, anti-NS1 antibody after incubating with NS1 protein in PB [n=7; y = -4.92x −35.41, R^2=0.99] (■), after incubation with PB only [n=6, y = -0.36x −2.4, R^2=0.91] (▼), and after incubation with BSA protein in PB. [n=9; y = -0.24x −2.6 R^2=0.53] (▲). Normalized response the immunosensors functionalized with BSA and Tween-20 but without antibody to incubation of NS1 protein in PB [n=8; y = -0.34x −2.9, R^2=0.93] (♦). The data points are the mean of measurements from independent immunosensors, and error bars represent ±1 standard deviation.

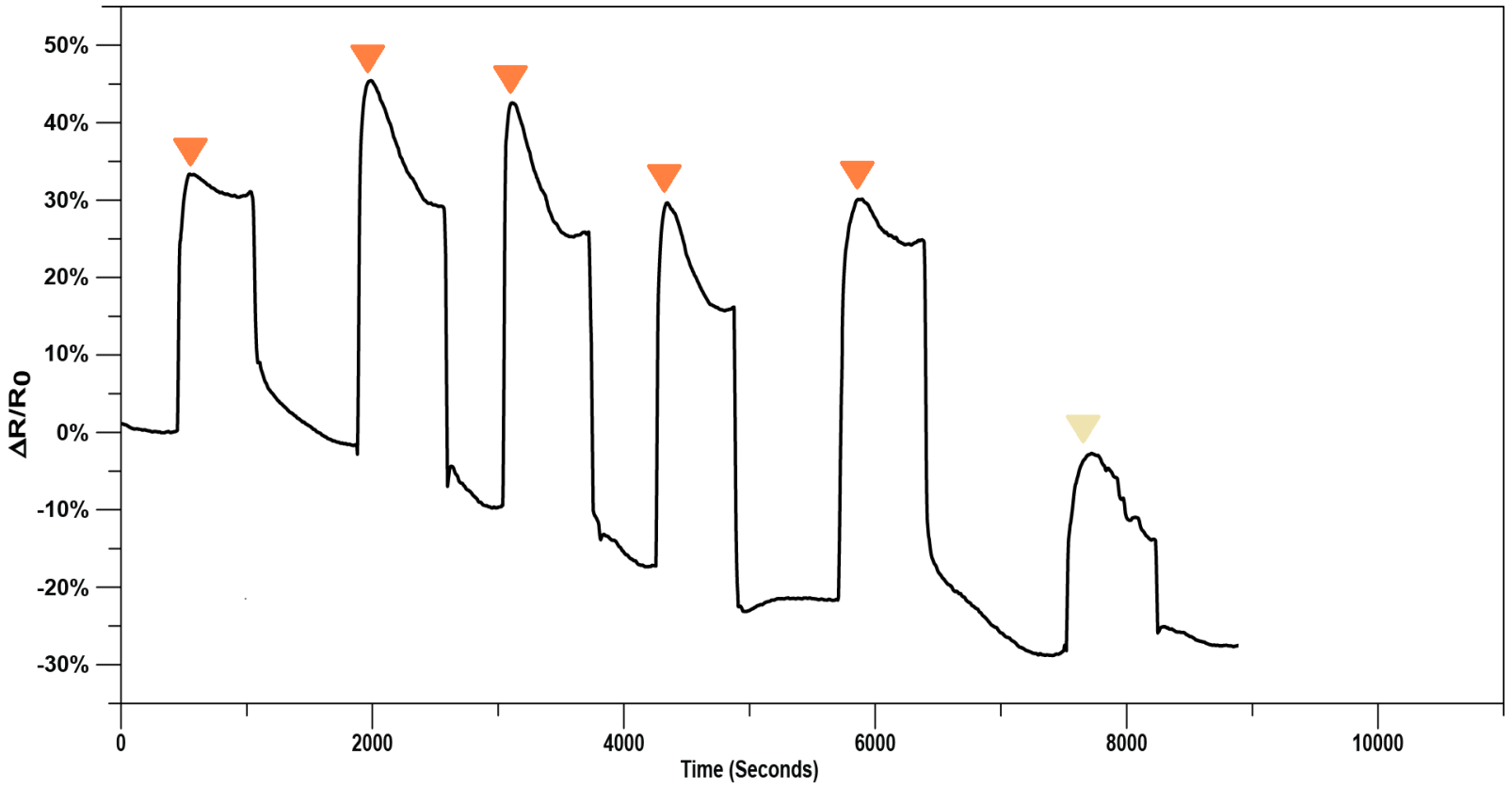

Figure 4.3 Normalized response of a single microelectrode to the incubation (▼), washing, and detection of NS1 protein in saliva or NS1 in PB (▼).

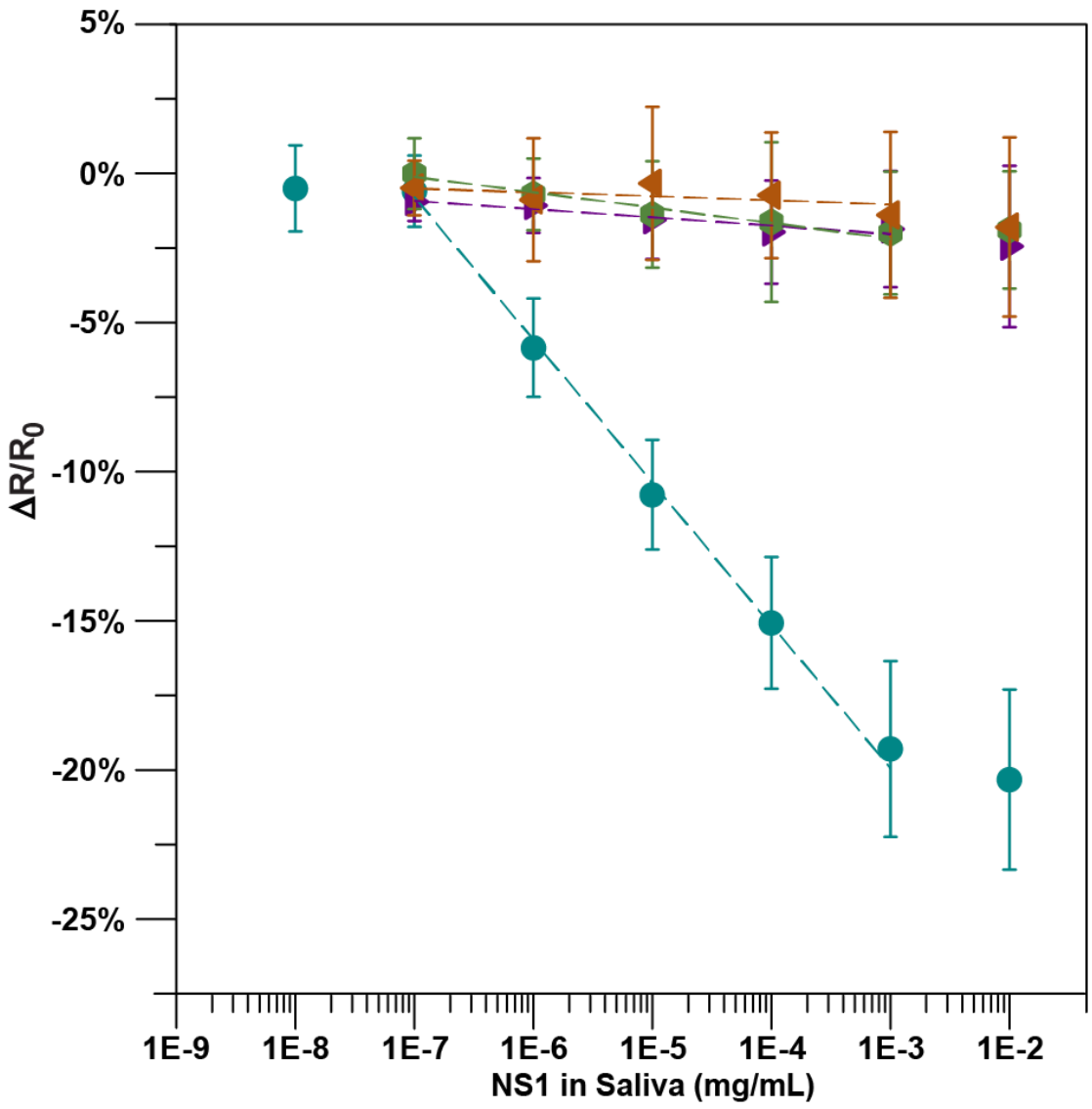

Figure 4.4 Calibration plot of the normalized response of the immunosensors functionalized with BSA, Tween-20, anti-NS1 antibody after incubating with NS1 protein in saliva [n=17; y = -4.79x −34.3, R^2=0.99] (●), after incubation with saliva only [n=6, y = -0.28x −2.9, R^2=0.92] (▶), and after incubation with BSA protein in saliva. [n=7; y = -0.14x −1.4 R^2=0.48] (◀). Normalized response the immunosensors functionalized with BSA and Tween-20 but without antibody to incubation of NS1 protein in saliva [n=5; y = -0.52x −3.7, R^2=0.96] (⬢). The data points are the mean of measurements from independent immunosensors, and error bars represent ±1 standard deviation.

4.7 References

Allonso, D., Meneses, M. D., Fernandes, C. A., Ferreira, D. F. and Mohana-Borges, R. (2014). "Assessing Positivity and Circulating Levels of NS1 in Samples from a 2012 Dengue Outbreak in Rio de Janeiro, Brazil." PLoS ONE **9** (11): e113634.

Anders, K. L., Nguyet, N. M., Quyen, N. T., Ngoc, T. V., Tram, T. V., Gan, T. T., Tung, N. T., Dung, N. T., Chau, N. V., Wills, B. and Simmons, C. P. (2012). "An Evaluation of Dried Blood Spots and Oral Swabs as Alternative Specimens for the Diagnosis of Dengue and Screening for Past Dengue Virus Exposure." Am J Trop Med Hyg **87** (1): 165-170.

Andries, A.-C., Duong, V., Ly, S., Cappelle, J., Kim, K. S., Lorn Try, P., Ros, S., Ong, S., Huy, R., Horwood, P., Flamand, M., Sakuntabhai, A., Tarantola, A. and Buchy, P. (2015). "Value of Routine Dengue Diagnostic Tests in Urine and Saliva Specimens." PLoS Negl Trop Dis **9** (9): e0004100.

Andries, A.-C., Duong, V., Ong, S., Ros, S., Sakuntabhai, A., Horwood, P., Dussart, P. and Buchy, P. (2016). "Evaluation of the performances of six commercial kits designed for dengue NS1 and anti-dengue IgM, IgG and IgA detection in urine and saliva clinical specimens." BMC Infect Dis **16** (1): 201.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J. and Hay, S. I. (2013). "The global distribution and burden of dengue." Nature **496** (7446): 504-507.

Blacksell, S. D. (2012). "Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs?" J Biomed Biotechnol **2012**: 12.

Blacksell, S. D., Jarman, R. G., Gibbons, R. V., Tanganuchitcharnchai, A., Mammen, M. P., Jr., Nisalak, A., Kalayanarooj, S., Bailey, M. S., Premaratna, R., de Silva, H. J., Day, N. P. and Lalloo, D. G. (2012). "Comparison of Seven Commercial Antigen and Antibody Enzyme-Linked Immunosorbent Assays for Detection of Acute Dengue Infection." Clin Vaccine Immunol **19** (5): 804-810.

Casenghi, M., Kosack, C., Li, R., Bastard, M., Ford, N. and Casenghi, M. (2014). "NS1 antigen detecting assays for diagnosing acute dengue infection in people living in or returning from endemic countries." Cochrane Database of Systematic Reviews (6).

Dawes, C., Pedersen, A. M., Villa, A., Ekstrom, J., Proctor, G. B., Vissink, A., Aframian, D., McGowan, R., Aliko, A., Narayana, N., Sia, Y. W., Joshi, R. K., Jensen, S. B.,

Kerr, A. R. and Wolff, A. (2015). "The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI." Arch Oral Biol **60** (6): 863-874.

Gruner, G. (2006). "Carbon nanotube transistors for biosensing applications." Anal Bioanal Chem **384** (2): 322-335.

Guzmán, M. G. and Harris, E. (2015). "Dengue." The Lancet **385** (9966): 453-465.

Inal, S. and Kelleci, M. (2012). "Distracting children during blood draw: looking through distraction cards is effective in pain relief of children during blood draw." Int J Nurs Pract **18** (2): 210-219.

Khanna, P. and Walt, D. R. (2015). "Salivary Diagnostics Using a Portable Point-of-Service Platform: A Review." Clin Ther **37** (3): 498-504.

Kim, J. P., Lee, B. Y., Lee, J., Hong, S. and Sim, S. J. (2009). "Enhancement of sensitivity and specificity by surface modification of carbon nanotubes in diagnosis of prostate cancer based on carbon nanotube field effect transistors." Biosensors and Bioelectronics **24** (11): 3372-3378.

Korhonen, E. M., Huhtamo, E., Virtala, A.-M., Kantele, A. and Vapalahti, O. (2014). "Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue." J Clin Virol **61** (3): 353-358.

Kutsuna, S., Kato, Y., Moi, M. L., Kotaki, A., Ota, M., Shinohara, K., Kobayashi, T., Yamamoto, K., Fujiya, Y., Mawatari, M., Sato, T., Kunimatsu, J., Takeshita, N., Hayakawa, K., Kanagawa, S., Takasaki, T. and Ohmagari, N. (2015). "Autochthonous Dengue Fever, Tokyo, Japan, 2014." Emerg Infect Dis **21** (3): 517-520.

Lee, C., Jang, E. J., Kwon, D., Choi, H., Park, J. W. and Bae, G.-R. (2016). "Laboratory-acquired dengue virus infection by needlestick injury: a case report, South Korea, 2014." Ann Occup Environ Med **28**: 16.

Mustafa, M. S., Rasotgi, V., Jain, S. and Gupta, V. (2015). "Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control." Med J Armed Forces India **71** (1): 67-70.

Nguyen, M. T., Ho, T. N., Nguyen, V. V., Nguyen, T. H., Ha, M. T., Ta, V. T., Nguyen, L. D., Phan, L., Han, K. Q., Duong, T. H., Tran, N. B., Wills, B., Wolbers, M. and Simmons, C. P. (2017). "An Evidence-Based Algorithm for Early Prognosis of Severe Dengue in the Outpatient Setting." Clin Infect Dis **64** (5): 656-663.

Paranavitane, S. A., Gomes, L., Kamaladasa, A., Adikari, T. N., Wickramasinghe, N., Jeewandara, C., Shyamali, N. L., Ogg, G. S. and Malavige, G. N. (2014). "Dengue NS1 antigen as a marker of severe clinical disease." BMC Infect Dis **14** (1): 570.

Parkash, O. and Shueb, R. H. (2015). "Diagnosis of Dengue Infection Using Conventional and Biosensor Based Techniques." Viruses **7** (10): 5410-5427.

Radzol, A. R. M., Lee-*Sr.*, K. Y. and Mansor, W. (2014). Classification of Salivary based NS1 from Raman Spectroscopy with Support Vector Machine. 2014 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society.

Radzol, A. R. M., Lee, K. Y. and Mansor, W. (2013). Nonstructural Protein 1 Characteristic Peak from NS1-Saliva Mixture with Surface-Enhanced Raman Spectroscopy. 2013 35th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC).

Radzol, A. R. M., Lee, K. Y., Mansor, W. and Azmin, N. H. R. (2015a). SVM-RBF Model PCA Criterion Selection for Detection of NS1 Molecule from Raman Spectra of Salivary Mixture. 2015 IET International Conference on Biomedical Image and Signal Processing (ICBISP 2015).

Radzol, A. R. M., Lee, K. Y., Mansor, W. and Othman, N. H. (2015b). Principal Component Analysis for Detection of NS1 Molecules from Raman Spectra of Saliva. 2015 IEEE 11th International Colloquium on Signal Processing & Its Applications (CSPA).

Rahim, F. (2017). "Acquired Dengue Virus Infection by Needlestick Injury: A Case Report." Case Study and Case Report **7** (1): 22-24.

Ramnani, P., Gao, Y., Ozsoz, M. and Mulchandani, A. (2013). "Electronic Detection of MicroRNA at Attomolar Level with High Specificity." Anal Chem **85** (17): 8061-8064.

Shamala, D. S. (2015). "Laboratory Diagnosis of Dengue: A Review." International Medical Journal Malaysia **14** (1): 17-28.

Shu, P.-Y., Yang, C.-F., Kao, J.-F., Su, C.-L., Chang, S.-F., Lin, C.-C., Yang, W.-C., Shih, H., Yang, S.-Y., Wu, P.-F., Wu, H.-S. and Huang, J.-H. (2009). "Application of the Dengue Virus NS1 Antigen Rapid Test for On-Site Detection of Imported Dengue Cases at Airports." Clin Vaccine Immunol **16** (4): 589-591.

Simmons, C. P., Farrar, J. J., Nguyen v, V. and Wills, B. (2012). "Dengue." The New England journal of medicine **366** (15): 1423-1432.

Souza, L. J., Pessanha, L. B., Mansur, L. C., de Souza, L. A., Ribeiro, M. B., da Silveira, M. d. V. and Souto Filho, J. T. (2013). "Comparison of clinical and laboratory characteristics between children and adults with dengue." Braz J Infect Dis **17** (1): 27-31.

Stefansson, S., Kwon, H. H. and Ahn, S. N. (2012). "Targeting Antibodies to Carbon Nanotube Field Effect Transistors by Pyrene Hydrazide Modification of Heavy Chain Carbohydrates." J. Nanotechnol. **2012**: 1-8.

Succo, T., Leparc-Goffart, I., Ferré, J. B., Roiz, D., Broche, B., Maquart, M., Noel, H., Catelinois, O., Entezam, F., Caire, D., Jourdain, F., Esteve-Moussion, I., Cochet, A., Paupy, C., Rousseau, C., Paty, M. C. and Golliot, F. (2016). "Autochthonous dengue outbreak in Nimes, South of France, July to September 2015." Euro Surveill **21** (21).

Tan, F., Saucedo, N. M., Ramnani, P. and Mulchandani, A. (2015). "Label-Free Electrical Immunosensor for Highly Sensitive and Specific Detection of Microcystin-LR in Water Samples." Environ Sci Technol **49** (15): 9256-9263.

Tarigan, L. H., Cifuentes, M., Quinn, M. and Kriebel, D. (2015). "Prevention of Needle-Stick Injuries in Healthcare Facilities: A Meta-Analysis." Infect Control Hosp Epidemiol **36** (7): 823-829.

Teets, F. D., Ramgopal, M. N., Sweeney, K. D., Graham, A. S., Michael, S. F. and Isern, S. (2014). "Origin of the dengue virus outbreak in Martin County, Florida, USA 2013." Virol Rep **1-2**: 2-8.

Thomas, S. J. (2015). "NS1: A corner piece in the dengue pathogenesis puzzle?" Sci Transl Med **7** (304): 304fs337.

Tlili, C., Cella, L. N., Myung, N. V., Shetty, V. and Mulchandani, A. (2010). "Single-walled carbon nanotube chemoresistive label-free immunosensor for salivary stress biomarkers." Analyst **135** (10): 2637-2642.

Tlili, C., Myung, N. V., Shetty, V. and Mulchandani, A. (2011). "Label-free, chemiresistor immunosensor for stress biomarker cortisol in saliva." Biosensors and Bioelectronics **26** (11): 4382-4386.

Wasik, D., Mulchandani, A. and Yates, M. V. (2017). "A Heparin-Functionalized Carbon Nanotube-Based Affinity Biosensor for Dengue Virus." Biosensors and Bioelectronics **91**: 811-816.

Weaver, S. C. and Lecuit, M. (2015). "Chikungunya Virus and the Global Spread of a Mosquito-Borne Disease." The New England journal of medicine **372** (13): 1231-1239.

Winkler, G., Maxwell, S. E., Ruemmler, C. and Stollar, V. (1989). "Newly Synthesized Dengue-2 Virus Nonstructural Protein NS1 is a Soluble Protein but Becomes Partially Hydrophobic and Membrane-Associated after Dimerization." Virology **171** (1): 302-305.

World Health Organization (2009). Dengue: Guidelines for Diagnosis Treatment Prevention and Control: New Edition. Geneva, World Health Organization.

Young, P. R., Hilditch, P. A., Bletchly, C. and Halloran, W. (2000). "An Antigen Capture Enzyme-Linked Immunosorbent Assay Reveals High Levels of the Dengue Virus Protein NS1 in the Sera of Infected Patients." J Clin Microbiol **38** (3): 1053-1057.

Zhang, Y., Bai, J. and Ying, J. Y. (2015). "A stacking flow immunoassay for the detection of dengue-specific immunoglobulins in salivary fluid." Lab Chip **15** (6): 1465-1471.

Chapter 5: Conclusions and Future Outlook

5.1 Conclusions

DENV has long been associated with tropical and subtropical regions, where ecological conditions make excellent habitats for the primary arboviral vectors, *Aedes aegypti* and *Aedes albopictus*. However, global warming, population growth, and other human activities have resulted in habitat expansion for the vector mosquitoes. Unfortunately, habitat modeling predicts further expansion of the mosquito habitat range which in turn will expand the range of DENV in both the sylvatic and human cycles.

The currently available DENV diagnostic and detections methods have a number of tradeoffs and drawbacks. Laboratory diagnostic methods have high sensitivity and selectivity but are complex and require a dedicated laboratory, high technical skills, a number of expensive sample treatments and reagents, costly equipment, and are not rapid. Unfortunately, these requirements prevent their use in routine surveillance and diagnostics in sylvatic and human cycles. RDTs are less expensive, rapid, and easily accessible to untrained personnel. However, RDTs compromise sensitivity and selectivity to achieve the increased accessibility which makes them impractical for everything but routine human infection diagnostics.

The chemiresistive biosensors presented here are prototyped devices that combine the rapidity and accessibility associated with RDTs with the sensitivity and selectivity of

laboratory diagnostics. The pseudo-1D-structure of the SWNT transducer are ideal for detection of a target analyte with ultra-low concentrations and high surface-to-volume ratio permits functionalization with a variety of bioreceptors. They are a rapid, inexpensive, and sensitive diagnostic method that is easily accessible by untrained personnel. Additionally, the electronic signal/response generated can then be stored and/or wirelessly transmitted to another device for data storage and analysis which makes the biosensors ideal for surveillance of the sylvatic cycle in remote locations.

In chapter 2, a heparin bioreceptor is used for the detection of whole DENV. Heparin is an analog of the heparan sulfate proteoglycans that are receptors for DENV. Heparin will maintain its structure and reactivity despite prolonged periods of direct sunlight and high temperatures; creating a robust and sensitive biosensor that can be used without refrigeration. The biosensor is compatible with viral culture which permits detection of DENV from the widest variety of samples; such as fluid or tissue samples from monkeys, vector mosquitos, and humans.

In chapter 3, anti-dengue NS1 monoclonal antibodies were used to detect DENV NS1 in mosquito homogenate. NS1 is a clinically accepted biomarker for DENV infection and is an excellent target analyte for early detection and diagnosis of the disease in *Aedes* mosquitos. Sylvatic DENV amplifications increase the risk of zoonotic transmission of DENV which can trigger outbreaks within the human cycle in nearby urban environments. Early detection gives time for medical personnel and government agencies prepare for a potential outbreak and implement disease prevention and control protocols.

The chemiresistive biosensor in chapter 4 utilizes anti-dengue NS1 monoclonal antibodies to detect DENV NS1 in human saliva. Currently available diagnostic methods rely on a blood draw for DENV diagnosis. This can be a source of bloodborne pathogens, needle-stick injuries, and pain for the patient. Saliva has recently been identified as a potential sample source for NS1 antigenic diagnosis of a DENV infection. Saliva sampling is a non-invasive, painless, simple collection method, even by minimally trained personnel.

Each of the biosensors presented in this work offer a facile, affordable, and label-free method for diagnosis of a DENV infection. Each was designed to meet the needs of a sensitive and accessible diagnostic tool. Additionally, they are useful for detection of DENV in both sylvatic and human cycles.

5.2 Future Outlook

The chemiresistive biosensors reported here are prototype devices that would need much larger studies before being accepted as a clinical diagnostic tool. The biosensors were highly sensitive and selective for their target analyte but they only target a single analyte per biosensor. Studies of the various commercially available DENV diagnostic RDTs have demonstrated that targeting a single analyte can report false negatives, especially when the target analyte is at low concentrations. However, the diagnostic accuracy of the RDTs have been significantly improved by combining serologic and antigenic detection methods into a single mutliplex assay (Blacksell et al., 2011).

Similar to RDTs, multiplexing on a single biosensor would increase the diagnostic accuracy. The sensing regions of the chemiresistive biosensors are small enough to combine multiple sensors onto a single device for multiplex detection. A dual-purpose biosensor that has 2 separate sensing regions, one functionalized for DENV virion detection with heparin and the other for NS1 detection with anti-NS1 antibodies would offer a powerful early diagnostic tool. Additionally, functionalization of multiple separate sensing regions with alternative antibodies and RNA bioreceptors may further combine virus serotyping, RNA detection, antigenic, and serologic detection on a single device that is able to diagnose a DENV infection at any stage of illness in any testing location.

5.3 References

Blacksell, S. D., Jarman, R. G., Bailey, M. S., Tanganuchitcharnchai, A., Jenjaroen, K., Gibbons, R. V., Paris, D. H., Premaratna, R., de Silva, H. J., Lalloo, D. G. and Day, N. P. (2011). "Evaluation of Six Commercial Point-of-Care Tests for Diagnosis of Acute Dengue Infections: the Need for Combining NS1 Antigen and IgM/IgG Antibody Detection to Achieve Acceptable Levels of Accuracy." Clin Vaccine Immunol **18** (12): 2095-2101.