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Current and nano-diagnostic tools for dengue infection

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1. ABSTRACT

Dengue is one of the infectious diseases that is widespread over global regions with yearly occurrence of epidemics and could be deadly in some cases. Thus the developments of rapid and specific diagnostic tools which can achieve early detection of dengue infection for disease control during epidemic situations and before complications occur are deemed highly desirable. This paper describes the current and advanced methods for diagnosis of dengue infection and discusses the advantages and disadvantages of these methods in terms of their analytical performances and clinical applicabilities. The current methods discussed herein include enzyme-linked immunosorbent assay and reverse transcriptase polymerase chain reaction. In addition, recent instrumental methods such as quartz crystal microbalance, surface plasmon resonance, photonic crystal and electrochemical impedance spectroscopy have shown promising results. Interesting developments in detection of dengue infection using nanosized materials including liposomes, nanowires and nanopores, coupled to conventional fluorescence, potentiometry and voltammetry methods are also described and could possibly point the way forward for the development of inexpensive diagnostic tools for use at point-of-care and in events of epidemic scale.

2. INTRODUCTION

Dengue is the most rapidly spreading, acute febrile mosquito-borne viral disease caused by the dengue virus. Over the past 50 years, the incidence of dengue has increased by approximately thirty folds, spreading across increasing number of countries as well as from the rural to urban regions. This disease is now endemic in most tropical and subtropical regions. An estimated number of about 50 million cases of dengue fever occur each year with 500,000 cases of dengue hemorrhagic fever and 22,000 deaths. Presently, about 2.5 billion people live in over 100 dengue endemic countries (1). The presence of dengue has inflicted significant health, social and economic burden on these endemic areas (2). Hence decreasing the number of outbreaks and if possible eliminating the disease will be of utmost importance. There are several factors that contribute to the occurrence of the disease, mainly the increases in international travel and human population, besides global climate change (3).

Dengue virus belongs to the flavivirus genus of the flaviviridae family. The dengue virus is transmitted to vertebrates by infected *Aedes aegypti* and *Aedes albopictus* mosquitoes when they feed on their blood. The dengue virus is divided into four antigenically related but distinct

serotypes; the Dengue 1, Dengue 2, Dengue 3 and Dengue 4. The mature dengue virus is spherical in shape and ~50 nm in size. It contains a single stranded RNA of ~11,000 nucleotide bases in length. This RNA resides within a nucleocapsid which is enclosed by a lipid bilayer containing three structural proteins (4-6). The RNA genome is organized into 2 basic and distinct regions. The first region codes for 3 structural proteins; capsid protein (C), membrane protein (prM) and envelope protein (E). The capsid protein is one of the main structural components of the nucleocapsid. The envelope protein is postulated to be responsible for the attachment of virus to the host cell as well as for the assembly of the virion. The next region codes for three large non-structural proteins: NS1, NS3 and NS5 and four smaller non-structural proteins: NS2a, NS2b, NS4a and NS4b. The NS1 protein is suspected to play a role in RNA replication and pathogenesis of the disease. The NS3 together with the NS2b protein form the protease domain of the virus, while the NS5 protein is the RNA polymerase responsible for viral replication in the host cell (5). Overall, the sequence of RNA genome is as follows: 5'-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3' in term of protein expression.

There are three major complexes within the flaviviridae family: the tick-borne encephalitis virus, Japanese encephalitis virus and dengue virus. All the flaviviruses share similar morphology and genomic structure, with a common antigenic determinant. As a consequence, the specific identification of a family member poses significant challenge for serological methods owing to extensive cross-reactivity. The situation is made worst in dengue virus since it comprises four serotypes. Despite this extensive cross-reactivity, infection by one of the dengue serotype usually confers lifelong immunity against the homologous serotype but not against the other three serotypes (4).

Dengue infection can be caused by any of the four serotypes with varying severity. Infection in human leads to a wide spectrum of illnesses, ranging from mild febrile illnesses to fatal hemorrhagic diseases. Dengue fever is usually characterized by a sudden onset of fever which normally last 2-7 days, together with a variety of non-specific signs and symptoms such as high fever, headache, nausea, vomiting, joint pain, fatigue and rashes. The entire course of illness can be broadly divided into three phases; febrile, critical and recovery phase (1). During the critical phase, some individuals may progress to severe plasma leakage in cases with dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), severe hemorrhage and severe organ involvement (1, 7). The warning signs for severe dengue include persistent vomiting, mucosal bleeding, lethargy, abdominal pain or tenderness, clinical fluid accumulation, restlessness, liver enlargement of more than 2 cm and an increase in hematocrit count with a rapid decrease in platelet count (1) Shock ensues when a critical volume of the plasma is lost through leakage. Prolonged shock may lead to coagulopathy and multi-organ failure. Careful monitoring of the blood counts, assessment of hemodynamic status and recognition of these warning signs allow early treatment intervention. Appropriate fluid management and supportive care are vital to the recovery.

Currently, there are no effective vaccines or drugs to prevent or treat dengue infection. Hence, the most effective way to reduce the spread of the disease is to control vector multiplication (6, 8). However, a global attention to this issue is required since migration and geographical expansion can lead to transportation of the virus from one place to another (5). Until a global cooperation of vector control can be achieved or an effective vaccine becomes available, early diagnosis remains an important tool for case confirmation and clinical care. In addition, isolation of the virus is also crucial for surveillance program, pathogenesis studies and vaccine research (9). Relying solely on clinical diagnosis can be problematic since the symptoms of dengue infection are non-specific, in particular during the early febrile phase (6). Laboratory diagnosis methods relying on genome and antigen detection as well as serological methods have been widely applied routinely. However, several disadvantages presently limit these methods. For example, antibodies take ~5 days to appear after an infection (1), thus antibody detection can contribute toward epidemic research but is less effective for early diagnosis compared to methods that detect virus or viral proteins directly. Likewise, the virus isolation process takes about one week, thus rendering it less effective for early diagnosis purposes. In contrast, the polymerase chain reaction method (PCR) can rapidly detect the viral RNA within hours, but is presently limited by possible contaminations by amplicons from previous amplification and generally requires specifically trained personnel (1). Alternative diagnostic systems that can detect dengue virus or related biomarkers during the early phase of infection with comparable sensitivity, selectivity, diagnosis time as current methods and can be operated by non-experts would be ideal. In this review, we will discuss some of the routine diagnostic methods currently in use followed by advanced nanotechnology-based methods (Figure 1) which are directed to achieve ideality.

3. CURRENT DIAGNOSTIC TOOLS FOR DENGUE INFECTION

Diagnosis of dengue was carried out using traditional methods including virus isolation, hemagglutination inhibition (HI) and plaque reduction neutralization test (PRNT). Needs for close monitoring of patients and early intervention to reduce mortality led to the development of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) which are current methods of choice for the diagnosis of dengue infection. For example, certain serological tests based on ELISA methods can give relatively rapid and accurate results using a single serum sample, unlike traditional methods such as HI test which requires paired serum samples collected during acute and convalescent phases of dengue infection, and PRNT which requires long analysis time of 4-7 days. For non-serological tests focusing on virus detection, PCR methods are highly preferred over virus isolation because of very short analysis time of hours compared to weeks in the latter.

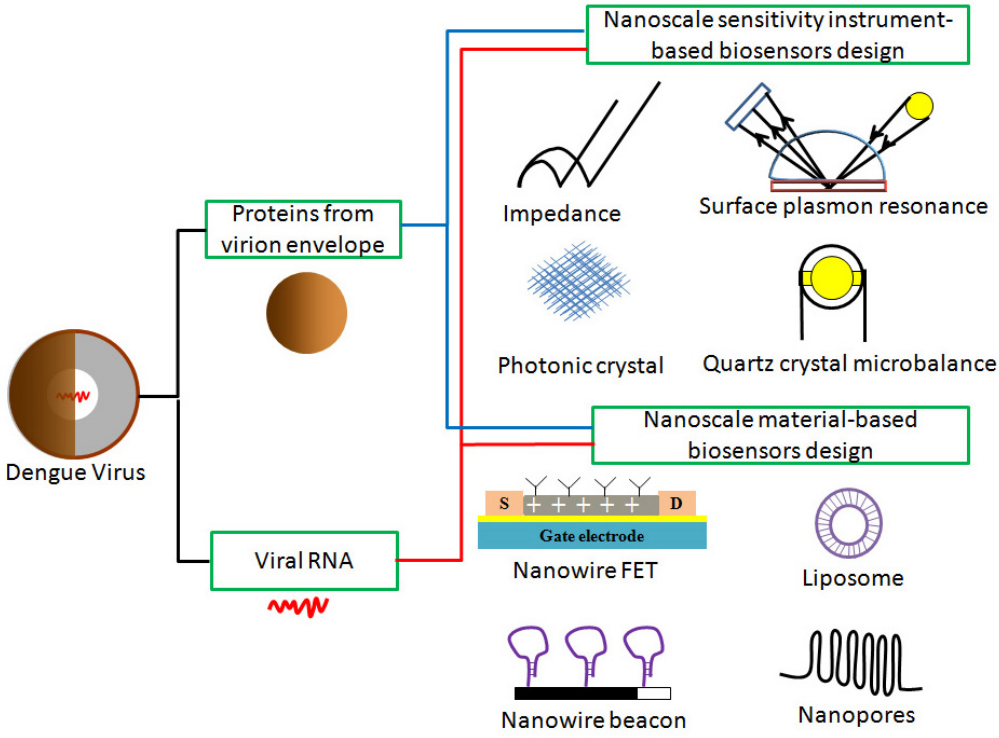

Figure 1. Overview of the nanoscale sensitivity instrument-based biosensors and nanoscale material-based biosensors used for the detection of dengue infection.

In the following, we classify ELISA and PCR methods as current methods because of their widespread use in many clinical laboratories today, while recent emerging methods which show potential applications in dengue diagnosis are discussed in Section 4 as advanced methods.

3.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a biochemical method applied in immunology to detect the presence of antigens or antibodies in the sample. There are 2 general forms of ELISA which are commonly carried out. The sandwich ELISA uses monoclonal antibodies to detect the presence of target antigens in the sample, followed by enzyme-labeled antibodies which will bind to the target antigens in a sandwich configuration. In contrast, the indirect ELISA uses immobilized antigens to detect the presence of target antibodies in the sample, followed by binding of the target antibodies to added enzyme-labeled antibodies. In both forms, after the formation of the immune-complexes, the enzyme substrate is added and subsequent reaction of the enzyme substrate changes the solution color which indicates a positive ELISA test.

Immunoglobulin M-antibody capture ELISA (MAC-ELISA) is the most common and widely used serological test. It is a sandwich ELISA method based on detecting the dengue specific immunoglobulin M (IgM) which appears ~5 days after the onset of fever and last ~30-60 days. Antibodies specific to IgM are coated onto a microplate to capture the total IgM in the serum sample. Next, dengue virus is added which will bind to the captured IgM. The detection of the immune-complexes formation is carried out by enzyme conjugated-monoclonal or polyclonal antibodies that will transform the colorless substrate into a colored product, measured with a spectrophotometer. This method is simple, rapid and requires basic equipment setup. The MAC-ELISA can be carried out with either one serum or paired sera from the patient (1). Positive diagnosis of the patient with one serum sample indicates a probable dengue case. Though MAC-ELISA using one serum sample does not confirm that the patient has dengue infection, the rapid analysis time allows immediate classification of suspected patients especially during epidemics. The sensitivity of this method often depends on the point of time during which the sample was tested. If the test was carried out too early, or carried out on elderly and immunocompromised patients, the level of IgM may be below detection limit. In addition, MAC-ELISA test with a single acute-phase serum sample has been reported to give significantly more false negative results compared to false positive results (5). The MAC-ELISA is also not able to identify the dengue serotypes owing to cross-reactivity of the antibodies (1).

An immunoglobulin G (IgG) ELISA using the indirect method can be developed to detect present dengue infection, similar as MAC-ELISA. The dengue specific immunoglobulin G (IgG) usually appears ~7 days after the onset of fever and last for months to years. Its advantage over MAC-ELISA is the ability to further identify past infection since IgG remains detectable in the infected

person for years (1). Thus, the simultaneous determination of IgG and IgM using IgG and IgM capture ELISA allows primary and secondary infections to be distinguished. In general, an IgM/IgG ratio greater than 1.2 or 1.4 (using patient's sera dilution of 1/100 or 1/20) is indicative of a primary infection and a ratio less than this reference number confirms a secondary infection (1).

A sandwich ELISA detection method for the non-structural protein 1(NS1), has also been used to confirm dengue infection cases (10). The NS1 proteins can be detected in the blood serum from day one after the onset of fever up to day nine. It can also be detected after the viremia period when RT-PCR shows negative test for viral RNA and when IgM antibodies are found in the serum sample (10). Thus, choosing NS1 as the biomarker for dengue infection has a clear advantage over the other serological methods which in addition, has shown good selectivity amongst other flaviviruses. However, NS1 detection presently cannot differentiate the different dengue serotypes (1), including the rapid sensing lateral flow dengue NS1 silver strip method employed by some local hospitals.

3.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

In this method, reverse transcriptase enzyme is added to produce the complementary DNA sequence from a specified region of the viral RNA genome. Subsequently, the complementary DNA is amplified through repeated denaturation, annealing and elongation processes using the polymerase chain reaction (6). The amplicons produced are separated by agarose gel electrophoresis and detected with ethidium bromide or fluorescence. This is a rapid and sensitive method that is applicable to most viruses. It has comparable cost and better sensitivity compared to the virus isolation method (1). In addition, the result of this method is less affected by the handling and storage of the serum samples as well as the presence of any antibodies (4). With a careful selection of the specific primers used, RT-PCR can be extremely useful for detecting and typing dengue virus, thus allows rapid identification of existing or new serotypes in endemic areas (6). Though the RT-PCR method is gradually replacing the virus isolation method, it is still far from being a simple diagnostic tool. Prior knowledge of the viral genome sequence must be known in order to synthesize the specific primer. Also, sample preparations must be done carefully to reduce false negative results caused by contaminations (6). Various modifications of this method such as nested RT-PCR, real-time RT-PCR and quantitative RT-PCR have also been used for the diagnosis of dengue infection. The advantages and disadvantages of these modified methods compared to the conventional RT-PCR have been clearly summarized by Ratcliff *et al.* (11).

The choice of diagnostic methods depends on the purpose of testing, the type of laboratory facilities, technical expertise available, and the analysis cost as well as the time of sample collection. During the first few days of infection also known as the viraemia stage (0-5 days), virus isolation, RT-PCR and antigen detection methods provide the most accurate result. After 4-5 days, MAC-ELISA is preferred over virus detection methods because of the appearance of IgM antibodies and significantly low amount of virus remaining in the blood serum. From day 6 onwards, a combination of ELISA and PCR based methods would be ideal for accurate results. In general, the identification of virus based on whole virus, viral RNA or viral antigen such as NS1, together with the detection of anti-dengue antibodies is preferred over either approach alone (1).

4. ADVANCED DIAGNOSTIC TOOLS FOR DENGUE INFECTION

Recent reports have described an increase use of high sensitivity methods for viral detection with detection limits in range of ng ml^{-1} (12, 13) or pg mm^{-2} (14, 15) and as such, have been broadly described as nanotechnological advancements (16). In general, these methods rely on optical, electrical or electrochemical signals to detect minute changes in the physico-chemical properties of the sensing elements which often comprise micrometer to nanometer size particulate, film or membranous materials. In the following review, we observe these methods can be further classified into those relying significantly on ultrasensitive instrument-based biosensor design as opposed to those which focus on nanoscale material-based biosensor design. However, this review does not purport to highlight all methods. Rather, we focus in particular, on nanoscale sensitivity instrument-based biosensors and nanoscale material-based biosensors which have demonstrated sensitivity at or below nanoscale level and have relied on nanosized materials in one or more dimensions, respectively. In addition, we have grouped the following discussion into analyte types under the respective detection methods since each analyte target such as IgM, IgG, whole virus, NS1 and nucleic acids can provide very useful information concerning past and present infections.

4.1. Nanoscale sensitivity instrument-based biosensor design

4.1.1. Quartz crystal microbalance (QCM)

Quartz crystal microbalance is a piezoelectric transducer which can measure sensitive mass changes in vacuum, gas and liquid phases (17). This method measures the change in mass per unit area by following the corresponding frequency change of the resonating quartz crystal when small amount of mass is added or removed from the crystal surface. With some simplifying assumptions, the change in frequency can be quantified and correlated with the change in mass on the crystal using the Sauerbrey's equation (18). Basically, a quartz crystal microbalance sensor comprises a thin quartz disk with thin electrode films plated on both sides of the quartz disk. A voltage is applied between the two electrodes to cause deformations of the crystal, allowing it to vibrate at its own resonant frequency which shifts in response toward changes in the mass adhered on the crystal surface (19, 20).

In general, quartz crystal microbalance sensors are low cost sensors designed for real-time mass determination. This method is highly sensitive and precise,

with potential for miniaturization owing to its small size. In addition, the method is simple to carry out, with a relatively fast response time, making it ideal for diagnostic purposes (21-23). The major disadvantages of the method are the lack of reproducibility on amount of antibodies immobilized onto the crystal surface and viscosity drag in the liquid phase (19). The quartz crystal microbalance has already been used in kinetic research, medical diagnosis and the detection of pathogenic microorganisms (19). For immuno-sensing, the antigens or antibodies are normally immobilized on the transducer carrier surface, followed by addition of blocking agents such as bovine serum albumin (BSA) in order to block all non-specific binding sites on the sensor surface (18).

Peng *et al.* developed a real-time and user-friendly QCM immunochip for the detection of dengue virus monoclonal antibodies against the dengue envelope protein and NS1 protein were immobilized onto the quartz crystal in a controlled orientation via protein A to produce the immunochip. Prior to the experiment, BSA was injected into the flow system leading to the immunochip sensor to block non-specific binding sites on the sensor. The sample was subsequently injected into the flow system, carried along with the circulating buffer to flow through the antibody-coated QCM. With the aid of sample pretreatment using the cibacron blue 3GA gel-heat denature method, the detection limit of the envelope protein and NS1 protein was reduced to 1.727 microg ml^{-1} and to 0.740 microg ml^{-1} respectively showing potential for future diagnosis of dengue clinical specimens (22).

In another work, Chih *et al.* developed a real-time and circulating-flow QCM useful for early diagnosis and epidemiology study of dengue infection using nucleic acids identification (23). Two types of gold nanoparticle probes with complementary sequences to the target sequence were constructed. Upon hybridization of probes to the target sequence, the gold nanoparticles were deposited onto the sensor area where the target sequence act as a linker for the layer-by-layer structuring of the gold nanoparticle probes. As such, increase in mass on the sensor was detected by a decrease in the quartz resonance frequency. They have demonstrated that this method has comparable sensitivity and specificity to the fluorescent real-time PCR method. In addition, the method does not require expensive instrumentation, is label-free and highly sensitive, with reported detection limit of 2 PFU ml^{-1} (23).

4.1.2. Photonic crystals (PC)

Photonic crystals are periodic optical nanostructure designed to affect the motion of the photons. It generally comprises periodic regions of high and low dielectric constant nanostructures that affect the propagation of electromagnetic waves by defining the allowed and forbidden electronic energy bands. The wavelength of light that is allowed to travel through the photonic crystals is known as the 'mode' while wavelength of light that is reflected is known as the 'band'. The range of wavelengths that are not permitted to pass through the crystal is called the 'photonic band gap' (24). Two conditions need to be satisfied in order to create this band gap in the photonic crystals. First, the crystal structure needs to be face-center cubic or diamond crystal structure and second, there must be a large difference in the refractive indices of the two materials used in constructing the crystal (25). Photonic crystals are classified into three basic types: one-dimensional, two-dimensional and three-dimensional depending on the direction in which light can propagate through (24). For example, one such interesting arrangement uses selective chemical modification of glassy spheres to generate surfaces specially tailored for assembly of micrometer-sized spheres from aqueous solution (26).

Photonic crystal is an appealing choice for making sensors since their optical properties can be modified under the influence of analytes. Detection is achieved by measuring the wavelength of peak reflectance as a function of time or space. A positive shift of the peak reflectance wavelength indicates a positive adsorption of the analyte onto the sensor surface. The magnitude of the shift is proportional to the deposited mass density. Thus the photonic crystal is a label-free method with typical detection limits in the range of pg mm^{-2}. Besides it is able to produce reliable results even when the sensing area is in the micrometer to millimeter range. This allows the crystals to be incorporated onto lab-on-chip devices for *in-situ* sensing of analytes (24, 27) including biological molecules (24).

Cunningham *et al.* have reported the detection of human anti-dengue antibodies from serum samples using compact optoelectronics biosensors (28). This novel biosensor demonstrated sufficient sensitivity for clinical-relevant assay, making it a potentially new platform for simplified and rapid point-of-care diagnostic tests for the detection of infectious diseases (28). Optical measurements were carried out using vertical cavity surface emitting laser and photonic crystals were bonded to the bottom of each well in a standard 96 wells microtiter plate. Dengue viral antigens were first incubated for 12 hours in the microtiter wells to allow the antigens to be absorbed onto the sensors' surface. Next, blocking agents were added to minimize non-specific bindings of the antibody. Subsequently, the serum sample was added and incubated for 4 hours . The optical wavelength shift was found to be proportional to the serum dilution concentration. The biosensor showed comparable detection sensitivity as ELISA but with shorter and simpler preparation steps. However, this method is not able to distinguish IgG and IgM antibodies binding owing to the lack of specificity (28).

Mandal *et al.* presented a nanoscale optofluidic sensor array for the detection of dengue virus which can carry out label-free, parallel detections of biomolecular interactions in aqueous environments with potential of achieving low mass detection limit (29). Their sensor design comprises a silicon waveguide with a one-dimensional photonic crystal micro-cavity (also known as the side resonator) aligned next to the waveguide. Their unique design allows multiplexing along a single waveguide by placing a number of resonators along the waveguide, each with a different resonant wavelength.

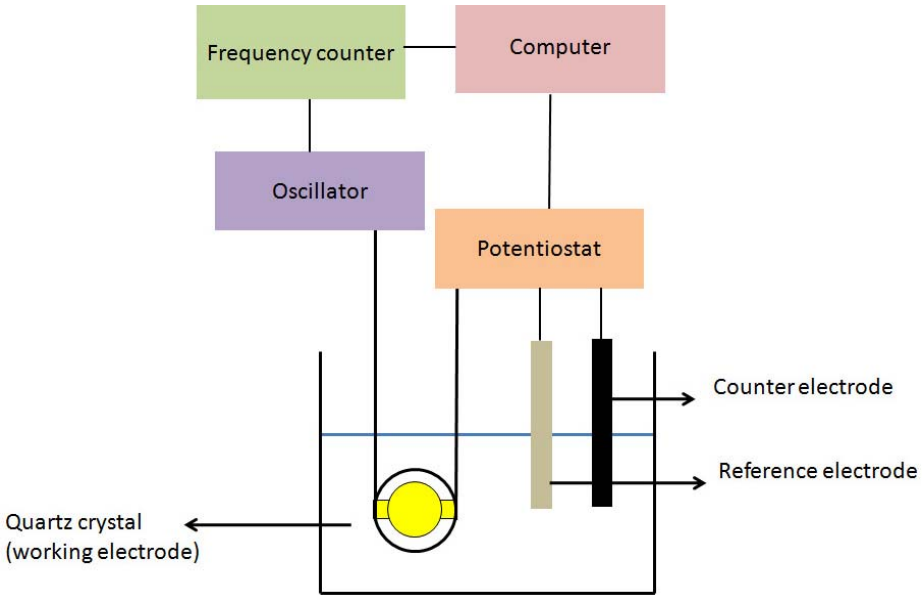

Figure 2. Schematic diagram of the electrochemical quartz crystal microbalance sensor.

They immobilized the oligonucleotide sequences of all four dengue serotypes onto four resonators. For specificity test, the synthetic DNA target sequence of Dengue 3 was flowed through the sensor and the shift in the wavelength was detected. The result showed red shifts for Dengue 1 and Dengue 3 resonators. The smaller red shift from Dengue 1 was attributed to cross-reactivity of the target sequence with the probe sequences (29).

4.1.3. Surface plasmon resonance (SPR)

Surface plasmon resonance is an optical method that depends on the coupling of light with plasmon on a metallic surface. At the interface of two materials of different refractive indices, light coming from the denser material will be partially reflected and refracted. However, at an incidence angle greater than the critical angle, most of the light will be reflected back into the denser material, while a small component penetrates into the material of smaller refractive index giving rise to evanescent wave. This evanescent wave is able to excite electrons, atoms or molecules near the interface. If the interface of the two materials is coated with a thin layer of metal such as gold and monochromatic light that is *p*-polarized, the evanescent wave can excite the freely oscillating electrons of the metal layer causing them to resonate, depending on characteristics of the metal film, its thickness, refractive indices of dielectrics on either side of the metal film and the angle of incidence (30). This surface plasmon resonance phenomenon causes loss of some light to the metal film, thus a sharp dip in reflectivity intensity when the incidence angle equals the resonance angle.

Surface plasmon resonance can be achieved by using either metal grating surface or a metal coated prism with the Kretschmann configuration. For the metal grating case, the sample solution needs to be transparent to allow illumination of the grating, limiting their uses. Thus, the Kretschmann configuration is usually the preferred choice for analysis (30, 31). The major advantages of the surface plasmon resonance sensor include being a label-free method, able to characterize binding reactions in real-time, generate reproducible results with little effort and time (32) and give excellent detection limit in the range of pg mm^{-2} (31). In addition, the method has shown several advantages over the ELISA method such as lower detection limits, less false positive and negative results and rapid analysis times (32, 33).

Kumbhat *et al.* demonstrated that the surface plasmon resonance method is a reliable, label-free and real-time method that could be made into a biosensor for clinical diagnosis and surveillance for the dengue virus (33). They used gold coated microscopic glass plate as the sensor chip, coated with a self-assembled monolayer of 11-mercaptoundecanoic acid with subsequent immobilization of the dengue antigen-bovine serum albumin conjugate. The dengue virus specific IgM antibodies were added and allowed to interact with the sensor chip for 15 min to achieve stable signal. The sensor surface could be regenerated by flowing pepsin solution to release the surface bound analytes and were stable even after 30 regenerations. They reported that the surface plasmon resonance method produced more sensitive results compared to the MAC-ELISA method (33).

4.1.4. Electrochemical impedance spectroscopy (EIS)

Electrochemical methods have always been an attractive method for the detection of biological molecules and monitoring of various cellular activities (34). Electrochemical biosensors allow label-free and real-time monitoring of electroactive and electro-inactive biological analytes using appropriate schemes (35) or by integration with other methods including quartz crystal microbalance (36) and surface plasmon resonance (37) (Figures 2 and 3).

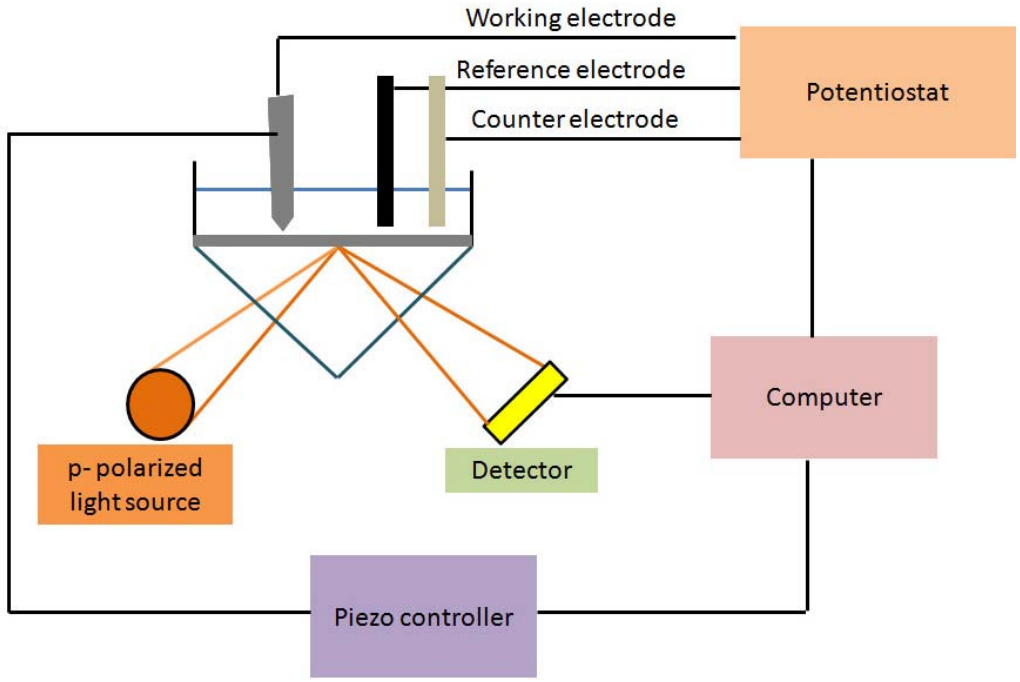

Figure 3. Schematic diagram of the electrochemical surface plasmon resonance.

Besides, the instrumentation is usually simple, economical and easy to miniaturize. Electrochemical detection of biological molecules can be carried out by measuring the current (Amperometry), potential (Potentiometry), resistance (Electrochemical impedance spectroscopy) or conductivity (Conductometry) of the system.

Among the electrochemical methods, electrochemical impedance spectroscopy has been applied for the detection of dengue infection (38, 39) In general, a small sinusoidal voltage signal is applied to the sensing element and the resulting sinusoidal signal output is analyzed to derive the impedance as a function of frequency (40, 41). The typical setup for the electrochemical impedance spectroscopy comprises working, reference and auxiliary electrodes. The electrolytes used usually include an electroactive redox couple with well defined oxidation and reduction properties such as the ferri/ferrocyanide redox couple. For immunology studies, when the respective antigen or antibody targets are added, binding to the surface immobilized probes cause changes in the electron transfer resistance across the electrode-solution interface, owing to increase difficulty for the electroactive species to reach the electrode surface blocked by large antigen-antibody complexes. The main attractiveness of such impedimetric immunosensors lies in the cost effective instrumentation, low accessories cost and the potential for miniaturization (41). However, precise control of surface immobilization procedure is often required in order to prevent non-specific interactions and multi-layer formation, thus confines this method to laboratory-based and well-defined experimental conditions.

Diniz *et al.* has presented an interesting method which allows identification of the various classes of dengue infection based on recognition of the glycoprotein pattern in the serum samples using a modified gold electrode (38). They immobilized concanavalin A on the gold electrode using gold nanoparticles and polyvinyl butyral. Polyvinyl butyral entrapped and immobilized the concanavalin A-gold nanoparticle aggregate onto the electrode surface. Then bovine serum albumin was added to block the remaining active site. The modified electrode exhibited specific impedimetric response toward the glycoproteins present in the serum. Three different samples were analyzed; the sera of volunteers with no dengue fever, patients with dengue fever and patients with dengue hemorrhagic fever. From the impedance plots obtained, it showed increase in the charge transfer resistance when the sensor was exposed to the infected sera. This indicates that in general, the non-infected samples gave less glycoprotein agglutination compared to the dengue infected samples, on the electrode surface. Variations in the charge transfer resistance and a complete analysis of the impedance data achieved differentiation among the three different types of sample. The variable response of the modified electrode to realize the differentiation of the three samples was attributed to concanavalin A capacity to detect distinct glycosylation pattern. This ability to distinguish infected and non-infected patients and between dengue fever and dengue hemorrhagic fever makes the method potentially attractive as a diagnostic tool during immediate clinical care (38).

A novel impedimetric immunosensor based on sol-gel derived strontium titanate thin film and interdigitated electrodes for the diagnosis of dengue

infection has been fabricated by Fang *et al.*(39). The electrode was pre-coated with a thin layer of strontium titanate. This layer was modified with 3-aminopropyltriethoxysilane and activated with glutaraldehyde to allow binding with the inactivated Dengue 2 virus. This modified electrode acts as a sensing probe for the dengue antibodies in the serum. The group was able to correctly identify the antibodies in the serum with good signal repeatability. The change in signal can be attributed to change in the surface conductivity upon interaction of the biomolecules at the electrode surface and change in the dipole induced interfacial polarization potential at the interface between the thin film and the biomolecules. The system is based on non-Faradaic processes, as such even deionised water can be used as the electrolyte. The promising results, together with the low electrode production cost, indicate potential use as a cheap, disposable, point-of-care diagnostic tool for dengue infection (39).

4.2. Nanoscale material-based biosensor design

In past decades, many scientists and engineers have been working toward improving the sensitivity and specificity of diagnostic tools as well as miniaturizing these systems such that they can be made portable for convenience use. This process of downscaling has led to the focus on the construction and utilization of materials with sizes in the nanometer regime. These nanomaterials have unique physical and chemical properties owing to their nanometer size in one or more dimensions. For example, the large surface-to-volume ratio of the nanomaterials allows large number of biorecognition molecules to be attached. The close range interactions between the nanomaterials (which also function as transducers) and the attached biorecognition molecules can influence the physico-chemical properties of these nanomaterials, especially in the presence of target analytes which bind to the biorecognition molecules (42, 43).

4.2.1. Nanowires

Nanowires are wires with diameters in the nanometer range. There are different types of nanowires including metallic, semi-conducting and insulating nanowires. Nanowires used in various sensing strategies including optical, electrical, mass-dependent and in particular electrochemical methods, have been reported (44). They are very attractive sensing materials owing to their small sizes and high surface-to-volume ratios with unique electronic, optical or magnetic properties (44). In addition, nanowires can be readily functionalized with various biochemicals using appropriate linkage chemistries to produce nanosensors and nanocarriers with enhanced properties (45). One such commonly employed nanowires are the silicon nanowires which are well characterized, highly reproducible and exhibit excellent electrical performances (46). Metal electrode arrays of nanoscale dimensions are also extremely useful for ultrasensitive detections owing to the large number of electrochemically active electrodes (47). Recent works on nanowire-based analysis of biomolecules have focused on barcoded nanowires (48) and nanowire-based field-effect transistors (49). In general, nanowire-based field-effect transistors use electrical methods, while barcoded nanowire relies on optical transducers. Specifically, barcoded nanowires are often used for multiplex analysis while nanowire-based field-effect transistors are used mainly for the analysis of single analytes.

In nanowire-based field-effect transistor biosensors, the sensing unit comprises the usual three-electrode transistor structure where the source and the drain electrodes bridge the receptor-coated semi-conductor channel while the underlying gate electrode modulates the channel conductance (49). The semi-conductor channel can be made of semiconducting nanowires with uniform conductance determined by the main carrier density in the nanowires. Generally, if the receptor-bound analytes carry a charge opposite to the main charge carriers, the charge carriers will accumulate in the channel causing an increase in the conductivity of the device. If the analytes are of similar charge as the charge carriers, depletion of main charge carriers occur, causing a decrease in the conductivity of the device (49) (Figure 4). As bioanalytes are affected by pH and ionic strength of the sample solution, such conditions need to be carefully considered. The main advantages of this technique are direct, label-free, real-time electrical signal transduction, high sensitivity, selectivity and low detection limit in femtomolar range (46, 49).

Zhang *et al.* make used of an innovative silicon nanowire-based sensor for label-free, specific, highly sensitive and rapid detection of RT-PCR product of Dengue 2 virus (50). The silicon nanowire was functionalized with aminopropyltriethoxysilane which was used to bind to glutaraldehyde, a bifunctional cross-linker for the subsequent covalent grafting of nucleic acid sequences complementary to that of the dengue virus. The RT-PCR products of the Dengue 2 virus were denatured to produce single strands and added to the detection cell. After the binding event, the resistance of the nanowire was measured. They observed an obvious increase in the resistance upon hybridization, which corresponded to the amount of hybridized RT-PCR products. Further experiments indicated good selectivity for Dengue 2 and good reproducibility between different nanowires. The silicon nanowire biosensor was able to detect lower than 10 fM concentration of amplicons within 30 min. In addition, the approach showed potential for integration of the nanowire biosensor in a point-of-care medical device to facilitate diagnostic applications (50).

Barcoded nanowires can be prepared from segmented nanowires in which different segments are derived from different metal materials. Two segments made of two different metals are optically distinguishable because of the difference in the reflectivity of the metals, thus the segment pattern on a nanowire can be readily recognized using optical imaging. For the detection of biomolecules, the barcoded nanowires are further functionalized with fluorescence probes. For multiplex sensing, barcoded nanowires of a specific segment pattern are coated with one particular fluorescence probe. A mixture of such barcoded nanowires of specific segment

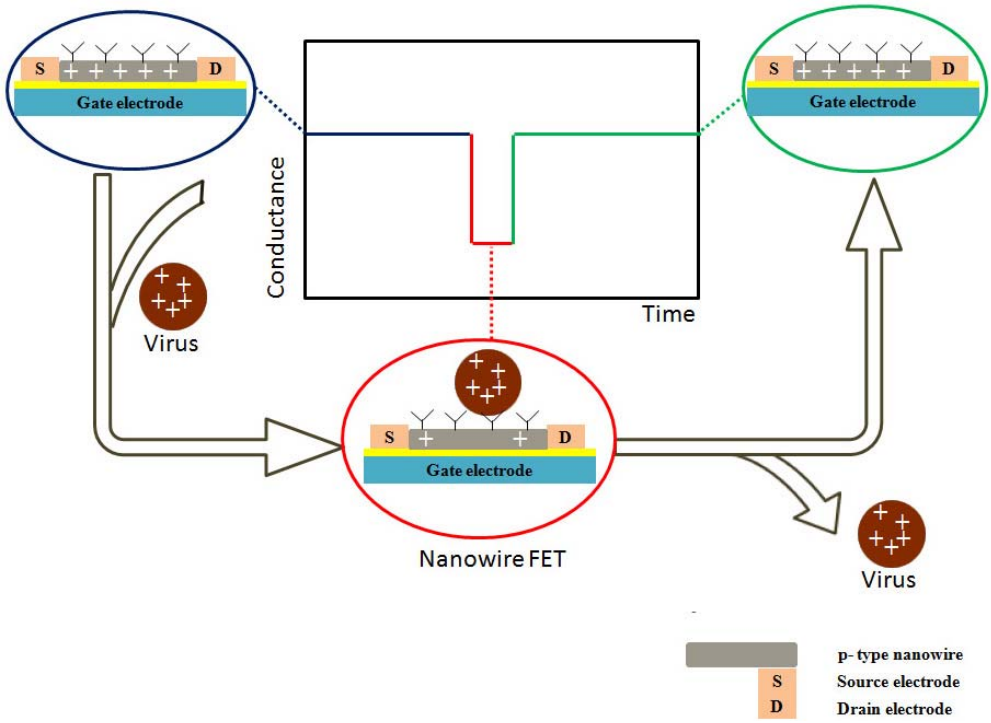

Figure 4. Schematic diagram showing conductance measurement using a nanowire-based field-effect transistor sensor.

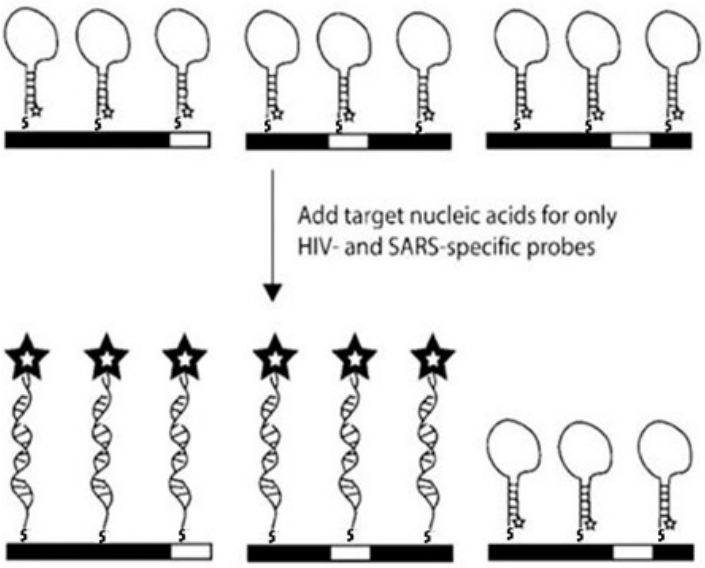

Figure 5. Schematic diagram illustrating multiplex detection of nuclei acid targets by barcoded nanowires functionalized with molecular beacon probes. Reproduced with permission from 48.

patterns and probes, allow simultaneous detection of several analytes by comparing the reflectivity pattern derived from the metallic nanowires and probe fluorescence signals on the nanowires (44).

For example, Keating *et al.* has synthesized oligonucleotide sequence probes for the simultaneous detection of various viruses including the Dengue 2 virus. The nanowire was able to recognize the complementary DNA target strands of Dengue 2 virus and illustrated the exciting capability of barcoded nanowires as a multiplexing agent (48) (Figure 5).

4.2.2 Liposomes

Liposomes are nanosized vesicles comprising one or more concentric lipid bilayers surrounding an internal

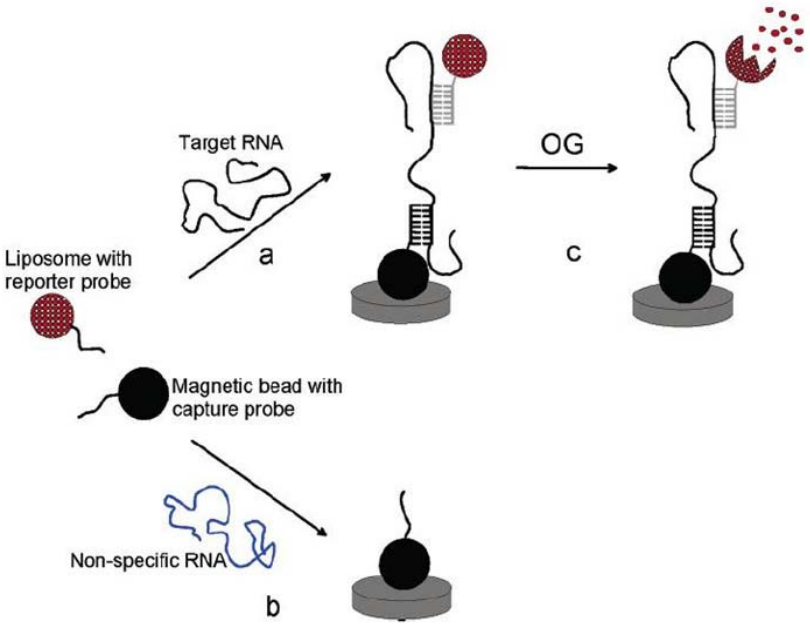

Figure 6. Schematic diagram illustrating the recognition and detection of Dengue RNA using a liposome-based biosensor. Reproduced with permission from 53.

aqueous compartment. They are formed spontaneously when phospholipids are dispersed in water. The morphology, structure and functionality of liposomes can be easily modified by changing the phospholipids composition, by altering the conditions during their formation and through insertion of substances. These molecules are also capable of encapsulating a wide range of substances including fluorophores, enzymes and drugs. Also, they are usually biocompatible, non-toxic and biodegradable. For application in the detection of microbes, the fluorophore-filled liposomes are first bound with the targets (microbes). Subsequently, proteins or detergents are added to cause lysis of the liposomes, leading to the release of the fluorophores which amplifies detection of each binding event. The mode of detection thus depends on the type of marker molecules encapsulated within the liposomes and thus may not be limited to optical detection (51, 52).

Baeumner *et al.* had created a microfluidic biosensor with fluorescence detection for the rapid, sensitive and serotype specific detection of the dengue virus (53) (Figure 6). The biosensor comprises a sandwich configuration of the target nucleic acid between magnetic beads and liposomes. The reporter nucleic acid probes that can recognize all four dengue serotypes were incorporated within the lipid bilayers of the liposomes during their formation. The same four serotype specific nucleic acid capture probes were also immobilized onto superparamagnetic beads via biotin-streptavidin conjugation. The reporter nucleic acid probe-incorporated liposomes, the nucleic acid capture probe-immobilized magnetic beads and the nucleic acid target were mixed in a microcentrifuge tube to achieve hybridization. After incubation, the mixture was injected into the microfluidic setup. The complexes formed between the liposomes, the complementary nucleic acid targets and the beads were attracted by the magnet at the microfluidic detection zone. Following that, detergent was flowed through to cause lysis of the liposomes. The fluorophores released allows fluorescence quantification of the hybridized complexes, with reported excellent detection limit of 50 pM and short analysis time of 20 min (53).

4.2.3. Nanopores

Nanometer-scale pores have been extensively utilized in recent times to solve urgent problems concerning health and environmental related issue. One obvious advantage of nanopore technology is the ability to utilize pore dimensions comparable with the size of many macromolecules to interrogate the interaction of these macromolecules with the porous materials, besides controlling their adsorption and transport behaviours. There are mainly two types of nanopores, the biological pores and the solid-state pores.

Among the biological pores are self-assembling peptides with low selectivity, large transmembrane ion channel proteins with high selectivity for specific ions and porins with porous channels (54). Ion channel proteins are ideal choice for sensing applications since these allow signal transduction and amplification (54). Recent advancements in protein engineering and grafting of functional groups have opened wide possibilities for applications of biological pores including high sensitivity detection, monitoring of chemical and biochemical reactions at single molecular level, targeted cytolysis of cancer cell and potential sequencing of long strand DNA or RNA (54). Bayley *et al.* (55) have used biological nanopores staphylococcal alpha-hemolysin and engineered pores based on alpha-hemolysin to detect analytes such as

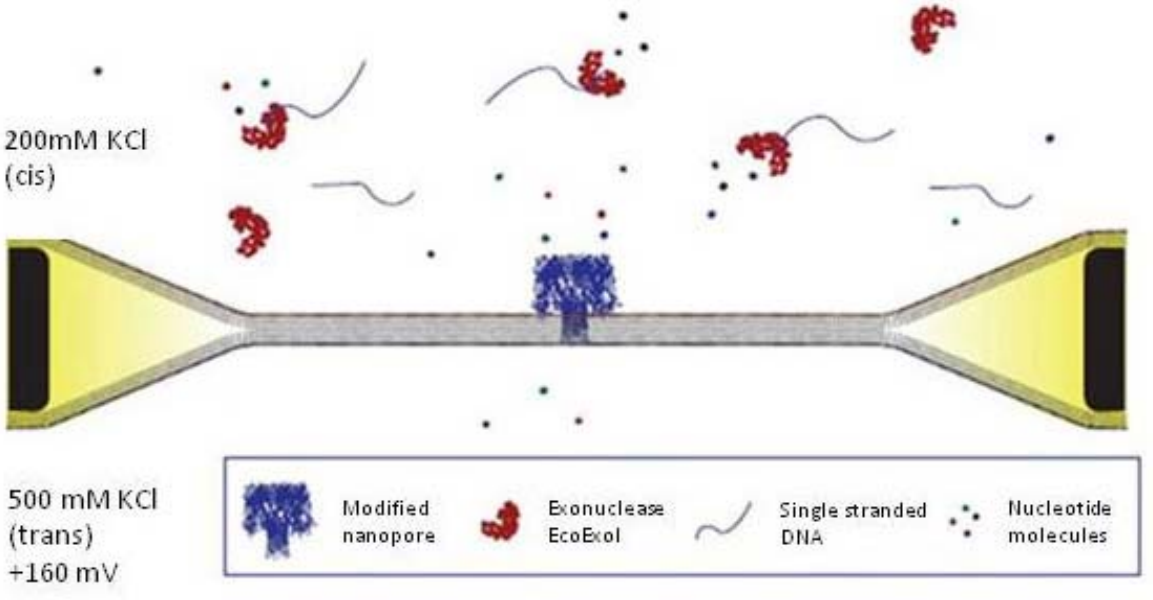

Figure 7. Schematic diagram of a biological nanopore for the molecular detection of nucleotides cleaved from single stranded DNA by exonuclease Reproduced with permission from 55.

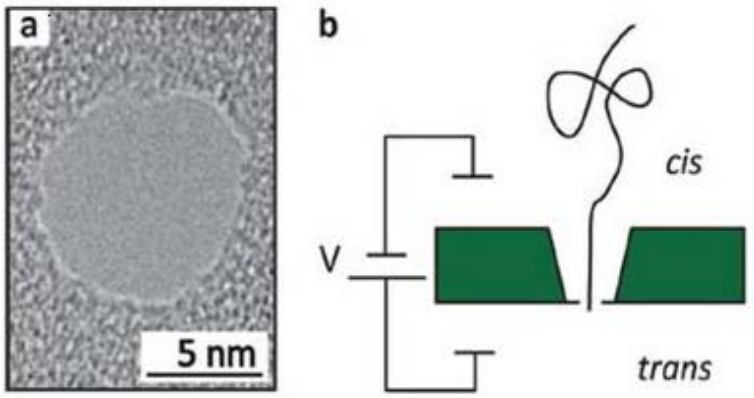

Figure 8. (a) A transmission electron micrograph of a single solid-state nanopore drilled into a silicon nitride membrane (b) Schematic diagram of the experimental setup used in the translocation of a single nucleic acid strand. Reproduced with permission from 59.

metal ions, organic compounds and in particular DNA using resistive-pulse sensing (Figure 7). The different strands of DNA can be identified using the signals produced as the DNA strands translocate through the nanopores. Recently, they have also attempted to sequence DNA using the nanopores (56). However, for most biological pore applications, these proteins must be reconstituted into lipid bilayer membrane. The stability of the lipid bilayer is known to be strongly affected by pH and concentration of the solutions (57) which somehow limits biological pores as a versatile diagnostic tool.

On the other hand, solid-state pores with adjustable lengths and diameters can be readily manipulated using appropriate pore formation conditions are highly attractive. In addition, these solid-state pores can be functionalized with different surface groups and exhibit significantly higher thermal, chemical and mechanical stabilities compared to biological pores (57) . These solid-state pores can be made of polymer, silicon, alumina, metallic or glass materials, using various top-down methods such as ion-beam sculpting, micromolding, latent track etching, and electron beam induced fine tuning (58). The applications of solid-state nanopores are similar to biological pores since they are fabricated largely to mimic the biological pores. The translocation and differentiation of DNA/RNA strands have been investigated using nanopores drilled into a silicon nitride membrane by Dekker *et al.* (57, 59) (Figure 8). Martin *et al.* (60) uses gold and alumina nanotubule membrane to detect analytes through a resistive-pulse sensing and biomimetic ion-gated channels mechanism. Recently, the

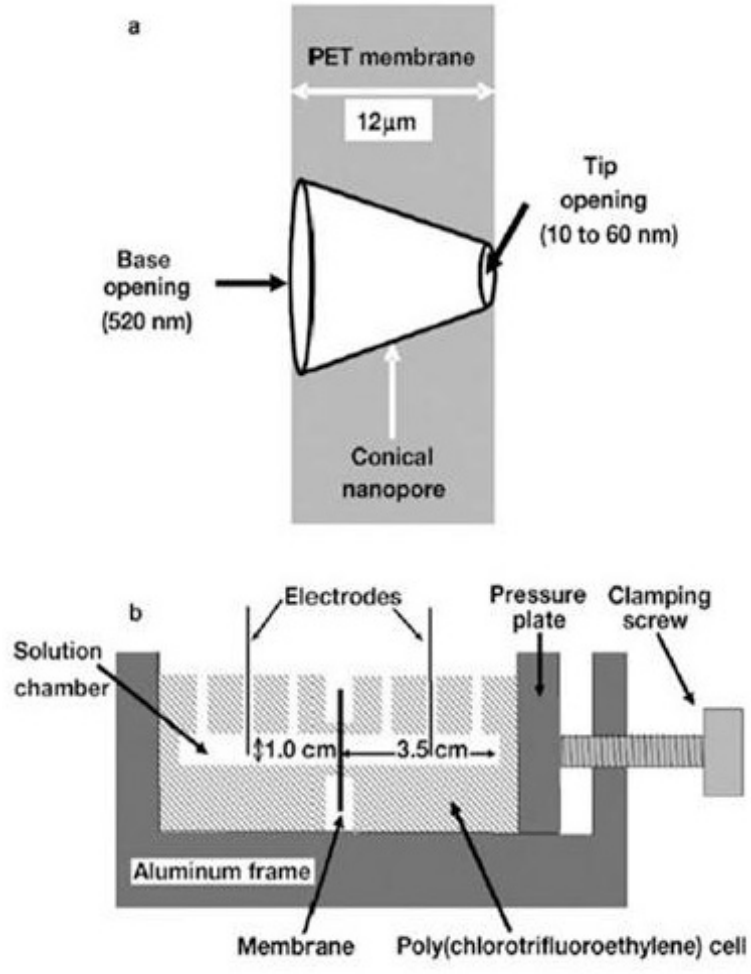

Figure 9. (a) Schematic diagram of a conical nanopore which mimics functions of biological nanopores formed by track-etch method in a polyethylene terephthalate (PET) membrane (b) Schematic diagram of the experimental setup used for electrochemical measurement of ion currents traversing across a single conical nanopore within a PET membrane. Reproduced with permission from 61.

group has also developed conically-shaped nanopores and nanotubes that can mimic functions of biological nanopores (61) (Figure 9). Sensing work can be carried out with these types of conical nanopore using molecular recognition elements that are bound to the nanopore tip. The tip is selectively blocked as the target analyte binds to the recognition element leading to a decrease in the ion current (62)

Other recent interesting works using solid-state pores include electrostatic gated transport using modified glass electrode (63), separation of analytes with muti-layer alumina membranes (64) and transportation of drugs through polypropylene membrane (65). Alternatively, solid state nanopores can also be fabricated using bottom-up methods such as sol gel process (66), template synthesis (67) and anodization (68). Previously, our group has synthesized an alumina membrane biosensor based on the anodization method and the nanobiosensor is sensitive toward whole viral particles for the detection of West Nile virus (69) IgM antibody probe molecules were physically absorbed onto the walls of the membrane nanochannels within the nanoporous alumina membrane. Subsequently, bovine serum albumin was added to block the non-specific binding site (Figure 10).

The sensing of whole viral particle was based on monitoring of the electrode's Faradic current response toward added ferrocenemethanol which is sensitive toward the formation of immune-complexes within the membrane nanochannels. The increase in the extent of the blockages of the nanopores caused a decrease in the mass transport rate of the ferrocenemethanol to the underlying sensing electrode, leading to a decrease in the signal. Applying this method, an extremely low detection limit of 2 pg ml^{-1} was obtained, which is comparable to PCR methods (69). However, it remains unclear whether the confined space within the nanopores will affect the binding affinity or the reactivity of the antibodies with the target antigens, which requires further confirmation. Nevertheless, this method

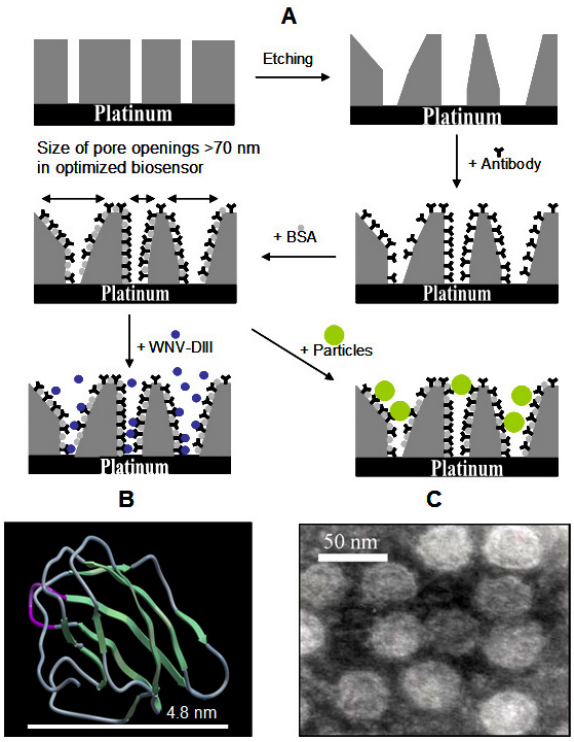

Figure 10. (a) Schematic diagram for the preparation of the membrane-based nanobiosensor for the detection of a flavivirus. (b) Homology model of the West Nile virus domain III protein. (c) Transmission electron microscope of the West Nile virus. Reproduced with permission from 69.

has shown potential to detect viruses directly without the need of analyzing paired serum samples and is thus a viable approach to achieve potentially rapid, ultra-sensitive detection of the dengue virus.

5. FUTURE OUTLOOK

While current methods used for diagnosis of dengue infection have relied on antibodies, cell cultures and nucleic acid-based PCR techniques, the advanced detection methods for dengue infection relying on nanoscale sensitivity instrument-based biosensor design have integrated some of these biological approaches and shown the potential to overcome certain limitations found in the current methods. For example, the EIS recently showed preliminary capability to differentiate dengue fever from dengue hemorrhagic fever which is difficult to achieve using the current choice methods, ELISA and RT-PCR. Interestingly, the highly versatile nanoscale material-based biosensor designs have shifted the focus to the construction of probes comprising functional biorecognition elements coupled with nanosized transducers with significantly less dependence on instrument. Overall, both nanoscale sensitivity instrument-based and nanoscale material-based designs can achieve low-cost, rapid detection time, small sample size and high sensitivity. Thus, it is foreseeable that more of these advanced methods may be developed for use at point-of-care, in particular during epidemic events, in the near future.

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Key Words: Dengue, Virus, Biosensor, Nanobiosensor, Diagnostic Tools, Review

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