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A novel method for dengue virus detection and antibody screening using a graphene-polymer based electrochemical biosensor

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Abstract

Dengue fever is a major disease that kills many people in the developing world every year. During early infection, a patient displays a high temperature without other signs. After this stage, and without proper treatment, serious damage to internal organs can happen, which occasionally leads to death. A rapid technique for the early detection of dengue virus (DENV) could reduce the number of fatalities. This study presents a new technique for the detection, classification and antibody screening of DENV based on electrochemical impedance spectroscopy (EIS). We found that the charge transfer resistance (R_{ct}) of a gold electrode coated with graphene oxide reinforced polymer was influenced by virus type and quantity exposed on the surface. Molecular recognition capability established during the GO-polymer composite preparation was used to explain this observation. The linear dependence of R_{ct} versus virus concentrations ranged from 1 to 2×10^3 pfu/mL DENV with a 0.12 pfu/mL detection limit.

Key words: Polymer matrix composite; Graphene; Dengue; Virus detection; Electrochemical impedance spectroscopy; Biosensor

Dengue hemorrhagic fever infects over 100 million people annually with a death rate of 2.5%.[1,2] The disease is caused by the dengue virus (DENV) and there are no approved drugs available. Dengue viruses are classified into four serotypes i.e. DENV-1, DENV-2, DENV-3, and DENV-4.[3] Normally, a patient infected for the first time will have immunity against the virus. However, subsequent infection by other serotypes often results in more serious symptoms.[4] Early detection of DENV infection means proper treatment will be provided which can reduce fatalities.[5] This means that the appearance of dengue fever symptoms, after three days of infection, is the primary means of diagnosis.[6] Although antibody testing could be the method of choice in screening, this approach is both expensive and requires extensive laboratory facilities.[7]

There have been a number of recent developments in biosensors for quantitative and qualitative detection of biomolecules including proteins and viruses based on polymer matrix composites (PMCs), unitizing different transducers, such as electrochemical,[8] optical,[9] electrical,[10] and physical (mass-sensitive) ones.[11] Some researchers use polymer matrix composites (PMCs) with nanostructured semi- or conducting materials, such as carbon nanotubes (CNTs),[12] graphene (G),[13] or metal nanoparticles[14] to functionalize biomolecule-assisted assembly of bridging nanoscale electrical circuits.[15,31] For example, the self-assembly induced electronic memory effect can happen by incorporating platinum nanoparticles (Pt) into tobacco mosaic virus (TMV). This TMV-Pt particle can be used in nanoelectronics when incorporated into a hybrid bio-inorganic composite layer based on a polyvinylalcohol (PVA) matrix.[16] Implanting poly(3,4-ethylenedioxythiophene) conducting nanowires into M13 virus particles makes the virus selectively recognize prostate-specific membrane antigen (PSMA).[17] In addition, an immunosensor based on poly(allylamine)-CNTs composites for NS1 protein of DENV has been reported.[18]

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Another example of a PMC application is the detection of inflammatory cytokine, interleukin-22 (IL-22) using anti-IL22 immobilized on poly(dimethyldiallylammonium chloride)-functionalized graphene/gold nanoparticles nanocomposite.[19]

Graphene oxide (GO) is a semiconducting material that can be incorporated into polymers to enhance the performance of biosensors for clinical diagnostics in terms of sensitivity and response time.[20] GO can be rapidly functionalized in water soluble polymers when it combines to form a polymeric material. GO filler exhibits high electrical conductivity,[21–25] high modulus,[26–28] and can be functionalized both covalently and non-covalently by coupling oxygen functionalities of GO with a reactive moiety present in a polymer.[26] Furthermore, GO-polymer composites can be simply furnished for the immobilization of enzymes, proteins, antibodies or viruses and fabricated into assorted platforms. These attributes make GO based PMCs attractive in designing and creating new medical biosensors.[29,30] For example, a glucose biosensor fabricated using polyethylene glycol (PEG) functionalized on the GO surface can immobilize enzymes for the detection of glucose by cyclic voltammetry (CV) with high sensitivity.[31]

In this work, we propose electrochemical impedance spectroscopy (EIS) as an alternative method for whole DENV detection and antibody assaying through protein recognition via a self-assembly process to PMCs. Direct DENV detection via the dengue surface protein recognition and antibody assay could provide a means to develop a more robust biosensor with high affinity and selectivity, and the first approach to detect entire dengue virions.[32]

Methods

Synthesis of graphene oxide (GO)

GO was synthesized by Hummers' method.[33] In the first step, graphite flakes (mesh size 300) were added to 90% sulfuric acid and 10% phosphoric acid and stirred. Then potassium permanganate was slowly added. The mixture was transferred to 150 mL of a 30:1 water and hydrogen peroxide mixture to obtain bright yellow color. The product was filtered (Nylon Membrane, 0.2 μm, GNWP, Merck Millipore Ltd.) and washed several times with water before centrifugation (KUBOTA3700, Bara Scientific Co., Ltd.) at 5000 rpm for 30 min to obtain GO.

Dengue virus (DENV) preparation and production of 4G2 monoclonal antibody

All the experiments described in this section were conducted in a BSL-2 laboratory with biosafety practice and procedures[34] at the Siriraj Center of Excellence in Biomedical Research (SiMR), Faculty of Medicine Siriraj Hospital, Mahidol University. Four serotypes of DENV including DENV-1 (strain Hawaii), DENV-2 (strain 16,681), DENV-3 (strain H87), and DENV-4 (strain H241) were propagated in mosquito cells, C6/36. Cells were cultured in L-15 media with 10% tryptose phosphate broth, 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 25 °C with 5% CO_2 atmosphere. On the day of infection, DENV was prepared in L-15 containing 1% FBS and added into the cells at multiplicity of infection (MOI) of 0.1. After 2 hours of incubation at 25 °C with 5% CO_2 atmosphere, the cells were washed gently and replaced with fresh media.

The culture was further incubated for 5 days before collecting the virus in culture supernatant. To remove cell debris, the culture supernatant was centrifuged at 1930 × g for 5 min at 4 °C. The supernatant containing DENV was gently transferred to fresh tubes and kept at −70 °C until the experiments were performed. To measure the amount of DENV in culture supernatant, plaque-forming unit (PFU) staining assay was carried out. Briefly, the Vero cells (monkey kidney cell line) were plated the day before the assay in minimum essential medium (MEM) with 10% (v/v) FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C with 5% CO_2 atmosphere. The culture supernatant containing DENV was prepared for serial dilution before adding to the monolayer of Vero cells. The cells were incubated for 2 hours at 37 °C with 5% CO_2 to allow absorption of DENV into the cells and then washed twice with fresh medium before overlaying the infected cell with medium containing 2% carboxymethylcelllulose (w/v). The plaque, a small circular zone in the monolayer, caused by DENV infection was visualized after 7-day incubation by staining the cell monolayer with 1% crystal violet in 20% ethanol.

4G2 hybridoma cells (ATCC: HB-112) were cultured in Hybri-Care medium (ATCC: 46-X) containing 10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. The sub-culture was carried out four times a week by centrifugation of the cells at 1500 rpm, 5 min, 22 °C, collection of supernatant and replacement with fresh medium. The culture supernatant containing 4G2 monoclonal antibody was collected to a new tube. The cell debris was removed by centrifugation 2500 rpm, 15 min, 4 °C. The antibody was stored at 4 °C until the experiment was carried out.

Preparation of PMC thin film electrode

An EIS electrode was made from PMCs coated on a gold surface electrode (AuE). The PMCs have three components: copolymers, GO, and DENV. We selected the monomer types as used in our previous work including acrylamide, methacrylic acid, methylmethacrylate, and N-vinylpyrrolidone.[35]

The monomers were mixed at the ratio of 5.0 mg of acrylamide (AAm, purchased from Acros), 12.0 mg of methacrylic acid (MAA, purchased from Sigma-Aldrich), 12.0 mg of methylmethacrylate (MMA, purchased from Sigma-Aldrich), 6.7 mg of N-vinylpyrrolidone (NVP, purchased from Sigma-Aldrich) respectively with 47 mg of N,N-(1,2-dihydroxyethylene) bisacrylamide (DHEBA, Alfa Aesar) as the cross-linker. This mixture was dissolved in 300 mL of dimethylsulfoxide (DMSO, Merck) containing 1.5 mg of 2,2′-azobis (isobutyronitrile) (AIBN, Sigma-Aldrich) as initiator. Afterwards, this was followed by pre-polymerization at 70 °C, just prior to reaching the gel point in approximately 20 min. Then 0.15 mg/mL GO solution was added to the pre-polymer in a mole ratio of 3:2.

As can be seen in Figure 1, GO-polymer mixtures were then transferred to AuE sheet (1 × 1 cm), which was cleaned by dipping for 5 min into Piranha solution, followed by cleaning via the potassium hydroxide potential sweep method[36] and finally

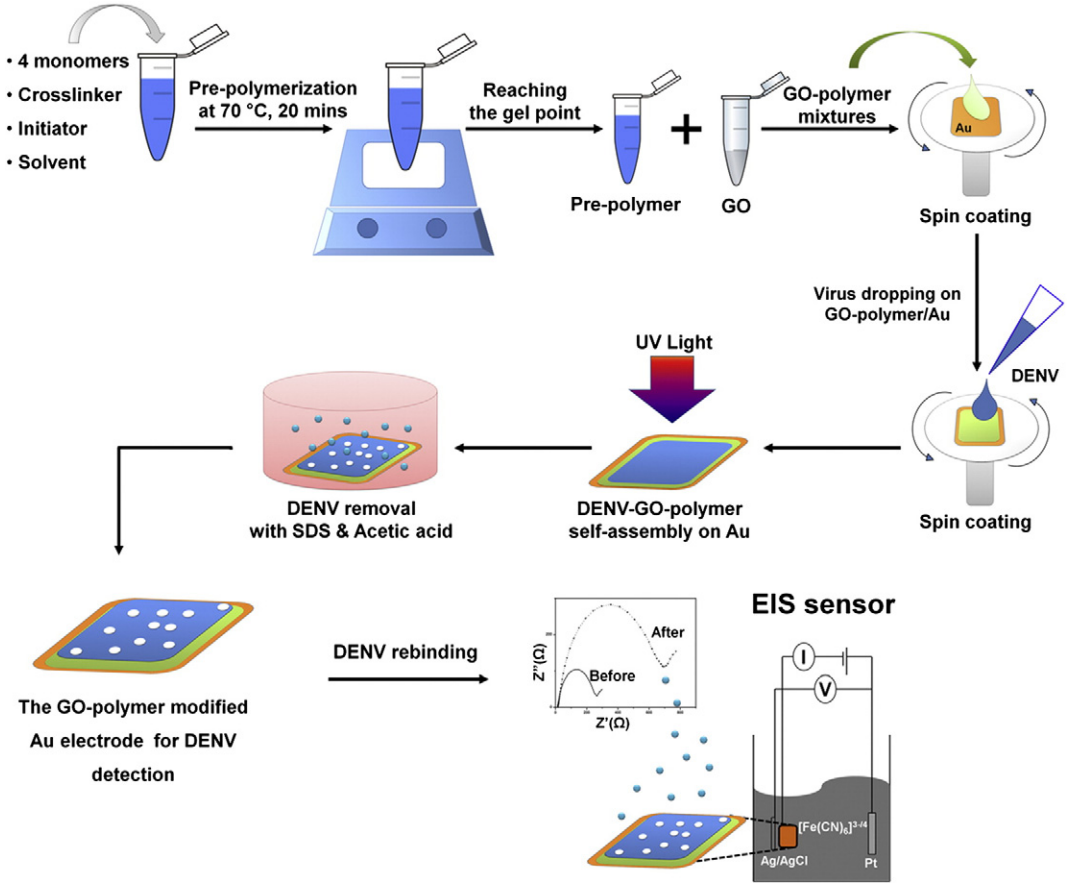

Figure 1. Schematic representation of the preparation of GO-polymer on gold electrode for DENV detection.

spin coating (AUTOLAB, UK) at 3000 rpm for 1 min. Subsequently, 5 μL of 1×10^3 pfu/mL DENV was added to allow for self-assembly of the GO-polymer on AuE followed by an additional spin at 3000 rpm for 10 seconds to result in a polymer layer of between 20 and 100 nm in thickness. Polymerization was achieved using ultraviolet (UV) light for 3 hours followed by heating overnight in an oven at 55 °C. Finally, the composite electrode was cleaned by rinsing with a 1:1 solution of 0.1% sodium dodecyl sulfate (SDS) and 10% acetic acid being left in water for 30 min to remove the polymer-bound virus particles to obtain GO-polymer/AuE electrode (Figure 1).

CV and EIS experiments

CV and EIS were performed by Autolab/PGSTAT302N using a 5 mM ferri-ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) redox indicator in 10 mM pH 7.40 ± 0.01 phosphate buffered saline (PBS) supporting electrolyte. A three-electrode electrochemical system was set up by connecting the GO-polymer/AuE as a working electrode, Ag/AgCl as a reference electrode, and platinum wire as a counter electrode. The CV potential was scanned from −0.45 V to +0.85 V at a 50 mV/s scan rate. The CV or impedance experiment started with adding 10 μL of analyte e.g. DENV, antibody etc. on the GO-polymer/AuE followed by 15 min drying before CV or EIS measurement.

The electrodes were regenerated by immersing them into a 1:1 solution of 0.1% SDS and 10% acetic acid for 5 min, then rinsing three times with distilled water. The data were monitored and analyzed using Autolab Nova software (Metrohm Autolab). The impedance spectra were recorded over a frequency range from 0.1 Hz to 10 kHz, using a sinusoidal excitation signal, superimposed on a DC potential of 0.2 V with 50 point impedance readings per decade with 10 mV amplitude. Impedance data were represented as Nyquist plots and fitted to an equivalent circuit by Autolab Nova software.

Measurement of antibody binding affinity

Binding affinity of the DENV serotypes with DENV 4G2 antibody were determined by slightly modifying the EIS measurement procedure as above. In the antibody binding assay, 10 μL of 1×10^3 pfu/mL of DENV was mixed with an equal volume of 4G2 antibody before incubated at 4 °C for 1 h. The mixture was added to the GO-polymer surface and incubating for 15 min at 25 °C before washing. After this they were ready to use. The experiment was carried out in series using 10 μL of DENV (fixed concentration at 1×10^3 pfu/mL) mixing with 10 μL 4G2 (1:1, 1:10, 1:100, 1:1000, 1:10,000 dilutions).

Atomic force microscope (AFM) and scanning electron microscope (SEM) pictures

AFM images were captured by an Asylum Research MFP-3D-BIO in contact mode using a silicon tip (Veeco

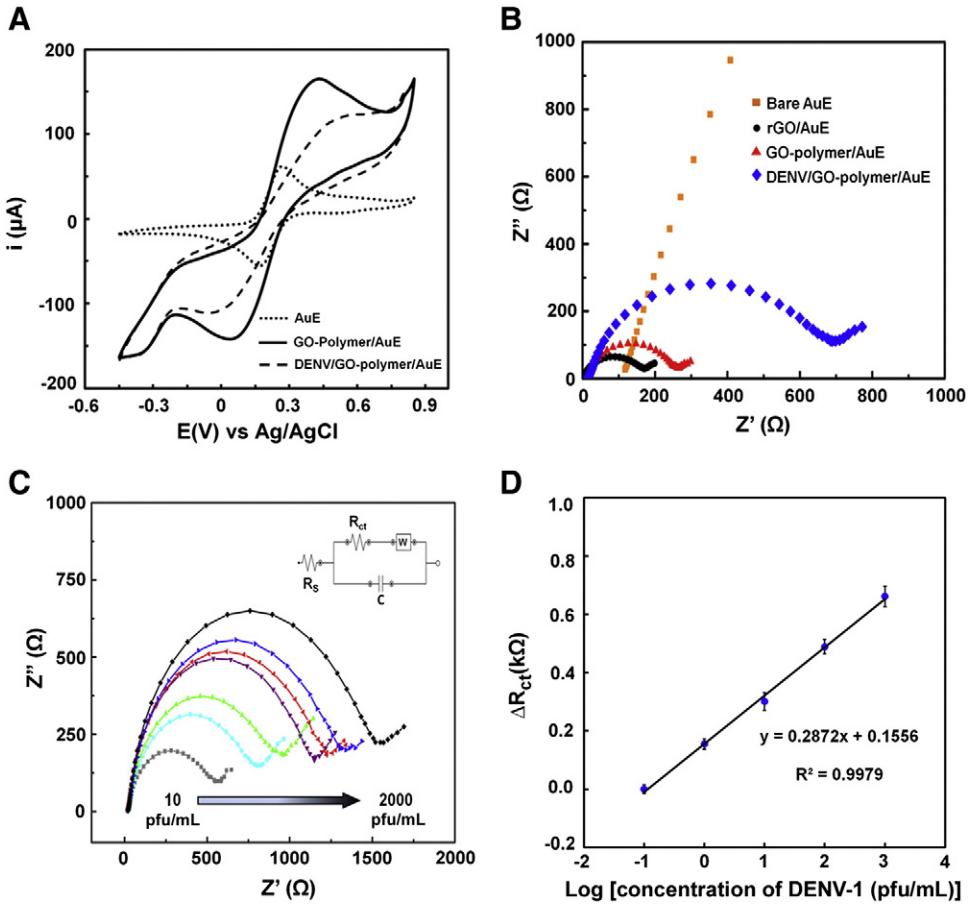

Figure 2. **(A)** CV spectra shows the effects of DENV on the charge transfer currents on GO-polymer/AuE. **(B)** EIS spectra show semicircular shape to confirm the GO-polymer composite coating on AuE (red color). Further absorption happened as DENV and made the semicircle wider as a result of increasing ΔR_{ct} (blue color). **(C)** EIS response of DENV/GO-polymer/AuE composite electrode as a function of DENV concentration with the simulated equivalent circuit. **(D)** A plot between the charge transfer resistance change (ΔR_{ct}) and log DENV concentration with error bars are 0.015, 0.018, 0.030, 0.025 and 0.035 at the concentration of 0.1, 1, 10, 1×10^2 and 1×10^3 pfu/mL DENV-1, respectively with the 95% CI (n = 5).

SNL-10) with a spring constant of 40 Nm and onset pressure corresponding to differential signal 1 V on a photodiode. Scanning electron microscopy was performed using a Quanta 450 FEI SEM at an operating voltage of 20.0 keV. All samples were coated with a thin layer of Au by sputter-coating prior to SEM examination (see Supplementary material).

Results

CV and EIS responses

The results obtained in this study show that we have succeeded in synthesizing a composite that is sensitive to DENV since its electrical conductivity shows a drop following DENV reabsorption (Figure 2, *A*). This can be achieved by the proper selection of the type and amount of ingredients used in generating the polymer prior to adding DENV (Figure 1). There are three ingredients used in fabricating the composite material, namely GO, polymers and DENV, each of which serves different purposes. In this work, GO makes copolymers electrically conductive (see also Figure 2, *A*). The negative charge on GO oxygen atoms also allows for functionalization of the GO surface to attract the DENV particles which have a net positive potential with zeta potential measurement in 10 mM PBS buffer at pH 7.40 (±0.01), 25 °C (Table 1) and the presence of positive charges on DENV surface.[37,38]

This allows the DENV particles to anchor onto the GO surface where self-assembly of the monomers around DENV can happen more effectively than when DENV is moving in solution. After virus removal the GO-polymers surface has areas that can recognize and selectively bind DENV which leads to an observable decrease in the conductivity. We can explain these phenomena the following way: When synthesizing the GO-polymer electrode, we add DENV into the GO and pre-polymers mixture and allow all these components to align themselves according to their interactions. Then DENV template is removed after GO-polymers are fixed by the cross-linking among the polymer chains. Some positions in GO-polymer networks optimized for the template virus that used to be occupied by DENV have left vacant. When the template DENV is reintroduced the position therefore can be reoccupied. However, for the other DENV viruses, even if they can bind to these sites the alignment and resistance contribution were not the same as that from the template DENV. It is generally believed that the virus particles bind preferably at the binding sites generated during

Table 1
Composition and characteristics of GO, copolymer, DENV and composites. Measurement of the average diameter and the average surface charge at the 95% CI, n = 5.

Materials	Average diameter (nm)	Average surface charge[a] (mV)	Morphology
GO	500 ± 250	−60.0 ± 1.1	Sheet
Copolymer	–	+25.8 ± 0.9	No pattern
GO + copolymer	–	−14.0 ± 0.6	–
GO + copolymer + DENV	–	+9.22 ± 0.4	–
DENV	68 ± 28	+42.0 ± 0.8	Spherical
GO − DENV	–	−1.2 ± 0.1	–

[a] The zeta potential value was determined with a Zetasizer Nano-ZS90.

Table 2
Linearity of DENV concentration on the GO-polymer composites for DENV detection with the 95% CI, n = 5 of LOD and LOQ.

	Linearity (n = 5)					
Serotypes	Linear working range (pfu/mL)	R^2	Slope	%RSD	LOD (pfu/mL)	LOQ (pfu/mL)
DENV-1	1 to 2 × 10^3	0.9979	0.2872	6.3	0.12	0.43
DENV-2	1 to 2 × 10^3	0.9894	0.2477	4.8	0.10	0.33
DENV-3	10 to 2 × 10^3	0.9878	0.2908	6.5	0.50	1.7
DENV-4	10 to 2 × 10^3	0.9935	0.2382	5.1	0.46	1.5

Abbreviations: %RSD, relative standard deviations percentage; LOD, limit of detection; LOQ, limit of quantitation.

electrode fabrication. This hypothesis is confirmed by AFM and SEM images showing that without the induced self-assembly pattern from DENV, the GO-polymer fails to consistently respond to the amount of virus present in solution (see Supplementary material Figures S2 and S4). In fact, it is possible that GO binds DENV non-selectively, which increase the binding efficiency of the sensor material. Actually, we were able to create a biosensor from GO alone to detect DENV, but the selectivity was too low to confirm presence of the virus in an unknown sample (see Supplementary material Figure S4). To some degree, the surface of GO could adsorb amino acids via electrostatic, hydrophobic, hydrogen bonding and π–π stacking interactions, such as arginine, histidine, lysine, tryptophan, tyrosine and phenylalanine.[39] However, these interactions cannot be optimized as the GO-polymer system explained earlier as molecular configurations of GO and dengue surface proteins are restricted.

The Nyquist plots shown in Figure 2, *B* and *C* provide visual insight into the system dynamics: they show that DENV added to the sensor will form a layer over GO-polymer surface at high frequency whereas at low frequency a diffusion based process is taking place. An equivalent circuit that fits to the obtained impedance scan is the Randles RC circuit type (shown in the inset in Figure 2, *C*). From the circuit corresponding to the Eq. (1),[40] R_{ct} is the charge-transfer resistance, which is inversely proportional to the rate of electron transfer; *C* is the double-layer capacitance; R_s is the solution phase resistance; and *w* is the Warburg impedance, which arises from mass-transfer limitations. For an analyte binding to a sensing layer on an Au electrode, the resistance R_{ct} is:

$$R_{ct} = R_{AuE} + R_{sens} + R_{anal} \quad (1)$$

where R_{AuE} is a Au electrode resistance, R_{sens} is a GO-polymer layer resistance, and R_{anal} is a DENV contribution. We use the same electrode for R_{ct} measurements at various amount of DENV loaded so the change in R_{ct} comes from R_{anal} contribution only as R_{AuE} and R_{sens} are fixed. In other words, ΔR_{ct} will depend on an amount of DENV added. By plotting ΔR_{ct} with log DENV concentration, linear relationships are obtained (Figure 2, *D*) which means the experiment can be used as DENV detection. The sensitivity of this method is 0.12 pfu/mL as calculated by the signal to noise (S/N) ratio = 3 at 95% confidence interval (CI) from the data used in Figure 2, *D* where linearity covers 1 to 2 × 10^3 pfu/mL range (Table 2).

It should be noted that measurement of R_{ct} requires charge transfer passing through the electrode surface. This requirement can be used to explain why we have to add GO as a component in our sensor. Without GO present on the Au electrode, CV scans did not reveal any significant observable signal indicating response of the electrode to the virus (see Figure 2, *A*). GO addition has increased polymer conductivity to the level that it can be noted when DENV is introduced.

DENV classification

The DENV serotype is classified based on the antigens on the virus surface. Their surface proteins are highly variable and thus constitute different serotypes. The samples used in our work accounted the four serotypes as classified by the PCR method[41] (see Supplementary note in Figures S5 and S6). These serotypes are similar in their geometry but have uniqueness in term of their antigenicity within the same serotype. According to our EIS measurements, we find that the signal response is highest after measuring the sample where DENV and the template serotype (DENV used to fabricate the electrode) are the same (see Figure 1). This implies that the DENV template used in EIS electrode preparation had produced specific antigen recognition sites. This tendency is consistent for all four serotypes of DENV as confirmed by the cross-selectivity results (Figure 3).

Sensitivity and selectivity of individual biosensors are shown in Figure 3 as ΔR_{ct} against virus concentration plots. To make it possible to compare both signal differences and selectivity, we report the results as relative effect i.e. the % ratio between ΔR_{ct} and $\Delta R_{ct,\ max}$ (example of obtained ratios was shown in Table S1). It can be seen from the ratios that individual composite biosensors gave the highest values only to the template DENV at fixed concentrations (Figure 4). The strongest signal from the virus used as the template allows us to use the experiment as a DENV subtype classification. Although other DENV serotypes, as the initially used one, can absorb on the surface of GO-polymer electrode, their contribution to resistance never reaches the same magnitude as the serotype used during synthesis as mentioned. In this manner, we can identify the virus serotype by comparing the responding ΔR_{ct} at several concentrations (Figure 3) which can be converted to relative effects R measurements (Figure 4).

Antibody assay

We demonstrate how our virus detection procedure can be modified to result in a DENV antibody assay. Figure 5, *A* shows

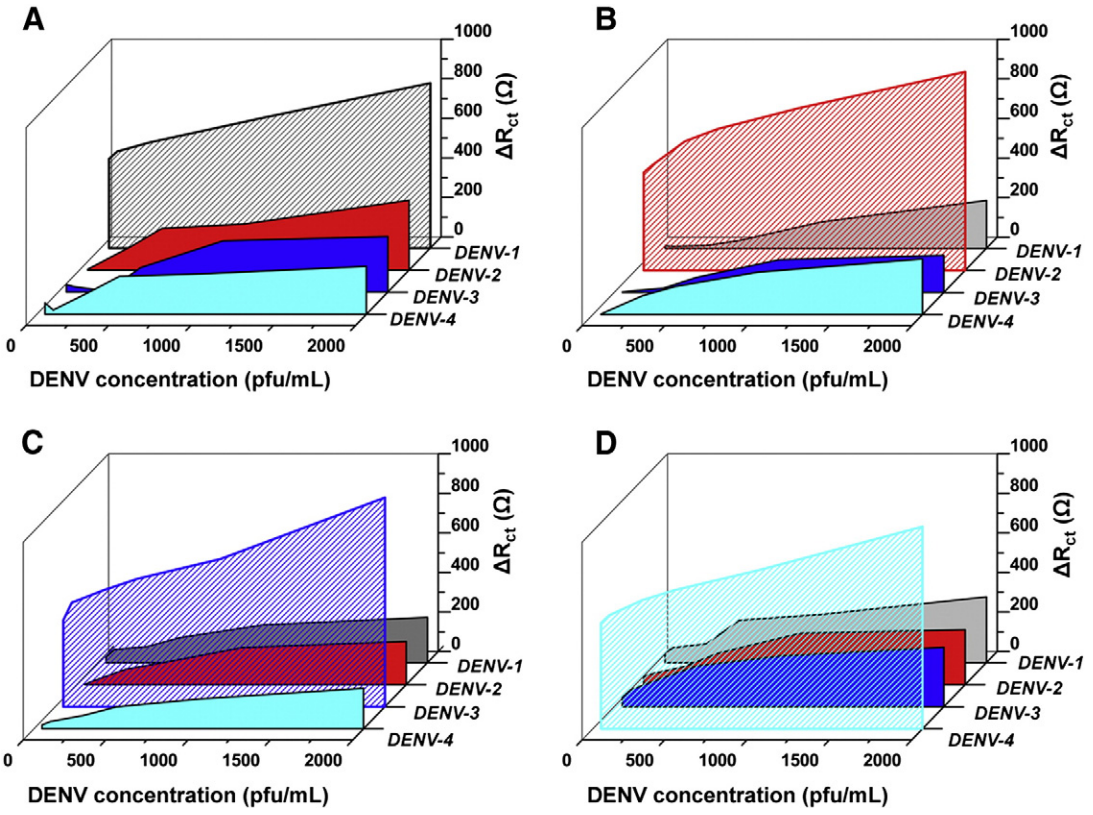

Figure 3. Three-dimensional histograms show the highest ΔR_{ct} signal response for the template dengue when used to detect all four dengue serotypes at the 95% CI (n = 5). The template dengue serotypes are **(A)** DENV-1, **(B)** DENV-2, **(C)** DENV-3, and **(D)** DENV-4.

that the ΔR_{ct} signal decreases when DENV was mixed with an antibody before loading onto the GO-polymer surface. Upon binding to DENV, the antibody will change the surface properties of DENV in terms of the shape, size and surface charge. As a result, we can observe signal changes under these conditions as compared to the signal where antibody is absent. In our case, it was found that adding 4G2 DENV antibody decreased the DENV signal where the highest effect can be seen in DENV-2. We have compared the result from this experiment with the result from enzyme-linked immunosorbent assay (ELISA) (see Figure S7) which indicates that 4G2 has the strongest binding affinity to DENV-2. In conclusion, the more the signal reduction, the higher the antibody binding affinity. As a result, we can detect antibody binding affinity to particular types of DENV. By plotting the relative response (%) $[(\Delta R_{ct,\ max}) - \Delta R_{ct})\ 100/\Delta R_{ct,\ max} - \Delta R_{ct,\ min}]$ versus log [4G2 dilution] we can see that the response (%) change covers a wide range of antibody dilutions (up to 3 orders of magnitude). The observed antibody concentration at 50% of response between individual minimum and maximum values or the term half maximal effective concentration (EC50) (see Figure 5, *B*) for DENV-1, DENV-2, DENV-3, DENV-4 is 0.018, 0.0071, 0.029, and 0.015 dilutions of 4G2 respectively. According to our experiment, the 4G2 affinity to DENV-2 is higher than those of DENV-1, DENV-3, and DENV-4 in which the affinity is approximately the same. The observed relative binding assay result was in a good agreement with ELISA results (see Supplementary material, Figure S7) and the data from the literature.[42]

Effects from other virus and electrode life-time

We have performed control experiments to investigate an effect of interference from the presence of DENV antibody and influenza A virus (Figure 6, *A*). DENV antibody selectively binds to this virus, which presumably affects binding to the electrode, while influenza A is an unrelated virus, and therefore represents an ideal negative control. In the first investigation, a DENV polyclonal antibody was mixed prior to sample loading on the electrode. It is found that the signal drop is proportional to antibody concentration (Figure 5, *A*), which obviously means that the antibody can bind to DENV, thereby changing its structure and thus selectivity for the electrode. However, when the influenza A sample is used no increase in the signal is observed. Figure 6, *B* shows that the reproducibility test of DENV-1 sensor at concentration of 10 pfu/mL DENV-1 can be used up to 20 times for 2 weeks in terms of precision, coefficient of variation (CV) is 0.049 (SD = 20.71, n = 30) and % recovery are 96% and 95% after virus rebinding 20 times for 2 weeks, respectively.

GO-polymer surface characteristics

Two experiments are used to confirm that virus addition into the GO-polymer mixture creates a distinct pattern on an Au surface which then makes the resulting electrode sensitive to DENV binding. The AFM images show that some dengue virus was embedded into the GO-polymer mixture, while some of

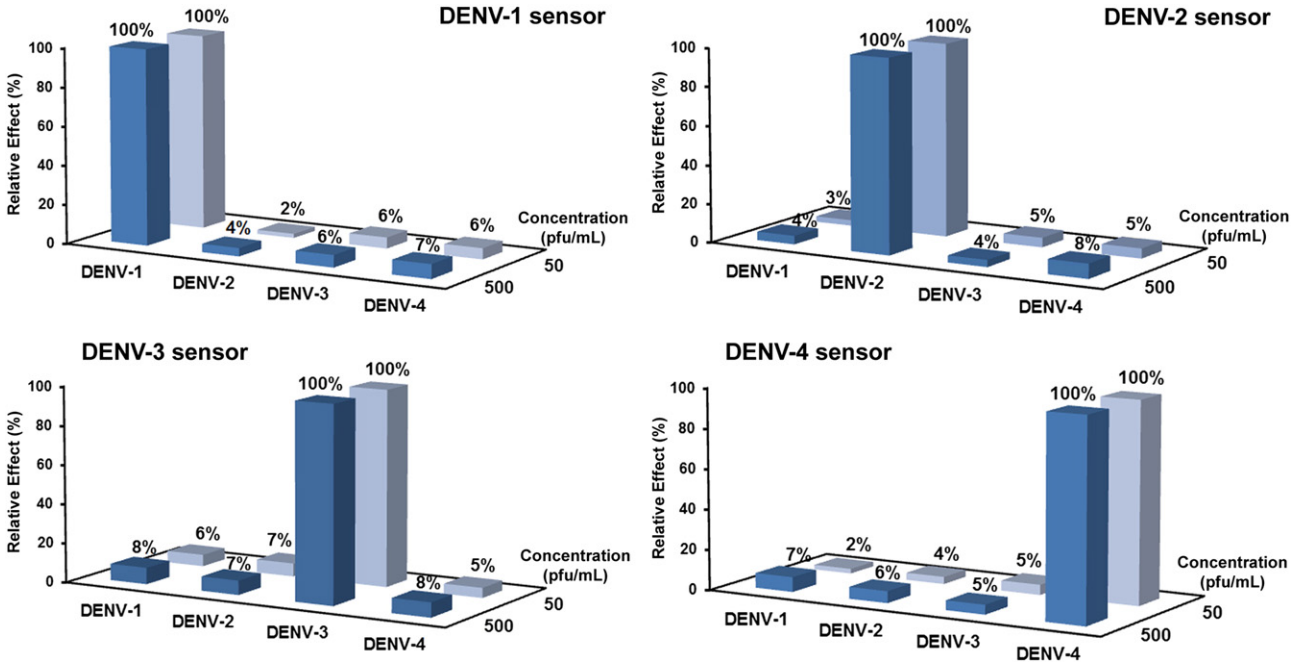

Figure 4. The selectivity of the biosensors can be visualized by comparing ΔR_{ct} and $\Delta R_{ct,\ max}$ at two DENV concentrations (from data used in Figure 3). The higher the ratio, the more response to the virus at this concentration. For example, relative effects (%) (see Figure 4, *A*) at 500 pfu/mL were 100%, 4%, 6%, and 7% for DENV-1, -2 -3, and -4 respectively.

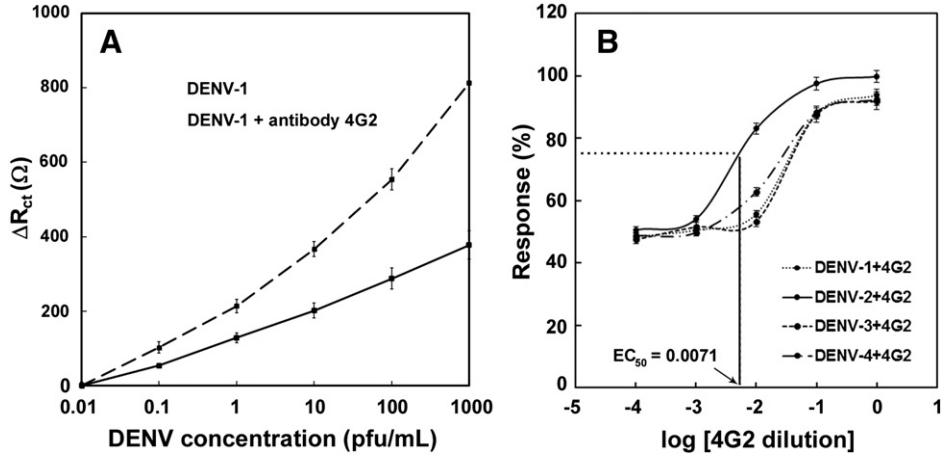

Figure 5. **(A)** Binding of 4G2 antibody on DENV-1 binding resulted in the lower value of the GO-polymer electrode resistance (95% CI, n = 3). **(B)** Binding assay results for four DENV serotypes with an example showing how the 50% binding of 4G2 to DENV-2 was calculated (95% CI, n = 3). The experiments in this part were carried out at 1×10^3 pfu/mL virus concentration.

them remain bound to the surface (Figure 7, *A* and *B*). We speculate that the virus has induced a self-assembly process of the copolymers similar to the surface imprinting since after virus removal a porous surface was observed in some areas (Figure 7, *C*).

Discussion

Whole DENV detection using an electrochemical technique has been reported[43] based on immobilized DENV antibody. This type of biosensor still requires a specific antibody as compared to conventional diagnostics and the sensitivity, selectivity and serotype classification are limited. A high sensitivity label-free method is more attractive. In recent years, the use of carbon nanostructure-polymer composites which can accommodate this requirement to detect small molecules or proteins has been reported[44] but the DENV and antibody detection from this type of sensors. We have adopted this approach by re-optimizing the polymer system and succeeded. In this work DENV serves as a component used to functionalize a GO-polymer surface by inducing a self-assembly process that makes the polymer surface more sensitive and selective to the virus. The use of GO as a dock for DENV means the surrounding monomers can assemble around the template virus to generate a surface by exploiting

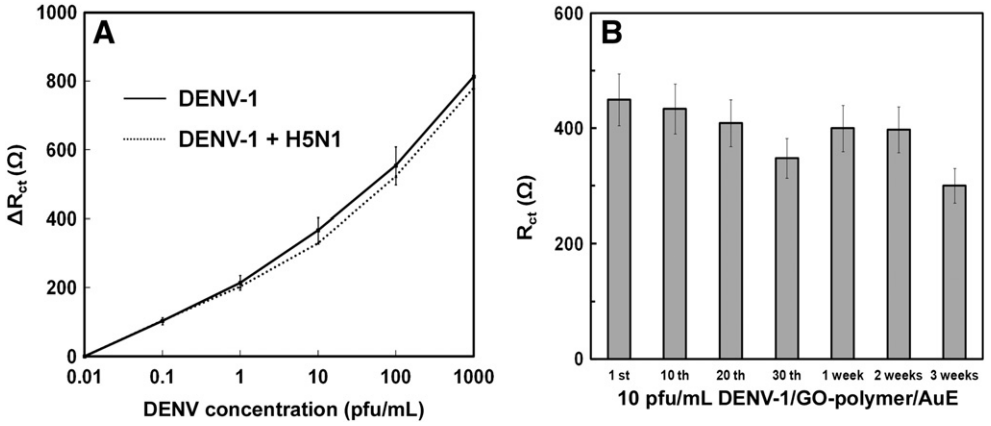

Figure 6. **(A)** The effects of influenza A H5N1 presented. The H5N1 had no effect at all as (.....) (95% CI, n = 3). **(B)** The first four columns show the reproducibility of the signal after the 1st, 10th, 20th and 30th measurements and the last three columns show the electrode stability during 1-3 weeks (electrode was kept in refrigerator at 4 ± 1 °C).

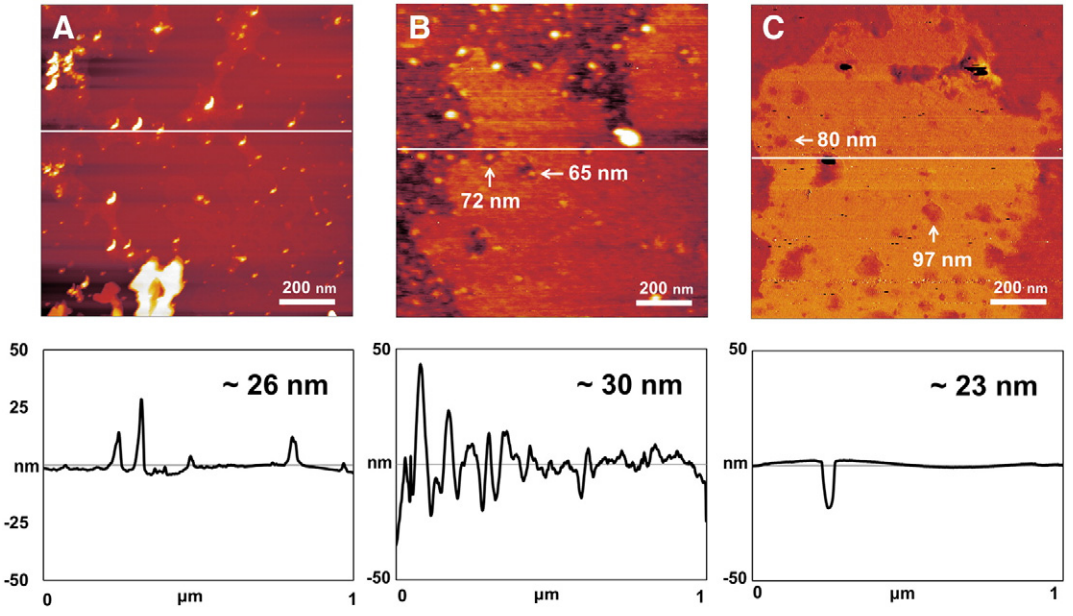

Figure 7. Two-dimension AFM images (1 × 1 μm) (top) and cross-section thicknesses (bottom) compare GO-polymer composite films at various conditions: **(A)** GO-polymer composite surface, **(B)** that of DENV particles about 50-100 nm in diameter as DENV-1 template self-assembly left over GO-polymer surface after polymerization, **(C)** after removing DENV-1 from the surface with recognition sites as virus particle size (the white-color lines are selected cross-section regions).

molecular recognition features unique to that virus. This surface can then be exposed by removing the original virus template.

A detector is constructed by applying the GO-polymer composite to an EIS electrode. The experimental protocol is relatively straightforward, with no need for expensive reagents or conditions yet it can detect the virus down to the limit of 0.12 pfu/mL ($P = 0.05$). At this detection limit, the method can confirm the presence of DENV at an early stage of infection. The method was able to discriminate between DENV and the other sub-types, as well as H5N1. Furthermore, our experiments also showed that when a DENV antibody is added to the corresponding DENV virus solution, it led to a decrease in resistance as comparing to the pure virus sample, indicating that the antibody inhibited virus binding to the GO-polymer. These results suggest that our method could be used to screen for dengue virus antibody and could be of general benefit in DENV vaccine research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.08.009.

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