

Serological Diagnosis of Dengue Infection in Blood Plasma Using Long-Range Surface Plasmon Waveguides

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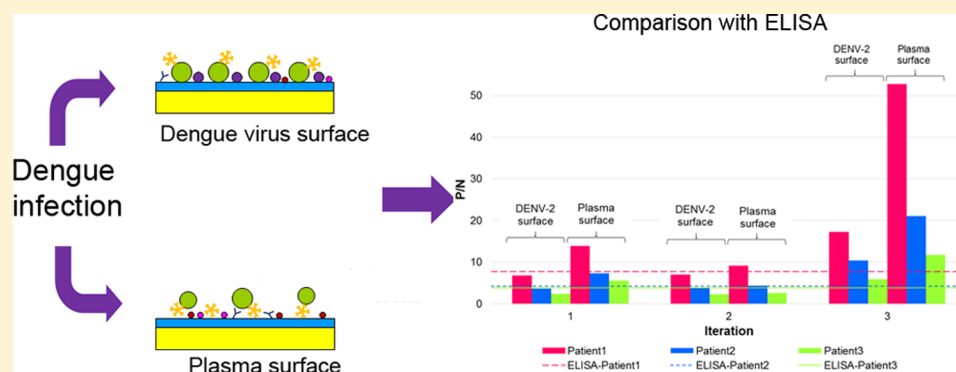
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S Supporting Information



ABSTRACT: We present a compact, cost-effective, label-free, real-time biosensor based on long-range surface plasmon polariton (LRSP) gold (Au) waveguides for the detection of dengue-specific immunoglobulin M (IgM) antibody, and we demonstrate detection in actual patient blood plasma samples. Two surface functionalization approaches are proposed and demonstrated: a dengue virus serotype 2 (DENV-2) functionalized surface to capture dengue-specific IgM antibody in blood plasma and the reverse, a blood plasma functionalized surface to capture DENV-2. The results obtained via these two surface functionalization approaches are comparable to, or of greater quality, than those collected by conventional IgM antibody capture enzyme linked immunosorbent assay (MAC-ELISA). Our second functionalization approach was found to minimize nonspecific binding, thus improving the sensitivity and accuracy of the test. We also demonstrate reuse of the biosensors by regenerating the sensing surface down to the virus (or antibody) level or down to the bare Au.

Dengue is a mosquito-borne disease, caused by one of the four serotypes of dengue virus, which can be found in over 100 tropical and subtropical countries around the world. Currently, there are over 3.6 billion people, or more than half of the world's population, at risk from dengue infection.¹ Every year it is estimated that there are 390 million dengue infections worldwide.² Unlike the more common and less dangerous dengue fever (DF), severe dengue hemorrhagic fever (DHF) has a case fatality rate in patients as high as 44%.³ Although there is no specific treatment for dengue infection, early diagnosis of the disease helps patients recover following fluid and electrolyte supportive therapy. Current laboratory methods for dengue diagnosis include direct virus detection via isolation of the virus in a cell culture or identification of viral nucleic acid or antigen and serological tests consisting of the detection of

virus-specific antibodies.^{4–6} Direct virus detection is not favorable for routine diagnosis of dengue infection because the detection procedures require expensive laboratory equipment and highly skilled lab technologists. Moreover, direct virus detection fails to be an ideal diagnostic test because live virus or viral components (RNA or antigen) can only be detected 0–7 days following the onset of symptoms;⁵ however, not all patients seek medical attention or diagnosis within this period. Serological tests are more commonly used in laboratory diagnosis of dengue infection because they are more easily and cheaply implemented. Patients infected by dengue virus will

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generate an immune response by producing immunoglobulins (IgM, IgG, and IgA) that are mainly specific to the virus envelope (E) protein. In particular, identification of dengue-specific immunoglobulin M (IgM) antibody is normally chosen as the preferred serological test because dengue-specific IgM is detectable earlier than dengue-specific IgG. Many immunoassay kits based on IgM antibody capture enzyme linked immunosorbent assay (MAC-ELISA) are commercially available,⁷ but they usually require long assay times and labor-intensive sample processing. Several rapid IgM-based dengue diagnostic tests have been developed for use at point of care, but they generally have lower sensitivity than the ELISA assay and so have limited clinical use.⁵

Taking into consideration these limitations, new methodologies and devices aimed at dengue diagnosis have been reported over the past decades.⁸ These biosensors can be classified based on the types of physical transduction mechanism exploited: piezoelectric (quartz crystal microbalance),^{9–13} optical (surface plasmon resonance,^{14,15} chemiluminescence,^{16,17} magnetoresistance,¹⁸ fluorescence,^{19–21} photonic crystals^{22,23}), and electrochemical.^{24–28} For successful diagnosis of dengue infection in tropical and developing countries, a biosensor should gather the advantages of simplicity, economy, rapidity, and accuracy.²⁹ These requirements have not been attained with any existing method, nor have any of the aforementioned studies demonstrated the detection of dengue infection in patient blood plasma.

Among all of the technologies, surface plasmon resonance (SPR) exhibits potential as a sensitive optical biosensor for real-time and label-free detection. Previous work reported detection of the dengue antibody using a commercial SPR instrument but not in a patient sample and using a complex sensing protocol involving dengue antigen-BSA conjugate.¹⁴ Although detection of antibodies have been demonstrated,³⁰ a broader literature search reveals that very little work has been done, in general, on the detection of antibodies in complex fluids such as patient blood samples using SPR techniques,^{31–33} and that additional preprocessing steps and features on the sensor (such as a reference channel) to minimize nonspecific binding are suggested.^{34–36}

In this paper we demonstrate a compact, cost-effective, label-free, real-time biosensor based on long-range surface plasmon polariton (LRSP) waveguides for the detection of dengue-specific IgM antibody in patient blood plasma. Two surface functionalization approaches are proposed: a dengue virus serotype 2 (DENV-2) functionalized surface to capture dengue-specific IgM antibody in blood plasma and the reverse, a blood plasma functionalized surface to capture DENV-2. The results obtained via these two surface functionalization approaches are comparable to, or of greater quality, than those collected by MAC-ELISA. Our second functionalization approach, which consists of immobilizing the patient's plasma on the surface, is straightforward, quickly carried out, and cost-effective and was found to minimize nonspecific binding when exposed to the virus solution, thus improving the sensitivity and accuracy of the test.

LRSPs are transverse magnetic (TM) polarized optical surface waves that propagate along a thin metal slab or stripe bounded by media of similar refractive index on all sides.³⁷ LRSPs can be easily excited by end-fire coupling (butt-coupling) an optical single-mode fiber to a thin and narrow metal stripe, which allows the miniaturization of LRSP biosensors. Although single-interface SPPs (as implemented

in commercial SPR instruments) are more surface sensitive, the greater propagation length of LRSPs produces greater overall sensitivity due to their much longer optical interaction length.³⁸ LRSPs on slabs have been used in sensing experiments in prism-coupled geometries.^{39–42}

The sensors used in this paper consist of straight gold (Au) stripe waveguides embedded in CYTOP (a fluoropolymer having a refractive index close to that of biologically compatible fluids) but exposed over a length in an etched microfluidic channel destined to confine the sensing fluid along the Au surface. Sensors were interrogated by coupling a polarization-maintaining (PM) optical fiber to the input, thereby launching light generated by a semiconductor laser diode into the sensor as propagating LRSPs. The optical output from the sensor was collimated and split into two parts: one was sent to an infrared camera for visual monitoring and the other to a power meter for real-time measurement of the output power. All experiments were carried out under a continuous flow rate of 20 $\mu\text{L}/\text{min}$, except for when the flow was stopped. Additional details on the sensor and experimental apparatus can be found in the Experimental Section and in the Supporting Information. Previously, we characterized such sensors by detecting changes in bulk refractive index, by detecting the adsorption of bovine serum albumin (BSA) on carboxyl- and polyethylene glycol-terminated surfaces and via the selective capture of human red blood cells.^{43,44} From previous work,⁴³ we demonstrated bulk sensing with a signal-to-noise ratio ($\Delta S/\sigma$) of ~ 1000 and a detection limit of 2.3×10^{-6} RIU. We also estimated the detection limit of our setup in terms of surface mass density to be $\Delta\Gamma \sim 12 \text{ pg}/\text{mm}^2$ (for $\Delta S/\sigma = 1$).

■ EXPERIMENTAL SECTION

Dengue Virus Serotypes 2 (DENV-2). Dengue virus serotype 2 (DENV-2) strain New Guinea C (NGC) was obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia. Briefly, virus was propagated in African green monkey kidney cells (VERO) in growth medium (20 mM hepes buffered RPMI 1640 with 10 mM nonessential amino acid, 2 mM glutamine, 10% fetal calf serum (FCS), 100 U/mL penicillin G sodium and 100 $\mu\text{g}/\text{mL}$ of streptomycin sulfate) by inoculating 1 mL of virus stock solution into a 50 cm^2 tissue culture flasks. The infected cells were then maintained at 37 $^\circ\text{C}$ in 5% CO_2 . The monolayer was harvested when 70% cytopathic effect (CPE) was seen. The cells were then trypsinized, pelleted by low speed centrifugation, washed with 0.01 M phosphate buffered saline (PBS), (Dulbecco "A" from Oxoid, U.K.) at pH 7.4 and aliquoted into 15 mL Falcon tubes at 5×10^7 cells per tube. The virus stock which has a viral concentration of 1×10^6 PFU/mL was stored at -20°C until use.

Plasma Samples. Dengue Positive Samples. Three patient plasma samples laboratory-confirmed as dengue positive were obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia (Ethical Clearance No. 782.90 from the University Malaya Medical Centre). The samples were collected and tested for dengue using a combination of hemeagglutination inhibition (HI), NS1 capture ELISA, IgG capture ELISA, and IgM capture ELISA prior to experimentation.⁴⁵

Dengue Negative Sample (Negative Control). A normal human plasma in K3 EDTA (lot no. PLE050412) was purchased from Zen-Bio, Inc. to serve as a negative control.

The donor is of U.S. origin, male, African American, and 49 years old.

Chemicals and Reagents. 16-Mercaptohexadecanoic acid (16-MHA), phosphate buffered saline (PBS) 0.01 M, pH 7.4, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), 2-isopropanol semiconductor grade (IPA), acetone HPLC grade $\geq 99.9\%$, sulfuric acid (H_2SO_4) 99.999%, hydrogen peroxide (H_2O_2) solution $\geq 30\%$, octane, and glycerol (electrophoresis grade) were obtained from Sigma-Aldrich. *N*-Hydroxysuccinimide sodium salt (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, $\geq 99\%$ (EDC) were purchased from Chem-Impex International, Inc. Distilled water was deionized using Millipore filtering membranes (Millipore, Milli-Q water system at $16\text{ M}\Omega\text{ cm}$).

In previous work,⁴³ we showed that the sensing device exhibits high sensitivity with a sensing buffer of $n = 1.3305$. This sensing buffer (PBS/Gly) was prepared by mixing standard PBS buffer with glycerol (7.235% w/w). DENV-2 and plasma samples were freshly diluted with PBS/Gly buffer at a 1:10 ratio prior to use. A $100\text{ }\mu\text{g/mL}$ BSA solution was prepared by mixing lyophilized BSA with PBS/Gly buffer. All the solutions except for the DENV-2 and plasma samples were filtered through Millex-GP filters (PES membrane $0.22\text{ }\mu\text{m}$).

Sensing Device and Experimental System. Sensors were fabricated by spin-coating CYTOP on a 4-in. silicon wafer as the bottom cladding. Au evaporation and lift-off were performed to define Au features using an optical lithography technique. Subsequently, a top cladding of CYTOP was coated. The top CYTOP cladding was etched down to the Au stripe surface to form a large fluidic channel of sensing length, $L = 1.65\text{ mm}$. A thick layer of photoresist was deposited onto the wafer before it was sent for dicing into ~ 300 dies per wafer. The detailed fabrication process of the sensors was described in previous work.^{46,47}

The sensor die ($3.8\text{ mm} \times 6.4\text{ mm}$) consists of 16 alternating straight Au waveguides and Mach–Zehnder interferometers (MZI), as shown in the microscope image as Figure 1. In this paper, only a single straight Au waveguide ($\sim 5\text{ }\mu\text{m}$ wide, 35 nm thick) located in the middle of a sensor die was used

throughout. Atomic force microscopy (XE-100 AFM, Park Systems) measurements were taken on a comparable sample to verify the dimensions and low roughness of the Au waveguides (see Figure S-1 in the Supporting Information). The experimental apparatus is shown in Figure S-2 in the Supporting Information, and its detailed description is provided in Supporting Information.

Device Preparation. To ensure the cleanliness of the waveguide facets for efficient optical input and output coupling, a fresh sensor die was cleaned by ultrasonication (FB-11201, Fisher Scientific) in octane for 5 min to remove any possible debris formed during dicing of the wafer. The sensor die was then left immersed in two sequential acetone baths for 5 and 30 min, respectively, to completely remove the dicing photoresist. After a thorough washing in IPA and drying with nitrogen gas (N_2), the sensor die was placed in a UV/ozone chamber (PSD-UV, Novascan) to remove any possible organic matter from the Au surface. The die was then washed intensely with distilled/deionized water (DDI H_2O) and IPA, followed by N_2 drying. A cleaned sensor die was incubated in a 1 mM IPA solution of 16-MHA overnight to allow self-assembled monolayer (SAM) formation. The SAM-modified sensor die was rinsed with IPA and dried with N_2 before being placed into the fluidic jig.

Upon completion of experimental runs for the same patient sample, the Au surface of the sensor die was fully regenerated by placing it in a UV/ozone chamber. After that, the sensor die was washed with plenty of DDI H_2O and IPA and dried with N_2 . The regenerated sensor die was again incubated in a 1 mM IPA solution of 16-MHA overnight to prepare the die for another experimental cycle.

RESULTS

Dengue Virus Serotype 2 (DENV-2) Functionalized Surface. Rapid dengue diagnosis requires single-step detection in clinical blood samples with a prefabricated sensor surface. We first present results relevant for this case, where dengue virus serotype 2 (DENV-2) was immobilized on the sensor surface to capture dengue-specific IgM antibody present in plasma samples. The functionalization process is illustrated in Figure 2A along with a sensing and regeneration run.

A sensor die was cleaned and functionalized with a 1 mM 2-isopropanol (IPA) solution of 16-mercaptohexadecanoic acid (16-MHA). The die was then placed into a fluidic jig with PBS/Gly buffer (a mixture of standard phosphate buffered saline (PBS) with glycerol 7.235% w/w) filling the etched channel. At the beginning of an experiment a baseline signal level was established while flowing PBS/Gly buffer for 10 min to ensure signal stability and low noise. The carboxyl group of 16-MHA was then activated *in situ* by injecting a mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in PBS/Gly buffer for 15 min. Next, the fluidic cell was flushed with PBS/Gly buffer to recover the baseline signal. DENV-2 diluted with PBS/Gly buffer in 1:10 ratio was then injected for 10 min to be covalently attached to the sensor surface. The drop in signal immediately after DENV-2 injection (Figure 2B) is caused by a bulk change in the refractive index, but the virus binding in real-time still can be observed over the time frame of 35–45 min. After the sensor was washed with PBS/Gly buffer to remove excess DENV-2, a new baseline power level corresponding to the formation of a DENV-2 adlayer on the surface was established (Figure 2B). Preliminary experiments with patient samples indicated that nonspecific binding can be

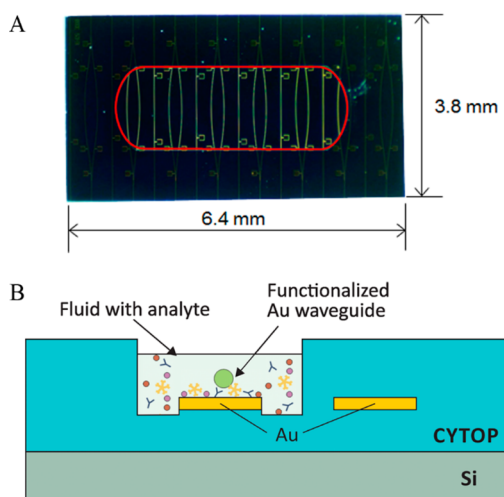


Figure 1. Long-range surface plasmon (LRSPC) based sensor: (A) Microscope image of a LRSPC based sensor. The yellow curves correspond to the Au waveguides. The area bounded in red is the etched fluidic channel. (B) Front cross-section of the device showing a functionalized waveguide and a CYTOP-embedded waveguide.

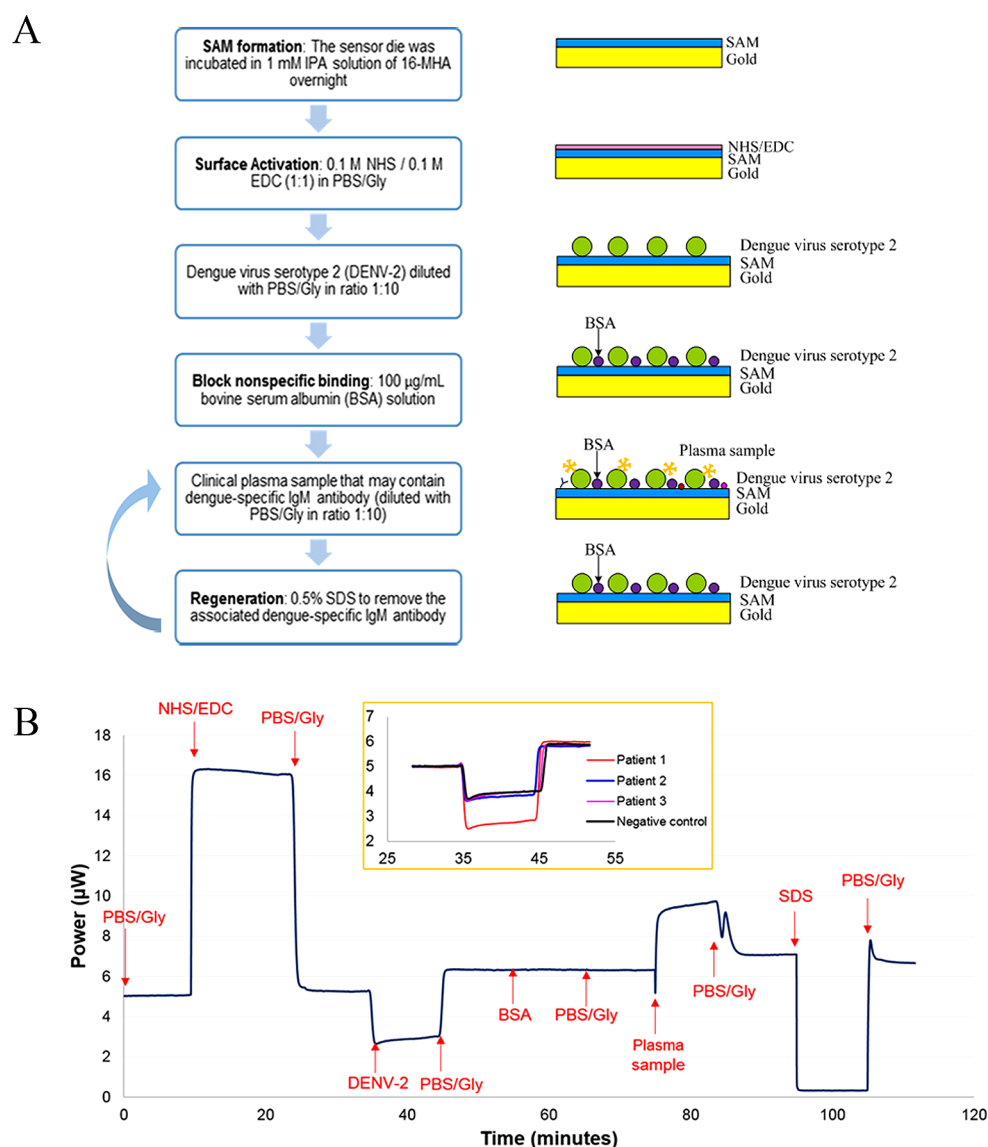


Figure 2. Surface functionalization of Au waveguide with dengue virus serotype 2 (DENV-2). (A) Schematic illustrating the functionalization of the sensor and a sensing run. When a plasma sample is injected over the DENV-2 surface, other proteins (represented by red and pink spheres) and other antibodies (represented as Y) in the complex fluid may nonspecifically adsorb onto the surface, in addition to the dengue-specific IgM (represented as pentamers) which adsorbs specifically. Regeneration of the surface at the virus level is also illustrated. (B) Real-time measurement showing the immobilization of DENV-2 onto the 16-MHA modified Au waveguide through a NHS/EDC reaction followed by the injection and capture of dengue-specific IgM antibodies. BSA was used as the nonspecific binding blocking agent before a plasma sample was injected. The surface was regenerated at the virus level with 0.5% SDS for repeated measurements. Inset: The zoom-in image shows the responses during DENV-2 attachment after fully regenerating a waveguide (down to the Au surface) between patient plasma samples.

significant for this functionalization approach and that it can affect the determination of results (data not shown). Thus, a 10-min flow of bovine serum albumin (BSA) solution was introduced to mitigate potential nonspecific binding; no measurable change in signal is observed in this case (Figure 2B) suggesting saturation of the surface with DENV-2. A clinical plasma sample diluted in a 1:10 ratio with PBS/Gly buffer was then injected and the flow was stopped after 5 min to allow reaction with DENV-2 on the surface. Stopping the flow reduces the consumption of plasma. The immunoreaction between immobilized DENV-2 and dengue-specific IgM antibody in the plasma sample was then followed by a PBS/Gly wash for 10 min to eliminate any loosely bound compounds. The same DENV-2 surface was reused to produce several measurements for the same patient sample (repeats).

This was accomplished by washing away the associated antibody through an injection of 0.5% sodium dodecyl sulfate (SDS). It has been shown previously^{48,49} that a sensor surface can be regenerated in this manner without affecting immunoreactivity. After the SDS flow, followed by a PBS/Gly wash, the signal level returned close to that prior to plasma injection, remaining slightly higher, indicating that some material remained bound to the surface (Figure 2B). Three binding/regeneration cycles were carried out in this manner for each patient plasma sample. The binding capacity of the DENV-2 surface was observed to drop slightly after every cycle, probably because some material remained bound after SDS injection; however, the sensor produced consistent results for all patients after three repeats for each.

Three positive samples with different concentrations of dengue-specific IgM antibody and normal plasma (negative control) were tested using the same Au waveguide on a sensor die. After a complete experimental cycle with one plasma sample (including repeats), the sensor surface was fully regenerated, down to the bare Au waveguide, by placing it in a UV/ozone chamber. The fully regenerated sensor die was then incubated in 16-MHA and another functionalization and experimental cycle was carried out with a different patient plasma sample. The inset to Figure 2B shows an expanded view of the responses observed during immobilization of DENV-2 onto the sensor surface after each full regeneration cycle. The power level measured before and after DENV-2 injection varies only very slightly from regeneration to regeneration, indicating that the sensor surface was not significantly degraded due to UV/ozone and that essentially the same virus coverage was achieved in all cases. This implies that the sequence in which the patients' plasma is investigated should be irrelevant. Nevertheless, we performed the first experimental run for each patient with the negative control.

Figure 3A summarizes the sensor responses of the first iteration carried out for each plasma sample (see Figure S-3 in

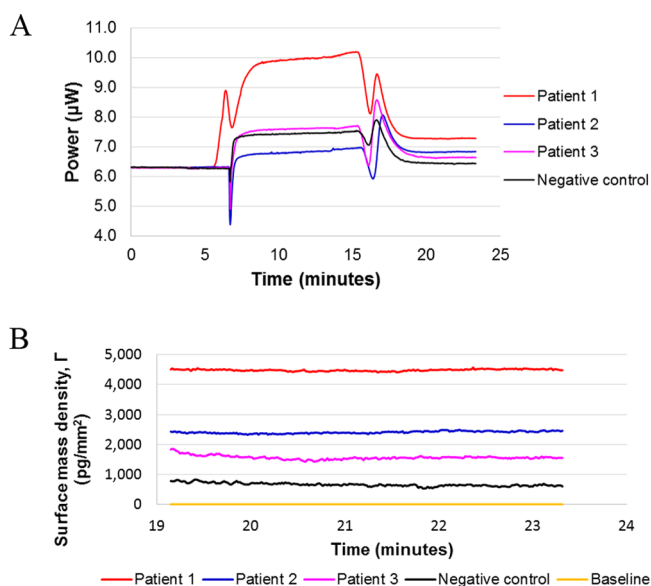


Figure 3. Dengue detection using a DENV-2 functionalized surface. (A) Real-time response data summarized for different plasma samples (patient 1, patient 2, patient 3, and negative control) injected over the DENV-2 surface. The difference in power measured before and after plasma injection is proportional to the mass of biomaterial adsorbed onto the sensor surface from the plasma sample. (B) Surface mass density adsorbed onto the sensor surface after plasma injection (the zero was set to the baseline level before injection).

the Supporting Information for consecutive iterations for each sample). During the injection of plasma samples, a spike in signal was observed due to the exchange of solution from PBS/Gly buffer to the more viscous plasma sample. The gradual increase in power after plasma injection indicates the real time binding of biomaterial to the DENV-2 surface. After 10 min, PBS/Gly buffer was injected to dissociate loosely bound biomaterial. The difference in power measured before and after plasma injection is proportional to the mass of biomaterial bound to the DENV-2 surface. The surface mass density of the

biomaterial on the sensor surface (in g/m²) is related to its equivalent optical parameters (n_a , P_{out}) through⁵⁰

$$\Gamma = \frac{(n_a - n_c)}{k \partial n / \partial c} \left(\frac{P_{out}(a)}{P_{out}(a_0)} - 1 \right) \quad (1)$$

where $n_c = 1.3303$ is the index of the sensing fluid, $P_{out}(a_0)$ is the power measured before plasma injection, and $P_{out}(a)$ is the power measured after plasma injection. Using $n_a = 1.5$, $k = 0.0318 \text{ nm}^{-1}$ (the determination of k can be found in the Supporting Information) and $\partial n / \partial c = 0.185 \text{ mm}^3/\text{mg}$,⁵⁰ the surface mass density of bound biomaterial is computed and plotted against time in Figure 3B; the zero of surface mass density was set to the baseline level before the injection of plasma (if the power measured after plasma injection equals that before injection, then no additional biomaterial was bound to the surface).

We observe in Figure 3B the accumulation of mass for the case of the negative control (surface mass density of 663 pg/mm²). We attribute this accumulation to residual nonspecific binding (due to the rather complex composition of plasma), which could not be completely mitigated by the BSA injection in our protocol. The increase in surface mass density for the three patient samples, relative to the negative control, is attributed to the binding of dengue-specific IgM antibody to the DENV-2 surface.

Plasma Functionalized Surface. The observation of nonspecific binding in the case of the negative control observed in Figure 3B led us to our second immobilization approach, which is inspired by the standard protocol in dengue diagnosis using conventional MAC-ELISA, where the plasma sample is immobilized onto the surface and the dengue virus is injected thereon. Figure 4A illustrates the functionalization and experimental procedures applied to the same sensor die as used in the previous section (after full regeneration via UV/ozone and piranha (3:1 solution of concentrated sulfuric acid (H₂SO₄) and 30% hydrogen peroxide (H₂O₂) cleaning).

After establishing a stable baseline with PBS/Gly buffer, the carboxyl groups of the SAM on the Au waveguide were activated by a flow of NHS/EDC. The refractive index mismatch between PBS/Gly buffer and NHS/EDC in PBS/Gly resulted in a sudden rise in signal as observed in Figure 4B. The baseline signal level recovered after a PBS/Gly wash and plasma samples were injected into the flow cell for covalent attachment to the sensor surface. Next, DENV-2 was introduced over the surface for 10 min. If there exists any dengue-specific IgM antibody on the sensor surface, DENV-2 will be captured resulting in a gradual rise in signal. Finally, PBS/Gly buffer was injected to wash away any loosely bound material. To regenerate the plasma surface for repeated measurements, the bound DENV-2 was washed away via a 10-min flow of SDS. Once the baseline signal level recovered to that observed before virus injection, DENV-2 was again introduced. Three repeats were obtained in this manner for each surface functionalized with different plasma samples (three positive samples with different concentrations of dengue-specific IgM antibody and a negative control). The experiments were performed in a similar sequence as in the previous section, starting with the negative control sample, to ensure consistency in the measurement process.

Figure 5A summarizes the sensor responses of the first iteration of DENV-2 injection on each plasma-functionalized sample (see Figure S-4 in the Supporting Information for

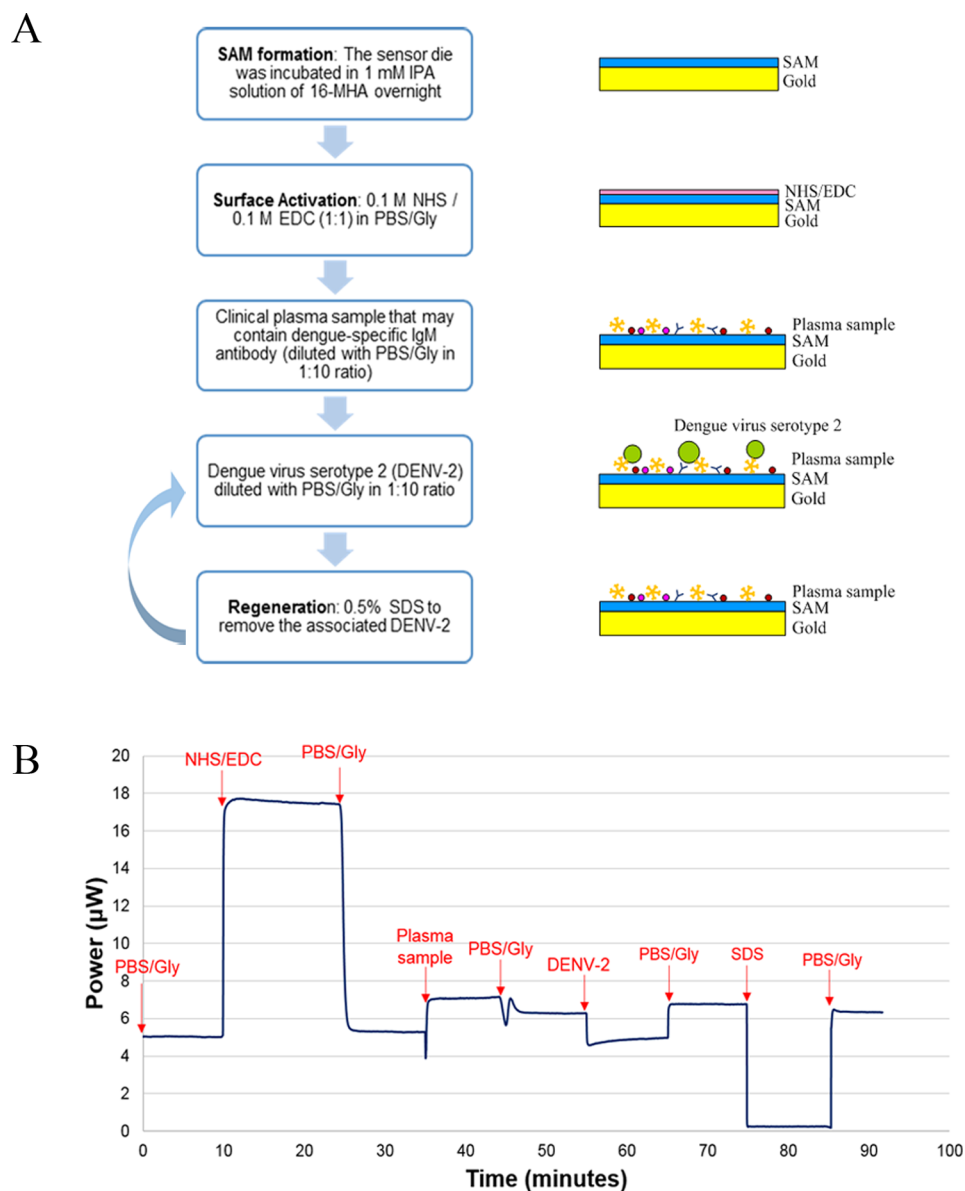


Figure 4. Surface functionalization of Au waveguide with a plasma sample. (A) Schematic illustrating the functionalization of the sensor and a sensing run. When a plasma sample is injected over the NHS/EDC-activated surface, other proteins (represented by red and pink spheres) and other antibodies (represented as Y) in the complex fluid may attach covalently to the surface in addition to the dengue-specific IgM (represented as pentamers). Regeneration of the surface at the plasma level is also illustrated. (B) Real-time measurement showing the immobilization of a plasma sample onto the 16-MHA modified Au waveguide through a NHS/EDC reaction, followed by the injection and capture of DENV-2 by dengue-specific IgM antibody on the surface. The surface was regenerated at the plasma level with 0.5% SDS for repeated measurements.

consecutive iterations for each sample). Generally, the solution of DENV-2 has a lower refractive index than PBS/Gly buffer which leads to a drop in power level during the solution exchange. As DENV-2 was captured by the dengue-specific IgM antibody on the sensor surface, the signal increased gradually. A new power level was established when PBS/Gly buffer was passed through the flow cell to wash away loosely bound material. The difference in power measured before and after DENV-2 injection is proportional to the mass of DENV-2 bound to the surface, which in turn is correlated to the actual amount of dengue-specific IgM antibody immobilized on the surface. Using eq 1, the surface mass density of the DENV-2 bound to the plasma surface was computed and plotted over time as Figure 5B. The surface mass density of the negative control in this case (Figure 5B) is about 3 times lower than in

the previous case (Figure 3B) indicating less nonspecific binding. The surface mass density on the positive plasma samples is also comparatively lower but not by a factor of 3, implying higher accuracy for this sensing approach.

DISCUSSION

In order to evaluate the performance of our approaches, we compare our results with those obtained by MAC-ELISA.⁵¹ In an ELISA assay, a positive-to-negative (P/N) ratio in terms of the optical density (OD) values obtained for a patient and a negative sample is generated. The OD (or absorbance) of a sample is a logarithmic ratio of the intensity of light that has passed through the sample to the intensity of the light incident thereon. According to Beer's law, the OD of a sample is proportional to its concentration in solution. A result with a P/

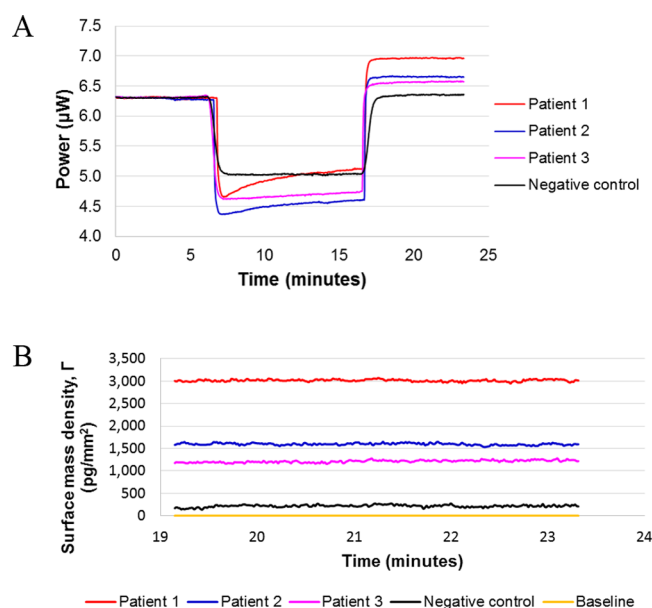


Figure 5. Dengue detection using a plasma functionalized surface. (A) Real-time response data summarized for different plasma samples (patient 1, patient 2, patient 3, and negative control) on the surface, during injection of DENV-2. The difference in power measured before and after DENV-2 injection is proportional to the mass of DENV-2 adsorbed onto the sensor surface, which is correlated to the amount of dengue-specific IgM antibody immobilized thereon. (B) Surface mass density adsorbed onto the sensor surface after DENV-2 injection (the zero was set to the baseline level before injection).

N ratio greater than or equal to 2.0 is considered positive. The same figure of merit was applied to our experimental results. The time-averaged surface mass density of a positive sample was divided by the time-averaged value of the negative control to obtain a P/N ratio:

$$P/N = \frac{\langle \Gamma_p \rangle}{\langle \Gamma_n \rangle} \quad (2)$$

where $\langle \Gamma_p \rangle$ is the time-averaged surface mass density of the positive sample and $\langle \Gamma_n \rangle$ is the time-averaged surface mass density of the negative control. The P/N ratios of the results using MAC-ELISA and our sensing approaches are summarized in Table 1. In general, higher surface mass densities are obtained on the DENV-2 functionalized surface than on a plasma functionalized surface. The plasma functionalized surface is again shown to reduce the nonspecific binding, probably due to the much less complex composition of the DENV-2 solution. Assuming independent and random Gaussian noises for the measurements, the error on the P/N ratio $\sigma_{P/N}$ can be computed as⁵²

$$\sigma_{P/N} = \frac{\langle \Gamma_p \rangle}{\langle \Gamma_n \rangle} \cdot \sqrt{\left(\frac{\sigma_p}{\langle \Gamma_p \rangle} \right)^2 + \left(\frac{\sigma_n}{\langle \Gamma_n \rangle} \right)^2} \quad (3)$$

where $\langle \Gamma_p \rangle$ is the time-averaged surface mass density of the positive sample, $\langle \Gamma_n \rangle$ is the time-averaged surface mass density of the negative control, σ_p is the error in surface mass density of the positive sample, and σ_n is the error in surface mass density of the negative sample. These errors were added to the data in Table 1.

The P/N ratios versus iteration number are summarized in Figure 6 for all patients, as measured using both surface

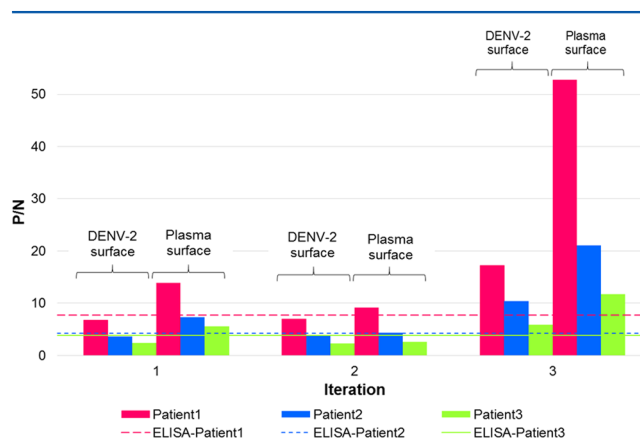


Figure 6. P/N versus iteration number for all patients, measured using MAC-ELISA, DENV-2 functionalized surface, and plasma functionalized surface.

functionalization approaches, and the results obtained using MAC-ELISA are indicated as the horizontal lines, for reference. In general, the P/N ratios obtained using our approaches are comparable to or greater than those obtained from ELISA. The P/N of the third iteration for the plasma functionalized surface is extremely high compared to the previous ones because the surface mass density of the negative control is very close to the baseline level (Figure S-4D in the Supporting Information), suggesting very low nonspecific binding in this case.

Although the P/N ratios for the DENV-2 functionalized surface are slightly lower than those of MAC-ELISA, a dengue positive result is easily concluded for all positive patients given the small standard deviation of the measured optical output power ($\sigma = 6.3$ nW). This functionalization approach has the advantage that sensor surfaces can be prepared in advance and detection of dengue-specific IgM antibody in plasma carried out in a single step. Label-free and real-time single-step detection is promising as a rapid IgM-based dengue diagnostic tool for use at point-of-care.

Table 1. Summary of Results in Terms of P/N Ratio Using MAC-ELISA, DENV-2 Functionalized Surface, and Plasma Functionalized Surface

P/N	MAC-ELISA	iteration 1		iteration 2		iteration 3	
		DENV-2 surface	plasma surface	DENV-2 surface	plasma surface	DENV-2 surface	plasma surface
patient 1	7.71	6.77 ± 0.58	13.88 ± 1.67	6.99 ± 0.60	9.21 ± 1.11	17.19 ± 1.63	52.83 ± 6.42
patient 2	4.20	3.63 ± 0.22	7.35 ± 0.57	3.74 ± 0.23	4.31 ± 0.34	10.39 ± 0.71	21.02 ± 1.87
patient 3	3.82	2.36 ± 0.55	5.59 ± 1.77	2.26 ± 0.52	2.56 ± 0.81	5.88 ± 1.38	11.71 ± 3.76
negative control	1.00	1.00 ± 0.12	1.00 ± 0.17	1.00 ± 0.08	1.00 ± 0.11	1.00 ± 0.33	1.00 ± 0.45

The second functionalization approach (plasma immobilization) exhibits almost 2-fold higher P/N ratios compared to the MAC-ELISA results, suggesting improved sensitivity and accuracy over the latter. Although very simple, this approach is probably better-suited to laboratory use given the multiple steps involved but is still quicker to carry out than MAC-ELISA, significantly reducing the time for dengue diagnosis. Furthermore, only a very small amount of plasma ($\sim 10 \mu\text{L}$) is required, even for repeated measurements to confirm a diagnosis. It is difficult, but not impossible,⁵³ to identify dengue serotypes through the immunoreaction between dengue virus and dengue-specific antibodies. Thus, there is also a possibility that the same plasma surface can be reused to identify the serotype of dengue virus infection.

Considering the largest signal change of $\Delta S = 0.66 \mu\text{W}$ due to the plasma sample of patient 1 using the second surface functionalization approach, the corresponding signal-to-noise ratio ($\Delta S/\sigma$) is ~ 135 . The standard deviation of the surface mass density calculated indicates the detection limit of dengue diagnosis using our setup, which is $\Delta\Gamma \sim 22 \text{ pg/mm}^2$. The determination of the detection limit is dependent on the dengue-specific IgM concentration in plasma sample. Therefore, it does not represent the ultimate detection limit of our setup. In fact, we estimated the detection limit of our setup as $\Delta\Gamma \sim 12 \text{ pg/mm}^2$ in previous work.⁴³

CONCLUSION

In conclusion, we have demonstrated the detection of dengue-specific IgM antibody in real-world complex samples of patient blood plasma using long-range surface plasmon waveguides in a simple interrogation setup and two different surface functionalization protocols: immobilized virus or immobilized plasma. The identification of positives by immobilizing virus (and flowing plasma) is almost as conclusive as using MAC-ELISA, whereas the identification of positives by immobilizing plasma (and flowing virus) is more conclusive by a factor of 2. Our proposed approaches can thus be used for the identification of dengue-specific IgM in complex media (plasma), in the presence of background interference (such as nonspecific binding). The sensor and functionalization approaches presented can likely be applied to improve the diagnosis of other virus-induced infections.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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