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Detection of dengue NS1 antigen using long-range surface plasmon waveguides

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ABSTRACT

The non-structural 1 (NS1) protein of the dengue virus circulates in infected patients' blood samples and can be used for early diagnosis of dengue infection. In this paper, we present the detection of naturally-occurring dengue NS1 antigen in infected patient blood plasma using straight long-range surface plasmon waveguides. Three commercially-available anti-NS1 monoclonal antibodies were used for recognition and their performance was compared and discussed. A similar figure of merit to the one used in conventional dengue NS1 capture using an enzyme-linked immunosorbent assay (ELISA) was applied to our results. In general, the positive patient samples can be clearly differentiated from the negative ones and the results agree with those obtained using ELISA. The largest signal-to-noise ratio observed during the experiments was 356 and the best detection limit observed is estimated as 5.73 pg/mm^2.

1. Introduction

Dengue is a tropical mosquito borne disease affecting over half of the world population (Beatty et al., 2010) with about 390 million cases annually (Bhatt et al., 2013). The diagnosis of dengue can be difficult because its symptoms are nonspecific and current laboratory techniques are expensive, time consuming, and require highly skilled lab personnel. Current laboratory dengue diagnosis techniques include virus isolation, detection of virus components (RNA or antigen), and detection of dengue-specific antibodies (IgM or IgG) (Peeling et al., 2010; Vorndam et al., 1997; World Health Organization, 2009). Over the past years, much research has been done on a dengue diagnostic tool through the detection of nucleic acid (Baeumner et al., 2002; Zaytseva et al., 2005; Zhang et al., 2006), antigen (Camara et al., 2013; Linares et al., 2013; Silva et al., 2014b; Tai et al., 2005) or antibodies (Kumbhat et al., 2010; Lee et al., 2009; Wong et al., 2014a). However, none of the studies are able to fulfill the requirement of an "ideal" dengue diagnostic test which should be sensitive regardless of the stage of infection (Peeling et al., 2010). We previously argued that not all patients seek medical attention during early onset of symptoms and therefore presented a dengue biosensor which is able to detect dengue-specific antibodies in blood plasma (Wong et al., 2014a). Recent studies suggest that the combined detection of dengue non-structural 1 (NS1) antigen and dengue-specific antibodies improves the diagnostic sensitivity (Blacksell et al., 2011; Fry et al., 2011).

Non-structural 1 (NS1) protein, which is approximately 45 kDa in molecular weight (Allonso et al., 2011; Zhao et al., 1987), is secreted from dengue virus infected cells. Dengue NS1 antigen is an important diagnostic biomarker found circulating in patient blood samples up to 9 days after the onset of symptoms (Alcon et al., 2002). Furthermore, dengue serotypes can be identified by using serotype-specific anti-NS1 monoclonal antibody (Ding et al., 2011). Much of the research on biosensors has been done on the detection of purified dengue NS1 antigen in buffer (Camara et al., 2013; Figueiredo et al., 2015; Hu et al., 2013; Mishra et al., 2014; Silva et al., 2014a; Singh, 2012; Su et al., 2003; Tai et al., 2005) or on spiked NS1 antigen in serum samples (Cecchetto et al., 2015; Dias et al., 2013; Silva et al., 2014b; Yen et al., 2015). The detection of naturally occurring dengue NS1 antigen in serum was reported using electrochemical (Cavalcanti et al., 2012; Parkash et al., 2014), fluorescence (Linares et al., 2013) and quartz crystal microbalance (Wu et al., 2005) biosensors. In this paper we present the detection of purified dengue NS1 antigen in buffer, and naturally-occurring dengue NS1 antigen in patient blood plasma, using straight long-range surface plasmon polariton (LRSPP) waveguides. Five

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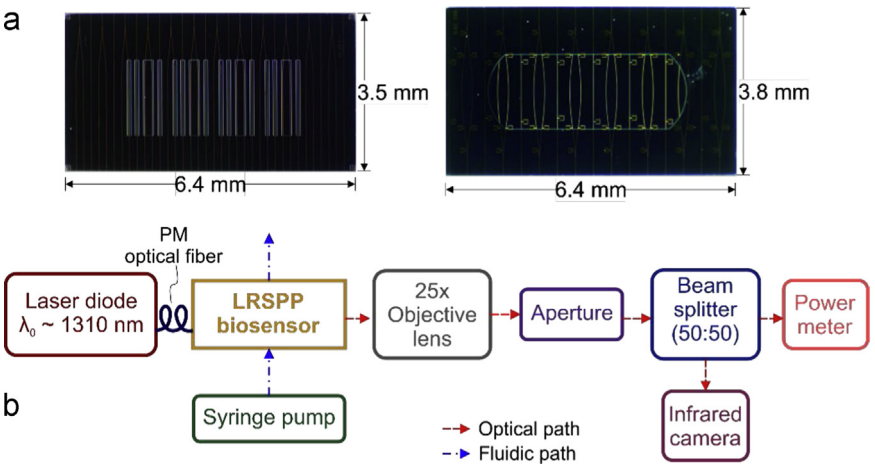

Fig. 1. (a) Microscope images of the sensor die used. (b) Schematic diagram of the experimental set-up.

clinical plasma samples were tested for dengue NS1 antigen. We also compare the performance of three commercially-available anti-NS1 monoclonal antibodies to detect dengue NS1 antigen. This is the first biosensor to demonstrate the detection of naturally occurring dengue NS1 antigen in blood plasma. The detection of dengue NS1 in blood plasma is more challenging than the detection of dengue-specific antibodies (Wong et al., 2014a) because NS1 has a smaller molecular weight and occurs in lower concentration in blood (0.01–2 μg/ml (Alcon et al., 2002)).

LRSPPs are transverse magnetic polarized optical surface waves propagating along a thin metal slab or stripe bounded by dielectrics of similar refractive index (Berini, 2009). The excitation of LRSPPs can be easily achieved by an optical fiber butt-coupled to the metal waveguide. The ease of LRSPP excitation enables compact and miniaturized biosensors. LRSPPs have reduced confinement and lower modal sensitivity than single-interface SPPs but its greater propagation length provides better overall sensitivity (Berini, 2008). For biosensing applications, low-index claddings are used to match the refractive index of biologically compatible sensing fluids ($\sim$1.32) which then maintains the optical symmetry of the mode. Fluoropolymers such as Cytop (Asahi) and Teflon (Dupont) are the most common materials used as low index claddings (Joo et al., 2010; Slavík and Homola, 2007; Wark et al., 2005). The sensor used throughout this paper consists of a straight gold (Au) stripe embedded in Cytop claddings with an etched microfluidic channel for sensing. The sensitivity of the straight waveguide as a biosensor was discussed previously (Wong et al., 2015a).

2. Materials and methods

2.1. Chemicals and reagents

16-Mercaptohexadecanioc acid (16-MHA), phosphate buffered saline (PBS) 0.01 M, pH 7.4, N-Hydroxysuccinimide sodium salt (NHS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), human IgG kappa antibody, sodium dodecyl sulfate (SDS), 2-Isopropanol semiconductor grade (IPA), acetone HPLC grade $\geq$99.9%, heptane and glycerol (electrophoresis grade) were obtained from Sigma-Aldrich. Distilled water was deionized using Millipore filtering membranes (Millipore, Milli-Q water system at 16 MΩ cm).

Dengue virus mouse anti-NS1 monoclonal antibody (clone D2N3) supplied as cell culture supernatant was purchased from Vivantis Technologies. Purified mouse anti-dengue virus NS1 glycoprotein antibodies were purchased from Abcam (ab138696) and ViroStat (#5162). Dengue virus NS1 full length glycoprotein (ab64456) was purchased from Abcam. All patient plasma samples (POS 1_3, POS 2_1, POS 2_2 and POS 2_3), laboratory-confirmed as dengue NS1-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). Normal human plasma in K3 EDTA (lot no. PLE050412) was purchased from Zen-Bio, Inc. to serve as a negative control. The dengue ELISA kit used is SD Dengue NS1 Ag ELISA from Standard Diagnostics Inc., South Korea.

In previous work (Wong et al., 2015a), it was shown that a sensing buffer (PBS/Gly) with a slightly higher refractive index than Cytop will result in a higher response for protein binding. Therefore, a sensing buffer with a refractive index of 1.338 was chosen and used throughout the experiments; this value is high enough to produce a sensitive response to protein binding but also low enough for the LRSPP mode to be below cut-off. The sensing buffer PBS/Gly having an index of 1.338 was prepared by mixing biological buffer (PBS, pH 7.4) with glycerol. The sensing buffer and the deionized water were filtered through Millex-GP filters (PES membrane 0.22 μm).

2.2. Sensing device and instrumentation

The sensor die consists of thin ($\sim$35 nm) narrow (width=5 μm) gold stripes embedded in Cytop claddings, with a fluidic channel of sensing length L=1.65 mm etched into the top cladding. Sensor dies with two different fluidic designs were used in the experiments, as shown in the microscope images of Fig. 1a (left and right). Although Y-junctions and Mach-Zehnder interferometers (MZIs) are also present on the sensor dies, only straight Au waveguides were used throughout.

A schematic of the experimental set-up is sketched in Fig. 1b. A polarization-maintaining (PM) optical fiber with a core diameter of 7 μm (PMJ-3AX-1300-7/125-1-1-1, OZ Optics) which carries the light generated by a semiconductor laser diode (NLK1356STG, λ_0 =1315.89 nm, NTT Electronics) was butt-coupled to the input facet of the LRSPP biosensor. Two multi-axis positioning stages (Thorlabs Inc.) were used to align the optical fiber to the input waveguide. The output signal from the sensing waveguide was magnified and collimated by a 25× objective lens (Melles Griot). Background light in the output signal was minimized using a pinhole aperture before it was sent to a 50:50 beam splitter (BSW12, Thorlabs Inc.). One portion of the output beam was sent to an infrared camera to visually monitor the emerging mode for ease of alignment while the other portion was sent to a power meter (81618A, Hewlett Packard) to record real-time changes in

the output signal during an experiment. A Labview script was written to perform data acquisition from the power meter. The fluid was injected over the sensor surface by a syringe pump (PicoPlus, Harvard Apparatus). The fluidic assembly of the sensor die in a fluidic jig was described previously (Krupin et al., 2013).

2.3. Device cleaning and surface preparation

After dicing, sensor die must be cleaned before use in biosensing experiments. The facets must be cleaned through ultrasonication for removal of dicing debris and efficient optical input and output coupling. The optimized settings of the ultrasonic cleaner (FB-11201, Fisher Scientific) were a frequency of 37 kHz and a power of 50% (of maximum) to provide sufficient agitation to remove the debris without destroying the Au waveguides. A fresh sensor die was picked from a wafer and cleaned by ultrasonication in heptane for 5 min. Photoresist applied to the wafer for protection during dicing was removed from sensor die through soaking in two sequential acetone baths for 5 and 30 min, respectively. Sensor die were then washed intensively with IPA and dried with nitrogen (N_2) gas. Removal of possible organic matter from the Au surface was performed by placing the sensor die in a UV/ozone chamber (PSD-UV, Novascan) for 15 min.

To prepare the sensor surface for biosensing, a cleaned sensor die was placed in 1 mM solution of 16-MHA in IPA overnight for the formation of a self-assembled monolayer (SAM) on the Au surface. After rinsing with IPA and drying with N_2, the SAM-modified sensor die was assembled into the experimental set-up.

2.4. Experimental procedures

2.4.1. Detection of dengue NS1 antigen in clean fluid

Clean fluid refers to the pure biomaterial solution (usually stored in buffer and some preservatives/stabilizers) without any other proteins or antibodies in the background. We first demonstrated the ability of our biosensor to detect purified dengue NS1 antigen in buffer using anti-NS1 monoclonal antibodies. The functionalization process is illustrated in Fig. 2I. After the SAM formation, the sensor die was placed into a fluidic jig with PBS/Gly buffer (a mixture of standard phosphate buffered saline (PBS) with glycerol (Gly)) filling the etched channel. After establishing a stable baseline signal with PBS/Gly, the carboxyl surface was activated by 0.1 M NHS/EDC for 15 min. Following a PBS/Gly wash, the dengue NS1 antigen (20 μg/ml) was passed through the fluidic channel to be covalently attached to the sensor surface. A high concentration of dengue NS1 antigen was selected to ensure saturation of the sensor surface. Human IgG kappa antibody (10 μg/

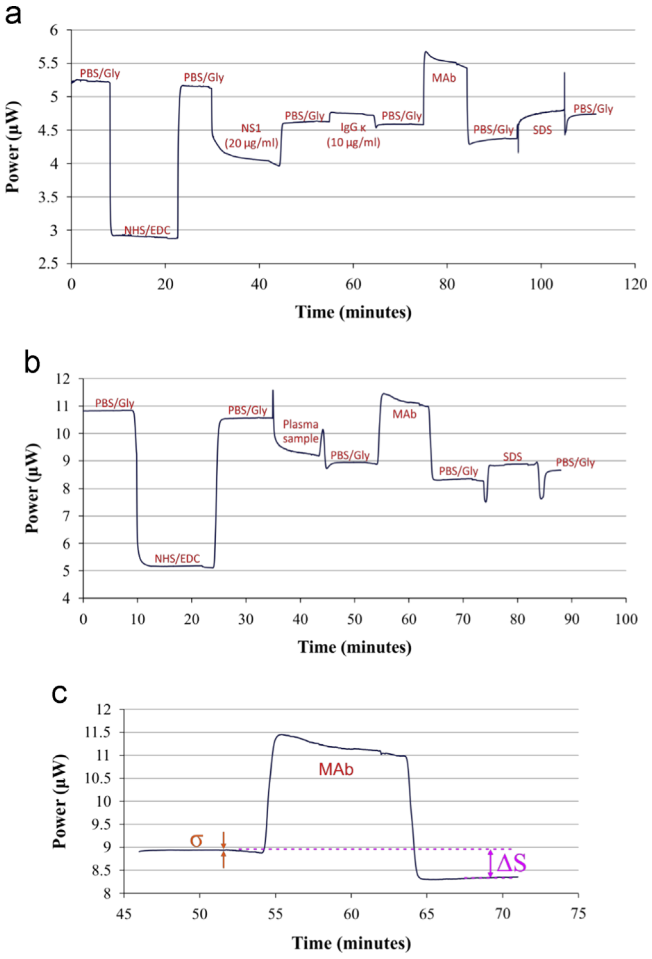

Fig. 3. Sensorgrams showing real-time sensing runs for the detection of dengue NS1 antigen in (a) clean fluid and (b) patient blood plasma. (c) Zoom-in image of (b) showing the change in output power ΔS and the noise level σ. A signal-to-noise ratio $\Delta S/\sigma$ is computed using such values.

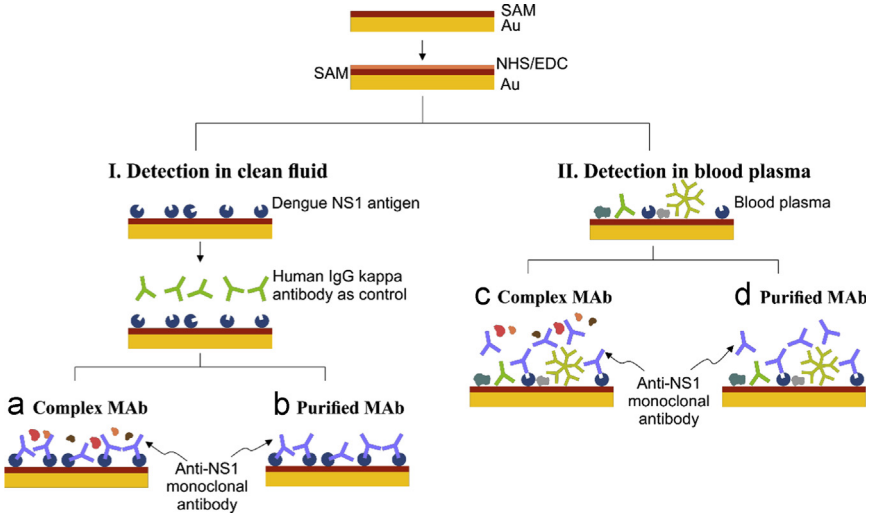

Fig. 2. Schematic illustrating the functionalization of the sensor and sensing runs for (a) detection of dengue NS1 antigen in clean fluid using complex monoclonal antibody (MAb); (b) detection of dengue NS1 antigen in clean fluid using purified MAb; (c) detection of dengue NS1 antigen in patient blood plasma using complex MAb; and (d) detection of dengue NS1 antigen in patient blood plasma using purified MAb.

ml) was first injected over the sensor surface as a negative control and also as a blocking agent of any unreacted sites. The complex anti-NS1 monoclonal antibody (MAb) (in cell culture supernatant) and the purified MAb were used to detect the dengue NS1 antigen on the sensor surface. The reason for using two different types of MAb will be apparent in Section 3.1.

Fig. 3a shows a full sensorgram for detection in a clean fluid. All experiments were carried out under a continuous flow rate of 20 µl/min, except for when the flow was stopped. During the injection of NHS/EDC in PBS/Gly solution (0.1 M NHS and 0.1 M EDC dissolved in PBS/Gly solution), there is a large drop in signal due to changes in the bulk refractive index. The *in situ* activation of the carboxyl-terminated surface causes a small change in the baseline signal ($t \sim 25$ min). A slight binding response was observed during the flow of human IgG kappa antibody over the NS1 functionalized surface but the baseline signal was recovered after washing with PBS/Gly. This observation indicates the response was mainly due to loosely nonspecific bound antibody on the sensor surface which dissociated upon washing with buffer. When anti-NS1 monoclonal antibody (MAb) was injected, a significant binding response was recorded after the fluid exchange which resulted in the change in baseline signal. In order to regenerate the surface for repeated measurements, the surface was washed with 0.5% SDS in PBS/Gly solution. The flow of human IgG kappa antibody and anti-NS1 MAb were repeated sequentially.

To reduce the consumption of biomaterials, the flow of NHS/EDC, dengue NS1 antigen, human IgG kappa antibody, and anti-NS1 MAb were stopped 5 min after fluid exchange. Due to the change in pressure during the restarting of the syringe pump, the slope of the binding response curve differs at $t \sim 40$, 60 and 80 min.

2.4.2. Detection of dengue NS1 antigen in human blood plasma

A similar surface functionalization approach was applied for the detection of dengue NS1 antigen in blood plasma samples, as illustrated in Fig. 2II. We demonstrated in previous work (Wong et al., 2014a) that a plasma functionalized surface is more efficient in minimizing nonspecific binding for detection in complex samples. Once the sensor surface was prepared with a carboxylated self-assembled monolayer (SAM), a human blood plasma sample was diluted 1:10 with PBS/Gly buffer and immobilized on the surface through amine coupling (using NHS/EDC in PBS/Gly solution). Two types of anti-NS1 MAb (in cell culture supernatant and purified) were injected to recognize NS1 antigen that might exist in the blood plasma.

The sensorgram for the detection of dengue NS1 antigen in a blood plasma sample is presented in Fig. 3b. Similarly, the experiments were carried out under a continuous flow rate of 20 µl/min, except when the flow of NHS/EDC, a plasma sample, and anti-NS1 MAb were stopped 5 min after fluid exchange. The flow stopping causes a slight change in the response curves during the restarting of the syringe pump ($t \sim 22$, 42 and 62 min). The complex composition of the plasma sample leads to a significant change in output signal after the PBS/Gly wash ($t \sim 50$ min). The change in baseline signal before and after injection of MAb, indicated by ΔS in Fig. 3c, is the result of interest, which is later related to the change in surface mass density on the waveguide (Section 3.1). 0.5% SDS in PBS/Gly solution was introduced to regenerate the plasma functionalized surface for repeated measurements.

3. Results

3.1. Detection of dengue NS1 antigen using monoclonal antibody

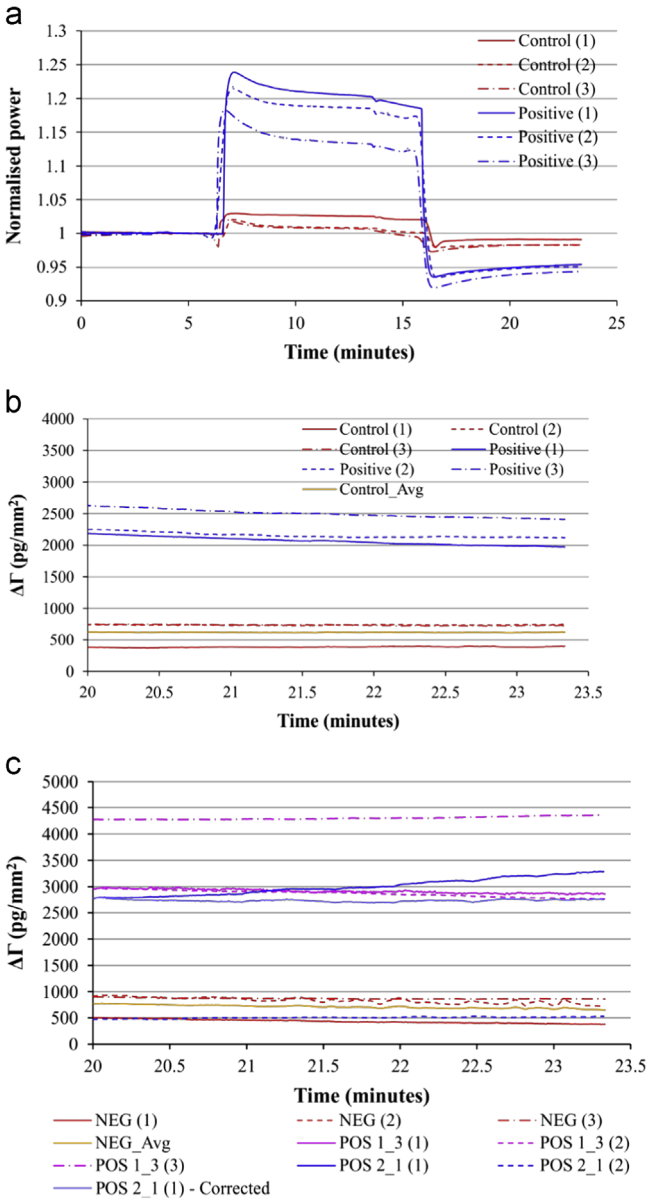

Fig. 4. Detection of dengue NS1 antigen using monoclonal antibody (MAb) in cell culture supernatant supplied by Vivantis Technologies. (a) Normalized responses during injection of anti-NS1 MAb (positive) and human IgG kappa (negative control) on a clean NS1 functionalised surface. The difference in power measured before and after injection is correlated to the amount of biomaterial adsorbed on the sensor surface. The number in parentheses indicates the iteration. (b) Surface mass density adsorbed onto a clean NS1 functionalized surface after injection of anti-NS1 MAb and human IgG kappa (control). The zero was set to the baseline level before injection. (c) Surface mass density adsorbed onto patient plasma functionalized surfaces after injection of anti-NS1 MAb. Two positive (POS 1_3 and POS 2_1) and one negative (NEG) plasma sample were used. The number in parentheses indicates the iteration. The zero was set to the baseline level before injection.

(MAb) in cell culture supernatant

The measurements obtained for the detection of NS1 antigen in clean fluid (Fig. 2a) and in patient blood plasma (Fig. 2c) using MAb in cell culture supernatant are summarized in Fig. 4. The anti-NS1 MAb was diluted 1:10 in PBS/Gly buffer. Fig. 4a shows the normalized power during the injection of antibodies (human IgG kappa antibody (control) or anti-NS1 MAb (positive)) over a clean NS1 functionalized surface. The measured power (in µW) was normalized to the baseline signal before the injection of

antibodies. We can clearly differentiate the change in power level due to the binding of anti-NS1 MAb to the dengue NS1 antigen immobilized on the waveguide, compared to the negative control (human IgG kappa antibody). The trend in the power signal during binding is opposite to those reported previously (Wong et al., 2014a). This is due to the use of a sensing buffer with a refractive index of 1.338, which is above Cytop, so the waveguide becomes more asymmetric during adlayer growth and thus producing a lower output power during and after binding (Wong et al., 2015a).

The power change due to the interaction between dengue NS1 antigen and MAb can be translated to a change in surface mass density $\Delta\Gamma$ (in pg/mm^2) using (Wong et al. 2015a):

$$\Delta\Gamma = \frac{1}{k_2}\frac{(n_a - n_c)}{\partial n/\partial c}\left(\frac{P_{out}(a_1)}{P_{out}(a_0)} - 1\right) \quad (1)$$

where n_a=1.5 is the index of the adlayer formed, n_c=1.338 is the index of the sensing fluid, P_{out} (a_0) is the power measured before antibody injection, and P_{out} (a_1) is the power measured after antibody injection. Using k_2=0.0204 nm^{-1} (the determination of k_2 can be found under Supporting information of Wong et al., 2014a) and $\partial n/\partial c$=0.185 mm^3/mg, the real-time $\Delta\Gamma$ of bound biomaterial is computed, as shown in Fig. 4b and c. The zero of $\Delta\Gamma$ is set to the average baseline level before the injection of antibodies. Eq. (1) shows that the change in surface mass density is related linearly to the output power $P_{out}(a_1)$. A linear model was also proposed relating the output power to the adlayer thickness a in our previous work (Krupin et al., 2013).

In Fig. 4b, the amount of anti-NS1 MAb (positive) bound to the clean NS1 functionalized surface is significantly higher than the nonspecific binding exhibited by the negative control. Since the negative control was also used as a blocking agent, we expected some nonspecific binding to unreacted sites. Three repeats were obtained for this clean fluid experiment by regenerating the NS1 functionalized surface through an injection of 0.5% SDS. A point-to-point average for the negative controls was computed for the ease of subsequent calculation of the positive-to-negative (P/N) ratio.

The change in surface mass density on plasma functionalized surfaces, after the injection of anti-NS1 Mab, is plotted against time in Fig. 4c. The amount of anti-NS1 MAb bound is correlated to the actual amount of dengue NS1 antigen that exists in the blood plasma samples. In Fig. 4b and c, the positives can be differentiated from the negatives, except for one case with positive plasma. The first positive patient (POS 1_3) produces a distinct response compared to the negative patient (NEG) for all three iterations (a point-to-point average for the negative control is plotted and will be used in the calculation of P/N ratios below). However, when we proceed to test for the second positive patient (POS 2_1), the second iteration is similar to those obtained for the negative patient. A difference between the two experiments (POS 2_1 (1) and POS 2_1 (2)) is the use of different sensor dies. After careful investigation, we deduce that there is a slight variation in performance of our sensor dies due to fabrication imperfections. Our previous theoretical work (Wong et al., 2014b) supports this deduction by demonstrating a difference in insertion loss response with adlayer formation for a slight difference (a few nanometers) in waveguide thickness. The difference in response between the third iteration of POS 1_3 compared with the previous two iterations in Fig. 4c is due to this reason. Each sensor die has a different binding capacity and sensitivity due to fabrication imperfection. Thus, to obtain quantitative results, each sensor die must be calibrated using clean fluids before testing patient samples. In order to distinguish positive patient samples from the negatives, the nonspecific binding must be minimized. We found that most of the nonspecific binding observed was caused by the complex composition of anti-NS1 MAb in cell culture supernatant. Therefore, we continued our experiments to detect dengue NS1 antigen using purified anti-NS1 Mab (following section).

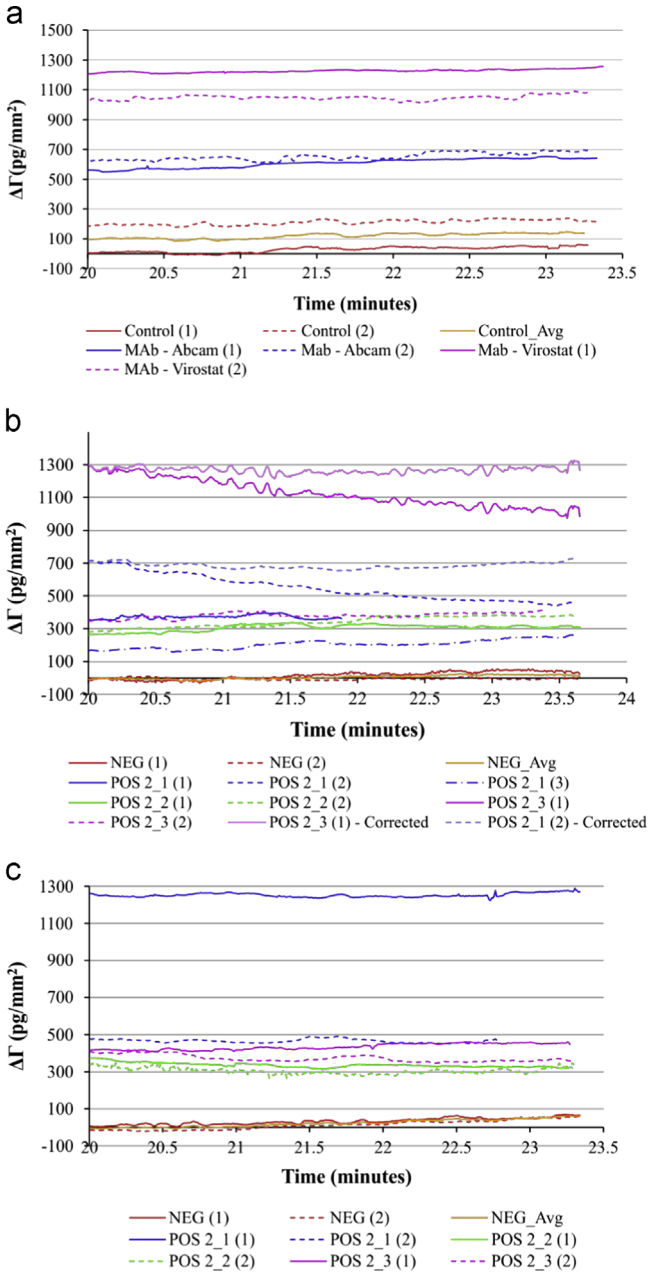

Fig. 5. Detection of dengue NS1 antigen using purified monoclonal antibody (MAb). In each case, the zero was set to the baseline level before injection. The number in parentheses indicates the iteration. (a) Surface mass density adsorbed onto a clean NS1 functionalized surface after injection of anti-NS1 MAb supplied by Abcam or Virostat, or human IgG kappa (negative control). (b) Surface mass density adsorbed onto patient plasma functionalized surfaces after injection of anti-NS1 MAb supplied by Abcam. (c) Surface mass density adsorbed onto patient plasma functionalized surfaces after injection of anti-NS1 MAb supplied by Virostat. In (b) and (c), three positive (POS 2_1, POS 2_2 and POS 2_3) and one negative (NEG) plasma samples were used.

3.2. Detection of dengue NS1 antigen using purified monoclonal antibody (MAb)

Fig. 5 shows the results obtained for the detection of dengue NS1 antigen in clean fluid (Fig. 2b) and in patient blood plasma (Fig. 2d) using purified MAb from two different sources (Abcam

and Virostat). The concentration of the anti-NS1 MAb was 10 μg/ml. In the clean fluid experiments, a similar surface functionalization process as described in Section 3.1 was adopted (Fig. 2b). In general, we observe a lower change in surface mass density $\Delta\Gamma$ after the injection of antibodies (human IgG kappa antibody (control) or anti-NS1 MAb (positive)) over the clean NS1 functionalized surface (Fig. 5a) than when using MAb in cell culture supernatant (Fig. 4b). We attribute the higher response in our previous observation (Fig. 4b) to non-specific binding due to the complex composition of the anti-NS1 MAb as cell culture supernatant. Anti-NS1 MAb supplied by Virostat yields slightly higher responses than those supplied by Abcam, as observed in Fig. 5a.

Three positive (POS 2_1, POS 2_2 and POS 2_3) and one negative (NEG) patient plasma samples were tested for dengue NS1 antigen using purified MAb, as shown in Fig. 5b and c. We obtained almost no response after the injection of MAbs over negative plasma functionalized surfaces. The three positive samples which have almost similar concentration of dengue NS1 antigen (according to ELISA results-discussed below) demonstrated a consistent change in surface mass density after the injection of anti-NS1 MAb. There is one exceptionally high response in Fig. 5b and c, which is due to the use of a sensor die with a different sensing performance (as discussed in Section 4.1). A point-to-point average for the negative controls/samples is plotted in the figures and this value will be used in the calculation of *P*/*N* ratios below.

It is observed from Fig. 5b that the responses for the second iteration of POS 2_1 and the first iteration of POS 2_3 drift over time. The drift is usually caused by a slight misalignment between the input fiber and the sensing waveguide. A minor signal drift will not affect the differentiation of positive samples from negatives. However, for the computation of positive-to-negative (*P*/*N*) and signal-to-noise (SNR) ratios (discussed in the next section) the drift should be removed. The signal drift was eliminated by fitting the responses to linear models and subtracting them from the raw data. The reference levels were taken at the time $t=20$ min, the time at which the MAbs were injected. The drift-corrected results were added alongside the raw data to Fig. 5b. (The response for the first iteration of POS 2_1 was similarly corrected in Fig. 4c.) Alternatively, a referenced sensor, such as a Y-junction sensor, can be used to eliminate the drift in the system directly, by dividing the output signal from the sensing waveguide with that from the reference waveguide (Wong et al., in press).

In an attempt to obtain a higher response for the detection of dengue NS1 antigen, we carried out an experiment using a plasma sample (POS 2_2) diluted 1:5 in PBS/Gly buffer and anti-NS1 MAb supplied by Virostat (results not shown). We hypothesized that a less-diluted plasma sample will contain a higher concentration of dengue NS1 antigen which would produce a larger change in output power. However, we found that the response was just slightly higher (not doubled) despite the higher concentration of plasma, probably because the sensing surface was saturated in both cases. Thus, we decided to continue the experiments using plasma diluted 1:10 in PBS/Gly buffer to minimize our consumption of blood samples.

4. Discussion

The results obtained using our biosensor were analyzed using a similar figure of merit as the one used in conventional dengue NS1 capture antigen enzyme-linked immunosorbent assay (ELISA) so that their performance can be compared. A test sample is considered positive if the time-averaged surface mass density is greater than twice the mean value of the negative samples. In other words, we define a positive-to-negative ratio as (Wong et al., 2014a):

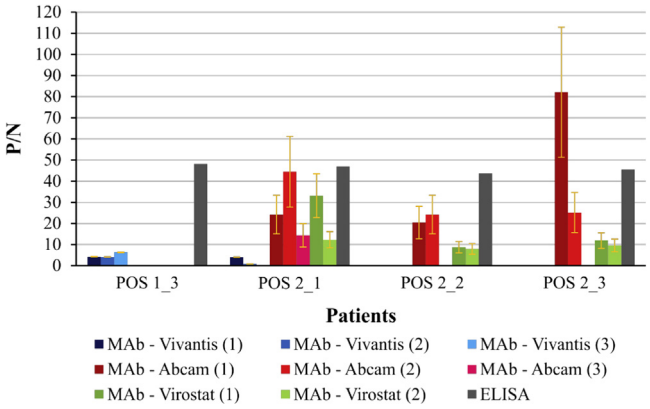

Fig. 6. Positive-to-negative (*P*/*N*) ratio for four positive patients using different sources of anti-NS1 Mab (Vivantis, Abcam and Virostat). The number in parentheses indicates the iteration number. The corresponding results determined using ELISA are also shown.

$$P/N = \frac{\langle \Gamma_{POS} \rangle}{\langle \Gamma_{NEG\_Avg} \rangle} \tag{2}$$

where $\langle \Gamma_{POS} \rangle$ is the time-averaged surface mass density of the positive sample and $\langle \Gamma_{NEG\_Avg} \rangle$ is the time-averaged mean value of the surface mass density of the negative samples (labeled as NEG_Avg in the figures). A sample producing a *P*/*N* ratio greater than or equal to 2.0 is classified as positive (Alcon et al., 2002; Shu et al., 2000). Fig. 6 summarizes the *P*/*N* ratios for all patients using different sources of anti-NS1 MAb, and the results measured by NS1 capture ELISA as reference. The error on the *P*/*N* ratio $\sigma_{P/N}$ is computed through (Taylor, 1997):

$$\sigma_{P/N} = \frac{\langle \Gamma_{POS} \rangle}{\langle \Gamma_{NEG\_Avg} \rangle} \sqrt{\left(\frac{\sigma_{POS}}{\langle \Gamma_{POS} \rangle} \right)^2 + \left(\frac{\sigma_{NEG\_Avg}}{\langle \Gamma_{NEG\_Avg} \rangle} \right)^2} \tag{3}$$

where σ_{POS} is the standard deviation of the surface mass density of the positive sample and σ_{NEG_AVG} is the standard deviation of the mean surface mass density of the negative samples.

The *P*/*N* ratios obtained using MAb in cell culture supernatant (Vivantis Technologies) are generally lower than those obtained using purified MAbs. The nonspecific binding due to the complex composition of the MAb solution reduces the distinction between the positive and negative samples. We also note the *P*/*N* of the second iteration for POS 2_1 sample is less than 2.0, which indicates a false negative. Switching to purified MAbs improves the dengue NS1 antigen detection results and produces higher *P*/*N* ratios.

Although the ratios using our biosensor are mostly lower than those obtained using ELISA, we are able to clearly distinguish the dengue NS1 positive patients using purified anti-NS1 MAbs. We also show in Fig. 6 that our biosensor can produce better detection results than ELISA (first iteration using MAb from Abcam for patient POS 2_3) if the thickness of the sensing waveguide is properly controlled during fabrication. Table S1 summarizes the *P*/*N* ratios of the results obtained with our biosensors using our different sources of anti-NS1 MAb.

The baseline noise (σ in Fig. 3c) observed during the experiments varies from 0.2 nW to 13.6 nW with a typical value of 3 nW. The variation in σ is mostly due to a slight misalignment of the input fiber in our setup, which can be easily corrected. The largest signal-to-noise ratio ($\Delta S/\sigma$) is found to be 356. The lowest detection limit of our setup in terms of surface mass density is estimated as 5.73 pg/mm^2 (for $\Delta S/\sigma=1$), for the first iteration of POS 2_3. This value agrees with the calculation in our previous work (Wong et al., 2015a).

5. Conclusion

The detection of dengue NS1 antigen has been demonstrated using a compact, cost-effective, label-free and real-time LRSPP based biosensor. The complex composition of anti-NS1 MAb in cell culture supernatant causes nonspecific binding which resulted in one false negative measurement. Using purified anti-NS1 MAb for recognition overcomes the problem by showing almost no response for negative samples and distinguishable results for all of our positive samples. Although the positive-to-negative ratio of our results is not as high as those measured using ELISA, our biosensor is able to identify the dengue NS1 antigen in clinical plasma samples with high reliability and in real-time. The results presented in this paper can be improved through careful design and fabrication of the biosensor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.11.030.

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