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Dengue immunoassay with an LSPR fiber optic sensor

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Abstract: Dengue fever is a viral disease that affects millions of people worldwide. Specific tests for dengue are not usually performed due to high costs, complicated procedures and, in some cases, long time to yield a result. For widespread use of specific tests to be possible, fast, reliable and fairly simple methods are needed. In this paper, we present a new dengue diagnostic method for the acute phase of the infection. The method proposed uses an all-optical fiber sensor based on Localized Surface Plasmon Resonance (LSPR) and specular reflection from gold nanoparticles (AuNPs). Dengue anti-NS1 antibody was immobilized on AuNPs deposited on the endface of a standard multimode fiber (62.5μm/125μm). The sensor is able to detect NS1 antigen at different concentrations, with limit of quantification estimated to be 0.074 μg/ml = 1.54 nM. These results indicate that the sensor could potentially be used for dengue diagnosis in the acute phase of the infection.

OCIS codes: (280.1415) Biological sensing and sensors; (060.2370) Fiber optics sensors; (240.6680) Surface plasmons.

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1. Introduction

Dengue fever is a tropical disease caused by the dengue virus, transmitted mainly by the female *Aedes aegypti* mosquito [1]. The World Health Organization (WHO) estimates that there are over 2.5 billion people at risk of dengue infection and that there are about 50 - 100 million new cases per year. Of these, over 500,000 would be cases of dengue hemorrhagic fever (severe dengue) [2]. Samir Bhatt et al [3], however, estimate that there are actually about 390 million dengue infections per year. In Brazil, for example, dengue is a major public health problem affecting thousands of people every year [4].

Dengue fever may be fatal if it evolves into hemorrhagic fever (severe dengue) or dengue shock syndrome, which occur in 5% of the cases. In fact, even non-severe dengue fever may be fatal, if untreated. In some Asian and Latin American countries, dengue is a leading cause of death among children [2]. Early detection and appropriate therapy increase the chance of survival; severe dengue mortality rates can be decreased from 20% down to 1% with proper support therapy from experienced healthcare providers [2]. Unfortunately, in some cases, the

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patient does not get the necessary support therapy in time to save his/her life, because the illness is initially misdiagnosed. One reason for this is that dengue symptoms can be easily mistaken with those of other illnesses, for example, flu, gastroenteritis or other viral infections. In many locations patients with suspicious symptoms are not routinely checked for dengue infection.

Usually, when a patient arrives at a hospital or clinic with dengue-like symptoms, the initial diagnosis performed is based mainly on observed and reported symptoms. The patient may be submitted to laboratory tests, but most of the times the only type of analyses used are white blood cell and platelet counts. While it is true that patients with dengue infection have low white blood cell and platelet counts, these are not specific to dengue infection. Despite that, specific tests for detection of dengue anti-NS1 antibody, dengue NS1 antigen, dengue viral RNA or dengue virus are rarely performed. In fact, even on the rare occasions when commercial specific tests are available at hospitals and clinics, these have several limitations. As reported by Blacksell [5], there are many challenges that still need to be addressed by researchers, manufacturers and legislators, including lack of regulations, geographical variation, differences between primary and secondary infections, sample type, etc. To make matters worse, the performance of many commercial tests has not been adequately evaluated [6].

The major laboratorial methods currently available for diagnosis of the disease are viral culture [7] and viral RNA detection by reverse transcriptase PCR (RT-PCR) [8], which require highly skilled personnel, laborious procedure and are time consuming [9]. Serological tests such as the frequently used immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA) have low sensitivity during the first four days of illness [10]. In fact, patients with dengue infection for the first time will not have anti-NS1 antibody until about day 5 after the onset of the infection [6, 11], which in some cases may be too late to administer the necessary care. Dengue virus and dengue antigen NS1 are present since day 1 of infection, but dengue virus testing is expensive and may take more than 1 week to yield a result, in addition to requiring expertise from the operator and expensive equipment/appropriate facilities [6]. On the other hand, dengue NS1 antigen detection methods are in general faster, cheaper and, therefore, ideal for early detection. In terms of early diagnosis and management, supplying tests based on NS1 antigen detection to healthcare providers in regions with high dengue incidence or during epidemics could save lives and help manage the disease as a public health problem [6]. Nonetheless, since NS1 antigen levels fall to undetectable levels after 5-6 days (secondary infection) or 8-9 days (primary infection) after the onset of the disease, these can only give a correct positive result during the first few days of infection [5].

Dengue diagnosis is thus complex and requires different solutions for different scenarios. Because current commercial solutions are not completely satisfactory, new diagnostic tools have been proposed in literature recently [12–14]. Among fast, reliable and fairly simple methods developed are solutions based on optical fiber sensors [15–17]. Depending on the method and setup used, optical fiber sensors offer several characteristics that could be advantageous if applied for dengue sensing: cheap sensing elements (optical fiber), portability, robustness, ease of handling and possibility of using a sample as small as a drop.

Localized Surface Plasmon Resonance (LSPR) is a viable phenomenon that can be used with optical fiber sensing in order to obtain a cheap, straightforward, fast and accurate diagnosis via NS1 antigen sensing. LSPR occurs when light impinges upon metal nanoparticles (NPs) surrounded by dielectric material. Due to LSPR, spectral changes in the resonant absorption of the NPs will occur with changes in the surrounding refractive index. This phenomenon can be employed in biosensing and sensing of liquids and gases [18]. In order to impart selectivity to a sensor based on LSPR, a material with affinity to the desired target-substance can be attached to the NPs.

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In this paper, we present a sensor for dengue NS1 antigen obtained by immobilizing anti-NS1 antibody on the gold nanoparticles (AuNPs) of an all-optical fiber sensor based on LSPR [19]. The sensor was shown to have a good correlation between wavelength shifts and NS1 antigen concentration, as well as negligible wavelength shift for zero concentration of NS1 antigen. The results discussed in this paper indicate that our sensor can be a powerful tool for sensing of dengue NS1 antigen in samples from patients who exhibit symptoms that fit the clinical presentation of dengue.

2. Materials and methods

2.1. Preparation of the sensing element

The sensing element is located at the tip of each fiber sample. It was prepared by following the first five steps described below, which involve forming and cleaning nanoparticles (NPs) on the fiber endface (Steps 1-2) and immobilizing anti-NS1 antibody on these NPs (Steps 3-5).

Step 1 (AuNPs preparation): Au nanoparticles were created on top of the endface of an optical fiber by sputter deposition of a 6 nm-thick gold thin film followed by a 4 minute-long annealing at 600°C. Figure 1(a) shows a Scanning Electron Microscope (SEM) image of the NPs on top of the fiber endface after the annealing. In the schematics in Fig. 1(b) the NPs are represented by pink spheres. The fibers used in this work were standard multimode fibers with core and cladding diameters equal to 62.5μm and 125μm, respectively.

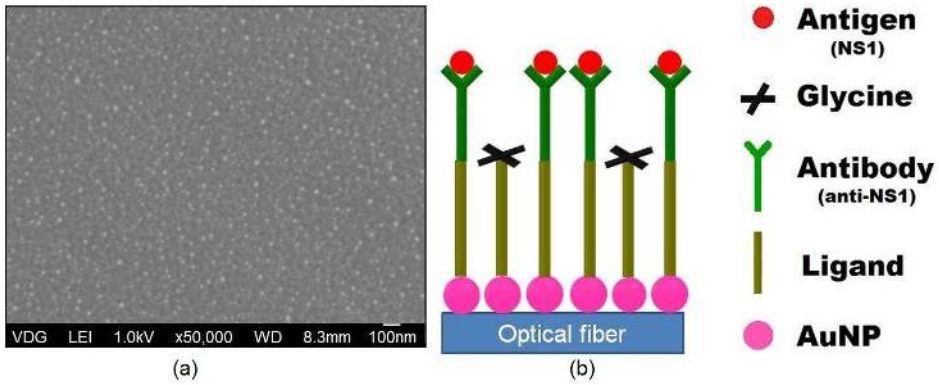

Fig. 1. (a) SEM image of AuNPs (white spots) on the endface of an optical fiber [courtesy of Van der Graaff Lab/PUC-Rio]. (b) Schematic diagram of the fiber endface with AuNPs, ligand, anti-NS1 antibody, Glycine and adsorbed dengue NS1 antigen.

Step 2 (AuNPs cleaning): The fiber tip was immersed in a 0.5 M solution of nitric acid (HNO_3) in water to treat/clean the NPs so that microorganisms would not affect the chemical reactions in the next steps.

Step 3 (ligand deposition/amine-fuctionalization): The fiber tip was immersed for at least 2 hours in a solution of ligand (cysteamine) in ethanol. The ligand allowed the adsorption of dengue anti-NS1 antibody to the NPs, and was represented by the dark yellow/olive lines in Fig. 1(b). Initially, two ligand solutions were tested: a 100mM solution of Mercaptopropionicacid (MPA) in ethanol and a 50 mM solution of Cysteamine (2-aminoethanethiol) in ethanol. Since the fibers with Cysteamine presented larger wavelength shifts and higher reproducibility, all fibers used in the experiments in this paper were prepared with Cysteamine.

Step 4 (anti-NS1 antibody immobilization): The fiber tip was immersed for 1 hour in a solution of anti-NS1 antibody in phosphate buffered saline (PBS) solution. The anti-NS1 antibody is represented by green Ys in Fig. 1(b). The PBS solution (pH7.4, 10 mM) was prepared by dissolving 0.2g KCl, 8.0g NaCl, 0.24g KH_2PO_4 and 1.44g Na_2HPO_4 in 1000mL

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of ultra-pure water. Anti-NS1 antibody binds to Cysteamine via amide bond; amine groups of Cysteamine form amide bonds with the carboxyl groups in the anti-NS1 antibody.

Step 5 (blocking with Glycine): The fiber tip was immersed for 1 hour in a 50mM aqueous solution of Glycine (represented by black crosses in Fig. 1(b)) to prevent NS1 antigen from binding free-amine groups of Cysteamine. By blocking, one ensures that NS1 antigen will interact only with anti-NS1 antibody.

The NS1 antigen used was dengue antigen nonstructural protein 1 (1 mg/mL, >90% purity) from *AbCAM*. All the other reagents used in this work were purchased from *Sigma-Aldrich Chemical*.

Step 5 is the last step involved in preparing the sensing element of the sensor, which is now ready for testing.

2.2. Sensor testing (NS1 antigen detection)

After the immobilization of anti-NS1 antibody and isolation of unbound ligand groups, the sensing element was ready to be used for sensing dengue NS1 antigen. In order to test if the sensor could detect the presence of NS1 antigen, the fiber tip was incubated by dipping it for 1 hour in a solution of NS1 antigen (red spheres in Fig. 1(b)) in PBS. Tests were also performed with shorter immersion times (approximately 30 min), yielding similar results.

The anti-NS1 antibody used was IgG.

2.3. Experimental setup

The fiber sample was spliced to the setup in Fig. 2, the all-optical fiber sensor based on LSPR [19].

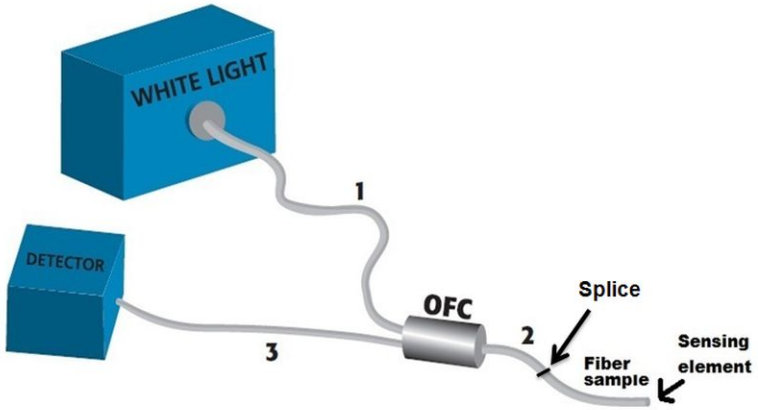

Fig. 2. LSPR-based all-optical fiber sensor: white light source, optical fiber coupler (OFC) and spectrum analyzer (detector). The fiber sample, with the sensing element on its tip, is spliced to Fiber 2.

The system consists of a white light source (*Ando AQ-4303B*), a detector (spectrum analyzer *OceanOptics®USB 2000*) and a 2x1 optical fiber coupler (OFC) with standard multimode fiber (62.5µm/125µm), custom made by *OptoLink Ind e Com Ltda*. The fiber sample was spliced to Fiber 2 of the OFC such that the sensing element was located on the free end (not spliced). Light coupled into Fiber 1 propagated through the coupler and Fiber 2, and into the fiber sample. The light back-reflected from its endface, where the sensing element is located, propagated back though the optical fiber coupler and into Fiber 3, which was connected to the detector/spectrum analyzer. The detected light contained the LSPR signal with information from the sensing element.

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3. Results and discussions

3.1 Dengue NS1 antigen sensing: detection

In order to test if our sensor was capable of detecting NS1 antigen, fifteen fiber samples were prepared. After each of the 5 steps described in section 2.1, the fiber sample was removed from the solution and spliced to the setup (Fig. 2) by the other side, i.e. the side that did not contain the sensing element. The reflected signal was then acquired with the sensing element in air. Figure 3(a) shows the reflected signals obtained for a typical fiber throughout the preparation of the sensing element.

Likewise, in order to test the sensor for NS1 antigen detection, the reflected signal was acquired with the fiber sample spliced to the setup after the procedure described in section 2.2. Figure 3(b) shows the reflected signal acquired and for comparison it also shows the reflected signal after the previous step (Step 5).

Note that all curves have a dip at lower wavelengths. This dip is present in reflection curves obtained with the optical fiber LSPR sensor when the medium surrounding the NPs has refractive index in the range from 1 to approximately 1.4, and exhibits a blue-shift as the refractive index increases [19].

In Figs. 3(a) and 3(b), the wavelength shifts between steps occur because the new component added at each step changed the medium surrounding the NPs, therefore changing its effective refractive index. Note that the wavelength shifts along the steps are not all in the same direction. This occurs because adding each new layer in the procedure will increase (blue-shift) or decrease (red-shift) the effective refractive index. No attempt was made to quantify the effective refractive index surrounding the NPs because this was not the focus of the experiment, however, in principle the model introduced in [19] could be used for this.

All curves in Fig. 3 have been smoothed and shifted vertically for clarity.

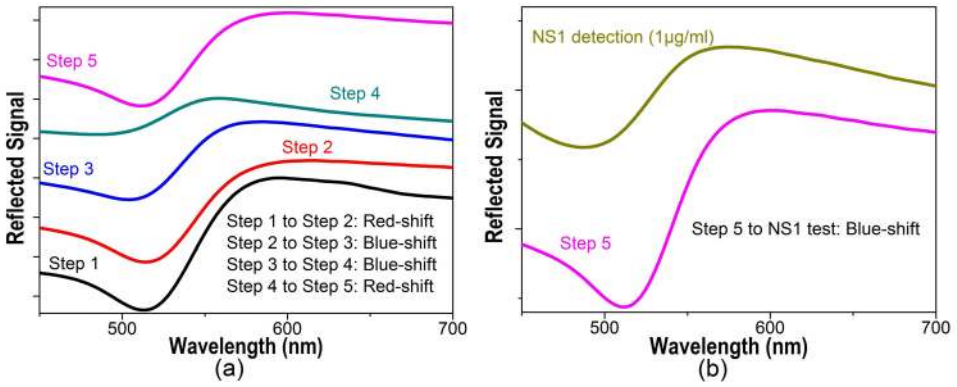

Fig. 3. Reflected signals for a typical fiber sample after (a) Steps 1 to 5 at ligand Cysteamine concentration of 1µg/ml (Step 3) and anti-NS1 antibody concentration of 1µg/ml (Step 4), and (b) when testing the sensor with NS1 antigen with concentration of 1µg/ml.

During the preparation of the sensing element, eleven of the fifteen fiber samples presented the wavelength shift pattern shown in Fig. 3(a): red-shift after the AuNPs cleaning (from Step 1 to Step 2); blue-shift after the ligand deposition/amine-functionalization (from Step 2 to Step 3); another blue-shift after the immobilization of anti-NS1 antibody (from Step 3 to Step 4); and red-shift after the blocking with Glycine (from Step 4 to Step 5). Since the other four fibers presented random wavelength shifts throughout the preparation of the sensing element, they were discarded.

All eleven fiber samples that followed the red-blue-blue-red wavelength shift pattern during the preparation of the sensing element presented a blue-shift when tested with NS1 antigen, as in Fig. 3(b). This result shows that our sensor is reliable for detecting the presence

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of dengue NS1 antigen, as long as the wavelength shifts during the preparation of the sensing element follow the red-blue-blue-red pattern.

3.2 Dengue NS1 antigen sensing: concentration

To investigate if the sensor could be used to assess the concentration of dengue NS1 antigen, solutions with different concentrations of NS1 antigen in PBS were prepared and used in the procedure described in section 2.2. A graph similar to the one shown in Fig. 3(b) was obtained for each measurement and the center of the LSPR dip was located for the reflected signals. The wavelength shift was then calculated as the difference ($\lambda_{NS1detection} - \lambda_{Step5}$).

The black squares in Fig. 4 represent the data of the wavelength shift ($\Delta\lambda$) as a function of NS1 antigen concentration (C_{NS1}) for three different fiber samples. It was possible to use each sample for more than one concentration because the measurements were performed from low concentration to high concentration.

Concentrations of NS1 antigen equal to 0.05μg/ml, 0.1μg/ml, 0.3μg/ml, 0.5μg/ml, 0.7μg/ml and 1.0μg/ml were used. The point (0,0) was added for fitting purposes, and is consistent with the result discussed in section 3.3. Note that $\Delta\lambda$ is negative because the wavelength shifts are blue-shifts, as discussed in the section 3.1.

The uncertainty of measurement ($U_\lambda = \pm 0.5$nm) is represented by black lines at each data point and was estimated using uncertainty Type B methods by taking into account the uncertainty of the detector and the uncertainty in determining the central wavelength. The uncertainty for concentration was not considered, since it was estimated to be small.

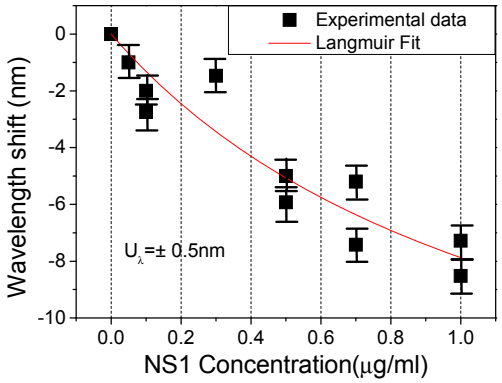

Fig. 4. LSPR wavelength shift ($\Delta\lambda$) at different concentrations of NS1 antigen (C_{NS1}) in PBS solutions (black squares) fitted by the Langmuir Isotherm (red curve).

The Langmuir isotherm equation can be applied to our LSPR sensor to fit the data in Fig. 4. The Langmuir adsorption model is frequently used to fit adsorption data as a function of concentration or partial pressure at a fixed temperature [20]. Among several other applications, this model can be applied to describe the antibody-antigen binding interaction when characterizing a biosensor. The Langmuir model can be applied to our LSPR sensor to characterize the antibody/antigen (anti-NS1/NS1) interaction, assuming the following requirements apply: all adsorption sites are equivalent, the adsorption is limited to only one monolayer, there is only one monolayer involved in adsorption, and the probability of a molecule being adsorbed by a site is independent of neighboring sites. In this case, the wavelength shift measured by our optical fiber LSPR sensor as a function of NS1 antigen concentration can be approximated by the Langmuir isotherm equation expressed as [21],

$$\Delta\lambda = \Delta\lambda_{MAX}\left(\frac{KC_{NS1}}{1+KC_{NS1}}\right) \quad \text{Langmuir isotherm equation} \qquad (1)$$

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where $\Delta\lambda$ is the LSPR wavelength shift due to binding of NS1 antigen to the layer of anti-NS1 antibody; $\Delta\lambda_{MAX}$ is the maximum value of the wavelength shift (at saturation level); K is the affinity constant between anti-NS1 antibody and NS1 antigen, equal to the ratio of bound to non-bound NS1 antigen at equilibrium (saturation); and the independent variable C_{NS1} is the concentration of NS1 antigen in PBS solution. Note that $\Delta\lambda$ and $\Delta\lambda_{MAX}$ are negative because the wavelength shifts are blue-shifts, as shown in Fig. 3(b).

The red curve in Fig. 4 shows the fitting of the experimental data obtained with Eq. (1). From the fitting, the maximum LSPR wavelength shift ($\Delta\lambda_{MAX} = -17.6$ nm) and the affinity constant ($K = 0.81$ ml/μg = 0.039 nM^{-1}) were determined. This result is in accordance with K values presented in [17]: K = 0.029 nM^{-1} for the Mach-Zehnder interferometer sensor and K = 0.063 nM^{-1} for the Michelson interferometer sensor.

The sensitivity of the sensor for NS1 antigen detection is given by [22],

$$S = \frac{|\Delta\lambda_{MAX}|}{\sigma_{MAX}} \tag{2}$$

where σ_{MAX} is the surface density of NS1 antigen when immobilized anti-NS1 antibody have all binding sites occupied, and is given by [22],

$$\sigma_{MAX} = \frac{M_{NS1}}{N_A P_{NS1}^2} \tag{3}$$

where $N_A = 6.02\text{x}10^{23}$ is Avogadro's number, and M_{NS1} and P_{NS1} are the molecular mass and average molecular length of NS1 antigen, respectively. Using $M_{NS1} \approx 48$kg/mol [23] and $P_{NS1} \approx 14$nm [17, 24] in Eq. (3), the surface density is $\sigma_{MAX} \approx 0.41$ ng/mm^2. Using this result in Eq. (2), the sensor sensitivity is equal to S ≈43 nm/(ng/mm^2).

An important procedure to establish the viability of the sensor is the determination of the lowest NS1 antigen concentration that can be quantitatively analyzed with reasonable reliability, known as the limit of quantification. By rewriting the Langmuir isotherm equation (Eq. (1), the limit of quantification ($C_{NS1} \rightarrow C_{lim}$) is given by [17, 25],

$$C_{\lim} = \frac{\Delta\lambda_{RES}}{K(\Delta\lambda_{MAX} - \Delta\lambda_{RES})} \tag{4}$$

where $\Delta\lambda_{RES}$ is the spectrometer resolution ($\Delta\lambda_{RES} \approx 1$nm). Using $\Delta\lambda_{MAX}$ and K obtained from fitting the experimental data, one can estimate that $C_{lim} = 0.074$ μg/ml = 1.54 nM. This quantification limit is in the range of NS1 antigen concentrations in serum samples of patients during the acute phase of the infection (up to 7 days) [10], which vary from 0.04 to 2 μg/ml. This result indicates that AuNP LSPR optical fiber sensor presents a real potential for dengue diagnosis.

3.3 Dengue NS1 antigen sensing: blank

To show that eventual changes in the value of the LSPR wavelength are negligible in absence of NS1 antigen, the fiber was immersed in a blank solution, i.e. a PBS solution without NS1 antigen ($C_{NS1} = 0$). Figure 5 shows the zoomed-in LSPR dip acquired with the fiber in air after Step 5 and after immersion in the blank solution ($C_{NS1} = 0$). The inset graph shows the full spectra.

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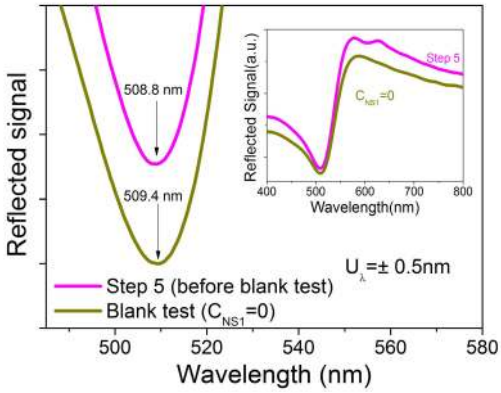

Fig. 5. Zoomed-in view of the LSPR dip after Step 5 (pink curve) and after a 60 min long immersion in PBS solution without NS1 antigen (C_{NS1} = 0, olive curve). Inset graph shows the full reflected spectra.

The uncertainty of measurement (U_λ = ± 0.5 nm) must be taken into account when calculating the wavelength shift in Fig. 5. After Step 5, the central wavelength is λ_{Step5} = 508.8nm ± 0.5 nm = [508.3 nm – 509.3 nm]. After immersion in the blank solution, the central wavelength is $\lambda_{NS1detection}$ = 509.4 nm ± 0.5 nm = [508.9 nm – 509.9 nm]. Since the intervals overlap considerably (40%), one can say that the wavelength shift for C_{NS1} = 0 was negligible or at least below our detection limit.

4. Conclusions

Dengue anti-NS1 antibody was immobilized on AuNPs to obtain an LSPR-based optical fiber sensor for NS1 antigen to be used during the acute phase of the infection. The sensor was shown to have good correlation between wavelength shifts and NS1 antigen concentration, as well as negligible wavelength shift when exposed to a blank solution (NS1 antigen concentration equal to zero). The sensing element is cheap and disposable, and was prepared in 5 straightforward steps. The NS1 antigen detection yields an accurate result in 1 hour, possibly less.

We showed that the sensor is able to detect and quantify dengue NS1 antigen with sensitivity of 43 nm/(ng/mm^2). The NS1 antigen concentration quantification limit (0.074 μg/ml = 1.54nM) was estimated. Considering that the level of secretory protein of dengue virus in the blood of an infected person in the acute phase is between 0.04 and 2.0 μg/ml, the proposed sensor can be a valuable tool for early diagnosis of the disease.

These results indicate that the sensor can be a powerful tool for sensing dengue NS1 antigen in samples from patients who exhibit symptoms that fit the clinical presentation of dengue. Future development of this sensor will involve attempting to use it to detect NS1 antigen in serum samples.

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