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Immunofluorescence–based biosensor for the determination of dengue virus NS1 in clinical samples

Nadiya T. Darwish [a], Shamala D. Sekaran [b], Yatimah Alias [a,c], Sook Mei Khor [a,c,*]

[a] Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Department of Medical microbiology, University of Malaya, 50603 Kuala Lumpur, Malaysia
[c] University Malaya Centre for Ionic Liquids (UMCiL), University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

The sharp increase in incidence of dengue infection has necessitated the development of methods for the rapid diagnosis of this deadly disease. Here we report the design and development of a reliable, sensitive, and specific optical immunosensor for the detection of the dengue nonstructural protein 1 (NS1) biomarker in clinical samples obtained during early stages of infection. The present optical NS1 immunosensor comprises a biosensing surface consisting of specific monoclonal NS1 antibody for immunofluorescence-based NS1 antigen determination using fluorescein isothiocyanate (FITC) conjugated to IgG antibody. The linear range of the optical immunosensor was from 15 – 500 ng mL^{-1}, with coefficient of determination (R^2) of 0.92, high reproducibility (the relative standard deviation obtained was 2%), good stability for 21 days at 4 °C, and low detection limit (LOD) at 15 ng mL^{-1}. Furthermore, the optical immunosensor was capable of detecting NS1 analytes in plasma specimens from patients infected with the dengue virus, with low cross – reaction with plasma specimens containing the Japanese encephalitis virus (JEV) and Zika virus. No studies have been performed on the reproducibility and cross-reactivity regarding NS1 specificity, which is thus a limitation for optical NS1 immunosensors. In contrast, the present study addressed these limitations carefully where these two important experiments were conducted to showcase the robustness of our newly developed optical-based fluorescence immunosensor, which can be practically used for direct NS1 determination in any untreated clinical sample.

1. Introduction

Dengue fever is a major public health crisis in tropical and subtropical regions of the world [1,2]. The rapid diagnosis of dengue virus (DENV) infection is crucial for limiting the spread of this disease [3,4]. ELISA (enzyme-linked immunosorbent assay) is currently the most frequently used diagnostic assay for DENV. This immunoassay is used to detect non-structural protein 1 (NS1), a DENV glycoprotein produced by infected host cells, or DENV-specific IgM and IgG antibodies (Abs) [5]. ELISAs are inexpensive and sensitive enough to detect analytes present at very low concentrations (0.1–2 μg L^{-1}) [6,7]. However, these assays are also time-consuming. For example, ELISAs measuring DENV specific IgM require about 1–2 days to perform. In addition, production of a chromogenic signal on enzyme-substrate interaction is a requisite in ELISA, and this enzyme-mediated reaction requires approximately 15 min to produce a detectable color change. However, this enzyme-mediated color change is not stable and can change indefinitely over a long period. Therefore, the resulting color intensity imprecisely reflects the quantity of primary antibody, and can yield false-positive results [6,8]. In traditional ELISA, enzymes such as horseradish peroxidase and alkaline phosphatase are often used for the amplification of the chromogenic signal. Nevertheless, the enzymes are usually costly and the catalytic activity of enzymes is sensitive to the environmental changes, e.g., temperature and pH. Enzyme denaturation caused by these environmental factors would compromise the accuracy of ELISA results and render them irreproducible [9,10]. Therefore, more stable indicators, such as fluorescence labels, are required to be developed. Fluorescence ELISAs [8] and immunospot assays [11] have been suggested as a means to overcome the limitations of conventional ELISAs. Fluorescence ELISAs have been conducted by Hosseini et al. using DENV – specific Ab bound to methacrylic microspheres via surface

* Corresponding author at: Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail address: naomikhor@um.edu.my (S.M. Khor).

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carboxyl groups on the microsphere surface. This composite was used to detect DENV using a sandwich ELISA with FITC – conjugated antibodies [8]. In addition, immunospot assays have been conducted by Linares et al. using nitrocellulose membrane as a platform for immunoreactions between NS1 antigen and anti-NS1 antibody coated fluorescent particles [11]. These studies have revealed that assays using fluorescent dyes have a high sensitivity, require a short processing time of only 45–60 min compared to the 3 – 5 h of classical ELISAs, and can be conducted using smaller sample volumes (4 μL) than commercial ELISA assays (100 μL). Another immunoassay reported by Ranzoni et al. involved the use of quantum dots and monoclonal antibody fragments linked to polystyrene nanoparticles [12]. This molecular architecture was used to detect NS1 antigen from DENV serotypes 1–4 in a buffer. This immunoassay displayed excellent sensitivity for NS1 and had a low limit of detection (LOD) of 1 ng mL^{-1}. However, a low LOD is not the ultimate goal when designing this assay, as the ability to detect DENV-NS1 analyte concentrations within the clinical range is of more practical importance. In the clinic, NS1 levels range from 0.04 to 2 μg mL^{-1} in primary infection sera and from 0.01 to 2 μg mL^{-1} in secondary infection sera [13]. Unfortunately, the three studies described above did not evaluate the cross-reactivity of the proposed immunoassays with other flaviviruses, e.g., Japanese encephalitis virus (JEV) and yellow fever virus. Therefore, because the main limitation of DENV serological tests is the occurrence of false-positives that are likely cross-reactive with co – circulating antibodies from other infecting flaviviruses, selectivity studies are necessary.

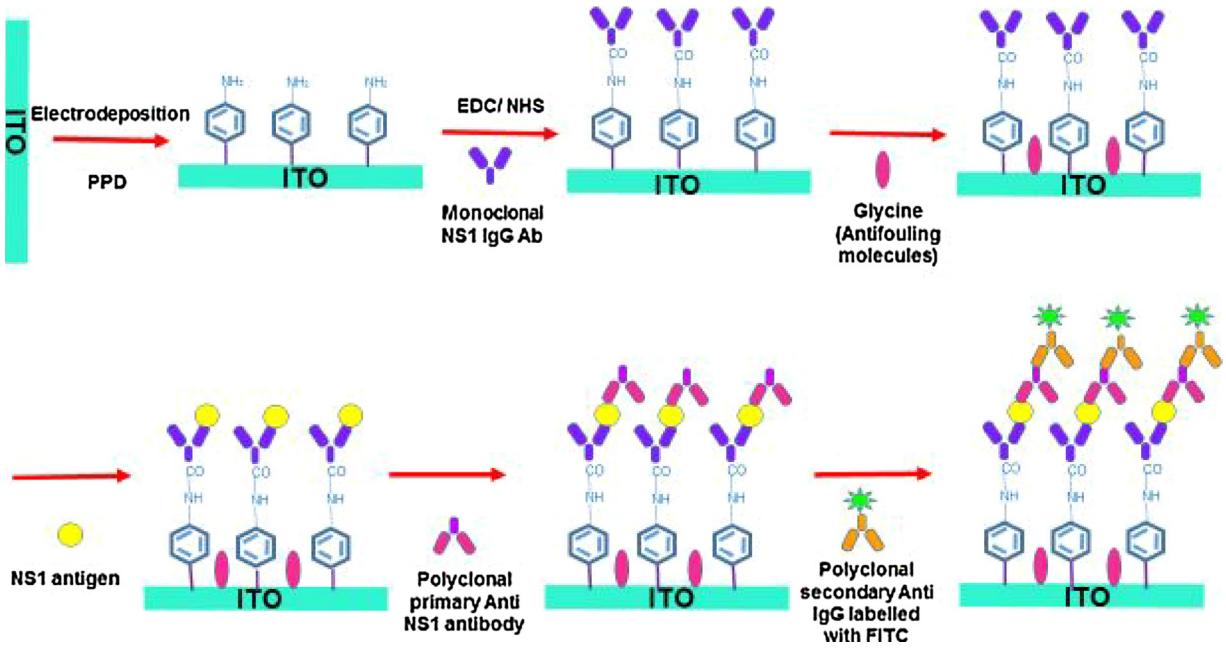
Immunochromatography testing (ICT), such as that used for qualitative measurement by the DENV NS1 Antigen STRIP Kit, is also currently used for diagnosing DENV infection. This diagnostic test can be easily performed in basic laboratories or patient homes, has good specificity, and enables early diagnosis of acute DENV infection. However, the results of this test when used to detect NS1 must be interpreted with caution for patients with secondary DENV infections or when testing patient samples obtained after the fifth day of illness, as the results are more likely to be negative in these patients despite DENV infection [14,15]. Furthermore, the ICT test is less sensitive than PCR (polymerase chain reaction) [15] as PCR allows detection of 1 plaque forming unit (pfu) of DENV [16]. Recently, electrochemical biosensors based on impedimetric [17], differential pulse voltammetry [18], and capacitance measurements [19], optical biosensors based on surface plasmon resonance (SPR) [20], fluorescence immunosensing [21], and piezoelectric biosensors using frequency monitoring quartz crystal microbalance [22] have received a great deal of attention owing to their high sensitivity, simplicity of use, and specificity [12,17,23,24].

Optical immunosensors based on liposome-labeled reporter probe and nucleic-acid sequence-based amplification (NASBA) have been described for DENV viral RNA detection. The use of the NASBA technique has been previously suggested [25,26] to eliminate the effect of background optical signals and therefore improve biosensor sensitivity for the detection of low concentrations of target analytes. However, this technique requires careful handling and storing of molecular targets such as viral nucleic acids. As a consequence, this technique limits the use of RNA amplification-based techniques for field applications such as epidemiological studies [27]. Label-free optical immunosensors based on surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR) have been developed for the immunodetection of various pathogens, including DENV. A biosensor model for NS1 detection, based on LSPR and specular reflection from gold nanoparticles (AuNPs), has been previously described [28]. DENV anti-NS1 antibody was immobilized on AuNPs deposited on the end face of a standard multimode fiber. SPR measurement was performed based on determination of the increment in the resonance angle of the surface containing the deposited sample in the presence of DENV. In another study, the dengue virus NS1 antigen (biorecognition molecule) conjugated with bovine serum albumin (BSA) was covalently immobilized on a gold sensor chip via activation of a self-assembled monolayer (SAM) of 11– mercaptoundecanoic acid by amide coupling. In this study, the presence of dengue virus-specific IgM antibodies in dengue-positive sera was monitored by measuring the increase in resonance angle via direct immunoassay using SPR [20]. The detection of dengue NS1 antigen using LSPR has additionally been reported in another previous study [29]. That biosensor model consists of a straight gold (Au) stripe embedded in Cytop cladding, with an etched microfluidic channel for sensing. The detection of NS1 antigen in clinical samples was estimated based on calculation of the change in surface mass density ΔG (in pg mm^{-2}) due to the interaction between dengue NS1 antigen and Mab, using straight long-range surface plasmon waveguides. The SPR technique is capable of providing typical signal resolution compared with optical fibers and other photonic devices; nevertheless, SPR measurement may be affected by background optical signals from the clinical sample. This limitation reduces the utility of the SPR technique for the detection of low levels of biomolecules, such as NS1, in clinical samples. In addition, SPR imaging has a limited resolution. Therefore, extended-resolution optical imaging techniques have been developed, e.g., SPR imaging [30] and widefield fluorescence microscopy [31].

Therefore, a simple, highly sensitive and selective, and reproducible fluorescent probe that serves as a powerful tool to assess and quantify NS1 in clinical human blood samples was proposed on the basis of a sandwich immunoassay. The present optical immunosensor has several advantages over other currently used diagnostic tests (e.g., NS1 Rapid Diagnostic Test [NS1 Ag Strip]) wherein the newly developed NS1 optical immunosensor can overcome the lack of sensitivity and quantitative ability, which are often encountered by most of the lateral flow tests. Improving analytical sensitivity and providing quantification measurements for NS1 biomarker are essential because they report the presence of DENV in complex clinical samples and determine disease severity.

The aim of the present study was to develop a simple, specific, sensitive, stable, and reproducible immunoassay to overcome the lack of reproducibility (due to surface binding chemistry) of electrochemiluminescence-based biosensors. The stable biosensing interface described in this work was prepared by electrochemical deposition of phenylenediamine, which enables covalent binding between 1,4 – phenylenediamine molecules and the ITO (indium tin oxide) substrate. The stability of the biosensing interface was additionally attributed to the binding between 1,4 – phenylenediamine molecules and the monoclonal NS1 antibody, based on 1 – ethyl – 3 – (3 – dimethylaminopropyl)carbodiimide (EDC) and N – hydroxysulfosuccinimide (NHS) chemistry (Scheme 1). Furthermore, the present immunoassay design was capable of providing data for the detected analyte, without the need for the complex data processing required for SPR measurement. The fluorescence intensity of the FITC – conjugated IgG antibody may be quantitatively determined by fluorescence microscopy.

In the present study, we focused on the development of an antibody-based immunofluorescence biosensor, as the use of antibodies as biorecognition elements enables selective and specific recognition of analytes, with the production of quantifiable signals [32,33]. The use of these monoclonal antibodies and FITC – conjugated antibodies, which provide high quantum yield, enhances the sensitivity of optical immunosensors [32,34]. The proposed immunofluorescence-based technique is simple, and may be easily performed in any laboratory equipped with basic instrumentation. Furthermore, unlike SPR-based detection, the present technique involves the production of visible light, which does not



Scheme 1. Schematic of the fabrication of immunofluorescence biosensor for determination of biomarker NS1 antigen in early stage of dengue virus infection.

require chemiluminescence reactions or complex data. Furthermore, immunofluorescence maybe used for the detection of NS1 antigens in clinical specimens without the need for sample pretreatment, which is required for detection using NASBA – based biosensors.

2. Materials and methodology

2.1. Chemicals, instrumentation and procedures

Indium tin oxide (ITO) slides were purchased from Sanyo (Japan). The following chemicals were purchased from Sigma – Aldrich (USA): 1,4 – phenylenediamine, sodium nitrite ($NaNO_2$), potassium chloride (KCl), potassium phosphate dibasic (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), hydrochloric acid (HCl), potassium ferricyanide ($K_3Fe(CN)_6$), and glycine. NS1 antigen from serotype 2 DENV and monoclonal anti-NS1 IgG antibody were purchased from GenScript (USA). Polyclonal DENV type 2 NS1 antibody and polyclonal anti rabbit IgG-FITC conjugate secondary antibody were purchased from Thermo Scientific (USA). All solutions were prepared using Milli-Q water (18 MΩ Cm^{-1}) (PURELAB, USA).

The deposition of 1,4 – phenylenediamine and electrochemical measurements were performed using an AutoLabIII potentiostat (Metrohm AutoLab, Netherlands) and a conventional three-electrode system consisting of an ITO working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl (3.0 M NaCl) as the reference electrode. All potentials were reported relative to that of the Ag/AgCl reference electrode. The stepwise fabrication studies of the modified sensing interface were performed in an aqueous solution containing redox probes $[Fe(CN)_6]^{4-/3-}$ (1 mM) composed of 0.05 M KCl, 0.05 M K_2HPO_4, and 0.05 M KH_2PO_4, adjusted to pH 7.0. Electrochemical impedance spectroscopy (EIS) techniques and Fourier Transform Infra – Red (FT – IR) Spectrum™ 400 (Perkin Elmer, USA) were used to confirm the deposition of linker layer (PPD), the biorecognition layer (monoclonal NS1 Ab), and antifouling molecules (glycine) on electrode surfaces. The electrodeposition of 1,4 – phenylenediamine layers onto the ITO electrode surface was performed using cyclic voltammetry at −0.6 V to 0.2 V at 100 mV s^{-1}. The EIS measurements were performed within the frequency range of 0.1–10^3 Hz and 10 points per decade of frequencies with a single sine wave type at 25 °C. Nova software (Metrohm AutoLab, Netherlands) was used to model the complex circuit. Atomic force microscopy (AFM) (Ambios, UK) was additionally used to study the morphology of monoclonal NS1 Ab and NS1 antigen layers, in the absence or presence of PPD, with scan size 10 × 10 μm and scan rate 1 Hz/sec. AFM images were analyzed using software (Q – Scope 250/400 Nomad, version 5). A fully automated fluorescence microscope (FM) (Leica, USA) was used to obtain images for the optical immunofluorescence biosensor at xy scan mode with fluo – filter cube 470; the exposure was set at 300 ms. Images (1024 × 1024 pixels) were captured at 8-bit resolution using the Las AF software (Origin) using an objective lens: 10.0/0.25 Dry. The gray value represents the fluorescence intensity. Naked-eye inspection of the targets is inaccurate because human eyes are insensitive to optical density variations at the same wavelength [35], especially at very low concentrations (ng mL^{-1}), as in the present study. Therefore, ImageJ (IJ – 1.48) software was used for image processing and quantification analysis in Java. Captured TIF format images were further analyzed using ImageJ (IJ – 1.48 g jar) software to determine the mean gray value after background subtraction.

2.2. Immunosensor fabrication for NS1 antigen detection

ITO slides were cleaned in an ultrasonicator using dichloromethane and then using 99% methanol for 10 min each, followed by washing with 0.5 M K_2CO_3 in a 3:1 methanol:Milli – Q water mixture for 30 min under sonication to eliminate residual organic contaminants. The ITO substrates were then washed with copious amounts of Milli Q water, dried, and stored in a nitrogen – filled container until use (M. Chockalingam et al., 2011). The bare ITO electrode was modified with 2 mM 1,4 phenylenediamine. For modification of the electrode surface, an in situ method was used in which aryl diazonium cations were electrochemically reduced with two times the molar amount of $NaNO_2$ in 0.05 M HCl. The modification solution was purged with nitrogen gas for 20 min before surface modification. Electrodeposition was performed using cyclic voltammetry at −0.6 V–0.2 V; the electrodes were scanned for 5 cycles at 100 mV s^{-1} to produce the first layer on the ITO electrode. Monoclonal anti–NS1 IgG antibodies

were incubated with EDC (200 mM) and NHS (50 mM) cross linkers for 30 min to activate carboxyl groups of the monoclonal antibodies [36]. Then, 50 μL of antibody solution was added to the 1,4 –phenylenediamine layer and incubated for 45 min at 25 °C to bind the monoclonal antibodies to the amine groups of 1,4–phenylenediamine. Next, the ITO substrate was rinsed with 50 mM NaCl to remove unbound antibodies on the electrode surface, and then washed 3 times with 10 mM phosphate buffer saline (PBS) at pH 7.4. Subsequently, the modified surface with anti–NS1 IgG monoclonal antibodies was incubated with 50 μL of 10 mM glycine to block non-specific sites (i.e., sites that did not contain monoclonal Ab) on the ITO–modified electrode. Glycine additionally possesses efficient and long-term antifouling properties in biological environments e.g., in human serum samples [37,38]. The modified surface thus developed served as the sensing interface for the detection of the NS1 antigen. The fabricated immunosensor was characterized using electrochemical measurements, AFM, and the FT–IR^{TM} 400 Spectrum.

2.3. Analytical performance of the optical immunofluorescence biosensor, as assessed by fluorescence microscopy

2.3.1. Sensitivity, reproducibility, and stability studies

The NS1 antigen was added to dilute human sera (10% in PBS, pH 7.4) at various concentrations in order to evaluate the sensitivity and determine the dynamic range and limit of detection (LOD) of the former. The NS1 antigen in human sera solution (50 μL) was pipetted onto the sensing surface. Then, the antigen was allowed to bind to the monoclonal NS1 antibody (used as the capturing antibody) – modified ITO slides for 45 min at 25 °C. This antigen–antibody complex was further incubated in 50 μL of polyclonal NS1 antibodies for 30 min to generate a sandwich immunoassay. The polyclonal NS1 antibodies (DENV type 2 NS1 antibody) used as the detecting antibody were diluted at a ratio of 1:100 in PBS, pH 7.4 (concentration 1 mg mL^{-1}). Finally, 50 μL of the secondary polyclonal FITC – conjugated antibody (at a ratio of 1:500, concentration 1.5 mg mL^{-1}) was added and incubated on the immunosensor for 30 min. Polyclonal anti rabbit IgG–FITC–conjugated secondary antibody as used in this study, as the labeling of primary polyclonal NS1 antibody changes antigen-binding features and may completely inactivate the antibody [39]. The immunosensors were rinsed thoroughly with PBS (pH 7.4) after each step of incubation, and signal measurement for the optical immunofluorescence biosensor was performed using a fluorescence microscope.

The reproducibility of the fabricated immunosensor was studied by testing five different modified slides. Then, the prepared immunosensors were incubated in a NS1 antigen solution (1 μg mL^{-1}) for 45 min. Next, the slides were soaked in PBS (pH 7.4) for 5 min and washed carefully with PBS (pH 7.4) to remove non-specifically adsorbed NS1 antigen. The slides were incubated with polyclonal NS1 antibodies to form a sandwich immunoassay, followed by the attachment of secondary polyclonal FITC – conjugated antibody. The fluorescence intensity (mean gray value) was measured for each slide, and the relative standard deviation for all five slides was calculated. For stability studies, twelve immunosensors were prepared following the procedure described earlier. Three of these immunosensors were examined on the same day for NS1 detection in human sera. The NS1 immunosensor was incubated in 50 μL of NS1 antigen (500 ng mL^{-1}) for 45 min. Then, the immunosensor was incubated with polyclonal NS1 antibodies to form a sandwich assay, followed by incubation with the secondary polyclonal FITC – conjugated antibody. The other nine immunosensors were stored in 10 mM PBS (pH 7.4) at 4 °C. Three of these immunosensors were examined each day at days 7 and 14 to test the stability of the modified slides over a 14 – day period. The fluorescence intensity (mean gray values) was measured for each slide using a fluorescence microscope, and the relative standard deviation was calculated for the tested slides.

2.3.2. Detection of the NS1 antigen in clinical samples and cross reactivity study

The fabricated immunosensor was further assessed using plasma samples from patients infected with DENV to evaluate its suitability for use in clinical diagnostic testing. First, the clinical samples were examined and confirmed positive for NS1 and negative for IgM and IgG based on ELISA – based serological assays and DENV NS1 Ag + Ab Combo tests. Five uninfected plasma samples were collected from patients with negative results for NS1, IgM, and IgG; these samples were used as controls. Both positive and negative plasma samples were diluted to various dilution factors (1:10, 1:100, 1:500, and 1:1000) using PBS (pH, 7.4). The fabricated immunosensors were incubated with 50 μL of diluted plasma samples at the various dilutions. After incubation for 45 min, the immunosensors were incubated with polyclonal DENV type 2 NS1 IgG antibodies and next with polyclonal anti rabbit IgG – FITC conjugate secondary antibody. Then, the fluorescence intensity of slides incubated with positive and negative plasma samples was compared.

The potential utility of the fabricated immunosensor in clinical diagnostics was further evaluated testing plasma samples containing Japanese encephalitis virus (JEV) and Zika virus for cross-reactivity studies. The fluorescence optical density of these plasma samples was compared with that of plasma samples containing NS1 antigen. All plasma samples were serially diluted to various dilution factors (1:50, 1:100 and 1:1000) using PBS (pH, 7.4). The modified slides were incubated with 50 μL of diluted sera at the various dilutions. After incubation for 30 min, the slides were examined using a fluorescence microscope.

3. Results and discussion

3.1. Characterization of the fabricated optical immunosensor

3.1.1. Electrochemical characterization of the modified electrodes

The cyclic voltammetry technique was used to modify the ITO electrode using 1,4 – phenylenediamine, which served as a linker molecule to bind monoclonal NS1 antibodies to the ITO substrate (supplementary material: Fig. S1A). The reduction in peak current in the second cycle of modification indicated that 1,4 phenylenediamine molecules were successfully deposited on the electrode surface. The basic mechanism involved in this modification is the generation of aryl radicals at the solution/electrode interface. This occurs via one – electron electro reduction of the corresponding diazonium salt, using twice the molar amount of $NaNO_2$ in 0.05 M HCl. Subsequently, the aryl radical attaches to the surface of the ITO electrode, which results in the formation of a covalent bond between the ITO substrate and the carbon atom of the modifier molecule.

The generation of a Nyquist plot of the electrochemical impedance spectrum represents an effective technique for monitoring the stepwise fabrication of the biosensor, using 1 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ as a redox probe [17,40–42]. The semicircle diameter of the Nyquist plot represents the electron-transfer resistance at the electrode surface. The formation of a well-organized layer on the modified electrode typically obstructs the diffusion of the redox probe, as an increase in the diameter of the semicircle causes high electron transfer resistance. A significant increment in the diameter of the semicircle on the Nyquist plot was observed after the modification of bare ITO with 1,4 – phenylenediamine molecules, as this organic layer barred the redox molecule from

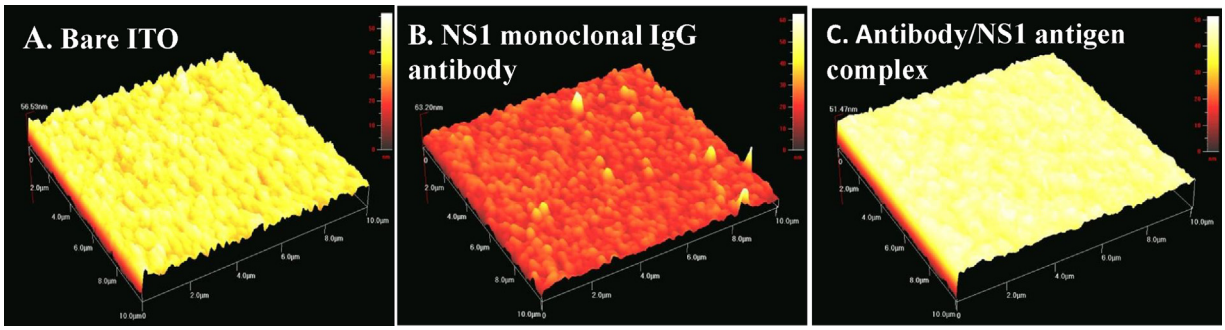


Fig. 1. Topographic atomic force microscope (AFM) images (A) a bare ITO surface, (B) the immobilized antibody (NS1 monoclonal IgG antibody), (C) AFM image of anti NS1 monoclonal IgG antibody/NS1 antigen complexes; the size of the scanning area is 10 × 10 μm and the scan rate 1 Hz/sec.

accessing the electrode surface (supplementary material: Fig. S1B.ii). Further increases in semicircle diameter were observed when the capturing antibody monoclonal anti – NS1 IgG antibodies were covalently bound to 1,4 – phenylenediamine linker molecules using EDC/NHS (Fig. S1B.iii). A slight increase in the Nyquist plot semicircle was observed when glycine was deposited to block non-specific sites on the modified ITO substrate (Fig. S1B.iv).

3.1.2. Morphological characterization of the developed immunosensor by atomic force microscopy

Antibody immobilization was confirmed by comparing atomic force microscopy images of ITO substrate before and after functionalization. The observed increase in roughness relative to bare ITO (Fig. 1A) suggested carbodiimide coupling of monoclonal NS1 Ab through the linker molecule 1,4 – phenylenediamine to the ITO surface (Fig. 1B). The root mean square roughness (*Rq*) values obtained by AFM were 4.4 for the bare ITO electrode and 74.2 for ITO functionalised with the monoclonal NS1 IgG antibody. After incubation with the NS1 antigen (Fig. 1C), the root mean square roughness (*Rq*) decreased to 3.17, which was attributed to the conglomeration of NS1 globular structures. This observation confirmed binding of the NS1 antigen to the monoclonal NS1 antibody. In addition, skewness (*RSk*) was used to measure the profile symmetry about the mean. In this form of analysis, if the height distribution is asymmetrical and the surface has more peaks than valleys, the skewness is positive. However, if the surface is more planar and valleys are predominant, the skewness is negative [43]. Therefore, the *RSk* value of −3.69 for the NS1 antigen layer confirmed binding of globular structures of NS1 to monoclonal NS1 IgG antibody.

3.1.3. Characterization of the modified electrodes by fourier transform infra-Red (FT-IR) spectrum

The FT–IR technique was used to characterize the fabricated immunosensor interface. The ITO electrode modified with 1,4–phenylenediamine (surface 2) (Fig. 2ii) shows specific peaks for amino functional group at 3317 cm^{-1} and aromatic rings at 1652 cm^{-1}. This observation confirmed that 1,4 – phenylenediamine was successfully deposited at the ITO electrode. In Fig. 2iii, the spectra show the peak for N—H stretching (at 3370 cm^{-1}) and the peak for carbonyl groups (at 1626 cm^{-1}). The band for C—N stretching (1300–1000 cm^{-1}) was clearly visible in the spectra for Surface 3; these bond signals confirm binding between the monoclonal anti-NS1 IgG antibody and amino groups of 1,4–phenylenediamine via the formation of amide bonds (—CONH). Surface 3 was further modified using glycine (antifouling molecule) (Fig. 2iv). Glycine bound the amine group of 1,4 – phenylenediamine molecules that did not bind the monoclonal anti-NS1 IgG antibody. The signal peak between 3050-

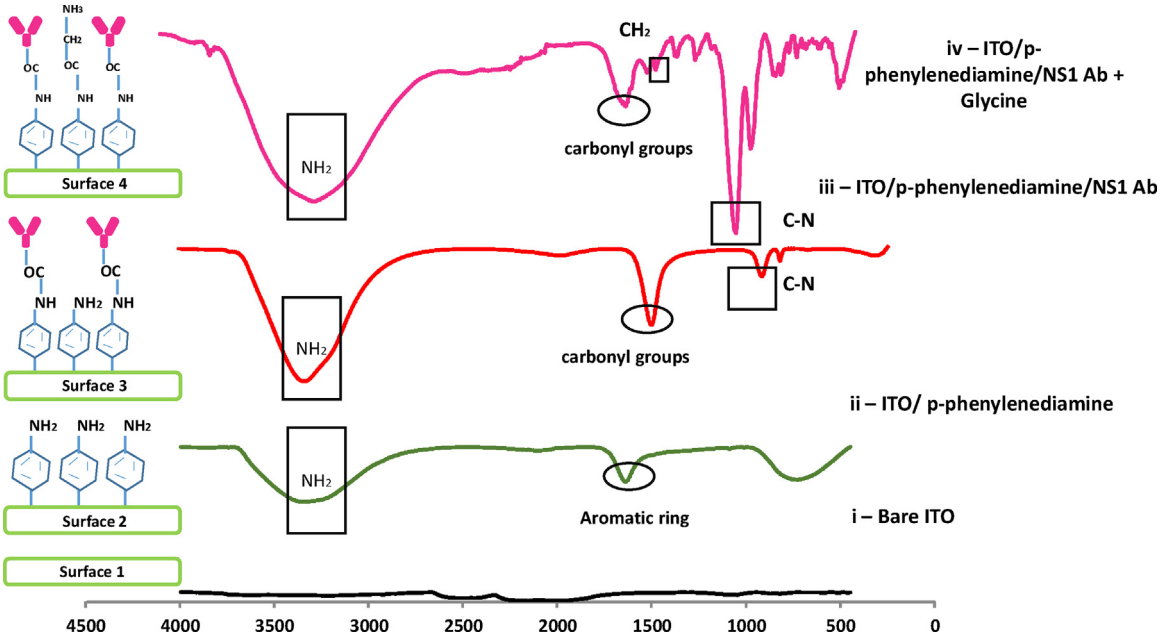


Fig. 2. FT-IR surface characterization of (i) the bare ITO surface, (ii) ITO/PPD, (iii) ITO/PPD/NS1 monoclonal IgG antibody (50 μg mL^{-1}) and (iv) ITO/PPD/NS1 monoclonal IgG antibody + Glycine.

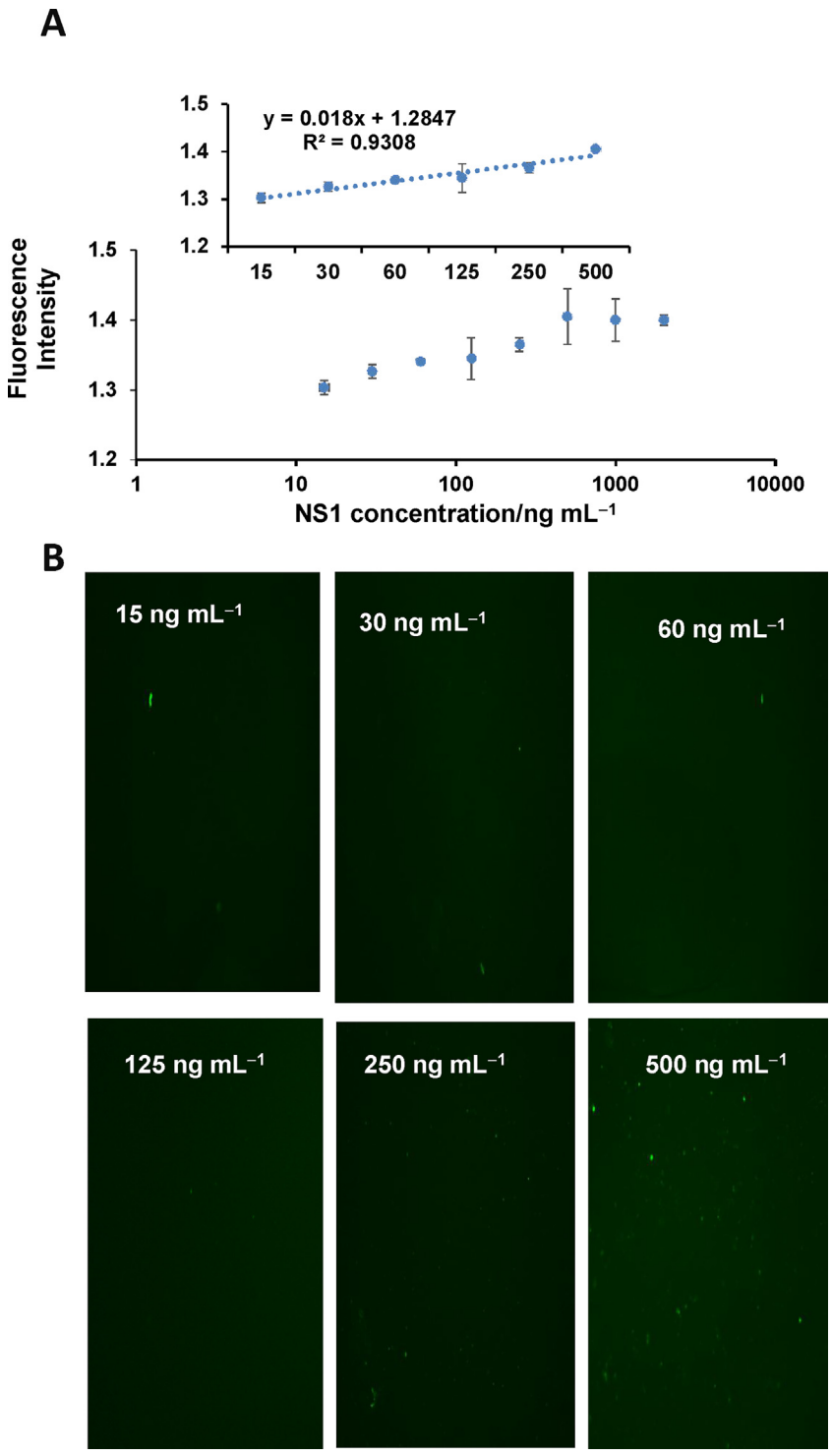


Fig. 3. (A) The calibration plot corresponding to the increase in fluorescence intensity (mean gray values) with various concentrations of NS1 antigen (15, 30, 60, 125, 250, and 500 ng mL^{-1}). (B) Fluorescence microscopy images of six different electrodes incubated with NS1 antigen concentrations of 15, 30, 60, 125, 250, and 500 ng mL^{-1}.

2675 cm^{-1} belonged to the NH broad band. The signal peak for carbonyl groups (at 1626 cm^{-1}) and the band for CN stretching (1300–1000 cm^{-1}) are attributed to amide bonds (—CONH) between the monoclonal anti—NS1 IgG antibody and amino groups of 1,4 phenylenediamine (PPD), and between carboxyl groups of glycine molecules and amino groups of 1,4–phenylenediamine. The signal peak at 1466 cm^{-1} corresponds to the CH bending vibrations of glycine molecules.

Fig. 2

3.2. Analytical performance of the optical immunofluorescence biosensor as investigated by fluorescence microscopy

3.2.1. Sensitivity, reproducibility, and stability studies

In order to assess the sensitivity and dynamic working range of the proposed immunoassay method, the fabricated immunosensor was incubated with various concentrations of NS1 antigen (ranging from 15 – 2000 ng mL^{-1}) in 10% human sera diluted with PBS (pH 7.4) (Fig. 3A). A calibration curve was plotted using the FITC flu-

orescence emission signal of the polyclonal anti rabbit IgG–FITC conjugated secondary antibody. Using ImageJ (IJ–1.48) software analysis, the increase in immunosensor FITC fluorescence emission of labeled IgG was found to be linear to the NS1 analyte concentration. The calibration curves were further used to determine the immunosensor essential parameters such as the coefficient of determination, sensitivity, and the lower detection limit. The coefficient of determination (R^2) obtained was 0.92; in addition, the optical immunosensor was shown to be capable of detecting the NS1 antigen in serum samples with a LOD of 15 ng mL^{-1} (Fig. 3A) and (Fig. 3B). Differences in optical densities were not perceptible to the unaided human eye, especially at very low concentrations (ng mL^{-1}). Therefore, ImageJ (IJ–1.48) software was used to determine NS1 analyte concentrations on the basis of fluorescence intensity.

The utility of immunosensors for clinical applications relies on their ability to detect the disease biomarker within the range of concentration in patient's sera. The LOD for the optical immunosensor presented in this study was 15 ng mL^{-1}, which is far below the NS1 levels detectable in patient sera, which range from 0.04 to 2 μg mL^{-1} in primary infection sera and from 0.01 to 2 μg mL^{-1} in secondary infection sera [13]. The present optical immunosensor shows higher sensitivity than previously developed LSPR-based biosensors [28]. The LOD for these previously reported assays was 74 ng mL^{-1} which is higher than the NS1 concentration detected in patient sera, in both primary and secondary infection [13]. The sensitivity of the present fluorescence – based optical immunosensor was however lower than that of optical biosensors for NS1 detection that utilize long-range surface plasmon waveguides [29]; the LOD of the latter was estimated to be 5.73 pg mm^{-2}. However, this reported detection limit was theoretically determined based on calculation of the estimated surface mass density.

The ability of the present optical immunosensor to detect NS1 at reasonably low detection limits is attributable to the FITC label of the secondary antibody, which is bound to antibodies that specifically bind NS1 antigen. Fluorescein-labeled antibodies (FITC) provide high quantum yield when conjugated with affinity – purified antibodies [44,45]. Furthermore, the present optical immunosensor is highly sensitive as NS1 detection was performed using indirect immunofluorescence, which enables signal amplification as multiple secondary antibodies binding to a single primary antibody [46].

The reproducibility of the optical immunosensor was evaluated by testing three different electrodes incubated with 0.125 μg mL^{-1} NS1 antigen. Good reproducibility was achieved with relative standard deviation (RSD) of 2%. Good reproducibility, relative to other electro-chemiluminescence-based biosensors for DENV RNA detection, represents another important advantage of the present optical immunosensor. The RSD of previously reported sensors was 2.6 for dengue serotype 1 detection, 1.7 for dengue serotype 2 detection, and 2.5 for dengue serotype 3 detection [47] or for the detection of DENV particles. These results demonstrate that the reproducibility of the chemiluminescence–based OFIS was lower than that of MAC–ELISA [48]. The acceptable degree of reproducibility obtained for the present fabricated NS1 immunosensor may be attributable to the immunosensor design and fabrication procedure utilized; the use of a monoclonal antibody for specific detection of the NS1 antigen enhances the reproducibility of the immunosensor. Therefore, the obtained FITC fluorescence signal was stable. Additionally, the reproducibility and the robustness of the fabricated optical immunosensor indicate that the monoclonal anti-NS1 antibodies were uniformly dispersed on the electrode substrate. The homogeneous distribution of biorecognition molecules over the ITO electrode results in stable fluorescence intensity <REFNUMLINK>[49].

The stability of the optical NS1 immunosensor was evaluated by testing at weekly intervals. The immunosensing interface retained 90.4% of its binding affinity towards NS1 antigen after being stored

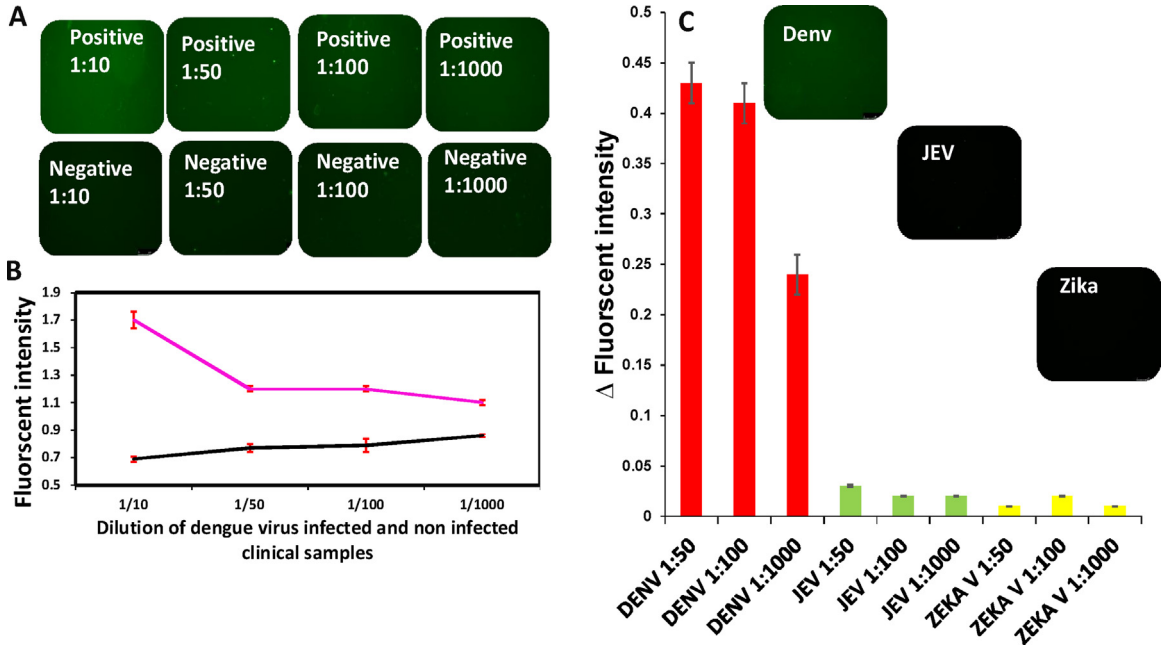


Fig. 4. (A) Fluorescence microscopy image for positive and negative human plasma samples at xy scan mode, 1024 × 1024 pixels, fluo-filter cube 470 and exposure sitting 300 ms. (B) Fluorescence intensity measurement for NS1 antigen in positive and negative human plasma samples at different dilution factors (1:10, 1:100, 1:500, and 1:1000) from a patient infected with the dengue virus. (A) Fluorescence microscopy images of human plasma samples positive and negative for NS1 antigen in XY scan mode, 1024 × 1024 pixels, fluo-filter cube 470, and exposure sitting 300 ms. (B) Quantification of fluorescence intensity of NS1 antigen in plasma samples from a patient infected with the dengue virus at dilutions of 1:10, 1:100, 1:500, and 1:1000. (C) Quantification of fluorescence intensity of plasma samples containing NS1 antigen, JEV, and Zika virus at sample dilutions of 1:50, 1:100, and 1:1000. Insert: Fluorescence microscopy images of human plasma samples infected with dengue, JEV, or Zika virus. Note 1: Fig. 4C, delta (Δ) represents the difference in fluorescence intensity of immunosensors incubated with positive clinical specimens compared to negative/control immunosensor slides.

Table 1
Comparison with previously reported optical biosensors for dengue virus determination.

Type of optical sensor.	Analyte	Analytical performance				Advantages	Limitations	Ref
		Linear range	Limit of detection	Reproducibility	Analysis of clinical samples			
The biosensor is a membrane – based DNA/RNA hybridization system using liposome amplification. Signals from liposomes are detected by a reflectometer	RNA nucleic acid	10 – 50 particles	10 plaque – forming units (pfu mL^{-1})	• The RSD* for dengue serotype 1 detection is 2.6 • The RSD for dengue serotype 2 detection is 1.7 • The RSD for dengue serotype 3 detection is 2.5 • The RSD for dengue serotype 4 is not available	Sensitive and specific for serotypes 1, 2, and 4. Weak positive signals were additionally generated for serotype 3 by biosensors designed for detection of dengue serotypes 1 and 4	Serotype – specific detection	• Prolonged sample pre-treatment • Useful only for viremia stage	[26]
The biosensor comprises an anti-NS1 antibody immobilized on the gold nanoparticles (AuNPs) of an optical fiber sensor. NS1 antigen detection is based on localized surface plasmon resonance (LSPR)	NS1 antigen	0.05 $\mu g\,mL^{-1}$ – 1.0 $\mu g\,mL^{-1}$	0.074 $\mu g\,mL^{-1}$	N.A	The ability of LSPR fiber optic to detect NS1 antigen in clinical samples was estimated using the Langmuir isotherm equation (the estimated quantification limit is $^{*}C_{lim} = 0.074\ \mu g\,mL^{-1}$)*	Real-time detection	• Prone to nonspecific binding • Useful only for viremia stage	[28]
The biosensor comprises immobilized anti-NS1 antibody on Micro – Spot with Integrated Pillars. NS1 antigen detection is based on immunofluorescent FITC – conjugated IgG antibody	NS1 antigen	$1{:}10^{1}$ – $1{:}10^{5}$ ng mL^{-1}	0.04×10^{-3} $\mu g\,mL^{-1}$	N.A	N.A	Micropillars provide more surface area and signal intensity	Useful only for viremia stage	[53]
The biosensor consists of a straight gold (Au) stripe embedded in Cytop claddings with an etched microfluidic channel for sensing. NS1 antigen detected based on localized surface plasmon resonance (LSPR)	NS1 antigen	N.A	5.73 pg mm^{-2}	N.A	The detection of NS1 antigen in clinical samples was estimated based on calculation of the change in surface mass density $\Delta\Gamma$ (in pg mm^{-2}) as a result of the interaction between dengue NS1 antigen and Mab	LSPR is sensitive because it produces a strong resonance absorbance peak	Prone to nonspecific binding	[29]

Table 1 (*Continued*)

Type of optical sensor.	Analyte	Analytical performance				Advantages	Limitations	Ref
		Linear range	Limit of detection	Reproducibility	Analysis of clinical samples			
The biosensor comprises antigen immobilized on a gold sensor chip via an activated self-assembled monolayer (SAM) of 11 – mercaptoundecanoic acid. IgM detected by monitoring increase in resonance angle based on SPR response	Dengue IgM antibodies	Dilution range 10–100% of positive control serum	N.A	N.A	SPR response is specific for dengue IgM	Real-time detection	• Useful only for convalescence stage • Prone to nonspecific binding	[20]
The chemiluminescence – based biosensor is an optical fiber immunosensor (OFIS) modified with anti – human IgM. The chemiluminescence signal generated by the goat anti-mouse IgG – HRP conjugate is measured using a photomultiplier tube (PMT)	Dengue IgM antibodies	$1{:}10^{1}$–$1{:}10^{7}$	$1{:}10^{6}$	The reproducibility of the chemiluminescence OFIS is lower than that of MAC-ELISA	Chemiluminescence OFIS provides high sensitivity and specificity for detection of dengue IgM in clinical samples	Very low optical background	• Lack of reproducibility • Useful only for convalescence stage	[48]
The optical immunosensor is a biosensing surface consisting of specific monoclonal NS1 antibody for NS1 antigen detection based on immunofluorescence using FITC – conjugated IgG antibody	NS1 antigen	$0.015\ \mu g\ mL^{-1}$ – $2.0\ \mu g\ mL^{-1}$	$0.015\ \mu g\ mL^{-1}$	Good reproducibility with RSD of 2	The present immunofluorescence biosensor provides high sensitivity and specificity for detection of dengue NS1 antigen in clinical samples	• Sensitive • Reproducible • Specific	Useful only for viremia stage	The present biosensor

*C_{lim} is concentration limit

for 21 days at 4 °C, which was attributed to the high storage stability of the monoclonal anti – NS1 IgG antibody used to modify the immunosensing interface (supplementary material: Fig. S2. The capability of developed immunosensor to retain its operational and storage properties may be attributed to the fabrication design. EDC and NHS tightly bind the antibody to the amine group of 1,4 – phenylenediamine, which is linked to the ITO substrate through a covalent bond [50], thereby greatly improving the stability of the modified electrode [36].

3.2.2. Detection of the NS1 antigen in clinical samples

The present NS1 optical immunosensor was used to detect the NS1 biomarker in plasma samples from patients infected with DENV. The clinical samples were first examined and confirmed positive for NS1 and negative for DENV–specific IgM and IgG at the University Malaya Medical Center (UMMC) using ELISA–based serological assays (PanBio Dengue ELISA) and DENV NS1 Ag + Ab Combo tests. This validated the data obtained from using our newly developed fluorescence immunosensor on real samples analyses. As a control, plasma samples from 5 uninfected people were collected and tested negative for NS1, IgM, and IgG. The images acquired for the positive plasma samples showed higher fluorescence intensity than those for optical immunosensors tested with negative plasma samples. As shown in Fig. 4B, the optical densities (ODs) of both positive and negative human plasma samples (Fig. 4A) were converted and plotted as a line chart to aid in quantitatively interpreting the data. There was an obvious decrease in the fluorescence intensity value for positive plasma samples; this was proportional to the dilution factor (Fig. 4B). However, no change in fluorescence intensity was detected for negative plasma samples at different dilution factors. The change in fluorescence intensity was attributed to the change in NS1 antigen concentration in the tested clinical samples. Further, regression analysis was conducted to study the correlation between the sample dilution factor and the obtained OD. This statistical analysis revealed a significant correlation between the dilution factor of NS1 in positive clinical samples and OD based on a multiple regression value of 0.99 and P–value of 0.006 ($P < 0.05$). Conversely, there was no significant correlation between the dilution factor of the negative clinical samples and OD with a P–value of 0.07 ($P > 0.05$). A minor fluctuation in fluorescence intensity signal was adequate for analyte detection and quantification. The present NS1 optical immunosensor shows that the fluorescent intensity of images acquired for the DENV-positive plasma samples is higher than that of optical immunosensors assessed using DENV-negative plasma samples with an arbitrary unit signal change of 1.1 at a dilution of 1:10 and an arbitrary unit signal change of 0.24 at a dilution of 1:1000.

Importantly, when clinical samples were tested, method validation showed that the optical immunosensor was equivalent to the lateral flow DENV NS1 Ag + Ab Combo test (tested in UMMC) in terms of discrimination between positive and negative plasma samples without false-negatives or –positives. In conclusion, the developed immunosensor is very promising for clinical applications based on analytical performance.

The present immunosensor exhibited high selectivity and specificity for DENV NS1 antigen detection, with reasonable differentiation from the negative clinical samples. These clinical samples were examined and confirmed positive for NS1 and negative for IgM and IgG based on ELISA-based serological assays and DENV NS1 Ag + Ab Combo tests, as described previously. The fluorescence-based immunosensor showed high specificity for the NS1 antigen, with low cross-reactivity with plasma samples containing JEV or Zika virus. The images of plasma samples containing the NS1 antigen showed higher fluorescence intensity than those from optical immunosensors tested with plasma samples contain JEV or Zika virus. Furthermore, there was a significant decrease in the fluorescence intensity value for plasma samples containing the NS1 antigen, which was proportional to the dilution factor at DEN 1:50, DEN 1:100, and DEN 1:1000. However, no significant change in fluorescence intensity values was observed when the biosensor was tested with plasma samples containing JEV or Zika virus at different dilution factors (Fig. 4C). Additionally, the images of plasma samples containing the DENV NS1 antigen showed higher fluorescence intensity than those from optical immunosensors tested with plasma samples containing Japanese encephalitis virus (JEV) or Zika virus. Virus RT-PCR analysis was performed at the University Malaya Medical Center (UMMC) to confirm the existence of Japanese encephalitis virus (JEV) or Zika virus in clinical samples. Differences in signal intensity between DENV and JEV virus were 0.40, 0.39, 0.22 at a dilution of 1:50, 1:100, 1:1000, respectively. Differences in signal intensity between DENV and Zika virus were 0.42, 0.39, and 0.23 at a dilution of 1:50, 1:100, and 1:1000, respectively. Therefore, the change in fluorescence intensity value was attributed to the change in NS1 antigen concentration in the tested clinical samples, implying that the biosensor displayed specificity for only the NS1 antigen and high selectivity for the NS1 biomarker.

Minor fluctuations in fluorescence intensity values of Fluorescein isothiocyanate (FITC) dye at low concentration has been also reported by Cheng et al. [51] and Huang et al. [52]. The changes in fluorescence intensity obtained in those two reported studies were found to be less than 0.1 arbitrary unit (a.u.), (0.09 and 0.07) in the presence of cyanide and IgG analytes at different concentrations ranging from 0 μM to 5 μM and from 0 $\mu g.mL^{-1}$ to 1.6 $\mu g.mL^{-1}$, respectively.

A previous study reported promising results for the serological diagnosis of dengue virus infection using SPR [20]. Therefore, SPR represents a useful technique for the testing of clinical samples, with significantly higher sensitivity than the dengue MAC – ELISA kit. However, in this previous study, only positive clinical samples were tested for comparison with the dengue MAC – ELISA kit. Therefore, it was not clear whether the observed results were attributable to the analyte being tested or to other nonspecific antigens (interferents) present in the samples tested [20]. Taken together, our data show that, compared with previously developed optical sensing platforms for dengue virus (as shown in Table 1), the present newly developed immunofluorescence-based biosensor shows good selectivity, reproducibility, and capability to detect NS1 in clinical samples. In addition, the present biosensor is also easy to use in any laboratory equipped with a standard fluorescence microscope, therefore demonstrating the great potential utility of this technique for dengue diagnosis in economically challenged developing countries.

4. Conclusion

In this study, a sensitive and specific optical immunosensor, with monoclonal anti–NS1 IgG antibody as a biorecognition molecule on the sensing ITO substrate and FITC–conjugated secondary antibody as the fluorescence signal label, was designed for the determination of the NS1 biomarker. The linear detection range for NS1 antigen was between 15 and 500 $ng\,mL^{-1}$, with coefficients of determination (R^2) = 0.92 and LOD of 15 $ng\,mL^{-1}$, which is far lower than that of a previously reported LSPR-based biosensor (74 $ng\,mL^{-1}$) for NS1 detection. The present optical NS1 immunosensor enabled good reproducibility, with a relative standard deviation of 2%, and high stability (up to 21 days) during storage at 4 °C. Furthermore, the present immunosensor effectively determined NS1 antigen in complex plasma specimens from patients infected with DENV, with high selectivity for the NS1 antigen and low cross–reactivity with JEV and Zika virus. The optical immunofluorescence-based biosensor fabricated in this

study therefore possesses the advantages of high sensitivity and specificity. In addition, the present biosensor is relatively simple to use compared with techniques based on SPR imaging, wide field fluorescence microscopy, or NASBA analysis, requiring only the availability of a standard fluorescence microscope; a laser source is not necessary for its application. The present study therefore reports the design and development of a novel immunofluorescence–based biosensor for facile, rapid, highly sensitive, and selective NS1 antigen determination, with potential utility in the clinical diagnosis of dengue infection.

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