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

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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

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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