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Short communication

# An impedimetric biosensor to test neat serum for dengue diagnosis

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ABSTRACT

NS1, a non-structural dengue protein, has been used as a biomarker for the detection of viremia in dengue diagnosis. In this work, an impedimetric label-free immunosensor based on an anti-NS1 modified gold electrode was developed. To immobilize anti-NS1, a mixed self-assembled monolayer consisting of 11-mercaptoundecanoic acid (for covalent anti-NS1 attachment) and 6-mercaptohexanol (as a spacer) was prepared. The impedance spectra were recorded in the presence of a redox probe ($[Fe(CN)_6]^{3-/4-}$) to monitor changes in charge-transfer resistance associated with target binding both in PBS and neat serum. A calibration curve was constructed in order to obtain analytical parameters. NS1 was diluted in PBS to final concentrations in the linear range of 0.01–2.00 μg mL$^{-1}$ with a sensitivity of 14.1 percentage decade$^{-1}$ ($R \sim 0.99$) and a limit of detection (LOD) of 3 ng mL$^{-1}$. A linear range 0.01–1.00 μg mL$^{-1}$ of NS1 diluted in neat serum was also obtained ($R \sim 0.98$) with a sensitivity of 10.4 percentage decade$^{-1}$ and a LOD of 30 ng mL$^{-1}$. The results open new insights into the potential use of this biosensor for point-of-care and bedside applications for dengue diagnosis.



## 1. Introduction

Dengue, a self-limiting and non-specific illness with a severe forms (hemorrhagic fever and shock syndrome), is a major public health problem in tropical and subtropical regions of the world. In 2013, 2.35 million cases of dengue were reported in America alone, with almost 40,000 cases of severe dengue [1]. A key point in avoiding the high mortality rate and reducing disease burden is diagnosing the disease at its initial stages. According to the World Health Organization, new tools for the diagnosis of diseases like dengue should provide affordable, sensitive, specific, user-friendly, rapid, and robust measurements that can be obtained using point-of-care and bedside approaches [2,3]. However, the laboratorial methods currently available for dengue diagnosis, viral culture and viral RNA detection by reverse transcriptase PCR (RT-PCR) [4], have limited scope as routine diagnostic procedures since they require highly skilled personnel and involve complicated, time-consuming procedures. Serological tests, such as the commonly used immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA), are characterized by low sensitivity during the first four days of illness. Additionally, antigenic cross-reactivity with other infections limits their clinical applications [5]. Another drawback of the currently available diagnostic methods is that early diagnosis of dengue in clinical samples can only be achieved when IgM analysis is combined with other assays, such as RNA detection [6,7]. In other words, the current methodologies are expensive and time-consuming, suffer from poor sensitivity, and yield false positive results.

NS1 is a non-structural dengue protein that is secreted from infected cells and has been used as an early surrogate biomarker for viremia and/or infected cell mass in patients. In addition, an NS1 antigen capture ELISA has been developed which revealed that secreted NS1 is present in the sera of infected patients during the acute phase of disease. This suggests that it can be used as a diagnostic marker for dengue [6]. Further, it has been reported that this test is able to detect all four dengue virus serotypes [8]. Thus, the confirmation of the presence of NS1 protein in patient blood can be used as diagnostic tool for dengue. Indeed, some studies have used NS1 detection assays in clinical samples during the acute phase of dengue infection [9,10]. Taken together, new methodologies that are a quick, cheap, and simple tool to confirm the presence of NS1 with a binary response (positive/negative) could be an attractive alternative to the current established techniques being used for the diagnosis of dengue.

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New biosensing techniques and methodologies to detect NS1 antigen have been developed with the goal to develop fully automated and portable diagnostic devices. Some of these non-commercial approaches reported in the literature provide potential for clinical applications, especially those based on optical and piezoelectric approaches [11]. Among these, electrochemical assays seem to be the most promising tools in terms of low cost, flexibility, sensitivity, and multiplexing capabilities.

Electrochemical impedance spectroscopy (EIS) can sensitively monitor the changes in capacitance or charge-transfer resistance associated with binding of targets to specifically prepared receptive electrode surfaces and requires no prior labeling [12]. It has been reported that the use of EIS in faradaic impedance measurements of classical antigen–antibody binding events shows good reproducibility and sensitivity [13–15]. In this paper, we developed an impedimetric immunosensor based on a modified anti-NS1 gold electrode that is able to detect the NS1 dengue biomarker at interesting clinically relevant levels in buffer as well as in neat serum. The modified electrode showed a linear response for the target protein at concentrations ranging from 0.01 to 2.00 $\mu g\,mL^{-1}$ and 0.01 to 1.00 $\mu g\,mL^{-1}$ for PBS and neat blood, respectively. This biosensor displays a better limit of detection (LOD) compared to previous sensors developed by Wu et al. [16] and Tai et al. [17] (3 $ng\,mL^{-1}$ in PBS versus 740 $ng\,mL^{-1}$ and 5 $ng\,mL^{-1}$, respectively) and has a LOD in neat serum that is useful for clinical applications (30 $ng\,mL^{-1}$) [6].

## 2. Material and methods

### 2.1. Reagents

11-Mercaptoundecanoic acid (MUA), 6-mercaptohexanol (6COH), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), fetuin, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]\cdot 3H_2O$, anhydrous ethanol, ethanolamine, bovine serum albumin (BSA) and human blood serum were purchased from Sigma–Aldrich and used without previous purification. NS1 antigen and anti-NS1 antibody were purchased from Abcam.

All solutions used in the analytical procedures were prepared with Milli-Q-purified water (Millipore) with a conductivity of 18.2 MΩ cm at 25 °C. The protein solutions were prepared in 10 mmol $L^{-1}$ phosphate-buffered saline (PBS; pH 7.4).

### 2.2. Surface engineering and anti-NS1 sensors functionalization

For all electrochemical measurements (EIS and Cyclic Voltammetry, CV), the gold electrode (2 mm, Metrohm) was cleaned and the surface area was determined as previously described [15] (details in Supplementary material). These measurements (~0.036–0.038 $cm^2$) were used to normalize the absolute recorded impedance. To immobilize anti-NS1, a mixed self-assembled monolayer (SAM) was prepared. The SAM was created by immersing the cleaned gold electrode for 16 h at 25 °C in a mixed solution containing 1 mmol $L^{-1}$ MUA (for covalent anti-NS1 attachment) and 1 mmol $L^{-1}$ 6COH (as a spacer) in anhydrous ethanol.

After SAM preparation, the electrode was washed in anhydrous ethanol and deionized water and dried in a flow of nitrogen gas. The carboxyl groups of MUA were activated for 30 min with an aqueous solution containing 0.4 mol $L^{-1}$ N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and 0.1 mol $L^{-1}$ N-hydroxysuccinimide (NHS). Subsequently, the electrode was washed using PBS, dried with nitrogen gas, and 50 µL of anti-NS1 solution (1 $\mu g\,mL^{-1}$) in PBS was applied to the mixed SAM. Following incubation for 1 h, the remaining NHS esters were deactivated by addition of a 1 mol $L^{-1}$ ethanolamine solution and after 5 min, the surface was thoroughly rinsed with deionized water. Finally, the anti-NS1 electrodes were immersed in a 0.1% BSA solution (in PBS) for 30 min to block unspecific sites. For experiments using serum, serum was used instead of BSA in the blocking step. All the steps of electrode functionalization are described in Fig. 1.

### 2.3. EIS analysis and standard curve

EIS and CV measurements were carried out on an AUTOLAB potentiostat PGSTAT30 by using NOVA software. A three-electrode setup consisting of a gold working electrode (2.0 mm diameter, Metrohm), a platinum mesh counter electrode, and an Ag|AgCl, 3 mol $L^{-1}$ KCl reference electrode was used for all procedures.

EIS and CV were used to monitor all steps of surface functionalization. EIS measurements were recorded in the presence of

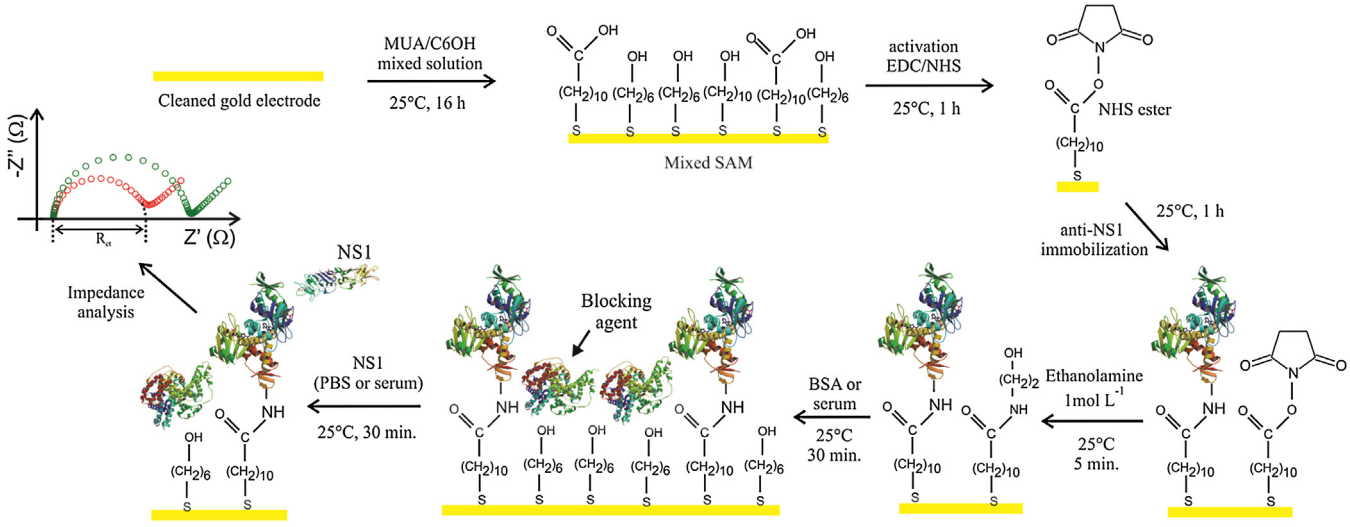


**Fig. 1.** Modified anti-NS1 gold electrode schematic construction representation (the molecules are not drawn to scale). The surface was constructed onto a gold surface using mixed thiol-SAM structures, in which 11-mercaptoundecanoic acid served as a receptor-supportive layer for anti-NS1 attachment, and 6-mercaptohexanol served as a spacer layer. Anti-NS1 immobilization was achieved through standard EDC/NHS bioconjugation, and the nonspecific sites were blocked with 0.1% BSA or neat serum. NS1 at various concentrations were quiescently lying on the interface at room temperature (25 °C) for 30 min, in pH 7.4. The faradaic impedance measurements were carried out using a redox probe in solution $[Fe(CN)_6]^{3-/4-}$. By the Nyquist plot, where $Z''$ is the imaginary and $Z'$ is the real part of impedance, $R_{ct}$ was measured from approximation of the diameter of the semi-circle of $Z'$.