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# Electrochemical Immunosensor Based on Antibody-Nanoparticle Hybrid for Specific Detection of the Dengue Virus NS1 Biomarker

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Dengue virus infection is a very deadly disease that threatens human lives in subtropical and tropical regions. Thus, for early and reliable diagnosis of Dengue virus infection, a sensitive, specific, and label-free electrochemical immunosensor was developed for the direct detection of the unstructured protein NS1. The NS1 biosensor was designed with a biosensing surface consisting of antifouling moieties and biorecognition molecules to enhance the specificity of the immunosensor for target analyte detection in complicated biological samples such as human sera. The immunosensor exhibited a wide detection range (5–4000 ng $mL^{-1}$) based on electrochemical impedance spectroscopy (EIS) measurements with a coefficient of determination ($R^2$) of 0.94, correlation coefficient (R) of 0.95, high reproducibility, and good stability for 21 days at 4°C. The developed immunosensor was able to detect the NS1 antigen in actual serum specimens from patients infected with Dengue virus. Moreover, the immunosensor is not only highly selective for the NS1 antigen, but also did not cross-react with human sera infected with malaria parasites.

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Because of the absence of an efficient treatment or vaccine for Dengue fever, a suitable diagnostic technique is necessary to detect the disease consistently and rapidly.[1] There are several types of diagnostic tests for the detection of the Dengue virus (DENV). Traditional diagnostic techniques for DENV include isolation in cell culture,[2] serological testing,[3] and polymerase chain reaction (PCR).[4,5] However, virus isolation is only effective if the sera samples are collected before the onset of fever.[2,6] Real time PCR (RT-PCR) often yields false-negative results.[5,7,8] Serological tests for DENV detection are not generally specific because the immunoglobulins for DENV are highly cross-reactive with other flavivirus antibodies (e.g., Japanese encephalitis virus and Powassan/Deer tick virus).[9] Compared with conventional tests for the detection of DENV, biosensors, and especially electrochemical immunosensors, have received increased attention because of their sensitivity, simplicity, and specificity.[10]

More recently, for Dengue disease diagnostics, the detection of the NS1 antigen biomarker has been considered because the NS1 antigen is secreted during the early phase of DENV infection with a concentration of up to 50 μg $mL^{-1}$,[11,12] even when viral RNA detection is negative.[13] Moreover, NS1 is measurable in sera of patients with Dengue from day 1 to day 18 after the onset of fever in both primary and secondary infections and in the Dengue hemorrhagic fever (DHF) stage.[13,14] This indicates that NS1 is suitable for early diagnosis and can be detected before Dengue antibodies (IgM and IgG) are present in patient sera.[14,15] Moreover, NS1 is a promising alternative for the serum glycoprotein[13] as it is stable, highly conserved, specific for DENV infection, and non-reactive with other viruses such as the West Nile, yellow fever,[16,17] and Japanese encephalitis viruses.[18] Because of these advantages of NS1 as a DENV biomarker, it was selected for use in this study.

Several electrochemical immunosensors have been reported for NS1 antigen detection. Silva and co-workers described an immunosensor that was fabricated on the basis of the immobilization of anti-NS1 antibodies onto an electrode surface by using a thin layer of poly(allylamine) assembled on carboxylated carbon nanotubes (CNTs).[19] The detection of NS1 by using this reported immunosensor was based on the electrochemical response to the immunoreaction, which was generated at a controlled potential by a reaction between hydrogen peroxide and peroxidase enzyme conjugated to anti-NS1 antibodies. The limit of detection for this immunosensor was 0.035 μg $mL^{-1}$, which is slightly lower than NS1 levels in sera of patients with primary infections (range of NS1 levels is 0.04–2 μg $mL^{-1}$) and higher than NS1 levels in sera of patients with secondary infections (range of NS1 levels is 0.01–2 μg $mL^{-1}$).[13] Another reported immunosensor for NS1 detection was fabricated based on CNT-screen printed electrodes (CNT-SPE).[20] This immunosensor exhibited a low detection limit of 12 ng $mL^{-1}$. Evaluation of NS1 concentration was based on the measurement of amperometric responses by using hydrogen peroxide reaction with peroxidase (HRP) conjugated to anti-NS1 antibodies. In the above mentioned studies, labeling techniques were used for the electrochemical biosensors to improve inadequate sensitivity results. However, the labeling process might induce structural changes to the labeled macromolecules.[19] Moreover, these techniques are costly and require increased sample preparation time and tedious detection procedures.[19] The operation of these immunosensors essentially depends on the detection of hydrogen peroxide. However, all biological samples contain a variety of electrochemically easily oxidizable reductants such as ascorbate and bilirubins. These reductants tend to be oxidized at similar potentials and generate undesired noise, which, in addition to the anodic current produced by the hydrogen peroxide, could reduce the sensitivity of biosensors.[21]

As a model of a label-free immunosensor, a thiophene-modified screen printed electrode (SPE) as reported by Saliva et al.[22] was developed for the detection of NS1 antigen. The incorporation of thiophene monomers with carbon ink enhanced the electroanalytical properties of the SPEs and increased their reproducibility and sensitivity (detection limit of 0.015 μg $mL^{-1}$ NS1). However, analytical performance studies of these immunosensors were conducted without examining selectivity or cross-reactivity, thus providing insufficient information about the specificity of these immunosensors for the specific detection of the NS1 antigen. Another label-free immunosensor was fabricated on the basis of gold film electrodes that were obtained from a recordable compact disk (CD-trode).[23] This immunosensor exhibited a linear response from 1 to 100 ng $mL^{-1}$ of NS1 detection in serum samples. However, the selectivity study for this immunosensor was conducted against bovine serum albumin, l-cysteine, and IgG of unknown origin. Using these molecules might not be representative of biomolecules typically found in human sera, as noted by Cavalcanti et al.[23] and in other reports of immunosensors developed for NS1 detection.[19,20,22] Even though most of the above mentioned techniques have shown reasonably good limit of detection and high sensitive sensoring interface for NS1 biomarker detection, however apart from the research work reported by Cecchetto et al.[1] and the studies conducted as described in this manuscript, most of the analytical responses and the calibration curves generated for other presented biosensors were obtained either in phosphate buffer saline[19,20,23] or in Dengue virus culture

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supernatant.[22] Therefore the ability of a biosensor to sense the NS1 biomarker in human sera samples is challenging but extremely important. This is not only to show the biosensor could really work well in real sample analysis for biomedical applications/clinical diagnostics and potentially to be prototyped and commercialized, but also to show the novelty and robustness of the biosensing interface being able to resist any non-specific absorption of proteins from the complex matrices onto the transducer surfaces and give false negative responses where the accuracy and precision of data obtained are being compromised. Therefore, the aim of this study was to develop a label-free, straight-forward, specific, and sensitive immunosensor utilizing zwitterionic antifouling molecules and based on nanoparticle-customized electrochemical techniques and monoclonal antibodies for the direct detection of NS1 antigens in blood specimens infected with DENV.

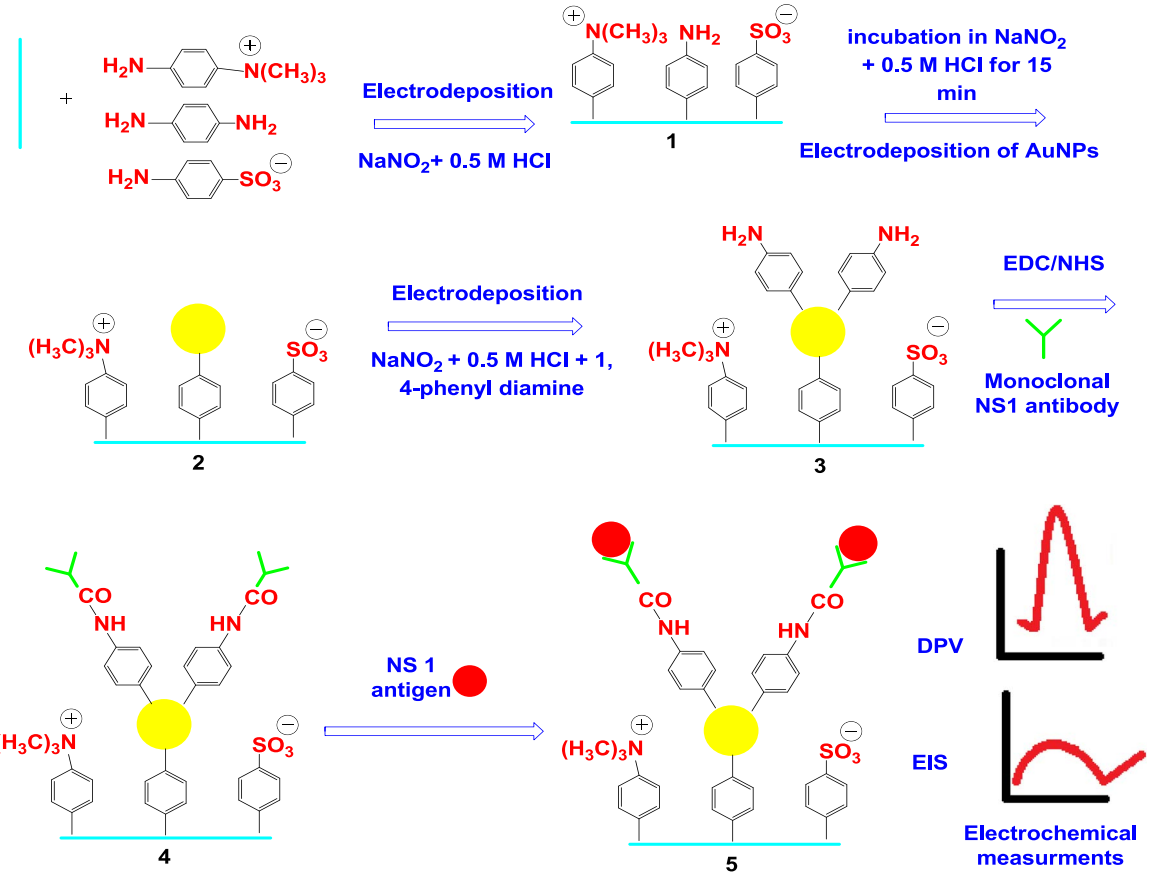
## Experimental

***Reagents and materials.—***ITO was purchased from Sanyo (Japan). The following were purchased from Sigma-Aldrich (USA): 4-sulfophenyl, 1,4-phenylenediamine, sodium nitrite ($NaNO_2$), potassium chloride (KCl), potassium dihydrogen phosphate ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), hydrochloric acid (HCl), potassium ferricyanide ($K_3Fe(CN)_6$), gold nanoparticles (AuNPs) (20 nm), human serum albumin (HSA), and sodium chloride (NaCl). Human IgG was obtained from Thermo Fisher Scientific (USA) and 4-trimethylammoniophenyl was obtained from Acros (USA). The NS1 antigen and monoclonal anti-NS1 IgG antibody were purchased from GenScript (USA). ELISA-based serological assays and Dengue NS1 Ag + Ab Combo tests (Standard diagnostics, Korea). All solutions were prepared using Milli-Q water (18 MΩ•cm$^{-1}$) (PURELAB, USA).

***Instrumentation and procedures.—***All 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 the Ag/AgCl reference electrode at 25°C. The surface studies on 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 differential pulse voltammetry (DPV) were used to probe the integrity of functionalized layers on electrode surfaces and to obtain the calibration curve for the NS1 antigen. The electrodeposition of different 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. The following parameters were used for the DPV measurements: initial potential (−0.2 V), end potential (0.6 V), modulation amplitude (25 mV), modulation time (0.05 s), interval time (0.5 s), and step potential (5 mV).

***Fabrication of a label-free electrochemical impedance immunosensor for direct detection of the NS1 antigen.—***ITO substrates were soaked and cleaned in an ultrasonicator using dichloromethane and then 99% methanol for 10 min each, followed by treatment with 0.5 M $K_2CO_3$ in a 3:1 methanol:Milli-Q water mixture for 30 min under sonication to remove any residual organic contaminants. The ITO substrates were then rinsed with copious amounts of Milli-Q water, dried, and stored in a nitrogen-filled container.[24] The bare ITO electrode was modified with a combination of 4-sulfophenyl, 4-trimethylammoniophenyl, and 1,4-phenylenediamine (SP:TMAP:PPD) at molar ratio of 0.5:1.5:0.37. This ratio was selected as it exhibited the best antifouling capabilities against positively and negatively charged proteins.[25] 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 the surface modification. The electrodeposition was performed using cyclic voltammetry at −0.6 V to 0.2 V; the electrodes were scanned for 5 cycles at 100 mV s$^{-1}$ to produce the first layer on the ITO electrode.[26,27] This surface is referred to as surface 1 (Scheme 1). Subsequently, the distal amine groups of 1,4-phenylenediamine were converted to diazonium groups by incubating surface 1 in $NaNO_2$ and 0.5 M HCl solution to produce a 4-phenyl diazonium chloride-modified interface (ITO-Ph-$N_2^+Cl^-$). Subsequently, AuNPs were immobilized on the interface by electrochemical reduction to give a 4-phenyl AuNP-modified interface (ITO-Ph-AuNP). The diazonium radicals link to the AuNPs via a covalent bond.[27] The ITO electrodes were first modified with 4-aminophenyl in mixture of SP:TMAP:PPD, and then the terminal amine of 4-aminophenyl was converted to diazonium groups by incubating the modified interface in $NaNO_2$ and HCl solution to form a 4-phenyl diazonium chloride-modified interface (ITO-Ph-N2$^+$Cl$^-$). Subsequently, AuNPs were immobilized on the interface by electrochemical reduction to give a 4-phenyl AuNP-modified interface (ITO-Ph-AuNP). The diazonium radicals link to the AuNPs via a covalent bond,[27] and this is referred to as surface 2 (Scheme 1). Note that AuNPs are used as they yield an enlargement of the electrode surface area, boost the interface with more bio-recognition molecules (as they have a high surface-to-volume ratio), exhibit advantageous redox properties, and are capable of sensing analytes in the pM range.[28–30] Next, 1,4-phenylenediamine (3 mM) was used to functionalize AuNPs on surface 2, allowing for the subsequent attachment of monoclonal anti-NS1 IgG antibodies onto the AuNPs. The AuNPs were modified with 1,4-phenylenediamine by using a scanning potential between 0.2 V and −0.6 V in a 0.5 M HCl solution containing 1 mM $NaNO_2$ and 3 mM 1,4-phenylenediamine for three cycles at a scan rate of 100 mV s$^{-1}$. This surface is referred to as surface 3 (Scheme 1). Monoclonal anti-NS1 IgG antibodies were incubated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (200 mM) and N-hydroxysulfosuccinimide (NHS) (50 mM) cross linkers for 30 min to activate carboxyl groups of the monoclonal antibodies.[31] Then, 50 μL of this solution was added to surface 3 and incubated for 45 min at 25°C to bind the monoclonal antibodies to the amine groups of 1,4-phenylenediamine. After, the ITO slides were washed with 50 mM NaCl to remove any extra unbound antibodies on the electrode surface, and then they were washed with 10 mM phosphate buffer solution (PBS) at pH 7.4. This surface is referred to as surface 4. Surface 4 is the sensing interface that was used for the detection of the NS1 antigen. The NS1 antigen was spiked into diluted human sera (10% in PBS, pH 7.4) at various concentrations. The NS1 antigen (50 μL) was pipetted onto surface 4, and the slides were incubated for 45 min at 25°C. Then, the immunosensors were rinsed thoroughly with PBS (pH 7.4) prior to DPV and EIS measurements.

***Analysis of reproducibility, stability, selectivity, and cross-reactivity of the NS1 immunosensor.—***The reproducibility of the fabricated immunosensor was examined by testing five different modified electrodes as mentioned earlier. Then, the prepared slides were incubated in a NS1 antigen solution (100 ng mL$^{-1}$) for 45 min. After, the slides were soaked in PBS (pH 7.4) for 5 min and washed carefully with PBS (pH 7.4) to remove any nonspecifically adsorbed NS1 antigen. The peak current of DPV was measured for each slide, and the relative standard deviation for the five slides was calculated. For the stability study, eighteen slides were modified as described earlier. Then, three of these slides were tested on the same day. The NS1 immunosensor was incubated in 50 μL of NS1 antigen (100 ng mL$^{-1}$) for 45 min. The other fifteen slides were stored in 10 mM PBS (pH 7.4) at 4°C. Thereafter, three slides were tested each day at days 7, 14, 21, 30, and 60 to determine the stability of the modified ITO over a 60-day period. Prior to each test, the NS1 immunosensor was rinsed with 10 mM PBS (pH 7.4). The peak current values were measured for each slide by using DPV, and the relative standard deviation




**Scheme 1.** Schematic of fabrication of an electrochemical immunosensor for direct detection of the biomarker NS1 antigen during early stages of Dengue virus infection.

was calculated. For the selectivity study, twelve modified slides were used. Three of these slides were incubated with 50 μL of NS1 antigen (50 ng $mL^{-1}$). Another three of the modified slides were incubated with 50 μL of HSA (50 ng $mL^{-1}$) from normal human sera. The last three modified slides were incubated with 50 μL of human IgG (50 ng $mL^{-1}$). After incubation for 45 min, the slides were electrochemically measured using EIS. Then, charge transfer resistance ($R_{ct}$) values of slides incubated with NS1 antigen were compared to $R_{ct}$ values of slides incubated with HSA and human IgG. For the cross-reactivity study, six modified slides were used. Three of these slides were incubated with 50 μL of 10% human sera diluted with PBS (pH 7.4) and spiked with NS1 antigen (1 μg $mL^{-1}$). Another three of the prepared slides were incubated with 50 μL of 10% human sera diluted with PBS (pH 7.4) taken from a patient infected with the malaria parasite. After incubation for 45 min, the slides were electrochemically measured using EIS. $R_{ct}$ values of slides incubated with the NS1 antigen were compared to $R_{ct}$ values of slides incubated with the infected sera with malaria parasites.

The potential use of the fabricated immunosensor in clinical diagnostics was further tested by using five actual sera samples from patients infected with DENV. Beforehand, these samples were tested and confirmed positive for NS1 and negative for IgM and IgG by both ELISA-based serological assays and Dengue NS1 Ag + Ab Combo tests. As control samples, five normal sera samples were collected from patients with negative results for NS1, IgM, and IgG. The positive and negative sera samples were serially diluted to various dilution factors (1:10, 1:100, 1:500, and 1:1000) by using PBS (pH, 7.4). The modified slides were incubated with 50 μL of diluted sera at the various dilutions. After incubation for 45 min, the slides were electrochemically measured using EIS. Then, $R_{ct}$ values of slides incubated with positive sera samples was compared to $R_{ct}$ values of slides incubated with negative sera samples.

## Results and Discussion

***An electrochemical immunosensor for direct detection of the NS1 antigen.***—EIS was used to monitor the stepwise fabrication of the biosensor by using 1 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. A Randles equivalent circuit was used to fit the impedance results and contains the following four elements: the resistance of the electrolyte solution ($R_S$), phase constant element (Q), Warburg impedance (W), and charge transfer resistance $R_{ct}$ (Table I). The $R_S$ and W elements relate to the properties of the electrolyte solution and the diffusion features of the redox probe, respectively. The diffusion of the redox probe is represented on a Nyquist plot by a straight line at low frequencies. The $R_{ct}$ and Q elements represent the dielectric and insulating characteristics at the electrode/electrolyte interface, and therefore, the values of these two elements are affected by the modification of the electrode surface. They are represented by the semicircle of the Nyquist plot. The semicircle diameter is typically proportional to the resistance of the deposited layers on the electrode surface. Forming a well-organized layer on the modified electrode generally hinders the diffusion of the redox probe, since an increase in the diameter of the semicircle causes a high electron transfer resistance. A substantial change in the diameter of the semicircle on the Nyquist plot, and therefore $R_{ct}$, was detected during the fabrication steps of the NS1 immunosensor (Figure 1a and Table I). After the modification of ITO with 4-sulfophenyl, 4-trimethylammoniophenyl, and 1,4-phenylenediamine molecules to produce surface 1 (Scheme 1), an increase in the $R_{ct}$ was observed as this organic layer prevented the redox molecule from accessing the electrode (Figure 1aII and Table I). However, when AuNPs were attached, there was a significant decrease in the $R_{ct}$ (Figure 1aIII and Table I), and this observation is consistent with other studies showing that AuNPs can decrease the resistance of charge transfer when they are attached to blocking layers.[32,33] The

**Table I. Values of equivalent circuit parameters of the fitting curve for the bottom-up stepwise fabrication of the NS1 electrochemical impedance immunosensor interface by Nova software.**

| Electrodes[a] | $R_s$ (Ω) | $R_{ct}$ (Ω) | CPE | N | $\chi^2$ |
|---|---|---|---|---|---|
| Bare ITO | 393.9.0 ± 5.4 | 279.3 ± 64.1 | $65701 \times 10^{-6} \pm 1.01 \times 10^{-6}$ | $0.9 \pm 3 \times 10^{-2}$ | $0.007 \pm 7 \times 10^{-3}$ |
| ITO/SP:TMAP:PPD | 34.0 ± 1.0 | 10072.2 ± 3075 | $6.4 \times 10^{-6} \pm 4.1 \times 10^{-6}$ | $0.9 \pm 9 \times 10^{-3}$ | $0.01 \pm 6 \times 10^{-3}$ |
| ITO/ SP:TMAP:PPD /GNPs | 39.6 ± 9.5 | 2012.1 ± 1174 | $77 \times 10^{-6} \pm 1.3 \times 10^{-6}$ | $0.9 \pm 2 \times 10^{-2}$ | $0.03 \pm 2 \times 10^{-3}$ |
| ITO/ SP:TMAP:PPD /GNPs/PPD | 30.3 ± 2.5 | 6537.4 ± 2555 | $7.5 \times 10^{-6} \pm 1.8 \times 10^{-6}$ | $0.9 \pm 1 \times 10^{-2}$ | $0.01 \pm 8 \times 10^{-3}$ |
| ITO/SP:TMAP:PPD/GNPs/PPD/Monoclonal anti-NS1 IgG antibody | 27.7 ± 1.7 | 7510.2 ± 1716 | $6 \times 10^{-6} \pm 0.76 \times 10^{-6}$ | $0.9 \pm 1 \times 10^{-2}$ | $0.004 \pm 2 \times 10^{-3}$ |

[a] Values correspond to the average of three separate measurements ± standard deviation.
$R_s$, the resistance of the electrolyte solution.
$R_{ct}$, charge transfer resistance.
CPE, constant phase element.
An n-value for CPE near 1.0 indicates a true capacitance.
$X^2$, estimated error.

deposition of 1,4-phenylenediamine caused an increase in the $R_{ct}$ (Figure 1aIV and Table I), and further increases in the $R_{ct}$ were detected when monoclonal anti-NS1 IgG antibodies were covalently bound to 1,4-phenylenediamine by using EDC/NHS (Figure 1aV and Table I). Moreover, an increase in the $R_{ct}$ was observed when the NS1 antigen bound specifically to the monoclonal anti-NS1 IgG antibody layer. In addition, electrochemical characterization of the fabricated NS1 immunosensor was performed using DPV to support our EIS results (supplementary information, Figure S1.a).

***Quantitative analysis of the NS1 antigen biomarker in human sera.***—A calibration curve was obtained using the NS1 immunosensor functionalized with monoclonal anti-NS1 IgG antibodies to detect the NS1 antigen at various concentrations in 10% human sera diluted with PBS (pH 7.4). The calibration curve obtained from a Nyquist plot of the impedance spectra showed a logarithmic relationship (log-linear model) between the $R_{ct}$ measurements and various concentrations of the NS1 antigen being spiked into the diluted samples of human sera (Figure 1b). The coefficient of determination ($R^2$) obtained was 0.94 for the dynamic range ranged from 5 to 4000 ng $mL^{-1}$. On the other hand, the correlation coefficient (R) calculated was 0.95 and this value indicates the strong relationship found between the Rct measurements and NS1 concentrations detected in human sera samples (Figure 1b). For statistical analysis purpose, a residual plot has been generated to assess the regression and relation between the Rct measurements and NS1 protein concentrations. Figure S2 (Supplementary material) shows all the points obtained in this residual plot were randomly dispersed around the horizontal axis which again has double confirmed the linear regression model for $R_{ct}$ and NS1 variables (5–4000 ng $mL^{-1}$) with *P* value of 0.4. The ability of the developed immunosensor to detect the NS1 antigen in serum samples with a detection limit of 5 ng $mL^{-1}$ is acceptable for clinical applications, since 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] In addition, to support our findings obtained from the EIS measurements, DPV methods were used to determine the relationship between the concentration of the NS1 antigen and the peak current of DPV (Supplementary material, Figure S1.b).

The ability of the NS1 immunosensor to approach such a low detection limit might result from the use of antifouling molecules. In our previous work,[25] it was shown that these antifouling molecules with charged terminal groups can counter the non-specific adsorption of the various proteins naturally found in human sera. Other reported immunosensors using AuNPs modified with polyvinyl butyral (PVB),[30] concanavalin A,[34] polyaniline hybrid,[35] or $Fe_3O_4$ nanoparticles[36] served as models for the detection of DENV. In these biosensors, lectins (proteins or glycoproteins from different sources) were immobilized on the biosensor surface to detect glycoproteins and the immune response produced in patients infected by DENV. Lectins are carbohydrate-binding proteins; therefore, they are able to bind to serum glycoproteins.[35,36] The majority of serum proteins are glycosylated when there is an infection, and glycoproteins will be changed to abnormal glycoproteins under conditions such as liver disease, cancer, or Dengue fever. These changes could provide a basis for clinical tests.[37] Unfortunately, the presence of these glycoproteins could be because of DENV infection or other diseases.

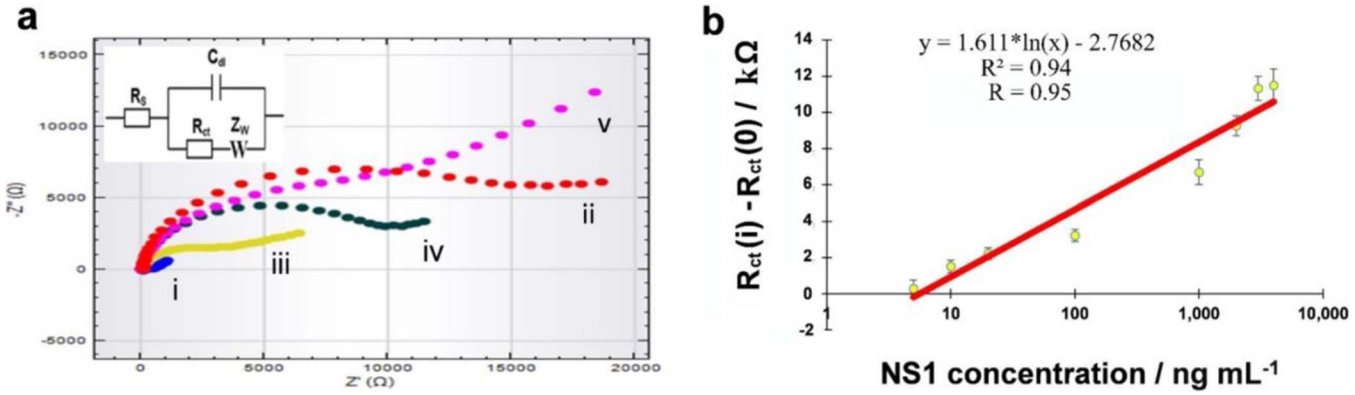



**Figure 1.** (a) Nyquist plots recorded in PBS containing 0.05 M KCl and 1 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ for impedance measurements of the stepwise surface modification of (i) bare ITO electrode, (ii) ITO/SP:TMAP:PPD, (iii) ITO/SP:TMAP:PPD/GNPs, (iv) ITO/SP:TMAP:PPD/GNPs/PPD, and (v) ITO/SP:TMAP:PPD/GNPs/PPD/NS1 monoclonal IgG antibody (50 μg $mL^{-1}$). (b) The calibration plot corresponding to the increase in electron transfer resistance of the immunosensor with various concentrations of NS1 antigen (5, 10, 20, 100, 1000, 2000, 3000, and 4000 ng $mL^{-1}$). Note: (a) inset is the Randles equivalent circuit for the impedance spectroscopy measurement.

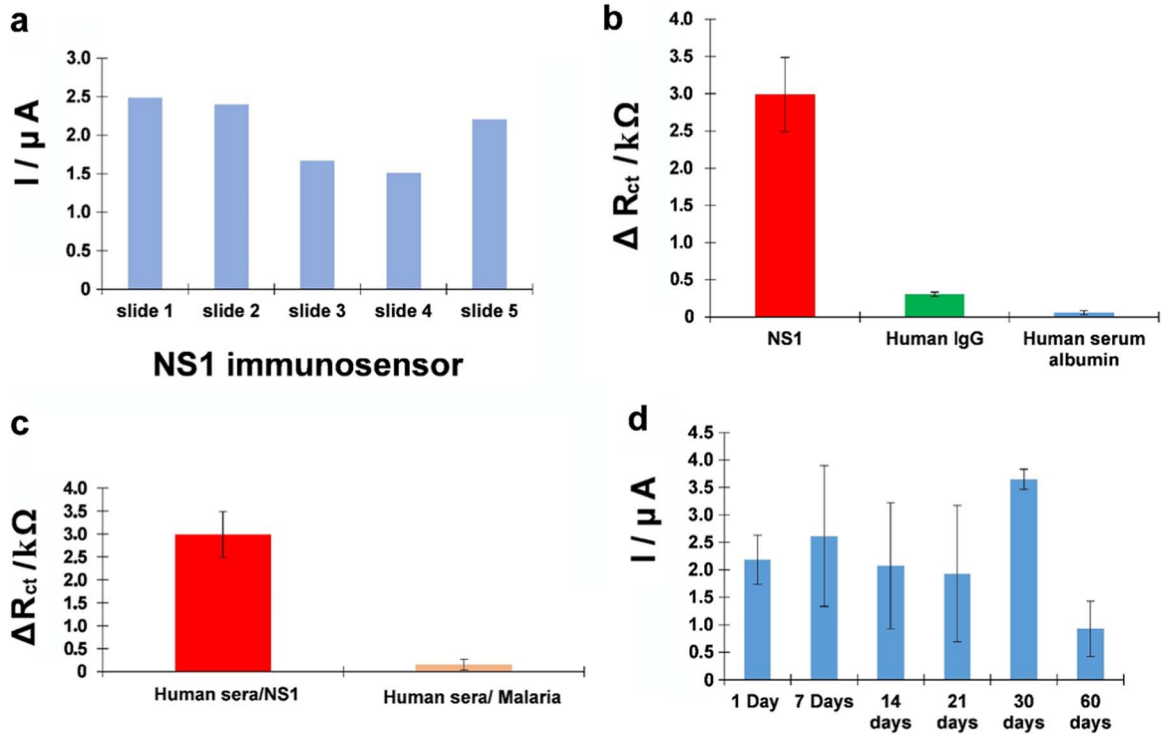



**Figure 2.** (a) The reproducibility study based on the peak height of DPV measurement for five different electrodes incubated in NS1 antigen solution (100 ng $mL^{-1}$) (the relative standard deviation obtained was 2.1%). (b) The changes of impedance observed in the selectivity study where the immunosensing interfaces were tested with 50 ng $mL^{-1}$ of target NS1 antigen, human IgG, and human serum albumin, respectively. (c) The changes of impedance observed in the cross-reactivity study where the immunosensing interfaces were incubated in samples of 10% human sera spiked with NS1 (1 µg $mL^{-1}$) and 10% human sera infected with malaria parasites, respectively. (d) The stability study where the peak height of DPV measurement was recorded and compared for electrodes tested with NS1 concentration of 100 ng $mL^{-1}$ for 60 days stored at 4°C. The error bars indicate the standard deviation of three replicates.

This indicates that these biosensors based on serum glycoproteins might not be highly specific to DENV infection, since serum glycoproteins could be detected in both infected and non-infected serum samples.[35]

***Analytical performance of the NS1 immunosensor: Reproducibility, selectivity, cross-reactivity, and stability studies.***—The reproducibility of the immunosensor was examined by testing five different electrodes incubated with 100 ng $mL^{-1}$ NS1 antigen. High reproducibility was achieved with relative standard deviation (RSD = 2.1%) (Figure 2a), indicating that the electrochemical measurements were more stable than those of previous immunosensors,[20,23] which had a reproducibility of RSD = 3.4% and RSD = 4.8%. Reproducibility is one of the most important challenges limiting the commercialization of any biosensor. The high reproducibility of the fabricated NS1 immunosensor might be attributable to the biosensor design and fabrication procedure.[38] Using a monoclonal antibody for the specific detection of the NS1 antigen could enhance the reproducibility of the immunosensor.[39] In addition, using antifouling molecules can control/resist the non-specific protein adsorption to the immunosensing interface. Consequently, the obtained current peak measurements were stable and proportional to the concentration of only the NS1 antigen. Furthermore, the reproducibility and the robustness of the fabricated immunosensor might indicate that the monoclonal anti-NS1 antibodies were uniformly distributed on the electrode matrix.[40] To investigate the selectivity/specificity of the developed NS1 immunosensor to the NS1 antigen, human IgG and HSA were tested to determine whether the NS1 immunosensor yields any positive response signals to these molecules in the absence of the NS1 antigen. An additional objective of this study was to rule out any possibility of human IgG and HSA inducing a biofouling effect and compromising the accuracy and reliability of the obtained analytical signal. Human IgG and HSA were tested as controls as they are major blood proteins found in human sera and will be the main components competing with the NS1 antigen or causing biofouling of the biosensor interface. Figure 2b shows that the NS1 immunosensor did not significantly respond to HSA and human IgG. In contrast, the NS1 immunosensor showed a much greater response and an increased $R_{ct}$ value only in response to the NS1 antigen. This result indicates that the NS1 immunosensor has a high specificity for the detection of only the NS1 antigen, and any response obtained corresponded to the NS1 antigen. Furthermore, the fabricated antifouling surface exhibited high resistance to the biofouling effect, which might be attributable to the non-specific protein adsorption of human IgG and HSA (Figure 2b). Using antifouling moieties (zwitterionic molecules) in the fabricated immunosensor might enhance its selectivity as this aided in overcoming the issue of protein fouling, which is the non-targeted molecular binding to the sensor interface, particularly when detecting biomarkers in biological samples such as human sera.

In some diagnostic methods that have been used to detect DENV infection, including the hemagglutination-inhibition (HAI) assay and the MAC-ELISA-IgM assay, false-positive readings have been reported.[41] These results have been ascribed mainly to cross-reactivity with co-circulating antibodies from either other flaviviruses such as the Japanese encephalitis virus or those that can be found in sera of patients with leptospirosis or chikungunya.[7,42] Cross-reactivity in patients with DENV infection who also test positive for malaria is a well-known issue in regions such as West Africa. Nevertheless, the relationship between these two diseases is not well defined. The cross-reactivity is considered an obstacle to commercially available serology kits and rapid diagnostic tests used for clinical diagnosis.[43,44] Therefore, in this study, an assessment of cross-reactivity was also conducted to determine if the sera taken from patients infected with malaria parasites displayed a positive response for NS1 antigen

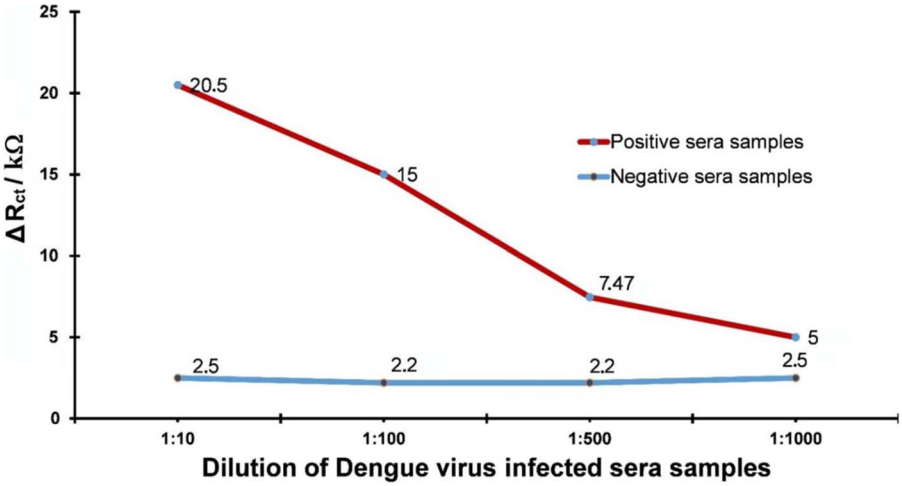



**Figure 3.** Response to the NS1 antigen in positive and negative human sera at different dilution factors (1:10, 1:100, 1:500, and 1:1000) from a patient infected with the Dengue virus.

detection. The NS1 immunosensor did not exhibit a significant response to malaria-infected sera (Figure 2c), but showed a much greater response to NS1 antigen. Selectivity is the most important characteristic of a biosensor and is another potential advantage of the immunosensor fabricated in this study compared with other reported biosensors that were not evaluated for sensor selectivity.[19,20,22] In research done by Cavalcanti et al.,[23] the selectivity study was carried out against bovine serum albumin, l-cysteine, and IgG of unknown origin. However, these might not represent biomolecules normally found in human sera. The high selectivity of the immunosensor fabricated in this study might be attributable to the high affinity between the biorecognition site (monoclonal Ab) and the target analyte (NS1 antigen), as the binding between antibody and antigen is the most promising methodology for detecting trace quantities of a specific biomarker in complex matrices.[45]

The stability of the developed NS1 immunosensor was investigated by carrying out experiments at weekly intervals. The fabricated immunosensing interface retains 90.4% of its activity after being stored for 21 days at 4°C, indicating that the monoclonal anti-NS1 IgG antibody used to modify the immunosensing interface has acceptable storage stability (Figure 2d). However, after 30 days, there was a reduction to 71.4% of the initial peak current (day 1). This decrease in peak current was expected, as the typical shelf life for antibodies is 30 days, and chemical modifications such as oxidation and proteolytic degradation can occur if these antibodies are stored for more than 30 days at 4°C.[46,47] The stability of a stock antibody can be prolonged to one year if they are stored at −20°C or to more than one year if they are stored at −80°C.[47] The operational and storage properties of the prepared NS1 immunosensor might be attributable to the fabrication procedure because using EDC and NHS could tightly bind the antibody to the amine group of 1,4-phenylenediamine attached on the AuNP surface through an amide bond, greatly improving the stability of the modified electrode.[31] In addition, the electrochemical deposition of the AuNPs was an effective method to obtain a film of AuNPs on the electrode. The AuNP film was distributed homogeneously and could tightly bind to the electrode, giving a powerful and stable immunosensing interface.[27,39] The fabrication design was further advantageous by maintaining immunosensor stability, which is essential for practical applications.

The developed NS1 immunosensor exhibited promising analytical performance for clinical diagnosis because it can be used directly to detect the NS1 protein in a single step in sera samples of patients infected with DENV. There was a noticeable decrease in the $R_{ct}$ value for positive sera samples, and this decrease was proportional to the dilution factor (Figure 3). However, no significant change in $R_{ct}$ value was detected for negative sera samples at different dilutions. The change in $R_{ct}$ value might be ascribed to the change in NS1 antigen concentration in the tested samples. The proposed immunosensor demonstrated selectivity for DENV NS1 protein detection with satisfactory differentiation from the negative sera.

## Conclusions

An electrochemical immunosensor for the specific and direct detection of the NS1 antigen was successfully developed. An immunosensor surface consisting of antifouling molecules and monoclonal anti-NS1 IgG antibodies was fabricated based on in situ-generated aryl diazonium cations. The dynamic range for NS1 antigen detection was 5–4000 ng $mL^{-1}$, indicating high analytical performance, with coefficients of determination ($R^2$) = 0.94 and correlation coefficient (R) = 0.95 as determined using the EIS technique. The developed NS1 immunosensor exhibited excellent reproducibility, with a relative standard deviation of 2.1% for the response of five independently prepared immunosensors to 100 ng $mL^{-1}$ NS1 antigen. The NS1 immunosensor showed high stability (up to 21 days with storage at 4°C), high selectivity to the NS1 antigen (no response to human IgG or HSA), and no cross-reactivity with malaria-infected sera. All of the experimental data described in this study demonstrate the potential use of aryl-diazonium-cation-derived zwitterionic antifouling molecules and monoclonal anti-NS1 IgG antibodies for the generation of a specific ITO immunosensing interface for direct detection of the NS1 antigen in human sera. Furthermore, this study highlights the capacity of the NS1 immunosensor as a potential clinical diagnostic test, since the immunosensor has been shown to be capable of specifically detecting the NS1 antigen in complex serum specimens from patients infected with DENV.

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## List of Symbols

| | |
|---|---|
| $Z_W$ | Warburg-Impedance, Ω |
| Cdl | Capacitance, F |
| Rs | The resistance of the electrolyte solution, Ω |
| Z | Impedance, Ω |
| $R_{ct}$ | Charge transfer resistance, Ω |

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