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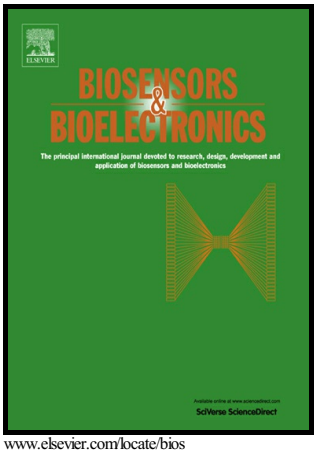

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Biosensor-based selective detection of *Zika virus* specific antibodies in infected individuals

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Abstract

Zika virus (ZIKV) recently emerged as a global threat subsequent to its global spread because it induces microencephaly and other brain damages in infants born to infected mothers. Epidemiological monitoring of infection has been hampered by the absence of reliable serological tests capable to distinguish between ZIKV and other Flavivirus infections, in particular Dengue virus (DENV). As both viruses are transmitted by the same mosquitoe-species, their distributions largely overlap and reliable serological distinction between the viruses is essential. Here we develop a novel biosensor which is based on recombinant forms of ZIKV non-structural protein 1 (NS1) and the domain III of the envelope protein (EDIII). Using electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV), we demonstrate that in addition to extremely sensitive detection of ZIKV-specific antibodies in serum and saliva, the biosensor promptly distinguished ZIKV and DENV-specific antibodies. Hence, this novel biosensor allows assessing ZIKV antibodies in blood and saliva and results are unaffected by presence of DENV virus-specific antibodies.

Keywords: Biosensor; Antibody detection; Zika-specific; Dengue

Introduction

Zika Virus (ZIKV) is a member of the Flaviviridae family and similarly to Dengue Virus (DENV) is a mosquito-borne pathogen transmitted to humans by infected Aedes mosquitoes ("WHO | Zika virus," 2018) (ECDC, 2016). In addition to insect vector transmission, recent studies have shown that ZIKV can also be transmitted by blood transfusion, maternal-fetal transmission and sexual intercourse, which poses additional challenges to the efficient control of viral spread (Turmel et al., 2016) (Seng et al., 2016)(Fiorentino and Montero, 2016). ZIKV was first identified in monkeys in the Zika forest 1947 in Uganda. The first human derived ZIKV sample was isolated a few years later in Nigeria. Until recently ZIKV mainly infected monkeys and disease in humans rarely occurred. However, ZIKV hit the news around the world after the outbreak which occurred in Brazil in 2015, especially since it was associated with neurological disorders, particularly microcephaly in neonates born to infected mothers (Heymann et al., 2016)(Johansson, Michael A. Mier‐y‐Teran‐Romero, Luis Reefhuis et al., 2009). Subsequently, ZIKV spread more worldwide and, in February 2016, the WHO declared it a Public Health Emergency of International Concern (WHO statement 2016). WHO also reported that ZIKV is not only affecting the Americas (including USA and Brazil) but also Oceania/Pacific Islands, Africa and Asia where Aedes species are also endemic.

ZIKV and DENV share substantial genetic similarity and antibodies raised after infection with these different viruses may cross-react and potentially affect disease severity and/or immune protection. Disease-enhancing cross-reactive antibodies are particularly well described for the different DENV serotypes for which previous infection with one of the four known serotypes is thought to predisposes to hemorrhagic fever in subjects infected with a different serotype. It is currently unclear whether infection with ZIKV also predisposes to hemorrhagic fever induced by DENV or vice versa, DENV-infection predisposes to ZIKV-induced hemorrhagic fever (Stettler et al., 2016) (Dejnirattisai et al., 2016)(Wong et al., 2016)(Kawiecki and Christofferson, 2016) (Barba-Spaeth et al., 2016) (Amorim et al., 2014). Thus, specific serological detection of both immunoglobulin M (IgM) and

immunoglobulin G (IgG) distinguishing the two viruses is an essential step to more precisely understand the epidemiology of the infection.

Detailed probing of the DENV and ZIKV envelope proteins with monoclonal antibodies (mAbs) isolated from infected humans have shown that most cross-reactive and disease enhancing antibodies are directed against domains I and II of the envelope protein (EDI/II) and that antibodies directed against EDIII domains are more strain-specific (Stettler et al., 2016). Furthermore, the NS1 protein represents another key antigen for the serological detection of both DENV (Amorim et al., 2014) (Hu et al., 2011) and ZIKV infections as it is immunogenic during natural infection and induces high levels of ZIKV-specific antibodies(Stettler et al., 2016). Currently, the standard methods for Zika detection include detection of nucleic acids (RNA Nucleic Acid Testing (RNA NAT), Trioplex rRT-PCR), or the virus itself (Afsahi et al., 2018). or ZIKV-specific IgM antibodies by Capture Enzyme-Linked Immunosorbent Assay (ZikaMAC-ELISA) in serum, or cerebrospinal fluid (Shukla et al., 2016). However these methods show technical problems such as cross-reactivity with other circulating viruses (DENGUE) and little information on the type of antigens used is provided (Shukla et al., 2016).

The successful development of new diagnostic technologies should ensure rapid sample processing, automatization, cost-effectiveness, high sensitivity and specificity and ease of use, especially by local staff in rural areas. Priye *et al* proposed for this purpose the development of a new platform for rapid detection of Zika, Chikungunya, and Dengue viruses with a smartphone. This is an interesting concept, but it relies on the use of different primers, which may hamper the cost-effectiveness of this approach (Priye et al., 2017).Thus, until now, there is no technique readily available to perform ZIKV diagnostics in real time and under field conditions.

Biosensors may turn out to be a valuable alternative in this context. Biosensors are usually developed using a biological component with an electronic device (G Cabral-Miranda et al., 2014a). A key goal when designing biosensors is that i) they maintain the best characteristic of traditional diagnostic

methods, which have high sensitivity and specificity, and ii) offer the possibility of creating portable and cost-effective devices in a more precise, non-invasive way with real-time detection (Gustavo Cabral-Miranda et al., 2014b),(Cardoso et al., 2017). However, few biosensors for ZIKV are characterized by these desired properties, as most of them are detecting different ZIKV antigens with traditional monoclonal antibodies casted on a given platform. This results in devices with a selectivity limited to the specificity of the antibodies used. For example, a recent study in this field proposed the development a graphene biosensor for early detection of ZIKV, relying on the high sensitivity generated by graphene-based supports but also on the specificity of the antibodies used therein (Afsahi et al., 2018).

Following a different approach, we report herein the development of a biosensor-based diagnostic method for detecting circulating antibodies instead of the antigens. The biosensor is based on a technology recently described by us for malaria antigens (Cardoso et al., 2017). The method consists on the application of two different antigens on a conductive carbon surface. Antibodies against both antigens could be measured in a sensitive way and from undiluted serum and saliva. The biosensor is based on recombinant structural protein domains; domain III of the envelope protein (EDIII) and a fragment of a non-structural (ΔNS1) protein. The choice of these two antigen allows to measure anti-ZIKV antibodies without cross-reactivity to DENV. Thus, this novel type of biosensor may be used in regions where both ZIKV and DENV circulate to enable rapid screening and specific identification of ZIKV-infected subjects.

Experimental Section

2.1 Ethics Statement

2.1.1 Procedures involving animals and human samples

All animals and procedures were used in accordance with the terms of the United Kingdom Home Office and under regulation of The Animals (Scientific Procedure) Act 1986. The Project License was approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2947). The mice were housed in ventilated cages, under specific pathogen free conditions, constant temperature, humidity and with a 12:12 light-dark cycle. For induction of short-term anaesthesia, mice were anaesthetized using vaporized IsoFlo. All animals were humanely sacrificed at the end of each experiment by an approved Schedule 1 method (cervical dislocation). Procedures involving Human samples were approved and all biological samples were collected under the approval of the Ethics Committee of the Institute of Biosciences of the University of São Paulo (CEPSH - Off.011616).

2.2 Construction, expression and purification of proteins

2.2.1 EDIII Protein

The coding sequence of the residues 299-407aa of the envelope protein domain III (E-DIII) and from Zika virus Brazil-ZKV2015 strain (GenBank: KY785450.1) were synthesized in codon optimized fashion and cloned into pET-21(+) (Novagen, Merck, Nottingham, UK) vector with a C-terminal His6-tag and a short C-terminal cysteine (GGC) containing linker. E. coli BL21 competent DE3 cells were then transformed with this plasmid and protein expression was induced with isopropyl-β-D-thiogalactopyranoside in a final concentration of 1 mM. Bacteria were harvested by centrifugation, lysed and inclusion bodies were washed and solubilized in 8 M urea, 100 mM Tris- Cl, 100 mM DTT, pH 8.0, followed by purification on a Ni Sepharose resin.

Purified proteins were refolded by three steps of dialysis. First was against 2 M urea, 50 mM NaH2PO4, 0.5 M arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, 10% glycerol, pH 8.5; followed with dialysis against 50 mM NaH2PO4, 0.5 M arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, 10% glycerol, pH 8.5, and concluding against 50 mMNaH2PO4, 10% glycerol, pH 8.5. Contaminating proteins were removed with Size-exclusion chromatography (SEC) using a HiPrepTM16/60 SephacrylTMS166HR column (GE Healthcare). For that were used 10 Column Volume of Phosphate Buffer Saline (PBS) (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4) in the equilibration step. The sample was eluted isocratically from a SEC column, using a PBS buffer and the flow rate in the both steps was 0.5 ml/min. We confirmed the protein identification, purity and concentration like above described. The ÄKTA purifier was using for IMAC and SEC process.

2.2.2 ΔNS1 Protein

Bacteria were transfected with a plasmid derived from pET28a+ (GenScript, USA), encoding the ZIKV ΔNS1 based in the Brazilian strain (GenBank reference number ALU33341), was introduced into chemically competent E. coli BL21- Codon Plus DEIII strain (Stratagene, USA). Bacterial cultures were induced to produce the ΔNS1 by the addition of a final concentration of 500 nM/L of isopropil-beta-D-tiogalactopiranosídeo (IPTG), for 18 hours, at 18 ºC. The ΔNS1 was obtained in the soluble fraction after cellular pressure lysis performed in a homogenizer (ARTEPEÇAS, Brazil) and the protein was purified with nickel column as describe above. Samples containing the ΔNS1 were pooled and submitted to a second purification step based on size exclusion chromatography. The purified proteins were submitted to a final concentration step by affinity chromatography. The purified protein was evaluated in 15% polyacrylamide gels to confirm the purity of the protein (above 95%). Quantification of the purified protein was determined by gel analysis using the IMAGELAB software (BIORAD LABORATORIES). The purified protein was stored in aliquots at -20 ºC for subsequent use.

2.3 Generation of murine and human serum samples

BALB/c mice were, immunized with protein E-DIII or ΔNS1 formulated in adjuvants and serum was harvested 2 weeks after the second boost.. The human serum samples were collected from ZIKV-infected persons living at the city of São Paulo following protocols approved by the Ethics committee of the Biomedical Science Institute in the University of São Paulo (CEPSH - Off.011616). The three ZIKV-positive samples were collected from symptomatic individuals that report recent travels to ZIKV-endemic regions at the Northeast of Brazil. The samples were confirmed to be ZIKV-positive by Real-Time PCR. Two of the three ZIKV-positive serum samples were also found to be DENV-positive after tested by DENV-specific immunochromatrography. The three DENV-convalescent samples were collected from asymptomatic individuals living in São Paulo. The double negative samples, collected from volunteers, were also used as controls. These samples were negative for both qRT-PCR and DENV-specific immunocromatrography. All serum samples were stored in aliquots at -80 ºC until usage, and transferred to -20ºC in 10µl aliquots for single use.

2.4 Biosensor Delineation

2.4.1 Apparatus

The electrochemical measurements were performed with a Metrohm Autolab, PGSTAT320N, potentiostat/galvanostat, controlled by NOVA 1.11 software. The carbon-screen printed electrodes (Carbon-SPEs) were of commercial origin, produced by ORION (OHT-000) – any other cabon SPEs may be applied herein. The three-electrode system of the carbon-SPEs contained: i) a counter electrode made of carbon; ii) a reference electrode made of silver; and iii) a carbon working electrode of 4 mm diameter. All electrical connections were made in silver. The Carbon-SPEs were interfaced with the potentiostat by means of a switch box (BioTID, Portugal).

All chemicals were of analytical grade and water was deionised (conductivity <0.1 μS/cm) or ultrapure Mili-Q laboratory grade. The chemical reagents including potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, were obtained from Riedel-deHäen; P-Phenyldiamine was obtained from Sigma, N'-ethyl-N'-(3-Dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was obtained from Alfa Aesar, N-Hydroxysuccinimide (NHS) was obtained from Fluka; N-Dimethylformamide (DMF) was obtained from Analar Normapur; Carboxylated carbon nanotubes were obtained from Sigma; and PBS was obtained from Amresco.

2.4.2 Solutions

All the solutions were prepared in PBS buffer and PBS was prepared in ultrapure water. An amount of 1 mg of carboxylated carbon nanotubes was suspended in 1ml DMF. The EDAC/NHS modification used 0.025 M EDAC and 0.05 M NHS solutions, freshly prepared in PBS. A 0.02M P-Phenyldiamine solution was also prepared in PBS. The electrical changes were followed by a solution with 5.0×10-3 M K3[Fe(CN)6] and 5.0×10-3 M K4[Fe(CN)6], prepared in PBS buffer, pH 7.4.

2.4.3 Electrochemical Procedures

The electrochemical studies involved Cyclic Voltammetry (CV), Electrochemical Impedance Spectroscopy (EIS) and Square Wave Voltammetry (SWV) assays, performed in the iron redox probe solution. CV assays were made by scanning potentials from -0.3 to +0.7 V, at 50 mV/s. EIS assays were made at a formal potential of +0.14V, using a sinusoidal potential perturbation with amplitude 0.01V, and 50 frequency values, logarithmically distributed from 0.1 to 100000 Hz. The EIS data was fitted to a Randles equivalent circuit using 1.11 Nova Software from Autolab. SWV

assays were conducted from -0.3 to +0.7 V, with a frequency of 25Hz and the step height of to 50mV.

Regarding specifically EIS data, Nyquist plots was used to plot the obtained spectra, showing the frequency response of the electrolyte system and plotting the imaginary component (Z'') of the impedance against its real component (Z'). EIS data was fitted herein into the typical Randle's equivalent circuit, which matched the physiochemical processes occurring at the carbon electrode surface. This circuit was composed of several elements: resistance of the solution phase (Rs), capacitance of the double layer (Cdl), charge-transfer resistance (Rct) at the electrode surface and Warburg diffusion element (W). Rct was given by the diameter of the semicircle obtained in the plot.

2.4.4 Synthesis of the sensing layer

The system used to build this biosensor is one of our biosensor construction previously applied to Malaria diagnostic which is earlier very well detailed in term of design and manufacture (G Cabral-Miranda et al., 2014). Following the previous biosensor delineation we applied the E-DIII or ΔNS1 protein from ZIKV as probes to make a bio-interface with carbon-screen printed electrodes (SPEs) (from Orion). The overall changes made on the carbon electrode area are presented in Figure 1. In brief, First, a suspension of carboxylated carbon nanotubes in DMF was dispersed in ultrasounds for 1h. After that, a volume of 6 µL of this suspension was casted on the carbon electrode area and let dry at 70 ºC for 30 minutes. This procedure was repeated three times. The carboxylic groups on the surface were activated by casting 6 µL of a solution containing EDAC (0.025M) and NHS (0.0125M), freshly mixed, for 1h30, at room temperature. This solution was removed and replace by 6 µL of a 0.02M -Phenylenediamine solution, casted for 60 minutes. Each protein (EDIII and NS1 protein) was immobilized by casting 10µL of a 4.5 µg/ml E-DIII and 1.5 µg/ml solution, prepared in PBS, pH 7.4. This solution was let stand for 45 minutes, at room temperature. The sensing layer was washed with PBS after that. The antibody binding was made by casting standard solutions of Anti-EDIII and Anti- NS1 of increasing concentrations. Each standard was incubated for 30 minutes at

37ºC. The positive serum samples were tested similarly, imposing several degrees of dilution to these and evaluating the response of the sensing layer to each degree of dilution.

2.5 *Assessment of anti-ZIKV EDIII and ΔNS1antibodies ELISA*

2.5.1 Zika virus specific antibodies detection by Enzyme-Linked Immunosorbent Assay (ELISA)

96-well microtiter ELISA plates (Thermo Scientific, Nottingham, UK) were coated with 100µL per well at 1µg/mL of EDIII or NS1 protein diluted in carbonate buffer (CBB) 50 mM at pH=9.6 and incubated overnight at 4°C. ELISA was then performed according to standard protocol using anti-human IgG diluted 1:2000 (Secondary Antibody, Horseradish peroxidase (HRP) conjugate (ThermoFisher, UK)) as a second stage.

Results and Discussion

3.1 EDIII and ΔNS1 ZIKV Proteins

Serological diagnosis of flaviviruses is a challenging task due to cross-reactivity between the anti-viral antibodies (Keasey et al., 2017). In places like Brazil and other endemic regions, where a variety of different flaviviruses co-exist, e.g. DENV, ZIKV and Yellow fever virus (YFV) (Marcondes et al., 2015), it is essential to distinguish serologically between different viruses in reliable manner. In order to avoid false positive diagnosis, the use of EDIII appears to be a promising strategy as mAbs isolated from infected patients reliably distinguish between DENV and ZIKV(Stettler et al., 2016). The NS1 protein is the main marker for serological diagnosis of DENV infection (Ambrose et al., 2017). After infection, the NS1 protein is expressed and transported to the cell surface where it either remains associated with the cell or is released in the blood stream, causing high IgG responses (Amorim et al., 2014). However, cross-reactivity of NS1-specific antibodies with other flaviviruses may interfere with specific detection. Indeed, as shown in this study, full-length

NS1 proteins of the 4 DENV serotypes strongly cross-react with ZIKV antibody positive sample causing false positive results (Figure S1). This urges the choice of more specific markers. Therefore, the use of a recombinant fragments derived of ZIKV NS1 (ΔNS1) may provide a reliable serological diagnostic tool in addition to EDIII.

The coding sequences for the EDIII and ΔNS1 domains were codon-optimized for bacterial expression and synthesized by GeneArt and GeneScript, respectively. The EDIII protein was expressed in E. coli as inclusion bodies and refolded subsequently. The purity of the proteins was assessed by Coomassie staining of SDS-PAGE and found to be around 95% after size exclusion chromatography. The ΔNS1 protein, encompassing the last 101 C-terminal amino acids of the ZIKV NS1 protein sequence, is currently used in a standardized diagnostic kit based on ELISA, reportedly capable to differentiate ZIKV antibodies from those generated by other flavivirusus infections (Zanotto et al., 2016). The ΔNS1-fragment was produced in E. coli and purified from the soluble fraction of cellular lysate. The purification procedure involved a 3-step procedure encompassing affinity and size exclusion chromatography, yielding a purified protein at purity level of approximately 95%. Proteins were subsequently used to immunize mice to generate sera for testing of both the biosensor and ELISA.

3.2. Biosensor Designing

Any device for screening a compound of interest requires the selection of biorecognition material of biological or synthetic nature, acting as sensitive recognition element, and its integration into a support in a compact analytical apparatus, acting as physicochemical transducer (Figure 1A). The overall device must be assembled in such a way that high sensitivity and selectivity are both equally achieved.

The main principle behind the construction of this novel biosensor was inspired by our previous work in malaria (Cardoso et al., 2017), where reproducibility, selectivity and sensitivity of antibody detection has proven to be exceptionally high. Herein, screen-printed electrodes (SPE) were

used and modified with carbon-nanotubes, to improve the electrical features of the final device. This layer was then modified with *p*-phenylenediamine to form an amine layer. The proteins ZIVK EDIII or ΔNS1were bound next to this layer by physical adsorption (Figure 1B).

In detail, the first stage of modification of the biosensor consisted of casting carboxylated carbon nanotubes onto the working electrode area (Figure 1BI). Only the working electrode was covered with the modifying solution, thereby avoiding changes on counter or reference electrodes. This procedure was repeated three times. Each drop was allowed to dry, before adding a new one. The final surface had good electrochemical feactures against an iron-redox probe, as evidenced in Figure S2-A. The resulting surface was then altered with the purpose of generating an amine layer. This was done in two stages. The first stage consisted on activating the –COOH functions of the carbon nanotubes by EDAC reaction, forming a highly reactive O-acylisourea intermediate. This was followed by the immediate reaction with NHS, which produced an activated ester that is sufficiently unstable to react with an amine group under mild conditions (Figure 1B-II) (Jiang et al., 2004). This chemical modification was followed by cyclic voltametry, as shown in Figure S2-B (the electrodes used for this control were discarded, as this unstable layer could be changed after electrochemical scanning). The second stage consisted of the amination of this surface (Figure 1B-III), by adding a di-amine compound to the activated carboxylic surface. This amine layer was now ready to accommodate the protein antigens on its surface (Figure 1B-IV). This was done simply by casting the corresponding solutions. The final cyclic voltammetry scan indicated a significant decrease in the current measured, thereby confirming the presence of adsorbed protein (Figure S2-C).

The detection of specific antibodies was made by incubating the sample for 30 minutes, washing and reading the resulting EIS and SWV electrochemical data of a solution with 5.0×10^{-3} M $K_3[Fe(CN)_6]$ and 5.0×10^{-3} M $K_4[Fe(CN)_6]$, prepared in PBS buffer, pH 7.4.

3.3.Preliminary tests using commercial antibodies

The newly assembled biosensor was tested against different sources of antibodies, to assess its response against standard (known) concentrations. For this purpose, serial dilutions were made of specific mAbs, which were used for calibration, as well as mice sera and the sensor was incubated with increasingly concentrated solutions for 30 minutes. After each incubation, the sensing layer was washed and the electrical data was obtained with a drop of iron redox probe prepared in PBS buffer pH 7.4. EIS and SWV data was obtained for this purpose. The EIS data is discussed next and the SWV data (which is consistent with the impedance data) is shown in the supplementary data.

Typical Nyquist plots obtained in EIS readings are presented in Figure 2A and signal the changes in resistance to charge-transfer (Rct) occurring for each concentration of a monoclonal antibody. As the antibodies are proteins and proteins have non-conductive properties, the resistance of the biorecognition element increases as antibodies bind to it. The sensor was able to detect antibodies in pure serum and serum diluted up to 17717700x times for EDIII, which corresponds to 53 fg/ml of mAb, and up to 53144100× times for ΔNS1, corresponding to 17 fg/ml of mAb. Within this concentration range, the relative Rct varied from ~0.97 to 2.75 Ω. This specific relative Rct range was considered valid to analyze sera containing a wide range of antibody concentrations.

3.4.Preliminary tests using sera from immunized mice

Next, the biosensor was tested with serum samples collected from mice immunized with EDIII or ΔNS1. Murine sera were serially diluted ranging from 900× to 53144100×. The Nyquist plots showed that increasing concentrations of Anti-EDIII and ΔNS1 increased the Rct values of the probe at the sensing layer (Figures 2A and 2C). This was observed by the increasing diameter of the semicircles in the Nyquist plots and caused by additional amounts of protein bound to the surface. Since each mouse serum had unknown antibody concentration, all sera samples were analysed by

ELISA and compared to mAbs used as reference estimate their concentrations. These concentrations were the ones used to plot the calibrations in EIS, shown in Figures 2B and 2D. The concentrations of these antibodies ranged from 8.7 mg/mL to 5.8 mg/mL in case of E-DIII and 9.6 mg/mL to 5.3 mg/mL for ΔNS1. Higher concentrations than these saturated the sensing layer, while lower concentrations were probably not detected, as little differences were observed between the signals of the blank and the first standard of the calibration curve. The reproducibility of the sensing system was excellent, as confirmed by performing two/three independent calibrations with two/three independent devices, assembled separately. For each specific antibody solution under study, the standard deviation of the slope of the calibration was ~1% (Figure 2B) and 3% (Figure 2D). Combining all calibrations (duplicates and different antibody solutions), the average slope values were -0.656 Ω/decade to E-DIII and -0.6528 Ω/decade to ΔNS1.

Overall, it was clear at this stage that EIS and SWV detected specific antibodies in a highly sensitive manner. The described sensing layer offered reproducible data and no significant differences were produced using different calibrating solutions. It is also important to highlight that the higher concentrations of antibody are located in the right side of the *x*-axis, meaning that the Rct signal decreased continuously over a 50`000-fold dilution range, indicating a very large window of sensitive antibody detection.

3.5.Testing the biosensor using human sera

Sera samples from naturally infected patients (*n*=3) were further tested using the biosensor, following the same dilution procedures. Convalescent patients had been diagnosed by PCR to be ZIKV-infected. The biosensor built with ΔNS1 fragment detected antibodies in serum from ZIKV-infected patients at a dilution up to 53,144.100×, when EIS was used as read-out (Figure 3A). The calibration corresponding to each of three patients are shown in Figure 3B (each curve represents

two independent assays). The standard deviation of the slope of the calibration was 1.615% (Figure 3B1), 0.46% (Figure 3B2) and 1.32% (Figure 3B3), thereby confirming the high reproducibility of the system. Combining all calibrations (duplicates and different antibody solutions), the slope values were -0.6919Ω/decade for Patient 1, -0.5532 Ω/decade for Patient 2 and -0.6333 Ω/decade for Patient 3.

In general, a similar behaviour was observed when using the EDIII-specific biosensor. Typical data of EIS is shown in (Figures 4A/B). Combining all calibrations (duplicates and different antibody solutions), the slope values were -0.5693Ω/decade for Patient 1, -0.5036 Ω/decade for Patient 2 and -0.5799 for Patient 3 (Fig 4D). Data generated by EIS was very similar to that of SWV and both techniques were able to detect antibodies in human samples in a dilution until 17,714.700× in all patients. (Figures S3 and S4).

As shown above, the sensitivity of the biosensor was very high. However, the specificity of the biosensor needed to be assessed as well. To this end, we used samples from negative control patients neither infected with DENV nor ZIKV. As seen in the Figure S5 the EDIII and ΔNS1 biosensors barely responded when tested with patient double negative for ZIKV and DENV.

3.6. Testing the biosensor in human saliva

The possibility of detecting specific ZIKV antibodies in saliva was tested next. For this purpose, a drop of saliva from ZIKV positive and negative (control) patients was tested in the biosensor. A positive response was yielded only by the ZIKV positive patient for both antigens using both EIS (Figure 5) and SWV (Figure S6). Indeed, the biosensor recognized antibodies against ΔNS1 and EDIII in saliva with high specificity. Moreover, using saliva from a negative patient, there was no electrical response. Hence, the here described biosensor may be a useful device for a non-invasive way to detect ZIKV antibodies in ongoing or previous infections.

3.7 Testing the cross-reactivity with anti-DENV-antibodies

Most critical was whether the biosensor would indeed be specific for ZIKV antibodies or cross-react with DENV antibodies. To check this possibility, we analysed samples from patients positive only for DENV and compaired their responses with samples positive only for ZIKV. Figure 6 shows the analytical data generated by incubating human serum from DENV or ZIKA-infected individual on the ΔNS1- or EDIII based biosensor. The results demonstrated that DENV+ sera failed to efficiently recognize the biosensor and that the detection system was highly specific for ZIKV. The remarkable sensitivity and selectivity observed in this biosensor opens the way for a new line of devices to be used in diagnosis at point-of-care to reliably detect ZIKV-specific antibodies.

3.8. Comparison with classical ELISA

To compare the biosensor sensitivity and specificity to a conventional ELISA assay, the same ΔNS1 and EDIII domains were coated on ELISA plates. The test was performed using HRP-conjugated anti human IgG (Thermo Fisher, UK), using the same patient sera. As shown in Figure S7, the 3 patients positive for ZIKV showed a more intense response than patients that were only DENV-positive. In fact, both negative and DENV-only positive patients were essentially negative for both ZIKV ΔNS1 and EDIII domains. Comparison with the biosensor data reveals that the biosensor is roughly 10`000 more sensitive than the ELISA, which had a limit of reliable detection of about 1:2700 only.

3.9. Using the Biosensor to detect ZIKV-specific IgG and IgM

A clinically relevant serological diagnosis needs to be able to differentiate acute from chronic or cleared infection. For this purpose, the detection of IgG and/or IgM is an attractive strategy, because

in an acute infection, levels of IgM are usually high within days after infection and continue to be detectable for several weeks. Hence, IgM antibodies are indicative of an acute infection. In contrast, IgG antibodies appear later in infection and continue to be detectable for long time-periods. IgG antibodies without IgM antibodies therefore indicate a low level chronic or cleared infection.

To detect ZIKV-specific IgG and IgM antibodies, the biosensors were first incubated with a standard serum diluted (218700×) and were subsequently exposed to a secondary antibody, namely anti-IgM (A, C) or anti-IgG (B, D). In general, IgG antibodies against NS1 or EDIII were detected in all patients using both electrochemical techniques, EIS (Figure S8) and SWV (Figure S9).

Although we do not know the exact time-points of infection, the ability to distinguish between ZIKV specific IgM and IgG shown for this biosensor seems to be promising to distinguish between acute/recent versus long-time ago infections, an interesting feature for the diagnosis of individual patients and a key asset for monitoring the development of ZIKV epidemiology.

Conclusions

A reliable serology for the monitoring and/or diagnosis of ZIKV infection remains a challenge with current technologies. PCR-based methods are complex to be used in rural areas and may not be useful to detect ZIKV after the virus has been cleared. ELISA-based methods are not very sensitive and little reliable information is available about their ability to distinguish between ZIKV and other Flaviviruses (Shukla et al., 2016).

We show here the development of a highly sensitive and specific biosensor for the detection of ZIKV antibodies in blood and saliva. The ability of the biosensor to detect antibodies in the saliva seems of particular interest, since no blood needs to be sampled from individuals to be tested. This represents a substantial advantage, since it is often difficult to obtain blood from volunteers in e.g. epidemiological studies. Furthermore, saliva testing would allow for rapid screening of people in

sensitive areas, as e.g. the border or airports during a ZIKV outbreak. Thus, this biosensor may be ideal for monitoring ZIKV infections in endemic areas.

Due to its high sensitivity, this novel biosensor may be used early during infection, when levels of circulating antibodies are still low and may not be detected by conventional ELISA methods. The technique therefore has the potential to become a valuable early diagnostic tool, in particular in regions were sensitive PCR methods are not readily available. This positions the biosensor as a potentially valuable tool both for monitoring the epidemiology of ZIKV at the level of the population as well as early diagnosis of ZIKV infection at individual levels.

The biosensor in its current form is only able to detect ZIKV specific antibodies and requires a relatively large machine for measurement of the electrical signals. Future work therefore focuses on miniaturizing the detector and the development of multivalent biosensors, covering a multitude of different viruses. Specifically, we are currently developing a biosensor with the ability to detect antibodies against ZIKV, the four DENV serotypes as well as Yellow fever virus. This would allow the simultaneous measurement of antibodies against of all major flaviviruses, adding an important tool for the diagnosis and monitoring of this important class of viruses.

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Figure 1*:* Schematic representation of a general biosensor (A) and of the biosensor developed here (B), containing ZIKV-derived proteins as biorecognition component (in the present case) bound to the carbon support material. (BI) Oxidation of carbon material; (BII) Activated carboxylic acid ends; (CIII) p-Phenylenediamine; (IV1) Incubation with ΔNS1 protein; (IV2) Incubation with EDIII protein; and (V) binding of the sensing layer to immune antibodies (Anti-ΔNS1 (V1) and Anti-EDIII (V2)).

Figure 2 Analysis of mice serum by EIS measurements. Nyquist plots for serial dilutions of (A) anti-EDIII and (C) anti-NS1 sera, along with the corresponding relative Rct values of two calibrations with two independent electrodes (B, D). Each serum dilution was incubated on the biosensor for 30 minutes, washed, and the Rct was obtained with a drop of 5.0×10^{-3} M $[Fe(CN)_6]^{3-}$ and 5.0×10^{-3} M $[Fe(CN)_6]^{4-}$, in PBS buffer pH 7.4, covering the three electrodes of the SPEs.

Figure 3 Analysis of ZIKV positive human sera by Anti-NS1 biosensor and EIS measurements. (A) Typical Nyquist plot for serial dilutions of a human serum sample (derived from patient 1). (B) Comparison of human sera from three patients with different dilutions; (B1) Patient 1; (B2) Patient 3; (B3) Patient 2. Each serum dilution was incubated on the biosensor for 30 minutes, washed and the Rct was obtained with a drop of 5.0×10^{-3} M $[Fe(CN)_6]^{3-}$ and 5.0×10^{-3} M $[Fe(CN)_6]^{4-}$, in PBS buffer pH 7.4, covering the three electrodes system of the SPEs.

Figure 4 Analysis of ZIKV-positive human sera by EDIII biosensor and EIS measurements. (A) Typical Nyquist plot for serial dilutions of a human serum sample (derived from patient 1). (B) Comparison of human sera from three patients with different dilutions ; (B3) Patient 1; (B2) Patient 2; (B1) Patient 3. Each serum dilution was incubated on the biosensor for 30 minutes, washed and the Rct was obtained with a drop of 5.0×10^{-3} M $[Fe(CN)_6]^{3-}$ and

5.0×10^{-3} M $[Fe(CN)_6]^{4-}$, in PBS buffer pH 7.4, covering the three electrodes system of the SPEs.

Figure 5 Specific detection of anti-ZIKV antibodies in saliva by EIS with (A) E-DIII and (B) ΔNS1 (B) proteins, testing positive and negative saliva samples. Each drop of saliva was incubated in the biosensor for 30 minutes, washed and the Rct obtained with a drop of 5.0×10^{-3} M $[Fe(CN)_6]^{3-}$ and 5.0×10^{-3} M $[Fe(CN)_6]^{4-}$, in PBS buffer pH 7.4, covering the three-electrode system of the SPEs. No pre-treating procedure was made.

Figure 6 Response of the E-DIII (A,C) and ΔNS1 (B,D) biosensor tested DENV and ZIKV sera. Each drop of pure serum was incubated on the biosensor for 30 minutes, washed and the Rct (A,B) and SWV (B,D) were obtained with a drop of 5.0×10-3 M [Fe(CN)6]3- and 5.0×10-3 M [Fe(CN)6]4-, in PBS buffer pH 7.4, covering the three-electrode system of the SPEs.

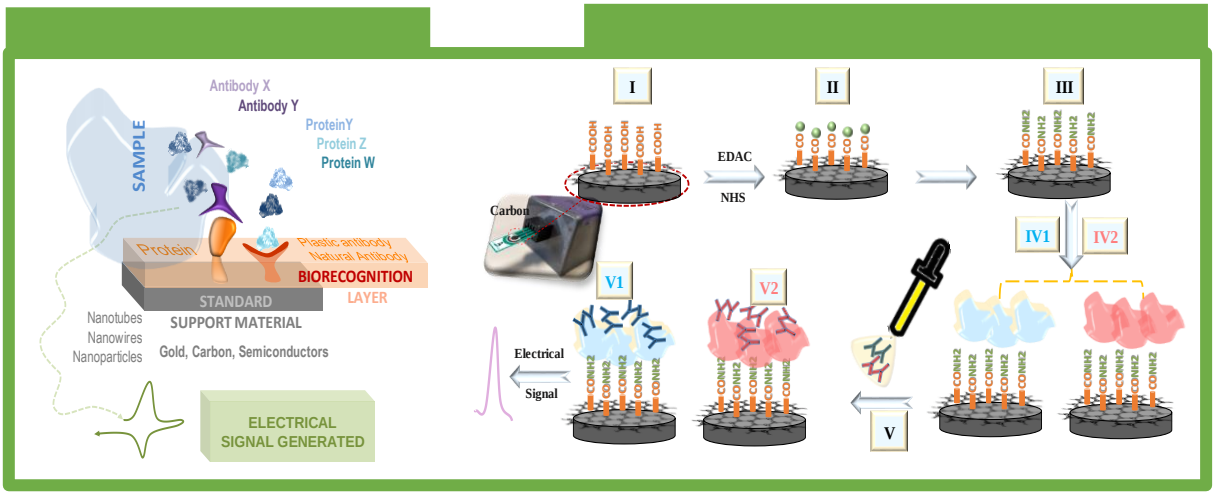

Figure 1

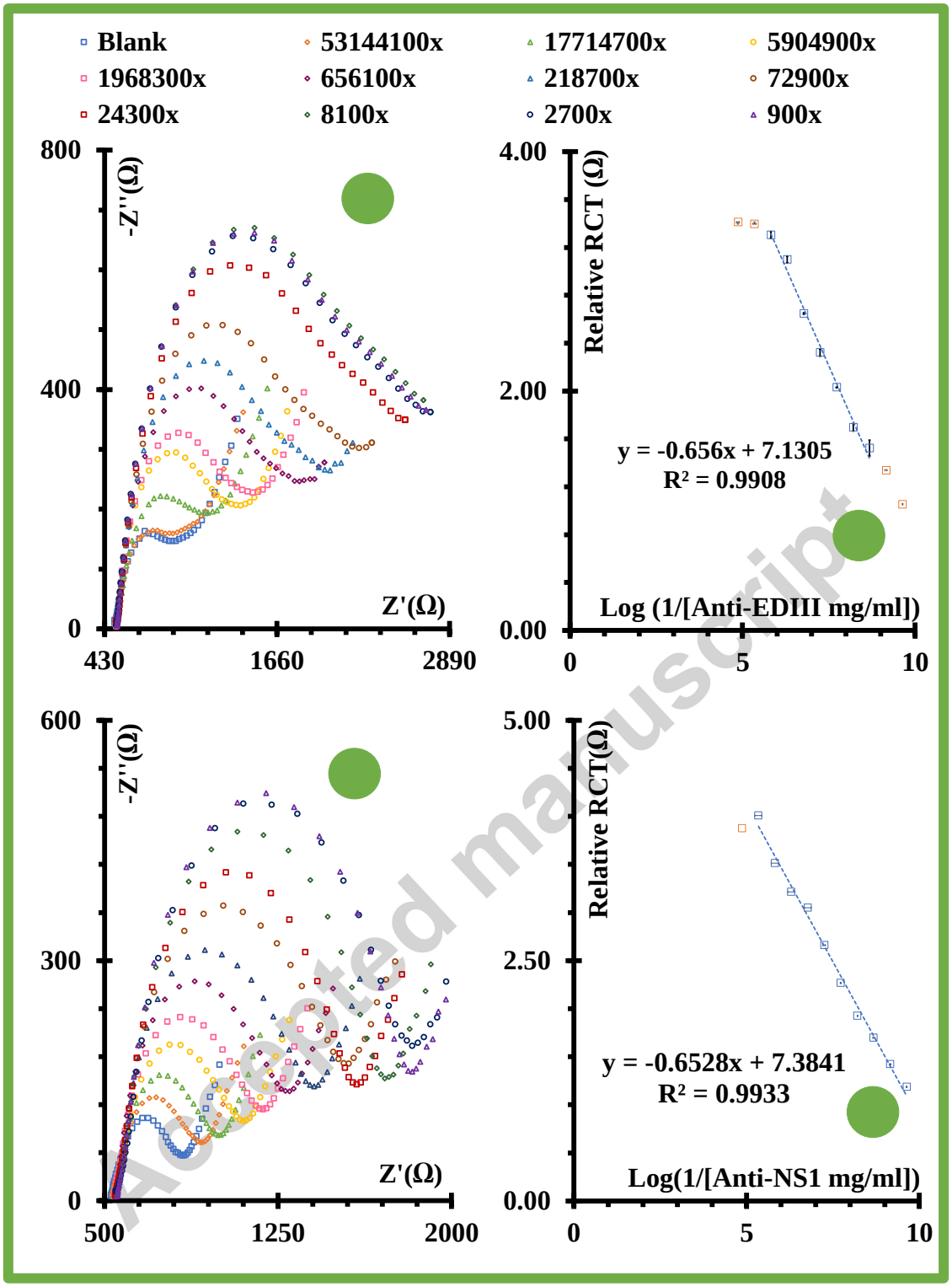

Figure 2

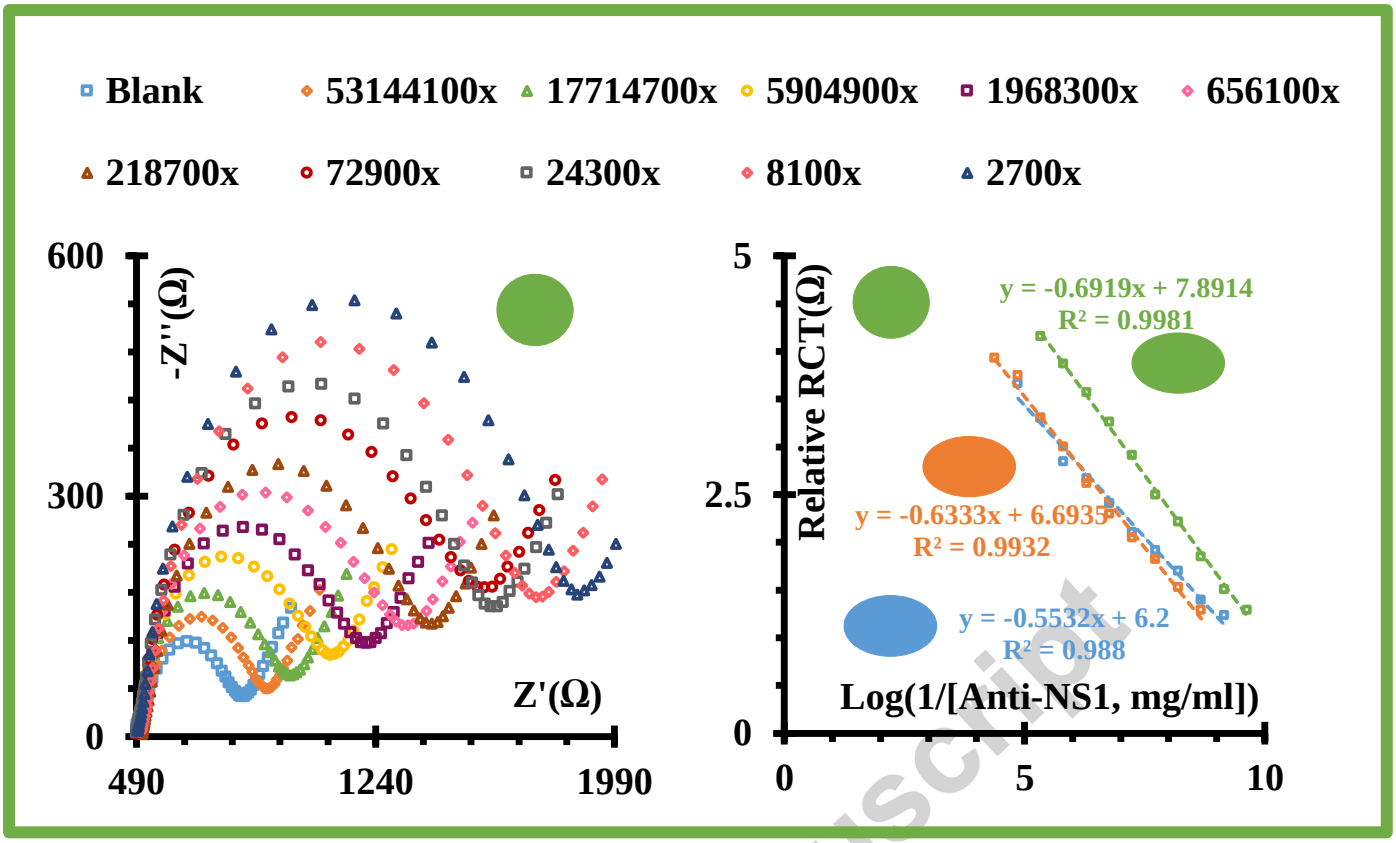

Figure 3

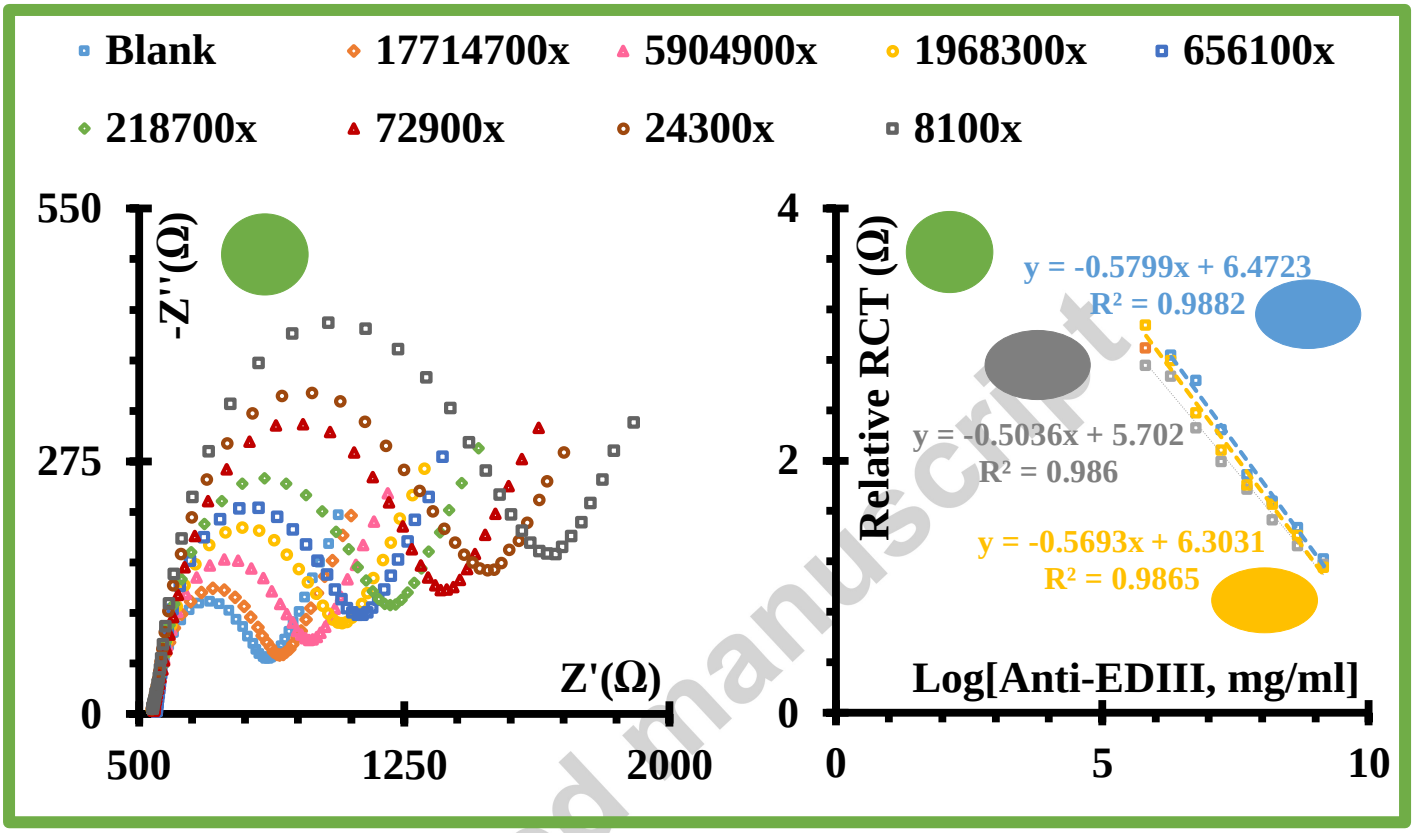

Figure 4

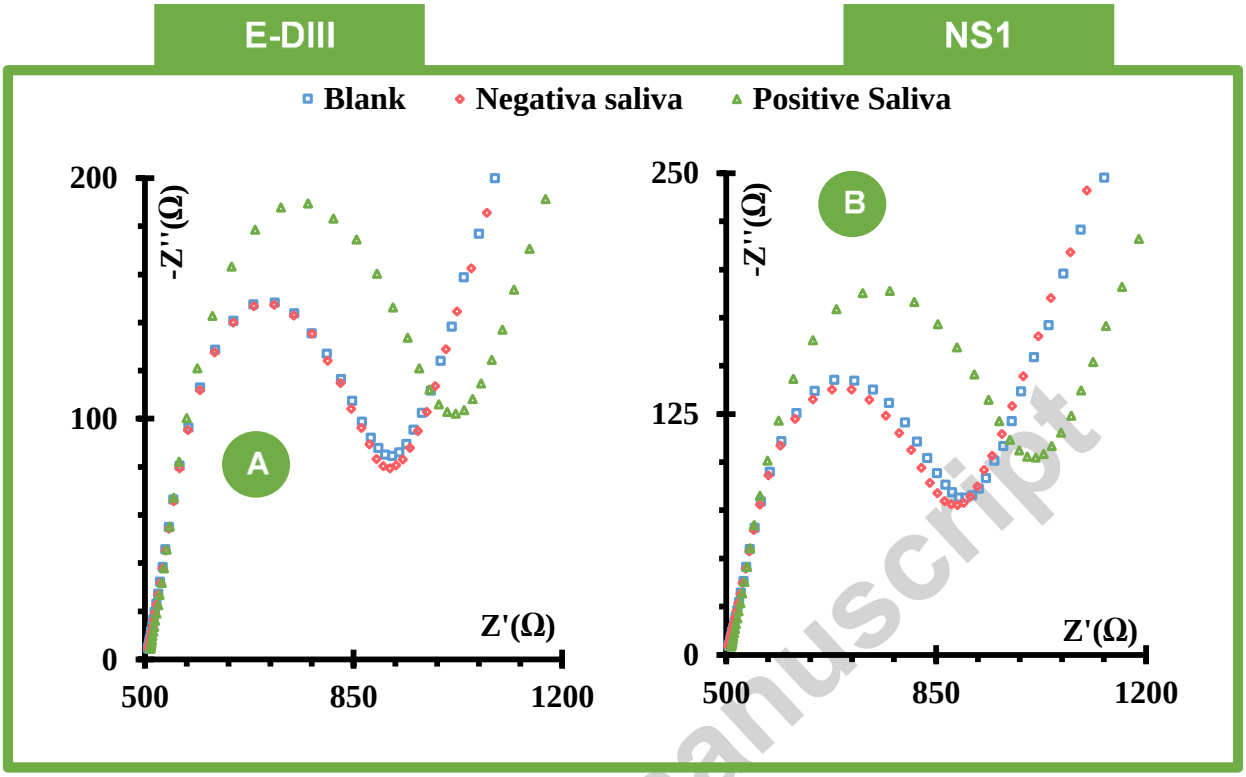

Figure 5

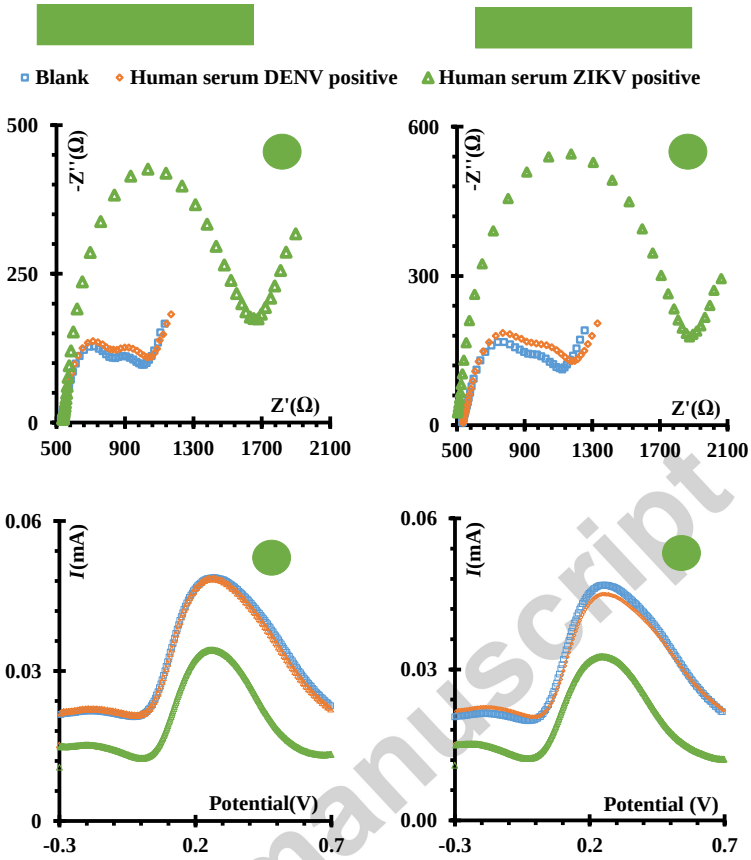

Figure 6

Highlights:

- A highly sensitive and specific biosensor for the detection of ZIKV antibodies in blood and saliva;
- Monitoring ZIKV infections in endemic areas;
- Valuable tool both for monitoring the epidemiology of ZIKV at the level of the population as well as early diagnosis of ZIKV infection at individual levels.