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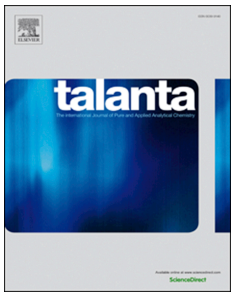

Early diagnosis of Zika infection using a ZnO nanostructures-based rapid electrochemical biosensor

Aline Macedo Faria, Talita Mazon

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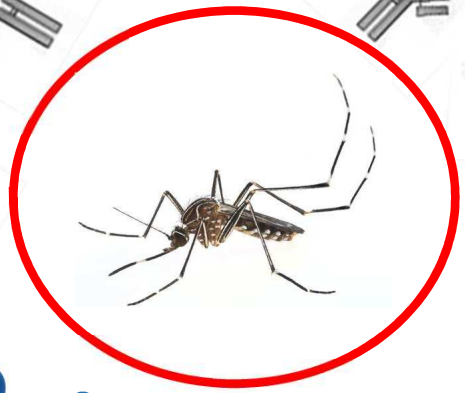

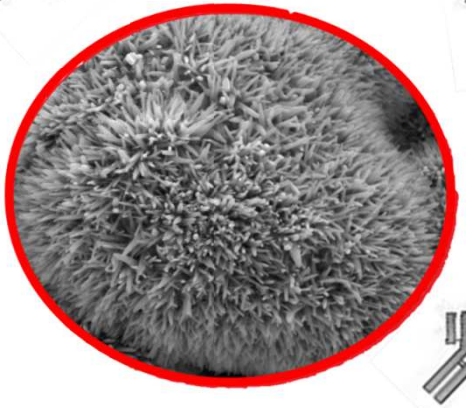

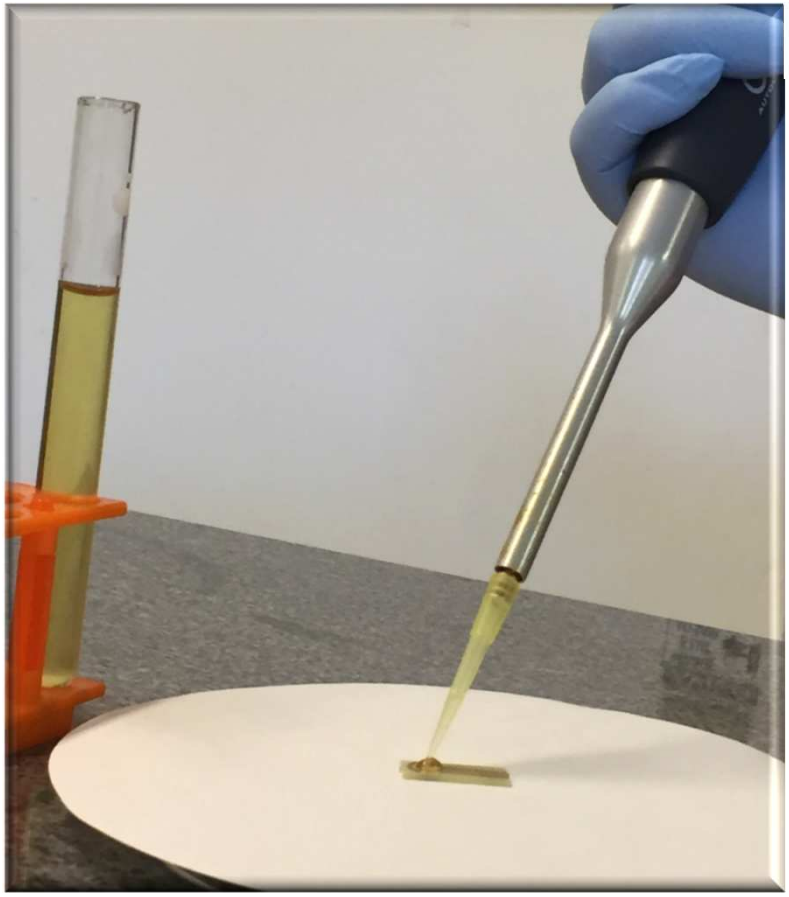

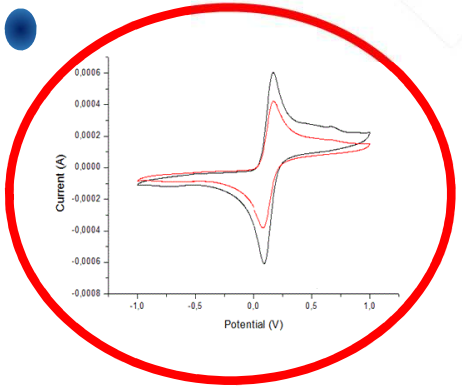
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Early Diagnosis of Zika Infection Using a ZnO Nanostructures-based Rapid Electrochemical Biosensor

Aline Macedo Faria, Talita Mazon*

Centro de Tecnologia da Informação Renato Archer, CTI, Rod. D. Pedro I, KM 143.6, 713069-901, Campinas, SP, Brazil

* Corresponding author: e-mail: talita.mazon@cti.gov.br; Centro de Tecnologia da Informação Renato Archer, CTI, Rod. D. Pedro I, KM 143.6, 13069-901, Campinas, SP, Brazil; Phone Number (+55) 19 3746-6185

ABSTRACT

In this work, we report a develop of electrochemical immunosensor based on ZnO nanostructures immobilized with ZIKV-NS1 antibody on Printed Circuit Board (PCB). ZnO nanostructures were grown on PCB by chemical bath deposition (CDB) and characterized by SEM. ZIKV-NS1 antibody was immobilized on the ZnO nanostructures surface via cystamine and glutaraldehyde. The samples were characterized by Immunofluorescence Confocal Microscopy and FTIR to identify the immobilization of the antibody to the sensor board. The analytical responses of the immunosensor were evaluated by Cyclic Voltammetry (CV). The biosensor developed here allows rapid detection of Zika virus in undiluted urine, without cross reactive with DENV-NS1 antigen, with linear range 0.1ng mL^{-1} to 100 ng mL^{-1}. The limit of detection is lower than 1.00 pg.mL^{-1}. The biosensor is portable, cost-effectiveness, and simple to use, which makes it ideal for point-of-care applications.

Keywords: Immunosensor; ZnO; Zika virus; dengue.

1. Introduction

Zika virus (ZIKV) is a member of family *Flaviviridae* transmitted through the bite of infected *Aedes aegypti* mosquito. People with ZIKV disease can have symptoms, including headaches, mild fever, artharalgia, myalgia, rash and conjunctivitis [1]. Differential diagnosis based on ZIKV symptoms is still challenging once their often overlap with Dengue, Chikungunya and Yellow Fever [2]. Although zika fever is mostly asymptomatic, direct correlation between ZIKV infection in pregnant women and brain defect in fetuses is a major concern [3]. Because of this, the development of ZIKV diagnostic reliable tests is a crucial issue extensively studied nowadays. The diagnosis of ZIKV normally has two approaches developed for detecting it. One is based on sequencing of viral genomes, and other is detection of IgM and IgG produced by patients in response to exposure to ZIKV [4].

RNA viral identified by genomic amplification (RT-PCR) [5,6], Elisa [7], CRISPR-Cas13 [8], reverse-transcription loop-mediated amplification (RT-LAMP) [9], immunochromatography [10], Plaque Reduction Neutralization Test [11], assays are

recommended for detecting ZIKV infection with high sensibility and selectivity. However, specificity in most ZIKV diagnosis is difficult to get due to cross reactive with Dengue virus. Besides, these techniques are expensive, take long time and require equipment and highly trained employees [12]. Considering the higher incidence of ZIKV infection is located in incoming countries, a specific, selective and sensible low-cost point-of-care test is needed.

Electrochemical biosensor technology has been considered promissory for point-of-care (POC) device [13]. In this technology, the material used as working electrode, and the molecules used as bioreceptors, are critical parameters to ensure a low limit of detection, high selectivity and specificity [14, 15]. Nanomaterials-based working electrode is a promise by affording low limits of detection and better selectivity than traditional assays [16, 17]. Among the promise nanomaterials, zinc oxide nanorods (ZnO NRs) are easily synthesized with different morphologies and large specific area [18,19]. As a semiconductor with high chemical stability, ZnO NRs provide a suitable surface for immobilizing antibodies and an ideal path for effective carrier transport [20]. We have recently demonstrated use of ZnO NRs-based working electrodes as a sensitive, and cost-effective working electrode [21].

With regard to bioreceptors, the use of Monoclonal antibodies makes biosensors more selective [22]. The non-structural protein 1 is an important molecule of the viruses in the flavivirus group, including Zika virus and Dengue [23 - 25]. Recent studies have shown that ZIKV-NS1 protein conformation has unique characteristics when compared to NS1 proteins from other flaviviruses [12, 23]. This suggest NS1 may be a potential target for the diagnosis selective of Zika infection without cross reactive with another flavivirus. ZIKV-NS1 protein can be detected in urine thus without invasive blood collection, since first symptoms until seventh day of infection, even before appearing specific antibody [5]. The major problem of using it is to find a NS1 protein specific to ZIKV without cross reaction with Dengue.

Herein, we combine ZnO NRs-based working electrode with anti-ZIKV-NS1 protein antibody to detect ZIKV-NS1 antigen in urine without cross-reaction with Dengue. The developed diagnostic test is specific, cost-effective, takes short time and may be used as point-of-care device. The test is made in urine without filtration, and the biosensor can be read in a portable potentiostat. These characteristics make it ideal for using in borders of ports and airports for travelers who return from endemic areas. The test could also be part

of prenatal care and used in health centers to identify patients with ZIKV. Furthermore, the obtained information by ZIKV biosensor could be used for management of the disease and identification of endemic areas.

2. EXPERIMENTAL

2.1. Materials and reagents

The bare-sensor board was made by using a Printed Circuit Board Technology (PCB). The three electrode system was built on an FR-4 (1.6 mm thick) sheet (Jiangsu Sunyuan Aerospace Material Co).

2.1.1. Chemical reagents and materials

Zinc acetate, Hexamethylenetetramine (HMTA), zinc nitrate ($Zn(NO_3)_2$), buffered phosphate saline (PBS) pH7.4, Ferrocianeto de Potássio ($K_4[Fe(CN)_6]$ Nitrato de Sódio ($NaNO_3$), glycerol, bromophenol blue, and dithiothreitol solution were purchased from Sigma (St. Louis, MO). Silver Conductive Epoxy, H2OE EPO-TEK, were purchase from Epoxy Tecnology. Graphene oxide (GO) was prepared by modified Hummers' method [26]. Cystamine dihydrochloride (Cys, Alfa Aesar), Glutaraldeyde (Glut) 2.5% (Electron Microscopy Sciences)Prestained protein marker (Color-coded,Cell Signalling Tecnology). Nitrocellulose membrane (Bio-Rad, Hercules, CA). Transfer buffer consisting of 50 mM Tris-HCl, pH 7.0, 380 mM glycine, 0.1% SDS, and 20% methanol. Tris-buffered saline with Tween and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz). Super Signal CL-HRP Substrate System (Pierce, Rockford, IL).

2.1.2. Biological reagents

Zika virus NS1 protein (Fitzgerald), mouse monoclonal anti-ZIKV-NS1 antibody (Fitzgerald); horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz); Dengue NS1 protein (DENV-NS1, Abcam – Cambridge).

2.2. Electrochemical bare-sensor board fabrication

Gold trails were used as working and counter electrodes, and silver trail as a reference electrode. Gold electrodes were deposited by an electrolytic method by using a current of 20 A. The silver electrode was deposited by screen printed by using a Silver Conductive

Epoxy, H2OE EPO-TEK and it was cured at 100°C for 2h. Figure S1 shows our electrochemical bare board made on FR-4 substrate.

2.3. Growth of ZnO NRs on working electrode

The samples were synthesized by CBD as previously described [26]. Firstly, a seeding layer, consisting of GO and zinc acetate films, was sprayed on the working electrode of the bare-sensor board by spray coating (Exacta Coat). Aiming to prepare it, we sprayed 12 layers of a 0.05g.L^{-1} GO solution followed by 12 layers of an ethanolic solution of 30 mmol.L^{-1} of zinc acetate. The spray parameters used during deposition were 5W, 1.00 KPa and 0.30 mL.min^{-1} at 100 ^{o}C.

After deposition of the seeding layer, samples were used for growing ZnO NRs by CBD. HMTA, and $Zn(NO_3)_2$ were mixed in the proportion 1:1 in a Polytetrafluoroethylene (PTFE) vessel. After that, the bare-sensor board was immersed in the precursor solution. The PTFE vessel was placed in silicone bath and the solution was stirred and heated at 90°C for 2 h, aiming to promote the growth of ZnO NRs.

2.4. Immobilization of the Zika NS1 antibody

The monoclonal ZIKV-NS1 antibody (Ab) was immobilized via Cys 20mM and Glut 2.5% on the surface of ZnO NRs. Twenty microliters of anti-ZIKV-NS1 monoclonal antibody solution were diluted in 0.1M PBS pH7.4. Three different concentrations of dilution (1:10000, 1:5000 and 1:1000) were tested. The diluted solution was dropped on the surface of the working electrode and incubated for 12 hours at 4°C in a moist chamber.

2.5. Electrochemical assays

The analytical responses of the immunosensor were evaluated by electrochemical measurements by using cyclic voltammetry (CV). During CV assays, the potential was scanned from -0.7 to 0.7 V at the scan rate of 100 mV.s^{-1} recorded in 10 mmol.L^{-1} $K_4[Fe(CN)_6]$ and 0.5 mol.L^{-1} $NaNO_3$ solution as mediator. All experiments were conducted in triplicate at room temperature.

2.6. Characterization methods

2.6.1 Structural and microstructural characterization

All samples were characterized by Scanning Electron Microscopy (SEM) in a FEI Inspect F50 SEM microscope. Immunofluorescence Confocal Microscopy and Fourier Transform Infrared Spectroscopy (FTIR) were used to identify antibody binding to the surface sensor after immobilization. For the Immunofluorescence Confocal Microscopy analyzes, the sensors were incubated with Alexa Fluor 594 (Life Technologies) fluorescent secondary antibody for 1h at room temperature. The sensors were evaluated by fluorescence intensity in a confocal microscope Leica, model TCS SP5 II. The experiments were performed in a moist chamber. FTIR measurements were performed using an Attenuated Total Reflectance (ATR) accessory of the Thermo Scientic Smart iTR Nicolet iS10.

2.6.2 Dot and Western blotting assays

ZIKV-NS1 protein commercially available was characterized by western blotting. For this test, 200ng of recombinant Zika virus NS1 protein was spiked in 20µL of PBS. The samples were previously boiled at 95°C, with a 5% glycerol/ 0.03% bromophenol blue/10 mM dithiothreitol solution, and loaded onto 10% SDS polyacrylamide gels. 10 µL of prestained protein marker were used as standard. After electrophoresis, proteins were transferred to nitrocellulose membrane in a transfer buffer. The membranes were then incubated overnight at 4°C with primary antibodies, 1:5.000 mouse monoclonal anti-ZIKV-NS1 antibody. The blots were subsequently washed in Tris-buffered saline with Tween and incubated with HRP-conjugated secondary antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System).

2.7. Immunosensor performance for ZIKV-NS1 detection

Immunosensor performance was evaluated by calibration curve and limit of detection (LoD). The ZIKV-NS1 standard was incubated in the immunosensor, and all analyses were performed according to followed protocol: after immobilization of the anti-ZIKV-NS1 antibody, the immunosensor was incubated with different concentrations of ZIKV-NS1 antigen (from 0.01-200 $ng.mL^{-1}$) at room temperature. Washing with PBS buffer was

realized after each step to remove antibody or antigen excess. The incubation steps were performed in a moist chamber. The evaluation was performed by CV analysis.

2.8. Immunosensor Specificity for ZIKV-NS1

The specificity of the ZIKV-NS1 immunosensor was assessed regarding Dengue NS1 protein (DENV-NS1) sample. 2.0µg.mL^{-1} DENV-NS1 was diluted in PBS buffer, dropped upon sensors, and incubated during 60 min. Afterward, the sensors were characterized by CV analysis.

Recombinant Zika virus NS1 protein and Recombinant Dengue virus 1 NS1 protein were characterized for dot blot analysis. A nitrocellulose membrane, and draw a grid by pencil were used to indicate the region we went blot. Using pipette tip, we spotted 2 µl of samples onto the nitrocellulose membrane at the center of the grid. The dots were made with 1.5µg; 0.15 µg; 0.015 µg, 1.5ng and 0.15ng. The membranes were let to dry for 30 min, and incubated overnight at 4°C with 1:5.000 mouse monoclonal anti-ZIKV-NS1 antibody. The blots were subsequently washed in Tris-buffered saline solution with Tween and incubated with HRP-conjugated secondary antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence method.

2.9. Urine samples analyses

The urine samples used here were supplied by the author herself. All methods were carried out in accordance with guidelines and regulations of the National Committee on Research Ethics, CONEP/CNS/MS, of the Brazil. All experimental protocols were approved by Comissão Nacional de Ética em Pesquisa – CONEP/CNS/MS. The human urine samples were collected in sterile means and immediately used. Before CV analysis, the undiluted urine sample was spiked with ZIKV-NS1 protein in urine with 02 different concentrations (0.01µg/mL^{-1} and 2.0µg/mL^{-1}). The sensors were incubated in the urine samples during 60 min and characterized by CV analysis. For western blotting, 200ng of recombinant Zika virus NS1 protein was spiked in 20µL of PBS, diluted urine in PBS (1:10) or undiluted urine. The western blotting was performed as described above.

3. RESULTS

3.1 Characterization of the bare board sensor

ZnO NRs are excellent semiconductors used for anchoring biomolecules, as antibodies, in its surface [21]. On the other hand, (Au) gold is another excellent material used for immobilize antibodies in biosensors. As our bare board sensor has working-electrode and trails made with Au film, we performed some immobilization tests in it, before growing ZnO NRs. The idea was to identify if the gold could contribute in the immobilization of antibodies. Figure 1a-b shows CV curves obtained for pure and incubated with Cys, Glut and anti-NS-1 antibody of ZIKV bare boards, respectively. No differences are observed in the curves and anodic peaks (Ipa) values in the presence of the ZIKV NS1 for both boards. That means there was no immobilization of the antibody in the bare board sensor before growing ZnO NRs. The lower surface area of the gold film may be contributed for it.

The stability of our homemade bare board sensor was evaluated by successive cyclic voltammograms performed in the presence of 10mmol.L^{-1} of $K_4[Fe(CN)_6]$ prepared in 0.5 mol.L^{-1} $NaNO_3$ electrolyte at 100mV.s^{-1} scanning rate and potential ranging from -0.7 to 7.0V (Figure 1c). The redox peaks were basically constant even after 10 cycles. Coefficient of variation, calculated based in Ipa, was 2.5%, validating the excellent stability of our homemade bare board sensor.

3.2 Growth of ZnO NRs on working electrode

After growing of ZnO NRs upon working electrode of the bare board, the samples were characterized by SEM (Figure 1d and e). Micrographs show ZnO NRs grow perpendicularly to the board with good density, as previously reported [21]. Because of the higher surface, using ZnO NRs can be a good path to immobilize antibodies and increase the selectivity and limit of detection of the biosensor.

Please insert Figure 1 here

3.3 Anti-ZIKV-NS1 immobilization

The sensor specificity is strongly related to the properties of the immobilized detection element [14]. The use of antibodies is an excellent tool for making biosensors because they are highly specifics [22].

Before anti-ZIKV-NS1 immobilization on the board sensor, it is necessary to evaluate the specificity of antibody-antigen linkage used here. For this, we performed a Western blotting analysis using a 200ng of isolate ZIKV-NS1 protein (Figure 2a). Only one band, around 50 kDa, appears in the analysis. The result indicates the purity of the ZIKV-NS1 protein and the high selectivity of antibody.

After testing the specificity of ZIKV-NS1 protein, we immobilized it via Cys and Glut 2.5% on the surface of ZnO NRs grown on the board sensor. Firstly, Cys (an organic amine disulfide) binds to oxygen dominant species on ZnO NRs surface via thiol groups, and modified it with amino groups. Subsequently, Glut covalently binds to amino groups and providing carbonyl groups on the sensor surface. Afterward, the amino groups (NH_2-Y) of the antibodies easily bind to the carbonyl groups.

One of the crucial parameters on developing low-cost biosensors is using small quantities of antibodies as possible. Therefore, we tested the immobilization of antibody with three different concentrations (1:1000; 1:5000 and 1:10000). The CV curves reveal a reduction in the Ipa values with addition of cys, glut or antibodies due to isolating nature of these compounds (Figure 2b). Incubating the sensor with lower antibody concentration (1: 5000 or 1: 10000) still resulted in a decrease of the Ipa compared to that immobilized just with cys and glu (Fig 2b). That means the immobilization of the antibody at the bare board sensor also occur in these concentrations. As the use of lower antibody concentration may reduce the cost of production, we choose the 1: 5000 for sensor assembly.

3.3.1 Characterization of Anti-ZIKV-NS1 immobilization on the board sensor

Antibody immobilization upon bare board sensor was confirmed by Confocal Fluorescence Microscopy. An intense fluorescence was observed in the board sensor with Ab immobilization (Figure 2e) when compared to sensor without Ab immobilization (Figure 2c and d).

Regarding the FTIR data, Figure 2f, the antibody immobilization was confirmed by the appearance of well define bands around 2093 cm^{-1} and 1352 cm^{-1} that could be attributed to the stretching vibration of the C≡N bound and N=O respectively [27]. The NS-1 ZIKV protein immobilization was confirmed by increase in peaks of band around 2093 cm^{-1} and 1352 cm^{-1}, suggesting a correct antibody orientation.

We evaluated the stability of immunosensor and observed a good repeatability of Ipa (6.5% coefficient of variation) in ten sequential voltammograms curves (Figure S2).

Another advantage of our immunosensor as a rapid test is reproducibility. We assessed this parameter in four ZIKV-NS1 immunosensors and observed Ipa values very similar (4.5% coefficient of variation) (Figure 2g).

Please insert Figure 2 here

3.4 Characterization of the ZIKV-NS1 Immunosensor

Under optimized experimental conditions, the calibration curve of immunosensor was performed with electrodes incubated in different concentrations of ZIKV-NS1 and submitted to CV analyses in presence of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ (10mM) in $NaNO_3$ (0.5 $mol.L^{-1}$). The results show that the Ipa increase by increasing of ZIKV-NS1 concentration in incubation solution (Figure 3a). Probably, a reaction between antibody with ZIKV-NS1 occurs with charge transfer. Good linearity in the calibration curve was obtained over the range 0.1 $ng.mL^{-1}$ to 100 $ng.mL^{-1}$ of ZIKV-NS1 ($r^2 = 0.9536$; n = 4) (Figure 3b). This linearity is suitable with ZIKV tests previously authorized by Food and Drug Administration (FDA) [28]. The purpose of our device is providing a rapid test to detect ZIKV-NS1 protein. As ZIKV-NS1 is an exogenous protein, not produced physiologically by the human body, using a fast qualitative test should be appropriated for detecting Zika without cross-reaction with Dengue.

The Limit of Detection is lower than $1pg.mL^{-1}$ (Figure 3c), indicating our ZnO NRs-based immunosensor is a good rapid test for detection of ZIKV in beginning of disease.

Some differences of potential can be seen in CV analyses. Probably, these differences are related with more random or oriented Ab immobilized on the surface of ZnO NRs. We worked with batches of samples and the surface pKa, temperature and humidity of the day may affect the orientation of the Ab onto ZnO NRs surface in these batches, since the immobilization occurs by adsorption. In addition, there are a number of intermediate states of immobilization that may either lead to reduced access of the cognate antigen to the Ab-binding site or link the Ab close to this site with an associated reduction in binding.

Despite these limitations, within the same batch, the samples show the identical behavior; that is, the Ipa increase by increasing of ZIKV-NS1 concentration. For each batch, we analyzed a control sample.

Please insert Figure 3 here

In order to determine the optimum incubation period of the ZIKV-NS1 antigen (Ag), we tested times of 10, 30 and 60 min for incubating 0.01µg.mL^{-1} ZIKV-NS-1. A higher anodic peak is observed for incubation periods higher than 30 min (Figure 4a). Incubation period higher than 30min favor a better charge transfer process during linkage of the antigen (Figure 4b).

Please insert Figure 4 here

3.5 ZIKV-NS1 Immunosensor Cross Reaction

The cross-reaction between DENV-NS1 and ZIKV-NS1 is very common since the protein sequence identities in the 53-56% range [29]. Because this DENV-NS1 was chosen to evaluated the specificity of immunosensor. The specificity of our ZIKV-NS1 immunosensor regarding dengue as assayed, and recovery experiments were carried out by adding 2.0 µg.mL^{-1} DENV-NS1 (Figure 5a). CV curves showed similar Ipa values even in the presence of high level of DENV-NS1 antigen. That means DENV-NS1 antigen didn't link with anti-ZIKV-NS1 antibody and confirm the specificity of our immunosensor. Dot-blot assay using ZIKV-NS1 and DENV-NS1 antigen incubated with anti-ZIKV-NS1 antibody (1:5000) (Figure 5b) also endorsed non-reactivity of our immunosensor with Dengue antigen.

Please insert Figure 5 here

3.6 Analyzis of spiked human urine samples

Previous groups have reported tests in urine to detect flavivirus, such as DENV [30], West Nile virus [31] and ZIKV [32, 33]. Using urine for disease diagnoses has some advantages, such as noninvasive sampling and ease of use. To confirm if the antibody-antigen binding remains in the urine samples, we performed the Western blotting assay (Figure 6a). Four samples were prepared: PBS spiked with 200ng ZIKV-NS1, Urine diluted in PBS (1:10) spiked with 200ng ZIKV-NS1, undiluted urine spiked with 200ng ZIKV-NS1 and urine. We observed a single band at 50KDa, revealing no interference by the other compounds of

the urine in antigen-antibody linking. Urine samples were assessed, and CV recovery experiments were carried out via the standard addition method. Assuming a null concentration of ZIKV-NS1 in the urine sample, CVs curves showed the possibility for detecting ZIKV-NS1 in urine without interference of its compounds (Figure 6b). To corroborate with this findings we analyze undiluted urine and undiluted urine spiked with 0.01μg/mL^{-1} and 2.0μg/mL^{-1} of ZIKV-NS1 and observed the immunosensor is capable to detect ZIKV-NS1 in urine even in the presence of interference compounds (Figure S3).

Please insert Figure 6 here

The diagnosis of ZIKV has been routinely carried out through the genome viral detection or by serological tests identifying IgG and IgM antibodies [4]. The disadvantages of these validation techniques in relation to our device is on the detection of IgG and IgM, which is done later (from the fourth to the 6th day after the onset of symptoms) [34]. In addition, the similarity of the DENV and ZIKV viruses allows the frequent occurrence of cross-reaction, evidencing the low specificity of the tests [34]. The techniques that evaluate the viral genome are accurate, RT-PCR is considering the gold standard for ZIKV detection, but like serology, they are carried out in clinical analysis laboratories. Therefore, they are expensive and time consuming. Our ZnO NRs-based electrochemical immunosensor can be used as a POC disposable, is able for detection ZIKV from the first day of symptoms, in a specific way (without cross-reaction with DENV), is selective, and the result can be obtained within one hour after collect of material. The sample used is undiluted urine with no processing required. The immunosensor can be read in a portable potentiostat and could be part of prenatal care and used in health centers to identify patients with ZIKV.

4. CONCLUSION

A ZnO NRs-based electrochemical immunosensor was successfully applied here as a rapid test for detection of ZIKV infection in urine. The biosensor showed high specificity to ZIKV-NS1, without cross-reactive with Dengue, and low limit of detection (1.00 pg.mL^{-1}). Dot-blot assay endorsed non-reactivity of our ZnO NRs-based immunosensor with Dengue antigen. Taking the test in urine enables the immunosensor to be performed as a rapid point-of-care test without need experts. The immunosensor here showed stability and

reproducibility suitable with other ZIKV tests. In the next steps, we are going to validate our immunosensor by analyzing patient urine samples. The obtained results will be compared to them gold standard test (RT-PCR). The immunosensor here developed has significant potential to improve early diagnostics of ZIKV, including weak healthcare infrastructure areas.

5. Author Contributions

Aline M. Faria: Methodoloy, Validation, Formal Analyses, Writing-Original Draft preparation. **Talita Mazon**: Conceptualization, Writing-Reviewing and Editing, Supervision.

6. Acknowledgements

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7. Competing Interests:

The authors declare that they have no competing interests.

8. Additional Information:

Supplementary information accompanies this paper.

9. References

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552 FIGURE CAPTIONS

553

554Figure 1: a) Cyclic voltammogram (CV) obtained for Bare board. b) CV obtained for Bare
555board plus antibody. c) CV obtained for FR-4 bare-sensor board with 10 scans. All cyclic
556voltammograms (CVs) were performed with a scan rate of 100mV.s^{-1}.d) SEM image of the
557ZnO nanostructures grown on sensor board, magnitude 5000x. e) SEM image of the ZnO
558nanostructures grown on sensor board, magnitude 50000x.

559

560Figure 2: a) Western blotting analyzes for ZIKV-NS1 showing the ZIKV-NS1 protein
561purity, 50KDa size, and the good selectivity of antibody. b) CVs obtained in different steps
562of preparing the immunosensor. c-e) Fluorescence Confocal Micrograph. c) Bare sensor
563board without GO/ZnO-NRs. d) Sensor board with GO/ZnO-NRs. e) sensor board with
564GO/ZnO-NRs plus antibody immobilization. All sensors were incubated with fluorescent
565secondary antibody. f) FTIR spectra in bare board (Black), in immunosensor (Blue) and in
566immunosensor incubated with NS-1 ZIKV protein (Red). g) CVs obtained for different
567immunosensors showing their reproducibility. All CVs were performed with a scan rate of
568100mV.s^{-1}.

569

570Figure 3: a) Voltammograms obtained for immunosensors in different ZIKV-NS1
571concentrations. b) Linearity of calibration curve obtained over the range 0.01 ng.mL^{-1} to
572100 ng.mL^{-1} of NS-1 Zika Virus protein (r=0.9536). c) CVs of immunosensor incubated
573with different ZIKV-NS1 concentrations. All CVs were performed with a scan rate of
574100mV.s^{-1}.

575

576Figure 4: a)CVs obtained for Zika–NS1 immunosensors incubated with antigen
577(Ag)(Zika–NS1 protein standard - 0.01µg.mL^{-1}); in black without Ag incubation; in red
57810 min of Ag incubation; 30 min of Ag incubation; green and blue 60 min of Ag
579incubation. All CVs were performed with a scan rate of 100mV.s^{-1}. b) Graphical
580representation of Ipa x time.

581

582Figure 5: a) Voltammograms obtained by CV analyzes of immunosensors in presence of
5832.0 µg.mL^{-1} of DENV-NS1 antigen at scanning rate of 100mV.s^{-1}. b) Dot blot for ZIKV-

584NS1 and DENV-NS1 antigen in different concentrations incubated with antibody anti-
585ZIKV-NS1(1:5000). All CVs were performed with a scan rate of 100mV.s^{-1}.
586
587Figure 6: a) Western blotting assay to access the ZIKV-NS1 expression: lane 1 - PBS
588spiked ZIKV-NS1(200ng); lane 2 - Urine Diluted in PBS (1:10) spiked ZIKV-NS1(200ng);
589lane 3 - Urine Diluted in PBS (1:10) spiked ZIKV-NS1(200ng); lane 4 - Urine. b)
590Voltammograms obtained by CV analyzes of immunosensors incubated with undiluted
591urine. In black CV of immunosensor incubated with urine without ZIKV-NS1; in red CV
592of imunosensor incubated with urine plus ZIKV-NS1 (0.01µg.mL^{-1}); in green CV of
593immunosensor incubated with urine plus ZIKV-NS1 (2.0µg.mL^{-1}). All CVs were
594performed with a scan rate of 100mV.s^{-1}.
595

596Supplementary Material: Figure S1: The bare-sensor board was made on FR-4 (1.6 mm
597thick) sheets using a Printed Circuit Board Technology (PCB). It was composed of gold
598trails (Au) as working and counter electrode, and silver (Ag) as a reference electrode, being
599configured each to be electrically isolated from the others. The working electrode is
600composed of ZnO nanostructures on the Au trail. Figure S2: Cyclic voltammogram of our
601immunosensor with 10 scans performed with a scan rate of 100mV.s^{-1}. Figure S3: Cyclic
602voltammogram of ZIKV-NS1 immunosensor incubated with urine (black) and urine +0.01
603µg.mL-1 ZIKV-1 (red) . Scan rate of 100mV.s^{-1}.
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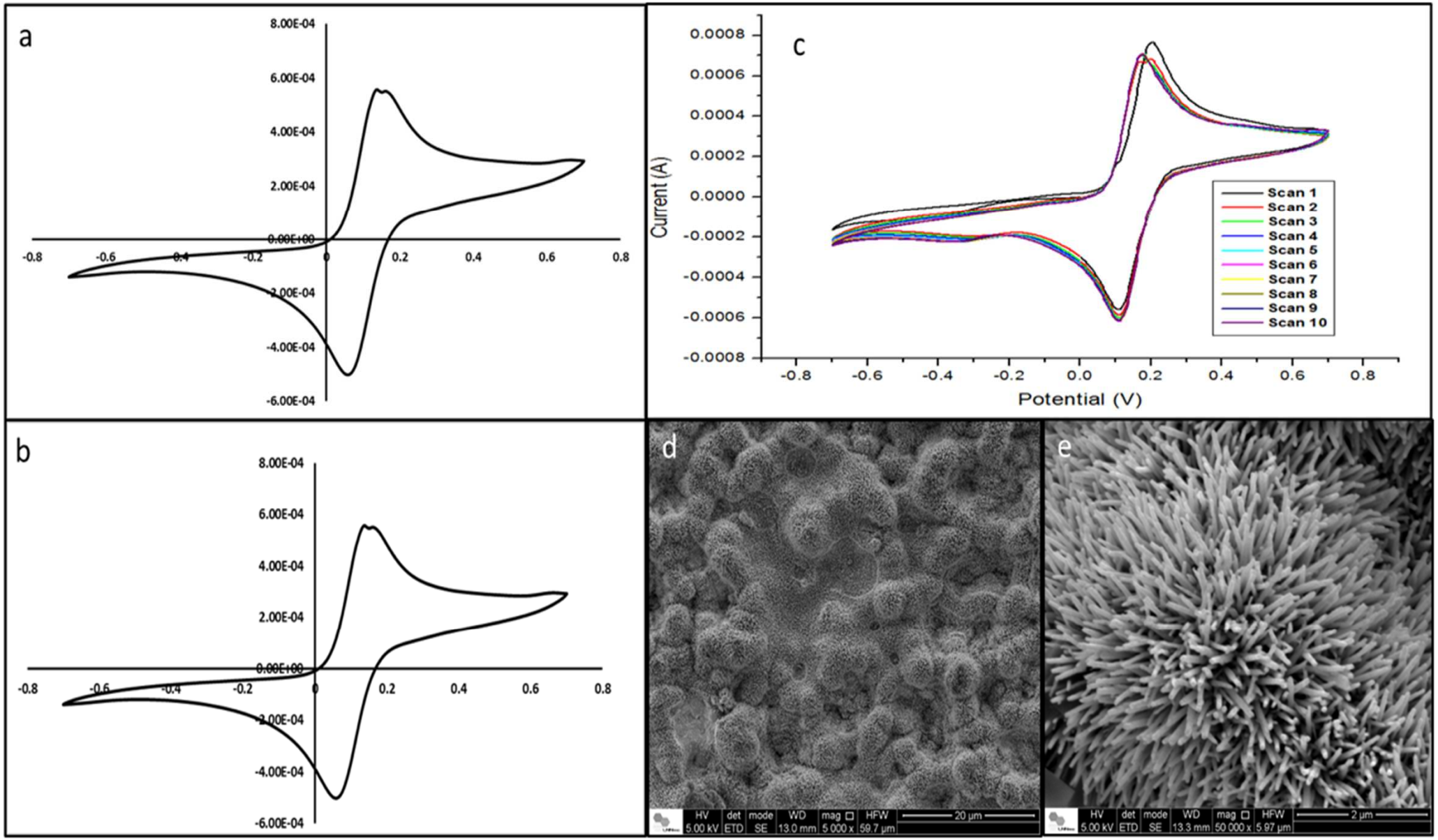
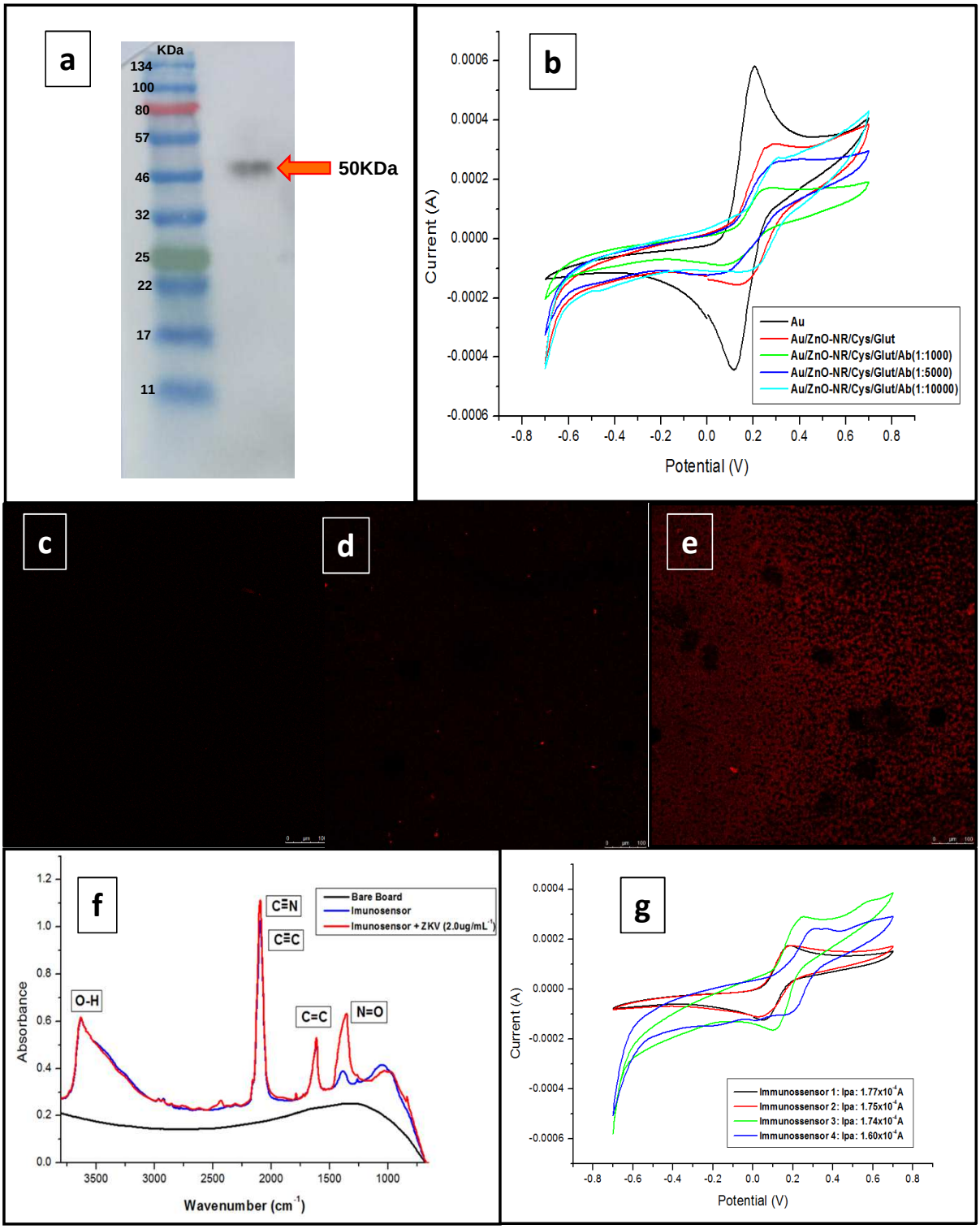
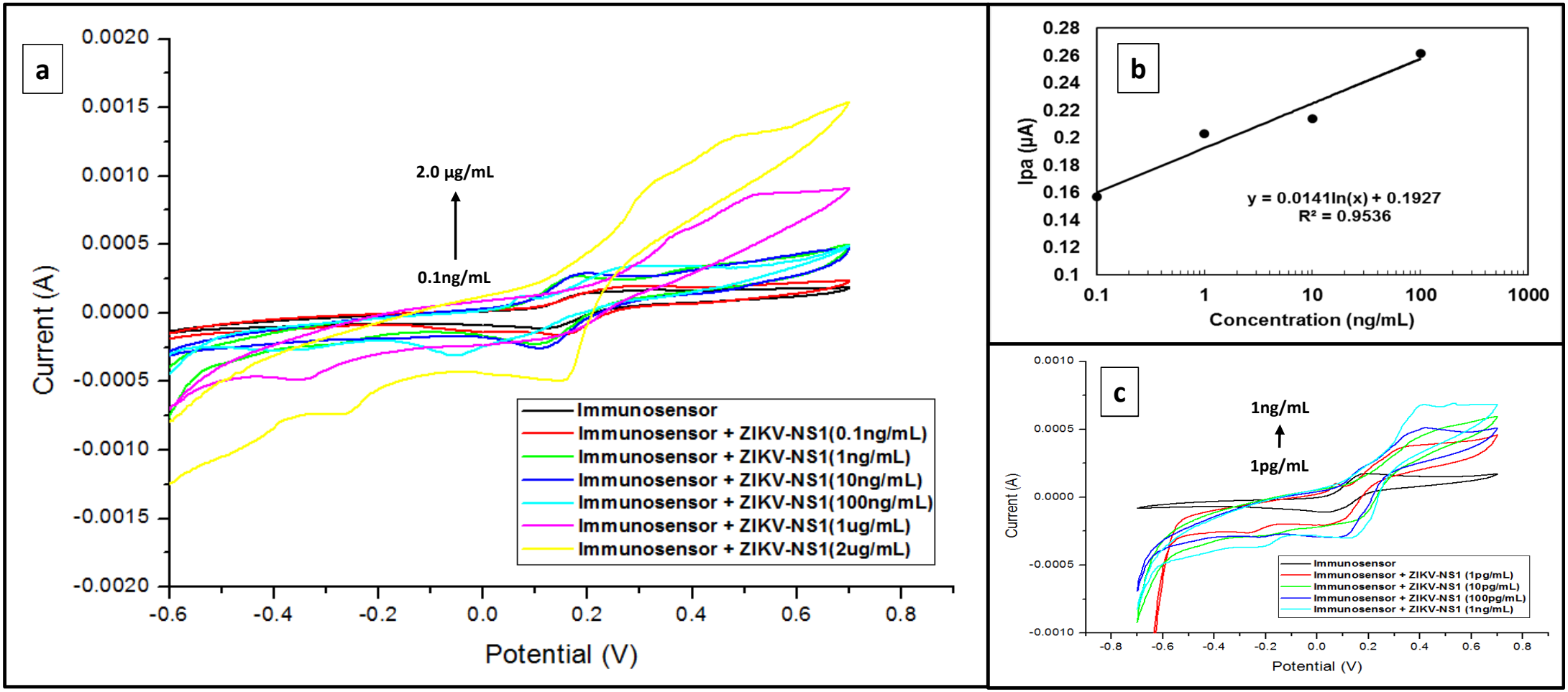
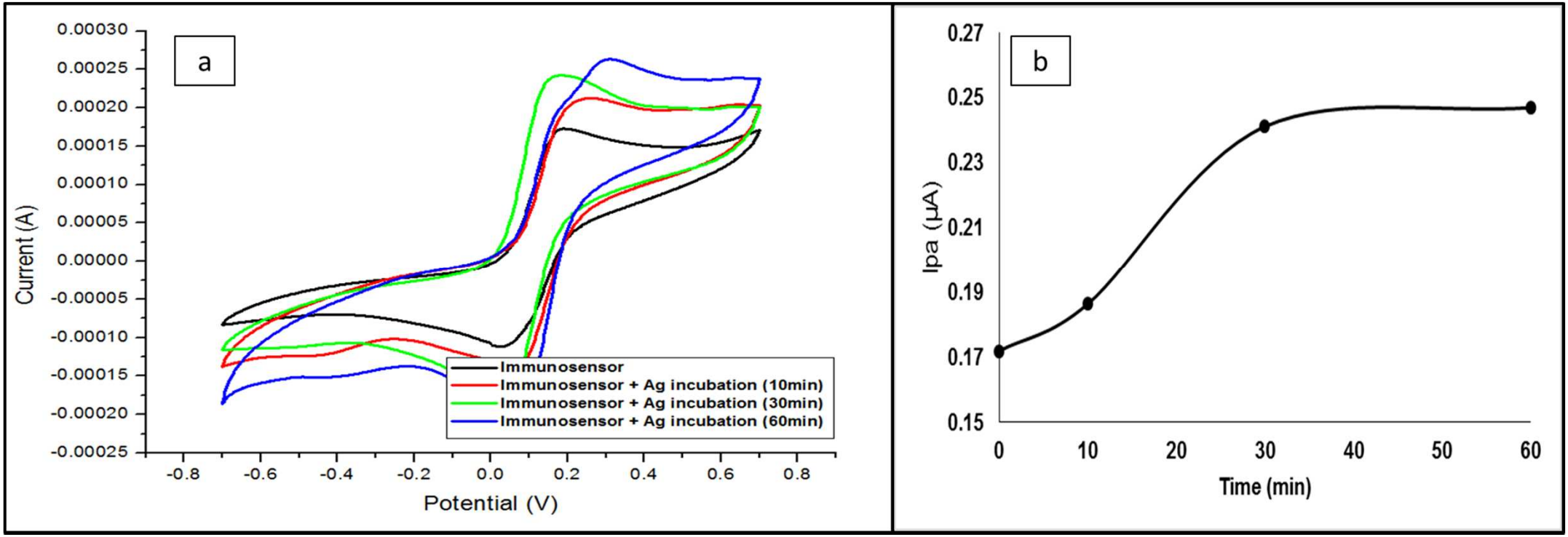
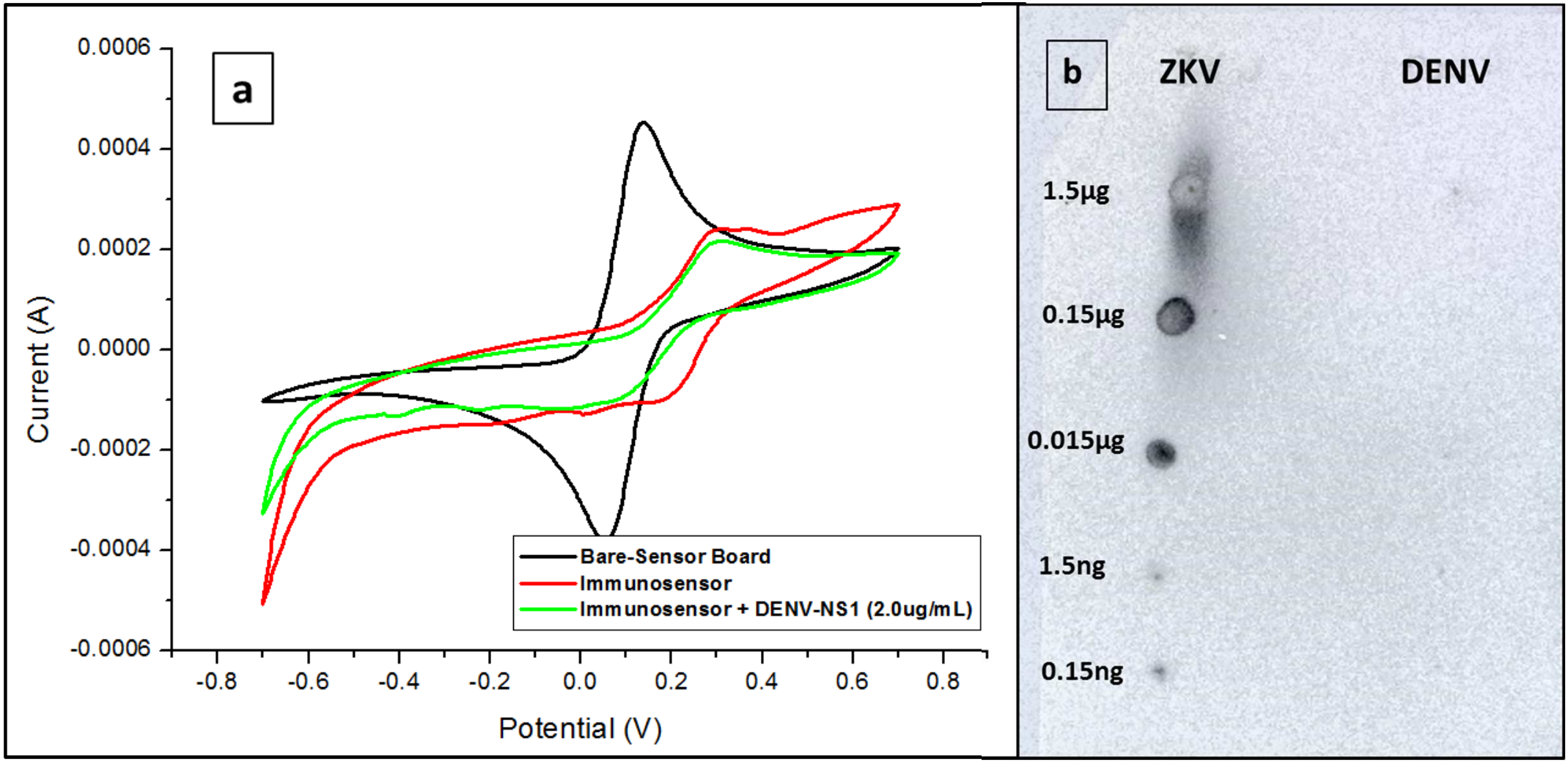
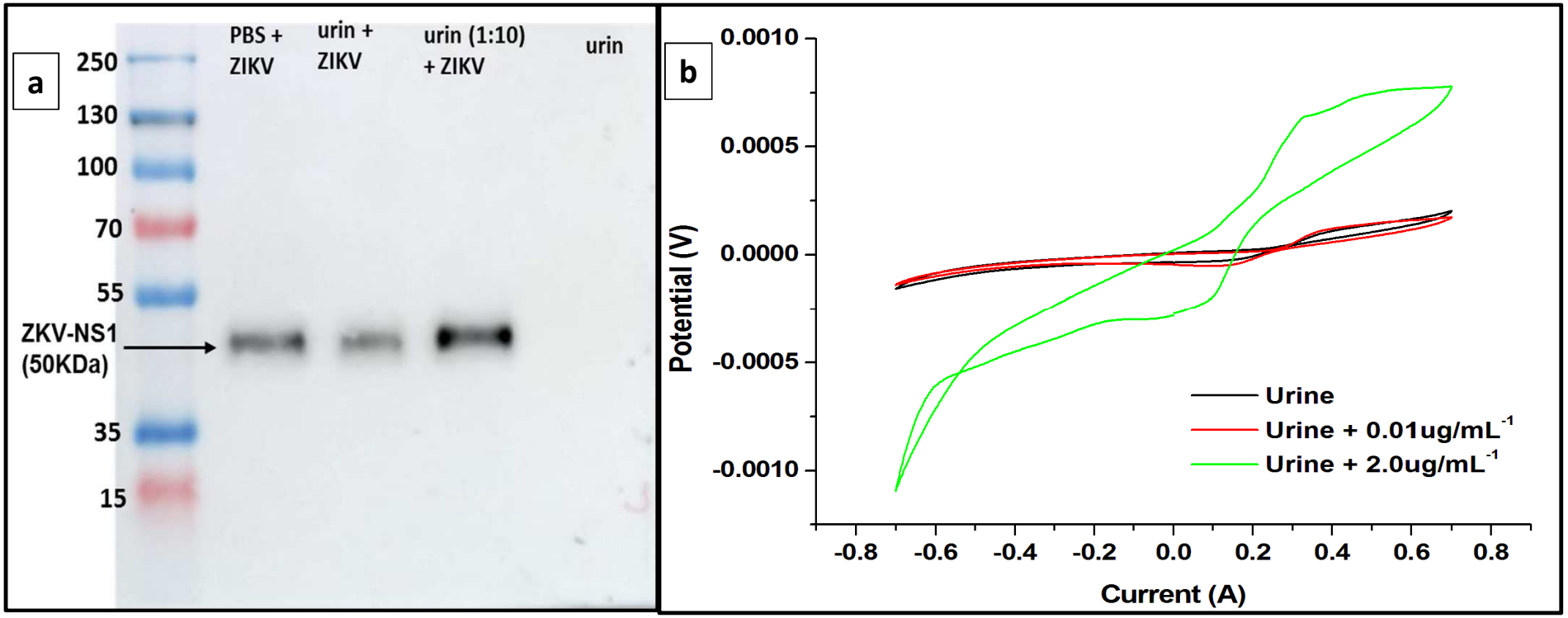

a
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
-2.00E-04
-4.00E-04
-6.00E-04
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
b
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
-2.00E-04
-4.00E-04
-6.00E-04
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
c
Current (A)
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.0006
-0.0008
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
Potential (V)
Scan 1
Scan 2
Scan 3
Scan 4
Scan 5
Scan 6
Scan 7
Scan 8
Scan 9
Scan 10
d
HV 5.00 kV
det ETD
mode SE
WD 13.0 mm
mag 5 000 x
HFW 59.7 µm
20 µm
e
HV 5.00 kV
det ETD
mode SE
WD 13.3 mm
mag 50 000 x
HFW 5.97 µm
2 µm


a
KDa
134
100
80
57
46
32
25
22
17
11
50KDa
b
Current (A)
Potential (V)
Au
Au/ZnO-NR/Cys/Glut
Au/ZnO-NR/Cys/Glut/Ab(1:1000)
Au/ZnO-NR/Cys/Glut/Ab(1:5000)
Au/ZnO-NR/Cys/Glut/Ab(1:10000)
c
d
e
f
Absorbance
Wavenumber (cm-1)
Bare Board
Imunosensor
Imunosensor + ZKV (2.0ug/mL-1)
C≡N
C≡C
O-H
C=C
N=O
g
Current (A)
Potential (V)
Immunossensor 1: Ipa: 1.77x10-4A
Immunossensor 2: Ipa: 1.75x10-4A
Immunossensor 3: Ipa: 1.74x10-4A
Immunossensor 4: Ipa: 1.60x10-4A


a
Current (A)
Potential (V)
2.0 µg/mL
0.1ng/mL
Immunosensor
Immunosensor + ZIKV-NS1(0.1ng/mL)
Immunosensor + ZIKV-NS1(1ng/mL)
Immunosensor + ZIKV-NS1(10ng/mL)
Immunosensor + ZIKV-NS1(100ng/mL)
Immunosensor + ZIKV-NS1(1ug/mL)
Immunosensor + ZIKV-NS1(2ug/mL)
b
Ipa (µA)
Concentration (ng/mL)
y = 0.0141ln(x) + 0.1927
R² = 0.9536
c
Current (A)
Potential (V)
1ng/mL
1pg/mL
Immunosensor
Immunosensor + ZIKV-NS1 (1pg/mL)
Immunosensor + ZIKV-NS1 (10pg/mL)
Immunosensor + ZIKV-NS1 (100pg/mL)
Immunosensor + ZIKV-NS1 (1ng/mL)


a
Current (A)
Potential (V)
Immunosensor
Immunosensor + Ag incubation (10min)
Immunosensor + Ag incubation (30min)
Immunosensor + Ag incubation (60min)
b
Ipa (µA)
Time (min)





HIGHLIGHTS

- Effective approach in using printed circuit board sensors and ZnO nanorods on making low-cost, low limit of detection, high-speed, high specificity electrochemical immunosensor.
- Zika fever diagnosis using Zika nonstructural protein 1 antigen as a biomarker.
- Evidence of lack of cross-reaction of the immunosensor with DENV-1 NS1.
- Detection of ZKV-NS1 in urine samples
- Early detection of virus infection and noninvasive rapid test.