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A sensor tip based on carbon nanotube-ink printed electrode for the dengue virus NS1 protein

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ABSTRACT

An immunosensor for the non-structural protein 1 (NS1) of the dengue virus based on carbon nanotube-screen printed electrodes (CNT-SPE) was successfully developed. A homogeneous mixture containing carboxylated carbon nanotubes was dispersed in carbon ink to prepare a screen printed working electrode. Anti-NS1 antibodies were covalently linked to CNT-SPE by an ethylenediamine film strategy. Amperometrical responses were generated at −0.5 V vs. Ag/AgCl by hydrogen peroxide reaction with peroxidase (HRP) conjugated to the anti-NS1. An excellent detection limit (in the order of 12 ng mL^{-1}) and a sensitivity of 85.59 μA mM^{-1} cm^{-2} were achieved permitting dengue diagnostic according to the clinical range required. The matrix effect, as well as the performance of the assays, was successfully evaluated using spiked blood serum sample obtaining excellent recovery values in the results. Carbon nanotubes incorporated to the carbon ink improved the reproducibility and sensitivity of the CNT-SPE immunosensor. This point-of-care approach represents a great potential value for use in epidemic situations and can facilitate the early screening of patients in acute phase of dengue virus.

1. Introduction

Dengue is considered a major public health problem in tropical and subtropical regions of the world and is endemically prevalent in approximately 112 countries (Gurugama et al., 2010). It is a self-limiting, non-specific illness characterized by fever, headache, myalgia, and constitutional symptoms. Its severe forms (hemorrhagic fever and shock syndrome) may lead to multisystem involvement and death, mostly amongst children. The incidence of this disease has increased over the last 50 years with 2.5 billion people living in areas where dengue is endemic (Smith et al., 2009). In view of the high mortality rate and to reduce the disease burden, it is desirable to have a rapid and practical diagnostic method for early detection of dengue virus (Singhi et al., 2007). The major laboratorial methods currently available for diagnosis of the disease are viral culture (Samuel and Tiyagi, 2006), viral RNA detection by reverse transcriptase PCR (RT-PCR) (Huhtamo et al., 2010) and serological tests such as an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA). The first two assays have restricted scope as a routine diagnostic procedure due to its requirement of highly skilled personnel, laborious procedure and time consumption (Huy et al., 2011). The MAC-ELISA, which is a commonly used assay, has a low sensitivity in the first four days of illness (Alcon et al., 2002). Therefore, early dengue diagnosis still remains a problem, as all these mentioned assays have their own pitfalls.

Dengue virus is an enveloped positive-sense RNA virus. The genomic RNA is approximately 11 kb in length and is composed of three structural protein genes that encode for nucleocapsid or core protein, a membrane-associated protein, an envelope protein and seven non-structural protein genes including NS1 protein (Shrivastava et al., 2011). Recently, ELISA assays specific to NS1 protein have been carried out showing that NS1 secretory protein is found at high concentrations during the early clinical phase of the disease, suggesting it as a predictive marker for dengue diagnosis and responsive to four serotypes (Lapphra et al., 2008). By aiming to achieve a practical diagnostic, rapid, immunochromatographic tests (RDTs) to NS1 detection have been proposed; however, they are limited due to their instability to provide qualitative responses and poor sensitivity on admission samples (Blacksell et al., 2006). Compared with RT-PCR analyzers and RDTs, biosensors present numerous advantages such as simpler management, easier miniaturization, faster and quantitative responses and, moreover, they can permit on-site monitoring (Dai et al., 2011). So far, only a few immunosensors have been developed to detect NS1 antigen and non-commercial approaches are available. Oliveira et al. (2011) developed a biosensor based on concanavalin A lectin as a bioreceptor; however, it is limited in the detection of NS1 due to its interaction with carbohydrates and

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glycoproteins that are present in the acute phase of dengue disease, including the cytokines, IFNα and others. Su et al. (2003) developed a quartz crystal microbalance immunosensor to simultaneously detect NS1 and envelope proteins in 1:10 diluted serum with 500 μg mL^{-1}. Although some progresses have been achieved, new attempts to obtain a practical and selective NS1 biosensor are required for diagnostic of dengue virus in acute phase.

Different types of transduction can be employed in a biosensor, after the analyte recognition through the sensing biomolecules. Although optical and piezoelectric transduction by using surface plasmon resonance (Dutra and Kubota, 2007) and quartz crystal microbalance (Mattos et al., 2012), respectively, have been more commonly employed for immunoassay, electrochemical transduction has received great attention, especially by using the screen printed electrodes (SPEs) (Kumbhat et al., 2010). SPEs have been shown to be more attractive since they combine a good strategy to accomplish disposable, safe and quantitative point-of-care testing (Silva et al., 2010). They are constructed by printing a conductive ink onto a solid support with significant advantages, like the feasibility to obtain printed electrodes in different sizes and designs, as well as the facility to incorporate diverse compounds in order to change their nature and electrochemical properties (Gornall et al., 2009). It is possible to develop SPEs with high performance, low background current and improved electron transfer kinetic, by simply adding conductive modifiers (Mohamed et al., 2010).

Nowadays, the important role that the carbon nanotubes play in the performance of electrochemical biosensors is well-known (Laschi et al., 2008). Due to their extraordinary chemical and physical properties, such as high electrical conductivity and good chemical stability, it is possible to obtain nanostructured electrodes with faster electron transfer reactions (Tam and Hieu, 2011). In addition, the carbon nanotubes can be functionalized with reactive groups to purposely attach biomolecules and other compounds (Li et al., 2010; Leng et al., 2011). Herein, caboxylated carbon nanotubes were incorporated into the carbon ink to produce SPEs with enhanced sensitivity and stability. A thin film containing amine groups was deposited on the caboxylated carbon nanotube-screen printed electrode (CNT-SPE) in order to perform a covalent and oriented immobilization of the anti-NS1 antibodies. This immunosensor showed to be an innovative electrochemical method for diagnosis of early clinical phase of dengue infection.

2. Experimental

2.1. Materials and reagents

Electrodag PF-407 C carbon ink was acquired from Acheson Henkel Corporation (USA). COOH-functionalized multi-walled carbon nanotubes (COOH-MWCNT), 95% purity degree, were obtained from Dropsens (Oviedo, SPA). Mouse monoclonal antibodies against NS1 glycoprotein of dengue virus (Anti-NS1) and Dengue Virus NS1 glycoprotein were purchased from Abcam (Cambridge, UK). Ethylenediamine (EDA) was acquired from Sigma-Aldrich (St. Louis, USA). Dimethylformamide (DMF) and hydrogen peroxide (H_2O_2) (30% w/v) were obtained from F. Maia (Cotia, BRA). Anti-NS1 antibody was labeled with horseradish peroxidase (HRP) according to Avrameas (1969). For the coupling of HRP to the anti-NS1, 12 mg of peroxidase was dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.8) containing 5 mg of anti-NS1 antibody. While the solution was gently stirred, 0.05 mL of a 1% aqueous solution of glutaraldehyde was added. The mixture was allowed to stand at room temperature (approximately 25 °C) for 2 h and then twice dialyzed against 5 L of PBS at 4 °C overnight. The precipitate was removed by centrifugation for 30 min at 20,000 rpm. This stock solution of peroxidase labeled-antibody was kept at +4 °C until used.

The pool of blood samples used in this work consisted of five serum samples from voluntary donors, kindly provided by Oswaldo Cruz Hospital of the Pernambuco University, according to the ethics committee's recommendations. All voluntary donors were found negative for dengue virus. The serum samples were collected from venous blood and immediately centrifuged for 120 s at 3000*g* and stored at −20 °C. The positive pool was spiked with NS1 fixing with a same volume at concentrations similar to those detected in the viremic dengue patients (Alcon et al., 2002).

Unless indicated, all the antibodies and antigen solutions was prepared in 0.01 mmol L^{-1} phosphate buffer saline (PBS) at pH 7.0. Ultrapure water (18 MΩ cm) used to prepare all solutions was obtained from a Milli-Q water purification system (Millipore Inc., Billerica, USA).

2.2. Apparatus

All the electrochemical experiments were performed in an Ivium Compact Stat potentiostat/galvanostat from Ivium Technologies (Eindhoven, The Netherlands) interfaced with a microcomputer and controlled by Ivium Soft software. A three-electrode system consisting of the CNT-SPE as the working electrode (4 mm diameter), an Ag/AgCl electrode as the reference electrode and a helical platinum wire as the counter electrode was used. The electrodes were set up in a glassy electrochemical cell with 5 mL volume.

The experiments to characterize the assembling of the CNT-SPE were conducted by using cyclic voltammetry in 5 mmol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ prepared in 0.1 mol L^{-1} KCl solution, at 0.1 V s^{-1} scan rate and potential ranging from −0.6 to 1.0 V.

The atomic force microscopy (AFM) technique was used for morphological and topographic characterization of the CNT-SPE. The micrographs were obtained using a WITec Alpha 300S AFM microscope (WITec Instruments, Ulm, Germany) operating in contact mode with a silicon tip at 0.2 N/m constant force.

2.3. Preparation of the CNT-SPE

The CNT-SPEs were obtained from a mixture containing carbon ink and COOH-MWCNT. Prior to mixing, 1 mg COOH-MWCNT was dispersed in 1 mL DMF solvent and sonicated in an ultrasonic batch for 2 h. After that, the CNT-SPEs were manufactured by squeezing the mixture over the adhesive plastic mold fixed on the rectangular support of polyethylene terephtalate (Fig. S1, supplementary information). Afterwards, the electrodes were cured at 60 °C for 20 min and finally, the adhesive plastic mold was removed. The circular area of the working electrode (approximately 4 mm of diameter) was delimited using adhesive tape resistant to chemical (electroplating and anodizing vinyl tape 470 supplied from 3M Co., USA). After they were ready, the CNT-SPEs were pretreated by cyclic voltammetry using 30 cycles at a scan rate of 0.1 V s^{-1}, potential ranging from −2.0 to 2.0 V and 2.44 mV step potential in 0.1 mol L^{-1} KCl solution as supporting electrolyte (Alonso-Lomillo et al., 2009).

2.4. Immobilization of the anti-NS1

Anti-NS1 antibodies were immobilized via EDA film deposited on the electrode surface. The pretreated CNT-SPEs were immersed in a 10% (v/v) EDA aqueous solution for 1 h and dried at room temperature (~25 °C) by forming EDA film. Afterwards, 10 μL of anti-NS1 (1 μg mL^{-1}) prepared in PBS was incubated on the