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A disposable chitosan-modified carbon fiber electrode for dengue virus envelope protein detection

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ABSTRACT

A chitosan-modified carbon fiber electrode (CFE) for dengue virus envelope protein (DENV) was developed. Antibodies against DENV were covalently immobilized on the chitosan (CHIT) matrix after activation with sodium periodate. Cyclic voltammetries and scanning electron microscopies analysis were performed to monitor steps involved in the CFE surface modification. Amperometric response of the competitive immunoassays was generated by hydrogen peroxide reaction with the peroxidase conjugated to DENV and 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as mediator. The immunosensor showed a lower limit of detection for DENV (0.94 ng mL^{-1}) than previously described and a linear range from 1.0 to 175 ng mL^{-1}, in concentration levels clinically relevant for dengue virus diagnosis. The intra- and inter-assay were respectively 5.8% and 3.6%. The unique and simple design of this immunoassay format provides an economical alternative for the manufacture of other sensitive sensors.

1. Introduction

Dengue virus is the causative agent of both classical dengue fever and the more severe dengue haemorrhagic fever and dengue shock syndrome and is mainly transmitted by Aedes infected mosquitoes [1]. Among viral proteins expresses of the dengue virus, envelope protein is responsible for virus attachment to the target cells. Envelope protein of dengue virus is present in the blood when the patient is in the viremic phase [2]. The rapid laboratory diagnostic of the acute infection is one of the key strategies to help the therapy and stop the disease progression to more severe and clinical forms. Currently, enzyme-linked immunosorbent assay (ELISA) and other techniques, such as polymerase chain reaction, have been used in laboratories to diagnose dengue infection [3]. However, these conventional methods are not practical to “in situ” diagnosis due to require sophisticated equipment, several experimental steps and they are time-consuming.

Biosensors have emerged as attractive alternatives overcoming all difficulties mentioned [4–7]. In literature, it has been published biosensors for DENV detection through viral RNA [8] and DNA [9]. Nevertheless, RNA genosensors are not practical and reliable because in dengue infections there are some drawbacks: the viral RNAs are packaged inside the cell requiring lyses before analysis; the RNA amounts are minimal and RNAs do not present very stable.

Due to unique recognition process and strong affinity of antibody–antigen interactions, immunosensors are highly selective and sensitive, and capable of identifying low abundance species from complex sample matrixes [10]. Among the available detection approaches, electrochemical methods are of particular interest owing to their simplicity, accuracy and high sensitivity, and also show a great compatibility with portable systems suitable for point-of-care testing [11]. In addition, electrochemical immunosensors, when compared to other transducers type (optical or mass-sensitive), have provided more advantages, for instance, disposability due to lower cost [12].

The chemically inert carbon fiber has outstanding mechanical and electrical properties and provides a great base substrate for electrochemical biosensor applications [13]. The carbon fiber electrodes have attractive features, such as a wide surface area (between 0.3 and 30 m^2 g^{-1}), excellent conductivity and a high porosity (>90%) [14]. They exempt the use of printing machines that are crucial to produce screen-printed electrodes with acceptable reproducibility and reliability [15,16].

The introduction of functional groups on sensing surface for stable and irreversible immobilization of biological molecules has been an important aspect in the development of immunosensors. The polymer functionalization on sensor platforms has become an alternative for conventional methods of immobilization [17]. CHIT is one of the most interesting biopolymer that can be easily handled in biomolecules immobilization due to its great film-forming

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ability, high permeability, mechanical strength, biocompatibility, and low cost, and also it is easily obtainable [18]. In additional, the CHIT structure contains amino and hydroxyl free functional groups, which can form chemical links to other biomolecules [19,20]. Due to all those, others have been investigating how to improve the CHIT properties for application in immunosensors, although only few advances have been reported.

Thus, the combined use of CHIT and carbon fibers has generated great expectations regarding the development of a new model of sensing surface, which could be more robust to the conjugation of proteins and use in electrochemical immunoassays, aggregating the main features of each component. In this article, a disposable CHIT-modified carbon fiber electrode was developed for the amperometric detection of DENV based on a competitive immunoassay.

2. Materials and methods

2.1. Reagents

The carbon fibers were acquired from the National Centre for Biotechnology Education (University of Reading, UK). CHIT flakes (85% deacetylated), 25% (v/v) glutaraldehyde (GLUT) solution, sodium periodate (SP), glycine and horseradish peroxidase (HRP) were acquired from Sigma–Aldrich Corp. (St. Louis, MO, USA). The potassium ferricyanide $[K_3Fe(CN)_6]$ and potassium ferrocyanide $[K_4Fe(CN)_6]$ were purchased from J.T. Baker (Philipsburg, NJ, USA). 2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 30% (v/v) hydrogen peroxide (H_2O_2) were obtained from KPL, Inc. (Baltimore, MD, USA) and Vetec (São Paulo, SP, Brazil), respectively. All other reagents used were of analytical grade. The phosphate buffer solution (PB) (10 mmol L^{-1}, pH 7.0) was used in all experiments as electrolyte support and prepared by mixing appropriate volumes of 20 mmol L^{-1} NaH_2PO_4 and 20 mmol L^{-1} Na_2HPO_4 to achieve pH 7.0 in deionized Milli-Q water from Millipore, Inc. (Bedford, MA, USA).

2.2. Immunomolecules obtaining

To obtain the monospecific antibodies against DENV (anti-DENV), aliquots of the virus envelope proteins (obtained from dengue infected patients) were subjected to SDS-PAGE. The band corresponded to the subunit protein of 55 kDa and related to the dengue virus envelope protein which was cut from the polyacrylamide gel and, with the aid of liquid nitrogen, was macerated in microtubes. It was then placed in NaCl 0.15 mol L^{-1}. This suspension was used to immunize subcutaneously New Zealand white rabbits with a concentration of 100 g in 0.5 mL NaCl (0.15 mol L^{-1}) in the absence of adjuvant. Whole blood was withdrawn via the marginal ear vein and centrifuged at 2500 × g for 20 min. This serum was purified on a column of 45 cm × 5 cm CNBR – activated Sepharose® 4 Fast Flow according to the standard procedure recommended by the manufacturer. The obtained monospecific purified antibodies were then tested for DENV antigen recognizable by ELISA methods.

DENV conjugated to HRP (DENV-HRP) was obtained according to the procedure using glutaraldehyde [21].

2.3. Apparatus

The electrochemical measurements were carried out by a computer-interfaced Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, Netherlands). The electrodes were incorporated into a conventional three-electrode electrochemical cell. The working electrode was the CHIT-modified carbon fiber, the auxiliary electrode was a platinum wire and an $Ag/AgCl,KCl_{(sat.)}$ was used as reference electrode. Electrochemical experiments were performed using an electrolyte volume of 10 mL at room temperature (±25 °C).

Scanning electron microscopy (SEM) was performed to characterize the carbon fiber surface. Microscopic images were obtained by a scanning electron microscope Philips XL-30 FEG and all images were taken at 15 kV.

2.4. Preparation of the CFE

The carbon fiber sheet (0.5 mm thickness) was cut in disk (0.2 cm^2 area) and was sandwiched between two rectangular polyethylene terephthalate sheets (1 cm × 0.5 cm), that were tightly held together by adhesive tape. The electrical contact was guaranteed by a copper wire directly inserted on the disk (see Electronic Supplementary Material, Fig. S1).

Prior to use, the carbon fiber surface was cleaned with 10% nitric acid solution in ultrasonic bath for 10 min and rinsed with distilled water. A suspension solution of CHIT in acetic acid was prepared by dissolving due amounts of CHIT flakes into a 1% aqueous acetic acid solution [22]. Then, 10 μL of CHIT was dropped onto CFE followed by adding 30 mmol L^{-1} NaOH solution. The CHIT film on the electrode surface was formed by heating at 60 °C for 30 min. Prior to anti-DENV immobilization, the CHIT-modified CFE was activated in 3 mmol L^{-1} aqueous SP solution for 45 min at room temperature. Subsequently, the coated electrode was incubated in 20 μL of anti-DENV solution (1 μg mL^{-1}) for 60 min. After that, the CFE was immersed in a glycine solution (50 mmol L^{-1}) for 45 min in order to block remaining active sites avoiding the non-specific adsorptions.

The stepwise of CFE assembling was accompanied by cyclic voltammetry (CV) at 100 mV s^{-1} scan rate using 5 mM $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ [1:1] in PB (10 mmol L^{-1}, pH 7.0) as redox-probe.

2.5. Immunoassay procedure

Competitive immunoassay was performed mixing sample of DENV (20 μL) and DENV-HRP (20 μL, 100 ng mL^{-1}) in sterile eppendorf® to obtain the incubation solution. Then, 10 μL of the incubation solution was pipetted on the CFEs and incubated for 40 min at room temperature (25 °C). Afterwards, the CFEs were thoroughly rinsed with PB and immersed into 10 mL electrolyte solution containing equal mixture (v/v) of 100 μmol L^{-1} H_2O_2 and 0.5 μmol L^{-1} ABTS. CVs were performed to evaluate the immunosensor response which was carried out in a potential ranging from −0.8 to +0.8 V, at 100 mV s^{-1} scan rate.

3. Results and discussion

3.1. Functionalization of the CFE

CHIT contains amino groups and a molecular arrangement that make it particularly susceptible to chemical interactions with biomolecules. Activation process of the CHIT polymer involving GLUT for immobilizing many enzymes has been widely described [23–26]. Herein, as a prerequisite for the immobilization of antibodies on the sensor surface, it was used SP instead GLUT to facilitate the anti-DENV antibodies immobilization. Fig. 1 shows the current responses of the CFEs in the immobilization of DENV-HRP (100 ng mL^{-1}) on the CHIT film modified with SP (3 mmol L^{-1}) and GLUT (2.5%, v/v). A higher increase of the current was observed in SP-treated CHIT than in GLUT-treated CHIT. This result suggests that a greater amount of the anti-DENV attached to CFE. The SP opens the monomeric structure of CHIT and induces formation of two aldehyde groups in the primary amine-containing cycle [27,28]. Whereas, when GLUT was used only one aldehyde group keeps exposed on the surface of CHIT. Thus, twice the amount of

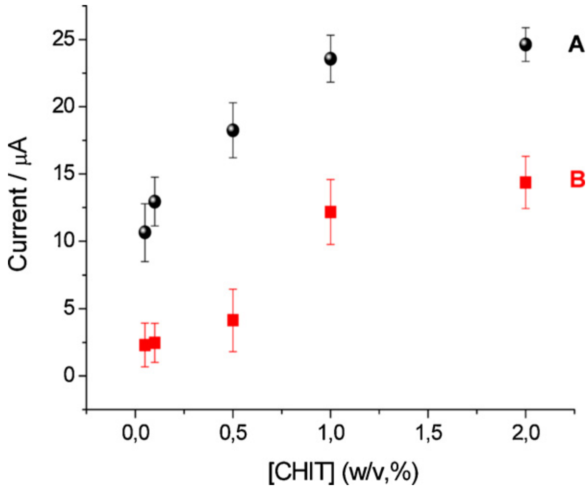

Fig. 1. Curves of the cathodic current peaks obtained from CHIT at different concentration activated with (a) SP, and (b) GLUT.

anti-DENV antibodies is linked to the CHIT film (Fig. 2). In this study, the influence of concentration of CHIT on performance of the CFE was also established (1% CHIT) when a plateau curve of current response was reached.

The degree of faradaic process inhibition is closely related to the electrochemical properties of films that are deposited on bare electrode surface. Considerable passivation can be observed after film formation. More insights into the electrochemical nature of the films were provided using the fast electron transfer redox pair $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and investigating the oxidative behavior of modified-CFEs in these redox species. After applying 1% CHIT on CFE, the redox peaks of CV were significantly reduced (see Electronic Supplementary Material, Fig. S2). From these CVs, the surface coverage (θ) was estimated in 91.5%. θ of CHIT film on a bare CFE can be calculated from the redox peak areas in terms of Eq. (1):

$$\Theta\% = \frac{Q_{CHIT}}{Q_{Bare}} \times 100 \qquad (1)$$

where Q_{CHIT} and Q_{Bare} denote the area of the redox-peaks of the CHIT-modified and bare CFEs, respectively. Subsequently, when anti-DENV was incubated on the CFE, the redox peaks were significantly reduced. This electrochemical behavior confirms that anti-DENV was attached to polymer CHIT which promotes reducing rates of interfacial electron transfer between the electrode surface and the electrolyte solution [29]. The results of CVs indicated that the modification by CHIT and the immobilization of anti-DENV on the electrode surface were successfully accomplished.

3.2. SEM characterization

SEM was employed to observe the morphology and arrangement of CFE. The obtained images are exposed in Fig. 3, which shows a clean arrangement of fibers, highlighting the empty spaces between them. At higher magnification, the carbon fiber was characterized by their cylindrical shape, smooth surface and the tiny grooves that extend along its axis. The fibers had a diameter of 7.2 µm calculated using the Image J program (version 1.43, developed at the National Institutes of Health, USA). After surface modification with CHIT (1%), it was observed the formation of a polymeric film covering the carbon fibers.

3.3. Influence of ABTS on the electrochemical responses of CFE

ABTS has been widely used with hydrogen peroxide substrate, acting as chromogene in the immunoenzymatic methods [30]. However, the ABTS role as an electron mediator in electrochemical systems is not well elucidated. Herein, when ABTS was added jointly to the H_2O_2 substrate, the voltammograms exhibited a higher current response in both anodic (+0.35 V) and cathodic (−0.07 V) peaks than in only H_2O_2 substrate use (Fig. 4). This increase is attributed to a more efficient process of electron transfer between the enzyme and the electrode surface (reaction (I)). ABTS can act as an electron donor and participate in the regeneration process of the HRP enzyme (reaction (II)) [30,31]. The reduction of $ABTS^{\bullet +}$ radical cation was confirmed by the reduction peak, which was used to quantify the concentration of DENV (reaction (III)). To confirm the reaction between ABTS and HRP, voltammetric analyses were performed in the absence of DENV-HRP (100 ng mL^{-1}). Redox peaks were not practically observed in the voltammogram C.

$$HRP_{(red)} + H_2O_2 \rightarrow HRP_{(ox)} + H_2O \qquad (I)$$

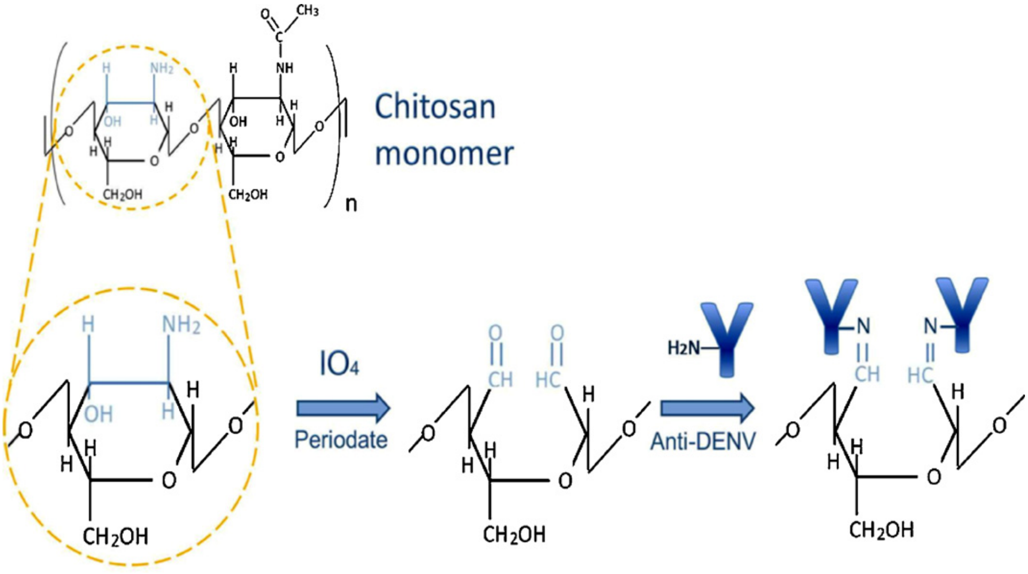

Fig. 2. Schematic illustration of reaction mechanism between CHIT and SP.

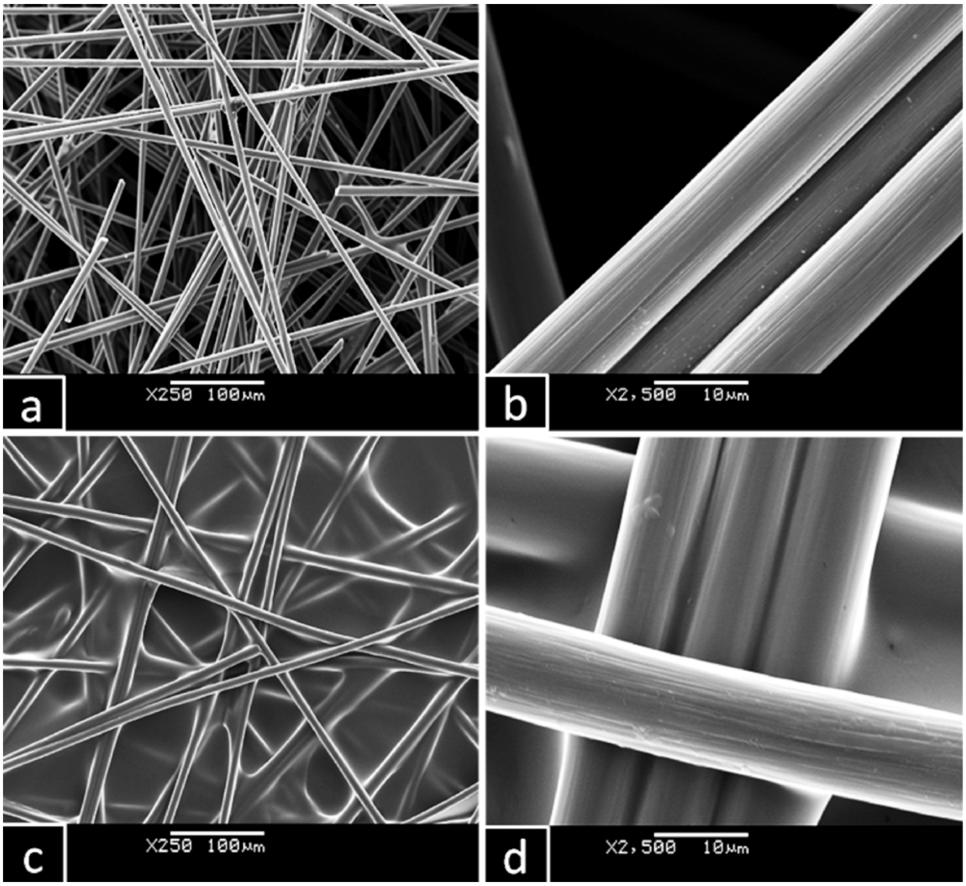

Fig. 3. SEM images of (a and b) bare carbon fiber and modified with (c and d) 1% CHIT film.

$$ABTS^0 + HRP_{(ox)} \rightarrow HRP_{(red)} + ABTS^{\bullet +} \quad \text{(II)}$$

$$ABTS^{\bullet +} + e^- \rightarrow ABTS^0 \quad \text{(III)}$$

3.4. Optimization of experimental conditions

The influence of H_2O_2 concentration on the catalytic activity of the conjugated enzyme is showed in Fig. 5a. The curve plotted by cathodic current peaks ($Ep_c = -0.1$ V) exhibited a hyperbolic profile in relation to the H_2O_2 concentrations, as expected by Michaelis–Menten kinetics [32]. The current response reached a maximum at 100 µmol L^{-1} H_2O_2, which was used in subsequent experiments.

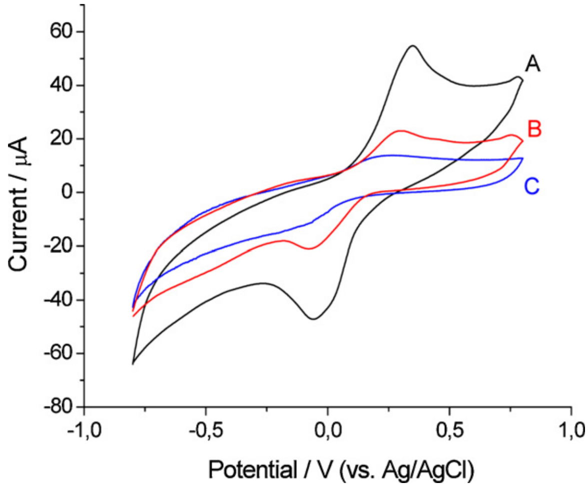

Fig. 4. Illustrative CVs of the CFEs response (a) with ABTS and (b) without ABTS (0.5 µmol L^{-1}). Curve (c) represents a control ABTS response (without DENV-HRP). CVs performed in PB (10 mmol L^{-1}, pH 7.0) using 100 µmol L^{-1} H_2O_2 at scan rate of 100 mV s^{-1}.

The effect of pH on the amperometric response of CFE was also investigated. The pH values of PB (10 mmol L^{-1}) used to dilute 100 ng mL^{-1} DENV antigen samples varied from 4.2 to 8.3. The optimal pH was established to be pH 7.0 (Fig. 5b), in agreement with those reported in the literature [33].

The incubation time on analytical response was studied by incubating anti-DENV-coated CFE in DENV-HRP solution (100 µg mL^{-1}) for 10, 20, 30, 40, 50, and 60 min. The CVs revealed a proportional increase in current signals of the cathodic peaks as function of incubation time. The plateau curve was reached at 40 min which was adopted to remaining experiments (see Electronic Supplementary Material, Fig. S3a).

The influence of the incubation temperature of the DENV-HRP (100 ng mL^{-1}) on the analytical performance of CFE was studied. The optimum incubation temperature was achieved at 37 °C which corresponds to the physiological temperature. When the temperature was above 37 °C the redox peaks decreased (see Electronic Supplementary Material, Fig. S3b). Although the optimum temperature was 37 °C, the room temperature (~25 °C) was adopted for all remaining experiments to the best convenience.

3.5. Competitive immunoassay and reproducibility of the CFE

In order to provide a competitive immunoassay offering an optimal amount of DENV-HRP for DENV to be measured, an experiment varying the concentration of DENV-HRP was performed. Then, the anti-DENV-coated CFEs were incubated in different DENV-HRP

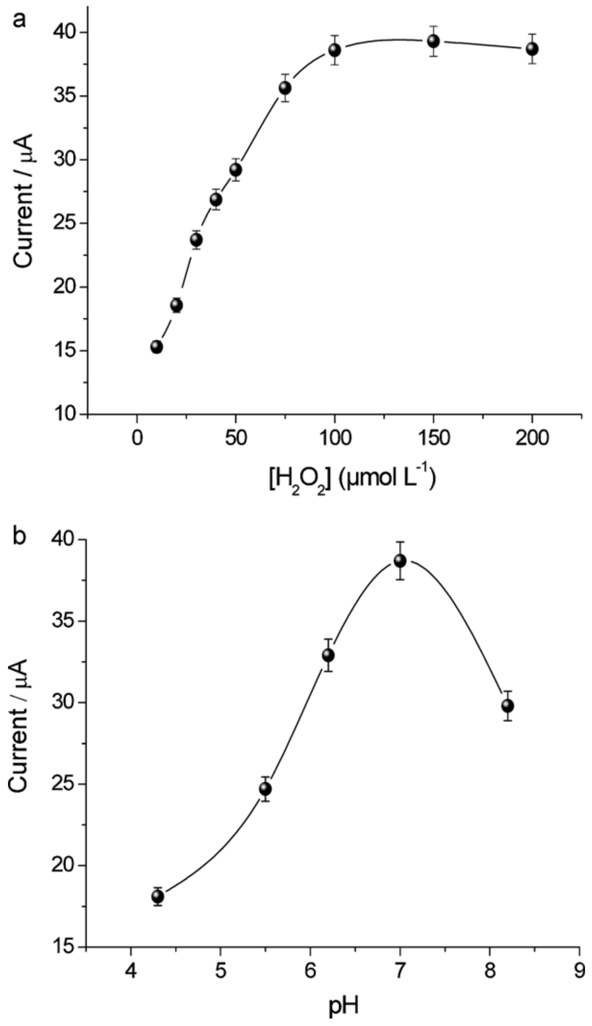

Fig. 5. Cathodic current peaks of the CFEs in PB (10 mmol L^{-1}, pH 7.0) containing 0.5 μmol L^{-1} ABTS as function of (a) H_2O_2 concentrations, (b) pH of the incubation solution. All cathodic current peaks were measured at −0.1 V constant potential by CV and at scan rate of 100 mV s^{-1}.

solutions (1–125 ng mL^{-1}) and subsequently subjected to CVs. It was observed an increase of cathodic peaks (at −0.1 V) proportional to DENV-HRP concentrations (Fig. 6a). The curve plateau was reached at approximately 100 ng mL^{-1} DENV-HRP. Thus, this concentration was established as optimal for a better performance of competitive immunoassay.

Analytical response were performed in a competitive assay incubating the immunosensor in a mixer solution containing 100 μL samples spiked with DENV and 100 μL DENV-HRP (100 ng mL^{-1}) for 40 min at room temperature (25 °C). Fig. 6b shows the cathodic current peaks of the competitive immunoassays from different electrodes. It was observed a decrease of the cathodic peaks inversely proportional to the DENV concentrations. It is shown a linear response between 1 and 175 ng mL^{-1} and a limit of detection (LOD) achieved of 0.94 ng mL^{-1} DENV. The LOD was determined by average of two replicates plus three times the standard deviations from minimal concentration of DENV measured. This limit of detection for DENV was lower than previously obtained in a piezoelectric immunosensor [34]. The LOD was also comparable to conventional enzyme linked immunosorbent assay in which the limit of detection has 4 ng mL^{-1} DENV for purified samples [35]. This approach has advantaging simplicity and low cost becoming a good alternative for determination of dengue virus antigens in serum.

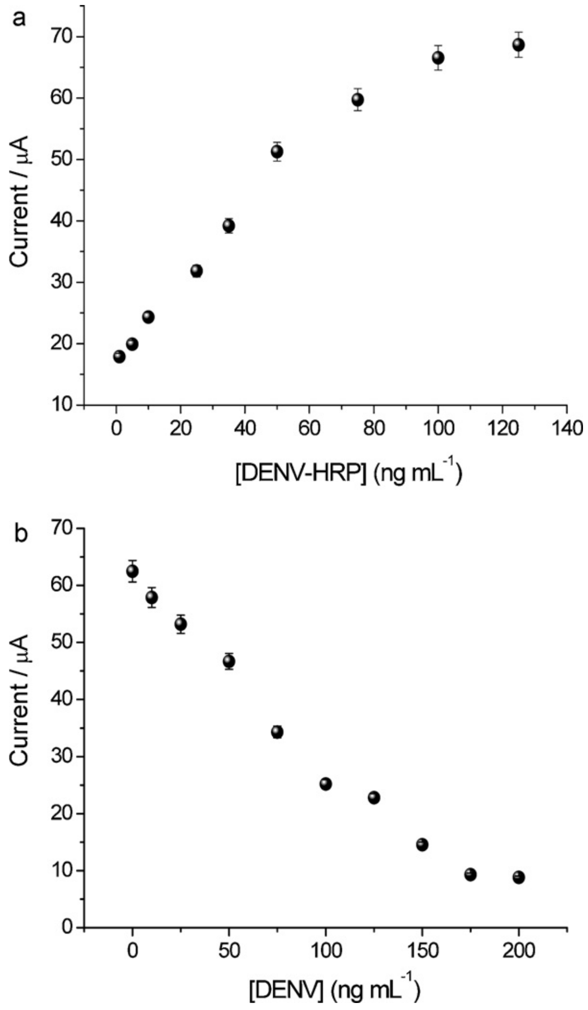

Fig. 6. Curves of the CFEs from cathodic current peaks (−0.1 V) (a) in different concentrations of the DENV-HRP; (b) in response to DENV.

The inter-assay variations of the immunosensor were evaluated using five modified CF electrodes. The inter-assay coefficient of variation was 5.8% when 10 ng mL^{-1} of DENV antigen was employed that indicate a good reproducibility of the CFEs. The intra-assay coefficient calculated in five replicates measurements was 3.6%; this value shows a satisfactory repeatability. Nevertheless, more studies involving real serum or whole blood is required for further use of this immunosensor in clinical routine due to these samples to be complex matrices subjected to interfering and other effects.

4. Conclusions

The use of carbon fiber to manufacture reproducible immunosensors was successfully supported. This CFE is cheaper than gold or platinum electrodes with additional advantage exempting printing machines, that are widely used to make disposable screen printed electrodes. The potential use of ABTS as mediator for the immunoassay based on conjugated peroxidase was demonstrated by the increase of the analytical response. This proposed CHIT/CFE immunosensor showed higher analytical performance when compared to conventional methods for DENV detection, such as ELISA, whereas has notable features that can be explored for developing of disposable device, for instance, the point-of-care testing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.002.

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