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New approach to immobilization and specific-sequence detection of nucleic acids based on poly(4-hydroxyphenylacetic acid)

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ABSTRACT

Immobilization and hybridization of oligonucleotides or specific-gene PCR product (DENV-1), a conserved genomic sequence of the dengue virus, onto graphite electrode modified with poly(4-hydroxyphenylacetic acid), were carried out with success using both direct electrochemical oxidation of guanine or redox electroactive indicator ethidium bromide.

Studies of oligonucleotides hybridization with the complementary target showed a decrease of both guanosine and adenosine current peaks, when compared with the peak previously obtained before the hybridization. Immobilized ssDNA, DENV-1, was hybridized with various concentrations of target DNA. The interaction between DENV-1 hybridized onto the modified graphite electrodes surface and the intercalator, ethidium bromide, was observed by differential pulse voltammetry, monitoring the current change generated to the DNA intercalator accumulated onto the modified electrode after DNA hybridization. For the determination of complementary target, the proposed method exhibited a good dynamic range (12–42 nmol L^{-1}) and a low detection limit (7.12 nmol L^{-1}).

AFM images showed that the oligonucleotides or single-stranded DNA, DENV-1, before hybridization, had roughness values lower than the double stranded obtained after hybridization.

The new surface obtained in these work, as well as the possibility of utilization of the same to monitor hybridization events is a promising strategy for the development of DNA electrochemical biosensors.

1. Introduction

The chemical modification of the surface of electrodes offers great potential to help in the identification between target compounds that have similar characteristic redox, increasing the efficiency and applicability of electrochemical sensors [1]. The ability to control and to modify the properties of the surfaces of the electrodes can provide a variety of attractive effects, which can effectively collaborate for the solution of problems presented by traditional electrochemical sensors [2]. The use of polymer films offers several advantages in the construction of sensors, since they are relatively cheap materials, the techniques for the production are simple, they can be deposited on various types of substrates and the choice of different molecular structures provides the construction of films with different characteristics [3]. The modification of the molecular composition of the electrode aims at improving sensitivity, selectivity and/or stability allowing the tailoring of its response in order to meet analytical needs [4–6].

The techniques currently used in the deposition of films on different areas are quite diverse. The methods of Langmuir–Blodgett and electrochemical polymerization are the most commonly used for the construction of biosensors [3,7]. The advantage of the last method is the control of the electrochemical deposition of the film as thickness, morphology and homogeneity in the formation of the chain, ranging up the potential and the number of scans on the work electrode [3,8].

The modification of surfaces with polymer films has been used in the development of biosensors to protect the surface of the electrodes against impurities, block interferents, incorporate mediators and provide biocompatibility [7]. The stage of the immobilization of biomolecules or indicators on the surface of the electrode plays an important role in obtaining the sensitivity, selectivity and stability of the biosensor. These characteristics are achieved by means of a chemical control and coverage of the area, thus ensuring reactivity, accessibility and stability to the biomolecules immobilized [9].

The use of conducting and non-conducting films is suitable for the immobilization of DNA probes [9]. This feature is due to the ability of being easily processed by the modified electrodes, with increase in the contact area of the biomolecules with the electrode, which allows greater accommodation of the molecule, simulating its natural

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environment, favoring the conversion of the biological signal to fast analytical signal with high stability and reproducibility [8,10]. Due to the various characteristics presented by polymer films, several studies are found in the literature aimed at the construction of biosensors [11–13].

Since, as a whole, DNA is highly redox-inactive, an electrochemical hybridization indicator is usually employed (indirect method). They can bind DNA through reversible physical intercalation between base pairs or through electrostatic interaction, in well-defined binding sites [14]. The effect is a differential accumulation of the indicator in the DNA layer near the surface of the electrode when a ssDNA or a dsDNA is attached, which correlates with different voltammetric peak currents [15]. The most common indicators are heterocyclic dyes (e.g., ethidium bromide, methylene blue, anthracyclines, phenotiazines and acridine derivatives), anticancer drugs (e.g., daunomycin) and organometallic complexes (mainly from Co, Fe, Os, Pt and Ru). DNA hybridization biosensor holds an enormous potential for pharmaceutical, clinical and forensic application and disease diagnosis [16].

Dengue virus is a member of the Flaviviridae family and is one of the most significant causes of arthropod-borne diseases on Earth. Dengue virus exists as four antigenically distinct serotypes (Dengue 1–4) and is transmitted among humans by the *Aedes aegypti* mosquito. Dengue is recognized in over 100 countries and territories, and the worldwide annual infection rate is estimated to be between 50 and 100 million infections per year [17].

Recently, we investigated the electropolymerization of 4-hydroxyphenylaceticacid on the surface of graphite electrodes, the electrochemical and morphological properties of the polymer film formed and their applications in incorporation and electrooxidation of nitrogenated bases of DNA. These electrodes modified with poly(4-hydroxyphenylacetic acid) were found to be efficient in immobilizing purine bases. Adenosine monophosphate and guanosine monophosphate presented an increase in the current values of the anodic potential peak when compared to bare graphite electrodes. This parameter is very important to guarantee the sensibility of the DNA biosensor [18].

In this work, poly(4-hydroxyphenylacetic acid) was used as matrix for the detection of a 18-mer synthetic oligonucleotide and a conserved genomic sequence of the dengue virus (dengue 1 or DENV1), aiming at contributing for biosensor development, once the prevention of the disease has widely focused on mosquito eradication strategies, which were of very limited success.

This is the first report on oligonucleotide and DNA fragment immobilization and detection of complementary target onto graphite electrode modified with poly(4-hydroxyphenylacetic acid).

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade. Ultra high purity water (Millipore Milli-Q system) was used in the preparation of the solutions. Oligonucleotide probes and target were synthesized by Invitrogen Life Technologies with the following sequences: probe: poly(G) 5′-GGGGGGGGGGGGGGG-3′, poly(A) 5′-AAAAAAAAAAAAAAA-3′, poly (C) 5′-CCCCCCCCCCCCCCC-3′, poly(T)5′-TTTTTTTTTTTTTTT-3′, poly (GA) 5′-GGGGGGGGAAAAAAAA A-3′, poly(CT) 3′-CCCCCCCCTTTTTTTT-5′. Stock solutions of the probe (6.4×10^{-2} mmol L^{-1}) and target oligonucleotides (1.8×10^{-1} mmol L^{-1}) were prepared in water and stored at −20 °C until use. Buffer components (CH_3COOH and CH_3COONa or Na_2HPO_4 and NaH_2PO_4) were purchased from Sigma-Aldrich Chemical, USA (ACS purity) and prepared at pH 4.7 and pH 7.4 respectively. Monomer solutions, 4-hydroxyphenylacetic acid, were prepared in 0.5 mol L^{-1} $HClO_4$ solution, immediately before their use. All reagents were used as received. The experiments were conducted at room temperature (25 ± 1 °C).

The gene-specific oligonucleotides for the dengue virus, DENV-1 (Probe and target) have as base sequences: DENV-1 (primer 1) 5′-CAA TAT GCT GAA ACG CGA GAG AAA CCG-3′ and DENV-1 (primer 2) 5′-AGC AGC ATA AGG AGC ATG GTC AC-3′. The DENV-1 (primer 1) was used for Reverse Transcriptase-Polymerase Chain Reaction, RT-PCR, and DENV-1 (primer 1 and primer 2) were used for Polymerase Chain Reaction, PCR reaction.

2.2. Apparatus

All electrochemical experiments were carried out using a potentiostat CH Instruments, model 760 C connected to a serial output program.

The electrochemical studies were carried using a graphite working electrode of 6 mm diameter, cut from a graphite rod (99.9995%, Alfa Aesar). Film morphology in absence or presence of biomolecules was assessed by atomic force microscopy (AFM) (Nanoscope IIIa, Digital Instruments). Polymerase chain reaction (PCR) was performed in a thermocycler (Eppendorf Mastercycler).

2.3. Production of poly(4-hydroxyphenylacetic acid) [poly(4-HPA)]

The electrochemical studies were performed in a three-compartment glass cell connected to a potentiostat. The graphite surface, prior to electropolymerization, was mechanically polished with alumina slurry (0.3 μm diameter), ultrasonicated, washed with distilled water and dried in the air. The monomer solutions were degassed with N_2 prior to electropolymerization. Poly(4-HPA) films were grown by means of potentiodynamic electropolymerization on graphite electrodes from 4-hydroxyphenylacetic acid solution (2.5 mmol L^{-1}) in $HClO_4$ solution (0.5 mol L^{-1}). The potential cycling was made between −0.70 V and +1.20 V vs. SCE at 50 mV s^{-1}. After electropo1ymerization, the modified e1ectrode was rinsed in deionized water to remove unreacted monomer. Preliminary studies of the formation and characterization of poly(4-HPA) were described previously by our group [18].

2.4. RT-PCR of dengue virus RNA, type 1, from human plasma

RNA from plasma specimens were isolated by using the Trizol Reagent (Invitrogen), according to the manufactures' instructions. First-strand cDNA was synthesized using a RT-PCR kit (Stratagene) with a primer 1 and the RNA total as a template. The reaction was carried out at 37 °C for 1 h. Double strand DNA (dsDNA) was amplified by PCR with the first strand cDNA as a template and two primers (primer 1 and primer 2).

PCR reactions contained 2 μL of cDNA, 2 μL forward and reverse primers (final concentration is 1 μM for each primer), 2.5 μL of PCR buffer (100 mmol L^{-1} Tris HCl [pH 9.0], 0.75 μL of 15 mmol L^{-1} $MgCl_2$, 0.5 μL of 10 mmol L^{-1} dNTP, 0.4 μL of 2 U Platinum Taq DNA polymerase (Invitrogen), and 13.85 μL distilled water in a total volume of 25 μL.

PCR conditions were as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and elongation at 72 °C for 1 min, and final extension at 72 °C for 3 min.

The PCR product was visualized in agarose gel stained with ethidium bromide under UV light. The size of the PCR product was 288 bp. Subsequently, the concentration of dsDNA was measured by the ultraviolet absorption at 260 nm (ε=6600 $L\cdot mol^{-1}\cdot cm^{-1}$).

2.5. Oligonucleotide and DENV-1 probes immobilization on graphite electrode modified with poly(4-HPA)

The immobilization of oligonucleotides was carried out by applying 15 μL of 6.4×10^{-2} mmol L^{-1} of poly(G), poly(A) or poly(GA).