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Short communication

An impedimetric biosensor for detection of dengue serotype at picomolar concentration based on gold nanoparticles-polyaniline hybrid composites

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ABSTRACT

In this work, we describe the preparation and characterization of a novel gold nanoparticles-polyaniline hybrid composite (AuNpPANI) with SH-terminal groups that, due to its ability of immobilizing dengue serotype-specific primers 1, 2 and 3 (ST1, ST2 and ST3), can be used for the development of biosensors. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were performed. CV and EIS results demonstrated that the AuNpPANI can immobilize ST1, ST2 and ST3, forming AuNpPANI-ST complexes. Well-defined cyclic voltammograms characteristic of a diffusion-limited redox process were observed both for the bare gold electrode and after these electrodes have been modified by the adsorption of AuNpPANI or AuNpPANI-ST. The AuNpPANI-ST(1–3) systems were able to recognize the dengue serotype of different patients at picomolar concentrations. Even when small volumes and low concentrations of the analyte were used, the CV and EIS results showed unequivocal evidence of an existing interaction between dengue serotype-specific primers and their complementary genomic DNA targets.

1. Introduction

The dengue virus causes more illness and death than any other arbovirus diseases, particularly in developing countries, where dengue viral infections are reaching plague proportions [1]. Detection and classification of the dengue virus requires 4–5 days for its isolation and cultivation. PCR methods can rapidly detect the viral RNA from the patients' serum in the viremis phase but their more widespread use is hindered not only due to the possibility of sample contamination, but also their characteristic demands of high technological support. Hence there is a need for diagnostic methods that, in a reliable manner, could identify the disease and allow the treatment of the dengue virus infection still in its initial stage [2]. Ideally, dengue biosensors should also be able to detect the presence of an analyte in minutes while using small volumes of sample [3].

Use of DNA biosensor for the detection of nucleic sequences holds an enormous possibility for disease diagnosis, drug screening, and forensic analysis [4]. Many techniques including electrochemistry, surface plasmon resonance and quartz crystal microbalance have been used for the development of DNA biosensors. Electrochemistry techniques offer sensitivity and selectivity at low cost for the detection of selected DNA sequences associated with human disease [5] and electrochemical biosensors for DNA hybridization present attractive capacity for converting the hybridization event into an analytical signal [6].

In the last two decades, conductive polymers have attracted much interest due to their high conductivity, ease of preparation, good environmental stability, and large variety of possible applications in areas such as light emitting, electronic devices, and chemical sensors [7]. Polyaniline (PANI) has been proven particularly useful in the development of biosensors [8]. In especial, polymer–nanoparticle composite materials have attracted the interest of a number of researchers, due to their synergistic and hybrid properties derived from their diverse components [9,10].

In this work, we describe the development a sensitive layer based on gold nanoparticles-polyaniline hybrid composite (AuNpPANI) (Fig. 1a) that can be used for monitoring the hybridization of dengue serotype virus, while requiring the use of small volumes and low concentrations of the analyte. We have applied self-assembly techniques based on the initial chemical adsorption of SH-terminal groups of AuNpPANI on the gold electrode surface and on the subsequent electrostatic interaction between

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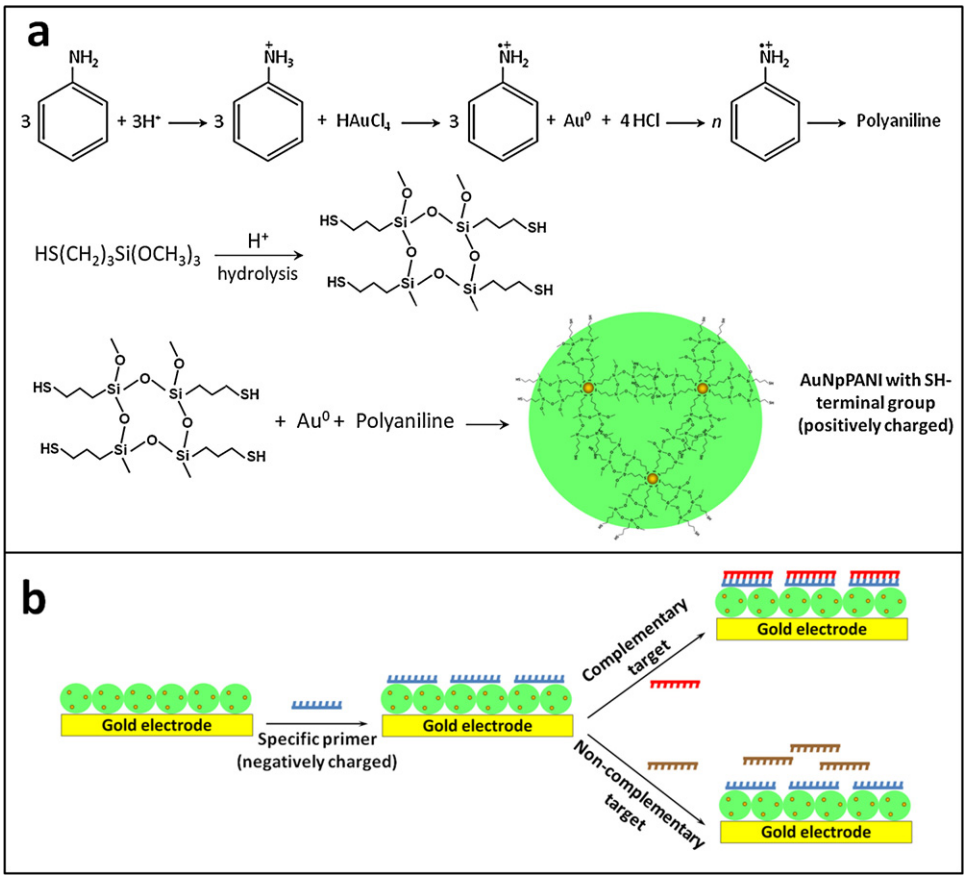

Fig. 1. Schematic representation of AuNpPANI synthesis (a) and biosensor construction (b).

the (positively charged) AuNpPANI and the (negatively charged) serotype-specific primers (Fig. 1b).

2. Experimental

2.1. Materials

Complementary sequence of human patient's genome classified serotype 1, 2 and 3 (DEN1, DEN2 and DEN3 genome sequences) and the serotype-specific primers were obtained from Instituto Aggeu Magalhães – FIOCRUZ (located in Recife, Brazil), which were obtained in according to Cordeiro and collaborators [11]. The serotype-specific base sequences (ST) for type 1, 2 and 3 [12] are, respectively:

- Serotype 1 (ST1): 5′-CGTCTCAGTGATCCGGGGG-3′
- Serotype 2 (ST2): 5′-CGCCACAAGGGCCATGAACAG-3′
- Serotype 3 (ST3): 5′-TAACATCATGAGACAGAGC-3′

$HAuCl_4{\cdot}3H_2O$ and 3-mercaptopropyl-trimethoxysilane (MPTS, $HS(CH_2)_3Si(OCH_3)_3$) were purchased from Sigma Chemical (St. Louis, MO, USA), while aniline and potassium ferri- and ferrocyanide were obtained from VETEC (Brazil). All chemicals and solvents were of analytical grade and they have been used as received, without further purification. Water used was obtained after passing through a Milli-Q plus (Billerica, USA) purification system.

2.2. AuNpPANI preparation

Hybrid organic–inorganic composites were obtained according to de Melo and collaborators [13]. The preparation of AuNpPANI was performed according to Refs. [13,14]. The preparation of AuNpPANI was performed in a round bottom glass flask containing ethanol (20 mL) and the compounds: aniline (0.030 mol/L), MPTS (6.46×10^{-2} mol/L), and $HAuCl_4{\cdot}3H_2O$ (0.81 mmol/L), which were subsequently added and subject to agitation (1100 rpm) for 48 h. Thus, the samples were centrifuged for 10 min at 12,000 RPM (to remove the excess of MPTS), and subsequently added 500 μL of 0.1 M HCl to obtain positively charged PANI and promotes the hydrolysis of MPTS. The H^+ ion from $HAuCl_4$ leads to the formation of anilinium cations ($PhNH^{3+}$), a process that involves the generation of a radical cation accompanied by the release of an electron. $[AuCl_4]^-$ acts as an oxidizing agent capable of oxidizing $PhNH^{3+}$ [14], resulting in polyaniline as the oxidative product (Fig. 1a). Each step during the polymerization process involved the release of electrons that reduce the $[AuCl_4]^-$ to form gold atoms, which are encapsulated and stabilized by PANI (Fig. 1a). While authors have prepared similar AuNpPANI composites [14–16], up to now no AuNpPANI systems with SH-terminal groups on the composite surface have been obtained. In our work, we have used MPTS to stabilize the formed nanocomposites due to the strong coordination of a mercapto group to Au. Subsequently, we promoted the hydrolysis of MPTS to obtain free thiol group on the surface [17].

2.3. Immobilization of probe ST-modified electrode and hybridization with genomic DNA target at picomolar concentration

The AuNpPANI-modified electrode was obtained by dropping AuNpPANI solution on the bare gold electrode surface at 25 °C for 2 min. Then, the AuNpPANI-modified electrode was rinsed with ethanol solution to remove non-adsorbed particles. The ST

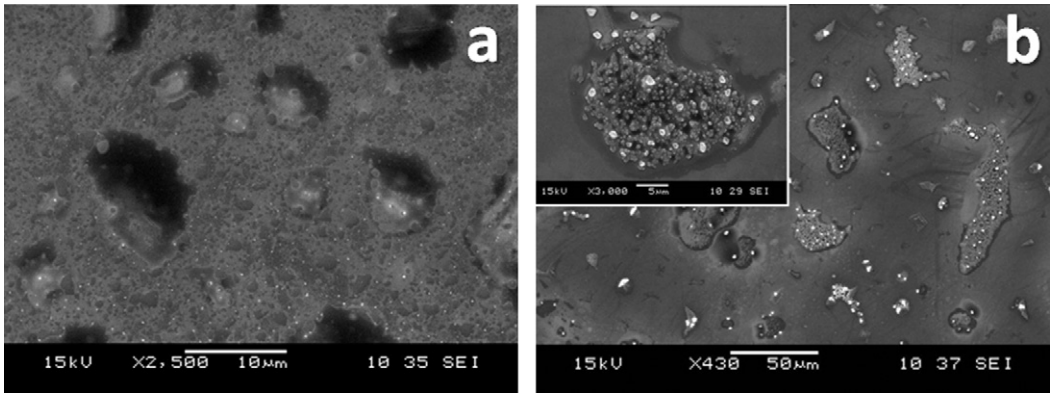

Fig. 2. Representative SEM images of the AuNpPANI-ST1 system (a) and AuNpPANI-ST1 exposed to serum from patient infected by dengue type I (b). In the inset we show the aggregation of AuNpPANI-ST1-DENcomplementary.

immobilization was performed with the AuNpPANI-modified electrode incubated into 0.05 pM probe ST in 10 mM phosphate buffer saline (PBS) solution for 5 min at room temperature. The immobilized electrode was then rinsed with PBS solution (pH = 7.0) and stored at 4 °C for the hybridization. The hybridization experiments were carried out by immersing the AuNpPANI-ST-modified electrode into different concentrations of genomic DNA target sequence in 10 mM PBS solution. Finally, the hybridized electrodes were rinsed with PBS. The scanning electron microscopy images were obtained from a JSM 5900 (JEOL Instruments, Japan) at an acceleration voltage of 15 kV and a working distance of 5 μm [18].

2.4. Electrochemical measurements

The impedance spectra were recorded in the frequency range of 100 MHz to 100 kHz (PGSTAT 302N, Autolab, The Netherlands). The amplitude of the applied sine wave potential was 10 mV. The corresponding values of the real and imaginary parts of the impedance (Z′ and Z″, respectively) were processed through the FRA software (Autolab, The Netherlands). CV was performed with a potential sweeping between −0.2 V and +0.7 V at a scan rate of 50 mV s^{-1}. A three-electrode setup with an Ag/AgCl (saturated KCl) reference electrode was employed throughout the investigation. A platinum wire and a modified gold disc (d = 2 mm) were used as auxiliary and working electrodes, respectively. CV and EIS measurements, performed in the presence of a 10 mM $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ (1:1) solution used as a redox probe in PBS, were carried out at different stages of the preparation of the modified electrode [19].

3. Results and discussion

3.1. SEM analysis

The SEM technique was employed to confirm the electrode surface modification. In Fig. 2a we show a SEM image of a gold surface coated with AuNpPANI-ST1 system, where a homogeneous distribution of the AuNpPANI composite can be seen. In Fig. 2b we could see that AuNpPANI agglomerates are present when the DEN1 genome sequence (complementary target) is hybridized with the dengue ST1. The formation of such agglomerates may be due to differences in DEN1, DEN2 and DEN3 genome sequences, since the serotype-specific base sequences for type 1 presents the longest genome sequence [20–22]. These results were later confirmed by both CV and EIS analyses, which indicated a higher hybridization process for the serotype 1 than for the serotypes 2 and 3.

3.2. Cyclic voltammetry of $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ at the AuNpPANI-ST-modified electrode surface

The electron transfer of ferricyanide through the modified electrode could be used as a valuable tool to monitor the different stages of the electrode fabrication process. Fig. 3 shows the cyclic voltammograms obtained for different systems: pure ST1 (Fig. 3a), ST2 (Fig. 3b) and ST3 (Fig. 3c) serotypes, measured at a scan rate of 50 mV s^{-1}. The bare gold electrode presents a pair of well-defined redox peaks, showing the reversible process of $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ in solution phase (Fig. 3, curve a). The assemblage of the AuNpPANI composite atop the electrode surface induced a decrease in the peak current (Fig. 3, curve b). Finally, curve c in Fig. 3 corresponds to the electrochemical behavior of redox pair after the probe ST has modified the electrode: here, the peak current is lower than that of curve b, in an indication that the probe ST for each dengue serotype has been successfully immobilized on the surface of the modified electrode. The decrease in the peak current could be assigned to the repulsion of $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ by the negatively charged phosphate backbone of probe ST(1–3). After the occurrence of the hybridization of the modified electrode with the target complementary DEN for ST(1–3), the peak current of redox pair was observed to suffer a further decrease (Fig. 3, curve d). The introduction of complementary genomic DNA increases the negative charge and therefore the repellence of the redox species. This change in charge upon hybridization induces a further increase in the resistance, in what constitutes the basis for the sensing mechanism of the detector. The AuNpPANI-ST(1–3) system responses to a non-complementary target can be visualized in curve e (Fig. 3), which shows no changes in the $K_4[Fe(CN)_6]^{4-}/K_3[Fe(CN)_6]^{3-}$ redox species.

3.3. Electrochemical impedance spectroscopy of modified electrode surface

Fig. 4a shows the Nyquist plots of the differently modified electrode when the frequency was varied from 100 MHz to 100 kHz. An almost straight line was observed for the bare gold electrode, which is a characteristic of an electron transfer process controlled by mass diffusion and a low R_{CT} (8.62×10^2 Ω). The assemblage of the AuNpPANI composite on the electrode surface induced an increase in the interfacial R_{CT} (curve b) as compared to the corresponding one of the bare gold electrode, in an indication of a blockage in the electron transfer. Results indicated that the R_{CT} has its value increased to 1.51 kΩ with the further assembly of the AuNpPANI composite.

The ST1 immobilization on the AuNpPANI-modified electrode results in an increase of R_{CT} from 1.51 kΩ to 2.12 kΩ (curve c),

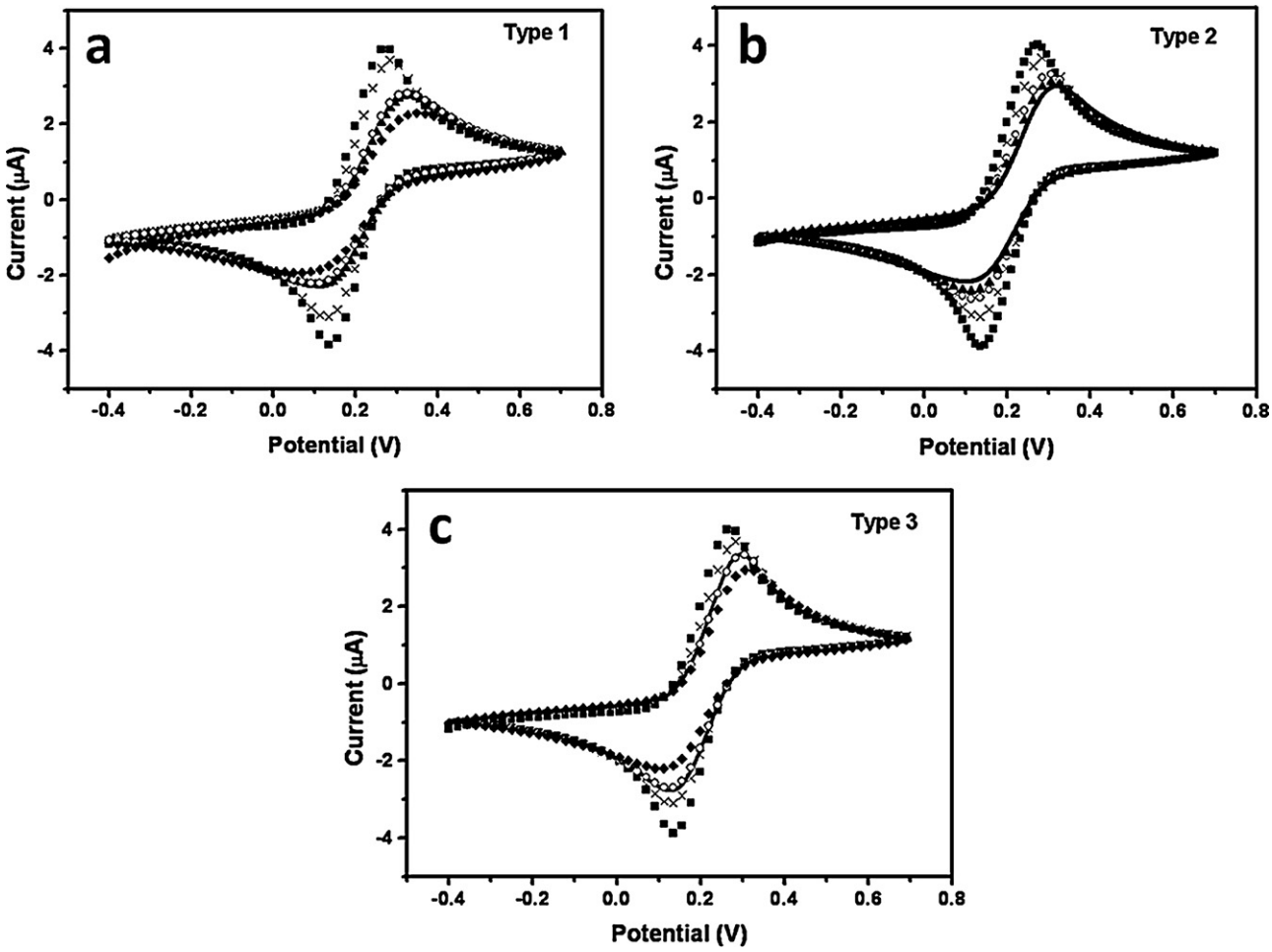

Fig. 3. Cyclic voltammograms of the electrode for serotype 1 (a), serotype 2 (b) and serotype 3 (c) at different stages of the process: bare gold electrode (■), AuNpPANI (×), AuNpPANI-ST (▲), AuNpPANI-ST-DENcomplementary (–) and AuNpPANI-ST-DENnon-complementary (○).

which could be ascribed to the repellence of redox probe from the approaching electrode surface by negative-charged phosphate skeletons of ST. After the ST1 probe hybridizes with the DEN1 genome sequence (complementary target), a new increase in the value of R_{CT} to 3.6 kΩ was obtained (curve e), indicating the occurrence of a specific biomolecular interaction. In fact, no similar results were observed for the case of the AuNpPANI-ST-DNAnon-complementary experiments (curve d). As observed before, the higher response obtained in the case of the dengue ST1 (as compared to the others serotypes) represents a specific response to the complementary genomic DNA dengue (DEN1) (see inset of Fig. 4a).

The highest R_{CT} value obtained could also be attributed to the low conductivity of the DNA structure, which contributes to a reduction of the redox pair. This also proved that the ST was immobilized on the gold surface, and that the target genomic DNA was hybridized with the ST probe.

The Randles model (Fig. 4b) [23] is used as an equivalent circuit to describe the data of electrochemical impedance measurements; the fitting of the measured spectra is shown in Fig. 4a, where a good agreement can be observed over the entire range of measured frequencies. The general equivalent electric circuit (Randles) [23] includes the ohmic resistance of the electrolyte solution (R_{Ω}), the Warburg impedance (W) resulting from ion diffusion from the bulk electrolyte to the electrode interface, the double layer (C_{dl}), and the charge-transfer resistance (R_{CT}), which are admitted to exist when the electrolyte solution contains a redox probe. In the AuNpPANI-ST-DEN(1–3) system, R_{Ω} and W denote, respectively, the bulk properties of the electrolyte solution and the diffusion features of the redox probe in the solution.

The best EIS parameters for this system were calculated from the data shown in Fig. 4a and are presented in Table 1. As discussed before, the stepwise increase of the R_{CT} for AuNpPANI, AuNpPANI-ST(1–3) and AuNpPANI-ST(1–3)-DENcomplementary indicate the formation of insulating films of AuNpPANI and DNA on the gold electrode surface. The C_{dl} value largely depends on the dielectric constant of the layer separating the ionic charges and the electrode surface, on the surface area of the electrode, and on the thickness of the separation layer. A large decrease in the C_{dl} value was observed when the AuNpPANI-TS1 system was introduced with the complementary target. Therefore, we observed that the value of R_{CT} depends on the insulating characteristics of the electrode/electrolyte interface.

Our results show the occurrence of a high R_{CT} value for the AuNpPANI-ST-DENcomplementary system at different dengue types. Since it is well known that high R_{CT} values are usually associated to the blockage of the electrode surface [23], we can assume that we have identified that a specific binding of target and probe exists in our system. Conversely, the low R_{CT} value obtained for the AuNpPANI-ST-DENnon-complementary seems to be due to non-specific binding. This impedance change could also be used for the characterization of DNA hybridization. The impedance experiments for differently modified electrodes were in good agreement with the results by CV experiments.

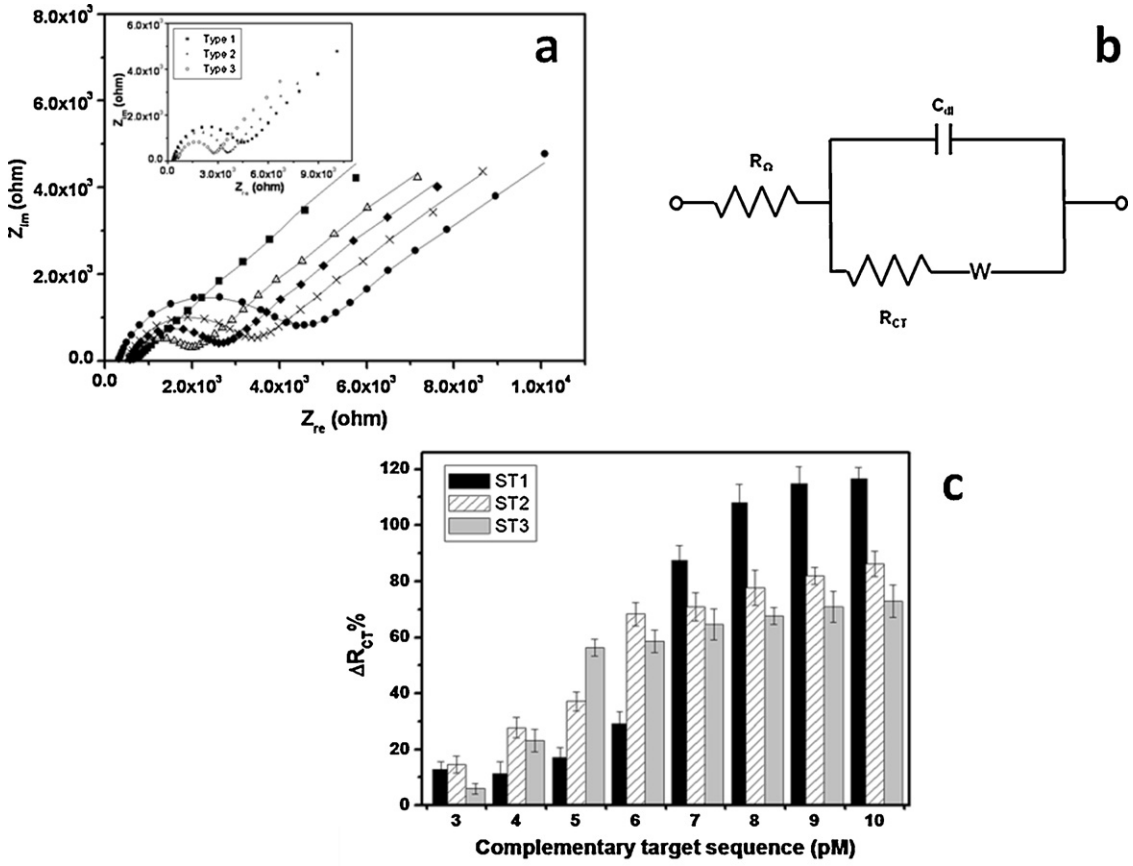

Fig. 4. Nyquist plots of the electrode at different stages (a), equivalent circuit adopted to fit the impedance data (b) and ΔR_{ct} versus complementary target concentration (c). Bare gold electrode (■), AuNpPANI (△), AuNpPANI-ST (♦), AuNpPANI-ST-DENcomplementary (●) and AuNpPANI-ST-DENnon-complementary (×). Inset: Nyquist plots of AuNpPANI-ST(1–3)-DENcomplementary.

The results presented in Fig. 4a clearly show that the DNA probe remain capable of recognizing the complementary target, as revealed by the increase of R_{CT}. Hence, the performance of the modified electrode for detection of dengue DNA target may be evaluated through the relative variation of this parameter (ΔR_{CT}), according to the equation:

$$\Delta R_{CT}(\%) = \left(\frac{R_{CT}(\text{ST-DENcomplementary}) - R_{CT}(\text{ST})}{R_{CT}(\text{ST})}\right) \times 100$$

Here, while R_{CT}(ST) is the value of the charge-transfer resistance of the AuNpPANI-ST-modified electrode, R_{CT}(ST-DENcomplementary) corresponds to the charge-transfer resistance of the AuNpPANI-ST after exposing it to the solution containing complementary and non-complementary genomic samples. The corresponding results are presented in Table 1, for fixed concentrations of DEN complementary and non-complementary samples (7 pM). The results obtained for ST(1–3) demonstrate the capability of recognition of specific targets via the hybridization process. The obtained value of ΔR_{CT} for ST1 (87.5%) was higher than for ST2 (71.03%) and ST3 (64%). These results allow us to suggest that AuNpPANI-TS biosensors can be successfully applied for biodetection of the genome of human patients.

To evaluate the hybridization reaction between AuNpPANI-TS1 and complementary target, we exposed this system to various concentrations of target type 1 (Fig. 4c). It was found that the ΔR_{CT} values increased with the concentrations. These results clearly confirm that the observed impedance changes originate from well-defined specific hybridization process.

The reproducibility of the biosensor system was evaluated from the ΔR_{CT} response (Fig. 4c) of three different patients for each

Table 1
Values of the equivalent circuit elements from fitted impedance results and relative charge transfer variation from impedance data.

Modified electrode	R_Ω (kΩ)	R_{CT} (kΩ)	C_{dl} (μF)	W (Ω cm^2) (×10^{-4})	ΔR_{CT} (%)
Bare gold electrode	0.69 ± 0.05	0.82 ± 0.06	10.38 ± 0.12	5.36 ± 0.07	–
AuNpPANI	0.63 ± 0.05	1.50 ± 0.05	5.48 ± 0.07	3.33 ± 0.05	–
AuNpPANI-ST1	0.63 ± 0.09	1.92 ± 0.06	6.12 ± 0.08	3.53 ± 0.07	–
AuNpPANI-ST1-DENcomplementary	0.58 ± 0.07	3.60 ± 0.08	5.30 ± 0.11	1.50 ± 0.04	87.50
AuNpPANI-ST1-DENnon-complementary	0.62 ± 0.04	2.14 ± 0.10	5.51 ± 0.07	3.08 ± 0.09	11.45
AuNpPANI-ST2	0.50 ± 0.08	1.83 ± 0.04	6.73 ± 0.03	4.40 ± 0.06	–
AuNpPANI-ST2-DENcomplementary	0.64 ± 0.06	3.13 ± 0.07	5.11 ± 0.13	4.25 ± 0.04	71.03
AuNpPANI-ST2-DENnon-complementary	0.61 ± 0.09	2.07 ± 0.09	6.61 ± 0.05	4.36 ± 0.09	13.11
AuNpPANI-ST3	0.61 ± 0.06	1.33 ± 0.04	5.53 ± 0.09	4.28 ± 0.05	–
AuNpPANI-ST3-DENcomplementary	0.55 ± 0.08	2.19 ± 0.09	5.18 ± 0.11	4.49 ± 0.12	64.70
AuNpPANI-ST3-DENnon-complementary	0.62 ± 0.10	1.50 ± 0.03	5.21 ± 0.06	4.40 ± 0.09	12.80

dengue serotype ST1, ST2 and ST3 at different biosensors ($n = 3$) and an acceptable R.S.D. values of 4.33%, 4.27% and 3.90% for complementary sequence of ST1, ST2 and ST3, respectively, were observed confirming the efficiency of the biosensor. These results indicate the suitability of the biosensor to practical applications. Our biosensor has valuable characteristic of effectively detecting the presence of the dengue virus in minutes while using small volumes of sample as compared to standard alternative techniques.

4. Conclusions

We have shown that AuNpPANI-ST(1–3) systems are capable of detecting dengue serotypes 1, 2 and 3 at picomolar concentrations with high specificity and reproducibility. EIS and CV measurements showed that redox probe reactions on the modified gold electrodes were partially blocked due the adsorption of AuNpPANI and a progressive blockage was observed at each modification step. The AuNpPANI-ST system exhibited a highly selectivity response to the complementary target of human patient's dengue genome; hence, we suggest that they could be used for the development of dengue serotype biosensors that are functional even in the presence of small volumes and low concentrations of the analyte.

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