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Concanavalin A and polyvinyl butyral use as a potential dengue electrochemical biosensor

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ABSTRACT

Immobilization of concanavalin A on gold electrode by means of gold nanoparticles and polyvinyl butyral was carried out and investigated by cyclic voltammetry and electrochemical impedance spectroscopy. The system was tested with sera from patients infected by dengue fever (DF) and dengue hemorrhagic fever (DHF). Electrochemical impedance spectroscopy (in the frequency range from 100 mHz to 100 KHz), and cyclic voltammetry (from −0.2 to 0.7 V vs. Ag/AgCl), was performed in phosphate buffer solution containing 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe. As biomolecules accumulated on the electrode surface the voltammetric response changed from a clear diffusional to an irreversible behavior. Impedance spectroscopy showed a clear increase of the electron-transfer resistance when the sensor is exposed to contaminated sera (DF or DHF) as compared to exposure to uncontaminated serum (NDF). The results were analyzed through an equivalent circuit and values of charge transfer resistance and capacitance were obtained. Variations in charge transfer resistance were used to distinguish the sensor response for the different sera investigated (DF, DHF and NDF). Alternatively, a three-dimensional graph gave the best response for differentiation of all three blood sera. The distinctive patterns of impedimetric responses observed were ascribed to different glycoprotein patterns in the sera investigated. Therefore, the lectin immobilization on electrode surface with gold nanoparticles and polyvinyl butyral, combined with the three-dimensional impedance analysis introduced herein are valuable tools in the development of a biosensor for immunological response to diseases.

1. Introduction

Dengue fever is a mosquito-borne virus disease that has reached plague proportions in developing tropical countries (Gibbons and Vaughn, 2002; Guzman and Kouri, 2002). It is caused by a virus belonging to the genus *Flavivirus*, family *Flaviviridae*. The *Aedes aegypti* mosquito has been found to be the most common transmitter of this epidemic. The dengue virus causes more illness and death than any other arbovirus diseases.

The diseases caused by the dengue viruses can range from an acute febrile illness called dengue fever (DF) to more severe, life-threatening illnesses, called dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The latter has a higher mortality rate (Cardosa, 2000; Rothman and Ennis, 1999; Gluber and Meltzer, 1999). Infection with the virus does not provide cross-protective immunity. A patient who is re-infected by a different serotype of the dengue virus, is likely to develop DHF or DSS (Wu et al., 2005).

The dengue virus nonstructural glycoprotein, NS1, may present different molecular mass depending on glycosilation. Some data, suggest conflicting results for the use of this protein as potential DHF predictor (Lemes et al., 2005). On the other hand, the level of some cytokines and soluble vascular cell adhesion molecule 1 (VCAM-1) in blood samples, may indicate a pattern for DF and DHF recognition (Chen et al., 2007)

The methods currently available to cultivate the virus or to detect the specific antibody are both cumbersome and time consuming. The PCR method, although rapid for detection in the viremia phase, suffers from sample contamination and high technological support demands (Wu et al., 2005).

A few immunosensors for DF based on crystal quartz microbalance have appeared in the literature in recent years. For instance, the immunochip developed by Wu et al., based on monoclonal antibodies for the detection of dengue E and NS1 (Wu et al., 2005). However, the distinction between DF and DHF, in laboratory is still a demanding issue.

Lectins are glycoproteins of plant, animal or bacterial origin that bind to cell surfaces through specific carbohydrate containing receptor sites (Sharon and Lis, 2003). The specificity of lectins to

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carbohydrate moieties should enable the development of specific sensors for sugars. Lectins have been used to evaluate serum glycoproteins in disease detection (Qiu et al., 2007), to detect markers of various chronic inflammatory diseases, including immunoglobulins in diseases of autoimmune (Novak et al., 2001) or infectious nature (Moore et al., 2005).

Concanavalin A, a lectin from jack bean, is a well-studied protein with a well-characterized molecular structure and with many medical applications (Brück et al., 2001; Schaeffer et al., 1982). Its ability to interact with a majority of glycoproteins present in serum (Yang and Hancock, 2004) makes it an ideal prototype for glycoprotein detection. For instance, Liljeblad and collaborators have developed an immunosensor for glycoprotein detection on cellular culture suspensions (Liljeblad et al., 2002).

The use of metallic nanoparticles for immobilization of proteins has been widely reported. Gole et al. pointed out the high surface-to-volume ratio as the main advantage of such systems (Gole et al., 2001). As a result, a larger amount of protein immobilization, as well as increased electrochemical activity are observed. The immobilization of proteins, such as concanavalin A on gold nanoparticles are generally ascribed to electrostatic interactions. In order to optimize the immobilization process, Tang et al. have successfully used polyvinyl butyral (PVB) and gold nanoparticles in the development of a immunosensor (Tang et al., 2004). According to the authors, PVB entraps the protein-nanoparticle aggregate on the electrode surface.

The electrochemical interactions of ConA modified electrodes with carbohydrates and glycoproteins has been the subject of studies in our laboratory (Diniz and Ueta, 2004; Ueta and Diniz, 2008; Oliveira et al., 2008). Using gold nanoparticle and PVB to immobilize the protein on gold electrode, it was possible to demonstrate that the lectin retained its biological activity toward sugars and glycoproteins. Electrochemical impedance spectroscopy (EIS) has been proved to be a sensitive and effective method to probe the interfacial properties of modified electrode (Houa et al., 2006; Navratilova and Skaladal, 2004). Moreover, impedance spectroscopy can be widely used for monitoring biological reactions and disease detection (Liu et al., 2008; La Belle et al., 2007; Pei et al., 2005). In this context, we are presenting here the experimental approaches for evaluation of a biosensor response for serum glycoproteins from patients infected by dengue virus using electrochemical techniques, to observe the behavior of gold electrodes modified with ConA immobilized on gold nanoparticles, with the perspective of having different responses for the three kinds of sera investigated: DHF, DF, and NDF.

2. Experimental

2.1. Materials

Concanavalin A, bovine serum albumin (BSA, 99%), PVB, and $HAuCl_4{\cdot}3H_2O$ were purchased from Sigma Chemical (St. Louis, MO, USA), and sera from three patients each, infected by DF, DHF, and negative for dengue fever (ND) were obtained from Laboratório Central do Estado de Pernambuco (LACEN). All chemicals and solvents used were of analytical grade, and were used as received without further purification. Water used was obtained from a Milli-Q plus (Billerica, USA) purification system.

Electrochemical measurements were carried out on an EG&G PAR 263A potentiostat interfaced with a 5210 EG&G lock in analyzer controlled by a computer. A three-electrode setup was employed throughout with an Ag/AgCl (saturated KCl) reference electrode. All potentials are referred to this electrode. A gold wire and modified gold disc ($d = 2$ mm) were used as auxiliary and working electrodes, respectively.

2.2. Lectin immobilization

Colloidal gold was prepared according to the literature (Oliveira et al., 2008), by adding 2 mL of 1% (w/w) sodium citrate solution into 50 mL of 1.0 % (w/w) $HAuCl_4$ boiling solution. The absorption maximum of the synthesized colloidal gold in the UV–vis spectrum was at 527 nm, and the solution was stored in a refrigerator in a dark-colored glass bottle before use.

The gold disc electrode was first mechanically polished with 0.05 μm α-Al_2O_3 powder, and washed ultrasonically in distilled water. The bare electrode was charged positively by holding it at a potential of +1.5 V in a 0.1 M NaOH stirred solution for 2 min followed by +0.5 V for 45 s in the same medium (Oliveira et al., 2008). A sol–gel method (Oliveira et al., 2008) was employed to modify the electrode. ConA solution (200 μg/mL) was mixed with 300 μL of colloidal gold into a beaker (4 °C) and kept under stirring for 10 minutes. Thereafter, 3 mL of polyvinyl butyral PVB–ethanol solution (2%, v/v) was added to the beaker quickly. The electrode was dipped into the above solution for 30 min and then removed. Then, the AuNp–ConA–PVB modified electrode was incubated in a phosphate buffer saline (PBS) with 0.2% BSA for 20 min at 37 °C in order to block the remaining active sites.

2.3. Serum–lectin immobilization

Lectin modified electrode (AuNp–ConA–PVB–BSA) was rinsed with water to remove unbound lectin and exposed to serum from patients infected by DF, DHF, as well as NDF, diluted in 10 mM pH 7.4 PBS solution (1:30 dilution) for 10 min at room temperature (ca. 26 °C).

2.4. Electrochemical measurements

The impedance spectra were recorded in the frequency range of 100 mHz to 100 kHz. The amplitude of the applied sine wave potential was 10 mV, while the direct current (dc) potential was limited at the open circuit potential measured just before the application of the sine wave potential. Cyclic voltammetry (CV) was performed with sweep potential between +0.7 to −0.2 V with scan rate of 50 mV s^{-1}. CV and EIS measurements were performed in the presence of a 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) solution, used as a redox probe in PBS (pH 7.4) and were carried out at different stages of the preparation of the modified electrode. Measurements in triplicate were carried out for each kind of sera from one patient only.

3. Results and discussion

3.1. Characterization by TEM

Fig. 1 shows TEM micrographs of the AuNp–ConA exposed to sera from patients contaminated with dengue hemorrhagic fever (1a) and non-contaminated (1b). It is seen an agglomeration of the nanoparticles in the case of DHF which is ascribed to the interactions of ConA with glycoproteins present in the serum. It will be shown below, that these results are in agreement with those obtained by electrochemical measurements. At this point, however, it is possible to say that the TEM images strongly suggest that the ConA immobilized on AuNp has a somewhat specific response to glycoproteins present in DHF infected serum.

3.2. Electrochemical detection of DF, DHF and NDF sera at AuNp–ConA–PVB modified electrode

In a previous work, it was shown that this modified electrode had specific response to glycoproteins (Oliveira et al., 2008).

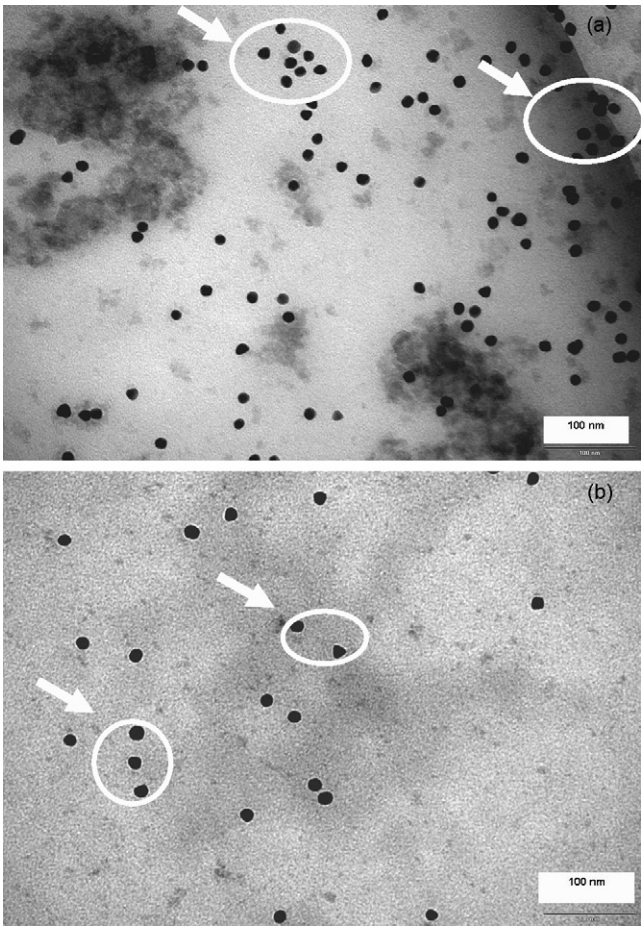

Fig. 1. Representative TEM images of the AuNp–ConA system: (a) exposed to serum from patient infected by dengue hemorrhagic fever; (b) exposed to serum from patient negative to dengue fever.

The resulted AuNp–ConA–PVB–BSA modified gold electrode, was obtained and further used as the sensing basis for electrochemical impedance and CV analysis of ConA–serum glycoproteins binding.

Fig. 2 shows the changes in voltammetric response of the electrode in PBS solution containing 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6$. As shown in this figure, the stepwise adsorption of different materials on the gold electrode is accompanied by a decrease in the amperometric response of the electrode and an increase in the peak-to-peak separation between the cathodic and anodic waves of the redox probe. The shapes of the curves vary from a clearly diffusional wave, towards an irreversible wave. Especially, after AuNp–ConA–PVB–BSA system interacted with DF serum, a clear disappearance of the current peaks was observed. This corresponds to a change from a clearly diffusion controlled process towards an electron-transfer controlled process. The main reason for this behavior is that the lectin–glycoprotein complex acts as an electron-transfer blocking layer, increasing charge transfer resistance.

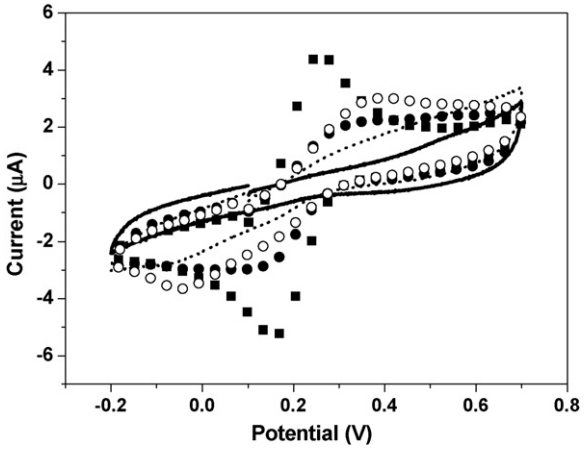

Fig. 2. Cyclic voltammograms at different stages of the electrode modification and exposure to sera: bare gold electrode (■), AuNp–ConA–PVB–BSA (○), AuNp–ConA–PVB–BSA–NDF (●), AuNp–ConA–PVB–BSA–DHF (□), AuNp–ConA–PVB–BSA–DF.

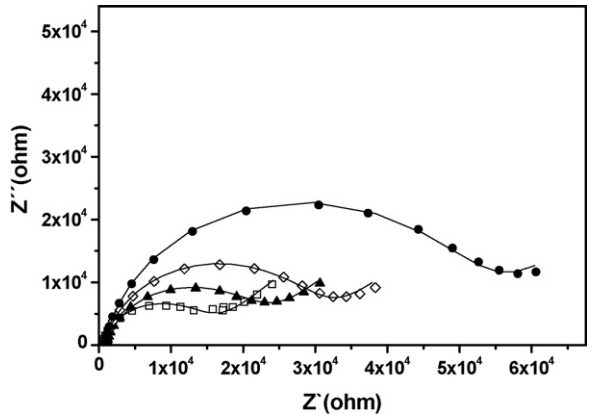

Fig. 3. Nyquist plot of the electrode/solution interface before and after exposure to different sera: AuNp–ConA–PVB–BSA modified electrode (□), AuNp–ConA–PVB–BSA–NDF (▲), AuNp–ConA–PVB–BSA–DHF (◊), AuNp–ConA–PVB–BSA–DF (●). Solid lines were obtained by fitting to equivalent circuit.

Fig. 3 shows the Nyquist plots for the modified electrode before and after exposure to different sera. The successive increase in diameter for the semi-circles in these plots, are related to the increase in charge transfer resistance for the redox probe, analogous to what could be seen in the cyclic voltammetric curves. Thus, the charge transfer resistance follows the sequence DF > DHF > NDF. A quantitative assessment of the charge transfer resistance can be obtained from the EIS data by a non-linear least squares fitting software (Boukamp, 1996). The equivalent circuit used in the fitting procedure is a modified Randles circuit largely used in analogous systems (for details of the circuit, see Oliveira et al., 2008). It consists essentially of a constant phase element, *Q*, in parallel with a faradaic impedance element (charge transfer resistance, R_{CT} in series with a Warburg impedance element). The quality of the fitting procedure (which yielded Chi squared values lower than 10^{-4}) can also be seen in Fig. 3. The fitting procedure yielded values of R_{CT}, *Q* and *n*. R_{CT} is the charge transfer resistance; *Q* and *n* are associated, respectively, with the reactance and dispersion of the constant phase element. Optimum serum dilution was found to be 1:30 (v:v).The results are given in Table 1. Since the aim of this study was to evaluate the capability of the modified electrode to distinguish the three kinds of sera investigated (DF, DHF, NDF), the data were handled according to two different procedures: via the relative variation in R_{CT} and through a three-dimensional plot of R_{CT} vs. *Q* vs. *n*.

For the relative change in charge transfer resistance the following equation was employed:

$$\Delta R_{CT}(\%) = \frac{R_{CT(after)} - R_{CT(before)}}{R_{CT(before)}} \times 100$$

where $R_{CT(before)}$ and $R_{CT(after)}$ are, respectively, the values of the charge transfer resistance of the electrode/solution interface before and after serum exposure. The results are given in Table 2. It can be seen that values of ΔR_{CT} are higher for sera contaminated with DF and DHF indicating the capability of ConA to recognize the glyco-

Table 1
Values of the equivalent circuit elements from fitted impedance results.

Modified electrode	R_{CT} (kΩ)	Q (μF)	n
AuNp–ConA–PVB–BSA	14.35	1.62	0.89
ConA system–DF1	33.80	3.94	0.79
ConA system–DF2	37.20	4.34	0.79
ConA system–DF3	38.90	4.54	0.78
ConA system–DF3,1*	33.79	2.99	0.80
ConA system–DF3,2*	38.34	3.42	0.80
ConA system–DF3,3*	43.73	3.59	0.79
ConA system–HDF1	32.00	2.72	0.85
ConA system–HDF2	29.10	2.47	0.86
ConA system–HDF3	27.86	2.36	0.86
ConA system–HDF1,1*	42.31	2.67	0.86
ConA system–HDF1,2*	37.01	2.35	0.86
ConA system–HDF1,3*	35.20	2.17	0.86
ConA system–DN1	19.22	1.63	0.88
ConA system–DN2	21.11	1.48	0.88
ConA system–DN3	20.19	1.55	0.88
ConA system–DN1,1*	19.51	1.67	0.88
ConA system–DN1,2*	21.15	1.68	0.88
ConA system–DN1,3*	20.34	1.58	0.88

DF/HDF/NDF 1–2–3 represents analysis of different patients and (*) their replicates.

Table 2
Relative charge transfer variation from impedance data.

Condition	Before	After DF	After HDF	After NDF
R_{CT} (kΩ)	14.3	37.6	33.9	20.2
ΔR_{CT} (%)	–	162.2 ± 21.3	136.3 ± 30.9	41.1 ± 4.6

protein pattern of these samples. However, the higher ΔR_{CT} values obtained for DF than for DHF sera are not statistically different on a 90% confidence level. Nonetheless, it can be said that ΔR_{CT} is a straightforward way of interpreting the impedance data.

The other way to handle these data is based on the observation that the three variables, R_{CT}, Q and n, seemed to have characteristic values for each serum type (DF, DHF, and NDF). Therefore, a three-dimensional plot with these parameters was prepared and is shown in Fig. 4. It is clear that the data are distributed in the plot, according to the serum type they belong to. Moreover, the data for DHF and DF sera are a little more scattered than the data for NDF. The NDF sera results are located in the region of lower R_{CT} and Q values, and of higher n values, which corresponds to a smaller blocking effect of the biosensor surface associated with smaller capacitive dispersion. In other words, there is little agglutination of glycoproteins from NDF samples, on the electrode surface. The DHF sera results are distributed at intermediate values of R_{CT} and n and higher values of Q, indicating intermediate dispersion and blocking of the biosensor surface. Finally, the data for DF sera are located at higher values of R_{CT}, and lower values of both n and Q. These are indication of larger blocking effect and smaller capacitive dispersion, resulting from higher agglutination of glycoproteins from the DF sera. The virtue of this 3-D graphical analysis is to display at once a clear separation of all three cases investigated. A numerical equivalent of this graph may be obtained by a statistical multivariate treatment. Since the number of samples is too small, such a treatment was not carried out.

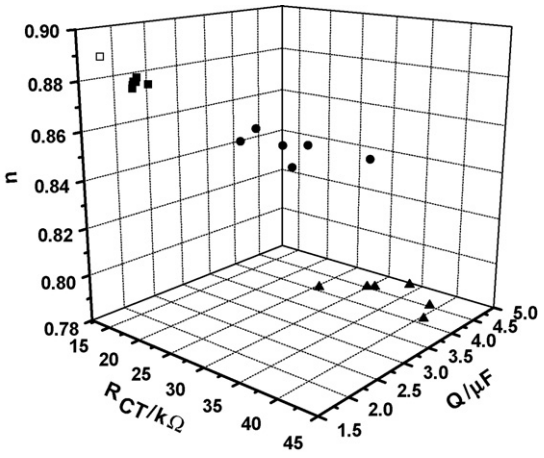

Fig. 4. Three-dimensional plot for values of R_{CT}, Q, and n given in Table 1. AuNp–ConA–PVB–BSA (□), AuNp–ConA–PVB–BSA–NDF (■), AuNp–ConA–PVB–BSA–DHF (●) and AuNp–ConA–PVB–BSA–DF (▲).

The ConA recognition for dengue sera may be attributed to differing patterns of glycoproteins in the sera. It is known that DHF contaminated serum demonstrates a higher expression of plasmatic glycoproteins like NS1, ICAM-1; cytokines IFNα and IFNγ; and VCAM-1, involved in hemorrhagic events (Hober et al., 1993; Chen et al., 2007). The higher levels of glycoproteins may explain the higher charge transfer resistances and capacitances shown by the DF and DHF samples. To understand the higher blocking effect indicated by the DHF samples, as compared to DF, is not a simple task. One possible explanation is the high level of VCAM-1 associated with DHF, as mentioned above. The presence of sialic acid in a terminal position of this protein plays important role in its interaction, with a consequent inhibition of agglutination with other biomolecules (Acheson et al., 1991; Abe et al., 1999). It is also known that ConA agglutination reaction is inhibited by the presence of sialic acid containing proteins (Rosangkima et al., 2008).

In summary, the variable response of the modified electrode allowing a differentiation among DHF, DF, and NDF is likely to be due to ConA capacity for detecting distinct glycosylation patterns. Since changes in metabolism and diseases revels different glycosilation in secreted glycoproteins by cells (Raju et al., 2000), this modified electrode may be useful as a biosensing system for disease diagnosis. The patterned response displayed in a three-dimensional graph may allow the distinction among sub-classes of an immunological response, such as the one found in Dengue virus contamination. Carbohydrate variations are of great interest as clinical markers for detecting disease in biological samples (Orntoft and Vestergaard, 1999). Although useful for predicting and monitoring the course of the disease once diagnosed, the detection of unusual carbohydrate structures using lectins, has proved possible for recognition of abnormal glycoproteins in blood serum (Sell, 1990).

4. Conclusions

In the present work, we have introduced a novel approach for fabrication of a biosensor based on the modification of electrode surface with nanoAu–ConA–PVB. Electrochemical impedance technique was used to investigate the interaction between ConA lectin and sera from patients infected by DF and DHF. The electrochemical response of a redox probe system ($K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$) was effective as a signal for the presence of agglutination reactions on the electrode. The resulting electrode exhibited different interactions to DHF, DF, and NDF. Variations in relative charge transfer resistance and a graphical analysis of the impedance data, trough R_{CT}, Q and n, allowed a clear separation of the results obtained with differentiation among DF, DHF, and NDF. This kind of electrode and data analysis, have a potential application as a biosensor for the recognition of different patterns of glycoproteins in blood serum.

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