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Impedimetric immunosensor for electronegative low density lipoprotein (LDL⁻) based on monoclonal antibody adsorbed on (polyvinyl formal)–gold nanoparticles matrix

Maria D.L. Oliveira a, Dulcinéia S.P. Abdalla b, Daniel F. Guilherme b, Tanize E.S. Faulin b, Cesar A.S. Andrade c, * 

ABSTRACT

Monoclonal antibodies (MAb) have been commonly applied to measure LDL in vivo and to characterize modifications of the lipids and apoprotein of the LDL particles. The electronegative low density lipoprotein (LDL⁻) has an apolipoprotein B-100 modified at oxidized events in vivo. In this work, a novel LDL⁻ electrochemical biosensor was developed by adsorption of anti-LDL⁻ MAb on (polyvinyl formal)–gold nanoparticles (PVF–AuNPs)-modified gold electrode. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used to characterize the recognition of LDL⁻. The interaction between MAb–LDL⁻ leads to a blockage in the electron transfer of the [Fe(CN)₆]⁴⁻/[Fe(CN)₆]³⁻ redox couple, which may could result in high change in the electron transfer resistance (R_CT) and decrease in the amperometric responses in CV analysis. The compact antibody–antigen complex introduces the insulating layer on the assembled surface, which increases the diameter of the semicircle, resulting in a high R_CT, and the charge transferring rate constant kₐ decreases from 18.2 × 10⁻⁶ m/s to 4.6 × 10⁻⁶ m/s. Our results suggest that the interaction between MAb and lipoprotein can be quantitatively assessed by the modified electrode. The PVF–AuNPs–MAb system exhibited a sensitive response to LDL⁻, which could be used as a biosensor to quantify plasmatic levels of LDL⁻.

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1. Introduction

The increased concentration of low density lipoprotein (LDL) cholesterol is a major risk factor for coronary heart disease (CHD) [1]. In addition, CHD is one of the leading causes of mortality not only in the developed world but also in some developing countries [2]. Most LDL pathogenicity becomes manifest after LDL oxidation [3]. Oxidized LDL (oxLDL), but not native LDL, induces endothelial vascular cell adhesion molecule 1 (VCAM1) expression in the presence of tumor necrosis factor-alpha (TNFα) [4]. It has been suggested that oxLDL is important in the development of atherosclerotic lesions and elevated markers of oxidation may be considered as emerging plasma biomarkers for the prediction of atherothrombotic events [5]. Thus oxidized LDL may play a key role in the pathogenesis of atherosclerosis [6].

A native form of LDL containing intermediated modified subfractions with higher electronegative charge, referred to as LDL⁻, has been identified and characterized [7]. LDL⁻ is a minimally modified LDL and contains oxidative modifications, similar to those of the well-described in vitro oxidatively modified LDL, including increased negative charge, increased content of conjugated dienes, thiobarbituric acid reactive substances (TBARS), and a decreased content of vitamin E [8,9]. Some authors [10] suggest that LDL⁻ may contribute to atherogenesis via several mechanisms, including its proinflammatory, proapoptotic and antiangiogenesis properties.

Monoclonal antibodies (MAb) have been commonly applied to measure oxidized LDL in vivo and to characterize modifications of the lipids and apoprotein of the LDL particles [11]. However, these antibodies show a poor specificity for LDL⁻ and are directed at highly modified particles. Although, Abdalla et al. [12] by immunizing mice with LDL⁻ isolated from human plasma using a HPLC, achieved 3D1036 MAb which binds to LDL⁻ and does not bind to native LDL. This MAb can be used for detecting LDL⁻ in rabbit, human plasma and atherosclerotic lesions [13]. A specific, simple, sensitive, and highly selective method for LDL⁻ detection is therefore highly desirable.

Im mobilizations of antibodies on different substrates have been widely used in many fields, such as environmental immunoassay,
diagnostic immunoassay, biochemical studies and immunosensors [14]. As the formation of biomaterial layers, organic compounds and macromolecules on conductive surfaces may block interfacial electron transfer, the latter can be monitored by different electrochemical techniques.

Electrochemical impedance spectroscopy (EIS) has long been established as a sensitive technique for monitoring the electrical response of a solid/liquid system subjected to the application of a periodic small amplitude AC signal. Analysis of the system response provides information on the solid/liquid interface and the possible occurrence of reactions at this local region [15,16]. On the other hand, cyclic voltammetry (CV) measurements are the current signals based on the electrochemical species consumed and/or generated during a biological and chemical interaction process of a biologically active substance and substrate [17].

The binding reaction of LDL at the MAb-immobilized surface is often insufficient to produce a large signal change for EIS measurements. To overcome this shortcoming, MAb molecules were immobilized by the self-assembled technique on the gold electrode modified colloidal gold and polyvinyl formal (PVF) as matrices via the large specific surface area and high surface free energy of gold nanoparticles (AuNPs), and the entrapped effect of PVF in the present paper. PVF has served primarily as microporous support matrices for the recognition element. The use of synthetic polymers provides several advantages such as higher mechanical strength, chemical resistance and the option to use complexing buffer components [18]. In addition, sol–gel chemistry provides an attractive, versatile, and simple route for the development of electrochemical biosensors.

In this paper we describe the development of an immunosensor that uses a MAb (3D1036) modified PVF–AuNPs electrode that relies on the sensitiveness of EIS and CV signals for LDL− detection with accuracy and specific detection. To our best knowledge, we believe we have shown the first time an impedimetric immunosensor for LDL−. To determine the ability of the MAb in recognizing LDL−, our assay was based on registering the differences in the biosensor responses in the absence and presence of these molecules in the testing media. The modified electrodes were characterized using EIS, CV and scanning electron microscopy (SEM).

2. Experimental

2.1. Materials

Polyvinyl formal (PVF) was obtained from SPI supplies (West Chester, PA, USA). Bovine serum albumin (BSA), fetuin, human serum albumin (HSA) and HAuCl₄·3H₂O were purchased from Sigma Chemical (St. Louis, MO, USA). Potassium ferri- and ferricyanide were obtained from VETEC (Brazil). All chemicals and solvents were of analytical grade and were used as received, without further purification. The water used was obtained from a Milli-Q water. A sol–gel method was employed to modify the electrode colloidal gold into a beaker (4°C) and, subsequently, another 500 g/mL polyvinyl formal–chboroform solution (0.1%, v/v) was added and kept under stirring for 10 min. After the electrode was incubated in PBS solution for 5 min and air-dried, it was ready for use in the LDL− detection investigation.

2.2. Apparatus

Electrochemical measurements were carried out on a PGSTAT 302N potentiostat (Autolab, Eco Chemie, The Netherlands) interfaced with an analyzer controlled by a computer. A three-electrode setup with an Ag/AgCl (saturated KCl) reference electrode was employed throughout the investigation. All potentials are referred to this electrode. A platinum wire and a modified gold disc (d=2 mm) were used as auxiliary and working electrodes, respectively. The scanning electron microscopy images were obtained from a JSM 5900 (JEOL Instruments, Japan) at an acceleration voltage of 5 kV and a working distance of 5 μm [19].

2.3. LDL− purification

MAb and LDL− were obtained as described in [20]. Briefly, male isogenic mice Balb/c 8 weeks of age received an intraperitoneal injection of 0.5 mL of culture medium containing 2 × 10⁶ hybridoma cells secreting anti-LDL− MAb. After 7 days, the ascitic fluid containing anti-LDL− MAb was collected and purified in a protein G column (GE Healthcare, Uppsala, Sweden). The concentration of MAb was determined by UV absorption at 280 nm (Beckman DU640 spectrophotometer, Beckman Instruments, Fullerton, CA, USA), using a molar extinction coefficient of 1.3 M⁻¹ cm⁻¹. The LDL fraction was separated by ultracentrifugation of human blood plasma from volunteers. LDL− was isolated from LDL by FPLC (BioLogic Duo Flow, Bio-Rad Laboratories Inc., Hercules, CA, USA) using an ion exchange column (Sepharose UNO Q-12, Bio-Rad Laboratories Inc.) and eluted with a gradient consisting of 20 mM TRIS, pH 7.4 (pump A) and 20 mM TRIS + 1 M NaCl pH 7.4 (pump B). The eluent was monitored by UV at 280 nm and the LDL− collected peak was visualized by agarose gel electrophoresis. The concentration of LDL− was determined by the Lowry method [21], using BSA as standard.

2.4. Preparation of colloidal gold

Colloidal gold was prepared according to the literature [22] by adding 2 mL of 1% (w/w) sodium citrate solution to 50 mL of 1.0% (w/w) HAuCl₄ boiling solution. The absorption maximum of the synthesized colloidal gold in the UV–vis spectrum was at 527 nm (data not shown), and the solution was stored in a refrigerator in a dark-colored glass bottle before use.

2.5. Preparation of PVF–AuNPs matrix

First, the gold disc electrode was mechanically polished with 0.05 μm α-Al₂O₃ powder and washed ultrasonically in distilled water. A sol–gel method was employed to modify the electrode [23,24]. 50 μL MAb solution (445 μg/mL) was mixed with 100 μL of colloidal gold into a beaker (4°C) and, subsequently, another 500 μL polyvinyl formal–chloroform solution (0.1%, v/v) was added and kept under stirring for 10 min. After the electrode was incubated in the above solution for 5 min and air-dried, it was ready for use in the LDL− detection investigation.

2.6. PVF–AuNPs–MAb–BSA–LDL−–modified gold electrode

The MAb modified electrode PVF–AuNPs–MAb (Fig. 1a) was rinsed with water to remove unbound protein, which after this modified electrode was put into contact with BSA solution (10 mM in phosphate buffered saline (PBS) pH 7.4) to obtain PVF–AuNPs–MAb–BSA system in order to block unspecific sites and eliminate the nonspecific binding effect. Next, this modified electrode was put into contact with BSA solution (10 mM in phosphate buffered saline (PBS) pH 7.4) to obtain PVF–AuNPs–MAb–BSA–LDL− system. To determine the ability of the MAb in recognizing LDL−, our assay was based on registering the differences in the biosensor responses in the absence and presence of these molecules in the testing media. The modified electrodes were characterized using EIS, CV and scanning electron microscopy (SEM).

2.7. 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. CV was performed with a potential...
sweeping between +0.7 and −0.2 V at a scan rate of 50 mV s⁻¹. CV and EIS measurements, performed in the presence of a 10 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] (1:1) solution used as a redox probe in PBS (pH 7.4), were carried out at different stages of the preparation of the modified electrode. All electrochemical measurements were performed at room temperature and inside a Faraday cage.

3. Results and discussion

3.1. PVF–AuNPs–MAb SEM characterization

The SEM technique was employed to confirm the fabrication process of the biosensor. Fig. 1b shows an image of the PVF–AuNPs-modified electrode and non-aggregation can be visualized. The PVF–AuNPs–MAb system possessed a heterogeneous system adsorption on the electrode surface (Fig. 1c). The PVF–AuNPs–MAb–LDL⁻ system demonstrated a biomolecular interaction based on antibody–antigen binding interactions, resulting in the preferential formation of clusters (Fig. 1d). These results were confirmed in CV and EIS experiments.

3.2. Electrochemical characteristics of PVF–AuNPs–MAb–BSA

Cyclic voltammetry experiments confirmed that the AuNPs and monoclonal antibody specific to LDL⁻ (MAb) were successfully adsorbed onto the gold electrode surface. When the electrode surface had been modified a number of materials, the electron transfer kinetics of [Fe(CN)₆]⁴⁻/K₄[Fe(CN)₆]³⁻ redox reactions was perturbed [25]. The free active sites of the biosensor were blocked with BSA to prevent LDL⁻ from nonspecific adsorption. The electrochemical characteristics of [Fe(CN)₆]⁴⁻/K₄[Fe(CN)₆]³⁻ on the modified electrodes are shown in Fig. 2a. Curve a represents the electrochemical response of the redox probe [Fe(CN)₆]⁴⁻/K₄[Fe(CN)₆]³⁻ on the bare gold electrode, curve b is the PVF-modified gold electrode and curve c is the PVF–AuNPs–MAb–BSA-modified gold electrode.

As can be seen, the assembly by the PVF and MAb on the gold electrode leads to a decrease in the amperometric response of the electrode and a slight increase in the peak-to-peak separation between the cathodic and anodic waves of the redox probe (Fig. 2a, curve c).

Antibody immobilization leads to a considerable decrease in the peak current and an increase in the potential separation between the cathodic and anodic peaks of the redox probe. This illustrates that the immobilization of antibody insulates the electrode and perturbs the interfacial electron transfer considerably. It can be observed in curve c that in low concentrations there is no interaction between MAb and LDL⁻. Curves d and e demonstrate the interaction between MAb and LDL⁻ (PVF–AuNPs–MAb–BSA–LDL⁻) at different concentrations (Fig. 2b). Moreover, at high concentrations the current anodic and cathodic peaks are seen to disappear as a result of the antibody–antigen interactions. In addition, the electron transfer of [Fe(CN)₆]⁴⁻/K₄[Fe(CN)₆]³⁻ redox couple was severely blocked, owing to the negative charge carried by the components of LDL⁻.

3.3. Detection of MAb and MAb–LDL⁻ interaction by impedance spectroscopy

EIS is an effective method for examining the interfacial properties of the sensor surface during the process of successive modifications. In EIS, the semicircle diameter of EIS equals the charge transfer resistance, R_CT. This resistance controls the electron transfer kinetics of the redox-probe at the electrode surface (Fig. 3). Impedance measurements were performed...
in the frequency range from 0.1 mHz to 100 kHz. Complex impedance plots of bare gold electrode (curve a), PVF-modified gold electrode (curve b), PVF–AuNPs–MAB–BSA–HSA (curve c), PVF–AuNPs–MAB–BSA–Fetuin (curve e), PVF–AuNPs–MAB–BSA–CBSS (curve f) and PVF–AuNPs–MAB–BSA–LDL (curve g) are shown in Fig. 3a. It can be seen that the bare gold electrode exhibits an almost straight line that is characteristic of a diffusional limiting step of the electrochemical process. It was found that there was no significant difference between the PVF–AuNPs–MAB–BSA, PVF–AuNPs–MAB–BSA–HSA and PVF–AuNPs–MAB–BSA–Fetuin diagrams, implying that blood proteins, such as HSA and fetuin, had no effect on the PVF–AuNPs–MAB–BSA system (Fig. 3a, curves c and e). Experiments on clinical blood serum samples from patients without previous LDL purification were conducted to demonstrate the ability of the PVF–AuNPs–MAB–BSA system to run a serodiagnostic test. Our results show that it is possible to detect LDL which is present in the human serum samples (Fig. 3a, curve f).

Fig. 3b shows the EIS responses of interaction between MAB and LDL concentrations. A gradual increase can be observed in the $R_{CT}$ at higher LDL concentration (curves b–f). These results indicated a sensing interaction and selective recognition of MAB through an increase in the electron transfer resistance value.

The impedance spectra follow the theoretical shapes and include a semicircle portion, observed at high frequencies, which corresponds to the electron transfer. The respective semicircle diameters at the high frequency, corresponding to the electron transfer resistance at the electrode surface, increase with the stepwise formation of a modifier to the electrode surface. In accord with our results, the general equivalent electric circuit (Randles) was used to fit the impedance spectroscopy. The Randles circuit [24] includes the ohmic resistance of the electrolyte solution ($R_{e}$), the Warburg impedance ($W$) resulting from ion diffusion from the bulk electrolyte to the electrode interface, the phase constant element ($Q$), and the charge-transfer resistance ($R_{CT}$), the existence of which is recognized when the electrolyte solution contains a redox probe.

In the PVF–AuNPs–MAB–BSA system, $R_{e}$ and $W$ denote, respectively, the bulk properties of the electrolyte solution and the diffusion features of the redox probe in the solution. The impedance data were fitted using the modified Randles equivalent circuit (see inset in Fig. 3b). This was found to suitably fit the data, yielding more detailed information on the impedance behavior of MAB. The Randles model [24] showed a good agreement that can be observed over the entire measurement frequency range.
The phase constant element changes are not as sensitive as the electron transfer resistance, which is one of the most directive and sensitive relations with the electron transfer rate. Thus, the change transfer resistance was used as the sensor signal. At high frequencies the semicircle demonstrates heterogeneous charge transfer kinetics, according to the equations below:

\[ R_{CT} = \frac{RT}{nFz_0C} \]  
\[ z_0 = nF^2\alpha_0C^2 \]

where \( R \) is the gas constant, \( T \) is the temperature, \( n \) is the electron transferring constant of the redox couple, \( \alpha \) is as for \( [\text{Fe(CN)}_6]^{4-}/[\text{Fe(CN)}_6]^{3-} \), \( F \) is Faraday current between the gold electrode and the redox couple, \( A \) is the area of gold electrode, \( \alpha \) is the charge transfer rate constant and \( C \) is the concentration of the redox couple. The charge transfer rate constant was obtained from the above equations. The curve simulated by the equivalent circuit is preferably close to the experimental impedance plots, which indicates that the equivalent circuit is accurate. The diameters of the semicircle represent the resistance of the layers and it is usually assumed that the electron transfer of \( [\text{Fe(CN)}_6]^{4-}/[\text{Fe(CN)}_6]^{3-} \) redox probe is blocked by the formation of highly organized layers on the electrode surface because these redox species do not penetrate the layers into the conductive electrode.

The compact antibody–antigen complex forms an insulating protein layer on the electrode surface, which increases the diameter of the semicircle, signifying a high electron transfer resistance. The diameter becomes larger and the charge transfer rate constant \( \alpha \) decreases in the following order: bare gold electrode, PVF-modified gold electrode, PVF–AuNPs–MAB–BSA-modified gold electrode and PVF–AuNPs–MAB–BSA–LDL–modified electrode (Table 1). \( \alpha \) decreased from 18.2 \( \times \) 10⁻⁶ m/s to 4.6 \( \times \) 10⁻⁶ m/s.

AC impedance results for the modified electrodes are listed in Table 1. This table shows the value of \( R_{CT} \) for each step. The electron transfer resistance of a bare gold electrode (curve a, Fig. 3b) is 0.32 kΩ, which is very small compared with the values of \( R_{CT} \) after the following stages of the construction of the electrode. This is in agreement with the fact that the electron transfer process on the bare gold surface is very rapid, and the immobilization of MAb results in an increase in \( R_{CT} \) (0.42 kΩ, curve b in Fig. 3b), by generating a PVF and an insulating protein layer on the electrode surface.

After the immune reaction of the immobilized electrode, there is a significant increase in \( R_{CT} \) from 0.54 kΩ to 1.64 kΩ at different LDL concentrations (curves c–f, Fig. 3b), reflecting an immune detection that leads to a very significant increase in mass on the electrode surface. The sequence of measurements for the pure PVF, PVF–AuNPs–MAB–BSA and for the PVF–AuNPs–MAB–BSA–LDL–modified electrodes is consistent with the occurrence of progressive blockage of the interface, confirming that the amount of material immobilized and/or adsorbed on the electrode surface directly correlates with the impedance.

With the present results, comments on the constant phase element parameters (\( Q \) and \( n \)) would be very speculative. The data for PVF–AuNPs–MAB–BSA–LDL at different concentrations showed a higher \( R_{CT} \) and \( Q \) values than the results in the absence of LDL. This is an indication effect of the LDL presence on the electrode surface contributes to the blocking effect of the biosensor surface associated with higher capacitive dispersion. However, some remarks should be made about the variation of \( n \). This parameter is close to one in most experiments, and close to 0.5 mainly for the MAB systems at low concentrations of LDL. This is an indication some diffusional aspect of the charger transfer process in the interface. In others words, besides bulk diffusional mass transport limitations, there is some diffusional limitations inside the material immobilized on the electrode surface. The bulk mass transport is accounted for by the Warburg impedance in the equivalent circuit, and appears in the low frequency part of EIS results.

The results presented in Fig. 3b clearly show that MAB remains capable of recognizing the LDL, as revealed by the increase in \( R_{CT} \). In general, the LDL conductivity is lower, so that small amounts of LDL on the electrode surface may result in substantial changes in \( R_{CT} \) [26]. Hence the performance of the modified electrode for detection of lipoprotein may be evaluated through the relative variation of this parameter (\( \Delta R_{CT} \)), according to the equation:

\[ \Delta R_{CT} (%) = \left( \frac{R_{CT(MAB)} - R_{CT(LDL^-)}}{R_{CT(MAB)}} \right) \times 100 \]
LDL\(^{-}\) concentration was found, ranging from 3.50 to 8.75 \(\mu g/mL\), an indication that the interactions between MAb and LDL\(^{-}\) can be detected by the modified electrode. In general, the reactivity of many antibodies produced against oxidized LDL decreases with cleavage of hydroporphic domains, and by fragmentation and aggregation of Apo-B. This MAb seems to recognize epitopes in the minimally oxidized LDL\[13\].

The performance of the PVF–AuNPs–MAb–BSA system is shown in Table 2, for a fixed LDL\(^{-}\) concentration, thereby confirming once again the ability of MAb to recognize LDL\(^{-}\) lipoprotein after adsorption. We draw call attention to the fact that the relative value to \(\Delta R_{CT}\) for LDL\(^{-}\) at low (28.6 \%) and high concentrations (290.0 \%) demonstrates the recognition capability of MAb. The detection limit of the immunosensor was 3.50 \(\mu g/mL\) lipoprotein. The PVF–AuNPs–MAb–BSA system detected a LDL\(^{-}\) concentration about 7.14 \(\mu g/mL\) (\(\Delta R_{CT} = 183\%\)) in the CBSS. In addition, the concentration of LDL\(^{-}\) in the CBSS is in the detection limit of the biosensor. Therefore, LDL\(^{-}\) was successfully detected in blood serum of normolipidemic subjects. Similar results were obtained by Damasceno et al.\[13\] detecting LDL\(^{-}\) from human blood plasma using ELISA and immunohistochemistry. These results are a strong indication that this system can be applicable to the construction of a biosensor for LDL\(^{-}\) levels of human blood serum.

These results demonstrate that after the adsorption of anti-LDL\(^{-}\) MAb on gold electrode, the remaining recognition capability can- not lead to a structural antibody modification. LDL\(^{-}\) has a higher \(\beta\)-strand and unfolds with a lower \(\alpha\)-helical structure, which may occur irrespective of oxidation when compared to native LDL\[27\]. The interaction between MAB-LDL\(^{-}\) is due to the recognition of epitopes in a modified Apo-B\[13\]. The apolipoprotein B-100 (apo-B100) modifications observed in LDL\(^{-}\) are related to a reduction in free amino groups due to their reaction with aldehydes formed by lipoperoxidation\[28\]. Among several studies to detect LDL\(^{-}\) in plasma, enzyme linked immunosorbent assays (ELISA) have been conducted to demonstrate the presence of LDL\(^{-}\) in the plasma of normolipidemic and hypercholesterolemic rabbits and humans. However, the detection of LDL\(^{-}\) in blood plasma by ELISA takes a great deal of time. We therefore demonstrated the importance of using electrochemical analysis to detect LDL\(^{-}\) with a good sensitivity and reproducibility. The PVF–AuNPs–MAb–BSA system can be used to detect LDL\(^{-}\) associated with the atherosclerotic process in the vascular intima.

4. Conclusions

In the present study the PVF–AuNPs–MAb–BSA-modified electrode has been used to fabricate a novel biosensor, combining the sensitivity and the versatility of electrochemical impedance spectroscopy and cyclic voltammetry. An LDL\(^{-}\) electrochemical biosensor was constructed by immobilizing anti-LDL\(^{-}\) monoclonal antibody on a PVF–AuNPs-modified gold electrode. The activity and stability of the immobilized MAb were maintained. It has been demonstrated that the PVF–AuNPs–MAb–BSA system can be used to detect the interaction of MAB–LDL\(^{-}\). The PVF–AuNPs–MAb–BSA may provide a new electrochemical platform for designing bioelectrochemical sensors of high sensitivity.

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**References**


Biographies

Dr. Maria Danielly Lima de Oliveira is Doctor of chemistry (PhD) from University Federal of Pernambuco (Recife, Brazil). She joined in the Oakland University as a postdoctoral research fellow. She is currently professor in departmental section of biochemistry, Center of Biology. Her research interests are electrochemistry, biosensors and lectins.

Dr. Dulcineia Saes Parra Abdalla graduated in pharmacy in 1978 at the University of São Paulo (USP). In 1988 she received the PhD in biological sciences from the USP. She joined in the University of Southern California as a postdoctoral research fellow. She is currently professor in Faculty of Pharmacy/USP. She has experience in hyperlipidemia, peroxidation and atherosclerosis.

Daniel Ferreira Guilherme is graduated in pharmacy at the Catholic University of Santos. His current research interests are concentrated on biosensors and lipoproteins.

Dr. Tanize do Espírito Santo Faulin is graduated in biomedicine. In 2010 she received the PhD in pharmacy at the University of São Paulo (USP). Her current research interests are concentrated on biosensors, lipoproteins and peroxidation.

Dr. César Augusto Souza de Andrade received his PhD in materials science from University Federal of Pernambuco (Recife, Brazil) in 2006. He joined in the Oakland University as a postdoctoral research fellow. Since 2006, he has been with UFPE University as professor. Currently, his research activities are focused on the development of biomolecular interfaces, biological sensor systems and novel nanomaterials for sensor applications.