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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,*]

[a] Departamento de Bioquímica, Universidade Federal de Pernambuco, 50670-901 Recife, PE, Brazil
[b] Departamento de Análises Clínicas e Toxicológicas, Universidade de São Paulo, 05508-900 São Paulo, SP, Brazil
[c] Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, 55608-680 Vitória de Santo Antão, PE, Brazil

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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 an (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 $[Fe(CN)_6]^{4-}/K_4[Fe(CN)_6]^{3-}$ 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 κ^0 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⁻.

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 atherotrombotic events [5]. Thus oxidized LDL may play a key role in the pathogenesis of atherosclerosis [6].

A native form of LDL containing intermediately 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.

Immobilizations of antibodies on different substrates have been widely used in many fields, such as environmental immunoassay,

* Corresponding author. Tel.: +55 81 3523 3351; fax: +55 81 3523 3351.
E-mail address: csrandrade@gmail.com (C.A.S. Andrade).

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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 $HAuCl_4{\cdot}3H_2O$ were purchased from Sigma Chemical (St. Louis, MO, USA). Potassium ferri- and ferrocyanide 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 plus (Billerica, USA) purification system. Clinical blood serum samples (CBSS) were collected in EDTA tubes, and plasma was isolated by centrifugation ($2000 \times g$ for 15 min).

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 2×10^6 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 (Bio-Logic 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) $HAuCl_4$ 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-Al_2O_3 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 system was exposed for 10 min to different LDL^- solutions, which were previously diluted in 10 mM PBS solution (pH 7.4), so as to obtain the final LDL^- concentrations in the 3.50 μg/mL to 175 μg/mL range. All the above procedures were performed at room temperature (ca. 26 °C).

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