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A fully integrated electrochemical biosensor platform fabrication process for cytokines detection

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ABSTRACT

Interleukin-1b (IL-1b) and interleukin-10 (IL-10) biomarkers are one of many antigens that are secreted in acute stages of inflammation after left ventricle assisted device (LVAD) implantation for patients suffering from heart failure (HF). In the present study, we have developed a fully integrated electrochemical biosensor platform for cytokine detection at minute concentrations. Using eight gold working microelectrodes (WEs) the design will increase the sensitivity of detection, decrease the time of measurements, and allow a simultaneous detection of varying cytokine biomarkers. The biosensor platform was fabricated onto silicon substrates using silicon technology. Monoclonal antibodies (mAb) of anti-human IL-1b and anti-human IL-10 were electroaddressed onto the gold WEs through functionalization with 4-carboxymethyl aryl diazonium (CMA). Cyclic voltammetry (CV) was applied during the WE functionalization process to characterize the gold WE surface properties. Finally, electrochemical impedance spectroscopy (EIS) characterized the modified gold WE. The biosensor platform was highly sensitive to the corresponding cytokines and no interference with other cytokines was observed. Both cytokines: IL-10 and IL-1b were detected within the range of 1 pg mL^{-1} to 15 pg mL^{-1}. The present electrochemical biosensor platform is very promising for multi-detection of biomolecules which can dramatically decrease the time of analysis. This can provide data to clinicians and doctors concerning cytokines secretion at minute concentrations and the prediction of the first signs of inflammation after LVAD implantation.

1. Introduction

Heart failure (HF), often called congestive heart failure (CHF) or congestive cardiac failure (CCF) occurs when the heart is unable to provide sufficient pump action to maintain blood flow to meet the needs of the body (Gullestad et al., 2012; Nymo et al., 2014; Slaughter et al., 2010). HF can cause a number of symptoms including shortness of breath, leg swelling, and exercise intolerance. Facing the difficulty of obtaining a sufficient number of donor organs, several bioelectronic devices such as pressure sensors and left ventricle assisted devices (LVADs) (Rohde et al., 2013) have been implanted into the patients' body in order to help the patient's heart and facilitate the continuation of patients' life until such a donor becomes available. The problem of organ biocompatibility as a direct cause to implanted LVADs can trigger increased pro- and anti-inflammatory cytokine levels (IL-10, IL-1, IL8, TNF-a, etc.) (Caruso et al., 2010; Kaptoge et al., 2014). This is amongst the principle origins of HF detection. Prior to LVAD implantation, Stumpf et al. (Stumpf et al., 2003) have reported on IL-10 plasma cytokine levels for 50 HF patients at 2.3 ± 1.9 pg mL^{-1}, while controls were measured at 5.2 ± 2.3 pg mL^{-1} ($P < 0.01$). In the same principle, Caruso et al. (Caruso et al., 2010) have reported on an exhaustive study for cytokines detection within survivors and non-survivors of 23 LVAD implanted patients. The collected data quantified exactly when patients were prone to higher levels of inflammation after cardiac surgery. Here, the authors have shown an early expression of human IL-10 and IL-1 peaked after LVAD implantation, when compared with other plasma samples analyzed within the 30 day period for survivors and non-survivors.

The standard technique for the detection of cardiac cytokines is based upon enzyme-linked immunosorbent assay (ELISA) (Navarri et al., 2015; Soman et al., 2011). Although this technique is highly sensitive and accurate, it is still time-consuming, requires specialized personnel, and requires at least 5 mL of sample for analysis. To overcome these problems, a highly sensitive biosensor and bio lab-on-chip were developed for the detection of the specific biomolecules (Benlarbi et al., 2012; Mazher-Iqbal and Desmulliez, 2011; Pui et al.,

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2011). These biosensors provide fast, real-time, and reliable medical diagnosis. This in turn, enhances the biomarkers detection at minute concentrations, thus, allowing the prediction of the first signs of inflammation. Several analytical analyses and techniques have been investigated for each developed biosensor, for e.g., optical, electromechanical, and electrochemical biosensors (Qureshi et al., 2012). These latter are widely reported as almost 50% of the biosensors used in the literature are based on electrochemical transducers (Dorothee et al., 2008; Kongsuphol et al., 2014). Previously, we have reported on electrochemical biosensors for TNF-α cytokine detection (Baraket et al., 2011). Here, the analyses were made by electrochemical impedance spectroscopy (EIS) onto a biocompatible and flexible material. This electrochemical technique is considered extremely sensitive to the weak variation changes that occur on the biosensor's surface. In the same principle, we have also reported on the detection of IL-10 by EIS using a new material substrate within complementary metal oxide semi-conductor (CMOS) technology (Lee et al., 2012). Here, the detection limit of IL-10 was 0.1 pg mL^{-1}. This can provide to clinicians, the first signs of cytokine secretion after LVAD transplantation.

The biofunctionalization of biosensors used mostly in the literature are based on self-assembled monolayers (SAMs) or cross-link to immobilize bioreceptors (Baraket et al., 2011; Baraket et al., 2016; Chebil et al., 2010; Hou et al., 2006; Prats-Alfonso et al., 2006). Although these techniques are highly adequate to support different techniques of detection, they remain limited by non-selectivity during biofunctionalization of an array of electrodes, for e.g., dropping the device in thiolated capture probe solution where thiol is adsorbed onto all working electrodes (WEs). To overcome this problem, an alternative technique was used in the literature to locally biofunctionalize microelectrodes. This is very important for multi-detection using several WEs in the same device (B. P. Corgier et al., 2005; Baraket et al., 2013; Corgier et al., 2007; Polsky et al., 2008).

In the present work, we report on the production of an electrochemical biosensor platform for multiple cytokine detection. This biosensor platform contains eight gold WEs allowing simultaneous and multiplex detection of different cytokines through electrically addressable diazonium-functionalized monoclonal antibodies (mAb's). Furthermore, this biosensor platform contains an integrated silver/silver chloride (Ag/AgCl) reference microelectrode (RE) and a platinum counter microelectrode (CE). The WEs, RE, and CE were fabricated onto a silicon substrate using silicon technology. Cyclic voltammetry (CV) was applied during the microelectrode functionalization process to confirm the mAb's immobilization and to characterize the gold microelectrodes surface properties. Finally, EIS characterization was applied onto the gold WEs for human IL-10 and IL-1b detection by the corresponding immobilized mAb, respectively.

2. Experimental

2.1. Reagents

4-carboxymethyl aryldiazonium(CMA), sodium nitrite ($NaNO_2$), hydrochloricacid (HCl) 37%, isopropyl alcohol (IPA) 96%, potassium chloride (KCl), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC), potassium ferrocyanide(II), potassium ferricyanide(III), and phosphate buffered saline (PBS) were purchased from *Sigma–Aldrich*, France. The immunoreagents: interleukin-10 (IL-10), interlukin-6 (IL-6), interleukin-1b (IL-1b) and mAb mouse anti-human IL-10/IL-6/IL-1b were purchased from *R & D Systems*, France.

2.2. Antibodies and cytokine preparation

Antibodies and cytokines were diluted in PBS buffer, aliquoted, and stored at −20 °C following the protocol of the supplier. The cytokines; IL-1, IL-6, and IL-10 were aliquoted before EIS measurements at different concentrations from 1, 5, 10, and 15 pg mL^{-1} respectively. These were stored at 4 °C. The antibodies; anti-IL-10 and anti-IL-1b were aliquoted at 10 mg mL^{-1}.

2.3. Instrumentation

The electrochemical experiments were carried out with a VMP3 multichannel potentiostat (Biologic-EC-Lab, France). CV technique was applied for diazonium-modified mAb deposition and EIS measurements were made to study the sensitivity and selectivity. All experiments were measured at room temperature. The modeling of the obtained EIS data was achieved by the EC-Lab software using the Randomize + Simplex method. Here, randomize was stopped on 100,000 iterations and the fit stopped on 5000 iterations.

2.4. Microelectronics technology

The devices were fabricated on 100 mm diameter silicon wafers. The metallic microelectrodes are isolated from the silicon substrate by a dielectric SiO_2 layer. It has a thickness of 800 nm and was grown by wet thermal oxidation. To fabricate the gold microelectrodes, a tri-layer of Ti (100 nm), Ni (150 nm), and Au (50 nm) was deposited by physical vapor deposition (PVD). The Ti layer ensures good adhesion to the SiO_2 and the Ni layer avoids intermixing of the Ti and Au. The microelectrode geometry was defined by photolithography and wet chemical etching. Next, the platinum microelectrodes were fabricated by PVD deposition of a metal bilayer of Ti (15 nm) plus Pt (150 nm), which was patterned by photolithography and a lift-off process. This was followed by the deposition of a dielectric passivation layer, obtained by plasma-enhanced chemical vapor deposition (PECVD) of SiO_2 (400 nm) plus Si_3N_4 (400 nm). The passivation layer was eliminated from the microelectrode areas and the contact pads by photolithography and dry reactive ion etching. Finally, silver for the reference electrodes was deposited by PVD as a bilayer of Ti (15 nm) plus Ag (150 nm) and patterned by photolithography plus lift-off. Fig. 1 shows the final image of the developed microelectrodes. Here, the chip integrates eight WEs in gold against a platinum CE and a silver RE.

Fig. 1(a) shows a topographic image of the chip taken by an optical 3D profilometer. The gold microelectrodes are detailed with a good definition. Measurements of electrical conduction showed the qualities of the metals deposited. The qualities of these metals play an important role in the process of chemical surface treatment and biofunctionalization of the chip after production.

The wafer was diced and the devices were glued to a printed circuit board (PCB) using an epoxy resin (Epo-Tek $H^{70}E-^{2}LC$, from *Epoxy Technology*) (Fig. 1b). Afterwards, the microelectrode pads were connected to the PCB board through aluminum wire (25 μm Ø) by wire-bonding (Kulicke & Soffa 4523 A) (Fig. 1c). Finally, the bonding area of the device, the bonding wires, and the gold tracks of the PCB were encapsulated using the same resin (Epo-Tek $H^{70}E-^{2}LC$) to protect them from the buffer or electrolyte solution (Fig. 1d). This resin is totally inert for any chemical reaction with buffers, electrolyte solution or other solvents, for e.g., acetone and ethanol.

2.5. Diazonium-antibody immobilization onto gold WEs

Before the electroaddressing of the CMA-modified mAb, the chip surface was cleaned with ethanol in an ultrasonic bath for 10 min and dried under nitrogen flow. The device was then placed under UV/O_3 for 20 min in order to remove all organic contaminants. After the cleaning procedure, the chip was incubated in 20 mg mL^{-1} of CMA-modified mAb's solution. Therefore, carboxyl diazonium (CMA) was previously covalently attached to the mAb's (Fig. 2(step1)) by EDC/NHS cross-linking with both at 0.1 M following the protocol used by P. Corgier et al. (B. P. Corgier et al., 2005). The CMA coupled to the mAb's was