Cytokine detection using diazonium modified gold microelectrodes onto polyimide substrates with integrated Ag/AgCl reference electrode

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Abstract

In this present study, we have analyzed one cytokine biomarker; interleukin-10 (IL-10), which is one of many antigens that is secreted in acute stages of inflammation after left ventricle assisted device (LVAD) implantation for patients suffering from heart failure (HF). Therefore, a biosensor was developed in order to increase the sensitivity of detection, decrease the time of measurements, and to simultaneously detect varying cytokine biomarkers using eight gold working electrodes (WE). This biosensor was fabricated onto a polyimide (PI) substrate using soft lithography technique. The anti-human IL-10 monoclonal antibodies (mAb) were immobilized onto gold microelectrodes through functionalization with carboxyl diazonium. Cyclic voltammetry (CV) was applied during the microelectrode fabrication process to characterize the gold microelectrode surface properties, while, electrochemical impedance spectroscopy (EIS) characterized the diazonium modification made on the gold microelectrodes, followed by the immobilization of IL-10 mAb. Here, a variation of the change transfer resistance (Rct) has confirmed that diazonium has modified the gold microelectrodes and that the mAb was successfully covalently bonded onto the surface.

1. Introduction

Cardiovascular disease (CVD) is one of the most increasing causes of death in western countries. Facing the difficulty of having a sufficient number of donor organs, several device-based therapeutic approaches

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roused the interest of scientific researchers in this field. However, the problem of multiple organ failure syndrome (MOFS) as a direct cause to implanted left ventricle assisted devices (LVADs) for heart failure (HF) patients is amongst the principle causes of patient mortality after cardiac surgery [1]. MOFS is influenced by the triggering of increased pro- and anti-inflammatory cytokine levels and accurate detection of an early inflammatory response has led to a growing interest in new point-of-care biological sensors that are capable of sensitive and specific detection of biomolecules at this early phase [2]. A large study was investigated in order to evaluate the first signs of inflammation and/or sepsis markers using biosensors for HF monitoring in LVAD patients. Recently we have reported the detection of tumour necrosis factor-α (TNF-α) that is another biomarker at weak concentrations [3], and IL-10 detection based on novel hafnium oxide (HfO₂) substrates [4]. Therefore, there is an increasing requirement for fast, real-time, and reliable medical diagnosis that can aid the clinician in administering therapeutic intervention before the onset of MOFS develops.

Here, we report in the present work, a method to manufacture a biosensor for multiple cytokine detection. This biosensor contains eight gold working microelectrodes (WE) which allows for the simultaneous detection of different cytokines through electrically addressable diazonium-immobilized monoclonal antibodies (mAb). Furthermore, this biosensor contains an integrated silver/silver chloride (Ag/AgCl) reference microelectrode (RE) and a gold counter microelectrode (CE). Microelectrodes were manufactured on polyimide (PI) substrate by soft lithography. Cyclic voltammetry (CV) was applied during the microelectrode fabrication process to characterize the gold microelectrode surface properties. Finally, electrochemical impedance spectroscopy (EIS) was used for observation of bare gold, diazonium modified gold, and immobilization of interleukin-10 (IL-10) through covalent bonding with diazonium.

2. Microelectrode fabrication

Gold microelectrodes were fabricated through a combination of soft lithography tools such as microcontact printing (μCP), self assembled monolayers (SAMs), and replica molding [5]. Therefore, an elastomeric stamp based on polydimethylsiloxane (PDMS) was fabricated by replica molding. Here, a mixture of PDMS and curing agent (10:1, w/w) was poured onto a silicon mold which contained microelectrodes engraved onto its surface which were transferred onto PDMS stamp surface. Afterwards, the PDMS stamp was inked with octadecanethiol (ODT) and brought immediately into conformal contact with the gold substrate on (PI). Here, a SAMs was formed onto the gold layer due to the high affinity sulfur has to gold and this monolayer acts as mask to protect the gold patterned by the structured PDMS stamp. Finally, after wet etching the gold microelectrodes were fabricated on the PI substrate (Fig. 1(I)).

![Fig. 1 (I): Eight gold WE on a PI substrate with Ag/AgCl integrated RE, and a gold CE, and (II): CV for the rejuvenation of the gold electrodes, where: (a) bare gold, (b) modified ODT gold (no heating), (c) modified ODT gold (3 min heating), (d) modified ODT gold (6 min heating), and (e) modified ODT gold (10 min heating).]
CV measurements were made in order to check if the ODT was successfully removed from all gold surfaces. All measurements were made by an external RE (Ag/AgCl) and a platinum (Pt) CE using a multichannel potentiostat (Biologic-EC-Lab VMP3) analyzer. The analysis was carried out using phosphate buffer saline (PBS) at pH 7.4 containing a mixture of antibody nitrate (AgNO₃) (5 mM) and ferricyanide (Fe(CN)₆³⁻) (5 mM). The electrodes were scanned at a rate of 100 mV/s from -0.4 to 0.6 mV. Fig. 1(II) illustrates the variation of CV in function of the gold electrode treatment by ODT, where, Fig 1(II)a shows the CV for bare gold. Then, after ODT treatment of the electrode, a considerable decrease in the amperometric response as a peak to peak separation between the cathodic and anodic waves of the redox probe was observed (Fig 1(II)b). This signifies that the entire gold electrode surface was covered by ODT and this prevented consequently the oxido/reduction reaction of ferro/ferricyanide with gold. Afterwards, the gold electrode was heated at 250°C on hotplate for 3, 6, and 10 min (Fig 1(II)c, d, and e) and then washed abundantly with deionized water. Therefore, the bonding between the thiol groups from ODT and the gold were made by its high affinity, and consequently the raised temperature with increased time has enabled the chemical bonds to break and thus allow the tracing back to the bare gold surface (Fig 1(II) e).

3. Reference electrode

Ag was deposited onto the fabricated gold electrode in order to formulate an integrated RE. Here, Ag was modified onto the RE by applying a redox potential of 0.3 to -0.5 mV with a scan rate of 20 mV/s for 16 s. The applied buffer used for silver deposition was formed with sodium nitrate (NaNO₃) (1 M) and silver nitrate (AgNO₃) (25 mM) at pH 1. Finally, the deposited Ag layer was chlorinated by incubation in hydrochloric acid (HCl) (1 M) solution overnight. This formulated an integrated Ag/AgCl RE (Fig. 1(I)).

4. Antibody immobilization on microelectrodes

The fabricated chip contains eight gold WE. This will enable eight different types of cytokine Ab to be selectively immobilized onto each gold WE. For this interest, carboxyl diazonium was electro-addressed to the different gold WE by applying CV (3 cycles) from 0.3 V to -1.4 V with a scan rate of 200 mV/s in a solution of ferricyanide (5mM) and potassium chloride (KCl) (0.1M) at pH 7.4. Afterwards, the mAb was covalently immobilized to the functionalized diazonium through N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N’-ethyl-carboimide hydrochloride (EDC) cross-linking with both concentrations made to 0.1 M [6].

5. Characterization by EIS measurements

Characterization of bare gold, diazonium modified gold, and immobilization of IL-10 mAb was made by EIS measurements. The analysis was observed by applying the same redox probe solution (Fe(CN)₆³⁻/⁴⁻, 5mM) previously described in section 2. The measurements were made in the frequency range of 100 mHz – 100 kHz, using a modulation voltage of 25 mV, and a fixed potential at -0.2 mV. The modelling of the obtained data was made by the software EC-Lab. All experiments were measured at room temperature and the EIS measurements were stable throughout the scanning process.

In Fig. 2, we observe a Nyquist impedance plot for IL-10 mAb immobilization though diazonium electro-addressing. The first semi-circle of the Nyquist plot corresponds to the bare gold microelectrodes (■). Then, after diazonium functionalization onto microelectrodes (●), the semicircle of the Nyquist plot raised up from the bare gold showing an increase of transfer charge resistance (Rct) [7]. Finally, after incubation of microelectrodes in a solution of IL-10 mAb (10 mg/mL) for one hour, the semi-circle continued to increase showing the augmentation of the Rct and confirming the immobilization of this antibody. The same principle of the Rct increasing was made for IL-10 cytokine detection, and the range of IL-10 detected was from 1pg/mL to 15pg/mL. Here, by EIS measurements we have successfully shown the immobilization of IL-10 mAb by electro-addressing, followed by detection of human IL-10 to the corresponding IL-10 mAb.
Fig. 2: Nyquist plot of an EIS measurement demonstrating surface modification of: (■) bare gold, (●) Diazonium modified gold, and (▲) IL-10 mAb immobilization.

6. Conclusion

In the present work, we have shown the feasibility of manufacturing a biosensor that is sensitive and capable of multiple simultaneous detections. Several combinations of soft lithography were investigated to manufacture this biosensor, such as μCP, replica molding, and SAMs. These techniques are generally very cost effective when compared to those used in classic lithography. The immobilization of mAb by electroaddressing opens the possibility to detect different cytokines at the same time. The developed biosensor will be used in the future as a chip for a small wearable potentiostat in order to detect several biomarkers secreted after LVAD implantation. This will provide critical information to the clinician after only a few hours post surgery and thus predict early post-implant release of inflammatory cytokines.

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