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Highly sensitive Electrochemical BioMEMS for TNF-α detection in humansaliva: Heart Failure

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Abstract

Prediction of disease progression using saliva as a diagnostic medium has roused the interest of scientific researchers in the 10 last past years. Potentially important biomarkers are increased in saliva during local and systemic inflammation. In the present study we have developed a highly sensitive biosensor for TNF-α detection in human saliva of patients suffering from heart failure. Therefore, a fully integrated electrochemical BioMEMS was developed in order to increase the sensitivity of detection, decrease the time of analysis, and to simultaneously detect varying cytokine biomarkers using eight gold working microelectrodes (WE). The monoclonal antibodies (mAb) anti-human Tumor Necrosis Factor alpha (anti-TNF-α) were immobilized onto gold microelectrodes through functionalization with carboxyl diazonium. Cyclic voltammetry (CV) was applied during the microelectrode functionalization process to characterize the gold microelectrode surface properties. Finally, electrochemical impedance spectroscopy (EIS) characterized the modified gold microelectrodes, and the detection range of TNF-α cytokines was from 1pg/mL to 15 pg/mL.

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1. Introduction

Heart failure (HF) is a condition where the heart fails in its duties of circulating blood through the lungs and back out to the tissues. Diagnosis of acute rejection is a complex and persistent problem in heart and ventricular assisted device (VAD) transplantation. To address this problem, measuring specific biomarkers can produce immediate information about the first signs of inflammation [1]. A wide spectrum of compounds (e.g. TNF-α cytokines) present in saliva may provide information for clinical diagnostic applications. Saliva is a good medium because its collection is not invasive and the donation process is relatively without stress. In recent years, different techniques have been used for the detection of protein biomarkers [2]. However, a majority of these techniques require adequate transducing elements, for instance fluorescent dyes or enzymes, to generate a signal which give rise of this interaction event. Increasing need for a fast, real-time and reliable medical diagnosis has led to growing interest in new point-of-care biological sensors capable for the sensitive and specific detection of biomolecules. For this interest we report in the present work on the fabrication of an electrochemical BioMEMS for multiple cytokine detection. This BioMEMS contains eight gold working microelectrodes (WEs) allowing a simultaneous detection (Fig. 1c). Cyclic voltammetry (CV) was applied during the microelectrode functionalization process to confirm the mAb immobilization and to characterize the gold microelectrode surface properties. Finally, EIS characterization was applied onto the gold WEs for TNF-α detection by the corresponding immobilized antibodies anti-TNF-α. The BioMEMS was highly sensitive to TNF-α within the range 1-15 pg/mL when compared to other interferences in artificial human saliva.

2. Experimental

2.1. Chemical and Reagent

Reagents used in this research are: 4-aminophenylacetic acid (4-carboxymethylaniline CMA), sodium nitrite ($NaNO_2$), hydrochloricacid (HCl), Ethanol, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), phosphatebuffered saline (PBS), phosphatebuffered saline Tween (PBSTween) and ($Fe^{2+}Fe^{3+}$) were purchased from Sigma Aldrich. Antibodies Tumor necrosis factor TNF-α, Recombinant Human TNF-α, Recombinant Human IL-10, Recombinant Human IL-8 and Recombinant Human IL-1, Antibodies TNF-α with fluorescence were purchased from R&D system France.Artificial saliva has been prepared by dissolving 0.6 g/L Na_2HPO_4, 0.6 g/L anhydrous $CaCl_2$,0.4 g/L KCl, 0.4 g/L NaCl, 4 g/L mucin and 4 g/L urea in deionized water, adjusted to pH 7.2, sterilized by autoclaving and stored in the refrigerator until use.

2.2. Antibodies and cytokine preparation

Antibodies and cytokines have been diluted in PBS buffer, aliquoted, and stored at −20°C following the protocol of the supplier. The cytokines TNF-α, IL-1 and IL-8 have been aliquoted before EIS measurements at different concentrations from 1, 5, 10 and 15 pg/mL respectively and stored at 4°C. For the detection in artificial saliva, the cytokine TNF-α has been diluted directly in artificial saliva.

2.3. Fluorescence microscopy

Fluorescence images were taken using a fluorescence micro-scope (Zeiss Axioplan 2 Imaging apparatus). Samples were observed by fluorescence light: Fluorescein, excitation was made with a 470 (±40) nm band-pass filter and fluorescence was observed with a 525 (±50) nm band-pass filter (Fig. 1a).

2.4. *Bio-Functionalization of Gold Surface*

Before functionalization, the surface of biosensor's WEs has been pre-cleaned by sonication for 10 min in acetone

followed by rinsing with ethanol, copious amount of deionized water, and finally it has been cleaned for 30 min under UV-ozone in order to remove all organic contaminations. To promote adhesion of the antibodies,CMA molecules has been electrochemicaly deposited onto gold WEs by using cyclic voltammetry (CV) technique. Fig. 1 shows cyclic voltammogramme performed in $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (5 mM) in phosphate buffered saline (PBS, pH 7.4)for gold WE before and after CMA deposition. The scan rate was measured at 80 $mV.s^{-1}$ and the switching potential was scanned between -0.3 to 0.6 mV. The oxidoreduction peaks of bare gold have totally desapeared after CMA depostion due to the weak electron transfer kinetics of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$caused by the CMA blocking layer.To immobilize the antibodies on the electrode surface, carboxylic acid groups of CMA molecules has been activated by incubation in EDC(0.4M)/NHS(0.1 M) in ethanol solution for 1 hour at room temperature. Afterward, the device has been washed with HCl 0,1M to remove the excess of EDC/NHS and immediately incubated in PBS solution containing TNF-α antibodies at 10µg/mL for 1hr at room temperature. Afterward,the remained active carboxylic acid groups have been deactivated with ethanol amine solution (1% in PBS) for 20 minutes at room temperature. This step is very important to prevent nonspecific bonding at the detection stage.

2.5. Sandwich of antibodies: optical and electrochemical characterization

The optical characterization has been made to test the correct biofunctionalization of the biosensor surface: therefore a proper antibodies adhesion onto WE and subsequently a proper antigens/antibodies bond. The biomolecules linked onto the WE surface have been checked performing an optical characterization by usingfluorescence microscope. The sandwich of antibody was composed by a primary antibody, antigen (TNF-α) and secondary antibody with a fluorescein fluorochrome. Therefore, the antibodies anti-TNF-α were immobilized onto WE as we have previously described. Afterward, the device has been incubated in artificial saliva containing TNF-α cytokines at 10pg/mLfor 30 minutes at 4°C, to saturate all the antibodies. The BioMEMS was then rinsed with PBS in order to remove the excess of TNF-α cytokines and incubated afterward at different concentrations of secondary antibodies anti-TNF-α from (1 pg/mL, 25 pg/mL, 2.5 ng/mL and 0.25 µg/mL) at 4°C.

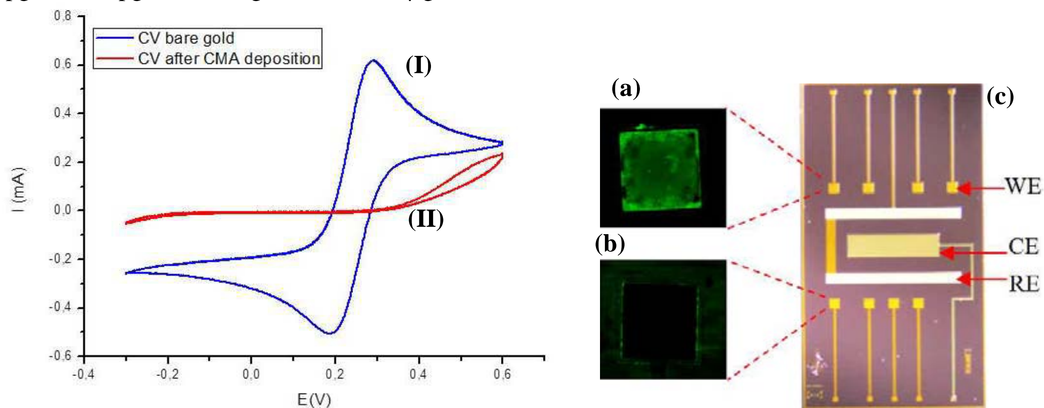

Fig. 1.Left : Cyclic voltammertry for (I) Bare gold WE(II) CMA modified WE. **Right**: Fluorescence optical images of (a) anti-TNF-α/TNF-α modified WE after incubation in secondary fluorescent TNF-α (b) no functionalized WE and (c) optical image of the fully integrated BioMEMS.

As expected, the surface of the nobiofunctionalized gold WE does not present any fluorescence when compared with anti-TNF-α modified WE (Fig. 1a and b). This confirms that we have successfully formed the sandwich antibody-antigen-antibody which confirm the biorecognition phenomenon.

2.6. Detection and Interference

To show measurement repeatability and to prove the sensitivity, the biosensor has been tested many times. After each incubation of the biofunctionalized WE in secondary antibody solution, the EIS measurements have been also performed to confirm the bonding of this secondary antibodies and to validate thus the optical test. For each concentration, the biosensor has been incubated for 30 minutes at 4°C(Fig. 2A). Here Nyquist plot semi-circles

increase by increasing the concentration of secondary antibodies anti-TNF-α which highlight the good sensitivity of the biosensor. In order to study the selectivity, the BioMEMS has been tested for other cytokines secreted in acute stage of inflammation.

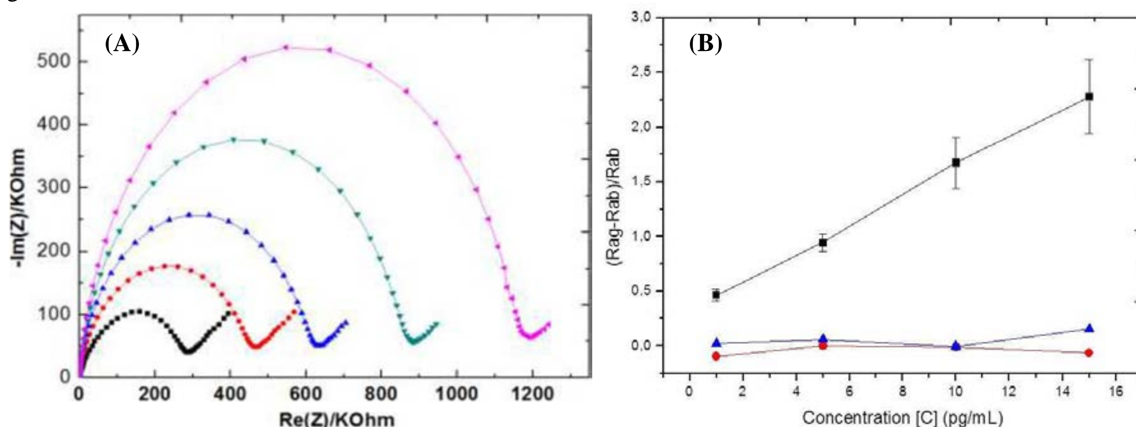

Fig. 2. **(A)** Nyquist impedanceplot (Zr vs Zi: at 5mM of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ in PBS pH7,4 solution) at various concentrations of secondary antibodies ani-TNF-α: (■) 0pg- mL; (●) 1pg/mL;(▲) 5pg/ mL; (▼) 10pg/ mL; (◀) 15pg/mL. **(B)** Sensitivity curves of the BioMEMS functionalized with (■)AbTNF-α for TNF-α antigen (●)AbTNF-α for the IL-1 antigen. (▲)AbTNF-α for the IL-8 antigen.

Not all these cytokines are present in saliva; however they have been used only to prove the selectivity of BioMEMS. To this end, the same previous procedure of biofunctionlization has been performed using other cytokines: IL-1 and IL-8 instead TNF-α. Here the BioMEMS was highly selective and sensitive to TNF-α cytokines when compared to other interferences IL-1 and IL-8 (Fig. 2B) which confirm the high selectivity of the BioMEMS.

3. Conclusion

A label-free, highly sensitive, accurate, fast EIS based BioMEMS for detection of TNF-α has been developed in the present study.A method to produce highly sensitive and specific biosensor for the detection of TNFα from diluted physiologic medium has been described in this research. The level of interferences attributable to non-specific binding was very minimal as a good selectivity was observed in presence of other inactive cytokines (IL-1 andIL-8).This BioMEMS is a promising bio-analytical tool for accurate quantification of TNFα when applied with commercial cytokines. Moreover, it would be important to be able to use saliva as a diagnostic test using the present electrochemical BioMEMS for heart disease in order to predict the first signs of inflammation..

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