Electrochemical Immunosensor for NT-proBNP Detection in Artificial Human Saliva: Heart Failure Biomedical Application [†]

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Abstract: Monitoring of circulating N-terminal proBNP (NT-proBNP) biomarkers is crucial for the diagnosis of people suffering from heart failure (HF). In this work, we describe a novel ultra-sensitive NT-proBNP immunosensor for NT-proBNP detection in artificial human saliva. The surface of the developed immunosensor based on gold working microelectrodes (WEs) was biofunctionalized through carboxyl diazonium to immobilize anti-NT-proBNP antibodies. The chemical surface modification of WEs was carried out by cyclic voltammetery CV whilst the quantification of NT-proBNP biomarkers was made by electrochemical impedance spectroscopy (EIS). The immunosensor has demonstrated a linear detection response within the range 1–20 pg/mL for NTproBNP detection in artificial human saliva with a good selectivity in the presence of other interferences.

Keywords: EIS; immunosensor; NT-ProBNP; heart failure

1. Introduction

Heart failure (HF) is a clinical syndrome where in cardiac output is insufficient to meet the metabolic demands of the body. This syndrome caused by a wide range of cardiovascular disorders, such as structural or functional abnormalities of the heart [1]. The early and quick diagnosis of HF is extremely important and crucial not for only patient survival but also saving cost and great deal of time in successful prognosis of the diseases. Existing methods of diagnosis for HF rely heavily on classical methods which are based on tests conducted in central laboratories that may take several hours or even days from when tests are ordered to when results are received [2]. To solve this problem, biosensors can play an important role in the early diagnosis without having to rely on hospital visits where expensive and time-consuming laboratory tests are recommended [3] one of the most commonly used biomedical approach for detection heart failure is determining the level of valuable specific biomarkers which are generated in different stage. Saliva analysis can represent a

very interesting biological matrix which has high potential for the surveillance of general health and disease, potentially important biomarkers are increased in saliva during local and systemic inflammation. These biomarkers are being detected through different techniques [4]. In the present study we have developed a fully integrated biosensor platform (Figure 1A) for N-terminal prohormone of brain natriuretic peptide (NT-proBNP) detection in artificial human saliva. The monoclonal antibodies (mAb) anti-NT-proBNP were immobilized onto gold WE 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) was used to characterize the modified gold microelectrodes as well as for the detection of NT-proBNP. The immunosensor has been used in artificial saliva and has demonstrated good sensitivity.

2. Materials and Methods

2.1. Chemical and Reagent

4-aminophenylacetic acid (4-carboxymethylaniline CMA), sodium nitrite ($NaNO_2$), hydrochloricacid (HCl) 37%, ethanol, N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine, phosphate buffered saline (PBS), phosphate buffered saline Tween (PBS Tween) and (Fe^{2+}/Fe^{3+}) were purchased from Sigma Aldrich (France). Antibodies N-terminal prohormone of brain natriuretic peptide (NT proBNP), NT proBNP were purchased from R&D System (France).

2.2. Artificial Saliva Preparation

Artificial saliva (AS) 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 by adding NaOH, sterilized by autoclaving and stored at −4 °C until use [5,6]. Antibodies and antigens were diluted in PBS buffer (pH 7.4), aliquoted at 5 mg/mL and 50 μg/mL respectively, then stored at −20 °C according to the protocol provided by the supplier.

2.3. NT-proBNP Detection in Artificial Saliva (AS)

Standard solutions used to detect NT-proBNP in AS were prepared by dissolving the appropriate amount of the stock solution (50 μg/mL) in AS, obtaining solutions with different concentrations of NT-proBNP (50, 60, 70, 80, 90 pg/mL).

2.4. Bio-Functionalization of Gold Surface

The biosensor platform (four working electrodes) has been pre-cleaned by sonication for 10 min in acetone, followed by rinsing with ethanol then deionized water, and dried with stream of nitrogen. Finally, the device has been cleaned for 30 min under UV-ozone to remove all organic contamination. Subsequently CMA molecules have been electrochemically deposited onto gold WEs by using cyclic voltammetry (CV) technique [5,6]. The biosensor platform was then rinsed and the terminal carboxylic acid (–COOH) groups of CMA were activated in a solution of NHS/EDC (0.1 M/0.4 M) for 1 h at room temperature. After rinsing with ethanol, the biosensor platform has incubated for 1 h in a 0.4 mg/mL solution of anti NT-proBNP antibodies. The terminal amine groups of the antibody forms amid bonding with the activated carboxylic functions of CMA. Finally, after rinsing with PBS, the biosensor platform has incubated for 20 min in ethanolamine. This blocks the remaining active carboxylic acidic groups to prevent nonspecific bonding at the detection stage.

2.5. Electrochemical Measurements

A multichannel potentiostat (Biologic-EC-Lab VMP3) analyser was used for CMA electrodeposition, CV, and EIS measurements. All experiments have been carried out at room temperature (20 ± 2 °C) in a Faraday box. CV measurements were used for WEs characterization using

$K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as analyte at 5 mM in PBS buffer at pH 7.4. This technique was also used for CMA deposition. Briefly; a 3 mM CMA solution was prepared in water with 1 M of HCl and 1 M $NaNO_2$. The solution was directly used to perform CMA electrodeposition. Ten CV cycles were applied to the biosensor platform inside the CMA solution. This was sufficient to cover all WEs surface with CMA. To evaluate the recognition properties of the biosensor platform in terms of sensitivity and selectivity, electrochemical impedance spectroscopy (EIS) measurements were used.

Data acquisition and analysis were accomplished using EC-Lab software. The device was then used to detect NT-proBNP in AS within the range 50 to 90 pg/mL. For analyte testing, the biosensor was subjected to successive incubations with standard solutions containing different concentrations of Ag NT-proBNP for 30 min at 4 °C for each incubation, followed by PBS washing. The impedance response was recorded for each concentration by immersing the biosensors in an electron mediator solution of 5 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS buffer at pH 7.4.

3. Results and Discussion

3.1. Characterization of the Functionalized Gold Surface

After thorough cleansing and functionalization, the surface of biosensor's WEs was characterized by CV at 80 mV/s and the switching potential was scanned between −0.2 to 0.6 mV). The CV was recorded before and after the CMA deposition, the results are shown in (Figure 1B). The Fe^{2+}/Fe^{3+} redox peaks have decreased after the CMA deposition. This can be attributed to the decrease of the electron transfer rate that was created by the CMA blocking layer.
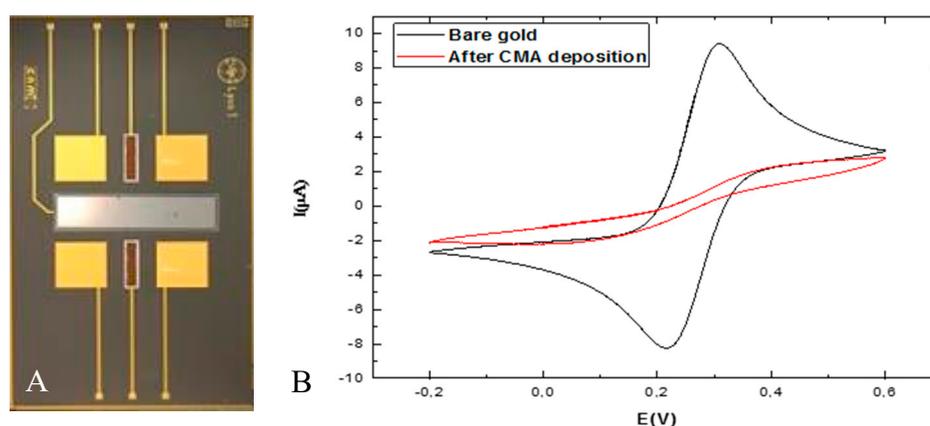

Figure 1. (**A**) Biosensor platform containing four gold WE, reference electrode (RE) based on Ag/AgCl and counter electrode (CE) based on platinum (**B**) Cyclic voltammograms of gold WE before (black) and after (red) CMA deposition.

3.2. Detection of NT-proBNP in Artificial Saliva

AS was used to simulate human real saliva and was prepared as described in the experimental section. Before detection process, the biosensors was tested for nonspecific adsorption. Firstly, the Ab-NT-proBNP modified biosensor was subject of several incubated for 30 min in AS without any NT-proBNP biomerkers. After each incubation, the biosensor was analyzed with EIS (Figure 2A). The first Nyquist plot semi-circle corresponds to the immobilized AbNT-proBNP. After the first incubation in AS, the second Nyquist plot semi-circle has increased from the firt showing an increase in impedance which means adsorption and not detection as there were no NT-proBNP in the solution. This unspecific adsorption was observed in all measurements performed on saliva samples, and thus considered as a constant. For all the other incubations in AS, there were no more increase in impedance and thus no more Heart failure (HF) is a clinical syndrome where in cardiac output is insufficient to meet the metabolic demands of the body. This syndrome caused by a wide range of cardiovascular disorders, such as structural or functional abnormalities of the heart [1]. The early and quick diagnosis of HF is extremely important and crucial not for only patient survival but also saving

cost and great deal of time in successful prognosis of the diseases. Existing methods of diagnosis for HF rely heavily on classical methods which are based on tests conducted in central laboratories that may take several hours or even days from when tests are ordered to when results are received [2]. To solve this problem, biosensors can play an important role in the early diagnosis without having to rely on hospital visits where expensive and time-consuming laboratory tests are recommended [3] one of the most commonly used biomedical approach for detection heart failure is determining the level of valuable specific biomarkers which are generated in different stage. Saliva analysis can represent plot semi-circles (Figure 2B) was observed after the first incubation of the biosensor in AS containing 50 pg/mL of NT-proBNP. This shift was not totally corresponded to NT-proBNP detection. It could be also attributable to the nonspecific adsorption phenomena, which was observed in the previous test. However, the Nyquist plot semi-circles have been increased by increasing NT-proBNP concentrations, highlighting thus the NT-proBNP detection in AS. Therefore, the biosensor was able to detect specifically NT-proBNP proteins within a complex artificial physiological medium.
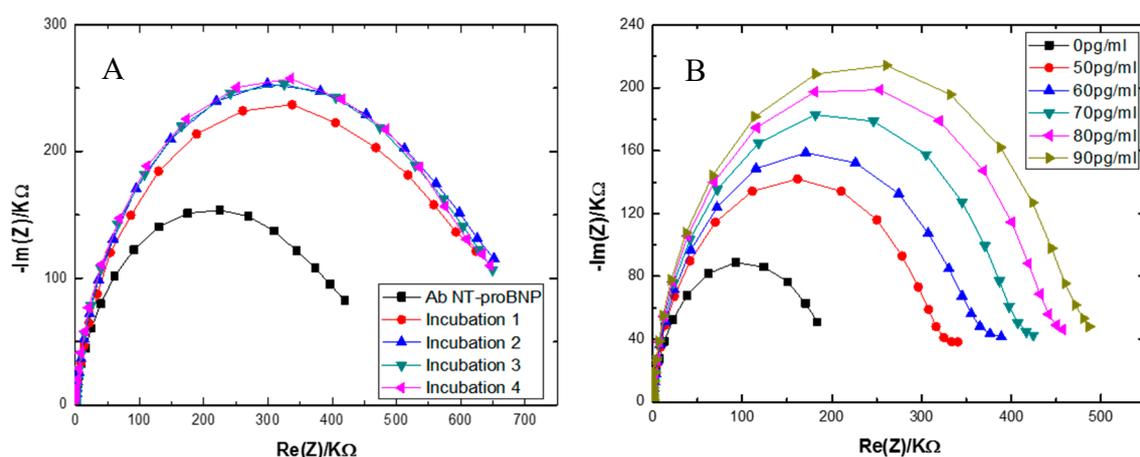

Figure 2. (**A**) EIS analyses for biosensor after several incubations in AS. (**B**) EIS analyses for biosensor after several incubations in AS spiked with different NT-proBNP concentrations (50–90 pg/mL).

4. Conclusions

An immunosensor for detection of NT-proBNP has been developed in this work. A method to produce highly sensitive and specific biosensor for the detection of NT-proBNP from diluted physiologic medium has been described. A good sensitivity was observed for preliminary performed in AS. This is very promising to use this device for cytokines and proteins detection in real human saliva.

Conflicts of Interest: The author do not have any conflict of interest to be declared.

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