Book Chapter

Electrochemical Immunosensor Prototype for N-Terminal Natriuretic Peptide Detection in Human Saliva: Heart Failure Biomedical Application

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Abstract

NT-proBNP is the gold standard biomarker for early diagnostics of heart failure, disease prevention, and stratified and individualized patient care. In this work, we aim to develop a novel ultra-sensitive immunosensor for direct NT-proBNP detection in human artificial saliva (AS), which represents an intriguing biological matrix potentially rich in biomarkers. The immunosensor will enhance the sensitivity of detection, reduce measurement time, and enable the simultaneous detection of various biomarkers. The developed biosensor, based on gold working microelectrodes (WEs), was biofunctionalized using 4-carboxymethyl aryl diazonium (CMA) to immobilize anti-NT-proBNP antibodies. The deposition of CMA onto the gold surface of the microelectrodes was accomplished using cyclic voltammetry (CV). The binding between NT-proBNP antibodies and NT-proBNP antigens was tracked using electrochemical impedance spectroscopy (EIS) in conjunction with the standard addition method. A linear detection response within the range of 1–20 pg/mL for NT-proBNP detection in PBS and artificial saliva was demonstrated, with good selectivity in the presence of other potential interfering biomarkers (interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 1 β (IL-1β)). The developed immunosensor shows great promise for rapid and accurate analysis in biomedical applications.

Keywords

Biosensor Platform; N-Terminal Brain Natriuretic Peptide; Saliva Analysis; Electrochemical Impedance Spectroscopy; Heart Failure

1. Introduction

Heart failure (HF) is a rapidly growing chronic cardiovascular disease and, according to the World Health Organization, it currently represents the main cause of mortality and major morbidity worldwide, particularly among older people [1–3]. HF is a complex clinical syndrome caused by a wide range of cardiovascular disorders, such as structural or functional abnormalities of the heart, which result in the impairment of the heart's ability to fill or pump out blood, eventually leading to the clinical syndrome of HF. In other words, HF can be defined as an abnormality of cardiac structure or

function that may, as a possible consequence, fail to deliver oxygen at a rate commensurate with the requirements of organs and tissues. One of the main symptoms of HF is shortness of breath, in addition to swelling of the legs and ankles, high jugular venous pressure, extreme fatigue, and exercise intolerance. Faced with the difficulty of obtaining enough donor organs, sleep disorders, and anorexia, all these symptoms combine to provide a very poor quality of life for patients with HF [4–7]. Nevertheless, heart failure patients often display ambiguous signs and symptoms that could be attributed to a broad range of conditions, making a diagnosis based only on clinical presentation problematic. This heterogeneity of presentation often results in delays in definitive diagnosis and treatment, and such delays are linked with poor prognosis [8,9]. These non-specific symptoms may lead to delays in accurate diagnosis and treatment, resulting in worse clinical outcomes and increased healthcare costs. Hence, more effective and rapid approaches are needed for HF diagnosis.

Biomarkers, measurable biological markers of a pathological process, have established a growing role in modern medical practice over the last fifty years. They are described as characteristics that provide information about various biological conditions, whether normal or pathological [10–15]. Recently, several HF biomarkers have been considered for HF management [16–18], but since 2008, the European guidelines have highlighted the role of natriuretic peptides (NPs), such as brain natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP), as markers of HF [19–21]. Increased plasma levels of circulating NPs have been described in patients with congestive HF and are directly proportional to the severity of congestive heart failure as classified by the New York Heart Association criteria [22,23]. Plasma or serum concentrations of BNP or NT-proBNP are currently the recommended biomarkers supporting clinical judgment for the diagnosis of HF [17,24–26].

BNP and NT-proBNP are generally quantified in blood by enzyme-linked immunosorbent assay (ELISA) [27–29], electrochemiluminescence immunoassay (ECLIA) [30], fluorescent immunochromatographic assay [31], radioimmunoassay (RIA) [32], and immunoradiometric assay (IRMA) [33]. Additionally, affinity chromatography [34] and chromatography coupled with tandem mass spectrometry methods [34,35] have recently been reported for NP

determination in plasma. However, all these methods have some disadvantages, such as the need for specialized personnel, expensive instruments, and long detection times. Nowadays, immunosensor devices with different sensing receptors and transducers can be considered more interesting and promising tools for determining biomarkers in biological fluids due to their increased sensitivity, decreased detection limit, cost-effectiveness, and easier usability [36–38].

This research introduces a biosensor with high sensitivity (Figure 1A) for identifying NT-proBNP in the saliva of individuals with HF. Saliva analysis offers several advantages compared to blood analysis because saliva can be easily and unobtrusively collected, even from critical subjects (e.g., children, older people, and disabled people), making it suitable for screening a large population. This bypasses several drawbacks such as invasiveness, psychological stress (especially if repeated sampling is needed), and possible health risks for patients and healthcare professionals [38–40].

Examining salivary biomarkers, NT-proBNP in particular, shows a promising technique for the non-invasive detection of heart failure. Nonetheless, present methodologies are constrained by their reproducibility and sensitivity as previously mentioned [41]. Consequently, recent research has increasingly focused on detecting salivary biomarkers as indicators of individual health status across various medical fields, including diabetes [42], cancer [43], and cardiovascular diseases. Studies have shown that NT-proBNP is present in saliva, with elevated levels in individuals suffering from heart failure compared to healthy individuals [44]. Here, the authors validated an analytical method for quantifying NT-proBNP in saliva samples using a commercial ELISA test, demonstrating promising sensitivity. Furthermore, emerging research supports the potential of salivary biomarker detection for identifying cardiovascular diseases, although this approach remains in the developmental stage [45]. These studies highlight the potential of the NT-proBNP detection in saliva as a non-invasive diagnostic tool, while emphasizing the need for more sensitive and specific methodologies, which our biosensor aims to achieve.

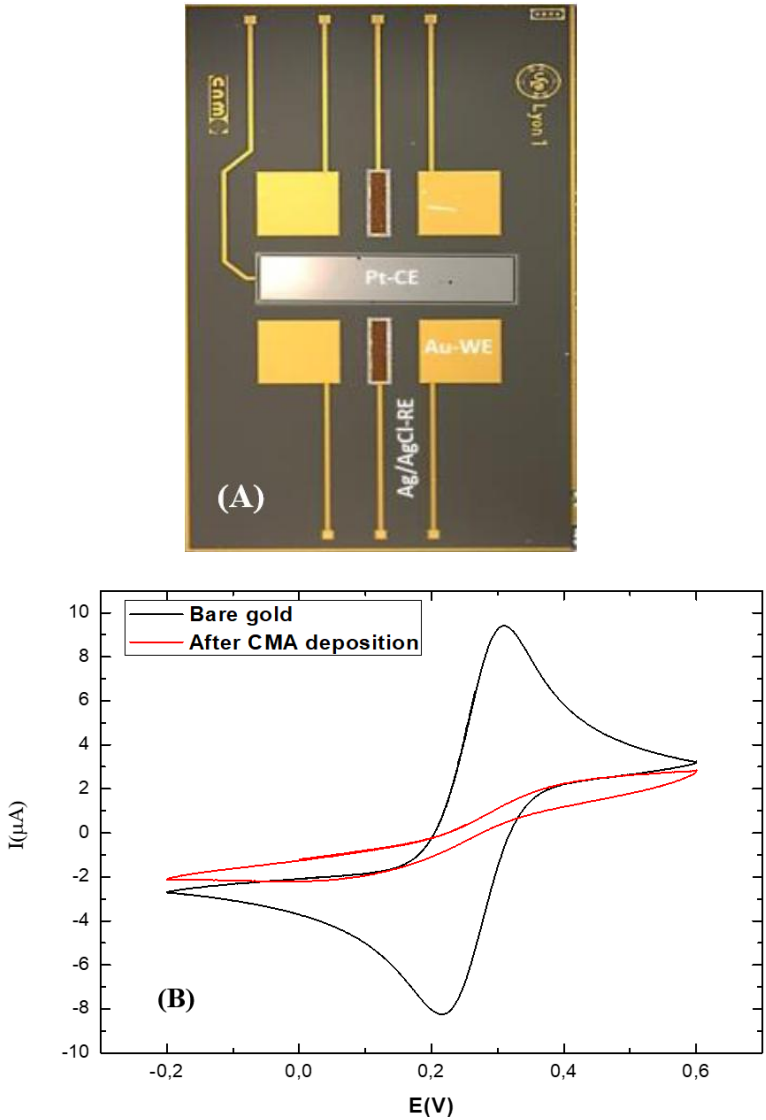

Figure 1: (**A**) Chip containing four Au-WEs, Ag/AgCl-RE, and Pt-CE. (**B**) Cyclic voltammograms of gold WE before (black) and after (red) CMA deposition.

For the creation of our biosensor, monoclonal antibodies (mAbs) targeting NT-proBNP were attached to gold working electrodes (WEs) using a process involving carboxyl diazonium. To validate the mAb attachment and analyze the surface characteristics of the gold

microelectrodes, cyclic voltammetry (CV) was employed throughout the microelectrode modification process. The immunosensor specificity was proven by analyzing different standard solutions containing other HF biomarkers, which may have represented possible interferences, such as the cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 10 (IL-10) [46–48]. Finally, electrochemical impedance spectroscopy (EIS) combined with the standard addition method was used to quantify the NT-proBNP in both standard solutions and artificial saliva, showing good sensitivity and selectivity.

2. Materials and Methods

2.1. Chemicals and Reagents

4-Aminophenylacetic acid (4-carboxymethylaniline, CMA), sodium nitrite ($NaNO_2$), hydrochloric acid (HCl) 37%, pure ethanol, N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine, phosphate-buffered saline (PBS), potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$), and potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$) were purchased from Sigma Aldrich (France). N-Terminal proBNP antibody (anti-NT-proBNP, Cat. No. ABIN191290) and NT-proBNP protein (Cat. No. ABIN2126406) were purchased from Anticorps-enligne.fr (France). Recombinant human IL-1 β protein (Cat. No. ab184595) and recombinant human IL-6 protein (Cat. No. ab119444) were from ABCAM (France). Recombinant human IL-10 with the reference (Cat. No. 217-IL) was purchased from R&D System (France). Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm) was produced by a Millipore Reagent Water System (Molsheim, France).

2.2. Antibodies and Standard Solutions of Antigens

Antibodies (Abs) were reconstituted according to the protocol provided by the supplier, aliquoted, and subsequently stored at −20 °C until use. The concentration of each aliquot was 5 mg/mL. Antigens (Ags, e.g., NT-proBNP, IL-1β, IL-6, and IL-10) were diluted in appropriate buffer following the protocol provided by the supplier, aliquoted into stock solutions with a concentration of 50 µg/mL, and then stored at −20◦ °C until use.

An Ab stock solution aliquot was thawed at 4 °C for 1 h each day of analysis and further diluted in PBS (10 mM, pH 7.4) to obtain 50 ng/mL solution.

Ag stock solutions were thawed at 4 °C for 1 h before being used to prepare standard solutions at different concentrations (1, 5, 10, 15, and 20 pg/mL) by dissolving the appropriate amount of the stock solution in PBS (10 mM, pH 7.4).

2.3. Sample Preparation

Artificial saliva (AS) was prepared as described by Ref. [12]. In summary, a solution was prepared by dissolving 0.6 g/L sodium phosphate dibasic, 0.6 g/L anhydrous calcium chloride, 0.4 g/L potassium chloride, 0.4 g/L sodium chloride, 4 g/L mucin, and 4 g/L urea in deionized water. The pH was then adjusted to 7.2 using 1 M sodium hydroxide. Finally, the solution was sterilized by autoclaving and stored at −4 °C until needed.

Appropriate amounts of NT-proBNP stock solution (50 µg/mL) were diluted in AS, obtaining samples with different concentrations (50, 60, 70, 80, 90 pg/mL). A sample containing 76 pg/mL of NT-proBNP was prepared in AS to simulate an unknown sample to be analyzed by performing the standard addition method. A constant volume (50 µL) of the unknown sample was added to each of the four 1 mL volumetric flasks. To achieve a final volume of 1 mL, the first flask (Level 0) was supplemented with 950 µL of AS. An NT-proBNP standard solution (100 pg/mL) was then added in increasing volumes (30, 60, 90 µL), corresponding to the additions of 3 (Level 1), 6 (Level 2), and 9 pg/mL (Level 3) of NT-proBNP to the subsequent flasks, and each flask was then made up to volume (1 mL) with AS.

2.4. Biofunctionalization of Working Electrode

The biosensor platform consists of four gold working electrodes (WEs), two Ag/AgCl reference electrodes (REs), and one platinum counter electrode (CE), as shown in Figure 1A. The device's development was already described by Ghedir et al. [49]. Before the chemical functionalization, the device was pre-cleaned by sonication for 10 min in acetone, followed by rinsing with ethanol then deionized water, and finally, it was placed into a UV/O_3 cleaner for 30 min to remove all organic contaminants. Subsequently, CMA molecules

were electrochemically deposited onto gold using the cyclic voltammetry (CV) technique as described in [10,12,38]. Briefly, a 3 mM CMA solution was prepared in water with 1 M HCl and 1 M $NaNO_2$. The device was placed into this solution and eight CV cycles were applied. The potential was scanned from 0.3 V to –1 V vs. Ag/AgCl at a scan rate of 80 mV/s and eight cycles were performed.

After CMA deposition, the biosensor platform was rinsed with a 1% HCl solution. Then, the terminal carboxylic acid (–COOH) groups were activated in an ethanolic solution of NHS/EDC (0.1 M/0.1 M) for 1 h at room temperature (22 ± 2 °C). Subsequently, the WE surface was rinsed with ethanol and promptly immersed in the anti-NT-proBNP solution for 1 h at 4 °C. This step was fundamental because the terminal amine groups on the antibody enable covalent bonding to occur through the activated carboxyl group from CMA functionalized in NHS/EDC. Finally, the WE was rinsed with PBS and the remaining active carboxylic acid groups were deactivated by incubation in ethanolamine solution (0.1% in PBS) for 20 min at 4 °C. This step is crucial in preventing non-specific bonding during the detection stage.

2.5. Electrochemical Measurements

All electrochemical measurements were carried out at room temperature (22 ± 2 °C) in a Faraday box using a VMP3 potentiostat (BioLogic Science Instruments, Seyssinet-Pariset (France)) controlled by EC-Lab software (version 10.40, BioLogic Science Instruments, Seyssinet-Pariset (France)). CV analysis was performed on both bare and functionalized electrodes by immersing the device into a 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS (10 mM, pH 7.4) solution as a redox couple. After thorough cleaning and functionalization, the WE surface was characterized by CV at 80 mV/s, and the switching potential was scanned between −0.2 and 0.6 V.

EIS measurements were carried out to evaluate the recognition properties of the biosensor platform in terms of sensitivity and selectivity, as well as to perform the quantification of NT-proBNP in both standard solutions and artificial saliva (AS). The EIS measurement was conducted with a potential set at 0.228 V relative to the built-in Ag/AgCl reference electrode, covering a frequency spectrum from 100 mHz to 100 kHz, and employing a modulation voltage of 25 mV [37,38]. The device was then used to detect NT-

proBNP within the range of 1 pg/mL to 20 pg/mL in PBS and for the standard addition method in AS. To assess the analyte, the biosensor underwent sequential exposures to standard solutions with varying NT-proBNP concentrations for 30 min at 4 °C, after which it was thoroughly rinsed with PBS. The impedance response was recorded for each concentration by immersing the biosensors in an electron mediator solution of 5mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS buffer at pH 7.4. The same procedure of EIS analyses was used for NT-proBNP detection in artificial saliva by performing the standard addition method.

3. Results and Discussion

3.1. Biosensor Biofunctionalization

Before CMA deposition onto the bare gold working electrode (WE), the Fe(II)/Fe(III) redox peaks were clearly visible in the cyclic voltammetry (CV) results. However, these peaks decreased after CMA deposition, as shown in Figure 1B, due to the reduced electron transfer rate caused by the CMA blocking layer. Adequate coverage of the gold WE, which was proportional to the decrease in redox peaks after CMA deposition, was crucial for the biofunctionalization step.

3.2. Biomarker Detection in PBS

The antibody (Ab) bond was then confirmed by performing electrochemical impedance spectroscopy (EIS) on the biofunctionalized working electrodes (WEs) before and after incubation in NT-proBNP standard solutions with increasing concentrations. Figure 2A shows Nyquist plots corresponding to an increase in the charge transfer resistance (Rct) as the concentration of NT-proBNP increased, which was due to the specific interaction between the antibody (Ab) and the antigen (Ag). The first Nyquist plot semicircle was recorded after incubating the functionalized device in a PBS solution in the absence of NT-proBNP to obtain the baseline. The device was then subsequently incubated in NT-proBNP standard solutions with increasing concentrations. The impedance measurements were carried out after the target substance was in equilibrium with the antibody-functionalized surface, in the presence of the redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. Indeed, the electron transfer of the redox probe from the electrolyte to the electrode

microelectrodes encountered the resistance of the sensitive layer (NT-proBNP antibodies+ NT-proBNP antigen) at equilibrium. The more biomarkers that were present on the surface of the sensitive layer, the more the average thickness of the sensitive layer increased. As the average thickness increased, the charge transfer resistance (Rct) also increased. Since the real part of impedance is related to charge transfer resistance, an increase in Rct leads to a corresponding increase in the real part of the impedance. Therefore, as a biomarker concentration increases, the increase in the real part of the impedance is represented by a Nyquist plot's semicircular diameter. Insert in Figure 2A illustrates the equivalent electrical circuit used to analyze the Nyquist plots, where Rs represents the resistance of the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox couple, Rct is the charge transfer resistance, W is the Warburg impedance, and Q1 is the constant phase element, an equivalent model of the double-layer capacitance [50]. A linear relationship between the Rct variation and NT-proBNP concentrations was found in the concentration range from 1 pg/mL to 20 pg/mL, with a correlation coefficient of $R^2 = 0.98$ (sensitivity S: 0.027 $(pg/mL)^{-1}$). The detection limit, calculated from the formula $3\sigma/S$ (s being the background variation and S the sensitivity), was 0.03 pg/mL. The selectivity was investigated by performing the same approach. Standard solutions containing three different heart failure biomarkers, IL-1β, IL-6, and IL-10 [51], were evaluated under identical experimental conditions and within the same concentration range. Figure 2B presents a comparison of the curves obtained for each of these biomarkers against that of the target analyte. The results indicated that our immunosensor exhibited significant selectivity for NT-proBNP, with negligible cross-reactivity toward the other evaluated biomarkers, thereby confirming its specificity. Moreover, our results are supported by other studies that have also demonstrated the absence of significant cross-reactivity between NT-proBNP and various cytokines [52,53]. These studies have confirmed that NT-proBNP is the most reliable and specific biomarker for heart failure diagnosis and monitoring [54,55], emphasizing the importance of our results and the necessity for highly selective detection methods. To the best of our knowledge, only a few studies have utilized electrochemical sensors for NT-proBNP detection at low concentrations. For instance, Pollok et al. [56] reported the detection of NT-proBNP at 0.58 nM in human serum using an electrochemical

biosensor based on a metalloimmunoassay on a paper electrode platform.

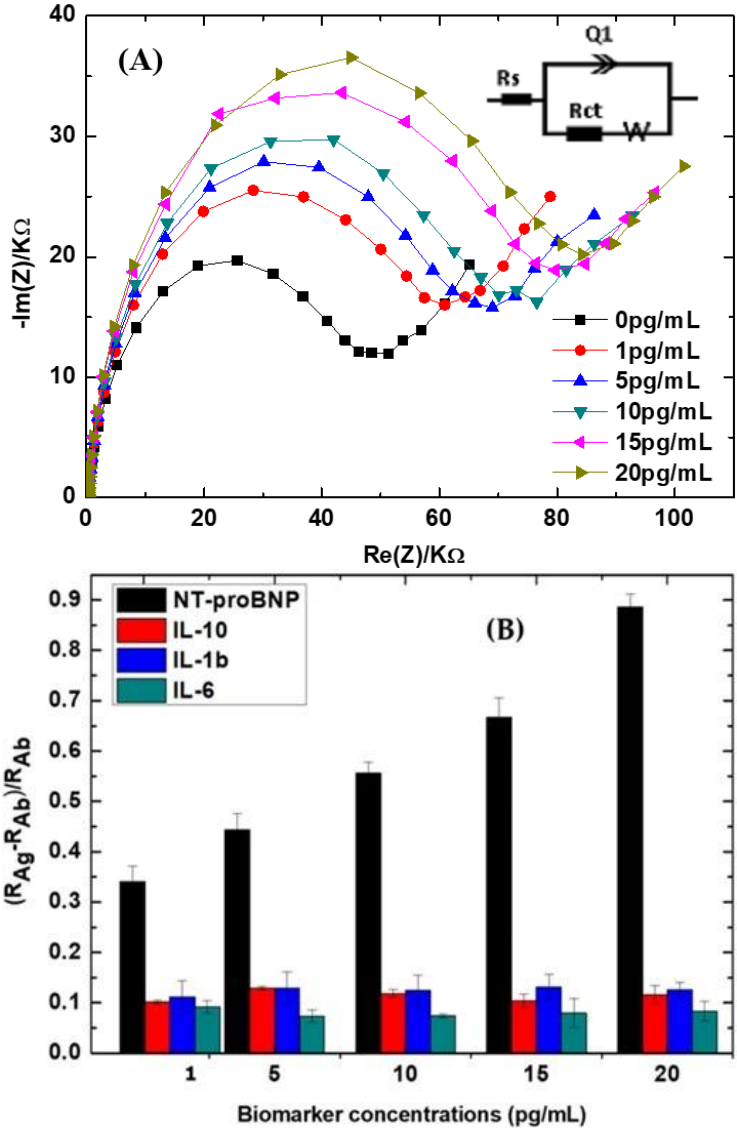

Figure 2: (**A**) Nyquist plot impedance (Zr vs. Zi: at 5mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS pH 7,4 solution) at various NT-proBNP concentrations. (**B**) Sensitivity of the biosensor functionalized with AbNT-proBNP for the detection of AgNT-proBNP and the IL-10, IL-1β, and IL-6 cytokines.

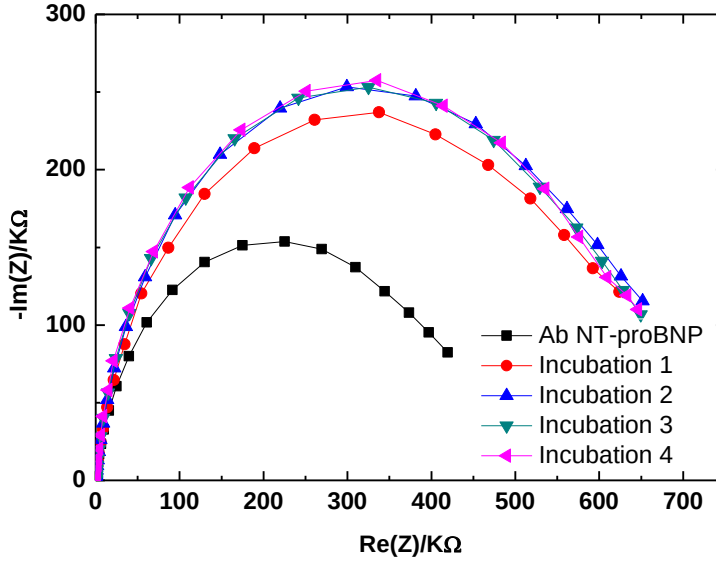

Figure 3: EIS analyses of the biosensor after several incubations in artificial saliva.

The analytical performance of this NT-proBNP immunosensor was compared to that of previously published NT-proBNP immunosensors using different methods of detection: electrochemical methods (anodic stripping voltammetry [56], electrochemical impedance spectroscopy on ISFET [52]), photoelectrochemistry [57], electroluminescence [58], SERS [59], and SPR [60] (Table 1). Compared to label-free immunosensors (EIS/ISFET, SPR), the developed sensor presented a lower detection limit. Compared to sandwich immunosensors (ASV, PEC, ECL, SERS), all immunosensors presented a lower detection limit, except the ASV-based immunosensor.

Table 1: Comparative analytical performances of various immunosensors for NT-ProBNP detection.

Method	**Recognition Element**	**Linear Range**	**LOD**	**Ref.**
Anodic stripping voltammetry	Sandwich immunodetection	0.58 nM–2.33 nM	0.58 nM	[56]
Electrochemical impedance spectroscopy on ISFET	Label-free immunodetection	0–50 pg/mL	1 pg/mL	[53]
Photoelectrochemistry F-Bi_2WO_6/Ag_2S/ITO	Sandwich immunodetection	0.1pg/mL - 100 ng/mL	0.03 pg/mL	[57]
Electrochemiluminescence TiO_2@CN-Au	Sandwich immunodetection	0.0001∼10 ng/mL	0.05 pg/mL	[58]
SERS $CoFe_2O_4$@AuNPs	Sandwich immunodetection	1 fg/mL - 1 ng/mL	0.75 fg/mL	[59]
SPR SiO_2/Ag	Label-free immunodetection	0–10 ng/mL	1 ng/mL	[60]
Electrochemical impedance spectroscopy	Label-free immunodetection	0–20 pg/mL	0.3 pg/mL	This work

3.3. Biomarker Detection in Human AS

Biosensor sensitivity was also tested in human artificial saliva (AS). Therefore, electrochemical impedance spectroscopy (EIS) measurements were performed after each incubation of the device in human AS not containing NT-proBNP (Figure 3). The first semicircle (black) corresponds to the immobilized anti-NT-proBNP antibody (AbNT-proBNP). After the first incubation in AS, the second (red) Nyquist plot semicircle increased from the first (black), showing a rise in impedance. This corresponds to non-specific adsorption and not to NT-proBNP detection, as there was no NT-proBNP in the solution.

This non-specific adsorption was observed in all measurements performed on saliva samples. After the second, third, and fourth incubations in AS, there was no further increase in impedance and thus no more adsorption (all Nyquist plots were superimposed). Therefore, the first gap was considered constant, as there was no more non-specific adsorption.

Subsequently, the same experiment was repeated by incubating the biosensor in different AS samples with increasing concentrations of the NT-proBNP biomarker. Following each incubation period, the biosensor's characteristics were assessed using EIS (Figure 4A). A significant shift between the first (black) and the second (red) Nyquist plot semicircles was detected after the first incubation of the biosensor in AS containing 50 pg/mL of NT-proBNP. This change is not solely due to NT-proBNP detection; it may have also been influenced by non-specific adsorption events that were noted in earlier experiments. However, the Nyquist plot semicircles increased with rising concentrations of NT-proBNP, highlighting the detection of NT-proBNP in AS when compared to the previous test. Therefore, the biosensor was able to specifically detect NT-proBNP biomarkers within a complex physiological medium. The sensitivity of the biosensor was calculated using the same equivalent circuit used in the detection in PBS. Figure 4B clearly shows the sensitivity toward NT-proBNP (R^2 = 0.97 with a sensitivity of 0.012 $(pg/mL)^{-1}$). This result confirms the sensitivity of the immunosensor toward NT-proBNP antigens in AS. Nevertheless, the sensitivity in AS was 0.44 times lower than in PBS, even after thorough rinsing with PBS. This point

shows the strong adsorption of some AS components, mainly proteins and salts. This strong adsorption would lead to lower values of NT-proBNP concentrations, bringing potential false-negative effects.

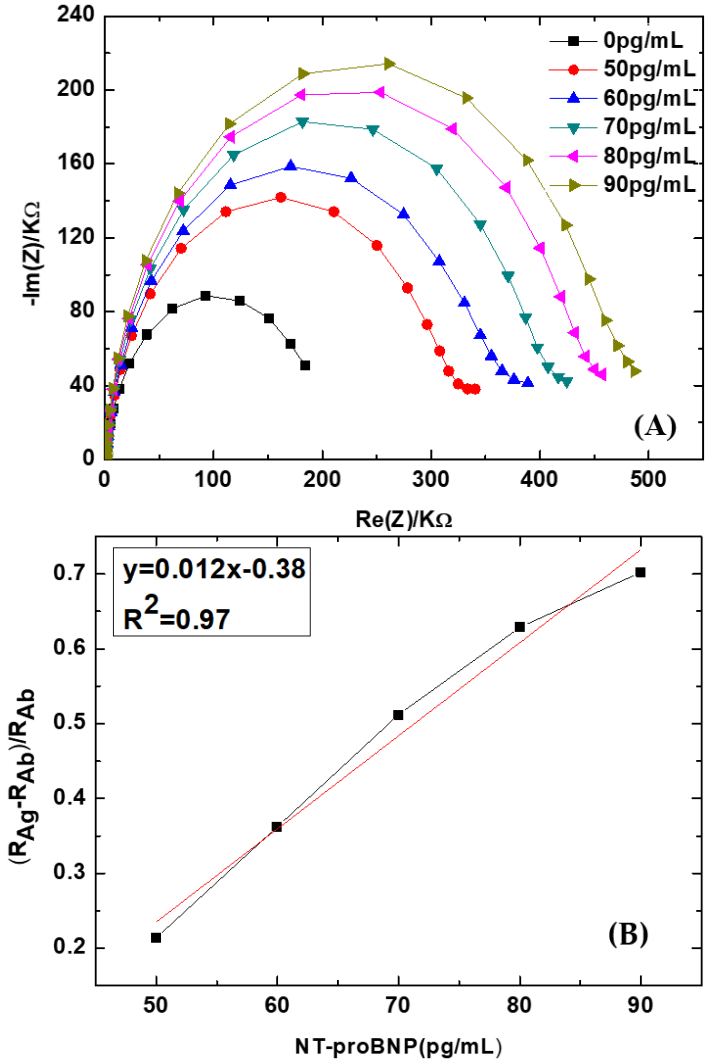

Figure 4: (**A**) EIS analyses of the biosensor after several incubations in artificial saliva spiked with different NT-proBNP concentrations (50–90 pg/mL). (**B**) Sensitivity curves of the biosensor functionalized with AbNT-proBNP for the detection of AgNT-proBNP in artificial saliva.

3.4. Biomarker Detection in AS Using Standard Addition Method

To mimic the detection of NT-proBNP in an unknown sample, the standard addition method was employed in conjunction with electrochemical impedance spectroscopy (EIS), following the procedure outlined in Section 2.3. As shown in Figure 5A, impedance measurements were carried out after each incubation of the biosensor in Level 1, Level 2, Level 3, and Level 4. Initially, there was a significant jump in the Nyquist plot semicircles (red), followed by a normal increase in the Nyquist plot semicircles with increasing NT-proBNP concentration, implying the detection of the analyte. The equation of the curve obtained by linear data fitting was $y = 0.27x + 1.33$, with $R^2 = 0.99$ (Figure 5B).

The analyte concentration in the unknown sample was determined by identifying where the extended calibration curve intersected the OX axis (abscissa) [12]. This resulted in a value of 97.6 pg/mL, taking into account the dilution factor. This value is higher than the expected concentration of 76 pg/mL of the unknown sample, which is possibly due to matrix effects [61]. To confirm this result and investigate the matrix effect, this technique (standard addition method) should be applied to a large batch of saliva samples from different patients of various genders to estimate the errors and matrix effects. Moreover, it is advisable to employ a standard method like the enzyme-linked immunosorbent assay (ELISA). This would help estimate the real concentrations of cytokines in saliva after EIS measurements. However, the obtained concentration is consistent with the salivary levels of this cytokine determined in saliva samples from groups of nominally healthy subjects [62,63].

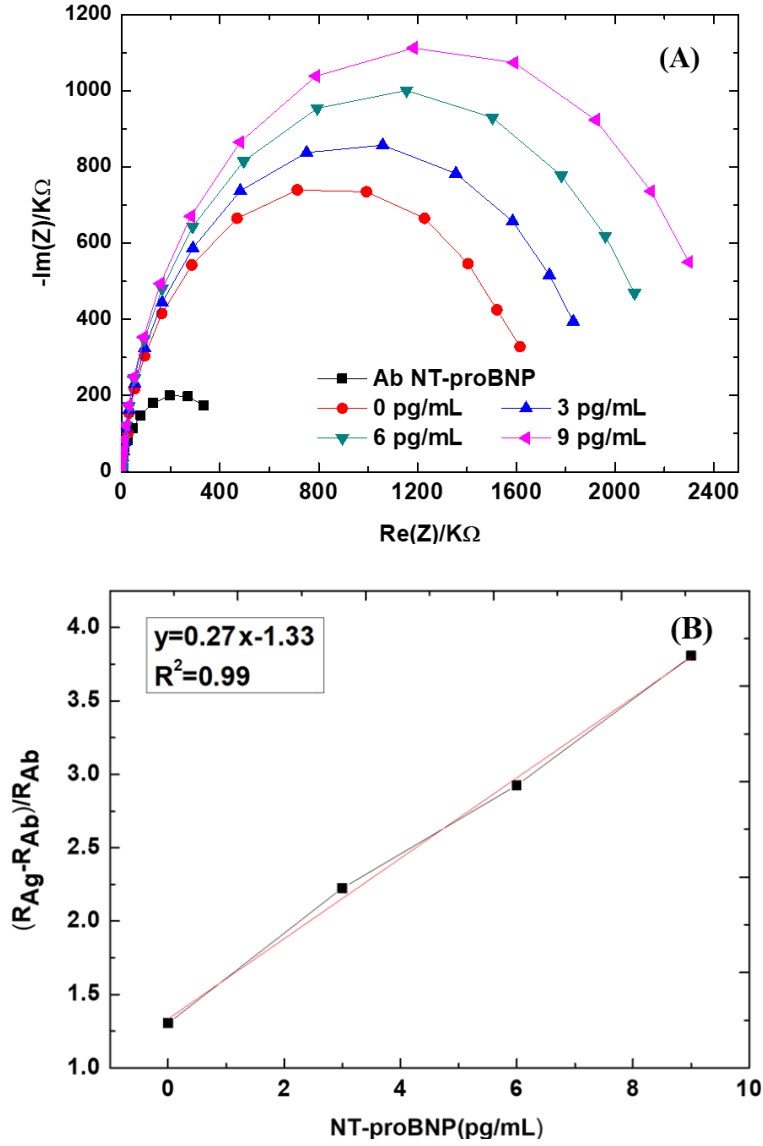

Figure 5: (**A**) Nyquist impedance plots (Zr vs. Zi: at 5 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$in PBS pH 7,4 solution) obtained from the standard addition method performed on artificial saliva, 0 pg/mL (corresponding to Level 1), 3 pg/mL (corresponding to Level 2), 6 pg/mL (corresponding to Level 3), and 9 pg/mL (corresponding to Level 4). (**B**) Sensitivity curve used to calculate the concentration of the analyte in the unknown sample by the standard addition method.

4. Conclusions

In this study, an immunosensor was developed for detecting NT-proBNP, a crucial biomarker for heart failure, in saliva. The biosensor, based on gold working microelectrodes, was biofunctionalized using carboxyl diazonium to immobilize anti-NT-proBNP antibodies, ensuring specific binding and enhanced sensitivity. The immunosensor demonstrated excellent sensitivity in the range of 1 pg/mL to 20 pg/mL, which is clinically relevant for early detection and monitoring of heart failure. Additionally, the sensor showed good selectivity in the presence of other interfering biomarkers such as IL-10, IL-6, and IL-1β, commonly found in biological samples.

Preliminary tests conducted in artificial saliva confirmed the device's capability to detect NT-proBNP in a complex physiological medium, validating its potential for real-world applications. Future work will focus on extending the analysis to real human saliva samples to thoroughly study the matrix effects and estimate the real concentrations of cytokines more accurately. Overall, this study highlights the potential of the developed immunosensor as a valuable tool for the non-invasive and convenient monitoring of heart failure biomarkers in saliva, offering promising applications in clinical settings for improved diagnosis and management of heart failure.

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