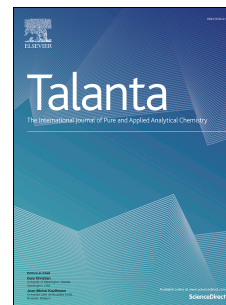


Journal Pre-proof

Capacitance electrochemical biosensor based on silicon nitride transducer for TNF- α cytokine detection in artificial human saliva: Heart failure (HF)

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PII: S0039-9140(19)31134-8

DOI: <https://doi.org/10.1016/j.talanta.2019.120501>

Reference: TAL 120501

To appear in: *Talanta*

Received Date: 24 July 2019

Revised Date: 19 October 2019

Accepted Date: 23 October 2019

Please cite this article as: M. Bahri, A. Baraket, N. Zine, M. Ben Ali, J. Bausells, A. Errachid, Capacitance electrochemical biosensor based on silicon nitride transducer for TNF- α cytokine detection in artificial human saliva: Heart failure (HF), *Talanta* (2019), doi: <https://doi.org/10.1016/j.talanta.2019.120501>.

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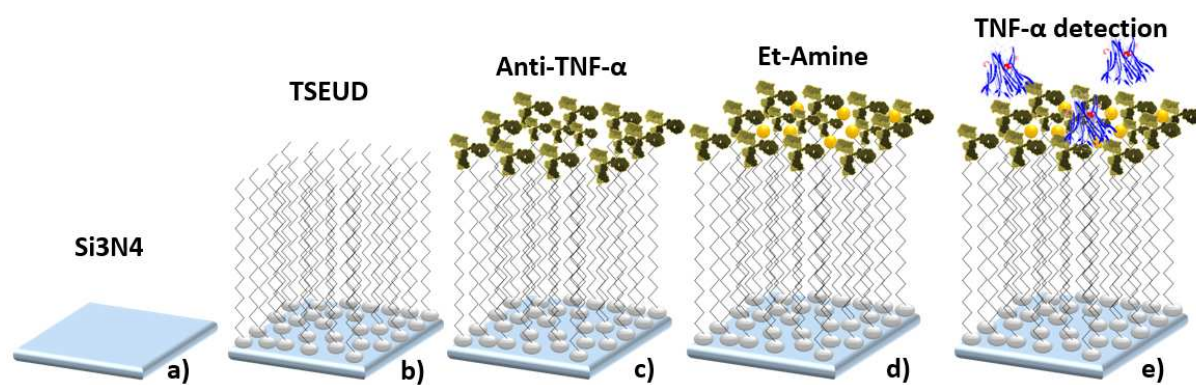


Fig. 1: Schematic illustrations of the chemical surface modification and bio-functionalization process of the immunosensor with antibodies Anti-TNF- α .

Capacitance Electrochemical Biosensor Based on Silicon Nitride Transducer for TNF- α Cytokine Detection in Artificial Human Saliva: Heart Failure (HF)

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Abstract:

In the present study, we have developed a capacitance electrochemical biosensor based on silicon nitride substrate (Si₃N₄/SiO₂/Si[P]/Al) for Tumor Necrosis Factor Alpha (TNF- α) cytokines detection. Micro-contact printing, Fluorescence microscopy characterization and contact angle measurement (CAM) were carried out during the bio-functionalization of the biosensor surface. Mott-Schottky analyses were applied for TNF- α detection within the range of 1 pg/mL to 30 pg/mL in which the immunosensor has exhibited a good linearity, a sensitivity of 4 mV.pM⁻¹ and 4.4 mV.pM⁻¹ in PBS and artificial saliva (AS) respectively. While the LOD was found at 0.38 pg/mL and 1 pg/mL in PBS and AS respectively. The developed immunosensor has also demonstrated a high and good selectivity for TNF- α detection in human AS when compared to other interferences like Cortisol and Interleukin-10. The performances of the developed biosensor are very promising for biomedical application to predict the first sign of inflammation.

Keywords: Electrochemical biosensor, Mott-schottky, Silicon nitride transducer, Tumour Necrosis Factor, Human artificial saliva.

1. Introduction:

Classical analyses for patients suffering from chronic disease were generally made by analysing blood or human plasma [1]. This was considered as very stressful and invasive analyses especially for elderly patients. During the last few decades, several challenges have aroused the interest of scientific researchers for chronic disease monitoring through non-invasive approaches for patient's suffering from heart failure (HF). For this interest, critical biomarkers secreted during local and systemic inflammation were analysed in human saliva

1 taking into account the advantages of painless, multiple sample collections by unskilled people, real-
2 time monitoring, non-invasive, and especially stress-free collection for the patient, etc [2–6]. Human
3 saliva contents reflect our body's health and about 20% of blood proteins are also present in saliva [7].
4 Besides, numerous studies had also proved that the concentration of TNF- α in saliva reflect those in
5 blood [8,9]. Tumour necrosis factor-alpha (TNF- α), widely considered as one of the biomarkers
6 detected during the acute stage of inflammation in both blood and saliva [10]. Nowadays, TNF- α is
7 considered as an indicator biomarker for HF diagnosis [11]. HF is becoming a priority global health
8 concern, affecting around 26 million people worldwide [12] and is estimated to possess about 26.819 \$
9 as a lifetime cost for HF patients [13]. Although there is no cure for HF disease [14], however, early
10 diagnosis can allow to take care of patients very quickly and to improve their health state.

11 Several techniques have been tested and used to quantify and detect TNF- α and other biomarkers to
12 predict the first signs of inflammation [15,16]. Enzyme-linked immunosorbent assays (ELISA)[17,18],
13 bioassays [19], radio-immunoassays (RIA) [20], surface plasmon resonance [21] and other methods
14 [22–27] were widely used in this interest and are considered as standard methods. Although, most of
15 these techniques are accurate and provide rapid screening and multiple analyses; they still limited by
16 the high cost and the necessity of a qualified person to carry these analyses. To overcome the beyond
17 limitations and satisfy the need of medical tools with high sensitivity, linear response, low cost and
18 especially a low limit of detection, many scientific researchers have developed various strategies for
19 biomarkers detection in human saliva. This latter contains a broad spectrum of biomarkers including
20 TNF- α , cortisol, interleukin-10 [28] and N-terminal proB-type natriuretic peptide (NT-proBNP) [6],
21 and their detection may afford relevant information's for clinical diagnosis [2,29]. In addition, TNF- α
22 concentrations in blood for healthy humans are generally below 40 pg/mL [38], while more than 80%
23 of patients with severe autoimmune diseases may maintain between 10 to 300 pg/mL [39]. For patients
24 suffering from chronic HF have an increased circulating level of TNF- α [40], tie with the severity of
25 disease [41]. Recently TNF- α cytokine was detected by electrochemical biosensors which were widely
26 used in the literature for biomarkers detections in saliva, urine and blood serum [30–32]. These
27 electrochemical biosensors are considered as a promising tool since they can be easily miniaturized
28 and require a small sample volume [33,34].

29 In the present study, we report the development of a capacitance electrochemical biosensors for TNF- α
30 detection based on silicon nitride transducer (Si_3N_4). This material based micro-fabrication
31 technology has been combined with biochemistry [35], allowing the fabrication of novel
32 biosensing devices with great selectivity and good sensitivity [36]. Antibodies Anti-TNF- α were
33 addressed onto the Si_3N_4 surface through covalent bonding of the aldehyde-silane (11-
34 (triethoxysilyl)undecanal) TESUD. The fabricated biosensor has exhibited an enhanced response for
35 TNF- α within a concentration range of 1-30 pg/mL, with a LOD of 1 pg/mL. Additional tests were

carried out to investigate the immunosensor selectivity in both PBS and artificial saliva (AS) using IL-10 and cortisol as interferences [4], which might be both detected in saliva [37].

2. Material and methods

2.1 Reagents and chemicals

Antibodies Anti-TNF- α (Ab-TNF- α), TNF- α protein (TNF- α), Interleukin-10 (IL-10), and hydrocortisone (Cortisol) were purchased from Abcam (France). ((11-Triethoxysilyl)Undecanal TESUD), octadecyltrichlorosilane (OTS), ethanol (98%), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), calcium chloride (CaCl_2), phosphate buffer solution (PBS) tablets, pure ethanol, urea and mucin were all purchased from Sigma-Aldrich. Acros Organics (France) supplied the sodium chloride. Ethanolamine (ETA), sulfuric acid (98%) (H_2SO_4) and hydrogen peroxide (30%) (H_2O_2) were purchased from Fluka (France). The polydimethylsiloxane (PDMS) was supplied from Dow Corning (France).

2.2. Silicon Nitride transducer (fabrication process)

The (Al/Si-p/SiO₂/Si₃N₄) transducer was fabricated by using <100> silicon wafer orientation as substrate. The fabrication process was carried out in Centre National de Microelectrónica (CNM-IMB, CSIC, Spain). The Si-p wafer was firstly doped with boron ($1 \times 10^{15} / \text{cm}^2$) and afterwards was thermally oxidized at 850 °C to get 78nm of SiO₂. Moreover, a thin layer of 100 nm thickness of Si₃N₄ was deposited onto SiO₂ by low-pressure chemical vapour deposition (CVD). Afterwards, 1 μm of aluminium was deposited on the backside of the wafer as an Ohmic contact [38]. Finally, the wafer was diced to individual squares of 1.2 cm² to be used as transducer of the biosensor.

2.3. Transducer bio-functionalization

Firstly, the silicon nitride substrates were cleaned by sonication in acetone for 15 min and then in ethanol for 15 min, then washed with distilled water and dried with nitrogen. This step was necessary to remove all organic contaminations provided from the remained protective resin layer. Then, the silicon nitride surface was activated with piranha solution (1/3 H₂O₂; 2/3 H₂SO₄) for 30 min by keeping the electrode backside (Aluminum layer) outward from the piranha solution to protect it from wet etching [38]. The activation step was used to generate the silanol and silylamine groups [39], which are necessary to obtain a perfect adhesion of bio/chemical substances onto the silicon nitride surface (Fig. 1.a). The obtained substrates were functionalized using 1% TESUD in ethanol solution overnight, rinsed gently with ethanol, dried with nitrogen and left in the oven at 100 °C for 1 h. Therefore, self-assembled monolayers (SAMs) of aldehyde-silane were formed onto the Si₃N₄ surface (Fig .1.b). The monoclonal Anti-TNF- α antibodies were covalently bonded to the surface through the acid amine linkage. So, after 3 h of incubation of antibodies TNF- α at room temperature (20 ± 2 °C) (Fig 1.c), the residual activated carboxylic acid groups of TESUD were blocked using 1% of

ethanolamine diluted in PBS for an additional 30 min in order to reduce the nonspecific binding during the detection process [40] (Fig.1d). Finally, the samples were neatly rinsed with PBS using micropipette and were ready for TNF- α detection step (Fig 1.e).

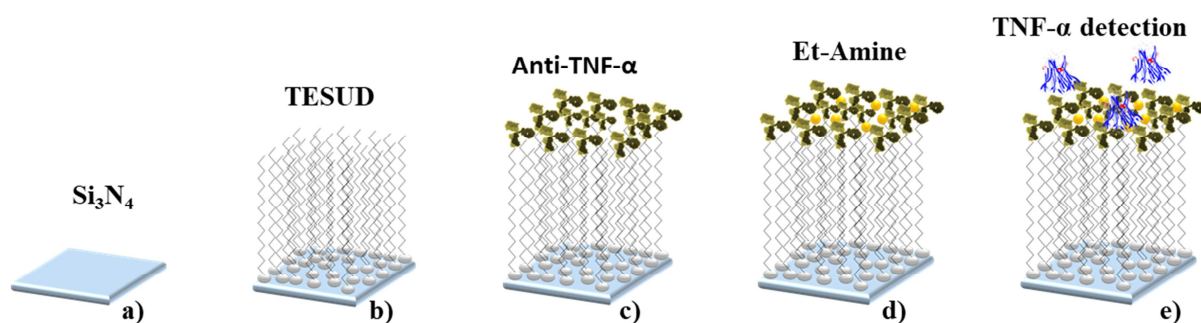


Fig. 1: Schematic illustrations of the chemical surface modification and bio-functionalization process of the immunosensor with antibodies Anti-TNF- α . a) Si_3N_4 surface cleaning and activation. b) Si_3N_4 surface functionalization with TESUD. c) Anti-TNF- α antibodies immobilization step d) Si_3N_4 surface blocking using 1% ethanolamine. e) Electrochemical TNF- α detection step.

2.4. Micro-contact printing and fluorescent microscopy

Micro-contact printing (μCP) technique was used for checking the successfully bio-functionalization surface and to ensure that the TNF- α antibodies were perfectly bonded onto the TESUD modified silicon nitride surface. The detailed process of μCP technique was previously published in our group [41]. Briefly, a negative elastomeric stamp based on polydimethylsiloxane (PDMS) with patterns of squares $10\text{ }\mu\text{m}^2$ was fabricated by replica moulding. Here, the pre-polymer PDMS and curing agent were mixed with a ratio of (10:1 w/w) respectively. Then the mixture was casted onto a positive silicon mould with micropillars on relief of its surface. The PDMS stamp was inked with OTS ($5\text{ }\mu\text{M}$) for 1 min and brought in conformal contact with the activated silicon surface. After peeling off the PDMS, a SAMs of OTS were formed on the silicon nitride surface as a blocking layer (Fig 2A). The obtained substrate was then immersed in ethanol solution with 1% of TESUD for 30 min, rinsed gently with ethanol, dried with a slight stream of nitrogen and placed in the oven for 1 h at $100\text{ }^\circ\text{C}$ for chemisorption of both silane OTS and TESUD.

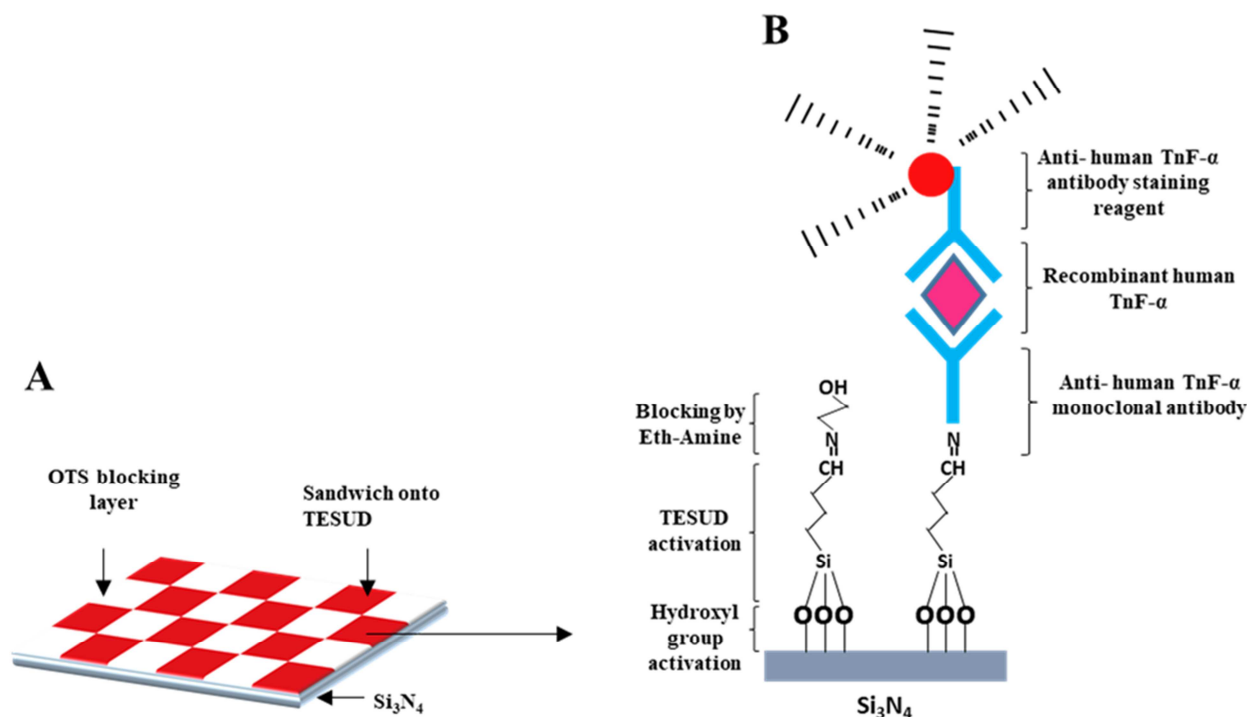


Fig. 2: A) Illustration of TESUD and OTS pattern after μ CP B) illustration of sandwich formation antibody-cytokine-antibody@Rh

Afterwards, Anti-TNF- α antibodies were immobilized onto TESUD, followed by Ethanol-Amine deactivation as previously described (Fig. 2B). The immunosensor was then incubated in PBS with 10 pg/mL of TNF- α cytokines for 30 min to allow the antibodies-antigens interaction. Then, the biosensor was rinsed with PBS to remove the unbound TNF- α cytokines and incubated again with antibodies Anti-TNF- α labelled with Rhodamine (Rh) for 1 h to form a sandwich antibody-cytokine-antibody@Rh (Fig. 2B). Finally, the sample was rinsed with distilled water and dried with nitrogen for fluorescence microscopy characterization.

Fluorescence microscopy (Zeissaxioplan 2 imaging apparatus, equipped with a monochrome camera, 10x and 40x lenses) was used to take the fluorescence images. Samples (positive and negative tests) were observed by fluorescent light. Antibodies Anti-TNF- α labelled with Rhodamine (Rh) were excited with a 550 ($\pm$ 25) nm band-pass filter and fluorescence from the sample was observed with a 605 ($\pm$ 70) nm band-pass filter.

2.5. Contact angle measurements

Data Physics Instruments digidrop (Germany) has been used to measure the contact angle measurements (CAM). A droplet of 5 μ L of deionized water was deposited on the silicon nitride surface, and the CAM was applied after each chemical surface modification. Five CAM were recorded for each chemical modification step.

2.6. Antigen dilution and artificial saliva preparation

The antibodies Anti-TNF- α , cytokines TNF- α , IL-10, and Cortisol have been reconstituted following the protocol of the supplier in PBS (10 mM, pH 7.4) and aliquoted at 20 μ L to get a final stock with a concentration of 125 μ g/mL, 33 μ g/mL, 10 μ g/mL and 50 μ g/mL respectively. Our standard solutions of the TNF- α cytokines as well as the interferences IL-10 and Cortisol were prepared within the same range of concentration of 1 pg/mL to 30 pg/mL by dissolving the appropriate quantity of cytokines in PBS (10 mM, pH 7.4). The same procedure was used for the AS test by dissolving the appropriate amount of cytokines stock solution in AS. This latter was accurately prepared by dissolving in 200 mL 0.12 g of Na₂HPO₄, 0.12 g of CaCl₂, 0.08 g of KCL, 0.08 g NaCl, 0.8 g of mucin and 0.8 g of urea in deionized water [28]. The pH of the obtained solution was gradually adjusted to 7.4 by appending NaOH and finally aseptically stored at -4 °C until use.

2.7. Mott-Schottky analysis

A three-electrode system has been used for electrochemical detection by using; a platinum counter electrode (Pt), a silver/silver chloride (Ag/AgCl) reference electrode and the biosensor acted as a working electrode. This latter was sandwiched between two parts of a conventional 1 mL electrochemical Teflon cell. Electrical contact was taken from the backside of the biosensor [41,42]. The Mott Schottky experiments were associated with a VMP3 potentiostat monitored by EC-Lab software (Biologic Science Instruments, France). The Mott-Schottky analyses were performed by sweeping the potential from -0.5 V to +4 V at a constant frequency of 10 KHz and a step rate of 25 mV. All the measurements were carried out in PBS (10 mM, pH 7.4) and in AS with a constant pH = 7.4, at room temperature (20 $\pm$ 2 °C) inside a Faraday cage.

3. Results and discussion

3.1 Contact angle measurements

Contact angle measurements (CAM) were used after each chemical surface modification of the transducer in order to follow the hydrophilic property of the surface and thus check the successful chemical functionalization of the silicon nitride Si₃N₄ surface. The CAM results were summarized in Fig. 3, and illustrate the CAM evolution as a function of chemical surface modification. The CAM of bare Si₃N₄ surface was recorded at 60.7° (Fig.3a), which is in a good agreement with the literature results [43]. The CAM has decreased to 33.7° (Fig. 3b) after piranha activation due to the hydroxyl groups formed onto the silicon nitride surface. Afterwards and after functionalization with TESUD, the CAM has increased again to 56.3° (Fig. 3c) highlighting thus the hydrophobic character of TESUD due to its hydrocarbon chain.

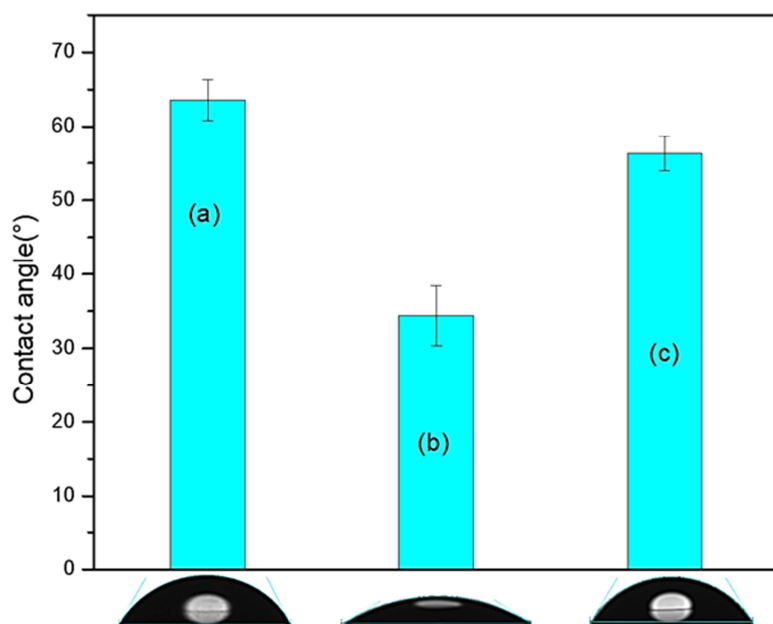


Fig. 3: CAM and images of water droplet evolution as a function of chemical surface modification of (a) bare Si_3N_4 surface after acetone and ethanol cleaning, (b) after piranha oxidation (c) and after TESUD formation.

3.2 Fluorescence characterization

Fluorescence characterization has been used as a rapid tool to ensure the bio-recognition process between the antibodies Anti-TNF- α and its corresponding cytokines TNF- α . This latter has been sandwiched between the first antibody Anti-TNF- α previously immobilized onto Si_3N_4 and the second antibody labelled with Rhodamine Anti-TNF- α @Rh to form a sandwich Anti-TNF- α <TNF- α >Anti-TNF- α @Rh (Fig.2B). The rhodamine outward from the surface indicates the well formation the sandwich and thus the successful biorecognition.

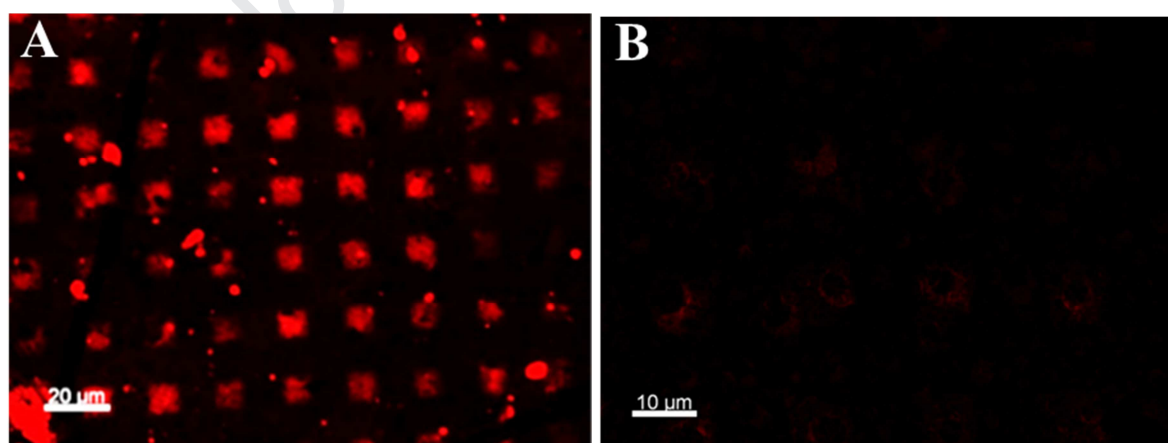


Fig. 4. Fluorescent images of A) the successful formation of the sandwich Anti-TNF- α <TNF- α >Anti-TNF- α @Rh and B) negative test by using IL-10 instead TNF- α cytokines.

The μCP has been used to create the required pattern through the PDMS stamp as previously described. Here the sandwich Anti-TNF- α <TNF- α >Anti-TNF- α @Rh (red squares Fig. 4A) will be formed only onto the patterns previously functionalized with TESUD. Fig. 4A shows a homogenous

pattern of the fluorescent Anti-TNF- α @Rh and demonstrates the successful immobilization of Anti-TNF- α antibodies onto Si₃N₄ and also the successful detection of TNF- α . No fluorescence was observed onto the blocked area with OTS which indicate the absence of nonspecific adsorption. Negative tests were made to prove that what was observed in the first test was well the biorecognition and not the only adsorption. The same procedure of sandwich was repeated by using this time IL-10 cytokines instead of TNF- α . Here no fluorescent has been observed as there was no recognition process (Fig.2B).

This fluorescence test was made before electrochemical detection to prove that what will be detected is well the biorecognition between Anti-TNF- α antibodies and their corresponding cytokines TNF- α and not the nonspecific adsorption.

3.3 Mott-Schottky results

3.3.1 Electrochemical parameters optimisation

Mott-Schottky analyses have been used to study the semiconducting behaviour of our biosensor based on silicon nitride substrate after TESUD functionalization and Anti-TNF- α immobilization. The capacitance measurements as a function of the applied potential with different frequencies ranging from 50 Hz to 10 KHz curves recorded in PBS buffer solution were presented in Fig. 5. The capacitance responses were typical with the appearance of the three known regions: the accumulation, the depletion and the inversion regions [41]. This test has been done to optimize the frequency and the potential, which were two specific and significant parameters for the Mott-Schottky analysis technique. The final potential range and frequency were chosen from -0.5 V to 2.5 V and at 10 KHz respectively as it gives a better capacitance behaviour.

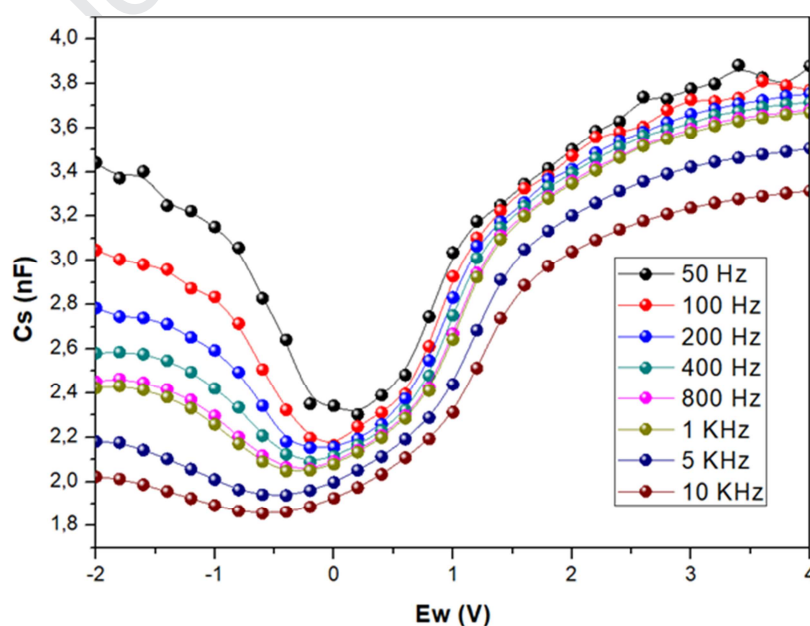


Fig. 5: Capacitive measurements as a function of the applied potential for different frequency ranges.

3.3.2 Detection and interference study of TNF- α in PBS

The detection of TNF- α cytokines at various concentrations was presented in Fig. 6A. Here, the capacitance response was normalized by dividing the capacitance of the substrate value (C_s) by the minimum value (C_{min}) of each measurement curves of C (V). The Capacitance response was presented as C_s/C_{min} in function of the applied potential of the working electrode (E_w). The first curve from left to right (Fig. 6A), corresponds to the biosensor with Anti-TNF- α before any detection. The biosensor was then left within the cell and was incubated in 1 pg/mL of TNF- α cytokines in PBS at 4 °C for 30 min. Then, the biosensor was rinsed gently with PBS to remove the excess of the cytokine TNF- α adsorbed onto the surface. Finally, the Teflon cell with the biosensor was placed again inside the faraday box for Mott-Schottky analyses (Fig. 6A). Here the second curve corresponding to 1 pg/mL has shifted from the first showing thus a difference of potential equivalent to a flat band voltage variation. By increasing the TNF- α concentration, the flat band voltage increases, also showing the detection phenomenon. The biosensor sensitivity was then obtained by measuring the slope of the potential variation in function of TNF- α cytokines concentration.

Fig. 6.B illustrates the calibration curve of the biosensor within a linear range of 1-30 pg/mL of TNF- α . E_0 is the potential of Anti-TNF- α without any cytokine concentration, and E is the extracted potential of different TNF- α concentration utilizing the tangential method. The fabricated biosensor provides a good sensitivity of 4 mV/pM and a correlation coefficient of 0.99. The LOD was calculated as $3.3 \times \text{SD}/\text{slope}$ and was found at 0.38 pg/mL. The specificity of the developed biosensor was also studied by using IL-10 and Cortisol instead TNF- α through the same structure and the same experimental process based on TESUD/Anti-TNF- α antibodies immobilization. The detection of the two interferences was made within the same linear range of TNF- α cytokines. The potential variation of IL-10 and Cortisol shows the specificity and sensitivity of the biosensor for TNF- α when compared to the interference.

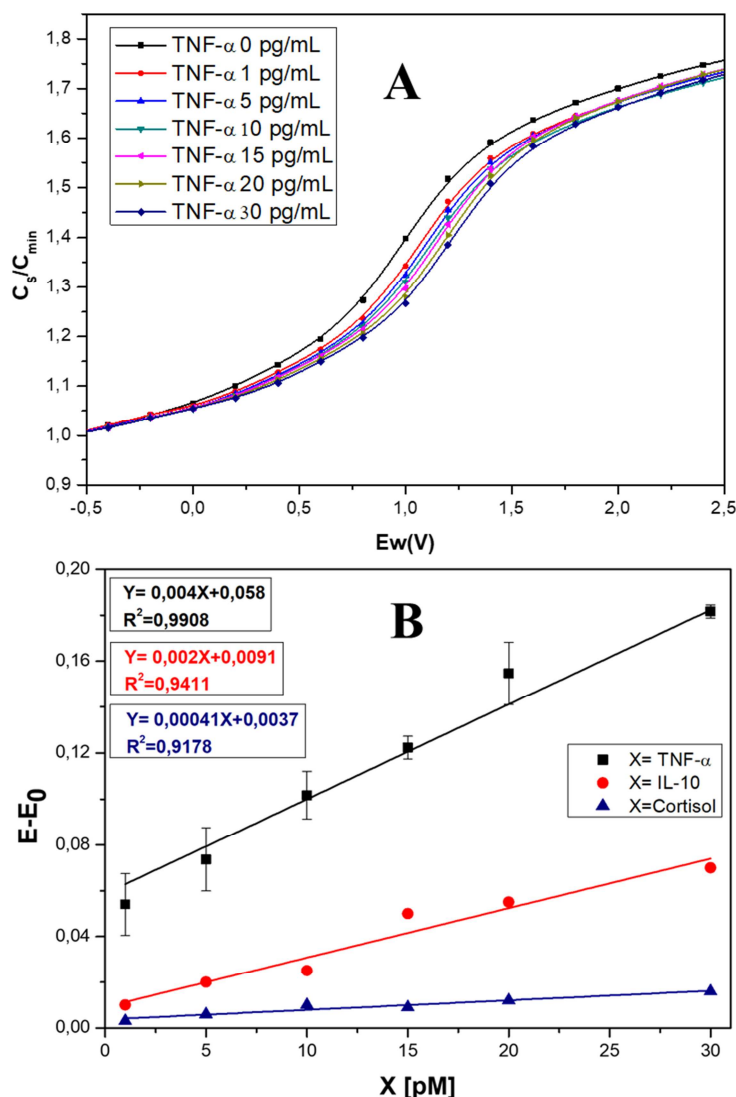


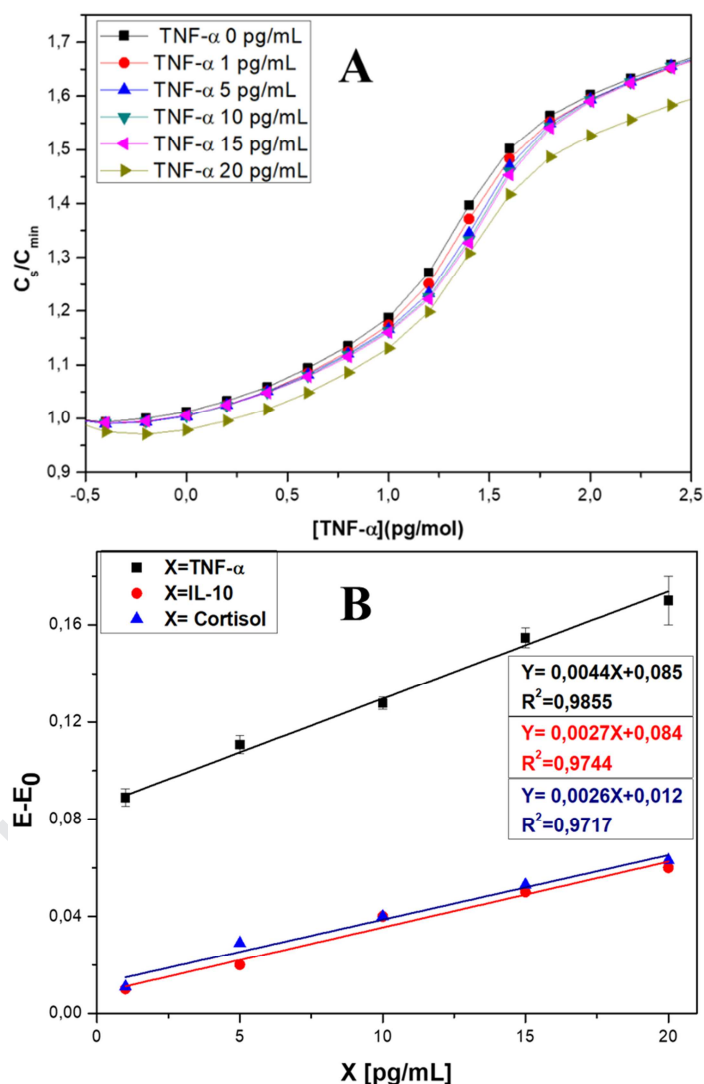
Fig. 6: (A) Mott-Schottky plots of $TNF-\alpha$ detection in PBS, **(B)** the detection sensitivity curves of $TNF-\alpha$, IL-10 cytokines and cortisol in PBS.

Until this stage, the interactivity of the developed biosensor and cytokines detection was achieved in PBS. In order to explore more the performances of the immunosensor, Mott-Schottky analysis was carried out in AS.

3.3.3 Detection and interference study of $TNF-\alpha$ in Artificial saliva

Different concentrations of $TNF-\alpha$ (1, 5, 10, 15 and 20 pg/mL) were prepared directly in AS instead PBS. The biosensor was then incubated for each concentration and followed by Mott-Schottky analysis for each concentration as previously described. Here, the working electrode was again sandwiched within the same Teflon cell and filled with 1 mL of PBS as electrolyte solution for electrochemical analyses. The normalized capacitance response (C_s/C_{min}) as a function of the applied potential for different $TNF-\alpha$ concentrations was presented in Fig. 7A. The evolution of the difference of flat band voltage variation as a function of cytokines concentration highlights the detection phenomenon in AS.

1 The calibration curve of TNF- α detection shows a linear regression equation $(E-E_0) = 0.0044[\text{TNF-}\alpha] + 0.085$ and an $R^2 = 0.985$ (Fig. 7B). The same study was also repeated with both interferences IL-10
 2 and Cortisol in AS. Here the biosensor was slightly more sensitivity toward TNF- α cytokines with a
 3 LOD of 1 pg/mL (with a sensitivity of 4.4 mV.pM⁻¹ and an $R^2 = 0.985$) when compared to IL-10
 4 (with a sensitivity of 2.7 mV.pM⁻¹ and an $R^2 = 0.974$) and Cortisol (with a sensitivity of 2.6
 5 mV.pM⁻¹ and an $R^2 = 0.971$).
 6



8 **Fig. 7: (A)** Mott-Schottky plots of Ag-TNF- α detection in AS, **(B)** detection sensitivity curves of Ag-TNF- α , IL-
 9 10 cytokine and cortisol in AS.

11 Table 1 lists a comparative study of different LOD reached, linear range and the technique used
 12 reported in the literature to measure TNF in both artificial and human saliva samples.

Table 1: Comparison of different electrochemical immunosensors for TNF- α detection

Technique used	Analyte	Linear range	LOD	Reference
Impedance spectroscopy	Artificial saliva	1–100 pg/mL	1 pg/mL	[28]
Amperometry	Artificial saliva	1–30 pg/mL	1 pg/mL	[4]
Amperometry	Artificial saliva	1–15 pg/mL	0.3 pg/mL	[44]
Impedance spectroscopy	PBS	0.01–2 pg/mL	3.7 fg/mL	[45]
Differential pulse voltammetry	Serum	0–1000 ng/mL	37 ng/mL	[46]
potentiometric	Serum	0.1–1 mg/L	0.015 mg/L	[47]
Amperometry	Saliva	1–200 pg/mL	0.85 pg/mL	[48]
Impedimetric	Artificial saliva	1–15 pg/mL	1 pg/mL	[5]
Mott Schottky	Artificial saliva	1–30 pg/mL	1 pg/mL	This work

Although the developed biosensor did not exhibit the best limit of detection as presented in table 1, however, it is still, an active competitor to the gold transducer in term of protocol complication and stability material.

Conclusion

The present study aimed to explore the development of a novel capacitance electrochemical biosensor based on $\text{Si}_3\text{N}_4/\text{SiO}_2/\text{Si}/\text{Al}$ structure utilizing Mott-Schottky analysis for the specific detection of TNF- α . Silicon nitride was used in this study to its known high stability in the literature, and it starts to become a good competitor to the gold transducer. Here, and under optimized conditions, the developed electrochemical capacitance biosensor provides a high sensitivity of 4 mV.pM^{-1} and 4.4 mV.pM^{-1} in PBS and in AS respectively and LOD of 0.38 pg/mL and 1 pg/mL in PBS and AS respectively. The immunosensor was highly selective in PBS when compared to AS. The selectivity degradation in AS compared might be explained by the matrix effect of saliva composition, which prevents the specific detection of TNF- α . Therefore, magnetic nanoparticles could be integrated in the future onto silicon nitride transducer [44,49,50] to enhance the sensitivity of this immunosensor within complex physiological mediums such as human saliva or blood samples

Acknowledgements:

The authors acknowledge the financial support from the European project KardiaTool [Grant No. 768686].

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- Fabrication of a capacitance electrochemical immunosensor based on silicon nitride transducer (Si₃N₄/SiO₂/Si[P]/Al).
- Silicon nitride surface was activated and chemically modified with TESUD to immobilize antibodies Anti-TNF- α .
- Micro-contact printing, Fluorescence microscopy and contact angle measurement (CAM) characterization were carried out during the bio-functionalization of the biosensor surface.
- Mott-Schottky electrochemical analyses were carried out for TNF- α cytokine's detection.
- The biosensor has exhibited a high sensitivity and a good selectivity for TNF- α cytokines when compared to the other potential interferences such as IL-10 and Cortisol.
- Interesting LOD of TNF- α cytokines was found at 0.38pg/mL in PBS and 1pg/mL-1 in AS.

CONFLICT OF INTEREST STATEMENT

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We the undersigned, Professor Abdelhamid Errachid, Head of Department Micro and Nano BioTechnology at Institut des Sciences Analytiques, Université Claude Bernard Lyon 1,

Attest that **we have no conflict of interest to declare** for the present article entitled “Capacitance Electrochemical Biosensor Based on Silicon Nitride Transducer for TNF- α Cytokine Detection in Artificial Human Saliva : Heart Failure (HF)”

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The authors names mentioned above certify that they have NO affiliations with or involvement in any organization or entity with any financial, or non-financial interest in the subject matter or materials discussed in this manuscript.

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