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# Electrochemical biosensor platform for TNF-α cytokines detection in both artificial and human saliva: Heart Failure

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## Highlights

- We manufactured an electrochemical biosensor platform for cytokines detection.
- Matrix effect has been studied in both artificial and real human saliva.
- Electrochemical detection of TNF-α cytokines has been carried out in artificial and human saliva.
- Electrochemical impedance spectroscopy (EIS) was used for human TNF-α cytokines detection.
- The immunosensor was highly sensitive and selective for TNF-α cytokines when compared to other interfering cytokines.

## Abstract

In the last decades, the possibility to assess physiological states, monitor disease onset and progression, as well as monitor post-treatment therapeutic outcomes through a non-invasive approach became one of the most desirable goals for healthcare research. In this respect, 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. In the present study we have developed a highly sensitive biosensor for TNF-α detection in human saliva. Therefore, a fully integrated electrochemical biosensor platform 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 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) combined with standard added method was used to characterize the modified gold microelectrodes as well as for

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the detection of TNF-α both in standard solution and in artificial saliva. The high selectivity of the biosensor platform was demonstrated by analysing solution containing other cytokines may representing possible interferences. TNF-α cytokines were analysed in PBS buffer, artificial saliva and real human saliva within the range 1-100 pg/mL which is the critical range for patients suffering from heart failure. The first preliminary results of EIS analyses in human saliva gave a result of 3.1 pg/mL which is very promising for rapid analysis for cytokines detection.

**Keywords**: Biosensor platform; heart failure; real human saliva; TNF-α cytokines, EIS.

## 1. Introduction

Heart failure (HF) is a complex clinical syndrome caused by a wide range of cardiovascular disorders, such as structural or functional abnormalities of the heart, that result in the impairment of the heart ability to fill or to pump out blood, which translated as an abnormality of cardiac structure or function leading to failure of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues [1]. Pathophysiology of HF is exceedingly complex and HF patients are also characterized by systemic inflammation, highlighted by raised circulating levels of several inflammatory cytokines (e.g. tumor necrosis factor-alpha, TNF-α, or interleukins, ILs) with increasing levels according to the degree of disease severity [2-5]. TNF-α and several ILs are involved in the remodelling and neurohormonal activation pathways, and evidence from both experimental and clinical trials indicates that inflammatory mediators are of importance in the pathogenesis of chronic HF contributing to cardiac remodelling and peripheral vascular disturbances [6].

The possibility to measure these specific biomarkers could result in an immediate information about the first signs of inflammation and it is well known that an early detection of disease plays a crucial role in successful therapy. Moreover, a wide spectrum of compounds (including TNF-α and ILs) are present in saliva, and their determination may provide information for clinical diagnostic applications [7-9]. Chronic HF patients have high circulating levels of TNF-α, which correlate with the severity of their disease [10]. Several studies had also demonstrated that concentrations of TNF-α in saliva may reflect those in serum [11, 12].

The advantages of saliva as a diagnostic tool, which offers distinctive advantages over serum (i.e. painless, non-invasive and stress-free collection, potentiality for real-time monitoring, etc.), are currently well known [7-9, 13, 14], thus the use of saliva for monitoring of diseases, including HF, and general health has become a highly desirable goal in healthcare research and promotion.

In recent years, different bio-sensing techniques have been used for the detection of biomarkers related to cardiovascular diseases [15]. However, a majority of these techniques require adequate transducing elements, for instance fluorescent dyes or enzymes, to generate a signal which gives 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

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detection of biomolecules. To overcome these problems, a highly sensitive biosensor were developed for the detection of the specific biomarkers [16-20]. These biosensors provide fast, real-time, and reliable medical diagnosis in order to enhance the biomarkers detection at minute concentrations allowing the prediction of the first signs of inflammation. We have already published studies on electrochemical biosensors for cytokines detection in different medium and using different transducers based on different materials [21-23].

In this paper we present preliminary results on the fabrication of an electrochemical biosensor platform biosensors for multiple cytokine detection. This contains eight gold working microelectrodes (WEs) allowing a simultaneous detection. Cyclic voltammetry (CV) was applied during the microelectrode functionalization process to confirm the mAb immobilization and to characterize the gold microelectrode surface properties. Then, Electrochemical Impedance Spectroscopy (EIS) characterization was applied onto the gold WEs for TNF-α detection by the corresponding immobilized antibodies anti-TNF-α (AbTNF-α).

Finally, the biosensor platform was used to detect TNF-α both in aqueous solution and artificial saliva. The biosensor was found to be highly sensitive to TNF-α within the range 1–15 pg/mL in PBS when compared to other interferences (e.g. other cytokines such as IL-1 and IL-8) in artificial human saliva. Preliminary test on real human saliva were also performed, and related results have proven that our biosensors can represent a promising tool for rapid cytokines detection.

## 2. Experimental

### 2.1 Chemical and Reagent

Reagents used in this research are: 4-aminophenylacetic acid (4-carboxymethylaniline CMA), sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), ethanol, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), ethanolamine, phosphate-buffered saline (PBS), phosphate-buffered saline Tween (PBS-Tween) and ($Fe^{2+}Fe^{3+}$) were purchased from Sigma Aldrich (France). Antibodies Tumor necrosis factor TNF-α, Recombinant Human TNF-α, Recombinant Human IL-8 and Recombinant Human IL-1, were purchased from R&D System (France).

### 2.2 Antibodies (Ab) and antigens (Ag) solutions

According to the protocol provided by the supplier, antibodies and antigens have been diluted in PBS buffer (pH 7.4) to obtain a stock solution with a final concentration of 10 µg/mL. This latter was then divided into aliquots and stored at -20°C. The standard solutions containing different concentrations of cytokines antigens (1, 5, 10, and 15pg/mL) used to investigate the sensitivity of the biosensors were prepared by dissolving the appropriate amount of the stock solution in PBS. All the standard solutions were prepared by weighing.

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### 2.3 TNF-α detection in artificial saliva

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 [24].

Standard solutions used to detect TNF-α in AS were prepared by dissolving the appropriate amount of the stock solution (10 μg/mL) in AS, obtaining solutions with different concentrations of AgTNF-α (40, 50, 60, 70 pg/mL).

To perform the standard addition method, a standard solution at 115 pg/mL of AgTNF-α was prepared by diluting the appropriate amount of the stock solution of 10 μg/mL of recombinant Human TNF-α in AS.

To prepare the *unknown sample*, the AgTNF-α stock solution of 10 μg/mL Ag-TNF-α was diluted in AS obtaining an *unknown sample* containing 103 pg/mL of AgTNF-α. A constant volume (50 μL) of the *unknown sample* was then added to each of five volumetric flasks of 1 mL. To reach a final volume of 1 mL, 950 μL of AS were added to the first flask containing the unknown sample (Level 1). The AgTNF-α standard solution of 115 pg/mL is then added in increasing volumes (50, 150, 350, and 500 μL, namely corresponding to the addition of 5, 15, 30 and 60 pg/mL of AgTNF-α) to the subsequent flasks and each flask was then made up to volume (1 mL) with AS. All the standard solutions and the *unknown sample* were prepared by weighing.

### 2.4 TNF-α detection in human saliva

To perform a preliminary test of biosensor platform with a real sample, a stimulated saliva sample was collected from a nominally healthy volunteer (female, 26 years) according to a sampling procedure described elsewhere [25]. Briefly, the saliva sampling was performed using the sterile synthetic swab Salivette® (Sarstedt, Germany) as sampling device. The volunteer was asked to move the swab 2 min in the oral cavity at a self-selected pace. Saliva was then recovered by centrifugation of the swabs at 5000 rpm for 5 min at room temperature (20 ± 2 °C). The sample volume was calculated from the different weights of the swab before and after sampling assuming the saliva density to be 1.0 g/mL [26].

A standard solution at 135 pg/mL of Ag TNF-α was prepared by diluting the appropriate amount of the AgTNF-α stock solution of 10 μg/mL.

Similarly to what have been done in 2.3 working with AS, a constant volume (2 μL) of the real sample was then added to each of three volumetric flasks of 1 mL. To reach a final volume of 1 mL, 998 μL of PBS were added to the first flask containing the real sample (Level 1). The standard solution containing the analyte was then added in increasing volumes (20 and 50μL, namely corresponding to

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the addition of 3 and 7pg/mL of AgTNF-α, respectively) to the subsequent flasks and each flask was then made up to volume (1 mL) with PBS. All the solutions were prepared by weighing.

### 2.5 Electrochemical characterization

All electrochemical measurements were carried out at room temperature (20± 2 °C) in a Faraday box using a VMP3 potentiostat (BioLogic Science Instruments, France) controlled by EC-Lab software (version 10.40, BioLogic Science Instruments, France). Cyclic Voltammetry (CV) was used for gold microelectrodes characterization using the analyst $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at 5 mM in PBS buffer at pH7.4. This technique was also used for CMA deposition, as described in [27,28]. Briefly, a 3 mM CMA solution was prepared in water with 15mM of HCl and 15 mM $NaNO_2$. The solution was kept in ice under stirring for 10 min and then was directly used to perform CMA electrodeposition. Four CV cycles were applied to the gold WEs placed inside the CMA solution, resulting sufficient to cover all WEs surface with CMA.

Electrochemical impedance spectroscopy (EIS) measurements were used to evaluate the recognition properties of the biosensor platform, in terms of sensitivity and selectivity. During the measurements the potential was kept at 0.228 V versus the integrated Ag/AgCl reference electrode [21]. Data acquisition and analysis were accomplished using EC-Lab software. The device was then used to detect TNF-α in PBS within the range 1 pg/mL to 15 pg/mL and within other TNF-α concentration ranges for AS and real saliva. Therefore, antibodies anti-TNF-α (AbTNF-α) were immobilized onto gold microelectrodes through CMA molecules. For analyte testing, the biosensor was subjected to successive incubations with standard solutions containing different concentrations of human TNF-α antigens (AgTNF-α), for 30 minutes 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 5mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS buffer at pH7.4.

## 3. Results

### 3.1 Bio-Functionalization of gold surface

The fully integrated biosensor platform (Fig. 1A) used for TNF-α detection was previously fabricated and characterized for many complex medium [20, 21]. The technology of its fabrication was recently detailed in previous paper [21]. 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. Antibodies anti-TNF-α were immobilized onto gold microelectrodes through CMA molecules. These have been electrochemicaly deposited onto gold WEs by using cyclic voltammetry (CV) technique [27]. Fig. 1B shows cyclic voltammogram performed in

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$K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (5 mM) in PBS (pH 7.4) for gold WE before and after CMA deposition. The scan rate was measured at 80 mV/s and the switching potential was scanned between -0.2 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 0.1 M to remove the excess of EDC/NHS and immediately incubated in PBS solution containing AbTNF-α at 10 µg/mL for 1hour at room temperature (20 ± 2 °C). Afterward, the remained active carboxylic acid groups have been deactivated by incubation in ethanolamine solution (1% in PBS) for 20 minutes at room temperature. This step is very important to prevent nonspecific bonding at the detection stage.

### 3.2 Detection and Interference of TNF-α in PBS

Complex impedance plots of the platform biosensor at different concentrations of TNF-α are shown in Fig. 2A in Nyquist plot presentation. The first semi-circle corresponds to the immobilized antibodies anti-TNF-α. After the incubation of the biofunctionalized WE in 1pg/mL of TNF-α, the second Nyquist plot semi-circle has increased from the first showing thus an increase in impedance which confirm the biorecognition between antibodies and cytokines (Fig. 2A). Nyquist plot semi-circles have continued to increase by increasing the concentration of TNF-α antigens (AgTNF-α) which highlight the good sensitivity of the biosensor. Nyquist plot semi circles were then fitted using Randles equivalent circuit model (Figire 2A inset) where Rs (solution resistance), Rct (charge-transfer resistance), W (Warburg impedance) and Q1 (constant phase element, an equivalent model of double-layer capacitance) [30]. The real part of impedance (Re(Z)) increased as function of cytokines concentration. This was due to the increase of the Rct between gold WE and electrolyte as the adsorption (detection) of cytokines increase also onto the WE. Therefore, the increase of Rct is directly related to the sensitivity of the biosensor. The normalized data were presented as ΔRct/Rct ( where ΔRct = Rct (cytokines) − Rct (antibodies) / Rct(antibodies)) as a function of TNF-α concentration is shown in Fig 2B. The biosensor was highly sensitive to TNF-α cytokines when compared to other interferences. Therefore, in order to investigate the selectivity, additional tests were performed using solutions containing other cytokines (IL-1 and IL-8) secreted in acute stage of inflammation (Fig. 2B).Not all these cytokines are present in saliva; however they have been used only to prove the selectivity of biosensor. To this end, new biosensors have been biofunctionalized with TNF-α antibodies using the procedure previously described, and then tested by EIS with other cytokines (e.g. IL-1 and IL-8) instead of TNF-α. Here the biosensor was highly selective and sensitive to TNF-α if compared to other cytokines such as IL-1 and IL-8 (Fig. 2B) which confirm the high selectivity of the biosensors. At this stage, the interactivity of the biosensor and cytokines detection

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was performed only in PBS buffer. In order to test the performance of this biosensor, EIS analyses were made in both artificial saliva and human saliva.

### 3.3 Detection of TNF-α in Artificial Saliva

Artificial saliva was prepared as described in the experimental section and used to simulate real saliva. Here the same principle of EIS analyses was made for TNF-α detection using artificial saliva as buffer. First, in order to test the no specific adsorption, the biosensor previously biofunctionalized with AbTNF-α was incubated for 30 min many times in artificial saliva without any TNF-α cytokines (Fig.3A). Here the first Nyquist plot semi-circle corresponds to the antibodies immobilized onto microelectrodes. After the first incubation in artificial saliva, the second Nyquist plot semi-circle has shifted from the first show an increase in impedance which mean adsorption (and not detection) as there is no TNF-α in the solution. The first shift is related to the matrix effect. This unspecific adsorption was observed in all measurements performed on saliva samples, and thus considered as a constant. We have recently published an article on cytokines detection in real human plasma for patients suffering from heart failure and we have observed the same phenomenon of the first shift related to matrix effect. After many test we have demonstrated that this shift could be considered as constant and it is reproducible [31].

The biosensor was rinsed abundantly with PBS and incubated another time in artificial saliva for 30 min in order to see if there is an increased phenomenon of adsorption. Fortunately there is no more adsorption as all Nyquist plot were superimposed even after the third incubation in artificial saliva (Fig. 3A). This means there is no more increase in impedance and thus no more adsorption.

In order to observe deeply the effect of artificial saliva on the EIS analyses, different concentration of TNF-α were prepared using artificial saliva as buffer to make dilution of these cytokines.

The big shift between the first and the second Nyquist plot semi-circles (Fig. 3B) was observed after the first incubation of the biosensor in artificial saliva containing 40 pg/mL of TNF-α. This shift does not correspond totally to TNF-α detection but it could be attributable to nonspecific adsorption phenomena, which were also observed in the previous test. However, the Nyquist plot semi-circles have also increased with increasing concentration of TNF-α, highlighting the TNF-α detection in artificial saliva if compared to the previous test. Therefore, the biosensor was able to detect specifically AgTNF-α within a complex physiological medium.

### 3.4 Detection of TNF-α in Human Saliva

The biosensor was biofunctionalized with anti-TNF-α and tested for real human saliva. As made for artificial saliva, here the biosensor was incubated different time in human saliva in order to have an idea about the nonspecific bonding in real physiological medium (Fig. 4A). The first Nyquist plot semi-circle corresponds to the antibodies anti-TNF-α. After the first incubation in human saliva, the

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second Nyquist plot semi-circle has shifted from the first showing an increase in impedance which means (adsorption and detection) as there is TNF-α in human saliva even for healthy people. The biosensor was rinsed abundantly with PBS and incubated another time in human saliva for 30 min in order to observe if there is an increased phenomenon of adsorption or detection. In the contrary of artificial saliva, here Nyquist plot has increased for the second and the third incubation maybe due to TNF-α detection. At this stage of analysis it is immature to affirm that the impedance increases due to the detection as there is other biomolecules in human saliva which can interfere with TNF-α.

The same previous experiment was repeated by using saliva diluted with PBST (PBS with tween surfactant) aiming to decrease no specific bonding if there is (Fig.4B). As shown in this figure, there is always increase in Nyquist plot semi-circles which could be certainly due the TNF-α detection.

In order to understand clearly the detection phenomenon of cytokines in human saliva, EIS analyses was combined with standard addition method in order to quantify the cytokines concentration in saliva.

### 3.5 Detection in artificial saliva by the standard addition method

The standard additions method can be used to determine the concentration of an analyte that is in a complex matrix (i.e. biological fluids, soil samples, etc.) once the analytical method was proven linear over the whole of the required working range. This method involves adding known quantities of a standard solution with a known concentration (the *standard solution*) to a sample of interest with unknown concentration (*unknown sample*) and measuring the *standard* solution's analytical signals in response to each *addition.*

The Z-fit analysis [30] was performed on each Nyquist impedance plot and the data were plotted with volume standard added in the x-axis and the $(R_{Ag}-R_{Ab})/R_{Ab}$ value in the y-axis where $R_{Ag}$ and $R_{Ab}$ are the transfer charge resistance of cytokines and antibodies respectively. Linear regression was performed and the slope (m) and y-intercept (b) of the calibration curve ($R^2 = 0.995$, Fig. 5B) were used to calculate the concentration of analyte in the *unknown sample*. The experimental concentration of Ag TNF-α in the *unknown sample* resulted 104 ± 3 pg/mL.

Fig. 5A shows Nyquist impedance plots at different volumes (and therefore at different concentrations) of TNF-α standard solution added to the *unknown sample*.

### 3.6 Detection in real saliva by the standard addition method

The standard additions method combined with EIS was used to estimate TNF-α concentration in a human saliva sample (Section 2.4). In this case, the amount of TNF-α was unknown in this sample as it was not previously analysed with other technique. A standard solution at 135 pg/mL of AgTNF-α

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was prepared by diluting the appropriate amount of the stock solution of 10 μg/mL of AgTNF-α in PBS.

Similarly to what have been done for tests in AS, a constant volume (2 μL) of the real sample was then added to each of three volumetric flasks of 1 mL. To reach a final volume of 1 mL, 998 μL of PBS were added to the first flask containing the unknown sample (Level 1). The standard solution containing the analyte was then added in increasing volumes (20 and 50μL, namely corresponding to the addition of 3 and 7 pg/mL of AgTNF-α, respectively) to the subsequent flasks and each flask was then made up to volume (1 mL) with PBS. All the solutions were prepared by weighing. The biosensor has been incubated for 30 minutes at 4 °C in each solution, and then EIS measurements were performed as has already been described in 2.5.

As shown in Fig. 6, the impedance increased with increasing the concentration of AgTNF-α, implying detection of the analyte and not adsorption phenomena attributable to the matrix. The slope (m) and y-intercept (b) of the resulting calibration curve ($R^2$ = 0.987) were used to calculate the concentration of analyte in the in the sample, that resulted 3.1 pg/mL. This result should be confirmed by a reference technique (i.e. enzyme-linked immunosorbent assay, ELISA), in order to evaluate/investigate the matrix effect and to estimate the real concentration of cytokines in saliva. However, this result is in line with the salivary levels of this cytokine determined in saliva samples from groups of nominally healthy subjects [31-33].

## 4. Conclusion

The measurement of salivary TNF-α levels can result an important tool for the screening, diagnosis, and long-term management of patients with HF. A label-free, highly sensitive, accurate, fast EIS based biosensors able to detect TNF-α in saliva has been developed in the present study. A method to produce highly sensitive and specific biosensor for the detection of TNF-α from diluted physiologic medium has been described in this research. The level of interferences attributable to non-specific binding resulted very minimal as a good selectivity was observed in presence of other cytokines (e.g. IL-1 and IL-8).

Preliminary tests were performed in both artificial and real saliva confirming the capability of the device to detect TNF-α, showing a linear relationship between increasing TNF-α concentrations and charge-transfer resistance. Further investigation will be performed in order to validate the device using real samples collected from HF patients.

This biosensors can thus represent a promising bioanalytical tool for accurate quantification of TNF-α in saliva to monitor symptoms of inflammation in HF patients.

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Horizon 2020 research and innovation programme under grant agreement No 643694), for the development and validation of analytical methods and the development of biosensor devices for the detection in biological fluids of biomarkers related to heart failure.

**Dr. Abdoullatif Baraket** received his Bachelors of Science degree in physics option electronics in 2004 and his Masters of Science in scientific computing in physics and experimental high energy from the University of Hassan II Casablanca, Morocco in 2006 and 2008, respectively. In 2009, he also received his Masters of Science in physics and nanomaterials from the University of Maine. Between 2009-2013, he obtained his Ph.D. at the Université de Lyon. Afterwards, he was a postdoctoral researcher at the Commissariat à l'énergieatomique (CEA) in Grenoble, France. He worked within the framework of national project for the fabrication and characterization of an 'Implantable Biofuel Cell" (IBFC). From 2015, he is involved in the Sea-On-Chip project for the packaging, biofunctionalization, and microelectrodes fabrication for Irgarol and sulfonamide detection by electrochemical impedance spectroscopy (EIS) analysis. Also, he is involved within H2020 HEARTEN and microMOLE European Union projects for the development of biosensor devices for the detection of biomarkers.

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**Dr. Monica Vatteroni** was born in La Spezia, Italy, in 1975. She received the M.S. degree in electrical engineering from the University of Pisa, Pisa, Italy, in 2001, and the Ph.D. degree in physics from the University of Trento, Trento, Italy, in 2008. She has 15 years of experience both in industry and academy. She has collaborated with companies like ST Microelectronics and EUROTECH S.p.A. In the research field, she has collaborated for more than 5 years with Scuola Superiore Sant'Anna in Pisa for the development of vision system and sensor platforms for biomedical applications. Since May 2015 she is with Université Claude Bernard, Lion, France, as a post doc research fellow with a Marie Skłodowska Curie Project for excellence in research to investigate on innovative readout technique for biosensors applications. She is also co-founder of 2 SMEs and author and co-author of 7 patents and more than 30 papers in international journals and conferences.

**Dr. Nadia Zine** received her B.S. in Chemistry from the University M. Ismail, Meknes in 1994 and her Ph.D. from the Centro Nacional de Microelectrónica (CNM) Barcelona in 2005. Her research interest is the chemical characterization of silicon chemical sensors, and specially, of ISFET devices.

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**Prof. Joan Bausells**graduated in Physics in 1980 and received a Ph.D. in Physicsin 1986, both from the University of Barcelona, Spain. After working for 5 yearsin the semiconductor industry, he joined the National Microelectronic Center(CNM) in 1986. At CNM he has been involved in the development of microsen-sors, MEMS devices and nanofabrication capabilities, and has been Vice-directorfrom 2008 to 2012. His current research activities are focused on nanofabrication,micro/nanomechanical devices and their integration in sensor systems.

**Dr. Fabio Di Francesco** graduated in physics and received a Ph.D. in bioengineering at the Interdepartmental Center "E. Piaggio" – University of Pisa, where he was involved in researches concerning multisensor systems for environmental and food applications. In 2000-2006 he worked at the Institute of Clinical Physiology - CNR, where he further extended his research on sensors to the biomedical field. Since 2006, he is researcher at the Department of Chemistry and Industrial Chemistry of the University of Pisa. His current research activity is focused on the development of chemical and physical sensors, wearable sensor systems, and the identification of chemical markers in breath, saliva and sweat for the diagnosis of disease and the monitoring of health conditions.

**Prof. Roger Fuoco** has been a full professor in Analytical Chemistry at the Department of Chemistry and Industrial Chemistry of the University of Pisa since 1994. His research activity began in 1977 as researcher at CNR (Italian National Research Council), and his main area has been the development of both analytical procedures and hyphenated instrumentation for the characterization of real systems. Prof. Fuoco has carried out environmental research both in industrialised areas (such as the Venice lagoon system and the river Arno) and remote areas (such as Antarctica). He has been the co-ordinator of a research programme on the development of analytical methodologies funded by the CNR for many years, and the scientific supervisor of research units granted by the CNR national project on the protection and conservation of Cultural Heritage and the special project "Safeguarding the Venice Lagoon". He has been also scientific supervisor of a research unit within the national project "Rapid and innovative analytical methods for the analysis and control of GMO, and food-stuffs containing or obtained from GMO" granted by MIUR and ISPESL. Due to his expertise in the above areas he has been invited to take part in several national (IRSA-CNR) and European (ex-BCR-CCE) committees for the standardisation of analytical methods and the certification of reference materials. Moreover, he has been a member both of the Editorial Board of Annali di Chimica (Rome) and the Executive Committee of the Analytical Chemistry Division of the S.C.I. (Italian Chemical Society). Recently, Prof. Fuoco became part of a group of experts at the Consortium for the co-ordination of the research activity in the Venice Lagoon System (Co.Ri.La.), whose task was to draw up a manual of analytical methods for monitoring the water and sediment quality in this lagoon. Prof. Fuoco is currently a member of the Editorial Board of "Applied Spectroscopy Reviews". He acts as a referee both for several government, Italian and international agencies with regard to assessing research projects, and also for many international journals. He has recently been appointed as an assessor for the CIVR Register of Experts. In addition, he is a scientific supervisor of a research unit within the National Programme of Antarctic Research (PNRA) and a member of the steering committee of the research project "Polar Chemistry" within the same programme. Since 2001 he has been the co-ordinator of a research project of national interest (PRIN) on the development of

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combined instrumentation and innovative analytical methodologies and their application for the characterization of complex environmental and biological systems, founded by the MIUR (2000-02, 2002-04, 2005-07 and 2007-09). In particular, the last two projects were focused on the evaluation of the metabolic response of GM plants to abiotic stress. He is involved in in the HEARTEN Project (funded from the European Union's Horizon 2020 research and innovation programme under grant agreement No 643694), for the development and validation of analytical methods for the detection in biological fluids of biomarkers related to heart failure.

Prof. Fuoco is the author of about one hundred scientific publications whose most significant findings have been presented in more than 150 national and international congresses. He has also given many seminars both in Italy and abroad, and has patented some analytical instrumentation.

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**Arianna Menciassi**received the M.Sc. degree in physics (Hons.) from the University of Pisa, Italy, in 1995, and the Ph.D. degree from the Scuola Superiore Sant'Anna (SSSA), Pisa, in 1999. She is Full Professor of biomedical robotics and bioengineering at the SSSA. Her main research interests include medical mechatronics, bio-hybrid systems, biomedical micro and nanodevices, and robotic surgery. She possesses extensive experience through European projects and international collaborative projects on topics related to robotic and microrobotic diagnosis, surgery and therapy. She is the author of about 370 international papers (Source: Scopus), with more than 200 ISI journals, one edited book, and six book chapters on medical devices and microtechnologies. She is also inventor of 25 patents, national and international.

**Prof. Abdelhamid Errachid** graduated in physics from the University M. Ismail, Meknes, 1992 and received a PhD degree in electronic engineering from the Universitat Autonoma de Barcelona, Spain, in 1998. Since 2008, he had joined Laboratory of Analytical Sciences in Claude Bernard University-Lyon 1 as professor. He is nowinvolved in several national and European projects. His current research activity is focused on bioelectronics, biofunctionalization and nanobiotechnology.

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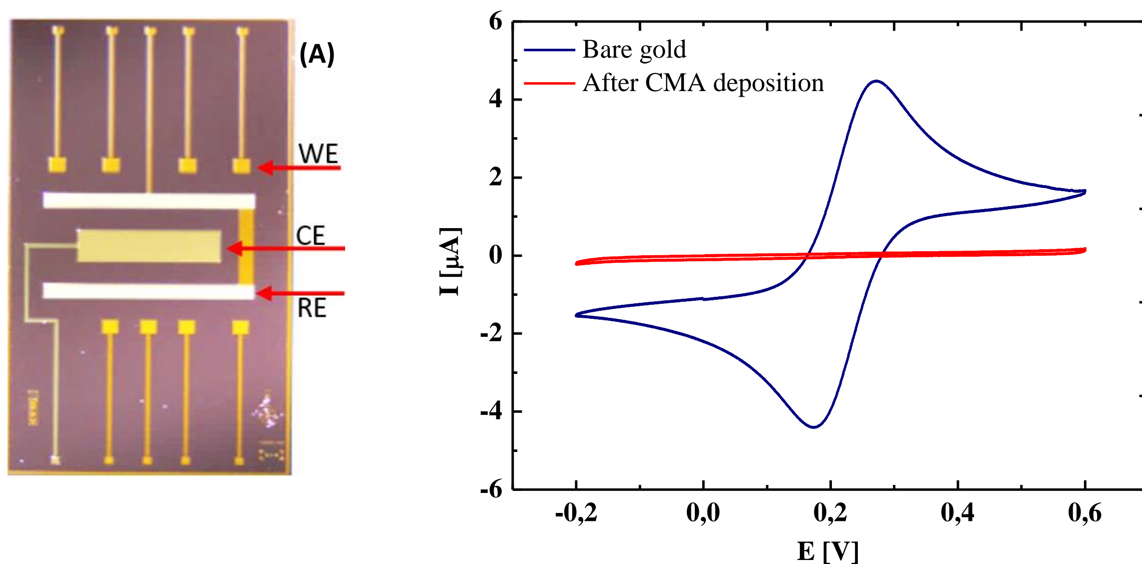


**Fig. 1:** **(A)** Biosensor platform containing eight gold working electrodes (WE), Ag/AgCl reference electrode (RE) and platinum counter electrode (CE). **(B)** Cyclic voltammograms of gold WE before (blue) and after (red) CMA deposition.

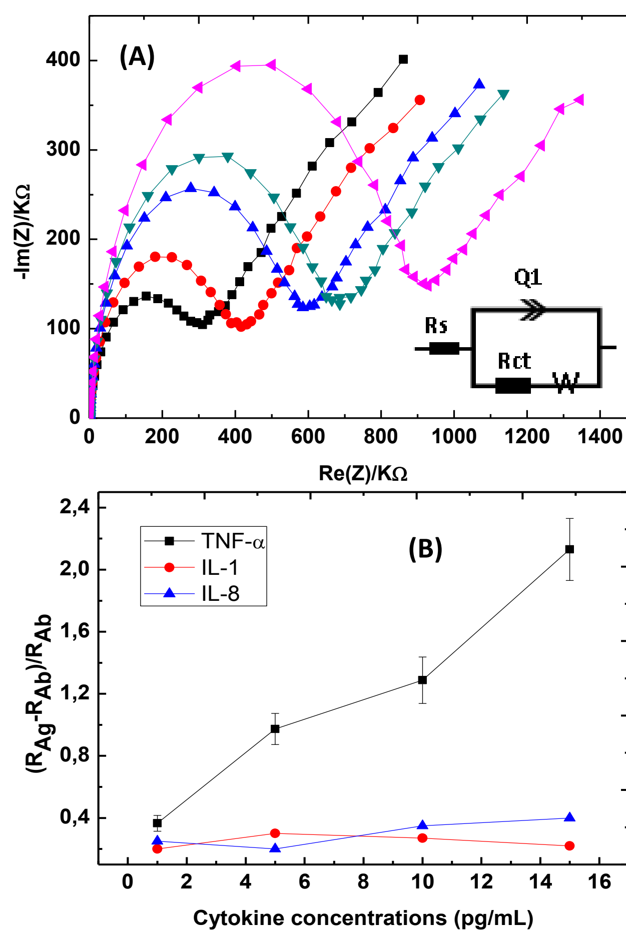


**Fig. 2.** **(A)** Nyquist impedance plot (Zr vs Zi: at 5mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS pH7.4 solution) at various concentrations of TNF-α: (■) 0 pg/mL; (●) 1 pg/mL;(▲) 5 pg/mL; (▼) 10 pg/mL; (◄) 15 pg/mL. **(B)** Sensitivity curves of the biosensor functionalized with AbTNF-α for the detection of (■) AgTNF-α (●) IL-1 and (▲) IL-8 cytokines.

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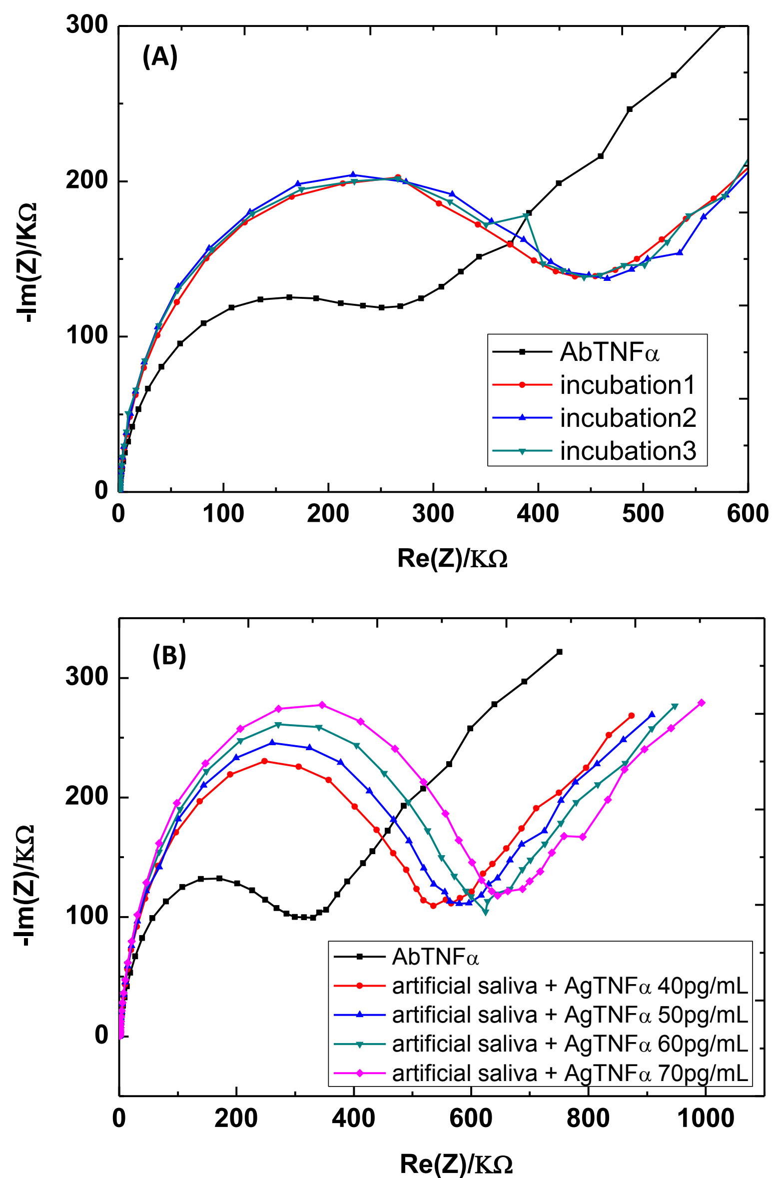


**Fig. 3**: EIS analyses for biosensor: **(A)** after several incubation in artificial saliva; **(B)** after several incubations in artificial saliva spiked with different TNF-α cytokines concentration.

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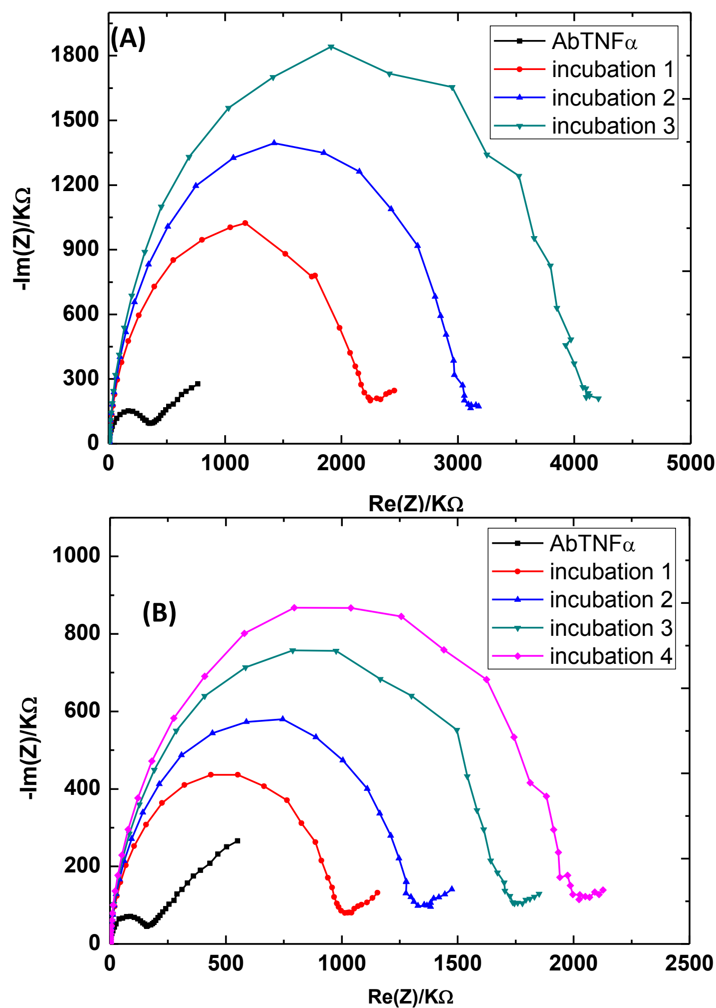
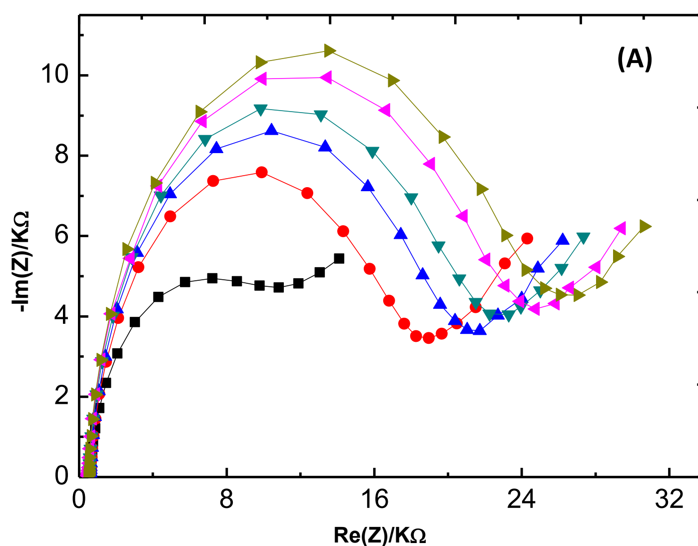



**Fig. 4**: (A) EIS measurements for different incubation of the biosensor in real human saliva. (B) EIS measurement for different incubation of the biosensor in real human saliva diluted in PBST (50mL of saliva + 950mL of PBST)



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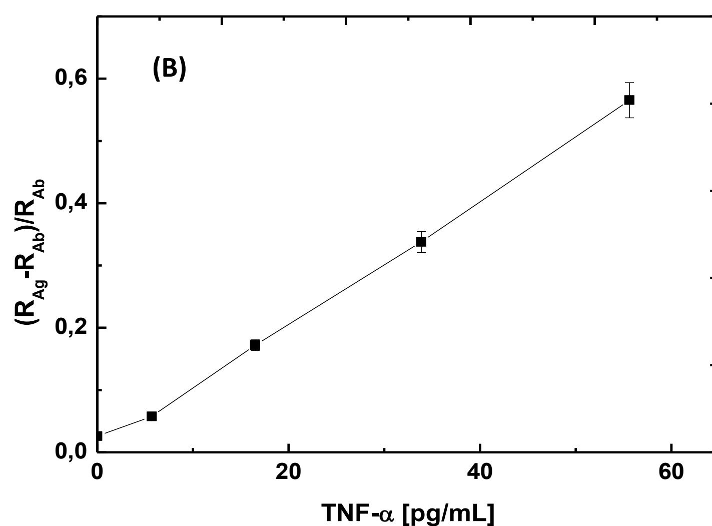


**Fig. 5**. **(A)** Nyquist impedance plots (Zr vs Zi: at 5mM of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ in PBS pH7,4 solution) of Ag TNF-α solutions analyzed by EIS to perform the standard addition method.(■)AbTNF-α for Ag TNF-α; (●) Level 1 (corresponding to an addition of 0pg/mL);(▲) Level 2 (addition of 5 pg/mL); (▼) Level 3 (addition of 15 pg/mL); (◄) Level4 (addition of 30 pg/mL); and (►) Level 5 (addition of 60 pg/mL). **(B)** Sensitivity curve used to calculate the concentration of analyte in the *unknown sample* by the standard addition method. Error bars represent the relative standard deviation on the measurements performed in triplicate using one device for each concentration level.

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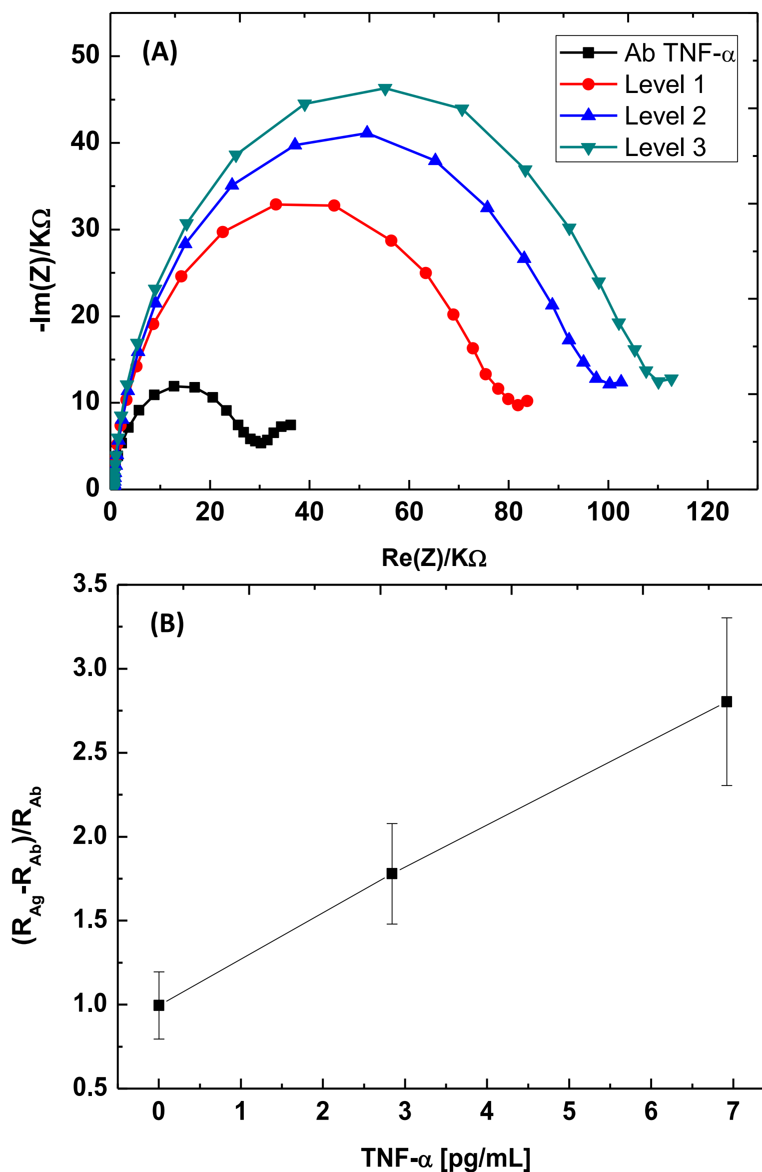


**Fig. 6.** **(A)** Nyquist impedance plots (Zr vs Zi: at 5 mM of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ in PBS pH 7,4 solution) obtained from the standard addition method performed on a real sample. (■) AbTNF-α for AgTNF-α; (●) Level 1 (corresponding to an addition of 0 pg/mL); (▲) Level 2 (addition of 3 pg/mL); (▼) Level 3 (addition of 7pg/mL). **(B)** Sensitivity curve used to calculate the concentration of analyte in the unknown sample by the standard addition method. Error bars represent the relative standard deviation on the measurements performed in triplicate using one device for each concentration level