Study of a Low-cost Sensitive Point-of-Care Testing System using Screen Printed Biosensors for Early Biomarkers Detection Related to Alzheimer Disease

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Abstract: Among neurodegenerative diseases, Alzheimer Disease (AD) represents one of the most serious pathology, for which an early diagnosis is still missing. A peculiar expression of an altered conformational isoform of p53 protein was reported to be a biomarker able to distinguish AD subjects from healthy population, quantifiable using a blood-based enzyme-linked immunosorbent assay (ELISA). In order to overcome ELISA limitations related to reliability and to improve sensitivity, this study aimed to realize a low cost highly sensitive portable point-of-care (PoC) testing system based on screen printed electrochemical sensors (SPES). The development of the platform specifically included both the design of the sensing probe and of the electronic circuit devoted to the conditioning and acquisition of the transduced electric signal. In particular, silver, carbon and silver-silver chloride were selected respectively to realize conductive tracks, working and counter electrodes, reference electrode in a three-electrodes configuration focusing on Anodic Stripping Voltammetry (ASV). The conditioning circuit was designed following the scheme for a common potentiostat, and produced as a Printed Circuit Board (PCB). Initial testing of the circuit were performed recording changes in the conductivity of NaCl solution and quantifying electrodes coating with antibodies using Electrochemical Impedance Spectroscopy (EIS) principle. Preliminary results obtained with saline solution, showed the ability of the circuit to give the best response corresponding to low changes in NaCl concentration (sensitivity 13 mA/(mg/ml)), suggesting a good sensitivity of the platform. Results from EIS showed the ability of the circuit to discriminate between different concentrations of antibodies coatings (sensitivity 70 mA/µg). The study is on-going and after a proper calibration, the circuit is intended to be optimized to quantify unknown concentration of unfolded p53 in samples of real patients, compared results with the one from ELISA analysis, aiming to realize a low cost, easy usable and highly precise platform.

1 RESEARCH PROBLEM

The number of people over 65 years is rapidly increasing; nowadays, in Europe, it represents the 16% of the whole population and this percentage will reach the 25% within the end of 2030. Ageing usually conveys several issues, which may include degenerative or chronic disorders. The impact of these diseases on the single person and on the whole society could become huge and difficult to support in the early future. In this perspective, the actual researches and innovations are pushing to improve the understanding of the causes and mechanisms underlying ageing and the associated diseases, thus to ensure elderlies with a healthy and active condition.

Among neurodegenerative diseases, Alzheimer Disease (AD) represents indeed one of the most investigated and serious pathologies, for which an early reliable diagnosis is still missing. Therapies currently available help in fact to alleviate the symptoms of this disease, but they are not able to specifically slow down the neurodegenerative process. AD presents a long pre-symptomatic period, that could last for 20 years, and which is characterized by biochemical and molecular events that are able to foresee the beginning of the disease. The ability to identify early reliable biomarkers (e.g. proteins) to effectively diagnose the pathology at an early stage is one of the actual priority of biomedical research in term of neurology and geriatrics.

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Recently, among several studies addressing this issue, different approaches to identify the specific AD biomarkers have been established and novel one discussed. To date, the most advanced and accepted methods to diagnose AD with high specificity and sensitivity are represented by enzyme-linked immunosorbent assay (ELISA) measurements in cerebrospinal fluid (CSF) and imaging biomarkers, like volumetric magnetic resonance imaging and positron emission tomography, evaluating glucose utilization or ligands binding to amyloid plaque. In addition to these methods, a great challenge is represented by the search for novel biomarkers in CSF and in blood by using modern potent methods, such as microarrays, mass spectrometry, bioinformatics, since only a combined analysis of several biomarkers seems the promising path to help in defining a patient-specific diagnose in the future (Thal et al., 2006; Humpel, 2011)

In this perspective, scientific literature recently reported how a peculiar expression of an altered conformational isoform of p53 protein in AD patients was able to distinguish them from healthy subjects with a sensitivity of 90% and a specificity of 77% (Buizza et al., 2012; Lanni et al., 2008; Uberti et al., 2006). On this basis, the levels of p53 unfolded could be an interesting starting point to a reliable blood-based ELISA performed with a specific conformational anti-p53 antibody. Even if ELISA assay actually represents the gold standard technique used for the detection and quantification of this p53 biomarker, some limitations are related to its diffusion. These issues specifically include high costs of the assay implementation, high operator dependence, lack of standardization and impossibility to lower the limit of detection, thus to detect the biomarker in the early stage of the disease when it would be more useful for the clinicians.

Biosensors represent the emerging technology that promises to address this challenge, bringing promising solutions in term of cost and sample use reduction, ease of use, high portability and sensitivity (Yager et al., 2008; Polese et al., 2014; Feng et al., 2015).

In light of this, the development of a portable point-of-care (PoC) testing system based on a screen printed electrochemical sensor (SPES), could represent an innovative and low cost solution to solve these problems, improving and bringing to a higher level of sensitivity the specific biomarkers detection and quantification, with a strong impact on the possibility to early diagnose AD (Dhawan et al., 2015).

2 OUTLINE OF OBJECTIVES

The main objective of this project addresses the design and development of a low-cost portable point-of-care testing platform for the detection and sensitive quantification of the unfolder p53, as biomarker for early detection of AD.

The development of the platform specifically includes both the design of the sensing probe, with particular attention to the choice of the materials and geometry, and of the electronic circuit devoted to the conditioning and acquisition of the electric signal. The electrodes were specifically designed to be easily implemented by means of screen printing methodology. In details, the outline of the project could be organized in the following phases:

1. preliminary characterization of the electrodes, and evaluation of compatibility of screen printing materials and substrate with wet lab practices;
2. design and production of the screen printed electrochemical sensor;
3. design and development of the conditioning electronical circuit to perform the electronical measurements, which are directly related to the concentration of the specific protein on the biological sample;
4. calibration of the sensors and evaluation of the circuit;
5. functionalization of the sensors and protein quantification using Electrochemical Impedance Spectroscopy (EIS) and Anodic Stripping Voltammetry (ASV).
6. optimization of the acquisition workflow.
7. validation of the sensor by using primary antibodies specific for p53 unfolded proteins and real samples from AD patients, compared to the ELISA assay.

Each reported phase addresses specific objectives.

3 STATE OF THE ART

One of the most pervasive challenges of the research in medical diagnosis is related to the ability to detect a specific pathology in its earliest development (Jacobs et al., 2014). To achieve this goal, in the last decades, new rapid, low cost and easily accessible methodologies have been increasingly investigated, supported by the interest toward customized medicine and toward rapid and home accessible diagnostic results (Chan et al., 2013).

For these reasons, the idea to identify and quantify specific biomarkers in the early stages of a disease appears to be really promising (Jr et al., 2010; Svobodova et al., 2012). Biomarkers represent an indicator of the biological status, which can give useful information concerning biological processes happening in physiological or pathological conditions and during different medical treatments.

As previously stated, in the specific case of AD, recent studies related to its diagnosis reported how the presence of a particular conformation of a specific protein (p53) could be able to discriminate patients affected by AD from healthy patients (Uberti et al., 2006; Lanni et al., 2008; Buizza et al., 2012).

In this perspective, clinicians require technologies able to identify quickly and with a high sensitivity specific biological biomarkers related to the disease.

Biosensors integrated in lab on a chip (LoC) devices could represent promising methods to reduce time, cost and sample needed to perform the analysis. (Song et al., 2014).

Thanks to their ability to be functionalized and customized for the detection of different analytes (e.g. DNA, proteins), electrochemical biosensors represent the ideal starting point to realize complete platforms, by integrating the sensor with the conditioning circuit needed for the electronical measurements, realizing portable and self-standing devices, useful for PoC applications (Yager et al., 2008).

Printed electronics represents a successful tool to realize low cost and sensitive biosensors for these specific applications. In particular, screen printing (also defined thick film printing) is the most used method to realize this kind of electrochemical sensors, as it arises in several works in the literature for the sensitive and specific quantification of different proteins (Silva et al., 2014; Elshafey et al., 2013; Yun et al., 2011). Protein detection and quantification is usually obtained by performing the same functionalization as used in the multiwells ELISA. More in details, the working electrode of the sensor is functionalized with the primary antibodies specific for the proteins that need to be quantified, thus allowing the formation of an immunocomplex. After that, the electrode is washed with the sample containing the proteins and with a solution containing a specific secondary antibody. After the immunocomplex is realized, different methods can be used to quantify the specific proteins.

A label free method is represented from EIS, which measures the changes of impedance deriving from a different electrons exchange between the functionalized surface of the electrode and a conductive solution (usually potassium ferricyanide), depending on the concentration of the recognized proteins (Silva et al., 2014; Elshafey et al., 2013). For this specific technique, 2–electrode conformation - including a working (WE) and a reference electrode (RE) - or 3- electrode conformation – including WE, RE and a counter electrode (CE) as reference for the current – have been used to ensure the correct acquisition of the proteins concentration, depending on the level of precision and sensitivity required.

A different technique often used is represented by voltammetry. In this case, the 3-electrodes conformation is usually adopted. The potential between RE and WE is varied arbitrarily - either step by step or continuously - and the actual current value between WE and CE is measured as the dependent variable. As reported in different works, this method can be performed both with the sensor completely immersed in a buffer solution (Elshafey et al. 2013) or by placing a drop of solution which cover the three electrodes (Escamilla-Gómez et al. 2009). Anodic stripping voltammetry (ASV) represents a very sensitive kind of voltammetry technique. Several groups optimized this measurement technique to detect both proteins (Escamilla-Gómez et al., 2009) or DNA sequences (Martínez-Paredes et al., 2010), with limit of detection in the order on ng/ml.

Using the catalyzing action of Alkaline Phosphatase (AP), ionic silver is selectively reduced in its metallic form only where the proteins are recognized, thus allowing the quantification of proteins converting the amount of deposited silver into a proportional peak of current during a stripping step, varying the potential between WE and RE.

To further improve the specificity and the sensitivity of this technology, limit of detection lower than 100 pg/ml have been achieved thanks to the integration of the SPES with nanostructured materials. Gold nanoparticles for example or carbon nanotubes or a combination of the two has been used to modify the surface of the working electrode allowed to better recognize antigens and DNA sequences (Escamilla-Gómez et al., 2009; Kara et al., 2010; Martínez-Paredes et al., 2010; Jeong et al., 2013).

4 METHODOLOGY

The experimental activities of the project are divided into 3 main parts:

- a first part dedicated to the evaluation of the compatibility of the screen printing materials and the circuit components with the wet lab practices.

- a second part dedicated to the design and realization of the sensor and the conditioning circuit.
- a final part dedicated to the calibration of the platform (sensor and circuit) and to p53 protein quantification.

4.1 Initial Sensors Characterization

Preliminary characterizations are required to assess the compatibility of the sensor with wet lab practices and to evaluate how antibodies can be coated on to electrode surface in order to select the best materials for the sensor production.

Alumina is identified as the optimal material used as substrate for the printing of the working electrodes. The adhesion of different concentration of antibodies solutions has to be evaluated, in order to optimize the best concentration for an efficient functionalization of the sensor. Two different techniques have been specifically chosen for the analysis: an electronic method, EIS, and an optical one, evaluating the light intensity thanks to fluorescent labels on the secondary antibody.

EIS measurements are performed in presence of Potassium Ferricyanide ($K_3[Fe(CN)_6]$) in a solution of KCl. This technique is based on the measurement of the impedance on a wide range of frequencies (200 Hz – 200 KHz), between a carbon WE and a silver-silver chloride RE, both immersed in an electrolytic solution of 5 mM $K_3[Fe(CN)_6]$ in 1 M KCl.

Different designs (single electrodes and multiwell electrodes) and materials (alumina, glass and polystyrene) are evaluated in this first optimization phase in order to better understand the technology to be implemented in the final layout of the sensor. For each of this different design, different protocols are adapted to obtain reliable and repeatable results.

Impedance measurements are specifically performed using an impedance analyzer (HP4194A), compared with the fluorescence measurements registered using a light intensity quantifier (Odyssey® Fc Dual-Mode Imaging System from LI-COR Biosciences).

4.2 Sensors Design and Production

The layout is designed using QCAD software. Each layer, corresponding to a different conducting material, is separately designed, in order to produce the masks required to screen print layer by layer the final structure of the sensor. A specific care should be put to realize conductive tracks with a resolution compatible with the printing performance of the screen printer employed (A2 Model, Baccini srl, Italy).

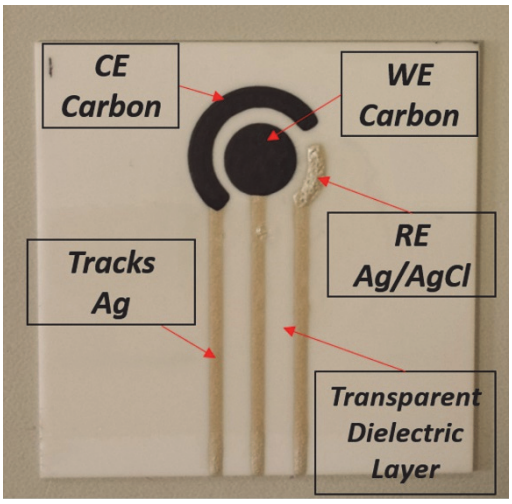

Figure 1: Final SPES layout.

Once the geometry is optimized - with a WE of 8.0 mm of diameter - it is printed on a lucid sheet by means of inkjet printing, thus to allow the realization by UV photolithography of the mask required for the screen printing process.

On a 0.4 mm thick alumina substrate, the 3 layers are consequently printed: firstly the silver for the conductive tracks, then carbon for the working electrode and finally silver-silver chloride for the reference electrode. In order to allow a better conduction of the signal, the conductive tracks are isolated using a conductive spray specific for printed circuit, leaving the terminal part of the tracks free for the connection with the conditioning circuit (Fig. 1)

The reproducibility of sensor geometry in the different printing processes is ensured thanks to a specific care in performing a standardized protocol while realizing each sensor. Each sensor is accurately observed under an optical microscope to evaluate the homogeneity of the printed layer and the resistance evaluated with a tester.

4.3 Circuit Design and Production

Parallelly to the realization of the electrochemical sensor, the conditioning circuit is designed in order to allow the production of a complete PoC testing platform.

The design of the circuit is performed following the scheme of a common potentiostat. Its aim is to control the potential between the functionalized WE and the RE, and then to measure the current flowing between WE and CE.

The electronic schematic is realized with OrCAD software (OrCAD©- Cadence Design Systems -San Jose, CA) whereas the design for the Printed Circuit Board is prepared using OrCAD Layout Plus tool (OrCAD©- Cadence Design Systems -San Jose, CA). The final PCB realized using OSH Park - community printed circuit board (PCB) order (Fig. 2).

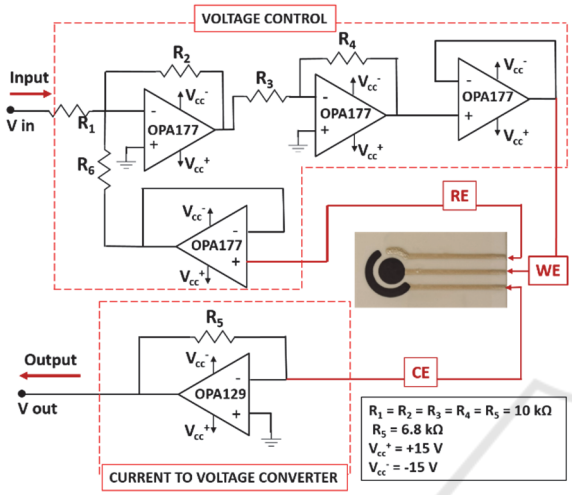

Figure 2: Schematic of the conditioning circuit.

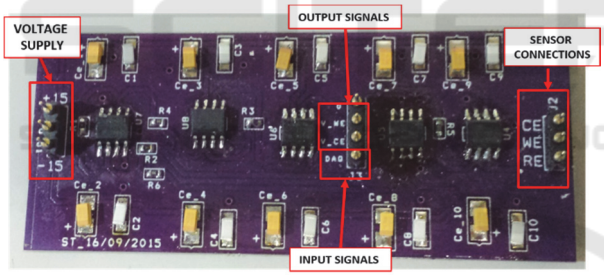

Figure 3: Final PCB of the conditioning circuit.

All the SMD electronic components are soldered and the board inserted in a metallic box to avoid noises on the signal recording and to improve the sensor sensitivity and precision.

4.4 Calibration and Measurements

After optimizing the design, the platform is tested following three different protocols, using before a saline solution, with different concentrations of NaCl, then performing EIS with Potassium Ferricyanide and after that performing ASV functionalizing the WE with proteins.

4.4.1 NaCL Solution Measurements

The first test of the circuit is performed using a saline solution, varying the concentration of NaCl in order to change the conductivity of the solution, and evaluating the ability of the circuit to quantify these changes as changes in the current peak flowing between WE and CE.

In the first test the concentrations of the solution evaluated are 0.0, 15.0, 22.5, 30.0, 37.5 and 45.0 mg/ml. After that, a second round of concentrations is tested, to evaluate the linearity of the circuit response in a range between 0.0 and 10.0 mg/ml, in particular: 0.0, 1.0, 2.2, 4.0, 5.5, 6.0, 10.4 mg/ml. Finally, the circuit is evaluated with concentrations lower than 1.0 mg/ml, in particular: 0.00, 0.44, 0.66, 0.88, 1.00 mg/ml.

In each experiment, drop of 2 ml of saline solution are dropped on WE, CE and RE, assuring that the drop stays in place with the help of a mask applied on top of the sensor.

For this analysis, the input signal is considered as a triangular wave, with amplitude 300 mV and frequency 40 mHz, obtained using a pulse generator (HP8116A pulse/function generator 50 MHz Hewlett-Packard)

The signal is then acquired using an oscilloscope (Tektronik TDS 1001B – two channel digital storage oscilloscope 40 MHz, 500 MS/s)).

Experiments are always performed in triplicate. All graphical and tabulated data are usually displayed as mean ± mean standard error.

4.4.2 Antibodies Quantification using EIS

EIS is also applied in order to measure changes of the current detected between WE and CE to quantify different concentrations of the primary antibodies released and adhered on WE surface. In particular, three antibodies concentrations were considered: 0, 4 and 8 µg/ml.

After an overnight incubation at 4 °C the measurements were performed in presence of a conductive electrolytic an solution of 5 mM K3[Fe(CN)6] in 1 M KCl. Once the functionalization was performed, a drop of 2 ml was placed in order to cover WE, RE and CE and allow current flow, and the electronic measurement were performed, giving a ramp as signal input, and recording the current between WE and CE using an oscilloscope. In particular, a first analysis was performed using triangular waves four different frequencies (40 mHz, 100 mHz, 200 mHz and 1 Hz) with an amplitude of 300 mV, and then a second one fixing the frequency to 50 mHz.

4.4.3 Protein Measurements

The protocol followed to quantify protein concentration, both for the preliminary test using a kit with human interleukin and for the real samples containing p53 proteins, is represented by the following steps:

- sensor wash with Phosphate Buffer Saline (PBS)
- WE coating with optimized primary antibody concentration (8 µg/ml).
- Overnight incubation at 4°C
- Block with a Bovine Serum Albumin (BSA) solution.
- 2 hours incubation with desired solution containing a defined concentration of proteins (in the calibration phase) or with the sample. Temperature mantained stable at 25°C
- Block with BSA solution.
- 1 hour incubation with biotin labelled secondary antibodies.
- Block with BSA solution.
- 30 min incubation with streptavidin labelled Alkaline Phosphatase.
- Block with BSA solution.
- 20 min incubation with a solution of 3 mM AA-p e 4 mM AgNO3, protected from light.

Once the functionalization is performed, a drop of 2 ml is placed in order to cover WE, RE and CE and allow current flow, and the electronic measurement are performed, giving a ramp as signal input, and recording the current between WE and CE using an oscilloscope, in the same way indicated in the previous paragraph.

5 EXPECTED OUTCOME

The complexities and the heterogeneity associated with AD, requires high precision and sensitivity in the reliable detection and quantification of specific biomarkers, able to allow an early diagnosis of the disease in the pre-symptomatic phase and to acquire additional information both from the biological and from the pathoclinical point of view.

The main expected outcome of this project is the realization of a self-standing portable point-of-care testing system, able to support clinicians to diagnose AD from its earliest stages.

The proposed methodology can be used in a routine automatized diagnosis technique, specifically quantifying the unfolded p53 biomarker. Following the development of the platform, this project inherently addresses different outcomes, specifically related to:

1. Optimized calibration of the sensor and conditioning/acquisition electronics (Fig. 3), thus to discriminate defined protein concentrations
2. Protein sensitive quantification modifying the sensor materials (e.g. using gold nanoparticles).
3. Final optimization of the platform to realize a self-standing point of care

Each step described addressed from one side an increasing in the sensitivity of the sensor itself and from the other the optimization of the portable point of care design, aiming to a low cost, easy usable and highly precise platform.

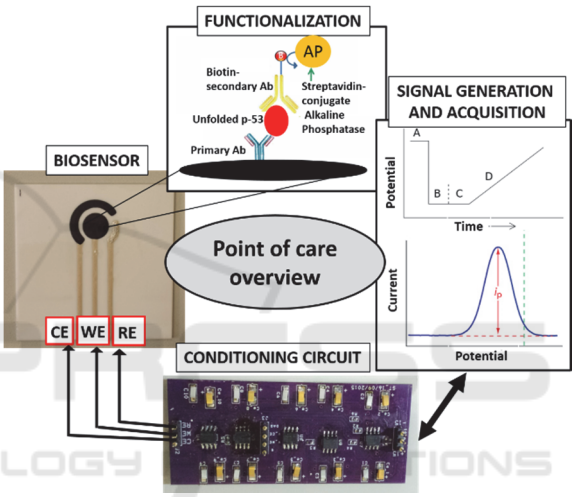

Figure 4: Point of care overview.

6 STAGE OF THE RESEARCH

6.1 Initial Sensors Characterization

Regarding the compatibility of the materials and of the printing process with wet lab practices, alumina substrate represented the optimal solution. Thanks to the intrinsic porosity of the material, electrodes printed in this substrate did not show any variation when washed with water-based solutions during functionalization steps. On the contrary, electrodes printed in glass and polystyrene, because of their low porosity, did not show an efficient adhesion, with critical modifications during the functionalization step, compromising the uniformity of the primary antibodies coating on the WE and the effective complex formation with the secondary antibody.

Among the different primary antibody concentrations evaluated (2.0, 2.6, 3.0, 4.0, 4.8, 6.0,

8.0, 10.0 µg/ml), 8.0 µg/ml was identified as the optimal one to achieve a homogeneous coating of the WE.

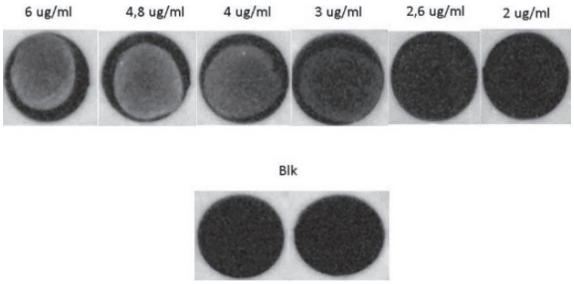

Figure 5: Image obtained from the optical analysis of WE coated with different primary antibody concentrations.

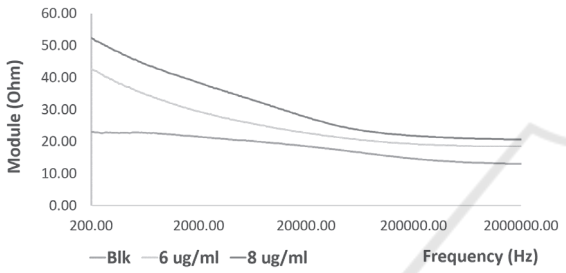

Figure 6: Electronical measurements (EIS) of different antibody concentration coatings.

The fluorescence signal recorded from the Odyssey showed a fluorescence intensity proportional to the concentration of primary antibody coated in the range between 2 and 6 µg/ml. (Fig. 4). Impedance measurements performed on the same electrodes, showed results in agreement with what previously evaluated with optical analysis. More specifically, the linearity observed for concentration of 0.0, 6.0, 8.0 µg/ml, could be observed with EIS as well.

Using electrodes printed on ceramic substrates, thanks to the good compatibility of the materials with wet lab practices, results obtained appeared to be repeatable and reliable for all the electrodes tested.

The impedance module measured for electrodes coated with 8.0 µg/ml primary antibody solution resulted to be superior in all the frequencies range evaluated, compared with the one of blank electrodes, treated with a buffer solution (mean 15.5 ± 4.6 Ohm between 2 and 20 kHz; max 30 Ohm at 2 kHz and minimum of 9 Ohm at 200 kHz). The impedance module measured with electrodes treated with 6.0 µg/ml showed a trend comprised between the previous two (Fig. 5). Using electrodes printed on glass substrates, the average difference between the impedance module of reference electrodes and electrodes treated with 8.0 µg/ml of primary antibody was 7.6 ± 1.1 Ohm, constant in the range between 200 and 2 MHz. Because of the poor adhesion and compatibility of the glass printing process with wet lab practices, measurements performed on electrodes coated with intermediate concentrations showed results compatible with the non-uniform coating highlighted from the fluorescence analysis.

6.2 Calibration and Measurements

The present stage of the research, after that the design and development of the sensor and conditioning/acquisition circuit has been optimized, is addressing the calibration of the sensor, firstly with NaCl solution, and then with human interleukin, in order to optimize the detection protocol and to prepare the platform for the following step of p53 proteins detection and quantification.

6.2.1 NaCl Solution Measurements

Results from the evaluation of circuit response to changes in saline solution conductivity showed a linear response for the specific ranges of concentrations evaluated. After evaluating the linearity using high concentration of NaCl, a narrower range of concentrations was evaluated in order to understand if the circuit was able to recognize small changes in solution conductivity and small current between WE and CE.

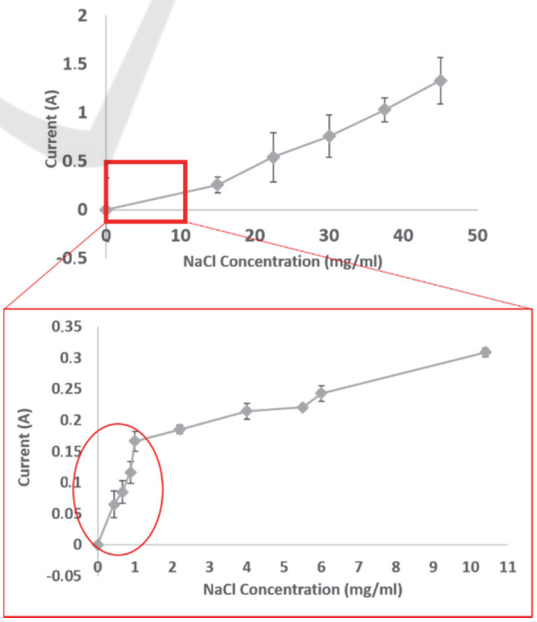

Figure 7: Calibration of SPES with NaCl solution.

Performing the same measures with lower concentration a particular behaviour could be noticed. Two different slopes could be observed respectively for concentration lower and higher than 1.0 mg/ml. In particular, a higher sensitivity was shown for the concentration lower than 1.0 mg/mg (13 mA/(mg/ml)), indicating a higher sensitivity of the sensor for small changes of conductivity and small currents (Fig. 6). This behaviour suggested that the range of concentration in which the circuit was able to give the best response corresponds to low changes in concentration, resulting in small ionic currents. On the contrary, high changes in concentration, causing high changes in conductivity, were discriminated with an inferior sensitivity (170 mA/(mg/ml)) because they brings to current which cause the circuit to saturate, and not to be able to discriminate the difference.

6.2.2 Antibodies Quantification using EIS

Results from EIS measurements showed a proportional decreasing of the peak of current flowing between CE and WE, indicating an increased impedance of the system due to an increasing concentration of antibodies coated on WE surfaces resulting in a reduced electrons exchange between WE surface and electrolytic solution (Fig 8 and 9).

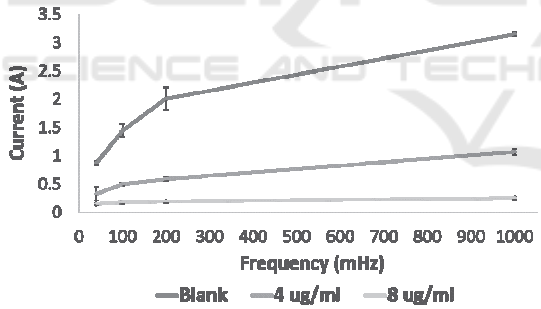

Figure 8: Difference in CE current measured at different frequencies, evaluating with EIS different concentration primary antibodies coatings.

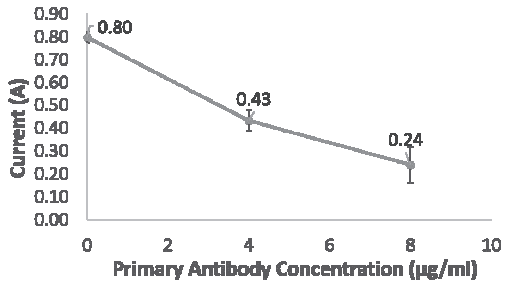

Figure 9: Difference in CE current measured at 50mHz, evaluating with EIS different concentration primary antibodies coatings.

The same behaviour was observed at all the frequencies evaluated. Increasing the concentration of antibodies coated on the WE resulted in reducing the differences of currents exchanged at different frequencies (Fig. 9). As showed in Fig. 9, the sensitivity of the sensor in detecting the change in antibodies coating concentration was of 70 mA/µg.

6.2.3 Protein Measurements

The activity actually going on refers to the implementation of the same protocol using interleukin protein, of dedicated kit DuoSet® development system for ELISA, Human CXCL8/IL-8. The different concentrations of proteins are going to be recognized with two different techniques:

- using EIS in presence of Potassium Ferricyanide in KCl solution.
- using ASV as described in the methodologies section.

This phase is essential for an effective calibration of the platform, in order to proceed with the quantification of unknown concentration of p53 proteins.

7 FUTURE OUTLOOKS AND CONCLUSION

In light of the positive results described, the activity actually going on refers to the implementation of the same protocol described in the materials and methods section using interleukin protein, of dedicated kit (DuoSet® development system for ELISA, Human CXCL8/IL-8). Different concentrations of proteins (order of ng/ml) will be recognized using both the techniques described (EIS and ASV). This phase is essential for an effective calibration of the platform, in order to proceed with the quantification of unknown concentration of p53 proteins. Specifically regarding ASV, the same protocol will be adopted to quantify protein concentrations, both for interleukin and for p53 proteins. It will be characterized by the use of immucomplexes of primary antibody-protein-secondary antibody labelled with Alkaline Phosphatase, as functionalization of the WE. In this way, through a selective chemical deposition of silver, the current flowing between WE and CE will be proportional to the amount of deposited silver, which in its turns will be proportional to the recognized protein. Before proceeding with unknown proteins concentrations, an accurate calibration of the biosensor will be performed. After the validation, the

proposed methodology and the platform design will be optimized in order to be easily accessible for a routine automatized diagnosis technique in the clinical environment. From these bases, particular attention will be then addressed to increase the sensitivity of the method itself, including both the introduction of nanostructured materials for the working electrodes and proper ASV measurements. All this, with the aim to realize an innovative self-standing portable point-of-care, a low cost, easy usable and highly precise platform able to support clinicians to diagnose AD from its earliest stages.

REFERENCES

Buizza, L. et al., 2012. Conformational Altered p53 as an Early Marker of Oxidative Stress in Alzheimer ' s Disease. , 7(1), pp.1–11.

Chan, K.C.A. et al., 2013. Cancer Genome Scanning in Plasma : Detection of Tumor-Associated Copy Number Aberrations , Single-Nucleotide Variants , and Tumoral Heterogeneity by Massively Parallel Sequencing. , 224, pp.211–224.

Dhawan, A.P. et al., 2015. Current and Future Challenges in Point-of-Care Technologies: A Paradigm-Shift in Affordable Global Healthcare With Personalized and Preventive Medicine. *Translational Engineering in Health and Medicine, IEEE Journal of*, 3, pp.1–10.

Elshafey, R. et al., 2013. Electrochemical impedance immunosensor based on gold nanoparticles-protein G for the detection of cancer marker epidermal growth factor receptor in human plasma and brain tissue. *Biosensors & bioelectronics*, 50, pp.143–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23850780.

Escamilla-Gómez, V. et al., 2009. Simultaneous detection of free and total prostate specific antigen on a screen-printed electrochemical dual sensor. *Biosensors and Bioelectronics*, 24, pp.2678–2683.

Feng, S., Roseng, L.E. & Dong, T., 2015. Quantitative detection of Escherichia coli and measurement of urinary tract infection diagnosis possibility by use of a portable, handheld sensor. *Medical Measurements and Applications (MeMeA), 2015 IEEE International Symposium on*, pp.586–589.

Humpel, C., 2011. Identifying and validating biomarkers for Alzheimer's disease. *Trends in Biotechnology*, 29(1), pp.26–32. Available at: http:// www.ncbi.nlm.nih.gov/pmc/articles/PMC3016495/.

Jacobs, J. et al., 2014. Harmonization of malaria rapid diagnostic tests : best practices in labelling including instructions for use. , pp.1–10.

Jeong, B. et al., 2013. Increased Electrocatalyzed Performance through Dendrimer- Encapsulated Gold Nanoparticles and Carbon Nanotube-Assisted Multiple Bienzymatic Labels: Highly Sensitive Electrochemical Immunosensor for Protein Detection.

Jr, C. R. J. et al., 2010. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. 9(1), pp.1–20.

Kara, P. et al., 2010. Aptamers based electrochemical biosensor for protein detection using carbon nanotubes platforms. *Biosensors and Bioelectronics*, 26, pp.1715–1718.

Lanni, C. et al., 2008. Conformationally altered p53 : a novel Alzheimer ' s disease marker ? , pp.641–647.

Martínez-Paredes, G., González-García, M.B. & Costa-García, A., 2010. Genosensor for detection of four pneumoniae bacteria using gold nanostructured screen-printed carbon electrodes as transducers. *Sensors and Actuators, B: Chemical*, 149, pp.329–335.

Polese, D. et al., 2014. Investigation on nanostructured biosensor for Biotin detection. *SENSORS, 2014 IEEE*, pp.1627–1630.

Silva, M.M.S. et al., 2014. A thiophene-modified screen printed electrode for detection of dengue virus NS1 protein. *Talanta*, 128, pp.505–510. Available at: http://dx.doi.org/10.1016/j.talanta.2014.06.009.

Song, M.J. et al., 2014. Electrochemical serotonin monitoring of poly(ethylenedioxythiophene): Poly(sodium 4-styrenesulfonate)-modified fluorine-doped tin oxide by predeposition of self-assembled 4-pyridylporphyrin. *Biosensors and Bioelectronics*, 52, pp.411–416. Available at: http://dx.doi.org/10.1016/j.bios.2013.08.040.

Svobodova, Z. et al., 2012. Development of a magnetic immunosorbent for on-chip preconcentration of amyloid b isoforms : Representatives of Alzheimer ' s disease biomarkers. , 024126, pp.1–12.

Thal, L.J. et al., 2006. The Role of Biomarkers in Clinical Trials for Alzheimer Disease. *Alzheimer disease and associated disorders*, 20(1), pp.6–15. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1820855/.

Uberti, D. et al., 2006. Identification of a mutant-like conformation of p53 in fibroblasts from sporadic Alzheimer ' s disease patients. , 27, pp.1193–1201.

Yager, P., Domingo, G.J. & Gerdes, J., 2008. Point-of-care diagnostics for global health. *Annual review of biomedical engineering*, 10, pp.107–144.

Yun, Y.H. et al., 2011. A glucose sensor fabricated by piezoelectric inkjet printing of conducting polymers and bienzymes. *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*, 27(4), p.375.