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Development of a new optical biosensor for the detection of sepsis using mid-regional pro-adrenomedullin as a biomarker.

Desarrollo de un biosensor óptico nuevo para la detección de sepsis utilizando como biomarcador la región medial de la pro-adrenomedulina.

Anabel Colom Dyke

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Universitat de Barcelona

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You are a world within a world: look within yourself, and you will see the whole creation.

Saint Nilus of Ancrya

I would like to thank first of all my tutor Juliana Fátima Giarola for her incredible guidance and her great patience. I would like to thank Laura Lechuga for allowing me to take part in her research group and discover the world of scientific discovery. I give special thanks to my university friends Ana and Paula who have stood by me from the beginning. This last year was made incredibly fruitful thanks to my friends Gaia, Paula, Lubomira and Anastasia. Last but not least, I would like to thank my family who have always provided me with unconditional love and support.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The *Institut Català de Nanociències i Nanotecnologia* (ICN2) institute is dedicated to sustainability and is trying to improve the way scientific research may impact the world around us. Of the five main topics comprising the Sustainable Development Goals, the institute is making great strides in the areas of People, Prosperity and Planet. The NB2A, the group of which I have been fortunate enough to be a part of these last few months, is researching the application of point of care devices to help doctors diagnose different diseases and pathologies in record time in order to improve the survival rates and medication prescription based on the needs of the patients. These points directly concern SDG number three, good health and well-being. The research I have been a part of is focussed on diagnosing sepsis within a 20-minute timeframe at the point-of-care. This condition affects millions of people every year, many of which die due to the slow diagnosis of this infection, which must be treated fast (see introduction). If this goal were to be reached, the outcome would be overwhelmingly positive for the survival rate of sepsis infected patients.

The ICN2 is also contributing to the ninth SDG, namely Industry and Innovation, seeing as its research in new technologies is promoting innovation in technology and scientific research. Several start-ups have emerged from the research undertaken by the different groups.

The research institute is looking into obtaining a *My Green Lab Certification for Lab Sustainability,* recognised by the United Nations Race to Zero Campaign as a key measure towards a zero-carbon future. This organization has advised the institute in sustainability, helping them to manage lab resources in a more efficient and environmentally friendly way. The ICN2 is actively engaged in improving energy efficiency and decarbonizing energy sources, reducing water consumption, improving waste management (both hazardous and non-hazardous), strengthening ICN2 community engagement in sustainability and developing a sustainable travel policy. Additionally, the ICN2 is committed to the European Commission's *Do No Harm Policy,* aimed at avoiding economic activities that do significant harm to any environmental objective. All these points cover the SDGs eleven, twelve and thirteen targeting sustainable cities and communities, responsible consumption and production, and climate action respectively.

NB2A is a group focused on gender equality and inclusivity in scientific research, being one of the few groups with a woman as its leader, professor Laura Lechuga. The ICN2 as an institute is equally committed to this goal, implementing policies ensuring equal treatment and opportunities, thus eliminating any form of discrimination, and embedding the principle of equal opportunities in its core policies.

CONTENTS

1. SUMMARY

Sepsis is one of the main causes of death worldwide. It is a serious condition in which an infection triggers an extreme immune inflammatory response in the body which may lead to organ dysfunction. If the blood pressure drops sufficiently, the condition may progress to multiple organ failure and, in extreme cases, death. This is called septic shock. Every hour sepsis goes without treatment represents an 8% increase in the risk of mortality. In the United States of America alone, 250,000 people die of sepsis every year.

This project has as its aim to develop a multiplexed point-of-care device capable of accurately diagnosing sepsis within a 20-minute timeframe. This would allow the appropriate treatment to be administered in record time, drastically augmenting the chances of survival of the patient. This technology would not require trained personnel, making it more accessible and economic than current detection methods.

This work focuses on the development of a new optical biosensor for the detection of mid-regional pro-adrenomedullin, a promising biomarker for sepsis. A comparison is made between using two different chip covering surfaces, gold and aluminium. Furthermore, a study of the behaviour of human serum on the proposed biosensor is performed, looking towards the future goal of detecting pro-adrenomedullin in real patient samples.

Keywords: sepsis, optical biosensor, pro-adrenomedullin.

2. RESUMEN

La sepsis es una de las causas principales de muerte en el mundo. Es una condición seria en la cual una infección desencadena una respuesta inmune inflamatoria extrema en el cuerpo, lo cual puede llevar a una disfunción de los órganos. Si la presión arterial baja excesivamente, la condición puede causar un fallo multiorgánico y, en caso extremo, la muerte. A eso se le llama choque séptico. Por cada hora que la sepsis progresa sin tratamiento, la probabilidad de muerte aumenta un 8%. En los Estados Unidos, al menos 250,000 personas mueren por esta condición anualmente.

Este proyecto tiene como meta el desarrollo de un dispositivo *point-of-care* multiplexado, permitiendo la diagnosis precisa de la sepsis en un tiempo de 20 minutos. Esto les permitiría a los profesionales administrar el tratamiento en un tiempo récord, drásticamente aumentando las posibilidades de supervivencia de los pacientes. Esta tecnología no requeriría personal entrenado, haciendo este método más accesible y económico que los métodos actuales.

Este trabajo se enfoca en el desarrollo de un nuevo biosensor óptico para la detección de la región media de la proteína pro-adrenomedulina, un biomarcador prometedor de la sepsis. Una comparación entre dos tipos de recubrimientos de los chips, oro y aluminio, fue establecida. Finalmente, se estudió el comportamiento del suero humano en el biosensor, de cara a la meta final de detectar cuantitativamente la concentración de pro-adrenomedulina en muestras de pacientes.

Palabras clave: sepsis, biosensor óptico, pro-adrenomedulina.

3. INTRODUCTION

Sepsis is a serious condition in which an infection triggers an extreme immune response in the body and is one of the primary causes of death worldwide [1]. This extensive inflammation may start affecting organ function. This stage is called severe sepsis. Septic shock occurs when the extreme bodily response causes a dramatic drop in blood pressure, possibly leading to multiple organ failure. If the damage is severe enough the patient may die. Early treatment of sepsis is key to improving chances of survival seeing as for a patient with septic shock, every hour that treatment is delayed represents an 8% increase in the risk of mortality [2]. In the United States of America alone, about 1.7 million adults develop sepsis every year of which at least 250,000 people die [3].

As mentioned, the early treatment of sepsis, and thus its early diagnosis is key to fighting this disease. There is currently no reference biomarker specific to the detection of sepsis. Doctors use a wide range of biomarkers and blood cultures to identify the pathogen infecting the patient [4]. All these tests take time and require trained personnel. This project is focussed on researching the possibility of detecting sepsis within a 20-minute time frame using a point-of-care device which would enable clinicians to diagnose and treat patients onsite, drastically augmenting the chances of survival of infected patients. This multiplexed technology would detect several biomarkers using the same patient sample. These biomarkers include the C reactive protein (CRP), procalcitonin (PCT), some cytokins (IL6, IL10) [5] and the mid-regional pro-adrenomedullin (proADM) [6]. This research will be focussed on the proADM protein as a biomarker for sepsis detection.

The adrenomedullin peptide (ADM) can be found in almost every tissue in the human body. The name is derived from the fact that it was first isolated in the 90s and was found to be abundant in the adrenal medulla. The ADM protein consists of a sequence of 52 amino acids and plays many roles in various physiological processes, primarily in the cardiovascular and endocrine systems. It has also been found to have immunomodulatory properties, which is why researchers have been looking into the possibility of using ADM as a prognostic tool for the detection of several infections and diseases. It has been determined that the monitoring of the ADM protein may be a valuable tool in evaluating the progression of a disease. However, the detection of ADM is challenging due to its short half-life (approximately 20 minutes) and high biological activity [7].

Pro-adrenomedullin is the precursor of the ADM protein, consisting of 185 amino acids. This precursor releases MR-proADM and ADM at a 1:1 ratio (figure 1). Mid-regional pro-adrenomedullin (referred to as MR-proADM in the literature and as proADM in the following work) is a fragment of 48 amino acids derived from the pro-adrenomedullin precursor. As mentioned, the concentration of MR-proADM proportionally represents that of the ADM protein. This fragment of the precursor presents a longer half-life and a lower biological activity, facilitating its monitoring [8]. This is why it has been used as a biomarker instead of ADM by many researchers.

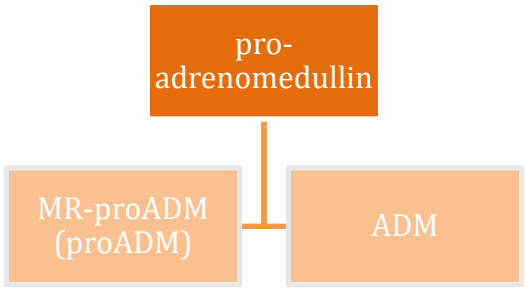

Figure 1: Derivatives of the pro-adrenomedullin precursor.

Mid-regional pro-adrenomedullin has been used as a biomarker for the detection of a wide variety of infections such as sepsis [4], organ failure resulting from infection [9], bacteriemia infection [10], septic shock due to community acquired pneumonia [11] and even COVID-19 [12]. The average concentration of proADM is 5 ng/mL in healthy patients, 13 ng/mL in patients with sepsis and 41 ng/mL in patients with septic shock [13].

As mentioned above, this research is focused on developing a point-of-care (POC) device capable of reliably diagnosising sepsis. POC biosensors allow testing to be performed close to the site of patient care where the treatment is applied. These tests provide rapid results allowing the administration of the correct response to be implemented promptly. These tests are also more economic seeing as they do not require trained personal or sample transportation from the patient care facility to the laboratories. POC devices are classified based on the testing modality into different groups such as testing strips, immunoassays, antigen-based testing and molecular POCT. The following research is conducted using the immunoassay modality [14].

3.1. BIOSENSORS

Biosensors are analytical devices capable of quantifying biological and chemical interactions. The first biosensor was developed by Leland C. Clark Jr. in 1956 for the detection of oxygen. He is considered the father of biosensors, and his invention still carries his name: the 'Clark electrode' [15]. Biosensors are comprised of four primary parts: a *bioreceptor*, a *transducer* capable of translating the biological or chemical interaction into a measurable signal, a *data acquisition system* preparing the transduced signal for display, and *the signal processing system* providing visual results. Biosensors may be classified based on the transduction system employed into *electrochemical biosensors* (monitoring electrochemical variations), *piezoelectric biosensors* (monitoring mass changes) and *optical biosensors* (monitoring optical signals such as absorbance, wavelength and reflectance). We will be focusing on the latter.

Optical biosensors are the most common type of biosensor. They offer great advantages such as label-free and real-time detection of the analyte. They offer a cost effective, highly specific and highly sensitive analysis. They are being studied for a wide range of applications in areas such as medicine, environment and the biotechnological industry. The most common optical biosensors are the surface plasmon resonance-based (SPR) biosensors [16].

3.1.1. SPR biosensors

These optical biosensors are based on an optical phenomenon first observed by Wood in the early 1900s. He observed an unusual dark and light pattern in the reflected light of a metal-backed diffraction grating. In the 1950s it was discovered that the excitation caused by the incident light of the free electrons on the metal surface forms surface plasmon polaritons which in turn generate an evanescent electromagnetic field. This effect happens on the interface between two media with dielectric constants of opposite signs (see figure 2A and appendix 1 for formulas and further explanation). The metals presenting optimal conditions for this phenomenon are gold, silver and aluminium [17].

As described, this phenomenon happens on the chip surface and exponentially decreases with distance (the evanescent wave reaches a depth of approximately 100 to 600 nm), which is favourable to avoid non-specific interactions caused by elements distant from the chip surface. As will be explained in the following paragraphs, it is on the interface that the bioreceptor-analyte interaction will take place.

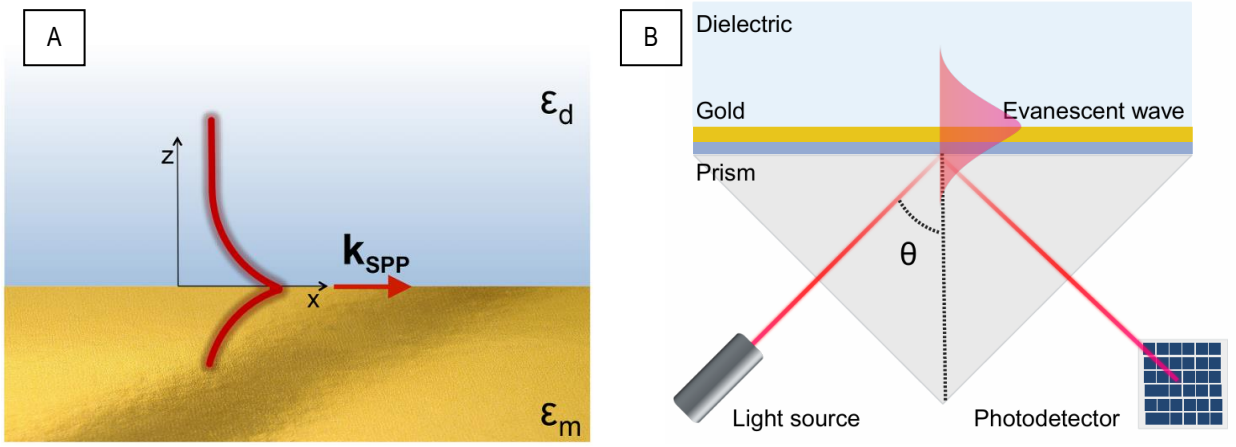

Figure 2: (A) Surface Plasmon Polariton vector on the interface [17]. (B) Kretschmann configuration [18].

The surface plasmons cannot be excited by direct illumination on smooth surfaces. Several different methods may be employed to correct this problem such as *prism coupling, grating coupling* and *waveguide coupling*. In the following experiments, the prism coupling system will be used, also known as the Kretschmann configuration (as shown on figure 2B).

The three main ways of using this phenomenon for the detection of an analyte are the *wavelength interrogation scheme, the intensity-based interrogation* and *the angle-based interrogation*. This work will focus on the two former ones, used to carry out the experiments.

3.1.1.1. Wavelength interrogation scheme

As seen in the previous section, part of the incident light is necessary to excite the surface plasmon polaritons and create the evanescent wave. This absorption of the light is crucial because it allows for the monitoring of what is called the plasmonic dip. This plasmonic dip happens at a specific wavelength (around 660 nm for gold, see figure 3A below). Any change on the chip surface will cause a change in the refractive index of the medium and thus cause a displacement in the wavelength of the plasmonic dip (figure 3B). This displacement is what is being monitored in the wavelength interrogation scheme and allows for real-time measuring. The angle of incidence of the light is fixed, the light source is polychromatic, and the detector employed is a spectrophotometer.

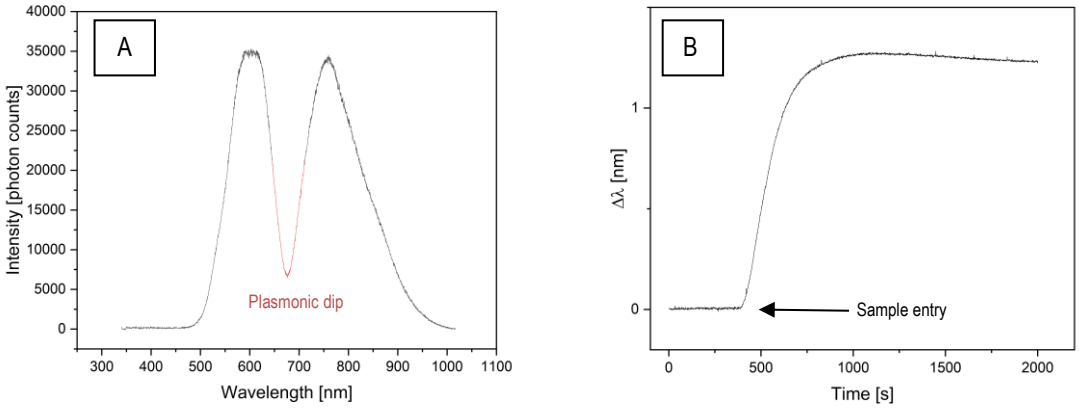

Figure 3: (A) SPR plasmonic dip. (B) Sensogram of a wavelength interrogation scheme biosensor after the injection of the analyte showing the displacement of the plasmonic dip as a function of time.

3.1.1.2. Intensity-based interrogation

Intensity-based interrogation biosensors operate on the same founding principles as the wavelength interrogation scheme biosensors. However, in this case what is being monitored is the percentage of reflectance of the incident light reaching the detector. The angle and the wavelength of the incident light are fixed; the light is thus monochromatic. Changes on the chip surface affect the Surface Plasmon Polaritons (SPP) propagation vectors, that is, the electromagnetic waves propagating along the interface between the metal and the dielectric medium and thus affect the amount of light reflected off the chip surface. The detector in this case is a photodiode. The sensogram looks the same, the only difference is the property on the y axis being percentage of reflectance instead of a difference in wavelength.

3.2. GOLD AND ALUMINIUM AS CHIP SURFACES

The most common metal used for the chip covering is gold. It has been thoroughly studied and is very known in the literature [19-20]. It is a very stable metal and can bond with thiol groups which is very useful when wanting to immobilize an antibody to the chip surface. Aluminium, on the other hand, is less studied and is less stable, being susceptible to oxidation and corrosion, if in contact with acids for example. The advantages it presents is its reduced cost and its easy implementation in the existing production chains in the industry. Using aluminium as a chip surface could greatly diminish the costs of the tests and thus make them more widely available. Furthermore, aluminium has three electrons on its conduction band compared to gold which only has one. This means it presents

more free electrons capable of being excited and creating the evanescent wave necessary for the monitoring of the surface plasmon resonance effect [19].

It is not possible to bond the bioreceptor directly to the metal surface. This is why surface modifications are necessary, the form of which will vary depending on the material. For example, the gold surface will be modified using alkanethiols (to construct a Self-Assembling Monolayer), whereas the aluminium surface will be treated using silicon oxides (silanization protocol).

3.2.1 Gold Self-Assembling Monolayer (SAM)

As mentioned above, to immobilize the bioreceptor to the chip surface, it is necessary to first build what is called a Self-Assembling Monolayer. This SAM will serve as a bridge between the metallic surface and the capture antibody. Gold forms stable covalent bonds with sulphur. This property allows us to bond thiol groups to the gold surface directly. This is why alkanethiols are used in this reaction. Indeed, this allows us to introduce the pertinent functional groups exposed on the chip surface. The thiol group reacts with the gold forming highly stable Au-S bonds, and the other functional group is left exposed. Interestingly, it has been shown that using a mixed SAM (utilising different alkanethiols simultaneously, some of which act as spacers and do not bond with the capture antibody) is beneficial to minimizing the non-specific binding, to avoiding steric hindrance in the immobilization step and to improve the signals obtained from the analyte [21]. Non-specific binding refers to the bonds formed between different molecules other than our analyte and the chip surface, leading to an increased signal attributed wrongly to the concentration of analyte in the sample. These interactions are to be avoided as much as possible.

In this case we are interested in having a carboxylic acid group on the SAM surface. This acid group will allow for the bonding of the capture antibody. In the following experiments a ratio of 3:7 of COOH:Sulfobetaine will be used to construct the Self Assembling Monolayer (see figure 4). COOH is an organic molecule which has a thiol functional group on one extreme and a carboxylic acid on the other. Sulfobetaine also has a thiol group on one end and has at its other extreme a zwitterionic group comprised of a quaternary amine (positively charged) and a unprotonated sulphonate group (negatively charged). This zwitterionic group has shown to minimise the non-specific binding which is inevitable when working in complex matrices like human serum [21].

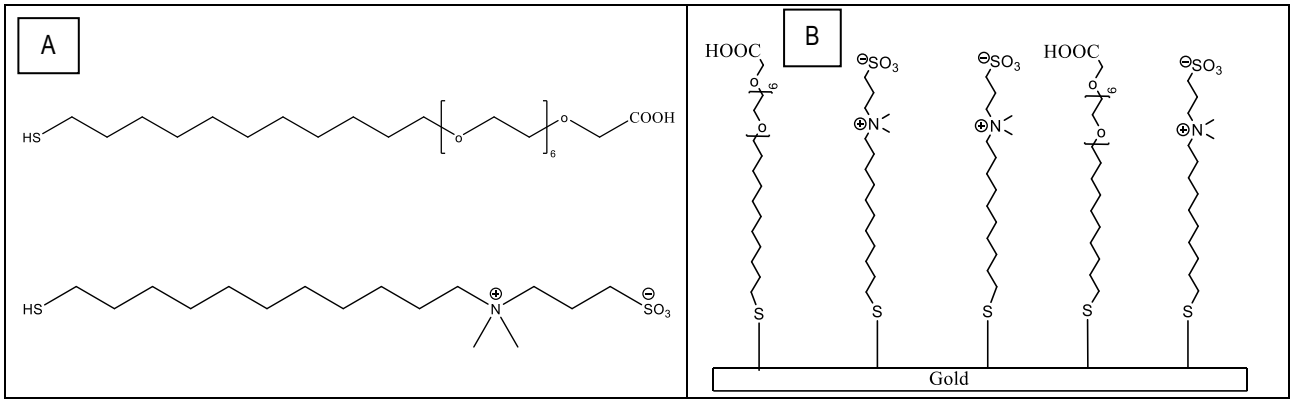

Figure 4: (A) Organic molecules employed in the SAM formation (COOH above and Sulfobetaine below). (B) Schematic representation of the resulting Self-Assembling Monolayer.

3.2.2 Aluminium silanization

Contrary to gold, aluminium does not form bonds with sulphur. Another method is thus applied called silanization. The aluminium layer is protected against corrosion by covering it with a layer of silicon dioxide (SiO_2). This layer allows for bonding with other silicon oxides such as APTES ((3-aminopropyl)triethoxysilane). As shown in figure 5A, this organic molecule presents on one side ethoxy groups and on the other a primary amine. The former bond with the silica layer, fixing the molecule to the chip surface (figure 5B), and the latter will serve for the bonding with the capture antibody. As can be observed on the figure below, the APTES may bond in different ways with the oxygen atoms of the chip surface. The exact position of each molecule is unknown however this is of no concern.

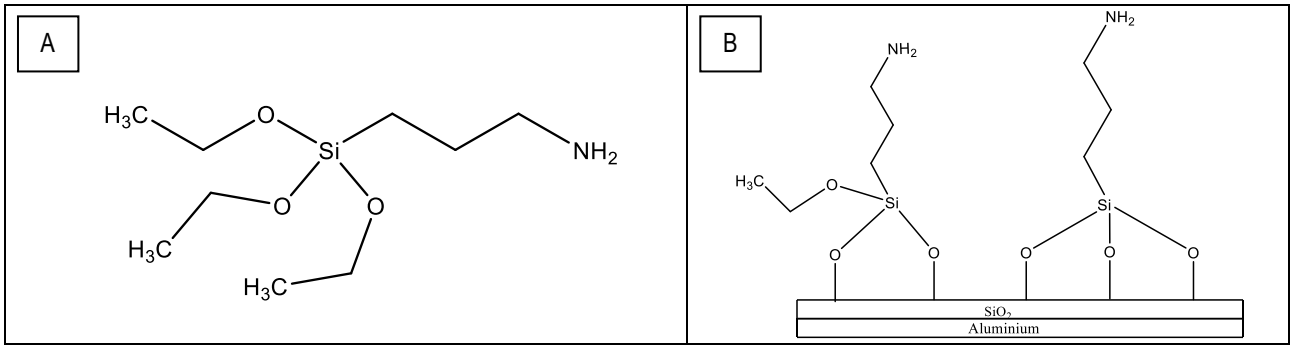

Figure 5: (A) APTES molecule. (B) Schematic representation of the resulting silanization.

3.3. ANTIBODY IMMOBILIZATION

3.3.1 Gold surface

The immobilization of the antibody on the chip surface happens through the formation of amide bonds between the exposed carboxylic acid groups on the SAM surface and the available amine groups on the antibody as shown on figure 6A.

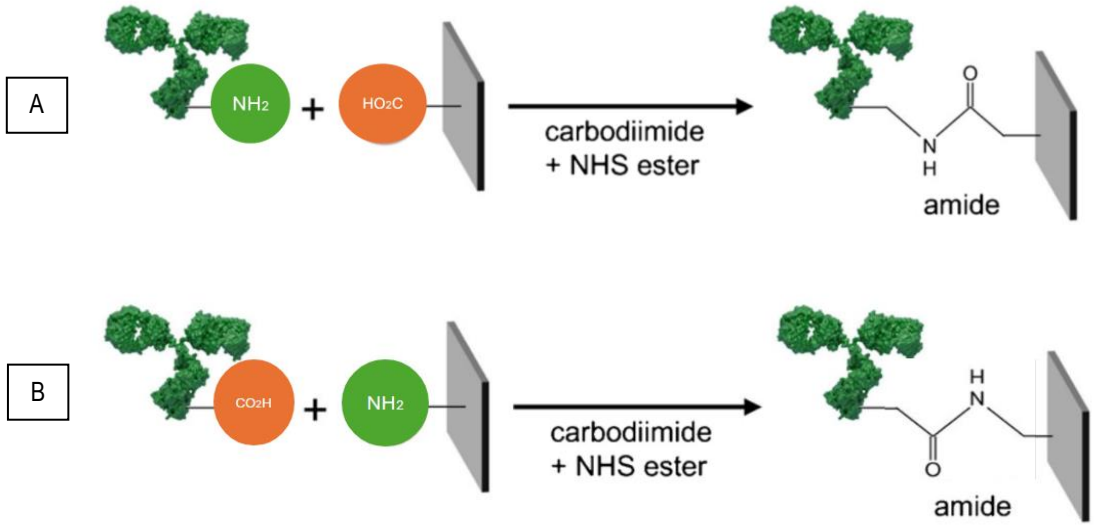

Figure 6: Schematic representation of the bonding reaction between the antibody and the functional groups on the chip surface [21]. (A) Gold. (B) Aluminium.

3.3.2 Aluminium surface

The immobilization on the aluminium surface works the opposite way. Thanks to the APTES, there are amine groups on the chip surface. These functional groups are the ones that will react with the carboxylic acid groups on the antibody surface as seen on figure 6B. In both cases, the result is the immobilization of the antibody on the chip surface through covalent bonds. The detection of the corresponding analyte may now be performed.

3.4. DETECTION METHODS

There are four main detection methods for the quantification of the analyte: *direct assay*, in which the analyte bonds directly with the capture antibody immobilized on the chip surface, *sandwich assay*, in which a secondary antibody is used to amplify the signal by increasing the size of the analyte, *direct competitive assay*, in which the analyte competes with its high-molecular-weight derivative (the signal is inversely proportional to the amount of analyte contained in the sample), and *indirect competitive assay* in which the

analyte or derivative is immobilized on the chip surface, meaning the fixed amount of antibody competitively bonds either with the analyte on the chip surface or the analyte in the sample [18]. This work will be focused on the first two types.

3.4.1 Direct assay and sandwich assay

A direct assay is one in which the protein is injected directly into the system and interacts with the antibody. These interactions are non-covalent bonds such as hydrogen bonds, Van der Waals forces and ionic bonds. As the analyte interacts with the bioreceptor, the difference in volume causes a change in the refractive index of the medium (figure 7A). This difference allows the instrument to quantify the amount of protein present on the chip surface due to its effect on the monitored property of the incident light.

A sandwich assay is necessary when the protein is too small to cause a significant signal on its own or when it is desired to increase the sensitivity of the method. In the case of the proADM protein, its small size (5kDa [22]) inhibits the use of a direct assay approach, as will be proved in the results discussion. For example, The CRP protein is measurable by direct assay and has a molecular weight of 120 kDa [23]. ProADM being 24 times smaller than CRP, it is necessary to use a secondary antibody which will bond to the available groups on the protein surface and thus effectively make the proADM protein larger. This increase in size means it is possible for the instrument to detect a significant change in the refractive index of the medium and the desired experiments may take place (figure 7B).

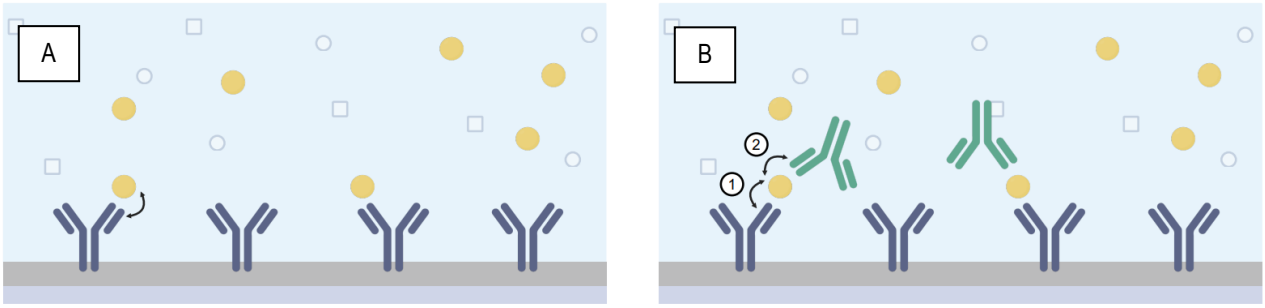

Figure 7: (A) Direct assay. (B) Sandwich assay. 1: bonding between capture antibody and protein. 2: bonding between protein and secondary antibody. [18]

4. OBJECTIVES

The objective of this work is to develop a new optical biosensor based on the surface plasmon resonance principle for MR pro-adrenomedullin, a promising sepsis biomarker, using two different surface materials. A comparison will be drawn between using gold and aluminium as chip covering surfaces. In each case, an analysis of the non-specific binding caused by human serum will be performed, considering the final aim will be to detect sepsis in real patient samples.

5. EXPERIMENTAL SECTION

5.1. EQUIPMENT AND MATERIALS

5.1.1 Equipment

Two different instruments were used to conduct this research. The first is a biosensor based on the wavelength interrogation scheme (it shall be referred to as SPR) and the second is an intensity-based interrogation (SENSIA).

As seen in the introduction, these two biosensors are capable of detecting minute changes of refractive index on the chip surface in real-time. In the case of SPR, the light is polychromatic. The SPR biosensor is composed of a light polarizer, a platform on which the chip is mounted (using the above mentioned Kretschmann configuration) and a spectrophotometer as a detector (figure 8A). The angle of incidence of the light is fixed and is set at the angle adjusted to the gold chip surface (at 70º). The signal obtained is a difference of nanometres, corresponding to the shift in plasmonic dip caused by the alteration on the chip surface.

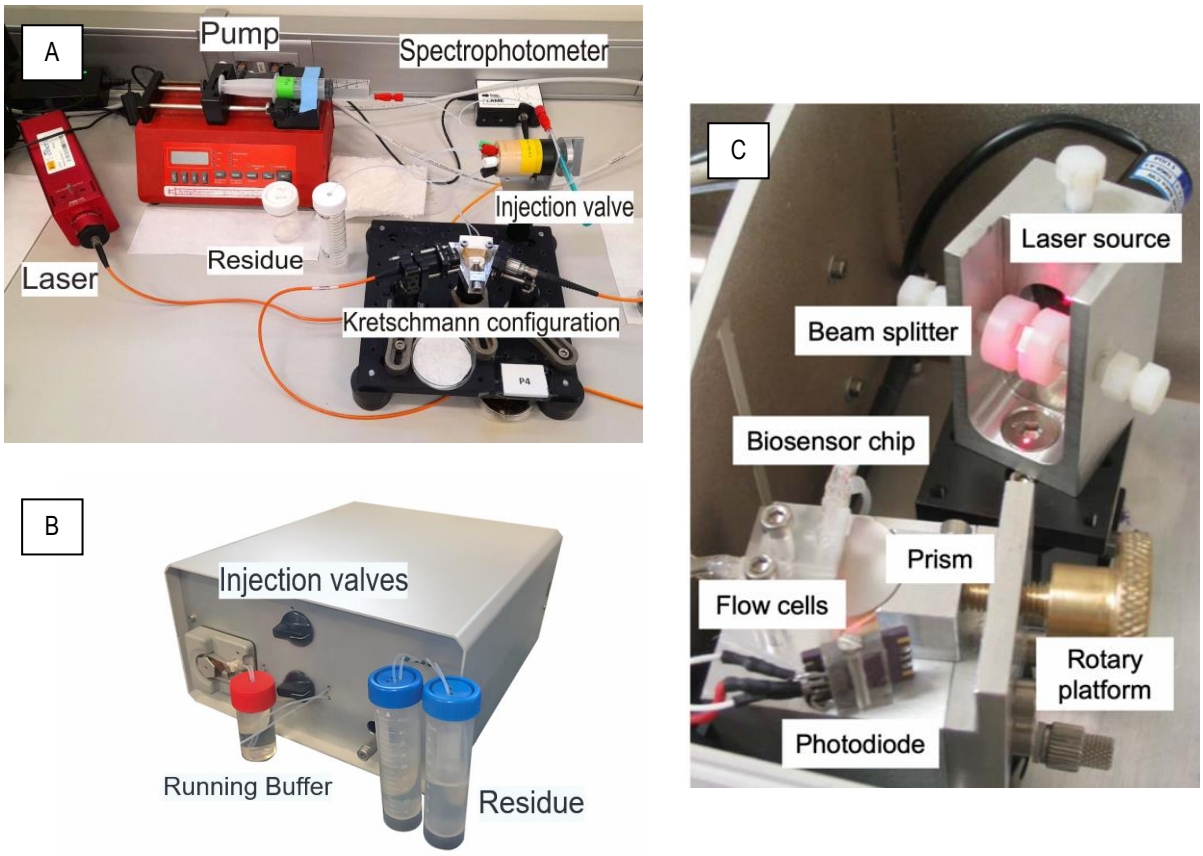

Figure 8: *(A) SPR biosensor. (B) SENSIA exterior [18]. (C) SENSIA interior [18].*

In the case of SENSIA (figure 8B), the signal obtained is a percentage of reflectance. The use of SENSIA was necessary to conduct the experiments on aluminium seeing as in this case, the optimal angle of incidence must be searched for using the rotary platform on which the chip is mounted. This sensor provides two beams of light at an exact wavelength thanks to the beam splitter, which allows us to study two independent channels simultaneously on the same chip. The detector used is a photodiode (figure 8C). When working with SENSIA, we must calibrate the instrument at its most sensitive point. This is done by representing the signal in front of the angle of the incident light (figure 9). Once this function is obtained, we may derive it and choose its minimum. At this point, the changes occurring on the chip surface will cause the maximum amount of difference in signal. Once the optimal angle has been determined, it is then fixed. From now on the only variant will be the percentage of reflected light that arrives to the detector, which is what will be monitored.

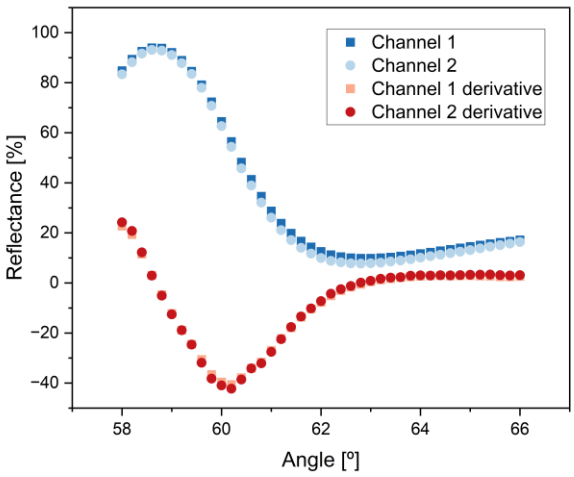

Figure 9: SENSIA calibration.

In both cases, the experiments are carried out with a continuous running buffer flowing through the system. The speed is controlled using a pump (a syringe pump in the case of SPR and a peristaltic pump in SENSIA).

5.1.2 Materials

The following buffers were used: MES 0.1M (pH 5.5), Acetic Acid/Acetate 6.4mM/3.7mM (pH 4.5), PBST 0.05% (v/v) (pH 7.4). The following solutions: NaOH 20mM, HCl 10mM and ethanolamine 1M (pH 8.0). The capture (H3) and secondary (G8) antibodies were commercially obtained through ThermoFischer. The proADM protein came from Biosynth (product code: PP17076, CAS number: 166798-69-2). For the SAM preparations: COOH (Prochimia Surfaces: TH003-m11.n6-0.2) and sulfobetaine (Prochimia Surfaces: ZI002m110.1) 1mM, at a ratio of COOH:Sulfo 3:7 in absolute ethanol. For the silanization: APTES obtained through Merck (product code: 440140, CAS number: 919-30-2).

5.2. CHIP PREPARATION

5.2.1 Gold (SAM)

A gold chip is cleaned successively using acetone, ethanol and milliQ water in a sonicator at 65ºC for one minute and is then left for 20 minutes in a UV/ozone cleaner. Once the chip is clean it is gently placed in a solution with the desired concentrations of COOH and sulfobetaine diluted in absolute ethanol. An activation step is then applied: the solution containing the chip is heated at 40ºC for 10 minutes and is then kept in storage at 20ºC.

5.2.2 Aluminium (silanization)

An aluminium chip is cleaned at 65ºC with acetone, ethanol and milliQ water successively in a sonicator for 5 minutes. It is then left for 40 minutes in a O_2 plasma cleaner. Once the cleaning process is finished, the chip is placed in 95% (v/v) EtOH/H_2O containing 1% APTES for one hour. The chip is then placed in the oven for 20'00 at 120ºC. It is then stored in the fridge.

5.3. ANTIBODY IMMOBILIZATION

The first step is the mounting of the chip on the platform of the desired instrument (SPR or SENSIA). Once the chip is in place, the pump may be turned on and the running buffer enters the system.

5.3.1 Gold

The first step is the activation of the carboxylic acid on the SAM surface. This is done by injecting a solution of EDC/NHS (0.2M/0.05M) in MES (pH 5.5), which deprotonates the acid and produces an amide which makes a better leaving group than the previous proton. The capture antibody is then injected at the desired concentration. The antibody's amine groups react with the

activated acid groups creating the corresponding amide bonds. These bonds are thus covalent, and the antibody is immobilized on the chip surface (see appendix 2 for reaction chemistry). An ethanolamine solution is then injected for two minutes. The ethanolamine, being such a small molecule, can react with any unreacted carboxylic acid groups on the SAM surface and thus prevent these groups from causing any non-specific binding when measuring samples. These two effects explain why sometimes, the ethanolamine leaves a positive signal, and other times leaves a negative signal compared to the one obtained with the antibody. Indeed, if many acid groups were left without reacting, the ethanolamine will take up space on the chip surface, increasing the refractive index of the medium and thus augmenting the signal. However, when it displaces the excess antibody which did not react, the signal decreases as the refractive index of the medium decreases. All of this is done with milliQ water as the running buffer.

5.3.2 Aluminium

Firstly, the instrument must be calibrated as seen in the experimental section. In the case of aluminium, the activation is done ex-situ. This is because following the silanization process, we are left with amine groups on the chip surface, meaning the carboxylic acid groups in need of activation are the ones present on the capture antibody. The activation is done by leaving the EDC/NHS and antibody in agitation at room temperature. Once this is done, the mixture is injected into the instrument and the reaction occurs between the activated carboxylic groups of the antibody and the available amine groups on the chip surface. Once again amide bonds are formed, and the antibody is immobilized on the chip surface. A small amount of diluted NaOH is then passed on the surface to wash away any loose antibody. All of this is done with milliQ water as a running buffer.

5.4. SAMPLE DETECTION

Now that the antibody is immobilized on the chip surface, the running buffer is changed to PBST which has shown to yield better results when measuring sample concentrations (effectively minimizing the non-specific bindings). In the case of working with SENSIA, the instrument must be calibrated again. Once this is done, the measuring of the different concentrations of protein diluted in PBST may be performed (the exact same one used as running buffer to create no greater signal difference than that caused specifically by the interaction of the protein with the antibody). As seen in the introduction, the proADM protein being so small, an additional step must be added to the sample preparation. A fixed amount of secondary antibody is diluted into the protein solution and is left in agitation for a specific amount of time, creating the sandwich effect and increasing the size of the analyte. In between sample injections, a regenerating step must be applied using either NaOH (20mM) or HCl (10mM) to wash away the protein on the chip surface. This process does not damage the immobilization of the antibody seeing as it is covalently bonded to the chip surface whereas the protein interacts through weak bonds with the antibody, explaining why it can be washed away. This enables us to use the same chip and the same immobilization for several measurements.

6. RESULTS AND DISCUSSION

6.1 SPR RESULTS

6.1.1 Optimal immobilization pH

The first test performed was the identification of the optimal pH for the immobilization of the antibody. This was done by injecting the capture antibody in the system, diluted in different buffer solutions at a concentration of 10 µg/mL, allowing it to flow on the non-activated SAM to determine the conditions in which the antibody seemed to have a greater affinity for the chip surface (figure 10). What is studied is of the attraction between the charges of the antibody and the charges of the SAM. Indeed, the different pH values affect the charges on the antibody as well as the charges on the SAM carboxylic acid groups. The effective charge on the antibody depends on its isoelectric point (which is unknown). At lower pH, the amine groups on the antibody will protonate and thus there will be positive charges present. At higher pH values, the COOH groups will deprotonate, and the antibody will present negative charges, as will the COOH groups on the SAM. As the pKa of carboxylic acids is around 5, it is expected that the SAM will be negatively charged in pH values above said value.

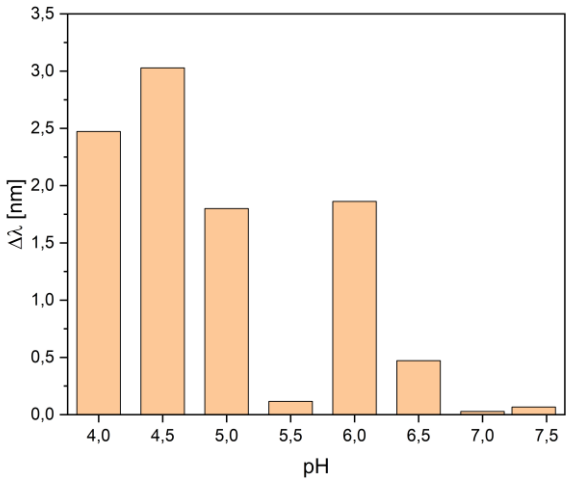

Figure 10: Signals obtained for the different pH buffer solutions containing 10 µg/mL of capture antibody. pH 4 and 4.5 were prepared using acetate, pH 5 to 6.5 in MES and pH 7 and 7.5 in PBS.

As can be observed, pH 4.5 using the acetate buffer gave the highest signal, meaning it has the greatest probability of immobilizing the bioreceptor on the chip surface and thus making it the optimal pH for the immobilization of the capture antibody. This will be the pH used in the following experiments during the immobilization step. At this pH, it is expected that the COOH and the amine groups (NH_3^+) will be protonated.

6.1.2 Capture antibody immobilization

Now that the optimal pH has been determined, the capture antibody is injected on the activated SAM surface (using the acetate buffer at a pH of 4.5). As seen on the sensogram (figure 11), the orange region shows the passing of the EDC/NHS in MES (pH 5.5), which activate the COOH groups on the SAM surface. The bulk represents the change in buffer solution, seeing as the MES buffer presents a higher refractive index than the milliQ water which is used as the solution running through the system. The next step, represented by the blue region, shows the reaction of the capture antibody with the activated surface. As can be seen, a stable signal of approximately 10 nm is obtained. The stability shows that an equilibrium has been reached between the antibody and the carboxylic acid groups on the SAM surface. The black region shows the passing of the ethanolamine solution. Its effect on the SAM surface is slightly negative. As seen in the experimental section, this may indicate there were some free antibodies on the chip surface that had not reacted and were washed away with the ethanolamine. The green region represents the changing of the running buffer from milliQ

water to PBST. This gives a positive signal seeing as PBST contains several different salts at different concentrations and thus presents a higher refractive index than water.

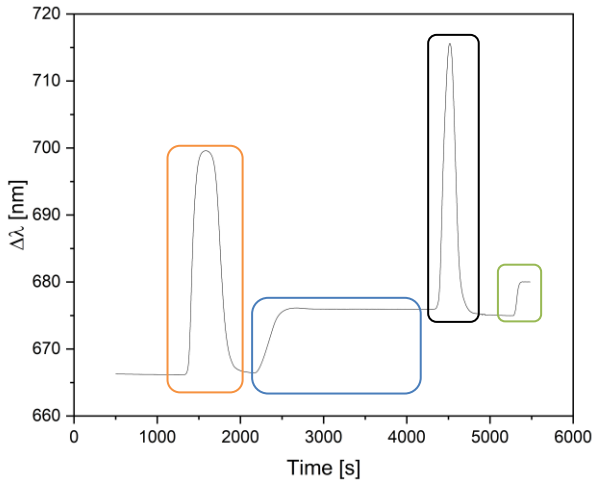

Figure 11: Sensogram showing the different stages of the immobilization process as a function of time.

The immobilization was successful (giving a signal of about 10nm). This means the bioreceptor is covalently bonded onto the chip surface and the protein measurements may now be performed.

6.1.3 Sandwich assay optimization

6.1.3.1 Agitation time

The next test performed was a study of the agitation time employed to carry out the sandwich mechanism. Indeed, this parameter affects the binding process of the proADM protein and the secondary antibody as can be seen on figure 12.

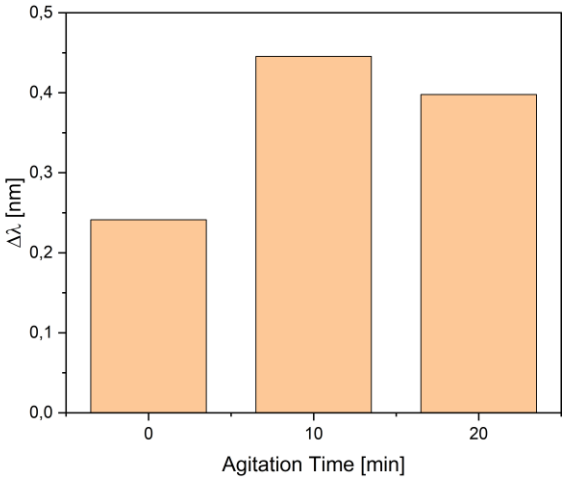

Figure 12: Signals obtained from different agitation times with fixed concentrations of proADM and secondary antibody (5 µg/mL and 4 µg/mL respectively).

The agitation time of 10 minutes at room temperature gives the highest signal. Indeed, the higher the signal the better, seeing as this means we will obtain a wider limit of linearity and improve the sensitivity of the method. It seems that the aggregates of protein and secondary antibody need these 10 minutes to form and stabilize. Leaving the mixture in agitation for longer doesn't improve the signal. The following experiments will thus be conducted using this optimized condition.

6.1.3.2 Secondary antibody concentration

It was deemed relevant to confirm the necessity of the sandwich mechanism and to determine the optimal concentration of secondary antibody for said mechanism. Two secondary antibody concentrations were tested, 2 µg/mL and 5 µg/mL (figure 13).

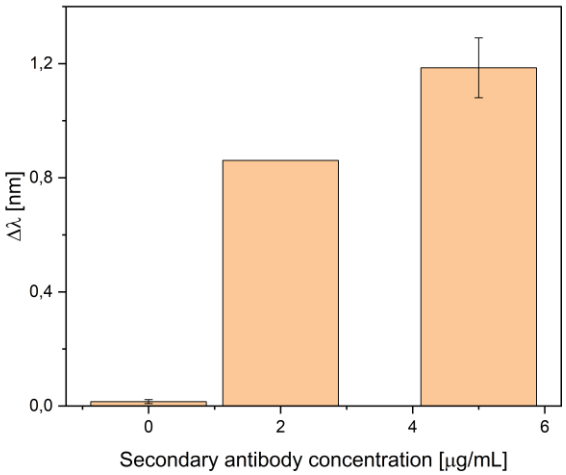

Figure 13: Signals obtained for different secondary antibody concentrations with 5 µg/mL of proADM.

The figure shows the signal obtained from 5 µg/mL of proADM protein without secondary antibody, almost zero, and the signals derived from performing the sandwich mechanism with two different concentrations of secondary antibody. The concentration of 5 µg/mL yielded a better result; this concentration will be used in the following experiments.

However, it is necessary to evaluate if this selected concentration affects the specificity of the biosensor. To this end, 5 µg/mL of the secondary antibody were injected, without any protein, and no significant signal was obtained. This means there is no non-specific binding between the two antibodies and thus the sandwich mechanism may be carried out without concern for an increased signal caused by the secondary antibody.

6.1.4 proADM calibration curve

A calibration curve was obtained by injecting varying concentrations of the proADM protein whilst using a constant concentration of 5 µg/mL of secondary antibody. As can be seen on figure 14, the curve has a saturation point of around 0.4 µg/mL. This happens because there is a limited amount of space on the chip surface for the protein to bind to the capture antibody.

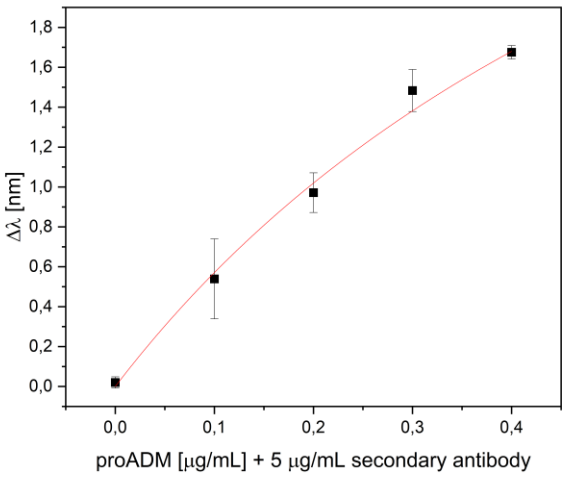

Figure 14: Calibration curve for different concentrations of proADM in PBST. Secondary antibody concentration 5 µg/mL.

It is interesting to note that the calibration curve is linear until the 0.3 µg/mL concentration. This is important to calculate the limit of detection (LOD) and the limit of quantification (LOQ). The limit of detection represents the lowest concentration of analyte which may be confidently distinguished from the blank. The limit of quantification is the lowest concentration of analyte that can be quantified by the method in a reliable way. These parameters are calculated using the following equations:

$$LOD = \frac{3 \cdot \bar{s}_x}{s} \qquad LOQ = \frac{10 \cdot \bar{s}_x}{s}$$

In which $\bar{s}_x$ represents the average standard deviation of the measurements [nm] and s represents the slope of the linear part of the calibration curve [nm·mL/µg]. In this case, the values obtained are LOD = 1.19 ng/mL and LOQ = 3.96 ng/mL. As can be seen, these values are slightly lower than those found in healthy and infected patients which means this calibration curve can potentially be used to quantify the amount of proADM in patient samples.

6.2 SENSIA RESULTS

As the calibration curve was successfully obtained in the prior set-up, the experiments were then continued on the second instrument, to conduct the gold and aluminium comparison. Indeed, the angle being fixed on the SPR setup up, another instrument is needed to study the aluminium surface seeing as the optimal angle for the incident light is different.

6.2.1 Au and Al sensitivity comparison

Using solutions with different concentrations of PBS, which have different salt concentrations and thus varying refractive indexes, a test was performed comparing the reflectance signals obtained on a clean gold surface (figure 15A) and on a clean aluminium surface (figure 15B).

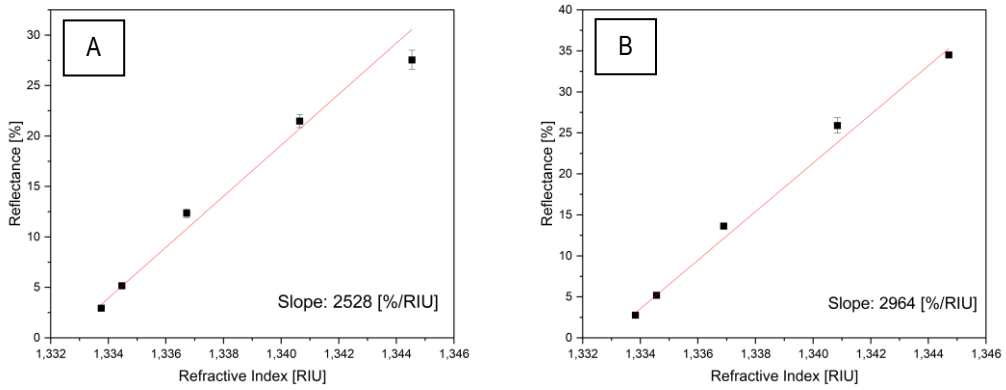

Figure 15: Results obtained from solutions of different refractive indexes. (A) gold and (B) aluminium.

The sensitivities are as follows: Au: 2528 [%/RIU], Al: 2964 [%/RIU]. From this data, it is clear to see that aluminium has a similar sensitivity to changes in the refractive index on the chip surface as gold. This is a favourable indicator for the potential of using aluminium as a chip surface seeing as it could reach a similar, or an even lower, limit of detection. Now that this test has been performed, the biofunctionalizations shall be compared on the two different surfaces.

6.2.2 Gold results

The protein behaviour on the gold chip must be studied anew seeing as the experiments are now being carried out on a different instrument. The prior results allow us to have a better idea of what to expect and the same optimized conditions will be used.

6.2.2.1 Capture antibody immobilization

A comparative study was accomplished comparing two concentrations of capture antibody: 20 µg/mL and 50 µg/mL. In a preliminary study, the two different concentrations were injected on the same chip, each in a different channel (figure 16).

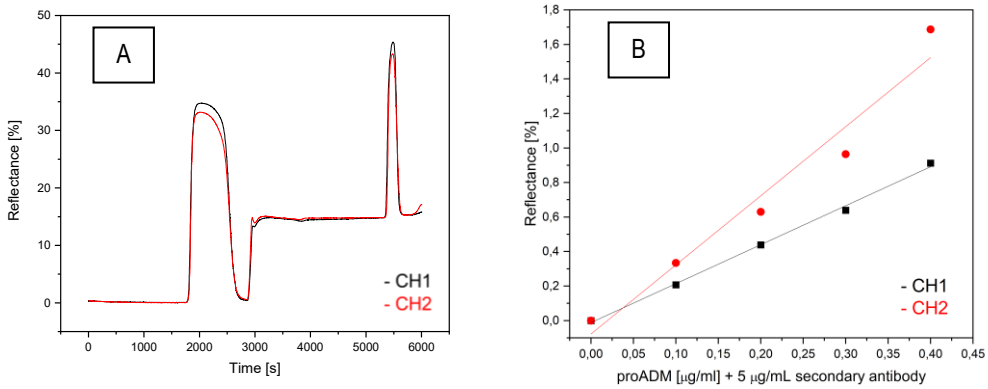

Figure 16: (A) Immobilization sensograms. (B) proADM calibration curves in PBST. CH1: 20 µg/mL and CH2: 50 µg/mL of capture antibody.

As can be observed on figure 16A, the two concentrations present almost the same exact immobilization pattern. This seems to mean that regardless of the concentration of the primary antibody injected, the amount of antibody immobilized is the same. However, a considerable difference is observed when comparing the calibration curves. The immobilization of 50 µg/mL yielded a higher sensitivity which appears to mean it would render better limits of quantification and detection compared to the 20 µg/mL concentration. Notwithstanding, these experiments needed to be repeated with sample duplicates on a different day to confirm the obtained results (figure 17).

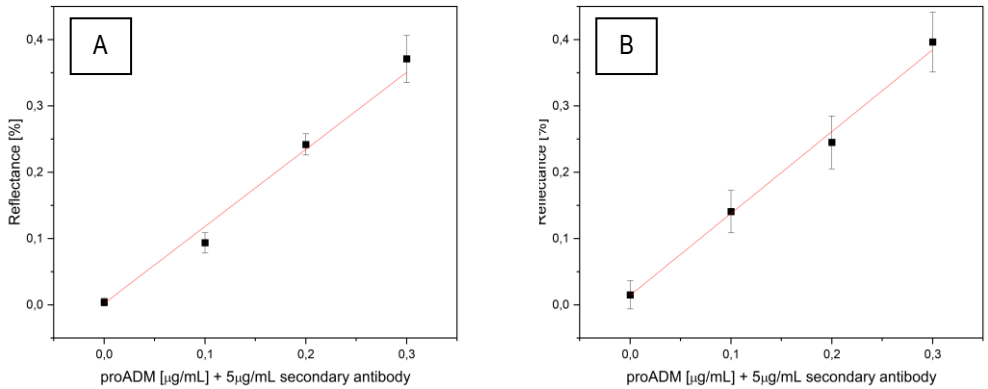

Figure 17: proADM calibrations curves in PBST. (A) 20 µg/mL and (B) 50 µg/mL of primary antibody.

As the figure shows, no significant difference is to be found when comparing the signals obtained in each case for the same protein concentration. The results are summarized in table 1.

Table 1: Summary of sensor results.

Capture antibody concentration	LOD (ng/mL)	LOQ (ng/mL)	Serum 1:10 (Reflectance [%])
20 µg/mL	7.95	26.5	1.65
50 µg/mL	5.37	17.9	1.84

The conclusion drawn from these results is that there is no significant difference in using 20 µg/mL or 50 µg/mL of antibody in the immobilization. The LODs in each case are very low and very similar.

Another important factor to consider is the non-specific binding caused by serum. It is important to keep in mind that the idea behind all these tests is to detect sepsis in real patient samples. The matrix, human serum, is thus a lot more complex and causes many complications. In this preliminary study, the effect of injecting a ten times diluted sample of human serum is considerable. If the experiments in patient samples were to be carried out at this dilution, it would be impossible for us to determine the concentration of proADM protein contained in the sample seeing as, just with the non-specific binding of the complex matrix, the signal greatly exceeds our limit of linearity. In the following experiments, the concentration of 20 µg/mL will be used seeing as it uses a lower amount of antibody whilst giving approximately the same results.

6.2.2.2 Serum dilution

The next step was to determine the serum concentration appropriate to conduct the protein samples quantification without the non-specific binding of the serum inhibiting the measurements. Table 2 shows the signals obtained from the different serum dilutions.

Table 2: Signal obtained from the different serum dilutions.

Serum dilution	Reflectance [%]
1:50	1.41
1:200	0.90
1:500	0.08

Based on these results, it seemed that a dilution of 1/500 is adequate for the measuring of the samples seeing as it gives a low enough signal that the effect caused by the proADM protein may still be studied.

6.2.2.3 Serum calibration curve attempt

Different concentrations of proADM protein diluted in a ratio of 1/500 of human serum were injected into the instrument and, unfortunately, as can be seen on figure 18, the signal was constant in all cases, which is also the same as the non-specific binding signal given by the serum 1/500. This test is done to mimic the real patient samples, which would come in a serum matrix.

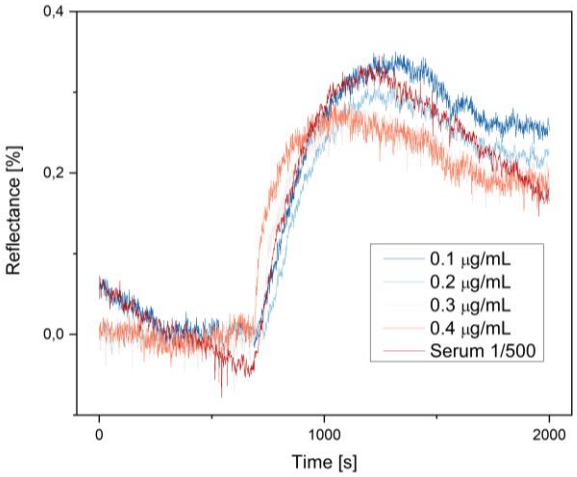

Figure 18: Sensograms obtained from different concentrations of proADM protein using a fixed concentrations of secondary antibody in a dilution of 1/500 of serum.

This behaviour indicates that the serum has a negative effect on the sandwich mechanism. This is to be expected given that serum contains all kinds of organic molecules, including for example albumin, which may also bond with the secondary antibody and the proADM protein and inhibit the formation of the proADM-G8 complex. This line of research will need careful study to optimize all the different parameters which may improve the signal and reach the aim of getting a calibration curve in serum, such as pH, changing the running buffer, etc. Once this is achieved, the quantification of proADM levels in real patient samples will be able to take place, which will probably bring its own set of challenges.

6.2.3 Aluminium results

6.2.3.1 Capture antibody immobilization

As seen in the experimental section above, the aluminium chip immobilization occurs in one step, due to the activation of the acid groups being completed ex-situ. Figure 19A shows a sensogram of the capture antibody immobilization. The orange region shows the passing of the EDC/NHS/capture antibody in acetate (pH 4.5). A signal of approximately 3% was obtained meaning some antibody was successfully immobilized on the chip surface, albeit a rather small amount. The bulk observed is caused by the buffer solution in which the EDC/NHS/capture antibody mixture is dissolved, seeing as it has a higher refractive index than the milliQ water. The blue region shows the effect of the NaOH. In this case, a slight loss of signal indicates the presence of antibody molecules that were not covalently bonded to the chip surface. The green region shows the change of running buffer from milliQ water to PBST 0.05%.

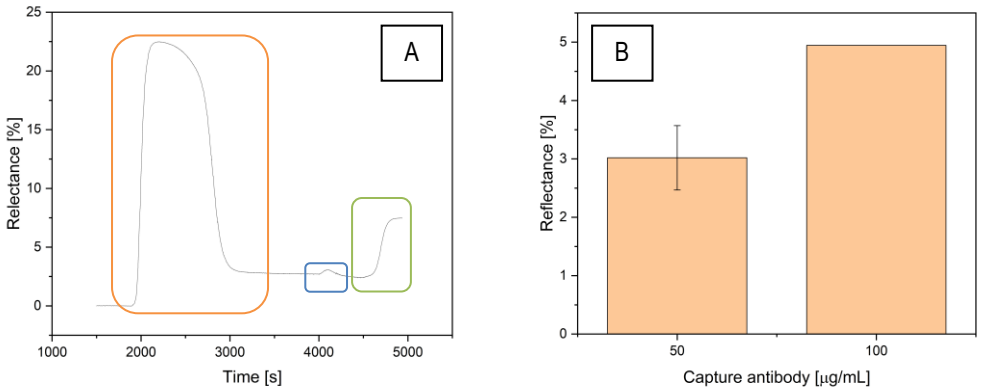

Figure 19: (A) Immobilization sensogram. (B) Immobilization signals obtained from different capture antibody concentrations.

A comparison was made between immobilizing 50 µg/mL and 100 µg/mL of primary antibody (figure 19B). As can be observed, the 50 µg/mL concentration achieved a lower immobilization. However, when comparing the non-specific binding caused by serum, it was noticed that the immobilization of 100 µg/mL presented a much greater value than that of 50 µg/mL. For a serum dilution of 1/300, the signals obtained are 0.14% and 0.06% respectively. This happens probably because, as there is more antibody on the chip surface, there is more opportunity for the molecules in the serum to bind to the antibody. The following test will thus be conducted using 50 µg/mL of capture antibody, even though 100 µg/mL provided a higher value of immobilization.

6.2.3.2 proADM detection

A calibration curve was obtained but, as can be observed in figure 20, the concentration of proADM protein needed to obtain a signal is ten times higher than in the case of gold, and the signals obtained are extremely low. Another problem is that these results are not reproducible, as the signal given by the same concentration varies from one day to the next.

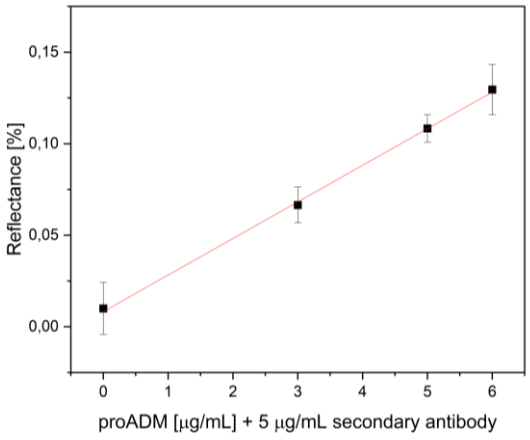

Figure 20: proADM calibration curve in PBST

The LOD and LOQ give values of 2.0 and 6.6 µg/mL respectively, a thousand times higher than those obtained with gold. This indicates that this calibration curve, in these conditions, cannot be used to measure samples. A study needs to be performed optimizing the different parameters such as the silanization protocol, the running buffer, the conditions for the immobilization, etc., to obtain a reproducible calibration curve with better signals and lower limits of detection and quantification.

As mentioned above, the signals obtained were not reproducible. This happens as well with the serum samples. This being said, some data was collected and is summarized in the following table 3.

Table 3: Signals obtained from the different serum dilutions.

Serum dilution	Reflectance [%]
1:50	0.46
1:100	0.26
1:200	0.18
1:300	0.06

From this data, it seems that aluminium presents lower non-specific binding than the gold chip surface (table 2), confirming what is said in the literature [19]. It seems that the 1:300 dilution would be adequate to measure protein samples. However, it would first be necessary to optimize the sample measurement without serum, before moving on to a more complex matrix.

To lower the LOD and the non-specific binding of the serum matrix, changes could be made to the running buffer, the immobilization pH, the silanization protocol (different silanes could be used other than APTES), the percentage of APTES, etc. It is seen that the same conditions that were optimized for the gold surface may not be applied to the aluminium surface.

7. CONCLUSIONS

From the results obtained by the SPR sensor, it may be concluded that the optimized conditions show extremely high potential to serve as a method for the quantitative detection of mid-regional pro-adrenomedullin. The immobilization is successful, the signals obtained are reproducible and the limit of detection is very low. Indeed, the limit of quantification of 4 ng/mL and the limit of linearity reaching 300 ng/mL provide a wide working range for the detection of sepsis. In a patient with sepsis, average values of proADM are 13 ng/mL as seen in the introduction, which falls within our working range.

The results obtained from the SENSIA instrument however show there is still a lot of work to be done before real patient samples can be tested. The limit of quantification in these conditions is 26.5 ng/mL. It is too high to detect patients with sepsis and is only adequate for diagnosing septic shock seeing as these patients have levels of about 41 ng/mL. The number of challenges only increases when looking into the effects of the complex matrix that is human serum. Indeed, the amount of non-specific binding in these conditions greatly exceeds our limit of linearity, making it impossible for the detection of the protein of interest. The only way to lower the non-specific binding whilst maintaining these conditions would be to dilute the human serum sample. By doing this, we make it impossible to detect the proADM levels seeing as they would be lower than our limit of quantification. This is clearly not an option.

In the case of the aluminium surface, the results are rather catastrophic. Many problems appeared such as low immobilization values, lack of reproducibility when analysing protein samples and high non-specific binding with serum (albeit lower than the ones observed in gold). Many factors need to be optimized such as the silanization protocol (trying different organic molecules, exploring the use of spacers), the conditions for immobilization (using of a different buffer, different pH) and the conditions for the protein measurements (using a different running buffer).

Overall, it may be concluded that the results are promising but that many more hours of work and research need to be invested into this project before this biosensing method can accurately measure proADM levels in patient samples. The immobilizations in all three cases were reproducible and successful, which is a promising start. The problems arise when the serum samples are introduced. Different methods can be applied to minimize the non-specific binding such as using different chip modifications (using different organic molecules, using different spacers), changing the running buffer throughout the system (increasing the level of Tween 20 in the PBST, adding other surfactants) and even applying different techniques to cover the chip surface (to mimic the role of the ethanolamine in gold for example). All these are potential factors to be explored that point towards a future in which the serum matrix does not impede the successful quantification of the proADM protein in real patient samples.

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9. ACRONYMS

APTES : (3-aminopropyl)triethoxysilane used in the modification of the aluminium surface.
COOH : alkanethiol used in the preparation of the gold SAM.
G8 : secondary antibody used for the sandwich mechanism.
H3 : capture antibody immobilized on the chip surface.
MES : (2-(N-morpholino)ethanesulfonic acid) is a buffer solution.
PBS : Phosphate-Buffered saline. This buffer contains 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2HPO_4. Its pH oscillates between 7 and 7.4.
PBST 0.05x : PBS containing 0.05% (v/v) of Tween 20 (a surfactant).
proADM : refers to the mid-regional pro-adrenomedullin protein which is released at a 1:1 ratio with the ADM protein by their precursor.
SAM : Self-Assembling Monolayer.
SPP : Surface Plasmon Polaritons.
SPR : Surface Plasmon Resonance.

APPENDICES

APPENDIX 1: SPR PHYSICS PRINCIPLES

The Surface Plasmon Polaritons are excited by the incident light and create an electromagnetic field called the evanescent wave. These SPP propagate along the interface of the two media with dielectric constants of opposite signs. The propagation component can be expressed using the following equation:

$$k_x{}^{SPP} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d}}$$

In which ω represents the angular frequency, c is the speed of light, ε_m is the dielectric function of the metal and ε_d is the dielectric constant of the medium which is directly correlated to its refractive index ($\varepsilon_d \approx n_d^2$). This relation between the propagation vector and the refractive index of the medium is fundamental to refractometric sensors.

The excitation of the SPP requires the coupling light's wavevector component parallel to the interface to match the propagation vector of the SPP:

$$k_x{}^{Light} = \frac{2\pi}{\lambda}\sqrt{\varepsilon_d}\,\sin\theta = k_x{}^{SPP}$$

This may not be achieved by direct illumination so a technique such as the Kretschmann configuration is applied. Once this condition is fulfilled, the evanescent wave is created and penetrates the metal film. The plasmon dip appears and the effects on the refracted light may be studied. For further information, see bibliography [17].

APPENDIX 2: EDC/NHS REACTION CHEMISTRY

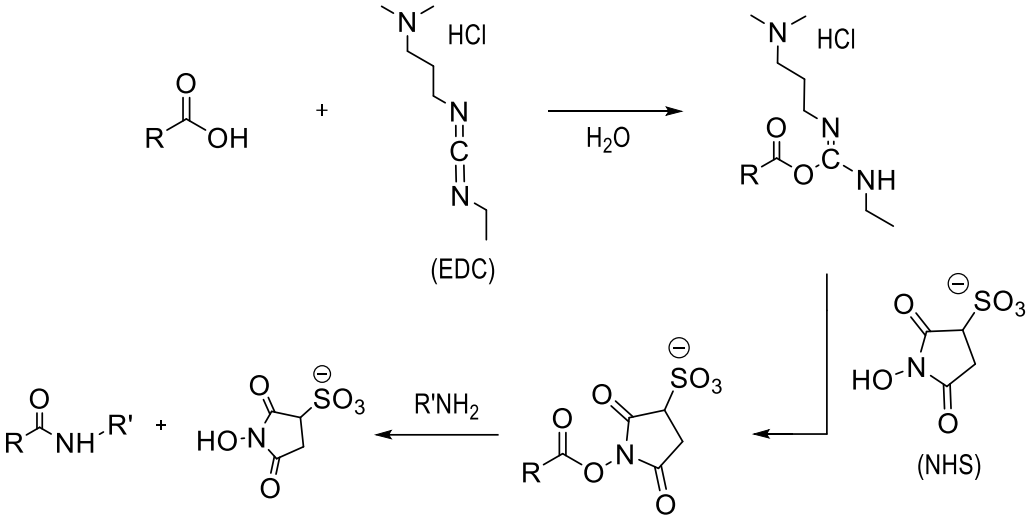

Figure 21: EDC/NHS reaction chemistry.

In the case of the gold surface, the RCOOH molecule represents the COOH organic molecule used for the SAM. R'NH2 represents the amine groups on the capture antibody which is then immobilized on the chip surface. As can be seen, the result is the covalent bonding between the COOH used for the SAM and the capture antibody.

In the case of aluminum/silica chips, RCOOH represents the acid groups on the capture antibody. $R'NH_2$ represents the amine groups available on the chip surface after the silanization protocol (the amine groups belonging to the APTES organic molecule). The result once again is the covalent bonding between the capture antibody and the chip surface.