Biosensors in forensic sciences: Application for SARS-CoV-2 detection

Master Thesis

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Abbreviations

ACE2 Angiotensin-converting enzyme 2

AMU University of Aix-Marseille

C12 Dodecyl spacer

COVID-19 Coronavirus disease

CWAs Chemical warfare agents

DNA Deoxyribonucleic acid

E Envelope

EDC 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide

NHS N-hydroxysuccinimide

ELISA Enzyme linked immunosorbent assay

FA Formamide

ICN2 Catalan Institute of Nanoscience and Nanotechnology

LOD Limit of detection

M Membrane

MHDA 16-mercaptohexadecanoic acid

MUOH 11-mercaptoundecanol

N Nucleocapsid

NH2- Amino

O/N Overnight

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

R Target binding response

RBD Receptor-binding domain

RNA Ribonucleic acid

RU Rack unit

S Spike

SAM Self-assembled monolayer

SARS-CoV Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV-2 Severe Acute Respiratory Syndrome Coronavirus 2

SDS Sodium dodecyl sulfate

SH- Thiol

SPR Surface Plasmon Resonance

SSC Saline sodium citrate

ssDNA Single stranded DNA

TMAC Tetramethyl ammonium chloride

TNT 2,4,6-trinitrotoluene

WHO World Health Organization

△R Increment target binding response

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0. Abstract

Background: Current methods in forensic analysis are usually time-consuming, require sample pre-treatment procedures and/or have low sensibility. The development of biosensors for biological and chemical determinations in medical diagnostics, environmental monitoring, drug detection and food safety analysis are taking advantage over traditional methods given their greater speed, ease of handling and sensitivity. The coronavirus disease-19 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has suddenly emerged as one of the major public health concerns because of its rapid spread and mortality. The actual methodologies for the early detection of the virus are quite slow and imprecise. The main objective of this work is to design a methodology for the detection of nucleic acids from SARS-CoV-2 by using a Surface Plasmon Resonance (SPR) biosensor in order to obtain a faster and more sensitive diagnostic tool. **Methods:** Two different biofunctionalization methods (chemisorption and covalent attachment) of the SPR biosensor chip, three conditions for probe incubation over the chip surface and five different hybridization buffers were tested for the optimization of the target detection. Additionally, we tested whether the use of lateral spacers during the chip biofunctionalization improves any aspect of the methodology. **Results:** Chemisorption allowed for a higher target detection compared with covalent attachment methods. Moreover, the incubation of the probe *ex-situ* and overnight over the chip surface showed a better sensibility when compared when *ex-situ* for three hours and *in-situ* overnight. The 5x SSC hybridization buffer showed higher hybridization efficiency than the other buffers tested. Finally, we found that the use of lateral spacers during the biofunctionalization allows for the use of lower target nucleic acids concentrations. **Conclusions:** The different strategies followed for the optimization of the SARS-CoV-2 nucleic acids detection point the use of SPR biosensors as a potential new faster, easier and more sensitive diagnostic tool.

Keywords: biosensor, SARS-Cov-2, nucleic acids.

1. INTRODUCTION

1.1 Biosensors in forensic sciences

Chemical and biological analyses are a frequent practice in forensic sciences. There are several screening analytical methods that are able to detect the presence of different compounds in different samples. Many of these methods are based on immunoassays using antigen-antibody reactions in which the antigen is the analyte to be detected. A very representative example is the enzyme-linked immunosorbent assay (ELISA), which is a widely used technique because its speed, high sensitivity and the scarce of sample manipulation[1,2]. However, the presence of false positives and negatives, and the lack of specificity are some of the potential disadvantages of this method, whose results usually require further confirmation with more complex analytical methodologies, such as mass spectrometry[3].

Under this scenario, the use of biosensors in forensic sciences could overcome many of the mentioned limitations. A biosensor is a device capable of providing specific quantitative analytical information by using a biological layer that is in direct contact with a transducer. The layer is covered by biological receptors such as proteins, antibodies or nucleic acids that are specifically designed to interact with the target compound in a sample. When the biological interaction occurs, a series of physicochemical changes in the layer's surface are detected by the transducer and converted into measurable signals (Figure 1)[4].

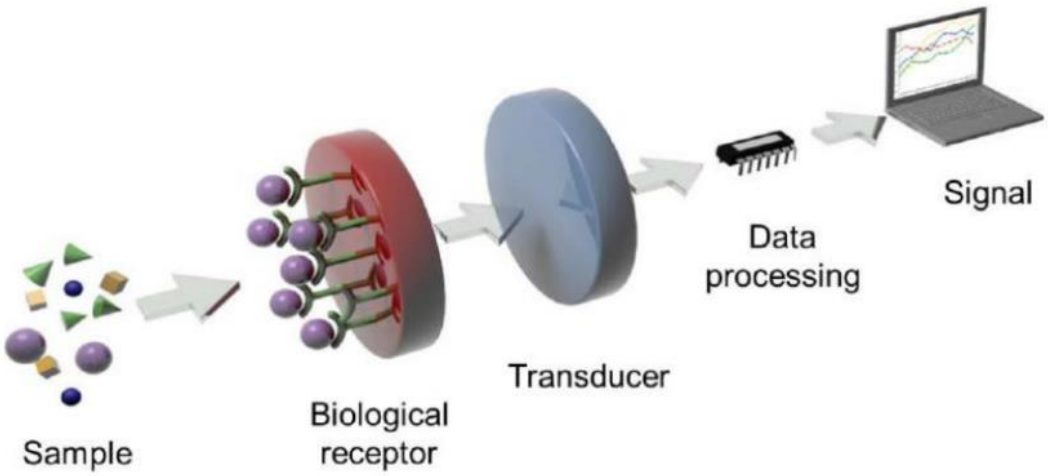

Figure 1. Schematic representation of a biosensor including the sample, the specific biological receptor, the transducer, the data processing system and the final signal. From NanoB2A group (ICN2).

The development of these biosensors has increased rapidly in the last years, and their use in the field of forensic analysis has been demonstrated with a large number of applications:

1) Detection of explosives such as 2,4,6-trinitrotoluene (TNT)[5].
2) Detection drugs, specifically methamphetamine in oral fluid samples[6].
3) Identification of five relevant body fluids at the same time (blood, semen, saliva, urine and sweat)[7].
4) Detection of chemical warfare agents (CWAs) which are defined as chemical substances that might be employed as a consequence of their direct toxic effects on humans, animals and plants[8]. For instance, a particularly dangerous class of CWAs are the nerve agents, which could cause rapid and severe effects on human health.
5) Detection of nucleic acids, which has recently gained much interest in diverse fields, such as disease diagnostics, food industry or environmental monitoring[4]. One of the most promising tools are the deoxyribonucleic acid (DNA)-based biosensors, which can detect the presence of pathogenic microorganisms or Single Nucleotide Polymorphism (SNPs)[4,9]. The present work is focused on the detection of these molecules using biosensors.

1.2 Types of biosensors

Biosensors can be classified depending on the type of transducer employed. Thus, we can distinguish optical, electrochemical or mechanical biosensors. For the detection of nucleic acids molecules, optical biosensors are the most commonly used, especially the Surface Plasmon Resonance (SPR) biosensor. They depend on sequence complementarity following Chargaff's rules of base pairing for DNA, A-T, G-C[4,10]. SPR biosensors allow real-time and label-free detection of biomolecular interactions measured as a SPR signal. Monitoring the change in the SPR signal over time produces a sensorgram, a plot of the target binding response (R) which measured as rack unit (RU), versus time that allows the visualization of different stages of a binding event[11]. RU value is directly proportional to the target detected in the sample.

Although the theorical basis of the SPR biosensor is not the main focus of the project, its working principle is going to be briefly described in the following lines (for more detailed information see Annex 1). SPR occurs when monochromatic light strikes an electrically conducting surface (usually gold) at the interface between two media, generating electron charge density waves called plasmons that reduce the intensity of the reflected light at a specific angle (resonance angle θ). Samples containing the target molecules in solution are injected over the surface of a previously biofunctionalized sensor chip through a series of flow cells, allowing their interaction with the receptor molecules immobilized on the surface. During the course of the interaction, light is directed toward the sensor surface and the resonance angle is detected. This angle changes as long as molecules bind and dissociate, and the interaction profile is then recorded in real time in a sensorgram (Figure 2)[12].

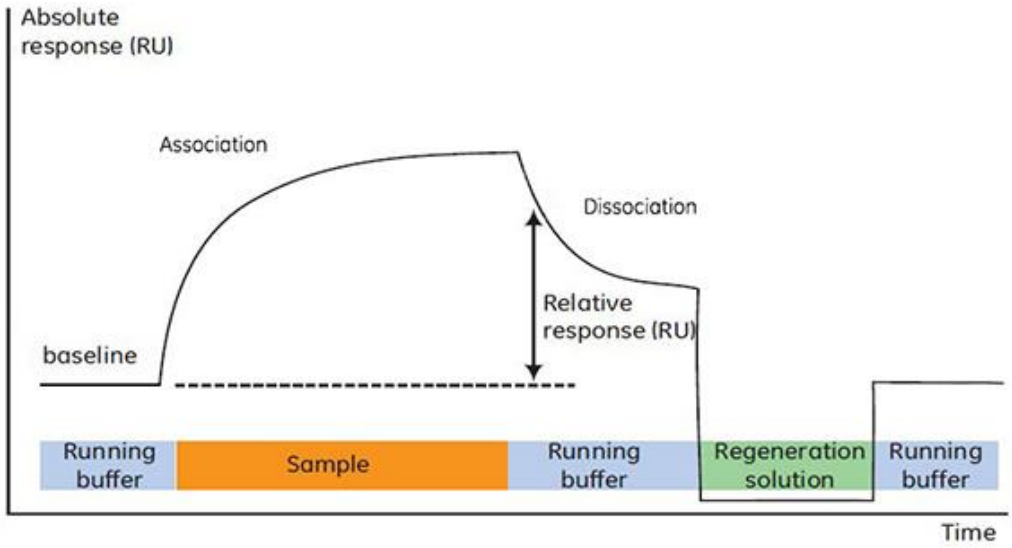

Figure 2. The schematic illustration of a sensorgram. The bars below the curve indicate the solutions that pass over the sensor surface: During sample injection, a positive response can be viewed in the sensorgram, as the target binds to the surface of the biofunctionalized chip. The response decreases when the sample is not flowing anymore and the signal stabilizes as the target is completely bound. After an analysis cycle is completed, regeneration solution is passed over the sensor chip, removing bound target and preparing for the next analysis cycle. Figue from Cytiva web.

1.3 Biofunctionalization of the layer surface

Previously to its use, the chip of the SPR biosensor should be biofunctionalized. The chip consists on a piece of glass coated with chromium and gold (Figure 3.A) and is the biofunctionalization of its layer surface consists of the immobilization of the desired receptor molecules to the gold surface (Figure 3.B). This procedure is especially important because the final sensitivity and specificity of the SPR biosensor are directly related to the activity of the immobilized molecules and the accessibility of their specific targets.

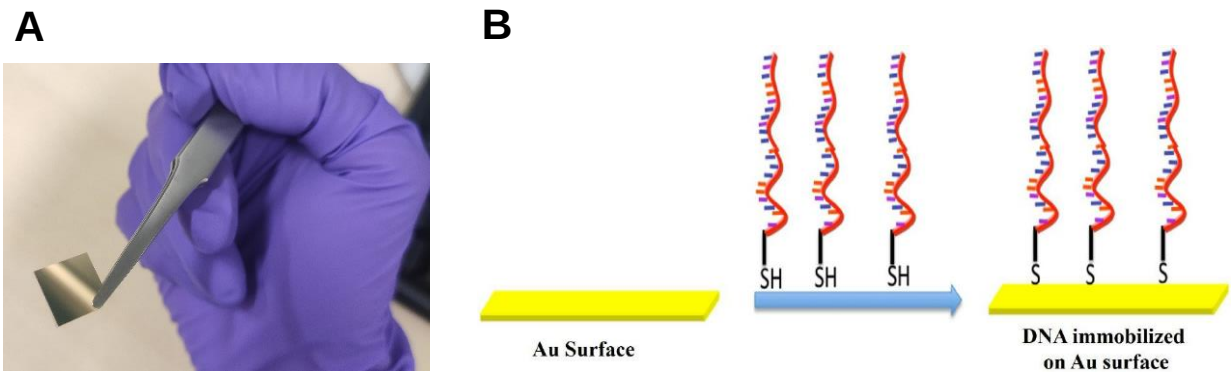

Figure 3. (A) Real image of a gold chip. Image taken by myself at our laboratory. **(B)** DNA immobilization on Au (Gold) surface. Figure from[13].

In the development of nucleic acid-based biosensors, there are two key steps: the DNA probe design and the surface biofunctionalization chemistry.

1.3.1 DNA probe design

The DNA probe, also called single stranded (ss)-DNA, is going to be immobilized to the layer surface of the chip and must provide affinity and selectivity for the target nucleic acid molecules in the sample and avoid non-specific hybridization with other molecules.

Two important elements that should be considered in the design of the ssDNA probes are the functional linker group and the vertical spacer. The functional linker group will allow the attachment of the probe to the sensor surface. The most widely used is the thiol (SH-) group. On the other hand, the vertical spacer is a poly-thymine sequences

that will improve the mobility of the probes already attached to the surface and their accessibility to the complementary target sequences (Figure 4)[14].

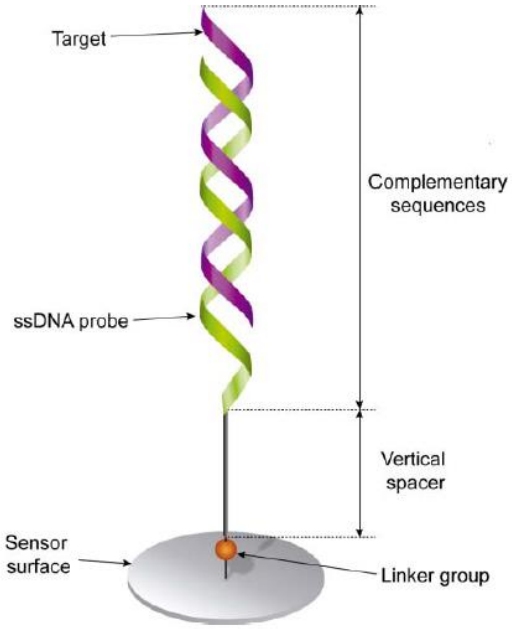

Figure 4: ssDNA probe employed as bioreceptor, where the functional linker group and the vertical spacer are indicated. From NanoB2A group (ICN2).

1.3.2 Surface biofunctionalization

There are different strategies to immobilize probes on gold surfaces. The most widely used approaches are the direct chemical grafting of DNA probes via a thiol linker, also known as chemisorption[15] and the covalent binding on a previously functionalized surface, named covalent attachment[16].

a) Chemisorption

Adsorption is the adhesion of atoms, ions or molecules from a gas, liquid or dissolved solid to a surface[17]. Chemical adsorption, also known as chemisorption, is a type of adsorption which involves a chemical reaction between the surface and the molecule. This technique takes advantage of the strong affinity of thiol molecules towards gold surfaces[18]. This type of immobilization generates self-assembled monolayers (SAMs) directly carrying the probe sequence (Figure 5) that will hybridize with the target nucleic acid sequence[19].

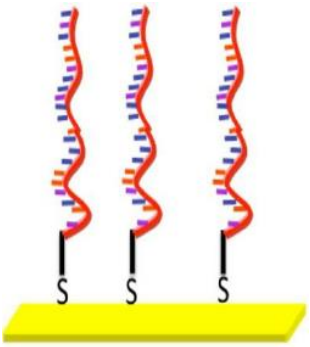

Figure 5. SH-DNA probes directly immobilized on gold surface by chemisorption where the thiol groups of the probes link to the surface through a covalent bond. Figure from[13]

During the chemisorption process, the probe-to-probe distance over all neighboring adsorbed DNA-probes continuously decreases and when a certain coverage has been exceeded lateral repulsions enter the scene, threatening the efficiency of the measurements (Figure 6)[15]. To solve this problem there are lateral spacers that optimize the DNA coverage at the surface, such as dodecyl spacer (C12), which promotes the correct organization of the probes [20].

Figure 6. Crowding effect on the DNA-DNA hybridization due to the high immobilization density of SH-DNA probes (red) difficulting the accessibility of the target DNA (purple). Figure from[13].

b) Covalent attachment

The surface of the chip can also be modified with a functional layer that carries various functional terminal groups, such as amine[21] or carboxyl[22] groups, creating a 3D matrix for the subsequent covalent immobilization of the probes (Figure 7). Due to the greater number of reactive sites, the 3D matrix permits the immobilization of a larger number of probes[23].

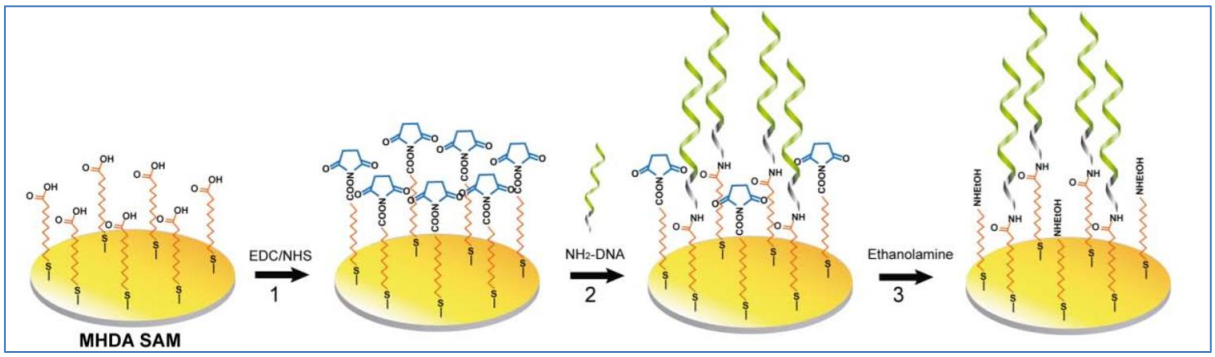

Figure 7. Scheme of the covalent immobilization of NH2-DNA probes. EDC/NHS: carbodiimide/ N-hydroxysuccinimide, MHDA: 16-mercaptohexadecanoic acid. The detailed process is going to be covered in the methods section. From NanoB2A group (ICN2).

Once the surface is biofunctionalized, either by chemisorption or covalent attachment, the next step is the detection of the target, in our case, sequences of ss-DNA . The hybridization efficiency of the DNA target with the immobilized ss-DNA probes is influenced by various factors, including the composition of the hybridization buffer, the time of probe immobilization and the quality of the surface layer, among others.

The final step in the target measurement process is the regeneration of the biosensor, which means the removal of the target DNA after the detection step without altering the immobilized probes, making possible to repeat analyses under the same conditions. To ensure the stability and integrity of the probe monolayer, it is crucial to apply a correct regeneration protocol. The use of formamide ($HCONH_2$) (FA) solutions reduces the melting temperature of the DNA and destabilize the bonds between the probe and the target double strand of DNA[24–26].

1.4 Biosensors as diagnostic tools: SARS-CoV-2 detection

In December 2019, a new type of coronavirus named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) emerged in Wuhan (China). Since then, the coronavirus disease (COVID-19) has been responsible for numerous deaths around the world, mainly through serious respiratory illness, such as pneumonia and lung failure[27,28]. While on January 30 2020 there were fewer than 100 cases and no deaths

outside of China, on August 3 2020 the number of cases and deaths increased to 17.5 million and 680,000 worldwide, respectively[29].

The rapid spread of the viral disease supposes a global public health concern. The lack of knowledge about several important aspects of SARS-CoV-2 infection, ranging from pathogen biology to host response and treatment options, has hampered the options to overcome the virus[30]. Because no specific drugs or vaccines for COVID-19 are yet available, early diagnosis is crucial for containing the outbreak[31]. Therefore, new rapid, accurate, and sensitive detection techniques are extremely needed for an early intervention. Currently, the methods for detecting the virus present some limitations: the detection of nucleic acids from SARS-CoV-2 is performed using the Polymerase Chain Reaction (PCR), a routine technique in clinical laboratories based on the amplification of ribonucleic acids (RNA). Although it is a highly specific and sensitive technique, PCR main limitations include the usual requirement of highly specialized personnel, the time of the technique, ranging from 2 up to 5 hours, and its high cost. On the other hand, the strategy of the immunochromatographic assay is based on the detection of whole virus by the use of specific antibodies directed to interact with the viral antigens or based on the detection of the presence of human antibodies produced in response to the infection. It is a really fast and low-cost technique in comparison to PCR. However, that advantages are overshadowed by the limited sensitivity that causes an amount of false negatives. Several technologies are being developed to overcome these limitations. Biosensor devices are taking notable advantage because of their great potential, providing high sensitivity and offering real-time measurements that can be used in primary care centres or even in emergency services.

On March 2020, the European Commission funded 17 research projects among which is the CoNVat project. It is the only one lead from Spain in collaboration with research groups from the University of Aix-Marseille (AMU) in France and the National Institute of Infectious Diseases (INMI) in Italy, and it has been carrying out at the Catalan Institute of Nanoscience and Nanotechnology (ICN2). CoNVat aims to contribute to the early diagnosis and clinical management of COVID-19 patients through the development of a biosensor allowing for direct, fast and specific SARS-CoV-2 identification. Since April 2020, I have been professionally implicated in the project,

reason why I decided to focus my master thesis on the development, optimization and use of nucleic acids biosensors to detect SARS-CoV-2.

1.4.1 SARS-CoV-2 structure

Taxonomically, SARS-CoV-2 is a strain of Severe Acute Respiratory Syndrome-Related Coronavirus (SARS-CoV) (Figure 8.A)[32]. It is believed to have zoonotic origins and has close genetic similarity to bat coronaviruses. SARS-CoV-2 genome contains four genes encoding for four structural proteins, named spike (S), envelope (E), membrane (M) and nucleocapsid (N) (Figure 8.B). It also comprises genes for eight accessory proteins (3a, 3b, p6, 7a, 7b, 8b, 9b, and orf14) [33]. The S protein contains a receptor-binding domain (RBD) that specifically recognizes angiotensin-converting enzyme 2 (ACE2) as its receptor, in humans[34]. This protein plays an important role to the pathogenesis of the virus because it binds to human ACE2 with 10–20 fold higher affinity than other coronavirus[35]. Another important part of the virus is the M protein which determines the shape of the virus[36]. N protein binds to the virus RNA and helps in host cell entry and interaction with cellular processes[37]. Finally, E protein is the smallest protein that can oligomerize and create an ion channel which plays a role in the viral replication cycle: viral assembly and virion release[38,39].

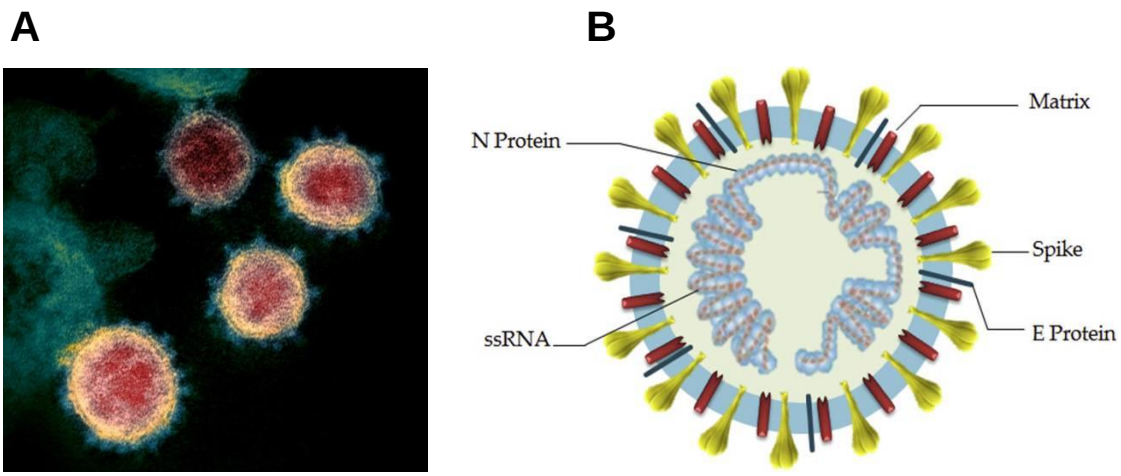

Figure 8. (A) SARS-CoV-2 real image made with a transmission electron microscope. From National Institute of Allergy and Infectious Diseases (NIAID). **(B)** SARS-CoV-2 structural proteins: S, E, N and M. Figure from[40].

2. Objectives

Given that:

- The number of infected and deceased caused by COVID-19 continue increasing worldwide.
- Currently there are only palliative treatments for which early detection is essential.
- More advanced, precise and faster techniques for SARS-CoV-2 detection are needed.

The **main objective** of this work is to optimize the development of sensitive and specific biosensor-based detection assays for specific SARS-COV-2 RNA sequences, in order to obtain a diagnostic tool for the rapid and precise virus detection and identification.

Secondary objectives include:

- Testing and selection of an efficient buffer for the hybridization of the target SARS-CoV-2 RNA sequences by using a SPR biosensor.
- Evaluation of different methodologies to biofunctionalize the chip.
- Analysis of several biofunctionalization strategies in terms of hybridization efficiency, stability and selectivity.

3. Materials and methods

3.1 Surface plasmon resonance biosensor

The SPR biosensor employed in this project was developed in our research group in 2004 and commercialized by Sensia S.L. Because the main interest of this project is the sensorgram plot obtained from the biosensor, the detailed description of the device will not be covered in this section. However, it could be found in Annex 2. Sensorgram reproduces the interaction events as an increase of the intensity of the reflected light (R %) versus time (s). Binding events lead to positive changes of the baseline while unbinding events lead to negative variations. Signals after the biological interaction are determined by measuring the difference of R (ΔR) between the baseline and the increase of the signal in the sensorgram. Data analysis was carried out using OriginPro 2018 software.

3.2 Chemical reagents and buffers composition

Solvents used for the cleaning of the sensor chips included acetone 99.5% and ethanol 99% (Panreac Applichem, Spain). Main salts and chemical reagents for the buffer preparation and biofunctionalization included sodium dodecyl sulfate (SDS),2-N-morpholino ethanesulfonic acid (MES), ethanolamine 1M pH 8.5, 50% FA solution in MilliQ water, cystamine dihydrochloryde, 3M Tetramethyl ammonium chloride (TMAC) buffer, 50mM Tris, 1mM EDTA and 0.1% SDS at pH 8.0 (Panreac Applichem, Spain). SAM formation required 16-mercaptohexadecanoic acid (MHDA) and 11-mercaptoundecanol (MUOH) (Panreac Applichem, Spain). Reagents for carboxylic group activation were 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) (Panreac Applichem, Spain). The amine-to-amine cross linker molecule was bis (sulfosuccinimidyl) suberate (BS3) (Thermofisher Scientific, Spain).

Several buffers and solvents were prepared either for biofunctionalization or target hybridization analysis: PBS 50 mM (50 mM Phosphate buffer, 0.75 mM NaCl –pH 7), buffer MES (0.1M MES + 0.5M NaCl pH 5.5-6), 20X SSC (0.3 M Na citrate, pH 7, 3 M

NaCl). For target hybridization analysis, 5 hybridization buffers with different compositions were employed (Table 1). Buffer solutions were prepared by using MilliQ water. All solid materials were autoclaved at 121ºC/20 min for plastic and 134ºC/10 min for glass.

Table 1. Hybridization buffers used for optimization of detection process.

Hybridization buffer
2x SSC + 0,5% SDS
2x SSC + 0,1% SDS
5x SSC + 20% FA
5x SSC + 3M TMAC
5x SSC

3.3 DNA sequences

For target detection, at the moment we are working with not virulent SARS-CoV-2 synthetic nucleic acids. Specifically, we are using DNA rather than RNA because of its higher stability. DNA sequences for probes and target design were provided by our partners from Aix-Marseille University (France) (Table 2) according to World Health Organization (WHO) recommendations for the design of the primers to detect RNA from SARS-CoV-2 with PCR. DNA probe sequences incorporate an SH- functional group at 5'-end to allow for direct coupling to the gold sensor surface, while they incorporate a NH2- when coupling to a previously generated functional monolayer.

Table 2. DNA probe sequences used for optimization of detection

Gene	DNA probe (5'->3')
E gene	SH/NH_2-CGA AGC GCA GTA AGG ATG GCT AGT GT
N gene	SH/NH_2-TGG CAA TGT TGT TCC TTG AGG AAG T
N1 gene	SH/NH_2-GGT CCA CCA AAC GTA ATG CGG GGT

3.4 Surface biofunctionalization

3.4.1 Chip cleaning

Gold chips were cleaned by consecutive sonication cycles (1 min) with solvents of decreasing polarity (acetone, ethanol and MilliQ water) previously heated up to their boiling point. After that, chips were dried under nitrogen flux and placed in an UV/O3 generator (BioForce Nanosciences, USA) for 20 min. Then, they were rinsed with ethanol and water, and dried under nitrogen flux.

3.4.2 Chemisorption: Thiol-DNA probes immobilization

Different methodologies of thiol (SH)-DNA probes immobilization were employed depending on the experiment. *Ex-situ* immobilization was performed outside the sensor apparatus by putting in contact the gold surface with 2µM of SH-DNA probes of E gene in PBS and incubated for 3h or overnight. Chips were rinsed with MilliQ water and dried under nitrogen stream. Afterwards, they were placed directly in the SPR biosensor. On the other hand, *in-situ* immobilization was carried out by placing the gold chip in the sensor apparatus and flowing 2uM of SH-DNA probes of E gene in PBS at a 7µL/min flow rate.

3.4.3 Covalent attachment: Amine-DNA probes immobilization

Two types of chemical matrix created on the surface of the chip were tested: one with carboxyl groups and another with amine groups. The formation of SAM of carboxylic-ended (Figure 7) was carried out by coating the sensor chip with 250µM of MHDA/MUOH (1:20) in absolute ethanol for 4h at room temperature. Once the SAM is formed, the chip was rinsed with ethanol and MilliQ water, dried under nitrogen stream and mounted on the sensor platform. MilliQ water was selected as running buffer for the immobilization procedure. The carboxylic groups activation was performed by flowing a 0.2M EDC /0.05M NHS solution in MES buffer 0.1M at a constant flow rate of 10µL/min. The immobilization of the probe was carried out at flow rate 7µL/min with 10µM of E gene NH2-probe solution in 1x PBS buffer. Finally, 1M ethanolamine solution (pH 8.5) was used to deactivate the unreacted carboxylic groups. For the formation of the SAM with amine-ended, 20mM cystamine dihydrochloryde in PBS was placed on the surface of the chip for 6 hours. The chip was then introduced into the sensor with a continuous MilliQ water flow. The BS3

crosslinker molecule in acetic acid 2mM was introduced at a flow rate of 10µL/min for 3 min. To immobilize the amino-DNA probe, 10µM of E gene probe-NH2 was introduced into the sensor and left overnight.

3.4.4 DNA hybridization

Target DNA hybridization was performed by injecting 300µL of DNA with hybridization buffer into the SPR biosensor at a 10 µL/min flow rate. Hybridization selectivity was always tested by using different target sequences that are not complementary to the probe after each experiment.

3.4.5 Regeneration of the chip surface

A 50% FA aqueous solution was injected at 13µL/min after each sample has been analyzed, in order to remove all the attached target DNA and allowing for the regeneration of the surface.

The workflow of the methodology used for optimization of detection were as it follows (Figure 9).

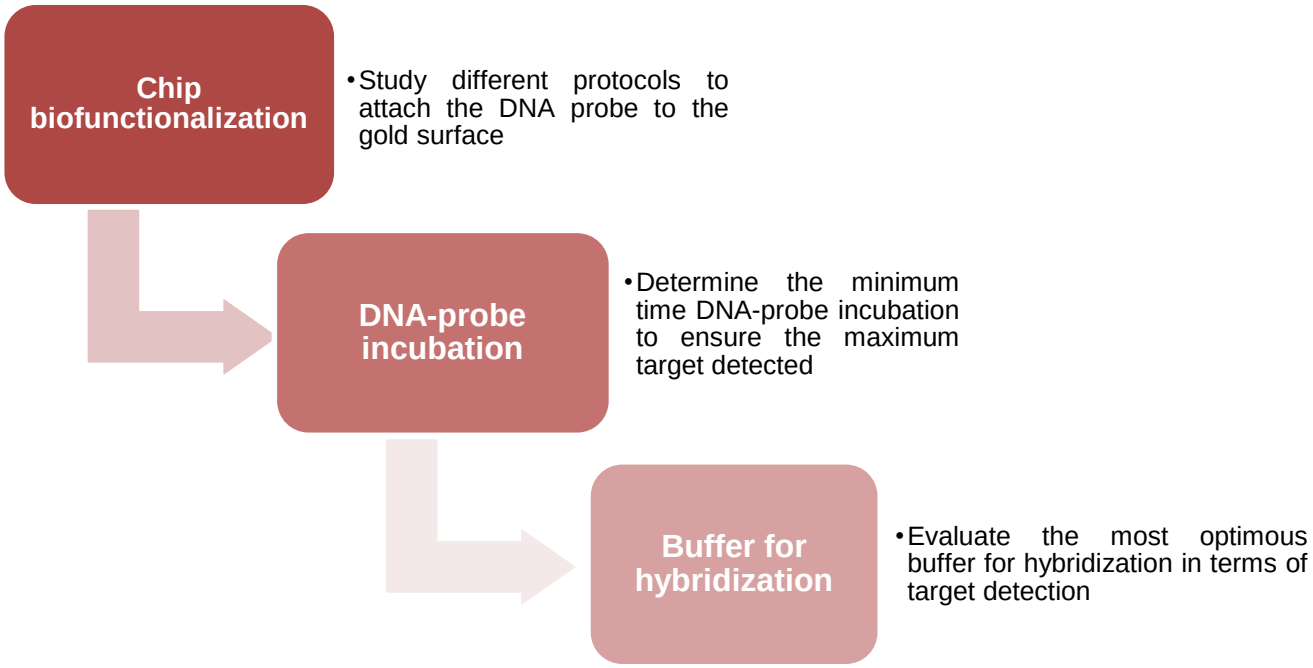

Figure 9. Workflow used for optimization of detection.

4. RESULTS

4.1 Chemisorption is a better biofunctionalization method for gold surfaces.

We tested different methods to biofunctionalize the surface of the chip: chemisorption and covalent attachment with cystamine or MHDA/MUOH. When injecting 100nM of E gene, a notorious improvement in the target detection was achieved when the chip was biofunctionalized by chemisorption in comparison with the covalent attachment (Figure 10). These results point that for chips with gold surfaces chemisorption is a more adequate method for biofunctionalization prior to target hybridization. Moreover, chemisorption process is faster and simpler than the covalent attachment.

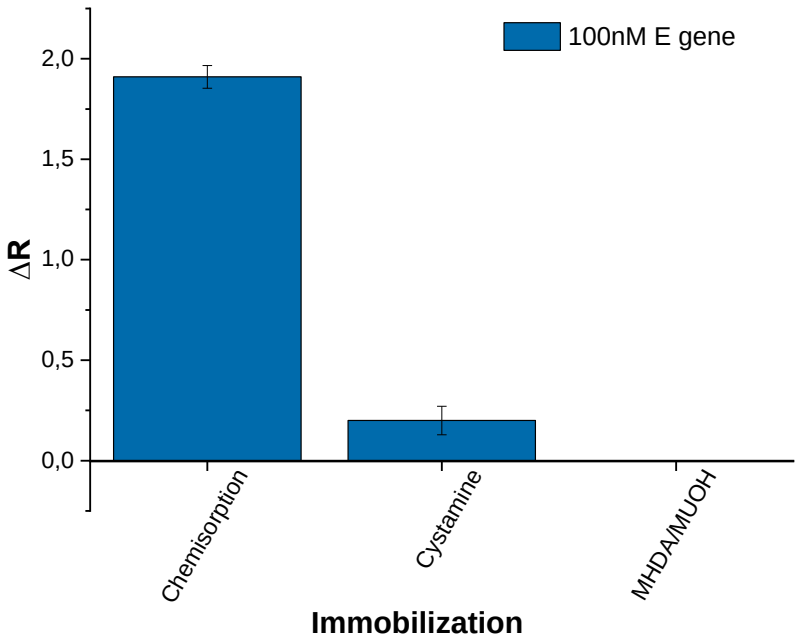

Figure 10. 100nM E gene target hybridization SPR signals for the different types of surface created on the chip.

4.2 *Ex-situ* immobilization overnight improves biosensor sensibility.

The way and the incubation time of the probe over the chip surface is directly related to the target detection signal. Next we tested three conditions in order to find the better sensibility for target detection: injecting the probe to the surface outside of the sensor (*ex-situ*) for 3 hours, ex-situ overnight (O/N) and injecting the probe to the surface while the chip is already put on the sensor (*in-situ*) O/N. We found that the incubation ex-situ O/N showed a higher △R in comparison with the other two conditions, indicating a better sensibility for target detection (Figure 11).

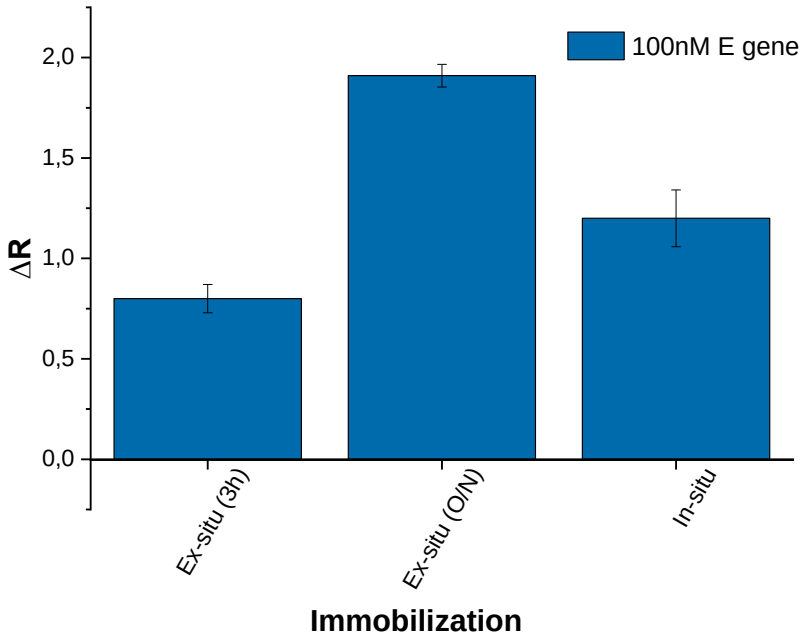

Figure 11. 100nM E gene target hybridization SPR signals for the different times and procedures of the

4.3 The 5x SSC buffer shows the highest hybridization efficiency.

We tried different running buffers in order to find the most efficient to hybridize the target DNA, which was 100nM of E gene DNA in all cases. The SPR biosensor allows the visualization of the target-probe hybridization through a sensorgram including the ΔR, which was directly proportional to the E gene detected in the injected sample. Among the five hybridization buffers tested, we obtained the highest ΔR value when using the 5x SSC buffer, indicating a remarkably better hybridization efficiency (Figure 12). By contrast, the addition of TMAC, FA or SDS to the hybridization buffer reduces the target detection. To note, it was not possible to detect any signal when using the buffer containing 0.5% of SDS because of the elevated background noise caused by bubbles into the biosensor tubes. The sensorgrams indicating the amount of target DNA bound to the probes when using the 5x SSC and the 5x SSC + 20%FA buffers are shown in Figure 13 as representative examples.

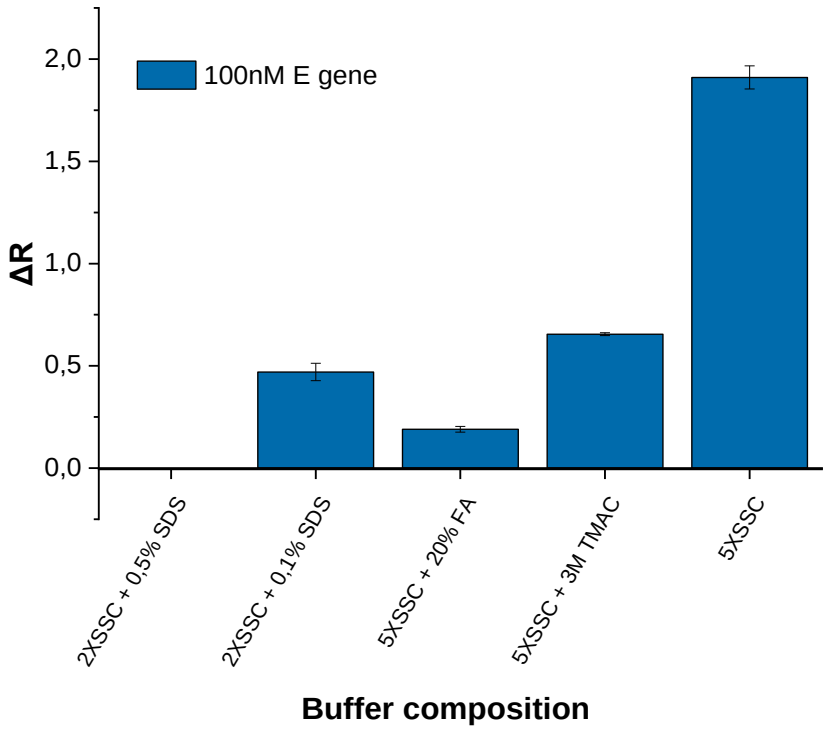

Figure 12. Sensor signal (ΔR) for the buffers 2x SSC + 0.5% SDS, 2x SSC + 0.1% SDS, 5x SSC + 20% FA, 5x SSC + 3M TMAC and 5x SSC when injecting a sample of 100nM of E gene.

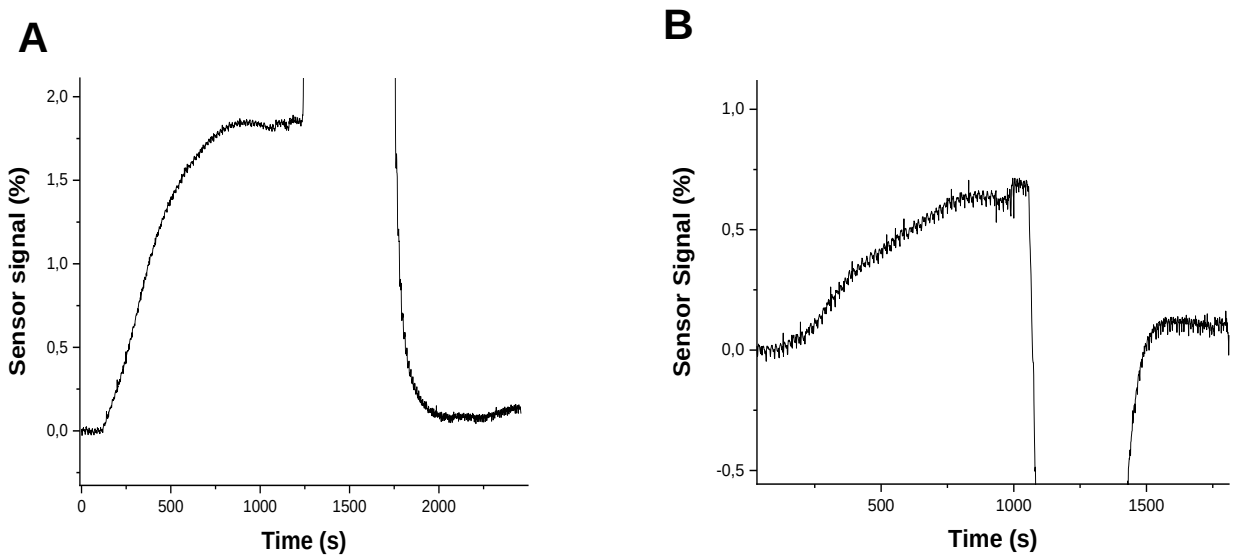

Figure 13. (A) Hybridization cycle when injecting a sample of 100nM of E in 5x SSC obtaining a ΔR of 1.85. followed by the regeneration cycle made with formamide 50% and finally, the return to the base line located at 0. **(B)** Hybridization cycle when injecting a sample of 100nM of E gene in 5x SSC + TMAC obtaining a ΔR of 0.65, followed by the regeneration cycle made with formamide 50% and finally, the return to the base line located at 0.

4.4 The use of lateral spacers allows for the use of lower target DNA concentrations.

The previous conditions led to an efficient immobilization of the probe on the gold surface of the SPR biosensor for the sequence of the E gene. In order to verify whether the methodology used was reproducible, we tested different probes (N1 gene and N gene) as well as different target concentrations (from 1µM to 100pM). N1 gene probe was tested without the lateral spacer C12, whereas N gene probe was tested with the lateral spacer C12. The limit of detection (LOD) was 4nM and 1nM, respectively. The E gene probe attachment was compared with and without lateral spacers. The sensor response is directly proportional to the target concentration in all cases (Figure 14). We observed that the use of lateral spacers showed better results in terms of target detection in the case of the N and E genes in comparison with N1 gene and E gene without lateral spacer. These data demonstrate that lower target concentration could be used for detection when lateral spacers were used in the biofunctionalization process.

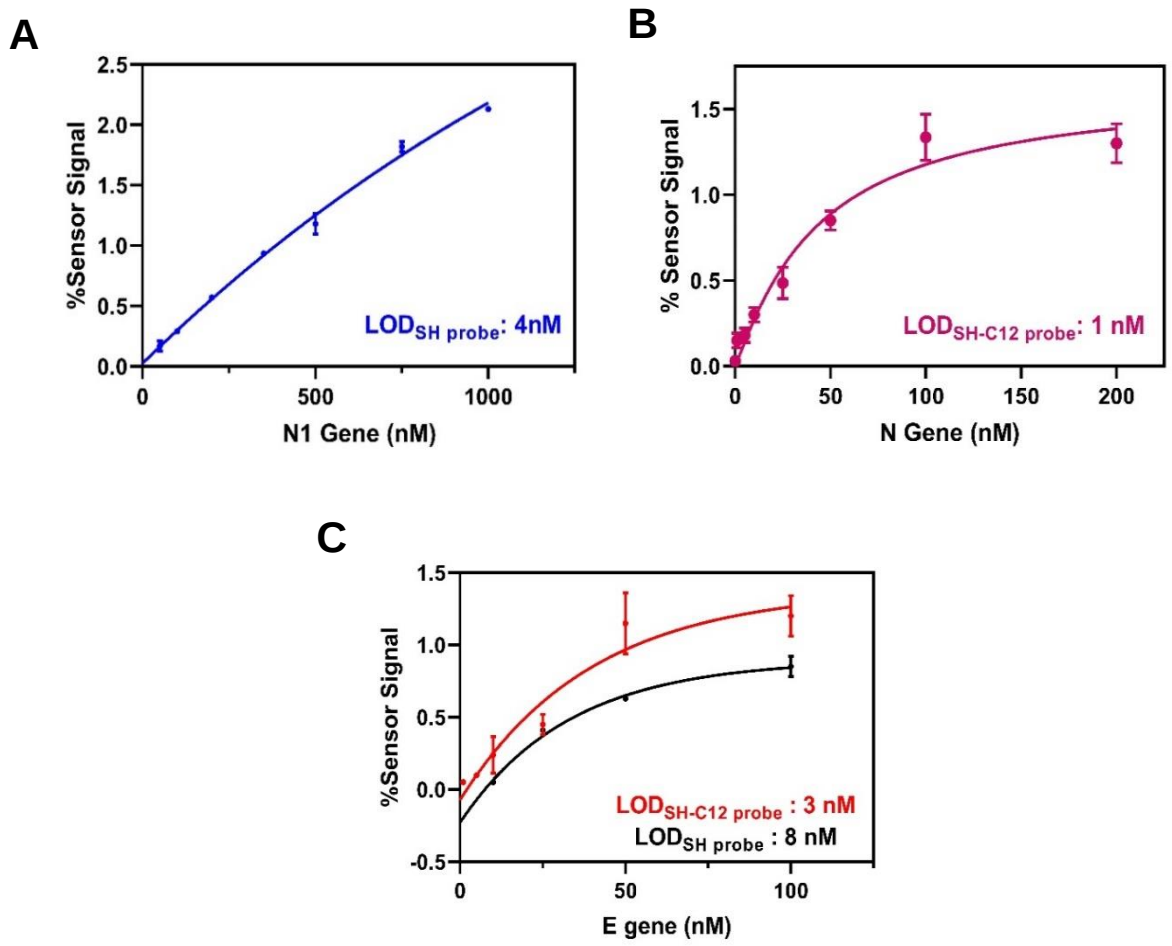

Figure 14. (A) Calibration curve for N1 gene with SH-DNA probe without C12. **(B)** Calibration curve for N gene with SH-DNA probe with C12. **(C)** Calibration curve for E gene with SH-DNA probe with C12 (red line) and without C12 (black line).

5. Discussion

Throughout this work we have studied different methods to optimize the detection of the genetic material of the SARS-CoV-2 virus. We demonstrated that the biofunctionalization of the chip by chemisorption exhibited better selectivity for the target binding in comparison to the covalent attachment. According with the findings of Yang et al., one possible explanation is that in the case of the covalent attachment method there is a lower accessibility of the probes and a slower diffusion of the target through the matrix[23]. Moreover, our results also highlight that the physicochemical structure of SPR chips affects the apparent binding behaviors of biomolecules, as it

was demonstrated by Yang et al., by testing different dimensional biofunctionalizations[23].

The *in-situ* immobilization allows the biofunctionalization of different probes for the simultaneous detection of independent target sequences, in contrast with *ex-situ* immobilization that only use one type of gene probe in each experiment. However, the level of target binding when *in-situ* immobilization is performed use to be scattered and not very reproducible[14]. We observed that *ex-situ* immobilization of the probes showed increased hybridization efficiency and more reproducible results than the *in-situ* counterpart, possibly due to the obtention of a better structured SAM.

For the hybridization buffer composition, we tested several additives that are usually used in PCR experiments to increase the efficiency and specificity of the reaction[41,42]. Following previous reports, FA was added to the hybridization buffer in order to avoid single base mismatches and improve the target detection[43]. Nonetheless, our results showed a decrease in target hybridization when adding FA 20%, which could be explained by the increased concentration of this compound which might affect the hybridization efficiency. This is not surpirising since it is the same reactive that was also used in the regeneration process of the biosensor, although at higher concentration, indicating that different %FA should be tested in the future for the optimization of the hybridization. On the other hand, TMAC was also tested in the hybridization buffer as it has been described to increase the PCR efficiency and specificity[41], although none study using it in SPR biosensors experiments was reported. We found that the addition of TMAC to the hybridization buffer allows for the target detection but at a lower rate than the other conditions tested. This could be in part due to the higher viscosity of this compound, which makes it difficult to pass through the small biosensor injection tubes. In this line, other studies demonstrated that buffers viscosity influence the DNA hybridization[44]. Although several studies showed an improvement in the DNA hybridization when it was used[42,45], the use of SDS was completely discarded in this study as it caused bubbles to form in the biosensor tubes, making it difficult to read any measurement.

Finally, we observed that the use of lateral spacers helped in reducing the amount of target DNA used in the analysis. Our hypothesis in that aspect is that a more spacious probe distribution on the surface when lateral spacers are incorporated make them more accessible for interaction, thus resulting in a more efficient recognition of the target. At the moment, we have just tested the use of lateral spacers in the E gene detection, but we are currently working in testing the N and N1 genes.

5.1 Strengths and limitations

SPR biosensors have demonstrated to be a very versatile tool given that they can detect a wide variety of different targets. The main strengths in the use of this devices are that the sample concentration needed is small, no sample modification is required (label-free) and the measurements can be followed in real-time. Moreover, although the biofunctionalization of the chip might be quite time consuming, once it is performed the analysis can be repeated for different samples with rapid results in approximately 20 minutes. On the other hand, one drawback is the cost of sensor chips which is difficult to assume in some cases. Additionally, although the use of lateral spacers in the biofunctionalization process has allowed for the use of lower DNA concentrations, the detection of very low concentrated target compounds at (pM or less) is quite difficult. In this aspect, the engineering crew of our research group is implementing a new Bimodal Waveguided Integrated (BiMW) biosensor with a lower detection limit, resulting in a more sensitive device. The use BiMW devices could also solve the issue of the high cost of the gold chips because they work better with silicon-based chips, which are cheaper.

5.2 Future perspectives

There is a considerable demand for improving health care in advanced societies. The field biosensors development is a growing area of research due to their potential to perform detection assays in a fast, sensitive and specific way. We demonstrated that use of SPR biosensors could be applied in the detection of the SARS-CoV-2 nucleic acids. Given that SPR biosensors can solve many of the limitations that are present when using traditional virus detection, the optimization of this technology opens the possibility for a faster, cheaper and more sensitive SARS-CoV-2 detection method for COVID-19 diagnostic.

Although the use of SPR biosensors in the forensic practice is still limited, recent literature supports their application in the analysis of forensic samples with better results than the traditional methods[5–7]. Along with our results, this data makes us to encourage the use of these devices in forensic sciences given the advantages that they have demonstrated. Moreover, SPR biosensors can be integrated into affordable, portable and easy-to-use point-of-care platforms, making them suitable to be used in a variety of fields such as clinical analysis, drug detection, environmental surveillance and food safety[46].

6. Conclusions

Chemisorption is the best method to biofunctionalize gold surface biosensor chips. Different conditions for the attachment of the probes on the chip surface lead to different target detection efficiency. In this aspect, *ex-situ* and overnight immobilization of the probes to the surface of the chip improves the biosensor sensibility. The use of 5x SSC hybridization buffer showed the highest hybridization efficiency. Finally, the use of lateral spacers during the biofunctionalization process allows the use of lower target DNA concentrations. Although more detailed studies are needed, these results suggest that the use of SPR biosensors for SARS-CoV-2 nucleic acids detection could be an efficient tool for COVID-19 diagnostics, and also highlight the great potential of these devices in different forensic analysis.

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Annex 1: SPR biosensor working principle

The biological interaction with the target is measured detecting variations in the optical properties of the light. The working principle of SPR biosensors is based on the generation of surface plasmon polaritons (SPP), which are collective oscillations of free electrons that occur at the interface between a dielectric and a thin film of a noble metal (usually 50 nm of gold). These oscillations generate an electromagnetic field propagating through the interface of both mediums. When illuminated with a laser of monochromatic light (λ = 670 nm) at a certain angle of incidence (θ), the SPP can be excited, absorbing part of the energy of the incident light (Figure 15.A) and consequently producing a decrease in the intensity of the collected light by the photodetector (Figure 15.B). The angle corresponding to the minimum reflected intensity is referred to as the resonance angle (SPR θ) and strongly depends on the refractive index (RI) at the interface between the metal and the dielectric. Therefore, any changes in the RI of the medium (i.e. biological interactions taking place on the metal surface) will produce a displacement of the resonance angle (Figure 15.C). Once the resonance angle has been found, it can be fixed in order to observe the subtlest changes in the reflected intensity over time at that exact position, allowing to monitor the binding events occurring on the sensor surface by a real-time sensorgram plot.

Sensorgram reproduces the interaction events as an increase of the normalized intensity of the reflected light (R %) versus time (s). R value is directly proportional to the target binding response. Signals are determined by measuring the absolute value of the baseline variation after the biological interaction as (△R). Data analysis was carried out using OriginPro 2018 software[47].

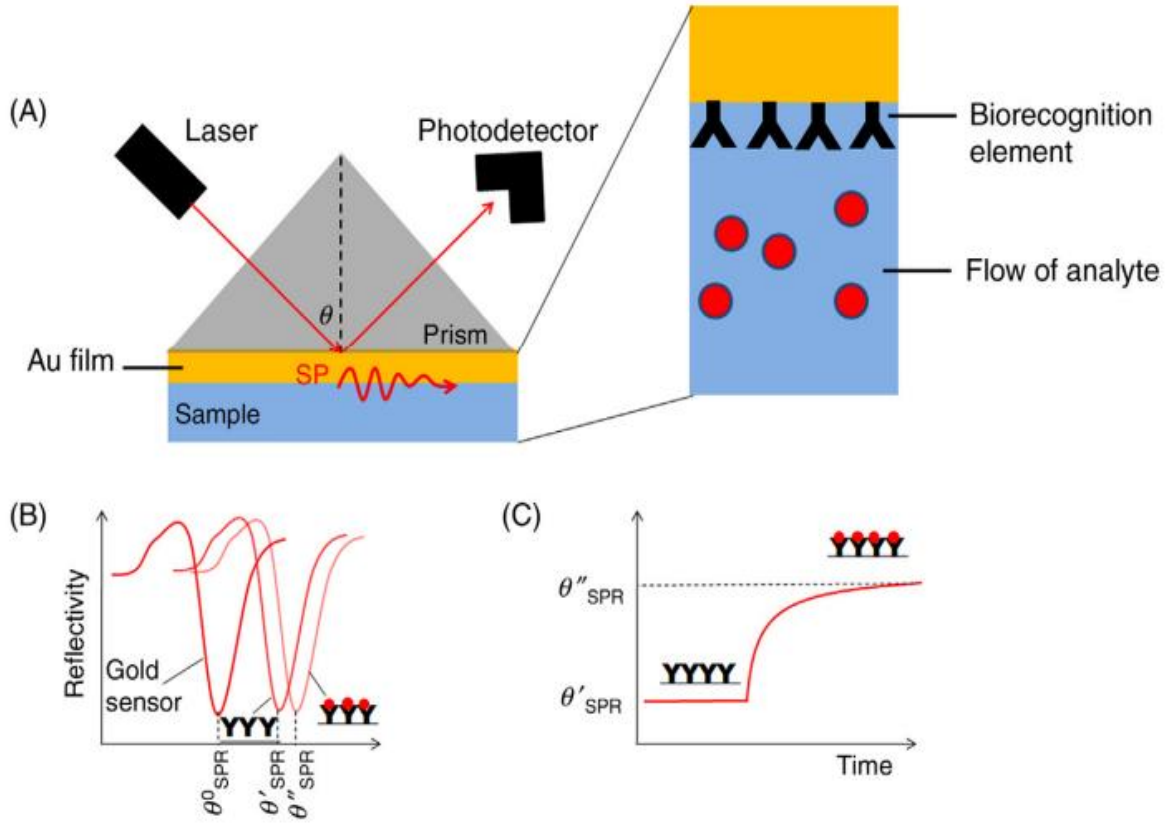

Figure 15. (A) Schematic representation of SPR setup. At a specific angle, part of the light energy is transferred to the surface causing a minimum intensity in the angular scanning, as can be seen in (B). **(B)** the angular scanning for three different situations (θ^0 SPR) pure gold sensor exhibiting; (θ' SPR) after the biological receptor immobilization on surface and (θ'' SPR) after detecting the target. **(C)** Sensorgram plot monitoring SPR angle variation as a function of time during the biomolecular interactions. Figure from[48]

Annex 2: SPR biosensor performance

The biosensor device is integrated in a platform and can be used as a portable device, also known as point-of-care (Figure 16.A). It allows the real-time monitoring of the intensity of the reflected light at a fixed angle of incidence. Light excitation is carried out with a diode laser (RS 194-032, Amidata, Spain), emitting at 670 nm. The laser beam is divided in two identical intensity beams using a light splitter (5 mm/side cube) to enable the simultaneous evaluation in two independent channels. The laser beams pass through a glass coupling prism with a refractive index (RI) of (n = 1.52), reaching the backside of the gold sensor chip via a RI matching oil (n ≈ 1.515) (Figure 16. B) The device also incorporates a fluidic system which consists on a peristaltic pump that

keeps a continuous flow through a set of tubes. A pair of valves linked to the biosensor tubes, allows the sample injection to the flow until arrive to the biofunctionalized chip[47].

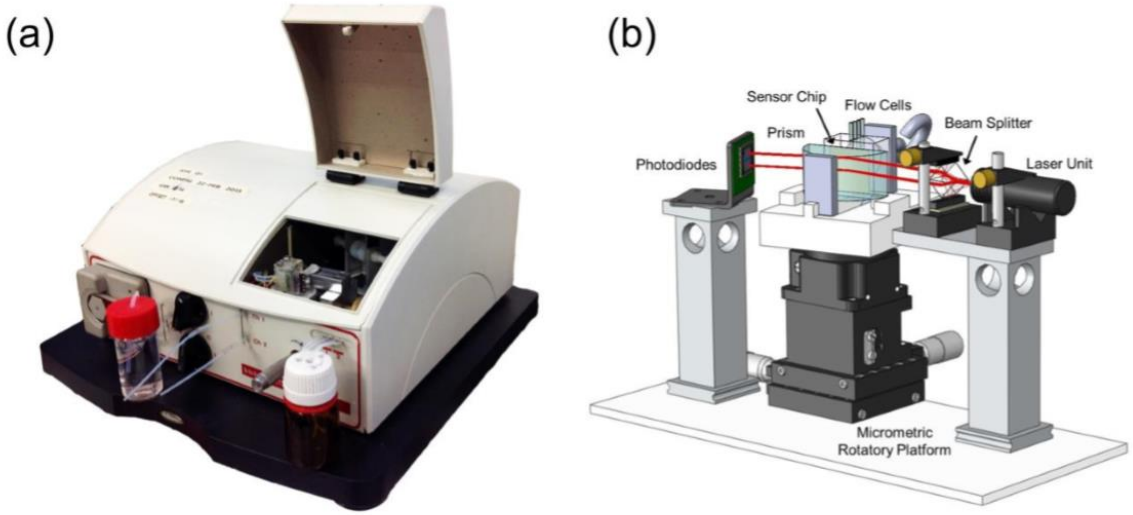

Figure 16. (A) SENSIA SPR Biosensor device. **(B)** Schematic representation of the sensor module. From NanoB2A group (ICN2).

The sensor chip consists of a glass surface (10 × 10 × 0.3 mm) coated with 2 nm of chromium and 45 nm of gold (Ssens, The Netherlands). The gold sensor surface contacts with two identical flow cells where the biomolecular interaction takes place and which are detected as changes in the reflected light intensity by a photodiode (S5870, Hamamatsu, Japan). This part of the setup is mounted on a rotary platform in order to select the optimum incidence angle that maximizes the changes of reflected light intensity. To monitor the biological interactions when the analyte is introduced, there is a software that produces the sensorgram[47].