QUANTIFICATION OF SECRETED CYTOKINES FROM AN INTESTINAL BOWEL DISEASE IN VITRO MODEL.

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BIOTECHNOLOGY DEGREE FINAL PROJECT

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In cooperation with: Institute for Bioengineering of Catalonia (IBEC). Biosensors for bioengineering.

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1. Centre information

The Institute for Bioengineering of Catalonia (IBEC) was created by the Government of Catalonia, the University of Barcelona (UB), and the Technical University of Catalonia (UPC). It received a Severo Ochoa Excellence Award from Spain's Ministry for Economy and Competitiveness in 2015. The main goal of this bioengineering research center is to confront, understand, and solve complex problems in biomedicine by intersecting life science and engineering. It currently has 22 research groups and this degree final project was developed working as part of the Biosensors for bioengineering group headed by Dr. Javier Ramón Azcón.(*Institute for Bioengineering of Catalonia (IBEC) – "Bioengineering Solutions for Health,"* n.d.).

2. Summary

Microengineered tissues represent a new paradigm in the field of cell-based in vitro assays providing systems with tissue-like characteristics and in vivo functionality. These new systems find applications in acquiring physiologically-resembling predictive data of cell behavior in microenvironments mimicking in vivo conditions. Thus, microengineered tissues represent a huge improvement of current reductionist cell culture monolayers, and open a new avenue of research providing more reliable tools for disease modelling and drug testing. With this in mind collaborations between two groups at IBEC have been developed a new microengineered device that mimics both the intestine structure and function to study epithelial restoration in the course of inflammatory bowel disease (IBD) combining recent developments in microfabrication, microfluidics, tissue-engineering and the unique self-organizing properties of stem cells. The present thesis has two main objectives, i) The firstly identification of biomarkers associated to this epithelial restoration processes by ELISA technique and ii) the in situ monitoring of those biomarkers by integrating in vitro IBD model to a SPR biosensing module with on-line monitoring capabilities to detect those biomarkers involved in this epithelial restoration process. With these results it is expected that this work could provide a unvaluable information about new potential therapeutic strategies for intestinal diseases.

Cytokines, SPR, sandwich ELISA, IBD, biosensors

3. Introduction

The following section moves on to present and describe the main topics this project covers including immune system, antibody structure and definition, a brief introduction of immunoassays, an explanation of enzyme-linked immunosorbent assay. Later a

presentation of the concept “biosensor”, focussing on optical biosensors and SPR. Concluding with the project “?” and a small context of the proteins to analyte.

3.1 Immune system. Antibodies

The immune system function is to protect against microorganisms or pathogens. This could be done by an adaptive immune response, that can confer protection against reinfection with the production of antibodies, or by innate immune response or cellular response, where phagocytic cells (macrophages) eliminate the pathogen without requiring a past infection. This last response is the first barrier against pathogens (Janeway, Charles; Travers, Paul; Walport, Mark; Shlomchik, 2007).

3.1.1 Antibodies

In 1890, Behring and Kitasato identified a substance in the blood that neutralizes specifically diphtheria toxin.(von Behring & Kitasato, 1890) This substance would be later called ‘antibody’, while the one that can induce the formation of the antibody is known as ‘antigen’.

Antibodies (Ab) or immunoglobulins are secreted by B cells and constructed by a paired heavy and light polypeptide chains. There are 5 different isoforms IgM, IgD, IgG, IgA and IgE. All of them have variable regions (which contain the antigen-binding site) and a constant region but have a different structure and function. To give a brief idea of the general structure of Ab, I will explain IgG (the most common isoform) as an example.

There are Y formed large molecules (150kDa), each one is composed of two heavy chains (50kDa) and two light chains (25kDa) that are identical, forming two identical antigen-biding sites and allowing the interaction of two antigens simultaneously. (Gao et al., 2018).

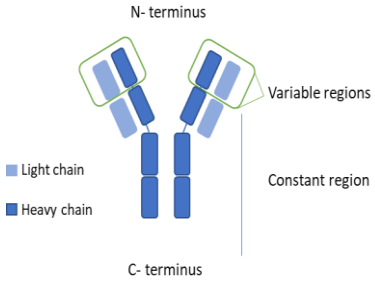


Figure 1. IgG structure representation. In light blue the identical light chain and in darker blue the identical heavy chain pair. The section circled in green corresponds to the variable regions or antigen-binding regions.

The variable regions of each Ab bind to a zone of the antigen, this zone is called epitope. Each B-cell produces Abs with an affinity for one specific epitope of the antigen. We can distinguish between monoclonal Ab (mAb) and polyclonal Ab (pAb). The former, mAb, are equal and recognize the same epitope, so they must be produced by identical B-cells. The latter, pAb, are a mix of different Ab with an affinity for different epitopes and are secreted by different B-cells. (Lipman et al., 2005).

This interaction antibody-antigen is not only important in the adaptative response, it is also the basis of most of the immunological techniques.

Immunoassays are based on the specificity and selectivity reaction of the antibody with the analyte. Are used to identify the presence or quantify the concentration of an analyte in a sample. There are different methods and the detection of the antibody can be measured in different ways, absorbance or fluorescence among others. (Van Emon, 1989). One of the most used immunoassays is the enzyme-linked immunosorbent assay.

3.2 Enzyme Linked Immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is an analytical biochemical technique used to detect the presence of antigen or antibody in a sample and quantify it. This technique was firstly described by Engvall and Perlmam (Engvall & Perlmann, 1971) and currently ELISA is a widely used diagnostic tool.

The liquid sample is placed on a solid surface where the antigen is immobilized, then the detection antibody is added and the immunocomplex (antigen-antibody) is formed. This antibody could be bioconjugated with an enzyme or could be detected by a bioconjugated secondary antibody. The enzyme will interact with an enzymatic substrate and produce a visible signal. Because this signal is directly proportional to the amount of antigen, it is also used as a quantitative technique to measure the concentration of antigen and antibody concentration.

To perform an ELISA assay is necessary to have a solid surface where the antigen is immobilized directly or indirectly, usually, this surface is presented in pre-treated ELISA plates. In this surface the antigen or antibody will passively be attached, this process is called adsorption or immobilization. After this step, a wash is needed to eliminate unbounded reagents and avoid non-specific reactions. To obtain a signal, an enzyme or enzyme conjugate (an enzyme attached to a protein, usually an Ab) will be added, this molecule will react to a low concentration of a substrate catalysing it, to promote a specific reaction. The resulting product of the enzymatic reaction turns from transparent

to a colour solution indicating the successful interaction between the antibodies and the antigen.

There are three different methods of ELISAs, all can be used to performed competition or inhibition ELISAs: direct, indirect and sandwich.

3.2.1 Direct ELISA

The antigen or sample is immobilized, and a conjugated detection antibody binds to the protein. It is less specific because only one Ab is used.

It is mainly used in competition and inhibition assays and when working with highly defined antigens.

3.2.2 Indirect ELISA

A first primary Ab binds to the antigen and a second conjugated Ab recognise the first one. The signal is amplificated compared to the direct ELISA.

3.2.3 Sandwich ELISA

Is based on the interaction of two Abs that recognize the same protein (antigen). In the solid surface Abs are attached (capture antibody). This Abs will bind specifically to the protein, immobilizing it in the well in a more specific way than with direct or indirect ELISA. Later a labelled Ab (direct ELISA) or a non-labelled Ab (indirect ELISA) will bind to the antigen. In the second one a secondary conjugated Ab must be added later.

The sandwich ELISA offers high specificity and sensitivity because of the use of two different Ab directed to the antigen and the amplification of the signal provided by the second antibody. Therefore, the ELISA assays performed in this project were direct sandwich ELISA.(Crowther, 2000).

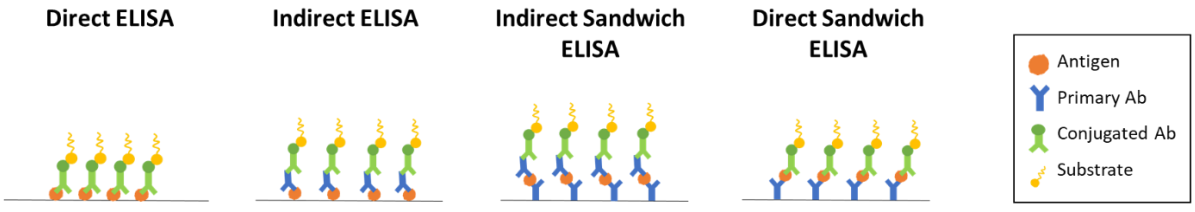


Figure 2. Direct and indirect ELISA and direct and indirect sandwich ELISA representation.

3.3 Biosensors. Optical biosensors. SPR

Biosensors allow the measurement of the analyte concentration by obtaining a proportionate signal. Their specificity and sensitivity make them a really useful tool in food industry (to detect toxic compounds, pesticides or identify an specific gene or molecule), medicine (to diagnose an illness in an early state, like a viral infection or measure glucose levels in blood), in fermentation processes (to monitor the reaction), plant biology (control de quantity of a molecule or monitor gene expression) and in vivo monitor of cellular metabolism. (Mehrotra, 2016)

The analyte (molecule of interest) is specifically recognized by a bioreceptor, this interaction generates a variation of one or more physical-chemical properties that must be converted into a measurable signal. This process is called signalization and is performed by a transducer that usually obtains optical or electrical signals*. These signals are processed by a complex electronic circuity and later quantify by the display. The display combines hardware and software obtaining the final desirable output, for instance, a graphic. (Metkar & Girigoswami, 2019).

*This is not applicable to paper biosensors

3.3.1 Classification by type of transducer:

-Mechanical biosensors. Measure masses, forces and motion variations derivate from the interaction of the bioreceptor with the analyte.

In static-mode surface stress sensing the binding of the antigen to the surface generates a surface stress that leads to a deflection of the cantilever (solid beam that can oscillate at one end). In dynamic mode devices, molecules are detected by their influence on resonance frequency of the cantilever. When molecules land on the cantilever they increase the mass of the cantilever and reduce de resonance frequency.

In quartz resonators, the resonance frequency is measured and related to the mass change induced by the biorecognition and immobilization of the analyte in the crystal surface. These biosensors are piezoelectric devices can directly measure their deformation and transform this pressure, acceleration, tension or strength into electrical signals. (Arlett et al., 2011)

-Electrochemical biosensors. Ab-antigen binding must be converted into a measurable signal the transducer is an electrode.(Maduraiveeran et al., 2018) Are powerful tools and highly used due to its sensitivity and selective rapid response, have been used to diagnose autoimmune diseases. (Florea et al., 2019)

There are different types of electrochemical biosensors. Amperometric biosensors measure the current generated from the electrochemical redox reaction of the bioreceptor with the analyte. And potentiometric biosensors measure the potential variation between working and reference electrode. (Gattani et al., 2019)

-Thermal biosensors. The total heat variation is measured by a temperature transducer that are highly sensitive, able to detect differentia of less than 10^{-5} degrees in some cases. This variation is proportional to the molar enthalpy change and the total number of moles of product molecules. This biosensor is not affected by the turbidity of the sample and do not have to be in direct contact with the fluid stream.(Lammers & Scheper, 1999)

-Magnetic biosensors. A magnetically labelled biomolecule interacting with a bioreceptor bound to a magnetic field receptor is expose to a magnetic field. Magnetic assays are reported to have a high sensitivity, stability on time in culture and do not have background noise. (Nabaei et al., 2018) In the recent years, micromagnetic tools to simulate and model have been developed. From now on the development of these biosensors can be related with the translation of magnetic biosensor into lab-on-chip portable devices.(Wu et al., 2019).

Thermal and magnetic biosensors are not as widely used as mechanical, electrochemical and optical biosensors.

-Optical biosensors can be divided into label-free, the signal is obtained directly to the interaction with the analyte, and label-based, detected by colorimetric, fluorescent or luminescent signal. I will focus on label-free biosensors, more specifically in surface plasmon resonance biosensors.

Surface plasmon resonance biosensors (SPR) is currently the predominant optical transducer. This is due to the capacity of this technique to measure binding, kinetics,

affinity, specificity and concentration, without the need of labels and allow to perform this in real time.

This phenomenon occurs when a photon of incident light (at a determinate angle or wavelength) hits a metallic surface, a portion of the light energy incidences through the metal coating and excites the electrons in the metal surface layer. The movements of the electrons are called plasmons and propagate through the metal surface. And the oscillation of the plasmons generates an electromagnetic field whose maxima are in the metal / dielectric interface and exhibits evanescent fields that decay exponentially in both media (a range in order of nm from the metal surface and the solution)

There are different coupling methods. Prism coupling, waveguide coupling and grating coupling. Some studies suggest that prism coupled plasmonic sensors have a less limited performance than grating coupled ones. But direct coupling, does not required bulky prism and gratings can be fabricated into plastic substrates and for that reason it can offer the possibility to obtain low-cost plasmonic sensors. If we coupled this fact to the small number of reports of biosensing based on plasmonic grating couples compared to prism couplers makes plasmonic sensors based on grating coupled a perfect candidate for performing low cost SPR biosensors.

A plasmonic biosensor is based on the prior immobilization of specific receptor molecules on the metallic thin film surface and the detection of the local refractive index changes that take place near this surface. When a biomolecular reaction occurs, it induces a local refractive index change that alters the plasmonic wavevector. This provides a quantitative methodology for label-free detection of biomolecular interactions.(Arturo López Muñoz Author Laura Lechuga Gómez Mª Carmen Estévez Alberola Director Co-Director DrEva Maria Pellicer Vilà Tutor, n.d.)(Piliarik et al., 2009).

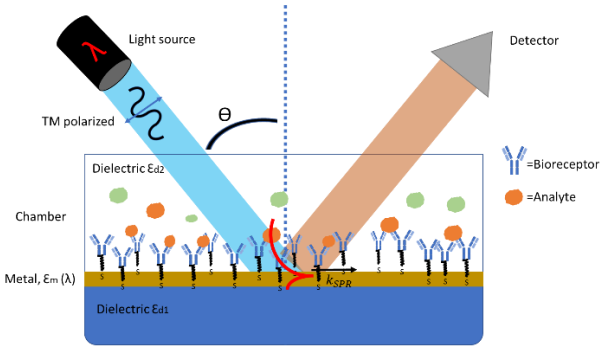


Figure 3. Grating coupled Surface Plasmon Resonance biosensing configuration

Summarizing, Surface Plasmon Resonance occurs on the surface of a conducting material (most frequently gold or silver because the physical-optical properties they offer) when it is illuminated by polarized light. A sensogram can obtain the shift of the angle or wavelengths against time. This method enables to obtain a signal proportional to the concentration of analyte on real time and label free. To do it a functional layer is needed, this layer allows the immobilization of the analyte on the sensor surface. This functional layer can be based on a self-assembled monolayer (SAM).

3.3.2 Gold functionalization strategies applied in optical biosensors

Performing the immobilization of the Ab in gold surface can easily lead to the formation of multilayers, this reduces the sensitivity and reproducibility of the sensing platform. As mentioned before a functional layer as SAM is needed. Self-assembled of alkyl molecules with a thiol group (-SH) at the end. The spontaneous formation of monolayers of thiolate molecules is directed mainly by the coordination between thiols or disulphides and gold, and Van der Waals interactions between alkyl chains. SAMs are easy to prepare, have very defined arrangement and relative inactivity.

Thiols are frequently used for the formation of SAM on top of gold and other metal surfaces. Some of the most commonly molecules used are 1-dodecanethiol (DDT), decanodithiol (DT) and 11-mercaptoundecanoic acid (MUA). The most popular systems are alkanethiols whose structure can be divided into a head group, the part of the molecule that will be attached to the substrate (thiol), it determines in which substrate it can be added, the chain, is formed by an alkane $(CH_2)_n$, and a terminal group, the superficial terminal group, it defines the interaction with the media and the receptor molecule.

However, the terminal carboxyl groups of the SAMs do not have a high affinity for the primary amino groups of the proteins. An intermediate reaction is needed to incorporate a better leaving group catalysing the reaction. The compound 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) forms a instable intermediate that activates the carboxyl groups, but the addition of N-Hydroxysuccinimide (NHS) is needed to transform this unstable compound to a semi-stable ester highly reactive to amines (Abs). This reaction is normally carried out in a single step where both compounds are added.

3.3 Project information. Bowel disease

The Biomimetic systems for cell engineering group Lead by Prof. Elena Martinez, in a close collaboration with Biosensors for bioengineering group have develop and validate a bioengineered model that mimics the intestine structure and function. The aim of the collaboration is to integrate this in vitro model with a multiplexed SPR sensing platform already developed by Prof. Javier Ramon's group, with the aim of study the epithelial restoration in inflammatory bowel disease (IBD). (This idea is already support by MINECO into the frame of the project INDUCT)

The group 'Biomimetic systems for cell engineering' has developed a cellular model composed of two mouse cellular lines: myofibroblasts and intestinal epithelium. The model structure is represented in figure 4. The epithelial layer is interrupted by a PDMS stencil that will be later extracted to study epithelial migration and reconstitution.

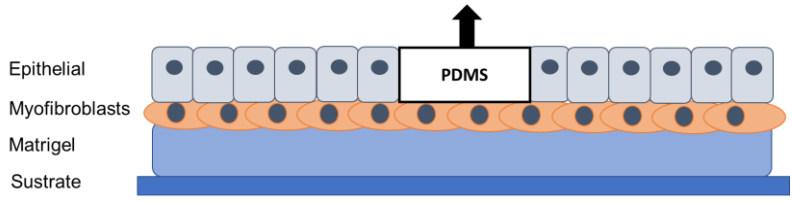


Figure 4. Cellular model of inflammatory bowel disease (IBD) performed by the group 'Biomimetic systems for cell engineering'. A first cellular layer of myofibroblasts seeded on Matrigel. Over this a layer of epithelial cells interrupted by a PDMS stencil.

IBD includes Crohn's disease (CD) and Ulcerative colitis (UC) chronic inflammatory conditions that appear in genetically susceptible individuals. In IBD the adaptative response of the immune system against the microbiota reducing the mucosa and increasing the epithelial permeability and integrity. (Kaser et al., 2010; Michielan & D'Incà, 2015)

The Biosensors for bioengineering is in charge of the monitoring of secreted cytokines from this model, developing the design, optimization, and manufacture of the multiplexed biosensing platform. The present work is focus mainly in those aspects of the project; firstly a quantitative screening of the cytokine profile of the in vitro model samples by ELISA technique and the later development of the SPR sensing platform to integrate it with the intestinal model and perform a continual monitoring of the most secreted cytokines.

3.4 Cytokines, chemokines and growth factors (TGF-β, IL-6, MCP-1, KC)

In the innate response, the activated macrophages will secrete cytokines and chemokines, initiating the inflammation(Janeway, Charles; Travers, Paul; Walport, Mark; Shlomchik, 2007). Cytokines are soluble proteins with an important role in intercellular communication. Are divided into classes: interferons, interleukins, tumor necrosis factors, and transforming growth factors. (Riera Romo et al., n.d.)

Interleukins (IL-6) stimulates the production of fibronectin in hepatocytes and proliferation of B lymphocytes. and Transforming growth factors TGF-β.

KC (CXCL1) and MCP-1 (CCL2) are chemokines, proteins whose concentration controls the migration and position of immune system cells. Chemokines have an important role in the innate response of the immune system and the interaction between innate and adaptative response. KC (GRO or MGSA or CXCL1) chemoattract and activate neutrophils and basophils. (Sokol & Luster, n.d.).

3.4.1 TGF-β

Transforming grow factor beta (TGF-β) has been reported to have a relation with intestinal homeostasis and that IBD patients showed higher levels of TGF-β. .(Ihara et al., 2017) One of the dysregulated mechanism in inflammatory Bowel Disease is the activation of proinflammatory mediators as macrophages, this can be induced by TGF-β.(Katsanos & Papadakis, 2017) Moreover, TGF-β signalling is important for Treg differentiation.(Ihara et al., 2017).

For measuring TGF-β is important to take in consideration that it is usually secreted in a latent form and is necessary to treat the samples with acidification before performing the measurement. (Areström et al., 2012).

3.4.2. IL-6

Interleukin 6 (IL-6) is a pleiotropic molecule, which means it has inflammatory and anti-inflammatory functions. IL-6 has been reported to contribute to intestinal damage in both Crohn's disease and ulcerative Colitis.(Sanchez-Muñoz et al., 2008). Dysregulation of IL-6 synthesis plays a pathological effect on inflammation and autoimmunity, but IL-6 signalling is essential for the maintenance of mucosal integrity and epithelial regeneration.(Hunter & Jones, 2015; Tanaka et al., 2014)

3.4.3. KC

The chemokine CXCL1 or Kreatinocyte chemoattractant (KC) is also called growth-regulated oncogene (GRO) is a pro-inflammatory chemokine, and it mediates the neutrophil trafficking . (Sawant et al., 2016)

3.4.4. MCP-1

Monocyte Chemoattractant Protein-1 (MCP-1) or CCL2 is one of the most studied chemokines and is produced by a high variety of cells The main function of this chemokine is regulate the migration of monocytes or macrophages.(Deshmane et al., 2009). MCP-1 participates in adaptative immunity controlling the differenciation of T helper (Th) into Th1 or Th2. It has been described an increase of expression of MCP-1 in IBD intestinal mucosa.(Bianconi et al., 2018).

4 Hypothesis and objectives

4.1 Hypothesis

The levels of IL-6, MCP-1 , KC and TGF-β in the supernatant of the in-vitro model of inflammatory bowel disease of mouse intestine will be quantified firstly by ELISA. It's expected that levels of cytokines involved in inflammatory process such as IL6 and also levels of TFG-β to be high. Both systems will be optimized into the SPR sensing platform. Finally, integration of the bio-engineered in vitro model with the SPR sensing platform will allow an in-situ monitoring of the secreted cytokines levels.

4.2 Objectives:

- Optimization and protocol development of a sandwich ELISA for TGF-β, KC, MCP-1, and IL-6 cytokines.

- Quantification by ELISA technique of cytokines level in samples from intestinal in vitro model for inflammatory bowel disease

- Optimization of SPR biosensor platform for the detection of the two highest secreted cytokines obtained in the previous objective.

- On-line multiplexed monitoring of samples by optimized SPR sensing platform.

5 Methodology

Two main protocols were followed for the experimental part, the sandwich ELISA protocol and the Biosensor operation protocol. For the biosensor operation, no biotinylated Ab and enzymatic treatment were needed.

5.1 Buffer preparation

5.1.1. Sandwich ELISA:

-Coating buffer is 0.05 M Na_2CO_3/$NaHCO_3$ at pH 9.6.

-PBS-T is 10mM of phosphate saline buffer at pH 7.5 and 0.05 % Tween 20.

-PBS 1%BSA is 10mM of phosphate saline buffer and 1% (w/v) bovine Serum Albumin.

-Citrate buffer is 0.04 M of sodium citrate at pH 5.4.

-Substrate solution is 0.004% (v/v) H_2O_2 and 0.96mg mL^{-1} of tetramethylbenzidine (TMB).

-Stop solution is 4N sulphuric acid.

5.1.2. Biosensor operation:

-MUA solution is 2.5mM 11-Mercaptoundecanoic acid and 2.6% (v/v) HCl diluted in ethanol 99%.

-EDC/sNHS solution is 11mM EDC (previously diluted in ethanol), 5.5mM s-NHS (previously diluted in 0.1M 2-(N-morpholino) ethanesulfonic acid, MES) and 25mM MES pH=6.1.

-25 mM MES solution.

-10mM PB solution is 6.2mM Sodium phosphate dibasic and 3.8mM sodium phosphate monobasic monohydrate.

-Blocking solution is ethanolamine at 1mg mL^{-1}.

All buffers were prepared with deionised water.

5.2 Chemical and immunochemical

Recombinant mouse IL-6 (CliniScience S.L, 200-02), Purified rat anti-mouse IL-6 clone MP5-20F3 (BD Pharmingen, 554400), Biotin rat anti-mouse IL-6 clone MP5-32C11 (BD Pharmingen, 554402), Recombinant murine JE/MCP-1 (CCL2) (PreproTech House, 250-10), Polyclonal rabbit anti-murine JE/MCP-1 (CCL2) (PreproTech House, 500-P113), Polyclonal rabbit biotinylated anti-murine JE/MCP-1 (CCL2) (PreproTech House, 500-P113BT), Recombinant Murin KC (CXCL1) (PreproTech House, 250-11), Polyclonal rabbit anti-murine KC (CXCL1) (PreproTech House, 500-P115), Polyclonal rabbit biotinylated anti-murine KC (CXCL1) (PreproTech House, 500-P115BT), Mouse TGF-beta 1 DuoSet ELISA (R&D Systems, DY1679-05), DuoSet ELISA Ancillary Reagent Kit 1 (R&D Systems, DY007), Sample Activation Kit 1 (R&D Systems, DY010).

All salts for buffer preparation were purchased in Sigma-Aldrich.

5.3 Sandwich ELISA

5.3.1 Sandwich ELISA method

In the assays performed the blocking protein was bovine serum albumin (BSA) a 66.5kDa widely used protein used to increase the sensitivity and decrease the background noise by reducing the possibility of a nonspecific binding to occur. Blocking buffers compete with nonspecific factors in the assay for available plastic sites. Other molecules that could act as part of a blocking solutions are other serum proteins and detergent as Tween 20. To choose blocking reagents the hole system must be taken in consideration and an adaptation may be needed. For example, BSA is an expensive highly used blocking reagent, but when the system has anti-BSA Ab it can't be used or a high background noise will be obtained (Crowther, 2000).

The conjugated Abs used were biotinylated Abs, Abs conjugated to biotin, a water-soluble vitamin. This biotin will be bind to streptavidin Poly- horseradish peroxidase (HRP) conjugate, a protein with polymers of HRP that will amplify the signal. HRP is an enzyme that catalyses the oxidation of various organic substrates and has two domains sandwich the hemin group. The reaction mechanisms and spectral changes catalysis are complex and HRP is widely used with hydrogen peroxide. The enzyme will reduce hydrogen peroxide by hydrogen donors that can be measured after oxidation as a color change. Tetramethyl benzidine (TMB) is added with hydrogen peroxide and will be oxidated when HRP is reduced. This oxidation will confer TMB a blue color and after the

stop solution is added, the acidification confers TMB a yellow color, therefore it will be measured at 450nm in the spectrophotometer.

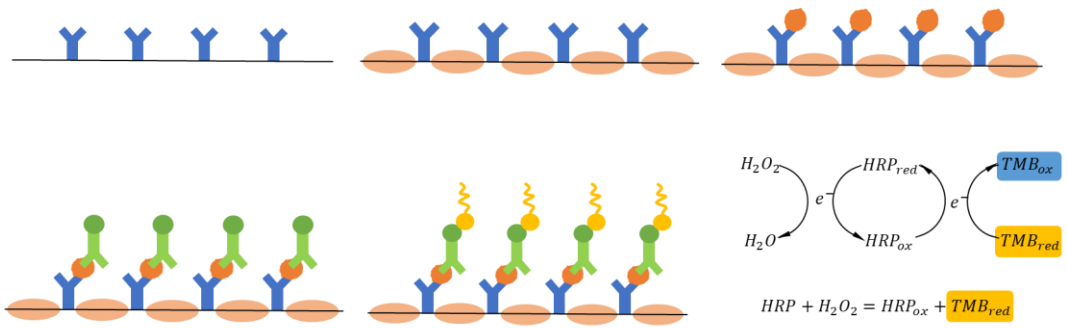


Figure 5. Sandwich ELISA step by step protocol. The capture Ab (blue) is attached to the surface. blocking solution was added (oval). Later, sample is added and the antigen (orange) binds to the capture Ab. Then a biotinilated Ab binds to the antigen and streptavidin-polyHRP (yellow) binds to the biotinilated Ab. Finally, sustrate reaction occures obtaining a color measurable signal

5.3.2 Sandwich ELISA protocol

Polystyrene microtiter plates (Nunc, Maxisorp) were coated with 100µL per well of the capture Ab solution was prepared in coating buffer and storage overnight at 4ºC. Then, microplates were washed with a 405 TS Washer microplate washer (Biotek Instruments Inc.) programmed to perform four washing cycles of 300µL per well of PBS-T. And 200µL per well of blocking solution was added at room temperature for 1h in agitation at 450rpm. After this step, microplates were washed, and protein or sample is added 100µL per well incubated for 1 hour at room temperature and 450rpm. Then, the microplates were washed, and the detection Ab is diluted in PBS-T buffer and 100µL per well were added and incubated for 1 hour at room temperature and 450rpm. Later, microplates were washed and Streptavidin-polyHRP (Pierce ThermoFisher S5512) solution at 0.1µg mL^{-1} in PBS-T was added (100µL per well) and incubated at room temperature,450rpm and avoiding light for 30 minutes. Finally, microplates were washed again, and 100µl per well of a substrate solution was added and incubated protected from light for 2-3 minutes at room temperature. After this time, the enzymatic reaction was stopped by the addition of 50µL per well of the stop solution. Absorbance was read at 450nm by Power Wave Microplate spectrophotometer reader (Biotek Instruments Inc.) using Gen5 (Biotek Instruments Inc.) software. Later, data Analysis was performed using GraphPad Prism 8 (GraphPad Software).

*The concentration of capture Ab, protein, or biotinylated Ab varies depending on the analyte and the specific assay. Moreover, protein solutions can be diluted in media or

assay solution. On the results and discussion chapter, these parameters will be specified.

5.4 SPR Biosensor operation

5.4.1 Nanostructured plasmonic biosensor based on Blu-ray discs.

The biosensor used for this project is a nanostructured plasmonic biosensor based on Blu-ray discs connected to a microfluidic system previously published in Biosensors and bioelectronics (López-Muñoz et al., 2017) and showed in figure 6.

An integrated nanoplasmonic sensor taking advantage of the periodic nanostructured array of commercial Blu-ray discs, that achieves a figure of merit (FOM) up to 35 nm−1 and a bulk limit of detection (LOD) up to 6.3×10−6 RIU (refractive index unit). And for label-free biosensing capability a LOD in the pM order was demonstrated. A prototype with high potential for integration in organ-on-a-chip platforms, given the simplicity, reproducibility and the low cost of the sensor fabrication process and the small chip size (only 1 cm^2)

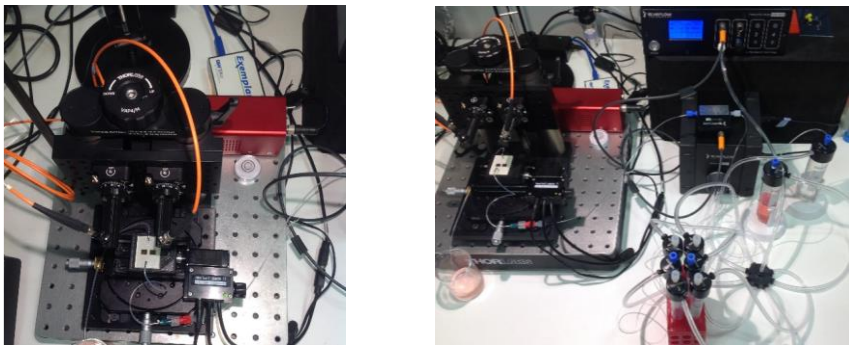


Figure 6. Nanostructured plasmon biosensor based on Blu-ray disc connected to a microfluidic system prepared for the biodetection.

5.4.2 SPR Biosensor protocol

Sensor fabrication was previously published and described in Lopez-muñoz work (López-Muñoz et al., 2017). Briefly a Blu-ray disc, whose aluminium layer was removed, was trimmed around the edges and immersed it in a hydrochloric acid solution (1 M CL) for 120 minutes. Then rinse with deionized water and ethanol and air dried. Later by electron beam deposition gold layers up to 100nm thickness were deposited. With a

conventional computer numerical control router individual plasmonic chips (size of 1 cm^2) were obtained by cutting the gold coated optical disc. The flow cell was fabricated by patterning a microfluidic channel using a cutting plotter in a 50 µm thick double-sided adhesive tape sheet.

The gold surface of the device is activated for 20 minutes in UV Ozone Cleaner ProCleaner (Bioforce, 1062). The device was incubated overnight avoiding light with MUA solution freshly prepared. Later, the device was rinsed 3 times with ethanol, dried with N2 and the EDC/sNHS solution was added for 40 minutes. After this time, the device is washed 2 times with 25mM MES and 1 time with 10mM PB. Then Ab immobilization was performed in PB 10mM pH 7,2 for 2 hours. The capture antibody concentration used was 50µg mL-1. Finally, the blocking solution was added and incubated for 15 minutes. The device was washed 3 times with PB 10mM and dried with N2. This drying process allowed the assembling with the top cover containing the microfluidics channels. A pressure pump controller software was used to deliver the solutions in a sequential an automatic way to the sensor. Zaber control software was used to control the motor who allows the movement of the platform to measure in a multiplexed way, BWSpec Spectral Acquisition Software (B&W Tek) was used to record the SPR signals in real-time and finally data obtained was processed with OriginPro and analized with GraphPad Prism software.

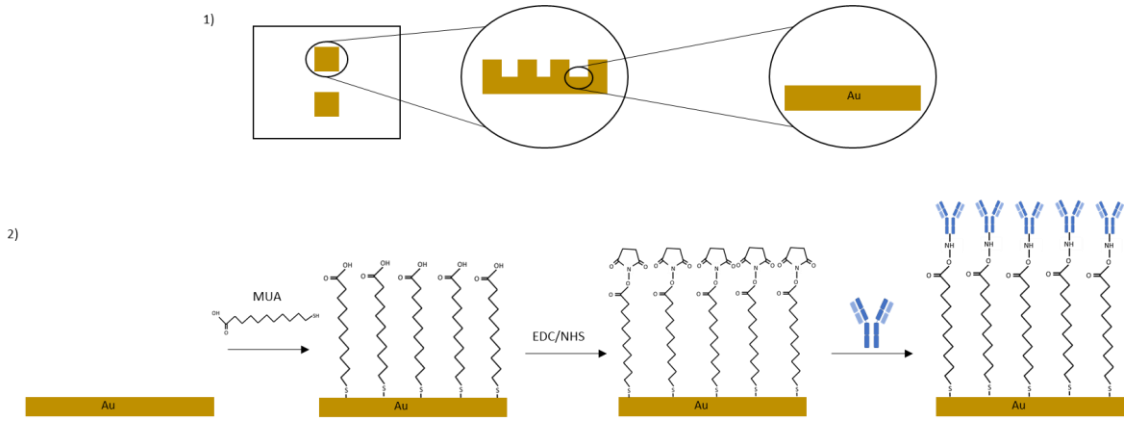


Figure 7. 1) Schematically representation of the chip structure. 2) Biosensor operation process. First, MUA is added to for a self-assembled monolayer (SAM). Then EDC/NHS to activate the SAM. Finally, Antibodies are added and immobilized.

5.5 Data Analysis

5.5.1. Sandwich ELISA.

The data Analysis was performed with GraphPad prism 8 Software. For the calibration curves it should follow a symmetrical sigmoidal shape, this is ELISA dose-response curve shape. For this reason, the chosen fitting was four-parameter logistic equation.

The detection limit (LOD) is the minimum signal detected by each platform and it will be calculated interpolating the 10% of the curve.

5.5.2. SPR biosensor data analysis.

In the assays performed in SPR biosensors the concentrations are additive. We have to take this in consideration to perform the curves. The data analysis is equal to the explained before with sandwich ELISA

6 Results and discussion

6.1 ELISA optimization

TGF-β was not possible to optimize with a previous stock of protein and Ab due to degradation of the protein and lack of specificity of the antigen-Ab interaction. For that reason, an ELISA kit was purchased and optimization wasn't needed.

6.1.1 Optimization of capture antibody and biotinylated antibody by a 2D ELISSA (IL-6, MCP-1 and KC)

A two dimensions (2D) assay was performed to see the range within with the lower concentration of capture or biotinylated antibody concentration we obtained the best signal (absorbance).

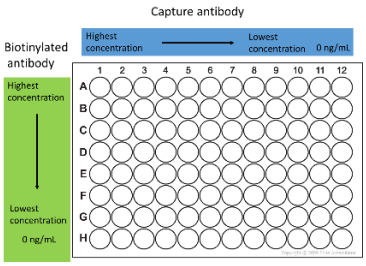


Figure 8. Two dimensions assay. Schematichal representation of the organization of a 96 wells plate.

The protocol followed is the same as described in 5.3.1. The concentration of capture Ab is different per each column, from the most concentrated to the les concentrated with a negative control, and the concentration of biotinylated Ab is different per each row, again, from the most concentrated to the less concentrated, as showed in figure 8. The objective of this assay is to observe which combination gives the best signal with less concentration.

IL-6

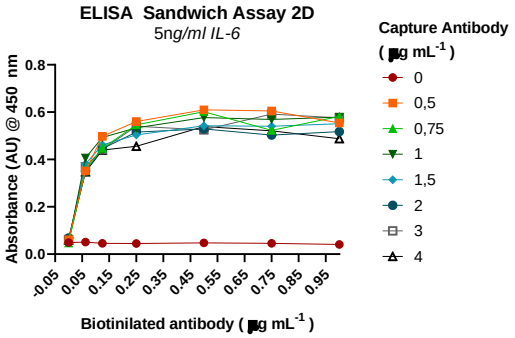


Figure 9. IL-6 ELISA sandwich 2D assay.

All the concentrations of capture ab showed in figure 9 follow a similar curve except for the negative control. So, the range of concentrations to be used will be from 0.5 to 4 µg mL^{-1}. For the capture ab it is important to cover as much surface as possible, which means, a higher concentration is preferred, but to avoid the interaction of one ab to another 3 and 4 µg mL^{-1} concentrations were discarded. So the concentration of capture Ab chose for the calibration curve was 2 µg mL^{-1}. Moreover in Fig.9,it is showed that with a concentration of biotinylated Ab of 0.25 µg mL^{-1} the curve is starting to be saturated. An excess of biotinylated Ab can lead to background noise so the concentration chose was 0.25 µg mL^{-1}.

MCP-1

The same assay was performed for MCP-1.

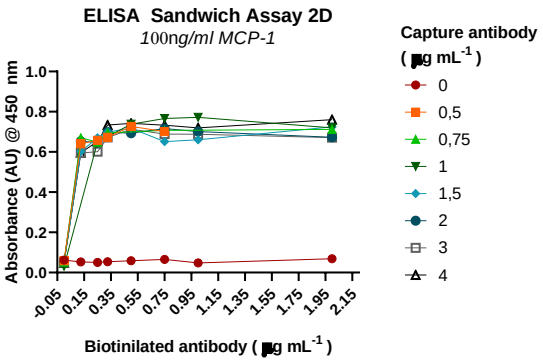


Figure 10. MCP-1 ELISA sandwich 2D assay

The graphic obtained for MCP-1 (fig.10) is similar to the obtained with IL-6 (fig.9). All the concentrations of capture antibody showed a similar curve, so following the same reasoning the concentration of capture ab to be used for the calibration curve is 2 µg mL^{-1}. The curves start to saturate at an earlier concentration, but this is related to the different protein concentration. As the concentrations to be measured are expected to be much lower the concentration of biotinylated Ab chose for the calibration curve is 1 µg mL^{-1}.

KC

The procedure was repeated for KC and the results are showed in figure 11.

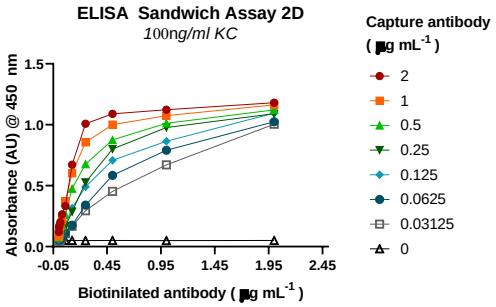


Figure 11. KC ELISA sandwich 2D assay

The graphic obtained for KC shows an evolution of the curve when the concentration of capture and biotinylated Ab increases. The system is not saturated, so the chosen concentration of capture Ab to performed the calibration curve would be the highest (2 µg mL^{-1}). In the curve corresponding to 2 µg mL^{-1} of capture Ab the slope of the curve tends to cero from 1 µg mL^{-1} of the biotinylated Ab. This is the concentration chose.

6.1.2 Calibration curves (IL-6, MCP-1 and KC)

For the three analytes a first calibration curve was performed in in phosphate buffer saline with tween 20 (PBST) and then the same calibration curve was performed to the celullar medium where the "?" samples are, Dulbecco's Modified Eagle Medium (DMEM) and complete media and growth factors (CM+GF)

IL-6

A calibration curve was obtained with 0.25µg mL^{-1} biotinylated Ab and 2µg mL^{-1} capture Ab. The limit of detection of the calibration curve, the smallest concentration that can be detected (LOD) was 0.120ng mL^{-1}. The calibration curve procedure was performed again in the two cellular media the samples "?" are and compare the obtained results.

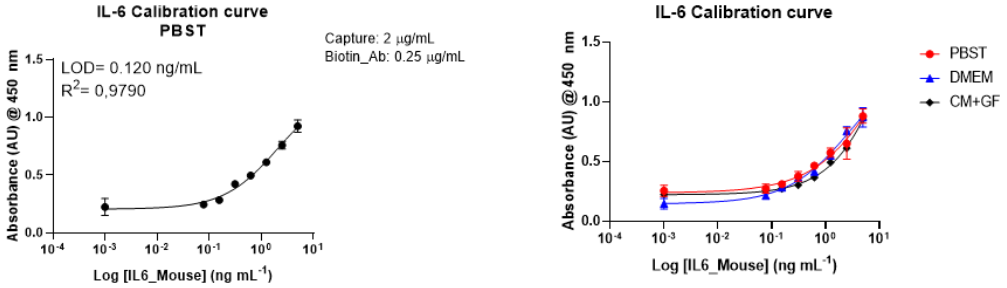


Figure 12. Left. IL-6 calibration curve in Phosphate Buffer Saline with Tween 20 (PBST). Right. Comparation of the IL-6 calibration curves in different media. PBST, Dulbecco's Modified Eagle Medium (DMEM) and complete media and growth factors (CM+GF)

It is observed that the matrix of the cellular media does not interfere with the detection of the protein and the slope of the calibration curve is almost the same.

MCP-1

A calibration curve was constructed with 1µg mL^{-1} biotinylated Ab and 2µg mL^{-1} capture Ab. The smallest concentration that can be detected by this calibration curve was 0.398ng mL^{-1}. As explained before, it is performed the calibration curve procedure in the two cellular media of "?" samples are and compare the result with the calibration curve obtained in PBST.

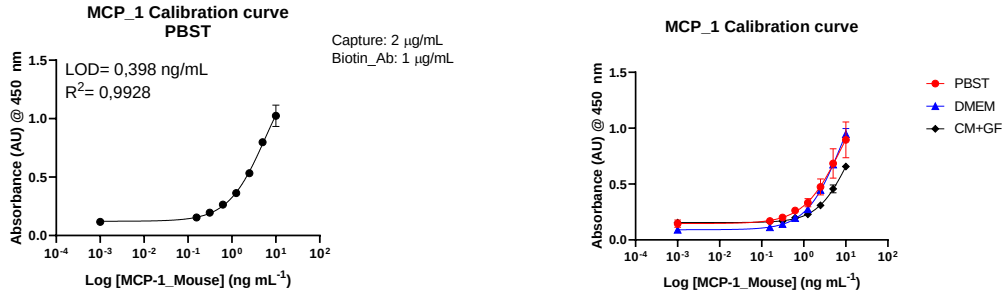


Figure 13. Left. MCP-1 calibration curve in Phosphate Buffer Saline with Tween 20 (PBST). Right. Comparation of the MCP-1 calibration curves in different media. PBST, Dulbecco's Modified Eagle Medium (DMEM) and complete media and growth factors (CM+GF)

As with IL-6 protein the matrix of the either cellular media affects the detection of the protein and the slope of the curve.

KC

To obtain a calibration curve 1µg mL^{-1} biotinylated Ab and 2µg mL^{-1} capture Ab were used.

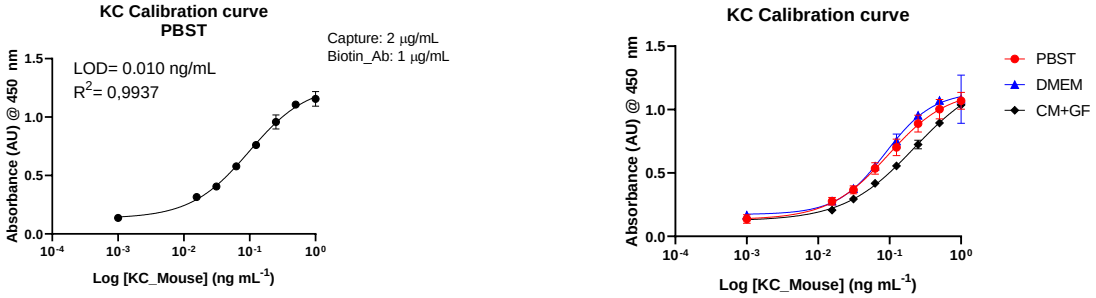


Figure 14. Left. MCP-1 calibration curve in Phosphate Buffer Saline with Tween 20 (PBST). Right. Comparation of the MCP-1 calibration curves in different media. PBST, Dulbecco's Modified Eagle Medium (DMEM) and complete media and growth factors (CM+GF)

The LOD of this calibration curve was 0.010ng mL^{-1}. We performed the same procedure with DMEM and CM+GF and compare these calibration curves with the first one performed in PBST, the calibration curve is similar, the change of media does not affect the calibration curve.

6.1.3 Cross reactivity of MCP-1 and IL-6

For a future functionalization of SPR sensor for a multiplex detection of MCP-1 and IL-6 a cross reactivity study is needed.

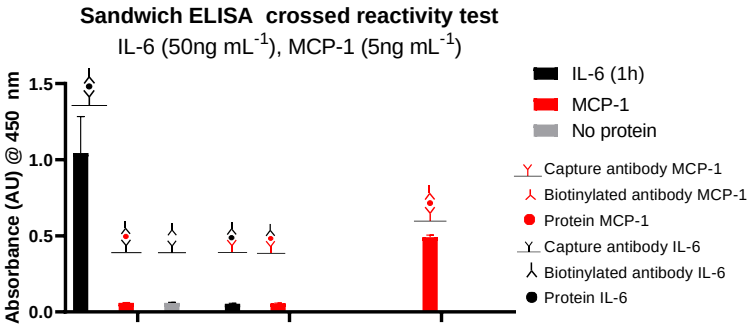


Figure 15. Sandwich ELISA crossed reactivity test between MCP-1 and IL-6.

There is not crossed reactivity between IL-6 and MCP-1, fig.16. For MCP-1 and IL-6 positive controls we obtain a high absorbance. The signal obtained for MCP-1 protein with IL-6 capture, biotinylated Ab or both is equal to the negative control (no protein). Moreover, MCP-1 capture antibody does not detect IL-6 protein.

6.1.4 Degradation in time (IL-6)

There was a problem with the detection of IL-6 protein performing an assay

To know if it was due to a degradation problem we compare the activity of the protein, capture and biotinylated antibody that were being used (defrosted one week before) with protein, capture and biotinylated ab that was just defrosted (one hour before).

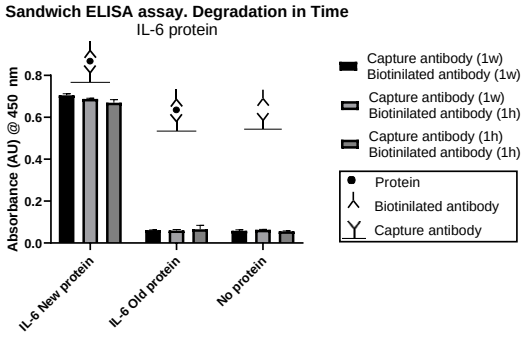


Figure 16. Degradation in time of IL-6 protein, capture and biotinylated antibody. In black, capture and biotinylated antibody was defrosted one week before the experiment. In light grey capture antibody was defrosted one week before the experiment and biotinylated antibody one hour before the experiment. In dark grey capture and biotinylated antibody were defrosted one hour before the experiment.

This assay showed that the absorbance obtained for IL-6 old protein (defrosted one week before the experiment) was the same as the obtained by the negative control. There was no recognition of the protein defrosted one week before the experiment.

Almost the same absorbance was obtained using capture and biotinylated Ab defrosted one week before comparing them with the ones just being defrosted. There was not degradation of the capture and biotinylated Ab.

6.2 Samples analysis of IL-6 and MCP-1

6.2.1 Samples "?" analysis of MCP-1, IL-6, KC and TGF-β.

The experiment consists in a cellular model contained two cellular layers, one of myofibroblast and one of epithelial cells on top of the myofibroblasts. The epithelial layer is interrupted by a PDMS stencil, this stencil is extracted, and the epithelial cells migrate to fill this "gap" or heal this wound. This process of epithelial reconstitution is monitored, and samples are extracted along the process as picture in figure 17. Moreover, of this cellular model a myofibroblast model is produced, as it has all the components of the cellular except for the epithelial cells and the PDMS stencil

We analyse the media of "?" in different stages of the epithelial reconstitution experiment. Fig.17. The first sample is extracted after 2 days in culture with the model with the PDMS stencil. Sample 2 is extracted one day later. Then, the PDMS stencil is extracted and 2 hours later sample 3 is extracted to analyse short term epithelial reconstitution. Sample 4 is extracted 3 days later, corresponding to long term epithelial reconstitution. All these are in complete media with growth factors (CM + GF) medium.

Sample 5 corresponds to DMEM 10%FCS 1%P/S medium 3 days in culture with the myofibroblasts control.

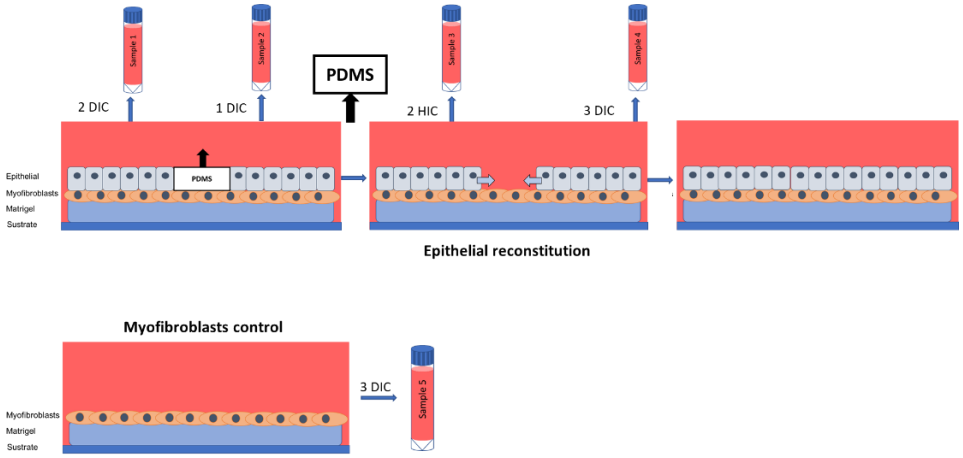
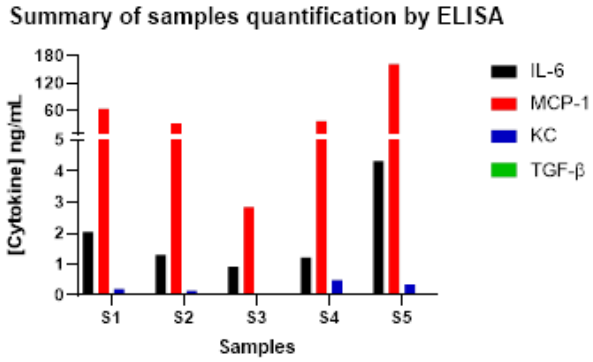


Figure 17. "?" Sample extraction of the cellular model. Sample 1, 2 days in culture of epithelial growth. Sample 2, 1 day in culture of epithelial growth. Sample 3, 2 hours in culture short term epithelial reconstitution. Sample 4, 3 days in culture long term epithelial reconstitution. Sample 5, 3 days in culture of myofibroblasts control (DIC. Days in culture. HIC. Hours in culture).*

The concentration of each analyte was measured by sandwich ELISA assay in each sample. A 96-well plate was used per analyte. In each plate it was performed a calibration curve on DMEM, a calibration curve on CM+GF and an analysis of the 5 samples without dilution and diluted 1/2, 1/4 and 1/8. The calibration curves enable the interpolation of the absorbances obtained for each sample to obtain the concentration of analyte.

From this assay we obtained the concentration of analyte in the samples.



	IL-6 (ng mL-1)	MCP-1 (ng mL-1)	KC (ng mL-1)	TGF-β (ng mL-1)
S1. Epithelial growth 2 days in culture	2,053	64,5	0,212	0,000656
S2. Epithelial growth 1 day in culture	1,302	33,1	0,159	0
S3. Short terrm epitelial reconstitution 2 hours in culture	0,928	2,847	0,019	0,01322
S4. Long term epitelial reconstitution 3 days in culture	1,229	37,8	0,497	0
S5. Fibroblasts control 3 days in culture	4,335	162,41	0,352	0,009108

Figure 18. Left, Concentration of each analyte (IL-6, MCP-1, KC and TGF-β) in "?" samples from 1 to 5 (S1-S5). Being S1, epithelial growth 2 days in culture, S2, epithelial growth 1 day in culture, S3 short term epithelial reconstitution 2 hours in culture, S4 long term epithelial reconstitution 3 days in culture, and S5 fibroblasts control 3 days in culture. Right, table with the obtained analyte concentrations in each sample.

The concentration of TGF-β is imperceptible in all the samples, on contrary of what was expected and KC concentration is less than 0.5ng/mL in the five samples but we can appreciate that the sample 3, long term epithelial reconstitution has the higher concentration (0.497ng mL^{-1}).

For MCP-1 the higher concentrations in the samples were obtained.Fig.18. Being sample 3 the less concentrated (2.847ng mL^{-1}), corresponding to 2 hours in culture short term epithelial reconstruction, and sample 1 the most concentrated in contact with the complete cellular model (64.500ng mL^{-1}), corresponding to 2 days in culture epithelial growth. This can be consequence of the high different of time the medium was in contact with the cellular model 2 hours (sample 3) and 2 days (sample 1).

IL-6 concentrations follow the same tendency as for MCP-1 but the values are lower, from 0.928ng mL^{-1}, the lowest, (sample 3) to 4.335 ng mL^{-1}, the highest, (sample 5).

The most concentrated analytes MCP-1 and IL-6 are the better candidates for the functionalization of the SPR biosensor.

As for IL-6 and MCP-1 the concentrations obtained in myofibroblast control (sample 5) are the highest. (4.335 ng mL^{-1} and 162.410 ng mL^{-1}), a second experiment was performed to analyse the reason of these concentrations of analytes.

6.2.2 Analysis of MCP-1 and IL-6 in myofibroblast control, myofibroblast treated and NIH/3T3 Fibroblasts.

The supernatants of the cell cultures where again proportionated by Biomimetic system for cell engineering. Correspond to Intestinal subepithelial myofibroblasts (ISEMF), ISEMF treated with the antifungal drug itraconazole (ITA), that has been reported to decrease the contractile activity of the myofibroblasts (Bollong et al., 2017), and NIH 3T3 fibroblast, a cell line of mouse embryonal fibroblast without contractile behaviour.

MCP-1 has been related with the contractile capacity of the myofibroblasts and their migration (Dagouassat et al., 2010).

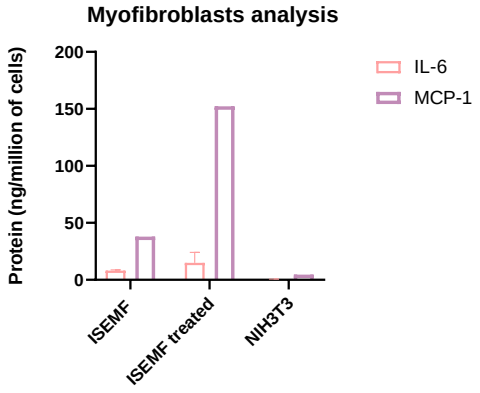


Figure 19. Myofibroblasts samples analysis of MCP-1 and IL-6. (ng per million of cells).

The hypothesis was that the myofibroblasts expose to ITA will decrease the secretion of MCP-1 due to the decrease in the contractile activity that the drug produces, and the levels of IL-6 increased due to the alteration. But on the contrary of expected the exposure of the cells to ITA has increase the secretion of MCP-1. Nevertheless, the low secretion of MCP-1 in NIH3T3, was expected and can support the hypothesis of the relation between this chemokine and the contractile activity of the myofibroblasts.

In conclusion, the results obtained in this experiment do not justify the high levels of MCP-1 in the myofibroblast control but express a high concentration of MCP-1 in the samples that were exposed to a stress (exposed to the drug). The same but less notable occurred with IL-6.

Moreover, the results obtained in this second experiment were normalize to the number of cells to obtain the secretion of analyte per million of cells. This was not possible in "?" samples due to the complexity of the cellular model, but the results may change if this normalization could be performed.

6.3 MCP-1 and IL-6 detection by SPR biosensor

6.3.1 MCP-1 and IL-6 Calibration curves

As mentioned before the two best candidates for the functionalization of SPR biosensor were MCP-1 and IL-6

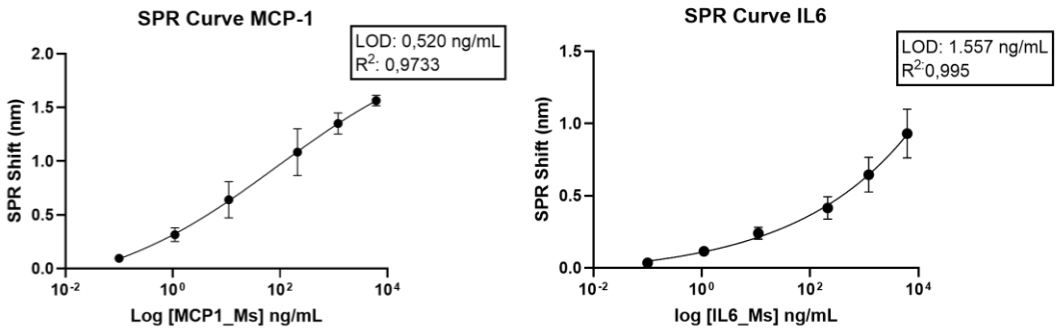


Figure 20. SPR biosensor calibration curve. Left, MCP-1 SPR calibration curve. Right, IL-6 SPR calibration curve

SPR shift indicates de quantity of protein attached to the biosensor. For MCP-1 the obtained LOD=0.520 ng mL^{-1} is lower than the one obtained for sandwich ELISA (Fig.13). This calibration curve has a higher sensibility and it can be used to the measurement of "?" samples because the concentrations obtained by sandwich ELISA assay are inside the values of the SPR calibration curve.

For IL-6 LOD=1.557 ng mL^{-1} is higher that the obtained in sandwich ELISA assay (Fig.12) but it is close to the concentration of IL-6 observed in "?" samples, so it is suitable for the measurement.

7 Conclusions

Optimization and protocol development of sandwich ELISA has been successfully performed for MCP-1, IL-6, and KC. For TGF-β was not possible, but an ELISA kit was purchased already optimized. Cross-reactivity and protein stability assays were showing

no cross reactivity between antibodies and proteins. Also, stability studies showed than proteins used in the calibration curves for quantification assays turns unstable after 1 week of been defrosted.

The quantification of the inflammatory bowel disease in vitro model samples was performed, and the highest secreted proteins were MCP-1 and IL-6. Contrary to the hypothesis the secretion of TGF-β was lower than other cytokines.

Control experiments to explain the high content of MCP-1 in the samples were performed. However, the reason why myofibroblasts control sample showed the concentration of IL-6 and MCP-1 higher than in the rest of the samples in unclear.

The optimization of SPR biosensor platform was performed for IL6 and MCP-1 cytokines. Sensing platform shows a LOD of 1.557 ng mL^{-1} for IL6 and 0.520 ng mL^{-1} for MCP1

Due to COVID-19 situation, it was not possible to integrate the in vitro model with the optimised sensing platform and monitor in situ IL6 and MCP1 secretion levels.

8 Future perspectives

Once the cross-reactivity test has been performed and the IL-6 and MCP-1 SPR calibration curves are obtained it is possible to functionalize one chip of the biosensor for the detection of IL-6 and the other for MCP-1. Measuring the concentration of both analytes in only one measurement procedure.

In the future, this biosensor can be connected directly to the cellular model obtaining real-time information of the analytes' concentrations. Moreover, other analytes or cytokines can be optimized for SPR biosensor obtaining a more complete secreted profile of this cellular model in real-time and it would be optimal to compare this profile with a cellular model without performing the extraction of the PDMS stencil.

9 Auto evaluation

Despite Covid-19 situation I decided to continue with this project and even thought at the begging it was really frustrating not knowing when we could continue to attend to the laboratory, now I feel It has worth the effort. I have learned a lot performing this project and I am grateful for having the opportunity to work in a laboratory for a few months.

It has also helped me realize that I need to improve my time and stress management skills.

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