POLITECNICO DI TORINO

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in Strumentazione Biomedica

Automated microfluidic platform of electrochemical immunosensor integrated with bioreactor for continual monitoring of mesenchymal stem cell secreted biomarkers

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Introduction

Mesenchymal Stem Cells (hMSC) may be considered as the best candidate for tissue engineering, gene therapy and regenerative medicine due to their broad differentiation potential and potent paracrine properties such as angiogenic capacity.

For this reason, studies focused on this kind of cells got increased and lots of research efforts, aimed at controlling the human Mesenchymal Stem Cells secretome for clinical applications, have explored multiple strategies including hypoxic, pharmacological, cytokine, or growth factor preconditioning, and/or genetic manipulations.

Important aspects of the hMSC microenvironment have been shown to influence growth and differentiation and they can play a significant role in regulating proangiogenic signaling.

These aspects interest the organism starting from the smallest elements and their micro-interactions up to all the interplay between tissues, organs and the total movement of the hole human body when it carries out normal activities during the daily life.

As micro-interaction: mechanical stimuli and physical characteristics of the extracellular matrix, the relatively small forces exchanged between every single cell and the neighborhood .

However, the precise role of these factors in directing proangiogenic signaling remains to be revealed.

The goal of this research field is to find strategies to increase these cells survival rate after transplantation and the angiogenic ability are of priority for the utility of MSCs.

This may be done only with the introduction of a 4D system which can mimic the real phenomena.

Therefore, in this project it has been fabricated a three-dimensional dynamic cyclic compression (which may be considered the fourth dimension) bioreactor in which hMSCs are cultured in 3D gelatin-methacrylate hydrogels to evaluate the influence of mechanical stimuli

and biophysical factors on MSC differentiation and secretion using eletrochemical biosensing integrated to bioreactor.
As a specific biomarker for in vitro study, Vascular endothelial growth factor(VEGF) has been selected since it has been found to be elevated during angiogenesis and osteogenesis where mechanical stimuli and physical factors play an important role in the fate decision.
Yes-associated protein (YAP), as mechanotransduction in regulating stem cell fate decisions, will be also employed to understand the mechanism behind how proangiogenic signaling of hMSCs can be regulated by these factors.

This work will prove useful information about the design of tailored model system that more efficiently direct proangiogenic signaling from hMSCs.
Therefore, the novelty of this project are:

- Use a bioreactor to examine the synergic role of physical factors and mechanical stimuli on MSCs differentiation and secretion.
- Evaluate the response of MSCs in a 4D system, which better mimics native tissue architecture compared to 2D cell culture.
- Produce an in vitro macroscale construct that exhibit angiogenesis that may be useful for further on-chip studies and for in vivo stem cell-based therapy.

In the first chapter of this thesis, general knowledge about mesenchymal stem cells and the influence of mechanotransduction on cell fate have been treated.
Consequentially, the approaches chosen for this study were later discussed and so two chapters were than reserved for microfluid bioreactor system and the analysis techniques which have been utilized during all the project.
Finally, the chapters materials, methods and results, discussion treat the core of the project and all the experiments and techniques used to accomplish the wanted results.

This project has been done in Ali Khademhosseini laboratory at the University of California, Los Angeles.

Chapter 1

Mesenchymal Stem Cells research and the role of mechanotrasduction on cell fate

1.1 Mesenchymal Stem Cells

The reason why the project was mainly focused on the study of Mesenchymal Stem Cells, has to be found on the properties of this particular kind of cells which can be considered as one of the best candidates for tissue engineering, gene therapy and regenerative medicine.
Mesenchymal Stem Cells (MSCs) have the quality to differentiate into different kind of cell type, they have good immunologic characteristic and they are able to migrate easily from a point of transplantation to an injury area.
"MSCs, which can be defined as multipotent mesenchymal stromal cells, are a heterogeneous population of cells that proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology, form colonies *in vitro* and can differentiate into bone, cartilage and fat cells" [18].

By the analysis of the definition multipotent mesenchymal stromal cell, it is possible to immediately understand the main characteristics and peculiarities of this kind of cells:

- Multipotent: cells ability to differentiate into other different cell types is called cell potency. Cells with a higher number of possible differentiation have an higher potential.

- Stromal: cells that form the boundary of each organ. Stromal cells adjacent to epidermis release growth factors, that are the proteins protagonist of this project and they have several roles like for example they promote cell division.

"Recent reports have revealed that stem progenitor cells, particularly those derived from bone marrow, promote tissue repair by secretion of factors that enhance regeneration of injured cells, stimulate proliferation and differentiation of endogenous stem like progenitors found in most tissues, decrease inflammatory and immune reactions, and, perhaps, by transfer of mitochondria". [33]
Basically, this means that more than the ability to differentiate, the characteristic of MSCs that make them capable to heal injured tissue is the secretion of soluble factors which they act on the surrounding micro-environment.

Here some *in vivo* and even *clinical* studies which explain the potentiality of MSC in several fields and why researchers are investing so much this field:

- "Marrow stromal cells (MSCs) were injected into the lateral ventricle of neonatal mice and it has been observed that multipotential mesenchymal progenitors from bone marrow can adopt neural cell fates when exposed to the brain environment" [20];

- "A well-characterized human mesenchymal stem cell population was transplanted into fetal sheep early in gestation. Mesenchymal stem cells maintain their multipotential capacity after transplantation, and showed to have unique immunologic characteristics that allow persistence in a xenogeneic environment." [22]

- "Systemic intravenous delivery of bone marrow MSCs to rats after myocardial infarction, although feasible, is limited by entrapment of the donor cells in the lungs. Direct left ventricular cavity infusion enhances migration and colonization of the cells preferentially to the ischemic myocardium." [6]

- "MSC were delivered into injured spinal cord of animals rendered paraplegic. After injury it has been obserbed a larger numbers of surviving cells than immediate treatment and significant improvements. MSC constitute an easily accessible, easily expandable source of cells that may prove useful in the establishment of spinal cord repair protocols." [16]

- "hMSCs were been administered intravenously on functional outcome after traumatic brain injury in adult rats. The transplanted cells successfully migrated into injured brain and were preferentially localized around the injury site. Some of the donor cells also expressed the neuronal and astrocytic markers." [25]

- "Allogeneic bone marrow transplantation in three children with osteogenesis imperfecta. Three months after osteoblast engraftment, representative specimens of trabecular bone showed histologic changes indicative of new dense bone formation." [17]

- "Blood supply is a crucial part of bone formation and fracture healing. Recent studies suggested that VEGF, a potent angiogenic stimulator, may play an important role during these processes in stimulating the restoration of blood flow to the fracture site, thus initiating the bone repair process."[27]

There is a multitude of scientific articles in which MSCs are described as a way to solve different diseases, to heal injured tissues and in general the goal is to use MSCs for therapeutic applications.
It has been demonstrated that it is possible to use MSCs for tissue regeneration, correct inherited disorders, mitigate chronic inflammation and use these cells as a vehicle to deliver some agents to a specific location.
Therefore, MSCs can migrate from blood, across endothelial cells to tissues.
In most of the studies, scientists found out that MSCs have a specific behavior when they are in the proximity of the damaged tissues and cells.
Therefore, they do not treat in the same way healthy and ill tissues, but it has been demonstrated that a injured tissue shows and expresses receptors and ligands that attract MSCs to go toward the location where the ill cells are and to interact with them.

1.2 Mechanical signals control cell behavior

Cells behavior is strictly related to the environment which surround them.
Environment means other cells neighborhood and even physical and mechanical stimuli which make cells react in some mysterious ways that are not completely analyzed and understood.
Extracellular matrix (ECM), a complex and dynamic cellular microenvironment, plays an important role in guiding behaviors of stem cells such as their growth, differentiation and secretome.
Model systems that modulate the physical characteristics of the ECM have proved useful in exploring how these signals regulate proangiogenic signaling.
However, the interplay between these physical factors and mechanical stimuli during proangiogenic signaling remains to be revealed.
Several researchers demonstrate that mechanical stimuli are translated into biochemical signals which influence cells to secrete for example specific proteins in higher or lower percentage, or to improve their viability and so they may grow better and healthier.
Moreover, mesenchymal stem cell, when they are subject to mechanical stimuli, show differentiation ability different from the one without any physical stimulation.

In this project, the aim of the study was to acquire important data about the different behavior of hMSCs when compression is applied to them.
The approaches were to check vascular endothelial growth factor (VEGF) secretion and Yorkie-homologues YAP (Yes-associated protein) expression.
" At present, the generation of viable tissue-engineered constructs larger than a few millimeters in size using mesenchymal or other stem cells is limited due to the lack of functional vasculature within the constructs.[...] This understanding is in turn the foundation for a rational approach to the design and optimization of prevascularized tissue-engineered constructs and essential for predicting optimal mechanical stabilization conditions for successful tissue regeneration." [47]

1.2.1 Vascular endothelial growth factor (VEGF)

One of the application in which multipotent stem cells could find an important role is for tissue regeneration as it is already explained in the previous section.
"Strategies to increase their survival rate after transplantation and the angiogenic ability are of priority for the utility of MSCs [...] mechanical stretch (10% extension, 30 cycles/min cyclic stretch) preconditioning increase the angiogenic capacity via VEGFA induction." [47]

"The secretion of trophic factors that promote angiogenesis from mesenchymal stem cells (MSCs) is a promising cell-based treatment."[1]
Regeneration takes place thanks mainly to the secretes of MSCs. Therefore, since it has been demonstrated that mechanical stimulation enhances protein secretion, the goal of this study was to find the correlation between physical and mechanical cues to VEGF secretion.

"Elevating the tissue levels of Vascular growth factor (VEGF) after acute myocardial infarction seems to be a promising reparative therapeutic approach." [14]
In that research VEGF, with and without MSC, was basically injected in the region of the scar area and good results on cardiac remodeling have been observed.

Due to the knowledge that matrix rigidity and physical stimulation increase the secretory profile of MSCs, the results expected from the stimulation by compression of cell-laden hydrogel are an increasing production of VEGF.
The question once this theory will be proved is how to use this increasing production of the angiogenic marker in order to heal and regenerate ill tissues.
One interesting approach could be to verify if preliminary stimulation by compression alters the behavior of MSCs when they got in the injured area. In this way, tissue reconstitution may be faster and it may reach goals that still are not possible to obtain.
Moreover, it is reasonable to think that the results obtained by T. Deuse et al. of a beneficial effect of MSC to a treat acute myocardial infarction was mainly due to the increase production of VEGF in turn due to the heartbeat, which is a very strong mechanical stimulation.

1.2.2 Yorkie-homologues YAP (Yes-associated protein)

There are different factors which influence single cell behavior, which in turn influences tissue properties like dimension, shape of the tissue, role inside the body.

Among them, cell-extracellular matrix (ECM) and forces which make interact one cell to the near cell.

"Every cell responds to the mechanical properties of its environment, such as the elasticity (or stiffness) of the ECM and traction or compression forces exerted by neighbouring cells." [15]

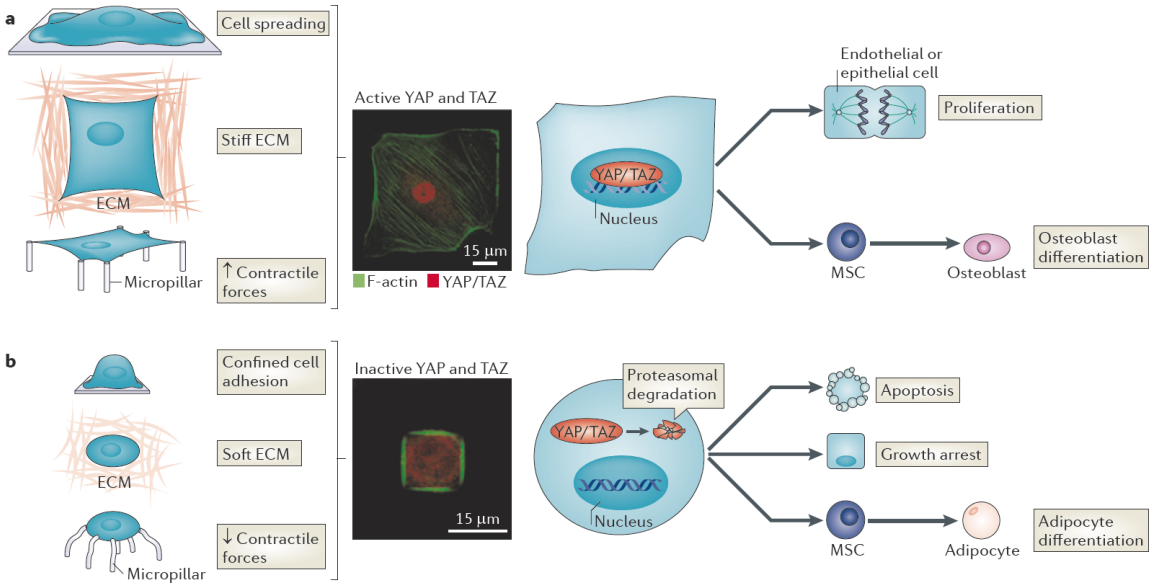

Figure 1.1: *"YAP as sensor and mediator of mechanical inputs from the extracellular matrix." [15]*

Each cell responds to mechanical stimulation regulating ECM elasticity and cytoskeletal tension.

Consequently, it has been proved that this alteration of geometry and shape of cells and so tissue play an important role in cell proliferation, death and differentiation.

Remaining in the field of mesechymal stem cells, one of the reason which makes these cells differentiate into osteoblasts is the cell-cell adhesion not too strong, in fact if the cells can easily spread they will be free to mute shape and acquire an oval shape.

On the contrary, MSCs become adipocytes when the shape remains rounded.

Yes associated protein seems to be correlated to mechanical signals. It may be considered as a mechanosensor which allows the translation of mechanical signals into biological outcomes and so the gene expression.

As explained schematically in figure 1.1, a stiff ECM and so high contractile forces make YAP active.

In this case, YAP goes inside the nucleus and the consequences might be proliferation and osteoblast differentiation.

Therefore, the expectation is an increasing secretion trend of the protein VEGF since cells go throw proliferation and wellness.

On the contrary, when contractile forces are low and cells show a soft ECM, YAP is inactive and it localizes in cytoplasm.

The consequences in this situation might be apoptosis, which is a way of dying of the cells, growth arrest of the cells and the differentiation goes towards adipocytes.

Chapter 2

Microfluidic bioreactor and organ-on-a-chip

Since the introduction of microelectromechanical system (MEMS) technologies, intensive research efforts have been devoted in the field of microfluidic.
The introduction of microfluidic bioreactor and organ-on-a-chip platforms have opened a new way to study biological phenomena in a three dimensional approach.
Researchers had improved the quality and reliability of their results since engineers started to work in collaboration with biologist and of course biomedical engineer seems to be the perfect figure, indeed he is able to approach to biological and medical problems with his skills on both the fields, medicine/physiology and engineering.
Microfluidic bioreactor and organ-on-a-chip can be found in several fields, such as tissue engineering, drug delivery, bioprocessing optimization and cell based screening studies.

The goal is to recreate with high fidelity what is really comparable to a real cell micro-environment. Of course, the first problem to deal with is the length scale of cells.
There are some techniques like photolithography, soft lithography, hot embossing, micro-injection molding and direct milling method, which allow us to make possible the realization of microfuidic device of dimension under the micro scale.
In few words, microfluidic technology has the potential to reliably and reproducibly mimic the *in vivo* microenvironment much better of what it is possible with a cells cultured in 2D monolayer petri dish.

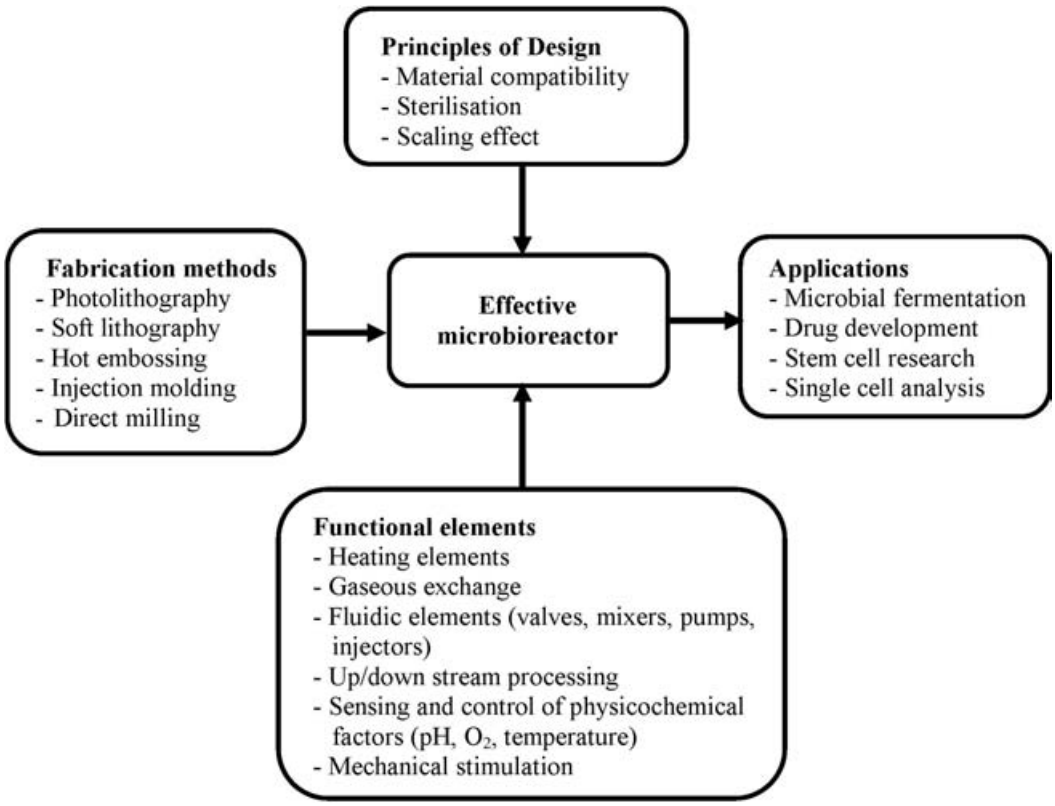

Figure 2.1: *Summary requirements for effective microbioreactor platforms. [31]*

The cell cultured 2D approach is mainly useful to obtain preliminary data in order to understand the main characteristics of the cell microenvironment, which influence the behavior of the cells object of the study.

"The in vivo cellular microenvironment is made up of a complex blend of various components which include: extracellular matrix (ECM), biochemical factors such as cell-cell and cell-matrix interactions, concentration gradient of soluble factors such as cytokines and glucose, O_2 availability and a combination of physical factors (hydrodynamic shear, mechanical compression or stretch) and electrical signals exerted on the cells." [31]
Therefore, with a bioreactor platform it is possible to manipulate the factors that govern the cell function by introducing fluidic, sensing, physiochemical, mechanical, electronic and optical components inside the micro-structure.

Bioreactor platform is a semi-closed system and this is one of the most important aspect that makes the system a remarkable way to culture and study cells.
As a semi-closed system, problems related to contamination occur with a low percentage in comparison with the common 2D culture in a petri dish.
It is also possible to make the system fully automated with a peristaltic pump for example and with the introduction of microfluidic channels, inlets and outlets in which medium and other specific solutions may flow in. In this way there are less chance to introduce viruses and contaminants in the microenvironment and to allow cells to grow properly thanks to the continuous replacement of waste material with nutrients.

Another advantage in developing bioreactors and organ-on-a-chip platforms has to be found in the detection approaches that are possible to be implemented in a automated system like a lab-on-a-chip device.
ELISA and mass spectroscopy were the best options to study a cell microenvironment until the introduction of the new technologies. The integration of immunosensors inside the microfluidic platform provided an easiest, faster, low cost and low volumes approach of protein detection.

2.1 Principles of Design

Bio-compatibility is the main problem when cells are involved in a system object of study. Of course, the material of which is made the bioreactor has to have some fundamental characteristics in terms of surface properties like cytotoxicity and it should present a good adherence with cells, without altering the well viability of the environment and affecting cellular growth.

Contamination is one of the main reason of failure in experiments which involve cells.
Therefore, sterilization by autoclaving all the elements needed for the experiments is one good way to remove all the bacteria, fungi, viruses that could influence the success of the experiment.
Though, not all the materials that are biocompatible have also the advantage of being resistant to high temperature and high pressure, which are essential for the autoclave system.

Techniques of sterilization require one or more of the following: heat, chemical, filtration and irradiation. Of course, everything is designed in agreement with the experiment in question. Basically, the sterilization technique has to be chosen based on the material characteristics and this last one has to be chosen based on the kind of experiment that it is needed to study a particular phenomena.

"Thermoplastics and elastomers have emerged as the preferred substrates for microbioreactor devices due to their thermal stability, biocompatibility, ease of fabrication, transparency, gas exchange and their potential to be used with high replication for low cost devices." [5]

2.1.1 Polydimethylsiloxane (PDMS)

Polydimethylsiloxane is a common choice as a material used for microfluidic circuitry.
It is a silicone elastomer and for this project it has been used PDMS Sylgard 184 (Dow Corning Corporation), which "[...] is a heat curable PDMS supplied as a two-part kit consisting of pre-polymer (base) and cross-linker (curing agent) components.
The manufacturer recommends that the prepolymer and cross-linker have to be mixed at a 10:1 weight ratio, respectively." [26]
"It is generally agreed that PDMS is among the most popular polymeric materials employed for the fabrication of microfluidic devices owing to a number of advantages: simple fabrication by replica molding, biocompatibility, nontoxicity, excellent optical transparency down to 280 nm, and permeability to gases." [43]
"In spite of these advantages, the strong hydrophobicity of PDMS surface always impedes PDMS based microfluidic devices from immediate use without any surface processing." [43]

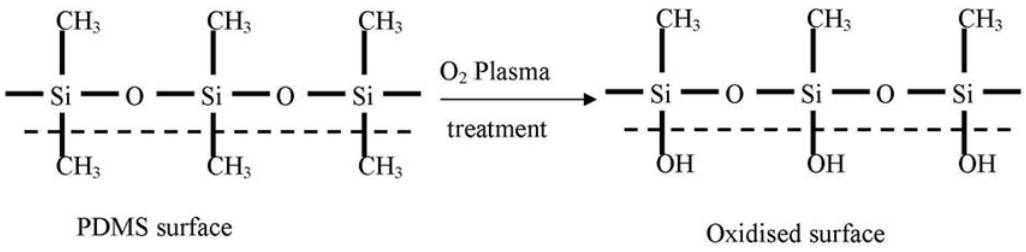

Figure 2.2: *"Surface modification of PDMS by oxygen-plasma treatment." [28]*

By plasma treatment it is possible to make the surface of PDMS hydrophilic. PDMS consists of repeating units of $OSi(CH3)_2$.
"By exposing PDMS to O_2 plasma, the CH3-groups on the PDMS-surface are removed and substituted by polar groups (OH) to create silanol (Si-OH) groups on the surfaces, thereby rendering them hydrophilic". [28]

2.2 Microfabrication techniques

There are several approach to fabricate a microfuidic system:

- Photolithograhy
- Soft Lithography
- Hot Embossing
- Micro-Injection Molding
- Direct milling method

The technique implemented in this project is the easiest and at the same time it gives precision and accuracy which might be not as good as the other methods.
The specifics of this project did not require an high level of precision and moreover the needed scale length was between hundred of μm and some mm, therefore direct milling method seemed to be the best choice in terms of efficiency, velocity of fabrication and costs.

2.2.1 Direct milling method

This method requires a hard material which is not sensitive to UV light, high temperature and high level of pressure and tensions. Therefore, polymer and metal are usually used.
The procedure consists in cutting the hard materials in order to obtain the wanted geometries and structures.
In this project as material Polymethyl methacrylate (PMMA)(McMaster Carr) sheets were cutted by Laser cutter (VersaLASER VLS2.30) and used as molds.

As software to design the structure was used CorelDraw, which is a simple software to create shapes in 2D.
Once the PMMA sheet was obtained in the right dimension and geometry, PDMS was spread on the mask.
After using a degassigator to remove the bubbles trapped inside the PDMS still in the liquid state, a woven at $80°C$ was used to make the PDMS cured.
After several tries in which the sealing between the different part of the bioreactor was not enough strong, an important improvement in the realization of the 3 layers of PDMS was found to solve the main problem. Indeed, a frame of PMMA was designed and used in order to sign the level of PDMS for each layer.
At the beginning simple petri dishes were used and PMMA molds were attached to the surface of the petri dish by glue. In this way, the distribution of the PDMS once spread on the mold was not accurate and homogeneous and moreover a contact angle between the PDMS and the plastic walls of the petri dish makes difficult the plasma bonding.
Therefore, the use of the frame was providential in terms of material distribution and if the PDMS layer overcomes as needed the frame no problem of contact angle occurred.

2.3 Applications

Microfluidic bioreactor and organ-on-a-chip made easier studies of several fields, like:

- Microbial fermentation
- Drug development
- Stem cell research
- Single cell analysis

The reason why the use of lab-on-a-chip got increased has to be found in the advantage of testing and analyzing cell behavior without *in vivo* study on animal and therefore without all the negative aspects as costs, ethical issues and not total compatibility of animal organism in comparison with human body.

Drug and toxicological screening are made to study the behavior of drugs specific for a disease and check the toxicological and therapeutic effects on the organ object of the treatment.

Therefore, the purpose is to verify and validate the efficiency of the drug and see the side effects in short and long time.

When a drug is inside the organism and it enters the systemic circulation, it effects not only the anatomic region of interest but also other organs which should not be treated.

Especially the liver is one of the main affected organ since its roles in the human body, for example it is the protagonist of some metabolic functions and detoxification.

Usually more bioreactors are connected together by a microfluidic sistem made of microchannel, microvalves, micromixers and pumps, in order to recreate a human body. Every bioreactor recreates an organ, therefore the design, material and microenvironment characteristics are made specifically to mimic the organ.

Once the organs are connected, it is possible to introduce in the microfluidic system a drug, for example chemotherapeutic medicine, and check if that drug gives problem to the organs which should not be treated.

Moreover, this systems may be used to test the functionality and efficiency of drugs before a clinical study.

For what concern stem cell research, the core of this study might be the simple purpose of obtaining a better understanding of this kind of cells behavior and how it is possible to use them in regenerative medicine and moreover an important step is to find "strategies to increase these cells survival rate after transplantation and the angiogenic ability are of priority for the utility of MSCs." [47]

This may be done only with the introduction of a 4D system which can mimic the real phenomena.

To obtain this kind of system, of course the only possible way is to design and fabricate a microfluidic bioreactor.

Therefore, in this project it has been fabricated a three-dimensional dynamic cyclic compression (which may be considered the fourth dimension) bioreactor in which hMSCs are cultured in 3D gelatin-methacrylate hydrogels to evaluate the influence of mechanical stimuli and biophysical factors on MSC differentiation and secretion using eletrochemical biosensing integrated to bioreactor.

Chapter 3

Analysis techniques

An important advantage in developing bioreactors and organ-on-a-chip platforms has to be found in the detection approaches that are possible to be implemented in an automated system like a lab-on-a-chip device.
ELISA and mass spectroscopy were the best options to study a cell microenvironment until the introduction of the new technologies.
The integration of immunosensors inside the microfluidic platform provided an easiest, faster, low cost and low volumes approach of protein detection.

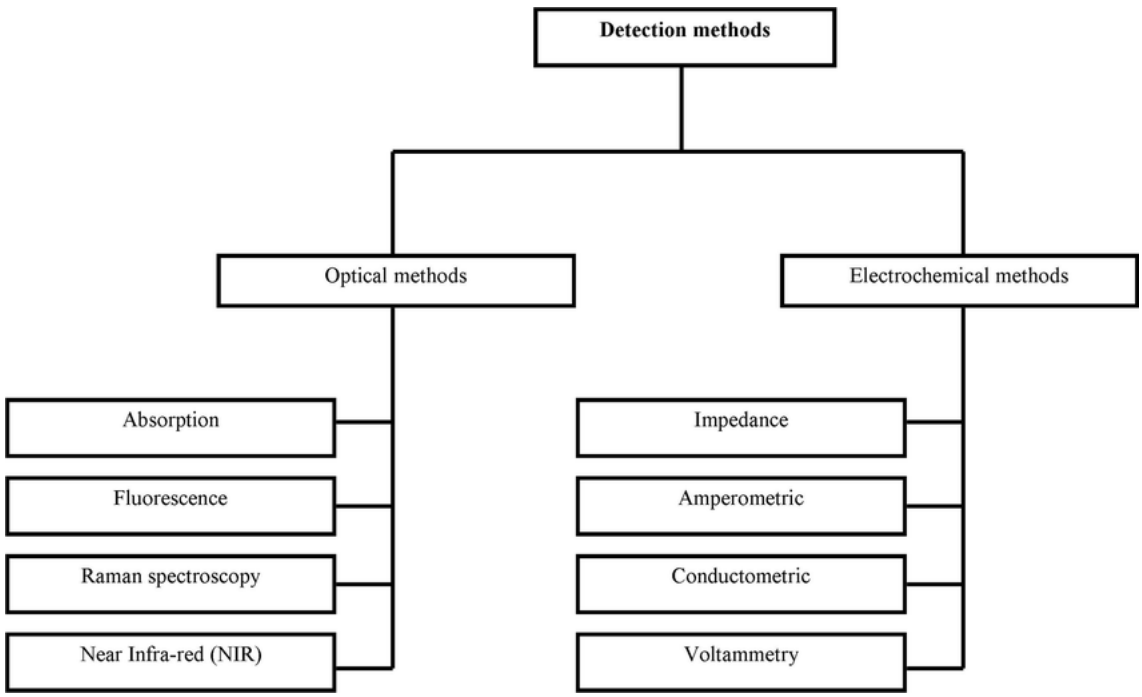

Figure 3.1: "*Common detection methods used in microbioreactor platforms." [31]*

3.1 Biochemistry assay techniques

By biochemical analysis are intended all the procedures which study a biological process or system in order to isolate and analyze the behavior of one specific biomelocule in a biological sample.

There are several approaches and all of them may include more than a single assay. Only two techniques have been selected for this project and so they are the only ones explained and debate in the following section.

3.1.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is the preferred method to measure antibodies, antigens, proteins and glycoproteins in biological samples.

This method takes place thanks to the strong interaction between antibody and antigen.

There are different kind of ELISA formats as shown in figure 3.2.

It is possible to distinguish ELISA assays from two aspects:

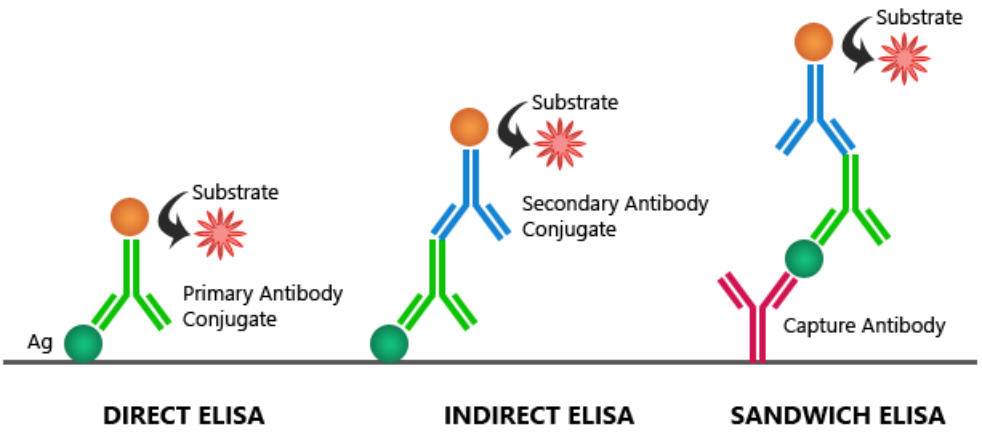

Figure 3.2: *Schematic representation of the most common ELISA assays*

1. Immobilization of the antigen of interest, which can attach to the assay plate or to the capture assay which is the one attached to the assay plate. The second type of immobilization is called Sandwich assay.

2. Where the detection enzyme is linked, that occurs on the primary antibody or secondary antibody which in turn recognizes the first antibody and it bonds to it. Therefore, it is possible to recognize a direct assay in the first case and indirect assay in the second one, as shown in figure 3.2.

The distinction between these two detection methods is very important in term of sensibility. The most common and preferred assays is the Sandwich assay since it is more stable, more reproducible and reliable. It consists of a capture antibody coated on the assay plate, which takes the antigen and it does not allow the antigen to detach from him.
Basically, the antigen is entrapped among two primary antibody, that are called capture antibody the first and detection antibody the second one.
The detection antibody on top of the antigen is in turn bonded to the second antibody.
It is important to focus mostly on the detection method because it widely influences the sensitivity of the ELISA technique and moreover based on this characterization, ELISA assay can be used in different applications.

For both direct and indirect ELISA detection there are different advantages and disadvantages:

Direct ELISA detection:

- Advantages:
    - It is faster, since less elements are involved and so, even the different steps of the procedure are in low quantity.
    - The result is influenced by only the reactivity of the primary antibody and not also the secondary antibody, therefore less variability and less percentage of failure.
- Disadvantages:
    - The signal amplification is minimal.
    - This method uses a labeled primary antibody that reacts directly with the antigen, therefore labeling antibody for each specific antigen is expensive and time consuming.

- From one experiment to another, the label antibody has to be changed so it is not possible to use the same ELISA plate given whit the kit.

Indirect ELISA detection:

- There is a much higher number of labeled secondary antibody than primary in commerce.
- Label an antibody makes its immunoreactivity lower. Therefore, since here the primary antibody is not labeled, this one maintains the maximum immunoreactivity.
- Higher sensitivity thanks to the amplification given by the bonding between primary and secondary antibodies.
- With the same primary antibody it is possible to switch the research of one antigen to another one.
- There are more elements and therefore more steps and this means more time to complete the procedure.
- Cross-reactivity can occur since the use of the secondary antibody.

A crucial step in ELISA assay success is the blocking of the remaining surface area of the ELISA plate. This step has an high impact on the sensitivity of the assay and if the blocking agent is properly chosen, it is possible to widely decrease the background signal and so to increase the signal-to-noise ratio.
The goal of the ELISA assays is to detect a specific protein and to not absorb another proteins present in the solution.
The blocking agent has the role to bind to all the sites of nonspecific interaction.
Of course, the blocking agent should not interfere and obscure the target protein detection.
Furthermore, even the washing step is crucial for the well execution of the experiment.
By washing after each step, it is possible to remove all the reagents that did not bind to the plate and of course reduce the impurities that may influence the result.
The final step of the ELISA assay requires an enzyme substrate which can emit a radioactive or fluorescent signal. The enzyme has the important role to convert the substrate in a detectable signal which can be read by proper devices.

This signal is basically proportional to the protein target concentration inside the solution.
An in depth explanation of all the steps of the ELISA assay procedure is reported in the following chapter.

3.1.2 Immunohistochemical (IHC) analysis

Immunohistochemical staining is a technique which takes place, as well as the ELISA assay, thanks to the antigen-antibody binding.
It is used to detect two or more antigens and also to discover the localization of the specific protein inside the cell, in order to obtain information about the interaction between morphology and function.
Therefore, it is an in situ study, which allows researchers to visualize by imaging the distribution and localization of a specific target protein.
It is not only used in biological study but it founds application even in diagnostic laboratories and in a lots of fields.
Therefore, it is not just an application which can be used in simple cell research, but it is possible to work with whole tissue and organ *in vitro* and even *in vivo*.
The first step is the staining of a thin tissue section.
Images of tissue can be taken by light or fluorescence microscopy.
In order to treat organ and tissue section, it is important to avoid breakdown of cellular protein and in turn the tissue degradation, otherwise the analysis will be altered by the bad condition of the sample.
Therefore, all the blood has to be removed from the tissue because it is reach of antigens which are not the wanted target antigen. After that, fixatives are used and they are usually chemical based.
When tissues and whole organs are under study, the complexity of the experiment is of course higher.
Since this project was basically focused on cell culture study, procedure of immunohistochemical study for simple cells agglomeration is going to be treated in the following chapter.

3.2 Integrated in-line sensors

"Integration of analytical detection methods with microfluidic can potentially improve the detection performance by reducing the analysis time, decreasing the consumption of liquid samples and reagents, and increasing reliability through standardization and automation."[5]
By the integration of sensors inside the platform is possible to carry out continual detection and so quantification of biomarkers secreted from the cells encapsulated in the bioreactor system.
The sensors has to be chosen among the different kind of sensors in commerce for their capability to work with high precision, efficiency, sensibility and for long time inside the system without material degradation.
Other detection approaches cannot fulfill the necessity of some kind of study in which continual monitoring is needed and moreover they are time consuming, expensive and unreliable.

The limit of detection has to be at least comparable as the one achieved by enzyme-linked immunosorbent assay (ELISA) and so it has to be $< 1ng/ml$.

3.2.1 Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy is an optimal technique in terms of limit of detection (LOD), low cost, accuracy, it fits properly in a microfluidic system, it allows continual and on line sensing and it can work with small volumes of samples and reagents.
Electrochemical impedance spectroscopy is a measurement provided in the frequency domain and it is acquired by stimulating a system with a sine wave excitation.
Therefore, the system has to be perturbed by a signal which is constant in amplitude but it changes the frequency in a range of possible values.
Since the different frequencies, what is obtained by this technique is a spectrum of impedance.
The device used to take this measurements can work as a potentiostat or as a galvanostat.
In the first case the signal applied to a system is a voltage signal, while in the second case what is regulated is the current amplitude.
Once the system has been perturbed, there is an other section of the device which has the role to obtain and analyze the response of the system in terms of phase and magnitude.

This measurement explains the electrochemical reaction which occurs at the interface of the electrode with the electrolyte.
It is possible to describe what happen on the surface which makes in contact the electrode with the solution by an equivalent electric circuit.
In order to measure the concentration of a protein inside a solution, which is the reason why this technique has been selected for this project, both electrode and electrolyte form the system of interest and it is possible to think this system as an electrochemical cell.
When a perturbation is applied, it involves all the elements inside the system and so the electrode and specifically counter electrode and working electrode and the solution.
At this point, if a voltage perturbation is applied, the system responds with a current variation at the same frequency of the voltage perturbation but with a phase which shifts the current signal from the voltage perturbation.
Therefore, the measurement of interest is the ratio between these two physical quantities:

$$R = \frac{V(t)}{I(t)} = \frac{V_0 sin(2\pi ft)}{I_0 sin(2\pi ft + \phi)} \tag{3.1}$$

V_0 and I_0 are the maximum value of the amplitude.

While in DC, the physical quantity which describes the opposition of the current inside a conductor is the resistance, in AC there is another physical quantity used to describe the same process that is the impedance Z.
The main difference is that the resistance is described by only the magnitude, while the impedance by magnitude and phase since it is a complex number.
The expression of the resistance in the real domain is not so useful, so it is better to work in the complex world. By using the Euler's theorem:

$$V = Re\{V_0 e^{(jwt)}\} \tag{3.2}$$

$$I = Re\{I_0 e^{(j\phi)} e^{(jwt)}\} \tag{3.3}$$

Therefore, finally here the impedance in the complex domain, which represents both resistance and capacitance of all the elements inside the system object of study:

$$Z = \frac{\bar{V}}{\bar{I}} = \frac{V_0}{I_0} e^{-(j\phi)} \tag{3.4}$$

Moreover, it is possible to obtain magnitude and phase by the following equation:

$$|Z| = \sqrt{(Z_r)^2 + (Z_j)^2} \tag{3.5}$$

$$\phi = tan^{-1}\left(\frac{Z_j}{Z_r}\right) \tag{3.6}$$

From the above equation, Nyquist plot or the Bode plot may be obtained, as shown in figure 3.3.

In the first one on the horizontal axis there is the real part of the impedance and on the vertical one there is the imaginary part of the impedance.

In this plot, each point of the curve represents an impedance value at one frequency.

For the bode plot instead, the representation is done by two plots: one is made by $log\,|Z|$ in function of log*f* and the other one is made by ϕ in function of log*f*.

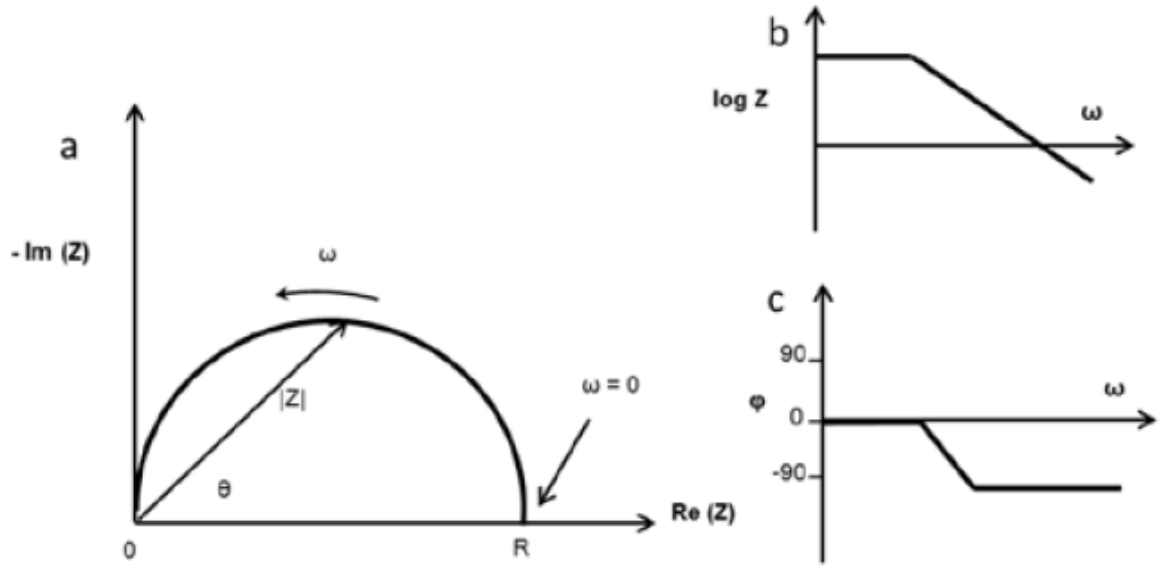

Figure 3.3: *EIS data in (a) Nyquist plot, (b)and (c) Bode plot. [36]*

By observing the Nyquist plot, the semicircular region toward the left side shows the coupling between double-layer capacitance and electrode kinetic effects at frequencies faster than the physical process of diffusion.

The diagonal “diffusive tail” on the right comes from diffusion effects observed at lower frequencies.

"The circuit used to represent the system depends on the kind of system investigated but usually a simplification of the circuit is made of: a resistance describing the solution resistance afforded by the ion concentration and the cell geometry R_S; charge transfer resistance R_ct it refers to current flow produced by redox reactions at the interface; the capacitance stored in the double layer C_dl and Warburg impedance W results from the impedance of the current due to diffusion from the bulk solution to the interface."[23]

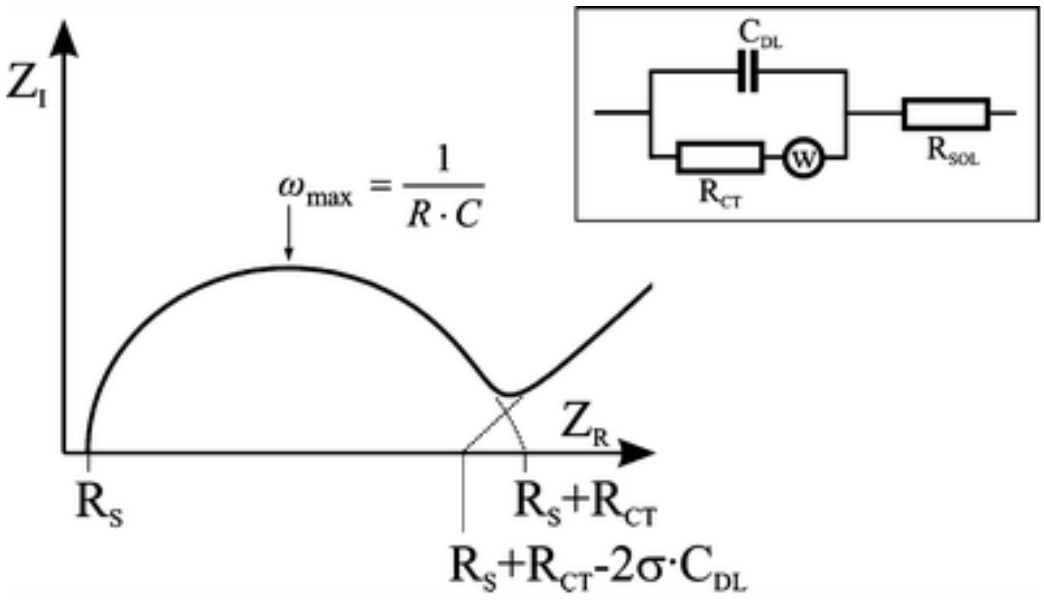

Figure 3.4: *"Randles equivalent circuit for an electrode in contact with an electrolyte and Nyquist plot". [23].*

The impedance values of each elements of the Randles equivalent circuit are:

$$Z = R \tag{3.7}$$

$$Z_C = \frac{1}{jwC} \tag{3.8}$$

$$Z_W = \frac{\sigma}{\sqrt{w}}(1 - j) \tag{3.9}$$

In which σ can be calculated by the equation:

$$\sigma = \frac{RT}{n^2F^2\sqrt{2}}\left(\frac{1}{\sqrt{D_0}c_0} + \frac{1}{\sqrt{D_R}c_R}\right) \tag{3.10}$$

To make easier the expression of the impedance of the equivalent circuit, the Warburg element can be neglected and only the element in parallel are considered:

$$Z(w) = R_s + \frac{R_{ct}}{1 + jwR_{ct}C_d} = Rs + \frac{R_{ct}}{1 + w^2R_{ct}^2C_d^2} - \frac{jw{R_{ct}}^2C_d}{1 + w^2R_{ct}^2C_d^2} = Z' + jZ'' \tag{3.11}$$

From the figure 3.4 of the Nyquist plot it is possible to measure the value of R_S and R_{ct} and C_{dl} from the w_{max} which is the time constant of the electrochemical reaction.
The intercept with the x-axis is $R_S + R_{ct} - 2\sigma C_{dl}$.

"The capacitance of the electrochemical double layer, C_{dl}, depends on all of the compounds present at the interface, which are predominantly solvent molecules, immobilised biomolecules, and optional films which promote the immobilisation and detection processes." [23]
Moreover, even R_{ct} gives a contribute in the measurement of the impedimetric detection of the analyte. This contribute is given by a redox reaction.

The purpose of this project was to detect a specific protein inside a media, therefore the EIS technique was chosen precisely in order to accomplish this objective.
The surface of the electrode has to be functional for the antigen which has to be detected and therefore it has to attach to the surface of the electrode and a change of the impedance value measured by the EIS technique will give the information needed about the concentration of the protein in a specific solution.
As in ELISA technique, the detection of an antigen is possible thanks to the bonding between antigen and antibody.
Of course the antibody cannot be coated on the surface of the electrode so a functionalization of the surface is required in order to provide an attachment of the antibody to the gold working electrode.

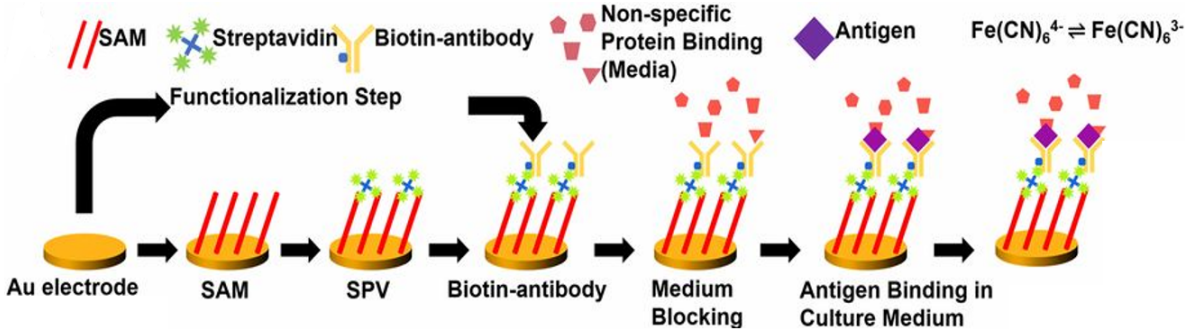

Figure 3.5: *Funzionalization process [46].*

The first step is to coat the surface of the electrode with a selfassembled monolayer (SAM) and this is done by $11-$mercaptoundecanoicacid ($11-MUA$).

"To make the alignment of the antibodies, Streptavidin (SPV) has to attach to the SAM.

Then by using N-ethyl$-N'-$(3-dimethylaminopropyl) carbodiimide (EDC) / N-hydroxysuccinimide (NHS) conjugation, a covalent bond can be formed between the surface and the self-assembled monolayer where the carboxylterminated alkyl surface was converted to an active NHS ester by reacting with $11-MUA$."[37]

Since the antibody is biotinylated, the biotin and streptavidin interact together and form a strong bonding.

The change in impedance is done by the change of electron transfer kinetics at the interface due to the redox reaction of $[Fe(CN)_6]^{4-/3-}$.

"With the attachment of more antigens, the measured the electron-transfer resistance (Rct) value of the electrode was found to increase due to shielding of the redox probe $[Fe(CN)_6]^{4-/3-}$ by antigens." [37]

Chapter 4

Materials and methods

In this project it has been fabricated a three-dimentional dynamic cyclic compression bioreactor in which hMSCs are cultured in 3D gelatin-methacrylate hydrogels to evaluate the influence of mechanical stimuli and biophysical factors on MSC differentiation and secretion, using eletrochemical biosensing integrated to bioreactor and other techniques as immunostaing and viability study.

As a specific biomarker for in vitro study, a represetative molecules, promoting angiogenesis, VEGF has been selected, since it has been found to be elevated during osteogenesis where mechanical stimuli and physical factor play an important role in the cell fate decision.
Mechanical stimuli were been applied to cell-laden hydrogels with tunable stiffness to promote secretion of proangiogenic factors from hMSCs.
Yes-associated protein (YAP) was also employed as mechanotransduction in regulating stem cell fate decisions to understand the mechanism behind how proangiogenic signaling of hMSCs can be regulated by these factors.

4.1 Human mesenchymal stem cell (hMSC) isolation and culture

Whole bone marrow was harvested from the iliac crest of healthy patients at the Case comprehensive Cancer Center Hematopoietic Biorepository and Cellular Therapy Core with approval from University Hospitals of Cleveland Institutional Review Board.

hMSCs were isolated from the marrow via a Percoll (Sigma) gradient and the differential cell adhesion method.
Only the adherent hMSCs were collected and cultured in Dulbecco's Modified Eagle's Medium - low glucose (DMEM-LG, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% Penicillin Streptomycin (PS, Invitrogen, Carlsbad, CA, USA) and 10 ng/ml recombinant human fibroblast growth factor-2 (rh-FGF-2; R&D Systems).
To osteogenically differentiate hMSCs, cells were cultured in DMEM-high glucose (DMEM-HG, Sigma) supplemented with 10% FBS, 1% PS, $10mM$ β-glycerophosphate (CalBiochem, Billerica, MA), 50 mM ascorbic acid (Wako USA, Richmond,VA), and 100 nM dexamethasone (MP Biomedicals, Solon, OH). Passage 3 hMSCs were used in the experiments.

4.2 Gelatin methacryloyl (GelMA) hydrogel

"In the last decade, research on cell-biomaterial interface has shifted to the third dimension."[19]
Cells cultured in a 3D matrix of gelatin-methacryloyl hydrogels can recreate a 3D biofrabricated tissue for bulk biofabrication.
"The interest on this structure and material is mostly related to the inherent bioactivity of gelatin and the physicochemical tailorability of photo-crosslinkable hydrogels"[19].
There are several advantages by using a 3D cultures than 2D. First of all, a three dimensional structure can recreate a shape that it is more representative of biological tissues and organs, therefore both physiology, mechanical integrity and anatomic characteristics may be recreated.
One of the main goal of using hydrogel is the chance to obtain a biofrabricating 3D constructs that can be used to repair and regenerate a tissue defect in vivo or even make artificial organs.
Therefore, GelMA is a versatile matrix that can be found in fields as tissue engineering, biomedical engineering, regenerative medicine or less specificly cell research like drug and gene delivery, cell signaling, sensing... Although, research is still trying to find new and better biomaterials, since characteristics as bio-functionality and mechanical tunability are not yet completely sufficient.

"GelMa hydrogels closely resemble some essential properties of native extracellular matrix (ECM) due to the precense of cell-attaching and matrix metalloproteinase, which allow cells to proliferate and spread in GelMA- based scaffolds." [45]

"Pure gelatin is water soluble, and it forms thermoreversible transparent hydrogels via physical interactions between collagen molecules."[24]

It is for this characteristic of traspacency that hydrogel is usefull in application like in vitro study, because it makes easier for reaserchers to observe cells incapsulated inside the matrix. For example, study of viability and observation of cells behavoir by microscope is much easier if the material in which cells spread and grow is transparent and clear.

Cells can be encapsulated in the hydrogel by adding them in the solution, trying to mantain the temperature of the solution at more or less $37°C$ to not cause thermic shock of the cells. When the mixture GelMA plus cells is made, a UV light source is used to crosslink the gelatin and make the mixture gel plus cells a cell-laden 3D hydrogel.

It has been demostrated in several study that the vibility of the cells when incapsulated in the GelMA hydrogel is quite succesfull, even if the crosslink is not completly biocompatible and moreover UV light exposure is not excellent to mantain the cells alive.

"GelMA hydrogel is biocompatible, it has a lower immunoresponse, better solubility and less antigienicity."[45]

A gelatin solution has the advantage to become solid crosslinked matrix when its temperature goes under a certain value.

Not only the temperature makes the solution acquires different mechanical properties, but also some chemical reactions that allow covalent bond between the particles.

"Introduction of methacryloyl substituent groups confers to gelatin the property of photocrosslinking with the assistance of a photoinitiator and exposure to light, due to the photopolymerization of the methacryloyl substituents." [41]

Therefore, mechanical and bio-compatible properties of GelMA hydrogel depend on the steps procedure and the concentration of each constituent, like the methacryloyl substitute, photo initiator concentrations and the photocrosslinking time.

4.2.1 Syntesis of GelMA

There are several protocols to synthesize GelMA, but due to the specifics of this project one procedure, with particular materials, has been selected in order to obtain the right physical and mechanical properties of the hydrogel.

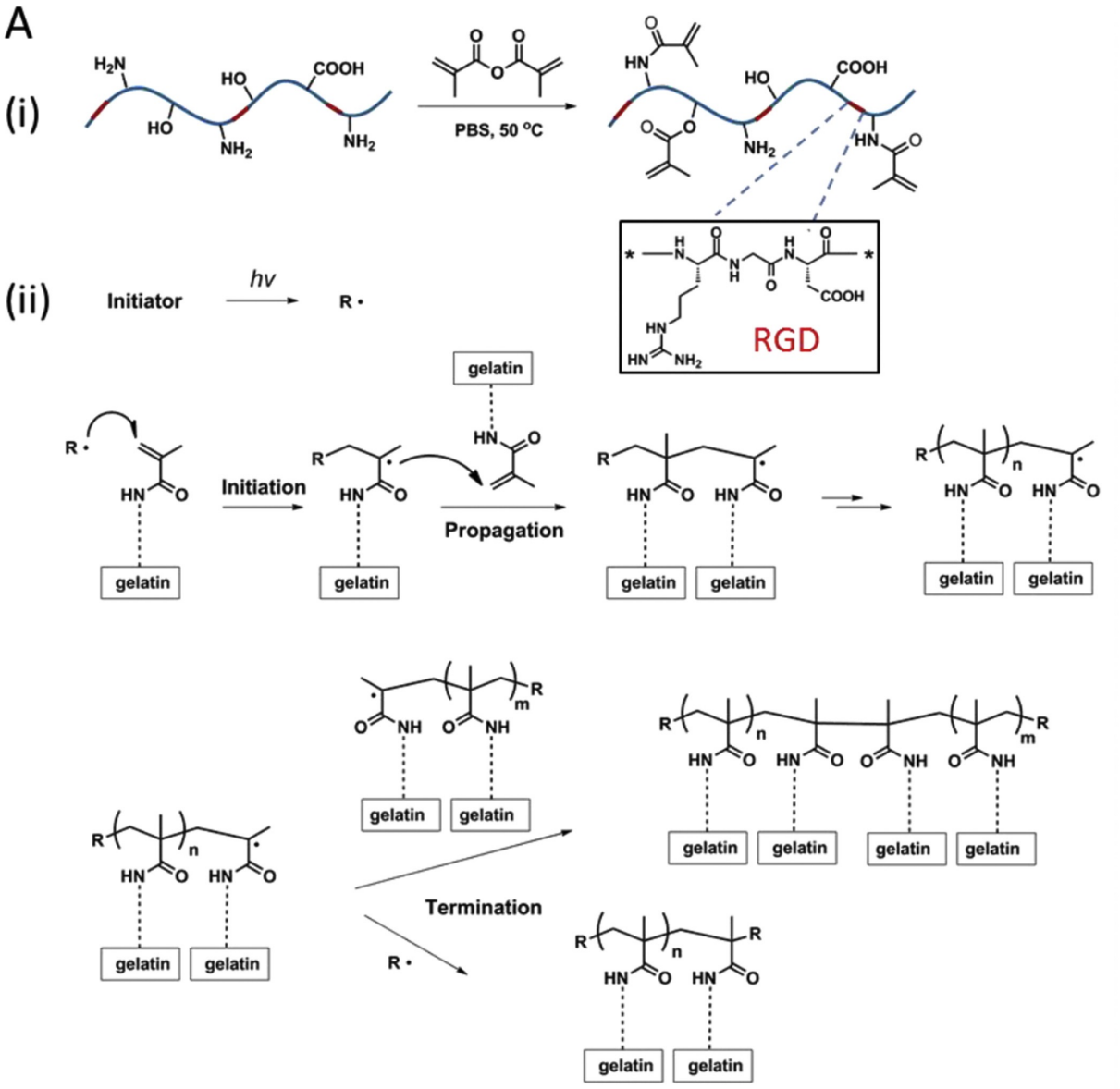

Figure 4.1: *Synthesis of GelMA hydrogel. (A) Scheme for preparation of photocrosslnked GelMA hydrogel. "(i)Reaction of gelatin and methacrylic anhydride for grafting of methacryloyl substitution groups. (ii) Representative reactions during the photocrosslinking of GelMA to form hydrogel networks."[45]*

The procedure requires 1-2 weeks to accomplish a well made synthesized polymer and to ensure stability and reproducibility.

"Gelatin from porcine skin (Sigma-Aldrich) was dissolved at 10% (w/v) in 100 mL Dulbecco's phosphate-buffered saline (PBS) at $50°C$ for 1 hour. Next, 8 mL methacrylic anhydride (Sigma-Aldrich) was slowly added to the gelatin solution and stirred at $50°C$ for 2 hours before an additional 100 mL of PBS was added and mixed at $50°C$ in order to stop the reaction.

The obtained solution was dialyzed at $40°C$ for 7 days using dialysis membranes in order to remove the low-molecular-weight impurities that may has a too high toxicity for the cells.(Mw cut off: 12–14 kDa, Fisher Scientific).

The purified solution was filtered using a vacuum filtration cup with 0.22μm pores (Millipore). This solution was frozen to $-80°C$ before freeze drying for 5 days." [13]

4.2.2 Cell-laden GelMA prepolymer solution

"Prepolymer solution of 10% GelMA for cell encapsulation were prepared with 0.5% PI (2- hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1- propanone 98%, (Sigma-Aldrich) in PBS." [13]

A low percentage of photoinitiator was chosen since the aim was to encapsulate cells in the hydrogel and since the photoinitiator is toxic, it affects the cells growth indeed.

hMSCs were trypsinized from the flask in order to detach the cells from the surface flask and make them available for the encapsulation in GelMA hydrogel.

The isolate hMSCs were then mixed with prepolymer solution at a cell density of $10^6 cells/mL$.

Therefore, 2 ml of PBS were mixed to 200 mg of GelMA to make 10% of stiffness and finally 10 mg of PI were added.

4.2.3 Mechanical properties of Hydrogel

"Mechanical properties of the hydrogels were calculated using Instron 5524 mechanical analyzer (Instron, Canton, MA) compressive tests with a 10 N load cell." [13]

The 3 concentrations under study were 5, 10 and 15 % w/v.

Therefore, 1 ml of each concentration was taken and placed on a glass plate.

The measurements has to be done in the same condition for the three concentrations so they may be comparable. It means for example, that all the three sample have to present the same thickness and dimensions.

Therefore, once the gel was placed on a glass plate, a spacer was used to make the right distance between the glass plate on the bottom and another glass plate placed on top of the gel and so on top even the spacer. In this way, the thickness of each GelMA was regulated. The following step was the crosslinking part by using 25 mW/cm^2 UV light for 30 seconds. Then, the solid gel was punched with a puncher of a precise and fixed dimension.

"Compressive modulus was calculated between 10 and 20% loading of 600μm thick samples with a 5 mm diameter with 6 replicates for each sample." [13]

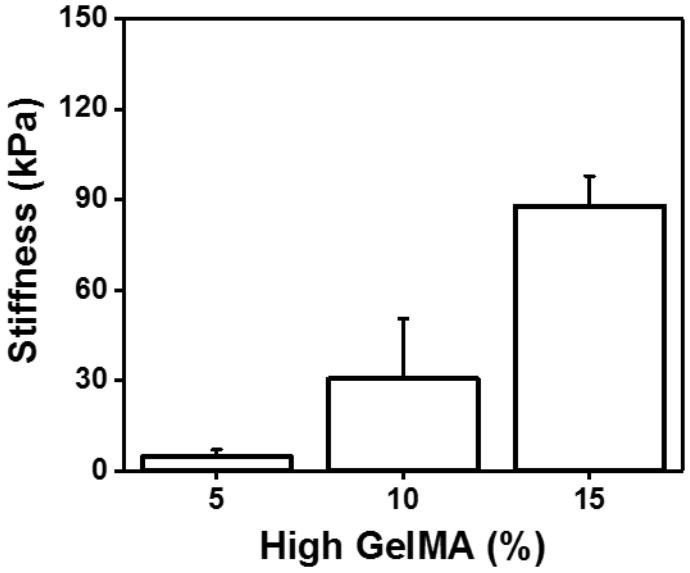

Figure 4.2: *Mechanical characterization*

4.3 Fabrication of Bioreactor

The bioreactor was composed of three different layers made of Polymethylsiloxane (PDMS) as shown in Figure4.3: nitrogen gas (N_2) pressure chamber, posts integrated on a thin membrane and media chamber.

In order to create three layers, Polymethyl methacrylate (PMMA) (McMaster Carr) sheets were cutted by Laser cutter (VersaLASER VLS2.30) and used as molds.

Previously, the geometry and design of the three molds were made by CorelDRAW and AutoCAD software.

The goal was to make more than one bioreactor always with characteristics as same as possible among each other.

To settle the same dimension and geometry it was important to find the best set of PMMA thickness, power and speed of the Laser cutter, which is the machine used to cut PMMA sheets.

Table 4.1 presents all the settings chosen to make the bioreactor a reliable system.

In the table are reported even the information to make frames of the three layer. The role and the importance of the frame is going to be explained later. PMMA molds were covered

Layers	thickness(mm)	Power(%)	Speed(%)
N_2 chamber	1.5	30	3
Frame	3	30	3
Membrane plus pistons	1	20	3
Frame	0.5	30	5
Media chamber	3	80	3
Frame	3	30	3

Table 4.1: PMMA thickness and the laser cutter setting (percentage of the maximum values of power and speed) for each layer in order to obtain a reliable procedure

with a liquid PDMS in a mixture of 1:10 base polymer: curing agent (Sylgard 184 Silicone Elastomer Kit, Dow Corning) and cured in the oven at $80°C$ for 1 hour.

Before the curing step, it was needed to remove the bubbles by using vacuum chamber.

The N_2 pressure chamber layer was composed of cylindrical chambers of different diameters (3, 5, 8 mm) in order to have respectably for each surface a proportional pressure.

Moreover, each chamber was connected to the others by channels to apply N_2 pressure through two inlets.

Due to the thin membrane (500μm) and the material flexibility, the integrated posts (1mm) were deflected upward by the dynamic pressure coming from the underlying N_2 chambers.

Four media chambers were designed for each of the three compressions and one more for the control 0 kPa compression.

The three layers were finally bonded together after being treated by Oxygen plasma (PlasmaEtch Inc.) for 60 seconds.

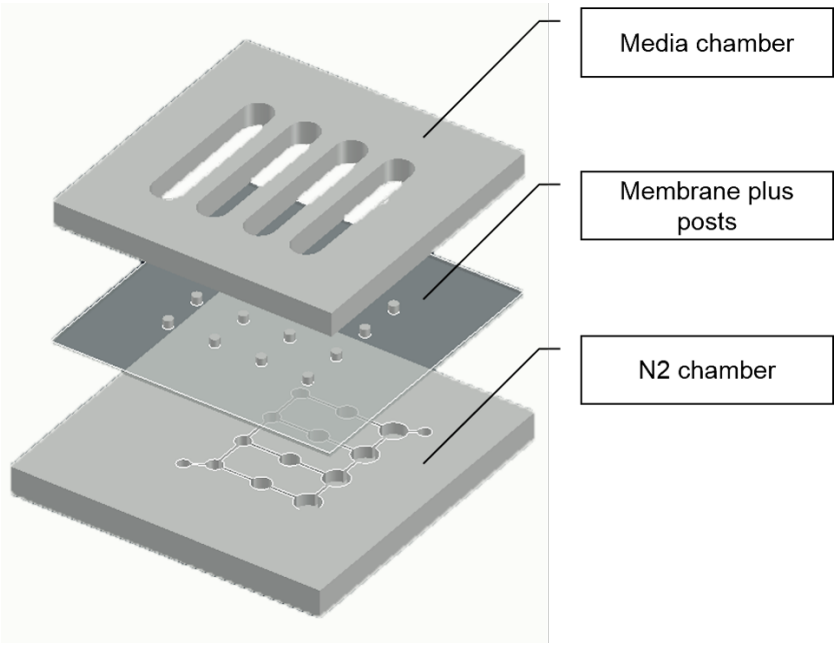

Figure 4.3: *Bioreactor design*

One important step to make a proper bonding is the cleaning part before the Oxygen treating. The two surfaces that needed to be bonded together were properly cleaned by using tape and ethanol to remove all the impurities between the layers, which otherwise would have made the bonding impossible.

Moreover, once each PDMS layer was removed from the plastic plate in which it was cured in, it was needed to cut the edges due to the property of liquid-gas to form a contact angle when they interact with a solid boundary. Once PDMS cures, it maintain the geometry of the volume that was filling, therefore it presented a concavity which resulted in a failing bonding when Plasma treating took place.

After several tries in which the sealing between the different part of the bioreactor was not enough strong, an important improvement in the realization of the 3 layers of PDMS was found to solve the main problem. Indeed, a frame of PMMA was designed and used in order to sign the level of PDMS for each layer.

At the beginning simple petri dishes were used and PMMA molds were attached to the sur-

face of the petri dish by glue. In this way, the distribution of the PDMS once spread on the mold was not accurate and homogeneous and moreover a contact angle between the PDMS and the plastic walls of the petri dish makes difficult the plasma bonding. Therefore, the use of the frame was providential in terms of material distribution and if the PDMS layer overcomes as needed the frame no problem of contact angle occurred.
The devise was finally autoclaved for sterilization.

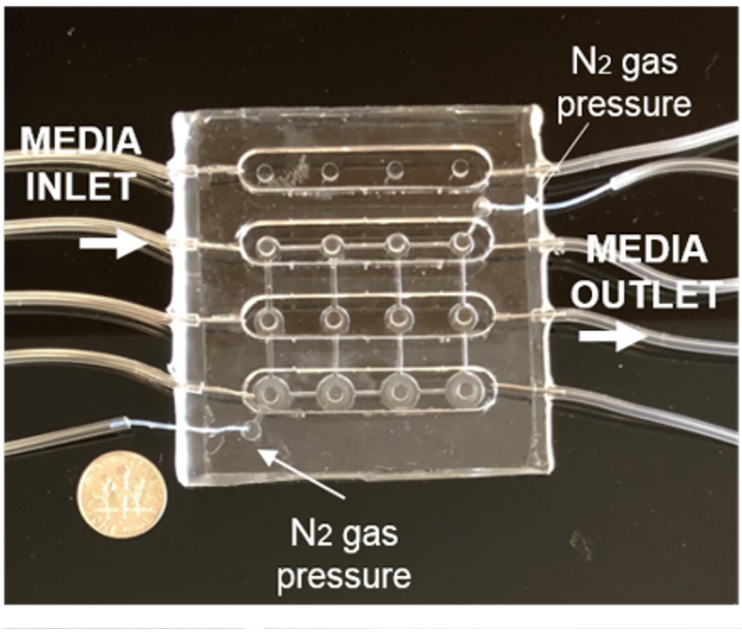

Figure 4.4: *Photograph of bioreactor with the integration of PTFE connectors for media and N_2 gas inlets and outlets. USA 10 cents coin used as a reference*

4.3.1 Calibration

Modulation of the applied compressive strains on the cell-laden hydrogel arrays was possible by regulating N_2 chambers diameter and the gas pressure of the tank connected to the Wago system (Valve Controller).
Of course, others factors influenced the corresponding compressive strain of each chamber, like the thickness of the PDMS membrane, the distance between posts and glass.
However a small error in the calibration could have been accepted since it does not influenced the final results.

The mechanism was basically a vertical displacement of the posts with on top of them there were cell-laden hydrogels.
Based on the equation:

$$P = \frac{F}{S} \tag{4.1}$$

Since the pressure remains constant because it has been set from the tank regulator, by inverting the formula to get the force it is easily understandable that with an higher chamber diameter and so an higher surface, the force transferred from the post to the hydrogel and finally to the glass would be proportionally bigger than the referential pressure $0Pa$.
The reason why the glass was coated by TMAPMA treatment is due to the prevention of lateral expansion of the hydrogel and following local deformation in the vertical direction.

In order to make the association between displacement of the post and the compressive strain, a bioreactor was built specifically to make measurement of displacements.
The procedure was not precise and it is based basically in taking pictures of the bioreactor in section when a pression is applied inside the N_2 chamber.
The posts are the objects of interest because it is from the vertical movement of the posts that it is possible to find out the relation between displacement and compressive strain.
To make easier the procedure of taking images of the posts, the bioreactor has to present only the two layers of N_2 chamber and membrane integrated with posts.

The vertical displacement of the posts was later measured by using the simple software Imagej which allows to make good measurements even of objects in the micro dimension.
One important adjustment is that when the pictures were taken, the operator had to be precise and avoid relative movements.
The camera was blocked and fixed in a stable position during all the experiment while the pressure of the tank had been regulated from 8 psi to 16 psi every 2 psi.

Therefore, because of the relative movement of the operator and also for the pressure which makes the reference move, first of all a point of the biorector was fixed and taken in consideration in all the measurements,

	h (inch)	Referement	Δh	Compressive strain (%)
16psi, D=3mm	3.75	3.17	0.58	18.29
16psi, D=5mm	3.3	2.4	0.9	37.5
16psi, D=8mm	3.33	1.6	1.73	108.12
14psi, D=3mm	2.67	2.33	0.34	14.59
14psi, D=5mm	1.75	1.25	0.5	32
14psi, D=8mm	3.08	1.75	1.33	76
12psi, D=3mm	1.58	1.41	0.17	12.05
12psi, D=5mm	2.08	1.67	0.41	24.55
12psi, D=8mm	2	1.25	0.75	60
10psi, D=3mm	1.6	1.5	0.1	6.66
10psi, D=5mm	1.75	1.5	0.25	16.66
10psi, D=8mm	2.58	1.75	0.83	47.42
8psi, D=3mm	1.45	1.36	0.09	6.62
8psi, D=5mm	1.5	1.33	0.17	12.78
8psi, D=8 mm	2.33	1.75	0.58	33.14

Figure 4.5: *Table of the data obtained by the measurement of the posts displacement and calculation of the compressive strain.*

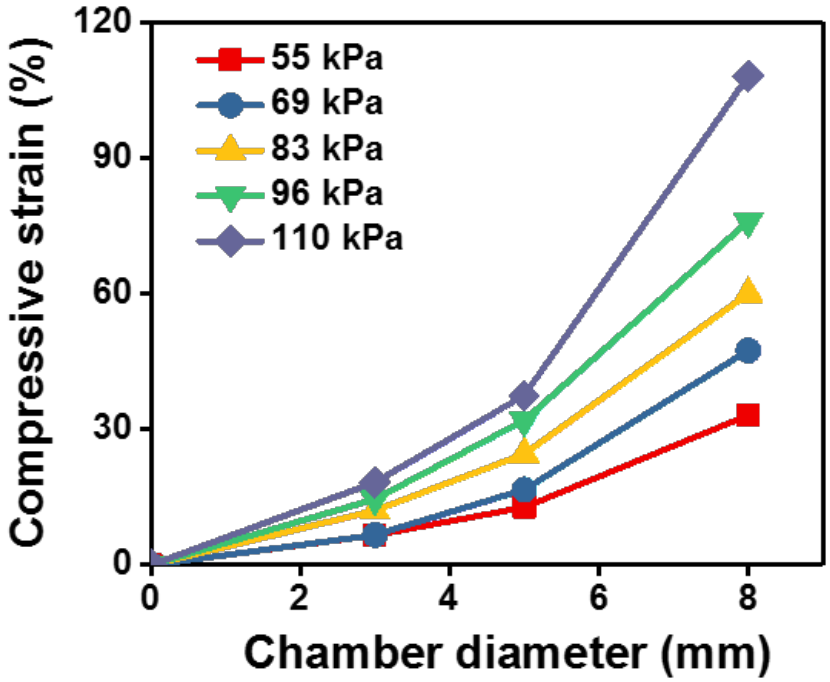

Figure 4.6: *Relationship between compressive strain (%) and N_2 chamber diameter when a variable gas pressure is applied.*

This point is static on the bioreactor but actually it moves inside the plane together with the bioreactor.
Once obtained the displacement of the reference point, the posts displacement was measured by the difference between the reference point and the top surface of the post which moved when the pressure is applied inside the N_2 chamber.
Two measurements were indeed taken for each combination of pressure set by the gas regulator and the chamber diameter of the bioreactor.
Finally the difference between the reference point and the top surface point of the post was taken for each combination and so the compressive strain was obtained:

$$\epsilon = \frac{decrease\,in\,length}{original\,length} \tag{4.2}$$

The data of the compressive strain calculated by the equation 4.2 are shown in figure 4.5 and 4.6.

4.3.2 Assembly and spotting materials

There were different approaches to achieve the goal of assembling the cell-laden hydrogels inside the bioreactor.
First of all, one approach was performed but it was found out that it costed material and an high amount of cells which are the most expansive element in terms of cost and time consuming since the operator takes a lot of time to culture them.
Therefore, the cell suspended GelMA prepolymer solutions were injected inside the bioreactor in a fluid state.
Once all the chambers were filled with this solution, a mask with specific and proper dimension and shape was placed on top of the bioreactor and a UV light source was used to crosslink and create the small cylindrical cell-laden hydrogels on top of the posts of the bioreactor.
The mask was made of a dark material like a black PMMA sheet and the geometry was designed by CorelDRAW software and than the sheet was cutted by Laser Cutter.
After the crosslinking step, to not waste material and cells, the liquid solution which was not subjected to UV light was pulled out and used in the following chamber.

Even though some material was saved by the pulling out procedure, still there were different problems to solve. For example, not few problems occurred when it was needed to align the mask in correspondence to the post of the bioreactor since it was done manually.
Consequently the crossinked hydrogels were not in the right position and even if it might be considered a small mistake, the experiment in this condition could not be completed.

In order to avoid the operator influence on the success of the experiment as much as possible, another approach was contrived.
Thanks to the TMSPMA glass treatment and the innate characteristic of the PDMS to be hydrophobic, it was possible to only drop a proper volume of solution on top of the posts and later just put the treated glass on top of the hydrogels.
After several attempts, the required volume of solution for each drop was found out to be 25 μl and this quantity was perfect in terms of adherence to the glass plate placed on top of the biorector and even for the size of the cell-laden hydrogels.

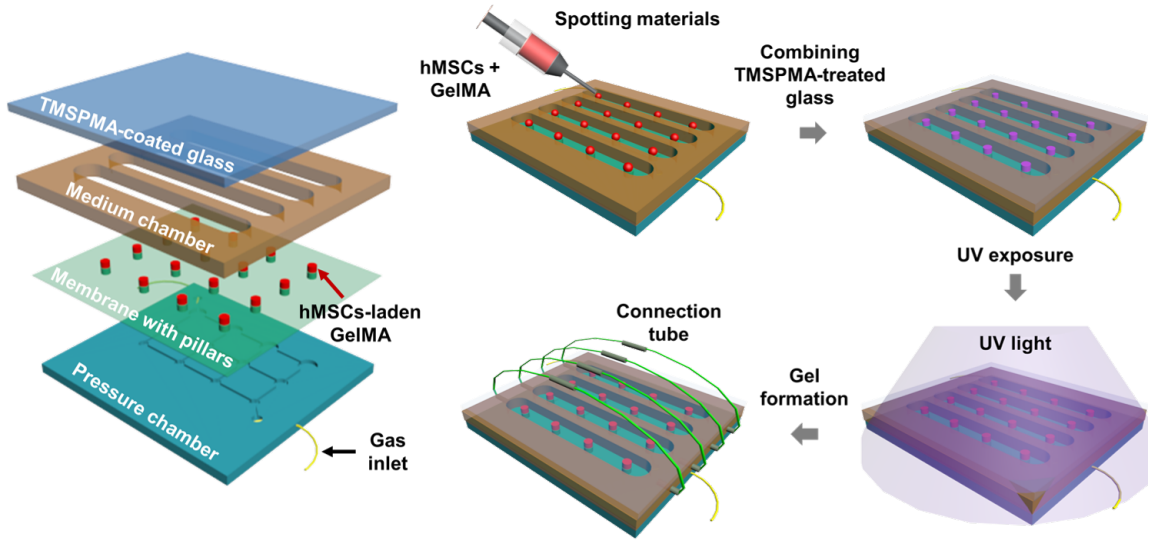

Figure 4.7: *Schematic procedure of the assembling biorector*

Once the hydrogels was fixed on top of the posts and under the glass plate, the easiest way to close and pressurize bioreactor, cell-laden hydrogels and glass plate was just a clumping method.
Lots of problems occurred at this time for linking problems which caused some delays.

TMSPMA glass treatment

To increase the stability of the cell laden 3D hydrogel arrays on top of the posts and avoid relative movements between GelMA arrays, glass and posts and following detachment, it was needed a glass treatment.
"3-(Trimethoxysilyl)propyl methacrylate was used to make glass substrates acrylated.
This surface tratment introduces terminal acryate funcional groups on the glass, providing anchoring sites for GelMA arrays." [29]
The procedure is the following:

1. A glass beaker was placed in a bucket of ice. The expected reaction is very exothermic, therefore it releases a lot of heat. 50 g of NaOH pellets was slowly added in 450 mL of distilled water in the beaker already introduced under the wood. In the same beaker, glass slides were added in a staggered manner but all of them were carefully distributed in a way that they were in contact with the NaOH solution.

2. The beaker was covered with a pyrex dish (Caution: do not use aluminum foil to cover as NaOH reacts with aluminum producing gaseous side products)

3. The beaker with inside glass slides and NaOH solution was left sit overnight in the fume hood.

4. 10% NaOH solution was discarded into the proper waste bottle. Each slide was thoroughly rinsed and rubbed one by one under distilled water.

5. Each slide was dipped in 3 distinct 100% reagent alcohol baths and let it air dry, it was faster under the hood.

6. The glass slides were wrapped with aluminum foil and bake for 1h at $80°C$. TMSPMA treatment (under the fume hood):

    a. Slides were stacked vertically and poor 3 mL of TMSPMA on top of the stack using a syringe.

    b. After 30 min, the stack was flipped upside down to get even coating.

    c. The beaker was covered with aluminum foil and bake the whole assembly in the beaker overnight at $80°C$.

7. The slides were cleaned in reagent alcohol again with 3 baths and air dry.

8. The glass slides were wrapped with aluminum foil and bake again for 1-2 hours at $80°C$.

9. TMSPMA coated glass slides were stored and wrapped in aluminum foil at room temperature.

4.3.3 Dynamic compression

Cell laden 3D hydrogel arrays were patterned between the posts and a 3-(trimethoxysilyl) propyl methacrilate (TMSPMA) treated glass substrate by photocrosslinked UV light.
UV crosslinking was carried out at $14mW/cm^2$ for 60s.
Media is then injected and the inlets and outlets were sealed.
Gas pressure was applied to the bioreactor and cyclic pressure was programmed by using multiple solenoid valves controlled by a MATLAB software tool and a WAGO controller.
The frequency set of the cyclic pressure was around 0.3 HZ.
The media was collected on Day 1, 3, 5 and 7 for Elisa analysis and fresh media was injected each time.
At this stage of the project, the whole device was not completely built, therefore the media was manually injected and extracted every 2 days. It was found that the amount of media in each chamber was around $100\mu L/min$ using a simple syringe.
Once the device will be finished, a peristaltic pump will be integrated and will take care automatically of the storing and changing media.

4.4 Immunostaining

All cell encapsulated hydrogels were washed with DPBS, later fixation was performes in 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 25 min.
Samples were stored overnight at $4°C$ in PBS
Samples were then incubated in 0.1% Triton x-100 (Sigma-Aldrich) at room temperature for 30 minutes to make cells permeable. Blocking was carried out in 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS at room temperature for 20 minutes.

The primary antibody for MSCs was diluted at 1:200 in PBS with 1% BSA and samples were incubated overnight at $4°C$ in a humidified chamber.
The samples were then washed with PBS. The sample was then treated with 2% Goat serum in 1% BSA and PBS for 10 minutes.
The secondary antibodies were added at a dilution of 1:200 in PBS with 1% BSA and incubated for 20 minutes at $37°C$. The stained cells were imaged using an inverted fluorescence microscope and a confocal microscope.

4.5 ELISA Assays

Sandwich ELISA kits are in vitro enzyme-linked immunosorbent assays for the quantitative measurements of soluble proteins in a variety of biological sample, sach as cell culture supernatant in this specific project .

4.5.1 Components

1. Antibody-coated ELISA Plate: 96 wells coated with specific capture antibody.
2. 20x Wash Buffer: 25 ml of 20x concentrated solution.
3. Target Protein Standard.
4. Assay/Sample Diluent Buffer/s.
5. Biotinylated Detection Antibody.
6. HRP-Streptavidin: 200μl of concentrated HRP-conjugated streptavidin.
7. ELISA Colorimetric TMB Reagent: 12 ml of 3, $3'$, 5, $5'$-tetramethylbenzidine.
8. ELISA Stop Solution: 8 ml of 0.2 M sulfuric acid. (TMB) in buffer solution.

4.5.2 Procedure

Human VEGF sandwich assay ELISA (Sigma-Aldrich) was carried out according to supplier's instructions, as below:

1. All the reagents and samples were brought to room temperature ($18 - 25°C$) before use.

2. Washing step: 100μL of each standard and sample were added to each well already coated with capture antibody and incubated for 2.5 hours at room temperature.
   After all the solution have been discarded, the washing step was accomplished by 1x Wash Solution. Therefore, each well was filled with Wash Buffer (300μl)using a multi-channel Pipette to make the procedure easier and faster.
   In order to get valuable results and a good performance of the ELISA kit, it was necessary to remove completely the liquid at each steps and to avoid any contact with the surface of the coated plate and the pipette to not remove or destroy the proteins attached to the surface.

   After the last wash, any remaining Wash Buffer was completely removed by aspirating and finally the plate was inverted and blotted against clean paper towels.

3. 100μL of Detection antibody was added in each well and left for 1 hours at room temperature with gentle shaking.

4. Washing procedure as step number 2.

5. 100μL of Streptavidin was added in each well and left for 45 minutes with again gentle shaking.

6. Washing procedure as step number 2.

7. 100μL of TMB One-Step Substrate Reagent was placed in the wells and incubated for 30 minutes at room temperature with gentle shaking in the dark since the the solution is sensitive to light.

8. Finally, 50μL stop solution.

9. The assay was measured at 450 nm using the plate reader.

4.6 Live/dead staining

Live/dead staining was performed over time (days 0, 3, 5 and 7) using a LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen). The kit is equipped with instruction given by the manifacturer.

Solution of 0.05% of Calcein and 0.2% ethidium homodimer-1 in PBS was added in each well and was incubated for 30 minutes.

Calcein and ethidium homodimer-1 are basically dye which reveal death and alive cells.

After three washes with PBS it was imaged under fluorescent microscope.

Calcein goes inside healthy and alive cells and it releases a uniform green fluorescence, therefore green is in few words the signal of live cells.

To obtain an image of only the "green" cells is just possible to set the wave length of the fluorescence microscope around $\sim$495 nm/$\sim$515 nm.

On the contrary, ethidium homodimer-1 doesn't enter inside healthy cells but only the ones which present a damaged membrane.

In this case EthD-1 releases a red florescence and so red color identify dead cells.

It is possible to obtain a picture of only the dead cells by the microscope setting of the waive length of $\sim$495 nm/$\sim$635 nm.

The images obtained from the microscope were analyzed by ImageJ software.

4.7 EIS measurements

"Integration of analytical detection methods with microfluidics can potentially improve the detection performance by reducing the analysis time, decreasing the consumption of liquid samples and reagents, and increasing reliability through standardization and automation."[5]

By the integration of sensors inside the platform is possible to carry out continual detection and so quantification of biomarkers secreted from the cells encapsulated in the bioreactor system.

Electrochemical impedance spectroscopy is an optimal technique in terms of limit of detection (LOD), low cost, accuracy, it fits properly in a microfluidic system, it allows continual and on line sensing and it can work with small volumes of samples and reagents.

4.7.1 Electrode for biosensing

In this project, disposable gold electrodes supplied by Metrohom DropSens company were used to perform EIS measurement. This commercial sensor is ideal to work with microvolumes.
About some characteristics:

- Ceramic substrate: L33 x W10 x H0.5 mm.
- Electric contacts: Silver.
- Working electrode size: 4mm diameter.

The electrochemical cell consists of:

- Working electrode: Gold
- Auxiliary electrode: Gold
- Reference electrode: Silver

EIS measurements were performed by electrochemical workstation CHI660E (CH instrument, Inc.). The surface of the sensor has to have a proper roughness since it is crucial for a well binding between the sensors and the self-assembled monolayer (SAM) coating and so it would improve the stability of the connection antigen-antibody, which in turn would make the sensitivity of the sensor increased.

4.7.2 Solution preparation

- Potassium ferricyanide (III) solution $K_3Fe(CN)_6$: 329.26 mg of $K_3Fe(CN)_6$ (Sigma-Aldrich 702587-250G) in 20ml of Deionized water.
- 11-Mercaptondecanoic acid (MUA acid) solution: 109.18 mg of 11-Mercaptoundecanoic acid (Sigma-Aldrich 450561-5G) dissolved in 50 ml of pure 99.5% Ethanol (Sigma-Aldrich 459844-500 ML). It was used for self-assembled monolayer (SAM)coating.
- H_2SO_4 solution: 10 mM of H_2SO_4, for cleaning.
- N-(3-Dimethylaminopropyl)$-N'-$ethylcarbodiimide Phosphate-buffered saline (PBS) solution without purification, for cleaning.

- N-Hydroxysuccinimide(NHS) solution: 5.75 mg of NHS in 1 ml of Citric buffer. This solution is sensible to high temperature, therefore during all the experiment the solution was taken in a ice box to maintain a proper temperature.
- Hydrochloride (EDC) solution: 9.55 mg of EDC solution in 1 ml of Citric buffer. It has the same problem of NHS regarded the temperature.
- Streptavidin solution (SPV).
- Antibody.
- Co-culture medium: Mesenchymal stem cell medium.

4.7.3 Parameters for electrochemical sensor measurements

Different combinations of the workstation parameters were performed and finally the parameters expressed in the following table were chosen for EIS measurements.

Parameter	Value	Etc.
Init E (V)	0.1	
High Frequency (Hz)	100000	
Low Frequency (Hz)	0.1	Minimum 0.05
Amplitude (V)	0.005	
Quiet times (sec)	2	
Sensitivity Scale Setting	Automatic	
Measurement Mode above 100 Hz	Single Freq.	
Avrg/Cycles	1	
Points/Decade Freq.	12	

Table 4.2: A.C. Impedance Parameters for EIS measurements

4.7.4 Protocol for EIS measurements

1. 1^{st} cleaning of bare electrodes: 60μL of H_2SO_4 was dropped on the surface of the sensor. The process was repeated 3 times and each time is strictly needed to not touch the surface of the sensor and to avoid contamination, therefore in every step tips were changed. This last advise it has to be considered even for the following points, especially after deposition and coating steps.
2. 2^{nd} cleaning of bare electrodes with 60μL of PBS solution for 3 times.
3. Drying step using N_2 blower.
4. 1^{st} measurement: 60μL of Potassium ferricyanide(III) solution before running the measurement. For every measurement step, it was needed to take at least 3 following measurements after gentle shaking, in order to find the maximum value and calculate average and value with standard deviation.
5. 1^{st} cleaning of bare electrodes as point 1.
6. 2^{nd} cleaning of bare electrodes as point 2.
7. Drying step using N_2 blower.
8. 3^{nd} cleaning of bare electrodes by 60μL of 100% Ethanol solution. Also this cleaning step was repeated 3 times.
9. Drying step using N_2 blower.
10. Self assembled monolayer (SAM) coating on the electrode surface: 60μL of 10 mM MUA acid solution was dropped on top of the electrode surface and left it for 1 hour. A glass slide was used to cover and prevent vaporization.
11. Cleaning step by 60μL of 100% ethanol after removing MUA acid solution. This step was repeated 5 times.
12. Drying step using N_2 blower.
13. EIS measurement as point 4.
14. Cleaning step with PBS as point 2.

15. EDC&NHS coating on SAM/electrodes: 60μL of mixed EDC and NHS with 1:1 ratio (e.g.700μ: 700μ).

16. Drying step using N_2 blower.

17. SPV solution: 60μL of solution for 30 minutes. This solution is needed for antibody bonding.

18. Cleaning step with PBS as point 2.

19. Drying step using N_2 blower.

20. Antibody for VEGF bonding: 60μL of solution for 30 minutes.

21. Cleaning step with PBS as point 2.

22. Drying step using N_2 blower.

23. Co-culture medium to block the reaction and remove all the regent that has not attached to the SAM: 60μL of solution for 30 minutes.

24. Cleaning step with Cleaning with ELISA buffer 3X solution after removing co-culture medium: 60μL of solution for 30 minutes. This cleaning steps was repeated 3 times.

25. Drying step using N_2 blower.

26. EIS measurement as point 4 to obtain Antibody/SPV/EDC & NHS/SAM/electrode EC measurement.

27. Cleaning step with PBS as point 2.

28. Drying step using N_2 blower.

29. Biomarker solution: 60μL of solution for 30 minutes.

30. Cleaning step with Cleaning with ELISA buffer 3X solution as point 24.

31. Drying step using N_2 blower.

32. EIS measurement as point 4 to obtain Biomarker/Antibody/SPV /EDC & NHS/SAM/electrode EC measurement.

33. Cleaning step with PBS as point 2.

34. Drying step using N_2 blower.

35. Repetition of the process from 29 to 34, using different concentration of biomarkers.

36. Repetition of all the point but instead of using Biomarker concentration from the ELISA kit, medium containing VEGF biomarker after tissue culturing for days.

Chapter 5

Results and discussion

5.1 Project set-up

The aim of the project was to build an automatic system which may evaluate hMSCs behavior in terms of proangiogenic signaling.

The final device should include multiple interconnected parts like a peristaltic pump, connectors, a medium reservoir for the fresh media, the bioreactor, microfluidic EC biosensor, a valve controller and a potentiostat.

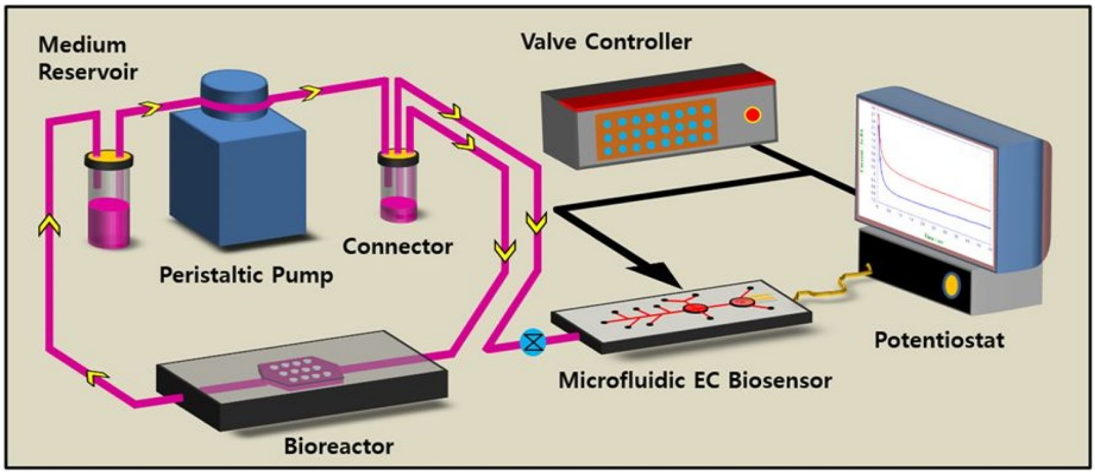

Figure 5.1: *Microfluidic system integrated with bioreactor [35]*

Each section has to work continuously for all the time needed for the success of the project. The peristaltic pump and the media reservoir will be used to continuously inject nutrients and oxygen into the bioreactor and to remove simultaneously all the waste produced by cells. Every parts is connected by tubes.

The valve controller will be connected to both bioreactor and microfluidic EC biosensor.
For what concerns the bioreactor, the valve controller has to manipulate the gas pressure to modify the dynamic compression regimes (0-42%). The microfuidic EC biosensor, as already explained in the previous chapter, is a platform which contains sensors for the EIS measurements and in order to make the sensor surface functional for the impedance spectroscopy measurements, different solutions have to be in contact with the sensor surface for a finite time and with a specific order.
The valve controller is a WAGO system, controlled by a MATLAB code.

5.2 Stiffness selection

To simplify and reduce the number of variables of the project, the stiffness selection was firstly performed in order to decide which stiffness could have been considered the best choice in terms of best viability and higher secretion of VEGF.

Circular cell-laden hydrogels of three different GelMA concentrations were made and stored in an incubator from day 1 to day 7, paying attention to change the media every 2 days and collecting the media to carry out some data from samples analysis.
Media was than collected on Day 1, 3, 5 and 7 for Elisa analysis.
Fresh media was added after each time.
On Day 7 YAP staining was performed.
Live/Dead staining was done for triplicates of each GelMA concentration on Day 1, 3 and 7.
In this way it was possible to choose the best GelMA stiffness and use that concentration of high GelMA in the bioreactor already built.

5.2.1 Viability

Solution of 0.05% of Calcein and 0.2% ethidium homodimer-1 in PBS was added in each well with samples of cell-laden GelMA and it was incubated for 30 minutes.
After three washes with PBS it was imaged under fluorescent microscope.

From the images taken by fluorescent microscope of figure 5.2, it is possible to simply recog-

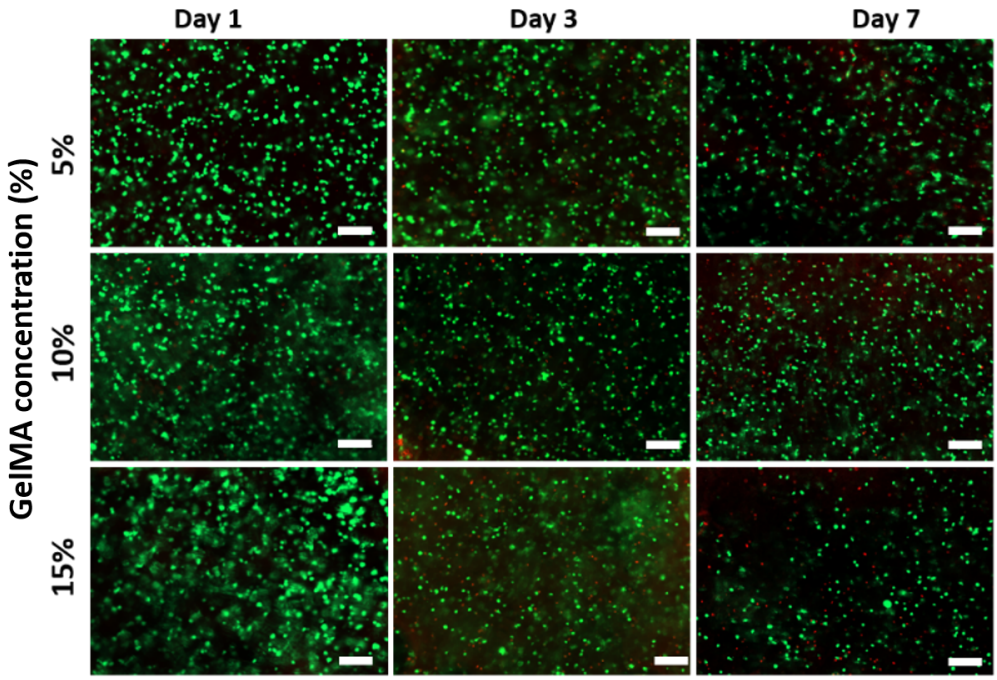

Figure 5.2: *Live/dead staining to select the best GelMA concentration (5, 10, 15%) in terms of viability*

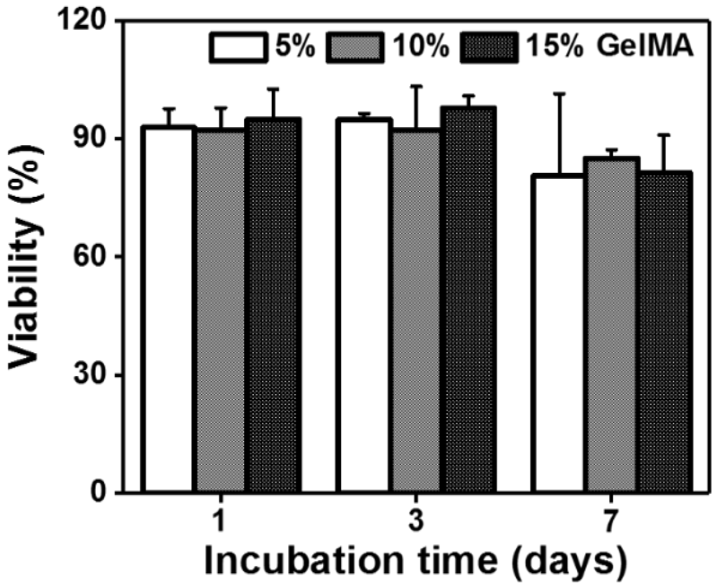

Figure 5.3: *Cell viability for each concentration of GelMA at different time points. hMSCs were mixed with prepolymer solution to have a concentration of 3 million/ml each hydrogel.*

nize that a decreasing number of alive cells occurred from day 1 to day 7 with a consequent increasing number of dead cells.
Thanks to Imagej, an open platform for scientific image analysis, it was possible to obtain a quantitative measure of the number of cells alive and dead. Therefore, figure 5.3 shows the diagram of the cell viability for each concentration of GelMA at different time points.

The goal of this preliminary study was to reduce the complexity of this project and make a selection of one stiffness between the three possible analyzed (5, 10 and 15 %).
Therefore, as it is showed in figure 5.3, the result is not so informative since there is not a GelMA percentage which shows a much better viability in comparison with the others.
The viability decreases with the incubation time but not a predominant difference was found between the different GelMA concentrations.

5.2.2 ELISA detection

"Prepolymer solutions of 5%, 10% and 15% GelMA for cell encapsulation were prepared with 0.5% PI (2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone 98%, Sigma-Aldrich) in PBS."[13] The isolated hMSCs were then mixed with prepolymer solution to have a concentration of 3 million/ml each hydrogel.
These cell-laden solutions were then crosslinked in a cylindrical mold of height 1mm. A biopsy punch of 5mm was used to make circular cell-laden hydrogel disks and were transferred to 96 well plate. 200μl of chondrogenic media was added to each triplicate sample and expansion media to the other triplicates of the three GelMA concentrations.

Following the procedure already reported in section 4.5.2, ELISA assays was performed after all the samples from day 1 to day 7 were taken.
The mean absorbance was calculated for each set of triplet standards, controls and samples and the average zero standard optical density was subtracted to all the mean absorbance.
In order to obtain the standard curve, the concentrate of VEGF biomarker, found in the ELISA kit, was opportunely diluted and eight concentrations of VEGF were obtained and distributed into the well plate.

Three measurements of the absorbance were obtained for each concentration in order to calculate the average and standard deviation.

	Concentration (ng/ml)								
	50	**6**	**2**	**0,6667**	**0,2222**	**0,074**	**0,024**	**0,00823**	**0**
Absorbance	1.957	1.046	0.586	0.231	0.161	0.104	0.076	0.09	0.064
	1.675	0.713	0.397	0.19	0.145	0.11	0.094	0.092	0.066
	1.067	0.879	0.726	0.218	0.117	0.093	0.073	0.071	0.058

Figure 5.4: *Three measurements of absorbance for each standard concentration in order to obtain the standard plot.*

The color of each cell of the table 5.4 shows the proportional absorbance and so the proportional concentration.

Three wells were reserved for 0 concentration in order to substrate the average zero standard optical density to all the average standard concentrations and therefore, the absorbance of the background of the well plate and the solution which filled the wells at the last step during the reading procedure, were removed.

Concentration(ng/ml)	Mean absorbance	Mean absorbance - average zero
50	1.566333333	1.503666667
6	0.879333333	0.816666667
2	0.569666667	0.507
0.6667	0.213	0.150333333
0.2222	0.141	0.078333333
0.074	0.102333333	0.039666667
0.024	0.081	0.018333333
0.00823	0.084333333	0.021666667
0	0.062666667	0

Table 5.1: *Subtraction of the mean value of the standard 0ng/ml to the absorbance of each concentration.*

The average of zero standards optical density was subtracted from the absorbance for each set of duplicate standards, control and samples. The plot of figure 5.5 shows the best-fit curve through the standard point.

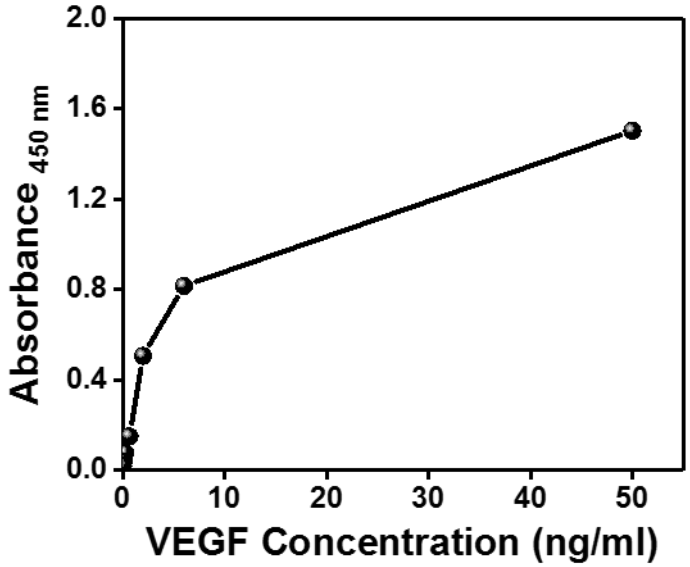

Figure 5.5: *Plot of the standard curve from Elisa analysis.*

Tables 5.6 show the absorbance value obtained for each media samples on 4 different time points.

As already explained, two different media were analyzed since normal media could have presented some traces of protein like grow factor which might have influenced the results.

Therefore, three wells of the ELISA plate were reserved for chondrogenic media and three more for Expansion media with the results of an average absorbance of 0.057 ± 0.0216 for Expansion media and 0.053 ± 0.0183 for Chondrogenic media.

It is indeed evident that there is not a substantial difference between the two media in terms of VEGF detection and none of them seem to alter the result of the ELISA study.

Thanks to this screening, it was possible to check even if one of the two media analyzed, Expansion and Chondrogenic, was a better choice in order to obtain an higher secretion of VEGF.

As shown in tables 5.6, samples treated with Chondrogenic media present lower absorbance values and so a lower concentration of VEGF secreted by the cells.

In the following step of the project Chondrogenic media was never used again since the neg-

	Day 1		
5% N	0.336	0.49	0.415
5% C	0.246	0.262	0.274
10% N	0.653	0.563	0.714
10% C	0.486	0.485	0.421
15% N	0.536	0.406	0.426
15% C	0.353	0.384	0.413

	Day 3		
5% N	0.854	0.888	0.805
5% C	0.197	0.185	0.169
10% N	0.789	0.975	0.87
10% C	0.235	0.345	0.255
15% N	0.84	0.729	0.673
15% C	0.239	0.265	0.264

	Day 5		
5% N	0.57	0.827	0.717
5% C	0.101	0.096	0.096
10% N	0.57	0.827	0.717
10% C	0.1	0.101	0.1
15% N	0.63	0.512	
15% C	0.094	0.109	

	Day 7		
5% N	0.291	0.436	
5% C	0.081	0.098	
10% N	0.458	0.508	
10% C	0.077	0.095	
15% N	0.19	0.186	
15% C	0.067	0.087	

Figure 5.6: *Absorbance value obtained for each media sample.*

ative impact which affected the VEGF secretion of the hMSCs.

The reason why the number of samples is different in day 5 in less part and in day 7, was just related to management issues based on the wells number of the ELISA kit plate.

On the top left of figure 5.7 there is the diagram obtained by the data of the ELISA essay which shows the concentration of VEGF detected from Expansion media treated cells every 2 days.

As shown, the maximum secretion of VEGF was found from day 1 to 3 and it got decreased from day 3 to 5 and again more from day 5 to 7. This can be explained by the reduction of cell alive in hydrogel.

On top right is showed the trend of the VEGF concentration from day 0 to day 7. It is evident that 10% GelMA presented the best fit, followed by 5% and the worse was 15% GelMA.

This result seems to validate even the theory which is the basis of this project: higher stiffness may be associated with higher tension between the cells.

For the following step of the project this result was taken always in consideration and therefore, for the bioreactor integration with cell-laden hydrogel, 10% high GelMA was chosen to maximize the success of the experiment.

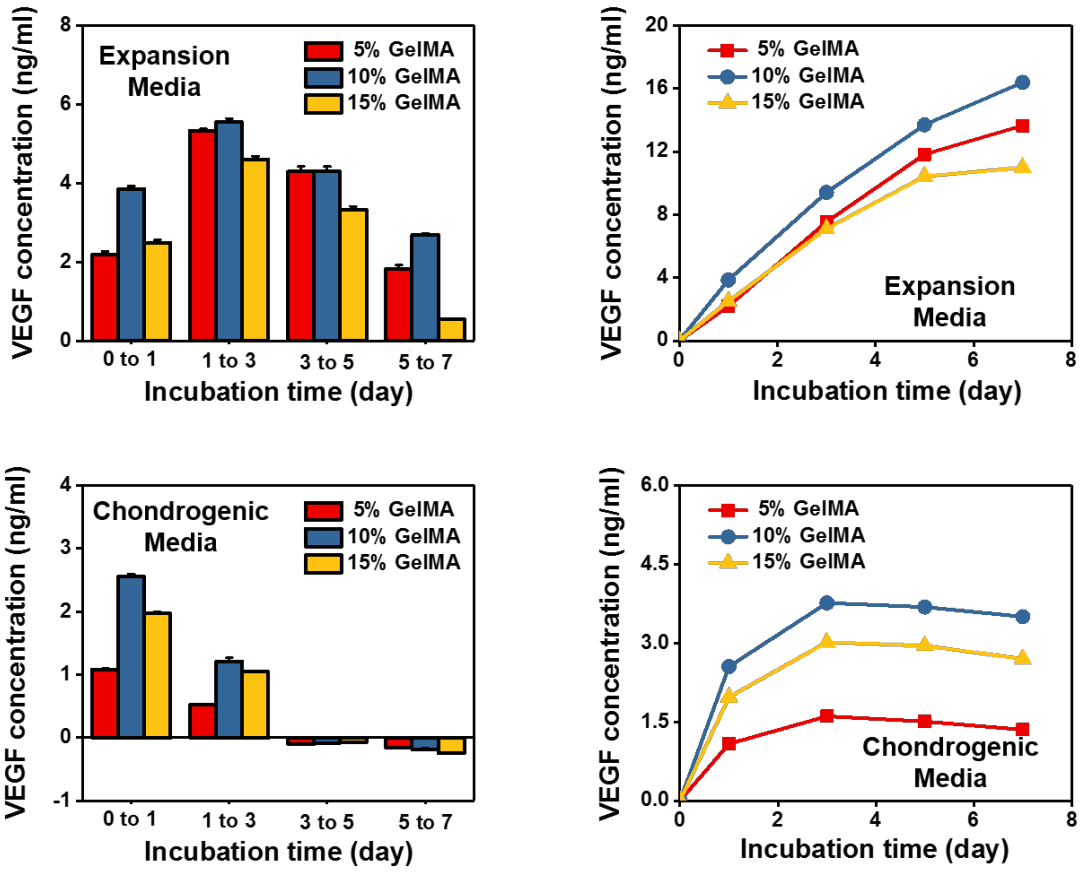

Figure 5.7: *On top VEGF concentration in Expansion Media for different GelMA stiffness at four time points and trend of VEGF secretion from day1 to day7. In the bottom, VEGF concentration in Chondrogenic Media for different GelMA stiffness at four time points and trend of VEGF secretion from day1 to day7.*

The result for chondrogenic media are much lower than those from expansion media and they are even under the limit of detection of ELISA assay as it is possible to observe from the negative value showed in the bottom left of figure 5.7 for incubation time 3-5 and 5-7. These results were not further taken in consideration and chondrogenic media was not used in the following step of the experiment because ,from the ELISA study, the results were bad.

5.3 EIS calibration curve

The goal of this project was to build and develop a label-free electrochemical immunobiosensor for continual detection of proteins.
First of all, it was important to check if in literature others researchers have already found out the concentration of VEGF secreted by hMSCs derived from bone morrow, as well as the cell population analyzed in this project.
Indeed, from Amable et.al, proteins secretion (expressed as mean $\pm$ standard deviation) of bone marrow mesenchymal stromal cells are reported.
For Angiogenic factors, it has been found that VEGF is secreted by hMSCs with a concentration of 24.80 pm 0.01 (pg/10^6cells/day).
Therefore, it is possible to state that this concentration is much higher than the limit of the detection of electrochemical impedance spectroscopy measurements and so this method can be used to detect the specific protein.

With these specifics of automatic system with high selectivity and sensitivity, a set of microfluidic tools and devices was required.
The microfluidic EC biosensor needs a well designed chip in which several reservoirs have to be reserved to each reagent and solution needed to obtain EIS measurements.
As explained in the section 4.7.4, the operator needs to follow a procedure to take a good measurement, therefore even the automated system needs to be designed and calibrated in order to follow an order of what reagent needs to be used, what is the right volume of solution for each step and for how long the solution has to interact with the sensor surface.
Furthermore, a regeneration step is required since saturation of the sensor surface occurs after few binding events.
An automated multiplexed microfuilidic chip with precise timed injection of solutions for protein detection has a complexity which makes impossible the realization in few months. Therefore, the present accomplishments are still far from what is going to be the final result.

At this stage of the project, EIS calibration curves for VEGF have been obtained.
Most of the experiments and measurements were conducted for analyzing and verifying the good behavior of the sensors chosen in commerce.

Therefore, as reported in figure 5.8, before and after the deposition of each layer (SAM, SPV, biotinylated anti-albumin, media blocking) impedance measurements were taken to check if the reagents added on the sensor surface were working in the proper way.

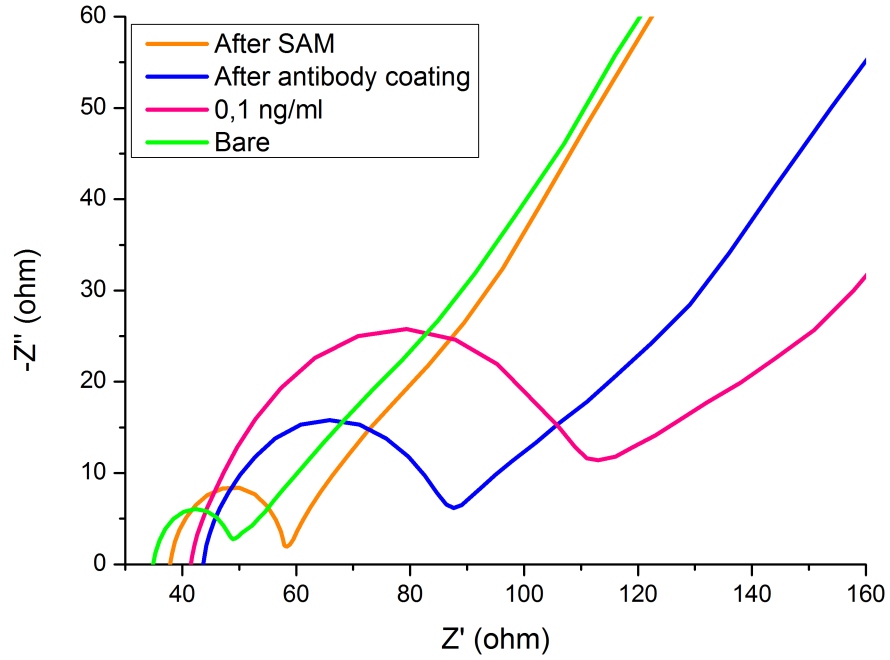

Figure 5.8: *Nyquist plots of the impedance measurements before and after each step of the deposition of each layer*

As already explained in 3.3.1, by the attachment of antigens on the surface sensor after proper surface functionalization, the measured impedance shows a proportional value to the number of detected proteins.

The calibration curve needs to be determined in order to find the concentration of a specific protein inside a solution which is linked to the impedance value measured for that concentration. This means that before taking the real measurement of an unknown concentration, some solution with a fixed amount of antigens inside have been used in order to obtain the respective curve for the known concentration.

The expected pattern of the impedance plot has to present a semicircle at high frequency and a linear behavior at low frequency which explains the limit of diffusion property of the charge.

Once the calibration curve were found, it is possible to analyze an unknown solution with a variable number of antigens inside and to measure the concentration by the relation between impedance and antigen concentration.

After each step of the process to make the sensor surface functional, a measurement of impedance was taken, or better three measurements of the same surface modification were taken, in order to make an average with a standard deviation to obtain reliable results.
The resulting figure of the impedance measurements of concentrations 0, 0.1, 0.01, 0.001, 0.0001 ng/ml are showed in 5.9.

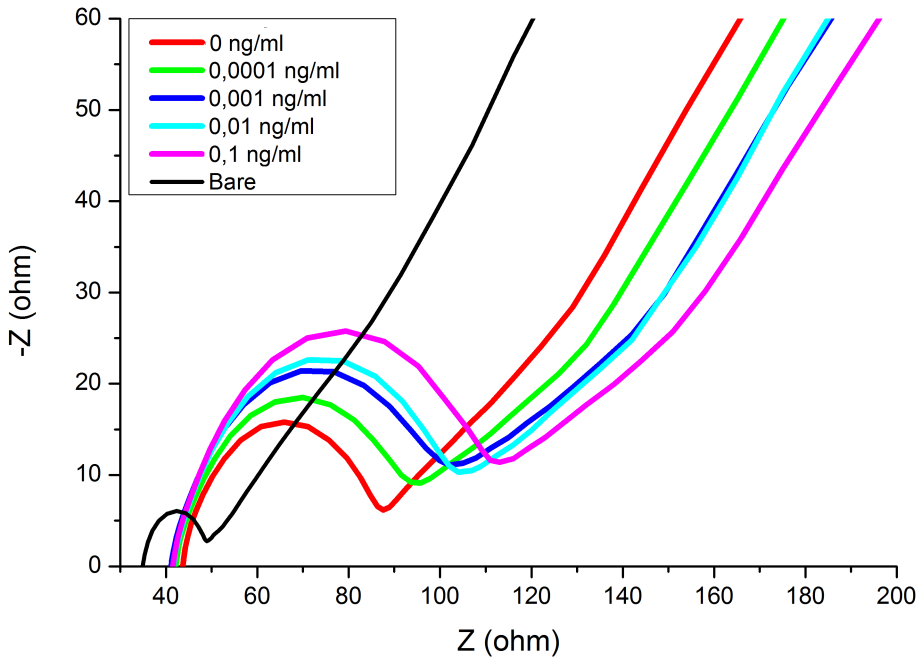

Figure 5.9: *Nyquist plots drawn for different standard VEGF concentrations.*

Once several impedance measurements of the known concentrations of the studied protein were taken, one point of the impedance pattern was chosen as a simple point to visualize on each curve. That is the maximum value reached by each semicircle curve without taking in consideration the Warburg impedance linear pattern.
At this point the maximum value of each plot was normalized by dividing R_{ct} value by R_{ct} media value.

By this curve it is possible to find the respective concentration value of a measured impedance value when the concentration of antigen inside the media is not already known.

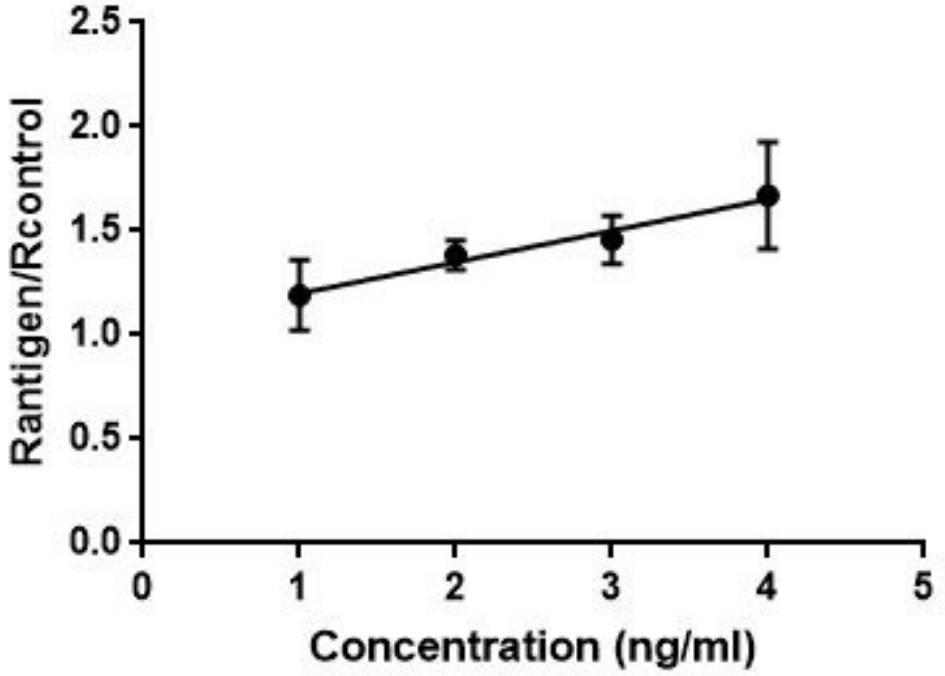

Figure 5.10: *VEGF calibration curve according to the normalized R_{ct} antigen/R_{ct} media values.*

The project is still going on, therefore once the calibration curves were obtained, the following step was to text the media taken out from the bioreactor to check if the concentration of VEGF got increased with the compression or not.
For liking problems of the bioreactor a delay occurred and members of the project team are still trying to make some improvement for the sailing part.

5.4 YAP and spreading

As explained schematically in figure 1.1, a stiff ECM and so high contractile forces make YAP active. In this case, YAP goes inside the nucleus and the consequences might be proliferation and osteoblast differentiation.
On the contrary, when contractile forces are low and cells show a soft ECM, YAP is inactive and it localizes in cytoplasm.
The consequences in this situation might be apoptosis, which is a way of dying of the cells, growth arrest of the cells and the differentiation goes towards adipocytes.
The previous study was conducted to analyze the influence of the GelMA stiffness on the YAP expression.

The immunostaining, in simple word, is performed by breaking and opening the cell membrane in order to give the chance to the antibodies to enter inside the cell and so on to the antigen which is in this case the YAP protein.

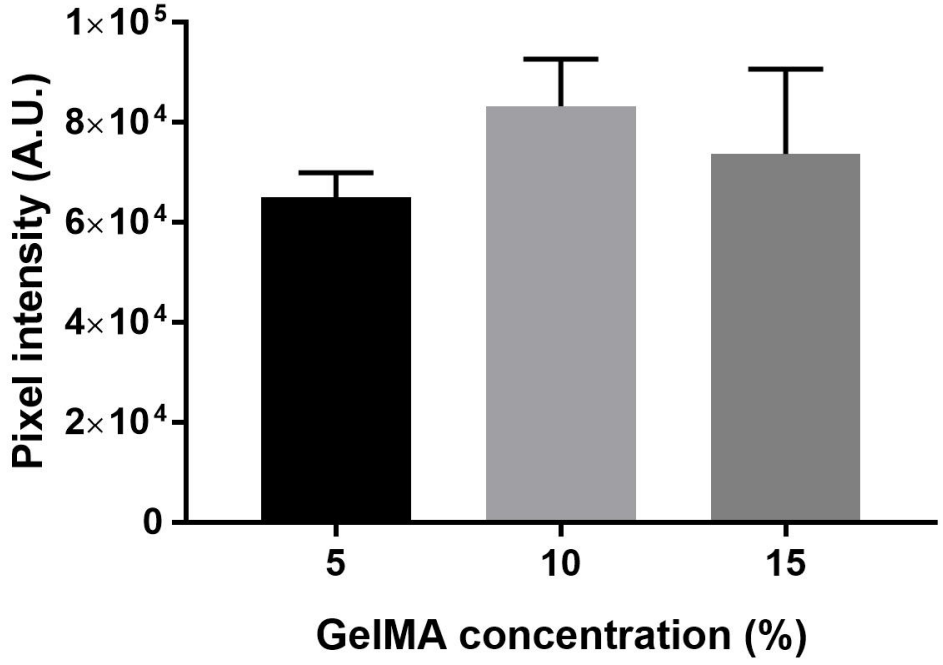

Figure 5.11: *YAP staining for hMSC encapsulated in different GelMA concentration*

To verify which concentration of GelMA may be considered the best option in terms of YAP expression, the pixel intensity was measured by ImageJ software which gives information about how much YAP was "absorbed" by the cells.
A same number of cells was considered for each concentration and the final result was the sum of all the pixel intensity measured for the three different GelMA concentrations.
As reported in the histogram of figure 5.11, the higher number of pixel intensity and so the proportional higher YAP concentration inside the cells was found for 10% GelMA.
The lower value was found for 5% and the middle result was for 15% GelMA concentration.
This result seems to be really similar to the VEGF concentration value found by the ELISA analysis when the stiffness selection was performed, as shown in section 5.2.2.
Therefore, polymer network of hydrogel affects YAP activation and 10% GelMA was considered the best concentration in terms of both VEGF secretion and YAP expression.

hMSCs were cultured for 7 days inside the bioreactor system and at the end of the 7 days, immunostaining analysis was performed.
In the figure 5.12 it is easily shown a different behavior of hMSCs encapsulated in GelMA hydrogel when 0% and 42% of compression are applied.

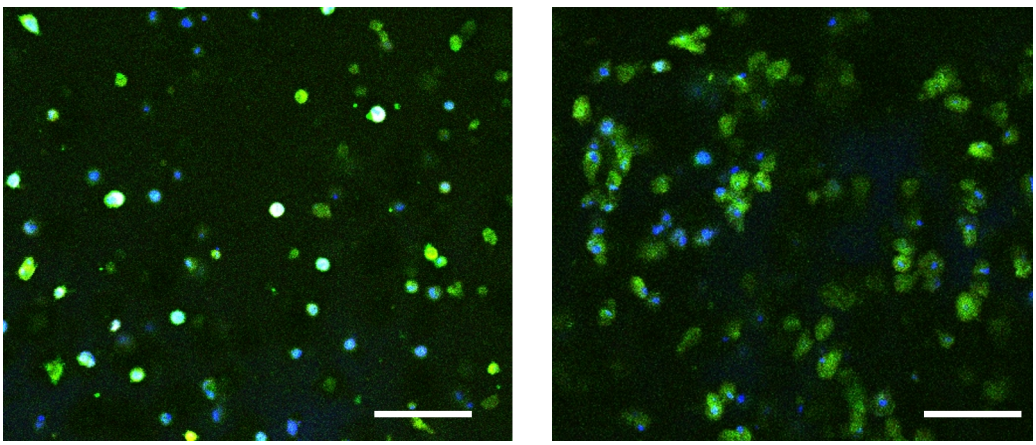

Figure 5.12: *Confocal images of the cell-laden hydrogels after YAP staining procedure. On the left side the hydrogel subject to 0% compression and on the right hydrogel subjected to 42% compression*

On the left image of figure 5.12, cells seem to be round, they spread and distribute in a more homogeneous way.

On the contrary, under 42% of compression on the right, the cells show a different distribution and shape.

External mechanical forces seem to influence not only the single cell's behavior but even the relation among one cell and the closest ones.

Cells agglomeration increased in response to increasing dynamic compressive strain, indeed. Cells under higher compression tended to agglomerate with each other and the distance between one cell to another got decreased.

Even the number of cells got increased and this shows a good response of the cell environment when it is submitted to mechanical stimulation.

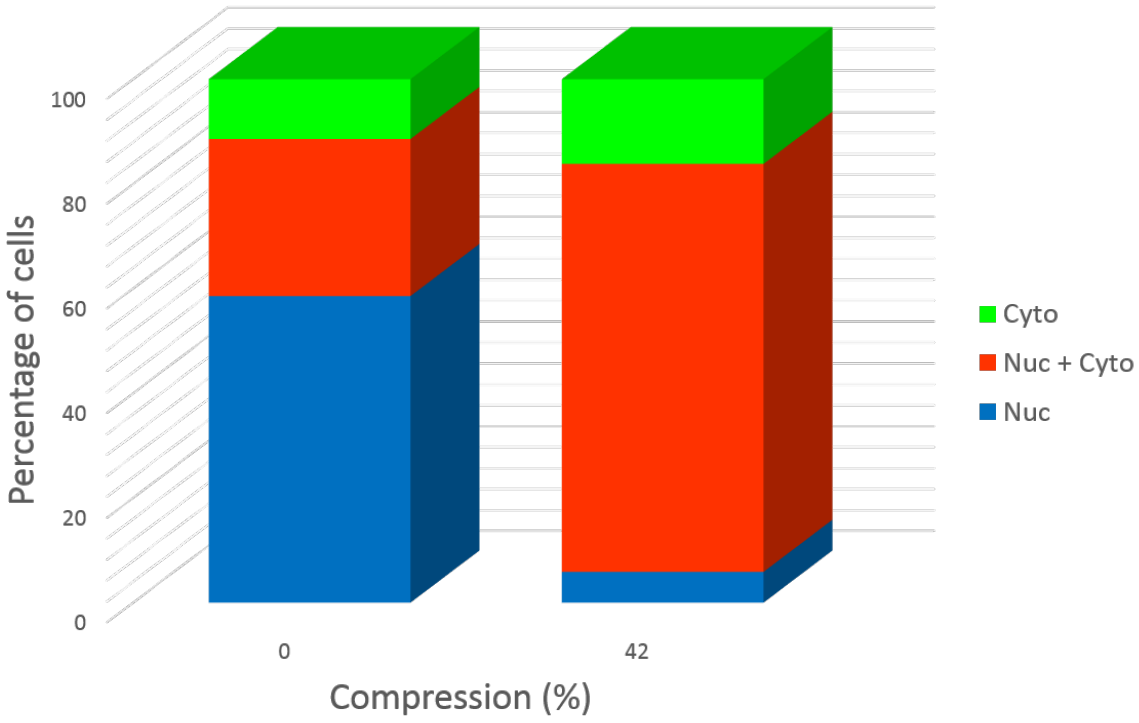

Figure 5.13: *YAP staining*

In order to understand the confocal images, it is important to know that the green color represents the YAP found in the cytoplasm, while the blue color represents the YAP inside the nucleus.

Therefore, the images give important information about the localization of the protein object of study inside the cell.
From the localization of the YAP inside the cell, it is possible to understand if the protein is active or not active for that cell.
As explained in the section 1.2.2, it is possible to state that for the cells subjected to 0% compression, the YAP looks inactive since it is mostly localized in the cytoplasm.
The consequences, as already studied by Piccolo et.al, could be Apoptosis and so cell death, growth arrest and the differentiation go toward Adipocyte.
The expected results in terms of vascular grow factor detection should be a decreasing concentration value since most of the cell faith will be death and arrest in terms of growth.
For cells subjected to high compression, the figure 5.12 shows a cell shape not regular and most of the cells present YAP in both nucleus and cytoplasm.
Less amount of cells present YAP only in the cytoplasm and even more less only in the nucleus.
As explained in figure 1.1, when YAP is active, the cell appears as the above results and the consequences should be proliferation and growth and osteoblast differentiation.
Therefore, the expected secretion pattern is an increasing VEGF concentration detection.

5.5 VEGF secretion under dynamic compression

The most important result of this study was obtained with the final experiment in which cell-laden hydrogels were stressed by different percentages of compression.
From an in depth study of the disposable literature, the hypothesis was an increasing VEGF secretion for cells subjected to a cyclic compression which mimics the mechanical elasticity of the extracellular matrix (ECM) and all the mechanical stimuli of the surrounding environment.
To validate this theory, the main experiment consisted of using three bioreactors and all of them were made opportunely by following the same fabrication procedure and of course same encapsulation of the cell-laden hydrogels protocol.
The reason of making three bioreactors has to be found in the huge difficulty of the operator to make a perfect and reliable system.
Even a small mistake in the fabrication and assembling procedure could have influenced the

result and success of the experiment. Therefore, using the materials and methods already explained in the previous chapter, three bioreactors were connected to the WAGO system and so 4 percentages of compression were applied.

Since each compression affected one single chamber of each bioreactor, at the end the samples analyzed were the media collected from each chamber which showed the influence of the mechanical stimulation on the cell behavior.

Due to some leaking problems of two of the built bioreactors, the data obtained cannot be considered as conclusive. Indeed, as shown in figure 5.14, it was not possible to show the standard deviation, as only one sample for each compression could have been collected. Anyway, even if the data obtained are not completely reliable, they give good results which

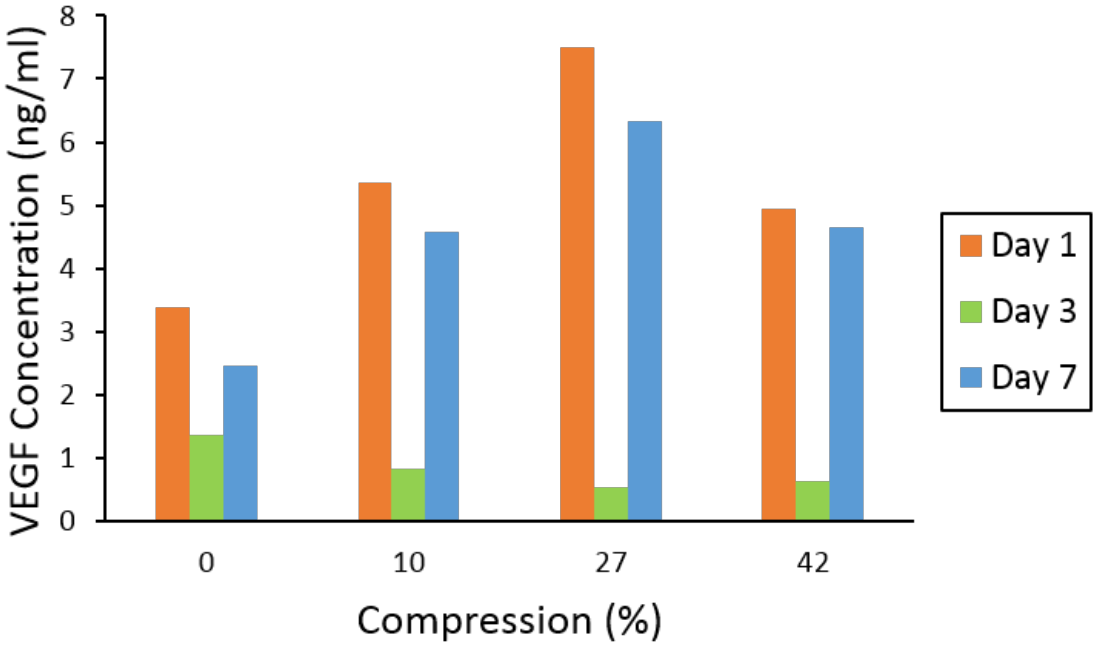

Figure 5.14: *VEGF concentration (ng/ml) for different percentage of compression at three time points*

are aligned to the initial hypothesis of a dependency between proangiogenic signaling of hMSCs and mechanotranstuction.

Indeed, it was decided that these results may be interesting for the purpose of this thesis but of course they have to be repeated for a possible publication.

Measurements of VEGF concentrations for each specific chamber in which different percentage of compression were applied, were taken by ELISA assay study.

Therefore, the media inserted in the chambers was extracted every 2 days and collected.
At the end of the seventh day, the procedure of ELISA assay, already explained previously, was followed accurately.
As shown in figure 5.15, higher values of VEGF were found for 27% of compression, followed by 10% and 42% in order.

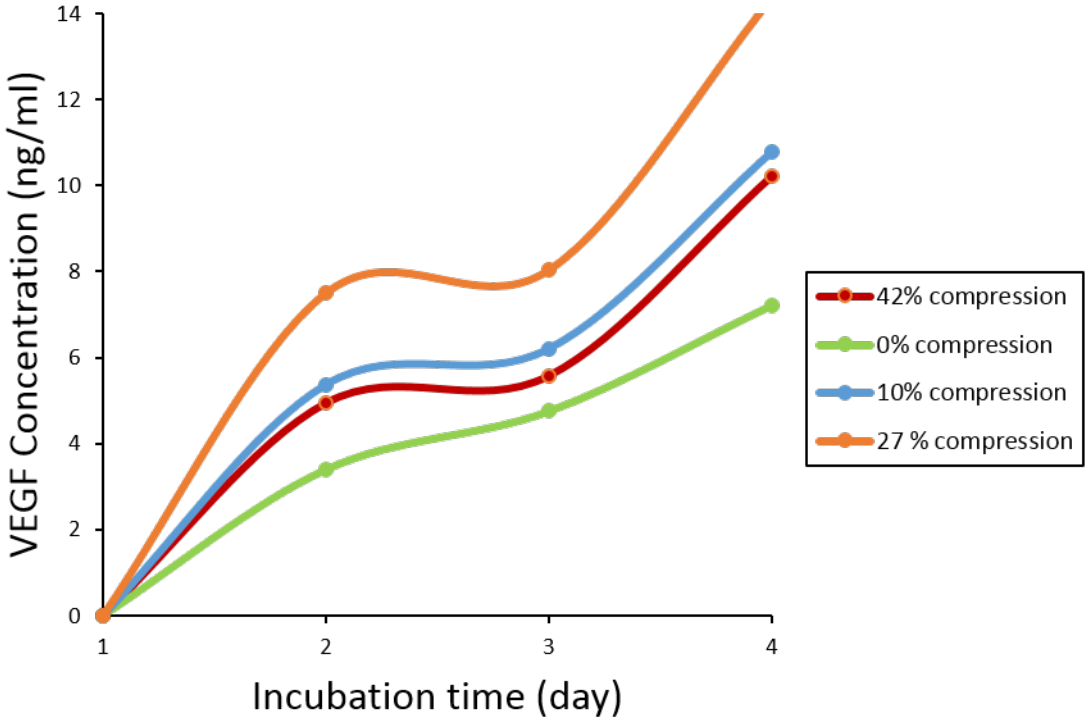

Figure 5.15: *Trend from day 1 to day 7 of VEGF concentration (ng/ml) for different percentage of compression*

From figure 5.14, the lowest values of VEGF concentration were found for cell not at all subjected to compression, except for the time point day 3 in which with the increasing compression, opposite results have been obtained and so a decreasing concentration.
For the results obtained, possible interpretations could be:

- With mechanical stimulation of hMCSs encapsulated in GelMA hydrogel, the concentration of the target protein VEGF got increased.

- It is possible to state that not every percentages of compression are eligible for increasing the secretion of the target protein. Indeed, from the figure above, it is possible to individualize 27% of compression as the best percentage and almost a linear pattern has been shown from 0% to 27% while on the contrary with higher percentage like 42% the VEGF concentration got decreased.

- The two points above are true for the time points day 1 and day 7 but they are invalid for day 3 in which the concentration follows a downward trend from 0% to 42%.

It is not simple to find a proper explanation of this last point, especially because, as already said, replicates of the experiment got wasted.
A possible hypothesis could be that cells, after the introduction in a new environment, the bioreactor, needed a time to get used to it and so, day 3 may be considered as a standby day in which the cell activities are kind of interrupted. This is of course just an hypothesis but it can be interesting to examine in depth this topic.

Chapter 6

Conclusion

Here, we fabricate a three-dimensional dynamic cyclic compression bioreactor in which human mesenchymal stem cells (hMSCs) are cultured in 3D gelatin-methacrylate hydrogels to evaluate the influence of mechanical stimuli and matrix stiffness on proangiogenic factor secretions of hMSCs.

Cells cultured in matrices mimicking mechanical elasticity of bone tissues show elevated secretion of vascular endothelial growth factor (VEGF), one of the representative signaling proteins promoting angiogenesis.

When cultured in the matrices promoting VEGF secretion, with different range of cyclic compressions, we demonstrate optimal microenvironments for controlling proangiogenic signaling of hMSCs.

Furthermore, we also show that matrix stiffness and cyclic compression modulate VEGF secretion of hMSCs through yes-associated protein (YAP) activity. Therefore, these results seem to validate the theory which lays the foundation of the all project, that is the the strong connection between hMSCs behavior and mechanotrasduction, which regulates cell fate in terms of viability and differentiation.

The final product of this study will be a interconnectable bioreactor which allows real time screening of hMSCs behavior in condition of dynamic compression and hydrogel compositions.

Data and results reported in this thesis are only the basic for the whole project and they can be considered as preliminary data.

Thanks to the accomplishments obtained at this stage of the project, lots of section of the

hole project have been already implemented and they are ready to be part of the final microfluidic system.
The bioreactor system has been already designed and fabricated. GelMA stiffness was selected in order to reduce the amount of variables and to make easier the following analysis.
The calibration curves, needed to make impedance measurements and so to acquire concentration values of the wanted protein inside the media, were obtained and they are ready to be used in the following steps when all the sections will be implemented and connected together to make the device functional.

This work will prove useful in the design of tailored model system, which more efficiently direct proangiogenic signaling from hMSCs.
The way to obtain the final device is still long and difficult, but this study will give lots of explanations about the stem cell properties and behaviors and ultimately it will lead to improved strategies for various stem cell-based therapeutic applications.

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