TECHNISCHE UNIVERSITÄT WIEN

Report on a Project Performed at the

Technische Universität Wien

In order to convalidate a Master's Thesis in Biomedical Engineering at University of Zaragoza

Course 2021/2022

Design and fabrication of bone-on-chip microfluidic devices for cell culture

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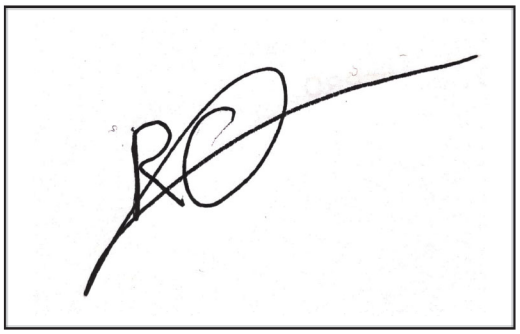

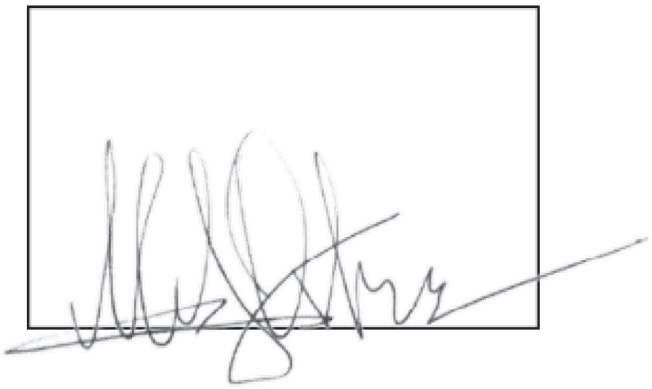

Design and fabrication of bone-on-chip microfluidic devices for cell culture

Summary

This project aimed to provide a basis for the development of a bottom up assembly of tissue within a microfluidic chip for a bone-on-a-chip model. The main purpose of the project was performing a cell culture in which human adipose-derived stem cells ("hASCs") spheroids could grow inside of the central channel of the device without invading the side channels. Concretely, highly porous buckyball microscaffolds, fabricated via two-photon polymerization technique, were employed as a structure to support the delivery of spheroids into the microfluidic device.

The vertical central inlet ("VCI") "SLA Accura® Xtreme™" mould was the best solution for the fabrication of the microfluidic devices in terms of surface quality and additionally because no leaking was observed during the test process.

The cell culture results showed that the central channel was not completely filled with spheroids due to the presence of air bubbles that were going inside the device during the pipetting. Additionally, cells were also growing inside the side channels which was not desired. The side channels are necessary for perfusion of nutrients, growth factors etc. Live/dead staining results showed that cells were still alive after 7 days of cell culture, which means that the protocol could be useful for further long-term culture studies like differentiation of stem cells into osteoblast cells.

Considering the mentioned results, another method for introducing the spheroids inside the microfluidic device must be chosen.

As future lines of investigation, more cell culture experiments should be done to verify if it is possible to completely fill the central channel with spheroids and create bone tissue inside without penetrating the side channels. Moreover, surface coating of the microfluidic devices could be performed to prevent cell attachment.

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Chapter 1

Introduction

1-1-Background information

Traditionally, cell culture has been done by using Petri dishes. However, experimental results by using this method cannot be extrapolated to humans' physiology due to several reasons, such as the difficulties of mimicking the complex extracellular matrix and the human body environmental conditions. On the other hand, the ethical and moral issues regarding in-vivo experimental models are some examples of the limitations of the other more human-mimicking alternative.

Microfluidics is a new field which combines principles of physics, material science, biology, chemistry, and fluid mechanics to obtain microfluidic devices. This new application can be a bridge to solve problems, as the ones stated before. A microfluidic chip is a set of micro-channels that are connected to achieve the desired features. These micro-channels relate to the outside by inlets and outlets as an interface between the macro- and microworld. It is through these channels that the cell medium, cells, and other substances are injected and removed from the chip via external systems (peristaltic pumps, syringe pump, pressure driven flow controller etc).

There are several applications for microfluidic devices. For example, diagnostic devices, cell culture, drug delivery systems, and nanomaterial synthesis platforms. One of the most innovative applications of this line of research is the development of tissue on a chip device.

The objective / aim of this project is to provide a basis for the development of a bottom up assembly of tissue within a microfluidic chip for a bone-on-a-chip model.

1.1.1-Petitioner

The petitioner of this project is the "Escuela de Ingeniería y Arquitectura (EINA)" of the "University of Zaragoza (Unizar)". It will be completed in collaboration with the "3D-printing and Additive Manufacturing Technologies" group of the "Technische Universität Wien (TU)".

1.1.2-Objective of the project

The main objective of this project is the design and fabrication of a bone-on-chip microfluidic device through additive manufacturing techniques, that complies with the tissue engineering principles. These principles consist in being able to design and fabricate 3D structures ("scaffolds") with suitable mechanical properties to promote extracellular matrix synthesis. It is on these scaffolds where the cells can proliferate, differentiate, and generate tissue in an appropriate environment (genetic control, mechanical factors etc.).

Moreover, human adipose stem cells ("hASCs") spheroids, in buckyballs, were injected to see if it is possible to fill the central channel of the microfluidic device and if the cells can survive after 7 days of culture.

1.1.3-Methodology

To achieve the objectives mentioned before, the following methodology was implemented:

- **State of the art.** The bibliography and literature related with microfluidic devices were used to obtain all the necessary knowledge for the development of this project.
- **Modelling of the bone-on-chip device.** Two models of the moulds of the bone-on-chip devices were designed with "Autodesk Fusion 360" CAD software. One has a vertical central inlet and the other a horizontal central inlet.
- **Manufacturing of the bone-on-chip-device.** Both models were manufactured with additive manufacturing techniques. Once the moulds were fabricated, the chips were obtained by pouring polydimethylsiloxane (PDMS) into the moulds.
- **Cell culture.** After the obtention of the chips, cell culture was performed. For this, hASCs in EGM-2 medium were introduced via the central inlet.
- **Discussion and conclusions.** The results obtained during the whole process were discussed and a conclusion was described.

1.1.4-Structure of the project

This project was divided into five chapters:

- **Chapter 1: Introduction.** In this chapter the context of the project and a brief description of microfluidic devices was shown. Moreover, a resume of fundamental concepts on the anatomy and physiology of bone tissue and a literature review on past research on bone-on-chip microfluidic devices was performed.
- **Chapter 2: Materials and methods.** First, the design aspects for the bone-in-chip models were resumed. Then, the following manufacturing

procedure for the obtention of the chips were described. Finally, the experimental planning for the cell culture was reported.

- **Chapter 3: Results and discussion.** The results obtained during the whole process were discussed.
- **Chapter 4: Conclusion.** A conclusion was described as well as possible future lines of investigation.
- **Bibliography.** All the references that were used in the present project were included.

1.2-Anatomy and physiology of bone tissue

1.2.1-Introduction

Bone tissue is a type of tissue which is in constant remodelling process. This means that it suffers destruction and formation processes. Additionally, bone tissue participates in homeostasis, protects our organs, and enables movement with the help of muscles and tendons.

In this subchapter, a brief description of the bone tissue structure and the differentiation process of stem cells was done.

1.2.2-Bone structure

Bone tissue is composed of cells and extracellular matrix ("ECM"). The ECM is also composed, in a nanometric scale, of an organic fraction and an inorganic fraction. The proportions of both are the following [1]:

- Approximately, 2 % is constituted by bone cells (most of the authors include bone cells in the organic fraction).
- The inorganic fraction represents between 60-70 % of the dry weight of bone, meanwhile the organic fraction approximately represents 35 %.

1.2.2.1-Cell component

Bone tissue is composed by three type of bone cells:

- ***Osteoblast cells.*** Mononucleated cells with pyriform aspect, so active and producers of elements of the organic fraction. These cells are located on the bone surface producing a matrix material called osteoid, a gelatinous substance made up of collagen and other proteins. This unmineralized organic tissue will eventually undergoes calcification and will be deposited as lamellae or layers in the bone matrix.

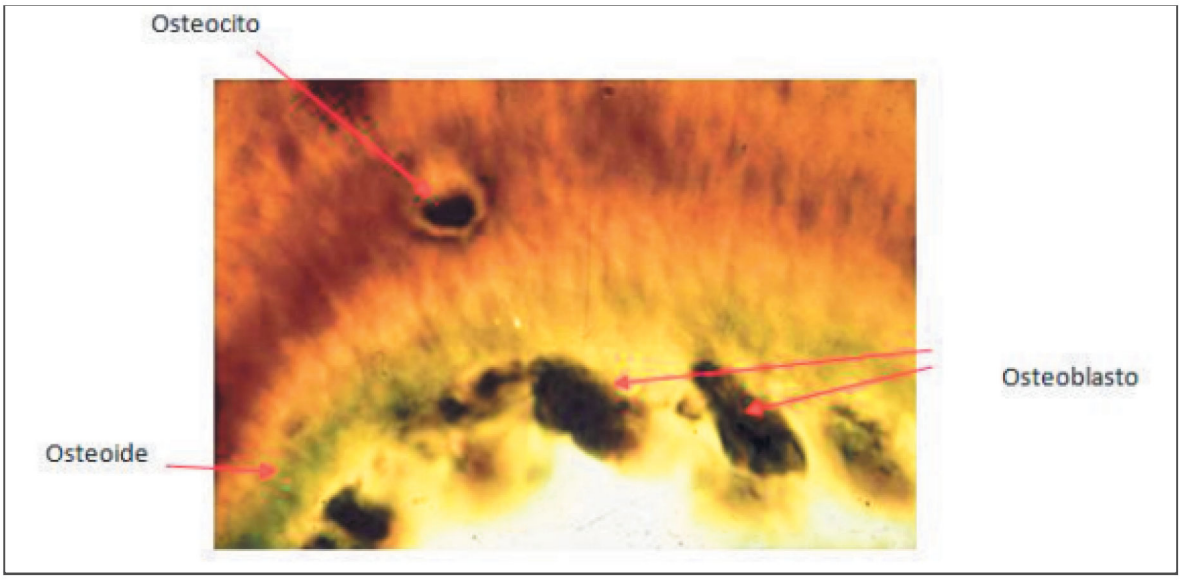

Figure 1: Microscope image of osteoblast, osteocyte cells and osteoid substance [1].

- ***Osteoclast cells.*** Multinucleated cells with irregular form. They are responsible for resorbing bone tissue so then it can be remodelled. Therefore, they participate in the calcium homeostasis process.

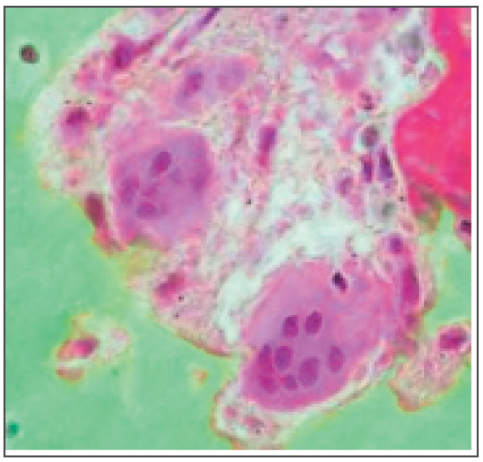

Figure 2: Microscope image of an osteoblast [1].

- ***Osteocyte cells.*** This type of cells are osteoblast cells which were embedded in the mineral bone tissue. Osteocytes also participate in the homeostasis of calcium. Only around 30 % of the osteoblasts can be transformed into osteocytes, the rest of them die by apoptosis.

1.2.2.2-Organic fraction

The organic fraction is constituted by 90 % of collagen, which is produced by osteoblasts and provides tensile strength for bone tissue. Collagen type I is the most important component of the organic fraction due to its contribution in the generation process of bone tissue. On the other hand, the rest of the organic fraction is composed by non-collagen proteins that act like biochemical markers for the bone activity and the homeostasis of calcium. Additionally, these proteins are used for osteoarthritis treatment and even to accelerate the bonding process of a fractured bone.

1.2.2.3-Inorganic fraction

Mineral deposits are located between the collagen fibres as hydroxyapatite crystals, which support the mineralization process. These crystals are principally constituted of tricalcium phosphate, but also of calcium carbonate and foreign bodies. Moreover, they are disposed around the collagen fibres forming a structure which provides great mechanical properties. Hydroxyapatite crystals contribute to the compression strength of bone tissue.

1.2.3-Stem cells for cell culture

Stem cells ("SCs") are undifferentiated cells with the capacity of self-renew and generate differentiated cells. This type of cells is so used in the tissue engineering scope as it has higher proliferation capacity and can support more passages than mature differentiated cells [3]. Moreover, SCs can be classified by their source, the tissue they are generated from and the stage during which they appear in the lifetime of the organism [2]:

-***Totipotent SCs:*** cells that can create a new organism by themselves (for example zygote cells in mammals).

-***Pluripotent SCs:*** cells that can generate cells from the three embryonic layers (ectoderm, mesoderm, and endoderm).

-***Multipotent SCs:*** cells that can generate specialised cells from a specific tissue (within one germ layer). This type of cells can be found in different types of tissues in a fully developed organism and in the umbilical cord blood (adipose tissue, nervous tissue, epithelial tissue etc.).

The stem cell hierarchy is represented in the next illustration:

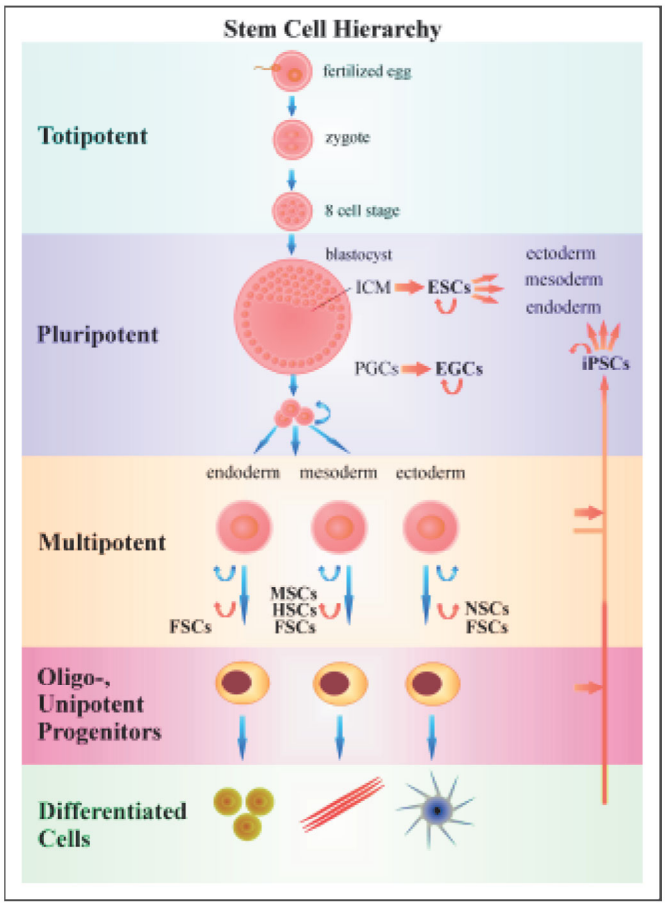

Figure 3: Stem cell hierarchy [2].

1.2.3.1-Bone marrow and adipose stem cells for bone tissue engineering

Bone marrow and adipose stem cells ("BMSCs", "ASCs") are the most common cells used in regenerative medicine.

"BMSCs" were the first stem cells to be successfully isolated and characterized. They can be aspirated from the sternum, pelvic during a diagnostic method for blood diseases or a bone marrow transplantation process. However, both techniques are uncomfortable and painful for patients and not so many stem cells can be collected. Even so, these multipotent cells are considered suitable for bone tissue engineering [3].

On the other hand, "ASCs" usually are more abundant in many patients than "BMSCs" and easy to access via subcutaneous adipose tissue. Moreover, it has been observed that "ASCs" and "BMSCs" have similar multilineage differentiation potential but, "ASCs" have better yield and a higher proliferation capacity [4] [5] [6].

"ASCs" can be used for bone repair due to their osteogenic differentiation into osteoblasts. Then, the differentiated cells can be seeded into a scaffold and subsequently transplanted into the human body to replace damaged bone or joint [7] [8].

Due to their mesodermal origin, the osteogenic differentiation process of the mesenchymal stem cells "MSCs" has been studied in detail. Moreover, it is important to know what are the different factors that influence the differentiation process of in vitro cell cultures. These factors can be classified into three groups:

1. ***Cell culture media.*** Growth factor, temperature, pH, oxygenation etc.
2. ***Mechanical properties of the scaffold.*** Type of structure, rigidity, roughness etc.
3. ***Mechanical and dynamic signals.*** Strain and shear stress, vibrations, electrical impulses.

Therefore, depending on these factors stem cells are going to respond in a certain way: change of their morphology, change of secretome, proliferation, differentiation into specialized cells and apoptosis [3].

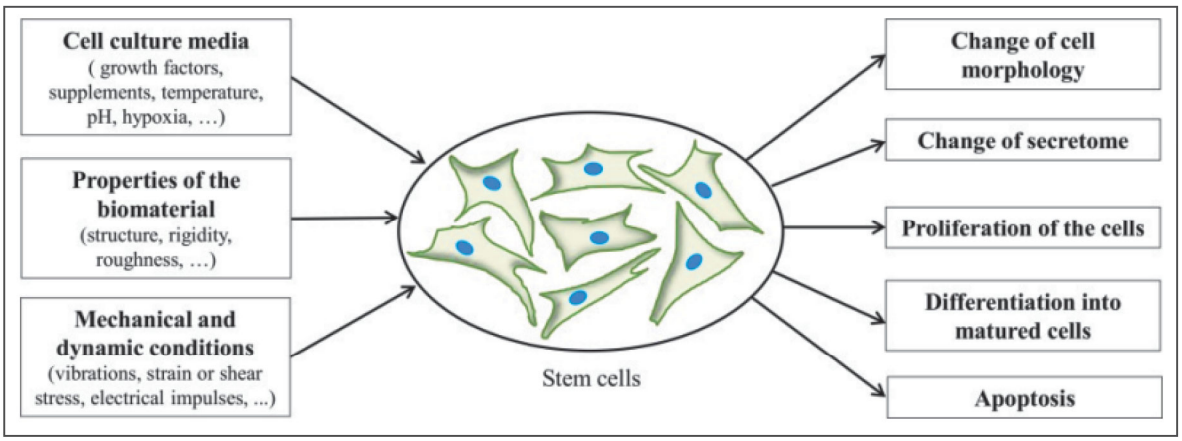

Figure 4: Influence of the cell culture conditions in cell response [3].

1.3-State of art

1.3.1-Introduction

Microfluidic technology, as George M. Whitesides described, has four parents: molecular biology, molecular analysis, national security and microelectronics [9], but the key factors that made the fabrication of microfluidic devices possible were the emerged of soft lithography and polydimethylsiloxane (PDMS). Soft lithography made it possible to use elastomeric moulds to transfer micropatterns into a wide range of materials (polymers, organic and biological molecules etc.). On the other hand, microfluidic devices were firstly made of silicon and glass. Nonetheless, these materials were not appropriate for microfluidics applied to biology since their low gas permeability and non-optically transparent, which is necessary for microscopy. The introduction of PDMS as material for microfluidics overcomes these issues since it is optically transparent and more biocompatible than the traditional materials [10].

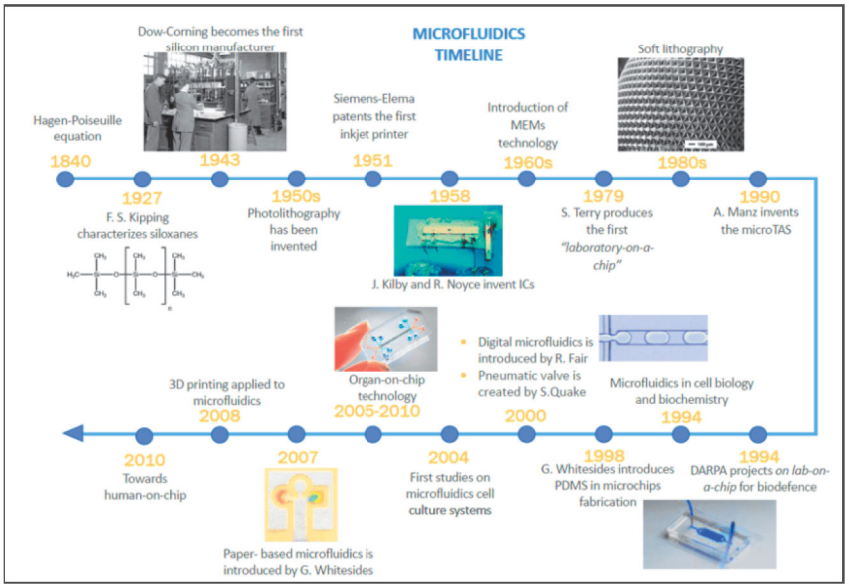

Figure 5: Microfluidics timeline [10].

Nowadays, due to the progress on additive manufacturing techniques and biomaterials it is possible to fabricate microfluidic devices with high resolution that can be used for studies on cell culture, diagnosis, therapies etc.

Microfluidic devices overcome the problems that in-vitro experiments on petri dishes could not solve. With these devices it is possible to provide a favourable environment to build a complex model by means of providing the fluidic compartments and the mechanical confinement where a cell culture strategy can be performed. Therefore, mimicking the complex extracellular matrix ("ECM"), where the adhesion, proliferation and differentiation processes occur, is achievable by

using these types of devices. Also, dynamics studies with flow control are achievable on these devices, which is fundamental for mimicking the body environment conditions.

In this subchapter a description of the predominant manufacturing techniques, biomaterials and cell types used in previous studies on bone-on-chip microfluidic devices will be done as well as their advantages, disadvantages, and applications.

1.3.2-Biomaterials for bone-on-chip microfluidic devices

A critical step in the fabrication of microfluidic devices is the selection of the optimal material. Moreover, on a microscale surface the properties are much more amplified so it is essential to ensure that they are suitable for their application. Some of these properties are biocompatibility, ease of fabrication, transparency, mechanical properties etc. Several biomaterials have been used in previous research on microfluidic devices, such as metals, ceramics, and polymers.

However, polymers have attracted more attention on the fabrication of bone-on-chip microfluidic devices. This is because polymers are inexpensive and can be manufactured with easier and lower cost techniques. Also, one of the main advantages is that some of them are optically transparent or semi-transparent.

For bone-on-chip applications the ones which are most used are polydimethylsiloxane ("PDMS") and polymethylmethacrylate ("PMMA"). PDMS is an elastomer with excellent microchip fabrication properties, easy to mould, cheap, optically transparent, good for prototyping, high elasticity and hydrophobic. These properties make PDMS suitable for long-term cell-culture. The main disadvantage of PDMS is that, due to its porosity, it is also an adsorptive material so many molecules can diffuse through it. Furthermore, water evaporation through channels will change the concentration solution. So, depending on the application another material must be chosen.

PMMA is a commonly used material in bone-on-chip devices. PMMA is an amorphous thermoplastic with interesting properties like optically transparent, good mechanical properties, no small molecule absorption and allows surface modification [11].

As mentioned before, PDMS and PMMA are normally used in bone-on-chip devices applications. However, for cell culture it is important to mimic the 3D ECM because it will result in better adhesion, proliferation, and differentiation results. Some studies have used gel, collagen [12] and other materials as coated materials on a rigid substrate to obtain an adhesion friendly surface and they demonstrate promising results. For this reason, the coating of the primary material could be a good strategy to ensure this.

One example of this strategy can be showed in this figure:

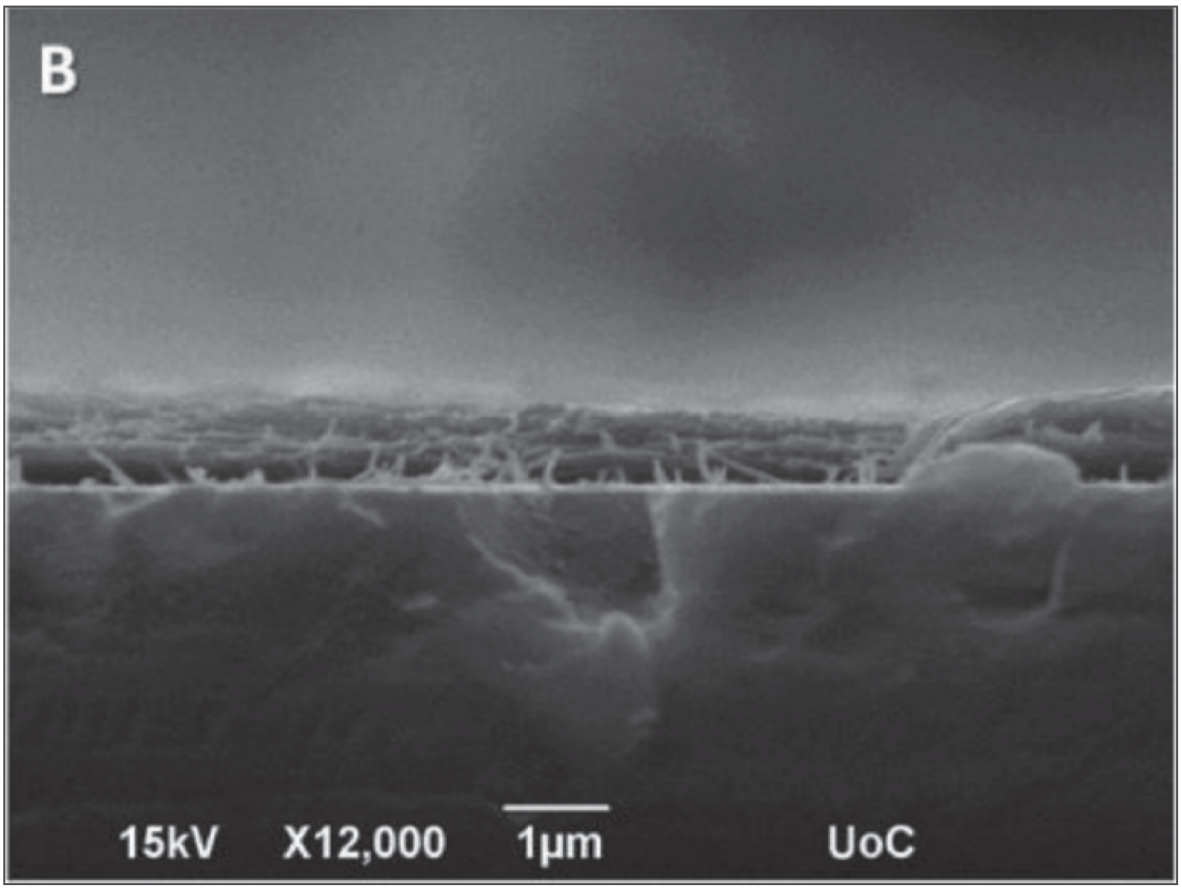

Figure 6: Scanning electron microscope ("SEM") image of a sample cross-section of collagen scaffold on a glass substrate. Approximately, a thickness of 700 nm for the collagen network is shown in the SEM image [12]

1.3.3-Manufacturing methods for bone-on-chip microfluidic devices

Several manufacturing methods have been employed on microfluidic devices. One key aspect is the selection of the optimal method, and this will depend on the characteristics of the biomaterial to be used and on the application. Another essential aspect for the selection of the fabrication technique is the cost. Microfluidic devices are normally used as disposables because they are difficult to clean, so the chosen method must be economically feasible for single-use chips [11].

The different techniques can be classified depending on the nature of the process and how the microfluidic device is created [13] [14]: material removing techniques and material depositing techniques. This is presented in the following table:

Table 1: Classification of fabrication techniques for microfluidic devices [13] [14].

	Material Removing Techniques	Material Depositing Techniques
Chemical processes	Electrochemical discharge machining [10] Wet etching [10] Dry etching [10]	Silicon surface micromachining [19] Lithography [60] Inkjet 3D printing [19] Powder 3D printing [19] Direct writing [19] Two-dimensional virtual hydrophilic channels [19]
Mechanical processes	Micro-milling [10] Micro-grinding [10] Micro-abrasive air-jet machining [10] Micro-abrasive water jet machining [10] Ultrasonic machining [10] Xurography [54]	Injection molding [54] Hot embossing [54]
Laser-based processes	Photothermal process [10] Ultra-short pulse process [10] Absorbent material process [10] Photochemical modification process [10] Laser direct machining [19]	Selective laser sintering [19] Stereolithography [54] Two-photon polymerization [54]
Other processes	Focused ion beam [10]	Forming process [10] Soft lithography [54,61] Layer-to-layer manufacturing [19] Layer-on-layer manufacturing [19] Fused deposition modeling [54] 2.5-Dimensional printing [19]

This project is focused on laser-based processes and soft lithography since these are the two most common fabrication techniques for bone-on-chip microfluidic devices [11].

1.3.3.1-Laser-based processes

Laser techniques are not the best solution for the fabrication of microfluidic devices due to the associated high cost. However, they are very convenient to produce moulds for soft lithography, since it is possible to easily create many prototypes and test them.

The most common additive manufacturing technique used in production of microfluidic devices is stereolithography ("SLA"), even if it has materials restrictions since it is only possible to fabricate with photopolymers. Photopolymers are polymers that cure, or become solid, when exposed to light.

Stereolithography is a classic 3D printing technique invented by Charles Hull in 1986. This method is ideal to fabricate structures with very fine features in a short time and with a great

surface quality. The process consists in generating the structure layer by layer by curing the liquid photopolymer selectively with laser or LED exposure.

An example of fabrication of ceramic substrates with microchannels by using SLA is presented in this figure:

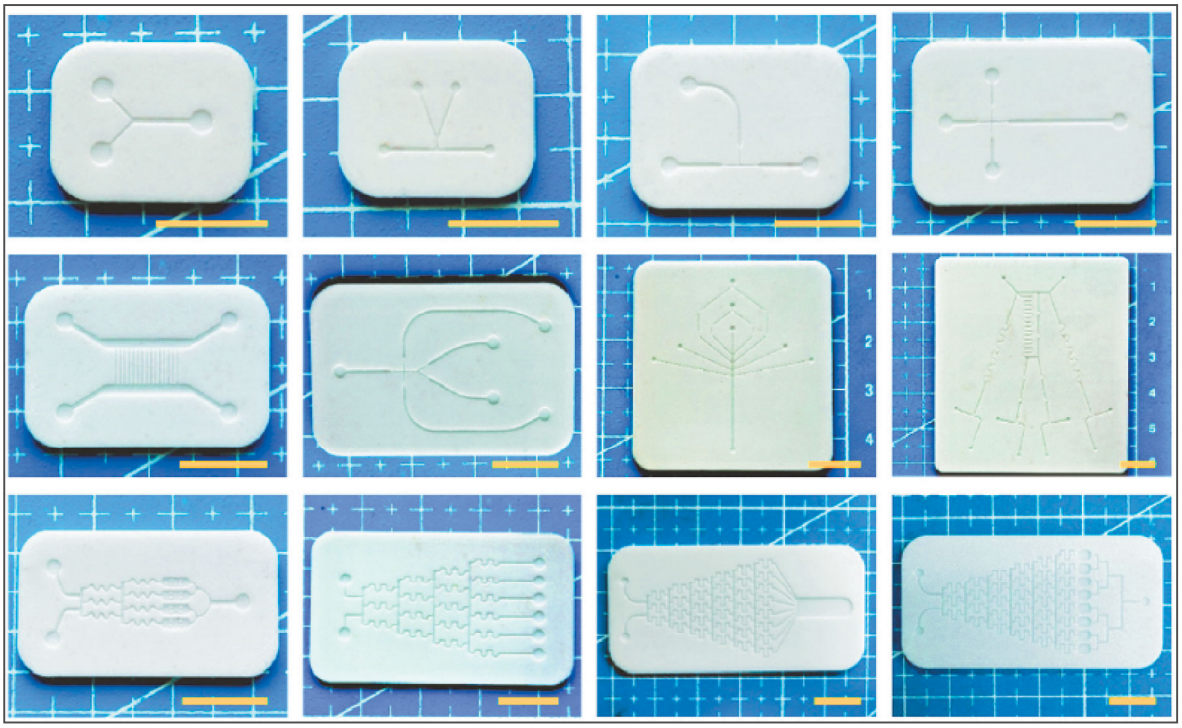

Figure 7: Ceramic substrates with microchannels fabricated via SLA. Scale bar=1 cm [15].

1.3.3.2-Soft lithography

Soft lithography is one of the most common techniques used to fabricate elastomeric polymers microfluidic devices [8] since it is a user-friendly and rapid production method compared to other ones. This technique belongs to the mechanical process family, and it is based on moulding. The manufacturing process consists of several steps: creation of the master mould (via SLA e.g.), pouring the liquid polymer directly into the mould, curing process and debonding of the polymer chip from the mould. The main advantages of this technique are the obtention of high-resolution replicas with an associated low cost and the possibility to generate valves and multiple layers. On the other hand, the main disadvantage is the possibility to break the master mould during the debonding process.

A schematic view of the process is shown in this figure:

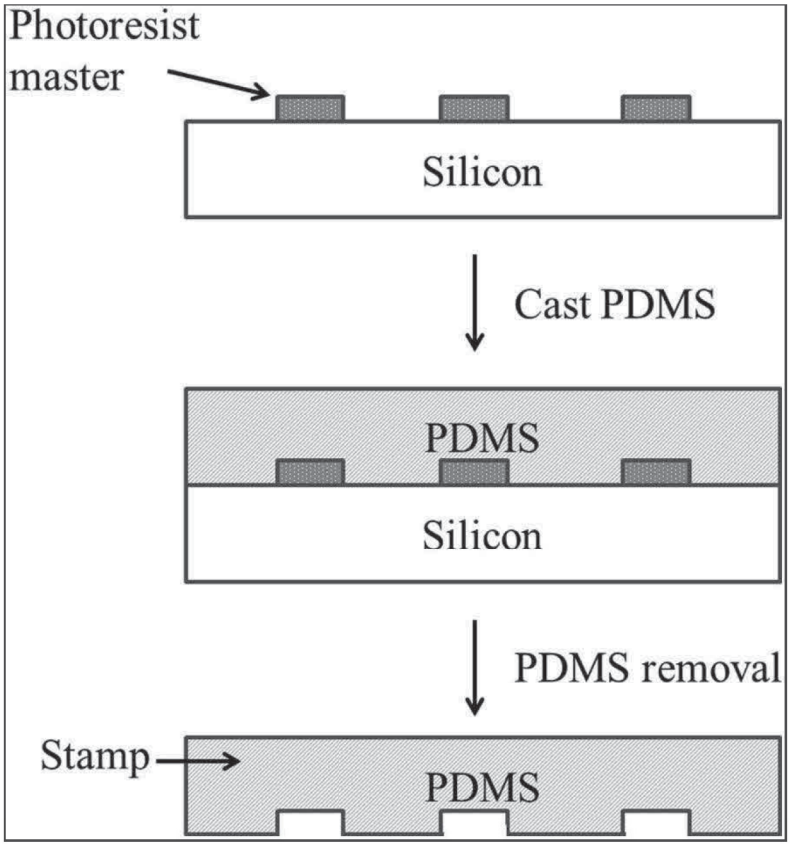

Figure 8: Schematic of the soft lithography process by using "PDMS" as biomaterial [16].

1.3.4-Applications

Bone-on-chip microfluidic devices have several applications depending on the purpose of the study. For example, it is possible to study the influence of mechanical signals on bone cell function. Previous research has demonstrated that shear stresses are essentials in the proliferation, differentiation, and maturation of osteoblast cells [17]. Microfluidic devices can be employed to study the influence of this type of stress since it is possible to control the flow conditions with an external system. In the study of (Babaliari et. al., 2018) [12] a microfluidic device system was made to investigate the influence of shear stresses in the orientation and capability to form "ECM" of pre-osteoblastic cells. Specifically, the microfluidic devices consisted in a glass base chip, which were coated with two different substrates (gelatin and collagen type I ("Figure 2")), and a "PDMS" chip. Also, a pressure pump was used to precisely control the flow conditions. Furthermore, the cell culture was performed with pre-osteoblastic cells from newborn mouse calvaria ("MC3T3-E1") and static and dynamic culture (flow rates of 30 and 50 μmL/min which are like small vessel rates [12] as cell culture conditions.

An illustration of the microfluidic device system is shown in the next figure:

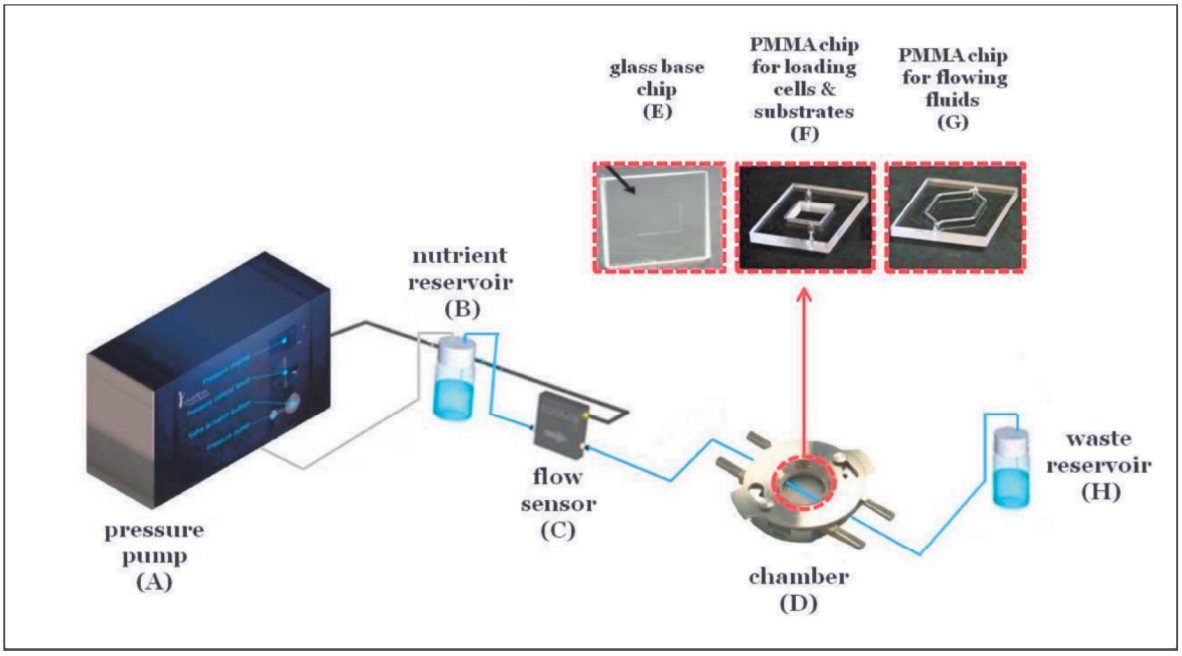

Figure 9: Illustration of the microfluidic device system [12].

After seven days of culture, the results showed that with flow rates of 50 µmL/min (shear stress of 0.5 $\frac{dyn}{cm^2}$) there was a 2.4-fold increase in cell proliferation compared to static conditions. With flow rates of 30 µmL/min (shear stress of 0.3 $\frac{dyn}{cm^2}$) non-significant differences compared to the static culture in terms of cell proliferation were shown. In contrast, the alkaline-phosphatase activity, an indicator of osteoblast differentiation activity, in the microfluidic devices exposed to flow rates of 30 µmL/min had a 1.6-fold increase compared to the static conditions while the ones which were exposed to flow rates of 50 µmL/min did not exhibit significant differences in comparison with static conditions.

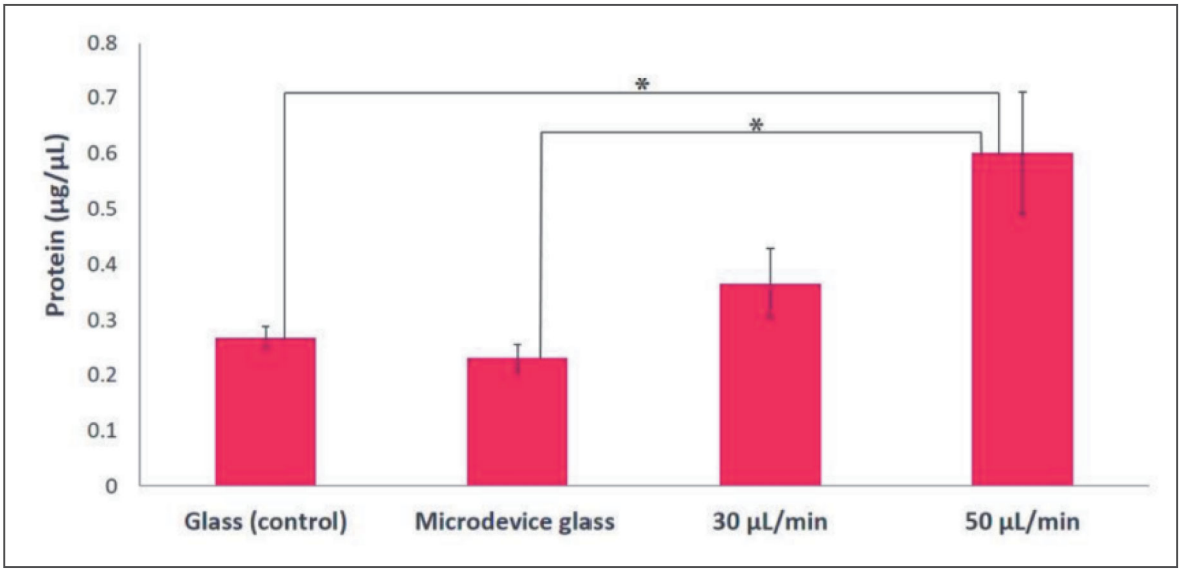

Figure 10: Protein concentration as proliferation indicator after 7 days of culture on collagen substrate. Glass and microdevice glass are static cell cultures while 30 µmL/min and 50 µmL/min are dynamic cell cultures [12].

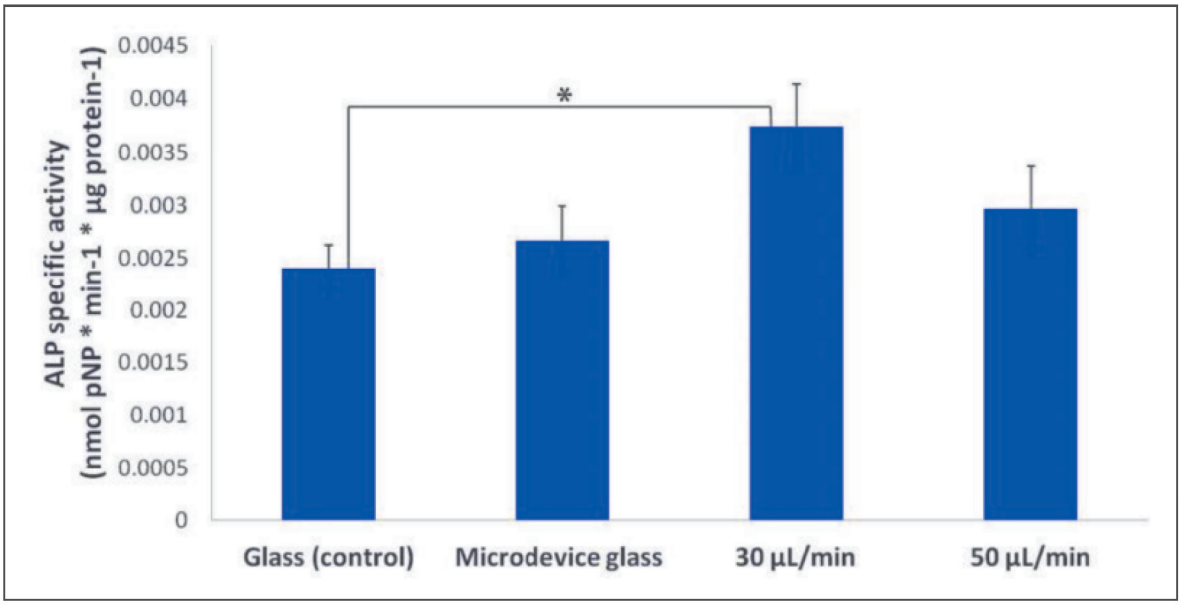

Figure 11: Alkaline phosphatase activity of MC3T3-E1 cells, on collagen substrate, as osteoblast differentiation indicator [12].

Therefore, in this study it was shown that dynamic cultures enhance the proliferation processes. One reason that can explain this is the improvements on mass and oxygen transport within the collagen fibers. Moreover, the introduction of flow rates could facilitate the removal of waste products [12].

On the other hand, the investigation of the interactions between cancer cells with other type of cells and cancer cells with tissue, in a bone microenvironment, is also achievable by using microfluidic devices. In the study of (Jeon, J. S. et. al., 2014) [18] a PDMS microfluidic device, fabricated via soft lithography technique, was used to analyse the extravasation process of breast cancer cells, through a microvasculature network, into a bone-mimicking microenvironment. The microfluidic device consisted in three channels: in the central channel they initially seeded osteoblast-differentiated cells (OBs), endothelial cells (ECs) and mesenchymal stem cells (MSCs) in gel to form a microvascular network in a bone marrow microenvironment. In the side channels breast cancer cells and some biochemical factors were introduced to flow across the microvascular network. This model can be showed in the next figure:

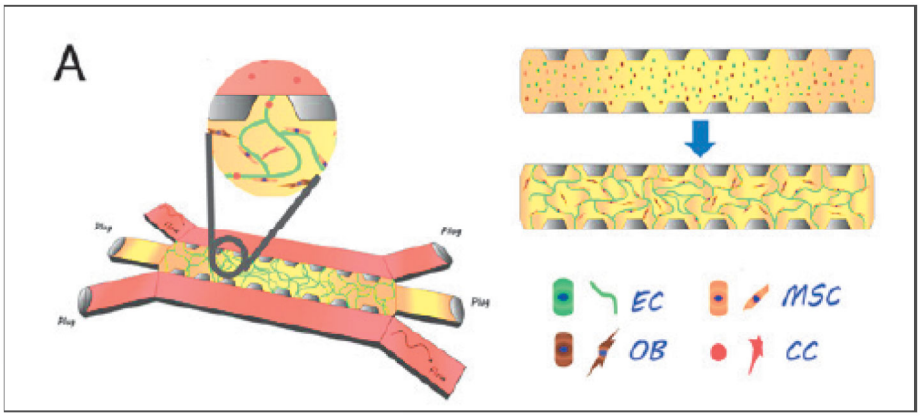

Figure 12: Illustration of the breast cancer cell extravasation model [18].

The results showed that the microvascular network, with a highly branched structure, and the bone marrow microenvironment was successfully formed. This was confirmed with the presence of osteocalcin and alkaline phosphatase, which are secreted by the osteoblastic differentiated cells.

This observation is showed in the figure below:

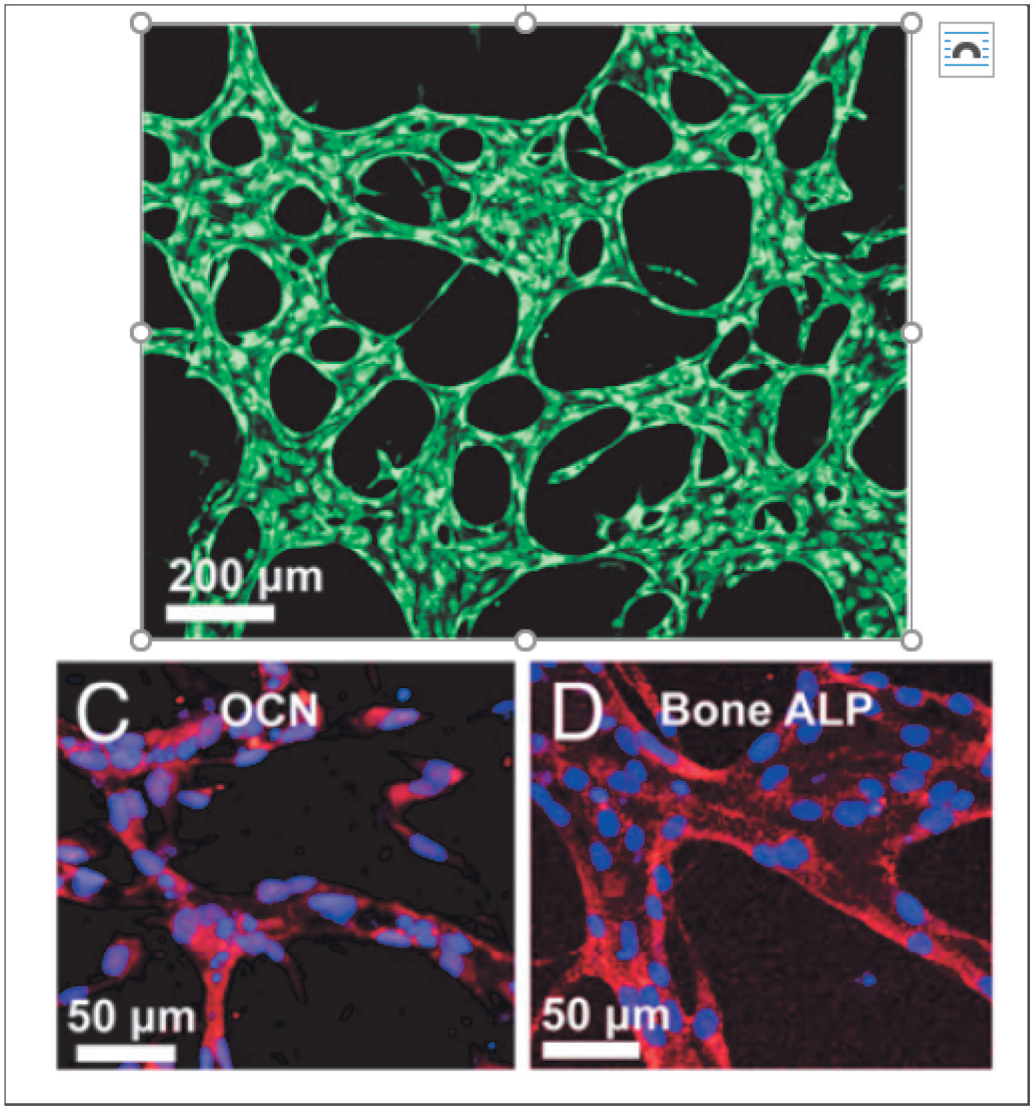

Figure 13: Confocal images of the microvascular network and the presence of osteocalcin (OCN) and alkaline phosphatase (ALP) [18].

As mentioned before, the main objective of this study was to examine the extravasation process of breast cancer cells in a bone marrow microenvironment. Three different microenvironments were performed to make a comparison: acellular, bone and muscle environments. The extravasation rates observed in the bone marrow microenvironment were significantly higher than the other ones. Specifically, the average extravasation rate in the bone marrow microenvironment was 3.8-fold higher than in the muscle microenvironment. Moreover, the vessel permeability study shows that the permeability was 2-fold higher in the muscle model

than in the bone marrow model, so this can explain that the extravasation process is mediated by several factors.

This can be showed in the following figure:

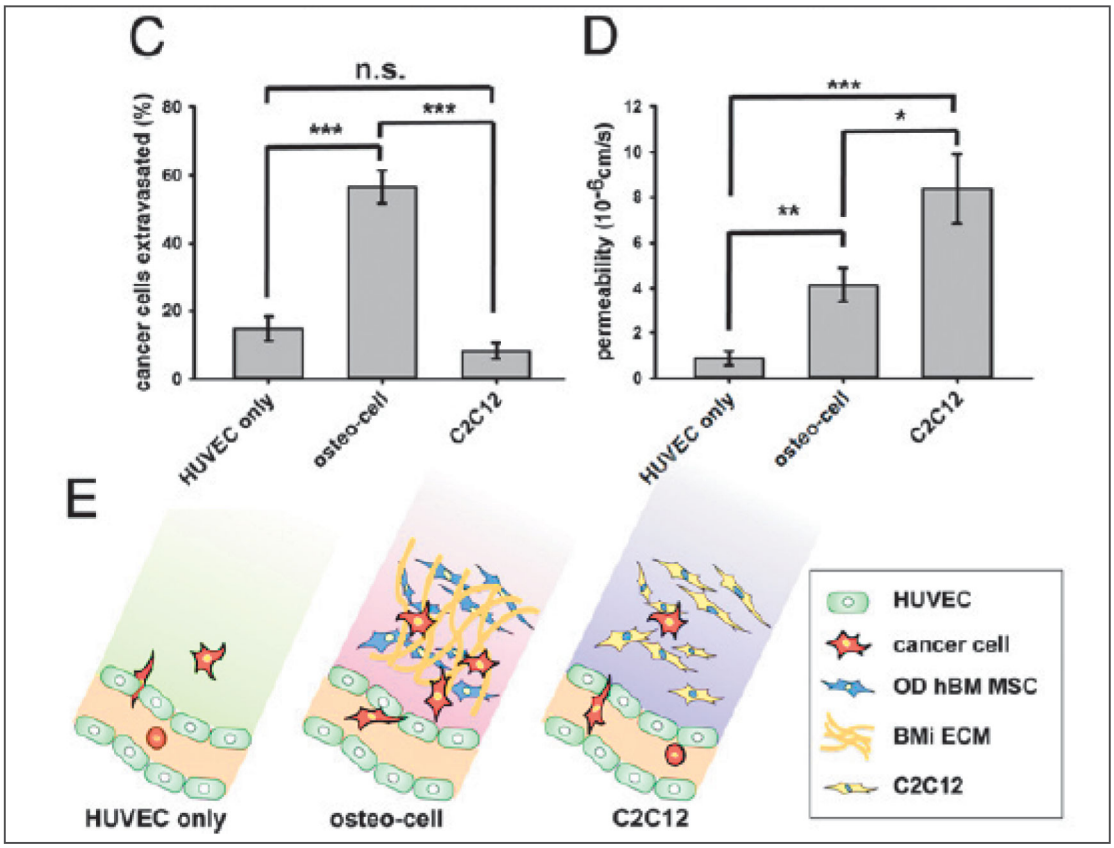

Figure 14: Extravasation rates of cancer cells and permeability in the acellular, bone and muscle microenvironments [18].

In previous studies it was demonstrated that breast cancer cells used to extravasate into the bone tissue [19], which is concordance with the results shown in "Figure 14".

In this project, two designs of microfluidic devices were manufactured, via soft lithography technique, in order to perform a cell culture inside with "hASC" spheroids. The objective consisted in checking if it is possible to completely fill the central channel with spheroids without penetrating the side channels and if the cells can survive after 7 days of culture.

Chapter 2

Materials and methods

2.1-Introduction

In this chapter a description of the bone-on-chip mould designs and the protocol for the fabrication of the microfluidic devices, buckyball microscaffolds, the leaking test and cell culture is explained.

Buckyball microscaffolds are structures with a suitable shape and highly porosity to allow spheroid fusion and simultaneously protect the individual spheroids from mechanical damage. Additionally, spheroids in buckyballs can provide a high initial cell density which means that can reduce the associated time cost for tissue formation [20].

Due to its highly porosity, the different struts which constitute the whole structure of the buckyball are only several microns wide. For this reason, two-photon polymerization technique was used to produce the buckyballs since it is a technique that enables the fabrication of structures in the nanoscale. In this laser-process technique the structures are formed by curing arbitrary spots within the material. The photopolymer is cured by two-photon absorption. This technique provides a higher resolution, about 100 nm or better than the stereolithography method.

2.2-Design and manufacturing process

2.2.1-CAD design

Two models of bone-on-chip microfluidic moulds were designed with "Autodesk Fusion 360" CAD software. Both models have three channels (see Figure 15). The central channel, where the spheroids are going to be contained, is 5.43 mm length and 1.38 mm width. The side channels are 4.52 mm long and 0.42 mm wide. The height of the channels is 0.7 mm. Moreover, both models have 3 inlets and 3 outlets. The main difference between the two designs lies in the central inlet: the first design has a vertical central inlet ("VCI" design) while the other one has a horizontal central inlet ("HCI" design). This was done in order to see which of them is more suitable for introducing the spheroids inside the central channel. It was expected that pipetting the spheroids with the "VCI" design would be easier, but some obstructions could occur in the bottom of the inlet as well. These obstructions could be avoided with the "HCI" design but at the same time the pipetting process would be more difficult. The diameters of the inlets and outlets in the "VCI" design are 1.5 mm,

while in the "HCI" design the diameter of the central inlet is 0.8 mm. Both mould designs are 25.81 mm long, 21.26 mm wide and 5 mm high.

The main objective of the microfluidic devices is keeping the buckyballs, with the spheroids, inside the central channel. According to this reason, pillars between the central and the side channels and a funnel shape outlet channel were designed.

Two illustrations of both designs are showed in the next figure:

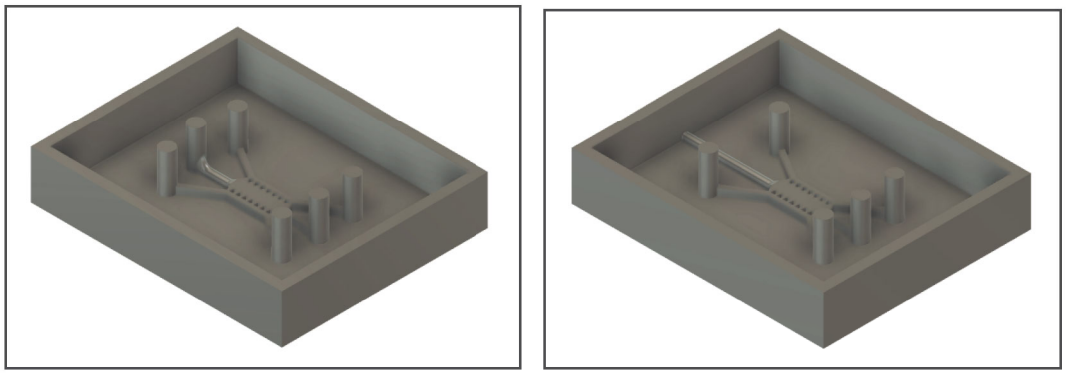

Figure 15: CAD design of "VCI" and "HCI" microfluidic moulds.

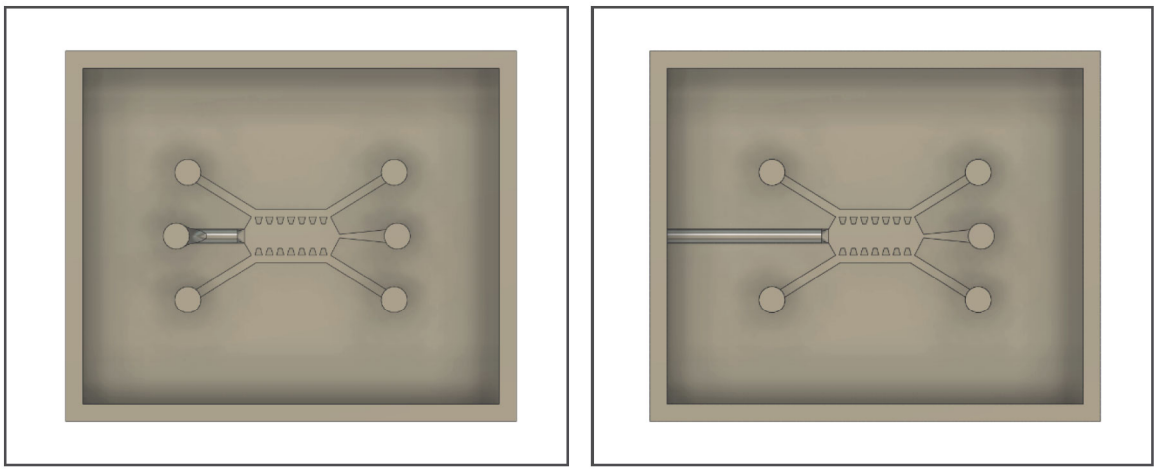

Figure 16: Top view of "VCI" and "HCI" microfluidic device moulds.

2.2.2-Manufacturing process

2.2.2.1-Moulds

The first step to fabricate the chips is obtaining the moulds. In this case, these were fabricated with two different materials and manufacturing processes in order to achieve good

surface quality and well-formed patterns. Specifically, the CAD designs were sent to an external group for the fabrication of the moulds.

The different materials and manufacturing processes used for the fabrication of the moulds are resumed in the next table:

Table 2: Materials and manufacturing processes for moulds fabrication.

Moulds fabrication			
Manufacturing process	**Material**	**Specifications**	**Number of moulds printed**
SLA	Rigid 4000 resin	Fabricated with TU printer	4 “VCI” and 4 “HCI” moulds
SLA	SLA Accura® Xtreme™	Fabricated by "Shapeways" company	1 "VCI" and 1 "HCI" moulds
Material jetting	Fine Detail Plastic	Fabricated by "Shapeways" company	1 "VCI" and 1 "HCI" moulds

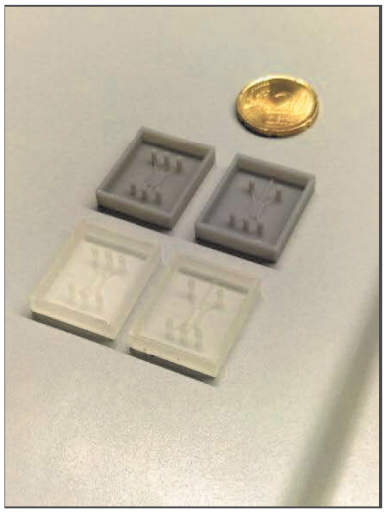

Figure 17: “VCI” and “HCI” microfluidic moulds.

2.2.2.2-Microfluidic chip devices

The microfluidic chips were fabricated via soft lithography technique. The material used was polydimethylsiloxane (SYLGARD™ 184 Silicone Elastomer Kit), also known as “PDMS”, mixed with 0.1 % wt initiator.

The fabrication protocol is described below:

1. PDMS was mixed with 0.1 % wt initiator, in a 50 mL falcon, by using a pipette.
2. Then the mixture was put in a vacuum chamber for 1 hour to eliminate the bubbles.
3. The surface of the moulds was washed with compressed air to avoid the presence of particles.
4. PDMS was poured directly into the moulds with a plastic pipette and then put again under vacuum for 1 hour.
5. Then, the PDMS was cured in an oven at 50 ° for 24 hours.
6. Finally, with the moulds submerged in isopropanol, the PDMS chips were extracted with a spatula.

2.2.2.3-Bonding process of the glass

Last step for the obtention of the microfluidic devices consists in bonding the glass to the PDMS chip. Oxygen plasma surface treatment is normally employed to achieve this. In the PDMS chips, silicon hydroxide bonds are created by replacing hydroxyl groups. On the other hand, in the glass surface silicon hydroxide bonds are formed. After the surface treatment process is done, both surfaces are brought into contact to be chemically bonded. Additionally, this process made temporally hydrophilic surfaces.

The bonding process protocol is described below:

1. The PDMS chip and a 24x32 mm coverslip were placed inside the oxygen plasma chamber and were treated for 10 min.
2. Then, they were brought together, without touching the surfaces with bare hands, to produce the chemical bonding.
3. Finally, the devices were resting for 3 days at room temperature to finish the bonding process.

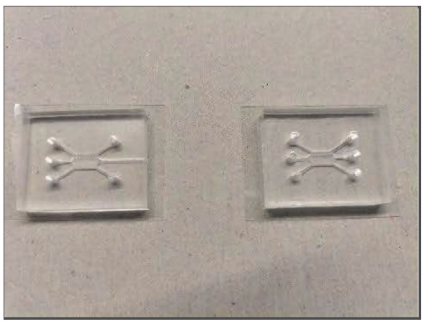

Figure 18: "VCI" and "HCI" microfluidic devices.

2.2.2.4-Protocol for printing buckyball microscaffolds

Buckyballs were used as microscaffolds to form spheroids. As the printing parameters were already established the expected outcome was printing buckyballs, via two-photon polymerization technique, with high shape accuracy. Zr hybrid photoresist with 0.5% wt photoinitiator (4-4 BIS) was used as material [21].

The methodology used is detailed below:

1. A metal holder was used to pour the sample inside. 2 coverslips were stuck to the holder by using two-component dentistry glue.
2. After pouring the material inside the holder the sample was resting overnight.
3. The set up for the printing process was the following:
    - Firstly, the material was placed into the printer holder.
    - Secondly, a calibration for the power of the printer was performed with a power meter.
    - Thirdly, the printing parameters and the buckyball STL file were set.
    - Lastly, the objective and the printer holder were placed into the zero position to start with the printing process. The zero position was established on the surface of the coverslip (Z axis) and in one of the corners of the sample holder (XY plane).
4. After the printing process, the buckyballs were washed in tetrahydrofuran solvent ("THF") for 1 hour and a half. The TFH was changed three times to avoid any kind of residual.
5. Last step was collecting all the buckyballs, on 70 % ethanol, inside a falcon.

Concretely, 2000 buckyballs were printed with "UpNano Nano One printer" using the printing parameters indicated below:

Table 3: Printing parameters for buckyballs.

Printing parameters for "Eve" printer	
Power (mW)	140
Velocity (mm/s)	600
Δxy (µm)	0,5
Δxz (µm)	2,5
Objective	10x
Printing mode	Top down in one block

2.2.2.5-Leaking and buckyballs test for microfluidic devices

This was made as a preliminary to observe if there is any leaking while introducing fluids into the microfluidic devices and if the buckyballs, without spheroids inside, are confined in the central channel without penetrating the other ones.

VCI and HCI microfluidic devices made of "SLA Accura® Xtreme™" and "Fine Detail Plastic" were tested (see "Table 2").

The test consisted in introducing buckyballs on 1-propanol via the central inlet by using a 10-100 µm micropipette.

2.3-Cell culture protocol

2.3.1-Introduction

In this subchapter the cell culture protocol for the microfluidic devices is described.

Firstly, the protocol to produce spheroids inside the buckyballs with V-bottom 96 well-plates was described.

Finally, the cell culture and the following monitoring process were explained.

2.3.2-Methodology to produce spheroids inside the buckyball microscaffolds

Spheroids were formed inside the buckyballs by using V-bottom well plates. These spheroids were employed in the microfluidic cell culture.

Human adipose stem cells ("hASCs") were cultured inside the Zr hybrid 0.5% wt photoinitiator (4-4 BIS) buckyballs to make spheroids and maintain their shape.

Two 96 V-bottom well plates were used to produce spheroids and on each well 1 buckyball was introduced so 192 spheroids in buckyballs were produced in total.

Three steps were done to complete the whole process:

- **Step 1.** 2 V-bottom well plates were coated by using a mixture of lipidure in 0,5% wt ethanol. Then the well plates were drying for two days.
- **Step 2.** Second step was introducing 100 µL of water to activate the surface and 1 buckyball on each well by pipetting with a 100 µL tip. Then the samples were centrifuged with 1000G for 5 minutes to make sure that the buckyballs were in the bottom of the well. After this, the microplates were left for 2 days in the laminar flow to dry so the water can evaporate.
- **Step 3.** Third step was introducing hASCs with 4000 cells/well in 100 µL of cell endothelial cell growth medium-2 ("EGM-2") per well and incubating for 2 days so the spheroids could be formed.

2.3.3-Methodology for cell culture inside the microfluidic device and the following monitoring process

The main purpose of the experiment was seeing if the spheroids could survive for 7 days in a microfluidic cell culture and if they were forming a tissue by merging together.

"hASCs" spheroids in buckyballs, in EGM-2 media, were collected in a falcon tube and then introduced into a microfluidic device by using a 100 µL micropipette. The microfluidic device was previously filled and covered with 70 % ethanol for 1 day to sterilize it.

The microfluidic device was rinsed with sterile water and then with phosphate buffered saline ("PBS") before introducing the spheroids.

192 spheroids were used to inject them into the central inlet. Then the central inlet and outlet were closed with tips covered with 2 component dentistry glue to prevent the spheroids to escape and the medium to leak. Additionally, 2 empty tips were introduced in one of the side channels and other 2 tips filled with 100 µL "EGM-2" were introduced into the other side channel so the cell medium can flow from one of the side channels to the other side channels by gravity. Also, the microfluidic device was inside a tip box with "PBS" inside to avoid the evaporation of the cell media inside the microfluidic device.

Pictures were taken every day with the 700 LSM light microscope (2.5x objective and 10x objective) and the medium was changed every day as well. After 7 days, live-dead staining was performed to discriminate, under fluorescence microscopy, between viable and non-viable cells. Green fluorescent calcein-AM will indicate intracellular esterase activity while red-fluorescent ethidium homodimer-1 will indicate loss of plasma integrity.

Chapter 3

Results and discussion

3.1-Surface quality of the microfluidic moulds

3.1.1-Moulds fabricated with TU printer

The "Rigid 4000 resin" moulds fabricated via stereolithography (see "Table 2") exhibited a rough surface and the pillars were not formed (see "Figure 19").

After seeing that the surface quality of the moulds was not suitable for cell culture experiments, they were washed again in isopropanol, but no better results were achieved.

This can be noticed in the following figure:

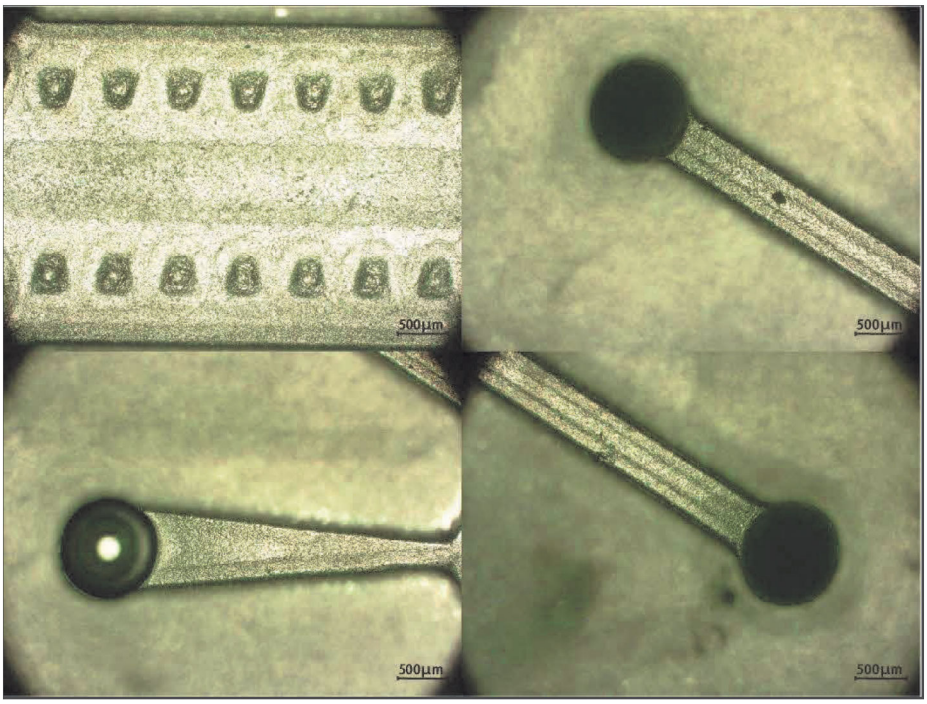

Figure 19: Surface quality of the "Rigid 4000 resin" mould fabricated via stereolithography technique with TU printer. Images were taken with "LSM 700" light microscopy. Scale bar is 500 µm.

3.1.2- Moulds fabricated by “Shapeways” company

The surface quality of the “Fine Detail Plastic” moulds was not suitable, rough instead of smooth, but all the patterns were well formed (see “Figure 20”). It is even possible to see the printer lines on the surface of the mould.

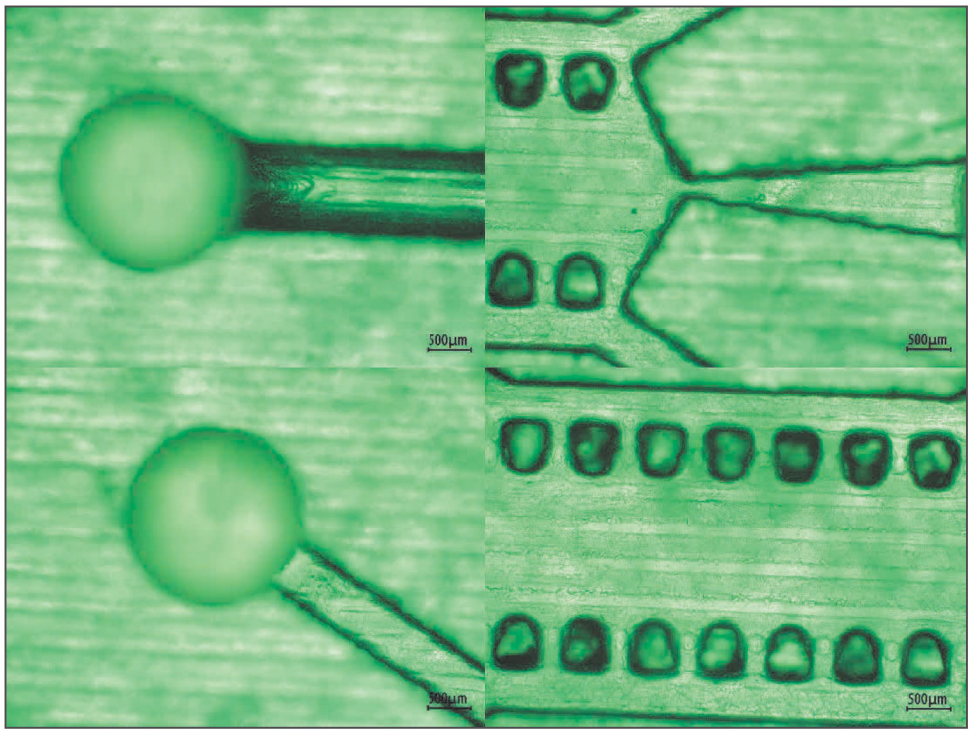

Figure 20: Surface quality of the “Fine Detail Plastic” mould fabricated via material jetting technique by “Shapeways” company. Images were taken with “LSM 700” light microscopy. Scale bar is 500 µm.

On the other hand, the surface quality of the "SLA Accura® Xtreme™" moulds were smoother than the "Fine Detail Plastic" moulds but still was not smooth at all since it was possible to see some roughness (see "Figure 21"). Regarding the pillars, in this case all of them were well formed. However, the central outlet resulted to be closed due to the manufacturing process so the fluids might not be able to exit through this channel. For the experiment intended, this might not represent a problem, as the liquid can escape through the side channels.

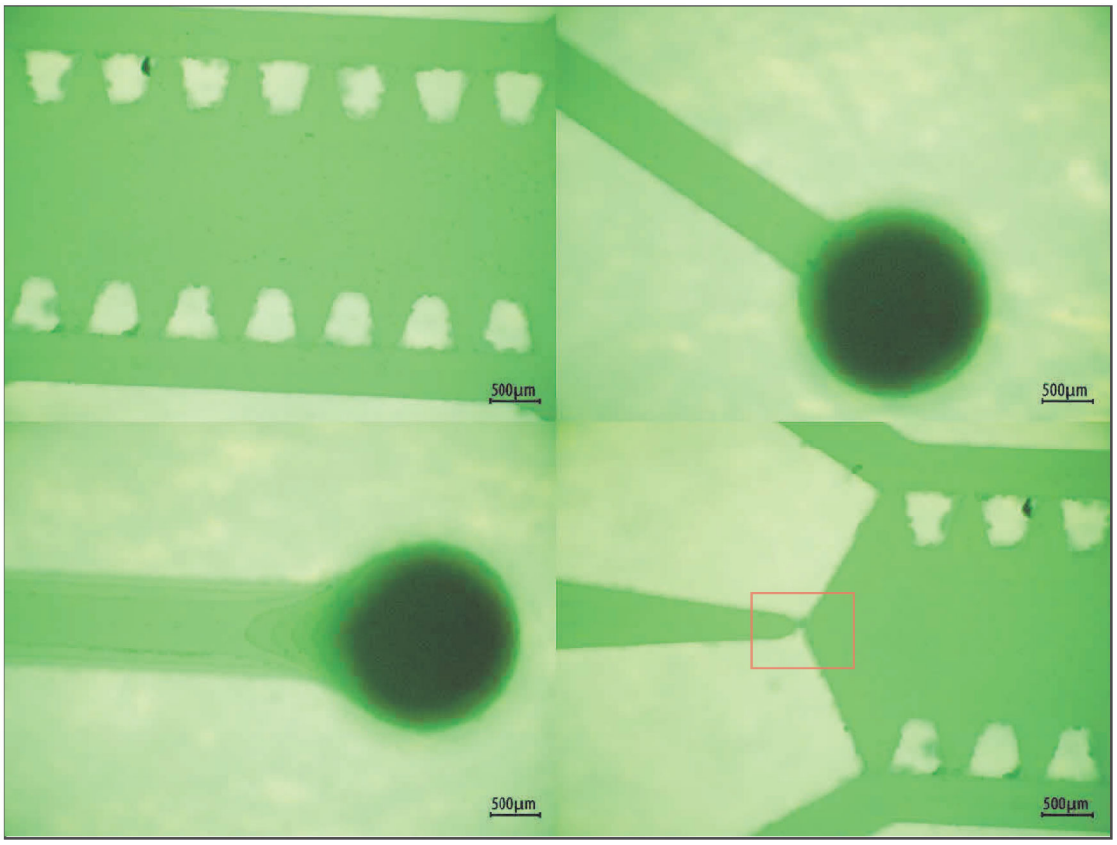

Figure 21: Surface quality of the "SLA Accura® Xtreme™" mould fabricated via stereolithography technique by "Shapeways" company. Images were taken with "LSM 700" light microscopy. Scale bar is 500 µm.

3.2-Leaking and buckyballs test for the microfluidic devices

Microfluidic devices with horizontal central inlet ("HCI") have debonded from the glass after introducing the micropipette into the central inlet. Also, the "Fine Detail Plastic" microfluidic device with vertical central inlet ("VCI") has debonded from the glass after the leaking test because the bad surface quality was not allowing sufficient adhesion.

On the other hand, the "SLA Accura® Xtreme™" microfluidic device with vertical central inlet ("VCI") did not show any leaking and could confine the buckyballs, without spheroids inside, in the central channel without penetrating the side channels. However, air bubbles were introduced as well, so the central channel was not completely filled with buckyballs (see "Figure 22").

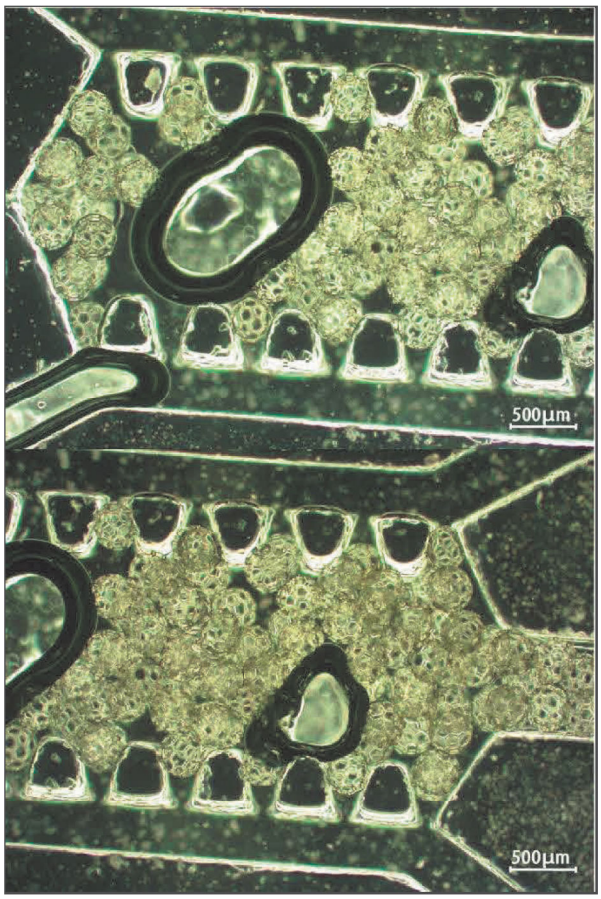

Figure 22: Leaking buckyballs test shows how the buckyballs, without spheroids inside, are confined in the central channel without reaching the side channels. Images were taken with "LSM 700" light microscopy. Scale bar is 500 µm.

3.3-Printing accuracy of the buckyball microscaffolds

The Zr hybrid photoresist with 0.5 % wt photoinitiator (4-4BIS) buckyballs showed a good shape accuracy after the fabrication process via two-photon polymerization technique (see "Figure 23").

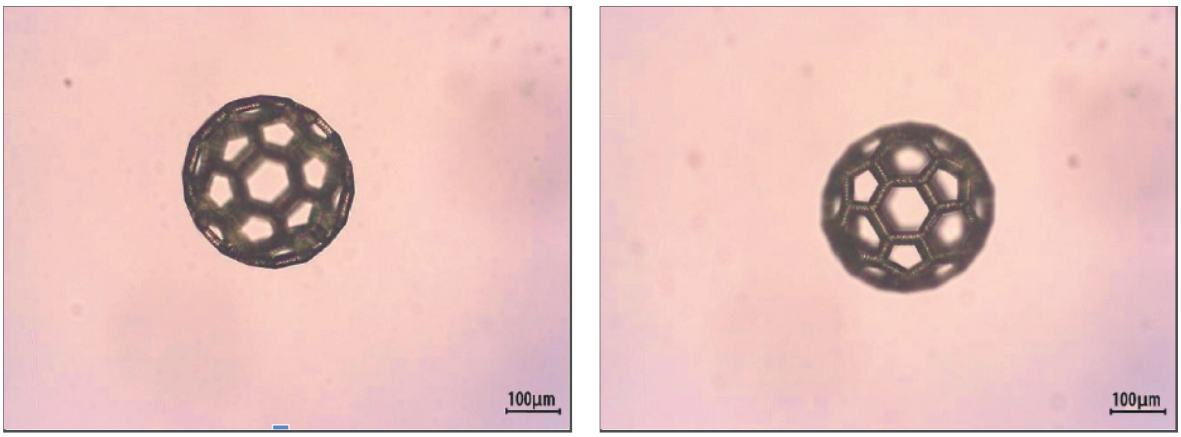

Figure 23: Shape accuracy of the 300 µm buckyball microscaffolds. Images were taken with "LSM 700" light microscopy. Scale bar is 100 µm. On the left image the focus is on the periphery of the buckyball while on the right image the focus is on the internal zone.

3.4-Cell culture results

As mentioned before in the "Subchapter 2.3.3", pictures of the microfluidic cell culture were taken every day, from day 0 to day 7. In this case, spheroids inside buckyballs were introduced on day 0. Moreover, on day 7 live-dead staining was done to discriminate, under fluorescence microscopy, between viable and non-viable cells.

The results of the cell culture are showed in the next figures:

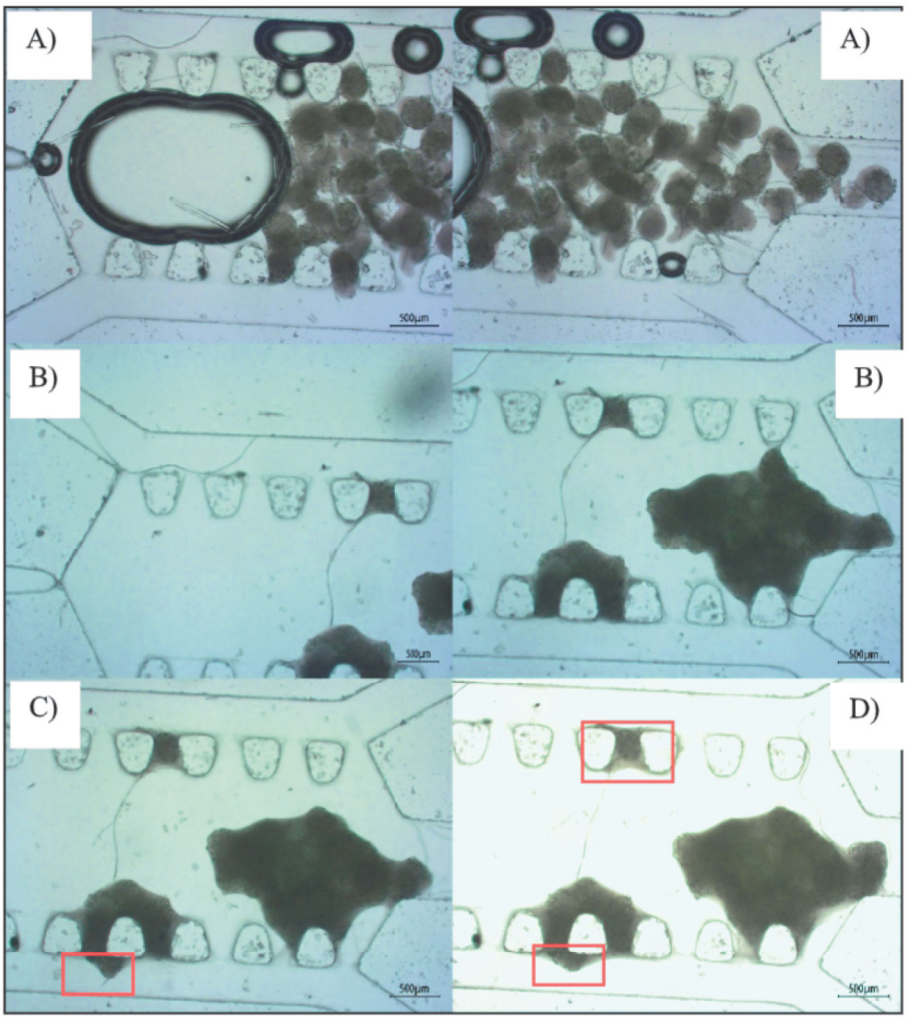

Figure 24: Cell culture results from experiment 2: A) day 0, B) day 3, C) day 5, D) day 7. Images were taken with "LSM 700" light microscopy. Scale bar is 500 µm.

Some aspects can be observed in the images shown in "Figure 24", "Figure 25" and "Figure 26":

1. On day 0 presence of air bubbles inside the microfluidic device is observed due to the difficulties of introducing the spheroids inside by using a micropipette. However, air bubbles were completely removed on day 3.
2. Spheroids are fused together so the volume occupied by them is reduced day by day. The buckyball microscaffolds should keep the volume constant to ensure a high initial density of cells, however it can be noticed how most of them were

destroyed after the pipetting process so the volume of cells could be altered. Also, it can be seen, on day 3, 5 and 7, how the cells are attached to the pillars.

3. On day 5 and 7, cells were growing on the side channels as well and adhering to the PDMS (see "Figure 25").

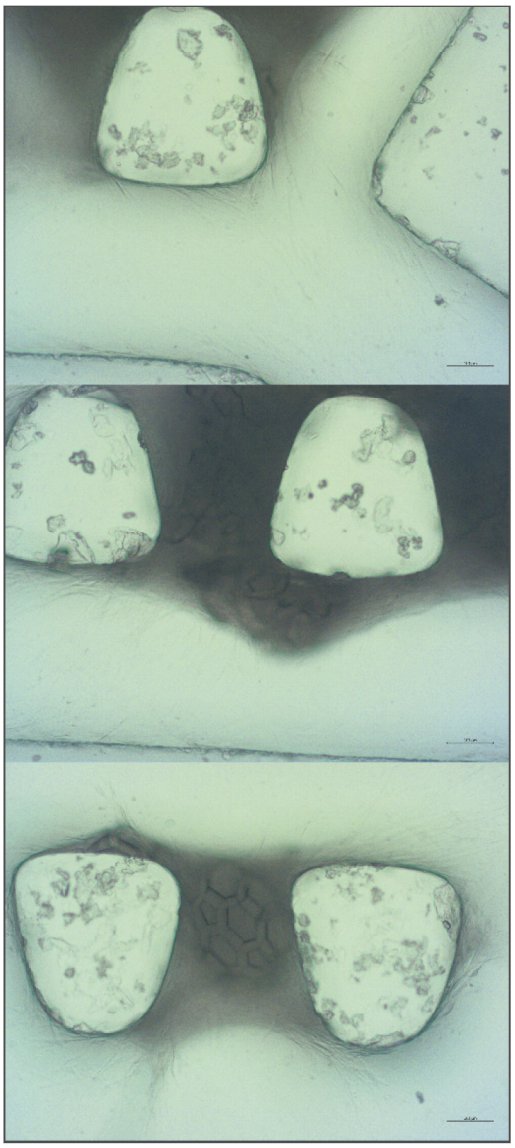

Figure 25: Cell culture results on day 7 from experiment 2. Images were taken with "LSM 700" light microscopy. Scale bar is 100 µm.

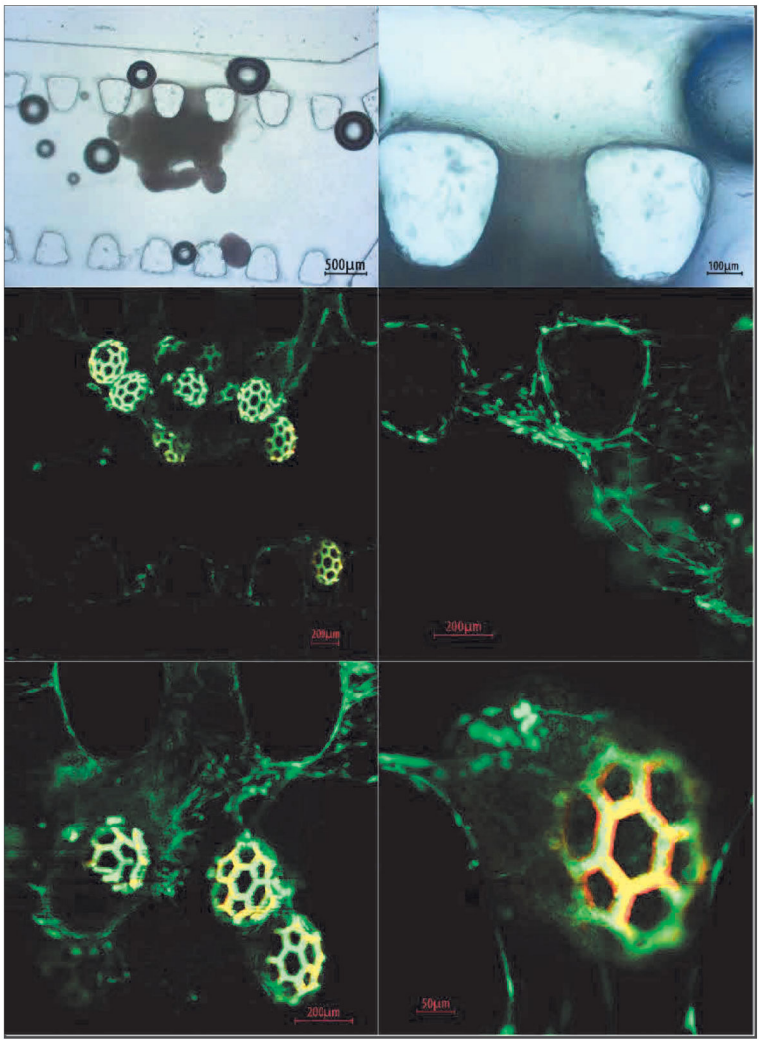

Figure 26: Cell culture results, from experiment 1, on day 7 and live-dead staining of the cells. Images were taken with "LSM 700" light microscopy and laser microscopy.

Images of "Figure 26" show that cells are still alive after 7 days of culture. Moreover, it is not possible to analyse the cells that are inside the buckyballs due to the high autofluorescence of the Zr hybrid buckyballs.

Chapter 4

Conclusion

4.1-Conclusion

According to the results of the surface quality of the moulds ("Figures 19, 20, 21") and the leaking test with buckyballs without spheroids inside, ("Figure 22"), the VCI "SLA Accura® Xtreme™" mould fabricated via stereolithography technique was the best solution for the obtention of the microfluidic devices. Microfluidic devices obtained with this mould did not show any leaking of fluids and have the smoothest surface in the microscale.

Additionally, several conclusions can be mentioned according to the results obtained from the cell culture experiments ("Figures 24, 25 and 26"):

- Firstly, it was not possible to completely fill the central channel with spheroids by pipetting due to the presence of air bubbles inside the microfluidic device. Air bubbles present interfacial stress so some of the spheroids go away through the central inlet due to this resistance. For this reason, another method must be chosen to avoid this problem: the use of a micropump, introducing spheroids by pipetting in an opened microfluidic device and then bond the glass with glue to the microfluidic chip or reduce the length of the channel so less spheroids would be needed to fill it.
- Secondly, it can be noticed that cells were also growing inside the side channels due to the presence of deformations. Most of the buckyballs were destroyed during the pipetting process due to the difficulties on filling the central channel, so the spheroids can be deformed and even cross to the side channels.
- Thirdly, the results of the live-dead staining show that cells were alive after 7 days of culture. This means that the selected protocol mentioned in "Subchapter 2.3.3" could work for the differentiation process of the stem cells into osteoblasts that takes 4 weeks to be done normally. On the other hand, it was shown that cells proliferated onto the PDMS, which was not expected. Therefore, treating the surface with lipidure could be an option to render the surface more hydrophobic and less prone to cell attachment.

As future lines of investigation, more cell culture experiments should be done to verify if it is possible to completely fill the central channel with spheroids, inside buckyballs, and create bone

tissue inside without reaching the side channels. The alkaline-phosphatase activity could be used as an indicator of osteoblast differentiation activity to verify that bone tissue was formed during the cell culture [12]. Moreover, surface coating of the microfluidic devices could be performed to prevent cell attachment.

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