THE ART OF JOINING THE SYNTHETIC AND THE NATURAL TOGETHER

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INTRODUCTION

Topic

Humans are social animals, we just have the need to constantly communicate. Personally, I have always adored to observe and analyze daily situations that occur and, especially, an admired concept of mine, **interactions**. The word *interaction* means "the action of having an effect from one subject to another".

Having that on mind, a topic to investigate was to pick for this research project, and I had a strong curiosity about how our inside was able to carry all of their processes when it was damaged: my interest was focused on **internal** interactions. However, that appeared complex to study until I bumped into bioengineering, and, especially, into tissue engineering and materials science.

After I became informed about these subjects, I noticed their complexity, and that developed into a kind of a challenge. I discovered I was constantly eager to know what could happen to our cells when biomaterials were implanted inside our body in any case, and how could both cells and these biomaterials change and react.

Moreover, my first thoughts were arisen from the fact that there is a lack of knowledge about these fascinating topics on our scholar environment, and a divulgation about these subjects from the same scholar environment would for sure help more people to discover them. My thoughts were that these topics deserved an investigation and latter divulgation.

However, why have I decided to apply tissue engineering to cell interactions? It is because bioengineering is the future of medicine and the future source of solutions for our current health problems. Getting a grasp on the effect biomaterials have in our body I think is key to understand the scope and the possibilities that biological engineering has.

Expectations

The basis on which I will investigate is getting to know what happens on cell-biomaterial interactions, considering biocompatibility and how the properties of the biomaterial and the cell change according to that interaction, researching its conditions.

I really expect to be granted a higher maturity level regarding science and a fulfilling first experience on writing and exposing an academic investigation project, apart from the amount of knowledge I am going to acquire on both theoretical and practical frameworks.

Curiosity is my motivational motor, and, by the same token, I am profoundly motivated to start my research.

Prudently, I can also foresee it will be a demanding investigation project, as many topics deserve a contextualization and their state-of-the-art, so brief introductory chapters are going to be exposed to contribute to the easy understanding of this project. Also, worth to mention, seeking for the interest of the readers in these topics is one of my main priorities.

Structure

To begin with, to first clarify all the concepts and then truly investigate, this project will be split into theoretical framework and practical framework.

First of all, the theoretical framework will consist of four main blocks:

I. An **introduction about tissue engineering**.
II. **Biomaterials**.
III. **Scaffolds**.
IV. The **interactions between cells and material surfaces**.

The intention of this structure is to gradually go from the exposure of the academic and theoretical knowledge on bioengineering to a presentation of creative applications that are already thought or yet to be discovered and plotted.

Shading light on this project's fieldwork or the practical framework, we have to expect six differentiated studies:

1. The **decellularization of a mouse's heart**, to show how an organ can be a natural scaffold.
2. The **fabrication of 3D scaffolds**, followed by a drug delivery technique, in two experiments.
3. A **CAM assay** is going to be done in order to induce the creation of new blood vessels on scaffolds or, in other words, angiogenesis. It is going to be checked out whether calcium is or is not a proangiogenic factor.
4. **PH and calcium measurements** and calculus to determine the calcium released by particles inserted in scaffolds.
5. A **fluorescent staining of cell-seeded scaffolds** will be carried out, in **two experiments** in different conditions and methods.
6. To shade light on bioethics, an **ethical consideration on biomaterial implants** regarding translational research[1] is going to be fulfilled.[2]

The materials used and the procedure parts of the studies will be implemented on the **Annexes** section, as well as two interviews, that have been carried out asking the opinion about the future and the current problematics and advances of bioengineering on two of the best bioengineers in history and even their creators, **Dr. Jeffrey Karp**, the world-leading researcher of the tissue adhesives, and **Dr. Robert Langer**, one of the founding fathers of tissue engineering and the founding father of drug delivery systems.

Objectives

These project's goals are going to be explained just before each of the two parts. Following this condition, now there are going to be enumerated the objectives of the theoretical framework section:

- ✓ **To absorb, comprehend and to be able to interpret clearly the fundaments of bioengineering and all the knowledge that surrounds these ideas.**
- ✓ **To be able to summarize what has been learnt.**
- ✓ **To communicate in an enjoyable manner what has been learnt.**

[1] Translational research applies findings from science to medical research, to solve a health problem. For biomaterials, apart from the investigation process, they undergo preclinical trials (tests on animals) and clinical trials (tests on humans) to eventually be implemented or not to the market for society.
[2] The sixth study will be implemented as an extra study in the **Annexes section** as well.

Just before starting with the theoretical framework, many new words have been introduced to us, and an introduction to bioengineering is going to be carried out set as a point of departure, being the **Preface**.

PREFACE

As you can see in FIGURE P.1, bioengineering (the even "metaphor" in which tissue engineering is included) consists of loads of topics, some of them correspond and fit perfectly with a specific science branch, and some, consequently, do not, for instance tissue engineering, biomaterials, or cellular interactions, the three topics I will relate and explain in this research project.

Bioengineers study biology from a perspective biologists do not: from the engineering perspective, studying not only chemistry or biology but also getting a grasp on electrical and mechanical engineering.

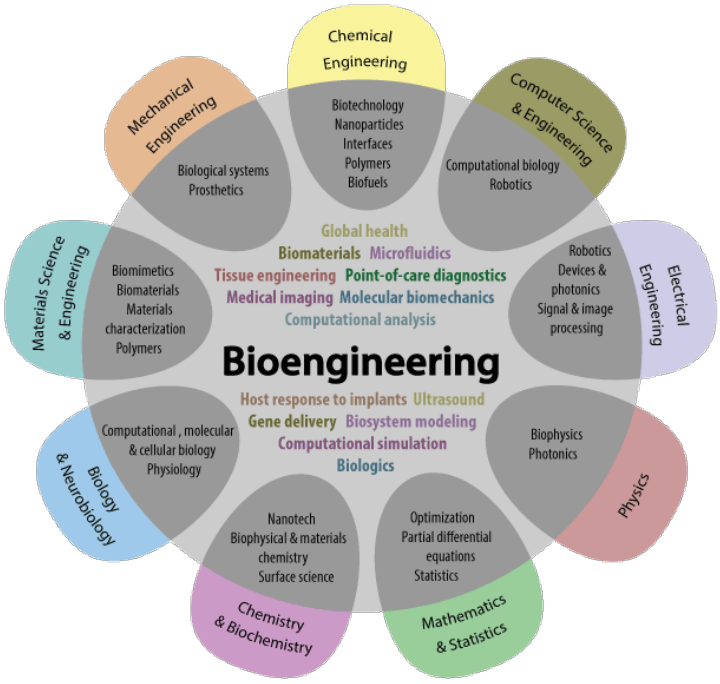


FIGURE P.1. Bioengineering is a huge multidisciplinary field that takes many different professionals to resolve complex society and health problems.

Biological engineers are everywhere: in ecological and environmental fields, in food processing and genetic engineering… and from different sides: academic, research and industrial.

These professionals want and are going to change the world, as rapid advances are being done all around the globe.

CHAPTER 1

INTRODUCTION TO TISSUE ENGINEERING

I. Interdisciplinary by necessity
II. Tissue paradigm
III. Classical tissue engineering
IV. Modern tissue engineering
V. Stem cells
VI. Repair vs regenerate
VII. How to stimulate regeneration

"The science of today is the technology of tomorrow."

-Edward Teller, American physicist.

I. INTERDISCIPLINARY BY NECESSITY

It is widely known that every day many surgical procedures are performed to replace or to repair damaged tissues when we suffer a disease or a trauma or even a malformation.

What comes next is the treatment, which generally consists in transplanting tissue from one site to another in the same patient (typically hair or skin, defined as an autograft), or this tissue could be transplanted from one subject to another (as an allograft), as it can be seen in FIGURE 1.1.

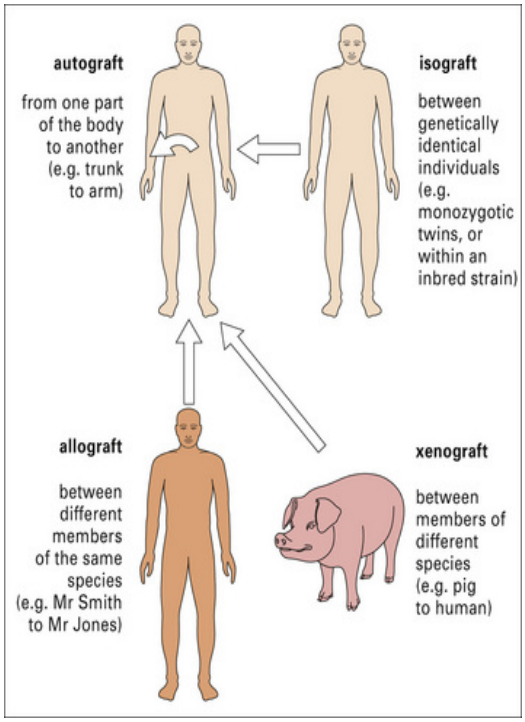


FIGURE 1.1. Visual scheme of the name given to the transplant depending on the genetic relations of the individuals.

These treatments, despite lifesaving, have some major complications, in both techniques:

Performing autografts is painful and have limitations related to our anatomy, as we suffer from infection and hematoma. On allografts there is not enough tissue available to transplant (see FIGURE 1.2.) and infection and diseases could be spread from one patient to another as well as a decrease of the endurance of the organ could happen, leading to even fracture over time.

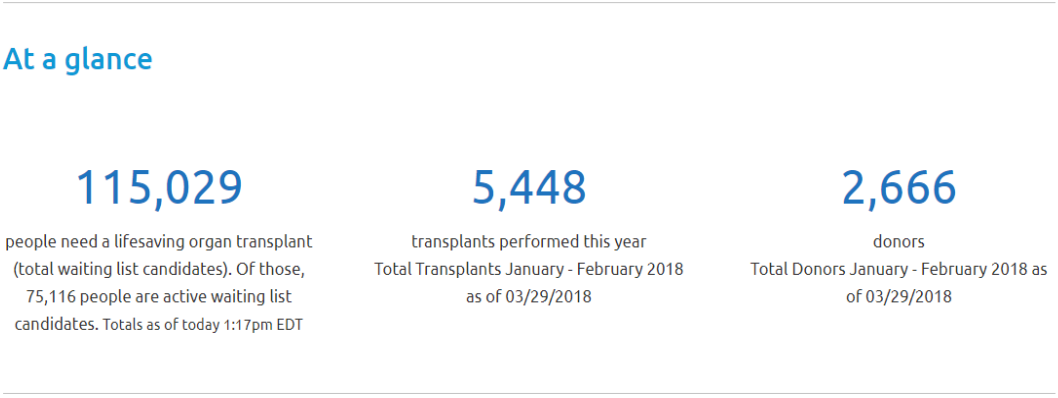


FIGURE 1.2. Screenshot taken on March 2018 of the statistics of February 2018 available at the UNOS website, the United Network for Organ Sharing.

And here is when tissue engineering comes into play as an alternative. The goal of tissue engineering is to understand the structural and functional relationship in normal and pathological tissue and to develop biological substitutes, new "artificial tissue" to substitute the limited amount of natural tissue.

This definition above appeared firstly at a National Science Foundation workshop in 1988, and the scientific community have entitled it as the standard definition of tissue engineering.

In this workshop it was also affirmed that tissue engineering is an **INTERDISCIPLINARY** field

which applies the principles and methods of engineering and life sciences.

An interdisciplinary field could be defined as a study that combines several academic disciplines, such as physics, material science, mathematics, biology… To develop a new thinking, normally based on fundamental sciences.

Usually when we think about a topic in science there is a tendency to fit that topic inside a tag, a category, for example: if we hear about statistics, we will automatically correspond it to mathematics. I believe that is a mistake, as, being a perfect example, tissue engineering brings together material scientists, geneticists, mechanical engineers, etc.

II. TISSUE PARADIGM

What is the route scientists describe for tissue bioengineering?

Firstly, as it can be appreciated on *FIGURE 1.3*, a biopsy is practiced to the patient whose tissue is damaged, keeping cells to its later proliferation, to, eventually, with the help of a scaffold, growth factors and mechanical stimuli, develop a tissue ready to be implanted on the same patient.

Referring to the growth factors, these are a huge and diverse group of proteins that regulate the growth of diverse cell types, depending on the organ we are mimicking.

This might seem quite easy at the beginning, and the cell culture and later proliferation could be, as well as performing a biopsy to create a cell source can be. The problem appears when it comes to the implantation part. Unfortunately, tissue engineering is not a commercial and established reality yet, and it is just because of this arrow above, in FIGURE 1.3.

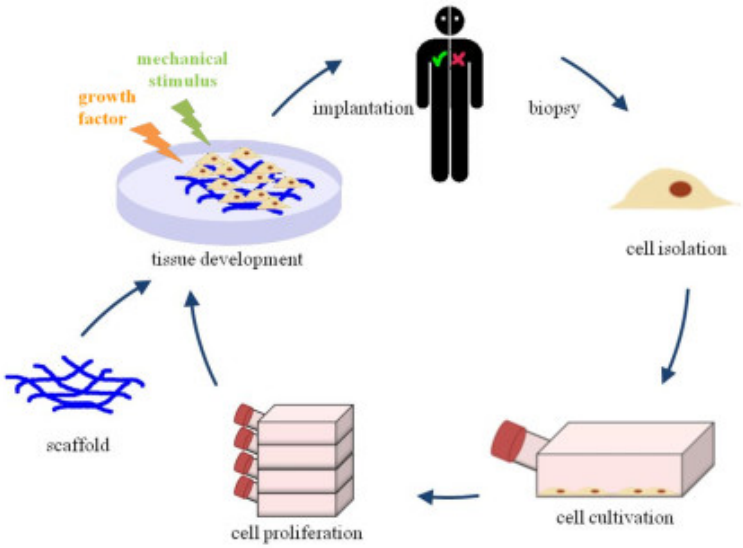


FIGURE 1.3. The tissue paradigm.

As the problematic comes from the tissue development, it can majorly be caused by the subject we know less about, in other words, the problem is mostly caused by the lack of knowledge on cell-scaffold[3] interactions.

Therefore, this idea already explained reinforces the need to know about biophysics.

III. CLASSICAL TISSUE ENGINEERING

To be capable of advancing in our society, we must always consider and learn about our past.

FIGURE 1.4. "Guarigione del diacono Giustiniano".

This painting (FIGURE 1.4.) is called *"Guarigione del diacono Giustiniano":* in English, "Healing of Justinian", painted in 1443 by Fra Angelico, an Italian Renaissance painter. This painting describes a limb transplant to a

[3] Still being an unknown word for us, the scaffold will be defined later.

wounded soldier. Historically, this piece of art has remained as the first reference to tissue engineering. However, in the Bible “one of the miracles God did” were actually transplants, then we could say that the Bible itself is the oldest written reference to tissue bioengineering.

Until this time of history, we could affirm that the generation of tissue and organs was even kind of a myth. One example of such is on the Greek mythology, for instance Prometheus’ generation, which was, supposedly, a generation of a person without sexual reproduction. Another one is the Biblical tale of Adam and Eve, both being born in the light of cloning.

Before tissue engineering being defined, in the European Renaissance many scientists observed alchemy as a tool to generate living organisms. We could conclude that tissue engineering was now being a dream. However, this was not just an idea to be left on their minds, as Theophrastus von Hohenheim, known as Paracelsus, tried without success to create human life mixing chemical substances.

The first real practical efforts were materialized when both clinical medicine (and specifically surgery) and biology (and more specifically biochemistry) advanced, leading to the discovery of biomaterials, materials that are designed to stay in contact with living tissues for a determined period of time, depending on the interests.

The first biomaterials used were metallic ones (FIGURE 1.5.). The first ones were designed for dental uses, with John Hunter (1728-1793) being a pioneer on this field.

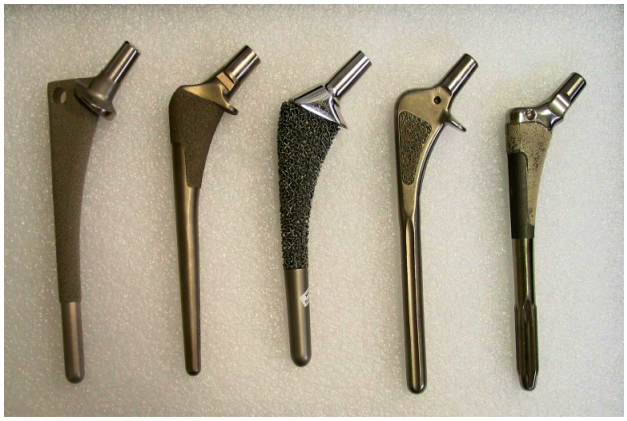


FIGURE 1.5. The first biomaterials were metallic. These are modern titanium hip prothesis.

As already mentioned in the Interdisciplinary by necessity section, the term tissue engineering was defined at the Keystone (Colorado) conference named “Tissue Engineering”, organized by the National Science Foundation. However, the term tissue engineering was not viewed as it is today.

The early years of tissue engineering were unsuccessful, as expected. However, that was not a failure, as they did come to the correct conclusion, affirming that with innovative biocompatible materials it would be possible to generate new tissue, by seeding cells to appropriate scaffolds.

Dr. Joseph Vacanti and Dr. Robert Langer are the founding fathers of tissue engineering, as they came up with an idea to design new scaffolds instead of inducing natural scaffolds, as physical and chemical properties could not be manipulated, resulting in unpredictable results.

All these experiments shape what I have named the classical tissue engineering, and all the experiments beyond these mentioned, are to be considered part of the modern tissue engineering.

IV. MODERN TISSUE ENGINEERING

After Vacanti studied how to generate functional tissue equivalents using a diverse amount of synthetic and biodegradable polymers as scaffolds with viable cells, he configured the most cited publication about tissue bioengineering, with the help of Dr. Langer. It was published on 1993.

From the milestone of Vacanti and Langer experiments, a large amount of research centers have focused their research on tissue bioengineering in the United States and Europe. The most significant first effort was in Boston in the early 90s, the Pittsburgh Tissue Engineering Initiative (PTEI). Outside the US, Dr. Polak, a stem cell biologist, focused her research in tissue engineering as well, in London.

The view of tissue engineering nowadays is:

"Tissue engineering is the application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function."

All the research on the next years after that definition was focused on tissues and cell culture.

Tissue engineering became of public awareness after a BBC broadcast, exploring the potential of this new science, and offering the image of a "mouse with a human ear", also known as the Vacanti Mouse, made by Charles Vacanti, brother of Joseph, in order to transfer the idea and concept of tissue engineering with an effective visual power.

Now referring to regenerative medicine[4], it has one of his bases on stem cells, the next section.

V. STEM CELLS

Stem cells are unique

Stem cells are unique, they are undifferentiated cells able to differentiate into a specialized cell.

They have general properties that differ from any other cell.

The specific factors that determine the unspecialized nature of stem cells are still unknown to scientists, and controlling many of the variables that can determine what specialized cell will

[4] Regenerative medicine and tissue engineering necessarily have to be combined, and that's why sometimes tissue engineering and regenerative medicine are referred as the same field, although tissue engineering tends to be included in regenerative medicine. We are going to be explained about regenerative medicine from the stem cells' perspective from a global perspective, including tissue engineering inside regenerative medicine.

that stem cell turn into, such as the substrate stiffness, is going to allow scientists to control cell-based therapies, as well as other important methods that could be developed into innovative applications.

They come from two main sources, from the embryos formed during the blastocyst phase of embryological development, they are called embryonic stem cells, and from adult tissue, and they are called adult stem cells. There are different kind of stem cells, worth to be mentioned, as in our practical framework we are going to work with one of them.

ADULT STEM CELLS

They are also called tissue-specific cells or somatic cells.

They exist in the body after embryonic development and are found in tissues such as the brain, blood, skeletal muscles… They remain in a non-dividing state until activated, by a disease or a tissue injury.

Adult stem cells can divide indefinitely, making them able to generate many cell types from the originating organ. It is thought that adult stem cells are limited in their ability to differentiate depending on their tissue of origin, but there is evidence that they can differentiate in other cell types.

One example of tissue-specific cells is the hematopoietic or blood-forming stem cells. They are in the bone marrow and they can give rise to red and white blood cells but not liver cells, for example, and vice versa, cells on our liver cannot form red blood cells, for instance.

- *MESENCHYMAL STEM CELLS*

These are the cells that will be used on the fieldwork. Abbreviated MSC, they refer to cells isolated from the stroma, the part of a tissue that has a connective or structural function. They are also called, more accurately, stromal cells, by many scientists. Mesenchymal stem cells are thought not to be all the same, and characteristics may be different depending on the tissue of origin.

EMBRYONIC STEM CELLS

They are derived from a four- or five-day-old embryo, in the blastocyst phase of development. The blastocyst phase is the phase in which the embryo presents a complex cellular structure of 200 cells approximately.

Usually, the embryos are extras, created in in vitro fertilization clinics. Several of them are fertilized in a test tube, but only one is implanted into a woman.

Embryonic cells are pluripotent, meaning they can give rise to any cell type on our body, with exception of the placenta and the umbilical cord. These are probably the best renewable cells to investigate.

INDUCED PLURIPOTENT STEM CELLS

Lastly, there are the induced pluripotent stem cells, or iPS cells. They are cells engineered in the lab by converting tissue-specific cells, such as skin cells, into cells that behave as embryonic stem cells.

Scientists investigate the differences between iPS cells and embryonic stem cells and what do these differences mean.

Stem cells proliferate

Another characteristic function of stem cells is their ability to proliferate. Our body can replicate its cells in two different ways, the first one is done by what is known as cell division or mitosis and meiosis.

And stem cells do so quite differently, they undergo through the process called asymmetric division, see FIGURE 1.6. They form two daughter cells, one is an exact replica of the first stem cell and the other one is a stem cell ready to differentiate. This process is also called self-renewal. This self-renewal allows the body to regenerate.

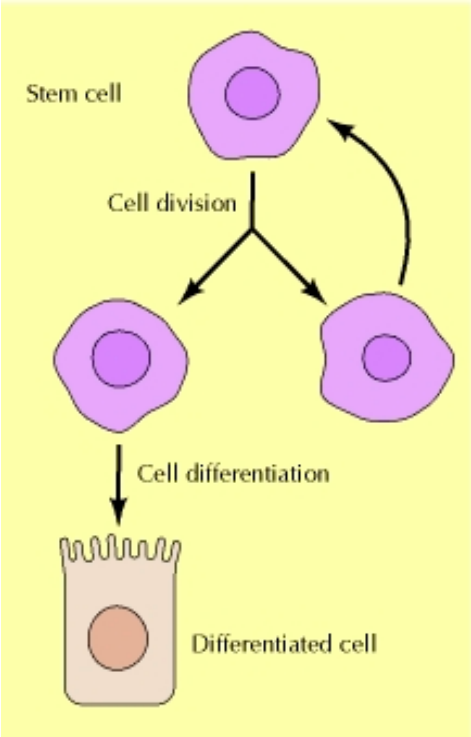


FIGURE 1.6. A stem cell proliferation scheme.

Stem cells have many potential uses

Stem cells yield us with a lot of information, and it is of major importance on research.

Scientists know that differentiation into a specific tissue is highly related to turning specific genes on and off. It is also of high interest the fact that diseases like cancer or birth defects are related to cell division and differentiation.

Currently, though, the research is focused on drug testing. New medications are being tested daily on the labs on differentiated cells. However, their first goal is to control the differentiation to induce, from a pluripotent stem cell, any kind of tissue cell.

The most promising application in stem cells is the cell-based therapy. Taking back an idea proposed on the first pages of this section, as the need of transplantable organs outnumbers the actual transplantable organs, stem cells can be an alternative source of regenerative therapy to damaged tissue, and this is the reason why regenerative medicine has its basis on stem cells.

Stem cells must overcome all these technical hurdles:

- ✓ Proliferate extensively and generate enough cells to regenerate a tissue. They are regenerative agents.
- ✓ Differentiate into a specific cell depending on the substrate and the physicochemical conditions (pressure, stiffness…)-
- ✓ Survive in a plastic recipient in favorable conditions.
- ✓ Integrate in the tissue after its transplant.
- ✓ Function correctly as a part of a tissue.

- ✓ Avoid harming the body.

Stem cells are regenerative agents

When damage occurs, our body needs a source of cells to quickly heal the wound, and it relies on the stem cells' ability to proliferate fast. This statement became a point of departure for scientists, that first came up with the idea of transplanting stem cells from the bone marrow to identical twins, and leukemia was cured from the one suffering it. This happened on 1956.

However, stem cells need to match: as the immune system has a way to notice foreign cells and strange behaviors, our body defends itself. This is the reason why at first stem cells' transplant was only limited to twins. Through the years, scientists discovered a protein on almost all the cells that allowed the body to know if the cell was a foreign cell or not. This type of protein is called nowadays compatibility marker and HLA (Human Leucocyte Antigens) allowed the first unrelated bone marrow transplant.

To sum up this section, the investigation on stem cells is a great research field, however, applications won't be efficient or viable without intensive years of research to overcome all the conditions to be part of an applied regenerative medicine.

By the way, what does exactly mean *to regenerate*?

VI. REPAIR VS REGENERATE

Regeneration and repair are two processes that are initiated upon injury. By definition, regeneration is the proliferation of cells and tissues to replace lost cells or tissue. In contrast, repair is the proliferation of fibers in response to a combination of regeneration and scar formation.
These two concepts are confused on numerous occasions and it is important to be able to differentiate both processes, in order to understand the importance of regeneration.
Firstly, **repair** implies a formation of new scar tissue where the wound was. It fills the space where the damaged tissue was, but it loses functionality.
Scar tissue is composed of the same tissue that it replaces; however, the loss of functionality is due to the alignment of collagen fibers. On the healthy tissue, the collagen fibers are random aligned, whereas on scar tissue, we can observe an oriented alignment on a single direction, see FIGURE 1.7.

Healthy Tissue Scar Tissue

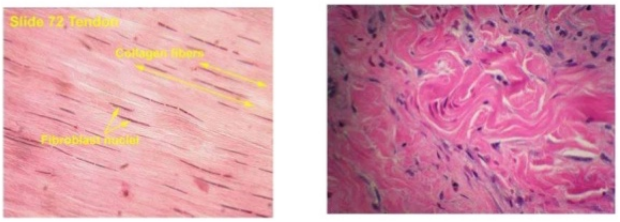


FIGURE 1.7. Comparation of the network of fibers of collagen in both healthy and scar tissue.

On the other hand, **regeneration** is the restoration of the tissue to both its original cellular and extracellular structure. Worth to be mentioned that collagen, mentioned before, acts as an extracellular matrix for cells to proliferate.
Quite clearly, we can understand why regenerative medicine is trying to induce regeneration.

Amazingly, human bodies are able to regenerate only bone and liver tissue correctly.

VII. HOW TO STIMULATE REGENERATION

And how pretends regenerative medicine to stimulate such a complex process?

On this last section, there will be explained the main cell therapies that are in development in order to do the latter mentioned.

Dedifferentiation. This concept refers to the cellular process in which a cell loses its function or reverts to its earlier development stage. There are different cells capable to do so in lower life forms, such as worms and amphibians.
This process is a great alternative in regenerative medicine, as, when the inverted tissue cells could be transplanted as stem cells, it wouldn't generate genetic incompatibility to that same patient.

Curiously, it has been observed that some specific tissue cells can dedifferentiate whereas others cannot, or some of them can dedifferentiate but cannot proliferate.

The phenomenon of dedifferentiation can be observed at the levels of gene, morphology and function. Development-related gene activity is repressed, and the genes that keep the cell undifferentiated are activated. Morphologically, dedifferentiated cells are smaller, and have fewer organelles. At the functional level, the cell can proliferate again.

Worth to say, this process is really poorly seen yet, although many researchers declare to have observed dedifferentiation, their findings included only one or two of the changes listed above.

Furthermore, it is not clear if these changes form the ultimate criterion for dedifferentiation, as this process is yet to be understood. Researchers currently are identifying common elements between species, in order to do so.

Transdifferentiating. This process is how the conversion of one cellular phenotype to another is referred to. It happens in limb regeneration in amphibia, and undergoes two phases. In the first phase, differentiated cells revert to dedifferentiated cells. The second phase involves the differentiated cells dedifferentiating into the new cell phenotypes.
However, this concept is not widely used, as it basically is a dedifferentiation-redifferentiation model.

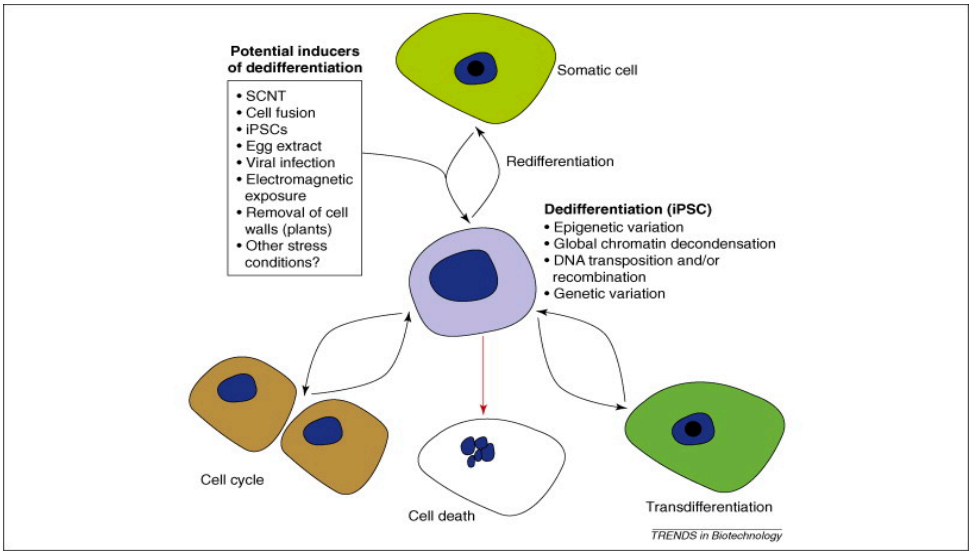
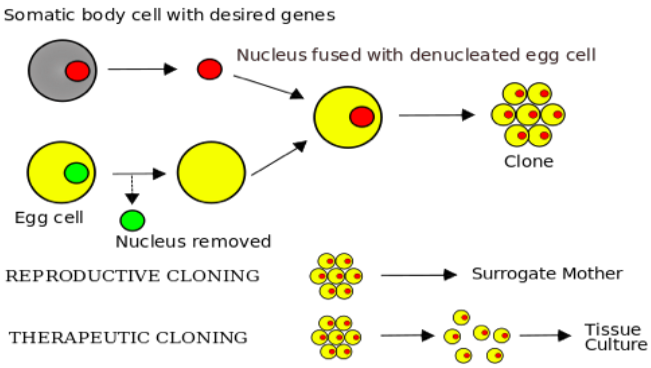


FIGURE 1.8. This image shows the processes listed above. Cell cycle implies continuing to proliferate as induced pluripotent stem cells.

To sum up, there are to be presented the most promising potential inducers of dedifferentiation.



Firstly, as indicated on FIGURE 1.8, SCNT, or somatic cell nuclear transfer. This process implies having a body cell nucleus implanted into an egg cell (FIGURE 1.9.), in order to clone that genetically modified cell.

FIGURE 1.9. A scheme of how SCNT works.

Cell fusion is another potential inducer of dedifferentiation, and thus of regeneration. It consists on having several uninuclear cells fused into a multinuclear cell, in order to maintain maturation throughout their growth. It has many challenges to overcome yet in order to be a therapeutic alternative.

iPSCs are the induced pluripotent stem cells, already explained before. Viral infection refers to causing such stress to the cell that forces it to reverse to its developmental stage.

Electromagnetic exposure could also be a dedifferentiation inducer. One example is the UV rays, that, basically, if a UV ray's proton hits a cell, it damages its DNA. The shorter the time of exposure is, the less harm the DNA suffers, and this is how we could genetically alter that cell.

Lastly, the removal of cell walls in plants is a good way of studying the membrane and the DNA alteration. Plant cells without cell walls are called protoplasts, and they are widely used to genetically vary plants, as the cell wall could possibly block the DNA's path inside the cell. Moreover, protoplasts can transform into an entire plant.

CHAPTER 2

BIOMATERIALS

I. **Bioactive materials.**
II. **Specific characteristics.**
III. **Synthesis.**
IV. **Surface analysis techniques.**
V. **Applications in medicine.**

“Any problem can be solved using the materials in the room “.

-Edwin Herbert Land, American inventor.

I. BIOACTIVE MATERIALS

Materials have been important since the beginning of humanity. A material is defined as that matter that conforms any composite. Everything is formed by materials. **But how about *biomaterials?***

The definition of biomaterials is widely varied, just as conventional materials, depending on the focus of research. For instance, when referring to the biomaterial science applied to medicine (see FIGURE 2.1.), a consensus of experts in the field doted the concept of a biomaterial in 1987 as:

"A nonviable[5] material used in a medical device, intended to interact with biological systems."

The most global definition, though, is that a biomaterial is any artificial material able to be in contact with biological systems, not just in a medical device.

Impliedly, however, they are going to be utilized to repair, assist or replace damaged or missing tissue, and these biomaterials will also need to be biocompatible.

Biocompatibility is understood as the quality of not inducing damaging or toxic effects towards the body's biological systems. However, it is important to know that biocompatibility is not either achieved or not, there are intermediate rates depending on the tissue reaction.

Bringing back the definitions, **in this project** we are going to refer biomaterials as **bioactive materials**, according to our necessities (to characterize cell-material interactions and for tissue engineering in general) later on.

If these materials induce certain physical and chemical behaviors, automatically, we are talking about bioactive materials. Bioactivity could be defined as the ability of the matter to conform a bond, to consequently adhere to tissues. The reactions caused vary depending on the material used and its composition, and this fact arose the science of biomaterials[6].

FIGURE 2.1. Biomaterials, applied to medicine, are going to be used to create constructs to regenerate damaged tissue (scaffolds).

A ceramic is a nonmetallic material that needs a specific thermal intervention to prepare it. The two most used ceramics in tissue engineering

[5] Nonviable in this case means no capable of living.

[6] Biomaterials science is the physical and biological study of materials and their interaction with their biological environment. The most intense research has been focused towards the biomaterials synthesis and optimisation, and the biology of the host-material interactions.

are hydroxyapatite (the mineral component of bone) and bioactive glasses[7], as Bio-glass (it contains SiO_2, Na_2O, CaO and P_2O_5 in determined proportions to induce certain properties).

About metals and metallic alloys, they are poorly used in tissue engineering, because they tend to have unwanted conditions, and are difficult to make the material satisfy biocompatibility conditions. However, they are widely used in dental implants and orthopedic applications, as well as in many medical applications, because of their mainly structural mechanical properties.

Polymers are maybe the most promising biomaterials. They are organic and thus really similar to the components in our bodies, as they are made of carbon, hydrogen, oxygen, phosphorus, sulfides… Able to be approached from many different ways, polymers can have different properties and reactivity depending on many conditions used.

Because of its versatility, polymers are the most used biomaterials[8] in tissue engineering and the focus of research.

To conclude this section, the most common classification methods are going to be exposed.

- The industrial classification:
  - → Ceramics.
  - → Metals.
  - → Polymers.
  - → Composites[9].
- The tissue response classification:
  - → Inert.
  - → Bioactive.
  - → Resorbable or biodegradable[10].
- Origin:
  - → Natural.
  - → Synthetic.

These classification methods are not used in all the materials, and are going to be explained as we advance on the diverse sections.

II. SPECIFIC CHARACTERISTICS

It is important to know the properties of biomaterials because they are going to later influence or even control the dynamic interactions in the tissue-implant interface. This is the reason why the most important mechanical properties of materials are going to be shortly defined.

[7] Bioactive glasses are a type of ceramics that will be described in the synthesis section.
[8] A biomaterial is not the same as a biological material. A biological material is that material that has his origin in a living being, just like a spiderweb.
[9] Composites are another type of biomaterial. However, they are not going to be considered in this project, as they are development plus they are mixtures of metals-ceramics, or ceramics-polymers, etc.
[10] Biodegradability is an important quality to consider in any biomaterial. It is defined as the resistance of a substance to be decomposed in their chemical elements by action of microscopical organisms, under environmental conditions.

MECHANICAL PROPERTIES

- **Brittleness**: Capacity to break or shatter without significant deformation when undergoing stress. It is the opposite of plasticity.
- **Creep**: The gradual deformation of an object with respect to time.
- **Ductility**: Capacity to deform under tensile load. Expressed in % of elongation.
- **Durability or hard-wearing**: Ability to undergo wear, pressure, or damage.
- **Fatigue limit**: Maximum stress a material can withstand. Expressed in MPa.
- **Flexibility or pliability**: Capacity to bend or deform in response an applied force.
- **Stiffness**: Ability to resist deformation in response to an applied force. Flexibility and stiffness are complementary.
- **Size.**
- **Strength of materials:** The general strength is determined as the relation of these various strengths.
    - **Specific strength:** Strength per unit of density. Expressed in N · m /kg.
    - **Compressive strength:** Maximum stress before compressive failure. Expressed in MPa.
    - **Tensile strength:** Maximum stress before failure. Expressed in MPa.
    - **Yield strength:** Maximum stress before a permanent deformation occurs. Expressed in MPa.
- **Toughness:** Capacity to absorb energy and plastically deform without fracturing. It is the combination of strength and plasticity.
- **Viscosity or thickness:** The fluid's resistance to gradual deformation by stress. This is really important in tissue engineering devices, to mimic blood's thickness, for instance.
- **Elasticity**: Ability to resist stress to then return to its original size and shape when stress is over.
- **Abrasion resistance**: Ability to withstand high temperatures.

Although not all of these mechanical properties are mentioned in the coming sections, they are fundamental to understand why bioengineering researchers are characterizing such conditions, trying to accomplish the perfect mixture of conditions depending on our necessities.

In order to enumerate the different characteristics of biomaterials, we should analyze them individually according to their industrial classification[11].

CERAMICS

First of all, within this section we include glass and ceramics. Ceramics are that compositions made of metallic and non-metallic elements.

Ceramics are used because of their:

- **Inertness inside the body**, that means they are stable and that there is little or no tissue reactivity when implanted.

[11] When talking about biomaterials, we can add the prefix bio- to ceramics, metals and even polymers.

- **Variability of shapes and porosities**. All the materials researched tend to adapt to many different shapes.
- **High compressive strength**.
- **Wear characteristics**. Just in some cases, ceramics tend to be more resistant to the gradual removal, deformation or damaging of themselves when implanted.

PROPERTIES	SPECIFIC MATERIALS
Bioactive	Hydroxyapatite, bioactive glasses
Nearly bioinert	Alumina (Al_2O_3), Zirconia (ZrO_2)
Resorbable or biodegradable	Tri-calcium phosphate (TCP)
Porous for tissue in-growth	Hydroxyapatite-coated materials, alumina (these are for the jaw bone, for instance)

TABLE 2.1. Properties of bio-ceramics with their specific material.

MATERIAL		TISSUE
TOXIC		**DEAD**
NON-TOXIC	**Nearly inert**	There is no interfacial bond with the bone. Instead, fibrous tissue of various thickness forms around the implant, like a biological isolation, as a protective mechanism.
	Bioactive	An interfacial bond forms with the bone. A common characteristic is the formation of hydroxy-carbonate apatite (HCA) layer (0.5-100 μm) on the surface, due to the different solubilities of the tissue and the implant.
	Soluble (resorbable or porous)	The surrounding tissue replaces it. For the successful application, the resorption rates of the material should match the body repair rate and the tissue mechanical properties, in order to induce a regeneration of the tissue.

TABLE 2.2. Classification depending on the reactivity.

Bio-ceramics, overall, are used to repair diseased or damaged parts of the musculoskeletal system. They include a wide range of different compositions. That can be observed on the TABLE 2.1.

Type of bio-ceramic	Type of attachment	Description
1	**Morphology fixation**	Dense, nonporous and nearly inert ceramics attach to bone (into surface irregularities).
2	**Biological fixation**	Porous nearly inert ceramic implants induce bone ingrowth, and these implants mechanically attach the bone to the materials.
3	**Bioactive fixation**	Dense, nonporous bioactive ceramics and glass-ceramics attach by chemical bonding with the bone.
4	**Slowly replaced (regeneration)**	Dense and nonporous or porous resorbable ceramics are fabricated with this goal, for the bone to slowly replace the bio-ceramic.

TABLE 2.3. Detailed types of attachments in ceramics.

Ceramics are also classified depending on their tissue-attachment mechanism, in other words, they are classified depending on their reactivity on the tissues, see TABLE 2.2.

Comparing TABLE 2.1. with TABLE 2.2, we can extract different examples of bio-ceramics and their reactivity. Furthermore, there are terms for the different attachment methods, referred to the bones[12], see TABLE 2.3.

It is still not demonstrated which of the four attachments work better. However, having a slowly replaced regeneration is thought to be less dangerous for the body, as the result is the same tissue there was before.

Concluding with this subsection, in order to implant these bio-ceramics and biomaterials overall in our body, these materials need to be:

- ✓ Biocompatible with host tissue (being non-toxic, non-allergic, non-carcinogenic and non-inflammatory).
- ✓ Able to stimulate bone's natural behavior.
- ✓ Resorbable following replacement of the bone.
- ✓ Radiopaque (impenetrability by the X-rays, in order to optically act as biological tissue as well).
- ✓ Capable of being sterilized.
- ✓ Inexpensive.
- ✓ Stable to the natural variation of temperature and humidity.
- ✓ To have enough porosity to allow bone growth.

[12] As already mentioned before, bio-ceramics are used mainly and almost exclusively to the musculoskeletal system.

To summarize bio-ceramics, see TABLE 2.4.

ADVANTAGES	FLAWS
High compression strength	Low strength in tension/stress conditions
Wear and corrosion resistance	Easy tendency to fracture
Can be both bioactive and bioinert	Difficult to fabricate
Variability of textures	Brittleness
Good biocompatibility	

TABLE 2.4. Balance of bio-ceramics.

As a brief conclusion of the bio-ceramics specific characteristics section, we observed how, for load bearing applications, we needed strong bio-inert ceramics, such as alumina or zirconia, however, we saw how these ceramics tend to have problems like corrosion or a high stress load on the elastic modulus, and bio-inertness itself (having only a physical connection with the host), along with brittle.
It is for these reasons that we, as tissue engineers or biomedical scientists in general, search for bioactive ceramics, like hydroxyapatite and bio-glasses, that show interfacial bonds with the host, and an equal elastic modulus compared with the natural bone.

Other bioactive bio-ceramic are bioactive glasses. Glasses begin to be considered as bioactive glasses if they follow these three rules of compositional range:

- <60 mole % SiO_2.
- High Na_2O and CaO content.
- High CaO moles: P_2O_5 moles ratio (CaO: P_2O_5).

We also mentioned how, still obtaining poor mechanical properties, bioactive ceramics are the most acceptable ones in terms of biocompatibility.

METALS

Metals and their alloys will be referred to within this section.

Both metals and alloys are used due to their electrical and thermal conductivity and their great mechanical properties.

The most important metals are: stainless steels, CoCr alloys and Ti and its alloys.

- **Stainless steel:**

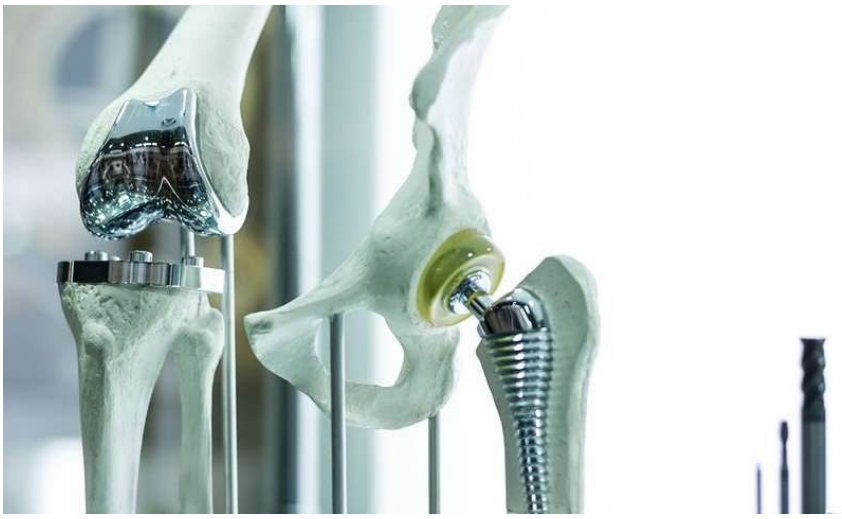


FIGURE 2.3. Kneel (left) and hip (right) stainless steel implants.

The first successful surgical material was the stainless steel, it can be seen in FIGURE 2.3. Even having implemented new techniques to the stainless steel such as adding molybdenum to improve corrosion resistance, stainless steels tend to corrode in the body under certain circumstances, making them able to uniquely be temporary implant devices.

However, stainless steel has several flaws, and these flaws are shared by another popular metal, gold. The most remarkable one is its biocompatibility. It has been proven that it tends to cause toxic reactions in our body, as well as allergies and general rejection from our body.

- **Cobalt-Chromium alloys**

Moving on to CoCr alloys or cobalt chromium alloys, they form a solid solution of up to 65% of cobalt. To that alloys usually molybdenum is added to produce finer grains, to result in higher strengths. The chromium is added in order to enhance corrosion resistance to the solid solution. An example of this is the dental implant shown on FIGURE 2.4. The concentrations vary depending on our necessities, adding for example nickel and lowering the concentration of cobalt to create a highly corrosion resistant to seawater alloy.

FIGURE 2.4. Example of alloy made of chromium (Cr), cobalt (Co) and molybdenum (Mo).

- **Titanium and its alloys**

The metal that has gotten a lot of attention lately in medicine has been titanium and its alloys. It has been because of its characteristics:

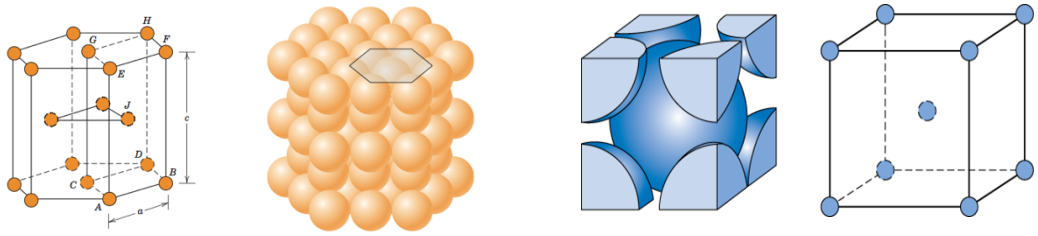
- Excellent biocompatibility.
- Light weight.
- Balance of mechanical properties.

- Excellent corrosion resistance.

Furthermore, titanium, in his elemental form, has a high melting point (1668ºC), and behaves in two different ways:

α) form → Up to 882.5ºC → Hexagonal closely packed structure (HCP) FIGURE 2.5.

β) form → Higher temperatures than 882.5ºC → Body centered cubic structure (BCC). FIGURE 2.6.



FIGURES 2.5-2.6. HCP form (left) and BCC form (right).

This is important to consider because adding other elements, such as Aluminum or Vanadium can ease the transformation from α to β. One example is the alloy Ti_6Al_4V, the most used to manufacture implants.

Although promising for orthopedics due to their mechanical properties just like low elastic modulus and a high specific strength, titanium and most of its alloys have low wear and abrasion resistance because of their hardness.

- **Surface modifications**

Worth to mention, surface modifications of metal alloys have been used in industry. Some examples of these surface implants are hydroxyapatite, bio-glass or oxide ceramics. However, these modifications have a high risk of delaminating or wearing, eventually. Moreover, another downplay is the additional cost to implant these surfaces, and the only ones taken afloat are the ones that show much superior advantages to the materials without those surfaces.

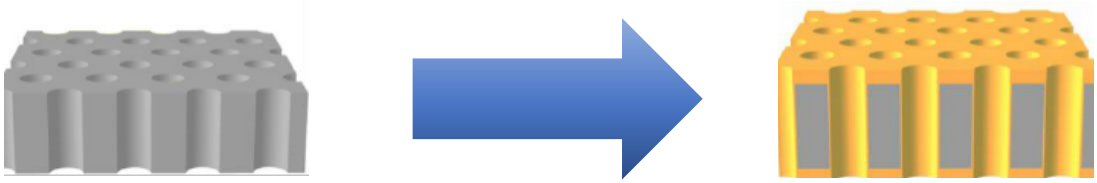


FIGURE 2.8. Scheme of a surface modification. The porous material is kept the same, but the external layer of the material is changed.

To balance bio-metals, see TABLE 2.5.

ADVANTAGES	FLAWS
Great mechanical properties	Tendency to corrosion
Rigid and strong materials	High density
Good biocompatibility depending on the circumstances	Difficult to fabricate
Load bearing devices	Low reactivity

TABLE 2.5. General balance of metallic biomaterials.

To accomplish the excellent mechanical properties bio-metals have not only we depend on its chemical composition, or the % of each metal to obtain an alloy, but also on its heat treatment and metal formation. We saw on the specific characteristics section that bio-metals can reach optimal mechanical and thermal properties, however, we also learnt its downplays, mainly corrosion, wear and low elastic modulus in some cases. This is why surface modifications are studied, to coat the metal with bio-ceramics, for instance.

POLYMERS

Polymers or macromolecules have assumed the most important research focus on biomaterials, as their properties have no competition from other types of biomaterials. To begin with, polymers are a chain of monomers, simpler chains of molecules. To be considered a polymer, it has to have a molecular weight of 10^3 g.

Polymers have drawn attention because of these characteristics:

- Flexibility.
- Resistance to biochemical substances.
- Much better biocompatibility.
- Can reach a much lower weight than other type of biomaterials.
- Availability of many different compositions, most of them with great mechanical properties, their dynamics tend to be similar to our tissue's.

There are two types of polymers, depending on their origin: **natural** or **synthetic**.

➢ NATURAL POLYMERS

Natural polymers are the one derived from living creatures. They are of substantial interest in all the biomedical fields, and, above all, biomaterial science. The two natural polymers that are to be explained are the most common on the field of tissue engineering. Respectively, they are collagen, a polymer derived from proteins, and alginate, a polymer derived from polysaccharides.

Collagen

The most common protein in mammals (25% of our protein mass). Is the provider of most of the strength of the tissue. The collagen molecule structure consists in a triple protein chain that form a helical structure, and in the body, as a macromolecule, they tend to form long fibers (see FIGURE 2.10.).

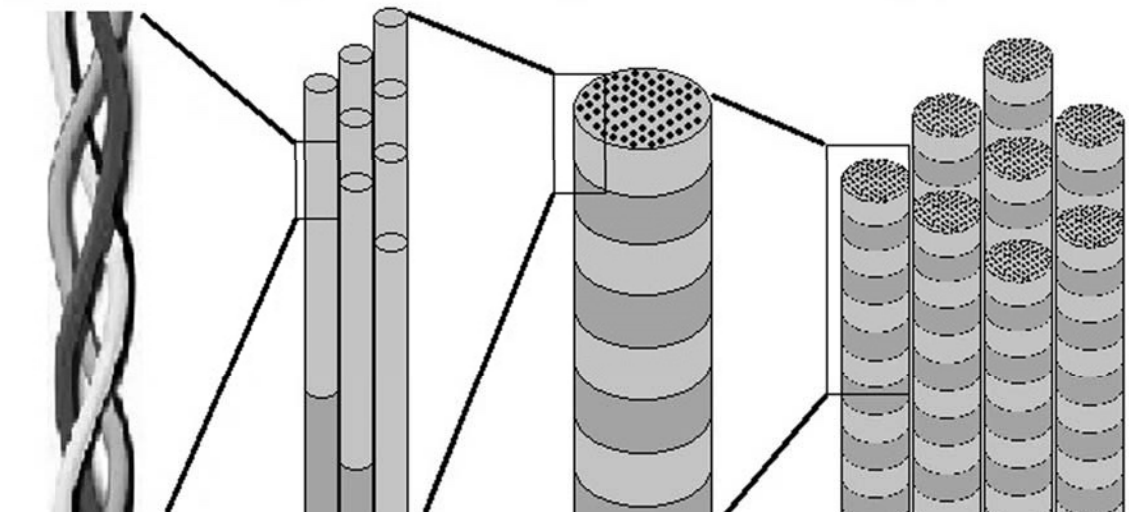


FIGURE 2.10. The collagen triple helix as a molecule and its consequent polymerized fibrillar forms.

These molecules will polymerize and give birth to collagen fibers, with varying length, thickness and pattern. There are 15 different types of collagen, with different properties to one another. The most used in the biomedical form, however, is the type I collagen, protein that can be found almost anywhere in our body.

Between the advantages of using collagen, there is the reabsorption into the body non-toxically, producing a minimal immune response. Also, collagen is great for attachment and biological interaction with cells. The collagen fibers can be manipulated to conform a huge variety of different formats, and can be cross-linked[13] with chemical compounds in order to alter their properties. This allows a great deal of applications in cosmetics, surgery and biomedical fields.

Important to consider for tissue engineering, cell grown in collagen act and behave in a closely similar way as natural organs do, and this is why collagen has been such a promising material to research.

Observing now its downplays, when collagen is implanted into a functional tissue, collagen do in some cases alter the cells' normal behavior, such as changes in growth or in development. Also, depending on the processing method of collagen, can reach inappropriate mechanical properties, leading to, potentially, contraction or fracture.

[13] To cross-link a polymer means to polymerize it and add that compound to its chain, achieving eventually a solid state solution.

Collagen is processed in two different forms, in both reaching a scaffold. The main problem of pure collagen is that as cells interact so easily with collagen, these cells can even change the collagen fibers' organization, causing a loss of shape. This is why collagen is normally cross-linked with other substances, to achieve better mechanical properties, or change cell behavior towards it.

When a biomaterial is cross-linked with other elements, we are talking about, in our case, of a ***collagen-based biomaterial***.

Alginate

Ending with the last of the most important natural polymers, alginate is a macromolecule derived from alginic acid, a polysaccharide found in the cell walls of brown algae, see FIGURE 2.11. Just like chitosan, alginate is found easy to process in water and it shines because of its great compatibility, being non-toxic and non-inflammatory, in such amount, that many countries already use them for wound healing techniques and in food products.

Continuing with the advantages of this polymer, alginate is also biodegradable and resorbable, and its porosity is controllable. It also can be treated to have its chemical side groups attached in a biologically active manner to other molecules, fixing to them.

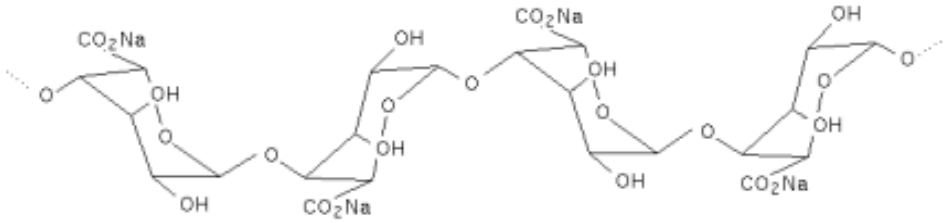


FIGURE 2.11. A fragment of the alginate structure.

Alginate is going to be used in the fieldwork because of its opportunities in scaffolding and drug delivery as well.

Again, its drawbacks are low mechanical properties and poor cell adhesion. We can conclude that these two are the common flaws of natural polymers. In the same way, they can be solved mixing alginate with other substances. In our case, alginate, there is a tendency to mix it with natural polymers like agarose or chitosan.

Worth to say, this natural polymer is really versatile and can be modified to be a drug delivery system and encapsulate cells, a porous scaffold to contain cells, micro-spheres that can are biodegradable... Alginate is able to accomplish great properties that can be promising for the future. However, nowadays alginate is not able to accomplish all the desired properties at the same time: biodegradability, bioactivity and fair mechanical properties.

➢ SYNTHETIC POLYMERS

Having the opportunity to choose monomers to polymerize and advancing in new synthesis methods offers a wide and even creative scenario where mimicking the behavior of natural tissues is the goal. From the natural polymers section, we have already seen how, in polymers,

the macromolecular chemistry is fundamental to determine the properties the biomaterial will have, along with its morphology.

There are two types of synthetic polymers. The classification is done dividing biodegradable and nonbiodegradable synthetic polymers, and it can already be supposed that nonbiodegradable ones will have more drawbacks associated to them. Furthermore, biodegradability is a property we are always searching for in tissue engineering.

Thus, nonbiodegradable synthetic polymers have extremely limited biomedical uses. However, there is still no engineered polymer that can substitute the mechanical properties of these nonbiodegradable synthetic polymers.

The synthetic polymers that are going to be exposed are the most used and the most important ones designed, see FIGURE 2.12.

BIODEGRADABLE	PLA or poly(lactic acid).
	PCL or poly(ε-caprolactone).
NONBIODEGRADABLE	PMMA or poly(methyl methacrylate).

FIGURE 2.12. Enumeration of the synthetic polymers that are to be exposed.

PLA, poly (lactic acid)

PLA is the polymerized form of lactic acid monomers. Since lactic acid is a chiral molecule, it has stereoisomers[14]: poly (L-lactide) (PLLA), poly (D-lactide) (PDLA) and poly (DL-lactide) (PDLLA). PLLA and PDLA are crystalline, whereas PDLLA is amorphous[15]. The majority of lactic acid in biological environments are found in the L-form, so the main fraction of PLA is PLLA.

PLA stands out from the other polymers because of its eco-friendly nature. That is because it is derived from 100% renewable resources (see FIGURE 2.13.), just like corn and rice, as it can also be seen in FIGURE 2.13., the PLA products' derivates are water and CO_2, and they are neither toxic or carcinogenic for our bodies. That leads to the second advantage of using PLA, its excellent biocompatibility.

Moreover, PLA is easily thermal processed in comparation of other biomaterials like poly (ethylene glycol) (PEG) or poly (ε-caprolactone) (PCL). These thermal properties also make PLA applicable for the textile industry or food packaging. Food and Drug Administration (FDA), authorized the use of PLA as a food additive, however in biomedical applications several factors have to be considered.

[14] Molecules that have the same molecular formula and the same sequence of atom bonding, however they differ in the three-dimensional organization of their atoms in the space.
[15] A solid state compound that has unordered molecules.

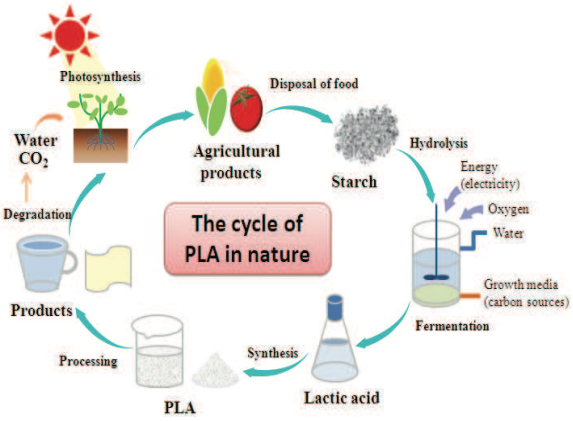


FIGURE 2.13. The natural cycle of PLA.

However, PLA also depends on its flaws. These are: that degradation rate of PLA is too slow, talking of even several years. Moreover, PLA is very brittle, with less than 10% of elongation at break, and that makes the mechanical properties of PLA poor for load bearing applications unless suitably modified. When used as a tissue-engineered material, PLA, as a hydrophobic polymer, represents a potential inflammatory product for tissues. Another drawback is its high cost.

Lastly, PLLA, the most common form of PLA, tends to be crystallized, and that can cause inflammatory reactions, and that is why PLLA is normally combined with D, L-lactic acid monomers, to then be rapidly degraded.

We used PLA in our fieldwork, as a scaffold made of fibers, see FIGURE 2.14.

FIGURE 2.14. PLA tissue-engineered scaffold.

PCL (poly ε-caprolactone)

Poly ε-caprolactone is the repetition of the monomer ε-caprolactone, see FIGURE 2.15.

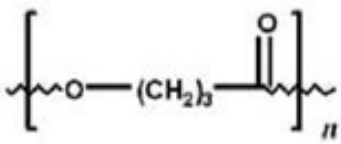
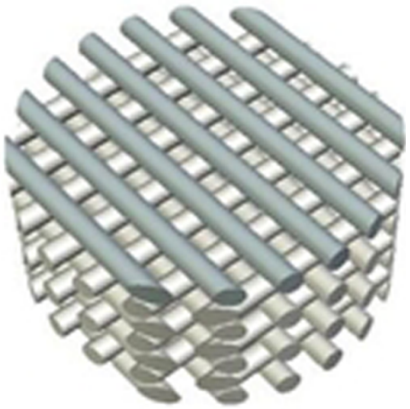


FIGURE 2.15. n times ε-caprolactone conform the PCL.

The main advantages of PCL are: firstly, the approval by the Food and Drug Administration (FDA) for some biomedical uses in humans, also, as biodegradable polymers, its easy absorption by the body, continuing with its compatibility with other polymers, its processability

enabling varied structures and forms, and, lastly, it results easy to process thanks to its thermal stability and low melting temperature.

Also, this polymer is relatively cheap, and it is broadly studied because of these excellent characteristics.



The physical, thermal and mechanical properties vary depending on its molecular weight, as every polymer. However, its viscoelastic properties are superior over other biodegradable polymers, fact that makes PCL an option for many devices. As other polymers, combining PCL with other polymers is of interest for many scientists, in order to improve mechanical properties, an ease of shaping and a better manufacturing.

We used PCL in our practical framework, as a porous 3D printed scaffold (see FIGURE 2.16).

FIGURE 2.16. Scheme of the PCL bio-printed scaffold.

PMMA (poly methyl methacrylate)

Beginning now with nonbiodegradable polymers, PMMA is normally known as Acrylics.

Acrylics have excellent mechanical properties, far better than biodegradable macromolecules. Between them there is its high light transmittance, its stability of mechanical and optical properties when exposed to moisture or water for long time lengths, as well as most laboratory chemicals. This structure can be seen in FIGURE 2. 17, where double bonds provide stability to the molecule.

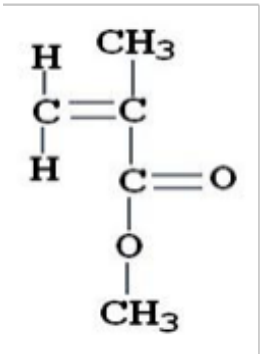


FIGURE 2.17. A methyl methacrylate molecule, PMMA is formed joining many of them.

Many shapes can be made with Acrylics, with various tints and colors.

On the other hand, PMMA is not normally contemplated for biomedical applications, since it can alter aromatic hydrocarbons, ketones or esters[16] and its biocompatibility is not so trusted.

[16] Different organic functional chemical groups.

Acrylics can also have cut surfaces, that, still being polished, offer stiff surfaces that, combined with its nonbiodegradability, make Acrylics an option for other fields, such as architecture, as its mechanical, electrical, thermal and optical properties suit best for sculptures or lenses.

Polymers are the most important biomaterials investigated nowadays. That is because they exhibit variable and versatile properties that allow scientists to tune their properties, always within biocompatible levels. However, between their flaws there was their poor mechanical properties, and specially their low strength.

There are two types of polymers, natural and synthetic. The natural polymers demonstrate an amazing compatibility with the body and a low immune response, however, they also show irregular behavior in some cases. The synthetic polymers are creative ways of joining monomers to achieve different types of characteristics, although the high cost limits these processing methods.

It was also learnt how there are two different types of synthetic polymers, biodegradable and nonbiodegradable. The former ones exhibit a higher biocompatibility level and our body do not reject them, however, mechanical properties are especially low, so mixtures with other substances and even with other polymers are needed.

The latter ones, nonbiodegradable polymers, have restricted uses in the biomedical field. They have fair biocompatibility, however its nonbiodegradability makes the body to cause rejection towards the polymers. They are important because they have much better mechanical properties than biodegradable polymers.

III. SYNTHESIS

This section will be focused on the bio-polymers production, because of its importance on the tissue engineering field.

Considering both **natural and synthetic polymers**, 2 synthesis methods will be explained. In polymers, their synthesis is called polymerization. These 2 methods are the most common ones.

- Polycondensation

  A popular type of polymerization is condensation polymerization or polycondensation. In other types of polymerization, the whole of the monomers become the polymer, however, in polycondensation, by-products remain such as water or HCl. It is called condensation polymerization because the polymer resulting has less mass than the original monomers.

  This polymerization produces in a step growth manner dimers, trimers, tetramers… until a termination reaction occurs. This last reaction is undergone by one of the molecules when, at the time of reacting, is completely consumed.

- *Addition reaction polymerization*

  There are three differentiated types of addition reaction polymerizations. They have in common that no elimination of small molecules occurs. These reactions generally take place in double or triple-bonded monomers.

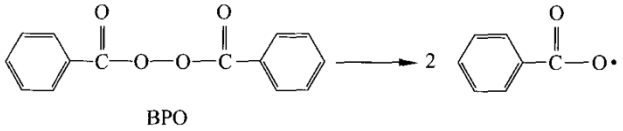
  - Ring-opening polymerization, free radical or chain reaction polymerization

  This is one of the most common methods.

This is a reaction that consists of 3 steps: initiation, propagation and termination. All the polymer is formed in less than a second.

Initiation

To start a ring-opening polymerization, a molecule called **initiator** is needed. Two typical initiators are AIBN (or 2,2'-azo-bis-isobutyrylnitrile) and benzoyl peroxide (or BPO). Heating these initiators, an unpaired electron appears, called free radical. BPO is in FIGURE 2.18.



BPO

FIGURE 2.18. BPO before and after being heated.

The new free radical will be bound to interact with monomers with unsaturated bonds. The carbon-carbon double bond in, for instance, ethylene (to later create polyethylene), is easily attacked by this free radical. The molecule would after look like FIGURE 2.19.

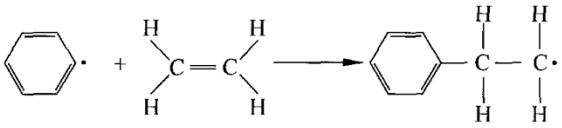


FIGURE 2.19. BPO attacking the ethylene monomer to form a free radical in the monomer.

Propagation

The new free radical monomer will react with another ethylene very similarly to the FIGURE 2.19 and, after a long succession, a chain is going to form, this is why these reactions are called chain reactions.

Termination

Radicals, however, are unstable, and they can either chain react, increasing the chain length, or they can react with another radical of a monomer. This latter option would mean no more radicals generated, and the chain would terminate (termination reaction).

There are several types of termination reactions. The simplest is the one mentioned. However, it is impossible in polyethylene a linear monomer chain with free radical polymerization, and at all times monomer branches will occur.

- Step reaction polymerization

  Is a variation that, when a *n* bond is broken, 2 new *m* bonds are formed. The next or previous monomer has to have a double or a triple bond.

- Ionic reaction polymerization

  As another variation of the chain addition reaction, instead of neutral, unpaired electrons, these electrons have either a positive or negative charge.

  To generate these charges, a co-catalyst is used, usually H_2O, to produce ionization, as the reaction cannot occur in dry conditions.

IV. SURFACE ANALYSIS TECHNIQUES

The surface of biomaterials is important because there are where biological reactions occur; cell adhesion, cell growth, protein absorption and blood compatibility.

However, the biological reactions will be studied in the **Cell-Material Interactions** chapter. In this subsection the most common methods to analyze the physical and chemical properties of biomaterials will be assessed.

The most common techniques are: X-ray photoelectron spectroscopy (XPS), to analyze chemical composition, and the atomic force microscopy (AFM) and the scanning electron microscopy (SEM) to analyze the topography of the surfaces.

X-ray photoelectron spectroscopy or XPS:

The typical XPS instrument includes an ultra-high vacuum system, a data acquisition system, an electron energy analyzer and an X-ray source. When the X-ray is emitted, photoelectrons are liberated, and their energy is calculated by this formula.

$$E_B = hv - KE$$

E_B (normally in eV[17], electron volts, represents the energy of the electron in the atom, **hv** is the energy of the X-ray source (see FIGURE 2.20.) and **KE** is the value of the kinetic energy of the emitted electrons measured.

Each element produces a different peak in the spectrum, and information about all chemical elements except helium and hydrogen is provided, as well as information about chemical bonding.

In the spectrum, the x-axis is the energy of the electron, and the y-axis is the intensity.

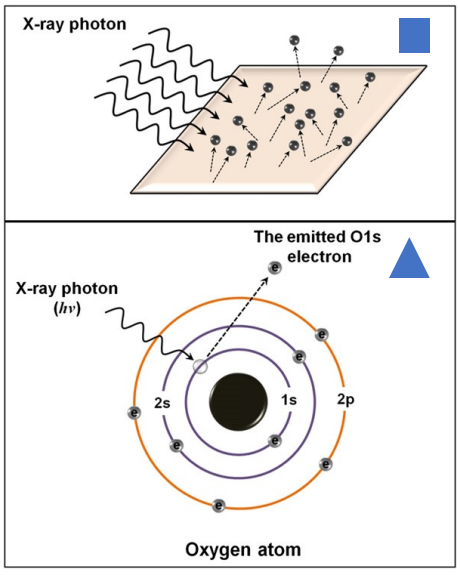


FIGURE 2.20. The x-ray photons hitting the surface of the biomaterial (square) and how the X-ray photon substitutes the electron of the atom (triangle).

Atomic force microscopy or AFM:

A commercial AFM instrument consists of a cantilever with a sharp tip (called probe) at its end that is used to scan the surface. The cantilever is normally made of silicon (100-200 mm long and 1 mm thick). The force between the tip and the surface leads to a deflection of the cantilever according to the Hooke's law or elastic spring law. This deflection is measured with a laser, that will transmit the ray the photodiodes, that will collect the

[17] An eV is a unit of energy that equals 1.6 x 10^{-19} joules in SI units.

information, see FIGURE 2.21. This is the typical methodology, however, other AFM variations and methods exist.

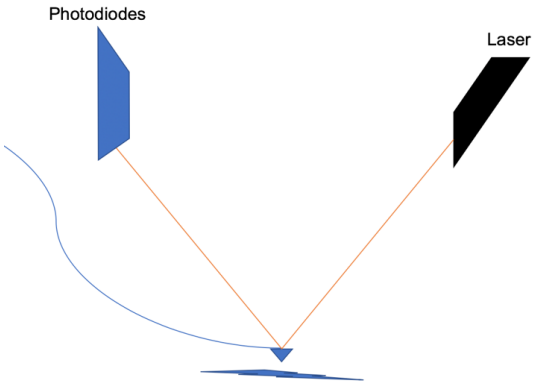
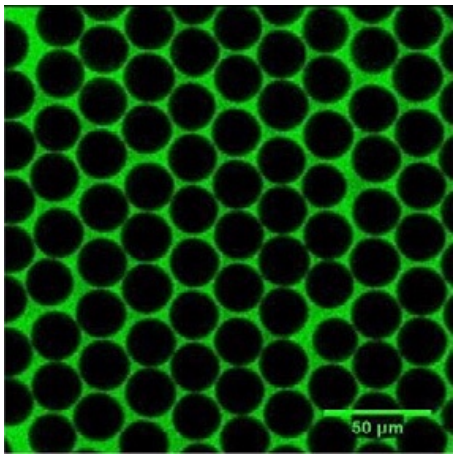


FIGURE 2.21. Basic scheme of the AFM procedure.

Scanning electron microscopy or SEM:

Scanning electron microscopy is a technique that images a surface by scanning an electron beam. This electron beam, focused by the electron gun, is formed by primary electrons (accelerated from 0.2 to 40000 eV), and these interact with the atoms of the surface, emitting varied signals.



Depending of these signals, a great deal of SEM models exists to characterize in different manners the topography of the sample. These SEM models differ from one another with the specialized detectors they use. However, they have in common the same cathode ray tube (CRT) to amplify the signals. The result of this amplification is an image that represents an intensity map of signals within the scanned area, see FIGURE 2.22.

FIGURE 2.22. Image of a hydrogel.

V. A NEW ERA FOR BIOMATERIALS

Having considered the most important information about biomaterial science, this last section intends to introduce the biomaterials application in tissue engineering. To regenerate damaged tissue, we needed either a transplant, causing immune response and rejection, or a way of inducing regeneration.

This last option could be carried out using the most promising new application of polymers and some other bioactive ceramics, the biomaterial constructs for tissue engineering, scaffolds.

The ultimate goal of these scaffolds is to find "the Holy Grail" of the biomaterials, and the most suitable way of inducing cells to proliferate and differentiate in the correspondent manner. In other words, scientists and engineers try to find the "best biomaterial" for each specific situation[18], and they also try to learn precisely the physical and chemical behaviors of natural tissue to mimic them. Tissue engineering, combined with the science of biomaterials, is believed to be the solution of many current health problems.

[18] There is no best biomaterial, what is intended to find is the best biomaterial for a specific tissue in a determined situation.

CHAPTER 3

SCAFFOLDS

I. **Biomaterial constructs.**
II. **Extracellular matrix.**
III. **Fabrication technologies.**
IV. **Vascularization.**

Design is not just what it looks like or how it feels like, it is how it works.

-Steve Jobs, American businessman.

I. BIOMATERIAL CONSTRUCTS

As mentioned on the last section of the second chapter, scaffolds are biomaterial constructs. However, they are more than just biomaterials. Scaffolds are artificial three-dimensional structures that serve as a mimic of the extracellular matrix to promote the adhesion of cells, as well as their proliferation and tissue regeneration. Also, their macro- and microstructure are defined copying the shape and structure of the regenerated tissue and organs, see FIGURE 3.1.

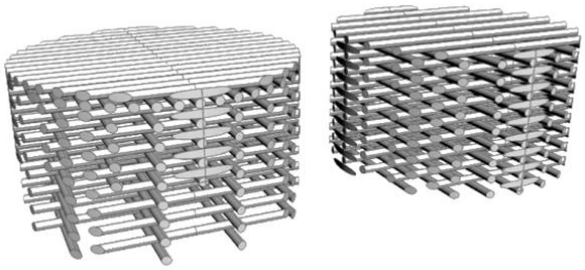


FIGURE 3.1. Scaffold are used as templates where cells will grow, mimicking the physiological shape of organs and tissues.

The ideal scaffold has to achieve:

- ✓ High biocompatibility to arise a minimum immune response.
- ✓ Three dimensions.
- ✓ Capable of regenerating tissue in their normal shape.
- ✓ A bioactive surface.
- ✓ Sufficient porosity to allow cell integration, growth and proliferation.
- ✓ Appropriate mechanical properties.
- ✓ A surface capable of being treated to allow cell attachment.
- ✓ Vascularity, to allow the receipt of nutrients.
- ✓ As structures, principles from, for instance, civil engineering have to be applied to scaffolds, such as tensegrity. This concept establishes that the mechanical forces have to be evenly distributed through all the areas of the scaffold, in order to be stable. So, structures with triangle, hexagon or pentagon shapes are preferred.
- ✓ Biodegradability. Scaffolds are not intended to be permanent implants.

Worth to be mentioned, there are two types of scaffolds that can be designed: solid-state scaffolds and hydrogel scaffolds. Different polymers allow one of the options, because of their properties. Hydrogels differ from the solid-state scaffolds in their huge capacity of absorbing water or biological fluids, as well as that they resemble the composition of tissues.

II. EXTRACELLULAR MATRIX

As scaffold mimic extracellular matrix, an introduction of it must be done. First of all, the extracellular matrix is a collection of molecules that surround cells.

All biological, chemical and physical behaviors of biological tissues are believed to depend on the composition of the extracellular matrix or ECM.

The ECM provides structural and biochemical support to their surrounding cells, apart from participating in all cell activities, including cell adhesion, growth and differentiation. The extracellular matrix does these by chemical signals, called ligands, that interact with cell receptors and mechanical stimuli.

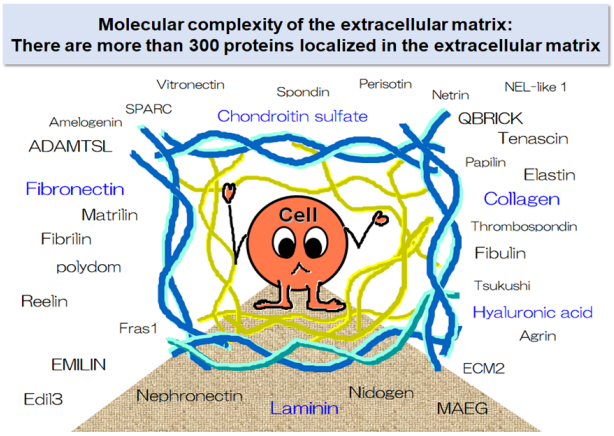


FIGURE 3.2. Cartoon image showing the complexity of the extracellular matrix.

A fundamental property of the ECM is its dynamism. The extracellular matrix undergoes through several remodeling processes with reciprocal interactions, and to transfer this characteristic to scaffolds, along with vascularization[19], is the most difficult part of designing them.

The ECM is composed by a large number of molecules (see FIGURE 3.2.), including fibrous proteins with structural functions (such as collagen), other specific proteins (such as growth factors or proteoglycans) and water.

III. FABRICATION TECHNOLOGIES

A few years after the foundation of tissue engineering occurred, Dr. Langer and Dr. Vacanti and their team enumerated the design techniques of the scaffolds, however, new technologies have substituted most of the former ones, just like the trends of thermal induced phase-separation (TIPS), electrospinning and three-dimensional bio-printing.

1. Thermal induced phase-separation (TIPS)

TIPS process has been widely used to form porous materials with a wide range of pore sizes. Another of the main advantages of the TIPS is the wide variety of the polymers that can be used. However, with time it has been substituted by the electrospinning technique, as in the TIPS method it is very difficult to control pore size.

TIPS method involves:

1. A homogenous polymer solution prepared by dissolving a polymer with a low melting point, meant to sublime it later on.
2. The polymer solution is shaped being cast in a mold with the desired shape, see FIGURE 3.3.

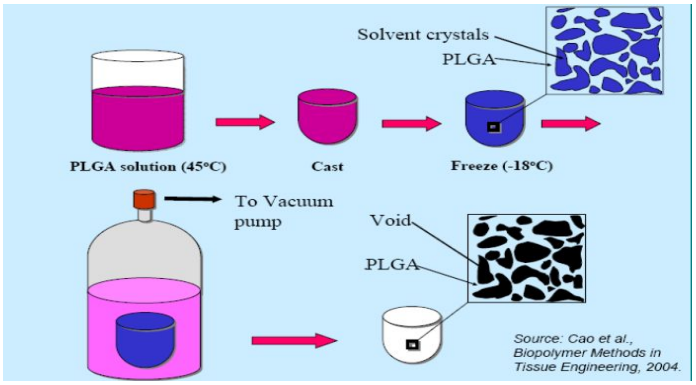


FIGURE 3.3. A PLGA polymer solution being molded in a desirable shape.

[19] This term is going to be described later on its own subsection.

3. Phase separation is induced by cooling down the solutions and adding a small quantity of water. Two different phases will be formed: polymer-rich and polymer-poor phases.
4. The solvent will yield a micro-porous structure scaffold (see FIGURE 3.4.) when cooled down the melting point and even some days of vacuum-dried.

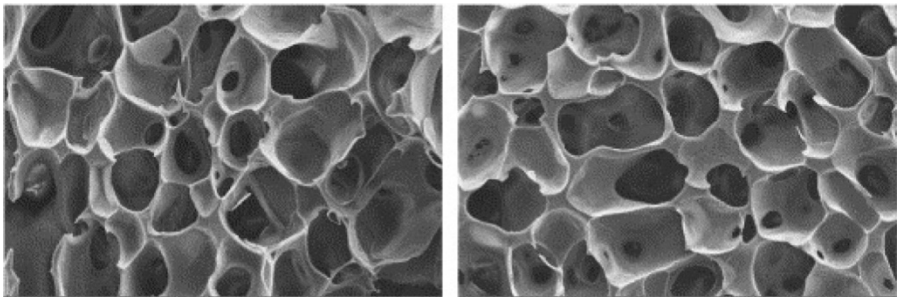


FIGURE 3.4. PLGA scaffolds made by TIPS.

2. Electrospinning

The electrospinning, also known as electrostatic spinning, is a very versatile technique that allows the formation from submicron to nanometer diameter fibers.

Between its advantages there is the major surface area of the final construct (because of its small pore size but high porosity) and the ease of the nanofiber composition.

Through a high electrical voltage and a syringe pump, the polymer solution jets the fibers that a lathe or collector will gather (see FIGURE 3.5.). Depending on the speed of the lathe spin, the resulting fibers will either be aligned or random oriented. If the lathe does not spin, the result will be random oriented fibers, whereas if it spins, an alignment of the fibers will be observed.

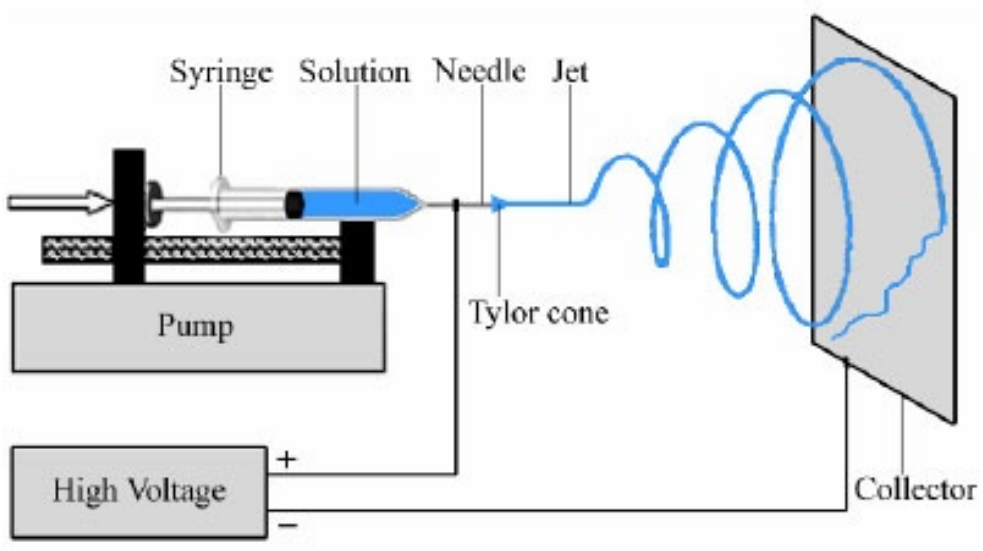


FIGURE 3.5. Electrospinning procedure scheme.

3. 3D bio-printing

CAM and CAD technologies permitted this method, CAM and CAD stand for Computer Assisted Manufacturing and Computer Assisted Design, respectively, see FIGURES 3.6-3.7.

FIGURES 3.6-3.7. CAM software used in bio-printers (left) and typical bio-printer (right).

This allows to precisely control the porosity and pore size. First the scaffold is designed and then the bio-ink forms the scaffold.

A bio-ink is a substance containing living cells that can be used to bio-print scaffolds. There are four types of bio-inks:

- → **Structural bio-ink**: They are the base of the bio-printed construct. On their own, they are not sufficient for a functional tissue, however, they give mechanical and degradation properties, as well as cell survivability.
- → **Sacrificial bio-ink**: This type of bio-inks generates void regions during the printing process, as they rapidly degrade, being auto-removed from the construct.
- → **Functional bio-ink**: These bio-inks direct cell differentiation and behavior towards the matrix. They normally contain growth factors or other biological agents. They can also provide different topographical cues to influence cells' organization.
- → **Supportive bio-ink**: Depending on the application, bio-printed constructs could be insufficient considering their mechanical properties, and supportive bio-inks protect the constructs until tissue development.

Once extruded the bio-ink with the 3D bio-printer, the result looks like FIGURE 3.8.

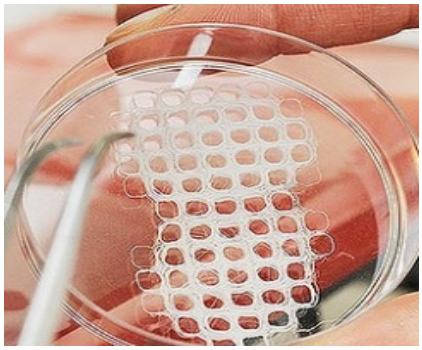


FIGURE 3.8. 3D bio-printed scaffold.

IV. VASCULARIZATION

This property is maybe the one that is keeping organ engineering from being an established commercial reality. Vascularization is the characteristic that allows blood vessels to communicate with biomaterial constructs. If a scaffold was not vascularized, oxygen would never reach it, causing hypoxia and later infection. Vascularization is a fundamental property for scaffolds implanted in vivo.

Due to their size, scaffolds need to have access to capillaries instead of thick vascular walls. A capillary is composed of endothelial cells (ECs), a basal membrane and pericytes. The physiology of the capillary is mainly regulated by ECs, however. To achieve vascularization in scaffolds, a formation of new blood vessels has to be induced. There are two types of formation of new blood vessels: vasculogenesis and angiogenesis, see FIGURE 3.9.

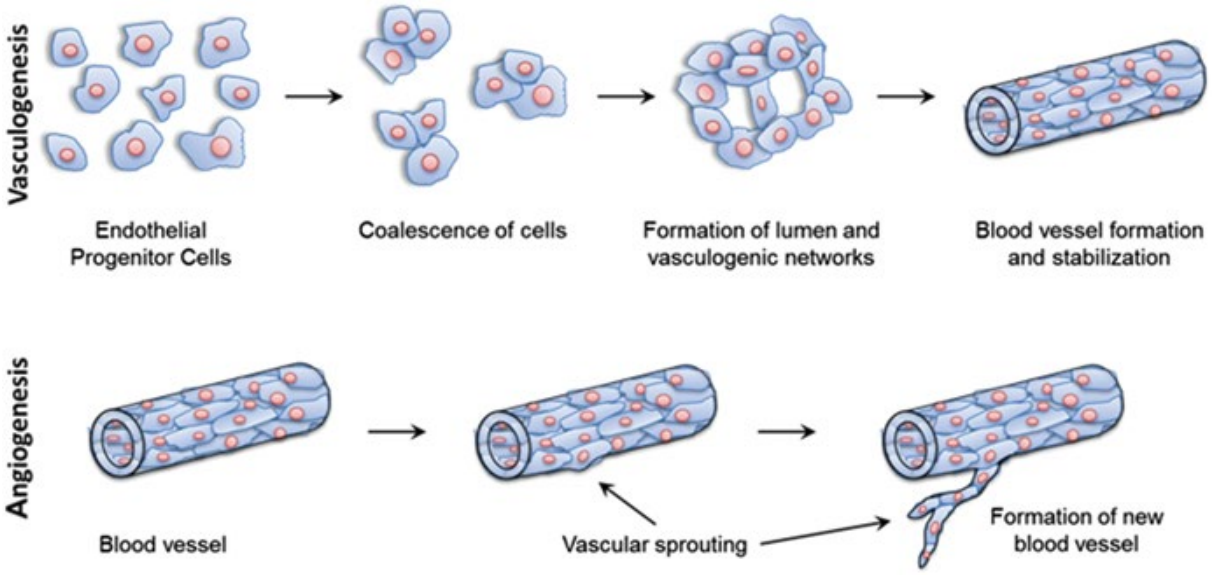


FIGURE 3.9. Vasculogenesis compared to angiogenesis.

Vasculogenesis means the differentiation undergone by mesodermal cells to form blood vessels. First, mesodermal cells differentiate into angioblasts, then into ECs and they organize to form a capillary, like in FIGURE 3.9. In another context, angiogenesis refers to the formation of blood vessels by sprouting from already-existing vessels. Whereas vasculogenesis only occurs in the formation of the embryo, angiogenesis can occur in the postnatal life, from pre-existing vessels.

Vascularization of biomaterial constructs can be promoted ex vivo through material design, culture conditions and cell source, to later be implanted in vivo and integrate with the host vasculature, using different biochemical signals. Scaffolds are normally seeded with these signals that will promote angiogenesis when implanted in vivo through proangiogenic factors.

So, to study these cases, there are several assays that can be carried out. The most common and effective one to study angiogenesis, however, is the CAM Assay, or Chorioallantoic Avian Membrane Assay.

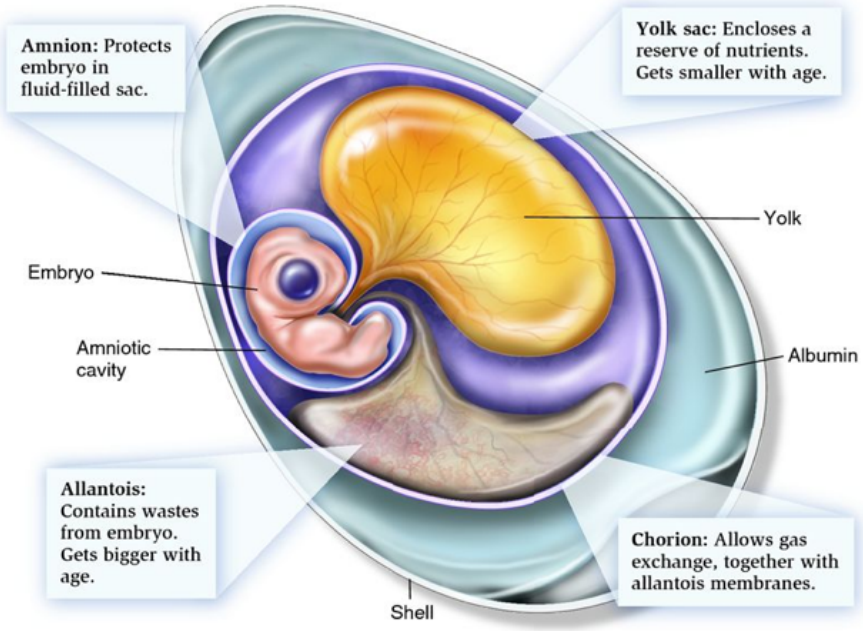


FIGURE 3.10. The chorioallantois in the egg of the avian embryo.

The chorioallantoic avian membrane (see FIGURE 3.10.) is the vascular membrane found in eggs of birds and reptiles, where it contains blood capillaries and sinuses. These membranes from developing chicken eggs are widely used to investigate proangiogenic and antiangiogenic factors. This membrane, in chicks, is formed during the fourth or fifth day of development, when the biomaterial is implanted.

Between the advantages of this model there is:

- High proliferation rate.
- Simplicity.
- Economical and time-efficient.
- Naturally immunodeficient host, so there is no immune response by the avian embryo.

This assay is majorly carried out to analyze the biophysics interactions between the implanted material and the membrane, as it is a way of checking effects of some elements. An example of this could be making a control group of empty biomaterials and an experimental group of biomaterials with a particular agent, to check which of them could inhibit or induce angiogenesis.

Inhibiting angiogenesis would be useful for tumors, for example, and to induce it would be useful for tissue-engineered implants.

CHAPTER 4

CELL-MATERIAL INTERACTIONS

I. Cell surface receptors.
II. The role of growth factors.
III. Foreign body reaction.
IV. Protein – Material interaction.
V. Photofunctionalization.

Everyone we interact with becomes a part of us.

-Jodi Aman, American psychotherapist.

I. CELL SURFACE RECEPTORS

Methods to control cell-material interactions are crucial in order to accomplish ideal biomaterials that can arise specific desirable responses from surrounding tissues. However, cell behavior in vivo changes drastically from in vitro. In vitro, quantifiable and controlled situations can be induced, while in vivo models have a complex cascade of cellular responses. The type of interactions that will be characterized, given its importance above the others, is cell adhesion.

As the majority of cell-substrate interactions are directed by biomolecules, to learn how cells adhere and interact with their own extracellular matrix (ECM) is necessary. There are several types of cell surface receptors, and this section will review integrins and immunoglobulins. These receptors are proteins that tend to have both a trans membranous and intracellular domains.

The transmembrane region is hydrophobic and the same thickness of the membrane, see FIGURE 4.1.

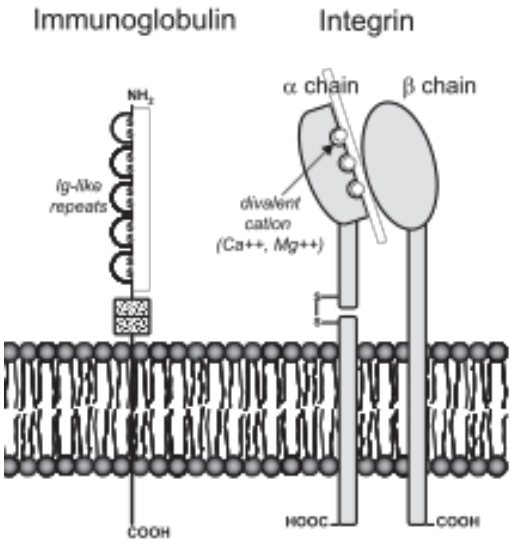


FIGURE 4.1. Immunoglobulins (left) and integrin(right). The COOH or carboxylic groups are the ones that interact with ECM ligands[20].

Most cellular interactions with the ECM occur with integrins, however, other receptors like immunoglobulins are looked to when designing scaffolds.

An important characteristic to consider for cell surface receptors is affinity. It is a measure of the binding strength between a lone ligand and a single receptor. An increase of affinity or avidity would mean that ligand binding could occur at lower ligand concentrations.

Integrins are the main receptors on mammalian cells for binding most of the ECM proteins. They are formed of one α-subunit and a ß-subunit. There are different types of integrins, as

[20] Ligands, or signals, are molecules that arise a response from one cell to another.

they are over 18 α-subunits and 6 ß-subunits. Their different combinations form what are called heterodimers.

Depending on the ligand (protein) that binds to integrins, different cell adhesions are promoted. For instance, the most important ligand sequence is the RGD sequence (Arginine - Glycine - Aspartic acid). The integrin recognition of this sequence occurs in most of the cell types, meanwhile the REDV sequence (Arginine - Glutamine - Aspartic acid - Valine) is recognized only for endothelial cells.

The **immunoglobulin** superfamily[21] is a large chain of proteins involved in cell recognition and later adhesion. Their structure is the same as antibodies' protein domains. The immunoglobulins will recognize cells for the immune system.

The proteins of the immunoglobulins conform a determining region important to recognize ligands, depending on the sequence of amino acids specific ligands will be recognized or not.

Referring now specifically to the extracellular matrix, cell-ECM adhesion is poorly understood, as it is a really complex interaction, characterized to have many signals acting. However, it is known that cell surface receptors as immunoglobulin or integrins anchor cells depending on the sequence of amino acids that the ligand contains and on the subfamily of receptors.

II. THE ROLE OF GROWTH FACTORS

Growth factors are protein molecules made by the body (although they can be genetic engineered), and they regulate cell division and cell survival.

Growth factors, in cells, act as transmembrane receptors' ligands. They bind to cell surface receptors, to activate internal cell signaling. Growth factors stimulate cell division in either numerous cell types or a specific cell-type, and they also promote cell growth, see FIGURE 4.2. In other words, they also are mitogens (growth stimulators) or growth inhibitors.

These proteins are also capable of inhibiting cell migration and modulating functions of cells. An example of the latter mentioned ability is the growth factors' involvement in apoptosis or angiogenesis of cells.

[21] A protein superfamily is a group of proteins related by evolution. They share structural properties; however, their sequence might be different.

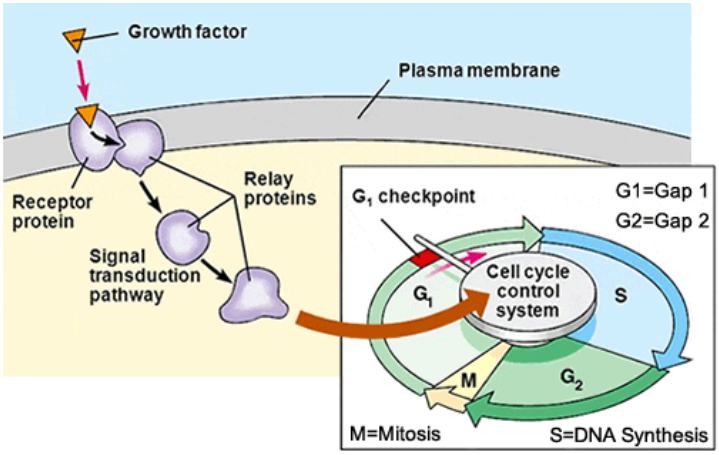


FIGURE 4.2. Visual scheme of the effect growth factors arise.

It could be concluded that growth factors are bioactive poly-peptides which function as hormones. Depending on their sequence of amino acids, growth factors are placed into families, the most important growth factors are Vascular Endothelial Growth Factors (VEGF) and Fibroblast Growth Factors (FGF).

VEGF are important in human development, they promote the formation of new blood vessels, naturally, in human embryos. In other words, they promote vasculogenesis.

FGF are a diverse family of growth factors. However, they, in a general manner, participate in angiogenesis, healing wounds, development of the embryo and endocrine signaling.

III. FOREIGN BODY REACTION

The inflammatory response (part of the foreign body reaction) towards an implant of a biomaterial is almost inevitable because of our immune system detecting it. Inflammation is actually a protective and necessary response against invading pathogens[22] or unknown substances. The interactions will be described as a whole process.

Process:

After being implanted, the biomaterial reacts with the body, in general ways of speaking, in this sequence (see FIGURE 4.3.).

[22] Pathogens are agents that can provoke diseases.

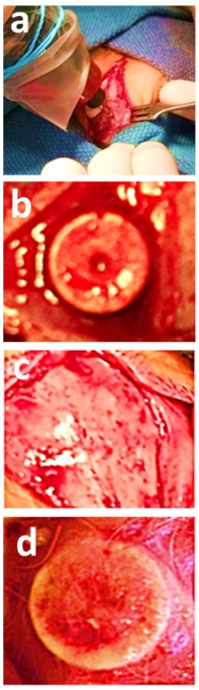


FIGURE 4.3. ***a*** *represents injury when implanting the material,* ***b*** *represents blood-material interaction,* ***c*** *represents inflammation and* ***d*** *represents the fibrous encapsulation.*

1. **Blood-material interaction.**
   The first reaction induced by the transplant is protein adsorption[23] onto the surface of the material. That will cause a provisional matrix. The properties of that matrix depend on the physical and chemical properties of the implant. This protein adsorption is not a static process, as proteins are adsorbed and resorbed in time.
   This provisional matrix is the first place where cells will react.

2. **Acute inflammation.**
   The acute inflammation is the result of the infiltration of polymorphonuclear leukocytes[24] into the provisional matrix, attracted by complement factors, a group of proteins adsorbed into the implant. This polymorphonuclear leukocytes, also called granular leukocytes, release granules (particles), that cause this inflammation.

3. **Chronic inflammation.**
   Chronic inflammation happens if the injury's extent is greater. It is the result of the migration of monocytes[25] (that will differentiate into macrophages[26]) to the implant.

[23] Protein adsorption is the process where proteins adhere to solid surfaces. Later on, more information will be exposed.
[24] Leukocytes are a type of white blood cell.
[25] Again, another type of white blood cell.
[26] An immune system cell specialized in recognizing, engulfing and destroying a determined target.

At the interface between tissue and material, these macrophages are going to secrete degradative enzymes, reactive oxygen species and acid to degrade the implant. Macrophages will, consequently, try to ingest the material.

4. **Foreign-body giant cells.**
   If that ingestion did not result, macrophages would fuse and foreign-body giant cells. Foreign-body giant cells are multi-nucleated cells that cause, exteriorly, damage that results in scar tissue.

5. **Fibrous encapsulation.**
   If the material still persists, a fibrotic connective tissue is formed around it, shielding it from the immune system and isolate it from surrounding tissues. This frequently leads to the device failing or losing efficiency.

   Even at this stage, where the implant is technically protected from the immune system, macrophages and foreign-body giant cells still try to attack and engulf the implant.

This is why biocompatibility is so important. If the body notices a foreign substance, it will attack that substance lowering their properties or even destroying that substance. Trying to avoid this response from the body is essential to develop functional drug delivery techniques or three-dimensional scaffolds.

IV. PROTEIN – MATERIAL INTERACTION

Every cell-material interaction is actually based on proteins and the surface of the material interacting, so proteins are maybe the most important biomolecules to consider.

When a material enters a biological environment, that material is exposed to a wide range of both general and specific proteins of that environment. Depending on many factors, as if the surface is hydrophilic or hydrophobic, for instance, it will react with a different number of proteins and that will determine the fate of the implant.

These proteins will be adsorbed by the surface and will display the provisional matrix. This matrix affects both bacterial and cell adhesion and blood coagulation.

As they are interacting with a surface, what is important of the biomolecules is their primary structure, in other words, their amino acid sequence.

Before stating the general process of the interaction that occurs, the important properties for the interaction of both proteins (TABLE 4.1.) and materials (TABLE 4.2.) will be mentioned, exposing how the interaction varies depending on those properties.

Property	*Effects*
Size	Larger proteins have more contact areas with the surface.
Charge	Molecules near their isoelectric point[27] adsorb more easily.
Structure stability	Less stable proteins (or less intramolecular cross-linking) can unfold more and form more contact points.
Structure unfolding rate	The speed of the unfolding means a faster creation of contact points.

TABLE 4.1. Property-effect of proteins.

Property	Effect
Topography	If the surface is stiffer more surface area is created to interact with proteins.
Composition	The chemical composition of the surface will determine the intermolecular forces that will occur.
Hydrophobicity	Hydrophobic surfaces tend to attract more proteins.
Potential	This will influence the distribution of ions when interacting with proteins.

TABLE 4.2. Property-effect of material surfaces.

Protein adsorption

In the last subsection, protein adsorption was already stated, however, this is going to deepen on this process.

Apart from depending on the properties of the surface and from the proteins themselves, adsorption depends on the number of proteins on the surface.

These proteins accumulate in the surface by four transport mechanisms: **1)** diffusion, **2)** thermal convection, **3)** flow and **4)** coupled transport (a mix of diffusion and convection). However, only diffusion, thermal convection and coupled transport or convection-diffusion will be explained to ease the reading of this chapter.

The variables that affect these four mechanisms involve, among others, concentration of the proteins, velocity of the biomolecules or their molecular size.

[27] The isoelectric point is the synonym of the no charge point.

1) Simple diffusion is explained by this equation:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}$$

EQUATION 4.1. Simple diffusion equation. Where C is the concentration, D the diffusion coefficient, and x the distance. This symbol ∂ represents the variation of a variable.

However, in such short times and different conditions, the equation varies to this one:

$$\frac{\partial n}{\partial t} = C_0\left(\frac{D}{\pi t}\right)^{\frac{1}{2}}$$

EQUATION 4.2. Protein adsorption diffusion equation. Where n is the surface concentration of protein, C_0 is the bulk concentration of protein[28]. D is the diffusion coefficient, and t is the time. The difference with EQUATION 4.1. is that in this equation a higher D and a higher n equals more proteins transporting to the surface.

2) Thermal convection or heat transfer coefficient is explained by this equation:

$$Q = U\,A(T_2 - T_1)$$

EQUATION 4.3. Typical heat transfer rate (Q) equation. Where U is the heat transfer coefficient, A is the surface area of the material, T_2 is the temperature of the biomolecule and T_1 is the temperature of the surface.

When both convection and diffusion are present, greater variables have to be considered, and convective diffusion or convection-diffusion results in this equation:

$$\frac{\partial T}{\partial t} = \nabla \cdot (\mathrm{D}\nabla \mathrm{T}) - \nabla \cdot (\bar{v}T) + R$$

EQUATION 4.4. General or typical equation of convection-diffusion. T is the temperature, t is time, ∇ is the divergence[29], v is velocity and R describes "sources" of T. In chemistry, if R<0 that means the reaction is destroying species and if R>0 it means the reaction is creating more of them.

Calculating each surface's heat transfer coefficient or diffusion rate helps scientists develop better solutions to avoid problematics in the protein adsorption and cell adhesion processes.

Once proteins are present on the surface, they interact with it via intermolecular forces: ionic

[28] The difference between surface concentration(Sc) and bulk concentration(Bc) is the units, Sc is expressed in units per area and Bc in units per volume, so, to change units, we follow Sc / t , being t the thickness of the surface, equals Bc.

[29] Divergence in this case means contrary to the flow.

bonding[30], hydrophobic interactions[31] and charge-transfer interactions[32], excluding hydrogen bonding.

V. PHOTOFUNCTIONALIZATION

Having seen already all the state-of-the-art of cell-materials, this last section will be dedicated to UV photofunctionalization, a chemical surface modification that has drawn the attention of scientists lately.

Photofunctionalization is the treatment given to titanium implants with ultraviolet (UV) light with a specific wavelength and strength, as a potential surface modification. There is not enough evidence to be a defined option to modify the implant's properties.

Titanium surfaces, in several studies, after being exposed to UV light, are more hydrophilic, accumulated hydrocarbons are decomposed and more proteins and cells are attracted. However, this information is not certain and more studies and more research have to be dedicated to this process.

This process was reviewed to show how science really advances, being divulgated and showing possibilities that they offer. Regenerative medicine and specifically tissue engineering are really young sciences and there is a lot of scientists and engineers making advances in a really quick manner. Also, it can be seen as this information cannot be fully trusted, as in science peer-reviewing (see FIGURE 4.4), citations and sufficient evidence is always needed.

FIGURE 4.4. A cartoon scheme of how peer review help articles to get to a journal and make a scientifically sound research.

Having finished this theoretical framework, the experimentation and investigation carried out is going to be detailed now.

[30] Chemical bonding that involves electrostatic attractions to oppositely charged ions.

[31] Chemical bonding that involves nonpolar substances bonding because of exclusion of water.

[32] Chemical bonding that involves two or more substances linking to each other transferring electronic charge between them.

FIELDWORK

EXPERIMENTATION AND INVESTIGATION

- **Approach and objectives.**
- **Study 1:** Decellularization of a heart.
- **Study 2:** Fabrication of 3D scaffolds and drug delivery method.
- **Study 3:** CAM model to induce angiogenesis.
- **Study 4:** PH and Calcium release measurements.
- **Study 5:** Fluorescent staining of cells within two experiments.

Nobody made a greater mistake than he who did nothing because he thought he could do only a little.

-Edmund Burke, Irish philosopher.

FORMULATION OF THE APPROACH AND GENERAL OBJECTIVES

PROJECT'S APPROACH

Having finished all the contextualization and state-of-the-art, it begins the real investigation.

Why this experimentation? We have already seen how the main problems of biomaterial science and tissue engineering are caused, in consequence, by the same factor, the lack of knowledge on biophysics and cell mechanics.

With this experimental procedure we are to determine how cells behave when they are in contact with different materials in different situations.

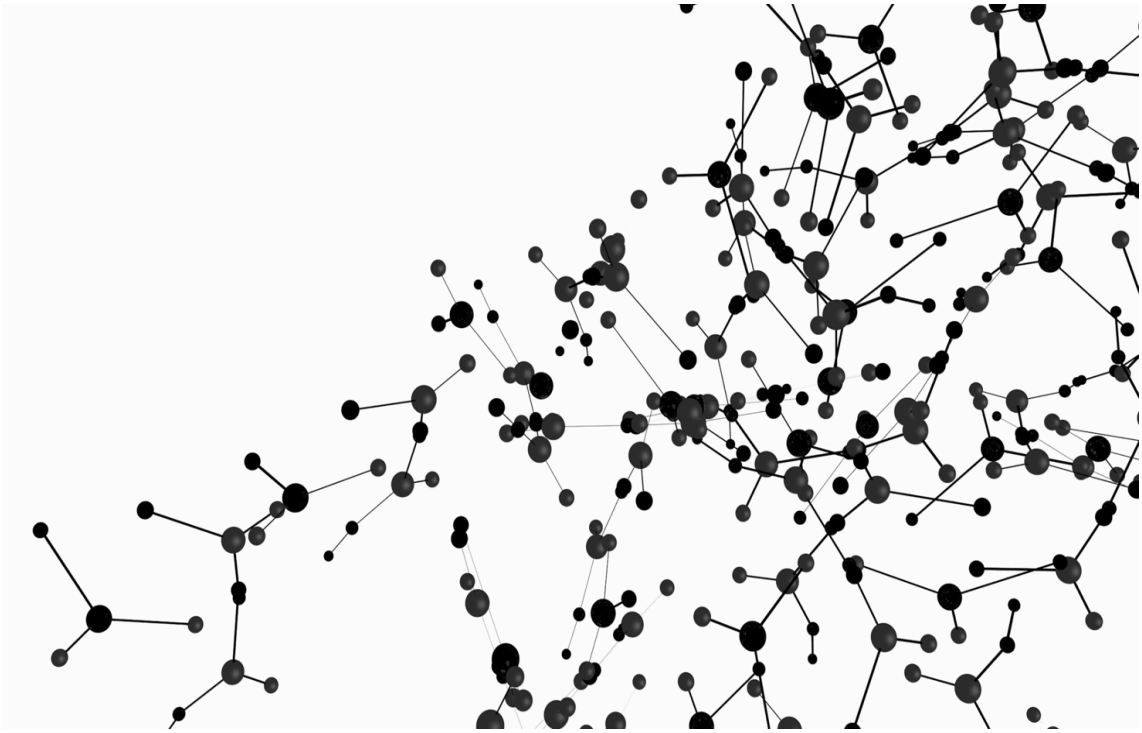


FIGURE F.1. Scheme of a molecular network.

Knowing how cells behave may lead to strong biophysical principles that would revolutionize tissue engineering as how we know it today.

Every study carried out on the laboratory will be structured in the same way:

- An **Introduction**; to explain why are we doing that specific experiment.
- The **Aim** of that experiment.
- **The Materials Used in our experiment will be put in Annexes.**
- **The Procedure carried out will be put in Annexes.**
- Our procedure's **Observations**.
- Finally, the **Results**.

GENERAL OBJECTIVES

- **Get a grasp on how cells behave in different contexts and observe how cells can adapt to different signals.** Learning how cells behave is going to allow new knowledge on many different fields, stem cells biology, tissue engineering, biomaterial science, biophysics… This knowledge will, consequently, allow new discoveries, applications and, eventually, solutions.

- **Demonstrate why biophysics and tissue engineering are the future of our society's health and quality of life.** Nowadays, biophysics and tissue engineering are being highly researched. This project intends to reassure why it is this way.

- **Use state-of-the-art techniques.** Only using the newest technologies and methods we will be able to measure and observe all the elements to analyze.

- **IN THE ANNEXES SECTION (Learn about the perspective of the best professionals in the world on bioengineering and its future.** Learning about the world pioneers is the best way of getting new knowledge or insights in these projects' fields. **)**

- **Raise consciousness and interest on these particular science fields.** If these fields become popular, eventually more money is going to be invested on new investigations and solutions to health problems will be faster.

Although there are more objectives to achieve, as they are specific ones, are going to be detailed just before explaining the experiments.

In order to check all the objectives mentioned above, we have carried out 6 different but related experiments, to study cell behavior in different contexts.

STUDY 1: DECELLULARIZATION OF A HEART

Introduction

Tissues are not only made of cells. A major part of their volume is composed by extracellular matrix, which is an intricate network of macromolecules made of a variety of proteins and polysaccharides that are secreted locally by cells and assembled into an organized meshwork. Collagen is the main component of these matrices.

However, the extracellular matrix (ECM), as we have already learnt, is not an inert filler material, but a dynamic, active component of all living tissues. Apart from providing structural support for the cells, it also guides division, growth and development. What is more, the extracellular matrix influences cell behavior, as well as how tissue looks like and its function.

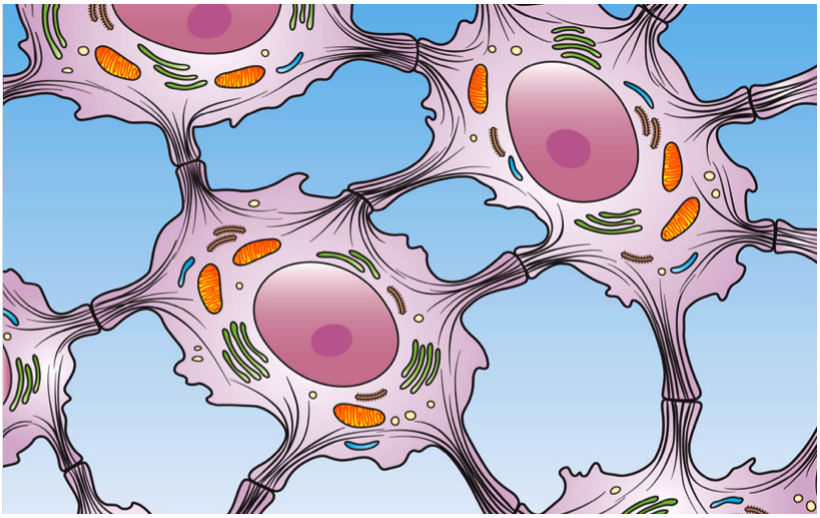


FIGURE F.2. Scheme that represents cells within their extracellular matrices' network.

And so, different tissues present a specific and characteristic extracellular matrix, with a defined composition and signals for cells, to guide a different behavior for them.

Scaffolds for tissue engineering are very frequently designed to mimic the ECM of the target tissue, thus providing the best environment of tissue regeneration. But, why don't we directly use natural ECM?

Researchers are able to take the tissue from a donor or cadaver, lyse and kill the cells within the tissue without damaging the extracellular components, and finish with a product that is the natural ECM scaffold that has the same physical and biochemical functions of the natural tissue. These acellular matrices have been successfully used in a number of different applications. Skin grafts are used in cosmetic surgery and burn care as mechanical support to the damaged area while supporting the development of host-derived connective tissue. ECM can also be used as a biocompatible ink for 3D printing, thus producing new shapes and structures. Additionally, the decellularization of a whole heart can provide an ECM scaffold which can be recellularized with patient's own cells, thus eliminating the adverse immune-response and avoiding rejection in organ or tissue transplantation.

Aim

To see how the heart progressively loses its cells.

To decellularize a whole mouse heart to obtain a natural scaffold, in other words, to obtain just the mouse's heart ECM.

Observations

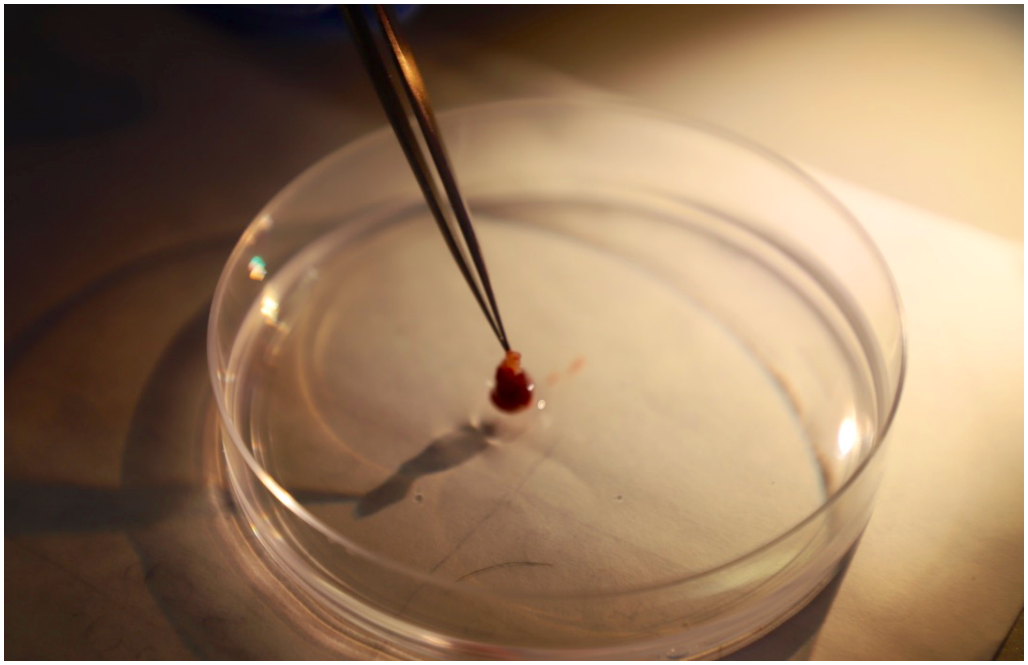


FIGURE F.3. Picture taken of our mouse's heart. Specifically, of when we were removing its blood.

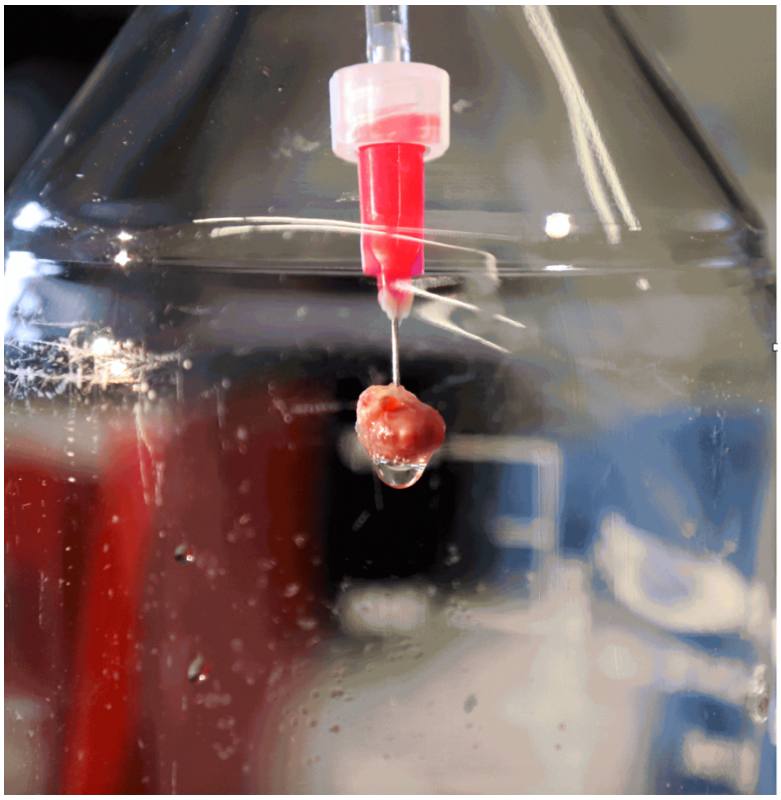
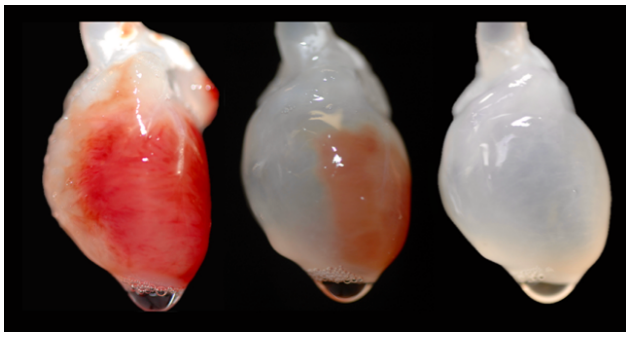


FIGURE F.4. SDS falling through our mouse's heart.

FIGURE F.5. External internet picture that shows the progression our mouse's heart went through.



Our experiment's progression was relatively slow compared to the other studies, as we had to wait until each of the heart cells were taken out. The last step took about 3 hours, as the perfusion pump was set a really low rate, in order to not damaging the organ. To check if we fully decellularized the heart, we should have pumped another decellularization agent (or another "detergent"), such as Triton X-100. Also, the perfusion time length should have been much larger, talking about, maybe, from 3 days to a week.

Results

Result as expected, we saw how the heart changed its color, meaning that its structure remains the same but its function and color is inhibited by the lack of cells. However, we have taken an internet photo (FIGURE F.5.) to show the whole heart decellularized because in our experiment it was not as clear as in this picture.

As stated on the **Observations** section, our heart may not have been fully decellularized, as we only used one decellularization agent and a really short time length.

Despite the fact that it probably was not fully decellularized, from this experiment we managed to see how an organ like the heart can be used in many ways, to study and to learn more about the inside of our organs' interactions. Some of the ways that a heart like the one investigated with could be the heart (ECM) as a natural scaffold, or the heart being used as a bio-ink, having yet more applications in the bio-printing techniques.

STUDY 2: FABRICATION OF 3D SCAFFOLDS

Introduction

Tissue engineering aims to replace or repair damaged tissues or organs. To do these different strategies can be used and mainly consist of the use of biomaterials, cells and/or signals (for instance growth factors) that stimulate the tissue regeneration in the injured area.

Cells can be used as "factories" producing signals that stimulate regeneration of damaged tissue. In several applications, they have been injected directly into the damaged tissue but this has been shown to have a low cell survival. Biomaterials can serve as vehicles for the administration of cells, improving their survival and promoting the success of the therapy. One example of a widely used biomaterial to encapsulate cells is **CALCIUM ALGINATE**.

Alginate is a polysaccharide extracted from brown algae widely used in the field of biomedicine. This material is biocompatible (does not cause unwanted reactions in the body) and biodegradable (is absorbed by the tissue) and can be easily modified both chemical and physically to give different structures, functions and properties. Alginate can be dissolved in water and can be transformed into gel when put in contact with a solution containing divalent ions such as calcium. Once cross linked with calcium, the materials created with alginate not only provide a 3D environment suitable for cell survival, but also, they can be used to protect the implanted cells from rejection by the patient's immune system, for instance when using cells from another person.

2 different experiments were carried out, the first one to fabricate a 3D hydrogel scaffold, and the other one to test a drug delivery technique with alginate encapsulation.

Aim

- IN THE EXPERIMENT I

To learn about fabrication of alginate blocks and to test the alginate gelation.

- IN THE EXPERIMENT II

To create spheres, encapsulating cells and observe the dissemination of biological factors released by these cells.

Experiment I: Fabrication of alginate blocks.

Introduction

Creation of calcium alginate blocks and compare how solutions containing different calcium concentrations affect the cross-linking of the alginate.

Observations:

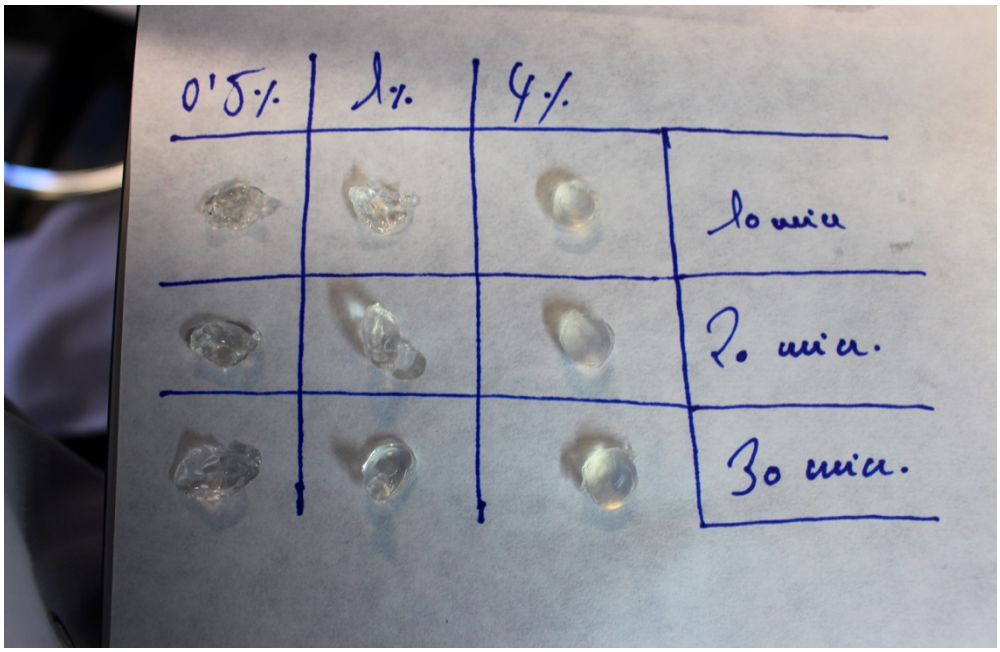


FIGURE F.6. Our samples when our experiment was finished.

When we finished our experiment, our samples (see FIGURE F.6.) had already changed their properties, as we were also evaluating them in time periods of 10, 20 and 30 minutes.

Results:

First of all, we observed differences between the samples at each time point. As the length of time was greater, our alginate blocks were stiffer, and their shape was more defined.

It was also observed how calcium concentration also affected the shape and stiffness of our samples. From the 0.5% of calcium chloride concentration, as it can be seen in FIGURE F.7., it had not successfully cross-linked, as some liquid was released when picked up with the tweezers.

In general, from the 0.5% on, the samples gradually changed this cross-linking capacity as both time and/or calcium chloride concentration increased.

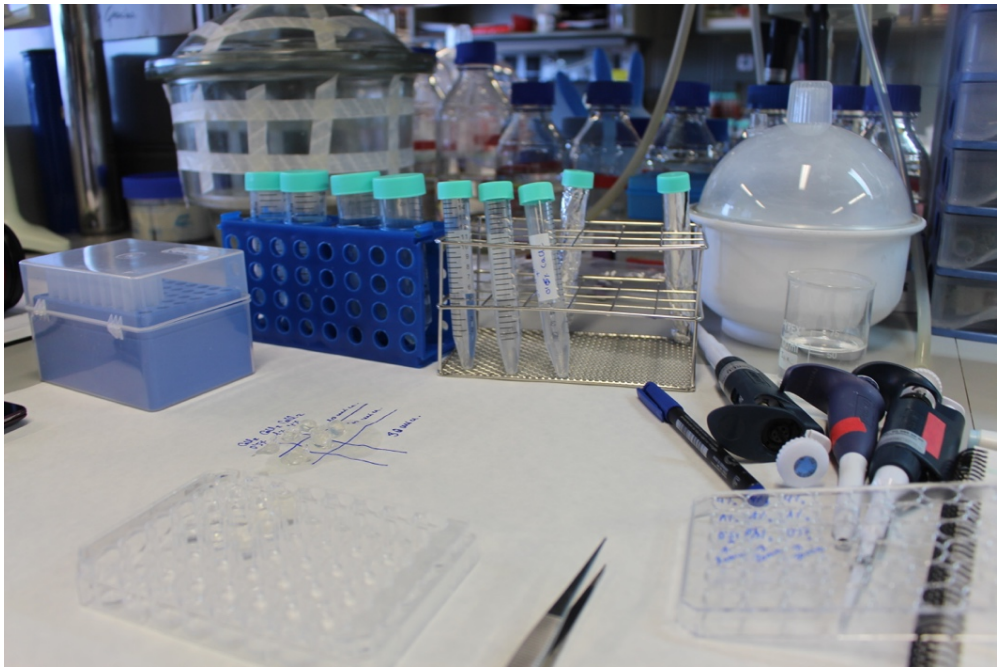


FIGURE F.7. Picture taken of the process of characterizing the macroscopic properties of the alginate blocks.

Experiment II: Generation of alginate spheres for the release of biological factors.

Introduction

We will stimulate the encapsulation of cells in calcium alginate spheres and observe how they release signaling factors. Because cell manipulation requires the use of special equipment and also practice handling them, we will not use real cells in this practicum. Instead of cells, we will use a chemical reagent called borax, which is a powder that when dissolved in water produces an alkaline pH.

After adding “our cells” (borax) to the alginate solution, we will generate drops that, when put in contact with a solution of calcium chloride ($CaCl_2$), will crosslink producing spheres. Then, we will isolate the borax-containing alginate spheres created and we will transfer them into a solution containing phenolphthalein, a pH indicator. As borax diffuses outside the spheres, we will observe a change in the medium color. This will simulate the release of proteins from cells encapsulated in the spheres, as could be the release of insulin from pancreatic beta cells in a diabetic patient, for instance.

Observations:

The spheres are created with a needle and a syringe, as seen on the FIGURE F.8. When we picked up the spheres with the paper filter, we added them on the phenolphthalein solution, and instantaneously changed its color. If it were colorless or orange, that would mean our solution would not be basic, and the biological factors would not have been released.

FIGURE F.8. With the help of a syringe and a needle, we created the spheres.

FIGURE F.9. The alkaline solution is brought to us with the pH indicator, phenolphthalein, staining the solution in fuchsia.

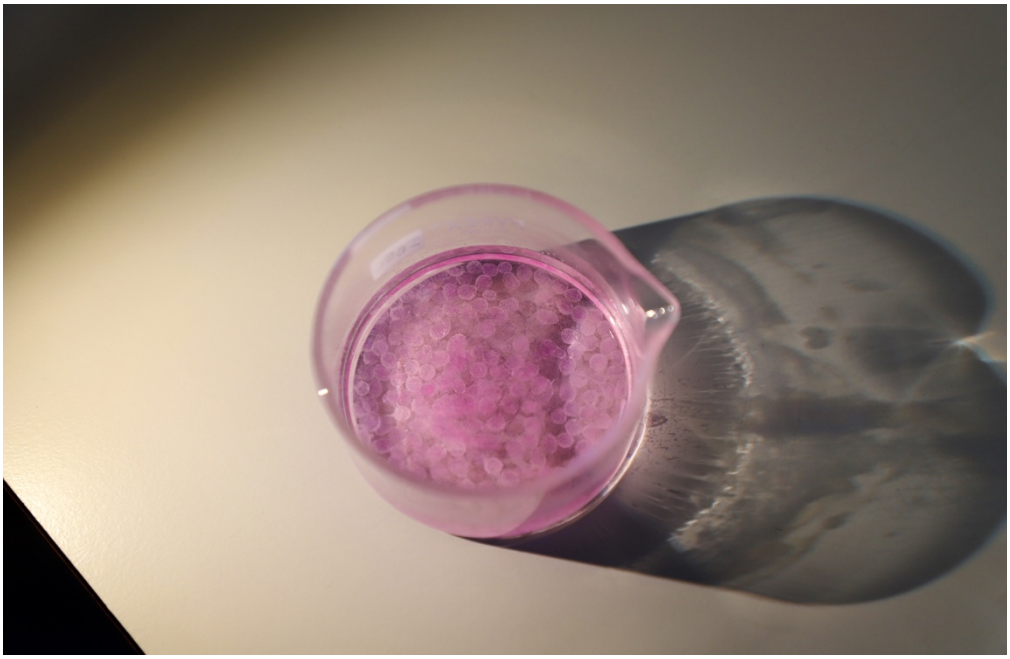


FIGURE F.10. Another image of the spheres added to the phenolphthalein solution.

Results:

This is an experiment to demonstrate the potential of drug delivery techniques. There are many ways of administrating drugs in a controlled system, and one of them is the encapsulation of spheres, just like in our case. We can see, as the solution changed to an alkaline pH, that borax has released these biological factors we were searching for, demonstrating how encapsulating drugs to achieve drug delivery inside our body is a reality in the near future.

Although it was not drugs, we have checked the functionality of this technique this chemical reagent.

STUDY 3: CAM Assay, Angiogenic assay with the ex ovo chick embryo model.

Introduction

Angiogenesis is the process by which new blood-vessels are generated from pre-existing vasculature. The study of substances that inhibit or stimulate angiogenesis is important in different biomedical fields. For instance, antiangiogenic factors have been studied as a new way of treating primary tumors and reducing their metastases. In tissue regeneration, stimulation of formation of new blood vessels in the implanted biomaterial is very important for the success of the implantation. The implanted biomaterial needs to be vascularized in order to allow the survival of the cells in the site and the formation of new tissue. Therefore, it is important to know what substances can induce or inhibit angiogenesis.

Different in vitro and in vivo models have been used to study the angiogenic or antiangiogenic properties of substances. Among these, the chick embryo chorioallantoic membrane (CAM) model has been widely used for being a robust, inexpensive, and flexible model, allowing multiple sample application.

The CAM model takes advantage of the chorioallantoic membrane present in the embryos of avian species. This membrane is a highly vascularized extra-embryonic tissue that allows for gas exchange between the embryo and the atmosphere surrounding the egg. In other words, it performs the function of a lung during the 21-day embryonic development of the chick.

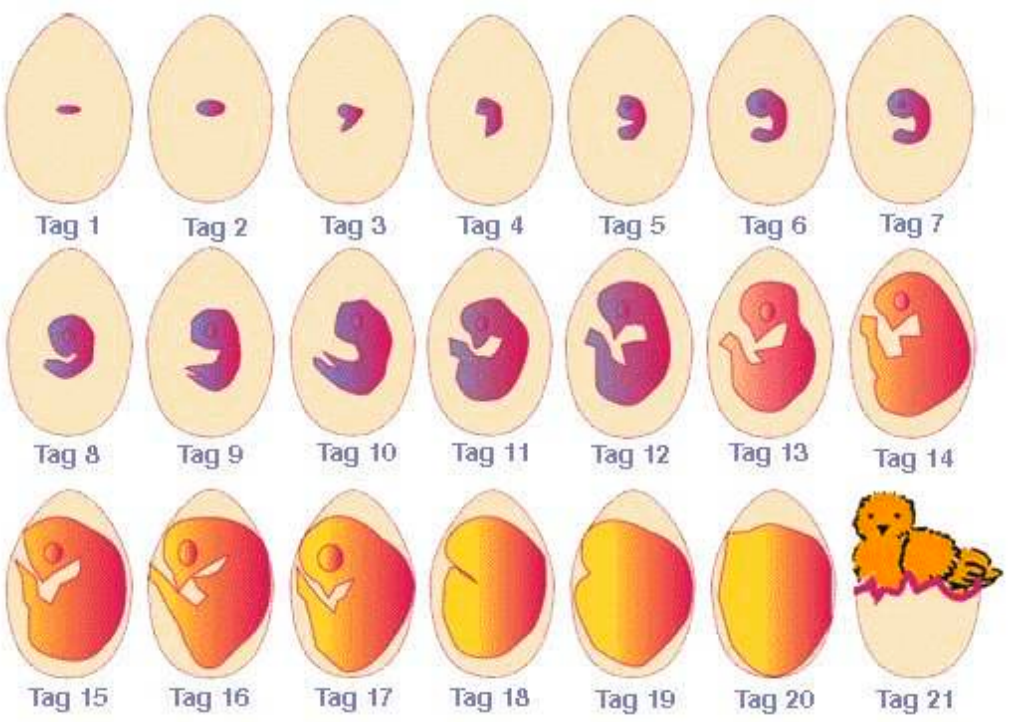


FIGURE F.11. Diagram in German that counts the days of the chick embryo development.

In the chick embryo, the chorioallantoic is formed between days 4 and 5 of development. The central portion of the CAM is fully developed by day 8 to 10, while the outskirts of the CAM are still developing and expanding. Also, between day 8 and 10, the developing CAM vasculature is ready to sprout in response to additional proangiogenic stimuli and, in turn, is very responsive to antiangiogenic factors. The vasoproliferative response will take place as soon as 72h after implantation with an increase in vessel density around the implant, with the vessels radially converging towards the center. For this reason, materials are added between 8-10 days and left until 12-13 days of development.

This model can be used both in ovo or ex ovo. In this practicum we used the ex ovo model. Briefly, eggs are cracked on day 3 of development and allow to develop in a Petri dish. Test materials are added on day 9 and CAMs are fixed on day 12.

Aim

To determine the angiogenic properties of calcium-releasing particles using the ex ovo CAM model. We want to test if calcium can stimulate an angiogenic effect. For that, the amount of vessel formed in the CAM around disks containing calcium-releasing particles (our experimental group) and disks without any stimulating agent (a negative control) are compared.

These experimental conditions will be added in methylcellulose disks which will serve as support materials. Methylcellulose disks are inert materials: they should not stimulate any effect by themselves, meaning that the effect that we will observe on the CAM will be stimulated only by tested substances.

Results and Observations

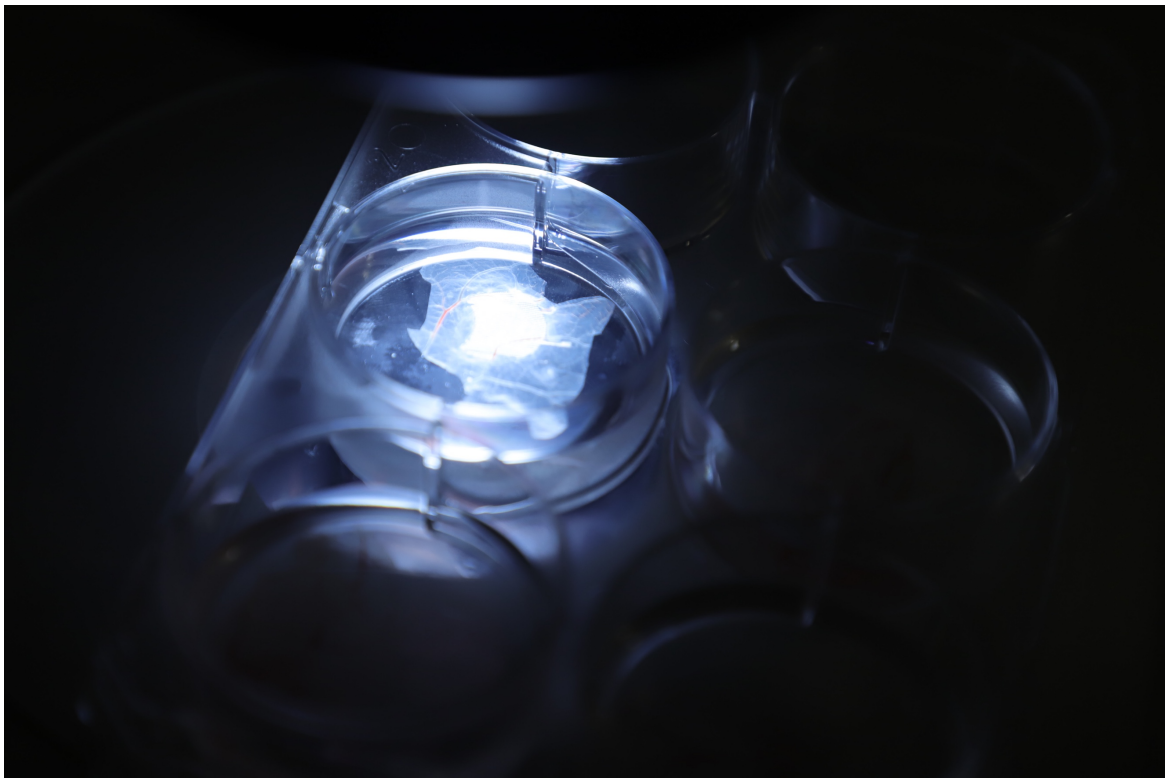


FIGURE F.12. How our resulting scaffolds looked like.

How was angiogenesis induced in scaffolds? We began our angiogenesis evaluation, quantifying the proangiogenic response from the basis of the *FIGURE F.13.*

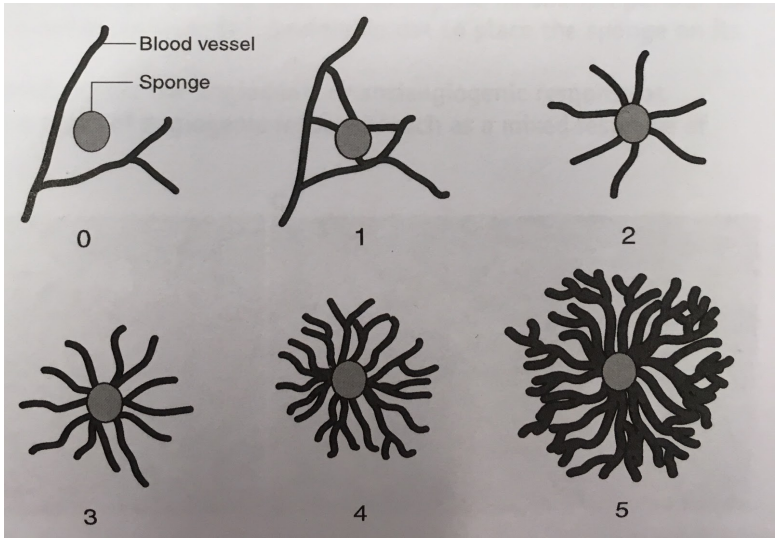


FIGURE F.13. We assigned the different numbers to 20 different scaffolds, numbered from A to T.

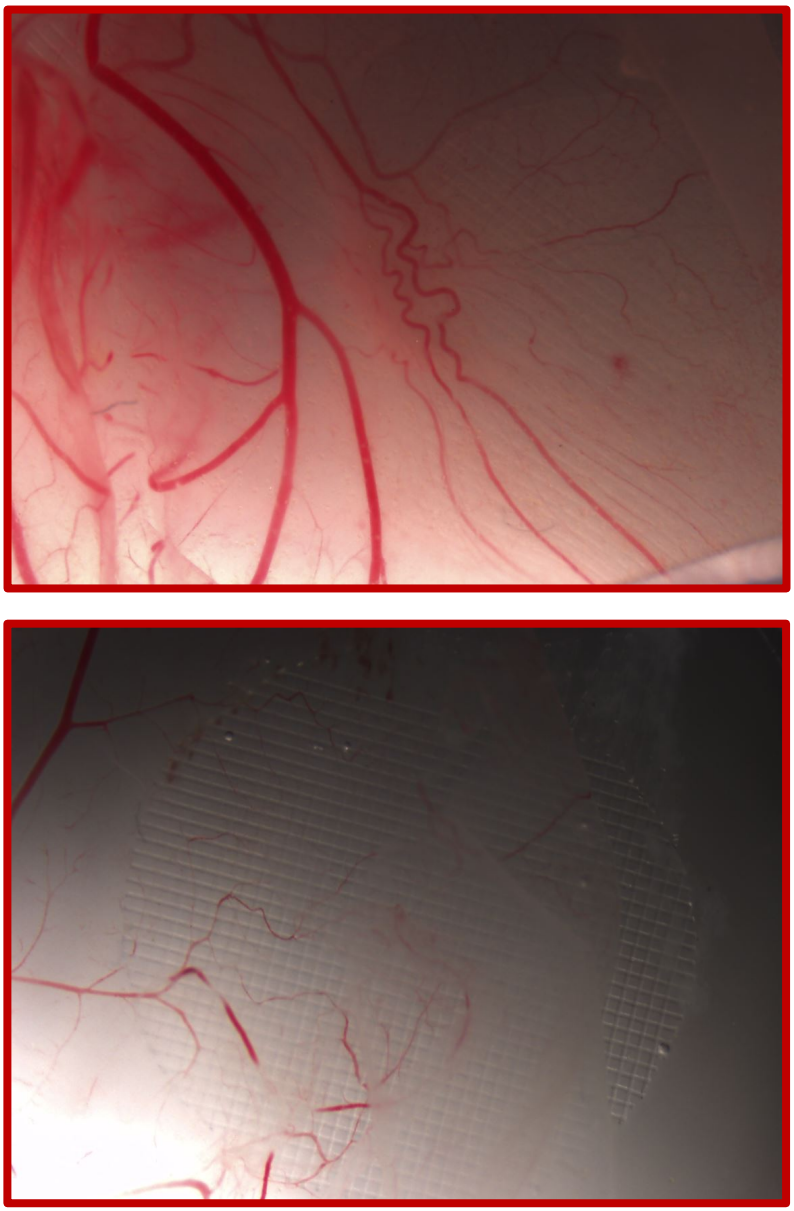


FIGURE F.14-15. Examples of experimental group and negative control group, respectively.

From unnamed scaffolds, done on purpose to not be biased on which were the calcium experiment and the negative control, we had to rate the angiogenesis achieved from 0 to 5, being a macroscopic semiquantitative scoring. In the *FIGURE F.14-15.*, it becomes quite clear which is the experimental group (being rated 4), and which is the negative control group (being rated 1).

When we rated all the scaffolds, we discovered that, from A to O, there were the calcium nanoparticles, so their results, in average, should have rounded 4 or 5, and from P to T they were the negative control, so the results should have rounded 0, 1 or 2.

From the evaluation seen on the *GRAPHIC F.1*, we can already see how, for example the scaffold named **"M"** was rated **1/5**, and the scaffold named **"T"** was rated **4/5**, and their result is not coherent with our hypothesis that calcium induces angiogenesis.

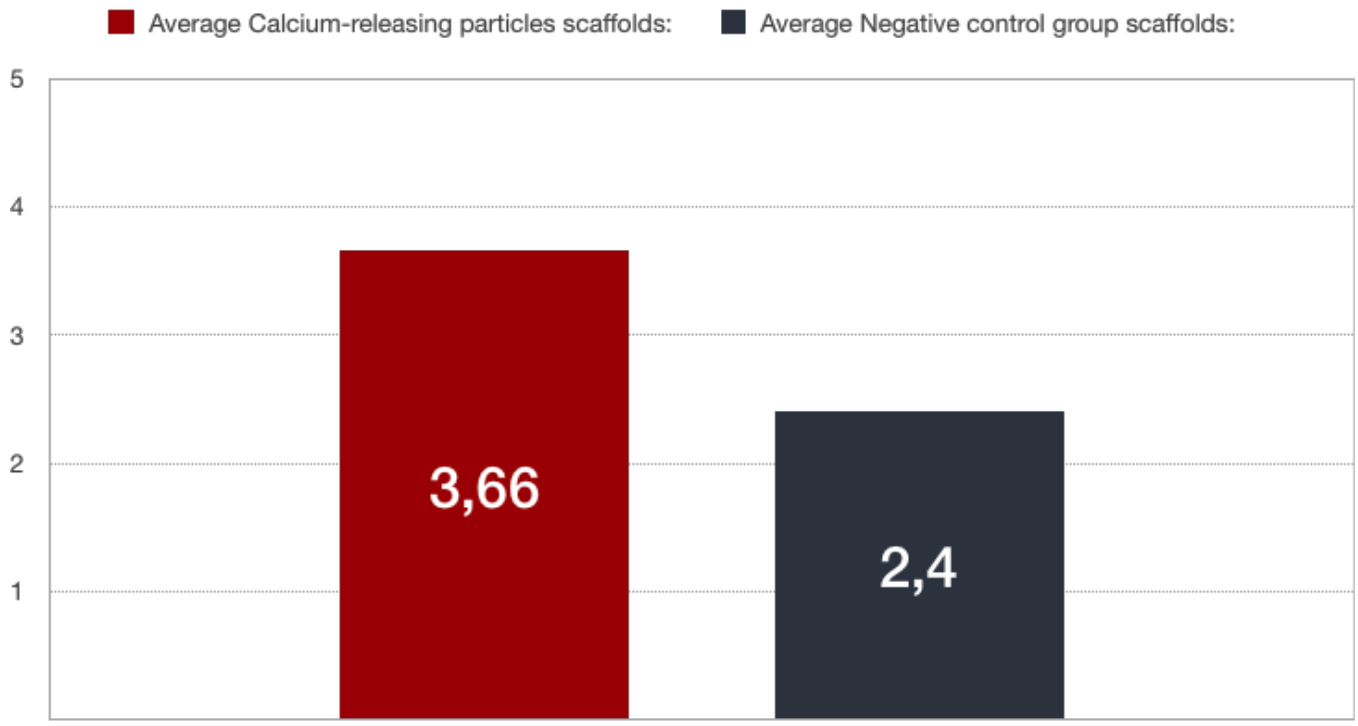
Moreover, our averages were different, and we had **3.66** for the disks containing **Ca^{2+} nanoparticles** and **2.4** for our **negative control** group of disks (see *GRAPHIC F.1*).

This represents a failed hypothesis, as we were expecting a higher rate of angiogenesis in the calcium-releasing nanoparticles, and we could observe how there is not that much difference between the values of our control and our calcium-releasing nanoparticle disks.

From failed hypothesis we can learn about things that we could not have considered before.

These results could have happened because of many factors: the concentration of the Ca^{2+} particles could not have been high enough (we had a 3.289 mM Ca^{2+}/ µL), maybe because of the manipulation of the disks when putting them to observe and taking images of them in the microscope and maybe even the temperature changes when manipulating them.

In any case, we can suppose that calcium is a proangiogenic factor as its average is higher, but to rule out mistakes or errors or just to check out if it actually is angiogenic, we should do a replica of this experiment. Another point would also be to equalize the number of blank and calcium scaffolds.



GRAPHIC F.1. Average of the two groups of the former graphic.

STUDY 4: PH AND CALCIUM RELEASE MEASUREMENTS

Introduction

The aim of this experiment is to measure the release of calcium from the particles we have used. This is essential to know the dose released in a case of implant, for instance. Too low concentrations might not have any bioactive effects on the body, while too high concentrations could be toxic. We will also determine the changes of the pH, in order to define if it would be a biocompatible scaffold or not.

To carry out this experiment, we are going to add a specific volume of water in an Eppendorf tube containing a known amount of the calcium-releasing particles, incubate them in water for 2 days at 37ºC and later we will measure the calcium concentration of the water. The water that we will use will contain HEPES, which acts as a buffer for the solution and prevents changes of the pH of the solution when a small or moderate amount of strong acid or base is added.

In our experiment, HEPES allows the buffering capacity that our body has. Cells and body functions are really sensitive to pH changes so having a buffering capacity is essential for their

normal function. Also, the pH solution is adjusted to 7.4, the pH of our body. Overall, with this solution and temperature of incubation are trying to simulate the physiologic conditions of the body so that the release that we will measure will be as similar as possible to the real context of application.

Measurement of the release of calcium

The method that we are going to use to quantify the calcium concentration is called the "O-Cresolphtalein Complexone Method". A reagent is that compound that causes a reaction in a system. Having that said, we will use the following reagents: the color reagent (CR) and the AMP Buffer.

The CR contains ortho-cresolphtalein complexone, which forms a purple-colored complex with calcium of our sample in an alkaline medium. This alkaline medium is provided by the AMP buffer solution. The reaction is instantaneous. Once the purple-colored complex has been formed, the intensity of the color can be measured with a spectrophotometer at 570 nm of absorbance. The spectrophotometer calculates the absorbance, the reflection or transmission of a sample at a determined wavelength.

Calibration curve

By including a calibration curve in our assay, we are able to extrapolate what the amount of calcium concentration of our samples is.

To make a right calibration curve, we must include at least 5 points in the curve. It has to be linear (it should not exceed the maximum sensitivity of the assay) and the measured values should fit at around half of the calibration curve. For our measurement, the points selected will be: 6-3-1.5-0.1-0.05-0. These are mM Ca^{2+}.

In the experiment, it is important to include a blank which might be the solvent used in the experiment measured to subtract the background from the measurement.

Justification and Results

In this experiment the words spectrophotometer and absorbance are widely used. First of all, the spectrophotometry measures quantitatively the capacity of the amount of light that colored compounds absorb (the absorbance) in a determined wavelength (in our case, of 570 nm).

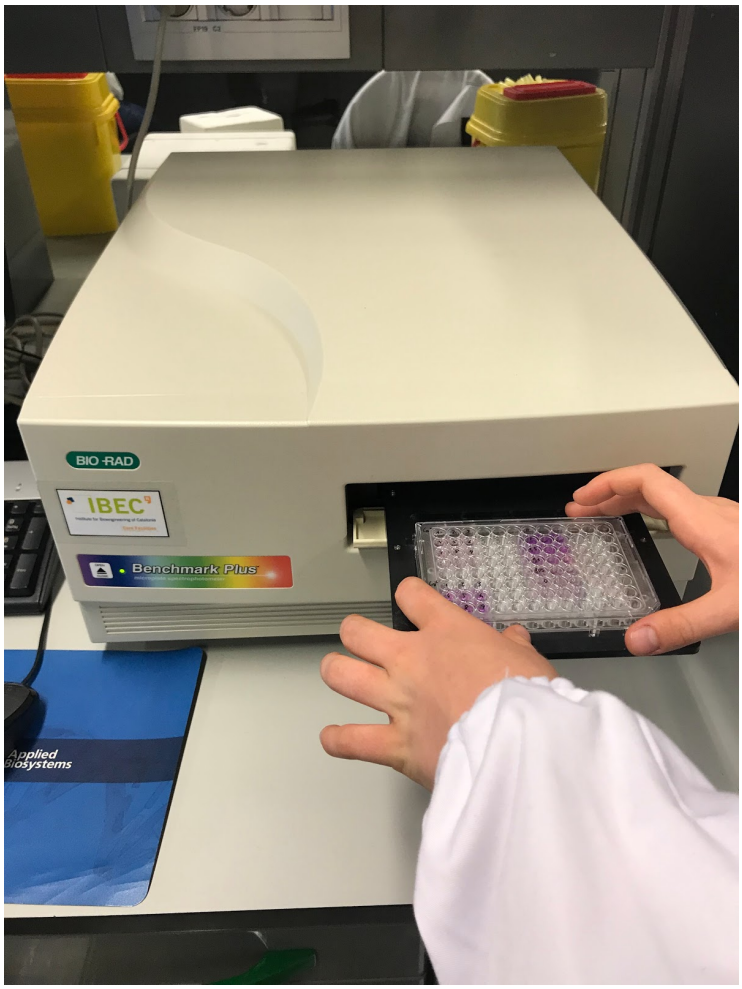


FIGURE F.16. The spectrophotometer used from the IBEC.

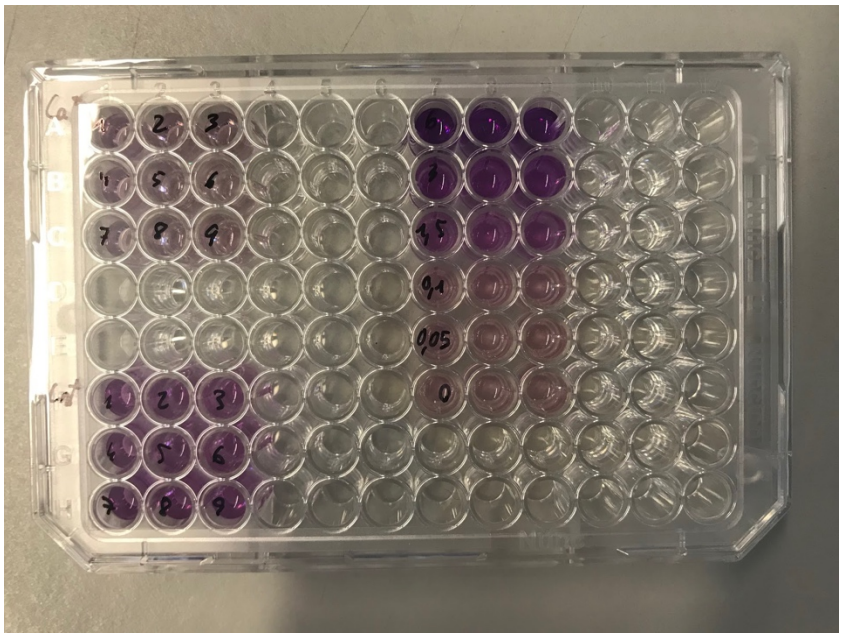
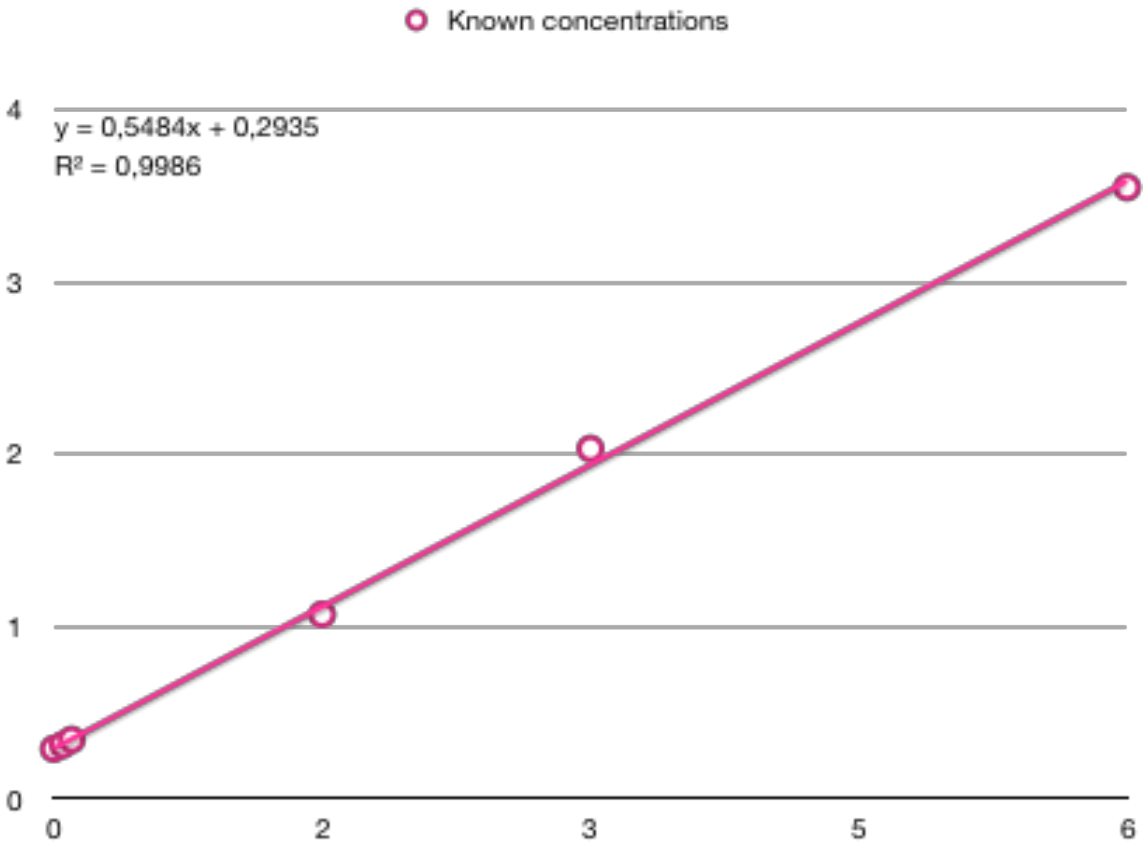


FIGURE F.17. Our samples.

In order to determine the concentration of calcium of our experimental samples, we first had to do a calibration curve with our known concentration's samples. To do this, we had to

compare the concentration of calcium with the absorbance of that sample. This will be done putting tables of the values, still in triplicates, to rule out errors, to then calculate their averages.

This data formed the calibration curve, the concentration was the X axis and the absorbance conformed the Y axis.



GRAPHIC F.2. Calibration curve.

Real concentration of the Ca^{2+} (In 3 mg)	/ 3	Real concentration of mM Ca per mg
0,955		0,318

TABLE F.1. Final result of the concentration of Ca2+ we will put in our next study, the CAM assay.

Results from our pH indicators:

FIGURE F.18. The pH indicators.

Before taking away the supernatant, we check the pH of our samples to see if the Ca^{2+} is biocompatible or not with the pH of our body (approximately 7.4).

Although the indicators from the FIGURE F.18. are almost dried, it can be rechecked in the picture that the pH was indeed between 6 and 8, being biocompatible.

STUDY 5: FLUORESCENT STAINING OF CELLS

Introduction

The fabrication of different scaffolds can be used as treatments for tissue engineering or regenerative medicine approaches. These scaffolds usually aim to stimulate cells in terms of proliferation so as they can be able to repopulate the damaged area of the tissue.

One of the first characterization steps after the fabrication of a designed scaffold is to test whether cells are able to attach and adhere to the surface of the structure of the scaffold. Additionally, we should also check for a normal cytoskeleton structure and a correct cell cytoplasm organization. Cells are transparent, so we are not able to see them directly through

a normal microscope, and this issue is even worse if cells are seeded on a thick and non-transparent scaffold. For this reason, it is usually needed a specific staining for cells or cellular structures, being the most common ones and the ones we are going to use phalloidin (Phal), for the actin cytoskeleton, and DAPI, for the nuclei.

Phalloidin is a seven amino acid peptide toxin from the mushroom *Amanita phalloides*, which binds specifically and with high affinity to the polymerized form of actin (F-actin). Phalloidin has been labelled with a proprietary fluorescent dye which allows it to be used to stain actin filaments in tissue cultured cells and tissue sections and cell-free preparations.

DAPI (or 4',6-diamidino-2phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, having less stain effectiveness on live cells.

In order to preserve the cellular components for the labelling, it is necessary to first fix the cells using paraformaldehyde. Paraformaldehyde is a polymerized form of formaldehyde, which fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine (Lys). Its effects are reversible by excess of water and it avoids formalin pigmentation.

Aim

To evaluate the suitability of different scaffolds to allow the attachment of cells and characterize cell morphology and structures. Also, another aim of this practicum is to learn how to prepare cells for a fluorescent staining.

Now there will be described the different scaffolds used in both cases. In the first time we used already prepared cells and scaffolds, and in the last time we ourselves prepared the cells for the scaffolds and sterilized the scaffolds and made them functional.

In both cases we were dealing with the same two different fabrication techniques, 3D bio-printing technique (see *FIGURE F.19.*) and 3D electrospinning technique (see *FIGURE F.20* and *F.21.*).

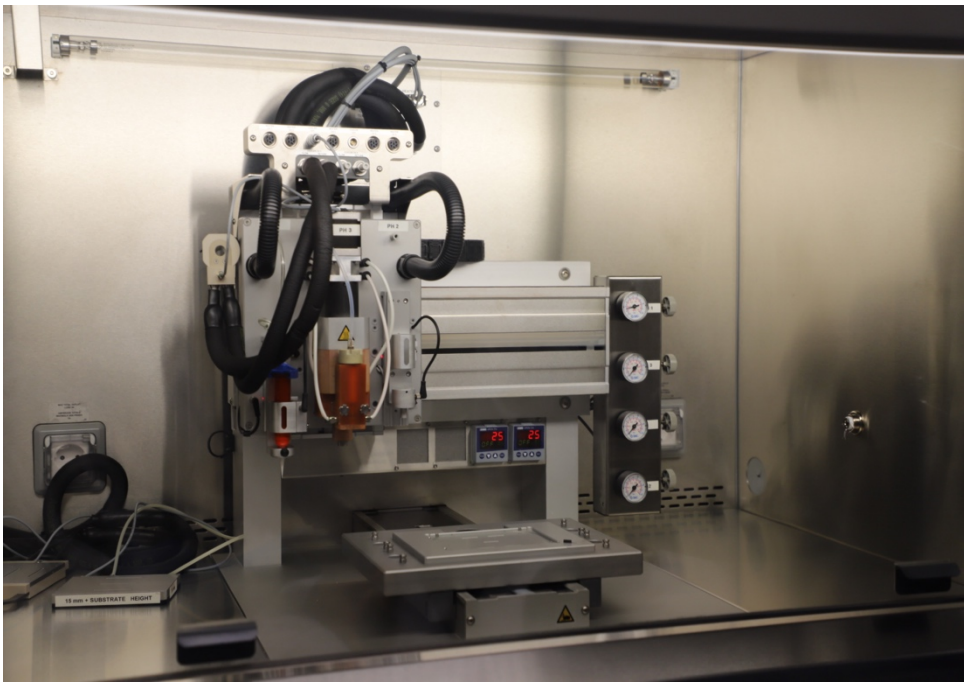


FIGURE F.19. This is the 3D bio-printer used for the bio-printing method.

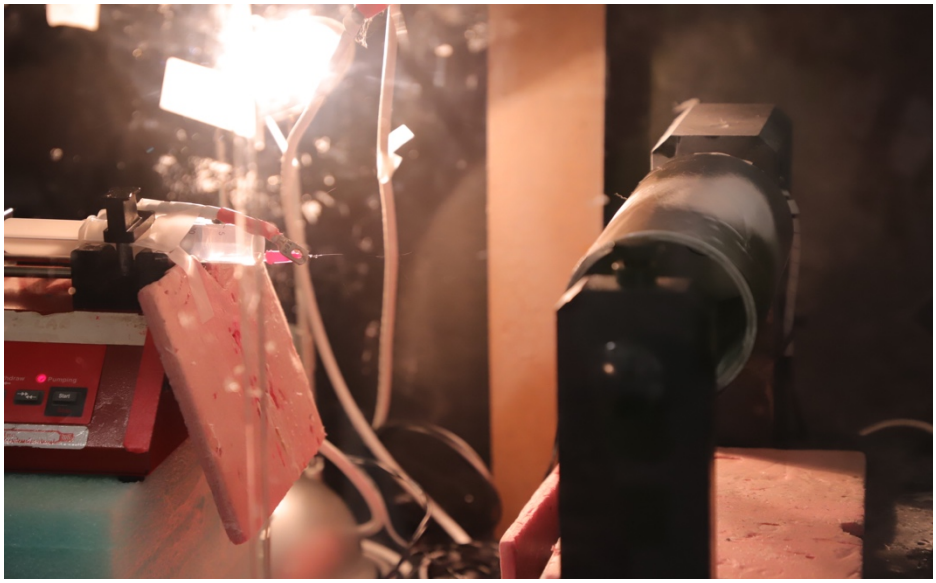


FIGURE F.20. This is the electrospinning method.

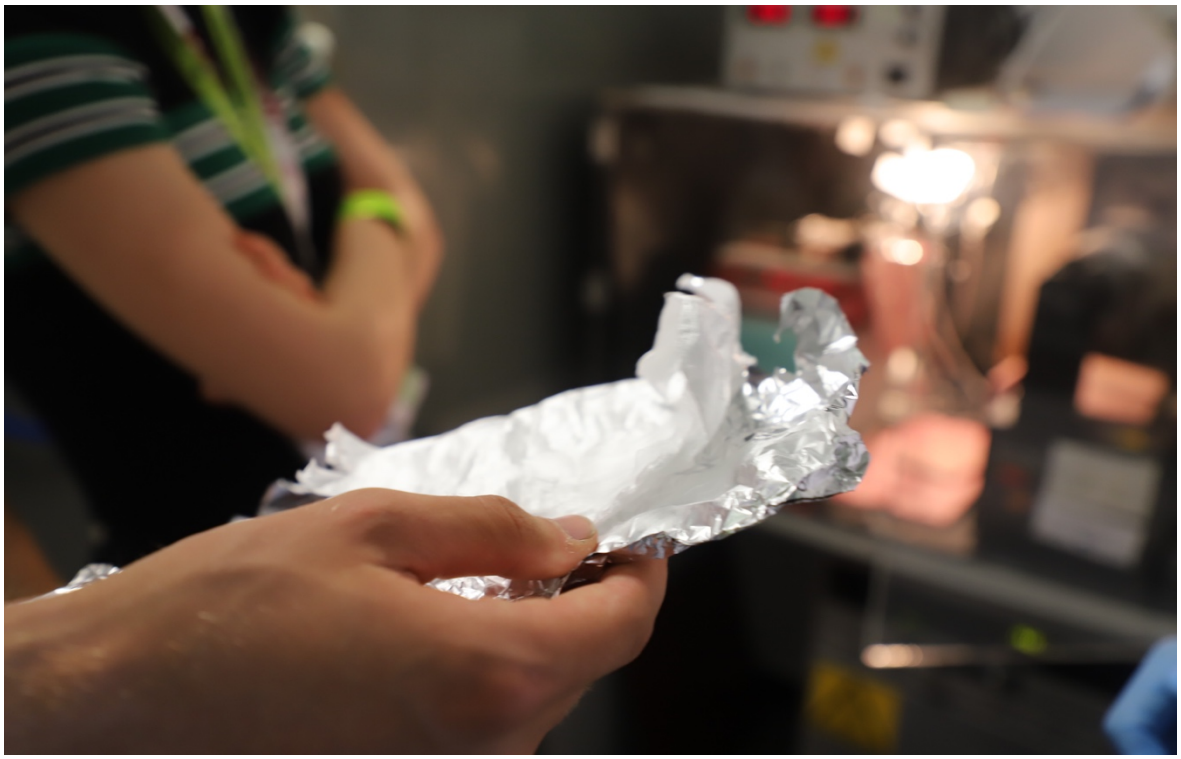


FIGURE F.21. This is how an electrospun scaffold looks like.

RESULTS

There are two experiments carried out based on the fluorescent staining method, the first one is an already prepared fluorescent staining, having all the scaffolds with its cells already prepared, and the other one is a fluorescent staining but with the cell and scaffold preparation done from the beginning.

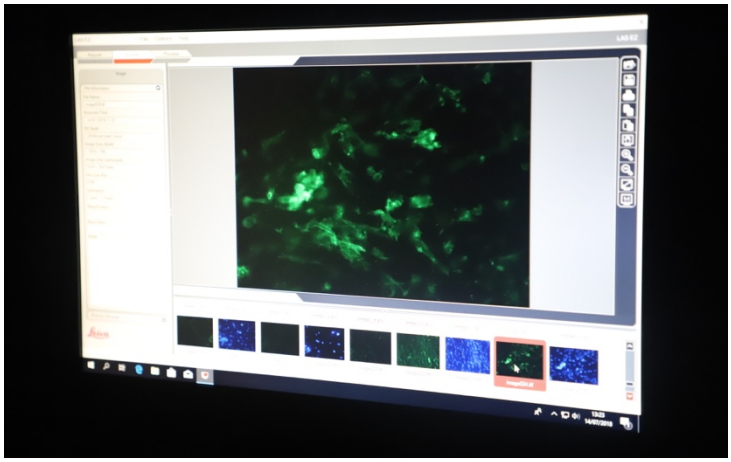


FIGURE F.22. This is the program that took the images from the microscope, Leica.

FROM THE ALREADY PREPARED FLUORESCENT STAINING:

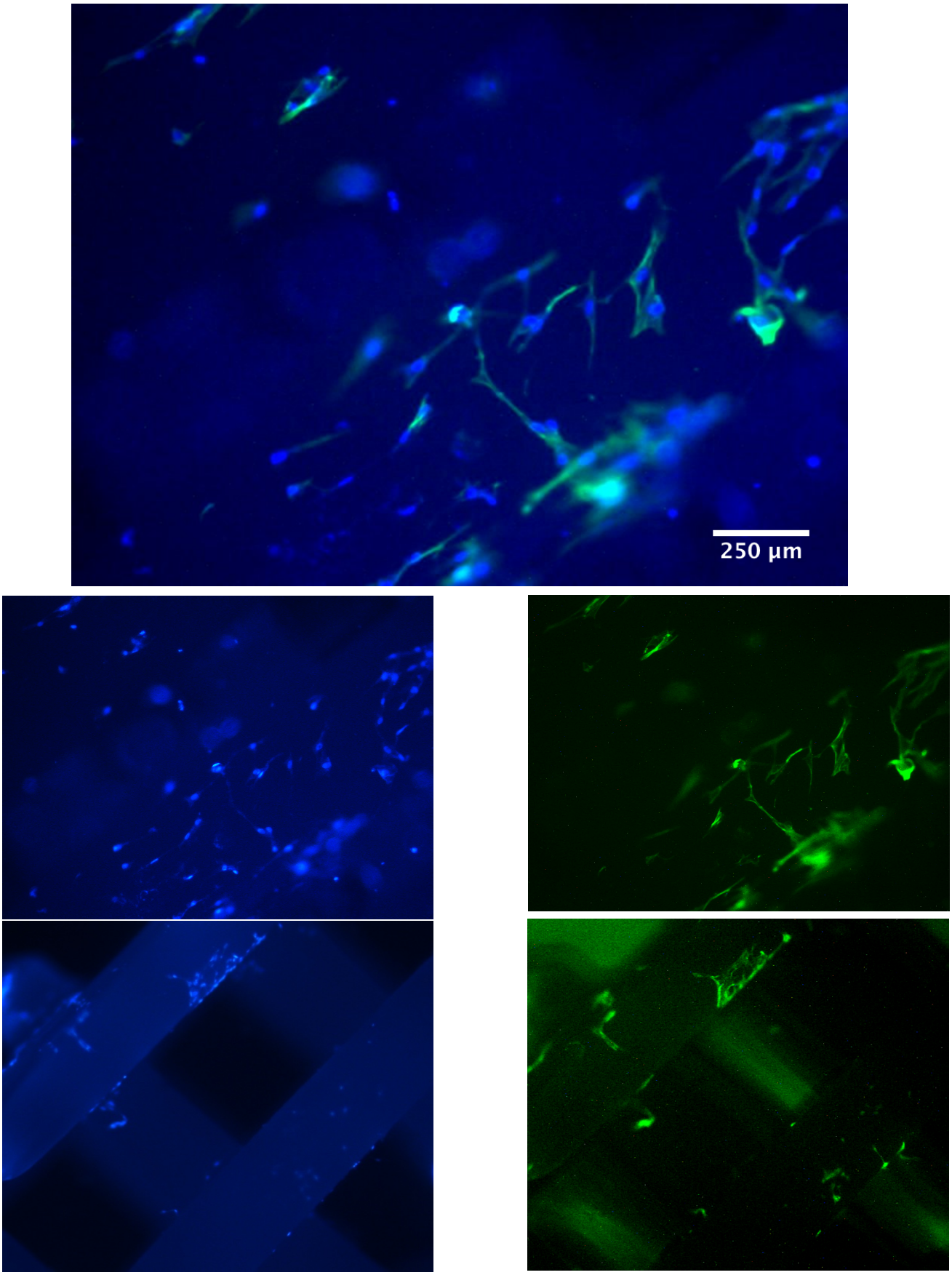
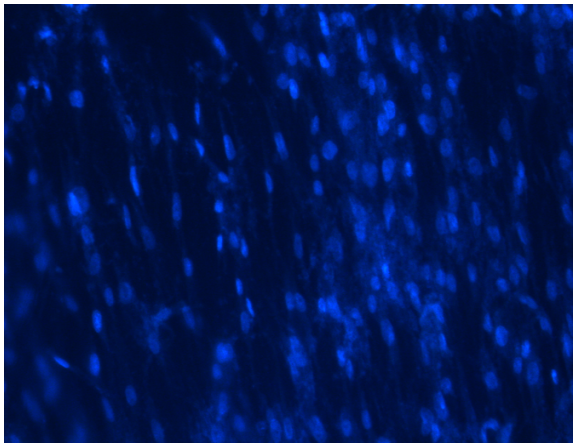
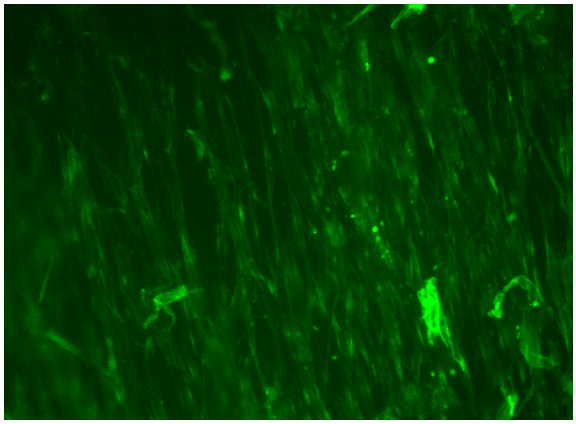


FIGURE F.23-24-25-26-27. From the 3D bio-printed scaffold we got these images.





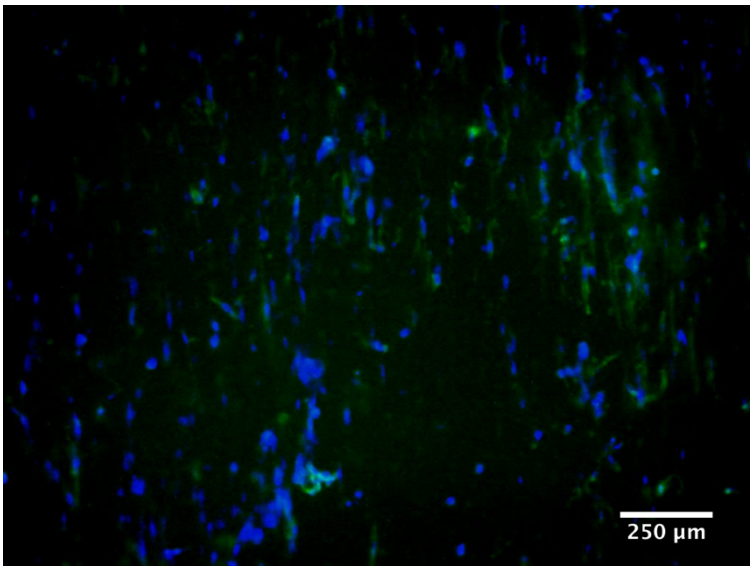


FIGURE F.28-29-30-31. Images from the scaffolds made of random oriented fibers.

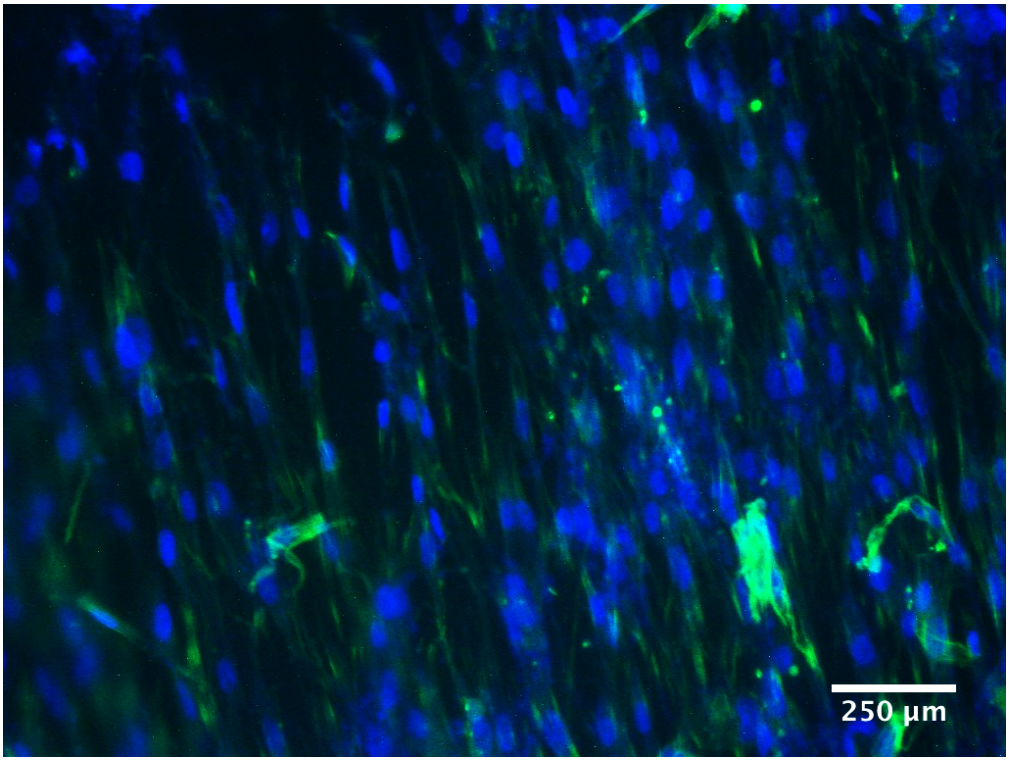


FIGURE F.32. Image from the scaffolds made of aligned fibers.

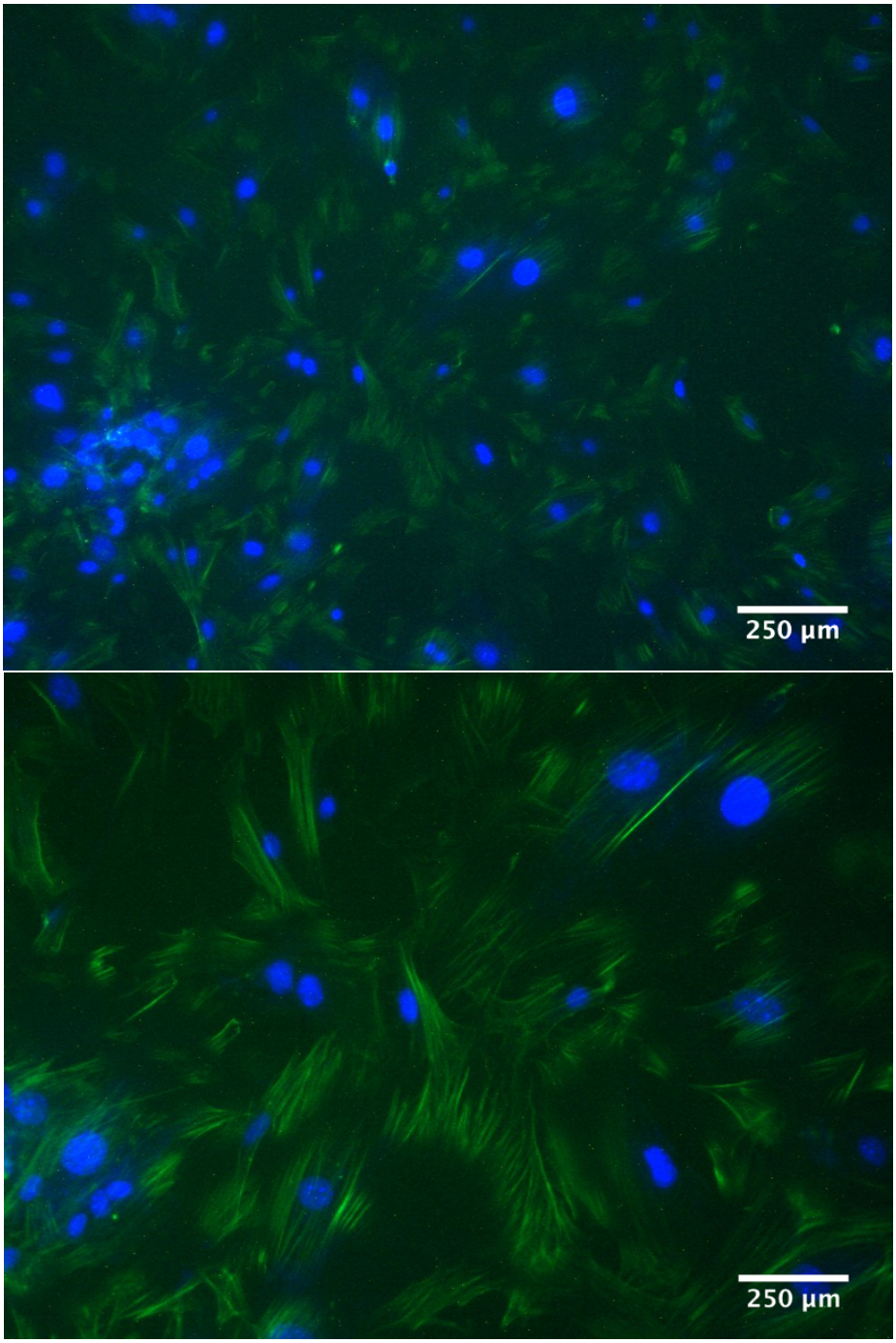


FIGURE F.33-34. The 2 control groups that were considered.

From all of these images, we can easily observe how cells tend to adapt their morphology, including cytoskeleton and cytoplasm, to their environment, in our case, to scaffolds. After finishing this experiment, we would then have to check if stem cells specialized into different cells, however, that would not be possible as our scaffolds were not vascularized (contrarily to our CAM model scaffolds). This is why the vascularization is so important in tissue engineering, our scaffolds' cells would not survive without nutrients.

FROM THE STAINING WE HAD TO PREPARE, IT INVOLVED ANOTHER SET OF EXPERIMENTAL PROCEDURE, THAT WILL NOW BE EXPLAINED.

The procedure of this staining is explained in the **ANNEXES chapter**.

We did the fluorescent staining method., exactly the same procedure and materials from before with the difference that we took images at the confocal microscope this time, and on the other experiment it was with a fluorescence microscope.

Also, the actin cytoskeleton was stained red this time. The nucleus was, as before, stained blue.

RESULTS

Firstly, all the merged-color channels on the bio-printed scaffolds:

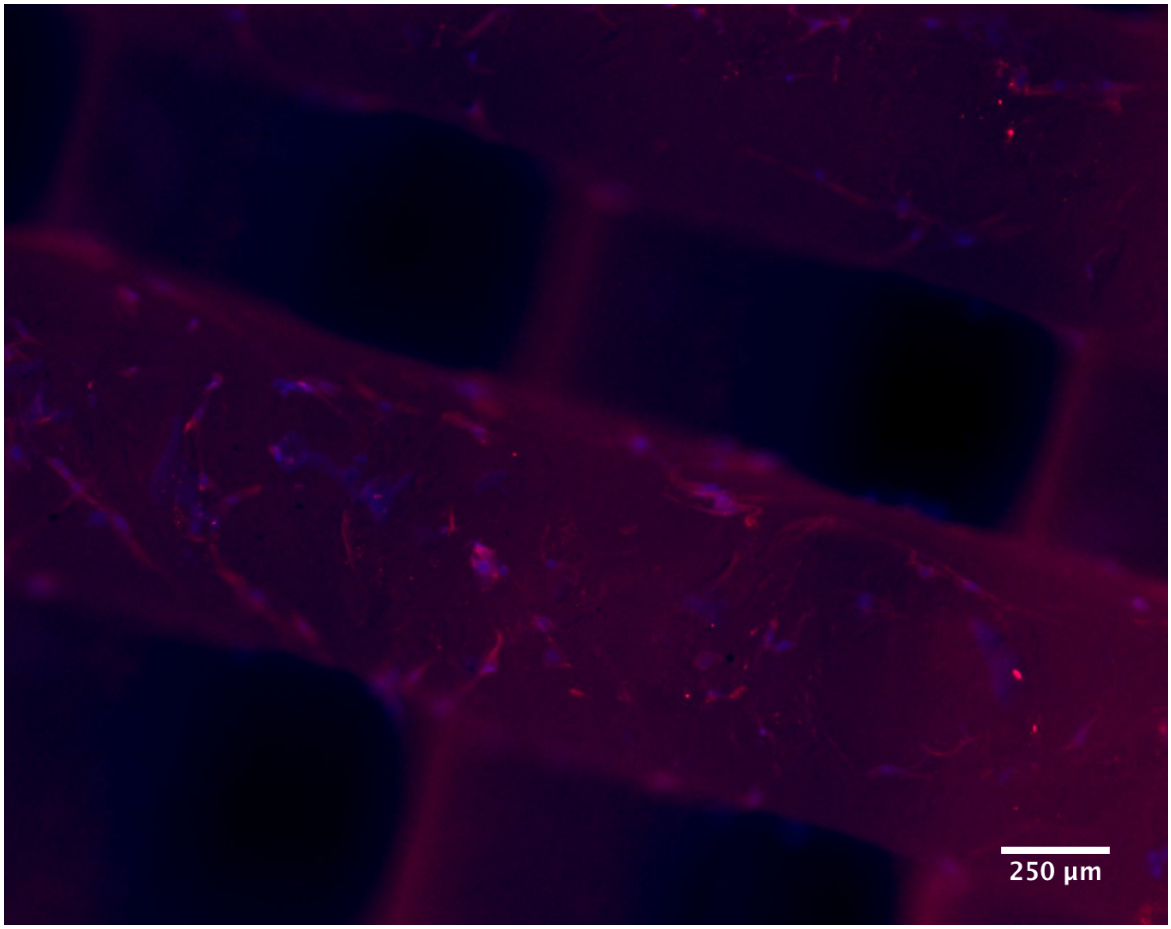


FIGURE F.35. Bio-printed scaffolds

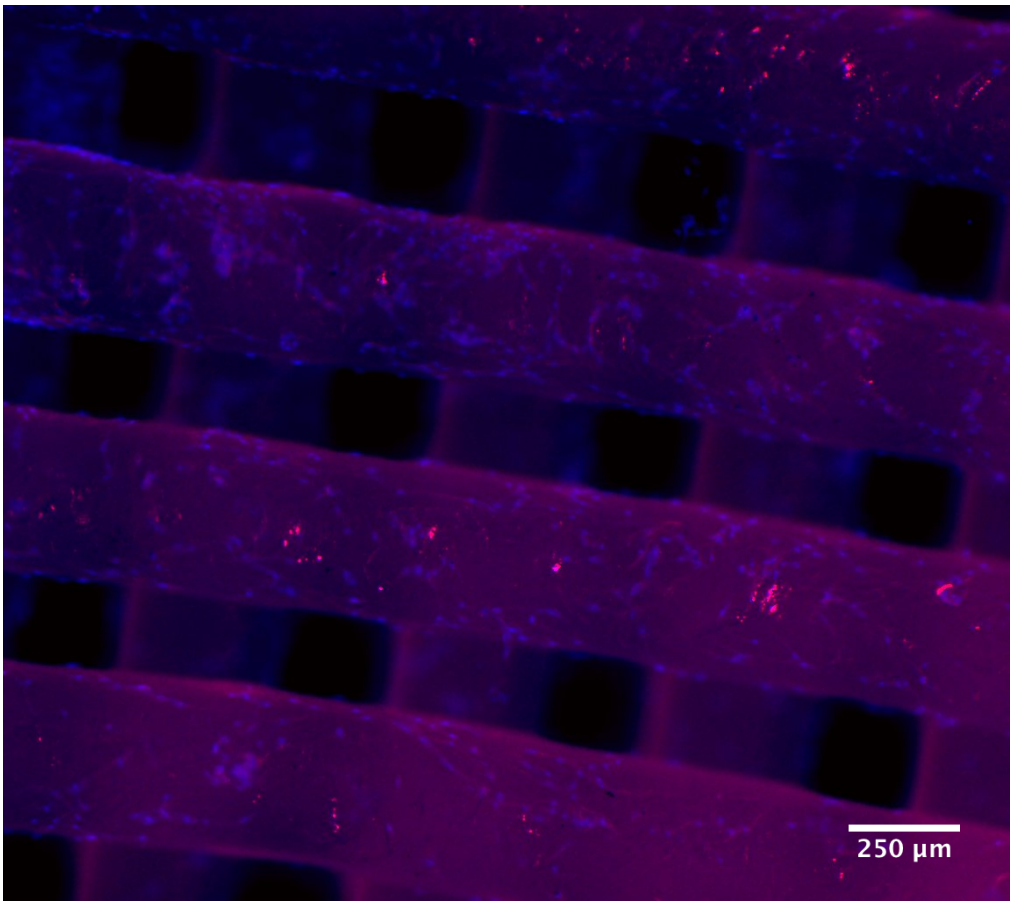


FIGURE F.36. Bio-printed scaffold from a smaller microscope magnification.

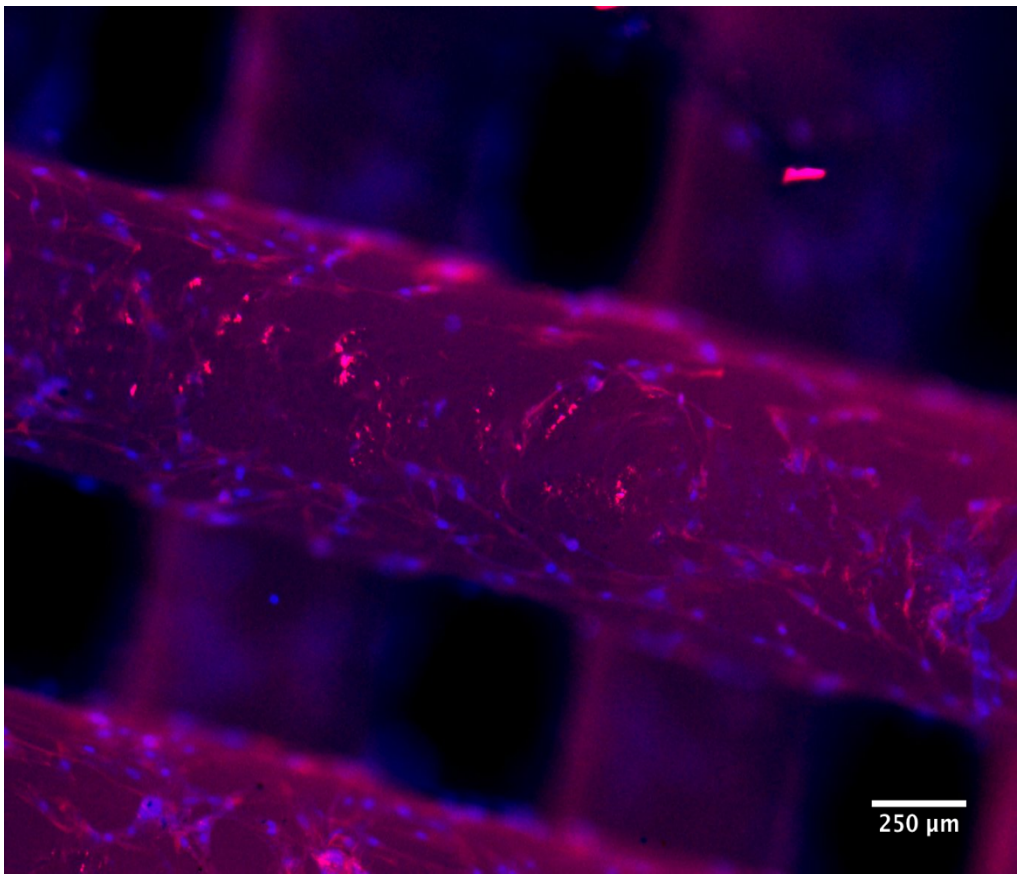


FIGURE F.37. Bio-printed scaffold with the foreground fibers being focused.

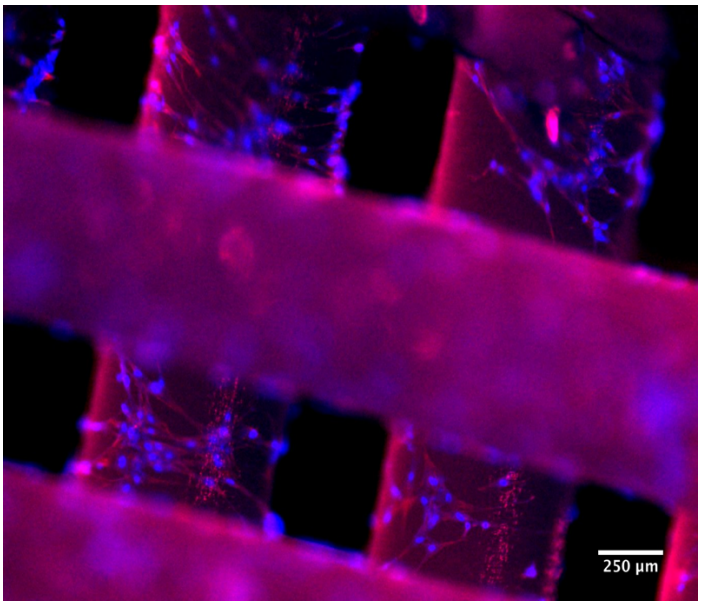
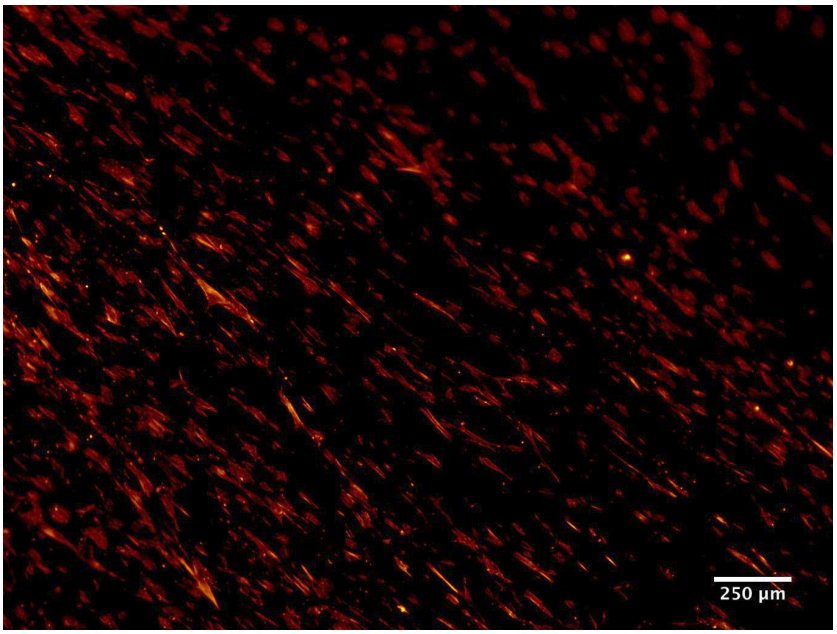


FIGURE F.38. Bio-printed scaffold with the fibers of the background focused.



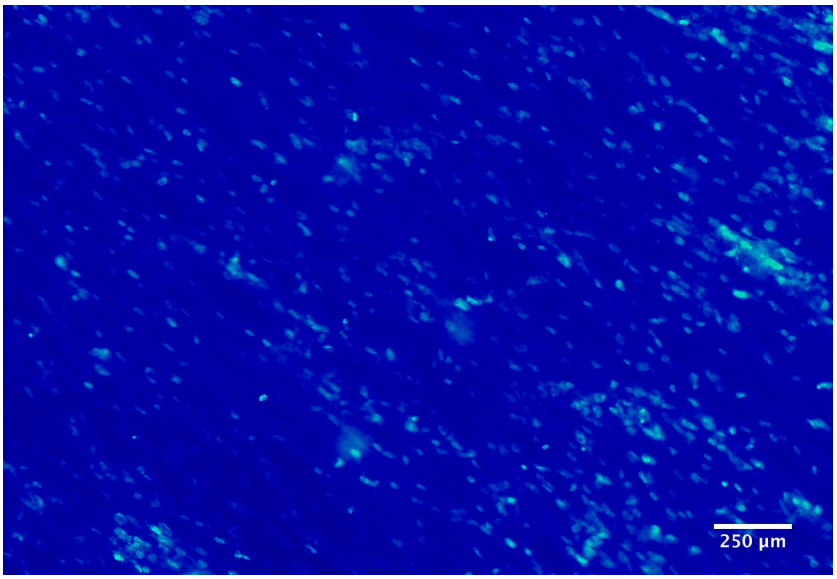


FIGURE F.39-40. Electrospun scaffold made up of aligned fibers with its color channels split.

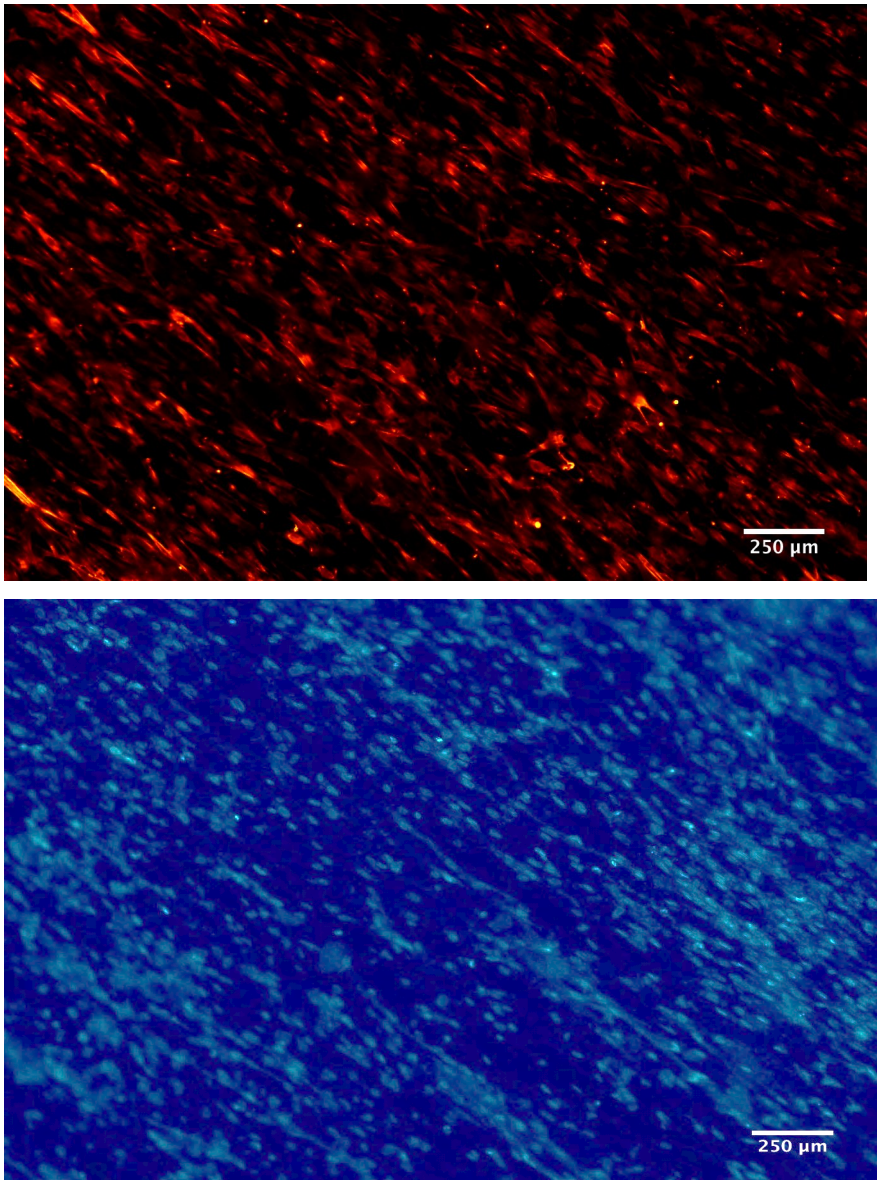


FIGURE F.41-42. Electrospun scaffold made up of aligned fibers with its color channels split.

So here we observed the same results as in the first time we carried out the fluorescent staining of cells, the cells' morphology adapt to their environment. However, we observed differences. From the already prepared fluorescent staining, cells were not that keen on adhering on our scaffolds, however, on the second experiment where we prepared the staining many more cells have been attached, comparing FIGURE F.23 with FIGURE F.37 on our bio-printed scaffolds, or doing the same with the electrospun scaffolds, FIGURE F.28 with FIGURE F.41-42.

However, the difference between our electrospun scaffolds is not that notorious. One supposition could be that electrospun scaffolds tend to adhere more to scaffolds fabricated by an electrospinning technique, or that maybe the porosity of the bio-printed scaffolds should be smaller in order to gather more cells.

Once again, to deepen in this topic, we should replicate the experiment and add more experimental groups.

DISCUSSION AND CONCLUSION

- **Explanation and analysis of the results**
- **Deductions and future perspectives**
- **Limitations**
- **Contribution to science**

It takes a lot of people to make a winning team. Everybody's contribution is important.

-Gary David Goldberg, American producer.

EXPLANATION AND ANALYSIS OF THE RESULTS

This subsection is going to be referred to the specific results of the fieldwork part of this project.

In **STUDY 1**, where we decellularized a mouse heart, we observed how with a specific procedure, we are able to use a heart as a natural extracellular matrix scaffold, avoiding or inducing minimal foreign host reactions if that decellularized heart was implanted into another mouse. However, we also saw how this decellularization process needs to be done even several times to take away the entirety of cells.

In **STUDY 2**, where we fabricated three-dimensional scaffolds in two experiments, we observed how different factors alter their nature and properties, as well as how biomaterials can also be used for other bioengineering techniques, such as drug delivery, and we got a grasp on how it worked. Our experimentation was successful as the alginate spheres did release biological factors, so we created a functional in vitro drug delivery system.

In **STUDY 3**, an angiogenic in vivo assay was carried out, the CAM assay. Not only we were able to crack successfully the eggs and maintain them alive giving them a humane treatment, but we also introduced and induced angiogenesis on scaffolds. We were able to do the angiogenic evaluation without being biased towards the negative or experimental controls with calcium releasing nanoparticles as we were unaware which were which until the end of the evaluation. However, this experiment showed a small tendency to create more blood vessels to our experimental group. Although this fact, we cannot affirm calcium is a proangiogenic factor, as this slight difference between the groups was not the expected. In this replica the concentration of calcium could be increased in order to observe a greater difference in the evaluation.

In **STUDY 4**, our goal was to measure the concentration of calcium of the calcium-releasing nanoparticles we used on the previous study. We measured it by preparing solutions of known concentrations of calcium and making a calibration curve absorbance-concentration. Also, we wanted to check if calcium nanoparticles were neutral pH, to check their biocompatibility, and they indeed were.

In **STUDY 5**, we evaluated the attachment of cells to PCL and PLA (more specifically PDLLA) scaffolds, using the fluorescent staining technique twice. Firstly, with a prepared staining and lastly starting from scratch. We observed how preparing ourselves the cells and the scaffolds more cells tended to adhere onto our scaffolds' fibers, in both scaffold types, electrospun and 3D bio-printed ones.

Now talking about **STUDY 1** and **STUDY 2**, they both are really promising techniques, and although simplified, if complex systems on both decellularization and drug delivery techniques are achieved, health solutions are going to be more feasible in the near future.

Although it could seem obvious, relating **STUDY 3** and **STUDY 5**, we can affirm that cells tend to adapt to their environment, in our cases, to biomaterials, and this is of great importance. However, we would need vascularization, in order for the scaffold's cells to get nutrients from the blood. For this reason, **STUDY 3** is a small study out of the great deal of studies that are being done to study vascularization.

DEDUCTIONS AND RECOMMENDATIONS/PERSPECTIVES FOR NEXT POTENTIAL INVESTIGATIONS

Having these studies done, the most important part is the "what about now" one. The future perspectives of bioengineering seem promising and game-changing, as well as rapidly-advancing.

Out of the 6 studies carried out (the last one in the **Annexes** section), we are able to establish and organize the ideas that have arisen from them:

- **In STUDY 1. Natural ECM scaffolds**. Have we found the solution to posttransplant complications and injury? Are they more efficient and effective than polymer scaffolds?
- **In STUDY 2. New drug delivery techniques**. The one carried out would not work on a living system. Are we able to avoid all drugs' secondary effects? Are we close to be able to do so?
- **In STUDY 3. A need for vascularization**. How is the ideal scaffold? How far are we from this one?
- **In STUDY 4. Calculating and deducing factors of our research**. Is computed monitoring possible on bioengineering research? Will it be?
- **In STUDY 5. Cell adhesion on surfaces**. Have we found the ideal surface modification treatment? How can we evaluate biophysical interactions in in vivo models?
- **What about the EXTRA STUDY: STUDY 6? Bioethics**. Knowledge over animal lives? How to accelerate preclinical trials and how to overcome clinical ones?

Much more questions emerge from these studies, and that is exactly what we were seeking. Knowledge leads to more potential knowledge, and divulgating this science is fundamental to achieve this knowledge that is already changing the way we attack health problems.

LIMITATIONS

To recognize a realist study we have to determine the limitations of the latter mentioned. The information received from, for instance:

> The decellularization of a heart was not actually a full, clean decellularization, as the final result was not as decellularized as the FIGURE F.5 showed.

However, the limitations of each study were mentioned on each study's specific section.

CONTRIBUTION TO SCIENCE

From my point of view, I believe this project has been a success.

Apart from all the knowledge gained from these incredible experiences, we got a grasp on a multidisciplinary field using the latest techniques combining biology, chemistry, physics and materials science.

Talking about the results, to elaborate or to simulate a deduction of the biophysical conducts of cells would be impossible. That is because this work has to have a highly backed evidence, so I feel my personal contribution to science does not come mostly from the purely scientific evidence of this project.

The purpose of this project was to demonstrate why biophysics and tissue engineering are changing and will change our quality of life and I think it has accomplished its goal meanwhile learning from the best bioengineering professionals there are.

The other main purpose of this project was to raise consciousness and interest on these science fields, and getting to the end of this project and having transmitted all my enthusiasm, that is from now on up to the readers.

In contrast about the scientific evidence, I believe this project contributes more to the divulgation and to a wider knowledge about bioengineering.

The following chapter is the **Formal Aspects** one, and then, to end this project, there are the **Annexes**, where the interviews, protocols and the sixth extra study are carried out.

FORMAL ASPECTS

- **Acknowledgements.**
- **REFERENCES: Bibliography.**
- **REFERENCES: Webs consulted.**
- **REFERENCES: Figures not from own origin.**

Gratitude is the most exquisite form of courtesy.

-Jacques Maritain, French philosopher.

ACKNOWLEDGEMENTS

Before finishing this enriching project, I wanted to thank some entities and people that I think they completely deserve this mention.

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ANNEXES

- **Interview on Mr. Jeffrey Karp and Mr. Robert Langer.**
- **Materials used and procedures carried out in Study 1.**
- **Materials used and procedures carried out in Study 2.**
- **Materials used and procedures carried out in Study 3.**
- **Materials used and procedures carried out in Study 4.**
- **Materials used and procedures carried out in Study 5.**
- **EXTRA PROCEDURE EXPLAINED: Processing of images with *ImageJ*.**
- **EXTRA STUDY: Study 6: Ethical considerations on biomaterial research regarding translational research.**

The difference between ordinary and extraordinary is that little extra.

-Jimmy Johnson, American coach.

INTERVIEW ON MR. KARP AND MR. LANGER

I have had the pleasure of interviewing two of the most influential and innovative multidisciplinary scientists of this century about their opinion on the future of bioengineering.

Mr. JEFFREY KARP

He is a world-leading researcher who has worked in the fields of drug delivery, medical devices, stem cells therapeutics and tissue adhesives for wound healing. He is currently an Associate Professor at Brigham and at Women's Hospital and at Harvard Medical School. He is also a Principal Faculty at the Harvard Stem Cell Institute and an affiliate faculty at the Broad Institute and at the Harvard-MIT Division of Health Sciences and Technology, where he teaches to MIT-Sloan business school students.

He has published more than 100 papers with more than 13,000 citations, and has given 250 international invited lectures and has more than 100 issued or pending patents. Several technologies developed in his lab have formed the foundation for products on the marked and under development and for the launch of six companies that have raised over 100 million dollars in funding. Three of this six companies are *Skintifique*, a skincare company, *Gecko Biomedical*, a tissue adhesive company and *Frequency Therapeutics*, a regenerative medicine company.

Karp's laboratory has been funded by multiple companies foundations, and governmental funding agencies, including *Bill and Melinda Gates Foundation*, *NFL*, *Johnson & Johnson*, *Phillips*, *Sanofi*, *UCB*, *NIH*, *DOD*, *US Army*, *Kenneth Rainin Foundation*, *Helmsley Trust*, *JDRF*, *Rheumatology Research Foundation*, *Coulter Foundation*, *Prostate Cancer Foundation*, *Brain Science Foundation*, *American Heart Association*, *Deshpande Foundation*, *Institute for Pediatric Innovation*, *Boston Children's Hospital*, *Brigham and Women's Hospital*, *the Government of India* and *the Government of Korea*. Karp also won an internal Shark Tank award.

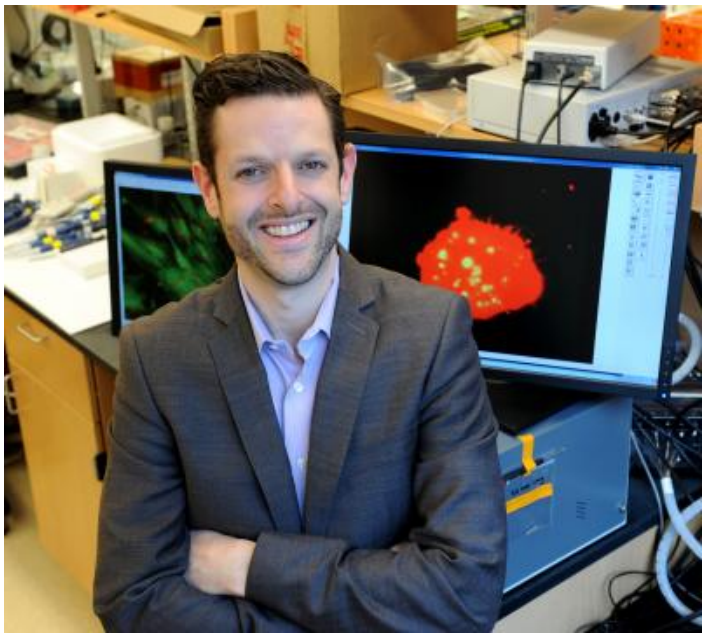


FIGURE A.1. Mr. Jeffrey Karp posing in his laboratory.

Karp's work has been published in high impact journals, just like *Proceedings of the National Academy of Sciences*, or *Cell Stem Cell*, or *Nature Nanotechnology*. His work has also been discussed in hundreds of newspapers, online websites, television newscasts and radio shows around the world, including *CNN*, *ABC News*, *BBC*, *National Geographic*, *The Guardian*, among many others.

Karp has had several notable achievements. In 2010, Karp was asked to serve a two-year term to co-lead the Brigham and Women's Hospital Regenerative Therapeutics Center, being the youngest faculty at this hospital to direct a research center. He also received the Society for Biomaterials Young Investigator Award and the Coulter Foundation Translational Young Investigator Award.

Karp is dedicated, as a research goal, to the career development of the next generation of bioengineers working at the forefront of regenerative medicine. Karp was selected as the Outstanding Faculty Undergraduate Mentor among all the faculty at MIT and he received the HST McMahon Mentoring award for being the top mentor of Harvard-MIT students.

Karp's mentor skills are more than demonstrated as, to date, 20 trainees from his laboratory have secured faculty positions. Karp also dedicates significant time to inspiring the next generation hosting groups of high school children to his laboratory for lectures and demonstrations.

INTERVIEW ON Dr. KARP

This interview was recorded with the program QuickTime Player, and the interview was held in FaceTime. Here the interview has been transcribed:

A- Magnus Dante
K- Dr. Jeffrey Karp, see FIGURE A.2.

FIGURE A.2. Jeffrey Karp giving a TEDMED talk about bioinspiration.

A- What would you categorize as your proudest publication as a scientist?

K- I think it is pretty challenging to answer that question. It is a question I even think I don't have an answer for. We try to focus on things that we like to work on and to have an impact on society. My favorite are all the publications that got translational from the lab to society to help patients.

A- You are now working on tissue adhesives and other medical devices. Could you summarize a bit their basis and explain us, up to the point you want, the experimentation you are currently doing? Or what properties or something that you are seeking on tissue adhesives? Are they only for the skin?

K- We are looking for materials that can attach and also that are non-toxic and can achieve relevant levels of adhesion to solve problems. Most of the work is for internal procedures, to work actually inside the body, sealing blood vessels or a beating heart, for instance. However, we also work with skin as well. Several projects are being advanced on skin along with internal procedures.

A- What drug delivery system do you find more promising for the future?

K- I think there is multiple applications and they require different solutions. I think that sometimes you need sustain release or pulsatile release, or a response for inflammation. So, it depends on the application you are referring to.

A- Currently, what is really keeping organ engineering away from being a reality?

K- The biggest challenge is the complexity of the solutions that are being developed, the cost is very high and their manufacture is difficult. It is basically complex, in every way you can refer to.

A- Have we found the perfect biomaterial for every situation?

K- No.

A- When is a biomaterial construct ready to undergo clinical trials?

K- In general, you want to have strong prove of data on preclinical models, and make some assumptions; ideally look to test as well as to learn.

A- How do you see tissue engineering in 10 years' time?

K- I am not sure actually, to be honest.

It is hard to say, what I can say is that there are a lot of experts and smart people working on many challenges: on the factory, regulatory and technology sides, trying to get the right cost-benefit and I hope there will be more progress, more translation of the technologies and we will probably see more investments in the field and more potential.

It is hard to say when millions of people will be benefiting from tissue engineering, not that far away, but it is hard to say it is in 5,10 or 20 years.

A- Will regenerative medicine kind of replace traditional medicine?

K- I don't think it will fully replace it, there will be a number of diseases that you don't actually need to regenerate tissues. However, I think it will be a primary modality within the clinics. We will still see a lot of other non-regenerative therapies.

A- Are all cell-biomaterial interactions possible to study? Could you name any example when this measurement is impossible?

K- I would say I don't think so currently, probably at some point in the future, with better models, I think one of the challenges is that in vitro models usually do not include all the complexity of, for instance, the dynamic changes that occur within our body or our immune system responses. However, I think overtime, with organ-on-a-chip we will get closer and closer to try to mimic what happens in the body. However, currently, there is nothing as in vivo models to study it.

A- Do you think we are ever going to formulate precise biophysical principles of our tissues or organs?

K- I think so, my sense is probably with machine learning and through artificial intelligence and big data and new tools I think we are getting there. All things are possible, it is all question of time and the resources that are gotten the time that it will take to achieve them.

A- One of the studies I have in mind to do in this research project is to simulate a code of ethics (ethical considerations) on biomaterial research. As an expert, what issues do you think are the most important to be mentioned?

K- I think there are key considerations to be considered, such as how patients could benefit, the potential risks to patients, what type of risks patients or caregivers are willing to take, the current therapeutic option for that patients. One could say that with current therapies that there is questionable ethics about, for instance, how therapy is administered to patients, because often patients are administered drugs that only work in the 30% of the patient population.

Having a non-personalized approach, one could question things around it, so 2/3 of the patients we know they won't get better.

I think the question of ethics is very challenging and personal and it really requires a lot of discussion to figure out.

Also, one could argue, moving to clinical trials, it could take 8-10 years to bring a new therapy forward, what about those patients that could potentially benefit today from that therapies? Should there be opportunity to have better approaches to experiment on humans where there is no current technology that can help them? I think it is hard to answer this question, and it requires different minds from different fields to assess the risks they are willing to take.

A- Which are the ethical limits and boundaries of biological engineering?

K- In many ways, it all comes down to the risks, costs, benefits, who's paying for it, purely experimental or does it actually work…

Every time you develop a new technology the cost is really high to recoup for the development cost, it can be hundreds of millions or even billions of dollars to recoup the cost to develop a new approach, so I think there is ethical questions, many different ones, that need to be considered.

Also, when a new technology is available, it may only be available to those who can afford it so there are ethical questions around that, and overtime if you consider the duration of a product that can be on the market, let's say it is a hundred years eventually it gets cheaper

and more accessible. The alternative to that is not getting it to the market and people not benefiting from it.

I think the question is really more about the greater good for society and how to maximize benefits to most of the people possible overtime.

A- Is science faster than government regulations?

K- I don't know about that, I mean I think they both are slow, in general.

A- Should bioethics be taught in every science field?

K- Everybody should have some grasps about it, and to be thinking about it constantly. I think it is important for people to have an appreciation and thinking about ethics in everything that they do.

A- Do you think we live in a society where common bioethical principles are applied globally?

K- I think it is different in different places, with organizations you have differences on how people think, different countries, different companies, I think it is different all over the place, even if you get 10 people in the room maybe every person has a different view on the same topic.

Mr. ROBERT LANGER

Robert S. Langer is one of the 13 Institute Professors at MIT. The position of Institute Professor is the highest honor that a faculty member can be awarded with. Dr. Langer has written more than 1,400 articles, and has over 1,300 issued and pending patents worldwide. Dr. Langer's patents have been licensed or sublicensed to over 350 pharmaceutical, biotechnology and medical device companies. He also is the most cited engineer in history (over 254,000 citations according to Google Scholar).

Dr. Langer has received over 220 awards, from 1982 to present days. He is one of the 4 people who have received both the US National Medal of Science (in 2006) and the US National Medal of Technology and Innovation (in 2011).

Many newspapers and journals, such as Forbes Magazine, Discover Magazine or CNN, name him one of the best innovators, medicine or biotechnology individuals in the world. Worth mentioning, he was selected by Parade Magazine (in 2004) as one of 6 “Heroes whose research may save your life.” He also has received 33 honorary doctorates.

Academically, he coursed his Bachelor’s Degree at Cornell University in 1972 and obtained his Doctor of Science at the Massachusetts Institute of Technology (MIT) in 1974, both being in Chemical Engineering.

FIGURE A.3. Robert Langer, posing after receiving the Queen Elizabeth Prize for Engineering.

He began his research being mentored by Judah Folkman, from 1974 to 1977, and he later consolidated as a biotechnology researcher, especially on the drug delivery methods and tissue engineering. He is considered the pioneer of many technologies, including the transdermal supply systems, a drug delivery technique, among others. He, along his team, was the creator of the muscular tissue’s vascularization engineering and the blood vessels vascularization engineering (in other words, a field treated on one of the studies of this project, angiogenesis).

Coincidentally, Dr. Karp and Dr. Langer have worked together, as FIGURE A.4. shows.

FIGURE A.4. Dr. Langer and Dr. Karp in an MIT lab 10 years ago.

INTERVIEW ON DR. LANGER

A- Magnus Dante
L- Dr. Robert Langer

A- As a founding father of tissue engineering and probably one of the most interdisciplinary scientists, what is your proudest publication?

L- My 1976 paper in Nature on Controlled Release of Macromolecules with Judah Folkman and my 1993 paper in Science on Tissue Engineering with Jay Vacanti.

FIGURE A.5. Dr. Langer receiving his US National Medal of Technology and Innovation on 2011 from the former president of the United States Barack Obama.

A- What is your opinion on the future of tissue engineering?

L- I think the future of tissue engineering and bioengineering in general are extremely bright.

A- Currently, what do you think is keeping organ engineering away from being a reality?
L- Its complexity including manufacturing, number of cell types and the need for vascularization.

A- You are also a founding father as well as a true pioneer on drug delivery systems, could you summarize a bit their basis to us?
L- Drug delivery systems are critical to making all drugs work, and they will be even more imperative and meaningful for the drugs of the future success.

A- Could you explain to us, up to the point you wanted, the drug delivery method you see more promising for the future?
L- I don't think there is just one. However, t I do think that nanotechnology is very important.

A- Do you think there is a need to put bioethical limits to the biomedical engineering field? For example, if bioengineered organs are a reality, everyone could just get a new organ, even if it was not necessary for them. Moreover, if people get new organs, we would be talking of a longer life longevity, and overpopulation would still be greater. What are your thoughts about that?
L- Yes. I think there will be government regulations.

A- One of the objectives I have in mind in this research project is to create a code of ethics on biomaterial research. As an expert, what issues do you think that are the most important to be written on or mentioned?
L- Stem cells and cost issues.

A- Research on human stem cells(hSCs) is considered so controversial, especially the use of human embryonic stem cells, because when they are used they later are destroyed. What is your opinion about investigating with hSCs?
L- I think one has to look at situations where they are destroyed anyhow, such as in vitro fertilization and how that's handled.

A- Do you believe in a personalized medicine? How do you think it is going to be?
L- Yes, I think that it will be excellent for patients.

A- What are your thoughts on the idea that "Bioethics should be taught as a fundamental basis of every science"?
L- I think it can be incorporated naturally into any existing classes.

We wanted to thank again Robert Langer and Jeffrey Karp for accessing to answer these interviews. As a future scientist it is a great pleasure to contact two of the best scientists there are and there have ever been on the field of the nanomedicine and the bioengineering.

STUDY 1: DECELLULARIZATION OF A HEART

Materials Used

- SDS 1% solution
- PBS solution
- Syringe and tube
- Perfusion pump
- Needle
- Tweezers
- Superglue
- Adult mouse heart
- Petri dish
- 0.5 L bottle

Procedure

1. First of all, we put the heart in a Petri dish with PBS to remove blood.
2. Then we identified the different parts of the heart and localized an artery.
3. We connected the tube with the needle, using an adaptor
4. With the use of the tweezers, we carefully inserted the needle into the artery, trying not to perforate the organ and not forcing the pass of the needle.
5. A drop of superglue was put on the insertion of the needle into the heart and we waited 3 minutes.
6. Having filled the syringe with 1% SDS solution and connected it to the tube, we placed the syringe into the peristaltic pump and the heart into the empty bottle (it can be fixed with tape).
7. We started pumping at a low rate and increased it until drops of SDS solution flowed through the heart.
8. It could eventually be observed the change in color of the heart tissue.

STUDY 2: FABRICATION OF 3D SCAFFOLDS

Experiment I

Materials used:

- Sodium alginate 5% (w/v) in Milli-Q water
- $CaCl_2$ 4% (w/v) in Milli-Q water
- $CaCl_2$ 1% (w/v) in Milli-Q water
- $CaCl_2$ 0.5% (w/v) in Milli-Q water
- 48 well plate
- Pipette for viscous solutions and tips

- Pipette (P1000) and tips
- Tweezers

Conditions (in triplicate):

I. 5% Alginate + 4% $CaCl_2$
II. 5% Alginate + 1% $CaCl_2$
III. 5% Alginate + 0.5% $CaCl_2$

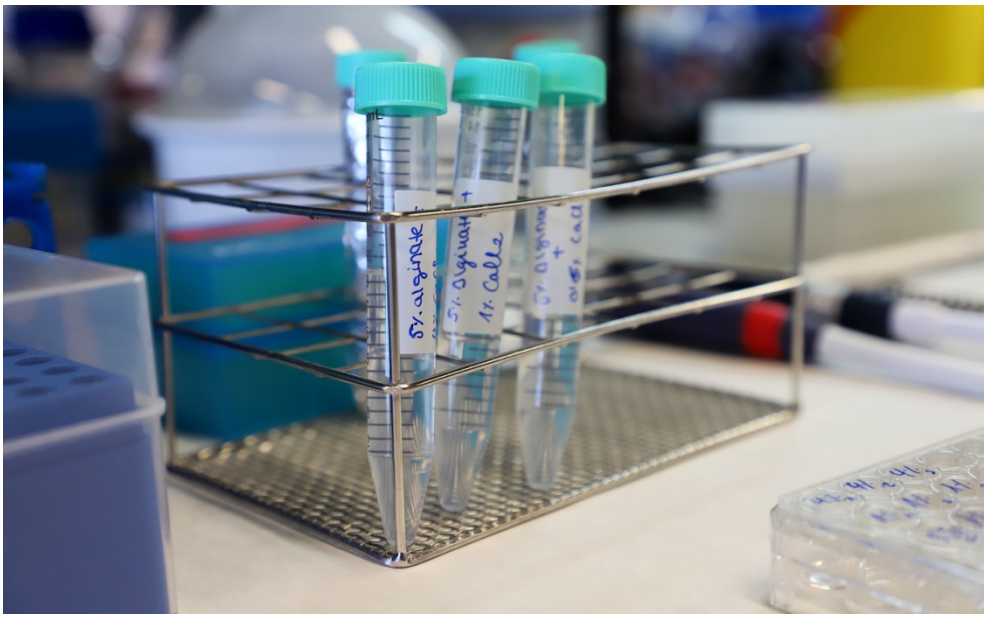


FIGURE A.6. Our three different conditions yet to be triplicated.

Procedure:

1. Add 200 µL of the alginate solution with the pipette for viscous solutions in a 48 well plate. Add in triplicates for each condition.
2. Move the plate to make sure that the solution covers all the base of the well and let it sit for a couple of minutes.
   Add 800 µL of the right calcium solution to each alginate solution carefully.
3. Put the timer on: at each time point (10 min, 20 min and 30 min), remove one of the gels of each condition with the tweezers and observe the differences between conditions.

Experiment II

Materials used:

- Solution 1: Sodium alginate (2%)
- Solution 2: $CaCl_2$ (4%)
- Solution 3: Phenolphthalein (pH indicator)
- Borax
- Magnetic stirrer and magnet

- Spoon
- 5 mL syringe
- Needle
- Plastic tubes
- Filter paper

Procedure:

1. Dissolve sodium alginate in Milli-Q water at 2% (w/v).
2. Prepare 200 mL of 4% $CaCl_2$ in Milli-Q water.
3. Prepare the phenolphthalein solution.
4. Add 1 spoon of borax of 10 mL in the sodium alginate solution.
5. Load 5 mL of the mix in a 5 mL syringe.
6. Place needle and generate drops over the $CaCl_2$ solution.
7. Prepare a filter with the paper and isolate spheres.
8. Add the isolated spheres in the phenolphthalein solution.

STUDY 3: CAM ASSAY

Conditions

The following 3 groups will be used in this experiment, being already cited, just to take them into account.

- **Negative control group :** Empty disks.
- **Experimental group:** Disk containing 1% (w/v) calcium-releasing particles.

Day 1 - Preparation of the material

Fabrication of disks made with Hydroxypropyl methylcellulose containing or not calcium-releasing particles.

Materials used:

- Hydroxypropyl methylcellulose powder
- Milli-Q water
- Magnetic stirrer and small magnets
- Beaker
- Glass vial
- Calcium-releasing particles
- Teflon mat
- Pipette for viscous solutions and tips (1000 µL and 100 µL)
- 30 mL plastic tubes.

Procedure:

1. We prepared 10 mL of 1.5% (g/100 mL) hydroxypropyl methylcellulose (0.15g) in Milli-Q water:

    a) We weighted 0.15g of methyl cellulose in a glass vial.
    b) We boiled Milli-Q water (using beaker) and added 10 mL on the methyl cellulose and put the lid on.
    c) Stir with magnet until cooled to room temperature (RT).

2. We prepared 3 mL of 1% solution of calcium-releasing particles in hydroxypropyl methylcellulose:

    a) We weighted 30 mg of calcium-releasing particles in small glass vial.
    b) We added 3 mL with the pipette.
    c) Particles were finally stirred until they were homogeneously distributed. If some particles remained in the bottom, we would then have to mix it with the pipette.

3. We pipetted 50 µL of solution on a clean Teflon mat.

4. We let it dry overnight at RT.

5. We sterilized under UV for 30min on both sides.

6. Finally, we used tweezers (sterilized with EtOH) to peel off disks.

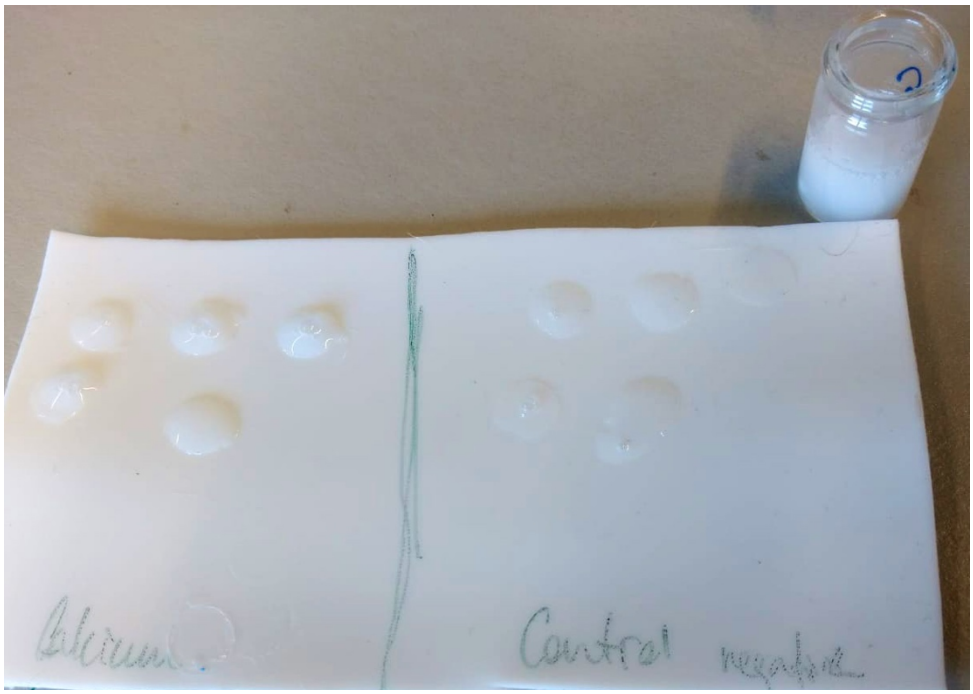


FIGURE A.7. Solutions with calcium-releasing particles and solutions with only Milli-Q water.

Day 2 - (Embryonic day 3: Eggs cracking)

After having the eggs incubated for 3 days in a horizontal position the fertilized eggs are cracked and placed in Petri dishes.

Materials used:

- Big Petri dish: (150 mm x 25 mm)
- Small Petri dish: (100 mm x 20 mm)
- Sterile phosphate buffer solution (PBS) bottle x3
- Scalpel
- Paper
- Marker
- Ethanol spray
- Aluminum foil

Procedure:

1. On the first place, the cabin was cleaned with EtOH.
2. Then we placed aluminum foil paper at the base of the cabin
3. We took the small Petri dish and we removed its lid.
4. We cleaned the egg with paper wet with EtOH, keeping the orientation of the egg present, the horizontal position. The break of the egg was done from the bottom part of the position in which it was incubated.
5. We broke the egg with the help of the sharp end of the scalpel, to later press the egg over this scalpel while moving it side to side. A crack was then created.
6. Quickly but in a careful manner, we opened the egg on the small dish.
7. We placed the small dish on the big dish, before covered with PBS (to maintain the moisture of the embryo).
8. The number of the egg, the name and the date were written on the eggs.

FIGURE A.8. Eggs being cracked.

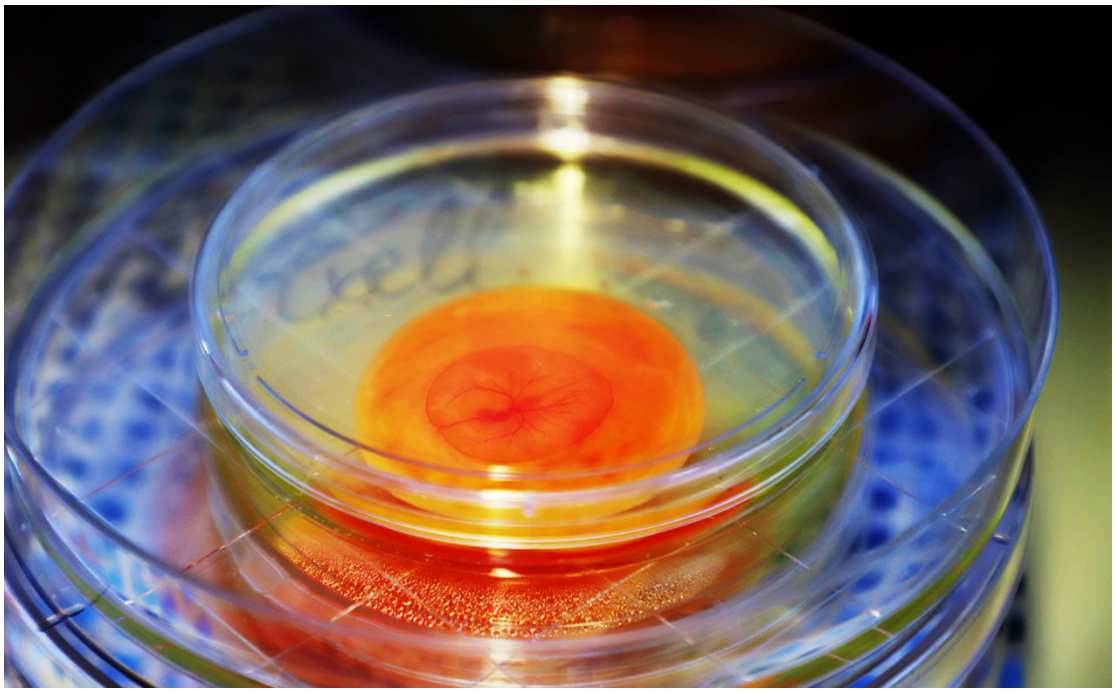


FIGURE A.9. Embryo on the third day of development, just after having the egg cracked. We can see the PBS surrounding the small Petri dish.

Day 3 - Embryonic day 9: Material Implantation

The test materials are added on the CAM.

Materials used:

- Hydroxypropyl methylcellulose disks (sterilized with UV).
- Tweezers (sterile)
- Marker
- VEGF solution (10µg/mL)
- Pipette and tips (sterile)

Procedure:

1. As always, we cleaned the cabin with EtOH.
2. We later placed 4-5 disks of the same group on each CAM: Carefully, we took the disks with the tweezers and we placed them on the CAM. Disks were placed halfway between the edge of the CAM and the chick, and close to big vessels but not over them. We also took care of the distance between the disks, to not be one too close to another. See FIGURE A.10.
3. We made sure trays and big Petri dishes had enough sterile PBS, and in some cases, we added more.
4. For the positive control only, we added 10 µL of 10 µg/mL on the disk after placing the disk over the membrane.

5. We marked on the lid the position of each disk and made another mark on the lid and on the base. This helped us to locate the disks at the end of the experiment.
6. We finally kept the dishes in the heater at 37ºC.

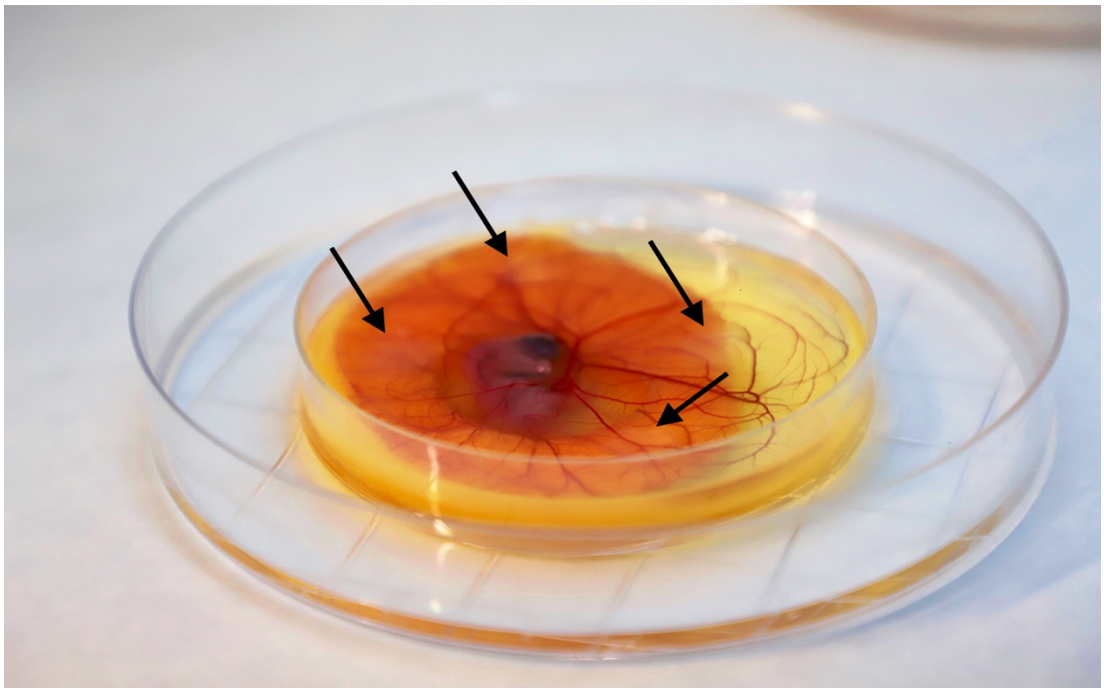


FIGURE A.10. Pointed with the arrows, the zones where the material was implanted. Cannot be so distinguished.

Day 4- Embryonic day 12: CAM fixation

On day 12 of development, chicks are sacrificed and CAMs are fixed with a fixing agent to retain blood in the vessels and preserve the tissue. The CAM around the disks will be carefully cut with scissors and placed in PBS in a Petri dish.

Materials used:

- 10% Formalin
- 10 mL serological pipettes
- Petri dish
- PBS
- Scissors
- Marker

Procedure

1. We marked a square in the lid over each area surrounding the disks on the lid, so we later would know where to cut.
2. We decapitated the embryos with scissors.
3. We added the fixing agent (Formalin) over the area of each disks (about 8 mL per embryo). See FIGURE A.11.

FIGURE A.11. The fixing agent we used, Formalin.

4. Then, we waited for 20 min.
5. Once we waited, we cut the area around each disk, taking a good part of CAM. See FIGURE A.12.
6. We transferred that area into a Petri dish with PBS.

7. Then we observed our results under a stereoscope microscope.

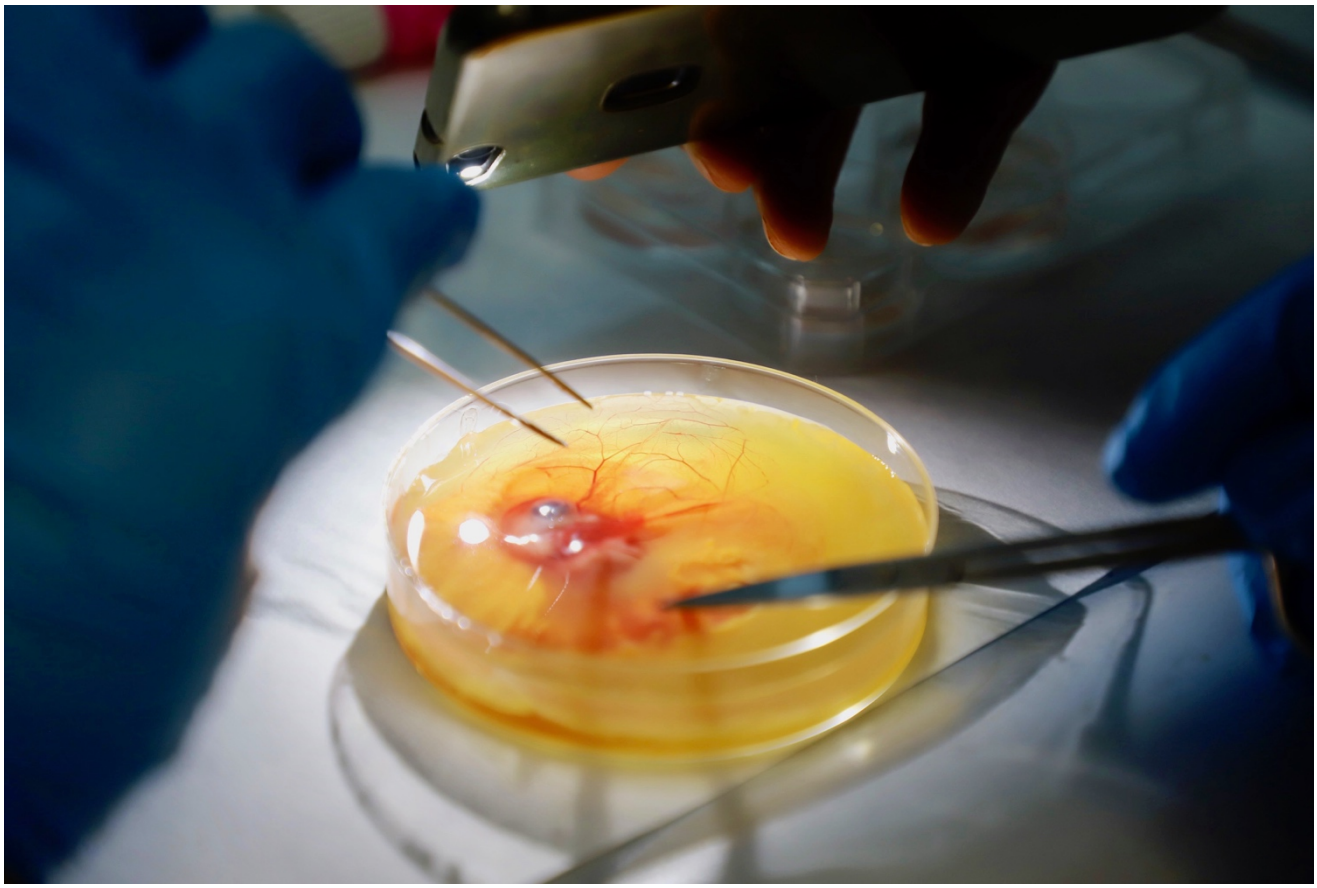


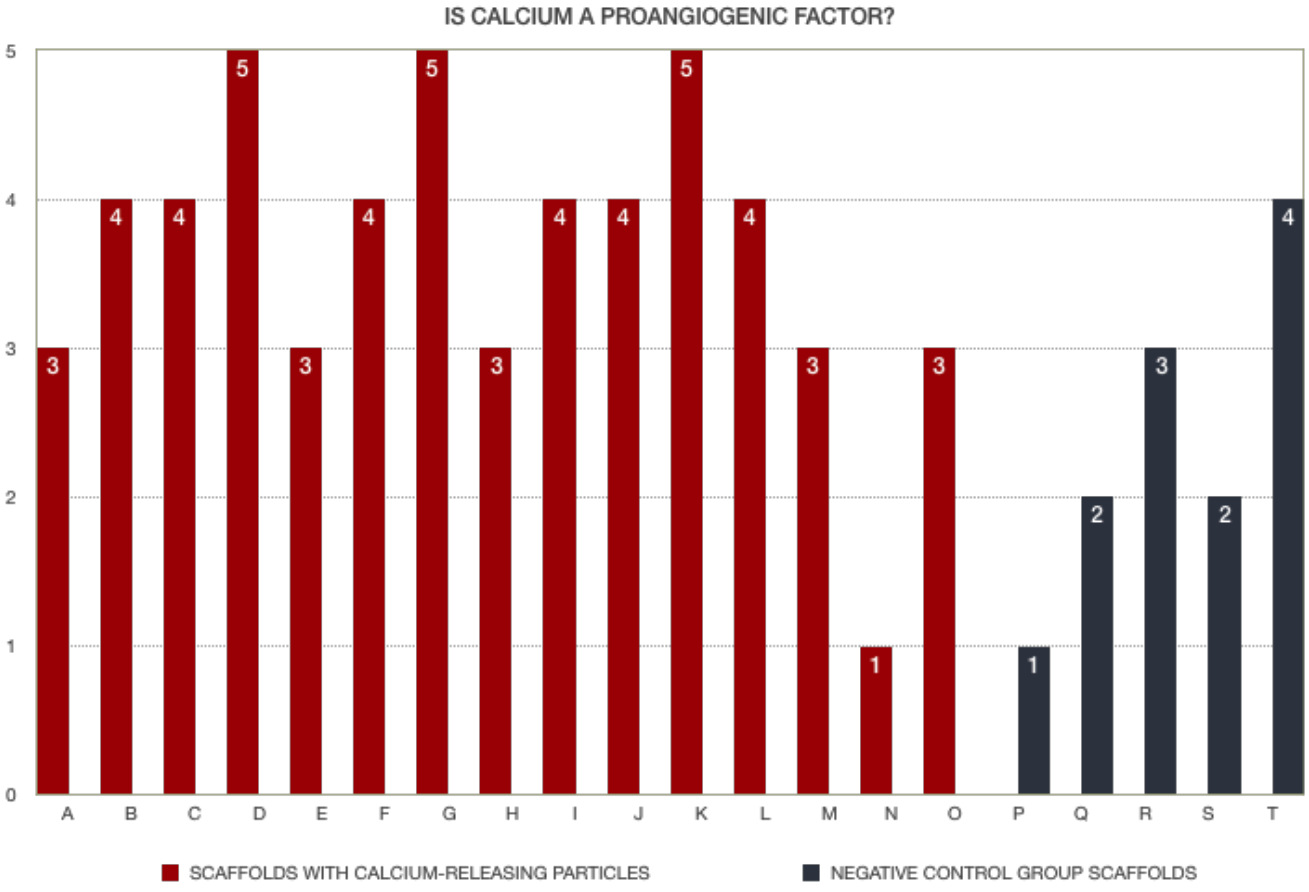
FIGURE A.12. Picture of how we extracted the area surrounding the disks.

On TABLE A.1. and GRAPHIC A.1. the evaluation of the angiogenesis and its graphical representation.

	SCAFFOLDS WITH CALCIUM-RELEASING PARTICLES	NEGATIVE CONTROL GROUP SCAFFOLDS
A	3	
B	4	
C	4	
D	5	
E	3	
F	4	
G	5	
H	3	

I	4	
J	4	
K	5	
L	4	
M	3	
N	1	
O	3	
P		1
Q		2
R		3
S		2
T		4

TABLE A.1. Scaffolds with calcium-releasing nanoparticles and scaffolds without them.



GRAPHIC A.1. Representation of the macroscopic angiogenesis evaluation.

STUDY 4: PH and Calcium Release Measurements

Day 1

Materials used:

- Calcium-releasing particles in Eppendorf tube (3 mg per Eppendorf).
- Release media: Milli-Q water with 10mM HEPES adjusted to pH 7.4.
- Pipette and tips.
- Eppendorf tubes.
- Rack for Eppendorf tubes.
- Vortex.

Procedure:

1. We labelled Eppendorf tube with the name of the conditions used:
    a) Calcium (contains the calcium-releasing particles).
    b) Blank (empty).
2. We then added 500 µL of release media.
3. We put our samples in a short time length at the Vortex.
4. We left it at 37ºC.

Day 2

Materials used:

- Samples (calcium and blank)
- Centrifuge
- Pipette and tips
- 96 well plate
- Marker
- Color reagent (CR)
- AMP Buffer
- Calcium standard: 100 mM
- Eppendorf tubes
- Rack for Eppendorf tubes
- Aluminum foil
- Spectrophotometer
- pH indicator paper

Procedure:

1. Firstly, we centrifuged our samples for 10min.
2. We later transferred their supernatants into new Eppendorf tubes (and we labelled them).

3. Here began our calculations to prepare a calibration curve with the 100 mM solution. The points already mentioned that we needed were: 6-3-1.5-0.1-0.05-0 (mM Ca^{2+}). We had the reference that we should have at least 100 µL of final volume for each concentration.
4. We wrote in the lid of a 96 well plate how the samples would be placed in the wells with a marker. We would need all the points prepared for the calibration curve and our samples, including blank ones, all in triplicate.
   We triplicated our samples in order to reduce the experimental error when pipetting.
5. Then we added 100 µL of the CR solution into each well.
6. Following the last step, we now had to add 5 µL of the samples to each well.
7. Later we added 100 µL of the AMP reagent to each well, mixing the solution pipetting up and down.
8. To finish the calcium measurement, we read the signal in the spectrophotometer, with an absorbance set to 570 nm.
9. We also did the pH measurement, cutting a piece of pH indicator and soaking one end in the sample to determine the pH.

Procedure (II)

1. Put experimental and known concentration of calcium in spectrophotometer, we know the absorbance of all of the samples.

Concentration In mM of Calcium (Ca^{2+})	Absorbance 1 No units	Absorbance 2 No units	Absorbance 3 No units
0,00	0,318	0,264	0,292
0,05	0,336	0,271	0,335
0,10	0,338	0,349	0,346
1,50	1,035	1,059	1,118
3,00	2,035	2,012	2,05
6,00	3,541	3,533	3,571

TABLE A.2. Absorbance of the known calcium concentrations group.

Concentration in mM Calcium (Ca^{2+})	Absorbances' average No units
0,00	0,291
0,05	0,314
0,10	0,344
1,50	1,071

3,00	2,032
6,00	3,548

TABLE A.3. Average of triplicate samples' absorbance.

This data formed the calibration curve, the concentration was the X axis and the absorbance conformed the Y axis.

Ca^{2+} (GROUP 1) No units	Ca^{2+} (GROUP 2) No units	Ca^{2+} (GROUP 3) No units
0,819	0,772	0,779
0,718	0,73	0,726
0,804	0,772	0,767

TABLE A.4. Absorbance of the unknown concentration of the calcium samples.

Neg. Control (GROUP 1)	Neg. Control (GROUP 2)	Neg. Control (GROUP 3)
0,278	0,264	0,261
0,248	0,213	0,215
0,183	0,238	0,274

TABLE A.5. Absorbance of the negative control groups.

2. With the known concentration group and its absorbance, we do a 2D scatter graphic, a calibration curve (see GRAPHIC A.2) , as stated above.

3. In some points of this calibration curve there will be our experimental group, so we take out an equation of the curve/line, see FIGURE A.13.

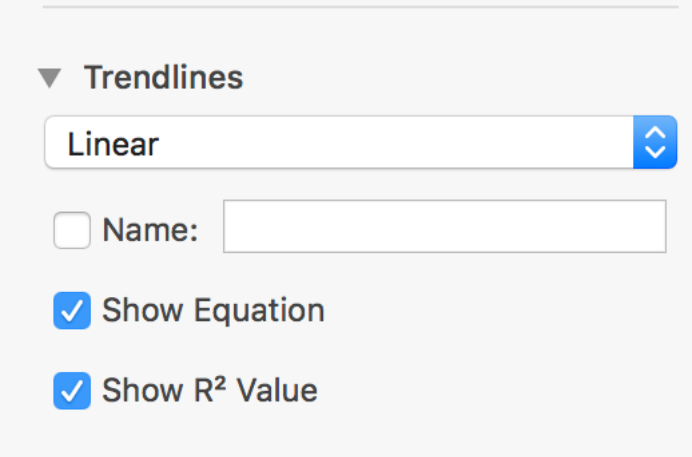
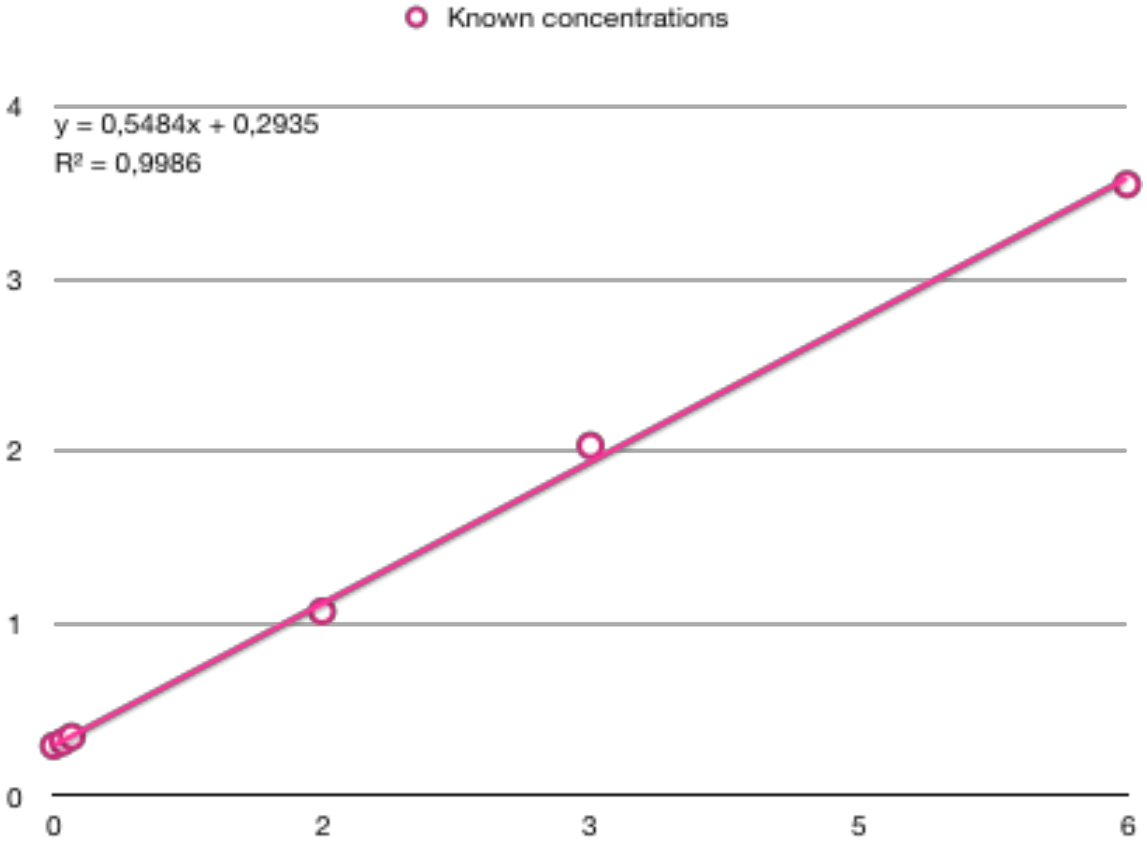


FIGURE A.13. The process used to show the equation of the calibration curve. The program used for the graphics was Numbers, from macOS High Sierra version 10.13.6.

4. We put as an unknown variable the concentration of the experimental calcium group, in other words, we isolate the X variable in the equation, and we calculate it. Everything it's done ruling out errors by doing an average of the triplicate samples.



GRAPHIC A.2. Calibration curve.

Concentration Ca^{2+} (mM) (GROUP 1)	Concentration Ca^{2+} (mM) (GROUP 2)	Concentration Ca^{2+} (mM) (GROUP 3)
0,958	0,873	0,885
0,774	0,796	0,789

0,931	0,873	0,863

TABLE A.6. Concentration of the experimental group extracted from the calibration curve's equation.

Concentration Neg. Control (GROUP 1)	Concentration Neg. Control (GROUP 2)	Concentration Neg. Control (GROUP 3)
-0,028	-0,054	-0,059
-0,083	-0,147	-0,143
-0,201	-0,101	-0,036

TABLE A.7. Concentration of the negative control group extracted from the calibration curve's equation.

Average concentration Ca^{2+} in mM
0,860
Average negative control concentration
-0,095

TABLE A.8. Average of the concentrations of the TABLES A.6. and A.7. above.

5. After getting our average concentration of the unknown samples from the equations, we subtract [0.860 – (-0,095)], because the -0,095 of the controls is the noise or background that is present on all of our samples, including our calcium ones.

Real concentration of the Ca^{2+} (In 3 mg)	/ 3	Real concentration of mM Ca per mg
0,955		0,318

TABLE A.9. Final result of the concentration of Ca2+ we will put in our next study, the CAM assay.

STUDY 5: FLUORESCENT STAINING OF CELLS

Before explaining the materials used and the procedure carried out on the fifth study, the protocols of the fabrication of scaffolds by both electrospinning and 3D bio-printing[33] will be implemented.

Electrospinning

1. Prepare 10 ml of a solution of 4% W/W of polylactic acid (PLDLA 70/38) in trifluoroethanol in the cabin hood.

2. Pour the solution into a 10 ml syringe and put a tip with a gauge of 25.

3. Couple the syringe in the infusion pump for electrospinning.

4. Configure the pump to run at 1 ml/h.

5. Prepare a sheet of greaseproof paper 29x20 cm and fix it in the rotary collector.

6. Configure the collector to run the minimum slower as possible.

7. Check that the collector is grounded.

8. Connect the tip of the syringe to the power source.

9. Run the pump until the liquid drops from the tip.

10. Gradually increase the power supply up to 12 kV.

11. Check that a fine jet is formed that finishes at the collector.

12. (Optional) The flow rate can be increased until 3 o 4 ml/h if jet is maintained.

13. Wait until the solution is completely electrospun cleaning the tip of the syringe when needed with the isolated stick (mind not to touch directly neither the syringe nor the collector).

14. Pick the formed mat up.

---

[33] Credits to the authors of the protocol: Celia Ximenes, Marc Batista and Oscar Castaño from the IBEC. The references they used are:

- M.A. Mateos-Timoneda, O. Castano, J.A. Planell, E. Engel, **Effect of structure, topography and chemistry on fibroblast adhesion and morphology**, J. Mater. Sci. Med. 25 (2014) 1781–1787.

- S. Yang, Z.-H. Wu, W. Yang, M.-B. Yang, **Thermal and mechanical properties of chemical crosslinked polylactide (PLA),** Polym. Test. 27 (2008) 957–963. doi:10.1016/J.POLYMERTESTING.2008.08.009.

- A. von Bomhard, A. Elsässer, L.M. Ritschl, S. Schwarz, N. Rotter, **Cryopreservation of Endothelial Cells in Various Cryoprotective Agents and Media – Vitrification versus Slow Freezing Methods**, PLoS One. 11 (2016) e0149660. doi:10.1371/journal.pone.0149660.

15. Punch several samples of 5 mm of diameter.

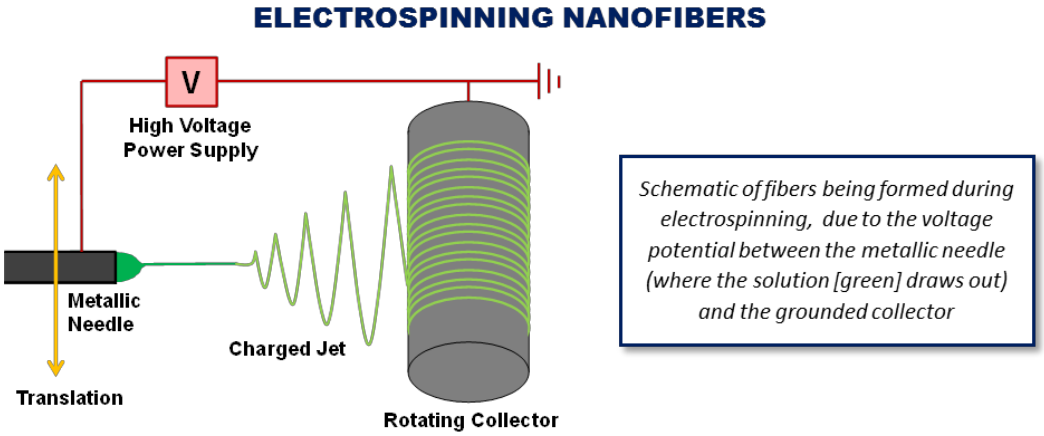


FIGURE A.14. Electrospinning scheme.

3D printing

Material preparation

1. A 50 ml solution 14% W/V of PLA in ethyl lactate has to be prepared.
    - Add 50 ml of ethyl lactate to 7 g of PLDLA (70/38) .
    - Let the mixture in a bath at 50-60 ºC in a hot plate.
    - Use the polytron (level 6) [this is a mixer/homogenizer] to finally homogenize the solution.
    - Now, the solution is ready to use if desired.

Scaffold fabrication

Syringe preparation

2. Preheat the mixture in a bath at 55-60ºC approx. For an hour.
3. Once the mixture is smooth, use the polytron (level 6) to homogenize the solution if required.
   o NOTE: It is highly recommended to homogenize the solution every time you will use them, since the PLA can “compact” to one side of the pot and separate from the ethyl lactate. This homogenization process has to be performed in a 55 to 60 ºC bath.
4. With a positive displacement micropipette, add 1 to 2,5 mL of the PLA into a syringe (Nordson, stored down the positive displacement micropipettes).
   o NOTE: Remember to use an end cap to block the PLA exit.
   o NOTE 2: It is recommended to prepare the syringe the day before to use it. Just place the PLA inside and close the extremes with and end cap and tip cap. Then, seal it with parafilm and stored at 4 ºC or -20ºC (at your preference, but 4ºC is ok). When the PLA solution is already cold, place the piston inside. Pre-warm at 55-60 degrees before you use it.

Scaffold 3D printing process

5. Set the syringe temperature around 60 degrees.
6. Replace the end cap by the desired tip. If not made before, put the piston inside the syringe and connect it to the compressed air valve (yellow). Place the syringe inside the supporter.
7. Set up the machine tool software and open the text document with your design.
   - o To open the document, click to the folder button.
   - o To edit the document, click to the notebook button.
   - o To turn the lights on of the Rapid Prototyping tool, click to "Vision" – "Lightning control".
   - o To start the control menu, click to the tools icon button.
     - ♣ There, you can control the syringe position (x, y and z coordinates, as well as performing the "homing"), and valve pleasure (ON/OFF as well as the 5-pressure value). It is important to perform the "x, y, z home" in order to calibrate the positions the first time.
   - o To start the printing control menu, click to the play icon button.
     - ♣ There, you can start the printing cycle, as well to stop it by pressing the abort button.
     - ♣ NOTE: when printing, press first the "cycle start" button before turning the pressure ON, and finish by turning OFF the pressure before aborting the printing process. That will make you prevent wasting material.
     - ♣ NOTE 2: Pressure, temperature and velocity are the parameters to optimize when printing. The tip diameter and height between layers ("dz") is also a thing to optimize. In the table below, you can find the ones used in PLA 14%, for a G27 tip. Material Tip Temperature Velocity Pressure PLA 14% in ethyl lactate. G27 (200 um diameter). For that tip, a "dz" distance of 0,1-0,11 mm is fine. 60 +/- 5 ºC. 3 mm/s. In the order of 20 PSI (pounds-force per square inch). *

     *usually, it is no need more than 30 PSI to print it.
8. To recover your print, use ethyl lactate/Milli-Q water progressive baths.
   - o NOTE: the most important is to start with a bath of 90:10 or 80:20 ethyl lactate: water. The water is used to precipitate the PLA, as it displaces the ethyl lactate. As the PLA is very elastic, if the material is not disposed properly at the surface (for that reason, is it better to print it at low velocities), or the solvent change is very abrupt, the PLA will contract and change the morphology of our scaffold. It is recommended to increment the % of water from 10 to 10, every three to five minutes.
9. When all ethyl lactate is displaced, lyophilize (low temperature dehydration method) the scaffold to avoid its degradation.

PREPARED STAINING

Materials used:

- Cells (rMSCs or rat mesenchymal stem cells) seeded on different scaffolds.

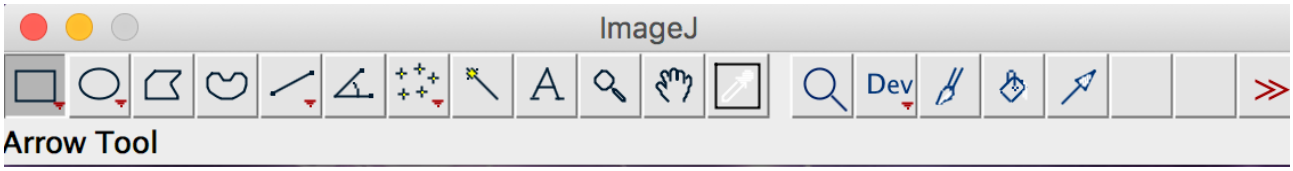
- Paraformaldehyde (or PFA) 4% solution
- PBS (or phosphate buffer solution)
- Phalloidin solution
- DAPI solution
- 1000 µL pipette and blue tips
- Timer
- Fluorescence microscope.

Procedure:

Considering that we could not let our cells dry, as they can be damaged, so cell wells always contained some liquid.

1. We completely removed cellular media from cell-containing wells.
2. Then we washed once in warm PBS solution (add 500 µL of PBS solution and immediately remove it).
3. Later we added 500 µL of PFA 4% solution in each well. This step was performed inside the hood, as PFA is toxic. We then incubated the cells for 10 mins.
4. After the fixation, cells were washed again with PBS.
5. Having added the PBS, we took it out again and we added 500 µL of phalloidin solution and we incubated the cells in the dark (we covered them with aluminum foil) for 30 mins, to preserve its fluorescence.
6. We then removed the phalloidin solution and we added 500 µL of DAPI. We incubated our cells in the dark again and we had them covered for 1 minute.
7. Finally, we washed the cells with PBS one last time and we maintained the cells with some PBS, we covered our samples and we could now take images using the fluorescent microscope.

Worth to say, this fluorescent staining method was carried out twice during my experimentation. In both procedures, once obtained the images, they were analyzed and processed with the program for microscopy edition called ***ImageJ*** (see *FIGURE A.15.*)



.*FIGURE A.15. This is how the **ImageJ** interface looks like.*

NON-PREPARED STAINING

Day 1

The first day we did the cell culture preparation.

Materials used

- Growth factors, 44 mL
- Calf serum, 4 mL
- Glutamine, 1 mL
- Antibiotic agents (penicillin) , 1 mL
- Pipette and tips
- Unfrozen rMSCs, 10º6 cells
- Flask
- Volumetric pipets (10 mL and 25 mL)
- Marker
- PipetBoy, pipette controller
- Incubator at 37ºC

Procedure

See FIGURE A.16.

1. The culture medium preparation. Added in a Falcon flask 44 mL of growth factors[34], 4mL of calf serum, 1 mL of glutamine[35] and 1 mL of antibiotic agents[36].
2. We took 10^6 rMSCs, rat mesenchymal stem cell. These cells had been frozen with the enzyme dMSO[37] .

[34] Growth factors are basically amino acids and enzymes where cells tend to adhere.
[35] Glutamine is a protein that was not included on the growth factors.
[36] The antibiotic agents were added to prevent any immunological problem that may have occurred to our cells.
[37] dMSO is an enzyme that avoids the crystallization of the cells. However, this enzyme is toxic for a determined amount of time exposure to cells.

3. We took 10 mL of the culture medium (or DMEM) and put it together with the 10^6 cells. We then marked them.

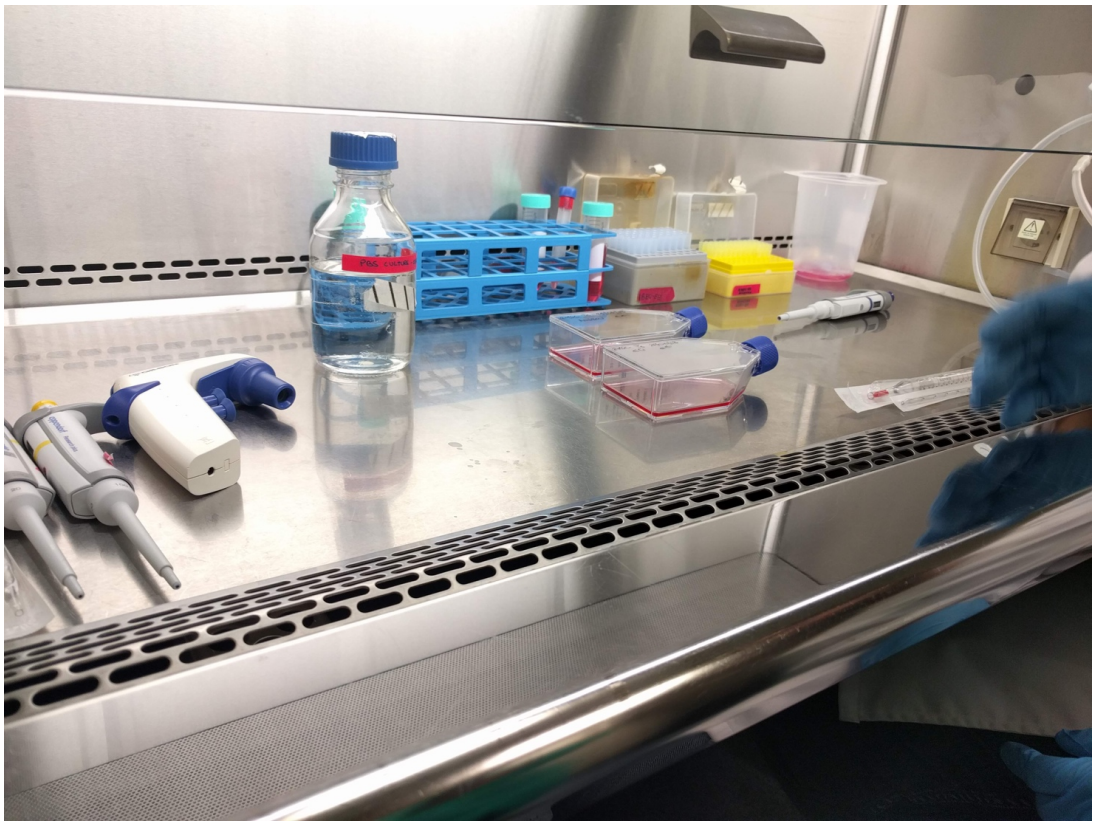


FIGURE A.16. Our hood was ready to begin our experiment, always being organized.

Day 2

Here we did a culture medium change, because of the dMSO already mentioned.

Materials used

- PBS or Phosphate Buffer Solution, 20 mL
- Culture medium, 10 mL
- Volumetric pipettes, (10 mL and 25 mL)
- PipetBoy, pipette controller
- Incubator at 37ºC

Procedure

1. First, a PBS wash was done (we needed a buffer in order to clean away dead cells on the freezing and unfreezing processes).
2. Then, 10 mL of the starting culture medium was added. We now observed how cells were adhered and were already reproducing.

Day 4

We had to wait 48 hours, as cells were through a lot of stress because of the freezing and unfreezing processes.

We did a new cell passaging, to passage 5. A passage consists in detaching cells and having them adhered again. The optimal number of passages is between 4 and 6, because when they are on passage 8 or even higher the cells have already reproduced too much, and their function is reduced.

Materials used:

- Volumetric pipet (10 mL and 25 mL)
- Pipettes and tips
- Centrifuge at 1000 rpm for 5 minutes
- “Falcon” flask
- Incubator at 37ºC
- Handheld manual cell counter, counted with the help of a hemocytometer or Neubauer chamber, see FIGURE A.17.
- Electrical microscope
- PipetBoy
- Culture medium with PBS solution (10 mL)
- Culture medium (1.3 mL)

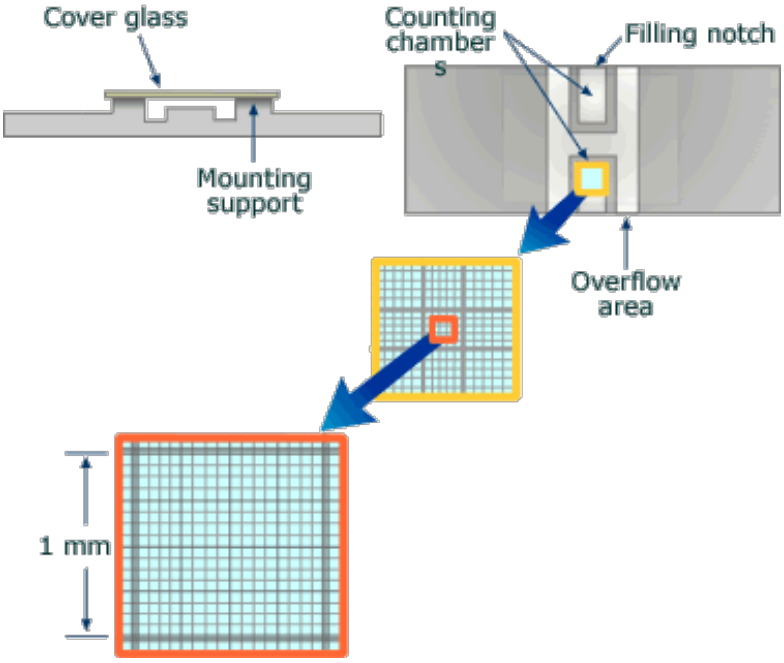


FIGURE A.17. Neubauer chamber or hemocytometer.

Procedure:

1. All the culture medium was taken away, see FIGURE A.18.
2. We put 5 mL of trypsin (digestive enzyme) + 10 mL of culture medium with PBS, letting it slide through the glass to remove the entirety of the cells.
3. We put the sample in a Falcon flask

4. We centrifuged for 5 minutes, to get the cells to the bottom forming a pellet.
5. Count the cells (just a third of the cells), the other two thirds were frozen*, in order to be taken advantage of in other future experiments.
6. The third of the cells was put in the incubator at 37ºC. We had two different sources of cells, so we had to different "thirds" of the initial number of cells. One of the thirds is going to be called A source and the other one B.
7. With the help of the hemocytometer and the manual cell counter, we counted the cells within a cube millimeter, and we calculated the cells that would be there within a cube centimeter, that is a milliliter.
8. So, having multiplicated by 10^3 our average of the four parts' count, our calculus exposed that the B source had 113.6 x 10^4 cells / mL and that our A source had 81 x 10^4 / 1.3 mL .
9. In order to put less cells into the electrospun scaffolds later on, we added to the A source (the cells that are going to be seeded on the scaffolds) 1.3 mL of cell medium, in order to dilute the concentration and to have 5.25 x 10^4 cells in 2.6 mL

- The freezing medium consisted on the two third of the cells, 100 µL of dMSO and 900 µL of calf serum.

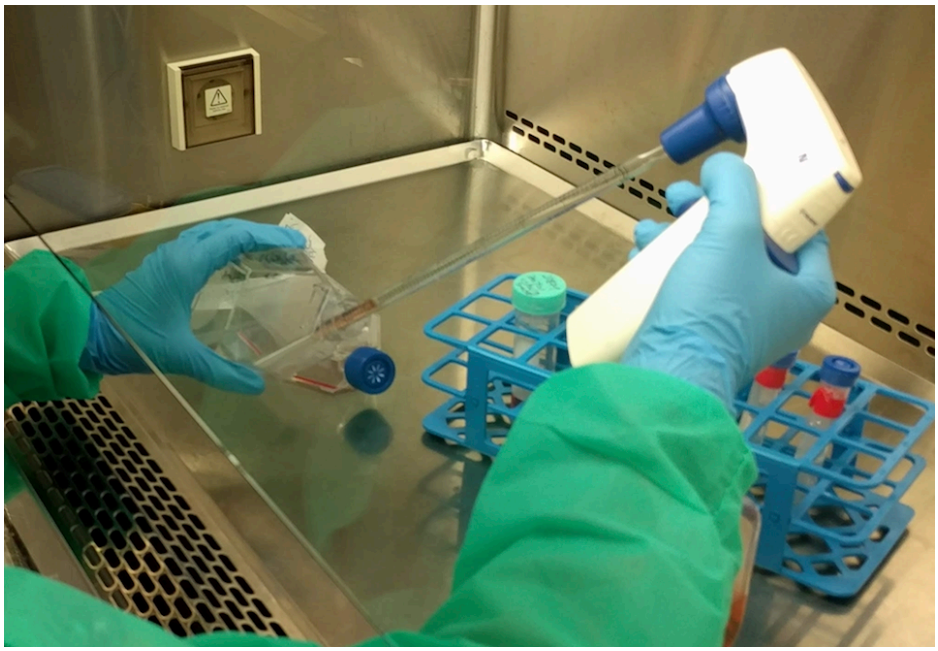


*FIGURE A.18. Step **1.**, when the cell media was being taken away.*

Day 5

Preparation of the scaffolds.

Materials used:

- Scissors
- Tweezers

- 6 mm diameter punch
- Set square
- Bio-printed PCL80 scaffolds
- Electrospun PLA scaffolds

Procedure:

We have two types of scaffolds, made by electrospinning (of the PLA, polylactic acid polymer) and ones made by 3D bio-printing (of the PCL80, polycaprolactone polymer).

We measured the back of the 36 well plate where we would put the electrospun scaffolds and we cut with the help of scissors a square of that measurements for each electrospun scaffold.

For the bio-printed scaffolds, we already had a rectangular network of bio-printed scaffold, we just had to take a 6 mm diameter punch and we took out circular-shaped bio-printed scaffolds.

Having shaped our scaffolds, they have to be first sterilized and then treated.

That is because our scaffolds are not bioactive at this point. To achieve bioactivity, a surface modification is done with the help of chemical functionalization. The method is called Carbodiimide Crosslinker Chemistry, and it involves 1-Ethyl- 3-(3-dimethylaminopropyl)-carbodiimide (or EDC) and N-hydroxysuccinimide (or NHS). For the activation we are going to use collagen type I and fibronectin.

Materials used (II)

- 10 mL of 0.1M and 0.01M of NaOH.
- Micropipettes of 20, 500 and 1000 µL.
- pH meter.
- Milli-Q water.
- Fibronectin source of 100 µg/mL.
- EDC and NHS source.
- PBS solution.

Procedure (II)

1. We prepared 10 ml solution of 0.1 (for the 3D printed scaffolds) and 0.01M (for electrospun scaffolds) of NaOH.
2. We also prepared 50 ml of a solution 0.1M of HCl.
3. As well as a 5 ml solution of EDC/NHS 0.1/0.02M of 5.5-6 pH.

   $M(EDC)$= 155.24 g/mol d=0.877 g/cm^3

M(NHS) =115.09 g/mol

We were using a pH meter continuously to get the proper pH. If it was not the proper pH, HCl or NaOH was added.

4. Also, a solution of 5 ml of fibronectin of 100 µg/mL were prepared.
5. The electrospun scaffolds were immersed 5s and the 3D printed scaffolds were immersed 30 minutes in the NaOH solution.
6. The scaffolds were washed with Milli-Q water.
7. We immersed the scaffolds in the filtered EDC/NHS solution for 10 minutes.
8. The scaffolds were rinsed with Milli-Q water.
9. Immerse the scaffold in the filtered solution with the protein for 10 minutes.
10. Lastly, we rinsed the scaffolds with PBS.

In FIGURE A.19., the surface's exact modification is shown:

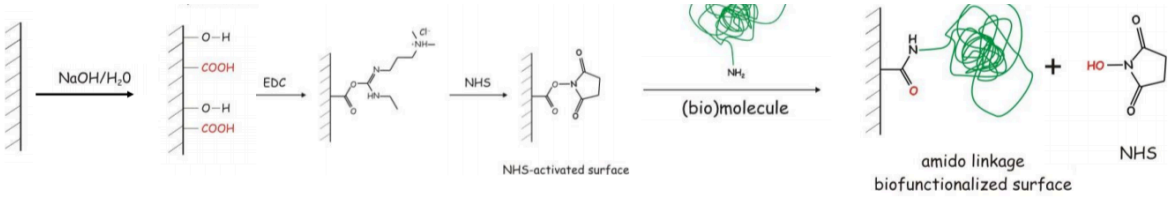


FIGURE A.19. Carbodiimide Crosslinker Chemistry.

Day 6

Functionalization of the scaffolds.

Materials used:

- 0.22 µm filter
- NaOH solution
- Trypsin solution
- Cell medium or DMEM
- Electrospun scaffolds
- Incubator at 37ºC
- A source of cells
- B source of cells
- Pipettes and tips
- Milli-Q water

Procedure:

1. We filtered Milli-Q water with a 0.22 µm filter.

2. Then we applied a bond treatment with 200 µL of NaOH at the bio-printed scaffolds, and we let it sit for 30 minutes.
3. We “trypsinized” the cells, in other words, the cell medium was taken of the samples and then 10 mL of trypsin was added.
4. We incubated the cells during 5 minutes at 37ºC.
5. After the 30 minutes, we added DMEM or cell medium to the electrospun scaffolds and put them at the incubator at 37ºC.
6. We added 50 µL of the A source of cells (113.6 x 10^4 cells / mL) to each of the 18 bio-printed scaffolds, and we completed it with 150 µL of cell medium, in order to let the cells survive.
7. Later, we incubated it at 37ºC.
8. To the electrospun scaffolds we added 288 µL of the B source of cells (5.25 x 10^4 cells / 2.6 mL, concentration already diluted), and we completed it with 600 µL of DMEM in each of the electrospun scaffolds.
9. We finally incubated it at 37ºC.

EXTRA PROCEDURE EXPLAINED: PROCESSING IMAGES IN THE MICROSCOPE

Introduction

In almost any experiment, to observe and analyze the particles, compounds or simply elements of a sample we need to process that images: adjust its brightness and contrast, merge their color channels and add a scale.

As stated before, for these images it was used the **ImageJ** program (see FIGURE A.20.), an open access software available for everybody as a universal tool.

FIGURE A.20. This is the logotype of the ImageJ software.

To explain the process, we will use as an example an electrospinning image, as it is so easy to observe all the phases done.

Procedure

1. We departed from this image, the original image from the *FIGURE A.21.* Although the actin cytoskeleton can already be distinguished, there is disturbing background that could be deleted.

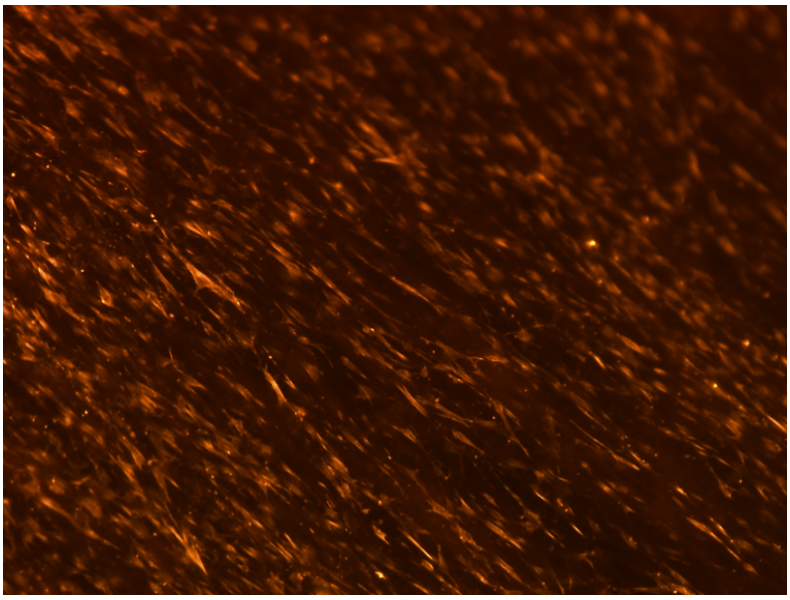


FIGURE A.21. Original electrospun image taken from the microscope.

2. To change our image brightness and contrast we take advantage of the B&C too, see FIGURE A.22.

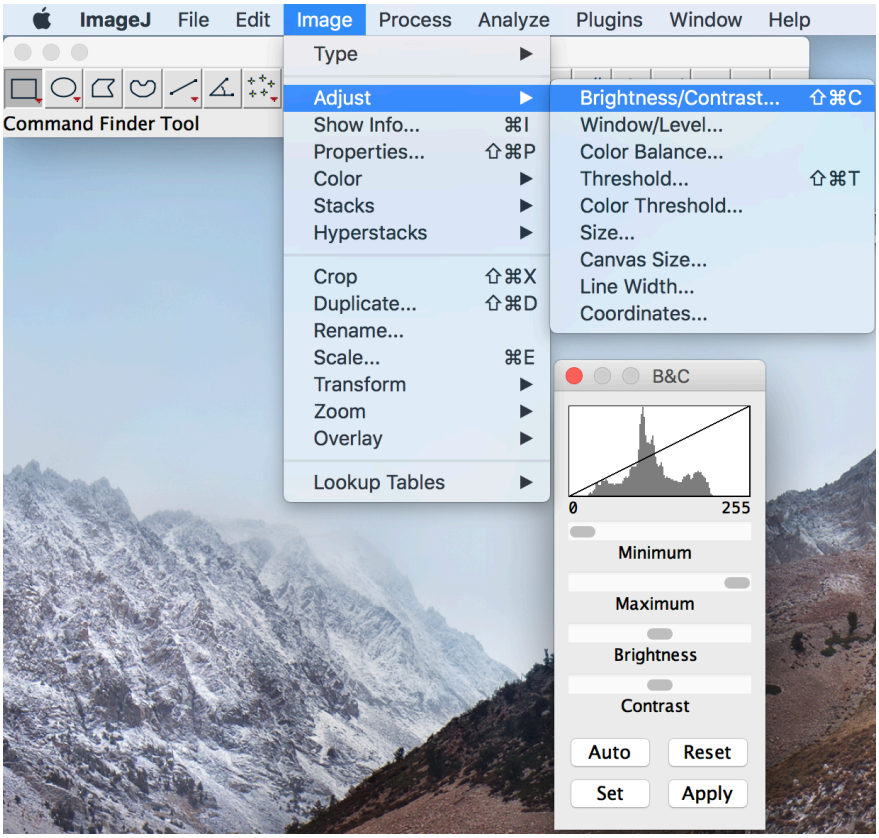


FIGURE A.22. B&C tool.

3. By trial and error, brightness and contrast are adjusted until we get an image without background, similar to FIGURE A.23., as there is no best way of processing these kind of images, it all depends on the necessities or what we want to observe.

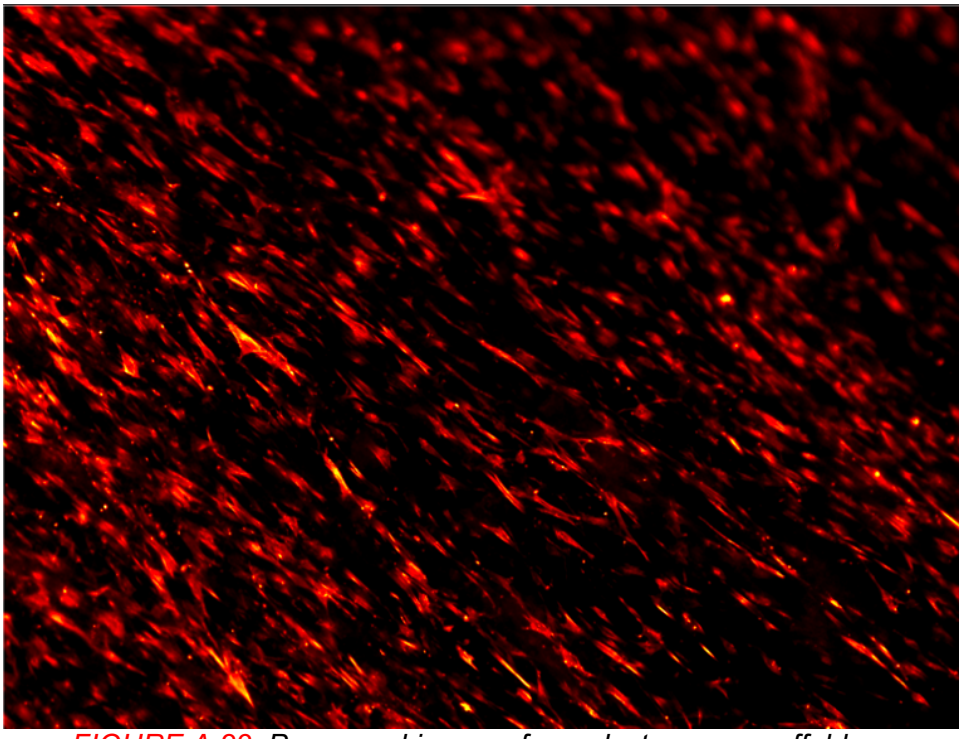


FIGURE A.23. Processed image of our electrospun scaffold.

4. However, our proccessing is not finished yet, as normally if we have different colour channels (in FIGURE A.24.) they tend to be merged.

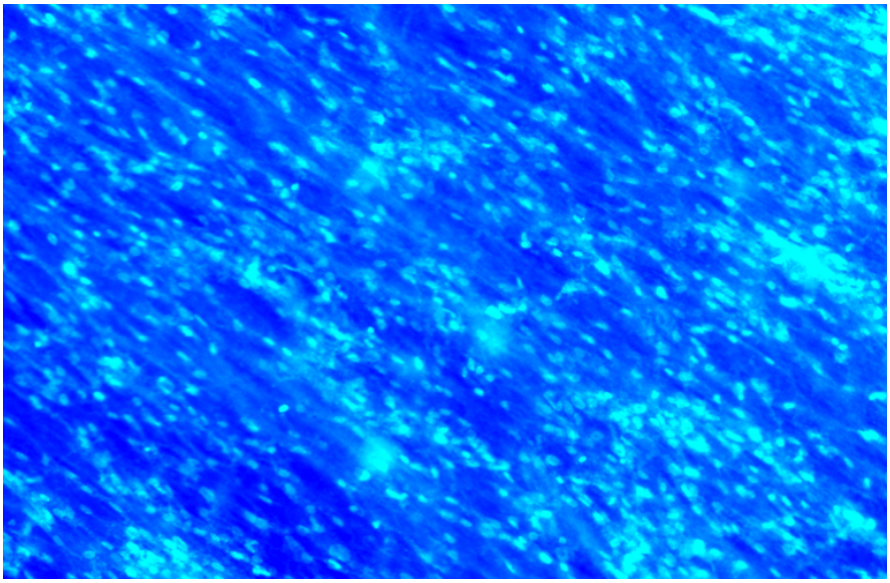


FIGURE A.24. The nuclei colour channel from the same electrospun scaffold, already processed.

5. Merging the two colour channels from our sample scaffold we observe why we do not always do so (in FIGURE A.25.), because the image becomes unclear and the elements we had to observe blurry.

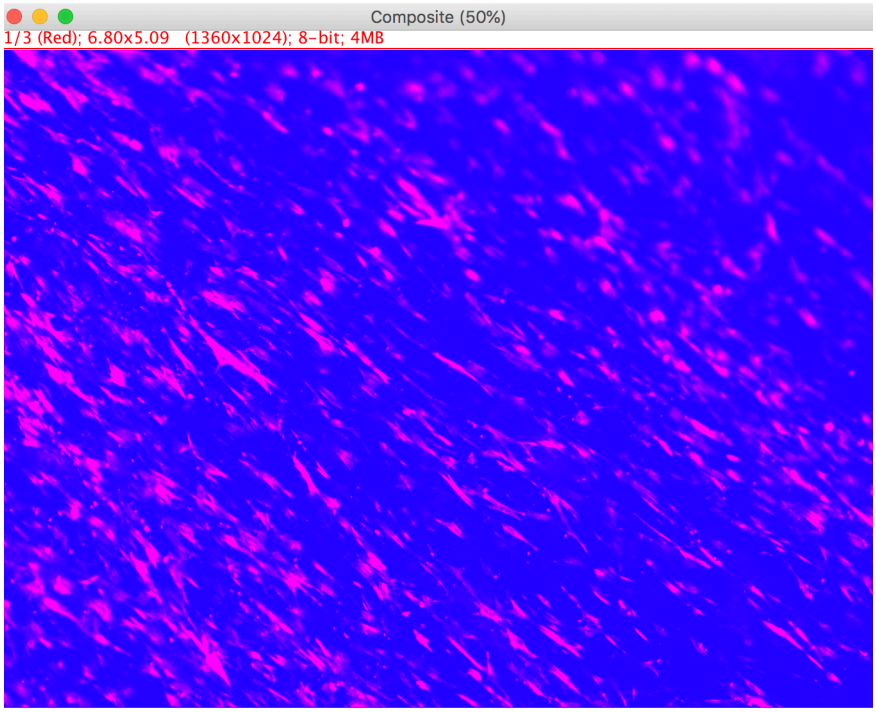


FIGURE A.25. Composite of the two colour channels merged.

6. An important part of post-microscope processing is to adjust and to put a scale bar. When images are calibrated (they always are when taking pictures from a microscope), it is as easy as selecting Analyze > Tools > Scale Bar…. However, we won't always have the scale put in µm, just like our case, so we took a microscope photo of a physical scale of 1 mm.

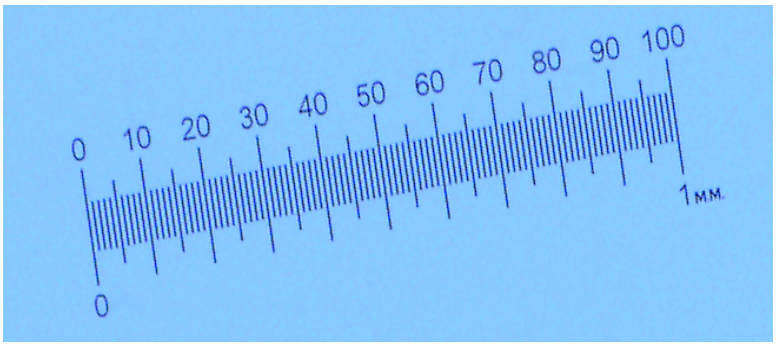


FIGURE A.26. Physical scale of 1 mm, amplified.

7. To adjust the scale in µm, we opened the *FIGURE A.26.* with *ImageJ*, and we drew a line from 0 to 1 mm. We later clicked on Analyze > Set Scale… See *FIGURE A.27.*

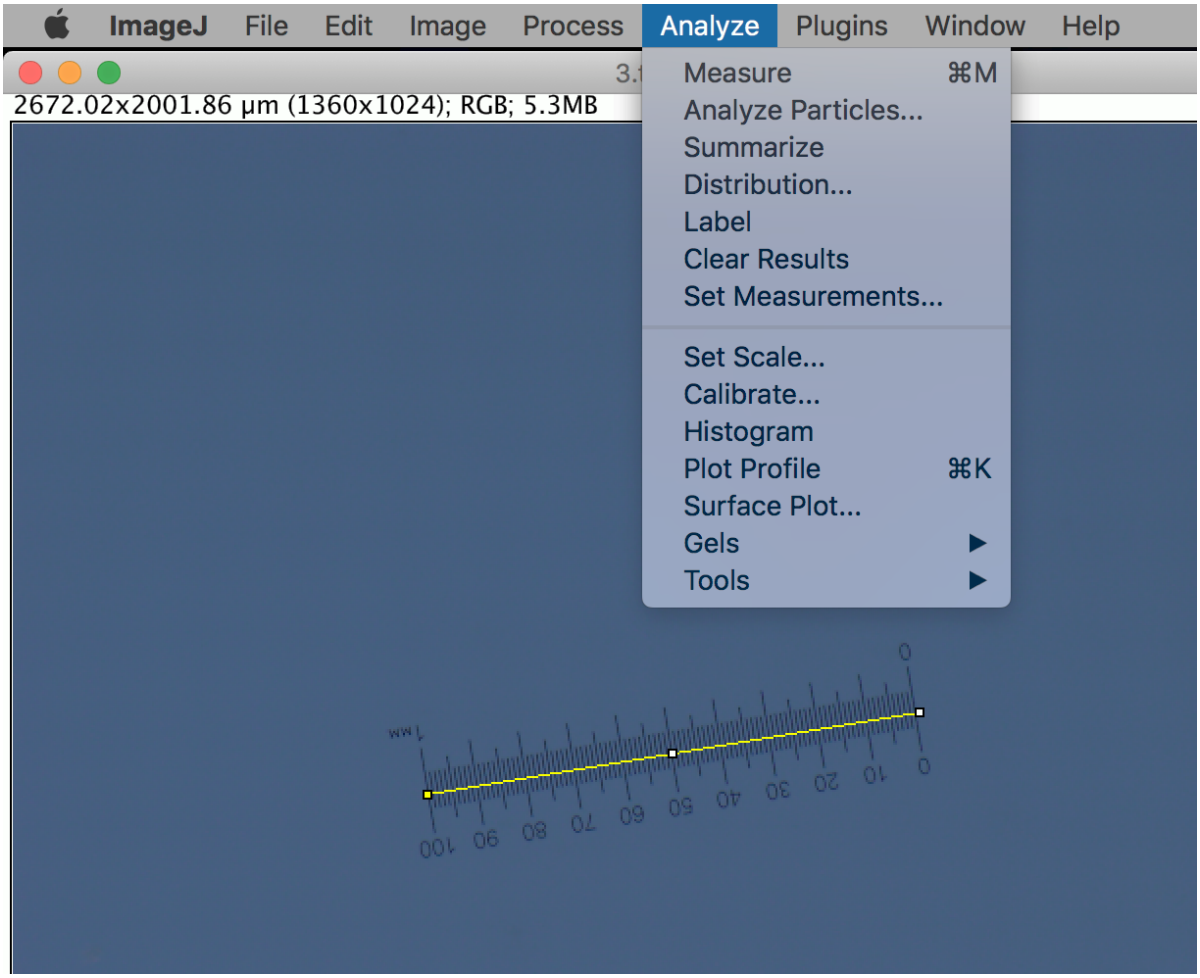


FIGURE A.27. The line drawn in the physical scale.

8. Finally, the unit length was changed from pixels to µm, and the known distance was set to 1000, as 1000 µm is 1 mm. By applying the Global condition, the scale applies to all the images opened after this one. See FIGURE A.28.

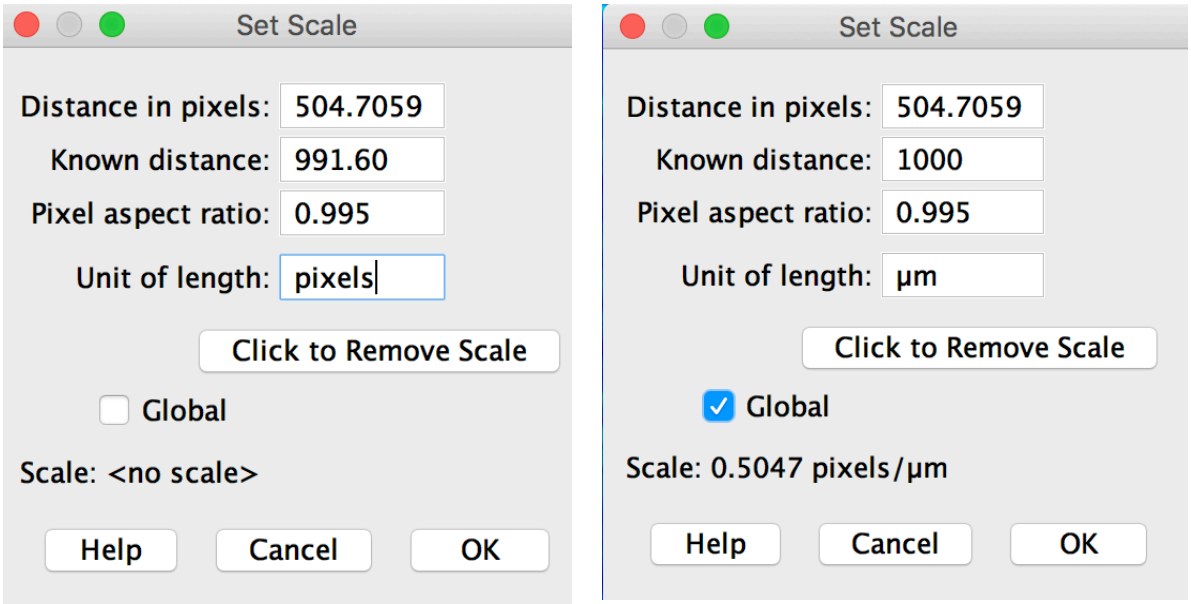


FIGURE A.28. The settings before and after being adjusted.

9. So going back to the step **6.**, we should now select Analyze > Tools > Scale Bar…. See FIGURE A.29.

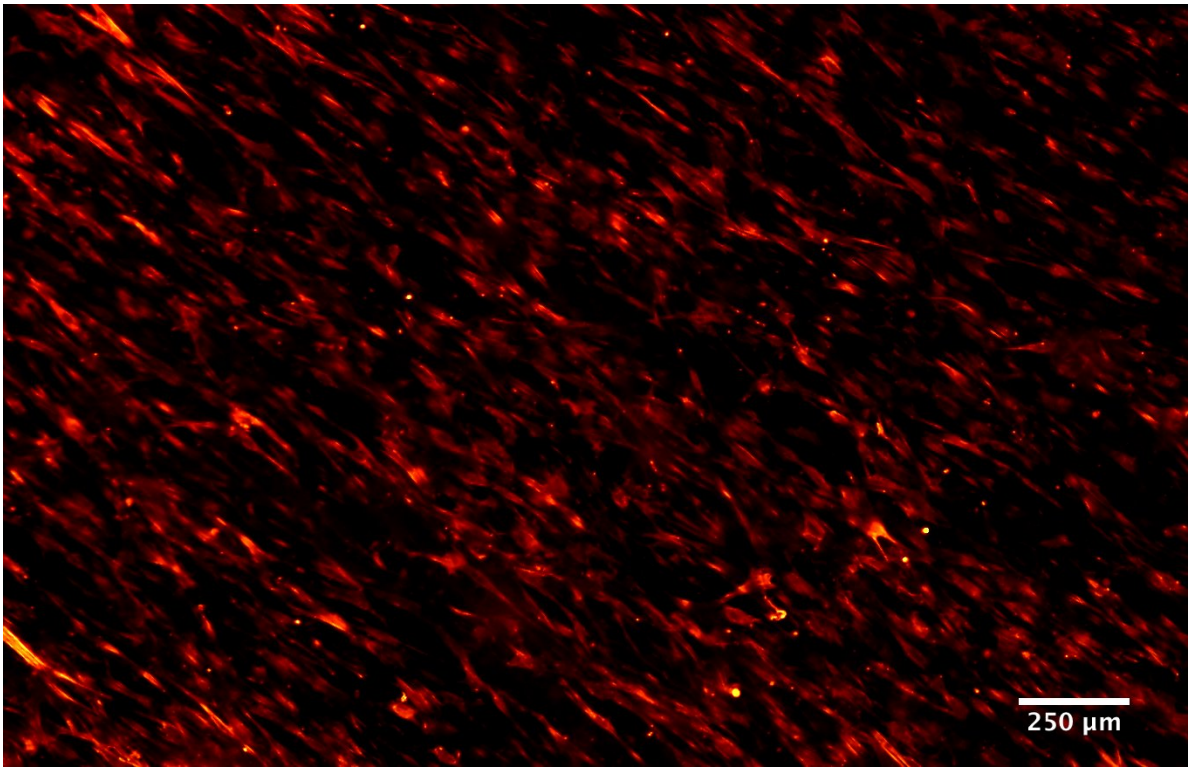


FIGURE A.29. This is the final result, the same from FIGURE A.21. The scale is pointed with the arrow.

EXTRA STUDY: STUDY 6: ETHICAL CONSIDERATIONS ON BIOMATERIAL RESEARCH REGARDING TRANSLATIONAL RESEARCH.

- Θ INTRODUCTION TO BIOETHICS.
- Θ TRANSLATIONAL RESEARCH AND CLINICAL TRIALS.
- Θ LIMITATIONS OF BIOMATERIAL IMPLANTS.
- Θ POPULATION PERSPECTIVE.
- Θ COMMON POINT OF DEPARTURE.

The art and science of asking questions is the source of all knowledge.

Thomas Berger, American novelist.

Θ Introduction to bioethics.

First of all, ethics. Ethics are defined as: “Norms of conduct to distinguish between acceptable and non-acceptable behavior”.

This is important in science because we want to search for a perspective or procedure that does the littlest harm to nature or ourselves, for instance.

Bioethics is the branch of ethics dedicated to the biomedical field, or biology and medicine. This study is done as a consequence of our fieldwork. To carry out those five studies, animals were sacrificed, specifically mice and chicken embryos. Although they were at all times treated humanely, avoiding harm to the animals, some ethical questions arose from these facts and also from the points of view given by Mr. Jeffrey Karp.

Doing some research, I discovered loads of institutions that work exclusively to ensure proper ethics when conducting research, examples of such are the Food and Drug Administration (FDA) or the Singapore Statement on Research Integrity (SSRI).

Between the **objectives** of this study there is:

- To learn the thinking of the population about ethics and biomaterials research.
- To learn how bioethicists work to ensure the greater good.
- To define the current regulations of our nearest organizations.
- Reach a common bioethical point of departure from the perspectives of the population.

Bioethics, though, compiles a lot of topics, the research on animals, how nature should be treated, how medical devices need to be, what types of stem cells can be used, what type of research is considered significative…

However, this study is going to focus on the ethics about biomaterial implants: how do they need to be considering the health of the patient, what risks should the patient be willing to take and when are implants ready to surpass clinical trials and actually be accessible for the market and the society.

This study will be done considering clinical trials and especially translational research, described hereunder.

Worth to mention, this study will consist on a review on the ethical limits of biomaterial implants, including questionnaires carried out to the population searching for their different bioethical opinions with controversial bioethics’ questions.

Θ Translational research and clinical trials.

What are clinical trials and translational research and why is bioethics related to them?

First of all, clinical trials are, according to its standard definition, scientific studies conducted to find better ways to treat or analyze disease, or even to show new medical approaches or treatments.

Every drug development process goes through a large research process, ending that process with a highly backed evidence in *in vivo* tests of, in our case, in animals, which are called pre-clinical trials.

Then, clinical trials begin in human tests, which are classified in four phases:

- Θ Phase I trials test an experimental biomaterial device (in our case) in a small group of people to evaluate the safety of it and how should this device be administered.
- Θ Phase II trials involve a larger number of people than in Phase I and it tests the design and the efficacy of the material. This phase usually lasts several years.
- Θ Phase III trials are large studies with a lot of patients. It compares the experimental treatment with a standard treatment and both safety and efficacy are checked again. The regulatory health authority, in the US and globally the most important is the Food and Drug Administration (FDA) will consider whether to approve or not the new treatment or device.
- Θ Phase IV trials are basically the post-approval part of the clinical trials. The results of the new treatment or device are monitored in hugely diverse populations.

The purpose of this order and this structure of the clinical trials are meant to ensure a relevant safety and efficacy test and proper measurements of the results obtained.

To sum up, this clinical process is the one that will allow the bridge between the laboratory hypotheses to useful treatments for patients. A lot of biomaterial devices are undergoing clinical trials, but they must be so strict to ensure the health of the future patients, as catastrophic or fatal reactions in the patient could arise from a device failure.

Translational research, a concept close to clinical trials, is that research that allows a link between academical knowledge and society. It basically consists of applying that knowledge learnt in the lab to design creative solutions meant to be applied in hospitals, for instance. It is a way of getting new discoveries and, combined with clinical trials, and bioethics, they establish the promising future that awaits for biomaterial devices.

The key of considering bioethics and clinical trials and translational research as a whole is to even philosophize about the best way of designing biomaterial implants, and, above all, establish their ethical limits to ensure patients' health at all times.

Θ Limitations of biomaterial implants.

Biomaterial implants need certain characteristics detailed along this project to be functional and compatible with the body. However, how do these biomaterials need to be to ensure the patient's health?

This is the grand question that remains unanswered, as the medical industry is guided by evidence and percentages and the limits of implants to accomplish a 100% secure implant are not clear. However, we can philosophize on the ethical limits we could establish.

However, that part will come up later, as it these ethical limits are not so easy to formulate. What we can try to establish, instead, is a common point of departure. That will be done formulating questionnaires carried out evaluating ethical controversies for the population to express their position on implants' ethics.

Many different opinions arise and within each country regulations vary.

The controversies evaluated on implants are, in the questions asked in the questionnaire in English and the local languages, like this:

Screenshot taken of the questionnaire carried out via Google Forms.

Bioengineering ethics: Biomaterial implants. / Ètica de la bioenginyeria: Implants biomaterials. / Ética de la bioingeniería: Implantes biomateriales.

*Compulsory

1. E-mail adress *

The e-mail address was compulsory to state as, otherwise, the same person could respond to the questionnaire multiple times. Despite the fact that the addresses were stated, the results have remained anonymous at all times.

2. **Bioethics is... / La bioètica és... / La bioética es...** *

Just mark an answer.

Searching a common point of departure. Buscant un punt de partida comú. Buscando un punto de partida común.

- The questionnaire will be focused on bioethical controversies.
- L'enquesta estarà enfocada en les controvèrsies bioètiques.
- El cuestionario estará enfocado en las controversias bioéticas.

3. **Is scientific knowledge above animal lives? /Està el coneixement científic per sobre de les vides animals? /¿Está el conocimiento científico por encima de las vidas animales?***

Just mark an answer.

4. **Should animal trials only be tested in investigations that lead to a solution? / Haurien els tests amb animals de ser portats a terme només en investigacions que porten a una solució? / ¿Deberían los tests con animales ser llevados a cabo solo en investigaciones que llevan a una solución? ***

Just mark an answer.

5. **Should animal experimentation be banned, skipping pre-clinical trials? / Hauria de ser prohibida l'experimentació amb animals, ometent les proves pre-clíniques? / ¿Debería ser prohibida la experimentación con animales, omitiendo las pruebas pre-clínicas? ***

Just mark an answer.

6. **Is an animal sacrifice worth to science if it is demonstrated that the animal does not feel pain? / Li val la pena a la ciència un sacrifici animal si es demostra que l'animal no pateix dolor? / ¿Le vale la pena a la ciencia un**

sacrificio animal si se demuestra que el animal no sufre dolor? *

Just mark an answer.

7. **Can the animal experimentation be substituted? / Pot l'experimentació animal ser substituïda? / ¿Puede la experimentación animal ser sustituida? ***

Just mark an answer.

8. **Should biomaterial implants, apart from heal or restore, ENHANCE a human ability? / Haurien els implants biomaterials, apart de curar o restaurar, de MILLORAR una habilitat humana? / ¿Deberían los implantes biomateriales, aparte de curar o restaurar, MEJORAR una habilidad humana? ***

Just mark an answer.

9. **If a biomaterial device shows up for being a really helpful and a revolutionary solution to a disease, it would not be used until tested and improved to a... / Si un dispositiu biomaterial destaca per mostrar-se molt útil i una solució revolucionària per una malaltia, aquest no hauria de ser utilitzat fins ser provat i millorat fins a un... / Si un dispositivo biomaterial destaca por mostrarse muy útil y una solución revolucionaria para una enfermedad, éste no debería ser utilizado hasta un... ***

Just mark an answer.

10. **Clinical trials should be transparent at all times, even if the first idea ends up being a failure. / Les proves clíniques han de ser sempre transparents, inclús si la primera idea acaba sent un fracàs. / Las pruebas clínicas deben ser siempre trasparentes, incluso si la primera idea acaba siendo un fracaso total. ***

Just mark an answer.

11. **A patient should decide whether or not to be implanted biomaterials, in whatever health conditions this subject may be. / Un pacient hauria de decidir si ser implantat o no un biomaterial, estigui en la condició que pugui estar aquest. / Un paciente debería decidir si ser implantado o no un biomaterial, esté en las condiciones que esté éste. ***

Just mark an answer.

12. **Biomaterial devices must remain as experimental treatments until demonstrated established, commercial, effective options. / Els dispositius biomaterials han de romandre tractaments experimentals fins ser demostrats com a opcions establertes, efectives i comercials. / Los dispositivos biomateriales deben permanecer como tratamientos experimentales hasta ser demostrados como opciones establecidas, efectivas y comerciales. ***

Just mark an answer.

13. **The environmental impact should always be minimized in the whole "life" of the biomaterial, in both manufacturing and implanting processes. / L'impacte ambiental hauria de ser sempre minimitzat en tota la "vida" del biomaterial, tant en manufactura com en l'implantació. / El impacto ambiental debería ser siempre minimizado en toda la "vida" del biomaterial, tanto en manufactura como en su implantación. ***

Just mark an answer.

14. **If the life of a patient depends of it, a biomaterial device treatment could be an emergency resource that could be taken profit of. / Si la vida d'un pacient en depèn, un tractament que inclogui un dispositiu biomaterial hauria de ser un recurs d'emergència que podria ser aprofitat. / Si la vida de un paciente depende de ello, un tratamiento que incluya un dispositivo biomaterial debería ser un recurso de emergencia que podría ser aprovechado.**

*

Just mark an answer.

15. **If the patient is considered not able to decide whether or not to be treated with a biomaterial implant, the closest family members should have the final say, not doctors or nurses. / Si un pacient és considerat incapaç de decidir si ser tractat o no amb un implant biomaterial, la decisió hauria de recaure als membres més propers de la família, i no als doctors o els infermers. / Si un paciente es considerado incapaz de decidir si será tratado o no con un implante biomaterial, la decisión debería recaer a los miembros más cercanos de la família, y no a los doctores o los enfermeros. ***

Just mark an answer.

The questionnaire's questions can be consulted in this link, clicking at **"See previous answers"**.

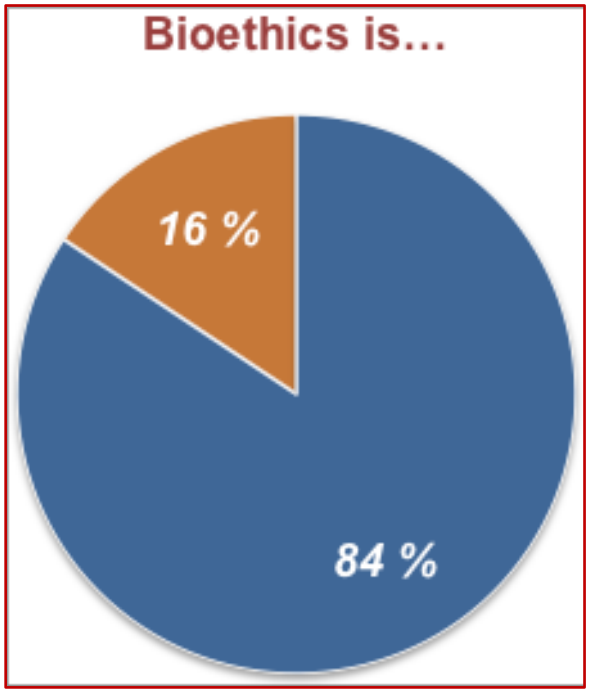
https://docs.google.com/forms/d/1_Y5jg6Fs6GZTNZ5PUr4gsOyKqqpdErqzddvkCVgCIT8/

Google Forms

Θ Population perspective.

Having carried out the questionnaire and being completed by 83 people, these are the results:

As a kind of introduction, the first question was to *contextualize the topic*. Although the respondents never knew the correct answer (biology, medicine and environment together) they could figure out to what it was correlated the most along the questionnaire.

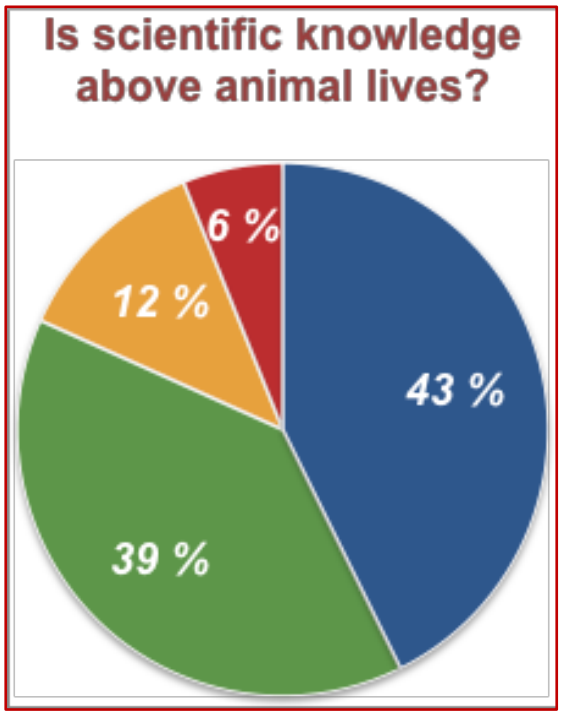


Bioethics is the branch of ethics that affects the area of biology and the environment.

Bioethics is the branch of ethics that affects the field of medicine.

Bioethics is the branch of ethics that affects biology, medicine and the environment together.

Moving on to the questionnaire itself, the first question was whether knowledge was above animal lives, the results here begin to be controversial and diverse.

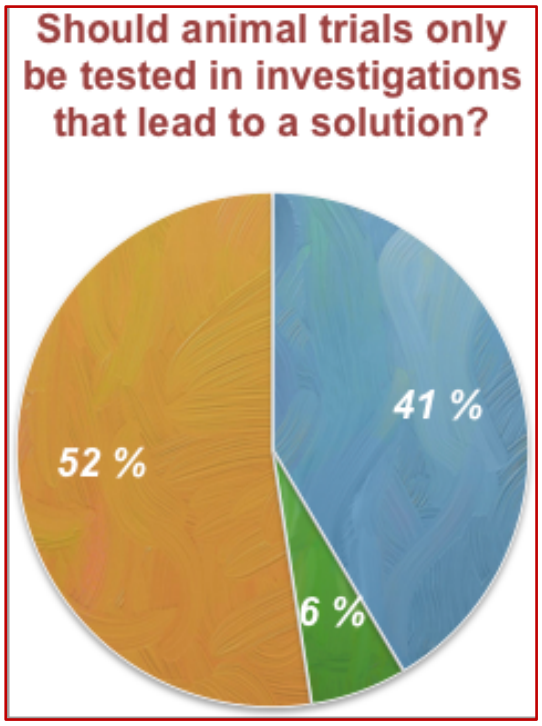


At all times.

Never.

It depends on the situation, but, overall: Knowledge > Animals lives.

It depends on the situation, but, overall: Knowledge < Animals lives.



Overall, we can already see how the thinking of the population is diverse, with an 49% of the survey respondents preferring knowledge over animal lives and a 51% preferring animal lives above animal sacrifices in science.

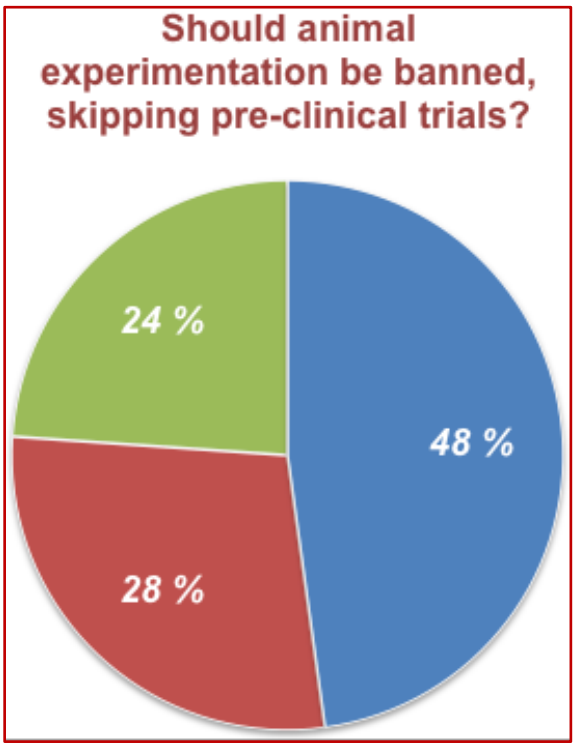
Yes, if it does not lead to a solution it is not worth to investigate with animals.

No, animal trials should be carried out in the same way.

Knowledge in basic science is always useful, although we cannot foresee a soluti on.

The third question was whether animal trials should only be carried out if the investigations were leading to a solution or not.

This question evaluated the importance that the population gave to preclinical trials (with animals) and basic science. Again, half of the respondents think radically different than the other half. However, yet subtle, more respondents consider basic science useful although not providing a rapid solution.



Never, for a treatment to go from a laboratory to a hospital is unthinkable.

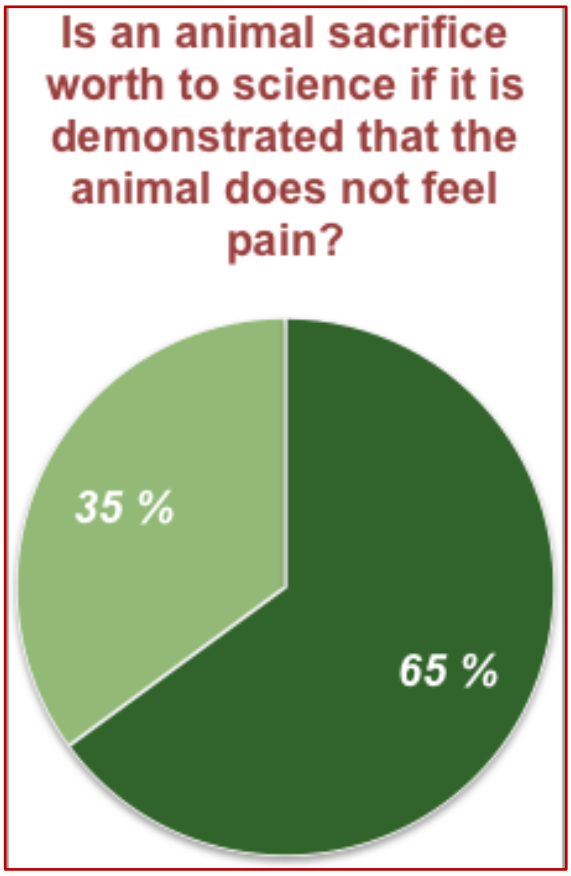
It could be banned in a future, but not nowadays.

Yes, animals should not be sacrificed.

The results of this question was a big surprise. In it, animal experimentation was doubted and its ban was questioned. Shockingly, a 28% of the respondents think that animals should never be sacrificed.

This could seem unreasonable, as if pre-clinical trials are not carried out, the ones that would test the drug or the device in the first place would be human people, causing probably several deaths. However, this probably was not their argument. What they would probably mean is to always minimize animal sacrifices.

However, this does not mean their position is right or wrong, since there are not correct answers to ethical questions. Nevertheless, it is interesting to think about our interests and priorities, as well as to contemplate different alternatives that could be arisen.



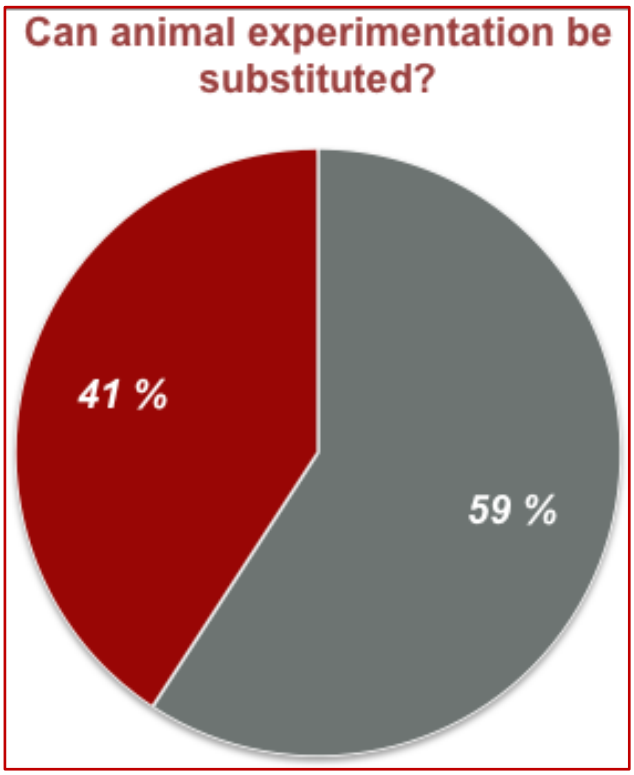
Although it could have been easy to predict the results of this question, the answers were not as expected.

A 35% of the respondents do not want animal sacrifices, even if the animal does not suffer.

Between the *Other* answers, highly appreciated, there are presented diverse arguments:

- The science advance must prevail over the suffer of animals.
- It is only worth if it has a real benefit for science.
- It is difficult to predict whether the animal will suffer or not, but it is essential before testing with people.
- Sincerely I believe that the animal suffering is really relative. It cannot be compared the suffering of an animal with the suffering of a human being.
- It depends on the importance of the investigation, however, although the animal does not suffer, it should not be done as you are preventing an animal from living and that represents an abuse to a living being.

It is interesting as both sides are really reasonable and it is difficult once having the two arguments to create a solid opinion on the topic.

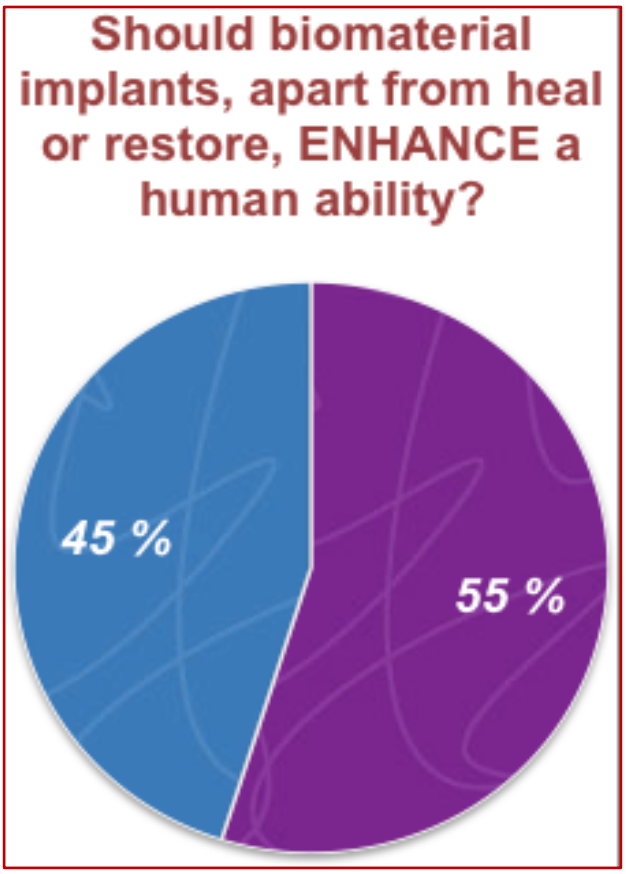


The sixth question went further. Not talking about banning experiments with animals or questioning the suffering of animals, this question asked whether animal experimentation could or not be substituted.

Here there was implicitly a comparation of in vitro and in vivo assays, and whether the former one could equal the first one on the amount of information the studies provide.

In the *Other* answers, the survey respondents expressed:

- Their ignorance on the topic.
- Their desire for the experiments to not be carried out with animals but with other methods.
- They also mention that it may be substituted in the future but not nowadays.



Yes.

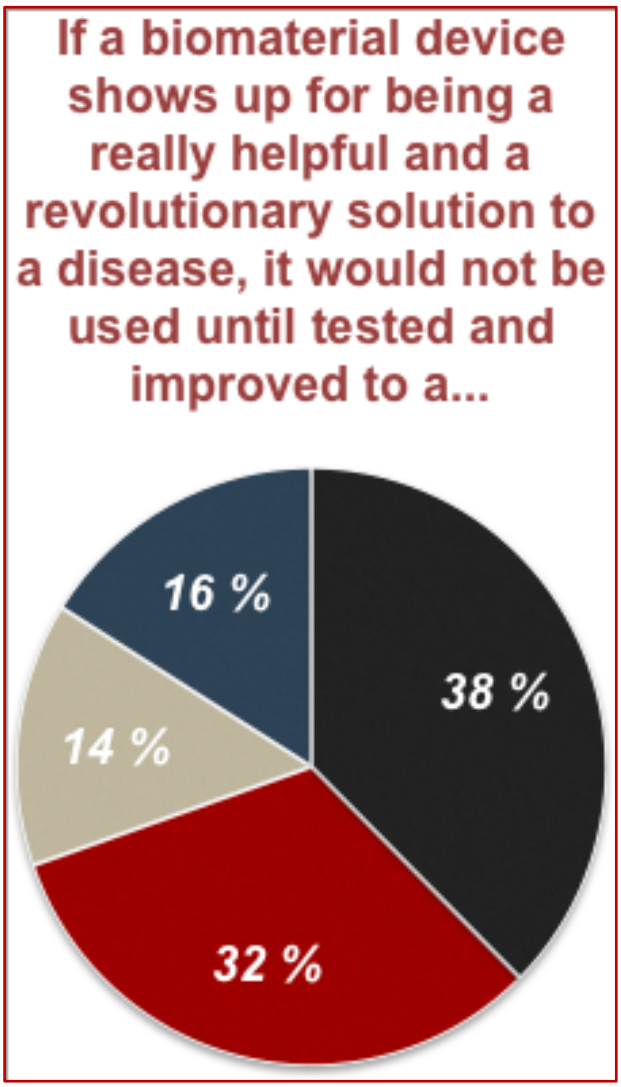
No.

This question refers to another topic inside bioethics. Apart from animal experimentation, the potential improvement of a human capacity or ability is also controversial.

Having to deal with the scarcity of resources and overpopulation, would it be good or bad for humanity if we dealt with “superhumans”, in a way?

On the other hand, we would have the capacity, for instance, to see or hear better improving our quality of life.

This topic was also controversial on the results of the survey, with a 55% agreeing on the enhancement of human capacities and a 45% disagreeing on it.



10% of success, being rapidly implemented to the market.

20% of success, after waiting for several years.

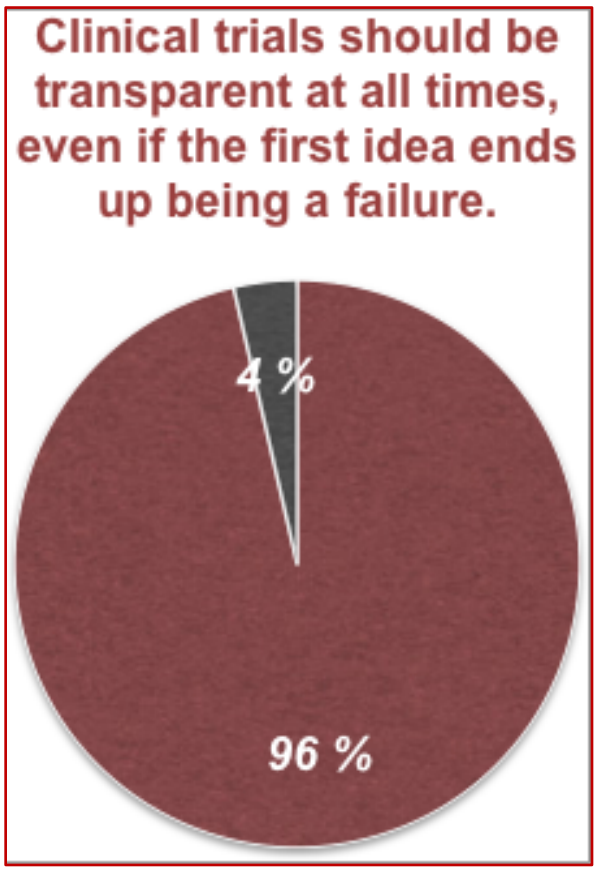
40% of success, after waiting 9-10 years of several failed device prototypes.

Minimum a 60% of success, although it would take a lot of years, the health of the patient must be the priority, even if these waiting period of time has taken lives.

This question represents one of the most important positions of the respondents. Here we were searching for a prioritization: safety over the implementation or to choose to risk to implement the device to the market.

A 70% of the survey respondents prioritized the safety, stating that either a 40% of success or a 60% of success was the minimum to be implemented to the market.

In the *Other* answers the respondents expressed, once again, their ignorance on the topic, and that it depended on the situation we were referring to.



Yes.

No.

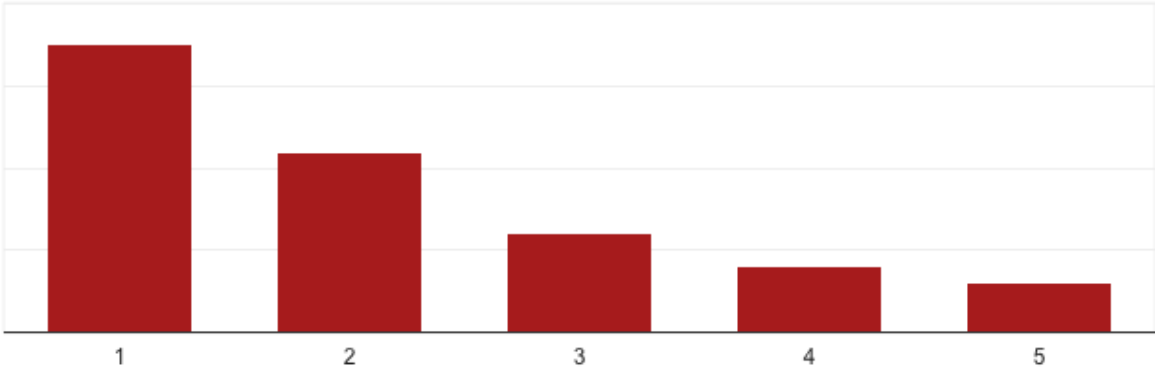
Quite obviously, almost the totality of the respondents agreed on the transparency of the clinical trials.

In the *Other* answers one respondent mentioned that not everybody was prepared to comprehend the truth and listen to it objectively.

A patient should decide whether or not to be implanted biomaterials, in whatever h ealth conditions this subject may be.

Strongly Agree	**(1):**	**42%**
Agree	**(2):**	**27%**
Neutral	**(3):**	**14%**
Disagree	**(4):**	**10%**
Strongly Disagree	**(5):**	**7%**

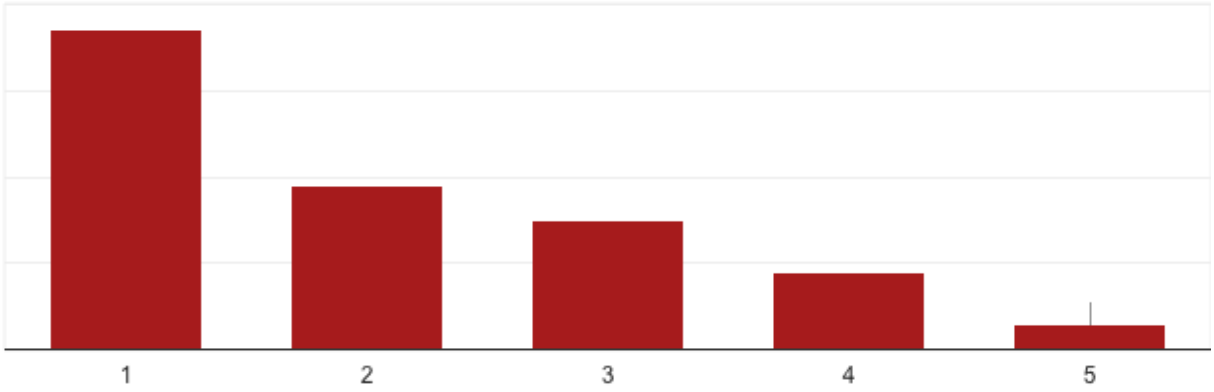
Varied answers emerged from the respondents, although the majority of them agreed on the responsibility the patient must have over himself. Related to the last question, one of the counterarguments that this sentence could have could be the insufficiently preparation to hear the truth objectively.

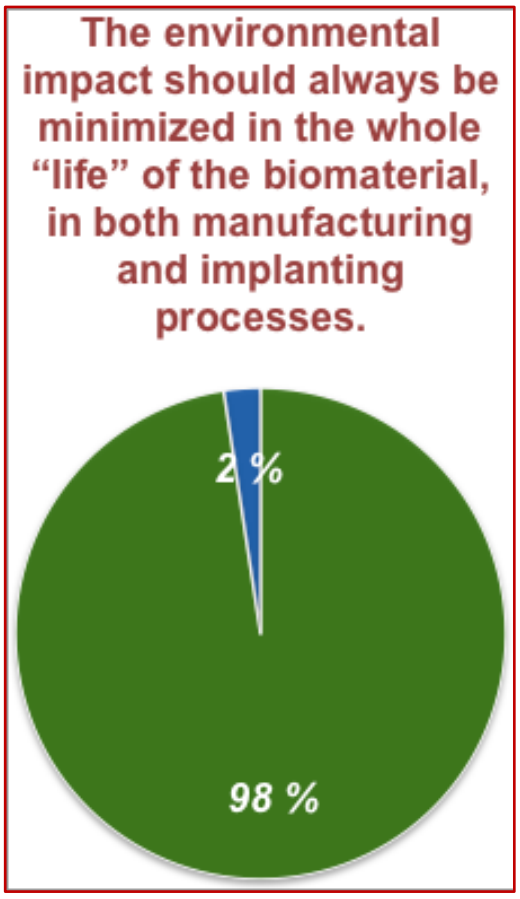


Biomaterial devices must remain as experimental treatments until demonstrated established, commercial, effective options.

Strongly Agree	(1):	44%
Agree	(2):	23%
Neutral	(3):	18%
Disagree	(4):	11%
Strongly Disagree	(5):	4%

Less diversity of opinions arose here. The majority of the respondents agree on having established, commercial, effective alternatives to the traditional methods in medicine. A 18% remain neutral on this topic. The pity of this question and the latter one is the impossibility to hear from *Other* answers.

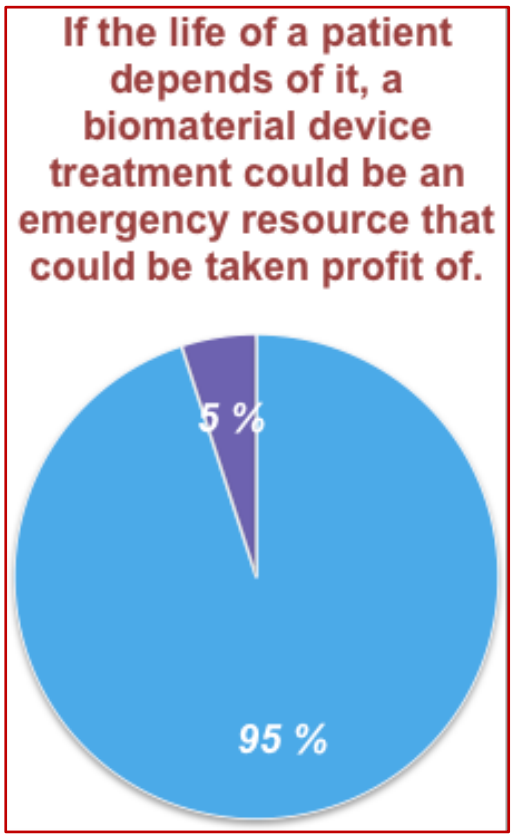




Yes.

No.

Another obvious sentence. A 98% of the respondents want the biomaterial to be eco-friendly, and although this sentence may seem too obvious, it is equally important to ensure less damage to our environment.

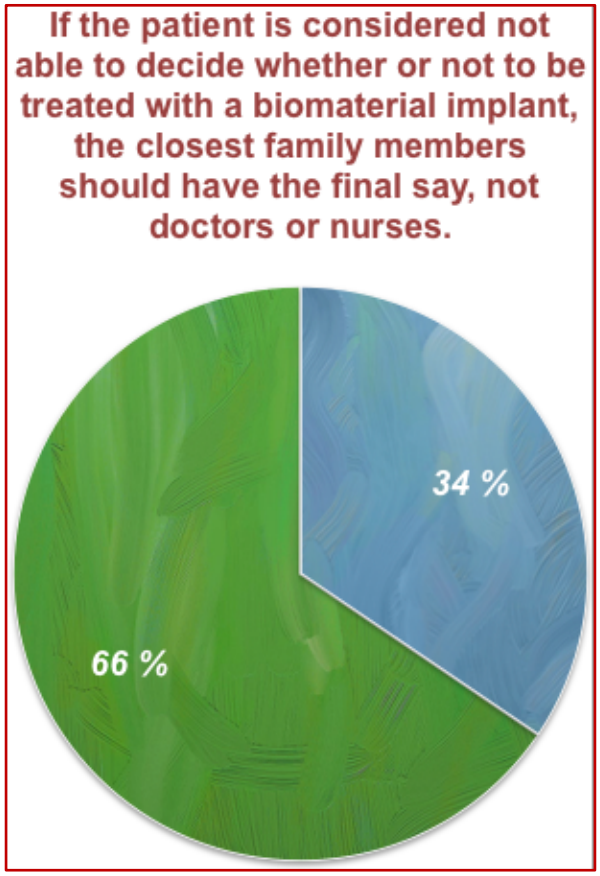


Yes.

No.

This question was a bit of a conclusion of the questionnaire. Again almost the totality of the respondents agree on having biomaterial device treatments as emergency resources.

Again that is important to hear, as this demonstrates the population wants more alternatives to improve their quality of life.



Decision taken by the family.

Decision taken by doctors/nurses.

Although the health system works in a way that doctors and nurses tell the truth to the family of the patient and they decide, this sentence questioned whether ones or the others should have the final say.

The majority agree on the system that is being carried out nowadays.

However, we need to appreciate the other 34%, as their main argument must be to be told the truth at all times.

Θ Common point of departure.

And to conclude the last study, a common point of departure of the ethical limits of biomaterial implants will be simulated.

However, as we have seen, there are questions where there are almost 50-50 over two answers. Given the difficulty to generalize between a wide range of opinions, this ethical point of departure will be equally general. However, this may not represent the actual reality of opinions, as the questionnaire was carried out to a wide range of ages but to 83 people and not a whole country, for instance.

1. The concept of bioethics should be learnt in schools, even if it was just a few notions it is important to think about our surroundings (environment, laboratories and hospitals).
2. Knowledge and animal lives are equally important and our interest for each of them should be the same. Therefore, we must care for both of them at all times.
3. Preclinical trials carried out with animals should be taken into account seriously and these trials should have the amount of animals used strictly regulated.
4. When animal trials had a similar alternative in terms of knowledge gained in the study, these trials should be tested with this new alternative.
5. Although the animal did not suffer, it must be taken into account that we are not playing with inert materials but with living beings and they must be manipulated humanely.
6. Experiments with animals must be personalized, and the conditions of the experiment should depend on the study that is being carried out.
7. The enhancement of a human ability is a enormously important step in medicine and health. This decision should not fall upon a single group of investigation given their controversy as even governments may have a say in this topic.
8. The safety of the patient is more important than the release of a product.
9. Clinical trials should be transparent at all times no matter the success of them.
10. The patient should be able to hear the whole truth of the experimental or already commercial treatment.
11. If biomaterial implant treatments become established, commercial and effective options, they can represent an alternatives to traditional treatments.
12. A biomaterial device development should remain eco-friendly from preclinical trials to their implementation.
13. A biomaterial device could be a last-resource option for patients.
14. The patient, the family and the medical staff should maintain a truthful and sincere relationship at all times.