ALMA MATER STUDIORUM - UNIVERSITÀ DI BOLOGNA
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# MONITORING THE EXTRACELLULAR MATRIX MINERALIZATION PROCESSES IN BIOLOGICAL SCAFFOLDS USING BIOREACTOR X-RAY μCT TECHNOLOGY AND 3D MODELING METHODS

TESI IN

MECCANICA DEI TESSUTI BIOLOGICI

Relatore

*Prof. Luca Cristofolini*

Presentata da

*Joseph Lovecchio*

Correlatore

*Prof. Paolo Gargiulo*
*Dott. Ólafur E. Sigurjónsson*

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*Ai miei Genitori…*

*"La geniale invenzione nasce sempre dall'uomo isolato,*
*ma solo l'opera tenace di pazienti ricercatori, con mezzi larghi e adatti,*
*può efficacemente svilupparla e utilizzarla ,,*
***B.M.***

# Abstract

To improve *in vitro* cell cultures, bioreactor systems have been widely used, e.g. in bone tissue engineering. Spinner flasks, rotating wall bioreactors, and flow perfusion systems have all been used, and each system has advantages and disadvantages. This thesis describes the development of a simple perfusion bioreactor system and the results from the assessment methodology employed which is based on x-ray $\mu$CT analysis and 3D Modeling Techniques.

A simple bioreactor with flow generator propeller was designed and built with the aim of improving differentiation of human embryonic derived mesenchymal stem cells (hES-MP) seeded on porous titanium scaffolds in order to improve mineralized matrix deposition. The bioreactor allows three types of flow: forward (clockwise), backward (counterclockwise) and pulse mode (back and forth) controlled by a micro-controller and a graphical interface.

We designed a simple model to calculate the pressure generated by the flow of the bioreactor on the scaffold ($3 \cdot 10^{-2}$ Pa).

We compared 3 scaffolds under static culture condition to 3 scaffolds within the bioreactor. The scaffolds were incubated for 21 days, fixed in paraformaldehyde (4% w/v) and subjected to acquisition by x-ray µCT.

Images obtained were processed using 3D imaging software; a “virtual" sectioning of the scaffolds was performed in order to obtain samples extracted from the surface and from the inside of the scaffolds in order to obtain the gray value gradient distribution.

This distribution is used to distinguish the various components present in the images, in this case scaffolds from the hypothetical cellular matrix.

The results show that scaffolds maintained in the bioreactor have higher density of gray values gradient distribution on the scaffold surface which suggests improved mineralized matrix deposition.

The studies coming from this bioreactor will be used to design a new version that will make it possible to analyze more than 20 scaffolds at the same time making it possible to further analyze the quality of the differentiation using molecular and histochemical methodology.

# Abstract

Al fine di migliorare le tecniche di coltura cellulare *in vitro*, sistemi a bioreattore sono sempre maggiormente utilizzati, e.g. ingegnerizzazione del tessuto osseo. Spinner Flasks, bioreattori rotanti e sistemi a perfusione di flusso sono oggi utilizzati e ogni sistema ha vantaggi e svantaggi.

Questo lavoro descrive lo sviluppo di un semplice bioreattore a perfusione ed i risultati della metodologia di valutazione impiegata, basata su analisi μCT a raggi-X e tecniche di modellizzazione 3D.

Un semplice bioreattore con generatore di flusso ad elica è stato progettato e costruito con l'obiettivo di migliorare la differenziazione di cellule staminali mesenchimali, provenienti da embrioni umani (HES-MP); le cellule sono state seminate su scaffold porosi di titanio che garantiscono una migliore adesione della matrice mineralizzata. Attraverso un microcontrollore e un'interfaccia grafica, il bioreattore genera tre tipi di flusso: in avanti (senso orario), indietro (senso antiorario) e una modalità a impulsi (avanti e indietro).

Un semplice modello è stato realizzato per stimare la pressione generata dal flusso negli scaffolds ($3 \cdot 10^{-2}$ Pa).

Sono stati comparati tre scaffolds in coltura statica e tre all'interno del bioreattore. Questi sono stati incubati per 21 giorni, fissati in paraformaldehyde (4% w/v) e sono stati soggetti ad acquisizione attraverso μCT a raggi-X.

Le immagini ottenute sono state poi elaborate mediante un software di imaging 3D; è stato effettuato un sezionamento "virtuale" degli scaffolds, al fine di ottenere la distribuzione del gradiente dei valori di grigio di campioni estratti dalla superficie e dall'interno di essi.

Tale distribuzione serve per distinguere le varie componenti presenti nelle immagini; in questo caso gli scaffolds dall'ipotetica matrice cellulare.

I risultati mostrano che sia sulla superficie che internamente agli scaffolds, mantenuti nel bioreattore, è presente una maggiore densità dei gradienti dei valori di grigio ciò suggerisce un migliore deposito della matrice mineralizzata.

Gli insegnamenti provenienti dalla realizzazione di questo bioreattore saranno utilizzati per progettare una nuova versione che renderà possibile l'analisi di più di 20 scaffolds contemporaneamente, permettendo un'ulteriore analisi della qualità della differenziazione usando metodologie molecolari ed istochimiche.

# 1. Introduction

Tissue Engineering (TE) is a discipline integrating biology with engineering to create tissues or cellular products outside the body (*ex vivo*) or to make use of gained knowledge to better manage the repair of tissues within the body (*in vivo*). This discipline requires understanding of different biological fields, including cell and molecular biology, physiology and systems integration, stem cell proliferation and differentiation with lineage attributes, extracellular matrix chemistry and compounds, and endocrinology. It also requires knowledge of many engineering fields, including biochemical and mechanical engineering, polymer sciences, bioreactor design and application, mass transfer analysis of gas and liquid metabolites, and biomaterials.

The combination of these sciences has spawned the field of regenerative medicine which has, at present, two strategic clinical aims [1]:

- Cell therapies for the repair of damaged tissues, involving injection or engraftment of cells or cellular suspensions, sometimes in combination with scaffolding material

- Establishment of tissue ex vivo for use as grafts or extracorporeal organs to assist or supplement ailing in vivo organs

Tissue Engineering has been defined first as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” [2]. Another definition is “understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use” [3].

Thus, Tissue Engineering has always been driven by the need to provide functional equivalents of native tissues that can be used for *in vivo* implantation in order to restore, maintain, or enhance tissue and organ physiology. To engineer living tissues, cultured cells are coaxed to grow on bioactive degradable scaffolds that provide the physical and chemical cues

to guide their differentiation and assembly into two- or three-dimensional tissues [4]. In contrast to classic biomaterial approach, it is based on the understanding of tissue formation and regeneration, and aims to induce new functional tissues, rather than just to implant new spare parts [5].

Tissue Engineering seeks to rebuild portions of tissue or whole organs through the manipulation of cells, using a wide variety of strategies [6]. These mainly involve the use of synthetic scaffolds, fabricated from biocompatible materials, to carry, support and guide cells towards tissue regeneration. The field of scaffold construction is still in its infancy, and many different approaches, both general and specific, are under investigation. Of particular interest is the ability to mimic the natural extracellular matrix surrounding cells in each tissue. Tissue specific functionality is largely maintained through the interaction of cells with matrix biological ligands, mechanical support and structural interaction. Matrix structure is particularly important for determining the flow of nutrients, signaling factors and waste products about cells, influencing their motility, protein expression and long-term differentiation [7]. In many cases it would be desirable to replace both small and large areas of diseased and damaged tissue with new healthy tissue, either fully generated outside the body, or at the site of damage through use of scaffolds engineered to support and encourage new tissue genesis. The scaffold must provide both the mechanical properties required by the regenerating tissue as well as the cues that cells require. This is a complex challenge. The organs of the body are highly sophisticated biological systems. Each maintains their own unique set of biological conditions, needed to sustain the function of tissue specific cells and enable self repair. These environments are difficult to mimic outside the body. One approach is to use the body as its own incubator, [8] drawing directly on the complexity of natural tissues to enable the generation of new tissue. For example an osteoconductive gel injected into the stem cell rich periostium layer in bone was used to create a space for the generation of new bone tissue, suitable for re-implantation [9]. Another approach is to use scaffolds that provide an appropriately sophisticated representation of the tissue environment, encouraging cells to generate new tissue (Figure 1) as the scaffold degrades

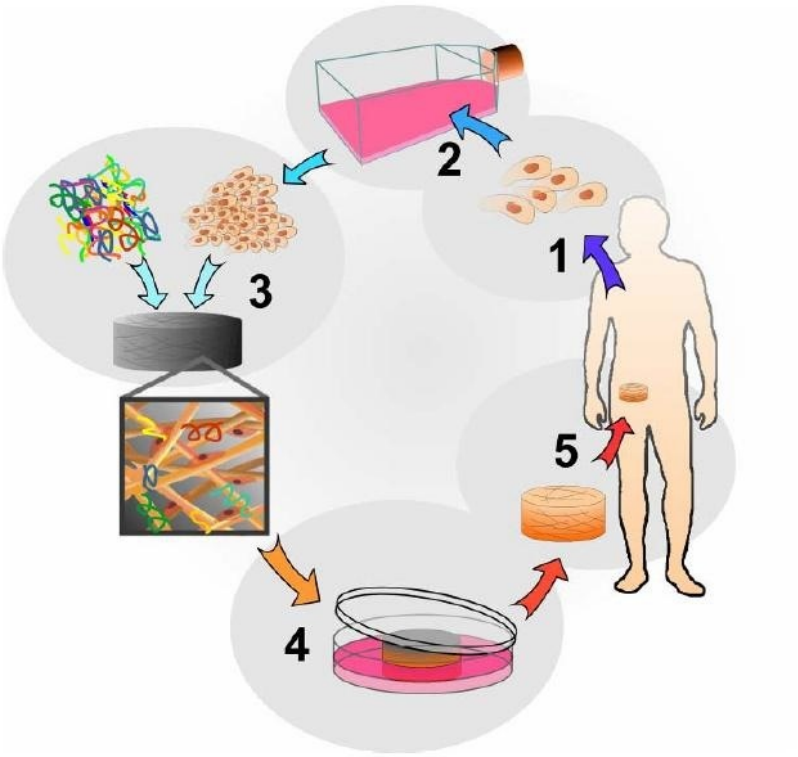



Figure 1 - The typical tissue engineering approach: 1. Remove cells; 2. Expand number in culture; 3. Seed onto an appropriate scaffold with suitable growth factors and cytokines; 4. Place into culture; 5. Re-implant engineered tissue repair damaged site [7]

There are three principal strategies for treating diseased or lost tissue in patients [10]:

- *In situ tissue regeneration*: new tissue formation is induced by specific scaffolds or external stimuli that are used to stimulate the body‘s own cells and promote local tissue repair

- *Implantation of freshly isolated or cultured cells*: individual cells or small cellular aggregates from the patient or a donor are injected directly into the damaged or lost region with a degradable scaffold

- *Implantation of bone like tissue assembled in vitro from cells and scaffold*: a complete three-dimensional tissue is grown in vitro using autologous or donor cells within a scaffold, which has to be implanted once it has reached “maturity”

Bone tissue engineering, as in most other Tissue Engineering areas, exploits living cells in a variety of ways to restore, maintain, or enhance tissue functions. The basic elements for tissue engineered bone are signaling molecules, cells and matrices for cell growth and differentiation. The combination of these three elements may need to be modified according to several variables such as patient age, gender, health, systemic conditions, habits, and anatomical shape of the implant. Furthermore that same strategy should also adapt itself to the area where the tissue engineering construct is needed, because different regions of the body will have different functional loads and vascularity. Up to now several strategies, from scaffolds alone to 3D matrices loaded with growth factors, have been proposed [5].

## 1.1 Stem Cell

Bioreactors utilize materials and cells that have already been proven effective for bone tissue engineering including polymer scaffolds that are biodegradable and mesenchymal stem cells (MSCs), a population of cells that exists in the bone marrow capable of differentiating into osteoblasts, chondrocytes, and adipocytes [11, 12]. This population represents only a small percentage of cells found in the bone marrow, thus expanding MSCs to clinically relevant numbers represents a significant hurdle to the implementation of a tissue engineering strategy utilizing these cells.

Stem cells are undifferentiated cells with a high proliferation capability, being able of self- renewal, multilineage differentiation and therefore the regeneration of tissues [5]. However, stem cells have varying degrees of differentiation potential. The most primitive derive from the fertilized oocyte (the zygote), more precisely from the very first descendants of the first divisions (two divisions). These cells are totipotent, because they are able to form the embryo and the trophoblast of the placenta. Some days later, these cells start to specialize, forming a hollow ball of cells, the blastocyst, and a cluster of cells called the Inner Cell Mass (ICM) from which the embryo derives. The ICM cells, also known as embryonic stem cells (ES), are

considered to be pluripotent (Figure 2). They can differentiate in almost all cells that arise from the three germ lines, but not the embryo because they are not able to give rise to the placenta and supporting tissues [13 ,14].

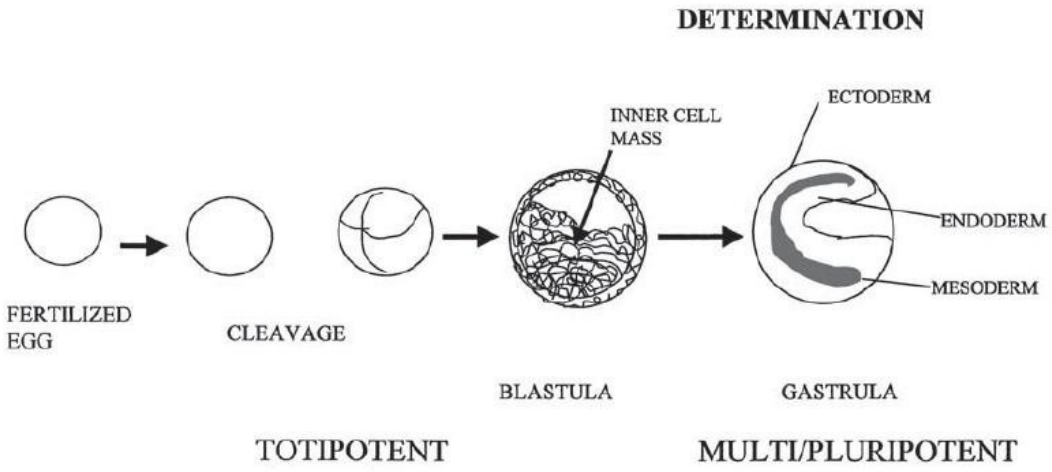



Figure 2 - Early development of embryo. Division of the fertilized egg results in the formation of a ball-like structure with a cavity on one end (the blastula). Until this stage, the cells divide by symmetric division, and all cells produced are totipotent. Invagination of one pole of the blastula leads to formation of the gastrula and establishment of the primitive germinal cell layers. During gastrulation and later formation of the fetus, the daughter cells lose potential as they gain specialized function. The process of the loss of potential and the gain of specialized function is known as determination [15].

Finally, we can find multipotent stem cells, also known as adult stem cells (ASC), in the fully differentiated tissues [13, 14, 16]. Theoretically, and opposing to ES cells, these would only be capable of producing a limited range of differentiated progeny, related to the embryonary origin of the tissue where they are located [13, 14]. However, these cells may have a higher degree of differentiation plasticity (differentiation into other cell lineages that are not related with the embryonary origin of the tissue were the ASCs are found) than expected. The biological mechanisms responsible by the broad developmental potential of stem cells are still not fully understood. Interaction with other cell types and the components of the extracellular matrix are believed to influence the survival and the development of stem cells to the committed lineages [5, 14, 16].

Tissue engineering strategies that combine scaffolds with cells capable of osteogenesis or bioactive proteins offer a potential alternative to bone grafting for treatment of large, clinically challenging bone defects. Several studies have demonstrated that delivery of osteoprogenitor cells or

osteoblasts significantly improves repair of long bone and cranial defects [17]. Tissue engineering has become a popular field of research for the treatment of many medical conditions. Strategies in tissue engineering for orthopedic disorders including those of bone, cartilage and muscle have been intensively studied. Stem cells are particularly attractive for TE applications due to their ability to self-renew, high proliferation capacity and ability to differentiate into many different cell types [18].

## 1.2 Scaffold

Scaffolds for tissue regeneration are defined as: “three dimensional open-cell porous structures synthesized from either natural or synthetic polymers which have the potential to support attachment, migration and multiplication of living cells”. These structural mimics of the extracellular matrix can be made of biodegradable synthetic polymers (e.g. polylactic and polyglycolic acid) or natural polymers (e.g. collagen, collagen– glycosaminoglycan copolymer). It has been demonstrated that scaffold pore size and shape, porosity, specific surface area, biodegradability and stiffness significantly influence cell functions. To control these features, various fabrication techniques have been developed for different biomaterials [19].

For bone tissue engineering, a wide choice of scaffold materials, including metals, natural and synthetic polymers, and ceramics has been proposed, each of them presenting different mechanical, chemical, and biological characteristics. Particular attention has been given to the manufacturing of porous bioceramics that mimic trabecular bone chemistry and structure. Calcium phosphate (CaP), hydroxyapatite (HA) ceramics and Porous Titanium (Ti) are considered among the most promising bone substitute for their bone-like chemical composition and mechanical properties [20].

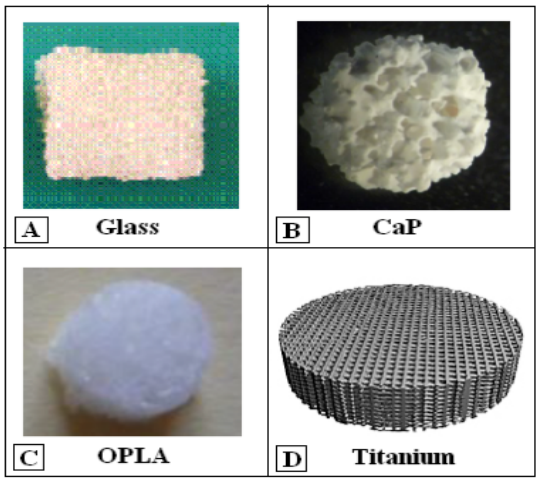



Figure 3. Represents 4 types of biocompatible scaffolds which are used in tissue engineering. A) Bioglass is bioactive glass; B) Calcium phosphate scaffold (CaP); C) Open-cell polylactic acid (OPLA); D) Titanium scaffold [21]

### 1.2.1 Temporary Matrices for Bone Growth

Scaffolds are temporary matrices for bone growth and provide a specific environment and architecture for tissue development [22]. Any tissue consists of a matrix and one, or usually, many cell types. The matrix is, in vivo, a 3D scaffold for cells, and provides them with a tissue specific environment and architecture. Furthermore, it serves as a reservoir of water, nutrients, cytokines, and growth factors. In this sense, and in order to restore function or regenerate tissues one needs a template, a scaffold that will act as a temporary matrix for cell proliferation and extracellular matrix deposition, with consequent bone in-growth until the new bony tissue is totally restored/regenerated. Moreover, would also act as a template for the vascularization of this neo-tissue and they could actively participate in the regenerative process through the release of growth/differentiation factors, present in its structure. It is then logical to say that an appropriate 3D scaffold is an essential component for a tissue engineering strategy [5].

However, it is important to realize that the latter must have a series of properties that make it suitable for TE purposes. Besides the choice of adequate materials the macro and micro-structural properties of the materials are of utmost importance. Such properties effect not only cell

survival, signaling, growth, propagation, and reorganization but also their gene expression and the preservation, or not, of their phenotype [5]. An optimal scaffold for tissue engineering application would mimic the properties of the extracellular matrix (ECM) of those tissues to be regenerated perfectly and completely [23].

### 1.2.2 Essential Properties

The following properties have been defined has being essential:

• *Biocompatibility*
Scaffolds should be well integrated in the host‘s tissue without eliciting an immune response [5].

• *Porosity*
Scaffolds must posses an open pore, fully interconnected geometry in a highly porous structure with large surface to area volume ratios that will allow cell in-growth and an accurate cell distribution throughout the porous structure, and will facilitate the neovascularization of the construct from the surrounding tissue. Furthermore, the scaffolds should also exhibit adequate microposity, in order to allow capillary in-growth. Porosity and interconnectivity are also important for an accurate diffusion of nutrients and gases and for the removal of metabolic waste resulting from the activity of the cells that had meanwhile grown into the scaffold. This is of particular importance regarding bone tissue engineering because, due to bone metabolic characteristics, high rates of mass transfer are expected to occur, even under in vitro culture conditions. However, the degree of porosity always influences other properties of the scaffolds such as its mechanical stability, so, its value, should always be balanced with the mechanical needs of the particular tissue that is going to be replaced [5, 24].

• *Pore Size*

Pore size is also a very important issue because, if the pores employed are too small, pore occlusion by the cells will happen. This will prevent cellular penetration, extracellular matrix production, and neovascularization of the inner areas of the scaffold. It is well accepted that for bone tissue engineering purposes, pore size should be within the 200–900 mm range. However, Holly et al. [25] reported a different concept. In the referred case the authors believe that bone reconstruction will only be achieved by having a 3D temporary matrix with a large macro-porous interconnected structure with pore size ranging from 1.2-2.0 mm. This later approach has evident advantages due to its high surface to volume ratios that will facilitate cell, tissue and blood vessels in-growth. However, this affects the mechanical properties avoiding its use in areas which are very demanding from the mechanical point of view [5].

• *Surface Properties*

Surface properties, both chemical and topographical, can control and affect cellular adhesion and proliferation. Chemical properties are related with the ability of cells to adhere to the material as well as with the protein interactions with the latter. Topographical properties are of particular interest when the topic is osteoconduction. As defined by Davies et al. [26] osteoconduction is the process by which osteogenic cells migrate to the surface of the scaffold trough a fibrin clot, which is established right after the material implantation. This migration of osteogenic cells trough the clot will cause retraction of the temporary fibrin matrix. Hence, it is of the utmost importance that the fibrin matrix is well secured to the scaffold or, otherwise, when osteogenic cells start to migrate the fibrin will detach from the scaffolds due to wound contraction. It has been previously shown [27, 28] that a more “rough“ surface will be able to imprison the fibrin matrix, better than a smooth surface, and hence facilitate the migration of osteogenic cells to the materials surface.

• *Osteoinductivity*

Osteoinduction is the process by which stem and osteoprogenitor cells are recruited to a bone healing site, and stimulated to undergo the osteogenic differentiation pathway. However, when the portion of bone to regenerate is large, natural osteoinduction combined with a biodegradable scaffold may be not enough. Because of this the scaffold should be osteoinductive by itself [5, 29].

### 1.2.3 Mechanical Properties and Biodegradability

In *vitro*, the scaffolds should have sufficient mechanical strength to withstand the hydrostatic pressures and to maintain the spaces required for cell in-growth and matrix production. In *vivo*, and because bone is always under continuous stress, the mechanical properties of the implanted construct should ideally match those of living bone, so that an early mobilization of the injured site can be made possible. Furthermore, the scaffolds degradation rate must be tuned appropriately with the growth rate of the neotissue, in such a way that by the time the injury site is totally regenerated the scaffold is totally degraded [5, 30].

### 1.2.4 Biomaterials used as Scaffolds

The selection of the most appropriate material to produce a scaffold to be used in bone tissue engineering applications is a very important step towards the construction of a tissue engineered product, since its properties will determine, to a great extent, the properties of the scaffold. Up to now several materials such as metals, ceramics and polymers from both natural or synthetic origins have been proposed [5].

However, metals and most of the ceramics are not biodegradable, which leaves the researcher‘s choice reduced to a small number of ceramics and to biodegradable polymers. Ceramics have been widely used in the biomedical engineering and bone substitution/regeneration field. They can

be from natural (e.g., coralline hydroxylapatite (HA)) origin or synthetic such as synthetic HA or b-tricalcium phosphate (b-TCP). Due to interesting properties, mainly the fact of being osteoconductive and osteoinductive, have been considered for bone tissue engineering applications [5]. Several works [5, 31–41] have shown that by using ceramics with or without bone marrow cells, good results regarding bone regeneration could be obtained. However, these materials have some major drawbacks.

To begin, ceramics materials are brittle and present a low mechanical stability, which prevents their use in the regeneration of large bone defects. Furthermore, due to factors that happen in vivo, such as osteoclastic activity, their degradation/dissolution rates are difficult to predict. This could present a problem because if degrades too fast will compromise the mechanical stability of the construct, which is low by itself. At the same time, this would dramatically increase the extracellular concentrations of Ca and P, which can cause cellular death, as demonstrated by Adams et al. [42].

As an alternative to the above referred materials, there are biodegradable polymers, which are believed to be the ideal materials for bone TE. These can be divided in two groups: natural and synthetic.
Natural biodegradable polymers are those obtained from natural sources, either from animal or vegetal source. Within these we can find, among others, collagen, fibrinogen, chitosan, starch, hyaluronic acid, and poly(hydroxybutyrate). The main advantages of these materials are their low immunogenic potential, the potential bioactive behavior and the capability of interacting with the host‘s tissue, chemical versatility, and in some cases their source, as in starch and chitosan, which is almost unlimited [5].

Synthetic biodegradable polymers are the ones that are more commonly used within the biomedical engineering field. Their chemical versatility and processability varies according to their structure and nature, and hence a direct comparison with the natural polymers can not be established. The most widely used are poly(a-hydroxy acids), poly(e-caprolactone), poly(propylene fumarates), poly(carbonates), poly-(phosphazenes), and poly(anhydrides) [5].

### 1.2.5 Processing Techniques

The next step after selecting the adequate materials is to develop or choose an adequate processing technique. In order to do so, and to be sure that all the scaffolds characteristics are fulfilled, the chosen processing technique should obey, in general terms, to the following criteria:

. 1) The processing methodology must not adversely affect the materials properties, namely their biocompatibility or chemical properties.

. 2) The technique should be accurate and consistent, regarding porosity, pore size, pore distribution and interconnectivity.

. 3) Different scaffold batches should exhibit minimal variations in their properties when processed from the same set of processing parameters and conditions [5].

Through the years a series of processing techniques such as solvent casting, phase inversion, fiber bonding, melt based technologies, high pressure based methods, freeze drying, and rapid prototyping technologies were developed with the aim of producing scaffolds with adequate properties for bone tissue engineering [5, 43-46]. These techniques typically produce stochastically ordered pores and have been used to engineer a variety of tissues. Manual intervention, inconsistent and inflexible processing procedures, use of toxic organic solvents, use of porogens, shape limitations and irreproducibility are the main limitations of these techniques [47].

### 1.2.6 Scaffold Coating

Current interest in regenerative medicine is focused on the three dimensional tissue formation through the interplay of biomaterials and cells. By using scaffolds, cells can be guided to grow into a certain three dimensional shape based on the shape of the scaffolds.

Titanium implants are commonly used in orthopedics today e.g. dental and hip implants. The problem of implant loosening with time and poor integration with the surrounding bone tissue has lead to experiments where cells are cultured on titanium surfaces in attempts to improve implant integration.
Coating porous titanium scaffolds with fibronectin, is an important step for better cell attachment after subsequent cell seeding.

Fibronectin (FN) is a multifunctional, extracellular matrix glycoprotein composed of two nearly identical disulfide-bound polypeptides of molecular weight 220 kDa. Cellular fibronectin is structurally and antigenically similar to cold insoluble globulin from plasma, therefore polyclonal antibodies to either form usually crossreact.

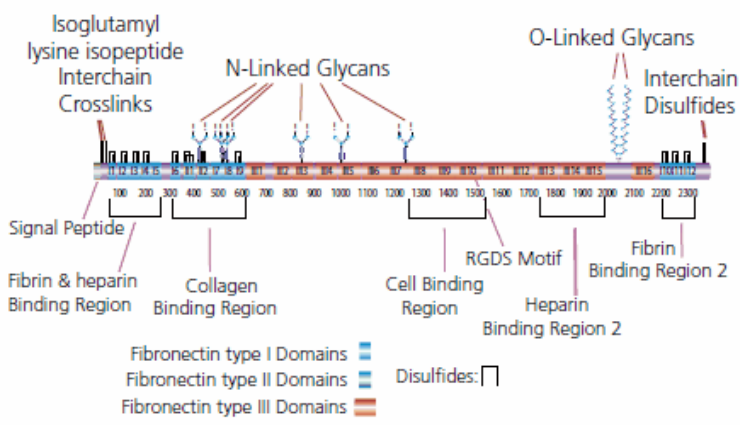



Figure 4 - Fibronectin, functionally and structurally distinct domain

Careful analysis of the fibronectin molecule indicates that it contains several functionally and structurally distinct domains which may bind to cell surfaces, collagen, fibrinogen or fibrin, complement, glycosaminoglycans, proteoglycans and heparin. Numerous studies have shown that fibronectin may enhance cell adhesion and spreading and affect the routes of cell migration both in vivo and in culture [48]. Moreover, it has been shown that upon malignant transformation many cells lose most of their surface bound fibronectin. Fibronectin has been shown to also play a role in cellular morphology, cytoskeletal organization, phagocytosis, hemostasis, embryonic differentiation and wound repair. Fibronectin is produced by a wide variety of epithelial and mesenchymal cells in vitro including: fibroblasts, chondrocytes,

myoblasts, Schwann cells, macrophages, hepatocytes and intestinal epithelial cells. Cellular fibronectin is present in many tissues including spleen, lymph node, tonsil, blood vessel walls, liver, kidney, muscle, skin, brain and peripheral nerves. It is found in basement membranes and in loose connective tissue stroma. It is also present in platelet α- granules and is expressed on the platelet surface after activation.

## 1.3 Bioreactor

In the overall cell-based bone tissue engineering strategy of expanding a stem cell source in vitro, culturing and differentiating this cell source on a three-dimensional scaffold, and implanting this scaffold *in vivo*, bioreactors can be used to enhance *in vitro* culture steps [49].

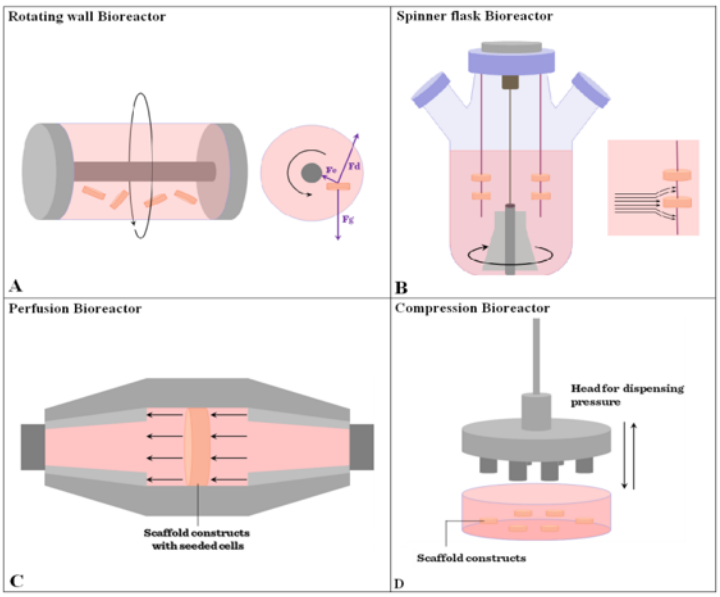



Figure 5 - Four types of a bioreactor which is used in bone tissue engineering. A) Properties of a rotating wall bioreactor; B) Properties of a stirring bioreactor; C) Principle of a perfusion bioreactor and D) Compression bioreactor

There have been lot of researches on the bioreactor in the past two decades. Lot of varying versions have been made for different kind of tissues. The most known bioreactor systems (Figure 5) are:

• *Rotating wall system*

Rotating wall system includes a cylindrical chamber which is filled with culture media and the scaffolds are moving freely inside. The outer wall of the chamber is capable to rotating slowly creating a hydrodynamic movement. Due to this drag force of movement the gravity force is neglected and the scaffold experience weightlessness inside the chamber. The weightlessness encourages the extracellular matrix expansion but do not increase cell proliferation [49,50].

• *Spinner flask system*

This system consists of a container and a magnetic stirring system. The magnetic stirrer is at the bottom inside the container which is standing on a plate. Inside the plate is a circulating magnetic source which rotates the stirrer inside the container. The scaffolds are fixed inside the container by a needle and the media is moving surround the scaffolds due to a flow in the media created be the magnetic stirring system. Due to flow of the media the nutrients are transported more inside the scaffold. This kind of stirring system increases the cell differentiation and proliferation [49, 50].

• *Perfusion system*

It may consider that the most effective system for bone tissue engineering is used by a flow perfusion bioreactor. The main principle of perfusion system is to get nutrition to the inside of the scaffold. It is done by a fluid flow through the centre of the scaffold. The scaffolds are fixed into a narrow tube filled with a culture media and a flow is applied by a pump or a motor driven propeller in one direction inside the tube [49, 50].

• *Compression and strain system*

## 1.4 Morphometric Analysis

A revolutionary invention in the field of medical imaging with X-rays occurred at the beginning of the 1970s when the first equipment for computed

tomography (CT) was developed. This method of imaging avoids several important limitations of conventional X-ray radiology. CT avoids the superimposition inherent to radiographic imaging of producing slices in the third dimension in a non-destructive way with contrast discrimination up to 1000 times better than that of a conventional radiograph.

MicroCT is similar to conventional CT systems usually employed in medical diagnoses and industrial applied research. Unlike these systems, which typically have a maximum spatial resolution of about 0.5 mm, advanced μCT is capable of achieving a spatial resolution above 0.3 μm, i.e. about three orders of magnitude lower. Such a high spatial resolution can be obtained only for samples of reduced size i.e. for dimensions in the range of a few cubic millimeters. The spatial resolution of the CT image is dependent on the number of parallel beam projections and the number of data points in each projection. A larger dataset means a more detailed description of the depicted object and hence more pixels and of smaller dimensions, i.e. better spatial resolution [51].

The development of high-resolution X-ray computed tomography (μCT) started in the early 1980s and has been used extensively to study the structure and architecture of bone tissue. Various parameters can be calculated with this technique, depending on the computational capability of the hardware and software. In addition, micro- CT is basically a nondestructive technique.

Researchers have employed μCT in the field of tissue engineering. The versatility of μCT has been demonstrated in the evaluation of scaffolds, because this single technique is capable of characterizing multiple aspects of the scaffolds. MicroCT enables to get three- dimensional (3D) images of the internal volume of a sample, and a detailed 3D view of pores at any depth. Further, different parameters may be calculated such as porosity, surface area to volume ratio, pore size, pore wall thickness, anisotropy, cross-sectional area, and permeability. MicroCT has been used for several polymer-based scaffolds that hold sufficient intrinsic contrast. For example, the internal geometry, pore network, and pore interconnectivity of poly-e-caprolactone scaffolds have been determined; in addition, the porosity, surface area to volume ratio, and interconnectivity of scaffolds made from a copolymer of poly ethylene glycol, poly-ecaprolactone, and polylactic acid

have been evaluated. Quantification of microarchitectural parameters, including volume fraction, density, thickness, spacing, and degree of anisotropy, of porous poly (L-lactide-co-DL- lactide) scaffolds with axially oriented macroporosity and random microporosity has also been reported. Scaffolds based on natural proteins such as collagen do not have the intrinsic X-ray attenuation capacity to be imaged by 3D microCT. Consequently, additional contrast has to be imposed upon such scaffolds [52]. Feldkamp et al. pioneered microCT when he developed an X-ray-based microtomographic system to analyze trabecular samples at a spatial resolution of 50 mm. Since then, microCT had been used extensively in the study of trabecular architecture and there are increasing applications of it in other areas. Its popularity can be attributed to its ability to provide precise quantitative and qualitative information on the 3D morphology of the specimen. The interior of the specimen can be studied in great detail without resorting to physical sectioning nor using toxic chemicals. Moreover, after scanning, the intact samples can be subjected to other tests, therefore resolving the problem of sample scarcity. As researchers began to recognize the potential of this radiographic technique, various biomedical applications are being explored which would include the assessment of scaffolds, regenerated tissue and vasculature networks.

In micro CT scanning, the specimen is divided into a series of 2D slices which are irradiated from the edges with X-rays. Upon transversing through the slice, the X-rays are attenuated and the emergent X-rays with reduced intensities are captured by the detector array. From the detector measurements, the X-ray paths are calculated and the attenuation coefficients are derived. A 2D pixel map is created from these computations and each pixel is denoted by a threshold value which corresponds to the attenuation coefficient measured at a similar location within the specimen. As the attenuation coefficient correlates to the material density, the resultant 2D maps reveal the material phases within the specimen. The quality of the 2D maps is dependent on the scanning resolution which ranges from 1 to 50 mm. At high resolutions, intricate details are imaged, however more time is required for high resolution scanning and the resultant large data set poses a challenge for data storage and processing.

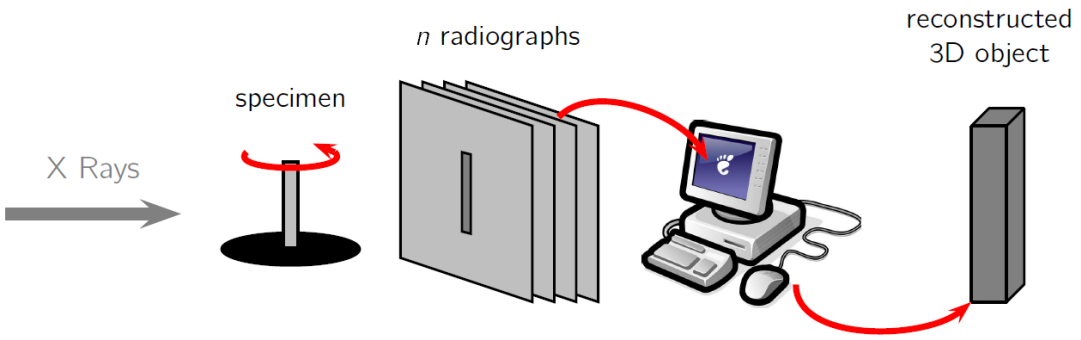



Figure 6 - MicroCT technique. In µCT to produce a three-dimensional CT image, a whole set of such two dimensional projections need to be acquired. These projections are usually taken in a setup in which the source and detector are at a fixed position and the object is rotated around its long axis. It can be shown that the number of projections taken over 180 degrees should be about twice the number of samples per projection to avoid aliasing artifacts. The two-dimensional projections can then be used to reconstruct a three-dimensional image

### 1.4.1 Scaffolds 3D Modeling

Image thresholding is a crucial step that has to be executed prior to 3D modeling and it affects the subsequent analysis and visualization. In the conventional approach, the thresholding range is selected via histographics and visual estimation and the problem arises when the scaffold composes of multiple materials whose thresholding ranges overlap and this renders the digital separation of these materials a difficult task. Moreover, as polychromatic X-ray beams are used in microCT, the lower energy rays would be readily attenuated by the sample resulting in a high exposure at the center of the scaffold. This effect is known as beam hardening and as a result thresholding is no longer dependent solely on radiodensity but also on the specimen size. MicroCT analysis is not suitable for scaffolds containing metals as X-rays are heavily attenuated by these metals. The presence of metals results in dark and bright grainy artifacts which obscure important details in the scan images. As microCT is a relatively new technology, improved algorithms and setups are anticipated, thus resolving such imaging errors [53].

# 2. Materials and Methods

## 2.1 Bioreactor Development

In this thesis, a flow perfusion bioreactor was developed from a prototype (Figure 7 and Figure 8) made by Department of Science of Landspítali University Hospital and the Institute of Biomedical and Neural Engineering of Reykjavík University.

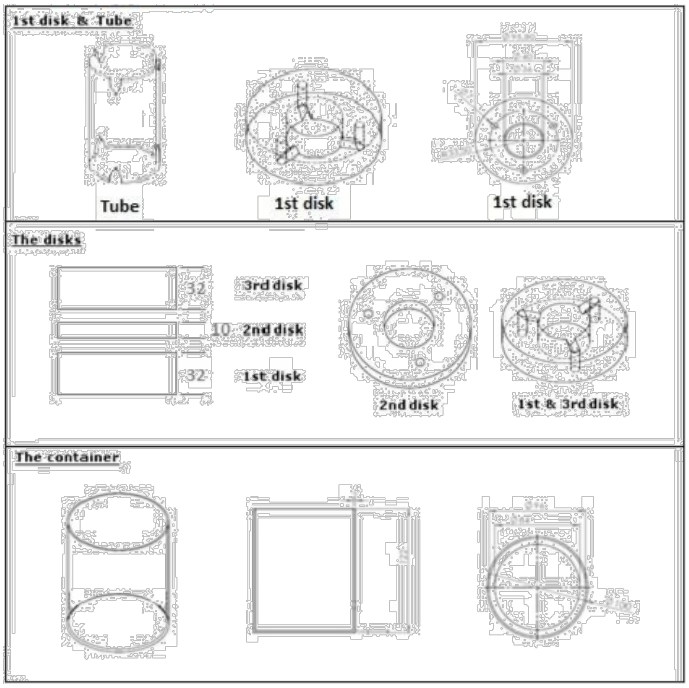



Figure 7 - The prototype consists out of one tube and three disks and a container

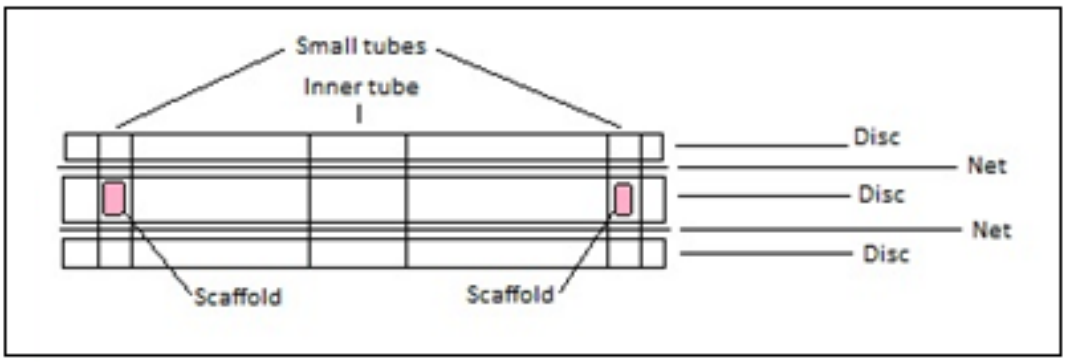



Figure 8 - Bioreactor system with the scaffolds. The small holes which are in the disks form small tubes.

This device, however, had different aspects to be improved, such as:

• *Replacing the DC Motor*

The used DC Motor corresponded to the small engines that often found in small appliances or toys for children. These motors provided a functioning also at low voltages, reduced storage space and limited performance. The motor in this prototype (DC Motor 1) had the following characteristics:

The replaced DC Motor (DC Motor 2) had better features instead. In particular it was controlled by a microcontroller that allowed to obtain a speed value between 0 and 255 svm, where 0 svm was about to 0 rpm and 255 svm was about to 5700 rpm.
The DC Motor replacement was necessary because the DC Motor 1 did not provide sufficient continuity of working for all 4 weeks of cell culture. To investigate the stability were performed two types of tests:

- Operation test over time (2-3 days of test)
- Operation test real-time (with digital multimeter)

The first test was carried out leaving the bioreactor in operation for 2-3 days, in the same operating conditions to be maintained during the cell culture.
The second test was performed by the use of a digital multimeter that has allowed to detect in real time the voltages across the DC Motor, in order to understand at what speed (rpm), the engine would guarantee the best performance.
For the DC Motor 1, the results were: for the first test, several blocks could be resolved only by restarting of operations; for the second test, a working speed had to be at least 220 svm.
For the DC Motor 2, the results were: for the first test, no blocking detectable; for the second test, a working speed equal to 90 svm, guaranteed good performance.
The following table shows the technical characteristics (Table 1) and the evaluation of the functioning (Table 2, Figure) of the DC Motor 2

| Motor Data | |
|---|---|
| **Name** | Motrax xDrive 2025-22 |
| **Rated voltage [V]** | 1.5 |
| **Working voltage [V]** | 1.5-3.0 |
| **No load Revolutions [rpm]** | 8000 |
| **No load Current [A]** | 0.2 |
| **Max load revolutions [rpm]** | 5700 |
| **Max load current [A]** | 0.5 |
| **Max Power [W]** | 0.24 |
| **Max torque [Ncm]** | 0.04 |
| **Efficiency [%]** | 31.4 |
| **Weight [g]** | 18 |

Table 1. Data for DC Motor 2

| Arduino Input Values [svm] | Output Values Forward [V] | Output Values Backward [V] |
|---|---|---|
| **90** | 0.36 | 0.37 |
| **100** | 0.40 | 0.41 |
| **110** | 0.44 | 0.45 |
| **120** | 0.48 | 0.49 |
| **130** | 0.52 | 0.54 |
| **140** | 0.56 | 0.58 |
| **150** | 0.60 | 0.62 |
| **160** | 0.64 | 0.67 |
| **170** | 0.68 | 0.71 |
| **180** | 0.72 | 0.75 |
| **190** | 0.76 | 0.79 |
| **200** | 0.80 | 0.84 |
| **210** | 0.83 | 0.87 |
| **220** | 0.87 | 0.92 |
| **230** | 0.90 | 0.96 |
| **240** | 0.94 | 1.00 |
| **255** | 1.04 | 1.11 |

Table 2. Average output values for Forward and Backward Method measured with multimeter across the DC Motor 2.

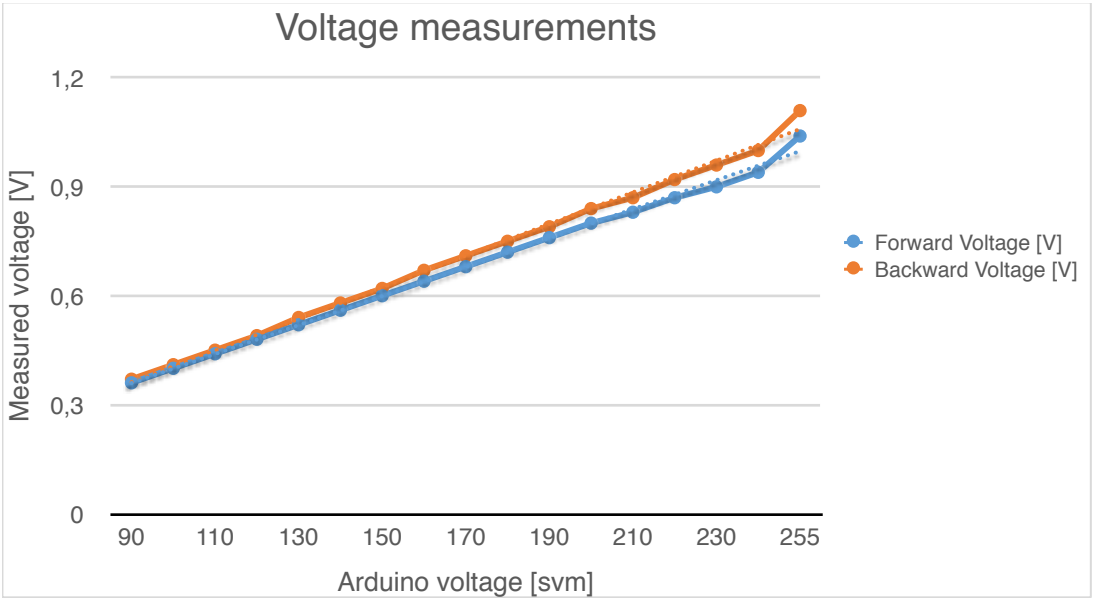
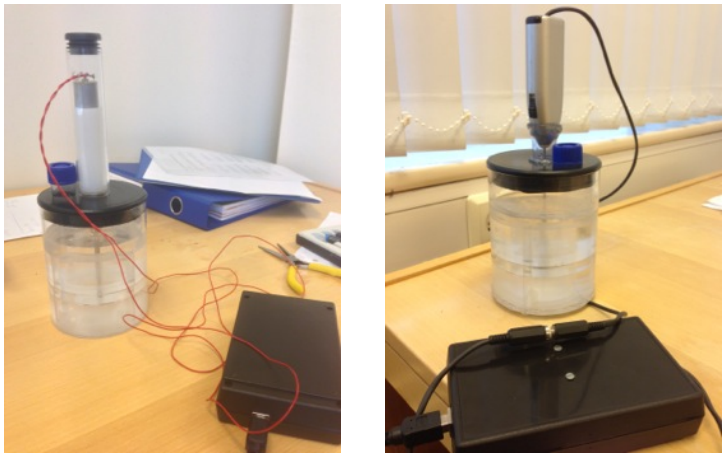



Figure 9 - The graph, obtained from the values present in the Table 2, show the stability of DC Motor 2

In particular, Table 2 shows that the DC Motor 2 has a greater stability, ensuring its operation for the entire period of cell culture, even at low speeds.

• *Replacing the axis and the stabilization system*

The axis with propeller, necessary to the realization of the flow inside of the bioreactor and the stabilization system (Figure 10) were replaced with a steel shaft and a new case of support. This was necessary to avoid oscillations and then a non-uniform flow.



**A** **B**

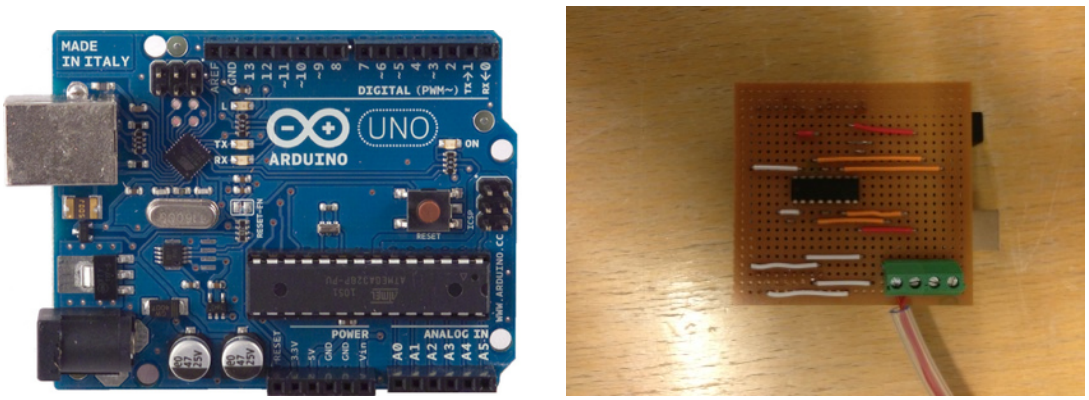
Figure 10 - A) Bioreactor before changes; B) Bioreactor after changes

- Creating a plug between the bioreactor and control unit

Finally, it was necessary to create a plug (shown in Figure 10.B) that would allow to connect and disconnect the bioreactor from the control unit quickly and easily. In particular this addition was very useful during the operations of exchange media; in fact it was possible to disconnect the control unit from the bioreactor and to extract only the bioreactor from the incubator without having to transport each time also inside the work hood.

### 2.1.1 Control Unit

The control unit was the heart of the entire bioreactor and thanks to this was possible to configure each operation that the bioreactor had to play. It consisted of a microcontroller (Arduino UNO) powered via USB transformer and required wiring to create various connections (Figure 11)




**A** **B**

Figure 11 - A) Arduino UNO; B) Base with the various connections needed

The Arduino Uno is a microcontroller board based on the ATmega328. It has 14 digital input/output pins (of which 6 can be used as PWM outputs), 6 analog inputs, a 16 MHz ceramic resonator, a USB connection, a power jack, an ICSP header, and a reset button.

Its programming was carried out through two software:

- Arduino

- Processing

“Arduino” is an application that uses the Java programming language and allows the implementation of the code realized directly on the microcontroller; in this study, this was useful when it was assumed that the operating characteristics of a device (e.g., speed, time, program, etc..) were set definitively. “Processing” is a software that exploits the bases of Arduino software and it allows the implementation of control interface; in this work, this was useful when it was necessary to configure and to control a device in real-time.

In this thesis, a code developed using the Arduino software was designed to allow the operation of the bioreactor during the 4 weeks of cell culture, this code implemented three operation methods (“Forward”, “Backward” and “Pulse”) at speeds of 170 svm.
In particular, the “Forward” and “Backward” methods allowed the propeller respectively a clockwise and couterclockwise, while the “Pulse” method allowed rotations very rapid (with a period of 150ms) alternately clockwise and counterclockwise. “Forward” and “Backward” methods were needed to allow the creation of two alternate streams, in order to improve the cell load above and internally to scaffolds. “Pulse” method was needed to maintain scaffolds in suspension and to create a light flow inside them.
A code, developed using the processing software, was realized for the creation of a user interface that would allow different operations in real-time during the use of the bioreactor.

### 2.1.2 Interface

The user interface (Figure 12), as already mentioned above, allowed to operate on the device in real-time. Its implementation was primarily useful for testing different phases of the bioreactor

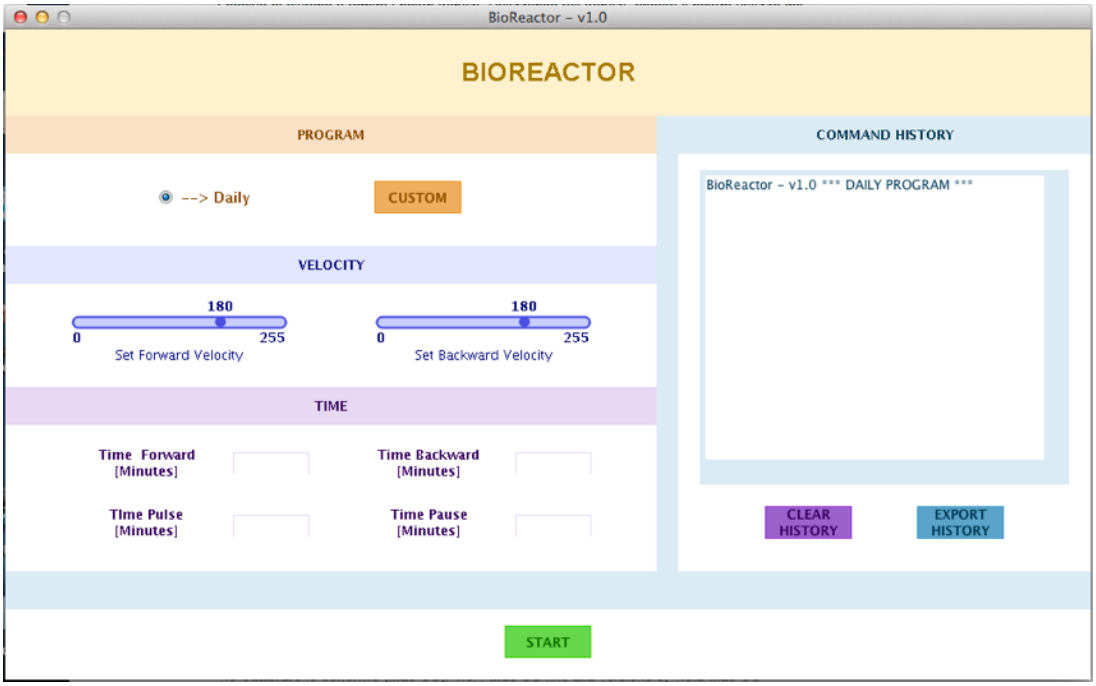



Figure 12 - User interface, daily program

The main available functions were:

- Set a default program (daily)

- Set a custom program

Through the daily program, the bioreactor, worked alternating the phases of “Forward”, “Backward” and “Pulse”, for a time and speed defined by the user; “Forward” and “Backward” speeds were the same that were utilized for “Pulse” phase; it was possible to define a “Pause” between the phases; this feature was helpful to use the device for short time (e.g. during a normal working day).

The custom program (Figure 13) allowed to setting the various phases in even greater detail, and this feature was helpful to use the device for long periods of time (e.g. 2-3 days or weeks).

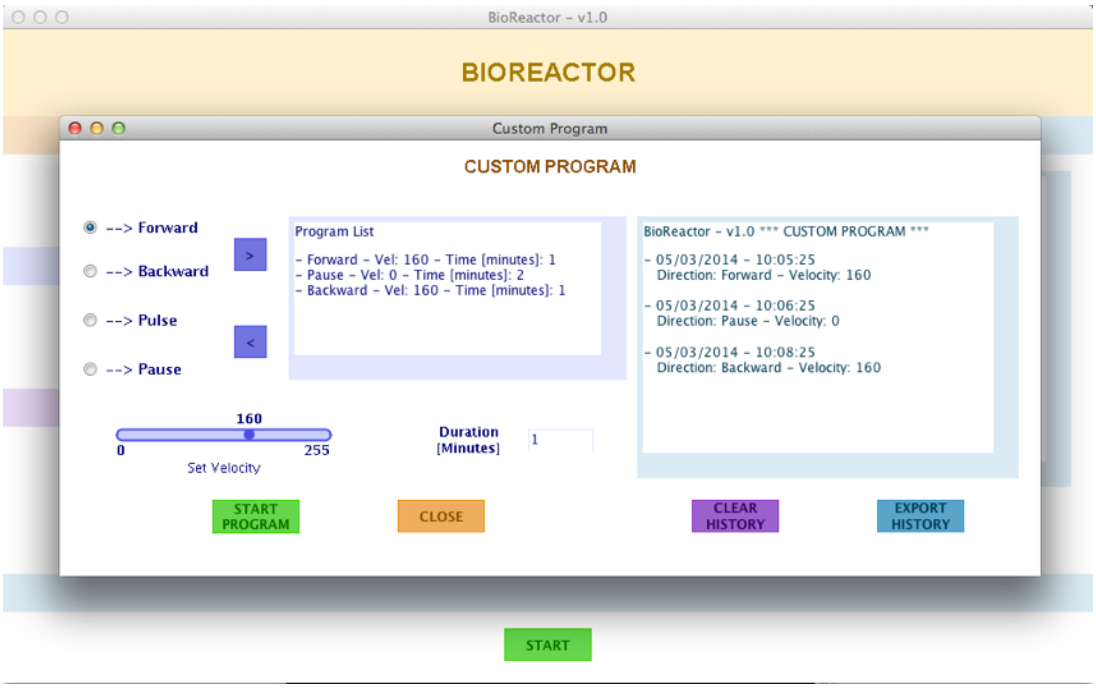



Figure 13 - User Interface, Custom Program

Finally for both functions, it was possible to extract a "Command History" (Figure 14) that was useful, for example, to check if the bioreactor had actually carried out, on schedule, all the set phases and then to monitor if any anomaly was intervened.

```
BioReactor - v1.0 *** CUSTOM PROGRAM ***

- 03/12/2013 - 11:17:47
   Direction: Forward - Velocity: 160

- 03/12/2013 - 11:18:47
   Direction: Pause - Velocity: 0

- 03/12/2013 - 11:20:47
   Direction: Backward - Velocity: 180
```

Figure 14 - Example of Command History

## 2.2 Computational Fluid Dynamic Modeling

The fluid dynamics is the part of mechanics that studies the motion of bodies within the fluids flowing. If a liquid flows in a pipe then it will define subject to an internal motion; if a body moves in a liquid this is called external motion.

In the field of fluid dynamics it is also necessary, given the numerous references, a brief definition of fluid that is defined as a substance whose molecules are so few adherents that can slide freely over each other, or they may move away from each other indefinitely. It is possible to divide fluids in two classes:

AERIFORMS: when the matter, consisting of molecules, tends to occupy the entire available volume, compressible

FLUIDS: liquid substances, having a proper mass and volume, assume the shape of the vessel that contains them, incompressible

Therefore there are two possible situations:

- Motion of fluids with the hypothesis of constant density (d), then incompressible fluid
- Motion of bodies inside of the fluids, taking into account of the resultant of all forces acting on the body, to study the behavior of the concerned body (drag force)

### 2.2.1 Viscosity

To talk about motion of a fluid within a body, it is also necessary to introduce the concept of viscosity, before in a particular case and then generalizing the definition, starting to Newton's law.

The viscosity is defined as the set of tangential forces between the surface and the fluid and between states of different fluid and it is opposed to the motion of these fluids respect to the surface, with velocity u (no constant).

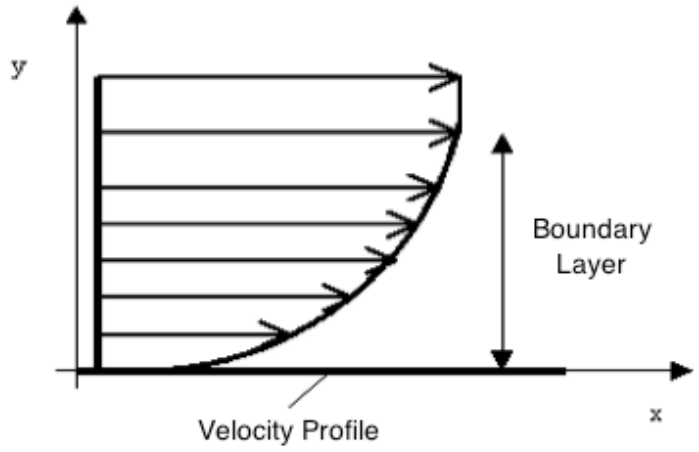



Figure 15 - Speed of a fluid in motion respect to a firm surface

In the graph, it is possible to note that, given a fluid in relative motion, respect to the solid surface, if a normal to the surface is plotted, after a certain interval of time, it is possible to observe the situation of the motion itself, which represents the speed of a fluid in motion, respect to a firm surface.

The particle in contact with the solid surface has not moved, so it is in the case of adherence, in fact in all fluids, the particles in direct contact with the solid boundaries do not flow, respect to same boundary.

Newton's law, relative to the fluids in motion on solid surfaces, binds tensions that are created with the velocity gradient of the fluid, through the constant of viscosity μ

$$\tau_{xy} = -\mu \frac{du_x}{dy}$$

τ is directed in the opposite direction respect to motion, along the x direction on the normal surface to y (in descending toward) and it is measured in Pascal [Pa].

μ is called viscosity characterizing each fluid.

It also defines the kinematic viscosity ratio

$$\nu = \frac{\mu}{\varrho} = \frac{[Pa]}{[Kg]} \frac{[s]}{[m^{-3}]} = \frac{[m^2]}{[s]}$$

with μ=dynamic viscosity and ρ=fluid density

The viscosity is not constant but it varies with temperature: in fact, increasing the temperature, the viscosity decreases, as it is possible to see in the following graphs

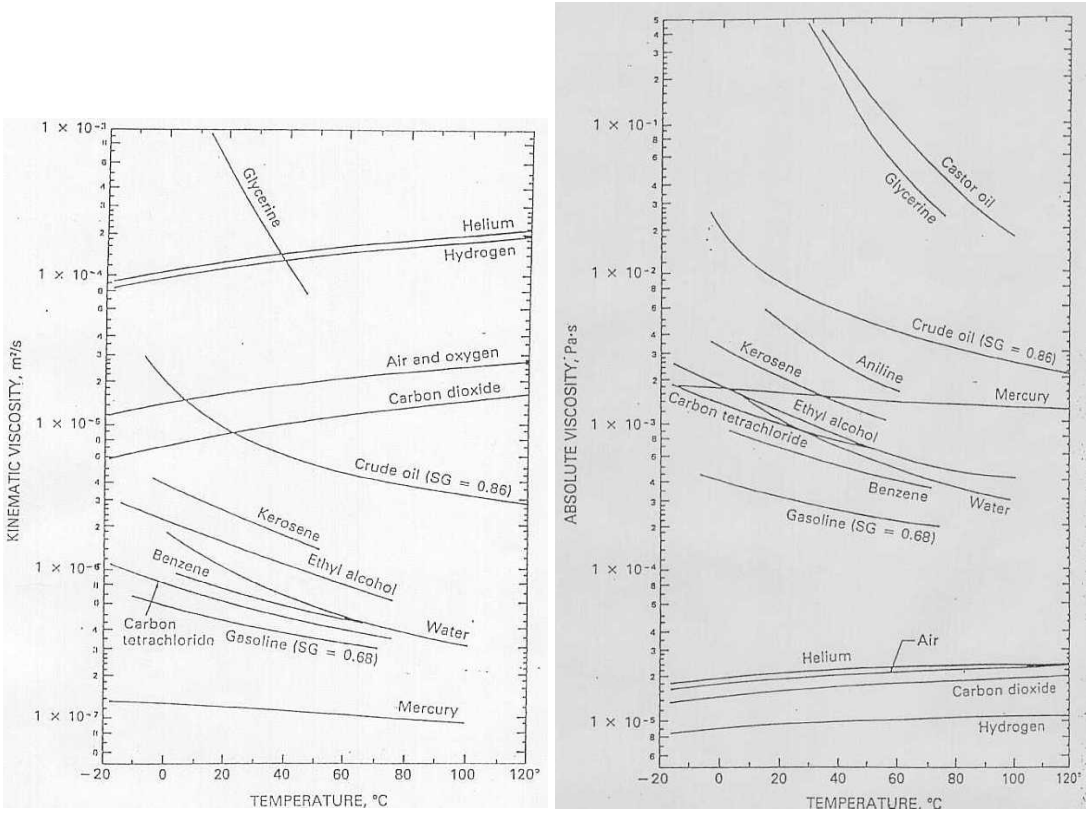



Figure 16 - The viscosity decreases with increasing temperature

### 2.2.2 Model

It is supposed to consider the motion of a fluid inside a tube of radius r with velocity u. By convention, this situation is studied on a volume element of the fluid with the following characteristics

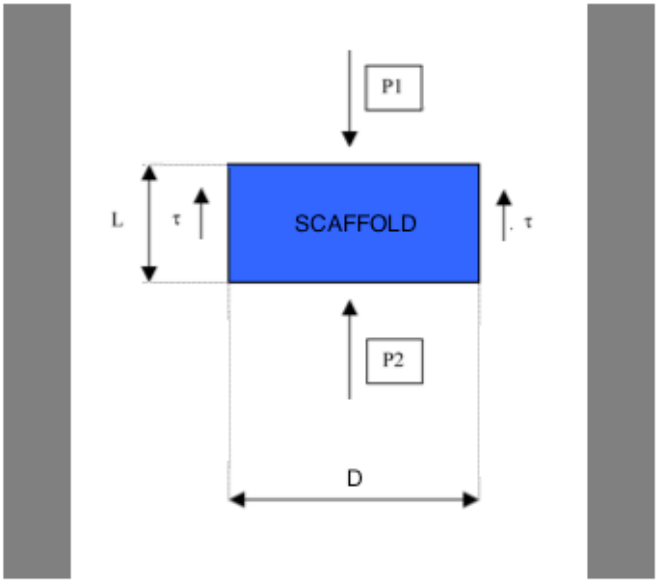



Figure 17 - Forces acting on the volume fluid element

L = length of considered volume element

D = diameter of the element

r = D/2 = radius of the element

τ = shear stress that curbs the fluid element in motion

Considering an equation that expresses the balance of the forces acting on the fluid element, it is possible to write:

$$p_1 A - p_2 A - \tau 2\pi r L = 0 \quad (1)$$

p1, p2 = pressures acting on the fluid element

A = area of the base circumference of the fluid element

Now calculating

$$\tau = \frac{du}{dr}(-\mu) \quad (2)$$

And replacing the (2) in the (1) , it is possible to write:

$$(p_1 - p_2)\pi r^2 = -\mu \frac{du_x}{dr} 2\pi r L$$

$$(p_1 - p_2) r \pi dr = -\mu du 2 L \pi$$

$$(p_1 - p_2)\pi \int_r^R r dr = -\mu \int_{u(r)}^{0} du 2\pi L$$

$$(p_1 - p_2)\frac{\pi}{2}(R^2 - r^2) = \mu u(r) 2L$$

$$u(r) = \frac{(p_1 - p_2)}{\mu}\frac{\pi}{4L}(R^2 - r^2)$$

The last obtained equation represents the velocity profile of the fluid in the tube. It is possible then to affirm:

$$w = \frac{\int_S u(r) ds}{S} = \frac{\int_{r=0}^{R} u(r) 2r\pi dr}{\pi R^2} = \frac{(p_1 - p_2) D^2}{32 \mu L}$$

$$u(r) = \frac{(p_1 - p_2)}{2\mu L}\frac{1}{2}(R^2 - r^2)$$

Then:

$$w = \frac{1}{R^2\pi}\int_0^R \frac{(p_1 - p_2)}{4L\mu}(R^2 - r^2)2\pi r dr$$

$$= \frac{1}{2\mu R^2 L}(p_1 - p_2)\int_0^R (R^2 r - r^3)dr =$$

$$= \frac{1}{2\mu R^2 L}(p_1 - p_2)(R^2\frac{r^2}{2} - \frac{r^4}{4}) \Big|_0^R = \frac{1}{2\mu R^2 L}(p_1 - p_2)(R^2\frac{R^2}{2} - \frac{R^4}{4}) =$$

$$= \frac{1}{2\mu R^2 L}(p_1 - p_2)(\frac{R^4}{4}) = \frac{1}{2\mu R^2 L}(p_1 - p_2)(\frac{R^4}{4}) = \frac{R^2(p_1 - p_2)}{8\mu L} =$$

$$= \frac{D^2(p_1 - p_2)}{32\mu L}$$

Ultimately:

$$w = \frac{D^2\Delta p}{32\mu L}$$

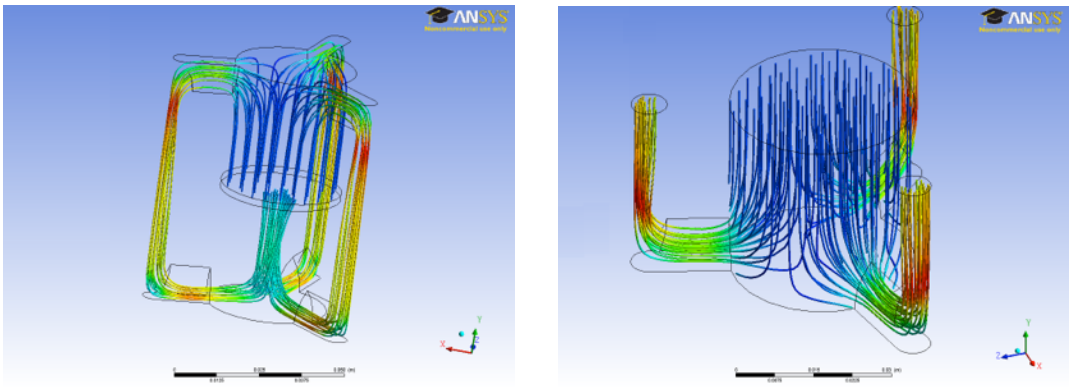
$$\Delta p = \frac{w32\mu L}{D^2}$$

### 2.2.3 Pressure in the Bioreactor

In order to know the stresses that would have occurred on the cells, stimulating them mechanically, it was assumed to approximate the scaffold (small, light, very porous, made in titanium) as an element of the fluid with the following characteristics

$$L = 0.0037\ m$$
$$D = 0.0059\ m$$
$$w = 0.009\ \frac{m}{s}$$
$$\mu = 0.001\ \frac{Kg}{m\ s^2}$$

where in particular the speed (w) was extrapolated, in first approximation, by measuring the distance covered by the scaffold and the number of times that this has covered it in one second.



**A** **B**

Figure 18 - A) Simulation of the flow circulating inside the bioreactor; B) Detail of the flow, at the height of scaffolds area, where the pressure was calculating

Then starting from

$$\Delta p = \frac{w32\mu L}{D^2}$$

The pressure, acting on each scaffold, was

$$\Delta P = 3 \cdot 10^{-2}\ Pa$$

This parameter was very important in order to test if use of the bioreactor, improved or not the growth of the mineralized matrix. In fact, studies focused on the effects of fluid shear on osteoblastic differentiation have shown that shear stress affects osteogenic signal expression of mesenchymal stem cells [54-59].

## 2.3 Stem Cell Culture

In this work hES-MP (human Embryonic Stem Cells-Mesenchymal Progenitor) were used. Human embryonic derived mesenchymal progenitor cells are cells that belong to the cell line hES-MP002.5, that was developed by Cellartis in Gothenburg, Sweden, 2009. These cells have been described to have high resemblance to primary adult mesenchymal stem cells (MSC) in terms of proliferation and differentiation. Handling and culture of hES-MP cells and MSC is almost the same but hES-MP cells require gelatin coating of the growth surface prior to seeding.
To obtain a single cell suspension via trypsination of adherent cell cultures in monolayer, some equipment and procedures were necessary (see *Appendix A*).

### 2.3.1 Counting Cells

Knowing the cell concentration was an important step for subsequent seeding cell. Cell concentration of viable cells in a solution was determined using hemocytometer.
Hemocytometer is a counting chamber made of glass especially designed for cell counting. The chamber has two counting grids that are each put

together with 9 “big“ squares that are further dived into 16 smaller squares. A cover slide is placed on top of the chamber and a sample of the cell solution placed under the cover slide on top of the grids. Each of the 9 bigger squares chambers has a surface area of 0,1 $mm^2$ and depth of 0,1 mm. The volume of liquid under the cover slide is thus 0,9 $mm^3$.

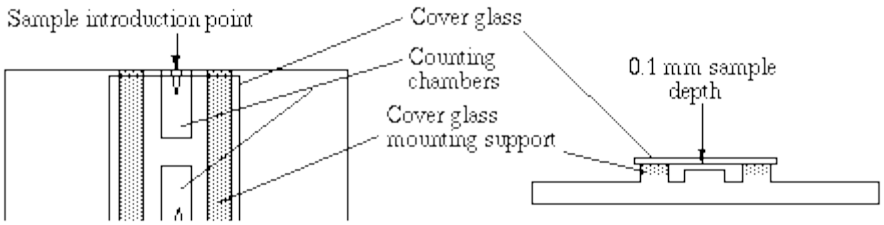



Figure 19 - Detail of Hemocytometer

A sample of the cell solution is stained with tryphan blue stain before counting. Cells that are live and viable will not take up the stain but dead cells and cell debris will stain blue and should be excluded from the count. Procedure to counting cells was described in *Appendix B*.

### 2.3.2 Scaffold Coating Preparation

Coating porous titanium scaffolds with fibronectin, is an important step for better cell attachment after subsequent cell seeding.
For scaffolds coating preparation, 6 scaffolds were used, 3 scaffolds for static culture and 3 scaffolds for bioreactor culture; 4 hours (+ overnight incubation), some equipment and procedures were necessary (see *Appendix C*).

### 2.3.3 Static and Bioreactor Culture

Bone metabolism is a complex process that involves the resorption of existing bone by osteoclasts and the subsequent formation of a new bone matrix by osteoblasts.

To start the Cell Culture 6 porous titanium scaffolds (previously coated with fibronectin, Figure) and following equipment and procedures were used and described in *Appendix D*. In particular, 3 scaffolds were used for Static Culture and 3 for Culture in Bioreactor.

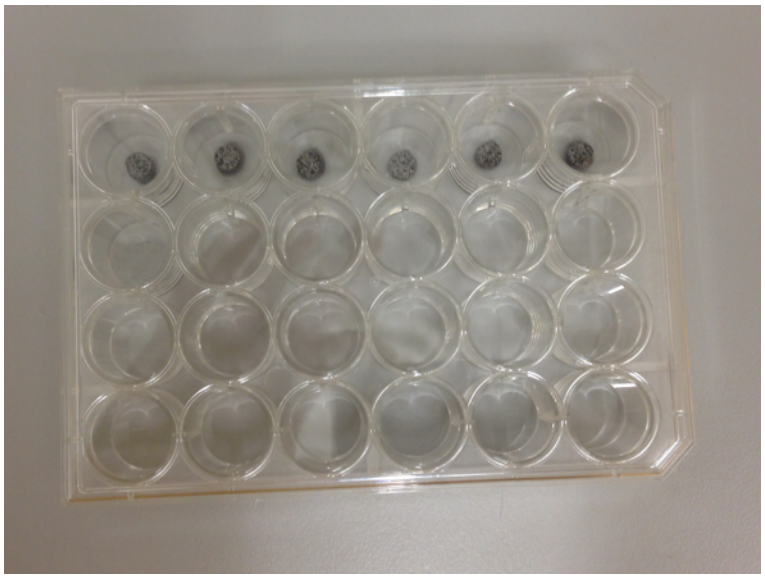


Figure 20 - Scaffolds coated with fibronectin, ready to start the static and bioreactor cell culture

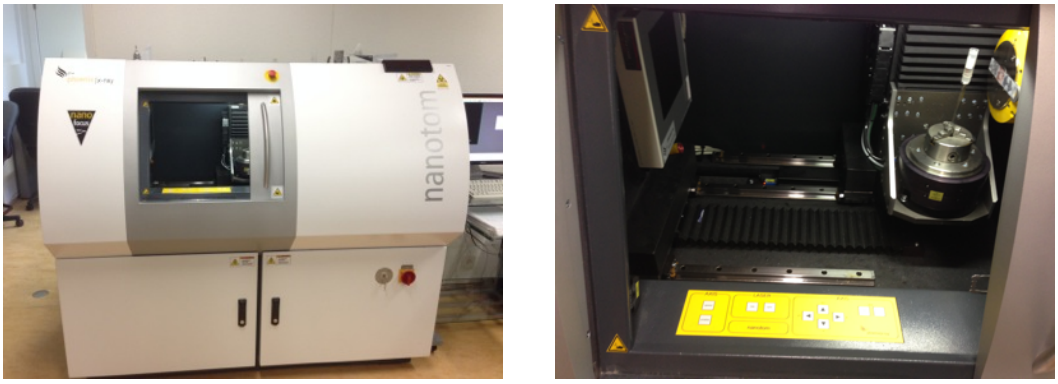
Both cultures were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO2. Differentiation, in bone tissue, was induced through the addition of Ascorbic Acid (Ascorbic Acid γ- irradiated, Sigma) and β-Glycerophosphate (Glycerol 2-phospate disodium salt hydrate, Sigma) to the growth medium containing Fetal Bovine Serum (FBS) and D-MEM (Dulbecco's Modified Eagle Medium, Gibco BRL). This solution was replaced every 7 days.

## 2.4 Data Acquisition

3D modeling programs such as Mimics (Materialize, Belgium), Velocity and Anatomics, stack the 2D maps to create 3D models, without these programs, visualization and analysis would have been impossible. As computation is inherent in this technique, the selection of software and hardware facilities would influence the efficiency and effectiveness of this radiographical

assessment. There are associated concerns despite of the numerous advantages of using microCT.

In this work 3D Models of 3 porous titanium scaffold maintained in static culture and of 3 porous titanium scaffold maintained in bioreactor culture were obtained by using µCT (phoenixlx-ray Systems, nanotom s, GE, Figure 21.A).




**A** **B**

Figure 21 - A) MicroCT system (phoenixlx-ray Systems, nanotom s, GE); B) Detail of the acquisition area

A µCT starts out by recording 2D-X-ray images of a sample. In the acquisition system a cone beam is used. In the process, the tube generates radiation which is partly absorbed by the sample. The number of transmitted photons is measured with a 2D detector. The detector integrates the radiation and transforms it into a digital image in order to save it on the computer‘s hard disk. The sample is placed on a rotating platform. The rotation is effected with motors. During recording, the sample is rotated step by step and one projection is recorded with every step. If too few projections are recorded, the quality of the reconstructed volume deteriorates and typical artefacts become visible. The imaging chain includes a detector to measure the intensity of the transmitted X-rays and acquisition electronics which digitize the detector signals and send them to a computer for reconstruction. The individual modules, X-ray control, CNC and data acquisition are controlled by the datoslx - acquisition program. The pixel size was 4.95µm, which seemed appropriate to image porous titanium scaffolds. Tube voltage was set to 120kV, current to 100 µA. Tube mode was set to 2. For each sample, 1080 radiographic images (or projections) with 2304 ×

2304 pixels were acquired at different angles of view between 0° and 180°. According to the design of the experiment the total acquisition time was approximately 180 minutes for sample.

After radiographic data acquisition, CT reconstruction was performed by means of a 3D filtered back-projection algorithm to retrieve the 3D scaffold structure image. For each sample, the reconstruction was limited to a 1250 × 1250 × 1000 voxel subvolume, which corresponded to a volume scaffold and included the background. The reconstruction time was approximately 10 minutes per sample. The program datoslx – reconstruction calculates a volume model from the recorded images of the sample. For this purpose, datoslx – reconstruction uses an optimized Feldkamp-algorithm resulting in an extraordinary reconstruction quality. This work was conducted in the Innovation Center in Iceland (http://www.nmi.is/).

## 2.5 Data Analysis

The scaffold structure was porous and had a cylindrical shape with diameter approximately of 5-5,5 mm and height of 3-3,5 mm

A detailed volumetric representation was obtained by scanning the scaffolds with slice distance and pixels size of 4,5 µm, each slice was represented with a matrix of 1250 x 1250 pixels. Every data set contained between 1000 and 1500 DICOM files, which summed up to a considerable amount of data for each scaffold (2 to 4 GB).

After this, the data were imported into special software called “Mimics” where 3D reconstruction, modeling and advance segmentation was performed.

Generally, in computer vision, segmentation refers to the process of partitioning a digital image into multiple regions (sets of pixels) and it produce a binary mask image which fulfills the segmentation criteria [60].
In the analysis of an image region is essential to distinguish between the region of interest and "the rest”. This latter group is also referred to as the “background”. The techniques used to find the objects of interest are usually

referred to as segmentation techniques: segmenting the foreground from background. Any segmentation result is a set of pixels that are related to each other in some sense, that is they belong to the same class (e.g. tissue class or an object in an image). This set of pixel is usually called segmentation mask or simply mask. Each pixel contains information which is represented through the gray value (GV). By grouping together gray values according to certain criteria (Thresholding), the image data can be segmented and the 3-Dimensional object created. An optimal thresholding is very important in order to obtain correct result from data analysis on the binarized data set [61].

The threshold was set to visualize the porous titanium scaffold; this threshold was represented in the GV interval [about 20000-30000].
The 3D Model reconstruction can be see in the following Figure 22

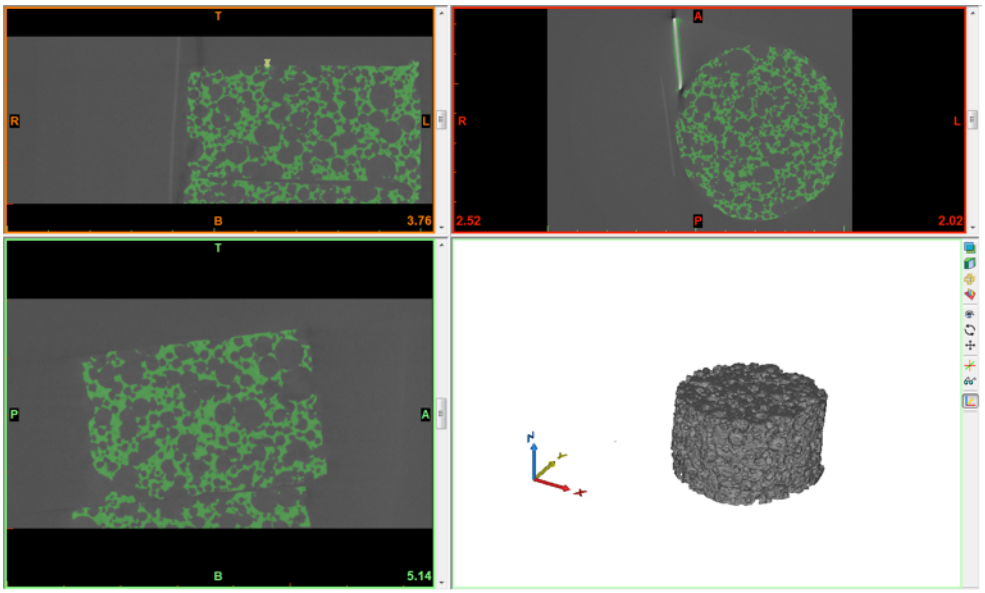



Figure 22 - Different views and 3D Model of porous titanium scaffold

To facilitate the data processing and to assess the GV homogeneity along the scaffold, every porous titanium scaffold was divided in segments (Figure 23), generating 20 “sub-scaffold” masks, 10 for the scaffold surface and 10 for the scaffold internal area. In this way 30 specimens coming from surface and 30 specimens coming from inside of scaffolds maintained in static culture were obtained. In a similar way 30 specimens coming from surface

and 30 specimens coming from inside of scaffolds maintained in bioreactor culture were obtained.

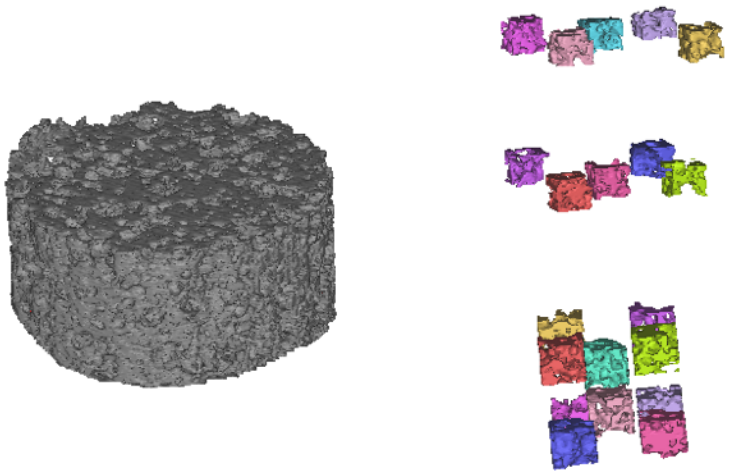


Figure 23 - Segmentation of titanium scaffold

To quantify on the scaffolds the extracellular matrix mineralization changes, GV distribution changes were analyzed. Therefore, starting to the GV distribution, the Gradient Distribution was calculated to visualize in 3D the extracellular matrix changes on the scaffolds; to do this a following criteria was applied.

Consider a cross section of the μCT data in which the scaffold composition was given by a scalar field GV, associated to each pixel, then to each point (x,y,z) of the mask. This point was displayed as "GV (x,y,z)". At each pixel in the image, the gradient of GV in that point showed the direction of most rapid changes of the GV in the scaffold. The magnitude of the gradient was determinate how fast the GV increased in that direction. To calculate and to display the Gradient Distribution, from the segmented scaffold, an algorithm was developed in Matlab (Matworks Inc).

First, the gradients were computed using a central finite difference approximation for each voxel. So for each point f(x, y, z), each gradient component was found by:

$$\frac{\partial f}{\partial x} = f\left(x + \frac{h_x}{2}\right) - f\left(x - \frac{h_x}{2}\right)$$

$$\frac{\partial f}{\partial y} = f\left(y + \frac{h_y}{2}\right) - f\left(y - \frac{h_y}{2}\right)$$

$$\frac{\partial f}{\partial z} = f\left(z + \frac{h_z}{2}\right) - f\left(z - \frac{h_z}{2}\right)$$

Where $h_x$, $h_y$ and $h_z$ were the separation between the adjacent points in x, y and z directions, respectively [62].

# 3. Results

## 3.1 Bone Tissue Formation

The static and bioreactor cell cultures foresaw a running time of four weeks for a good cell growth. In the third week of culture, an interruption was necessary due to a bacterial infection in a bioreactor.

There are many possible reasons why the bioreactor might have gotten infected. Some factors were more likely than others and included:

• *Underlying infection since the bioreactor assembly*
This was without a doubt the most risky stage to get infected due to the many components that needed to be handled and putted into the bioreactor.

• *Infection during feeding*
*The fact that the whole bioreactor needed to be removed from the incubator to change the media was undesirable and increased the risk of infection each time. The lid of the bioreactor needed to be removed each time, leaving large surface area exposed and vulnerable for short time, during the change. Furthermore, the propeller that went right into the culture media, needed to be removed from the bioreactor as well (since it was attached to the lid) and putted aside giving it enough time to catch something before it was returned to the bioreactor.*

• *Infection during transfer between the hood and the incubator*
The junction between the motor and the lid was not tight. The glue was not able to seal it securely and sticky tape had to be used to stabilize the motor when it was shaking. Therefore the infection might originate from the transfer.

- *Infection from the incubator*

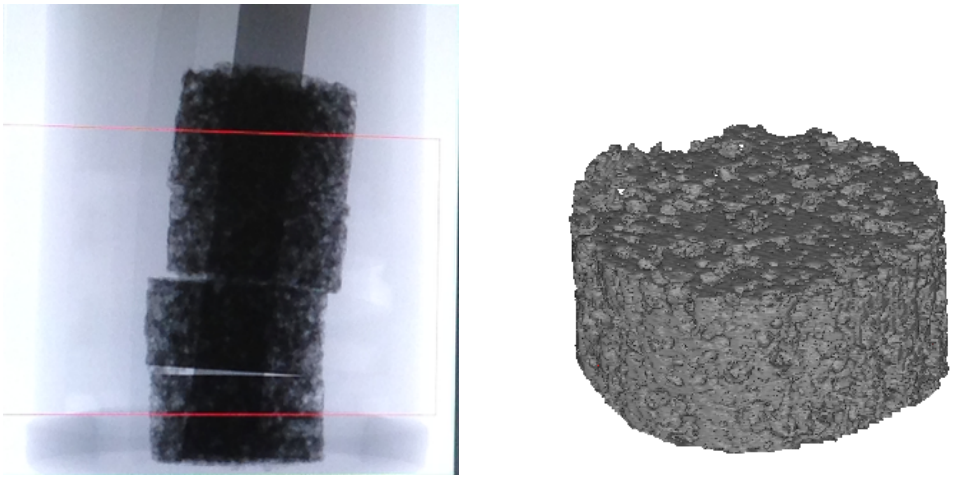
Possible but unlikely. Neither the static cultures or any other cultures in the incubator at the same time got infected.

During a long term culture even the smallest infection can become something big, since the cell culture conditions are obviously the best conditions for infectious organisms such as fungi as well as for cells. Even though the best working procedures were applied there were so many factors that were hard to control and so obtain an infection was probable.

After 3 weeks of culture, then, cells on scaffolds were fixed in Formalin. The scaffolds were washed several times with PBS afterwards to remove formalin and then were used for µCT-scanned.

## 3.2 Static and Bioreactor Culture Comparison

After the µCT scanning 3D Model of each scaffold was created through the software “Mimics” (see section 2.6).



**A** **B**

Figure 24 - A) Scaffolds during µCT scanning; B) 3D Model of Scaffold

Subsequently, each model was sectioned in order to obtain 10 specimens taken from the surface and 10 specimens taken from the inside of it. In this way were obtained:

- for scaffolds maintained in static culture, 30 specimens coming from the surface and 30 specimens coming from inside

- for scaffolds maintained in the bioreactor, 30 specimens coming from the surface and 30 specimens coming from inside

Then, 120 specimens were obtained. An analysis of specimens was carried out observing the Gray Value Gradient Distribution and performing a statistical analysis by T-Test.

The results of these two studies were useful to understand if, in scaffolds maintained within the bioreactor, there was a greater mineralization of the extracellular matrix respect to the scaffolds maintained in the classical static culture.

### 3.2.1 Gray Values Gradient Distribution

The specimens obtained by the sectioning of the scaffolds was processed in order to obtain the Gray Value Gradient Distribution (see section 2.5). A specimens coming from an empty scaffold was used as a tester. Then a comparison was performed

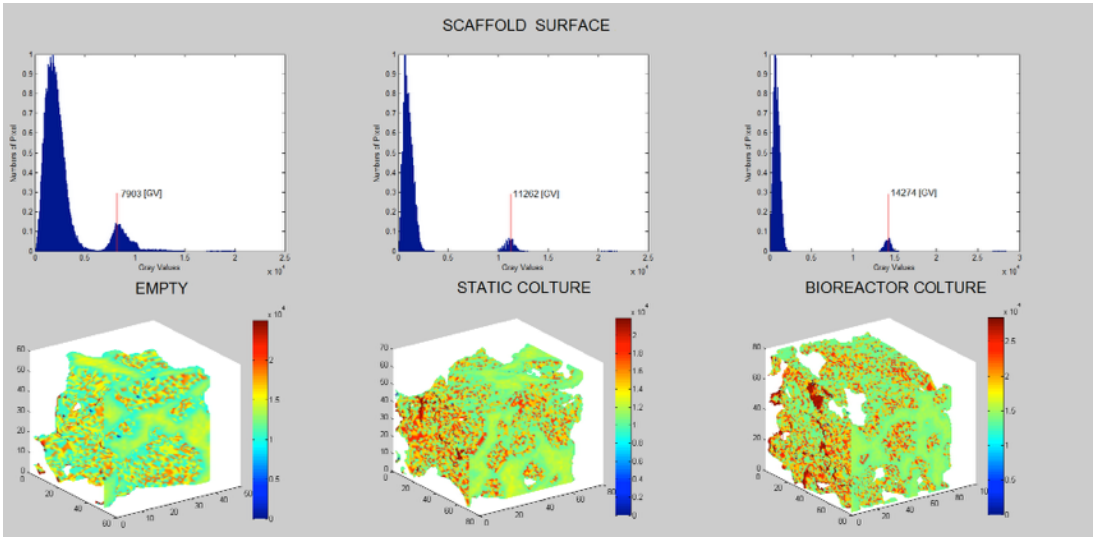



Figure 25 - Comparison between sample coming from surface of the scaffolds

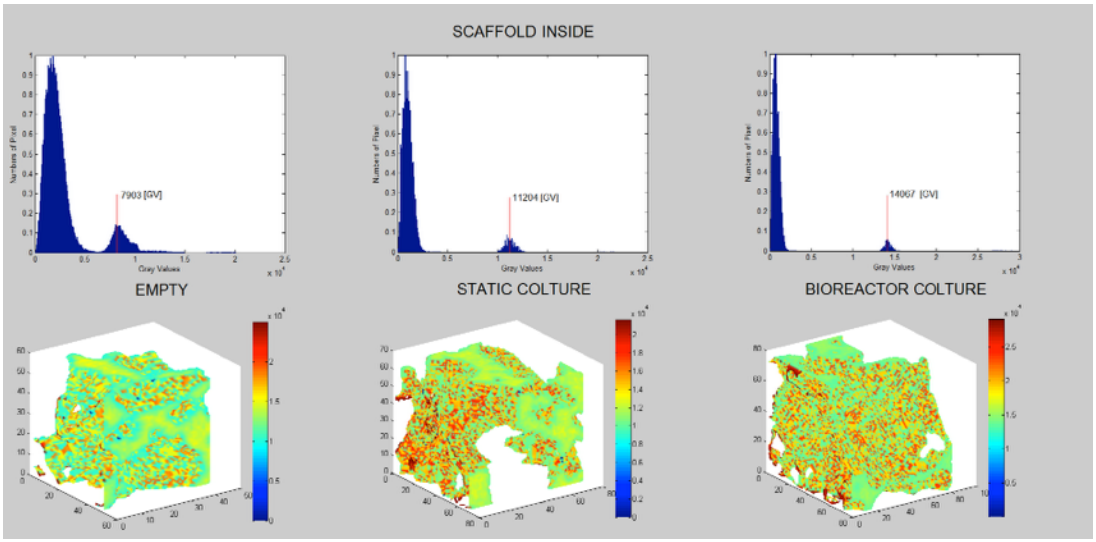



Figure 26 - Comparison between sample coming from inside of the scaffolds

In both figures, it was possible to note that three groups of specimens (empty, static, bioreactor) had similar GV Gradient Distribution (top in figures). In particular, the center of smaller Gaussian distribution had a tendency to increase respectively in the specimens.

The same results were observed in the 3D visualization of the GV Gradient Distribution (bottom in figures); gradient intensity was coded with colors, in blue was displayed low gradient voxels and in red was displayed high gradient voxels.
Changes of this distribution could indicate possible structural changes in the scaffolds maintained in the bioreactor.

### 3.2.2 Statistical Analysis

In order to understand, in a statistical way, if the use of the bioreactor had actually improved the structural changes, the t-test was used to perform: three different types of comparison between scaffolds maintained in static culture and scaffolds maintained in bioreactor culture; two different types of comparison between samples of scaffolds coming from surface and inside of the static and bioreactor culture.

- Comparison between Static and Bioreactor Culture considering 60 specimens (30 Static, 30 Bioreactor) taken from the surface of scaffolds of respective cultures

| Cell Culture | Mean | STD | Sp | $t_0$ | $t_{0.05,58} = 1.671$ |
|---|---|---|---|---|---|
| **Static** | 23034 | 75.075 | 1432.5 | **11.627** | **H1** |
| **Bioreactor** | 27335 | 1430.5 | | | |

Table 3 - T-Test between surface samples

- Comparison between Static and Bioreactor Culture considering 60 specimens (30 Static, 30 Bioreactor) taken from the inside of scaffolds of respective cultures

| Cell Culture | Mean | STD | Sp | $t_0$ | $t_{0.05,58} = 1.671$ |
|---|---|---|---|---|---|
| **Static** | 22708 | 151.75 | 1357.8 | **12.543** | **H1** |
| **Bioreactor** | 27105 | 1349.3 | | | |

Table 4 - T-Test between inside samples

- Comparison between Static and Bioreactor Culture considering 120 specimens (60 Static, 60 Bioreactor) taken from the scaffolds of respective cultures

| Cell Culture | Mean | STD | Sp | $t_0$ | $t_{0.05,118} = 1.658$ |
|---|---|---|---|---|---|
| **Static** | 22871 | 113.29 | 1393.4 | **17.093** | **H1** |
| **Bioreactor** | 27220 | 1388.8 | | | |

Table 5 - T-Test between whole samples

- Comparison between Static and Bioreactor Culture considering 60 samples, 30 taken from the surface and 30 taken from the inside of scaffolds in static culture

| Static Cell Culture | Mean | STD | Sp | $t_0$ | $t_{0.05,58} = 1.671$ |
|---|---|---|---|---|---|
| **Surface** | 23034 | 75.075 | 169.31 | **7.4649** | **H1** |
| **Inside** | 22708 | 151.75 | | | |

Table 6 - T-Test between surface and inside samples of static culture

- Comparison between Static and Bioreactor Culture considering 60 samples, 30 taken from the surface and 30 taken from the inside of scaffolds in bioreactor culture

| Bioreactor Cell Culture | Mean | STD | Sp | $t_0$ | $t_{0.05,58} = 1.671$ |
|---|---|---|---|---|---|
| **Surface** | 27335 | 1430.5 | 1966.5 | **452** | **H0** |
| **Inside** | 27106 | 1349.3 | | | |

Table 7 - T-Test between surface and inside samples of bioreactor culture

The results of T-Test show that in first three comparisons, the hypothesis H1 could be accepted, namely, the use of the bioreactor had made significant improvements to the structural changes in the scaffolds contained in it. Furthermore, the hypothesis H1 could be accepted also for the T-Test performed between the samples taken from the surface and inside of static culture, demonstrating that the structural changes occurred in non homogenous way (likely more in the surface than in the inside); this result could not be observed with the analysis of GV Gradient Distribution. Indeed, in the T-Test performed between the samples taken from surface and inside of bioreactor culture, the hypothesis H0 could be accepted, demonstrating that the structural changes occurred in homogenous way.

# 4. Discussion

In most clinical cases, crucial bone defects are impossible to cure due to a big gap in the bone. These cases are caused for example by cancer, accidents or serious infections and many other sicknesses. Healing of these big gaps naturally is often hard or impossible. In those cases, bone grafting is often implemented. There are two kinds of methods which are used for bone grafting. These are “autografting" and “allografting”. In case of “autografting”, the injured bone is replaced with a bone taken from the patient’s own body. If the gap is so big that “autografting” is not possible, the “allografting” approach is done and the injured bone is replaced with a bone from a cadaver. Both these methods have disadvantages: in case of “autografting”, there are medical complications after surgery; in case of “allografting”, a risk of immune response and possibility of infection. To solve this problem, researchers have been dealing with tissue engineering to try to create a bone tissue. Bone tissue strategy is mainly about creating or form bone tissue by cell expansion and differentiation on 3-dimensional scaffolds. The 3-dimensional scaffolds give the tissues physical form and a support for the new bone tissue formation. Studies, made *in vivo* and *in vitro*, show that physical forces such as fluid flow and mechanical loading stimulate bone factor metabolism. The mechanical stimulation can inspire cells to create an extracellular matrix in a shorter time period and also in a more homogeneous manner than in static culture.

To create the most likely biological environment for cells, a perfusion bioreactor was designed and tested. The main aim of this bioreactor was:

- To get nutrition to cells everywhere on the 3-dimensional scaffolds
- To provide mechanical stimulation that could inspire cells to create an extracellular matrix in a shorter time period
- To obtain a more homogeneous growth than in static culture

To calculate the mechanical stimulation on scaffolds in bioreactor, a theoretical model was used, with the appropriate approximations, and comparable results with the values present in the literature [49] were obtained. During cell culture, to improve extracellular matrix growth and calcium deposition, six porous titanium scaffolds were used (three in static culture and three in bioreactor culture); they were seeded with osteoblastic progenitor cells (hES-MP) and perfused with osteogenic medium. After three weeks, the culture was interrupted and scaffolds were fixed in formalina and afterwards washed several times with PBS to remove it and to obtain scaffolds usable for scanning by µCT.

For tissue engineering applications, microCT imaging may provide an efficient, non destructive tool to quantitatively measure the amount and distribution of mineralized matrix formation throughout 3D construct *in vitro* and *in vivo* [63].

The tomographic image data obtained, in DICOM format, were imported into the “Mimics” software and they were appropriately segmented, in order to obtain, from each of two type of culture, 30 samples coming from surface and 30 samples coming from inside of the scaffolds. A Matlab script was implemented to compare tomographic data relative to scaffolds in different conditions (three maintained in static culture and three in bioreactor culture, compared with one empty as tester). The script highlighted the evolution of the GV gradient in segmented scaffolds and from it a GV Gradient Distribution was obtained. All diagrams showed two Gaussian distribution; one big and one small. In particular, the center of smaller Gaussian distribution had a tendency to increase, greater in bioreactor samples respect to the static samples. Same results were observed in the 3D visualization of the GV Gradient Distribution, where the intensity of color changed greater in bioreactor samples. Changes of this distribution could indicate possible structural changes in scaffolds maintained in the bioreactor.

To confirm these results, statistical analysis was performed by five T-Test confirming that:

- Use of the bioreactor had made significant improvements to the structural changes in scaffolds maintained in it

- In the specimens taken from the surface and inside of static culture, structural changes occurred in non homogenous way (likely more in the surface than in the inside) and this result could not be observed with the analysis of GV Gradient Distribution

- In the samples taken from surface and inside of bioreactor culture, the structural changes occurred in homogenous way

- The results coming from use of GV Gradient Distribution was statistically significant

Although good results were obtained, limitations are still present due to:

- The high number of parts to be manipulated to assemble the bioreactor

- The replacement of the media non-automated

- The lack of sensors useful for monitoring parameters such as the speed of the flow

- The limited number of scaffolds analyzed

In particular, the first two limitations are those that increase the risk of infection; the other limitations, instead, if resolved would improve the quality of results.

## 4.1 Future Directions

The studies, coming from use of bioreactor discussed in this thesis, will be used to design a new version that will make it possible to prepare and test more than 20 scaffolds at the same time making it possible to further analyze the quality of the differentiation using molecular and histochemical methodology.

To do this, different changing should be applied in order to improve the structure and usability of the device. Possible changing might be: adding useful tools to monitoring parameters as e.g. the flow velocity, decrease number of parts to manipulate during the device assembly, automate the media replacing. In particular, these last two change might help to decrease the bacteria infection risk.

# 5. Conclusion

Bioreactors have been shown to be used to improve cell seeding efficiency [64-66], cell proliferation [67-69], and mesenchymal stem cell osteoblastic differentiation [70-77]. In addition, to enhancing differentiation and proliferation, perhaps the most notable contribution of bioreactor systems to a bone tissue engineering strategy is the possibility of automation. A clinically relevant strategy must greatly minimize the risk of contamination from bacteria and other cells, reduce labor intensity, and reduce costs associated with *in vitro* cell culture. Bioreactor systems have the potential to minimize all of these aspects through automated cell culture.

Furthermore, scaffolds with stem cells obtained through the use of bioreactors, at the end of the culture period, might be implanted into the patients, affected by cancer, accidents or of serious infections and many other sicknesses, in order to replace bone defects.

# Appendixes

## Appendix A

*Equipment*

| Reagent | Manifacturer |
|---|---|
| **DMEM F12 + Glutamax medium** | Gibco, Grand Island, NY, USA |
| **Penicillin-Streptomycin** | Gibco |
| **Fetal Bovine Serum (heat inactivated or MSC screened)** | Gibco |
| **10% Platelet lysate media** | Bloodbank, Reykjavik, Iceland |
| **PBS** | Gibco |
| **Confluent hES-MP cells in monolayer** | Cellartis, Gothenburg, Sweden |
| **0.1% Gelatin** | Sigma Aldrich, St. Louis, MO, USA |
| **0.25% Trypsin** | Gibco |

Table 1 - Reagents used for harvesting stems cells

| Component | Manifacturer |
|---|---|
| **75cm cell culture flasks** | Nunc, Penfield, NY, USA |
| **Tubes (15 and 50 ml)** | Falcon, Franklin Lakes, NJ, USA |
| **Pipettes and tips** | Gilson, Middleton, WI, USA |

Table 2 - Components used for harvesting stems cells

| Device | Manifacturer |
|---|---|
| **Centrifuge** | Thermo Scientific, Vantaa, Finland |
| **Incubator (5% $CO_2$, 95% $H_2O$)** | N/A |

Table 3 - Devices used for harvesting stems cells

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times.

**hES-MP002.5 are cells of human origin. Same procedures that apply to other tissue of human origin such as blood should be followed.**

| Component | Storage | Hazards and Precautions |
|---|---|---|
| **DMEM F12 +glutamax** | 4-8°C | Not dangerous |
| **Penicillin Streptomycin** | -20°C | **Danger**<br>Skin corrosion, serious eyes damage, respiratory and skin sensitization, reproductive toxicity |
| **Fetal bovine serum** | -20°C | Not dangerous |
| **Platelet lysate** | -20°C | Not dangerous |
| **Sterile Gelatin** | 4-8°C | Not dangerous |
| **Sterile PBS** | RT | Not dangerous |
| **Trypsin** | -20°C | Not dangerous |

Table 4 - Precautions for the components used during harvesting stems cells

*Procedure*

*Preparation of solutions*

For the preparation of cell culture media, the following protocols was applied:

- Preparation of Culture media with 10% Platelet lysate in DMEM F12 +glutamax.
- Preparation of Culture media with 10% FBS in DMEM F12 +glutamax.

*Preparation of 0.1% gelatin solution*

- Bringing 2% gelatin stock solution to room temperature.
- Working under sterile conditions in a clean hood with the fan on.
- In a 50 ml tube mixing 47,5 ml sterile $H_2O$ with 2,5 ml 2% gelatin.
- Storing at 4-8°C.

*Coating cell culture flasks and preparation*

- Blending media or obtaining already prepared media
  - Making sure that media has 0.1% Penicillin streptomycin and 10% supplement (either PL or FBS)
  - If using platelet lysates (PL) making sure that media has 4IU/ml of heparin
- Bringing the media and the trypsin to 37°C in the shaking plate incubator
- If preparing cells to be reseeded, working sterile and add 5 ml of 0.1% gelatin solution to a sterile 75$cm^2$ cell culture flask (67µl/$cm^2$) or other culture vessel of your choice.
- Close the flask, allowing the gelatin to completely float over the culture surface and keeping at 4-8°C for at least 30 min.
- Making sure that your flask is carefully labeled with your name, date, cell type, media type and passage number.

*Harvesting cells*

- Preparing the laminar flow hood with clean o Pipettes and tips
  - Waste container with chlorinated water o Empty lab tubes
  - Reagents to be used
- Obtaining the cell culture from the incubator and placing inside the hood.
- Loosening the cap and pouring the media into the waste container.

- Transfering 5 ml sterile PBS (67µl/cm$^2$) into the cell culture flask. Close the flask and allowing the PBS to float over the culture surface by rocking the flask gently.
  - This will wash excess media from the culture flask and ensure better effectiveness of the trypsin in next steps.
- Pouring the PBS off and then putting 5 ml of warmed Trypsin into the flask.
- Close the flask and putting it into the incubator for 5 min
  - The trypsin, as a digestive enzyme, will release the cells from the plastic surface.
  - When the cells are released they will appear small, round and bright in the microscope and float around creating a “snow storm” impression.
  - **In this step the cells should be monitored carefully since prolonged trypsin digestion can harm the cells. When the cells are released proceed immediately to next step.**
- Returning the cell culture flask into the hood and adding 5 ml of pre-warmed media into the flask.
  - The supplemented media will help neutralize the trypsin and stop the digestion.
- With the aid of a pipettboy, using the cell solution inside the flask to wash the culture surface and then transferring the solution to a 15 ml tube.
- Centrifugating for 5 min at 1750 rpm.
- Pouring of the supernatant into the waste container and resuspending the cells in 1 ml culture media.
  - If the cell solution is dense, like when combining cells from many culture flasks into a single tube, resuspend the cells in as many ml as the number of culture flasks. 1 culture flask = 1 ml.
- Proceed to cell counting. Seeing the following protocol:
  - Counting cells with hemocytometer
- When the number of cells has been obtained they can be reseeded, frozen or used for experimentation.
- If reseeding, using gelatin coated culture flask and using 5500 cells/cm$^2$ seeding density.

*Precautions*

**Use sterile working techniques without exceptions when handling cells for culture.**

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Always use a clean water-bath. Mark the medium bottle as supplemented and write your name and date (as for all solutions you work with). Always take the volume you expect to need from the a stock bottle for media or PBS and place in a different sterile container (like 50 ml plastic tubes) before continuing work. This is to prevent contaminating your medium bottle. Do not spray ethanol on culture flasks because the cells are sensitive to ethanol.

# Appendix B

*Equipment*

| Reagent | Manifacturer |
|---|---|
| **Trypan Blue** | Gibco, Grand Island, NY, USA |
| **PBS** | Gibco |
| **Cell solution of unknown concentration** | |

Table 1 - Reagents used for counting cells

| Component | Manifacturer |
|---|---|
| **Microtubes** | Sarstedt, Nümbrech, Germany |
| **Pipettes and tips** | Gilson, Middleton, WI, USA |

Table 2 - Components used for counting cells

| Device | Type | Producer |
|---|---|---|
| **Hemacytometer** | Neubauer | Assistant, Munich, Germany |
| **Cell counter** | | N/A |
| **Microscope** | | Lumenera, Capella court, Ottawa, ON, Canada |
| **Calculator** | | N/A |

Table 3 - Devices used for counting cells

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times.

| Component | Storage | Hazards and Precautions |
|---|---|---|
| **Trypan Blue** | RT | **Danger**<br><br>May cause cancer<br>Suspected of causing genetic defects<br>Suspected of damaging fertility or the unborn child |
| **PBS** | RT | Not dangerous |

Table 4 - Precautions for the components used during counting cells

*Procedure*

*Preparation of stained cell solution*

- To a microtube adding
  - 50µl Trypan Blue
  - 30 µl PBS
  - 20µl Cell solution.
- Mixing well
- **Note! Never bring your cell solution outside of the cell hood.**
  o You want your cell solution to remain sterile. Simply transfer a sample of the cell solution into the staining solution inside the hood under sterile operating procedures.
- Afterwards, the staining solution containing the cell sample can be brought outside the hood.

*Cell Counting*

- Preparing the counting chamber
  - Making sure the counting surface of the Neubauer hemacytometer is clean
  - Placing the cover slide on top of the counting surface
    - The cover slide will stay in place on the counting chamber via capillary

force. Trying to breathe gently on the glass and then placing it immediately on the counting chamber.

- With a pipette transferring a small amount of the cell solution into the hemocytometer by placing the tip at the end of the cover slide and gently allowing the solution to be drawn under cover slide until the area is covered. Do not overfill.
- Counting all the cells in the four big corner squares. Excluding cells that stain blue.
- If more than 200 cells are counted per big square, diluting the solution and repeating.

*Calculation*

- The volume of each square is 1 $mm^3$ or $10^{-3}$ $cm^3$. 1 $cm^3$ equals 1 ml so the count can be found with the following formula:

Cells/ml = Average count per square * dilution factor * $10^4$

- If the protocol above is followed the total cell count is divided by 4 and the dilution factor is 5

$$\frac{cells}{ml} = \frac{Total\ cell\ count}{4} \cdot 5 \cdot 10^4$$

- To obtain the total cell count of the original solution, the outcome is simply multiplied by the original volume of the solution
- It's recommended to count each stained solution few times (2-4 times) and mix thoroughly between counts to obtain more accurate results.

Figure 1 - The figure show in the centre the Microscope, bottom right the cell counter

*Precautions*

**Use sterile working techniques without exceptions when handling materials intended for cell culture.**

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Tryphan blue is a histological dye to stain tissues. Wearing protective gloves and safety googles is recommended since both fingers and eyes are covered with tissue that you don't want to stain!

Wear lab coat, the stain will not wash out of clothes.

# Appendix C

*Equipment*

| Reagent | Manifacturer |
|---|---|
| **Fibronectin (5 µg/ml)** | Sigma Aldrich, St. Louis, MO, USA |
| **PBS** | Gibco, Grand Island, NY, USA |
| **Cell culture media (e.g. a-MEM, DMEM)** | Gibco |

Table 1 - Reagents used for scaffolds preparation

| Component | Manifacturer |
|---|---|
| **20 ml syringe** | Braun, Melsungen, DE |
| **Syringe-driven sterifilter** | Millipore, Billerica, MA, USA |
| **Pipettes and tips** | Gilson, Middleton, WI, USA |
| **24 well plate – non-tissue treated** | BD Falcon, Franklin Lakes, NJ, USA |
| **Sterile porous titanium scaffolds** | Icelandic Innovation Center, Reykjavik, Iceland |

Table 2 - Components used for scaffolds preparation

| Device | Type | Producer |
|---|---|---|
| **Warming/shaking incubator** | Unimax 1010 | Heidolph, Schwabach, DE |
| **Pincett** | Gibco, Grand Island, NY, USA | N/A |

Table 3 - Devices used for scaffolds preparation

Protective equipment should be worn while handling the components of this assay. Wear lab coat and protective gloves.

| Component | Storage | Hazards and Precautions |
|---|---|---|
| **Cell culture media** | 4-8°C | Not dangerous |
| **Fibronectin** | -20°C | Not dangerous |
| **PBS** | 22°C | Not dangerous |

Table 4 - Precautions for the components used during scaffolds preparation

*Procedure (Figure 1)*

- Preparing a 5µg/ml fibronectin solution in PBS.
  - 1 ml per scaffold is needed + surpluses for the infiltration step
- Adding 1 ml of fibronectin solution into a 24 well plate, 1 well per scaffold.
- Opening the syringe and placing the scaffolds in it.
- Drawing fibronectin solution into the syringe, enough to completely submerging the scaffolds in and pushing out any air in the syringe.
- Attaching a syringe-driven steril filter to the syringe.
- Putting your finger on the tip of the steril filter and pulling the plunger to allow air to be drawn out of the scaffolds.
- Releasing the plunger and allowing the fibronectin solution to infiltrate the scaffolds.
- Repeating few times, pushing out any air that accumulates in the syringe.
- When the scaffolds have been thoroughly infiltrated with the fibronectin solution, transferring them into the 24 well plate.
- Covering the scaffolds with a fibronectin solution, using the rest of the syringe solution if necessary.
- Putting on a shaker for 3 hours at 37°C.
- After incubation, replace the fibronectin solution with cell culture media.
- Allowing to rest in a culture incubator overnight.

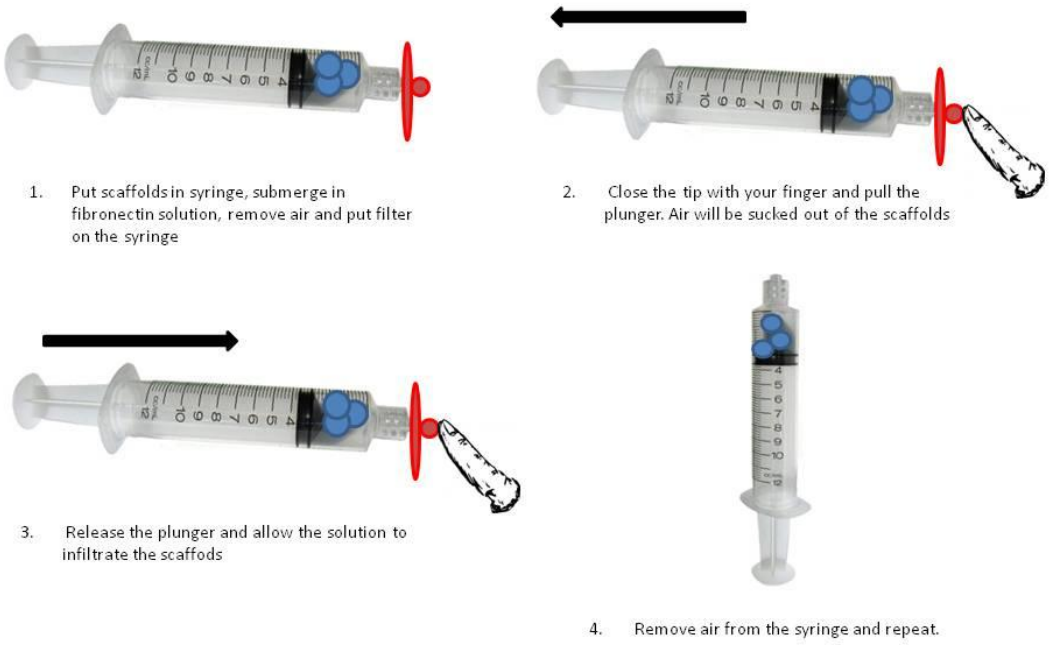



Figure 1 - Procedure followed to treat the scaffolds with fibronectin

*Precautions*

**Use sterile working techniques without exceptions when handling materials intended for cell culture.**

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

# Appendix D

*Equipment*

| Reagent | Manifacturer |
|---|---|
| **Cell culture media (e.g. a-MEM, DMEM)** | Gibco, Grand Island, NY, USA |
| **Cell culture supplement (e.g. FBS, hPL)** | |
| **Cell solution (e.g. MC3TE, MSC or hES-MP)** | |

Table 1 - Reagents used for cell culture

| Component | Manifacturer |
|---|---|
| **24 well plate – non-tissue treated** | BD Falcon, Franklin Lakes, NJ, USA |
| **Sterile porous titanium scaffolds** | Icelandic Innovation Center, Reykjavik, IS |
| **Pipettes and tips** | Gilson, Middleton, WI, USA |

Table 2 - Components used for cell culture

| Device | Producer |
|---|---|
| **Centrifuge** | Thermo Scientific, Vantaa, Finland |
| **Incubator (5% $CO_2$, 95% $H_2O$)** | N/A |

Table 3 - Devices used for cell culture

Protective equipment should be worn while handling the components of this assay. Wear lab coat, protective gloves and safety googles at all times.

*Procedures*

- Obtaining the plate with 6 coated scaffolds in cell culture medium from the overnight incubation.
- Removing the scaffolds from the medium and placing in a new 24-well plate.

- Allowing the scaffolds to dry for 1 hour at 37°C in the bench top incubator
- Adjusting your cell solution to 4000 cells/µl (100.000 cells/ 25µl)
    - Obtaining the correct amount of cells needed in a test tube.
        - You need 100.000 cells per scaffold
    - Centrifuging for 5 min at 1750 rpm (545 x g)
    - Discarding the supernatant
    - Resuspending the cells in 25µl of cell culture media for each scaffold.

- Seeding 25 µl (100.000 cells) drop by drop on each scaffold and incubating in the $CO_2$ incubator for 15 min.
    - **Mixing the cell solution well prior to seeding each scaffold.**
- After 15 min, removing the scaffold-plate from the incubator and aspirating and reseeding any medium that has leaked from the scaffolds.
- Returning the plate to the incubator for another 15 min.
- Repeating for a total of 4 times.
- At the end of the $4^{th}$ seeding
    - Getting 3 of 6 scaffolds and add it into the 3 wells of 24-well plate; after adding 1 ml media for each well.
    - Getting the remaining 3 scaffolds and adding it into the bioreactor; after adding the necessary media.
- Returning to the incubator for culture.

*Precautions*

**Use sterile working techniques without exceptions when handling cells for culture.**

This includes cleaning everything with ethanol prior to entering the hood, including gloved hands.

Mixing the cell solution prior to seeding is important. No blending will result in inaccurate seeding density and negatively affect the experiment.

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