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DEVELOPMENT AND FINE TUNING OF A GMP PRODUCTION PROCESS FOR THE MANUFACTURING OF 2D/3D SCAFFOLDS FOR REGENERATIVE MEDICINE

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Anno Accademico 2013 – 2014

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SUMMARY

Tissue engineering constitutes an important support to regenerative medicine to repair damaged tissues towards the use of polymeric biomaterials. Synthetic and natural polymers are employed to produce three-dimensional highly porous fibers-made scaffolds in order to guide cells organization and tissue reconstruction.

In the initial part of this thesis project an overview about the state of the literature and the different available methods used in the field of tissue engineering was obtained at SUPSI (Manno, TI).

Therefore, the attention was focused on the aims and main treatment options of cartilage repair, in particular the use of biomaterials to produce hollow microfibers that allow the cells used in this kind of applications, mesenchymal stem cells, to adhere to the surface and grew inside the cavity of the fibers.

In order to create fiber-based scaffolds for cartilage tissue repair, two artificial biomaterials were investigated: poly-L-lactic acid (PLLA) and poly-caprolactone (PCL).

The apparatus used in this study was realized by Gimac (Castronno, Italy) and is based on the dry-wet spinning technique to produce different types of porous fibers through the control of process parameters.

This production process requires the determination of the appropriate operative conditions to allow phase inversion, in order to obtain fibers with the suitable porosity for their final use. Therefore, on the basis of the theory behind polymeric mixtures, the experiments were conduced using different polymeric solutions and non-solvents.

Then, through a purposeful procedure, two arrangements of the hollow fibers (random and ordered) were used to produce different types of scaffolds.

For regenerative medicine aims, it is necessary that these scaffolds allow cells adhesion on the target tissue to ensure significant clinical results.

For this reason, mesenchymal stem cells were seeded on the matrices produced to investigate their adhesion on the surface of biomaterial used, their vitality and growth.

Biological characterization of the samples was conduced, through in vitro tests, at University of Pisa, to evaluate the effective functioning of scaffolds realized during this project.

SOMMARIO

L'ingegneria dei tessuti costituisce un valido supporto per la medicina rigenerativa per riparare i tessuti danneggiati attraverso l'uso di biomateriali polimerici. I polimeri sintetici e naturali sono impiegati per produrre scaffolds tridimensionali costituiti da fibre porose al fine di guidare l'organizzazione cellulare e la ricostruzione del tessuto.

Nella parte iniziale di questo progetto di tesi, svolta presso la SUPSI (Manno, TI), è stata fatta una panoramica riguardante la letteratura e i differenti metodi disponibili nell'ambito dell'ingegneria dei tessuti.

Sono stati quindi analizzati gli scopi e le principali opzioni di trattamento della riparazione della cartilagine, in particolare l'uso di biomateriali per produrre fibre cave che consentano alle cellule usate per questo tipo di applicazioni, cellule staminali mesenchimali, di aderire alla superficie e crescere all'interno della cavità delle fibre.

Sono stati considerati due biomateriali artificiali per la produzione di fiber-based scaffolds per riparare i tessuti cartilaginei: acido polilattico (PLLA) e policaprolattone (PCL).

Il macchinario usato in questo lavoro è stato realizzato dalla ditta Gimac (Castronno, Italia) e il suo funzionamento è basato sulla tecnica della filatura secco-umido per produrre differenti tipi di fibre porose tramite il controllo dei parametri di processo.

Il processo di produzione richiede la determinazione di appropriate condizioni operative per permettere l'inversione di fase, in modo da ottenere fibre con porosità adeguata per il loro uso finale. Quindi, sulla

base della teoria sulle miscele polimeriche, sono state condotte delle prove sperimentali utilizzando diverse soluzioni polimeriche e non solventi.
Utilizzando quindi una procedura specifica, sono state realizzate due disposizioni delle fibre cave (disordinata e ordinata) per produrre differenti tipologie di scaffolds.
Per gli obiettivi della medicina rigenerativa è necessario che gli scaffolds consentano l'adesione cellulare sui tessuti bersaglio al fine di ottenere risultati clinici significativi.
Per questo motivo, le matrici prodotte sono state caricate con cellule staminali mesenchimali per verificarne l'adesione sulla superficie del biomateriale utilizzato, la loro vitalità e crescita.
La caratterizzazione biologica dei campioni è stata condotta, attraverso test in vitro, presso l'Università di Pisa, per valutare l'effettivo funzionamento degli scaffolds realizzati durante questo progetto.

1. INTRODUCTION

1.1 Tissue Engineering

Tissue engineering is an emerging interdisciplinary field that combines the principles of life sciences and engineering to develop biological artificial substitutes to repair, maintain or improve deteriorated tissue or failed organ functions [Langer et al., 1993].

In 1930s Bisceglie gave one of the first demonstration that tissue engineering was possible and an early example of cells encapsulation was showed: he placed mouse's tumor cells in a polymer membrane and then put them into a pig's abdominal cavity. With these studies he could show that cells could survive and not be destroyed by the immune system [Ma et al., 2006]. The term Tissue Engineering was used for the first time at the end of 1980s, when this discipline moved its first step; after that it first grew slowly but then accelerated rapidly: nowadays many tissues, for example skin, cartilage, bones and others can be regenerated with manmade biodegradable carriers [Jayo et al., 2008].

Quite often the term regenerative medicine is used as synonymous of tissue engineering because they both are research fields focusing their attention on production of artificial support to repair and regenerate damaged tissues, but regenerative medicine is more concentrated on the use of stem cells to produce tissues [Greco, 2008].

Tissue engineering usually applies three main approaches: the first one uses isolated cells or cell substitutes to replace those cells that supply the

needed function; the second takes advantage of the distribution of tissue-inducing substances, such as growth and differentiation factors, to specific locations; the third one provides the growth of cells in three-dimensional scaffolds [Comeau, 2007].

The third method is the most important and used because the other two methods can be applied only when the defect is small. It can be applied to treat larger injuries, in fact it uses three-dimensional scaffolds, which should be tailored in size and shape to the damaged area (fig.1), to allow different cells types to grow in the proper orientation and differentiate appropriately [Comeau, 2007].

Traditionally the aim of tissue engineering was to repair or replace injured tissue, but its development produced a culture model system that controls biochemical and mechanical factors, cells and materials [Greco, 2008].

The main challenge of tissue engineering is to increase healing of a defect by using body's own mechanism, toward the production of bio-artificial implants that occur with the deposition of appropriate cells on an artificial extracellular matrix or scaffold, to guide their growth [Yang et al., 2001].

The main research of tissue engineering is driven by the necessity of having new tissues, external to human body, and by the decreasing availability of donor organs for therapeutic aims. In fact, it provides in vitro culture of cells that allows development and organization of a new tissue with the same characteristics of the original one. To reach this goal, it is mainly necessary to give the proper three-dimensional support, in addition to nutritive substances and growth factors [Cancedda, 2002].

Tissue engineering provides *in vitro* culture conditions to mimic the biochemical and physical signals to regulate *in vivo* functional tissue development towards bioreactor culture vessels. The system composed by cells, polymer and bioreactor controls tissue development towards *in vitro*

studies to create implants for *in vivo* tissue regeneration. In this kind of studies, cells should first proliferate and then differentiate *in vitro* to control their capacity of reproduction [Atala et al., 2002].

Natural tissues are characterized by a structure composed by connective tissue to link the cells to form the organ. Connective tissue is composed by structural molecules, such as collagen type I, II, III, IV, and other molecules with adhesive or regulative function; many of these molecules are used to create biomaterial to produce bio-artificial support (scaffold), where cells can grow in vitro [Cancedda, 2002].

In this study scaffolds of synthetic polymers were created to guide cells growth to repair connective tissue, in particular cartilage tissue.

The implantation of scaffold alone to regenerate adjacent tissues is successfully used only in bone repair but not in cartilage repair and this is due to the fact that the chondrocytes have a low capacity to proliferate and migrate. For this reason the scaffolds are associated with the use of cells or growth factors [Atala et al., 2002].

There are many causes and types of cartilage injuries that use different strategies in the field of tissue replacement. Generally cartilage repair is necessary when articular, nasal, auricular, rib cartilage, intervertebral disc and meniscus trauma occurs. Cartilage has a low capacity to repair itself, that's why loss of cartilage tissue is difficult to treat. This is the reason why research has focused its attention on the field of cartilage tissue engineering for the last 20 years [Atala et al., 2002].

Tissue engineering usually involves four main steps:

1. Identify, isolate and produce an appropriate cell source in sufficient amount;
2. Produce an appropriate biocompatible cell carrier into the desired

shape and dimensions;

3. Uniformly seed the cells onto or into the carrier and incubate for a predetermined period in a bioreactor;
4. Implant the seeded carrier in the proper animal model [Ma et al., 2006].

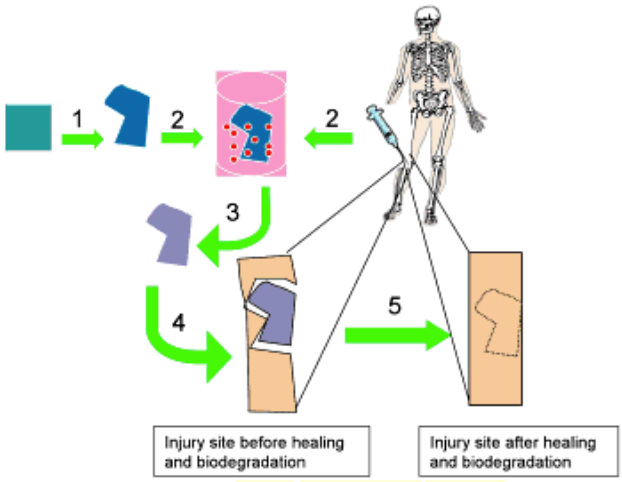

Figure 1. General tissue engineering scaffold approach [Comeau, 2007]

1.2 Aim of the present study

The aim of this study is the tuning of a GMP (Good Manufacturing Practice) production process to produce biodegradable polymeric matrices, which can be used as scaffolds for applications of regenerative medicine, in particular to repair connective tissue, specifically for cartilage repair.

One of the main challenges of tissue engineering and regenerative medicine is represented by the use of cell therapies based on stem cells.

It is necessary the use of appropriate bio-artificial structures that allow stem cells to remain in the destination site and do not disperse in the circulatory flow.

This project is focused on these ideas, in fact a process to produce scaffolds to seed mesenchymal stem cells (MSC) for regenerative medicine of connective tissue was studied. The main problem of using this type of cells to repair tissues is the adhesion of the cells to the target tissue. Hence the need of using specific scaffolds to allow cells to adhere to the target tissue to ensure engraftment with significant clinical results.

During this study, the state of the literature and of the different available methods was studied at University of Applied Sciences and Arts of Southern Switzerland (Manno, TI).

Then the technical state of the art relative to the machinery to use, a dry-wet spinning apparatus, produced by Gimac (Castronno, Italy), was analyzed.

It was then conduced a period of tests designed to parameterize the chemical-physical characteristics of the products to obtain, in function of the operative conditions of the process and the optimal process parameters have been tuned.

Different kind of polymeric scaffolds were produced and due to the need of integration with the tissue, they should be degradable in times and ways compatible with the final aim and specific for stem cells adhesion.

The response of effective functioning of matrices developed during this study is based on cellular tests on the samples produced.

2. SCAFFOLDS FOR CARTILAGE REPAIR

2.1 Cartilage Repair

2.1.1 Articular cartilage

Cartilage is a connective tissue composed of specialized cells called chondrocytes that produce a large amount of extracellular matrix (ECM) composed of collagen fibers, abundant ground substance rich in proteoglycan, and elastin fibers [Mescher, 2013].

The extracellular matrix has a high concentration of glycosaminoglycan (GAG) chain(s) and proteoglycans, which interact with collagen and elastic fibers. Chondrocytes synthesize and maintain ECM components and are located in matrix cavities called lacunae.

Variations in the composition of these matrix components and cells produce three types of cartilage adapted to local biomechanical needs.

The principal macromolecules present in all types of cartilage matrix are collagen, hyaluronic acid, proteoglycans, and various glycoproteins [Mescher, 2013].

There are three types of adult cartilage distributed in many areas of the skeleton, particularly in joints and where pliable support is useful, as in the ribs, ears, and nose (fig. 2a).

The firm consistency of the cartilage ECM allows the tissue to bear mechanical stresses without permanent distortion. In the respiratory tract,

ears, and nose, cartilage forms the framework supporting soft tissues. Because of its smooth, lubricated surface and resiliency, cartilage provides shock-absorbing and sliding regions within joints and facilitates bone movements.

There are three types of cartilage, each varying somewhat in matrix composition:

1. *Hyaline cartilage* is the most common form and type II collagen is the principal collagen type (fig. 2b)
2. *Fibrocartilage* is characterized by a matrix containing a dense network of coarse type I collagen fibers and is present in body regions subjected to pulling forces (fig. 2c).
3. *Elastic cartilage* is the more pliable and distensible form, it possesses abundant elastic fibers in addition to collagen type II (fig. 2d).

In all three forms, cartilage is *avascular* and receives nutrients by diffusion from capillaries in adjacent connective tissue (perichondrium). In some instances, large blood vessels traverse cartilage to supply other tissues, but these vessels release few nutrients to the cartilage.

The physical properties of cartilage depend on electrostatic bonds between the flexible collagen, elastin fibers and the GAGs on densely packed proteoglycans. This allows cartilage to serve as a shock absorber, a role of major functional importance [Mescher, 2013].

Articular cartilage is composed by two different phases, the liquid phase constituted of aqueous solution of electrolytes and the solid phase composed by collagen and proteoglycans matrix.

Macroscopic properties of cartilage can be determined towards different

experiments to analyze the amount of different phases [Cancedda et al., 2002].

The mechanical properties of cartilage are influenced by several factors, such as, for example, collagen cross-link density, collagen fiber diameter, orientation of collagen fibers, number of zones of layers, proteoglycan content, rate of deformation and source of tissue. Proteoglycans have a high negative surface charge that amplifies the charge density of collagen matrix; this contributes to better mechanical properties because this high concentration of charges donates osmotic pressure within the collagen matrix [Silver, 1994].

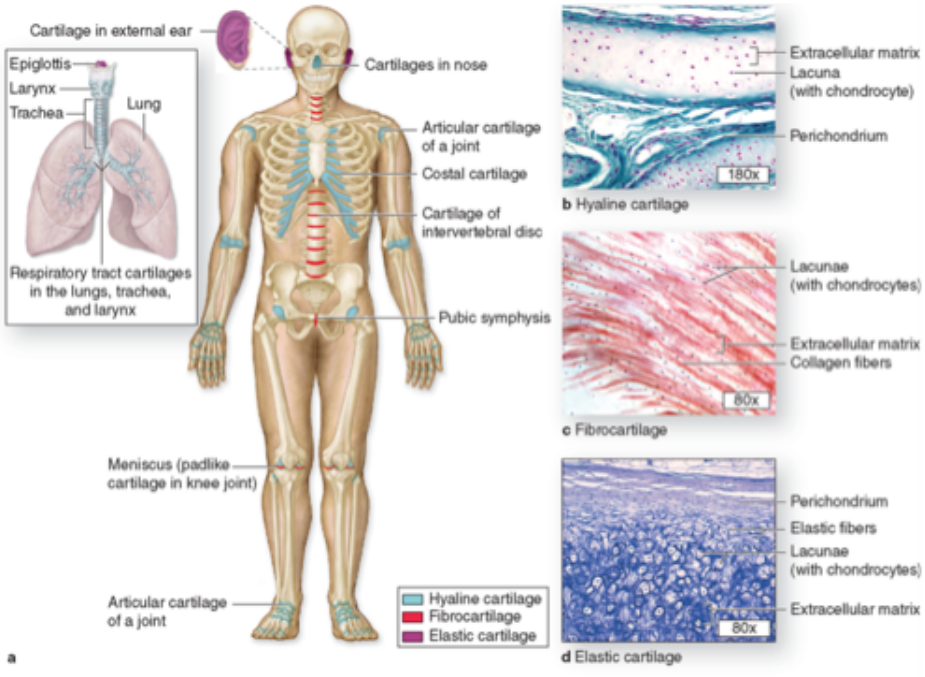

Figure 2. Cartilage [Mescher, 2013]

Natural cartilage is a highly hydrated porous gel; when a load is applied to

ECM, chondrocytes have a change of osmotic pressure, electric field, stress, deformation and hydrodynamic flow in space and time, due to interstitial flows. The outflow of interstitial liquid provokes a deformation of cartilage and its mechanical properties decrease with increasing the amount of liquid that can outflow and be resorbed from cartilage matrix. Extracellular fluid bears the compression load applied to articular cartilage; when the outflow velocity is higher, the rate of cartilage deformation is higher, so that the load is not uniformly distributed on the adjacent bone [Cancedda et al., 2002].

Hyaline cartilage is mainly composed by type II collagen fibers, which let the cartilage be strong and resistant, and proteoglycans, which give cartilage the possibility to swell under compression, thanks to their capacity to keep water. An example is constituted by articular cartilage, a particular kind of hyaline cartilage that covers bone joints; when a compressive load is applied, the high amount of water allows cartilage to uniformly dispose the load to the surrounding bone (fig. 3). Cartilage is softer than bone so, when a load is applied, it shows a higher deformation increasing contact area beneath the joints and reduces stress [Standring, 2009].

Articular cartilage can be injured as a result of *mechanical destruction,* which is a direct trauma that can injure the articular cartilage, or *mechanical degeneration*, the progressive loss of the normal cartilage structure and function [Romanelli et al., 2008].

Articular cartilage cells can sometimes heal, depending on the extent of the damage and the location of the injury. This is due to the fact that articular cartilage is avascular, so that it has no capacity to heal itself, and to the fact that adult chondrocytes don't migrate or replicate to fill defects.

When mechanical degeneration occurs, the initial loss begins with cartilage softening then progresses to fragmentation. As the loss of the articular cartilage lining continues, the underlying bone has no protection from the normal wear and tear of daily living and begins to break down, leading to osteoarthritis [Romanelli et al., 2008].

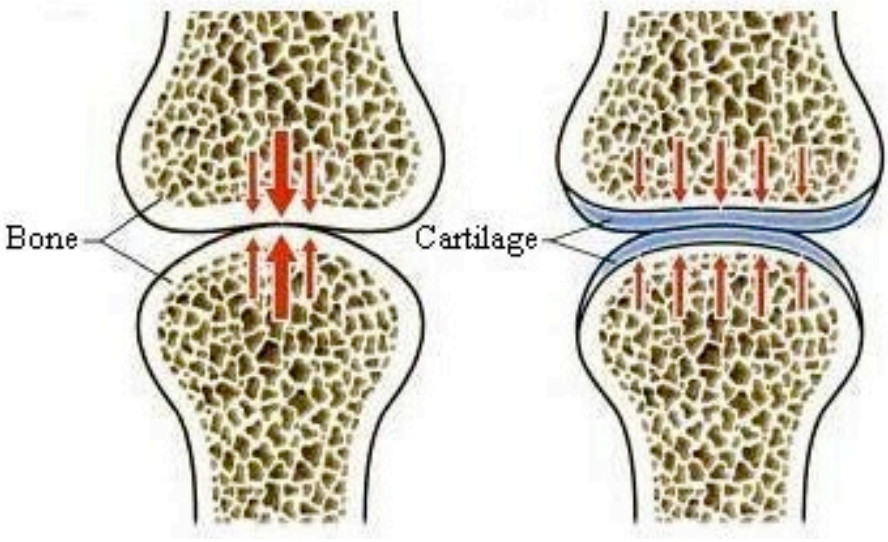

Figure 3. Mechanical behaviour of cartilage [Standring, 2009]

2.1.2 Goals of Cartilage Repair

Articular cartilage is a connective tissue that provides a friction-free surface between joints, which can uniformly distribute loads but has a low

capacity to regenerate itself once damaged because it contains no vascular supply and chondrocytes are imprisoned in a dense extracellular matrix that restrains their possibility to move and contribute to heal the injury [Cancedda et al., 2002].

For this reason tissue engineering focuses its attention on cartilage regeneration in vitro to develop new possibilities of restoration of damaged hyaline cartilage in the joints, for example as a consequence of different events such as traumatic sport injuries, repetitive use of the joint, congenital malformation that affect normal joint structure and hormonal disorders that affect bone and joint development [Cancedda et al., 2002].

The primary challenges of cartilage repair are the restoration of smooth articular cartilage surface, the alleviation of patient symptoms and improvement of function, the equalization of biomechanical and biochemical properties of normal hyaline cartilage and prevention or slowing down the progression of focal chondral injury to end-stage arthritis [www.fda.gov].

The main goals in cartilage repair methods are to fill the defect and to restore the articular damaged surface [Bos et al., 2010].

There are different procedures for cartilage repair and regeneration that are designed to heal the cartilage by filling the cartilage defect. The choice of the procedure depends on the size and location of the defect.

One approach to cartilage repair consists of developing functional cartilaginous systems that can be implanted to fill the defect [Cancedda et al., 2002].

Cartilaginous systems are generated in vitro with combination of three components: chondrogenic cells, polymeric ‘scaffolds’ that provides a three-dimensional structure for tissue development and bioreactors that constitute an advantageous environment to control chondrogenesis.

To prevent the degeneration of joints and let pain-free movement, two different factors are necessary: biomechanical properties similar to hyaline cartilage that last for a long period and the proper integration with the surrounding articular cartilage [Bos et al., 2010].

2.1.3 Treatment options

Articular cartilage can be commonly damaged as a consequence of traumatic mechanical events, continuous repetitive micro-traumas of cartilage and applied overloads [Mantero et al., 2009].
These events, sometimes common even in young people, can lead to localized damages and progression of joint degeneration that often brings to osteoarthritis.
During the past 30 years, several surgical techniques have been developed to solve or palliate these problems.
The different surgical treatment methods used in cartilage repair include symptomatic treatments (e.g. debridement and lavage), mosaicplasty, osteochondral allograft/autograft transplantation, sub-chondral marrow stimulation techniques (e.g. microfracture), autologous chondrocytes implantation (ACI) and several techniques of tissue engineering that use cells and biomaterials to repair the injury of cartilage and bone [Bos et al., 2010].
The first approach to cartilage repair is *debridement and lavage*, with the main aim of improvement the shape of the cartilage defect by shaving the superficial cartilage layers and the lavage of the entire joint cavity with several lavage fluids [Lanza et al., 2000].

The *mosaicplasty* is a method introduced for the first time in 1990s and it utilizes individual own bone portions with the above cartilage withdrawn from the injured joint and grafts them in the cartilage defect. This technique is utilized when the defect is small, in particular the size should be between 1 and 4 cm^2.

The disadvantages of this method are the low availability of donor tissue and the possibility of donor site diffused damage [Bos et al., 2010].

When the wound involves the sub-chondral bone, the entire autologous bone-cartilage unit should be reintegrated towards *osteochondral autograft* (fig.4). The main advantage is the combination between hyaline-like cartilage with living chondrocytes and a bone part with the role of support and anchorage.

If the dimension of the injury is too big, *osteochondral allograft* is used; from the immunologic point of view, this implantation is tolerated because extracellular matrix protects donor chondrocytes from host immunological system.

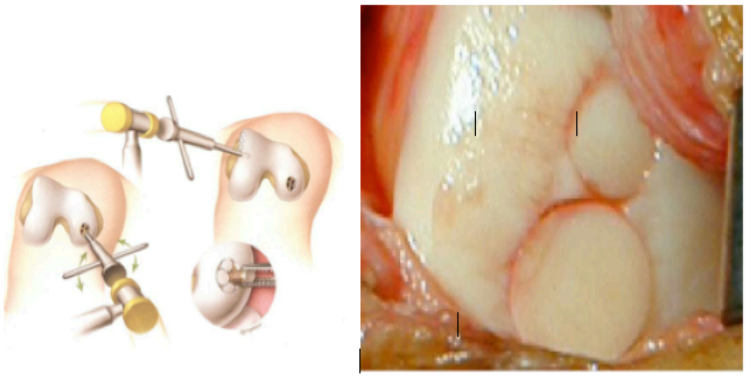

Figure 4. Osteochondral autograft [www.fda.gov]

On the contrary bone cells cannot survive to transplantation and they are substituted with host own new cells [Cabitza, 2013].

The *marrow stimulation method* (fig.5) occurs with the removal (abrasion) and/or drilling (microfracture) of exposed sub-chondral marrow due to the loss of cartilage. This method forms a blood clot containing marrow progenitors elements that gradually fill the chondral wound. The success of this technique is due to the fact that the procedure is relatively simple and reproducible, it has low invasiveness (it can be performed by arthroscopy), is inexpensive and has a long history of clinical use.

On the other hand this method has several disadvantages, in fact it creates fibrocartilage that has low wear characteristic with respect to hyaline cartilage and is more effective on smaller defects (less than 4 cm^2) [Cabitza, 2013].

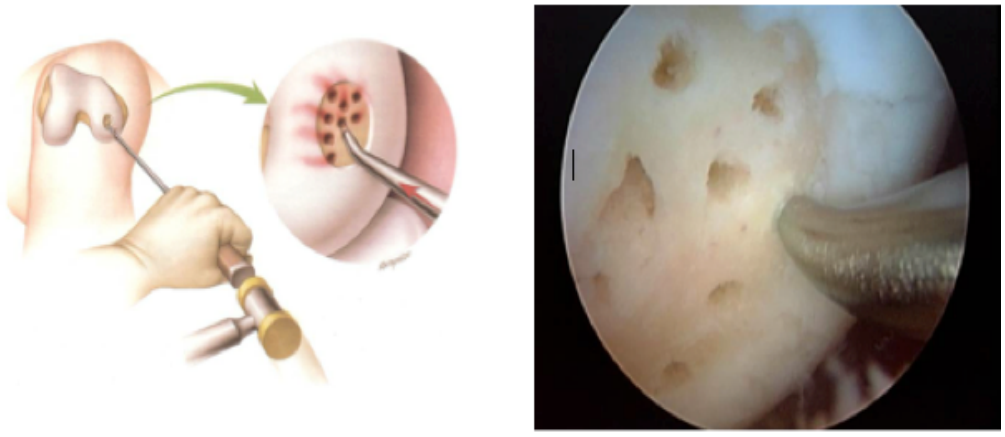

Figure 5. Cartilage damage treated with microfracture of subchondral marrow [www.fda.gov]

The aim of autologous chondrocytes implantation (ACI) is to the repair but especially to regenerate articular cartilage using living cells that synthetize extracellular matrix.

This method consists of three different phases (fig.6): the first one withdraws autologous chondrocytes from cartilaginous healthy zone; the

second one consists of isolating chondrocyte and culture them *in vitro* in laboratory following the GMP (Good Manufacturing Practice) rules; the last phase replants chondrocytes grown in laboratory [Cabitza, 2013].

With respect to microfracture, this treatment has the advantage of producing hyaline-like cartilage and it is not limited by defect size. On the contrary it is more expensive and invasive than microfracture, in addition it requires a longer recovery period.

Autologous chondrocytes implantation showed better clinical, functional and long-term (almost 10 years) results than traditional techniques. However it shows some technical limits, such as nonhomogeneous distribution of chondrocytes in the implantation site and the risk of their outflow [Rosa et al., 2002].

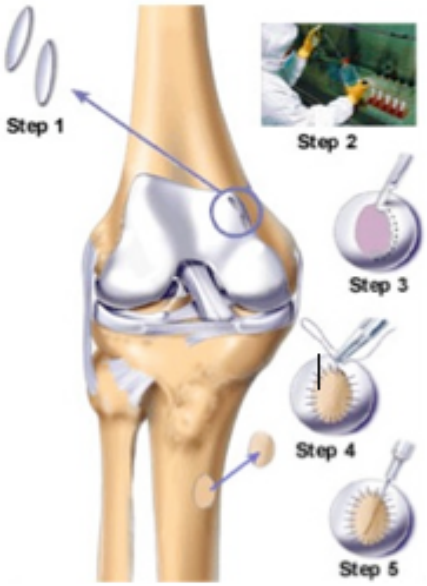

Figure 6. Steps of ACI technique [www.cartilaginedelginocchio.it].

For this reason an innovative technique of tissue bioengineering was introduced: matrix associated autologous chondrocytes transplantation

(MACT), where a three-dimensional biodegradable structure is used to improve structural and biological characteristics of the implant; in fact it creates an advantageous environment for the proliferation and differentiation of chondrocytes.

Several biomaterials were introduced to serve as a scaffold for transplantation of autologous cultured cells, so there are different MACT products:

- MACI® (Matrix-induced Autologous Chondrocytes Implantation)
- Hyalograft C®
- CaReS® (Cartilage Regeneration System)
- Bioseed C®.

In MACI® technique the scaffold is constituted by porcine type I/ type III collagen membrane that shows a smooth surface and a rough side where the chondrocytes are seeded. Collagen membrane offers a lot of advantages because it can be applied only utilizing fibrin glue without any suture system [Fu, 2013; Rosa et al., 2002].

As shown in fig. 7, MACI® method consists of different steps:

1. The defect is ascertained and injured cartilage removed
2. Membrane is cut according to the size and shape of the defect
3. The proper membrane orientation is chosen
4. Fibrin glue is applied to the defect and to the bone
5. MACI® is held in place with low pressure and fixed by fibrin glue [Miller et al., 2011].

Hyalograft C® utilizes hyaluronic acid derived biomaterial to produce a three-dimensional structure to culture autologous chondrocytes and opposed to MACI, it is a softer biomaterial and is not stable enough for sufficient suturing.

However it can be applied on both sides to the defect ground, due to the homogenous material characteristics and the cell distribution [Fu, 2013].

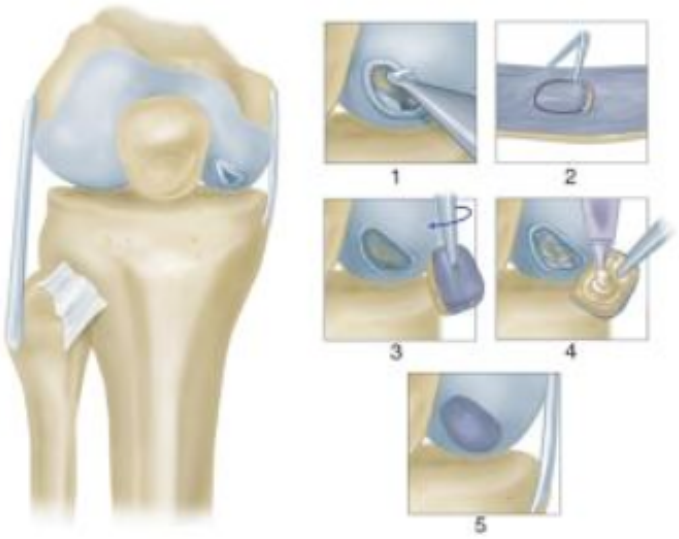

Figure 7. Overview of the MACI technique [Miller et al., 2011]

Another product is CaReS® (Cartilage Regeneration System), which is biologically purified type I collagen three-dimensional matrix, derived from rat-tail collagen [Minas, 2011].

The seeding of autologous chondrocytes and cultivation process is performed two weeks after cell extraction from the original cartilage biopsy, necessary for cell culture. The implant should be cut to exactly fit into the defect and fixation will be achieved with fibrin glue [Fu, 2013].

Bioseed C® is a cartilage graft obtained with a combination of autologous chondrocytes and bioresorbable two-component gel-polymer scaffold. The

first step of this procedure is the arthroscopic harvest of the cartilage; then cells are isolated, expanded ex vivo and loaded in three-dimensional environment. The chondrocytes are seeded on the scaffold three weeks before transplantation and the fixation is achieved with resorbable suture [Doral, 2012].

2.2 Medical Devices in Tissue Engineering

The US Federal Food and Drug Administration (FDA) defines the world "device" as any instrument, apparatus, machine implement, in vitro reagent, implant or a combination of these elements, that is designated for diagnosis, prevention or treatment of a disease [Silver, 1994].

In the past the use of polymers, metals, ceramics and composite materials as medical devices was substituted from plant and animal skins for the betterment of pathological conditions resulting from mechanical, chemical or pathogenic injuries for tissues or organs; nowadays polymers, metals, ceramics and composite materials are not used by themselves, but approved from FDA to be formulated in an end-use device.

The development of materials used in medical devices is a complicated process, particularly for what regards design criteria, in fact it requires the ability to synthesize anatomical and physiological data for replicating the structure and properties of natural tissues.

Depending on the end-use application, according to the ASTM Medical Devices Standards, different types of biological test methods are used for materials and medical devices [Silver, 1994].

A biomaterial intended for use in a medical device must follow safety and effectiveness criteria. The FDA is required to establish a system of classes of controls over medical devices with varying requirements based on the relative risk presented.

There are three classifications of medical devices depending on the amount of risk and invasiveness associated to their end-use (Tab.1):

- Class I of devices requires that, before the commercialization of the product, the manufacturer informs the FDA so that any performance standards are considered.
- Class II of devices makes a list of performance standards to avoid risk to the patient.
- Class III of devices includes all devices that could lead to injury or risk to the patient if used inappropriately.

Table 1. FDA classification of medical devices [Silver, 1994]

Class	*Types of devices*
I	Crutches, bedpans, depressors, adhesive bandages, hospital beds
II	Hearing aids, blood pumps, catheters, contact lenses, electrodes
III	Cardiac pacemakers, intrauterine devices, intraocular lenses, heart valves, orthopedic devices

2.2.1 Biomaterials

American National Institute of Health defines biomaterials as ‘‘any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual’’ [Bergmann et al., 2013].

Synthetic materials made from polymers, ceramics, metals and materials of biological origin, have been use as biomaterials when used in contact with human living cells.

Polymeric materials have some advantages over the others because of their flexibility in physical properties [Reis et al., 2001].

A disadvantage of polymers is their limited mechanical strength when used in orthopedic and oral devices. In these circumstances, ceramics and metal are favored, although their modulus is higher than the one of bone and tooth tissues and this results in stress of shielding to the tissues in contact [Reis et al., 2001].

Biomaterials constitute scaffolds for cells support, but they can modify the cellular response and change both chemical and mechanical properties with time [Palsson et al., 2004].

Biomaterials are characterized by surface, bulk and mechanical properties:

- *Surface properties* consist of the factors that determine the interactions between materials and cells depending on nonspecific interactions (e.g. electrostatic repulsion), on specific interactions (e.g. binding interactions mediated by cell-surface receptors) and on surface topology. Specific interaction can be "engineered" to apply specific adhesive properties.
- *Bulk properties* consist of all different types of interaction bonding such as ionic, covalent and metallic.
- *Mechanical properties* represent all the properties that can be explained by theories of rubber elasticity and viscoelasticity. Materials having uniform properties in all direction are called isotropic, instead materials that have properties that vary with

direction are called anisotropic. Hook's law explains elastic behavior through classic linear stress-strain curve (fig. 8) [Palsson et al., 2004].

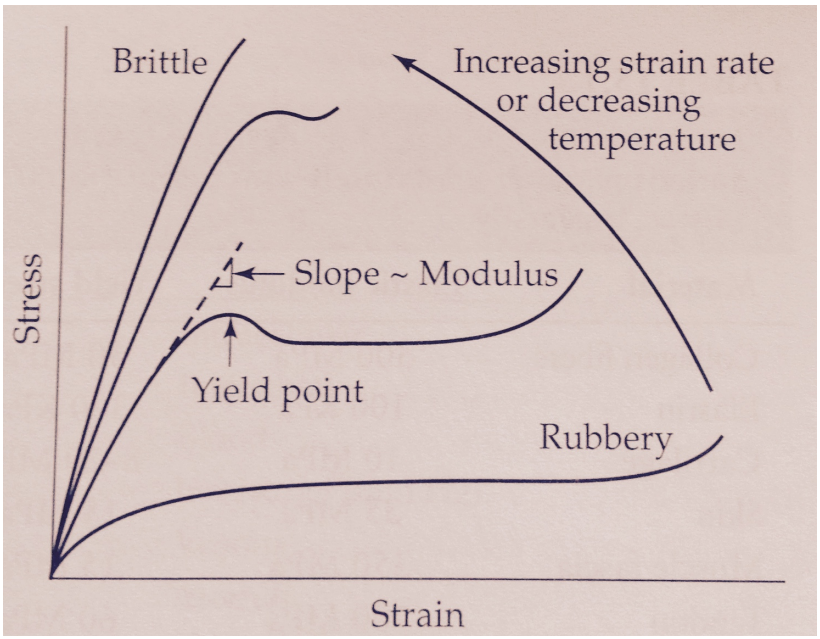

Figure 8. Mechanical properties of polymeric biomaterials [Pallson et al., 2004]

Stress is defined as the force on the unit of area, while strain represents the change in length/original length. Biomaterials in linear regime of stress-strain response behave like a rubber band, but they deform themselves plastically when the regime is non linear.

When a mechanical load is applied to a biomaterial, its response has a time-dependent component. As matter of fact, it causes an internal segment of polymer chains movement and biomaterial recovers when the load is removed. The response is termed viscoelastic if material completely recovers at long time scales; instead the response is viscoelastic-viscoplastic when material does not completely recover.

Mechanical properties of biomaterials must be equalized with those of the biological tissues; they have the elastic moduli highly nonlinear, but increase with strains. In particular, non-fibrous tissues show more nonlinear behaviour than fibrous tissues; the challenge is matching the mechanical properties of biomaterials with those of the surrounding tissues that can vary with respect to the kind of tissue (tab.2).

Table 2. Mechanical properties of tissues [Palsson et al., 2004]

Material	*Elastic modulus*	*Yield stress*	*Maximum strain*
Collagen fibers	500 MPa	50 MPa	10%
Elastin	100 KPa	300 KPa	300%
Cartilage	10 MPa	8-20 MPa	70-200%
Skin	35 MPa	15 MPa	100%
Muscle fascia	350 MPa	15 MPa	170%
Tendon	700 MPa	60 MPa	10%

2.2.2 Scaffolds

Scaffolds are defined as a temporary supporting structure for growing cells and tissues, and are also called synthetic extracellular matrix [Collins et al., 2012].

Several matrix materials have been used to keep cells in three-dimensional culture and to procure biochemical and organizational connections to these cells [Atala et al., 2002].

Chemical characteristics and the way in which the materials are processed play an important role in determining physical properties of the scaffolds and the way in which cells should interact with these bio-artificial carriers [Atala et al., 2002].

Scaffolds for tissue engineering have different design criteria:

- They should permit cells adhesion, promote their growth, allow different cells functions and have the ability to carry drugs or growth factors;
- They should be biocompatible, in fact their degradation shouldn't provoke inflammation or toxicity to human body;
- They should be biodegradable in synchronization with the repair or regeneration of the damaged tissue and in some cases they should be easily removed. The degradation kinetics of biodegradable scaffold is critical to determinate for two reasons: the first one is that, if the scaffold degraded too fast, it would not provide the right mechanical support for the new tissue formation. On the other hand, the second one is that if the scaffold degraded in a very long time, it would be dangerous to the tissue engineering endeavor;
- They must have sufficient mechanical integrity from the time of implantation to the end of the treatment;
- They should have adequate mechanical properties to provide the correct stimuli to cells, so that they can show improved ECM formation and tissue regeneration. With respect to the final

application of the scaffold, its mechanical properties should be tested to perform the kind of final loading conditions. For example, in cartilage repair, scaffolds should be tested under compression;

- They should be highly porous to give sufficient space for cell adhesion, extra-cellular matrix regeneration;
- They should have the correct pore size range and the pore structure should allow even homogeneous tissue regeneration. Pore size may determine the kind of cells that cannot enter the interior of the scaffold;
- They should be highly permeable to facilitate diffusion, so the pores should be interconnected: diffusion is important for the entry of nutrients to cells into the scaffold;
- They should be made with reproducible and processable into three-dimensional structures material [Collins et al., 2012; Reis et al. 2002].

The types of scaffolds usually used in tissue engineering can be classified into three categories: hydrogels, natural polymers and synthetic scaffolds [Atala et al., 2002].

Hydrogels are composed by highly hydrated polymer network that is formed by physical, ionic or covalent interactions. Temperature changes, ionic changes, chemical cross-linkers and radiation can form cross-links. Thanks to their high water amount (70-99%), hydrogels allow high biocompatibility and they should be used when mass transport from or to a cell population is necessary [Atala et al., 2002; Palsson et al., 2004].

Naturally derived polymers represent another kind of material used as a scaffold material. The most traditional natural polymer is, for example,

type I collagen; it is used for different applications thanks to the easiness of cell delivery. Natural polymer scaffolds support chondrocytes growth for articular cartilage repair and for the engineering of intervertebral discs and meniscus. Natural polymers cannot be very often used in medical practices because of their moduli that are not high enough to let them have the right mechanical performances.

Another example of natural polymer scaffold widely used in cartilage repair is fibrin network [Atala et al., 2002].

Synthetic polymers are often used in medicine and in tissue engineering because they can be easily synthesized to be non-adhesive, resorbable and tissue-like hydrogels.

Synthetic polymers should be biodegradable and it means that they should be bioerodible (polymers that degrade by addition of water), bioresorbable (polymers that require cellular activity) and biodegradable (they require enzymatic activity).

There is a kind of polyesters that degrade to monomers of hydroxyacids (lactic and glycolic acids) and these polymers with their copolymer are often used in tissue engineering. Degradation can occur with two different mechanism: bulk erosion, when the water penetration rate is higher than the rate at which polymer is solubilized, and surface erosion, when the water penetration rate is less than the polymer dissolution rate.

Some example of synthetic polymers and their characteristics are shown in tab. 3, referred to the specific medical application [Palsson et al., 2004].

Table 3. Example of syntetic polymers with tissue engineering applications [Palsson et al., 2004]

Polymer	*Physical characteristics*	*Potential clinic application*
Poly(esters): • Poly(glycolic acid) • **Poly(lactic acid)** • **Poly(caprolactone)**	Solid-fiber, tube, sponge, screw, etc.	**Cartilage**, bone, muscle, nerve, blood vessel, valves, bladder, drug delivery, liver, cardiac tissue
Poly(anhydride)	Solid, cross-linked network	Bone, drug delivery
Poly(propylene fumarate)	Solid, copolymer hydrogel	Bone, cardiovascular
Poly(vinyl alcohol)	Solid sponge, hydrogel	Cartilage, nerve
Poly(ethylene glycol)	Hydrogel, coating, solid	Cartilage

Traditionally cartilage tissue engineering uses different kind of synthetic scaffolds such as poly-caprolactone (PCL), poly-glycolic acid (PGA), poly-lactic acid (PLA), and poly-lactic-co-glycolic acid (PLGA) copolymers. The selection of a scaffold material is a critical and difficult choice and passes through the FDA acceptance [Atala et al., 2002].

2.3 Mesenchymal Stem Cells

Regenerative medicine and tissue engineering have the main aim of repair and regenerate tissues and damaged organs derived from different traumas. In recent years the potential use of stem cells, both adult and embryonic, as therapeutic agents was proposed for this type of applications [Cancedda et al., 2002].

Stem cells are cells that can renew by themselves and are able to differentiate along one or more lineages and specialized cell types.

Considering their potential of differentiating, they can be classified as [Mantero et al., 2009; Pallson et al., 2004]:

- *Totipotent* stem cells, which can produce all cell types, are produced by the fusion of an egg and sperm cells. These cells can differentiate into embryonic and extra-embryonic cell types;
- *Unipotent* stem cells, which can produce only one cell type;
- *Pluripotent* stem cells, which are the descendants of totipotent cells, can produce many cell types (fig. 9).

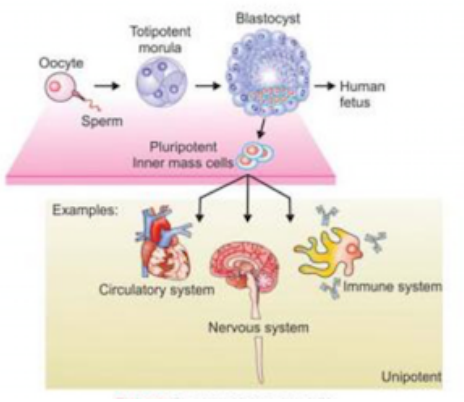

Figure 9. Differentiation potential of stem cells [Cherian et al., 2011]

There are four main types of stem cells that can derive from different sources:

- Embryonic tissues
- Fetal tissues
- Cord blood
- Adult tissues.

Adult stem cells are able of producing identical copies of themselves and making progenitor or precursor cells that can develop into specific cell lines. These cells have been found in different organs and tissues such as, for example, blood, cornea, bone marrow, brain, skeletal muscle, but there are only few cells in each tissue and they usually generate the cell types of the tissue where they reside [Pavlovic et al., 2013].
Initial studies on adult stem cells determined the existence, in many tissues, of unipotent stem cells that can produce only the cell type of the tissue to which they belong [Mantero et al., 2009].
More recent studies have demonstrated the presence of pluripotent stem cells in many tissues.
In particular in the bone marrow there are mainly two types of pluripotent stem cells:

- *Hematopoetic* stem cells (HSCs), which are blood-forming cells that can produce all the cell types specific of this tissue and sustain the formation of the cellular immune system;
- *Mesenchymal* stem cells (MSCs), which can be use to regenerate and repair tissues because they can produce a huge variety of cells

such as, for example, bone tissue cells (osteocytes), cartilage tissue cells (chondrocytes), fat cells (adipocytes), connective tissue cells, etc.

For the first time in 1976, Friedenstein described mesenchymal stem cells as a source of the osteoblastic, adipogenic and chondrogenic cell lines differentiated in vitro depending on the microenvironment in which they reside [Sorensen, 2008].

MSC are characterized by a high potential to proliferate and a high capacity to differentiate in cellular phenotypes of many tissues (fig.10) not only the one where they reside (high degree of plasticity) [Cancedda et al., 2002].

This type of stem cells have not been easily isolated only from the bone marrow, but also from the blood, the umbilical cord blood, the placenta, the amniotic fluid, the scalp tissue, the fetal tissue and the adipose tissue [Sorensen, 2008].

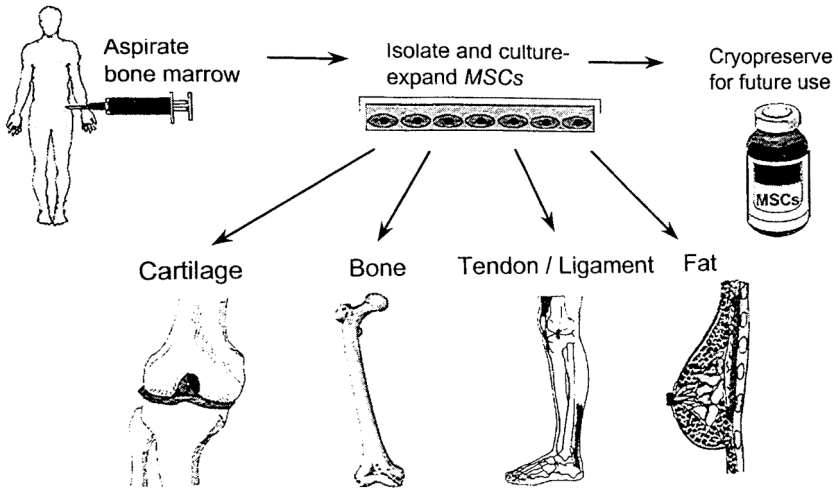

Figure 10. Regenerative tissue MSC therapy [Lanza et al., 2000]

In recent years the use of mesenchymal stem cells was proposed for regenerative medicine applications, such as regeneration and repair of damaged organs and failed tissues.

In particular, a construct composed by a polymeric structure (scaffold) and mesenchymal stem cells (MSC) is required as a great alternative for the reconstruction of cartilaginous tissues as a consequence of traumas and pathological conditions. The main reasons of the use of MSC are their advantages such as the fact that they can be easily harvested from different kind of tissues, their long-term self-renewal capability, their potential of differentiating and their expansion capacity [Sorensen, 2008].

3. MANUFACTURING FIBERS TECHNOLOGY

3.1 Fiber bonding

During the last 30 years, a lot of biodegradable polymeric materials have been investigated for biomedical applications. As matter of fact, they are the best candidates for the production of porous three-dimensional structures like scaffolds. The main challenge is the replacement of standard device generally used in temporary therapeutic applications with biodegradable device able to repair and regenerate damaged tissues [Nair et al., 2007].

In the field of tissue engineering, biodegradable polymers are widely used to produce scaffold because of their high biocompatibility and biodegradability. These properties allow cells adhesion and growth into the matrix and eliminate the need for a second surgery to remove the external device [Tian et al., 2011].

Several techniques have been investigated to produce fiber-based scaffolds in order to provide the characteristics required to the final aim. A scaffold should be biocompatible, have a controlled degradation rate, have mechanical properties similar to those of the tissue to be replaced and be porous enough to promote cells adhesion, growth and differentiation [Salgado et al., 2013].

The production of fiber-based scaffolds can be achieved by the fiber bonding technique. With this method, a predefined amount of fibers, produced by the spinning process, is randomly or orderly collocated in

Teflon net; then this product is usually heated at a predefined temperature and time period, under compression, to bond the fibers together on the contact points.

The most important advantage of this method is the production of scaffolds with high surface area and high porosity for cell adhesion and rapid diffusion of cells and nutrients.

3.2 Spinning of polymers into fibers

Three-dimensional scaffolds created from different biomaterials are one of the main essential elements of tissue engineering and they can be used as potential substitutes for natural ECM. Nowadays the researchers are focusing their attention on fiber-based scaffolds production, due to the fibrous nature of natural ECM [Salgado et al., 2013].

Fibers for scaffolds production can be created with different spinning techniques and an important help to comprehend the technology behind fibers production is provided by the spiders and the silkworm. In fact, these animals use the following steps to extrude thin continuous filaments: acquirement of spinnable liquid, jet production and jet hardening. If a bobbin winding is added to these steps, a yarn state of manufactured fibers is achieved [Gupta et al., 1997].

In the production process of polymeric fibers, the polymers in the form of a melt or a solution are injected through orifices and the polymeric fluid comes out from the orifice in the form of a continuous filament, that is rapidly pulled on the bobbin winding and is stretched and solidified to a thin fiber [Gupta et al., 1997].

There are two main process of spinning to convert the fiber-forming polymers into filaments: melt-spinning process and solution-spinning process.

In *melt-spinning* process the polymer is heated above its melting point and extruded through the spinneret hole (fig.11a). The melted material comes out through the extrusion head and is air cooled and solidified to obtain continuous filament line. This process usually utilizes thermoplastic polymers, which are not affected by high temperatures required by the

melt spinning [Ratner et al., 2012].

This method is easier and more economic than solution spinning, because in this case solidification of the melted material involves only heat transfer and extensional deformation; instead, the other technique, adds mass transfer and diffusion [Baird et al., 2014]. It is preferred over solution spinning if the polymer melts under appropriate conditions, because no solvent is used, so there are no all the problems associated with its presence: its removal, its recovery and environmental concerns [Gupta et al., 1997].

The *solution spinning* is used when the polymer degrades before the melting point and the melt is thermally unstable, because the polymer doesn't have suitable viscosity for stable fiber formation [Schultz et al., 2006].

There are many types of this process and they differ on how the solvent is removed:

- *Dry-spinning* (fig. 11b): the polymer is dissolved in a solvent that is removed by evaporation by heating. This process is used when the solvent is highly volatile and it can be evaporated rapidly during fiber formation [Schultz et al., 2006].
- *Wet-spinning* (fig. 11c): this process is used when the polymer is dissolved in a non volatile solvent that is removed by coagulation in another fluid, which is miscible with the solvent but it is not a solvent for the polymer [Gupta et al., 1997].

A combination of dry-spinning and wet spinning is called *gel spinning* and it is used to prepare high performance polymeric fibers with specific characteristics.

This last process will be described in the next section and is applied, for example, when polymers are converted into a fiber-based drug delivery system [Ratner et al., 2012].

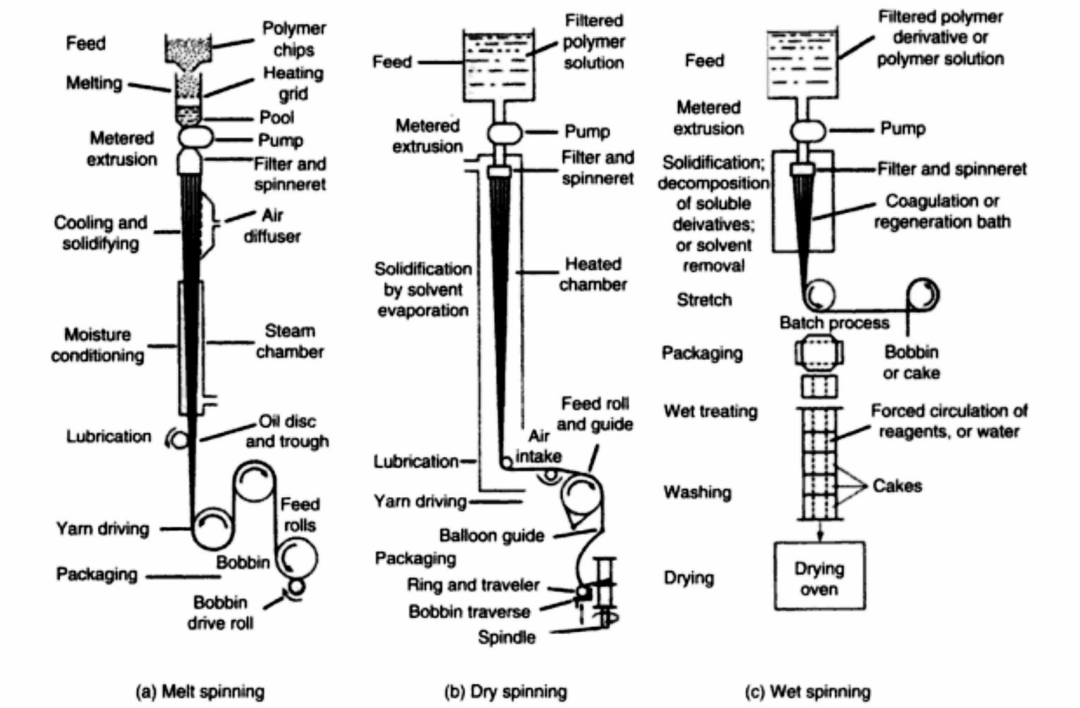

Figure 11. Three main types of spinning process [Schultz et al., 2006].

Another process recently investigated is the electrospinning (fig.12) to produce nano-fibers. This technique is used when fibers with a thin diameter are required. As matter of fact, the diameter of fibers thread by melt spinning and solution spinning are determined by the size of the orifice in the spinneret and the stretch applied before the wind up. This method consists in exposing polymer solution or melt to a electrostatic field created by high voltage, which overcomes the surface tension of the polymer and accelerates fine jets of the liquid polymer [Ratner et al., 2009].

The diameter of the filaments depends on the chemical characteristic of the polymer, the viscosity of polymer solution, the strength and uniformity of the applied electric field and on the operating conditions of the process.

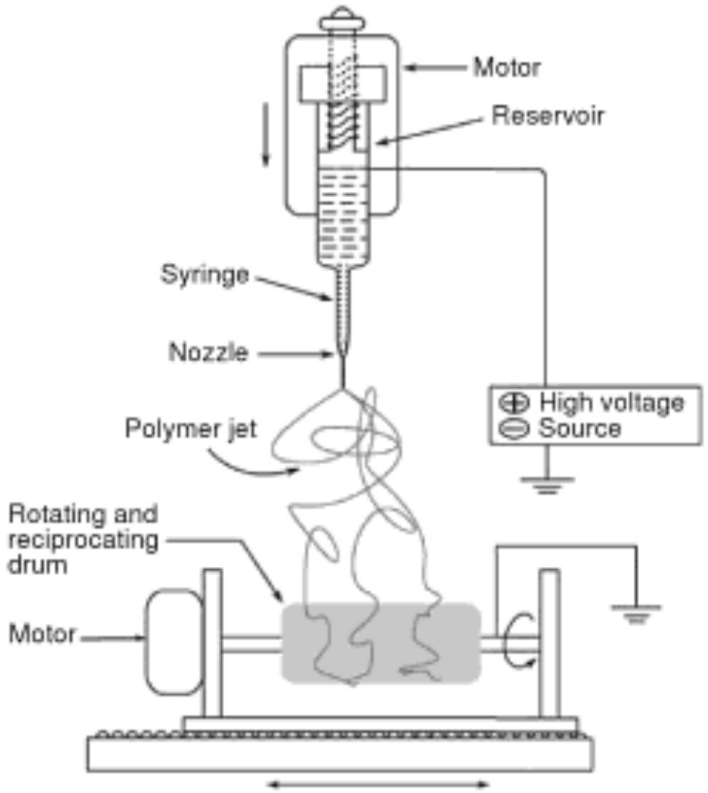

Figure 12. Electrospinning process [Ratner et al., 2009]

This method has been largely used in tissue engineering applications, in particular in the production of scaffolds. In fact, non woven scaffolds were produced using type I collagen and synthetic polymers such as poly(L-lactide), poly(lactide-co-glycolide), poly(vinyl alcohol), poly(ethylene-co-vinyl acetate), poly(ethylene oxide), poly-urethanes and polycarbonates [Ratner et al., 2009].

3.2.1 Dry-wet spinning

Dry-wet spinning, also known as gel-spinning (fig.13), is a process used to prepare high performance polymer fibers. Gel spinning differs from wet spinning, described in the above section, because it does not use a liquid solution of polymer during extrusion, but the polymer forms a gel characterized by polymer chains bound together in various point to create a liquid crystalline structure that results in filaments with strong inter-chain forces and a high degree of orientation [Gajjar et al., 2014].

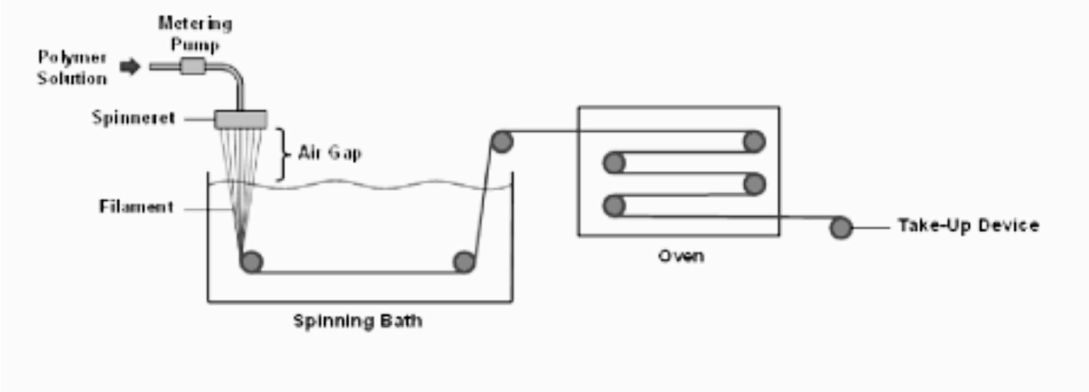

Figure 13. Scheme of a basic gel spinning process[Zhang, 2014]

As shown in the previous figure, the polymer solution is pumped into the spinneret, which is localized above the spinning bath to create an air gap. In the first step, the extruded polymer passes through the air gap, in the same way of dry-spinning process; then a spinning bath occurs, like in wet-spinning. These characteristics are the main reason why this process is called dry-wet spinning [Zhang, 2014].

Gel spinning usually provides gel filaments that still contain an appreciable amount of solvent forming swollen networks joined by small crystalline regions (fig.14), so they are transferred into an oven to be drawn to high-performance fibers by removing the solvent.

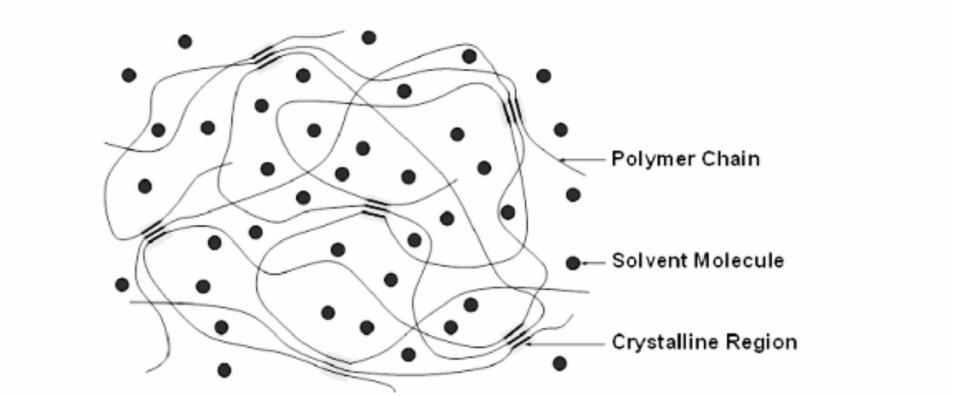

Figure 14. Swollen network structure in a gel filament [Zhang, 2014]

3.3 Dry-wet spinning apparatus

The dry-wet spinning machine used for this study was designed and realized by Gimac, a world leader on the extrusion field, headquartered in Castronno (Italy).

This apparatus is necessary for the production of hollow fiber-based scaffolds for tissue engineering. These scaffolds are an artificial extracellular matrix where cells are seeded to analyze their growth and differentiation with the final aim of repairing damaged connective tissue like, in particular, cartilage.

The machine is composed by steel (fig.15). The main body of this structure is characterized of three axes with the ability of moving in the three spatial directions. Vertical axis contains a couple of steel syringes, which end in an extrusion head. This axis moves in the translational direction in order to collect a deposition of extruded fibers on a rotating roll, situated under the axes.

There are different kinds of syringes, which correspond to different capacity: 20, 50 and 100 cc. The different types can be used in various combinations to favour process requirements.

A polymer is first dissolved in a solvent and then loaded in a syringe; the other syringe is loaded with a non-solvent. Polymer solution and non-solvent converge to the extrusion head, which is composed by a double anulus exit hole. The polymer solution goes to the outer side of the hole, while non-solvent passes through a central needle to create a hollow fiber. The supplied needles were two, one with diameter of 0.3 mm and the other one with diameter of 0.68 mm, so that fibers with different dimensions of the cavity could be created (fig.16).

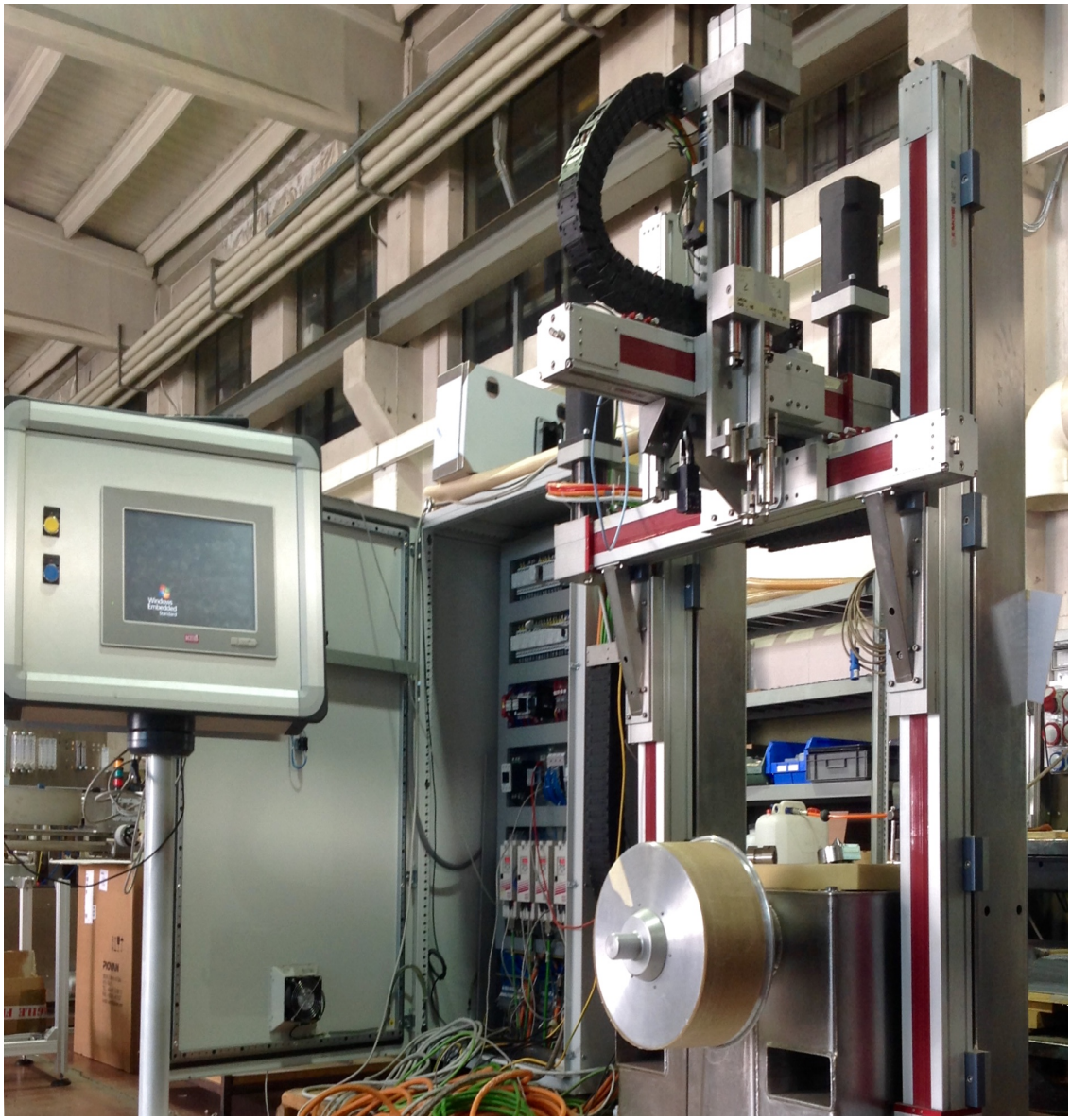

Figure 15. Dry-wet spinning apparatus

The cavity inside the fiber could represent a need for the final application; in fact, these kind of hollow fibers are usually used as drug delivery systems or to create a scaffold to seed cells for applications of tissue engineering.

The extrusion head can be easily disassemble to facilitate cleaning operations.

Polymers have a viscoelastic behavior, so they can “die-swell” during the extrusion process because they try to recover the shape and volume they had before the spinneret. For this reason, the extrusion head has a conical way-out to reduce the “die-swell” during the fibers formation.

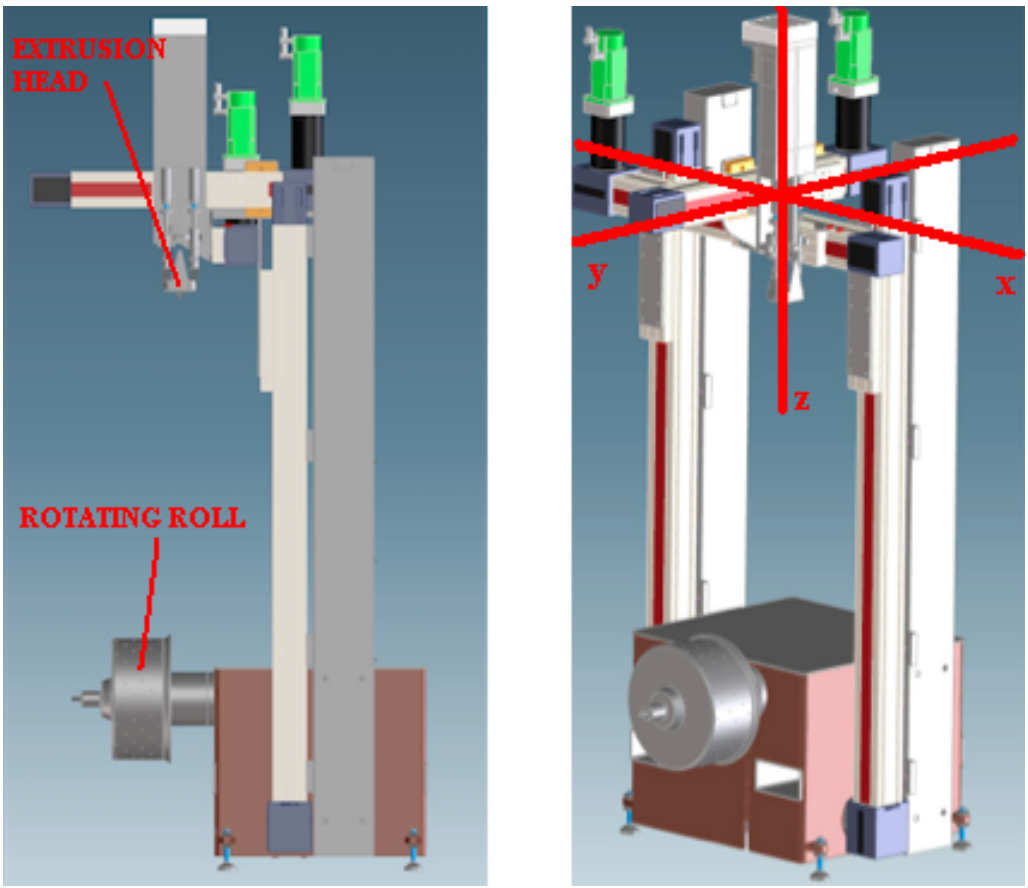

Figure 16. Dry-wet spinning apparatus design

Fibers extruded from the head fall down on a rotating roll, situated in the lower side of the machine, which is covered with a Teflon sheet where fibers are collected and solidified by solvent evaporation. A motor gives

the roll a rotational motion with a rate between 50 and 500 rpm in order to obtain a different degree of stretching of the fibers as a consequence of the rotational rate.

The motor unit is driven by a control board and it is connected to a PC to allow the set up of operative conditions, like the rate of pistons of the syringes, and as a consequence the volumetric flow rate of the polymer solution and the non-solvent, the rotational rate of the roll and the translational rate of the axis (fig. 17).

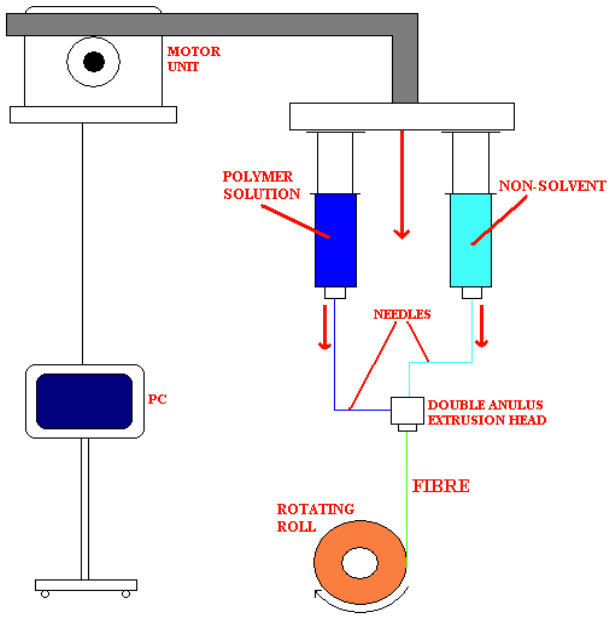

Figure 17. Dry-wet spinning technique

Describing the procedure in this way, it could seem a typical dry-spinning process because it passes through the formation of fiber from a polymer solution with solvent evaporation on air. But the non-solvent that is conducted inside the fiber behaves like a coagulation bath and this is the reason why this process can be described as a dry-wet spinning technique, described in the above section.

3.4 Biomaterials

Biomaterials are usually defined as materials able to interface with biological tissue to treat, repair or regenerate damaged function rather than act simply as a static replacement. They play an important role in the development and design of tissue engineering products [Lanza et al., 2000].

A lot of natural or synthetic materials have been used as biomaterial to produce scaffolds for tissue engineering application.

The most important kind of biomaterials is represented by biopolymers and they can be natural or synthetics. Natural polymers, for example collagen, fibrin, hyaluronic acid and its derivatives, could be very promising but they are not available in huge amount. For this reason, the attention has focused on synthetic polymers, in particular to biodegradable polymers. Several examples of biodegradable and bioresorbable synthetic polymers that have been approved by FDA are collagen, poly-glycolic acid (PGA), poly-lactic acid (PLLA or PDLA), poly-caprolactone (PCL) and few else.

3.4.1 Poly-caprolactone

Poly-caprolactone (PCL) is a biodegradable polyester characterized by low melting point (about 60°C) and glass transition temperature of about -60°C, it is soluble in a wide range of organic solvents. It has a high thermal stability, in fact aliphatic polyesters usually have a decomposition

temperatures between 235 and 255°C, while PCL degrades at a temperature of 350°C [Lanza et al., 2014].

PCL has a low tensile strenght (about 23MPa) and high elongation at breakage (>700%) [Nair et al., 2007].

Production of PCL passes through anionic ring-opening polymerization of ε-caprolactone in the presence of a catalyst. In the fig.18 the structure of poly-caprolactone is shown [McKeen, 2012].

One of the main properties of PCL is its attitude to create compatible blends with several other polymers. In addition, ε-caprolactone can be copolymerized with different monomers; a remarkable example is the copolymer composed by ε-caprolactone and lactic acid. PCL and copolymers with PLA can be processed to form nanofibers-based scaffolds that can be use for tissue engineering applications [Lanza et al., 2014].

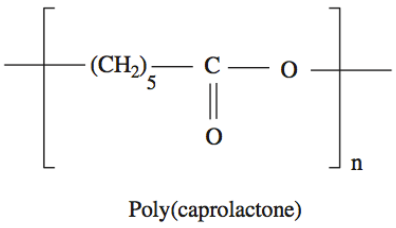

Figure 18. Structure of poly-caprolactone [Nair et al., 2007]

Thinking about practical applications of a biocompatible polymeric material, it is necessary to analyze the rate and the mechanism of its biodegradation. The rate of degradation of a biomaterial depends on several factors such as, for example, molecular weight, the shape and size of the implant, composition, monomer conversion, external factors and methods of processing and sterilization [Nakamura et al., 1989].

Due to its high degree of crystallinity and hydrophobicity, high molecular weight PCL shows an appreciable in vivo degradation time. The degradation occurs by hydrolysis because its nature of aliphatic polyester.
It was initially studied as a drug delivery system due to his slow rate of degradation, high permeability to many drugs and non-toxicity [Nair et al., 2007].
The physiological conditions in the human body permit the degradation of the polymer by hydrolysis of its ester linkages. The complete breakdown of the polymer occurs in a time period of about 2/3 years, depending on the degree of crystallinity. This is one of the reasons why PCL has been studied and used as long-term implantable biomaterial for the production of scaffolds for biomedical application [McKeen, 2012].

3.4.2 Poly-L-Lactic Acid

Poly-lactic acid (PLA) is a thermoplastic, high-strength, high modulus polymer widely used in the field of biocompatible and bioasborbable medical devices [McKeen, 2012].
For the first time in 1845 Pelouze produced condensed lactic acid by distillation of water and low molecular weight PLA and the cyclic dimer of lactic acid, lactide, were formed. PLA has been known for over 100 years, but its commercial viability or usefulness had not been practical. Consequently, Cargill began to study the manufacturing, processing and cost issues and, in 1997, Cargill and The Dow Chemical Company formed Cargill Dow LLC in order to develop and bring to full commercialization the PLA technology and products [Gruber et al., 2002].

PLA can be prepared by both direct condensation of lactic acid and by the ring-opening polymerization of the cyclic lactide dimer to remove the water molecule, which could limit the production of high molecular weight polymers (fig.19).

PLA is a chiral molecule that can be isolated into thee different forms, two optically active, (fig.20), and one optically inactive, respectively:

- L-lactic acid (PLLA)
- D-lactic acid (PDLA)
- Mixture of DL-lactic acid (PDLLA, which is an amorphous polymer).

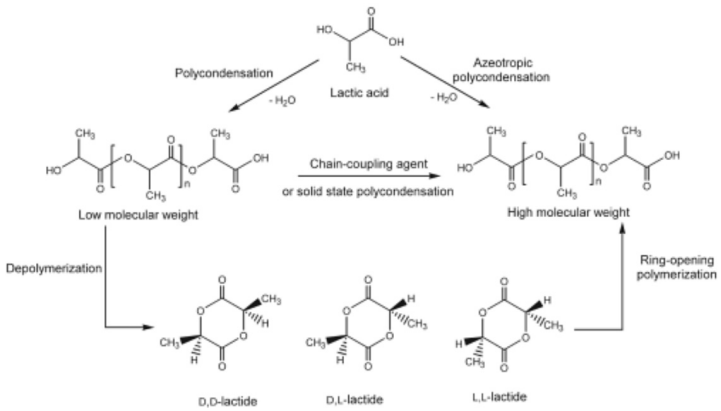

Figure 19. Different ways of high molecular weight PLA production [Sharma et al., 2011]

Among these monomers, PLLA is the naturally occurring isomer.

PLA can be produced in two different ways: the first one is a petrochemical route, through hydrolysis of lactonitrile with a strong acid, forming a racemic mixture of optically inattive lactic acid; the second is bacterial fermentation of a carbohydrate carbon source of lactic acid, which is the most chosen method. With this last method PLA can be

produced with renewable and low cost substances, for example corn, potatoes, rice bran and other biomass, etc. [Sharma et al., 2011].

PLLA has a chemical structure similar to that of poly-glycolic acid (PGA) with the addition of a methyl group. As PGA, PLLA is also a semi-crystalline polymer, with degree of crystallinity of about 37%, it depends on the molecular weight and the parameters of the processing method [Nair et al., 2007].

PLLA has a glass transition temperature of 60-65°C and a melting point of 175°C.

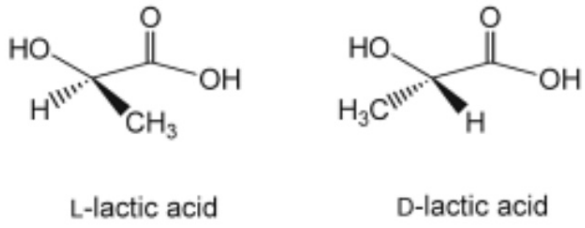

Figure 20. Stereoisomeric structures of lactic acid [Sharma et al., 2011].

Typically, PLLA experiences bulk, hydrolytic ester-linkage degradation, and it decomposes to lactic acid. This is eliminated from the body in the form of carbon dioxide and water through respiratory system [Fischer et al., 2007].

PLLA usually degrades slowly compared to PGA and this is due to the presence of methyl group that impedes the ester bond breakage.

PLLA has good tensile strength (about 3-4 GPa), low extension at breakage (about 4%) and a high modulus (approximately 4.8 GPa), that is the reason why it is an ideal material for fiber production and fiber-based devices for biomedical applications, for example orthopaedic fixation devices [Nair et al., 2007].

Different kind of cells can be seeded in PLLA matrix used as support, such as, for example, nerve stem cells to regenerate neurons and human bladder smooth muscle cells, which show the normal metabolic functions and cells growth.

PLLA can also be used as a scaffold to support chondrocytes to form extracellular matrix, in fact cartilage tissue engineering showed that it could be produced by collagen, glycosaminoglycan and elastin [Fischer et al., 2007].

4. MATERIALS AND METHODS

4.1 Materials and Instruments

The materials used during fibers production were:

- Dichloromethane, Sigma-Aldrich (Israel) (CAS Number 75-09-2)
- Distilled water, purchased from Gimac, Castronno (Italy)
- Ethyl alcohol, Carlo Erba Reagenti (Italy) (CAS Number 64-17-5)
- Gelatin, Merck (CAS Number 9000-70-8)
- Isopropyl alcohol, Carlo Erba Reagenti (Italy) (CAS Number 67-63-0)
- PCL, Sigma-Aldrich (United Kingdom); Mn≈80000; (CAS Number 24980-41-4)
- PLLA, Purac (Netherlands); Mw ≈ 200000; i.v. = 1,4

The materials used during cells characterization were:

- Fetal Bovine Serum, Invitrogen (Carlsbad, CA, USA)

- Fluconazole, Diflucan (Pfizer, NY, USA)
- L-glutamine, Sigma-Aldrich
- Low glucose D-MEM, Sigma-Aldrich
- Penicillin, Sigma-Aldrich
- Streptomycin, Sigma-Aldrich
- Trypan Blue, Sigma-Aldrich
- Trypsin, Sigma-Aldrich

The Instruments used to characterize the fibers were:

- AlamarBlue® colorimetric test, Serotec Ltd., (Kidlington, UK),
- Burker Chamber
- Camera for the digital macro (Canon Ixus) with 1×, 2× and 4× enlargements.
- Neutral Red test (NR), Sigma-Aldrich
- Plates of ninety-six wells
- Spectrophotometer (Victor 3; PerkinElmer, Waltham, MA, USA)

4.2 Polymer solution

The study of kinetics and thermodynamics of phase separation constitutes a useful instrument to analyze the effect of different parameters on the final morphology of a porous structure. These parameters are, for example, the concentration of the polymer, the type of solvent, the rate of cooling and the final temperature.

Porous matrices for scaffolds production should be created by thermodynamic separation of the system composed by the polymer and the solvent into two phases: the first one is rich in polymer and the other one is rich in solvent.

In this study, the system is composed by the polymer, which is PLLA or PCL, dissolved in a solvent, dichloromethane.

Phase separation can be of two different types:

- *Chemically induced*: for example the polymerization of a monomer in a solution leads to a solubility loss of the polymer and therefore to a separation.
- *Physically induced*: the driving force has a thermic or diffusive nature. When diffusion is the driving force, like in this study, a non-solvent is introduced in the system to reduce the solvent power. After separation of the solution in a phase rich in the polymer and one rich in the solvent, solvent and non-solvent are removed by extraction, evaporation or sublimation. The space previously

occupied by the solvent and non-solvent will be substituted with empty pores.

Porous structure, created during the process of solvent removing, should be preserved to avoid its collapse. There are different techniques to obtain this purpose:

- *Freeze-drying*: the solvent is frozen and removed by vacuum sublimation.
- *Freeze-extraction*: a polymeric solution is frozen and then is immersed in a non-solvent bath with a low freezing point. Because the non-solvent is liquid, while the solvent is already frozen, there is an exchange of the frozen solvent with the liquid non-solvent at a temperature lower than the freezing point of the polymer solution. Solvent is thus extracted and non-solvent can be removed with air-drying or freeze-drying [Mattiasson et al., 2010].
- *Immersion precipitation*: a homogeneous polymer solution is immersed in a non-solvent bath and the solvent diffuses into the bath while the non-solvent penetrates into the solution. The solubility of non-solvent in the polymer is limited, so, when it reaches its critical concentration, it precipitates [Gad, 2008].

4.2.1 Thermodynamics of polymer solution

The miscibility of a polymer solution can be quantitative evaluated through the change of Gibbs free energy associated to the mixing process:

$$\Delta G^{Mix}=\Delta H^{Mix}-T\Delta S^{Mix} \quad (1)$$

where:

- ΔH^{Mix} is the enthalpy of mixing
- ΔS^{Mix} is the entropy of mixing
- T is the temperature.

When temperature and pressure are constants three different states are possible to distinguish:

- If $\Delta G^{Mix}<0$, the mixing occurs spontaneously. This is a necessary but non-sufficient condition for the system stability (one phase constituted of an homogeneous solution);
- $\Delta G^{Mix}>0$ unstable state (inside the miscibility gap), two phases in equilibrium;
- $\Delta G^{Mix}=0$ equilibrium state, on the spinodal curve. [Nesterov et al., 1997].

The typical equilibrium diagram for phase separation of a binary system (polymer-solvent) or a quasi-binary system (polymer/solvent/non-solvent, where the composition of one of the three components is constant) is shown in the next figure (fig.21).
The internal curve of the miscibility gap represents the spinodal curve, while the external is the binodal curve. Under the spinodal curve there is an area of instability, where two phases are present, and over the binodal

curve there is a stable area, where one phase is present and homogeneous solution is formed. Between the curves there is a metastable area [Robeson, 2014].

The polymer-rich phase and solvent-rich phase can be separated through two mechanisms:

- *Binodal decomposition* (nucleation and growth): it occurs in the metastable area and an energy barrier must be exceeded to reach nucleation;
- *Spinodal decomposition*: this process occurs in the spinodal area (instable) and there is no an energy barrier [Harrats, 2009].

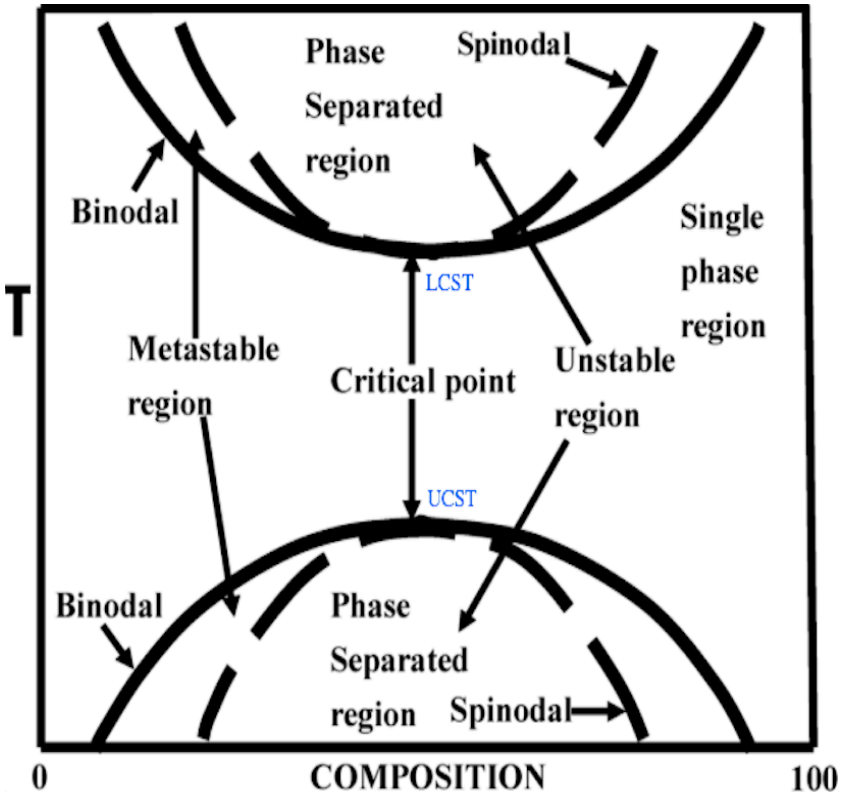

Figura 21. Phase diagram for binary polymer blend (Temperature versus Composition) [Robeson, 2014]

The structures obtained with these two mechanisms are different from each other, in fact the morphology developed as a consequence of the phase separation process, depends on the separation route, which is shown in the next thermodynamic diagram (fig.22).

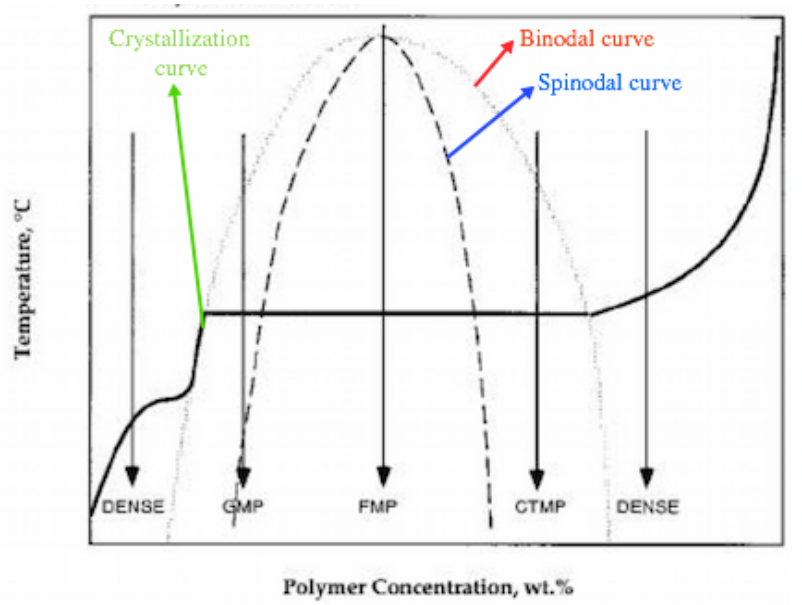

Figura 22. Thermodynamic diagram: Binodal, Spinodal and Crystallization curve [Robeson, 2014]

Complete miscibility between the polymer and the solvent may exist above the upper critical solution temperature (UCST) and under the lower critical solution temperature (LCST) [Aguilera et al., 1999].

If separation occurs with spinodal decomposition, resulting structures are fibrous and micro-porous (FMP). Instead, when the concentration of the

polymer is very low or very high, the polymer crystallizes directly into the solution and resulting morphologies are dense.

4.2.2 Flory-Huggins theory

The easiest theory to understand polymer solution was proposed in 1942 by Flory and Huggins [Chung, 1996].

A polymer solution differs from a solution of small molecules, in fact it can be considered like an entanglement of molecules, with length-to-diameter ratio of 10000:1, dissolved in a solvent. The miscibility conditions of a solution polymer-solvent are theoretically treated in the same way of dilute ideal solution, with $\Delta H^{Mix}=0$ (athermal mixing), of small molecules [Aguilera et al., 1999].

It is necessary the calculation of the values of entropy and enthalpy to evaluate the Gibbs free energy of mixing. The Flory-Huggins theory considers the polymeric solution like a network, where a polymer segment or a solvent molecule occupies each network site [Aguilera et al., 1999].

Flory-Huggins equation describes the change of free energy of mixing for polymeric solution; for a binary system it can be expressed as follow:

$$\frac{\Delta G^{Mix}}{N} = RT(\chi_{12}\phi_1\phi_2 + \phi_1 ln\phi_1 + \frac{\phi_2}{x} ln\phi_2) \qquad (2)$$

where:

- ϕ_1 is the volume fraction of solvent
- ϕ_2 is the volume fraction of polymer

- x is the number of segments per polymer molecule
- χ_{12} is the polymer-solvent interaction parameter. It can be evaluated with the following equation:

$$\chi_{12} = \frac{\Delta H^{Mix}}{RTN_1\phi_2} \quad (3)$$

where ΔH^{Mix} is the excess energy involved in neighbor interaction; N_1 is the number of moles of solvent; R is the gas constant and ϕ_2 is the volume fraction of the polymer. For this reason the interaction parameter can be defined as the ratio between energy involved in the interaction with neighbor molecules and the thermal energy (energy involved in the intermolecular bonds at a certain temperature). When χ_{12} is positive, it indicates repulsion and the mixing is endothermic, while the negative value means that the mixing is exothermic and indicates miscibility [Aguilera, 1999].

In equation (2), the first term on the right site in brackets represents the enthalpic contribution or interaction energy between solvent molecule and polymer segments; the last two terms represent the entropic contribution that results from placement that polymer occupies in the network with respect to the solvent.

The Flory-Huggins theory analyzes the polymer-solvent equilibrium system and it has specific limitations and application fields describing binary systems, but it is not very accurate describing ternary solutions.

The Flory-Huggins theory can be extended for ternary systems composed by non-solvent, solvent and polymer (respectively the subscripts 1,2,3 in the equation), with the following expression [Chung, 1996]:

$$\frac{\Delta G^{Mix}}{RT} = n_1 ln\phi_1 + n_2 ln\phi_2 + n_3 ln\phi_3 + \chi_{12} n_1 \phi_2 + \chi_{13} n_1 \phi_3 + \chi_{23} n_2 \phi_3 \qquad (4)$$

where:

- R is the gas constant
- n_i is the number of moles of component i
- ϕ_i is the volume fraction of component i
- χ_{12} is the non-solvent/solvent interaction parameter
- χ_{23} is the solvent/polymer interaction parameter
- χ_{13} is the non-solvent/polymer interaction parameter.

In equation (4), the first three terms on the right side represent the entropy of mixing for athermal polymeric solutions, while the last three represent the heats of mixing. This equation is valid only at constant temperature [Chung, 1996].

The equation written above was developed for asymmetric flat membrane formation and there are many limitations when this theory is applied to describe the Gibbs free energy of solutions during hollow-fibers formation process, because it is very difficult to simulate the conditions adopted for asymmetric flat membranes.

If the fiber is spun isothermally, it is necessary to add at least two terms to the equation (4): this is due to the fact that hollow-fibers are extruded with a hot spinneret with an air-gap distance and a speed necessary to increase the production and reduce fibers diameter and this process is not

isothermal; for this reason one terms that should be added is the work done by external stresses and the other is an extra change of the entropy induced by these stresses [Chung, 1996].

In this study, a quasi-ternary system composed by a polymer, a solvent and a non-solvent was analyzed. Usually it is not possible to consider the system as a real ternary system because polymer shows usually a molecular weight distribution, it is practically considered as a multicomponent system. The concept is the same when a polymeric solution is immersed in a non-solvent bath. The polymeric solution can be spun as a porous hollow-fiber, so in the inside or outside it can be touched with a non-solvent. Consequently, this kind of system can be analyzed with a ternary diagram, as explained in the following section.

4.2.3 The Gibbs Triangle

The treatment of ternary phase behavior requires adequate graphical representation. The most frequently employed graphical representation is the Gibbs phase triangle [Deiters et al., 2012].

The Gibbs triangle consists of an equilateral triangle, where the three pure substances are disposed at the corners: A, B and C (fig. 23).

Binary systems are plotted along the sides of the figure, so ternary mixtures are marked within the area of the triangle, for example point P in the next figure (fig.23).

Three lines, through the point P and parallel to each side of the triangle, are plotted to get three small equilateral triangles (aaa, bbb and ccc). It is possible to observe:

- The sum of the lengths of the nine sides of the three triangles is equal to the sum of the lengths of the three sides of the triangle ABC;
- The sum of the lengths of one side of the aaa, bbb and ccc triangles is equal to the length of a side of the major triangle;
- The sum of the altitudes of the minor triangles is equal to the altitude of the ABC triangle.

Each side of the Gibbs triangle can be divided into ten equal parts, which represent the percentage on the binary composition scale. Moreover, this percentage scale can be used to determine the composition at point P (fig. 24).

For this reason, the Gibbs triangle is divided with lines parallel to each of the three sides, so the percentage amount of the components A, B and C can be read simply.

The length of side a, measured in percent parts, represents the amount of the component A in the mixture at point P; equally the length of side b represents the percentage of B in P and the length of side c represents the percentage of C at point P.

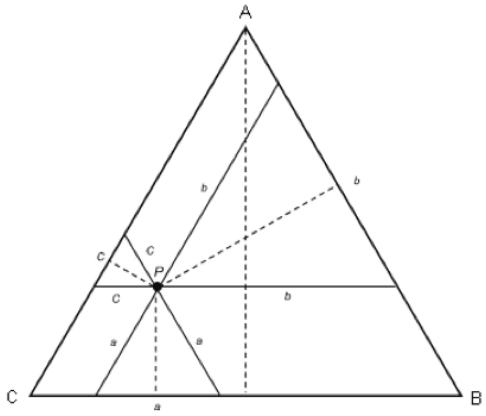

Figure 23. The Gibbsian equilateral triangle [Campbell, 2012]

For example, at point P the A percentage is given by Pa (or Pa' or aa') straight line, which measured 20 units, so a similar consideration can be done with B and C compositions and this expression is obtained:

$$P = 20\% \text{ of } A + 70\% \text{ of } B + 10\% \text{ of } C$$

The sum of the three sides a, b and c, which is equal to the length of a major side of the triangle, must be equivalent to 100% of the mixture composition [Campbell, 2012].

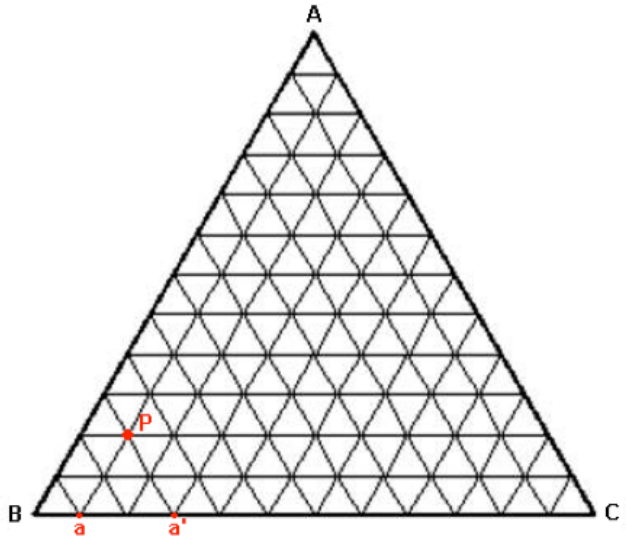

Figure 24. The Gibbs triangle with composition lines inside

Ternary systems are not always homogeneous, in fact they can present some binary subsystems, called miscibility gaps, which delimits the

heterogeneous mixture using a line that connects the saturation points (binodal curve) [Sattler et al., 1995].

As shown in the next figure (fig. 25), the easier ternary cross section (with constant temperature and pressure) is represented by two miscible binary subsystems and one immiscible binary subsystem. The miscibility gap of the binary mixture (B+C) expands from the side BC into the Gibbs triangle, so it decreases until it disappears with increasing amount of B, because the binary subsystems A+B and A+C are completely miscible.

The lines, which connect two coexisting phases in equilibrium, are called connodes and they are usually not parallel.

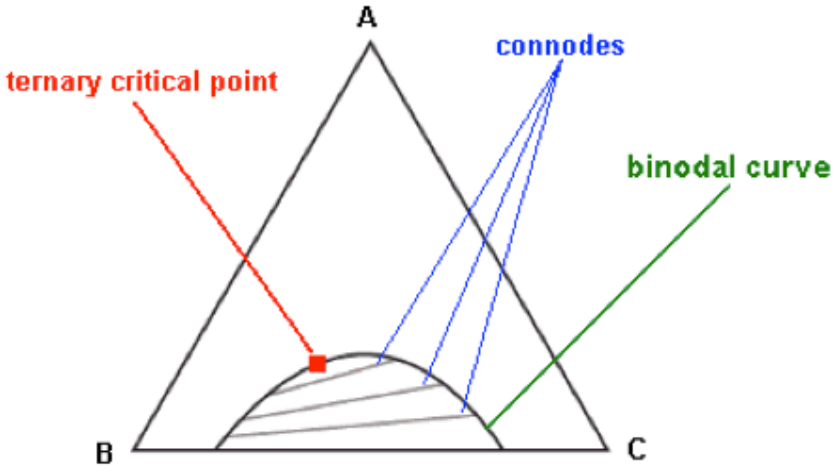

Figure 25. Isothermal-Isobaric section of a ternary phase diagram with miscibility gap

A mixture of components A, B and C, whose composition is described by point P located inside miscibility gap, is divided into two phases (described by points D and E). The relative amounts of the two phases can be estimate by the level rule (fig.26).

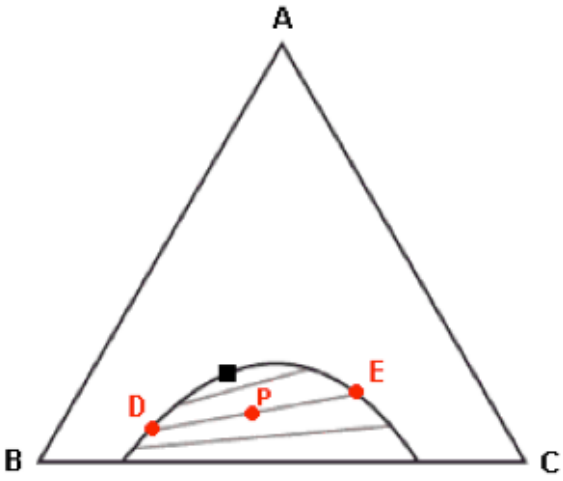

Figure 26. Immiscible subsystem into Gibbs triangle

The next figure (fig. 27a) shows another ternary cross-section, which is characterized by one miscible binary subsystem and two immiscible binary subsystems. The size and shapes of the two miscibility gaps, where two phases occur, change with pressure and temperature, so sometimes the two-phase areas can collide and the two ternary critical points meet in one point (fig. 27b); at last the two-phase regions form a continuous band, where the connodes have to change continuously from the connodes of the subsystem (A+C) to those of the subsystem (A+B) (fig. 28).

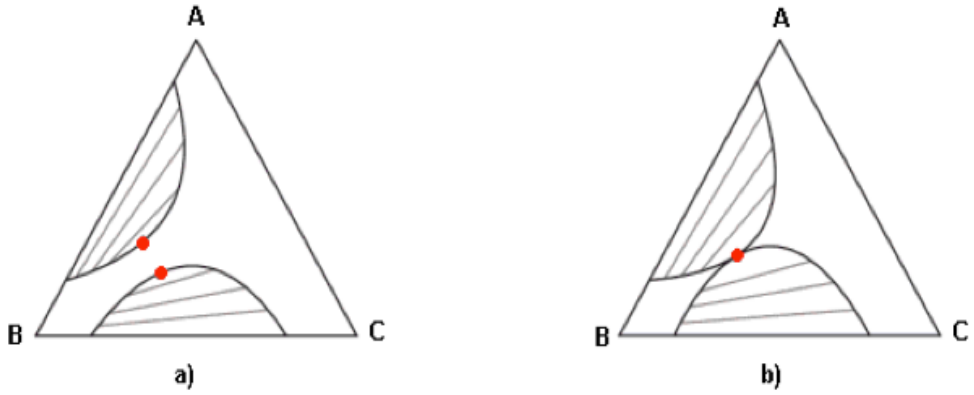

Figure 27. Cross section of a ternary phase diagram with two partially immiscible subsystems

Finally, the most complicated ternary cross section is composed by three immiscible binary subsystems. As in the previous system, it is possible to have different configurations for the miscibility gaps into the Gibbsian equilateral triangle.

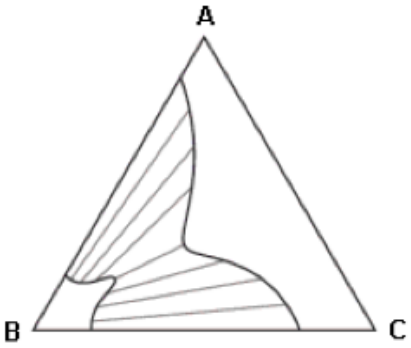

Figure 28. Ternary diagram with two-phase regions forming a continuous band [Deiters et al., 2012].

As shown in the next figure (fig. 29a), the first diagram is formed by three-phase areas that are not connected to each other. The second Gibbs triangle (fig. 29b) is composed by a band, where two-phase regions are connected, and one unconnected. At least, if all miscible gaps are connected, a three-phase triangle is represented inside the diagram [Deiters et al. 2012].

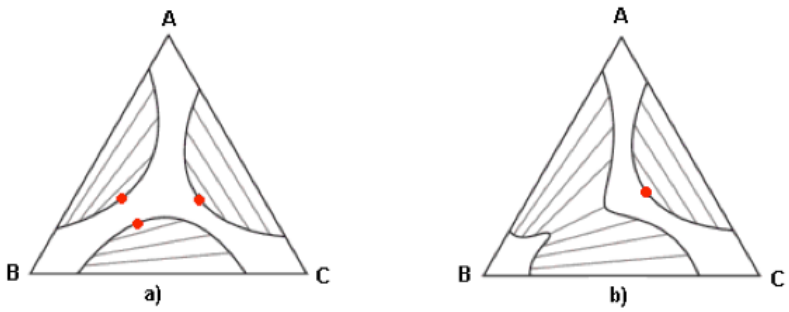

Figure 29. Three partially immiscible subsystems in a ternary phase diagram [Deiters et al., 2012].

In this study, a quasi-ternary system composed by a polymer, a solvent and a non-solvent was analyzed. A polymer shows usually a molecular weight distribution, so it is not possible to consider the system as a real ternary system, but it is practically considered as a multicomponent system. The concept is the same when a polymeric solution is immersed in a non-solvent bath. The polymeric solution can be spun as a porous hollow-fiber, so in the inside or outside it can be touched with a non-solvent. Consequently, the exchange between solvent and non-solvent occurs by diffusion and in each point of the polymeric solution the composition of polymer/solvent/non-solvent system will change as a function of time (fig.30). This change can be represented with a ternary diagram [Ronner et al., 1988].

The diagram schematically represents the composition paths due to the mass transfer occurring between non-solvent in the cast solution and between the solvent in non-solvent bath.

In figure 30 a qualitative representation of a liquid-liquid demixing gap is shown, which is divided into four regions: one region of spinodal demixing, two regions of nucleation and growth (located between the binodal and spinodal area) and one gelation region.

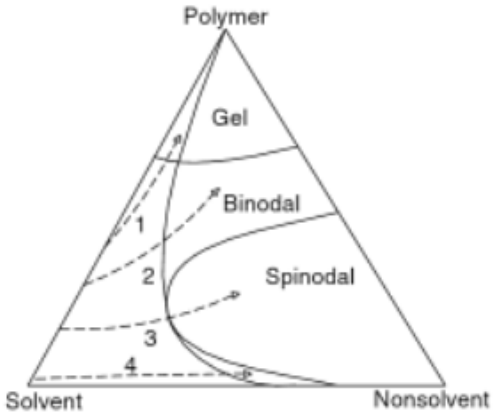

Figura 30. Ternary phase diagram of a generic polymer/solvent/non-solvent system

Starting from the polymer-solvent side of the triangle, different ways of changing composition during the process can be followed and some examples are shown by the arrows numbered from 1 to 4 [Drioli et al., 2010]:

1. This path shows an increasing concentration of the polymer in the solution because the inflow of non-solvent is slower than outflow of the solvent, so the polymer solidifies by gelation and/or crystallization into a dense structure without the creation of biphasic system (phase inversion).
2. This composition arrives in the metastable region (between the binodal and spinodal curve) where nucleation and growth of polymer-poor droplets occur with solidification of the polymer-rich phase. Depending on the polymer/solvent/non-solvent system, two different morphologies can be obtained: porous or dense.
3. Starting from a homogeneous polymeric solution, adding a non-solvent, a biphasic system is reached: one solid polymer-rich phase, constituting the fiber matrix, is obtained; the other one is a liquid non-solvent rich phase, which leads to the formation of pores inside the structure.
4. This path arrives between the binodal and spinodal curve (metastable region) and leads to nucleation and growth of polymer-rich droplets with solidification of the polymer-rich phase. A particulate structure is obtained [Drioli et al., 2010].

Finally Flory-Huggins theory provides only a macroscopic overview of the equilibrium of the system but does not allow the prediction of the

porous structure of the fiber. Dimensions and distribution of the pores depend on kinetic parameters and chemical and physical properties of the system.

The porosity into the fibers can be described with Flory-Huggins theory taking account only the interaction between polymer solution and non-solvent. Indeed, by changing the casting and the non-solvent bath conditions a variety of fibers structures can be obtained. For this reason different non-solvents were investigated: in particular ethyl alcohol (95%vol) and an aqueous solution with 1%w of gelatin and 50% (vol.) of isopropanol were used during the fiber production.

4.3 Fibers Production

The wet-spinning apparatus previously described in section 3.3 permitted the production of hollow fibers with diameters with order of magnitude of few micrometers. These fibers were then used to create three-dimensional scaffolds to seed stem cells and to analyze their adhesion, growth and proliferation in order to use them in cartilage tissue repair.

The first part of the experiments was the preparation of polymer solution. Polymers used are poly-L-lactic acid (PLLA) and poly-caprolactone (PCL) and they were dissolved in an appropriate solvent, in this work it was dichloromethane (DCM). For this purpose a mixer was used.

The machine is provided with two different syringes; the polymer solution was loaded in one of them and non-solvent in the other, in order to behave like a coagulation bath to remove the solvent. Different non-solvents were use to achieve this aim: ethyl alcohol and an aqueous solution of gelatin with isopropyl alcohol.

Polymer solution and non-solvent converge to the extrusion head, where they come out respectively from the outer hole and from the internal needle in order to create a cavity inside the fibers.

When the polymer solution comes out from the spinneret, polymeric fiber solidifies due to the evaporation of the solvent and to the coagulation bath. The hollow fibers extruded were collected on the rotating roll.

4.3.1 PLLA Fibers

The first polymer that has been tested was poly-L-lactic acid (PLLA). The solvent used to dissolve the polymer was dichloromethane in the ratio of 1:3.5 w/v.

Polymer and solvent were loaded in the chosen syringe covered with a top made by polytetrafluoroethylene (PTFE) and then dissolved using a mixer.

Two different PLLA fibers were created using two different non-solvents:

- Ethanol 95% (fig.31)

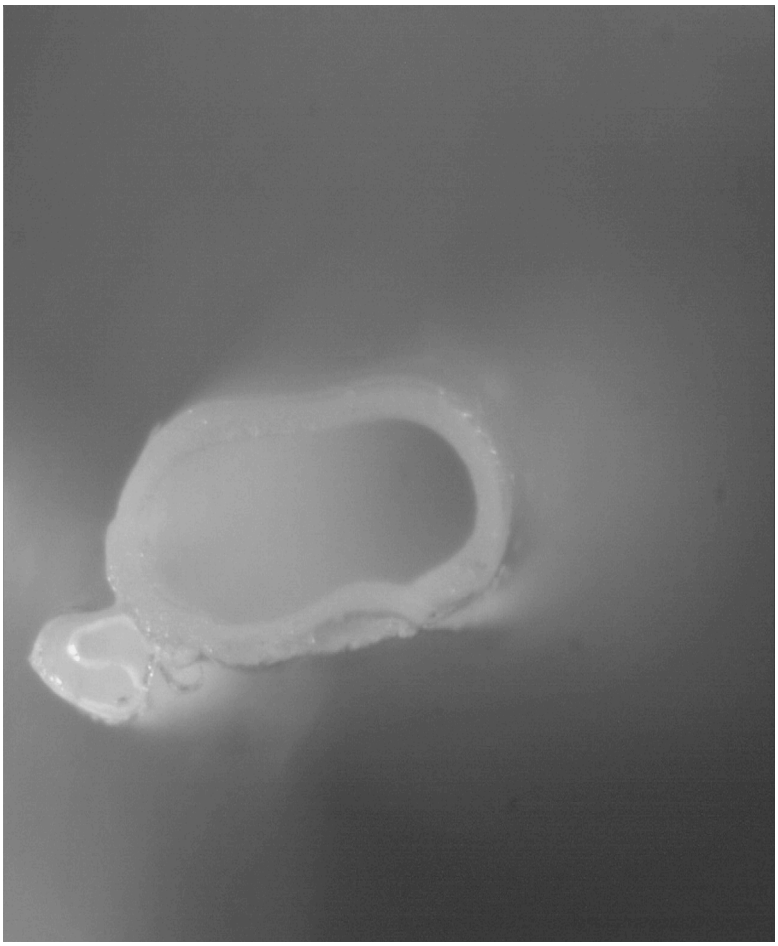

Figura 31. PLLA fiber; non-solvent: ethanol

- Aqueous solution with 1%w of gelatin and 50% (vol.) of isopropanol (fig.32).

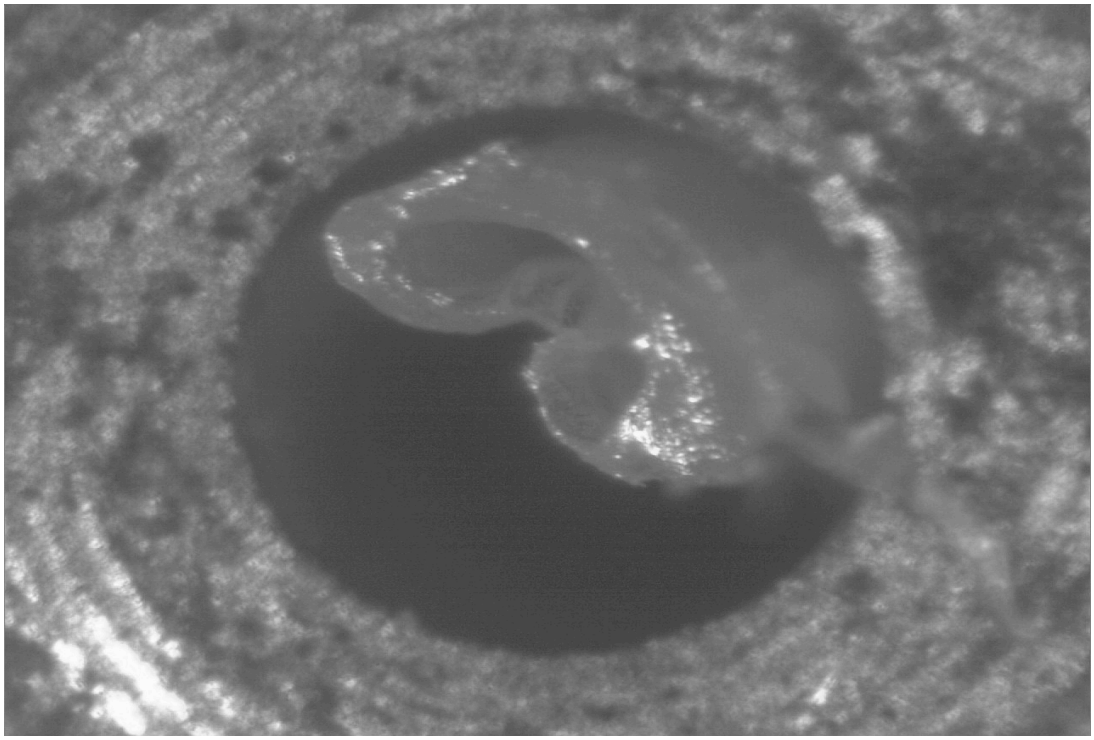

Figura 32. PLLA fiber; non-solvent: aqueous solution of gelatin and isopropanol

This solution was prepared using an agitated heating bath (fig.33) at 50°C to dissolve 1g of gelatin in 50 ml of distilled water; then 50 ml of isopropanol were slowly added.

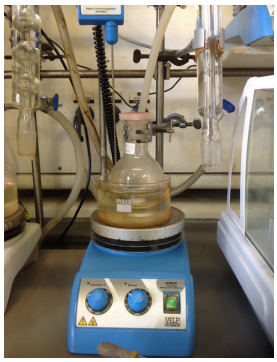

Figura 33. Heating bath

4.3.2 PCL Fibers

The other polymer that has been tested was polycaprolactone (PCL). The solvent used to dissolve the polymer was dichloromethane in the ratio of 1:4 w/v.

Polymer and solvent were loaded in the chosen syringe covered with a top made by polytetrafluoroethylene (PTFE) and then dissolved using a mixer.

Two different PCL fibers were created using two different non-solvents:

- Ethanol 95% (fig.34)

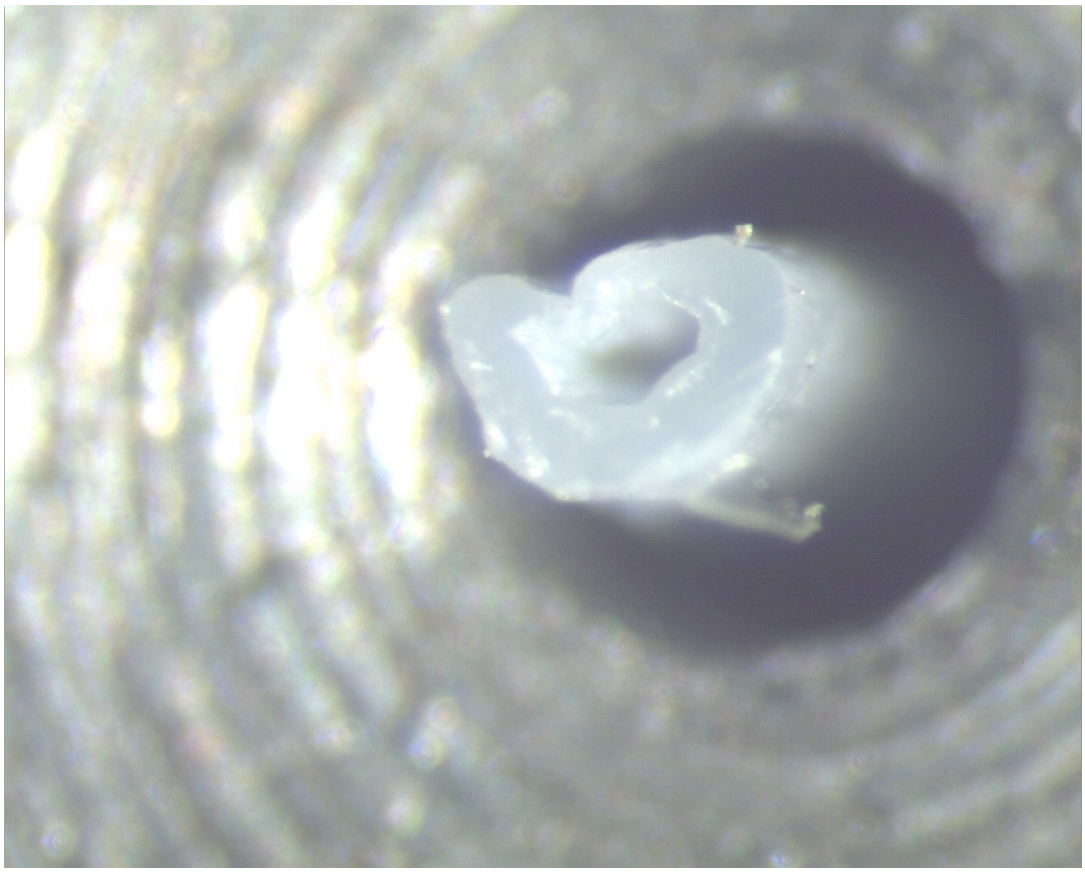

Figura 34. PCL fiber; non-solvent: ethanol

- Aqueous solution with 1%w of gelatin and 50% (vol.) of isopropanol (fig.35)

This solution was prepared using an agitated heating bath at 50°C to dissolve 1g of gelatin in 50 ml of distilled water; then 50 ml of isopropanol were slowly added.

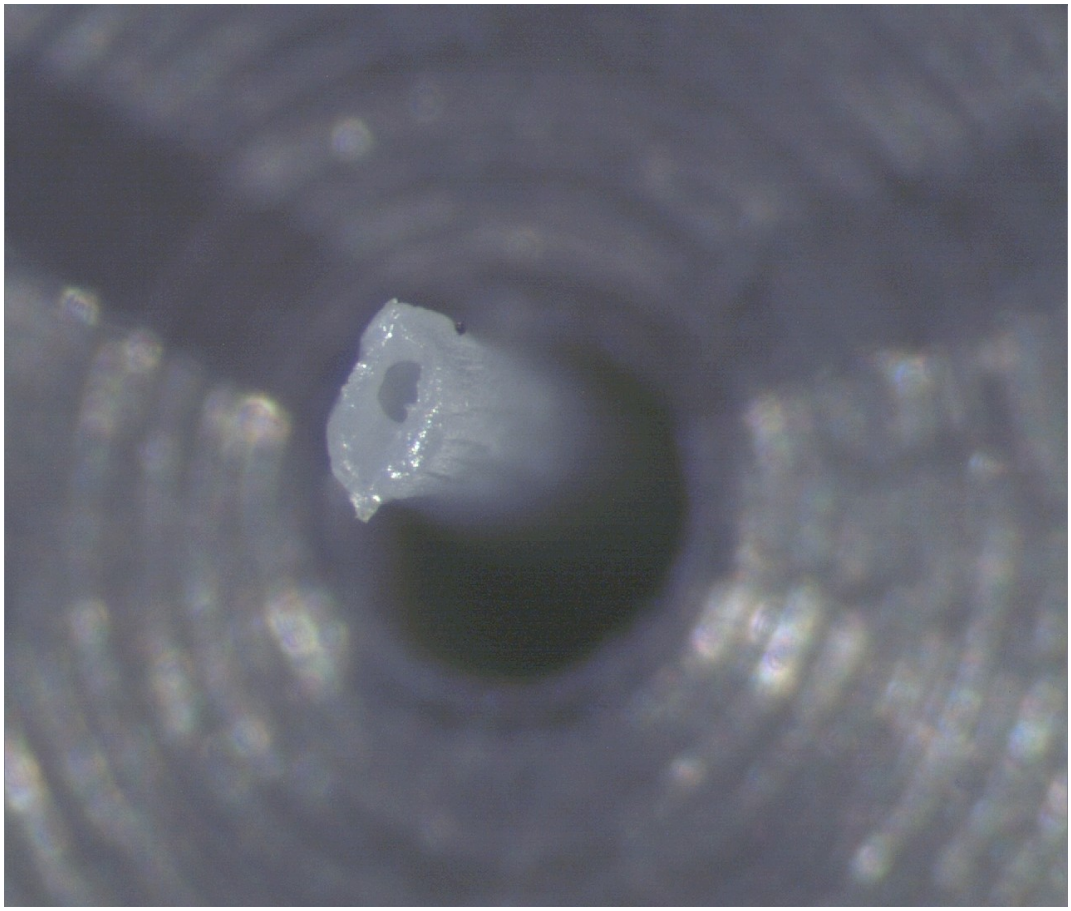

Figura 35. PCL fiber; non-solvent: aqueous solution of gelatin and isopropanol

4.4 Scaffolds production

Polymeric fibers produced by the dry-wet spinning apparatus previously described, were used to hand-make hollow fiber-based scaffold through different simple steps.

In the first step, fibers were taken out the rotating roll and arranged on a Teflon sheet. Two different arrangement were realized:

- *Random*: fibers were wound up by hand into a random shape and disposed on small fine Teflon net. Then they were covered with another Teflon net and compressed using clips.
- *Ordered*: five layers of fibers were arranged on a small fine Teflon net, rotating each layer 45° compared to the previous one. Then they were covered with another Teflon net and compressed using clips.

The second step of the procedure provides the creation of points of junctions between the fibers. For this purpose the arrangements were heated in an oven at a predefined temperature and time period to bond the fibers together on the contact points.

PLLA fibers were heated at 150°C for about 1 hour, while PCL fibers were heated at 60°C for about 3 minutes.

Scaffolds can be produced in different shapes depending on the final application. The aim of this production is the biological characterization, so a shape of small disk, usually used for this purpose, was chosen; a steel puncher was used to create scaffolds with a diameter of about 15 mm and a thickness of about 2 mm.

5. BIOLOGICAL CHARACTERIZATION

5.1 Cells culture

Human Mesenchymal Stem Cells (hMSCs) were isolated from the bone marrow aspirates of orthopaedic patients. These samples were collected after informed consent and anonymously treated, in conformity with the ethical principles of the declaration of Helsinki on human trials. Cells cultures were conduced, as reported in previous studies [Trombi et al., 2008], at the "Dipartimento di Bioingegneria Otologica (OTOLab), Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica, Università di Pisa".

Briefly, bone marrow aspirate was diluted with sterile saline in the ratio of 1:3; then the mononuclear cell layer, containing the MSC, was isolated with density gradient (Lymphoprep, Axis-Shield, Norway).

Mononuclear cells were treated with a physiologic solution with 0.2% of Trypan Blue to estimate their vitality and they were counted with a Burker Chamber. Therefore $0.2 \cdot 10^5/cm^2$ viable cells were plated in cell culture flasks using a culture medium (CM) containing low glucose D-MEM, 2 mM L-glutamine, 100 UI/ml of penicillin, 100 mg/ml of streptomycin and 10%(vol.) fetal bovine serum (FBS).

After 24 hours, non-adherent cells were removed from cell culture by washing with saline, so that MSC could be selected because adherent cells. When cultured had reached about 70-80% of confluence, MSCs were

removed by using 0.2% of Trypsin and plated again in culture medium with a density of 10^3 cells/cm^2.

All the cultures were conduced in standard conditions of 37°C, humidity 95% and a mix CO_2/Air of 5%/95%.

Cells at first step and 70% of confluence were trypsinized and used to seed the samples of scaffold produced.

Three scaffolds of each type were sterilized by immersion in absolute ethanol overnight and then exposed to UV for 1 hour. After two washes of 10 minutes each with a solution 2x of penicillin-streptomycin and fluconazole in sterile physiologic, scaffolds were immersed in FBS for 30 minutes. The excess of FBS was aspirated before the seeding. MSCs were detached from the plates with trypsin, counted and seeded with a density of 500000 cells per scaffold, suspended in 20 μl of CM.

After the seeding, the constructs MSCs/scaffolds were put in incubator for 1 hour to promote cells adhesion and then were covered with 5 ml of CM.

The constructs were cultivated for 18 days in standards conditions using plates of six wells each and changing the CM every three days.

The vitality of MSCs on the scaffolds were monitored during the time of culture using the alamarBlue® Assay.

At final time, one sample of each kind of scaffolds was processed for staining with Neutral Red Test to evaluate the colonization of the scaffolds produced.

5.1.1 Vitality of the MSC/scaffold system: alamarBlue® test

The cells metabolic activity was monitored during the culture period using the alamarBlue® colorimetric test. The dye includes a redox indicator, which cause a CM color change according to cells metabolic activity. In addition, the colorimetric test can be used several times on the same sample because the chosen dye is not toxic. The trial was performed following the protocol provided by the manufacturer, as reported in previous studies [D'Alessandro et al., 2014].

As by experimental protocol, three samples for each scaffold type and other samples without cells were incubated in CM in presence of diluted dye (3 ml for each sample) for three hours at 37°C. The test was performed every three days starting from cell seeding day, so it was done on the third, sixth, ninth, twelfth and fifteenth day. Every time 100 µl of sample or check supernatant were loaded into platelets, which are composed by ninety-six little wells, and excess supernatant was removed to replace it with new CM. The supernatants absorbance (λ) was measured by spectrophotometer; a double reading of the wavelengths (570 nm and 600 nm) was performed. As the protocol requires, experimental data were expressed by reduced percentage of alamarBlue® (%ABred), which is obtained correlating absorbance values with the molar extinction coefficients of the dye calculated at the two wavelengths, using the following expression (5):

$$\%AB_{red} = 100 \cdot \frac{(117{,}216 \cdot (sample@570\ nm - 80{,}586 \cdot (sample@600\ nm)}{(155{,}677 \cdot (control@600\ nm - 14{,}652 \cdot (control@570\ nm)} \qquad (5)$$

5.1.2 Vitality of the MSC/scaffold system: Neutral Red test

As reported in previous studies [D'Alessandro et al., 2014; Danti et al., 2014], cell vitality and colonization of the scaffolds' upper surface on experiment final time (day 18) was quantitatively evaluated using Neutral Red test. The dye is metabolized by viable cells and is placed into lysosomes and the cell cytoplasm becomes red. The NR is also a pH indicator: if the pH is less then 6.8 it becomes red and if the pH is higher than 8 it becomes yellow. Briefly, one construct for each type of scaffold was incubated with 50 μg/ml of NR on CM for two hours. The samples were rinsed using sterile saline and their surface was photographed using a camera for the digital macro (Canon Ixus) with 1×, 2× and 4× enlargements.

5.2 Results and discussion

The results of alamarBlue® test are entirely shown in figure 36.

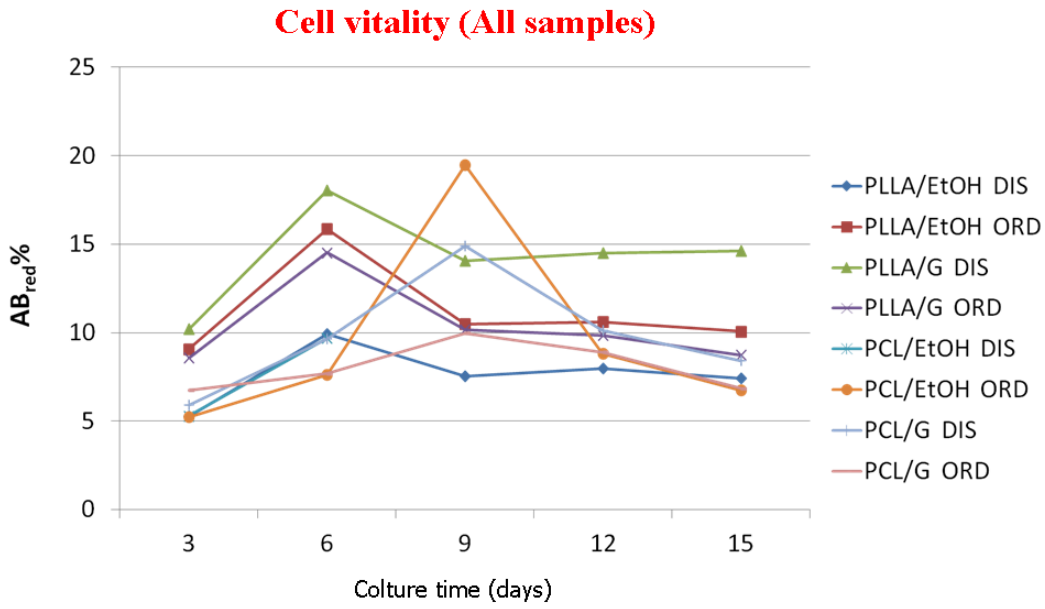

Figure 36. Cell vitality of all samples

On average, constructs metabolic activity (%ABred) was included in the 5.23-10.20% range at day 3, in the 6.73-14.62% range at day 15, so PCL/EtOH_ORD has shown a maximum value of 19.45% at day 9 (tab.4). To get a clearer view of the scaffolds performance, they were divided according to the polymer type: PLLA and PCL.

The figure 37 shows the %ABred values, including its standard deviations, for the four PLLA construct types (G = gelatin; EtOH = ethanol; DIS = random arrangement; ORD = ordered arrangement). The trends are similar

and show a vitality increase at day 5, then it stabilizes at approximately constant values until day 15. On average, PLLA samples vitality was G_DIS > EtOH_ORD ≥ G_ORD > EtOH_DIS for all the culture period.

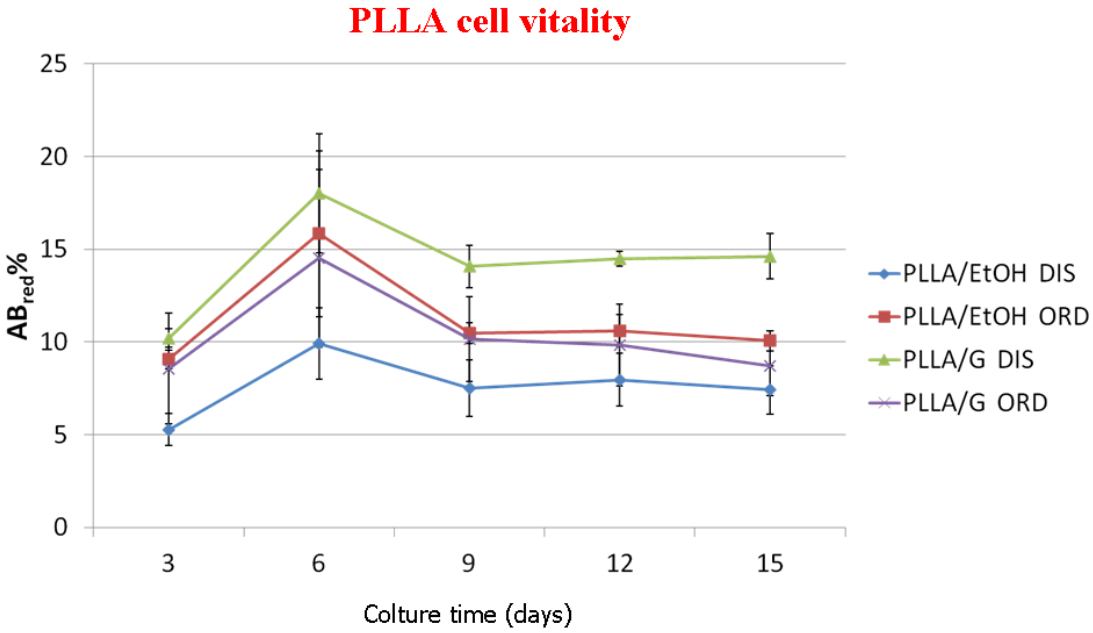

Figura 37. PLLA cell vitality

Particularly, PLLA/G_DIS samples show the best vitality to both the final time to the initial time, respectively 14.62 ± 1.23 % and 10.20 ± 0.51 % (tab. 4).

The PCL samples vitality is shown in figure 38. All PCL/EtOH_DIS samples were infected by mold between day 6 and day 9, for this reason only a few initial data were taken, which are not reliable (only one sample was survived at day 6).

Table 4. Experimental vitality data expressed by reduced percentage of alamarBlue® (%ABred)

%ABred	Average					Standard Deviation				
Day	3	6	9	12	15	3	6	9	12	15
PLLA/EtOH DIS	5,281166	9,914409	7,526211	7,965194	7,411764	0,848908	1,916232	1,528321	1,429063	1,321363
PLLA/EtOH ORD	9,066891	15,85273	10,47629	10,61422	10,06115	0,493952	4,473181	0,577124	0,864231	0,541401
PLLA/G DIS	10,20452	18,02615	14,07287	14,50596	14,62164	0,507431	3,213341	1,144708	0,401542	1,226149
PLLA/G ORD	8,573271	14,51617	10,16076	9,839937	8,730142	2,991434	4,797289	2,272531	2,207721	1,620873
PCL/EtOH DIS	5,288213	9,682371				0,559665	1,210648			
PCL/EtOH ORD	5,230724	7,623231	19,4553	8,809865	6,753868	0,345761	0,696577	2,825597	0,66559	0,253415
PCL/G DIS	5,910373	9,649082	14,89798	10,11948	8,416407	0,235217	1,094118	1,570476	1,459433	0,842795
PCL/G ORD	6,730742	7,67541	9,978313	8,893848	6,83962	1,192679	0,67589	2,284541	0,55903	0,648136

The three PCL types showed a similar trend: %ABred maximum at day 9 and then decreases. PCL/EtOH_ORD showed the best vitality, in fact it was 19.45 ± 2.82% at day 9; but at final time, PCL/G_ORD showed the highest average vitality: 8.41 ± 0.84 % (tab. 4).

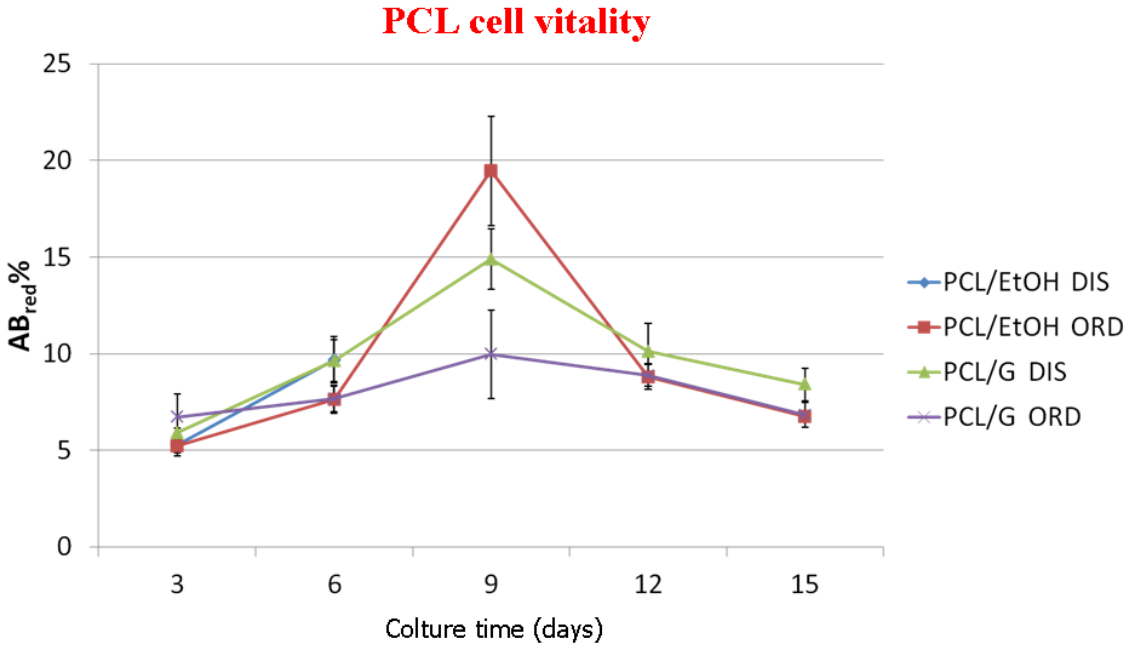

Figura 38. PCL cell vitality

The alamarBlue® test experimental data seem to resemble respect to Neutral Red test values at day 18 (culture final time). The viable cells are represented by bright red areas, instead dye non-specificity is indicated by orange frustules.

Figure 39 and figure 40 show respectively PLLA/EtOH and PLLA/G samples colored by NR.

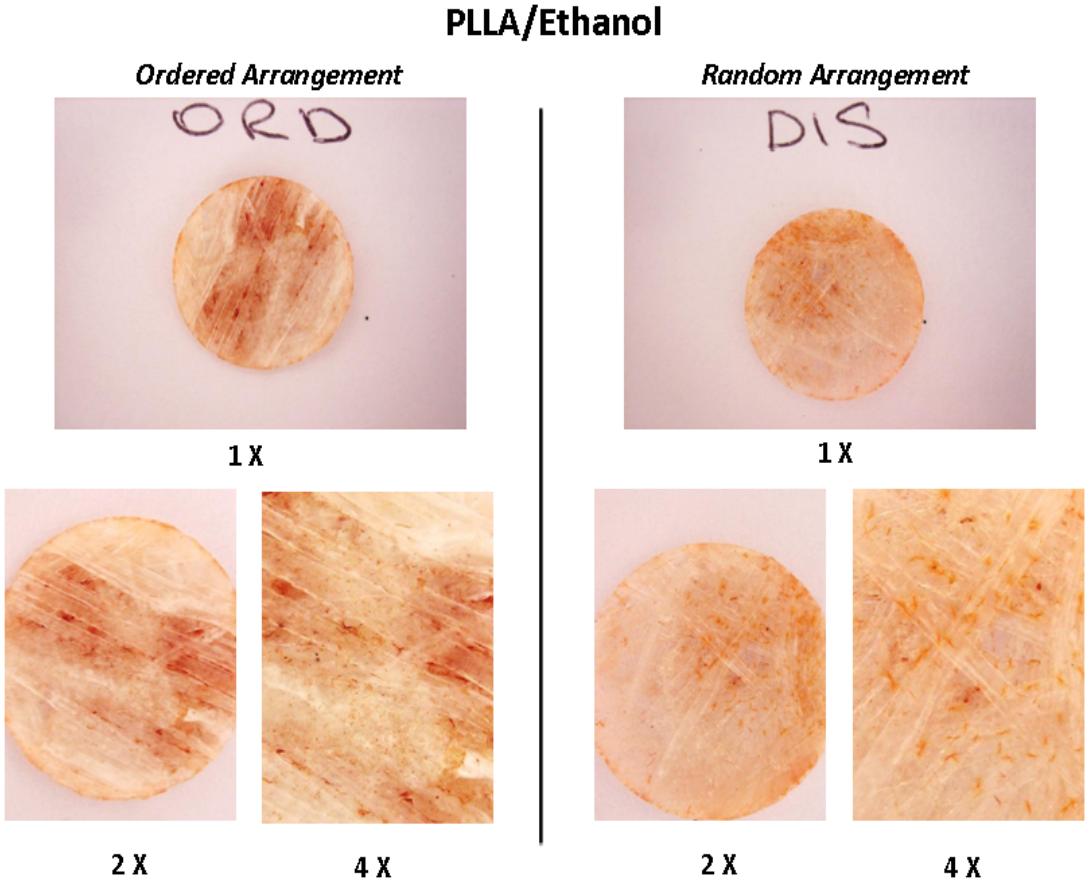

Figura 39. PLLA/Ethanol scaffolds

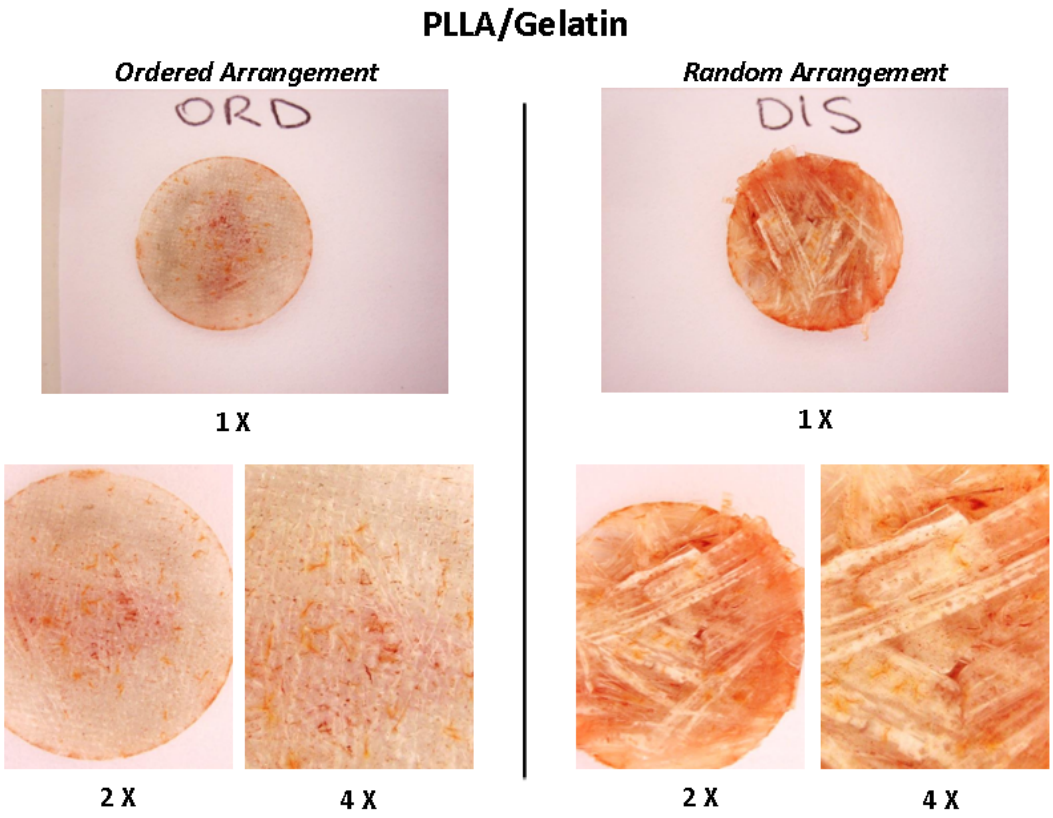

Figura 40. PLLA/Gelatin Scaffolds

It is possible to observe random and incomplete samples colonization, except for PLLA/G_DIS samples, which result the best. Also PLLA/EtOH_ORD samples show a few densely populated areas.

Figure 41 and figure 42 show respectively PCL/EtOH and PCL/G samples colored by NR.

All PCL samples are colored by intense yellow, which probably represents a very basic environment and poor cells colonization into this sample types.

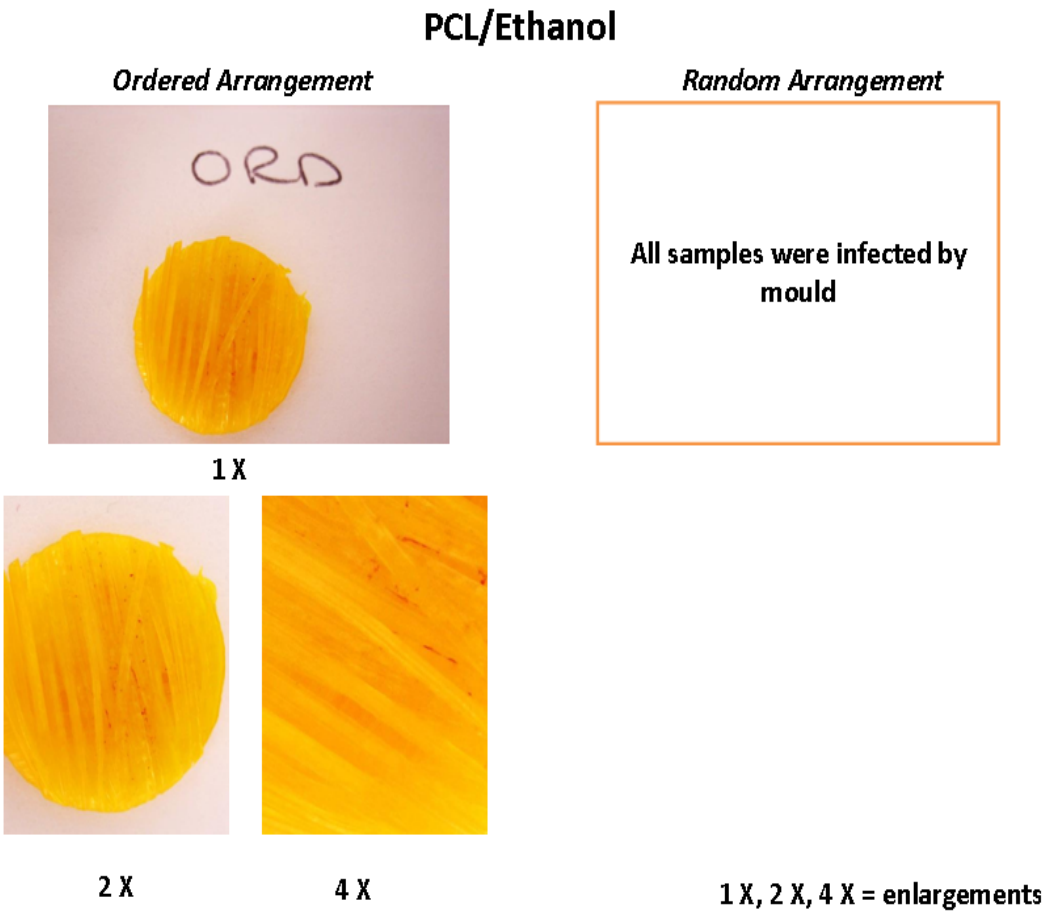

Figura 41. PCL/Ethanol scaffolds

From a qualitatively point of view, PCL/EtOH_ORD samples are the best PCL construct because the cells colonization is more uniformly distributed, according to alamarBlue® test.

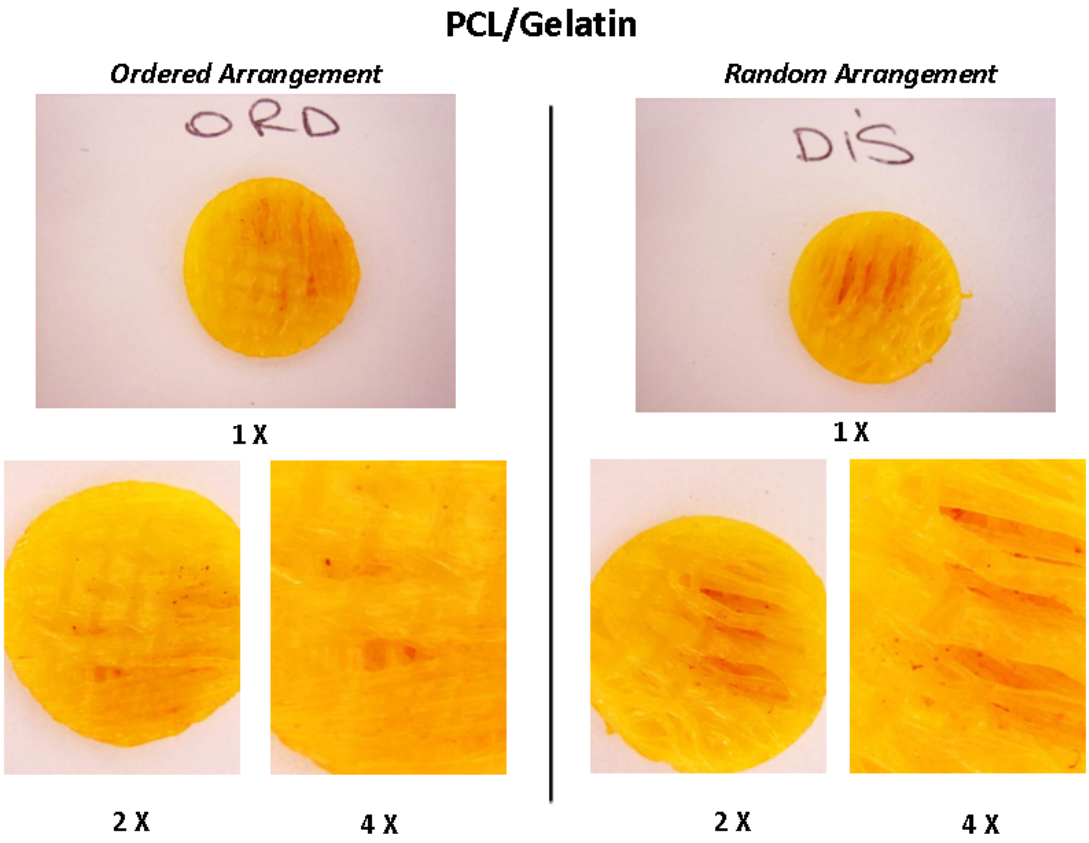

Figure 42. PCL/Gelatin Scaffolds

Between the analyzed scaffolds, PLLA/G_DIS samples have shown the best metabolic activity, cells vitality and colonization. To confirm the results of these experiments, other studies are in progress.

6. CONCLUSIONS

The main focus of this thesis project has been the tuning of a dry-wet spinning apparatus, developed by Gimac (Castronno, Italy), to create polymeric fibers through the control of process parameters.

This production process required the determination of the appropriate operative conditions to allow phase inversion, in order to obtain fibers with the suitable porosity for their final use. The aim is the production of biodegradable scaffolds that behave as support for cells in regenerative medicine applications, in particular in regeneration and repair of damaged connective tissue, specifically for cartilage tissue repair.

In the initial part of this thesis, an overview about tissue engineering and the main challenges of this field of research was obtained to analyze the strategies behind scaffolds production.

Therefore, the attention was focused on the goals and main treatment options of cartilage repair, in particular the use of biomaterials to produce scaffolds, which constitute together with adequate cells a needed construct to repair an injured tissue. Biomaterials investigated in this study were poly-L-lactic acid (PLLA) and poly-caprolactone (PCL) in order to comprehend their behavior in porous fibers production.

The main manufacturing fibers technique is the spinning-process, for this reason a review of the literature on this method was treated. However, to understand the characteristics of the apparatus used in this project, the dry-wet spinning technique was studied.

On the basis of the theory behind polymeric solutions, the experiments were conduced in order to produce different kind of hollow fibers to create several scaffolds structures.

Two different polymeric solutions were proposed to create the fibers and the cavity inside them was obtain with different non-solvents. Then, through a purposeful procedure, two arrangements of the hollow fibers (random and ordered) were used in order to produce different types of scaffolds.

Mesenchymal stem cells were seeded on the matrices produced in order to investigate their adhesion on the surface of biomaterial used, their vitality and growth.

Biological characterization on the scaffolds, conduced at University of Pisa, showed, through the results of in vitro tests, that the matrices produced allow cells adhesion and growth. In particular, the constructs that showed the best cells colonization have been the samples composed by PLLA and gelatin solution used as non-solvent, randomly arranged.

The results of the preliminary biological characterization showed that the fiber-based scaffolds produced with the dry-wet spinning apparatus analyzed in this study are promising supports for cell adhesion and growth.

These scaffolds are required to be degradable in times and ways compatible with the final aim due to the need of integration with the tissue; for this reason degradation studies should be conduced to verify scaffolds ability of having proper degradation rates.

The gel spinning apparatus studied permits the production of hollow-fibers with different structural characteristics in order to allow a huge field of final applications.

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RINGRAZIAMENTI

Desideriamo ringraziare tutti coloro che ci hanno aiutato nella stesura della tesi con suggerimenti, critiche ed osservazioni.
Ringraziamo anzitutto il professor Maurizio Masi, Relatore di questa tesi, il professor Giuseppe Perale e il professor Gianni Pertici, Co-relatori di questa tesi, per il supporto e la continua disponibilità.
Un ringraziamento particolare alla ditta Gimac per averci permesso di poter fare questa esperienza presso la loro Azienda, con il costante aiuto e l'infinita disponibilità di tutte le persone che ci lavorano. In particolare ringraziamo i signori Giorgio e Marina Maccagnan per i sapienti consigli, Sebastiano, Tiziano, Mauro e Alberto per l'aiuto pratico, Simone e Davide Maccagnan per le preziose informazioni e la signora Barbara per la cortesia dimostrataci.
Desideriamo, inoltre, ringraziare il "Dipartimento di Bioingegneria Otologica (OTOLab), Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica" dell'Università di Pisa, in particolare la dott.ssa Serena Danti, la dott.ssa Luisa Trombi e il dott. Claudio Ricci, che si sono occupati dell'isolamento delle MSC e delle colture cellulari.
Vorremmo, infine, ringraziare le persone a noi più care: i nostri amici e le nostre famiglie, a cui questo lavoro è dedicato.