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List of abbreviations

CIBER-BBN	Centro de investigacion biomédica en red, en Bioingenieria, biomateriales y nanomedicina
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
E. Coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraacetic acid
FIOBI-HULP	Bone physiopathology and biomaterilas, Hospital universitario La Paz
GFP	Green fluorescence protein
Gly	Glycine
hMSC	Human mesemchymal stem cells
IB's	Inclusion bodies
IPTG	Iso propyl-D-thiogalactoside
LB	Luria Bertani
NANOMOL	Molecular nanoscience and organic materials
OD	Optical density
PCL	Poly caprolactone
PGA	Poly (glycolic) acid
PLA	Poly lactic acid
PLGA	Poly lactic acid co-glicolid
P_W	Working pressure
RT	Room temperature
SCFs	Supercritical compressed fluids
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microcopy
Ser	Serine
Tg	Glass transition temperature
TIPS	Thermally induced phase separation
Tw	Working temperature
Tyr	Tyrosine
UAB	Autonumus university of Barcelone

1. ABSTRACT

Regenerative medicine integrates different biomedical approaches to restore normal function in damaged tissues using a combination of cell/molecular biology and materials engineering. One of the most promising alternatives is to design a biodegradable and porous scaffold decorated with inductive factors that promote cell colonization and proliferation. In this context, an appropriate processing of the polymer is required in order to obtain a scaffold that acts as a support for tissue growth.

In this work is developed a new route for the preparation of porous matrices with appropriate physico-chemical and biological properties using supercritical fluid technology. In special, we investigate the PLA polymer to obtain 3D porous scaffolds and its decoration with bacterial inclusion bodies. IB's have been revealed as adhesive, mechanically and biocompatible protein materials that can be used to favour cell colonization and proliferation when used to decorated flat surfaces.

The synthesis of new matrices with improved properties concerning porosity, interconnectivity and mechanical properties has been possible using supercritical fluid technology for the processing of PLA. In addition, the decoration of PLA porous scaffolds with bacterial inclusion bodies promotes cell adhesion and colonization. Inclusion bodies do not result toxic to the cells and contrarily improve cell proliferation on the surface. The hybrid polymeric-IB's porous matrices obtained for the first time is this work are promising platforms to be used in bone tissue engineering.

2. RESUMEN

La medicina regenerativa integra diferentes estrategias biomédicas con el objetivo de restablecer la función normal en tejidos dañados usando una combinación entre la biología celular y molecular y la Ingeniería de materiales. Una de las alternativas mas prometedoras es diseñar un soporte, poroso y biodegradable, decorado con factores inductivos que promuevan la colonización y proliferación celular. En este sentido, es necesario un correcto procesado del polímero para obtener una estructura que pueda actuar como soporte para el crecimiento de tejidos.

En este trabajo, se desarrolla una nueva ruta para la preparación de matrices porosas con características físico-químicas y biológicas adecuadas, usando la tecnología de fluidos supercrítica. En particular, investigamos el ácido poli lactido para la obtención de matrices porosas y su posterior decoración con cuerpos de inclusión (IB's). Estos cuerpos de inclusión son materiales proteicos biocompatibles y adhesivos que pueden ser empleados para favorecer la colonización y proliferación celular cuando son usados para decorar superficies.

La síntesis de nuevas matrices con propiedades mejoradas en cuanto a porosidad, interconectividad y características mecánicas ha sido posible usando la tecnología de fluidos supercrítica para el procesado del polímero PLA. Además, la decoración de las matrices porosas de PLA con cuerpos de inclusión promueve la adhesión y colonización celular. Los cuerpos de inclusión no resultan tóxicos para las células y, contrariamente, mejoran la proliferación celular en la superficie. La matrices porosas híbridas, polímero-IB's, obtenidas por primera vez en este trabajo constituyen una plataforma prometedora para ser usada en Ingeniería de tejido óseo.

3. INTRODUCTION

3.1. Regenerative medicine

Humanity has been exposed to disease and injury since the beginning of its existence. While many diseases such as bacterial infections and injuries can be cured by drugs and surgery, many others remain incurable. When such incurable diseases become severe, they are often treated by transplantation of tissues and organs from a donor. However, such cases pose the problems of preservation of the organs to be transplanted and dealing with rejection of the transplanted organs. Regenerative medicine was devised as a revolutionary treatment method for solving such problems.

There are four areas of interest in the field of regenerative medicine:

- **Medical devices and artificial organs**. There is a critical shortage of donor organs of all type. This approach is based in the fabrication of artificial biomedical devices that perform the function of natural organ or tissue.
- **Cell therapy**. This approach is directed to restore a tissue or organ by introducing cells capable of developing the function of the mentioned tissue or by stimulating other cells to do it.
- **Tissue engineering**. This term refers to methods that promote the growth of cells lost due to trauma or disease. Tissue engineering uses many strategies, including the manipulation of artificial and natural materials that provide structure to cells for growing into specific kinds of tissues. These materials are called scaffolds.
- **Gene therapy**. It consists in the application of genetic engineering to inserting one or more corrective genes into genetic material of a patient's cells in order to cure a disease caused by a genetic defect. The expression of the new gene can then alter the DNA or RNA transcripts used to synthesis proteins and therefore correct the disease. This therapy is still in the experimental stages.

3.2. Bone tissue engineering

Despite its constant ability to repair small damages in response to injury, bone is not always able to achieve a complete regeneration of the injured area by itself. In this cases, tissue engineering emerges as a promising technology that integrates different strategies in order to restore, maintain, replace and enhance tissue normal function using the combination of biology, engineering, material science and medicine. The main advantage of this technology is that can provide a permanent solution to the problem of organ function (Khademhosseine et al, 2006).

In the past years, two important strategies have been developed in this field:

- Implantation of tissues, which have been pre-cultured in vitro. In this route, 3D biocompatible material called scaffold is designed to promote bone formation as an

ex situ template on which cells are seeded and cultured, followed by a subsequent implantation.

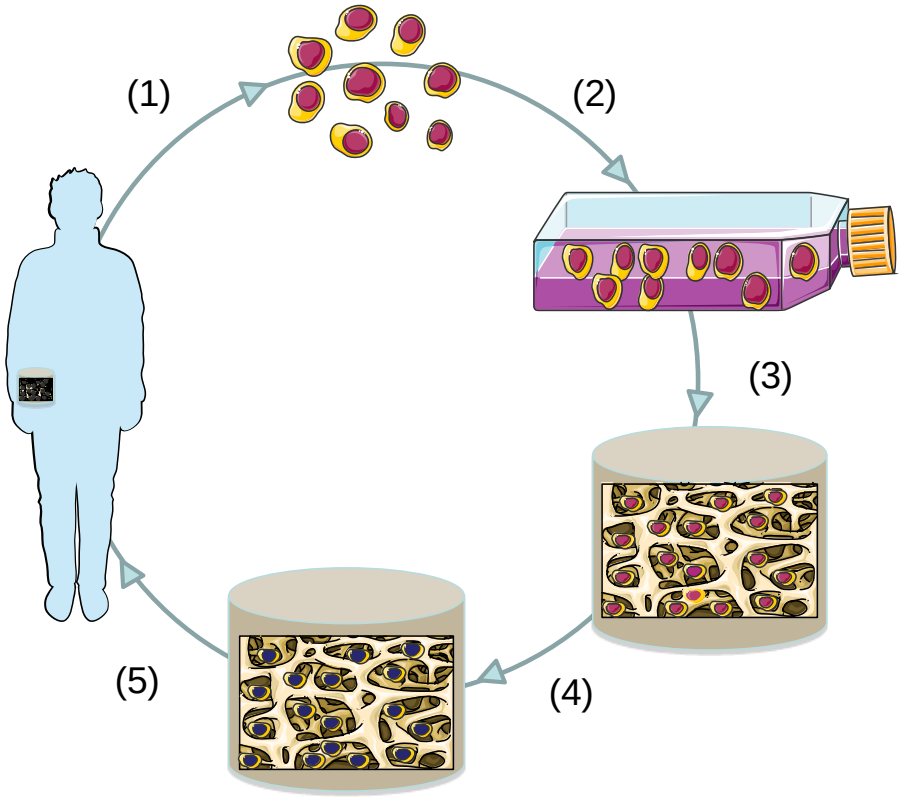


Figure 1: Tissue engineering *ex situ* approach. 1: extraction of cells from the patient, 2: culture of cells *in vitro*, 3: cell seeding on the 3D porous scaffold, 4: cell proliferation and differentiation on scaffold, 5: transplantation of scaffold to repair or regenerate the damaged tissue (Sachot, N., 2014; figure made using images from *www.servier.fr*).

- Insertion of a 3D porous scaffold directly *in vivo* with the purpose to stimulate and to direct tissue formation *in situ*. The advantage of this approach is the reduced number of operations needed.

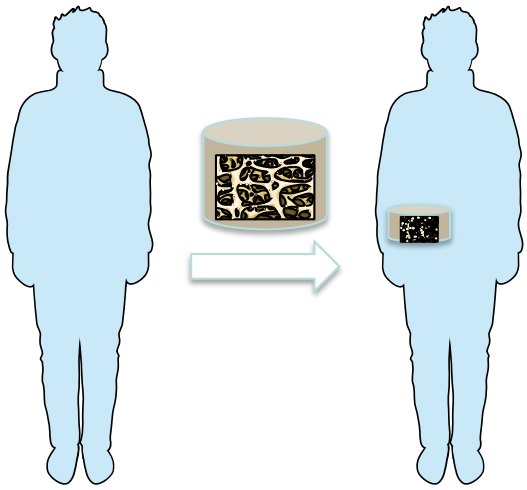


Figure 2: Tissue engineering *in situ* approach (figure made using images from *www.servier.fr*)

Although both approaches may appear very different, they have the same requirements, which are the followings (Barry J.J.A., et al., 2006):

- Cells, which will proliferate to create the tissue.
- Growth factors that signal the cells to proliferate.
- A method of delivery and stabilizing the cells in the bone defects. The vehicle for delivering cells is called scaffold.

Moreover, these two strategies support the principle that cell seeded in 3D biocompatible scaffolds are able to reassembly into functional structures similar to normal tissue. (Shi et al., 2010). Consequently, cells and scaffolds are the two essential components, and an adequate combination between them is required for the regenerative process.

Cells

Bone is a complex system formed by different cell types. Stem cells are undifferentiated cells that have unique capability of self-renewal and multipotential differentiation to serve as a cell source (Zhao C. et al., 2013). Mammals present two different kinds of stem cells, embryonic an adult. The difference between them is that embryonic can generate all cell types and adult only a few lineages. In bone tissue engineering, the most commonly cellular line is adult, in particular, meshenchymal stem cells.

Scaffolds for bone tissue engineering

The success of a scaffold-based strategy is dependent not only in providing a structural support for the cells, but also in establishing a microenvironment to maintain and regulate cell behaviour and function.

A scaffold can be a polymeric structure, which acts as a support for cell adherence, growth and proliferation during repair and regeneration of damaged tissue. For a long time, two-dimensional scaffolds composed of natural polymer such as collagen have been used as scaffolds for tissue engineering. However, there is an important limitation in the number of cells that can be attached. By using three dimensional porous materials as scaffolds, the number of cells that can be loaded in the material increases. In addition, the natural polymer can cause manufacturing and immunologic problems (Langer, R. 2009).

From a general point of view, scaffolds have to possess many different characteristics to be considered as good candidates for bone tissue engineering. As a three dimensional structure they provide mechanical integrity and a surface responsible of the chemical and architectural guidance for regenerating tissues. The most important characteristics are the followings:

- Be biocompatible and biodegradable without releasing toxic by products.
- Act as a template to support cell adhesion, colonization, proliferation and differentiation.
- Have a high degree of porosity and interconnection between pores to favour cell migration towards the centre of the material structure. An adequate cell attachment,

colonization and, as consequence, tissue regeneration, can be possible throughout a 3D matrix, promoting the integration of the scaffold and facilitating cell migration and nutrient transfer during tissue formation.
- Stimulate simultaneously osteogenesis and angiogenesis
- Resorb at the same time than the new bone is formed
- Possess mechanical properties and keep a structural integrity at the beginning of the regeneration process.
- Be sterilizable.

Nowadays, there are two types of materials widely used in the fabrication of porous scaffold: polymers (naturals or synthetics) such as polysaccharides, hydrogels or thermoplastic elastomers and bioactive ceramics, such as calcium phosphates and bioactives glasses.

3.2.1. Biodegradable polymers for porous materials preparation in tissue engineering

The use of degradable polymers is desirable because scaffolds are designed as temporary structures (Shoichet, M., 2010). Biodegradable polymers are separated in two groups: natural polymers, including polysaccharides (starch, alginate, chitin/chitosan, hyaluronic acid derivates) or proteins (soy, collagen, fibrin gels, silk), and synthetic polymers. This second group present many advantages such as production under controlled conditions, predictable and reproducible mechanical and physical properties and control of material impurities. Indeed, toxicity, immunogenecity and infections are lower for synthetic polymers.

The most commonly biodegradable synthetic polymers in tissue engineering are saturated aliphatic polyesters: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), their copolymer poly(lactic-co-glycolide) (PLGA) and poly(ε-caprolactone) (PCL) (Rezwan et. al., 2006).

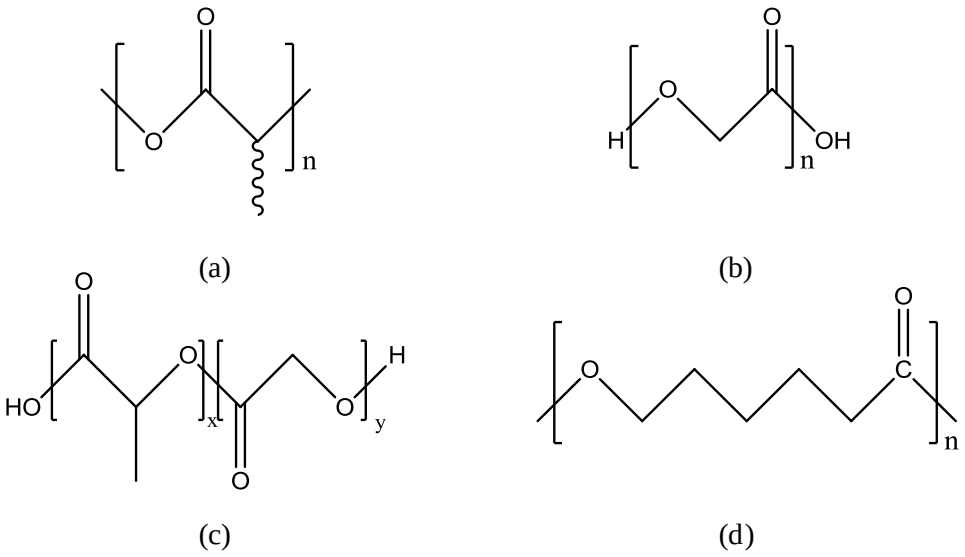


Figure 3: Biodegradable polymers used in tissue engineering. (a) PLA structure, (b) PGA structure, (c) PLGA structure and (d) PCL structure.

PLA has unique properties like good appearance, high mechanical strength, low toxicity and good barrier properties. This polymer exists in three forms: $_{L}$-PLA (PLLA), $_{D}$-PLA (PDLA), and racemic mixture of $_{DL}$-PLA (PDLLA). Crystalline state is given by L-phase of the polymer while amorphous, is given by D-phase.

One of most important parameters is the degradation rate in order to know that the polymer will be stable enough time to fulfil its purpose as a temporary structure. In general, degradation takes longer in crystalline (PLLA) than amorphous (PDLA and PDLLA) polymers because crystalline parts are chemically more stable that amorphous parts (Rezwan K. et al., 2006)

These polymers can be degraded through de-esterification into their monomeric components (lactic and glycolic acids) and completely removed from the body by natural ways. PLA, for example, can be eliminated through the tricarboxylic acid cycle.

3.3. Inclusion bodies as cell growth promoters

Bacterial inclusion bodies (IB's) are protein aggregates formed in bacteria during recombinant protein overexpression (Carrió 2002). IB's are in the size range of a few hundred nanometers and formed by polypeptide chains that can retain a certain amount of native or native-like structure (García-Fruitós, E. et al., 2009)

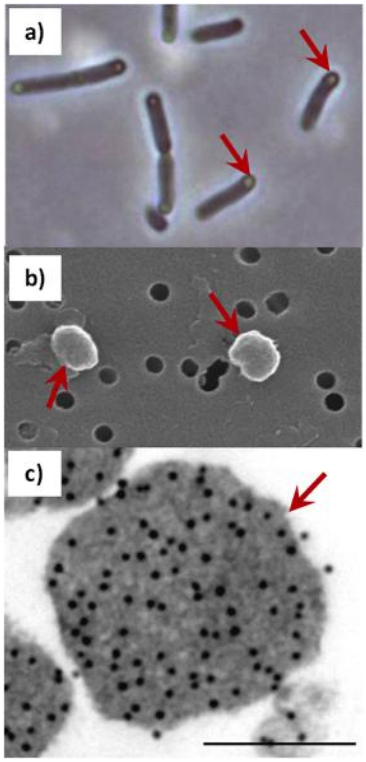


Figure 4: SEM images of bacterial inclusion bodies; (a) (Elena García Fruitós et al., not published), (b) (Joaquin Seras-Franzoso et al. not published) and (c) (Joaquin Serás-Franzoso et al. not published)

Inclusion bodies have been regarded as a major obstacle in protein product process by recombinant DNA technologies. At first, these aggregates were described as inactive proteins formed by misfolded polypeptides and considered as non-desired products of the recombinant DNA production process (García fruitós, E. et al, 2012). However, several studies showing the capacity of the IB's to retain certain grade of the biological activity of the forming proteins have changed this perception. In this sense, potential applications for these protein aggregates have raised such as their use to act as immobilized biocatalysts or more recently their use as a biocompatible biomaterial with application in regenerative medicine and tissue engineering approach.

IB's are commonly found in the bacterial cytoplasm. In recombinant *E. coli*, the target protein is the main IB component but other cell proteins can be found in lower concentration.

In this work, bacterial IB's formed by Green fluorescence protein (GFP) linked to major capsid protein of the foot and mouth disease virus (VP1) were employed. GFP is an active protein that emits green light following excitation of an internal fluorophore composed of a Ser-Tyr-Gly sequence positioned near the protein's amino terminus (Feilmeier, 2000). This protein is stable, species-independent, and can be monitored noninvasively in living cells using fluorescence microscopy (Bizarri, 2009). The VP1 protein conducts and facilitates the GFP aggregation as Inclusion bodies.

Recent studies on the applications of IB's have demonstrated their potential as biomaterial in regenerative medicine, where cell proliferation can be stimulated through the topographical modification of the material surfaces with these protein aggregates (García-fruitós, E. 2009 and Diez-Gil, C., 2010). IB's cannot only stimulate cell proliferation but also direct cell growth to specific regions when a surface is decorated or patterned with these aggregates, providing an appropriate environment for cells. In this regard, recent results show that cells cultivated on supports functionalized with IB's are adhered to the IB's areas, aligned and elongated according to specific patterns (Tatkiewicz, W.I et al, 2013), proving again their potential as protein-based nanomaterials for cell growth promotion.

3.3.1. IB's production by recombinant DNA technology

DNA keeps all the information needed for the normal function of a living organism. This information is codified in polynucleotide chains combining 4 distinct nucleotides. These DNA chains are firstly transcribed into mRNA and then translated into proteins carrying out all functions that support life in an organism. The direct connection between DNA sequences and protein sequences implies that potentially new and different protein can be created by changing the DNA sequence.

Recombinant DNA technology involves all procedures that are used to join DNA segments from two different sources to produce the desired protein. The most common approach inside

this technology consists on the introduction of a DNA fragment from one organism into a second organism. The basic procedure of this technology is described in the following figure.

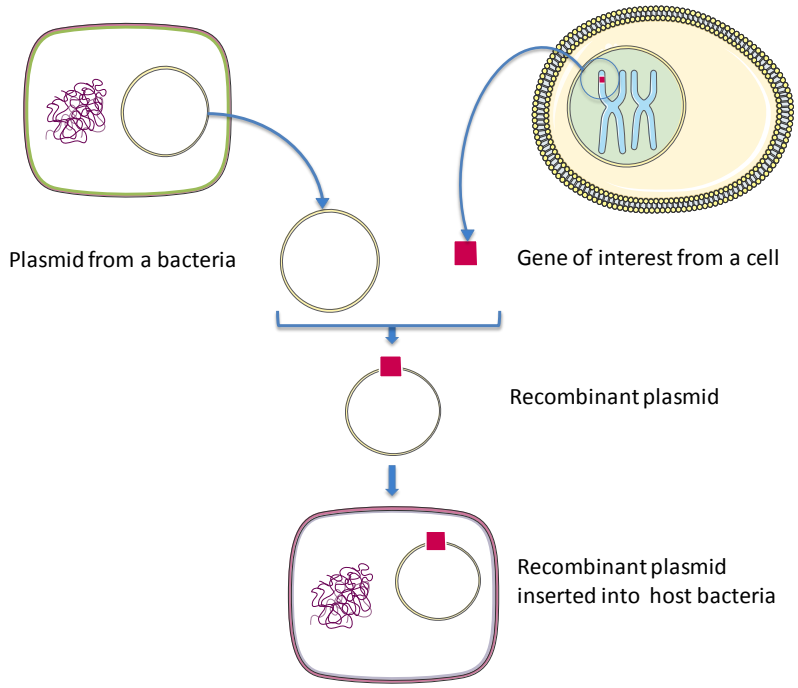


Figure 5: Recombinant DNA technology (figure made using images from *www.servier.fr*)

As it is described in Figure 5, a recombinant protein could be produced when a fragment of DNA isolated and purified, which contains the gene of interest, is cut by the action of restriction enzymes at the recognition site. These fragments are then inserted into a vector, forming a recombinant plasmid that is after placed into the bacterial host. Once the recombinant vector is transformed, bacterial cells grow forming colonies that contain the desired fragment of DNA.

The main objective of recombinant DNA technology is to produce a high amount of the desired protein. For achieving this objective, the host organism is normally forced to produce above its capacity, leading to the formation of protein aggregates, which often gives raise to inclusion bodies formation (Peternel 2011). Stress conditions such as high growth temperature or overproduction of foreign polypeptides cause the deficiency of the cellular quality control system producing a failure of folding and holding activities (García-Fruitós, E. et al., 2005). DnaK and their co-chaperones are involved in the protein folding. In cell devoid of functional DnaK, overproduction of foreign proteins results into inclusion bodies formation.

a) IB's production in *E. Coli*

Choosing and optimal expression system is crucial for an efficient protein production process. Bacterial host systems are very attractive, as they are usually genetically well characterized having a large number of cloning vectors and mutant host strains available. Indeed, they grow rapidly at high density on inexpensive substrates. In this regard, *E. coli* is

the most common host used in the biotechnology industry for the production of recombinant proteins.

E. coli is a Gram-negative enterobacteria, generally found in animal intestines being part of the normal intestinal flora and is involved in the nutrients uptake. These cells grow fast in a simple and inexpensive growth medium. Its genetics is well understood and the gene transfer mechanisms are easy to perform. Therefore, it can be easily manipulated.

There are many factors that play an important role in the protein production in *E. coli*, including the expression vector and strains.

Expression vector

A vector is a DNA molecule that can be used to transport foreign DNA segments from one organism to another. The most commonly used vectors in *E. Coli* are plasmids.
Commonly protein expression vector contain 3 main structures: a *replication origin*, allows the maintenance of the DNA molecule in the host organism through an autoreplicative process; a *selection marker*, since protein production and the vector maintenance suppose an extra metabolic load for the microbial host it is necessary to favour the growth of the recombinant bacteria through the application of a selective pressure. This external pressure consists usually in the presence of antibiotics while the selection markers are usually genes codifying for the resistance to the mentioned antibiotics. Finally, a protein expression vector needs a *promoter* to regulate the recombinant protein expression.

Strains

At present, a higher variety of *E. coli* strains are available for their use in recombinant protein technology. Some examples of *E. coli* are: BL21(DE3) is the most widely used host for protein expression being deficient in the Lon and OmpT proteases *(Sorense, H.P., and Mortensen, K.K., 2005)*. Rosetta host strains are BL21 derivatives designed to enhance the expression of proteins that contain condons rarely used in *E. Coli (Tegel, H., et al., 2010)* Origami host strains are K-12 derivatives that have mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which enhance disulfude bond formation in the cytoplasm *(Zhang, L., et al., 2011).*

K-12 derivatives are the last example, which are deficient in DnaK, ClpA and ClpP protein quality modulators. MC4100 strain is an example of K-12 derivatives and will be used in this work.

3.4. Current methods for polymeric scaffolds fabrication

Fabrication methods of polymeric scaffolds have to have a high control of final material porosity, pore size and mechanical properties. Additionally, they have to keep material biocompatibility. At present the most widely used preparation methods are described below:

- **Solvent casting.and particulated leaching**
  Solvent casting an particulated leaching (SCPL) method involves the dissolution of the polymer in an organic solvent, mixing with porogen granules, like sugar, inorganic salt (salt leaching), paraffin spheres and casting the solution into a predefined 3D mould *(Ravichandran, R. et al., 2012).* The organic solvent is then evaporated, resulting in a solid as final product. It is simple, easy and inexpensive and does not require any large specialized equipment. However, organic solvents must be fully removed in order to avoid any possible damage to cells seeded on scaffolds . Pore uniformity and pore interconnectivity can be enhanced with the combination of SCPL with centrifugation.
  This method has application in bone and cartilage tissue engineering.

- **Thermally induced phase separation (TIPS)**
  In this method, the porous material is obtained due to a phase change. This phase change is produced by a temperature gradient that separates the polymeric solution in two phases, one having the lower polymer concentration and other, having the high. *(Akbarzadeh, R. et al., 2014).* The first step is to make a homogenous polymer solution. The polymer is dissolved in solvent by heating the mixture for a certain period of time. The solution is then cooled down to the desired quenching temperature. The final step is removing the solvent that yields the final porous structure. Changing the quantity of solvent in the mixture can regulate porosity.
  The main disadvantage of this method is the use of organic solvent. In addition, a user and equipment sensitive are needed.

- **Freeze drying**
  In this method, polymer is dissolved in a solvent to form a solution with desired concentration. The solution is then frozen and solvent is removed by lyophilisation under the high vacuum. Nevertheless, this process generates various stresses during freezing and drying steps, and material characteristics are difficult to control.
  When emulsion is frozen, a solid is obtained with the same porous morphology as the emulsion template.
  As it happened in phase separation, this method implies the use of organic solvent in order to dissolve the polymer, being incompatible with proteins, drugs and gens.

- **Compression moulding**
  The compression moulding process is a method of moulding in which a pre-heated polymer is placed into an open and heated mould cavity. The mould is closed and pressure is applied to force the material to contact all areas of the mould. Throughout the process, heat and pressure are maintained until the polymer has cured. This method does not involve any organic solvents during the scaffold fabrication process *(Oh, S.H., et al., 2003).* The main problem of this method is the high temperature required.

Table 1: Comparison between methods for scaffold preparation

Method	Organic solvent	Compatibility with proteins, gens and drugs	Cost	Advantages	Disadvantages
Solvent casting and particulated leaching	Yes	No	Cheap	Interconnected pores	Poor control over pores Remaining salt
Thermally induced Phase separation	Yes	Possible	Cheap	Highly and interconnected porous scaffolds Control over pore morphology	Limited to certain polymers Use of organic solvent
Freeze drying	Yes	No	Cheap	Easy	Use of solvents Difficult to control material characteristics
Compression moulding	No	No	Expensive	No use of organic solvent Fabrication of complex geometries Easy process	High temperature required Limited to certain polymers

As it can be seen in the Table 1, all above described methods are not adequate for the one-step preparation of scaffolds loaded with biological components (drugs, proteins and genes). Solvent casting, phase separation and freeze drying are the cheapest but they imply the use of organic solvents during the process. An operation for removing all organic solvent is needed in order to avoid any contamination and this could be very difficult.
In the case of phase separation, an elevated processing temperature is required which can prohibit their use in the preparation of scaffolds.

3.5. Supercritical fluid technology for 3D porous scaffold preparation

During the past two decades, supercritical fluid technology has attached interest for the preparation of 3D polymeric scaffold unloaded and loaded biological.

In the case of tissue engineering, this technology has been used for developing new strategies, including the encapsulation of growth factors, pharmaceuticals and plasmids in order to develop tissues; the elimination of residual solvents, the improvement of tissue biointegration and, finally, the preparation of 3D scaffolds with appropriated properties such as high

porosity and interconnected pore structure. Carbon dioxide is the most common fluid used as compressed fluid for this application.

A supercritical fluid (SCF) is formed when its critical pressure and temperature are exceeded. Under these conditions, SCFs have special properties: its density is similar to a liquid but diffusivity and viscosity are comparable with a gas. In the case of $SCCO_2$, the combination of gas-like diffusivity and liquid-like density makes it a unique medium for polymer synthesis and processing (Cooper 2001). Its properties can be adjusted by small manipulation in pressure and temperature.

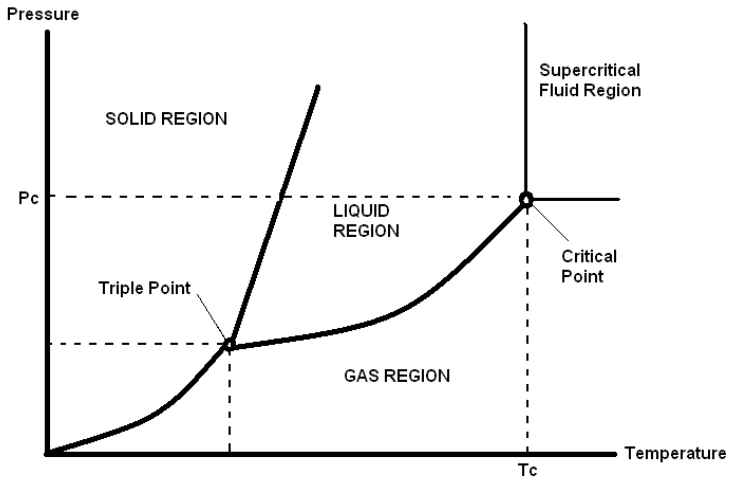


Figure 6: Idealized phase diagram

In regenerative medicine, processing with SCFs, especially $SCCO_2$, has been used for different applications, including (Davis, 2008):

- 3D porous polymer scaffolds production.
- Encapsulation of growth factors and cells for tissue engineering.
- Removal of residual solvent from pre-fabricated scaffolds.
- Sterilization of implantable materials.

SCCO2 is the most commonly used SCF for the preparation of 3D polymeric scaffolds.

3.5.1. Scaffold production with $SCCO_2$

$SCCO_2$ is the most investigated SCF because it presents many advantages not only economics but also environmental. It has emerged as an attractive alternative of water and organic solvent in synthesis as well as other processing areas for polymers. CO_2 is non-toxic, non-flammable and chemically inert, therefore is considered a "green solvent". Moreover, its critical conditions are easy to achieve and a large amount of CO_2 is available from NH_3 and ethanol industries and refineries. It can be removed by simple depressurization.

Critical conditions for the CO_2 are 304 K and 7,38 MPa. In supercritical region, properties of fluids are intermediate between those of liquid and gas. Density of a supercritical fluid is similar to liquid. They have a high diffusivity and low viscosity.

3D porous scaffolds can be produced using a foaming technique, which is based on plasticising action of the CO_2. Polymer foams are formed when a polymer, plasticized by saturation in a supercritical fluid is rapidly depressurized at a constant temperature. (Davies et al., 2008). When CO_2 reaches its critical parameters, it can dissolve into the amorphous region of polymers, plasticising them at low temperature in a solvent-free environment. (Ginty. P.J. et al., 2008). Under these conditions, the glass transition temperature of the polymer (Tg) is reduced, resulting in the formation of foams.

For instance, a common procedure for the preparation of polymeric scaffold with $sCCO_2$ is described below and schematized in the Figure 7.

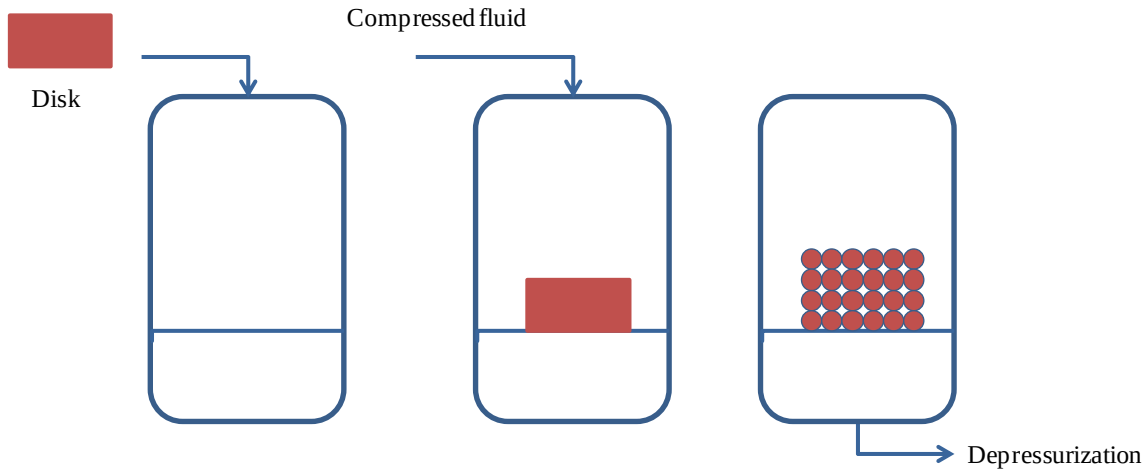


Figure 7: Schema of experiment for 3D scaffolds preparation

- This experiment starts with the preparation of a polymeric disk using a special mould.

- Disk is then placed into high pressure vessel pre-heated at the working temperature.

- After, a high pressure pump is employed to introduce $SCCO_2$ into the vessel to reach the working pressure. The polymer/compressed fluid mixture is maintained at working pressure during the soak time, producing polymer saturation with CO_2. This first step produces the plasticization and a decrease in the Tg of the polymer. Moreover, polymer will expand due to the plasticization process.

- Depressurization step. This step induces phase separation and nucleation due to an alteration of thermodynamic equilibrium. At this point, the amount of CO_2 present in the polymer decreases and the Tg of the polymer starts to increase again, returning to the glassy state. When Tg for the polymer is higher than the foaming temperature, the porous structure is fixed (Davies et al., 2008).
  Depressurization occurs at constant rate. As a result, porous scaffolds are obtained.

PLA porous matrices obtained with this technology can be observed in the Figure 8:

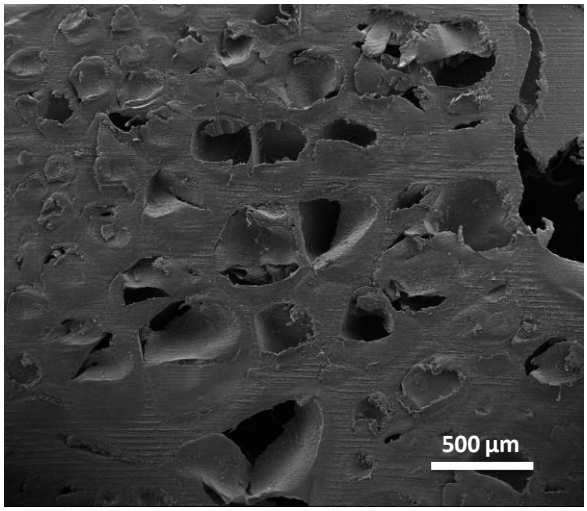


Figure 8: SEM image for PLA porous scaffold obtained with $SCCO_2$

3.5.2. Scaffold production with Freon R-134a.

The use of $SCCO_2$ in scaffold preparation could be restricted by the high-pressure equipment required and the low solubility of many compounds restricts its potential (Gimeno, 2005).

Recently, NANOMOL group of CSIC and CIBER-BBN have studied the use of 1,1,1,2-tetrafluoroethane as an alternative to $SCCO_2$ in porous polymer fabrication. Commercially known as R-143a, this fluid belongs to the group of freons, a group of halogenated hydrocarbons that are characterized by their high stability and safety, as well as, they are non-fammable, odourless and colourless. It has been used in refrigeration and in auto air conditioning system.

The use of R-134a presents numerous advantages, including a lower pressure to become liquid (< 2 MPa, RT) if we compare with the 7,38 MPa, RT for the CO_2. Although it remains more expensive, the lower pressure for the liquid phase could result in a lower cost of equipment because equips that tolerate high pressure are not necessary. In addition, pumps are not needed for achieving a high pressure, resulting in a lower initial investment.
The risks associated with working at high pressure are also reduced.

As can be observed in SEM image of Figure 9, by using compressed Freon R-134a, it is also possible to prepare porous matrices of PLA.

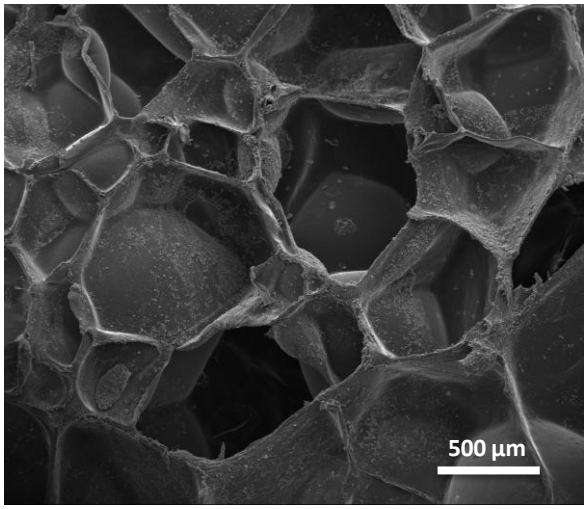


Figure 9: SEM image for PLA porous scaffold obtained with compressed R-134a

4. OBJECTIVE

An adequate combination between the biology and material engineering can be used for the regeneration of damaged tissue employing not only a biodegradable support where cell can growth and proliferate but also a biological agent to stimulate cell proliferation. Following this well accepted approach, the main objective of this work is to study the influence of polymer nature in structural properties obtained, Inclusion bodies presence and processing conditions on the behaviour on 3D porous matrices as scaffolds for bone tissue engineering.

5. EXPERIMENTAL

In this work, the experimental methodology summarized in the diagram of Figure 10 has been followed, in order to achieve the proposed objectives. This methodology includes:

- Production of Inlusion Bodies (IB's) composed by VP1GFP protein by recombinant DNA technology.
- Production of 3D porous scaffolds employing Supercritical fluid technology.
- Functionalization of 3D scaffolds with the bacterial inclusion bodies purified and quantified.
- Cultivation of 3D porous scaffolds unloaded and loaded with human mesenchymal stem cells for studying cell viability and proliferation and, finally, IB's impact.

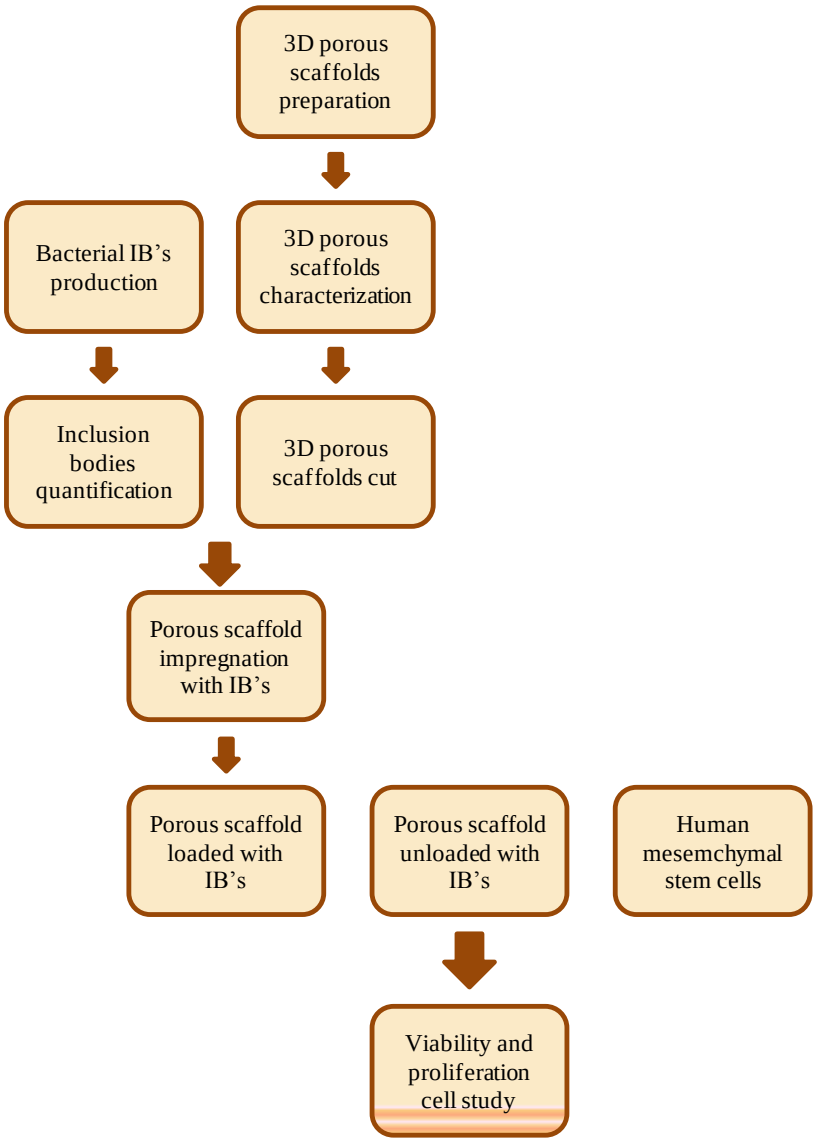


Figure 10: Experimental methodology

5.1. Inclusion bodies production and purification

Bacterial IB's formed by VP1GFP protein were produced by Recombinant DNA technology at the laboratory of Prof. Antonio Villaverde (IBB-UAB), following the procedure describe in Figure 11.

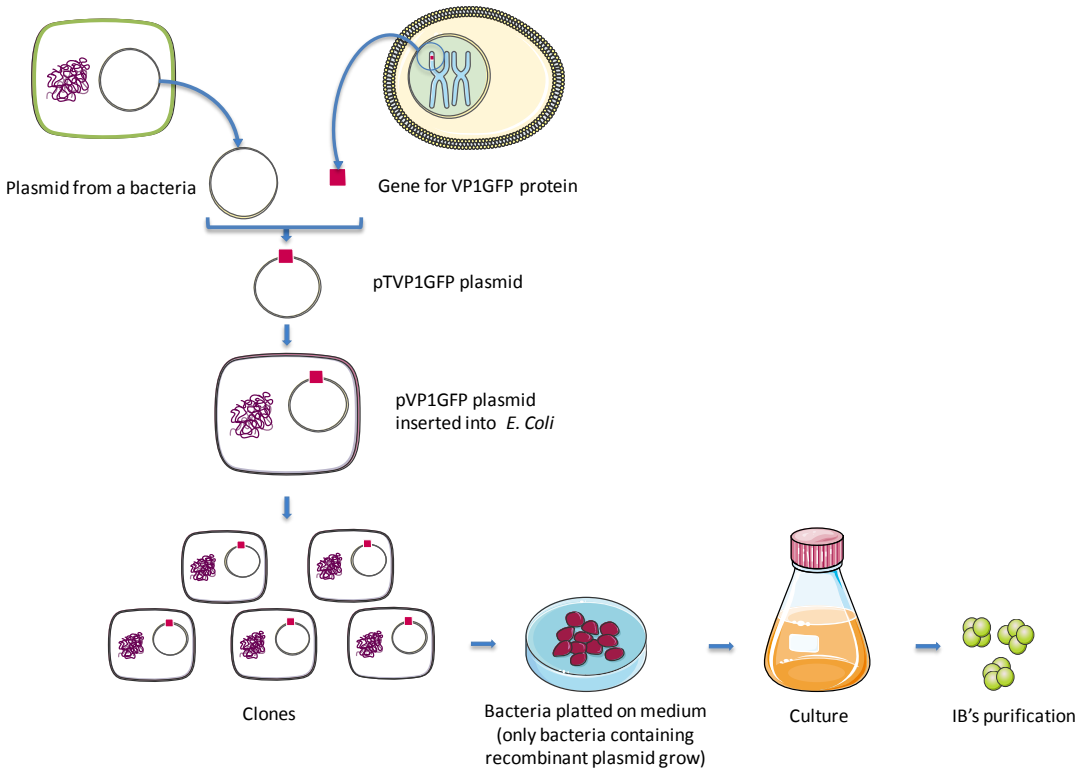


Figure 11: Diagram of recombinant DNA technology

As it can be seen en the figure 11, first step in this technology is to select a fragment of DNA to be inserted into a vector. Employing different restrictive enzymes, the fragment selected is cut and placed into the vector using a DNA ligase. For this work, gene for VP1GFP protein was inserted into a plasmid which was then inserted into a host cell. This process is called transformation and requires a selectable marker which allows the identification of recombinant molecules. Selectable markers can be for antibiotic resistance, colour changes or any other characteristics which can distinguish transformed from untransformed hosts. Normally, an antibiotic marker is used.

In our case, we'll use *Escherichia coli* as a host cell and a plasmid as a vector.

Experimental procedure has been also made following the protocol supplied by Professor Antonio Villaverde, from the Applied microbiology group (Institut de Biomedicina i biotecnologia, UAB).

5.1.1. Bacterial cell culture: strains and plasmids

The *E. Coli* strains used for the production of Inclusion bodies were MC4100 and its derivative JGT20 which is deficient in DnaK. Those bacteria were transformed with pTVP1GFP plasmid that contains the gen for the VP1GFP protein.

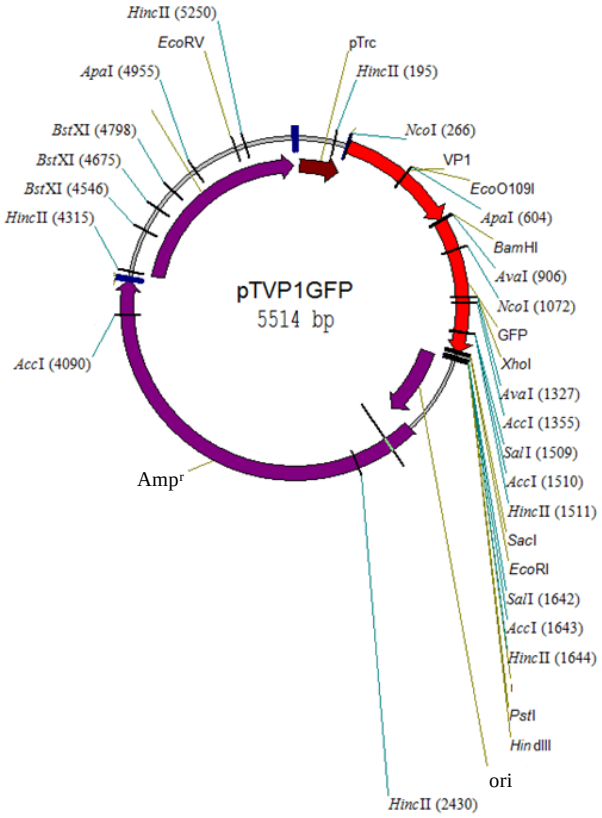


Figure 12: Structure of pTVP1GFP plasmid.

The gene of interest was under the control of P_{TRC} promoter, a DNA region situated upstream of the gen VP1GFP. In addition, this vector contains the gen coding for the resistance to ampicillin as a marker.

5.1.2. Inclusion bodies production

Starting with bacterial inoculums which contain transformed *E. Coli* with pTVP1GFP, inclusion bodies were produced in 1 l of Luria Bertani medium (LB) divided in two shake flasks of 500 ml.

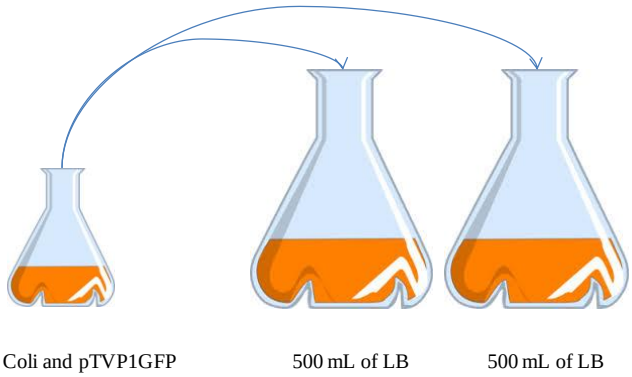


Figure 13: Inclusion bodies production

- 4,67 mL of the initial inoculum were added to 500 mL of LB medium in the two flasks at an optical density of 0,05 at 550 nm (OD_{550})
- These cultures were then cultured in a shaker flask at 37º C and 250 r.p.m using LB medium. The LB is a rich medium designed for growth of pure cultures and it is used for maintaining and cultivating recombinant strains of *E. Coli*. Indeed, this medium is supplemented with the corresponding antibiotics: ampicillin for plasmid maintenance, streptomycin as a strain selection marker and tetracycline.
- Bacterial strains were cultured in the conditions mentioned above until they reach an OD_{550} of 0.5.
  At this point, bacterial cultures were in exponential phase. This is the optima phase because bacteria are in rapidly growing and dividing, showing an active metabolism essential for a good protein. The culture reaches the maximum growth rate and the number of bacteria increases exponentially.
- The iso propyl-D-thiogalactoside (IPTG) was then added in order to induce gene expression and protein deposition as IB's. IPTG is the gene expression inductor and responsible for activating the promoter.
- The culture is then incubated for 3 hours at 37ºC and 250 rpm.

5.1.3. Inclusion bodies purification

After protein production, bacterial inclusion bodies were isolated by a combination of enzymatic and mechanical cell lysis, followed by extensive sample washing in the presence of detergents.

Table 2: Material for inclusion bodies purification

Product	Supplier	Reference
Complete EDTA free	Roche	05 056 489 001
PMSF	Roche	11 359 061 001
Lysozyme (0,5 mg/ml)	Roche	10 837 059 001
Triton X-100	Roche	10 789 704 001
NP-40	Roche	11 754 599 001
$MgSO_4$ 1M	n.a.	n.a.
DNasa I (a 1 mg/ml)	Roche	10 104 159 001
Lysis buffer + Triton X-100	n.a.	n.a.
PBS buffer	n.a.	n.a.

- Once induction was finished, three reagents were added directly to the culture: complete EDTA free, PMSF and lysozyme for cell wall digestion. The culture was incubated during 2 hours, at 37ºC and 250 rpm and after, was placed in a beaker for freezing at -80ºC overnight. After, culture was thawed at room temperature (RT).
  This freeze/thaw cycle was performed in order to disrupt the partially digested bacteria to release the inclusion bodies.
- Triton X100 (0,4 vol. %) was added to culture and incubated during 1 hour at ambient temperature with stirring. Culture was then frozen at -80ºC for two hours.
- Once culture was thawed, a sterility control was made. To this end, 100 µL of culture were seeded in LB plate (without antibiotics) at 37ºC. This culture was then frozen at -80ºC overnight and after, thawed at RT. In this point, contamination degree was checked by counting the number of colonies. This step was repeated until no colonies are shown on the plate.
- Once culture was free from colonies, NP-40 (0,025 vol.%) reagent was added and incubated during 1 hour with stirring.
- Two reagents were then added to samples ($MgSO_4$ and DNAsa) and incubated 1 hour at 37ºC with stirring. Culture was centrifuged at 15.000 g, for 4 minutes at 4ºC. Supernatant is discarded and pellet is re-suspended.
  This DNase treatment and washing with detergent was carried out for removing bacterial DNA and cell membrane contaminats.
- 100 µL of culture were seeded in LB plate (without antibiotics) at 37ºC overnight. This culture is then centrifuged at 15.000 g for 15 minutes at 4ºC. Supernatant was discarded and pellet is re-suspended.
- This final step was repeated and pellets were kept at -80ºC until their use.

Once purification is finished, inclusion bodies quantification is made using SDS-PAGE and Western-Blot method.

5.1.4. Inclusion bodies quantification: SDS-PAGE and Western-Blot method

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot is one of the most widely used laboratory techniques to detect proteins. SDS-PAGE relies on the migration of charged molecules in a gel matrix in response to an electrical field. This technique facilitates the separation and resolution of a mixture of proteins according to the molecular weight. Western-blot method transfers proteins from SDS-PAGE gel to a solid supporting membrane.

a) SDS-PAGE electrophoresis

Two types of gels are employed in this electrphoresis: a “staking gel” and a “running gel”.

- “Staking gel” is located on top of the running gel. It has a lower degree of crosslinking and is the section of the support in which the sample wells are located.

- "Running gel" is the support used for the electrophoretic separation of substances in the sample. This gel is formed first and is located throughout the middle and lower section of the system.

After the sample has been placed in the wells and an electric field has been applied, analytes travel quickly through the stacking gel until they reach its boundary with the running gel.

Sample preparation

Samples were dissolved in the Laemli solution and then, heated at 98ºC during 25 minutes. We used the *Precision Plus Prtoein TM All blue standards* as a standard (Ref. 161-0373 BioRad).
Samples were prepared in the presence of loading buffer that contains glycerol to increase the density of samples and their deposition on the bottom of the wells. The loading buffer also contains a dye, blue of bromophenol that indicates the position of the samples during the electrophoresis.

SDS-PAGE Electrophoresis assembly

SDS-PAGE electrophoresis starts with the preparation of "running" and "stacking" gels that will be placed between two glasses.

Table 3: Composition of SDS polyacrylamide gel

Reactives	Running gel Polyacrylamide 15%	Staking gel Polyacrylamide 3.5%
H_20 MQ	9,86 mL	4,124 mL
Solution A	5 mL	0,7 mL
Solution B 0,4 g SDS 18,2 g Tris base pH=8,8	5 mL	
Solution C 0,4 g SDS 6 g Tris base pH= 6,8		1.575 mL
APS	120 µL	70 µL
Temed	16 µL	7 µL

- The top and separator glasses were fixed to the support with a space between them of 0,75 mm, approximately.
- This is a discontinuous electrophoresis, so running gel was prepared first.
- 9,86 ml of utltrapure water were added followed by 5 ml of the running gel buffer at pH= 8,8 (solution B).

- The, mixture of acrylamide/bisacrylamide was added followed by the SDS.
- Then, polymerization was started by the addition of 60 µl ammonium persulfate, APS, a free radical generation.
- Finally, 8 µl the reagent TEMED was added in order to complete the polymerization.

The final mixture was well stirred for achieving a complete homogenization and, then, placed in the space between the two glasses. This mixture was added until it occupies the third of the total volume. In the upper part, a thin layer of ultrapure water was added to prevent the contact with the air. Once the polymerization has finished, water was removed and running gel is ready.
Now, the stacking gel is prepared. The procedure is similar to running gel but modifying the proportions in order to obtain a larger pore size.

- 4,124 ml of ultrapure water were added followed by 1,575 ml of the stacking gel buffer at pH= 6,8. (solution B)
- The, mixture of acrylamide/bisacrylamide was added followed by the SDS.
- Then, polymerization has started by the addition of 70 µl ammonium persulfate, APS, a free radical generation.
- Finally, 7 µl the reagent TEMED was added in order to complete the polymerization.

The stacking gel was located at the top and its function is to concentrate the protein prior to separation.
Finally, a plastic comb was placed for allowing the formation of the wells where the samples will be charged. SDS-PAGE system was ready and placed in the electrophoresis cuvette.

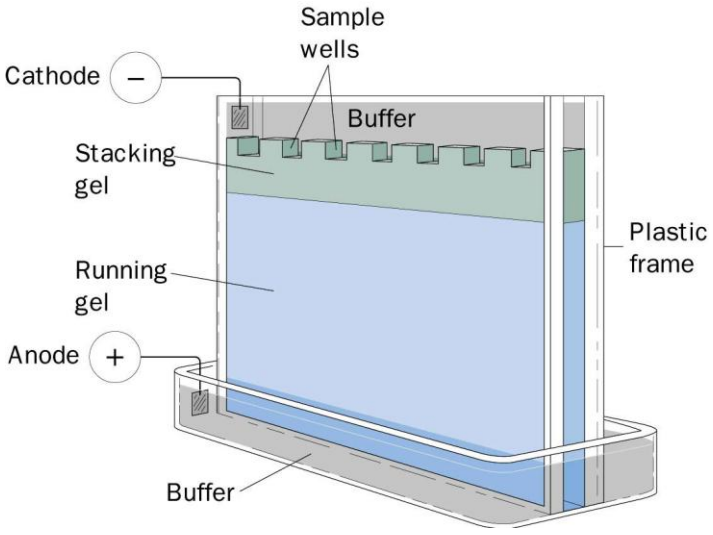


Figure 14: SDS-PAGE electrophoresis system

The support contains the electrodes, which supply the current once the electrophoresis buffer is in contact. Once the system was in cuvette, the electrophoresis buffer was added until it covers the gel.

Table 4: Electrophoresis buffer composition

Reactives	V(mL)		
Phoresis tampon 10X	80	100	300
SDS 10X	8	10	30
H_2Od	712	890	2670
V_{final}	800	1000	3000

The comb is removed and samples are loaded into the wells.
In order to begin the electrophoresis, the electrodes are connected to an electrical source and the appropriate voltage is selected. In our case, selected voltage is 100 V and intensity is 40 mA. The electric field generated causes the protein migration from the top to the bottom, causing their separation by size. The gel will be running until samples reach the final of the glass.

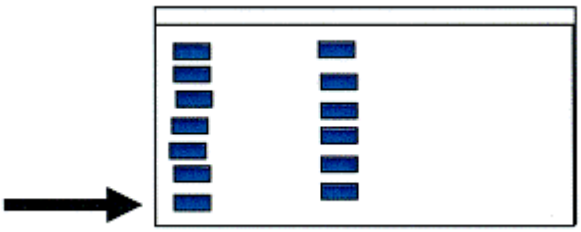


Figure 15: Electrophoresis end

Once the migration is ended, the current is turned off and the electrophoresis buffer is removed.

Table 5: Transference buffer composition

Reactives	V(mL)		
Phoresis tampon 10X	70	100	200
Methanol	140	200	400
H_2Od	490	700	1400
V_{final}	700	1000	2000

b) Western-blot method

When gel electrophoresis has finished, analytes bands can be detected in two different ways: directly on the gel or transferred to a different support for detection. In our case, we'll use the second way, in particular, a blotting method, which consists in transfer a portion of the

analyte bands to a second support, where they are reacted with a labelled agent. This method is called Western-Blot and we are going to use Watman membrane as a second support.

In this technique, proteins separated by electrophoresis are blotted onto a second support. This support is then treated with labelled antibodies that can specifically bind the proteins of interest. After antibodies and proteins have been allowed to form complexes, any extra antibodies are washed away and the remaining bound antibodies are detected through their labels.

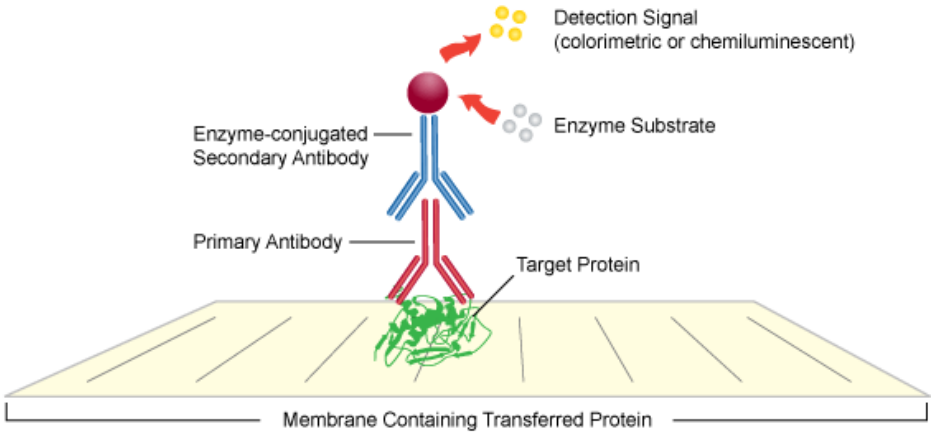


Figure 16: Western-Blot detection

Before the addition of antibodies, the gel has to be transfer to the second support. Steps for transference are the followings:

- The gel is cut removing the staking part.

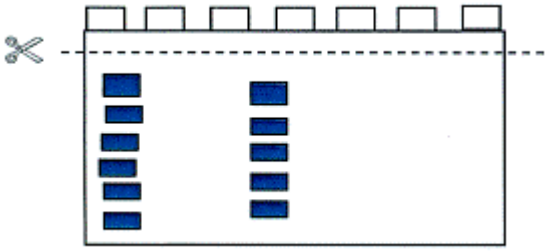


Figure 17: Removal of staking gel

- The rest of the gel is then placed in a cuvette with a little of transference buffer

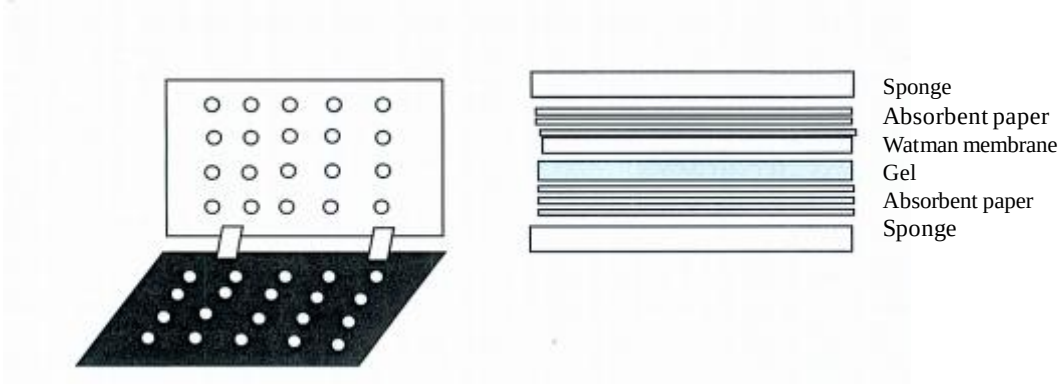


Figure 18: Transference assembly

- The gel transference conditions were 100 V and 190 mA during 1 hour.
- The transferred membrane was finally placed with 40 mL of PBS and 2 gr. of milk in a continuous swinging overnight.
- 20 µl of the first antibody (SC-17767, 200 µg/ml, Santa Cruz Biotechnology) were added to 10 ml of the mixture PBS+milk (dilution 1/5000).
  This mixture was maintained during 2 hours with the first antibody at RT.
- The transferred membrane was then washed with PBS.
- After, the membrane was washed again two times with a mixture of PBS-Tween, during 15 minutes each washing.
- 5 µl of the second antibody was added with 10 ml of PBS. The membrane was then incubated during 1 hour at ambient temperature.
- Then, a fast washing with PBS was made.
- The membrane was washed again with a mixture of PBS-tween, during 15 minutes.
- This mixture was removed and PBS-tween was added again for a new washing during 15 minutes.
- Finally, revealed solution was added to the gel.

Table 6: Composition of revealed solution

Reactives	Composition
4-cloranaftol	13 mg.
Cold methanol (-20ºC)	5 ml.
H_2O_2	40 µl.
PBS	20 ml.

- The membrane was maintained in the revealed solution until the signal is clearly visible.
- The membrane was after washed in distilled water and placed on paper until dry.

5.2. Scaffold fabrication by supercritical fluid technology

5.2.1. Materials

In this study, the polilactic acid $P_{L,DL}LA$, with inherent viscosity between 5,7-6,5 dl/g, RESOMER LR708, was purchased from Evonik Röhm GmbH (Darmstadt, Germany), in bags of 100 g and in "pellet" form. This polymer is kept at 4ºC until its use and has the following structure:

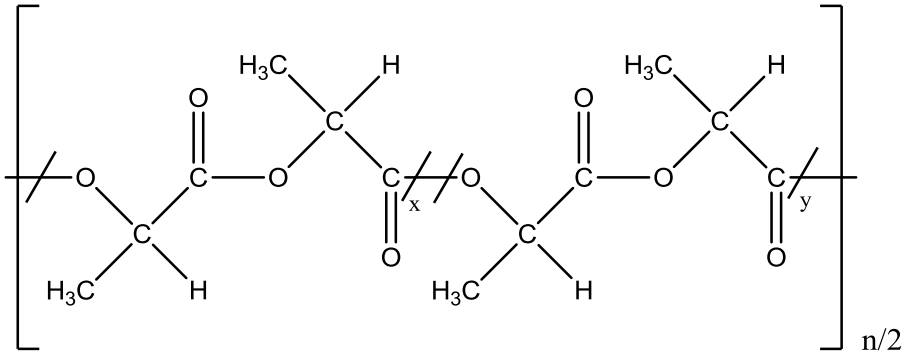


Figure 19: PLA structure used for this work

The polilactic co-glicolic acid, PLGA, with inherent viscosity between 0, 32- 0,44 dl/g, RESOMER RG503, was also purchased from Evonik Röhm GmbH.

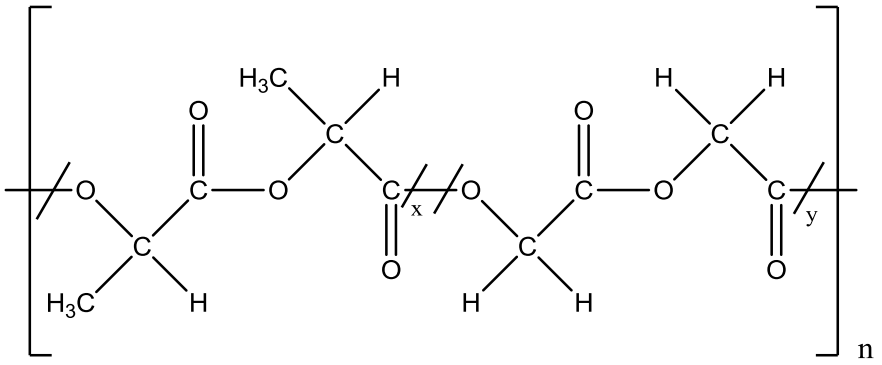


Figure 20: Structure of PLGA used for this work

CO_2 (purity 99,995%) with 62730 reference and Freon R134a with 62730 reference were supplied by Carburos metálicos-Air products S.A. (Barcelona, Spain).

5.2.2. Equipment and preparation methods

The scaffold preparation process with supercritical fluid technology can be divided in two different steps: first, the preparation of polymer disk and, second, the 3D porous scaffold production by processing of the polymer disks with supercritical fluid technology.

a) Equipment and protocol for polymer disk preparation.

Polymer disks were prepared using a special mould of 13 mm of diameter, which is showed in the following figure:

Figure 21: Mould used for disk preparation

This mould has two detachable parts to allow removal the disk after fabrication.

Protocol for PLA disk preparation

The PLA used for this work contains a crystalline and amorphous part. The first one is done for the L phase of the polymer, while the amorphous is done for the DL phase. In previous work (Parera, M., 2013) , it has been demostrated that crystalline part is more difficult to be expanded that the amorphous one, consequently its elimination is mandatory. A thermal pre treatment of the polymer is done in a oven following the protocol described bellow:

- Starting with the polymer in pellet form, PLA disks have been prepared with a desired mass *m* of polymer , 13 mm of diameter and 3 mm of thikness.
- Oven was estabilished at working temperature during one hour before the experiment.
- The desired mass *m* of polymer was weighed in a precision lab scale (0,1 mg.), in order to achieve a final disk of *m* grames.
- The polymer was then introduced into the oven during 15 minutes for ensuring that all material reaches the working temperature.
- After 15 minutes, the material was placed into the special mould with a diameter of 13 mm.
- Using a piston and a hidraulic press of 10 tones (Perkin Elmer), the mass was compressed with 3 tones during 20 seconds.

Figure 22: Perkin Elmer hidraulic press.

- Disk formed was removed from the mould using the two detachable parts.

Protocol for PLGA disk preparation

Since PLGA used does not contain a crystalline part, thermal pre-tretament was not necessary.
Protocol for the preparation of PLGA disks is the same as described for PLA disks but eliminating the part corresponding to thermal treatment.

- A desirable mass *m* of PLGA, weighed in a precision lab scale, was directly placed in the mould with a diameter of 13 mm.
- Polymeric mass *m* was compressed with 3 tones during 20 seconds using a piston and a hidraulic press of 10 tones (Perkin Elmer).
- PLGA disk was removed from the mould throught the two detachable parts.

Both polymeric disks were stored in the friged at 4ºC untill their use.

b) Equipment and protocol for 3D porous scaffolds preparation with compressed fluids.

For the preparation of 3D porous scaffolds, a high-pressure plant at laboratory scale has been used. This plant belongs to CIBER-BBN instrumental and is schematized in the Figure 23:

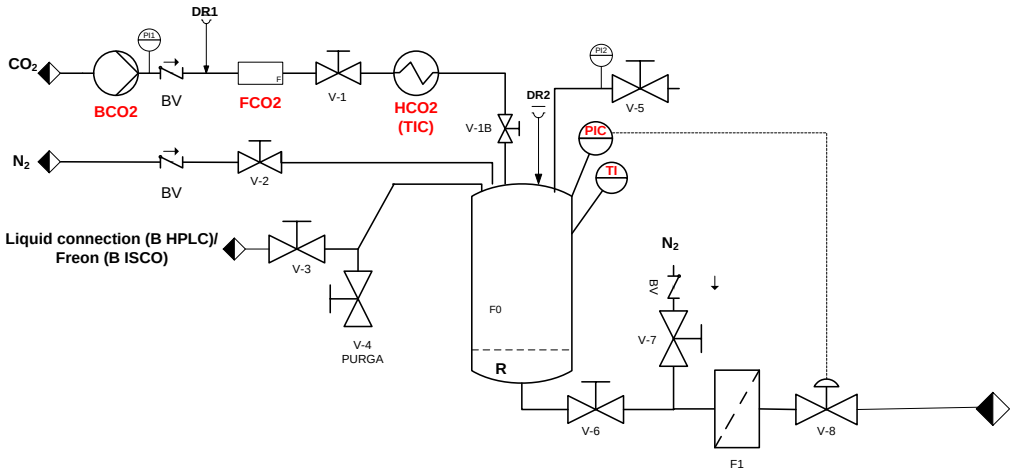


Figure 23: Experimental system diagram. This system consists of one entry for CO_2, other for N_2 and liquid connection, a liquid pump (BCO2), check-valves (BV), mass flow (FCO2), regulation valves for gaz entry (V-1, V-2, V-3, V-1B), vent valve (V-4), manual decompression valve (V-5, V-6), back-pressure valve (V-8), rupture disk (DR1, DR2)

Three addition lines form this system: one for the CO_2, other for N_2 and the last one, for Freon R-134.

This plant is equipped with a high-pressure vessel of 300 mL with maximum working pressure of 19,5 MPa at 523 K.

(A)

(B)

(C)

Figure 24: 300 ml high-pressure plant (a) and reactor detail (b), (c).

The temperature vessel is regulated with a water-ethilenglicol mixture that circulates through a jacket around the vessel at working temperature, T_W. A Haake is used for controlling the temperature of this mixture and propelling it through the circuit.
Temperature is measured with a type K thermocouple that is introduced inside the high-pressure vessel, in direct contact with compressed fluid.

The system controller registers process variables once every 4 seconds with an acquisition programme.

The polymer disks prepared in previous step are placed into the high-pressure vessel using a special stainless steel basket.

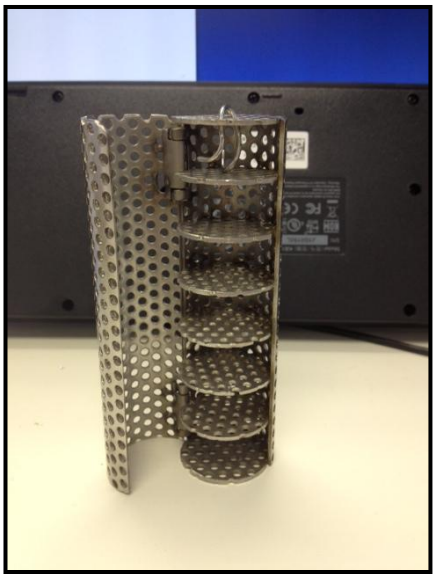


Figure 25: Stainless steal basket

This basket consists of different levels where disks are placed. On the extremes, experimental conditions can be different then disks are not put in the higher and lower level. Only intermediated levels are used for experiments. In each experiment, 4 are placed in this basket.

Protocol for the preparation of 3D porous scaffolds

The operation procedure for porous polymer production using high-pressure plant consists on different steps that are summarized below..

- High-pressure vessel was stabilized at working temperature, T_W.
- The basket with polymer disks was then placed inside the vessel. Disks were weighed before introduction in the basket.
- Pressure vessel was closed, making sure the Teflon gasket was properly positioned. The reactor was screwed with a screwdriver and after with a torque wrench.
- The system was pressurized through fluid addition (CO_2 or Freon R-143). (See below specific operational protocol followed for each fluid)
- When addition was finished, the pressure vessel was depressurized trough V-8 valve. This valve can work in two different operational modes: manual and automatic.
  The manual consists of setting a valve opening grade value between 0 (closed) and 95 (totally opened) to open/close/regulate valve, which will remain at the set value during depressurization process.
  The automatic option (back-pressure) consists of setting a constant pressure value. When the system detects that the pressure is exceeded inside the vessel, valve V-8 is opened in order to maintain the pressure settle in the set value.
  For those experiments, depressurization was made with V-8 in manual option with a desired period of time.
- Then, temperature control was stopped and porous scaffolds were removed from the reactor.
- Scaffolds were weighed and kept at 4ºC in the refrigerator.

Procedure for CO_2 addition

- Before starting the CO_2 addition, ensured that valves V-1B, V-2, V-3, V-4, V-5 and V-8 are closed. V-6 will be opened during the experiment.
- To perform vessel pressurization, V-1B was opened slowly to let CO_2 enter in the vessel until line pressure is reached, normally 50-60 bar.
- For reaching the working pressure, P_W, a BCO_2 pump was used with a flow of 200 ml/hr.
- When working pressure was achieved, pump was stopped and V-1B is closed.
- The system was then stabilized during a period of time, t.

Procedure for Freon R-134 addition

- Freon addition was made through V-3 valve. In this case, a syringe pump (B_{ISCO}) was used to perform vessel pressurization.
- First, Freon volume was measured and registered in the ISCO pump, at constant pressure and temperature (20 bar and 10ºC).
- Before starting the addition, ensured that valves V-1B, V-2, V-4, V-5 and V-8 were closed. V-3 and V-6 will be opened during the experiment.
- Freon addition was made at 25ml/min., trough V-3 valve until deposit exceeds liquid-vapour equilibrium region.
- For reaching the working pressure, Freon was added slowly.
- When working pressure was achieved, V-3 was closed and Freon volume was measured and registered, at constant pressure and temperature (20 bar and 10ºC).
- The system was then stabilized during a period of time, t.

5.3. Scaffold characterization

Scaffolds produced with Supercritical fluid technology have been characterized studying their solid density and porosity, morphology and qualitative IB's loading. The different techniques used for each characterization are described below.

5.3.1. Solid phase density and porosity

Solid phase density and porosity of scaffolds were measured using a helium picnometer. This equipment measures skeletal density of a solid which is defined as the ratio of dry specimen mass to volume of its solid part.

Figure 26: He picnometer used in measures

This technique is based on measuring the volume of helium displaced by the volume of solid introduced. Helium is used because is an inert gas, does not affect samples, its atomic ratio is very small as a result it has a great capacity for penetration in the porous matrix.

The equipment used for the experiment is an Ultrapycnometer 1200e from Quantachrome Instruments, using the small cell for measurements.

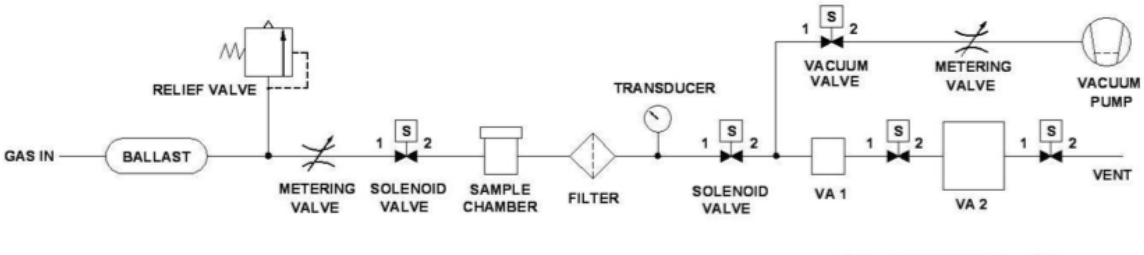


Figure 27: ULTRAPYC flow diagram

The variables employed are the followings:

- Small measurement cell
- Purge time to remove air from the system: 5 minutes.
- Helium pressure: 180 psia
- Stabilization time: automatic mode.
- Measures number for average: 5
- Standard deviation: 0,005%.

$$V_p = V_C + \frac{V_A}{1 - \left({}^{P_2}/_{P_3}\right)}$$

Where,

- V_C is the cell volume
- V_A is the volume added
- P_2 is the pressure in the chamber before opening the valve that connects with V_A

- P_3 is the pressure after opening the valve that connects with V_A

For obtaining solid density, scaffolds were weighed before introduction in the pycnometer. This mass is necessary for density calculation, using the following equation:

$$\rho_{sample} = \frac{mass_{sample}}{V_P}$$

The equipment make a measurement report and saves it in USB storage device in .txt. Format. It is possible to estimate the porosity of scaffold samples with remarkable accuracy, speed and using gas pycnometry. Subtraction the sample volume from the geometric volume yields the total pore volume of the sample. The pore volume can be expressed as a percentage of geometric volume.

$$Porosity = \frac{(V_g - V_p)}{V_g} \cdot 100$$

Where,

- V_g is the geometric volume, calculated as a cylinder: $V_g = \pi \cdot r^2 \cdot h$
- V_P is the solid phase volume calculated with He picnometry.

5.3.2. Morphology and pore size.

In this work, micro and nanoscopic morphology of the porous polymeric matrices was analysed by scanning electron microscopy. This microscope produces images by scanning the sample with a high-energy beam of electrons. The electrons in the beam interact with the sample, producing various signals that can be used to obtain information about the surface topography.

Comparing with optical microscopy, electron microscopy has a higher resolution and is therefore also able of a higher magnification. In addition, it is possible to view the three-dimensional external shape of the samples and they have a greater depth of field.

Polymers used are non-conductive, thus a metallization process is needed before SEM study. This metallization was made in evaporator Emitech K550X during four minutes at 20 mA, in the electron microscopy service from the Universidad Autonoma de Barcelona (UAB). In order to achieve a homogeneous metallization of the scaffolds surface, the procedure was repeated two times with different inclination from the horizontal (-45ºC and 45ºC) because gold deposition is made mainly in the horizontal plane. A correct metallization is desired in order to avoid electric charge of sample during microscopic inspection.

5.3.3. Qualitative IB's loading in scaffolds.

A qualitative measure of IB's retained in porous matrices can be done with an optical microscopy in order to evaluate the efficiency of impregnation process. In this case, OLYMPUS BX51 microscope has been used.

Figure 28: Optical microscopy

The microscope configuration used for these measurements was the following:

- Ultraviolet light produced in mercury lamp.
- Light filter allowing passage of the wavelength corresponding to green.
- Specific shutter speed.

Only matrices decorated with IB's were analysed using this technique.

5.4. Culture of human mesenchyme stem cells: viability cell tests

Cell health can be monitored by numerous methods. Plasma membrane integrity, enzyme activity, presence of ATP and cellular reducing conditions are known indicators of cell viability and cell death. In this present work, the reagent used for cell viability test is alamarBlue® that uses the reducing power of living cells to quantitatively measure the proliferation of cells. When cells are alive they maintain a reducing environment within the cytosol of the cell. Resazurin, the active ingredient of alamarBlue reagente, is a non-toxic, cell permeable compound that is blue and non fluorescence. Upon entering cells, resazurin is reduced to resofurin, a compound that is red in color and highly fluorescence. Viable cells continuously convert resazurin to resofurin, increasing the overall fluorescence and color of the media surrounding cells.

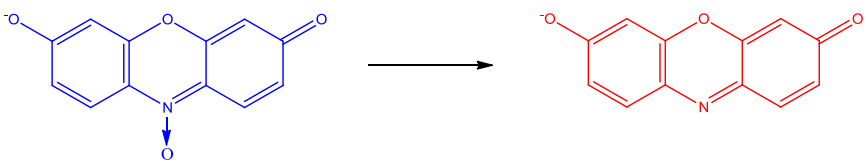


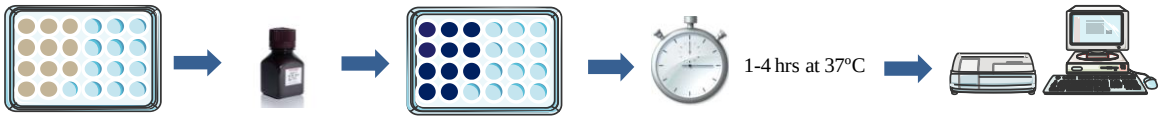
Figure 29: Resazurin reduction to resofurin

It incorporates a fluorimetric/colorimetric growth indicator based on detection of metabolic activity.

5.4.1 Protocol for measuring cell viability using alamarBlue®

All cell viability test of this work were performed at.Hospital Universitario La Paz, in collaboration with FIOBI-HULP group.

Protocol for cell viability test is schematized in the following figure



a) Materials are placed and cells are seeded AlamarBlue® b) Add reagent to cell c) Incubation d) Read fluorescence

Figure 30: Alamarblue cell viability assay protocol

- Materials scaffolds were placed in a 24 well plate.
- Cells were, then, seeded over material scaffolds.

- AlamarBlue dissolved in DMEM (10%) red phenol free, was directly added to each well.
- The plates were incubated at 37ºC to allow cells to convert resazurin to resofurin during 3 hours.
- Fluorescence at 590 nm was measured after excitation at 530 nm with a spectrofluorimeter “Synergy” (BioTek Instruments, Winooski, VT, EE.UU.)
- As higher is fluorescence emission, higher is the number of cells living.

6. RESULTS AND DISCUSSION

In the present work, it was studied the influence of different structural characteristics of 3D polymeric porous materials on their behaviour as scaffolds for mesenchymal stem cells growth. Inclusion bodies presence was also evaluated as promoting agents that can stimulate cell proliferation on porous scaffolds.

To achieve this objective, different series of materials have been prepared, physic-chemically characterized and tested as scaffold for cell growth.

6.1. Inclusion bodies production

In the present work, a batch of IB's was prepared for further decoration of PLA based scaffolds. These protein aggregates composed by Green Fluorescence protein were produced in 1 litre of LB medium using recombinant DNA technology. The host organism was *Escherichia Coli*, in particular, MC4100 strain and its derivative JGT20 (dnak756 thr::tn10, Sm^R , Tc^R). *Escherichia Coli* was then transformed with pTVP1GFP plasmid in order to obtain the inclusion bodies formed by the protein of interest, following the procedure described in the experimental section.

Inclusion bodies were produced inside the bacteria, consequently a purification treatment was needed for obtaining the protein aggregates. The procedure was describe in the experimental section and is schematized in the following figure.

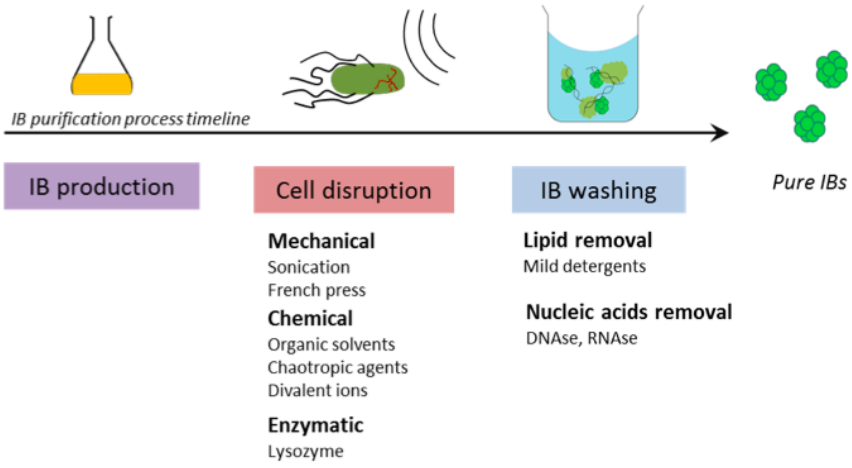


Figure 31: Protocol for inclusion bodies purification (Seras Franzoso, J., 2012)

Once inclusion bodies were purified, Western-Blot was used in order to quantify them. For this method, polyacrylamide gel was prepared using the protocol described in the section 4. It starts with the protein separation by SDS-PAGE, followed by the protein transfer to a membrane (Watman membrane), block of binging sites and finally, protein detection. The polyacrylamide gel was charged as it can see in the figure 32:

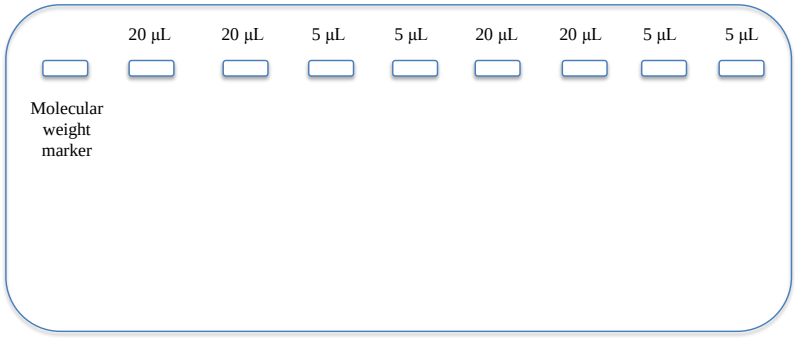


Figure 32: Polyacrylamide gel charged for Western-Blot detection

The gel was first loaded with the molecular weight marker, followed by four known amounts of the protein to be detected. These amounts were, placed in this order on the gel, 500, 250, 125 and 62,5 ng. of protein and they were used for the creation of a line pattern. Finally, the last four corresponds to protein that we want to quantify.

We want to quantify the amount of IB's that we have in 1 aliquot. The quantity of IB's present in 1 aliquot has been resuspended in 1 ml and two different dilutions have been prepared: 1/40 y 1/20. From each dilution, we loaded 20 and 5 µL.

Once SDS-PAGE has finished, 2 mL of first antibody was added for starting the quantification of the IB's. We are using polyclonal antibodies produced in rabbits. The green fluorescent protein is injected in these animals and they produce the correspondent antibody. The result can be seen in the next figure:

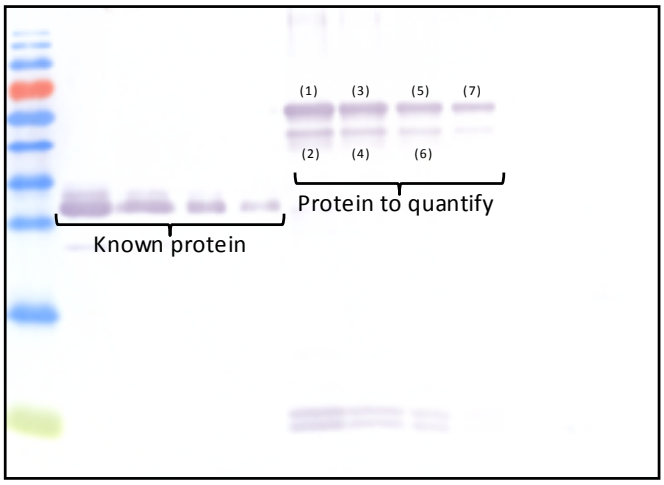


Figure 33: Result for Western-Blot detection

The quantification of the inclusion bodies present in aliquot is based on the comparison between the bands obtained after membrane development. Starting from the known amounts of proteins and their producing bands in the membrane, we can construct a calibration curve where the amount of protein is represented against the integrated density provide by each. This area was calculated by Image J software. The calibration curved is represented in the figure:

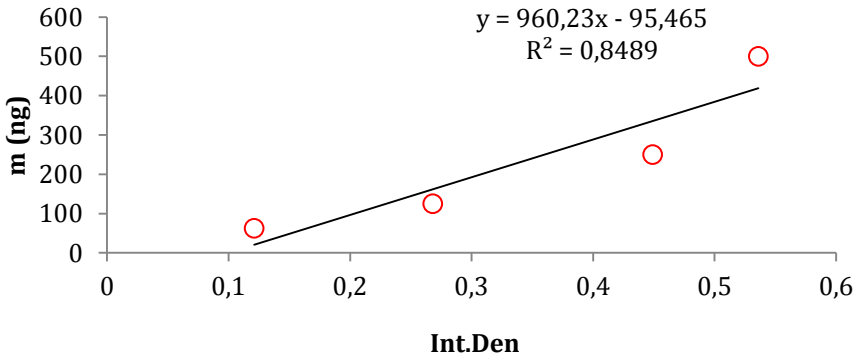


Figure 34: Calibration curve

First, we calculate the integrated density of the different bands obtained and then, using the calibration curve, we can estimate the quantity of protein in each charge.

Table 7: Calculation of IB's quantity in 1 aliquoat

Sample	Point	Area		M (ng)	C_{charge} (ng/µL)	$C_{aliquoat}$ (ng/µL)	$M_{aliquoat}$ (µg)
20 µL (1/20)	1	0,465	0,595	475,87	23,79	475,87	475,87
	2	0,13					
20 µL (1/40)	3	0,376	0,453	339,52	16,97	679,04	679,04
	4	0,077					
5 µL (1/20)	5	0,26	0,272	165,72	33,14	662,87	662,87
	6	0,012					
5 µL (1/40)	7	0,164	0,164	62,01	12,40	496,10	496,10

Finally, taking the results obtained in table, the quantity of IB's present in each aliquoat is 578,47 ± 107, 31 µg.

6.1.1. IB's size determination

Particle size of bacterial inclusion bodies produced was analysed using dynamic light scattering instrument (DLS). The equipment used for this determination is the Zetasizer Nano ZS.
The particle size measured in a DLS is the diameter of the sphere that diffuses at the same speed as the particle being measured. This system determines the size by first measuring the Brownian motion of the particles in a sample using dynamic light scattering. The procedure followed is described below:

- 1 aliquoat of Inclusion bodies were resuspended in 1 ml of ultrapure water and sonicated during 10 minutes to facilitate the formation of an appropriate particle suspension, using the same conditions as in the preparation of suspension.
- DLS measurements were carried out in triplicate.

The result is showed in figure 35:

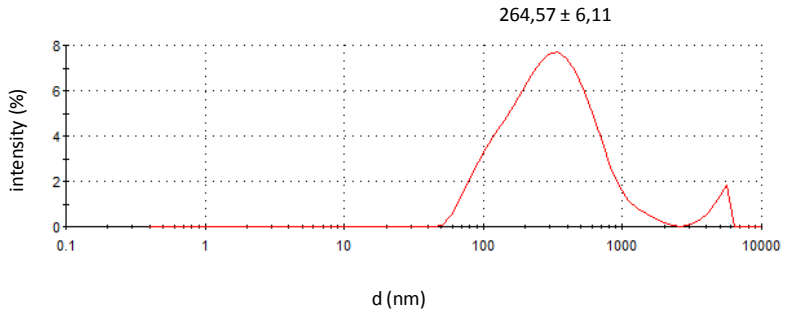


Figure 35: IB's size determination by DLS

This batch name IB-VP1GFP has a particle size of 264.57 nm and will be used in this work for scaffold decoration.

6.2. Development of a cutting scaffold procedure

SCF processing of PLA based polymer yields porous materials which have a non-porous skin layer. Porous have to be accessible for cell attachment. In order to improve the behaviour of these materials as scaffolds, it is necessary to remove this skin layer.

In the present work it was developed a suitable procedure, based on the use of a saw, in order to obtain disks of porous PLA with reproducible dimensions and free of the skin layer.

There are many advantages in using the diamond saw, such a fabrication of porous disks with desired thickness, good repeatability and reproducibility and lower risk level to the user.

Figure 36: Diamond saw

Below is detailed the cutting method designed in this present work for scaffolds disks fabrication from porous materials prepared with compressed fluids.

- Porous materials were pasted in the holder of the diamond saw. Before starting the cutting, make sure that diamond has enough water as lubricant. Water was used to refrigerate diamond saw during the cutting.
- For ensuring that cuts were parallels and, for irregular nature given of the porous sample, a first cutting was made in order to obtain a perpendicular plane to the rotation axis of the saw. This cutting also allowed the removal of the first non-porous layer. This layer was always rejected.
- After, the cutting position was adjusted with the micrometer of the saw for fixing the desired thickness in disks, 3,5 mm.

One of the most important parameters during the cutting is the rotation speed of the saw. An excessive speed could overheat the samples. We used 200 r.p.m.

Figure 37: Final scaffold after cutting method

- Finally, in order to remove the non-porous part around the disks, a die of 15 mm of diameter was used. Therefore, porous disks with a diameter of 15 mm were obtained
- Porous disks were kept at 4ºC in the refrigerator until its use.

6.3. Influence of polymer nature on cell growth over 3D scaffolds

In this section is compared the cell growth observed over two scaffolds composed by different polymers (PLGA and PLA) but processed with the same compressed fluid, Freon R-134. These materials are the followings:

- PLGA (RG-503) processed with Freon R-134a
- PLA (LR-708) processed with Freon R-134a

6.3.1. Preparation of 3D porous materials

Following the procedure describe with detail in section 5.2.2. of Experimental Part, and schematized in Figure 38 , porous scaffolds of PLA (LR-708) and PLGA (RG-503H) were prepared.

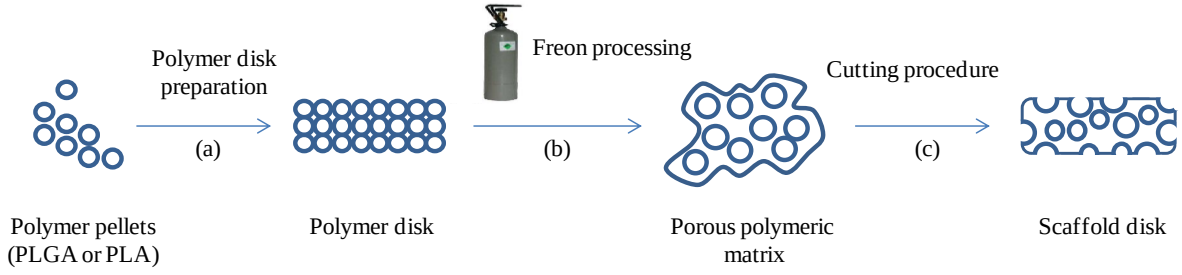


Figure 38: Procedure for 3D scaffolds obtaining with Freon R-134

As schema depicts, there are three operations required:

a) Polymer disk preparation
b) Freon processing
c) Cutting procedure

The variable values used for the preparation of both porous scaffolds with different polymeric nature are detailed in Table 8, for polymer disk preparation, and in Table 9 for processing polymeric disk with Freon R-134.

Table 8: Conditions for polymer disk preparation

Polymer	Thermal Treatment	Tª (ºC)	M (g)	Applied Pressure (Tn)
PLGA	No	-	0,3	0,5
PLA	Yes	150	0,4	3

Table 9: Experimental conditions for 3D porous materials preparation

Polymer	Tª (ºC)	P (BAR)	Incubation time (h)	Opening degree (%)	Porous matrices?
PLGA	35	20	2	60	Yes
PLA	40	20	3	60	Yes

After experiment, disks were inflated leading to the formation of porous matrix. There is a clear difference between first disk and final scaffold that it can be seen in Figure 39:

(a)

(b)

Figure 39: Comparison between initial disk and final scaffold. (A) Corresponds to PLGA and B) to PLA.

Figure 39 shows the difference between the initial polymer disk and final scaffolds for two materials tested. PLGA and PLA processed with Freon have inflated producing a porous matrix. PLA processed with Freon presents a structure formed by bubbles.

By the experimental conditions detailed in Tables 8 and 9, several batches of scaffolds disk were prepared. In all of them, the same structural characteristics were achieved.

6.3.2. Physic-chemical characterization of 3D porous material

The structural characterization of representative porous materials of PLA and PLGA is described below.

A) Mass variation study

Mass of scaffolds obtained were measure before the experiment, directly after the experiment and after 7 days of stabilization. The mass of fluid retained in scaffolds can be studied with these values, as it can be seen in the next figure. Figure 40 shows the increase in the mass of the scaffolds in these two different moments: directly after the experiment and after 7 days of stabilization.

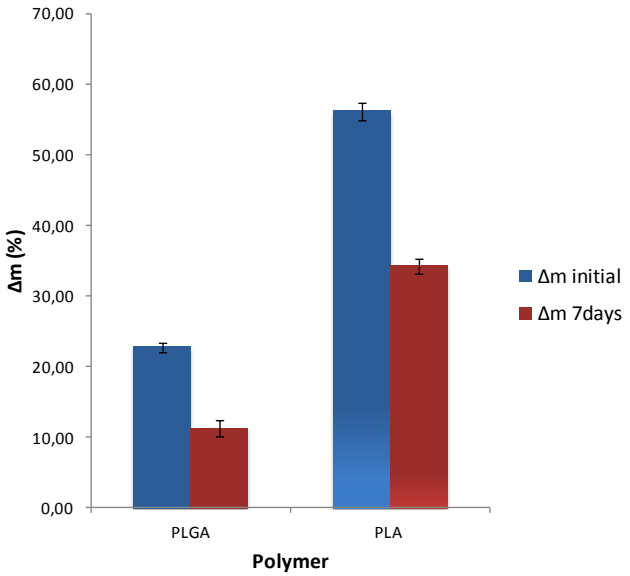


Figure 40: Mass variation in the scaffolds processed with compressed fluids

In the two materials tested, an increase of mass is produce after the experiments made with Freon. The increase of the mass is higher is the case of the PLA scaffold than in the PLGA.

After 7 days of stabilization after experiment, the graphic shows that polymers processed with Freon accumulate a high amount of residual Freon. This fact indicates that these scaffolds have a lot of closed pores and, as a consequence, Freon cannot escape from them.

If we compare two polymers processed with Freon, the graphic shows that in the case of PLA the retention of the fluid is higher than in the case of the PLGA. The PLA has a very high viscosity in comparison with the PLGA, consequently going out from the structure of the scaffold is more difficult for Freon in the case of the PLA based scaffold.

B) Solid density study

The solid density of the two different materials has been measured using Helium picnometry following the procedure describe in Experimental section.

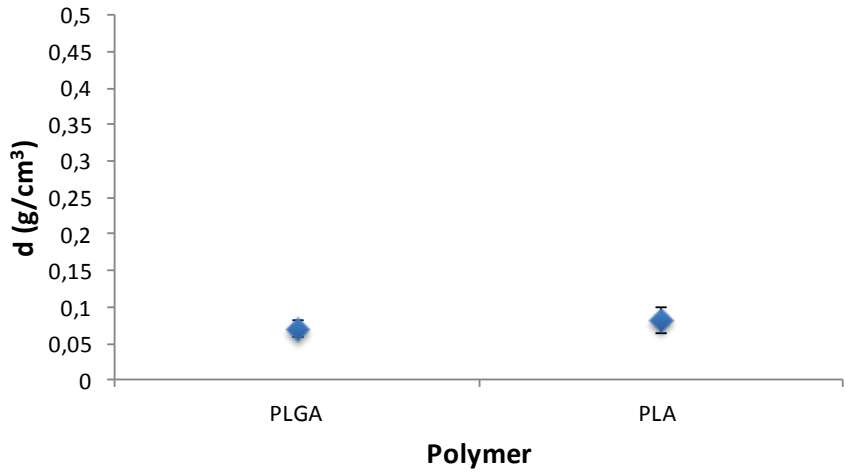


Figure 41: Solid density for the three different materials

In Figure 41, it is observed that solid density is higher in PLA scaffolds than in scaffolds composed by PLGA.
As we can see in visual inspection, Freon creates larger pores. Polymers processed with compressed Freon increase their volume, resulting in the formation of larges pores and lower solid density.

C) Porosity study

Porosity is a morphological property necessary for bone tissue regeneration (Karageorgiou, V. et al, 2005). Pores allow migration and proliferation of osteoblastos and mesenchymal cells, as well as the proper vascularization of the implant. (Gentile P., et al, 2012).

The characterization of the porosity of scaffolds can be a problem sometimes. Studies found in literature normally report the measurement of the porosity and pore size using mercury intrusion (Sarazin, P. et al., 2004; Heang Oh, S. et al., 2003). However, if we are working with a non-rigid material, this technique is not recommended. This technique requires high pressure to perform the measurements. When this force is applied to materials, the pore arrangement can be modified. This means that this technique does not measure the initial pore size and porosity of the material as produced and consequently, the measurements are not reliable.

For this reason, porosity of the two different materials was estimated using the equation described in section 5.3.1. The following graph shows results obtained for these samples

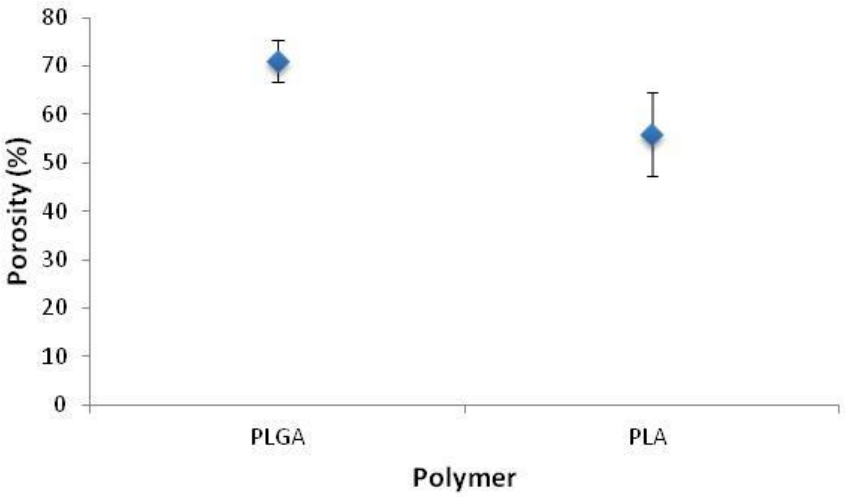


Figure 42: Porosity for the two different materials

Porosity is higher in scaffolds formed by PLGA than in scaffolds formed by PLA, following the tendency showed in the density. This result demonstrated that all scaffolds produced show a porous structure which is crucial for cell adhesion and proliferation, being good candidates for bone regeneration.

D) Morphology and pore size study.

Morphology of scaffolds obtained can be studied with scanning electron microscopy as well as the size of pores formed during the experiments with Freon R-134a.

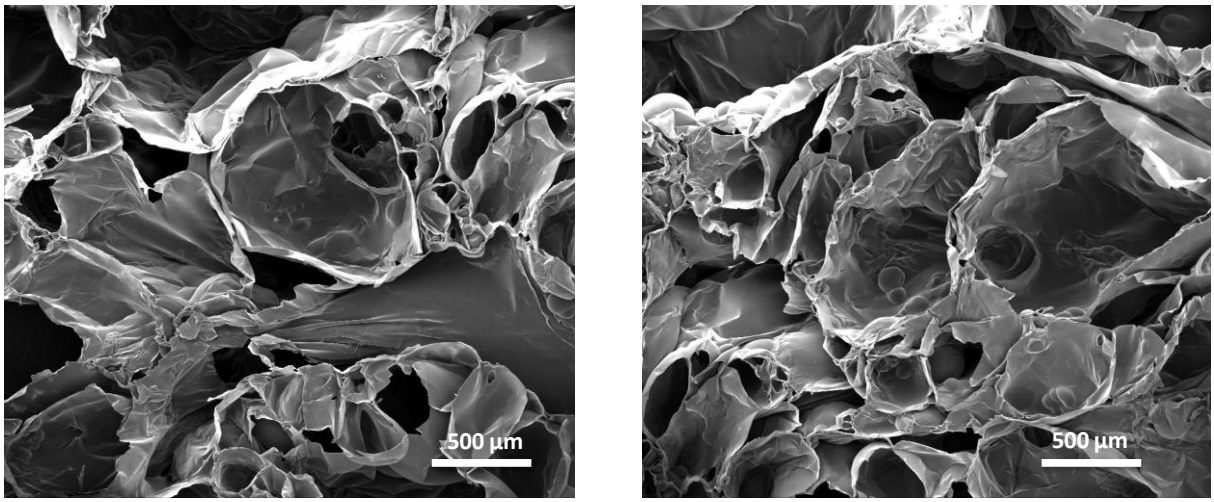


Figure 43: SEM images for PLGA scaffolds processed with Freon R-134a

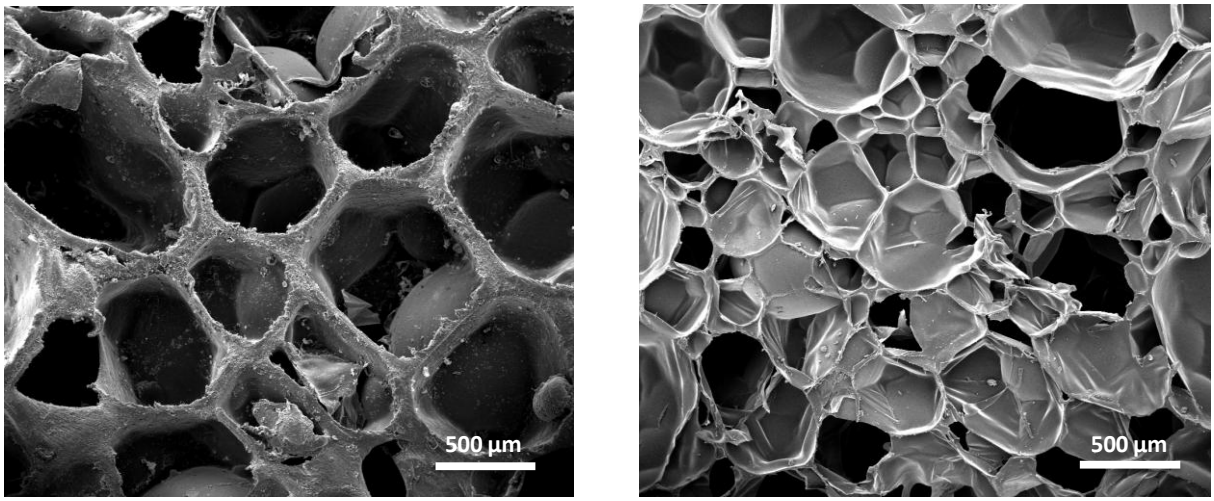


Figure 44: SEM images for PLA scaffolds processed with Freon R-134a

SEM images confirm porosity results. Pores formed in PLA based scaffolds are lower than those PLGA based scaffolds. Pore size is higher in the case of PLGA scaffolds, followed by PLA scaffolds. Most of pores in PLGA based scaffolds have a size above 800 µm, but smaller pores can be also showed in these matrices with a size between 200 and 300 µm. In the case of PLA based scaffolds, pore size is situated between 200 and 800 µm. There are a few pores with a pore size higher, around 1mm.

There is a minimum pore size recommended for a scaffolds and it is 100 µm (Hulbert, S.F., et al, 1970), due to cell size, migration requirements and transport. But recent studies have showed better bone regeneration in scaffolds with pores > 300 µm (Karageorgiou, V., et al., 2005; Kuboki , Y., et al., 2001; Tsuruga, E., et al., 1997).

In general, high degree of porosity and larger pores favor direct bone regeneration, thus PLGA could be the best candidate for its use as scaffolds. However, there is a limitation in porosity and pore size and it is associated with mechanical properties. As we have said, a highly porous structure is preferred but normally, it is achieved at the expense of mechanical properties (Gentile P., et al, 2012). When the void volume increases, mechanical strength of scaffold is reduced.

Taking these considerations into account, PLGA scaffolds present higher porosity and pore size but its mechanical properties are not the desirable's one for a scaffold. For this reason, PLA matrices are, *a priori*, the best candidates for their use as scaffolds.

6.3.3. Cell viability test

Procedure for viability cell assay has been developed by FIOBI-HULP group, from Hospital Universitario La Paz and is divided in the following steps:

- Porous disks were sterilised during 1 hour for each face with ultraviolet light and then, placed in 24 well plate.

- These disks float in culture medium and do not cover completely space in the wells, producing cells settle in the bottom when they are added for cultivation. In order to avoid this problem, plastic devices showed in Figure 45 that allow the immobilization of materials were used (Scaffdex, tampere, Finlandia).

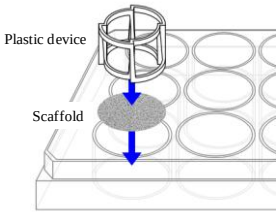


Figure 45: Scaffdex plastic device

- Disks were then pre-incubated in a medium culture during 20-24 hours at 37ºC, in an atmosphere with 5% of CO_2 and 95% of relative humidity in DMEM medium ("Dulbecco's Modified Eagle medium", Invitrogen, Barcelona, Spain) supplemented with 15% of fetal bovine serum, penicillin and streptomycin (Invitrogen, Carlsbad, CA, EE.UU).
- As cellular lines, commercial human meshenquymal cells, isolated from bone narrow donor between 21 and 30 years, were used (hMSCs, Cambrex, Bio Science, Verviers, Belgium). In this case, cell viability was measured using cell from two donors (hMSCs1 and hMSCs2). Each material was tested in duplicate; as a result, four disks from each material were used for test.

Following the procotol described in Experimental Section, cellular viability was tested using AlamarBlue® reagent. 400.000 cells were added to each well where scaffolds disks were placed. This 24 well plate was incubated with alamarBlue reagent during 3-4 hours. Results for this cell viability test are represented in Figure 46.

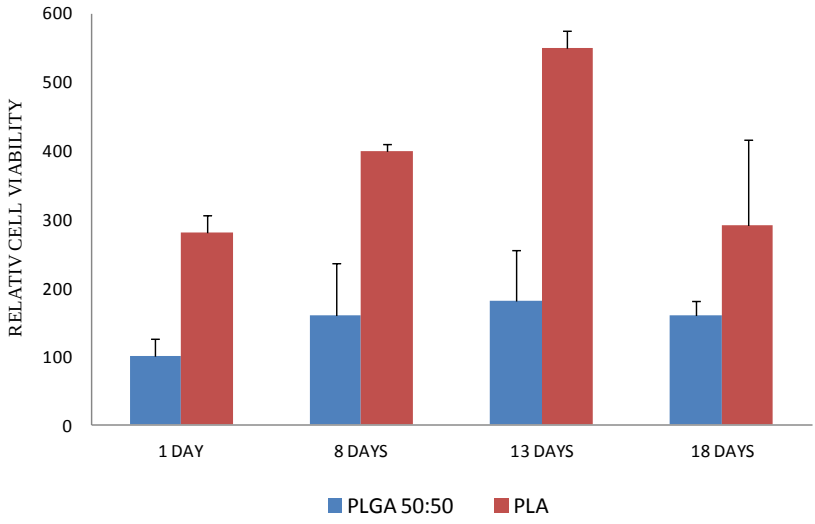


Figure 46: Relative cell viability for the two different materials

Figure 46 shows that cells are viable for two materials tested but the viability is higher in the PLA than PLGA scaffolds. From 1 to 13 day of measures, cell viability increases over PLA based materials, contrarily to 50:50 PLGA scaffolds where only little changes are observed.

But, after 18 days in culture, it is observed that cell viability did not increase. This fact indicates that cells have colonized the entire available surface of the material and begins to die for lack of surface available for growth.

As a conclusion of this test, and taking into account the physico-chemical characterization of these two materials, PLA based scaffolds processed with Freon is a better candidate for it use as scaffold.

6.4. Variation of cell growth over materials CO_2 or Freon processed

In this section is compared the cell growth observed over two scaffolds composed by the same polymer but processed with two different compressed fluids, Freon R-134 and sCO_2. These materials are the followings:

- PLA (LR-708) processed with Freon R-134a
- PLA (LR-708) processed with sCO_2

6.4.1. Preparation of 3D PLA based scaffolds.

Following the procedure describe with detail in section 5.2.2. of Experimental Part, and schematized in Figure 47 , PLA (LR-708) based porous scaffolds were prepared.

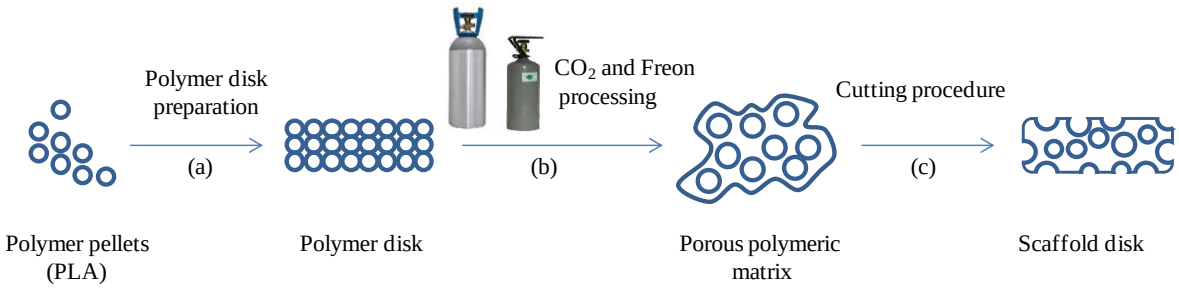


Figure 47: Procedure for 3D PLA based scaffold obtaining using Freon and sCO_2

As it is represented in Figure 47, there are three operations required:

a) Polymer disk preparation
b) Freon R-134a or sCO_2 processing
c) Cutting procedure

The variable values used for the preparation of both porous scaffolds using Freon or sCO_2 in processing are detailed in Table 10, for polymer disk preparation, and in Table 11 for processing polymeric disk with Freon R-134 and sCO_2.

Table 10: Conditions for polymer disk preparation

Polymer	Thermal Treatment	Tª (ºC)	M (g)	Applied Pressure (Tn)
PLA	Yes	150	0,4	0,5
PLA	Yes	150	0,8	3

Table 11: Experimental conditions for the preparation of 3D PLA based materials

Compressed fluid	Tª (ºC)	P (BAR)	Incubation time (h)	Opening degree (%)	Porous matrices?
Freon R-134a	40	20	3	60	Yes
sCO_2	35	100	2	60	Yes

After experiment, disks were inflated leading to the formation of porous matrix, as can be seen in Figure 48.

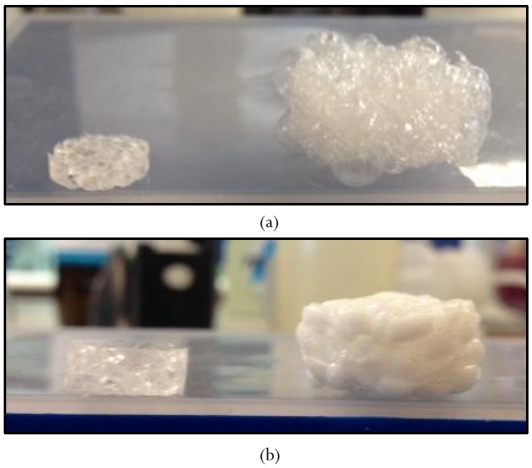


Figure 48: Comparison between initial disk and final scaffold. (a) Corresponds to PLA processed with Freon and (b) to PLA processed with sCO_2.

6.4.2. Physico-chemical characterization of 3D porous materials.

A) Mass variation study

The mass of compressed Freon and sCO_2 retained in scaffolds can be studied by measuring the mass of scaffolds before the experiment, directly after the processing with compressed

fluids and, finally, after 7 days of stabilization. Figure 49 shows the increase in the mass of the porous materials directly after the experiment and after 7 days of stabilization.

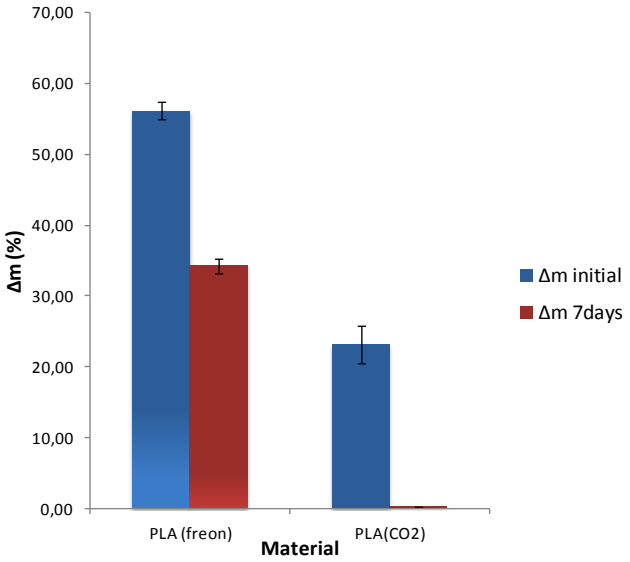


Figure 49: Mass variation of PLA based scaffolds processed with Freon and CO_2

In the two PLA based materials tested, an increase of mass is produce after the experiments made with Freon and with Supercritical CO_2. The increase of the mass is higher is the case of the PLA scaffold processed with Freon.

After 7 days of stabilization after experiment, the graphic shows that polymers processed with Freon accumulate a high amount of residual Freon. This fact indicates that these scaffolds have a lot of closed pores and, as a consequence, Freon cannot scape from the scaffold. In the case of the PLA processed with CO_2, tendency is oppose: these scaffolds do not retain a large amount of residual CO_2. These samples do not show retention of the gas after 7 days. This fact can be explained due to the higher diffusivity of the CO_2 in comparison with the Freon.

B) Solid density study

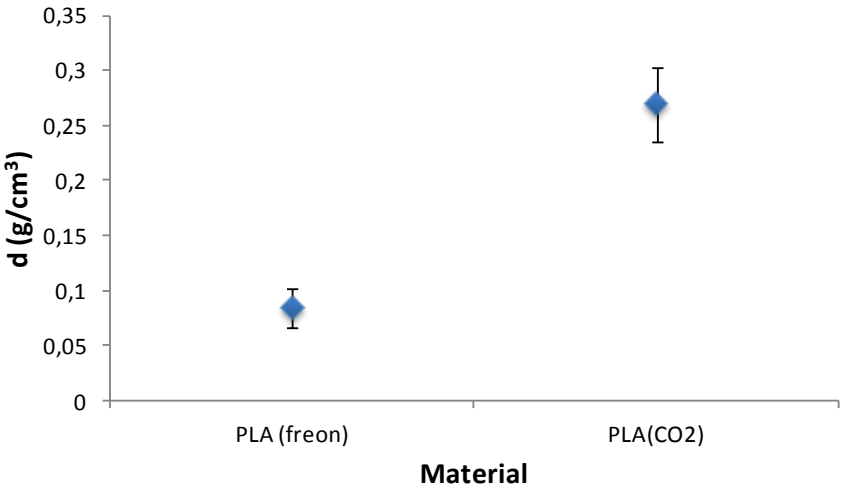


Figure 50: Solid density for PLA based scaffolds

Figure 50, it is observed that solid density is higher in PLA scaffolds processed with CO_2 than in PLA scaffolds processed with Freon R-134a. As we can see in visual inspection, Freon creates larger pores than those created by supercritical CO_2. Polymers processed with compressed Freon increase their volume more during the experiment resulting in the formation of larges pores and, consequently, lower solid density.

C) Porosity study

Porosity of two PLA bases scaffolds has been estimated using the equation described in Experimental Section.

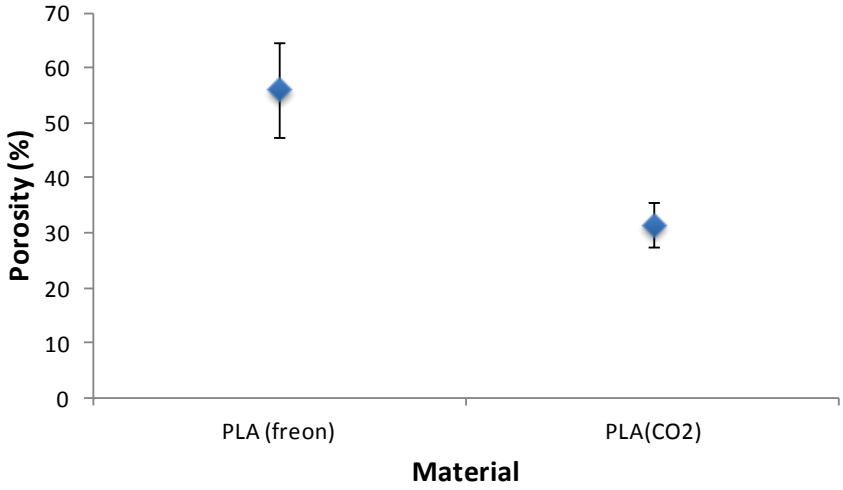


Figure 51: Porosity for two PLA based scaffolds

Figure 51 shows that porosity is higher for PLA scaffolds processed with Freon R-134a, following again the tendency showed for solid density. This result demonstrates that PLA based scaffolds have a porous structure which is a property necessary for cell adhesion and proliferation, being also good candidates for bone tissue regeneration.

D) Morphology and pore size study

Morphology and pore size of PLA bases scaffolds was studied using scanning electron microscopy.

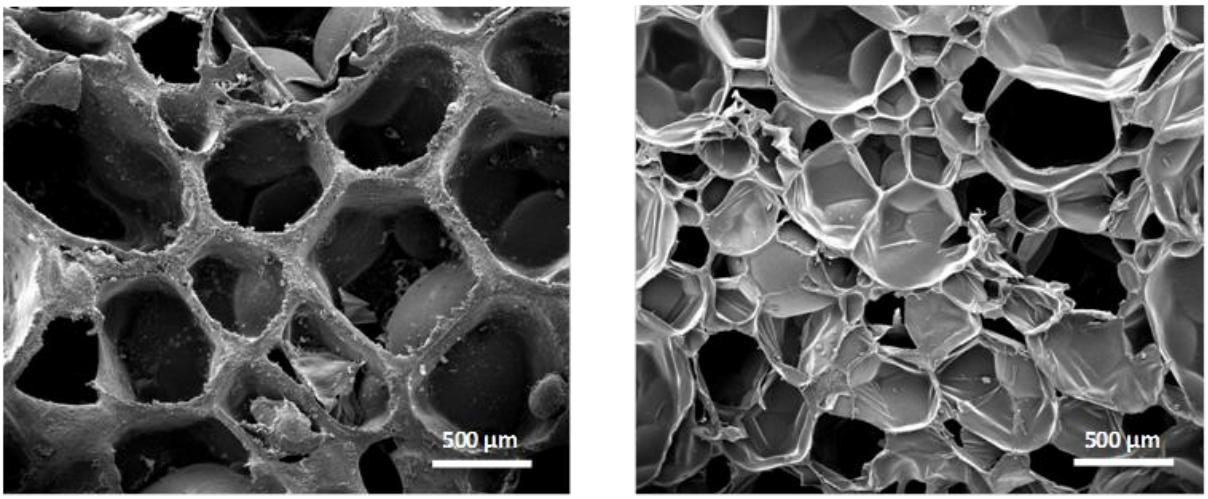


Figure 52: SEM images for PLA scaffolds processed with Freon R-134a

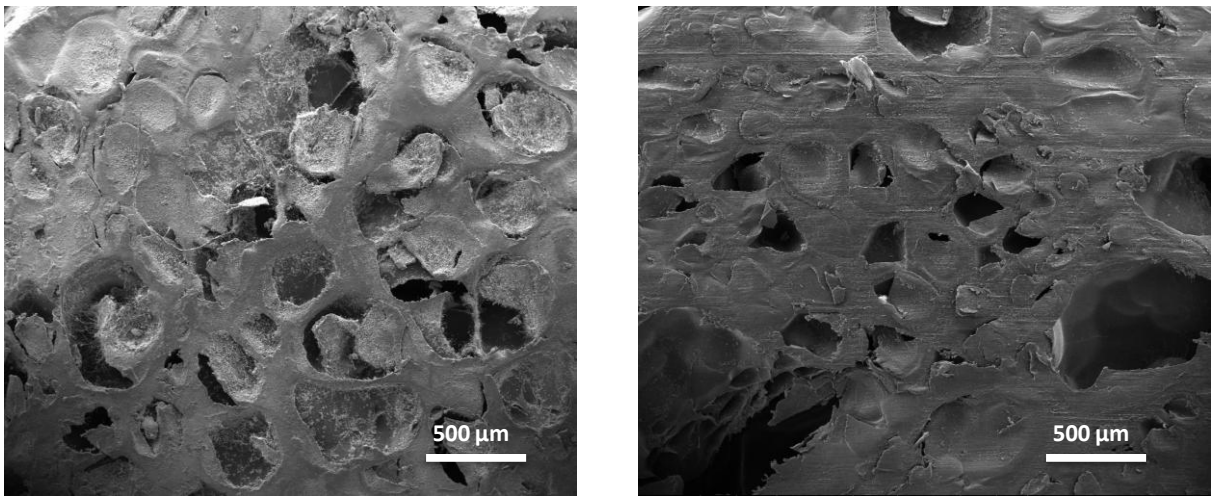


Figure 53: SEM images for PLA scaffolds processed with sCO2.

SEM images confirm again porosity results. Pores formed in PLA based scaffolds processed with Freon are higher than those processed with sCO_2. In the case of PLA based scaffolds processed with Freon, pore size is situated between 200 and 800 µm. There are a few pores with a pore size higher, around 1mm. Pore size for PLA scaffolds processed with sCO_2 is situated between 150 and 600 µm. These two PLA based scaffolds present pores with recommended size for scaffolds (>300 µm), consequently they are also good candidates for their use in bone tissue regeneration.

6.4.3 Cell viability test

Following the procedure developed by FIOBI-HULP group and described in detail in Section 6.3.3., cell growth over two materials prepared was tested.

As cellular lines, commercial human meshenquymal cells were used (hMSCs1 and hMSCs2). These cells have been isolated from bone narrow donor between 21 and 30 years (Cambrex,

Bio Science, Verviers, Belgium). Each material was tested in duplicate; therefore four disks from each material were used for test.

Cellular viability was tested using the protocol for alamarBlue® described in Section 4.4.1. 400.000 cells were added to each well where scaffolds disks were placed. This 24 well plate was incubated with alamarBlue reagent and after 3-4 hours, fluorescence was measure. The results of this cell viability study are represented in Figure 54

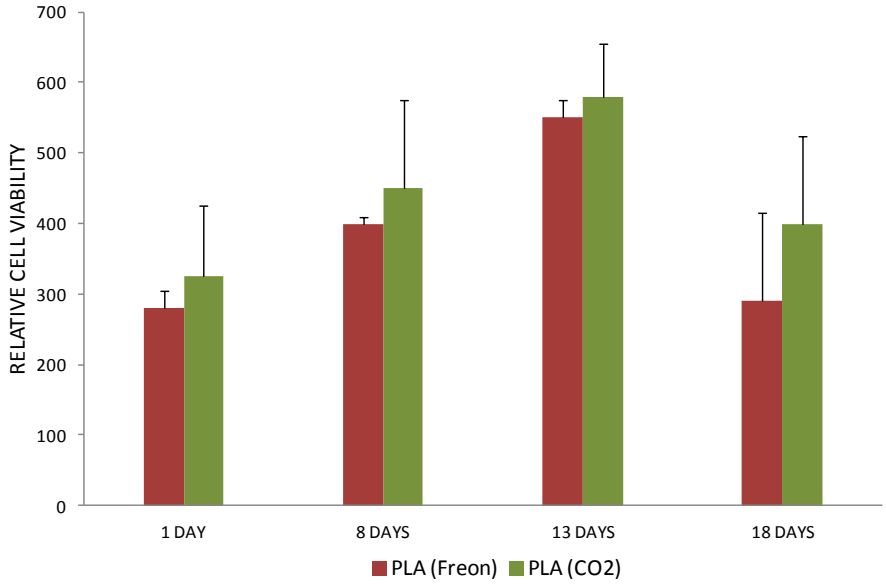


Figure 54: cell viability test for two PLA based scaffolds

Figure 54 shows that cells are viable for two materials tested but the viability is a bit higher in the PLA processed with CO_2. From 1 to 13 day of measures, cell viability increases over two PLA based materials.

But, after 18 days in culture, it is observed that cell viability did not increase. This fact indicates again that cells have colonized the entire available surface of the material and begins to die for lack of surface available for growth.

In this case, and regarding the physic-chemical characterization, the two PLA based scaffolds are good candidates for their use as support for cell colonization.

6.5. Variation of cell growth over IB's decorated and non decorated scaffolds

As it is widely explained in the introduction, Inclusion bodies (IB's) are protein aggregates, which have recently used to enhance cell growth mainly on 2D scaffolds. Scaffolds based on PLA have been prepared because they are more widely used by the scientific community working in this field. In addition, the use of PLA CO_2 processed scaffolds, will allow a comparison of IB's with other cell growth stimulators already used to decorate these CO_2 processed polymeric matrices.

In this section, it is studied the impact of IB's on cell growth over 3D porous PLA based scaffolds.

6.5.1. Development of IB's scaffold disk impregnation method.

In order to carry on properly the above described study, in the present work it was developed a suitable methodology for 3D porous scaffold impregnation with IB's.

Three different alternatives have been explored for matrix impregnation using inclusion bodies prepared following the procedure described in Experimental part. Those alternatives are the followings:

a) Suspension addition before scaffold formation
b) Immersion
c) Filtration.

a) Suspension addition before scaffold preparation

In this method, IB's are directly added to polymer as it is schematized in Figure 55

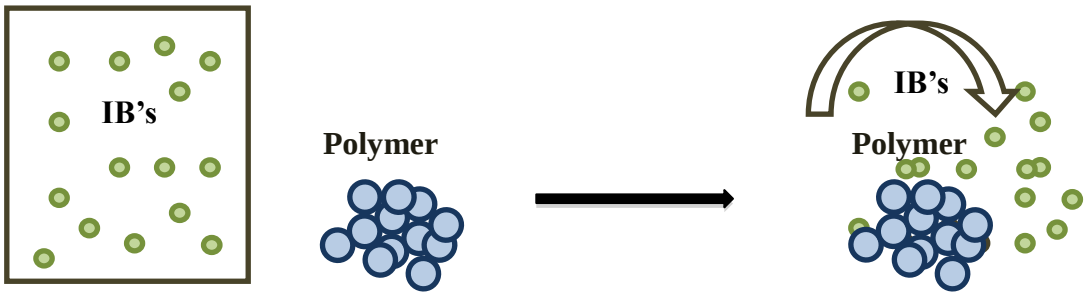


Figure 55: Suspension addition method

For this method, it is necessary that Inclusion bodies tolerate working pressure and temperature because inclusion bodies are added before de polymer disk formation.

b) Immersion

This method consists of immersing the porous disk (disk obtained by cutting scaffold) in the inclusion bodies suspension.

Figure 56: Immersion method

c) Filtration

In this technique, the porous disk, obtained by cutting the scaffold, is placed in a filtration equip, formed by a kitasato flask, a funnel and a vacuum pump.

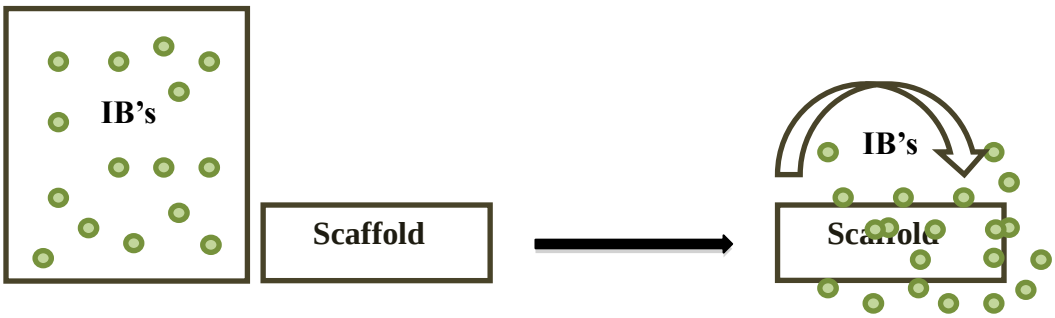


Figure 57: Filtration method

Results obtained from these studies show the following conclusion:

- The major disadvantage of the inclusion bodies addition before forming the polymer disk is the thermal pre-treatment needed for removing the crystalline part because a temperature of 150ºC is required, therefore IB's have to tolerate this high temperature. Immersion and filtration techniques are applied after thermal pre-treatment, consequently, inclusion bodies are not exposed to high temperatures.

- If immersion and filtration are compared, the second one presents a greater efficiency regarding the inclusion bodies adherence by optical microscopy. Results in this technique are more homogenous on the scaffold surface and inclusion bodies have greater penetration.

For this reason, filtration technique has been selected as scaffold impregnation technique and will be used in this work.

6.5.2. Preparation of PLA matrices processed with sCO_2 and decorated with IB's

Following the protocol described in section 5.2.2, PLA based scaffolds have been prepared using sCO_2. As it has been described and schematized in Figure 58, this procedure is divided in three different steps: it starts with the preparation of the polymer disk. Then, PLA porous matrix is produced using sCO_2 and, finally, PLA scaffold disks are generated with a cutting methodology.

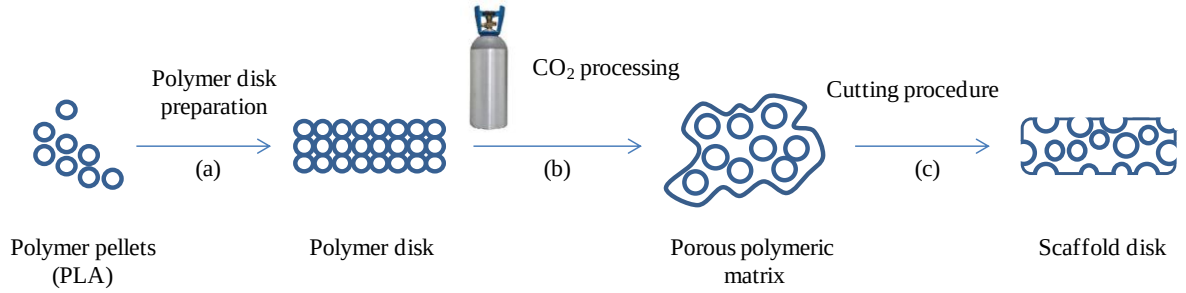


Figure 58: Procedure for 3D porous scaffolds preparation using sCO_2

For the preparation of polymer disk and following the protocol described in Experimental part, 0.8 g. of PLA (LR-708) were weight and put in the oven for thermal treatment, during 15 minutes. Polymer was then placed into a special mould for giving to disk a round form with a diameter of 15 mm by applying 3 tons. Then, PLA disks were placed inside high-pressure plant, at 10 MPa and 35ºC for the preparation of 3D porous scaffolds.

Finally, the porous matrices where cut following the procedure describe in Section 6.2, in order to obtain scaffold disks with 3,5 mm of thickness and 15 mm of diameter. From 1 porous scaffold, two disks were obtained after cutting method.

PLA porous scaffold impregnation with inclusion bodies was made using aqueous suspensions of the protein aggregates prepared by the procedure described in Section 5.1.
611 µg of inclusion bodies were re suspended in 20 ml of PBS 1x (c = 30,55 µg/ml). In order to avoid possible contamination of the scaffolds during its manipulation, 1,6 ml of three antibiotics were added to the suspension. Those antibiotics are tetracyclin, kanamycin and chloramphenicol. Finally, suspension was sonicated during 10 minutes.

A kitasato flask, a funnel and a vacuum pump form filtration equipment. The porous disks were placed on the funnel as it can be seen in the following figure:

Figure 59: Scaffold position during filtration

Once the scaffold disk was placed and the filtration equipment was mounted, vacuum pump was connected to the system. The final montage can be seen in Figure 60.

Figure 60: Filtration equip

In order to increase the process efficiency, filtration procedure was repeated 3 times with 5 mL of suspension. Scaffolds disks were then removed and dried with compressed air. Scaffolds were weight before and after filtration, and then after drying process. Finally, they were conserved at -20ºC.

Following the above described procedure, 8 disks of PLA scaffolds free of IB's and 8 disks of PLA scaffolds loaded with IB's were prepared and properly characterized.

As it has been describe in Experimental part, section 5.3.3. , IB's loading in scaffolds can be qualitatively estimated using optical microscopy. For this estimation, scaffolds were looked at the upper face, which first receives suspension during filtration process; and at the lower face, the one receiving in last place.

In Figure 61 is shown IB's observation by Optical microscopy of a representative disk of all set prepared.

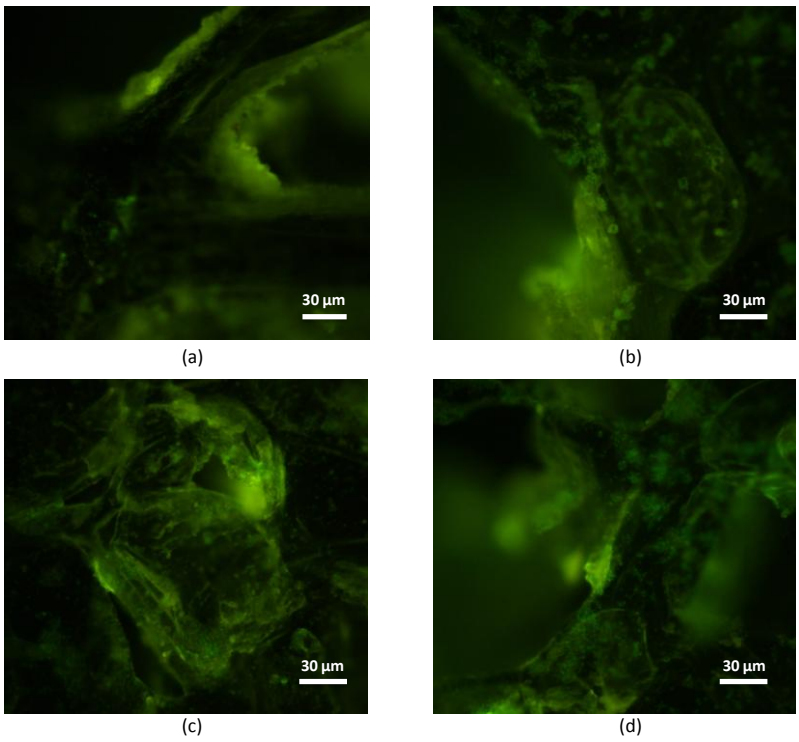


Figure 61: Optical microscopy for PLA scaffolds processed with CO_2. (a) and (b) corresponds to upper face; (c) and (d) to the lower face.

PLA based scaffolds processed with sCO_2 present higher quantity of Inclusion bodies, not only in the upper face but also in the lower face.

6.5.3. Cell viability test

Cell viability test was made following the protocol developed by FIOBI-HULP group and properly described in Section 6.3.3.

As cellular lines, commercial human meshenquymal cells were used (hMSCs3). These cells have been isolated from bone narrow donor between 21 and 30 years (Cambrex, Bio Science, Verviers, Belgium).

Cellular viability was tested using the protocol for alamarBlue® described in Section 5.4.1. 400.000 cells were added to each well where scaffolds disks were placed. This 24 well plate was incubated with alamarBlue reagent and after 3-4 hours fluorescence was measured. The results of this cell viability study are represented in Figure 62.

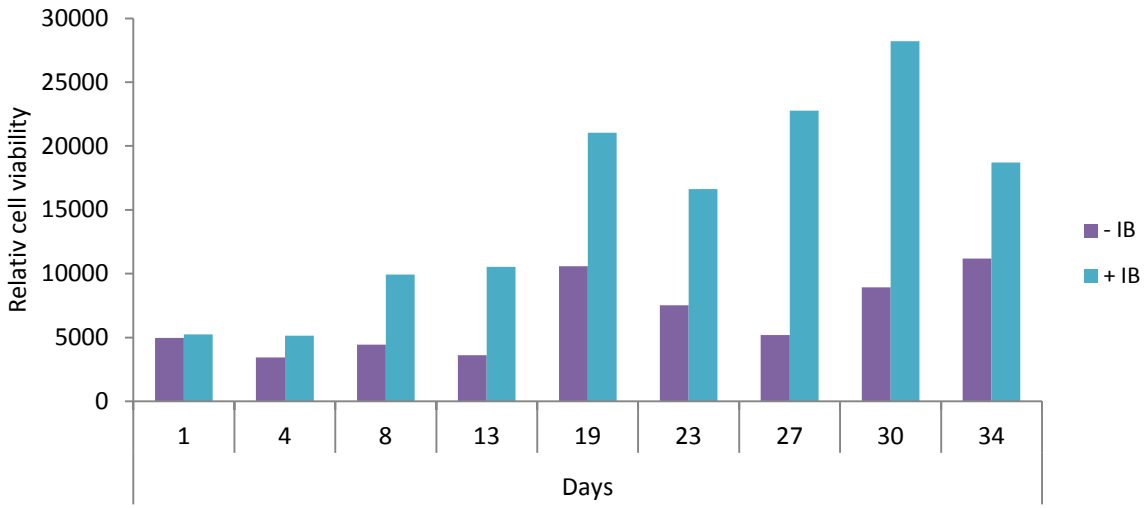


Figure 62: Cell viability test for PLA scaffolds

As it can be seen in the figure, human meshemquimals stem cells are adhered to all the scaffolds and they grow on them, both scaffolds containing inclusion bodies and those who do not.

But, cells grow significantly faster in the scaffolds decorated with bacterial IB's.

6.6. Relative importance of IB's decoration, polymer nature and processed conditions over the behaviour of 3D polymeric matrices as scaffolds.

On the basis of the promising results achieved with PLA IB's loaded scaffolds, a new set of experiments was designed in order to confirm the relationship between mesenchymal cell growth stimulation and structural characteristics described in previous sections, and to try to quantify the relative importance of them. The followings materials form this new set of experiments:

- PLGA (RG-503) processed with compressed Freon and without IB's
- PLA (LR-708) processed with compressed Freon and without IB's.
- PLA (LR-708) processed with compressed Freon and functionalized with IB's.
- PLA (LR-708) processed with supercritical CO_2 and without IB's.
- PLA (LR-708) processed with supercritical CO_2 and functionalized with IB's.

The main idea for this part of the work is not only repeat the experiment made using PLA matrices processed with CO_2 and decorated with IB's but also study IB's decoration in PLA matrices processed with Freon and compare them with those that are not decorated. Indeed, PLGA will be study for comparing all materials tested during the realization of this work.

6.6.1. Preparation of 3D porous matrices and their impregnation with IB's

This new set of porous scaffolds has been prepared using protocol widely described in Section 5.2.2 of Experimental part, and schematized in Figure 63.

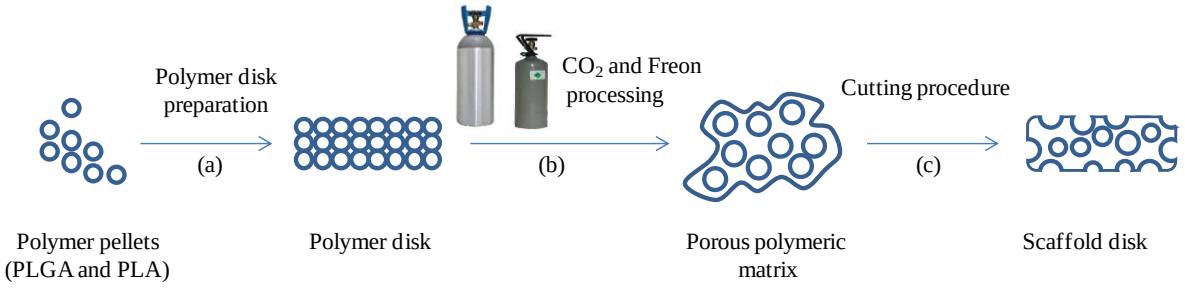


Figure 63: procedure for 3D porous scaffolds preparation using compressed fluids.

As it is showed in Figure 63, procedure for 3D porous scaffolds is formed by three steps:

a) PLGA and PLA polymer disk preparation
b) Freon R-134a and sCO_2 processing
c) Cutting procedure

The different values used for the preparation of both porous scaffolds with different polymeric nature are detailed in Table 12, for polymer disk preparation, and in Table 13 for processing polymeric disk with Freon R-134 and sCO_2.

Table 12: Conditions for polymer disk preparation

Polymer	Thermal Treatment	Tª (ºC)	M (g)	Applied Pressure (Tn)
PLGA	No	-	0,3	0,5
PLA	Yes	150	0,4	3
PLA	Yes	150	0,8	3

Table 13: Experimental conditions for 3D porous material preparation

Polymer	Compressed fluid	Tª (ºC)	P (BAR)	Incubation time (h)	Opening degree (%)	Porous matrices?
PLGA	Freon R-134a	35	20	2	60	Yes
PLA	Freon R-134a	40	20	3	60	Yes
PLA	sCO_2	35	100	2	60	Yes

Following the above described procedure, 7 disks of each material were prepared and properly characterized (See annexe for more information).

Contrary to experiment described in Section 6.5.2, in this large experiment, all materials were treated with antibiotics (tetracycline, kanamicine and chloramphenicol) and not only those loaded with IB's in order to avoid any possible contamination; then two different suspensions were prepared.

- Materials, which were not functionalized with IB's received a suspension formed by PBS and three antibiotics. For preparing this suspension, 1,6 ml of each antibiotics were added to 20 ml of PBS. 5 ml of this suspension was filtrated over scaffold three times.
- Materials, which were functionalized, received a suspension formed by PBS, IB's and three antibiotics. This suspension contained 20 ml of PBS, 1,6 ml of each antibiotics and 611 μg of IB's.

Following this procedure, we avoid any contamination in all materials treated and, in addition, the only difference between them is the presence or absence of IB's.

In Figure 64 and Figure 65 are shown IB's observation by optical microscopy of a representative disk of all set prepared.

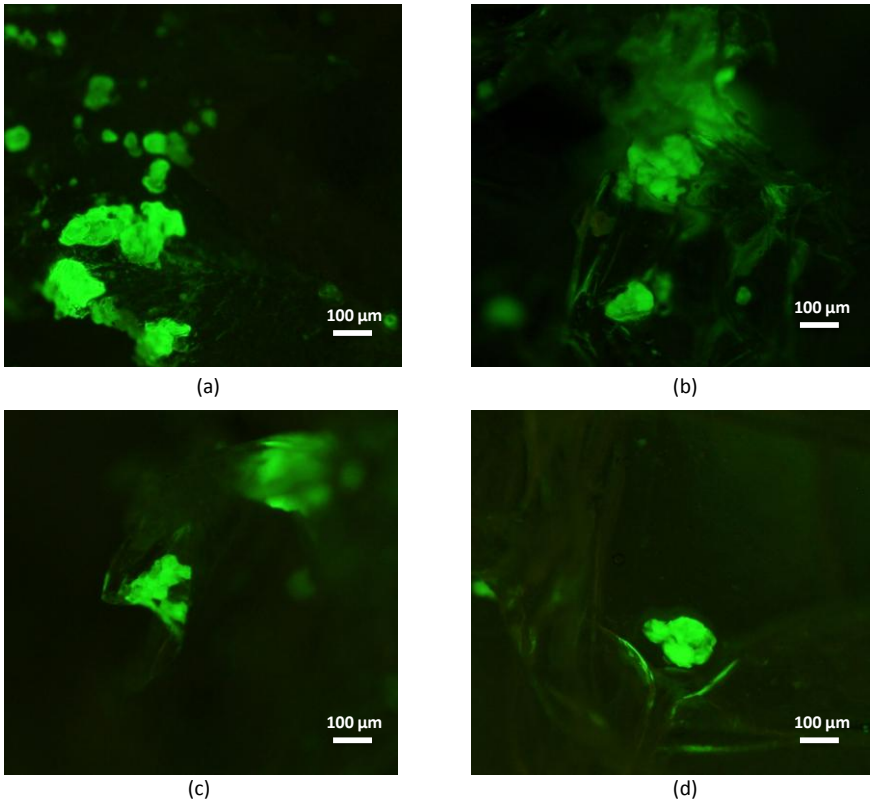


Figure 64: Optical microscopy for PLA based scaffolds disks processed with Freon R-134a. (a) and (b) corresponds to face which receives first the suspension during filtration; (c) and (d) corresponds to face which receives suspension in last place.

In this figure, result for impregnation with IB's of PLA based scaffolds processed with Freon can be seen, showing that face which is not directly exposed to IB's suspension during filtration accumulates less quantity of inclusion bodies. In the other hand, and as it was expected, face that receives the IB's suspension first, accumulates more quantity of these protein aggregates. Indeed, distribution is not homogenous in the entire surface, accumulating in specific regions of scaffold.

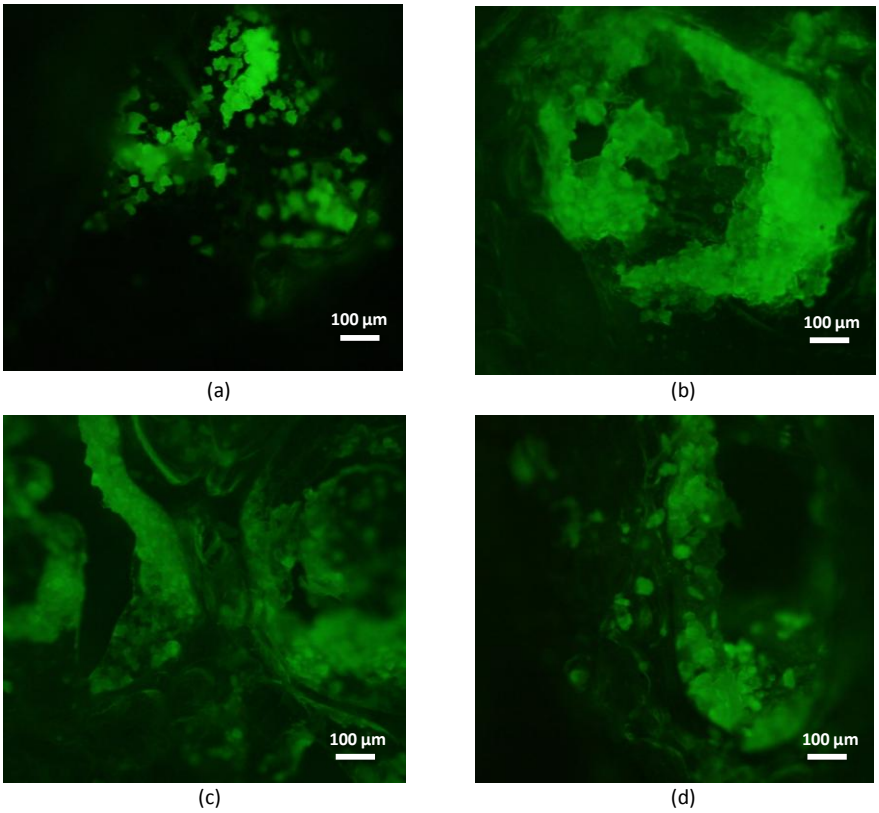


Figure 65: Optical microscopy for PLA scaffolds processed with CO_2. (a) and (b) corresponds to face which receives first the suspension during filtration; (c) and (d) corresponds to face which receives suspension in last place

As it can be observed by optical microscopy, PLA based scaffolds processed with sCO_2 present higher quantity of Inclusion bodies, not only in the upper face but also in the lower face. Protein aggregates are accumulated in specific region, especially around pores.

Comparing two PLA based scaffolds, the presence of Inclusion bodies is higher in scaffolds processed with sCO_2.

6.6.2 Estimation of the quantity of IB's retained in scaffolds.

Quantity of Incusion bodies can be estimated using a fluorescence spectrophotometer. Inclusion bodies are formed by a fluorescent component and this property can be used in order to know the quantity retained comparing signals before and after filtration. Concentration of IB's suspension is known, therefore signal obtained before filtration corresponds to this value. With these information and signal obtained after filtration, final concentration of Inclusion bodies can be estimated and, as a consequence, the quantity of IB's retained in matrices.

a) Protocol for estimating the quantity of IB's retained in scaffolds

This procedure starts with the preparation of 20 ml of IB's suspension, with a concentration of 30, 55 µg/ml.

- 200 µl of suspensión were taken before filtration process. This signal corresponds to initial concentration of suspension. All matrices receive the same initial quantity of IB's that was estimated with the filtration volume (5 ml) and initial concentration. Quantity of IB's added to matrices was estimated in 152, 75 µg.
- After filtration, the collected filtrate was brought to a final volume of 5 ml. From this volume, 1 ml was taken in order to measure the fluorescence.
- Knowing the initial concentration of the suspension and comparing these two signals, the concentration of the collected filtrate could be estimate.

Estimation of IB's retained in porous matrices was done for this last large experiment and results are shown in the Figure 66:

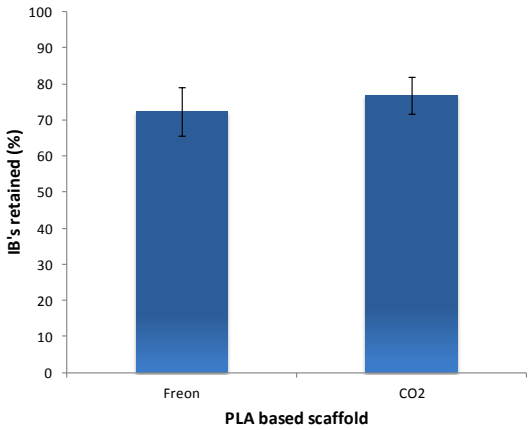


Figure 66: % of IB's retained in PLA matrices processed with Freon and with CO_2

As the figure shows, the quantity of IB's retained in PLA matrices processed with CO_2 is higher than the quantity retained in PLA matrices processed with Freon. This fact could explain why scaffolds processed with CO_2 present high cell viability.

Based on the idea that IB's stimulate cell growth as it has been demonstrated in the experiment done with PLA scaffolds disks processed with CO_2, PLA scaffold disks which retain more quantity of IB's, should present faster cell growth than those which are less loaded. Figure 66 shows that the quantity of IB's retained is higher in PLA scaffold disks processed with CO_2; as a result cell growth should be faster in those matrices. And this is what shows Figure 66, PLA scaffold disks processed with CO_2 grow faster than PLA scaffold disks processed with Freon.

However, these fluorimetric measures are made in a suspension not in a solution and starting from initial quantities of protein estimated by Western-Blot; consequently, result obtained is only estimation that could be used as a possible explanation of why cells grow faster in matrices processed with CO_2.

6.6.3 Confocal microscopy

An important parameter when studying matrices impregnation with IB's is their penetrability through the porous zones. The penetrability of IB's can be analysed using confocal microscopy. This technology allows us to analyse different parts of the material in order to see how deep the IB's have reached during filtration method. We have studied the two different matrices impregnated with IB's: PLA scaffolds processed with Freon R-134a and sCO_2.
First, we needed to cut a cross section of each porous scaffold using a diamond wire saw. Cut with this type of saw is more precise and samples are less degraded due to lower friction during the cutting. Cut made in matrices appear schematized in Figure 67:

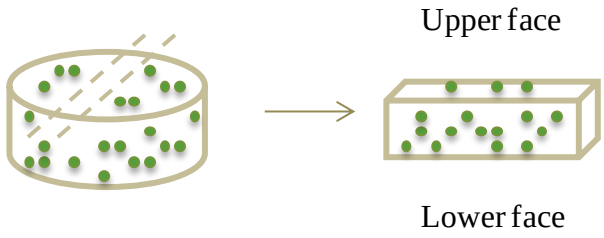


Figure 67: Portion of scaffold used for confocal studies

Inclusion bodies were observed both on the surface and inside the PLA scaffold processed with Freon

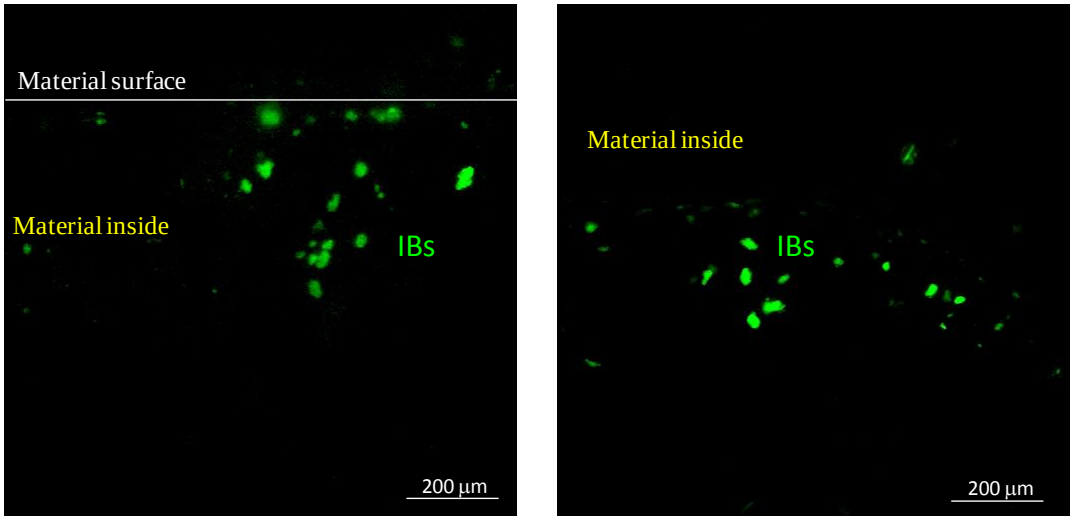


Figure 68: Confocal microscopy images for PLA scaffolds processed with Freon R-134a

After microscopic examination of different areas of the sample, it is observed that the IB's colonize inside the material through the pores. Indeed, the distribution of the IB's in the material is not homogeneous, accumulating randomly in certain regions.

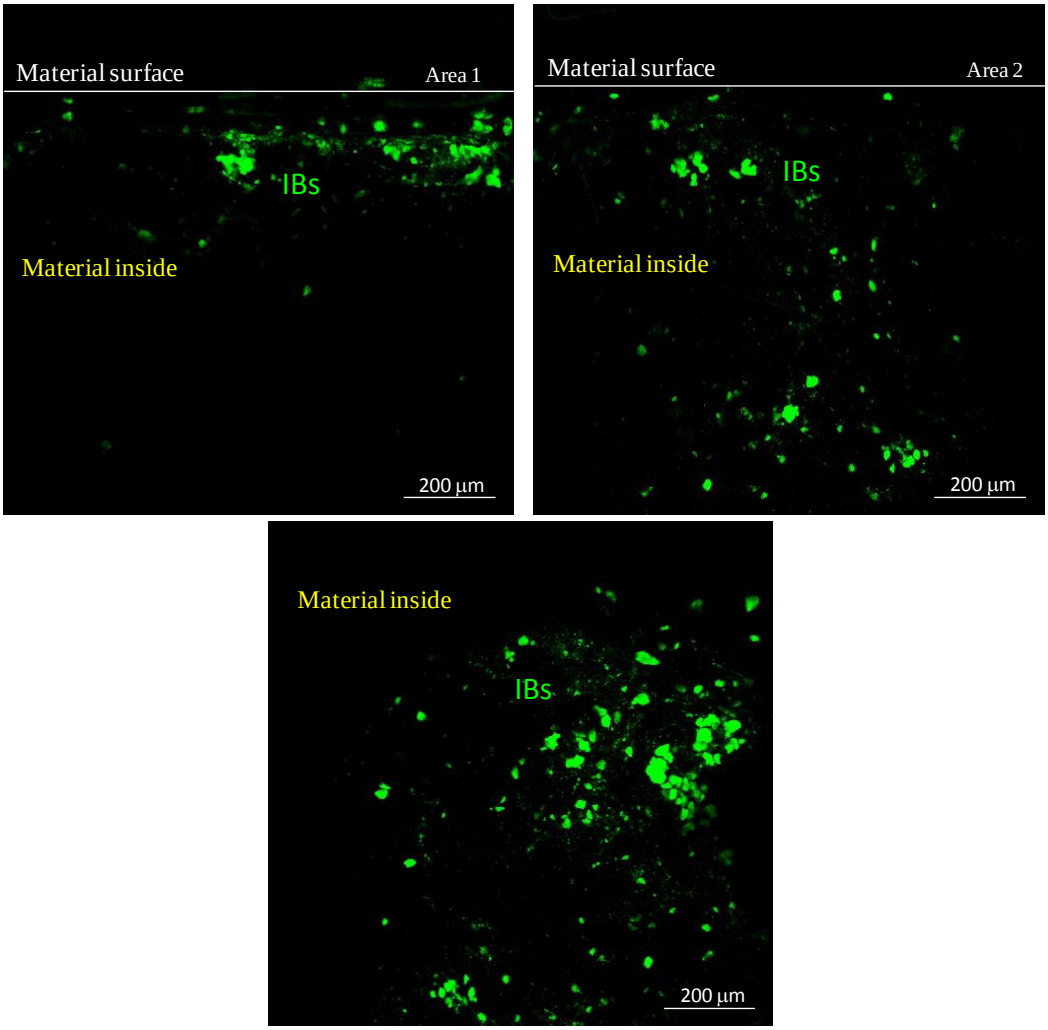


Figure 69: Confocal microscopy images for PLA scaffolds processed with sCO_2

In the case of the PLA scaffolds processed with sCO_2, there are some areas where IB's are located exclusively on the surface of the material (area 1) and others, where the IB's penetrate inside the materials through the pores (area 2).

Also, the accumulation of IB's is observed inside the material. As in the PLA scaffolds processed with Freon, the distribution of IB's in the material is not homogenous and they are accumulated in certain areas.

If we compared the two different materials, the higher concentration of Inclusion bodies is observed in PLA scaffolds processed with sCO_2, both on the surface and inside the material. This could be an explanation of why cells seeded on PLA scaffolds processed with sCO2 growth faster, because these matrices accumulate more quantity of IB's.

6.6.4 Cell viability test

Following the procedure developed by FIOBI-HULP and protocol described in Section 5.4.1., of Experimental Part, cell viability was measure for this large experiment.

In this case, cell viability was measured using cell from two donors different from the employees in the first test (hMSCs4 and hMSCs5). Each material was tested in duplicate; therefore four disks from each material were used for test.

In Table 14, there are detailed some of the most relevant physic-chemical characteristics of disks.

Table 14: Physico-chemical characteristics for scaffold disks prepared

Material	m (g)	Porosity (%)	IB's quantity (µg)	IB's /m (µg/g)
PLGA	20,50	77,29		
	21,02	68,82		
	22,16	67,40		
	32,41	71,26		
PLA (Freon/-IB's)	199,26	55,75		
	189,31	53,88		
	197,94	53,31		
	203,96	44,12		
PLA (Freon/+IB's)	138,49	64,37	76,91	0,56
	180,18	63,19	70,83	0,39
	164,46	50,64	63,08	0,38
	205,93	51,84	66,64	0,32
PLA (CO_2/-IB's)	173,65	30,03		
	200,11	32,83		
	215,33	36,36		
	175,37	36,08		
PLA (CO_2/+IB's)	166,83	34,56	120,61	0,72
	157,02	37,20	118,84	0,76
	209,11	28,23	117,65	0,56
	156,77	32,70	134,09	0,86

Cellular viability was tested using the protocol for alamarBlue® described in Section 4.4.1. of Experimental Part. 400.000 cells were added to each well where porous scaffolds disks were placed. As it is described in Section 6.3.3., scaffolds processed with Freon (PLGA and PLA), materials do not cover completely the space in the wells, then a plastic device has to be used. We think that cells could also be bind to the small proportion of this plastic device, then we are considering those cells for viability test and not only those that are adhere to scaffolds disks. For this reason, all materials tested have changed to a new 24 well plat prior to incubation with the alamarBlue reagent. In this way, we quantify only the cells adhered to material under study. After 3-4 hours of incubation, fluorescence was measured.

The results of this cell viability study are represented in Figure 70.

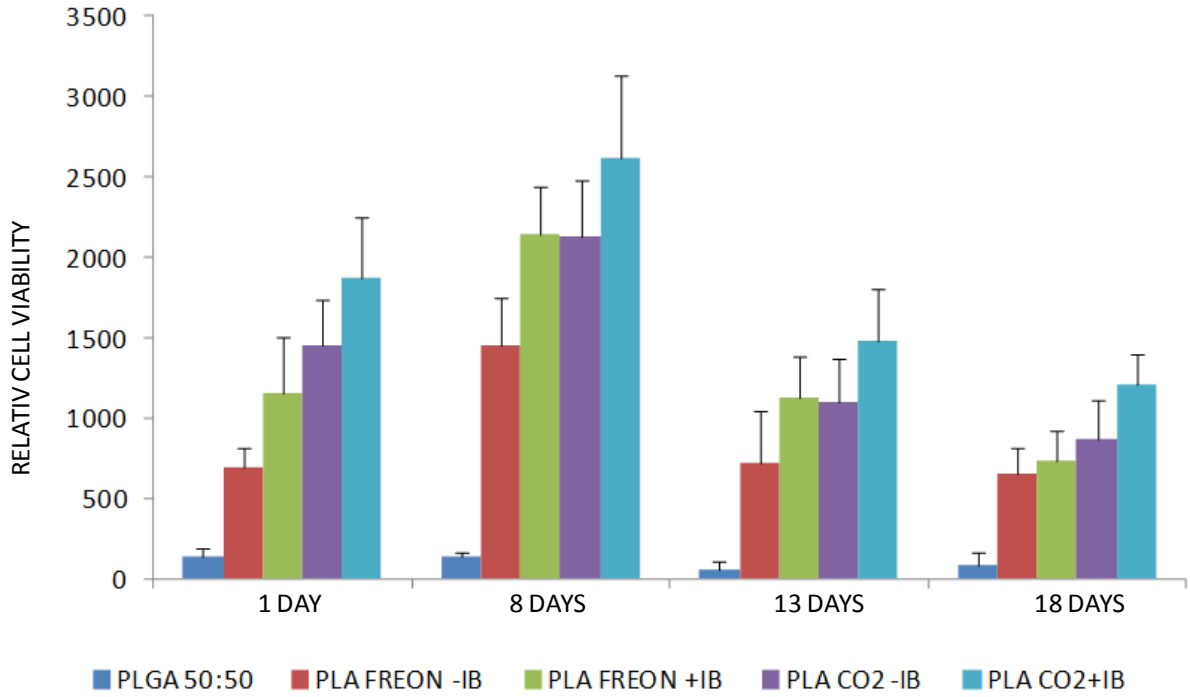


Figure 70: Cell viability test results

The figure shows that, as in the first viability test, cells are viable for all materials tested. As it happened in the first assay, cell viability is much higher in the PLA scaffolds than in PLGA.

During the 8 first days of measures, the cell viability increases in all materials, except for the PLGA where even decreases. After 13 days in culture, it is observed that cell viability decreases, indicating that cells have colonized the whole available surface on the material and begins to die for lack of surface available for growing.
If we compare two tests, in the first one, the decrease in cell viability was observed on day 18 while, in the second one, was detected on day 13. These differences may be due to differences in the growth rate of cells from different donors, as well as the passage number used was not the same in all cases.

In this second test, cell viability is higher in the PLA scaffolds processed with sCO_2 compared to PLA scaffolds processed with Freon confirming results obtained in previous cell viability test.

Regarding IB's impact on cell proliferation, their presence improved cell viability in both PLA based materials, confirming result obtained in first experiment made with only PLA scaffolds processed with sCO_2.

7 CONCLUSION

The conclusions that can be after the realization of this work are the followings:

- SCF processing gives porous structures which are adequate for bone tissue regeneration. Pores allow migration and proliferation of osteoblastos and mesenchymal cells, as well as the proper vascularization of the implant

- Comparing the physic-chemical characteristics, such porosity, pore size and morphology, and relative cell viability between PLGA and PLA, PLGA porous materials are not good candidates for their use as scaffolds. Degradation rate is faster than in the case of PLA scaffolds and cells do not growth over these materials. Therefore, PLGA was rejected as scaffold for bone tissue regeneration

- Contrary to PLGA, PLA based scaffolds are good materials for their use as support for cell attachment, colonization and proliferation. In both cases, cells can grow over these materials. Cell viability is higher in the case of PLA processed with CO_2, being the best option for a scaffold.

- The presence of IB's in scaffolds stimulates cell adhesion and colonization, as it can be seen in cell viability test results. Inclusion bodies do not result toxic to the cells and contrarily improve cell proliferation on the surface. Cells grow faster in scaffolds decorated with these protein aggregates, being promising platforms to be used in bone tissue engineering.

- Cell grow stimulation is higher in the case of PLA base scaffold disk processed with sCO_2 and decorated with IB's. This material (CO_2+IB's) is the best scaffold to stimulate bone grow and, consequently, regeneration.

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"Si he logrado ver mas lejos, ha sido porque me he subido a hombros de gigantes"

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10 ANEXE

10.1 Physico-chemical characterization for large set of experiment

a) Mass variation study

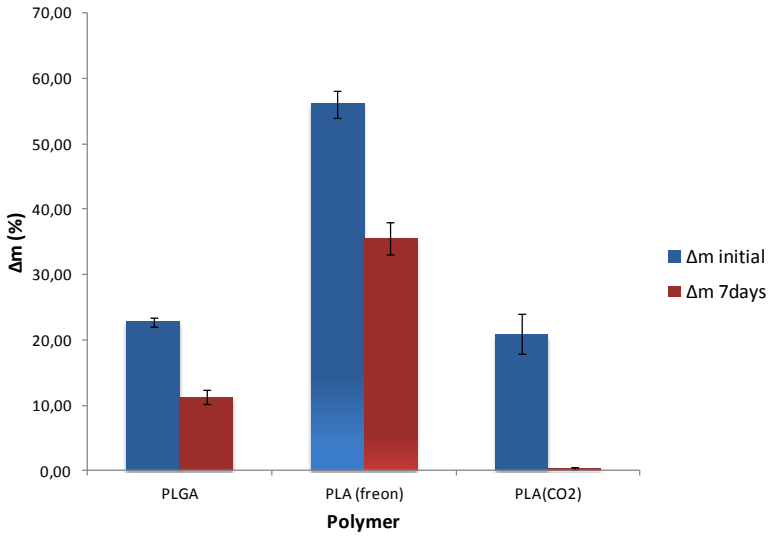

Figure 1: mass variation in scaffolds processed with compressed fluid

Tendency observed in this case is the same to that in the first experiment. After experiment, all materials have increased their mass, being higher in the case of scaffolds processed with Freon.

As it saw in previous experiment, after 7 days of stabilization polymers processed with Freon accumulate a high amount of residual Freon. In the case of the PLA processed with CO_2, tendency is oppose: these scaffolds do not retain a large amount of residual CO_2 which can be explained due to the higher diffusivity of the CO_2 in comparison with the Freon. This figure also shows that in the case of PLA the retention of the fluid is higher than in the case of the PLGA. The PLA has a very high viscosity in comparison with the PLGA, so going out from the structure of the scaffold is more difficult for Freon in the case of the polilactic-acid polymer based scaffold.

b) Solid density study

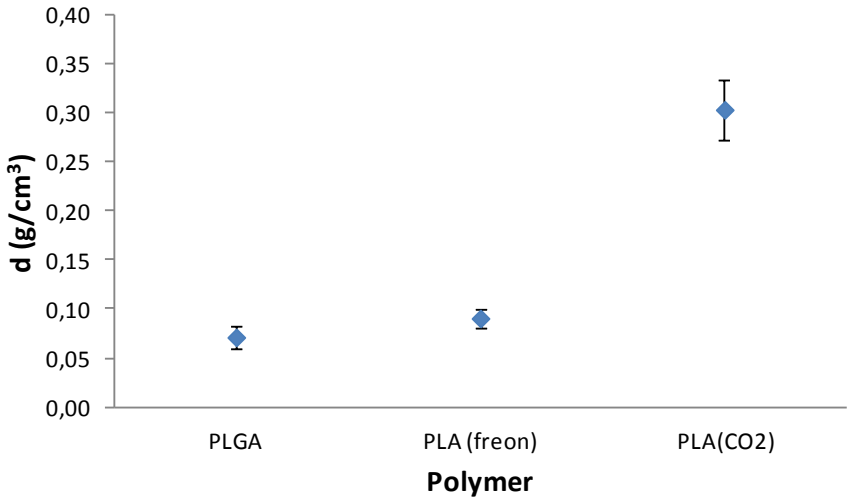

Figure 2: solid density study for three materials

This figure confirms that solid density is higher in the case of PLA scaffolds processed with sCO_2. PLGA scaffolds have the lower solid density.

c) Porosity

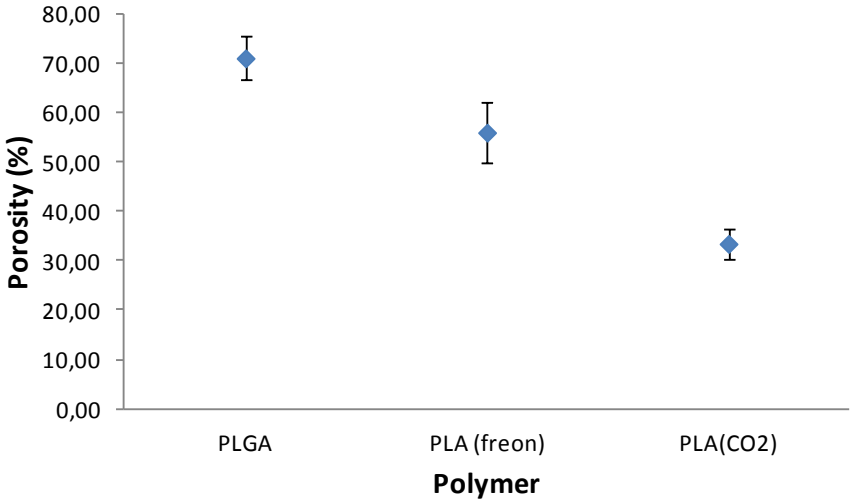

Figure 3: porosity for three materials

This figure corroborates the results showed in previous experiment and also the initial visual inspection. Pores showed in PLGA scaffolds are larger, so porosity has to be higher for this matrix. PLA scaffolds processed with CO_2 have the lowest porosity; pore size is smaller for this material.

d) Morphology

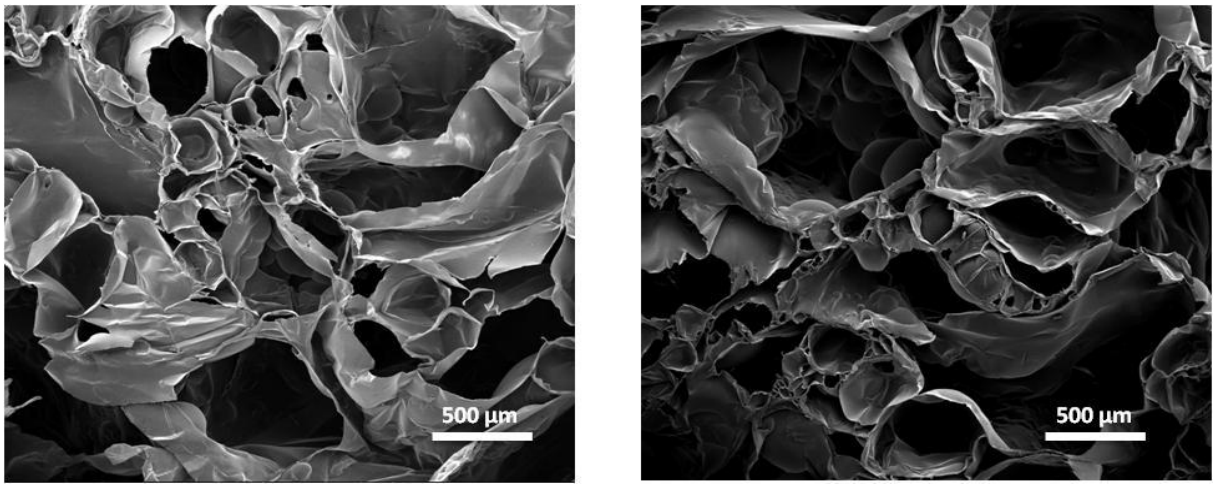

Figure 4: SEM images for PLGA scaffolds processed with Freon R-134a

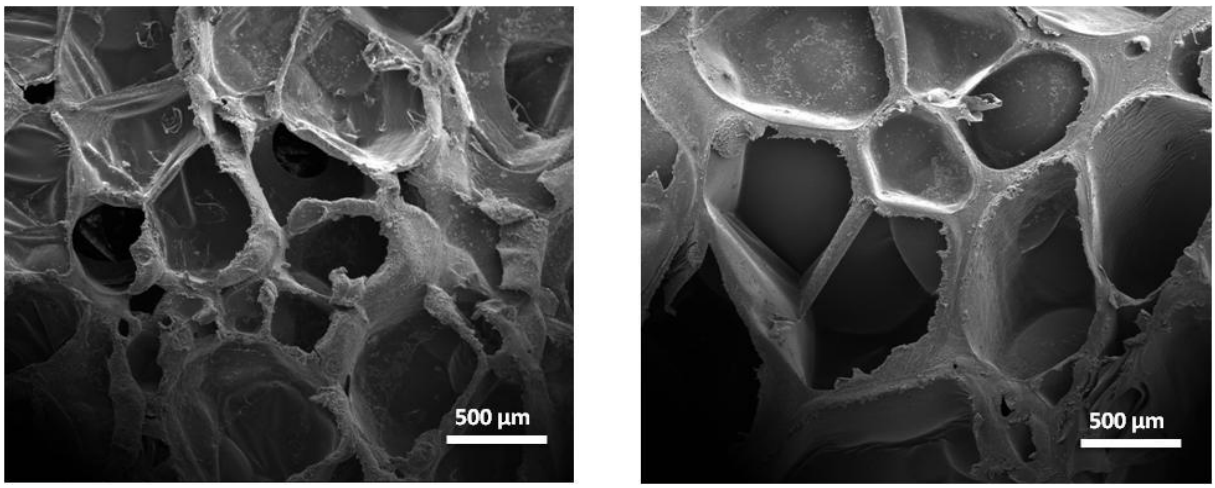

Figure 5: SEM images for PLA scaffolds processed with Freon R-134a

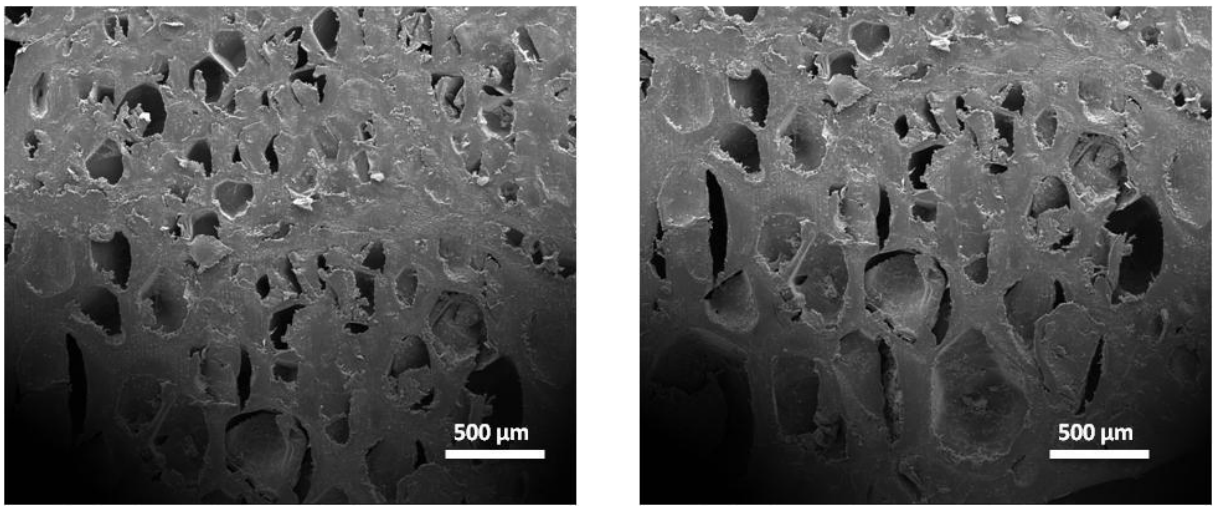

Figure 6: SEM images for PLA scaffolds processed with sCO_2