MASTER THESIS

Master's degree in Chemical Engineering

ANTIBACTERIAL SCAFFOLDS CONSTITUTED BY TRILAYERS OF MICROFIBERS BASED ON POLYLACTIDE AND POLY (3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE)

Report

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RESUM

L'objectiu d'aquest treball final de màster va ser la fabricació de fibres poroses uniaxials i amb tricapa que presentés funcions antibacterianes i potencialment d'altres funcionalitats (antitumoral, antioxidant, antibaterial...). Les fibres es van fabricar mitjançant la tècnica de l'electrospinning a partir d'una dissolució de polímers i sempre treballant amb un polímer comercial, el PHB. El disseny de les matrius va consistir en una capa de mescla PLA/PEG als extrems i PHBV al mig, amb configuració similar a un tipus sandvitx. La droga antibacterial es va incorporar també a la capa intermitja, mesclada amb el PHBV. A més, van afegir també partícules d'hidroxiapatita per formar complexes Matriu-Hidroxiapatita i així poder estudiar la alliberació de droga.

Per tal d'obtenir les matrius amb les condicions òptimes, es van modificar els paràmetres durant l'electrospinning de fibres amb una sola capa i es van provar diferents concentracions i diferents dissolvents. Un cop obtinguts els valors òptims dels paràmetres i el dissolvent adequat, es va procedir a crear diferent matrius amb diferents característiques i components per el seu posterior estudi. En el desenvolupament del projecte es van dur a terme diferents caracteritzacions, tant físiques com químiques de les matrius creades.

Primer es va estudiar els angles de contacte, en funció del comportament de l'aigua al estar en contacte amb la superfície de les matrius. Tots els resultats obtinguts van ser superiors a 120º per tant, es va considerar el polímer estudiat era de tipus hidrofòbic, ja que presentava valors superiors al 90º. Posteriorment, es van analitzar els diàmetres de les fibres que s'obtenien a les matrius mitjançant microscòpia electrònica de rastreig (SEM) i també es va obtenir l'espectre de cadascuna dels components que es van utilitzar per muntar les matrius (per espectroscòpia infraroig) per tal d'obtenir les absorcions dels components que formaven part d'elles.

Per últim es va realitzar un estudi de l'alliberació de la droga, dissolent una mostra de les matrius que contenien la droga (per triplicat) amb un medi utilitzat per creixement biològic (PBS). L'estudi es va realitzar en un temps total de 48h, realitzant mostreigs periòdics i finalment, analitzant les absorbàncies obtingudes mitjançant l'espectrometria UV-Vis per obtenir concentracions finals i amb les seves regressions, obtenir dades sobre l'alliberació en el temps de la droga.

El treball realitzat al laboratori inclou el muntatge experimental, la preparació de les dissolucions de polímers per dur a terme cadascuna de les tècniques i la realització dels experiments mitjançant cadascuna de les tècniques mencionades. Finalment, a partir de les diferents proves realitzades es va poder determinar la naturalesa del polímer emprat, la seva morfologia, el seu comportament químic així com la capacitat d'alliberament de droga que presenta vers el temps.

RESUMEN

El objetivo de este trabajo final de máster fue la fabricación de fibras porosas uniaxiales y con tricapa que presentase funciones antibacterianas y potencialmente otras funcionalidades (antitumoral, antioxidante, antibacterianas...). Las fibras se fabricaron mediante la técnica de la electrospinning a partir de una disolución de polímeros y siempre trabajando con un polímero comercial, el PHB. El diseño de las matrices consistió en una capa de mezcla PLA/PEG en los extremos y PHBV en medio, con configuración similar a un tipo sándwich. La droga antibacteriana se incorporó también a la capa intermedia, mezclada con el PHBV. Además, se añadieron también partículas de hidroxiapatita para formar complejos Matriz-Hidroxiapatita y así poder estudiar la liberación de droga.

Para obtener las matrices con las condiciones óptimas, se modificaron los parámetros durante el electrospinning de fibras con una sola capa y se probaron diferentes concentraciones y diferentes disolventes. Una vez obtenidos los valores óptimos de los parámetros y el disolvente adecuado, se procedió a crear diferentes matrices con diferentes características y componentes para su posterior estudio. En el desarrollo del proyecto se llevaron a cabo diferentes caracterizaciones, tanto físicas como químicas de las matrices creadas.

Primero se estudió los ángulos de contacto, en función del comportamiento del agua al estar en contacto con la superficie de las matrices. Todos los resultados obtenidos fueron superiores a 120° por lo tanto, se consideró que el polímero estudiado era de tipo hidrofóbico, puesto que presentaba valores superiores al 90°. Posteriormente, se analizaron los diámetros de las fibras que se obtenían mediante microscopia electrónica de rastreo (SEM) y también se obtuvo el espectro de cada uno de los componentes que se utilizaron para montar las matrices (por espectroscopia infrarrojo) para obtener las absorciones de los componentes que formaban parte de ellas.

Por último, se realizó un estudio de la liberación de la droga, disolviendo una muestra de las matrices que contenían la droga (por triplicado) con un medio utilizado para crecimiento biológico (PBS). El estudio se realizó durante un tiempo total de 48h, realizando muestreos periódicos y finalmente, analizando las absorbancias obtenidas mediante la espectrometría UV-Vis, para obtener concentraciones finales y con sus regresiones, obtener datos sobre la liberación de la droga en el tiempo.

El trabajo realizado en el laboratorio incluye el montaje experimental, la preparación de las disoluciones de polímeros para llevar a cabo cada una de las técnicas y la realización de los experimentos mediante cada una de las técnicas mencionadas. Finalmente, a partir de las diferentes pruebas realizadas se pudo determinar la naturaleza del polímero empleado, su morfología, su comportamiento químico, así como la capacidad de liberación de droga presentaba respecto al tiempo.

ABSTRACT

The objective of this final master thesis was the fabrication of porous uniaxial trilayer fibers that present antibacterial functions and other potential functionalities (antitumoral, antioxidant, antibacterial...). Fibers were made by means of electrospinning technique, from a polymer solution and aways working with a commercial polymer type, PHB. Scaffolds design consists of a mixture PLA/PEG applied in a layer on extremes and PHBV at the middle, with a sandwich seems configuration. Antibacterial drug was added to intermediate layer, mixed with PHBV. Additionally, it was added particles of hydroxyapatite to form Scaffold-Hydroxyapatite complexes and allow to study drug liberation.

To obtain scaffolds in optimum conditions, it was modified the parameters during electrospinning process with unilayer scaffolds and there were tested different concentrations and solvents. Once optimal parameters were obtained and the suitable solvent, different scaffolds were created with different characteristics and components for its posterior study. During the development of the project, different physical and chemical characterizations were made on the created polymer scaffolds.

Firstly, contact angle of the scaffolds was evaluated in function of water behavior been in contact with surface scaffold. All angles obtained were higher than 120º and, for so, it was considered that the polymer analyzed was hydrophobic, as it has values higher than 90º. Subsequently, it was analyzed the diameters of fibers by means of scanning electron microscopy (SEM) and spectra were obtained for each of the components that were used to make the scaffolds (by FTIR spectroscopy) and obtain the absorbances of the components that be part of them.

Finally, it was made a liberation of the drug study, dissolving a sample of the scaffold that contains the drug per triplicated, with a biological grown media (PBS). The study was done during a total time of 48h, making periodical samplings and, finally, analyzing obtained absorbances by means of UV-Vis spectroscopy, to obtain the final concentrations and its regressions, obtaining data about drug release pending time.

The laboratory tasks made, include experimental setup, preparation of polymer solutions to carry out each of the techniques and the perform of the experiments by means of all the mentioned techniques.

Finally, from the different tests performed, it was possible to stablish the nature of the polymer used, its morphology, chemical behavior, and its capacity to drug release in time.

ACKNOWLEDGEMENTS

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GLOSSARY

PHA – Polyhidroxialcanoates

PHB – Polyhidroxibutyrate

PHBV – Polyhidroxibutyrate – valerate

PP – Polypropylene

PE – Polyethylene

PLA – Polylactic acid

FTIR – Infrared spectroscopy

SEM – Scanning Electron Spectroscopy

NMR – Nuclear magnetic resonance

HAP- Hydroxyapatite

PBS – Phosphate buffered saline

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1. CHAPTER 1. INTRODUCTION

1.1. Aim of the project

The main objective of this project was the fabrication of scaffolds by porous uniaxial trilayer fibers with antibacterial functions using electrospinning technique by means of polymer solution blend with a solvent suitable for the technique.

In this project, two different types of scaffolds have been prepared (unilayer and trilayer) to understand its morphology and physical and chemical behavior, as well as its properties when other components are added, such as antibacterial drugs.

1.2. Scope of the project

To achieve the general objective of scaffold fabrication, other objectives are defined:

- Obtain the most suitable parameters for fiber fabrication
- Scaffolds characterizations by means of optical microscopy and electronic microscopy
- Drug release study among time
- Trilayer scaffolds behavior

1.3. Motivation of the project

Electrospinning technique is a widely used technique to create nowadays tissue scaffolds in medicine or other outstanding applications. Motivation of the project come from the interest of understand the technique and to understand all the information that can be extracted from its creation.

2. CHAPTER 2. THEORETICAL BACKGROUND

2.1. Polyhidroxialcanoates (PHA)

Polyhidroxialcanoates are a family of biodegradable and biocompatible polyesters, that are generated via fermentation processes at different scales. PHA are completely biosynthesized, and bio polymerized, summing up to over 150 monomer variations, as the most common monomers can be used to form homopolymers, random copolymers or block copolymers.

PHA production is fractioned in different stages, as strain develop laboratory, and pilot fermenter studies and finally, all these trials are scaled up in industrial production.

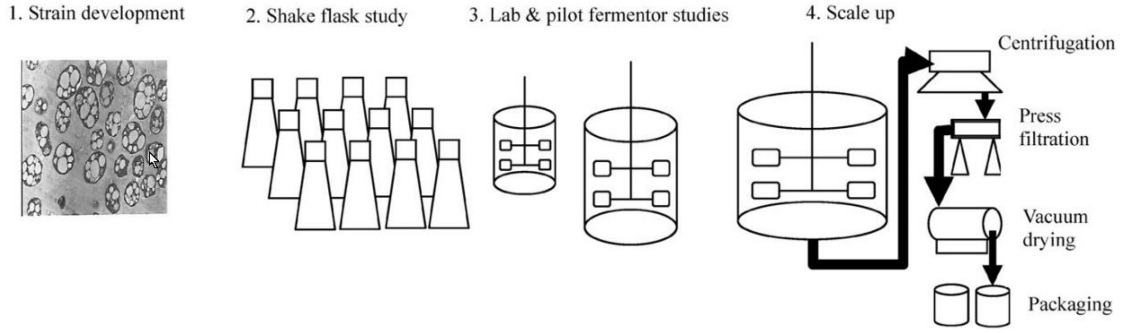

Figure 1. Process development for industrial production of PHA. Source: [1]

Most commercial PHA polymers are poly hydroxybutyrate and poly hydroxybutyrate. In terms of prices, PHA's are less competitive than common low-cost petroleum-based plastics, as the very limiting market. However, PHA have been considered environmentally friendly bioplastics both in production and processing of the polymers.

General applications of PHA polymers cover a huge range in different industries and fields, thanks to its great features.

Table 1. Applications of polyhidroxialcanoates. Source: [1]

Applications	Examples
Packaging industry	Packaging that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances…
Printing	Heat sensitive adhesives, latex, smart gels.
Plastic processing	Processing aids for plastic processing.
Textile industry	Like nylons, PHA can be processed into fibers.
Fine chemical industry	Can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals.
Medical implant biomaterials	As PHA have great biocompatibility and can be developed into medical implant materials.
Medical	Therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement.

Healthy food additives	PHA can be used as food supplements to obtain ketone bodies
Industrial microbiology	PHA used as a metabolic regulator or resistance enhancer to improve performances of industrial microbial strains.

Degradation of the PHA's (changes in its chemical structure) is principally biological, but the most common ones are:

- Thermal degradation: by means of the temperature
- Hydrolytic degradation: by means of the water contact
- Photodegradation: by means of solar light
- Biodegradation: by means of microorganisms

2.1.1. Poly-hydroxybutirate (P3HB)

PHB was the first isolated and characterized PHA. It's a highly crystalline polymer due to its linear chain structure and can contain both amorphous and crystalline phases.

PHB present similar characteristics than PP and PE, but it's typically more stiffer and brittle, with low thermal stability and high degree of crystallinity. Also its mechanical properties can be easily modified to obtain different applications.

The main applications of the poly-hydroxybutyrate are in pharmaceutical and chemical industries because it's high degradability and packaging applications. Biomedical applications have also high importance as its high biocompatibility with human tissues (can be used as suture thread, pericardial substitutes, or drug delivery systems).

Some limitations with PHB are present nowadays, as the high costs of production, low yield, high susceptibility of degradation and extraction difficulties.

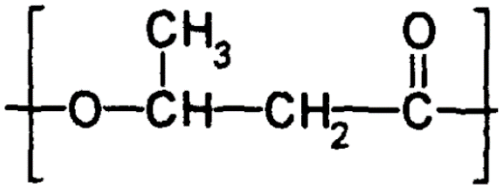

Figure 2. PHB structure

2.1.2. Polyhydroxibutirate-valerate (PHBV)

PHBV is considered a copolymer of the poly-hydroxybutyrate, also obtained by biomechanical means and biodegradable, biocompatible and renewable. The structure of the PHBV depends on the relationship between the carbon and oxygen atoms, acquiring high levels of crystallinity and a hydrophobic behavior and high resistance to organic solvents.

PHBV is produced following a controlled bacteria growing by means of simple substrates. That makes a loss of crystallinity of the PHBV regarding the PHB and an enhance in its processability and flexibility.

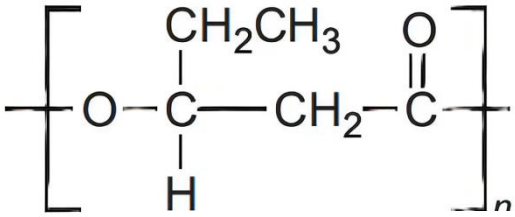

Figure 3. PHBV structure

2.2. Electrospinning

Electrospinning is a simple and robust technique that consists in electrostatic fiber formation which utilizes electrical forces to produce polymer fibers with diameters from 2nm to several micrometers, using polymer solutions that can be either natural or synthetic origin. This technique is the most effective one to produce novel nanofibers with controlled pore structure and specifications. [1]

Electro spun fibers usually present smaller pores and higher surface area than regular non-electro spun fibers and that have allowed them to be applied in different fields, such as nano catalysis, pharmaceutical and chemical field, biotechnology, and tissue engineering scaffolds.

2.2.1. Advantages and disadvantages of electrospinning technique

Electrospinning started to be used almost 60 years ago but, in recent years it has been an increase of interests for exploiting this technique. Although is a very widely used technique and have a lot of advantages, it also presents some disadvantages.

Advantages

- Simple process
- Controllable micro/nano-sized fibers
- Tunable properties and functionality (as porosity, malleability and in consequence, shape, and size of the fibers)
- High surface-to-volume ratio

Disadvantages

- Use of toxic solvents as chloroform or dichloromethane
- Higher fiber diameter distribution

2.2.2. Applications of electrospinning

Fibers obtained by electrospinning can be used in different applications as they provide several advantages as high surface to volume ratio, high porosity, and enhanced physic-mechanical properties as manipulation of the process parameters, that allow to obtain the desired morphology of the fibers.

Most common uses of electro spun fibers are the following [2]:

- Biomedical applications as almost all human tissues are deposited in nanofibrous forms or structures (bones, skin, dentin, collagen, cartilage), is easy for electrospun fibers to be

introduced in different biomedical areas, such as drug delivery, tissue engineering, wound dressings or even cosmetics.

- Functional materials and devices to reinforce the structures of the different commercial materials. Most common applications include the composite reinforcement, enhancement of commercial filters or even protective clothing and smart textiles creations.
- Energy and electronics Electrospun fibers have been widely used in new sustainable devices to generate energy showing an outstanding potential being applied in supercapacitors, lithium cells, fuel cells, transistors…
- Sensors electrospun fibers are applied in different kind of sensors as thermal, biochemical, optical, or chemical as fibers offer high surface area and porous structure and this makes them appliable for sensitive and fast sensing.
- Catalyst as electrospun fibers can be considered to provide an attractive solid support for enzymes and other catalysts. Also, this type of catalysts can offer other advantages, such as feasibility of adapting to any geometry or low resistance to the flow of liquid or gases.

2.2.3. Polymers used in electrospinning

A wide range of polymers are used in electrospinning process, but they must be able to form nanofibers. Electrospun fibers can be created with polymers that are synthetic, natural or a blend of both types. These polymers also can include proteins, nucleic acids, drugs, and polysaccharides in the solution. [6]

- Natural polymers. Generally, exhibit better biocompatibility and low immunogenicity. Some examples can be collagen, chitosan, gelatin, casein, silk protein...
- Synthetic polymers. Sometimes offer many advantages over natural polymers as can be tunned some of their mechanical properties. Typical synthetic polymers are polycaprolactone (PCL), polylactic acid (PLA), polyurethane (PU)…
- Copolymers. Copolymers enhance the polymeric materials, for instance it's thermal stability, mechanical strength, barrier properties… Electrospun fibers of copolymers generate new materials of desirable properties. The most common copolymers used in electrospinning technique are poly methyl methacrylate (PMMA), ethylene-co-vinyl alcohol (PEVA)…

2.2.4. Solvents used for electrospinning

Solvents used in electrospinning technique has relevant importance as it's a significant element of the solution and has relevant influence on the spinnability. [6][3]

Principal characteristics of the solvents is that they need to have good volatility, vapor pressure, boiling point and have to maintain the integrity of the polymer solution. All these characteristics have significant importance in electrospinning process, for instance, good volatility is important in terms of rapid solvent evaporation, that plays a significant role in the formation of nanostructures. Solvent vapor pressure it's also important as determine the evaporation rate and the drying time.

Table 2. Most common used solvents in electrospinning and it's properties. [3]

Solvents	Surface tension (mN/m)	Dielectric constant	Boiling point (ºC)	Density (g/ml)
Chloroform	26.5	4.8	61.6	1.498
Dimethyl formamide	37.1	38.3	153	0.994
Hexafloruro isopropanol	16.1	16.7	58.2	1.596
Trifloruro ethanol	26.4	7.5	66	0.886
Acetone	21.1	27	78	1.393
Water	25.20	21	56.1	0.786
Methanol	72.8	80	100	1.000
Acetic acid	22.3	33	64.5	0.791
Formic acid	26.9	6.2	118.1	1.049
Dicholorometane	37	58	100	1.21
Ethanol	27.2	9.1	40	1.326
Trifloruro acetic acid	21.9	24	78.3	0.789

2.2.5. Electrospinning process

Electrospinning process consists of a spinning technique, using electrostatic forces to produce fine fibers from different polymer solutions. These fibers are characterized by having thin diameters (from nano to micro scale) and high surface area.

Generally, electrospinning is conducted at room temperature with atmospheric conditions. The system setup is very simple, it only consists in three major components [4]:

- High voltage power supply. It is needed a DC voltage connected to the system to provide the necessary current to carry out electrospinning process. The range of this voltage is from 0 to 80 kW approximately.
- Dosification pump. A dosification pump is necessary to set the rate at which the solution is ejected and the volume of solution that is inside the syringe.
- Syringe with a needle tip. There is necessary a syringe to charge the solution quantity desired to be electro spun and a metallic needle with blunt tip for the correct dosification of the solution. The needle is connected to the current.
- Collecting plate. The plate is usually in metal where is connected also the current. This collector must present conductive properties.

The setup can be either horizontal or vertical one, depending on which position the syringe is placed.

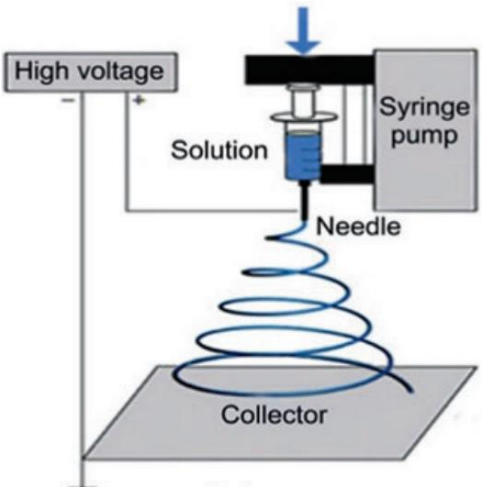

Figure 4. Schematic view of downward electrospinning setup. Source: [18]

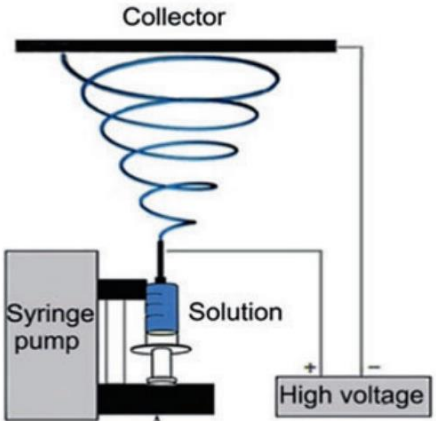

Figure 5. Schematic view of upward electrospinning setup. Source: [18]

In the electrospinning process, the polymer solution held by its own surface tension is subjected to the electric field generated and an electric charge then is induced to the liquid surface of the solution due the electrical field. This electrostatic force has been regarded as the driving force that is necessary to initiate the electrospinning force.

Then, when the electrostatic field generated achieves the critical voltage (V_c) the balance of repulsive forces is broken, and a charged jet of the polymer solution is ejected from the tip of the needle, where a Taylor cone is forming, and a whipping of the jet occurs in the space between the collecting plate and the tip of the needle, which leads to an evaporation of the solvent, leaving the polymer behind, spreading on the collecting plate. The jet is only stable at the tip of the needle.

The critical voltage for electrospinning is given by the following expression:

$$V_c{}^2 = 4\frac{H^2}{h^2}\left(\ln\left(\frac{2h}{R}\right) - 1.5\right)(1.3\pi R\gamma)(0.09)\ [18]$$

Where H (cm) is the distance from the spinneret tip to the collector, h(cm) is the length of the liquid column, R (cm) is the inner radius of the spinneret and γ $(\frac{dyn}{cm})$ is the surface tension of the spinning solution. The factor 0.09 is indicated to predict the voltage.

2.2.6. Parameters that affect electrospinning

There are different parameters that can affect electrospinning results, despite it's an easy and affordable technique. Depending on the element of the setup it can be differenced [3]:

- **Environment parameters**: humidity and temperature.
- **Solution parameters**: Concentration, conductivity, viscosity, molecular weight, solvent volatility, molecular structure, surface tension.
- **Electrospinning parameters** (process): Distance, voltage, flowrate, collector.

Each of these parameters, affect significantly to the fiber's morphology obtained in electrospinning process and, the proper manipulation of those can led to the obtention of the desired morphology of the fibers as a result of the electrospinning, for example, in form of nanofibers with desired diameter and morphology.

2.2.6.1. Concentration

In electrospinning technique, for fiber formation, a minimum solution concentration is required. At low concentrations, a mixture of beads and fibers is formed and as the concentration rises, a uniform bundle of fibers is formed as the diameters increase as well, thanks to a higher viscosity resistance. For determining the right concentration, it has to be taken into account the solution surface tension and the viscosity of the solution.

There exists some relationship between the solution concentration and the fiber diameter, as increasing the concentration of the solution also increases the fiber diameter.

2.2.6.2. Molecular weight

Molecular weight reflects the number of entanglements of the polymer chains of the solution, playing an important role in the process of electrospinning. This parameter has significant effect on rheological and electrical properties of the polymers, such as the viscosity, surface tension, conductivity, and dielectric strength. High molecular weight solutions give fibers with larger average diameter and are more suitable for electrospinning, as they provide the desired viscosity for fiber generation.

However, high molecular weights are not always essential if intermolecular polymer interactions can provide a substitute of interchain connectivity through chain entanglements.

2.2.6.3. Viscosity

Viscosity plays a fundamental role in determining the fiber size and the morphology of the fibers during the spinning process. At very low viscosity values, there is no continuous fiber formation and, otherwise, at high viscosity values, there exists some difficulties to eject the jets of the solution. For this issue, it is important to find the optimal viscosity of the polymer for the electrospinning process. This parameter also plays an important role in determining the range of concentrations form which the fibers can be obtained.

Viscosity, polymer concentration and molecular weight of the polymers are correlated to each other, as viscosity is strongly related with the concentration of the solution.

An increase in solution viscosity or concentration, give a rise to a larger and more uniform fiber diameter.

2.2.6.4. Surface tension

Surface tension is in relation with solvent composition and helps the fibers obtained be more uniform, determining the upper and lower boundaries of the electrospinning window if the other variables are constant. High surface tension tends to inhibit the electrospinning process due the instability of the jets.

2.2.6.5. Conductivity

Solution conductivity depends mainly on the polymer type, the solvent used and the availability of ionizable salts. If conductivity increases, there is a significant decrease of the diameter of the fibers, whereas at low conductivity of the solutions, results to an insufficient elongation of a jet.
Nanofibers with small diameters can be obtained with high electrical conductivities, and the jet radius varied inversely with the cube root of electrical conductivity of the solution.

2.2.6.6. Applied voltage

Applied voltage is a critical parameter in electrospinning process. When higher voltages are applied, greater stretching of the fibers is produced due to the strong electric field. Also, increasing the applied voltage the fiber diameter tends to decrease, the level of significance of the applied voltage effect depends also on fiber diameter, polymer solution concentration and the distance between the tip and the collector.

2.2.6.7. Flowrate

The flowrate of the polymer through the syringe is an important parameter as it influences the jet velocity and the material transfer rate. Lower feed rate is desirable as the solvent has enough time for evaporation until it reaches the collection plate, but it's important to always have a minimum flow. On the other hand, high flow rates don't allow to proper drying the solvent prior to reach the collector, and beaded fibers can be obtained.

2.2.6.8. Tip to collector distance

For obtaining the correct morphology, a minimum distance to the collector is required to give the fibers sufficient time to dry before reaching the collector, otherwise beads can be observed. However, the effect of the tip to collector distance is not as significant as other parameters but it's necessary to find the optimum distance between the tip and collector that favor the evaporation of solvent from the nanofibers.

2.2.6.9. Ambient parameters

Ambient parameters include principally humidity and temperature. There is an inverse relationship between viscosity and temperature. At very low humidity, volatile solvents dry rapidly as the evaporation of the solvent is faster while high values of humidity can help the discharge of the electrospinning fibers.

2.2.7. Electrospinning characterizations

Characterization of electrospinning fibers is one of the most difficult tasks as the probability to get single fibers is so infrequent. Different type of characterizations is carried out, generally in fields of physical or structural, mechanical, and chemical characterizations.

2.2.7.1. Geometrical characterizations

Geometrical characterizations are related with nanofibers internal structure, that determine its physical and mechanical properties. The most important physical characterizations of the nanofibers include fiber diameter, diameter distribution, fiber orientations, fiber morphology, porosity (determining pore diameters) …

Most important techniques for geometrical characterizations are scanning electron microscopy (SEM), field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM).

2.2.7.2. Chemical characterizations

Chemical characterizations are related with the study of the inter molecular interactions and the structure that can be stablished when two polymers are blended for the fabrication of nanofibers. For this issue, molecular weight of the polymers, composition, bonding or even repeating unit information is necessary to be known to fully understand chemical characterization results.

Most important techniques for chemical characterizations are Fourier transform infra-red (FTIR) and nuclear magnetic resonance (NRM).

2.2.7.3. Mechanical characterizations

Mechanical characterizations are related with physical properties of the fibers and its behavior and strength. The most important parameters controlled by mechanical characterizations are the determination of fiber diameter, tensile strength of the fibers, or viscoelasticity properties. It is important to avoid damages during fiber or scaffold samples manipulation or even mounting the nanofibers, as they are very sensitive to changes.

Most important mechanical characterizations include nanoindentation, bending tests, resonance frequency measurements and microscale tension tests.

2.3. Scaffolds characterization

2.3.1. FTIR spectroscopy [8]

Fourier Transform Infrared Spectroscopy (FTIR) is a wide known technique that is used in areas of determination of molecular structure, identification of chemical species (quantitative or qualitatively) and other properties.

This technique is useful to determine the changes in molecular vibrations within the bonds that form the molecule. It works by the examination of the infrared radiation that absorbs a sample.

As each chemical compound has a characteristic set of absorption bands, this technique is very useful to distinguish between them.

FTIR can analyze samples either in aqueous solution, in non-aqueous solution and in dry or powder state. Its instrumentation is relatively economic and the software to perform the technique is widely developed.

Nowadays FTIR spectroscopy is widely used in biopharmaceutical applications as a qualitative tool but can be also used as quantitative analysis for structural components in proteins.

2.3.2. UV-VIS spectroscopy

Ultraviolet Visible spectroscopy is very important for the determination of the product characteristics and concentrations. These concentrations are obtained quantitatively in a solution according to Beer-Lambert law:

$$A = \mathrm{Log}_{10}(I_0/I) = \varepsilon c L$$

Where A measures the absorbance I0 is the intensity of the incident light at a given wavelength, I is the transmitted intensity, L is the path length through the sample, c is the concentration of the absorbing species, and ε is a constant known as the molar absorptivity or extinction coefficient for each species and wavelength.

The complete UV-Vis spectrum can provide a lot of information about a molecule, by means of scanning through UV-Vis wavelengths (comprised between 190 – 400 nm) into the visible spectra region (400-700 nm). The spectra obtained, can be used to determine the maximum absorption at a particular wavelength or give an approximate profile of a particular sample.

2.3.3. Contact angle

Contact angle is known as the analysis of the wetting of a solid by a liquid. The wettability interferes in the behaviour of the solid, to form a common interface with the liquid that is in contact with the surface.

Contact angle allows us to know the surface tension of the liquid and the surface free energy of the solid. The three-phase system (solid, liquid and gas) is regulated by the Young's equation. This equation allows to calculate the energy that is generated between liquid and solid phases and also to classify the nature of the surface, that means to classify them either if they are hydrophilic or hydrophobic.

2.3.4. SEM [9]

Scanning electron microscopy (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid elements. Signals that derive from electron-sample interactions reveal information about the sample that is analyzed, as the external morphology (for example, it's texture), chemical composition, or the crystalline structure and orientation of the materials that make up the sample.

SEM can both perform analysis of the whole sample and a selected point location on the sample, specially when qualitatively and semi-quantitatively determination of chemical compositions is required.

Areas from 1cm to 5 microns in width can be imaged in a scanning mode using conventional SEM (ranging from 20X to 30000X of magnifications and spatial resolution of 50 to 100nm).

2.3.5. Nuclear Magnetic Resonance (NMR) [10]

Nuclear Magnetic Resonance has been used to characterize condensed matter. This technique studies principally relaxation studies of kinetic processes and gives a three-dimensional imaging to probe properties of gases, liquids, and solids.

It can also be used to study porous media, focusing on measurements of the fluids in the pore space.

The principle of Nuclear Magnetic Resonance is based in the principle of the magnetic shielding interaction, that gives rise to the perturbation of the energy levels which yield frequency shifts related the specific electronic and chemical environment of the nucleus.

Drug delivery system consists in the method or the process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals.

In terms of drug delivery systems, polymer scaffolds are known as a platform able to load active compounds and that releases them in a target tissue to enhance the efficiency of the drug.

Usually, scaffolds are implanted on a site-specific bases, with a controlled spatial-temporal release of the active compound that contains. They also show ideal conditions for cell attachment and proliferation of microbials as the physical bonds between the biological parts and the scaffolds lead an integration of the tissue.

In addition, scaffolds can provide drug protection in the body before the release and that prevent side effects during a controlled release and fluctuations of drug levels on administration.

2.3.6. Drug release

2.3.6.1. Drug and polymers [11]

For drug delivery systems, the drug and the polymer must be conjugated in a covalent manner. This way of bonding has advantages over drug-loaded nano and microparticles, as it allows a precise control of the quantity of the incorporated drug. Also, provides strong stability, preventing drug aggregation, quenching the burst release, and avoiding drug loss at sterilization and purification of the scaffolds. [14]

Most common chemical conjugation between drug and polymers involve process where esterification, oxidation and amination reactions are present. The most common way to perform this process is by means of blending.

Drugs can also be introduced into polymers preloading the drug into nanoparticles in order to embedding them later into the polymer scaffolds. This method consists in loading the drug in the polymers and then confining in within the scaffold, once the polymer is well homogenised in the adequate solvent. Drug particles have to be added to polymer solution under stirring until complete dispersion.

Finally, another types of drug encapsulation are soaking-load and drug-polymer conjugation. Both consist in a heterogeneous distribution of the dug into the matrix and generally the behaviour of the drug and its release is controlled.

2.3.6.2. Drug release process

Kinetics of drug delivery process from polymer scaffolds is governed by desorption, dissolution, diffusion of the drugs or, in the other hand, erosion, swelling and degradation of the matrix.

Drug release rates can be tuned with different parameters, as methods of drug encapsulation, nature of the polymer, method of the scaffold preparation, degree of the network of the scaffold, multiple layers… [13]

The drug-matrix interaction has an important role in the drug release process. Polymer and drug molecules can interact at physical and chemical levels by Van der Waals interactions, hydrogen bonds, electrostatic interactions, and covalent bonds.

Regarding physical interactions, when scaffolds present pores at its surface, they intensify bulk drug release due to water infiltration. Additionally, if functional groups of association with the drug are added, it can decrease the release rate of the drug.

On the other hand, chemical interactions comprise the cleavage of chemical groups of the drug and its kinetics. This groups, enable drug release in a way to enzymes, redox species, pH, light…

Another chemical way to release the drug is by the degradation of the matrix. The drug release rate will be in this case function of the nature of the polymer which degradation time vary from minutes to years.

3. CHAPTER 3: EXPERIMENTAL SECTION

3.1. Introduction to experimental part

Experimental part of the project was focused on the fabrication of uniaxial fiber scaffolds, constituted by poly(3-hydroxybutyrate) (P3HB) and, as the commercial polymer didn't render high efficiency, it was changed by commercial poly-hydroxybutyrate-valerate (PHBV). This uniaxial fiber scaffolds are intended to be tri layer, with superficial shells constituted by different ratios of polylactide (PLA) and polyethylene glycol (PEG). Once obtained the trilayer scaffolds, hydroxyapatite (Hap) nano capsules were incorporated to test the antibacterial capacity of the drug, in this case, streptomycin, that is known as an antibiotic used to treat a wide range of principally bacterial infections.

Different physical and chemical methods have been performed during the experimental part, as SEM (Scanning Electron Microscopy), UV-Vis spectroscopy, FTIR (Infrared spectroscopy) and some bioactivity tests.

3.2. Optimization of electrospinning parameters

For the obtention of the optimum parameter conditions, fibers need to be obtained and for so, electrospinning method was performed. This technique needs a polymer solution to be prepared in order to properly create the scaffolds.

Different polymers were tested at different concentrations with also different solvents to find the most suitable solution to evaluate the optimum parameters (voltage and flowrate principally). All solvents used must be stabilized with amylene.

Solution preparation was performed by dissolving solid polymers (previously weighted) in solvent. Once prepared, before use, solutions must be left overnight under light agitation to assure a correct dissolution of the polymer in the solvent.

First polymer tests were performed with P3HB (Polyhydroxy-butyrate). Composition of the commercial polymer was unknown.

Figure 6. Pellets of P3HB used in experimental part

Second polymer tested was PHBV (Polyhydroxy-butyrate valerate). It was used on the last tests to obtain trilayer scaffolds, along with a mixture of PLA (polylactic acid) and a sacrificial polymer, in this case, PEG (polyethylene glycol).

3.2.1. Solution preparation

First solutions were prepared for obtention of one-layer scaffolds, with the objective to determine the optimum concentration of polymer solution.

Solutions were prepared at 10%, 20%, 30% and 40% by weight, weighting 1, 2, 3 and 4g respectively in 10mL of solvent.

Solvents used to prepare the solutions where dichloromethane, chloroform and trichloroacetic acid.

Solutions prepared with chloroform need to be previously heated on a stove (that reaches approximately 75ºC) over 50 minutes and then, before using in electrospinning setup, need to be left in the agitator overnight at a 33ºC of temperature. Solution made with dichloromethane and chloroform were prepared the same way and trichloroacetic ones doesn't need to be heated previously in the stove, only agitation under 33ºC in the agitator. All solutions under these conditions were properly dissolved and ready to be used.

3.3. Electrospinning technique

Electrospinning technique was performed in the laboratory setup as it follows. Instrumentation consists in a needle pump, placed on the top of the box. This pump allows the user to select the parameters as the rate flow and the total volume that the syringe contains once the type of syringe used is defined (in this case, BD with 5mL capacity).

To start the tests for the best parameter conditions, volume selected in the pump was 5mL (top of the syringe) and 5mL/h as flowrate. Then, when first scaffolds were obtained, these parameters were changed higher or lower after microscopic observation of a scaffold sample.

The needle used in the electrospinning was a blunt needle and the collector plate was fixed at 14,5cm of the tip of the needle.

Humidity and temperature could be checked at the top of the column.

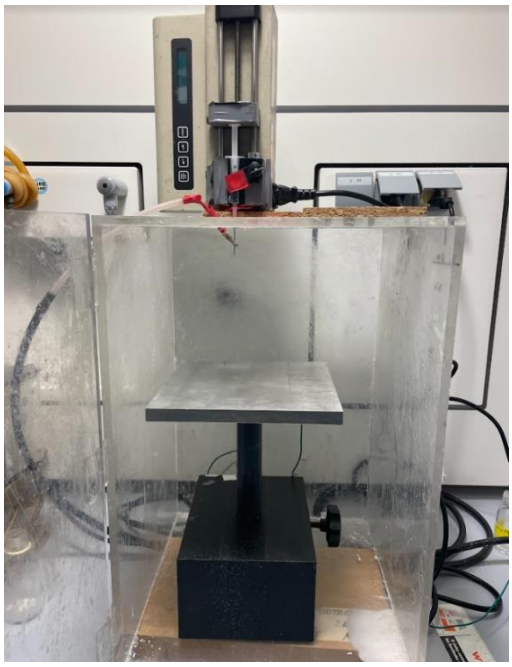

Figure 7. Setup configuration used to perform electrospinning technique

To start the testing, collection plate was covered with aluminium foil, for picking the scaffold once formed. Then, the syringe loaded with the polymer solution was placed on the pump and correctly adjusted to ensure it's locked. Then, parameters were selected in the pump and then, then wire that connects the voltage font was attached to the tip of the needle.

When setup is ready, pump was settled up and, when first drop of polymer solution fell from the syringe, voltage font was turned on and fibers begin to form.

Figure 8. Fibers formed in collector plate

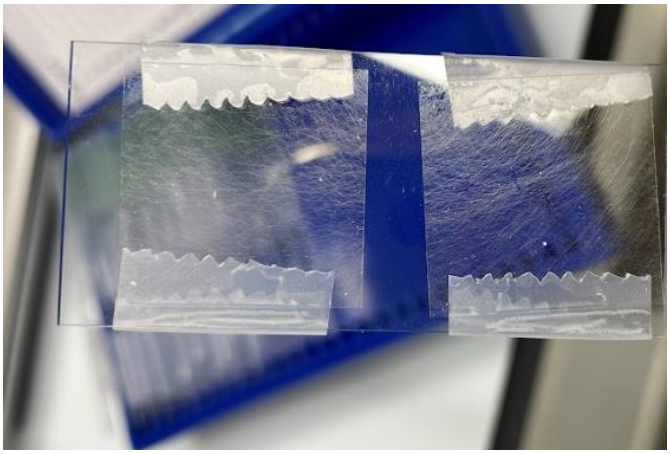

Figure 9. Samples of fibers collection

Once the sample of the fibers are collected, optical microscopy evaluation of them were necessary to decide which parameters were necessary to tune for enhancing the result of the scaffolds.
Both chloroform and dichloromethane were suitable solvents for fiber obtention, although characteristics of the fibers in terms of entangling and morphology present several appreciable differences.
Electrospinning technique was used to prepare either monolayer and multilayer scaffolds.

3.4. Contact angle

Contact angle measurement is used for evaluating the surface of a scaffold, in order to determine if it presents hydrophobic or hydrophilic characteristics. For experimental procedure it was used the drop shape analysis method, that is a technique based in loading a syringe with a chosen solvent and let the drop of the solvent falls into the scaffold surface to evaluate, by means of a software connected to the equipment, the behaviour of this drop in the surface and take a picture of it when drop reaches a baseline previously defined.
Measuring equipment presents a very simple configuration. It consists of a vertical column support to place a syringe where it will be placed the chosen liquid to put in contact with the scaffolds and a surface plate, where a small piece of scaffold is attached. Then, it is necessary to allow light pass through to start the measurements.
It is important prior to testing, to select wisely the solvent to use according to its viscosity, because as higher the viscosity, higher the contact angle. In this case, the chosen solvent was water. There are other parameters to consider, for instance, porosity of the sample or the chemical interaction of the sample and the solvent.
Equipment presents a very simple configuration. It consists of a vertical support where syringe is placed that allows drop by drop dosification of the solvent, a plate, were attached the sample of scaffold, and a light source, to allow see the drop falling from the syringe and be in contact with the scaffold in the connected software.

Figure 10. Contact angle equipment

Before starting the measurements, it is necessary to remove air bubbles that may present the syringe with the liquid and that the needle is shown in the software camera at the centre of the image and the drop focused. Then, it is necessary to select the commercial type of the syringe to adapt the force and accomplish the desired rate for drop to fall. Dosing rate was selected at 2uL/s with a maximum dosing volume of 0.6uL.

When dispensing of the drop takes place, a picture of it touching the surface is taken manually mode and a baseline is placed as a reference, because it allows to calculate the final volume of the drop and that explains how the solvent is evaporating.

As distribution is never homogeneous, it is necessary to perform more than one sampling to obtain the average value.

Contact angle test was performed only on monolayer scaffolds, as in trilayer scaffolds it will be only shown the most superficial layer.

3.5. Diameter obtention

For diameter obtention of the fibers that conform monolyer scaffolds it is necessary, prior to take the measures of the diameter, to analyse a sample of scaffold in SEM (Scanning Electron Microscopy) technique.

To scan the samples, they have to be previously treated with an automatic carbon coater, in order to apply a thin layer to the surface of the samples with a carbon coat to low it's conductive atomic number and allow the microscopy to obtain the images of the fibers, thanks to a signal that derives from electron-sample interactions within the microscope and the scaffold introduced. Once passed

through the microscope, the images reveal information about the morphology of the sample, it's texture and the orientation of the fibers and entanglements within them. Samples have to be taken quickly as the carbon layer can degrade and that implies that the SEM microscope is not able to give images of the fibers in the scaffolds.

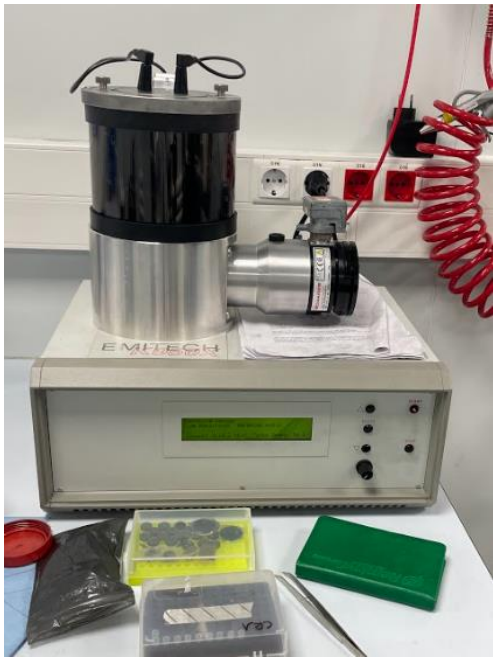

Figure 12. Automatic carbon coater used for preparing the samples before entering to the SEM microscopy.

Figure 11. Samples treated prior entering SEM microscopy. It can be appreciated the thin layer above consisting in carbon particles

Figure 13. SEM microscopy

Once obtained the images of the fibers in nano and microscale, these images were treated with the "Diameter J" software. This program allows to settle a representative scale with the image and to measure perpendicularly different fibers that appear in the image. As these measures need to be representative, it is necessary to measure minimum 50 fibers to determine the diameter range of the majority of them.

3.6. FTIR

For FTIR graphical obtention, it was used a spectrophotometer. The objective was to obtain the absorbance profiles of each sample analyzed in a wavelength range from 500 to 4000 cm-.

Samples of every multilayer and monolayer scaffolds were carefully folded to fit the sensor that allows the absorbance to be read. Graphical results of the FTIR method, allows to obtain the molecular structure of the materials used in the scaffolds and to see the predominance of the raw materials or the intermediate compounds.

The software linked to the equipment allows to recreate the profiles of the components with all the data collected. All intermediate linking and stretching zones are reflected on the absorbance peaks obtained.

3.7. Trilayer scaffolds obtention

Once the optimum conditions for a single PHB scaffold were obtained, single layer and trilayer scaffolds were mounted. All trilayer scaffolds were prepared with two external layers of a solution PLA/PEG (Polilactide/Polyethylene glycol), containing on the inside the respective layer mounted with PHBV and hydroxyapatite or streptomycin, if applies. PLA has to be in amorphous phase and the PEG selected was the one that have high molecular weight (35000g/mol). Chloroform was the selected polymer solvent for all the different scaffolds.

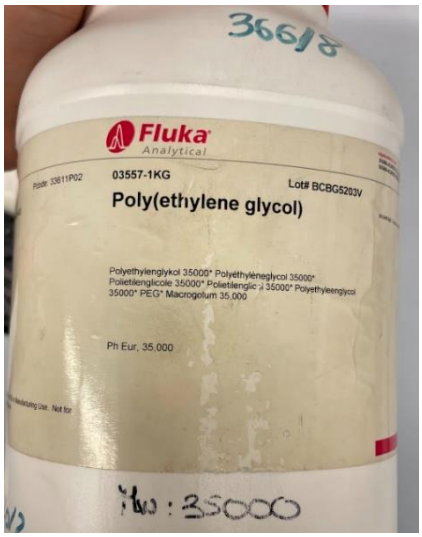

Figure 14. Polyetilene glycol used

Firstly, monolayer scaffolds were prepared. Monolayer scaffolds were used mainly for characterization purposes. The method of preparation and the quantities of each component are detailed below.

3.7.1. PLA-PEG scaffold

PLA-PEG scaffold solution preparation consisted of a mixture of 90% PLA and 10% PEG. The chosen solvent was chloroform, mixed with acetone at 3% by volume (proportion 3:1), to conform the solvent solution.

0,9 g of PLA (2002D) were weighted and dissolved in 7mL of solution and 0,1 of PEG (35.000 g/mol) were weighted and dissolved in 3 mL of solution.

Each solution was prepared separately, and it was left overnight. For mixing both solutions it was used a Vortex agitator once solutions were put together.

Once prepared, it was accumulated a total of 2 ml of solution at 5ml/h rate and 15kV of voltage during electrospinning process. The distance between the tip of the syringe and the collector was 15 cm.

3.7.2. PHBV scaffold

PHBV scaffold solution preparation consisted of a mixture of 10% w/v solution. The chosen solvent was chloroform, mixed with acetone at 3% by volume (proportion 3:1), to conform the solvent solution.

1g of PHBV was weighted and dissolved in 10mL of solvent solution. Then, the mixture was carried out at 50ºC to achieve homogeneous blending, and, before electrospinning process, it was added 1 mL of formic acid (mixed with the Vortex) to enhance and optimize the process.

Once prepared, it was accumulated a total of 2 ml of solution at 5ml/h rate and 15kV of voltage during electrospinning process. The distance between the tip of the syringe and the collector was 15 cm.

3.7.3. PHBV / HAP scaffold

PHBV/HAP scaffold solution preparation consisted of a mixture of 90% by weight of PHBV with 10% by weight of hydroxyapatite. The chosen solvent was chloroform, mixed with acetone at 3% by volume (proportion 3:1), to conform the solvent solution.

1 g of PHBV wea weighted and dissolved in 10 ml of solvent solution. Then, the mixture was carried out at 50ºC to achieve homogeneous blending, and 1 mL of formic acid was added. After mixing

with the Vortex the solution, 0.11g of hydroxyapatite were added. Before, electrospinning process, solution with hydroxyapatite was mixed again with the Vortex.

Once prepared, it was accumulated a total of 2 ml of solution at 5ml/h rate and 15kV of voltage during electrospinning process. The distance between the tip of the syringe and the collector was 15 cm.

3.7.4. PHBV /streptomycin scaffold

PHBV/Streptomycin scaffold solution preparation consisted of a mixture of 95% by weight of PHBV with 5% by weight of streptomycin. The chosen solvent was chloroform, mixed with acetone at 3% by volume (proportion 3:1), to conform the solvent solution.

1 g of PHBV wea weighted and dissolved in 10 ml of solvent solution. Then, the mixture was carried out at 50ºC to achieve homogeneous blending, and 1 mL of formic acid was added. After mixing with the Vortex the solution, 0.05263g of streptomycin were added. Before, electrospinning process, solution with hydroxyapatite was mixed again with the Vortex.

Once prepared, it was accumulated a total of 2 ml of solution at 5ml/h rate and 15kV of voltage during electrospinning process. The distance between the tip of the syringe and the collector was 15 cm.

3.7.5. PHBV /streptomycin/HAP scaffold

PHBV/Streptomycin/HAP scaffold solution preparation consisted of a mixture of 85% by weight of PHBV, 5% by weight of streptomycin and 10% by weight of hydroxyapatite. The chosen solvent was chloroform, mixed with acetone at 3% by volume (proportion 3:1), to conform the solvent solution.

1 g of PHBV wea weighted and dissolved in 10 ml of solvent solution. Then, the mixture was carried out at 50ºC to achieve homogeneous blending, and 1 mL of formic acid was added. After mixing with the Vortex the solution, 0.11g of hydroxyapatite were added and the solution was mixed again on the vortex. Then, 0.05263g of streptomycin were added and, before starting electrospinning process, solution with hydroxyapatite and streptomycin was mixed again with the Vortex.

Once prepared, it was accumulated a total of 2 ml of solution at 5ml/h rate and 15kV of voltage during electrospinning process. The distance between the tip of the syringe and the collector was 15 cm.

3.7.6. Multilayer scaffolds

For multilayer scaffolds construction there were constructed using layers of PLA and a combination of the different monolayer scaffolds prepared. For all the cases, 2mL was accumulated for each layer of the scaffolds and the electrospinning processes were the same for all the cases, 5ml/h rate

and 15kV of voltage during the process. The distance between the tip of the syringe and the collector was 15 cm.

In summary, multilayer scaffolds prepared were the following:

- PLA/PEG + PHBV + PLA/PEG
- PLA/PEG + PHBV + HAP + PLA/PEG
- PLA/PEG + PHBV+ Streptomycin + PLA/PEG
- PLA/PEG + PHBV+ Streptomycin +HAP+ PLA/PEG

All these multilayer scaffolds were used to study drug release capacities and to analyze their FTIR graphs.

4. CHAPTER 4: RESULTS AND DISCUSSION

4.1. Optimization of the fibers

Results for electrospinning technique result favourable for chloroform as a solvent. On the other hand, polymer solutions with trichloroacetic acid, although were uniformly dissolved, it didn't have the ability to form scaffolds, so it was rejected.

Also, solutions prepared at a 40% concentration, both for dichloromethane and chloroform were not suitable for electrospinning as it was so concentrated that the solution didn't pass the syringe and it was impossible to perform electrospinning.

Observation at different conditions analysed by electrospinning allow to determine the optimum conditions for scaffolds formation. Representative examples have been chosen to compare the different experimental conditions. All these tests were performed with optical microscopy, only with the intention to settle common parameters

First, it was compared the effect of the parameters if, at 30% of concentration, it remains constant the flowrate and only changes the voltage applied during electrospinning process. Microscope images were taken at 20x augmentations.

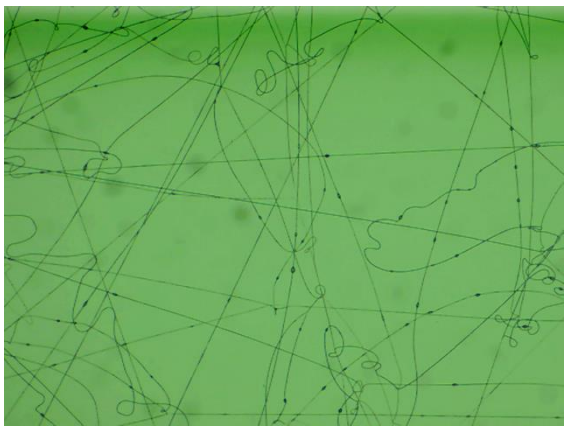

Figure 16. P3HB solved in chloroform at 30% 5ml/h and 15kV

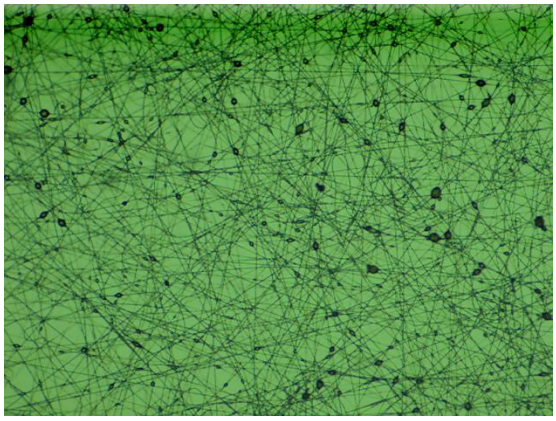

Figure 15. P3HB solved in chloroform at 30% 5ml/h and 30kV

As it can be observed, at less kV applied less entanglements appears in the fibers and less bits due to liquid trapped.

Then, it was tested the scaffold obtentions if it was maintained the concentration at 30% and, at the best voltage (15kV, obtained previously) it was changed the flowrate of the electrospinning technique. Microscope images were taken at 20x augmentations.

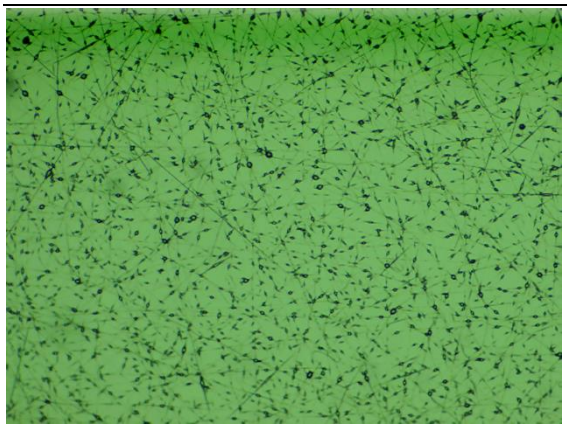

Figure 18. P3HB solved in chloroform at 30% 2ml/h and 15kV

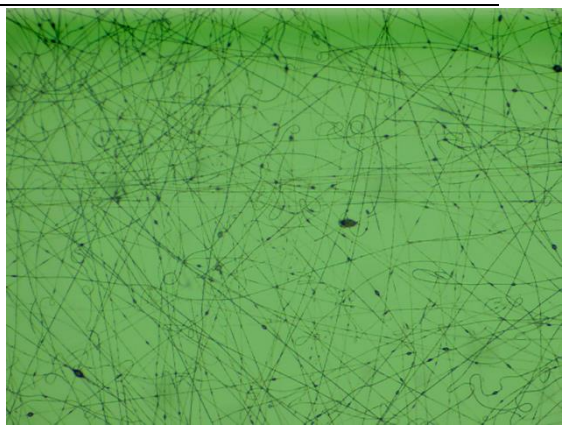

Figure 17. P3HB solved in chloroform at 30% 4ml/h and 15kV

As it can be observed, at less flowrate it starts to appear bits, as the solvent didn't evaporate correctly. So, higher rates are better for electrospinning technique.

As a conclusion, regarding the images obtained by optical microscopy of different parameter tunning while changing performing electrospinning technique, the parameters selected to construct monolayer and multilayer scaffolds where 5 ml/h and 15 kV.

4.2. Characterization of the fibers

4.2.1. SEM microscopy

SEM microscopy was useful to obtain the fiber images at microscale, regarding its morphology and determine from them the mean diameter of all these fibers. For SEM analysis only monolayerr scaffolds were taken into account as it is a fiber characterization technique and, as it only analyze the surface of the sample, only external layer of trilayer scaffold will have been analyzed.

Some images of the morphology of fibers obtained from the electronic microscopy are shown below.

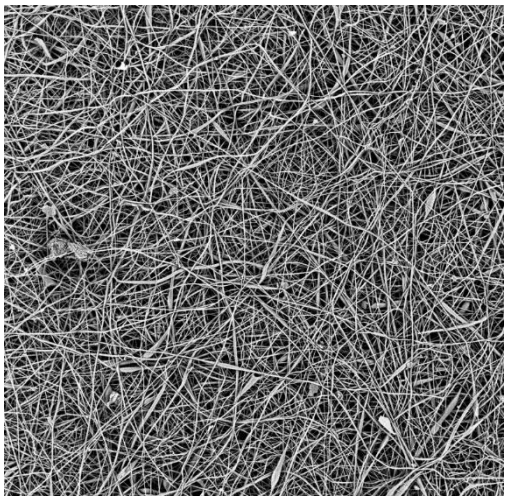

Figure 20. SEM image of PLA/PEEG scaffolds at x780 magnifications

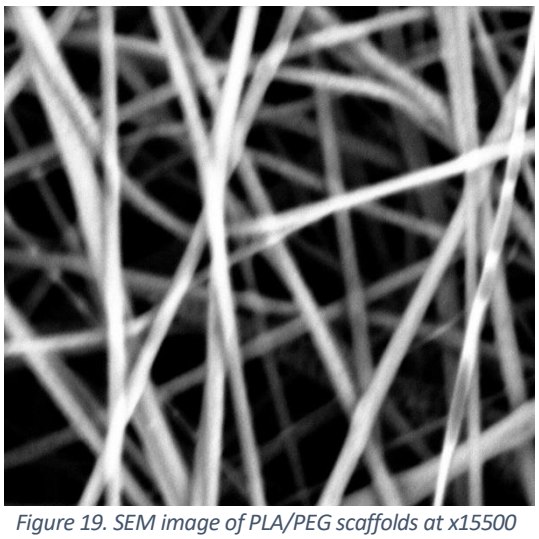

Figure 19. SEM image of PLA/PEG scaffolds at x15500 magnifications

It can be observed than PLA/PEG fibers are thin and smooth, and they are well ordered in the space. Magnitude of the fibers were in micro scale.

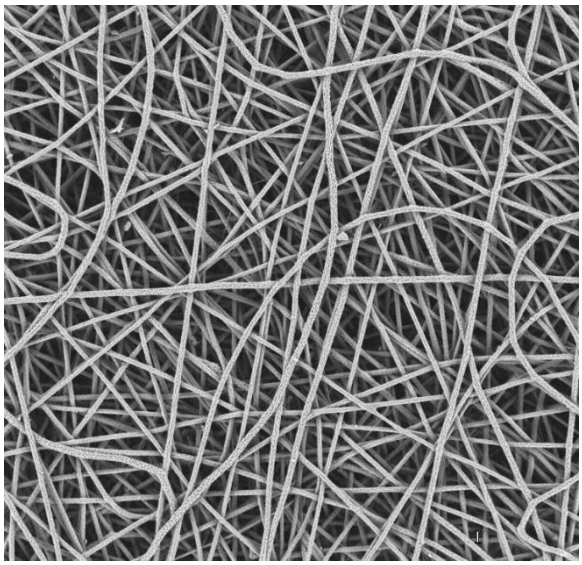

Figure 21. SEM image of PHBV scaffolds at x790 magnifications

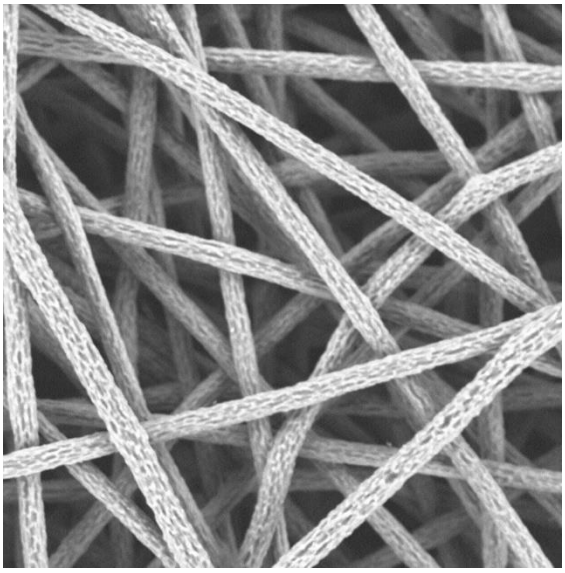

Figure 22. SEM image of PHBV scaffolds at x3900 magnifications

PHBV fibers are thicker than only PLA/PEG fibers, and they are also well ordered in the space. Additionally, it can be observed that they present porous structure in the surface. Magnitude of the fibers were in micro scale.

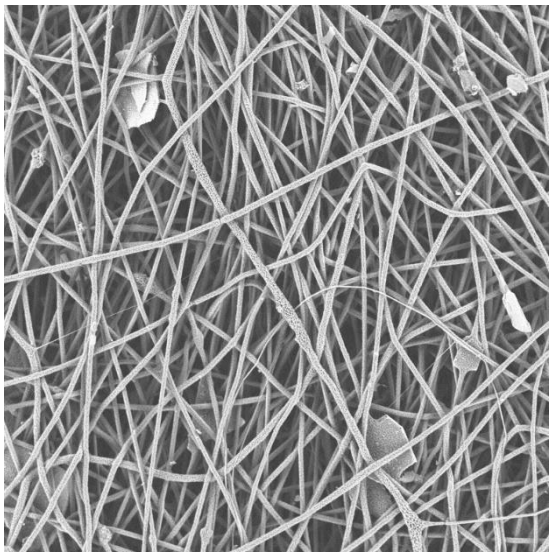

Figure 24. SEM image of PHBV+HAP scaffolds at x860 magnifications

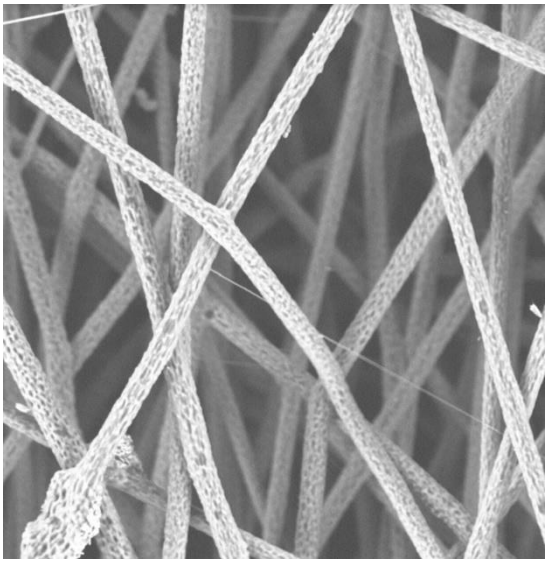

Figure 23. SEM image of PHBV scaffolds at x3700 magnifications

PHBV+HAP fibers are similar thick as PHBV fibers, and they are also well ordered in the space. Additionally, it can be observed that they present porous structure in the surface, with punctual accumulations in some fibers (as granules) of hydroxyapatite. Magnitude of the fibers were in micro scale.

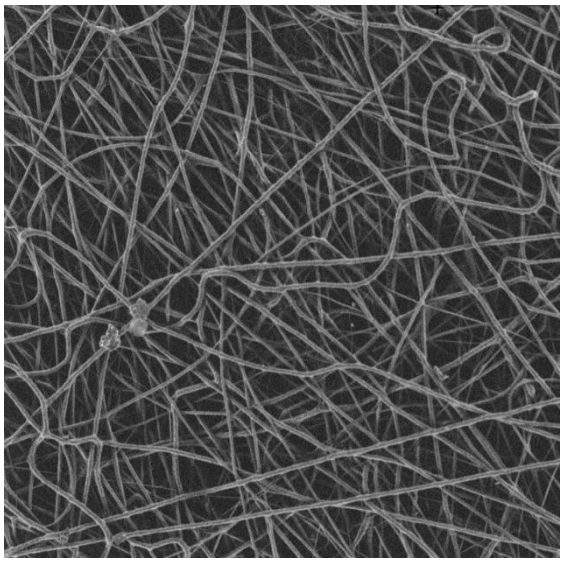

Figure 26. SEM image of PHBV+Strep scaffolds at x840 magnifications

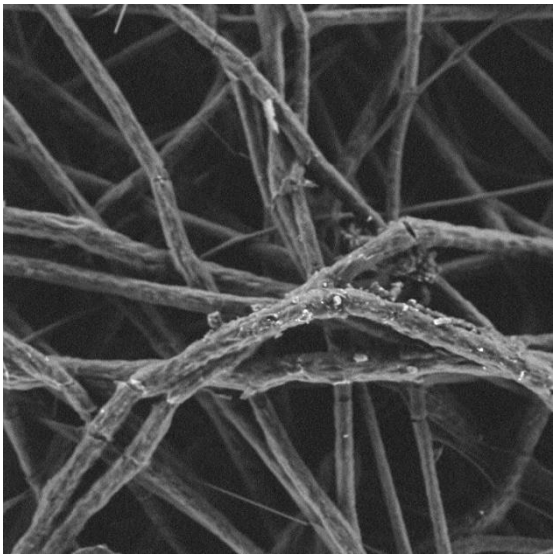

Figure 25. SEM image of PHBV+Strep scaffolds at x3900 magnifications

PHBV+Strep fibers present some irregularities on the thickness and surface as streptomycin is a drug that tends to create more add ons on the fibers in granulate state. Fibers were less porous than PHBV with hydroxyapatite but enough to accumulate the streptomycin. Magnitude of the fibers were in micro scale.

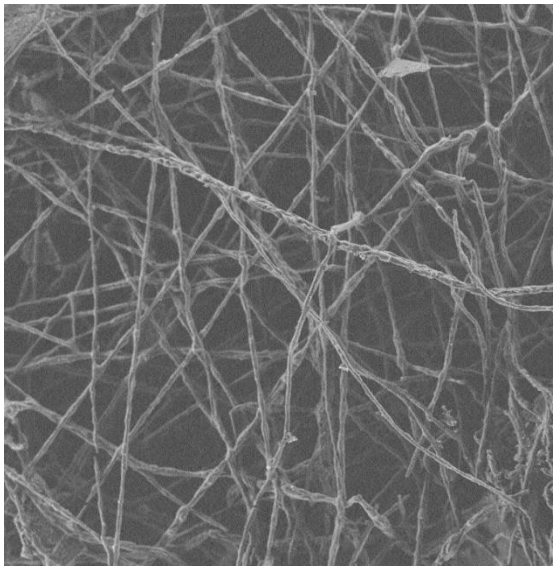

Figure 27. SEM image of PHBV+Strep scaffolds at x830 magnifications

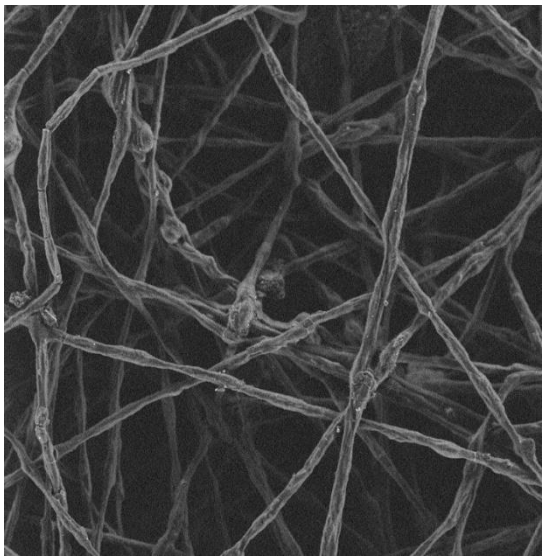

Figure 28. SEM image of PHBV+Strep scaffolds at x1650 magnifications

PHBV+Strep+hydroxiapatite fibers present more irregularities in the fiber morphology that any other monolayer scaffold analyzed as fibers have the add ons of both streptomycin and hydroxyapatite. Fibers were disordered and present high porosity, as can contain high quantity of ganules. Magnitude of the fibers were in micro scale.

4.2.2. FTIR

For monolayer scaffolds there were measured the absorbances of the different components in wavelengths comprises between 600 and 400 cm-. The result shows in scaffolds that contains more than one element which is the predominant, and the observed peaks characteristics of every specie.

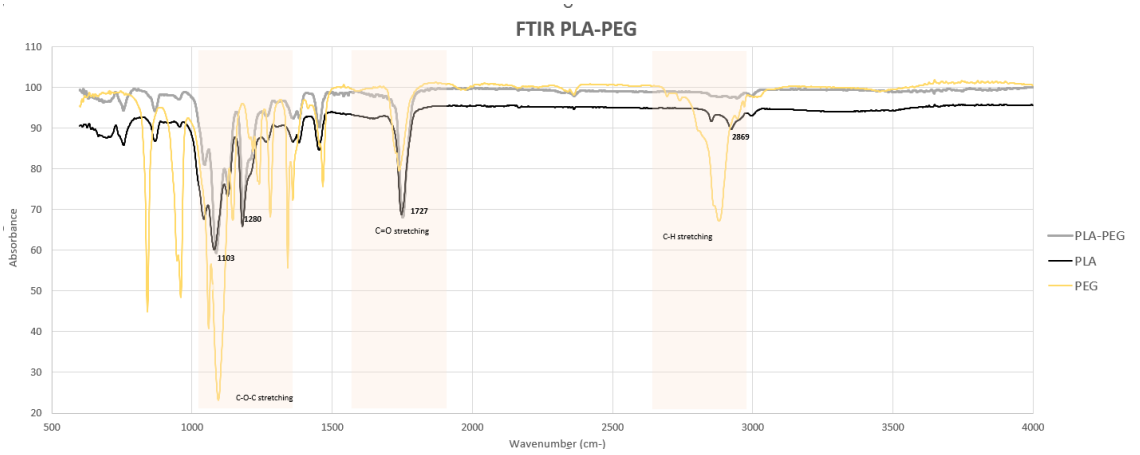

Figure 29. FTIR of PLA-PEG

For FTIR of PLA-PEG, it can be seen from the graphical representation of the absorbance that the PLA-PEG profile is more like solely PLA, and the stretching zones, where the bonding of each element was formed.

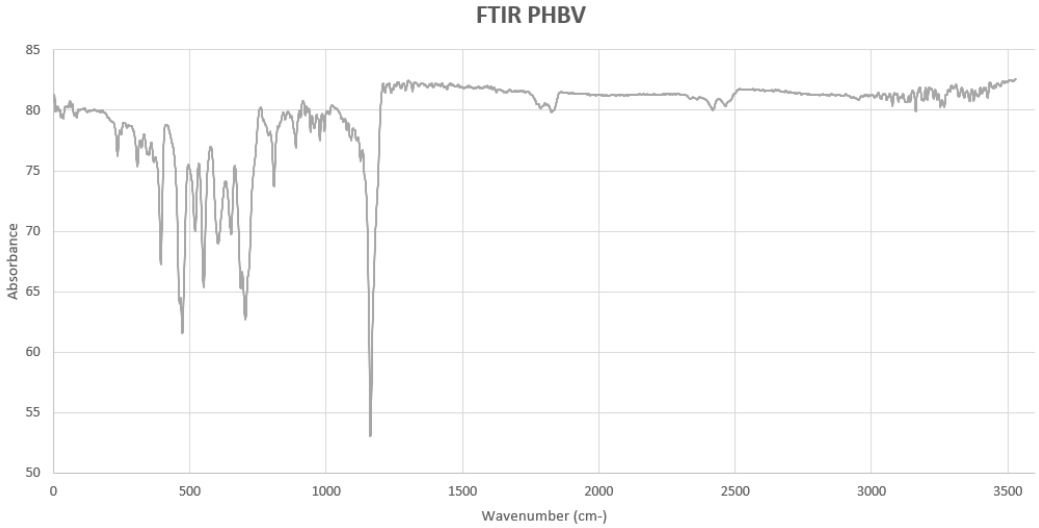

Figure 30. FTIR of PHBV

As there is a monolayer matrix with only PHBV, FTIR of only PHBV was represented to understand then it when it has other elements blended the behavior of the absorbances.

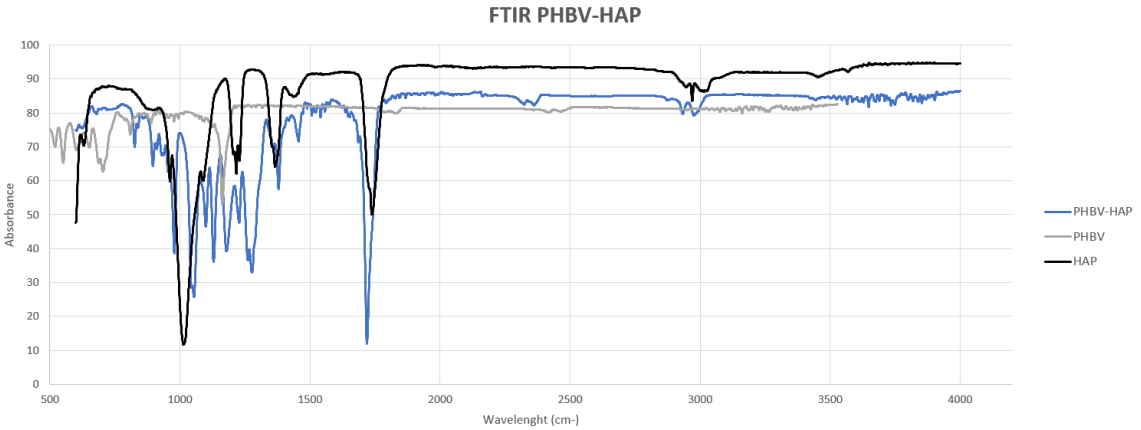

Figure 31. FTIR of PHBV and hydroxyapatite

For FTIR of PHBV-HAP it can be seen from the graphical representation of the absorbance that the PHBV-HAP profile is a combination of both PHBV and HAP, as present some similarities with HAP profile, but between 600 to 1200 cm- wavelength the profile of the absorbance is totally independent.

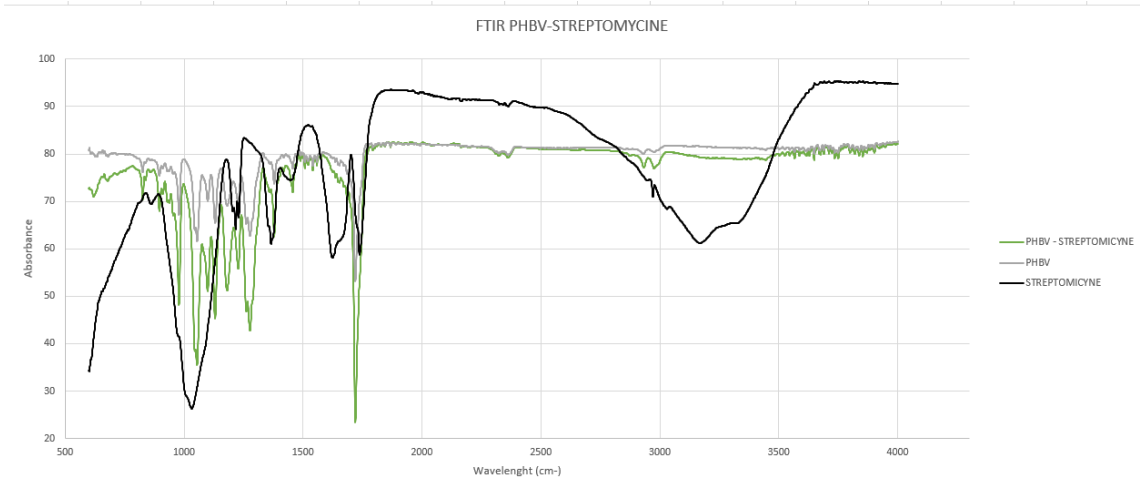

Figure 32. FTIR of PHBV and streptomycin

For FTIR of PHBV and streptomycin it can be seen from the graphical representation of the absorbance that the PHBV-Streptomycin profile is a more likely to PHBV profile, with almost any coincidence with the streptomycin profile and having an independent absorbance peak at approximately 1700 cm-.

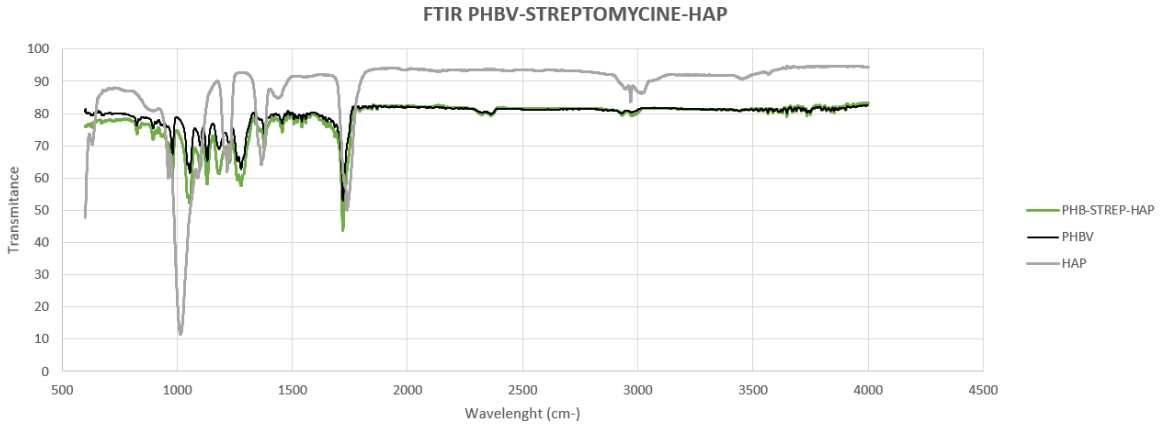

Figure 33. FTIR of PHBV, streptomycin and hydroxyapatite

Finally, for FTIR of PHBV streptomycin and HAP it can be seen from the graphical representation of the absorbance that the PHBV streptomycin and HAP profile is very close to the PHBV profile, with almost any coincidence with the streptomycin.

So, for PLA-PEG, the predominant specie is PLA, whereas in different PHBV scaffolds with add-ons of drug or hydroxyapatite, PHBV absorbance profile is the one that remains more evident, with some irregularities corresponding to the other substances.

4.2.3. Diameter obtention

Once obtained SEM microscopy images, diameter of the fibers for each of the monolayer scaffolds was calculated with "Diameter J" software, and they were classified by range groups. All diameters were in microscale. A total of 50 samplings were taken to see the distribution segmentation.

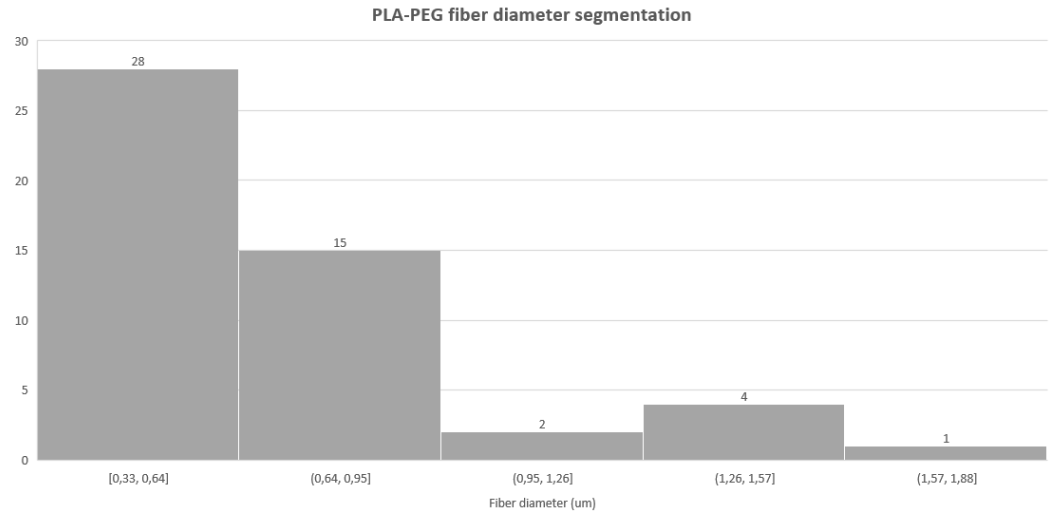

Figure 34. Diameter segmentation for PLA-PEG scaffold

For PLA/PEG case, dimeters of the fibers were mostly between 0,33 and 0,64 microns, as they didn't have any add on substance, neither PHBV.

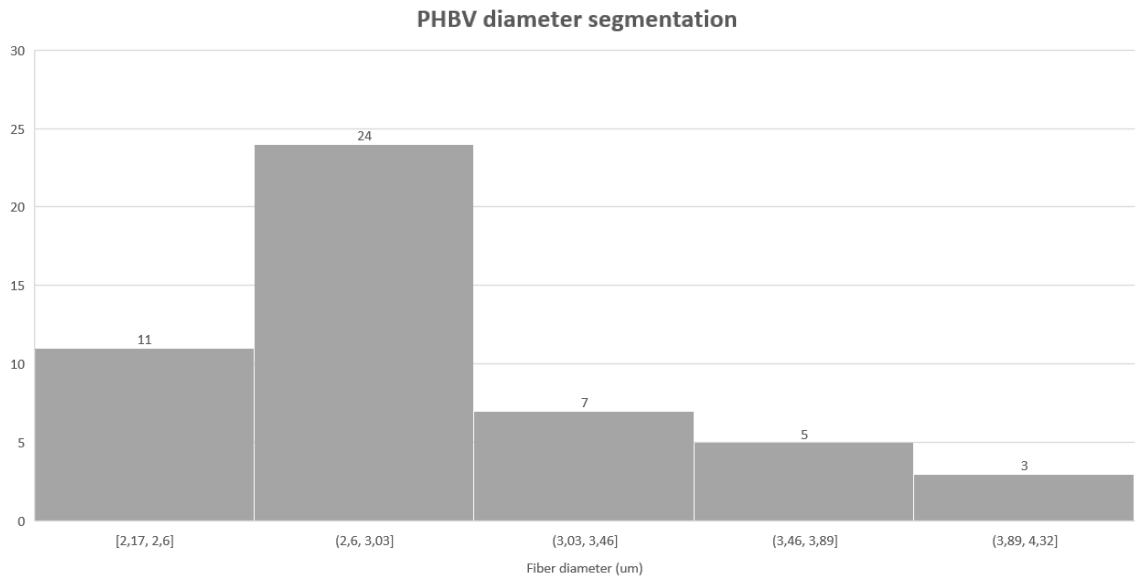

Figure 35. Diameter segmentation for PHBV scaffold

For PHBV case, dimeters of the fibers were mostly between 2,6 and 3,03 microns, much more thicker than PLA/PEG alone.

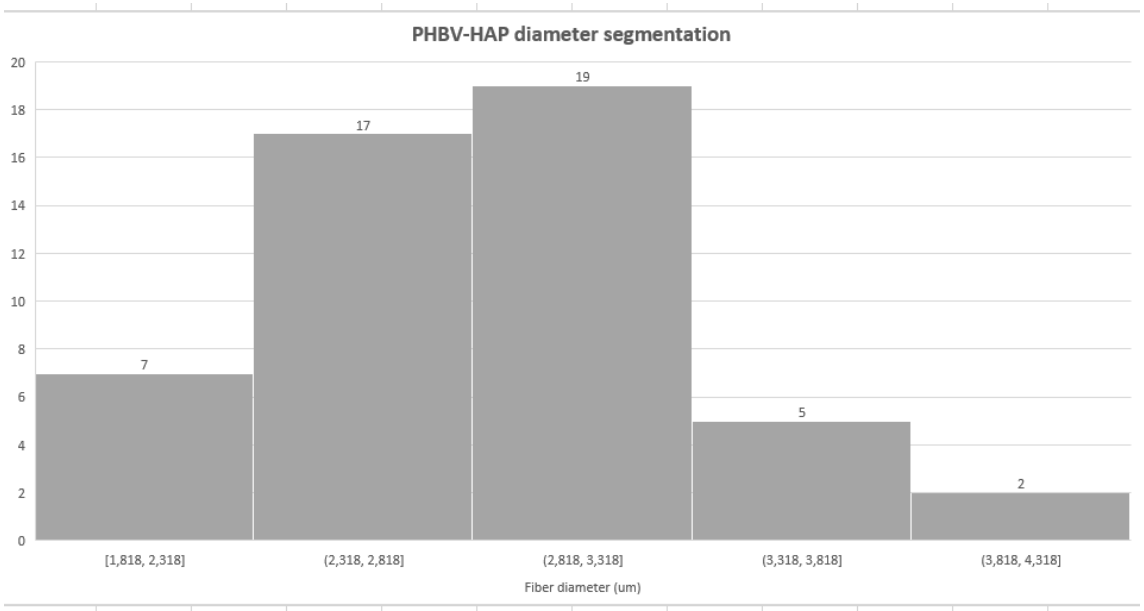

Figure 36. Diameter segmentation for PHBV HAP scaffold

It can be appreciated that, when hidroxyapatite is added to the porous scaffold formed with PHBV, its diameter increase, as the particles are attached to the fibers and make them thicker. In the case of PHBV-HAP, the most common range is situated between 2,818 and 3,318 microns.

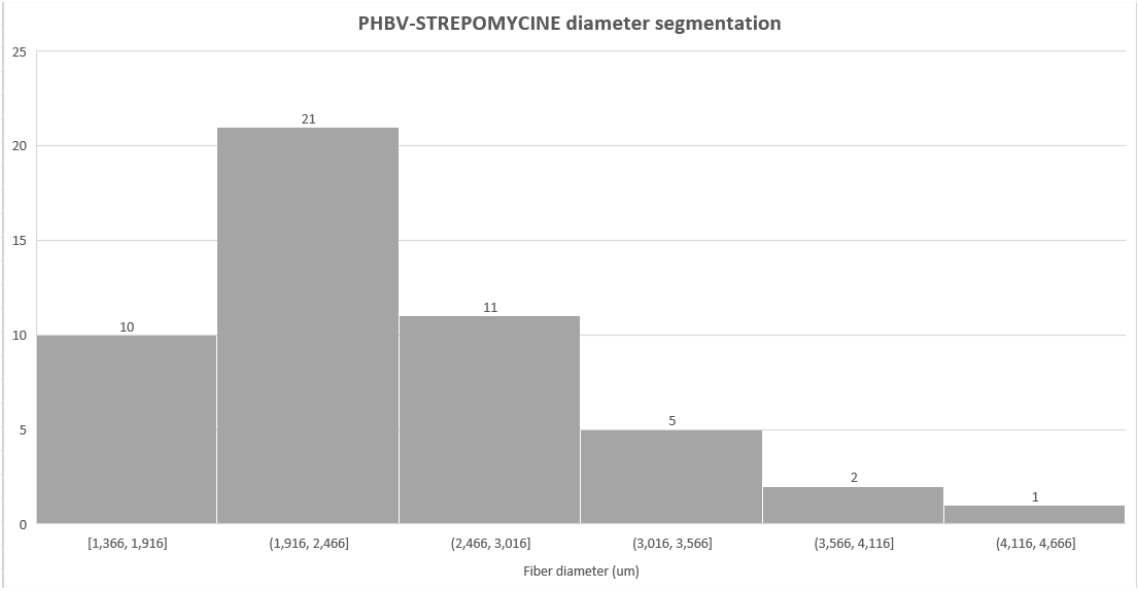

Figure 37. Diameter segmentation for PHBV Streptomycine scaffold

For PHBV and streptomycine case, dimeters of the fibers were more or less as PHBV alone values, as the add on of the drug does not impact much on the diameter thickness.

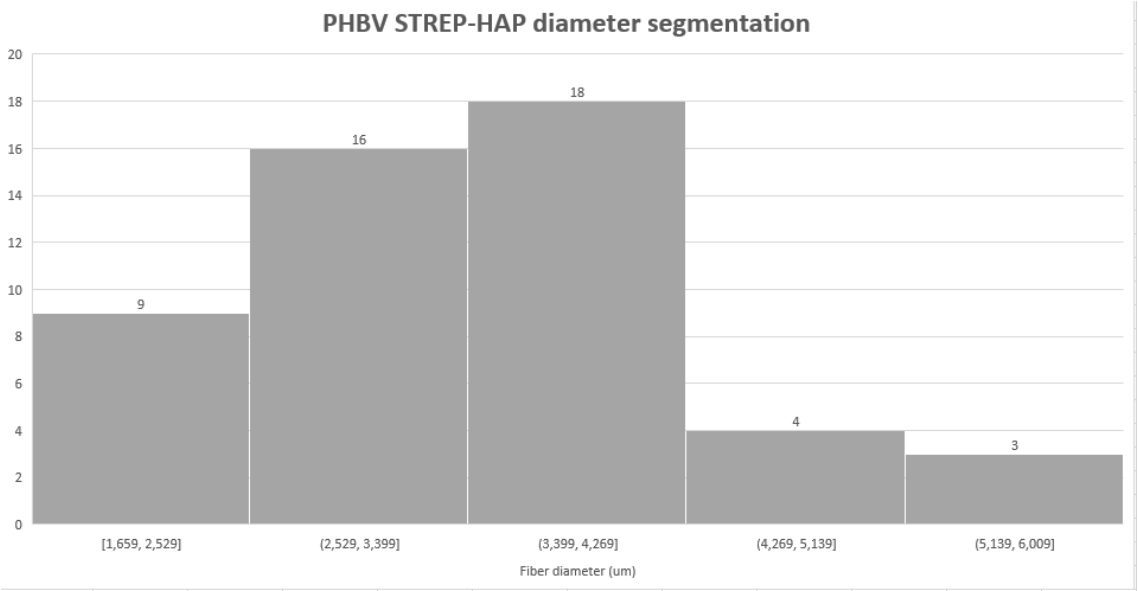

Figure 38. Diameter segmentation for PHBV Streptomycine scaffold

Finally, for PHBV streptomycine and hidroxyapatite it can be appreciated an increase of the diameter range, the thicker of all the cases, with values between 3,399 and 4,269 microns.

4.2.4. Contact angle

For each monolayer scaffolds it was also measured the contact angle, to see how the behavior of a water drop once touches the scaffold surface and as the picture was taken, it was measured the angle that forms the drop with the baseline stablished on the software.

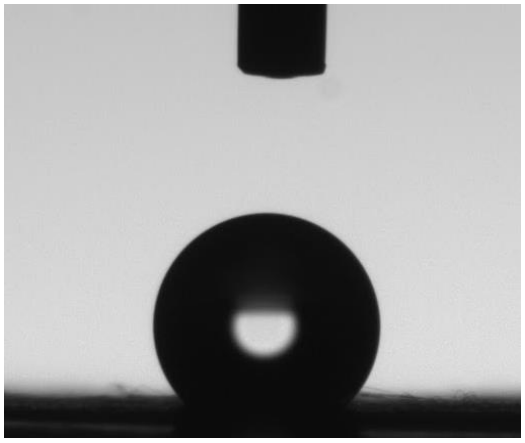

Figure 39. Contact angle image for PLA-PEG scaffold

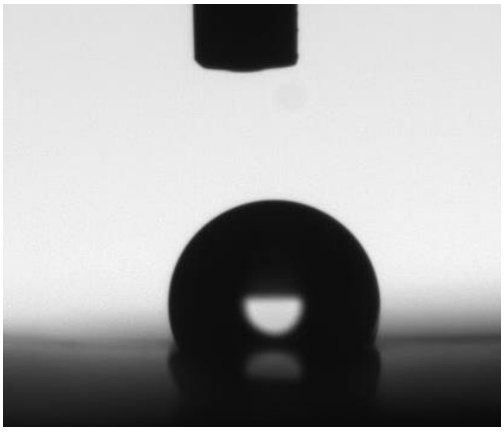

Figure 40. Contact angle for PHBV + Streptomycine scaffold

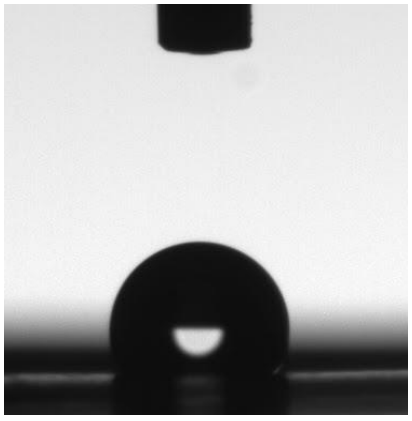

Figure 41. Contact angle for PHBV

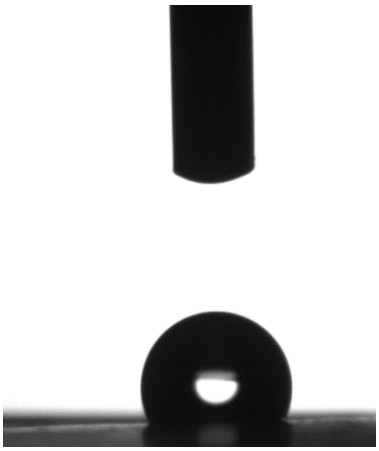

Figure 42. Contact angle for PHBV + HAP

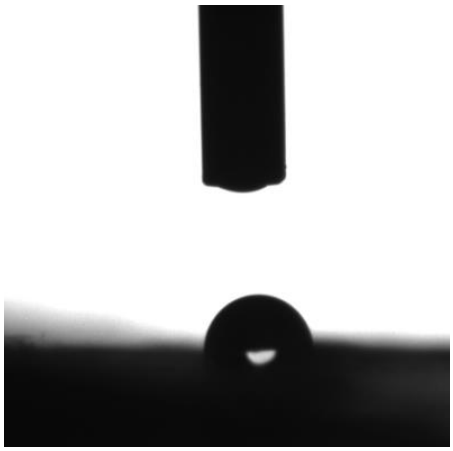

Figure 43. Contact angle for PHBV + HAP

Results of mean angles obtained for each scaffold and it's deviation can be summarized as:

Table 3. Summary of contact angles and its deviation

Polymer	Mean angle (º)	Deviation
PHBV	124,4564453	2,2579171
PLA-PEG	134,0171692	2,54778128
PHBV-Strep	120,6702431	3,90433101
PHBV-HAP	123,2564453	3,1545023
PHBV-HAP-Strep	125,9564453	4,13874241

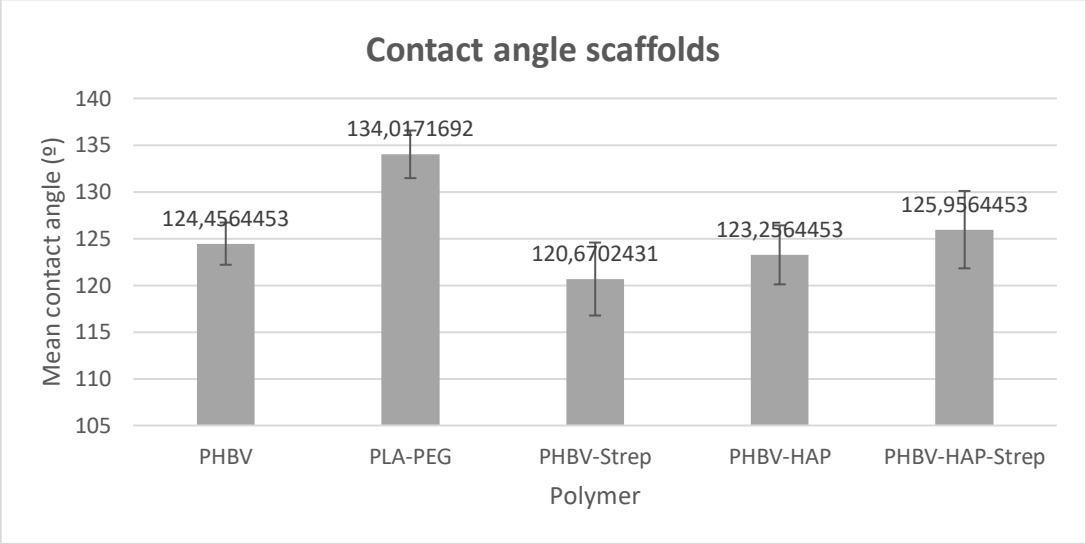

Figure 44. Mean contact angle summarized for all scaffolds tested with its deviations

As seen in images and the mean angle results, PHBV polymer does not have a good wettability, so, as the mean values are around 120º, value higher than 90º, it is considered that the PHBV polymer is a hydrophobic type of polymer, and for that, the water drop does not merge with the scaffold when they are in contact.

4.3. Scaffolds

4.3.1. FTIR

For trilayer scaffolds there were also measured the absorbances of the different components in wavelengths comprises between 600 and 400 cm-. The result shows in scaffolds that contains more than one element which is the predominant, and the observed peaks characteristics of every specie. For trilayer scaffolds, the absorbances obtained were the following:

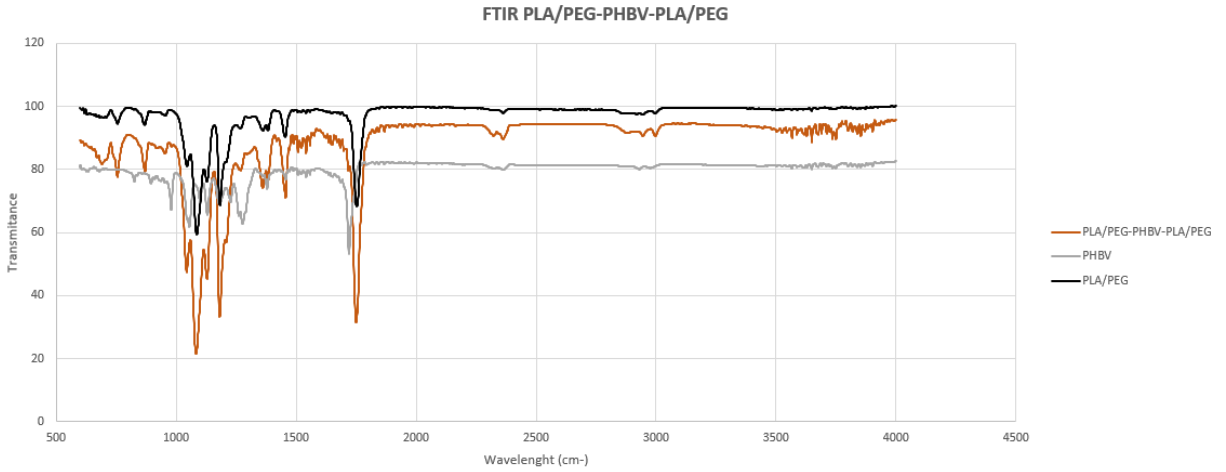

Figure 47. FTIR of trilayer scaffold PLA/PEG-PHBV-PLA/PEG

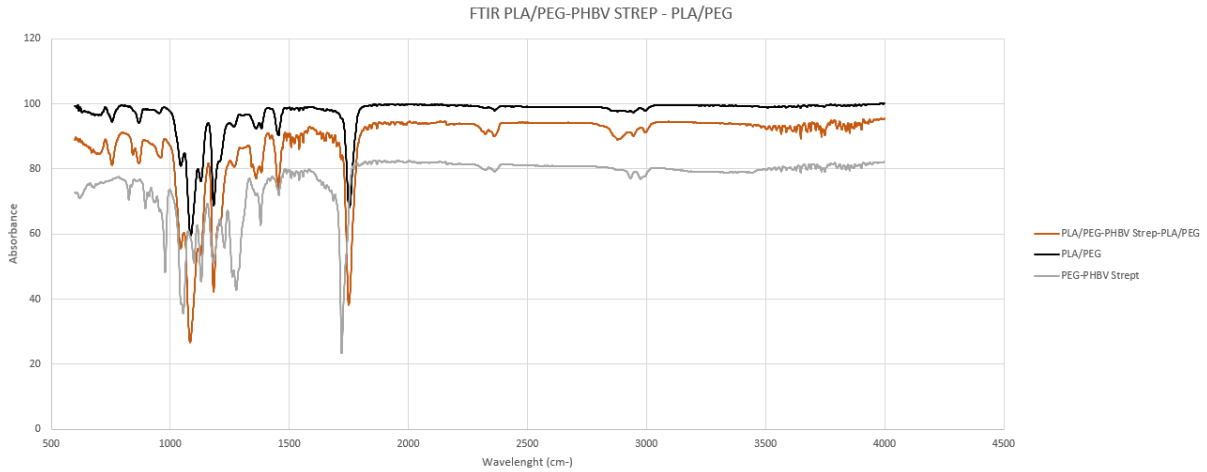

Figure 46. FTIR of trilayer scaffold PLA/PEG-PHBV-Streptomycine-PLA/PEG

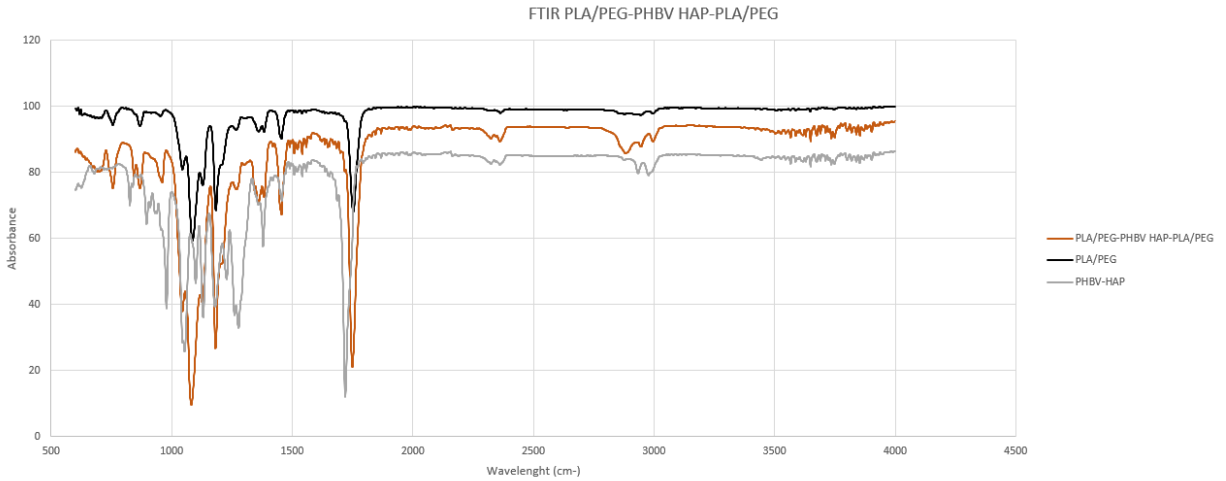

Figure 45. FTIR of trilayer scaffold PLA/PEG-PHBV-Streptomycin-PLA/PEG

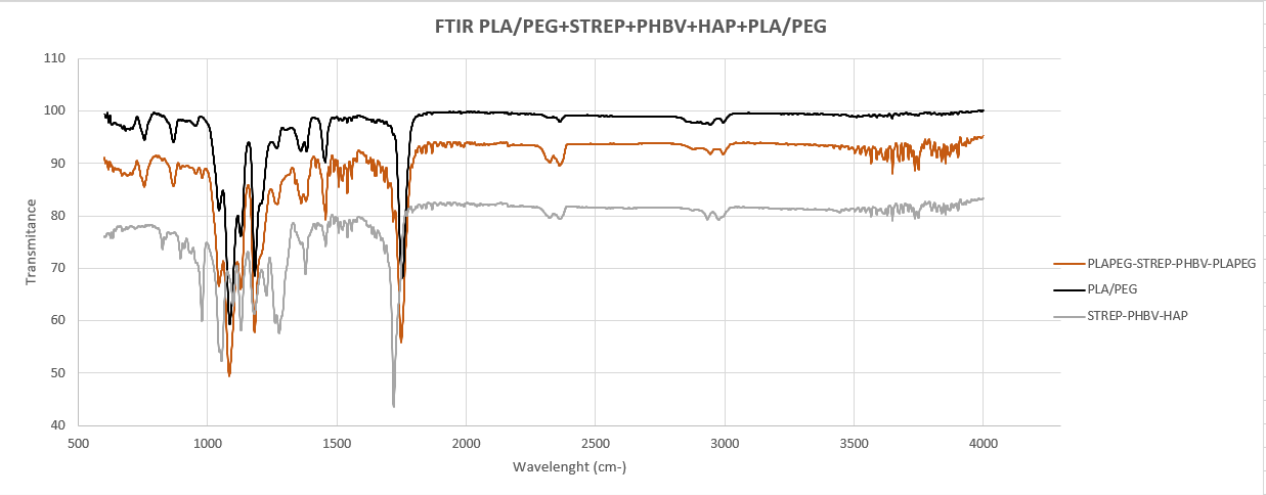

Figure 48. FTIR of trilayer scaffold PLA/PEG-PHBV-Streptomycin-HAP-PLA/PEG

As it can be seen on the graphs, all trilayer scaffolds follow the same profile than the content on the middle layer of the scaffold, and they are not affected by the two external layers containing PLA/PEG.

4.3.2. Drug release

Drug release test was performed to study the liberation of the drug among the time. The only scaffolds subjected to this test were the ones that contain streptomycin, as it was the antibacterial drug selected. This test was performed triplicated to obtain more accurate results.

To prepare the suitable solutions to analyze via UV-Vis spectroscopy, it was prepared a buffer solution with PBS (Phosphate Buffered Saline) at 7.2 pH and at 0,02% by weight concentration. Then, a sample of each scaffold was introduced in a tube, correctly labelled and was soaked in 5mL total of PBS media. Then, tubes were brought to a tube rotator, were they stay until sampling times happen. Rotary speed was maintained at 30 rpm.

Samples of the tubes were taken at the following hours since scaffolds are soaked: 1h, 2h, 3h, 4h, 5h, 24h and 48h. Every time a sample is needed to be taken, tubes were carried out to a laminar flux cabinet, and, with a micropipette, 1 mL sample was taken from the tube and introduced to an Eppendorf. Then, volume was filled out until 5mL with bulk buffer solution of PBS media. Samples on Eppendorf needed to be preserved on the fridge, as otherwise, solution could be damaged due to degradation.

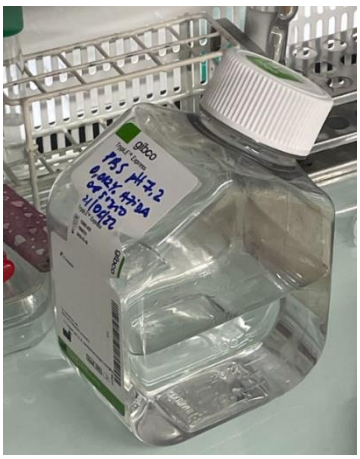

Figure 50. PBS medium

Figure 49. Sampling environment in laminar flux cabinet

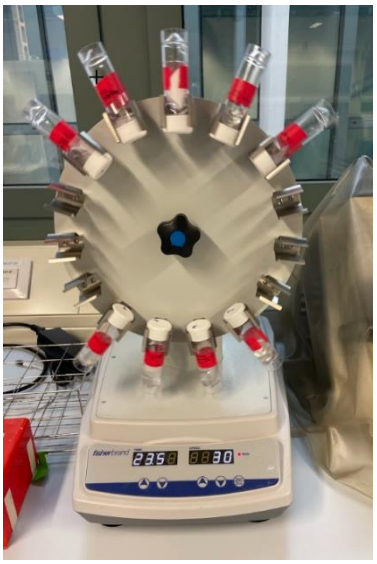

Figure 51. Rotary speed of the tubes containing the samples with PBS

When all samples were collected, solutions in the Eppendorf were analyzed in the UV-Vis spectrophotometer, to obtain the data of the release. Absorbances were calculated between 200 and 500 nm of wavelength.

First, it was necessary to create a basis with a solution with streptomycin to obtain the curves and consequently, the peaks of the basis where it could be analyzed the degradation. Concentrations calculated for the basis were stablished at 0.1 mg/ml, 0.2 mg/l, 0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml, 3.2 mg/ml and 6.4 mg/ml.

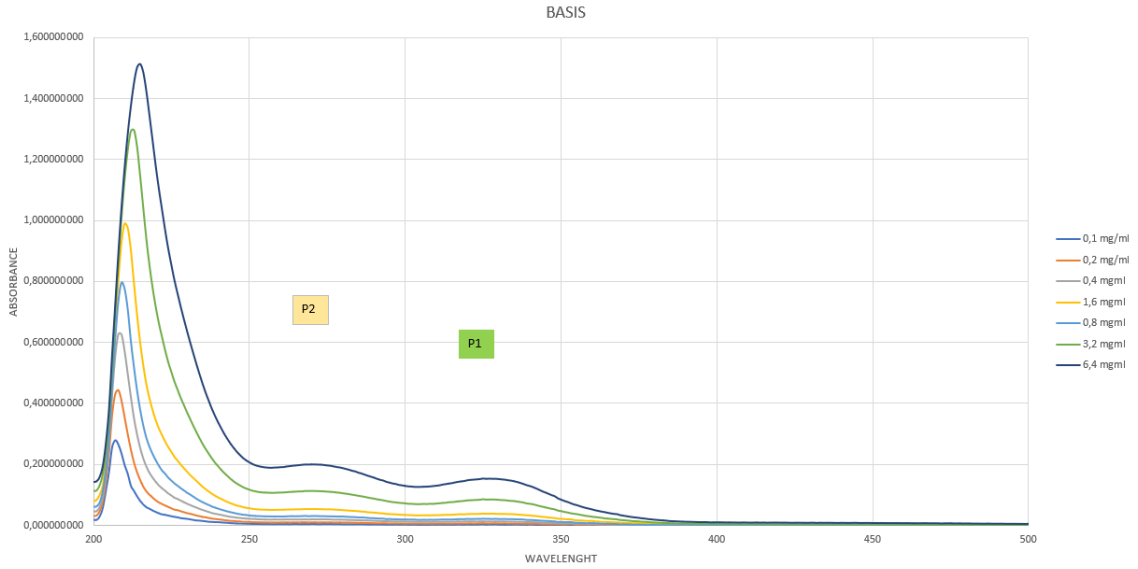

Figure 52. Basis of the curves at different concentrations of streptomycin

As it can be seen on the graph, there were two different peaks that could be analyzed, P1 and P2. In this project, only P1 was studied, that corresponds to a wavelength of 329nm. Once absorbance values were obtained, it was necessary to plot the values of concentration against absorbances to obtain a linear regression and subsequently, a line equation of the basis.

Table 4. Values of concentration against absorbances

Cx (mg/ml)	abs
0,1	0,004937258
0,2	0,00706964
0,4	0,01319359
0,8	0,02177605
1,6	0,03675631
3,2	0,084979363
6,4	0,152185828
0	0

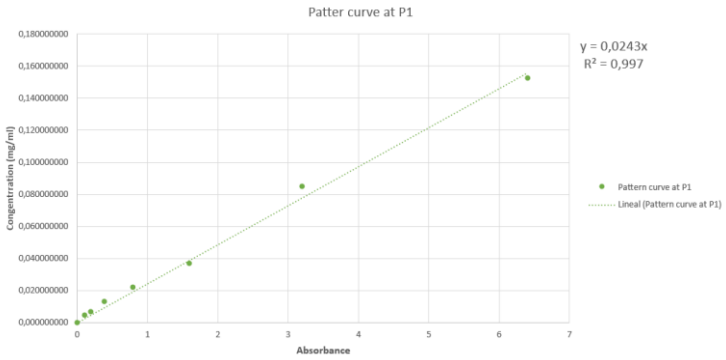

Figure 53. Linear regression of the pattern curve with the final linear equation

Once this equation is obtained, it was calculated for every sample analyzed the percentage of liberated drug and the deviation on the calculation.

As an calculation example, for Samples 1, 2 and 3 corresponding to PHBV- Streptomycin there were obtained the following results:

Table 5. Calculation results for samples 1, 2 and 3 corresponding to PHBV+ streptomycin

PATTERN CURVE 1		y= 0,0243x		DEFINED AT 329 nm				
TIME (h)	ABS+CORR	CONC	DRUG (mg)	%LIB	%M2	%M3	Average	Deviation
0	0	0	0	0	0	0	0	0
1	0,0156539	0,0003804	0,001902	15,287822	58,339468	69,287174	47,638155	28,545947
2	0,0813892	0,0019778	0,0098888	79,485796	96,428111	80,377223	85,430377	9,5347405
3	0,08158204	0,0019824	0,0099122	79,674123	84,971822	73,694268	79,446737	5,6422144
4	0,068598129	0,0016669	0,0083347	66,99386	62,830377	63,140625	64,321621	2,3194203
5	0,062852591	0,0015273	0,0076366	61,38269	59,036349	61,783516	60,734185	1,4839648
24	0,071441509	0,001736	0,0086801	69,770743	67,254666	49,205995	62,077135	11,217502
48	0,0760113	0,0018471	0,0092354	74,233663	62,229503	74,072922	70,178696	6,8846725
120	0,086406574	0,0020997	0,0104984	84,385828	76,849743	56,71241	72,649327	14,306891
144	7,18E-02	0,0017451	0,0087253	70,133818	59,838383	79,792463	69,921554	9,9787334
AFTER	0,079940386	0,0019426	0,0019426					
MAXIMUM WEIGHT			0,012441					

Calculation of "%lib" corresponds to the 1 sample. Same calculations were performed for sample 2 and sample 3 and data was brought to this table with name "%M2" for sample 2 and "%M3" for sample 3. The calculation of the percentages is calculated taking into account the final value "After", that corresponds to the value once the last sampling was take.

Values of absorbance are taken directly from software data.

Concentration value was calculated considering the equation of the linear regression of the basis. For 1h time:

$$y = 0{,}0243 \cdot x$$
$$y = 0{,}0243 \cdot 0{,}0156539$$
$$y = 0{,}0003804$$

mg of drug was calculated taking into account that 5mL was the volume were the drug was contained, so, for 1h time:

$$mg\ of\ drug = concentration \cdot 5$$
$$mg\ of\ drug = 0{,}0003804 \cdot 5$$
$$mg\ of\ drug = 0{,}001902$$

Percentages of liberation were calculated taking into account the value after the last sample was collected. For 1h time:

$$\%\ liberation = \frac{mg\ of drug}{maximum\ weight} \cdot 100$$
$$\%\ liberation = \frac{0{,}001902}{0{,}012441} \cdot 100$$
$$\%\ liberation = 15{,}28\ \%$$

Finally, an average and the deviation were calculate between the other samples corresponding to the same scaffold (as it was done triplicated). Results of the release during time was plotted in the following graph.

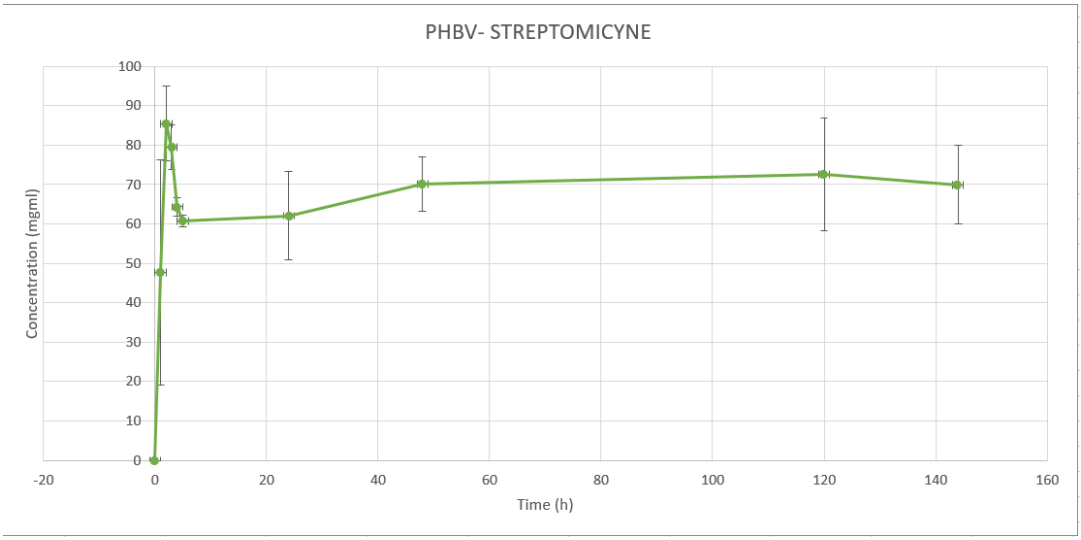

Figure 54. Evolution of the concentration of streptomycin against time for PHBV - streptomycin scaffold

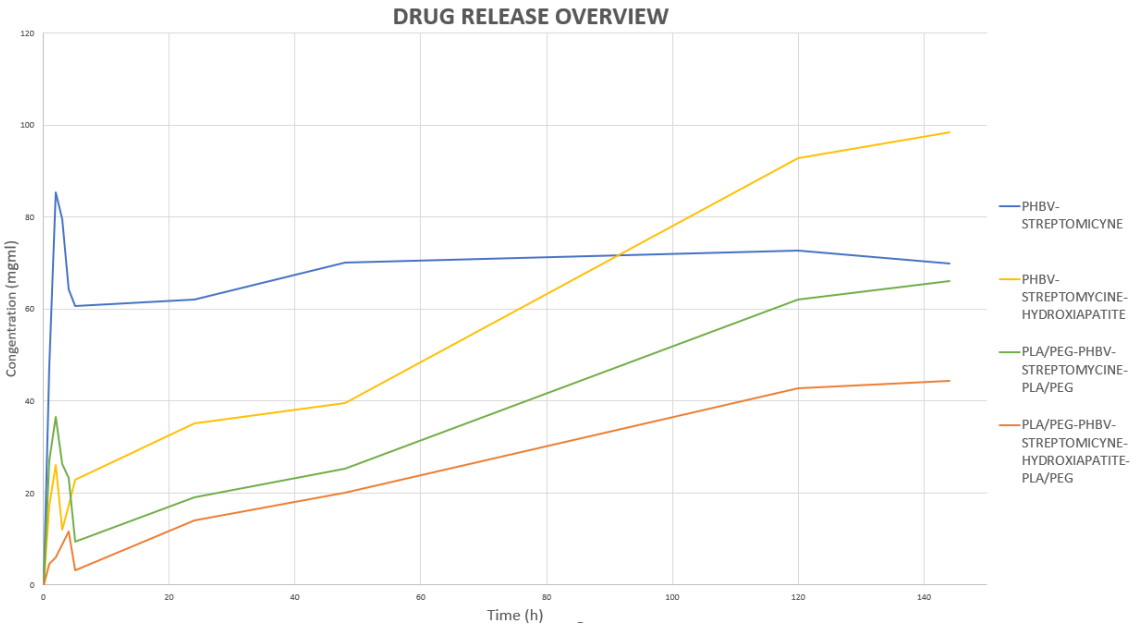

Figure 55. Overview of final drug release for every scaffold tested

Finally, all graphs of the evolution of concentration against time were plotted together. The maximum liberation drug scaffold corresponds to PHBV-streptomycin-hydroxyapatite, as the evolution of PHBV remains constant pending the time and trilayer scaffolds, present lower rates of drug release than monolayer scaffolds.

5. CHAPTER 5: CONCLUSIONS

In this project it has been analyzed different characterization of the fabricated monolayer and trilayer scaffolds, and it has also been studied a drug release test to prove scaffolds capacity.

At this point, it has been observed that, as contact angle results indicate, the studied polymer presents hydrophobic behavior. Also it have been obtained the different ranges of diameter for the fibers, and that PHBV + Streptomycin +Hydroxyapatite are the ones that present thicker morphology, with diameters between 3,39 and 4,26 microns.

Finally, studying the capacity of the scaffolds to drug releasing, it has been observed that the scaffold that has the most capacity to drug release is PHBV-streptomycin-hydroxyapatite scaffold, followed by PHBV-streptomycin scaffold and lastly the trilayer scaffolds, that present very low capacity to drug release.

6. CHAPTER 6: PLANNING

6.1. Timeline

Table 6. Timeline of the tasks to perform

Codification	Task	Description	Time spent (h)
A	Project acceptance	Acceptance of the project to be carried out	2
B	Determination of the objectives	Identification of the main objectives of the project and how it will be accomplished	20
C	Research of the information needed for the project	Research of different papers and information about the topic of the project	60
D	First tests with monolayer scaffolds	Preparation of monolayer scaffolds and determination of the best working conditions	75
E	Trilayer preparation scaffolds	Fabrication of the trilayer scaffolds once known the best electrospinning conditions	26
F	Characterization tests	Characterization tests of the monolayer scaffolds with the different techniques mentioned	250
G	Trilayer scaffold tests	Drug release tests and spectroscopy of the scaffolds	265
H.1	Writing of theoretical report	Writing the final draft of theoretical report with all the concepts and researched information summarized	30
H.2	Writing of experimental part of the project	Writing the final draft with all the results obtained once the experiments are finished.	98
H.3	Writing of the budget	Final price calculation of the project	12
I	Report delivery	Upload of the report via Atenea	1
TOTAL			**839**

6.2. Gantt chart

Codification	Activity	February				March				April				June				July				August				September			
A	Project acceptance																												
B	Determination of the objectives																												
C	Research of the information needed for the project																												
D	First tests with monolayer scaffolds																												
E	Trilayer preparation scaffolds																												
F	Characterization tests																												
G	Trilayer scaffold tests																												
H.1	Writing of theoretical report																												
H.2	Writing of experimental part of the project																												
H.3	Writing of the budget																												
I	Report delivery																												

7. CHAPTER 7: ECONOMIC ANALYSIS

All the materials and the resources that were necessary to carry out the project were supplied by Chemical Engineering department of EEBE laboratories. Therefore, this budget represents an estimation of the cost if it will not be able to access to the instrumentation and reactants.

7.1. Materials and reagents cost

Table 7. Material cost

Material	Price/unit (€)	Amount	Total price (€)
Volumetric flask	4,6	6	27,6
Syringes	0,5	60	30
Needles	0,8	60	48
Tubes	1,5	24	36
Eppendorf	0,2	50	10
Fly for stirring	2,1	3	6,3
Wash bottle	6,5	1	6,5
Coverslips	0,05	250	12,5
Slides	0,08	250	20
Solution bottles	1	20	20
Volumetric flask	10	3	30
Pipettes	60	2	120
Pipette tips	0,05	50	2,5
TOTAL			**369,4**

Table 8. Reagent costs

Reagent	Price/unit (€)	Distributor	Amount	Total price (€)
Formic acid 85% 25L	3,96 €/L	-	0,0025	0,099
Chloroform	94€/L	Fisher	0,5	47
Hydroxyapatite	50€/kg	-	0,07	3,5
Streptomycin	15€/kg	Sigma Aldrich	0,1	1,5
PHB	30€/kg	-	0,250	7,5

Reagent	Price/unit (€)	Distributor	Amount	Total price (€)
PLA	18€/kg	Technopackaging	0,120	2,16
PEG	99,72€/kg	Sigma Aldrich	0,05	4,98
TOTAL				**66,745**

7.2. Personnel cost

Table 9. Pesonnel cost

Role	Category	Price/hour	Persons	Hour worked	Total price
Researcher	Chemical engineer	12	1	839	10068
Project management	Doctor of chemistry	45	2	60	5400
TOTAL					15468

7.3. Equipment cost

Table 10. Equipment cost

Equipment	Useful life	Time used	Price (€)	Total(€)
Oven	10 years	2 months	5200	**86,6**
Pump for electrospinning	10 years	5 months	590	**24,58**
Optical microscope	10 years	5 months	2000	**83,3**
SEM microscopy	10 years	2 days	160.000	**87,6**
Contact angle	10 years	1 week	6500	**12,46**
FTIR	10 years	1 week	20000	**38,35**
UV-Vis	10 years	2 weeks	7400	**28,38**
Stirrer	10 years	2 months	300	**5**
Analytic balance	10 years	5 months	3000	**125**
TOTAL				**461,27**

7.4. Total cost

Table 11. Total cost of the project

Costs	Total(€)
Reagents	**66,745**
Materials	**369,4**
Equipment	491,27
Personnel	15468
Total	**16395,415**

8. CHAPTER 8: FUTURE PROSPECTS

8.1. Environmental impact analysis

The environmental impact is defined as any change to the environment, whether adverse or beneficial, resulting from a facility's activities, products, or services. In other words, it is the effect that people's actions have on the environment [15].

In this project mainly all the products used were not harmful fo environment. Otherwise, some solvents were used that could be harmful if there were not treated correctly and, for that, waste management formation about how to dispose all the solvents and materials used during the experiments was obtained and applied.

8.2. Proposals for future projects

Based on the results obtained in this project, the following is recommended for possible future work:

- Test the same experiments but with another solvent, for example with dichloromethane.
- Try the same experiments with coaxial fibers
- Change the conditions of the polymer scaffolds fabrication

9. CHAPTER 9: REFERENCES

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