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RESUM

Aquest projecte s'ha centrat en la producció d’una membrana polimèrica formada per chitosà i poly(etilene)oxide per la prevenció de la adherència abdominal post-quirúrgica en pacients que pateixen una hernia abdominal.

La tècnica utilitzada per produir aquesta membrana ha sigut “l’electrospinning”. “L’electrospinning” o “electrofilat” és un sistema relativaument senzill que permet la producció d’una membrana o teixit a partir de nano-fibres.

Partint d’estudis preliminars i mitjançant diversos assajos, s’han optimitzat els paràmetres del sistema per produir nano-fibres regulars, consistentes i sense defectes. Un cop produïdes, les membranes han estat reticulades exitosament fins a tres formes diferents: tèrmicament, mitjançant l’addició del reactiu glutaraldehyde i mitjançant l’addició de genipina. Mitjançant la reticulació les membranes han adquirit estabilitat i consistència. La reticulació química amb la genipina ha permès a la membrana persistir durant una setmana dins de medis àcids pH 4.

Els estudis in Vitro efectuats han mostrat una vitalitat cel·lular insuficient i no ha mostrat cap disminució en l’adherència cel·lular, tot i això, han estat considerats invàlids.
RESUMEN
Este proyecto se ha centrado en la producción de una membrana polimérica formada por quitosano y oxido de poly(etileno) para la prevención de la adherencia abdominal post-quirúrgica en pacientes que sufren una hernia abdominal y requieren la implantación de una malla. La técnica utilizada para producir esta membrana ha sido el “electrospinning”. El “electrospinning” o “electrofilado” es un sistema relativamente sencillo que permite la producción de una membrana o tejido a partir de nanofibras.

Partiendo de estudios preliminares y mediante varios ensayos, se han optimizado los parámetros del sistema para producir nano-fibras regulares, consistentes y sin defectos. Una vez producidas, las membranas han sido reticuladas exitosamente de tres formas diferentes: térmicamente, mediante la adición del reactivo químico glutaraldehido y mediante la adición de genipina. Mediante la reticulación las membranas han adquirido estabilidad y consistencia.

La reticulación química con la genipina ha permitido a la membrana persistir durante una semana dentro de medios ácidos de pH 4.

Los estudios in vitro efectuados han mostrado una vitalidad celular insuficiente y no ha mostrado ninguna disminución en la adherencia celular; sin embargo, han sido considerados inválidos.
ABSTRACT

This project is focused in the yielding of a polymeric membrane composed by chitosan and poly(ethilen) oxide in order to prevent de post-surgical abdominal adhesion in patients which suffer an abdominal hernia.

After previous researches and essays carried out, parameters had been optimized to produce defect-free, beadless, and geometrically uniform nanofibers. Once they were produced, membranes were successfully cross-linked by three different ways: thermical, by adding the chemical reactive glutaraldehyde and by adding genipin. Membranes presented and improvement in their stability once they were cross-linked. Genipin crosslinking has allowed membranes to persist in pH 4 conditions.

*In vitro* studies revealed low vitality activity and did not show a decrease of cell adherence. However, these results have been considered unreliable.
CHAPTER 1:
INTRODUCTION
Electrospinning is a method in which materials in solution or melt are formed into nano- or micro-sized continuous fibers. It has become the most used technique for fabrication of nanofibers, due to its cost-effectiveness, flexibility, potential to scale up, and ability to spin a broad range of polymers.

In this project, we have produced electrospun membranes with the addition of different chemical products in order to compare their physical and chemical properties. Although these types of membranes could have more than one application, we have been focused in the production of electrospun membranes in order to prevent the post-surgical adhesion of the abdominal wall.

Abdominal hernia repair is one of the most common surgical procedures in hospitals. A typical procedure in order to repair this issue consists in an introduction of a mesh between the damaged tissues and the abdominal wall. Due to the advances in research, patients tend to be completely recovered among the first days after surgery. Nevertheless, there are some complications which might appear after surgery such as abdominal adhesion, swelling, or displacement of the mesh. So that is why the following weeks after surgery are crucial. After some weeks, the risk of any side effect decreases with the time (as long as the mesh remains properly). However, abdominal adhesions can become larger and tighter thorough the time, sometimes causing problems years after surgery. All these complications have one point in common: they are painful for patient.

Among patients who undergo abdominal surgery (as for example abdominal hernia repair), more than 90 percent develop abdominal adhesions. Our biomaterial membrane could be placed in the surface of the mesh in order to reduce the occurrence of abdominal adhesion after hernia surgical repair.

To sum up, the main objective of this project is:

- Yielding of different biomaterial membrane by electrospinning process made by:
  o A solution of chitosan with poly-ethylene(oxide)

In order to achieve this goal we have to:

  o Review the state of art in producing biomaterials to treat the abdominal adhesion
  o Acquire a basic understanding of electrospinning process and the influence of parameters in the yielding of membranes, looking for best conditions in yielding membranes.
Treat the membranes: In the willing of improving their mechanical and biodegradable properties, three different types of cross-linking.

Establish a comparison of the different types of cross-linking by the characterization of membranes.

Determination of which type(s) might constitute a possible and accurate membrane in the purpose of this project

1.1 Abdominal hernias

A hernia is an outpunching of the parietal peritoneum through a preformed or secondarily established cavity (Holzheimer & Mannick, 2001). This is caused by a weakness or defect in the abdominal wall. If the outpunching is limited to peritoneal pockets, it is known as an internal hernia. Depending on the size of the outpunching, it is called complete (total) or incomplete (partial) hernia. A hernia could be strangulated if the intestine is trapped in it causing a decrease of the blood supply. In this case, a surgical emergency is immediately necessary.

An inguinal hernia is a protrusion of abdominal contents into the inguinal canal through an abdominal wall defect. If hernia is reducible, then it can be pushed back into the opening. When intestine or abdominal tissue fills the hernia sac and cannot be pushed back, it is called irreducible or incarcerated. Most inguinal hernias, however, are less dangerous, and elective surgery is often performed to correct the defect.

There is not an only cause for abdominal hernia. Usually, abdominal hernia is caused by several factors. Among all the risk factors are: older age (because muscles become weaker); obesity (it derives in an increased pressure on abdominal muscle), sudden twist, pulls or strains; chronic straining; congenital problem (family history); connective tissue disorders or pregnancy (it is estimated that 1 in 2000 women develop a hernia during pregnancy).

It is estimated that about 10% of the population in EEUU may develop some type of hernia during their lifetime. (Assar A Rather, 2014).

From all the hernias diagnosis, approximately 75% are inguinal, 14% are umbilical, 10% are incisional or ventral and only 3-5% of hernias are femoral.

In 2007, about 3,5 % of all surgical procedures was hernia repairs (2,1% inguinal or femoral hernia and 1,4 other hernias(Fingar, Stocks, Weiss, & Steiner, 2014)
Over 1 million abdominal hernia repairs are performed each year, with inguinal hernia repairs constituting nearly 770,000 of these cases only in North-America. (Assar A Rather, 2014)

In Catalonia, according to the report of activity of 2009 and 2010, abdominal was reported to be one of the most common diagnosed diseases with 28.002 (2009) and 27.990 cases, representing 2,9 % of total diagnosis reaching second position, just below of cataract disease. The number of conventional hospitalization of inguinal and femoral hernia in 2010 represented 3,7% of total with 10928 patients. The number of surgical procedures of inguinal and femoral hernia was reported in 6.060 procedures, reaching fourth position of most common surgeries with 3,1% of cases. 85,6% among all inguinal and femoral hernia diagnosis were men as it could be preview (noticing that these diseases have bigger impact in men population rather than women). Mean period of hospitalization was estimated in 1,7 days with only 0,3% of mortality. (CatSalut, 2012)

1.1.1 Methods of hernia repair

At present, two different major procedures of repair are established: mesh-free hernia repair and tension-free hernia repair with mesh. These can be performed using an open approach or laparoscopically.

**Tension-Free Mesh**

Surgical mesh is basically a medical device that is used to provide additional support to weakened or damaged tissue. The majority of surgical mesh devices currently available are constructed from synthetic materials or animal tissue.

The "tension-free" mesh technique or Lichtenstein technique was pioneered by the Lichtenstein Hernia Institute in 1984 and constitutes the current standard repair of hernias. In this procedure, repair is accomplished by covering the opening of the hernia with a patch of mesh, instead of sewing the edge of the hole together. That is why it is called “tension-free” as no tension force is added to muscles of injured zone. The surgical mesh only acts as a scaffold tissue to reinforce the abdominal wall.

For an open mesh repair the hernia sac is removed. Then, mesh is placed over the hernia site. The mesh is attached using sutures and sewed into stronger tissue of the surroundings (Figure 1: ). It can also be placed a mesh plug into the hernia space. The mesh plug fills the open site and is sutured similarly. In some cases, an additional mesh patch is applied and may or may not be sutured. The site is closed using sutures, staples or surgical glue.
The use of surgical mesh improves patient outcomes by decreasing operative time and minimizing recovery time. However, recovery time depends on the type of hernia, the surgical approach, and the patient’s condition both before and after surgery.

Information found in medical literature has consistently demonstrated a reduced hernia recurrence rate when surgical mesh is used to repair the hernia compared to hernia repair without surgical mesh. For example, inguinal hernia recurrence is higher with open repair using sutures (primary closure) than with mesh repair. (American College of Surgeons, 2009)

Despite reduced rates of recurrence, there are situations where the use of surgical mesh for hernia repair may not be recommended. However, due to improvement throughout last decades, tension-free mesh has become the golden standard of hernia surgical repair.

The cumulating long-term mesh complications could be: degradation of mesh, erosion of neighboring structures such as blood vessels, spermatic cord, or bladder, adhesion- and fistula-formation, and a possible reduction of the abdominal wall mobility.

1.1.2 Abdominal adhesion

The incidence and severity of abdominal adhesions varies between surgical specialties and procedures.

Abdominal adhesions are bands of fibrous tissue that can be formed between abdominal tissues and organs. Normally, internal tissues and organs have slippery surfaces, preventing them from sticking together as the body moves. However, in abdominal adhesions, tissues and organs of the abdominal cavity attach together.
The most frequent surgery-related causes of abdominal adhesions causes according to the NIH (National Institute of Diabetis and Kidney Disease, 2013) are:

- cuts involving internal organs
- handling of internal organs
- drying out of internal organs and tissues
- contact of internal tissues with foreign materials, such as gauze, surgical gloves, and stitches
- blood or blood clots that were not rinsed away during surgery

Most adhesions are painless and do not cause complications. However, adhesions cause about 60% of small bowel obstructions in adults and contribute to the development of chronic pelvic pain. In extreme cases, adhesions may form fibrous bands around a segment of an intestine. (Casey & Taylor, 2013) This constricts blood flow and leads to tissue death.

**Figure 2:** Forming of tissue between both walls
1.2 State of art

The potential of therapeutic agents in order to treat abdominal adhesions has increased dramatically during the last years. Thus, pharmacological treatments have become the most commonly treatment in order to prevent postoperative adhesions. Various pharmacological approaches to treat adhesion have been assessed such as the use of fibrinolytic agents like anticoagulants, anti-inflammatory agents (both steroidal and non-steroidal), antibiotics or agents that interfere with specific cytokines or vascular permeability (Chang, Yen-hsien, & Chien, 2012)

Pharmacological treatments have proven to be effective against adhesion, inflammatory reactions or fibrosis proliferation. But different drugs are required to be administrated because different reactions are needed to be defeated, which turn out in an increase of harmful side effects. In addition, their effectiveness decreases in long-term therapies.

On the other hand, physical barrier between injured site and host tissue has also been tested. Various materials made of animal tissues have been used as physical barrier due to their biocompatibility but biological materials and synthetic polymers have also been reported to be effective both in animal models and clinical practices.

Jung-Jhih Chang et al (Chang, Yen-hsien, & Chien, 2012) have carried out an electrospun anti-adhesion barrier made of chitosan alginate for reducing peritoneal adhesions. They have reported that about 40% of the rat model treated with this kind of membrane exhibited no tissue adhesion between injured peritoneum and cecum suggesting that chitosan alginate might be effective in reducing the formation of tissue adhesion.

In summary, physical barriers manage to become a satisfactory method in adherent post-surgical prevention and they avoid undesirable damages as no drug is required. However, they have no effects in other typical issues as for example inflammatory reaction or in the removing fibrin already established.

Combination between physical barriers and pharmacological delivery are in most current investigations. N. Bölgen et al (Bolgen, Korkusuz, & Vargel, 2006) made a poly (e-caprolactone) (PCL) membrane embedded with the antibiotic Biteral® processed by electrospinning. Their issue showed that the use of these barriers reduces the extent, type and tenacity of adhesion. With the addition of antibiotic, their embedded membranes significantly eliminated post-surgical abdominal adhesions and also improved healing. However, the application of PCL in anti-adhesion products has been limited because of its inflexibility and hydrophobicity.
Nowadays, some of the most ambitious investigations have attempted developing biodegradable membranes as barriers that can protect tissue when they are implanted but dissolve when they are no longer needed. As all biodegradable prosthesis, this sort of membrane has a great advantage from permanent prosthesis because second surgery is avoided. The risk of infection usually increases dramatically whether more surgeries are required.

Interceed, made by Johnson & Johnson and Seprafilm, made by Genzyme Corporation are two common examples. Both of them dissolve and form gel barriers after their placement. Studies showed that the formation of postoperative adhesions could be reduced by these barriers. However, the efficacies of Interceed may be significantly reduced in the presence of blood.

Although Seprafilm succeed in abdominal adhesion prevention, it is not recommended in healing procedures that takes long time of release, as for example peritendinous adhesion (Chen, Chen, & Teng, 2014). Furthermore, Seprafilm is difficult to place in the peritoneal cavity because the sheets are not deformable and can easily become stuck when they wet.

Katsunori Takagi et al have compared their biomembrane, composed of aldehyde dextran and ε-poly(L-lysine), with Interceed and Seprafilm. They reported equivalent efficacy to that two commercial anti-adhesion materials. (Takagi K., Araki, Fukuoka, Takeshita, & Hidaka, 2013)

### 1.3 Electrospinning

#### 1.3.1 Taylor Cone

Taylor cone was described by G. I. Taylor in 1964. He observed the behavior of liquids in a medium with electromagnetic field.

When the bubble is exposed to an electric field, the shape of bubble is stretched upward and deformed from spherical to oval shape and finally to conical shape. From the conical bubble a jet or numerous jets can be ejected. If the applied voltage reaches the threshold value, the bubble in the electric field becomes instable and breaks.
1.3.2 Electrospinning process

A typical electrospinning setup consists of a capillary through which the liquid to be electrospun is forced; a high voltage source with positive or negative polarity, which injects charge into the liquid; and a grounded collector.

The basis of electrospinning is the application of a strong electric field. The polymer solution or melt is hosted in a syringe pump. When a high voltage is applied, usually between 1 and 30 kV, the pendant drop of polymer solution will become highly electrified and the induced charges are distributed over the surface. As it was explained in the chapter before, the liquid drop will be deformed into a conical object by the electrostatic field, the Taylor cone.

When the voltage reaches a threshold value, the electric force overcomes the surface tension of the droplet and one or multiple charged jets of the solution are ejected from the tip of the droplet depending on the electric field intensity. As the jet moves toward a collecting metal screen (counter electrode), solvent evaporates. While it is elongating, small bends in the fiber can be formed due to electrostatic repulsion until it is finally deposited on the grounded collector.

The elongation and thinning of the fiber leads to the formation of a uniform fiber with micro and nanometer scale diameters, which can be collected in various orientations to create unique structures with different composition and mechanical properties.

Despite the relative ease of use of electrospinning, there are several factors that have to be optimized in order to prepare an accurate membrane. These factors can greatly affect fiber formation and structure. Suboptimal parameterization could lead to bead defects in the spun fibers or even failure in jet formation.
Among these factors, the most important parameters that have to be considered are capillary-collector distance, voltage applied and flow rate. The way these parameters affect in solutions depends on the type of solution that we are working with. However, besides exceptions, there are general relations between parameters and results.

1.3.3 Electrospinning parameters

Surface tension

When a very small drop of water falls through the air, the droplet generally is an approximately spherical shape. In the absence of other forces, including gravity, drops of all liquids would be perfectly spherical. The liquid surface property that causes this phenomenon is known as surface tension.

For a liquid molecule submerged within the solution, there is a net attractive forces exerted on it by other liquid molecules in the surroundings. However, for a liquid molecule at the surface of the solution, there is a net downward force because the liquid molecules below exert a greater attractive force than the gas molecules above.

The net effect of the pulling of all the surface liquid molecules causes the liquid surface to contract by reducing its surface area. The shape which minimizes the surface area of volume ratio is a spherical shape.

![Attractive forces between the liquid molecules are stronger than air molecules](image)

In electrospinning, the charges on the polymer solution in the surface must be high enough to overcome the surface tension of the solution.

As the solution jet accelerates from the tip of the source to the collector, the solution is stretched. However, surface tension of the solution may cause the solution to breakup into droplets meanwhile. When droplets are collected, a different process called electrospraying is taking place rather than electrospinning, where fibers are collected instead. Surface tension has also been attributed to the formation of beads on the electrospun fibers. For
all of that, it is important to understand the role of surface tension in a fluid.

Surface tension also has a slightly relation with concentration and viscosity of the solvent, which are detailed in following sections.

With a high concentration of free solvent molecules, there is a greater tendency for the solvent molecules to congregate and adopt a spherical shape due to surface tension.

When there is a high viscosity in solution, it means that there is stronger interaction between the solvent and polymer molecules. Thus, when the solution is stretched under the influence of the voltage applied, the solvent molecules will tend to spread over the polymer molecules. So that it reduce the tendency for solvent molecules to come together under the influence of surface tension, which creates undesirable bead defects.

![Figure 5](image)

**Figure 5**: At high viscosity, the solvent molecules are distributed uniformly over the polymer molecules (A). With a lower viscosity, the solvent molecules tend to congregate due the action of surface tension (B)

Another way to reduce the surface tension is to add a surfactant to the solution. The addition of surfactant was found to yield more uniform fibers. (Jing Zeng, 2003)

**Molecular Weight and Solution Viscosity**

The molecular weight of the polymer represents the length of the polymer chain, which have an effect on the viscosity of the solution. Generally, when a polymer of higher molecular weight is dissolved in a solvent, its viscosity tends to be higher than same solutions with lower molecular weight. In fact, the polymer length determines the amount of entanglement of the polymer chains in the solvent which at the same time determines its viscosity.
Another way to increase the viscosity of the solution is to increase the polymer concentration. In fact, there is a close relation among molecular weight, polymer concentration and viscosity. Concentration and molecular weight are directly proportional with viscosity.

Regardless whether low viscosity is due to its low molecular weight, low solution concentration or both, it has been observed that a solution with low viscosity tends to form beads rather than fibers. In order to maintain the continuity of the jet during electrospinning, it is necessary to increase its viscosity. When the viscosity increases, there is a gradual change in the shape of the beads from spherical to spindle-like until a fiber is obtained. In addition, by increasing the concentration or weight, greater polymer chain entanglements will be established within the solution and fibers will be produced with larger average diameter.

However, a too high viscosity will make it very difficult to pump the solution through the syringe needle (Kameoka, et al., 2003) Moreover, the solution may dries at the tip of the needle before electrospinning can be initiated (Zong, et al., 2002) and it might appear beads defects, similarly with the case of too low viscosity.

In conclusion, an optimum range of polymer concentrations, molecular weight and viscosity exist in which fibers can be electrospun when all other parameters are held constant.

**Dielectric Effect of Solvent**

The dielectric constant of a solvent has a significant influence on electrospinning. Generally, a solution with a greater dielectric property reduces the beads formation and the diameter of the resultant electrospun fiber.

The bending instability of the electrospinning jet also increases with higher dielectric constant. This may also facilitate the reduction of the fiber diameter due to the increased jet path.

However, if a solvent of higher dielectric constant is added to a solution to improve the electrospinnability of the solution, the interaction between the mixtures such as the solubility of the polymer may changes, having an impact on the morphology of the resultant fibers.

**Voltage**

Deitzel et al. (Deitzel, Kleinmeyer, Harris, & Tan, 2001) tested a polyethylene oxide (PEO)/water system and found that changes in applied voltage altered the shape of the surface at which the Taylor cone and fiber jet were formed.
In low applied voltage, a pendant drop is formed at the tip of the capillary. As it was exposed before, the Taylor cone then is formed at the tip of the pendant drop. If the applied voltage increases, the greater amount of charges accelerates the jet and more volume of solution is drawn from the tip of the needle to the collector. This may result in a smaller and less stable Taylor Cone.

The strength of the applied electric field controls formation of fibers from several microns in diameter to the scale of nanometers. Suboptimal field strength could lead to bead defects in the spun fibers or even failure in jet formation. In most cases, a higher voltage lead to greater stretching of the solution due to the greater columbic forces in the jet as well as the stronger electric field. It reduces the diameter of the fibers and allows faster solvent evaporation to produce drier fibers. When a solution of lower viscosity is used, a higher voltage may favor the formation of secondary jets during electrospinning which also reduce the fiber diameter.

Another factor that may influence the diameter of the fiber is the flight time of the electrospinning jet. A longer flight time might allow more time for the fibers to stretch and elongates before it is deposited on the collection plate. Thus, at a lower voltage, the reduced acceleration of the jet and the weaker electric field may increase the flight time of the electrospinning jet which may favor the formation of finer fibers. In this case, a voltage close to the critical voltage for electrospinning may be favorable to obtain finer fibers.

Flow-rate

Polymer flow rate also has an impact on fiber size, and additionally can influence fiber porosity as well as fiber shape. The flow-rate will determine the amount of solution available for electrospinning. For a given voltage, there is a corresponding flow-rate if a stable Taylor cone is maintained. When the flow-rate is increased, there is a corresponding increase in the fiber diameter or beads size.

However, there is a limit to the increase in the diameter of the fiber due to higher flow-rate.

Due to the greater volume of solution drawn from the needle tip when flow-rate is higher, the jet needs longer time to dry. As a result, the solvents in the deposited fibers may not have enough time to evaporate given the same flight time. The residual solvents may cause the fibers to make together forming webs. A lower flow-rate is more desirable because the solvent will have more time for evaporation.
Voltage, flow rate and concentration are parameters which have great impact in the morphology of yielded fibers. The Figure 6 represents a scheme of the effect on solution in changing these parameters. At very low concentrations fabricated particles collapse into rings, discs, etc. By increasing the concentration, spherical particles can be formed with different sizes by changing the flow rate and applied voltage. When the concentration is higher, it starts to produce particle-tail structures or beaded fibers depending on the processing condition. When a critical concentration is reached, uniform fibers are produced. Although these general principles are applicable for most electrospinning conditions, it has to be remarked that solutions with specific properties such as high conductivity may behave differently.

**Solution Conductivity**

Electrospinning involves stretching of the solution caused by repulsion of the charges at its surface. Thus, solutions with high conductivity will have a greater charge capacity than solutions with low conductivity. Moreover, the fiber jet of highly conductive solutions will be subjected to a greater tensile force in the presence of an electric field than would a fiber jet from a solution with a low conductivity.

The conductivity of the solution can be increased by the addition of ions. Since the presence of ions increases the conductivity of the solution, the critical voltage for electrospinning to occur is also reduced.

When a small amount of salt or polyelectrolyte is added to the solution, the increased charges of the solution will increase the stretching of the solution.
Finally, the increased in the stretching of the solution tend to produce fibers of smaller diameter (Zong, et al., 2002).

Another way to increase the conductivity of the solution is by changing the pH of the solution. In some cases, the addition of ionic salt may cause an increase in the viscosity of the solution. Thus, although the conductivity of the solution is improved, the viscoelastic force is stronger than the columbic force resulting in an increase of the fiber diameter instead.

**Temperature**

The temperature of the solution has both the effect of increasing its evaporation rate and reducing the viscosity of the polymer solution.

When polyurethane is electrospun at a high temperature, the fibers produced have a more uniform diameter. This may be due to the lower viscosity of the solution and greater solubility of the polymer in the solvent which allows more even stretching of the solution.

However, in cases where biological substances such as enzymes and proteins are added to the solution for electrospinning, the use of high temperature may cause the substance to lose its functionality.

**Collector**

There must be an electric field between the source and the collector for electrospinning to initiate. Thus, in most electrospinning setup the collector plate is made out of conductive material such as aluminum foil which is electrically grounded so that there is a stable potential difference between the source and the collector.

In the case when a nonconducting material is used as a collector, charges on the electrospinning jet will quickly accumulates on the collector which will result in fewer fibers deposited.

Fibers that are collected on the non-conducting material usually have a lower packing density compared to those collected on a conducting surface. This is caused by the repulsive forces of the accumulated charges on the collector as more fibers are deposited. For a conducting collector, charges on the fibers are dissipated allowing more fibers to be attracted to the collector.

The porosity of the collector seems to have an effect on the deposited fibers. Experiments with porous collector such as paper and metal mesh had shown that the fiber mesh collected had a lower packing density than smooth surfaces such as metal foils.
This can be attributed to the diffusion and rate of evaporation of the residual solvents on the fibers collected. In a porous target, there is faster evaporation of residual fibers due to higher surface area while smooth surfaces may cause an accumulation of solvents around the fibers due to slow evaporation rate. However, on a smooth surface, the residual solvents will encourage the residual charges to be conducted away to the collector.

The fact whether the collector is static or moving also has an effect on the electrospinning process. While rotating collector has been used to collect aligned fibers, it was found to assist in producing fibers that are dry. This is useful because certain solvents which are good for electrospinning but have a high boiling point that may result in the fibers being wet when they are collected. A rotating collector will give the solvent more time to evaporate (Wannatong & Sirivat, 2004) and also increase the rate of evaporation of the solvents on the fibers.

The internal diameter of the needle or the pipette orifice has a certain effect on the electrospinning process. A smaller internal diameter was found to reduce the clogging as well as the amount of beads on the electrospun fibers (Mo, Xu, Kotaki, & Ramakrishna, 2004).

**Distance between Tip and Collector**

When the distance between the tip and the collector is reduced, the jet will have a shorter distance to travel before it reaches the collector plate. Moreover, the electric field strength will also increase at the same time and this will increase the acceleration of the jet to the collector. As a result, there may not have there may not have enough time for the solvents to evaporate when it hits the collector. Decreasing the distance has the same effect as increasing the voltage supplied and this will cause an increased in the field strength. Thus, beads are formed when the distance is too short. At longer distances, the fiber diameter increases. This is due to the decrease in the electrostatic field strength resulting in less stretching of the fibers.

To sum up, in table 1 it is represented a resume of how changes in most important parameter effect on fiber morphology.
**Table 1: Parameters with their effects on morphology**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Effect on fiber morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied voltage ↑</td>
<td>Fiber diameter ↓ initially, then ↑ (not monotonic)</td>
</tr>
<tr>
<td>Flow rate ↑</td>
<td>Fiber diameter ↑ (beaded morphologies occur if flow rate is too high)</td>
</tr>
<tr>
<td>Distance between capillary and collector ↑</td>
<td>Fiber diameter ↓ (beaded morphologies occur if the distance is too short)</td>
</tr>
<tr>
<td>Polymer concentration (viscosity) ↑</td>
<td>Fiber diameter ↑ (within optimal range)</td>
</tr>
<tr>
<td>Solution conductivity ↑</td>
<td>Fiber diameter ↓ (broad diameter distribution)</td>
</tr>
<tr>
<td>Solvent volatility ↑</td>
<td>Fibers exhibit microtexture (pores on their surfaces, which increase surface area. Hollow fibers)</td>
</tr>
</tbody>
</table>

**Humidity**

The humidity of the electrospinning environment has also an influence. At high humidity, it is likely that water condenses on the surface of the fiber when electrospinning is carried out. In suitable humidity condition (normally below 35%) the membranes produced are soft. Nevertheless, when the humidity is a formation of craters and holes tends to appear in the surface. These is a phenomenon that we have actually experimented throughout so good environmental conditions have to be considered in order to produce membranes with soft surfaces (which is highly recommended as the surface of membranes are able to change adhesive properties of material. These defeats can be attributed to capillary instabilities, which caused the breakup of the jet into droplets. Thus, greater humidity also causes the formation of more bead defeats. (Bonino, Efi, Jeong, & Krebs, 2012)

1.3.4 Electrospinning in drug delivery system

Electrospinning technique is widely used in drug delivery systems as it takes significantly advantages from conventional techniques. Electrospinning allows a direct encapsulation of drugs into the electrospun fibers. The high surface area of nanofibers and open porous structure reduce the limitation towards drug diffusion, resulting in a more efficient system. In addition, local drug delivery by electrospun fibers can decrease the minimum required dosage of the drug, reducing unwanted side effects. Electrospun membranes also can be cut to any size and shape, making them suitable for target clinical application. All these features provide
Electrospinning the opportunity of being one of the most accurate techniques in giving finer control over drug release kinetics.

1.3.5 Electrospinning in tissue engineering

All biological tissues (blood vessels, cartilage, bone, nerves, skin, etc.) are composed by nanofiber structures. In the field of tissue engineering and biomaterials one of biggest challenge is to produce artificial extracellular matrixes (ECM) which can mimic the structure and function of natural ECM in order to heal tissues or dysfunctional organs. Human cells are able to be attached and organized among fibers with fewer diameters. This allows nanofibers to be a suitable support for cells in order to migrate and develop. But the scaffold must have some properties in order to support cell attachment as well as to facilitate nutrient transport throughout the assembly. Therefore, both a high surface area and an interconnected three dimensional (3D) porous structure are essential.

Proper geometry and pore dimensions are also factors that can significantly affect cellular behavior (Khorshidi, et al., 2015)

1.4 Materials proposed

1.4.1 Chitosan

Chitosan (CHT) is the N-deacetylated derivative of Chitin. Chitin is a naturally abundant mucopolysacharide commonly used as supporting material of several crustacean, insects, etc. It consist of 2 2-acetamido-2-deoxy-β-D-glucose through a betha(1→4) linkage. (Figure 7) In chitin, the degree of deacetylation (which means the percentatge of group acetyl removed) is typically 0.1 indicating that some amount of deacetylation might take place during extraction from natural resources.

When the degree of deacetylation of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. It also becomes a charged positive molecule. Because of its solubility in aqueous solutions and polarity charge, it is largely used in different applications as solutions, gels, or films and fibers.

![Structure of chitosan molecule](image-url)

*Figure 7: structure of chitosan molecule*
It is demonstrated that Chitosan has also biocompatible and biodegradable properties, besides other accurate properties such as antitumoral and antibacterial activities. (Lifeng & Zirong, 2006) (Benhabiles, et al., 2012) (Jarmila & Vavríková, 2011) Thus, it is not surprising the fact that Chitosan has become into a typical material for medical applications as newer applications has been discovered dramatically during this latest decades.

The biocompatibility and antibacterial proprieties allow chitosan to be an appropriated possible candidate to develop a membrane through the prosthesis, avoiding painful or unexpected reactions in the host.

It has already been proven that the crosslinking between chitosan with other polymers has an anti-biofouling performance in terms of the anti-adhesion and anti-bacteria effects avoiding the colonization of bacteria. Jing Fu et al (Fu, Ā, Yuan, & Shen, Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan, 2005) have made an antibacterial and anti-adhesive membrane onto a PET membrane by the assembly of chitosan and heparin. (Liu, Zhang, He, & Zhao, 2010).

One of the main reasons we have chosen chitosan in this project is to produce an anti-fouling membrane to avoid bacterial colonization and to create an anti-adherent membrane between the prosthesis and host tissue.

**Electrospinning of chitosan**

Producing a pure membrane of CHT by electrospinning has been a big challenge for researchers but not clear succeed has been reported. Chitosan has high viscosity due to its strong molecular chain and its crystalline structure caused by the presence of amino and hydroxyl group. They have been some attempts in electrospinning sole CHT but results showed that the "electrospinnability" of sole CHT remains unclear.

One of the firsts to report a membrane with almost pure CHT were Spasova et al (Spasova, Manolova, & Paneva, 2004). They carried out the electrospinning of a solution made by chitosan and polyethylene oxide (PEO) blend. Their results showed that the electrospinning of this blend is possible with a chitosan/PEO mass ratio of <1. Since then, several experiments with chitosan-PEO have been reported and PEO has become a typical polymer used to improve electrospun properties of chitosan. (ref A fundamental study of chitosan/PEO electrospinning) .
1.4.2 PEO

Polyethylene oxide (PEO) and polyethylene glycol (PEG) are polymers with the subunit C-O-C (Lee, Venable, Mackerell, Jr, & D, 2008). In general, poly(ethylene glycol) refers to polyols, an alcohol containing multiple hydroxyl groups) of molecular weights below 20,000, poly(ethylene oxide) refers to higher molecular weight polymers. PEO can be dissolved in chloroform, ethanol, dimethylformamide and water (Keun, Ho, Seung, & Ho, 2004). PEO has several proprieties which make it an interesting biomaterial. PEO is a nontoxic, hydrophilic and biocompatible polymer. Thus, PEO has attracted tremendous attention in the biomedical field, especially in developing the scaffolds for tissue engineering and for controlled drug release. In drug release, PEO has been extensively used as a hydrogel or as a matrix, due to its capability in altering pharmacokinetics. As long as we will manage to carry out an anti-adherent membrane for an abdominal prosthesis, it must not be forgotten our horizons in developing an anti-adherent membranes which could be able also to perform as drug carrier.

PEO is also one of the most common high molecular weight polymers cross-linked with chitosan. Due to its high molecular weight, and as Spasova et al demonstrated, (Spasova, Manolova, & Paneva, 2004) it is possible to create a solution CHT-PEO with low concentration of PEO.

1.5 Crosslinking of CHT-PEO

The fibers tend to collapse in aqueous medium. This is an effect undesirable as during the surgical implantation of the mesh a lot of blood gets in contact with the mesh. Cross-linking nanofibers is necessary for preserve their stability in aqueous medium. Moreover, cross-linked nanofibers present better mechanical properties and low degradation ratio. Biodegradable polymers such as chitosan need to be cross-linked in order to modulate their general properties and to last long enough for delivering drugs over a desired period of time.

1.5.1 Glutaraldehyde


In Figure 8 is represented the cross-linking that may occur between chitosan and glutaraldehyde. GTA can react with free amino groups of chitosan, leading to the formation of imines.
Cross-linking enables more intermolecular interactions among the chains of chitosan. Therefore, greater tensile force will be required in order to break the same amount of polymer chains so as there is a theoretical increase in mechanical properties of membrane. However, one of the biggest challenges is the fact that glutaraldehyde is considered a toxic chemical product and is not recommended for medical purpose. The test in vitro will verify its toxicity.

1.5.2 Genipin

Genipin is a desirable cross-linking agent because it has been proven that among all cross-linking agents tested with chitosan, genipin are among the agents with less cytotoxic. Yuan et al (Yuan, Chesnutt, Utturkar, Haggard, & Yang, 2007) demonstrated that genipin is about 10,000 times less cytotoxic than glutaraldehyde. They have recently reported genipin-chitosan cross-linked material using chitosan and chitosan-glutaraldehyde membranes as control.

It has been demonstrated that chitosan membranes show less swelling reaction, slow degradation and faster healing times when cross-linked with genipin compared with glutaraldehyde. (YC, HC, RN, & HW, 2001) They have found that cross-linking of chitosan membrane using genipin increased its ultimate tensile strength but significantly reduced its strain-at-fracture comparing with glutaraldehyde and chitosan membrane without genipin, suggesting that genipin crosslinked agent change the mechanical properties of chitosan as other studies had already shown (Bvariya, et al., 2013). They reported no significant difference in antimicrobial activity between chitosan membrane with and without genipin.

Figure 9 shows the reaction mechanism for the synthesis of genipin crosslinked chitosan network. The carboxymethyl group of genipin firstly reacts with amino group of chitosan to form secondary amide. Then, nucleophilic attack by amino group of chitosan occurs on the third carbon of genipin followed to form heterocyclic amine.

Under basic conditions, genipin underwent a ring-opening polymerization prior to crosslinking with chitosan (Fu, A, Yuan, & Shen, Characterization of...
Ring-Opening Polymerization of Genipin and pH-Dependent Cross-Linking Reactions Between Chitosan and Genipin, 2005). Then, more engagements are formed within fibers so it is an increase of mechanical properties.

**Figure 9:** Crosslinking reaction between chitosan and genipin
CHAPTER 2: MATERIALS AND METHODS
2.1 Materials
PEO with a molecular weight (Mv) of 900,000 g mol\(^{-1}\), Chitosan of low Molecular weight and glutaraldehyde water dilution of 50/50 were purchased from Sigma–Aldrich Incorporation (St. Louis, MO, United States).

Genipin of molecular weight of 226.23 g mol\(^{-1}\) was purchased at Wako chemicals (Osaka, Japan).

Acetic acid with a purity of 99-100% was used as a solvent. It was purchased from Chem-Lab (Zedelgem, Belgium).

Distilled water was also used in several experiments.

2.2 Methods
In this chapter, all the methods followed during the experiments are summarized. The first part corresponds to the preparation of solutions.

Once solutions were prepared, we proceeded to the yielding of membranes which it is explained in “Electrospinning process”. In this part, optimal conditions are detailed and compared with suboptimal conditions (slight changes in the parameters). The morphology of membranes was observed in order to determine whether parameters chosen are accurate or not.

When membranes were produced, three different types of cross-linking were carried out with the willing of improving their mechanical properties. (which are explained in “Cross-linking of membranes” part)

Different test of characterization were used in order to determinate which reactions are involved during cross-linking as well as to establish a comparison between the three kinds of cross-linking. (see “Characterisation” part)

Finally, stability of the different kind of membranes was tested in “Degradation test”. Moreover, a study in vitro was performed so as to investigate the biocompatibility and cellular adherence of membranes.

Some tests in attaching membranes into textile surface were accomplished. As further tests are required, it was decided to not include these tests in this chapter. However, a small discussion is included in “Conclusions” chapter.
2.2.1 Preparation of chitosan- polyethylene oxide solution

3.5 % of (CHT)/polyethylene oxide (PEO) mixtures (ratio 9:1) were dissolved in 90%(v/v) acetic acid aqueous solution.

In order to ensure the complete dissolution of the polymers and to obtain homogeneous solutions, solutions were constantly mixed throughout 12 hours by a magnetic agitator at 300 rpm. In some solutions tends to appear some bubbles because of the agitation. As they might constitute some problems for the electrospinning, solutions were treated with ultrasonic bath at ambient temperature.

2.2.2 Electrospinning of Chitosan-PEO

Fibrous scaffolds were produced with an electrospinning set-up, assembled in-house, comprising:

A high voltage power supply(Figure 12: High voltage supply) provides a continuous tension with a maximum voltage of 20 kV (provided by Iseg company, model T1CP 300 304p).

Anode and cathode electrodes were connected to the collector and the tip of metal capillary, respectively.

The needle is hold by a support which allows it to move in parallel to the surface of the collector. The needle is connected to a syringe by a plastic tube. The syringe, which contains the solution, is pushed by a pump. Figure 11 (provided by Fisher company). This pump motorizes the flow rate and total volume to inject depending on the diameter of syringe.

Fibers were collected on a cylindrical rotating metallic mandrel with a rotating speed going from 50rpm to 1000rpm. The collector, connected to the mass, was covered by aluminum folder.

In table 2 general parameters used for the yielding of all membranes are summarized.

<table>
<thead>
<tr>
<th>Needle diameter (mm)</th>
<th>Syringe diameter (mm)</th>
<th>Speed of collector rpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>12</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 3, summarizes most important parameters of solution with their respective optimal range of values.

**Table 3: Parameters of CHT-PEO electrospinning**

<table>
<thead>
<tr>
<th>Voltage (kV)</th>
<th>Flow rate (ml/h)</th>
<th>Distance between collector and tip (mm)</th>
<th>Temperature(°C)</th>
<th>Relative humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-13</td>
<td>0.2-0.3</td>
<td>200-220</td>
<td>15-35</td>
<td>&lt;35</td>
</tr>
</tbody>
</table>

The system was automatized by “Electrospinning computer program” (Figure 10). Parameters such as speed of needle displacement, speed of collector, stop and start of the system, kind of system (dynamic or static) were motorized by computer. In static conditions, the system could be motorized manually with an external analog hardware which modifies the speed of collector. Voltage supplied was monitored by the analog power supply.

![Figure 10: Digital electrospinning system](image)

Humidity was under suitable parameters (below 35%) by the use of silica gel with cobalt sulfate which change of color when it is hydrated so that it is easy to verify whether it has to be changed or not. They recover their desiccation properties by reheating.
Figure 11: Pump and syringe of electrospinning system

Figure 12: High voltage supply

Figure 13: Needle, support, humidity sensor and collector
2.2.3 Crosslinking of the membranes

In order to verify whether the membrane is crosslinked or not, membranes were introduced in an aqueous medium. If crosslinking reaction occurs, the membrane holds its structure when is putted into aqueous medium. Otherwise, membranes tend dissolve instead of keeping their physical structure which means that it undergoes a high degradation.

Three different methods of cross-linking have been carried out. These methods are:

- Crosslinking with glutaraldehyde (GA)
- Crosslinking with genipin (Gnp)
- Thermal crosslinking

In the following chapters the procedure followed in order to achieve these different cross-linking is explained.

**Glutaraldehyde cross-linking**

We have considered glutaraldehyde as a possible candidate to carry out the cross-linking process.

For the GA cross-linking of CHT-PEO membrane, a solution of glutaraldehyde concentrated at 50% in water was placed in the lower compartment of vacuum desiccator. On the upper compartment, membrane was placed so as the liquid evaporated is the only element of solution which takes contact with the membrane.
We have performed some cross-linked membranes with GA at different exposition times: 4 and 24 hours.

**Genipin cross-linking**

Other sort of cross-linking considered is a chemical cross-linking with genipin as crosslinking agent.

Genipin tends to react after approximately 5 hours of exposure into chitosan. Once reaction is produced, the solution is not able to be electrospun. Genipin with a concentration of 0.1% was mixed with CHT-PEO solution few minutes before the electrospinning process. After 1.5 ml of membrane produced (which corresponds to 5 hours of electrospining with a flow rate of 0.3ml/h) solution was renewed.

Once the membranes were yielded, it was activated in order to induce cross-linking reaction. For the activation, membranes where deposed in a dissecator containing distilled water throughout approximately 24 hours at a stable temperature of 37°C. Only evaporated water was in contact with the membrane.

**Thermal cross-linking**

Membranes were put in 90°C throughout 24 hours. Other membranes were placed in 140°C throughout 4 hours. These temperatures do not constitute any chemical change in the membrane because they are lower than the degradation temperature of both polymers (this fact will be corroborated in the thermal analysis).

2.2.4 Characterisation of membranes

In order to verify the properties that we are looking for some techniques of characterization have been carried out. These techniques have two general purposes. Firstly, some of them can provide interesting information in order to understand which kind of chemical or physical reactions are produced during each cross-linking methods. Moreover, they constitute useful tools to observe and compare all cross-linking methods and determine whether there is a type of membrane that should be discarded or constitutes damage to the host.

**Scanning electron microscope (SEM)**

Owing to the SEM, the morphologies of the obtained membranes could be observed and useful information of which parameters could be considered optimal and which parameters should be changed are provided.

It is an efficient technique to evaluate diameters of fibers, presences or absence of bead defects or other types of defects.
Nanofibers morphologies of the electrospun nanofibers were investigated with scanning electron microscopy (SEM, Hitachi S-4700). The samples were cut with a scalpel and placed on the platinum support with an adhesive tape and a thin chrome layer (100 Å) was deposited in order to make the surface of nanofibres conductive.

**Fourier transform infrared spectrometry**

Fourier transform infrared spectroscopy (FTIR spectroscopy) is a technique used to obtain an infrared spectrum of absorbance of chemical compounds. This tool was used in order to better clarify what kind of reactions might occur by the different types of cross-linking.

The information on structural changes which took place during the crosslinking was collected by a transmission spectrum at room temperature against an air background. Powders and obtained nanofibres crosslinked or not were scanned at wave numbers ranging from 4000 to 700 cm\(^{-1}\) with a 4.0 cm\(^{-1}\) resolution.

**Swelling test**

Swelling behavior of the membranes was performed by immersing the membranes in distilled water at 37 °C. The swollen sample weights were measured after removing the excess of water in surface by tapping the surface with filter water paper. Water absorption was determined from the differences in weight of the swollen membrane and the initial mass (in dried state) using Eq. (1). Experiment was repeated three times for each sample, and the average value was considered to be the water absorbance ratio. All crosslinked membranes has been tested throughout 2h, 4h, 24h and 48h.

The capacities of water absorption were tested by immersing gnp-crosslinked, GA crosslinked and Thermal-crosslinked (at 90°C and 140°C) nanofibrous CTS samples in a distilled water at 37°C for 2, 4, 24, 48 hours with gentle shaking. Subsequently, the weight of the swollen membrane was measured and the swelling ratio calculated according to equation as follows:

\[
Swelling(\%) = \frac{W_o - W(t)}{W(t)} \times 100
\]  

(1)

**Thermal properties:** thermogravimetric analysis

Thermogravimetric analysis (TGA) is a method of thermal analysis which measure physical or chemical changes of material as a function of temperature. This technique has realized in order to figure out the maximum temperature in which membranes are able to withstand. Being aware of the maximum temperature is essential to choose accurate range of
temperatures in techniques which requires heat, as for example in differential scanning calorimetry.

TGA was carried out to all the different types of cross-linking, non cross-linked membrane, chitosan and PEO powder. They were subjected in a range from -80°C to 800°C. Temperature increased lineally under a constant heating rate of 10°C/min. During this process the mass of membrane was constantly measured and compared to initial mass in order to determinate the loss of mass (degradation) until all membrane was evaporated. Graphs represented the weight percentage (from initial weight) during the increase of temperatures.

**Thermal analysis: Differential scanning calorimetry**

Differential scanning calorimetry (DSC) is a thermo analytical technique used to study the thermal transition of a sample. It measures the difference in the amount of heat required to increase the temperature of this sample and a reference as a function of temperature.

DSC was accomplished to the same substances tested in TGA. The DSC curves were obtained under nitrogen flow. All cross-linked membranes were washing with distilled water and data was collected before and after washing.

Range of temperatures is determined by the results of TGA so that depending on the sample analyzed the range varies.

Similarly to thermogravimetric analysis, temperature increased from -80 to the maximum temperature of work (depending on the sample) with a constant heating rate of 10°C/min. After reaching the maximum point, temperature decreases until -80°C and the process restarts. Graphs resultants include both complete cycle of the flow heat depending on the temperature.

2.2.5 Degradation study of membranes

**Degradation test in vitro of CHT-PEO**

As it has been mentioned in the introduction, prepared membranes are biodegradable. In order to evaluate the degradation time, an in vitro test of degradation must be performed. It consists on evaluating the weight loss during several days.

Moreover, as it was explained in the previous section, these degradation tests could provide us information about how environment affects the membranes and see whether there is a difference in behavior with the different sorts of crosslinking.
The electrospun nanofibres mats were cut and each cut specimen were measured as an initial weight (~ 10mg) and then placed in a test vial contained 10mL of aqueous medium (Phosphate buffered saline solution 0.1M (pH7.4), or acidic medium (pH4)). Vials were placed under stirring for 37°C for 28 days. After each time interval, samples were removed, rinsed several times with distilled water and kept at room temperature to dry. The mass loss was determined by gravimetric method by comparing the dry weight remaining at specific time with the initial weight according to the following equation:

\[
\text{degradation} = \frac{W_0 - W(t)}{W(t)} \times 100(\%)
\]

Where \( W_0 \) indicates the initial mass and \( W(t) \) indicates mass in period \( t \).

2.2.6 In vitro test

Two different kinds of test in vitro have been applied to CHT-PEO membrane: vitality test and adhesion test. Commercial cell line of epithelial cells has been exposed to membranes. Hence, we were able to simulate human conditions in order to predict the behavior of membranes in these conditions.

Cells were incubated in EARl’s minimum essential medium (MEM) supplemented with 5% of fetal calf serum containing 50µg ml\(^{-1}\) of gentamicin at 37°C.

Samples (11mm diameter disks) were sterilized under UV irradiation for 30minutes (15 minutes for each face) and they were placed at the bottom of a 24-wells cell culture plates (COSTAR® Starlab) and then a Viton® rings were added to maintain the samples at the bottom.

**Adhesion test**

3 cell culture plates were prepared, one for each incubation time (30, 60 and 120 minutes). 6 samples disks were introduced into the cell plates. The wells without samples were used as the control groups. 0,6ml of the culture medium and 0.4ml of the cells solution were added to each well in order to obtain 40000 cells per well.

After each incubation time, media was removed and wells were rinsed two times with PBS. Then, 300µl of para-nitrophenylphosphate (pNPP) was added to each well. After 3 hours of incubation, reaction was stopped by adding 150µl of NaOH solution (1M). 200µl of each well were transferred to a 96-wells cell culture and the absorption at 405nm of each well was measured by using an ELISA lecture.
An increment in absorbance means that more irradiation of the spectrum is absorbed by the sample. This means that there is an increment of particles in the sample which does not allow the light to cross through the sample to the detector. Thus, this increment of particles is in fact an increment of cells in the culture which is the opposite effect expected.

**Vitality test**

2 cell culture plates were prepared, one for each incubation time (1 and 3 days). 10000 cells were seeded in each well. Cell vitality was assessed using the non-toxic dye Alamar Blue®. 1 and 3 days after cell seeding the culture medium was removed and 500*L of diluted Alamar Blue® was added to each sample. After 3h of incubation time, 100µl of this solution was transferred to a 96-well plate and the fluorescence measured with twinkle LB 970™ fluorometer at an exciting wavelength of 510nm and an emission wavelength of 590nm.

By testing membranes viability, we are able to obtain an estimation of the toxicity of membranes. As it has been explained in this project, crosslinking between CHT-PEO and glutaraldehyde had to be studied carefully due to glutaraldehyde high toxicity so a vitality test can give information about the viability in using glutaraldehyde. Moreover, some publications had reported that genipin is an inert crosslinking agent so that this test could give us information to confirm or deny it.

All materials used were previously sterilized. Both methods were evaluated according to ISO 10993-5 Standard.
CHAPTER 3: RESULTS AND DISCUSSION
3.1 Morphology of the obtained membranes

Several research groups have studied the electrospinning of chitosan (Bhattarai et al., 2005; Bizarria et al., 2014; Cheng et al., 2015; Elsabee et al., 2012). The most of these studies showed that electrospinning of neat chitosan is a real challenge because of the highly viscous behavior of chitosan in aqueous medium. However, this obstacle could be overcome by electrospinning chitosan mixed with another polymer like PEO. In this work, we succeeded to produce based chitosan nanofibres by using a blend solution of chitosan and PEO with a mass ratio of 9/1. The solution parameters and electrospinning parameters were optimized previously.

Figure 15 shows the microscopic image of the nanofibers produced by CHT-PEO solution. With a concentration of 3.5%, defect-free, beadless, and geometrically uniform nanofibers were observed.

![Microscopic image of CHT-PEO nanofibers](image)

**Figure 15: Nanofibers of CHT-PEO without crosslinking**

Following what it was explained in the introduction, when the voltage is lower than 12 kV less volume is ejected causing an increase of the size of Taylor cone. If the size of Taylor cone is big enough, gravity force is greater than electric forces which cause that cone falls as gout to the membrane. Then, holes are observed with naked eye ruining the membrane. (Figure 16)
Figure 16: Improperly conditions cause the formation of beads

When the humidity conditions were above 35% some protuberances visible by the naked eye appeared in the mat surface (Figure 17). They were attributed to the breakup of the jet due to the instability of the jet (Bonino, Efi, Jeong, & Krebs, 2012)

Figure 17: On the left, mats with accurate cross-linking. On the right, protuberances are observed with higher humidity

To improve the stability of chitosane-based membranes in aqueous medium, nanofibres were crosslinked with different ways.

All membranes that were cross-linked presented rougher and more consistent structure that can be a consequence of an improvement in their physical properties. A change of color was also appreciated in the case of chemical cross-linkings. Un-crosslinked electrospun membrane is white in color, but the Genipin-crosslinked electrospun CHT membrane showed a dark blue color whereas the GA-crosslinked electrospun membrane became yellow (Figure 18). Hence, different chromophores groups appeared after cross-linking reaction depending on the crosslinking agent used. The change of color in genipin might be associated with the oxygen radical-induced polymerization of genipin as well as its reaction with amino groups of CHT
(ref). Thermal-treatment did not change the color of membranes so that there is not chemical reaction involved in this type of crosslinking. However, membranes heated at 140°C slightly changed into toasted-color.

![Figure 18. From left to right: GA-electrospun, Genipin-electrospun, thermal-crosslinked at 90°C and thermal-crosslinked membrane at 140°C](image)

As a result of its low concentration in solution (0.1%), the addition of genipin has not an significant effect in fibers morphology so the electrospinning parameters were not changed.

Figure 19 show SEM images of CHT-PEO with genipin. Fibers experimented and increase of their diameter and a large amount of attachments were produced amount fibers. That engagements formed could increase the mechanical properties of mats. A change of shape is clearly reported from regular cylindrical form in the case of non-crosslinked fibers to an irregular shape.
**Figure 19:** SEM images of genipin-crosslinked nanofibers

Figure 20 shows SEM images of nanofibers after thermal cross-linking at 90°C and 140°C respectively. As it is shown, there were no significant changes in morphology of membranes either in their diameter size.

**Figure 20:** Image SEM of thermal-crosslinked nanofibers. On the right, fibers treated at 90°C. On the left, fibers treated at 140°C

Figure 21 show SEM images of glutaraldehyde-crosslinked mats. Morphology remains regular but attachments appeared among fibres.
Scanning electron micrographs were taken before and after the mats were washed with water. Images of non-crosslinked nanofibers after washing were not possible to take as the membrane was highly degraded. Membranes that were crosslinked did manage to persist in aqueous solution, so that it is clearly appreciable that crosslinking gives more stability to the structure of membranes. However, as observed in Table 4 the morphology of the after-washed fibers changes depending on the type of crosslinking. Thermal-crosslinked fibers turns into an irregular shape (rather than cylindrical) than the original structure whereas in genipin and GA crosslinking, the structure of membranes is almost maintained. Thus, chemical cross-linkings seems to have a stronger influence on the nanofibers stability.

In all cases, attachments among fibers appeared which causes a loss in porosity.
<table>
<thead>
<tr>
<th>Type of crosslinking</th>
<th>Before washing</th>
<th>After washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non crosslinked</td>
<td><img src="image1" alt="SEM image" /></td>
<td><img src="image2" alt="SEM image" /></td>
</tr>
<tr>
<td>Thermal</td>
<td><img src="image3" alt="SEM image" /></td>
<td><img src="image4" alt="SEM image" /></td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td><img src="image5" alt="SEM image" /></td>
<td><img src="image6" alt="SEM image" /></td>
</tr>
</tbody>
</table>
3.2 Fourier transform infrared spectroscopy

Figure 22 (a) presents FTIR spectra of powder chitosan. Chitosan exhibits an OH stretching band at 3352 cm$^{-1}$, C–H stretching peak at 2870 cm$^{-1}$, C–O–C stretching signal between 1150 cm$^{-1}$ and 1027 cm$^{-1}$.

The peak located at 1653 cm$^{-1}$ is attributed to –C=O vibration of its primary amine groups; while the peak at 1595 cm$^{-1}$ is related to the stretching of the -NH$_2$ secondary amino groups.

Figure 22 (b) presents FTIR spectra of powder PEO. The major absorption peak of PEO appeared at 1100 cm$^{-1}$ are due to the C-O-C symmetric bending vibration and sharp absorption bands at 1340 and 1359 cm$^{-1}$ are due to the vibrations of CH$_2$ groups in crystalline phase.

This is in accordance with IR characteristic bands of chitosan and PEO found in the literature. (Bostan, Mutlu, Kazak, & Sinan Keskin, 2012). (El Ichi, et al., 2015)

With the electrospinning of CHT-PEO mat, the bands of amino (1588.5 cm$^{-1}$) and hydroxyl (3358 cm$^{-1}$) shifted to 1557 and 3363.97 cm$^{-1}$, respectively. In addition; the vibration peaks of CH$_2$ of PEO changed to a lower frequency. The changes may have been due to the formation of new bonds of hydrogen among amino and hydrogen groups.
Figure 22: FTIR spectrum of CHT-PEO nanofibers (red), powder PEO (blue) and powder CHT (black)

Figure 23 shows the spectra of CHT-PEO mat with addition of genipin compared with non-crosslinked mat. As it was said in the introduction, the cross-linking mechanism with genipin involves the reaction of amino groups of chitosan which results in an amide linkage and a heterocyclic amine. Due to the replacement of the C=O stretching band of chitosan with the C=C stretching in the cyclic structure of genipin, the amide I band becomes slightly broader (Austero, Donius, Wegst, & Schauer, 2012).
After crosslinking with GA vapor (Figure 24), the primary amine peak intensity decreased, and a new peak for C=N imine appeared. This appeared as a strong split peak at 1663.25 cm$^{-1}$ and the peak at 1557 cm$^{-1}$ disappeared because of the loss of free amines in the GA-crosslinked nanofibers.

Figures 23: FTIR spectrum of CHT-PEO noncrosslinked mats (red) and Gnp-crosslinked mats (blue)
Figure 24: FTIR spectra of CHT-PEO non-crosslinked mats (red), GA-crosslinked mats (blue)

Figure 25 shows the spectra of non-crosslinked membranes in comparison to the thermal-crosslinked membranes treated at 90°C and 140°C. Thermal crosslinking does not report significant changes from non-crosslinked spectra. However, a decrease of absorbance in the case of thermal-crosslinking appeared around 3365 and 1385.73 cm⁻¹. This might be a consequence of the formation of the hydrogen links due to the curing.
3.3 Thermal properties

3.3.1 Thermogravimetric analysis

Figure 26 shows thermogravimetric analysis of powders and cross-linked membranes. In the case of chitosan, a slightly decrease of mass around the temperature of 100°C is observed. It is attributed to the evaporation of water associated with the hydroxyl groups present in the polymer chains. The melting point of chitosan (maximum pick of derivated weight) is located at 295°C as the percentage of mass highly decreases from 90% to 10% whereas the degradation point of PEO and CHT-PEO mats are located at 270°C and 210°C respectively.

In general terms, thermogravimetric analysis shows a considerable decrease of degradation temperature on the nanofibers (regardless if they are cross-linked or not) in comparison to powder chitosan and PEO.

Concerning the genipin-nanofibers, its rapidly degradation displayed on the graph might be due to a too high rate of degradation. Thus, further test with lower degradation rate (for example 5°C/min) must be tested.
3.3.2 Differential Scanning Calorimetry

CHT-PEO mats nanofibers without cross-linking and CHT-PEO mats thermal cross-linked before and after washed mats were characterized. PEO powder and chitosan powder were also characterized for comparison.

The Figure 27 represents the DSC analysis of chitosan powder. The sampling process starts at -80°C. From -80°C to approximately 40°C, the flow rate decrease slowly. Around 100°C a broad endothermic peak clearly defined. Endothermic peaks around 100°C are normally attributed to the loss of water associated with the hydroxyl groups present in the polymer chains (Pakravan, Heuzey, & Ajji, 2012). Therefore, this peak disappears in the second cycle supporting the view that water evaporation occurred during the first DSC run. This fact corroborates the loss of mass appreciated in the TGA of CHT is due to the evaporation of water.
For the neat PEO powder (Figure 28), two sharp endothermic peaks around 60-70°C are observed (one for each heating cycle). According to some authors it is related to its melting point (Neto, et al., 2005). The difference between both peaks might reside in the fact that in the first cycle PEO powder could has some impurities or some conditions which affect to its melting point, the second peak is more reliable about the melting point of pure PEO. An exothermic peak is observed around 40°C which corresponds to the temperature of crystallization of PEO.
All peaks observed in CHT neat and PEO neat are as well observed in CHT-PEO non-crosslinked nanofibers: melting point of PEO, crystallization of PEO, evaporation of water in CHT (Figure 29). However, in the case of PEO, the peaks observed are lower on CHT-PEO nanofibers. The reason of this might be attributed to the the low concentration of PEO in solution, which only corresponds a 10% of the total mass of solution. Furthermore, interactions between chitosan and PEO chains in the nanofibers that hinder the crystallization of PEO (Pakravan, Heuzey, & Ajji, 2012). Hence, less heat is needed in order to melt the membranes.

**Figure 29: DSC of chitosan-peo nanofibers**

Thermal crosslinked membranes have similar characteristics than non crosslinked membrane. However, at the melting point of PEO a double peak appears rather than one peak.
PEO endothermic and exothermic peaks disappear once the membranes are washed (figure 16) and the spectra obtained is that of neat chitosan (Figure 27). Therefore it can be concluded that after CHT-PEO washing, PEO is removed and the nanofibrous membrane is exclusively made of chitosan. Thus, chitosan persist in the membrane once it is washed.

Figure 30: DSC of chitosan-peo thermal crosslinked membrane

Figure 31: DSC of CHT-PEO thermal crosslinked mats after washing

DSC graph of genipin-crosslinked and GA-crosslinked membranes do not report significant changes.
Figure 32: DSC of genipin-crosslinked mats

Figure 33: DSC of glutaraldehyde-crosslinked mats

Table 5: comparison of endothermic peak temperatures of samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>PEO endothermic peak temperature (°C)</th>
<th>CHT endothermic peak temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO powder</td>
<td>68.38/71.07</td>
<td>-</td>
</tr>
<tr>
<td>CHT powder</td>
<td>-</td>
<td>106.8</td>
</tr>
<tr>
<td>CHT-PEO nanofibers</td>
<td>50.06/50.04</td>
<td>118.49</td>
</tr>
<tr>
<td>thermal crosslinked</td>
<td>53.5/60.05</td>
<td>118.59</td>
</tr>
<tr>
<td>nanofibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gnp-crosslinked</td>
<td>54.02/62.4</td>
<td>124</td>
</tr>
<tr>
<td>nanofibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA-crosslinked</td>
<td>56.39/61.34</td>
<td>129.3</td>
</tr>
<tr>
<td>nanofibers</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5 shows endothermic peaks of chitosan and there exist differences in the peak position. In the case of nanofiber, the peak position was moved to higher temperature, indicating that the water interaction with this network is stronger than with powder chitosan. It could be due to that nanofibres are more hygroscopic than chitosan powder.

In the case of glutaraldehyde and genipin cross-linking reactions, the amino group of chitosan is involved to the reaction. So, the cross-linked material will have less amino groups available to form hydrogen bonds with water molecules. As a consequence, most of water molecules will be bound to chitosan hydroxyl groups instead of amino groups. Hydrogen bonds with the hydroxyl groups of chitosan are stronger than the ones with the amino groups, higher temperature would be necessary to remove such water molecules. (Neto, et al., 2005)

3.4 Swelling test

Results in Figure 34 have revealed a strong influence of the crosslinking on the swelling volume. Membranes thermal-treated at 90°C gain about 400% of their initial weight. However, genipin-crosslinked membranes gain approximately 200%. That fact is attributed to a more rigid network formed by the inter-intra polymer chain reactions that have occurred, reducing the flexibility and number of hydrophilic groups of mats which is unfavorable to the swelling rate (De Souza Costa, Pereira, & Mansur, 2009). However, there exists an important increase in the swelling of GA-crosslinked membranes, which have a swelling ratio of 700%, contrary to what it is found in some articles (FL, YC, HC, RN, & HW, 2001)
Swelling test

![Swelling test graph](image)

**Figure 34: Swelling behaviour of membranes**

3.5 Degradation test in vitro CHT-PEO

3.5.1 Degradation test in PBS

By using PBS, we tested the behavior of our membranes in similar conditions that it could be found in a human body.

Figure 35 shows the evolution of the percentage of mass in time (represented in days) for the two different types of cross-linking. After the first day, a rapid degradation in CHT-PEO crosslinked with genipin is observed whereas the decrease in percentage of mass in thermal treated membrane is slight. Afterwards, the mass of both membranes remains practically constant. The reason of this decrement might be attributed to the fact that there is still a large amount of genipin had not reacted with CHT-PEO membrane and was removed by the contact of PBS. Moreover, following the results obtained in DSC this high degradation can be attributed to the loss of PEO after washing.
Figure 35: Degradation test of membranes in PBS medium

The pictures in Figure 36 and Figure 37 were taken after two weeks of test. As it is illustrated, all membranes conserve their structure regardless the type of crosslinking without breaking in multiple parts as it will be observed in the following chapter.

Figure 36: Membranes of genipin after 2 weeks
3.5.2 Degradation test in acid

By using acid acetic, our membranes were tested in extreme conditions that, despite being improbable, we could find in our body.

One hour after the introduction of membranes into acid medium, membranes which were thermal fixed were totally degraded. However, membranes with genipin remained in the acid medium.

So far, the addition of chemical agent did not give any advantage respect of thermal crosslinking. Although it has been proven that the addition of genipin does not produce any effect on the toxicity of the membrane, adding chemical agents is always a point of risk. As long as thermal crosslinking demonstrates the same or better properties, there is no reason in keeping the consideration of using genipin. Hence, the fact that genipin are able to persist in acidic conditions gives the first advantage to genipin cross-linking.
Figure 38: Degradation test of genipin in acidic medium

Figure 39 represents the percentage of degradation of the CHT-PEO with genipin membranes tested (as there was no data for thermal fixation). In the first measure (taken the 4th day) membranes started to break when they were put into drying conditions. As is it not clear whether the weakening of the membranes is caused by the acid, repetitive dryings or both, the methodology was reconsidered.

Figure 39: Genipin mats started to break after 4th day
3.6 In vitro test

3.6.1 Vitality test

Results for our first test in vitro attempt are showed in Figure 40: Vitality test of membranes. All membranes have showed good results after 24 hours, especially membranes with glutaraldehyde cross-linking. However, after 78 hours their vitality has decreased rapidly. Almost all cells did not persist after 78 hours in the case of glutaraldehyde cross-linking. This might be caused by its toxicity. In other cases, we considered that results were worse that they had been expected. A possible explanation is that the protocol followed could be inaccurate or the membranes were not well sterilized.

Because of its low vitality, cross-linking by using glutaraldehyde did not report any advantage from the other kinds of it.

![Figure 40: Vitality test of membranes](image)
3.6.2 Adhesion test

The following figure represents the results for the protocol attempted. The three membranes were compared to a positive sample control of adhesion.

Samples were exposed into an acidic tampon medium pH 5.5. In those conditions, membranes were not able to persist. Hence, membranes were partially/totally degraded into the medium during the performance of the test giving wrong measures of absorbance in ELISA test. In conclusion, one considers that these results are no reliable.

![Adherence test](image)

*Figure 41: adherence test of samples*
CHAPTER 4: ELECTROSPINNING OF PEO
4.1 Materials

PEO with a molecular weight (Mv) of 900,000 g mol\(^{-1}\) and pentaerythritol triacrylate with 298.29 g/ mol were purchased from Sigma–Aldrich Incorporation (St. Louis, MO, United States).

4.2 Methods and results

4.2.1 Preparation of solution

By the adaptation of the work of Chengjun Zhou et al, 4% PEO-PETA mixtures of mass ratio 8/2 were dissolved in deionized water. Solutions were constantly mixed throughout 12 hours by a magnetic agitator at 300 rpm and treated with ultrasonic bath at ambient temperature.(Zhou, Wang, & Wu, 2012).

The solution was electrospun with the following parameters: flow rate of 0,5 ml/h; voltage around 8-9 kV and distance between collector and jet of 200 mm.

4.2.2 Crosslinking of PEO

Once the membranes were produced, we have proceeded to UV-crosslinking. Membranes were deposed into a cage which contains a UV lamp. They were irradiated throughout 4 hours and a half. Below this time, all samples tested became gelatinous in aqueous environment which notifies that they were not completely crosslinked. Figure 42 shows images SEM of PEO-PETA membranes after four hours of UV-treatment. It is clearly displayed how fibers attaches among them giving stronger stability.
4.2.3 Fourier transform infrared spectrometry

Specrometry data was collected by a transmission spectrum at room temperature against an air background. PEO-PETA membranes were scanned at wave numbers ranging from 4000 to 700 cm\(^{-1}\) with a 4.0 cm\(^{-1}\) resolution.

Figure 43 (a) presents FTIR spectra of PEO-PETA electrospun membranes whereas figure b) presents spectra of PEO powder. Only a significant peak located at 1731 cm\(^{-1}\) differs from both graphs. This peak corresponds to PETA polymer according to the spectrum of the provider Sigma-Aldrich- (Figure 44)
Figure 43: FTIR of PEO-PETA membranes. a) PEO-PETA membranes b) PEO powder

Figure 44: Spectra of PETA. Source: Sigma Aldrich Inc.
4.2.4 Degradation test

The electrospun nanofibres mats were cut and each cut specimen were measured as an initial weight (~ 5mg) and then placed in a test vial contained 10mL of distilled water. Vials were placed under stirring for 37°C for one week. Measures were taken throughout 1 hour, 4 hours, 24 hours, 3, 5 and 7 days.

Figure 45 shows the results for this test. Throughout the first day of experiment, there was a strong degradation of the samples. When they were extracted from medium, all samples have a slightly gelatinous structure which means that a high degradation occurred. After that, the percentage is stabilized reaching its lowest point after one week with almost 40% of membrane degraded.

Regarding of CHT-PEO in PBS medium, which have a similar pH than water; it is observed that PEO-PETA did not persist as well as the other membrane. However its quick degradation might be due to an incomplete cross-linking between both solvents. In order to verify this, membranes were exposed to higher irradiance UV power.

![Figure 45: Degradation test of PEO-PETA](image-url)
CHAPTER 5: BUDGET
During this chapter all expenses involved in the project are enumerated. They have been classified depending on the type of expense and summarized at the final cost.

5.1 Materials budget

This part identifies all the expenses from the material used during the experiments, besides equipments. Price rate was estimated according to the prizes of Sigma Aldrich website. Prices change depending on the set chosen so the set purchased of each product was checked in order to be as accurate as possible.

All experiments carried out were saved into a database, so that the quantity of material used has been estimated in accordance with it.

Table 6: List of chemicals used during all experiments. includes a list of chemicals used throughout this research, regardless whether the membranes were well or bad produced. Acetone, ethanol and a part were used to clean and sterilize the recipients.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Price rate</th>
<th>Cost (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>21.73 g (69)</td>
<td>87.75€/50g</td>
<td>38.13</td>
</tr>
<tr>
<td>PEO</td>
<td>1.03 g (0-69)+7.2 g (69-87)</td>
<td>131€/250g</td>
<td>4.31</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>690ml (69)+120 ml</td>
<td>19.46€</td>
<td>15.76</td>
</tr>
<tr>
<td>Acetone</td>
<td>2.3 L</td>
<td>11.88€</td>
<td>27.33</td>
</tr>
<tr>
<td>PETA</td>
<td>1.44ml (69-87)</td>
<td>60.50€/100ml</td>
<td>0.87</td>
</tr>
<tr>
<td>Genipin</td>
<td>0.08 g (0-16)</td>
<td>369.50€/125mg</td>
<td>236</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.4 L</td>
<td>75.70€/1 L</td>
<td>30.16</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.5 L</td>
<td>110 €/20 L</td>
<td>13.76</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.18 L(69-87)+ 5L</td>
<td>0.15 €/L</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>Total cost</strong></td>
<td></td>
<td></td>
<td><strong>366</strong></td>
</tr>
</tbody>
</table>

5.2 Amortization of equipments

This part shows the cost of the equipments used in order to analyze the membranes obtained in the project. However, these equipments are not only used for this project so that they have been already used and will be used. The total cost of equipments is distributed during the machines life cycle. Only the cost of equipments corresponding to the period of time of this project is included.

This cost or value is called amortization and can be estimated by the following equation:

\[
Amortization = \frac{(\text{Real cost} - \text{Residual value})}{\text{life cycle}}
\]
Where:

- **Real cost**: Total cost of the product sold.
- **Residual value**: value of the product once they are useless. In this case, it is considered 0 as the machine is used until the end of their life cycle
- **Life cycle**: It is considered an average of 15 years for all equipments.

<table>
<thead>
<tr>
<th><strong>Product</strong></th>
<th><strong>Cost (€)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CPU</td>
<td>1200</td>
</tr>
<tr>
<td>Single Syringe Pump</td>
<td>1567</td>
</tr>
<tr>
<td>Aluminum cylinder</td>
<td>217</td>
</tr>
<tr>
<td>HV generator mini 40Kv@</td>
<td>1570</td>
</tr>
<tr>
<td>FTIR spectrophotometer</td>
<td>7450</td>
</tr>
<tr>
<td>SEM</td>
<td>75000</td>
</tr>
<tr>
<td>ELYSA TESTER</td>
<td>4700</td>
</tr>
<tr>
<td>Weighting scales</td>
<td>210</td>
</tr>
<tr>
<td>Furnace with mechanic agitation</td>
<td>920</td>
</tr>
<tr>
<td>Differential scanning calorimeter</td>
<td>15345</td>
</tr>
<tr>
<td>Thermal galvanic analyst</td>
<td>6430</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>114610</strong></td>
</tr>
</tbody>
</table>

*Table 7: List of equipments used during the project*

\[
Year \text{ amortization} = \frac{114610}{15} = 7640
\]

However, the project lasted only five months, and therefore, the use of the equipment. Thus the final repayment is left with:

\[
Project \text{ amortization} = \frac{5}{12} \times 7640 = 3183 \text{ €}
\]
5.3 Personnel cost

Table 8 summarizes the amount of hours spent on every task, with a separate cost for the student and the thesis director.

<table>
<thead>
<tr>
<th>Task</th>
<th>Hours</th>
<th>Cost (€/h)</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research and investigation</td>
<td>120</td>
<td>20</td>
<td>2400</td>
</tr>
<tr>
<td>Experiments</td>
<td>300</td>
<td>20</td>
<td>6000</td>
</tr>
<tr>
<td>Analysis and results</td>
<td>200</td>
<td>30</td>
<td>6000</td>
</tr>
<tr>
<td>Report writing</td>
<td>80</td>
<td>30</td>
<td>2400</td>
</tr>
<tr>
<td><strong>Total amount</strong></td>
<td></td>
<td></td>
<td><strong>16800</strong></td>
</tr>
</tbody>
</table>

*Table 8: Tasks performed by the undergraduate researcher*

<table>
<thead>
<tr>
<th>Task</th>
<th>Hours</th>
<th>Cost (€/h)</th>
<th>Total (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meetings with student</td>
<td>20</td>
<td>35</td>
<td>700</td>
</tr>
<tr>
<td>Project guidance</td>
<td>40</td>
<td>35</td>
<td>1400</td>
</tr>
<tr>
<td><strong>Total amount</strong></td>
<td></td>
<td></td>
<td><strong>2100</strong></td>
</tr>
</tbody>
</table>

5.4 Total cost

*Total cost = Materials budget + Amortization budget + Personnel budget = 366 + 3183 + 16800 + 2100 = 22459 ≈ 22500 euros*

The total amount of the project is stipulated on TWENTY-TWO THOUSAND AND FIVE HUNDRED Euros.
CHAPTER 6: CONCLUSIONS
In this project we have produced nanofibers mats using the technique of crosslinking. Although these membranes may have some applications, we were focused on creating an anti-adherent covering for abdominal meshes in order to prevent the adhesion of the abdominal wall. SEM images corroborate that we succeed in the yielding of defect-free and bead-less membranes. In order to achieve an improvement in the mechanical properties of mats, they were cross-linked by three different methods. By SEM images, it is reported that there was a certainly benefit to retain the nanofibrous morphology in aqueous medium.

FTIR reflected significant changes in the transmittance spectra of nanofibers mats in comparison of the powder substances, revealing that there had already been an important change in structure during electrospinning process. Moreover, other changes were observed specially in imine and amide group between non-crosslinked mats and chemical crosslinked mats which provided us an overseeing of the molecular reactions produced between polymers. These singularities observed on the FTIR spectrum agree with other publications which confirm that we succeed in the crosslinked of CHT-PEO mats with genipin and GA agents.

Thermal analysis revealed once a membrane is exposed to an aqueous medium, a significant quantity of PEO is degraded into the medium. Other studies like UV-spectroscopy were carried out in order to demonstrate it. However, although in some cases this fact was corroborated, data provided was found unreliable so that one decided to do not include it onto this project.

Regarding the swelling test, the reduction in hydroxyl groups (as it was observed in the FTIR) also induced a reduction in the swelling capacities of membranes. However, this was not observable in the case of GA-electrospun mat.

The vitality test showed that the addition of genipin and the thermal treatment do not contribute to increase the toxicity of the membranes. However, as the results are below 70% in vitality we cannot guarantee that these membranes do not produce any damages or hazards to their host. It was clearly demonstrated that the addition of GA agent must be discarded as an option due to its extremely low vitality. For this reason, this is why glutaraldehyde was not included in other researches such as the degradation test. Adhesion test did not report the results expected as it was observed that the adhesion between cells and the surface of membrane increased. Nevertheless, it is not clearly demonstrated whether the increase of adhesion was due to the properties of membrane or due to some errors that appeared during the procedure. As thermal-membrane was degraded by the addition of acid, it would be required to carry out further tests in vitro changing the protocol.
During all the experiments, glutaraldehyde has been used as a control rather than a candidate. This polymer has been widely investigated onto the literature so that it constitutes an excellent indicator in order to determine the reliability of our test. We had been awarded about its high toxicity before vitality test and this is why we did not include GA in the degradation test.

Degradation test exhibits that membranes were able to persist in long-term into an aqueous medium similarly with the medium that might be found into the human body. Genipin, as opposed to thermal-crosslinked mat, did persist throughout a fully week into an acid medium.

We have carried out some experiments to attach membranes on the surface of the mesh. By a surface modification and a neutralization of the mesh, an attachment was observed between both products. However, it was not a guarantee that this attachment would persist in long-terms once the mesh would be incorporated onto the host. As some characterization tests have to be carried out in order to corroborate it, it was decided to do not include this section into the project.

In conclusion, although we succeed in the yielding of electrospun membranes, further researches have to be carried out in order to corroborate whether they can be use to prevent the post surgical abdominal adhesion.

For the second part of the present work, we have produced another type of biomaterial. PEO were crosslinked with other substances polymers like acid citric and other photoindicators like Darcum. However, we failed in the electrospinning of those membranes until we succeed in the yielding of PEO-PETA. This sort of cross-linked mats was not broadly studied in comparison to the first part of the project, which make up a challenge for us.

6.1 Prospects

- For the first part of the project, it is proposed to:
  o Use of lower frates during thermogravimetric analysis
  o Higher sterilization of membranes before vitality test
  o Change in protocol of adhesion test, avoiding the use of acids
  o Study of diameters among all types of crosslinking in order to corroborate the broaden of diameters
  o Test in vivo of membranes
- UV-spectroscopy monitoring the degradation of samples
- Mechanical test to corroborate an accurate attachment between membranes and meshes.
- Incorporation of a drug delivery system in order to release the pain in patients

- For the second part:
  - Further test of characterization
  - Test in vitro
7.1 Bibliographical reference


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7.2 Consultation bibliography