POLITECNICO DI TORINO

FACULTY OF ENGINEERING

Master's degree in Biomedical Engineering

MASTER'S THESIS

DIPARTIMENTO DI INGEGNERIA MECCANICA E AEREOSPAZIALE

PRELIMINARY STUDIES FOR CELL-LADEN HYDROGELS DEVELOPEMENT

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Academic year 2017/2018

To my family and friends,

Abstract

The following document presents a Master's Thesis based on preliminary studies for the development of cell-laden hydrogels and their biological characterisation. The aim of this hydrogels elaboration is the peripheral nerve regeneration, in this case mainly related to the regeneration of the sense of touch. The results obtained through the experimentation of cells encapsulation would serve to know which are the best conditions and the preferable environments where cells can live and reproduce. It would definitely set a step forward for the in vivo studies and the real regeneration of the peripheral nerves.

In first place, there is a preface, where the main reasons to develop this project and the importance that hydrogels have in the current investigation for tissue regeneration are explained. In second place, the objectives of this project and its scope are defined.

Then, an introduction and description of the materials being used in the experiments is done. The developed hydrogels are chitosan based with a phosphate salt as gelling agent. The cells being used for the encapsulation are Schwann cells, which are the cells in the peripheral nervous system, residing at sensory nerve endings.

Next, the summarisation of the executed experiments can be found. The first point made is the description of the obtaining process of hydrogels and the characterisation of the selected ones for doing the cell encapsulation, while the second point made is the description of the experimentation with cells, its complications and results.

The hydrogels finally obtained will be used in further investigations and this project will give an idea from where to focus the attention in the future and on how to continue with the experimentation.

The hydrogels characterisation and the experiments done at the Politecnico di Torino brought to light the best hydrogel in terms of gelation time, stability and pH are the CS 95/100 2.5% w/v R=4 with β-GP and the CS 95/100 2% w/v R=4.5 with G1-P. The experimentation with cells developed in San Luigi Hospital, in Orbassano, Torino, were based in the study of the behaviour of cells to these two hydrogels and its components.

The results showed that the hydrogel CS 95/100 2.5% w/v R=4 with β-GP presents a better response in the experiments of cells encapsulation and cells on the top, for the concentrations defined. On the contrary, the hydrogel CS 95/100 2% w/v R=4.5 with G1-P presents better cells behaviour when working with lower concentrations of salt. Further investigations will be performed to define the optimal hydrogel composition.

POLITECNICO DI TORINO

ETSEIB

Table of contents

1. Glossary

UPC	Universitat Politècnica de Catalunya
ETSEIB	Escola Tècnica Superior d'Enginyeria Industrial de Barcelona
CS	Chitosan
CS 95/100	Chitosan 95% deacetilate, 100kDa molecular mass
CS 80/100	Chitosan 80% deacetilate, 100kDa molecular mass
β-GP	beta-glycerol phosphate
GP	Glycerol phosphate
G1-P	glycerol 1-phosphate
ECM	Extracellular matrix
T	Temperature
P	Pressure
PBS	Phosphate-buffered saline
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle's medium
Q	glutamine
P/S	penicillin stractomisin

2. Preface

In this first section it can be found the opening of the Master Thesis itself, as well as the reason and origin of this study and the motivation that brought the author to choose this field of study and this theme. Furthermore, the project main goals and scope can also be read in this section.

2.1. Origin of the project

The advance in cells treatment and the discoveries made in this field of study on the last 40 years, together with the growth and maturation of the 3D printing technology, made it possible to develop the bioprinting. This technique allows the printing of engineered tissues made of biomaterials (materials containing cells) that mimic accurately the characteristics of the natural tissues.

The biomaterial most promising, which gained an exponential interest for investigators through the last 20 years of research, is the hydrogel. This biomaterial composition works very well as a natural extra-cellular matrix because of its similarity with the tissue components of the body, being soft, biocompatible and having a microporous structure.

The research in the regenerative medicine caught the attention of the investigators of the Industrial Bioengineering Department at Politecnico di Torino, who got involved in the research ten years ago. The investigation is directed towards the regeneration of the peripheral nerve with the final goal of the regeneration of injured nerves. There were written different papers during these years, from more general to more precise, where there were studied biodegradable and natural based materials, scaffolds for peripheral nerve regeneration and different hydrogels.

Nowadays, the part of the investigation in this institution related to the biomaterials and bioprinting is centred in the development of chitosan-based hydrogels, which could be printed to build a scaffold and could keep cells alive inside. In particular, this master thesis is focused in the second purpose, having as main goal the study of the chitosan-based hydrogel and the conditions in which the cells could be encapsulated to obtain the highest ratio of cells survival, doing at the same time the biological study of them.

This work is a preliminary study about cells encapsulation that would permit future more deep investigations about that and the unification with the parallel study that is currently taking place (in another Master Thesis), where there are being studied the best conditions to print a proper hydrogel. In this way it is going to be possible to print the proper hydrogel

with cells encapsulated inside, which would permit the investigation of its application on peripheral nerve regeneration.

2.2. Motivation

The election of this theme for the Master Thesis is grounded in several arguments. Firstly, it was chosen because of the interest for the biomedical engineering field, being the specialty of the author at the Master in Industrial Engineering at the Polytechnic University of Catalonia. This project pretends to wider the knowledge acquired at the University about the hydrogels topic and, at the same time, to go deeper into biology, that is a field not especially treated during the studies.

Secondly, this project was developed at the Politecnico di Torino. Even though it represents a challenge not only for the language, but also for the rigorousness that this prestigious university demands, it supposes also a fantastic opportunity to get to know other cultures, institutions and ways of working; what is of great importance in the current globalised world.

And lastly, this project is a collaboration between the Politecnico di Torino and the NICO, Neuroscience Institute Cavalieri Ottolenghi, placed at the San Luigi Hospital in Orbassano, Torino. This institution is needed for the work with cells that cannot take place in the Polito yet. The fact of working in a different environment, with biologists, doctors and people from not engineering fields is a point in favour, since it gives the opportunity of seeing the biomedical area from a different point of view.

2.3. Requirements

This project requires basic knowledge on biomaterials and more specifically, on hydrogels, since it is extremely important to know the composition and behaviour of this biomaterial for the correct development of the project, for the correct treatment while encapsulating cells inside and for the analysis of the best conditions and parameters that allow the highest survival of cells.

In this work there are applied the knowledge acquired at the *Biomaterials* course of the Master in Industrial Engineering at the UPC, the knowledge of professionals in biology, bioengineering and medicine working on the research centres and universities, where it takes place, and the knowledge gained through the interpretation of the literature that can be found on internet about this area of investigation.

Moreover, as the project forms part of a student exchange programme, it was written and developed in English. Therefore, it is required at least an intermediate level of English for the right elaboration of this Thesis and for a good comprehension of the information

obtained through Internet (journals, papers and new, for example), which are published around the world.

2.4. Main goals

This work is going to contribute to the investigation about the regeneration of the peripheral nerve, with the last aim on the regeneration of the sense of touch. As it is a considerably broad and complicated subject, which englobes lots of disciplines and areas of research, it was subdivided into subtopics, as for example concerning the biomaterial: the study of the parameters that influence the preparation of hydrogels, the study of its printability and the encapsulation of cells.

In this case, the main goal of this Master Thesis is the preliminary study of the cells encapsulation into chitosan-based hydrogels. It aims to respond to the most important questions on the development of a biomaterial: Is it biocompatible? Do the cells survive in contact to the hydrogel? Which is the ratio of survival and proliferation?

To achieve the above-mentioned purpose, the following objectives are set:

1. Study in depth the hydrogels and learn the procedure of elaboration of them, at the same time as understanding the parameters that influence their behaviour.
2. Find the most proper one for the experimentation, which must have the following main properties:
   - Be printable
   - Have a low gelation time
   - Have a pH similar to the natural pH of the body (=7.4)
3. Experiment with cells at the NICO, Neuroscience Institute Cavalieri Ottolenghi.
4. Do the biological characterization

2.5. Project scope

This project consists in a scientific investigation about the cells encapsulation into the hydrogels and, as all the scientific investigations, is based on the scientific method. The structure of this project is similar to the Master Thesis made by Rosanna Fusaro, the student that worked with chitosan-based hydrogels and their sensibility and effect to some parameters the semester before (September-February 2017/18). This structure consists of an Abstract, Introduction, Materials and Methods and Results, as the scientific papers usually include.

Being the period of development of this project short, 6 months, the objectives of it are specific and concise with the aim of making the project feasible and the results reliable. This

is the reason why the project is centred only in the experimentation with cells without involving neither other topics of the hydrogels, such as printability, nor other topics of its application by the building of the scaffold and its conditions for the application as peripheral nerve for the regeneration of the sense of touch. These topics remain out of this Maser Thesis, but are being studied in parallel or are future steps in this investigation.

3. Introduction

During the 20th century medicine has had an impressive advance and has gained many important successes, such as the development of antibiotics, of insulin for diabetic people, of immunosuppressive drugs for organ transplants, of several vaccines for various diseases (polio, tetanus, rubella, etc) and the development and use of X-ray, for example. However, there are still many pathologies as Cancer, AIDS or Arthritis, between others, that cannot be treated by preserving the affected part.

The palliative treatment of diseases has allowed patients to prolong their lives by enlarging the course of the disease, but has not solved the root cause of it. The excessive increase of chronic degenerative diseases in the last years has focused interest on the research of the cure of the degradation and destruction of cells and the loss of tissue function, and is causing the evolution of medical treatments and the regeneration of damaged tissues, branch of medicine called regenerative medicine. [1] [2]

Regenerative medicine is an area of the medicine that deals with the process of replacing, engineering, regenerating and repairing human cells, tissues or organs that were irreparable through the stimulation of the body repair mechanisms to cure the damaged ones. This area includes three different branches. The first one is the cell therapy, in which the stem cells or progenitor cells are injected in pathologic tissues. A very important example of this technique can be found in the transplant of bone marrow, succeeded the first time in 1968, which is used to cure the leukaemia. [3] The second branch is the gene therapy, which uses the study of the genes to treat or prevent diseases by replacing or inactivating a mutated gene or introducing a new gene. And the third one is the tissue engineering, which consists in the transplantation of in vitro grown organs and tissues.

The combination of the first and the third branches of the regenerative medicine would allow the creation of human tissues and organs that could substitute the donated organs and solve the existing problems of its shortage and rejection. This branch of the science is known as Tissue Engineering. [4] The cells that can be used for this procedure are differentiated autologous harvested cells, meaning cells removed directly from the tissue of the patient, or undifferentiated stem cells.

On the first hand, harvesting autologous cells is the favourite option, since it does not cause immune response, that is the reaction of the cells and fluids of the body towards a not recognised substance. However, it is difficult to get a big number of these cells in vitro, because they are put away from the natural environment that they need to proliferate, and sometimes the cells are not appropriate for transplantation for example due to the advanced age of the patient. [2] [5]

On the other hand, the stem cells (SC), discovered on the 1978, are a promising possibility, since they are able to proliferate widely and indefinitely, to regenerate themselves (self-renew) and to keep the undifferentiated state until they are stimulated to differentiate into more specific cell types. They can have several origins: autologous if they are removed directly from the patient, allogeneic if they come from a human donor and xenogeneic if they come from an animal.

The cells in which SC can be differentiated depends on the point of extraction of the body. Adult stem cells (ASCs), are undifferentiated cells that can be found in all the body, since their function is to substitute dying cells and restore damaged tissues. They are multipotent, what means that they can differentiate in several kinds of cells on the human body, and they can be found in bodies of all ages. Among this group there are two main types, the hematopoietic stem cells (HSCs), which are found in the bone marrow and umbilical cord blood, and the mesenchymal stem cells (MSCs), which are found in the bone marrow, placenta, adipose tissue, lung and blood. Both types are multipotent and widely used because of their therapeutic properties.

There exist also the embryonic stem cells (ESCs), which are derived from the inner cell mass of an embryo and are pluripotent, that is that they differentiate in all cell types except the placenta ones. Although its high potential, they entail ethical and moral issues as the interruption of the development of an embryo is required for their acquisition. The same properties of the ESC can be obtained by the reprogramming of adult cells, obtaining induced pluripotent stem cells (iPSCs). [2] This kind of cells were obtained from adult embryonic and adult fibroblast cultures for the first time just on the 2006. [6]

The development of an engineered tissue *in vitro* requires the development of a matrix, as similar as possible to the extracellular matrix (ECM) of the body tissue, where the cells must be added. The best results with engineered tissues were made with primary cells, but the limitations that this kind of cells present (explained above) have promoted the use of stem cells. The sources of stem cells used in tissue engineering are the ESCs, which can generate all kind tissues as the skin, cornea and circulatory system for example [7], and the MSCs, which are mostly used for the regeneration of bone and cartilage and also extensively studied in the last years for other applications.
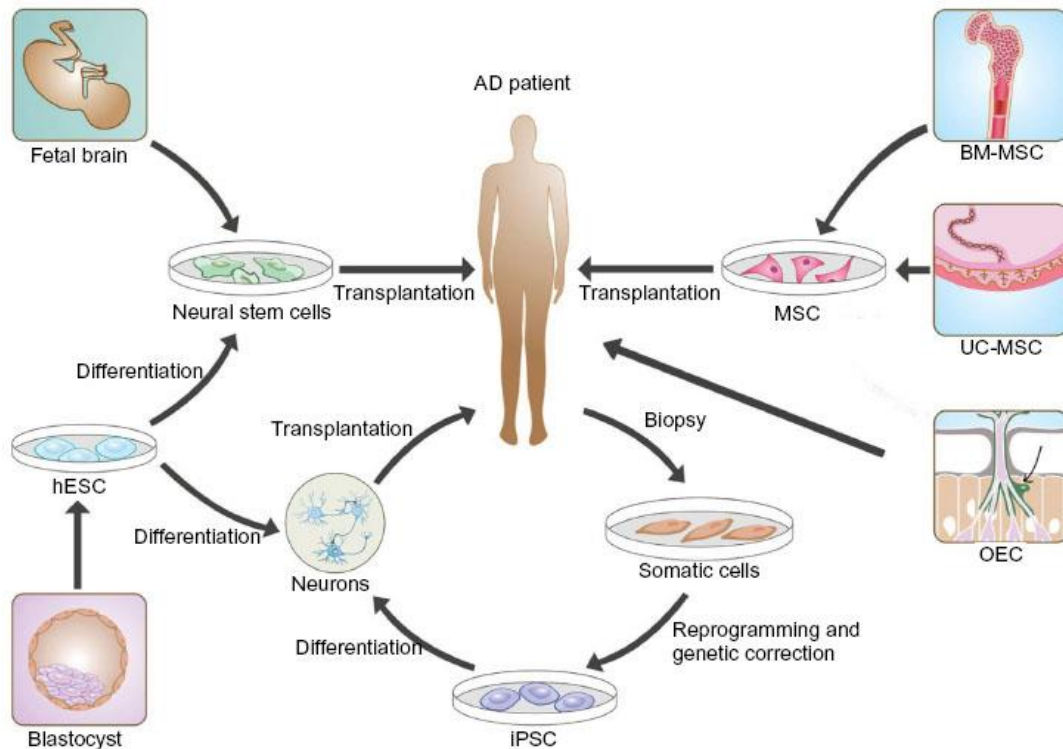
Figure 1: Regenerative medicine. Sources and types of stem cells. [8]

It is of major importance the use of differentiated cells for tissue engineering in order to potentiate the tissue formation and to prevent the teratoma formation (a tumour). Therefore, one of the critical steps by using stem cells for regenerative medicine is the control of the differentiation of cells to the desired cells for each kind of tissue.

The final purpose of this thesis is the regeneration of the peripheral nerve with the final goal of the regeneration of the sensory recovery after traumatic nerve transection. This goal was motivated from the fact that there are more than 1 million people per year that suffer from injuries in the peripheral nerve, but the health still has not managed to give a solution. For this aim the most optimal cells are the Schwann cells because of their release of growth factors that contribute to the regeneration of axons, which are the nerve fibres that transmit information to different neurons, muscles and glands. [9] Moreover, it was proven that Schwann cells can be obtained by the differentiation of bone marrow stem cells, as for example the Georgia Institute of Technology and Emory University states. [10] Therefore, the selected cells for the experiments are the RT4-D6P2T, that are a cell-line derived from Schwann cells.

The other main component of the engineered tissue constructs, apart from the cells, is the artificial extracellular matrix (ECM), also known as scaffold, which provides the structural and biochemical functions of the natural extracellular matrix. The composition of these scaffolds are biomaterials, where cells are encapsulated (enclosed). They are used to build

solid 3D structures used as mechanical support, they have a degradation rate that matches with the cell remodelling rate of the original tissue and they are biocompatible and bioactive, since they need to support the cell adhesion and growth, proliferation, migration and differentiation. Not only has the biomaterial to perform the desired function without eliciting any undesirable effect in the body (biocompatible), but also has it to generate the most appropriate beneficial response, eliciting a controlled reaction in the physiological environment (bioactive).

On the 8th of August 1984, Charles W 'Chuck' Hull patented the *Apparatus for production of three-dimensional objects by stereolithography*, which was the first 3D printer in the world, based on the creation of solid objects by printing successive layers of material mimicking computer modelled shapes. Nowadays, the technology has grown a lot and is applied in several fields, such as electronics, automotive, aerospace and even medical engineering. The adoption of the technology in this last field, medical engineering, was stimulated by the success of the first laboratory-grown and implanted organ, which was a urinary bladder. In this case, the scaffold was built by hand with collagen and synthetic polymer seeded with cells, but this process was time-consuming and heavy. [11] Therefore, there was recognised the necessity of automate the process. [12]

By the adoption of the 3D printing technology in the medical field was born the bioprinting, which is a technology that uses a device to model an organ by the accurate deposition of cells and biomaterials. This technology allows the automatic production of scaffolds for tissue engineering and the combination of the treatment with stem cells, subsequently differentiated, and the biomaterials, giving them shape for the temporal replacement of human tissues in order to promote the tissue regeneration. Several applications have already been demonstrated, such as printing of ears, bones, vascular tissues, cartilage and others, although they are not yet ready for clinical use. [13] There were investigated different bioprinting methods during the last years, including extrusion-based, droplet-based and laser-based technologies.

The extrusion-based technologies consist in a nozzle, through which the bioink flows following the pattern created by a CAD software connected to the bioprinter. The flow of the extruded biomaterial is continuous thanks to the control of the mechanical or pneumatic forces over the fluid. Among the droplet-based technologies stands out the inkjet bioprinter. This method imitates also a CAD design, but in this case the biomaterial is deposited drop-by-drop, responding to thermal, piezoelectric or electromagnetic forces. Finally, among the laser-based bioprinting technologies the stereolithography and the laser-assisted bioprinting are the most interesting ones. The stereolithography uses a UV light directed to the model made of photosensitive material, causing the crosslinking of the polymer and creating a hardened layer, while the laser-assisted bioprinting directs a laser pulse to a

ribbon, where the temperature and the pressure increases provoking the deposition of droplets on the surface under the ribbon. [14]

The critical point of this technology is to find the proper "bioink", the biomaterial that needs to be printable, economic, has to maintain the structure and has to be biocompatible and bioactive. Many new biomaterials were developed and tested in the last years, confirming that hydrogels are the ones with biggest potential for bioprinting, as the exponential increasing research on them indicates, because they mimic the natural extracellular matrix (ECM), have mechanical properties similar to the soft tissues and can support cell adhesion and protein sequestration. [15]

3.1. Hydrogels

The first material similar to the hydrogels, as they are nowadays understood, is the polyhydroxyethylmethacrylate (PHEMA), introduced in 1960 with the goal of being in constant contact to the inside of the patient. Since then, the story of the hydrogels has enlarged considerably and they have evolved until the point they are now. Hydrogels are polymeric materials, characterised by their hydrophilic structure, since they can absorb large amounts of water while keeping their dimensional stability. Once the hydrogel is formed (swollen state), they maintain their structure thanks to the crosslinking, which can be either physical or chemical. The crosslinking points make it possible to reach the equilibrium point between the solubility, present because of the thermodynamic compatibility of the polymer chains and water, and the elasticity of the network.

Figure 2: Hydrogel in swollen state. [16]

The chemical crosslinking is based on the action of crosslinking agents, which create covalent bounding between polymer chains, resulting in a very stable and strong structure that cannot be further modified. Moreover, these crosslinking agents are generally toxic and dangerous for humans. On the other hand, physical crosslinking is reversible, can be modified, is created by the casting of a solvent or thermal processing and does not need

the addition of crosslinking agents, but it has weaker mechanical properties due to its non-covalent bounding.

Hydrogels can be classified in two main groups, from natural and from synthetic origin, having each of them advantages and disadvantages. Hydrogels exist in nature since always and they can be found, for example, in bacterial biofilms, plant structures, ECM components, gelatine and agar. The hydrogels made of natural polymers several advantages for tissue engineering since they are biocompatible, have good viscoelastic biomechanical properties and swelling properties, have fibrillar microarchitecture like ECM and are biodegradable by natural enzymes of the body.

The main problems that hydrogels from natural origin present are their weakness in comparison to the original tissue, originated because of the random alignment of their fibres and their porosity, and their high variation batch to batch. On the other hand, synthetic hydrogels solve the problem from the variability from batch to batch by using biologically modified hydrogels, which have a better alignment of fibres, resulting in stronger structures. The last steps in this field indicate that there can be produced smart hydrogels, whose swelling behaviour depends on the external environment, reacting to changes of pH, temperature and electrical and magnetic stimuli, for example. [17]

Hydrogels have proven useful for cell culture applications because of their special properties, that make possible the adhesion of cells and their proliferation, migration and differentiation. However, there are several properties that need to be controlled when forming hydrogels for cellular experiments, which can be modified through crosslinking. These properties are the following:

- Mechanical properties: define the stability of the biomaterial with cells encapsulated or adhered on the top and affect the cellular mechanotransduction, the sensitivity towards the flux of information from the environment to the biochemistry of the material, influencing at the same time the cells behaviour. These properties are commonly analysed with the shear modulus (G) or elastic modulus (E), which are usually measured using rheology, since it depends on the time and the gelation ratio.
- Swelling properties: understood as the amount of water or buffer solution, which is an aqueous solution formed by an acid and its conjugate base, that can be accepted into the hydrogel. It is useful to know the hydrophilicity of the network and the density of crosslinking, and it is measured by drying the hydrogels and calculating the equilibrium water content and the weight loss.
- Network porosity: influences the transfer of information inside the matrix, the diffusion of nutrients and waste, and is defined by the mesh size. It is tightly linked

with the swelling and mechanical properties, as a high modulus and low swelling ratio means a small mesh size.

- Hydrogel degradation: is the loss of mechanical properties and swelling ratio over time, what definitely influences the cell behaviour. It can be hydrolytic or enzymatic degradation, depending on the presence of hydrolytically unstable bonds or enzymes. The velocity and degree of degradation desired depends on the application of the hydrogel.

Hydrogels can be formed and printed alone, without cells, for subsequently seeding them with cells on the surface, or can be bioprinted with cells in suspension into the hydrogel, forming the bioink. When working with cells, it has to be considered that it is essential to sterilise the solution prior to cell culture and, in case of encapsulating cells into the hydrogel, it has to be done prior to the hydrogel gelation. This sterilization can be executed through filtering or through UV irradiation, always attending to not lose physical properties during the process. [18]

The desired hydrogel can be obtained not only by modifying and adjusting the properties above, but also analysing the best technology and conditions of printability, such as composition of the hydrogel, the printing temperature, pressure and embedding.

It is common to print thermosensitive sterilised hydrogels already seeded with cells by means of extrusion-based technologies, building a scaffold. This kind of hydrogels undergo the transition from solution to gel when the temperature exceeds the critical point (Lower critical solution temperature, LCST), which is usually set around 37ºC, the physiological body temperature. These systems offer several advantages: (i) they are easy to manufacture, (ii) easy to administrate, since they become gel and maintain the solid stable structure seconds after being printed, and (iii) the fact of having physical crosslinking makes it possible to prevent toxic crosslinking agents.

Hydrogels based on both natural and synthetic polymers can form thermosensitive hydrogels, either in combination with other substances or in their original or modified state. Taking benefit of the multiple advantages that natural polymers have, natural biocompatibility, biodegradable and very similar to the ECM, lots of studies have developed thermosensitive hydrogels from natural original throughout the last years. These hydrogels were prepared mainly with collagen, fibrin and alginate, but also with other polymers that involve polymer synthesis prior to the formation of the hydrogel, such as chitosan and silk. [18]

Chitosan was used in lots of medical applications, including peripheral nerve repair, because of its biodegradability, biocompatibility, antibacterial activity and physicochemical properties. What is more, a recent study has proven the survival of Schwann cells over

chitosan membranes. [19] There are several examples of chitosan-based hydrogels in the literature, theme that is going to be treated more in deep in the following subsegment.

3.2. Chitosan-based hydrogels

Chitosan (CS) is a linear polysaccharide made by treating the chitin, obtained from crustacean shells, with an alkaline substance in what is known as the deacetylation process. It is composed of glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units). The degree of deacetylation (%DD) is determined through spectroscopy, going from 60 to 100% for commercial chitosans, and the average molecular weight range of them is 3800-20000 Daltons.
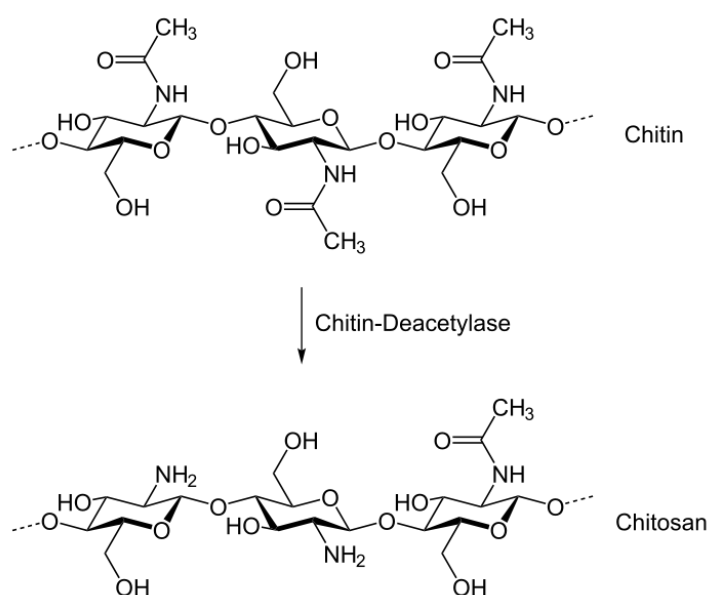

Figure 3: Chitosan synthesis from deacetylation of chitin

Thanks to its well-known properties, like biocompatibility, non-toxicity and biodegradability, chitosan has multiple possible biomedical uses. It presents good properties for clotting blood, and it is hypoallergenic and has also natural antibacterial properties, what makes it usable in bandages and other antihemorrhagics. CS is also widely used for drug delivery systems, since it is mucoadhesive, reactive and has positive charge in acidic environments, meaning that it degrades and releases the medicine when it arrives to the acidic condition. It was already proven and used for the transport of insulin. [20] It can also be combined with other materials, and it is being used for the research and development of new nanomaterials, bioadhesives and edible coatings. Finally, it can also be used for bioprinting and tissue engineering, that is the application that is being studied in this thesis.

CS is only soluble in acidic aqueous solutions, with a pH lower than the pk_a of the CS, which is around the value of 6.2. At low pH, the free amino groups are protonated, leading to an increase of the solubility because of the repulsion originated between the polymer chains. On the contrary, at high pHs (neutral and basic environments) the solution becomes insoluble, non-hydrolytically degradable, maintaining its structure and losing its possible applicability by drug delivery and biodegradable hydrogels formation.

It was not until the 2000, when the first thermogelling system was developed by Chenite *et al*. This system consisted of chitosan and β-glycerolphosphate (β-GP), which formed a thermosensitive gel-forming aqueous solution after the addition drop-by-drop of a cold GP solution to the acidic solution of the chitosan. The final CS/β-GP solution increased the pH, but remained liquid at low temperatures and became a semisolid gel when heated at 37ºC. This new gelling system opened up doors in many possibilities in this area of research, motivating the experimentation and characterization during the last two decades.

The negative charges of phosphate react with the positive charges of the CS in acidic environments giving place to a neutral and homogeneous solution, forming a protective hydration layer around the chitosan molecules; this gelation mechanism is extensively explained in the following subsegment. The main concern of CS/β-GP hydrogels is their instability at room temperature and even at reduced temperatures, as the transition sol/gel occurs after a few days, what makes them impossible to storage during a considerable time for being ready-to-use. For this reason, other gelling agents were investigated, for instance, the glucose 1-phosphate (G1-P), glucose 6-phosphate (G6-P) and inorganic phosphate salt (without polyol group). From these ones, the one that presented the most similar behavior and results to the commonly used β-GP was the G1-P.

In this thesis, chitosan-based hydrogels with different gelling agents, namely the β-GP and the G1-P (*Figure 4*) were developed and characterised. The first one is a phosphoric ester of glycerol with lots of uses in biomedicine, outstanding especially on its function of driving osteogenic differentiation of bone marrow stem cells in vitro [21] and with more than proven biocompatibility [22], whereas the second one is a glucose molecule with a phosphate group on the 1'-carbon. Despite its similarity to the β-GP, the hydrogels made of CS/G1-P were not as deeply investigated and have not any specific application.
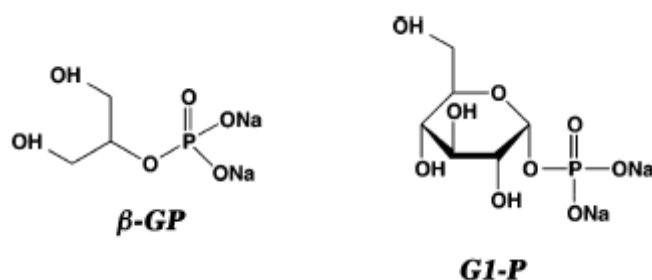
Figure 4: Molecular structures of the gelling agents studied: β-GP and G1-P

From the few investigations related to the G1-P, there should be noted the one lead by Stephanie Supper and al. [23], where there were presented the gelling properties of CS/G1-P and their ability to turn CS solutions into thermosensitive systems, and the one, also lead by Stephanie Supper [24], where it was analysed the CS/G1-P as new system for controlled drug delivery, it was pointed that the G1-P is more stable due to its thicker protective water layer around CS and it was stated that indeed it can be used as alternative gelling agent for CS-based solutions.

3.3. Gelation mechanism

Although the properties of the CS/salt solution were extensively studied, the exact working of the gelation mechanism still not well defined and understood. Several investigations were done, but one of the most convincing explanations of this mechanism was again the given by Supper et al. in 2014 [24], in which they studied the CS/β-GP gelation and they exposed the following interpretation.

The mechanism is based on multiple intermolecular interactions, that determine the solution/gel transition. These interactions are described in *Figure 5* and include:

a) At the beginning, the chitosan solution is solubilised because of the electrostatic repulsion between its positive chains, keeping them apart and allowing their mobility through the space. The solubility of the molecules of CS in acidic conditions is ensured thanks to this electrostatic repulsion.

b) When the gelling agent is introduced, there appears an electrostatic attraction between the amino groups of the CS (+) and the phosphate groups of the salts (-), causing their movement and electrostatic interactions. Once this action has taken place, the polymer chains are almost neutral and the reducing of the repulsion charges between the chains stimulates the formation of a precipitate in gel form. However, the presence of polyol in the gelling agent delays this precipitation and keeps the chitosan soluble. The polyols surround the chitosan molecules, protecting the CS molecules against thermal degradation, and at the same time water

molecules form hydrogen bonds with the polyols and between each other, forming a hydration protective layer. This layer remains stable at low temperature and neutral pH and prevents the formation of the gel.

c) The increasing of the temperature over the critical temperature, usually around 37⁰C, which is the body natural temperature, has several effects on the molecular structure of the CS/gelling agent solution, since it breaks the polyol layer and allows the interaction of polymers. More specifically, it stimulates the transfer of protons from CS to the β-GP, deriving in the neutralization of the CS and in less electrostatic interactions between the polymer and the gelling agent. This also results in the destruction of some hydrogen bonds, what induces the cohesion of the polyol protective shells around the chitosan molecules. The loss of these hydrogen bonds (originated for the increase of thermal energy) and the change in the pK_{ap} provoke the reduction of the CS chains hydration, impeding their solubilization in the aqueous phase and inducing their gelation by means of hydrophobic interactions.
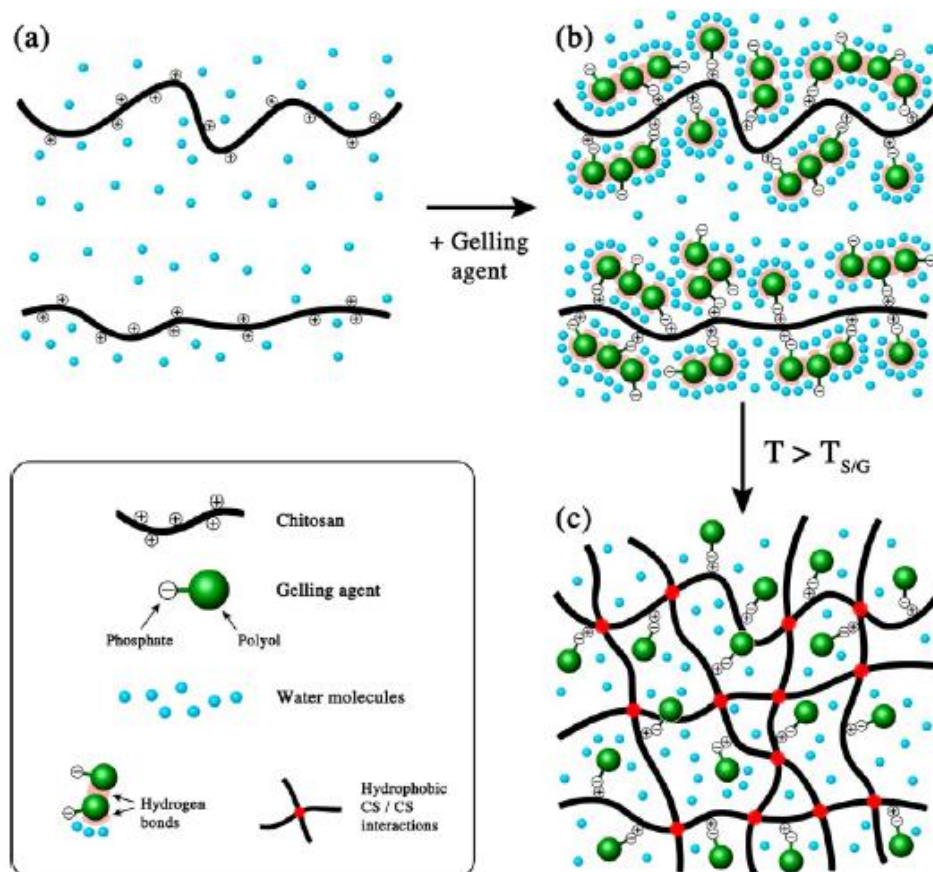

Figure 5: Representation of the Gelation Mechanism of CS/Polyol-phosphate Solutions

The study also concluded that the chemical structure and size of polyols are very important in the gelation mechanism, because they form the protective layer around the CS chains that subsequently is broken through the influence of temperature, inducing gelation. The

size of the polyol affects the stability of this hydration layer and has an impact on the transition temperature ($T_{S/G}$) and duration.

4. Materials and methods

In this segment of the project there can be found the materials and procedures used in the experimental process. The sections that it contains were subdivided in two different parts, since there were done two main groups of experiments. In the first one there were tested the development and properties of different hydrogels with the goal of finding the most proper one to experiment with cells, while the second one involves the cells investigation taken place at NICO.

4.1. Materials

4.1.1. Hydrogel development

For the procedure of elaborating and testing the hydrogels there were used two different kinds of chitosan, called CS 80/100 and CS 95/100, with a medium molecular weight of 100 kDa and a degree of deacetylation of 80% and 95% (*Heppe Medical Chitosan, Germany*), respectively. Being the chitosan in powder form, it is necessary to dissolve it to obtain a solution. For this purpose, it was used hydrochloric acid (*HCl, 37% v/v, Merk, Austria*) and distilled water, obtained through the QMillipore purification system (*Millipore Direct Q UV3, USA*).

There have also been used two types of gelling agent: the *β-Glycerophoshate Disodium Salt Pentahydrate* ($C_3H_7PNa_2*5H_2O$- *Santa Cruz Biotechnology, USA*) (*β* -GP) and the Glucose 1-phosphate ($C_6H_{11}Na_2O_9P*4H_2O$- *ThermoFisher, Germany*) (G1-P). These salts can also be found in powder form, but in this case for the dissolution was used medium DMEM (*Dulbecco Modified Eagle Medium*) with 2% of FBS (*Fetal Bovine Serum*), 1% of Q (*Glutamine*) and 1% of P/S (*Penicillin/Streptomycin Solution*) to simulate an environment more similar to the body one.

The degradation test was done in contact to PBS (*Phosphate-buffered saline solution, Sigma-Aldrich*), with a pH of 7.4. This buffer solution is commonly used in biological research because it is a water-based salt solution, that helps to maintain a constant pH and is isotonic and non-toxic to most cells. [25]

4.1.2. Cells experimentation

For the cells experiments the cell line that was used is the RT4-D6P2T from the American Type Culture Collection (ATCC), since it is a resistant cell line, has a fast velocity of proliferation and is easy to treat.

During the defrosting and manipulation of cells, the same DMEM used by the formation of the hydrogel is needed, which is the DMEM (*Sigma Aldrich, USA*) with 4.5 g of glucose, 10% of FBS (*Fetal Bovine Serum; Invitrogen, USA*), sodium pyruvate 1 mM (*Sigma Aldrich, USA*), 4 mM of Q (L-glutamine*; Sigma Aldrich, USA*) and 100 U/ml of P (*Penicillin; Sigma Aldrich, USA*) and 0.1 mg/ml of S (*Streptomycin Solution; Sigma Aldrich, USA*), where the cells can be suspended and can proliferate.

For the cell division process, it is needed a dissociation agent that allows the detachment of the cells from the surface (petri dish). The dissociation agent used in this case is the trypsin (0.05%) - EDTA (0.02%), which is a body natural-produced enzyme needed to feed the cells. During this process it is also needed the PBS solution (without Ca and Mg), as a washing fluid, and the same DMEM than in the other operations.

Finally, for the freezing of cells it needs to be added the 90% of FBS and a 10% of DMSO (Dimethyl sulfoxide), which is necessary to avoid the crystallization of the solution.

4.2. Development of chitosan-based hydrogels

4.2.1. Preparation of the chitosan solution

Considering the results obtained by Rosanna Fusaro on her master thesis [26], some hydrogels with different composition regarding not only the type of chitosan and its concentration, but also the type of salt (β-GP or G1-P), were prepared and tested with the objective of identifying the proper one for the experimentation with the cells.

The solubility of the chitosan powder CS 80/100 and CS 90/100 into the acidic solution of HCl 0.2 was tested on the same master thesis [26], where it was stablished that the maximum concentration that could be solubilized was 4.5% w/v (weight/volume) for the CS 80/100 in HCl 0.2M and 3.6% w/v for the CS 95/100 in HCl 0.2M. These are the concentrations used to prepare the chitosan solution within this work.

In the first place, the acidic solution was prepared by mixing 1.66% v/v (volume / volume) of HCl with 98.34% v/v of distilled water to obtain the HCl solution 0.2M during 10min. And in second place, the 4.5% w/v of CS 80/100 or 3.6% w/v of CS 95/100 in powder form was added and mixed for 48h at room temperature at 80-120 rpm until it became homogeneous. The mixer used was a magnetic stirrer (*IKA, Germany*). The chitosan solution obtained was stored in the fridge (T~4ºC) until the day of the conformation of the hydrogel.

4.2.2. Preparation of the CS/salt solution

For the conformation of the hydrogel it is required to solubilize the salt into liquid. In this case, the solubilization was done in water. The mixture is done with the help of the magnetic stirrer, modifying the velocity according to the degree of solubilization in each moment.

Once the salt is solubilized in water to obtain the desired concentration and ratio, and the solution acquires a temperature of around 4ºC (fridge), the union of the chitosan solution and the salt solution can start.

For this procedure, the right amount of chitosan must rest overnight in the fridge (~4ºC) to release the bubbles inside, and afterwards this one needs to be placed inside a cold bath (with ice cubes and water), which maintains its temperature between 4ºC and 6ºC. The temperature should be controlled with the sensor of temperature provided by the magnetic stirrer, adding more ice cubes in case of necessity. This magnetic stirrer also supplies the rotation movement for the mixing, beginning at the lowest velocity of 50 rpm, due to the high viscosity of the chitosan, and increasing it gradually with the time. The salt solution is added slowly but continuously, drop by drop, to the chitosan solution. The homogeneity of the final hydrogel solution is controlled through visual supervision.

4.2.3. Parameter definition

By following the same line as the previous master thesis [26], different tests were performed to the chitosan-based hydrogels and there were analysed the changes in its behaviour by modifying the following parameters:

- The chitosan type, in powder form, used for the elaboration of the solution: CS 80/100 or CS 95/100
- The salt type used for the salt solution: β-GP or G1-P.
- The concentration in weight/volume (% w/v) of chitosan in the final chitosan/salt solution. The lower or higher concentration of chitosan in the final solution can be achieved by dissolving the salt into more or less DMEM, respectively.
- The ratio (R), which indicates the relation between the moles of salt and the moles of amino groups at the final solution. By the variation of the ratio it gets modified the quantity of salt (g of β-GP or G1-P) needed to be dissolved in the DMEM and after added at the chitosan solution.

When the final solution was obtained, the pH was measured and the homogeneity was controlled through visual supervision.

Stating with a chitosan concentration of 4.5% w/v for CS 80/100 or 3.6 % w/v for CS 95/100, it can be calculated the amount of water or DMEM in which it is required to solubilize the

salt to obtain the concentration desired. For example, starting with a concentration of 3.6% w/v of CS 95/100 and working with an initial volume of 5ml of CS, there would be needed 2.2 ml of water or DMEM in order to obtain a final concentration of 2.5% w/v. The calculations to achieve this result are the following:

$$\frac{3.6 \; g \; of \; CS}{100 \; ml \; of \; solution} = \frac{x}{5 \; ml \; of \; solution} \; ; x = 0.18g \; of \; CS \; in \; the \; solution \qquad \text{(Eq. 4.1)}$$

$$\frac{2.5 \; g \; of \; CS}{100 \; ml \; of \; solution} = \frac{0.18 \; g \; of \; CS}{y \; ml \; of \; solution} \; ; y = 7.2 \; ml \; of \; solution$$

(Eq. 4.2)

$$7.2 \; ml \; of \; solution = \; 5ml \; of \; CS \; solution + 2.2 \; ml \; of \; salt \; solution$$

The range of concentrations treated in the experiments was between 1.5% w/v and 3% w/v of chitosan in the final solution.

The ratio (R), as mentioned before, indicates the relationship between the moles of the salt and the moles of amino groups of the chitosan:

$$moles_{salt} = R \cdot moles_{NH_2} \qquad \text{(Eq. 4.3)}$$

From the previous master thesis on hydrogels [26], the following data were obtained:

Chitosan solution type (5 ml)	Number of moles of the amino group ($mole_{NH2}$)
CS 80/100 4.5% w/v HCl 0.1 M	$0.950*10^{-3}$
CS 95/100 3.6% w/v HCl 0.2 M	$0.902*10^{-3}$

Table 1: Number of moles of the amino groups for 5ml of each type of chitosan solution

Salt type	Molar mass (g/mol)
B-GP	306.120
G1-P	376.172

Table 2: Molar mass (g) of each type of salt

Knowing the ratio, the number of moles in the volume of chitosan treated and the molar mass of the salts (β-GP or G1-P), the weight of salt needed for the dissolution can be calculated by means of the following equation:

$$mass_{salt} = R \cdot moles_{NH_2} \cdot M_{w\ salt}$$ (Eq. 4.4)

In the case of a hydrogel composed by CS 95/100 2.5% w/v and R=4 with β-GP salt, the mass of salt that necessary to conform 5 ml of this hydrogel is:

$$mass_{salt} = 4 \cdot 0.902 \cdot 10^{-3} \cdot 306.120 = 1.1045 \text{ g}$$ (Eq. 4.5)

4.3. Characterisation of hydrogels

Without having lost sight of the purpose of the investigation with hydrogels, this is, its characterization and the discovery of the most proper one for the cells experiment, in this section can be found the different analyses that were done to them.

Considering the final goal of these hydrogels, which is the application of them in the human body as engineered tissues for the regeneration of the peripheral nerve and the sense of touch, it is primordial that they accomplish the following points:

- The pH of the hydrogel needs to be between 7 and 7.4 [27], since it is the range of pH where cells can survive.
- The gelation time of the temperature-sensitive hydrogel needs to be low in order to achieve the desired structure while printing
- The stability in the fridge must be enough for allowing the storage of the hydrogel solution without being jellified before printing
- The velocity of degradation of the hydrogel in contact to liquid needs to match with the velocity of regeneration of the native tissue that is being treated. As the aim is to introduce it inside the human body, it would be in contact with a high volume of water (65% of adult human body is water).
- The hydrogels must have proper properties to be printed. For this reason, the viscosity of the hydrogel and the printing conditions need to be studied and adjusted. This theme is treated in the parallel thesis conducted by Marina Flores.

Taking into account the stated points, there were conducted several tests to verify the accomplishment of them, such as the measurement of the hydrogel solution (chitosan/salt final solution) pH and the evolution of it while gelation, the measurement of the gelation time of the hydrogel at 37ºC, the test of compression and the degradation test.

For a deep comprehension of the behaviour of the hydrogels and its good characterisation, diverse experiments were done with different chitosan type, chitosan concentration in the final solution and ratios. The first group of experiments was performed with CS 95/100 (3.6% w/v, HCl 0.2M) and β-GP salt. This first hydrogel was analysed in the previous thesis about hydrogels [26], where there were obtained the final concentrations of chitosan with the values of 1.5% w/v, 2% w/v and 2.5% w/v, through the addition of different amount of water into the salt solution (7 ml, 4 ml and 2.2 ml). For each concentration there were analysed the ratios with value 1, 1.5, 2, 2.5, 3, 3.5 and 4. In addition, there were also studied the solution of 2.75% w/v of chitosan concentration for an R of 2.5 and the solution of 3% w/v for an R of 2 and 2.5.

The second group of experiments was done with CS 80/100 (4.5% w/v, HCl 0.2M) and β-GP salt. Through the addition of 10ml, 6.25ml, 4ml, 2.5ml, 1.429ml and 0.625ml of total volume of salt solution to 5ml of chitosan solution overnighted in the fridge, there were obtained diverse final concentrations of chitosan, which are 1.5% w/v, 2% w/v, 2.5% w/v, 3% w/v, 3.5% w/v and 4% w/v. All of them, unless the experimentation with the concentration of 1.5% w/v, were also elaborated during the development of previous thesis [26]. Fixing these concentrations, there were executed tests for the R of 1, 1.5, 2, 2.5, 4, 5, 6, 7 and 8 depending on the case and following the line showed by the results with the CS 95/100.

The third group of experiments was conducted with CS 95/100 (3.6% w/v, HCl 0.2M) and G1-P salt. Also following the experiments already made with the other salt and chitosan type, there were developed the hydrogels that where more interesting in terms of solubility of the salt, value of the pH and gelation time. In this case, there were selected for the experimentation the hydrogels with 2% w/v of chitosan and a ratio of 2.5, 3, 3.5, 4 and 4.5, and the hydrogels with 2.5% w/v of chitosan and a ratio of 2.5 and 3. Furthermore, there was also made a trial with a concentration of 1.75% w/v and a ratio of 4.5.

Lastly, the fourth group of experiments were performed with the chitosan CS 80/100 (4.5% w/v, HCl 0.2M) and the G1-P salt. The tests were done with the concentration of 1.5% w/v and with the ratios of 5 and 7.

For the solution of each group that best fit the requirements, the experiments were repeated but dissolving the salt into medium (DMEM) instead of water, since it is the most similar liquid to the body environment and since it prevents the stress that the cells could suffer in a sudden contact with water. By this way, it was possible to ensure that the difference on the behaviour between dissolving the salt into water or medium (DMEM) is not significant.

4.3.1.   Gelation time

The gelation time of the final solution, based on chitosan and salt, was tested through the Method of the Tube Inversion at 37ºC, since it is approximately the temperature of the human body. This test consists in measuring the time in which the content (hydrogel) inside an Eppendorf does not flow, under its own pressure, when inverting it. The difference of time between the observation of this phenomenon and the insertion of the Eppendorf in the incubator does the gelation time.

The temperature of 37ºC was achieved by means of an incubator (*Fratelli Galli, Italy*) and the container chosen was an Eppendorf of 1.5 ml, with a quantity of 0.5ml of hydrogel solution inside. The inversion of the Eppendorf was done at different time intervals, starting with small time intervals, of 1-2 min and increasing or decreasing it with time in relation to the fluidity observed.

4.3.2.   pH analysis

The cells can only survive in fluids with pH values between 7 and 7.4 and, for this reason, not only the pH of the final solution was measured, but also the evolution of this pH during the gelation procedure was analysed (4.3.4).

The pH of the final solution was measured by means of a pH meter (HI 9124/25 pH Meter, Hanna Instruments, Italy), which measures the hydrogen-ion activity in water-based solutions. The pH was measured in the same container, where the mixture of chitosan solution and salt solution were placed.

4.3.3.   Degradation

The degradation test was executed to understand the amount of hydrogel that is dissolved into the PBS (7.4 of pH) in different time points at 37ºC. The samples consisted of 0.8 ml of Hydrogel after 24h at 37ºC, for a complete gelation of them, inserted in a lab bijou of 18mm of diameter and 7 ml of capacity (*Thermo Scientific™ Sterilin™*). The samples were weighted before and after the gelation (W_{bijou} and $W_{hydrogel}$).

Then, 1.6ml of PBS were added on the top of the jellified hydrogel in each bijou. These bijous were put again in the incubator and they were analysed at different periods of time (1h, 1 day, 4 day, 7 days and 14 days). In each analysis, the PBS was removed from the samples and was stored in a different bijou. The pH of this PBS was measured at room temperature and the samples with the hydrogel (with the PBS completely removed) were weighted ($W_{hydrogel\_PBS}$).

The samples with the hydrogel were frozen for at least one night and they were lyophilised at vacuum for 24h (*Scanvac, CoolSafe™, Denmark)*. The samples were weighted again ($W_{lyophilised}$) in order to know the content of water that these samples had. The calculus used to know the content of water at equilibrium (EWC, *Equilibrium Water Content*) is the following:

$$EWC\ (\%) = \frac{W_{hydrogel_{PBS}} - W_{liophilised}}{W_{hydrogel_{PBS}}} * 100 \qquad \text{(Eq. 4.6)}$$

The weight lost (WL) was calculated as follows:

$$WL\ (\%) = \frac{W_{lyophilised\_theo} - W_{liophilised}}{W_{lyophilised\_theo}} * 100 \qquad \text{(Eq. 4.7)}$$

where the $W_{lyophilised\_theo}$ is the lyophilised weight of a sample that was not in contact with aqueous medium (PBS) prior to lyophilisation. To determine this value, three samples of 0.8 ml of hydrogel were prepared. The empty bijou was weighted and also the bijou after lyophilisation, allowing the calculation of the lyophilised content. The mean of the three samples was done and the value was defined as the theoretical lyophilised weight. The experiment was developed for both hydrogels studied (one with β-GP as gelation agent and the other one with G1-P).

The test was done on 3 samples for each hydrogel and each interval of time. The hydrogel studied are the ones that had best results regarding the gelation time and the pH, which are CS 95/100 2.5% w/v R=4 with β-GP salt, CS 80/100 1.5% w/v R=8 with β-GP salt and CS 95/100 2% w/v R=4.5 with G1-P salt.

4.3.4. pH evolution

The evolution of the pH during gelation was also monitored using the pH meter. In this case, 0.5 ml of the final solution were put in an Eppendorf and this one was placed in the incubator at 37ºC with the pH meter inside. Values at different time intervals were measured, starting by taken values each 15 seconds, since the variation of the pH is rapid during the first minutes and increasing it with time when detecting a diminution of the rate of change. The procedure lasts until the value of pH is stable for 5-10 minutes.

The values of gelation time and the pH are the most relevant ones to know if it makes sense to continue experimenting with it or if it cannot be used for the regeneration of the peripheral nerve. In general, when a hydrogel has not accomplished the requirements of time or pH, any other experiment was done with it. For this reason, the pH evolution was not performed with all the samples but only with the most interesting ones of each group of experiments

(CS 95/100 2.5% w/v R=4 with β-GP salt, CS 80/100 1.5% w/v R=8 with β-GP salt and CS 95/100 2% w/v R=4.5 with G1-P salt).

4.4. Cells experimentation

In this section there are described the steps and methodology used for the experimentation with cells. The selected hydrogels were the CS 95/100 2.5% w/v R=4 with β-GP and the CS 95/100 2% w/v R=4.5 with G1-P salt, since they have good properties regarding the characterization made at the laboratory of the Politecnico di Torino, particularly regarding gelation time and pH. As POLITO does not have yet a laboratory prepared for cell treatment, with its proper sterilization, the cells experimentation has taken place in the laboratory of Human anatomy, Department of Clinical and Biological Sciences, San Luigi's Hospital.

The cell line used for the experiments is the RT4-D6P2T from the American Type Culture Collection (ATCC, # CRL-2768), which is a glial cell line derived from a rat schwannoma induced by treatment with N-ethyl-N-nitrosurea. This cell line was selected for its glial nature, given the regenerative application of the developed hydrogels. The regeneration of the peripheral nerve in fact is due to the Schwann cells fundamental role in guiding the regrowth of the new neurites. Furthermore, this cell line has a considerably good response by cell culturing because of its resistance and proliferation velocity. [28]

As the objective of the cells experimentation is to know if cells survive in contact to the hydrogel, it is important to decide whether to culture cells in 2D (at the top of the hydrogel) or in 3D (encapsulated into the hydrogel). Even if this choice depends majorly on the kind of application that the hydrogel is going to have, each configuration presents its points in favour and in contra. The culture of cells in 2D environments reduces the confine that they suffer, giving them more mobility and space for their processes, while the 3D environments mimic more accurately the tissues of the body and more realistic cellular responses can be obtained. Therefore, it was decided to analyse both kind of environments, with different percentage of contact between the cells and the hydrogel, and get to know the behaviour of cells in both cases.

It is extremely important to work under sterile conditions when working with cells, since any kind of contamination could cause them damage or kill them. That is why during the development of all the experiments in San Luigi, the hydrogel was fabricated controlling its sterility all the time. The recipients used were sterilised previously with the autoclave (*Melag Autoklav 24, Germany*) and also the magnets and the powder of chitosan. The salt solution was sterilised by filtration, using a syringe with a needle (*Terumo, Philippines*) to take the solution, and a filter (*Sartorius stedim biotech, Germany*). Finally, the conformation and manipulation of the hydrogel was always done under the biosafety cabinet.

The experiments were developed in order: i) test the response of the cells to the salt, ii) introduce the indirect contact of them with the hydrogel and iii) put the cells in direct contact with the hydrogel by encapsulating them or culturing them on the top of the hydrogel. In this way a progressive study can be done and different conclusions can be drawn.

4.4.1.   Treatment of cells

The cells need to be prepared and manipulated for a good treatment of them, to work with the desired concentration of cells and the desired conformation, and for a posterior good analysis. For this reason, several procedures were done before using cells for experimentation, which are explained in this subsection.

The frozen cells were thawed in a water bath at 37 °C (*Savatec Strumenti scientifici, Italy*). Once thawed, DMEM 10% FBS was added, drop by drop, to the cell resuspension that was subsequently centrifuged at 900 rpm for 4 minutes at room temperature (*Centrifuge 5702, Eppendorf, Germany*), in order to eliminate the DMSO used to freeze. The supernatant was removed and complete medium was added to the pellet by means of a pipette (*Sarstedt, Germany*). Finally, the cells were plated and kept in culture. [29]

The cells are cultured in petri dishes (*Sarstedt, Germany*) with DMEM and FBS, where they grow and divide until they fill all the surface available. At this point, a nutrient depletion and pH change in the growth media starts to appear, as well as a cell-to-cell contact that can cause the stop of the cell division. For this reason, it is necessary to divide the cells during the experiment in order to avoid the contamination and cells dead. [30]

The splitting method consists in removing the old medium and detaching the cells from the surface of the petri dish with the action of 1.5 ml of trypsin (0.05%) - EDTA (0.02%) at 37ºC (incubator) for 1.5 seconds. Afterwards, a small number of cells can seed a new culture, meaning that a small volume of the cells in suspension into new DMEM is put in a new petri dish, which is filled also with DMEM to reach the 10ml. The most typical splitting ratio of these cells is 1/16, since they grow very fast. This procedure needs to be done at least once a week.

The cells that remain unused after the splitting can be thrown or frozen and stored. For this last option, the storage is made in a cryovial. The cells in suspension are spun (4 min at 900 rpm at room temperature) and deposited at the bottom of the falcon. The medium is removed and the cells are resuspended with a 90% of FBS and a 10% of DMSO. The cells are frozen at -80ºC for 2 days and then definitely transferred to liquid nitrogen.

Cells need to be fixed in order to perform immunohistochemical reactions and be visualized. This process involves the removing of the medium (DMEM) from the samples, with which cells had been cultured, the washing of the cells twice with PBS and the addition of

paraformaldehyde 4% (PAF 4%), which acts as fixative solution. After resting 15 min in PAF, it is removed and another two washes in PBS are done before storing it in the fridge with PBS with 1% of sodium-azide on the samples.

4.4.2. Observation of cells vitality cultured in presence of β-GP and G1-P salts

The first experiment developed with cells was the observation of the cells vitality after different time-points into a DMEM medium that contained either β-GP or G1-P. By this way, it is possible to observe how cells react to the presence of salts and if it causes any damage or detracts the cells wellness somehow.

In this experiment two different cell lines were analysed to compare the behaviour of the two kinds of cells under the presence of the salts. The cell lines that were chosen are the RT4-D6P2T, and the NIH3T3, that is a cell line derived from mouse fibroblast.

For the good attachment and proliferation of cells before the addition of the salt, the cells were plated in plates of 9 cm^2 with a concentration of 200000 cells/plate for the RT4-D6P2T cell line and 100000 cells/plate for the NIH3T3 cell line and cultured for 2 days before the experimentation.

For the correct development of the experiment and to compare the effect of the two salt into the cells, the same concentration has to be achieved for all the plates with the same kind of cells. Therefore, it is crucial to know with high precision the number of cells put at the beginning of the experiment in each plate. The initial concentration of the cells solution into the petri dish where they were cultured was measured by means of a Bürker's chamber, which is a device used for cell counting and has the shape showed in *Figure 6*.
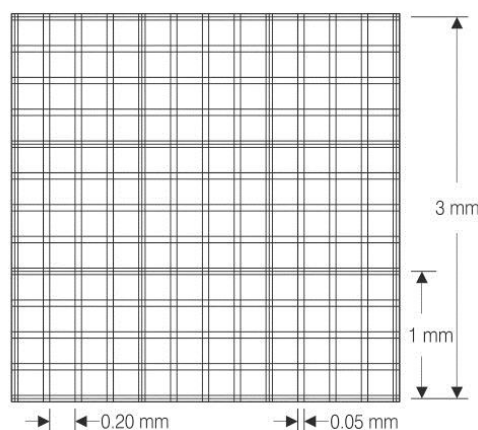

Figure 6: Burker's counting chamber

The chamber is composed by 9 squares of 1 mm^2 each, which are subdivided by double lines into 16 group squares. The cells are counted in 4 or 5 of the 9 squares and a mean of the number obtained in each square is done, obtaining for the case of RT4-D6P2T a result of 30.25 cells. This number multiplied by 10^4 indicates the concentration of cells in 1 ml of medium:

$$\text{Concentration of cells} = 30.25 * 10^4 = 302500 \text{ cells/ml} \qquad \text{(Eq. 4.8)}$$

Knowing the concentration of cells desired in the plates, in this case 200000 cells/plate, the volume desired in each plate, in this case 1 ml, and the number of plates that have to be filled for each kind of experiment, 6 plates in total: 2 plates with RT4-D6P2T and β-GP, 2 plates with RT4-D6P2T and G1-P and 2 plates for control (without salt), it is possible to know the volume of the petri dish that needs to be dissolved with medium to achieve the desired concentration in each plate:

$$200000 \ \frac{\text{cells}}{\text{plate}} \cdot 6 \text{ plates} \cdot \frac{1 \text{ ml}}{302500 \text{ cells}} = 3.97 \text{ ml} + 8.03 \text{ ml of medium} \qquad \text{(Eq. 4.9)}$$

The volume of 2 ml was put in each plate and they were deposited in the incubator at 37ºC for 2 days. After this time, the medium is removed and the salt solution is being added over the attached cells. Two different concentrations of salt were analysed, one corresponding to the 50% of salt content in 1 ml of hydrogel and one corresponding to the 30% of the salt content in 1 ml of hydrogel. The 50% of salt is the approximate amount of salt that is released after gelation, while the 30% of salt content is used to analyse if the cells survival is dependent on the amount of salt that is released by the hydrogel. In summary, the experiment with RT4-D6P2T line cell is made up of:

- 1 plate for RT4-D6P2T and the 50% of β-GP from 1 ml of CS 95/100 2.5% w/v R=4 with β-GP hydrogel solution
- 1 plate for RT4-D6P2T and the 30% of β-GP from 1 ml of CS 95/100 2.5% w/v R=4 with β-GP hydrogel solution
- 1 plate for RT4-D6P2T and the 50% of G1-P from 1 ml of CS 95/100 2% w/v R=4.5 with G1-P hydrogel solution
- 1 plate for RT4-D6P2T and the 30% of G1-P from 1 ml of CS 95/100 2% w/v R=4.5 with G1-P hydrogel solution
- 2 plates for RT4-D6P2T control

The amount of salt needed to conform 5 ml of hydrogel, calculated with the equation 4.4, is 1.1045 g of β-GP for the CS 95/100 2.5% w/v R=4 hydrogel solution and 1.5269 g of G1-P for the CS 95/100 2% w/v R=4.5 hydrogel solution. The 50% and 30% of the amount of these salts for 1 ml can be found in the following table (*Table 3*):

In 1 ml of hydrogel	CS 95/100 2.5% w/v R=4 with β-GP hydrogel solution	CS 95/100 2% w/v R=4.5 with G1-P hydrogel solution
50% of salt (g)	0.077	0.085
30% of salt (g)	0.046	0.051

Table 3: Amount of salt dissolved in medium for the observation of cells vitality cultured in presence of salt

The previous amount of salt was dissolved in each case with 2 ml of DMEM. The old DMEM is removed and the new salt dissolution was put in the plates over the cells, as it can be seen in the *Figure 7*. Pictures of the cells on the third day under this environment were taken and a comparison between both kind of salts and its effects on cells is done.
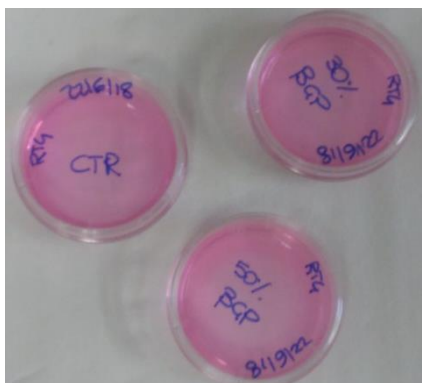
Figure 7: Plates for the experiment of the observation of the vitality of cells cultured in presence of beta-GP salt in different concentrations and in medium (control)

For the other cell line, the NIH3T3, the concentration of cells in each plate was 100000 cells/plate and it was calculated using the same methodology as for the RT4-D6P2T. First calculated with the Bürker chamber the concentration of cells in the petri dish, where they were cultured, and then dissolving the right volume of cells with medium to acquire the desired concentration to plate.

For this cell line there were analysed 2 plates for each salt concentration and kind of salt and two extra for the control. The day of the experiment, the medium was removed and the new medium with salt dissolved in it (2 ml in total with the amount of salt in *Table 3*) was added on the cells. Then, in this case pictures were taken after three days.

4.4.3. Proliferation test in supernatant

The proliferation of cells is a natural and healthy process by which cells grow and divide. Contrasting the ratios of proliferation of healthy cells or cells in control with the ratio of the

cells in contact with the hydrogel, either encapsulated or on the top, insight of the cells health may be provided. Before putting the cells in direct contact to the hydrogel, it was considered opportune to develop a proliferation test with the cells in indirect contact to the hydrogel. This indirect contact was achieved by culturing cells in a medium put in contact with the formed hydrogel (after gelation) during one day of incubation. This experiment has the aim of proving if the hydrogel releases any component or particle that could detriment the RT4-D6P2T line or cause any damage.

The experiment was performed for the two kinds of hydrogels being studied, both composed by CS 95/100 but with different gelation agents (β-GP and G1-P). The procedure used for the quantification of cells consists in counting the number of cells at time 0 and counting the cells in different time points, in order to build a proliferation curve and contrast the natural evolution of cells in their natural environment (medium for cell culturing) with the evolution of cells in other environments.

Cells were counted with the Bürker's chamber, following the procedure described in section 4.4.2. The results from the counting of cells were analysed and the proliferation curve of cells in both kinds of supernatants was computed and compared.

4.4.3.1. CS 95/100 2.5% w/v R=4 with β-GP

The first thing by the development of this experiment was to conform 12.8 ml of hydrogel, following the procedure described in sections **¡Error! No se encuentra el origen de la r eferencia.** and **¡Error! No se encuentra el origen de la referencia.**, by mixing 8.88 ml of CS with 3.9 ml of β-GP solution (with 2.334 g of β-GP; after filtration). The volume of hydrogel was distributed in a multiwell with 12 wells (*Becton Dickinson, USA*), where each well has a surface of 3.66 cm^2 and was filled with 1 ml of hydrogel, and in a multiwell of 6 wells (*Becton Dickinson, USA)* with a surface of 9.6 cm^2, where only one well was filled with 2 ml of hydrogel.

The filled multiwells have rested for 30 min in the incubator (37ºC), after which time the double volume of medium (DMEM) was added over the hydrogel surface. This medium (supernatant) was collected after 1 day in the incubator and was filtered.

The second part of the experiment consists in culturing cells in the previously described dissolution medium in plates for cell culture of 9 cm^2 of surface. The desired concentration is 10000 cells/cm^2 and the procedure to calculate the amount of cells solution necessary to be dissolved in order to achieve the desired concentration is the same than in the previous experiment. The concentration of cells in the petri dish was calculated through the Bürker's chamber, obtaining a value of 648000 cells/ml (Eq. 4.8).

The desired concentration in each plate was determined using the area and the desired concentration per cm^2:

$$\text{Concentration in each plate} = 10000 \ \frac{\text{cells}}{cm^2} \cdot 9 \ cm^2 = 90000 \ \text{cells/plate} \qquad \text{(Eq. 4.10)}$$

By means of these two values and the quantity of plates that need to be filled (12 for the experiment with supernatant and 12 for controls), it is possible to compute the volume of cells that should be taken and dissolved in new medium to achieve the desired concentration in each plate (as in the eq. 8). 1 ml of cells was plated with 1 ml of medium (DMEM with 2% of FBS) in each plate.

The evolution of the cells after 1 day, 2 days and 6 days in culture with supernatant was analysed. The samples need to be compared with cells cultured in normal medium, which are called control samples. For a more complete study, for the experiment of the hydrogel composed of CS 95/100 and β-GP, 4 samples with cells in culture with supernatant were analysed for each time point, and also other 4 samples for controls.

4.4.3.2. CS 95/100 2% w/v R=4.5 with G1-P

For this experiment the hydrogel was fabricated with 6.6 ml of CS and 5.4 ml of G1-P dissolved in medium and filtrated. The volume of hydrogel solution was distributed in 12 wells of a multiwell (*Becton Dickinson, USA*), where each well has a surface of 3.66 cm^2 and was filled with 1 ml of hydrogel. After the gelation of the hydrogel during 30 min in the incubator, 2 ml of medium were added over the filled wells.

The multiwell rested for 24 hours in the incubator at 37ºC, after this period the supernatant was collected and filtrated. This fluid was used as cell culture medium, following the same procedure as for the previous hydrogel.

The desired concentration of cells in each plate is also in this case 90000 cells/plate and the initial concentration of cells in the petri dish is 660000 cells/ml. Knowing these both concentrations and the number of plates that need to be filled (9 for supernatant and 9 for controls), it can be calculated the right volume of cells to be dissolved to achieve the desired concentration in each plate. Six samples for each time-point, 3 for cells in culture with supernatant and 3 for cells in culture with normal medium (controls) were analysed. The time-points at 1 day, 2 day and 6 days were also analysed.

4.4.4.   Direct contact test

Once an idea of the behaviour of cells by culturing them in salt dissolutions was obtained, it is necessary to analyse the behaviour of cells in direct contact to the hydrogel. It is

essential to develop an experiment where cells can come in direct contact with the hydrogel, since this is going to be the case by the temporal substitution of a nerve for its regeneration inside the body.

As it was mentioned at the beginning of the section **¡Error! No se encuentra el origen de l a referencia.**, there are different configurations for cells in contact with the hydrogel. The culture of cells can be done in 2D or in 3D, by putting them on the surface of the formed hydrogel or by conforming the hydrogel directly with cells encapsulated in it, respectively. The culture of cells in 2D gives the cells a higher mobility and can give information about the release of any component that can interfere with the cell vitality, whereas the encapsulation of cells can provide information about the matrix that forms the hydrogel and if it allows the execution of the basic processes of cells. For this reason, both experiments were done with RT4-D6P2T cells and with both kinds of hydrogels (containing β-GP or G1-P as gelation agent). The two configurations that were analysed are schematized in *Figure 8: Position of the cells in relation to the hydrogel*.
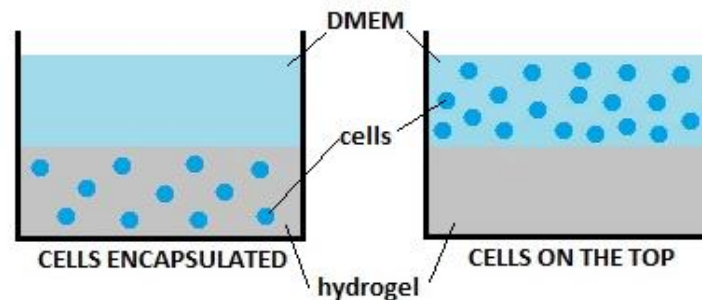
Figure 8: Position of the cells in relation to the hydrogel

First of all, the hydrogel was fabricated, as explained in the sections **¡Error! No se e ncuentra el origen de la referencia.** and **¡Error! No se encuentra el origen de la referencia.**, by first conforming the chitosan solution and then adding the salt solution within a controlled temperature. After that, the treatment of the hydrogel solution and the seeding of cells begins.

The procedure for the encapsulation consists in suspending the cells with the help of a pipette into the hydrogel in solution state (before gelation), absorbing and discharging it several times ensuring a homogeneous distribution of the cells throughout the volume. Cell resuspension was plated and incubated for 30 min at 37ºC. After gelation, the medium was put over the hydrogel, simulating the conditions in contact to aqueous medium present inside the body.

The experiment with the cells on the top consists in adding the pellet from centrifugated cells (at 90 rpm during 4 min) resuspended in 200 µl of medium over the jellified hydrogel

(30 min in incubator), letting them 30 min in the incubator for the proper attachment of the cells on the surface of the hydrogel. After this time some DMEM is added over them. The presence of DMEM over the hydrogel in theory should affect it the same way as the PBS does in the degradation test, because the composition of both solutions is similar.

The first step before developing a proper proliferation test for cells in direct contact with the hydrogel is to analyse the viability by means of a viability test, in which it is defined qualitatively if cells are able to live and develop in contact with the hydrogel. A very practical and simple type of viability assay is the live/dead assay (L3224, LOT1873203), that is the one used to analyse the samples in this experiment. It consists in a two-colour fluorescence cell test based on the simultaneous determination of live and dead cells with two probes that measured specific parameters of cell viability. The parameters that are recognised by these probes are the intracellular esterase activity and the plasma membrane integrity. [31]

To apply this live and dead assay and to be able to study and analyse the cells under the microscope they need to be placed out of the multiwell or transwell, where the experiment is done. For an easier manipulation of the samples with cells and in order not to lose information by destroying them when transferring them from one surface to the other, a small squared glass is places on the bottom of the wells. The cells and the hydrogel were adhered to its surface and when they were analysed, they were placed in another recipient (see *Figure 9*). For the analysis of the samples with the confocal microscope (LSM800, Zeiss, Germany) cells were fixed (following the section 4.4.1) and mounted on glass coverslip

Figure 9: Glass surfaces where hydrogel and cells are adhered to make the process of application of teh live/dead assay and its analyses with the confocal microscope easier.

4.4.4.1. CS 95/100 2.5% w/v R=4 with β-GP

The hydrogel was fabricated with 12 ml of CS 95/100 and 5.27 ml of β-GP dissolution into medium. This experiment was performed both within a transwell and in a multiwell. The transwell is the support used commonly for the migration test of cells, and in this case, it is used since it allows the addition of a very low quantity of hydrogel inside and its contact over and under with medium. In this way, cells can grow in an environment more similar to the one of the body, where it exists a higher exchange with the surroundings.

The desired cells concentration was 10000 cells/cm^2, that for an area of 3.66 cm^2 result in a concentration of 36600 cells/well. By means of the Bürker's chamber the concentration of the initial cells dissolution can be obtained, which is 445000 cells/ml. Considering the number of wells that need to be filled from each configuration, the volume of cells needed can be calculated as follows:

$$36600 \ \frac{\text{cells}}{\text{well}} \cdot 4.5 \ \text{wells} \cdot \frac{1 \ \text{ml}}{445000 \ \text{cells}} = 0.37 \ \text{ml} \qquad \text{(Eq. 4.11)}$$

The calculations were done for 4.5 wells instead of 4 wells because of the manual error. This volume of cells was the volume required for each kind of experimental design (encapsulated, top and control; 4 samples of each). These volumes were soaked separated in falcons, with 3 ml of DMEM in each, and they were spun at 90 rpm during 4 min to form a pellet. The medium was removed and the cells are resuspended in 4.5 ml of hydrogel for the encapsulation experiment, and the other two falcons in 4.5 ml of medium each for cells on the top and controls.

For the multiwell with a distribution of 4x3 wells, which have a surface of 3.66 cm^2 each, the filling of the wells was the following:

- 4 with 1 ml of hydrogel with encapsulated cells (covering all the surface), which rested for 30 min in the incubator (at 37ºC). After this time, 2 ml of medium was put over the hydrogel and the multiwell was placed again inside the incubator.
- 4 with 1ml of hydrogel without cells, which was in the incubator for 30 min to achieve the gelation. After that, the centrifuged cells were placed over the hydrogel surface, which also rest 30 min in the incubator for a good attachment. Finally, 2 ml of medium were added on the top the multiwell was placed again inside the incubator.
- 4 with control cells resuspended in DMEM 2% FBS.

The analyses of the vitality of the cells (live/dead assay) were done at two different time-points, 1 day and 2 days, and in each time-point 2 samples from each configuration were studied.

The distribution was done in two different multiwells for each time-point, as it is shown in the *Figure 10*.
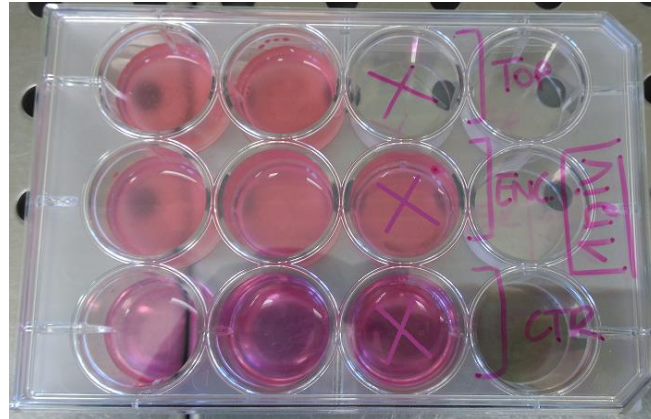

Figure 10: Distribution of the experiments in the multiwell for the analysis after 1 day (1 div) of incubation. The hydrogel studied is the CS 95/100 2.5% w/v R=4 with β-GP.

A second experiment using the same procedure and analyses was perfotmed with the transwell support from 6x4 wells, with 8.0 μm of pore size of the membrane and an area of 1 cm^2.

The desired concentration of cells also in this case is 10000 cells/cm^2, that for an area of 1 cm^2 results in a concentration of 10000 cells/well. The initial concentration on the petri dish (counted with the Bürker's chamber) is 445000 cells/ml. The calculations of the cells volume that should be taken to achieve the desired concentration in each well were:

$$10000 \ \frac{\text{cells}}{\text{well}} \cdot 4.5 \ \text{wells} \cdot \frac{1 \ \text{ml}}{445000 \ \text{cells}} = 0.101 \ \text{ml} \qquad \text{(Eq. 4.12)}$$

This volume of cells is the volume required for each kind of configuration (encapsulated and top; 4 samples of each). These volumes were soaked separated in falcons, with 3 ml of DMEM in each, and they are spun at 90rpm during 4 min to form a pellet. The medium was removed and the cells were resuspended in 0.9 ml of hydrogel for the encapsulation experiment, and in 0.225 ml of medium for cells on the top.

The filling of the wells for the transwell was the following:

- 4 wells with 0.2 ml of hydrogel with encapsulated cells. Once the suspension of the cells inside the hydrogel was done, it was incubated for 30 min at 37ºC and after this, 0.3 ml of medium were added on the bottom part of the transwell (under the membrane) and 0.1 ml was added on the top of the formed hydrogel.
- 4 wells with 0.2 ml of hydrogel and cells on the top. The hydrogel rested 30 min in the incubator and after this time the cells were added. Cells were incubated for 30

min for a proper attachment and finally the medium was distributed around; 0.1 ml on the top and 0.3 ml on the bottom of the well.

Also for this support the analysis (live/dead assay) was performed in to different time-points: after 1 day in culture and after 2 days in culture, studying 2 samples from each configuration each time-point.

4.4.4.2. CS 95/100 2% w/v R=4.5 with G1-P

For the development of the experiment with the second type of hydrogel, the CS 95/100 2% w/v R=4.5 with G1-P, the same steps as in section 4.4.3.1 were followed. The hydrogel was produced with 3.75 ml of CS and 3 ml of G1-P solution (in medium) after filtration.

The support used for this experiment was a multiwell of 12 wells, with a surface of 3.66 cm^2 each, and the desired concentration is 10000 cells/cm^2. Therefore, also in this case the final concentration in each well is 36600 cells/well. Knowing the number of wells for each configuration (3 wells) and the initial concentration in the petri dish where cells had been in culture, which is 547500 cells/ml, the resultant volume of 200 µl for each configuration can be determined.

For cells on the top, the 200 µl were dissolved with other 100 µl of medium in order to add 100 µl in each well drop by drop. 1 ml of hydrogel was added in 3 wells and after its gelation during 30 min in the incubator, the addition of the cells on the surface was done. It rested other 30 min in the incubator, after which 1 ml of medium was added over the cells.

For the encapsulation of cells, the 200 µl were resuspended several times in 3 ml of hydrogel to achieve a uniform homogeneity and 1 ml of this dissolution was poured in each well. The wells have rested 30 min in the incubator, after which 1 more ml of medium was added on the surface.

For the control cells, the 200 µl of cells were resuspended in 3 ml of DMEM 2%FBS for a further addition of 1 ml in each well.

All the samples rested in the multiwell at the incubator for 1 day, after which the analysis (live/dead assay) was performed.

4.5. Statistical analysis

The experimental data were analysed by means of the Excel 2016 for Windows (*Microsoft Software*). The graphics were plot using this software and they are represented as the average value ± standard deviation (S.D.).

5. Results

5.1. Hydrogel development and characterisation

In this section, the results of the experiments performed to characterise the hydrogels are presented and discussed.

5.1.1. Gelation time and pH

The gelation at 37ºC was monitored through visual inspection, since the sample changes the consistency and colour when it jellifies, becoming whiter and thicker, as it can be observed in the *Figure 11: Tube inversion method*. The moment in which the change of colour occurred and the content did not flow anymore was the moment in which the gelation time was taken as meaningful value.

Figure 11: Tube inversion method

For the first group of experiments, the gelation time and pH of the chitosan 95/100-based hydrogel with β-GP salt as gelling agent, the gelation time and pH was analysed for the ratios of 1, 1.5, 2, 2.5, 3, 3.5 and 4 and for different concentrations. The experiments were conducted and sampled in the previous thesis [26], thanks to which it can be concluded that from this group of experiments, the solution that best accomplishes the points mentioned in section **¡Error! No se encuentra el origen de la referencia.** is the CS/β-GP solution with a concentration of 2.5% w/v of CS 95/100 and a ratio of 4. The characteristics of this solutions are a pH of 7.08 and a gelation time of 5 min at 37ºC (for a sample of 0.5 ml).

It was considered appropriate to prove if the behaviour of the solution changes by dissolving the salt into medium instead of into water. For this reason, once the best option from the first group was identified, the analyses of the pH and gelation time were repeated with the new CS/β-GP solution (β-GP dissolved into medium), and the results were compared with the results got dissolving it into water (*Graphic 1*).

From the result was perceived that the behaviour is not exactly the same with both kind of dissolutions. There was a significant decrease on the value of the gelation time and also a slight variation on the pH.
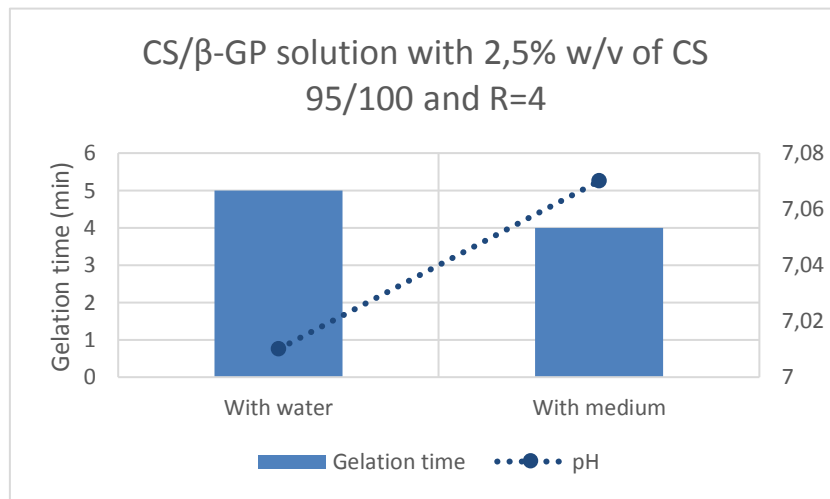

Graphic 1: Comparison between the CS/ β-GP solution with 2.5% w/v of CS 95/100 and R=4 when dissolving the β-GP with water and with medium. Analysis of the pH (T: 4-6 ºC) and the gelation time at 37ºC.

For the hydrogel composed of CS 80/100 and β-GP, several concentrations were analysed (1.5, 2, 2.5, 3, 3.5, 4) with a range of ratios from 1 to 8. *Graphic 2* demonstrates that the pH increases with an elevation of the ratio for hydrogels with the same concentration. The tendency can be very well approximated to a linear equation with positive gradient, since the R value proves (being it proximal to 1).
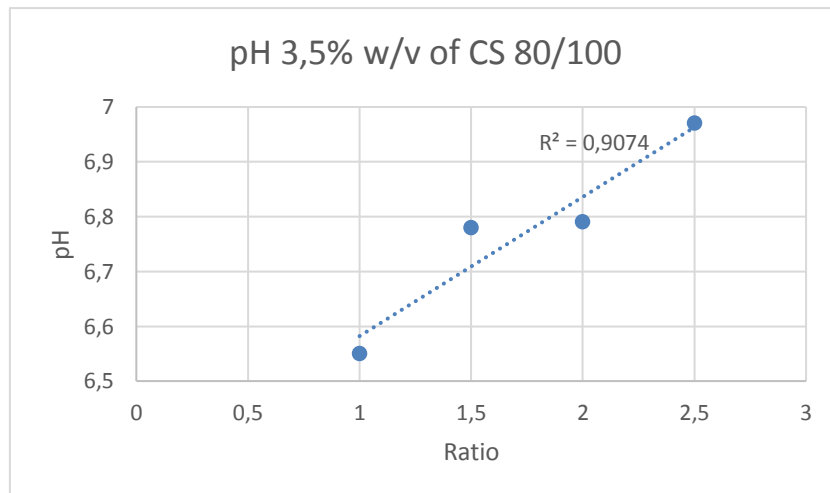
Graphic 2: Evolution of the pH (T: 4-6 ºC) of CS/β-GP solution with a concentration of CS 80/100 of 3.5% w/v with different ratios (R), developed from a solution of CS 80/100, 4.5% w/v, HCl 0.2 M.

However, fixing the ratio and changing the concentration the result was the opposite to the one observed in the CS 95/100 with β-GP, since with the CS 80/100 a decrease on the concentration derivates in a smaller gelation time (*Graphic 3*).
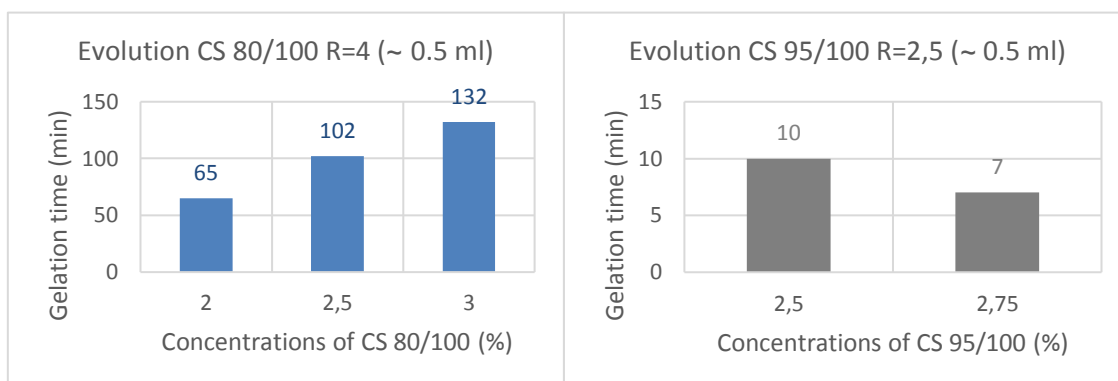

Graphic 3: Comparative between CS/ β-GP solution developed with CS 80/100 and CS 95/100 by fixing the ratio and changing the concentrations. Gelation time measured with a sample of 0.5 ml in the incubator at 37ºC.

For this reason, it was decided to proceed with the analyses of the concentration of 1.5% w/v, expecting a lower gelation time. With this concentration a complete analysis was made, so that it could be studied not only the gelation time, but also the pH by changing the ratio (*Graphic 4*). The results were compared with the results for a concentration of 2% and it can be seen that regarding the gelation time it is clearly preferable the concentration of 1.5% w/v because of its lower gelation times. Regarding the pH both hydrogels work, since almost all the solutions were on the range of pH where cells can survive.
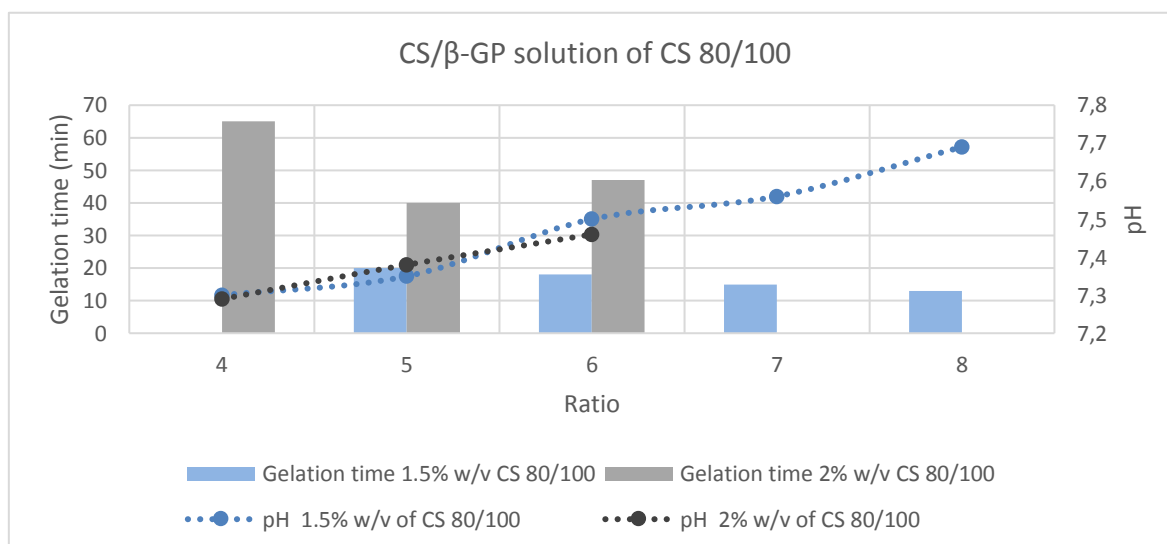
Graphic 4: Analysis of the pH (T: 4-6 ºC) and the gelation time at 37ºC of CS/β-GP solution with a concentration of CS 80/100 of 3.5% w/v with different ratios (R), developed from a solution of CS 80/100, 4.5% w/v, HCl 0.2 M.

The best choice would be the CS/β-GP solution of 1.5% w/v of CS 80/100 and R=8, since it had the lowest gelation time, only if the pH would be a bit lower, with a maximum value around 7.4 [27]. As the experience by the dissolution of the salt with medium indicated a variation on the pH, it was conducted an experiment for this solution by changing the dissolution agent. It can be seen on the *Graphic 5* that also in this case the gelation time and the pH are decreased, following the same behaviour than in *Graphic 1*. In this condition, the pH was in the range of cells survival, having a value of 7.42, and the gelation time is 5 min.
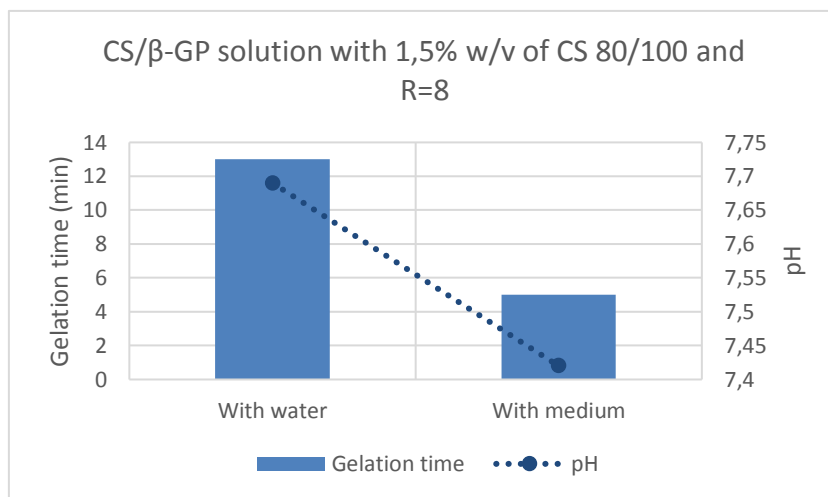
Graphic 5: Comparison between the CS/ β-GP solution with 2.5% w/v of CS 80/100 and R=8 when dissolving the β-GP with water and with medium. Analysis of the pH (T: 4-6 ºC) and the gelation time at 37ºC.

The concentration must not be further reduced because the solution becomes aqueous in excess and the gelation is not uniform. In consequence, any other experiment was conducted for a lower CS concentration and it was concluded that the best solution from this group of experiments is the CS/β-GP solution with 1.5% w/v of CS 80/100 and R=8, with 7.42 of pH and 5 min of gelation time as reference.

The third group of experiments consists on the investigation with the solution made of CS 95/100 and G1-P salt. In the literature it can be found that the stability of the solutions made with this salt should be higher, they should have a longer duration, than the solutions with the β-GP. It was necessary though to prove if there exists a relationship between a longer stability and a longer gelation time at 37ºC, as well as a difference on the pH.

The *Graphic 6* shows that this solution presents the same behaviour as the solution of the second group of experiments (CS 80/100 + β-GP), decreasing the gelation time with a diminution of the concentration.
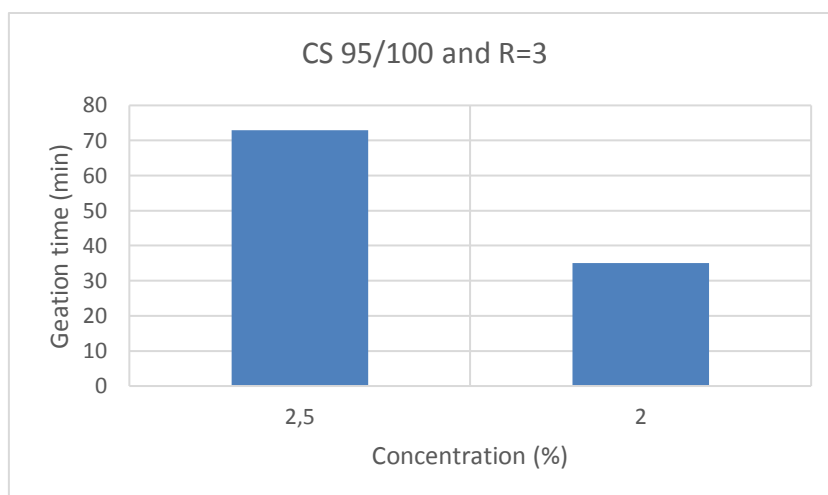
Graphic 6: CS/ G1-P developed with CS 95/100. Analysis of the behaviour by fixing the ratio and changing the concentrations. Gelation time measured with a sample of 0.5 ml in the incubator at 37ºC.

For the concentration of 2% w/v, since it was not possible to reduce it more for the solution consistency as the experimentation with 1.75% w/v confirmed, there were done experiments with different concentrations. The relationship between the ratio and the gelation time followed the same pattern as the previous studies, the gelation time was lower with a high ratio, but for the pH no pattern was extracted (*Graphic 7*).
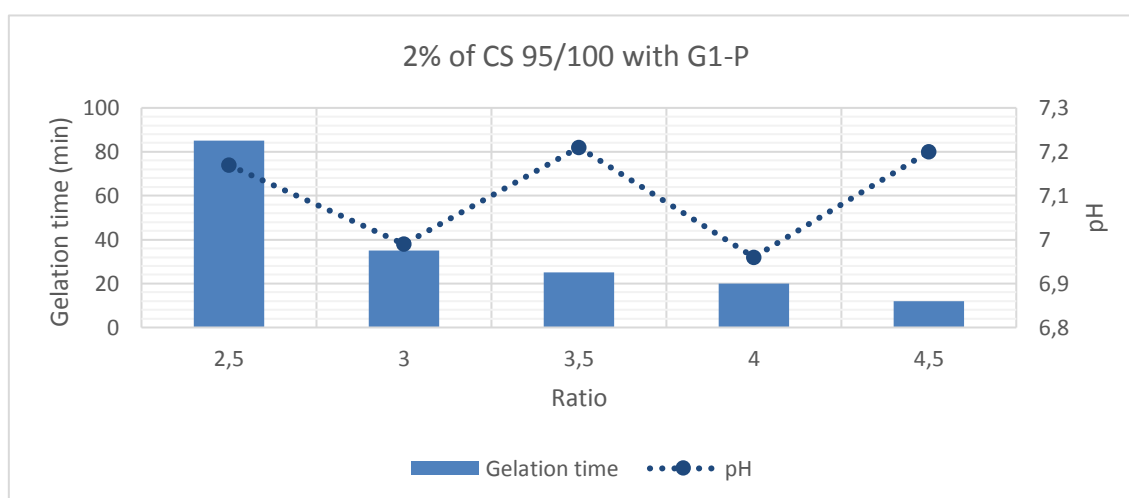
Graphic 7: Analysis of the pH (T: 4-6 ºC) and the gelation time at 37ºC of CS/β-GP solution with a concentration of CS 95/100 of 2% w/v with different ratios (R), developed from a solution of CS 95/100, 3.6% w/v, HCl 0.2 M

As it was seen in the graphics, the solution that best matched the requirements of the pH and gelation time was the CS/G1-P solution with 2% w/v of CS 95/100 with a ratio of 4.5 of G1-P. For this solution, the tests were repeated dissolving the salt into medium instead of water, and the final values obtained were a pH=7.02 and a gelation time of 10 min.

Finally, among the last group of experiment, with CS 80/100 and G1-P, only a couple of samples were analysed. Working with 1.5% w/v of CS 80/100 concentration and analysing the results with solutions of ratios 5 and 7, it was easily and rapidly detected that the gelation time increases significantly (more than 6 times) the value obtained with the other salt, and that the conformation of the hydrogel with these two main components did not accomplish the requirements of this project.

5.1.2.   Water uptake and degradation

The soaking of samples into physiological solutions allowed the investigation of the capacity of absorbing water and the weight loss of the different kind of hydrogels when immersed into a physiological fluid at 37ºC. The reason of conducting this experiment was to analyse the behaviour of the hydrogel once inserted into the human body, because there it would be in contact to other human tissues and fluids, which have a high content of water and a temperature of around 37ºC. It was also possible to measure the change on the pH of the surrounding liquid (PBS).

The next graphic (*Graphic 8*) shows the water content in equilibrium (EWC %) of the hydrogels after the incubation with PBS at 37ºC during different time points.
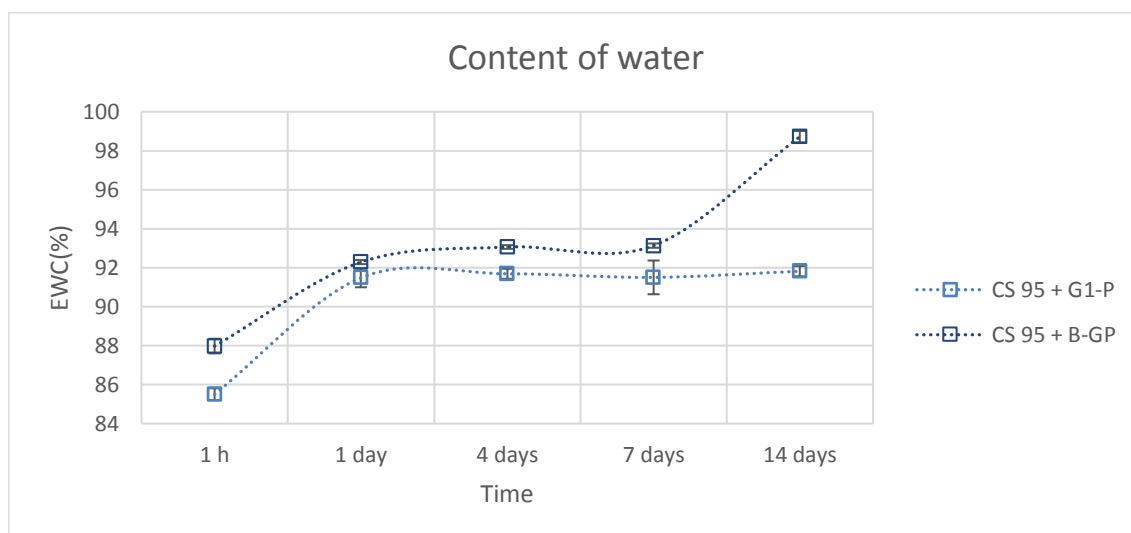
Graphic 8: Equilibrium water content (EWC %) of different hydrogels in function of the incubation time in PBS at 37ºC. The represented value is the mean value ± S.D. (n=3)

The studied hydrogels were highly hydrated systems, but the content of water became even higher in aqueous solution. In the *Graphic 8*, the content of water is reported showing an increase of 85-95% for the hydrogel CS/G1-P with 2% w/v of CS 95/100 and R=4.5 and the increase is progressive with time, existing an important difference between the first hour and the first day of incubation.

The results for the experiment of the theoretical lyophilised weight estimation are summed up in the following table:

	CS 95/100 2.5% w/v R=4 with B-GP hydrogel solution	CS 95/100 2% w/v R=4.5 with G1-P hydrogel solution
Theoretical lyophilised weight	0,12 ± 0,005	0,144 ± 0,006

Table 4: Theoretical lyophilised weight of different hydrogels. The represented value is the mean value ± S.D. (n=3)

The weight loss trend of the hydrogel fabricated with G1-P salt as gelation agent is represented in the *Graphic 9*:
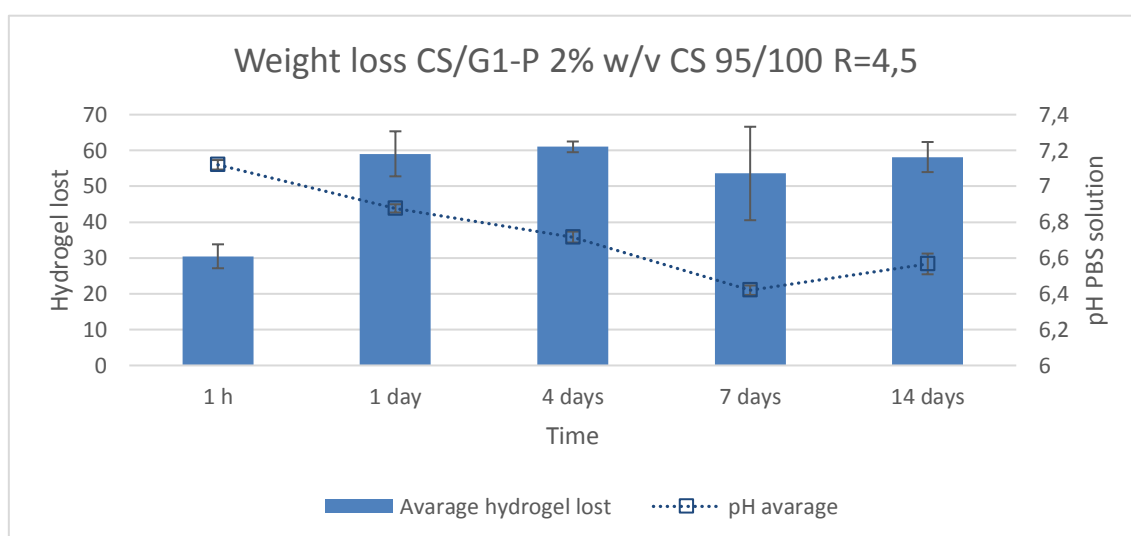
Graphic 9: Weight loss (WL %) of the hydrogel CS/G1-P 2% w/v of CS 95/100 and R=4.5 in function of the time of incubation in PBS at 37ºC. The value represented is the average value ± S.D. (n=3)

It can be observed that the weight loss increased with time, achieving the around 60% of weight loss after 24 hours in contact to the PBS and keeping this value constant until 14 days after. The increasing weight loss during the first 24 hours indicates a progressive release of degradation products until it achieves the plateau (~60%).

The weight loss from the CS/β-GP with 2.5% w/v of CS 95/100 and R=4 followed also the same pattern. In this case the maximum amount of hydrogel lost in contact to the aqueous environment (PBS) is also around 60%.
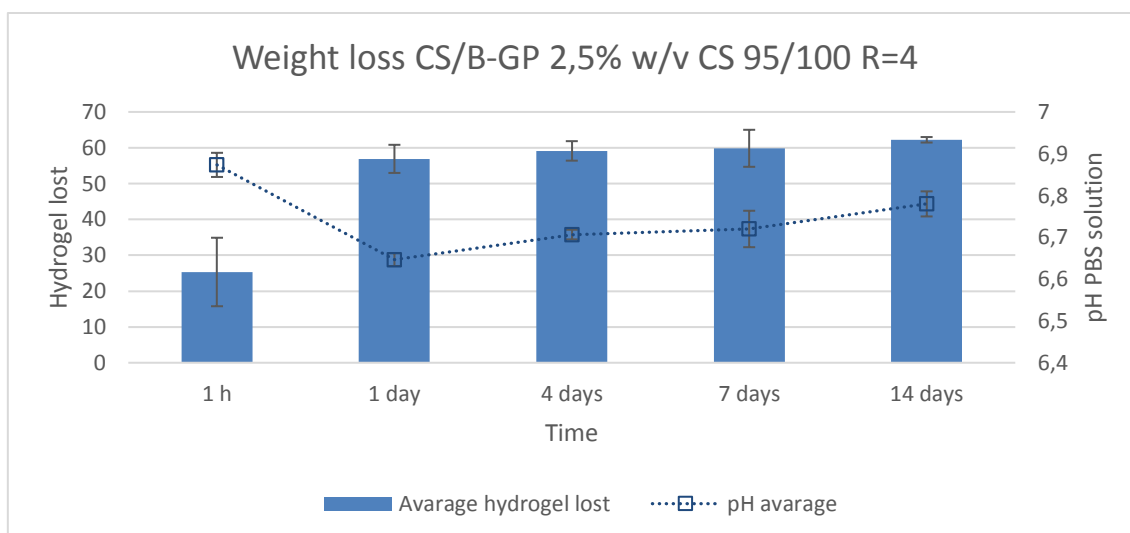
Graphic 10: Weight loss (WL %) of the hydrogel CS/B-GP 2.5% w/v of CS 95/100 and R=4 in function of the time of incubation in PBS at 37ºC. The value represented is the average value ± S.D. (n=3)

The progressive increasing of the weight loss and the big difference between the first hour and the first day of incubation, could be explained by an increasing release of salt throughout the time. The moles of salt into the hydrogel is R=4.5 (for the G1-P hydrogel) and R=4 (for the β-GP) times higher, as the ratio indicates, than the moles of amino groups in the chitosan. This suggested that not all the molecules of salt match with an amino group to form the hydrogel, being some of them released and moved to the surface. This release might be progressive, and therefore, the weight loss may increase with the time. It can be extracted from the graphics that both hydrogels follow the same pattern in terms of weight loss and that both salts affect similarly.

Graphic 9 and *Graphic 10* reported the decrease on the pH of the PBS, being it different for the hydrogel made of G1-P and the one made of β-GP. In the case of the G1-P the decrease of the pH was more progressive in the time, for then increasing after one week, but in the case of the β-GP there is a sudden drop after 1 day of incubation and then the pH increases slowly. The reason of this behaviour might also be the release of salt from the hydrogel, since the surplus of salt might be dissolved into the PBS. The salt might induce an increment of H_3O^+ in the aqueous solution and, therefore, the decrement of pH.

The further increase of the pH could be caused by the reaction of the released salt with other ions present at the PBS, such as the sodium (Na^+), the chlorine (Cl^-) or the potassium (K^+). The difference between the behaviour of both salts could be explained by the interaction of each salt with the previous mentioned ions in the PBS.

The hydrogel CS/β-GP with 1.5% w/v of CS 80/100 and R=8 does not appear in the graphics since its degradation test failed. The hydrogel was too aqueous and it lost its

structure in contact to liquid. For this reason, it was not possible to weight it nor measure the pH. On the other side, the two hydrogels analysed had a high stability in hydrogel form in contact to an aqueous solution and no extra swelling or any other effect on the hydrogels was observed.

5.1.3.   pH evolution

For the best samples in each group of experiments, except for the hydrogel formed with CS 80/100 because of its poor stability in aqueous conditions (as the degradation test showed), the evolution of the pH during the gelation process was studied. As it can be observed in the *Graphic 11*, there was a reduction on the pH value while the gelation took place, until it achieved a stable value when the gelation was complete. This reduction could be caused by the release of the polyols that were not linked to a proton of the chitosan chains forming the hydrogel structure. The diminution of the pH in all cases was around 0.3 of pH.
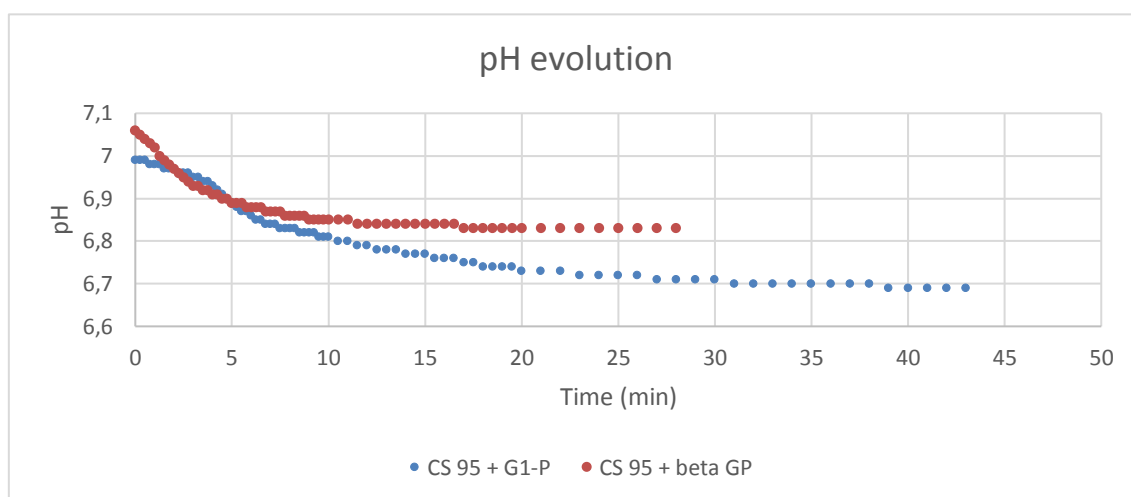
Graphic 11: pH evolution while gelation at 37ºC

5.2. Cells experimentation

In this section the results of the experiments with cells performed in San Luigi Hospital are presented and discussed.

5.2.1.   Observation of cells vitality cultured in presence of β-GP and G1-P salts

The vitality allowed to know the behaviour of cells when cultured in medium in presence of β-GP and G1-P salts at two different concentration (50% and 30%), which according to literature should not present a cytotoxic effect on cells.

The experiments were performed using two different cell lines. The results for the experiment performed with RT4-D6P2T cell line are reported in *Figure 12*. Images acquired at the microscope (DC100, Leica, Germany) with a magnification of 20x, showed that cells suffered a cytotoxic effect in presence of 50% of salt (both G1-P and β-GP), that is the usual amount of salt that the hydrogel releases when conformed. It can be seen that a high percentage of cells were dead and did not proliferate.

On the other side, cells presented a better response in presence of 30% of salt. Although there was a big difference between the response of cells under the presence of both kind of salts, it can be observed that the quantity of cells in the plates in both cases was higher than with the concentration of 50%.
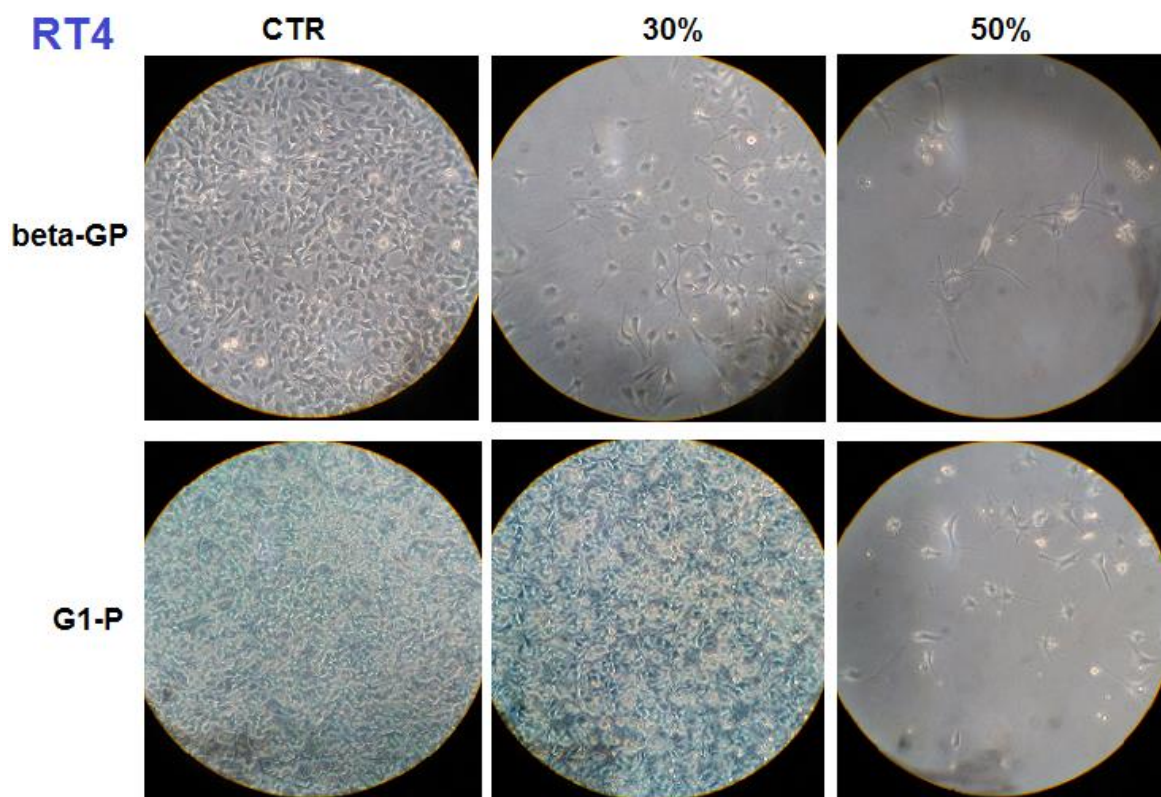

Figure 12: Observation of RT4-D6P2T cells vitality cultured in presence of β-GP and G1-P salts for different concentrations (30% and 50%)

The response of cells towards a dissolution of G1-P in a 30% of concentration showed that cells can perfectly survive and proliferate without suffering any damage. As it could be observed on the corresponding image (*Figure 12*), the confluence of cells in 30% of G1-P salt was very similar to the confluence of cells in control condition, while cells cultured in 30% of β-GP were less confluent than the control, meaning that they later died in a higher rate.

The results of the experiment performed with NIH3T3 cell line can be analysed in *Figure 13*. According to literature and based on experience, this cell line should be resistant, since they are fibroblasts, but also in this case it could be seen a cytotoxic effect in cells. Images showed that, the cytotoxic effect under of salts was very similar, presenting a diminution of confluence by increasing the concentration of salts.
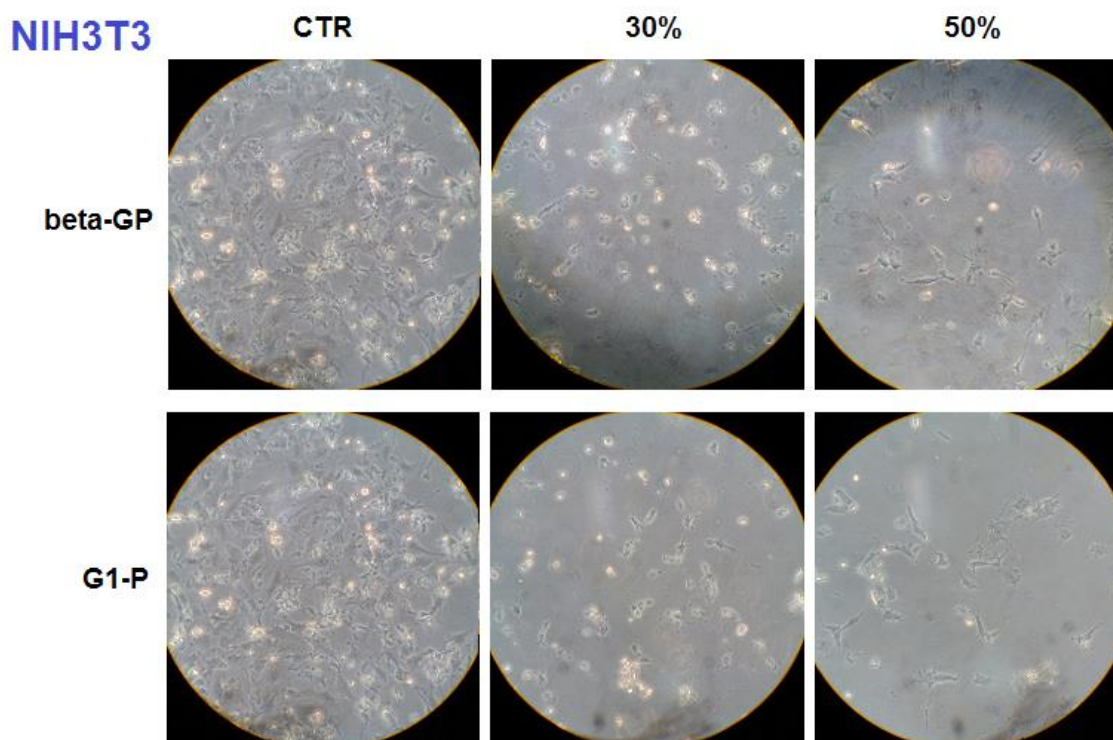

Figure 13: Observation of NIH cells vitality cultured in presence of β-GP and G1-P salts for different concentrations (30% and 50%)

From these experiments, it could be concluded that a high dependency on the salt concentration for cells survival and proliferation exists.

5.2.2. Proliferation test in supernatant

The proliferation test was carried out with culture medium that had been over the hydrogel for 24 hours in the incubator (37ºC). The medium enriched with 24 hours of dissolution products was collected, filtered and used as culture medium for RT4-D6P2T cells. The experiment was performed with both hydrogels studied.

The results seen in the previous subsection (5.2.1) made it possible to conclude that cells cannot survive in an environment with a presence of 50% of the salt with which the hydrogel was composed. Considering this, also a cytotoxic effect in the proliferation curve was expected. This was confirmed by the results of this experiment, reported in *Graphic 12*. The
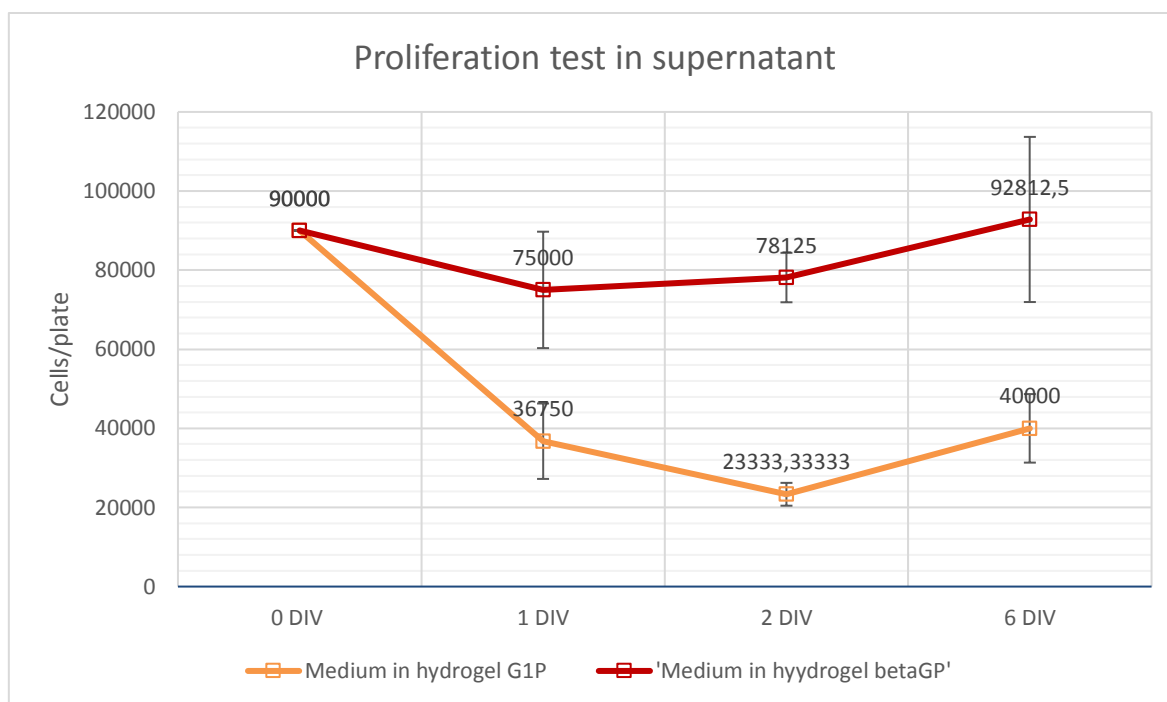
proliferation curve of both hydrogels was much lower than the proliferation curve of the controls.
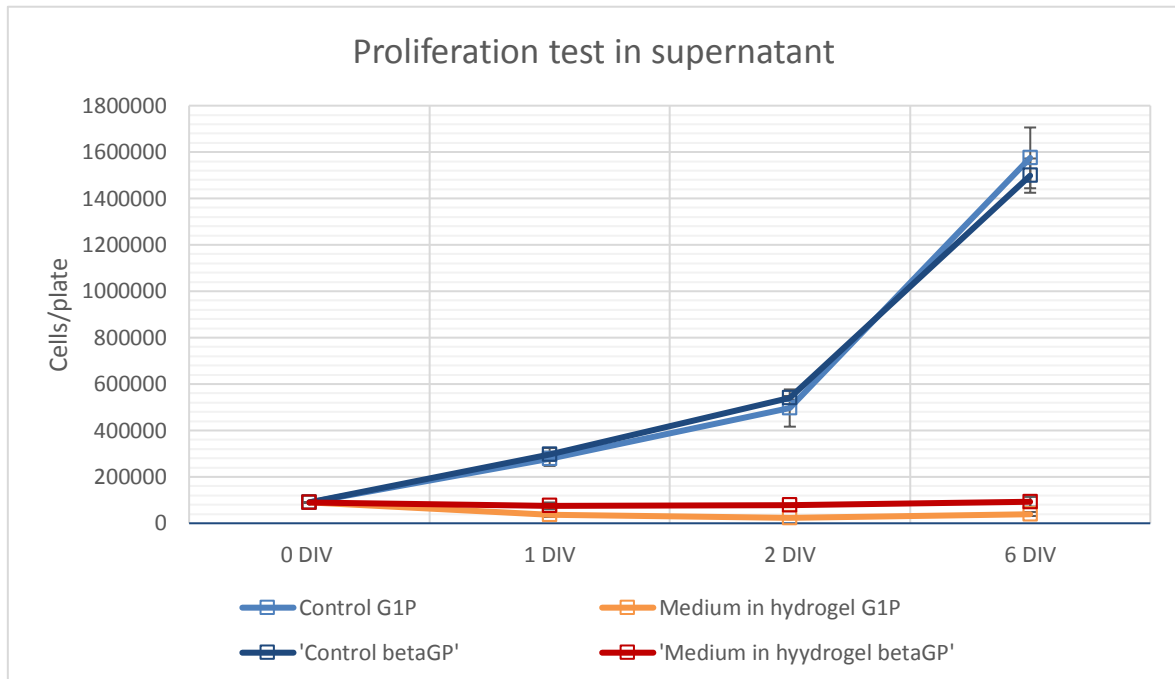
Graphic 12: Proliferation test of cells cultured in a medium that had been in contact to the hydrogels for 24 hours and controls. The value represented is the concentration (cells/plate) in function of the time of incubation at 37ºC, and it is shown as the average value ± S.D. (n=3 for the β-GP and 4 for the G1-P)

In the following graphic a comparison between the effect of both hydrogels on the cell proliferation was done. It could be recognised that there was an adaptation period, since at the beginning cells suffer from the effect of the salt, but then the number of cells increases with both salts, meaning that cells were getting used to the presence of the salt and they start proliferating.

It can be detected also that the proliferation curve of the supernatant with β-GP was slightly better than the proliferation curve of the supernatant that had been in contact to the hydrogel with G1-P.

Graphic 13: Proliferation test of cells cultured in a medium that had been in contact to the hydrogels for 24 hours. The value represented is the concentration (cells/plate) in function of the time of incubation at 37ºC, and it is shown as the average value ± S.D. (n=3 for the β-GP and 4 for the G1-P)

5.2.3.   Direct contact test

The results of the experiment with the encapsulation and cells on the top of the hydrogels were divided in two sections, the results for the CS 95/100 2.5% w/v R=4 with β-GP and the results for the CS 95/100 2% w/v R=4.5 with G1-P.

5.2.3.1. CS 95/100 2.5% w/v R=4 with β-GP

For each time-point (1 and 2 days, the live/dead assay was applied and the samples were fixed following the procedure explained in section 4.4.1 and analysed by means of a confocal microscope (LSM800, Zeiss, Germany) with a magnification of 10x.

Figure 14, reported that the cells on the top were alive and that the quantity of cells on the surface was approximately the same as in the control, meaning that the cells had a good response in 2D contact with the hydrogel composed by CS and beta-GP. This behaviour was unexpected, since the previous experiment showed a cytotoxic effect on cells, but the reason of a better response in the direct contact could be explained by a higher exchange between cells and the environment, accelerating the adaptation period and also their proliferation.

By the analysis of the picture of encapsulated cells in the multiwell, it could be observed that although in less quantity, cells were also alive. The decrease of cells number was totally expected, because of the change in conformation. In this condition, cells are spread out into the hydrogel matrix and they could be found distributed in different layers, whereas at the experiment with cells on the top, all cells could be observed on the surface. In the picture, only the first layer with encapsulated cells could be observed.
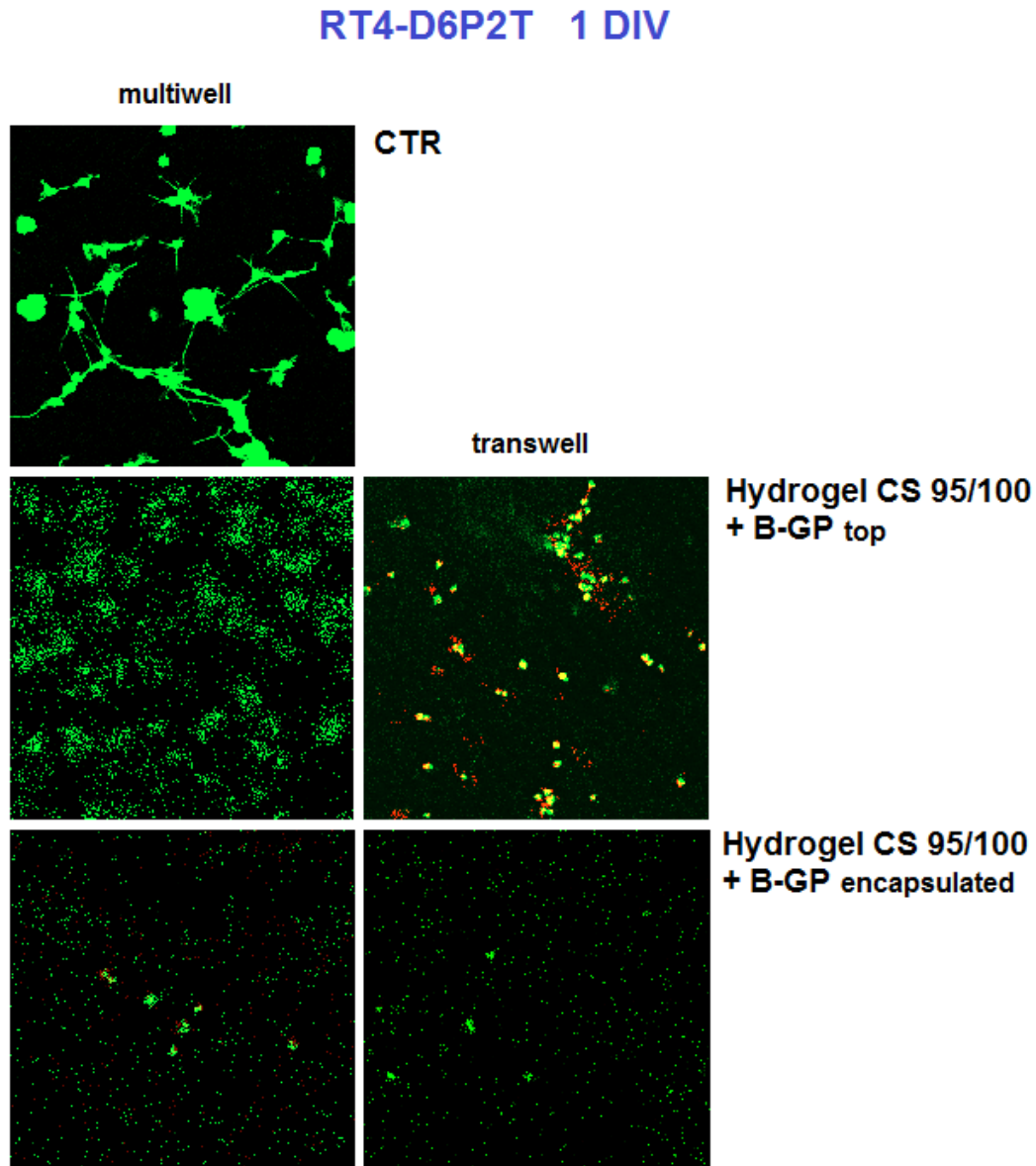

Figure 14: Direct contact test for CS 95/100 2.5% w/v R=4 with β-GP after 1 day of incubation at 37ºC. Test developed for different conformations (top and encapsulation) and in different supports (transwell and multiwell)

Comparing the images obtained in the multiwell and in the transwell, the behaviour in both cases was positive and similar, since the cells on the top in the transwell were also more confluent than the encapsulated (for the configuration), but both survived in contact to the

hydrogel. By this experiment it was ensured that cells could adapt themselves to the environment containing the hydrogels degradation products, because no component induced cytotoxic effects, and that the 3D matrix of the hydrogel allowed the transfer of nutrients and the development of cells basic processes.
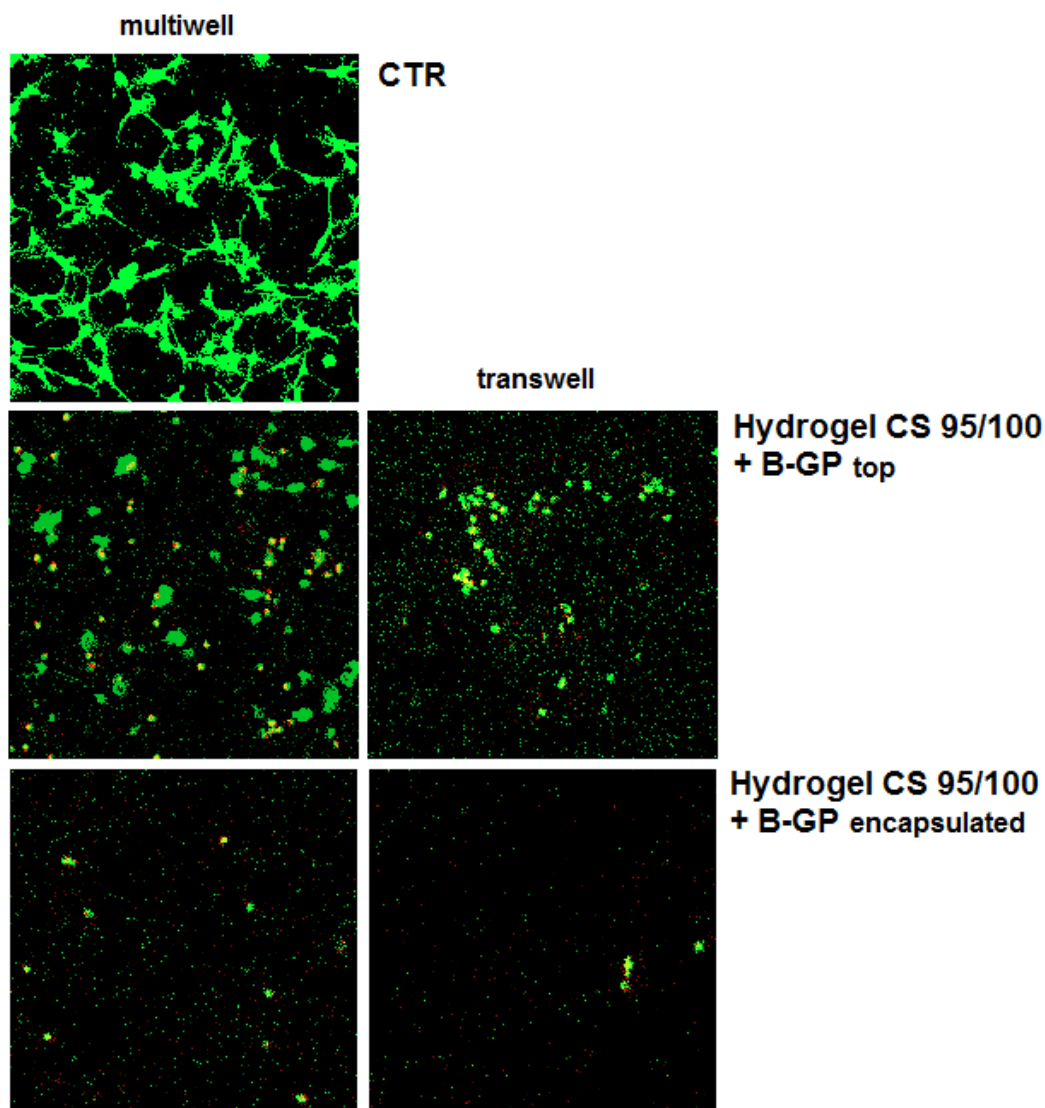

Figure 15: Direct contact test for CS 95/100 2.5% w/v R=4 with β-GP after 2 days of incubation at 37ºC. Test developed for different conformations (top and encapsulation) and in different supports (transwell and multiwell)

In *Figure 15*, it could be noted that the number of cells cultured on the top increased in respect to the number of cells after 1 day of culture. This indicated that cells could be proliferating and acting normally in contact to the hydrogel.

Cells alive were also seen in the first layer of the hydrogel with cells encapsulated in the case of the two days in culture. This could mean that the 3D matrix of the hydrogel does not present a mortal effect on cells. A more extended analysis (with more time-points) would be required to determine if the matrix allows the natural growing and proliferation of cells.

The thickness of the hydrogel and its opacity made it impossible to achieve better resolution images and also to acquire an image composed of different layers (as the stack option of the confocal microscope does) for the encapsulated cells experiment.

5.2.3.2. CS 95/100 2% w/v R=4.5 with G1-P

The live and dead assay was performed for the hydrogel CS 95/100 2% w/v R=4.5 with G1-P after 1 day in culture, but the result was the expected in relation to the results obtained in the previous experiments. The salt G1-P showed a cytotoxic effect, as it was reported in the Figure 12, the Figure 13 and the Graphic 12, and the results from the encapsulation and cells on the top for this hydrogel confirmed it. The cells suffered a severe damage after 1 day in culture, impeding its survival and showing all the cells in red colour after the live and dead assay.

This experiment indicated for now that the amount of salt from the hydrogel solution CS 95/100 2% w/v R=4.5 with G1-P, which is 0.85 g for 5 ml, was too elevated for its application in cells encapsulation. This hydrogel released around the 50% of this salt when it was prepared and the components released were detrimental for cells. It could be concluded that this hydrogel, with this concentration and salt ratios is not suitable for regeneration of human tissues.

A possible solution could be the reduction of salt concentration, in view of the results seen in *Figure 12*, but the pH and the gelation time of the hydrogel should be studied because it is probable that the pH is too low for cells survival and the gelation time too high for being printable. These issues should be studied in further investigations. As an alternative, the blending with other degree of deacetylation of the chitosan or other natural polymers could be investigated.

As it was proved in in vivo experiments, found in literature [24], that G1-P can be used as gelation agent in hydrogels without cytotoxic effect on the rats, further experiments should be developed before dismissing its use.

POLITECNICO DI TORINO

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6. Conclusions

In this thesis, chitosan-based hydrogels were studied as biomaterials for cells encapsulation through the experimentation with cells in different configurations. Chitosan-based hydrogels, widely known in scientific literature, are ideal candidates for nervous regeneration for their numerous qualities: antibacterial, biocompatible, non-immunogenic and non-toxic, derived fundamentally from the chitosan, and their ability to become a gel at physiological temperature. Nevertheless, the results obtained are preliminary results and further investigations are required to optimise this system.

The main objective of this thesis was the study of the cells encapsulation, which required a first selection of the hydrogel, where to encapsulate the cells. The investigation and characterisation of the hydrogels developed in the POLITO succeeded, since it exposed the properties of different hydrogels, such as pH, gelation time and degradation, and made it possible to select the ones that best fit the requirements: CS 95/100 2.5% w/v R=4 with β-GP and CS 95/100 2% w/v R=4.5 with G1-P.

The experimentation with cells performed at San Luigi Hospital in Orbassano evidenced that the survival of cells in a salty environment, similar to the one that would appear around a hydrogel after gelation because of the release of salt, is highly dependent on the concentration of salt. Experiments revealed that a high concentration of salt in the culture medium (50%) was damaging for cells, having a cytotoxic effect on cells also proven in the proliferation curve, while a lower concentration allowed a perfect proliferation of cells, especially with the G1-P as gelation agent.

The results for the experiment of the direct contact of cells to the hydrogel were a surprise, as these data were in contrast with published literature. It could be expected a negative response of cells to the direct contact, based on the negative effect that the dissolution with the 50% of salt had on cells. But, on the contrary, the experiment demonstrated that cells survival was possible in the case of the hydrogel CS 95/100 2.5% w/v R=4 with β-GP for the culture in 2D, as well as in 3D.

A possible explanation of this fact could be the highest exchange of components due to a higher percentage of contact between cells and the environment, which could suppose a more rapid adaptation.

In the case of the culture in contact to the hydrogel CS 95/100 2% w/v R=4.5 with G1-P, the result was the expected since the cells did not survive to the high concentration of salt in the environment. Further tests should be performed with this salt, by reducing the

concentration or changing the hydrogel composition, since it was proven that cells could respond perfectly to the G1-P salt in lower concentrations.

The hydrogels developed, all in all, present high potential for being used for cells encapsulation, since the response of cells to the salty environment was in some cases positive and for hydrogels with β-GP as gelation agent the suitable matrix and composition ensured its possibilities.

This thesis is added to the wide spectrum of investigations and efforts dedicated to biomaterials, in specific to hydrogels. The development of this thesis has been useful to indicate that the cells culture in presence of salts is delicate, but also that it is interesting to continue with the investigation since for some concentrations cells grow and proliferate in perfect conditions. The investigation has ensured once again the potential that chitosan-based hydrogels have on the regenerative field.

Acknowledgements

This project has been possible thanks to the Erasmus+ Programme, which is the EU's programme that supports education and provides opportunities for students to study train and gain experience abroad, and to the agreement between the 'Universitat Politecnica de Catalunya' and the 'Politecnico di Torino' universities.

I am grateful to professor Gianluca Ciardelli, from this second institution, for giving me the opportunity of developing this work in collaboration to the DIMEAS department at the POLITO and for the possibility of widening my knowledge about hydrogels and their applicability in the biomedical field of study. I would like to thank also the professor Chiara Tonda Turo, who guided the work and helped me on the development of the thesis with her knowledge and experience.

I thank to Rosanna Fusaro, the master's thesis student that worked with hydrogels the last semester and that passed on all her knowledge about the theme and helped me getting introduced to the world of the hydrogels.

I would like to name also Federica Fregnan, biologist and investigator at San Luigi's Hospital in Orbassano (Turin), and the members of the team in appreciation for the help offered, guidance by working with cells and patience.

I would also like to express my gratitude to Marina Flores for the great work did together and the constant help we have given to each other.

Finally, thanks to my family and friends for their unconditional support.

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