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ESCUELA TÉCNICA SUPERIOR INGENIEROS INDUSTRIALES VALENCIA

MASTER THESIS ON BIOMEDICAL ENGINEERING

DEVELOPMENT OF BIOMIMETIC INJECTABLE HYDROGELS FOR HEPATIC CELL CULTURE

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ABSTRACT

The liver is a vital organ which is responsible for a wide range of functions. End-stage liver diseases have a very high morbidity and mortality due to associated complications, and liver transplantation represent the only current effective treatment. However, organ transplantation is hampered by the scarcity of donors, thus, over the past years, new alternative approaches have been explored to answer this need.

Hepatic cell therapy has been used to recover and stabilize the lost metabolic functions due to different diseases. However, despite its promising results, it has not been considered as a definitive treatment because the scarcity of cells and the low cell engraftment and survival of the transplanted cells.

Tissue-mimic cell culture methods, which promote liver-specific functions for a long period, are promising tools for liver tissue engineering. They are based on the use of biomaterials as three-dimensional cell culture platforms that try to recapitulate cell-cell and cell-matrix interaction. They can mimic the composition and stiffness of the liver extracellular matrix, by the combination of a protein and a polysaccharide, as a strategy to promote hepatic functionality of cultured cells. Furthermore, if biomaterials are injectable, they result very advantageous, as they allow the homogeneous encapsulation of cells, the scalability of the *in vitro* culture by bioprinting and the implantation of the cell-biomaterial construct by minimally invasive surgeries for liver regeneration. The aim of this Master Thesis is the development of biomimetic injectable hydrogels based on the combination of gelatin and hyaluronic acid and two crosslinking chemistries, to explore the optimal for cell encapsulation and liver tissue engineering. In part I we study the suitability of enzymatically crosslinked hydrogels (previously developed in my bachelor thesis) for the encapsulation of primary human hepatocytes. We found an enhanced hepatic functionality (ureogenesis, albumin secretion and activity of drug metabolic enzymes (P450)) when the hepatocytes were within the hydrogels than in monolayer, which makes this system very promising for the future. Part II is dedicated to optimize the synthesis conditions of hydrogels based on the covalent reaction between aldehyde groups of oxidized hyaluronic acid (oHA) and amino groups of gelatin (Gel) through imine bond formation. Experiments demonstrate that 100 mg/mL solutions of oHA and Gel mixed at a ratio oHA: Gel 1:3 produce stable hydrogels. Furthermore, hydrogel crosslinking with fibroblasts is not cytotoxic, as most of the cells remained alive after 1 and 7 days of culture, with adequate level of metabolic activity after 7 days within the hydrogel. This system is advantageous over the enzymatically crosslinking, as no catalyzer is used and the buffer to dissolve the hydrogel precursors molecules is more cell friendly. Future experiments with human hepatocytes could allow us to propose it as a good alternative for the tissue engineering of liver.

Keywords: liver tissue engineering, injectable hydrogels, hyaluronic acid, gelatin, tyramine conjugates, oxidized polysaccharides and hepatic functionality.

RESUMEN

El hígado es un órgano vital responsable de una amplia gama de funciones. Las enfermedades hepáticas en fase terminal tienen una morbilidad y mortalidad muy alta debido a las complicaciones asociadas, y el trasplante de hígado representa el único tratamiento efectivo actual. Sin embargo, el trasplante de órganos se ve obstaculizado por la escasez de donantes, por lo que en los últimos años se han estudiado nuevos enfoques alternativos para responder a esta necesidad.

La terapia celular hepática se ha utilizado para recuperar y estabilizar las funciones metabólicas perdidas debido a diferentes enfermedades. Sin embargo, a pesar de sus resultados prometedores, no se ha considerado como un tratamiento definitivo debido a la escasez de células y al bajo nivel de injerto celular y supervivencia de las células trasplantadas.

Los métodos de cultivo de células que imitan el tejido, promoviendo funciones hepáticas específicas durante un largo período, son herramientas prometedoras para la ingeniería de tejidos hepáticos. Se basan en el uso de biomateriales como plataformas de cultivo celular tridimensional que tratan de recapitular la interacción célula-célula y célula-matriz. Pueden imitar la composición y la rigidez de la matriz extracelular del hígado, mediante la combinación de una proteína y un polisacárido, como estrategia para promover la funcionalidad hepática de las células cultivadas. Además, si los biomateriales son inyectables, resultan muy ventajosos, ya que permiten la encapsulación homogénea de las células, la escalabilidad del cultivo *in vitro* por bioimpresión y la implantación del constructo célula-biomaterial mediante cirugías mínimamente invasivas para la regeneración del hígado. El objetivo de este Trabajo Final de Máster es el desarrollo de hidrogeles inyectables biomiméticos basados en la combinación de gelatina y ácido hialurónico y dos químicas de reticulación, para explorar la óptima para la encapsulación de células y la ingeniería tisular de hígado. En la parte I estudiamos la idoneidad de los hidrogeles reticulados enzimáticamente (desarrollados previamente en mi Trabajo Final de Grado) para la encapsulación de hepatocitos humanos primarios. Encontramos una funcionalidad hepática mejorada (ureogénesis, secreción de albúmina y actividad de enzimas del metabolismo de fármacos (P450)) cuando los hepatocitos están en los hidrogeles que en monocapa, lo que hace que este sistema sea muy prometedor para el futuro. La segunda parte está dedicada a optimizar las condiciones de síntesis de los hidrogeles basada en la reacción covalente entre grupos aldehído de ácido hialurónico oxidado (oHA) y grupos amino de la gelatina (Gel) mediante la formación de enlaces imina. Los experimentos demuestran que disoluciones de 100 mg/mL de oHA y Gel mezclados en una proporción oHA:Gel 1:3 producen hidrogeles estables. Además, el entrecruzamiento del hidrogel en presencia de fibroblastos no es citotóxico, ya que la mayoría de las células permanecieron vivas después de 1 y 7 días de cultivo, con un nivel adecuado de actividad metabólica después de 7 días en el hidrogel. Este sistema es ventajoso sobre el reticulado enzimáticamente, ya que no se utiliza ningún catalizador y el tampón para disolver las moléculas precursoras del hidrogel es más favorable para las células. Los futuros experimentos con hepatocitos humanos pueden permitirnos proponerlo como una buena alternativa para la ingeniería tisular del hígado.

Palabras clave: ingeniería tisular de hígado, hidrogeles inyectables, ácido hialurónico, gelatina, conjugados de tiramina, polisacáridos oxidados y funcionalidad hepática.

RESUM

El fetge és un òrgan vital que és responsable d'una àmplia gamma de funcions. Les malalties hepàtiques en fase terminal tenen una morbilitat i mortalitat molt alta a causa de les complicacions associades, i el trasplantament de fetge representa l'únic tractament efectiu actual. No obstant això, el trasplantament d'òrgans es veu obstaculitzat per l'escassetat de donants, per la qual cosa en els últims anys s'han estudiat nous enfocaments alternatius per a respondre a aquesta necessitat.

La teràpia de cèl·lules hepàtiques s'ha utilitzat per a recuperar i estabilitzar les funcions metabòliques perdudes a causa de diferents malalties. No obstant això, malgrat els resultats prometedors, no s'ha considerat com un tractament definitiu a causa de l'escassetat de cèl·lules i el baix nivell d'empelt cel·lular i supervivència de les cèl·lules trasplantades.

Els mètodes de cultiu de cèl·lules que imiten el teixit, promovent funcions hepàtiques específiques durant un llarg període, són eines prometedores per a l'enginyeria de teixits hepàtics. Es basen en l'ús de biomaterials com a plataformes de cultiu cel·lular tridimensional que tracten de recapitular la interacció cèl·lula-cèl·lula i cèl·lula-matriu. Poden imitar la composició i la rigidesa de la matriu extracelul·lar del fetge, mitjançant la combinació d'una proteïna i un polisacàrid, com a estratègia per a promoure la funcionalitat hepàtica de les cèl·lules cultivades. A més, si els biomaterials són injectables, resulten molt avantatjosos, ja que permeten l'encapsulament homogeni de les cèl·lules, l'escalabilitat del cultiu *in vitro* per bioimpresió i la implantació del constructe cèl·lula-biomaterial mitjançant cirurgies mínimament invasives per a la regeneració del fetge. L'objectiu d'aquest Treball Fi de Màster és el desenvolupament d'hidrogels injectables biomimètics basats en la combinació de gelatina i àcid hialurònic i dues químiques de reticulació, per a explorar l'òptima per a l'encapsulament de cèl·lules i l'enginyeria tissular de fetge. En la part I estudiem la idoneïtat dels hidrogels reticulats enzimàticament (desenvolupats prèviament en el meu Treball Fi de Grau) per a l'encapsulació d'hepatòcits humans primaris. Trobem una funcionalitat hepàtica millorada (ureogénesi, secreció d'albúmina i activitat d'enzims del metabolisme de fàrmacs (P450)) quan els hepatòcits són en els hidrogels que en monocapa, la qual cosa fa que aquest sistema siga molt prometedor per al futur. La segona part està dedicada a optimitzar les condicions de síntesi dels hidrogels basats en la reacció covalent entre grups aldehid d'àcid hialurònic oxidat (oHA) i grups amino de la gelatina (Gel) mitjançant la formació d'enllaços imina. Els experiments demostren que solucions de 100 mg/mL de oHA i Gel mesclats en una proporció oHA:Gel 1:3 produeixen hidrogels estables. A més, la reticulació de l'hidrogel en presència de fibroblastos no és citotòxic, ja que la majoria de les cèl·lules romanen vives després d'1 i 7 dies de cultiu, amb un nivell adequat d'activitat metabòlica després de 7 dies a l'hidrogel. Aquest sistema és avantatjós sobre el reticulat enzimàtic, ja que no s'utilitza cap catalitzador i el tampó per a dissoldre les molècules precursores de l'hidrogel és més favorable per a les cèl·lules. Els futurs experiments amb hepatòcits humans poden permetre'ns proposar-ho com una bona alternativa per a l'enginyeria tissular del fetge.

Paraules clau: enginyeria tissular de fetge, hidrogels injectables, àcid hialurònic, gelatina, conjugats de tiramina, polisacàrids oxidats i funcionalitat hepàtica.

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ABBREVIATURE INDEX

ACLF – Acute-on-Chronic Liver Failure

ALD – Alcoholic Liver Disease

ALF – Acute Liver Failure

BSA – Bovine Serum Albumin

CFDA – 5(6)-Carboxyfluoresceine Diacetate

CF-KRB – Calcium-Free Krebs Ringer Buffer

CS-AT – Chitosan-graft Aniline-Tetramer

CYP's – Polymorphic Cytochrome P450 Enzymes

DMEM – Dulbecco's Modified Eagle's Medium

dPBS – Dulbecco's Phosphate Buffered Saline

ECM – Extracellular matrix

EDC – 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

ELISA – Enzyme-Linked ImmunoSorbent Assay

ESLD – End Stage Liver Diseases

EWC – Equilibrium Water Content

FBS – Fetal Bovine Serum

GAG's – Glycosaminoglycans

Gel-Tyr – tyramine conjugate of Gelatin

H_2O_2 – Hydrogen Peroxide

HA – Hyaluronic Acid

HA-CA – Catechol-modified Hyaluronic Acid

HA-Tyr – tyramine conjugate of Hyaluronic acid

HCl – Hydrochloric Acid

HCT – Hepatic Cell Therapy

HRP- Horseradish Peroxidase

iPSC's – Induced Pluripotent Stem Cells

KCl – Potassium Chloride

LSPC's – Liver Stem Progenitor Cells

MES – 2-(N-Morpholino)Ethanesulfonic Acid

MSC's – Mesenchymal Stem Cells

MWCO – Molecular Weight Cut-Off

mTG – microbial transglutaminase

NaCl – Sodium Chloride

NAFLD – Non-alcoholic Fatty Liver Disease

$NaIO_4$ – Sodium Periodate

NaOH – Sodium Hydroxide

NH_3OHCl – Hydroxylammonium Chloride

NH_4^+ – Ammonium

NHS – N-Hydroxysuccinimide

NPC's – Neural Progenitor Cells

OCT – Optimal Cutting Temperature

oHA-Gel – Oxidised hyaluronic acid and gelatin hydrogels

oHA – oxidised Hyaluronic Acid

PEG – Polyethylene Glycol

PEG-DA – Dibenziladehyde-terminated Poly(Ethylene Glycol)

PLGA – Poly(Lactic-co-Glycolic Acid

PNIPAM - Poly(N-isopropylacrylamide)

PSF – Penicillin-Streptomycin-Fungizone

RGD – Cell attachment sequence

SCW's – Schwann Cells

SDS – Dodecylsulphate Sodic

w – Mass of water per unit mass of dry polymer

mTG - Transglutaminase

TMB – 3,3',5,5'-Tetramethylbenzidine

TNBSA – 2,4,6-Trinitrobenzene Sulfonic Acid

t(HA-Gel) – Tyramine conjugate hydrogels

1. MOTIVATION

I was first interested in tissue engineering after I suffered an anterior cruciate ligament injury. After the surgery, my surgeon told me about tissue engineering, and I have been fascinated ever since. This gave me an extra motivation to choose a career specialisation with the focus on tissue engineering. Last year I finished the degree on biomedical engineering, and I could work in the development of hydrogels as supports for hepatic tissue engineering during my Bachelor Thesis. This period provided me with an invaluable experience and convinced me to start a research career. I decided to continue exploring the same topic, as the results I obtained were very promising. The present Master Thesis is the result of our last research in which the hydrogels I developed during my bachelor period were explored as suitable matrices for the culture of primary human hepatocytes. Furthermore, alternative hydrogels that do not need catalysers to react are here presented, with similar composition but different crosslinking reaction. This system was developed in international collaboration and thanks to a summer internship of two months I could made in Germany in the lab of Prof. Thomas Groth, my second co-tutor. During the time I was with him I learnt to work in a different lab, out of my comfort zone, in a foreign language, and could enjoy the experience and the little satisfactions the science sometimes brings.

The Master studies allowed me to explore other interesting fields, but among them, the one I liked the most was again tissue engineering. This year the course was focused on how to bring research products to the market, which was very interesting to learn.

Nonetheless, my motivation will not end with this project, and I will try to consolidate my research studies in a future pre-doctoral period to become PhD.

During the Master's Thesis I acquired the following knowledge:

- Synthesis and fundaments of self-crosslinkable hydrogels
- Physical characterisation: Equilibrium water content and gel content
- Human primary hepatocytes and fibroblasts culture
- Functional assessment of encapsulated cells
- Immunohistochemistry techniques
- Classification of medical devices
- Risk analysis of medical devices

2. OBJECTIVES

Tissue engineering applied to liver tissue is a consequence of the major limitations that exist today: the increased mortality associated to patients with end-stage liver diseases, the increment of the incidence of these diseases in recent years and the shortage of donor organs.

Hydrogels have been widely used for other tissues and different pathologies and the potential of injectable hydrogels has been evident since their discovery thanks to the applicability by non-invasive surgeries. In the case of hepatic tissue, research has focused on the use of hydrogels as three-dimensional cell culture matrices, stem cell differentiation platforms and *in vitro* models for drug screening. In liver tissue engineering is very important to find materials able to stimulate the hepatic functionality of the cells once they have been encapsulated, as well as to overcome the limitations of cell therapy such as the low cell engraftment in the host liver. The idea is to look for alternatives to liver transplantation that allow the recovery of lost functions or help keeping the patient alive while a liver donor is available.

Injectable hydrogels are implanted in aqueous solutions compatible with cells and are able to crosslink and become solid within the body with cells in them encapsulated. This allows the cells to better target the tissue and the place of interest. The hydrogel matrix mimics the extracellular matrix (ECM) of the tissue and provides three dimensionality to the cells enhancing hepatic functionality. If the composition of the 3D hydrogels has components of the native ECM the hepatic environment for the hepatocytes is better recreated and the therapy will have better outcomes. Consequently, in this study we explored two crosslinking chemistries to combine hyaluronic acid and gelatin to propose them as injectable hydrogels for liver tissue engineering, since both components are present in the extracellular matrix of the tissue.

Thus, the main purpose of this project is to compare two different ways to synthesize injectable hydrogels for human hepatocytes culture. The first system is an enzyme-mediated crosslinking, which was characterised in my Bachelor Thesis and checked with a cell line. It is here explored as a suitable matrix for the encapsulation of primary human hepatocytes to move forward to a more realistic application. The second system is an auto crosslinking method based on the reaction of amino and carboxylic groups forming a Schiff's base, free of catalysers. It is proposed as an alternative of the enzymatic hydrogel. As this system is new, the aim of this part was to optimise the synthesis to find compositions compatible with cells. The cell culture experiments with this second hydrogel were performed with fibroblasts, as a proof of concept before starting with hepatocytes. The aim is to characterize equilibrium water content, gel content and biological performance by cell culture. Functionality of encapsulated hepatocytes was characterised by analysing the activity of drug metabolism enzymes, ureogenic capacity and albumin secretion. Viability of encapsulated fibroblasts was assessed to proof the cytocompatibility for the second method. It is worth mention that my intention was to encapsulate liver cells in the second system, activity that was interrupted due to the current pandemic situation. The results here presented correspond to the experimental work done from the last summer (July 2019 when I did my summer internship in Germany) until the lockdown, March 2020.

3. BACKGROUND

Tissue engineering is the part of the science that explores new ways to treat and deal with the current limitations of the medicine in terms of damaged tissues or organs. It is based on three main pillars acting together within damaged tissues for their proper regeneration: cells, scaffold and signalling. They are selected according to the tissue, trying to mimic the natural environment found in the healthy organism.

The liver is one of the most relevant organs within the body, being the main metabolic centre, with vital functions such as metabolism, bile production, plasma protein synthesis, detoxification, vitamin storage, glycolysis, and gluconeogenesis. Nowadays, there is not an effective treatment against end-stage liver diseases, and only liver transplantation is considered as an effective treatment. However, the shortage of organs, the high incidence of complications and the long-term immunosuppressive therapy lead to look for alternative therapies to face these challenges.

In this introduction, fundamental concepts such as the liver anatomy, the alternatives for liver transplantation, the hydrogels that are considered as scaffolds for liver regeneration and the necessary concepts for the chemistry I followed in this Master thesis will be explained.

3.1. Liver

The liver is the biggest internal organ inside the body, which weighs around 1.5 kg. Located in the upper right and part of the left abdominal quadrants, it is formed by two main lobes called right liver and left liver and two smaller lobes called quadrate and caudate (Adrian, 2020). The four lobes are also divided into 8 anatomical lobes. Not only its functions make the liver a special organ, but its blood supply is also unique and comes from two different pathways: portal vein and hepatic artery, providing a 75% and 25% of the total blood volume, respectively (Kalra et al., 2018) (Figure 1).

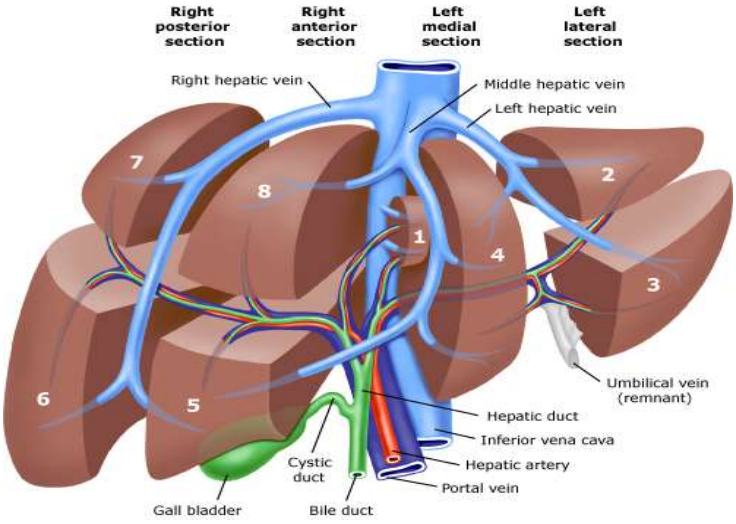

Figure 1.Schematic representation of the liver anatomy: lobes, vasculature and bile ducts from the gallbladder. (Davoodabadi et al., 2018)

The hepatic vascular system is represented by the portal vein, the hepatic vein and the hepatic artery with their respective subunits (i.e. central vein, terminal hepatic vein and hepatic arteriole) and the bile ducts coming from the gallbladder (Figure 1). In a hepatic lobule three ducts are found, forming the "portal triad": the bile duct, the hepatic arteriole and the portal vein. On the other hand, in all lobes there is a central vein. The blood flows from the portal triad in order to feed hepatocytes and remove waste substances. All these central veins come from the hepatic vein (Bismuth, 1982).

Hepatocytes are the major cell type of the liver and represent approximately 80% of hepatic volume. Additionally, the liver is composed by other parenchymal (cholangiocytes) and non-parenchymal cells (Kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells) that also play important functions in the liver. Hepatic sinusoids are described as cords that connect all the hepatocytes to each portal triad and the terminal central vein (Figure 2). The portal triad is a structure formed by three different tubules: bile duct, portal vein and hepatic arteriole, located in one of the sinusoid sides. The branch of the hepatic artery supplies the nutrients and oxygen required by the cells. The diffusion occurs through the fenestrated endothelial cells allowing a better blood perfusion. Besides, there are cells which are lining this sinusoid, the endothelial cells which have little fenestrations to allow a better blood perfusion (Gerber et al., 1987; Krishna et al., 2013). Between the hepatocytes and the endothelial cells there is the space of Disse (Figure 2). It is the microenvironment where hepatic stellate cells, better known as "Ito cells", are located. (Kordes et al., 2015; Häussinger et al., 2019).

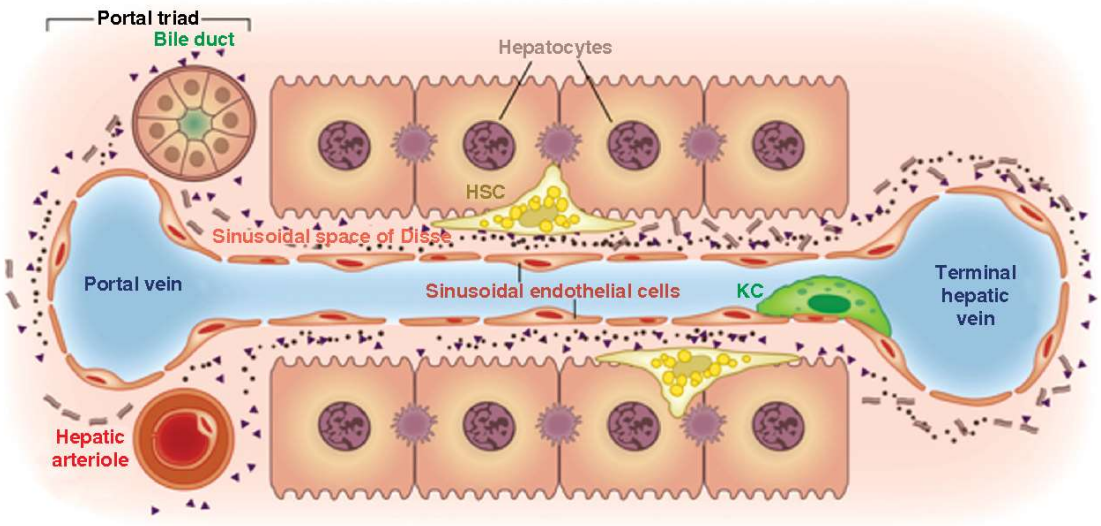

Figure 2. Representation of the organisation of hepatic sinusoid structures and hepatic cells: hepatocytes, hepatic stellate cells (HSC), endothelial cells and Kupffer cells (KC). (Puche et al., 2013)

Hepatocytes have polygonal shape and sizes between 25 to 40 µm. In addition, their functions depend on their location within the organ, due to the gradient of nutrients and oxygen which is produced at the hepatic sinusoid (Wardle, 1987). Among liver functions, metabolism, and transport of drugs, ureogenic capacity and protein synthesis and secretion are particularly

relevant for regenerative medicine purposes. The principal ones are summarised in Table 1, differentiating among distinct cell types.

Table 1. Hepatic cells and their functions

Cell type	Function	References
Hepatocytes	Ureogenesis*, detoxification*, protein synthesis and secretion*, gluconeogenesis, glycogen synthesis from lactate, bile formation, glycolysis, ketogenesis and glutamine formation.	(Trefts et al., 2017)
Hepatic stellate cells (Ito cells)	Secretion of lipoproteins, cytokines and growth factors; immunoregulation, role on liver development, retinoid metabolism (vitamin A), enzymes for detoxification, pH regulation and generation of	(S. L. Friedman, 2008)
Endothelial cells	Protective barrier, cellular communication, cooperation and signal exchange to coordinate the	(Margreet De Leeuw et al., 1990)
Biliary epithelial cells (cholangiocytes)	Bile secretion and absorption of ions, amino acids, glucose and bile acids.	(Tabibian et al., 2013)
Kupffer cells	Phagocytosis of particulate debris, removal of bacteria from blood, metabolism of iron and bilirubin, uptake and catabolism of lipoproteins and defense against virus.	(Wardle, 1987)
Pit cells	Natural killer activity.	(Wisse et al., 1997)

*Functions assessed in this Master Thesis. We evaluated 8 isoforms of P450 enzymes, the ureogenic capacity and albumin secretion of human hepatocytes.

Detoxification is a process associated with the liver metabolism that facilitates the elimination of determined substances like drugs or xenobiotics. In general, the process consists of making the molecules more soluble to improve its excretion by the kidneys. This process comprises three different phases mediated by different enzymes. Phase I enzymes, located in the cytosol of the cells, convert the target molecule into a more polar one by adding new functional groups that increase their hydrosolubility. There are two families of phase I enzymes: cytochrome P450 (CYP) and Flavin-Containing Monooxygenase enzymes. Phase II enzymes perform the covalent bonding of phase I-metabolites to increase the hydrosolubility with a decrease of its pharmacological activity or toxicological effects. Both enzymes improve the renal or biliary excretion, which is considered phase III, but not all the substances require to pass through both phases (Olinga et al., 2008; Jancova et al., 2010) (Figure 3).

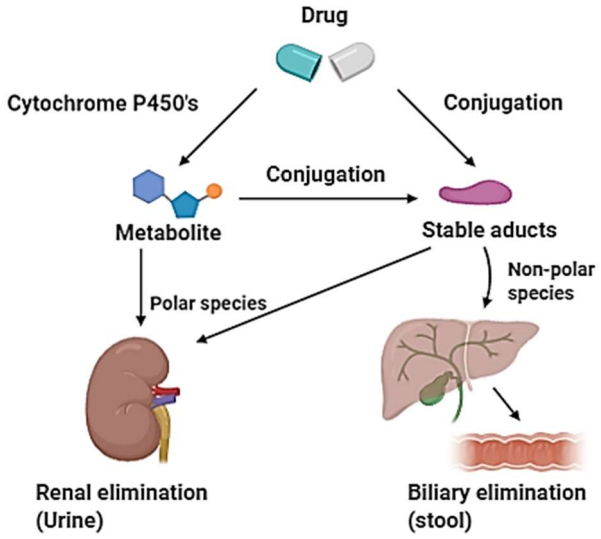

Figure 3. Schematic representation for drug metabolism in liver mediated by enzymes. Drawing made by the author of this Master Thesis.

Ureogenesis is defined as the capacity to convert nitrogenated substances into urea, due to a metabolic process called *"urea cycle"*, starting the conversion in the mitochondria and finishing at the cytoplasm. During the cycle the amino acids or NH_4^+ molecules are metabolised and are removed from the body by the kidneys through the urea (Atkinson et al., 1984). Hepatocytes are also responsible for the 85% of the circulating protein volume, being albumin the most abundant (55% of the total). Albumin is essential since it is the transporter molecule of hormones, drugs and enzymes. Hepatocytes also synthesise other relevant proteins such as blood coagulation proteins (Tennant, 1958; Trefts et al., 2017).

At a microanatomological level, the liver cells are organised in polyhedral structures, mainly with hexagonal shape, called hepatic lobules and where the cells are arranged in cords radiating from the central vein (Figure 4).

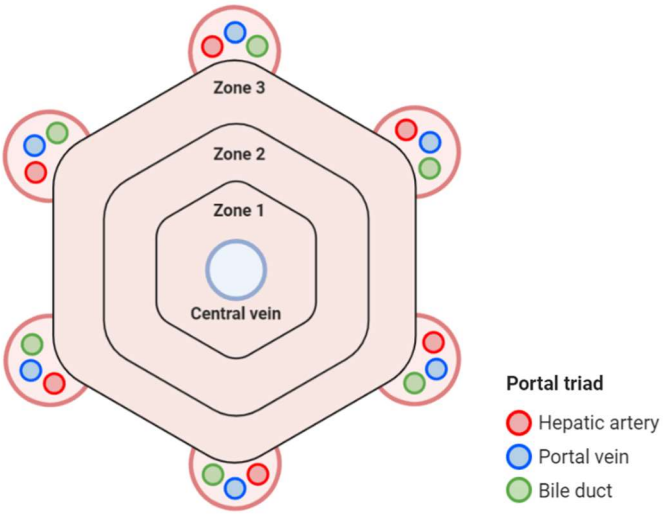

Figure 4. Scheme from hepatic lobule. Drawing made by the author of this Master Thesis

On the other hand, there is a complementary description of the hepatic microanatomy, it is called liver acinus. Divided in 3 zones, it is based on the gradient of nutrients and oxygen in the hepatic sinusoids, the hepatocytes which are closer to the portal triad are receiving blood with higher concentrations of these substances than the ones closer to the central vein. This fact leads to hepatocytes with the same genome to express it differently and subsequently to carry different functions (Figure 5).

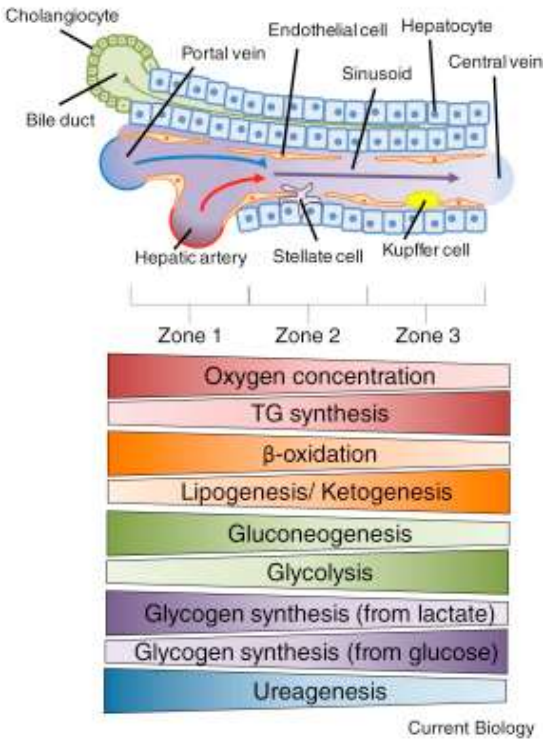

Figure 5. Schematic representation of sinusoid and the "metabolic zonation" concept. (Trefts et al., 2017)

Liver extracellular matrix (ECM) plays a relevant role in cell behaviour providing mechanical support, enabling cell-cell contacts and promoting cell differentiation, migration and proliferation (Baiocchini et al., 2016). It is commonly described by two different histological units, capsule of Glisson and stroma. Glisson's capsule is a dense connective tissue composed of collagen type I that surrounds the external surface of the liver and some of the main blood circulatory structures like the central veins and branches from hepatic artery (Walker, 1966). Stroma is the extension of Glisson's capsule, presented along the sinusoids and formed by collagen type III. The liver's ECM is composed by other molecules: collagen type IV and V with laminin are found on the basement membrane of bile ducts and vessels. Other glycoproteins apart from laminin are also found in the connective tissue of the liver ECM, like fibronectin and tenascin in the basal membrane and the perisinusoidal space (Gerber, 1987).

Proteoglycans or also called glycosaminoglycans (GAG's) such as hyaluronic acid (HA), heparan sulphate, dermatan sulphate, keratan sulphate and chondroitin sulphate are quite relevant (Bedossa et al., 2003). These GAG's have a discontinuous distribution between the sinusoidal endothelium and hepatocytes. Only heparan sulphate is found in the basement membrane (Maher et al., 1993). Despite all GAGs in the liver ECM are important, HA is the

only one that is not sulphated and the only one negatively charged. It has special properties. For instance, HA molecules strongly interact with water molecules, retaining them when deforming, which confers stiffness. HA regulates cell aggregation, cell migration, cell motility and growth factor responsiveness (Allison et al., 2006). In addition, HA contributes to keep cell morphology and the hepatic phenotype in a better way than monolayer cultures (Deegan et al., 2016).

3.2. Liver diseases

Over the last century, hepatic diseases have been a challenge for the medicine. Different liver pathologies like acute liver failure (ALF), acute-on-chronic liver failure (ACLF) or metabolic pathologies, also known as end-stage liver diseases (ESLD) can lead to liver transplantation. These medical conditions imply a high cost to the health care systems and, despite the promising therapeutic approaches, liver transplantation remains as the main effective treatment (Tolosa et al., 2016). Damage to the liver is either caused by a trauma or an acute disease in a short term, or the slow effect over the years of chronic diseases. The most common medical end for ESLD is cirrhosis when the scar tissue replaces healthy liver until the liver cannot work properly. The most common diseases which can lead into an ESLD are different types of chronic viral hepatitis, diseases related with an increased level of triglycerides and inherited metabolic disorders (Table 2).

Table 2. Hepatic pathologies classified by types

Type of disease	Metabolic	Viral	Acquired disorders
	Wilson disease	Hepatitis B	Acute liver failure
	G6ase complex deficit	Hepatitis C	Acute-on-chronic liver failure
	Glycogen storage (Type I)	Autoimmune hepatitis	Non-alcoholic fatty liver
	Urea cycle defects	-	Alcoholic fatty liver
	Refsum disease	-	-

Metabolic disorders are medical conditions with a genetic origin. They have a negative effect over a protein or an enzyme that inhibit one or several metabolic processes, leading into a lack of functionality. These are developed during the first months after birth, but the incidence is relatively low with 1 every 800 child (Pico et al., 2008). The most relevant metabolic disorders are Wilson's disease, glycogen storage type I disease, defects on urea cycle, hemochromatosis and deficit on factor VI.

Wilson's disease is an inherited disorder with recessive autosomal behaviour. Through the blood and targeting the brain and the liver, the copper starts to store in these tissues and would induce toxic damage to the organism. Glycogen storage disease (type I) has the same autosomal behaviour, but it affects the metabolism of carbohydrates. This causes a lack on the G6Pase

complex, which carries processes like the glycolysis or gluconeogenesis, characterised by hypoglycaemia due to the extra storage of glucose in certain tissues such as the liver or the kidneys. Finally, hemochromatosis is similar to Wilson's disease, where the iron is stored instead of copper; although, the medical complications are related with cardiac dysfunctions (Taddei et al., 2009). The common fact among different metabolic diseases is the need of replacing one single function, which is lacking on the liver. Since the only curative treatment considered at present is the liver transplantation, the research is looking for non-invasive therapies, and, hepatic cell therapy can contribute to this objective. For example, regarding with inborn metabolic liver diseases, only replacing a 5-10% of the liver mass would be enough to correct the metabolic pathology (Struecker et al., 2014).

Regarding viral liver diseases, there are three distinct types of hepatitis disease. Hepatitis C is considered the major cause of chronic hepatic damage in the world. Therefore, it is one of the main reasons for liver transplantation. After virus infection, the body starts an intermediate immune response that induces the destruction of liver cells, ending in fibrosis. Nowadays, there is an effective treatment which eradicates the virus in 60% of the cases and reduces cirrhosis in more developed cases. Despite this, the cases and deaths keep increasing due to poor efforts to detect and treat hepatitis C (Poynard et al., 2003). Hepatitis B damages chronically the liver as well, but it has less cases worldwide. The people who is carrier of the virus (which means positive to the surface antigen) for more than 6 months have more probabilities to develop cirrhosis and liver cancer, and at the end they need a liver transplant. There are different ways to treat this pathology, but *interferon* that is a drug with antiviral, antiproliferative and immunomodulatory effects, induces liver disease remission in a specific group of the cases (Lok et al., 2001). There is a final type of hepatitis that is not closely related with the presence of a virus, or not directly, called autoimmune hepatitis. It is characterised by mononuclear-cell infiltration into the tissue, reaching the parenchyma and causing necrosis that can finally lead to cirrhosis and require a liver donor (Krawitt, 2006). It is not exactly known what triggers autoimmune hepatitis, although there is evidence that has relationship with some types of virus such as measles virus, cytomegalovirus, hepatitis and Epstein-Barr. Other authors suggest that it can be developed after the intake of some drugs that replicates the autoimmune hepatitis, but it is still unclear.

Finally, among the most predominant liver diseases is liver steatosis, which can be defined as the increase of triglycerides in the hepatic tissue over 5% of the liver mass. When the origin is not based on an exacerbated alcohol consumption and even without any indicator of hepatitis, is known as *"non-alcoholic fatty liver disease (NAFLD)"*. The symptomatology is not significant, any sign of liver disease, just slight complications like fatigue, sensation of fullness or discomfort on the upper right side of the abdomen. Therefore, diagnosis requires a combination of non-invasive and invasive test, being the liver biopsy the most sensitive, because allows analysing the lipidic content in the hepatocytes (Angulo, 2002; Bellentani et al., 2010; Clark et al., 2009; Matteoni et al., 1999). If the disease has an ethology based on an unusual alcohol consumption it is called *"alcoholic liver disease (ALD)"*. The spectrum of the disease is the same. NAFLD can be a simple steatosis or a serious cirrhosis. The diagnosis is

different depending on the clinical evidence of alcoholic overconsumption and test which can indicate liver abnormalities (Madayam et al., 2018; O'Shea et al., 2010). Despite both diseases have different origins, they lead into the same disease spectrum. Both, the less harmful consequence (hepatic steatosis) and the worst clinical complication (cirrhosis) are now only cured by organ transplantation.

3.3. Alternatives to liver transplantation

Liver transplantation is the unique effective treatment for ESLD that have a huge economic impact on the health care systems (Tolosa et al., 2016). Due to the low number of liver donors, the demand surpasses the offer and it is not possible to reach all patients. According to the *European Liver Transplant Registry*, 127,646 liver transplantations were performed in the period between 1968 and 2013. The patients must wait months, even years, till a compatible liver is available, and the mortality in the waiting list is increasing. Criteria to classify the patients and asses the priority to receive the liver were implemented, which consist of features such as the age, previous pathologies and life-quality expectance. In addition, this type of surgery is really invasive and harmful, causing a huge damage on the patients, even though not all patients survive after the surgery due to the complications like fibrosis.

The science has been putting all the efforts in this field, towards the exploration of new alternatives, which allow facing all the inconveniences produced by the liver transplantation surgery or the scarcity of donors. These alternatives are not based on the removal of the whole liver. One of the surgical alternatives is Liver Donor Liver Transplantation, based on the transplant of a hepatic split from a donor to the patient after a partial hepatectomy. This split should carry the minimum functionality to ensure the patient survival. Although this option is commonly rejected due to the inherent risks for the donor. The success of the transplant is regulated by the mass ratio between the donor graft and patient's liver, it is required to transplant a minimum of 5% of the liver's patient mass in order to obtain enough functionality (Thalheimer et al., 2002).

Despite these invasive alternatives, non-invasive treatments like extracorporeal liver support systems, liver-directed gene therapy, xenotransplantation or cell therapy have been also proposed. But not all of them are really promising because of different limitations. For example, xenotransplantation produces immune response with a hyperacute rejection of the graft. Although immunosuppression can be used, it has many drawbacks. Other alternatives like gene therapy have shown to be more successful, but the effect is not sufficient to be considered as an effective treatment (Thalheimer et al, 2002; Sgroi, et al., 2009). Among the different alternatives proposed, hepatic cell therapy emerged as the most promising one.

3.3.1. Hepatic cell therapy

Nowadays, hepatic cell therapy (HCT) is considered a promising option to stabilise and recover the functions lost in cases of ALF and ESLD, or in patients where liver transplant is not a suitable option. The procedure is quite simple and based on the transfusion of donor cells to the

damaged tissue in order to regenerate or replace it. It reduces the potential risks inherent to liver transplantation, the mortality associated to the disease and allows the preservation of the native liver, reasons why it is worth researching (Alwahsh et al., 2018).

Various cell sources such as human hepatocytes, immortalised cells, adult and embryonic stem cells, tumor-derived and cell-derived hepatocyte-like cells have been suggested to be used in cell therapy (Lee et al., 2015). The first attempts were based on the use of primary human hepatocytes due to their compatibility and functionality, although one of the major limitations is the lack of proliferation that does not allow their *in vitro* expansion before the infusion.

From a clinical point of view, transplantation of human hepatocytes could signify an alternative to whole organ transplantation for correcting genetic disorders that result in metabolically deficient states, in ALF or ESLD, or for maintaining liver function in patients who do not meet the clinical eligibility criteria to be candidates for liver transplantation due to different reasons (i.e. advanced age, other diseases or cardiovascular factors). The wider application of cell transplantation would imply lower patient morbidity, improved quality of life and better survival rates (Tolosa et al., 2016). Hepatocyte transplantation has been clinically applied in pediatric patients with liver-based inborn metabolic disorders (i.e. Crigler-Najjar disease, Wilson's Disease, tyrosinemia, urea cycle defects and CPS1 deficiency) and has been also suggested as a metabolic support in patients with ALF or ACLF, which could serve as a bridge to liver transplantation (Hansel et al., 2014). Moreover, patients with ESLD and no indication of LT could benefit from cell therapy which could delay disease progression (Soltys et al., 2017). Although clinical studies have clearly established a proof of principle for this therapy as a treatment for patients with acquired or inherited liver disease, there are still some specific limitations, which, if overcome, could greatly enhance the efficacy and implementation of HCT.

Not only basic research has been working towards HCT, there is a company called Promethera (Belgium) which has been doing clinical trials on phase I/II. They used Heterologous Human Adult Liver-derived Progenitor Cells with trademark HepaStem, derived from MSC's from the parenchymal fraction of a non-transplantable liver. The aim of Promethera studies were the evaluation of the safety and dosage of the cell therapy on paediatric patients with urea cycle deficit. The results showed a safe cell therapy administration and any rejection to HepaStem. Despite the good results, several patients presented adverse events related with the hospitalization, immobilization and prolonged transhepatic characterisation. They confirmed the tolerability on these patients, allowing to study deeper on HepaStem (Smets et al., 2019).

Cells used in HCT are mainly infused in three different ways: intrasplenically, through the portal vein or ectopically. The intrasplenic procedure was chosen because the spleen is connected to the liver by the splenic vein. Additionally, the portal vein is also a way to infuse the cells to the hepatic sinusoids, because is the main vein of the liver and is a direct pathway to the liver sinusoids. These ways are underpinned on the translocation of the cells from portal pedicles to the liver microenvironment, disrupting the endothelial cells and moving through the space of Disse till the adjacent host hepatocytes. However, the transplanted hepatocytes are

cleared from these areas sometime later, so it supposes a relevant drawback to consider. In addition, a complication commonly found after the infusion is the thrombosis at the portal vein, which can lead into liver failure and portal hypertension. Finally, the ectopic infusion considers allocating the cells on an ectopic place, for example, intraperitoneal cavity, lung parenchyma, pancreas, the kidney capsule, etc. Ectopic transplantation provides more space for donor cells, even allows distinguishing between host hepatocytes from donor hepatocytes (Ohashi, et al., 2001; Sgroi et al., 2009; Deward et al., 2014).

Despite the potential and the good results of the clinical approaches, important limitations have been observed, including low functionality after transplantation, complex isolation, effects on the cells after cryopreservation, low engraftment and the lack of an unlimited source of functional cells. These limitations lead to not consider cell therapy as a definitive replacement for liver transplantation, but it has been possible to improve the quality of life of patients who are on the wait list for a donor.

Regarding the cell sources, at present, treatment is limited by the scarce availability of suitable donor liver tissue for hepatocyte isolation. Livers with severe steatosis, prolonged cold ischaemia time, older donors that make the tissue unsuitable for organ transplantation are currently the main sources of human hepatocytes (Bhogal et al., 2011; Allameh et al., 2012). The quality and viability of hepatocytes obtained from these livers are often poor and not sufficient for their clinical application. On the other hand, the isolation procedure is the first cause of hepatocyte injury, probably due to oxidative stress. In addition, cryopreservation and thawing procedures can adversely lead to both structural and metabolic damage of hepatocytes, which often renders them unsuitable for clinical use (Stéphenne et al., 2010).

The lack of primary hepatocytes has led to explore alternative cell sources which provide an unlimited number of readily available cells. At the moment the generation of induced pluripotent stem cells (iPSC's) that have proliferation capacity and that can be also differentiated to a variety of phenotypes is being investigated as a promising alternative. iPSCs possess the unique properties of hepatic differentiation, self-renewal and *in vitro* expansion which makes them a very promising cell source for generating large-scale production of suitable functional hepatocyte-like cells (Figure 6). Despite the fact that, hepatocyte like cells-derived from iPSCs have been proved in different animal models of liver disease, up at the moment they have not been applied in humans because some important issues regarding their safety and efficacy should be firstly addressed (Chiang et al., 2015).

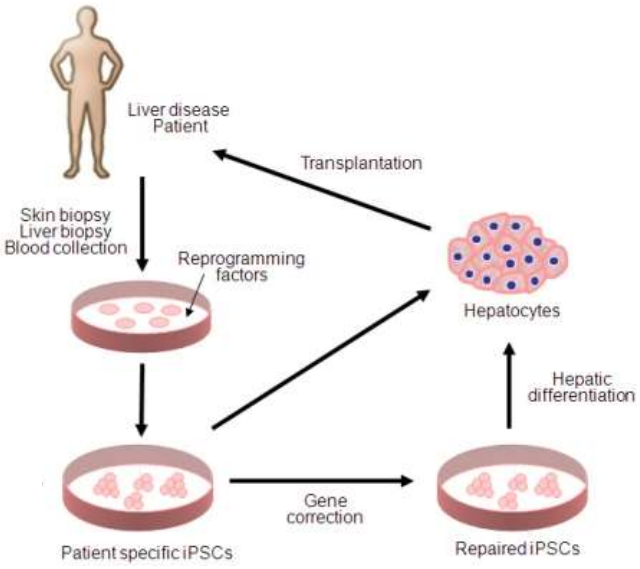

Figure 6. Scheme for hepatic iPSC cell differentiation and gene editing for cell therapy. (Chun et al., 2010)

Another major limitation of hepatic cell therapy is the low engraftment of cells, since the final outcome depends on the hepatocytes grafted to the host liver. After 1-2 days from cell delivery, around 80-90% of the population is cleared due to immune system clearance, which produce only short-term effects of HCT. Because of this, the outcome is always partial or transitory and the application of the therapy does not change the need of a liver donor. Different experimental techniques, based on pre-conditioning the recipient's liver, have been proposed to improve engraftment after cell transplantation. On the one hand, vasodilator drugs have been widely used for this purpose, the most used are nitroglycerine and phentolamine. For instance, nitroglycerine reduces consequences of the HCT like ischemia and inflammation. It has been tested on rodent animal models where cells reached zone II from the liver acinus avoiding to be stacked at the periportal zone (zone I) (Slehria et al., 2002; Bahde et al., 2013). Another of the alternatives proposed is to generate a regenerative stimulus in the recipient's liver by temporary embolization of the portal vein or liver irradiation. This is based on the concept where two different cells with diverse proliferating rate grow in the same tissue, the one with higher rate will lead to the death of the other one. Thus, when the liver is irradiated the host hepatocytes stop to grow and allow the ones which come from the donor to attach and proliferate, in humans this rates are quite low but still being enough to have differences and be an improvement in HCT (Hughes et al., 2012; Yamanouchi et al., 2009).

Nonetheless, tissue engineering is looking for alternatives where the patient would not need to be prepared before the treatment. In fact, the idea is to increase the delivery and attachment of the cells by using different tissue engineering approaches.

3.3.2. Liver tissue engineering

Liver tissue engineering comprises the use of biomaterials as hydrogels, decellularized organs, extracorporeal devices that mimic the functions of the organs and scaffolds, which can be created manually or using of bioprinters, to implant cells for the regeneration of the liver.

Decellularized organs bring the opportunity to use the native tissue as a scaffold, preserving the 3D organisation of the liver ECM. The benefits of this practice are the composition of the scaffold, which is biologically equal, and the 3D architecture that plays a relevant role to maintain the phenotype of hepatic cells. However, the low availability of organs to create those decellularized scaffolds limits the clinical approaches. Different researchers have used this approach, showing promising results in mice models with human liver ECM and human hepatic cells (Mazza et al., 2017; Perez et al., 2017). On the other hand, the use of extracorporeal devices is quite interesting, but limited, since its clinical applications are not into the replacement or treatment of the liver, and it is considered as an external support.

Other solutions that propose the combination of HCT with biomaterials are for instance based on injectable delivery matrices, microspheres for cell encapsulation or patches. These platforms are not only destined for cell therapy, but also for *in vitro* modelling. Among these proposals, the most promising one is the use of injectable matrices they can overcome the low engraftment of cells. Theoretically, they are proposed to mimic the liver extracellular matrix composition and modify the properties to control parameters such as the crosslinking time, stiffness, cell attachment, etc. In addition, injectable matrices provide the 3D environment to the cells, facilitating the spatial organisation of cells and the newly secreted matrix to preserve cell phenotype, functionality and viability. Commercial injectable matrices as Matrigel® are good alternatives because of the good *in vitro* and in animal model performances, but difficult to transfer to humans for the presence of proteins derived from mouse tumours. Then, tissue engineering is proposed as an alternative to liver transplantation and is considered as an improvement of cell therapy to be used in the future (Suuronen et al., 2008; Perez et al., 2017).

3.4. Injectable hydrogels for liver tissue engineering

The essence of life is polymeric, almost all the components form the human body are polymers. Then, the use of polymers in medicine has sense, especially when these polymers are biological and can be degraded by the organism when they have realized their function. Hydrogels were among the first polymeric biomaterials used in humans, mainly due to the role of water in them. They are able to swell large amounts of water without dissolving, being the water mass fraction in them within the range of 90% to 99%. They can be synthetic or made of biological macromolecules (Kopecek, 2007). Peppas precisely defined them as follows: *"Hydrogels are water-swollen, cross-linked polymeric structures containing covalent bonds produced by the reaction of one or more comonomers, physical cross-links due to chain entanglements, association bonds including hydrogen bonds or strong van der Waals interactions between chains or crystallites bringing together two or more macromolecular chains"* (Peppas et al., 2000).

Hydrogels have more advantages compared with other biomaterials thanks to their ability to better interact with the host tissue. Properties such as the water content, the consistency that goes from soft to elastic hydrogels and low interfacial tension, provides excellent features to be used within the human body. The focus of this project is about biological hydrogels, they are mainly composed by either proteins, polysaccharides or a combination of both. Also, it is well

known that the ECM of the tissues regulates processes like cell adhesion, protein synthesis and secretion, cell migration, distribution of growth factors, ions and drugs. The processes are regulated either by the mechanical properties and the composition of the ECM. Then, it is feasible to affirm that biological hydrogels made with a mixture of proteins and polysaccharides can reproduce this ECM behaviour if the composition is mimicked (Ratner et al., 1976; Lieleg et al., 2011).

One way to classify hydrogels considers if the hydrogel is formed before or during the use in the organism, and according to this they are called *"pre-formed"* and *"in-situ / injectable"*. Injectable hydrogels allow focusing on non-invasive approaches due to its nature, they can fill or adapt its shape to the defect or the tissue where it is going to be injected and often they crosslink with the host ECM of the surrounding healthy tissue. The objective is to introduce in the organism an aqueous solution containing the precursor macromolecules together with the cells; then crosslinking of the hydrogel takes place in the tissue defect and the matrix increases its consistency and mechanical properties after a determined time, which depends on composition and nature of the crosslinker. The process where the liquid compound becomes solid after a certain time is called crosslinking, the molecules start to attach between them by physical or chemical interactions. Crosslinks between chains or molecules has a direct effect on the physical properties such as viscosity, increased strength and toughness, elasticity, insolubility of the polymer, etc.

Table 3. Some examples of hydrogels of the two main types. (Bahram et al. , 2016)

Type of crosslinking	Examples	References
Physical crosslinking	PLGA-PEG-PLGA	(Zentner et al., 2002)
	PNIPAM (Stimuli responsive)	(Feil et al., 1993)
	Elastin-like polypeptides	(Urry, 1992)
	Alginate/Ca^{2+} (Self-assembly)	(Van Vlierberghe et al., 2011)
Chemical crosslinking	Hyaluronic acid-Gelatin (enzyme mediated)	(Sanmartín-Masiá, et al., 2017)
	Collagen-Hyaluronic acid (Carbodiimide)	(Wang et al., 2009)
	Chitosan-Hyaluronic acid (Aldehyde-Amine)	(Tan et al.,2009)
	Fibrin (enzyme mediated)	(Stabenfeldt et al., 2012)

Table 3 shows some examples of hydrogels of the two main types, physical or chemical (Overstreet et al., 2012). Firstly, physical hydrogels are formed when the mixture is exposed to

temperature, pH or other environmental factors, triggering a response which makes the hydrogel pass from liquid to solid by non-covalent interactions. On the other hand, chemical crosslinking is based on covalent bonds between molecules, this provides higher stability to degradation and better mechanical properties (Bahram et al., 2016).

Injectable hydrogel matrices have been proposed as cell supports that can solve the problems of the low engraftment of cells in cell therapy as they can *in situ* crosslink with the cells in them entrapped close to the host tissue. During the last decades they have been used for a variety of target tissues. They were used for central nervous system cell therapy; the approach was based on delivering neural progenitor cells (NPC's) and Schwann cells (SCW's) into the damaged area. The defects on the brain are variable on size and shape, then injectable biological hydrogels were considered the best option (Pakulska et al., 2012). Compounds like Matrigel®, which contains collagen and laminin, were used in animal models with improved NPC's survival after delivery against conventional cell therapy with good outcomes (Jin et al., 2010). In addition, SCW's survival with Matrigel® was also studied and the results showed a survival over 36% of transplanted cells (Patel et al., 2010). Other authors have created compounds trying to mimic the brain ECM with fibrin/fibronectin and they obtained even better results than with collagen matrixes. This reaffirms the relevance of mimetic hydrogel compositions to obtain better cell responses and regeneration (King et al., 2010). Other authors have been using injectable hydrogel for cardiac cell therapy. The hydrogel was composed by chitosan-graft-aniline tetramer (CS-AT) and was chemically crosslinked in presence of dibenziladehyde-terminated poly(ethylene glycol) (PEG-DA), thanks to the reaction of amino groups of the CS-AT and the aldehyde of the PEG-DA. The cells used were the immortalised cell line of myoblast from rat (C2C12). Results showed a rapid self-healing ability, good viability and proliferation, concluding that injectable hydrogels were a key point on the success of the cell therapy (Dong et al., 2016).

Injectable hydrogels have been also important as supports for HCT. Several authors have published very interesting studies. For instance, it was at the King's Collage in London where the firsts human hepatocytes were encapsulated into alginate microbeads. The crosslinking method was based on the ionic interaction between alginate and Ca^{2+}. They performed *in vivo* experiments in animals with promising results regarding cell viability, human albumin expression/secretion and no immune response (Jitraruch et al., 2014). Other authors proposed a catechol-modified hyaluronic acid (HA-CA) as injectable matrix to improve HCT. This modification has been reported as non-cytotoxic and does not provoke inflammatory response. They also proved how catechol groups helped to attach the hydrogel to the tissue surface through interactions with surface proteins, allowing to inject the matrix to the defected tissue. The hydrogel, previously seeded with human hepatocytes, was injected and placed on top of the liver lobe. After 2 weeks, cells were well distributed along the matrix and just at the third day human albumin was detected that was considered an index of cell survival and functionality. In addition, when the injection was performed after a partial hepatectomy, human albumin was found further than 2 weeks in blood serum (Shin et al., 2015). The results confirmed the potential of injectable matrixes for HCT support.

3.5. Hyaluronic acid

Hyaluronic acid, also known as hyaluronan. is a glycosaminoglycan. Formed by two monosaccharide units that are N-acetylglucosamine and D-glucuronic acid linked by a β1,3-glycosidic bond (Figure 7). Each disaccharide unit is linked to the other by β1,4-glycosidic bonds and repeated up to 10^4 times (Turner et al., 2007). In addition, hyaluronic acid is biocompatible, and is present in almost all ECM from the human organism playing key roles in cell signalling transduction, stimulation of angiogenesis, modulation of inflammatory response and interaction with cell receptors and wound healing, specifically on ECMs with large amounts of hyaluronic acid. Also, it is metabolised and synthesized by a lot of cell types and tissues by common physiological processes (Darr et al., 2009; Zhou et al., 2012; Turley et al, 2002).

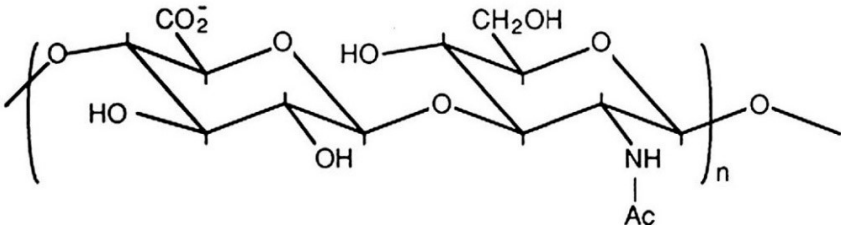

Figure 7. Chemical structure of the hyaluronic acid. (Allison et al., 2006)

Hyaluronic acid is considered one of the most promising molecules due to its biocompatibility and degradation within the body. It can form itself hydrogels by hydrogen bonds between its own carboxylic groups and N-acetyl groups, one of the reasons for its high stiffness (Ortega et al., 2012). One of the main drawbacks is that most of crosslinking chemistries are cytotoxic, such as those based on the use of divinylsulfone or in esterification reactions, provoking cell death. That is why new crosslinking methods have been proposed in order to overcome this toxicity, methods like enzyme-mediated, disulfide or thiol-reactive are non-toxic ways to create hydrogels (Darr et al., 2009; Khanmohammadi et al., 2016).

Among the applications of the hyaluronic acid into medicine and tissue engineering, the first steps were injecting it into joints to improve lubrication and in cosmetic surgery (Friedman et al., 2002; Kim et al., 2001). Applications for brain demand ECM-like scaffolds, and hyaluronic acid was recognised as an important compound on brain formation. Then, for traumatic brain damaged HA was used, modified with polylysine on his side chains and crosslinked using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. It was tested on animal models with neuronal cells from neonatal rats with promising results about cell ingrowth and angiogenesis. (Tian et al., 2005). Other authors claim the relevance to mix hyaluronic acid with collagen to create scaffolds by EDC crosslinking, in order to promote better outcomes and improve the use of neural stem cells from animal as well. The outcomes were excellent achieving *in vitro* differentiation of neuronal stem cells to neuronal cells (Wang et al., 2009). In addition, drug delivery systems have been using hyaluronic acid modified with tyramine, producing an injectable compound that can be crosslinked *in vivo* in the presence of the enzyme horseradish peroxidase. This allows to have a more efficient control of the drug supplied and better directed therapies (Kurisawa et al., 2005). Moreover, injectable hydrogels for cartilage defects have been developed. The possibility to fill the defect with non-invasive surgery was an attractive idea. Hybrid compositions of oxidised hyaluronic acid with aldehyde groups that

react by Schiff's base linkage with chitosan modified with amino groups have been proposed. The *in vitro* results demonstrated that this combination formed a suitable injectable hydrogel for chondrocyte culture that promoted cell survival and supported cell attachment (Tan et al., 2009).

3.6. Gelatin

Gelatin (Figure 8) is a protein derived from collagen, obtained by partial hydrolysis of collagen. It has the same composition of amino acids as collagen, the propellers obtained by hydrolysis contain heterogeneous mixtures of 300 to 4000 amino acids. Therefore, gelatin could retain protein domains of collagen that regulate or establish cell differentiation, cell adhesion and cell growth. Gelatin was reported to be non-cytotoxic and biocompatible, which led to its wide use and characterization. Gelatin can be obtained mainly from animals such as pigs, bovine and fish, there are a variety of protocols for extracting the gelatin and the quality depends on them (Zhou et al., 2012; Rose et al., 2014).

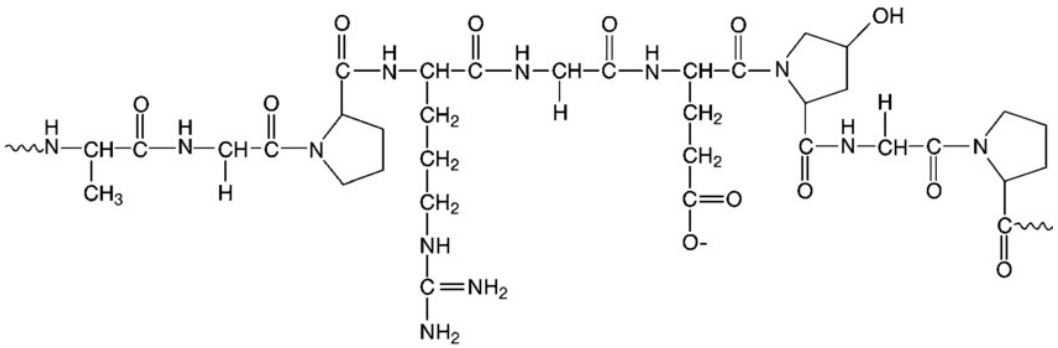

Figure 8. Chemical structure of gelatin. (Kommareddy et al., 2007)

Based on the process for the obtention, there are two types of gelatin either as Type-A or Type-B. If the collagen was under acidic treatment gelatin Type-A is obtained and if the treatment is alkaline Type-B is obtained. Each one has a different isoelectric point (pI), acidic treatments provide a pI between 7 and 9, then alkaline treatments between 4.8 and 5.0 (Kommareddy et al., 2007). Other relevant characteristic from the manufacture to obtain gelatin is the Bloom strength, considered as a measurement of gelation properties and stiffness. It is an index that stands for the renaturation degree from the gelatin and henceforth it is correlated with the ability to adopt big molecule conformations. The results on the characterisation showed that higher Bloom values describe stiffer hydrogels as well as more insoluble compounds reflected on the swelling degree (Bigi et al., 2004).

Gelatin can form a physical hydrogel below the melting point (≈ 23ºC), which is reversible, meaning that at higher temperatures (for instance the body temperature) it becomes liquid. Mechanical properties of gelatin hydrogels depend on its bloom value, type of gelatin, the concentration and the crosslink methodology, and in general they are very poor compared to other hydrogels. That is why crosslinking of gelatin with other compounds can be a good strategy to enhance its mechanical properties. Gelatin is usually crosslinked mainly by chemical

reactions, for example reactions with carbodiimides, divinylsulfone and glutaraldehyde (Grover et al., 2012; Alarake et al., 2017).

Despite the low mechanical properties, it has been widely used in tissue engineering, most of the times combined with other molecules. For example, it has been used for ocular tissue engineering due to the softness of the tissue, alone or mixed with chitosan, as is the case of limbal epithelium or corneal stroma regeneration (Rose et al., 2014). It has been proposed as a matrix for cartilage regeneration, resembling one of the components of the tissue ECM. In this case it also is proposed alone, microcarriers with trade mark Cultisphere® for cell and substance delivery (Sommar et al., 2010), or mixed with chitosan to create scaffolds to implant isolated chondrocytes (Xia et al., 2004). Bone tissue regeneration has also been using gelatin, despite the low mechanical properties (Liu et al., 2009). These examples confirm the inherent potential that gelatin has for tissue engineering purposes, especially when it is combined with other molecules that improve the mechanical properties.

3.7. Crosslinking methods used in this study

The aim of the Master Thesis has been to compare two different crosslinking methodologies for human hepatocytes cell culture. One is based on enzyme-mediated crosslink of tyramine conjugates of hyaluronic acid (HA-tyr) and gelatin (Gel-tyr) by horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2), and the other is based on Schiff's base linkage between oxidised hyaluronic acid (oHA) and non-modified gelatin (Gel).

In the first systems, a first reaction allows producing tyramine conjugates of HA and Gel, obtained through a modification of the chains of both components, mediated by EDC and N-Hydroxysuccinimide (NHS), by reacting the carboxylic groups with the amine groups of tyramine. This modification allows a subsequent crosslinking reaction by oxidation of the phenol groups of grafted tyramine in the presence of HRP and H_2O_2.

The combined hydrogels have chains of gelatin crosslinked to other gelatin chains, crosslinks between two HA chains or combined crosslinks between one Gel and HA, as observed in Figure 9. Peroxidase-catalysed oxidation reactions are common in the human body, for example in melanin formation, and this crosslinking is not cytotoxic for cells (Kurisawa et al., 2005). Henceforth, this hydrogels will be called t(HA-Gel) to differentiate them.

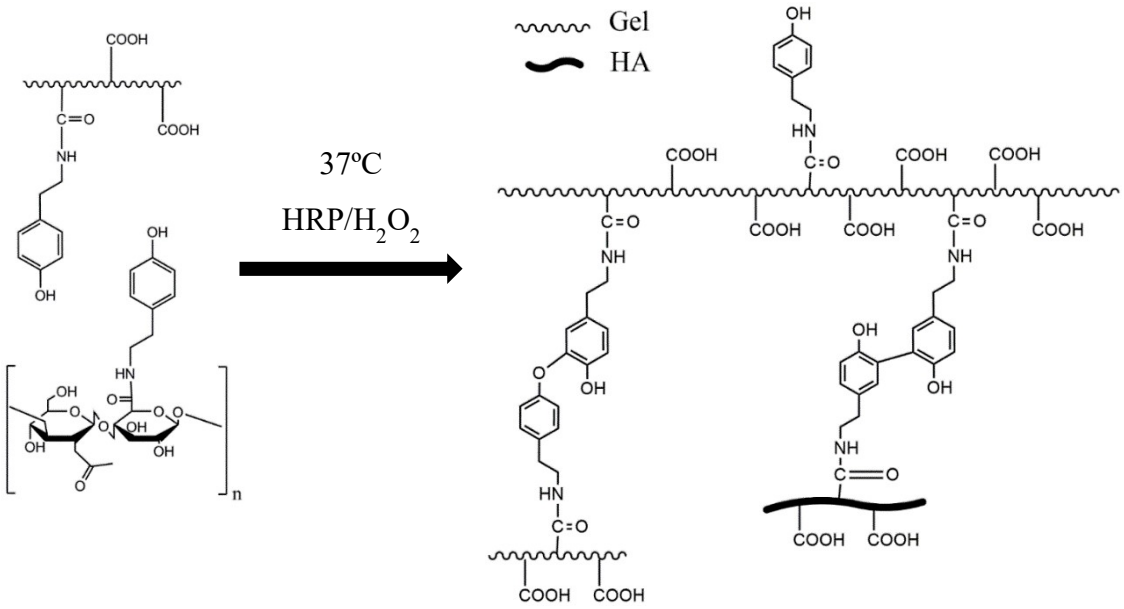

Figure 9. Chemical scheme for enzyme-mediated crosslinking of t(HA-Gel) tyramine conjugates. Scheme modified from (Poveda-Reyes et al., 2016)

In the second system we use Schiff's base reaction because it is non-cytotoxic and easy to produce. The chemical reaction occurs between aldehyde groups, previously created in hyaluronic acid by oxidation, and amino groups of the gelatin. The reaction is spontaneous, does not need a catalyser, and occurs by mixing both components forming an imine bond to from a Schiff's base (Figure 10) (Cordes et al., 1962). Recent literature reports about the use of Schiff's base to create injectable hydrogels of Gel and HA; both molecules where modified, hydrazide-modified (NH_2) gelatin and oxidised-modified (-CHO) hyaluronic acid (Hozumi et al., 2018). Hereafter, this hydrogels will be called as oHA-Gel hydrogels.

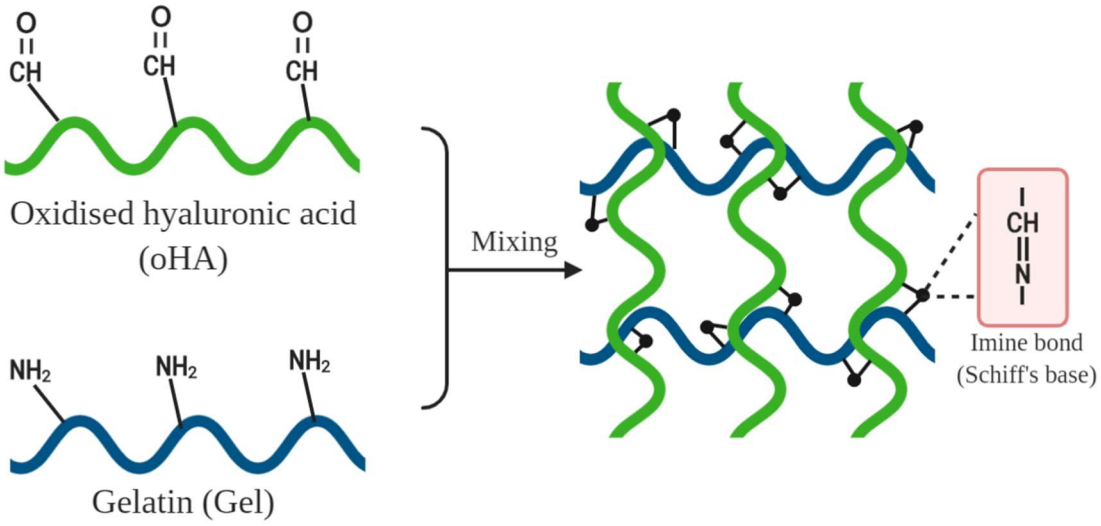

Figure 10. Chemical scheme for Schiff's base crosslinking for aldehyde groups. Drawing made by the author of the Master Thesis

4. JUSTIFICATION

During the last years, medicine and engineering have started to settle bases for a common research to solve the limitations of the current medicine. Biomaterials have been a complete innovation and have allowed the improvement of a myriad of medical products. New families of biomaterials have appeared on the demand for alternative cell therapy treatments. Injectable hydrogels are very promising biomaterials for their ability to be implanted by minimally invasive surgeries, the homogeneous encapsulation of cells by non-cytotoxic reactions and the good integration with host tissue.

The current project aiming hepatic tissue engineering is focused on biopolymers inspired in the composition of the hepatic extracellular matrix, which is mainly composed of collagen type I and glycosaminoglycans, in minor amounts. The molecules selected were gelatin, derived from collagen type I, representing the protein component, and hyaluronic acid as the glycosaminoglycan component. The proportion was selected according to previous experimentation, being the major component hyaluronic acid to provide mechanical stiffness and hydration, to ensure the diffusion of molecules to the encapsulated cells. Gelatin confers cell adhesion thanks to the presence of RGD sequences. Both components in the same polymeric matrix have already been used as chondrogenic matrices stimulating the chondrogenic differentiation of mesenchymal stem cells (MSC's).

Liver transplantation is the unique curative treatment for end stage liver diseases. The systems here presented are intended as alternatives to liver transplantation or can be used in patients waiting for a liver donor. They pretend to overcome the low cell engraftment of the current hepatic cell therapy. The lack of cell attachment in cell therapy produces a loss of 80% of transplanted cells after 2-3 days of injection. We hypothesise the hydrogels here developed will provide three-dimensionality to the cells in them encapsulated and keep them in the host tissue by the entanglement of the hydrogel with the surrounding tissue.

The aim of the project was to compare two different ways of synthesizing injectable hydrogels. One is based on the use of tyramine conjugates (hyaluronic acid and gelatin) in order to produce enzyme-mediated crosslinks by non-cytotoxic reaction. The other is based on the oxidation of polysaccharides (hyaluronic acid), modifying its carboxylic groups into aldehyde groups, able to spontaneously react with the amino groups present in the proteins (gelatin) producing the crosslinking by non-cytotoxic reaction.

In this document we present the synthesis and some characterisation of both hydrogels. We evaluated the functionality of human hepatocytes encapsulated in the enzymatically crosslinked hydrogels by ureogenic capacity, albumin expression and CYP's activity. For the oxidised-polysaccharide hydrogels we optimized the synthesis and checked their suitability to encapsulate cells with human fibroblasts, evaluating the cell viability and metabolic activity.

5. REGULATION

The regulations in which the experimental part of this work is framed, are extracted from the National Institute of Safety and Health at Work. The experiments will be governed by Royal Decrees and Laws related to the handling and storage of biological and chemical substances and waste.

- Law 31/1995 of 8 November 1995 on the Prevention of Occupational Risks.

- Law 14/2007 of 3 July 2007 on the biomedical research

- ROYAL DECREE 833/1988, of 20th July, approving the Regulations for the execution of Law 20/1986, the basic law on toxic and hazardous waste.

- ROYAL DECREE 374/2001, of 6th April, on the protection of the health and safety of workers against risks related to chemical agents at work (Article 5.3).

- ROYAL DECREE 374/2001, of 6 April, on the protection of the health and safety of workers against risks related to chemical agents at work.

- ROYAL DECREE 485/1997, of 14 April, on minimum requirements for safety and health signs at work (Annex I.2; II.1; III.3).

- ROYAL DECREE 664/1997 of 12 May 1997 on the protection of workers against risks related to exposure to biological agents at work (BOE 124, 24 May 1997, pp. 16100-16115)

- ROYAL DECREE 374/2001, of 6 April, on the protection of the health and safety of workers from the risks related to chemical agents at work (Articles 5.2.c, 7.4.c).

- ROYAL DECREE 614/2001, of 8 June, on minimum requirements for the protection of the health and safety of workers from electrical risks.

- ORDER ESS/1451/2013, of 29 July, establishing provisions for the prevention of injuries caused by cutting and sharp instruments in the health and hospital sector.

- Royal Decree 53/2013, of 1 February, establishing the basic rules applicable to the protection of animals used in experimentation and other scientific purposes, including teaching

- Order ECC/566/2015, of 20 March, establishing the training requirements to be met by personnel handling animals used, bred or supplied for experimental and other scientific purposes, including teaching.

- UNE-EN ISO 14971 “Application of risk management to medical devices”

- Regulation (UE) 2017/745 (Medical devices)

5.1. Classification by Regulation (UE) 2017/745

To commercialize an injectable hydrogel we need to find its classification in the *"Regulation (UE) 2017/745"* according to 4 possible categories: class I (low risk), class IIa (medium risk), class IIb (medium risk with high invasive potential) and class III (high risk). This allows to correctly apply the legislation regarding the documents and tests which are necessary to bring a cell therapy product to the market.

Following the annex VII from this regulation, the rule 7 says *"All surgically invasive devices intended for short-term use are classified as class IIa unless they..."*, also, rule 8 says *"All surgically invasive devices intended for long-term use are classified as class IIb unless they…"*. At this point, the difference between the rules is the time the device is within the body, the degradability of the injectable hydrogel proposed remains unclear. It would be difficult to classify, but both rules match in this fact *"unless they have a biological effect or are wholly or mainly absorbed in which case they are classified as class III"*. On the other hand, it is also classified as "class III" by the rule 18, which is also included in the special rules. It says, *"All devices manufactured utilising tissues or cells of human or animal origin, or their derivatives, which are non-viable or rendered non-viable are classified as class III"*. Based on this we can conclude that the injectable hydrogels we propose here are considered class III (high risk).

5.2. Risk analysis by UNE-EN ISO 14971

Injectable hydrogels would also have to be assessed in terms of inherent risks of the device, by following the UNE-EN ISO 14971 it is possible to do different analysis. After the analysis, the risks which belongs to the device have to be controlled, allowing to modify the device in a safer way. The following table explains the semi qualitative parameters used for the risk analysis (Table 4 and Table 5).

Table 4. Probability and risk matrix

Probability		**Impact**	
Grade	Value	Grade	Value
1	Exceptional	1	Insignificant
2	Rare	2	Minor
3	It is probable	3	Moderated
4	Quite probable	4	Major
5	Highly probable	5	Catastrophic

Table 5. Risk severity matrix according the probability and impact

Probability 5	Moderated	High	Very high	Very high	Very high
Probability 4	Moderated	Moderated	High	Very high	Very high
Probability 3	Low	Moderated	High	High	Very high
Probability 2	Low	Moderated	Moderated	Moderated	High
Probability 1	Low	Low	Low	Moderated	Moderated
	Impact 1	Impact 2	Impact 3	Impact 4	Impact 5

Once defined the different grades of probability and impact, the next table gathers the risks, the values of impact and probability and the safe design proposed for the hydrogels (Table 6). The final risk is obtained by multiplying the value of probability.

Table 6. Risk analysis for hydrogels on hepatic cell therapy

Risk	Probability	Value (Prob)	Impact	Value (Imp)	Total risk	Safe desing
Fast degradation of the hydrogel	Rare	2	Moderated	3	6 (Moderated)	Degradation tests in human body conditions
Occlusion of vein after crosslinking	Rare	2	Catastrophic	5	10 (High)	Calculus of the crosslinking time in human body conditions and after the cell therapy an exploratory exam to discard occlusions
Hydrogel produce foreing body reaction	Exceptional	1	Catastrophic	5	5 (Moderated)	Use of natural polymers which form the ECM from the target tissue

The risks have been selected in the case of a hypothetical application on the human body, at the moment there is no evidence of hydrogels supporting hepatic cell therapy in humans.

6. METHODOLOGY

6.1. Part I: tyramine crosslinked hydrogels

6.1.1. Reagents

The following substances, materials and reagents were used for the part I which is dedicated to the synthesis of tyramine conjugated t(HA-Gel) hydrogels, the viability test and the functionality tests carried on them with human hepatocytes.

t(HA-Gel) hydrogel synthesis required porcine gelatin (type A, gel strength 300), hyaluronic acid from streptococcus, horseradish peroxidase (HRP) type IV, hydrogen peroxide (30% w/w in H_2O_2), tyramine hydrochloride (Tyr-98%), 2-(N-morpholino)ethanesulfonic acid (MES - 99%), N-hydroxysuccinimide (NHS - 98%), dialysis membranes 12.400 MWCO and 3500 MWCO, hydrochloric acid, sodium hydroxide, potassium chloride, sodium dihydrogen phosphate, HEPES salt, sodium chloride, glucose solution 1 M, bovine serum albumin (BSA), Hoescht 33342 and propidium iodide, were all purchased from Sigma-Aldrich (USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) was obtained from Iris Biotech GmbH (Germany).

On the other hand, for cell viability assay and hepatic functionality testing trypsin, F12 cell culture medium, L15 cell culture medium, cell culture flasks and Dulbecco's phosphate buffered saline (dPBS) purchased from Gibco, ThermoFisher (USA), were needed. P48 well plates were bought from VWR (USA). L-Glutamine and fetal bovine serum (FBS) were purchased from Biochrome AG (Germany). Bicarbonate 1 M was obtained from Grifols (Spain). Penicillin-streptomycin-fungizone (PSF) was purchased from Promocell (Germany). QuantiChrom kit was obtained from Bioassay System (USA). ELISA human albumin kit was obtained from Bethyl Laboratories (USA).

Calcium-Free Krebs Ringer Buffer (CF-KRB) used on t(HA-Gel) hydrogel synthesis was handmade, and all the components were obtained from Sigma-Aldrich (Germany). The buffer is composed by milliQ water adding 5 mM of potassium chloride, 25 mM of HEPES salt, 1 mM potassium dihydrogen phosphate and 115 mM sodium chloride. In addition, for primary hepatocyte cell culture a specific culture medium explained in the annex was prepared.

6.1.2. Synthesis of HA-Tyr

The procedure to obtain tyramine grafted hyaluronic acid was described by Sakai (Sakai et al., 2014), based on the same chemistry as Kurisawa applied for tyramine grafted gelatin (Kurisawa et al., 2005). During the reaction, the carboxylic groups (-COOH) of the hyaluronic acid are activated and linked to the amine groups ($-NH_2$) of the tyramine, forming amide bonds, as observed in Figure 11.

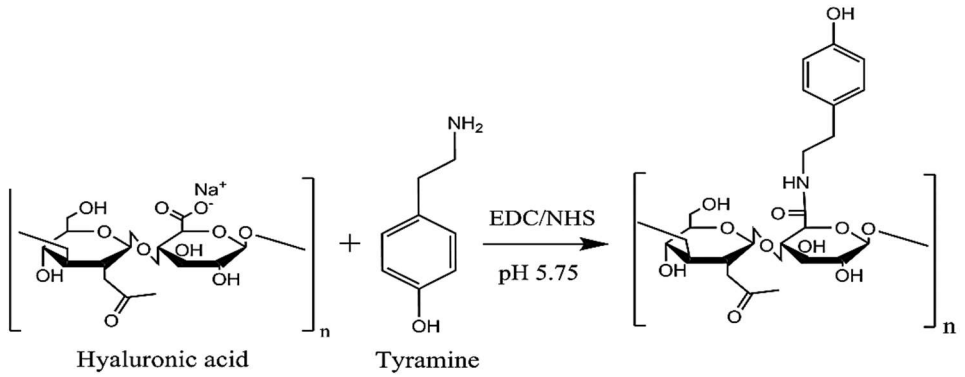

Figure 11. Tyramine grafting on hyaluronic acid chains through EDC/NHS chemistry (Poveda-Reyes et al., 2016).

Before starting the reaction for the tyramine grafting, the molecular weight of pristine hyaluronic acid was reduced by acidic degradation. For this, a solution of HA was firstly performed by dissolving 500 mg of hyaluronic acid in 50 mL of milliQ water, stirring for 24 h at 4 ºC. Then, the pH was adjusted to 0.5 by the addition of HCl, and acidic degradation took place for 24 h at 37 ºC. In this step, the molecular weight was reduced from 1.2 MDa to 350 kDa (Poveda-Reyes et al., 2016). After this, the pH was adjusted to 7 by adding NaOH and the product was purified by dialysing against deionized water for 3 days with a membrane of 3500 MWCO. Water was changed 3 times per day. At that point, low molecular weight HA solution was frozen at -80 ºC for 24 h and then freeze-dried for 3-4 days.

20 mL of a solution of low molecular weight HA was performed by adding 100 mg of HA in a mQ water solution containing 175.3 mg of sodium chloride (NaCl), 1.08 mg of MES (acting as a pH stabilizer) and 0.3 mL of NaOH, adjusted to pH 5.75 before adding HA. The solution was kept in agitation at room temperature for 4 h.

Once dissolved, 85.64 mg of HCl-Tyr were added, stirring at room temperature for 20 min, and the solution was again adjusted to a pH of 5.75. After this, 47.77 mg of EDC and 2.87 mg of NHS were added, with agitation at 37 ºC for 24 h. In this step the grafting of tyramine molecules onto the hyaluronic acid chains took place. The amounts of reagents were selected respecting the same molar ratios as in (Poveda-Reyes et al., 2016); 2:1 for Tyr:COOH, 1:1 for EDC:COOH, 1:2 for EDC:Tyr and 1:10 for NHS:EDC.

The solution was purified for 2 days using a 3500 MWCO dialysis membrane. The first day against a 150 mM NaCl solution and the second day against distilled water. The solution was then frozen at -80ºC and subsequently freeze-dried for 4 days.

The particularity of the HA tyramine grafting is that all the processes must be carried out with the containers covered with aluminium foil since it is photosensitive.

6.1.3. Synthesis of Gel-Tyr

The grafting of tyramine in the gelatin is also due to the reaction of the carboxyl groups (-COOH) in its chains and the amine groups (-NH_2) of the tyramine, as observed in Figure 12. The procedure was adapted from that of Kurisawa (Kurisawa et al., 2005).

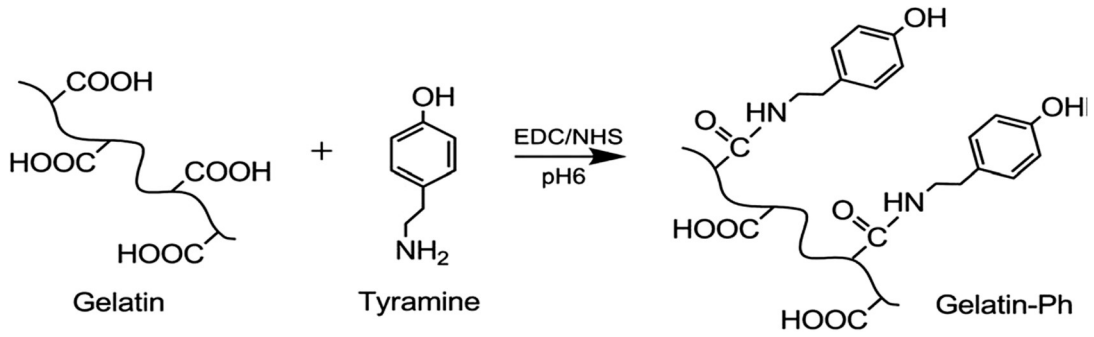

Figure 12. Tyramine graft on gelatin chains through EDC/NHS chemistry (Sakai, 2009).

We used the same molar ratios as Poveda-Reyes (Poveda-Reyes et al., 2016), 2:1 for Tyr:COOH, 2:1 for EDC:COOH, 1:1 for EDC:Tyr and 1:10 for NHS:EDC.

First, 0.4 g of gelatin were dissolved into 20 mL of mQ water (20 mg/mL) and then 195.24 mg of MES were added to stabilize the pH. The solution was stirred for 30 min at 60 ºC. After that, 113.13 mg of tyramine hydrochloride were added and stirred for 20 min at room temperature. The pH was adjusted to 6 by adding drops of a 0.1 M solution of NaOH. Subsequently, 7.36 mg of NHS were added and stirred for 30 min, before adding 122.8 mg of EDC. This solution was left 24 h at 37 ºC under stirring.

After the tyramine grafting was completed, the product was purified by dialysis against deionized water with a membrane of 12400 MWCO for 2 days and changing the water 3 times per day.

Finally, the tyramine conjugate of gelatin (Gel-Tyr) was frozen during 24 h at -80 ºC and then freeze-dried for 3 days for further use.

6.1.4. Formation of t(HA-Gel) hydrogels

The ratios and volumes chosen were proved as the optimal system in my Bachelor thesis. Hydrogels synthesized with F12 culture media as the solvent, shown a remarkable shear modulus 459 Pa and a gelation time of 13 min. On the other hand, HepG2 cells were cultured within them to prove their potential with good outcomes in terms of cell viability and ureogenesis as a functionality test.

Hydrogels were prepared with volumetric hyaluronic acid-gelatin ratio of 80/20 (HA-Gel). As gelling media, F12 culture medium was used, due to its compatibility with cells. Precursor solutions of gelatin and hyaluronic acid were prepared by dissolving the previously prepared products, Gel-try and HA-tyr, in F12 at 2% w/v.

Whereas gelatin dissolution was prepared at 37 ºC for 40 minutes, hyaluronic acid was dissolved at 4 ºC a minimum of five hours before the hydrogel preparation. They were mixed in the volumetric proportion 80/20 HA-Gel to obtain the hydrogel mixture once the pure components were dissolved.

Crosslinking was performed by adding 10% volume of F12 culture media (respect the total volume of the hydrogel) with 12.5 U/mL of horseradish peroxidase enzyme and 10% volume

of F12 culture media with 20 mM of H_2O_2. The hydrogels were left for 30 minutes until cross-linking reaction was completed. The aspect of the hydrogels can be seen in Figure 13. Hydrogel droplets of a total volume of 100 µL were prepared for the cell culture.

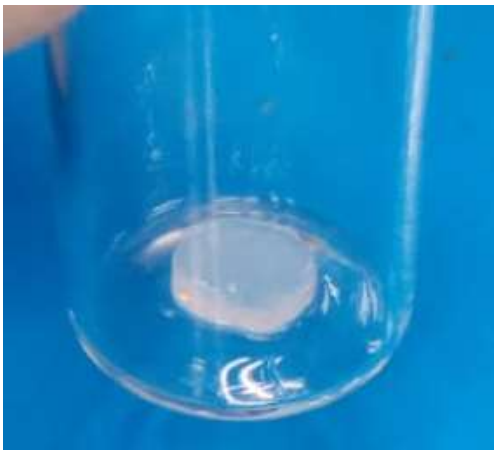

Figure 13. Example of t(HA-Gel) (80/20) hydrogel after crosslinking.

6.1.5. Equilibrium water content

The hydrogels were characterised by the amount of water they absorbed after 24 h of immersion in an aqueous solution of dPBS. The results were expressed in two ways, by the mass of water per unit mass of dry polymer (*w*, %) and the equilibrium water content (mass of water per unit mass of the hydrogel, or water mass fraction within the swollen hydrogel, EWC, %).

t(HA-Gel) hydrogels were prepared on homemade cylindrical agarose moulds and after 1 h of crosslinking, they were transferred to a p24 well-plate and 500 µL of dPBS were added to cover them. After 24 h of swelling they were weighed (w_s) and weighed again after a freeze-dry step to obtain their dry weight (w_d). The following equation (equation 1) determines *w* (Sanmartín-Masiá et al., 2017):

$$w = \frac{w_s - w_d}{w_d} \times 100 \quad (1)$$

Equation 1. *Mass of water per unit mass of dry polymer*. (Sanmartín-Masiá et al., 2017)

Equilibrium water content was determined in the same way, but using equation 2 (Yang et al., 2016):

$$EWC = \frac{w_s - w_d}{w_s} \times 100 \quad (2)$$

Equation 2. Equilibrium water content equation (Yang et al., 2016)

6.1.6. Primary hepatic cell culture

Liver samples were obtained in agreement with the rules of the hospital's Ethics Committee. Human hepatocytes were isolated in the Hepatic Cell Therapy Unit from IIS La Fe from whole cadaveric livers using a two-step collagenase perfusion technique. Cellular viability was

assessed by the dye exclusion test with 0.4% trypan blue. Hepatocytes were seeded and cultured as previously described in detail (Bonora-Centelles et al., 2010). Only hepatocytes with a viability >75% were cryopreserved. They were pelleted and suspended at a density of 10^7 viable cells/mL in ice-cold CellBanker cryopreservation medium. The freezing protocol consisted in ten segments from 4.0 ºC to -140.0 ºC by using a controller-rate freezer. Then, cells were stored in liquid nitrogen at -196 ºC. For thawing, cryovials were quickly placed at 37 ºC for 1-2 minutes and resuspended in 18 mL of pre-warmed biopsy medium, which is mainly composed by two different base media Williams E and F12 (Annex 1 for composition), supplemented with 10% FBS. Cells were centrifuged at 50 xg for 5 min and resuspended in 5 mL of sterile dPBS. After thawing, cellular viability was assessed by the trypan blue test and the total cell number determined by counting the cells in a Neubauer chamber.

Inside each hydrogel 200.000 cells (cell density 2×10^6 cells/mL) were introduced and crosslinked as explained in the point 6.1.4. Once the mixture of liquid hyaluronic acid and gelatin was prepared and filtered with 0.2 µm pore size filters for sterilization, the pellet with the cells required for each hydrogel was resuspended into the t(HA-Gel) solution already containing the required amount of HRP. A drop of 100 µL was poured on the bottom of cell culture wells. Then, the required amount of H_2O_2 was added and the hydrogels were left until complete crosslinking (30 min approximately). 450 mL of biopsy cell culture medium were added in each well of a p48 well-plate. In addition, the control 2D was performed seeding human hepatocytes cultured in p24 well plates with a collagen/fibronectin coating at the same cell density 2×10^6 cells/mL on each well plate.

Culture medium was replaced every two days. Moreover, after the first 24 h of cell culture instead of simply including veal serum, it was mixed with Dexamethasone 10^{-7} M.

6.1.7. Viability test: live dead assay

As a measure of viability, hydrogels containing the cells were incubated with two fluorescent probes: Hoechst 33342 and propidium iodide. Hoechst is a permeable DNA intercalation agent and stains all the cells with an emission wavelength in blue and propidium iodide is a non-permeable stain which mark the DNA of dead cells with an emission wavelength in orange. To perform the staining, cells were incubated with biopsy medium containing Hoechst and propidium iodide in at 1.5 µg/mL for 30 min at 37 ºC in dark.

After incubation with fluorescent probes, cells were imaged using the INCELL 6000 Analyzer system (GE Healthcare, USA). In order to acquire enough cells (>500) for the analysis, 12 fields per well were imaged. The 20X objective was used to collect images for the distinct fluorescence channels. Dyes were excited and their fluorescence was monitored at the excitation and emission wavelengths with appropriate filter settings. The collected images were analysed using the INCELL analysis Workstation module that allows the simultaneous quantification of subcellular structures, which are stained by different fluorescent probes and measure the fluorescence intensity associated with predefined nuclear and cytoplasmic compartments.

After this, the image analysis starts with a background correction, which is always applied before quantifying the number of cells. In addition, cell nuclei are chosen as size reference, which is obtained through a Top-Hat algorithm where things bigger than the size reference are not taken into account for the analysis. The total number of cells is quantified using the Hoechst probe and a cell exclusion is performed with the Propidium probe to obtain viability.

Thus, data is provided through the software interface for calculate the cell viability percentage.

6.1.8. Functionality tests

It is necessary to test the most important hepatic specific functions to prove the influence of the environment and its potential for human hepatocytes cell culture. It was decided to evaluate: ureogenic capacity, secretion of proteins (albumin) and the metabolic activity of cytochromes.

- Functionality tests: ureogenic capacity

The ureogenic capacity was assessed by the exposure of human hepatocytes to NH_4^+ and analysing the amount of urea produced. After 1 and 7 days of cell culture the culture media was removed and 250 µL of a Hepes saline solution with a final concentration of 1 mM of calcium chloride and 2 mM of ammonium chloride were added to each well. Cells were incubated for 120 min at 37 ºC, and then, the medium was removed and centrifuged for 5 minutes at 5000 rpm to discard aggregates. Then, the cleaned supernatant was transferred to another Eppendorf and frozen at -20 ºC till the evaluation.

A standard curve with urea was prepared to quantify the results, following the kit instructions (Bioassys Kit: QuantiChrome). 1/5 of the standard (50 mg/dL) is diluted in H_2O to obtain a use solution of 0.1 mg/mL. By mixing different volumes of the use solution with water the urea calibration curve could be obtained. Final concentrations of this curve ranged between 0.00 and 4.00 mg/mL, as shown in (Table 7).

Table 7. Calibration curve proportions for urea assay

Volume	0 µL	5 µL	10 µL	20 µL	30 µL	40 µL
Urea 0.1 mg/mL	0.0 µg/mL	0.5 µg/mL	1 µg/mL	2 µg/mL	3 µg/mL	4 µg/mL
Deionized water	50 µL	45 µL	40 µL	30 µL	20 µL	10 µL

The absorbance of the different solutions containing urea was recorded to build the standard curve (Figure 14). The linear adjustment was used to quantify the samples (Equation 3).

$$[abs] = (0{,}06914 * [urea]) + 0{,}1805 \quad (3)$$

Equation 3. Experimental equation for urea calculation

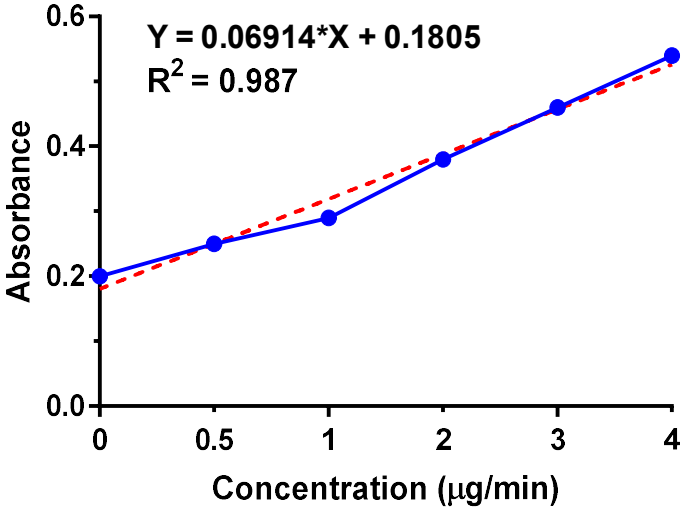

Figure 14. Urea standard curve to quantify ureogenesis.

The samples were thawed and centrifuged at 8000 rpm for 3 min and 50 µL of the supernatant were pipetted into a well of a p96 plate. For the blanks, the same volume was pipetted in other wells, consisting of NH_4Cl in saline Hepes that is the solution previously used for the incubation of the cultured hydrogels.

Two reagents A and B, non-specified composition, were used to produce a colorimetric change on the solution, by the reaction of the *o*-phthaladehyde/primaquine with urea (Jung et al., 1975; Zawada et al., 2009). A 1:1 solution of the reagents was prepared, taking into account that there is a 20 min window of stability of the mixture. 200 µL of this solution were added to each well, and kept at room temperature for 30 min, protecting the plate from light to avoid interference.

Samples were measured in the spectrometer at 520 nm, obtaining the absorbance. By using the standard curve and taking into account the time the reagents were in presence of the cells, we could calculate the ureogenic capacity of hepatocytes in µg/min.

- Immunohistochemistry: albumin expression

Immunohistochemistry allowed assessing albumin expression of cells within the hydrogel quantitatively. We obtained cryo-cuts from the hydrogels before the immune assay.

After the cell culture the media was removed and washed with dPBS (1X). 200 µL of formalin (4%) were added and samples were kept for 20 minutes under an extraction hood. After this fixing step, the formalin was removed, and hydrogels were washed twice with dPBS (1X). The samples can be sealed with parafilm in a mixture of dPBS (1X) and 0.02% azide and be used later.

The following step consists of immersing the samples in a sucrose solution (30%) overnight at 4 ºC (initially the samples can float, but they will eventually deposit). Then, the hydrogels were soaked in Optimal Cutting Temperature (OCT) medium and frozen at -80ºC. Cuts between 8 and 12 µm were performed on the cryostat (Leica CM 1950, Leica Biosystems, Germany) at -20 ºC, placed into glass slides and stored at -20 ºC.

The OCT was removed by washing once with formalin (4%) and twice with dPBS (1X). The perimeter of the sample was marked with a hydrophobic marker so that the antibodies could later be incorporated onto the slide without spillage. Triton (x100) at 0.5% dPBS was added for 20 min followed by 3 washings with dPBS (1X).

A blockade with BSA (3%) on dPBS (1X) was firstly performed to decrease the possible background staining due to non-specific antibody binding. It was incubated for 1 hour at room temperature and the BSA was removed. Primary albumin antibody (goat anti-human) diluted to 1% in dPBS/BSA was added and incubated overnight at 4 °C. The following day three washings were performed with PBS/BSA and Tween (0.1%), to remove the excess of primary antibody and to avoid protein-protein interactions that would cause background. Subsequently, secondary antibody (Donkey anti-goat 488) was added to the sample, diluted in dPBS/BSA at 1% and incubated in the dark at room temperature. It will interact with the goat-anti-human antigen attached to the albumin and give rise to fluorescence. Then, the secondary antibody was removed, washed with dPBS/BSA (1%) once, and incubated with Hoechst for 5 minutes to mark the DNA of human hepatocytes. Finally, each sample was washed twice with dPBS/BSA (1%) mixed with Tween (0.1%) and covered until imaging with the INCELL 6000 analyser device. 20x pictures of each staining were taken and mounted with Adobe Photoshop software.

- Functionality tests: albumin secretion

Protein synthesis and secretion is a specific hepatocyte function. We quantified the secretion in 3D structures and compared to 2D cell culture. ELISA (*Enzyme-Linked ImmunoSorbent Assay*) method was used following the protocol provided by the manufacturer, Bethyl Laboratories Inc.

First, 100 µL of the blank (dilution buffer) and the samples (culture medium) were added to the wells (run in duplicate). To prepare the standard solution and standard curve, 500 ng hAlbumin was reconstituted with 1 mL of 1X Dilution Buffer C, provided in the kit.

Table 8. Calibration curve proportions for human albumin assay

hAlbumin 500 ng/mL	500 ng/mL	167 ng/mL	55.6 ng/mL	18.5 ng/mL	6.17 ng/mL	2.06 ng/mL	0.69 ng/mL	0 ng/mL

After this, 7 Eppendorf were prepared, one for each standard curve, the solution represents the top point 500 ng/mL and the last tube which contains 300 µL of 1X Dilution Buffer C represents the blank. The middle points were obtained preparing serial dilutions 1:3 starting from the top (Table 8). Then, the plate was covered and incubated at room temperature for 1 h.

Subsequently, the adhesive cover was removed, and the plate washed four times with 1X Wash Buffer. Then, 100 µL of anti-albumin detection antibody were added to each well, properly mixed by tapping the well plate and covered again with adhesive for an incubation at room temperature for 1 h. After this, the cover was carefully removed, and the wells washed four times again with 1X Wash Buffer. Then, 100 µL of HRP solution A were added to samples and blank, and an adhesive cover was attached. Samples were incubated for 30 min at 20-25 ºC, and

then, washed four times with 1X Wash Buffer. Subsequently, 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and the enzymatic reaction was incubated at room temperature and for 30 min in the dark, turning the solution into blue. The plate was not covered during the incubation. To stop the reaction, 100 µL of Stop Solution was poured in each well and the plate tapped to mix, the blue colour changed from blue to yellow. After this, and within the next 30 min the absorbance was measured on a plate reader at 450 nm. Finally, the albumin secreted by the cells was quantified according the standard curve shown in Figure 15.

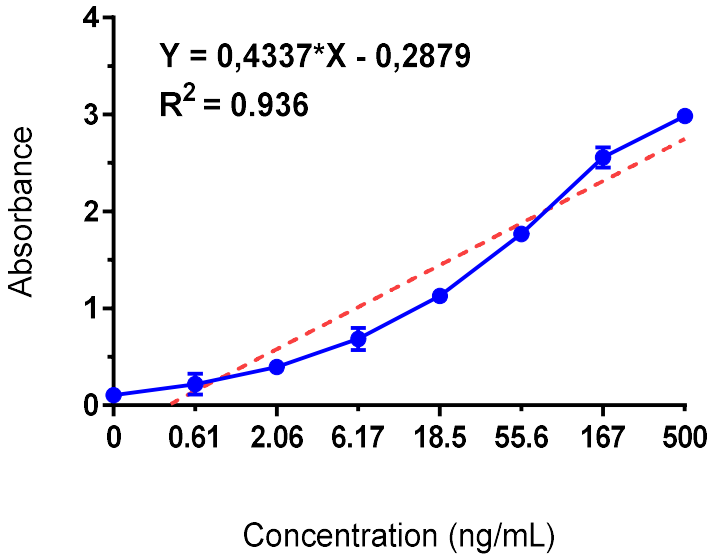

Figure 15. Example of the standard curve for human albumin assay.

- Functionality tests: cytochrome P450 (CYPs) activity

Primary hepatic cells can carry basic and advanced functions, but the metabolic activity is one of major ones. The evaluation of CYPs activities starts preparing a cocktail of 8 substrates of 8 different specific isoforms of CYPs (table 9). These substrates will be metabolised by each specific isoform in the cells, and the quantity of metabolites produced will be determined and are indicative of the activity of these CYPs. The cocktail was prepared according the concentrations presented in the protocol made by Lahoz (Lahoz et al., 2007).

Table 9. Cocktail of substrates to evaluate metabolic activity

CYP	Substrate	Final concentration	Metabolite
1A2	Phenacetin	10 µM	Acetaminophen
2A6	Coumarin	5 µM	7-H0-Coumarin
2B6	Bupropion	10 µM	H0-Bupropion

2C19	Mephenytoin	50 µM	4´-H0-Mephenytoin
2C9	Diclofenac	10 µM	4´-H0-Diclofenac
2D6	Bufararol	10 µM	Hydroxybufuralol
2E1	Chlorzoxazone	50 µM	6-H0-Closzoxazone
3A4	Midazolam	5 µM	1´-H0-Midazolam

First, each 5 µL of the cocktail of substrates was blended with 1 mL of Hepes saline, taking 200 µL to prepare a blank of AL4. After this, culture media is removed and 200 µL of the Hepes-AL4 mixture was poured, incubated for 20' and 120' at 37 ºC. Subsequently, each sample was collected at the time marked, then, it was centrifuged for 3 min at 5000 rpm. Finally, the samples were transferred to an Eppendorf and frozen at -80 ºC until evaluation. The final evaluation was carried by the Analytic Service from the IISLAFE, which used the Ultra Performance Liquid Chromatography-Mass Spectrometer (UPLC-MS/MS) methodology to assess the hydroxylation of the substrates which indicates the activity of Phase I enzymes. The activity for each specific isoform was referred as pmol of the metabolites produced in the period of incubation.

6.2. Part II: oxidised polysaccharide hydrogels

6.2.1. Reagents

Sodium hyaluronate (Mw ~1.2 MDa) was provided by Kraeber & Co GmbH (Germany). Sodium periodate ($NaIO_4 \geq 99.8\%$) was purchased from Sigma Aldrich (Germany). Spectra/Por® dialysis membrane (MWCO 3.5 kDa), sodium hydroxide (NaOH), hydrochloric acid (HCl) and potassium chloride (KCl) were obtained from Carl Roth GmbH & Co KG (Germany). Hydroxylammonium chloride was purchased from Merck KGaA (Germany). Dulbecco's Modified Eagle's Medium (DMEM) and Bicarbonate Buffer for cell culture were obtained from Lonza (Belgium). Fetal Bovine Serum (FBS) and trypsin were received from Biochrome AG (Germany). Penicillin-streptomycin-fungizone (PSF) was purchased from Promocell (Germany). 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) was bought from ThermoFisher (USA).

6.2.2. Synthesis of oHA

This second type of hydrogels is based on the use of oxidised hyaluronic acid and their ability to spontaneously react with amine groups. The oxidation process was carried out with sodium periodate that is able to open HA rings and give rise to two aldehyde groups, as observed in Figure 16. The protocol was adapted to hyaluronic acid, following the previous literature (Gomez 2007; Khorshidi, 2016).

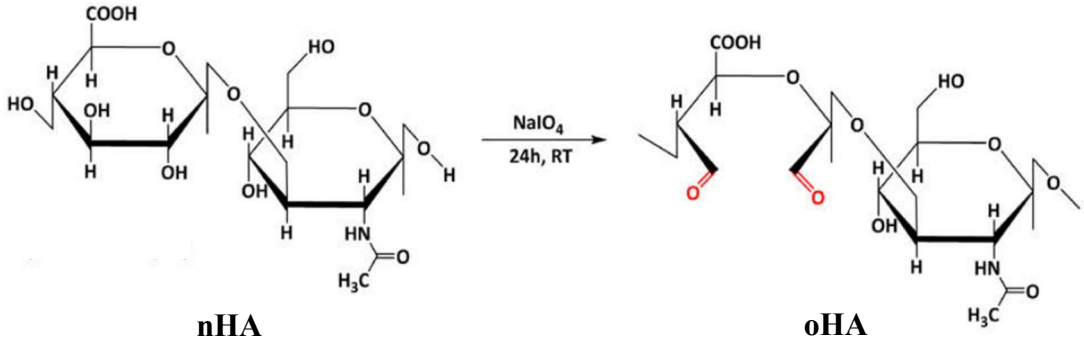

Figure 16. Scheme of the formation of oxidized hyaluronic acid (oHA) from non-oxidised hyaluronic acid (nHA) (Su et al., 2010)

Firstly, 1 g of hyaluronic acid was dissolved in 200 mL mQ water adding the hyaluronic acid slowly, avoiding the aggregation. Approximately this step took from 2 h to 3 h. After it was completely dissolved, 0.63 g of $NaIO_4$ were added. It is possible to obtain different oxidation degrees depending on the proportion of sodium periodate. In order to obtain a theoretical 100% oxidation degree, a percentage of 120% of $NaIO_4$ related to the amount of HA was added. The percentage means the moles of $NaIO_4$ that make react with the moles of HA. According to that we used 1.2 moles of $NaIO_4$ per 1 mol of HA. The solution was stirred for 72 h at room temperature. Subsequently, the oxidised HA (oHA) was dialysed for 3 days changing the water twice per day. It was freeze-dried for 24 h for further use.

6.2.3. Oxidation degree of oHA

Oxidation degree was characterized because it gives an indication of the susceptible crosslinking points the polysaccharide has. The most common procedure to obtain the oxidation degree is by titration. A solution of oHA is put in contact with hydroxylammonium chloride (NH_3OHCl) that reacts with the aldehyde groups of the oHA releasing HCl (as observed in Figure 16). The moles of HCl are equal to the moles of aldehyde groups. The titration consists in calculating the volume of a NaOH solution of known molarity necessary for the sample (oHA dissolved in NH_3OHCl) to reach the pH of a previously prepared blank.

$$R_2C{=}O + NH_3OH{\cdot}Cl \rightarrow R_2C{=}NOH + HCl{+}H_2O$$

Figure 17. Chemical reaction of hydroxylammonium chloride with oHA.

The blank was prepared by mixing 25 mL of mQ water with 20 mL of a 0.4 M aqueous solution of hydroxylammonium chloride. The pH of this solution was determined. The samples were prepared by dissolving 60 mg of oHA in 25 mL of mQ water and adjusting the pH to 7 with NaOH 0.01 M. Then 20 mL of hydroxylammonium chloride 0.4 M were poured and stirred for 3 h at room temperature. When this hydroxylammonium is poured into the sample, the solution has a lower pH than the blank due to the release of HCl, which is quantified in the following titration.

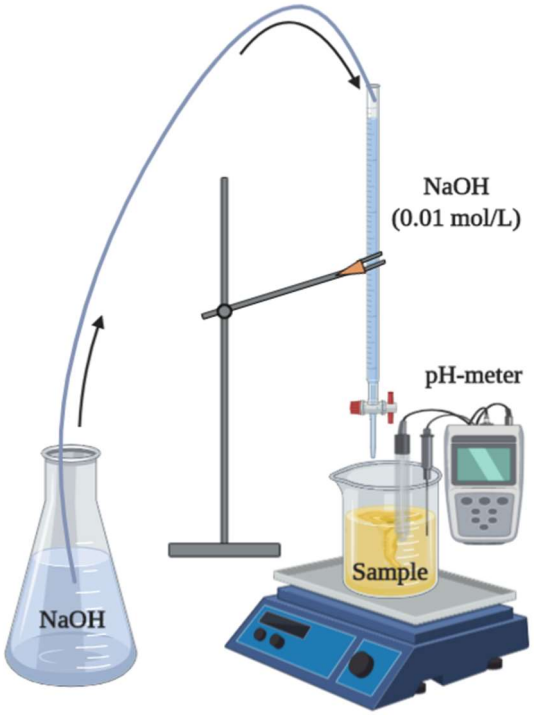

Figure 18. Scheme of titration procedure setup. Drawing made by the author of the Master Thesis

The lab equipment of Figure 18 was prepared for the titration, consisting of a pH-meter, a Pyrex glass containing the sample, a magnetic stirrer and a buret. The buret was cleaned with NaOH 0.01 M, because it is the reagent used to titrate the sample. The buret was filled with NaOH 0.01 M, which was slowly poured into the sample until the pH of the sample was equal to that of the blank. The volume of poured NaOH solution provided the moles of NaOH, that are equal to the moles of aldehyde groups within the samples, connected with the oxidation degree. A minimum of 3 samples per batch were measured.

The final calculation required to obtain the oxidation degree consists of dividing the experimental number of aldehyde groups by the absolute theoretical number of aldehyde groups (Equation 4). This last value stands the maximum number of aldehyde groups which can be present in the case of 100% oxidation degree, the CHO absolute theoretical concentration was previously calculated by the group of Prof. Groth.

$$Oxidation\ degree = \frac{Experimental\ CHO\ concentration\ (\frac{mol}{g})}{Absolute\ theoretical\ CHO\ concentration\ (\frac{mol}{g})} * 100 \quad (4)$$

Equation 4. Experimental oxidation degree equation.

6.2.4. Primary amino group quantification

Amino group content of gelatin is relevant for hydrogels formation because they need to be available to react with the aldehyde groups of oHA to achieve successful gelations. We

measured three types of type A gelatin from porcine skin (characterized to their bloom value) to select the best. The three different bloom values were 80, 175 and 300.

The method used to quantify gelatin amino groups is by the reaction of this groups with the compound 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) (Figure 19). When both components are blended, an orange colour starts to appear into the solution, measuring the absorbance of the mixture in a spectrophotometer and depending on the amino content the absorbance will change. The protocol used is from the company ThermoFisher Scientific.

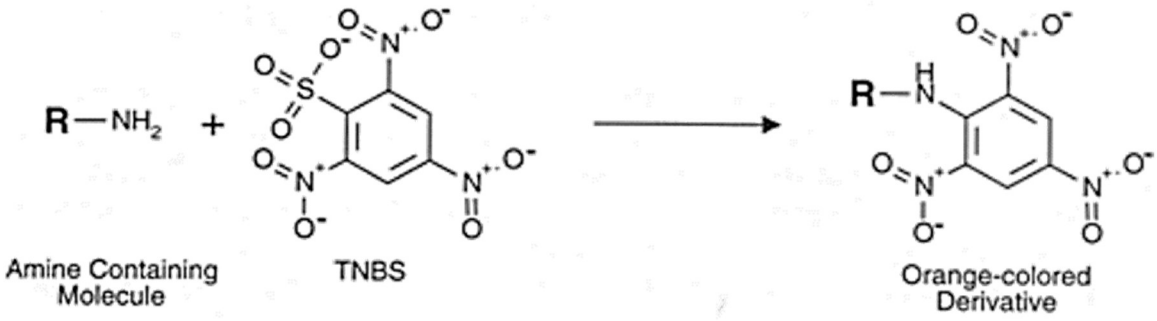

Figure 19. Chemical reaction of TNBSA with an amino group to produce coloured derivates. Image obtained from Sigma-Aldrich protocol.

All the dilutions were done with the same reaction buffer that would be used in the hydrogel formation, 0.1 M bicarbonate buffer. The calibration curve was done with lysine. Lysine was dissolved in bicarbonate buffer at different concentrations ranging from 2 µg/mL to 20 µg/mL. Gelatin was dissolved in the buffer, but with different range of concentrations, from 20 µg/mL to 200 µg/mL, due to the minor quantity of amino acids. The TNBSA reagent was prepared at 0.01% (w/v) in bicarbonate buffer.

To 0.5 mL of gelatin or calibration curve sample, 0.25 mL of TNBSA were poured and properly mixed. They were incubated for 2 h at 37 ºC. Then 0.25 of SDS at 10% (w/v) and 0.125 mL of 1 N HCl were added and the absorbance at 335 nm was measured on an ultraviolet/visible (UV/VIS) spectrophotometer (Specord200, Analytik Jena AG, Jena).

Three replicates per samples were measured.

6.2.5. Formation of hydrogels (oHA-Gel)

Before defining the concentrations of hydrogel precursor solutions and buffer, thanks to previous experimentation at different conditions (results not published) we could obtain the optimal conditions. Bloom 300 gelatin was chosen since the hydrogels were stiffer than lower Bloom values hydrogels. Then, 10% w/v polymer concentration was required to have enough aldehyde and amine groups to crosslink. The bicarbonate buffer was the solvent which conferred better crosslinking. In addition, the ratio oHA-Gel 1:3 was selected because in the literature free aldehyde groups from oxidised polysaccharides were reported as cytotoxic and was decided to increase the amount of gelatin to have enough amine groups to fill them.

The procedure started by dissolving oHA and Gel into the bicarbonate buffer (which is suitable for cell culture). Gel took 2 h for dissolving and oHA was left 24 h to dissolve. Whereas the hyaluronic acid was prepared at room temperature, gelatin was dissolved at 37ºC.

Hydrogels of 100 µL were prepared. After joining both solutions they were mixed with the tip of the micropipette to ensure hydrogel homogeneity. Hydrogel drops (Figure 20) were left for 30 min to 60 min for crosslinking at 37ºC avoiding the physical linkage which happens at room temperature.

Figure 20. Example of oHA-Gel hydrogels (1:3 ratio) synthesized with bicarbonate buffer and Bloom 300 gelatin.

6.2.6. Physical characterisation

- Equilibrium water content

The oHA-Gel hydrogels were characterised by the same procedure as tyramine conjugates. We calculated the mass of water per unit mass of polymer (in %) and the equilibrium water content (in %), using the equations 1 and 2of section 6.1.5.

Hydrogels were prepared on a hydrophobic surface and after 1 h of crosslinking they were transferred to a p24 well-plate and 500 µL of dPBS were added to swell them. After 24 h of swelling they were weighed (w_s) and weighed again after a freeze-drying step to obtain their dry weight (w_d).

- Gel content

Gel content is defined as the crosslinked mass remaining after swelling, assuming that non crosslinked molecules will be released to the swelling medium during the first 24 h of swelling provoking a weight loss.

Hydrogels were prepared on a hydrophobic surface for 1 h. Then, they were freeze-dried and weighted, to determine the initial dry weight (w_{di}). After that, the hydrogels were immersed in dPBS for 24 h and freeze-dried again, to determine the final dry weight (w_{df}). The following equation allows determining the gel content (equation 5)

$$Gel\ content = \frac{w_{df}}{w_{di}} \times 100 \quad (5)$$

Equation 5. Gel content definition

- Stability test

Hydrogels stability was evaluated in culture media with different proportions of fetal bovine serum (FBS). 2 replicates per proportion of FBS (0%, 1%, 5% and 10%) were prepared.

Hydrogels were prepared in bicarbonate buffer on top of a hydrophobic surface and left to crosslink for 1 h. Then, culture media (with and without FBS) was added. The volume used to cover the hydrogels was 500 µL.

The stability of the hydrogels at different time points was evaluated by tilting the hydrogels with a spatula once they were taken off the plate.

6.2.7. Cell culture in oHA-Gel hydrogels

The non-cytotoxicity of hydrogels was assessed by the encapsulation of fibroblasts. For this, gelatin (bloom 300) and oHA were dissolved in bicarbonate buffer (pH 8.1) at 100 mg/mL and kept warm at 37 ºC. Both components were filtrated through filters with a pores size from 0.22 µm to 0.45 µm. Taking into account that highly concentrated gelatin is very viscous, all the equipment (syringe and filters) were heated to 37 ºC in order to get a lower viscosity and facilitate the filtration.

The cells were detached form the culturing flasks by adding 1 mL of trypsin and incubating for 5 min at 37ºC. Then, were added 3ml of F12 cell culture media inactivating the trypsin. Thereafter, fibroblasts were transferred into an Eppendorf and centrifuging for 3 minutes at 250 g, the supernatant was removed, and the cell pellet was transferred into a Neubauer chamber to select cell density. Each hydrogel was seeded with 50.000 cells (cell density 0.5×10^6 cells/mL), the cell pellet was resuspended in the gelatin solution because oHA can produce cytotoxicity due to the free aldehyde groups. Cells could not be resuspended in the oHA solution to avoid a possible cytotoxic effect of the aldehyde groups. Both components were mixed with a tip and gelation was left for 1 h. After that time, the culture media was added.

Results were compared to hydrogels made of gelatin crosslinked with transglutaminase (mTG), as a positive control of biocompatibility (Gel-TG). This system has been widely characterised and used for tissue engineering as platform for breast carcinoma modelling (Fang et al., 2014), as a promising *in vitro* environment for cardiac cell culture (McCain et al., 2014) and as bioprinting ink for smooth muscle cell culture (Tijore et al., 2018). The hydrogels were composed by 98.75 µL of a 10 % w/v solution of gelatin dissolved in bicarbonate buffer were mixed with 1.25 µL of a 10% solution of transglutaminase dissolved in dPBS (Alarake et al., 2017).

6.2.8. Viability test: CFDA staining

Oxidised polysaccharides have a drawback due to the cytotoxicity produced by the free aldehyde groups. Therefore, a cell viability study was carried out to confirm the suitability of hydrogels for cell culture and see if the crosslink does not let any free aldehyde group. Being

one of the most well-known fluorometric methods to assay life-dead on cells, 5(6)-carboxyfluoresceine diacetate (CFDA) was used to mark alive cells in green and To-Pro 3 to stain the nuclei of dead cells in blue. This assessment was performed at 1 day and 7 days of cell culture also comparing between Gel-TG and oHA-Gel.

First, cell culture medium was removed, and a mixture of CFDA (1:200) and to-pro (1:2000) in colourless DMEM (to avoid phenol red interferences) was added to cover all the hydrogels. Hydrogels were incubated for 10 mins at 37 ºC. After this, the staining solution was removed, and the hydrogels were covered again with DMEM colourless. Two pictures for each hydrogel were visualised with a fluorescence microscope (Axiovert 100, 488 nm excitation and 530 nm emission filters, Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with a CCD camera (Sony, MC-3254, AVT-Horn, Aalen, Germany) and image analysing software KS300 (Carl Zeiss, Oberkochen, Germany). At least 2 images per sample using a 10x objective were taken.

6.2.9. Functionality test: metabolic activity by Q-Blue test

Q-Blue reagent was used to assess the metabolic performance through the conversion of the redox dye resazurin, which is not fluorescent, into a highly fluorescent product called resorufin. Then, after 1 day of cell culture the culture media was removed and Q-Blue reagent at 10% in DMEM colourless was added.

Usually, incubation takes 2 h at 37 ºC, but, since the cell culture is made in hydrogels, we left it for 5 h. After the incubation, the supernatant that had turned blue was removed, placed into a p96 black well-plate, and the fluorescence was read with the ultraviolet/visible (UV/VIS) spectrophotometer (Specord200, Analytik Jena AG, Jena, Germany), setting 544 nm of excitation wavelength and 590 nm of emission wavelength. In addition, a blank of Q-Blue in DMEM without phenol red was prepared to validate the experiment. 3 samples for oHA-Gel hydrogels and 2 for Gel-TG were prepared.

7. RESULTS AND DISCUSSION

The Master Thesis had the purpose of comparing two different ways to synthesize hydrogels, for their use on hepatic cell therapy and hepatic cell culture. However, the current situation due to the global pandemic since March made impossible to continue and suddenly stopped all the experiments. Because of this, the results here presented are from the work done till that date. Firstly, t(HA-Gel) hydrogels were characterized by their swelling, ability to encapsulate primary human hepatocytes and support hepatocyte functionality. Second, the novel system based on the reaction of aldehyde and amine groups, oHA-Gel, has been studied in terms of hydrogel synthesis, swelling and ability to encapsulate human fibroblasts keeping them alive.

7.1. Part I: tyramine crosslinked hydrogels

7.1.1. Physical characterisation

We determined the mass of water per mass of dry polymer (*w*) and the equilibrium water content (EWC) of the hydrogels after 24 h of immersion in dPBS.

- Hydrogel swelling

The water uptake of hydrogels can be expressed as *w* or EWC. *w* is the mass fraction of water within the hydrogel referred to the mass of dry polymer and EWC is the mass fraction of water within the hydrogel referred to the total mass of hydrogel (water and dry polymer). The values obtained are presented in the following table (Table 11).

Table 10. Values for *w* and EWC of t(HA-Gel) hydrogels after 1 day of swelling in dPBS.

	w	**EWC**
Sample 1	5690 %	98.3 %
Sample 2	5693 %	98.3 %
Sample 3	4023 %	97.5 %
Sample 4	3642 %	97.3 %
Sample 5	3437 %	97.1 %
Average	4497 ± 1110%	97.7 ± 0.5%

The results of *w* were compared with those obtained by (Sanmartín-Masiá et al., 2017), where different compositions of t(HA-Gel) where characterised. Hydrogels had the same polymer concentration of 2%, but the ratio between HA and Gel was slightly different. In our study it was 80-20, while in the hydrogels made by Sanmartín-Masià it was 70-30. Despite the differences in composition our results are very similar to those previously obtained, 4497 ± 1110% compared with 5774 ± 553%. This allows us to confirm the correct synthesis of hydrogels as well as the consistency on the swelling.

7.1.2. Viability test: live dead assay

During my Bachelor Thesis I worked with a hepatic cell line, HepG2, to choose the optimal composition of hydrogels for liver tissue engineering. In this Master Thesis, we pretend to advance and get closer to the clinical application, by the culture of primary human hepatocytes that are more delicate than cell lines. Two crosslinking buffers were used to encapsulate the cells, F12 cell culture media and calcium-free Krebs ringer buffer (CF-KRB).

Live/dead pictures in Figure 21 show the results after 1 day of culture in which dead cells are marked in orange/red and all the cells are stained in blue. Apparently, it seems that more dead cells are found in the hydrogels prepared in CF-KRB, i.e. F12 produces a lower damage to human hepatocytes. This result was confirmed after 7 days of culture, as seen in Figure 22.

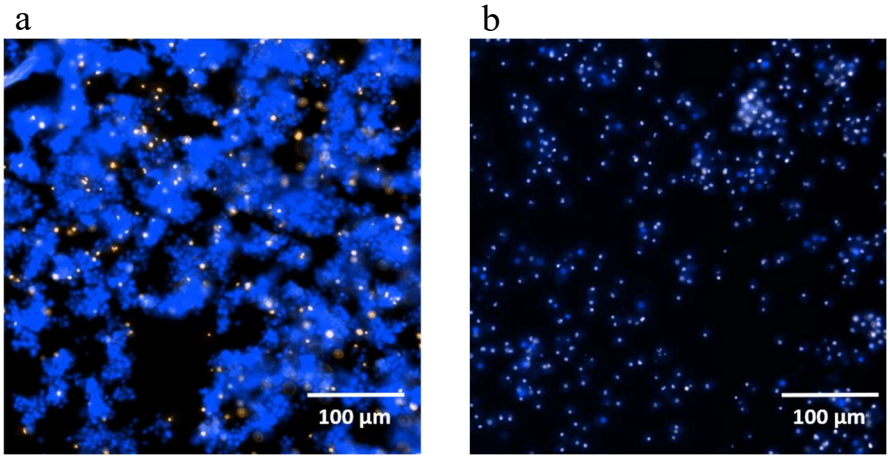

Figure 21. Representative pictures (20x) of live/dead assay on human hepatocytes cultured within t(HA-Gel) hydrogels in p48 non treated well plates with biopsy medium. a) F12 medium t(HA-Gel) hydrogel after 1 day. b) CF-KRB t(HA-Gel) hydrogel after 1 day

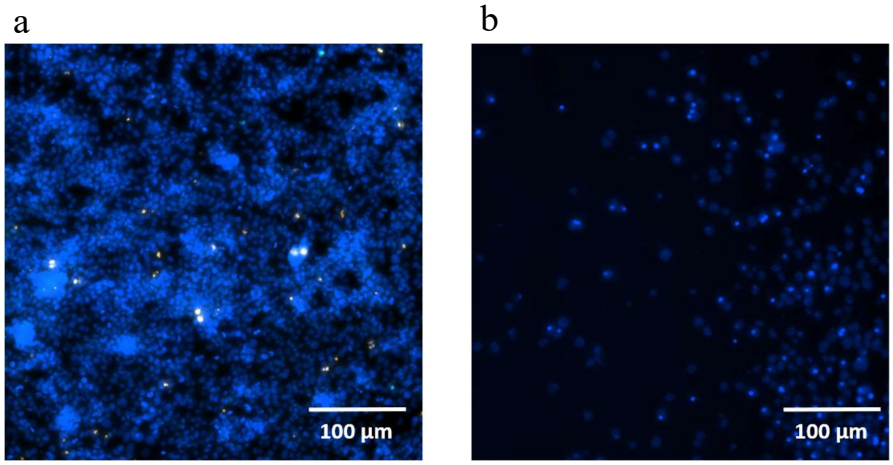

Figure 22. Representative pictures (20x) of live/dead assay on human hepatocytes cultured within t(HA-Gel) hydrogels in p48 non treated well plates with biopsy medium. a) F12 medium t(HA-Gel) hydrogel after 7 days. b) CF-KRB t(HA-Gel) hydrogel after 7 days

The image analysis performed with the INCELL Workstation, allowed identifying single cells and through the use of intensity algorithms the proportion of alive and dead cells. The data confirmed the observations of the representative images (Figure 23). After 1 day the viability

was higher on F12 hydrogels with a 75% of viability whereas in the CF-KRB hydrogels the viability was 23%. After 7 days, the viability in the CF-KRB hydrogels was very reduced (0.5%) while in the F12 hydrogels it was still quite high, 67%. It is worth noting that this is a good result, as human hepatocytes have low viability from the beginning of the experiment due to the stress they suffer during cryopreservation and thawing before cell culture (Stéphenne et al., 2010). Viability test allowed concluding that F12 culture can be used for the encapsulation of human hepatocytes within the t(HA-Gel) despite the crosslinking degree is not as high as in the CF-KRB (Rodriguez-Fernandez et al., 2018; Vaca-González et al., 2020). The statistics showed high statistical differences in the cell viability between CF-KRB and F12 hydrogels either at 1 day or 7 days of cell culture (p-value < 0.0001).

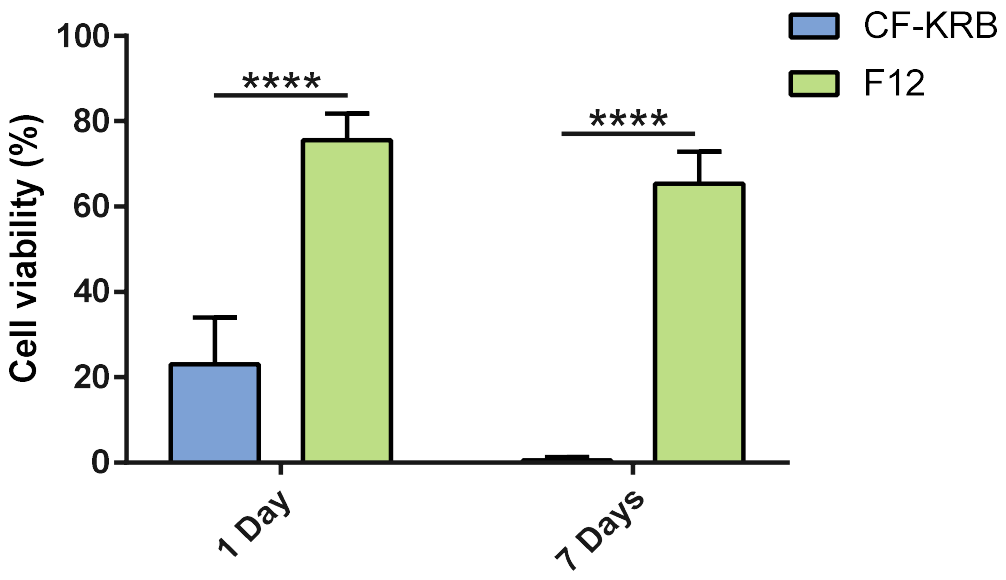

Figure 23. Human hepatocyte viability graph comparing t(HA-Gel) hydrogels synthesized with CF-KRB and F12 culture medium after 1 day and 7 days of cell culture with biopsy medium in p48 non-treated well plates.

7.1.3. Functionality test: ureogenic capacity

A major function of the liver is metabolism of ammonia into urea. Measurement of urea synthesis is another important quality assay, particularly in cases of HCT for patients with urea cycle disorders (Horslen et al., 2003). After the addition of ammonia to hepatocytes in culture or suspension, the formation of urea can be measured (Donato et al., 2008). The ureogenic capacity of the human hepatocytes was assessed by an exposure of them to a mixture which contains ammonium (NH_4^+). The aim of this test is to observe the capacity of the cells to transform the ammonium to urea. (Figure 24).

Hepatocytes within the Gel-HA hydrogels showed a higher ureogenic capacity (2.89 µg/min) than those in cultured in monolayer (2.23 µg /min) after 1 day of culture. Monolayer culture has no results after 7 days of culture due to low viability of hepatocytes in monolayer over long periods. Even though, the ureogenic capacity was higher after 7 days with 3.34 µg/min than after 1 day of culture. Even though, the ureogenic capacity was even higher after 7 days with 3.34 µg/min . Thus, confirming our hypothesis that the 3D environment in which the cell-cell

and cell-matrix interactions are recreated provides better conditions to keep hepatocyte functionality.

Firstly, the homoscedasticity was analysed with a F-test (Fisher) to assess the statistical differences on the standard deviation and no differences were found. Then, an ANOVA test was applied to compare the means by the Post-Tukey method, finding statistical differences between all de data with different grade of significance (p-value = 0.05).

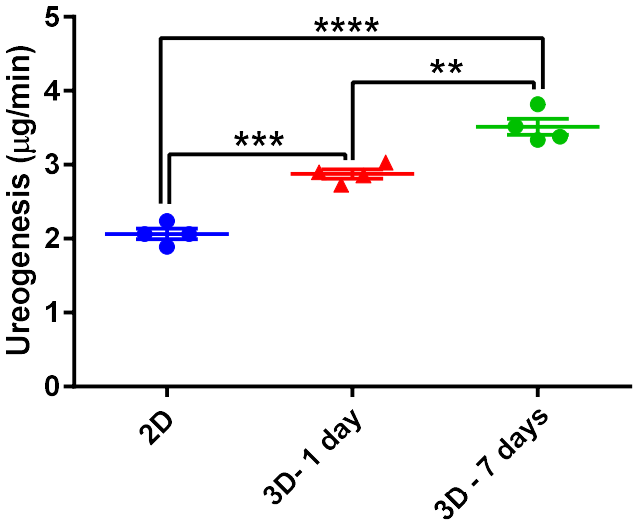

Figure 24. Graphic representation of human hepatocyte ureogenic capacity on 2D and 3D t(HA-Gel) cell culture after 1 day and 7 days of cell culture with biopsy medium. Hydrogels were made on a p48 non-treated well plate and 2D was done in p24 well plate coated with collagen/fibronectin.

7.1.4. Immunohistochemistry: albumin expression

In order to assess the maintenance of the hepatic phenotype after culture in Gel-Ha hydrogels, the expression of albumin, a hepatocyte marker, was determined by means of immunofluorescence.

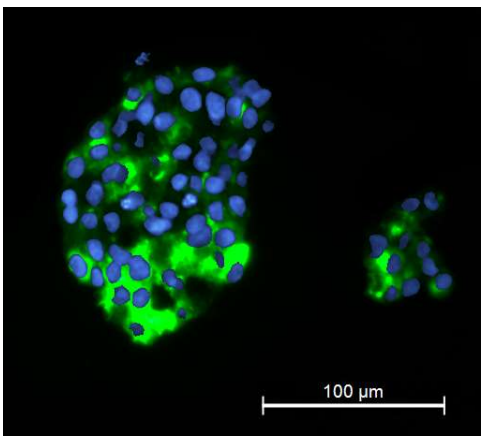

Figure 25. Immunohistochemistry picture for albumin expression of human hepatocytes in cryocuts of t(HA-Gel) hydrogels after 1 day of cell culture with biopsy medium. Alive cells nuclei marked with Hoechst (blue) and albumin marked by ELISA assay (green).

The picture from above (Figure 25) shows that the cells expressed albumin. Not being a quantitative analysis, the result is quite satisfactory since apart from confirming albumin expression the picture shows how cells organize in clusters within the hydrogels, resembling the situation within the tissue.

7.1.5. Functionality test: albumin secretion

Protein synthesis and secretion is one of the specific hepatocyte functions. Once the albumin expression by the human hepatocytes was demonstrated by immunohistochemistry, we quantified the production and secretion albumin in the 3D hydrogels and compared the results with the cells cultured in monolayer. The protocol is explained in section 6.1.9 and is based on the use of an ELISA assay to quantify the albumin secreted. The results have been represented in the following graph, again comparing 2D cell culture between 3D cell culture (Figure 26). The results show better outcomes in hepatocytes cultured on 3D environments confirming the ureogenesis results that showed increased hepatocyte functionality of the cells within the hydrogels.

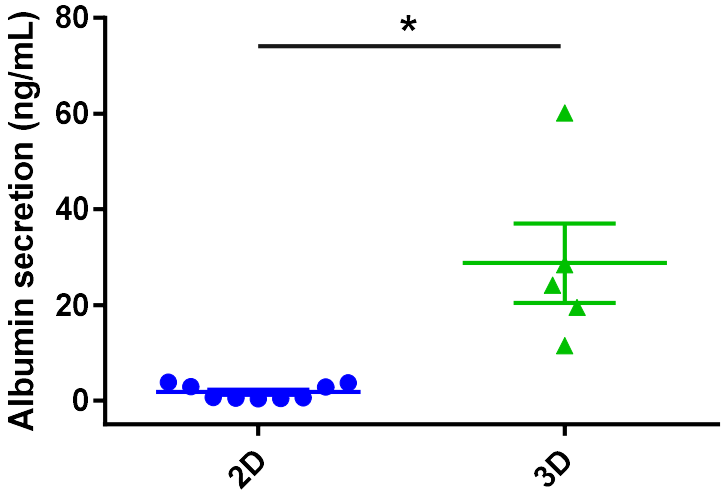

Figure 26. Graphic representation of human hepatocyte albumin secretion in 2D and 3D t(HA-Gel) cell culture after 1 day of cell culture with biopsy medium Hydrogels were made on a p48 non-treated well plate and 2D was done in p24 well plate coated with collagen/fibronectin.

The statistical analysis was performed slightly different since statistical differences were found on the standard deviation. A T-student with the Welch's correction was applied due to the non-sensibility to the variance and significant differences were observed on the means (p-value = 0.03).

7.1.6. Functionality test: CYP's activity

Finally, the drug metabolic capacity of cultured hepatocytes was determined. Assessment of the drug-metabolising function seems to be of special interest for the clinical setting of HCT in which recipients receive extensive medication. P450 enzymes, and particularly CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4, are mostly responsible for the oxidative metabolism of most drugs in clinical use (Lammert et al., 2010). The assessment of P450 activities was notably

simplified by the application of mass spectrophotometry (Lahoz et al., 2007). The method is based on the incubation with a combination of substrates that are specifically metabolised by CYP enzymes, producing a metabolite. These specific metabolites are determined by liquid chromatography tandem mass spectrometry (Figure 27).

Once the specific results for each cytochrome were obtained, a statistical analysis of student t was applied as in the previous results for comparison of means. However, homoscedasticity was taken into account which was not met in the first two cytochromes CYP1A2 and CYP2C19.

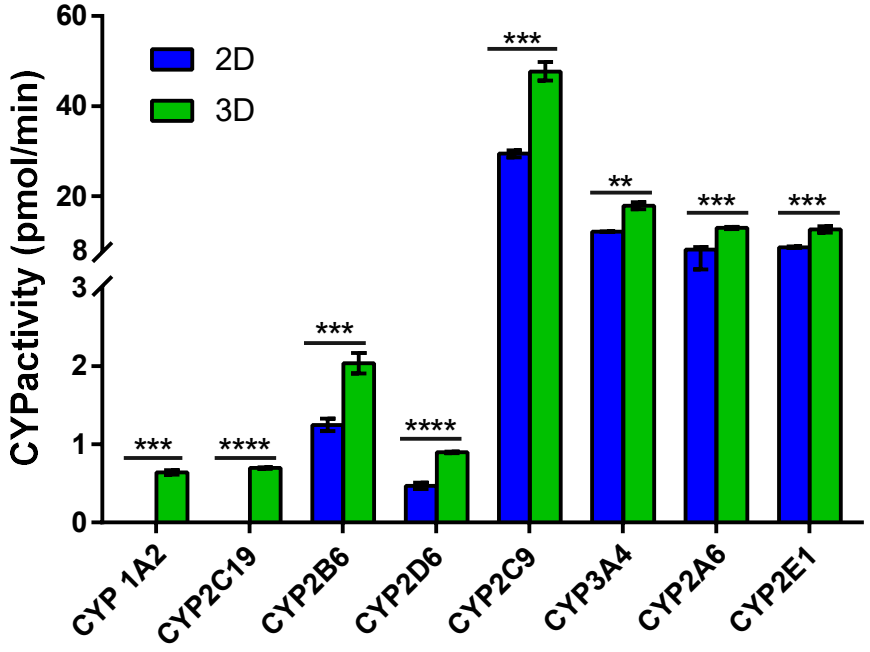

Figure 27. Metabolic quantification of human hepatocyte CYP's activity on t(HA-Gel) hydrogels after 1 day of cell culture with biopsy medium Hydrogels were made on a p48 non-treated well plate and 2D was done in p24 well plate coated with collagen/fibronectin.

This is due to the fact that activities in 2D were undetectable, so the student t was corrected by the Welch test. Significant differences were found in all of them with different degrees of significance. The conclusion to be drawn from the results is the difference observed in three-dimensional cultures with respect to conventional single-layer cultures. All this indicates that the 3D configuration of hepatocytes conferred by the hydrogel and the biomimetic composition of the hydrogel stimulated hepatic functionality and improved the inherent metabolism of human hepatocytes.

7.2. Part II: oxidised polysaccharide hydrogels

7.2.1. Oxidation degree of oHA

The oxidation degree from oHA was measured in order to know the quantity of aldehyde groups available for the crosslink with the amino groups. After titrating the three samples of each batch, the moles of aldehyde groups were obtained. The value was calculated from the volume needed

to titrate the samples till the original pH. Then referring the number of moles per milliliter to the sample mass, the moles per gram were obtained. Finally, to obtain the oxidation degree, the moles per gram of aldehyde groups was divided by the absolute theoretical number of moles that could be in the molecule. The next table (Table 12) summarizes the experimental values obtained for each sample from two batches.

Table 11. Aldehyde content for oHA obtained by titration with hydroxylammonium chloride

	NaOH vol	[CHO] (mol/mL)	[CHO] (mol/g)
Batch 1 – Nº1	12.9	$1.29 * 10^{-4}$	$2.22 * 10^{-3}$
Batch 1 – Nº2	13.3	$1.33 * 10^{-4}$	$2.12 * 10^{-3}$
Batch 1 – Nº3	12.5	$1.25 * 10^{-4}$	$2.08 * 10^{-3}$
Batch 2 – Nº1	13.9	$1.39 * 10^{-4}$	$2.31 * 10^{-3}$
Batch 2 – Nº2	12.7	$1.27 * 10^{-4}$	$2.11 * 10^{-3}$
Batch 2 – Nº3	12.9	$1.29 * 10^{-4}$	$2.14 * 10^{-3}$

Subsequently, after calculating the experimental aldehyde content, applying equation 5 and averaging three samples, the degree of oxidation of each batch was obtained (Table 13).

Table 12. Oxidation degree for each batch of oHA

	Batch Nº1	Batch Nº2
Sample Nº1	42.5%	44.2%
Sample Nº2	40.6 %	40.4%
Sample Nº3	39.9 %	40.9 %
Average	41 ± 1 %	42 ± 2%

Oxidized polysaccharides have been widely characterized; the results obtained are satisfactory since similar results to those of other authors were obtained. Weng in 2007 described the oHA for self-crosslinkable hydrogels with gelatin, obtaining a 44% oxidation degree as the highest value with an unspecified ratio (Weng et al., 2007). Then, Su in 2011 using a similar molar ratio HA-$NaIO_4$ (1:1) obtained 44% oxidation degree (Su et al., 2011). Finally, Li in 2014 has synthesized oHA with lower ratios (3:5) for injectable hydrogels with chitosan. He achieved a maximum oxidation degree for HA of 34% that is close to our results (Li et al., 2014).

7.2.2. Primary amine groups quantification

The aim of this assay was to quantify the free amino groups from the different gelatin types in terms of Bloom value. After performing the procedure explained at 6.2.4, the results showed

and inversely relation between bloom values and amino content. The values were gathered in the following table (Table 14).

Table 13. Free amino group content value for each type of gelatin assessed by TNBSA reagent

	Amino content [mol/g]
Bloom value 80	$8.71 * 10^{-3}$
Bloom value 175	$8.06 * 10^{-3}$
Bloom value 300	$7.29 * 10^{-3}$

The higher the bloom values the larger and more complex the gelatin molecules are (Eysturskarð, 2010). Lower Bloom values provided more amino groups available to be bonded with aldehyde groups and backwards. Even though there are other factors which affect the free amino groups available such as the pH, ionic strength or the nature of the macromolecule itself. The aim of this assay was to evidence the best type of gelatin for the crosslinking with oHA. Despite that lower values show higher concentration of free amino groups, gelation experiments demonstrated very poor consistency and mechanical properties of these hydrogels although the gelation was carried to be fully chemical crosslinking (Figure 28). That is why we decided to use Bloom 300 gelatin for the following experiments because despite the lower number of amino groups, the mechanical consistency was higher, probably due to the conformation of gelatin.

Figure 28. oHA-Gel hydrogels synthesized with bicarbonate buffer, ratio 1:3 (oHA-Gel) and made with different gelatin Bloom values (80, 175 and 300). Crosslinking time 1h at 37°C.

7.2.3. Physical characterisation

- Hydrogel swelling

In the same way as above in section 7.1.1, the physical characterisation consisted of the calculus of the *w* and EWC parameters. The procedure is explained in section 6.2.6 and the hydrogels where under freeze-dry and swelling steps. The *w* and EWC values are listed in the next table (Table 15)

Table 14. Values for w and EWC of oHA-Gel hydrogels after 1 day of swelling in dPBS

	w	**EWC**
Sample 1	1898 %	94.9 %
Sample 2	2132 %	95.5 %

Sample 3	2673 %	96.4 %
Sample 4	2643 %	96.3 %
Average	2337 ± 383%	96 ± 0.7%

The mass fraction of water within the hydrogels referred to the mass of dry polymer (*w*) are quite low compared to those obtained for the t(HA-Gel) hydrogels, either ours or those from other authors. One explanation for this is that we used very concentrated hydrogel solutions to prepare the samples, in fact the concentration for t(HA-Gel) was 2% and in the case of oHA-Gel we used 10%. Consequently, the hydrogels were stiffer and with lower water uptake.

- Gel content

Complete hydrogel cross-linking is rarely obtained, and some hydrogel molecules are lost when the hydrogel is immersed in water after the crosslinking. We decided to calculate the mass that had been cross-linked in oHA-Gel as a measure of cross-linking efficiency. The results obtained, calculated as explained in section 6.2.6, are compiled in table (Table 16). We observe that about 76% of the mass is effectively crosslinked and the rest of molecules are released from the hydrogel after 24 h of immersion in the buffer.

Table 15. Values of gel content for oHA-Gel hydrogels after 1 day of swelling in dPBS

	Gel content
Sample 1	75.3 %
Sample 2	75.6 %
Sample 3	79.8 %
Sample 4	72.2 %
Average	75.7 ± 3%

- Stability test

The oHA-Gel hydrogels are quite novel and are not fully characterized. For instance, their stability in cell culture conditions is still in doubt. That is why we decided to submerge the hydrogels in F12 cell culture media with different concentrations of FBS (1%, 5% and 10%), conditions that are frequent in cell culture experiments. A control without FBS was used for comparison (see section 6.2.6). Hydrogels were observed daily, and it was checked their integrity for 7 days. FBS concentration affected on the hydrogel's stability. Hydrogels that were soaked with high amounts of FBS were dissolved after 4 days and 5 days with FBS at 10% and 5%, respectively. On the other hand, lower concentrations of FBS (control and 1%) allowed the hydrogels to keep their integrity. In addition, between oHA and gel chain there is formed an imine bond which its equilibrium can be affected by the concentration, pH, temperature and others like steric or electronic factors. This equilibrium can be driven into the reverse direction

undoing the imine bond, the main reaction is the hydrolysis where a proton acceptor like OH^- can pull an electron form the C=N link and undo imine bond (Belowichet al., 2012). Then, it is believed that the integrity is affected by either the FBS proteins which can interact with the free aldehyde groups, the pH which can unbalance the equilibrium or by hydrolysis of imine bonds.

The stability was assessed taking off the hydrogels from the plates with a metallic spatula and tilting it, the pictures are representative of what a stable hydrogel was considered (Figure 29).

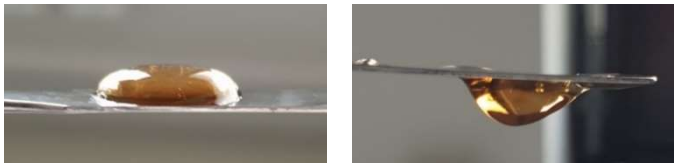

Figure 29. Representative image of oHA-Gel stable hydrogels after 7 days of soaking in F12 culture media with 1% of FBS in non-treated p24 non-treated well plates.

7.2.4. Viability test: CFDA staining

Once the hydrogels had been seeded with the human fibroblasts, we studied the viability of cells as explained in section 6.2.8. We obtained very promising results. The following images were obtained from a confocal microscope which has the proper module to observe fluorescent staining (alive cells are in green and dead cells in blue).

After 1 day of cell culture we observe differences between the cells in our hydrogel and a control of pure gelatin (Figure 30). In the control of Gel-TG more cells were found and a bit elongated. Which means a better affinity for the environment in terms of cell-matrix interactions. On the other hand, a lower number of cells in total were observed in oHA-Gel, maybe due to the gelation time which is higher than in the Gel-TG hydrogels. The longer gelation time would allow the cells to migrate out of the hydrogel during crosslinking

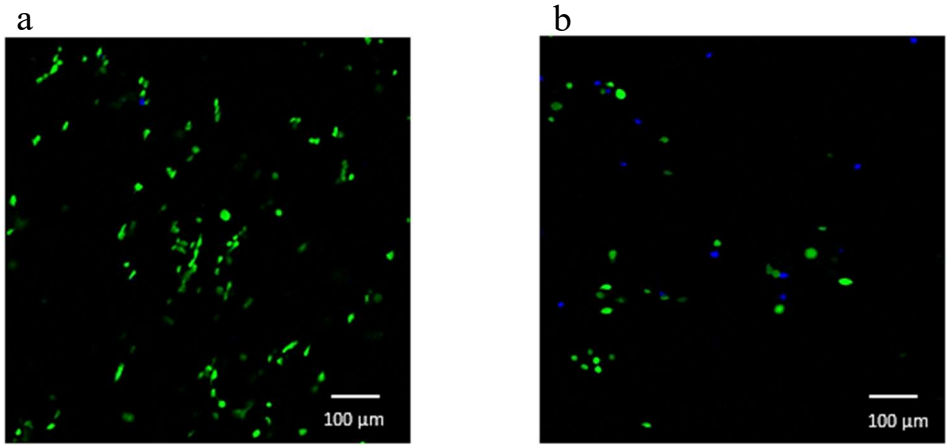

Figure 30. Representative pictures (10x) of human fibroblast culture on Gel-TG control hydrogel and oHA-Gel hydrogels in p24 non-treated well plates with F12 culture medium (1% FBS). Alive cells marked with CFDA (green) and dead cells with TO-PRO 3 (blue) A) Gel-TG after 1-day b) oHA-Gel after 1-day

The positive results are that most of the cells are alive inside the oHA-Gel hydrogels being the population of dead cells scarce, which confirms that the crosslinking in the presence of cells is not cytotoxicity. We repeated the experiment after 7 days of culture and very good results were obtained (Figure 30). The cell culture after 7 days showed the good outcome on the control of

Gel-TG hydrogel since this system was used before for cell culture (Figure 31), no dead cells were observed within the hydrogels. oHA-Gel hydrogel promoted cell spreading and human fibroblasts had elongated shape. However, there was considered rare the fact that fibroblasts were more elongated in oHA-Gel hydrogels and firstly was explained by the cell migration and attachment to the bottom of the well. But it was confirmed that the fibroblasts remained within the hydrogel, the adjacent substrate was checked and the area where the cells were located was scanned in the Z-axis to confirm its 3D configuration.

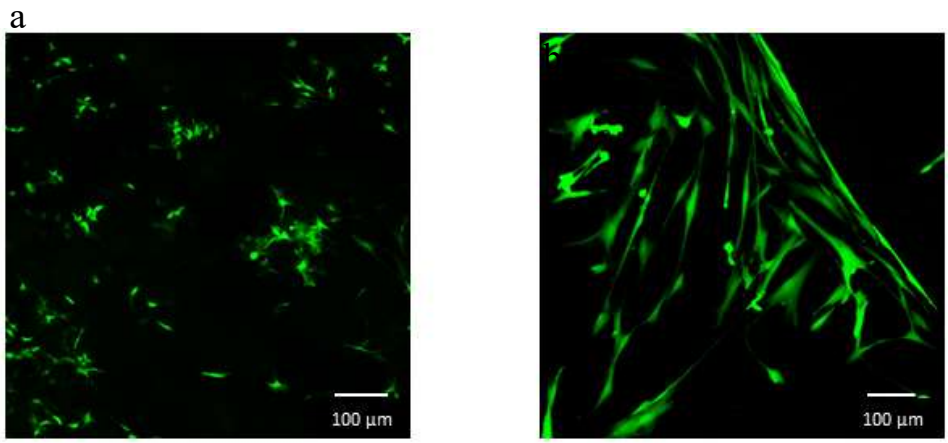

Figure 31. Representative pictures of human fibroblast culture on Gel-TG and oHA-Gel hydrogels in p24 non-treated well plates with F12 culture medium (1% FBS). Alive cells marked with CFDA (green) and dead cells with TO-PRO 3 (blue). a) Gel-TG after 7-days b) oHA-Gel after 7-days

7.2.5. Metabolic test: Q-Blue

The metabolic assessment was performed with Q-Blue colorimetric test, as explained in section 6.2.9. Only two replicates could be used for Gel-TG hydrogels due to experimental problems, for the oHA-Gel three samples were used. The results obtained from the spectrophotometer were corrected from Q-blue reagent blanks. The graphic (Figure 32) summarize the results indicating the quantification of the metabolic activity referred to the control Gel-TG.

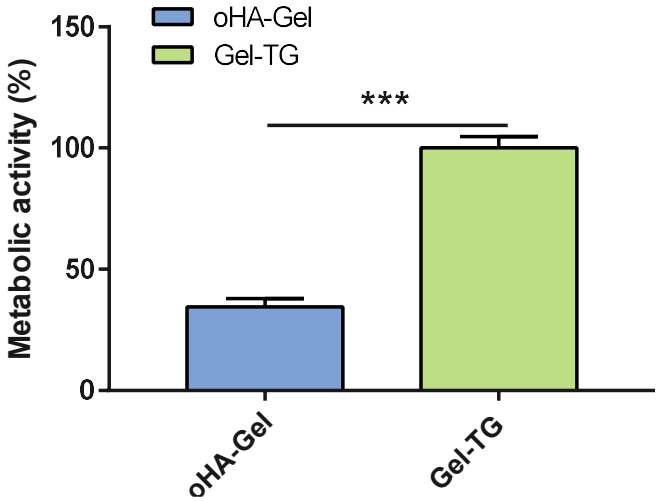

Figure 32. Graph for metabolic activity of the cells after 1 day within the oHA-Gel hydrogels and within a positive control of pure gelatin (Gel-TG). Both cultured in a p24 non-treated well plate with F12 culture medium (1% FBS).

The Gel-TG had the better outcome with a really high metabolic performance compared with oHA-Gel hydrogels, which is normal for a positive control as Gel-TG. Despite that, the cells in the oHA-Gel still demonstrated a good level of metabolic activity. This result opens the possibility to explore these hydrogels for hepatocyte culture in the next future.

Statistical analysis was performed, and a T-student revealed statistical differences on means (p-value = 0.009).

8. CONCLUSIONS

The aim of this Master Thesis was to compare two different systems and the conclusions after the experimentation are:

t(HA-Gel) hydrogels enzymatically crosslinked and synthesized with F12 culture media allowed to culture primary hepatocytes up to 7 days. However, the same type of hydrogels but synthesized with CF-KRB did not keep the cells viable after 7 days.

Human hepatocytes within t(HA-Gel) 3D constructs are more functional than those cultured in 2D forms. Cell-cell and cell-matrix contacts are promoted in 3D and hepatocytes show improved ureogenic capacity, cytochrome P450 metabolic activity and albumin secretion.

Oxidised hyaluronic acid with a 41% of oxidation degree have formed stable hydrogels with gelatin. In addition, oHA-Gel hydrogels kept integrity with low FBS concentrations.

The equilibrium water content in t(HA-Gel) hydrogels is higher than oHA-Gel due to different polymer concentrations. Higher concentrations imply less porous hydrogels with lower water uptake.

Crosslinking based on reaction between oHA aldehydes and gelatin amino groups was non-cytotoxic demonstrated by the live/dead assay.

Fibroblast's metabolic activity within oHA-Gel hydrogels is lower than in the positive Gel-TG hydrogel but still promising. Future results will confirm the suitability of this system for the encapsulation of cells.

9. BIBLIOGRAPHY

Adrian, R. (2020). Liver histology: Structure, cells and characteristics. Retrieved May 7, 2020, from https://www.kenhub.com/en/library/anatomy/liver-histology

Alarake, N. Z., Frohberg, P., Groth, T., & Pietzsch, M. (2017). Mechanical properties and biocompatibility of in situ enzymatically cross-linked gelatin hydrogels. *International Journal of Artificial Organs*, *40*(4), 159–168. https://doi.org/10.5301/ijao.5000553

Allameh, A., & Kazemnejad, S. (2012). Safety evaluation of stem cells used for clinical cell therapy in chronic liver diseases; with emphasize on biochemical markers. *Clinical Biochemistry*, *45*(6), 385–396. https://doi.org/10.1016/j.clinbiochem.2012.01.017

Allison, D. D., & Grande-Allen, K. J. (2006). Review. Hyaluronan: A powerful tissue engineering tool. *Tissue Engineering*, *12*(8), 2131–2140. https://doi.org/10.1089/ten.2006.12.2131

Alwahsh, S. M., Rashidi, H., & Hay, D. C. (2018). Liver cell therapy: is this the end of the beginning? *Cellular and Molecular Life Sciences*, *75*(8), 1307–1324. https://doi.org/10.1007/s00018-017-2713-8

Angulo, P. (2002). Nonalcoholic Fatty Liver Disease. *Medical Progress*, *346*(16), 1221–1231.

Atkinson, D. E., & Bourke, E. (1984). The role of ureagenesis in pH homeostasis. *Trends in Biochemical Sciences*, *9*(7), 297–300. https://doi.org/10.1016/0968-0004(84)90293-7

Bahde, R., Kapoor, S., Bandi, S., Bhargava, K. K., Palestro, C. J., & Gupta, S. (2013). Directly acting drugs prostacyclin or nitroglycerine and endothelin receptor blocker bosentan improve cell engraftment in rodent liver. *Hepatology*, *57*(1), 320–330. https://doi.org/10.1002/hep.26005

Bahram, M., Mohseni, N., & Moghtader, M. (2016). An Introduction to Hydrogels and Some Recent Applications. *Emerging Concepts in Analysis and Applications of Hydrogels*. https://doi.org/10.5772/64301

Baiocchini, A., Montaldo, C., Conigliaro, A., Grimaldi, A., Correani, V., Mura, F., … Mancone, C. (2016). Extracellular matrix molecular remodeling in human liver fibrosis evolution. *PLoS ONE*, *11*(3), 1–14. https://doi.org/10.1371/journal.pone.0151736

Bedossa, P., & Paradis, V. (2003). Liver extracellular matrix in health and disease. *Journal of Pathology*, *200*(4), 504–515. https://doi.org/10.1002/path.1397

Bellentani, S., Scaglioni, F., Marino, M., & Bedogni, G. (2010). Epidemiology of non-alcoholic fatty liver disease. *Digestive Diseases*, *28*(1), 155–161. https://doi.org/10.1159/000282080

Belowich, M. E., & Stoddart, J. F. (2012). Dynamic imine chemistry. *Chemical Society Reviews*, *41*(6), 2003–2024. https://doi.org/10.1039/c2cs15305j

Bhogal, R. H., Hodson, J., Bartlett, D. C., Weston, C. J., Curbishley, S. M., Haughton, E., … Afford, S. C. (2011). Isolation of primary human hepatocytes from normal and diseased liver tissue: A one hundred liver experience. *PLoS ONE*, *6*(3), 1–8. https://doi.org/10.1371/journal.pone.0018222

Bigi, A., Panzavolta, S., & Rubini, K. (2004). Relationship between triple-helix content and mechanical properties of gelatin films. *Biomaterials*, *25*(25), 5675–5680.

https://doi.org/10.1016/j.biomaterials.2004.01.033

Bismuth, H. (1982). Surgical anatomy and anatomical surgery of the liver. *World Journal of Surgery*, *6*(1), 3–9. https://doi.org/10.1007/BF01656368

Bonora-Centelles, A., Donato, M. T., Lahoz, A., Pareja, E., Mir, J., Castell, J. V., & Gómez-Lechón, M. J. (2010). Functional characterization of hepatocytes for cell transplantation: Customized cell preparation for each receptor. *Cell Transplantation*, *19*(1), 21–28. https://doi.org/10.3727/096368909X474267

Chiang, C. H., Wu, W. W., Li, H. Y., Chien, Y., Sun, C. C., Peng, C. H., … Wang, C. Y. (2015). Enhanced antioxidant capacity of dental pulp-derived iPSC-differentiated hepatocytes and liver regeneration by injectable HGF-releasing hydrogel in fulminant hepatic failure. *Cell Transplantation*, *24*(3), 541–559. https://doi.org/10.3727/096368915X686986

Chun, Y. S., Chaudhari, P., & Jang, Y. Y. (2010). Applications of patient-specific induced pluripotent stem cells; focused on disease modeling, drug screening and therapeutic potentials for liver disease. *International Journal of Biological Sciences*, *6*(7), 796–805. https://doi.org/10.7150/ijbs.6.796

Clark, J. M., Brancati, F. L., & Mae Diehl, A. (2009). Nonalcoholic Fatty Liver Disease. *Textbook of Gastroenterology, Fifth Edition*, *2*, 2274–2301. https://doi.org/10.1002/9781444303254.ch89

Cordes, E. H., & Jencks, W. P. (1962). On the Mechanism of Schiff Base Formation and Hydrolysis. *Journal of the American Chemical Society*, *84*(5), 832–837. https://doi.org/10.1021/ja00864a031

Darr, A., & Calabro, A. (2009). Synthesis and characterization of tyramine-based hyaluronan hydrogels. *Journal of Materials Science: Materials in Medicine*, *20*(1), 33–44. https://doi.org/10.1007/s10856-008-3540-0

Davoodabadi, A., Abdourrahimkashi, E., Khamehchian, T., Akbari, H., Alizadeh, M., Hosseinpour, M., & Fakharian, E. (2018). Effects of Right Hepatic Artery Ligation. *Trauma Mon*, *23*(3), 63240. https://doi.org/10.5812/traumamon.63240

Deegan, D. B., Zimmerman, C., Skardal, A., Atala, A., & Shupe, T. D. (2016). Stiffness of hyaluronic acid gels containing liver extracellular matrix supports human hepatocyte function and alters cell morphology. *Journal of the Mechanical Behavior of Biomedical Materials*, *55*, 87–103. https://doi.org/10.1016/j.jmbbm.2015.10.016

Deward, A. D., Komori, J., & Lagasse, E. (2014). Ectopic transplantation sites for cell-based therapy. *Current Opinion in Organ Transplantation*, *19*(2), 169–174. https://doi.org/10.1097/MOT.0000000000000050

Donato, M. T., Lahoz, A., Montero, S., Bonora, A., Pareja, E., Mir, J., … Gómez-Lechón, M. J. (2008). Functional assessment of the quality of human hepatocyte preparations for cell transplantation. *Cell Transplantation*, *17*(10–11), 1211–1219. https://doi.org/10.3727/096368908787236620

Dong, R., Zhao, X., Guo, B., & Ma, P. X. (2016). Self-Healing Conductive Injectable Hydrogels with Antibacterial Activity as Cell Delivery Carrier for Cardiac Cell Therapy. *ACS Applied Materials and Interfaces*, *8*(27), 17138–17150. https://doi.org/10.1021/acsami.6b04911

Eysturskarð, J. (2010). *Mechanical Properties of Gelatin Gels ; Effect of Molecular Weight and*

Molecular Doctoral thesis at NTNU ,. NTNU.

Fang, J. Y., Tan, S. J., Yang, Z., Tayag, C., & Han, B. (2014). Tumor bioengineering using a transglutaminase crosslinked hydrogel. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0105616

Feil, H., Bae, Y. H., Feijen, J., & Kim, S. W. (1993). Effect of Comonomer Hydrophilicity and Ionization on the Lower Critical Solution Temperature of N-Isopropylacrylamide Copolymers. *Macromolecules*, *26*(10), 2496–2500. https://doi.org/10.1021/ma00062a016

Friedman, P. M., Mafong, E. A., Kauvar, A. N. B., & Geronemus, R. G. (2002). Safety data of injectable nonanimal stabilized hyaluronic acid gel for soft tissue augmentation. *Dermatologic Surgery*, *28*(6), 491–494. https://doi.org/10.1046/j.1524-4725.2002.01251.x

Friedman, S. L. (2008). Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiological Reviews*, *88*(1), 125–172. https://doi.org/10.1152/physrev.00013.2007

Gerber, M. A. (1987). *Histology for Pathologists Histology of the Liver*. *11*(9), 709–722.

Gomez, C. G., Rinaudo, M., & Villar, M. A. (2007). Oxidation of sodium alginate and characterization of the oxidized derivatives. *Carbohydrate Polymers*, *67*(3), 296–304. https://doi.org/10.1016/j.carbpol.2006.05.025

Grover, C. N., Gwynne, J. H., Pugh, N., Hamaia, S., Farndale, R. W., Best, S. M., & Cameron, R. E. (2012). Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films. *Acta Biomaterialia*, *8*(8), 3080–3090. https://doi.org/10.1016/j.actbio.2012.05.006

Hansel, M. C., Gramignoli, R., Skvorak, K. J., Dorko, K., Marongiu, F., Blake, W., … Strom, S. C. (2014). The history and use of human hepatocytes for the treatment of liver diseases: The first 100 patients. *Current Protocols in Toxicology*, *2014*(November), 14.12.1-14.12.23. https://doi.org/10.1002/0471140856.tx1412s62

Häussinger, D., & Kordes, C. (2019). Space of Disse: A stem cell niche in the liver. *Biological Chemistry*. https://doi.org/10.1515/hsz-2019-0283

Horslen, S. P., Mccowan, T. C., Goertzen, T. C., Warkentin, P. I., Cai, H. B., Strom, S. C., & Fox, I. J. (2003). Cycle Disorder. *Pediatrics*, *111*(6).

Hozumi, T., Kageyama, T., Ohta, S., Fukuda, J., & Ito, T. (2018). Injectable Hydrogel with Slow Degradability Composed of Gelatin and Hyaluronic Acid Cross-Linked by Schiff's Base Formation. *Biomacromolecules*, *19*(2), 288–297. https://doi.org/10.1021/acs.biomac.7b01133

Hughes, R. D., Mitry, R. R., & Dhawan, A. (2012). Current status of hepatocyte transplantation. *Transplantation*, *93*(4), 342–347. https://doi.org/10.1097/TP.0b013e31823b72d6

Jancova, P., Anzenbacher, P., & Anzenbacherova, E. (2010). Phase II drug metabolizing enzymes. *Biomedical Papers*, *154*(2), 103–116. https://doi.org/10.5507/bp.2010.017

Jin, K., Mao, X., Xie, L., Galvan, V., Lai, B., Wang, Y., … Greenberg, D. A. (2010). Transplantation of human neural precursor cells in Matrigel scaffolding improves outcome from focal cerebral ischemia after delayed postischemic treatment in rats. *Journal of Cerebral Blood Flow and Metabolism*, *30*(3), 534–544.

https://doi.org/10.1038/jcbfm.2009.219

Jitraruch, S., Dhawan, A., Hughes, R. D., Filippi, C., Soong, D., Philippeos, C., … Mitry, R. R. (2014). Alginate microencapsulated hepatocytes optimised for transplantation in acute liver failure. *PLoS ONE*, *9*(12), 1–23. https://doi.org/10.1371/journal.pone.0113609

Jung, D., Biggs, H., Erikson, J., & Ledyard, P. U. (1975). New colorimetric reaction for end point, continuous flow, and kinetic measurement of urea. *Clinical Chemistry*, *21*(8), 1136–1140. https://doi.org/10.1093/clinchem/21.8.1136

Kalra, A., & Tuma, F. (2018). Physiology, Liver. In *StatPearls*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/30571059

Khanmohammadi, M., Sakai, S., Ashida, T., & Taya, M. (2016). Production of hyaluronic-acid-based cell-enclosing microparticles and microcapsules via enzymatic reaction using a microfluidic system. *Journal of Applied Polymer Science*, *133*(16), 1–8. https://doi.org/10.1002/app.43107

Khorshidi, S., & Karkhaneh, A. (2016). A self-crosslinking tri-component hydrogel based on functionalized polysaccharides and gelatin for tissue engineering applications. *Materials Letters*, *164*, 468–471. https://doi.org/10.1016/j.matlet.2015.11.041

Kim, C. H., Lee, B. J., Yoon, J., Seo, K. M., Park, J. H., Lee, J. W., … Park, J. H. (2001). Therapeutic Effect of Hyaluronic Acid on Experimental Osteoarthrosis of Ovine Temporomandibular Joint. *Journal of Veterinary Medical Science*, *63*(10), 1083–1089. https://doi.org/10.1292/jvms.63.1083

King, V. R., Alovskaya, A., Wei, D. Y. T., Brown, R. A., & Priestley, J. V. (2010). The use of injectable forms of fibrin and fibronectin to support axonal ingrowth after spinal cord injury. *Biomaterials*, *31*(15), 4447–4456. https://doi.org/10.1016/j.biomaterials.2010.02.018

Kommareddy, S., Shenoy, D. B., & Amiji, M. M. (2007). Gelatin Nanoparticles and Their Biofunctionalization. *Nanotechnologies for the Life Sciences*, *1*, 330–352. https://doi.org/10.1002/9783527610419.ntls0011

Kopecek, J. (2007). Hydrogel biomaterials: a smart future? *Biomaterials*, *28*(34), 5185–5192. https://doi.org/10.1016/j.biomaterials.2007.07.044

Kordes, C., Sawitza, I., Götze, S., Schumacher, E., & Häussinger, D. (2015). Beyond fibrosis: Stellate cells as liver stem cells. *Zeitschrift Fur Gastroenterologie*, *53*(12), 1425–1431. https://doi.org/10.1055/s-0035-1566895

Krawitt, E. L. (2006). Autoimmune hepatitis. *Frontiers of Medicine*, *9*(2), 187–219. https://doi.org/10.1007/s11684-015-0386-y

Krishna, M. (2013). Microscopic anatomy of the liver. *Clinical Liver Disease*, *2*(SUPPL. 1), 4–7. https://doi.org/10.1002/cld.147

Kurisawa, M., Chung, J. E., Yang, Y. Y., Gao, S. J., & Uyama, H. (2005a). Injectable biodegradable hydrogels composed of hyaluronic acid-tyramine conjugates for drug delivery and tissue engineering. *Chemical Communications*, (34), 4312–4314. https://doi.org/10.1039/b506989k

Kurisawa, M., Chung, J. E., Yang, Y. Y., Gao, S. J., & Uyama, H. (2005b). Injectable biodegradable hydrogels composed of hyaluronic acid-tyramine conjugates for drug

delivery and tissue engineering. *Chemical Communications*, (34), 4312–4314. https://doi.org/10.1039/b506989k

Lahoz, A., Donato, M. T., Picazo, L., Gómez-Lechón, M. J., & Castell, J. V. (2007). Determination of major human cytochrome P450s activities in 96-well plates using liquid chromatography tandem mass spectrometry. *Toxicology in Vitro*, *21*(7), 1247–1252. https://doi.org/10.1016/j.tiv.2007.03.022

Lammert, C., Bjornsson, E., Niklasson, A., & Chalasani, N. (2010). Oral medications with significant hepatic metabolism at higher risk for hepatic adverse events. *Hepatology*, *51*(2), 615–620. https://doi.org/10.1002/hep.23317

Lee, S. Y., Kim, H. J., & Choi, D. (2015). Cell sources, liver support systems and liver tissue engineering: Alternatives to liver transplantation. *International Journal of Stem Cells*, *8*(1), 36–47. https://doi.org/10.15283/ijsc.2015.8.1.36

Li, L., Wang, N., Jin, X., Deng, R., Nie, S., Sun, L., … Gong, C. (2014). Biodegradable and injectable in situ cross-linking chitosan-hyaluronic acid based hydrogels for postoperative adhesion prevention. *Biomaterials*, *35*(12), 3903–3917. https://doi.org/10.1016/j.biomaterials.2014.01.050

Lieleg, O., & Ribbeck, K. (2011). Biological hydrogels as selective diffusion barriers. *Trends in Cell Biology*, *21*(9), 543–551. https://doi.org/10.1016/j.tcb.2011.06.002

Liu, X., Smith, L. A., Hu, J., & Ma, P. X. (2009). Biomimetic nanofibrous gelatin/apatite composite scaffolds for bone tissue engineering. *Biomaterials*, *30*(12), 2252–2258. https://doi.org/10.1016/j.biomaterials.2008.12.068

Lok, A. S. F., & McMahon, B. J. (2001). Chronic hepatitis B. *Hepatology*, *34*(6), 1225–1241. https://doi.org/10.1053/jhep.2001.29401

Madayam, S., Mazen Jamal, M., & Morgan, T. R. (2018). Epidemiology of alcoholic liver disease. *Clinical Epidemiology of Chronic Liver Diseases*, 75–98. https://doi.org/10.1007/978-3-319-94355-8_7

Maher, J. J., & Montgomery Bissell, D. (1993). Cell-matrix interactions in liver. *Seminars in Cell and Developmental Biology*, *4*(3), 189–201. https://doi.org/10.1006/scel.1993.1023

Margreet De Leeuw, A., Brouwer, A., & Knook, D. L. (1990). Sinusoidal endothelial cells of the liver: Fine structure and function in relation to age. *Journal of Electron Microscopy Technique*, *14*(3), 218–236. https://doi.org/10.1002/jemt.1060140304

Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Yao Chang Liu, & McCullough, A. J. (1999). Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology*, *116*(6), 1413–1419. https://doi.org/10.1016/S0016-5085(99)70506-8

Mazza, G., Al-Akkad, W., Rombouts, K., & Pinzani, M. (2017). Liver tissue engineering: From implantable tissue to whole organ engineering. *Hepatology Communications*, *2*(2), 131–141. https://doi.org/10.1002/hep4.1136

McCain, M. L., Agarwal, A., Nesmith, H. W., Nesmith, A. P., & Parker, K. K. (2014). Micromolded gelatin hydrogels for extended culture of engineered cardiac tissues. *Biomaterials*, *35*(21), 5462–5471. https://doi.org/10.1016/j.biomaterials.2014.03.052

O'Shea, R. S., Dasarathy, S., McCullough, A. J., Shuhart, M. C., Davis, G. L., Franco, J., …

Zuckerman, M. J. (2010). Alcoholic liver disease. *Hepatology*, *51*(1), 307–328. https://doi.org/10.1002/hep.23258

Ohashi, K., Park, F., & Kay, M. A. (2001). Hepatocyte transplantation: Clinical and experimental application. *Journal of Molecular Medicine*, *79*(11), 617–630. https://doi.org/10.1007/s001090100260

Olinga, P., Elferink, M. G. L., Draaisma, A. L., Merema, M. T., Castell, J. V., Pérez, G., & Groothuis, G. M. M. (2008). Coordinated induction of drug transporters and phase I and II metabolism in human liver slices. *European Journal of Pharmaceutical Sciences*, *33*(4–5), 380–389. https://doi.org/10.1016/j.ejps.2008.01.008

Ortega, F. R., Rodríguez, G., & Aguilar, M. R. (2012). *Comportamiento reológico de geles biodegradables para aplicaciones en medicina regenerativa*. *20*, 7–19.

Overstreet, D. J., Dutta, D., Stabenfeldt, S. E., & Vernon, B. L. (2012). Injectable hydrogels. *Journal of Polymer Science, Part B: Polymer Physics*, *50*(13), 881–903. https://doi.org/10.1002/polb.23081

Pakulska, M. M., Ballios, B. G., & Shoichet, M. S. (2012). Injectable hydrogels for central nervous system therapy. *Biomedical Materials*, *7*(2). https://doi.org/10.1088/1748-6041/7/2/024101

Patel, V., Joseph, G., Patel, A., Patel, S., Bustin, D., Mawson, D., … Pearse, D. D. (2010). Suspension matrices for improved Schwann-cell survival after implantation into the injured rat spinal cord. *Journal of Neurotrauma*, *27*(5), 789–801. https://doi.org/10.1089/neu.2008.0809

Peppas, N. A., Bures, P., Leobandung, W., & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, *50*(1), 27–46. https://doi.org/10.1016/S0939-6411(00)00090-4

Perez, R. A., Jung, C. R., & Kim, H. W. (2017). Biomaterials and Culture Technologies for Regenerative Therapy of Liver Tissue. *Advanced Healthcare Materials*, *6*(2). https://doi.org/10.1002/adhm.201600791

Pico, M. L. C., Lorenzo, J. R. F., & Bermúdez, J. M. F. (2008). Enfermedades congénitas del metabolismo en el período neonatal. *Protocolos de Neonatología*, 434–442.

Poveda-Reyes, S., Moulisova, V., Sanmartín-Masiá, E., Quintanilla-Sierra, L., Salmerón-Sánchez, M., & Ferrer, G. G. (2016). Gelatin—Hyaluronic Acid Hydrogels with Tuned Stiffness to Counterbalance Cellular Forces and Promote Cell Differentiation. *Macromolecular Bioscience*, 1311–1324. https://doi.org/10.1002/mabi.201500469

Poynard, T., Yuen, M. F., Ratziu, V., & Lung Lai, C. (2003). Viral hepatitis C. *Lancet*, *362*(9401), 2095–2100. https://doi.org/10.1016/S0140-6736(03)15109-4

Puche, J. E., Saiman, Y., & Friedman, S. L. (2013). Hepatic stellate cells and liver fibrosis. *Comprehensive Physiology*, *3*(4), 1473–1492. https://doi.org/10.1002/cphy.c120035

Ratner, B. D., & Hoffman, A. S. (1976). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, *64*(SUPPL.), 18–23. https://doi.org/10.1016/j.addr.2012.09.010

Rodriguez Fernandez, J., Gallego Ferrer, G., & Tolosa, L. (2018). *ESTUDIO DEL COMPORTAMIENTO DE HEPATOCITOS EN HIDROGELES DE ORIGEN NATURAL PARA TERAPIA CELULAR HEPÁTICA.* (Universitat Politécnica de Valencia). Retrieved

from http://hdl.handle.net/10251/123116

Rose, J. B., Pacelli, S., El Haj, A. J., Dua, H. S., Hopkinson, A., White, L. J., & Rose, F. R. A. J. (2014). Gelatin-based materials in ocular tissue engineering. *Materials*, *7*(4), 3106–3135. https://doi.org/10.3390/ma7043106

Sakai, S., Ashida, T., Ogino, S., Taya, M., Rose, J. B., Pacelli, S., … Uyama, H. (2014). Injectable biodegradable hydrogels composed of hyaluronic acid-tyramine conjugates for drug delivery and tissue engineering. *Journal of Microencapsulation*, *7*(4), 4312–4314. https://doi.org/10.1039/b506989k

Sakai, S., Hirose, K., Taguchi, K., Ogushi, Y., & Kawakami, K. (2009). An injectable, in situ enzymatically gellable, gelatin derivative for drug delivery and tissue engineering. *Biomaterials*, *30*(20), 3371–3377. https://doi.org/10.1016/j.biomaterials.2009.03.030

Sanmartín-Masiá, E., Poveda-Reyes, S., & Gallego Ferrer, G. (2017). Extracellular matrix–inspired gelatin/hyaluronic acid injectable hydrogels. *International Journal of Polymeric Materials and Polymeric Biomaterials*, *66*(6), 280–288. https://doi.org/10.1080/00914037.2016.1201828

Sgroi, A., Serre-Beinier, V., Morel, P., & Bühler, L. (2009). What clinical alternatives to whole liver transplantation? current status of artificial devices and hepatocyte transplantation. *Transplantation*, *87*(4), 457–466. https://doi.org/10.1097/TP.0b013e3181963ad3

Shin, J., Lee, J. S., Lee, C., Park, H. J., Yang, K., Jin, Y., … Cho, S. W. (2015). Tissue Adhesive Catechol-Modified Hyaluronic Acid Hydrogel for Effective, Minimally Invasive Cell Therapy. *Advanced Functional Materials*, *25*(25), 3814–3824. https://doi.org/10.1002/adfm.201500006

Slehria, S., Rajvanshi, P., Ito, Y., Sokhi, R. P., Bhargava, K. K., Palestro, C. J., … Gupta, S. (2002). Hepatic sinusoidal vasodilators improve transplanted cell engraftment and ameliorate microcirculatory perturbations in the liver. *Hepatology*, *35*(6), 1320–1328. https://doi.org/10.1053/jhep.2002.33201

Smets, F., Dobbelaere, D., McKiernan, P., Dionisi-Vici, C., Broué, P., Jacquemin, E., … Sokal, E. (2019). Phase I/II Trial of Liver Derived Mesenchymal Stem Cells in Pediatric Liver Based Metabolic Disorders. *Transplantation*, *00*(00), 1. https://doi.org/10.1097/tp.0000000000002605

Soltys, K. A., Setoyama, K., Tafaleng, E. N., Soto Gutiérrez, A., Fong, J., Fukumitsu, K., … Fox, I. J. (2017). Host conditioning and rejection monitoring in hepatocyte transplantation in humans. *Journal of Hepatology*, *66*(5), 987–1000. https://doi.org/10.1016/j.jhep.2016.12.017

Sommar, P., Pettersson, S., Ness, C., Johnson, H., Kratz, G., & Junker, J. P. E. (2010). Engineering three-dimensional cartilage- and bone-like tissues using human dermal fibroblasts and macroporous gelatine microcarriers. *Journal of Plastic, Reconstructive and Aesthetic Surgery*, *63*(6), 1036–1046. https://doi.org/10.1016/j.bjps.2009.02.072

Stabenfeldt, S. E., Gourley, M., Krishnan, L., Hoying, J. B., & Barker, T. H. (2012). Engineering fibrin polymers through engagement of alternative polymerization mechanisms. *Biomaterials*, *33*(2), 535–544. https://doi.org/10.1016/j.biomaterials.2011.09.079

Stéphenne, X., Najimi, M., & Sokal, E. M. (2010). Hepatocyte cryopreservation: Is it time to

change the strategy? *World Journal of Gastroenterology*, *16*(1), 1–14. https://doi.org/10.3748/wjg.v16.i1.1

Struecker, B., Raschzok, N., & Sauer, I. M. (2014). Liver support strategies: Cutting-edge technologies. *Nature Reviews Gastroenterology and Hepatology*, *11*(3), 166–176. https://doi.org/10.1038/nrgastro.2013.204

Su, W. Y., Chen, K. H., Chen, Y. C., Lee, Y. H., Tseng, C. L., & Lin, F. H. (2011). An injectable oxidated hyaluronic acid/adipic acid dihydrazide hydrogel as a vitreous substitute. *Journal of Biomaterials Science, Polymer Edition*, *22*(13), 1777–1797. https://doi.org/10.1163/092050610X522729

Su, W. Y., Chen, Y. C., & Lin, F. H. (2010). Injectable oxidized hyaluronic acid/adipic acid dihydrazide hydrogel for nucleus pulposus regeneration. *Acta Biomaterialia*, *6*(8), 3044–3055. https://doi.org/10.1016/j.actbio.2010.02.037

Suuronen, E. J., Kuraitis, D., & Ruel, M. (2008). Improving Cell Engraftment with Tissue Engineering. *Seminars in Thoracic and Cardiovascular Surgery*, *20*(2), 110–114. https://doi.org/10.1053/j.semtcvs.2008.03.005

Tabibian, J. H., Masyuk, A. I., Masyuk, T. V., O'Hara, S. P., & LaRusso, N. F. (2013). Physiology of cholangiocytes. *Comprehensive Physiology*, *3*(1), 541–565. https://doi.org/10.1002/cphy.c120019

Taddei, T., Mistry, P., & Schilsky, M. L. (2009). Inherited metabolic disease of the liver. *Current Opinion in Gastroenterology*, *25*(3), 209–214. https://doi.org/10.1097/MOG.0b013e328329e13d

Tan, H., Chu, C. R., Payne, K. A., & Marra, K. G. (2009). Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*(13), 2499–2506. https://doi.org/10.1016/j.biomaterials.2008.12.080

Tennant, B. C. (1958). Hepatic function. *Postgraduate Medicine*, *23*(4), 441–445. https://doi.org/10.1080/00325481.1958.11692062

Thalheimer, U., & Capra, F. (2002). Liver transplantation: Making the best out of what we have. *Digestive Diseases and Sciences*, *47*(5), 945–953. https://doi.org/10.1023/A:1015004616884

Tian, W. M., Hou, S. P., Ma, J., Zhang, C. L., Xu, Q. Y., Lee, I. S., … Cui, F. Z. (2005). Hyaluronic acid-poly-D-lysine-based three-dimensional hydrogel for traumatic brain injury. *Tissue Engineering*, *11*(3–4), 513–525. https://doi.org/10.1089/ten.2005.11.513

Tijore, A., Behr, J. M., Irvine, S. A., Baisane, V., & Venkatraman, S. (2018). Bioprinted gelatin hydrogel platform promotes smooth muscle cell contractile phenotype maintenance. *Biomedical Microdevices*, *20*(2). https://doi.org/10.1007/s10544-018-0274-8

Tolosa, L., Pareja, E., & Gómez-lechón, M. J. (2016). *Clinical Application of Pluripotent Stem Cells : An Alternative Cell-Based Therapy for Treating Liver Diseases ? 100*(12). https://doi.org/10.1097/TP.0000000000001426

Trefts, E., Gannon, M., & Wasserman, D. H. (2017). The liver. *Current Biology*, *27*(21), R1147–R1151. https://doi.org/10.1016/j.cub.2017.09.019

Turner, W. S., Schmelzer, E., McClelland, R., Wauthie, E., Chen, W., & Reid, L. M. (2007). Human Hepatoblast Phenotype Maintained by Hyaluronan Hydrogels. *Journal of*

Biomedical Materials Research. Part B, Applied Biomaterials, *83*(2), 340–344. https://doi.org/10.1002/jbm.b.30801

Urry, D. W. (1992). Free energy transduction in polypeptides and proteins based on inverse temperature transitions. *Progress in Biophysics and Molecular Biology*, *57*(1), 23–57. https://doi.org/10.1016/0079-6107(92)90003-O

Vaca-González, J. J., Clara-Trujillo, S., Guillot-Ferriols, M., Ródenas-Rochina, J., Sanchis, M. J., Ribelles, J. L. G., … Ferrer, G. G. (2020). Effect of electrical stimulation on chondrogenic differentiation of mesenchymal stem cells cultured in hyaluronic acid – Gelatin injectable hydrogels. *Bioelectrochemistry*, *134*. https://doi.org/10.1016/j.bioelechem.2020.107536

Van Vlierberghe, S., Dubruel, P., & Schacht, E. (2011). Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review. *Biomacromolecules*, *12*(5), 1387–1408. https://doi.org/10.1021/bm200083n

Walker, R. M. (1966). Francis Glisson and his capsule. *Annals of the Royal College of Surgeons of England*, *38*(2), 71–91.

Wang, T. W., & Spector, M. (2009). Development of hyaluronic acid-based scaffolds for brain tissue engineering. *Acta Biomaterialia*, *5*(7), 2371–2384. https://doi.org/10.1016/j.actbio.2009.03.033

Wardle, E. N. (1987). Kupffer cells and their function. *Liver*, *7*(2), 63–75. https://doi.org/10.1111/j.1600-0676.1987.tb00319.x

Weng, L., Pan, H., & Chen, W. (2007). Self-crosslinkable hydrogels composed of partially oxidized hyaluronan and gelatin: In vitro and in vivo responses. *Journal of Biomedical Materials Research - Part A*, *85*(2), 352–365. https://doi.org/10.1002/jbm.a.31491

Wisse, E., Luo, D., Vermijlen, D., Kanellopoulou, C., De Zanger, R., & Braet, F. (1997). On the function of pit cells, the liver-specific natural killer cells. *Seminars in Liver Disease*, *17*(4), 265–286. https://doi.org/10.1055/s-2007-1007204

Xia, W., Liu, W., Cui, L., Liu, Y., Zhong, W., Liu, D., … Cao, Y. (2004). Tissue engineering of cartilage with the use of chitosan-gelatin complex scaffolds. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *71*(2), 373–380. https://doi.org/10.1002/jbm.b.30087

Yamanouchi, K., Zhou, H., Roy-Chowdhury, N., Macaluso, F., Liu, L., Yamamoto, T., … Guha, C. (2009). Hepatic irradiation augments engraftment of donor cells following hepatocyte transplantation. *Hepatology*, *49*(1), 258–267. https://doi.org/10.1002/hep.22573

Yang, X., Huang, L., Zhou, L., Xu, H., & Yi, Z. (2016). A photochromic copolymer hydrogel contact lens: From synthesis to application. *International Journal of Polymer Science*, *2016*(Cd). https://doi.org/10.1155/2016/4374060

Zawada, R. J. X., Kwan, P., Olszewski, K. L., Llinas, M., & Huang, S. G. (2009). Quantitative determination of urea concentrations in cell culture medium. *Biochemistry and Cell Biology*, *87*(3), 541–544. https://doi.org/10.1139/O09-011

Zentner, G. M., Rathi, R., Shih, C., McRea, J. C., Seo, M.-H., Oh, H., … Weitman, S. (2002). Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. *Journal of Controlled Release*, *3*(2), 779–811. https://doi.org/10.3390/polym3020779

Zhou, Z., Yang, Z., Kong, L., Liu, L., Liu, Q., Zhao, Y., … Cao, D. (2012). Preparation and Characterization of Hyaluronic Acid Hydrogel Blends with Gelatin. *Journal of Macromolecular Science, Part B*, *51*(12), 2392–2400. https://doi.org/10.1080/00222348.2012.676355

ANNEXES

Composition of biopsy cell culture medium

Hepatic cell culture requires a specific composition on its culture medium, it is composed by two base media, Williams E and F12 both in 1:1 ratio. Since they are commercialised it is already containing glucose 10 mM and bicarbonate 20 mM. Therefore, F12 includes 1mM of L-glutamine. In order to prepare the "complete" medium is necessary the precise concentration of the following components (Table 17).

Table 16. Complete composition for biopsy medium

Component	Quantity for 500 mL	Final concentration
Insulin	75 µL	$9.03x10^{-8}$ M
Transferrin	2500 µL	0,025 mg/mL
Ethanolamine	200 µL	66,8 µM
L-Glutamine	5 mL	2.5mM
Linoleic acid	500 µL	7,17 µM
Glucose	3500 µL	17 mM
Ascorbic acid	3100 µL	0,62 mM
NAME	800 µL	0,64mM
PSS	5 mL	1%
BSA	5 mL	1 g/L

DOCUMENT II:

BUDGET

BUDGET INDEX

1. BUDGET INTRODUCTION

This document gathers the calculus made in order to obtain the budget of the present Master Thesis, with the unique purpose of study how economically feasible is the project. The 6th point developed, which corresponds to "Methodology", is the base of this document, taking into account all the costs associated to it. The concepts taken into account to organise the budget are labour/personnel, materials and equipment. From this it was obtained the decomposed price table and subsequently the budget for each part of the project. To end, material execution budget was calculated and obtained from it, the general budget.

Each resource was codified to create a proper organisation, the following table explains this codification (Table 1).

Table 17. Codification for the Master Thesis resources

Code	Description
LP	Labour/Personnel
EQP	Equipment
MAT	Materials
IPE	Individual Protection Equipment

2. LABOUR/PERSONNEL PRICE TABLE

Labour budget was made considering the author of the Master Thesis as a biomedical engineer and is necessary to clarify that was not earned any type of salary for the labour. The following price table (Table 2) resume the time spent for the personnel associated to the project.

Table 18. Labour/Personnel budget for the Master Thesis

Nº	Code	Unit	Description	Cost		
				Price (€)	Quantity	Total price (€)
1	LP.1	h	Biomedical engineer	12,00	416,25	**4995,00**
2	LP.2	h	Principal supervisor of the project	45,00	90,00	**4050,00**
3	LP.3	h	Co-supervisor of the project Nº1	45,00	90,00	**4050,00**
4	LP.4	h	Co-supervisor of the project Nº2	45,00	25,00	**1125,00**
5	LP.5	h	Laboratory assistant	9,00	23,50	**211,50**
Total budget "Labour/Personnel" (€)						**10381,50**

3. MATERIALS PRICE TABLE

The following table 19 summarizes the materials budget required to develop the Master Thesis experiments.

Table 19 Materials budget for the Master Thesis

Nº	Code	Unit	Description	Cost		
				Price (€)	Quantity	Total Price (€)
1	IPE.1	ud	Laboratory coat	22,50	2,00	**45,00**
2	IPE.2	ud	Latex gloves box	5,95	8,00	**47,60**
3	IPE.3	ud	Protection googles	8,95	2,00	**17,90**
4	IPE.4	ud	Gas mask	30,50	2,00	**61,00**
5	IPE.5	ud	Particle mask	7,25	2,00	**14,50**
6	MAT.1	g	Hyaluronic acid	27,63	0,10	**2,76**
7	MAT.2	g	Sodium hyaluronate	0,70	0,20	**0,14**
8	MAT.3	g	EDC	62,30	0,34	**21,18**
9	MAT.4	g	Gelatin (Bloom 300)	0,43	0,82	**0,35**
10	MAT.5	g	MES	2,82	2,56	**7,22**
11	MAT.6	g	NHS	3,84	0,01	**0,05**
12	MAT.7	g	Hepes	0,80	0,94	**0,75**
13	MAT.8	g	Monopotassium phosphate	0,08	0,06	**0,00**
14	MAT.9	g	Potassium chloride	0,16	0,12	**0,02**
15	MAT.10	g	Sodium periodate	15,10	0,18	**2,72**
16	MAT.11	g	Sodium chloride	0,15	174,14	**26,12**
17	MAT.12	g	Tyramine Hydrochloride (Tyr)	15,10	0,20	**2,96**
18	MAT.13	g	Lysine	52,20	0,04	**2,09**
19	MAT.14	g	Hydroxylammonium chloride	0,41	0,12	**0,05**
20	MAT.15	mg	Primary antibody	175,00	0,00	**0,18**
21	MAT.16	mg	Secondary antibody	212,00	0,00	**0,21**
22	MAT.17	m	Dialysis membrane (12.400 MWCO)	32,00	0,06	**1,92**
23	MAT.18	m	Dialysis membrane (3.500 MWCO)	30,00	0,04	**1,20**
24	MAT.19	m	Dialysis membrane (wet 3.500 MWCO)	38,70	0,02	**0,77**
25	MAT.20	L	MilliQ water	0,20	0,83	**0,17**
26	MAT.21	L	Distilled water	0,20	126,00	**25,20**
27	MAT.22	L	Williams E media	74,78	0,25	**18,70**
28	MAT.23	L	F12 culture media	115,50	0,25	**28,88**
29	MAT.24	L	Bicarbonate Buffer	111,30	0,03	**3,78**
30	MAT.25	L	Sodium hydroxide	294,00	0,10	**29,40**
31	MAT.26	L	TNBSA reagent	3030,00	0,01	**30,30**
32	MAT.27	L	Absolute ethanol	3,93	0,50	**1,97**
33	MAT.28	L	Trypsin	71,80	0,01	**0,86**

34	MAT.29	L	L-Glutamine	160,59	0,005	**0,80**
35	MAT.30	L	Glucose	40,20	0,004	**0,14**
36	MAT.31	L	Bicarbonate	125,10	0,002	**0,25**
37	MAT.32	L	Penicillin-Streptomycin	151,70	0,007	**1,06**
38	MAT.33	L	Ethanolamine	25,00	0,0002	**0,01**
39	MAT.34	L	BSA	125,00	0,007	**0,88**
40	MAT.35	L	Horseradish peroxidase	38,60	0,024	**0,93**
41	MAT.36	L	Hydrogen peroxide	7,17	0,024	**0,17**
42	MAT.37	L	Fetal Bovine Serum	1246,00	0,00	**3,74**
43	MAT.38	L	Linoleic acid	46,80	0,0005	**0,02**
44	MAT.39	L	Ascorbic acid	44,70	0,003	**0,13**
45	MAT.40	L	Insulin	1830,00	0,000075	**0,14**
46	MAT.41	L	NAME	192,00	0,0008	**0,15**
47	MAT.42	L	Transferrin	578,00	0,0025	**1,45**
48	MAT.43	L	dPBS	175,00	0,045	**7,88**
49	MAT.44	L	Trypan blue	84,60	0,002	**0,17**
50	MAT.45	L	Calcium Chloride	67,80	0,0001	**0,01**
51	MAT.46	L	Ammonium Chloride	122,00	0,0001	**0,01**
52	MAT.47	L	Hoechst 33442	1580,00	0,001	**0,95**
53	MAT.48	L	Iodide propidium	991,00	0,001	**0,59**
54	MAT.49	L	SDS	4,26	0,002	**0,01**
55	MAT.50	L	HCl	147,60	0,020	**2,95**
56	MAT.51	L	CFDA	2750,00	0,015	**41,25**
57	MAT.52	L	DMEM	34,20	0,100	**3,42**
58	MAT.53	L	Q-Blue	27,63	0,100	**2,76**
59	MAT.54	L	Cocktail AL4	2000,00	0,0002	**0,40**
60	MAT.55	L	Formalin	62,80	0,0001	**0,01**
61	MAT.56	L	OCT	20,00	0,02	**0,40**
62	MAT.57	L	BSA	91,70	0,002	**0,18**
63	MAT.58	L	Tween	55,50	0,001	**0,06**
64	MAT.59	L	Sucrose	30,20	0,001	**0,03**
65	MAT.60	L	Triton	46,70	0,005	**0,23**
66	MAT.61	ud	Laboratory tweezers	8,73	1,000	**8,73**
67	MAT.62	ud	p48 culture plate	2,81	6,000	**16,86**
68	MAT.63	ud	p96 black culture plate	1,01	1,000	**1,01**
69	MAT.64	ud	Metallic spatula	3,66	1,000	**3,66**
70	MAT.65	ud	Filter 0.22	18,00	2,61	**46,98**
71	MAT.66	ud	Laboratory tweezers	8,73	1,00	**8,73**
72	MAT.67	ud	p48 culture plate	2,81	6,00	**16,86**
73	MAT.68	ud	p96 black culture plate	1,01	1,00	**1,01**
74	MAT.69	ud	Well plate p48	2,81	6,00	**16,86**
75	MAT.70	ud	Falcon tubes 50 mL	0,12	2,00	**0,24**
76	MAT.71	ud	Falcon tubes 15 mL	0,10	6,00	**0,60**
77	MAT.72	ud	Centrifugal tube (50 mL)	0,10	3,00	**0,30**
78	MAT.73	ud	Micropipette tips 1000 µL	65,25	3,00	**195,75**
79	MAT.74	ud	Micropipette tips 200 µL	39,16	2,00	**78,32**

80	MAT.75	ud	Micropipette tips 20 µL	60,20	3,00	**180,60**
81	MAT.76	ud	Micropipette 1000 µL (Thermo)	215,00	1,00	**215,00**
82	MAT.77	ud	Micropipette 200 µL (Thermo)	215,00	1,00	**215,00**
83	MAT.78	ud	Micropipette 20 µL (Thermo)	215,00	1,00	**215,00**
84	MAT.79	ud	Pyrex 3L	5,83	5,00	**29,15**
85	MAT.80	ud	Pyrex 1L	4,62	2,00	**9,24**
86	MAT.81	ud	Pyrex 25 mL	3,56	2,00	**7,12**
87	MAT.82	ud	Urea kit (QuantiChrom)	362,00	1,00	**362,00**
88	MAT.83	ud	Human albumin kit (Sigma)	354,00	1,00	**354,00**
89	MAT.84	ud	Eppendorf 2 mL	0,08	210,00	**16,80**
90	MAT.85	ud	Eppendorf 1,5 mL	0,07	284,00	**19,88**
91	MAT.86	ud	Eppendorf 0,5 mL	0,06	342,00	**20,52**
			Total budget "Materials" (€)			**2507,34**

4. EQUIPMENT PRICE TABLE

The equipment used on the Master Thesis project has been gathered in the following table 20. The prices of the laboratory equipment have been calculated taking into account the depreciation of the equipment as shown in the following equation 6. The amortised time has been estimated at 12 years. The equation consists of the time the equipment has been used (T_{eq}), the amortized period (P_{am}) and the acquisition cost of each device (C_{eq})

$$Equipment\ price = \frac{C_{eq} * T_{eq}}{P_{am}}\ (VI)$$

Equation 6. Depreciation estimation for laboratory equipment

Table 20. Equipment budget for the Master Thesis

Nº	Code	Unit	Description	Cost		
				Price (€)	Quantity	Total Price (€)
1	MQ.1	h	PH-meter XS instruments, model pH510	0,30	1,00	**0,30**
2	MQ.2	h	Multiprobe stirrer VELP SCIENTIFICA AM4	0,20	386,00	**77,20**
3	MQ.3	h	Magnetic stirrer with probe. Heidolph, model MR Hei-standard with temperature control	0,40	48,00	**19,20**
4	MQ.4	h	Liophylizer (-80º)	9,46	336,00	**3178,56**
5	MQ.5	h	Precision balance, Mettler Toledo, model XS105 dualrange	0,15	12,00	**1,80**
6	MQ.6	h	Spectrophotometer Technologies Cary 60 Uv-vis	0,80	1,00	**0,80**
7	MQ.7	h	Laminar flow hood Faster TWO30	0,15	10,00	**1,50**
8	MQ.8	h	In-cell analyzer 6000	120,00	3,25	**390,00**
9	MQ.9	h	Centrifuge Thermo Scientific Megafuge402	0,40	0,15	**0,06**
10	MQ.10	h	Cryostat Leica CM 1950	5,00	4,00	**20,00**
11	MQ.11	h	Stove WTC Binder	0,10	18,06	**1,81**
12	MQ.12	h	Centrifuge Orto Arlesa Biocen20	0,20	0,23	**0,05**
13	MQ.13	h	UPLC-MS (QToF) Acquity-Synapt G2-Si Waters	95,00	1,00	**95,00**

14	MQ.14	h	pH meter 7710 (Isolab)	0,50	8,00	**4,00**
15	MQ.15	h	lyophilizer alpha 1-2 LO plus (Christ)	10,57	24,00	**253,68**
16	MQ.16	h	Stirrer Advanced VMS-C7 (VWR) with temperature control	1,15	144,00	**165,60**
17	MQ.17	h	Multistirrer Multipoint HP Electronicrührer (Variomag)	0,65	16,00	**10,40**
18	MQ.18	h	Cell culture hood Biowizard (Kojair)	0,50	15,00	**7,50**
19	MQ.19	h	Ultraviolet/visible (UV/VIS) spectrophotometer (Specord200)	5,75	4,00	**23,00**
20	MQ.20	h	Fluorescence microscope (Axiovert 100)	25,00	4,00	**100,00**
			Total budget "Equipment" (€)			**4350,45**

5. DECOMPOSED PRICES

The decomposed price section develops in chapters and subchapters the most relevant procedures needed for the Master Thesis. The next table 21 shows the budget required for each procedure.

Table 21. Decomposed prices for the Master Thesis

Nº Code	Chapter description					
	Tyramine conjugated hydrogels					
	1. Synthesis Gel-Tyr					
1.1	Tyramine Gelatine Graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	0,50	h	0,15	0,08	
	Magnetic stirrer Heidolph	4,00	h	0,40	1,60	
	Magnetic stirrer VELP	25,00	h	0,20	5,00	
	pH meter XS	0,50	h	0,30	0,15	
	Tyramine hydrochloride	0,11	g	15,10	1,66	
	Gelatin (Bloom 300)	0,40	g	0,43	0,17	
	MES	0,20	g	0,34	0,07	
	NHS	0,01	g	1,30	0,01	
	EDC	0,12	g	0,50	0,06	
	milliQ water	0,02	L	0,20	0,00	
	Biomedical engineer	5,00	h	12,00	60,00	
	Indirect costs	3,00	%	68,80	2,06	
	Auxiliary resources	3,00	%	70,86	2,13	
						72,99
1.2	Purification of gelatine with tyramine graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Magnetic stirrer VELP	48,00	h	0,20	9,60	
	Dialysis membrane (12.400 MWCO)	0,01	m	32,00	0,32	
	Distilled water	18,00	L	0,20	3,60	
	Biomedical engineer	1,00	h	12,00	12,00	
	Indirect costs	3,00	%	25,52	0,77	
	Auxiliary resources	3,00	%	26,29	0,79	
						27,07

1.3	Freeze-drying of gelatine with tyramine graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Liophylizer	72,00	h	9,46	681,12	
	Biomedical engineer	0,50	h	12,00	6,00	
	Indirect costs	3,00	%	687,12	20,61	
	Auxiliary resources	3,00	%	707,73	21,23	
						728,97

2. Synthesis HA-Tyr

2.1	Hyaluronic acid tyramine graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	1,50	h	0,15	0,23	
	Magnetic stirrer Heidolph	24,00	h	0,40	9,60	
	Liophylizer	72,00	h	9,46	681,12	
	Hyaluronic acid	0,10	g	27,63	2,76	
	Tyramine hydrochloride	0,09	g	15,10	1,30	
	pH meter	0,50	h	0,40	0,20	
	MES	1,08	g	0,34	0,37	
	NHS	0,003	g	1,30	0,00	
	EDC	0,05	g	0,50	0,02	
	MilliQ water	0,10	L	0,20	0,02	
	Biomedical engineer	12,00	h	12,00	144,00	
	Indirect costs	3,00	%	839,62	25,19	
	Auxiliary resources	3,00	%	864,81	25,94	
						890,75

2.2	Hyaluronic acid purification with tyramine graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Dialysis membrane (3.500 MWCO)	0,02	m	30,00	0,45	
	Dialysis membrane (12.400 MWCO)	0,02	m	32,00	0,48	
	Distilled water	45,00	L	0,20	9,00	
	Sodium chloride	86,00	g	0,15	12,90	
	Magnetic stirrer	120,00	h	0,20	24,00	
	Biomedical engineer	4,00	h	12,00	48,00	
	Indirect costs	3,00	%	94,83	2,84	
	Auxiliary resources	3,00	%	97,67	2,93	
						100,61

2.3	Hyaluronic acid freeze-drying with tyramine graft (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Liophylizer	192,00	h	9,46	1816,32	
	Biomedical engineer	1,00	h	12,00	12,00	
	Indirect costs	3,00	%	1828,32	54,85	
	Auxiliary resources	3,00	%	1883,17	56,50	
						1939,66

3. Buffer preparation and culture medium

3.1	Preparation CF-KRB (80 mL)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Sodium chloride	1,07	g	0,15	0,16	
	Potassium chloride	0,06	g	0,16	0,01	
	Hepes	0,47	g	0,80	0,38	
	Monopotassium phosphate	0,03	g	0,08	0,00	
	MilliQ water	0,08	L	0,20	0,02	
	Biomedical engineer	0,50	h	12,00	6,00	
	Indirect costs	3,00	%	6,56	0,20	
	Auxiliary resources	3,00	%	6,76	0,20	
						6,96

3.2	Medium biopsy preparation for human hepatocytes (500 mL)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Williams E media	0,25	L	74,78	18,70	
	F12 media	0,25	L	115,50	28,88	
	L-glutamine	0,005	L	160,59	0,80	
	Glucose	0,0035	L	40,20	0,14	
	Ethanolamine	0,0002	L	25,00	0,01	
	Linoleic acid	0,0005	L	46,80	0,02	
	Ascorbic acid	0,003	L	44,70	0,14	
	Transferrin	0,0025	L	578,00	1,45	
	Insulin	0,000075	L	1830,00	0,14	
	NAME	0,0008	L	192,00	0,15	
	Penicillin-Streptomycin	0,005	L	151,70	0,76	
	BSA	0,005	L	125,00	0,63	
	Biomedical engineer	0,75	h	12,00	9,00	
	Indirect costs	3,00	%	60,80	1,82	
	Auxiliary resources	3,00	%	62,62	1,88	
						64,50

4. Physical characterisation t(HA-Gel) hydrogels

4.1	Physical characterisation t(HA-Gel) hydrogels (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	0,50	h	0,15	0,08	
	Module 3.1	1,00	ud	6,96	6,96	
	Modulo 5.1	1,0000	ud	6,79	6,79	
	Laminar flow hood	0,50	h	0,15	0,08	
	Liophylizer	24,00	h	9,46	227,04	
	Biomedical engineer	4,00	h	12,00	48,00	
	Indirect costs	3,00	%	288,94	8,67	
	Auxiliary resources	3,00	%	297,61	8,93	
						306,54

5. Cellular encapsulation t(HA-Gel) hydrogels

5.1	Aliquot preparation for crosslinking (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Module 4.1	0,002	L	86,88	0,17	
	F12 media	0,002	L	115,50	0,23	
	Horseradish peroxidase	0,001	L	38,60	0,04	
	Hydrogen peroxide	0,001	L	7,17	0,01	
	Laminar flow hood TWO 30	0,50	h	0,15	0,08	
	Biomedical engineer	0,50	h	12,00	6,00	
	Indirect costs	3,00	%	6,53	0,20	
	Auxiliary resources	3,00	%	6,72	0,20	
						6,92
5.2	Human hepatocyte cell preparation for encapsulation (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Centrifuge Thermo	0,05	h	0,40	0,02	
	Centrifuge Orto Arlesa	0,05	h	0,20	0,01	
	Laminar flow hood TWO 30	1,00	h	0,15	0,15	
	Stove WTC Binder	0,02	h	0,10	0,00	
	Module 4.2	0,018	L	163,00	2,93	
	dPBS	0,005	L	175,00	0,88	
	Trypan blue	0,002	L	84,60	0,17	
	Trypsin	0,004	L	71,80	0,29	
	Biomedical engineer	1,00	h	12,00	12,00	
	Indirect costs	3,00	%	16,45	0,49	
	Auxiliary resources	3,00	%	16,94	0,51	
						17,45

5.3	Encapsulation of Human hepatocytes in hydrogel (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Laminar flow hood TWO 30	1,00	h	0,15	0,15	
	Stove WTC Binder	0,50	h	0,10	0,05	
	Well plate p48	2,00	ud	2,81	5,62	
	Filter (0.22 µm)	4,00	ud	2,61	10,44	
	Biomedical engineer	1,00	h	12,00	12,00	
	Indirect costs	3,00	%	29,26	0,88	
	Auxiliary resources	3,00	%	30,14	0,90	
						31,04

6. Cell culture characterization t(HA-Gel) hydrogels

6.1	Viability assessment using the Live-Dead (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	In-cell analyzer 6000	0,25	h	120,00	30,00	
	Stove WTC Binder	0,50	h	0,15	0,08	
	Hoechst	0,0001	L	1580,00	0,16	
	Propidium iodide	0,0001	L	991,00	0,10	
	Laminar flow hood TWO 30	0,50	h	0,15	0,08	
	Biomedical engineer	0,50	h	12,00	6,00	
	Laboratory assistant	0,50	h	9,00	4,50	
	Indirect costs	3,00	%	40,91	1,23	
	Auxiliary resources	3,00	%	42,13	1,26	
						43,40

6.2	Functional evaluation by ureogenesis (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Centrifuge Orto Arlesa	0,05	h	0,20	0,01	
	Laminar flow hood TWO 30	0,50	h	0,15	0,08	
	Stove WTC Binder	4,00	h	0,10	0,40	
	Calcium chloride	0,0001	L	67,80	0,01	
	Hepes	0,001	L	0,80	0,00	
	Ammonium Chloride	0,0001	L	122,00	0,01	
	Urea evaluation kit	1,00	ud	362,00	362,00	
	Biomedical engineer	3,00	h	12,00	36,00	
	Laboratory assistant	3,00	h	9,00	27,00	
	Indirect costs	3,00	%	425,50	12,77	
	Auxiliary resources	3,00	%	438,27	13,15	
						451,42

6.3	Functional evaluation using albumin synthesis (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)

	Spectrophotometer Cary 60	1,00	h	0,80	0,80	
	Laminar flow hood TWO 30	0,50	h	0,15	0,08	
	Human albumin kit	1,00	ud	476,00	476,00	
	Biomedical engineer	6,00	h	12,00	72,00	
	Laboratory assistant	6,00	h	9,00	54,00	
	Indirect costs	3,00	%	602,88	18,09	
	Auxiliary resources	3,00	%	620,96	18,63	
						639,59
6.4	Immunohistochemistry albumin expression (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	In-cell analyzer 6000	3,00	h	120,00	360,00	
	Cryostat Leica	4,00	h	5,00	20,00	
	Primary antibody	0,001	mg	175,00	0,175	
	Secondary antibody	0,001	mg	212,00	0,21	
	Formalin	0,0001	L	62,80	0,01	
	dPBS	0,03	L	41,40	1,24	
	OCT	0,02	L	20,00	0,40	
	BSA	0,002	L	91,70	0,18	
	Tween	0,001	L	55,50	0,06	
	Sucrose	0,001	L	30,20	0,03	
	Triton	0,005	L	46,70	0,23	
	Biomedical engineer	12,00	h	12,00	144,00	
	Laboratory assistant	12,00	h	9,00	108,00	
	Indirect costs	3,00	%	634,54	19,04	
	Auxiliary resources	3,00	%	653,57	19,61	
						673,18
6.5	Functional evaluation using CYP's activity (3 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	External service - UPLC-MS	1,00	ud	90,00	90,00	
	Laminar flow hood TWO 30	0,50	h	0,15	0,08	
	Centrifuge Orto Arlesa	0,03	h	0,20	0,01	
	Cocktail Al4	0,0002	L	2000,00	0,40	
	Hepes	0,001	L	0,80	0,00	
	Biomedical engineer	2,00	h	12,00	24,00	
	Laboratory assistant	2,00	h	9,00	18,00	
	Indirect costs	3,00	%	132,48	3,97	
	Auxiliary resources	3,00	%	136,46	4,09	
						140,55
	Oxidised polysaccharide hydrogels					
	7. Synthesis oHA					

7.1	Hyaluronic acid oxidation (80 gr)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	1,00	h	0,15	0,15	
	Stirrer Advanced	72,00	h	1,15	82,80	
	Sodium hyaluronate	0,10	g	27,63	2,76	
	Sodium periodate	0,09	g	15,10	1,30	
	Dialysis membrane (wet 3.500 MWCO)	0,02	m	38,70	0,77	
	MilliQ water	0,10	L	0,20	0,02	
	Biomedical engineer	6,00	h	12,00	72,00	
	Indirect costs	3,00	%	159,81	4,79	
	Auxiliary resources	3,00	%	164,60	4,94	
						169,54
7.2	Oxidised hyaluronic acid freeze-drying (80 gr)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Liophylizer alpha 1-2 LO plus	24,00	h	10,17	244,08	
	Biomedical engineer	1,00	h	12,00	12,00	
	Indirect costs	3,00	%	256,08	7,68	
	Auxiliary resources	3,00	%	263,76	7,91	
						271,68

8. Oxidation degree oHA

8.1	Measurement oxidation degree oHA (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	1,50	h	0,15	0,23	
	Multistirrer Multipoint	8,00	h	0,65	5,20	
	pH meter 7710	4,00	h	0,50	2,00	
	oxidised HA (Module 7)	0,18	g	5,52	0,99	
	Hydroxylammonium chloride	0,06	g	0,41	0,02	
	Sodium hydroxide	0,10	L	294,00	29,40	
	MilliQ water	0,075	L	0,20	0,02	
	Biomedical engineer	8,00	h	12,00	96,00	
	Indirect costs	3,00	%	133,86	4,02	
	Auxiliary resources	3,00	%	137,87	4,14	
						142,01

9. Primary amino group quantification

9.1	Amino group content analysis (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	0,50	h	0,15	0,08	

	UV/VIS spectrophotometer Specord200	2,00	h	5,75	11,50	
	Stove WTC Binder	2,00	h	0,10	0,20	
	TNBSA reagent	0,01	L	3030,00	30,30	
	Gelatin (Bloom 300)	0,01	g	0,43	0,004	
	Bicarbonate buffer	0,012	L	111,30	1,34	
	SDS	0,001	L	4,26	0,004	
	HCl	0,01	L	147,60	1,48	
	Lysine	0,02	g	52,20	1,04	
	Biomedical engineer	2,00	h	12,00	24,00	
	Indirect costs	3,00	%	69,94	2,10	
	Auxiliary resources	3,00	%	72,04	2,16	
						74,20

10. oHA-Gel hydrogel synthesis

10.1	Aliquot preparation for oHA-Gel hydrogel (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	0,50	h	0,15	0,08	
	Bicarbonate buffer	0,005	L	111,30	0,56	
	Hyaluronic acid (Module 7)	0,01	g	4,82	0,05	
	Gelatin (Bloom 300)	0,01	g	0,43	0,004	
	Biomedical engineer	2,00	h	12,00	24,00	
	Indirect costs	3,00	%	24,68	0,74	
	Auxiliary resources	3,00	%	25,42	0,76	
						26,19

11. Physical characterisation oHA-Gel hydrogels

11.1	Physical characterisation oHA-Gel hydrogels (ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Precision balance Mettler	0,50	h	0,15	0,08	
	Module 3.1	1,00	ud	6,96	6,96	
	Module 5.1	1,0000	ud	6,79	6,79	
	Liophylizer	24,00	h	9,46	227,04	
	Biomedical engineer	4,00	h	12,00	48,00	
	Indirect costs	3,00	%	288,87	8,67	
	Auxiliary resources	3,00	%	297,53	8,93	
						306,46

12. Cell encapsulation oHA-Gel hydrogels

12.1	Human fibroblast encapsulation within oHA-Gel and Gel-TG hydrogel (6 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Cell culture hood Biowizard	5,00	h	0,50	2,50	
	Stove WTC Binder	5,00	h	0,10	0,50	
	Module 10.1	1,00	ud	26,19	26,19	
	Filter (0.22 µm)	2,00	ud	2,61	5,22	
	Fetal Bovine Serum	0,001	L	1246,00	1,25	
	Penicillin-Streptomycin	0,01	L	151,70	1,52	
	F12 media	0,01	L	115,50	1,16	
	Biomedical engineer	5,00	h	12,00	60,00	
	Indirect costs	3,00	%	98,33	2,95	
	Auxiliary resources	3,00	%	101,28	3,04	
						104,31

13. Cell culture characterization oHA-Gel hydrogels

13.1	Viability assessment by CFDA staining (6 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Fluorescence microscope (Axiovert 100)	4,00	h	27,00	108,00	
	CFDA	0,015	g	2750,00	41,25	
	DMEM (Colourless)	0,05	L	34,20	1,71	
	Module 12.1	1,00	ud	102,08	102,08	
	Biomedical engineer	6,00	h	12,00	72,00	
	Indirect costs	3,00	%	325,04	9,75	
	Auxiliary resources	3,00	%	334,79	10,04	
						344,83
13.2	Metabolic assessment by Q-Blue assay (6 ud)	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	UV/VIS spectrophotometer Specord200	2,00	h	5,75	11,50	
	Stove WTC Binder	5,00	h	0,50	2,50	
	Module 12.1	1,00	ud	102,08	102,08	
	Q-Blue reagent	0,10	L	27,63	2,76	
	DMEM (Colourless)	0,05	L	34,20	1,71	
	p96 black culture plate	1,00	ud	1,01	1,01	
	Biomedical engineer	12,00	h	12,00	144,00	
	Indirect costs	3,00	%	265,56	7,97	
	Auxiliary resources	3,00	%	273,53	8,21	
						281,74

14. Personal protection and safety equipment

14.1	IPE's	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Gas mask	2,00	ud	30,50	61,00	
	Particle mask	2,00	ud	7,25	14,50	
	Laboratory coat	3,00	ud	22,50	67,50	
	Latex glove box	8,00	ud	5,95	47,60	
	Protection googles	2,00	ud	8,95	17,90	
	Indirect costs	3,00	%	208,50	2,79	
						211,29

15. Inventory/Fungible material

15.1	Inventory/Fungible material	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Pyrex 3L	2,00	ud	5,83	11,66	
	Pyrex 1L	4,00	ud	4,62	18,48	
	Pyrex 25 mL	8,00	ud	3,56	28,48	
	Micropipette 1000 µL	1,00	ud	215,00	215,00	
	Micropipette 200 µL	1,00	ud	215,00	215,00	
	Micropipette 20 µL	1,00	ud	215,00	215,00	
	Tweezers	2,00	ud	8,73	17,46	
	Falcon Tube 50 mL	8,00	ud	0,12	0,96	
	Falcon Tube 15 mL	40,00	ud	0,10	4,00	
	Centrifugal tube	12,00	ud	0,10	1,20	
	Metallic spatula	1,00	ud	3,66	3,66	
	Micropipette tip box 1000 µL	4,00	ud	65,25	261,00	
	Micropipette tip box 200 µL	3,00	ud	39,16	117,48	
	Micropipette tip box 20 µL	4,00	ud	60,20	240,80	
	Eppendorf 2 mL	320,00	ud	0,08	25,60	
	Eppendorf 1,5 mL	260,00	ud	0,07	18,20	
	Eppendorf 0,5 mL	310,00	ud	0,05	15,50	
	Indirect costs	3,00	%	1409,48	42,28	
						1451,76

16. Project meetings

16.1	Planning meetings	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Biomedical engineer	45,00	h	12,00	540,00	
	Thesis director	45,00	h	45,00	2025,00	
	Thesis co-supervisor (1)	45,00	h	45,00	2025,00	
	Thesis co-supervisor (2)	10,00	h	45,00	450,00	
	Indirect costs	3,00	%	5040,00	151,20	

	Auxiliary resources	3,00	%	5191,20	155,74	
						5346,94
16.2	Results meetings	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Biomedical engineer	45,00	h	12,00	540,00	
	Thesis director	45,00	h	45,00	2025,00	
	Thesis co-supervisor (1)	45,00	h	45,00	2025,00	
	Thesis co-supervisor (2)	15,00	h	45,00	675,00	
	Indirect costs	3,00	%	5265,00	157,95	
	Auxiliary resources	3,00	%	5422,95	162,69	
						5585,64
16.2	Writing and corrections	Quantity	Unit	Unit price (€/u)	Partial price (€)	Total price (€)
	Biomedical engineer	180,00	h	12,00	2160,00	
	Indirect costs	3,00	%	2160,00	64,80	
	Auxiliary resources	3,00	%	2224,80	66,74	
						2291,54

6. PARTIAL BUDGETS

The partial budget gathers the information developed in the section 5 in chapters (Table 22)

Table 22. Partial budgets for the Master Thesis

Nº Code	Chapter Description				
	Tyramine conjugated hydrogels				
1	**1. Synthesis Gel-Tyr**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
1.1	Tyramine Gelatine Graft	ud	2	72,99	145,98
1.2	Purification of gelatine with tyramine graft	ud	2	27,07	54,14
1.3	Freeze-drying of gelatine with tyramine graft	ud	1	728,97	728,97
	Partial budget "Chapter 1"				**929,09**
2	**2. Synthesis HA-Tyr**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
2.1	Hyaluronic acid tyramine graft	ud	2	890,75	1781,50
2.2	Hyaluronic acid purification with tyramine graft	ud	2	100,61	201,22
2.3	Hyaluronic acid freeze-drying with tyramine graft	ud	1	1939,66	1939,66
	Partial budget "Chapter 2"				**3922,38**
3	**3. Buffer preparation and culture medium**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
3.1	Preparation CF-KRB	ud	2	6,96	13,92
3.2	Medium biopsy preparation for human hepatocytes	ud	1	64,50	64,50
	Partial budget "Chapter 3"				**78,42**
4	**4. Physical characterisation t(HA-Gel)**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
4.1	Physical characterisation t(HA-Gel) hydrogels	ud	2	306,54	613,08
	Partial budget "Chapter 4"				**613,08**

5	5. Cell encapsulation t(HA-Gel) hydrogels				
	Description	Unit	Quantity	Price (€)	Total Price (€)
5.1	Aliquot preparation for crosslinking	ud	3	6,92	20,76
5.2	Human hepatocyte cell preparation for encapsulation	ud	3	17,45	52,35
5.3	Encapsulation of Human hepatocytes in hydrogel	ud	3	31,04	93,12
	Partial budget "Chapter 5"				**166,23**
6	**6. Cell culture characterization t(HA-Gel) hydrogels**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
6.1	Viability assessment using the Live-Dead	ud	1	43,40	43,40
6.2	Functional evaluation by ureogenesis	ud	1	451,42	451,42
6.3	Functional evaluation using albumin synthesis	ud	1	639,59	639,59
6.4	Immunohistochemistry albumin expression	ud	1	673,18	673,18
6.5	Functional evaluation using CYP's activity	ud	1	140,55	140,55
	Partial budget "Chapter 6"				**1948,14**
	Oxidised polysaccharide hydrogels				
7	**7. Synthesis oHA**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
7.1	Hyaluronic acid oxidation	ud	2	169,54	339,08
7.2	Oxidised hyaluronic acid freeze-drying	ud	1	271,68	271,68
	Partial budget "Chapter 7"				**610,76**
8	**8. Oxidation degree oHA**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
8.1	Measurement oxidation degree oHA	ud	2	142,01	284,02
	Partial budget "Chapter 8"				**284,02**
9	**9. Primary amino group quantification**				

	Description	Unit	Quantity	Price (€)	Total Price (€)
9.1	Amino group content analysis	ud	2	74,20	148,40
	Partial budget "Chapter 9"				**148,40**
10	**10. oHA-Gel hydrogel synthesis**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
10.1	Aliquot preparation for oHA-Gel hydrogel	ud	2	26,19	52,38
	Partial budget "Chapter 10"				**52,38**
11	**11. Physical characterisation oHA-Gel hydrogels**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
11.1	Physical characterisation oHA-Gel hydrogels	ud	2	306,54	613,08
	Partial budget "Chapter 11"				**613,08**
12	**12. Cell encapsulation oHA-Gel hydrogels**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
12.1	Human fibroblast encapsulation within oHA-Gel and Gel-TG hydrogel	ud	3	104,31	312,93
	Partial budget "Chapter 12"				**312,93**
13	**13. Cell culture characterization oHA-Gel hydrogels**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
13.1	Viability assessment by CFDA staining	ud	1	344,83	344,83
13.2	Metabolic assessment by Q-Blue assay	ud	1	281,74	281,74
	Partial budget "Chapter 13"				**626,57**
14	**14. Personal protection and safety equipment**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
14.1	IPE's	ud	1	211,29	211,29
	Partial budget "Chapter 14"				**211,29**
15	**15. Inventory/Fungible material**				

	Description	Unit	Quantity	Price (€)	Total Price (€)
15.1	Inventory/Fungible material	ud	1	1451,76	1451,76
	Partial budget "Chapter 15"				**1451,76**
16	**16. Project Meetings**				
	Description	Unit	Quantity	Price (€)	Total Price (€)
16.1	Planning meetings	ud	1	5346,94	5346,94
16.2	Results meetings	ud	1	5585,64	5585,64
16.3	Writing and corrections	ud	1	2291,54	2291,54
	Partial budget "Chapter 16"				**13224,12**

7. MATERIAL IMPLEMENTATION BUDGET, CONTRACTUAL AND TENDER BUDGET

Tyramine conjugated hydrogels	
1. Synthesis Gel-Tyr	929,09 €
2. Synthesis HA-Tyr	3.922,38 €
3. Buffer preparation and culture medium	78,42 €
4. Physical characterisation t(HA-Gel) hydrogels	613,08 €
5. Cell encapsulation t(HA-Gel) hydrogels	166,23 €
6. Cell culture characterization t(HA-Gel) hydrogels	1.948,14 €
Oxidised polysaccharide hydrogels	
7. Synthesis oHA	610,76 €
8. Oxidation degree oHA	284,02 €
9. Primary amino group quantification	148,40 €
10. oHA-Gel hydrogel synthesis	52,38 €
11. Physical characterisation oHA-Gel hydrogels	613,08 €
12. Cell encapsulation oHA-Gel hydrogels	312,93 €
13. Cell culture characterization oHA-Gel hydrogels	626,57 €
14. Personal protection and safety equipment	211,29 €
15. Inventory/Fungible material	1.451,76 €
16. Project Meetings	13.224,12 €
Total:	**25.192,65 €**

The material implementation budget amounts to **TWENTY-FIVE THOUSAND ONE HUNDRED NINETY-TWO EUROS WITH SIXTY-FIVE CENTS**

Material implementation budget	25.192,65 €
General costs (13%)	3.275,04 €
Industrial benefit (0%)	0,00 €
Contractual budget	28.467,69 €

The contractual budget amounts to **TWENTY-EIGHT THOUSAND FOUR HUNDRED SIXTY-SEVEN EUROS WITH SIXTY-NINE CENTS**

Contractual budget	28.467,69 €
IVA (21%)	5.978,22 €
Tender budget	34.445,91 €

The tender budget amounts to **THIRTY-FOUR THOUSAND FOUR HUNDRED FOURTY-FIVE EUROS WITH NINETY-ONE CENTS**