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From semi-synthetic to natural biomaterials: a journey to the development of an optimized *in-vitro* model for liver tissue engineering

Elisa Tarsitano

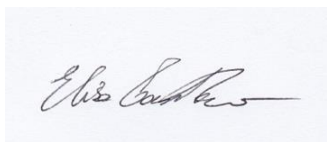
Thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy (PhD)

School of Pharmacy
University of Nottingham
October 2021

Declaration

I, Elisa Tarsitano, declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Signed:

A handwritten signature in black ink, appearing to read 'Elisa Tarsitano', is written over a light blue rectangular background.

Date: _____29/10/2021_____

Acknowledgment

First and foremost, I want to thank my mum and dad. They have made this journey possible and without their constant support and encouragement I would not be where I am right now.

I have taken this opportunity, but they have laid the foundation that guided me to succeed. I will be forever grateful for all the opportunities they have given me in my life so far. So, really, Grazie.

I also want to thank my sister, Francu, she has made some beautiful drawings for some Figures and Captions that are in this thesis. They have really helped simplify and better visualise aspects of my work. She is remarkably bright and talented. Thank you Francu for the beautiful drawings!

To my Cicciobear, I particularly thank you for the countless hours of reassuring me it was going to be fine. For listening to my problems day after day, for always offering a shoulder to cry, for never complaining about any of my meltdowns, for cooking hundreds of dinners because I was late finishing an experiment. You were and you are my rock. I could always count on you to put a smile on my face at the end of a rough day. I am truly thankful to have shared this journey with you. Thank you.

I would like to seize this opportunity to thank all my supervisors for their support. Their suggestions and feedback have greatly improved the quality of this work.

I am deeply thankful to Dr. Lisa White for allowing me to become one of her late 'PhD student additions' due to a change of direction of the project. I am grateful for her feedback and guidance she provided me during different aspects of my PhD and for really steering this PhD to the finish line. I could always count on her when problem arose, but she would always find a solution.

I would like to thank Dr. Andrew Bennett for welcoming me in the FRAME lab and allowing me to use equipment's and expertise that allowed this work to be successful. Thank you for the guidance you provided me during my studies.

Much appreciation goes to each member of the FRAME lab. You have made me feel welcome and it was a brilliant environment to work and grow as a person. Every time I had a question or some issues, I only had to ask, and there was always someone willing to help me.

Particularly, I want to thank Dr. Fatima Abukunna, she has trained me in the art of 'organoid culture' and taught me all different techniques that I was then able to apply to this work. Thank you for your support and for sharing your knowledge with me.

Many thanks to each member of Lisa's group, although I have not spent much time in the lab alongside you, I have enjoyed every PhD meme, conversations and BDI-gossip we have shared through our WhatsApp group.

A special thanks to Josh Jones, thank you for teaching me the decellularization process and many more characterisation techniques which have been used to analysed this work. Many components of this work and data have had your full contributions and I am extremely grateful for it.

I would like to express my gratitude to the RMCT group, but especially to Mahi, Jopeth, Francesco and Inchi. I will never forget our coffee breaks, where we exchange knowledge and try and figure out what was not working in our experiments. You all truly became more than work colleagues for me, but friends for life.

Special thanks to Jopeth, you are true inspiration to me, I really admire your work and your leadership attitude, you not only helped me with many of my chemistry experiments, but you also encourage me to take personal development courses that really helped build up my confidence. Although, none of the data we worked together ended up in this work, I have truly learned a lot from you, and I do believe that my broad range of skills that I acquired thanks to you, have helped me getting the job I am about to start. Thank you!

To Mahi, you are a true role model, I have admired you from the beginning, I knew you were smart, fair, and always ready to help or answer any of my questions. Thank you for making my time in ex-CBS truly special and memorable.

To Francesco, my 'compatriota', it was amazing working alongside you, we have shared our love for Italy and food, as much as, our love for science. Thank you for the wonderfully delicious Tiramisu SOP.

Finally, to Inchi, our PhD's did not really plan out and went as smoothly as we would have hoped, but we stucked together and helped each other through those rough times. I am truthfully grateful for your support as a colleague but most of all as a friend. I cannot thank you enough for listening to all my ups and downs during the last stretch. You have truly encouraged me and inspired me during this journey. You are a fantastic leader and hardworking person with a huge heart, I am certain you will do great things in you next chapter. No matter how many adversities get thrown at you, you always get up and fight back, nothing can get you down. I really admire your strength and some hard days I relied on your strength to get through. Thank you, I truly believed without you by myside, your constant encouragement and understanding, I would have not made it this far. Shukran lak Inchi!

Abstract

Within the liver, tissue resident cells exist in a state of dynamic reciprocity with the extracellular matrix (ECM) whereby the ECM provides structural and biochemical cues to influence cellular functions and cells response by modifying the ECM. To direct cell behaviour, generation of a scaffold with similar biochemical properties to the ECM is therefore highly desirable. One of the main challenges of producing such a scaffold is to find suitable biomaterials that can replicate liver ECM microarchitecture and biochemical complexity.

This study investigated the suitability of both natural and semi-synthetic biomaterials to be used as a scaffold for culturing hepatic cells for the development of a 3D *in-vitro* liver model to use for drug investigation.

Alginate, although biocompatible, once chemically conjugated with small molecules it became unsuitable to use as a potential liver scaffold, due to the compound's cytotoxicity. Both HepG2 and primary hepatocytes encapsulated in the chemically conjugated alginates died within 24 and 16 hours, respectively. Also, DNA content and both Protein and Albumin secretion were highly effected.

Porcine livers were decellularized using an enzymatic and detergent washing process. More than 89% of cellular components were removed and sulphated GAGs were still present post decellularisation. Decellularised livers were digested and solubilised to form ECM-derived hydrogels. Both mechanical and viscoelastic proprieties from a range of liver ECM concentration were tested and compared with the derived Basement Membrane Matrix (BME2) matrix, which is the gold standard material to culture and expand progenitor liver organoids. No significant difference in terms of storage moduli and stiffness was found between 2, 3 mg/ml liver ECM gels and BME2 matrix. Therefore, these concentrations were used to culture and expand progenitor liver cells.

Adult progenitor liver cells were taken from human liver biopsies and sorted by the presence of the EpCAM marker. Progenitor cells were encapsulated in a variety of liver ECM concentrations and compared to the animal-derive gold standard BME2.

Progenitor live cells proliferated in lower concentration ECM hydrogels (2 and 3mg/ml ECM), but fail to proliferate in higher concentration of liver ECM hydrogels, indicating the significant role of stiffness in cell culture.

Ultimately, utilising the lower ECM hydrogels concentrations and home-made differentiation media, progenitor liver cells were differentiated towards both a hepatic and cholangiocyte phenotype.

This present work show that the liver ECM matrix has a major role in directing progenitor cells differentiation towards a hepatocyte's lineage. Many hepatic genes were upregulated at the RNA level when hepatic progenitor cells were encapsulated in liver ECM hydrogels. Cells encapsulated in the BME2 matrix were found to be biased towards a cholangiocyte phenotype with cholangiocyte markers upregulated at the RNA level. This study emphasises the importance of the careful considerations of biomaterials composition that is required for differentiating liver progenitor cells. The combination of matrix composition and differentiation medium thereby influence the successful development of an *in-vitro* liver model.

Conferences and Awards

The following awards have resulted from the work presented in this thesis.

- 1st Prize for best oral presentation, titled: 'Assessing the elastic properties and cytocompatibility of porcine liver decellularized ECM gels for a 3D in-vitro model' at the Future Leaders Virtual Conference – a joint CDT and UK Society for Biomaterials conference held 25-26 June 2020. The participant Universities included Keele University, Sheffield University, Loughborough University, University of Nottingham, Imperial College, University of Glasgow, The University of Manchester, University of Leeds, University of Liverpool, UCL London.
- Winning image from the Future Leaders in Regenerative Medicine: Joint CDT & UKSB image contest (2021). The image was featured in the newsletter of Bioactive materials Journal.

<http://www.keaipublishing.com/en/journals/bioactive-materials/news-from-bioactive-materials/cdt-best-image-competition-winner/>

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Abbreviations

3Rs	Reduction Refinement and Replacement
AFP	Alpha-fetoprotein
ALF	Acute Liver Failure
APO_A1	Apolipoprotein A-I
ASBT	Apical Sodium-dependent Bile acid Transporter
ASC	Adult Stem Cell
ATP	Adenosine Triphosphate
BEC	Biliary Epithelial Cell
BME2	Basement Membrane Matrix type 2
BMP7	Bone morphogenetic protein 7
c-AMP	Cyclic Adenosine Monophosphate
CDMT	2-Chloro-4,6-dimethoxy-1,3,5-triazine
cDNA	Complementary DNA
CFD	Computational Fluid Dynamics
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CT	Cycle Threshold
CuAAC	Copper-Catalized Azide-Alkyne Cycloaddition
CuI	Copper(I)-Iodide
CYP2B6	Cytochrome P450 2B6

CYP3A4	Cytochrome P450 3A4
DAPT	3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
dECM	Decellularized Extra Cellular Matrix
DEPC	Diethylpyrocarbonate
DILI	Drugs Induced Liver Injury
DLM	Decellularized Liver Matrix
DM	Differentiation Media
DMAPs	Damage-Associated-Molecular-Patterns
DMEM	Dulbecco's Modified Eagle Medium
DMMB	Dimethyl methylene Blue
DMSO	Dimethyl Sulfoxide
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
EM	Expansion Media
EpCAM	Epithelial Cell Adhesion Molecule
ESC	Embryonic Stem Cell
Et3N	Triethylamine
FA	Food Alginate
FGF	Fibroblast growth factor
FGF19	Fibroblast growth factor 19

FSK	Forskolin
GAGs	Glycosaminoglycans
GC	Galactolysed Chitosan
GelMA	Gelatine Methacryloyl
GMP	Good Manufacturing Practices
GRGDS	Glycine-Arginine-Glycine-Aspartic Acid-Serine
H&E	Haematoxylin and Eosin
HAMA	methacrylate hyaluronic acid
HBV	Hepatitis B Virus
HEPES	2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
Hnf4 α	Hepatocyte nuclear factor 4 alpha
HPC	Hepatocytes Progenitor Cell
HPC	Human Progenitor Cell
HU	Housefield Units
HUVEC	Human Umbilical Vein Endothelial Cell
IFN γ	Interferon gamma
IL-10	Interleukin-10
IPSC	Induced Pluripotent Stem Cell
ITS	Insulin Transferrin Selenious Acid
Krt18/CK18	Cytokeratin-18

Krt19/CK19	Cytokeratin-19
Krt7/CK7	Cytokeratin-7
Lgr5	Leucine-rich repeat-containing G-protein coupled Receptor 5
LPC	Liver Progenitor Cell
LSEC	Liver Sinusoidal Endothelial Cell
MGA	Medical Grade Alginate
MRP2	Multidrug resistance-associated protein 2
mTORC1	mammalian Target Of Rapamycin Complex 1
NEC	non-reverse transcriptase
NMM	4-methylmorpholine
NTC	non-template control
PAA	Paracetic Acid
PBS	Phosphate Buffer Saline
PCTS	Precision-Cut Tissue Slices
PEG	PolyEthylene Glycol
PEGDA	PEG-diacrylamide
PEO	Polyethylene Oxide
PET	Polyethylene Terephthalate
PGA	Poly (Glycolic Acid)
PHH	Primary Human Hepatocytes
PLA	Polylactic Acid

PLGA	Poly Lactic-co-Glycolic Acid
PPO	Poly (Phenylene Oxide)
PSMA	Poly (Styrene-co-Maleic Acid)
PVDF	Poly (Vinylidene difluoride)
RGD	Arginine-Glycine-Aspartic Acid
RIN	RNA Integrity Number
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT	Room Temperature
Rt-PCR	real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SIS	Small Intestine Submucosa
SOX9	SRY-Box Transcription Factor 9
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methylamine
TGF- β	Transforming Growth Factor beta
TNF- α	Tumour Necrosis Factor alpha
TWEAK	TNF-Like Weak Inducer of Apoptosis
UBM	Urinary Bladder Matrix
UTS	Ultimate Tensile Strength
Wnt	Wingless-related integration site
YAP	Yes-Associated Protein

Chapter I - Introduction

1.1 Liver toxicology models

The liver performs many functions such as protein metabolism, carbohydrate metabolism as well as lipids and ions synthesis. Furthermore, it acts as a blood filter and detoxifier (Sendensky and Dufour, 2011). Due to its numerous metabolic activities, liver, is one of the primary organs that pharmaceuticals company test their drugs on (Dasgupta and Wahed, 2014). Drug-induced hepatotoxicity is the reason why most drugs pull out from the market (Raschi and Ponti, 2015), and researchers are trying to design liver models to identify drugs induced liver injury (Gomez-Lechon et al., 2008).

1.1.1 In-vivo animal models

An increased number of animals have been employed globally in research to advance medical technologies, from around 58.3 million in 2005 to 192.1 million in 2015 (Alvarez and Taylor, 2019). Although debates on ethics and animal distress are ongoing, animal experimentation is used all over the world. Despite many disadvantages, *in-vivo* models remain the preferred method of evaluating drugs and chemicals (Doke and Dhawale, 2015).

Animal models are expensive to maintain, but their main shortcomings are their unreliability (Norman, 2019). Laboratory environmental factors such as captivity and artificial habitat can lead to the animal being in distress and behaving abnormally, which can influence the results of an experiment (Bracken, 2008). Furthermore, the physical and genetic differences between humans and animals are substantial (Langley, 2009). Gene' activation and regulation can differ between species, and genes upregulated by a drug in a rat model is different from a human model (Akhtar, 2015). Thus, any differences are amplified, and this species variation might lead to not correctly translating laboratory results to humans (Toutain et al., 2010). Therefore, pharmacokinetics reliability is limited, and many drugs fail to enter the market from inadequately modelling human response. The overall probability of success for a drug entering Phase I clinical trial is around 13.8%, which means that more than 86 % do not pass a preclinical test, including an initial animal test (Hay et al., 2014; Wong et al., 2019). The main reasons for this failure are the substantial genetic and physical differences between animals and humans. Therefore, re-creating a more representative 3D *in-vitro* model using scaffolds and cells is crucial to reduce drugs failure and increase drugs success in entering the market.

1.1.2 Alternative to *in-vivo* animal models

Animal testing comes with significant ethical concerns and skilled man force, lengthy protocols, and, as mentioned above, high cost (Gruber and Hartung, 2004).

The 3Rs (reduction, refinement, and replacement) is a code of practice that can be applied in laboratory environments to reduce the use of animal models for testing. The use of animals must be planned and refined so that stress and pain can be avoided. If possible, the animal must be replaced with alternative solutions and methods (Hubrecht and Carter, 2019).

Because of the many limitations of *in-vivo* animal models, there has been a drive to develop complex *in-vitro* liver models for toxicology screening in recent years.

Alternative methods can be suggested to help reduce and avoid animal experimentation. One example could be computer models (Doke and Dhawale, 2015). Computer-generated simulation can help predict different biological outcomes and the potential toxic effect of a drug (Patil, 2008). Different cell culture conditions and parameters such as culture media composition, cell density and multiple cell system culture can inform experimental data on the optimal conditions and parameter to use in the model, reducing the animal needed in the study as some conditions would have been already rolled out by the computer-generated simulations. One advantage of computer models is the speed and their reasonably inexpensive procedures.

Cell and tissue cultures also represent a potential alternative to animal testing. They are used to screen drugs and potentially toxic compounds. Other organisms like zebrafish or invertebrates can be used to ‘refine’ the use of animal models in research (Sonvane et al., 2020). Computer models (Passini et al., 2017), bioinformatics, *in-vitro* cell cultures (Kendall Powell, 2018), combined with modern analytical techniques, could provide alternative tools to reduce and replace animal testing in the future.

In-vitro cell culture models are a more rapid and cost-effective form for testing drug toxicity. More robust models should be developed as they have the advantage of actively monitoring the progress of toxic damage of a drug and key events which are not easily observed in an *in-vivo* setup (Rim, 2019). An initial test should be implemented to understand the fundamental biological properties of the drug. A further study should be carried out where more complex tissue or cell components would be added to try and replicate the *in-vivo* environment. Similarly, *in-vitro* models can be employed to recreate a tumour microenvironment. The

combination of 3D cell culture, biomaterials, and microfabrication have enabled tumour models to become more complex, allowing the study of the tumour microenvironment by adjusting variables and facilitating quantitative analysis (Moriah E. Katt et al., 2016).

1.2 *In-vitro* liver models

In-vitro liver models are used not only in drug screening (Serras et al., 2021) but also in disease modelling, such as fibrotic liver models (Grunsvén, 2017) and to investigate anti-cancer drugs for liver tumours (Yip and Cho, 2013). Over the years, different *in-vitro* models both 2D and 3D have been developed to assess drugs' adverse effects, including liver slices, immortalised cell lines, and primary hepatocytes (Soldatow et al., 2013). One of the significant shortcomings of a 2D *in-vitro* liver model is the failure to represent a complex system. 2D *in-vitro* models cannot represent liver-specific metabolism, cell organisation and cell-cell signalling, oxygen gradients within the liver tissue, and the liver architecture (Collins et al., 2019).

For this thesis, from now on, a liver model will be defined as a 3D construct consisting of two major components: a cellular part and a biomaterial part acting as support while preserving different aspects of the *in-vivo* environment. In this case, the word scaffold describes a frame where cells can attach to, and scaffolds can be fabricated from different biomaterials.

1.2.1 Challenges in creating *in-vitro* liver models

Replicating an *in-vitro* liver model present many challenges which can be categorised below:

- A functional challenge
- A structural challenge: the ECM
- A cellular composition challenge: different cell population that reside in the liver
- Durability challenges

Tissue engineering is the construction of tissue utilising implantation of suitable cells isolated from donor tissue and biocompatible biomaterial to provide a scaffold for cell integration and repopulation. This combination of biomaterial and cells is a suitable approach for replicating an *in-vitro* model (Berthiaume and Yarmusha, 2003). Tissue engineering principles can be used to develop and construct an *in-vitro* liver model Figure 1.1.

Functional Challenge

One of the most significant challenges of tissue engineering is to imitate the complexity of tissues and organs. A particularly challenging organ is the liver, because of the complexity of all its metabolic functions, lipids, and carbohydrates metabolisms and lipids and ions synthesis (Sendensky and Dufour, 2011).

Structural Challenge

Another factor to consider is the type of biomaterial to use to fabricate scaffolds for *in-vitro* liver models. This scaffold would have 3D structures able to create a suitable environment for the cell to grow, attach and proliferate (Bokhari et al., 2007; Drury and Mooney, 2003). One of the main challenges of producing such a scaffold is finding a suitable replacement biomaterial that best mimics the extracellular matrix and the biological organisation of the tissue (Malda et al., 2013). This environment needs to be porous to allow cell penetration, biodegradable, biocompatible, and non-toxic. The cells can organise themselves in a 3D manner to reproduce the desired tissue (Chen et al., 2002; Yang et al., 2001).

The liver extracellular matrix (ECM) plays a fundamental role as an active foundation where hepatocytes can adhere, interact, proliferate, and maintain their polarity and function. It comprises collagens, adhesion proteins such as fibronectin, and proteoglycans (Martinez-Hernandez and Amenta, 1993a). The ECM is known to predispose the cells by changes in the chemistry of the matrix (Reid et al., 1992), in particular, a gradient in the matrix has been reported by Martinez-Hernandez in the space of Disse, where cells and matrix composition in their quiescent and regenerative state were investigated (Martinez-Hernandez and Amenta, 1993b) as shown in Figure 1.2.

The complex liver microarchitecture with its ECM components and its parenchymal and non-parenchymal cells is yet to be achieved by researchers. Such a model is complicated to recreate, it would have to match the stiffness of a healthy liver between 4.8 and 5.4 KPa (Colombo et al., 2011). A Fibro scan device can assess the stiffness of the liver non inversely via transient elastography. A value of liver stiffness is given in kPa, which is calculated from the time the wave takes to travel a particular volume depth within the liver. This complex liver model would have to mimic the spatial patterning as well as the ratio of the liver parenchymal and non-parenchymal cells through an additive fabrication method such as bioprinting or microfluidics (Tekin et al., 2012). The biomaterial choice to mimic the liver ECM would be very challenging since many criteria within the physicochemical property of the liver need to be met.

Cellular Composition Challenge

Hepatocytes have a considerable role in the correct functioning of the liver. Although hepatocytes constitute 80% of the total organ, they need to be supported by non-parenchymal cells to function correctly (Bale et al., 2016; Bedossa and Paradis, 2003). Liver non-parenchymal cells comprised of: Kupffer cells which produce cytokines when stimulated by inflammatory stimuli, sinusoidal endothelial cells mainly responsible for vascular endothelial factor (VEGF) secretion and stellate cells, known as fat-storing cell playing an important role in ECM production and matrix remodelling (Malik et al., 2002).

The challenge of creating a functional *in-vitro* liver model comes from the co-culture of primary hepatocytes with the non-parenchymal cell types, as they require different media composition and culture conditions.

Durability Challenge

Another challenge for an *in-vitro* model is durability. Long-term *in-vitro* liver models are complicated if not yet possible to achieve due to the short-term primary hepatocytes' viability and functionality. Long-term *in-vitro* hepatocyte monolayer cultures are challenging to maintain due to the rapid loss of hepatocyte morphology and polarity (Bachmann et al., 2015); The optimum hepatic model would be a 3D structure containing hepatocytes as close as possible to their mature state without undergoing dedifferentiation and with non-parenchymal cell types to support them. Hence researchers have employed many strategies to maintain hepatocyte morphology and functionality

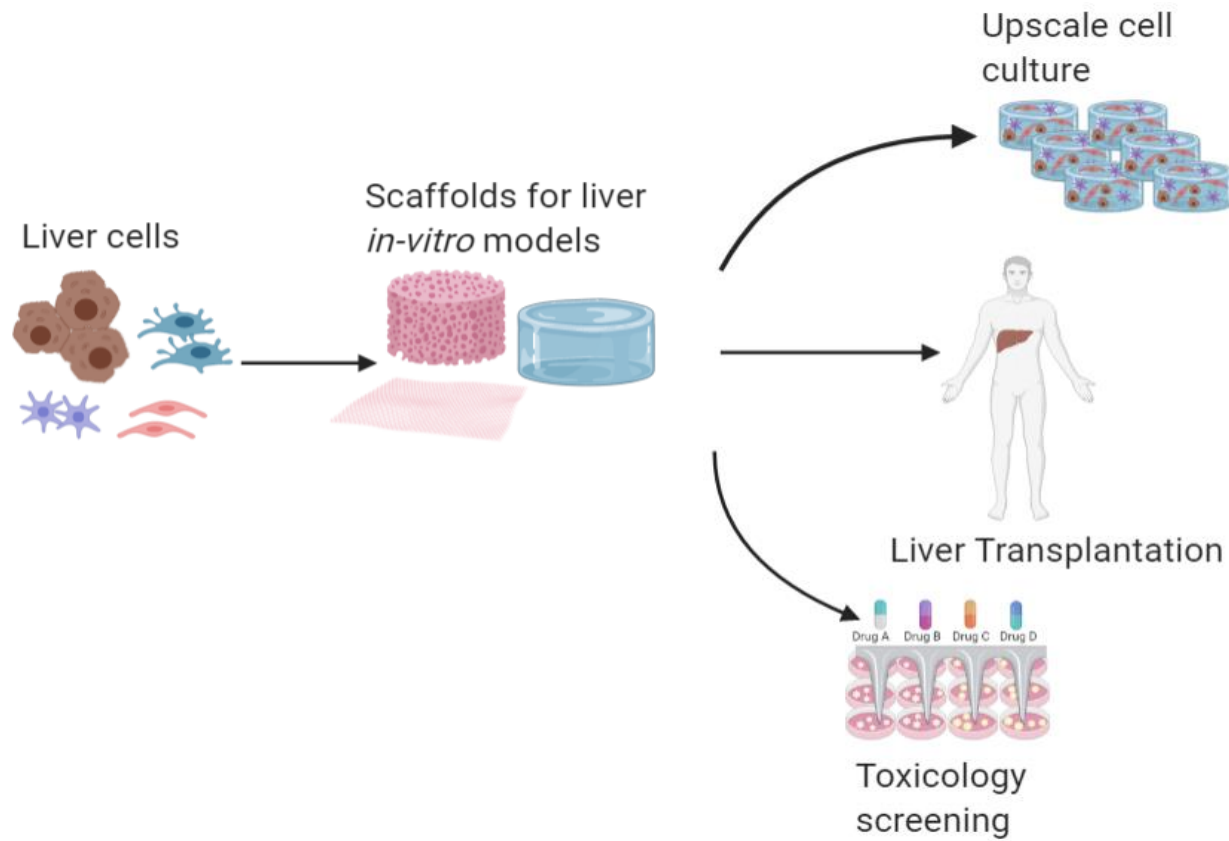


Figure 1.1 Schematic representation of 3D liver models and their application in Tissue Engineering. Drawing created using Biorender.com

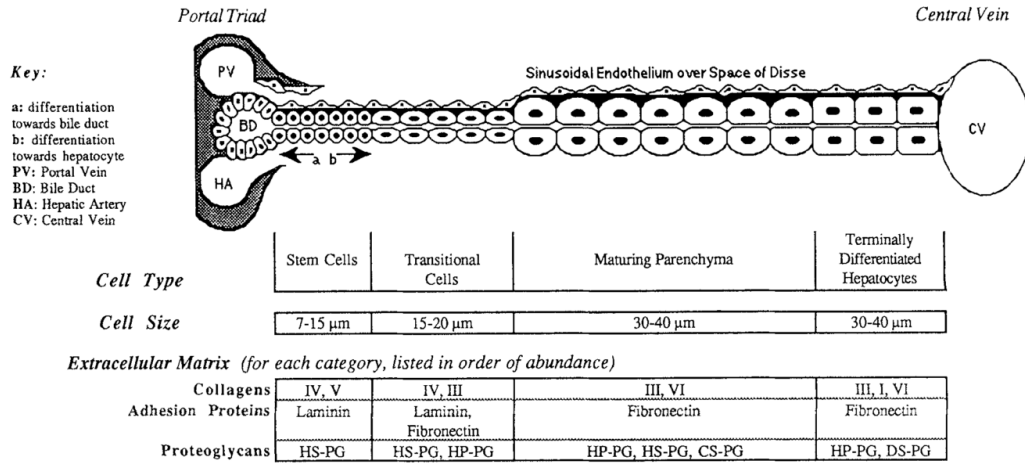


Figure 1.2 Diagram represents the liver gradient in terms of cells and matrix composition. HS-PG= heparan sulfate, HP-PG = heparin PG, CS-PG = chondroitin sulfate PG; DS-PG = dermatan sulfate PG. Copyright 1992, Reid,L. Extracellular Matrix Gradients in the Space of Disse: Relevance to Liver Biology. *Hepatology*, 15(6): 1198-1203.

1.3 Cellular components used for *in-vitro* liver models

Researchers have engineered the ECM matrix in different architecture, for example as a sandwich structure involving Collagen and Matrigel matrices (Choi and Choi, 2013; Deharde et al., 2016; Moghe et al., 1996; Sellaro et al., 2010; Shulman and Yaakov, 2013; Wang et al., 2004), as hepatic spheroids assembly (Acikgöz et al., 2013; Bell et al., 2016; Desai et al., 2017; Meng et al., 2006; Peshwa et al., 1994; Semino et al., 2003; Wu et al., 1996; Yan et al., 2015) and as self-assembly peptides (Wang et al., 2008). Different non-parenchymal cells (stellate, Kpuffer and sinusoidal epithelial) have been employed to study cell-to-cell interactions and their matrix support (Bale et al., 2016, 2015; Kaihara et al., 2000; Krause et al., 2009; Malik et al., 2002; Rose et al., 2017). Additionally, different hepatic cell lines such as HepG2, HepaRG, IPSc and hepatic progenitor cells (HPC) have been cultured to assess their response in relation to their microenvironment (Español-Suñer et al., 2012; Gomez-Lechon et al., 2008; Guillouzo et al., 2007; Lu et al., 2015; Mann, 2014; Sauer et al., 2014; Sison-Young et al., 2017; Yoon et al., 2010).

1.3.1 Primary Hepatocytes

Primary hepatocytes are still the gold standard for drug testing. Although they have been widely used for liver models and toxicology studies, they are challenging to maintain in monolayer culture. Once cultured, they start undergoing dedifferentiation and consequent changes in structure, gene expression, functions and morphology (Hu and Li, 2015). Primary hepatocytes' main drawback is the decline in P450 enzyme function and reduced albumin production (Valerie Y et al., 2013). Wilkening et al. (2003) made a comparison study between HepG2, a hepatic cancerous cell line, and primary hepatocytes to assess their biotransformation properties. Gene expression of primary hepatocytes was comparable to similar findings, but HepG2 genes were significantly different. Therefore, human primary hepatocytes are a more robust model for assessing bio-transportations in the liver (Wilkening et al., 2003). Although primary hepatocytes are still used in 2D for initial drug screening tests, Schyschka et al. (2013) demonstrated that acetaminophen-induced liver toxicity was observed in hepatocyte 3D cultures leading to cell death. However, no cell death was reported in the 2D system. However, higher activity of membrane transport protein was observed in 3D culture, demonstrating the higher metabolic functionality of 3D hepatocyte culture over 2D (Schyschka et al., 2013).

Due to these drawbacks, researchers have tried to develop a solution to increase the longevity and maintain the functionality of these primary hepatocytes cell cultures.

1.3.1.1 3D Primary hepatocytes sandwich structure as an in-vitro liver model

Sandwich hepatocytes structure is a well-established technique that involves the culture of freshly isolated primary hepatocytes between two layers of gelled collagen (Shulman and Yaakov, 2013). This configuration was adapted to successfully maintain hepatocytes' polarity and functions for a longer time. A further study conducted by Deharde et al. (2016) demonstrated functional bile canaliculi and gap junction formation in the collagen sandwich configuration. Still, different morphologies were observed when hepatocytes were overlaid with Matrigel. The combination of hepatocytes cultured on a Matrigel layer and overlaid with a collagen layer gave the most interconnected canaliculi network (Deharde et al., 2016). However, bile leakage and canaliculi disruption were found in the sandwich structure, which could limit its application for long-term toxicity screening. Wang et al. (2004) conducted a study where freshly isolated hepatocytes from piglets were encapsulated in collagen I sandwich structure or mixed homogeneously with media and collagen I. Both conditions worked in creating a robust sandwich model, and albumin secretion was seen on day 7. Although albumin secretion was not quantified, the possibility of mixing collagen I and media could have the potential for bioartificial liver studies (Wang et al., 2004). In another study,

Foster et al. (2015), compared the use of a ‘hybrid’ heparin-PEG hydrogel sandwich structure to the conventional Collagen I to assess the long-term functionality of hepatocytes over 24 days of culture. Hepatocytes in heparin-PEG hydrogel sustain a higher release of albumin and express a higher activity of cytochrome P450 (Foster et al., 2015). One of the main advantages of this developed system is using the heparin-PEG hydrogel, a synthetic-natural material with tuneable mechanical properties and controlled degradation rate, which holds potential for tuneable *in-vitro* liver models.

As well as Collagen I sandwich structure, Matrigel culture has been used to extend the longevity and functionality of hepatocytes. Researchers have demonstrated that the composite sandwich structure containing Collagen I and Matrigel reinstated some missing markers in the collagen sandwich structure, and hepatocyte polarity can be tuned (Moghe et al., 1996). Ultimately, although still widely used, Collagen I sandwich and Matrigel cultures present many limitations. For example, hepatocytes embedded between two collagen layers might be subjected to a limited supply of oxygen and nutrients (Foster et al., 2015), leading to changes in the hepatocytes phenotype.

When looking at synthetic sandwich hepatocyte’s structure, Du et al. (2008) developed a sandwich structure comprising hepatocytes embedded between a top layer of a glycine-arginine-glycine-aspartic acid-serine (GRGDS) modified polyethylene terephthalate (PET) and a bottom layer of galactose modified PET. Compared with the Collagen sandwich structure, the synthetic alternative exhibited better hepatocyte polarity and differentiation over the 14 days in culture (Du et al., 2008). This artificial sandwich culture could provide an option for an *in-vitro* liver model. Although batch-to-batch variation could be limited, and mechanical properties can be tuned, it still lacks the 3D microenvironment complexity desired in hepatocytes’ *in-vitro* culture model.

1.3.1.2 Self-Assembly peptide

In a study conducted by Genové et al., 2009 hepatocytes were cultured using a self-assembly peptide. RAD16-I was combined with a self-assembly peptide with an integrin-binding sequence (GRGDSP). Hepatocytes culture in this sandwich self-assembly peptide was compared with the gold standard of Collagen sandwich structure. Albumin and urea secretion was analysed as well as hepatic genes. Both forms exhibited bile canaliculi formation, but more effort was needed to induce liver-specific functions. The tuneable geometry of the self-spheroid structure can be modified, which presents an advantage over the collagen sandwich model for creating more controlled *in-vitro* liver models (Genové et al., 2009).

Also, Wang et al. (2008) have used a self-assembly peptide with nanofibrils characteristics to produce hepatic spheroids. PuraMatrix is a peptide hydrogel with the capability of self-

assemble into a 3D nanofibers network. In this study, PuraMatrix, Collagen sandwich, and Matrigel sandwich were evaluated. PuraMatrix exhibited higher urea and albumin expression in the first week of culture and a higher P450IA1 cytochrome activity than the other two constructs. It was suggested that PuraMatrix could produce hepatocytes spheroids that better preserve metabolic activity in early cultures for liver models (Wang et al., 2008).

Self-Assembly structures are synthetic alternatives for *in-vitro* hepatocytes culture. They offer many advantages over Collagen sandwich and Matrigel. They allow architectural control of the scaffold and have the potential for translational studies due to controlling and fully characterising their composition.

1.3.1.3 Hepatocytes spheroids assembly

Spheroids assembly is another effective way to culture primary hepatocytes in 3-dimensional packed aggregates exhibiting enhanced and sustained liver functions (Bell et al., 2016). Many studies have shown that hepatic spheroids displayed improved liver functions and an increased hepatocytes differentiation state. Furthermore, hepatocyte spheroids assembly shows similarities in the morphology and structure of the liver lobule (Peshwa et al., 1994).

In one of the first studies of spheroids assembly, primary rat hepatocytes were cultured in spheroids aggregates using two different methods, a spinner culture and static culture. Hepatic spheroids cultured in spinner flasks exhibited high viability and constant urea and albumin production during the six days of culture. A spinner flask with polyurethane foam was a fast method to form hepatocytes spheroids due to the high surface area, which allowed higher spheroids attachment (Wu et al., 1996).

In a study conducted by Meng et al. (2006) hepatocytes-spheroids were cultured in a hollow fibre bioreactor with soluble collagen. The presence of Collagen in the dynamic culture aided the formation of hepatic spheroids. Those spheroids maintain liver-specific function for eight days, as demonstrated by the urea and albumin profiles. This method can facilitate hepatic spheroids formation for *in-vitro* liver models (Meng et al., 2006).

In another study, Ikeda et al. (2012) used a microfabricated array to form hepatic spheroids. Rat primary hepatocytes cultured in conjunction with feeder cells showed and maintained a spheroid morphology for 42 days. The spheroids' cytochrome activities were assessed as well as their albumin production. This long-term model could be employed in an *in-vitro* liver model to screen drug toxicity (Ikeda et al., 2012).

Yan et al. (2015) employed a highly porous scaffold modified with RGD and ligands at a different concentration to establish the optimal condition for hepatic spheroids culture.

Different pore sizes and densities of electrospun polystyrene and poly(styrene-co-maleic acid) (PSMA) mesh were fabricated to study the effect of spheroids formation. Albumin and urea secretion were evaluated, as well as cell morphology and the cytotoxic responses of hepatic spheroids within the scaffolds. Scaffold pore size and ligands and RGD concentration significantly impacted the hepatic spheroids culture, where hepatic spheroids exhibited a greater sensitivity for cytochrome activity and toxic responses (Yan et al., 2015).

In another hepatic spheroid model, developed by Bell et al. (2016), primary human hepatocytes were cultured, and a proteomic approach was used to characterise their phenotypes and functionality. The hepatic spheroids were maintained in serum-free condition for five weeks, and spheroids formation was seen within seven days. They demonstrate the hepatic phenotype of the spheroids by ATP levels, albumin secretion, and cytochrome activities. In addition, the spheroids model could have the potential to study different liver pathology, such as hepatitis, cholestasis, and steatosis (Bell et al., 2016).

1.3.3.4 Co-culture of Hepatocytes with non-parenchymal cells

Cells in the body communicate and function through a complex signal cascade event (Kudo et al., 2019), which leads to signals being transmitted and received from and to other cells. The liver includes different cell population which supports and maintain hepatocytes functions. This population consists of non-parenchymal cells such as Kupffer cells (liver macrophages), sinusoidal endothelial cells, and stellate cells (Malik et al., 2002). Hepatocytes co-cultures with these cells type is a successful *in-vitro* model for long-term maintenance of hepatocytes phenotype and function (Begue et al., 1984; Kaihara et al., 2000). In many different studies, other cell types such as endothelial cells and mouse fibroblast NIH3T3 were employed to develop *in-vitro* liver models (Lu et al., 2005; Toyoda et al., 2017).

In their study, Krause et al. (2009) demonstrated the maintenance of hepatocytes in a differentiated state using stellate cells as co-culture conditions in an *in-vitro* model. Hepatic stellate cells could maintain hepatocytes in a differentiated state either by cell-to-cell interaction or by elements presented in the conditioned media of stellate cells. However, to maintain carboxykinase activity, a more significant number of stellate cells was needed compared to hepatocytes in great proximity to stellate cells, where cell-to-cell interaction was present. As well as the cell to cell interactions, lipids from hepatic stellate cells culture also contributed to maintaining hepatocytes morphology and functions (Krause et al., 2009).

In a study conducted by Bale et al. (2015), liver sinusoidal endothelial cells (LSECs) were co-cultured with primary hepatocytes in different collagen sandwich conditions. Hepatocytes were co-cultured with sinusoidal cells at the bottom, between two collagen layers, sinusoidal

cells at the top between two collagen layers, or in a 50% mixture with hepatocytes in between two collagen layers. The study wanted to determine which conditions would result in better cytochrome activity, albumin secretion, and maintenance of primary hepatocyte morphology. Mixing the hepatocytes and LSECs resulted in maintenance of hepatocyte phenotype and metabolic activity and albumin secretion up to 4 weeks. However, when LSECs were co-cultured at the bottom, primary hepatocytes showed 15 times higher albumin production than hepatocytes culture in collagen sandwich alone (Bale et al., 2015).

In a similar study conducted by Kim et al. (2010), LSECs were co-cultured with primary rat hepatocytes and human umbilical vein endothelial cells (HUVEC) in a 3D multi-layered structure. The polyelectrolytes chitosan-hyaluronic acid structure was employed to mimic the space of Disse. Over 12 days of culture, the 3D co-culture system demonstrated an enhanced hepatocyte's function and increased albumin production. Furthermore, bile canaliculi were only found in the 3D polyelectrolyte scaffold co-culture. Overall the results showed a model that mimics the morphology and function of the hepatic sinusoids (Kim and Rajagopalan, 2010).

In a different study conducted by Rose et al. (2017), Kupffer cells were utilised to develop an inflammatory co-cultures system. Monolayer culture of hepatocytes and a co-culture model of hepatocytes and Kupffer were tested on a different compound to address their hepatotoxicity, cytochrome response, and viability. Results had shown that when co-culture systems were exposed to polysaccharides and specific compounds, cytochrome Cyp3a4 increased, and interleukin-6 decreased. This model could investigate the effects of the different compounds, making it a valuable tool for investigating immune responses in an *in-vitro* liver model (Rose et al., 2017).

Bale et al. (2016), developed a model to isolate the parenchymal and non-parenchymal cells from a whole rat liver. Hepatocytes and stellate cells were co-cultured in a collagen gels configuration with LSECs and Kupffer cells on a thin layer of fibronectin in a transwells configuration. Stellate cells and Kupffer cells maintain their phenotype and high viability, whereas LSECs exhibited a decrease in expression of SE-1, suggesting they did not maintain their phenotype. Furthermore, the co-culture model in response to lipopolysaccharide exposure expressed the cytokines: TNF- α and IL-10, and a modulatory response was seen in the albumin activity from the hepatocytes (Bale et al., 2016). This complex *in-vitro* liver model, incorporating non-parenchymal cell type, holds potential in successfully mimic physiological liver functions.

1.3.2 Hepatocyte cell lines and iPSCs use in *in-vitro* liver models

Hepatic cells lines derived from hepatic cancers have been developed over several decades (Lin and Khetani, 2016), and their use in *in-vitro* liver models is well established. However, iPSCs have increasingly been employed for toxicological screening and liver models in the last decade due to their pluripotency, plasticity, and stem-cell-like characteristics Table 1.1.

Table 1.1 Summary of hepatic cells used as *in-vitro* liver models with their advantages and limitations.

Cell types	Advantages	Disadvantages	References
HepG2	• Unlimited source • Easy to culture	• Inaccurately model the complex physiological system, • Low gene expression and CYP activities level • Not representative of patient population due to a single genotype	(Gomez-Lechon et al., 2008),(Gerets et al., 2012)& (Sison-Young et al., 2017)
HepaRG	• Unlimited source • Some liver-specific functions and P540s enzymes expression	• Cancerous cell line • Lower P450s expression then primary hepatocytes • Not representative of patient population due to single genotype	(Guillouzo et al., 2007),(Andersson, 2008) & (Aninat et al., 2006)
iPSCs-HLCs	• Pluripotency and plasticity • no-ethical issues • Patient-specific 'personalised medicine'	• Potential tumour growth driven by provirus integrations • Foetal hepatocytes-like characteristics • Fast decline of liver-specific genes	(Takahashi and Yamanaka, 2006),(Szkolnicka and Hay, 2016),(Sauer et al., 2014),(Mann, 2014),(Yu et al., 2014), (Sakurai et al., 2017) & (Ware et al., 2015)
aSCs	• Committed to the lineage from the tissue they came from • Genetically stable • No reprogramming required	• Limited tissue biopsies and biobanks available	(Drost and Clevers, 2017), (Kim and Koo, 2020), (Tran et al., 2020)

1.3.2.1 HepG2

HepG2 is an immortalised human liver cancer cell line. Their morphology resembles epithelial cells, and they adhere and proliferate in monolayer or small cells aggregates. It is widely used due to its unlimited source, especially in early screening for new drugs. Although HepG2 expresses multiple cytokines genes (Stonans et al., 1998), it does not shows the liver activities of cytochromes P450s (Gomez-Lechon et al., 2008) effectively. Gerets et al. (2012) used the HepG2 cell line and compared their gene expression and P450 cytochrome with HepaRG cells and primary human hepatocytes. They found that HepaRG and HepG2 had very low sensitivity compared to primary hepatocytes; in particular, HepG2 had the weakest responses from different inducers in both CYP activity and gene expression (Gerets et al., 2012). Although HepG2 is a widely used human liver cell line, studies have shown that their metabolic drug profile is much lower than the gold standard of primary hepatocytes (Sison-Young et al., 2017), making this cell line a less attractive alternative to use as a cell candidate for *in-vitro* liver models.

1.3.2.2 HepaRG

HepaRG is another liver cell line derived from human hepatoma and expresses similarities with differentiated hepatocytes (Guillouzo et al., 2007). If cultured at low density, two different cell morphologies can be observed from HepaRG cells. One type presents a flat morphology, and the other surrounds it and shows a more epithelial-like look, similar to hepatocytes (Andersson, 2008). Compared to the HepG2 cell line, HepaRG expresses many liver-specific genes (P540) and nuclear receptors similar to primary hepatocytes (Guillouzo et al., 2007). Andersson et al. (2008) presented a study comparing drugs' mRNA profiles and metabolic properties between HepaRG and primary hepatocytes. Dimethyl sulfoxide (DMSO) was used to induced differentiation and to maintain the hepatocyte-like function of HepaRG. Their stability and DMSO tolerance were assessed in different periods. Over six weeks, HepaRG had a steady P450s enzymes expression and nuclear transcription factors and transporters. Although some of the compounds' metabolism was low compared to the primary hepatocytes, HepaRG is believed to be a better cell line than HepG2 for drug metabolism studies (Andersson, 2008).

Aninat et al. (2006) used HepaRG to compare drug metabolism and toxicity with human hepatocytes in a similar study. When HepaRG were seeded at low density, they started dividing and expressing a more hepatocyte-like morphology. Whereas when seeding at high density, the hepatocyte-like cluster kept their differentiated state. It was demonstrated that levels of P450 activities in primary hepatocytes were comparable to the differentiated HepaRG cells. Furthermore, in the presence of DMSO, HepaRG cell functions

reached the highest level, not only it induced a more differentiated and improved several enzyme expressions (Aninat et al., 2006).

1.3.2.3 Induced pluripotent stem cells derived hepatocytes-like-cells (iPSC - HLCs)

The discovery made by Yamanaka and Takahashi (2006), of introducing four transcription factors (Oct3/4, c-Myc, Sox2, Klf4) to reprogram adult fibroblast into Induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006) led to numerous studies to determine the best and most efficient way of reprogramming somatic cells into iPSCs (Szkolnicka and Hay, 2016). Pluripotent stem cells have many advantages over other cell lines. Firstly, they allow the development of personalised medicine with the possibility of being patient-specific. Secondly, patients' cells can be corrected from their genetic mutation and use for organ regeneration, bypassing the immune compatibility issue. After the germ layer specification (endoderm or ectoderm), cells can be cultured in 3D systems, which give rise to 'cell aggregates' that can emulate developmental steps *ex vivo* (Huch and Koo, 2015).

Furthermore, iPSCs possess plasticity and pluripotency characteristics like embryonic stem cells (ESC) but avoid the ethical concerns of embryo disruption (Sauer et al., 2014). Finally, iPSCs have demonstrated long-term viability and sustained function in culture conditions compared to primary human hepatocytes. They have been used as an *in-vitro* liver model and explore the consequences of long-term *in-vitro* toxic exposure (Mann, 2014). iPSCs can generate hepatocytes-like cells through a series of steps involving the administrations of specific growth factors agents at a particular time point. Firstly, iPSCs are induced toward an endoderm phenotype by being exposed to the growth factor (TGF- β). Then iPSCs are induced to become hepatic progenitor cells by adding specific growth factors. Finally, by adding endostatin, cytokines and glucocorticoids, the hepatocytes undergo maturation and become hepatocyte-like cells (Sauer et al., 2014; Yu et al., 2014).

Compared to hepatoma cell lines, iPSCs-HLCs had a better immune response from HBV induction, and it was demonstrated that iPS-HLCs is a robust *in-vitro* HBV model (Sakurai et al., 2017). Another study conducted by Ware et al. (2015) showed that micropatterned co-culture of induced pluripotent stem cells into hepatocytes-like murine mouse fibroblast is a potential tool for screening DILI in early drug studies. Co-cultures were treated with 47 different drugs for six days. The model successfully detected 24 out of the 37 hepatotoxic drugs (with ten non-toxic drugs) correctly as non-toxic (Ware et al., 2015). One of the main advantages of this model is the potential for high-throughput screening in early drug development due to the unlimited supply of iPSC cells and well-established high throughput culture models (Scott et al., 2013).

However, there are limitations when culturing iPSc as they can give rise to tumours due to provirus integrations. They are phenotypically and functionally similar to fetal-like cells, which required further differentiation protocols (Aoi, 2016; Baxter et al., 2015; Medvedev et al., 2010).

1.3.2.4 Adult stem cells (ASCs)

Adult stem cells (ASC's) are stem cells found in small numbers in adult tissues and are tissue specific ASCs. ASCs are generated from biopsies from the organ of interest, and they are comprised mainly of cells found in the epithelium lining. ASCs do not require a complicated reprogramming step like iPSCs, as they are already committed to the lineage of the organ they were acquired from. ASCs can be expanded *in-vitro* for the long term and remain genetically stable over long periods (Drost and Clevers, 2017). This type of stem cell has been used as an alternative to different models. ASCs represent a better human-cell-based model for understanding and modelling human diseases and creating living biobanks for biomedical research and precision medicine (Kim and Koo, 2020; Tran et al., 2020).

1.3.3 Hepatic stem progenitor cells (HPCs)

HPCs are thought to be oval-shaped cells expressing markers of both cholangiocytes and hepatocytes, which suggest that they retain bipotential differentiation capabilities (Miyajima et al., 2014). The origins of HPCs are still debated due to the limited knowledge regarding this subject (Ko et al., 2020). Some researchers believe that HPCs contribute to the activation of new hepatocytes and cholangiocytes during liver injuries, making them responsible for tissue regeneration (So et al., 2020). Others, such as Schaub et al. (2014) showed that after inducing chronic liver injury in mice, the new hepatocytes did not originate from HPCs (Schaub et al., 2014). They are in a quiescent state but activated in response to a chronic liver injury (Kaur et al., 2015). Espanol-Suner et al. (2012) successfully reported the *in-vivo* differentiation of HPCs into hepatocytes. A developed chronic injury mouse model found that liver progenitor cells successfully repopulate the injury site with the new hepatocytes forming biliary canaliculi and expressing hepatocytes specific markers. This is evidence of the contribution role of HPC in liver regeneration during liver damage, but they play no role in a normal liver regeneration process (Español-Suñer et al., 2012). In a similar study, Lu et al. (2015) developed a mouse model where necrosis, senescence, or apoptosis was induced in 98% of hepatocytes, leading to the activation of HPCs throughout the liver. A population of HPCs was isolated and transplanted back into the *in-vivo* mouse model. This triggered HPCs proliferation and differentiation, which resulted in liver regeneration (Lu et al., 2015). This

study suggests that HPCs might be a future cell source for liver transplantation as an alternative to primary hepatocytes.

1.3.3.1 Hepatic progenitor cells as organoids ‘clusters’

For this research, the organoids of interest are those composed of hepatic stem progenitor cell clusters, which can also be referred as spheroids. Over the years, spheroids and organoids definitions have been debated extensively in the scientific community. In the last decade, the terms "organoid" can be described using various definitions (Simian and Bissell, 2017). For simplicity, we will define an organoid following Cleavers' perspective, which is very similar to a spheroid: "An organoid is now defined as a 3D structure grown from stem cells and consisting of organo-specific cell typed that self-organises through cell sorting and spatially restricted lineages commitment ..." (Clevers, 2016). In this piece of work, I interchangeably classify organoids and spheroids as the same, following Cleavers' definition mentioned above.

Therefore, we can consider organoids as a 3D cell cluster capable of self-renewal and displaying similar functionality of the tissue of origin (Fatehullah et al., 2016). Although more complex cell culture systems have been developed, scientists are still trying to answer the fundamental questions: what biochemical cues regulate the formation and the stability of the organoids' differentiated state, and can we replicate organoids morphogenesis in culture?

This gave rise to an interest in modelling diseases and developmental stages using organoids, as they exhibit self-organising properties which reflect essential functional and structural properties of multiple organs. Organoid's culture can be established for different organs such as the kidney, pancreas, liver, intestine, lung, and brain (Broutier et al., 2016; Chiaradia and Lancaster, 2020; Epithelium et al., 2011; Georgakopoulos et al., 2020; Romero-guevara et al., 2020). Organoid culture can be initiated through embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells committed to their organ of origin (ASCs).

1.3.3.2 Hepatic progenitor as a new source of hepatocytes – like cells for in-vitro models

Huch and Clevers' group has set the standard for organoids research. Huch and Clevers have established organoids culture from various organs during the years, setting the threshold for many research groups (Huch et al., 2013a; Huch and Doll, 2016). Initially, Huch et al. (2013) reported that intestinal stem cells positive for Lgr5⁺ marker could build crypt-like organoid structures without the need for an epithelial niche to sustain their development. The epithelial crypts could reorganise in a crypt-like organoid with villus-like luminal domains (Huch et al., 2013b). This was the first step that highlighted the presence of a 'new' stem cell-like cell able to proliferate and self-renew and sustain long-term culture (Sato et al., 2009). Huch et al.

(2013) paved the path for employing liver stem cells Lgr5+, adult-derived stem cell progenitor, and primary liver cancer-derived organoids in liver models, cancer therapy, gene editing, and toxicology studies (Broutier et al., 2016; Prior et al., 2019).

Hepatic progenitor cells (HPCs) have been recently investigated as a new potential source for hepatocytes when differentiated (Hindley et al., 2016; Huch et al., 2017). Huch et al. (2013b) described Lgr5+ liver stem cells to form epithelial organoids with sustained expansions and *in-vitro* cultures and differentiate into hepatocyte-like cells (Huch et al., 2013b). In another study, Huch et al. (2015) extracted these bipotent cells from the human liver and then sorted them for EpCAM marker. The ductal EpCAM+ cells formed organoids with high efficiency, whereas EpCAM- (hepatocytes) did not develop any organoids. Ductal EpCAM+ cells are stable in culture for up to 3 months and can differentiate both *in-vitro* and *in-vivo*. In expansion media, human liver cultures did not express albumin but did express hepatocyte marker (Hnf4a) as well as ductal marker (KR19) and progenitor markers (Lgr5 and SOX9). However, when progenitor liver cells were cultured in differentiation media, their albumin and CYP activity increased slightly. Conclusively, primary human bile duct cells can be expanded as bipotent cells that can give rise to 3D organoids and differentiate into hepatocyte-like cells (Huch et al., 2015).

1.4 Biomaterials used in *in-vitro* liver models

To successfully create an *in-vitro* liver model, a suitable biomaterial needs to be able to support and integrate cellular components. Biomaterials need to cover different roles during the development of an *in-vitro* liver model. Biomaterials need to be able to be fabricated into a structure capable of supporting and integrating cells (Khoo and Shin, 2012; Mazza et al., 2018). The biomaterial structure needs to be mechanically stable long enough for the cells to start secreting their own matrix and degrading the biomaterial (Wang et al., 2020). Biomaterials should contain specific biochemical cues to be able to influence cell behaviour (Kim et al., 2021; Li et al., 2021). Overall, any structure fabricated from any biomaterials should mimic the 3D *in-vivo* liver microenvironment. However, one of the biggest limitations is the liver tissue complexity. The liver architectures, the different cell populations and the liver tissue biochemical composition are all required components to be able to imitate the liver microenvironment. Other parameters to consider in the development of biomaterials are: the porosity and the architectural interconnectivity, which will aid in the transport of nutrients and oxygen (Jain et al., 2013). An ideal biomaterial for an *in-vitro* liver model would have to replicate the physiochemical properties of the tissue its representing. The mechanical

properties of the tissue are also challenging to reproduce since it is complicated to closely control the composition of natural materials such as collagen and Matrigel. Contrarily, synthetic materials are easy to tune and manipulate but lack biological moieties important for cell signalling. These cues would have to be chemically conjugated to the synthetic biomaterial to allow cell attachment. This biomaterial should provide the optimal condition for cells integration, spreading, and differentiation. Cells should be producing their own ECM, and the initial biomaterial should be degrading at the same rate as the ECM being produced. Choosing the right biomaterial for the 3D liver model would determine its success in tissue engineering applications (Hammond et al., 2006).

The liver microenvironment is very complex and involves different ECM components such as collagens and fibronectin, different non-parenchymal cell types, bile canaliculi, and blood flow. Therefore, creating an *in-vitro* liver cell culture model that takes into consideration perfusion, cell-to-cell, and cell-to-matrix interactions has yet to be achieved.

Hydrogels have an important role in tissue engineering and regenerative medicine because their high-water content closely mimics the natural ECM structure (Babo et al., 2020). They are 3D networks composed of homopolymers, copolymers, or macromolecules which have been cross-linked to form an insoluble structure. The structure is held together by tie points: strong covalent bonds, temporarily physical entanglements, or weak hydrogen bonds (Ahmed, 2015).

Hydrogels have been vastly explored due to their biocompatibility, degradability, and similar environment and mechanical properties, which mimic the natural ECM (Drury and Mooney, 2003; Lee and Mooney, 2001; M.El-Sherbiny and Yacoub, 2015). Furthermore, they are well studied candidates for additive manufacturing, and they have been used as bioinks in 3D printing due to their cell-friendly characteristics (He et al., 2016).

Hydrogels derived from different biomaterials have been used as a scaffold for long-term maintenance of hepatocytes function (Foster et al., 2015), as liver toxicology models (Skardal et al., 2015b), and for bioartificial liver (No et al., 2014).

1.4.1 Natural Hydrogels - derived biomaterials for *in-vitro* liver models.

Natural derived hydrogel biomaterials have the advantages of already possessing biochemical cues in the matrix, necessary for cell-to-cell signalling and cell-to-matrix interaction (Babo et al., 2020). The physical cues already present in naturally derived biomaterials, drive cell adhesion, proliferation, and differentiation (Gaharwar et al., 2020; Xing et al., 2019).

However, two major drawbacks are the poor mechanical properties and the batch-to-batch material variation (Catoira et al., 2019).

1.4.1.1 Collagen I

Collagen I is the most common natural material used in hepatocytes culture. Collagen I is one of the main constituents of the liver ECM along with fibronectin (Martinez-Hernandez and Amenta, 1993a). Collagen is composed of three polypeptide chains, which together form a triple helix. It has been vastly exploited in the field as a hydrogel scaffold because of its biocompatibility and cell-adherent characteristics. This is due to many integrin binding domains present in the structure (Gullberg and Lundgren-Akerlund, 2002). As previously discussed in Section 1.3.1.1, Collagen I has been used in a sandwich configuration or as a scaffold to maintain primary hepatocytes phenotype (Choi and Choi, 2013; Martin et al., 2016; Zeigerer et al., 2017). Although many biological functions such as moieties, ligands, and integrins facilitate cell attachment and proliferation, Collagen I can trigger an immune response, has variable enzymatic degradation rate, its highly hydrophilic, its costly and many parameters such as fiber size, impurities and crosslinking density aid its variability (Lee et al., 2001).

In a study conducted by Rad et al., 2014 conductive scaffolds were utilised to study hepatocytes viability. The conductive scaffolds containing a mixture of Collagen I, hyaluronan, gelatine, and Chitosan were electrically stimulated resulting in an increase in proliferation and attachment of the hepatoma cell line (Rad et al., 2014).

1.4.1.2 Matrigel

Matrigel is a tumour-derived basement membrane containing laminin, collagens, growth factors, and proteoglycans (Benton et al., 2011; Kleinman and Martin, 2005), with multiple biological complex molecules and components distributed in an unknown quantity due to manufacturing restrictions. This drawback leads to batch-to-batch variations and poor reproducibility of in vitro results (Berthiaume et al., 1996). This natural-derived hydrogel has been used as previously described for long-term culture and maintenance of primary hepatocytes and their phenotype (Guzelian et al., 1988; Molina-Jimenez et al., 2012). One of its unique characteristics is that it closely mimics the ECM compositions of most tissues, allowing for a more representative physiological condition. However, as previously mentioned, significant variability is introduced not only with batch-to-batch variation but also with the unknown compositions of Matrigel. Furthermore, physical properties of the hydrogel, such as elasticity, stiffness, and swelling, cannot be controlled, or tuned (Aisenbrey and Murphy, 2020). These drawbacks and its derivation from murine sarcoma cells, make Matrigel inappropriate for clinical applications (Chae et al., 2018).

1.4.1.3 Hyaluronic acid

Hyaluronic acid is a natural non-sulphated glycosaminoglycan that can be found in most connective tissues. It is a component of the ECM, and it promotes angiogenesis and is non-immunogenic (Panwar and Tan, 2016). Due to its high viscosity, hyaluronic acid is used as a supportive hydrogel. Its main applications are cartilage tissue engineering, cardiac repair, valve engineering, and microdevices (Burdick and Prestwich, 2011). Although it has been shown to promote cell proliferation, hyaluronic acid has a very fast degradation rate, low shape fidelity, and poor mechanical properties (Ozbolat and Hospodiuk, 2016; Panwar and Tan, 2016). There have been attempts to increase the shape fidelity of hyaluronic acid by using different crosslinking methods (Ouyang et al., 2016), using photochemical crosslinking to successfully produce methacrylate hyaluronic acid (HAMA). In a study conducted by Skardal et al. (2010). HAMA was partially photo-crosslinked to GelMA to obtain a gel-like fluid for bioprinting. This new composite two-phase crosslinked hydrogel maintained high HepG2 and fibroblast attachment and proliferation (Skardal et al., 2010). In another study hyaluronic acid and liver-ECM were mixed to form a hydrogel-like structure which showed support and function of human hepatocytes (Deegan et al., 2015).

1.4.1.4 Chitosan

Chitosan is a copolymer of mainly D-glucosamine and a small proportion of N- acetyl-D-glucosamine with β -linkage and is obtained by alkaline or enzymatic deacetylation of chitin (Ozbolat and Hospodiuk, 2016). Chitosan has excellent antibacterial and non-immunogenic properties. However, chitosan has a high viscosity, but it is non-cytotoxic and biocompatible. It lacks stability without modification and possesses weak mechanical properties and a slow gelation rate. Due to its poor mechanical properties, the structure of chitosan is very fragile and structurally unstable. A solution to increase its stability is to blend chitosan with other biopolymers such as alginate and agarose (Panwar and Tan, 2016). Chitosan has been used in hepatocytes culture to produce a porous scaffold to assess hepatocytes' functional activity such as albumin and urea (Li et al., 2003; Rajendran et al., 2017).

1.4.1.5 Gelatin

Gelatin is a protein derived from the denaturation of Collagen I; it is obtained by breaking down the alpha chain strands into single molecules. It possesses favourable biological properties, such as natural cell-binding domains that allow cell attachments and proliferation (Panwar and Tan, 2016). Gelatin is a thermosensitive material, and it will change from a solid state to a gel state with an applied change in temperature. Gelatin can be employed as a sacrificial material to create vascularised networks (Lee et al., 2010), which allows the maximum exchange of nutrients and growth factors throughout a tissue. Although gelatin is biocompatible, biodegradable, and expresses non-immunogenic characteristics, it has poor

mechanical properties and low viscosity at physiological conditions (Ozbolat and Hospodiuk, 2016). It is possible to stabilise gelatin at 37 °C once it has been functionalised with a methacrylamide group, forming GelMa.

In liver tissue engineering, gelatine has been used as a hydrogel alone or in combination with other materials to study hepatocytes viability and functionality (Jiankang et al., 2009; Li et al., 2005), as well as being employed as microcarriers to study the metabolic activity of rat hepatocytes (Tao et al., 2003).

1.4.1.6 Alginate

Alginate is a readily produced polysaccharide derived from brown algae with versatile mechanical properties. It has been used for cell encapsulation as a drug delivery vehicle, cell-laden fibres, and wound dressing (Lee and Mooney, 2012). Alginate structure is composed of alternating or repetitive sequences of (1,4) -linked β -D mannuronate (M) and α -L-guluronate (G) (Drury and Mooney, 2003) as depicted in Figure 1.3. The distribution and amount of M and G blocks determine how strong and porous the gel can be. One of the main advantages of alginate is the ease of the crosslinking process. Alginate can be crosslinked and form a gel by creating ionic bridges when divalent ions contact the G blocks (Rowley et al., 1999). Over time it has been shown that the ionic chain breaks and alginate stiffness decrease (Mistry et al., 2017). Tougher alginate gels can be fabricated by covalent crosslinking using PEG and carbodiimide chemistry to enhance alginate mechanical properties (Lee and Mooney, 2012).

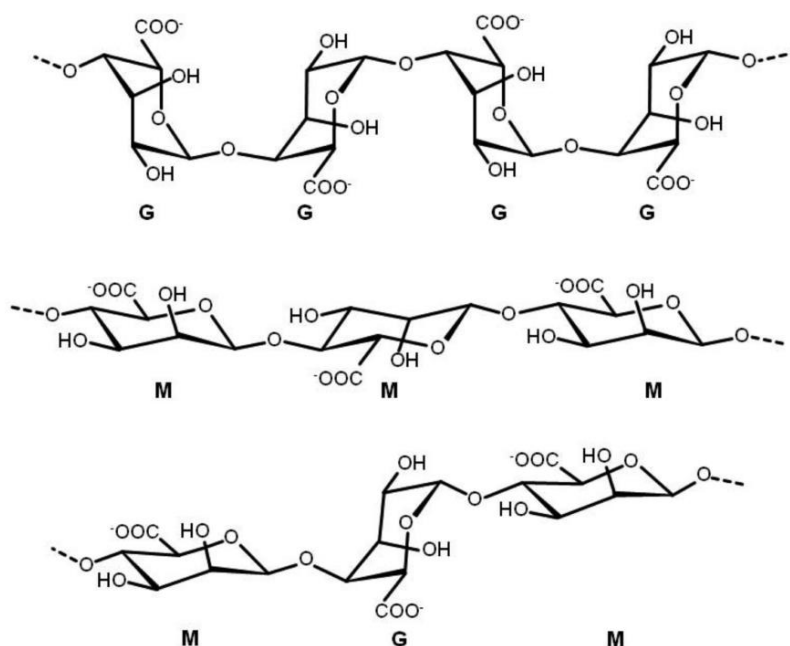


Figure 1.3 Representation of Alginate chemical structure of M and G blocks, either repeated or alternated. Copyright 2013, Mooney, D. Alginate: properties and biomedical applications. *Prog Polym Sci.*, 37(1): 106-126.

Furthermore, alginate possesses low toxicity and is biodegradable (Drury and Mooney, 2003). It can be used as hydrogel to create controlled porous structures for tissue regeneration, as demonstrated by a study conducted by Tabriz et al. (2015) where various 3D bioprinted alginate structures were assessed over their stability for 11 days. The viability of tumour cells was evaluated at different crosslinked stages, the printed construct's shape fidelity, and its stiffness (Tabriz et al., 2015). Alginate retains tuneable mechanical properties governed by the gelation process and the concentration of cations. Kaklamani et al. (2014) have established that the elastic modulus for Alginate not containing cells can range from 150-550 KPa. However, this value will change depending on the density of the cells within the hydrogel (Kaklamani et al., 2014). Much is known about alginate under compression and shear; however, not much is known about alginate tensile properties. In a study conducted by Drury et al. (2004), the tensile properties of alginate were tested extensively. Alginate tensile properties were dependent on its gelation, its concentration, and strain rate. The UTS, tensile modulus, and strain decreased by increasing the phosphate concentration and the saline buffer solution. The data showed that the alginate UTS and elastic modulus were both in the region of kPa, which is much too weak to replace living tissue constructs such as ligands, skin, and tendons which can reach MPa (Drury et al., 2004). However, it can have an application for softer tissues such as the brain and liver.

Alginate has been employed as a biomaterial for liver tissue engineering, mainly as a cell carrier and hepatocytes spheroids for bioartificial liver studies (Khalil et al., 2001; Lou et al., 2016; Naghib et al., 2017). However, Yang et al. (2002) developed an alginate scaffold covalently coupled with galactose to immobilised hepatocytes as a hybrid artificial liver *in-vitro* model. Hepatocytes could spread with 0.15 wt% galactose hydrogel, whereas the hepatocytes remained rounded in the 0.5% galactose hydrogel. Alginate-galactose capsules containing primary hepatocytes exhibit high liver-specific functions and viability (Yang et al., 2002). Similarly, Jitraruch et al. (2014) used microencapsulated hepatocytes in GMP alginate as an *in-vivo* model for acute liver failure and established their potential for the clinics. No immune response was seen when retrieving the microcapsules and hepatocytes, which were viable and had preserved their function (Jitraruch et al., 2014). Glicklis et al. (2000) developed a 3D highly porous alginate model and seeded primary rat hepatocytes. Cells distributed in the sponge-like scaffold assumed a spheroid form due to the absence of binding domain in alginate. The cell aggregates start producing albumin and urea similarly to the control collagen gels (Glicklis et al., 2000).

Alginate presents many advantages as a natural hydrogel as it is biocompatible and non-toxic. However, it lacks cell-binding domains, which results in poor cell adhesion to its structure (Panwar and Tan, 2016). Peptides, moieties, and different proteins have been chemically conjugated onto the alginate structure to increase cell attachment (Pawar and Edgar, 2013, 2012; Rowley et al., 1999).

Alginate chemical functionalisation with protein and small molecules

As discussed above, alginate is an inert biomaterial without any RGD domain for the cells to attach (Drury and Mooney, 2003; Malda et al., 2013). Hepatocytes are adherent cells that require a substrate to remain polarised and functional. They rely on biochemical cues from the native liver ECM components such as collagens and fibronectin and growth factors being released (BEN-ZE'EV et al., 1988). Alginate alone cannot provide a favourable environment for these cells to grow. Therefore, researchers have utilised specific chemistries to functionalised alginate and create an active site where moieties, peptides, and chemical groups could be attached. In many studies, alginate is primarily used as a microencapsulation device for hepatocytes (Jitraruch et al., 2014; Lou et al., 2016), but not on its own as a potential *in-vitro* liver model for hepatocytes culture. Mooney et al. (1999) conducted one of the first pilot studies in functionalised alginate using GRGDY peptide and aqueous carbodiimide chemistries. The optimised procedure reached an 80% degree of substitution. Myoblast was cultured on alginate GRGDY modified surfaces and compared with normal alginate. Myoblast

cultured on modified alginate expressed the myosin marker, whereas no marker was observed in normal alginate. This was the first step to show how ‘synthetic’ alginate can become a potential candidate for cell tissue engineering (Rowley et al., 1999).

An alginate scaffold modified with galactolysed Chitosan (GC) was compared to alginate only in a study conducted by Chung et al. (2002) to assess hepatocytes viability. In the study, GC alginate scaffold enhanced hepatocytes signal and the mechanical properties of the sponge scaffold. They also showed that changes in alginate GC led to changes in hepatocytes morphology when the freezing temperature, the molecular weight of alginate, and GC concentration were varied (Chung et al., 2002).

Alginate has been chemically improved using several different chemistries, either by modifying the hydroxyl groups, using: oxidation, reductive-amination, sulfation, copolymerisation, or by modifying the carboxyl groups using: esterification, ugi reaction, or amidation (Yang et al., 2011). Breger et al. (2015) employed “click” chemistry to synthesised alginate hydrogel capsule as a cell carrier for implantable therapies. Firstly, Alginate was functionalised with an alkyne or an azide and then reacted through click chemistry to create an alginate microcapsule. Functionalised alginate beads compared to the chemically crosslinked ones with Ca^{+2} showed better stability in EDTA and better permeability, suggesting a great potential as encapsulation devices for primary hepatocytes (Breger et al., 2015).

Vegas et al. (2016) created a library of chemically modified alginate to identify which ones would have the most negligible foreign body immune response. A high throughput screening method was used to find the materials that reduce the immune response in fibrosis models using mice and non-human primates. Alginate was covalently modified utilising a range of chemistries such as alginate amidation, alginate esterification, and cycloaddition or ‘click’ chemistry. 774 alginate combinations were prepared, from which 16 top polymers and 53 different analogues were selected to produce barium alginate microcapsule. From the top ten, three modified alginates: Z2-Y12, Z1-Y15, and Z1-Y19, produced the lower fibrotic response after implantation. Alginate with the triazole modification yielded the best *in-vivo* response, suggesting that triazole modification could be used as a small molecule to reduce immune response (Vegas et al., 2016).

1.4.2 Synthetic hydrogel scaffold-derived biomaterials for *in-vitro* liver models

Most synthetic hydrogels have a known composition that can be easily tuned or modified (Spicer, 2020). Their crosslinking time and concentration can be optimised. Adjusting the synthetic biomaterial concentration allows for a precise prediction of the polymer degradation rate and its mechanical properties and porosity, which can be tuned by changing the molecular concentration. Although polysaccharide synthetic biomaterials present many advantages, they do not have any inherent bioactivity (Madduma-bandarage, 2021) and lack of cell adhesive ligands (Li et al., 2018). Many synthetic hydrogels have been used in liver tissue engineering to improve hepatocytes sensitivity in 3D cultures (Shen et al., 2014) as a novel microfabrication system for hepatocytes culture (Fukuda et al., 2006) and for assessing hepatic spheroids formation (Acikgöz et al., 2013).

1.4.2.1 Polyethylene glycol (PEG)

PEG is a hydrophilic branched polymer used in various applications such as surface modification, drug screening, and soft tissue engineering (Zhu, 2010). Although it offers tailored mechanical properties and good hydrophilicity, it lacks cell-binding domains, making it a good candidate for cell encapsulation applications (Ozbolat and Hospodiuk, 2016). PEG is biocompatible, non-immunogenic and it is sometimes used as a crosslinker to increase the mechanical stability of hydrogels (Prestwich, 2011). PEG has low cell adhesion and proliferation rates due to the lack of cell binding domains. Cell interactions can increase by chemically modifying PEG with an arginine-glycine-aspartic acid (RGD) domain. In liver tissue engineering, PEG has a significant role in preserving hepatocytes in PEG capsules (Yarmush and Uygun, 2015) and as a liver tissue for maintaining long-term hepatic function (Ng et al., 2017).

PEG-acrylamide (PEGDA) is a modified synthetic hydrogel with photocurable characteristics (Shi et al., 2015), and it has been used extensively in hepatic tissue engineering (Stevens et al., 2015; Underhill et al., 2007).

1.4.2.2 Pluronic

Pluronic is a copolymer composed of two blocks of PEO and one of PPO (Ozbolat and Hospodiuk, 2016). Pluronic is thermosensitive and can undergo a gel-sol transition with an applied change in temperature (Panwar and Tan, 2016). It has poor mechanical properties, a fast degradation rate, and is water-soluble. That is why researchers use it as a sacrificial material to create vascular networks, a key factor for the successful transport of oxygen and nutrients within a liver model (Kolesky et al., 2014; Miller et al., 2012). It has not been

extensively used in liver tissue engineering. However, it has been investigated in combination with galactose as an absorbable coating to assess rat hepatocytes attachments and functions (Lu et al., 2003). Lu et al. (2003), used a galactose-derived pluronic-F68 hydrogel on top of poly(vinylidene difluoride) PVDF membrane to assess hepatocytes attachment and functions. Hepatocytes spheroids cultured on the modified pluronic-galactose scaffold exhibited better albumin synthesis and cytochrome P450 function than the un-modified scaffold (Lu et al., 2003).

1.4.2.3 Poly-L-lactic acid (PLLA) & poly(lactic-co-glycolic acid) PLGA

PLLA and PLGA are biodegradable polyesters that have been used in tissue engineering for hepatocytes culture models and hepatocyte implantation. Many studies have been carried out using these polymers, but limited success has been achieved (Jain et al., 2013).

One of the first pivotal studies of hepatocytes culture on synthetic scaffolds was conducted by Cima et al. (1991). PLGA or a 50:50 blend of PLGA and PLLA was used to study primary rat hepatocyte' function and biochemical activities compared to Matrigel and collagen substrates. Both synthetic scaffolds were suitable for long-term culture and retained a longer albumin secretion than collagen and Matrigel substrate, which declined over a few days. In both synthetic substrates, hepatocytes demonstrated retention to a differentiated state and comparable DNA synthesis to the natural substrates (Cima et al., 1991).

PLGA-PLA biomaterials have been used as composite foams to support hepatocytes after implantation on a rat model (Mooney et al., 1996). PLGA-PLA has been used as a supportive matrix in a flow bioreactor system which promoted hepatocytes spheroids formation (Torok et al., 2011). However, PLGA may cause an acidic environment upon scaffold degradation, which can lead to inflammation and limited tissue engraftment (Jain et al., 2013).

Kaufmann et al. (1997) also used a porous synthetic material to evaluate hepatocytes viability and functionality. 3D PLLA sponge-like scaffolds were fabricated using the particulate leaching technique. Cells immobilised on PLLA sponge coated with collagen showed an increase in albumin secretion and improvement in cell viability (Kaufmann et al., 1997). In a similar study, primary rat hepatocytes were cultured in a collagen coated PLLA nanofibrous scaffold for seven days. High viability and constant albumin secretion were recorded, as well as expression of cytochrome enzymes. Hepatocytes remained viable for 14 days; nevertheless, the albumin secretion was deficient and decreased over the culture period.

1.5 Tissue derived ECM as a biomaterial for an *in-vitro* liver model

Tissue derived ECM can be obtained through a decellularization process whereby cellular components are stripped off the tissue or organ leaving behind the structural architecture composed of collagens, laminins, fibronectin and glycosaminoglycans (GAGs) which form macromolecular network that surrounds cells with structural and biochemical cues (Hussey et al., 2018).

As previously discussed, an engineered liver model would require an optimised distribution of ECM components in a gradient layout where hepatocytes can modulate and function within their microenvironment and facilitate heterotypic cell interactions (Jain et al., 2013; Michalopoulos et al., 1999). ECM has also been employed to engineer *in-vitro* liver models. The combination of biochemical and biophysical cues within a bioresponsive material, such as ECM, can recruit cells to develop an *in-vitro* liver model (Gaharwar et al., 2020). Therefore, liver decellularised derived-ECM matrix is a good candidate to be used in developing an *in-vitro* liver model. The combination of ECM biomaterial and cells have been used in a variety of tissue models such as brain spheroids (Gu et al., 2021), skin (Won et al., 2019), small-intestine submucosa hydrogels for endodermal spheroids culture (Giobbe et al., 2019). Furthermore, tissue solubilised ECM has been used for myocardial infarction regeneration (Gershlak et al., 2014; Sullivan et al., 2014) and as cartilage hydrogels for support, encapsulation and proliferation of chondrocytes (Wu et al., 2015).

1.5.1 Native ECM matrix

The ECM is a fibrous network of a combination of functional proteins and biomolecules organised in a self-assemble 3D structure. The native ECM contains structural and functional proteins secreted by the cells in each tissue and organ (Arriazu et al., 2014). This architecture provides cells with signalling cues, elastic and mechanical stimuli, and the exchange of nutrients and oxygen (Hussey et al., 2018). Cell integration in the ECM is complemented by the continuous remodelling of ECM into a specific 3D microenvironment. Cells constantly secrete ECM components in response to different stimuli, such as mechanical and chemical. In return, the ECM sends a mechanical and chemical signal to the cells' surface receptors, resulting in a signalling cascade effect and determine changes in the cell phenotype. This mechanism is called dynamic reciprocity, where both cells and ECM influence one another (Bissell et al., 1982).

Native ECM matrix also possesses bio inductive properties as its degradation, combined with the release of growth factors and cytokines, modulate the immune response (Morwood and Nicholson, 2006). ECM-derived material also displays antimicrobial properties; in many preclinical studies, its bacterial infection resistance has been demonstrated (Badylak et al., 1994; Badylak and Gilbert, 2008). In a study conducted by Sarikaya et al. (2002), ECM antimicrobial peptides were retained after decellularisation. Both urinary bladder and small intestine submucosa showed complete growth inhibition of *Escherichia coli* and *Staphylococcus* (Sarikaya et al., 2002). This finding created many opportunities to use ECM tissue in cancer therapies and regenerative medicine (Hinderer et al., 2015).

1.5.2 Native liver ECM components

The native liver ECM contains functional and structural proteins secreted by cells and make up the majority of the cell microenvironment (Sawkins et al., 2018). Most liver ECM is found between endothelial and epithelial cells (Martinez-Hernandez and Amenta, 1993b). The liver lobule does not have a basement membrane, and the ECM is comprised mainly of fibronectin and Collagen I, while collagen III, IV, V, and VI are found in small quantities (Martinez-Hernandez and Amenta, 1993a; Okazaki et al., 1996; Ueno et al., 2003). The ECM also contains non-collagenous proteins such as proteoglycans (GAGs), including fibronectin, laminin, and tenascin, as well as heparan sulfate, perlecan, and syndecan (Yada et al., 2003) as shown in Figure 1.4, which have all been shown to play an essential role in driving stem cell fate (Gattazzo et al., 2014).

Laminins are responsible for the formation of networks in the basement membrane (Okazaki et al., 1996). Fibronectin, a high molecular weight glycoprotein, is involved in cell adhesion, migrations, and cytoskeleton organisation. Notably, in the space of Disse, fibronectin also connects the endothelial cells and hepatocytes to Collagen I bundle, integrating all the components in one single unit. In addition, many macromolecules are also found in the ECM, and they are cell adhesion molecules, growth factors from the TGF β family, metalloproteases, and processing degradation enzymes (Okazaki et al., 1996). The integrin receptors in the ECM play a significant role in mechanotransduction and transmitting mechanical and biochemical signalling to the cell's cytoskeleton. In return, cells regulate ECM mechanics and encourage mechanical homeostasis so that the tissue can sustain its structure and functional integrity (Humphrey et al., 2015).

Although representing only around 10% of the total liver volume in a healthy liver (McQuitty et al. 2020), liver ECM principal structural components: Collagen and fibronectin, play a crucial role in the mechanical interaction between cells and tissue. Collagen IV and laminin have been found where the progenitor cells reside (Terada and Nakanuma, 1994), and their role in cell to matrix interaction is vital in controlling stem cell behaviour (Sánchez-romero et al., 2019).

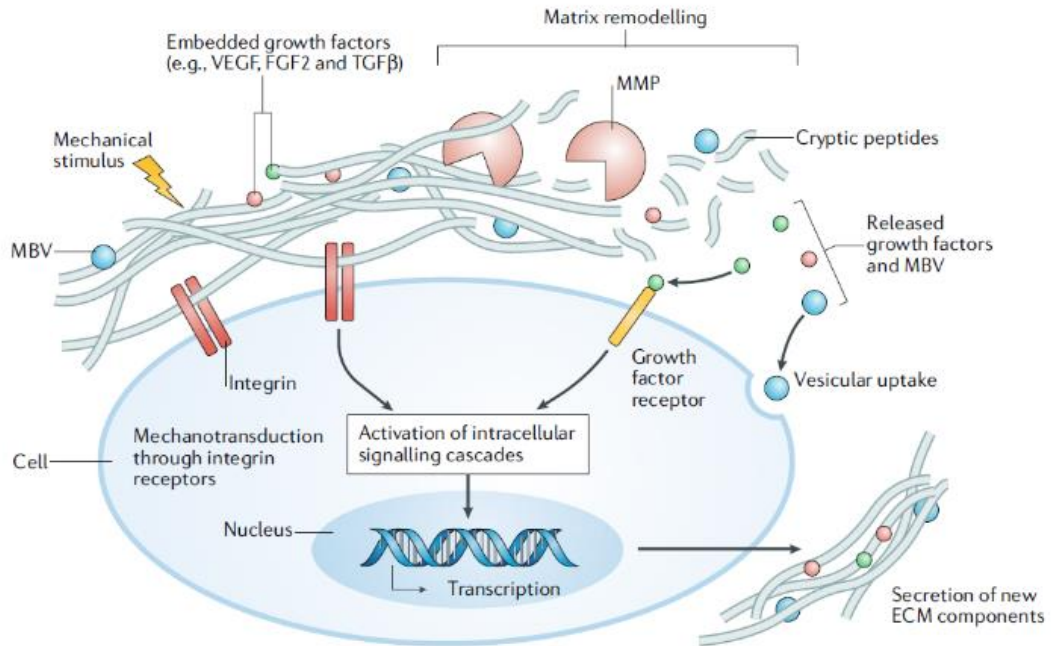


Figure 1.4 Diagram depicting cell-extracellular matrix interactions. Integrin receptors on cell surfaces communicate with the ECM and growth factors; this activates a signalling cascade of events that results in the cell secreting new ECM components and remodelling the matrix. Reproduced from Hussey et al. (2018).

1.5.3 Liver decellularisation process

The ECM-derived tissue can be obtained through decellularisation, where all the cell components are stripped off the tissue or organ. Many tissues and organs have been successfully decellularised, and some have been used in clinical and preclinical studies (Badylak et al., 2010; Sawkins et al., 2018). It is essential to maintain a balance when decellularising tissues; decellularisation must be achieved while preserving the ECM components and structure at their best. Urinary bladder matrix (UBM) and small intestine submucosa (SIS) can be decellularised by mechanical scraping and agitation in a peracetic acid solution. Other organs such as porcine liver, heart valves and cartilage, require the use of enzymatic agents and chemical detergents (Badylak et al., 2009; Gilbert et al., 2006; Gilpin and Yang, 2017; Keane et al., 2015; LIN et al., 2004).

One approach to decellularise the whole liver is by perfusion with enzymes and chemical reagents to produce a derived extracellular matrix scaffold ready to be reseeded with new cells (Faulk et al., 2015; Gupta et al., 2018). The perfusion approach is favourable as it preserves the vasculature of the organ and the liver architecture for successful hepatocytes recellularization to achieve good cell engraftment and viability (Lang et al., 2011; Rossi et al., 2019). Retaining all these components after the liver decellularisation is essential for successfully imitating the tissue microenvironmental niche (Badylak et al., 2011).

It is desirable to remove all cellular components but leave behind the ECM architecture, which still need to maintain the organ's structure. One of the approaches in decellularisation methods is a combination of physical, chemical, and enzymatic treatment (Gilbert et al., 2006). One challenge is ensuring the biochemical components and mechanical properties are retained and native tissue disruption is minimised, providing a good quality material post decellularisation (White et al., 2016). Although the effectiveness of decellularisation might be high, the alteration of the ECM may vary depending on the tissue of interest (Mendibil et al., 2020).

Limitations of tissue decellularisation include batch to batch variability and no standardised methodology for decellularised tissues, contributing to the variability in the methodology reported in the literature (Damodaran and Vermette, 2018). Furthermore, in the case of transplantation, a significant drawback is the potential for tissue rejection. An acellular scaffold should not pose a threat to the recipient immune systems; however, many *in-vitro* models have shown a triggered immune response in both allogeneic and xenogeneic transplanted scaffolds (Bilodeau et al., 2019).

Geerts et al. (2016) have combined the traditional decellularisation technique with a non-invasive method to monitor the extent of the cell removal. CT scan was employed to look at the different steps of decellularisation of rat liver to verify the success in the decellularisation by retaining the GAGs content but removing the cellular components (Geerts et al., 2016).

Mazza and his group have also tried to optimise the liver decellularisation methods by decellularising liver tissue using two different approaches with and without the Ethylenediaminetetraacetic acid (EDTA) enzyme. The DNA and proteomics were assessed, showing a reduction of DNA when EDTA has added and preserved the microarchitecture and ECM components. Furthermore, they injected HepG2 into the scaffold and showed cellular engraftment after two weeks. Overall, the addition of EDTA created a more compact matrix which could be used for scaling up and tissue transplantation (Maghsoudlou et al., 2016).

In another similar experiment, Mazza et al. (2017) reported a new methodology for fabricating acellular liver cubes using high shear stress oscillation-decellularization. ECM proteins, the 3D architecture, and their physicochemical properties were retained after decellularisation.

Furthermore, the repopulation potential was investigated by seeding HepG2, stellate cells, and HUVEC, as well as primary hepatocytes. Both parenchyma and non-parenchyma cells grown in acellular liver cubes show different gene expressions to their 2D counterpart. Furthermore, HUVEC migrated in the ECM spontaneously. This unique system showed the potential of creating a complex system comprised of decellularised liver scaffold and cells as a solid *in-vitro* liver model for liver tissue engineering applications (Mazza et al., 2017).

In a different study, Shimoda et al. (2019) evaluated the potential of an acellular liver scaffold in supporting the repair and regeneration after partial hepatectomy. Decellularised acellular liver tissue was implanted into the damaged liver and induced the formation of a blood-like vessel. Cellular infiltration in the scaffold was checked on days 10 and 28, suggesting that the implanted decellularised liver tissue promoted the reorganisation of the liver after resection (Shimoda et al., 2019).

1.5.4 ECM tissue as an *in-vitro* liver model

More specifically, liver ECM models have recently gained interest as they can be employed to study liver tumour microenvironment (Ferreira et al., 2020) as well as being utilised for liver drug metabolism studies (Robertson et al., 2018) and engineering cell function (Xing et al., 2019).

It has been shown that ECM utilised as *in-vitro* models play a crucial role in guiding stem cell differentiation (Hoshiba et al., 2016). However, McQuitty et al. (2020) have also shown *in-vivo* ECM response. When chronic liver damage occurs, it is accompanied by fibrosis, an uncontrolled accumulation of ECM and proteins. Consequently, ECM proteins and DMAPS are involved in the pro and anti-inflammatory response in chronic liver disease (Mcquitty et al., 2020). Therefore, it is thought that ECM has an immunomodulatory role in liver damage and regeneration *in-vivo* (Klaas et al., 2016).

Many research groups over the years have used liver tissue-derived-ECM to study hepatocyte infiltration and functionality (Coronado et al., 2018), liver injury (Rupert et al., 2020), as well as constructing hepatic microenvironments to closely investigate niches for hepatocytes (Grant et al., 2019).

Zhou et al. (2012) demonstrated the capability of maintaining albumin and CYP P450 of primary hepatocyte mixed with a decellularised liver matrix comparable to freshly isolated

hepatocyte. This illustrates the potential of liver ECM as a long-term environment suitable for primary hepatocyte culture (Zhou et al., 2012).

Similarly, Bual et al. (2019) showed that liver-specific ECM matrix scaffold helped to maintain hepatocyte function *in-vitro*. Hepatocytes cultured in liver ECM scaffolds expressed higher liver-specific functions, and hepatocyte spreading was wider across the scaffold (Bual and Ijima, 2019).

Minami et al. (2019) demonstrated that the combination of HiPSCs and rat decellularised liver serving as a scaffold yielded a successful recellularisation which led to albumin expression and secretion in culture medium and cyp3a4 gene activation (Minami et al., 2019).

Asadi et al. (2021) created an engineered construct comprised of the thermoresponsive pNIPAAm hydrogel and decellularised ECM. The structural and functional cues of hepatocyte-like cells were investigated when incorporated in this hybrid scaffold. Hepatocyte-like cells cultured in this hybrid scaffold expressed albumin both at the mRNA and protein level. Furthermore, urea secretion was also expressed, and other hepatic genes like AFP and CK18 were also upregulated (Asadi et al., 2021).

Lee et al. (2017) had developed a bioink from liver decellularised ECM (ECM) to use in the 3D cell printing application. The printability of the dECM bioink was assessed, and the HepG2 cell functions post-printing. HepG2 encapsulated in dECM bioink was also compared with commercial collagen bioink. dECM bioink promoted stem cell differentiation and enhanced HepG2 functions (Lee et al., 2017).

Conclusively, decellularised liver ECM has been proven successful in various applications, from guiding stem cell differentiation to helping maintain hepatocyte functionality. It has also been used as a bioink proving to be a potential candidate for 3D bioprinting (Lee et al., 2017). Furthermore, due to the liver ECM involvement in liver regeneration and damage, many models of liver fibrosis have been reported, and its immunomodulatory role has been investigated (Baiocchi et al., 2016; Mazza et al., 2015; Miyauchi et al., 2017). Liver decellularised ECM tissue has been proven to be an exciting biomaterial to construct and evaluate as a biomaterial for the development of an *in-vitro* liver model.

1.5.5 Tissue derived ECM hydrogels

Liver ECM has been employed for *in-vitro* research in different forms: as an acellular scaffold (Mazza et al., 2017), as a powdered form for coating, or media supplement (Sarika et al., 2020), and as a derived hydrogel (Saheli et al., 2017).

Freytes et al. (2008), discovered the possibility to fabricate a gel from SIS-derived ECM for the growth and support of smooth muscle cells (Freytes et al., 2008). This breakthrough made possible the further characterisation and development of other ECM-derived tissue hydrogels. It has been proved that pepsin cleaves telopeptides and triple helix bodies during enzymatic tissue digestion. This produces a monomeric sequence and breaks down the ECM disrupting the collagen fibre structure. Once solubilised, the homogeneous ECM-viscous solution is ready for the gelation step and the formation of the hydrogel. Gelation is achieved by the self-assembly of the collagen fibres when the temperature is raised to 37 °C. This is an entropy-driven process at the end of which the ECM pre-gel has achieved a 'hydrogel form' (Sawkins et al., 2018).

ECM hydrogels have been derived from different tissues (Sackett et al., 2018) and have also been used in *in-vivo* applications as injectable material following minimally invasive procedures (Spang and Christman, 2018). One of the advantages of ECM-derived hydrogels is retaining water and creating a 3D environment for the cells to integrate. ECM hydrogels are biocompatible, and many cell types retain their morphology when embedded in them (Crapo and Wang, 2010).

In a study conducted by Fernandez-Perez et al. (2019), hydrogels were fabricated from porcine ECM cornea. ECM-derived hydrogels retained fibrillar and porous structure after being formed. Human corneal stromal cells were embedded in them to assess the cornea-derived-ECM hydrogel cytotoxicity. Hydrogels fabricated using SDS during decellularisation were cytotoxic, while the other employed method, freeze-thawing, and triton-X-100, produced cytocompatible hydrogels (Fernández-pérez and Ahearne, 2019).

The advantages of retaining bioactivity, tissue microarchitecture, and a nanofibrous structure (Claudio-Rizo et al., 2018; Sackett et al., 2018; Sawkins et al., 2013; Viswanath et al., 2017), gives the tissue derived-ECM hydrogels an edge to be considered as a possible biomaterial for an *in-vitro* liver model.

1.6 Overarching Hypothesis

The overarching hypothesis is that biomaterials could be used to support hepatic progenitor cell culture and differentiation to advance a 3D *in-vitro* liver model for toxicology studies.

Chemically modified alginate hydrogels could be fabricated for the support of hepatic cells. However, they resulted cytotoxic to the cells and unsuitable for the development of a 3D *in-vitro* liver model. From this part of the research, the importance of chemical substrates on cell behaviour has been demonstrated. As a result of this work, it was decided to explore other potential natural biomaterials which already present moieties and chemical cues within their structure with no need for a further chemical conjugation step.

Thus, we hypothesised that the fundamental components present in native ECM could provide natural biochemical cues and we chose ECM derived biomaterials to be a more suitable candidate for encapsulating hepatocytes cells. Liver-ECM hydrogels could be fabricated from porcine liver decellularized tissue and used for the support of long-term hepatic progenitor cells culture and differentiation towards a hepatic lineage.

The aims of this thesis are outlined below:

- Fabrication of modified alginate by chemical conjugation with small molecules through a ‘click chemistry’ reaction.
- Chemical characterisation of the modified alginate hydrogels and cytotoxicity assessment of encapsulated hepatocytes cells.
- Decellularisation of porcine liver tissue and characterisation of the porcine liver-derived hydrogels, by investigating:
 - The residual ds DNA pre- and post-liver decellularisation
 - Biologically active components pre- and post- liver decellularisation
 - Viscoelastic properties of decellularised ECM hydrogels
- Investigation of porcine liver derived-ECM hydrogels as a biomaterial for culturing and differentiating human hepatic progenitor cells (hHPC) into hepatocyte-like cells.

Chapter II - Materials & Methods

2.1 Materials

Reagents, antibodies, chemicals, general consumables, cell lines and assay kits used in this thesis are listed in Table 2.1. Used equipment are listed in Table 2.2

Table 2.1 List of consumables, chemicals, assay kits, reagents and cell lines used in the project

Item	Supplier	Item Code
<i>T75 Flasks</i>	Fisher	TKT-130190W
<i>1,9-dimethylmethylene blue</i>	Sigma	341088
<i>1-Bromo-3-chloro-propane</i>	Sigma	B9673
<i>2-Chloro-4,6-dimethoxy-1,3,5-triazine</i>	Sigma	375217
<i>2-phospho-l-ascorbic acid trisodium salt</i>	Sigma	49752
<i>3-Ethoxybenzaldehyde</i>	Sigma	516317
<i>4',6-diamidino-2-phenylindole (DAPI)</i>	Sigma	32678
<i>4-methyl morpholine</i>	Sigma	M56557
<i>50 ml tubes</i>	Greiner	210270
<i>96 well plate</i>	Fisher	TKT-180-070U
<i>A83-01</i>	Sigma	SML0788
<i>Acetonitrile</i>	Sigma	70-05-8
<i>Advanced DMEM/F-12</i>	Life Technologies	12634-028
<i>AffinityScript Multiple Temperature cDNA Synthesis Kit</i>	Agilent	200436
<i>Alamar Blue</i>	Invitrogen	A50100
<i>Alginate (food grade)</i>	FMC Biopolymer	
<i>Alginate lyase</i>	Sigma	A1603
<i>Alginate, Pronova UPVLVG</i>	NovaMatrix	4200501
<i>allyl-but-2-ynyl-amine</i>	Sigma	CDS018913
<i>anti KRT19</i>	Sigma	SAB2101302
<i>Anti-EpCAM</i>	Abcam	ab71916
<i>B27 supplement</i>	Life Technologies	17504-044
<i>BME2 organoid matrix, Type 2, RGF</i>	R&D System	3532-010-02

<i>BMP7</i>	Peprtech	120-03
<i>Bradford Reagent</i>	Bio-Rad	5000201
<i>Brilliant III UF MM SYBR QPCR/ Low ROX</i>	Agilent	600837
<i>Calcium chlorides dehydrate</i>	Sigma	223506
<i>Chondroitin – 1,6 – sulphate</i>	Sigma	C4384
<i>Collagenase islet Isolation from Clostridium histolyticum</i>	Bioreagent	9001-12-1
<i>Collagenase NB 4G Proved Grade</i>	Serva	17465.03
<i>Collagenase Type IV</i>	Life Technologies	17104-019
<i>Copper(I) iodide</i>	Sigma	03140
<i>Coverslip</i>	VWR	631-0156
<i>DAPT</i>	Sigma	D5942
<i>Deoxycholic acid solution</i>	Sigma	D2510
<i>Deuterium oxide</i>	Sigma	7789-20-0
<i>Dexamethasone</i>	Sigma	D4902
<i>Dibenzocyclooctyne-amine (DBCO)</i>	Sigma	761540
<i>Dimethyl sulfoxide</i>	Sigma	D5879
<i>Dispase</i>	Invitrogen Corporation	17105-041
<i>DMEM</i>	Fisher Scientific	10749764
<i>donkey anti-rabbit Alexa Fluor® 568</i>	LifeTechn	A10042
<i>Eagles' minimal essential medium</i>	Sigma	M4655
<i>Earle's balanced salt solution HyClone</i>	Fisher	SH30029.02
<i>EDTA</i>	Fisher Sci	D/0700/53
<i>EGF</i>	Peprtech	AF-100-15
<i>FBS</i>	Fisher Scientific	10664083
<i>FGF</i>	Peprtech EC	100-18
<i>FGF10</i>	Peprtech	100-26
<i>FGF19</i>	Peprtech	100-32
<i>FSK</i>	Tocris	1099
<i>Gastrin</i>	Sigma	G9020
<i>Glucose</i>	Fisher Sci	50-99-7

<i>Glycogen</i>	ThermoFisher	R0561
<i>goat anti-rabbit CY5</i>	Abcam	ab6564
<i>Harris Haematoxylin</i>	Sigma	HHS32
<i>HEPES Buffer</i>	Sigma	H4034
<i>HepG2</i>	ATCC	2019
<i>HGF</i>	Peprtech	100-39
<i>Human Albumin ELISA Kit</i>	Abcam	ab179887
<i>Human skin fibroblast cells (HS137)</i>	ATCC	
<i>hydroquinone</i>	Sigma	123-31-9
<i>Isopropanol</i>	Fisher Sci	C3H8O
<i>ITS + premix</i>	Sigma	I3146
<i>L Wnt-3A cells</i>	ATCC	CRL-2647
<i>L/Glutamine</i>	Invitrogen	25030-024
<i>L-cysteine</i>	Sigma	C1276-50G
<i>LIVE/DEAD™ Viability/Cytotoxicity Kit</i>	Invitrogen	L3224
<i>Low attachment 48-well plates</i>	Greiner	677970
<i>Methanol</i>	Fisher Scientific	67-56-1
<i>N2 supplement</i>	Life Technologies	17502-048
<i>N-Acetylcysteine</i>	Sigma	A9165-5G
<i>Nicotinamide</i>	Sigma	N0636
<i>Noggin</i>	Peprtech	120-10C
<i>Non-Essential Amino Acids</i>	Gibco	11140-035
<i>OCT</i>	VWR	25608-930
<i>P450-Glo™ CYP3A4 Assay</i>	Promega	V9001
<i>Papain</i>	Sigma	P3375-25MG
<i>Paraformaldehyde (PFA)</i>	Sigma	P6148
<i>PCRBIO Probe Mix Lo-ROX</i>	PCR Biosystem	PB20.21-20
<i>Pen/Strep</i>	Invitrogen	15070-063
<i>Penicillin and Streptomycin</i>	Invitrogen	15070-063
<i>Pepsin</i>	Sigma	P7012-250MG
<i>Peracetic acid</i>	Acros Organics	257755000
<i>Phenol/Chloroform/Isoamyl</i>	Sigma	77617
<i>Phosphate buffered saline PBS</i>	Invitrogen	14190-169
<i>Prestoblue</i>	Fisher Scientific	A13262

<i>Propargylamine</i>	Sigma	P50900
<i>Proteinase K</i>	Invitrogen	25530-049
<i>Quant-iT™ PicoGreen™ dsDNA Assay</i>	ThermoFisher	P11496
<i>Rabbit Anti-Ki67 antibody</i>	Abcam	ab16667
<i>Random Primers</i>	Promega	C1181
<i>Rb anti-human Ki67</i>	Invitrogen	MA514520
<i>Recombinant collagenase COL H</i>	Abiel Biotech	
<i>Recovery™ Cell Culture Freezing Medium</i>	Life Technologies	12648-010
<i>RIPA Lysis and Extraction Buffer</i>	ThermoFisher	89900
<i>RNAlater</i>	Invitrogen	AM7020
<i>RNase-Free DNase Set</i>	Qiagen	79254
<i>RSpo1-Fc 293T cells</i>	Stanford University	
<i>Sodium bicarbonate</i>	Sigma	S8761
<i>Sodium dodecyl sulfate</i>	Sigma	L3771
<i>Sodium Hydroxide</i>	Fisher Sci	S/4930/17
<i>Sodium Pyruvate</i>	Fisher Sci	11360070
<i>Sterile 10ml pipettes</i>	Fisher	PMK-345-092Q
<i>Sterile 25 ml pipettes</i>	Fisher	PMK-345-111P
<i>Sterile 5ml pipettes</i>	Fisher	PMK-345-067P
<i>Trichrome Stain (Masson) Kit</i>	Sigma	HT15
<i>Triethylamine</i>	Sigma	471283
<i>Tri-reagent</i>	Sigma	T9424
<i>Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methylamine</i>	Sigma	678937
<i>Triton X-100</i>	Sigma	T8787
<i>Trizol</i>	Sigma	T9424
<i>Trypan Blue</i>	Sigma	72-57-1
<i>Trypsin</i>	Invitrogen	25200-056
<i>Trypsin 0.25%</i>	Invitrogen	25200-056
<i>Tween-20</i>	Sigma	P9416
<i>Tyramine</i>	Sigma	W421501

VECTASHIELD	Vector Laboratories	H-1400
William's E Medium	US Biological	38210000
WY-14643, Pirinixic Acid	Selleckchem.com	
Xylenes	Sigma	534056
Y-27632	Sigma	Y0503

Table 2.2 List of equipment used in the project

Item	Supplier
Beckman Coulter FC500 flow cytometer	Beckam
2100 Bioanalyzer Instrument	Agilent Technologies
AriaMx Real-Time PCR System (RT-PCR)	Agilent Technologies
Coffee grinder	Krups, F203
Confocal Microscope, Zeiss LSM 880	Zeiss
Cryostat, CM2018	Leica
DM4000B light microscope digital camera	Leica
Fluorescence Microscope, Zeiss 200M	Zeiss
FT-IR	Agilent Technologies
H^1 -NMR Bruker 400MHz	Bruker
Heating Block, TRIO-ThermoBlock	Biometra,
Homogeniser Model PRO 200	PRO Scientific Inc.
Kitchen mandalin	Chef's inspiration Premium V-blade Mandolin, Amazon
Leica DM IRB	Leica
NanoDrop2000, Spectrophotometer	Fisher Scientific
Orbital shaker	OHAUS
Plate Reader, FLUOstar Omega	BMG LABTECH
Rheometer	Anton Paar, Physica MCR 301
Tecan plate reader, Infinite M200	Tecan
Texture Analyser	TA.HD.plus, Stable Micro System
TP1020 Tissue Processor	Leica
Leica 2245 microtome	Leica

2.2 Cell culture

2.2.1 HepG2

HepG2 cells were cultured at 37 °C with 5% CO₂ and used until passage 20. Briefly, hepatocytes cells were cultured in Eagles minimal essential medium with 10% foetal bovine serum (FBS), 1% glutamine, and 1% non-essential amino acids.

2.2.2 HS137

Human skin fibroblast cells (HS137) P3-7 were cultured at 37 °C with 5% CO₂. Briefly, human skin fibroblasts were cultured in Eagles minimal essential medium with 10% foetal bovine serum and 1% glutamine.

2.2.3 EPCAM⁺ sorted human progenitor liver cells

Progenitor liver cells L252, L264, L266, and L269 EPCAM⁺ single sorted cells using Flow cytometry were cultured in 48 low attachments well plate at 37 °C with 5% CO₂ and used until passage 10. Briefly, organoids were formed and maintained in the BME2 matrix from single cells dissociation. Expansion Media (EM) was refreshed every three days, and cells passaged every 7-10 days as the matrix filled large organoids. Single cells are first placed in Starting media (SM) which is supplemented with 25 ng/ml Noggin, 10% RSPO1 conditioned media, 30% Wnt3a conditioned medium and 10 µM Y27632 for the first 3-4 days to support organoids formation. The homemade described conditioned media was prepared as per Fatima instructions followed in her thesis materials and method section. Then the media is changed to Expansion media (EM). Expansion media (EM) is made up with Advanced DMEM/F12 and supplemented with 1% N2, 1% B27, 1.25 mM N-Acetylcysteine, 10 nM gastrin, 10% RSPO1 homemade conditioned media, 10 mM Nicotinamide, 5 µM A83.01, 10 µM FSK and the growth factors: EGF 50 ng/ml, FGF10 100 ng/ml and HGF 25 ng/ml all purchased from. The components of the media with the relevant working concentration can be found in Table 2.3.

Human Tissue and human-derived cells extracted from liver tissues are covered under 04/Q2403/70 ethical approval under which we worked with the tissues in the Lab and used the consent from the School of Clinical Sciences, Tissue Bank in the QMC. The human

derived- cells (human progenitor liver cells) were extracted from L252, L264, L266 and L269 liver donor tissue.

Table 2.3 Components used in the media to differentiate hepatic progenitor cells towards a hepatocyte-like phenotype.

Components	Working concentration
Total volume DMEM F12	-
B27	(1x)
N2 supplements	(1x)
HGF	(25 ng/ml)
EGF	(50 ng/ml)
Gastrin	(10 nM)
A83 01 (TGF B inhibitor)	(5 μ M)
FGF 19	(100 ng/ml)
BMP7	(25ng/ml)
DAPT	(10 μ M)
Dexamethasone	(30 μ M)

Hepatocyte differentiation media (Hep-DM)

Differentiation media is made up of some Expansion media components with the addition of the growth factors: FGF19 and BMP7 at a concentration of 100 ng/ml and 25 ng/ml, respectively and the small molecules DAPT and Dexamethasone at a concentration of 10 μ M and 30 μ M respectively.

Cholangiocyte differentiation media (Chol-DM)

Hepatic progenitor cells were also differentiated towards a cholangiocyte phenotype using the components and working concentrations listed in the Table 2.4

Table 2.4 Components used in the media to differentiate hepatic progenitor cells towards a cholangiocyte-like phenotype.

Components	Stock Concentration	Working Concentration
		-
William's E Medium	5 X	1X
Glucose	1M	14mM
Sodium bicarbonate 7.5%	893mM	17mM
NicotinAmide	1M	10mM
Dexamethasone	0.1mM	0.1µM
ITS + premix (1:100 recommended dilution)	100X	1%
Sodium Pyruvate	100mM (100X)	6.3mM
HEPES Buffer	1 M	20mM
The 2-phospho-l-ascorbic acid trisodium salt	10mg to (0.2M)	0.2mM
EGF	20µg/ml	20ng/ml

2.3 Alginate synthesis

2.3.1 Alginate amidation

Alginate (Pronova UPVLVG from NovaMatrix, 1 Equiv., 100 mg = 0.52 mmol of COOH available for reaction) was dissolved as a 2% alginate solution in a 3:2 water: acetonitrile mixture (5 ml total volume). Amine [11-azido-3,6,9-trioxaundecan-1-amine, Dibenzocyclooctyne amine (DBCO)-MGA 56 and Tyramine+DBCO (1:1)-MGA 57] (1 Equiv.,) were then added to the mixture along with the coupling agent 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 0.5 Equiv., 45 mg) and 4-methyl morpholine (NMM, one Equiv., 56µl). The mixture was stirred at 55 °C overnight, and the solvent was removed under reduced pressure. The resulting solid was dissolved in 5ml water and dialysed against a 10,000 MWCO dialysis membrane overnight with DI water to purify the polymer further. The resulting solution was then lyophilised to get purified compound.

2.3.2 Huisgen cycloaddition

In another reaction, alginate (1 Equiv., 100 mg = 0.52 mmol of COOH available for reaction) was dissolved as a 2% alginate and reacted with 11-azido-3,6,9-trioxaundecan-1-amine dissolved in a solution of water: methanol 1:1 (5 ml total). Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methylamine (TBTA, 0.2 equiv, 50 mg), triethylamine (0.25 equiv., 15 μ l), Copper(I)-Iodide (0.25 equiv, 5 mg) were added as coupling agents. Then 0.51 mmol of the respective alkyne [propargylamine (MGA 42), allyl-but-2-ynyl-amine. HCl (MGA 53) and 3-ethynyl benzaldehyde (MGA 54)] was added, and the mixture was stirred at 55 °C overnight. The solvent was removed under reduced pressure. The obtained solid was re-dissolved in 5 ml of water and dialysed against a 10,000 MWCO dialysis membrane overnight with DI water to further purify the polymer. The resulting solution was then lyophilised to obtain purified compound. This protocol was adapted from Vegas et al. (2016).

2.3.3 H^1 - NMR of alginates

4-5 mg of alginate and each modified alginates were dissolved in 600 μ l of deuterium oxide (D_2O). The samples were then placed in specific H-NMR tubes, and they were run on H^1 -NMR Bruker 400MHz instrument, following set up instructions.

Estimation of PEG_3-N_3 group substitution on alginate polymer

4 mg of N_3-PEG_3-ALG was dissolved in 700 μ l of D_2O to which 0.275 mg of hydroquinone was added. The peak of hydroquinone at 6.7 ppm (4H) was used as a reference to calculate the concentration of N_3-PEG_3 groups in the system as shown in Figure 2.1.

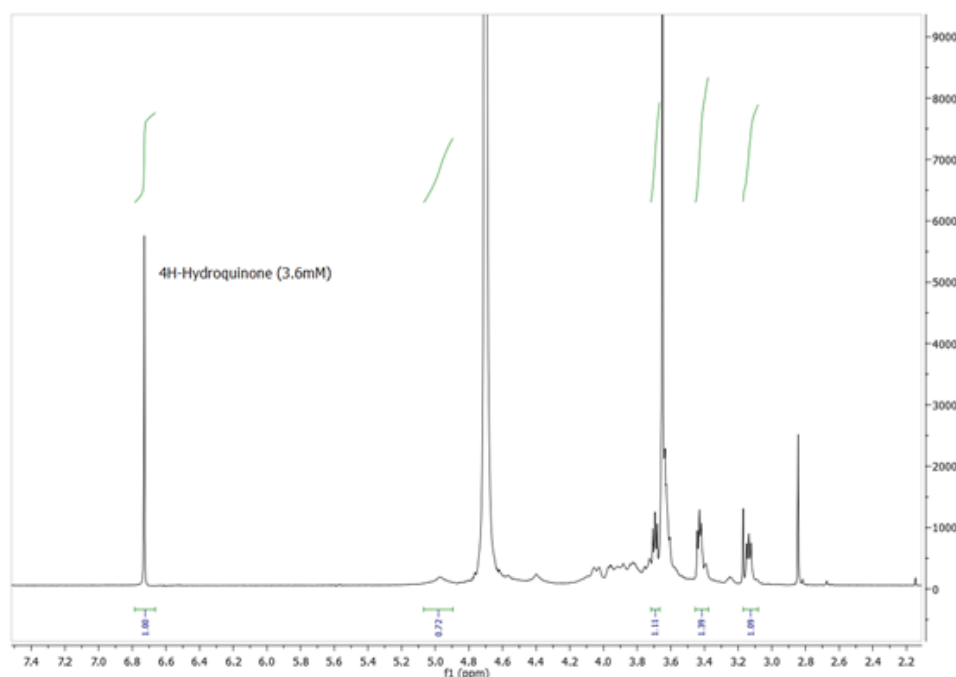


Figure 2.1 ^1H NMR spectra of 4mg $\text{N}_3\text{-PEG}_3\text{-ALG}$ / 700 μl D_2O along with a known concentration of an internal standard (hydroquinone, 3.6 Mm).

The amount of $\text{NH}_2\text{-PEG}_3\text{-N}_3$ appended to alginate was estimated to be 0.1 $\mu\text{mole}/\text{mg}$ of polymer (approx. 20% substitution) using ^1H NMR compared with an internal standard (hydroquinone) of known concentration. The free azide groups were then quantitatively clicked with propargylamine, giving click induced hydrophobic flank (triazole+ $\text{CH}_2\text{-NH}_2$) to form MGA 42. So MGA42 has approximately 20% substitution of the $\text{PEG}_3\text{-triazole ring-CH}_2\text{NH}_2$ and 80% free carboxylic acid. Below is the FTIR spectra of $\text{ALGPEG}_3\text{N}_3$ and MGA42.

2.3.4 FTIR of alginates

A small amount of dry sample from medical grade alginate and the five modified alginates (MGA42,53,54,56 and 57) were places on the FTIR instrument. The instrument crystal was then lowered until it touched the samples and readings were recorded as per manufacture instructions.

2.3.5 Fabrication of chemically modified alginate hydrogels

Food grade alginate (FA), medical-grade alginate (MGA) and modified alginates solutions were prepared by dissolving 12-14mg in 1 ml of sterile Dulbecco modified Eagles medium to obtain a final working concentration of $\sim 1.2\%$ w/v. These solutions were filtered, UV sterilised and sonicated for 30 minutes before cells encapsulation. Calcium chloride dehydrates (CaCl_2) was used to ionically crosslinking all alginate hydrogels with encapsulated

HepG2 cells. A final concentration of 100 mM was prepared in deionised water and added onto the encapsulated alginates hydrogels for 1-2 minutes until fully crosslinked.

2.4 Liver ECM preparation

The porcine liver was purchased from R B Elliott's and Sons in Calow as a whole as shown in Figure 2.2 (A). The organ is then cut up in big chunks (40-60g) and froze until needed. A portion of the frozen tissue is then cut into 3-4 cm strips. The porcine liver was sliced using a kitchen mandolin into thin slices using the thinnest settings and then washed as shown in Figure 2.2 (B) Figure 2.2 (C) respectively.

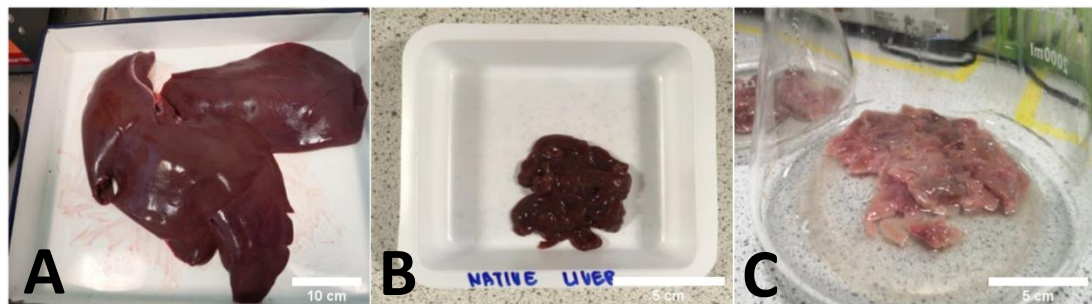


Figure 2.2 (A) Entire Porcine liver before preparation, scale bar 10 cm. (B) Sliced porcine liver with a mandolin tool, scale bar 5cm. (C) Porcine liver slice after a few washes to remove excess blood, scale bar 5cm.

2.4.1 Porcine liver decellularisation process

The porcine liver slices were placed into a 2L conical flask with 1.5L of dH₂O and agitated on a mechanical shaker at 200 rpm for 30 minutes as shown in Figure 2.3 (A). The process is repeated three times, and the tissue is gently pressed against a mesh after every wash. Dr Simon Kellaway, Dr Rabea Loczenski and Josh Jones have optimised the method for decellularisation following previously published methods (Loneker et al., 2016; Mazza et al., 2015). On the first detergent wash, the tissue is placed in a conical flask with a 0.02%trypsin/0.05%EDTA solution for 1 hour on a mechanical shaker at 200 rpm as shown in Figure 2.3 (B). The tissue strips were washed three times for 15 minutes with dH₂O. On the second detergent wash, the tissue was placed in the 3% Triton-X-100 solution and agitated at 300 rpm for 1 hour on a mechanical shaker as shown in Figure 2.3 (C). The tissue strips were washed three times for 15 minutes with dH₂O. On the last detergent wash, the tissue strips were placed in 4% Deoxycholic acid solution (98% HPLC) and agitated with a mechanical shaker at 200 rpm for 1 hour Figure 2.3 (D). The tissue strips were then washed three times

for 15 minutes with dH₂O. To disinfect the tissue, a treatment with peracetic acid (PAA) was applied. The decellularised liver material was added into 0.1% PAA solution and placed on a magnetic stirrer for 2 hours at medium speed Figure 2.3 (E). The material was washed with PBS and placed back on the mechanical stirrer at 200 rpm for 15 minutes three times. The tissue was then snapped, froze, and lyophilised for three days.

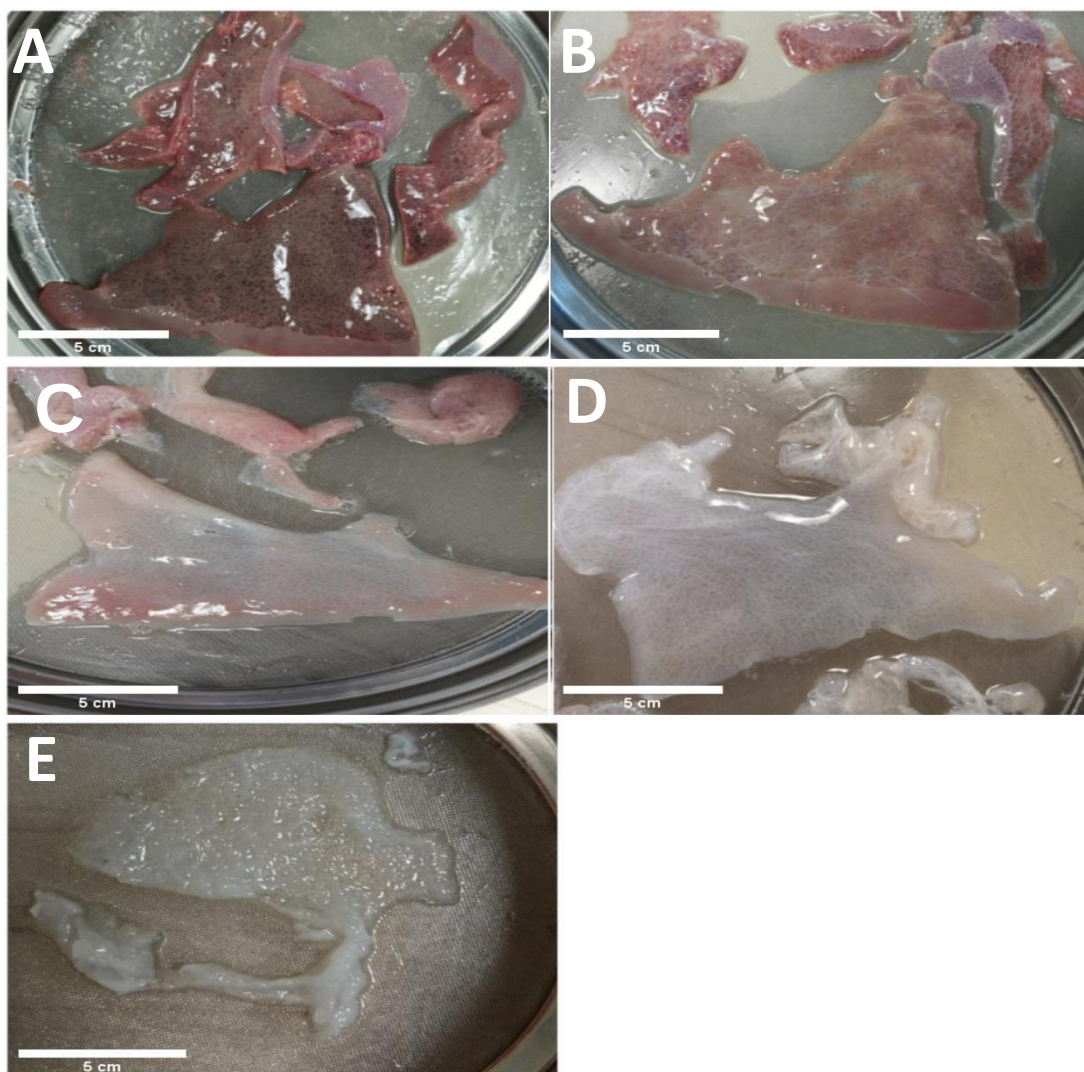


Figure 2.3 (A) Liver tissue after three washes, scale bar 5 cm. (B) Liver tissue after the trypsin/EDTA treatment, scale bar 5 cm. (C) Liver tissue slices after 3% Triton-X-100 solution, scale bar 5 cm. (D) Liver tissue slices after 4% Deoxycholic acid solution, scale bar 5 cm. (E) Liver tissue slices after PAA treatment, scale bar 5 cm.

2.4.2 Liver dECM hydrogels fabrication

After obtaining a lyophilised form of decellularised ECM, the ECM was ground using a ground coffee miller in small pieces as shown in Figure 2.4 (A). To solubilise the liver, the ECM Freytes method was employed (Freytes et al., 2008), and 1 g of dry ECM was mixed with 100 mg of pepsin in a 100 mL of 0.01N HCL solution. The resulted digest was then left to stir for 2-4 days at room temperature. This will yield a final concentration of 10 mg/ml stock ECM digest as shown in Figure 2.4 (B). From this stock, gels can be formed at different concentrations once neutralised with the neutralisation buffer. The gels are placed in a mould and left in the incubator for 40 min to solidify as shown in Figure 2.4 (C).

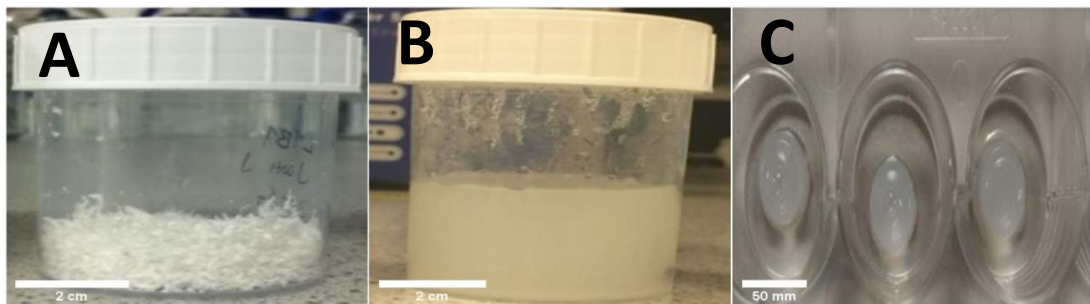


Figure 2.4 (A) Lyophilized and milled liver dECM, scale bar 2cm. (B) Liver dECM tissue digest (10mg/ml concentration), scale bar 2cm. (C) Liver dECM hydrogels once gelation has occurred and solid hydrogels are created, scale bar 50mm.

2.4.3 Neutralisation buffer composition and dECM hydrogel fabrication

To form ECM hydrogels a neutralisation buffer is mixed with the pre-gel digest as shown in Figure 2.4 (B). The neutralisation buffer is prepared by using a combination of 1xPBS, 0.1N NaOH and x10 MEM.

The required volume of PBS, 10xMEM and 0.1N NaOH was added together to an appropriate container and kept on ice until the ECM digest stock (10mg/ml) was added to fabricate the ECM hydrogels at the required concentration. ECM stock and Neutralisation buffer were added together and mixed. Gelation was initiated once the samples were placed at 37 °C.

2.5 Liver ECM characterisation

2.5.1 Liver tissue digestion for DNA quantification

100 mg of liver tissue or ECM-derived matrix was weighted into a 15 ml tube after being lyophilised and cut into small pieces. The buffer solution was made up of 1M Tris -HCl (pH 8.0), 5M NaCl, 0.5 M EDTA (pH 8.0), 10% SDS and deionised water. 5 µl of proteinase K per 1ml of buffer solution was made to make the proteinase K buffer. The tissue was digested by adding 2 ml of proteinase K buffer at 50 °C for 24 hours or until no visible tissue remained. These samples were utilised to analyse the DNA amount in the liver tissue pre and post decellularization

2.5.2 dsDNA quantification

2 ml of phenol/chloroform/IAA (25:24:1) was added to purify the proteinase K digested samples which were centrifuged afterwards at 10'000 rcf for 10 minutes at 4 °C. The aqueous phase was removed and placed in another tube. Once no more debris was observed, 200 µl of 3 M Sodium Acetate (pH 5.2) was added to each sample. Next, 100% ice-cold ethanol was added to each sample and centrifuged for 10 minutes at 10'000 rcf. The supernatant was discarded, and the empty tubes were centrifuged again at 10'000rcf for 1 minute at 4 °C. The pellet was left to dry for 10 minutes at room temperature. Liver samples were diluted 1:1000, and TE buffer was added to the required number of samples. The PicoGreen reagent was diluted 200-fold as per manufacture instructions. Finally, a DNA standard curve was prepared per manufacture instructions. Samples and DNA standards were added on a 96 well black flat bottom plate, and 100 µl of Quant-iT PicoGreen reagent was added to each sample. The plate was protected from light and incubated for 5 minutes at room temperature. The plate was read using a fluorescence plate reader with an excitation wavelength of 480 nm and 520 nm.

2.5.3 Glycosaminoglycan quantification

A papain solution was prepared by adding 125 µg/ml of papain, 10mM L-cysteine, 10mM EDTA and 100 mM Na₂HPO₄. Liver ECM dried samples were weighed and digested by adding 1 ml of papain solution per 10mg of ECM sample. The samples were vortexed and placed in the oven at 65 °C until fully solubilised. The Dimethyl methylene Blue reagent (DMMB) was prepared by dissolving 16 mg/L of DMMB, 40mM glycine and 40 mM NaCl. The solution was then adjusted to a pH of 1.5 with hydrochloric acid; this was chosen to minimise the non-sGAGs artefact such as hyaluronic acid (HA) and DNA solutions that can generate a signal at pH 3 (Zheng and Levenston, 2015). The standards were prepared by creating a serial dilution of chondroitin sulphate. 20 µl of standards and samples were added into the plate in triplicates, and 200 µl of DMMB reagent was added to each sample. Samples and standards were analysed using a plate reader within 10 minutes, and the absorbance was read using a wavelength of 525nm and a reference wavelength of 595 nm.

2.5.4 Liver ECM mechanical characterisation

Rheology

Crosslinked ECM derived hydrogels were fabricated at different concentrations and the storage and loss moduli were measured using a rheometer. ECM derived samples were compared to BME2 gels. A Rheological test was performed using a Rheometer with a parallel plate probe. A rotation of 1000 1/s was applied before each test run. The test was carried out at an amplitude gamma of 0.5 % and increasing angular frequency from 0.01 to 200 rad/s. Four different tests were carried out: a time sweep, a frequency sweep, an amplitude sweep and a temperature sweep as depicted in Table 2.5.

Table 2.5 Parameters and tests used to measure the viscoelastic properties of liver ECM hydrogels at different concentrations and BME2 matrix.

	Time Sweep	Frequency Sweep	Amplitude Sweep	Temperature Sweep
Parameters	T= 37 °C γ=1% ω=1 rad/s	T= 37 °C γ=1% ω=0.01-200 rad/s	T= 37 °C γ=0.01-200% ω=1 rad/s	T= 4-25 °C γ=1% ω=1 rad/s

Texture analyser

The Texture analyser was used to measure the crosslinked ECM derived hydrogels' stiffness at different concentrations. ECM gels were prepared at 4, 6 and 8 mg/ml concentrations by injecting 250 µl of ECM pre-gel into a Teflon mould of 1 cm in diameter which was left in the incubator for 40 minutes. A compression method was used to obtain Young's Modulus. The samples were placed underneath the compression probe and the test was carried out up to 50% strain, 0.05 mm/s test speed, and with a load cell sensitivity of 0.005 N. Only the linear region of the curve was considered to obtain the modulus. Samples were done in quadruplicate.

Liver dECM hydrogel swelling

250 µl of decellularised porcine liver ECM solution was placed in circular moulds. ECM samples were made from 2, 4 and 8 mg/ml ECM concentrations to study a range of swelling behaviour. All samples were left in the incubator to solidify for around 40 minutes. The weight of the gels was measured at 0, 2,4,6,24,96 hours and 7 and 14 days. Gels were left incubating in DMEM media solution for the time points specified. After each time point, the gels were removed from the media solution and any remaining media on top of the gels was gently taken up by filter paper to minimise the weighting error and weighted samples.

The swelling ratio (Q) at each data point was calculated using the following equation (1) from the work of Raquel Silva (Silva et al., 2018) .

$$Q = \frac{(W_f - W_i)}{W_i} \quad (1)$$

W_f = weight of the sample at each timepoint

W_i = weight of the sample at 0 hour

Q = Swelling ratio

2.5.5 Liver ECM histology

PFA fixation and paraffin embedding of porcine liver samples

Both ECM and native liver pieces were fixed with 4% PFA solution overnight. Samples were transferred in cassettes and placed in a tissue processor, where they were transferred from container to container to be processed. This was done with an automated process set up by the user. The process of paraffin embedding involves three steps. Firstly, specimens are immersed in a series of ethanol solutions of increasing concentration (from 50% until pure). This dehydration step is necessary to remove water in the specimen to be then allowed to infiltrate with wax. The second step involves a clearing phase, where a solvent (xylene) is involved in rendering both ethanol and paraffin wax miscible. Specimens will be displaced in xylene solution for a clearing sequence until ethanol is displaced completely. Thirdly, the tissue can now be infiltrated with paraffin wax to be sectioned. Paraffin-embedded tissues were formed into a block. This step allows for the correct orientation of the sample in the mould, and a cassette is placed on top, which is topped up with more wax and placed on a cold plate to solidify.

Sectioning of paraffin-embedded liver samples

When the wax was solidified, the block could be removed, and the cassette containing the tissue was ready to be sliced with a microtome. Specimens were sliced at a 10 µm thickness and stored for further processing.

Haematoxylin and Eosin staining of Porcine liver sections

Liver sections slides were deparaffinised and rehydrated through a graded series of alcohol washes at different concentrations. Initially, slides were immersed in xylene for 5 minutes, then in the fresh xylene for 2 minutes, twice in 100% ethanol for 40 seconds, then in 95%, 70% and 50% ethanol for 40 seconds each. The slides were then submerged in tap water for 2 minutes. The sections were stained with Harris Haematoxylin solution for 5 minutes and then washed with running tap water for a further 5 minutes until clear. The sections were immersed in 1% acetic acid for 10 seconds, then in running tap water for 1 minute, and finally, they were dipped in Scott's tap water for 15 seconds. The sections were then counterstained with Eosin solution for 5 minutes, followed by a final wash with tap water. Slides were mounted with mounting media, and images were taken using a light microscope with digital camera (Q-imaging MircroPublisher 3.3 RTV software).

Masson trichrome staining of liver sections

Initially, slides were immersed in xylene for 5 minutes, then in the fresh xylene for 2 minutes, twice in 100% ethanol, then in 95%, 70% and 50% ethanol for 40 seconds each. The slides were then submerged in tap water for 2 minutes. The slides were added in a glass container suited for the microwave, and Bouin solution was poured in. The slides were microwaved at 600W for 25 seconds. The sections were then left for 10 minutes to incubate prior to a washing step. Then, Haematoxylin of Gill was added into the container with the slides and microwaved at 800W for 5 seconds. The slides were washed carefully for 60 seconds. Scott's water was added to the slides for 1 minute. Biebrich scarlet acid fuchsin solution was added to the slides and then microwaved at 600W for 20 seconds. The slides were then incubated for 2 minutes and frequently washed with water. Phosphomolybdic acid solution was added to the slides and microwaved at 600W for 20 seconds. Slides were carefully washed with water a few times. Finally, aniline blue solution was added to the slides and microwaved at 600W for 15 sec. The slides were then placed into 1% acetic acid solution for 30-60 seconds and washed thoroughly. A coverslip with mounting media was added and sealed with nail polish.

2.6 Cell/hydrogel constructs

2.6.1 2D seeding of HepG2 on top of liver dECM hydrogels

ECM-derived scaffolds were made at two different concentrations, 4 and 6 mg/ml, respectively. Scaffolds were fabricated using Teflon moulds and sterilised by UV for 30 minutes and 24 hours incubation with media containing 1% AB/AM before seeding. The cells were seeded at a concentration of 53'000 cells/cm², which was enough to cover the ECM hydrogels surface. Media was changed every two days, and on day three, cells were stain with a live and dead kit for cytotoxicity assessment. Bright-field images to check cell morphology and spreading were taken with a microscope Leica DM IRB after 6, 24 and 72 hours.

2.6.2 3D encapsulation of HepG2 in liver dECM hydrogels

HepG2 cells were detached from the flask, spun down, and counted. HepG2 cells were encapsulated in 25 µl of BME2 matrix or 2 and 3 mg/ml ECM at a concentration of either 5'000 or 20'000 cells per gel. The seeding density were chosen based on the high proliferation potential of HepG2 with a late and early time point analysis, respectively. Gels were allowed to be set in the incubator for 25-40 minutes (depending on gel concentration) before 250 µl of Eagles minimal essential medium was added to each well. Media was exchanged every 3-4 days.

2.6.3 3D encapsulation of HS137 in liver dECM hydrogels

Human skin fibroblast cells P6 were detached from the flask, spun down and counted. HS137 cells were encapsulated in 25 µl of BME2 matrix or 2 and 3 mg/ml ECM at a concentration of 2'500 cells per gel. Gels were allowed to be set in the incubator for 25-40 minutes (depending on gel concentration) before 250 µl of Eagles minimal essential medium was added to each well. Media was exchanged every 3-4 days.

2.6.4 3D encapsulation of EpCAM + Human progenitor liver cells in liver dECM hydrogel

Briefly, single-cell suspension of L252, L264, L266 and L269 human progenitor liver cells were sorted for EpCAM marker using Flow cytometry. These cells were only used between passage 3-10. Single sorted cells were encapsulated in 25 µl of BME2 matrix or 1,2, 3,4,6, and 8 mg/ml ECM-derived hydrogel at a concentration between 8'500- 20'000 cells per gel. Gels were allowed to be set in the incubator for 25-40 minutes (depending on gel concentration) before 250 µl of the starting media (SM) was added to each well. After three days, media was replaced with expansion media (EM) and changed every 3-4 days.

2.6.5 Differentiation protocol – Organoid's differentiation toward hepatocyte phenotype

Liver organoids were seeded in BME2 and liver ECM hydrogels at different concentrations. Organoids were expanded for seven days, and then the Expansion media was changed to Expansion media supplemented with BMP7 (25 ng/ml). Organoids were kept in this media

for 2-5 days. After that, the EM+BMP7 was exchanged for hepatocyte differentiation medium (Hep-DM) for a range of days varying between 7- and 11-days Figure 2.5. The Hep-DM was changed every three days. Subsequently to the differentiation protocol, gene expression of progenitor organoids for mature hepatocytes genes was measured.

Hep-DM Protocol Pipeline

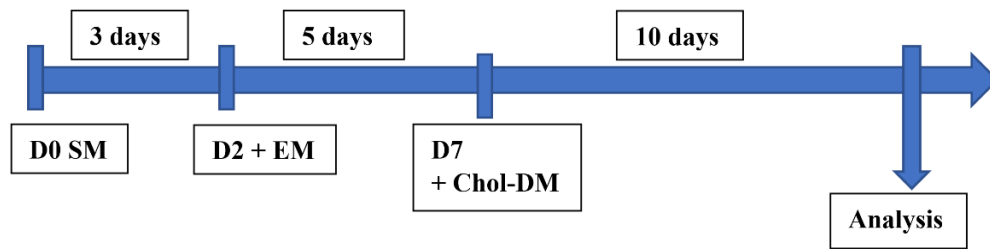


Figure 2.5 A pipeline of the differentiation protocol is used to differentiate hepatic progenitor towards hepatocytes like phenotype.

2.6.6 Differentiation protocol – Organoid's differentiation toward Cholangiocyte phenotype

Liver organoids were seeded in BME2 and liver ECM hydrogels at different concentrations. Organoids were expanded for 7-8 days, and then the Expansion media was changed to Cholangiocyte differentiation media (Chol-DM) for ten days Figure 2.6. Chol-DM media was changed every three days. Subsequently to the differentiation protocol, gene expression of progenitor organoids for mature cholangiocyte genes was measured.

Chol-DM Protocol Pipeline

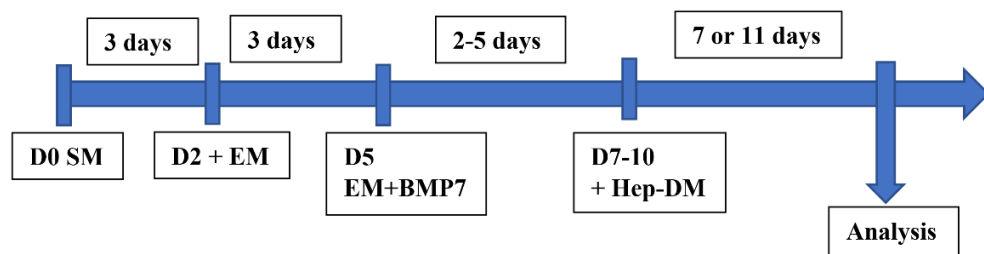


Figure 2.6 Pipeline of the differentiation protocol used to differentiate hepatic progenitor towards a cholangiocyte-like phenotype.

2.6.7 HepG2 encapsulation in Chemically modified Alginate's hydrogels

Firstly, cells were trypsinised as per standard protocol and counted. A final cell density of 10'000 cells per well was achieved. Briefly, cells were resuspended in media containing the desired cell density. The supernatant was aspirated. The cell pellet was mixed with alginate hydrogels to achieve a final volume of 30µl per well containing 10'000 cells. 80µl of 100 mM CaCl₂ solution was added drop by drop to crosslink each alginate/cells mixture and then left at room temperature for 1-2 minutes to crosslink. The CaCl₂ solution was then removed, and a solution of Eagles minimal essential medium with 1% antibiotic/antimitotic was added to the alginate hydrogels as a washing step. After two washing steps, the washing solution was discarded, and 200µl of culture media was added to each well. The encapsulated alginate hydrogels were culture in 96 well plates and incubated at 37 °C in a 5% CO₂ humidified environment; media was exchanged every two days. FA and MGA encapsulated alginate hydrogels were used as controls. A total of three replicates were fabricated for each condition.

2.6.8 Digestion of dECM hydrogels for the retrieval and subculture of progenitor liver cells

Liver progenitor organoids in liver ECM hydrogels were cultured as per standard protocol (Method section 2.6.4). After 7-8 days, when the ECM was full of organoids, 5 different Collagenase solutions were prepared to disrupt the matrix and retrieve the organoids.

- Dispase (1.8 U/mg)
- Collagenase Type IV (160 U/mg)
- Collagenase islet Isolation from *Clostridium histolyticum* (125 U/mg)
- Collagenase NB 4G Proved Grade from *Clostridium histolyticum* (0.13 U/mg)
- Recombinant collagenase COL H (50 U/mg)

All the collagenase mentioned above were reconstituted with DMEMF12 at a given concentration. 300 µl was added to each well. The samples were incubated on a shaker for 1-2 hours in an incubator at 37 °C to extract the organoids from the matrix fully. Once extracted, organoids were either fixed or subculture in new liver ECM hydrogels.

2.7 Cell assays

2.7.1 Live/dead viability fluorescent staining

Live/Dead staining was used to assess the cell viability of HepG2 following culture in chemically modified alginates and on the 4 and 6 mg/ml ECM-derived scaffolds at different timepoints. Briefly, cells were washed with PBS and incubate for 20 minutes with 1 ml of DMEM containing 3 μ M of EthD-1 and 0.75 μ M of Calcein-AM. Next, cells were imaged with a fluorescence microscope at 5x and 10x magnification. The experiment was performed in duplicates for each condition, and three images per scaffold were taken at three different positions within the scaffold. The percentage of viable cells was calculated by dividing the number of live cells by the total sum of live and dead cells. Mean and standard deviation was extrapolated from six images taken per condition.

Similarly, Live/Dead staining was used to assess cell viability of progenitor liver cells L252, L264, L266 and L269 EPCAM⁺ following their encapsulation and culture in BME2 matrix and the ECM-derived scaffolds at 1,2,3,4,6 and 8 mg/ml at different concentration. Briefly, on the day of the staining, cells and scaffold were washed with PBS and incubate for 40 minutes with 100 μ l of starting media containing three μ M of EthD-1 and 0.75 μ M of Calcein-AM. Next, cells were imaged with a fluorescence microscope at 5x and 10x magnification.

2.7.2 Alginate digestion

Alginate lyase stock solution was prepared to a 4 mg/ml final working concentration. To remove the alginates and acquire the cell pellet, alginate lyase in PBS was added at 1:100 to the culture media to make a final volume of 200 μ l per well. The samples were placed on a rocker and incubated at 37 °C for 2 hours to enzymatically digest the alginate hydrogels. Once the alginates samples had been defrosted, 200 μ l of the digestion solution was added into each Eppendorf and centrifuged to allow the samples to floating in the digestion solution. RIPA buffer was then used to lysate the cells once the alginates had been digested.

2.7.3 dsDNA quantification using PicoGreen™ assay

Once the alginate matrix has been digested adequately with alginate lyase (2.7.2 Alginate digestion), dsDNA of HepG2 encapsulated in alginates was quantified using Quant-iT™ PicoGreen™ dsDNA assay kit, all reagents, samples, and standards were prepared per manufacturer instructions. Fluorescence was read at an excitation of 480 nm and emission of 520 nm wavelength with a plate reader.

2.7.4 Bradford protein quantification

Total protein quantity secreted by HepG2 encapsulated in alginates was measured using Quickstart Bradford 1x Reagent. Media from each alginate condition was collected at 24 and 72 hours, centrifuged and diluted 1:100 as recommended by the protocol. A 1:1 ratio of media to reagent dye was added to all samples and standards. All samples and standards were incubated for 5 minutes at room temperature and shielded from light. The absorbance was read at 595 with a plate reader.

2.7.5 Albumin ELISA assay of HepG2 encapsulate in modified alginates

The same media collected at 24 and 72 hours was used to quantify the samples' albumin secretion using an enzyme-linked immunosorbent assay (ELISA) kit. All reagents, samples, controls, and standards were prepared following the manufacturer protocols. The absorbance was read immediately at 450 with the plate reader.

2.7.6 Resorufin metabolic activity assay (Alamar blue)

Briefly, expansion media was removed on days 4 and 7 from the progenitor liver cells EPCAM⁺ encapsulated in BME2, 4,6 and 8 mg/ml gels. Gels were washed with warm PBS, and 250 µl of Alamar Blue 10% working solution was added to each condition. The samples were left to incubate for 3 hours until a slight pink change of colour was observed. 50 µl of the solution for each sample was transferred into a 96 well plate in 3 technical replicates. The fluorescence was read using a plate reader with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The experiment was performed in triplicates with three technical replicates.

2.7.7 CYP3a4 colorimetric assay

Organoids were culture as per standard protocol in both BME2 and ECM hydrogels at different concentrations following 2.6.4 Methods section. At the end of the differentiation period, organoids were incubated with 100 μ M PPra ligand inducer (WY-14643), for 24 hours. The next day, organoids in BME2 were extracted from the matrix and incubated for 4 hours with the substrate (3 μ M Luciferin-IPA). Similarly, ECM hydrogels were collagenase digested with Collagenase NB 4G Proved Grade at a concentration of 0.13 U/mg for 2 hours to loosen the matrix but not fully to release the organoids. Subsequently, the ECM/organoids samples were incubated for 4 hours with the substrate (3 μ M Luciferin-IPA). After the incubation time, 25 μ l of media or buffer from each well was transferred to a white luminometer plate. 25 μ l of the Luciferin detection reagent was added to initiate the luminescent reaction per manufacture protocol. The plate was incubated on a shaker at room temperature shielded from light for 20 min. Afterwards, the plate was read with a luminescence measurement at an integration time of 1s with an Omega plate reader. The net signal was calculated by subtracting the blank to each reading, and an induction relative to untreated samples was calculated by dividing the treated samples with the untreated samples.

A similar protocol was used to induce the HepG2 with the ligand, but the incubation time with the substrate was 1 hour as per manufacture instruction for 2D samples.

2.7.8 RNA extraction optimization

RNA extraction results for each method can be found in Appendices C, Figure AppC 5.5-5.8.

Method 1: 500 μ l of Trizol was added to each sample. Vigorous mixing and pipetting directly onto ECM samples was applied until samples were fully dissolved. The process could take longer than 30 minutes.

Method 2: The recombinant collagenase solution Collagenase H (ColH), at a stock concentration of 200 U/ml was diluted to 50 U/ml (follow manufacture protocol).

The ECM samples were transferred into Eppendorf, and 250 μ l of 50U/ml recombinant collagenase was added. Samples were incubated for at least 30 minutes or until almost all samples had dissolved. Eppendorf was centrifuged at 10 000 g for 5 minutes, and the supernatant was discarded. 500 μ l of Trizol was added to each sample and mixed to dissolve the pellet. Samples were stored at -80 °C until further processing.

Method 3: Samples were transferred in Eppendorf tubes and immersed in liquid Nitrogen until the whole of the sample was frozen. 500 μ l of Trizol was added to each sample and

homogenised for 45 seconds on ice using the homogeniser. Samples were then stored at -80 °C until further processing.

Method 4: 300 µl of RNAlater solution was added to each sample after removing media to stop the experiment. Samples were allowed to sit in the fridge overnight at 4 °C. The next day samples were removed from the RNAlater solution and snap freeze using liquid Nitrogen. Trizol was added to the frozen samples, and a homogeniser was used to homogenise each sample. Homogenised samples in Trizol were stored in the freezer at -80 °C until further processing.

RNA extraction (optimised protocol)

To extract RNA from progenitor liver cells encapsulated in BME2 and liver ECM gels, a Tri Reagent™ method was utilised. Initially, media was removed, and 300 µl of RNAlater solution was added to each gel (optimised Method 4). Samples were left overnight in the fridge. The following day samples were transferred in labelled Eppendorfs. After snap freezing in liquid Nitrogen, 500 µl of Tri Reagent™ was added to each gel and homogenised with a homogeniser for 45 sec on ice. 200 µl of 1-Bromo-3-chloro-propane (BCP) per 1 ml of monophasic lysis reagent was added to each sample and vigorously vortexed. Samples were centrifuged at 17 000 g for 15 min at 4 °C until the sample separated into two distinct mixtures. The upper aqueous solution was transferred into a fresh tube. To allow the RNA to precipitate from the aqueous phase, 0.5 ml of isopropanol was added for every ml of monophasic lysis reagent. After thorough mixing, due to the anticipation of the recovery of a small amount of RNA, 1 µl of Glycogen [20mg/ml] was added to each sample. The samples were then placed in the freezer -20 °C overnight. The next day the collected RNA from each sample was centrifuged at 17 000 g for 15 min at 4 °C. Samples were washed with 75% ethanol twice and centrifuged again. Samples were left aside to allow the remaining alcohol to evaporate without allowing the pellet to dry completely. After adding 87.5 µl of DEPC treated water to each sample, the heating block was used to heat the RNA for 5 minutes at 65 °C. To further purify the RNA, 2.5µl of DNase I and 10µl of RDD buffer were added to each sample. The samples were incubated at room temperature for 10 minutes. The volume was adjusted to 400 µl with DEPC-treated water, and an equal volume (400µl) of phenol/ chloroform/ isoamyl alcohol was added. After vigorous mixing, the samples were centrifuged at 10,000g for 10 minutes at 4 °C. The upper aqueous phase was placed into a fresh Eppendorf. Pure RNA was precipitated with the addition of 1/10 of the volume (40µl) of 3M sodium acetate, pH 5.2 and 2 volumes (800µl) of absolute ethanol. The samples were placed in the freezer -20 °C overnight after adding 1 µl of Glycogen [20mg/ml]. The RNA was pelleted by centrifugation at maximum speed for 10 minutes. RNA was then washed twice in 75% ethanol and allowed

to dry, but not completely. RNA was dissolved in 20-30 µl of DEPC-treated water. The final RNA concentration was measured using a NanoDrop2000, Spectrophotometer.

2.7.9 RNA Integrity and purity assessment

1 µl of RNA from each sample was used to analyse the integrity of the RNA after the RNA was extracted from progenitor cells encapsulated in liver ECM gels. The analysis was carried out following manufacturing instructions using the 2100 Bioanalyzer Instrument. Samples with a RIN (RNA Integrity Number) value below 6.5 were discarded.

2.7.10 Reverse transcription synthesis of RNA to cDNA

According to the manufacturer's instructions, RNA from each sample was reverse transcribed to cDNA using the AffinityScript Multiple Temperature cDNA Synthesis Kit. An initial quantity of 200ng total RNA was used in each reaction. Random Primers were used in the reaction. Time conditions and temperatures are shown in the Table 2.6.

Table 2.6 Time and Temperature used for the cDNA synthesis using the AffinityScript Multiple Temperature cDNA Synthesis Kit.

Temperature, °C	Time, minutes
65	5
Cooling down	10
25	10
50	60
70	15
4	Hold

2.7.11 RT-PCR TaqMAN™ Gene expression

The expression level of interesting genes was quantified using the AriaMx Real-Time PCR System (RT-PCR). The Taqman RT-PCR system uses a pair of primer and a probe composed of a fluorophore covalently bound to the 5'-end of the probe and a quencher at the 3'end. The probe is designed to anneal to a DNA region amplified by a specific pair of primers. During the amplification step, the probe is degraded by the Taq polymerase, and the fluorophore is quenched, releasing a fluorescent signal detected by the machine. Therefore, the amount of fluorescence detected in each sample during the PCR thermal cycles is directly proportional

to the quenched fluorophore and the RNA present. Finally, the fluorescent intensity can be used to measure the expression level of a gene of interest.

To ensure a lack of contamination and reasonable accuracy of the mRNA product, a non-template control (NTC) was created and included in each plate. In the NTC sample, the cDNA template was substituted with DEPC treated HPLC grade water. A non-reverse transcriptase (NEC) was also utilised to check the specificity of the primers and probes to each gene of interest and DNA contamination. NEC samples were made from a pool of RNA from the samples of interest without incorporating the reverse transcriptase during the reverse transcription reaction. Plates showing NTC amplification were discarded, and repeats were done to ensure accuracy.

A Taqman standard curve method was used to create a reliable standard curve for each gene. The gene expression of each sample is evaluated as the quantity in the sample relative to a neat sample.

Briefly, the neat was created by mixing a pool of cDNA from each sample, and a 5-point standard curve at 1:4 series dilution from the neat was prepared to complete the standard curve. The relative gene expression of the samples of interest is determined from the standard curve, and all the quantities are expressed as relative to the neat sample. A master mix for each gene of interest was prepared by mixing: Taqman universal master mix, forward primer, reverse primer, and probe at 10 μ M with DEPC treated HPLC grade water as depicted in Table 2.7. 10 μ l of MasterMix and 3 μ l of a diluted sample (1:16) were placed in each well in triplicate. The plate was sealed and spanned down before being amplified. Finally, amplification cycles were performed as described in Table 2.9. Primers and probes sequences used in the project are shown in Table 2.12. The universal mastermix and other components used for Sy-green are depicted in Table 2.8 and the amplification step for Sy-green is shown in Table 2.10.

Table 2.7 Components of the master mix and their amount per sample used in the amplification reaction.

Components of the MasterMix	Amount per sample (μ l)
(PCRBIO Probe Mix Lo-ROX) (PCR Biosystem) Master Mix PCR 2x	6.5
Forward Primer	0.4
Reverse Primer	0.4
Probe	0.25
DEPC water	2.5

Table 2.8 Components used for the SyGreen reaction to check the gene expression of the SOX9 gene.

Components of the MasterMix for SyGreen	Amount per sample (µl)
(Brilliant III UF MM SYBR QPCR/ Low ROX) (Agilent Technologies) Master Mix PCR 2x	6.5
Forward Primer	0.4
Reverse Primer	0.4
DEPC water	2.5

Samples and standards, as well as NTC and NEC, are assayed in duplicates. The cycle threshold (Ct) value of a reaction corresponds to the cycle number when the fluorescence intensity from the fluorophore from the PCR product can be detected above the background signal. A standard curve can be created by plotting the standard curve Ct Values and the log of the quantity of the standard sample. This standard curve can be a measure of the effectiveness and accuracy of the semi-quantitative RT-PCR.

The aim for each standard curve was to be between -3.32 and -3.6 (90-100% efficient). Any curve that fell outside this range was not used. Cycling conditions used in the qPCR reaction are detailed in Table 2.7 and Table 2.8.

Table 2.9 Amplification condition for qPCR using designated primers and probes. Samples were amplified for 40 cycles.

Temperature, °C	Time (minutes: seconds)
95	2:00
95	0:05
60	0:20

Table 2.10 Amplification condition for qPCR using Sox9 gene. Samples were amplified for 40 cycles.

Temperature, °C	Time (minutes: seconds)
95	3:00
95	0:05
60	0:10

2.7.12 Primers and Probes Sequence Design

The primers and probes used in this thesis have been designed following the recommendations from the Table 2.11.

Table 2.11 Requisites that are necessary to successfully design highly specific primers and probes for quantitative Real-Time PCR (Rodríguez et al., 2015).

Requisites	Primers	Probes
GC content	30–80 %	
Calculated primer/probe T_m	50–60 °C (always >55 °C) T_m of primers should not differ >2 °C	68–70 °C (8–10 °C above T_m primer)
Runs of identical nucleotides	Maximum 3 (No G bases)	
Primer/probe length	15–30 bp	
PCR product length	50–150 bp (optimum <80 bp)	
Distance forward primer to probe	50 bp	
Primer-dimers, hairpins	Avoid	Avoid
3' end rule (3' instability)	Maximum two G or C in the last 5 bp	–
Autoquenching	–	No G on the 5' end
GC ratio	–	C>G
Degree of degeneracy of bases	Avoid	Avoid

Table 2.12 DNA sequences of primer and probes designed for specific genes used in the Taqman based qPCR reaction. Each probe was labelled with FAM fluorescent dye and ROX as reference dye except for the SOX9 gene.

Gene	Forward sequence 5'→3'	Reverse sequence 5'→3'	Probe sequence 5'→3'
β-actin	CCTGGCACCCAG CACAAT	GCCGATCCAC ACGGAGTACT	ATCAAGATCATTGCTC CTCCTGAGCGC
EpCAM	CTC GCG TTC GGG CTT CT	TGT AGT TTT CAC AGA CAC ATT CTT CCT	ACG GCG ACT TTT GCC GCA GC
CK19	GTT CAC CAG CCG GAC TGA A	GTAACCTCGG ACCTGCTCATC T	TCGCTGGCCACACGGA G
HNF4α	GGGTGTCCATAC GCATCCTT	CCTTGGCATCT GGGTCAAAG	ACGAGCTGGTGCTGCC CTTCCA
Lgr5	CAG CTA AGA CAC GTA CCC ACA GA	GCA GGC CAC TGA AAC AGC TT	CAA TCC CTG CGT CTG GAT GCT AAC CA
Albumin	TCGATGAACTTC GGGATGAAG	AGCTACTGCCC ATGCTTTGAA	TCGTCTGCCAAACAGA GACTCAAGTGTGC
Cyp3a4	TGGCCACTCACC CTGATGT	GTCAAGATACT CCATCTGTAGC ACAGT	TTTACCCAATAAGGCA CCACCCACCTATGA
MRP2	GAAGACGATGAC TATGGGCTGAT	TGGACCTAGA ACTGCGGCTA A	AGAGATCCCCGAAGAT GCAGCCTCC
APO-A1	TGTACGTGGATG TGCTCAAAGAC	GGAGCTTTAG GTTTAGCTGTT TTCC	ACTATGTGTCCCAGTT TGAAGGCTCCGC
Cyp2b6	AAT ACC CTC ATG TTG CAG AGA GAG T	TGC CTC TGT GTA TGG CAT TTT G	CAC ATC GCC CTC CAG AGC TTC ATG A
SOX9 (syGreen)	CTCTGGAGACTT CTGAACGAGAG	CCTTGAAGATG GCGTTGGGG	N/A

CK7	TGCTGCCTACAT GAGCAAGGT	TCTGTCAACTC CGTCTCATTGA G	ATGAGATCAACTTCCT CAGGA
CK18	TGGCGAGGACTT TAATCTTGGT	ACCACTTTGCC ATCCACTATCC	ACCATCCAAAAGACCA CCACCCGC
CFTR	AGTAGGTCTTTG GCATTAGGAGCT T	TGGTCTGGTCC AGCTGAAAAA	AGGGACCCCAGCGCCC GA
ASBT	ATGGACCTGAGC GTCAGCAT	GAGGCACAGC GGCATCAT	TGCTCCACACTGCTTG CCCTCG

2.8 Immunofluorescence staining

2.8.1 EpCAM⁺ human liver progenitor cells staining

PFA fixed organoids were washed twice with PBS, permeabilised using 1% Triton in PBS for 10 minutes on a shaker, blocked with 5% horse serum in buffer 1 (PBS-1% BSA and 0.1% Tween-20) for 30 minutes. Primary antibodies were added at appropriate dilutions EpCAM 1:100, Ki67 1:50, CK19 1:100 and organoids were kept overnight at 4 °C on a shaker. The following day the primary antibodies were decanted, and organoids were washed twice with buffer 1 each of 5 minutes on a shaker.

Appropriate secondary antibodies (1:500 dilution donkey anti-rabbit Alexa Fluor® 568 or 1:1000 dilution goat anti-rabbit CY5) were added to the organoids and kept for 1 hour at room temperature on the shaker, washed with buffer 1 for 5 minutes before adding DAPI at a dilution of 1:1000 in PBS for 1-2 minutes followed by a couple of brief washes with PBS and water respectively. The organoids were then transferred into mounting medium-containing slides vectashield, covered with coverslips, sealed with nail polish, and left to dry in the dark. Similarly, cryo-sectioned slices of progenitor liver cells encapsulated in ECM were stained following the same procedure as whole organoids.

2.8.2 EpCAM⁺ human liver progenitor cells DAPI stained for proliferation in dECM hydrogels

PFA fixed organoids still embedded in dECM hydrogels were washed twice with PBS, permeabilised using 1% Triton in PBS for 10 minutes on a shaker. Once permeabilised, the gels were washed twice in PBS and stained with DAPI at a dilution of 1:1000 in PBS for 1-2 minutes followed by a couple of brief washes with PBS and water, respectively. Gels were transferred into medium containing slides and pressed flat with a coverslip. The coverslip was then sealed and left to dry. At least ten images from different positions were taken for each of the dECM gels.

2.8.3 Cryo-sectioning of EpCAM⁺ human progenitor liver organoids encapsulated in dECM liver hydrogels

Liver ECM hydrogels of different concentrations embedded with liver organoids were fixed in 4% PFA overnight in the fridge. Samples were washed twice with PBS before adding 15% sucrose solution for two days, after which the solution was exchanged with 30% sucrose and left in the fridge for another two days. Then samples were dipped in OCT medium, which is liquid at room temperature and start solidifying at 0 °C. Once the samples were solidified, they were mounted on a probe and placed in the cryostat chamber, ready to be sectioned. Specimens were sliced at a 10 µm thickness and stored at -20 °C for later processing.

2.9 Imaging

2.9.1 Fluorescence Microscope

A Zeiss 200M upright microscope (5x,10x and 20x) was used to image fluorescent-stained cells in 3D dECM gels and BME2 matrix and cryo-sectioned stained slices of liver progenitor organoids embedded in liver dECM hydrogel.

2.9.2 Confocal Microscope

A Confocal microscope (Zeiss LSM 880) was also used to capture Z-stack images of fluorescent labelled whole organoid encapsulated in dECM hydrogels and BME2 matrix.

2.10 Image Analysis

2.10.1 Macro-automated DAPI nuclei stain count

A plugin-macro installed into Fiji was utilised to automatically count the nuclei over time to assess the proliferation potential of the organoids embedded in liver dECM hydrogels. At least ten images for each time point were taken per dECM hydrogel. Nuclei were stained in blue with DAPI and counted.

2.11 Statistical analysis

The data for each experimental study is expressed as mean $\pm$ standard deviation (SD). The error bars are derived from the SD of the mean, and n represents the technical replicates performed within each experiment. All analysis of normality was performed using analysis of variance One way-ANOVA with Bonferroni multiple comparisons, Two way-ANOVA with Bonferroni multiple comparison or un-paired t-Test in GraphPad Prism (version 9.1.0) software. Significance was considered at $p < 0.05$.

Chapter III - Chemical characterisation and cytotoxicity assessment of hepatocytes cells encapsulated in chemically modified alginate hydrogels.

3.1 Introduction

One of the challenges in tissue engineering is to develop *in-vitro* models capable of mimicking or simulating the responses that different drugs might induce in the tissue of interest (LeCluyse et al., 2012). Researchers have focused their efforts on the development of complex 3D liver *in-vitro* models that employ: 3D scaffolds (Mazza et al., 2015), co-cultures of parenchymal cells and non-parenchymal cells (Wei et al., 2018), liver organoids (Pang et al., 2020), hepatocytes sandwich cultures (Brandon et al., 2010).

Hydrogels are widely used in tissue engineering because of their versatility in their applications (Das, 2013). Hydrogels can be classified as three dimensional, porous, hydrophilic polymers networks capable of retaining very high water content (Shivani et al., 2015). They provide a 3D environment where cells experience greater cell-to-cell interaction and cell-to-materials interactions. Researchers have taken advantage of this ability to develop 3D scaffolds in cell culture (Caliari and Burdick, 2016; Zimmermann and Shirley, 2007). Furthermore, hydrogels degrade either by hydrolysis or by an enzymatic mechanism which results in a change in mechanical properties, affecting cell motility and behaviour (Khetan et al., 2013).

Alginate is a plant-derived polysaccharide from brown algae. It uses calcium ions to crosslink in an ‘egg box’ configuration. It has been widely used in tissue engineering because of its biocompatibility, low toxicity, and easiness of crosslinking to form a hydrogel structure. Alginate is one of the first materials used in tissue engineering for a variety of applications, from 3D printing (Axpe and Oyen, 2016) to primary cell cryopreservation (Camboni et al., 2013) to scaffold fabrication (Farokhi et al., 2019).

Furthermore, alginate can be easily functionalised by employing different chemistries and adding proteins and ligands, making it a semi-synthetic biomaterial alternative. As thoroughly discussed in the introduction, alginate, although biocompatible, does not present any RGD domain that mammalian cells recognise, making it difficult for the cells to attach, proliferate and function. This shortcoming can be resolved by chemically modifying the alginate structure

with known moieties and proteins with defined and expected cell response or unknown compounds, which could trigger and induce a different cell behaviour or phenotypical change.

3.1.1 Alginate chemical modification methods

Over the years, research has utilised alginates as an abundant biocompatible biomaterial easily prepared into hydrogel construct. By the addition of ‘small molecules’ and proteins through derivatisation reactions on the polysaccharide backbone, alginates with ‘added-values’ have been fabricated (Pawar and Edgar, 2012). This has been achieved by creating alginate synthetic derivatives (Nataraj and Reddy, 2020) utilising controlled biomolecule conjugation (Spicer et al., 2018). Alginate can be modified either at the -OH or at the -COOH terminal (Pawar, 2017) Table 3.1 and with carbodiimide chemistry to form construct for *in-vitro* liver applications Table 3.2.

Table 3.1 Types of chemical conjugation employed to modify hydroxyl and carboxyl groups highlighting their advantages and disadvantages.

	Types	Benefits	Disadvantages	References
Modification of Hydroxyl groups	Oxidation	• More reactive groups available • Faster degradation	• Light sensitive reaction	(Boontheekul et al., 2005) (Gomez et al., 2007) (Bouhadir et al., 2001)
	Reductive-amination of oxidised Alginate	• New groups for chemical modification • Fast reaction • Physiological ph range • Amphiphilic characteristic • Chain flexibility • No by-products formation • High reproducibility	• Slow kinetics • Low site-selectivity • Potential toxicity from aldehydes	(Huamani-palomino et al., 2021) (Dalheim et al., 2016) (Spicer et al., 2018)

	Sulfation	• Lower charge density • More protein interactions • Unimpaired crosslinking of G-block increases sulphated alginate strengths and stability	• Less heterogenous substitution • Limitations in sulfation degree • Deterioration of gelling ability	(Arlov and Skjåk-bræk, 2017) (Freeman et al., 2008) (Mhanna et al., 2014)
	Free radical polymerisation	• Robustness • Synthetic ease • Water tolerance • Diversity of monomer to conjugates	• Monomer toxicity • A slow rate of chain growth • Reactive species formation • Highly sensitive to oxygen	(Kapishon et al., 2015) (Yoshii, 1996) (Delplace and Nicolas, 2015)
	Cyclodextrin-linked Alginates	• Increased solubility • Improve stability • Enhance bioavailability	• Toxicity at high dosage	(Omtvedt et al., 2019) (Pluemsab et al., 2007) (Gidwani and Vyas, 2015) (Martin et al., 2018) (Przybyla et al., 2020) (Tiwari et al., 2010)

	Types	Benefits	Disadvantages	References
Modification of Carboxyl groups	Esterification	• Simplicity • Increase hydrophobicity	• Thermodynamic reversibility • Slow reaction • In strong acids, tertiary alcohol undergo dehydration • Catalyst use to speed up reaction	(Rajabi et al., 2016) (Leonard et al., 2004) (Yang et al., 2007)
	Amidation	• Ease to use • Reagent availability • Robust • Reliable	• Low site-selectivity • Yield effected by side reactions • Heterogenous products	(Spicer et al., 2018) (Joubert et al., 2010) (Yang et al., 2011)
	‘Click’ chemistry or Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)	• High chemo-selectivity • Easy access to functional groups	• By-products from the reaction • Copper potentially toxic • Toxicity from residual metal • Alkynes/Azide need to be biomolecules • High loading amount of catalysts • Disruption of peptide and protein structures • Generation of ROS	(Link and Tirrell, 2003) (Kennedy et al., 2011) (Bahsis et al., 2020)(Vegas et al., 2016) (Breger et al., 2015) (Desai et al., 2015)
	Carbodiimide Coupling	• No harsh acids or bases	• By-products from the reaction • Hydrolysis	(Bax et al., 2017) (Donati et al., 2003) (Jeon et al., 2010)

Table 3.2 Alginate construct with or without chemical modification employed in the study of hepatocyte function for liver model applications.

	Alginate Chemical Modification	Molecule chemically added	Cells	Application	References
<i>Alginate constructs for in-vitro liver applications</i>	N.A	N.A	Rat primary hepatocytes	Human hepatocytes microbeads for ALF	(Jitraruch et al., 2014)
	N.A	N.A	HepG2 and Rat primary hepatocytes	Cell entrapment and immobilisation for bioreactor culture	(Murtas et al., 2005)
	N.A	N.A	Rat adult hepatocytes	Alginate sponge scaffold for <i>in-vitro</i> cell culture	(Glicklis et al., 2000)
<i>Chemically modified Alginate constructs for in-vitro liver applications</i>	Carbodiimide Coupling	Galactose	Rat Primary hepatocytes	<i>In-vitro</i> scaffold	(Yang et al., 2002)
	Carbodiimide Coupling	Galactose/chitosan	Mouse Primary hepatocytes	<i>In-vitro</i> alginate sponge scaffold	(Chung et al., 2002)
	Carbodiimide Coupling	Galactose/H.A.	Rat primary hepatocytes	<i>In-vitro</i> scaffold for 3D culture	(Fan et al., 2010)
	Carbodiimide Coupling	Galactose/chitosan	Human foetal Hepatocyte cell line L02	3D microcapsule for hepatocytes	(Tian et al., 2014)
	Reductive Amination and Carbodiimide Coupling	Galactose moieties/chitosan	Rat primary hepatocyte	3D microcapsule for Hepatocytes	(Lou et al., 2016)

3.1.2 Alginate chemical characterisation techniques: HNMR and FTIR

Depending on the application intended to use alginate as biomaterials, different types of characterisations may be needed. Nuclear Magnetic Resonance (NMR) is a standard method used to analyse organic compounds. It can determine the entire structure without requiring a large amount of sample. Hydrogen 1 (^1H), as it contains an odd atomic number and an odd mass, can be used in NMR spectroscopy. Its principle relies on the resonance frequency of the particle (atoms) of a substance directly proportional to the strength of the magnetic field applied (Sanders, 2008). Due to the high natural abundance of (^1H) in several organic compounds, proton NMR is the most used method. This spectroscopy method uses light as a function of its wave, which is reflected when passing through a solid, liquid, or gaseous substance. When a magnetic field has applied, the atoms of the compounds emit electromagnetic radiations in the frequency region of 4 to 900 MHz. These radiations induce a transition between the nuclear spin of different samples in the magnetic field (Diehl, 2008). The principle of NMR involves three sequential steps: the polarisation or alignment of the nuclear spins under a constant magnetic field, the perturbation of the alignment by an oscillating magnetic field and the detection of the signal after the radiofrequency (R.F.) pulse (Muller, 1941).

Alginate has been characterised thoroughly using (^1H) NMR. ^1H NMR is the reference method to find the ratio between the M/G units of the alginate backbone. The alginate peaks are close to each other on the HNMR spectrum, mostly between 4.35-5.25 (Jensen and Larsen, 2015). A variety of species of alginates have been studied using both ^1H NMR and FTIR techniques as they are employed in the food industry and the medical field (Belattmania et al., 2020). Fourier Transform Infrared spectroscopy (FTIR) is another well-known technique used to characterise molecules. It uses the interactions of radiation with the vibrations of the molecules of a compound (Spragg, 1999). The infrared spectrum can be used to identify a 'fingerprint' unique to a molecule and many functional groups (Colthup, 1999). Alginate has been fully characterised with the FTIR technique (Fenoradosa et al., 2010; Fertah et al., 2017). It becomes trickier and more challenging to identify the peaks when alginate is conjugated with another compound or molecules as some bands may overlap, and a second FTIR derivative can be employed (Huamani-palomino et al., 2021).

3.1.3 Alginate hydrogels as liver *in-vitro* model

Alginate is an inert biomaterial without any RGD domain for the cells to attach (Drury and Mooney, 2003; Malda et al., 2013). Hepatocytes are adherent cells that need a substrate to remain polarised and functional. They rely on biochemical cues from the ECM components such as collagens and fibronectin and growth factors released in the environment (BENZ'EV et al., 1988). Alginate on its own cannot provide a favourable environment for these cells to grow. Therefore researchers have utilised specific chemistries to functionalised alginate and create an active site where moieties, peptides and chemical groups could be attached (Carre et al., 1991; Schulz et al., 2019). In many studies, alginate is primarily used as a microencapsulation device for hepatocytes (Jitraruch et al., 2014; Lou et al., 2016) , but only a few studies report alginate as a potential scaffold for hepatocytes culture (Dvir-Ginzberg et al., 2003).

Many papers report alginate modification with galactose as a potential liver scaffold for hepatocytes maintenance because of galactose adhesive properties Table 3.2 (Chung et al., 2002; Yang et al., 2002). No papers have yet reported the chemical functionalisation of different small molecules onto the alginate backbone to develop an *in-vitro* liver construct. These small molecule additions onto the alginate backbone might direct and influence cell behaviour and can potentially differentiate hepatic cells into mature-like hepatocytes.

3.2 Purpose of the work

The purpose of this work was to characterise the composition of chemically modified alginate hydrogels and assess the cytocompatibility of HepG2 and human primary hepatocytes embedded in them. This chapter assessed HepG2 and human primary hepatocytes cytotoxicity and morphology when encapsulated in five modified alginates hydrogels chosen from a library of alginates. The goal was to identify a hit material that could be brought forward for fabricating an *in-vitro* liver model.

The aims of this chapter are as follow:

- Chemically characterise the selected five chemically modified alginates using proton nuclear magnetic resonance (HNMR) to identify the chemically attached small molecules and determine their structure.
- Use Fourier Transform Infrared Spectroscopy (FTIR) to identify chemical bonds in the five chemically modified alginates.
- Fabricate hydrogels from the five selected modified alginates and assess their cytotoxicity using HepG2 and human primary hepatocytes.
- Assess the functionality of HepG2 encapsulated in the selected five chemically modified alginate using the quantification of dsDNA over time and albumin secretion

3.3 Experimental Design

A library of hundreds of chemically modified alginates with different combinations of the substrate was created by Nishant Singh PDRA. The combination of alginate and substrate that formed the alginate library is depicted in Figure 3.3. From that library, five modified alginate were selected for further characterisation and hydrogel fabrication. The experimental design is depicted in Figure 3.1.

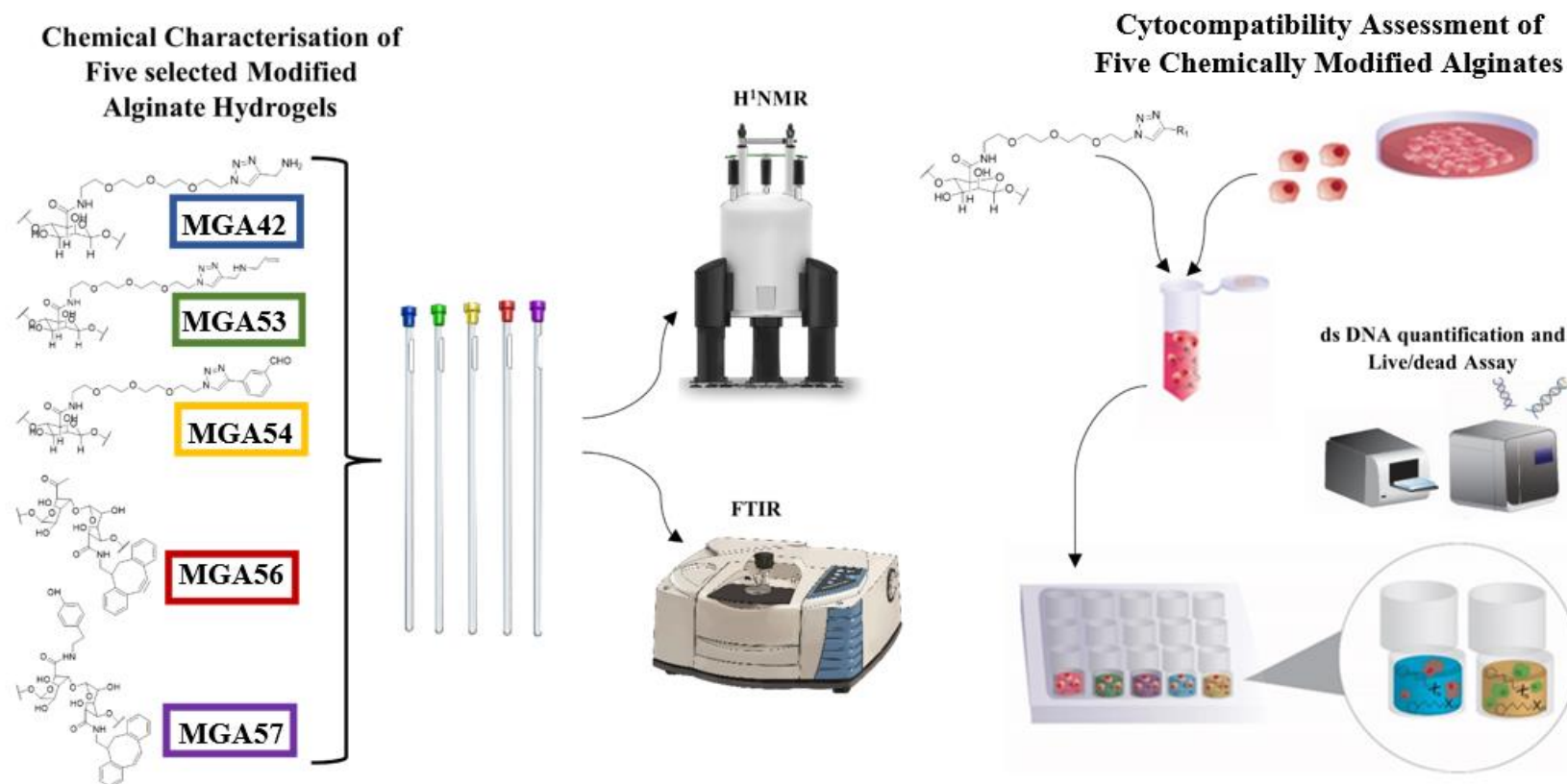


Figure 3.1 Schematic representation of the experimental design utilised to characterise the cytocompatibility and chemical composition of the selected five modified alginate MGA42-57. Drawings made by Francesca Tarsitano.

3.3.1 Functionalisation Method used to synthesise the Five selected Modified Alginates

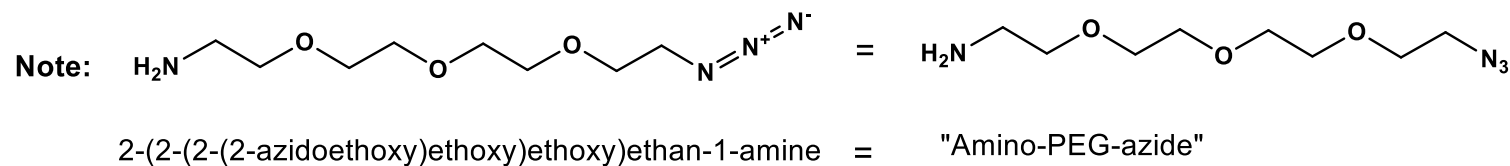
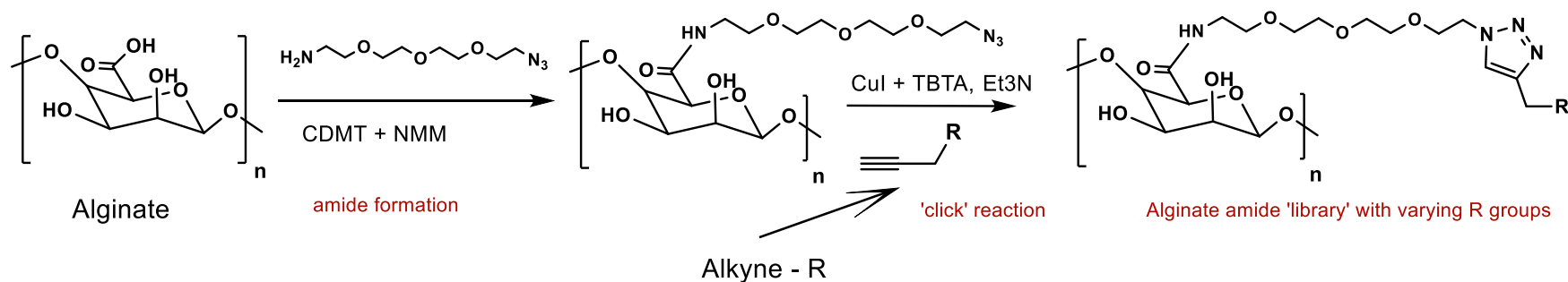


Figure 3.2 Schematic representation of alginate chemical functionalisation. The synthetic chemistry steps, fully described in Chapter 2 Materials and Methods (Section 2.2 alginate synthesis and small molecule conjugation), have been utilised to create a large library of modified alginate. These chemically modified alginate has been synthesised by Postdoc Dr Nishant Singh.

3.3.2 Library of Structure of Substituted Alginates

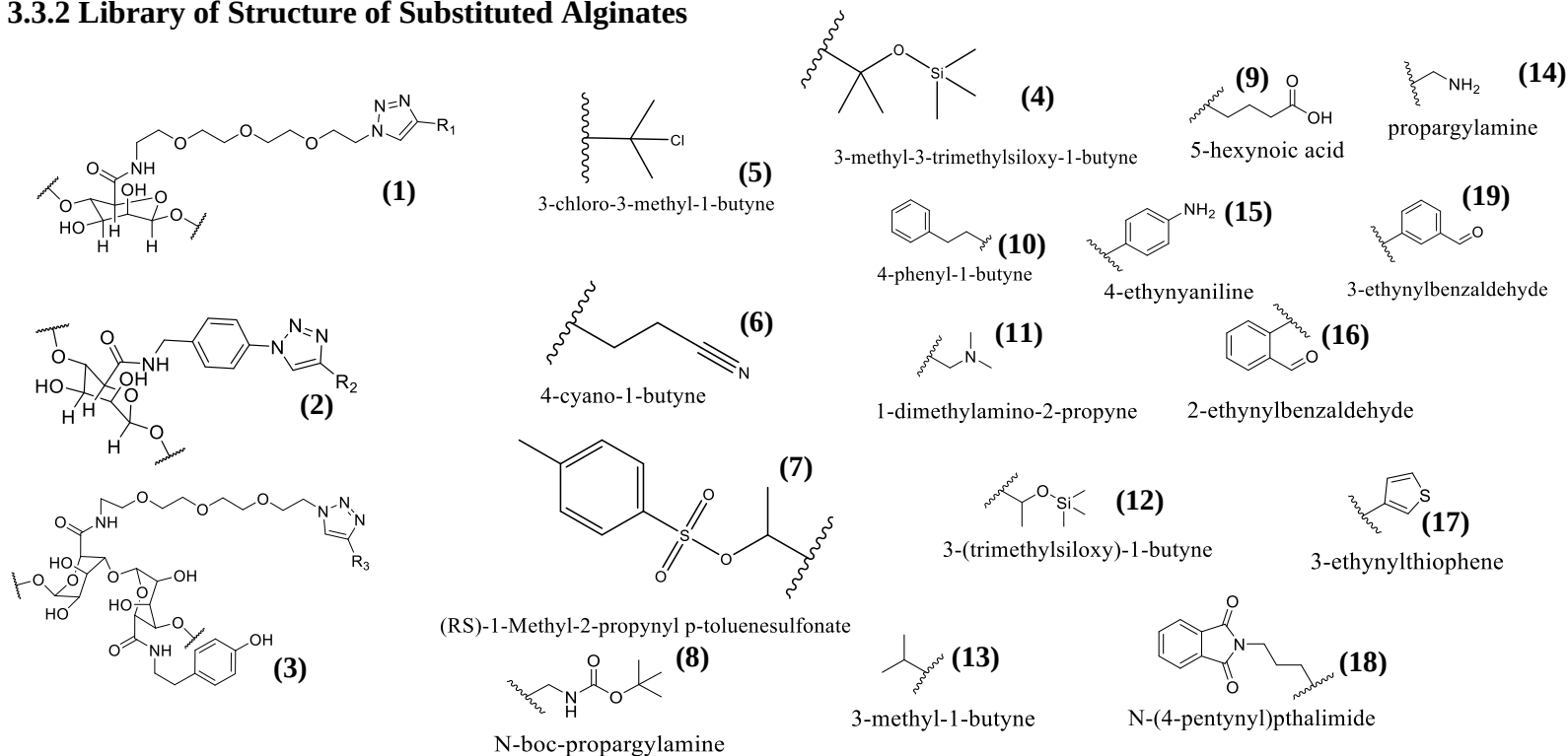


Figure 3.3 The chemically modified alginate library created using the synthetic step reaction previously described. The combination of the alginate Backbone and ligands ((1), (2), (3)) (using amidation reaction) and the small molecule (4-19) will be chemically attached ('click reaction') onto (R₁, R₂, R₃) in an array format and a variety of combination of these small molecules.

3.4 Results

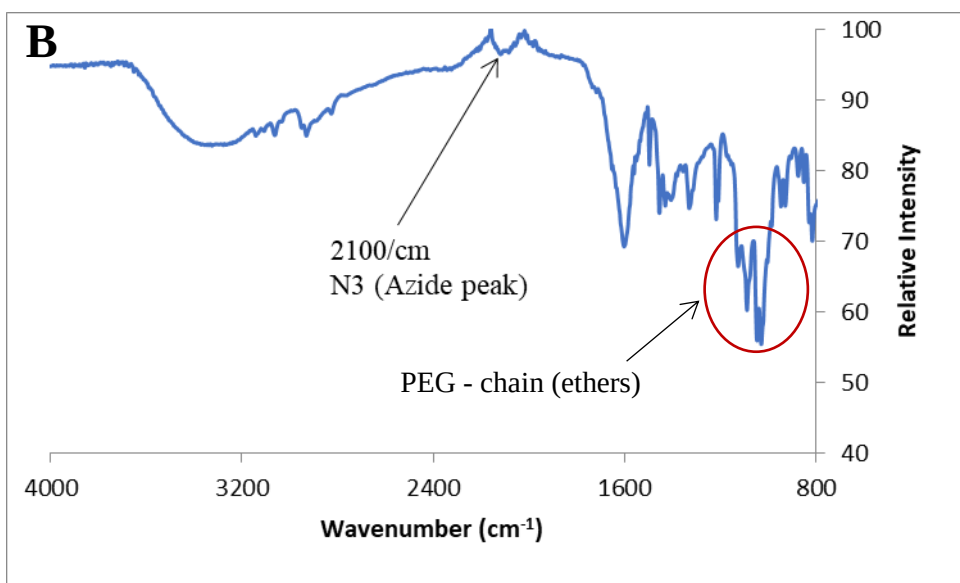
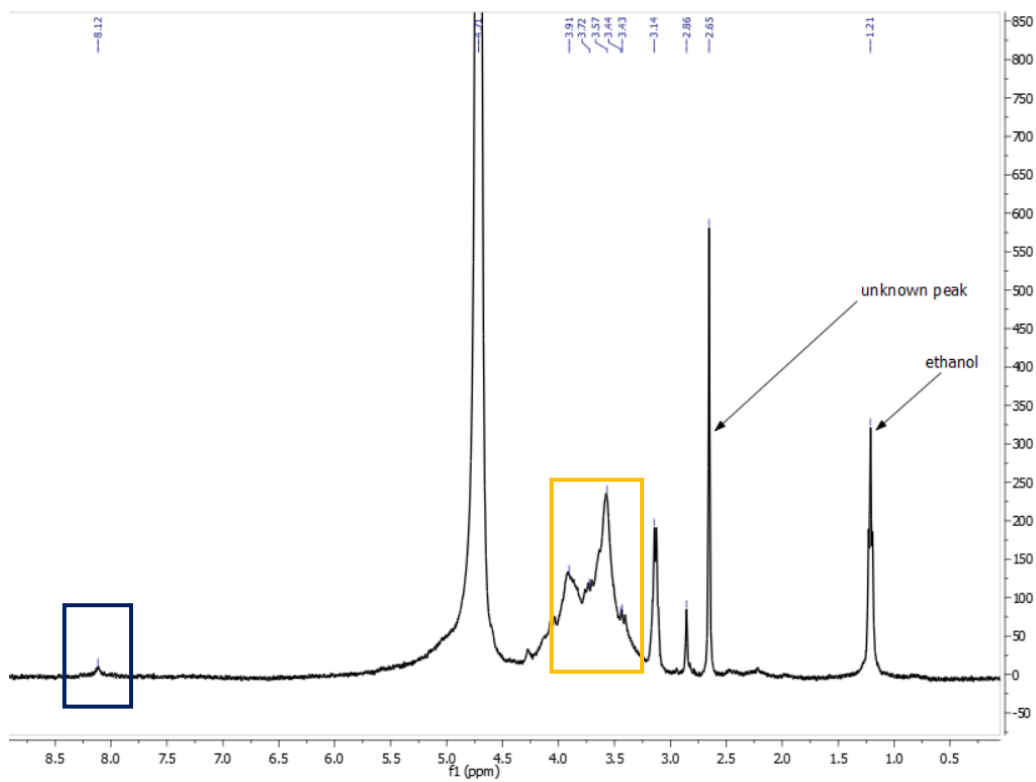
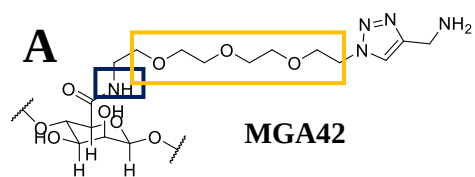
3.4.1 Chemical Characterization of Structures of substituted Alginates

From the work of Vegas et al., (2016), five chemically modified alginates were selected from a library of hundreds of chemically modified alginates created by the Postdoc Dr. Nishant Singh, as shown in Figure 3.3. The five modified alginates are labelled as follows: MGA42, MGA53, MGA54, MGA56 and MGA57. These selected chemically modified alginates belonged to two subgroups. MGA42, MGA53 and MGA54 modified alginates were synthesised by an amidation step and subsequently a ‘click reaction’ or Huisgen cycloaddition to attach the selected chemicals substrate. In this case: (14) propargylamine, 1-dimethylamino-2-propyne (11), and 3-ethyl benzaldehyde (20) for MGA42,53 and 54 respectively. MGA56 and MGA57 were fabricated using only the ‘click’ reaction and a small molecule. The synthetic chemistry steps utilised to fabricate the five modified alginates are represented in Figure 3.2.

The five modified alginates have been fully characterised using both FTIR and HNMR. In the HNMR spectra of MGA42, MGA53 and MGA54, it was possible to identify many peaks and confirm the success of the reaction (Figure 3.4 - Figure 3.8). In MGA42, MGA53 and MGA54, the PEG³ linker (O-CH²-CH²-O) was successfully conjugated onto the alginate backbone, as demonstrated by the range of peaks between 3.3 and 3.5 ppm. It was not possible to identify the amine peak at the end of the PEG-azide linker with the HNMR for the MGA42 modified alginate as shown in Figure 3.4 (A), but in the FTIR spectra (Figure 3.4 (C)), it was possible to see the C-N stretch of the aliphatic amine at 1215 cm⁻¹, which indicates the successful reaction in fabricating the ‘amino-PEG-azide’. HNMR of MGA53 shows a peak around 7.4 ppm, confirming the PEG-azide. Also the peak present at 2100/cm corresponding to N₃(azide) group in ALGPEG3N₃ spectra (Figure 3.4 (B)) disappears completely in MGA42 spectra due to the consumption of azide by reaction with alkyne group of propargylamine after click reaction forming triazole ring.

Furthermore, the peaks at 5.8 and 5.4 ppm are confirmed to be the protons peaks of the substitute as depicted in the yellow box shown in Figure 3.5 (A). HNMR of MGA54 (Figure 3.6 (A)) showed the PEG³ linker (O-CH²-CH²-O) successfully conjugated onto the Alginate backbone as well as the proton peak from the aldehyde at 9.9 ppm and the aromatic protons from the benzaldehyde ranging from 8.5 ppm to 7.6 ppm. Unfortunately, it was hard to characterise the remaining two modified alginates, MGA56 and MGA57, as the substituted

compound might be a mixture of conjugated and unconjugated components. It was possible to identify only a few peaks from Figure 3.7 (A) and Figure 3.8 (A), which have been confirmed with the Chemdraw software (Appendix A, Figure AppA 3.2 and 3.3). Furthermore, it was difficult to identify the peaks in the NMR, as most of the alginate backbone peaks overlapped with the water peak and the substrates peaks. It is also possible to detect a trace of ethanol in MGA42 at 1.21 ppm and an unidentified peak at 2.6 ppm (Figure 3.4 (A)). Most of the proton's peaks of each substituted alginate were possible to identify when the HNMR of the structure was predicted using the software ChemDraw Professional. The chemical shift was seen in some peaks, as expected. The predicted HNMR of all substituted structures can be found in Appendix A, Figure AppA 3.1-3.3.



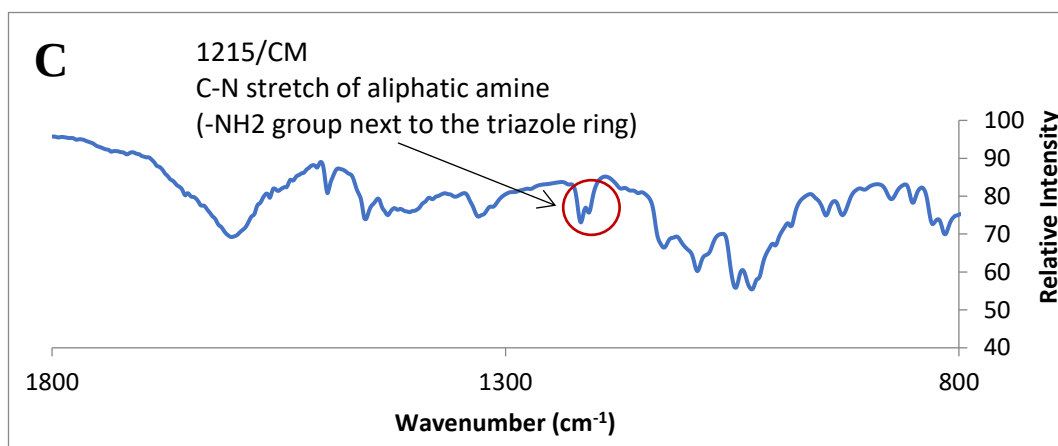


Figure 3.4 HNMR and FTIR spectra of MGA42 (A) HNMR of modified alginate MGA42. Depicting the peg chain linker (PEG³- O-CH² -CH² - O) in the yellow box. (B) FTIR spectra of modified alginate MGA42 showing the azide peak around 2100\cm. (C) Zoomed in FTIR spectra of MGA42. Red circle indicating the C-N stretch of the aliphatic amine. The presence of chemical bonds proves that the “click” reaction has successfully taken place, and the NH₂ group has been attached to the modified alginate.

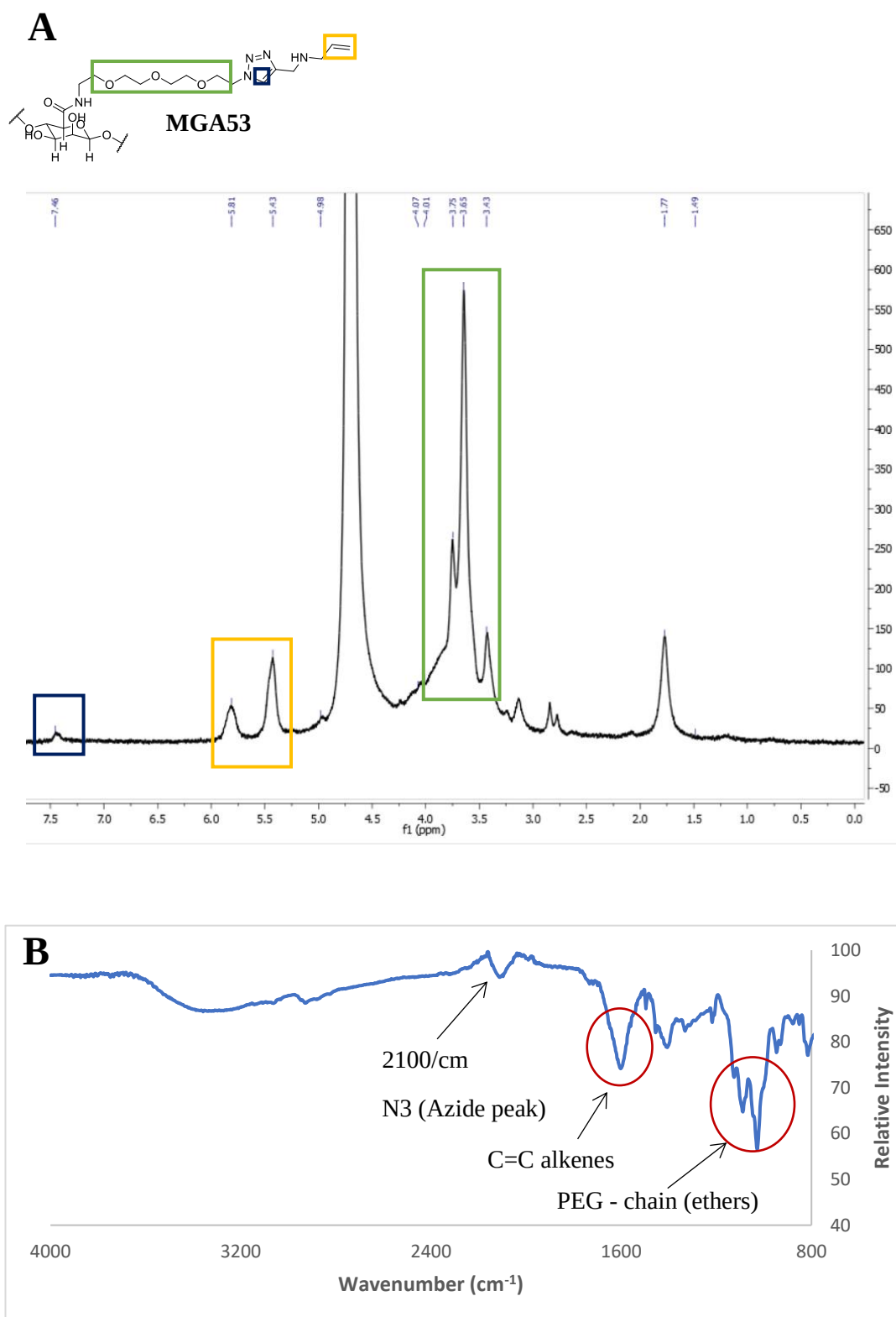


Figure 3.5 HNMR and FTIR spectra of MGA53. (A) HNMR spectra of MGA53 with peaks associated with the PEG chain linker (PEG³- O-CH²-CH² - O) in the green box. (B) I.R. spectra showing the alkenes peaks and the PEG chains (ethers) and the azide peak at around 2100 /cm⁻¹.

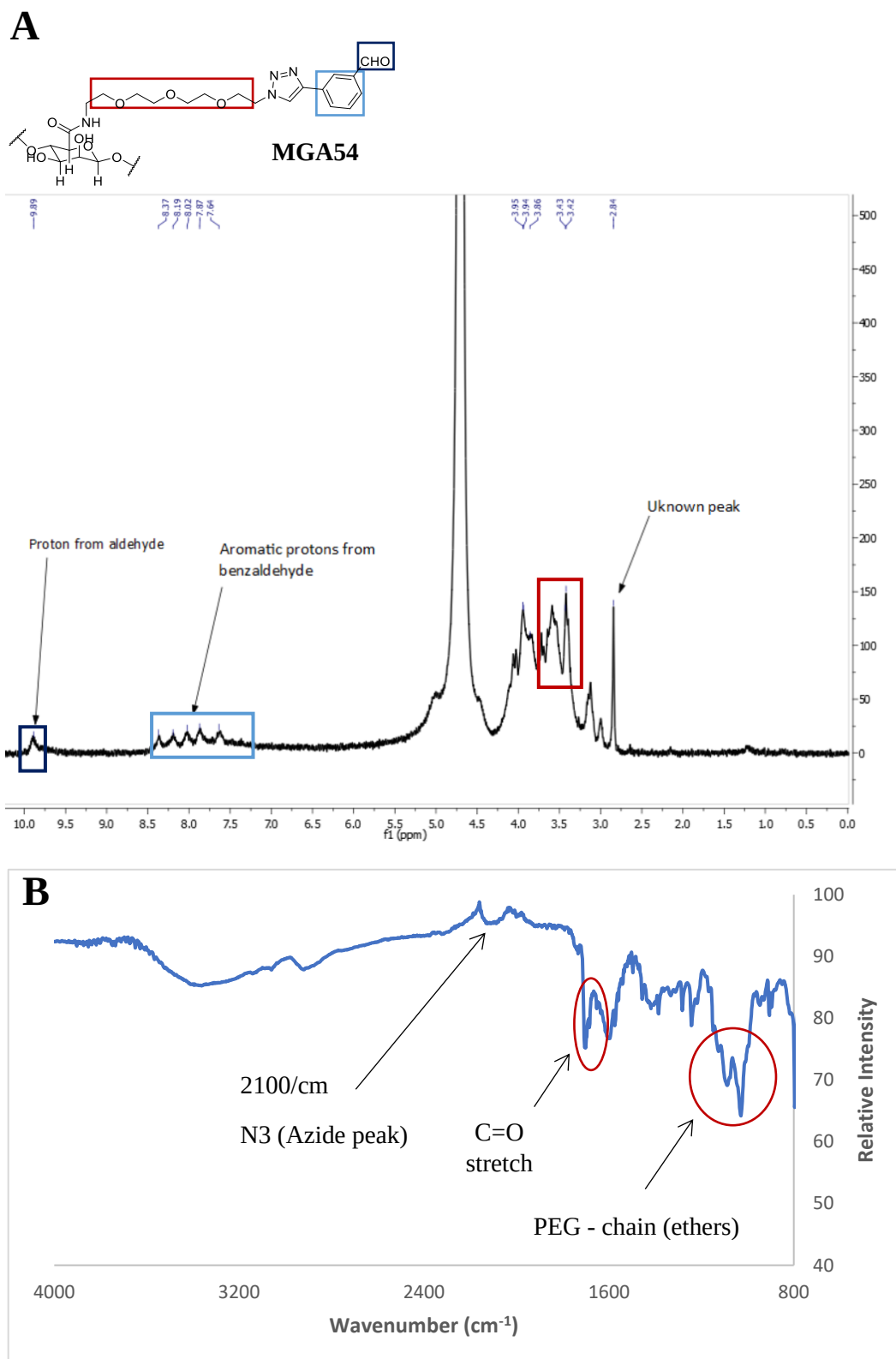


Figure 3.6 HNMR and FTIR spectra of MGA54. (A) HNMR spectra of MGA54. It depicts the chain linker (PEG³- O-CH²-CH² - O) in the red box. It is also possible to observe the proton peak from the aldehyde at 9.9 ppm and the aromatic protons from the benzaldehyde ranging from 8.5 ppm to 7.6 ppm. (B) FTIR. spectra show the C=O stretch at around 1699 /cm, the PEG chains (ethers) and azide peak at around 2100 /cm⁻¹.

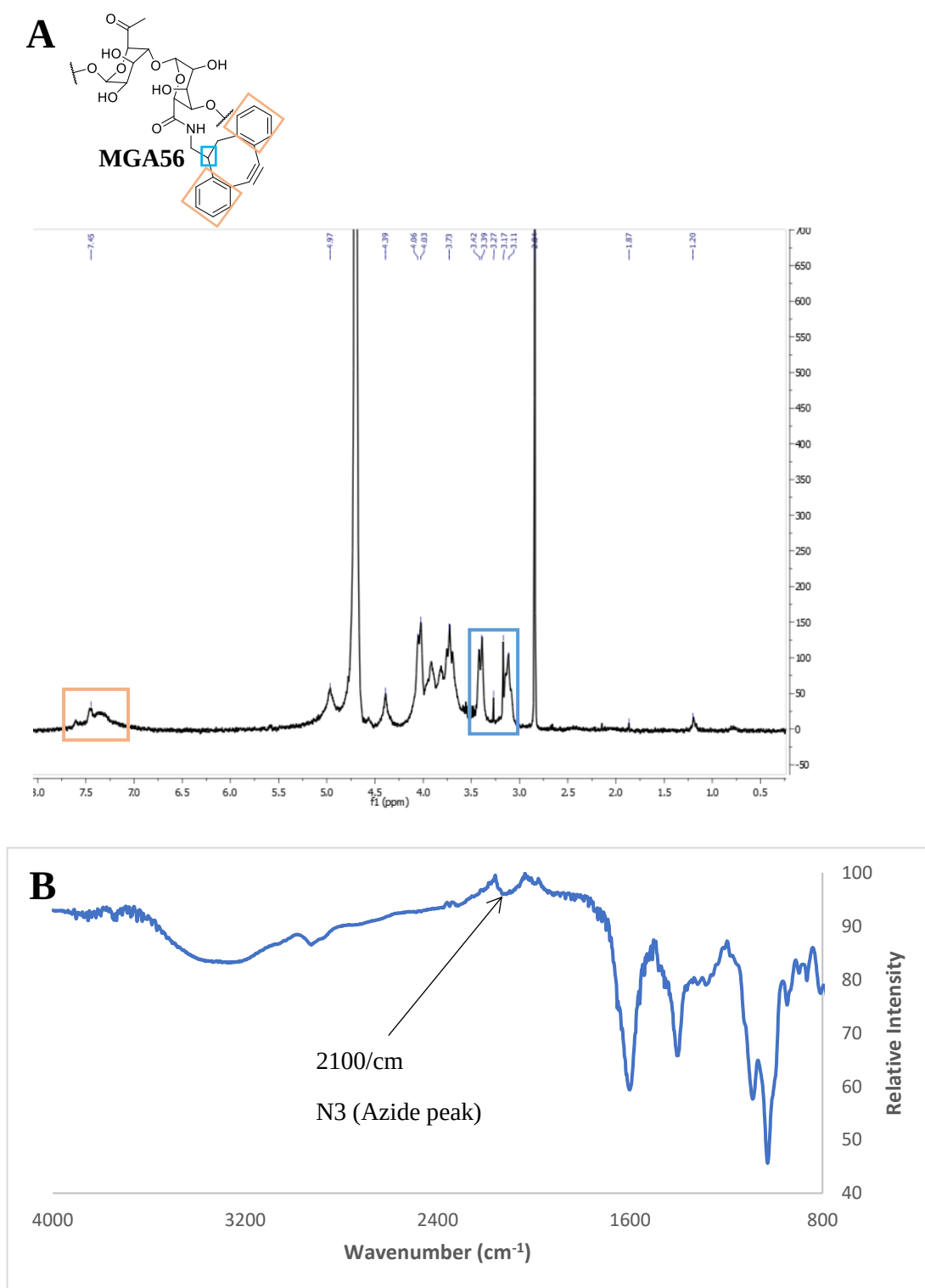


Figure 3.7 HNMR and FTIR spectra of MGA56. (A) HNMR spectra of MGA56 showing the predicted HNMR peaks (B) I.R. spectra showing the chemical profile of the modified alginate MGA56, but unable to identify peaks in FTIR spectra.

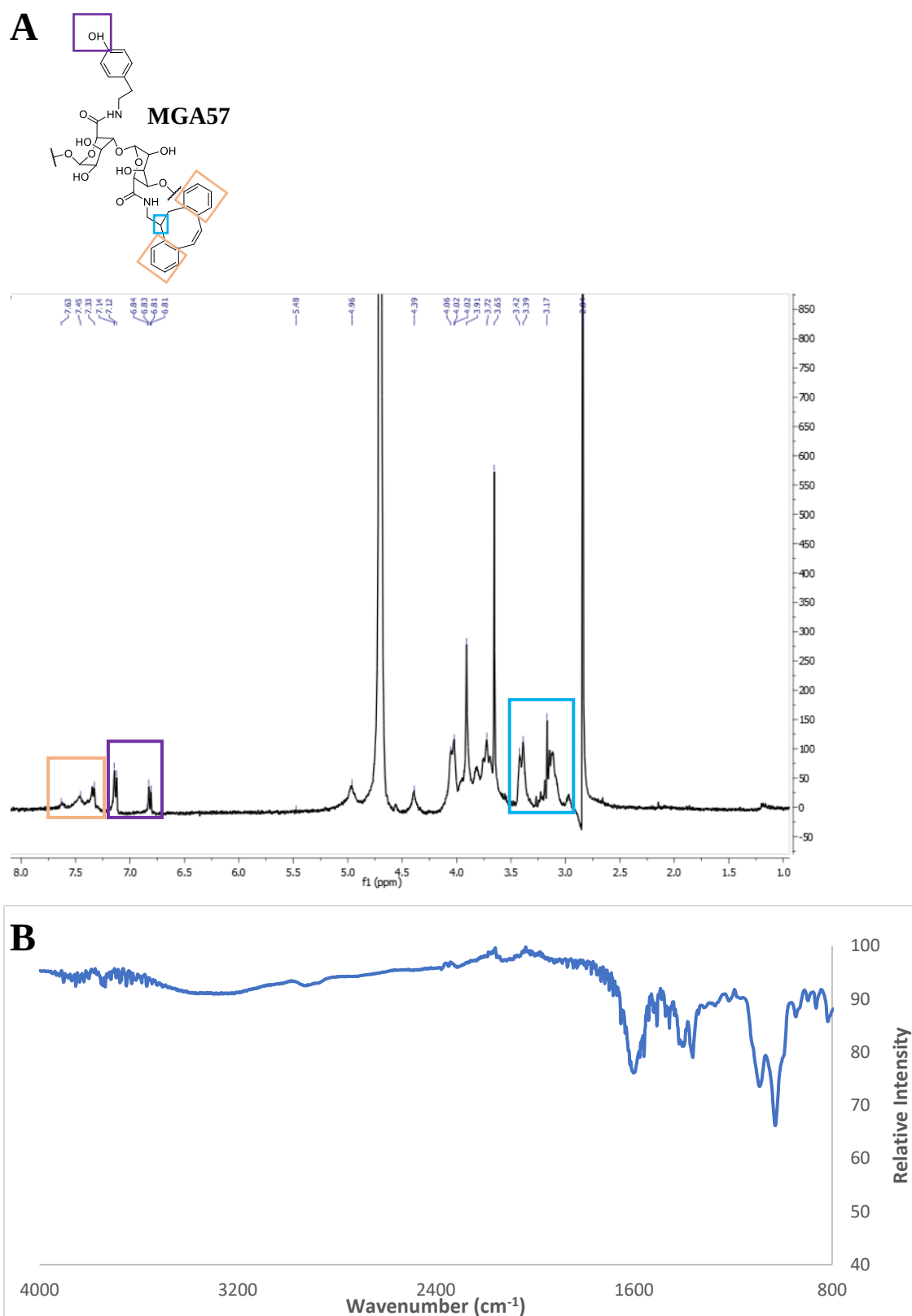


Figure 3.8 HNMR and FTIR spectra of MGA57. (A) HNMR spectra of MGA57 showing the predicted HNMR peaks (B) FTIR. spectra showing the complex chemical profile of the modified alginate MGA57, but unable to identify peaks in FTIR spectra.

3.4.2 Assessing the biocompatibility of modified alginate hydrogels using HepG2 and human primary hepatocytes

10' 000 HepG2 and primary human hepatocytes were encapsulated in the five modified alginates and controls (Food grade Alginate (FA) and Medical grade alginate (MGA)) Figure 3.9 and their viability in the alginate hydrogels was assessed.

Cell viability at 8, 24 and 72 hours was measured using a developed macro on ImageJ. Three separate images were taken from three different section within each alginate hydrogel sample. After staining, cell viability was measured as a percentage of live cells (green fluorescent) over the total number of cells (green and red fluorescent) (Figure 3.12). Viability was maintained over 72 hours for the food alginate (FA), medical-grade alginate (MGA) and MGA42 modification with the viability of above 79% (Figure 3.10). At 8, 24 and 72 hours, a slight drop of (viability dropped 76%) was recorded for the MGA control. This data shows that the HepG2 cell line in FA, MGA and MGA 42 remained viable for at least 72 hours. However, HepG2 encapsulated in the remaining modified alginates show a drastic drop in cell viability from an average of 55 % to less than 5% over 72 hours, suggesting that the addition of the different chemical substrate have had a cytotoxic effect on the HepG2. These results are consistent with the live/dead images shown in Figure 3.12.

Similarly, the viability of human primary hepatocyte extracted from a resected liver after hepatectomy was measured at 16, 24 and 72 hours using a live ad dead assay and counting the live/dead cells. Primary hepatocytes were counted using an in house developed macro on ImageJ. Three separate images were taken for each of the three alginate hydrogels (MGA, MAG42 and MGA57). After staining, cell viability was measured as a percentage of live cells (green fluorescent) over the total number of cells (green and red fluorescent). Viability in human primary hepatocytes seems to drop drastically after 16 hours, even though HepG2 encapsulated in the modified MGA42 alginate remained viable over 72 hours. No biological compatibility was seen in the human primary hepatocytes when encapsulated in the MGA42. Viability dropped around 25% for the MGA42 after 24 hours, having started from approximately 75%. Primary hepatocytes encapsulated in MGA57 showed no viable cells after 16 hours, as seen in Figure 3.13. The chemistries/substrates added in the MGA42 and MGA57 must have had a cytotoxic effect on the primary hepatocytes, as shown in Figures 3.11 and 3.13. Human primary hepatocytes encapsulated in control medical-grade alginate (MGA) maintained viability at around 70% over the 72 hours (Figure 3.11).

HepG2 proliferation in chemically modified alginate and controls was quantified by performing a Picogreen dsDNA assay. This fluorescent assay quantifies the amount of double-stranded DNA present in a given sample. Firstly, alginate lyase was added to the alginate hydrogels to digest them enzymatically. Results were calculated as a relative fold increase to 8 hours for each alginate sample. An increase of DNA was seen in MGA 42 modified alginate over 72 hours compared to FA and MGA alginate controls. An almost 3-fold increase in dsDNA quantity was recorded in the MGA 42 samples, indicating an increase in proliferation activity of the HepG2 over 72 hours (Figure 3.14).

However, as shown in the viability graph (Figure 3.10), HepG2 encapsulated in the other modified alginates experienced a huge drop in viability, in agreement with the results shown in the Picogreen analysis (Figure 3.14). HepG2 encapsulated in the remaining modified alginates failed to increase. No significant difference in dsDNA quantity was found over 72 hours between the control groups (MGA and FA Alginates) (Figure 3.14).

An estimation of the total protein present in each sample was carried out using a Bradford assay test. The media was collected, and the test was carried out as per manufacture instructions. No significant difference in total protein secretion was seen over 72 hours across each chemically modified alginates and controls (Figure 3.15).

Albumin secretion is a crucial parameter for testing hepatocytes *in-vitro* functionality. To assess the functionality of the HepG2 encapsulated in the chemically modified alginates, albumin secretion was measured using an Albumin ELISA kit. Media was collected, and the assay was carried out following manufacture instructions.

Results show no significant difference in the increase in albumin production for the controls and the MGA 42 between 24 and 72 hours. A significantly higher difference was seen at 72 hours when comparing albumin secretion of the MGA 42 alginate with MGA 53, 54 and 56 Figure 3.16.

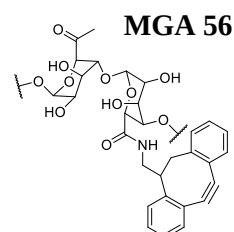
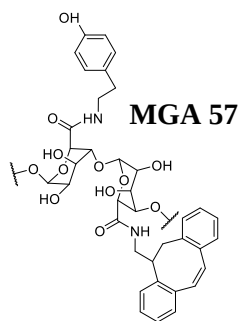
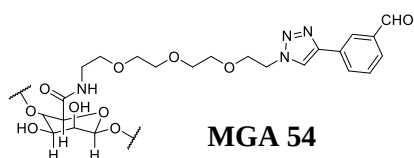
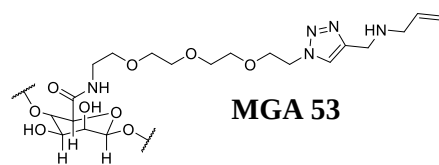
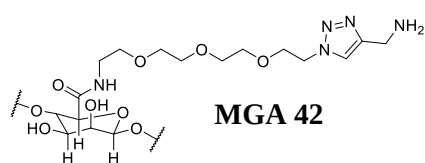


Figure 3.9 Chemical structure of the 5 selected modified alginates. Chemical structures drew utilising ChemDraw Professionals software.

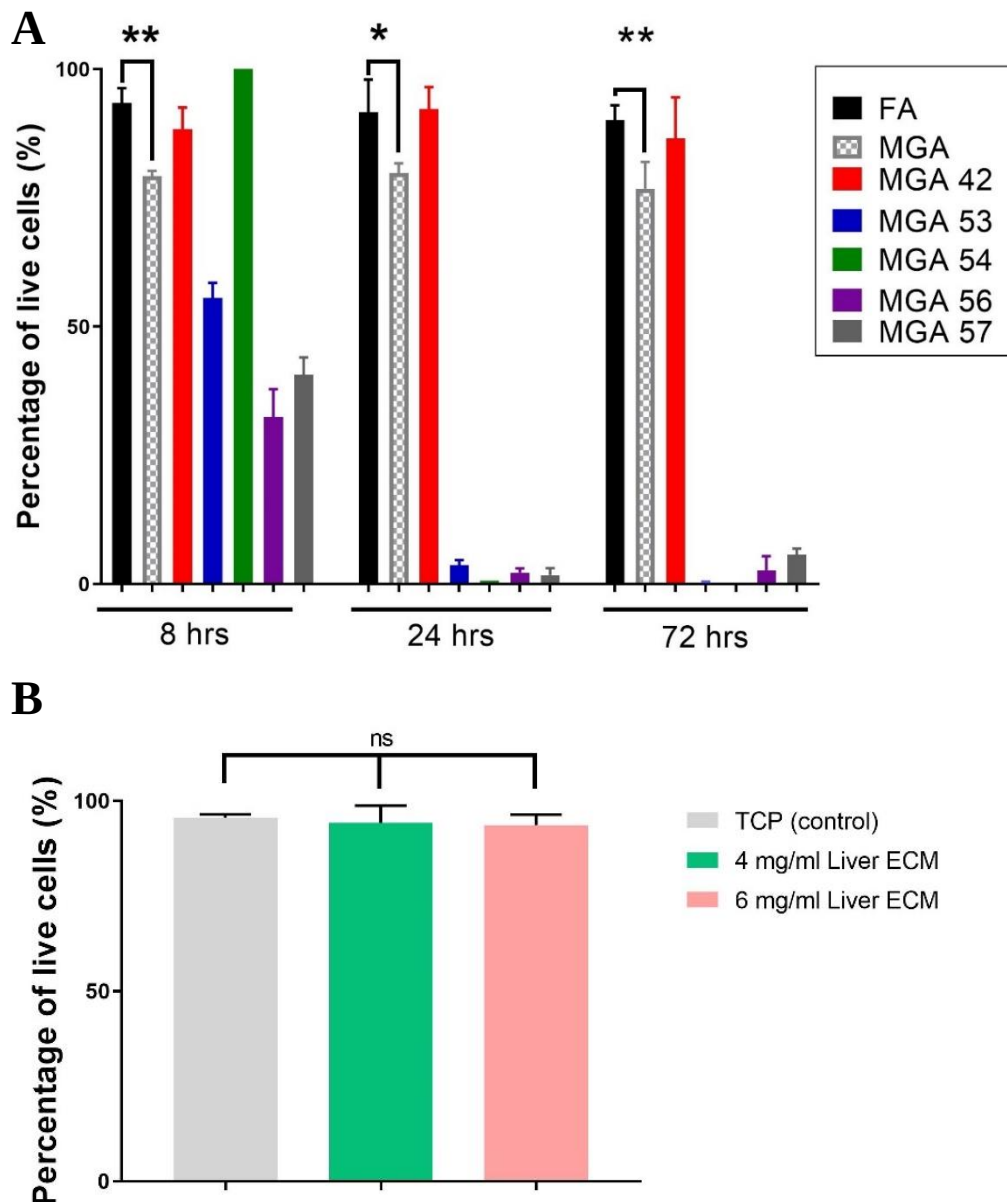


Figure 3.10 (A) Cell viability of HepG2 in controls (FA., MGA) and selected modified alginate (MGA 42,53,54,56,57). Cell viability of HepG2 in each alginate hydrogels was analysed at 8, 24 and 72 hours. (*) indicates statistical significance between FA. control and MGA control. Statistical analysis was carried out with One-way Anova multiple comparison tests with Bonferroni post hoc test, $p < 0.05$ was considered significant. No statistical significance was seen in HepG2 encapsulated in MGA42 over 72 hours. Data represented as means $\pm$ S.D., $n = 3$ replicate samples per condition. (B) The histograms plots represent the percentage of viable HepG2 after 72 hours cultured on 4 and 6 mg/ml ECM hydrogels. The error bars are calculated from $n = 3$ technical replicates. Statistical significance was assessed using Ordinary One-Way Anova with Tukey multiple comparisons (ns: $p = 0.25$). Similar viability is found between HepG2 cells cultured in ECM hydrogel, Alginate control and MGA42.

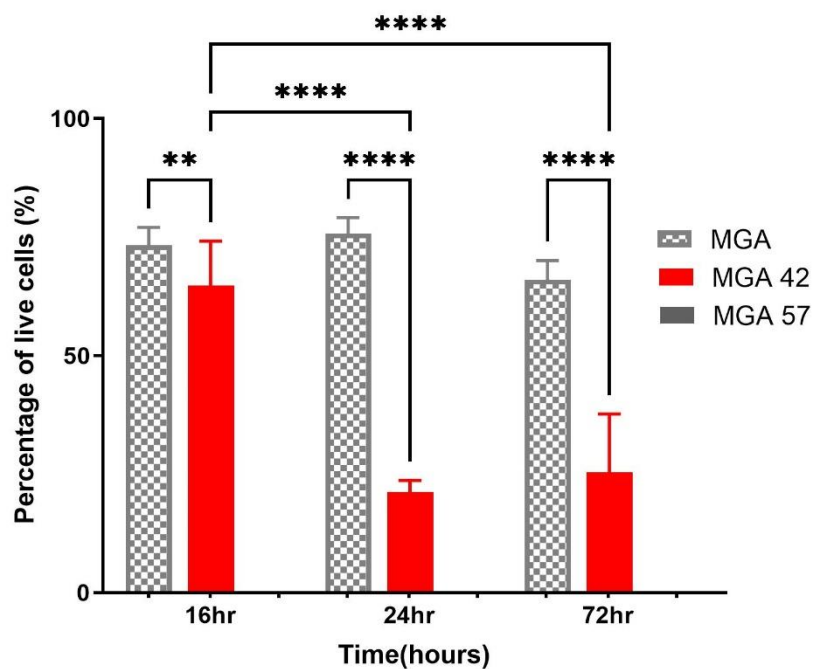


Figure 3.11 Cell viability of encapsulated human primary hepatocytes in control (MGA) and modified alginate (MGA 42 & 57). Cells viability was analysed at 16, 24 and 72 hours. (*) indicates the statistical significance of MGA42 and MGA between time points. Medical grade alginate showed a consistent cell viability of 70% over the 24 hours, with a slight drop at 72 hours. Statistical analysis was carried out with One-way Anova multiple comparison tests with Bonferroni post hoc test, $p < 0.05$ was considered significant. Data represented as means $\pm$ S.D., $n = 3$ replicate samples per condition.

3.4.2.1 HepG2 cytotoxicity in modified alginate hydrogels

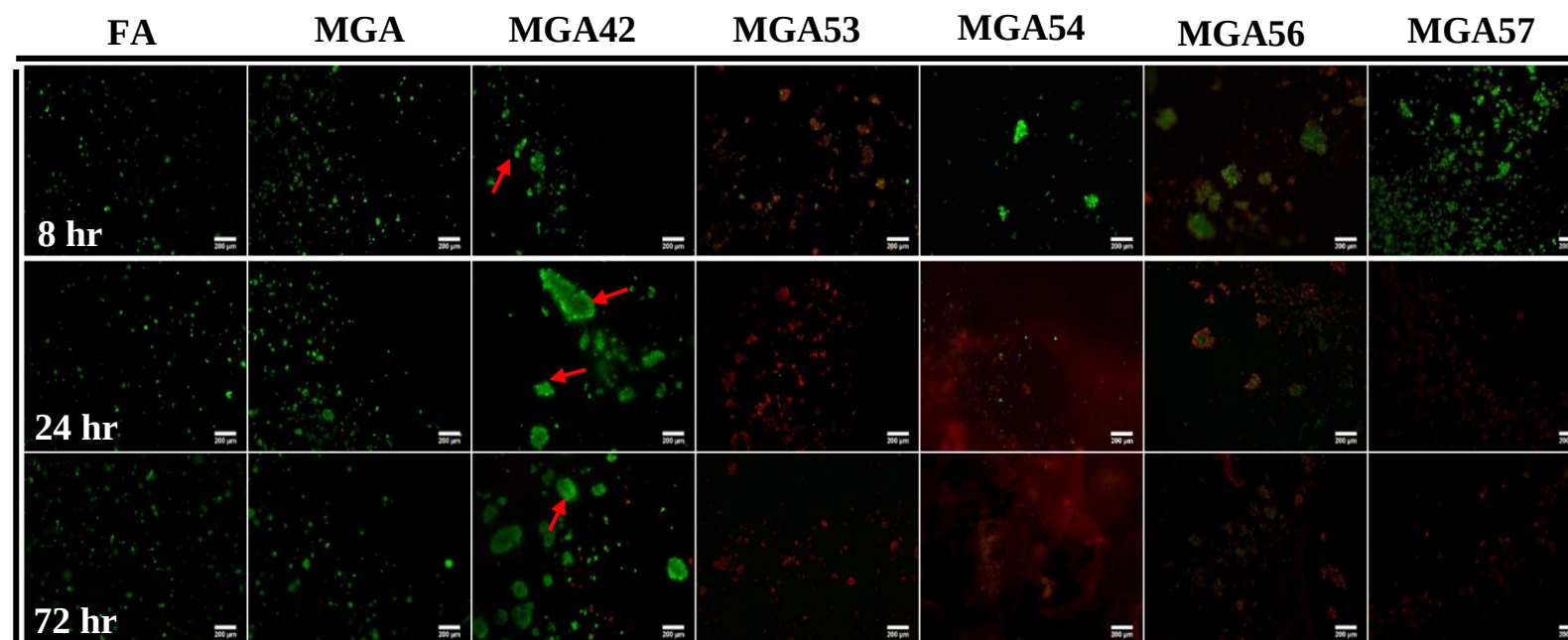


Figure 3.12 Live/dead staining of HepG2 encapsulated in controls (F.A. and MGA) and chemically modified alginates (MGA 42,53,54,56 & 57). Images show the viability of HepG2 encapsulated in both controls and modified alginates over 72 hours. Green (calcein-AM) represents alive and intact cells, while red (ethidium) represent dead cells. Cells appear rounded in morphology or clustered (indicated by red arrows). Images show that HepG2 cells encapsulated in F.A., MGA and MGA42 remained viable within the hydrogels over 72 hours. HepG2 encapsulated in MGA 42 modified Alginate showed an increase in cell clustering over 72 hours. Scale bar 200 μ m.

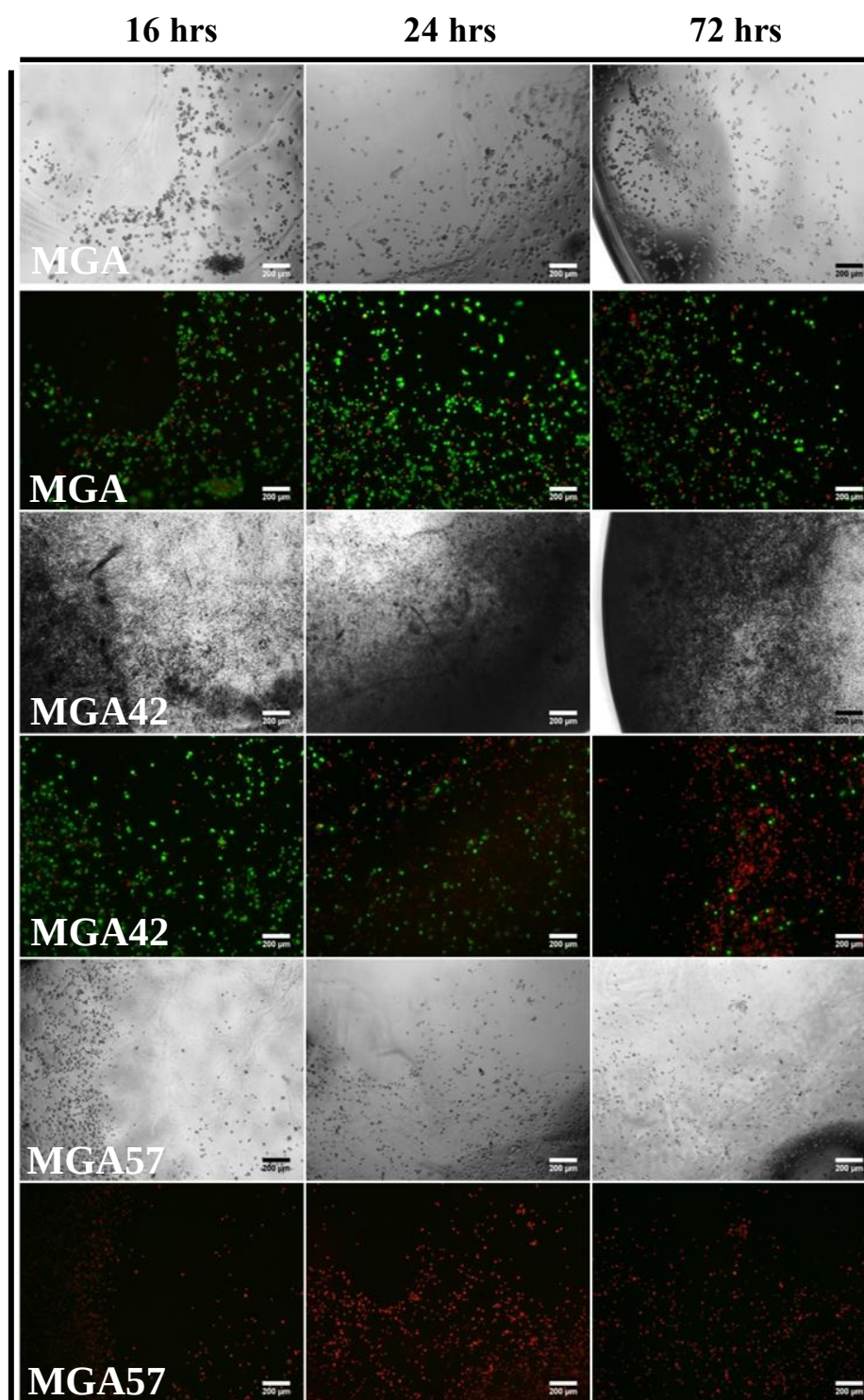


Figure 3.13 Live/dead staining of Primary human hepatocytes encapsulated in control (MGA) and chemically modified alginates (MGA 42 & 57). Cells appear rounded in morphology in both controls and modified alginates. Viability drops at different rates, with MGA57 the quickest, followed by MGA42 and MGA control. Within 72 hours, some cells were still viable in both control MGA and MGA42, but no cells appeared viable in MGA57. Scale bar 200 μ m.

3.4.2.2 HepG2 cells proliferation in the five selected modified alginates

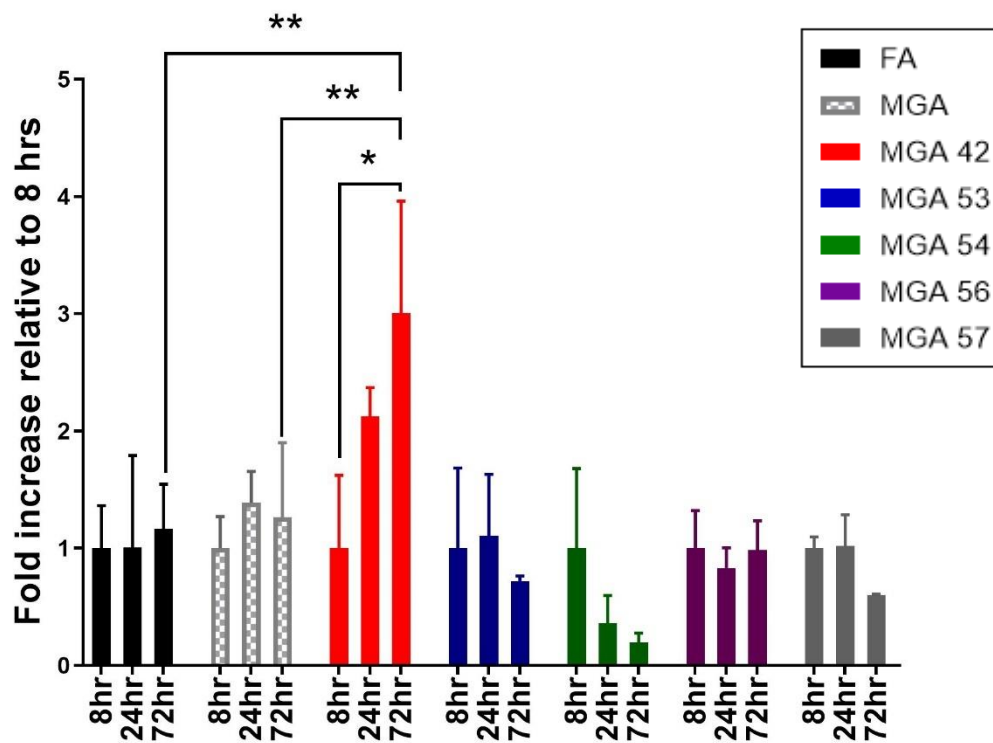


Figure 3.14 Relative DNA content normalised to 8 hours for each alginate group. Ds DNA quantification of HepG2 encapsulated in modified alginates and controls. Statistical analysis was carried out with One-way Anova multiple comparison tests with Bonferroni post hoc test, $p < 0.05$ was considered significant. Data represented as means $\pm$ S.D., $n = 3$ replicate samples per condition.

3.4.2.3 Total amount of protein secreted by HepG2 encapsulated in modified alginates

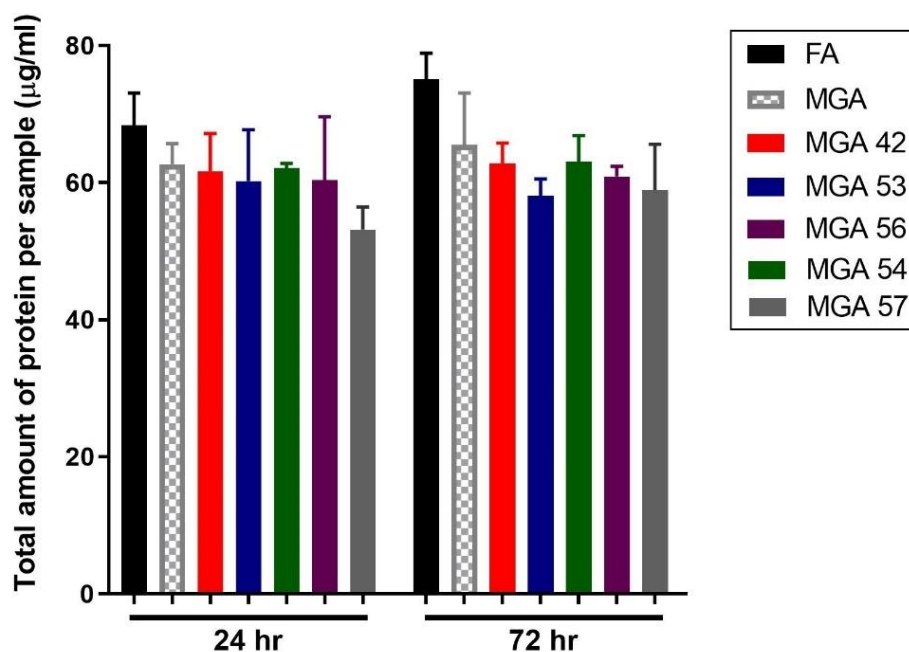


Figure 3.15 Total content of protein present per sample. The total amount of protein was measured at 24 and 72 hours for each modified alginate and controls (cells are cultured in a serum-containing medium). Statistical analysis was carried out with One-way Anova multiple comparison tests with Bonferroni post hoc test, $p < 0.05$ was considered significant. Data represented as means $\pm$ S.D., $n = 3$ replicate samples per condition.

3.4.2.4 Albumin secretion of HepG2 encapsulated in modified alginates

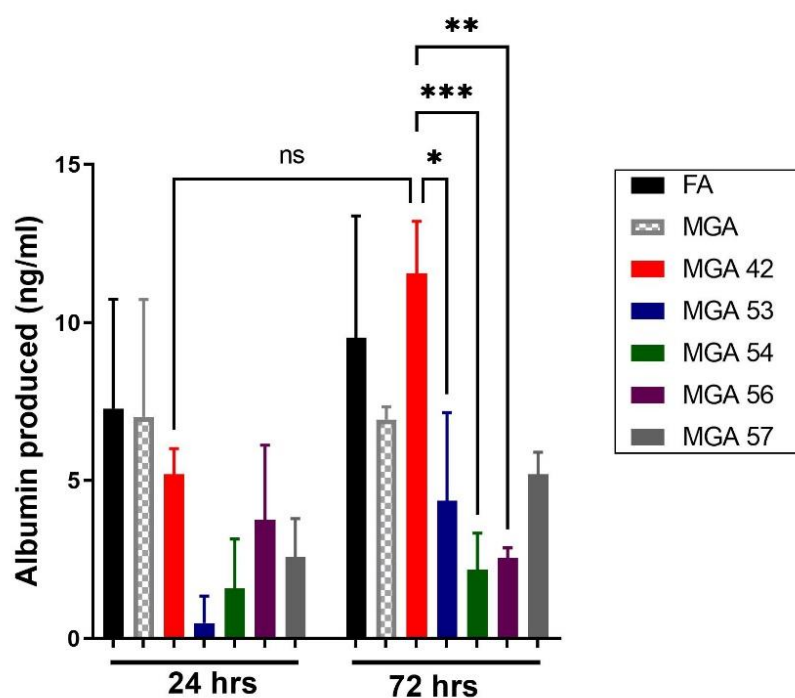


Figure 3.16 Albumin secretion of encapsulated HepG2 over 72 hours. Albumin secretion of HepG2 encapsulated in selected modified alginates and controls at 24 and 72 hours. Statistical analysis was carried out with One-way Anova multiple comparison tests with Bonferroni post hoc test, $p < 0.05$ was considered significant. Data represented as means $\pm$ S.D., $n = 3$ replicate samples per condition.

3.5 Discussion

Alginate is an inert biomaterial without any RGD domain for the cells to attach (Lee and Mooney, 2012; Malda et al., 2013). Hepatocytes are adherent cells that rely on biochemical cues from ECM components such as collagens and fibronectin and growth factors released to attach (BEN-ZE'EV et al., 1988). Therefore researchers have utilised specific chemistries to functionalised alginate and create active sites where moieties, peptides and chemical groups could be attached (Chung et al., 2002). In these preliminary experiments, different small molecules (Vegas et al., 2016) from a large library have been chemically attached to the alginate backbone through a click chemistry reaction to finding out what effect this substrate would have on cells attachment morphology and proliferation. Vegas et al. (2016a) also employed a high throughput approach to determine which best-modified alginates mitigated foreign body response (Vegas et al., 2016).

The five chosen modified alginates have different substrates attached and different chemical cues that the cells are exposed to. MGA 42 substrate contains an amine group (NH_2), and the chemical name is propargylamine. This functional group is hydrophilic, a potential characteristic that could have increased cell proliferation and clustering. Furthermore, the cell clustering ability in MGA42 might have been due to the cation content. A hydrophilic scaffold can provide a substrate for cell adhesion and proliferation (Birhanu et al., 2018; Mirhosseini et al., 2016). It has also been reported in different papers that the primary amine components have an improved effect on cell attachment and proliferation on plasma-treated surfaces (Aziz et al., 2015; Černochová et al., 2020; Yang et al., 2014). The difference with this chemical conjugation is that the chemical substrate (propargylamine) is conjugated to alginate, which is then made into a hydrogel, which provides a 3D environment instead of a 2D surface. Therefore, cells might have had access to the substrate more easily and abundantly.

Similarly, FA and MGA are hydrophilic hydrogels with high water retention capability, and results demonstrate that HepG2 were viable and rounded in morphology. However, no significant cell clustering was observed compared to the MGA 42. The other hydrophilic substrate attached in one of the modified alginates was a phenol group. MGA 57 contains a phenol group reported to be cytotoxic and harmful to cells in several papers (Pace and Elliott, 1962; Pedersen et al., 2002). My work confirmed this finding as cell viability dropped dramatically within 24 hours of culture, as shown in Figure 3.10 and Figure 3.12.

Furthermore, MGA57 may be partly toxic due to oxidant residues and potentially be a mixture of conjugated and unconjugated components left behind during the synthesis steps. The

remaining modified alginates have hydrophobic substrates attached. MGA 53 has an amide functional group that seems to have a cytotoxic effect on HepG2. However, more research is needed to fully understand why the amide functional group has this toxic effect on cells. MGA 54 has a benzaldehyde ring, and the chemical substrates are called 3-ethynyl benzaldehyde. A paper reported the toxicity of 4 different aldehydes on two hepatoma cell lines. Other toxicities were found between the two different cell lines when exposed to various chemicals. However, aldehyde, in general, are considered carcinogenic and can influence cells growth and activity (Bassi et al., 1997). In this study, the cytotoxicity of 3-ethynyl benzaldehyde was found to be detrimental to the HepG2 cell viability. Further experiments should confirm at what concentration or percentage this chemical might be harmful to the cells. Finally, MGA 56 had a toxic effect on cell viability (Figure 3.12), where most of the cells died within 24 hours. The chemical has a Dibenzocyclooctyne-amine substrate group, which contains the previously discussed benzene ring, proven toxic to the cells (Moriarty et al., 2010). A correlation between hydrophilicity and cell viability might be present. Speculation can be made that anchorage-dependent cells, as HepG2, prefer a hydrophilic environment to remain viable and aggregate.

In fact, the small molecule tethered to alginate in MGA 42 is hydrophilic; it seems to have an essential role in inducing cell proliferation compared to the other hydrophilic alginate used as controls. Contrarily, when human primary hepatocytes were encapsulated in MGA42, a cytotoxic effect was seen within 16 hours (Figure 3.13). Alginate beads are primarily used to help cryopreserve human primary hepatocytes to maintain their function and viability better once revived (Rialland et al., 2000) as well as to aid potential intraperitoneal transplantation to provide short term regeneration function for acute liver failure (ALF) (Dhawan et al., 2020; Jitraruch et al., 2014). As shown in the preliminary results, although MGA42 was cytocompatible with the HepG2 cell line, it triggered a cytotoxic effect on the human primary hepatocytes leading to complete cell death within 72 hours (Figure 3.11). Isolated human primary hepatocytes are susceptible cells and difficult to maintain and culture due to their rapid depolarisation and dedifferentiation when the culture is in 2D substrates (Shulman and Yaakov, 2013). They lose their cuboidal morphology and liver-specific function. Due to these shortcomings, they might have been more fragile and susceptible to the presence of the moieties attached to the alginate, and those moieties might have caused a cytotoxic effect leading to cell death.

However, the unmodified medical-grade alginate seems to maintain the cell viability slightly better than its modified form, at around 60% after 24 hours. Primary hepatocytes are not proliferative cells, as they go through a cycle of division once plated on collagen sandwich and then stay quiescent (Cho et al., 2015). Therefore, proliferation in the alginate was not

expected. Furthermore, many studies have shown that primary hepatocytes can be maintained in Collagen or Matrigel sandwich culture over a short period (Yang et al., 2016). Collagen and Matrigel provide the primary hepatocytes with a mimical environment and biochemical cues released from the ECM matrix (Sellaro et al., 2010). The inert, plant-derived natural material, alginate, cannot compete.

Furthermore, future studies involving these chemically modified alginates will need to maintain cell viability for longer to assess cell development and functionality *in-vitro*.

Looking more closely at the HepG2 cell line, when HepG2 are exposed to a 3D environment, cells start to form clusters as shown in the control group and the MGA 42 modification in Figure 3.12. Cells exposed and surrounded by a matrix as in a 3D environment form spheroids because of cell-to-cell interaction. This behaviour can be seen in the control alginates and large cell clusters seen in the modified Alginate MGA 42. The NH₂ substrate, which was chemically conjugated to Alginate, might have played an essential role in enhancing cell clustering within the gels. The cell to cell signalling pathway might have been increased because of exposure to this new chemical cue. As reported in Figure 3.14, the dsDNA quantification data shows an almost 3-fold increase in dsDNA in the MGA 42 alginate after 72 hours of culture compared to the controls, where cells did not seem to proliferate.

Similarly, Lan et al. (2010) studied the morphology and viability of HepG2 encapsulated in simple alginate gels. HepG2 cells in 3D did not proliferate over 14 days. Cells numbers remained constant, and cells remained viable over that period. Furthermore, cells in their study were reported rounded in morphology which is consistent with my finding' in the alginate controls (Lan et al., 2010). This rounded morphology could be due to cells being entrapped in the surrounding matrix, which impedes the cells to spread. In addition, integrin proteins on the cells surface were unable to recognise the material due to alginate lacking mammalian cells binding motifs that allow cells to interact with the matrix. In another study conducted by Coward et al. (2005), HepG2 proliferation rates encapsulated in alginates beads were compared between a static and dynamic culture. The HepG2 cultured in the dynamic culture showed an increase in proliferation, suggesting that not only chemical cues but external micro forces and stimuli can trigger an increase in cell proliferation (Coward et al., 2005). In this experiment, HepG2 encapsulated in the other modified alginates showed a drastic decrease in cell viability after 8 hours, as shown in Figure 3.10, suggesting the chemical conjugation might have a cytotoxic effect leading to cells apoptosis. After 8 hours, almost 100 % of the cell were dead in MGA 53, 54, 56 and 57. Their respective chemical conjugation with an amide group, a benzaldehyde, a dibenzocyclooctyne-amine group, and a phenol group had proven to be toxic for cells, as previously stated.

The dsDNA results for the MGA 53, 54 56 and 57 alginates in Figure 3.14 are consistent with the results of the cell viability. dsDNA did not increase after 8 hours for all the modified alginates apart from the hit alginate MGA 42. Alginate hydrogels appearance look significantly different when comparing the controls with the modified alginates. Most of the chemically modified alginate appears to have uncrosslinked materials within the hydrogel (darker patches) (Appendices A, Figure AppA 3.4 and 3.5). Another challenge was encountered in the crosslinking process. The alginates gelation occurred very fast. Due to the 'egg block' configuration (Rowley et al., 1999), which occurs around the calcium ions as alginates are crosslinked, it undergoes a fast and uncontrolled significant shrinkage.

Furthermore, due to hysteresis, cells encapsulated in the hydrogel were found to be deposited at the edge of the well, leaving few or no cells in the middle of the well. This needs optimisation to allow for a more uniform distribution of cells within the alginate gel. A slower and more controlled crosslinking process might be the solution and can be achieved by changing the concentration of CaCl_2 , the crosslinking time and the crosslinking reagents (Alizadeh Sardroud et al., 2017; Kurowiak et al., 2020), the methodology to control better the crosslinking process (Dodero et al., 2019) or by chemically modifying the Alginate to be crosslinked using a photoinitiator (Jeon et al., 2009).

Albumin secretion is one of the most common parameters tested on hepatocytes to prove their validity in terms of functionality. HepG2, as discussed in the introduction, poorly express liver-specific function such as gene expression and CYP activity. In this report, albumin secretion was measured with an Albumin ELISA kit. An increase in albumin secretion over 72 hours was observed for the MGA 42 compared to the other modified alginate, where a decrease in albumin secretion was seen but not significant. These results are consistent with the Picogreen assay, whereas expected, with an increase in cell proliferation for the MGA42, a respective increase in albumin secretion was seen, although not significantly different. In future experiments, more time points can be selected. No statistically significant difference was seen in MGA and FA between 24 and 72 hours, suggesting another more extended time point is needed to view the albumin secretion profile fully. Many papers report albumin secretion of HepG2 in alginate beads with time points up to 14 days, where a significant increase in albumin can be detected (Lan et al., 2010; Sun et al., 2018). Due to the limited quantity of the materials, the preliminary time points were chosen to be 24 and 72 hours.

3.6 Conclusion

Different chemical substrates were added to the alginate backbone through amidation or Huisgen cycloaddition to verify if these chemical cues influenced cell behaviour. The modified alginate chemistries were characterised using HNMR and FTIR technologies to assess the chemical composition. HepG2 encapsulated in alginates have been tested for cytotoxicity, Albumin secretion and DNA quantification. From the data reported, almost all of the chemically modified Alginate has been proven cytotoxic (except MGA42) for both HepG2 cells and human primary hepatocytes. Therefore, it was concluded that these specific four selected chemically modified Alginate were not suitable for encapsulating hepatocytes. Further study could investigate the potential of MGA42 over longer time points and using different cell lines to see if an increase in cell proliferation and clustering is observed. Overall, none of the chemically modified Alginate hydrogels was suitable for producing an *in-vitro* liver model.

Although results from MGA42 modified alginate were promising and showed some positive effect on HepG2 cells, alginate, as a biomaterial, lack important chemical moieties and RGD domain which are necessary if we are trying to mimic the liver microenvironment. Cells require those biochemical cues which can be found in liver ECM. From this part of the research, the importance of chemical cues on cell behaviour has been demonstrated. Cues within a microenvironment are necessary if the end goal is to mimic the liver niche.

As a result of this work, it was decided to explore other potential natural biomaterials which already present moieties and chemical cues within their structure with no need for a further chemical conjugation step.

Thus, we hypothesised that the fundamental components present in native ECM could provide natural biochemical cues and we chose ECM derived biomaterials to be a more suitable candidate for encapsulating hepatocytes cells.

We then wanted to explore a way of retaining all these critical ECM components and chemical cues without compromising our goal to create a structure that would encapsulate liver cells for the sought-after 3D *in-vitro* liver model. Hence, we choose a decellularisation method to strip off all the cellular components from the liver tissue, aiming to be left with an acellular scaffold with preserved chemical cues, growth factors, proteins and glycosaminoglycans.

Chapter IV - Decellularisation and characterisation of porcine liver ECM

4.1 Introduction

Decellularisation aims to remove antigenic and cellular material from the native organ while preserving the ECM structure and relevant biochemical components (Gilbert et al., 2006). This acellular scaffold is immunogenic and with preserved proteins and glycosaminoglycans based on how it has been processed (Choudhury et al., 2020).

The main goal of liver decellularisation is to maintain the tissue biochemical component such as ECM molecules and growth factors while providing a suitable environment for hepatic cells to integrate and repopulate the decellularised tissue (Baptista et al., 2013; Sellaro et al., 2010).

In the last decade, the use of decellularised liver ECM has expanded as its application has been diversified. Liver dECM can be solubilised and formed into hydrogels (Lewis et al., 2018). It can be used as bioinks for 3D extrusion printing (Lee et al., 2017; Mao et al., 2020), and it has been shown to influence hepatic cells migration, activation, survival and differentiation (Hussein et al., 2020; Loneker et al., 2016).

After tissue decellularisation, a bioactive scaffold is produced. Such dECM scaffold retained growth factors and proteins contributing to tissue remodelling and cellular engraftment (Crapo et al., 2011). A biologically active scaffold is left behind with preserved architecture and structure and vesicles and proteoglycans (Gilbert et al., 2006; Gilpin and Yang, 2017; Hussey et al., 2018). Thus, dECM scaffolds can be implemented and used in *in-vitro* (Mazza et al., 2017, 2015) and *in-vivo* applications (Spang and Christman, 2018).

4.1.1 Liver decellularisation techniques

One of the liver decellularisation method for transplantation of whole livers is whole organ perfusion. Detergents and enzymes are flushed through the hepatic artery and portal vein (Schurink et al., 2019). Recently, perfusion decellularisation has been coupled with oscillation pressure, which aims to mimic the intraabdominal conditions during respirations (Hillebrandt et al., 2015; Struecker et al., 2014). The main aim is to maintain the whole organ architecture and all ECM components as a potential reseeding scaffold for liver parenchyma

and non-parenchyma cells (Uygun et al., 2010) Table 4.1. This addresses organ shortage and the use of decellularised liver as a possible solution (Yujia Wang et al., 2017).

Other methods of liver decellularisation have used thinly sliced liver tissue and combinations of detergent/enzyme and mechanical agitation methods to create dECM liver discs for hepatic cell culture (Lang et al., 2011). Furthermore, solubilised liver dECM can be formed into hydrogels, and it has been found to promote hepatocyte *in-vitro* functions (Ijima et al., 2019).

Table 4.1 Liver decellularisation techniques were employed to decellularised whole organs or liver pieces, highlighting the advantages and disadvantages.

Liver Decellularization Techniques	Application	Advantages	Disadvantages	Papers
Perfusion (Detergents and Enzymes)	Whole organ re-cellularisation and transplantation	• Maintenance of liver architecture • Liver cells engraftment • Maintenance of vasculature • Retention of ECM components	• Long decellularisation time significantly bigger species, e.g. Human and porcine livers • Mainly used for rat's liver • Due to lengthy perfusion time, ECM components can be compromised	(Verstegen et al., 2017) (Schurink et al., 2019) (Maghsoudlou et al., 2016) (Uygun et al., 2010) (Schliamser et al., 2016)
Perfusion + Oscillation (Detergents and Enzymes)	Whole organ re-cellularisation and transplantation	• Maintenance of liver architecture • Liver cells engraftment • Maintenance of vasculature • Retention of ECM components • Shorten perfusion time • Homogeneous decellularisation • More effective perfusion	• Mainly used for rat's liver • Risk of damaging or thinning of vasculature if perfusion rate and pressure are too high	(Struecker et al., 2014) (Mazza et al., 2017) (Hillebrandt et al., 2015)
Immersion +Agitation (Detergents and Enzymes)	Hydrogels Bioinks ECM discs	• 3D printing applications • 3D culture • Retention of ECM components • Adjustable mechanical properties and gelation kinetics	• Lost of architecture and vasculature. • Hypoxic environment in 3D hydrogels • Batch to batch ECM hydrogel variation	(Mattei et al., 2013) (Ijima et al., 2019) (Lee et al., 2013) (Lang et al., 2011) (Skardal et al., 2015a)

One of the quality criteria set out for a successful decellularisation is the removal of DNA and nuclear material from tissues (Rossi et al., 2019). The removal of cellular components can be achieved through several methods: with enzymes and nuclease agents, chemically with detergents, with acids/bases solutions, and physically by mechanical agitation (Mendibil et al., 2020). Many research groups have used a combination of all three methods to successfully decellularise liver tissue (Mazza et al., 2017; Soto-gutierrez et al., 2011; Uygun et al., 2010). The removal of nuclear DNA material is essential to avoid an immune or inflammatory response in an immunocompetent host (Bilodeau et al., 2019).

Although many decellularisation protocols include a DNase treatment, even commercially available scaffolds still retain some DNA fragments, which can trigger an immune response and result in rejection or loss of scaffold function (Gilbert et al., 2009). Therefore, the successful decellularisation of tissues relies on the almost complete removal of DNA materials (Badylak and Gilbert, 2008; Bilodeau et al., 2019). Keane et al. (2012) showed a correlation between fragments of DNA left post decellularisation and the release of pro-inflammatory molecules from M1 macrophages (Keane et al., 2012). In the literature, the three accepted standards for evaluating liver ECM decellularised product are:

1. < 50 ng of DNA / mg ECM dry weight
2. the DNA fragment to be less than 200 bp
3. the absence of nuclei content using either 4',6-diamidino-2-phenylindole (DAPI) or haematoxylin and eosin staining (H&E) (Crapo et al., 2011; Gilbert et al., 2009).

A delicate balance between removing DNA and antigenic material from the liver tissue and retaining its biochemical ECM components needs to be achieved to account for a successful decellularisation. The use of certain detergents, e.g. Triton x-100 and sodium dodecyl sulphate (SDS), have been shown to have some disruptive effects during ECM decellularisation (White et al., 2016). The length of time of decellularisation and the concentrations of detergents and enzymes are all contributing factors that need to be carefully optimised to achieve the maximum removal of DNA material without irreversibly disrupting ECM components (Rossi et al., 2019; White et al., 2016; Yagi et al., 2013).

Post liver decellularisation, the integrity of many ECM components, thus laminin, fibronectin, Collagen I, Collagen IV, cytokines and growth factors need to be evaluated (Bedossa and Paradis, 2003). Basement protein as laminin, fibronectins, and glycosaminoglycan are directly responsible for the integrin signal cascade of the hepatic cell network, thus modulating hepatocytes functions (Kim et al., 2011). Therefore, the importance of retaining ECM components because of their direct involvement in hepatic architecture and stem cell fate differentiation cannot be underestimated (Yunfang Wang et al., 2011).

Detergents are used to dissolve cell membranes and disrupt cell cytoplasm. Therefore, we must assume that any decellularisation process is not without harm, and it is crucial to evaluate the degree of retention and condition of ECM components post decellularisation (Yujia Wang et al., 2017). Many researchers use histological analysis such as H&E staining and specific staining including Sirius Red and Masson-trichrome (Hussein et al., 2020; Mao et al., 2020). Lang et al. (2011) went even further by characterised the presence of different ECM components: Collagen I, III, IV, Laminin, Elastin and Fibronectin through immunohistochemical staining before and after liver tissue decellularisation, to confirm the retention of these critical ECM components after decellularisation (Lang et al., 2011).

Another more quantitative technique to determine the successful retention of sulphated GAGs post decellularisation is Dimethylmethylene blue assay (DMMB) (Gupta et al., 2018; Sackett et al., 2018; Struecker et al., 2014). The sulphated GAGs concentration is measured with this colourimetric assay, resulting from a change of the DMMB dye shifting to a violet colour when it binds to polyanionic substrates (Templeton, 1988). There are debates whether the Ph value used to quantify sulphated GAGs is subjected to interference from polyanions such DNA and RNA. Zheng et al. (2015) suggested that reducing the Ph to 1.5 from 3 when using the DMMB assay results in more accurate quantification of sGAGs without compromising assay sensitivity (Zheng and Levenston, 2015).

4.1.2 Liver ECM hydrogels characterisation

After liver decellularisation, decellularised liver ECM pieces can be comminuted, solubilised and manipulated to fabricate hydrogels for *in-vitro* (Hussein et al., 2020; Mao et al., 2020; Sellaro et al., 2010) and *in-vivo* applications (Lee et al., 2013). ECM components can be solubilised using enzymatic digestion at acidic pH (Sawkins et al., 2018). The monomeric components spontaneously reform intramolecular bonds when exposed to a change in pH or temperature generating 3D hydrogels (Claudio-Rizo et al., 2018; Saldin et al., 2017) .

Particularly, collagen fibres self-assemble in the long fibrillar structure under the influence of specific parameters such as temperature, pH and concentration (Theocharis et al., 2016). The kinetics of polymerisation of the fibrillar collagen gel network consequently influence the swelling, mechanics, degradation and biological response of ECM hydrogels (Claudio-Rizo et al., 2018).

4.1.2.1 Liver ECM hydrogels under compression

A universal test-frame instrument is used to study materials behaviour under compression and tension. Most frames are uni-axial, meaning the motion and the force are applied along a single axis (Oyen, 2014). The stress-strain response of ECM derived hydrogels can be studied to assess the yield stress and the elastic modulus to elucidate its mechanical strength response (Giobbe et al., 2019; Ijima et al., 2018; Mao et al., 2020; Wu et al., 2015).

Furthermore, it can be used in combination with rheology to study the tissue mechanics of normal and fibrotic livers. Perepelyuk et al. (2016) developed a decellularised liver rat model. The apparent Young's modulus was measured by a series of studies using compressive, extensional and shear strain. It was found that storage and loss moduli increased in progressive fibrosis (Perepelyuk et al., 2016).

This technique can be used to approximate and compare the stiffness of the *in-vitro* liver model with actual liver physiological stiffness (Colombo et al., 2011). Consequently, it can be an indicator to be used in the development of *in-vitro* liver diseased models. It can be applied to identify liver stiffnesses to diagnose liver diseases (Huang et al., 2014; Mueller and Sandrin, 2010).

4.1.2.2 Rheology

Rheology commonly used outputs are the storage (G') and loss moduli (G''), which indicate the hydrogel stiffness and viscoelastic behaviour (Saldin et al., 2017). The storage moduli (G') can be defined as the material's ability to store energy elastically, whereas the loss moduli (G'') relates to the material's ability to dissipate stress. The ECM pre-gel solution is characterised by a low viscosity, which increases with ECM concentration (Freytes et al., 2008).

Frequency-based hydrogel testing uses a rheometer. The sample is loaded into the instrument, and different 'sweeps' are performed. Other parameters are measured, including a frequency sweep, where the sinusoidal oscillation is fixed but increases the angular frequency (Oyen, 2014). A temperature sweep can also be performed where the temperature is increased from 4 °C to 37 °C, and it is possible to observe that the rate of gelation is greater with increase ECM hydrogel concentration. (Lee et al., 2013; Massensini et al., 2015). When the gelation occurs, the viscous gel has transitioned from a liquid to a solid (Karoyo and Wilson, 2017). The maximum storage modulus can be associated with hydrogel stiffness. The transition to its solid-like behaviour is verified when the G' is above or greater than the G'' of around one order of magnitude (Freytes et al., 2008).

One of the most desirable characteristics of ECM pre-gel is its shear thinning behaviour, an increase in shear rate is accompanied by a decrease in viscosity (Shin et al., 2018), making it an ideal candidate in injection delivery system (Kadiyala et al., 2018) and 3D printing (Pati et al., 2014). With increased liver ECM concentration, an increase in storage modulus can be observed (Ijima et al., 2018; Lee et al., 2013; Loneker et al., 2016). However, due to different testing methods and decellularisation techniques the values reported for storage and loss moduli are significantly different between research groups and therefore not comparable.

4.1.2.3 Swelling behaviour

One of the hydrogels innate capabilities is to retain water and swell (Malda et al., 2013; Shivani et al., 2015). The swelling capacity of a hydrogel is calculated by the amount of aqueous media that can incorporate (Claudio-Rizo et al., 2018; Silva et al., 2018; Vlierberghe et al., 2014), and it is an indicator of the swelling profile, and the volume changes the hydrogel experience (Shivani et al., 2015). As well as being able to retain a large volume of water in a swollen state, hydrogels have also the capability of de-swelling reversibly in water (Ahmed,

2015). The significant changes in hydrogels volumes occur in the first 24 hours, after which usually the swollen or de-swollen state stabilise (Wu et al., 2020).

Mao et al. (2020) studied the swelling behaviour of a combination of porcine dECM and GelMA. An increase in swelling ratio was observed in the GelMA+ dECM hydrogels compared to the GelMA alone. Furthermore, the degradation profile was assessed under enzyme action, leading to faster degradation of the composite hydrogel (Mao et al., 2020).

4.2 Purpose of the work

The purpose of this work was to develop decellularised liver materials and to characterise these using a combination of mechanical and biochemical assay. Three critical key criteria to consider for liver dECM applications are:

- 1) Residual dsDNA material left post decellularisation.
- 2) Biologically active components such as sulphated GAGs.
- 3) Viscoelastic properties of the dECM fabricated hydrogels.

The fabricated liver dECM hydrogels will then be used to culture and differentiate human liver progenitor cells into hepatocyte-like cells for the fabrication of the liver *in-vitro* model

The aims of this chapter can be categorised as follows:

- Quantify the remaining amount of ds DNA left after decellulization.
- Qualitatively assess the presence of any residual ds DNA through H&E staining of decellularised and native liver tissue.
- Confirm the presence of sulphated GAGs retained after decellularization.
- Fabricate liver ECM hydrogels with a range of concentrations.
- Characterise the liver ECM hydrogels viscoelastic properties at different concentrations using a Rheometer.
- Characterise liver ECM hydrogels mechanical properties under compression at different concentrations.
- Characterise Liver ECM hydrogels swelling behaviour.

4.3 Experimental design

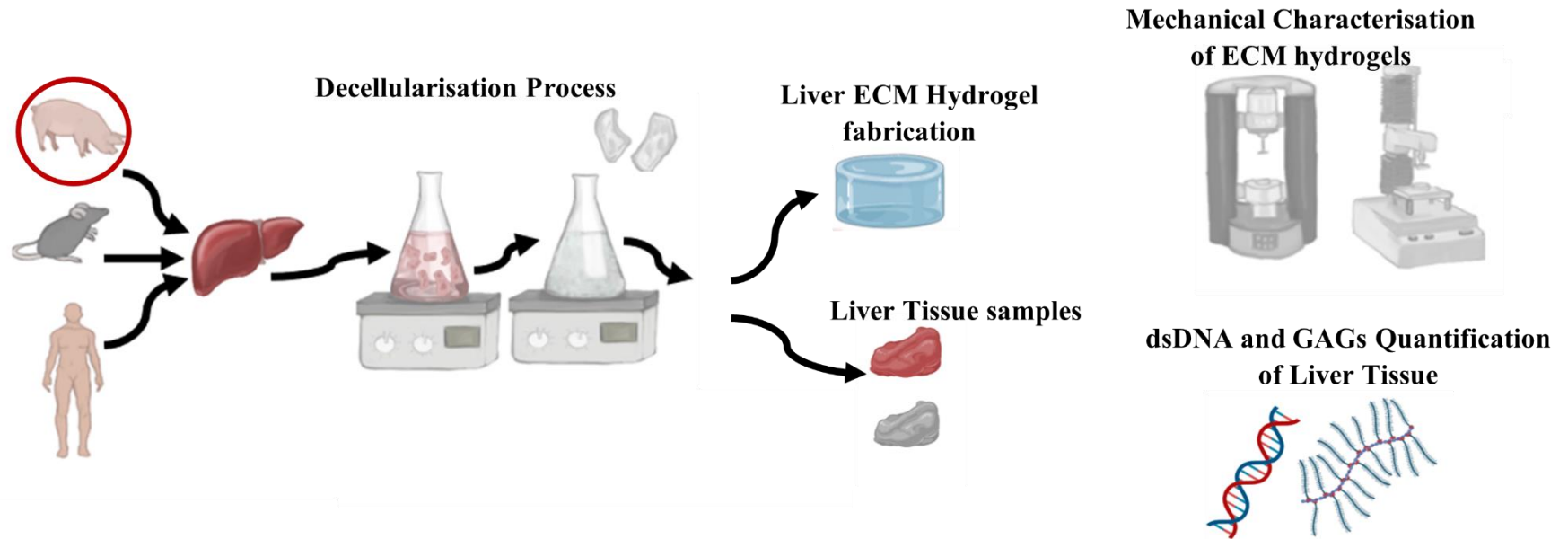


Figure 4.1 Experimental design map showing liver (derived from porcine, red circle) decellularisation process, liver tissue and liver ECM hydrogel physicochemical and mechanical characterisation. Drawings were made by Francesca Tarsitano, and it was created using BioRender.com.

4.4 Results

4.4.1 Physiochemical characterisation of porcine decellularised tissue

The decellularisation process is described in section 2.8. In this project, liver 2 (L2) was used, and tissue material was collected into three independent batches called L2B1, L2B2 and L2B3. Josh Jones and Dr Fatima Abukunna have collaborated in the preparation and characterisation of all three liver batches.

Once the liver tissue had been decellularised, three assays were run to determine the success of the decellularisation process: dsDNA quantification, DMMB assay and H&E.

Following liver decellularisation, a Picogreen assay was used to quantify the DNA content of the native liver tissue (Figure 4.2 (A)) and after decellularisation Figure (4.2 (B)). Batch L2B1 yielded ds DNA content of 250.1 ± 102.7 ng/mg of dry tissue resulting in an estimated removal of 92% of dsDNA (Figure 4.3 (A)). Batch L2B2 yielded ds DNA content of $566.5.1 \pm 75.12$ ng/mg of dry tissue resulting in an estimated removal of 82% of dsDNA (Figure 4.3 (A)). Batch L2B3 yielded ds DNA content of 236.1 ± 26.09 ng/mg of dry tissue resulting in an estimated removal of 92.4 % of dsDNA (Figure 4.3 (A)). To assess the preservation of sulphated GAGs, a dimethylmethylene blue assay was performed in all three batches. Figure 4.3 (B) shows the sulphated GAGs were retained in all batches and there was no statistical significance in GAGs maintain in Native liver with decellularised batches.

The further biochemical characterisation was confirmed qualitatively using Hematoxylin Eosin and Masson Trichrome staining. For a full detailed description, refer to Material and Methods section 2.5.5 Liver ECM hystology. Figure 4.4 indicate H&E and Masson Trichrome staining of native liver tissue (NTV), decellularised liver tissue (DLT) and lyophilised decellularised live tissue (LDLT). In Figure 4.4 (A), NLT presents densely populated nuclei depicted in dark purple, whereas DLT Figure 4.4 (B) shows that most nuclei have been successfully removed. Although some nuclei remain present, around 88.8% of nuclei material was eliminated between all the liver batches, from 2813 ng/mg to 363 ng/mg. Figure 4.4 (D) depicts a Masson trichrome stain of the liver lobule with dark blue representing collagens and dark red/pink indicating cytoplasm material. In Figure 4.4 (E), it is possible to see the collagens staining retained post decellularisation.

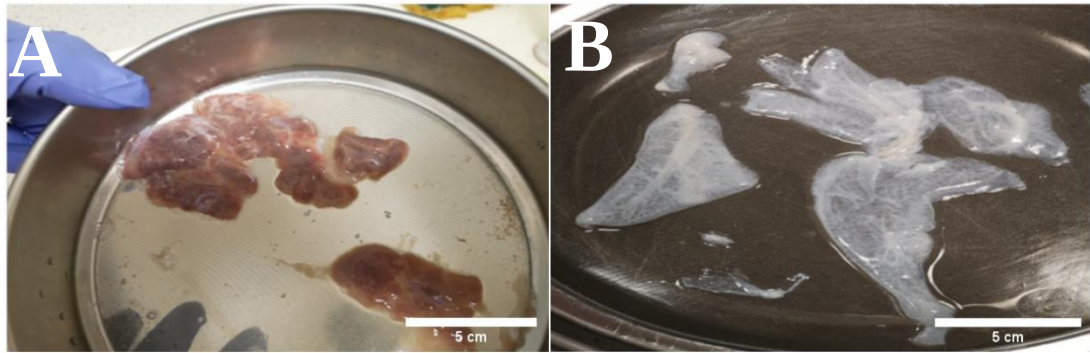


Figure 4.2 (A) Sliced liver tissue before the start of the decellularisation process. Scale bar 5cm. (B) Decellularized liver tissue. Scale bar 5cm.

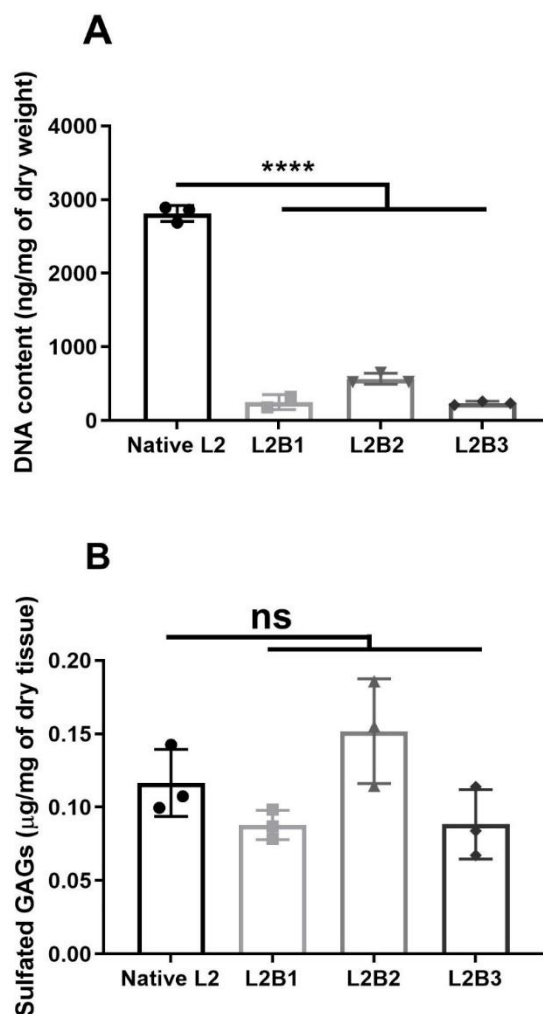


Figure 4.3 (A) Quantification of DNA in the native and decellularised liver (ECM). The DNA content in native liver L2 and decellularised batches L2B1, L2B2 & L2B3. DNA content in native and decellularised liver was normalised to the initial dry weight of each sample. **** $p < 0.0001$; values are expressed as mean $\pm$ SD, $n = 3$ /group except for L2B1 where $n=2$. (B) Quantification of sulphated GAGs in the native and decellularised liver (ECM). GAGs content in native and decellularised liver was normalised to the initial dry weight of each sample. $p > 0.05$; values are expressed as mean $\pm$ SD, $n = 3$ /group

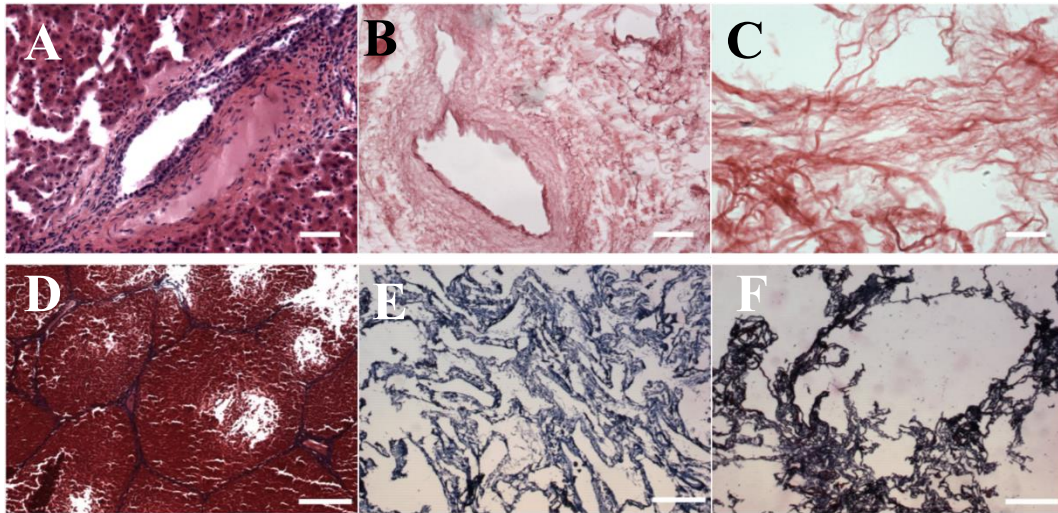


Figure 4.4 Histological staining of native and decellularised porcine liver tissue. (A) Porcine normal liver tissue (NLT), (B) decellularised liver tissue (DLT) and (C) lyophilised decellularised liver tissues (LDLT) were stained for Haematoxylin and Eosin (H&E) staining and Masson Trichrome staining (D), (E) and (F), respectively. In H&E dark purple stain indicate cells nuclei presence, and pink stains indicate the extracellular matrix and cytoplasm. For Masson trichrome staining, dark blue indicates the presence of collagens, and red stain shows cytoplasm. Scale bar 50 μm & 500 μm for H&E and Masson's Trichrome.

4.4.2 Porcine ECM-derived hydrogels fabrication

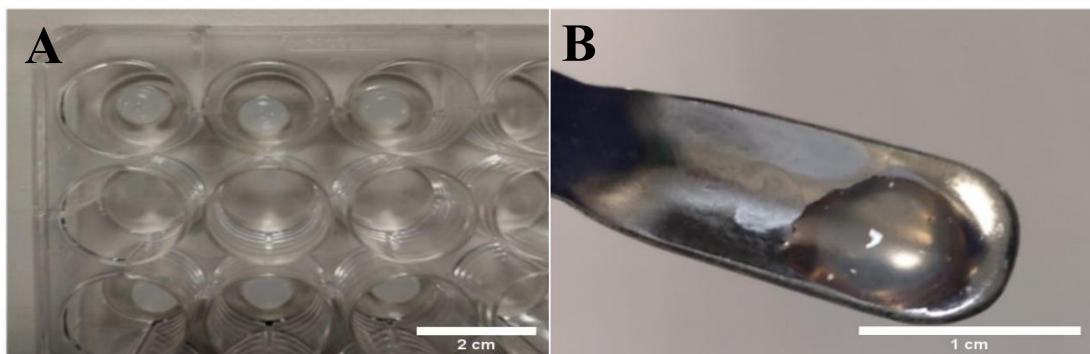


Figure 4.5 Porcine dECM hydrogel formation. (A) 8mg/ml Porcine dECM hydrogel cast in a 24 well-plate. (C) 2mg/ml Porcine dECM hydrogel is still robust and easy to manipulate.

dECM lyophilised powder was solubilised using pepsin to form a porcine dECM digest. To form dECM hydrogel, the solubilised digest is then neutralised to achieve a physiological pH of 7.4 using the neutralisation buffer solution fully described in Materials and Methods section 2.4.3. This neutralisation step, followed by an incubation period of 40 minutes at 37 $^{\circ}\text{C}$, yielded a dECM hydrogel Figure 4.5 (A). dECM hydrogels were fabricated from as low as 2mg/ml, and they remained easy to handle and manipulate (Figure 4.5 (B)).

4.4.3 Mechanical characterisation of porcine liver ECM-derived hydrogels

Porcine dECM-derived hydrogels were characterised using both a compression test to establish the strength of the material and a rheological test to assess the elastic-like response of the ECM hydrogels and determine if they behave like a solid-like or liquid-like hydrogel. A compression test was carried out initially for 4, 6 and 8mg/ml porcine dECM hydrogels. Hydrogels at the given concentration were formed and quickly hydrated in media before being placed in the texture analyser stand to begin testing. Figure 4.6 reveals a gradual increase of stiffness (Young's Modulus) with increased porcine dECM concentration. Specifically, 4 mg/ml dECM had a Young's modulus of 2.98 ± 0.4 kPa, 6mg/ml dECM of 5.86 ± 0.5 kPa and 8 mg/ml dECM of 8.43 ± 1.4 kPa.

Rheological profiling of ECM hydrogels was performed on a parallel plate rheometer. Porcine dECM hydrogels were fabricated at five chosen concentrations: 2,3,4,6 and 8 mg/ml and compared to the commercially available matrix BME2. The time sweeps profiles (Figure 4.9 (A)) shows that concentrations 2 and 3mg/ml immediately display a solid-like behaviour. For both 2 and 3mg/ml ECM concentrations, the loss modulus (G'') was briefly above the storage modulus (G') before the 1.6-minute mark, showing liquid-like behaviour. After 1.6 minutes, the storage modulus (G') was above the Loss modulus (G''), demonstrating a solid-like behaviour. Similar gelation kinetics were found between 2 and 3 mg/ml ECM and BME2 matrix. The gelation profile was complex as the storage modulus did not plateau over time, but it seemed it kept rising throughout the 20 minutes of test time. For the BME2 matrix, the loss modulus was above the storage modulus for twice as long (about 3.5 minutes). This showed that the BME2 matrix displayed a liquid-like behaviour for longer than the ECM hydrogels. Similarly, the temperature profile Figure 4.9 (D) showed ECM concentrations of 2 and 3 mg/ml to have storage moduli above the loss moduli even at low temperature (4 °C). Contrarily, the BME2 matrix remained in a liquid-like state until the temperature reached 12 °C, from which the storage modulus exceeded the loss modulus and the gelation process started (Figure 4.9 (D)).

A frequency sweep as shown in Figure 4.8 (A) and Figure 4.9 (B) was tested after a time sweep. A constant strain was kept at 1%, with the angular frequency increasing from 0.01 to 200 rad/s. With an increase of angular frequency, a decrease in viscosity (η^*) is observed. The storage modulus of 2 and 3 mg/ml ECM and BME2 remained linear throughout the test (Figure 4.9 (B)), whereas the storage modulus for 4,6 and 8 mg/ml ECM seemed to increase with angular frequency (from around 52 rad/s) as shown in Figure 4.8 (A).

Subsequently to the frequency sweep, an amplitude sweep was implemented (Figure 4.8 (B) and Figure 4.9 (C)). A constant frequency was applied at 1 rad/s. Both ECM at 2 and 3 mg/ml and BME2 matrix exhibited a viscoelastic behaviour until the critical strain was reached. After that, a slight increase in the modulus was observed (Figure 4.9 (C)), indicating a strain stiffening effect. After the critical strain was reached (around 95%) (Figure 4.9 (C)), the hydrogels weakened, and the storage moduli values dropped below the linear viscoelastic region.

The average storage modulus (G') is depicted in Figure 4.7. The average storage modulus for ECM at 8mg/ml is 470 ± 140 Pa, 6 mg/ml is 202 ± 29 Pa, 4 mg/ml is 163 ± 31 Pa, 3 mg/ml is 81 ± 29 and 2 mg/ml is 73 ± 23 . The average storage modulus of the BME2 matrix is 68 ± 6 Pa. The storage modulus of the BME2 matrix is significantly lower than the average modulus of 6 and 8 mg/ml ECM hydrogel. However, no statistical significance was found when comparing BME2 storage modulus with 2 and 3 and 4 mg/ml ECM storage modulus.

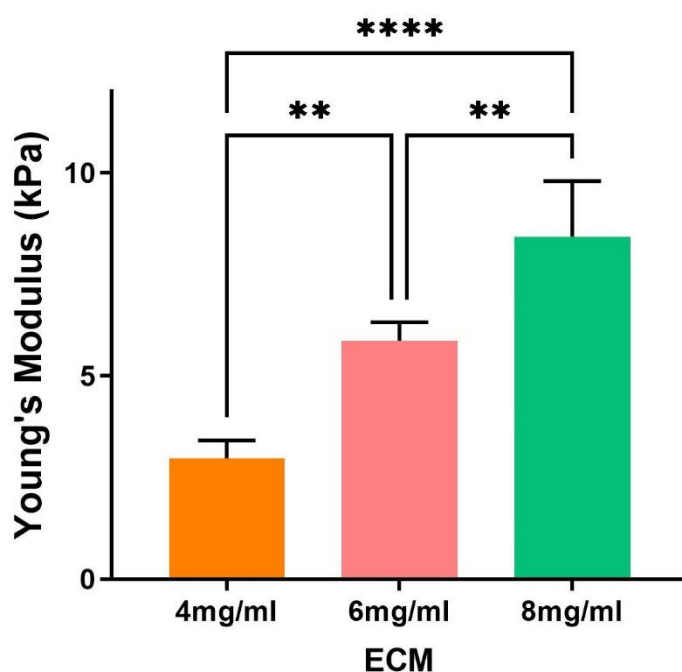


Figure 4.6 Compressive testing data representing the Youngs' modulus average for each decellularised ECM gel concentration as displayed. A compression test was performed using the texture analyser. The samples geometry was measured, and the test was carried out at 50% strain, 0.05 mm/s test speed, and with an applied force of 0.005 N. The error bars are calculated from $n=4$ independent samples. Statistical significance was assessed using Ordinary One-Way Anova with Tukey multiple comparisons (**: $p < 0.01$, ****: $p < 0.0001$).

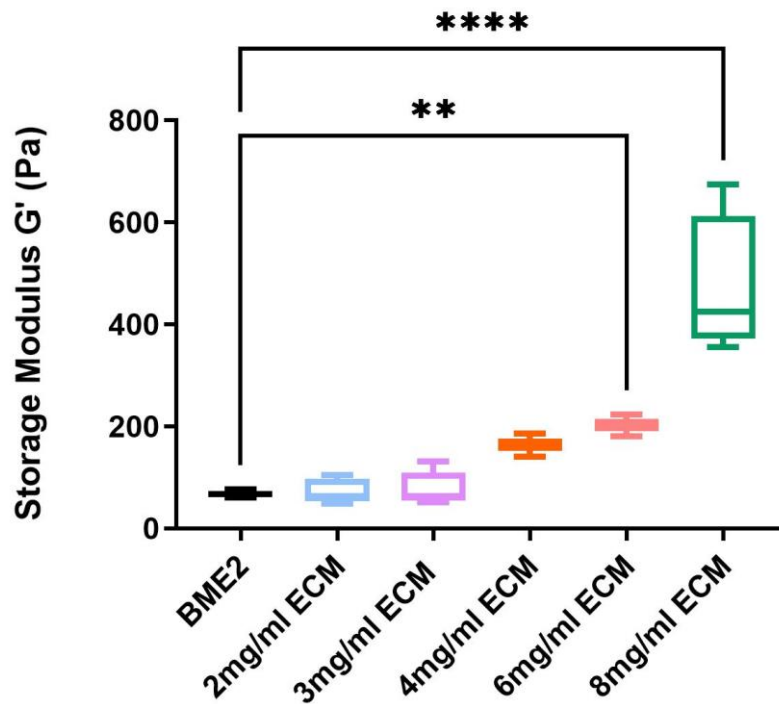


Figure 4.7 Storage modulus (G') of BME2 decellularised ECM hydrogels at a given concentration. The test was carried out with an increase in strain from 0.01 to 100 % at a constant angular frequency of 1 rad/s. The error bars are calculated from $n=2$ independent samples for 4, 6 mg/ml ECM and $n=4$ independent samples for 8 mg/ml ECM hydrogels, $p<0.05$. The error bars for BME2 were calculated from $n=2$ independent samples from 3 independent experiments, 2 and 3 mg/ml ECM were calculated from $n=5$ independent samples from 3 independent experiments. Statistical significance was assessed using Ordinary One-Way Anova and comparing the BME2 with each concentration of ECM liver tissue.

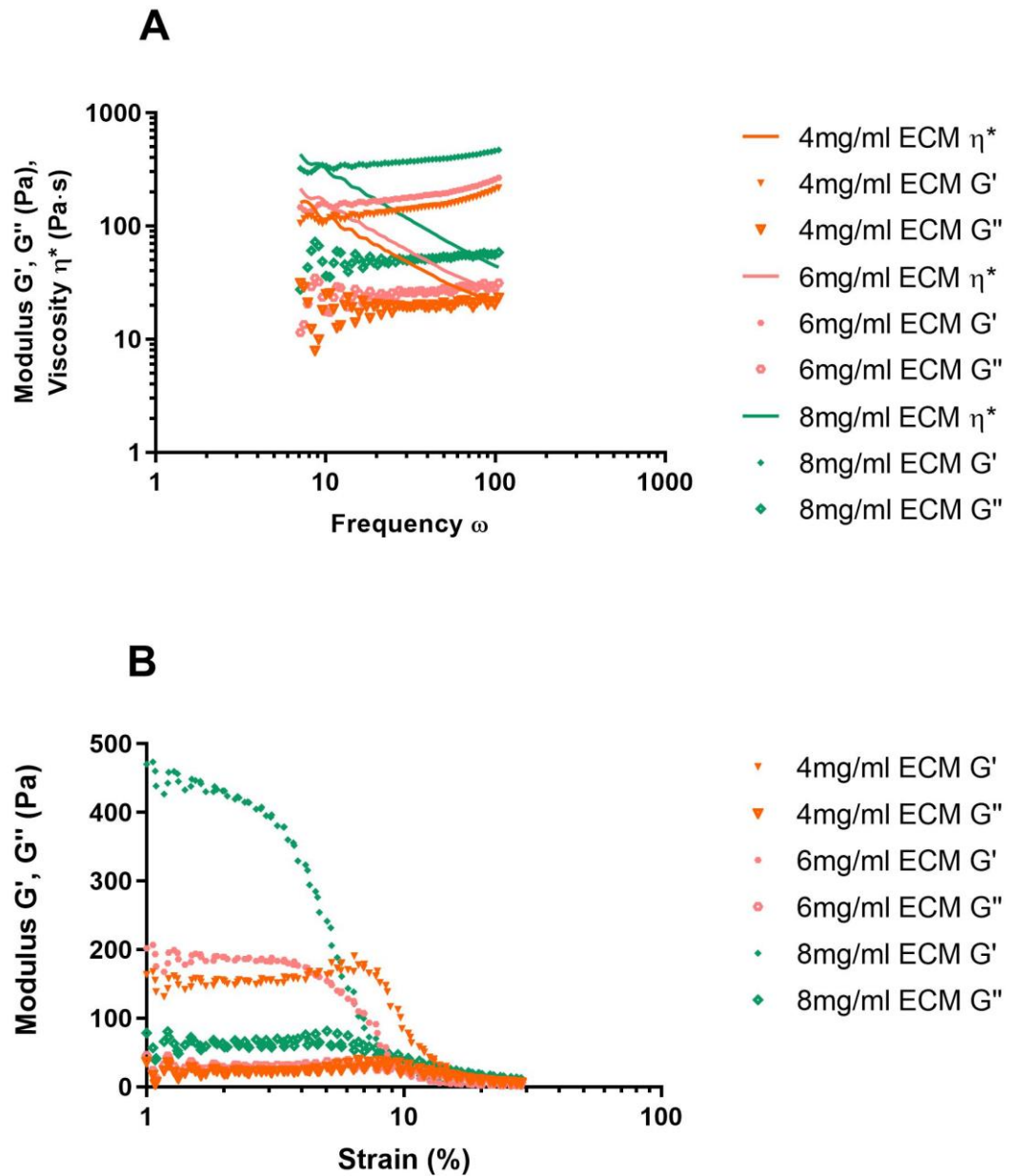
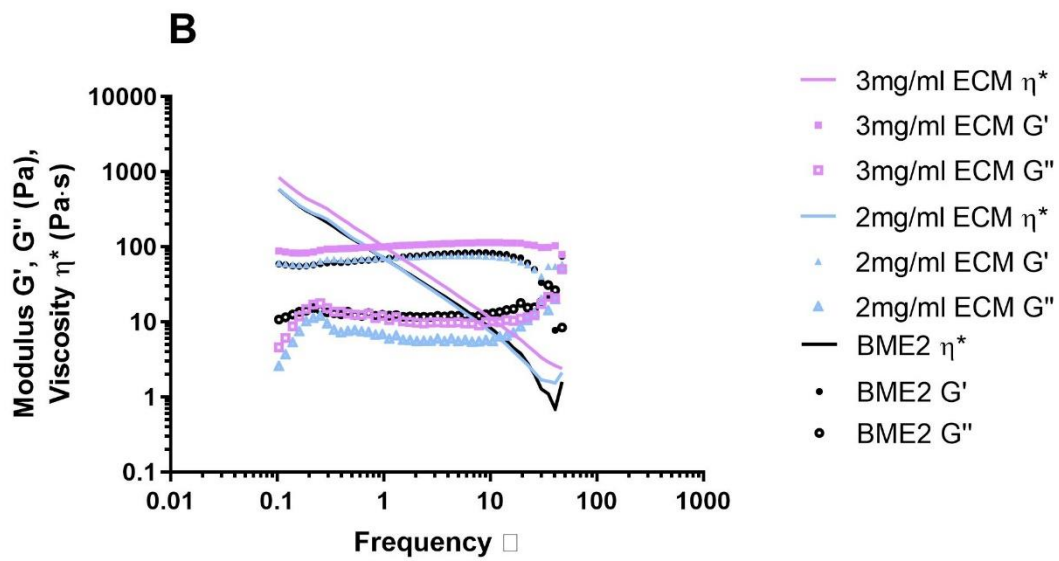
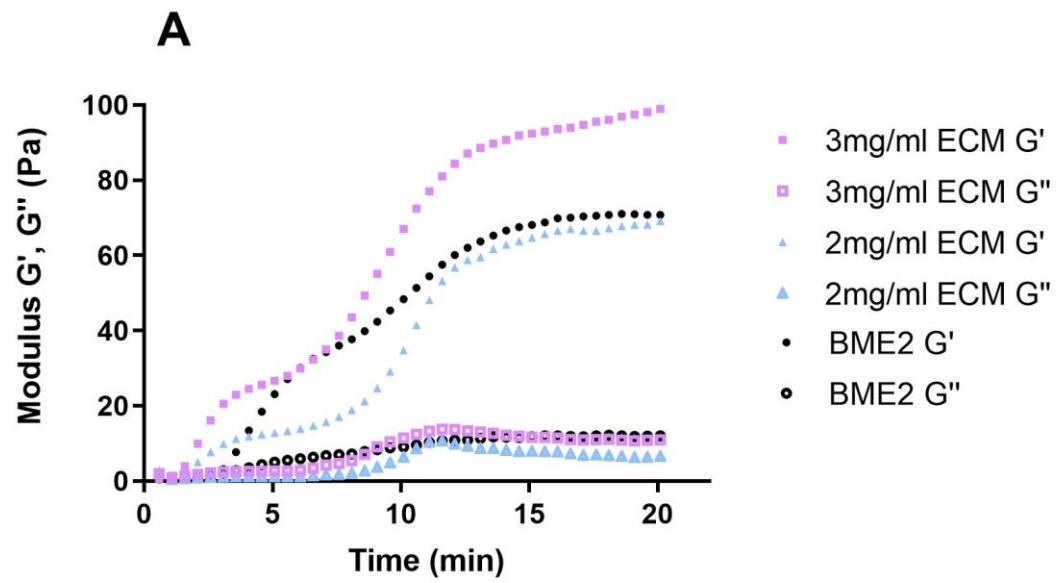


Figure 4.8 (A) Frequency Sweep of 8,6 and 4 mg/ml ECM derived hydrogels. Representative curves depict the rheological profile of different ECM concentrations. An initial shear rate of 1000 1/s was applied to remove any bubbles from the top of the hydrogels. A frequency sweep was performed between 0.01- 200rad/s at an amplitude gamma of 1% with a constant temperature of 37 °C. (B) Strain sweep of 8,6 and 4 mg/ml ECM derived hydrogels. Representative curves depict the strain profile of different ECM concentrations. The measurements were carried out at a constant angular frequency of 1 rad/s with an applied strain from 0.01 to 200%.



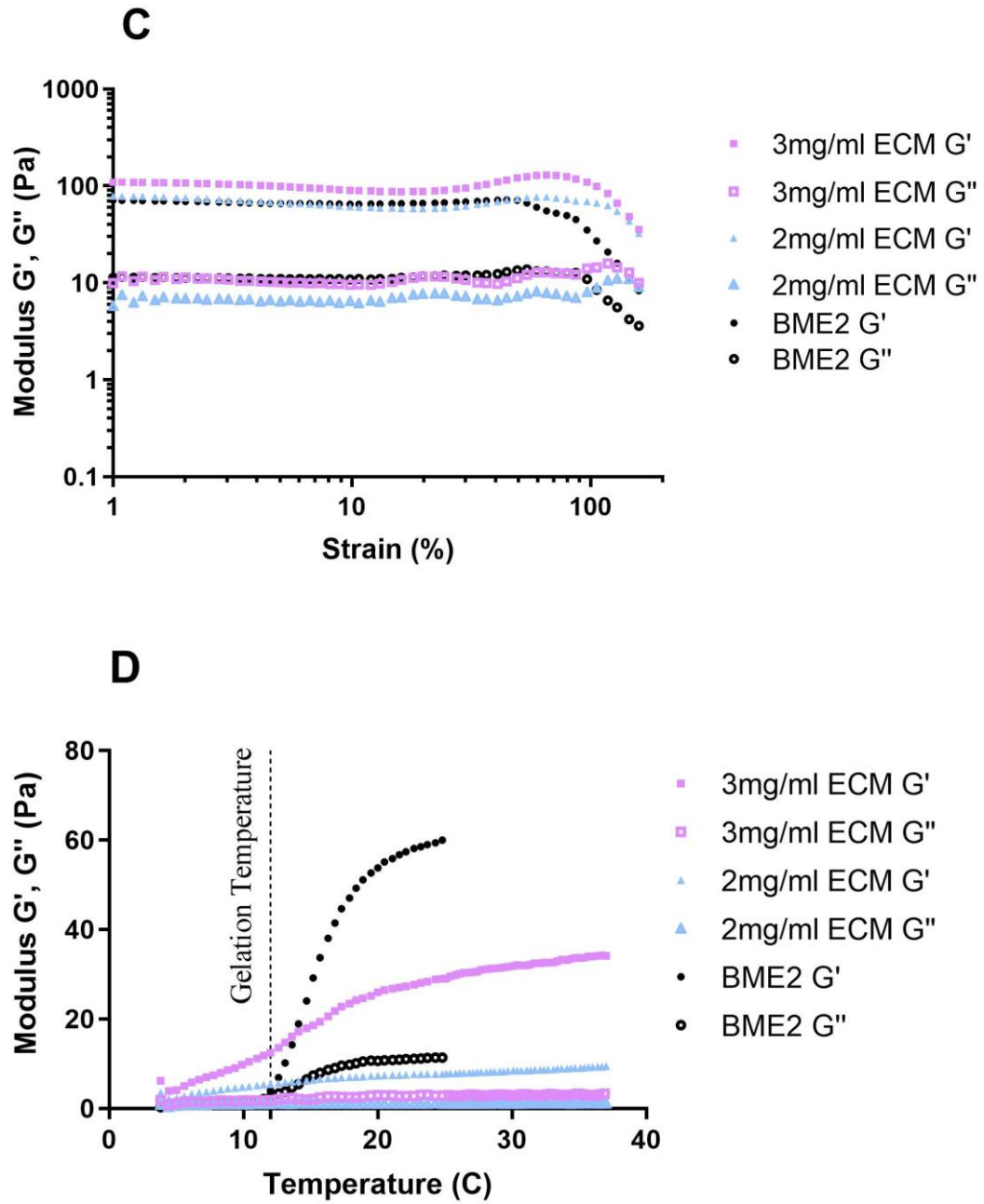


Figure 4.9 (A) Time Sweep of BME2 and each ECM derived hydrogels at the displayed concentration (BME2, 2 and 3 mg/ml liver ECM). Representative curves depict the rheological profile of different ECM concentrations. (B) Frequency Sweep of BME2 and each ECM derived hydrogels at the displayed concentration (BME2, 2 and 3 mg/ml liver ECM). (C) Strain Sweep of BME2 and each ECM derived hydrogels at the displayed concentration (BME2, 2 and 3 mg/ml liver ECM). (D) Temperature sweep of BME2 and each ECM derived hydrogels at the displayed concentration (BME2 and 3 mg/ml liver ECM), BME2 matrix start gelation around 12 °C. $n=4$ individual samples for BME2 matrix and $n=2$ individual samples for each ECM concentration.

4.4.5 Swelling characterisation of porcine ECM-derived hydrogels

The swelling behaviour of ECM hydrogels was assessed at different time points after media incubation at 37 °C.

The percentage of weight/media loss at each data point was calculated using the following equation (Silva et al., 2018):

$$\text{Weight Loss (\%)} = \frac{(W_f - W_i)}{W_i} \times 100$$

W_f = weight of the swollen sample at each timepoint

W_i = weight of the initial sample at 0 hour

W = Weight Loss (%)

Three ECM hydrogel were chosen to represent a good range of concentrations, 2, 4 and 8 mg/ml. ECM hydrogels maintained their weight throughout the 14 days of incubation time (Figure 4.10 (A)). No significant weight loss is observed over time for each ECM hydrogel concentrations with the weight stabilising after 6 hours for each ECM concentration (Figure 4.10 (A)). The ECM hydrogels do not seem to swell after incubation in media.

A significant difference was observed when comparing the weight of both 2&8 and 2&4 mg/ml ECM gels at each time point. Furthermore, the lower the concentration of the ECM, the more transparent the ECM looked (Appendices B, Figure AppB 4.1). All ECM concentrations displayed some form of weight loss over time (Figure 4.10 (B)), with the 2mg/ml losing almost 20% of its weight by day 14. The higher the concentration, the more stable the ECM was, and less ECM mass was lost (<10%, for the 8mg/ml ECM hydrogel). The swelling behaviour of the BME2 matrix was not characterised due to the difficulty encounter in manipulating BME2 matrix hydrogels.

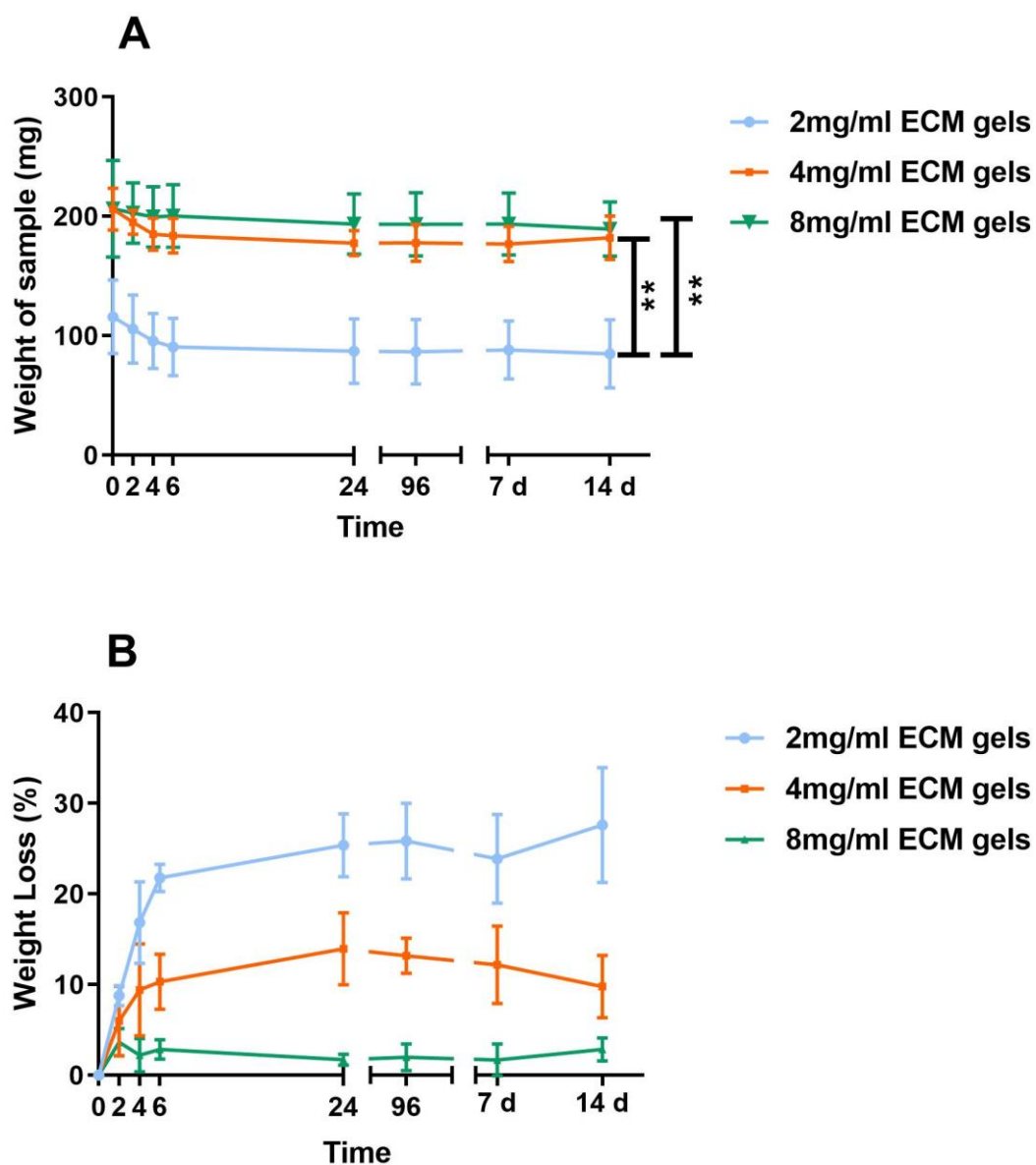


Figure 4.10 (A) Weight change of 2,4 and 8 mg/ml ECM hydrogels weighted over 14 days (B) Weight Loss (%) of the ECM hydrogels at given concentration over 14 days over time. Statistical significance was assessed using One Way Anova with Tukey's multiple comparisons (**: $p < 0.0001$), $n=5$ independent samples for each concentration condition.

4.5 Discussion

When decellularising liver, both biochemical and mechanical components need careful considerations, a successful decellularisation is considered when less than 50ng of dsDNA per mg of ECM dry weight is removed or complete cell removal and elimination of at least 90% of host DNA (Gilbert et al., 2009, Gilpin et al., 2017). The decellularisation process impacts biomaterial immunogenicity (Sullivan et al., 2013) and the state of which sGAGs are preserved. The liver is composed of more than 90% of the cellular component, including parenchyma and non-parenchyma cells (Vekemans and Braet, 2006). Furthermore, liver ECM is only a small component of the liver (J.M and Seyer, 1980; Rojkind and Martinez-palomo, 1976; Rojkind and Ponce-noyola, 1982) compared to other tissues, but it provides an architecture and structure for the maintenance of hepatocyte polarity and function (Martinez-Hernandez and Amenta, 1993a).

All batches of liver dECM underwent an almost complete decellularisation process with the removal of a combined 89% of cellular components such as dsDNA and debris (Figure 4.3 (A)). The long detergent and enzymatic treatment with EDTA and trypsin, Triton-X-100 and sodium deoxycholate were responsible for the substantial removal of cellular components in the remaining scaffold. But other factors, such as the thickness of liver slices and the mechanical agitation speed, might have impacted the successful removal of dsDNA, which was slightly less (89%) than the gold standard of 90%. Other contributing factors that need to be considered during mechanical agitation are the speed of the agitation and the conical flask itself. Better removal of dsDNA could potentially be achieved by using baffles conical flask. Li et al. (2013) have simulated using computational fluid dynamics (CFD) the unsteady turbulent flow in baffled flasks. There were many advantages in mass transfer capacity and shear formation with increased shaking frequency. The results were validated using the cultivation of filamentous fungus (Li et al., 2013).

Further analysis of the nuclear material removal during liver decellularisation was achieved using H&E staining. Although, a qualitative method, it is possible to confirm the removal of nuclei (dark blue) while retaining the ECM components (pink) (Figure 4.4 (A), (B), (C)). It is possible to notice that after instant freeze-drying (Figure 4.4 (C)), the dECM tissue seemed to have lost its architecture, which seemed still intact in Figure 4.3 (B). The instant freeze-drying process appeared to have altered the dECM tissue architecture and disrupted the collagen fibres (Sheridan et al., 2013). In this case, we were not interested in preserving the architecture as we would solubilise the dECM and fabricate hydrogels. Nonetheless, careful considerations

should be taken in the freeze-drying process if dECM is going to be used as a tissue to prevent the ECM disruption and inferior mechanical properties (Sheridan et al., 2013).

Although a maximum reduction of DNA content is preferred in liver decellularisation, a delicate balance needs to be achieved to preserve intact and functional GAGs and proteins in liver tissue (Gilbert et al., 2009; Hussey et al., 2018).

The presence of sulphated GAGs was assessed using the DMMB assay. In each independent batch (Figure 4.3 (B)), GAGs were retained and comparable with the amount found in liver native tissue. Furthermore, the collagen structure remained intact, as seen in the histological analysis, and collagens were still present post decellularisation (Figure 4.4 (E)).

To measure the viscoelastic proprieties of hydrogels, pre-gel viscosity, gelation kinetics, and gel stiffness are the primary parameters investigated (Saldin et al., 2017). Uniaxial compression is also used to measure the bulk stiffness of hydrogels (Pilipchuk et al., 2013; Wu et al., 2015). A uniaxial compression test can be utilised to compare physiological liver stiffness and identify the cut off value for fibrotic and cirrhosis liver. Real-time shear elastography is a medical device used to estimate liver fibrosis severity and set a threshold for normal range liver stiffnesses. Many studies have established parameters to detect healthy liver stiffness in the ranged between 2 kPa and 6 kPa (Huang et al., 2014), while the cut off value for liver fibrosis was set around 7.9 kPa and liver cirrhosis to around 14 kPa. Furthermore, different stages of fibrosis can be identified with an increase in liver stiffnesses (Colombo et al., 2011; Mueller and Sandrin, 2010).

dECM hydrogels were fabricated at three different concentrations, 4, 6 and 8 mg/ml, respectively, to be tested for uniaxial compression. A correlation was found between dECM hydrogel concentration and Young's Modulus. 4 mg/ml dECM hydrogel has Young's modulus of 2.9 kPa (Figure 4.6), which is an indication of normal physiological liver stiffness, but other papers do report liver physiological stiffnesses as low as 2 kPa because it is dependent on the measurement technique (Mueller and Sandrin, 2010). 6 mg/ml ECM hydrogel has a Young's Modulus of 5.8 kPa (Figure 4.6), which again is still in the range of a normal liver stiffness. However, 8 mg/ml dECM hydrogel has a Young's Modulus of 7.9 kPa (Figure 4.6), which is the cut off value for early-stage liver fibrosis. It is possible to change the concentration of dECM liver hydrogel to create an *in-vitro* model for the healthy and diseased liver. The mechanosensing of substrate thickness and influence of stiffnesses in cells and stem cells fate is well known (Tusan et al., 2018). Therefore, dECM hydrogels at different concentrations can be utilised for liver disease model applications.

Particularly important in determining how collagen fibrils crosslink is the gelation profile observed in liver ECM. Its complexity may be attributed to the presence of a variety of ECM components; thus: laminin, collagens, fibronectin, proteoglycan, which depending on their abundance and distribution, play a key role in the gelation kinetics of liver ECM.

After powder solubilisation, liver dECM hydrogels were formed at five different concentrations. Their gelation kinetics and storage moduli were compared to the commercially available BME2 matrix. All ECM hydrogels displayed a solid-like behaviour, where the storage moduli were above the loss moduli (Figure 4.8 (B) and Figure 4.9 (C)). They also exhibited a shear thinning behaviour as the viscosity decreased with increased frequency (Figure 4.8 (A) and Figure 4.9 (B)). These findings are consistent with shear-thinning ECM behaviour also reported in urinary bladder matrix (UBM) ECM (Massensini et al., 2015) and small intestine submucosa (SIS) derived ECM (Giobbe et al., 2019). ECM hydrogels formed a sigmoidal curve indicating a 90% gelation within 13 minutes (Figure 4.9 (A)). All concentrations of liver ECM gel and BME2 exhibited gel-like properties when exposed to 37 °C with the Storage moduli (G') above the loss moduli (G''). A concentration dependency between storage moduli (G') and ECM concentration was found.

The linear viscoelastic region was present only up to a certain point (Figure 4.8 (A)), then 4, 6 and 8 mg/ml ECM gels started to weaken and showed an increase in G' as a function of frequency, displaying a non-linear viscoelastic behaviour. However, 2 and 3 mg/ml ECM and BME2 matrix exhibited a storage modulus independent of frequency (Figure 4.9 (B)). This material characteristic indicates a more stable and robust gel with a more strongly connected polymer network of crosslinked collagen fibres. Similarly, Claudio-Rizo et al. (2017) have shown different ECM matrices; thus, rat tail, bovine Achilles tendon, and porcine small intestine submucosa exhibit non-linear viscoelastic behaviour (Claudio-rizo et al., 2017).

ECM gels at 4, 3 and 2 mg/ml concentrations and BME2 matrix exhibited a strain stiffening behaviour. It has been found that Collagen I also exhibited a strain stiffening behaviour, where it was found that when the stiffening occurred earlier, the yield strain was lower. Furthermore, the degree of strain stiffening is smaller in higher concentration gels or those who have thick fibril bundles (Kaufman and Motte, 2012). These findings are consistent with what we see in the liver ECM hydrogels and BME2 matrix (Figure 4.9 (B) and Figure 4.10 (C)).

The storage modulus of 2 and 3 mg/ml ECM was comparable with the storage moduli of BME2 matrix (Figure 4.7) or Matrigel (Zuidema et al., 2014). Following the same criteria adopted by Giobbe et al. (2019), although using a different decellularised tissue, the lower concentration gels 2 and 3 mg/ml ECM liver hydrogel were preferentially used for cell culture

purposes as they exhibited a similar rheological profile to the BME2 matrix, including storage, loss modulus and viscosity (Giobbe et al., 2019).

With a temperature sweep, it was possible to determine the gelation temperature at which the hydrogel started to crosslink. Only the BME2 matrix was found to behave like a liquid ($G'' > G'$) (<12 °C) before it began the gelation process ($G'' > G'$) (Figure 4.9 (D)). After the temperature had reached 12 °C, the values of the storage modulus were above the loss modulus, indicating that the gelation process had started. This was confirmed with the time sweep (Figure 4.9 (A)); after 15 minutes, the storage modulus seemed to have stabilised, and the gel was fully crosslinked.

Contrarily, the storage modulus of both 2 and 3 mg/ml ECM hydrogels was always above the loss modulus (Figure 4.9 (A)). It appeared that liver ECM hydrogels at these concentrations behaved already as a solid-like material at temperature as low as 4 °C; these findings are consistent with the work of Ijima et al. (2018) where it was found that both high and low concentration liver ECM hydrogels and Collagen I exhibited a solid-like behaviour from the beginning of the test and never crossed over (Ijima et al., 2018). The rate of gelation is greater with increasing concentration in liver ECM hydrogels (Lee et al., 2013). One possible explanation could be that the ECM main component is collagen I; therefore, we see this behaviour in ECM but not in Matrigel or BME2, which is known to contain mostly laminin (60%), Collagen IV (30%) and entactin (8%) (Borries et al., 2020; Kane et al., 2018).

It appears that BME2 behaves as a non-Newtonian viscoelastic fluid that displays a lower critical solution temperature of around 12 °C. However, liver ECM gels also exhibit a non-Newtonian viscoelastic profile. Still, they do not seem to have a lower critical solution temperature below which they behave as liquid-like, as we could not detect it. However, liver ECM hydrogels have a crossover point when the loss modulus is above the storage modulus during the time sweep (Figure 4.9 (A)). This crossover time is shorter with increased ECM concentration and BME2 having almost double and three times longer crossover points than 2 and 3 mg/ml ECM, respectively (Appendix B, Figure AppB 4.4).

In the literature scientific groups utilised different liver decellularisation protocols therefore it is difficult to appropriately compare the viscoelastic and mechanical properties of dECM liver hydrogels. There is no standardised protocol for liver decellularisation as many research groups use a combination of detergent and enzymes. This leads to batch-to-batch variation and inconsistencies (Gilpin and Yang, 2017). However, it is established that tissue derived ECM hydrogels exhibit a non-Newtonian shear thinning viscoelastic behaviour and can be susceptible to strain stiffening effect (Pati et al., 2014).

Hydrogels' swelling and contraction behaviour is another essential property to understand the physiochemical characteristics of biomaterials and needs to be accounted for when designing tissue constructs (Malda et al., 2013). Not many swelling/degradation studies have been reported for liver ECM hydrogels (Ijima et al., 2018). In an *in-vivo* setting, the swelling of a biomaterial is a critical parameter for assessing the ability of an implant to degrade over time. In the case of an *ex-vivo* model, cells secrete new ECM replacing the original ECM scaffold. Typically, the degradation rate of the ECM will need to match the new ECM tissue regeneration in both an *in-vivo* and *ex-vivo* environment (Lee and Mooney, 2001).

It appears that liver ECM hydrogels at 2, 4 and 8 mg/ml concentration did not exhibit the usual swollen profile typically seen in hydrogels (Purwada et al., 2014; Zhang et al., 2020). 8 mg/ml ECM was the best ECM hydrogel at maintaining its weight, followed by 4 mg/ml and then 2 mg/ml. All ECM hydrogels seemed to have a burst degradation within 6 hours, with 2 mg/ml ECM losing up to 20% of its original weight (Figure 4.10 (B)). Overall, the weight of each hydrogel condition remained constant after the first 6 hours for up to 14 days in media (Figure 4.10 (A)). This condition makes them a suitable candidate for cells encapsulation. Furthermore, we can assume that the small degradation is due to hydrolysis as we did not use an enzyme (collagenase) to digest the ECM during the swelling studies. It was found that the weaker the ECM gel (lowest concentration), the more efficiently it underwent biodegradation; this finding is consistent with a study of UBM-ECM hydrogel in restoring brain tissue in a rat model (Ghuman et al., 2018).

Hydrolytic degradation of pure collagen hydrogels appears to be quick; within 14 days, only around 40% of the total collagen mass is retained (Takallu and Mirzaei, 2019). However, after 14 days, 8 mg/ml liver ECM derived hydrogels still retained 91% of its original mass, 4 mg/ml gels retained 90% and 2 mg/ml gels retained 73%, suggesting only partial collagen hydrolytic degradation. Unfortunately, we could not study the swelling/degradation profile of the BME2 matrix as the gels were too fragile to handle and would break upon manipulation.

4.6 Conclusion

Extensive rheological, mechanical, and swelling analyses of both dECM hydrogels and the BME2 matrix have been carried out. Liver decellularisation was successfully achieved with 89% of native dsDNA removed while retaining sGAG and collagens. Stiffnesses, viscoelastic behaviour and strain stiffening response, led us to choose the lower ECM hydrogels concentrations as they were comparable with the BME2 matrix response.

Potential structural damage to proteins might have occurred during the liver decellularisation process, compromising the integrity and activity of proteins and glycosaminoglycan. Hence, the capability of liver ECM hydrogels to sustain and support cellular function such as migration and proliferation needed to be investigated. Moving forward, the cytocompatibility of each ECM concentration was addressed, but only the lower ECM concentrations of 2 and 3mg/ml were utilised for progenitor liver cell culture and functional assessment.

Chapter V - Bi-potential human adult progenitor liver cells cultured and differentiated in liver ECM hydrogel scaffolds

5.1 Introduction

Although initially, ECM was thought to be an inert supportive scaffold, its role has changed over the years (Gattazzo et al., 2014). It has been recognised to be involved in critical aspects of cell biology, thus: cell motility and adhesion (Hynes, 2009). It was found that ECM is dynamic and versatile. Furthermore, it can influence the production, degradation and remodelling of its constituents (Lu et al., 2011; Page-McCaw et al., 2007). The structure and biochemical components present in the ECM give a fingerprint to its biochemical and biomechanical properties (Gattazzo et al., 2014). Physical characteristics such as porosity, rigidity and topography are responsible for influencing cell migration, division and polarity (Lu et al., 2012). ECM stiffness is an important property responsible for exerting external forces which the cells sense in reaction to their environment. These properties are connected and can influence one another, and this process is called mechanotransduction (Humphrey et al., 2015; Martino et al., 2018). The 3D ECM microenvironment holds biochemical cues necessary for cell-cell interaction and cell-to-matrix interactions (Yue, 2014). Mainly, stem cells reside in a specialised microenvironment called 'niche'. The niche regulates the stem cells behaviour by maintaining their quiescent state or triggering self-renewal or differentiation towards the tissue of interest (Gattazzo et al., 2014). It is thought that, although stem cells have high proliferative potential, the niche can maintain the cells in a quiescent state (Orford and Scadden, 2008). The ECM macromolecules and soluble factors are also responsible for maintaining stem cell homeostasis (Discher et al., 2012; Peerani and Zandstra, 2010).

Adult liver progenitor cells are descendants of stem cells and usually lack the self-renewal capacity of stem cells. However, Turner et al. (2011) proposed a model where the types of progenitor cells are dependent on the stage of liver development (Turner et al., 2011). It was identified that hepatic stem cells retain some stemness and can be isolated utilising specific cell surface marker molecules (Tanaka and Miyajima, 2012). Two main differences are the size and their concentrations in the liver. One population represents around 1% of the multipotent liver residents; it can be sorted for EpCAM and is approximately 8 μm in size (Lombard et al., 2013). This population also express hematopoietic, endothelial, and mesenchymal markers but little to no albumin. Hepatoblast liver stem cells of around 11 μm are bipotential cells derived from hepatic stem cells. They can differentiate towards both a

hepatic and cholangiocyte lineage (Lombard et al., 2013), and they represent 0.01% of the liver stem cell population. Finally, the largest liver progenitor cells (13 μ m) are the least plastic and committed to differentiating towards the hepatic and cholangiocyte lineage. They still have good proliferative capacity *in-vitro* but eventually, reach senescence and stop proliferating (Lombard et al., 2013). Their role as a source of hepatocyte cells still needs to be exploited. Being stem-like cells, there is always a risk associated with tumour development (Tang et al., 2008) and GMP conditions for upscale production are not yet established (McClelland et al., 2008).

However, liver progenitor cells are genetically more stable than potential hepatic lineages derived from iPSC. iPSCs application in cell replacement therapies presents many challenges firstly, retroviral DNA integration into the host cell genome is random therefore, it could potentially introduce variations and alter coding sequences and ultimately affect transcription. Secondly, the transcription efficiency of certain genes (Oct4, Sox2 and Nanog) might decrease as cells transform into a pluripotent state. Thirdly, there is a high probability that Oct4, Sox2, Klf4 and c-Myc genes can resume expression, making it impossible to use retrovirus to form iPSCs in clinical trial settings (Medvedev et al., 2010). With the right environmental and biochemical cues, progenitor liver stem cells can be differentiated into both hepatocyte and cholangiocyte-like cells making them a more suitable model for our study without the drawbacks of iPSCs (Dorrell et al., 2011; Español-Suñer et al., 2012; Ko et al., 2020).

5.1.1 *In-vitro* liver models

In-vitro liver models are not only essential to understand liver diseases but are also a crucial tool used for preclinical stages of drug development (Collins et al., 2019). As the liver is a heterogenous structure composed mainly but not only of hepatocytes, it is vital to study the cell-to-cell interactions and the matrix to cell interactions. A variety of *in-vitro* liver models are utilised to study the cellular components in a well-controlled and reproducible manner. Conventional 2D models rely on primary liver cells cultured and supported on matrix or plastic surfaces. Primary hepatocytes are very difficult to maintain polarised, and they dedifferentiate quickly in 2D (Kiamehr et al., 2019). Primary hepatocytes can be cultured in a sandwich-like structure between two collagen layers, it has been shown they polarised correctly, and the bile network is functional (LeCluyse et al., 1994). Secretion of albumin and bile acid can also be maintained for a longer time (Dunn et al., 1991). Immortalised cell lines such as HepG2 and HepRG have been selected due to the rapid proliferation, easy culture, and resistance to senesce. However, one of the most significant shortcomings is their reduced liver-specific

functions (Ramboer et al., 2015). The biggest drawback of 2D *in-vitro* liver models is the lack of heterogeneity found in the hepatic sinusoid or an *in-vivo* animal model (Collins et al., 2019). 3D *in-vitro* models allow complex interactions between cells and the surrounding matrix. 3D *in-vitro* models comprise of precision-cut tissue slices (PCTS) (Graaf et al., 2010; Olinga and Schuppan, 2013), tissue explants (Piera et al., 2016), spheroids (Nath and Devi, 2016), organoids (Clevers, 2016) and organ on a chip device (Bhise et al., 2016).

5.1.2 Hepatic progenitor cells in 3D hydrogels

The gold standard for culturing primary hepatocytes remains a sandwich structure between two collagen layers (Dunn et al., 1991; Wang et al., 2004). It has been shown that primary hepatocytes remained polarised and functional (Moghe et al., 1996). However, they cannot be cultured over long periods (LeCluyse et al., 1994). Over the years, different matrix-based hydrogels have been used to culture and maintain primary hepatocytes (Deharde et al., 2016; Foster et al., 2015; Kim et al., 2010; Li et al., 2005), but they all lacked the prospect of the long term culture.

Therefore, there was a need to replace primary hepatocytes cells with an alternative that could be differentiated into a hepatocyte-like cell. This was when organoids started to look like a potential solution to a lack of a primary hepatocytes source (Harrison et al., 2021; Yu Wang et al., 2017). They would be used as a source for mature hepatocytes and used to study liver diseases (Fatehullah et al., 2016; Wu et al., 2019).

Hepatic organoids have been cultured in 3D hydrogels composed of matrix-like proteins. The current gold standard for culturing hepatic organoids relies on the tumour derived mouse matrix Matrigel (Huch et al., 2015, 2013b) or the basement membrane extract (BME2) Cultrex® (Broguiere et al., 2018; Broutier et al., 2018, 2016; Nuciforo et al., 2018). One of the main limitations is the unknown batch composition of the matrix. However, we do know that Matrigel and BME2 matrix is composed of around 60% laminin (Benton et al., 2011; Borries et al., 2020; Kane et al., 2018). The liver ECM components, however, are very unrepresented within both Matrigel and BME2 matrices. In the liver ECM, the most abundant proteins are Collagens (type I, III, IV and V). Little laminin is present, responsible for creating a low-density basement membrane (Bedossa and Paradis, 2003). Furthermore, the matrix chemistry associated with mature hepatocytes phenotype is composed of fibronectin, a high ratio of Collagen I and III and sulphated glycosaminoglycan (Martinez-Hernandez and Amenta, 1993a; Martinez-Hernandez A et al., 1991), while there is no laminin (Freser et al., 1997; McClelland et al., 2008), hyaluronans or collagen IV present (Gebhardt, 1992).

A study of lineage tracing conducted by Suner et al. (2012) showed that inhibition of laminin during HPC regeneration resulted in immediate HPC differentiation into hepatocytes (Español-Suñer et al., 2012). These results align with the idea that laminin maintains liver progenitor cell phenotype and promotes biliary specification (Hul et al., 2009; Lorenzini et al., 2010).

Liver ECM can be obtained through a decellularisation process where the liver tissue is stripped away of its cellular components, leaving behind proteins, collagens and proteoglycans (Gilpin and Yang, 2017). This ECM matrix environment is a more suitable alternative for culturing organoids and hepatic cells.

5.2 Adult bi-potential progenitor liver cells organoids

As previously described, progenitor liver cells origins and their role in liver regeneration are still a matter of debate (So et al., 2020). Evidence suggests that liver regeneration can be achieved by the proliferation of pre-existing hepatocytes or by the proliferation of hepatocytes differentiated from liver progenitor cells (Carpino et al., 2016; Shin et al., 2013).

It is thought that bipotential adult progenitor cells reside within the canals of Herring or intrahepatic ductules Figure 5.1 (Stanger, 2015) and activate in response to a severe liver injury (Duncan et al., 2009; Miyajima et al., 2014). Because of their bipotential nature, LPC expressed both hepatocytes (Krt8, Kr18 and Albumin) (Dan et al., 2006; Xiao et al., 2003) and biliary epithelial cells (BECs) (Kr7, Krt19, EpCAM and SOX9) markers (Furuyama et al., 2010; Okabe et al., 2009; Xiao et al., 2003). The expression of these different markers suggests that liver progenitor cells are heterogenous (Pepe-mooney et al., 2019).

Huch et al. (2013) cultured progenitor liver organoids and expanded them utilising defined culture conditions. Firstly, Wnt target Lgr5 was identified as a marker from proliferating cells following mouse liver damage, and these cells had the capability to differentiate into both a hepatic and cholangiocyte lineage (Huch et al., 2013b). Following up, studies were able to sort human organoids EpCAM⁺ cells from a human biopsy. They activated the cAMP signalling pathway and inhibited TGF- β , successfully culturing these cells, which displayed a mixture of ductal and Hepatocyte markers (Huch et al., 2015).

Different research groups over the years have established what markers should be used to detect these bi-potential liver progenitor cells (Bria et al., 2017; Dorrell et al., 2014; Furuyama et al., 2010; Huch et al., 2015, 2013b; Shin et al., 2011; So et al., 2020).

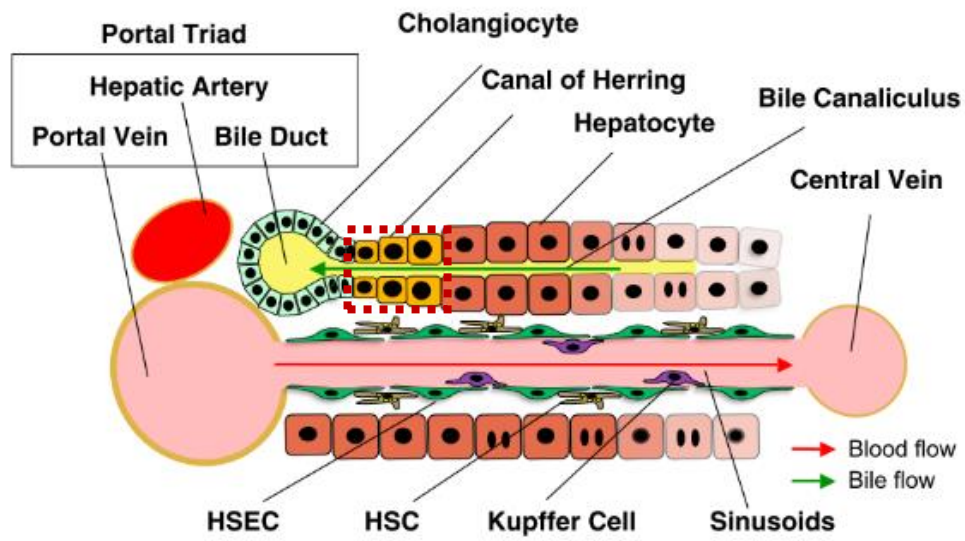


Figure 5.1 Graphical representation of the liver lobule with emphasises the location of hepatic progenitor cells (red dotted), situated in the Canal of Herring close to the portal Triad (Hepatic Artery, Portal vein and Bile duct). Adapted from (Miyajima et al., 2014).

5.2.1 Role of hepatic progenitor in liver regeneration

Although there are diverse routes to liver regeneration as shown in Figure 5.2, a debate is still ongoing regarding whether primary hepatocytes undergo proliferation or if hepatic progenitor cells differentiate into hepatocytes and repopulate the liver's loss mass. The signalling pathway driving this regeneration are depicted in Figure 5.3 (Bria et al., 2017; Huch and Doll, 2016; Miyajima et al., 2014).

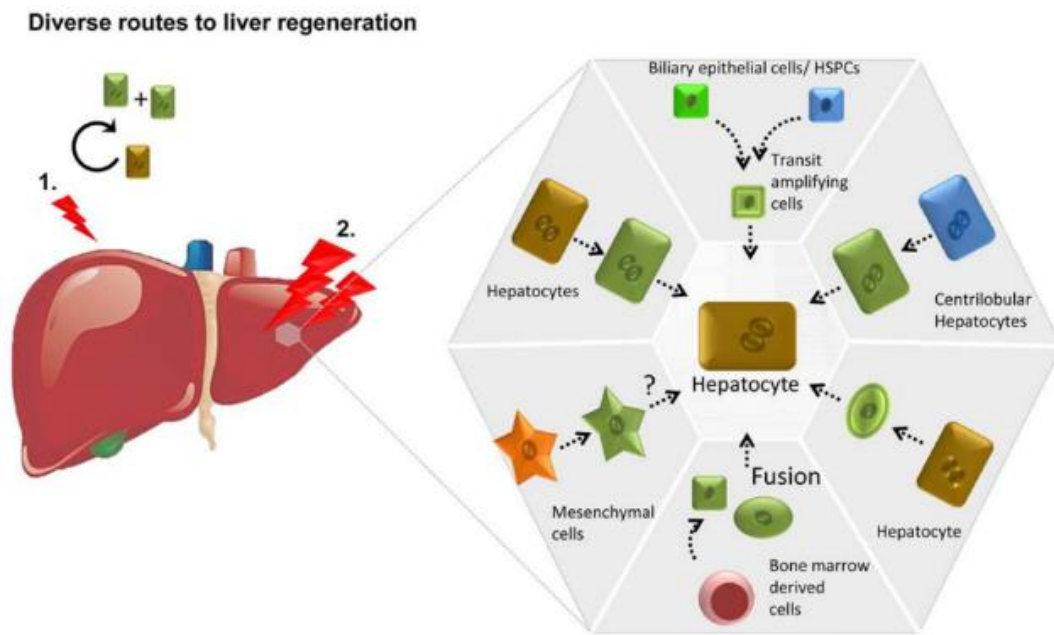


Figure 5.2 Huch and Doll (2016, p.655) gave a detailed schematic showing the diverse route that liver can regenerate and what type of cells are involved in the process.

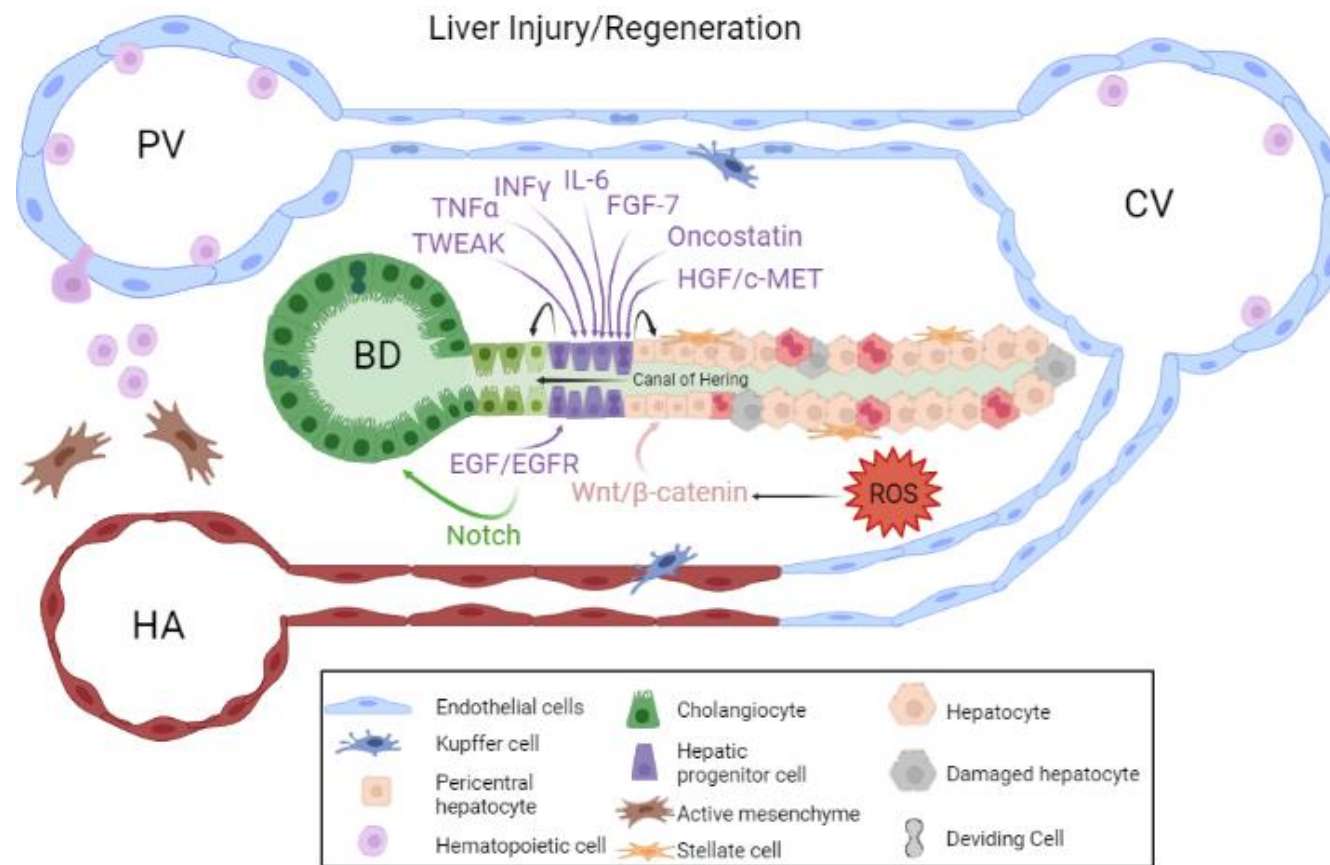


Figure 5.3 Schematic drawings of the differentiation signalling pathway occurring during liver damage and regeneration, involving the hepatic progenitor cells (HPCs) which originate in the Canal of Herring. HPCs are bipotential cells expressing both hepatocytes and cholangiocyte markers. When liver damage occurs, different factors such as TWEAK, TNF α and others induce the activation and differentiation of the HPC. Furthermore, hepatocytes themselves undergo a cycle of proliferation to repopulate the liver mass lost in case of acute liver injury (Bello et al., 2018; Hindley et al., 2016). Portal vein (PV), Central vein (CV), hepatic artery (HA), Bile duct (BD), Reactive oxygen species (ROS). Image created using BioRender.

5.2.2 Molecular mechanism of progenitor liver cells activation

Although the mechanism of hepatic specification during hepatoblast development is increasingly understood, the pathway of lineage differentiation of HPCs in the adult liver is yet to be identified (Ko et al., 2020; So et al., 2020). The HPC activation is also known as ductular reaction (Bria et al., 2017; Ko et al., 2020) and involves LPC proliferation in the periportal regions of the liver. Many studies have reported that the c-Met, an HGF receptor, is required for the HPC commitment into hepatocytes (Bria et al., 2017; Ishikawa et al., 2012). Both Notch1 or Notch2 receptors are activated with the interaction with Jagged1. This Notch pathway is used to develop HPC into cholangiocytes and is inhibited during the regeneration of hepatocytes through ligase Numb (Boulter et al., 2013). During HPC differentiation into hepatocytes, Numb transcription is activated by B-catenin. By this point, Notch and Wnt pathways can interact and specify to either lineage (Boulter et al., 2013). The presence of macrophages was found to be important in driving Wnt signalling and hepatocyte lineage specification. Phagocytosis of hepatocytes debris promotes Wnt upregulation in macrophages, closely linked to hepatic cell death and HPC hepatocyte specification (Kinases et al., 2015). As mentioned before, this activation mechanism has been studied using animal models, and more recent reports found that both YAP and mTORC1 are crucial in LPC activation. They regulate the expansion of BEC derived organoids *in-vitro* and proliferation of BEC in mice *in-vivo* (Aloia et al., 2019; Planas-paz et al., 2019). Furthermore, macrophages secrete tumour necrosis factor (TNF), which promotes cell apoptosis (TWEAK) and is part of the proinflammatory TNF family. Consequently, TWEAK binds to its receptor expressed on LPCs (Jakubowski et al., 2005). It has been found that overexpression of TWEAK is responsible for the stimulation and proliferation of HPC across the nuclear factor-kB (NF-kB) pathway (Jakubowski et al., 2005; Tirnitz-parker et al., 2010). Other signalling pathways involved in HPC activation are TNF- α (Knight et al., 2000), IFN γ (Knight et al., 2007) and interleukin-6 (IL-6) (Yeoh et al., 2007).

5.2.3 Role of ECM components in HPC proliferation

The liver ECM and its macromolecules, even if not abundant in liver tissue, play a crucial role in hepatic cells migration, adhesion, and differentiation (Yue, 2014).

One of the most recent hepatic models consists of adult ductal cells encapsulated in ECM derived matrix and differentiated into hepatocyte-like cells (Huch et al., 2015, 2013b). Huch et al. (2015) demonstrated that bile duct derived bipotent progenitor cells from the human liver could be expanded in culture and be differentiated into hepatocytes *in-vitro* and *in-vivo* upon transplantation (Huch et al., 2015).

The theory of laminin being an essential liver ECM protein responsible for the activation and proliferation of HPC is supported by different researchers (Hul et al., 2009; Lorenzini et al., 2010). Kallis et al. (2011) showed that in advanced liver injury, the hepatic progenitor population (HPC) activates and proliferates to initiate hepatic regeneration. A proliferative response from HPC was associated with high laminin deposition, which resulted from induced liver injury. After a chronic fibrotic liver injury, ECM failed to remodel, prompting the activation of HPC. Furthermore, laminin-HPC interaction is critical for HPC activation and proliferation (Kallis et al., 2011) as shown in Figure 5.4.

Zhu et al. (2012) also suggested that laminin, collagen III and collagen IV in liver ECM aided HPC in proliferation after growth factors were added to the media. Collagen I and fibronectin were associated with a more differentiated phenotype under the same media condition (Zhu et al., 2013). Kaylan et al. (2016) demonstrated that Notch ligands and ECM proteins were responsible for the fate commitment of bipotential mouse embryonic progenitor cells. Specifically, fibronectin and laminin had the highest number of positive albumin cells demonstrating that the combination of ECM protein is the most beneficial for HPC fate specification (Kaylan et al., 2016).

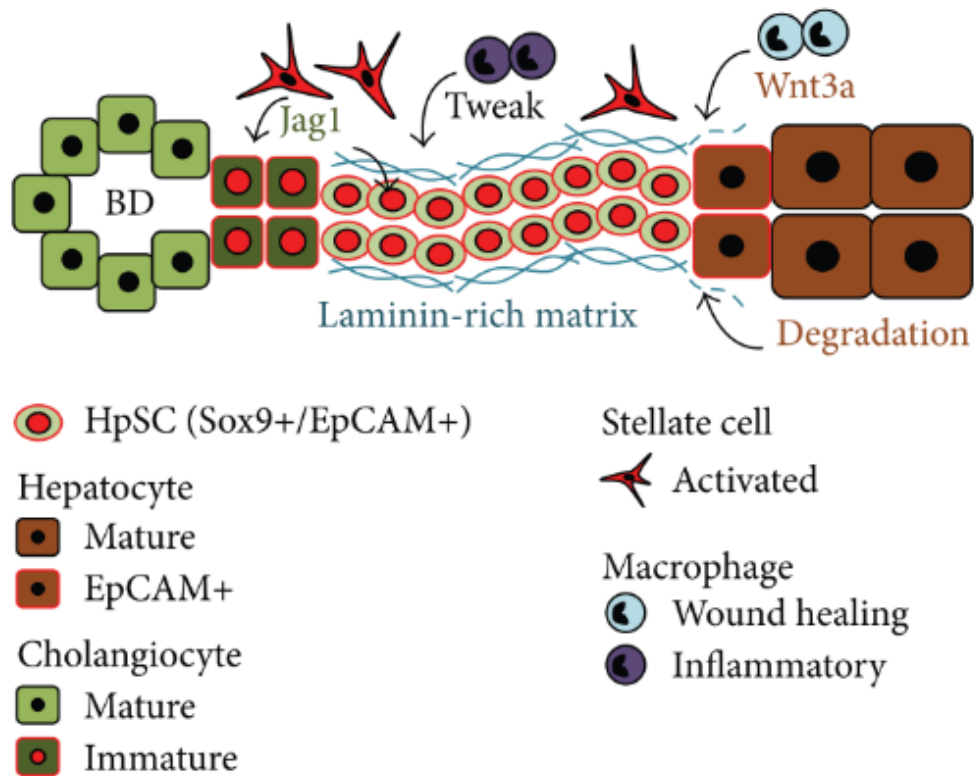


Figure 5.4 Hepatic progenitor stem cells (HpSC) activation upon niche signalling. The matrix surrounding the HpSC is rich in laminin, hyaluronans, types III and IV collagens. This microenvironment maintains the stem/progenitor/biliary phenotype and inhibits hepatic differentiation. A niche environment with less laminin and more sulphate proteoglycans are necessary to start the differentiation into hepatocytes. Adapted from (Carpino et al., 2016).

5.3 Purpose of the work

The purpose of this work was to assess the cytocompatibility, culture and differentiation potential of EpCAM⁺ adult progenitor liver cells/organoids embedded in liver dECM hydrogels as shown in Figure 5.5. This chapter evaluates the proliferation capability and morphology of progenitor cells encapsulated in dECM hydrogels and compares it to the gold standard matrix, BME2. We chose the best organoids based on their proliferation potential from five potential liver donors and cultured them in a range of dECM hydrogel concentrations, checking their proliferation capacity and some hepatic markers through immunostaining. Finally, a differentiation medium was applied to verify the capabilities of these cells to differentiate towards a hepatocyte phenotype and their functionality was assessed with a Cyp induction assay. The gene expression of both stem and mature hepatocyte markers was checked. Also, a few mature cholangiocyte genes were designed to verify that the combination of the gold standard BME2 matrix and a cholangiocyte differentiation medium would promote the differentiation of this HPC towards a cholangiocyte phenotype.

The aims of this chapter can be categorised as follow:

- Determine the liver ECM hydrogels biocompatibility using different cell lines.
- Encapsulate human adult hepatic progenitor liver cells (sorted for EpCAM⁺) in ECM hydrogels at different concentrations and check cytocompatibility and proliferation potential, comparing it with the gold standard of the BME2 matrix.
- Assess the presence of some hepatic markers during progenitor liver expansion through immunostaining.
- Differentiate hepatic progenitor liver cells encapsulated in both BME2 matrix and ECM hydrogels into hepatocyte-like cells and assess their functionality with a Cyp induction assay
- Differentiate hepatic progenitor liver cells encapsulated in both BME2 matrix and ECM hydrogels into cholangiocyte-like cells
- Undertake RT-PCR of both hepatocytes and cholangiocytes genes of interest for differentiated hepatic progenitor liver cells

Hypothesis: The combination of hepatic differentiation media and liver micro environmental factors secreted by the liver-derived ECM will drive the hepatic progenitor cells towards a more mature hepatocyte-like phenotype. Additionally, combining cholangiocyte differentiation media and BME2 matrix would drive the cells towards a bile-duct phenotype.

5.4 Experimental design

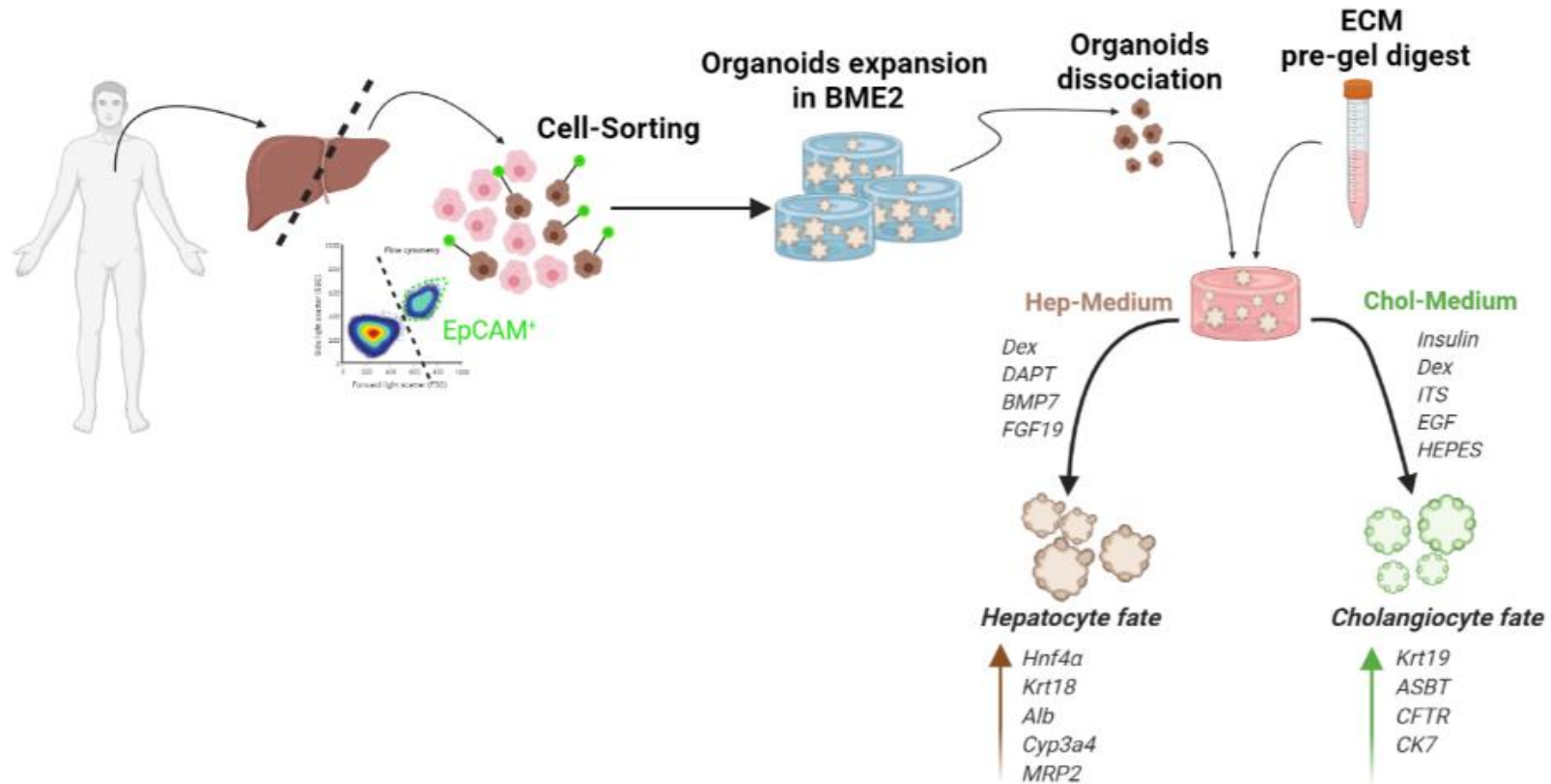


Figure 5.5 Schematic illustration of the experimental design from EpCAM cell sorting to bipotential cell encapsulation in liver dECM for hepatic or cholangiocyte differentiation. Liver ECM hydrogels were fabricated, and human hepatic progenitor liver cells were encapsulated for cytocompatibility and proliferation analysis. The aim is to differentiate bipotential human liver progenitor, encapsulated in dECM scaffolds, towards a hepatocyte or cholangiocyte fate. The combination of growth factors in the media and liver dECM microenvironment aims to direct the hepatic progenitor cells towards a more committed lineage. Created with BioRender.com

5.5 Results

5.5.1 Mediated cell contraction in liver-derived ECM hydrogels

Once liver dECM has been solubilised, dECM-derived hydrogels were fabricated and incubated with and without cells to assess the contraction of ECM hydrogels. Liver batches used for all the following cell-culture work were primarily L2B2 and L2B3. 2 and 3 mg/ml ECM hydrogels were fabricated and incubated in media for up to 15 days. Qualitative images assessment (Figure 5.6 (A) and (B)) showed no contraction of ECM hydrogels when incubated in media over 15 days. Acellular ECM hydrogel droplets remained circular and did not change shape. However, contraction was observed when cells were encapsulated in liver ECM hydrogels as shown in Figure 5.6 (C) whereby a drastic reduction in hydrogels size was found after seven days in culture. Further microscopic images taken on days 3 and 7 and shown in Figure 5.7 revealed the mediated cell contraction in liver-derived ECM; however, organoids embedded in the BME2 matrix showed little to no gel contraction. Organoids embedded in ECM hydrogels seemed to shrink the liver ECM hydrogels dynamically, but organoids remained morphologically comparable to those cultured in the BME2 matrix.

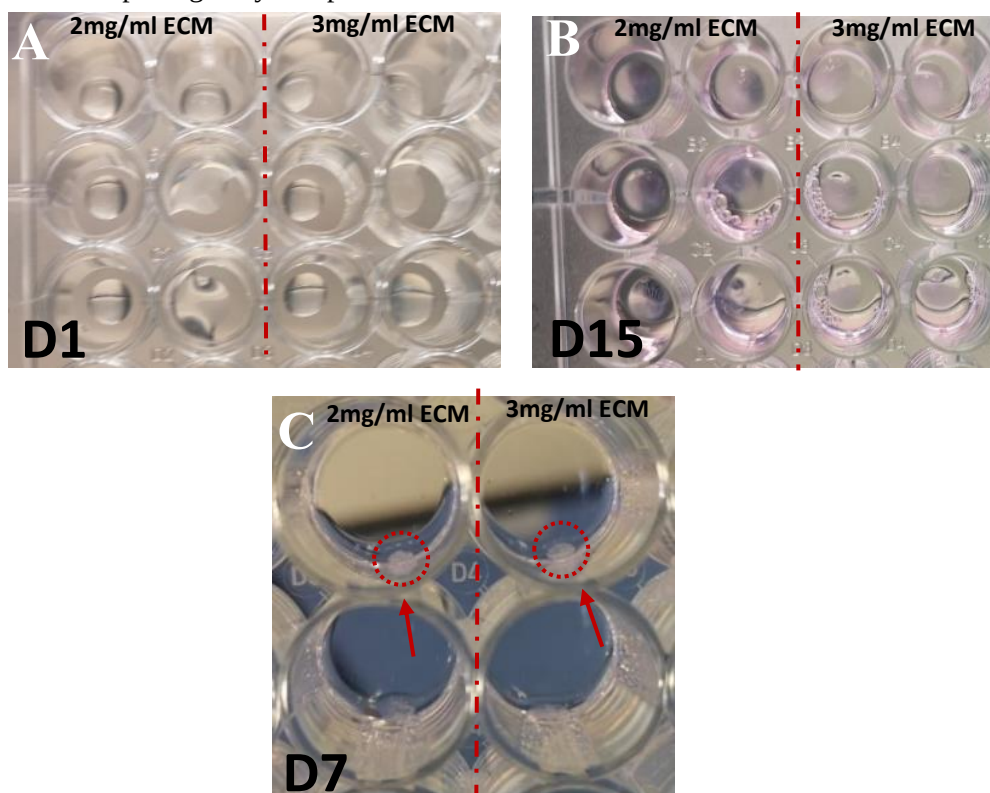


Figure 5.6 Image of liver dECM gels. (A) Day1 (D1) and (B) Day15 (D15) at given concentrations showing no sign of shrinkage when cells are not present. (C) Gels + cells. Shrinkage appears if cells are encapsulated after seven days of incubation in expansion media (EM).

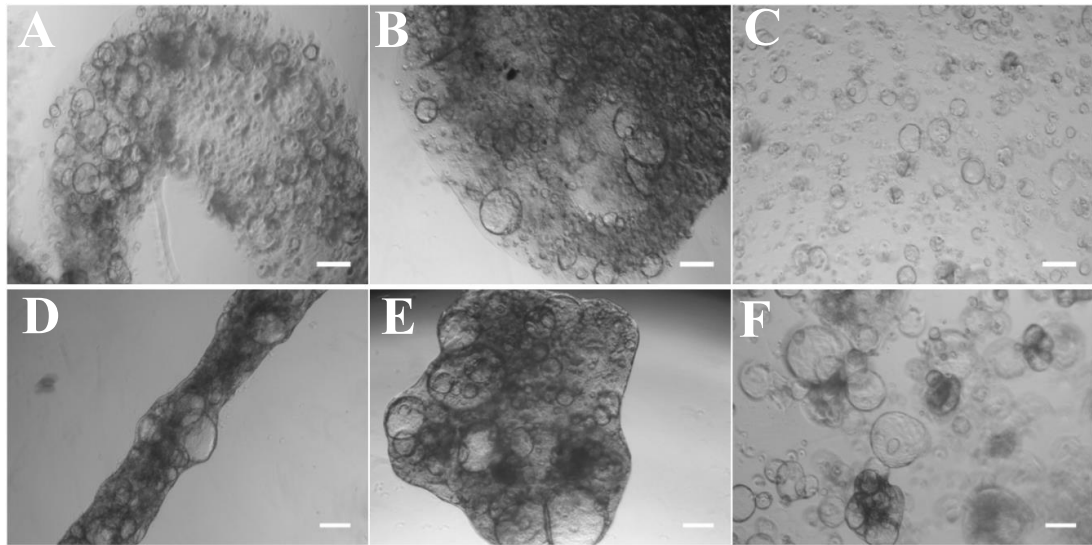


Figure 5.7 Microscopic images of progenitor liver organoids encapsulated in (A) 2 mg/ml ECM, (B) 3 mg/ml liver ECM gels and (C) BME2 matrix at day 3 and (D) 2 mg/ml ECM, (E) 3 mg/ml liver ECM and (F) BME2 matrix at day 7. Organoids encapsulated in 2mg/ml ECM contracted allowed the gel to contract and form tubular gel structures (A) and (D). Organoids in 3mg/ml ECM gels induced the gels to contract but the gel structure remained rounded (B) and (F). Organoids encapsulated in BME2 did not seem to be able to contract the BME2 matrix, so the matrix remained rounded and spread out (C) and (F). 200 µm scale bar.

5.5.2 Progenitor liver cells response in 3D porcine liver ECM gels

Before assessing human progenitor liver cells biocompatibility and functional activity in ECM hydrogels, HepG2 in 2D and 3D, as well as HS137 were cultured in liver ECM hydrogels. Refer to Appendices C (Figure AppC 5.2, 5.3 and 5.4). HepG2 cytotoxicity was assessed in 2D and 3D on ECM of different concentrations. They remained viable over seven days and proliferated as shown in Figure AppC 5.2 and Figure AppC 5.3. Human skin fibroblast HS137 were also cultured in ECM hydrogels as shown in Figure AppC 5.4, cells proliferated and remained viable over seven days. Furthermore, HS137 encapsulated in liver ECM hydrogels contracted from day one (D1) of encapsulation. However, HS137 embedded in the BME2 matrix failed to contract the gels.

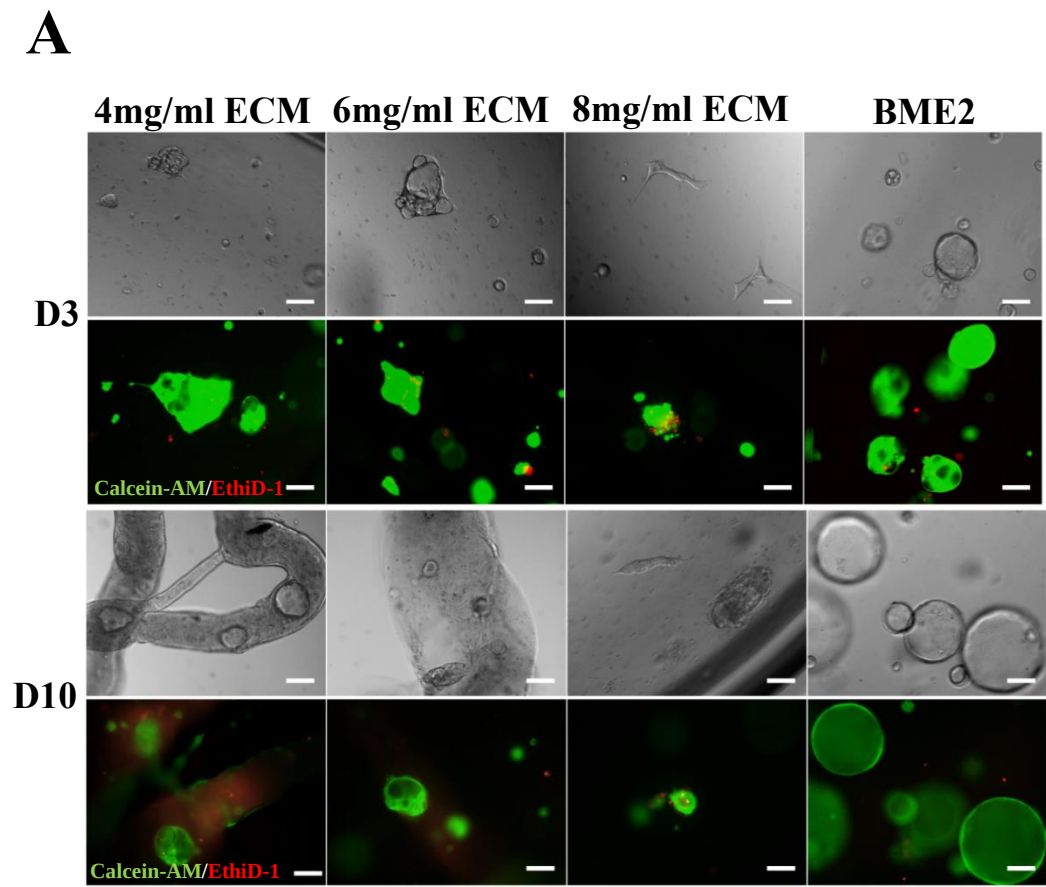
EpCAM⁺ sorted progenitor liver cells viability, metabolic activity and proliferation were assessed in 3D ECM gels at 1,2,3,4,6, and 8 mg/ml concentration. ECM hydrogels at 4,6 and 8 mg/ml were initially used based on liver ECM hydrogels literature (Lewis et al., 2018; Loneker et al., 2016). Progenitor liver cells encapsulated in ECM derived hydrogels remained viable over ten days Figure 5.8, but the organoids formed in 4,6 and 8 mg/ml were smaller than the organoids formed in the BME2 matrix as shown in Figure 5.8. Organoids metabolic

activity was measured with an Alamar blue assay on day four and day 7 (Figure 5.8 (B)). A pattern of decreasing metabolic activity with increasing ECM hydrogel concentration can be observed (Figure 5.8 (B)). Progenitor liver cells proliferation in hydrogels was assessed at day 10 with a Picogreen assay. Organoids encapsulated in the BME2 matrix had grown over ten days, and a significant amount of DNA was retrieved on day ten compared to organoids encapsulated in 4, 6 and 8 mg/ml ECM hydrogels as shown in Figure 5.8(C). Furthermore, immunocytochemistry of organoids embedded in BME2 matrix and ECM derived hydrogels revealed proliferation marker ki67, present in the nuclei of organoids embedded in the BME2 matrix but not in organoids embedded in ECM hydrogels as shown in Figure 5.9. This further confirmed the lack of proliferation of organoids encapsulated in ECM hydrogels. However, even after expansions and cell-passage, both organoids embedded in ECM hydrogels and BME2 matrix retained the marker EpCAM, from which they were originally sorted (Figure 5.9).

Due to the failure of liver organoids to proliferate in higher concentrations of liver ECM, it was decided to lower the concentration of liver ECM-derived hydrogels to 1, 2 and 3 mg/ml. Organoids embedded in lower concentration ECM are similar in shape and size to the organoids embedded in the gold standard BME2 matrix as shown in Figure 5.10. However, the contraction is also seen in lower concentration ECM hydrogels as shown in Figure 5.10. Cytocompatibility was assessed for the lower concentration ECM hydrogels, and organoids remained viable for up to 7 days in culture (Figure 5.11 (A-C)). Proliferative marker ki67 was found in organoids embedded in the BME2 matrix, but it was not possible to see clearly for 2 and 3 mg/ml ECM (Figure 5.11 (D-E)). Organoids in 1, 2 and 3 mg/ml ECM were stained with nuclei marker DAPI over seven days (Figure 5.12) at different time points to assess the proliferation.

Proliferation potential was assessed in organoids from three different liver donors: L265, L266 and L269. These three liver donors have been previously selected from a pool of 5 liver donors cultured over two weeks. They yielded the best organoid formation and proliferation Figure AppC 5. 1. All organoids from the three donors show increasing proliferation over time (Figure 5.13), a significant increase in proliferation is seen between D5 and D7 for each organoids' donor and each ECM concentration. Further differentiation studies carried out by the Postdoc Dr Fatima Abukunna suggested that the progenitor organoids from L266 donor had the best potential to be differentiated into hepatocyte-like cells. Therefore, from this point onward, all cell studies have been carried out using L266 donors. The proliferation study was repeated for L266 in the lower three concentrations of ECM hydrogels. It was observed that the higher the ECM concentration, the lower the number of nuclei were present as shown in

Figure 5.14. However, organoids embedded in each ECM concentration proliferated over seven days, with 1 mg/ml ECM showing the most proliferation potential (Figure 5.14). However, due to the long-time of incubation for the 1 mg/ml ECM gel to set, it was decided to proceed only with the 2 and 3 mg/ml ECM concentrations.



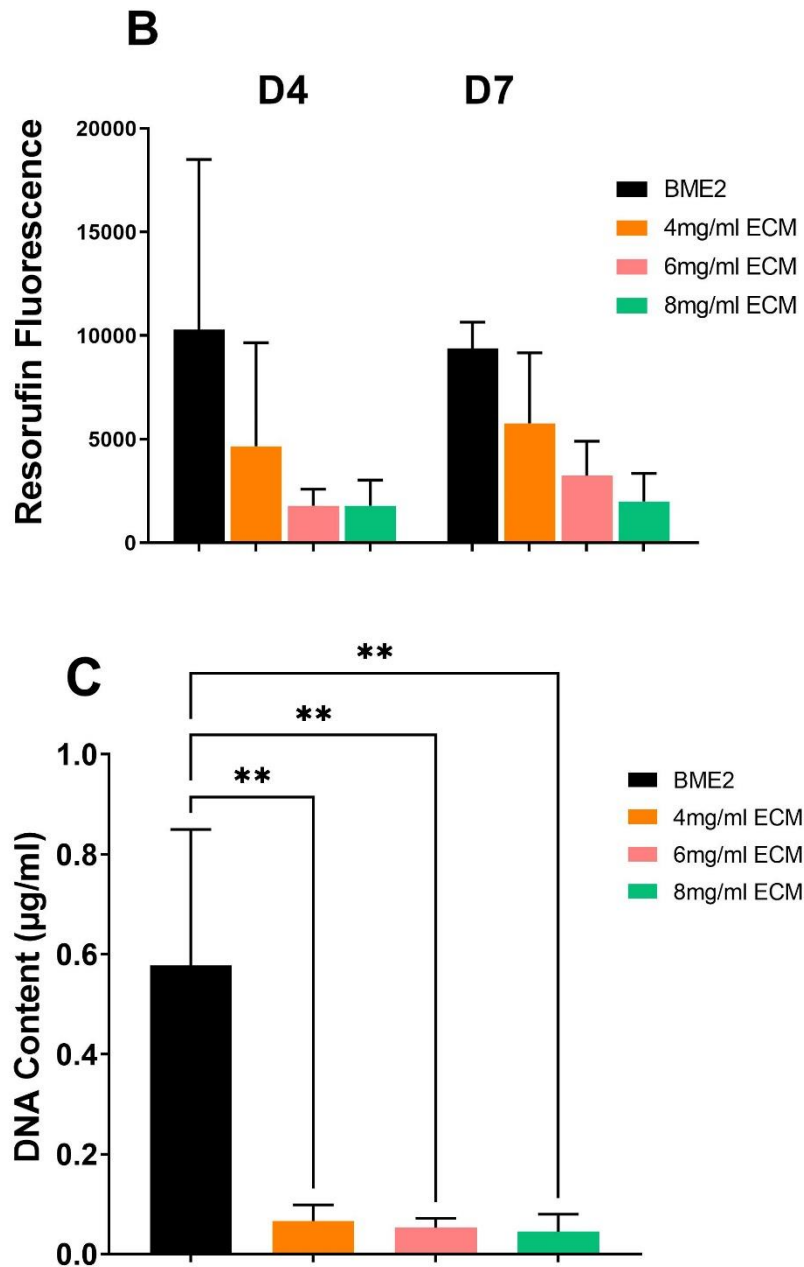


Figure 5.8 (A) Bright-field and fluorescence images of human organoids encapsulated in: 4g/ml ECM, 6mg/ml ECM, 8mg/ml ECM and BME2. Images were captured at day 3 and day 10 as depicted. Live cells were stained with Calcein-AM (green), while dead cells with Ethidium homodimer (red). Scale bar is equal to 100 µm. (B) The histogram plots represent the organoids' metabolic activity embedded in each experimental condition, as displayed. The error bars are calculated from n=3 technical replicates. Statistical significance was assessed using Ordinary One-Way Anova with Tukey multiple comparisons (ns: $p>0.05$). (C) Picogreen analysis of dsDNA content of 3D organoids grown in 4,6,8 mg/ml Porcine dECM hydrogels and BME2 matrix extracted at day 10. The error bars are calculated from n=3 technical replicates. Statistical significance was assessed using Ordinary One-Way Anova with Tukey multiple comparisons (**: $p<0.01$)

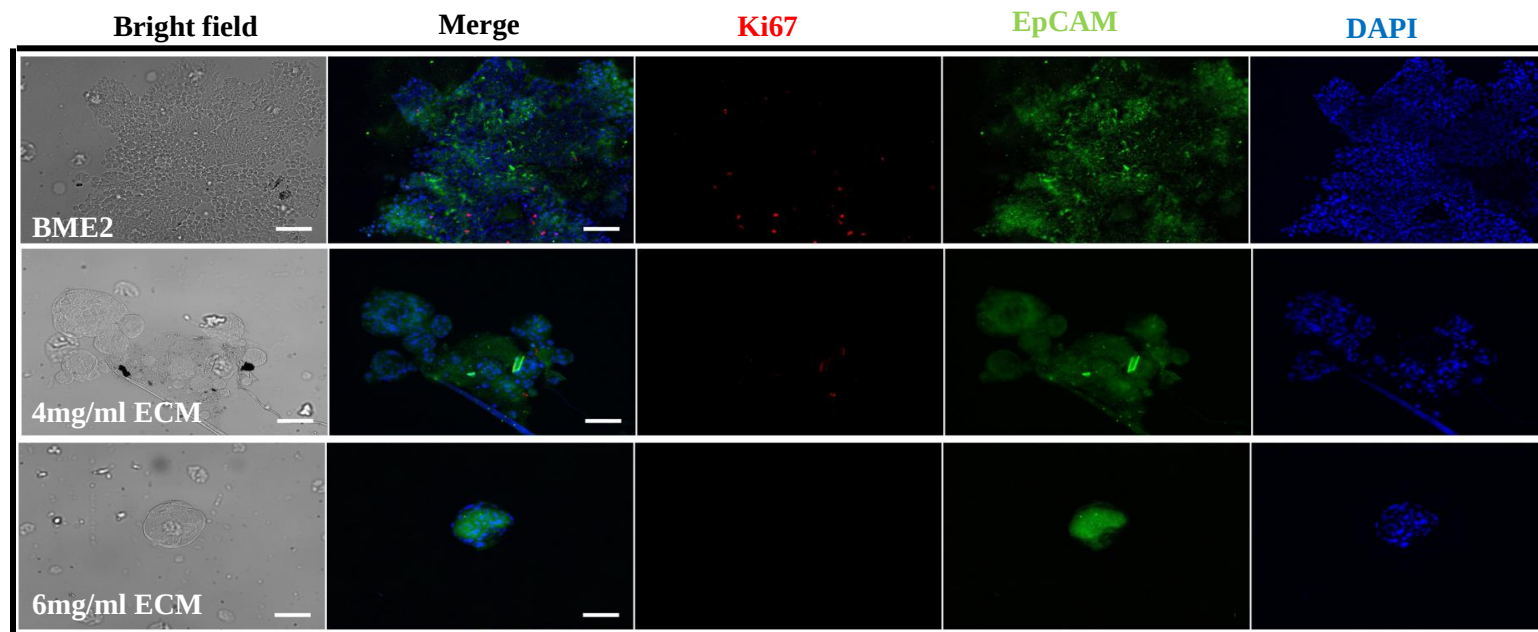


Figure 5.9 Immunostaining of progenitor liver cells grown in 3D in BME2, 4 and 6 mg/ml ECM at D10 triple stained for Ki67 (1:50) (proliferation marker), EpCAM (1:100) (progenitor epithelial marker) and DAPI (1:1000) (nuclei). Scale bar is equal to 100 μ m.

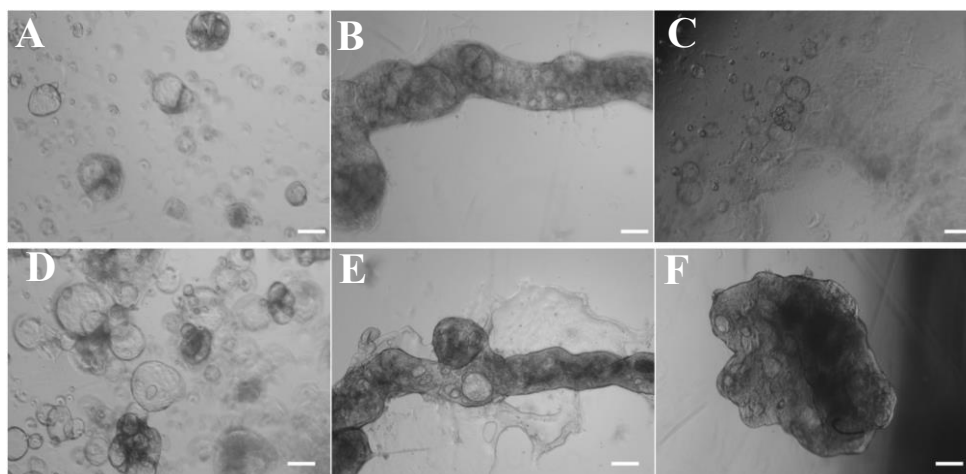


Figure 5.10 Representative images of organoids seeded initially as single cells (12'500 cells per well) in 25 µl of (A) BME2, (B) 2mg/ml ECM, (C) 3mg/ml ECM at day 3 and (D) BME2, (E) 2mg/ml ECM, (F) 3mg/ml ECM at day 7. Scale bar is equal to 200 µm.

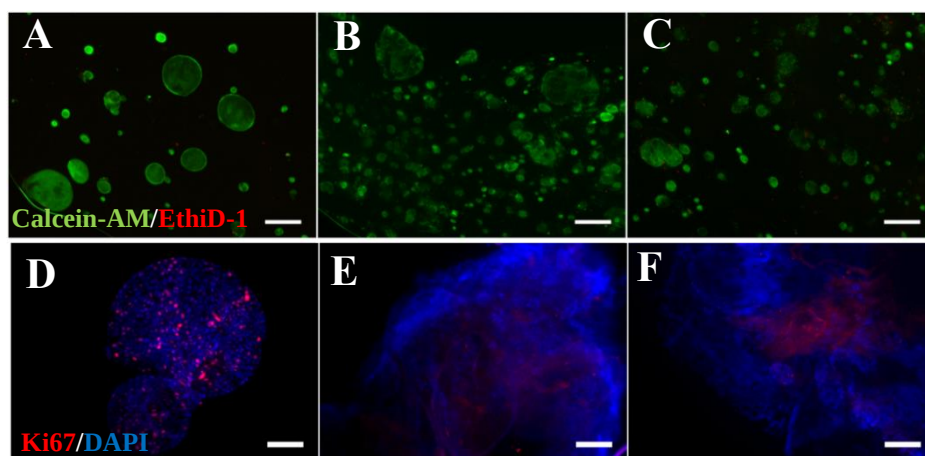


Figure 5.11 Representative images of progenitor liver cells encapsulated in (A) BME2, (B) 2mg/ml ECM, (C) 3mg/ml ECM. Cells were stained with Calcein (green) and Ethidium (red) at day 7. Scale bar 100 µm. Immunostaining of progenitor liver cells encapsulated in (D) BME2 and (E) 2 mg/ml ECM & (F) 3 mg/ml ECM gels for proliferation marker Ki67. The cells were then incubated with Ki67 primary antibody at (1:100 dilution) overnight at +4 °C, followed by a further incubation at room temperature for 1 hour with a donkey anti-rabbit Alexa Fluor® 568 at (1:1000) (shown in red). Nuclear DNA was labelled in blue with DAPI at (1:1000). Scale bar is equal to 100 µm.

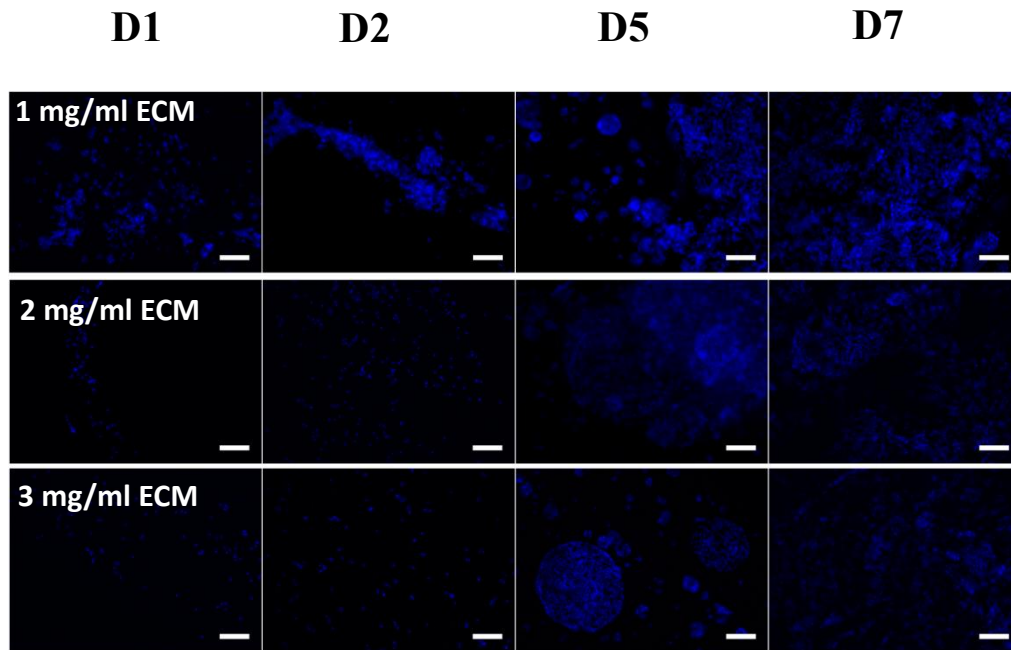


Figure 5.12 Representative images of DAPI stained progenitor liver cells encapsulated in 1,2 & 3 mg/ml ECM gels. The cells were incubated with DAPI (1:1000) labelled in blue. Scale bar is equal to 100 μ m.

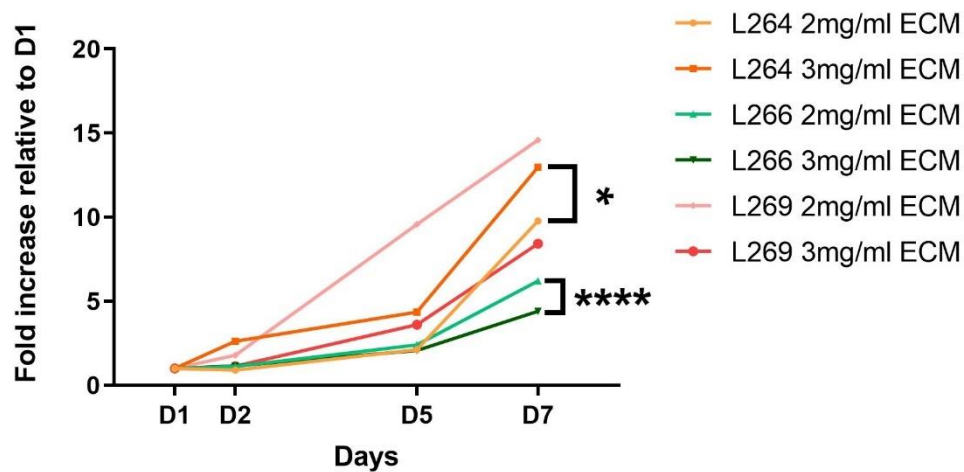


Figure 5.13 Proliferation curve of three liver donors L264, L266 and L269 in two different ECM concentrations 2 and 3 mg/ml (2ECM and 3ECM, respectively). Fold increase in nuclei relative to D1. Statistical significance was assessed using Ordinary One-Way Anova with Tukey's multiple comparisons (*: $p < 0.04$, ****: $p < 0.0001$).

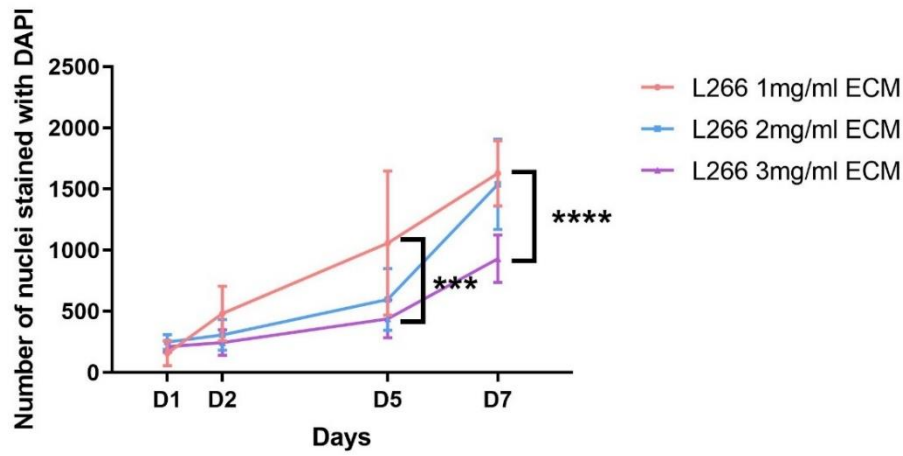


Figure 5.14 Proliferation curve of L266 donor in different ECM concentration, 1,2,3 mg/ml, respectively. Curve obtained from counting the number of nuclei with Fiji and standardizing it to D1. Statistical significance was assessed using Ordinary One-Way Anova with Tukey's multiple comparisons (***: $p < 0.001$, ****: $p < 0.0001$).

5.5.3 Hepatic progenitor markers for progenitor liver organoids

Immunostaining for epithelial stem marker, EpCAM and bile duct marker, CK19 was carried out on organoids embedded in BME2 matrix and ECM hydrogels while cultured in expansion media (EM). Both organoids embedded in BME2 matrix and ECM concentrations expressed the bile duct marker CK19 as seen in Figure 5.15 and retained the epithelial stem marker EpCAM as shown in Figure 5.16.

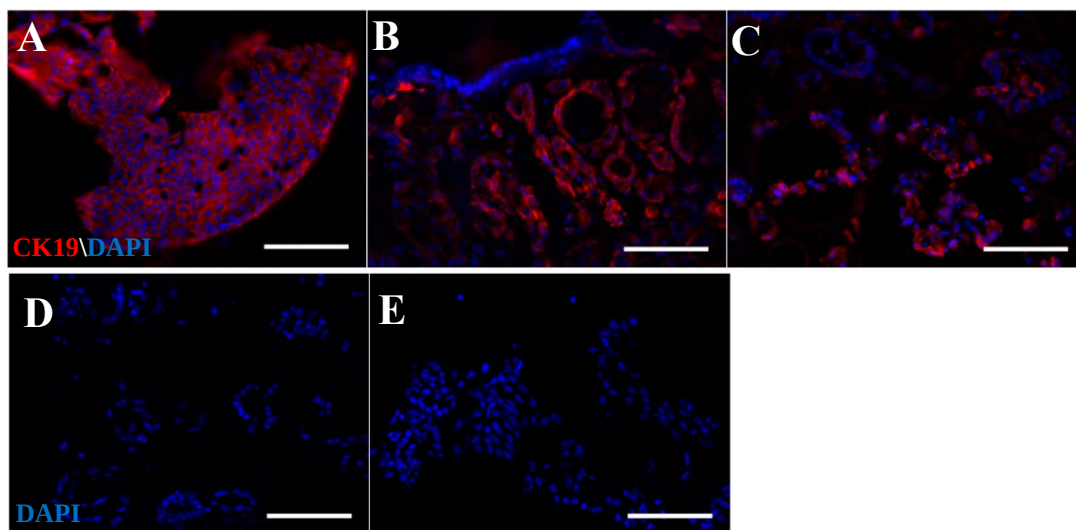


Figure 5.15 Immunostaining of CK19 in progenitor liver cells encapsulated in (A) BME2, (B) 2mg/ml ECM, (C) 3mg/ml ECM (D) BME2 DAPI stained minus antibody, (E) 2mg/ml ECM (- primary antibodies, + secondary antibodies). The cells were then incubated with CK19 primary antibody at (1:100 dilution) overnight at +4°C, followed by a further incubation at room temperature for 1h with a donkey anti-rabbit Alexa Fluor 568 at (1:500) (shown in red). Nuclear DNA was labelled in blue with DAPI at (1:1000). Scale bar is equal to 100 μ m.

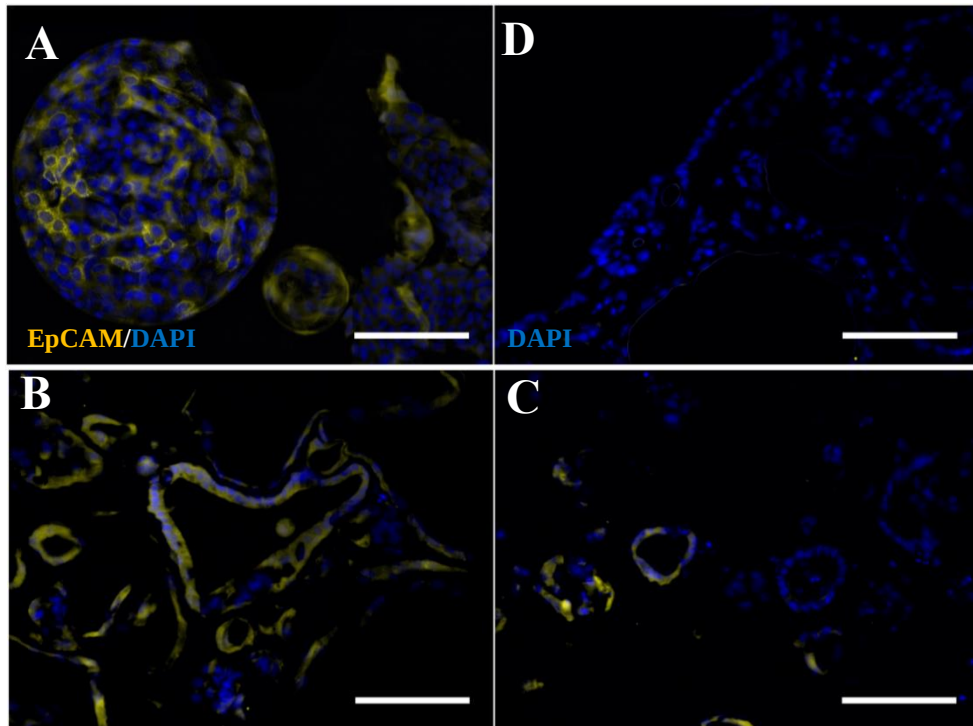


Figure 5.16 Immunostaining of EpCAM marker in progenitor liver cells encapsulated in (A) BME2, (B) 2mg/ml ECM, (C) 3mg/ml ECM and (D) 2mg/ml ECM (- primary antibodies, + secondary antibodies). The cells were then incubated with human EpCAM primary antibody at (1:100 dilution) overnight at +4 °C, followed by a further incubation at room temperature for 1h with Cy5 ab6564 (shown in yellow). Nuclear DNA was labelled in blue with DAPI at (1:1000). Scale bar is equal to 100 μ m.

5.5.4 Differentiation of bipotential progenitor liver cells into both hepatocyte and cholangiocyte lineage

Organoids embedded in ECM hydrogels and BME2 matrix were expanded in expansion media for around seven days before the differentiation protocol was applied. Once organoids were formed and filled the matrix, two protocols were implemented to differentiate progenitor cells into hepatocyte-like cells and cholangiocyte-like cells. All differentiation information can be found in the Material and method sections: 2.6.5 and 2.6.6. Once the organoids were differentiated into each lineage, an optimisation method was carried out to extract RNA from organoids embedded in ECM hydrogels. Different methodologies for RNA extraction were implemented (Figure AppC 5.5-5.7), until the best combination was found (Figure AppC 5.8). The combination of RNAlater solution, liquid Nitrogen, snap freezing and homogenisation, yielded the best quality RNA, which was checked using the Bioanalyser instrument (Figure

AppC 5.8). Once the RNA was reverse transcribed to cDNA, the level of expression of each interest gene was quantified using the Aria Mx Real-Time PCR System. Organoids embedded in both BME2 matrix and ECM hydrogel underwent morphological changes when differentiating in both Hepatocyte media (Hep-DM) and cholangiocyte media (Chol-DM) Figure 5.17. Organoids embedded in the BME2 matrix and differentiated in Chol-DM display a thick lumen as shown in Figure 5.17 (G), (J). Similarly, organoids embedded in 3 mg/ml ECM hydrogel display a thicker lumen in Chol-DM and Hep-DM compared to EM (Figure 5.17 (H), (I), (K), (L)). However, organoids embedded in the BME2 matrix and 2 mg/ml ECM in Hep-DM exhibited an epithelial-like morphology as shown in Figure 5.17 (D)& (E).

Expression of genes of interest for both mature Hepatocyte and mature cholangiocyte markers were assessed. Mature hepatic markers: Albumin and phase I drug metabolism enzymes CYP3a4 and CYP2b6 appeared to be upregulated in Hep-DM, and a higher expression at the RNA level was seen in organoids embedded in 2 and 3 mg/ml ECM after seven days in Hep-DM (Figure 5.18 (A), (B), (F)). Similarly, the MRP2 gene from Phase II drug metabolism seemed to have a higher expression in organoids embedded in liver ECM hydrogels after seven days in Hep-DM than organoids embedded in the BME2 matrix (Figure 5.18 (D)). APO_A1, another enzyme responsible for lipid metabolism, was assessed. APO_A1 was also more highly expressed in organoids embedded in ECM compared to the BME2 matrix as shown in Figure 5.18 (E). Other hepatic genes, thus Hnf4a, was also higher expressed in organoids in ECM cultured in Hep-DM for seven days. CK18 hepatic liver gene was also expressed in both organoids' cultures in Hep-DM and Chol-DM (Figure 5.19 (A)). Bile duct marker CK19 gene remained expressed in organoids embedded in ECM and BME2 and for both differentiation media conditions (Figure 5.18 (G)). Epithelial cell adhesion molecule EpCAM gene remained expressed in all organoid's conditions and after differentiation (Figure 5.18 (H)). Stem marker Lgr5 was expressed in all organoids in Expansion media but was not detectable when organoids were differentiated in both lineages (Figure 5.18 (I)). SOX9 gene expression was retained in all organoids for all conditions and in both un-differentiated and differentiated organoids (Figure 5.18 (J)). Furthermore, mature cholangiocytes gene expression was investigated in both Chol-DM and Hep-DM. CK7, ASBT and CFTR were examined in both organoids differentiated toward a cholangiocyte and hepatic lineage. CK7 cholangiocyte gene was expressed in organoids embedded in both BME2 matrix and ECM in Hep-DM (Figure 5.19 (B)). Cystic fibrosis transmembrane conductance regulator (CFTR) was only expressed

in organoids cultured in Chol-DM, and significantly higher expression was seen in organoids cultured in BME2 matrix compared to organoids cultured in ECM (Figure 5.19 (C)). Acid bile salt transporter (ASBT), also known as Solute Carrier Family 10 Member 2, was expressed in organoids differentiated in both lineages. But a significantly higher expression at the RNA level was seen in organoids cultured in the BME2 matrix in both Hep-DM and Chol-DM (Figure 5.19 (D)).

Mature cholangiocyte genes: CK18, CK7, CFTR and ASBT were designed with PrimersDesign Software, and their efficiency and expression were checked with established cell lines HeLa and CaCO2 cells Figure AppC 5.9.

Finally, the functionality of differentiated progenitor organoids towards a hepatic lineage was assessed with a CYP540 assay (Figure 5.20 (A)). The activity of the CYP3a4 enzyme was measured once the organoids were induced with WY- 14643 ligand. It is possible to see a trend with increased expression in organoids treated with the ligand compared to untreated organoids. However, a significant increase in Cyp activity was seen only in organoids treated with the ligand and encapsulated in both 2 and 3 mg/ml liver ECM. No difference in activity was seen in organoids treated with the ligand and embedded in the BME2 matrix as shown in Figure 5.20 (A). However, some activity was also seen in the vehicle (DMSO 0.1% treated cells) (Figure 5.20 (A)). When the experiment was repeated, no significant increase in Cyp activity was seen in organoids treated with the WY ligand in all conditions. However, the trend of slightly increased Cyp activity could still be seen (Figure 5.20 (B)). The assay requires further optimization to account for the cells encapsulated in a matrix environment.

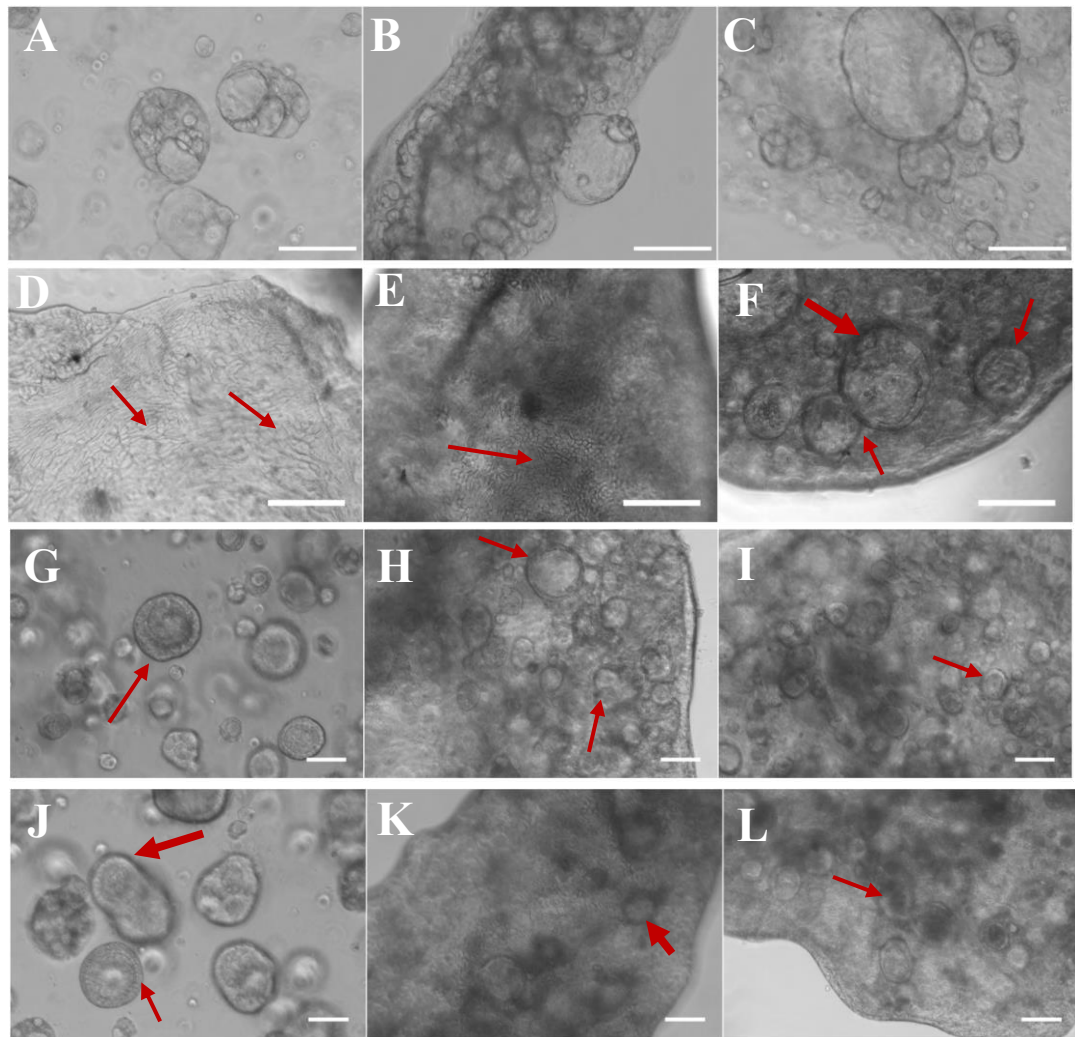


Figure 5.17 Bipotential cells encapsulated in ECM hydrogels and BME2 with different media conditions. Expansion media 7 days: (A) BME2, (B) 2mg/ml ECM, (C) 3mg/ml ECM, Hepatocyte differentiation media 11 days: (D) BME2, (E) 2mg/ml ECM, (F) 3mg/ml ECM, Cholangiocyte differentiation media 4 days: (G) BME2, (H) 2mg/ml ECM, (I) 3mg/ml ECM Cholangiocyte differentiation media 10 days: (J) BME2, (K) 2mg/ml ECM, (L) 3mg/ml ECM. Scale bar of (A)-(F) is equal to 200 μ m, scale bar of (G)-(N) is equal to 100 μ m.

Legend of hepatic progenitor culture conditions:

Expansion Media (EM) N=2
 Differentiation media 7 days (DM7) N=3
 Differentiation media 11 days (DM11) N=3
 Cholangiocyte differentiation media (Chol-DM)

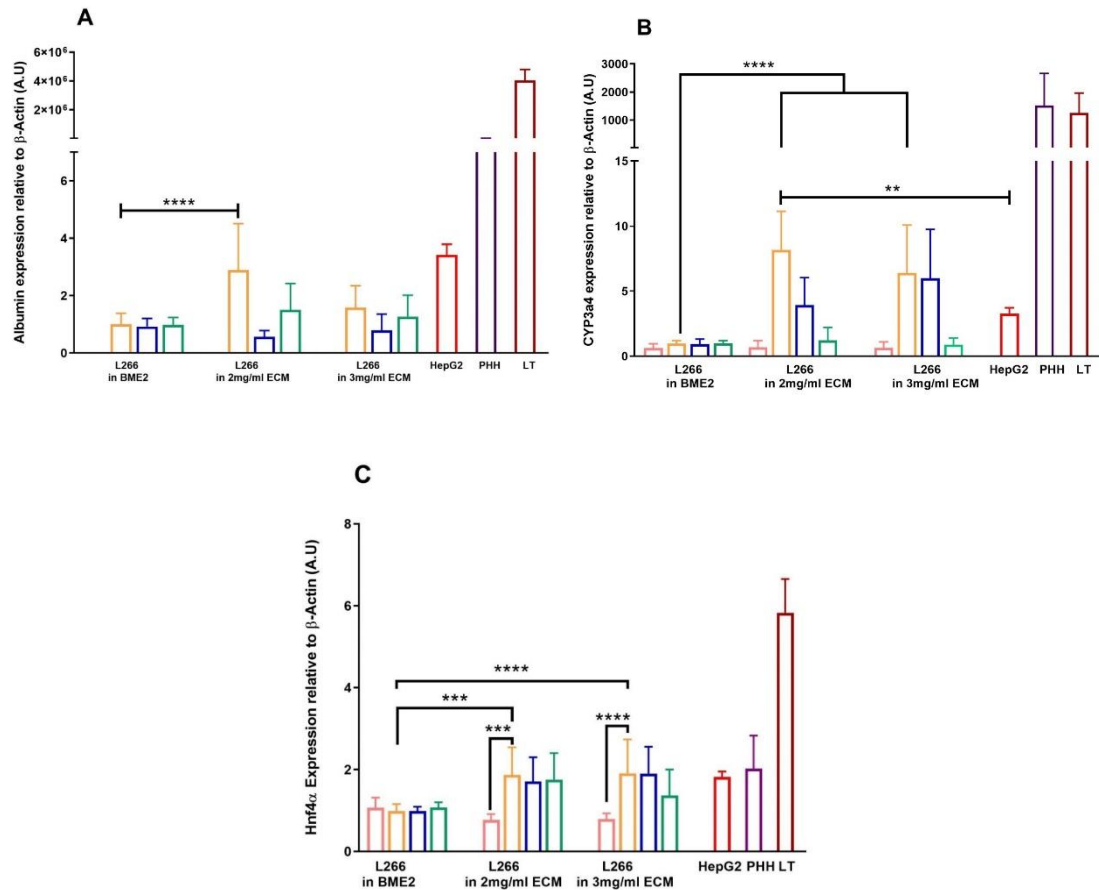


Figure 5.18 Bipotential hepatic progenitor cells encapsulated in 2,3 mg/ml ECM and BME2 cells expressing hepatic markers relative to control gene, β -Actin: (A) Albumin, (B) CYP3a4 and (C) Hnf4 α gene expression of hepatic progenitor encapsulated in ECM and BME2 with primary human hepatocytes, HepG2 and Liver tissue as controls. Hepatic progenitors were cultured in the highlighted condition specified in the legend. EM, N=2 with 5 independent samples for each condition. DM7, N=3 with at least 3 independent samples for each condition. DM11, N=3 with at least 3 independent samples for each condition. Chol-DM, N=3 with at least 3 independent samples for each condition. Statistical significance was assessed using Ordinary One -Way Anova with Bonferroni multiple comparison (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

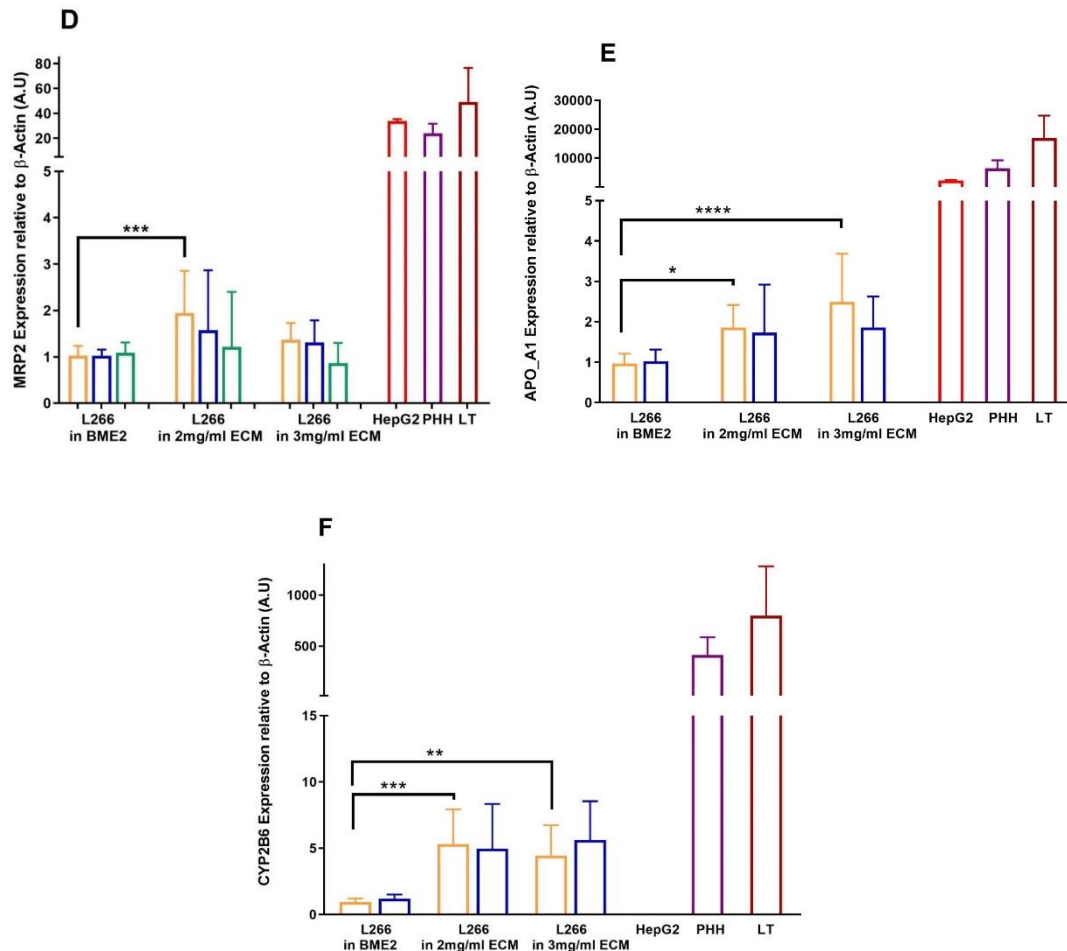
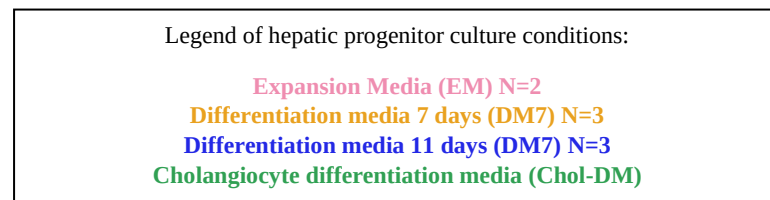


Figure 5.19 Bipotential hepatic progenitor cells encapsulated in 2,3 mg/ml ECM and BME2 cells expressing late hepatic markers relative to control gene, β -Actin: (D) MRP2, (E) APO_A1 and (F) CYP2B6 gene expression of hepatic progenitor encapsulated in ECM and BME2 with primary human hepatocytes, HepG2 and Liver tissue as controls. Hepatic progenitors were cultured in the highlighted condition specified in the legend. EM, N=2 with 5 independent samples for each condition. DM7, N=3 with at least 3 independent samples for each condition. DM11, N=3 with at least 3 independent samples for each condition. Chol-DM, N=3 with at least 3 independent samples for each condition. Statistical significance was assessed using Ordinary One -Way Anova with Bonferroni multiple comparison (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

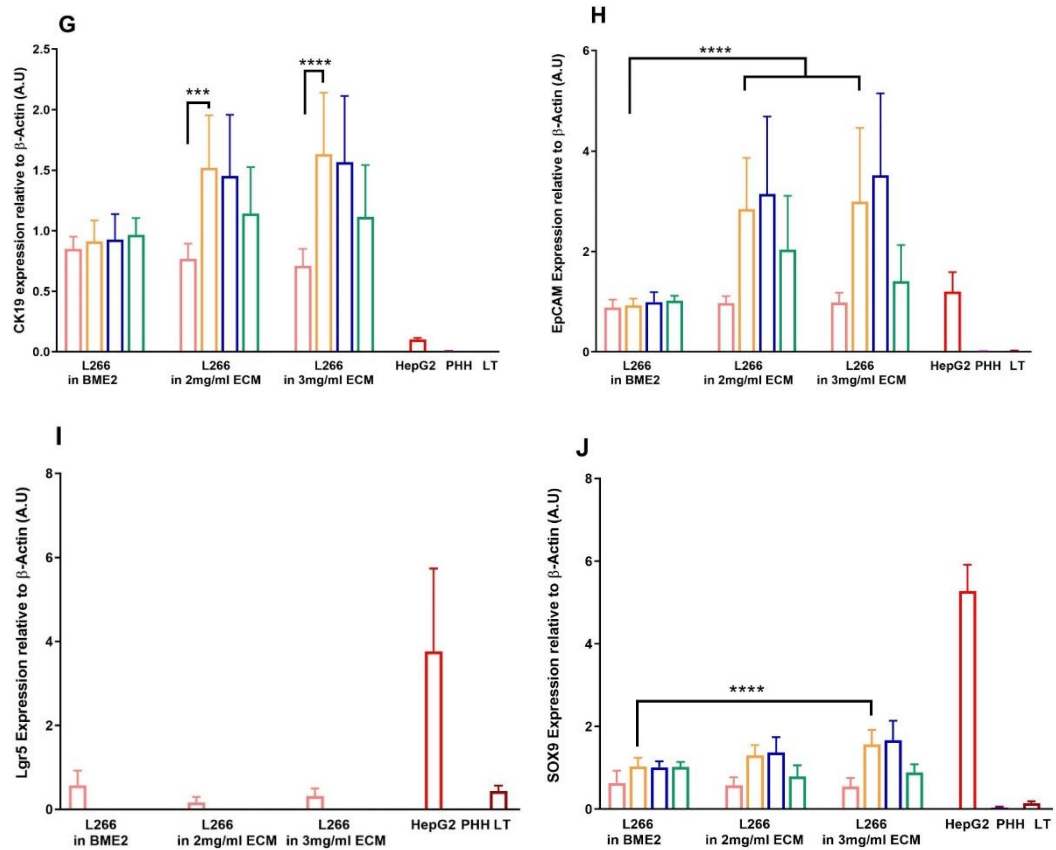
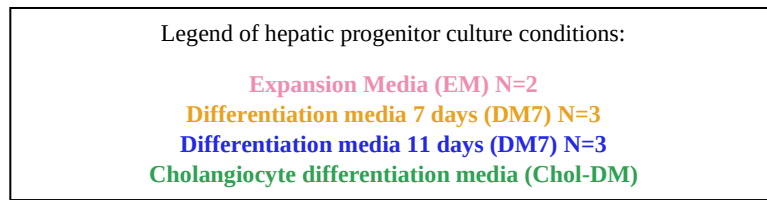


Figure 5.20 Bipotential hepatic progenitor cells encapsulated in 2,3 mg/ml ECM and BME2 cells expressing bile and epithelial stem markers relative to control gene, β -Actin: (G) CK19, (H) EpCAM, (I) Lgr5 and (J) SOX9 gene expression of hepatic progenitor encapsulated in ECM and BME2 with primary human hepatocytes, HepG2 and Liver tissue as controls. Hepatic progenitors were cultured in the highlighted condition specified in the legend. EM, N=2 with 5 independent samples for each condition. DM7, N=3 with at least 3 independent samples for each condition. DM11, N=3 with at least 3 independent samples for each condition. Chol-DM, N=3 with at least 3 independent samples for each condition. Statistical significance was assessed using Ordinary One -Way Anova with Bonferroni multiple comparison (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

Legend of hepatic progenitor culture conditions:

Differentiation media 7 days (DM7) N=3
Cholangiocyte differentiation media (Chol-DM) N=3

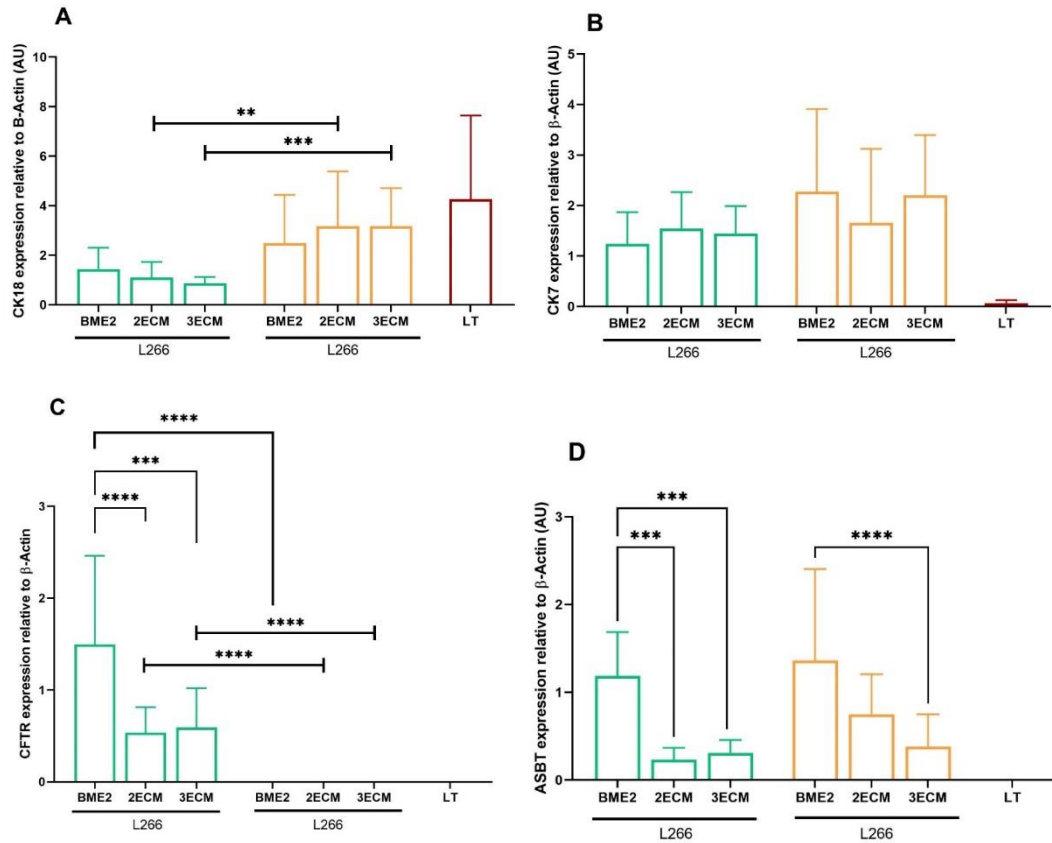


Figure 5.21 Bipotential hepatic progenitor cells encapsulated in 2,3 mg/ml ECM and BME2 cells expressing liver bile duct and differentiated cholangiocytes markers relative to control gene, β -Actin (A) CK18, (B) CK7, (C) CFTR and (D) ASBT gene expression of bipotential progenitor liver cells encapsulated in BME2 and 2 and 3 mg/ml ECM with liver tissue as control. Hepatic progenitors were cultured in the highlighted condition specified in the legend. DM7, N=3 with at least 3 independent samples for each condition. Chol-DM, N=3 with at least 3 independent samples for each condition. Statistical significance was assessed using Ordinary One -Way Anova with Bonferroni multiple comparisons (**: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

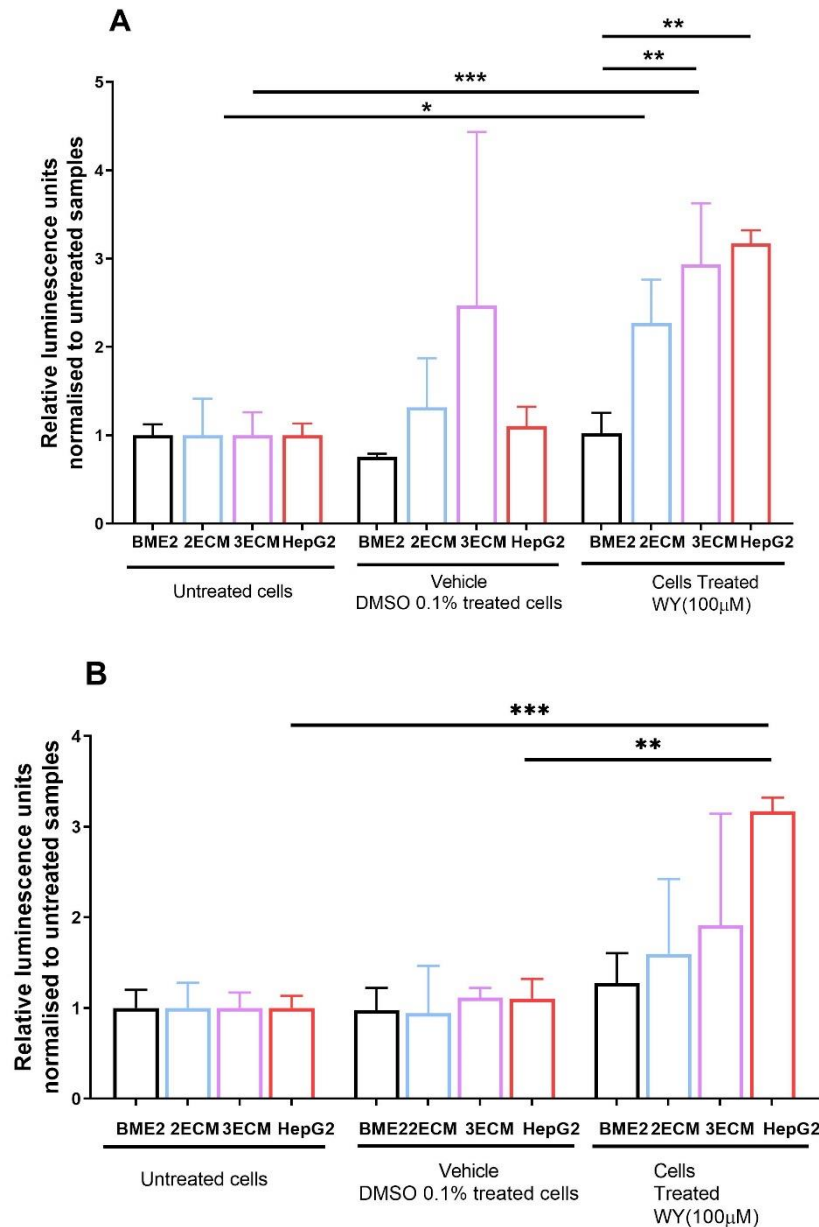


Figure 5.22 (A) Cyp3a4 Induction of progenitor liver organoids encapsulated in BME2, 2 and 3 mg/ml ECM, 2ECM and 3ECM, respectively. Organoids were expanded and differentiated in the hepatocytes' differentiation medium for 7 days (DM7) than the organoids were induced with WY- 14643 (100 μ M) for 24 hours before the addition of the substrate. The cells were incubated with the substrate for 4 hours before detection using a plate reader under luminescence conditions. N=1, n=3 independent samples for each condition. Statistical significance was assessed using One-way Anova with Bonferroni post-Hoc test. $p < 0.05$ was considered significant. (B) Same induction of Cyp3a4 using WY- 14643 small molecule. This time data included two independent experiments N=2, with n=3 independent samples for each condition. Statistical significance was assessed using One-way Anova with Bonferroni post-Hoc test. $p < 0.05$ was considered significant.

5.5.5 Subculturing of hepatic organoids in liver ECM hydrogels

To assess the subculturing potential of liver organoids embedded in liver dECM derived hydrogels, hepatic organoids were cultured in 3 mg/ml ECM hydrogels until many organoids were formed (7-8 days). After optimising a collagenase digestion protocol (Figure AppC 5.10), organoids cultured in ECM were collagenase dissociated and resuspended in fresh dECM hydrogels. Cytotoxicity of the collagenase process was assessed straight after encapsulation in the new ECM with live/dead staining. Organoids have retained their shape and are alive after the process, with few to no dead cells after 0 hours of re encapsulation in fresh ECM (Figure 5.21 (A-D)). Viability in the ECM after collagenase digestion was assessed after 72 hours of culture; even though the hydrogel shrunk dramatically, liver progenitor organoids remained alive for up to 72 hours (Figure 5.22 (A-C)).

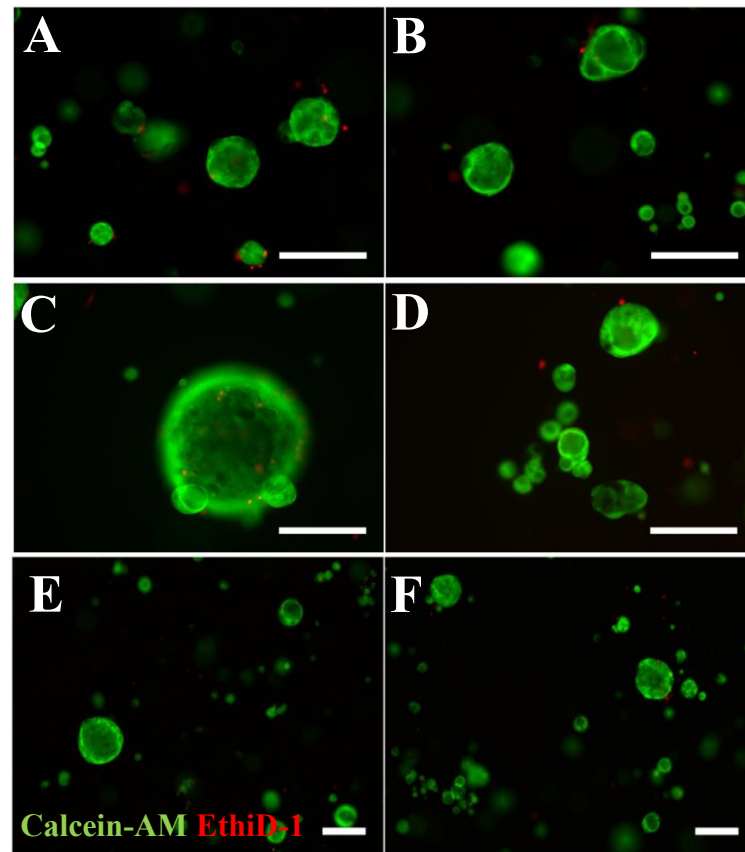


Figure 5.23 (A-D) Live-dead staining of bipotential liver progenitor organoids after 0hr of being re-seeded in 3mg/ml ECM gels after collagenase dissociation (A-D) images were taken at x10 magnification. Scale bar is 200 μm. (E and F) images were taken at 5x magnification. Scale bar is equal to 100 μm.

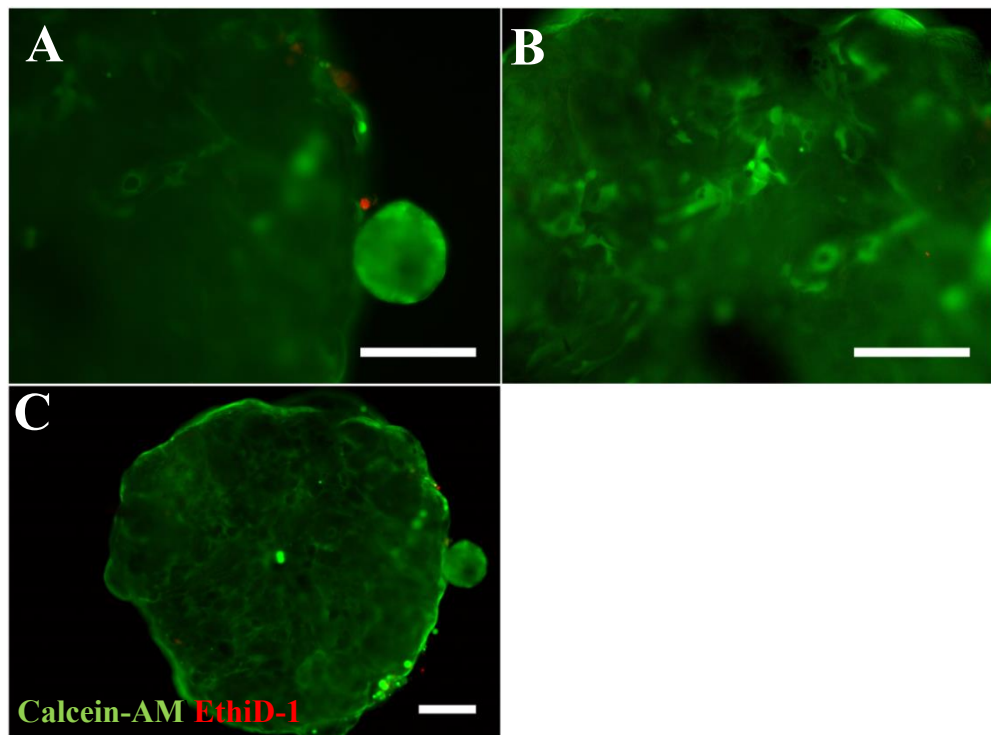


Figure 5.24 (A-C) Live-dead staining of bipotential liver progenitor organoids cultured for 72 hours after being re-seeded in 3mg/ml ECM gels following collagenase dissociation from ECM gels. Scale bar is equal to 200 μ m at 10x magnification (A and B). Scale bar is equal to 100 μ m at 5x magnification (C).

5.6 Discussion

Mediated cell contraction was observed when different cell types were encapsulated in ECM gels. Mechanosensing is a well-established phenomenon in which cells are influenced by their substrates and strain energy (Panzetta et al., 2019). Progenitor liver cells shown in Figure 5.7 and 5.10 as well as cancerous hepatic cell line HepG2 (Figure AppC 5.3 (H), (J), (L), (M)) and Human skin fibroblast (HS137) (Figure AppC 5.4 (E), (F), (H), (I)) caused a drastic liver ECM gel contraction and cell spreading upon encapsulation. These findings are consistent with many reports of fibroblast contracting in collagen gels (Zhang et al., 2019; ZHU et al., 2001). However, when these same cells were encapsulated in the BME2 matrix, little to no contraction was recorded as shown in Figure AppC 5.3 (A), (D), (G), (K) and Figure AppC 5.4 (A), (D), (G), leading to speculate that fibronectin and Collagen I are the most influential protein in ECM matrices responsible for mediating cell contraction (Kelynack et al., 2000; Nakamura et al., 2003). This speculation is consistent with the commercially available BME2 matrix composition, which comprises 60-65% laminin and no contraction is observed (Benton et al., 2011; Borries et al., 2020; Kane et al., 2018). Huch et al. (2015) cultured Lgr5⁺ bile duct derived bipotent organoids embedded in the BME2 matrix, and there was no mention of matrix shrinkage over time (Huch et al., 2015). Nuciforo et al. (2018) also cultured liver cancer-derived organoids in BME2, and there was no mention of mediated cell shrinkage (Nuciforo et al., 2018). Shrinkage of liver ECM gels was observed in all ECM concentrations when organoids were encapsulated, suggesting that cell mediated ECM contraction is not dependent on ECM concentration but rather ECM composition and interaction with embedded cells.

Liver ECM derived hydrogels were biocompatible and non-toxic to cell lines as seen in Figure AppC 5.3 and Figure AppC 5.4 and adult bipotential progenitor cells as shown in Figure 5.8 (M-P) and Figure 5.11 (A-C). To assess if adult progenitor liver cells embedded in higher concentration liver ECM hydrogels (4,6 and 8mg/ml) behave similarly to progenitor embedded in BME2, a metabolic assay and proliferation assay was carried out. Considerable variations in metabolic activity were found as the assay was optimised for 2D cell culture rather than 3D. It was found unreliable in 3D culture and not carried over. A proliferation assay was instead carried out. Organoids embedded in the BME2 matrix had significantly higher DNA content (Figure 5.8 (A)), suggesting a greater cell proliferation occurred in BME2 matrices. This was also confirmed with immunostaining of proliferation marker Ki67 (Figure

5.9 (A)). Is it well established that ECM contractile forces influence cell behaviour (Tse and Engler, 2011). The motility of the organoids could have been impaired due to the high stiffness of the liver ECM hydrogels, which would have influenced cell functionality. Organoids-organoids signalling could also have been impaired due to the restricted motility.

Progenitor liver cells were encapsulated in 4,6 and 8 mg/ml ECM hydrogels but failed to proliferate, spread and form homogeneous organoids, possibly due to the high stiffness of the hydrogels which impeded cells migration and proliferation (Razinia et al., 2017; Xu et al., 2017).

As the ECM concentration was lowered to 1,2 and 3 mg/ml ECM, as well as remaining viable as seen in Figure 5.11 (B), (C), organoids started proliferating (Figure 5.12). Morphologically, organoids embedded in the BME2 matrix are similar in shape and size (Figure 5.10) to organoids embedded in ECM hydrogels. Furthermore, the morphology of cyst-like structure is reported in many liver organoids studies (Gómez et al., 2020; Huch et al., 2015), and it is consistent with what we have found. Suggesting that adult progenitor organoids in ECM hydrogel at lower concentrations proliferated and developed similarly to organoids embedded in the BME2 matrix. These findings indicated that substrate stiffness modulates cell motility, proliferation and reprogramming (Ali and Chuang, 2014; Kalli and Stylianopoulos, 2018; Razinia et al., 2017; Sorrentino et al., 2020; Yu Wang et al., 2011).

Proliferation capabilities in ECM hydrogels were assessed on organoids from three different donors (Figure 5.13). The lower the concentrations of the ECM hydrogels, the higher the amount of the nuclei was present. It was hypothesised a correlation between the concentration of the ECM and the number of nuclei. However, after some parallel studies conducted by the postdoc Dr Fatima Abukunna it was discovered that the L266 donor yielded the best chances of differentiating into a hepatocyte-like phenotype with the required differentiation protocol. Therefore, L266 donor was carried over for further investigation and studies. Unfortunately, although the adult progenitor encapsulated in 1 mg/ml ECM hydrogel yielded the best proliferation as shown in Figure 5.14, the lack of homogenisation in the fabrication process and the lengthy incubation time for the gel to develop lead us to abandon this concentration and carry on with 2 and 3 mg/ml ECM gels. Organoids cultured in expansion media (EM) were stained for epithelial stem marker EpCAM and bile duct marker CK19 (Figure 5.15 and 5.16). Organoids embedded in both matrices retained both the epithelial marker EpCAM and expressed the bile duct marker CK19. Similar studies have also reported these markers being

present in bipotential adult progenitor liver organoids (Broutier et al., 2016; Huch et al., 2015; Russell et al., 2019; Steenbeek et al., 2020).

After expanding progenitor liver organoids in both BME2 matrix and ECM derived hydrogels, a differentiation protocol was designed and adapted from Huch group (Huch et al., 2015, 2013b) to differentiate the cells towards a hepatocyte-like phenotype. Progenitor organoids were differentiated in Hepatocyte's medium (Hep-DM) for 7, 11 and 13 days. Similarly, once expanded, a differentiation protocol towards a cholangiocyte fate was applied following the work of Dr Fatima Abukunna, Sampaziotis et al. (2015) and Tanimizu et al. (2007) (Sampaziotis et al., 2015; Tanimizu et al., 2007). Progenitor organoids were differentiated in cholangiocytes medium (Chol-DM) for ten days. Morphological analysis of organoids differentiated in both cholangiocytes and hepatocytes medium showed a thickening if the lumen layer surrounded the organoids (Figure 5.17 (D-L)) compared with undifferentiated organoids (Figure 5.17 (A-C)). This thickening in the lumen is consistent with other reports of differentiated liver organoids (Gómez et al., 2020). Gene expression of hepatocytes, cholangiocytes and stem cell genes was compared across differentiation conditions and between organoids cultured in BME2 matrix and ECM hydrogels as shown in Figure 5.18 and 5.19.

Mature hepatocytes genes, thus albumin (Figure 5.18 (A)), phase II enzyme MRP2 (Figure 5.19 (D)), APO_A1 apolipoprotein, involved in lipids metabolisms (Jiang et al., 2013), (Figure 5.19 (E)) and phase I enzymes CYP3a4 and CYP2b6 (Figure 5.18 (B) and Figure 5.19 (F)) seemed all to be higher expressed in organoids embedded in ECM hydrogels compared to organoids in BME2. CYP2b6 phase I enzyme did not seem to be expressed in HepG2 cells. However, it is well reported that levels of Cyps enzymes at the transcript levels are low in the human hepatoma cancerous cell line, HepG2 (Steinbrecht and Kammerer, 2019; Westerink and Schoonen, 2007). Other studies reported low to none expression of cyp2b6 at the mRNA level (Berger et al., 2016). Only by using lentiviral transfection, Xuan et al. (2016) produce a transfected Hepg2 cell line able to express at the mRNA level most of the Cyps phase I enzymes (Xuan et al., 2016).

Furthermore, liver genes Hnf4a (Figure 5.18 (C)) and CK18 (Figure 5.21 (A)) are also expressed in organoids encapsulated in both BME2 and ECM hydrogels when differentiated in Hep-DM. These hepatic markers are reported to be expressed in similar studies, where liver

progenitor cells were differentiated into hepatocyte-like cells (Gómez et al., 2020; Huch et al., 2015; Segal et al., 2019; Steenbeek et al., 2020).

Upon differentiation, only the Lgr5 stem cell marker was downregulated and failed to be expressed in differentiated conditions as shown in Figure 5.20 (I), which is consistent with Huch findings (Huch et al., 2015) and others (Gómez et al., 2020), however, EpCAM and SOX9 stem markers as seen in Figure 5.20 (H) and (J), respectively, are still retained after the application of both differentiation media conditions, suggesting a heterogeneous population of liver progenitor (Pepe-mooney et al., 2019) is present. Not all progenitor cells have successfully differentiated. In other studies, the expression of EpCAM and Sox9 is considered to be common in bile epithelial cells (BEC) (Furuyama et al., 2010; Okabe et al., 2009; Xiao et al., 2003). Hence, the gene expression of these progenitor cells is not defined. However, it is possible to conclude that although the Lgr5 stem marker was not expressed in differentiated conditions, EpCAM and Sox9 were still present, and progenitor cells still retained some cellular plasticity (Huch and Doll, 2016). Indicating one marker to check the stemness is not enough to conclude that the progenitor cells have fully differentiated.

To assess the liver progenitor cell functionality, once differentiated towards a hepatocyte-like phenotype, progenitor organoids were induced with WY-14643 (100 μ M) ligand. Even if we saw upregulation of the Cyp3a4 enzyme at the mRNA level for progenitor encapsulated in 2 mg/ml ECM hydrogels, the enzyme activity was not consistent (Figure 5.22 (A), (B)). The colorimetric assay was optimised for 2D culture, and therefore even if a longer incubation time with the substrate was tried, it was not consistent in producing a consistent readout. Further optimisation of the assay in 3D is needed to confirm the functionality of the differentiated progenitor liver cells.

As suggested in the introduction, it was hypothesised that a laminin rich environment would maintain the liver progenitor phenotype and promote biliary specification (Hul et al., 2009; Lorenzini et al., 2010; Suárez et al., 2012). BME2 matrix comprises up to 65% of laminin. Therefore, to try this hypothesis, we differentiated the progenitor liver organoids towards a cholangiocyte phenotype using a cholangiocyte medium. This hypothesis is partially supported, as we see an upregulation of bile duct and mature cholangiocyte genes in organoids embedded in the BME2 matrix (Figure 5.21 (A-D)). CK19 bile duct marker (Figure 5.20 (G)) was present in all conditions and in both matrices, unsurprisingly as it seems to be upregulated in progenitor liver cells as it is considered an adult biliary marker (Bria et al., 2017). Mature

adult biliary marker CK7 (Figure 5.21 (B)) was present in both differentiated conditions, suggesting the presence of an heterogeneous population of liver progenitors. Acid bile salt transporter (ASBT) mature cholangiocyte gene (Kip et al., 2004) (Figure 5.21 (D)) was present in both differentiated conditions, but it had a significantly higher expression in organoids embedded in the BME2 matrix. However, CFTR (Figure 5.20 (C)), a mature cholangiocyte marker, was only expressed in organoids differentiated towards a cholangiocyte phenotype (Cohn et al., 1993; Roos et al., 2021; Venter et al., 2016). Furthermore, it was significantly higher expressed in organoids embedded in BME2 matrices, confirming the original hypothesis that a laminin-rich environment promotes biliary specification (Lorenzini et al., 2010). These results suggest that not only the cholangiocyte medium but also the BME2 matrix play a crucial role in the specification of progenitor liver cells into a cholangiocyte phenotype, and the matrix composition is partially responsible for phenotype regulation and cell differentiation (Ahmed and French-Constant, 2016; Smith et al., 2017).

Lastly, the capability of subculturing the progenitor organoids in the ECM matrix was investigated. Organoids were successfully dissociated from the ECM and re-encapsulated in new ECM matrices. Organoids were viable after dissociation (Figure 5.23) and for up to an additional three-day (Figure 5.24). These results open the possibility to use ECM matrix as a substitute for culturing and differentiating liver progenitor organoids into hepatocyte-like cells.

5.7 Conclusion

We successfully cultured and differentiated EpCAM⁺ sorted progenitor cells into both a hepatic and cholangiocyte lineage. We demonstrated the potential of liver ECM hydrogels as an alternative matrix to subculture progenitor liver organoids. Liver progenitor cells in high concentration liver ECM hydrogels were unable to proliferate. However, as the concentration was lowered, progenitor cells proliferated at a similar rate to cells embedded in the BME2 matrix.

A differentiation protocol was applied to direct the progenitor organoids towards either a cholangiocyte or a hepatocyte phenotype. Gene expression of mature hepatic and cholangiocyte markers revealed a significant increase in expression of mature hepatocytes genes for progenitor organoids embedded in liver ECM hydrogels compared to organoids encapsulated in the BEM2 matrix. However, upon differentiation in cholangiocyte media, mature cholangiocyte markers were significantly higher expressed in organoids embedded in the BME2 matrix compared to organoids encapsulated in liver ECM hydrogels. These findings suggest that the matrix composition has a direct effect in influencing liver progenitor cells fate.

Chapter VI - General discussion and future work

6.1 General Discussion

Developing an *in-vitro* liver model relies on the successful integration of cellular components within a 3D porous scaffold. Isolated hepatocytes grown in monolayer undergo rapid dedifferentiation and depolarisation (Guillouzo, 1998). When designing a liver scaffold, the choice of the material will have a significant influence on its success. Hepatocytes bind to the ECM via transmembrane integrin receptors, subsequently mediating cell migration and functionality (S. J. Hammond et al., 2006). Therefore, a liver scaffold needs to be able to mimic the environment that hepatocytes experience *in-vivo*. Another aspect to consider is the heterogeneity of liver tissue, which is why it is very difficult to replicate in an *in-vitro* model. Non-parenchymal cells are responsible for intercellular interaction; therefore, a co-culture system is highly desirable to ensure better hepatocyte functionality.

Furthermore, *in-vivo* the liver experiences a gradient of oxygen, hormones, nutrients and matrix composition, which results in zonal variations of hepatocyte subpopulations (Jungermann and Kietzmann, 1996). The type of liver cells utilised to develop *in-vitro* liver models has been thoroughly reviewed (Collins et al., 2019). Stem cells have the ability to proliferate and differentiate into more specialised cells. Thus they can restore and maintain functions of damaged cells, tissue and organoids (Manohar and Lagasse, 2014). However, the genetic instability of hESCs and iPSCs cultures raise concerns about their suitability as cell-therapies (Bayart and Cohen-haguenauer, 2013; Medvedev et al., 2010; Tang et al., 2008).

In recent years, tissue-derived stem cells have grown in popularity as a new source of hepatocytes-like cells (Faris et al., 2001). Notably, a population of bipotential progenitor liver cells which reside in the canal of Herring and is capable of differentiating into both hepatocytes and bile duct phenotype both *in-vitro* and *in-vivo* (Huch et al., 2015, 2013b; Lu et al., 2015; Okabe et al., 2009).

6.1.1 Progenitor liver cell response in ECM matrices hydrogels

Within the liver, tissue-resident cells exist in dynamic reciprocity with the ECM (Bissell et al., 1982). The ECM provides structural and biochemical cues to influence cellular functions, and cells respond by modifying the ECM (Xu et al., 2009). The generation of a scaffold with similar biochemical properties to the ECM is highly desirable to direct cell behaviour. One of the main challenges of producing such a scaffold is to find suitable biomaterials that can replicate the liver ECM microarchitecture and biochemical complexity. Synthetic and naturally derived hydrogels have previously been explored due to their biocompatibility, degradability, and mechanical properties similar to the ECM (Croce et al., 2019; Kazemnejad, 2009). Given the right cues and a three-dimensional environment, progenitor liver cells can differentiate into functional hepatocyte-like cells.

To our knowledge, this is the first time that EpCAM⁺ progenitor liver cells have been cultured in decellularised ECM from porcine liver and differentiated towards both a hepatic and cholangiocyte lineage. EpCAM⁺ isolated from human liver tissue were able to proliferate and form organoids in ECM hydrogels. EpCAM⁺ progenitor liver cells embedded in ECM liver hydrogels were later differentiated towards both a cholangiocyte and hepatocytes phenotype. The hepatocyte differentiation medium containing BMP7, FGF19 and Dexamethasone was deprived of R-spondin, a WNT signalling pathway antagonist (Huch et al., 2015). However, only progenitor cells in ECM hydrogel expressed higher hepatocyte-specific markers (Albumin, CYps, Hnf4a) when compared with progenitor organoids embedded in the BME2 matrix. This leads us to consider how important the ECM is in regulating liver progenitor organoids differentiation (Lu et al., 2011; Page-McCaw et al., 2007). However, bile duct markers such as CK19 and stem cell markers such as SOX9 were still present after hepatic differentiation.

Additionally, the differentiation status of these progenitor cells seems to be limited compared to the gene expression of both primary human hepatocytes and liver tissue. It could be considered that these EpCAM⁺ sorted cells are biased towards a more bile-duct epithelial phenotype. They express many bile duct markers (EpCAM, SOX9, CK7, CK19) in expansion and differentiation conditions (Furuyama et al., 2010; Okabe et al., 2009; Xiao et al., 2003). After all, EpCAM cell marker is not exclusively expressed in hepatic progenitor cells. Therefore the isolated cells were possibly composed of a mixture of cells already committed

to a biliary-duct lineage (De Boer et al., 1999; Yovchev et al., 2007). When EpCAM⁺ progenitor liver cells were differentiated towards a more biliary epithelial phenotype, organoids embedded in the BME2 matrix showed a higher expression of mature cholangiocyte markers (CFTR, ASBT). With the CFTR gene being completely downregulated in organoids cultured in hepatocyte differentiation media. It confirmed the idea that these cells might be biased towards a biliary fate.

Further work in designing the composition of differentiation media could be carried on. However, there is an incomplete understanding of the activation pathways of progenitor liver cells; a more accurate sorting for other markers could be applied to identify a population of liver progenitors cells more committed towards a hepatic lineage (Segal et al., 2019). Segal et al. (2019) suggested that linear restriction of EpCAM⁺/NCAM⁻ and TROP⁻² could give rise to hepatocytes while EpCAM⁺/NCAM⁻ and TROP⁻² could generate biliary epithelial cells (Segal et al., 2019).

6.1.2 Role of liver ECM in liver progenitor stem cell fate and differentiation

It is well established that ECM components not only have a supportive role in tissues but are also involved in a variety of critical aspects in cell biology, thus: motility, attachment, proliferation and differentiation (Martinez-Hernandez and Amenta, 1993; Martinez-Hernandez A et al., 1991; Sánchez-Romero et al., 2019b; Yue, 2014). ECM environment is dynamic, and it can influence the degradation and remodelling of its constituents upon tissue damage (Klaas et al., 2016; Lu et al., 2011; Page-McCaw et al., 2007). Although the liver ECM is only a small component of the liver itself (J.M and Seyer, 1980; Rojkind and Martinez-palomo, 1976), it is comprised of a thin basement membrane with Collagen Type IV and laminin present (Mak and Mei, 2017; Martinez-Hernandez, 1984), but mostly ECM Collagens (I, III, fibronectin) (Martinez-Hernandez, 1984).

We have reported that ECM stiffness influence progenitor liver cells proliferation. When cells were cultured in stiffer ECM matrices they failed to proliferate. This led to the speculation that these cells did not thrive in a stiff environment and this work is consistent with the literature on how collagens and collagen contraction regulates cell survival (ZHU et al., 2001).

Progenitor liver cells reside in the basement membrane region of bile ducts, and laminin helps them maintaining an undifferentiated state (Boulter et al., 2013; Carpino et al., 2016; Lorenzini et al., 2010; Miyajima et al., 2014). Likewise, it supports the expression of biliary genes and inhibits the expression of early hepatocytes genes, proving the role of ECM components in liver progenitor stem cell fate (Hul et al., 2009; Lorenzini et al., 2010; Sánchez-Romero et al., 2019b).

The commercially gold standard BME2 matrix comprises of 60-65% laminin and a small percentage of Collagen IV, entactin and heparan sulphate proteoglycans (Benton et al., 2014, 2011, 2009; Borries et al., 2020; Kane et al., 2018). Our study showed lower gene expression of hepatic markers in liver progenitor embedded in BME2 matrix, even after Hepatic differentiation. However, higher expression of mature cholangiocyte genes is reported in progenitor liver cells embedded in a laminin rich matrices (Huang et al., 2020). These results confirm that laminin inhibits hepatocytes genes and promotes liver progenitor cells towards a biliary epithelial fate. Our study also highlighted structural and dynamic differences between liver ECM and BME2 matrix. Although they have comparable storage and loss moduli, no matrix contraction is seen when cells are encapsulated in the BME2 matrix. However, ECM gels undergo a drastic contraction, which can be appointed to the presence of Collagen I and the interaction of cell-matrix mediated by integrin binding sites (Kelyack et al., 2000; Zhang et al., 2019; ZHU et al., 2001). The dynamics of mechanotransduction (Humphrey et al., 2014; Martino et al., 2018) in progenitor cells encapsulated in live ECM but not in BME2 matrix gels might have played a role in the differentiation of the progenitor liver cells towards a hepatic phenotype.

It is clear that the matrix composition is an essential parameter which is partially responsible for directing progenitor liver cell differentiation. This claim is supported by literature and many studies where it has been found that laminin, collagen III, IV and hyaluronic acid, present in the liver ECM matrix, help to maintain progenitor liver cells in an undifferentiated state and promoting progenitor liver cells proliferation. Whereas collagen I and fibronectin, are associated with a more differentiated phenotype (Zhu et al., 2013). This was observed herein when progenitor liver cells were cultured in liver ECM derived hydrogels. Further analysis of the derived ECM matrices composition for each collagen components needs to be carried out to further support this claim. Additionally, the porosity and topography of ECM matrices could also have had an effect and further studies are required to support this.

The importance of reproducing the liver microenvironment cannot be underestimated. As shown in chapter 3, there was an attempt to encapsulate hepatocytes cells in the natural derived biomaterial, alginate. However, despite being biocompatible and high hydrophilic it lacked of integrins and cell-active domains, making alginate a non-adhesive biomaterial (Neves et al., 2020). For this reason, it was decided to chemically conjugate small molecules onto the alginate backbone and study their response. However, without the rationale as to why and how those molecules could influence cell behaviour, the extremely challenging and ambitious task to design an *in-vitro* liver model was unsuccessful.

6.2 Conclusion and Future work

The present study demonstrated the importance of choosing the right biomaterial to replicate the microenvironment that liver cells experience *in-vivo* and how to successfully fabricate a liver 3D *in-vitro* model. Alginate was found unsuitable due to the lack of RGDs domains necessary to maintain cellular functions. Chemically modified alginate was also unsuitable due to the complex synthetic steps required for the conjugation of small molecules and the toxic by-product formation after the synthesis.

The study demonstrated the feasibility of porcine liver ECM derived hydrogels from being used as a possible substitute for culturing and expanding the EpCAM⁺ sorted human liver progenitor organoids as the organoids' passage from the ECM matrix to the new ECM matrix was successful. Furthermore, we speculated these sorted progenitors to be a heterogeneous population with the capacity to differentiate (although limited) into hepatocytes-like and cholangiocyte-like cells. It was proven that specific proteins in the ECM matrix are responsible for directing progenitor liver cell differentiation. Liver ECM derived matrix is a more suitable biomaterial to use as a scaffold to differentiate progenitor liver cells towards a hepatocyte phenotype.

To fabricate a more accurate liver model, further improvements of the liver decellularisation process needs to occur. A more standardised protocol should be adopted with common enzymatic and detergents reagents, with washing time and steps standardised for liver tissue ECM production. This would solve the variability of the tissue and reduce batch to batch variations.

Vascularisation of the ECM derived tissue should also be considered, and improvement in the scaffold repopulation methods to guarantee a co-culture of both HPCs and non-parenchymal liver cells. Human liver tissue has been already decellularised successfully as a proof of concept. The next step would be to inject a co-population of sorted progenitor liver cells and non-parenchymal cells into human decellularised liver tissue. Furthermore, human liver ECM hydrogels could be fabricated, and human progenitor liver cells could be encapsulated in them and differentiate towards hepatocytes. This study would be interesting and could provide a direct comparison between the two different species of liver tissue.

Liver ECM tissue was successfully decellularized and characterised, progenitor liver cells were encapsulated in different concentration of ECM hydrogels and were partially differentiated towards both a hepatocyte and cholangiocyte phenotype.

This novel approach could be utilised to produce a 3D microenvironment that closely resembles the liver stem-cell niche to co-culture non-parenchymal liver cells and progenitor liver cells. Where the final aim would be to differentiate HPCs into functional hepatocyte-like cells producing an accurate human-like model which could be used to study liver diseases and for drug toxicity testing.

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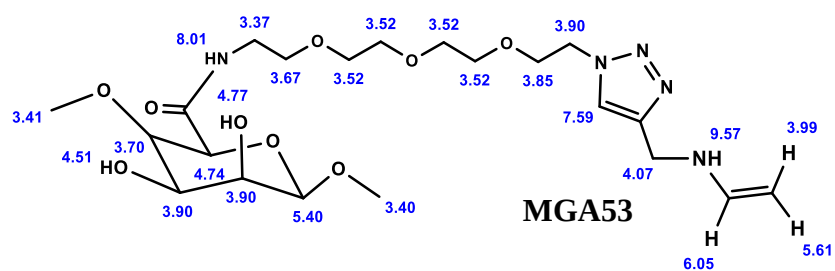
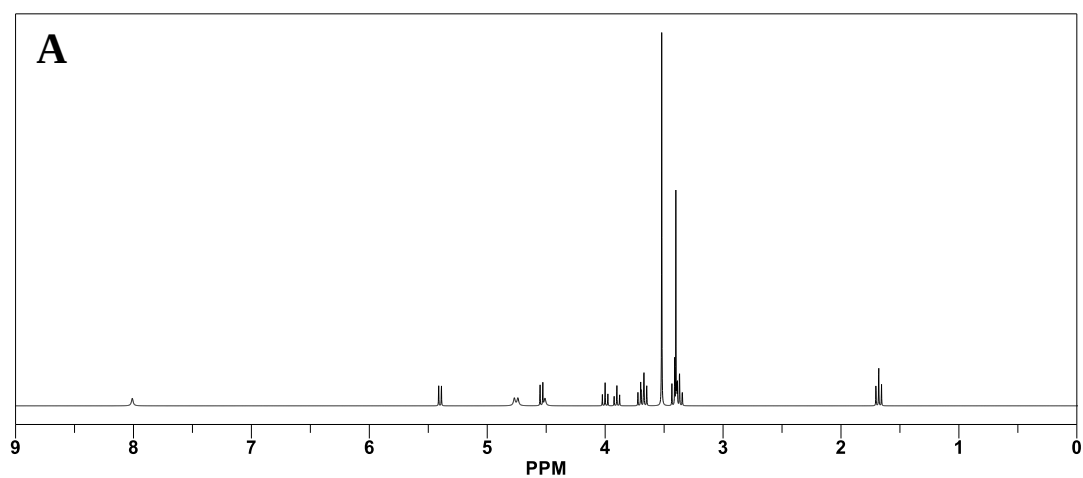
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Estimation quality is indicated by color: **good**, **medium**, **rough**



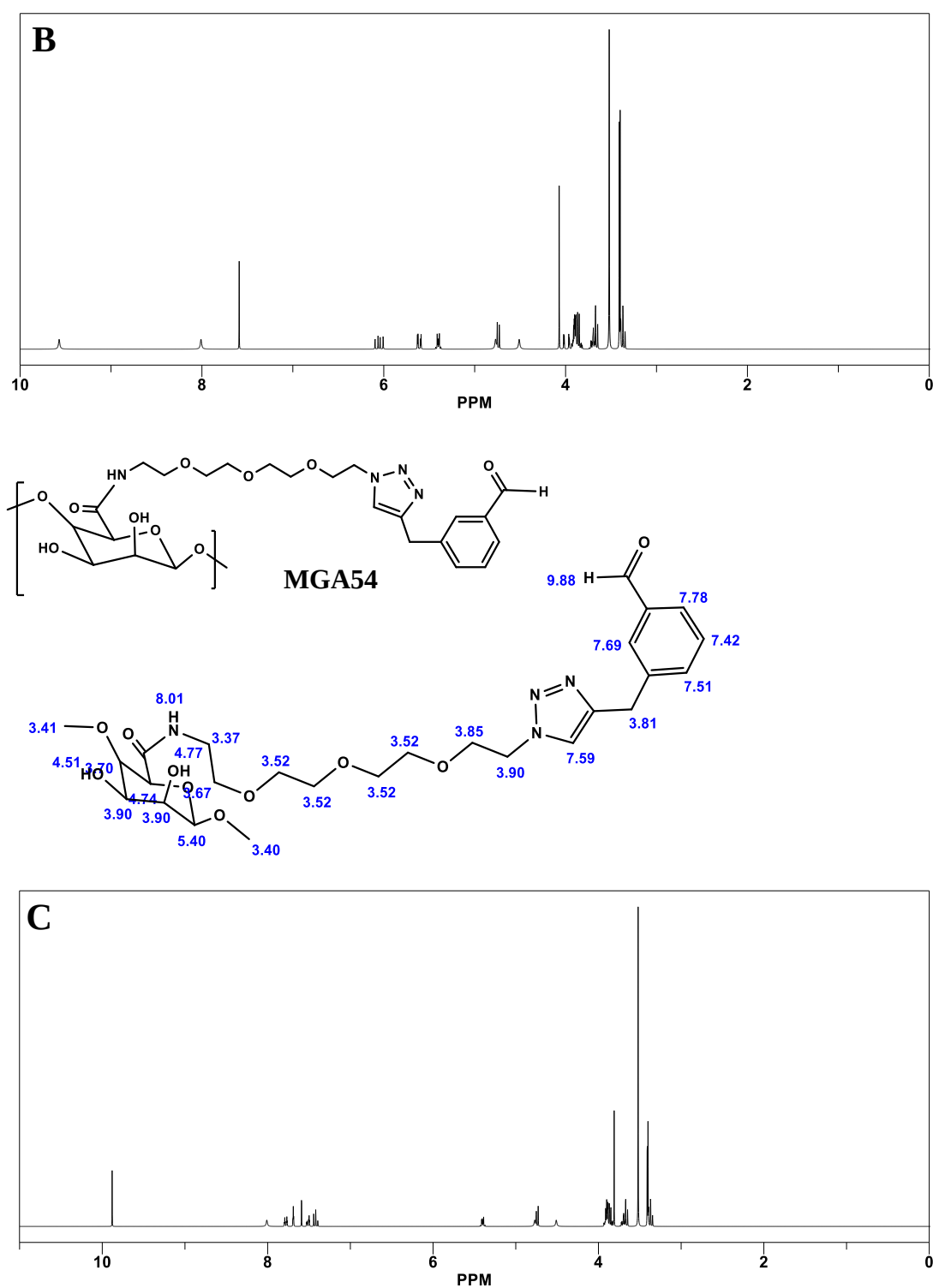
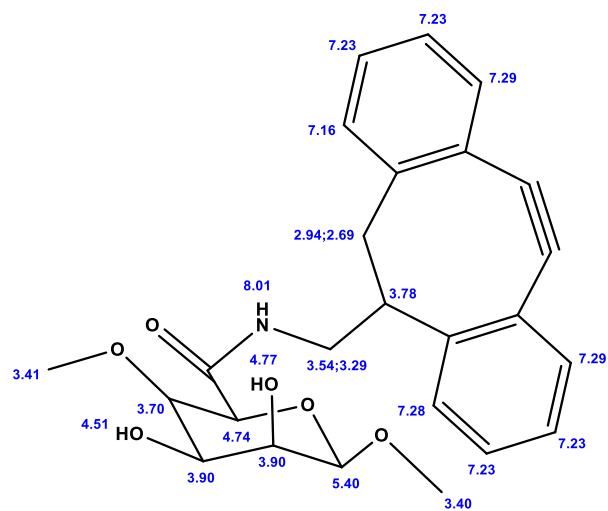


Figure AppA 3.1 Predicted HNMR spectra of chemically modified Alginates. (A) MGA42, (B) MGA53 and (C) MGA54.

ChemNMR ¹H Estimation



Estimation quality is indicated by color: **good**, **medium**, **rough**

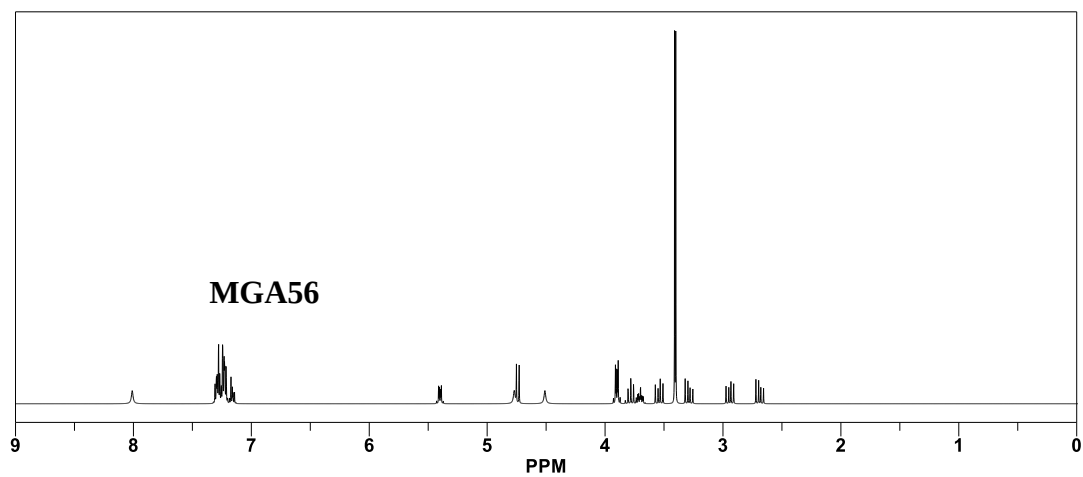
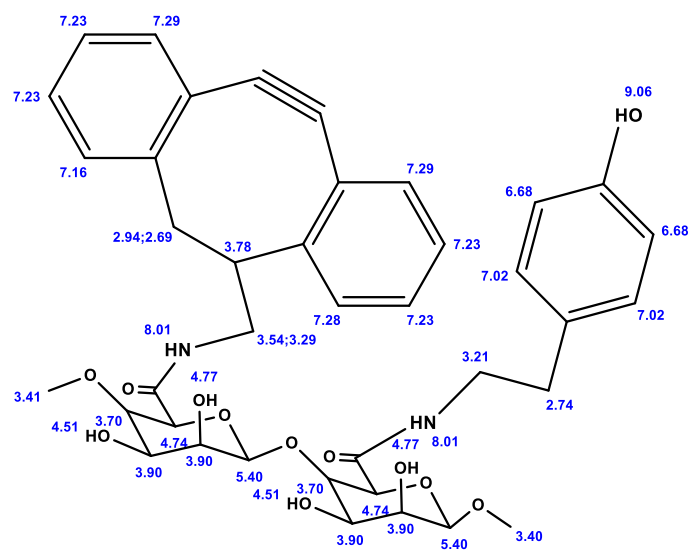


Figure AppA 3.2 Predicted HNMR spectra of chemically modified Alginate MGA56.

ChemNMR ¹H Estimation



Estimation quality is indicated by color: **good**, **medium**, **rough**

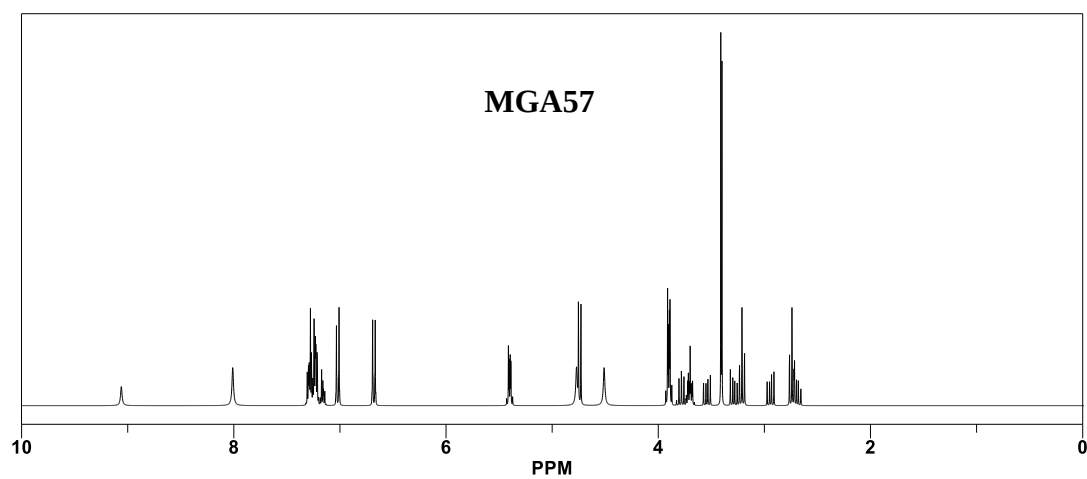


Figure AppA 3.3 Predicted HNMR spectra of chemically modified Alginate MGA57.

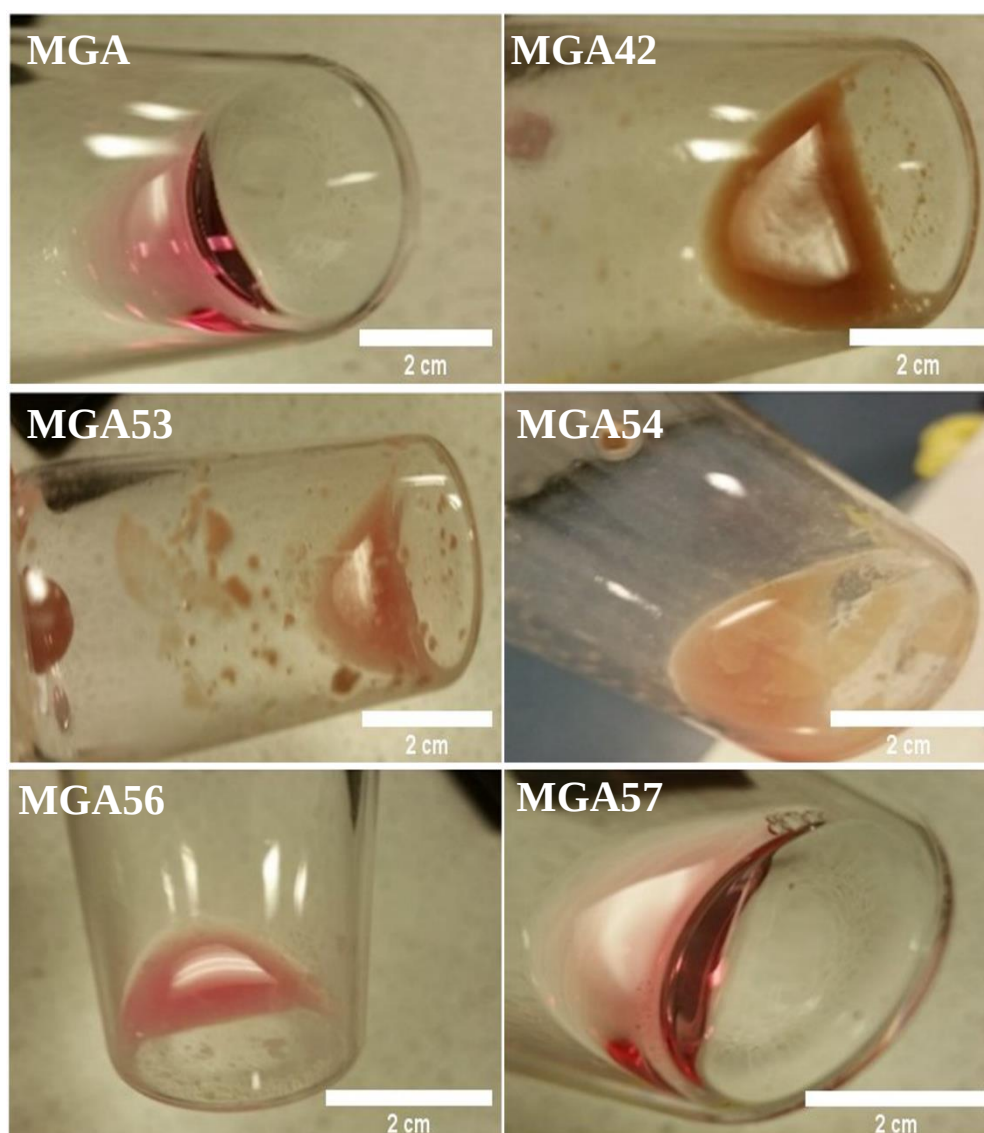


Figure AppA 3.4 Image capturing appearance of the pre-crosslinked modified Alginate before being mixed with cells and cast into a hydrogel form. Some modified alginates appear opaque and not transparent. Scale bar 2cm.

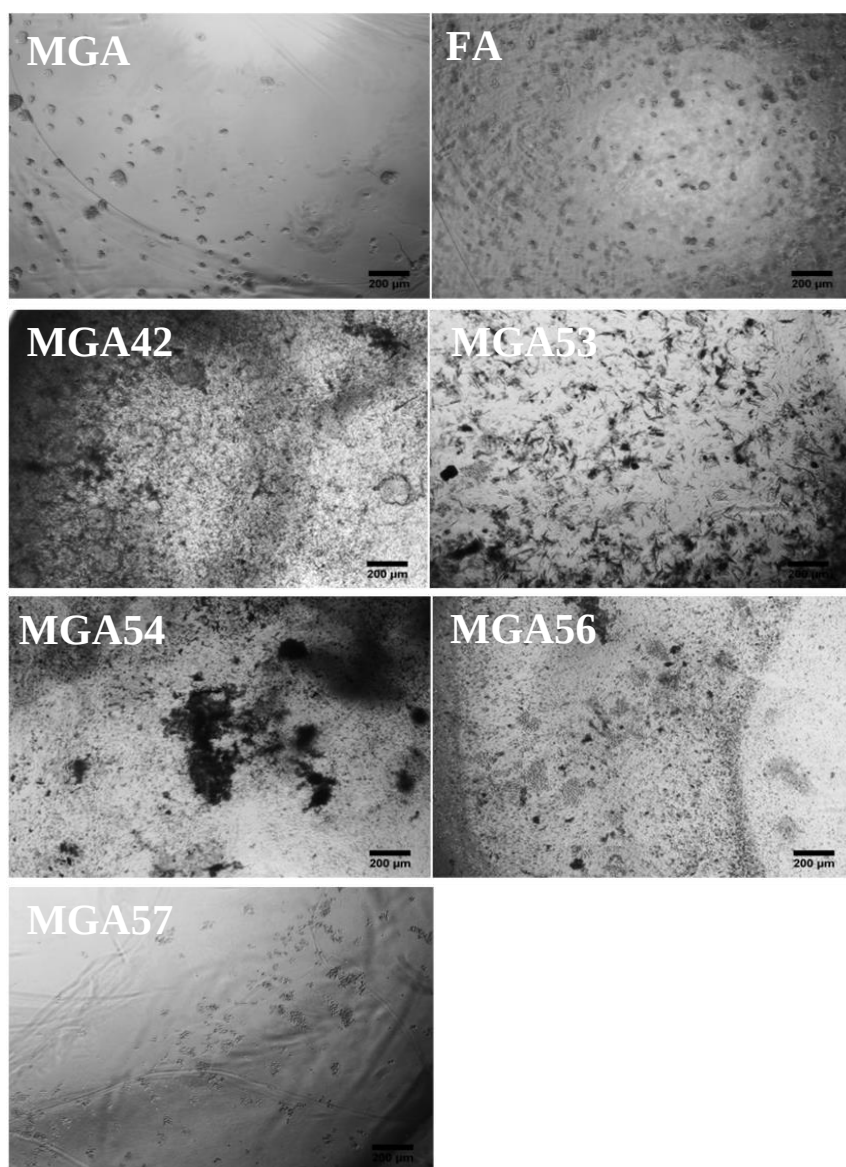


Figure AppA 3.5 Bright-field images of Chemically modified alginate and controls 72 hours post encapsulation. Scale bar 200µm.

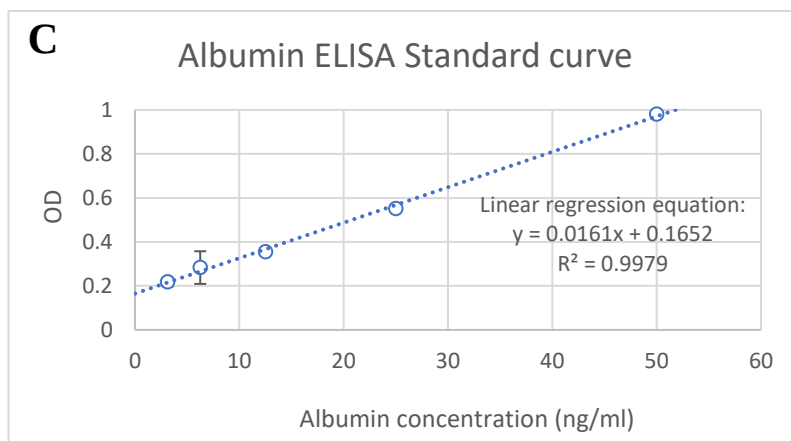
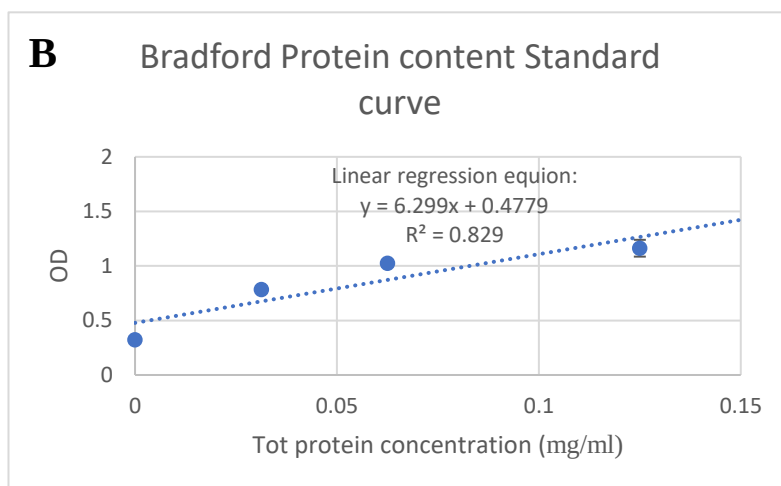
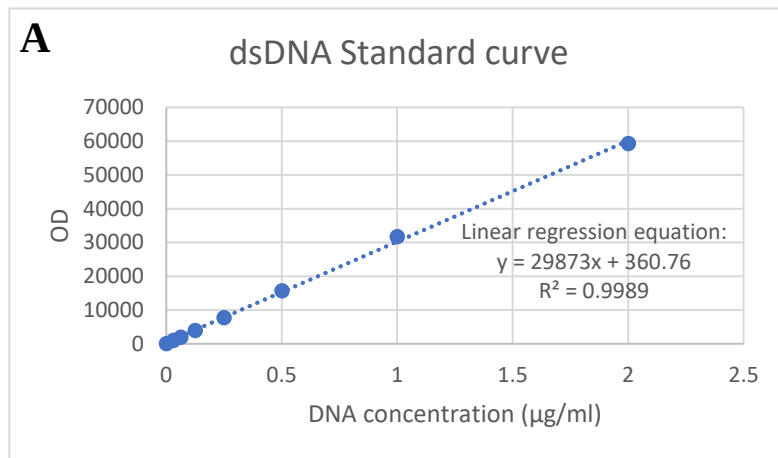


Figure AppA 3.6 Standards curve of different biological assay. (A) Picogreen standard curve, (B) Bradford Standard curve (C) Albumin ELISA standard curve

Appendices B

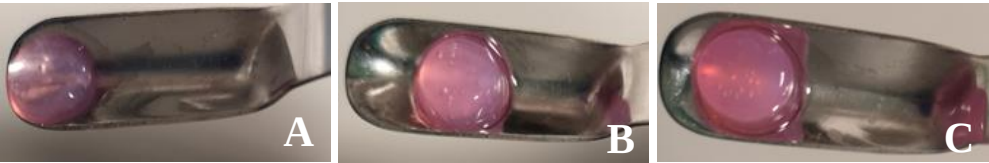


Figure AppB 4.1 (A) 2 mg/ml ECM Hydrogel ECM hydrogel, (B) 4 mg/ml ECM hydrogel and (C) 8 mg/ml ECM hydrogel ECM, 24 hours after media incubation at 37 °C.

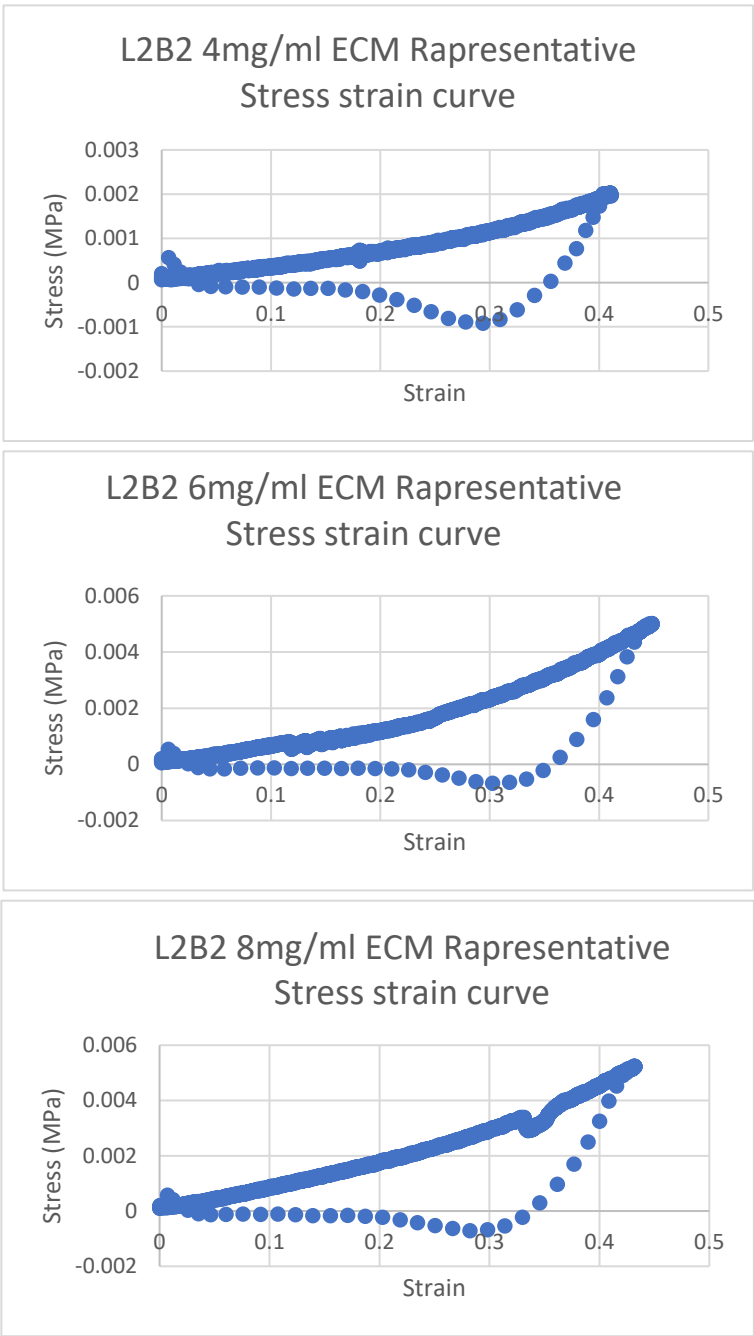


Figure AppB 4.2 Representative stress-strain curves of ECM hydrogels at a different concentration is displayed. The hydrogel is compressed at 50% strain, and the rupture/failing point can be seen before the 50% strain is applied.

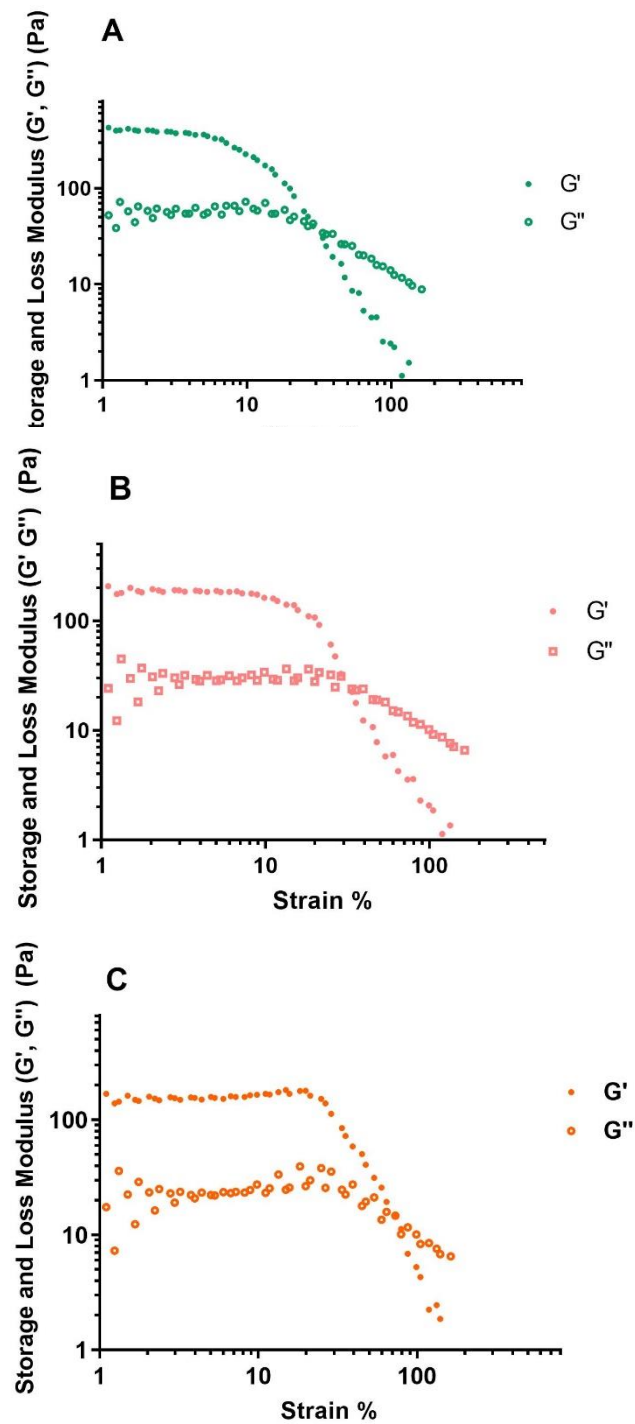
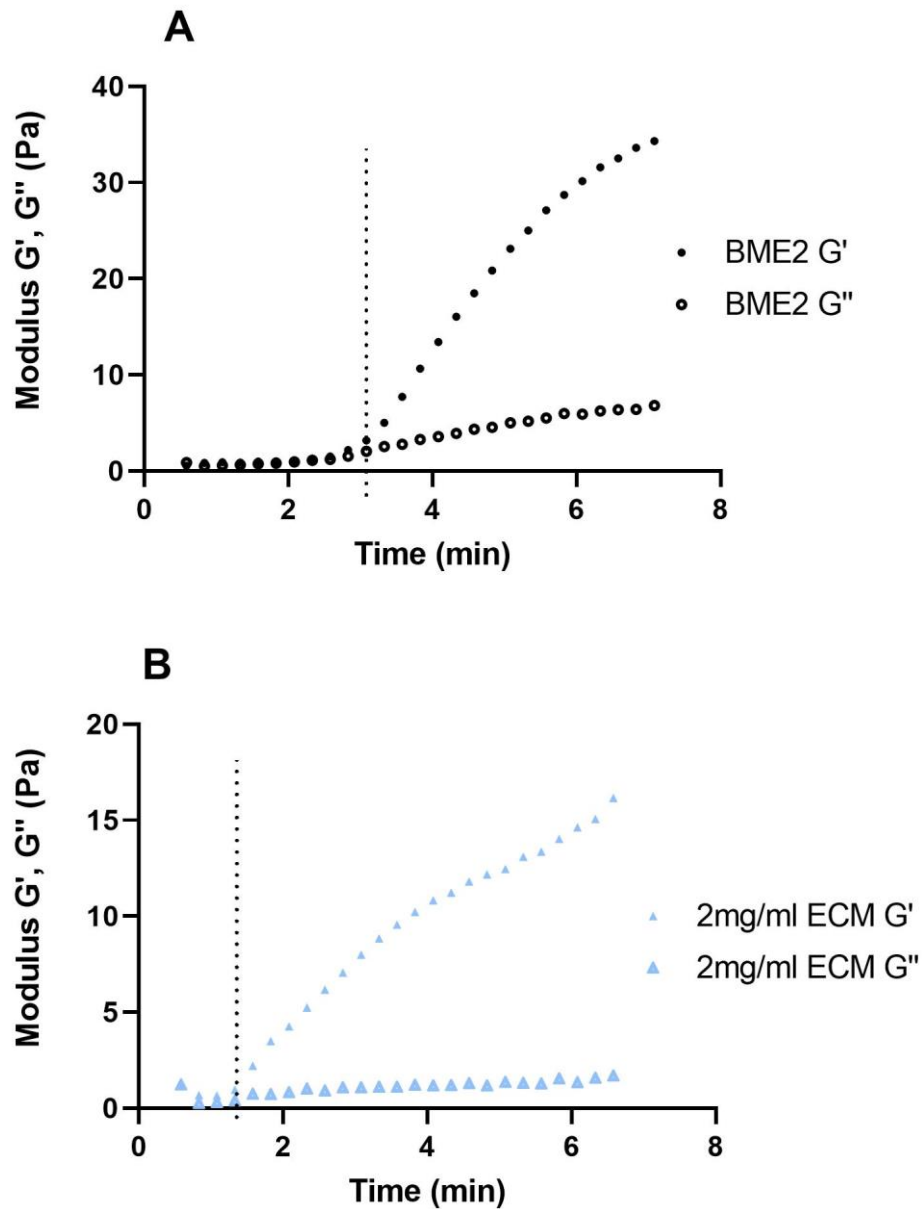


Figure AppB 4.3 (A-C) Strain sweep of ECM hydrogels already formed. They are kept in the chamber at 37 °C while carrying out the test. The crossover between G' and G'' is when the hydrogels move from a solid-like state (G' curve above G'' curve) to a liquid-like behaviour (G'' is now above the G'). The earlier in the strain test the interchange occurs, the more solid like the hydrogels behave.



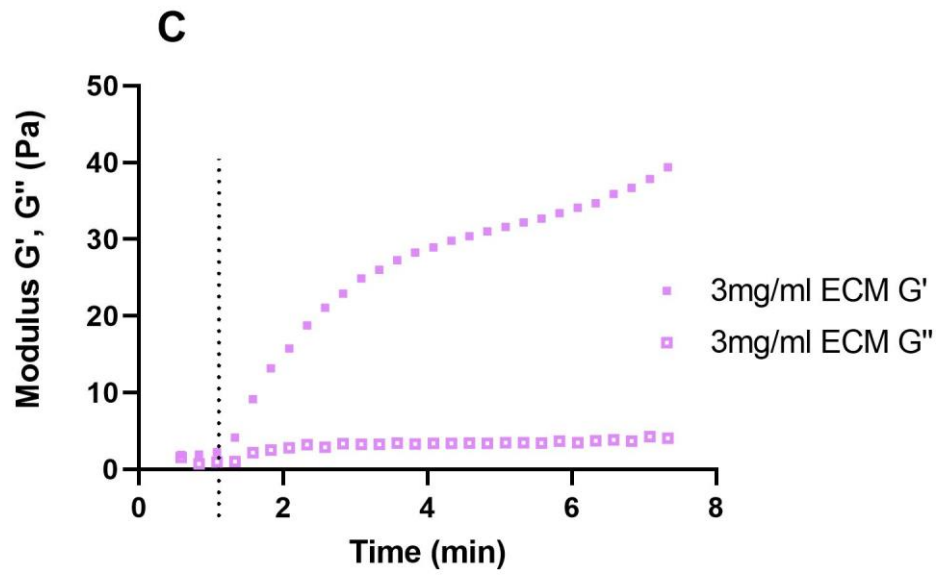


Figure AppB 4.4 Time sweep of ECM hydrogels and BME2 matrix. The dotted line represents the crossover point when from liquid-like, it starts crosslinking. The crossover point for BME2 is at 3.1 minutes, for 2mg/ml ECM and 3mg/ml ECM are 1.33 and 0.83 minutes, respectively.

Appendices C

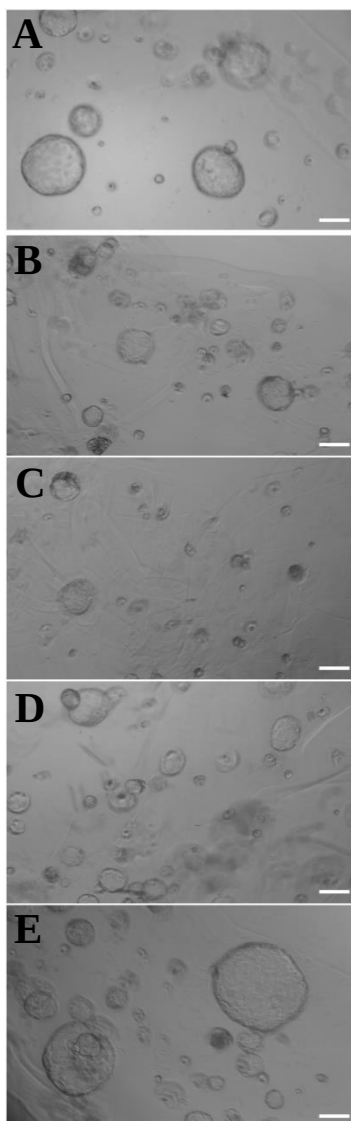


Figure AppC 5.1 Images of liver organoids encapsulated in BME2 and cultured for 7 days in expansion media. Five different donors as depicted. (A) L260, (B) L264, (C) L265, (D) L266 and (E) L269. Scale bar 200 μm .

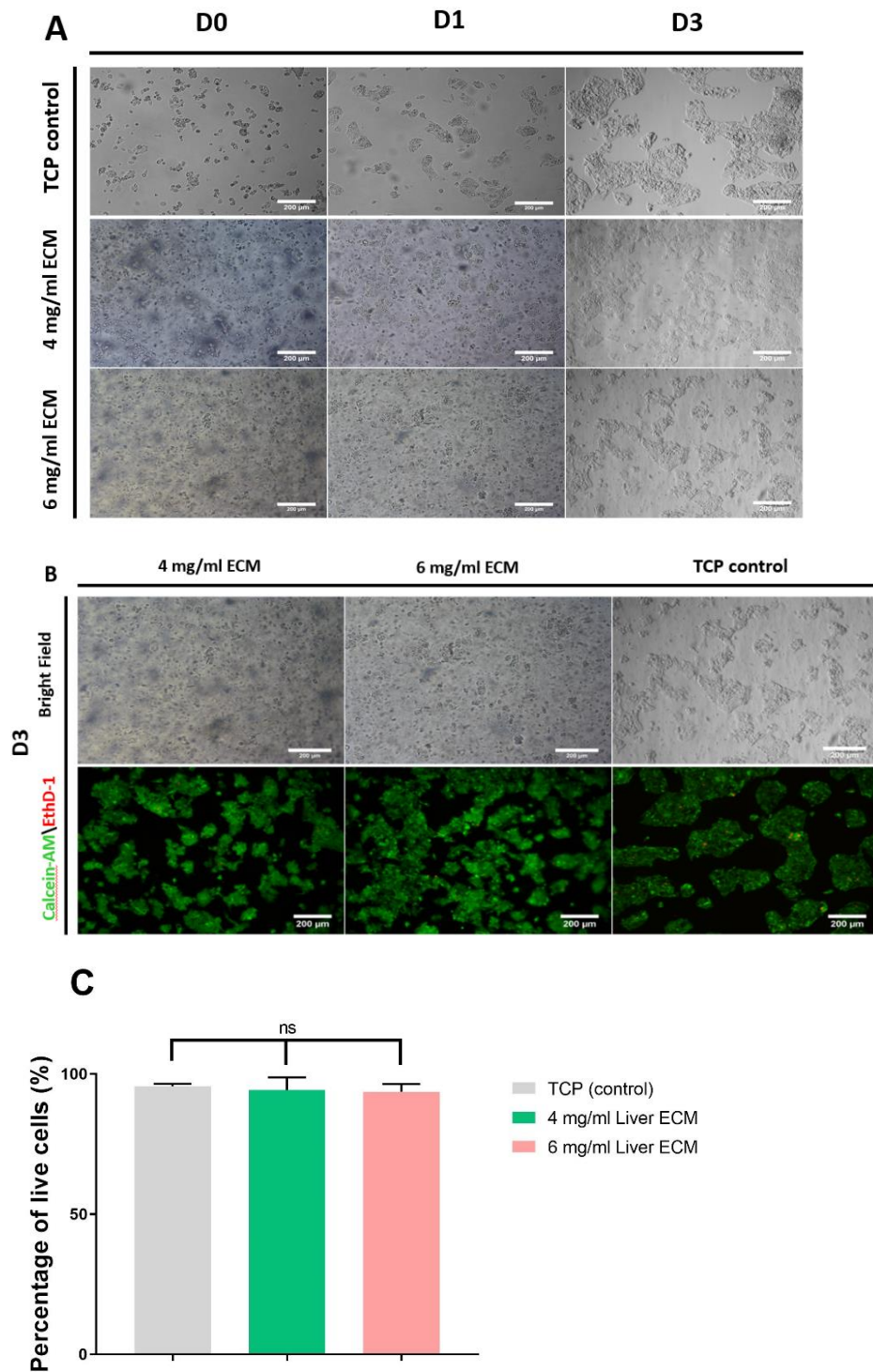


Figure AppC 5.2 (A) Bright-field images of HepG2 cultured on top of ECM hydrogels display and TCP over 3 days. (B) Bright-field (top) and Fluorescence (bottom) images of HepG2 at day 3 of culture. Live cells were stained with Calcein-AM (green), while dead cells with Ethidium homodimer (red). Scale bar 200 μ m. (C) The histograms plots represent the average percentage of live cells for each experimental condition as displayed. The error bars are calculated from n=3 technical replicates. Statistical significance was assessed using Ordinary One-Way Anova with Tukey multiple comparisons (ns: p=0.25).

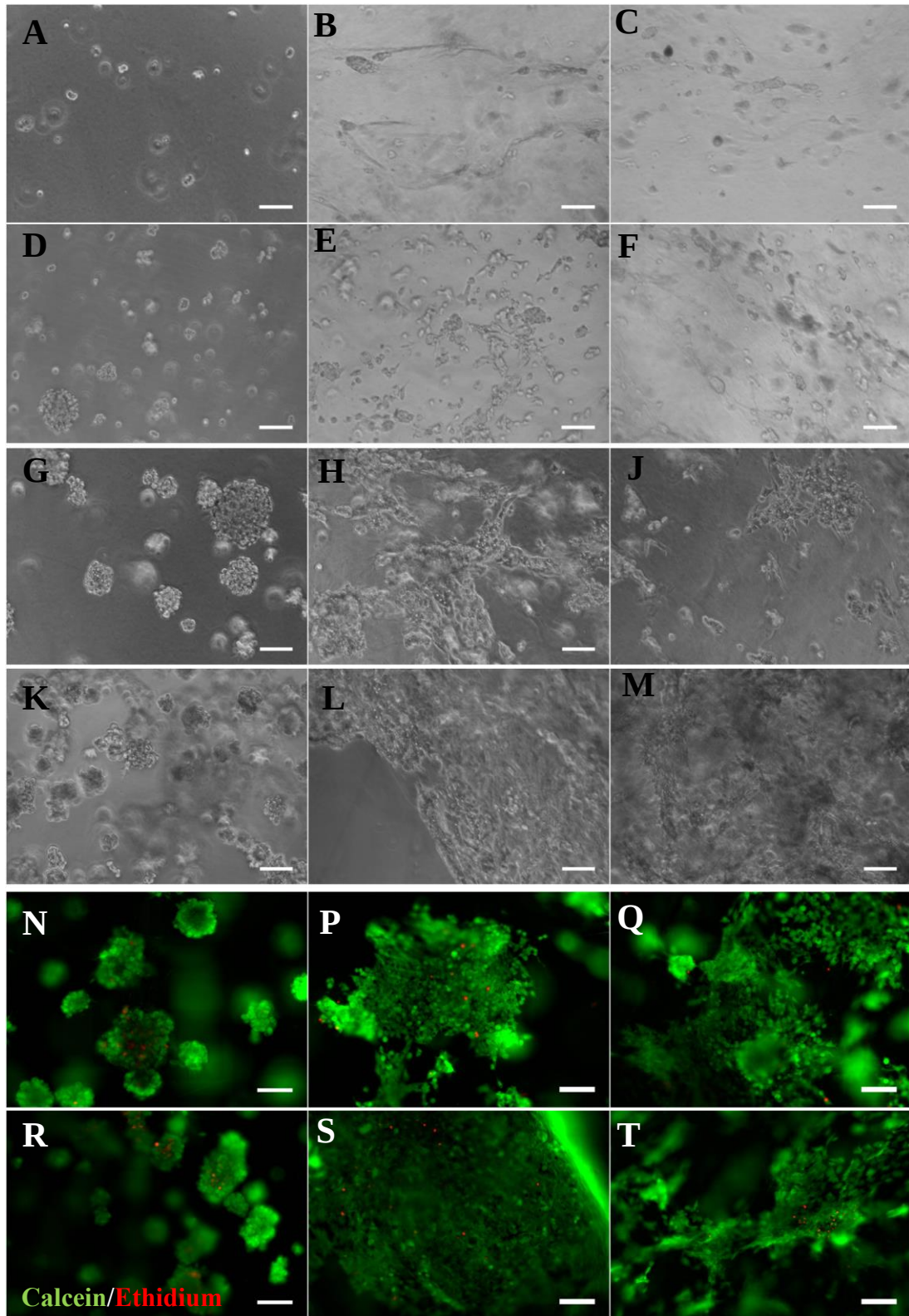


Figure AppC 5.3 Morphology and cell Viability assessment of HepG2. (A) HepG2 in BME2, (B) in 2mg/ml ECM (C) in 3mg/ml ECM at Day 1 and 5000 cells per gel. (D) HepG2 in BME2, (E) in 2mg/ml ECM (F) in 3mg/ml ECM at Day 1 and 25000 cells per gel. (G) HepG2 in BME2, (H) in 2mg/ml ECM (J) in 3mg/ml ECM at Day 5 and 5000 cells per gel. (K) HepG2 in BME2, (L) in 2mg/ml ECM (M) in 3mg/ml ECM at Day 5 and 25000 cells per gel. Live cells were stained at Day 7 with Calcein-AM (green), while dead cells with Ethidium homodimer (red). (N) HepG2 in BME2, (P) in 2mg/ml ECM (Q) in 3mg/ml ECM at 5000 cells and (R) HepG2 in BME2, (S) in 2mg/ml ECM (T) in 3mg/ml ECM at 25000 cells per gel. Scale bar 100 μ m.

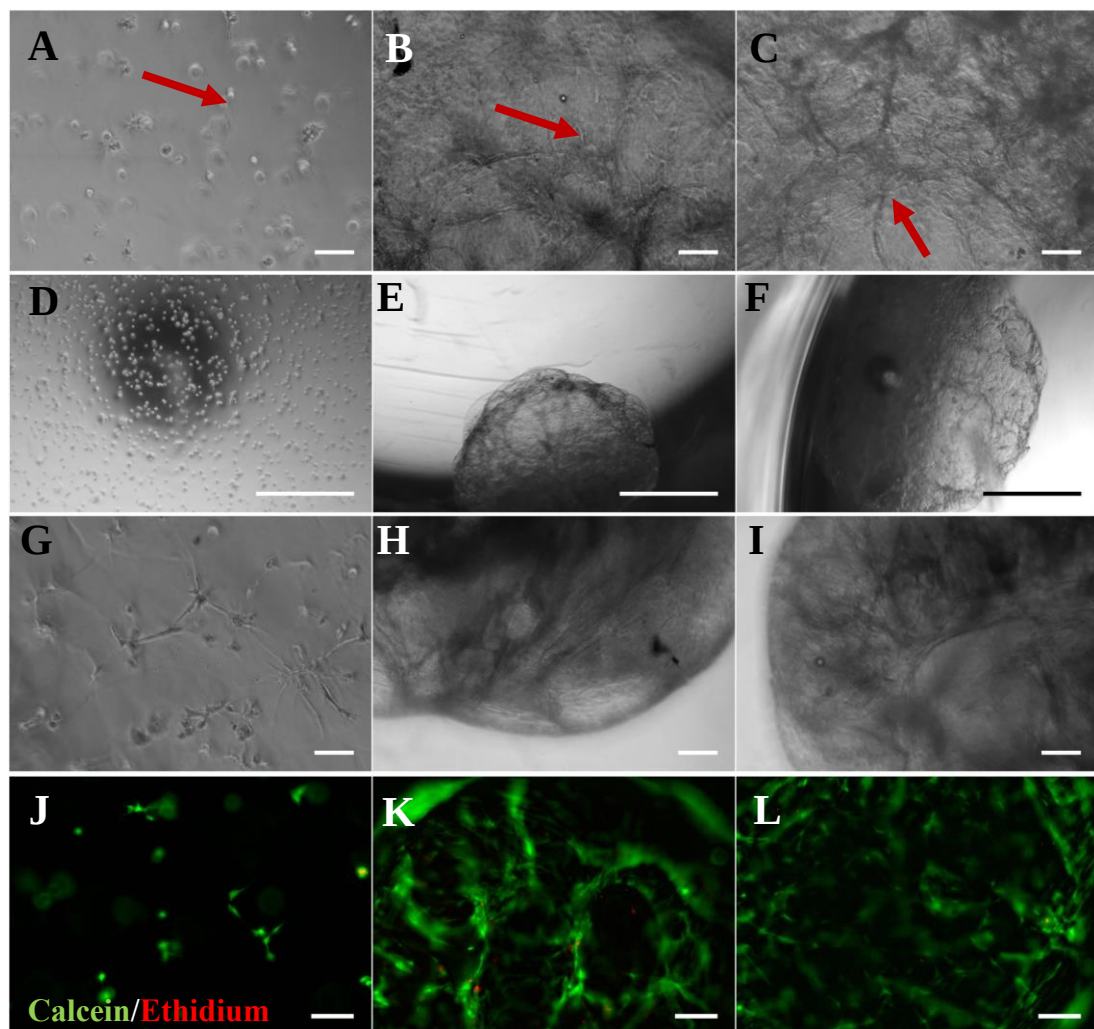


Figure AppC 5.4 Morphology and cell Viability assessment of HS137 (human skin fibroblast). (A) HS137 in BME2, (B) in 2mg/ml ECM (C) in 3mg/ml ECM at Day 1 and 10x magnification. (D) HepG2 in BME2, (E) in 2mg/ml ECM (F) in 3mg/ml ECM at Day 1 and 2.5x magnification. (G) HepG2 in BME2, (H) in 2mg/ml ECM (I) in 3mg/ml ECM at Day 5 and 10x. Live dead staining of (J) HepG2 in BME2, (K) in 2mg/ml ECM (L) in 3mg/ml ECM at Day 7. The scale bar at 2.5x magnification is 1000 μ m while at 10x is 100 μ m.

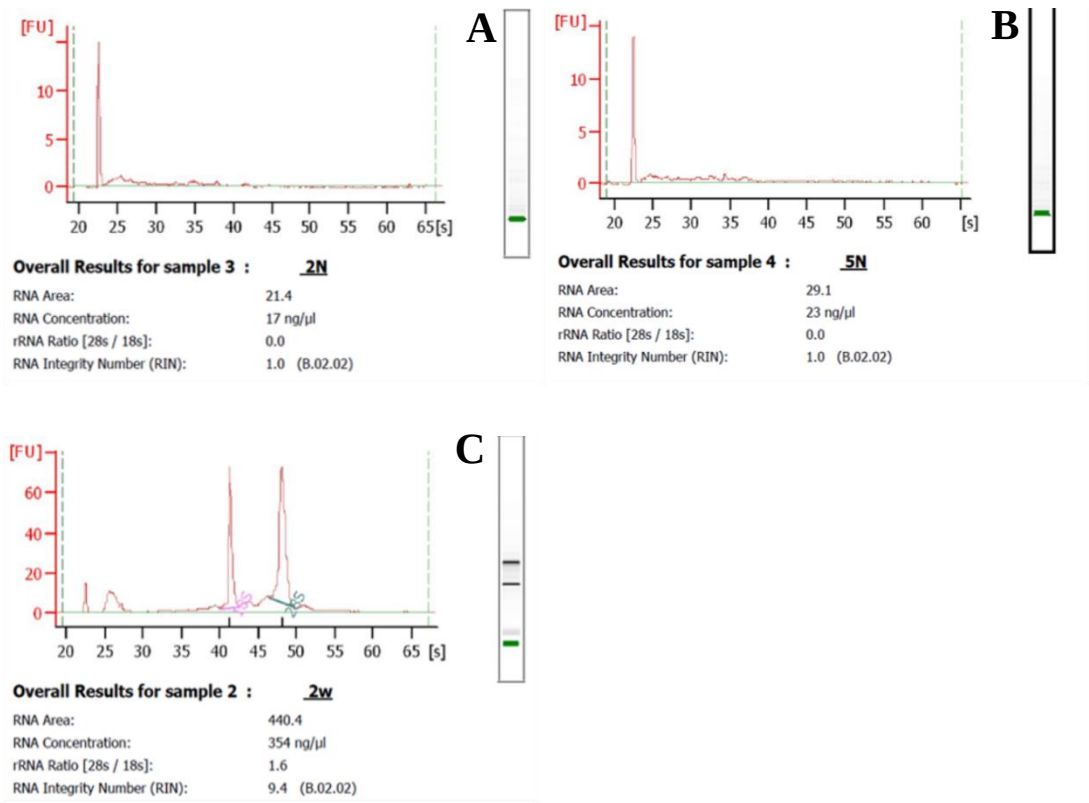


Figure AppC 5.5 (A) & (B) Electropherogram of RNA extracted from organoids embedded in 2 and 3 mg/ml ECM hydrogels, respectively. RNA was extracted using Method 1 as fully described in Method section 2.7.8 (C) Electropherogram of cells cultured in 2D as control.

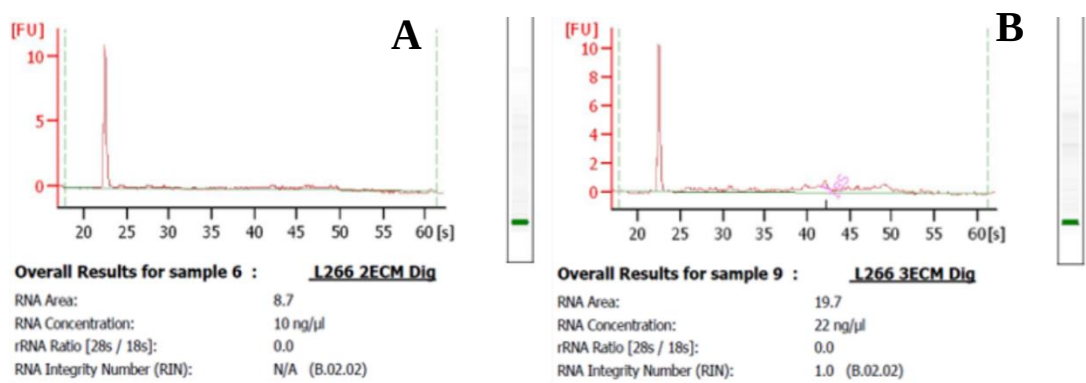


Figure AppC 5.6(A) & (B) Electropherogram of RNA extracted from organoids embedded in 2 and 3 mg/ml ECM hydrogels, respectively, using Method 2 as fully described in Method section 2.7.8.

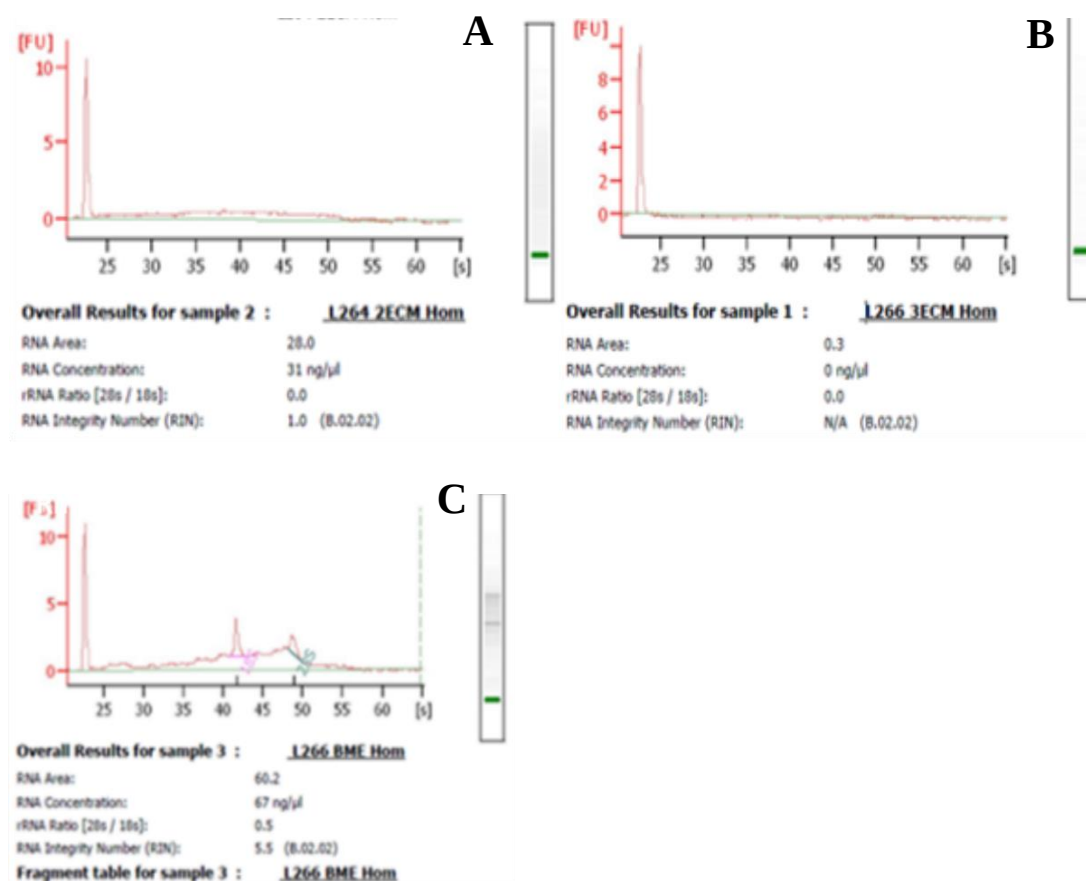


Figure AppC 5.7 (A) & (B) Electropherogram of RNA extracted from organoids embedded in 2 and 3 mg/ml ECM hydrogels, respectively. (C) Electropherogram of RNA extracted from organoids encapsulated in BME2 matrix extracted RNA was extracted utilising Method 3 as fully described in Method section 2.7.8.

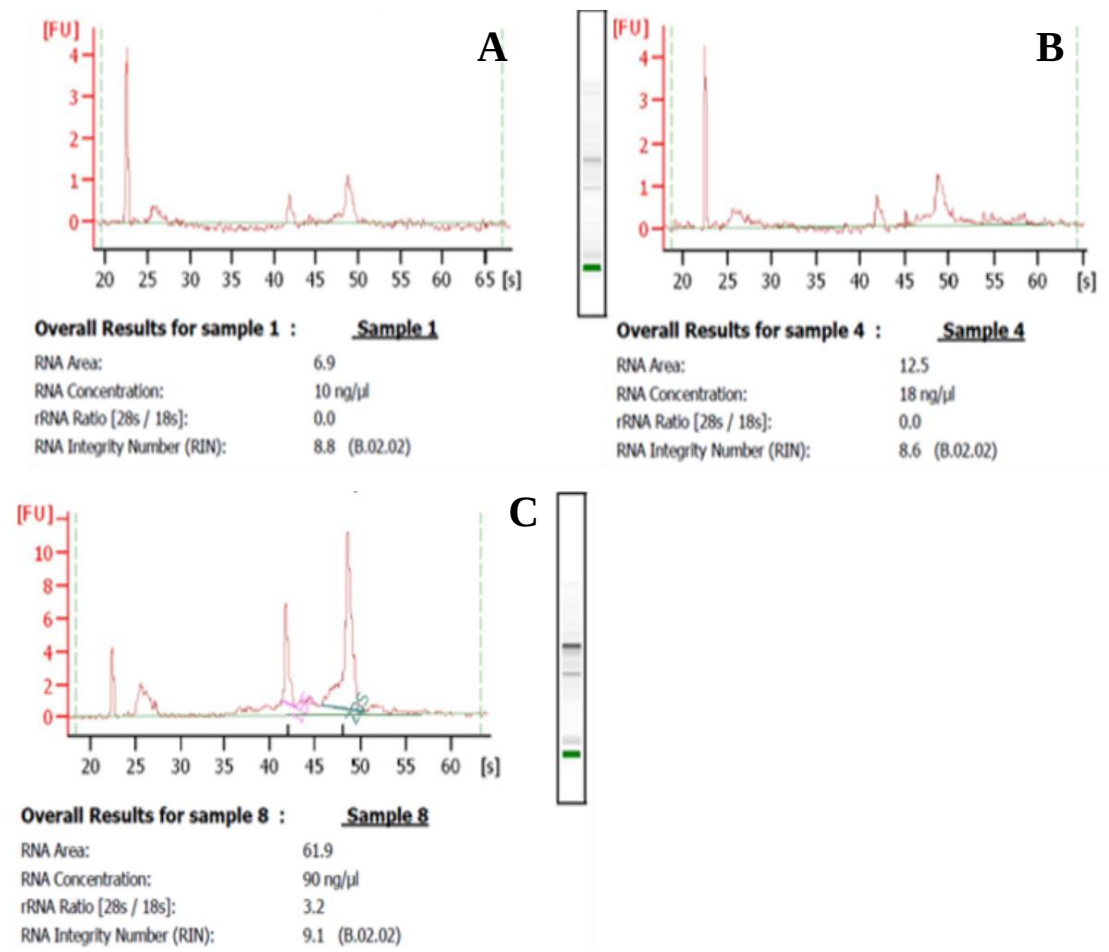


Figure AppC 5.8 (A) & (B) Electropherogram of RNA extracted from organoids embedded in 2 and 3 mg/ml ECM hydrogels, respectively. (C) Electropherogram of RNA extracted from organoids encapsulated in BME2 matrix. RNA was extracted utilising Method 4 3 as fully described in Method section 2.7.8.

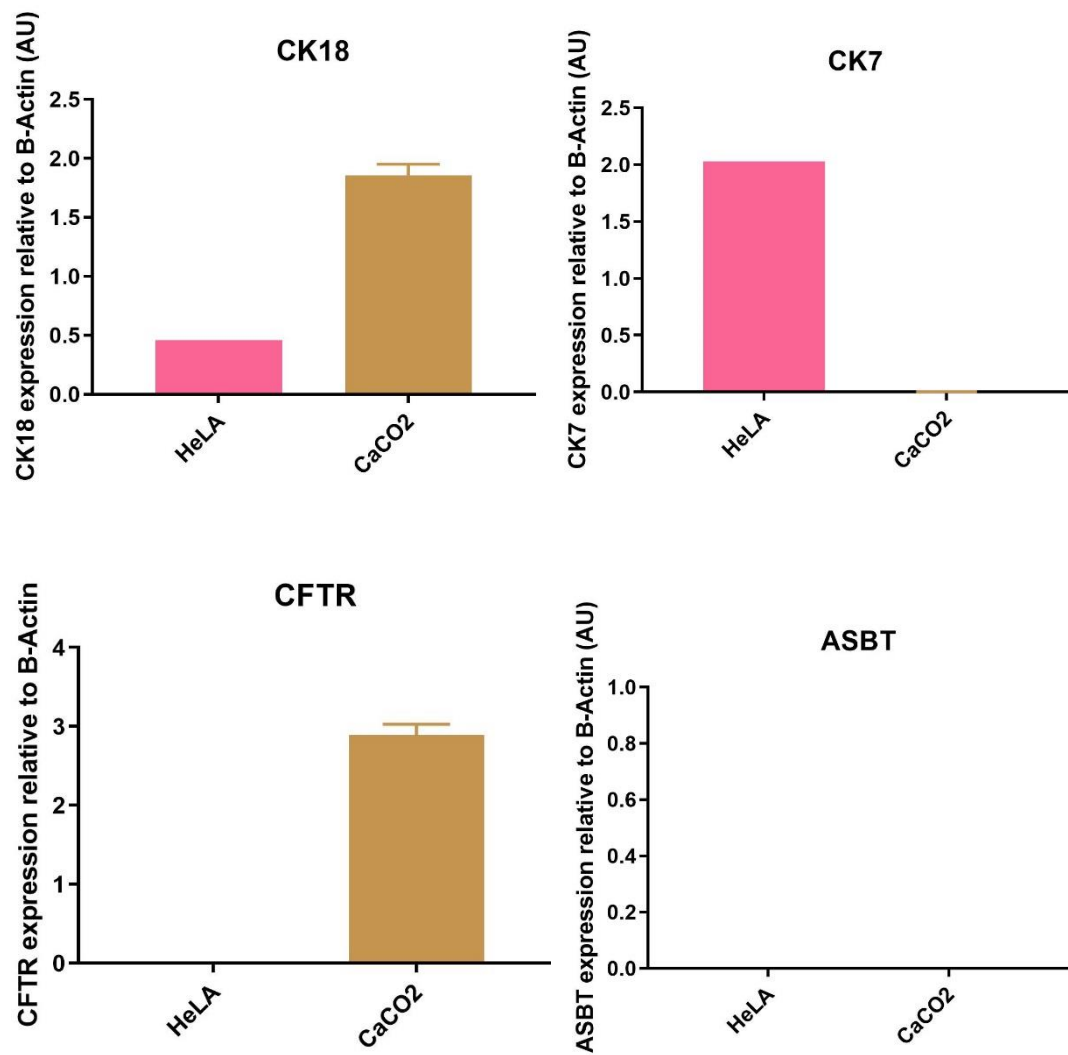


Figure AppC 5.9 Gene expression of Cholangiocyte markers checked on CaCo2 and HeLa cell lines. Caco2 and HeLa cell lines were used to check the efficiency and specificity of the designed primers and probes for the depicted genes.

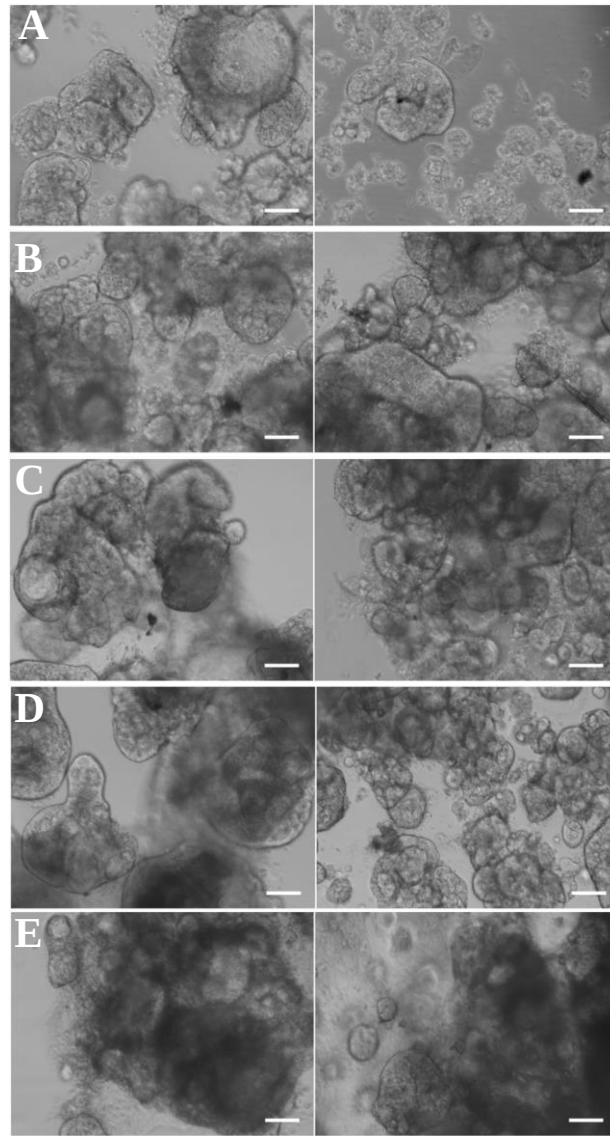


Figure AppC 5.10 Collagenase digestion of ECM hydrogels at the given types of collagenases. (A) Collagenase at a concentration of 0.25 U/mg, (B) Collagenase Type IV at a concentration of 160U/mg, (C) Collagenase islet isolation at a concentration of 125U/mg. (D) Recombinant Collagenase at a concentration of 50U/mg and (E) Dispase at a concentration of 1.8 U/mg. Scale bar 100μm.