



**University of
Nottingham**

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Engineering ECM-Inspired Hydrogel Scaffolds with Complex Geometries for Hepatic Tissue Models through Projection Micro-Stereolithography

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Ana Valeria Gonzalez Abrego

20404623

Supervised by

**Richard Hague
Yinfeng He
Lisa White
Ricky Wildman**

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Abstract

The liver’s extracellular matrix (ECM) exhibits a highly heterogeneous and hierarchical architecture that is essential for regulating hepatocyte organisation, mechanotransduction, and metabolic function. Reproducing these complex structural cues *in-vitro* remains a major challenge for hepatic tissue engineering, where most existing scaffolds rely on oversimplified geometries that limit functional maturation. This thesis integrates computational design, ECM analysis, and high-resolution biofabrication to investigate how ECM-inspired 3D architectures influence hepatocellular behaviour, with the overarching aim of establishing design principles for next-generation liver models.

Decellularised liver ECM was first characterised to extract quantitative architectural descriptors which were subsequently abstracted into four representative scaffold geometries: Stochastic (ECM-mimetic), Isotruss, Gyroid, and Cubic. To fabricate these designs with micron-scale fidelity, a hybrid GelMA–PEGDA hydrogel was developed and systematically optimised for projection micro-stereolithography (P μ SLA). The resulting bioresin enabled reproducible fabrication of highly resolved lattices with tunable stiffness and excellent cytocompatibility.

Using these scaffolds, HepG2 hepatocytes were cultured for 14 days to assess geometry-dependent differences in viability, organisation, and func-

tional maturation. The Stochastic scaffold, capturing the irregular, biomimetic features of native ECM, promoted rapid formation of multicellular aggregates, reduced HNF6 expression, and early upregulation of hepatic function. This was reflected in significantly enhanced urea synthesis and albumin secretion at days 3 and 7, along with sustained performance through day 14. In contrast, the periodic Isotruss and Gyroid geometries showed modest early activity but exhibited marked functional improvements by day 14, including strong albumin production and elevated CYP3A4 enzymatic activity. These findings suggest that ordered lattices support a slower but ultimately effective maturation trajectory as tissue consolidation progresses.

Genetic expression analysis indicates that the heterogeneous microenvironment of the Stochastic scaffold might reduce cytoskeletal tension and enhances cell–cell junction formation, enabling early activation of hepatic transcription factors such as HNF4 α and downstream metabolic pathways. Together, these results demonstrate that scaffold architecture influences not only the magnitude of hepatocyte function but also the temporal progression of maturation.

This work establishes a clear structure–function relationship linking ECM-inspired architectural cues, bioresin optimisation, and high-resolution P μ SLA fabrication to hepatocellular performance. The insights generated offer a rational foundation for designing biomimetic scaffolds that more faithfully recapitulate hepatic microenvironments, advancing the development of physiologically relevant liver models for drug screening, disease modelling, and regenerative medicine.

Declaration of Authorship

Unless otherwise specified in the respective captions, the candidate, Ana Valeria Gonzalez Abrego, is the sole author of all figures, tables, data, and associated graphics used in this thesis. Figures were created using the illustration software BioRender in accordance with the required licensing agreement. Any figures, tables, or data not generated by the candidate are appropriately referenced and acknowledged in their captions and in the List of References.

Contents

Acknowledgements	i
Abstract	iii
List of Tables	xiii
List of Figures	xiv
Abbreviations	xxvi
Chapter 1 Literature Review	1
1.1 Introduction to Liver Tissue Engineering	2
1.1.1 The Liver: The Body's Metabolic and Detoxification Core	2
1.1.2 The Challenge of Drug-Induced Liver Injury (DILI) .	3
1.2 Native Liver Architecture and ECM Biology	5
1.2.1 The Hepatic Lobule: The Structural and Functional Unit	5
1.2.2 Cellular Composition and Spatial Organization . . .	6
1.2.3 Liver Sinusoidal Endothelial Cells (LSECs)	7
1.2.4 The Perisinusoidal Space of Disse and the ECM . . .	9
1.3 Decellularised ECM architecture as a Seeding Platform . . .	10
1.3.1 Why is dECM the "gold standard"?	10
1.3.2 Evolution of Decellularization Protocols	11
1.3.3 The Limitations of dECM as a seeding platform . . .	12
1.4 Projection Micro-Stereolithography (P μ SLA) and Light-Based Hydrogels	14
1.4.1 Fundamentals of P μ SLA	15

1.4.2	Photocurable Hydrogels for Biofabrication	18
1.4.3	The GelMA-PEGDA Hybrid Hydrogel: a Comple- mentary System	19
1.5	Lattice Design Paradigms	21
1.5.1	Evolution of Scaffold Design: From Simple Pores to Engineered Lattices	21
1.5.2	Periodic Architectures: Order and Predictability . . .	22
1.5.3	Stochastic Architectures: Biomimetic Randomness . .	23
1.6	Interfacing Geometry and Biology in Tissue Engineering . .	25
1.6.1	Mechanotransduction: How Cells sense Their Envi- ronment	26
1.6.2	The Influence of Pore Size, Porosity, and Intercon- nectivity	27
1.6.3	The Influence of Curvature	28
1.7	Determination of Functional Hepatic Maturation	30
1.7.1	Assessing Synthetic Functions	30
1.7.2	Assessing Metabolic and Detoxification Function . . .	31
1.7.3	Assessing Structural and Polarity-Dependent Function	32
1.8	Current 3D Hepatic Culture Platforms	33
1.8.1	Scaffold-Free Systems: Spheroids and Organoids . . .	34
1.8.2	Perfused Systems: Microfluidic Organs-on-Chips De- vices	35
1.8.3	Scaffold-Based Systems: Additive Manufacturing for Tissue Engineering Lattices	36
1.8.4	3D Printing for Hepatic Tissue Models	40
1.9	Gaps in Current Work Addressed by This Thesis	46
1.9.1	The Biochemical Gap: The Need for Defined, Tune- able Native Recapitulation	46

1.9.2	The Architectural Gap: The Need for High-Resolution, Bio-inspired Geometry	48
1.9.3	The Integration Gap: The Synergy of Material and Manufacturing	49
1.10	Summary and Research Objectives	50
1.10.1	Summary of the State-of-the-Art and Knowledge Gaps	50
1.10.2	Thesis Aims and Hypothesis	51
Chapter 2	Methodology	54
2.1	Introduction	55
2.2	Hydrogel Formulation, Printing and Characterization	55
2.2.1	GelMA Synthesis	55
2.2.2	GelMA Degree of Methacrylation	57
2.2.3	GelMA Hydrogel Composition	58
2.2.4	GelMA-PEGDA Hydrogel Composition	58
2.2.5	Rheology and Tilt Test	59
2.2.6	Stereolithographic 3D Printing Optimization, Fidelity and Photopolymerization Kinetics	60
2.2.7	Fourier Transform Infrared (FTIR)	60
2.2.8	Cryo-Scanning Electron Microscopy (SEM)	62
2.2.9	Drying Rate, Swelling Test and Degradability of Hy- drogel	64
2.2.10	Compression Test	64
2.2.11	Cytotoxicity	65
2.2.12	Sterilization Optimization and Cell Attachment	66
2.3	Lattice Design and Optimization Based in Decellularised Liver ECM	68
2.3.1	Systematic Review with Quantitative Analysis and Characterization of dLECM	68

2.3.2	Scaffold Design	69
2.3.3	Lattice Printing	74
2.3.4	Lattice Analysis and Characterization	79
2.4	Performance of dLECM Based Lattice as Liver <i>in-vitro</i> Model	81
2.4.1	Seeding Optimization	81
2.4.2	Quantification of Double-Stranded DNA (dsDNA) . .	83
2.4.3	LIVE/DEAD	84
2.4.4	Lactate Dehydrogenase (LDH)	85
2.4.5	Albumin Quantification	85
2.4.6	Urea Quantification	86
2.4.7	CYP3A4 Quantification	87
2.4.8	Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)	88
2.4.9	Data Analysis and Statistical Analysis	89
Chapter 3	Development and Characterization of a GelMA- PEGDA Hydrogel for PμSLA-Based Liver Tissue Scaffolds	90
3.1	Introduction	91
3.2	Results and Discussion	93
3.2.1	Enabling Consistent and High-Degree Methacryla- tion of GelMA	93
3.2.2	Enhanced Room-Temperature Flow Behaviour of Fish GelMA for P μ SLA	96
3.2.3	Achieving Complex Bio-inspired architecture and Struc- tural Integrity through tuneable Hydrogel Stiffness .	98
3.2.4	Optimized Photopolymerization for Accurate Hydro- gel Scaffold Printing	100
3.2.5	Avoiding Cytotoxicity Through Post-Processing . . .	104

3.2.6	Cryo-SEM Optimization Enabled Accurate Visualization of Hydrogel Microstructure	106
3.2.7	Swelling Behaviour and Degradation Kinetics Enable Long-Term Tissue Support	110
3.2.8	Confirmed Biocompatibility of GelMA-PEGDA for Liver Tissue Engineering	115
3.2.9	Promoted Biocompatibility through Sterilization and Surface Conditioning protocol optimization	116
3.3	Conclusions	119
Chapter 4	Translating ECM Architecture into 3D Printed Lattices for Liver Tissue Engineering	122
4.1	Introduction	123
4.2	Results and Discussion	127
4.2.1	ECM Characterization of Morphometric features as a Inspiration for Scaffolds Parameters	127
4.2.2	Translating Native ECM Heterogeneity into Scalable Scaffold Parameters	133
4.2.3	Implicit Design of Periodic and Stochastic Scaffolds for dLECM-Mimetic Architectures	135
4.2.4	Scaffold Printability	138
4.2.5	High-Resolution Printing of designed Lattices with GelMA-PEGDA Through Intensity Tuning	142
4.2.6	Architectural Performance of Periodic and Stochastic Scaffolds	145
4.2.7	Geometric Replication of Designed Lattices in Hydrogel Constructs	148
4.3	Conclusions	151
Chapter 5	Biological Performance of dLECM-Inspired Lattices for Liver <i>in-vitro</i> Modelling	153

5.1	Introduction	154
5.2	Seeding HepG2 into dL-ECM like constructs	157
5.2.1	Seeding optimization	157
5.2.2	Temporal Dynamics of Cell Proliferation and Viability in Hepatic 3D Scaffold Cultures	163
5.3	Scaffold Microarchitecture Dictates Cellular Spatial Organisation and Viability	165
5.4	Liver gene expression	169
5.4.1	RT-qPCR	169
5.5	Functional Assays	176
5.5.1	Urea Synthesis Evaluation	176
5.5.2	Albumin Production Assessment	179
5.6	Drug Metabolism	182
5.6.1	CYP3A4 Activity Evaluation	182
5.7	Conclusions	184
Chapter 6	Conclusions and Future Work	187
6.1	Conclusions	188
6.2	Central Conclusion and Significance	189
6.3	Limitations and Future Work	190
	Bibliography	192

List of Tables

1.1	Summary of Key Studies on Scaffold Geometry and Cellular Function	29
1.2	Comparative Analysis of 3D Hepatic Culture Platforms . . .	38
1.3	Summary of Key Studies on 3D Printing of Hepatic Tissue Models	44
2.1	Composition of GelMA Hydrogel	58
2.2	Composition of GelMA-PEGDA Hydrogel.	59
3.1	Comparative table of Degree of methacrylation on fish GelMA between batches and synthesis methods	94
3.2	Liver tissue stiffness in literature	100
3.3	Summary table with hydrogel rates and percentage in relation with water	114
4.1	Data summary of image analysis and characterization of decellularised liver ECM	130
4.2	Quantitative assessment of printing fidelity across scaffold geometries based on measured pore sizes. The designed pore size was $\sim 70\mu\text{m}$ for all geometries. Fidelity was calculated as the percentage deviation from the target pore size, with Isotruss showing the highest agreement (99.57%) and Simple Cubic the lowest (97.57%).	150

List of Figures

1.1	Conventional Drug Development Pipeline. This schematic illustrates the four main stages of bringing a new therapeutic drug to market. The process begins with (1) Research and Development (3-6 years) to identify drug targets and lead compounds, followed by (2) Preclinical Studies (1 year) to assess safety and toxicity <i>in-vitro</i> and <i>in-vivo</i> . Promising candidates advance to (3) Clinical Trials (4-7 years) to evaluate efficacy and safety in humans across three phases. Finally, the drug undergoes (4) Review and Approval (1-2 years) by regulatory authorities. The entire pipeline is a lengthy and high-risk endeavour, with an estimated overall probability of success of only around 5% . Adapted from Sun et al. (2022).	4
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1.2	Hierarchical Architecture of the Liver. This schematic illustrates the multi-scale organisation of hepatic tissue, from the whole organ down to the molecular level. The liver is composed of repeating hexagonal functional units known as hepatic lobules, which are arranged around a central vein with portal triads at the vertices. These lobules consist primarily of hepatocytes, the main parenchymal cells, which are supported by non-parenchymal cells including hepatic stellate cells, Kupffer cells, and liver sinusoidal endothelial cells. All cells are embedded within a complex ECM containing structural and adhesion proteins such as collagens, elastin, fibronectin, and proteoglycans, which form the critical microenvironment for cellular function.	7
1.3	Schematic of the P μ SLA system. A UV beam is modulated through a dynamic digital mask and projected using a DMD via a lens into a photocurable resin bath. The beam delivery feedback ensures precise patterning, while the X-Y-Z stage enables layer-by-layer fabrication of complex 3D structures. .	16
1.4	Research aim and stages.	52
2.1	Schematic illustration of the two methods used for GelMA synthesis. Method A was adopted from Shirahama et al. (2016) while method B was adopted from Van Den Bulcke et al. (2000)	56
2.2	Mask projection used in P μ SLA compared to the actual print used to determine printing fidelity.	61
2.3	Cytotoxicity evaluation experimental plan detailing time points and assays proposed	66

2.4	Sterilization and post printing processing optimization protocol	67
2.5	Building blocks for Stochastic lattice construction in nTopology	73
2.6	Building blocks for periodic lattice construction in nTopology	74
2.7	Workflow for mesh post-processing prior to 3D printing. . . .	76
2.8	Screenshot of the BMF S130 3D printing system interface during hydrogel scaffold fabrication.	78
2.9	Seeding optimization protocol	82
3.1	NMR Spectra of GelMa and gelatine showing successful methacrylation of gelatine	95
3.2	Tilt test comparing flow-ability of GelMA prepared from fish-derived and pork-derived gelatin at 24°C. Only the fish GelMA demonstrated fluid behaviour under room-temperature conditions, indicating better suitability for stereolithographic hydrogel printing.	97
3.3	Hydrogel Stiffness graph showing Young Modulus of pure GelMA and GelMA-PEGDA hydrogel. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$) between samples. $N = 5$. . .	99
3.4	GelMA and GelMA-PEGDA Hydrogel Printing Optimization showing printing fidelity in % in the bottom left corner	101
3.5	Successful print of microscopic features using GelMA-PEGDA hydrogel. A) CAD design of gyroid lattice. Top (B), bottom (C) and side (D) view of the printed gyroid lattice. E) 20x of printed gyroid lattice showing pore and strut size. . . .	103
3.6	FTIR spectra of uncured resin (red), freshly print sample (blue) and post-processed printed sample (cyan)	105

3.7	Test hydrogel with different preparation techniques for Cryo-SEM. A-C) Prepared with slush nitrogen. D-F) Prepared with metal mirror freezing	108
3.8	GelMA hydrogel Cryo-SEM using metal mirror freezing A)10k Magnification B)25K Magnification	109
3.9	GelMA-PEGDA hydrogel swelling ratio and polymeric matrix mechanics	111
3.10	GelMA-PEGDA hydrogel drying curve over time. Scale bar = 500 μm	111
3.11	Degradation of printed hydrogel samples over 14 days	112
3.12	GelMA-PEGDA hydrogel swelling measurement in xyz . . .	113
3.13	Cytotoxicity test for GelMA-PEGDA Hydrogel on L929 cells. A) Metabolic Activity of cells exposed to GelMA-PEGDA hydrogel extract compared to fresh cell media. B)LIVE/DEAD staining on cells exposed to GelMA-PEGDA hydrogel extract compared to fresh cell media for live control and IMS for dead control. Green stained cells represent live cells while red stained cells represent dead cells. 10X Scale bar= 100 μm . 20X Scale bar= 50 μm	115
3.14	LIVE/DEAD staining in HepG2 cells after exposing the hydrogel prints to different sterilization and pre-seeding protocols compared with tissue culture plastic (TCP). Green represents live cells while red represent dead cells	118

4.1	Scaffold unit cell geometries used for periodic lattice design. A) Simple Cubic lattice with 90° strut intersections (top and side views). B) Isotruss lattice with diagonal struts intersecting at 45° and 60° angles (top, side, and 45° views). C) Gyroid surface-based lattice showing smooth curvature and multi-angled surface intersections (top, side, and 45° views) .	126
4.2	Recompilation of dLECM images for parametric analysis . .	128
4.3	Example of dLECM Image analysis. A) Pore measurement and B) strut measurement of Yagi et al. (2013) using Image J measuring tool. Thin white lines were used to measure the pores and strut and to keep a log of the ones that were already measured	128
4.4	Example of dLECM image analysis of volume fraction measurement using threshold tool in Image J, Red area represents the ECM against the void space	129
4.5	Designs of periodic scaffolds generated from selected unit cells using nTopology A) Isotruss B) Simple Cubic and C) Gyroid. Scale bar = 1mm	136
4.6	Voronoi-based stochastic scaffolds with increasing pore size ranges: A) 70 μ m, B) 140 μ m, and C) 210 μ m. These models reflect variable openness and surface-to-volume ratios. Scale bar= 1mm	137

4.7	Representative top-view cross-sectional slices of the scaffold architectures. For stochastic scaffolds, slices are shown for pore sizes of 70, 140, and 210 μm . For the periodic architectures (Simple Cubic, Gyroid, and Isotruss), each pair of images corresponds to different slice depths within the same scaffold, illustrating how internal pore morphology varies throughout the structure. All geometries were cleaned and sliced prior to fabrication	139
4.8	Fabrication results of scaffold designs printed using BMF HTL resin.	140
4.9	Optimization of hydrogel scaffold printing using Isotruss geometry as a test structure. Scaffolds were printed at different light intensities. Scale bars: 500 μm	143
4.10	Hydrogel printing of all scaffold geometries using optimized exposure conditions. Low magnification(5X): Scale bar= 500 μm . High magnification (10X: Scale bar= 250 μm	144
4.11	Geometric characterization of CAD scaffolds.	146
4.12	Assessment of printing fidelity in GelMA-PEGDA stochastic scaffolds. A) Confocal maximum intensity projections from the top (0 μm), middle (250 μm), and bottom (500 μm) sections of the scaffold. B) Quantitative distribution of pore sizes by depth. C) Mean and standard deviation of pore sizes across scaffold height. Pore sizes decreased with depth, though all values remained within the designed stochastic range (140 $\pm$ 30 μm), indicating overall fidelity in geometry replication.	149

5.1	Functional assessment strategy for hepatic characterization. The diagram maps the primary liver functions of interest (Metabolism, Plasma Protein Production, Detoxification, and Drug Metabolism) to the specific quantitative assays performed to assess cellular performance.	155
5.2	HepG2 cells labelled with MitoTracker™ Green FM on day 1 in Isotruss and Stochastic scaffold after A) agitation seeding protocol with and without FBS afterwash and B) after pipette tip entrapment with and without FBS afterwash. Scale bar = 100 μ m.	159
5.3	HepG2 cells labelled with MitoTracker™ Green FM on day 1 and 3 in Isotruss and Stochastic scaffold after pipette tip entrapment with and without FBS afterwash. Scale bar = 100 μ m.	161
5.4	A) Schematic representation of the final seeding protocol. Scaffolds were prewetted with 100% FBS for 30 minutes to enhance surface protein availability, followed by a PBS rinse and static seeding of HepG2 cells in an enclosed volume. Scaffolds were incubated under static conditions for 12 hours prior to media exchange. B) Seeding efficiency assessed by dsDNA quantification after 24 hours. All scaffold types showed high seeding efficiency ($\geq 95\%$) with no statistically significant differences between geometries (one-way ANOVA, $p \geq 0.05$), indicating protocol reproducibility and architecture-independent cell retention at day 1.	162

5.5	Quantification of cell number and metabolic activity across scaffold geometries and timepoints. (A) dsDNA quantification on days 3, 7, and 14 shows a geometry- and time-dependent increase in cell number, with Stochastic scaffolds supporting the highest levels by day 14. (B) LDH assay reflects non-significant changes in metabolic activity, showing cell viability greater than 98% in all geometries during the 14 days of culture. Data are presented as mean $\pm$ standard error of the mean ($N = 3$; $n = 3$). Statistical analysis by one-way ANOVA indicated significant effects scaffold geometry (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)	164
5.6	Live Dead microscopy Images of HepG2 cells in different geometry scaffolds	166
5.7	Schematic of cell distribution in different scaffold geometries. Cells formed dense aggregates in Stochastic scaffolds, aligned along linear struts in Cubic scaffolds, concentrated around vertices in Isotruss scaffolds, and stayed confined in void spaces in Gyroid scaffolds	167
5.8	HNF6 expression as an indicator of hepatic maturity. A) A schematic illustrating the principle that HNF6 is a marker of cellular immaturity; its expression is high in immature cells and low in mature cells. B) Fold change of HNF6 to TBP as housekeeping gene and normalized to TCP expression of HepG2 cells in different geometry scaffolds throughout 14 days. Data are presented as mean $\pm$ SEM (*: $p < 0.05$). . . .	170

5.9	Intercellular Junctions in Hepatocyte Aggregates. This schematic illustrates the specialized cell-cell junctions that is hypothesized to re-established when hepatocytes self-aggregate in a 3D culture, especially in the Stochastic scaffold. These connections might include adherens junctions, which link the cytoskeletons to provide mechanical strength; tight junctions (composed of proteins like claudin and occludin), which form a seal to create polarized structures such as bile canaliculi; and gap junctions, which form channels for direct intercellular communication. The formation of this complex junctional architecture could be a hallmark of a mature, functional hepatic phenotype, enabling the aggregate to behave as a coordinated tissue.	172
5.10	G6PC1 expression as an indicator of hepatic maturity. A) A schematic illustrating the principle that G6PC1 expression is positively correlated with cellular maturity. B) Fold change of G6PC1 to TBP as housekeeping gene and normalized to TCP expression of HepG2 cells in different geometry scaffolds throughout 14 days. Data are presented as mean $\pm$ SEM. One-way ANOVA showed no significant differences between groups	175

- 5.11 Comparative analysis of Urea synthesis on HepG2 cells cultured on different scaffold geometries versus TCP assessed at multiple time points. Tests were normalized to cell count with dsDNA content and measured at days 3, 7 and 14. All 3D geometries were significantly different to TCP ($p < 0.0001$) at all time points. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, *****: $p < 0.00001$) between samples. N=3, n=3. 177
- 5.12 Albumin secretion by HepG2 cells cultured in 3D scaffolds of varying geometry over 14 days. Albumin levels were quantified at days 3, 7, and 14 using ELISA from culture supernatants. All 3D scaffolds showed increased albumin production over time, with Stochastic scaffolds exhibiting significant differences to all geometries at day 3 and 7. Although at day 14, hepatocytes in Isotrusss seemly produce more urea than any other in day 4, only the Stochastic scaffolds presented a significant difference compared to Cubic at day 14. All 3D geometries were significantly different to TCP ($p < 0.0001$) at all time points. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, *****: $p < 0.00001$) between samples. N=3, n=3. 180
- 5.13 Assessment of CYP3A4 enzymatic activity in HepG2 cells cultured on different scaffold geometries. Data presented relative to tissue culture plastic (TCP). Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$) between samples. N=3, n=3. 183

Abbreviations

KH₂PO₄ monopotassium phosphate.

Na₂HPO₄ disodium phosphate.

2D two-dimensional.

2PP 2-photon printing.

3D three-dimensional.

ALF acute liver failure.

AM additive manufacturing.

ANOVA analysis of variance.

BMF Boston Micro Fabrication.

CAD computer-aided design.

cDNA complementary DNA.

CYP cytochrome P450.

dECM Decellularised ECM.

dH₂O distilled water.

DILI drug-induced liver injury.

dLECM decellularised liver ECM.

DM degree of methacrylation.

DMD Digital Micromirror Device.

DMEM Dulbecco's Modified Eagle Medium.

DMSO Dimethyl sulfoxide.

DNA deoxyribonucleic acid.

dsDNA Double-Stranded DNA.

ECM extracellular matrix.

ELISAs Enzyme-linked immunosorbent assays.

FBS Fetal Bovine Serum.

FTIR Fourier Transform Infrared.

G6PC1 Glucose-6-Phosphatase Catalytic Subunit 1.

GelMA Gelatin Methacrylate.

GelNB gelatin-norbornene.

HdL-ECM human decellularised liver matrix.

hiPSCs induced pluripotent stem cells.

hrs hours.

HSCs Hepatic Stellate Cells.

HTS high-throughput screening.

IMS Industrial Methylated Spirits.

KCs Kupffer Cells.

LAP Lithium phenyl- 2,4,6-trimethylbenzoylphosphinate.

LDH Lactate Dehydrogenase.

LSECs Liver Sinusoidal Endothelial Cells.

MM Metal Mirror.

MMA methacrylic anhydride.

MMPs matrix metalloproteinases.

MSCs mesenchymal stem cells.

NaOH sodium hydroxide.

NMR Proton nuclear magnetic resonance.

NPCs non-parenchymal cells.

P μ SLA Projection Micro-Stereolithography.

PBS Phosphate-Buffered Saline.

PEGDA Poly(ethylene glycol) Diacrylate.

Pen-Step Penicillin-Streptomycin.

PHHs human hepatocytes.

PNG Portable Network Graphics.

RGD Arginine-Glycine-Aspartic acid.

RGD arginylglycylaspartic acid.

RNA Ribonucleic acid.

RT-qPCR Reverse Transcription Quantitative Polymerase Chain Reaction.

SD standard deviation.

SDS sodium dodecyl sulphate.

TAZ PDZ-binding motif.

TBP TATA-box binding protein.

TBP TATA-binding protein.

TCP tissue culture plastic.

TMB 3,3',5,5'-Tetramethylbenzidine.

TPMS Triply Periodic Minimal Surfaces.

YAP Yes-associated protein.

Chapter 1

Literature Review

1.1 Introduction to Liver Tissue Engineering

1.1.1 The Liver: The Body's Metabolic and Detoxification Core

The liver stands as the largest internal organ and a central nexus of metabolic activity, indispensable for maintaining physiological homeostasis in the human body. Its functions are vast and complex, encompassing the metabolism of proteins, carbohydrates, and lipids; the synthesis of critical plasma proteins such as albumin and clotting factors; the production and excretion of bile for digestion and urea for detoxification; and the storage of vital biomolecules like glycogen (Heydari et al., 2020; Kalra et al., 2023). Perhaps its most pharmacologically significant role is that of the body's primary filtration and detoxification system. The liver is responsible for the biotransformation of a myriad of endogenous substances and exogenous compounds, or xenobiotics, including therapeutic drugs and environmental toxins (Buratti and Lavine, 2002). Hepatocytes, the main parenchymal cells of the liver, are equipped with a sophisticated enzymatic machinery, most notably the cytochrome P450 (CYP) family, which modifies these compounds, typically rendering them more water-soluble for excretion (Dash et al., 2009). This metabolic processing is a double-edged sword: it is essential for activating certain prodrugs and for clearing most pharmaceuticals from the body, but it can also generate reactive metabolic intermediates that are toxic to the liver itself. Consequently, the liver is not only the principal determinant of a drug's pharmacokinetic profile but also the primary target for drug-induced toxicity, placing it at the epicentre of pharmacological and toxicological research (Buratti and Lavine, 2002).

1.1.2 The Challenge of Drug-Induced Liver Injury (DILI)

The central role of the liver in drug metabolism makes it uniquely vulnerable to injury from medications, a phenomenon known as drug-induced liver injury (DILI). DILI represents a major clinical and industrial challenge, standing as the leading cause of acute liver failure (ALF) in many Western countries and accounting for a substantial portion (approximately 30%) of post-marketing drug withdrawals (Holm et al., 2025). The economic and human cost of DILI is immense, derailing promising therapeutic candidates late in the development pipeline and posing significant risks to patients. A fundamental driver of this problem is the profound inadequacy of traditional preclinical models to predict human hepatotoxicity (Dash et al., 2009). For decades, the pharmaceutical industry has relied on a combination of animal models and simple two-dimensional (2D) *in-vitro* cultures of hepatocytes. However, data from these systems often fail to translate to human clinical outcomes (Dash et al., 2009) and cost time and money tending to fail at late stages of the drug discovery process as illustrated in Figure 1.1 (Sun et al., 2022). Animal models, though mandated by regulatory agencies, frequently exhibit species-specific differences in drug metabolism pathways that do not reflect human responses (Janani et al., 2022). For instance, a drug may be safely metabolized by a rodent but produce toxic byproducts in humans, or vice versa. Concurrently, conventional 2D monolayer cultures of primary human hepatocytes (PHHs), while using the correct cell type, are plagued by the rapid loss of differentiated phenotype and function. Within hours to days of being plated on rigid plastic surfaces, PHHs de-differentiate, downregulating the expression and activity of crucial metabolic enzymes like CYPs (Lauschke et al., 2019). This functional decline renders them unsuitable for the long-term or

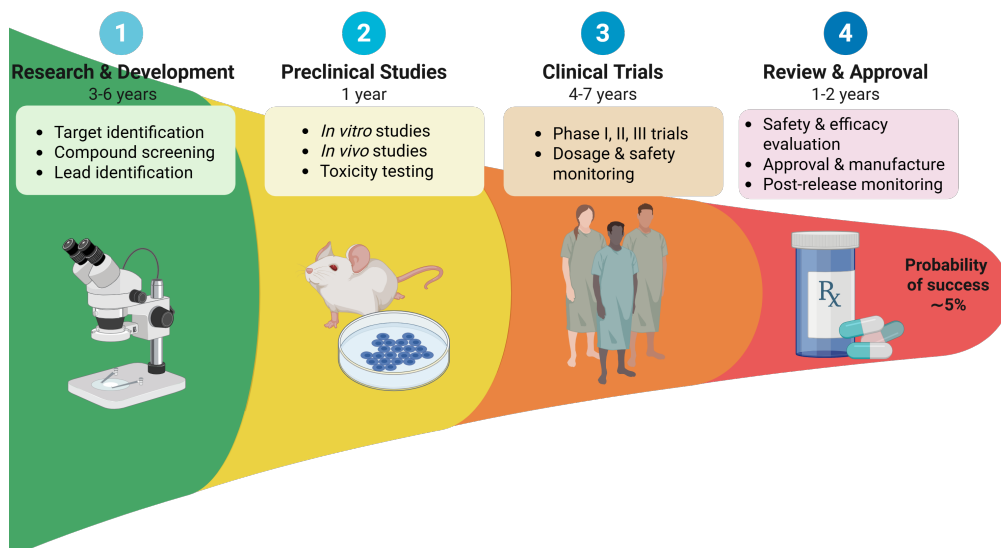


Figure 1.1: Conventional Drug Development Pipeline. This schematic illustrates the four main stages of bringing a new therapeutic drug to market. The process begins with (1) Research and Development (3-6 years) to identify drug targets and lead compounds, followed by (2) Preclinical Studies (1 year) to assess safety and toxicity *in-vitro* and *in-vivo*. Promising candidates advance to (3) Clinical Trials (4-7 years) to evaluate efficacy and safety in humans across three phases. Finally, the drug undergoes (4) Review and Approval (1-2 years) by regulatory authorities. The entire pipeline is a lengthy and high-risk endeavour, with an estimated overall probability of success of only around 5% . Adapted from Sun et al. (2022).

repeated-dose studies necessary to detect many forms of DILI, particularly those that manifest after chronic exposure (Dash et al., 2009).

This predictive failure is especially pronounced for idiosyncratic DILI, a complex form of hepatotoxicity that is not strictly dose-dependent and may involve the interplay of multiple liver cell types and the patient's immune system (Dash et al., 2009). Such intricate interactions are impossible to recapitulate in simplistic monoculture systems. The persistent failure of these gold-standard preclinical models has created a compelling industrial and regulatory imperative to develop more physiologically relevant, human-based *in-vitro* platforms. The goal is to create systems that can more accurately predict DILI early in the drug discovery process, thereby reducing late-stage attrition, minimizing development costs, and enhanc-

ing patient safety. This need has catalysed a paradigm shift away from 2D cultures and toward three-dimensional (3D) biofabricated models that can better mimic the complex architecture and multicellular interactions of the human liver (Holm et al., 2025).

1.2 Native Liver Architecture and ECM Biology

To recapitulate *in-vitro* a functional liver, one must first understand its native design. The remarkable capabilities of the liver are not merely a product of its constituent cells but are deeply rooted in their precise spatial organization and their dynamic interaction with a specialized extracellular matrix (ECM). The fundamental blueprint for hepatic tissue engineering is therefore the liver’s own microanatomy, from the lobular architecture down to the arrangement and composition of the fibres and proteins found in the ECM.

1.2.1 The Hepatic Lobule: The Structural and Functional Unit

The liver parenchyma is exquisitely organized into repeating structural and functional units known as lobules (Janani et al., 2022; Kalra et al., 2023). The classic model describes the lobule as a roughly hexagonal prism of tissue, with a terminal hepatic venule (or central vein) at its axis and portal triads located at each of the hexagon’s corners (Wang et al., 2023b). Each portal triad is a connective tissue sheath containing three primary structures: a branch of the portal vein, a branch of the hepatic artery, and a

bile duct (Krishna, 2013). Blood from both the portal vein (nutrient-rich, oxygen-poor) and the hepatic artery (nutrient-poor, oxygen-rich) enters the lobule at the portal triads and percolates through a network of specialized capillaries, called the sinusoids, flowing radially inward to drain into the central vein (Janani et al., 2022). These details are illustrated in Figure 1.2. This entire cellular arrangement is embedded within a complex ECM structure. This non-cellular network, rich in proteins such as collagens and fibronectin, provides not only essential structural support but also critical biochemical and mechanical cues that regulate hepatocyte function and maintain tissue homeostasis. The successful replication of these architectural details is a major goal, and a significant challenge, for advanced liver tissue engineering.

1.2.2 Cellular Composition and Spatial Organization

The liver is a multicellular organ where parenchymal cells (hepatocytes) and various non-parenchymal cells (NPCs) are arranged in a specific architecture, enabling their complex intercellular communication.

Hepatocytes

These are the primary functional cells of the liver, comprising about 80% of its volume (Tomaz et al., 2022). They are arranged in interconnected plates or cords, typically one to two cells thick, that radiate from the central vein towards the portal triads (Krishna, 2013). Hepatocytes are highly polarized epithelial cells. Their basolateral surface, rich in microvilli, faces the sinusoidal blood supply to facilitate the exchange of molecules. Their apical surfaces face each other, forming a network of tiny channels called

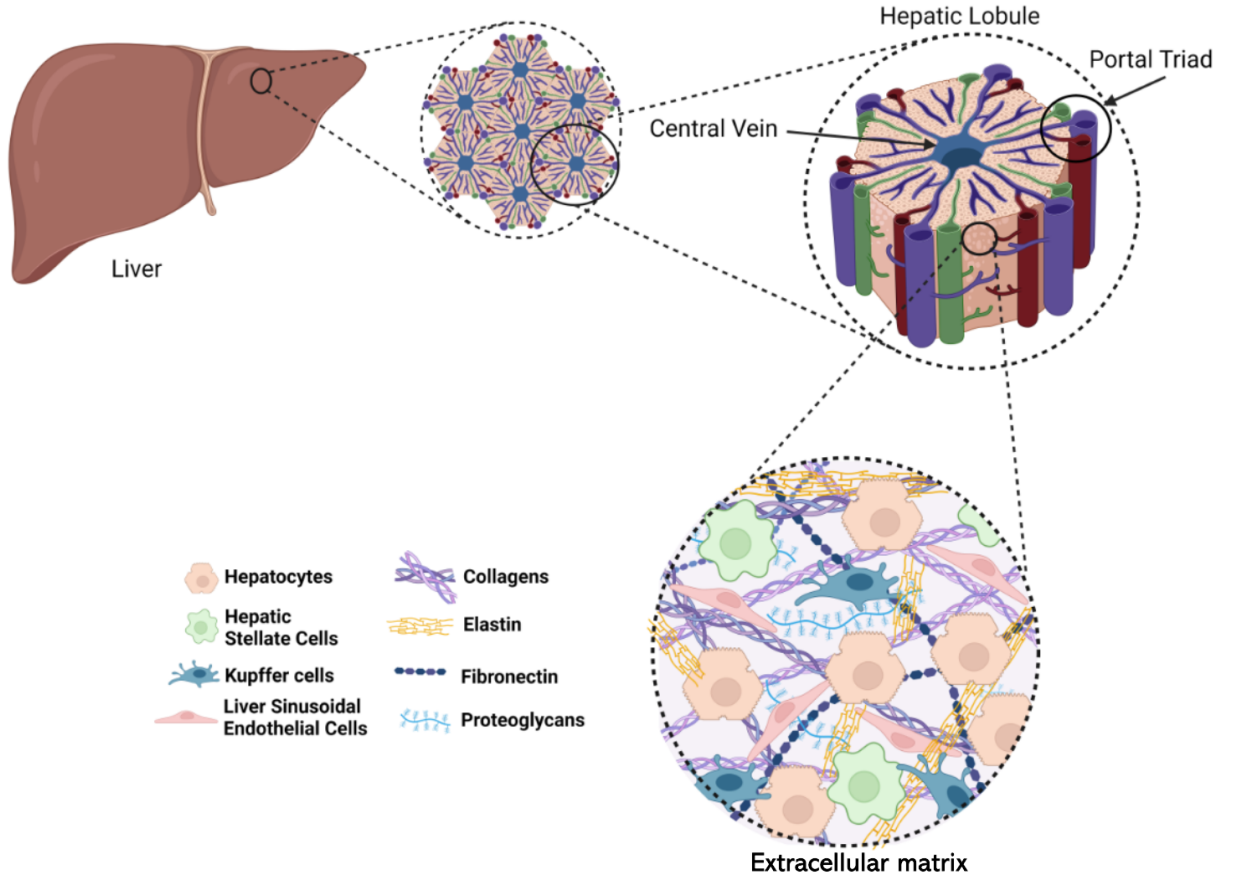


Figure 1.2: Hierarchical Architecture of the Liver. This schematic illustrates the multi-scale organisation of hepatic tissue, from the whole organ down to the molecular level. The liver is composed of repeating hexagonal functional units known as hepatic lobules, which are arranged around a central vein with portal triads at the vertices. These lobules consist primarily of hepatocytes, the main parenchymal cells, which are supported by non-parenchymal cells including hepatic stellate cells, Kupffer cells, and liver sinusoidal endothelial cells. All cells are embedded within a complex ECM containing structural and adhesion proteins such as collagens, elastin, fibronectin, and proteoglycans, which form the critical microenvironment for cellular function.

bile canaliculi, into which bile is secreted (Kalra et al., 2023). This polarity is essential for the liver’s vectorial transport functions.

1.2.3 Liver Sinusoidal Endothelial Cells (LSECs)

These unique endothelial cells form the lining of the hepatic sinusoids. They are characterized by open fenestrae (transcellular pores lacking a

diaphragm) that allow for the free passage of blood, plasma and macromolecules, excluding red blood cells, into the underlying space of Disse (Janani et al., 2022). This feature is critical for the efficient exchange between blood and hepatocytes.

Hepatic Stellate Cells (HSCs)

Also known as Ito cells, these reside in the perisinusoidal space of Disse (Janani et al., 2022). In their quiescent state, they are the primary storage site for vitamin A in the body. However, upon liver injury, HSCs become activated, transforming into myofibroblast-like cells. These activated HSCs are the main producers of the dense, scar-like ECM that characterizes liver fibrosis (Shi et al., 2024).

Kupffer Cells (KCs)

These are specialized, liver-resident macrophages that are located within the sinusoidal lumen, often adhering to LSECs (Krishna, 2013). As part of the mononuclear phagocyte system, they play a crucial role in clearing pathogens, cellular debris, and endotoxins from the portal blood, acting as the liver’s first line of immune defence (Nair and Weiskirchen, 2024).

The functional state of the liver depends on the orchestrated crosstalk between these cell types. NPCs provide critical support for hepatocyte survival and function, and this heterotypic interaction is a key element that must be incorporated into physiologically relevant models.

1.2.4 The Perisinusoidal Space of Disse and the ECM

Nestled between the fenestrated LSECs and the microvilli-covered surface of hepatocytes lies the Space of Disse, a unique microenvironment critical to liver function (Janani and Mandal, 2021). This space is not empty but is filled with blood plasma that has filtered through the sinusoidal fenestrae, allowing for intimate contact between the plasma and hepatocytes (Sanz-García et al., 2021). It also contains a specialized, low-density ECM that provides structural support without forming a significant barrier to mass transport.

In a healthy liver, the ECM constitutes a very small fraction of the total tissue volume, less than 3% (Agarwal et al., 2019). The ECM within the Space of Disse is a delicate, basement membrane-like structure composed primarily of collagen type IV, laminin, fibronectin, and various proteoglycans (Sanz-García et al., 2021). This composition creates a soft, compliant microenvironment with a stiffness typically in the range of 0.1-1 kPa. This mechanical and biochemical niche is now understood to be critical for maintaining the differentiated, quiescent phenotype of hepatocytes and HSCs.(Liu et al., 2012)

This delicate homeostasis is dramatically disrupted in liver disease. In response to chronic injury, HSCs activate and proliferate, initiating a fibrotic cascade. They remodel the ECM, degrading the normal basement membrane-like matrix and replacing it with a dense, scar-like matrix composed predominantly of fibrillar collagens, mainly type I and type III (Liu et al., 2012). This pathological accumulation of ECM leads to a progressive stiffening of the liver tissue, with stiffness values increasing tenfold or more, often exceeding 10-20 kPa in cirrhotic tissue (Guo et al., 2023a). This altered microenvironment is not merely a passive consequence of disease; it

is an active driver of its progression. The stiff, fibrotic matrix further promotes hepatocyte dysfunction and perpetuates HSC activation, creating a vicious feedback loop that culminates in cirrhosis and organ failure (Liu et al., 2012). This bidirectional relationship between cells and their matrix underscores a fundamental principle for liver tissue engineering: replicating the liver’s function requires replicating not only its cellular and biochemical composition but also its specific mechanical and architectural properties.

1.3 Decellularised ECM architecture as a Seeding Platform

In the quest to replicate the complex hepatic microenvironment, one of the most intuitive and compelling strategies has been to use the native liver’s own extracellular matrix as a scaffold. This approach, centred on the use of Decellularised ECM (dECM), is founded on the principle that the tissue-specific composition and architecture of the ECM provide the ideal set of biological cues to guide cell behaviour and promote tissue regeneration Isaeva et al. (2022). For over a decade, dECM has been explored as a potential ”gold standard” biomaterial for liver tissue engineering, evolving from simple matrix coatings to whole-organ scaffolds.

1.3.1 Why is dECM the ”gold standard”?

The central hypothesis underpinning the use of dECM is that it retains the intricate cocktail of structural proteins (e.g., collagens, elastin), adhesion molecules (e.g., fibronectin, laminin), glycosaminoglycans (GAGs), and sequestered growth factors that are unique to the liver’s native mi-

environment as well as maintain its architectural details in whole organ decellularization (Kasturi and Vasanthan, 2024). This complex environment is thought to provide organ-specific signals that are superior to those from single-component biomaterials or non-specific matrices like Matrigel (Jaramillo et al., 2018). Numerous studies have validated this hypothesis, demonstrating that when hepatocytes or pluripotent stem cells are cultured on or within liver dECM, they exhibit enhanced attachment, proliferation, differentiation, and maintenance of mature hepatic functions compared to conventional culture substrates. (Mir et al., 2023). For instance, Jaramillo et al. (2018) showed that differentiating human induced pluripotent stem cells (hiPSCs) on human decellularised liver matrix (HdL-ECM) resulted in significantly higher expression of mature hepatocyte markers compared to differentiation on Matrigel or collagen I, (Jaramillo et al., 2018). The dECM scaffold effectively provides the necessary cues to recapitulate aspects of the *in-vivo* niche, guiding cells toward a more physiologically relevant phenotype (Jaramillo et al., 2018). But decellularization techniques play an important role on how cells react to this type of scaffolds (Gilpin and Yang, 2017).

1.3.2 Evolution of Decellularization Protocols

The goal of decellularization is to completely remove all cellular and nuclear material from a tissue or organ while maximally preserving the 3D architecture, composition, and mechanical integrity of the ECM (Stoian et al., 2024). A variety of protocols have been developed, typically involving a sequence of physical, chemical, and enzymatic treatments, (Kasturi and Vasanthan, 2024). For whole organs like the liver, perfusion-based methods are preferred, as they utilize the native vascular network to ef-

ficiently deliver decellularizing agents throughout the tissue (Kasturi and Vasanthan, 2024).

A typical perfusion protocol involves cannulating a major vessel (e.g., the portal vein) and sequentially perfusing the organ with:

1. **A hypotonic buffer or water** to induce osmotic cell lysis (a physical treatment).
2. **Detergents** to solubilize cell membranes and proteins. Both ionic detergents like sodium dodecyl sulphate (SDS) and non-ionic detergents like Triton X-100 are commonly used (Maghsoudlou et al., 2016).
3. **Enzymes** like DNase and RNase to digest and remove nucleic acids, which are a primary source of immunogenicity (Maghsoudlou et al., 2016).
4. **Extensive washing steps** with saline or buffer to remove residual detergents and cellular debris.

The success of decellularization is typically assessed by histological analysis (e.g., H&E and DAPI staining to confirm the absence of nuclei) and quantitative DNA analysis, with the generally accepted criteria for adequate decellularization being less than 50 ng of double-stranded DNA per mg of dry tissue weight and DNA fragment lengths less than 200 base pairs (Crapo et al., 2011).

1.3.3 The Limitations of dECM as a seeding platform

Despite its powerful biological rationale, the dECM approach is fraught with significant challenges that have made its widespread adoption and

clinical translation complicated. These limitations stem from the very process of its creation and its inherent biological complexity.

Structural and Biochemical Damage

The harsh chemical and enzymatic treatments required for decellularization also have undesirable effects on the tissue. While effective at removing cells, they inevitably cause collateral damage to the delicate ECM structure. Detergents like SDS are known to disrupt collagen fibril architecture and can significantly reduce the content of vital components like GAGs, proteoglycans, and growth factors (Isaeva et al., 2022). This damage can alter the mechanical properties and compromise the biochemical and structural signalling capacity of the resulting scaffold, partially defeating the purpose of using dECM in the first place (Maghsoudlou et al., 2016).

Batch-to-Batch Variability and Lack of Standardization

This is perhaps the most significant barrier. The final properties of a dECM scaffold are highly dependent on a multitude of uncontrolled variables, including the species, age, sex, and health status of the source animal (Isaeva et al., 2022). Furthermore, subtle variations in the decellularization protocol, sterilization methods, and processing can lead to profound differences in the final product, even when using the same techniques (Isaeva et al., 2022). This inherent lack of consistency and reproducibility makes it extremely difficult to generate a standardized, off-the-shelf product suitable for regulated clinical applications or as a reliable high-throughput drug screening platforms (Shi et al., 2024).

Poor Mechanical Properties and Printability

While whole-organ dECM scaffolds retain macroscopic shape, they are often mechanically weak. To be used in modern biofabrication techniques like 3D bioprinting, dECM is typically processed into a powder and then reconstituted as a hydrogel. These dECM hydrogels often exhibit poor mechanical properties and low viscosity, making them challenging to print with high fidelity and structural stability (Shi et al., 2024). Consequently, they are frequently blended with synthetic polymers to improve their printability, but even then, their batch to batch variability makes these properties vary significantly (Shi et al., 2024).

Immunogenicity and Pathogen Risk

Even after extensive processing, residual cellular components, like MHC antigens, or xenogeneic proteins, such as those from animal sources, can remain, potentially triggering an adverse immune response upon implantation (Isaeva et al., 2022). Additionally, the use of animal tissues, particularly from porcine sources, carries a non-zero risk of transmitting zoonotic diseases (Isaeva et al., 2022; Fishman et al., 2012) and often brings restrictions due to ethical or religious concerns (Rodger and Blackshaw, 2019; Eriksson et al., 2013).

1.4 Projection Micro-Stereolithography ($P\mu$ SLA) and Light-Based Hydrogels

The ambition to engineer tissues with the architectural fidelity of their native counterparts requires not only advanced biomaterials but also ad-

vanced fabrication technologies capable of shaping those materials with micro-scale precision. The convergence of photocurable hydrogels with high-resolution additive manufacturing techniques, particularly Projection Micro-Stereolithography ($P\mu$ SLA), represents a technological groundwork for the next generation of tissue engineering. This combination provides an unprecedented ability to systematically deconstruct and rebuild the hepatic microenvironment, allowing for rigorous investigation into the interplay between scaffold architecture and cellular function.

1.4.1 Fundamentals of $P\mu$ SLA

$P\mu$ SLA is a vat photopolymerization AM technology that enables the rapid fabrication of complex 3D structures with exceptionally high resolution (Gauvin et al., 2012; Ma et al., 2022). Unlike extrusion-based printing which deposits material point-by-point, or laser-based stereolithography which traces a pattern, $P\mu$ SLA utilizes a digital light source, typically a UV projector, to cure an entire two-dimensional layer of a liquid photopolymer resin simultaneously (Ge et al., 2020).

As illustrated in Figure 1.3, the core of the technology is a Digital Micromirror Device (DMD), an array of hundreds of thousands to millions of individually controlled microscopic mirrors (Ge et al., 2020). For each layer of the 3D model, the DMD creates a corresponding light pattern, a virtual photomask, which is projected on to the surface of the resin vat. The illuminated areas of the resin are rapidly cross-linked and solidified. After a layer is cured, the build platform moves by a specified layer thickness, often on the order of microns, a fresh layer of liquid resin flows over the solidified structure before the next projection occurs. (Gauvin et al., 2012; Ma et al., 2022; Ge et al., 2020).

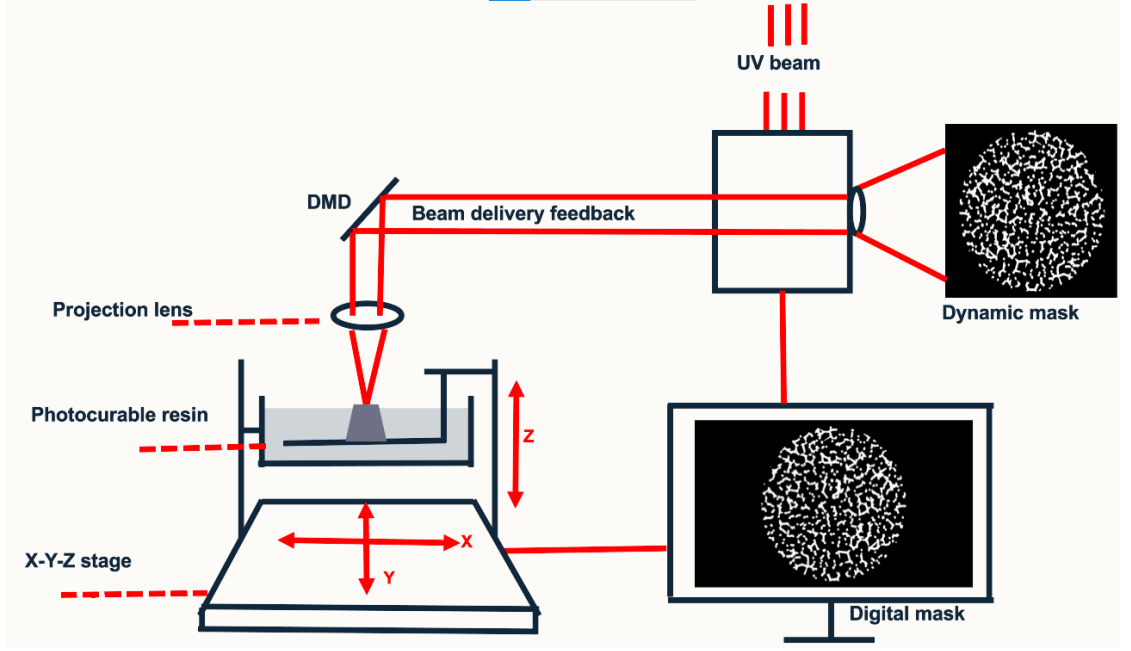


Figure 1.3: Schematic of the $P\mu$ SLA system. A UV beam is modulated through a dynamic digital mask and projected using a DMD via a lens into a photocurable resin bath. The beam delivery feedback ensures precise patterning, while the X-Y-Z stage enables layer-by-layer fabrication of complex 3D structures.

In conventional $P\mu$ SLA systems, this recoating step is typically achieved using a wiper or blade that spreads a thin layer of resin across the vat (Lakkala et al., 2023). However, the Boston Micro Fabrication (BMF) S130 system employed in this work adopts a film-based recoating mechanism, which is distinctive among μ SLA platforms. In this configuration, a transparent and flexible film continuously advances below the build area, acting as an interface between the resin and the projected light source. A controlled volume of resin is deposited on the film to form a uniform layer, thereby reducing shear stresses on delicate printed features and preventing adhesion between the cured layer and the vat surface (Pan et al., 2017). This approach ensures highly consistent layer thickness, promotes efficient resin renewal, and contributes to the precision characteristic of the BMF system. This re-coating process is then repeated iteratively until the full 3D object is constructed.

$P\mu$ SLA offers several key advantages that make it particularly well-suited for fabricating tissue engineering scaffolds:

High Resolution: $P\mu$ SLA systems can achieve features in the range of 2-10 μm and vertical resolutions on the order of 5-10 μm , enabling the creation of intricate micro-architectures that can recapitulate biological structures such as fibres found in ECM (Ge et al., 2020).

High Speed: When operating within the projection size limits, $P\mu$ SLA can polymerize an entire layer in a single exposure, which is considerably faster than serial writing techniques such as 2-photon printing (2PP). This parallel curing capability greatly improves printing times throughput, making the fabrication of large or geometrically complex structures more practical and faster (Pingali et al., 2024).

Architectural Freedom: As an additive manufacturing technique and a digital, layer-based process driven by a CAD model, $P\mu$ SLA enables the fabrication of scaffolds with high geometric complexity, including interconnected porous networks, internal channels, and precisely controlled pore architectures (Ma et al., 2022). Although certain geometries in light-based printing can require support structures to prevent deformation or delamination during fabrication, $P\mu$ SLA is particularly well suited for the design of self-supported architectures. The high spatial resolution and accurate layer registration inherent to this technique allow the production of lattices and porous networks that maintain structural integrity throughout the printing process without the need for additional supports. This capability facilitates the fabrication of complex yet cleanly resolved microstructures, which is especially advantageous for scaffold fabrication where open porosity and unobstructed internal features are essential.

Material Versatility: The process is compatible with a wide range of photopolymerizable materials, including the soft, biocompatible hydrogels necessary for cell culture. Although there is a limited availability of commercial hydrogels for this technique, it provides the freedom to experiment with a wide range of custom-made materials and tune them properly for P μ SLA.

1.4.2 Photocurable Hydrogels for Biofabrication

To leverage the power of P μ SLA for tissue engineering, the liquid resin must be a "bioink", material that is not only photocurable but also biocompatible and capable of supporting cell survival and function (Unagolla and Jayasuriya, 2020). Hydrogels are the material class of choice, as their high water content and soft mechanical properties closely resemble those of native soft tissues (Zhang et al., 2024). For light-based bioprinting, two materials have become particularly prominent: Gelatin Methacrylate (GelMA) and Poly(ethylene glycol) Diacrylate (PEGDA).

Gelatin Methacrylate (GelMA)

GelMA is a semi-synthetic biomaterial derived from gelatin, which is made of collagen, the most abundant protein in the ECM (Yue et al., 2015). The gelatin backbone is chemically modified with methacrylate groups, which allows it to be covalently cross-linked into a stable hydrogel network upon exposure to UV or visible light in the presence of a photoinitiator (Ma et al., 2022). GelMA is highly valued in tissue engineering for its intrinsic bioactivity. It retains crucial biological motifs from its parent collagen, including Arginine-Glycine-Aspartic acid (RGD) sequences that

mediate cell adhesion via integrin binding, and sites that are susceptible to degradation by matrix metalloproteinases (MMPs) (Cuvellier et al., 2021). This allows cells to adhere to, interact with, and actively remodel their scaffold microenvironment. However, pure GelMA hydrogels often suffer from poor mechanical strength and can be thermally unstable, limiting their use in fabricating robust, self-supporting structures (Cui et al., 2019).

Poly(ethylene glycol) Diacrylate (PEGDA)

PEGDA is a synthetic polymer that has long been a workhorse in biomaterials science (Peppas et al., 2006). It consists of a hydrophilic poly(ethylene glycol) core functionalized with acrylate groups at each end, enabling rapid photocrosslinking (He et al., 2022). In contrast to GelMA, PEGDA is largely considered bio-inert; it resists non-specific protein adsorption and does not possess native cell adhesion ligands (Tan et al., 2021). Its primary advantage lies in its highly tuneable and predictable physical properties. The stiffness of a PEGDA hydrogel can be precisely controlled over several orders of magnitude by varying the PEG molecular weight and concentration (He et al., 2022). This mechanical tunability, combined with its excellent biocompatibility and print stability, makes it an ideal structural component for engineered scaffolds (Fowler et al., 2025).

1.4.3 The GelMA-PEGDA Hybrid Hydrogel: a Complementary System

The limitations of GelMA and PEGDA as stand-alone materials naturally lead to their combination in a hybrid hydrogel system. By blending GelMA and PEGDA prepolymers, it is possible to create a bioink that possesses

the biological functionality of GelMA and the mechanical tunability and structural integrity of PEGDA (Duan et al., 2022). In this composite network, the GelMA component provides the necessary biochemical cues to support cell adhesion, viability, and function, while the PEGDA provides the mechanical strength required to print high-fidelity, complex architectures that do not collapse under their own weight (Duan et al., 2022). This synergy is critical. The ability to independently tune the concentration of each component allows for the decoupling of a scaffold’s biological and mechanical properties. For example, one can maintain a constant GelMA concentration having a constant ligand density, while varying the PEGDA concentration to create scaffolds of identical bio-functionality but different stiffnesses, such as the work undertaken by He et al. (2022). This level of control is essential for systematic studies of mechanobiology and to mimics the intended tissue stiffness. The resulting GelMA-PEGDA bioink is both highly biocompatible and highly printable, making it an ideal material platform for use with high-resolution $P\mu$ SLA to investigate the impact of precisely defined micro-architectures on hepatic function (Gao et al., 2023). This powerful combination of a tuneable, bioactive material shown to be functional (Liang et al., 2016a; Tan et al., 2021; He et al., 2022; Duan et al., 2022; Gao et al., 2023; Fowler et al., 2025) and a high-fidelity fabrication technology, as $P\mu$ SLA, makes it possible to move beyond simply observing that geometry has an impact and toward developing quantitative design rules for functional tissue engineering and quantifying cell behaviour in this 3D printed hydrogel scaffolds.

1.5 Lattice Design Paradigms

The advent of high-resolution additive manufacturing has transformed scaffold design from a process limited by fabrication constraints into a discipline of computational architecture. Engineers and biologists can now design and create scaffolds with nearly any conceivable geometry, moving from the random, uncontrolled pores of early biomaterials (Kanwar et al., 2022) to mathematically defined, complex lattices (Guo et al., 2023b; Zhou et al., 2019; Todd et al., 2024). This has opened a new frontier of investigation focused on understanding which architectures are optimal for guiding tissue regeneration. However at the forefront of this exploration is a fundamental dichotomy between two design philosophies: highly ordered, predictable periodic structures and biomimetic, heterogeneous stochastic structures.

1.5.1 Evolution of Scaffold Design: From Simple Pores to Engineered Lattices

The first generation of tissue engineering scaffolds were often fabricated using techniques like solvent casting, particulate leaching, gas foaming, or freeze-drying (Lewis et al., 2018). These methods produced porous structures, but with little to no control over the key architectural parameters of pore size, shape, or interconnectivity, so the resulting architectures were inherently stochastic, but in an uncontrolled and often irreproducible manner (Ranucci et al., 2000; Lee et al., 2005; Lowery et al., 2010; Lien et al., 2009). With the rise of 3D printing, the first forays into designed architectures involved simple, repetitive geometries. For liver tissue engineering, this often meant creating grid-like or hexagonal patterns in an attempt to mimic the standardized structure of the hepatic lobule (Hammond et al.,

2006). While an improvement in control, these simple rectilinear or honey-comb designs lack the geometric complexity and surface characteristics of native tissue micro-architectures.

1.5.2 Periodic Architectures: Order and Predictability

A periodic architecture is a structure built from the tessellation of a single, repeating unit cell in a 3D space (Ahmadi et al., 2015; Park et al., 2021). This approach yields highly ordered, predictable lattices whose bulk properties, like mechanical stiffness and permeability, can be readily modelled and controlled by modifying the design of the unit cell.

Many of the most advanced periodic structures are described by implicit mathematical functions known as level-set equations. A particularly powerful and popular class of these are the Triply Periodic Minimal Surfaces (TPMS). TPMS are surfaces with a mean curvature of zero at every point, meaning they locally minimize surface area, and they are periodic in three independent directions (Vijayavenkataraman et al., 2018; Kapfer et al., 2011). They are notable for their smooth, continuous surfaces that lack the sharp corners and stress concentrations found in strut-based lattices. Prominent examples used in tissue engineering include the Gyroid, Schwarz Diamond, and Schwarz Primitive surfaces (Kanwar et al., 2022). The Gyroid structure, for example, can be mathematically defined by the equation 1.1:

$$\sin\left(\frac{L}{2}\pi x\right) \cos\left(\frac{L}{2}\pi y\right) + \sin\left(\frac{L}{2}\pi y\right) \cos\left(\frac{L}{2}\pi z\right) + \sin\left(\frac{L}{2}\pi z\right) \cos\left(\frac{L}{2}\pi x\right) = t \quad (1.1)$$

In this equation, L represents the size of the unit cell, and the threshold parameter t controls the solid volume fraction, and thus the porosity, of the resulting lattice (Kanwar et al., 2022).

Periodic lattices, and TPMS in particular, have been extensively investigated, primarily in bone tissue engineering (Guerra et al., 2019; Flores-Jimenez and Fuentes-Aguilar, 2021; Baumer et al., 2024; Do Cha et al., 2015). Their appeal comes from their high surface-area-to-volume ratio, excellent interconnectivity, and highly predictable mechanical properties that can be precisely tuned by adjusting the parameters of the defining equation (Vijayavenkataraman et al., 2018). This makes them an ideal platform for engineers aiming to design scaffolds with specific, reproducible mechanical characteristics; however, they can also introduce rigidity and structural features that do not accurately represent the geometry of the target tissue.

1.5.3 Stochastic Architectures: Biomimetic Randomness

In contrast to the perfect order of periodic lattices, stochastic architectures are characterized by a lack of long-range translational symmetry. Their structural elements, such as struts, pores, and surfaces, exhibit random or pseudo-random variations in size, orientation, and connectivity (Kanwar et al., 2022). The rationale for exploring these designs is compellingly biomimetic since most native biological tissues, from the random orientation of ECM fibres to the sinusoidal microvasculature of the liver, are not perfectly periodic but are inherently stochastic and heterogeneous (Kanwar et al., 2022). To generate these type of structures, several computational methods have been developed.

Voronoi Tessellation This is a widely used geometric construct fundamentally formed by a set of "seed" points that are randomly distributed within a volume. The space is then partitioned into polyhedral cells, where each cell consists of all points closer to its seed than to any other. The edges and faces of these polyhedra can then be used to define the struts and walls of a stochastic scaffold (Deering et al., 2021; Herath et al., 2021; Du et al., 2020; Todd et al., 2024).

Randomized Level-Set Methods This more sophisticated approach combines the mathematical elegance of periodic functions with randomization. As described by Kanwar et al. (2022), a volume is first populated with random seed points, which are used to partition the domain into sub-regions. Within each sub-region, a base periodic lattice is generated but with a randomized orientation (Kanwar et al., 2022). These individually oriented lattices are then smoothly blended at the boundaries between sub-regions using a weighted function, resulting in a single, continuous, yet globally aperiodic structure.

While less explored than their periodic counterparts, stochastic scaffolds are gaining significant interest. The central hypothesis is that their biomimetic randomness may provide a more natural and effective microenvironment for cells. In bone tissue engineering, several studies have now shown that stochastic lattices can promote superior cellular behaviour and osteogenesis compared to periodic lattices with similar porosity or surface area (Kechagias et al., 2023; Wang et al., 2021; Liang et al., 2022; Torres-Sanchez et al., 2022). This finding from the field of bone tissue engineering provides a powerful motivation to investigate whether the same principle applies to soft tissues like the liver.

The ability to computationally design and physically fabricate these two distinct classes of architecture sets the stage for a direct, rigorous comparison. It raises a fundamental question at the intersection of engineering and biology to investigate whether the predictable, engineered order of periodic lattices is superior for guiding cell function, or the inherent, biomimetic heterogeneity of stochastic structures provides a more potent set of cues for tissue regeneration. Perusing this path is critical for establishing evidence-based design principles for the next generation of tissue-engineered constructs.

1.6 Interfacing Geometry and Biology in Tissue Engineering

The architecture of a tissue engineering scaffold is far more than an inert support structure; it is an active participant in directing cell fate. Cells are sensitive to the physical and geometric features of their surroundings, a phenomenon governed by the process of mechanotransduction (Janson and Putnam, 2015; Ingber, 2003; Urbanczyk et al., 2020). Through this process, geometric cues like pore size, interconnectivity, and surface curvature are translated into intracellular biochemical signals that regulate gene expression and ultimately determine cell behaviour, including adhesion, migration, proliferation, and differentiation (Loh and Choong, 2013; Blanquer et al., 2017). Understanding this intricate geometry-biology interplay is paramount for designing scaffolds that do not merely house cells, but actively instruct them to form functional tissue.

1.6.1 Mechanotransduction: How Cells sense Their Environment

Mechanotransduction is the molecular mechanism by which cells sense mechanical forces and geometric constraints in their microenvironment and convert these physical stimuli into biochemical signals (Kim et al., 2021). The process typically begins at the cell-matrix interface. Transmembrane proteins called integrins bind to specific ligands on the ECM. This binding triggers the recruitment of dozens of other proteins to form complex adhesion structures known as focal adhesions (Luo et al., 2021). These focal adhesions act as mechanical anchor points, connecting the ECM to the cell's internal actin cytoskeleton (Ingber, 2003). Forces generated by the cell's own contractile machinery, such as actomyosin stress fibres, or applied externally are transmitted through this integrin-cytoskeleton linkage. This mechanical information propagates through the cell to the nucleus, where it can induce changes in chromatin structure and regulate the activity of key transcription factors (Han et al., 2023). A central pathway in this process is the Hippo signaling pathway, whose downstream effectors, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), are master regulators of mechanosensing. When cytoskeletal tension is high (e.g., on a stiff or flat surface that allows for cell spreading), YAP/TAZ translocate to the nucleus and activate pro-growth and differentiation genes. When tension is low (e.g., in soft matrices or confined geometries), YAP/TAZ are phosphorylated and retained in the cytoplasm, often promoting stemness or quiescence (Swanson et al., 2022). Thus, the geometry of a scaffold, by dictating how a cell can spread and exert force, directly controls these powerful signalling pathways.

1.6.2 The Influence of Pore Size, Porosity, and Interconnectivity

The most fundamental architectural properties of a scaffold are its porosity, the percentage of void space, and the size and interconnectivity of its pores. These features are critical for ensuring cell infiltration and the transport of nutrients and waste, with diffusion limits typically constraining tissue construct thickness to 100-200 μm in the absence of vascularization (Agarwal et al., 2021). However, these parameters also provide direct mechanobiological cues.

A seminal study by Ranucci et al. (2000), using hepatocytes cultured in collagen foams, revealed a complex, non-linear relationship between pore size and function (Ranucci et al., 2000). They observed that hepatocytes maintained high albumin secretion in two distinct regimes: in very small pores (10 μm), which constrained cell spreading and forced a compact, cuboidal morphology, and in large pores (82 μm), which were large enough to permit the formation of 3D multicellular spheroid like aggregates. Conversely, function was significantly repressed in intermediate-sized pores (18 μm), which seemed to promote excessive 2D-like cell spreading without enabling 3D aggregation (Ranucci et al., 2000). This landmark study demonstrated that pore size is not a simple variable but rather a complex cue that influences cell morphology and intercellular organization.

More recently, the advent of 3D printing has allowed for the deconstruction of pore architecture into its constituent parts, such as interconnectivity. Lewis et al. (2018) used extrusion-based 3D printing to fabricate gelatin scaffolds with identical material chemistry and pore size but with two different pore geometries: a simple grid (90° strut angle) and a more tortuous, interconnected hexagonal-like geometry (60° strut angle) (Lewis

et al., 2018). They found that while both scaffolds supported high cell viability, the more interconnected 60° geometry led to significantly enhanced hepatocyte-specific functions, including higher albumin secretion, increased CYP enzyme activity, and more robust formation of bile canaliculi (Lewis et al., 2018). This work provided direct evidence that scaffold geometry, independent of material chemistry or pore size, is a potent modulator of hepatocyte function, likely by influencing the extent of 3D cell-cell contacts and aggregation.

1.6.3 The Influence of Curvature

Beyond pore size and connectivity, cells are also sensitive to more subtle geometric features like surface curvature, which they can sense at both the microscale, as the shape of a pore, and the nanoscale, as the topography of a surface (Luo et al., 2021). A groundbreaking study by Swanson et al. (2022), though focused on mesenchymal stem cells (MSCs) for bone tissue engineering, established a clear mechanistic link between curvature and cell fate (Swanson et al., 2022). Using scaffolds with varying pore sizes, they demonstrated that high principal curvature found in small pores facilitated cell aggregation and maintained MSC stemness by promoting the cytoplasmic retention (inactivation) of YAP. In contrast, low principal curvature found in large pores, allowed for cell spreading, which led to YAP nuclear translocation (activation) and subsequent osteogenic differentiation (Swanson et al., 2022). Pharmacological inhibition of the actin cytoskeleton reversed the effects in low-curvature environments, confirming that the response was mediated through cytoskeletal tension and mechanotransduction (Swanson et al., 2022).

While this study was not performed on hepatocytes, it provides a powerful

conceptual framework and a testable hypothesis for liver tissue engineering. It establishes that curvature is a fundamental geometric cue that can be used to control a master regulatory pathway of cell fate. The principles of mechanotransduction are broadly conserved across cell types (Kim et al., 2021), but the specific responses of epithelial cells like hepatocytes, which exist in a much softer native environment than MSCs, to complex curvature profiles remain a critical and under explored area of research. This knowledge gap is particularly relevant when considering the complex, multi-scale curvature inherent in the design of stochastic lattices. The collective evidence strongly suggests that scaffold architecture can be used as to guide and influence cell behaviour, transforming the scaffold from a passive support to an active signalling component in the engineered tissue.

To synthesize the key findings in this area, Table 1.1 summarizes seminal studies that have linked scaffold geometry to cellular outcomes.

Table 1.1: Summary of Key Studies on Scaffold Geometry and Cellular Function

Reference	Fabrication Method	Cell Type	Geometric Parameters	Key Functional Findings
Ranucci et al. (2000)	Collagen Foam	Rat Hepatocytes	Pore Size (10, 18, 82 μm)	Non-linear effect: high function (albumin secretion) in small (10 μm) and large (82 μm) pores, but low function in intermediate (18 μm) pores.
Lewis et al. (2018)	3D-Printed Gelatin	HUH7 Hepatoma Cells	Pore Geometry & Interconnectivity (60° vs. 90° strut angle)	More interconnected 60° geometry significantly enhanced albumin secretion, CYP450 activity, and bile canaliculi formation compared to 90° geometry.
Swanson et al. (2022)	3D-Printed PLGA	MSCs	Pore Curvature and Size	High curvature (small pores) maintained stemness via YAP inactivation; low curvature (large pores) induced osteogenic differentiation via YAP activation.
Kim et al. (2015)	Nanopatterned Substrates	Primary Hepatocytes	Nanotopography (400, 1400, 4000 nm pitch)	400 nm pitch patterns significantly improved hepatocyte attachment and function (albumin expression) compared to larger pitches or planar surfaces.

1.7 Determination of Functional Hepatic Maturation

The ultimate goal of liver tissue engineering is to create constructs that faithfully recapitulate the functions of the native organ. Therefore, the ability to accurately assess the functional state and maturity of engineered hepatic tissue is of great importance. A successful evaluation relies on a suite of robust, quantitative assays that probe the diverse physiological roles of the liver, from its synthetic capacity to its complex metabolic and detoxification functions. Relying on a single biomarker is insufficient; a comprehensive, multi-faceted assessment is required to make a credible claim of a liver-like phenotype. Furthermore, the literature reveals a critical distinction between gene expression and actual protein function, underscoring the necessity of functional assays over purely transcriptomic analyses.

1.7.1 Assessing Synthetic Functions

The liver is a protein factory, and its capacity for synthesis is a primary indicator of its health and functional state. Its most important synthetic products are described as follows.

Albumin Secretion: Albumin is the most abundant protein in blood plasma and is synthesized exclusively by hepatocytes and its rate of secretion is a universally accepted gold-standard marker for mature hepatocyte function (Kalra et al., 2023). Enzyme-linked immunosorbent assays (ELISAs) are routinely used to quantify the concentration of albumin secreted into the culture medium over time, providing a reliable measure of

the synthetic health of the engineered construct (Holm et al., 2025).

Urea Synthesis: A key detoxification function of the liver is the conversion of toxic ammonia, produced as byproduct of protein catabolism, into urea via the urea cycle. The rate of urea production, typically measured with colorimetric assays, serves as a crucial functional readout of this metabolic pathway (Janani and Mandal, 2021).

1.7.2 Assessing Metabolic and Detoxification Function

The liver’s role in drug metabolism is central to its importance in toxicology and pharmacology. Assessing this function is critical for any model intended for DILI screening.

Cytochrome P450 (CYP) Enzyme Activity: The CYP superfamily of enzymes is responsible for the Phase I metabolism of the vast majority of clinical drugs and other xenobiotics (Buratti and Lavine, 2002). Measuring the activity of key isoforms is essential. CYP3A4 is arguably the most important, as it is involved in the metabolism of approximately 50% of all drugs on the market. Other clinically relevant isoforms include CYP1A2, CYP2C9, CYP2C19, and CYP2D6 Milton et al. (2024). Its functional activity is typically assessed in one of two ways. The first involves incubating the cells with a specific drug substrate and then using liquid chromatography-mass spectrometry to quantify the formation of its specific metabolite (e.g., 1-hydroxymidazolam) (Holm et al., 2025). The second method uses commercially available kits with probe substrates that are converted into luminescent or fluorescent products, allowing for a more high-throughput readout (Kim et al., 2015). A hallmark of a mature

and responsive hepatic phenotype is the ability to upregulate CYP enzyme expression in response to chemical inducers. For example, treating cells with rifampicin should lead to a measurable increase in CYP3A4 activity. This inducibility is a critical functional endpoint for a predictive toxicology model (Nakamura et al., 2011).

Following Phase I metabolism, Phase II enzymes like uridine diphosphate glucuronosyltransferases, or Glutathione S-transferases, conjugate molecules to increase their water solubility for excretion. Assessing the expression and activity of these enzymes is necessary for a complete metabolic profile (Uygun et al., 2010).

1.7.3 Assessing Structural and Polarity-Dependent Function

Many of the liver's functions depend on the correct 3D organization and polarization of hepatocytes. The ability of hepatocytes to synthesize and store glycogen is a key metabolic function and a sign of maturation. This can be visualized histologically using Periodic acid-Schiff staining, which stains glycogen granules a bright magenta color (Kim et al., 2015). It can also be assessed by quantifying gene upregulation with genes such as Glucose-6-Phosphatase Catalytic Subunit 1 (G6PC1). This is a key hepatic gene that serves as a robust marker of late-stage hepatocyte maturation. It encodes a catalytic component of the glucose-6-phosphatase complex, which is essential for glucose homeostasis and glycogen storage (van Schaftingen and Gerin, 2002). G6PC1 expression is tightly regulated and requires metabolic induction, typically becoming prominent only in fully differentiated hepatocytes (Zabulica et al., 2019).

The formation of a sealed, functional network of bile canaliculi at the apical surfaces of adjacent hepatocytes is a definitive marker of correct cellular polarity. This can be assessed functionally by incubating the engineered tissue with a fluorescent bile salt analogue, such as cholyl-lysyl-fluorescein. In a properly polarized culture, the cholyl-lysyl-fluorescein will be taken up by hepatocytes and actively transported into the canalicular network, where it accumulates and can be visualized as bright, linear structures using confocal microscopy (Lewis et al., 2018).

A critical takeaway from the literature is the potential disconnect between gene expression, measured by qPCR, and actual protein function, measured by activity assays. The work by Lewis et al. (2018) highlighted this disparity, showing that hepatocytes in 2D culture expressed high levels of CYP3A4 mRNA but exhibited very low functional enzyme activity (Lewis et al., 2018). This suggests that while the gene was being transcribed, the cellular environment lacked the necessary 3D cues for proper protein translation, folding, or localization to yield a functional enzyme. This finding powerfully demonstrates that functional assays are not merely confirmatory; they are essential for revealing the true physiological state of an engineered tissue and are superior to transcriptomic data alone for judging the success of a tissue engineering strategy.

1.8 Current 3D Hepatic Culture Platforms

The recognition that 2D culture systems are fundamentally inadequate for maintaining hepatocyte phenotype has encouraged the development of a diverse array of 3D culture platforms. These technologies aim, to varying degrees, to recapitulate the key features of the native liver microenviron-

ment, including cell-cell and cell-matrix interactions, 3D architecture, and dynamic perfusion. Each platform possesses a unique set of advantages and disadvantages, creating a complex technological landscape with inherent compromises between physiological relevance, throughput, and experimental complexity. A comparative analysis of these systems is essential to contextualize the current research and identify the specific gaps that new approaches aim to fill.

1.8.1 Scaffold-Free Systems: Spheroids and Organoids

These systems rely on the innate ability of cells to self-aggregate and, in some cases, self-organize into tissue-like structures without the need for an exogenous scaffold (Duval et al., 2017; Rodrigues et al., 2018).

Spheroids are the simplest form of 3D culture, consisting of multicellular aggregates formed through self-assembly (Yang et al., 2023). They are typically generated using methods that prevent cell adhesion to a substrate, such as hanging drop plates or culture dishes coated with ultra-low attachment polymers (Yang et al., 2023). Their primary strengths are simplicity, low cost, and scalability, making them highly amenable to high-throughput screening (HTS) applications in drug discovery (Xie et al., 2024). The formation of tight cell-cell junctions in spheroids significantly improves hepatocyte viability and function compared to 2D monolayers (Yang et al., 2023). Although it has its advantages, spheroid formation offers no control over the final architecture or size of the aggregate. This often leads to the formation of large spheroids (200-500 μm) with hypoxic and necrotic cores due to diffusion limitations of oxygen and nutrients (Asthana and Kisaalita, 2012; Hirschhaeuser et al., 2010). While they promote cell-cell contact, they lack the crucial cell-ECM interactions that are provided by a

scaffold, and they do not recapitulate the higher-order tissue organization of the liver lobule (Lauschke et al., 2019; Duval et al., 2017).

Liver organoids represent a more sophisticated scaffold-free system. Derived from pluripotent stem cells (iPSCs or ESCs) or adult tissue stem cells, organoids have the capacity for self-renewal and self-organization, enabling them to recapitulate aspects of organ development and form structures containing multiple hepatic cell types in a somewhat organized fashion (Saxton and Stevens, 2023). One of their advantages is that they can model developmental processes and genetic diseases in a patient-specific manner (Tutty et al., 2022). They achieve a higher degree of biological organization than spheroids, sometimes forming rudimentary ductal and hepatocytic domains (Xu, 2021). But liver organoids are often phenotypically immature, resembling fetal rather than adult tissue (Zhang and Yang, 2025). They critically lack a vascular network and integrated immune cells, limiting their physiological relevance and size. Their generation is complex, and they suffer from significant batch-to-batch variability (Zhang and Yang, 2025).

1.8.2 Perfused Systems: Microfluidic Organs-on-Chips Devices

These platforms aim to recreate the dynamic *in-vivo* environment by culturing cells within micro-scale devices under continuous fluid flow. Liver-on-a-chip systems are microfluidic devices containing chambers where liver cells, often in a 2D monolayer or simple 3D hydrogel, are cultured. Micro-channels allow for the continuous perfusion of culture medium, mimicking blood flow through the sinusoids (U.S. Food and Drug Administration, 2017; Lee and Cho, 2016; Fetah et al., 2016). The perfusion these systems incorporate provides physiological fluid shear stress, establishes stable nu-

trient and oxygen gradients, and allows for efficient waste removal. This dynamic environment can maintain high levels of hepatocyte function for weeks, far longer than static cultures (Dash et al., 2009). These systems are ideal for studying pharmacokinetics and can be linked to other "organ" chips to model systemic effects (U.S. Food and Drug Administration, 2017; Lee and Cho, 2016). Their mayor disadvantage is that microfluidic systems are technically complex to design and operate and often require specialized equipment to run or to fabricate. They are also typically low-throughput, making them less suitable for large-scale screening. Another disadvantage is that cell numbers are small, and most current designs still rely on overly simplistic 2D or homogenous 3D cell arrangements, failing to capture the complex micro-architecture of the liver lobule (Xu, 2021).

1.8.3 Scaffold-Based Systems: Additive Manufacturing for Tissue Engineering Lattices

The inherent limitations of traditional 2D culture and scaffold-free 3D models have driven the field towards additive manufacturing to create hepatic constructs with greater physiological relevance (Duval et al., 2017; Lauschke et al., 2019). In contrast to the self-assembly seen in spheroids and homogenous 3D cell arrangements in organ-on-chip devices, scaffold-based approaches use additive manufacturing to build a predefined architecture that is then seeded with cells (Mota et al., 2015; Duval et al., 2017; Hutmacher, 2001). Technologies such as extrusion, inkjet, or light-based methods (like P μ SLA) are used to deposit bioinks into precise, pre-designed 3D structures or lattices (Xu, 2021).

The principal advantage of this approach is the unparalleled control over both macro and, potentially, dependent on the AM system used, micro-

architecture. This allows for the fabrication of scaffolds with defined porosity, pore geometry, and mechanical properties, and offers a direct route to incorporating perfusable vascular channels (Lewis et al., 2018). This yields highly reproducible constructs, which is essential for systematic studies and screening applications (Lewis et al., 2018).

The major challenges lie in the development of suitable bioinks that are simultaneously biocompatible, bioactive, and possess the specific rheological properties required for a given printing technology (Mancha Sánchez et al., 2020). Cell viability can be compromised by the mechanical stresses (in extrusion) or light exposure (in photopolymerization) during the printing process (Unagolla and Jayasuriya, 2020). Furthermore, achieving the sub-50 μm resolution needed to print fine features remains a significant technological hurdle due to the lack of specialized 3D printing technology available in tissue engineering laboratories (Agarwal et al., 2019).

No single platform is currently sufficient as addressed in Table 1.2. Spheroids offer throughput but lack architecture. Organoids offer self-organization but lack control and maturity. Microfluidics offers a dynamic environment but lacks throughput and architectural complexity. Bioprinting offers architectural control but faces bioink and resolution challenges. This analysis reveals a clear opportunity for a hybrid approach that integrates the architectural control of high-resolution bioprinting with the dynamic environment of perfusion, using advanced bioinks that provide both the necessary biochemical cues and mechanical properties.

Table 1.2: Comparative Analysis of 3D Hepatic Culture Platforms

Platform	Principle	Architectural Control	Key Advantages	Key Limitations
Spheroids	Scaffold-free; self-aggregation of cells.	None to very low; size and shape are uncontrolled.	Low cost, simple to implement. Simplicity, scalability for HTS.	Necrotic cores, no architectural control, no defined ECM interaction, lacks complex 3D tissue architecture.
Organoids	Scaffold-free; stem cell self-organization and differentiation.	Low; relies on intrinsic self-organization, resulting in high variability.	High cost, complex and lengthy protocols. Patient-specific disease modeling, recapitulates development.	Immaturity, lack of vasculature, high variability, reliance on undefined matrix (Matrigel).
Bioprinted Lattices	Scaffold-based; precise deposition of cell-laden bioink into a pre-designed structure.	High; precise, user-defined control over macro- and micro-geometry from a CAD file.	Moderate to high cost (printer), but can be automated. Bioink development is complex. Unparalleled architectural control, reproducibility, potential for vascularization.	Bioink development, printing resolution limits, potential for cell damage during printing.

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Table 1.2 continued from previous page

Platform	Principle	Architectural Control	Key Advantages	Key Limitations
Organ-on-Chip	Dynamic culture of cells under continuous fluid flow in a micro-device.	Low to moderate; controls channel geometry but often uses simple 2D/3D cell layouts.	High cost (chip fabrication, pumps), technically demanding to operate. Dynamic perfusion, long-term functional maintenance, multi-organ integration.	Low throughput, technical complexity, often lacks cell-cell and cell-ECM interactions in complex 3D arrangements.

1.8.4 3D Printing for Hepatic Tissue Models

Recent research has made considerable progress in tackling the challenges mentioned earlier and using 3D printing to develop hepatic tissue models. This section reviews key studies that have utilised 3D printing to advance the state-of-the-art in liver tissue engineering, focusing on the influence of scaffold architecture, innovations in bioink development, and the creation of multi-cellular, complex constructs.

Early work in scaffold-based tissue engineering established that architectural cues like pore size and interconnectivity are critical determinants of cellular behaviour (Ranucci et al., 2000; Loh and Choong, 2013). A key study by Lewis et al. (2018) systematically demonstrated that scaffold architecture directly modulates hepatocyte function. By using extrusion-based 3D printing to create gelatin scaffolds with identical porosity but different pore geometries, they showed that a more interconnected network significantly enhanced metabolic outputs like albumin and urea secretion. This work was crucial in isolating the influence of physical cues from material chemistry.

While synthetic and simple natural polymers provide excellent printability, they often lack the tissue-specific biochemical cues of the native liver. To address this, many researchers have focused on developing bioinks from dECM, which retains the complex mixture of proteins and growth factors of the source tissue (Isaeva et al., 2022).

Pioneering work by Pati et al. (2014) was instrumental in establishing dECM as a viable bioink for 3D printing (Pati et al., 2014). They developed a printable hydrogel from dECM that supported cell viability and promoted tissue-specific function, representing a significant advance towards

creating more biomimetic chemical microenvironments. Nevertheless, this study also highlighted a primary limitation of pure dECM bioinks, their inherently poor mechanical properties and low viscosity, which constrain the fabrication of complex, high-fidelity structures. Building on this, Lee et al. (2017) specifically developed and characterized a liver dECM bioink for extrusion printing, confirming that hepatocytes printed within it showed significantly higher expression of mature hepatic markers compared to those in a standard collagen bioink (Lee et al., 2017).

To overcome some of these fabrication challenges, Mao et al. (2020) employed DLP, a vat photopolymerization technique, to fabricate liver dECM-based constructs (Mao et al., 2020). This approach enabled the rapid creation of dECM hydrogels with greater geometric control, though a potential drawback is lack of complexity in the design due to the lack of a printing platform with enough resolution.

Further efforts have focused on improving the functionality and printability of these biomimetic inks. The work of Cuvellier et al. (2021) optimised a GelMA bioink for the co-printing of HepaRG progenitor cells with stellate cells, developing a robust platform for long-term toxicity studies.

To address the poor printability of dECM, Tabatabaei Rezaei et al. (2025) developed a photo-crosslinkable dECM-GelMA composite bioink, which combines the biomimetic advantages of dECM with the superior fabrication capabilities needed for high-resolution stereolithography .

Using P μ SLA, Sphabmixay et al. (2021) developed a perfusable, meso-scale 3D liver model using high-resolution stereolithography that could be maintained in culture for up to four weeks (Sphabmixay et al., 2021). They successfully used P μ SLA to fabricate a large scaffold ($\approx 1 \text{ cm}^3$) with an integrated, perfusable vascular network, recapitulating hepatic lobules. This

design integrated in a microchip device overcame the common limitation of nutrient and oxygen transport that prevents the long-term culture of larger engineered tissues. They also demonstrated the model's utility by inducing a disease state, thereby successfully recapitulating key features of steatosis and insulin resistance, demonstrating that the model is a functionally responsive platform for studying metabolic diseases (Sphabmixay et al., 2021).

Sphabmixay et al. (2021) demonstrated the feasibility of using specialised and precise printing systems to recapitulate geometries found in the organ tissue. The incorporation of these complex geometries enhanced hepatic behaviour, in this case, it maintained its activity and functionality for long-term culture models.

Regarding complexity, another way of achieving the mimicking of tissue in hepatic tissue engineering is to recapitulate the complex, multi-cellular organisation of the native liver lobule (Krishna, 2013). A landmark study by Ma et al. (2016) took a significant step in this direction by using P μ SLA (Ma et al., 2016). They successfully printed iPSC-derived hepatocytes with endothelial and mesenchymal support cells in a hexagonal, lobule-mimicking pattern, demonstrating the feasibility of deterministic multi-cellular positioning. Whilst architecturally sophisticated, the model's application was limited to the micro-scale and utilized a synthetic PEGDA hydrogel, which lacks the complex biochemical cues of a natural matrix.

Focusing on vascularization, Lee et al. (2016) developed a tri-culture system by co-printing hepatocytes with endothelial cells and adipose-derived stem cells (Lee et al., 2016). This approach successfully promoted angiogenesis, leading to the formation of vessel-like networks within the construct that correlated with enhanced liver-specific functions. A limitation of this

method, however, was its reliance on spontaneous self-organization for vascular network formation, which offers less control over the final vascular architecture. To more precisely engineer the interface between different cell types, Taymour et al. (2021) utilized a co-axial (core-shell) printing technique (Taymour et al., 2021). By printing hepatocytes in the core of a filament and fibroblasts in the shell, they ensured direct heterotypic cell-cell contact, which markedly improved hepatocyte functionality. While effective at the micro-scale, this method produces simple filamentous structures and has not yet been scaled to create a complex, organ-like architecture.

The work of Janani & Mandal represents a significant step in combining these multi-cellular and biomimetic ink approaches. They printed a construct using a silk-gelatin-dECM bioink that guided cells into forming zone-specific patterns, effectively beginning to replicate the metabolic zonation of the native liver lobule (Janani et al., 2022).

However, as highlighted in a recent systematic review by Ali et al. (2024), a significant lack of standardisation in reporting printing parameters and functional outcomes across these studies remains a major barrier to progress, hindering direct comparisons and slowing clinical translation (Ali et al., 2024).

In summary, 3D printing for liver tissue engineering has progressed from establishing the influence of scaffold architecture on cell function to developing sophisticated bioinks like dECM and creating multi-cellular, vascularised constructs. While significant achievements have been made, each approach carries a unique profile of advantages and limitations regarding architectural control, physiological relevance, and technical feasibility. To provide a clear overview of how these 3D printing strategies compare to other modern culture platforms, Table 1.3 offers a comparative analysis.

Table 1.3: Summary of Key Studies on 3D Printing of Hepatic Tissue Models

Author(s) & Year	Material & Fab. Method	Cell Type(s)	Architectural Feature	Key Findings / Achievements
Pati et al. (2014)	dECM (Extrusion)	HepG2	—————	Pioneered dECM as a bioink.
Ma et al. (2016)	PEGDA Hydrogel (PpSLA)	iPSC-Hepatocytes, HUVECs, MSCs	Hexagonal	Achieved multi-cellular patterning at the micro-scale enhancing function.
Lee et al. (2016)	dECM Hydrogel (Extrusion)	Primary Hepatocytes, HUVECs, ADSCs	—————	Promoted angiogenesis and the formation of vessel-like networks.
Lewis et al. (2018)	Gelatin (Extrusion)	HepG2	Pore Geometry (0°/90° vs. 0°/45°)	Showed that a more interconnected pore geometry enhanced albumin and urea secretion
Mao et al. (2020)	Liver dECM (DLP)	hiHep Cells	Vili-like ring	Successfully printed liver dECM using DLP

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Table 1.3 continued from previous page

Author(s) & Year	Material & Method	Cell Type(s)	Architectural Feature	Key Findings / Achievements
Taymour et al. (2021)	Alginate/Gelatin (Co-axial Extrusion)	HepaRG Cells, Fibroblasts	Core-shell (hepatocyte- fibroblast) filaments	Engineered direct heterotypic cell contact.
Cuvellier et al. (2021)	GelMA (Extrusion)	HepaRG, LX-2 (stellate) cells	—————	Developed a robust platform for long-term viability and mature hepatocyte function
Sphabmixay et al. (2021)	Custom Photopolymer (P μ SLA)	Primary Human Hepatocytes	Meso-scale scaffold with perfusable vascular network	4-week culture of a larger tissue volume and modelled fatty liver disease.
Janani et al. (2022)	Silk-Gelatin- dECM (Extrusion)	Hepatocytes, HUVECs, ADSCs	Zonal gradient architecture	Mimicked liver lobule zonation.
Tabatabaei Rezaei et al. (2025)	dECM-GelMA (SLA)	—————	—————	Addressed the poor printability of dECM by creating a composite hydrogel suitable for SLA printing.

1.9 Gaps in Current Work Addressed by This Thesis

The preceding review of the state-of-the-art in hepatic tissue engineering reveals a field rich with activity yet constrained by specific, interconnected challenges. While significant progress has been made in moving beyond simplistic 2D cultures, current 3D platforms each present a distinct set of compromises, leaving critical gaps in the ability to fully replicate and systematically study the liver microenvironment. This thesis is designed to address three fundamental gaps, biochemical, architectural, and integrational, by proposing a novel platform that synergistically combines advanced materials science with high-fidelity additive manufacturing to enable a new level of control over the engineered tissue niche.

1.9.1 The Biochemical Gap: The Need for Defined, Tuneable Native Recapitulation

The first major gap lies in the biomaterials used to construct the scaffolds. The field is caught between two imperfect options. On one hand, decellularised ECM (dECM) offers the complete, tissue-specific biochemical milieu, making it the theoretical "gold standard" for biomimicry (Isaeva et al., 2022). While decellularised tissue products have seen widespread and significant clinical success (Parmaksiz et al., 2016; Liu et al., 2024), translating their use to high-precision applications like 3D bioprinting introduces distinct challenges. The derivation of dECM from biological sources can lead to inherent batch-to-batch variability in composition and rheological properties (Pati and Cho, 2017). This variability, alongside processing steps that may alter the matrix structure, presents a significant hurdle

for achieving the high level of reproducibility and material consistency required for controlled 3D printing processes (Zhe et al., 2023). On the other hand, purely synthetic polymers like PEGDA offer excellent reproducibility and mechanical tunability but are bio-inert and lack the essential biological cues needed to support hepatocyte adhesion and function (Tan et al., 2021). Additionally, GelMA alone does not have the mechanical properties to replicate complex geometries that mimic hepatic micro-environments (Tan et al., 2021).

After reviewing the current state-of-the-art in hepatic tissue models a gap was identified. There is a critical need for a bioink suitable for high-resolution light-based printing such as P μ SLA that bridges this divide, offering the biological relevance of the native ECM within a defined, reproducible, and mechanically tuneable hydrogel system for hepatic tissue models. This thesis will address this gap by developing and utilizing an GelMA-PEGDA hydrogel. Although it is currently being used for other tissues and printing techniques (Wang et al., 2018; Vila et al., 2020; Wang et al., 2020b; Sheng et al., 2021; Duan et al., 2022; Yu et al., 2022; Gao et al., 2023; Ferchichi et al., 2024a; Fowler et al., 2025), its use for hepatic tissue models using P μ SLA is still unknown. This hybrid system is fundamentally defined and controllable. The GelMA component provides essential cell adhesion such as RGD, and remodelling (MMP-sensitive) sites, while the PEGDA component provides tuneable mechanical stiffness and structural stability. This additive manufacturing approach aims to capture the key biochemical functionalities of the ECM without inheriting the variability and processing damage associated with the dECM scaffold approach, thus creating an enhanced material for systematic biological studies.

1.9.2 The Architectural Gap: The Need for High-Resolution, Bio-inspired Geometry

The second gap concerns the physical architecture of the scaffold. It is now well-established that scaffold geometry is a potent regulator of cell function, with parameters like pore size, interconnectivity, and curvature directly influencing hepatocyte behaviour (Ranucci et al., 2000; Lewis et al., 2018; Swanson et al., 2022). However, most studies have been limited by fabrication methods that produce overly simplistic or random geometries. While the superiority of bio-inspired, stochastic architectures over engineered, periodic ones has been demonstrated in bone tissue engineering (Gómez et al., 2016; Frayssinet et al., 2022; Kanwar and Vijayavenkataraman, 2022; Kechagias et al., 2023), this principle has not been systematically tested for soft tissues like the liver.

A rigorous, direct comparison of the biological impact of periodic versus stochastic scaffold architectures on hepatocyte function is lacking. It is unknown whether replicating ECM with the complex, multi-scale curvature and heterogeneous nature of a stochastic lattice provides a superior microenvironment for hepatic maturation compared to a highly ordered, predictable periodic lattice. This thesis will address this gap by leveraging the power of P μ SLA to fabricate a library of scaffolds with precisely defined yet architecturally distinct geometries. By using computational design tools, scaffolds with mathematically defined periodic and stochastic (Voronoi-based) architectures will be created. The recent finding that the native liver’s micro-architecture is accurately described by Voronoi diagrams, a type of stochastic geometry, provides a strong, data-driven rationale for applying this design principle to hepatic tissue engineering (Lau et al., 2021). P μ SLA’s high resolution will ensure these complex designs are

fabricated with high fidelity, enabling a direct comparison of the two types of design paradigms while keeping other variables like material composition and overall porosity constant.

1.9.3 The Integration Gap: The Synergy of Material and Manufacturing

The third and most crucial gap is the lack of integration between advanced materials and advanced manufacturing to create a platform for systematic mechanobiology. The true potential of the field can only be unlocked by combining a highly controllable fabrication process with a highly tuneable material system. While individual components exist, few studies have leveraged their synergy to deconstruct the complex relationship between a scaffold's physical design and the resulting biological function. There is a need for an integrated experimental platform that can systematically and independently vary the biochemical, mechanical, and geometric properties of the cellular microenvironment to dissect their individual and combined effects on hepatocyte fate. The central premise of this thesis is to create and exploit such an integrated platform by combining P μ SLA, implicit design tools and GelMA-PEGDA bioink. This platform is uniquely powerful because it allows for the decoupling of key variables. For instance, one can print scaffolds with identical stochastic geometries but with different stiffnesses (by varying the PEGDA concentration) to study the role of mechanics. Alternatively, one can print scaffolds with the exact same stiffness but with different geometries (periodic vs. stochastic) to isolate the role of architecture. This systematic approach moves the field beyond qualitative observations and toward the development of quantitative, evidence-based design principles for functional hepatic tissue engineering. By filling these

biochemical, architectural, and integrational gaps, this work aims to provide fundamental new insights into how to rationally design biomaterials and scaffolds to guide liver tissue formation.

1.10 Summary and Research Objectives

1.10.1 Summary of the State-of-the-Art and Knowledge Gaps

The field of liver tissue engineering is driven by the dual imperatives of creating predictive *in-vitro* models to combat drug-induced liver injury and developing therapeutic tissues to address the chronic shortage of donor organs. The success of both attempts hinges on the ability to recapitulate the complex microenvironment of the native liver, which governs hepatocyte function through an intricate interplay of multicellular interactions, ECM geometry, and physical cues. Decades of research have established that simple two-dimensional cultures fail to maintain the mature hepatocyte phenotype, necessitating the move to three-dimensional systems. Current 3D platforms, including scaffold-free spheroids and organoids, scaffold-based bioprinted constructs, and perfused microfluidic chips, each offer distinct advantages but also suffer from significant limitations in control, reproducibility, and physiological fidelity. A promising path forward lies in leveraging the native ECM as a design template. However, using decellularised ECM directly is plagued by processing-induced damage and batch-to-batch variability. A more robust strategy is to engineer bioinks for additive manufacturing, combining key biological motifs with the tuneable and reproducible properties of synthetic polymers. This approach, however, requires an equally advanced fabrication technology. The emer-

gence of high-resolution additive manufacturing, particularly P μ SLA, now allows for the creation of scaffolds with precisely defined, complex micro-architectures. This technological convergence has opened a new frontier of inquiry focused on using architecture as a guidance, where the geometry of a scaffold is recognized as a potent regulator of cell fate via mechanotransduction. While the influence of simple geometric features like pore size and interconnectivity has been demonstrated, the behaviour of hepatocytes in an ECM-like geometry has not yet been investigated. Specifically, it is unknown whether bio-inspired, heterogeneous stochastic geometries, replicating the native ECM geometry found in liver tissue, offer a functional advantage over highly ordered, engineered periodic geometries in the context of soft tissue engineering. This lack of a systematic, comparative investigation represents a critical knowledge gap that prevents the establishment of rational design principles for functional liver tissue.

1.10.2 Thesis Aims and Hypothesis

This thesis aims to bridge these gaps by developing and utilizing an integrated platform that combines photocurable hydrogels with high-resolution P μ SLA printing to systematically investigate the influence of ECM-like scaffold architecture on hepatocyte maturation and function.

The central hypothesis of this work is that 3D hydrogel scaffolds fabricated with bio-inspired, stochastic architectures will promote superior hepatocyte maturation and function compared to scaffolds with mathematically periodic geometries, due to the recapitulation of more native-like and heterogeneous mechanotransductive cues provided naturally by ECM. To test this hypothesis, the following specific research objectives, illustrated in Figure 1.4, will be pursued.

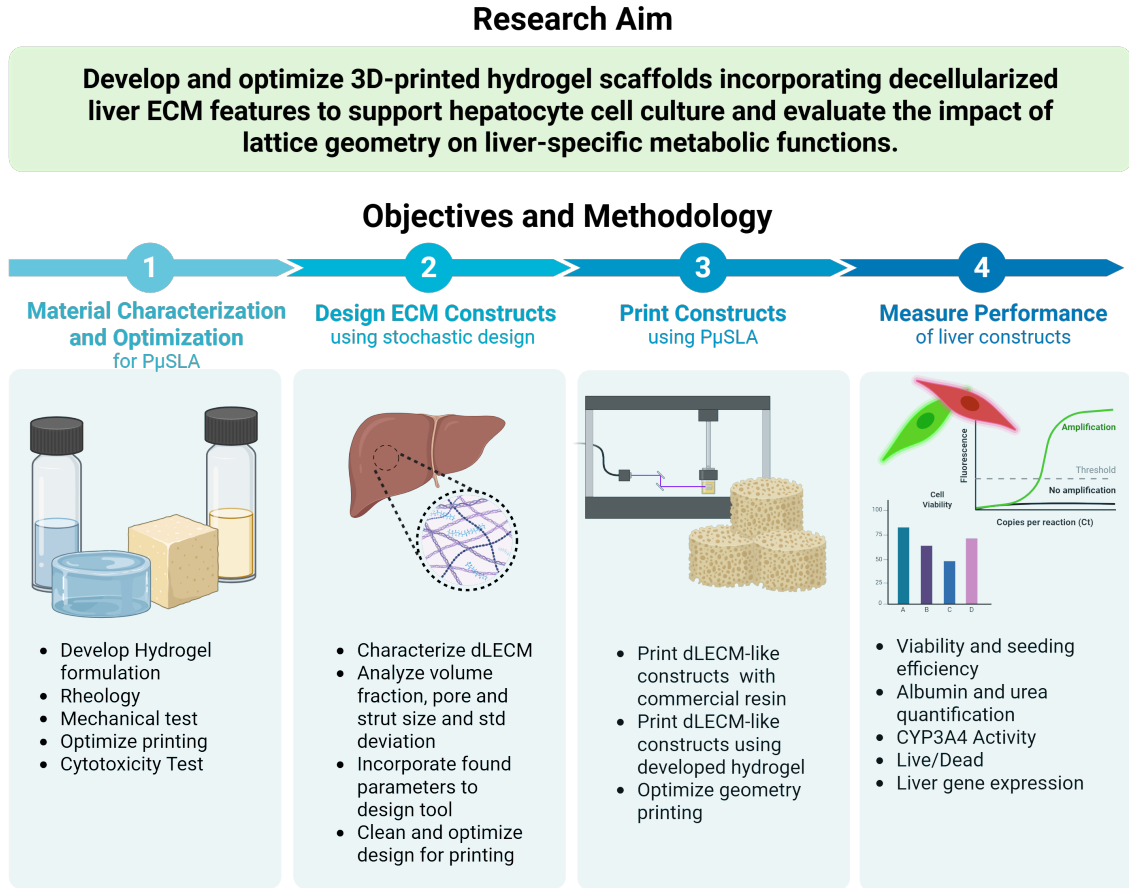


Figure 1.4: Research aim and stages.

Objective 1: To develop and characterise a photocurable GelMA-PEGDA hydrogel bioink suitable for high-resolution PμSLA. This objective addresses the need for a defined and mechanically tuneable bioink. The hydrogel will be characterised for its rheological, mechanical, and swelling properties, and its cytocompatibility will be confirmed to ensure the hydrogel is printable, non-toxic, and mechanically analogous to liver tissue.

Objective 2: To design biomimetic scaffolds by using quantitative architectural data from native liver ECM to inform the geometry of both periodic and stochastic lattice models.

Objective 3: To fabricate the designed scaffolds using high-resolution

P μ SLA and to validate that the developed hydrogel can be printed with high fidelity. This step is critical to validating whether the hydrogel formulation developed could withstand the photopolymerization process while preserving the complexity of the intended design.

Objective 4: To systematically evaluate how scaffold geometry influences hepatocyte function by performing a comprehensive suite of assays. These assessments aim not only to confirm the biocompatibility of the materials but also to provide insights into how scaffold geometry influences liver-specific cellular functions.

Together, these objectives and methodology provide a robust framework for investigating how ECM-inspired lattice geometry and material formulation influence scaffold performance and hepatocyte function in 3D-printed liver tissue models. Each step was intentionally designed to bridge the gap between biomaterial development, architectural design, and biological outcome, ensuring that experimental decisions are grounded in both engineering principles and biological relevance.

Chapter 2

Methodology

2.1 Introduction

This chapter outlines the methodological framework employed to achieve the research objectives, providing a comprehensive description of the experimental design, materials, and analytical techniques used throughout the study following the research objectives described in section 2.10.2 and illustrated in Figure 1.4.

2.2 Hydrogel Formulation, Printing and Characterization

2.2.1 GelMA Synthesis

Two types of protocols were tried for GelMa synthesis, aiming for a higher degree of methacrylation and consistency between batches.

Methodology A: Synthesis was done according to protocol on Shirahama et al. (2016). Gelatine from cold water fish skin (Sigma-Aldrich, G7041) or porcine skin (Sigma-Aldrich, G2500) (10% w/v) were dissolved in .25 M carbonate-bicarbonate buffer at 60°C with continuous stirring in a Erlenmeyer flask to avoid phase separation. pH was then adjusted to 9.0 using 5.0 NaOH. While stirring at 60°C, .2 ml of methacrylic anhydride (MMA) (94%, 276685, Sigma-Aldrich) per gram of gelatin were added dropwise and let to react for 2 hours. To neutralize the reaction, 5M sodium hydroxide (NaOH) was used to bring the mix to a 7.4 pH. Once neutralized, the solution was precipitated dropwise in ten times of the reaction mixture volume of Industrial Methylated Spirits (IMS) (74 OP. M/4550/17 Fisher). When finished, the precipitated GelMA was filtered to remove the excess of IMS

2.2. HYDROGEL FORMULATION, PRINTING AND CHARACTERIZATION

by compressing it. GelMA was then redissolved in 50 ml of distilled water (dH_2O) at 60°C while stirring. Precipitation was made two more times to make sure unwanted components were removed. After final precipitation GelMA was left in a vacuum oven for 12 hours to then be resuspended in distilled water and transferred to a freezer overnight. Next day the frozen samples were freeze-dried for 48 hrs. to remove water. Freeze-dried GelMA was then ground into powder and stored at -20°C . The whole process is illustrated in section A in Figure 2.1.

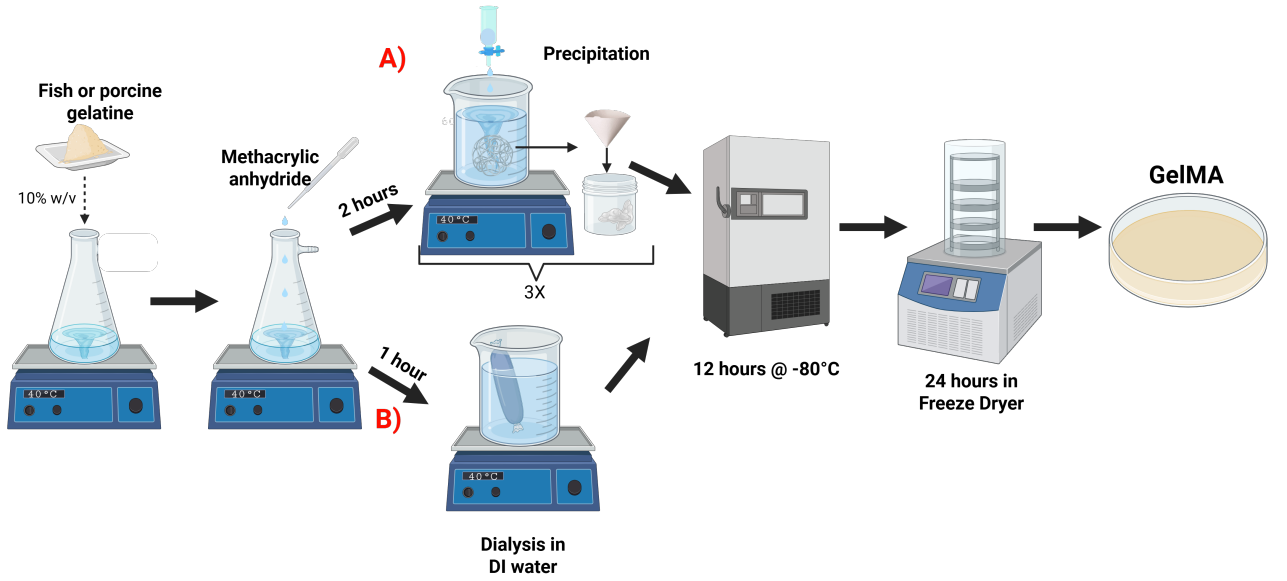


Figure 2.1: Schematic illustration of the two methods used for GelMA synthesis. Method A was adopted from Shirahama et al. (2016) while method B was adopted from Van Den Bulcke et al. (2000)

Methodology B: Following the protocol from Van Den Bulcke et al. (2000), a phosphate buffer was prepared using 17.8 g of disodium phosphate (Na_2HPO_4) and 6.8 g of monopotassium phosphate (KH_2PO_4) in 1L of Milli-Q water, pH was brought to 7.8 using NaOH and left stirring at 40°C at 400 rpm. When the buffer was heated and dissolved, 100 gr of fish gelatine were dissolved in the buffer at 60°C while slowly sprinkling the gelatine in the buffer

solution until it was all dissolved. When dissolved, 15 ml of methacrylic anhydride were added to the mixture and left 1 hr. to react at 40°C at 650 RPM. After the reaction time had passed, 1 L of Milli-Q water was added to the mixture and the solution was dialyzed in Milli-RO water for 24 hrs at 40°C during which water was changed 5 times. After dialysis, the pH solution is adjusted to 7.4 using NaOH and transferred to Petri dishes to the -20°C freezer. When completely frozen, Petri dishes were freeze dried for 48 hrs. The product was then stored in the -80°C freezer.

2.2.2 GelMA Degree of Methacrylation

The degree of methacrylation (DM) refers to the extent to which methacryloyl groups are introduced into gelatin molecules during the methacrylation process. This parameter is crucial as it directly influences the mechanical properties, crosslinking density, and overall performance of the resulting GelMA. Proton nuclear magnetic resonance (NMR) spectra was used to find out the DM of the three batches prepared during this project.

.017 g of Fish GelMA as well as cold water fish skin gelatin were diluted in 700 μ l of deuterated Dimethyl sulfoxide (DMSO)(Sigma-Aldrich) and pipetted into a 5 mm NMR tube. The tube was subjected to a proton and nitrogen-16 NMR test. To determine the DM of Fish GelMA, both gelatine and GelMA spectra were baselined corrected and normalized integrating the phenylalanine signal between 7.4 and 6.9. Then GelMA's lysine methylene signal between 2.9 and 3.1 was integrated and compared with gelatine's. Equation 2.1 was used to determine GelMA's DM.

$$DM(\%) = 1 - \left(\frac{\text{Area of lysine methylene of fish GelMA}}{\text{Area of lysine methylene of unmodified gelatine}} \right) \times 100 \quad (2.1)$$

2.2.3 GelMA Hydrogel Composition

GelMA Hydrogel was made according to the composition found in Table 2.1. Fish or Porcine GelMA were diluted in ultra pure water at 60°C while stirring constantly at 500 RPM. When diluted, Lithium phenyl- 2,4,6-trimethylbenzoylphosphinate (LAP) (Sigma Aldrich, LAP, $\geq 95\%$, 900889) and tartrazine (Sigma Aldrich, Dye content $\geq 85\%$, Powder, T0388) were incorporated and left to stir for 20 min. protecting the ink formulation from direct light exposure while mixing. Hydrogel formulations were then stored at room temperature and used within 1 week.

Chemical	Function	Concentration
GelMA	Polymeric matrix	12.50 %
LAP	Photoinitiator	0.50 %
Tartrazine	Photoabsorber	0.25%

Table 2.1: Composition of GelMA Hydrogel

2.2.4 GelMA-PEGDA Hydrogel Composition

GelMA-PEGDA hydrogel was formulated with the composition seen in Table 2.2. Fish or Porcine GelMA were diluted in water at 60°C while stirring constantly at 500 RPM. When GelMA was totally dissolved, PEGDA (Sigma Aldrich, average Mn 700, 455008) was introduced to the mixture and left stirring for 5 min. The right amount according to Table 2.2 of LAP and Tartrazine were incorporated into the mixture protecting the ink

formulation from direct light exposure while mixing for 20 min. Hydrogel was then stored at room temperature and used within 1 week.

Chemical	Function	Concentration
GelMA	Cell Attachment	7.50 %
PEGDA	Structural Strength	5.00%
LAP	Photoinitiator	0.50 %
Tartrazine	Photoabsorber	0.25%

Table 2.2: Composition of GelMA-PEGDA Hydrogel.

2.2.5 Rheology and Tilt Test

Rheological measurements were conducted using an Anton Paar MCR302 rheometer equipped with a cone plate (CP25-1). The cone plate had a diameter of 25 mm and a preset gap of 0.051 mm, with the temperature maintained at 24°C throughout the measurements to ensure consistency.

A volume of 85 μl of hydrogel sample was carefully loaded onto the plate to minimize air bubble formation and ensure even distribution. The shear rate was progressively increased following a logarithmic ramp from 0.1 to 100 s^{-1} to assess the viscosity profile and shear-thinning behaviour of the hydrogel. Data acquisition was performed continuously during the ramp to capture detailed viscosity changes as a function of shear rate.

All measurements were conducted 5 times to ensure reproducibility, and the data were analysed to obtain viscosity curves and evaluate the shear-thinning properties of the hydrogel.

To assess the room-temperature flowability of different source derived GelMA formulations for suitability in light-based 3D printing, a qualitative tilt test was performed. Two GelMA formulations, one synthesized from pork-derived gelatin and the other from fish-derived gelatin, were prepared at

60°C at the same concentration (12% w/v in Phosphate-Buffered Saline (PBS)) and allowed to equilibrate at 24°C for 10 minutes. Each solution was transferred into a clean 7 ml glass vial, sealed, and then tilted at a 45° angle to observe flow behaviour. The test was used as a practical screening method to determine whether the solution retained a liquid-like behaviour suitable for PµSLA platform re-coating.

2.2.6 Stereolithographic 3D Printing Optimization, Fidelity and Photopolymerization Kinetics

To test printability, a TPMS structure was printed in a MicroArch S130 3D PµSLA (Boston Micro Fabrication (BMF), USA) printer using both formulations, GelMA Hydrogel and GelMA-PEGDA Hydrogel. Light intensity was varied to find optimum parameters. For both hydrogels, the starting point was the maximum intensity (165 mW/cm²) and 5 seconds of exposure time; from there the intensity was decreased by 82.5 mW/cm² maintaining the same exposure time. After printing, optical microscope images were taken of the samples and compared to the Portable Network Graphics (PNG) images used as the printing mask to measure printing fidelity by comparing the area of the mask projected to the area of the printed sample just as shown in Figure 2.2.

2.2.7 Fourier Transform Infrared (FTIR)

Liquid samples of the unpolymerized resin, a freshly printed hydrogel sample and a post processed printed hydrogel sample were prepared separately. For the resin, approximately 10 µl of the liquid precursor was directly placed onto the diamond crystal of a PerkinElmer Frontier FTIR spec-

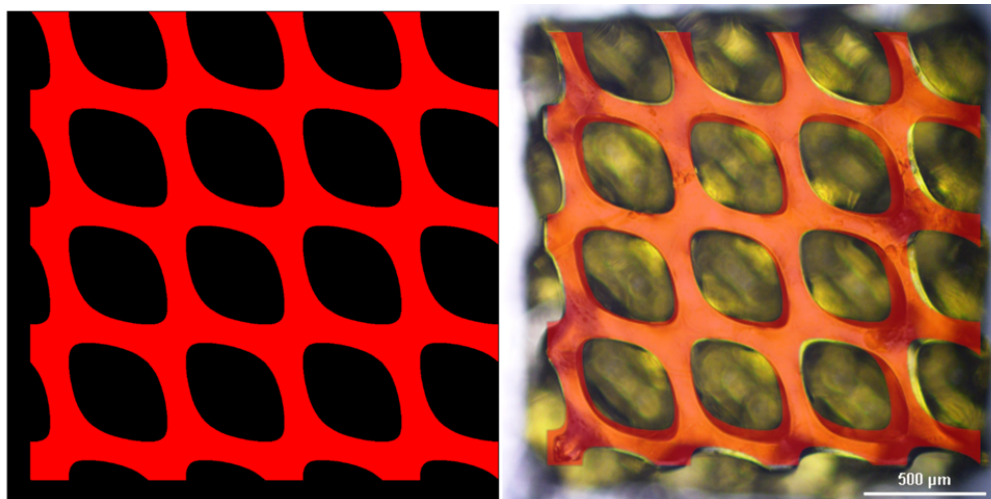


Figure 2.2: Mask projection used in PμSLA compared to the actual print used to determine printing fidelity.

trometer. Spectra were collected in the range of $1000\text{--}600\text{ cm}^{-1}$ averaging 32 scans to reduce noise. After printing, the freshly printed hydrogel was thoroughly washed with distilled water to remove any unreacted monomer. The printed sample was blotted dry to remove excess liquid and then placed onto the crystal for analysis under the same spectral settings. The same was undertaken for the post processed sample after being washed for 12 hours in distilled water. The FTIR spectra obtained from all three samples were processed to correct the baseline. Automated baseline correction was performed using Spectragryph software to minimize background noise and ensure accurate peak integration. The acrylate-specific peak at $820\text{--}800\text{ cm}^{-1}$ was identified, corresponding to the out-of-plane deformation of $=\text{C}\text{--}\text{H}$ in methacrylate groups. Peak integration was performed by setting the integration limits specifically between 820 and 800 cm^{-1} . The area under the curve was calculated to quantify the intensity of the unreacted acrylate signal. To ensure consistency between spectra, normalization was conducted using the 950 cm^{-1} peak which is commonly associated with the $\text{C}\text{--}\text{O}$ stretching vibration of methacrylate groups, which remains relatively stable before and after polymerization. The ratio of the acrylate peak area

to the reference peak area was calculated to account for any variations in sample thickness or measurement conditions. The percentage of unreacted acrylate change was determined by comparing the normalized peak area of the freshly printed sample and post processed sample to that of the liquid resin.

2.2.8 Cryo-Scanning Electron Microscopy (SEM)

To preserve the hydrated microstructure of GelMA-PEGDA hydrogels for high-resolution imaging, two cryogenic freezing techniques were employed to find out an optimal imaging technique for hydrogels: metal mirror freezing and slush nitrogen freezing. These methods enable ultra-rapid vitrification of the aqueous phase in the hydrogel, minimizing ice crystal formation and structural artifacts associated with conventional dehydration protocols. 2.5% gelatin-norbornene (GelNB) printed samples were prepared with two different techniques, metal mirror freezing and slush nitrogen freezing to find the optimal technique.

Metal Mirror Freezing

Hydrogel samples were kept hydrated prior to freezing. The mirror-polished copper block of a Leica EM MM80 E Metal Mirror Freezer was pre-cooled in liquid nitrogen for 10-15 minutes to ensure thermal equilibrium at cryogenic temperatures until it reached -196°C and stabilized. Using pre-cooled forceps, hydrogel samples were transferred onto the surface of the metal mirror and samples were quickly pressed using a second pre-cooled flat copper tool to ensure immediate and complete contact. The sample remained in contact with the metal surface for 10–15 seconds

to allow complete vitrification. Once frozen, samples were immediately submerged in liquid nitrogen and transferred to a cryo-transfer holder for fracture, sublimation, and imaging (McDonald et al., 2007).

Slush Nitrogen Freezing

Hydrogel samples were held within the slush for 20–30 seconds to ensure complete and uniform vitrification. Following freezing, the specimens were transferred under liquid nitrogen into pre-cooled storage vials or directly into a cryo-transfer stage for subsequent processing (McDonald et al., 2007).

Imaging

Once initially frozen, samples were transferred into a Quorum 3010 preparation system, sublimed at -90°C for 35 min and brought back to -150°C , then samples were sputter coated with platinum for 45 seconds. Samples were then transferred into a ZEISS Crossbeam 550 SEM, and imaged using an electron high voltage of 2.00 KV and a working distance of 5.7 mm. Once an optimal technique was found, GelMA-PEGDA Hydrogel printed samples were imaged using metal mirror freezing under the same conditions stated above.

2.2.9 Drying Rate, Swelling Test and Degradability of Hydrogel

Drying and swelling rate

To find its drying rate, GelMA-PEGDA hydrogel was cast as 1 cm³ cubes, left at room temperature and weighted every 5 min the first 15 min, doubling up time until the first hour and then at timepoints 5 hrs, 24 hrs, 72, hrs and 96 hrs. When samples were totally dehydrated, they were immersed in ultra-pure water and measured in each dimension every 5 min until they recovered their initial dimensions. This was undertaken to ascertain swelling data.

Degradability

Casted GelMA-PEGDA samples were submerged in PBS and left at room temperature and 37°C for 14 days, and the samples were weighted every 24 hrs. to determine the materials degradability. All measurements were undertaken in triplicate

2.2.10 Compression Test

Compression tests were conducted to evaluate hydrogel stiffness of the GelMA and GelMA-PEGDA Hydrogels. Tests were performed using a TA HD Plus Texture Analyser equipped with a 5000 g load cell. Casted 13 mm diameter 4 mm height cylinders of each formulation were carefully positioned between the compression plates, ensuring proper alignment to minimize uneven loading. A trigger force of 5 g was applied to establish contact between the sample and the plates before initiating the test. The

compression speed was set at 1.2 mm/min to ensure consistent deformation throughout the testing process. The test was conducted in strain-controlled mode, with a target strain of 50% to evaluate the compressive mechanical properties. Force and displacement data were continuously recorded until the target strain was reached. Five replicates of each formulation were tested to ensure reproducibility. The compressive modulus was calculated from the linear region of the stress-strain curve. Statistical analysis was performed to compare the mechanical performance across different formulations.

2.2.11 Cytotoxicity

Cytotoxicity testing was undertaken following ISO 10993-5 standards for indirect contact testing using extract from GelMA-PEGDA printed samples. Using a 6 ml/cm² extract ratio, sterilized samples were left in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, D5030) media at 37 °C for 24 hrs to prepare extract media. When L929 cells were sub-confluent they were transferred into a 96-well plate at a seeding density of 7,000 cells per well, left for 6 hrs and then exposed to extract media for 24 hrs.

PrestoBlue

PrestoBlue™ (Invitrogen A13261, USA) assay was used according to the manufacturer's instructions to determine cellular metabolic activity. Cells were exposed to PrestoBlue™ solution for 1 hr. at 37°C. After 1 hr., 10 μ l of a PrestoBlue media were taken in triplicates into the plate reader to determine metabolic activity through fluorescence.

LIVE/DEAD

LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Invitrogen L3224, USA) solution was added to the samples and were incubated 10 min at 37°C. Fluorescent microscope Nikon Eclipse Ts2 was then used with an exposure time of 100 ms to image cells in red and green channels. LIVE/DEAD ratio was then calculated using ImageJ. 20 min of IMS exposure was used as dead control while fresh DMEM media was used as live control. Assays were undertaken in triplicates (n=3) each with 2 biological replicates (N=2). This is all illustrated in Figure 2.3.

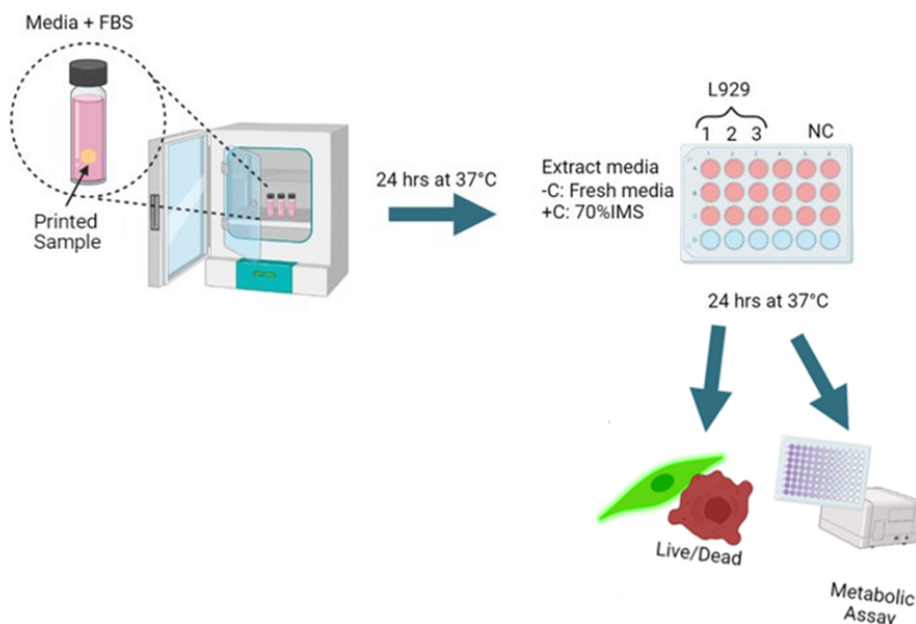


Figure 2.3: Cytotoxicity evaluation experimental plan detailing time points and assays proposed

2.2.12 Sterilization Optimization and Cell Attachment

Since constructs are printed under non-sterile conditions, before being in contact with cells they must be sterilized. A total of 4 methods were tested to explore the effect of sterilization solutions, soaking solution and time

2.2. HYDROGEL FORMULATION, PRINTING AND CHARACTERIZATION

exposed to them on cell attachment and viability. Simple 20 mm diameter 2 mm height cylinders were printed using the DLP Luminex printer. As a first attempt, after printing, constructs were left in water overnight, next day they were exposed to 405 nm UV light 20 min and then left in Penicillin-Streptomycin (Pen-Step) 10000 U/ml for sterilization for 48 hours to let it permeate the whole scaffold. Following the waiting time, Pen-Strep was aspirated and constructs were washed twice with PBS and left in PBS for another 48 hrs to wash out all the antibiotics previously used. The first attempt to sterilize ended up with long waiting periods so after using it a couple of times, the protocol was further optimized. Although this protocol worked, reducing washing times was a priority. 70% IMS was also tried and washing time was reduced to 12 hrs. After washing constructs in media at 150 RPM three times, they were left in either media or Fetal Bovine Serum (FBS) as soaking solutions. Protocol optimization is illustrated in 2.4.

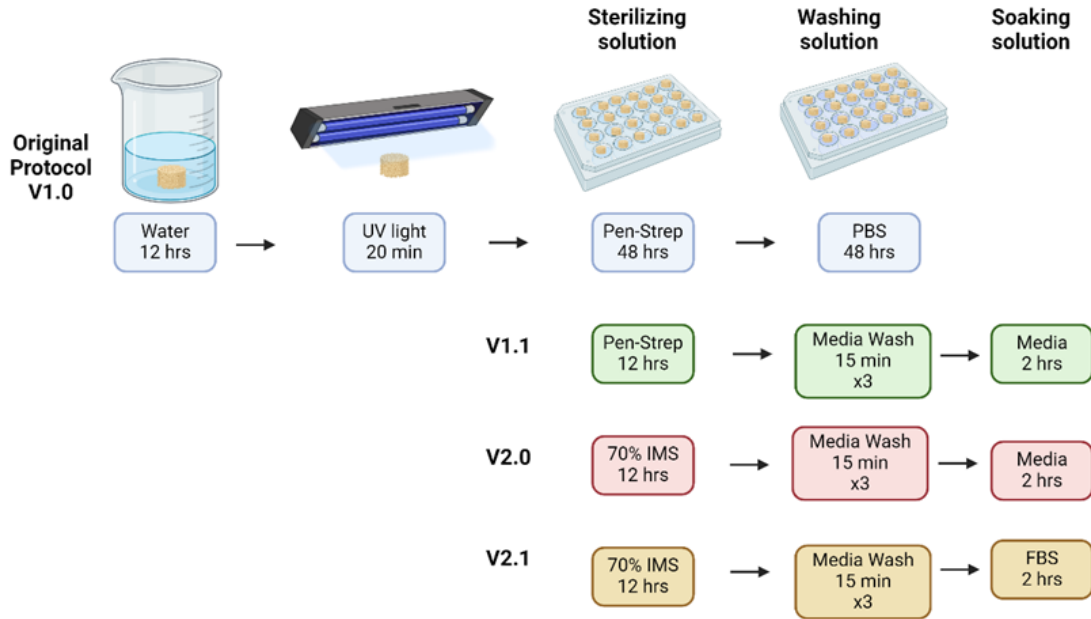


Figure 2.4: Sterilization and post printing processing optimization protocol

After sterilization and being left 2 hrs in their respective soaking solution, HepG2 cells were seeded on the samples at a seeding density of 100,000 cells/scaffold, left for 24 hrs and then observed through bright field microscopy

and LIVE/DEAD assay was also used to determine cell viability.

2.3 Lattice Design and Optimization Based in Decellularised Liver ECM

To replicate the structural features of decellularised liver ECM (dLECM), a series of scaffold geometries were computationally designed using nTopology 3.8 (nTop, New York City, USA). Design parameters, including pore size, strut thickness, and unit cell topology, were informed by quantitative analysis of dLECM images. The resulting models were optimized and exported for manufacturing using P μ SLA system BMF S130 in conjunction the GelMA-PEGDA hydrogel developed .

2.3.1 Systematic Review with Quantitative Analysis and Characterization of dLECM

Image Acquisition

SEM images of dLECM were obtained from peer-reviewed literature without a publication time restriction. The selection criteria included studies that involved dLECM from any species and utilized SEM for structural characterization. Only images with clearly distinguishable pores and struts, as well as a visible scale bar for accurate measurement were included. Images with low resolution or unclear visualization of structural features were excluded from analysis. This exclusion is essential to ensure accurate quantification and reproducibility.

Image Processing and Quantitative Analysis

Image analysis was performed using FIJI software (Schindelin et al., 2012). Images were manually analysed without any preprocessing. To ensure quality and consistency, only images in which pores and struts could be visually distinguished were used.

The quantitative analysis focused on measuring pore size, strut size, volume fraction, and the standard deviation of pore and strut sizes. Measurements were performed manually using ImageJ, with at least 150 pores and struts quantified per image, all within the same plane. Manual tracing was employed to delineate the features of interest. The scale bar present in each image was used for calibration to ensure accurate and consistent measurements across different magnifications. The parameters gathered from the image analysis were subsequently used as input parameters to design both stochastic and periodic scaffolds. Volume fraction was also measured using the threshold tool in FIJI (Schindelin et al., 2012), quantifying this way the space the ECM fibres took up in the total image space.

2.3.2 Scaffold Design

In this study, nTopology 3.8 (nTop, New York City, USA) software was employed to design and optimize the scaffolds used. nTopology offers advanced generative design capabilities that allow for the creation of highly intricate geometries that are difficult to achieve through traditional CAD software. One of the key strengths of nTopology is its use of implicit design, which enables the creation of complex, continuous structures that maintain a high degree of flexibility and adaptability. Implicit design in nTopology allows for the definition of geometry through a set of mathematical func-

tions and fields, rather than traditional mesh-based approaches, leading to more seamless and efficient designs. This approach is particularly valuable in biofabrication, where scaffold design is critical for cellular growth, nutrient delivery, and tissue engineering. Using nTopology’s powerful parametric design tools, various scaffold architectures were explored and optimized based on specific criteria such as pore and strut size, porosity distribution, and volume fraction.

Unit Cell Geometry and Distribution

Two design strategies approaches were employed. The first one had as a primary goal to create scaffolds that exhibit pore and strut size variation and a stochastic distribution that resembles the native dLECM. The second approach involved designing commonly used periodic unit scaffolds to assess the differences between incorporating stochasticity and utilizing distinct unit cell types in a periodic array.

The choice of specific unit cells was based on their reported effects on hepatocyte performance. IsoTruss which has a 60° angle connection within its struts has shown favourable impact on gene expression and metabolic activity, as reported by (Lewis et al., 2018) compared to a cubic array with 90° angle within struts. While gyroid geometries were included to explore the influence of more complex and interconnected structures. Studies have shown how triply periodic minimal surface geometries enhance different cell type behaviour (Flores-Jiménez et al., 2023). This approach allowed for a systematic comparison of scaffold architectures and their capacity to support hepatic functions, ultimately informing the selection of optimal designs for biofabrication of hepatic tissue constructs.

Stochastic Scaffolds Design

Stochastic scaffolds were generated using the nTopology platform to replicate the irregular and stochastic nature of native dLECM. The primary objective was to create scaffolds with varied pore and strut sizes while maintaining a spatially random distribution characteristic of hepatic ECM. The scaffold design employed the Voronoi Volume Lattice approach, leveraging random point distributions within a cylindrical geometry. Initially, a cylindrical implicit body was defined with a diameter of 2 mm and 0.5 mm height. The scaffold architecture was constructed using the Voronoi Volume Lattice block, configured to generate seed points by distributing them randomly within the bounding box of the cylindrical volume. This ensured the spatial heterogeneity necessary to replicate native ECM structures. To control the variation in pore size, a Linear Map function was applied, which facilitated a gradual and randomized transition between smaller and larger pores. The scalar field was mapped to the cylindrical geometry, maintaining a consistent structural shape with specific scalar field parameters set to achieve the desired randomness. The minimum input value was set to 0 mm, while the maximum input value was 1.5 mm, creating a broad range for structural variation in the pore and strut size. Similarly, the output minimum and maximum were defined as 0.045 mm and 0.095 mm, respectively, to establish variability in pore and strut dimensions according to the standard deviation shown in the dL-ECM characterization. To ensure reproducibility without sacrificing randomness, the random seed was fixed at 1. Additionally, the thickness of the lattice struts was defined as 0.03 mm to match wanted strut size .

The initial stochastic scaffold design, generated using parameters derived from dLECM characterization, exhibited a high volume fraction of 89.4%,

resulting in a highly closed structure with limited porosity. This characterization reflects an environment in which cells were introduced before ECM deposition, allowing cells to generate their own matrix within the scaffold. However, in the context of this study, cells are incorporated after scaffold fabrication, necessitating sufficient pore size to facilitate cell seeding and migration. Therefore, two additional designs were created with increased pore sizes, approximately double and triple the originally intended dimensions, changing the pore size output minimum and maximum in the Linear map function to result in pore ranges of 110–170 μm and 200–240 μm , respectively. Importantly, the standard deviation derived from the initial dLECM characterization was preserved in these alternative designs to maintain consistency in pore size variability while enhancing the overall scaffold porosity.

Finally, the generated Stochastic scaffold geometries were exported as an .STL file for subsequent printing and characterization. The building block and functions used in this design process are shown in Figure 2.5

Periodic Scaffolds Design

Periodic scaffolds were generated using the nTopology 3.8 platform to create geometrically consistent and repeatable architectures, facilitating a comparative analysis against Stochastic scaffolds. The primary goal was to evaluate how different periodic unit cells influence hepatocyte behaviour and scaffold performance. Three types of periodic lattice structures were designed: IsoTruss, Simple Cubic, and Gyroid, each offering distinct spatial configurations. The scaffold construction began by defining a cylindrical implicit body with dimensions matching the Stochastic design. The periodic lattice structures were then generated using the Periodic Lattice block,

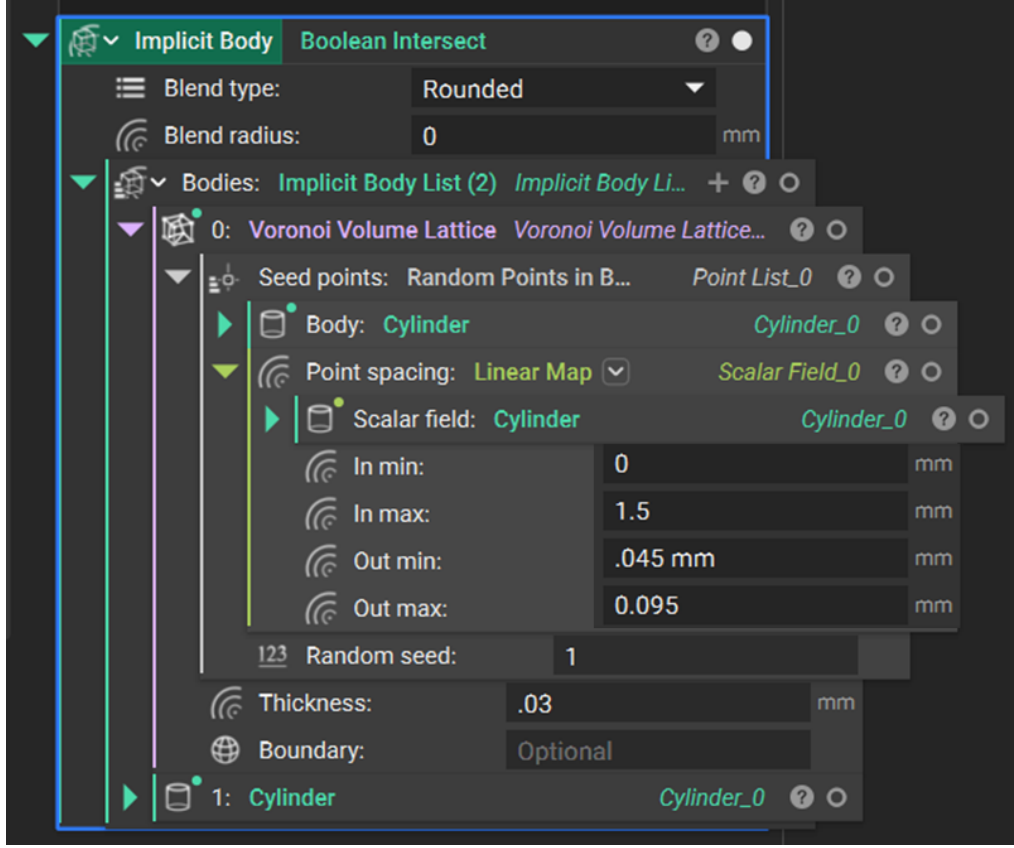


Figure 2.5: Building blocks for Stochastic lattice construction in nTopology

selecting the Walled TPMS Unit Cell for the gyroid lattice, and the the Isotruss and Simple Cubic unit cells respectively. To map the lattice configuration accurately, a rectangular cell map was applied to the cylindrical volume, maintaining consistent alignment throughout the structure. The cell size for all unit types was standardized at $0.35 \text{ mm} \times 0.35 \text{ mm} \times 0.35 \text{ mm}$ to facilitate a fair comparison and maintain the desired mean pore size from the dLECM characterization. The strut thickness for the IsoTruss and Simple Cubic designs was set to $30 \text{ }\mu\text{m}$ to balance mechanical stability with permeability. The gyroid lattice, inherently smooth and continuous, was rendered with an approximate thickness of 0.02 mm to maintain structural coherence while maximizing surface area for cell attachment. The generated scaffold geometries were subsequently exported as .STL files for 3D printing and experimental validation. The building block and functions

used in this design process are shown in Figure 2.6

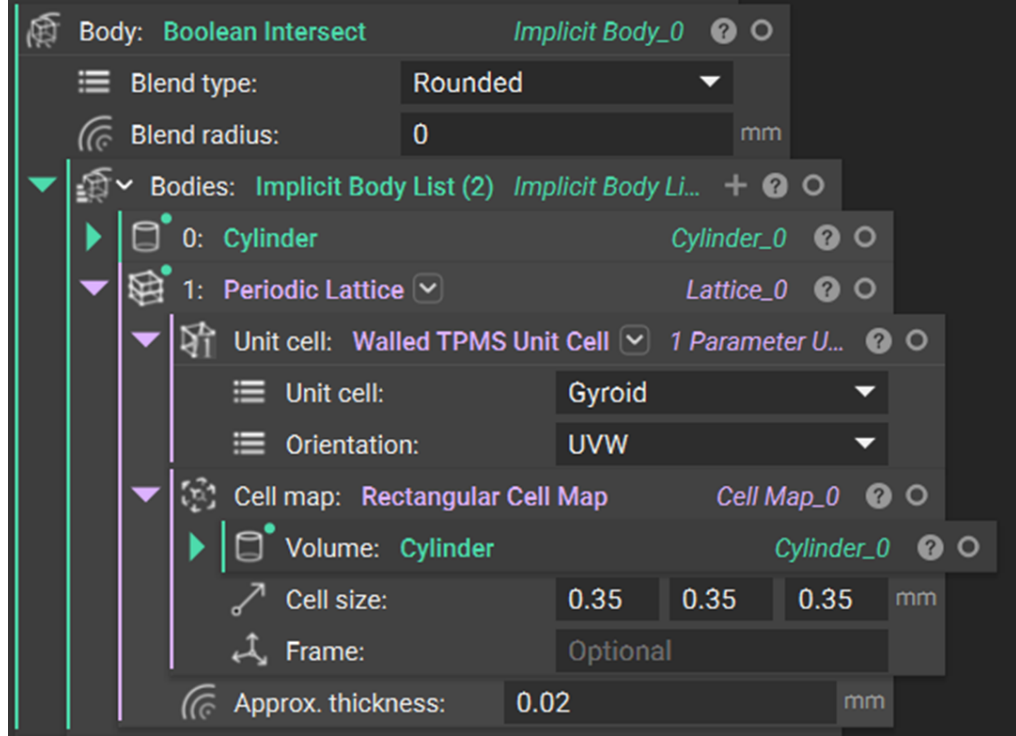


Figure 2.6: Building blocks for periodic lattice construction in nTopology

By employing these structured and periodic designs, the study aimed to delineate the impact of controlled geometrical parameters on hepatocyte functionality, contrasting with the irregularity inherent in Stochastic scaffolds.

2.3.3 Lattice Printing

Cleaning and slicing the lattice for printing

Prior to slicing and printing, the STL files of the scaffold designs were meticulously processed to ensure high-quality and accurate prints. Before 3D printing, STL files must be carefully processed and cleaned to ensure that the digital model accurately represents a printable geometry. Since the STL format describes the surface of an object as a collection of tri-

angular meshes without information on scale, topology, or physical units, errors can easily occur during model generation, conversion, or export from CAD software. Typical defects include non-manifold edges, holes, inverted normals, intersecting or duplicated triangles, and open surfaces. These imperfections can prevent proper slicing, result in incorrect toolpaths, or lead to printing artefacts such as gaps, distortions, or incomplete structures.

Cleaning and repairing STL files, through mesh optimization, normal vector correction, and watertightness verification, ensures that the model forms a continuous, closed surface suitable for layer-by-layer fabrication. This step is particularly important in high-precision techniques such as P μ SLA, where even micron-scale discontinuities in the mesh can compromise feature fidelity or cause printing failure. Therefore, STL processing represents a critical stage in the digital workflow, bridging CAD design and successful physical fabrication. The cleaning procedure was performed using MeshLab 2023.12 (Cignoni et al., 2008), an open-source software specialized in mesh processing and manipulation. The cleaning workflow included four essential steps explained below and illustrated in Figure 2.7.

1. Simple Quadratic Edge Collapse Decimation: This step aimed to reduce the file size while preserving the overall geometry and structural features of the scaffold. The decimation algorithm efficiently decreased the number of mesh vertices and faces, resulting in a more manageable file for slicing without compromising the scaffold's design integrity.
2. Repair Non-Manifold Edges: To eliminate topological inconsistencies that could hinder successful printing, non-manifold edges were identified and repaired. This step ensured that the mesh remained watertight and suitable for 3D printing, preventing potential slicing errors or structural weaknesses in the final print.

3. Remove Isolated Pieces: Any small, disconnected mesh fragments that could not contribute to the overall structure were detected and removed. These isolated pieces often result from design imperfections or post-processing errors and can interfere with the slicing process or reduce print quality.
4. Close Holes: The final cleaning step involved detecting and closing any holes or gaps within the mesh. This operation ensured the structural continuity of the scaffold and enhanced the printability by producing a sealed, continuous surface.

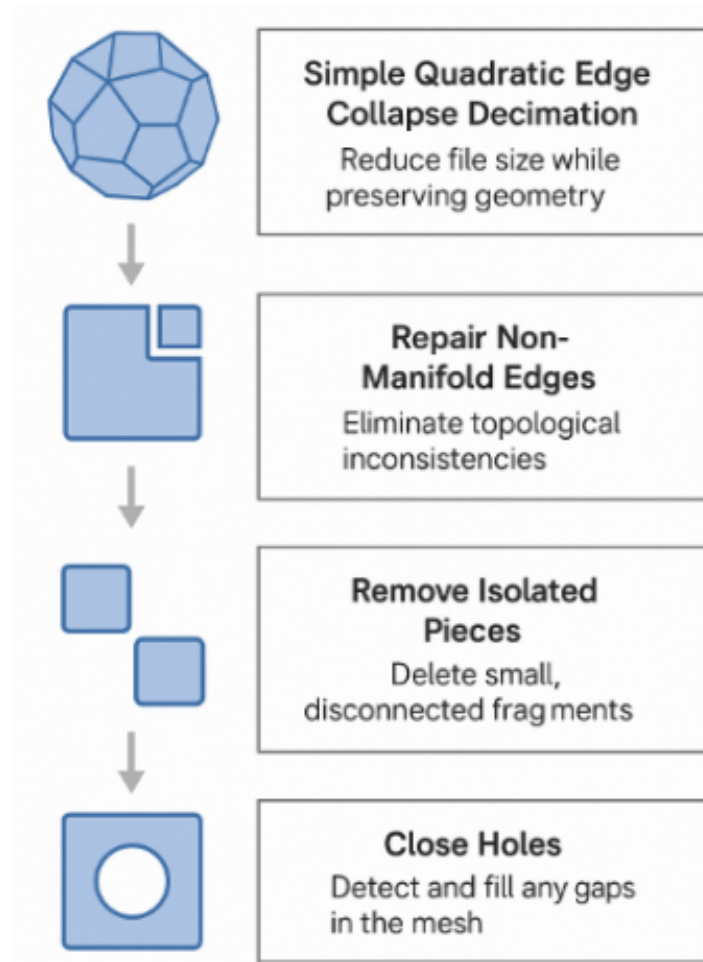


Figure 2.7: Workflow for mesh post-processing prior to 3D printing.

Following the completion of these cleaning procedures, the processed STL files were thoroughly inspected to verify the correctness and completeness

of the modifications. The cleaned files were then exported and prepared for slicing, ensuring optimal performance during the subsequent printing steps.

Slicing was performed using a cross-sectional method, where each scaffold was digitally "cut" along the z-axis to generate planar layers corresponding to the intended image projected by the μ SLA printer. Slicing was undertaken using the Boston Microfabrication slicing software and geometry was sliced every 5 μm with a total of 101 slices per scaffold.

Printing with commercial ink

The scaffolds were initially printed using HTF resin (BMF, USA) to evaluate the fidelity and structural integrity of the designs. The printing process was carried out on the small vat (7 ml) of the microArch S130 BMF system. Prints were undertaken at an intensity setting of 60, corresponding to an energy output of 49.42 mW/cm² from a 405 nm UV light source. An exposure time of 3 seconds was used during the printing process. The resin was loaded into the system, and the pre-slide geometry was introduced into to the printer's software.

Post-processing involved washing the printed scaffolds in isopropanol to remove unpolymerized ink, followed by curing under UV light for complete polymerization and stabilization. The printed scaffolds were then visually inspected under a Nikon Eclipse LV100ND optical microscope and measured to ensure dimensional accuracy and structural integrity. The printing job and specifications are shown in Figure 2.8

2.3. LATTICE DESIGN AND OPTIMIZATION BASED IN DECELLULARISED LIVER ECM

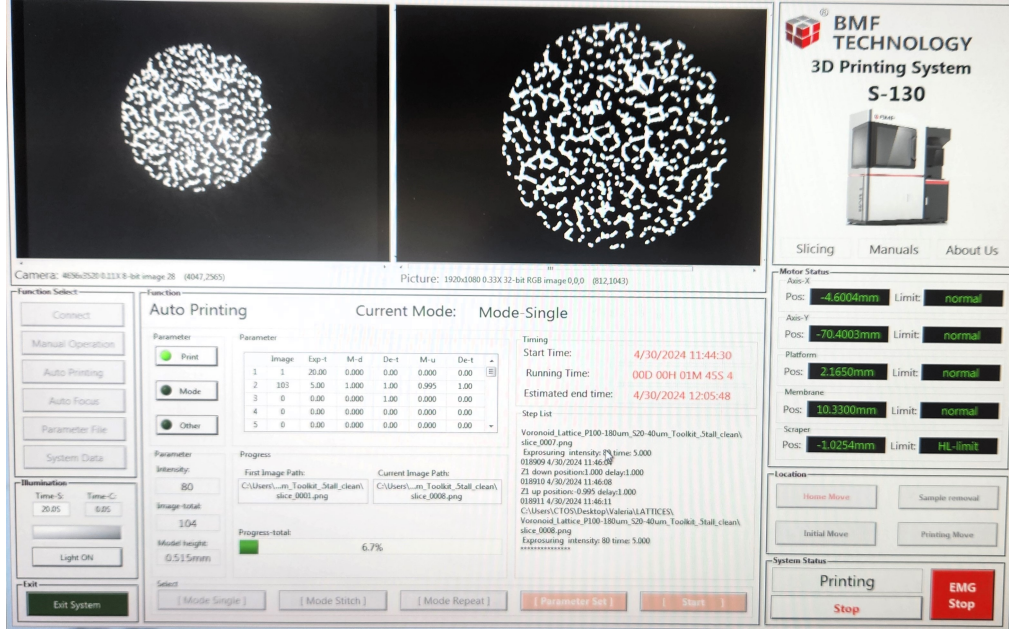


Figure 2.8: Screenshot of the BMF S130 3D printing system interface during hydrogel scaffold fabrication.

Printing with GelMA-PEGDA Hydrogel

To optimize the printing parameters for the custom hydrogel formulation, a series of trials were conducted to determine the most suitable intensity and exposure settings. The intensities tested were 60, 65, 75, and 80, corresponding to 49.42 mW/cm^2 , 53.53 mW/cm^2 , 61.77 mW/cm^2 , and 65.89 mW/cm^2 , respectively. An exposure time of 5 seconds was maintained throughout the trials. The hydrogel formulation was carefully prepared and loaded into the microArch S130 system, and the Isotruss scaffold design was used to maintain consistency during the trial. Following each print, scaffolds were left in water to keep them hydrated and to remove unreacted resin from the structure. The printed hydrogel scaffolds were assessed for structural fidelity under a Eclipse LV100ND (Nikon Metrology NV, Japan) optical microscope. The optimal intensity setting was selected based on the achieved print quality and structural integrity.

2.3.4 Lattice Analysis and Characterization

CAD Lattices

To characterize the designed lattice structures, the Lattice Analyzer module from the Osseointegrative Structures Toolkit, developed by Christopher Cho was utilised. This tool enabled a comprehensive quantitative analysis of the scaffold architectures, providing critical metrics essential for evaluating their suitability for hepatocyte culture and tissue engineering applications. The characterization process involved importing the 3D scaffold models into the Lattice Analyzer, where the tool automatically calculated key morphological parameters.

The primary metrics obtained included the volume fraction, which is essential for assessing the scaffold's potential for cell attachment and nutrient diffusion, and the pore size distribution, which directly influences cell infiltration and tissue ingrowth. These parameters were particularly crucial for comparing the stochastic and periodic scaffold designs, as well as for evaluating the impact of variations in pore size and strut thickness across different lattice geometries.

Printed Lattice

The fidelity of the printed lattice scaffolds was characterized by comparing the original design features with the printed structures to assess dimensional accuracy and consistency. Different approaches were employed for periodic and stochastic scaffolds to ensure precise evaluation.

Stochastic Lattice Scaffolds: For the Stochastic lattice scaffolds, characterization involved staining the printed constructs with Ethidium Homodimer-1 to enhance visualization of the scaffold geometry. Confocal microscopy was used to obtain a full 3D reconstruction of the scaffold by capturing a z-stack of the whole geometry. To ensure comprehensive analysis, three images were taken from each of the top (0 μm), middle (250 μm), and bottom (500 μm) sections of the scaffold. Manual measurements of pore sizes were performed on these images to evaluate whether the actual pore sizes aligned with the intended dimensions and variability range.

The results of these analyses provided a comprehensive understanding of how well the printed scaffolds matched the designed parameters, allowing for the optimization of the printing process and identification of potential sources of geometric deviation.

Periodic Lattice Scaffolds: For the periodic lattice scaffolds, printing fidelity was assessed by quantitatively comparing the pore sizes observed in the printed structures to the dimensions defined in the original CAD design. High-resolution microscopy images of the printed lattices were obtained under standardized imaging conditions to ensure consistency. From these images, pore diameters were measured manually using FIJI (Schindelin et al., 2012). These values were then compared to the nominal pore sizes defined in the design files to evaluate dimensional accuracy. The percentage deviation between the measured and intended values was used as a quantitative indicator of printing fidelity. This approach allowed for the identification of structural discrepancies introduced during the printing process and provided a metric to assess the reproducibility and resolution of each lattice geometry.

2.4 Performance of dLECM Based Lattice as Liver *in-vitro* Model

2.4.1 Seeding Optimization

The seeding optimization methods involved a series of protocols to assess the most effective approach for ensuring cell survival to the seeding protocol and attachment to the hydrogel scaffolds. The process is illustrated in Figure 2.9. The process was tested in one stochastic design and one periodic design to ensure efficacy in both types of scaffolds. Stochastic scaffolds with a mean pore size of 140 and Isotruss were utilized in this optimization process. After being exposed to UV light 20 min, both scaffold types were sterilized using 70% industrial methylated spirits (IMS) for 12 hours. After sterilization, scaffolds were washed three time with culture media for 15 minutes each under agitation (150 RPM) to remove any residual IMS.

Following media washes, scaffolds were soaked in FBS to enhance cell attachment by pre-conditioning the scaffold surface with adhesion-promoting proteins for 30 min. Some scaffolds were directly seeded after FBS treatment, while others underwent an additional PBS wash before seeding to assess whether removing excess FBS influenced attachment efficiency. After that scaffolds underwent one of two seeding procedures: (1) cells were seeded directly in the well with the scaffolds and exposed to gentle orbital agitation for 15 min at 50 RPM, or (2) the wide end of 200 μ L plastic pipette tips was cut to create a chamber for scaffold entrapment, cells were then seeded onto the scaffolds using this entrapment method to ensure close contact with the scaffold during this initial stage. These variations were designed to optimize cell penetration and adherence by preventing aggregation and improving distribution across the scaffold surface. The effective-

2.4. PERFORMANCE OF DLECM BASED LATTICE AS LIVER IN-VITRO MODEL

ness of each protocol was later evaluated through live cell MitoTracker™ Green FM staining and imaging them on day 1 and 3 to determine which combination of pre-treatment and seeding strategy yielded the highest cell viability and attachment efficiency.

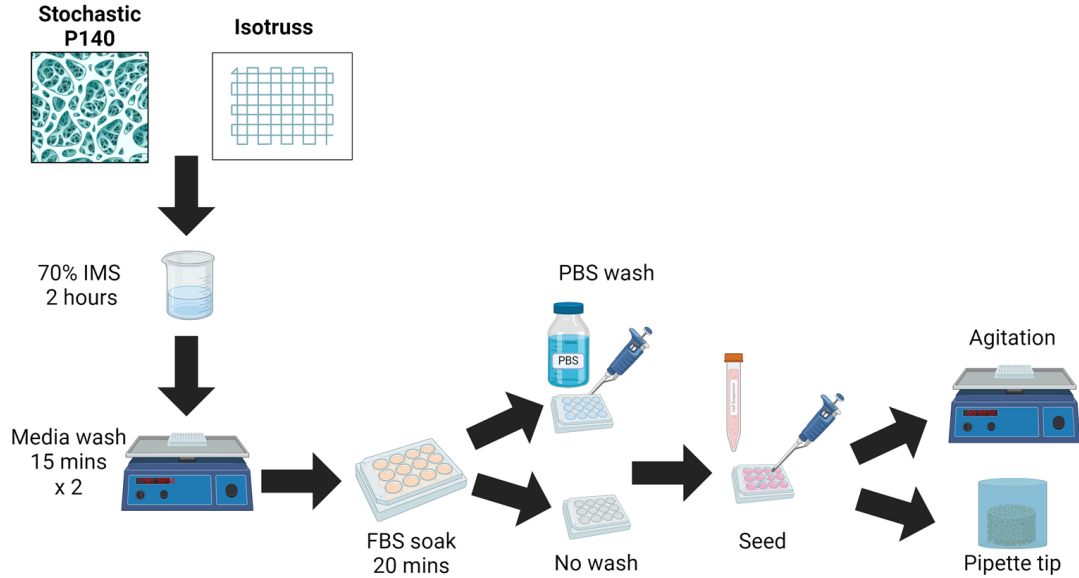


Figure 2.9: Seeding optimization protocol

Resulting seeding protocol

To ensure sterility, scaffolds were exposed to 180 nm UV light for 20 minutes, followed by immersion in 70% IMS for 12 hours. After incubation, IMS was aspirated, and the scaffolds were washed with media three times for 15 minutes under constant stirring at 150 RPM. Following post-printing processing and sterilization, scaffolds were incubated in FBS under agitation for 30 minutes, washed with PBS, and then air-dried in a biosafety cabinet for 20 minutes.

HepG2 cells were sub-cultured at least once before seeding and maintained in alpha-MEM (Gibco, USA) supplemented with 10% FBS, 1% NEAA (Gibco, USA), and 1% L-glutamine in a cell culture incubator at 37°C and

5% CO₂ until 70% confluency, at which point they were seeded. A 1×10^6 cell suspension was carefully deposited dropwise onto each scaffold using the entrapment method and then incubated overnight. The next day, the entrapment was removed and fresh media was added to each well containing the scaffolds. The seeded scaffolds were then maintained in the incubator at 37°C and 5% CO₂ until the designated time points.

2.4.2 Quantification of Double-Stranded DNA (ds-DNA)

To assess cell content retained within the printed scaffolds, dsDNA was quantified using the Quant-iT™ dsDNA Broad Range (BR) Assay Kit (Invitrogen Q33130, USA), following the manufacturer's protocol. This assay was performed on cell lysates prepared using a custom lysis buffer adapted from established protocols.

Cell Lysis Procedure For cell digestion and lysis, a custom cell assay buffer (CAB) was prepared by dissolving zinc chloride and magnesium chloride to a final concentration of 0.67 mM in 0.15 M Trizma-HCl, pH 8.0. This buffer can be stored at 4°C for extended periods. A fresh cell digestion buffer (CDB) was then prepared by diluting CAB 15% v/v in ultrapure water to achieve final concentrations of 0.15 M Trizma-HCl, 100 μ M zinc chloride, 100 μ M magnesium chloride, and 1% Triton X-100. This working buffer was prepared freshly before each use and was not reused.

To begin digestion, scaffolds were washed twice with PBS to remove media and unattached cells. 300 μ l of CDB was added to fully cover the scaffold surface. Samples were refrigerated overnight at 4°C to allow enzymatic lysis, then subjected to three freeze-thaw cycles (−80°C for 10 min followed

by 37°C for 15 min). Contents were scraped into microcentrifuge tubes, vortexed briefly, and centrifuged at $1,000 \times g$ for 5 minutes to remove debris. The resulting supernatant was collected as the cell lysate and used for deoxyribonucleic acid (DNA) quantification.

Double-Stranded DNA (dsDNA) Quantification A standard curve for cell number and DNA content was generated using the provided DNA standards included in the Quant-iT™ BR assay kit. 20 μ l of cell lysate were incubated with the Quant-iT™ reagent according to the manufacturer's instructions in a black 96-well microplate. Fluorescence intensity was measured using a microplate reader (excitation/emission: 485/530 nm). The dsDNA concentration of each sample was calculated based on the standard curves, and values were normalized to tissue culture plastic (TCP), when necessary.

This method allowed for accurate quantification of total cellular DNA retained within the scaffold matrix, serving as a proxy for cell number and retention efficiency.

2.4.3 LIVE/DEAD

Cell viability is a crucial metric for assessing the biocompatibility of liver tissue models. The LIVE/DEAD Viability Kit (Invitrogen) distinguishes between live and dead cells according to membrane integrity, using Calcein-AM as live cell stain and ethidium homodimer-1 as a dead cell stain. To assess cell viability, a working solution was prepared by diluting Calcein-AM and ethidium homodimer-1 in PBS as recommended by the manufacturer. Scaffolds were incubated with the working solution for 15 minutes in the dark. The stained samples were then imaged using a fluorescence micro-

scope with excitation/emission wavelengths of 488 nm for Calcein-AM and 590 nm for ethidium homodimer-1.

2.4.4 Lactate Dehydrogenase (LDH)

LDH is a cytosolic enzyme released into the extracellular medium upon cell membrane damage, making it a reliable marker for cytotoxicity in hepatic tissue models. A CyQUANT™ LDH Cytotoxicity Assay (Invitrogen) was used to quantify LDH release in culture supernatants. Supernatants from cell cultures were collected and centrifuged at $1,000 \times g$ for 5 minutes to remove debris. 50 μ l of each sample were added to a 96-well plate, followed by 50 μ l of LDH reaction mixture. The plate was incubated at room temperature for 30 minutes in the dark. Absorbance was read at 490 nm using a microplate reader, background was subtracted from all measurements, and cytotoxicity values calculated as manufacturer instructions were used to get viability values of each sample.

2.4.5 Albumin Quantification

Albumin is the most abundant protein produced by the liver and is critical for maintaining oncotic pressure and transporting various substances in the bloodstream. Measuring albumin secretion is essential for evaluating hepatic function and hepatocyte maturation rate in liver models. The Human Serum Albumin Enzyme-linked immunosorbent assays (ELISAs) Kit (Thermo Fisher Scientific, EHALB) was used to determine albumin levels in cell culture supernatants quantitatively. Supernatants were collected, centrifuged at $10,000 \times g$ for 5 minutes, and stored at -80°C until use. A 96-well plate pre-coated with anti-human albumin antibodies was used,

where 50 μ l of standards, samples, and controls were added to the appropriate wells. The plate was incubated at room temperature for 2 hours. The wells were washed three times with a wash buffer before the enzyme-conjugated detection antibody was added. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and incubated for 30 minutes in the dark, and the reaction was stopped using the provided stop solution. Absorbance was read at 450 nm using a microplate reader, and albumin concentration was determined using a standard curve and normalized to cell number.

2.4.6 Urea Quantification

Urea quantification is a key metabolic assay for assessing liver function, as the liver plays a central role in nitrogen metabolism by converting ammonia into urea for excretion. Elevated urea levels can indicate hepatocyte maturity in liver models. The QuantiChrom Urea Assay Kit (BioAssay Systems DIUR-100, USA) was used to measure urea concentration in cell culture supernatants. Cell culture supernatants were collected on days 3, 7, and 14 and centrifuged at $10,000 \times g$ for 5 minutes to remove debris. Working reagents were prepared according to the manufacturer's protocol. 50 μ l of each sample or urea standard were added to a 96-well plate, followed by 200 μ l of the working reagent. The plate was incubated at room temperature for 20 minutes, and absorbance was read at 520 nm using a microplate reader. Urea concentration was determined using a standard curve generated from known urea concentrations and normalized to cell number.

2.4.7 CYP3A4 Quantification

Cytochrome P450 3A4 (CYP3A4) is one of the most abundant and functionally significant enzymes in the human liver, responsible for the oxidative metabolism of a wide range of endogenous and exogenous compounds. It plays a central role in hepatic detoxification, contributing to the biotransformation of approximately 50 % of clinically used drugs, as well as steroids, fatty acids, and bile acids (Guengerich, 2008). The activity of CYP3A4 is therefore a key indicator of hepatic metabolic competence and is commonly used as a marker of liver-specific functionality *in-vitro*. Assessing CYP3A4 expression and activity in hepatocyte models provides critical insights into their maturity, drug-metabolizing capacity, and overall physiological relevance to native liver tissue (Guengerich, 2008)

The CYP3A4 activity was assessed using the P450-Glo™ CYP3A4 Assay (Promega V9001, USA), which measures the bioluminescent signal generated by the conversion of a specific substrate (luciferin-IPA) into a luciferase substrate. The non-lytic protocol was used to ensure cell viability during the assay.

At each timepoint normal media was changed to 50 μ l of media with 1:1000 Luciferin-IPA and incubated 60 min at 37°C. Subsequently, 25 μ l of media were added into a 96-well opaque white luminometer-compatible plate at room temperature, and 25 μ l of Luciferin Detection Reagent were added to initiate a luminescent reaction. Samples were incubated at room temperature for 20 minutes, then luminescence was read using a Tecan Infinite 200 plate reader. Net signals were calculated by subtracting background luminescence values from no-cell control samples. Percent change was calculated by dividing net treated values by net untreated values and multiplying by 100.

The resulting luminescence signal is proportional to the CYP3A4 activity, with higher values corresponding to greater enzyme activity.

2.4.8 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Quantifying gene expressions is essential for evaluating hepatic maturation and function. Hepatocyte Nuclear Factor 6 (HNF6) plays a key role in liver development and function, and it ceases to be present when hepatocytes are mature. Meanwhile, Glucose-6-Phosphatase Catalytic Subunit 1 (G6PC1) is critical for glucose homeostasis and indicates the functional path hepatocytes take when they mature. RT-qPCR is a sensitive and precise method for assessing their expression levels in hepatic models. Total Ribonucleic acid (RNA) was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific, 12183018A) following the manufacturer's protocol. RNA purity and concentration were determined using a Nanodrop spectrophotometer. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to synthesize complementary DNA (cDNA) from 1 μ g of total RNA in a 20 μ l reaction volume. qPCR was carried out using SYBR Green Master Mix (Applied Biosystems) in a 96-well plate format with gene-specific primers for HNF6 and G6PC1. Each reaction contained 10 μ L of SYBR Green Master Mix, 1 μ L of each primer (final concentration: 0.2 μ M), 2 μ L of cDNA, and 7 μ L of nuclease-free water. Amplification was performed using a QuantStudio ViiA 7 Real-Time PCR System under the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression was calculated using the $\Delta\Delta C_t$ method, normalizing to TATA-box binding protein (TBP) as the housekeeping gene.

and TCP as the control.

2.4.9 Data Analysis and Statistical Analysis

Statistical analyses and graphical plotting of the data were performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, USA). Data from compression tests are presented as the mean $\pm$ standard deviation (SD), with N=5. Statistical significance was determined using a one-way analysis of variance (ANOVA) to compare three or more datasets. For cell culture data, the mean $\pm$ SEM is shown, with N=3 and n=3 unless otherwise indicated. A p-value of < 0.05 (*) was considered statistically significant, with further significance levels evaluated as **p < 0.01 , ***p < 0.001 , and ****p < 0.0001 .

Chapter 3

Development and Characterization of a GelMA-PEGDA Hydrogel for P_{μ} SLA-Based Liver Tissue Scaffolds

3.1 Introduction

The fabrication of intricate liver tissue models using P μ SLA requires the development of specialized photopolymerizable resins capable of reproducing the complex ECM geometries and mechanical properties of hepatic tissue. In this chapter, we explore the formulation, characterization, and printing of a GelMA–PEGDA hydrogel designed for this purpose. GelMA and PEGDA were selected for their complementary biological and mechanical attributes, which together yield an effective material system for liver tissue engineering.

GelMA, derived from gelatin, is renowned for its biocompatibility and ability to support cell adhesion and growth, making it an ideal matrix for tissue engineering (Liu and Chan-Park, 2010; Loessner et al., 2016; Peplanova et al., 2018; Schuurman et al., 2013). Its tuneable mechanical properties, achieved by varying the degree of methacrylation or its concentration, (Wang et al., 2014) allow for the customization of the hydrogel’s stiffness to match that of liver tissue. Furthermore, GelMA’s inherent biological cues promote cellular activities, essential for creating functional tissue models.

PEGDA, on the other hand, provides the mechanical robustness required for the structural integrity of the printed constructs. Its hydrophilic nature contributes to the formation of a stable hydrogel network, enhancing the overall mechanical strength and stability of the composite material (Della Sala et al., 2020; Nakamura et al., 2010). The combination of GelMA and PEGDA thus creates a hydrogel that balances biological functionality with mechanical resilience, ensuring the resulting tissue models are both physiologically relevant and structurally sound (Ferchichi et al., 2024b; Wang et al., 2020b) .

In addition to resin formulation, it is essential to preserve the intrinsic microarchitecture of the printed hydrogels during characterization. Cryogenic techniques are commonly employed for this purpose. Slush-nitrogen freezing involves cooling samples in a mixture of liquid nitrogen and nitrogen vapour, producing a semi-solid “slush” phase that rapidly freezes the material. Metal-mirror (MM) freezing is a vitrification-based approach in which samples are placed on a highly conductive metal surface and cooled at rates exceeding 10,000 K/s. Unlike conventional freezing, which generates large ice crystals and leads to structural distortion, MM vitrification minimizes crystallization and preserves the native hydrogel architecture. Incorporating these techniques ensures that microstructural analyses accurately reflect the true morphology of the printed constructs.

To make this formulation suitable for photopolymerization, LAP was used as the photoinitiator due to its high photoreactivity, water solubility, and biocompatibility (Elkhoury et al., 2023; Lim et al., 2020). Its activity across 365–500 nm (Elkhoury et al., 2023) enables efficient crosslinking of GelMA and PEGDA under the 405 nm exposure of the BMF S130 printer. Tartrazine was included as a photoabsorber to regulate light penetration, improving resolution and structural fidelity by limiting overexposure and mitigating internal light scattering (Yu et al., 2020).

This chapter details the development and optimization of the GelMA–PEGDA hydrogel resin, demonstrating its suitability for advanced tissue engineering through its biological relevance and capacity to produce accurate, structurally robust microfabricated constructs.

3.2 Results and Discussion

3.2.1 Enabling Consistent and High-Degree Methacrylation of GelMA

Two distinct synthesis methods, Method A and B as illustrated in Figure 2.1 and explained in section 2.1.1, were evaluated to achieve optimal methacrylation of gelatin.

Two batches were created using Method A, but the degree of methacrylation (DM) was not consistent since it presented a 20.1% deviation from batch to batch as presented in Table 3.1. Suspecting buffer instability was the reason for this variation, Method B was used to ensure a more reproducible fish GelMA batches with higher DM.

^1H NMR spectroscopy was used to analyze the resulting materials, confirming key spectral differences between the synthesis methods as shown in the spectra in Figure 3.1. To ensure accurate quantification, the aromatic protons of phenylalanine (7.2–7.4 ppm) were used for normalization across all spectra, as these residues remain unaffected by methacrylation and provide a stable internal reference.

In the unmodified gelatin spectrum, the absence of the characteristic methacrylate vinyl proton signals at 5.3–5.7 ppm was evident. These peaks correspond to the vinyl protons of the methacrylate group introduced during the reaction with methacrylic anhydride. Their appearance in all GelMA spectra confirms successful functionalization of gelatin.

Additional assignments within the spectra correspond to characteristic amino acid residues in gelatin. The methylene protons of lysine $\epsilon\text{-CH}_2$

groups appear in the region of 2.8–3.2 ppm and are commonly used to quantify the degree of methacrylation, as methacrylation occurs primarily at the ϵ -amine groups of lysine residues. Variations in the intensity of this lysine-associated signal were observed when comparing the spectra of the first two batches synthesized using Method A. These differences were reflected in the calculated degree of methacrylation presented in Table 3.1, where a difference of 20.1% was observed between Batches 1 and 2, highlighting the inconsistency of Method A.

In contrast, Batch 3, synthesized using Method B, exhibited a strong presence of the methacrylate vinyl peaks, consistent with a higher degree of functionalization. This observation aligns with the calculated degree of methacrylation of 99.1% shown in Table 3.1. These findings demonstrate that Method B enables a more efficient methacrylation reaction, making it the preferred method for further applications.

Table 3.1: Comparative table of Degree of methacrylation on fish GelMA between batches and synthesis methods

Batch	Synthesis Method	Degree of Methacrylation (%)
1	A	90.9
2	A	70.8
3	B	99.1

The differences in methacrylation efficiency between the two methods are primarily attributed to buffer stability and pH control during the reaction. The amine groups of lysine in gelatin have a pKa of approximately 10.5. Below this pH they remain protonated and are therefore less nucleophilic, whereas at slightly basic pH they become deprotonated and more reactive toward methacrylic anhydride. If the buffer does not maintain a stable pH throughout the reaction, fluctuations toward lower pH can reduce the availability of nucleophilic amine groups, decreasing methacrylation efficiency. In addition, methacrylic anhydride is susceptible to hydrolysis in aqueous

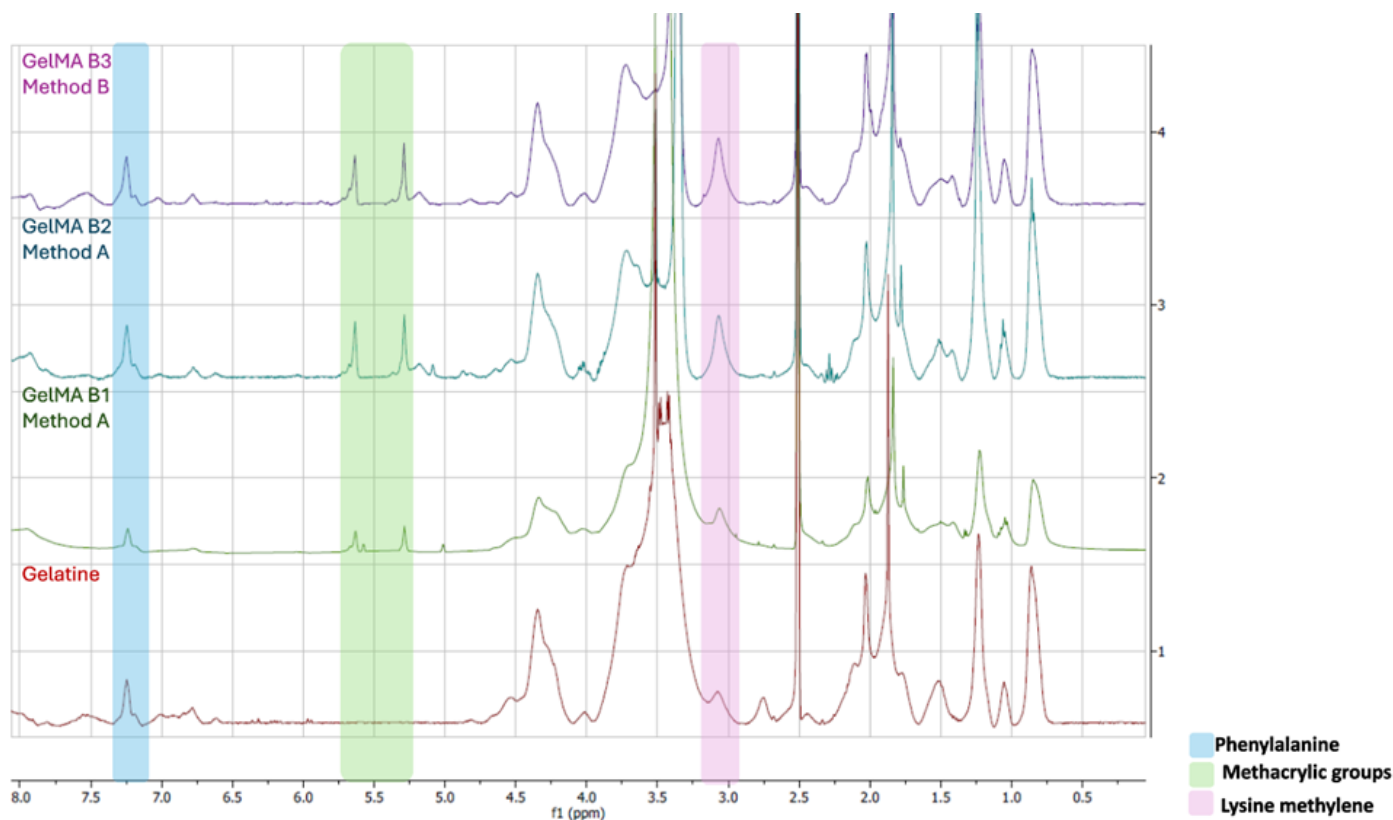


Figure 3.1: NMR Spectra of GelMa and gelatine showing successful methacrylation of gelatine

basic environments, which competes with the desired reaction with gelatin amines and can further reduce functionalization efficiency. In Method A, the buffer system did not maintain a stable pH during the reaction, mainly due to methacrylic acid produced in hydrolysis, likely leading to reduced amine reactivity. In contrast, Method B maintained a stable pH, promoting consistent amine availability and more efficient methacrylation, resulting in higher yields consistent with previous reports in the literature (Zhu et al., 2019)

An additional critical factor contributing to the success of Method B was the inclusion of a dialysis purification step, which was omitted in Method A. This step is essential for the effective removal of unreacted methacrylic anhydride (MA) and low molecular weight reaction byproducts. Its importance has been widely emphasized in prior studies, as it directly affects

the chemical purity, consistency, and ultimately the biocompatibility of the resulting GelMA hydrogels (Zidan et al., 2021; Elkhoury et al., 2021).

Achieving a high degree of methacrylation with minimal residual contaminants is vital for maximizing the availability of photocrosslinkable functional groups. The increase between 28.3-8.2% and the incorporation of the dialysis step for purification makes Method B the superior choice to continue using during the next experimental procedures. This aspect also directly impacts the mechanical integrity of the hydrogel during stereolithographic printing and the overall cytocompatibility of the scaffold giving it more support and better cell viability (Zhu et al., 2019).

3.2.2 Enhanced Room-Temperature Flow Behaviour of Fish GelMA for P μ SLA

In P μ SLA, a critical requirement for the prepolymer is to possess rheological properties suitable for the layer-by-layer fabrication. To achieve precise and consistent curing, the resin must remain in a fully liquid state at room temperature throughout the print to facilitate a uniform layer recoating, enabling accurate light penetration, and preventing scattering or incomplete crosslinking. All of these are essential for achieving high-resolution and high-fidelity structures. Conversely, partially gelled or highly viscous formulations can obstruct optical clarity and flow dynamics, leading to print defects such as delamination, under-curing, or feature collapse. Therefore, ensuring the formulation remains fluid under ambient conditions is critical for maintaining the printing accuracy and reproducibility required for the fabrication of complex tissue engineering scaffolds.

As shown in Figure 3.2, the GelMA solution prepared from pork gelatin

displayed gel-like behaviour at room temperature, as evidenced by the absence of flow upon tilting. In contrast, the fish-derived GelMA remained fully liquid under the same conditions, as demonstrated by the downward flow and slanting of the hydrogel volume along the vial wall. This qualitative test clearly distinguished the thermal gelation behaviour of the two formulations under ambient conditions relevant to the printing process.

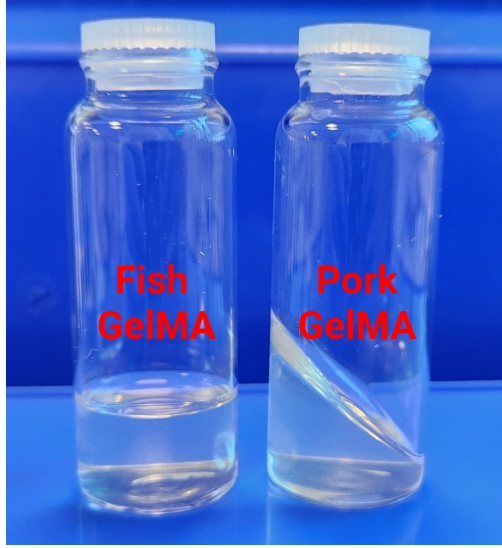


Figure 3.2: Tilt test comparing flow-ability of GelMA prepared from fish-derived and pork-derived gelatin at 24°C. Only the fish GelMA demonstrated fluid behaviour under room-temperature conditions, indicating better suitability for stereolithographic hydrogel printing.

The room-temperature viscosity behaviour of gelatin-based hydrogels is primarily governed by the source-dependent amino acid composition and gelling temperature of the precursor gelatin. Pork-derived GelMA exhibits thermal gelation around 25–30°C, which is close to room temperature, leading to gel-like behaviour during handling and potentially interfering with resin levelling and light penetration during layer-by-layer printing. In contrast, fish gelatin, particularly from cold-water species, has a significantly lower gelation point (10–15°C) and remains in a liquid state at room temperature, as previously reported in the literature for cold-set gels used in bioprinting (Yoon et al., 2016; Wang et al., 2017).

For stereolithographic applications, maintaining the hydrogel in a low-viscosity, flowable state at ambient temperatures is essential to ensure reliable resin re-coating and minimize layer delamination. The flow-ability observed in the fish-derived GelMA indicates its suitability for PμSLA printing workflows without requiring elevated temperature handling or system heating, which can complicate setup. Therefore, based on its favourable flow-ability profile at room temperature, fish GelMA was selected as the gelatin source for all subsequent hydrogel formulations.

3.2.3 Achieving Complex Bio-inspired architecture and Structural Integrity through tuneable Hydrogel Stiffness

The mechanical properties of hydrogels play a crucial role in their ability to replicate ECM architecture found in native tissues, directly influencing cell behaviour, adhesion, and differentiation (Janson and Putnam, 2015; Urbanczyk et al., 2020). The Young's modulus results from compression test shown in Figure 3.3 indicate that the GelMA-PEGDA hydrogel exhibits significantly higher stiffness compared to the pure GelMA hydrogel. This increase in stiffness can be attributed to the presence of PEGDA. The formation of a randomized co-polymer between GelMA and PEGDA enhances the hydrogel's mechanical integrity by increasing network density and reducing polymer chain mobility (Wang et al., 2018).

Comparing these values to liver tissue stiffness reported in the literature (Table 3.2), it is evident that the GelMA-PEGDA hydrogel approaches the stiffness range of ex vivo human liver (0.59 – 1.73 kPa), whereas the softer GelMA formulation remains closer to the lower end of this range. Matching

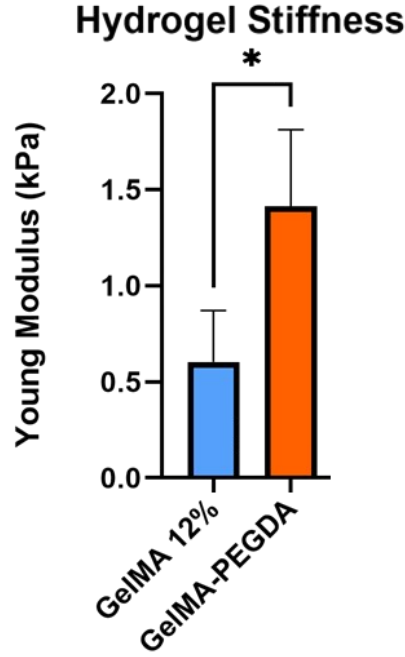


Figure 3.3: Hydrogel Stiffness graph showing Young Modulus of pure GelMA and GelMA-PEGDA hydrogel. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$) between samples. $N = 5$.

the stiffness of the target tissue is essential, as mechanical cues significantly influence cellular functions such as proliferation, migration, and differentiation as shown in literature (Janson and Putnam, 2015). In liver tissue engineering, achieving physiologically relevant stiffness is particularly important for hepatocyte functionality, as liver cells exhibit mechanosensitive behaviour that affects their metabolic activity (Huang et al., 2013; Wells, 2005). Studies have shown that that hepatocytes cells maintain their normal function and behaviour in soft hydrogels (1kPa) more than in stiff gels (12kPa) (Huang et al., 2013). Although the in-vivo stiffness of human liver (7 kPa) has shown to be higher, this might potentially be due to vascularization, cellular interactions, and dynamic loading conditions in a living system. Nevertheless, the enhanced stiffness of the GelMA-PEGDA formulation suggests a more robust structural environment suitable for liver tissue scaffolding and it can be considered a soft hydrogel, according to

Huang et al. (2013) study, and as such, it is expected to support cell viability and function while maintaining the necessary biomechanical cues for proper cellular behaviour.

Table 3.2: Liver tissue stiffness in literature

Reference	Tissue type	Young Modulus (kPa)
Chen et al. (1996)	Bovine Liver	0.3 - 1.6
Wen-Chun Yeh et al. (2001)	Human liver (<i>ex-vivo</i>)	0.59 to 1.73
Huwart et al. (2006)	Human Liver (<i>in-vivo</i>)	7

In addition to its biological relevance, stiffness plays an important role in the printing process. The incorporation of PEGDA into the GelMA hydrogel formulation is expected to enhance performance in stereolithographic printing because of increased stiffness and crosslinking density. Compared to pure GelMA, the GelMA-PEGDA composite theoretically forms a more mechanically robust network, which may help maintain structural integrity during the layer-by-layer fabrication process. This enhanced stiffness is likely to reduce deformation, improve feature resolution, and support the formation of self-standing architectures during printing (Dong et al., 2022).

3.2.4 Optimized Photopolymerization for Accurate Hydrogel Scaffold Printing

To optimize the printing of the GelMA and GelMA-PEGDA hydrogels, the light intensity was tuned while the exposure time was held constant at 5 seconds per layer. This method revealed distinct trends in under-curing and over-curing effects until the optimal parameters were found. A TPMS lattice with 100 μm pores was used as a standard printing geometry to test printing fidelity. Printing fidelity was calculated as explained in section 2.2.6. As shown in Figure 3.4, GelMA alone failed to reproduce the intended structure across all tested intensities, with severe under-

curing at 20.59 mW/cm², resulting in complete polymerization failure (0% fidelity). As intensity increased to 45.30 mW/cm², partial polymerization was observed (26.2%), though structures remained weak and incomplete. At 127.66 mW/cm², polymerization improved slightly (31.6%), but the resulting structures lacked well-defined features, suggesting that GelMA alone does not efficiently crosslink under these conditions. Even at 210.02 mW/cm², significant structural deformation persisted, with uneven and incomplete polymerization leading to feature loss and lack of structure (70.3% fidelity).

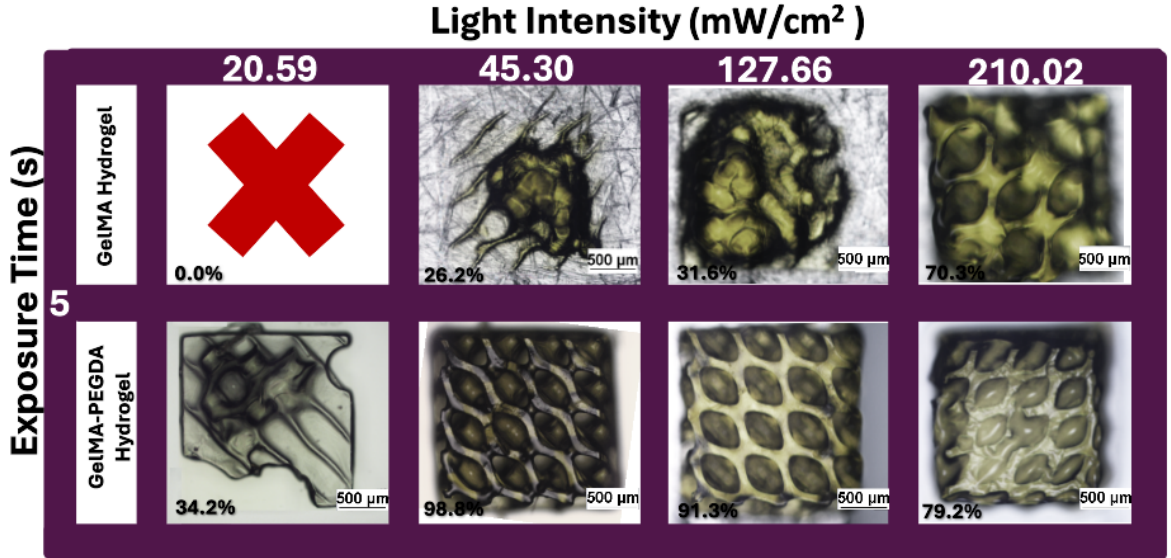


Figure 3.4: GelMA and GelMA-PEGDA Hydrogel Printing Optimization showing printing fidelity in % in the bottom left corner

In contrast, GelMA-PEGDA hydrogel exhibited significantly improved printability. At 20.59 mW/cm², partial polymerization occurred (34.2% fidelity), but under-curing led to weak and incomplete structures. However, at 45.30 mW/cm², near-perfect structural fidelity (98.8%) was achieved, with well-defined features and minimal distortion, indicating an optimal balance between polymerization and material resolution. Further increases in intensity to 127.66 mW/cm² resulted in overcuring effects (91.3%), where excessive crosslinking led to strut thickening and pore deformation. At

210.02 mW/cm², overcuring was more pronounced (79.2%), with fused features and loss of intended pore structure.

The improved printability of the GelMA-PEGDA hydrogel over GelMA alone can be attributed to differences in crosslinking efficiency, polymer network formation, and mechanical stability. GelMA relies on methacrylate-functionalized gelatin for photocrosslinking, but its polymerization is hindered by its relatively low degree of functionalization and high water content, which led to incomplete network formation under light based printing conditions (Lim et al., 2020; Pepelanova et al., 2018). This resulted in poor structural fidelity, especially at lower light intensities where insufficient radical formation leads to under-curing. Even at higher intensities, GelMA alone did not exhibit complete polymerization nor could it replicate the intended printing features. Additionally, GelMA’s hydrogel network lacked the mechanical rigidity required to sustain complex geometries during layer-by-layer curing, leading to soft, unstable structures with compromised fidelity. The moderate mechanical stiffness found in compression tests further explains this behaviour as there is insufficient initial modulus to support the intended printing structure.

In contrast, the addition of PEGDA significantly enhanced photopolymerization efficiency and structural integrity. This enhanced photopolymerization response can be primarily attributed to the higher density of acrylate groups introduced by PEGDA, which, as reported by Kloosterboer et al. (1986) and Wang et al. (2018), increased the formation of photoinitiated radicals and promoted rapid and uniform network crosslinking improving gel stiffness, rapid polymerization kinetics, and overall printing resolution (Kloosterboer et al., 1986; Wang et al., 2018). As shown in Figure 3.4, this higher functionality contributed to faster and more uniform polymerization, reducing the effects of under curing at low intensities. Furthermore,

PEGDA's hydrophilic nature helped maintain water retention, preventing excessive shrinkage during polymerization. The combination of GelMA and PEGDA thus enabled higher fidelity printing with well-defined features, as evidenced by the successful fabrication of the gyroid scaffold in Figure 3.5, demonstrating that the incorporation of PEGDA in the hydrogel composition effectively enhanced both printability and mechanical stability in SLA-based hydrogel systems.

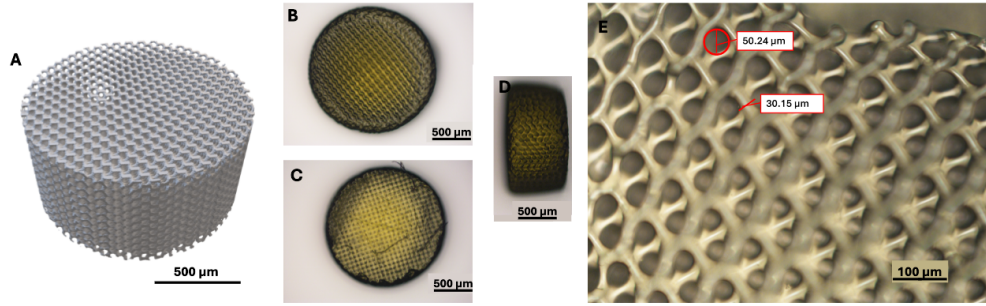


Figure 3.5: Successful print of microscopic features using GelMA-PEGDA hydrogel. A) CAD design of gyroid lattice. Top (B), bottom (C) and side (D) view of the printed gyroid lattice. E) 20x of printed gyroid lattice showing pore and strut size.

Collectively, these findings highlight the material-dependent kinetics of photopolymerization and their critical influence on printing fidelity. While GelMA alone may remain suitable for applications requiring softer hydrogels or thermo-responsive behaviour, the addition of PEGDA significantly improves control over spatial resolution, mechanical robustness, and polymerization efficiency. The optimal printing condition for GelMA-PEGDA hydrogels was identified at 45.30 mW/cm^2 , achieving high-fidelity reproduction of simple geometries with minimal over curing or structural collapse, and enabling the successful replication of complex gyroid structures with feature sizes aligned with the targeted scaffold parameters in this study as shown in Figure 3.5. These results established a robust photopolymerization window for GelMA-PEGDA systems and provided a solid foundation for fabricating architecturally complex scaffolds using stereolithography.

3.2.5 Avoiding Cytotoxicity Through Post-Processing

Fourier-transform infrared spectroscopy (FTIR) was performed to evaluate the reduction of acrylate groups throughout the printing and post-processing stages. The spectra of the uncured resin (red), freshly printed sample (blue), and post-processed sample (cyan) found in Figure 3.6 reveal key differences in acrylate conversion. A significant reduction in absorbance intensity at the 810–830 cm^{-1} region (highlighted), which corresponds to the C=C out-of-plane bending of acrylate groups, indicates progressive polymerization. This peak corresponds to the twisting or wagging vibration of the =C-H bond on the acrylate’s vinyl group. As the carbon-carbon double bond is consumed during the polymerization reaction, the intensity of this sharp peak decreases (Decker and Moussa, 1989). The transition from resin to print results in a 68.5% reduction in acrylate groups present, demonstrating substantial but incomplete acrylate group elimination during the initial photopolymerization step. The post-processing step, which included an additional prolonged washing step, further enhanced acrylate elimination, reaching a total reduction of 94.2% compared to the uncured resin.

The FTIR spectra confirms that post-processing successfully minimized the presence of reactive species, leading to a more stable and cell-safe material. The post-printing washing step ensured the complete removal of any uncured prepolymer, leaving a final construct composed solely of the polymerized hydrogel network. Therefore, the effective post-printing removal of residual acrylates, as demonstrated here, is critical for maintaining a cytocompatible environment in the intended hepatic tissue model.

Photopolymerization in P μ SLA consumes acrylate groups through a radical-induced chain reaction initiated by light exposure. When a photoinitiator

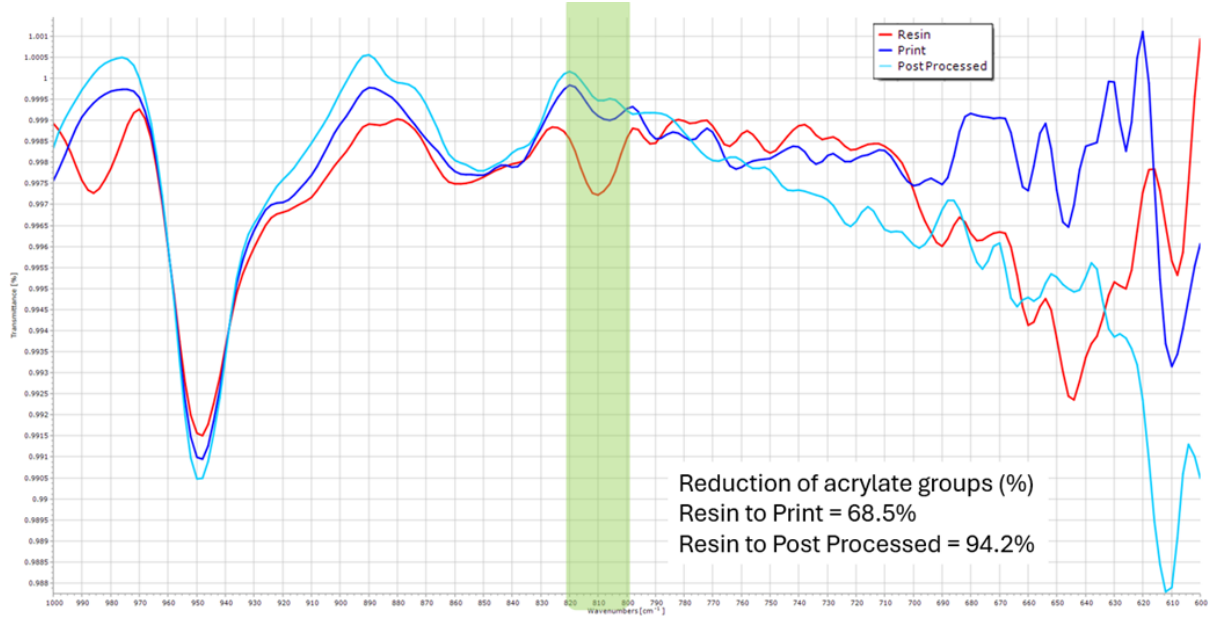


Figure 3.6: FTIR spectra of uncured resin (red), freshly print sample (blue) and post-processed printed sample (cyan)

absorbs light at the appropriate wavelength, it generates free radicals that react with the carbon-carbon double bonds ($C=C$) in acrylate groups, leading to polymer chain formation. This crosslinking process converts liquid monomers into a solid polymer network by incorporating acrylate groups into the growing polymer structure. The efficiency of this reaction depends on factors such as light intensity, exposure time, and the presence of oxygen, which can inhibit radical propagation (Ligon et al., 2017).

Incomplete polymerization can leave residual unreacted acrylate groups trapped within the polymer network. Furthermore, because the construct is surrounded by uncured prepolymer throughout the printing process, inadequate post-process washing can result in a layer of this unreacted material remaining on the scaffold's surface. In either case, these residual acrylate moieties remain chemically reactive and can leach from the scaffold over time, potentially causing cytotoxicity when used in tissue engineering applications. Free acrylates are highly reactive electrophiles capable of forming covalent bonds with cellular macromolecules, including proteins, lipids,

and nucleic acids (Kreß et al., 2020). The effective removal of residual acrylate groups is particularly important, as these unreacted moieties are chemically reactive and cytotoxic, as they participate in secondary radical generation or induce oxidative stress, thereby compromising cell membrane integrity, impairing protein function through oxidative modification, and promoting DNA damage that may lead to mutagenesis (Qamar and Udit, 2022). This is especially problematic in tissue engineering, where material biocompatibility is essential for supporting cell viability and function. Therefore, achieving a high degree of acrylate conversion through optimized photopolymerization and making sure there is no uncured prepolymer thorough post-processing is critical to minimizing cytotoxicity and ensuring the safe use of printed hydrogels in biological applications.

Additionally, this data underscores the importance of optimizing light exposure and post-curing conditions to balance print fidelity and biocompatibility, ensuring the hydrogel is suitable for biomedical applications.

3.2.6 Cryo-SEM Optimization Enabled Accurate Visualization of Hydrogel Microstructure

Keeping hydrogel samples hydrated while imaging them is crucial for preserving their natural structure and functionality, as dehydration during traditional SEM preparation can cause significant distortion, collapse, or shrinkage of the gel network (Martinez-Garcia et al., 2022). Hydrogels, by definition, are water-based and rely on hydration to maintain their 3D structure, porosity, and mechanical properties. Without proper hydration, the intricate morphology of hydrogels, such as the pore network and gel matrix, may be irreversibly altered, leading to misleading or inaccurate representations. Therefore, cryo-SEM, which involves imaging samples at

cryogenic temperatures, offers a solution by freezing the hydrogel in its hydrated state, preventing dehydration and preserving the native structure during high-resolution imaging. This technique ensures a more accurate assessment of the hydrogel's properties, making it essential for studies in areas like tissue engineering and regenerative medicine.

To optimize the Cryo-SEM preparation technique for hydrogel imaging, two freezing methods were evaluated: slush nitrogen freezing and Metal Mirror (MM) freezing. The results of both methods are shown in Figure 3.7 demonstrating that MM freezing produced a more uniform and well-preserved hydrogel structure, while slush nitrogen freezing led to significant damage to the intrinsic hydrogel mesh. The latter technique resulted in uneven pore sizes and disrupted network formation due to ice crystal formation. As reported by Liang et al. (2016b), ice crystal formation is one of the most common artifacts that compromise the fidelity of hydrogel imaging. This structural distortion prevented an accurate representation of the native hydrogel architecture, making MM freezing a better method for sample preparation. By rapidly vitrifying the hydrogel without the formation of large ice crystals, MM freezing maintained the integrity of the porous network, preserving a more representative microstructure. In contrast, hydrogels frozen with slush nitrogen displayed irregular pores, collapsed fibrillar networks, and disrupted architectures due to ice-induced damage. The freezing conditions directly influenced pore morphology, where slower freezing (as in slush nitrogen) led to larger, more irregular pores due to ice expansion, whereas MM freezing resulted in a finer, more consistent network. Because of this MM freezing was chosen for GelMA-PEGDA hydrogel to ensure accurate visualization of its microstructure while avoiding artifacts caused by its preparation technique.

After optimizing Cryo-SEM sample preparation, samples were frozen using

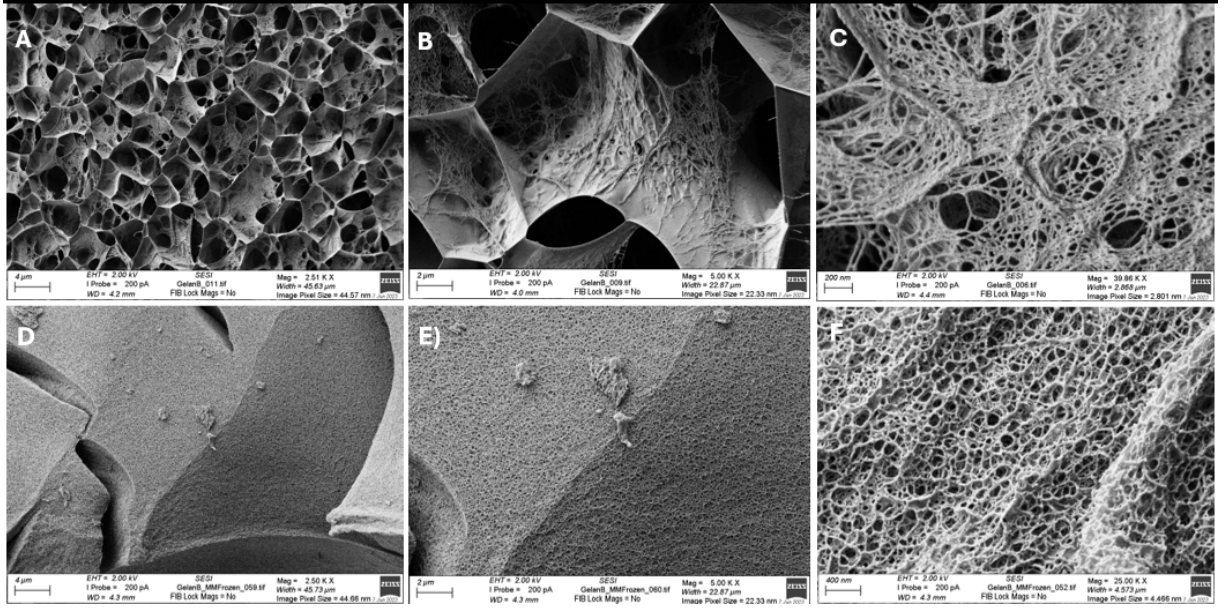


Figure 3.7: Test hydrogel with different preparation techniques for Cryo-SEM. A-C) Prepared with slush nitrogen. D-F) Prepared with metal mirror freezing

MM freezing and Cryo-SEM images were used to assess the microstructural organization of the GelMA-PEGDA hydrogel. Representative images shown in Figure 3.8 revealed a highly interconnected porous mesh network, free of ice crystal artifacts, and closely resembling the native hydrated state of the material.

Quantitative analysis of the pore architecture, based on image segmentation and scale calibration against the 400 nm reference bar, determined an average pore size of approximately 55 nm, with a standard deviation of 29 nm. This nanometric mesh structure indicates a dense but highly permeable internal network, characteristic of well-formed hydrogels optimized for biomedical applications.

This nanoscale mesh architecture observed in the GelMA-PEGDA hydrogel is highly favourable for supporting mass transport critical to cellular function and tissue development. With an average pore size of approximately 55 nm, the hydrogel network is ideally placed to accommodate

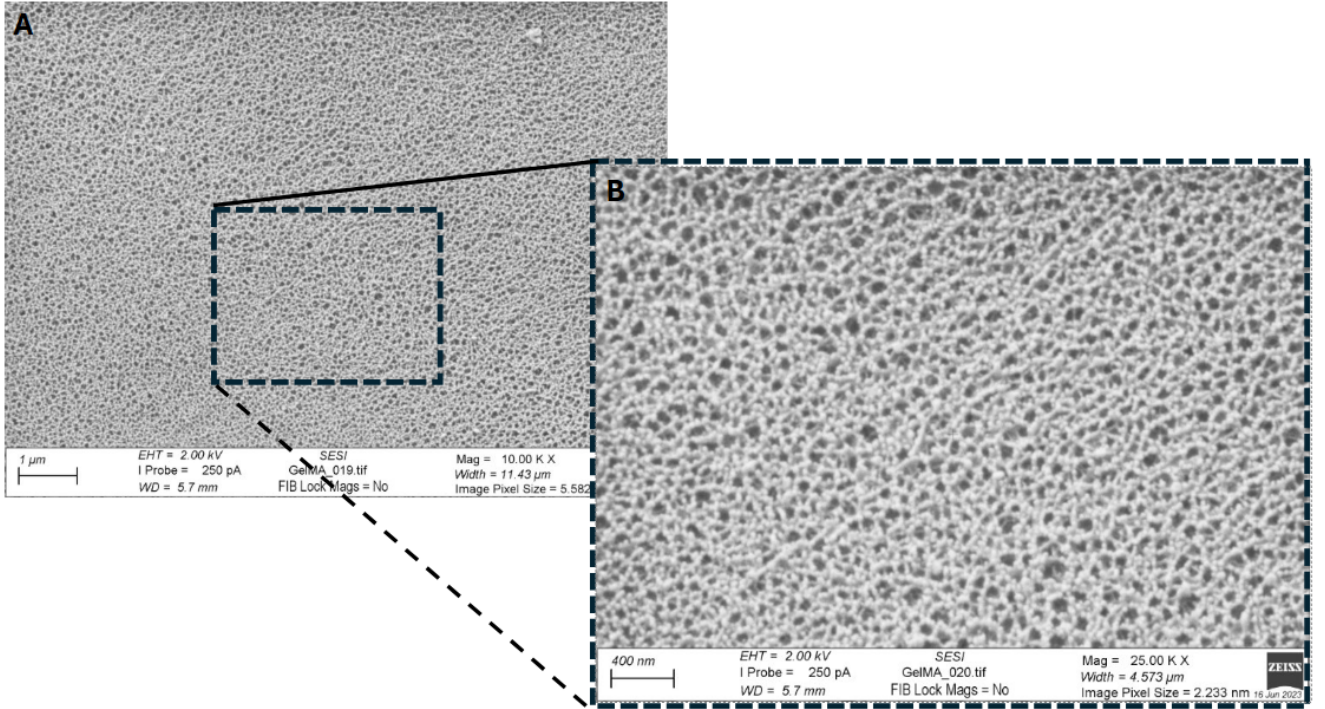


Figure 3.8: GelMA hydrogel Cryo-SEM using metal mirror freezing A)10k Magnification B)25K Magnification

the diffusion of essential small molecules, including oxygen, carbon dioxide, glucose, and amino acids, as well as larger biomolecules such as growth factors and serum proteins like albumin (Barlow, 1980). Given that these biologically relevant molecules are significantly smaller than the characteristic pore dimensions, it is expected that the hydrogel will facilitate efficient exchange of nutrients, oxygen, metabolic waste, and signalling molecules throughout the construct. This balance is vital in three-dimensional tissue constructs, where the diffusion of nutrients and the removal of waste must occur over larger distances compared to traditional two-dimensional cultures. This permeability is critical not only for maintaining cell viability within three-dimensional scaffolds but also for supporting processes such as proliferation, differentiation, and matrix remodelling. In addition to the critical role it plays in the diffusion of nutrients and waste, the mesh provides a biomimetic microenvironment that supports cell adhesion and matrix remodelling, processes crucial for tissue formation (Woo et al.,

2003). Thus, the confirmed nanoscale porosity of the GelMA-PEGDA hydrogel directly contributes to creating a potentially biologically supportive microenvironment for engineered tissue and ensures mechanical integrity while maintaining sufficient permeability, a balance that mimics the ECM architecture found in native soft tissues such as liver. The Cryo-SEM analysis thereby strengthens the case for the GelMA-PEGDA system as a promising platform for the development of advanced biofabricated tissues.

3.2.7 Swelling Behaviour and Degradation Kinetics Enable Long-Term Tissue Support

A hydrogel scaffold's performance is fundamentally defined by its interaction with the surrounding aqueous environment. The material's swelling behaviour determines its final structural and mechanical integrity and governs its porosity, as illustrated in Figure 3.9. This is critical for the mass transport of nutrients and metabolic waste to seeded cells in the hydrogel environment. The hydrogel's interaction with water was analysed through drying rate, swelling behaviour, and degradation studies.

The drying curve shown in Figure 3.10 showed a slow initial weight loss within the first hour, followed by a significant decrease in weight between 50 and 60 minutes until being completely dry when measured 20 hrs later. The inset images confirm the physical shrinking of the hydrogel during drying and a total water content representing 92.7% of the hydrogel's weight. This behaviour suggests that the hydrogel retains a significant amount of water initially but gradually loses it as evaporation progresses, which is indicative of a highly hydrated network with moderate water retention capacity.

The degradation study in Figure 3.11 compares weight loss over 14 days

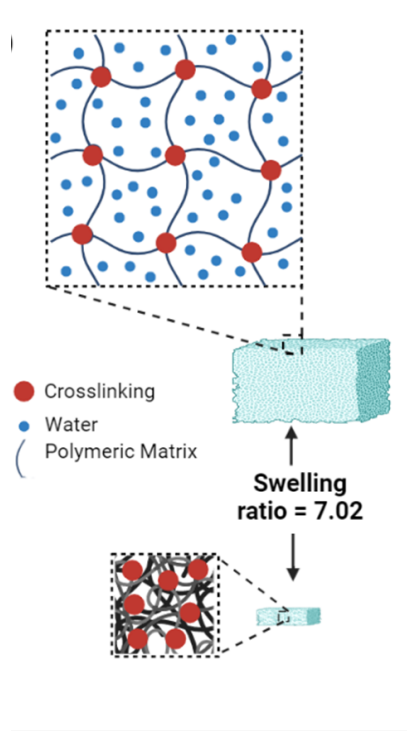


Figure 3.9: GelMA-PEGDA hydrogel swelling ratio and polymeric matrix mechanics

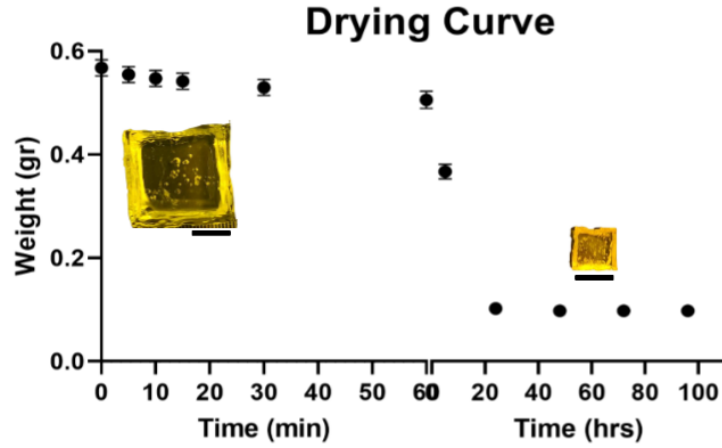


Figure 3.10: GelMA-PEGDA hydrogel drying curve over time. Scale bar = 500 μm

at room temperature and 37°C. Although the hydrogel seems to degrade faster at room temperature during the first 7 days, there is no significant difference in weight loss at the end of day 14 with approximately 16% total weight loss at 37°C compared to 14% at room temperature, as indicated in Table 3.3. Furthermore, this degradation rate aligns well with *in-vitro* liver model timescales proposed by other groups (Kim et al., 2023; Gijbels

et al., 2021; Kang et al., 2020; Ma et al., 2025).

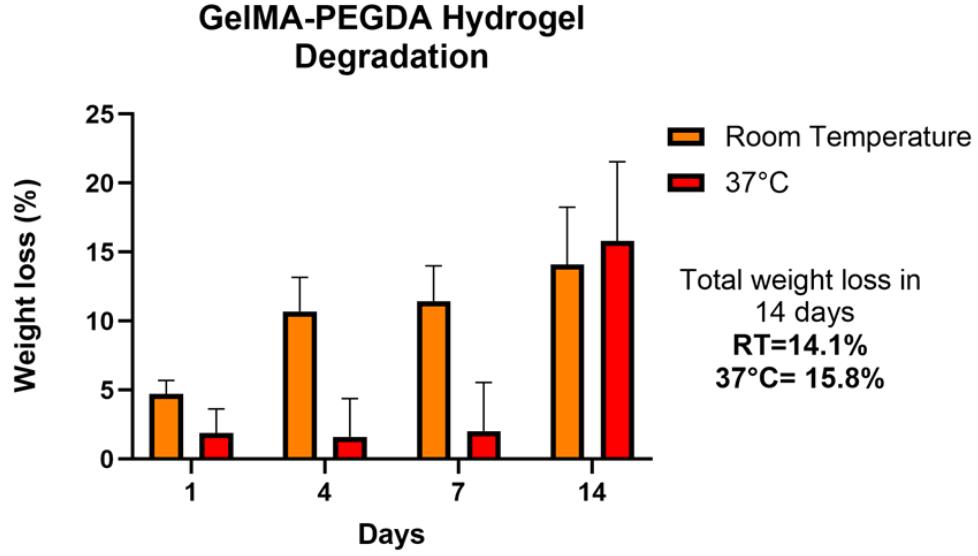


Figure 3.11: Degradation of printed hydrogel samples over 14 days

The faster degradation of the hydrogel at room temperature during the first seven days can be attributed to the initial surface erosion and water diffusion dynamics (Mndlovu et al., 2024). The slower degradation of the hydrogel at 37°C during the first seven days compared to room temperature can be attributed to differences in water retention, cross-link stability, and degradation mechanisms (Garcia-Garcia et al., 2024). Consistent with previous reports, increases in temperature shifted the hydrophilic–hydrophobic balance of polymer chains, and promoted transient polymer–polymer associations and network contraction; this thermal strengthening temporarily stabilized the network and reduced early mass loss (Feng and Wang, 2023; Lu et al., 2024). In contrast, at room temperature, hydrogels often exhibit greater water mobility and more accessible surfaces, which led to diffusion-driven mass loss and more pronounced surface erosion during the initial degradation period (Garcia-Garcia et al., 2024). Nevertheless, by day 14, the hydrogel’s structural components reached a critical threshold where the remaining polymer network resisted further breakdown at similar rates in

both conditions, suggesting soluble degradation of by-products may have stabilized over time, leading to a plateau effect that minimized further discrepancies between the two temperature conditions.

Swelling behaviour shown in Figure 3.12 demonstrated anisotropic expansion, with the X and Y dimensions increasing more significantly than the Z direction. The anisotropic swelling behaviour observed in Figure 3.12, where the X and Y dimensions expand more than the Z direction, suggested that the hydrogel's internal structure imposed directional mechanical constraints. This could be due to factors such as polymer chain alignment during synthesis, cross-linking density variations, or differences in the degree of swelling resistance in different planes. Hydrogels with anisotropic swelling are particularly relevant in tissue engineering, as many biological tissues exhibit direction-dependent mechanical properties, such as muscle fibres, cartilage, and skin, where controlled expansion can help mimic native behaviour (Thé et al., 2006; Khuu et al., 2021). This anisotropic swelling is advantageous in providing mechanical cues that support cell growth, orientation, and organization within three-dimensional constructs.

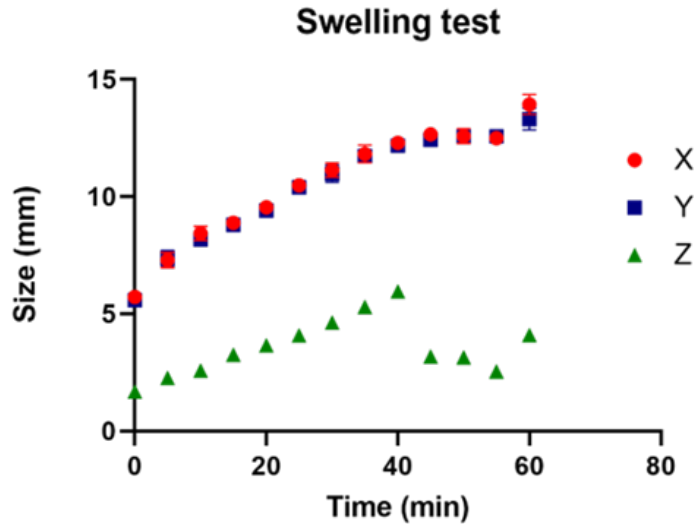


Figure 3.12: GelMA-PEGDA hydrogel swelling measurement in xyz

The high swelling ratio of 7.02 (Figure 3.9) and the significant water content of 92.7%, shown in Figure 3.10 and summarized in Table 3.3, indicated a strong ability to retain moisture, which is crucial for maintaining a hydrated environment conducive to cell viability and function. The rapid absorption rate of 121 $\mu\text{l}/\text{min}$ further ensures that the hydrogel can quickly rehydrate and integrate with surrounding tissues effectively. However, the controlled degradation behaviour (Figure 3.11) ensures that the material does not dissolve prematurely, maintaining structural integrity for a sufficient period to prolonged cell culture periods to reach maturity of the expected tissue. The interplay between swelling, water retention, and degradation kinetics underscores the hydrogel’s potential for tissue engineering where precise hydration control is essential as mentioned on Yin et al. (2023). Together, these hydrogel-water interaction properties, summarized in Table 3.3, are critical for tuning mass transport, providing mechanical and biochemical support, and ensuring scaffold stability throughout the culture period. These findings confirm that the GelMA-PEGDA hydrogel formulation is well-suited for sustaining viable and functional liver tissue constructs over relevant biological timescales.

Table 3.3: Summary table with hydrogel rates and percentage in relation with water

Water content (%)	92.7 ± 0.1
Water loss rate ($\mu\text{l}/\text{hr}$)	56.8 ± 16.0
Absorption rate ($\mu\text{l}/\text{min}$)	121.0 ± 6.0
Total weight loss at RT (%)	14.1 ± 4.1
Total weight loss at 37°C (%)	15.8 ± 5.8

3.2.8 Confirmed Biocompatibility of GelMA-PEGDA for Liver Tissue Engineering

The biocompatibility of the GelMA-PEGDA hydrogel was assessed through metabolic activity assays and LIVE/DEAD staining, following the ISO 10993 standards for biological evaluation of medical devices (International Organization for Standardization, 2009b). The metabolic activity results, show in Figure 3.13 A indicated a viability of 95% in the presence of the GelMA-PEGDA hydrogel extract media, compared to 100% in fresh culture media, demonstrating that the hydrogel does not significantly affect cell metabolism.

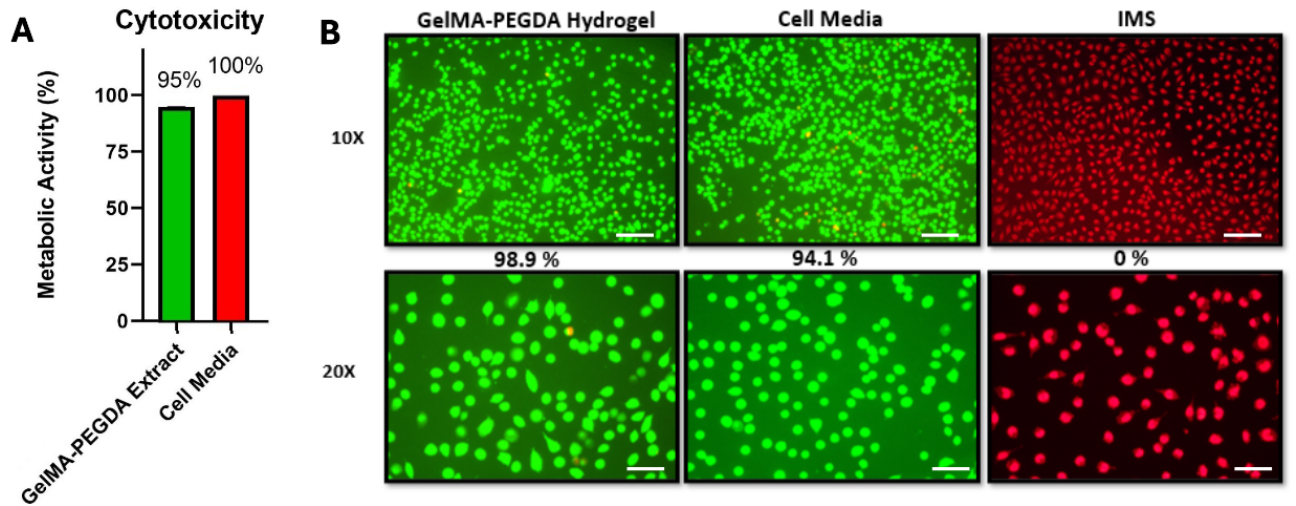


Figure 3.13: Cytotoxicity test for GelMA-PEGDA Hydrogel on L929 cells. A) Metabolic Activity of cells exposed to GelMA-PEGDA hydrogel extract compared to fresh cell media. B) LIVE/DEAD staining on cells exposed to GelMA-PEGDA hydrogel extract compared to fresh cell media for live control and IMS for dead control. Green stained cells represent live cells while red stained cells represent dead cells. 10X Scale bar= 100 μm . 20X Scale bar= 50 μm

LIVE/DEAD staining further confirmed this, with 98.9% of cells remaining viable in GelMA-PEGDA hydrogel extract media, a percentage even slightly higher than the 94.1% observed in the fresh culture media control,

and significantly higher than the 0% viability in the IMS-treated (dead control) group. These results strongly supported the non-cytotoxic nature of this hydrogel, meeting the ISO 10993 cytotoxicity threshold, which requires cell viability above 70% for biomaterials intended for biomedical applications (International Organization for Standardization, 2009b). Given that the GelMA-PEGDA hydrogel exhibited only minimal viability reduction, well within the acceptable range, it was classified as non-cytotoxic and was therefore suitable to be used in liver tissue engineering applications. These findings confirmed that the material formulation supports both mechanical integrity and a biologically permissive environment, essential for sustaining hepatic cell culture and functional tissue development.

3.2.9 Promoted Biocompatibility through Sterilization and Surface Conditioning protocol optimization

To optimize the sterilization process and enhance cell attachment to the GelMA-PEGDA hydrogel scaffolds, different combinations of washing and soaking solutions were tested as illustrated in Figure 2.4. The initial sterilization method (V1.0 shown in Figure 2.4) required long waiting periods, delaying the seeding process and potentially affecting cell viability as seen in the lack of cells in the Pen-Strep/PBS image in Figure 3.14 . Therefore, alternative protocols were evaluated to minimize preparation time while maintaining a sterile environment conducive to cell attachment. Following sterilization techniques proposed by Caliri and Burdick. (Caliri and Burdick, 2016), several sterilization methods were evaluated for their effectiveness in preserving hydrogel integrity and supporting subsequent cell culture. A total of two sterilization solutions including Penicillin-Streptomycin

(Pen-Strep) or 70% Industrial Methylated Spirit (IMS), two washing agents included phosphate-buffered saline (PBS) or culture media, and 2 soaking solutions, either culture media or fetal bovine serum (FBS) were used. These conditions were compared to standard tissue culture plastic (TCP) as a control to assess baseline cell behaviour and sterility.

The LIVE/DEAD fluorescence images seen in Figure 3.14 demonstrate clear differences in cell viability and attachment between the tested sterilization and post processing conditions. The IMS-Media (V2.0) and IMS-FBS (V2.1) conditions resulted in high cell attachment and viability, similar to the TCP control, indicating their effectiveness in preserving cell health. In contrast, the Pen-Strep-PBS (V1.0) condition showed lower cell coverage, likely due to residual PBS affecting initial cell adhesion. The Pen-Strep-Media (V1.1) condition improved cell attachment compared to PBS but was still less effective than the IMS-based methods. IMS treatment, particularly when followed by FBS soaking, provided an optimal balance between rapid sterilization and maintaining a favourable surface for cell adhesion.

The absence of contamination across all tested conditions confirms the effectiveness of the sterilization methods employed. The use of antibiotics, such as Pen-Strep, effectively eliminated bacterial contamination by inhibiting cell wall synthesis and protein production in potential pathogens. Additionally, ethanol-based agents, such as 70% ethanol or IMS, are widely used for sterilization due to their effectiveness against fungi, bacteria, and common viruses. Their antimicrobial action primarily results from disruption of cellular membranes and protein coagulation, both leading to organism inactivation and death. In this study, IMS treatment successfully sterilized the printed hydrogel constructs, as evidenced by the absence of contamination following cell seeding. Immersion in 70% IMS likely contributed

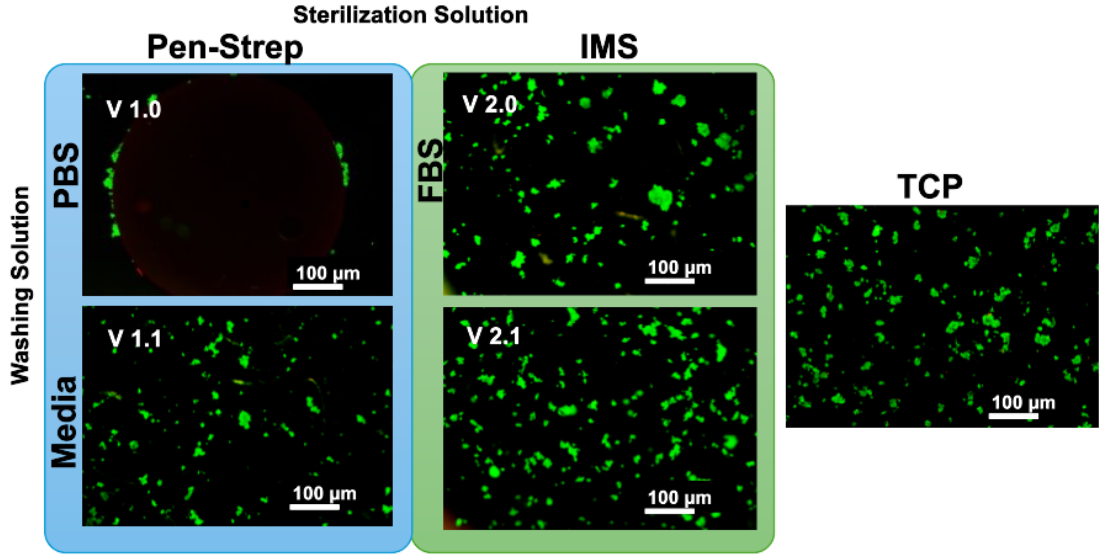


Figure 3.14: LIVE/DEAD staining in HepG2 cells after exposing the hydrogel prints to different sterilization and pre-seeding protocols compared with tissue culture plastic (TCP). Green represents live cells while red represent dead cells

to microbial inactivation through protein denaturation and membrane disruption. The washing steps with PBS and media further removed residual washing agents, ensuring a suitable environment for cell culture.

Soaking the hydrogel samples in fetal bovine serum (FBS) before cell seeding can significantly enhance cell attachment by adsorbing essential proteins onto the scaffold surface. FBS contains a rich composition of ECM proteins, including fibronectin, vitronectin, and laminin, which provide integrin-binding sites that facilitate cell adhesion (Lin et al., 2014; Felgueiras et al., 2018a). When the hydrogel is pre-soaked in FBS, these proteins form a bioactive layer, mimicking the natural ECM environment and improving cell-scaffold interactions (Camci-Unal et al., 2012). Additionally, FBS contains growth factors that support initial cell survival and spreading by promoting cytoskeletal organization and integrin-mediated signalling. The presence of serum proteins can also modulate the hydrogel's surface properties, reducing hydrophobicity and enhancing wettability, which further sup-

ports cell attachment (Felgueiras et al., 2018a). This is particularly important for synthetic or semi-synthetic biomaterials, like the GelMA-PEGDA hydrogel used in this study, which may lack intrinsic cell-recognition motifs. By providing an optimal biochemical microenvironment, FBS soaking ensures more effective initial cell anchorage, leading to improved viability, proliferation, and function in tissue engineering applications. Furthermore, the subsequent FBS soaking step played a crucial role in enhancing cell attachment. By pre-conditioning the hydrogel with FBS, a more biologically favourable microenvironment was created, facilitating initial cell adhesion and spreading.

These findings highlight the importance of selecting appropriate sterilization and soaking protocols for hydrogel-based hepatic tissue models. The chosen methodology that incorporates IMS and FBS soaking ensures an efficient sterilization and scaffold cell attachment and biocompatibility while minimizing delays in the experimental workflow. The optimized IMS-FBS protocol not only reduced preparation time but also promoted strong cell attachment, making it a promising approach for this study.

3.3 Conclusions

The primary objective of this chapter was to develop, optimize, and characterize a GelMA-PEGDA-based hydrogel system suitable for P μ SLA printing of architecturally complex scaffolds, with specific application toward liver tissue engineering. The formulation strategy was grounded in achieving a balance between biological compatibility, mimicking mechanical liver integrity, and fabrication precision. Key outcomes included successful methacrylation of gelatin using an improved synthesis method (Method B), incorpo-

ration of PEGDA to enhance photocrosslinking and structural robustness, optimization of light exposure parameters for high-fidelity printing, and validation of structural, mechanical, and biological performance through an array of analytical techniques.

Mechanical testing demonstrated that the incorporation of PEGDA significantly increased hydrogel stiffness, closely matching the mechanical properties of *ex-vivo* liver tissue. This mechanical tuning not only supports biologically relevant cellular responses but also enhances print fidelity by reducing deformation during fabrication. Photopolymerization experiments confirmed that the GelMA-PEGDA formulation exhibited superior printing performance compared to GelMA alone, achieving high-resolution structures at optimized light intensities. FTIR analysis further validated the effective removal of residual acrylates post-printing, ensuring the material's biocompatibility.

Cryo-SEM imaging, optimized through metal mirror freezing, revealed a uniform nanoscale mesh network with an average pore size of approximately 55 nm. This nanostructure is highly favourable for mass transport of nutrients, oxygen, and signalling molecules, critical for sustaining cell viability and function in three-dimensional constructs. Swelling and degradation studies further demonstrated that the hydrogel maintains scaffold stability and permeability over a 14-day period, aligning well with the intended *in-vitro* culture time frames for hepatic models.

Biological evaluation showed excellent cytocompatibility, with cell viability exceeding 95%, well within the non-cytotoxic threshold defined by ISO 10993-5 standards. Additionally, sterilization optimization using IMS-FBS treatment proved critical for ensuring scaffold sterility while enhancing initial cell attachment and spreading.

Collectively, the results presented in this chapter confirm that the GelMA-PEGDA hydrogel formulation offers a robust, biologically supportive, and fabrication-ready platform for the development of advanced liver tissue engineering scaffolds. The combined mechanical, structural, and biological properties achieved position this hydrogel as a promising candidate for future studies involving hepatic cell culture, tissue regeneration, and disease modelling applications.

Chapter 4

Translating ECM Architecture into 3D Printed Lattices for Liver Tissue Engineering

4.1 Introduction

The ECM is a dynamic, multifunctional network of proteins and polysaccharides that profoundly influences cell behaviour through biochemical, mechanical, and structural interactions (Frantz et al., 2010). It regulates fundamental cellular processes such as adhesion, migration, differentiation, and survival, while also serving as a reservoir for bioactive molecules (Wang et al., 2020a; Chaudhuri et al., 2020; Urbanczyk et al., 2020). In hepatic tissue, the ECM provides not only a structural scaffold for hepatocytes and sinusoidal endothelial cells, but also a complex topographical and mechanical landscape that modulates liver-specific functions, including metabolic activity, zonation, and drug detoxification (Wells, 2005).

However, replicating the intricate microarchitecture of native ECM in synthetic scaffolds remains a major challenge in tissue engineering. As discussed in Chapter 1, decellularised tissues provide a reference for biologically relevant ECM geometry, but their direct use as scaffolds is often limited by batch variability, immunogenic risk, and poor control over porosity and shape (Wang et al., 2015a; Neishabouri et al., 2022). Moreover, the irregular and heterogeneous structure of ECM is difficult to recapitulate using traditional computer-aided design (CAD) approaches, which rely heavily on parametric symmetry and manual modelling.

Recent advances in computational design tools and additive manufacturing have made it possible to generate complex bio-inspired structures with high spatial resolution (Chouhan and Murali, 2024). Software platforms such as SolidWorks, Rhino/Grasshopper, and Autodesk Fusion 360 have enabled the design of simple lattice-based scaffolds; however, they often lack the flexibility to generate spatially heterogeneous or topologically irregular constructs. In this context, implicit modelling tools such as nTopology

represent a significant advancement for biofabrication.

nTopology enables geometry creation based on mathematical fields rather than discrete surfaces, allowing for the design of highly complex, functionally graded, and procedurally defined lattice architectures. nTopology supports volumetric Boolean operations, field-driven patterns, and Voronoi tessellations which makes it particularly suited for replicating the architectural features observed in dLECM. Furthermore, nTopology avoids common issues such as mesh distortion or Boolean failures during solid modelling, which are often encountered in bio-inspired scaffold design. Moreover, the software's integrated lattice generation toolkit facilitates precise control over design parameters, such as unit cell size, density, and connectivity, enabling a high degree of reproducibility and structural fidelity in exported CAD models

To systematically explore how scaffold geometry influences hepatocyte performance, a set of scaffold designs were generated using periodic unit cell-based lattices and stochastic geometry based in the characterization of dLECM. The periodic designs incorporated three different unit cell types: Simple Cubic, Isotruss, and Gyroid. These unit cells were selected for their variation in strut connectivity patterns, intersection angles, and porosity distribution. These designs were compared against a Voronoi-tessellated stochastic lattice, developed to emulate the irregularity observed in dLECM.

A) Simple Cubic: A regular orthogonal structure with struts intersecting at 90° , forming a uniform cubic lattice. This geometry was chosen as a baseline control, offering high manufacturability and predictable mechanical behaviour. Simple Cubic lattices are widely used in mechanical testing due to their regularity, though they exhibit lower surface-area-to-volume

ratios and lack microarchitectural cues present in native ECM (Lei et al., 2019). As such, this geometry serves to benchmark deviations introduced by more complex lattice designs.

B) Isotruss: A triangulated lattice composed of struts intersecting at approximately 45° and 60° , arranged in a spatially reinforced, helical configuration. This design was selected to assess how acute-angle intersections affect cell behaviour. Isotruss geometries have been shown to exhibit superior mechanical efficiency, with high strength-to-weight ratios and angular anisotropy that may enhance directional guidance of hepatic cells and enhanced liver functions in hepatocytes compared to cubic scaffolds (Lewis et al., 2018). Their spatial reinforcement and interfacial complexity also allow for localized mechanical stimuli that could modulate cytoskeletal organization.

C) Gyroid: A triply periodic minimal surface (TPMS) structure without discrete struts, featuring continuous curvature and gradual transitions. In this context, its pore walls form oblique surfaces with non-linear, multi-angled intersections. TPMS geometries such as the Gyroid offer enhanced isotropic permeability, high surface-to-volume ratios, and interconnected void spaces that improve perfusion and nutrient transport in engineered tissues (Baumer et al., 2024). Furthermore, their smooth topologies are well-suited for soft cell-laden hydrogels and hepatocyte aggregates that require non-abrasive environments.

This three unit-cell types, shown in Figure 4.1, were modelled to represent different geometries in periodic scaffolds. All periodic lattices were designed using these unit cells, enabling control over strut intersection angle as a variable influencing mechanical and biological performance. The diversity of geometrical features among these lattices allows for interrogation

of how structural regularity, angular variation, and continuous curvature affect scaffold behaviour *in-vitro* and give us a solid ground to compare this repeating unit geometries with the stochastic distribution proposed later on.

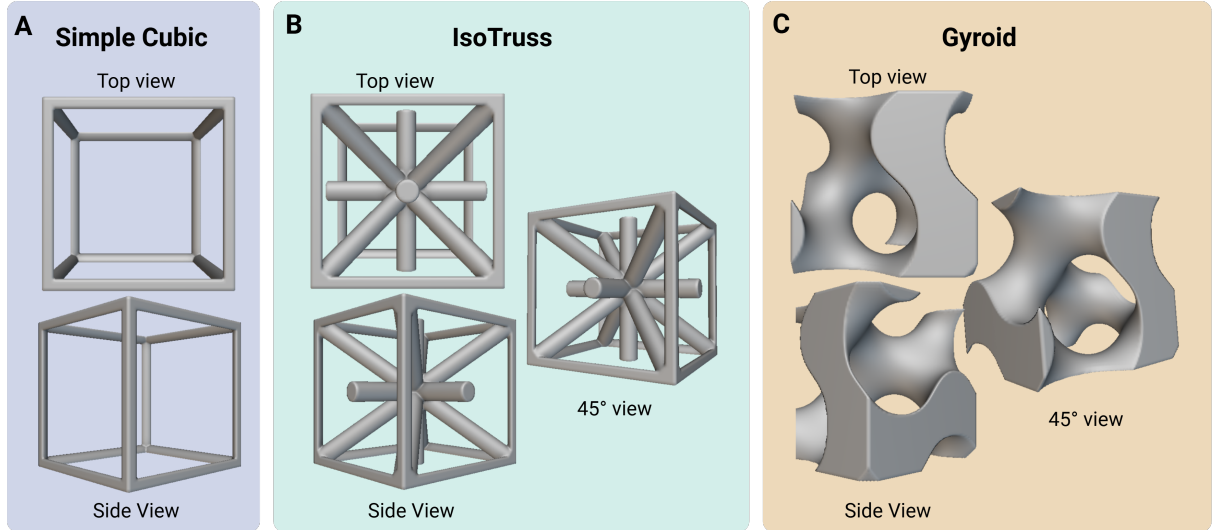


Figure 4.1: Scaffold unit cell geometries used for periodic lattice design. A) Simple Cubic lattice with 90° strut intersections (top and side views). B) IsoTruss lattice with diagonal struts intersecting at 45° and 60° angles (top, side, and 45° views). C) Gyroid surface-based lattice showing smooth curvature and multi-angled surface intersections (top, side, and 45° views)

In contrast to the periodic designs, the Stochastic scaffold was constructed using a Voronoi tessellation algorithm, in which seed points were randomly distributed in three-dimensional space and connected to form a spatially irregular, non-repeating lattice. This design lacked an underlying unit cell and instead reproduced the stochastic variation in pore and strut dimensions observed in native ECM characterization explained in further sections.

The resulting Voronoi geometry introduced randomized strut angles, pore interconnectivity, and variable spacing, more closely reflecting the architectural complexity of natural hepatic tissue. Such irregular geometries have been shown to more effectively mimic tissue anisotropy helping replicate better native tissue in other tissue types such as bone tissue (Lau et al.,

2021). Unlike periodic scaffolds, the Voronoi design was not limited by symmetry constraints, enabling exploration of how non-uniform topology impacts functional properties such as diffusive transport, mechanical stress distribution, and cell guidance.

This chapter presents a systematic approach to scaffold design based on microarchitectural features extracted from dLECM as discussed in Section 2.3. By integrating ECM-informed morphometric analysis with computational modelling in nTopology, a series of periodic and stochastic scaffold designs were developed, analysed, and optimized for fabrication in P μ SLA. This chapter evaluates how geometric variation impacts printing fidelity, surface-to-volume ratio, and bio-inspired models, providing design insights for the development of functionally relevant hepatic scaffolds.

4.2 Results and Discussion

4.2.1 ECM Characterization of Morphometric features as a Inspiration for Scaffolds Parameters

To establish a design target for the bioinspired scaffolds, a curated set of SEM images of dLECM was assembled from peer-reviewed publications. The resulting image collection, which visually defines the target microarchitecture, is presented in Figure 4.2. The selected images span multiple tissue sources, including human (Mazza et al., 2015), porcine (Mirmalek-Sani et al., 2013; Wang et al., 2015b; Yagi et al., 2013), rabbit (Nari et al., 2013), and rodent (Lu et al., 2018; Damania et al., 2018; Wang et al., 2023b; Shirakigawa et al., 2012; Uygun et al., 2010; Ren et al., 2013; Soto-Gutierrez et al., 2011; Maghsoudlou et al., 2016; Zhou et al., 2016; Bao et al., 2011;

Hussein et al., 2016; Jiang et al., 2014) liver, and reflect a variety of decellularization techniques and SEM preparation protocols, providing a broad morphological overview of pore organization, strut formation, and ECM topology across spatial and species-specific contexts.

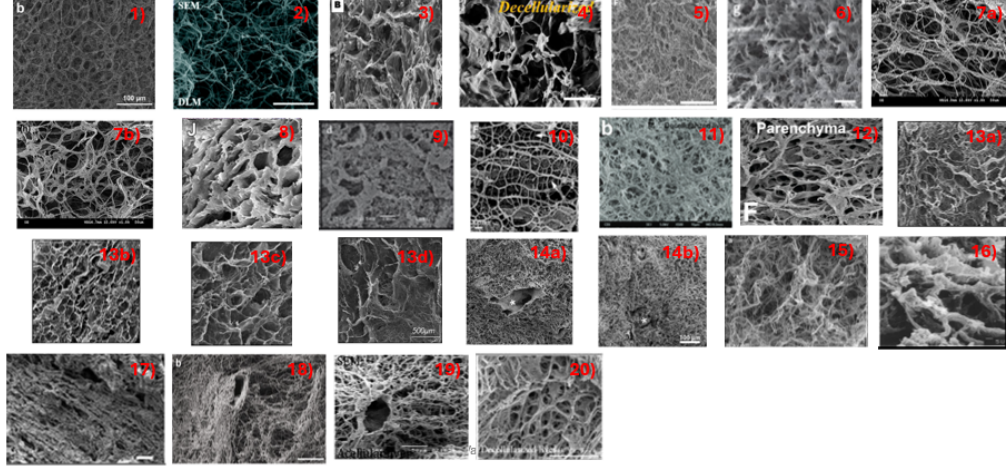


Figure 4.2: Recompilation of dLECM images for parametric analysis

This visual dataset served as the foundation for subsequent qualitative and quantitative analyses. To extract quantitative data, each image was analysed using ImageJ software. A minimum of 150 pores and 150 struts were measured per sample to quantify their respective sizes, thicknesses, and variability as described in section 2.3.1. An example of this measurement process is presented in Figure 4.3.

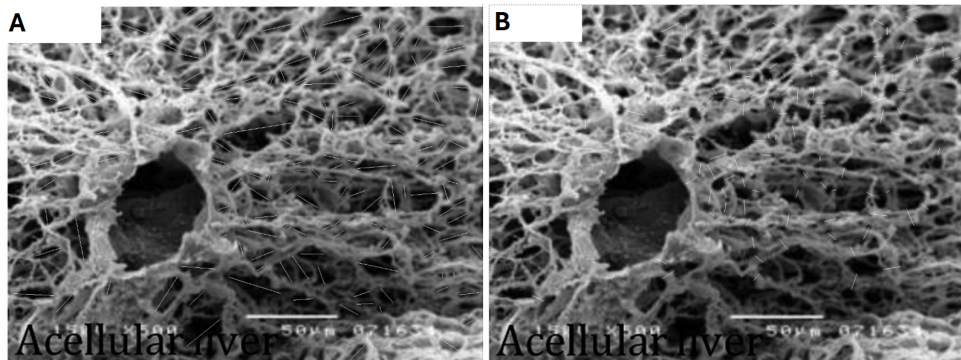


Figure 4.3: Example of dLECM Image analysis. A) Pore measurement and B) strut measurement of Yagi et al. (2013) using Image J measuring tool. Thin white lines were used to measure the pores and strut and to keep a log of the ones that were already measured

In addition to these two parameters, the volume fraction was also calculated. For this, the threshold tool in ImageJ was used to determine the percentage of the image area occupied by fibres, as shown in Figure 4.4. The numerical data from this analysis provided the key reference parameters for the subsequent scaffold designs. A summary of the measured pore size, strut thickness, their variabilities, and the volume fraction is presented in Table 4.1.

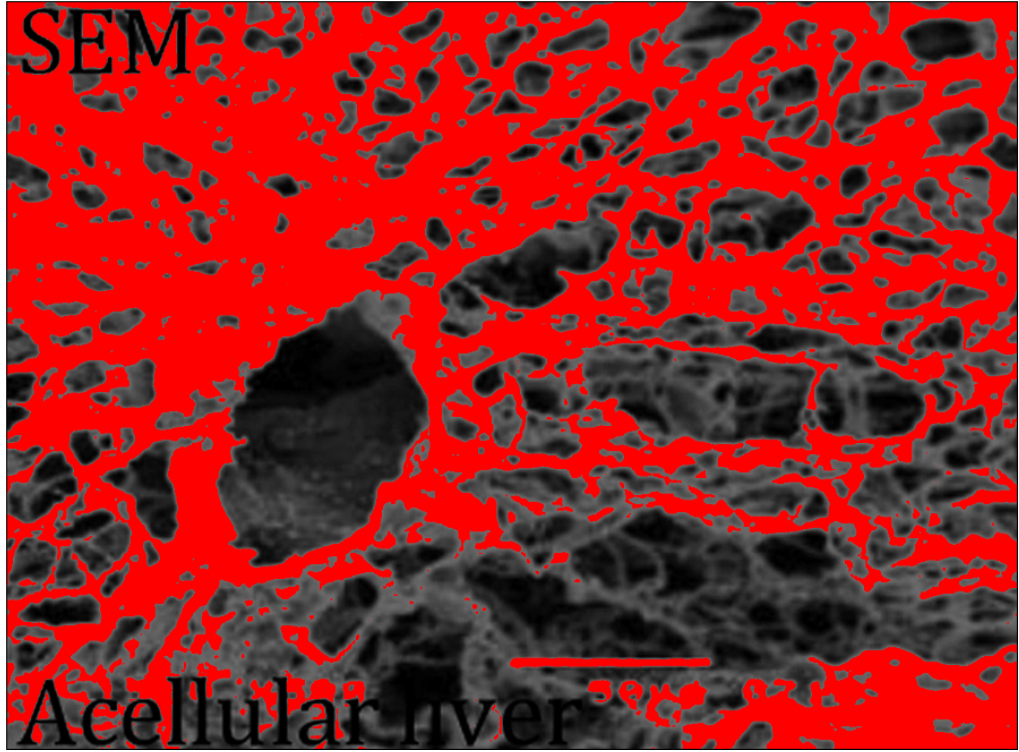


Figure 4.4: Example of dLECM image analysis of volume fraction measurement using threshold tool in Image J, Red area represents the ECM against the void space

Table 4.1: Data summary of image analysis and characterization of decellularised liver ECM

ID	Pore (μm)		Volume fraction (%)	Strut (μm)		Tissue Source	SEM Preparation	Reference
1	27.71	± 12.75	68.88	10.07	± 4.39	Human	CPD	Mazza et al. (2015)
2	10.85	± 6.26	56.19	2.24	± 0.86	Rat	Lyophilized	Lu et al. (2018)
3	23.77	± 17.47	60.61	10.35	± 5.29	Rat	vacuum dry	Damania et al. (2018)
4	33.82	± 20.38	50.55	1.747	± 0.90	Rat	-----	Wang et al. (2023a)
5	11.77	± 5.87	65.04	3.95	± 1.73	Rat	-----	Shirakigawa et al.(2012)
6	6.55	± 3.85	50.93	3.51	± 1.73	Rat	CPD	Uygun et al. (2010)
7a	8.423	± 4.47	69.81	2.42	± 1.45	Rat	Air-dried	Ren et al. (2013)
7b	4.22	± 5.26	68.72	2.33	± 1.37			
9	3.09	± 2.45	57.72	1.57	± 0.88	Rabbit	-----	Nari et al. (2013)
11	8.00	± 2.55	55.53	9.22	± 18.00	Rat	CPD	Soto-Gutierrez et al. (2011)
12	11.58	± 7.85	62.48	6.48	± 3.44	Pig	CPD	Mirmalek-Sani et al. (2013)
14a	19.68	± 7.91	56.09	6.98	± 2.87	Rat	CPD	Maghsoudlou et al. (2016)
14b	14.41	± 7.33	65.02	5.69	± 2.13			
15	11.51	± 6.72	78.03	5.14	± 2.06	Rat	CPD	Zhou et al. (2016)
16	6.60	± 6.07	68.51	3.24	± 1.98	Rat	CPD	Bao et al. (2011)
17	6.45	± 3.72	62.35	3.94	± 1.55	Pig	-----	Wang et al. (2015b)
18	19.50	± 14.19	69.18	7.09	± 2.77	Rat	CPD	Hussein et al. (2016)
19	9.92	± 5.17	68.30	5.63	± 2.19	Mouse	CPD	Jiang et al. (2014)
20	6.15	± 3.98	66.29	4.09	± 1.76	Pig	CPD	Yagi et al. (2013)

Notably, the inclusion of images from various species and decellularization methods allowed for a comparative morphological analysis, highlighting how processing conditions can influence ECM integrity and architecture (Peloso et al., 2016; Gilpin and Yang, 2017). The diversity observed, from dense collagen bundles to fenestrated porous networks, reflects the inherent complexity of the hepatic ECM and emphasizes the importance of quantitative morphometric parameters in scaffold design.

Overall, this image repository not only captures the architectural heterogeneity of dLECM but also establishes a data-driven foundation for reverse engineering biologically inspired scaffolds. By anchoring lattice design to empirical ECM features, the approach transcends arbitrary geometries and facilitates the development of functionally relevant tissue models.

Strut Size and Its Correlation with Pore Architecture

To examine the mechanical and morphological interdependence of matrix features, a correlation analysis between pore size and strut thickness was performed. A moderate positive correlation was observed as tissues with larger pore diameters generally exhibited thicker struts. This architectural scaling relationship reflects a biological need to balance porosity and mechanical stability, particularly in large lobular zones or sinusoidal regions.

For example, some samples with pores exceeding $18\text{ }\mu\text{m}$ presented strut sizes above $1.5\text{ }\mu\text{m}$, while smaller-pored tissues (like rabbit) displayed thinner struts ($1.5\text{ }\mu\text{m}$). However, this relationship was not strictly linear. Certain rat samples demonstrated large pores ($33.82\text{ }\mu\text{m}$) with thin struts ($1.74\text{ }\mu\text{m}$), whereas others retained high strut thickness despite modest pore dimensions. This suggests that fibre bundling, matrix composition, or partial collapse during processing may introduce architectural decoupling.

These observations support the notion that pore-strut ratios vary non-linearly, influenced by species-specific architecture, ECM remodelling post-decellularization, and imaging technique. Consequently, scaffold designs mimicking liver ECM should not enforce fixed pore-strut scaling laws, but instead draw from distributions reflective of native variability.

Influence of SEM Preparation on Microarchitectural Metrics

The reported variability in pore and strut dimensions was also examined in the context of SEM preparation methods, including drying technique and metal coating. Critical Point Drying (CPD) was the most frequently used protocol across studies and generally yielded consistent measurements with moderate pore and strut variability. CPD preserves ultrastructure by avoiding capillary collapse during dehydration, which is crucial for capturing fibrillar ECM networks (Gilpin and Yang, 2017).

In contrast, air-dried or lyophilized samples showed pronounced distortions. For instance, a rat sample processed via air-drying (Ren et al., entry 7b) displayed abnormally large pores ($4.22 \pm 5.26 \mu\text{m}$) with relatively thick struts ($2.33 \pm 1.37 \mu\text{m}$), likely reflecting shrinkage-induced compaction due to the freeze-drying process. Similarly, vacuum-dried samples (Damania et al., entry 3) exhibited higher variability in both pore ($23.77 \pm 17.47 \mu\text{m}$) and strut ($10.35 \pm 5.29 \mu\text{m}$) sizes, suggesting deformation artifacts.

The metallic coating used for SEM imaging (Au/Pd, gold, chromium, palladium) may have influenced image clarity and resolution, particularly in detecting fine struts. However, no consistent bias was identified across coatings, indicating that preparation-related dehydration is a more critical determinant of morphological fidelity than conductive layer selection.

In summary, SEM preparation method exerts a significant influence on reported geometrical metrics. Careful selection of drying techniques and consistent sample handling are essential to ensure reliable structural measurements for scaffold modelling.

4.2.2 Translating Native ECM Heterogeneity into Scalable Scaffold Parameters

While the literature characterization undertaken on dLECM revealed pore diameters predominantly ranging from 3 to 34 μm and strut thicknesses from ~ 1.5 to 10.35 μm , with a mean pore size of $12.4 \mu\text{m} \pm 8.4 \mu\text{m}$ and a mean strut thickness of $5.1 \mu\text{m} \pm 2.7 \mu\text{m}$, a parameter translation had to be performed to accommodate the requirements of this study. The native ECM pore dimensions, although reflecting acellular tissue, they are optimized for microvasculature and sinusoidal perfusion and produced with cells already in place. However, in biofabricated scaffolds, cell seeding, infiltration, and nutrient diffusion require larger pore sizes than those observed in static dLECM snapshots. Similarly, strut dimensions in native ECM correspond to thin collagen fibrils and fibronectin strands, but biofabricated scaffolds require greater mechanical robustness and structural fidelity.

The final scaffold design used enlarged architectural features, with pore diameters centred at $70 \mu\text{m} \pm 30 \mu\text{m}$ and strut diameters at $27 \mu\text{m} \pm 14 \mu\text{m}$, maintaining the 2.5 ratio between pore size and strut thickness and the corresponding variability ratio for both pore size and strut thickness. Maintaining also the volume fraction found in the characterization is vital, designing scaffolds with approximately 63.2 % volume fraction.

The selection of 70 μm as the mean pore diameter in the final scaffold design is further supported by literature demonstrating enhanced hepatocyte function within this dimensional range. Kukla et al. (2020) reported that porous silk scaffolds with an average pore size of $73 \pm 25 \mu\text{m}$ promoted the formation of larger primary human hepatocyte (PHH) spheroids, which in turn resulted in significantly improved liver-specific functions. Similarly, Tripathi and Melo (2015) showed that agarose–chitosan sponges featuring interconnected pores between 40–70 μm supported high cell viability and functional activity in hepatocyte cultures. These findings underscore that pore sizes around 70 μm are not only structurally permissive for hepatic cell infiltration but also functionally optimal for *in-vitro* liver tissue models. The adoption of $27 \mu\text{m} \pm 14 \mu\text{m}$ struts allows for reliable fabrication via P μ SLA, while mimicking the relative ratios and spatial disorder seen in hepatic tissue ECM. Importantly, this value reflects the 2.5 ratio scaling factor relationship with pore size found in the previous image assessment.

Though absolute dimensions were increased, the coefficient of variation in the design (approximately 43% for pores and 52% for struts) mirrors the high variability found in the examination. This was undertaken with the intention of preserving biologically realistic heterogeneity in one of the scaffolds design, which is crucial for replicating non-uniform diffusion and mechanical gradients inherent in liver tissue.

This approach reflects the broader tissue engineering paradigm, in which functional bio-inspired geometries, rather than exact microstructural replication, guides scaffold development.

4.2.3 Implicit Design of Periodic and Stochastic Scaffolds for dLECM-Mimetic Architectures

Having established the key microstructural and architectural parameters of the native dLECM, the next stage involved translating this biological blueprint into a reproducible scaffold design. The quantitative data from the characterization (such as pore and strut size and volume fraction) served as direct design inputs for a computational model. This section details the design process used to create a bio-inspired scaffold that geometrically represents the target dLECM features.

Engineering Periodic Scaffolds for Controlled Topological Features

The periodic scaffolds were generated by applying volumetric repetition of the previously selected unit cells (Simple Cubic, Isotruss and Gyroid) within a cylindrical scaffold. Original designs had a 2000 μm height and 2000 μm diameter, as shown in Figure 4.5. These scaffolds were later reduces to 500 μm height to save printing time. The lattice generation in nTopology preserved the unit cell symmetry and connectivity, with each design corresponding to one of the three architectural types.

In all three cases, nTopology's lattice generation module allowed tight control over unit cell size, lattice density, and strut diameter, ensuring that the final designs conformed to the target pore size (70 μm) and strut size (27 μm) without incorporating any variability.

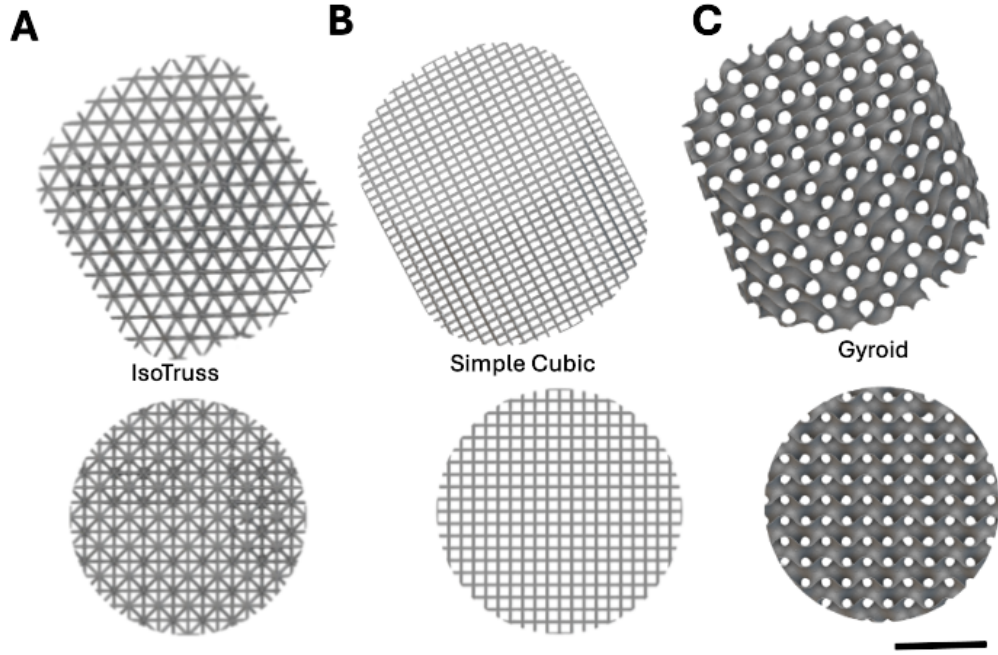


Figure 4.5: Designs of periodic scaffolds generated from selected unit cells using nTopology A) IsoTruss B) Simple Cubic and C) Gyroid. Scale bar = 1mm

Designing Stochastic Scaffolds to Replicate ECM Heterogeneity

The stochastic scaffold series was generated using Voronoi tessellation workflows within nTopology as mentioned previously in the methodology section, wherein random seed point distributions governed the spatial generation of interconnected voids. Resulting geometries are shown in Figure 4.6. Unlike the periodic designs, these scaffolds lack repeating unit cells and instead rely on controlled randomness to emulate the irregularity of natural liver ECM.

Voronoi-based lattice designs are increasingly recognized for their ability to recapitulate the heterogeneous and anisotropic architecture of biological tissues (Frayssinet et al., 2022). By allowing spatially irregular pore formation, these structures better capture the complexity and variability of native ECM, which is critical in tissues like liver where heterogeneity directly impact mass transport and cell behaviour.

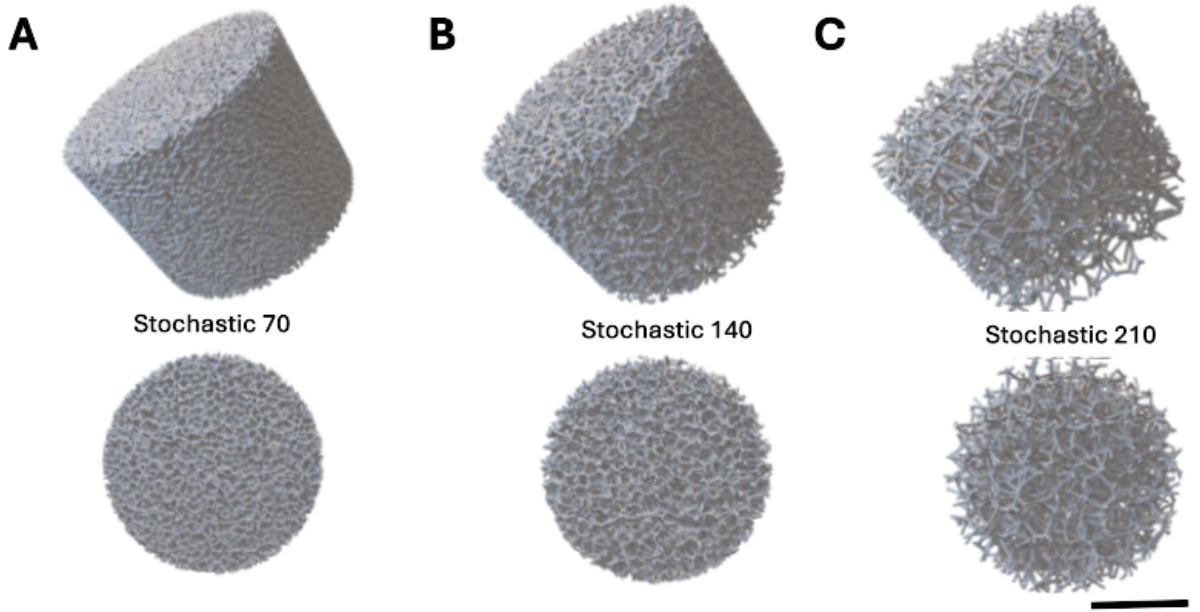


Figure 4.6: Voronoi-based stochastic scaffolds with increasing pore size ranges: A) $70\mu\text{m}$, B) $140\mu\text{m}$, and C) $210\mu\text{m}$. These models reflect variable openness and surface-to-volume ratios. Scale bar= 1mm

To address a key limitation observed during preliminary results (namely, high volume fraction of the initial stochastic scaffold with a $70\mu\text{m}$ mean pore size) a design sweep across pore sizes was performed. Three variants incorporating pore sizes two and three times the proposed pore size were developed to test the effect of pore enlargement on structural openness and its ability to print. Variability was also included in enlarged designs by maintaining the standard deviation found in the characterization section.

(A) **Stochastic $70\mu\text{m}$** : This design represents the lowest end of the pore range, yielding a dense, fibrous structure with high interconnectivity but low internal void fraction. Such densely packed networks may enhance cellular adhesion, but risk limited perfusion and nutrient diffusion (Han et al., 2021).

(B) **Stochastic $140\mu\text{m}$** : This intermediate design presents pores larger than the target pore size ($70\mu\text{m}$ mean), but with enhanced open volume

and better-defined channels. The increased void fraction improves scaffold permeability while maintaining a heterogeneous microenvironment suitable for cell attachment and polarity (Loh and Choong, 2013).

(C) **Stochastic 210 μm** : This upper-range design prioritized maximal void space and higher surface exposure, better accommodating limitations of resin penetration during printing. Large, irregular pores have been associated with improved material printability and perfusability in microfluidic systems and tissue models (Loh and Choong, 2013).

The stochastic designs revealed how adjusting pore size within the same topological family could be used to tune architectural performance, particularly in terms of permeability and interface accessibility. These variations also highlighted one of the strengths of Voronoi-based design: the ability to span a morphometric spectrum while preserving biological realism. In this way, stochastic design enables a progression of scaffold behaviours, offering a flexible design space for optimizing cell-scaffold interactions in hepatic biofabrication.

4.2.4 Scaffold Printability

Mesh Cleaning and Slicing Workflow for Print-Ready Scaffold Generation

Following lattice generation in nTopology, all scaffold models underwent a postprocessing workflow to prepare them for fabrication, as mentioned in the methodology section, to eliminate elements that could interfere with slicing and printing.

Representative 2D slices of the scaffolds shown in Figure 4.7 highlight the

internal structural patterns generated by different architectures, including Stochastic, Isotruss, Simple Cubic, and Gyroid designs. The slice images illustrate the cross-sectional complexity and pore distribution within each scaffold, confirming the successful preservation of target geometries across different topologies after slicing. This preparatory step ensured that the final scaffold models were manufacturing-ready, with clean, contiguous geometries optimized for 3D printing.

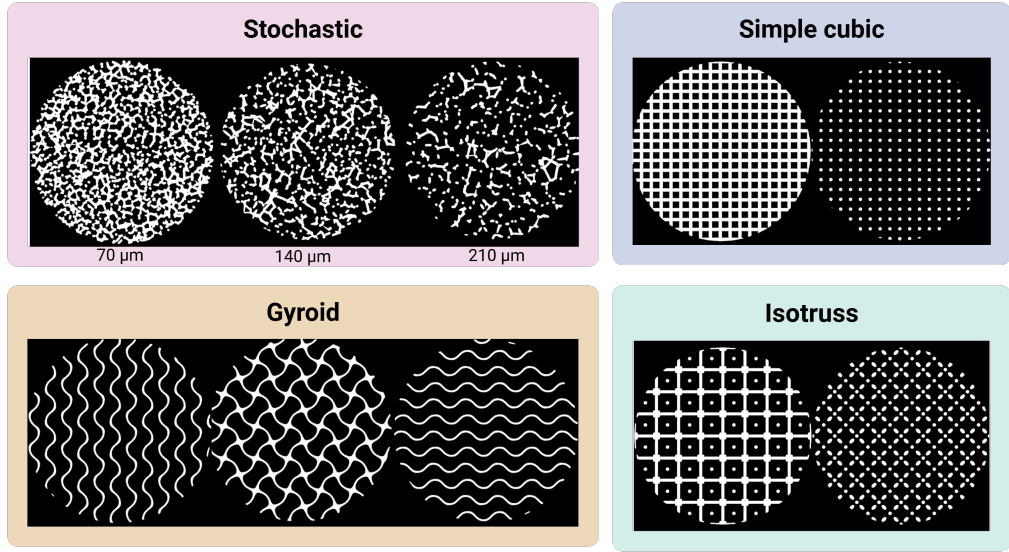


Figure 4.7: Representative top-view cross-sectional slices of the scaffold architectures. For stochastic scaffolds, slices are shown for pore sizes of 70, 140, and 210 μm . For the periodic architectures (Simple Cubic, Gyroid, and Isotruss), each pair of images corresponds to different slice depths within the same scaffold, illustrating how internal pore morphology varies throughout the structure. All geometries were cleaned and sliced prior to fabrication

Evaluation of Print Fidelity for Architected Scaffolds Using Standard Photopolymer Resin

Following model preparation and slicing, all scaffold designs were fabricated using a commercial photopolymer resin (HTL Resin, BMF Material Technology) via high-resolution micro-stereolithography (μSLA). Scaffold printing was performed according to the parameters detailed in Section

3.3.6, ensuring consistency in layer thickness, exposure settings, and post-processing conditions.

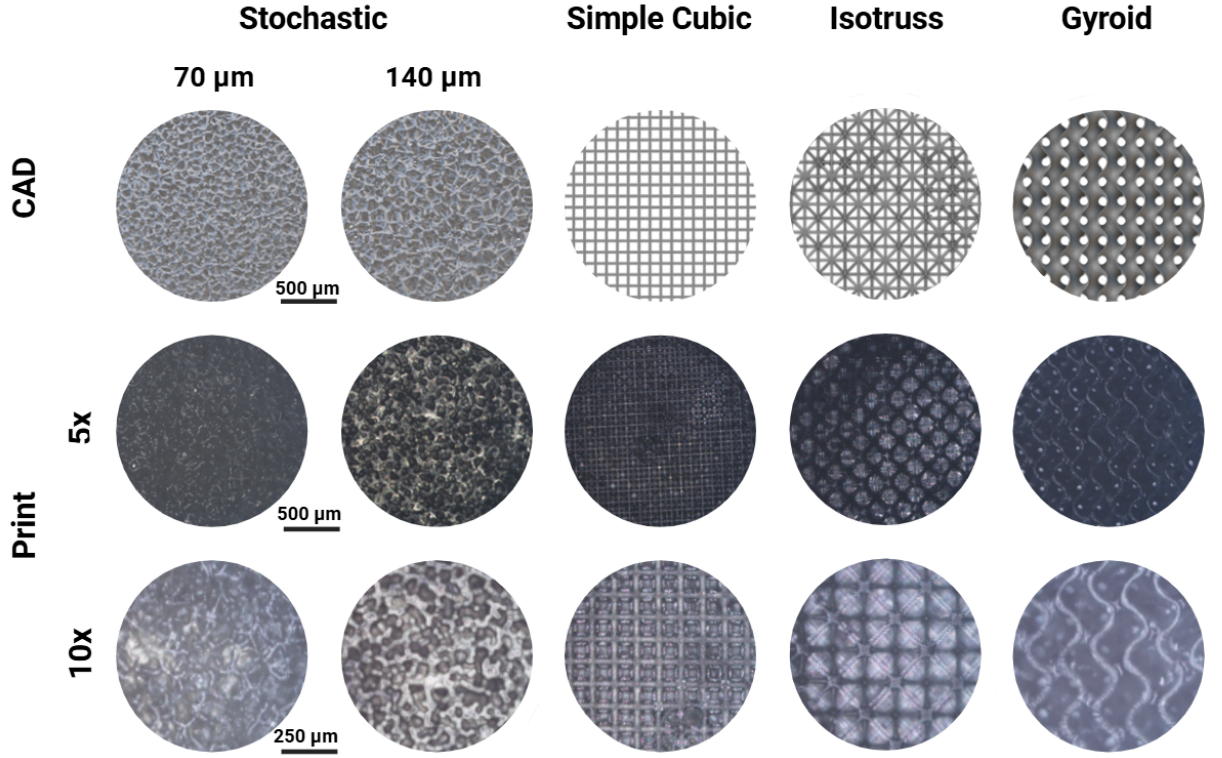


Figure 4.8: Fabrication results of scaffold designs printed using BMF HTL resin.

The top panel of Figure 4.8 presents the original digital CAD models for each scaffold type, while the middle and bottom panels display the corresponding fabricated scaffolds under optical microscopy. The printing process successfully replicated the intended architectural features across most designs, with varying degrees of fidelity depending on geometry:

Stochastic (70 μm and 140 μm) Both stochastic variants exhibited dense, highly interconnected porous networks. The smaller-pore (70 μm) scaffold appeared more compact and opaque, whereas the larger-pore (140 μm) scaffold presented greater openness and a more discernible internal structure. Minor fusion between adjacent struts was observed, particularly in the 70 μm variant, likely due to the close pore interconnection.

Isotruss The Isotruss lattice retained its triangulated pattern, with struts intersecting at acute angles (45° and 60°). Minimal merging of struts at intersection points was observed, which may reflect challenges in resin drainage and local overcuring inherent to highly interconnected geometries.

Simple Cubic The Cubic lattice scaffold demonstrated excellent geometric fidelity. Orthogonal 90° strut intersections were well preserved, resulting in a uniform grid with clearly defined channels along the principal axis.

Gyroid The Gyroid scaffold exhibited a smooth, continuous surface morphology, successfully replicating the complex minimal surface structure.

Importantly, the stochastic scaffold designed with a $210\mu\text{m}$ mean pore size could not be successfully printed. The high openness and minimal strut connectivity in this design as well as the material properties, resulted in insufficient support during printing, leading to structural collapse and failure as also found in other work such as in Weisgrab et al. (2020). This highlights a critical limitation in lattice design, where excessively large pores, thin connections and build orientation exceed the physical resolution and support capabilities of the μSLA system.

This fabrication failure using a mechanically robust commercial acrylate resin was particularly insightful. It demonstrated that the $210\mu\text{m}$ stochastic design, with its extensive open volume and minimal strut connectivity, pushed beyond the fundamental physical limits of the μSLA system and another printing technique as 2-Photon printing (Weisgrab et al., 2020), might be needed for this type of geometries. The failure of the mechanically robust commercial resin to produce this geometry demonstrated that the design exceeded the fabrication limits of the μSLA system. This finding

strongly suggested that a hydrogel, with a significantly lower mechanical modulus, would be incapable of maintaining structural integrity for this specific architecture.

On the other hand, the successful fabrication of the other designs confirmed that their geometries possessed sufficient structural integrity for the P μ SLA process. Consequently, the next stage of the research involved transitioning from the commercial resin to the custom-formulated GelMA-PEGDA hydrogel, with the knowledge that these specific architectures were stable and interconnected enough to be printed with the resolution and printing capabilities of P μ SLA .

4.2.5 High-Resolution Printing of designed Lattices with GelMA–PEGDA Through Intensity Tuning

To successfully fabricate high-resolution scaffolds using a photopolymerizable GelMA-PEGDA hydrogel, a refined printing optimization process was necessary. The initial parameters applied for this purpose were those established in Section 3.2.4, where the exposure conditions were tuned using simple geometries. However, while those settings enabled an acceptable curing of basic shapes, they were insufficient when applied to more architecturally complex lattice designs. In particular, the fabrication of the Isotruss and Stochastic lattices, with its intricate angled struts and highly interconnected topology, resulted in low-fidelity or incomplete prints, prompting the need for further optimization.

The Isotruss geometry was selected as the optimization model, given its sensitivity to photopolymerization accuracy. A series of test prints were per-

formed at increasing light intensities, ranging from 49.4 to 65.9 mW/cm², to identify the optimal exposure condition. As seen in Figure 4.9, at 49.4 mW/cm², under-curing was evident, with diffuse features, weak strut formation, and minimal retention of the lattice pattern on all the structure. Increasing the intensity to 53.5 mW/cm² improved strut visibility slightly, but structural discontinuities and blurring persisted, indicating that energy input remained below optimal. At 61.8 mW/cm², the internal geometry became more consistent and most of the struts were polymerized throughout the full depth of the scaffold, although minor under-curing artifacts, such as in the edge of the scaffold where visible. Finally, at 65.9 mW/cm², the scaffold exhibited sharply defined lattice nodes, consistent strut dimensions, and excellent geometric fidelity across the construct. This intensity provided a well-balanced exposure level, allowing complete polymerization of the hydrogel while preserving the fine resolution needed to replicate the original CAD geometry. Based on these outcomes, 65.9 mW/cm² was selected as the standard exposure intensity for all subsequent scaffold printing using this hydrogel formulation.

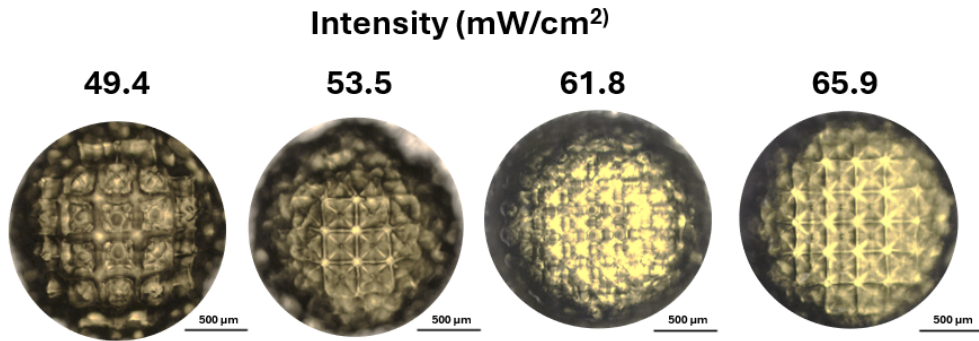


Figure 4.9: Optimization of hydrogel scaffold printing using Isotruss geometry as a test structure. Scaffolds were printed at different light intensities. Scale bars: 500µm.

Notably, as seen in Figure 4.10 the optimized conditions proved transferable to other scaffold topologies which were successfully fabricated with-

out further adjustment. Following the successful optimization of printing parameters using the Isotruss geometry, the same exposure conditions ($65.9\text{mW}/\text{cm}^2$) were applied to fabricate all other scaffold types using the GelMA-PEGDA hydrogel system. As shown in Figure 4.10, this parameter set enabled consistent and reproducible printing of both stochastic and periodic lattices, validating the robustness of the optimized protocol across topologies with distinct architectural complexity.

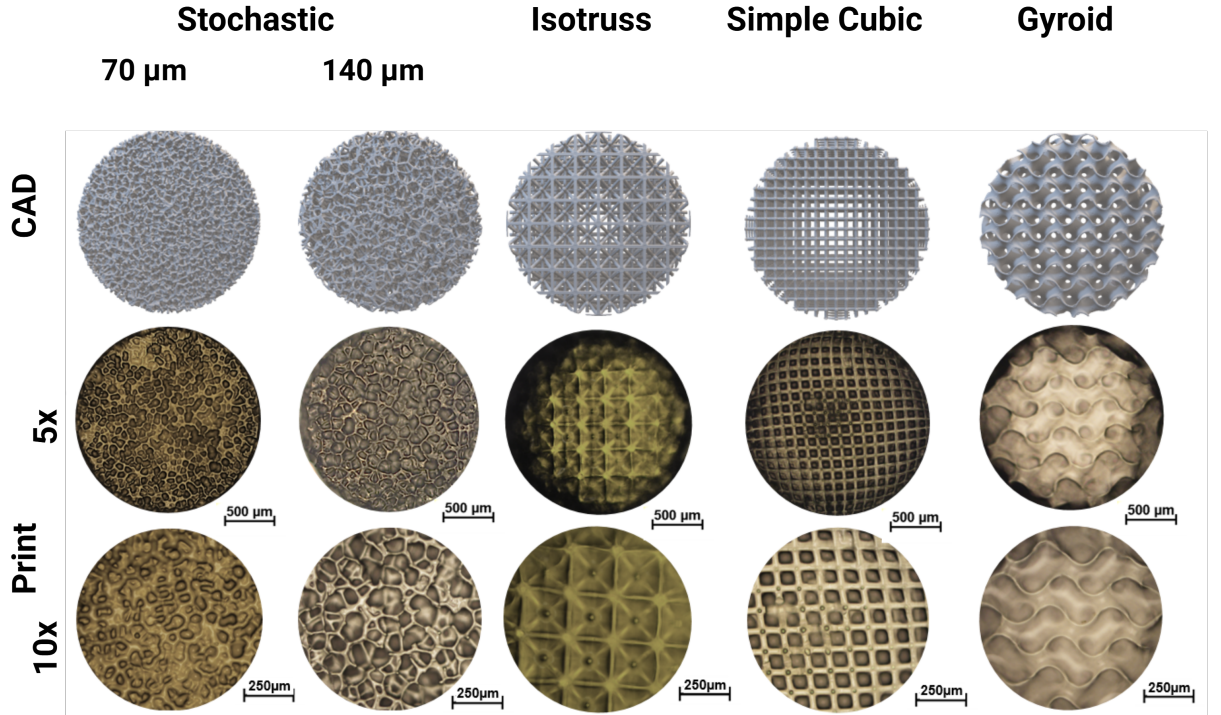


Figure 4.10: Hydrogel printing of all scaffold geometries using optimized exposure conditions. Low magnification(5X): Scale bar= $500\text{ }\mu\text{m}$. High magnification (10X: Scale bar= $250\text{ }\mu\text{m}$).

All designs, ranging from the randomly organized Stochastic lattices to the highly ordered Simple Cubic, Isotruss and Gyroid surfaces, were printed with high structural fidelity, as shown in both 5X and 10X magnification images (Figure 4.10). Stochastic scaffolds exhibited their characteristic irregular pore networks with clear interconnectivity, while the periodic

structures showed well-preserved unit cell geometries, Simple Cubic lattices maintained crisp orthogonal intersections, the Isotruss again showed diagonally connected struts with minimal distortion, and the Gyroid demonstrated smooth curvature and continuous surface morphology.

As seen in Figure 4.10, the hydrogel constructs retained their form after printing and exhibited no significant collapse or warping, confirming that the material system and light intensity were well-matched to the resolution demands of the designs. Together, these results confirm that a single set of optimized parameters can be successfully used to fabricate a wide range of architecturally distinct scaffolds in this tailored GelMA-PEGDA hydrogel, enabling a standardized yet versatile workflow for this project.

4.2.6 Architectural Performance of Periodic and Stochastic Scaffolds

Characterization of CAD Designed Scaffold Architectures

Following scaffold design in nTopology, a parameter analysis of the resulting lattices was performed to quantitatively assess geometric fidelity, dimensional accuracy, and volume fraction characteristics. Five different scaffold types were evaluated, including both stochastic and periodic architectures, using visual inspection and computational geometry analysis, as explained in the section 2.3.4. Results are shown in Figure 4.11.

All scaffold models were limited to cylindrical shapes with a height of 0.5 mm and a diameter of 2 mm, consistent between designs. Visual side views confirmed that all scaffolds maintained high-dimensional fidelity, without warping or having any significant irregularity in external shape.

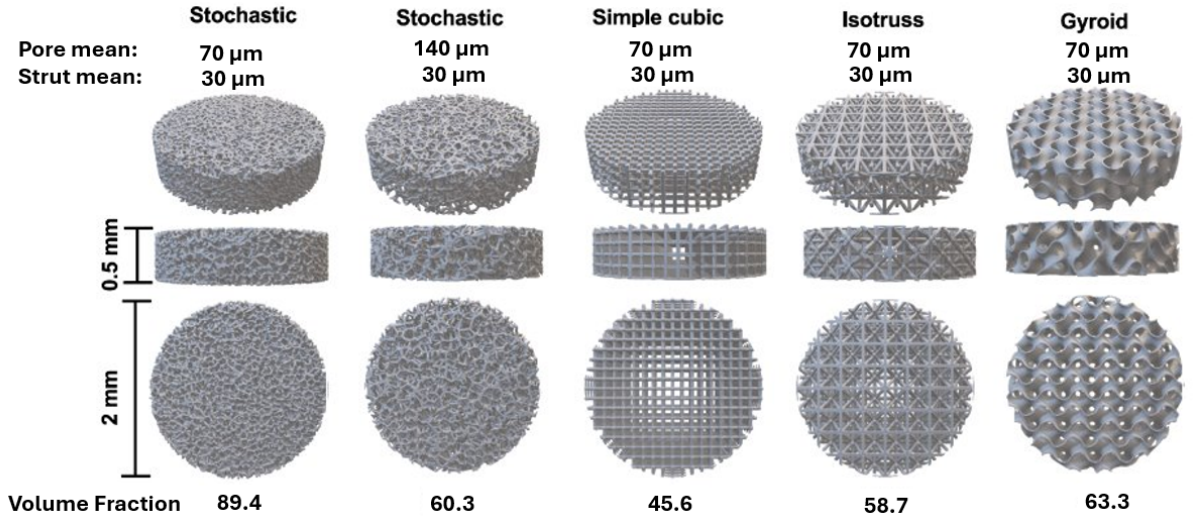


Figure 4.11: Geometric characterization of CAD scaffolds.

Pore and Strut Analysis

Despite differences in internal complexity, the scaffolds' macroscopic dimensions remained controlled and under the pore and strut parameters wanted, demonstrating the feasibility of using nTopology's lattice workflows to produce highly varied internal architectures without affecting gross scaffold parameters. The different scaffold types illustrate how topology inherently governs internal pathway complexity, pore interconnectivity, and mechanical anisotropy.

Volume Fraction Analysis

A critical parameter for tissue engineering applications is the volume fraction, as it directly affects the available area for cell attachment, the rates of nutrient diffusion, and the efficiency of mass transport (Nguyen et al., 2019). Computational volume fraction measurements were performed across scaffold designs, revealing differences among architectures, as shown in Figure 4.11, but within the desirable range of the dLECM characterization.

The stochastic scaffold with larger pores (140 μm) exhibited a low volume fraction compared to the smaller-pore stochastic scaffold (70 μm), confirming that increasing pore size markedly enhances internal surface exposure. A high-volume fraction in the initial stochastic design limited available surface for cell adhesion and impaired potential for nutrient transport. This led to the elimination of the stochastic geometry with a 70 μm mean pore size from further testing, and experimental efforts focused on the 140 μm pore variant.

Among the periodic lattices, the Gyroid architecture exhibited the highest volume fraction (63.3), owing to its continuous curvature and minimal surface nature. These geometries provide extensive surface exposure without introducing sharp corners or strut junctions, which can otherwise become stress concentrators (Kapfer et al., 2011). The smooth topology and isotropic pore connectivity of Gyroid scaffolds make them well-suited for applications requiring enhanced perfusion and homogeneous cell distribution (Vijayavenkataraman et al., 2018).

The Simple Cubic scaffold, despite its orthogonal simplicity, achieved a moderate volume fraction (45.6), while the Isotruss lattice reached 58.7, reflecting its higher strut density and increased internal interface area. These differences underscore how architectural features, such as the orientation of the strut, the density of the nodes, and the curvature, directly influence the internal surface area of the scaffold.

These findings emphasize the strong dependency of the volume fraction on the scaffold topology. Notably, certain stochastic designs can outperform periodic architectures in surface exposure when pore dimensions are appropriately scaled. This reinforces the biomimetic advantage of Voronoi-based stochastic lattices, which not only capture ECM-inspired irregularity

but also offer tuneable surface exposure beneficial for hepatocyte seeding, attachment, and metabolic activity.

Overall, the analysis highlights that volume fraction should be considered a design priority in scaffold engineering, particularly when fabricating systems for highly metabolic tissues like liver, where cell-scaffold interactions and mass transport are key elements. These geometries have a similar volume fraction to the dLECM scaffolds characterized as 63.2.

4.2.7 Geometric Replication of Designed Lattices in Hydrogel Constructs

Stochastic Lattice

To evaluate the structural fidelity of the printed stochastic scaffold with a mean pore size of $140\ \mu\text{m}$, pore size distributions were measured at three vertical levels along the scaffold height, top ($0\ \mu\text{m}$), middle ($250\ \mu\text{m}$), and bottom ($500\ \mu\text{m}$), using confocal microscopy. As shown in Figure 4.12, panel A displays representative maximum intensity projections at each level, while panel B summarizes the quantitative comparison of pore size distribution across the z-stack. Corresponding numerical values are reported in panel C.

The analysis revealed a clear axial gradient in pore dimensions, with the top region exhibiting the largest average pore size ($139.2 \pm 31.6\ \mu\text{m}$), followed by the middle layer ($120.7 \pm 28.3\ \mu\text{m}$), and the bottom region ($107.3 \pm 24.4\ \mu\text{m}$). Despite this progressive reduction in pore size with depth, the measured values remained within the designed stochastic range (centred around $140\ \mu\text{m} \pm 30\ \mu\text{m}$), confirming that the overall geometric variability

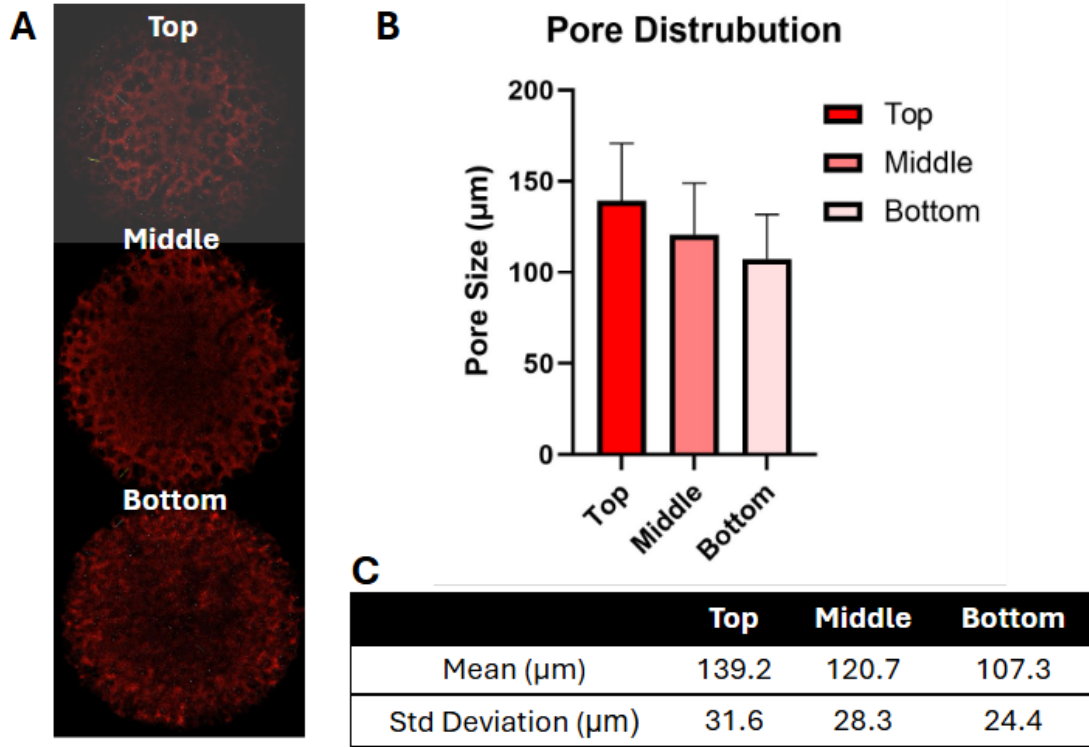


Figure 4.12: Assessment of printing fidelity in GelMA-PEGDA stochastic scaffolds. A) Confocal maximum intensity projections from the top (0 μm), middle (250 μm), and bottom (500 μm) sections of the scaffold. B) Quantitative distribution of pore sizes by depth. C) Mean and standard deviation of pore sizes across scaffold height. Pore sizes decreased with depth, though all values remained within the designed stochastic range (140 ± 30 μm), indicating overall fidelity in geometry replication.

was largely preserved. This suggests that the fabrication process successfully replicated the complex spatial irregularity of the original CAD design, particularly in the upper and middle layers of the construct.

The observed reduction in pore size from top to bottom within the printed stochastic scaffolds is consistent with common defect in photopolymerisation during the P μ SLA printing process. Since the bottom of the scaffold is the first printed part of it, mechanical weight and inaccuracy of light projection on the platform tends to cause minor fidelity issues. Additionally, oxygen inhibition near the base layer may affect curing asymmetries.

Such axial variations are well-documented in layer-based photopolymer sys-

tems (Harmatys et al., 2025; Badanova et al., 2022; Msallem et al., 2024), and while they do not significantly compromise the stochastic character of the scaffold, they highlight the importance of z-axis calibration and adaptive exposure compensation in future print protocols.

Periodic Lattice

To evaluate the printing fidelity of the periodic scaffold designs, pore sizes were quantitatively measured from microscopy images of Figure 4.10 and compared against the target design value of $70\text{ }\mu\text{m}$. The results, summarized in Table 4.2, show high concordance between designed and printed features across all three geometries, with fidelity values exceeding 97%.

Geometry	Pore Sized (μm)	Std Deviation (μm)	Printing fidelity (%)
Isotruss	70.3	0.2	99.6
Simple Cubic	71.7	0.1	97.6
Gyroid	68.8	0.2	98.3

Table 4.2: Quantitative assessment of printing fidelity across scaffold geometries based on measured pore sizes. The designed pore size was $\sim 70\mu\text{m}$ for all geometries. Fidelity was calculated as the percentage deviation from the target pore size, with Isotruss showing the highest agreement (99.57%) and Simple Cubic the lowest (97.57%).

Among the tested designs, the Isotruss scaffold demonstrated the highest fidelity, with a mean pore size of $70.3\text{ }\mu\text{m}$ and a calculated fidelity of 99.6%. This close agreement may reflect the high structural redundancy and angular strut distribution of the Isotruss geometry, which provides mechanical stability during layer-by-layer polymerization and reduces the likelihood of localized deformation. Additionally, its triangulated topology likely distributes curing stresses more uniformly across the structure, mitigating distortion during resin crosslinking.

The Simple Cubic scaffold exhibited the lowest fidelity among the three

designs, at 97.6%. The printed pore size ($71.7\ \mu\text{m}$) exceeded the designed value by $1.7\ \mu\text{m}$. This overgrowth may be attributed to its largely open architecture, which offers minimal constraint on polymer expansion during curing, coupled with slight over-curing of struts. While this did not compromise the structural integrity of the scaffold, it likely resulted in marginally wider spacing between struts and reduced lateral constraint during photopolymerization.

The Gyroid scaffold showed a slightly lower fidelity of 98.3%, with an average pore size of $68.8\ \mu\text{m}$. This minor undershoot may be attributed to under-polymerization at the curved Gyroid surfaces. Nonetheless, the Gyroid's smooth topology and isotropic properties contribute to consistent printing outcomes with a low standard deviation and high dimensional accuracy.

Overall, these results indicate that all three periodic scaffolds can be reliably fabricated with high geometric accuracy in relation to the CAD model. Notably, complex geometries such as Isotruss and Gyroid, despite their spatial intricacy, can achieve higher fidelity than simpler orthogonal lattices, provided that their strut continuity and surface transitions are well-resolved during printing.

4.3 Conclusions

This chapter presented a multi-scale design strategy for engineering hepatic scaffolds using periodic and stochastic lattice geometries informed by the microarchitecture of dLECM. Through the integration of systematic image analysis, quantitative morphological extraction, and computational modelling in nTopology, a diverse set of scaffold geometries was developed.

The periodic scaffold designs, based on Isotruss, Simple Cubic, and Gyroid unit cells, demonstrated high fabrication fidelity, with pore size deviations under 2.5% from the target value. Notably, the Isotruss geometry exhibited the highest dimensional accuracy, suggesting that triangulated frameworks with angular connectivity may resist photopolymerization-induced deformation more effectively than orthogonal grids. In contrast, the Gyroid scaffold, while slightly under the target pore size, provided the volume fraction value that most closely resembles the dLECM characterization, mainly due to its continuous curvature, highlighting its potential in applications requiring extensive cell–scaffold interaction and mass transport.

Stochastic scaffolds generated via Voronoi tessellation captured the architectural irregularity of native hepatic tissue. The tunability of pore size within the same stochastic framework was demonstrated to strongly influence surface exposure and internal porosity. In particular, increasing the mean pore size to 140 μm significantly decrease its volume fraction, enhancing the design’s suitability for hepatic cell culture and nutrient diffusion.

Comparative analysis of all geometries revealed that scaffold performance metrics, such as volume fraction, printing fidelity, and architectural realism, are highly sensitive to both the base unit geometry and pore size scaling. While periodic lattices offer manufacturing predictability and structural regularity, stochastic designs exhibit greater biomimetic complexity and tuneable functionality. Ultimately, this design framework demonstrates that the combination of data-driven ECM characterization with lattice optimization tools enables the rational development of hepatic scaffolds that are both structurally precise and biologically relevant. These insights provide a foundation for future work that integrates these designs into a printable environment that aims to model liver physiology.

Chapter 5

Biological Performance of dLECM-Inspired Lattices for Liver *in-vitro* Modelling

5.1 Introduction

In-vitro liver models are critical tools for studying hepatic physiology, drug metabolism, and liver-specific pathologies, offering alternatives to animal testing and enabling high-throughput screening of pharmaceuticals. However, the fidelity of these models depends strongly on their ability to represent the microenvironment of the native liver. Tissue architecture is an important part of this microenvironment, particularly the ECM, which regulates cell behaviour through mechanochemical and structural cues (Ingber, 2003; Janson and Putnam, 2015; Chaudhuri et al., 2020; Urbanczyk et al., 2020). Traditional 2D cultures fail to recapitulate the spatial organization, mechanical properties, and diffusional profiles that govern hepatocyte function *in-vivo*. Consequently, 3D scaffold-based systems have emerged as promising platforms to support hepatocyte growth, organization, and function by providing bio-inspired physical and chemical environments (Lewis et al., 2018; Kukla et al., 2020; Underhill and Khetani, 2018).

Chapter 3 established the material foundation for this work by developing a custom GelMA-PEGDA photoresin, ensuring the bioink possessed the necessary printability and biocompatibility for creating hepatic constructs. Subsequently, Chapter 4 defined the architectural blueprint by designing and characterising a library of lattice geometries, including a bio-inspired Stochastic design informed by dLECM analysis and three periodic designs (Isotruss, Gyroid, and Cubic) with precisely controlled structural features.

Building upon these foundations, this chapter now evaluates how these engineered microenvironments, fabricated using P μ SLA, affect the behaviour of HepG2 cells. The focus shifts from material and structural characterisation to a systematic analysis of how the interplay between the developed bioink and the selected scaffold geometries influences hepatocyte viability,

spatial organisation, and liver-specific functions.

Hepatic functions were assessed as illustrated in Figure 5.1. Together, this series of tests targets the most critical hepatic functions. Collectively, the data provides a comprehensive view of how scaffold geometry modulates hepatocyte behaviour in 3D, revealing the interplay between architecture-driven micro-environmental cues and liver-specific cellular functions.

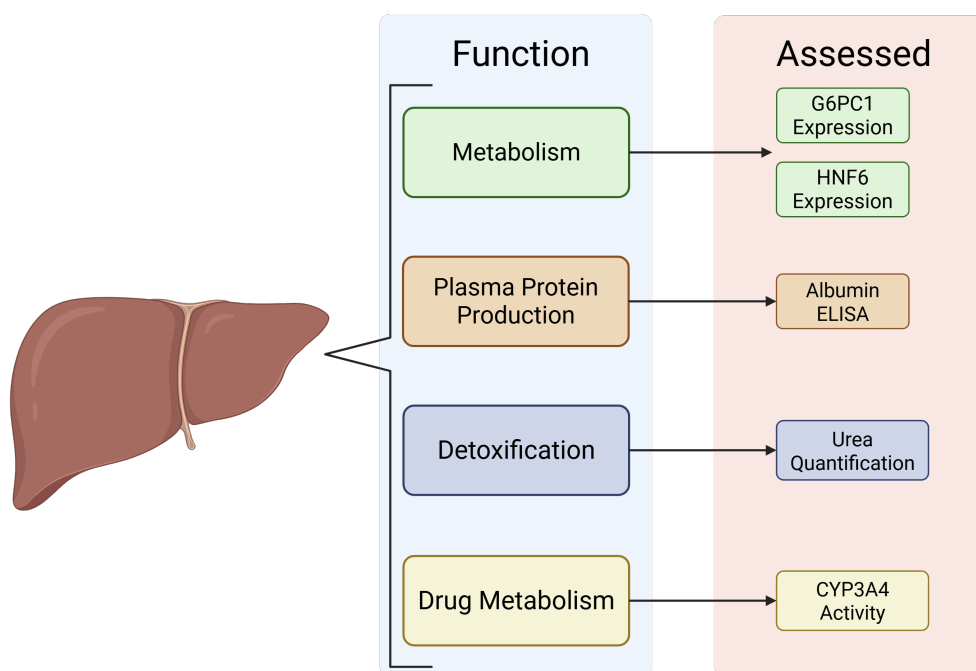


Figure 5.1: Functional assessment strategy for hepatic characterization. The diagram maps the primary liver functions of interest (Metabolism, Plasma Protein Production, Detoxification, and Drug Metabolism) to the specific quantitative assays performed to assess cellular performance.

Glucose-6-Phosphatase Catalytic Subunit 1 (G6PC1) is a key hepatic gene that serves as a robust marker of late-stage hepatocyte maturation. It encodes a catalytic component of the glucose-6-phosphatase complex, which is essential for glucose homeostasis through its role in gluconeogenesis and glycogenolysis (van Schaftingen and Gerin, 2002). G6PC1 expression is tightly regulated and requires metabolic induction, typically becoming prominent only in fully differentiated hepatocytes (Zabulica et al., 2019). As such, its upregulation reflects advanced metabolic functionality and the

acquisition of mature hepatic phenotypes.

Hepatocyte Nuclear Factor 6 (HNF6), also known as Onecut-1, is a transcription factor essential for early liver development and the regulation of genes associated with hepatocellular identity (Clotman et al., 2002). It serves as a marker of immaturity in hepatic cells and is prominently expressed during the early stages of hepatogenesis as illustrated in Figure 5.8 A. HNF6 is progressively downregulated during maturation, making it a valuable indicator of developmental progression in hepatocyte cultures (Rowe et al., 2013). Functionally, HNF6 influences the expression of genes critical for liver-specific functions, including those involved in cell polarity, bile duct morphogenesis, and metabolic regulation (Clotman et al., 2002).

Albumin is the most abundant protein in blood plasma and is synthesized exclusively by hepatocytes, making its secretion a key functional indicator of hepatic biosynthetic capacity and cellular maturity (Kalra et al., 2023). Urea synthesis is a central hepatic function reflective of nitrogen metabolism and detoxification capacity. As hepatocytes convert ammonia into urea, the measurement of extracellular urea provides a direct read-out of metabolic functionality and cellular maturity (Kalra et al., 2023; Lauschke et al., 2019).

A critical function of the liver is the detoxification and metabolism of xenobiotics, a process largely mediated by the cytochrome P450 (CYP) enzyme family (Dash et al., 2009). The CYP3A4 isoform is particularly significant, as it is responsible for the metabolism of a large proportion of clinically used drugs. Consequently, measuring CYP3A4 activity serves as a crucial benchmark for assessing the metabolic competency and functional maturity of engineered hepatic constructs

By assessing both short-term viability and long-term functional markers,

this chapter aims to determine which geometries best support the establishment of a robust and mature hepatic phenotype when using HepG2 cells. In doing so, it contributes to the broader objective of engineering liver-mimetic scaffolds that are structurally and functionally relevant for advanced *in-vitro* applications.

5.2 Seeding HepG2 into dL-ECM like constructs

5.2.1 Seeding optimization

Efficient cell seeding is a critical determinant of scaffold-based tissue engineering, directly influencing initial cell attachment, distribution, and long-term viability (Melchels et al., 2011, 2010; Maghsoudlou et al., 2016). In the context of 3D hepatic models, achieving stable cell integration within porous constructs is particularly important to ensure functional maturation and reproducibility. Variations in scaffold architecture, surface wettability, and micro-scale geometry can markedly affect cell retention during the early stages of culture (Felgueiras et al., 2018b; Janson and Putnam, 2015). Therefore, prior to conducting functional assessments, a systematic seeding optimization was carried out to identify conditions that maximized HepG2 cell retention across the scaffolds, using Stochastic and Isotruss as sample geometries. The Stochastic and Isotruss geometries were selected for seeding optimization as representative examples of the two main architectural classes investigated in this study, disordered and periodic lattices. The Stochastic geometry was chosen as the only one in its group, constructed to mimic the irregular, fibrous network of the native extracellular

matrix, providing a biomimetic baseline. The Isotruss geometry was selected as a representative of the periodic group as it offers a higher degree of structural complexity and interconnectivity than the simple Cubic lattice, yet avoids the intricate, curved surfaces of the Gyroid, making it an ideal intermediate model to assess cell behaviour in an ordered, non-simple architecture.

Figure 5.2 compares two different seeding techniques, agitation versus pipette tip entrapment. Within each method, the effect of washing with PBS after Fetal Bovine Serum soaking was assessed. The agitation method reflects that cells might need movement to penetrate through the scaffolds architecture (Goldstein et al., 2001), while the entrapped seeding also called "droplet seeding" looked into using a small volume to physically confine, or "entrap," the cells within the porous architecture, maximizing the initial cell-scaffold contact time and preventing cell loss to the rest of the culture dish (Ishaug et al., 1997).

The images in Figure 5.2 reveal that agitation-based seeding led to limited cell attachment, particularly in the absence of washing, where very few cells are present based on the green fluorescence observed due to MitoTracker staining. Washing improved attachment in both scaffold types but remained suboptimal in the agitation technique. This improvement is attributed to the removal of excess FBS from the environment. While FBS is a necessary supplement in culture media for cell survival and proliferation, excessive concentrations can introduce cytotoxic factors (Cui et al., 2004); therefore, washing away the excess appears to have improved initial cell attachment.

In contrast, the pipette tip entrapment method resulted in enhanced cell adhesion, which was visually confirmed by the greater presence of green

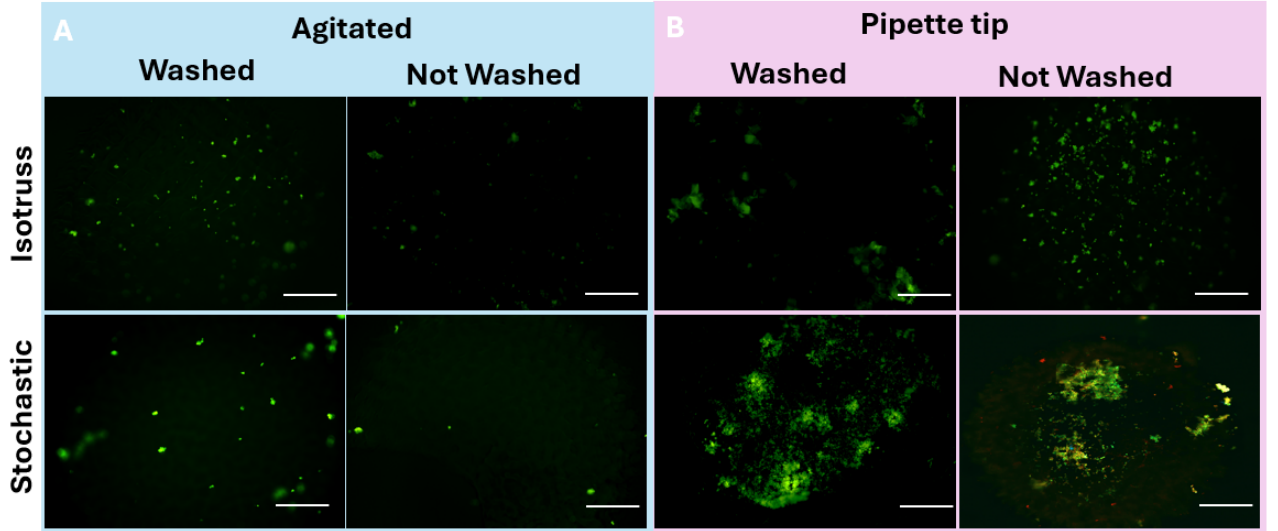


Figure 5.2: HepG2 cells labelled with MitoTracker™ Green FM on day 1 in Isotruss and Stochastic scaffold after A) agitation seeding protocol with and without FBS afterwash and B) after pipette tip entrapment with and without FBS afterwash. Scale bar = 100 μ m.

fluorescence, particularly in the washed condition (Figure 5.2). This superior cell retention is likely attributed to the prolonged and stable contact time between the cells and the scaffold's surface. Based on these initial observations, only scaffolds seeded via the entrapment method were selected for continued culture and subsequent imaging on day 3.

Figure 5.3 assesses cell retention over time and shows cells during day 1 and day 3 using the pipette tip entrapment method. Washed conditions resulted in better-distributed cells with moderate survival over three days, especially in the Stochastic scaffold, where a larger area remained covered with cells. Meanwhile, in the not-washed condition, significant aggregation and detachment were observed by day 3, particularly in the Isotruss scaffold. The slight decrease in fluorescence seen from day 1 to day 3 is a normal phenomenon explained by the multi-stage process of cell adhesion to a biomaterial surface. According to the principles outlined by Garcia et al. (2005), initial cell attachment is a rapid but weak and reversible process (García, 2005). Over the subsequent hours and days, cells must actively

strengthen this bond by spreading and forming stable focal adhesions; cells that fail to complete this transition are washed away during routine media changes, resulting in a lower cell count at the later time point (García, 2005). While there was a decrease in the cells observed by day 3, the significantly higher cell retention observed in the entrapment group supports this method as the most effective seeding approach.

According to Felgueiras et al. (2018), the interaction between a cell and a biomaterial is not a direct physical contact, but is instead mediated by an intervening layer of proteins adsorbed onto the material’s surface. This crucial protein interface, which can be enhanced by pre-soaking scaffolds in FBS, dictates subsequent cellular responses. Cells use receptors like integrins to biochemically recognize and bind to this layer containing cell-binding motifs such as arginyglycylaspartic acid (RGD) sequence and fibronectin and vitronectin; this process governs initial cell adhesion and activation (Felgueiras et al., 2018b).

However, this contact is not only for biochemical binding; it is also for physical sensing. Anisotropy or directionality of the microenvironment provides powerful instructional cues that govern a cell’s internal organization and polarity. This process of sensing the surface geometry and reorganizing the cytoskeleton requires stable, uninterrupted contact (Thé et al., 2006).

This dual requirement for both biochemical binding and physical sensing strengthens the case for an entrapment seeding method in conjunction with the FBS soaking and washing. This technique likely provides the stable, prolonged exposure necessary for cells to first form strong biochemical adhesions due to the presence of motifs left by the FBS soak and then subsequently sense the scaffold’s architecture and organize themselves accordingly. In contrast, while agitation may improve initial cell penetration,

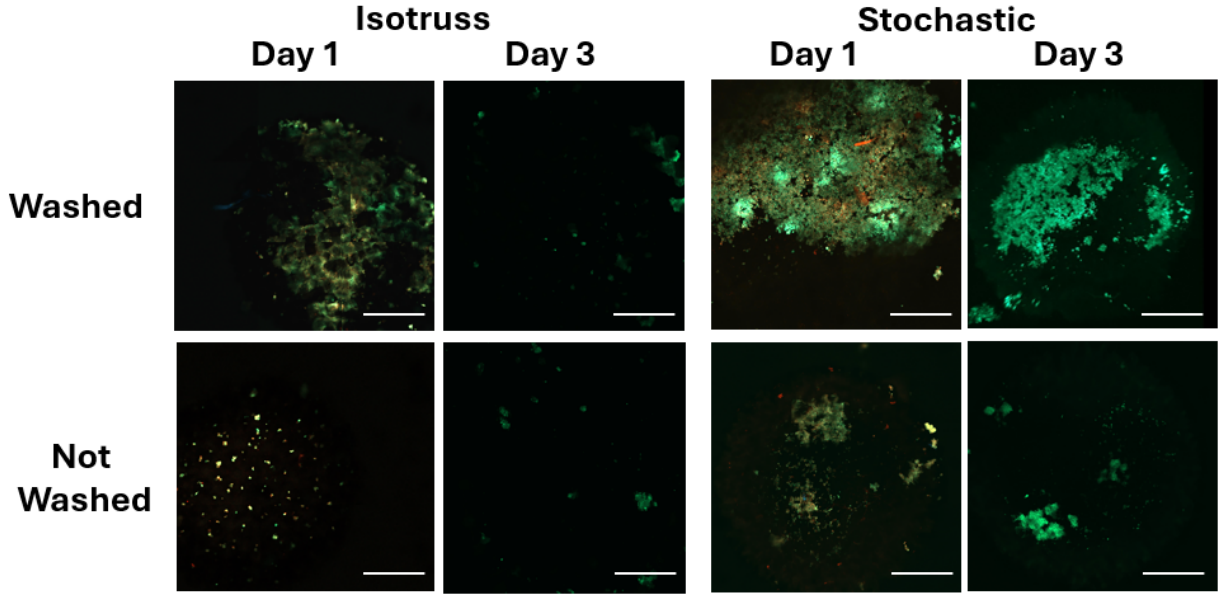


Figure 5.3: HepG2 cells labelled with MitoTracker™ Green FM on day 1 and 3 in Isotruss and Stochastic scaffold after pipette tip entrapment with and without FBS afterwash. Scale bar = 100 μm .

the transient nature of the cell-surface contact is likely insufficient for the cells to fully engage in these critical sensing and organizational processes, leading to poorer initial adhesion and retention. Therefore, it can be concluded that entrapment seeding, followed by a washing step after being soaked, leads to more uniform cell distribution, greater initial adhesion, and superior cell retention over time. The resulting optimized seeding protocol is illustrated in Figure 5.4A and was used for all cell studies.

HepG2 cells were seeded and after 24 hours of static incubation, DNA content was quantified using a dsDNA assay to determine seeding efficiency. Quantification revealed high and consistent cell retention across all scaffold types, with no significant differences among geometries compared to TCP (Figure 5.4B). On average, seeding efficiencies exceeded 95% in all tested groups, as measured in three biological replicates with internal technical replicates. These results validate the seeding method as both reproducible and geometry-independent at early time points. Achieving high initial cell retention is critical for the success of long-term 3D hepatic cul-

tures, particularly when working with porous and architecturally complex scaffolds.

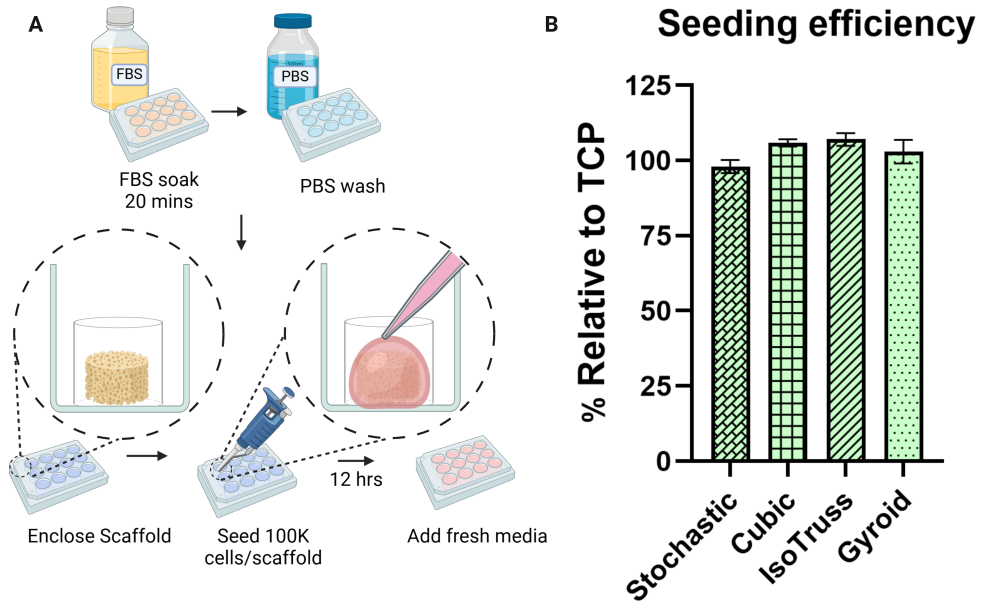


Figure 5.4: A) Schematic representation of the final seeding protocol. Scaffolds were prewetted with 100% FBS for 30 minutes to enhance surface protein availability, followed by a PBS rinse and static seeding of HepG2 cells in an enclosed volume. Scaffolds were incubated under static conditions for 12 hours prior to media exchange. B) Seeding efficiency assessed by ds-DNA quantification after 24 hours. All scaffold types showed high seeding efficiency ($\geq 95\%$) with no statistically significant differences between geometries (one-way ANOVA, $p \geq 0.05$), indicating protocol reproducibility and architecture-independent cell retention at day 1.

The observed lack of significant variation across scaffold types suggests that, under this protocol, seeding efficiency is not hindered by micro-architectural differences at this early stage. This is particularly important for ensuring fair comparative analysis across groups in downstream functional assays. Moreover, consistent DNA quantification across biological and technical replicates supports the robustness and reproducibility of the method, making it a suitable protocol for subsequent high-throughput hepatic tissue engineering studies.

5.2.2 Temporal Dynamics of Cell Proliferation and Viability in Hepatic 3D Scaffold Cultures

To determine how different scaffold geometries support cell growth, cell proliferation and viability were tracked over 14 days using dsDNA quantification and lactate dehydrogenase (LDH) assays (Figure 5.5). The observed growth kinetics on the 2D tissue culture plastic (TCP) control, characterized by a phase of rapid proliferation up to day 7 followed by a significant decline by day 14, is a well-documented and expected outcome of cells reaching confluence. As adherent cells expand to cover the entire available surface area, they establish extensive cell-cell contacts, which triggers a natural regulatory process known as contact inhibition of proliferation that arrests the cell cycle (Hanahan and Weinberg, 2011). Once this confluent monolayer is formed, the culture environment begins to degrade. The high cell density leads to the rapid depletion of essential nutrients from the medium and, concurrently, the accumulation of toxic metabolic byproducts (Ozturk et al., 1992). This combination of growth arrest, nutrient starvation, and increasing toxicity creates an unsustainable environment, ultimately inducing widespread apoptosis and leading to the observed decline in the viable cell population.

In contrast, all 3D scaffolds supported continuous cell growth, with the most substantial increase in dsDNA content occurring between days 7 and 14. Notably, the Stochastic scaffolds achieved the highest cell count by day 14, suggesting the stochastic scaffold provided a superior microenvironment for long-term proliferation compared to both the 2D control and other 3D geometries (Bokhari et al., 2007; Asthana et al., 2018).

LDH release remained consistently low for all scaffold types at days 3, 7 and 14, confirming the biocompatibility of the GelMA-PEGDA hydrogel.

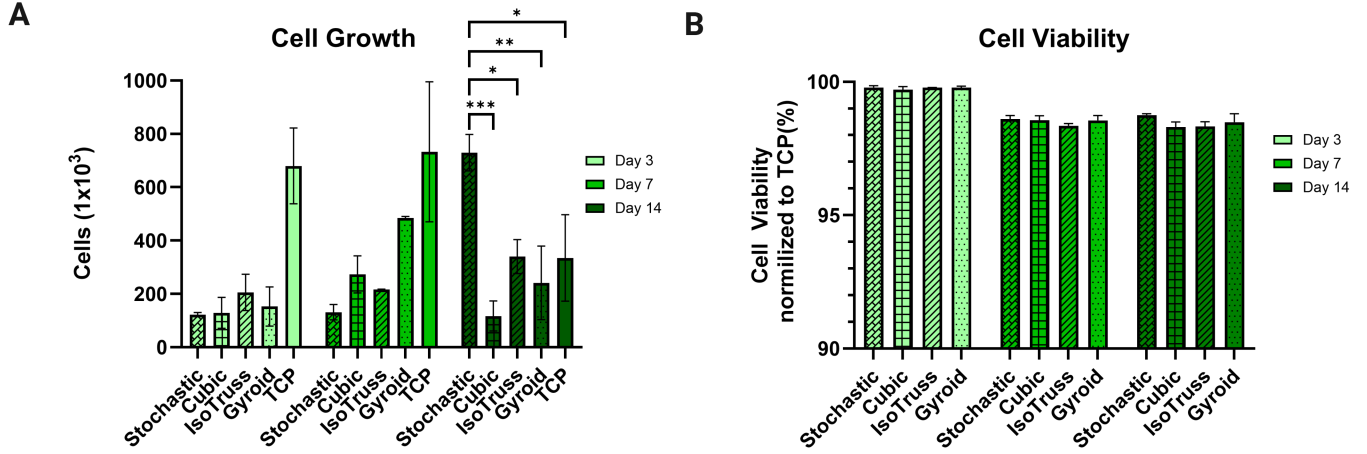


Figure 5.5: Quantification of cell number and metabolic activity across scaffold geometries and timepoints. (A) dsDNA quantification on days 3, 7, and 14 shows a geometry- and time-dependent increase in cell number, with Stochastic scaffolds supporting the highest levels by day 14. (B) LDH assay reflects non-significant changes in metabolic activity, showing cell viability greater than 98% in all geometries during the 14 days of culture. Data are presented as mean $\pm$ standard error of the mean ($N = 3$; $n = 3$). Statistical analysis by one-way ANOVA indicated significant effects scaffold geometry (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

The slight increase in LDH levels observed at later time points by the decrease in viability to $\approx 98\%$ remains within the expected range for proliferating and metabolically active cultures (International Organization for Standardization, 2009a). The results indicated that all printed geometries successfully maintained viable cell populations throughout the culture period. No significant decline in viability was observed, confirming that the fabrication strategy, post processing, seeding and scaffold designs created a stable and supportive micro-environment for hepatocytes.

5.3 Scaffold Microarchitecture Dictates Cellular Spatial Organisation and Viability

LIVE/DEAD labelling was conducted to visualise how cells colonize the different scaffold geometries over time. This revealed that the microarchitecture of each scaffold provided distinct topographical cues for hepatocytes. Figure 5.6 shows representative images of the spatial distribution and viability of HepG2 cells within the different scaffold geometries over time.

Brightfield images showcase the distinct architecture of each scaffold, while fluorescence microscopy highlights the resulting differences in cell organisation and viability at days 3, 7, and 14. Across all geometries, high green fluorescence signals indicate robust cell viability, with minimal red fluorescence suggesting negligible cytotoxicity, which is consistent with the quantitative data from previous sections.

While cell arrangement appears similar across Stochastic, Isotruss and Gyroid on day 3, by day 7, distinct, architecture-driven patterns emerge. As illustrated in Figure 5.7, these architectural differences directly influenced whether cells formed dense aggregates (Stochastic), aligned along linear struts (Cubic), concentrate around vertices (Isotruss), or stayed confined in void spaces (Gyroid). This highlights the critical role of scaffold design in actively guiding patterns of tissue formation, rather than simply acting as a passive support structure.

A key observation across the non-linear geometries is the promotion of spheroid-like cell clusters, which can be explained by the innate behaviour of hepatocytes. Liver cells possess a strong, intrinsic tendency to self aggregate through powerful cell-cell adhesion molecules (cadherins) (Duval

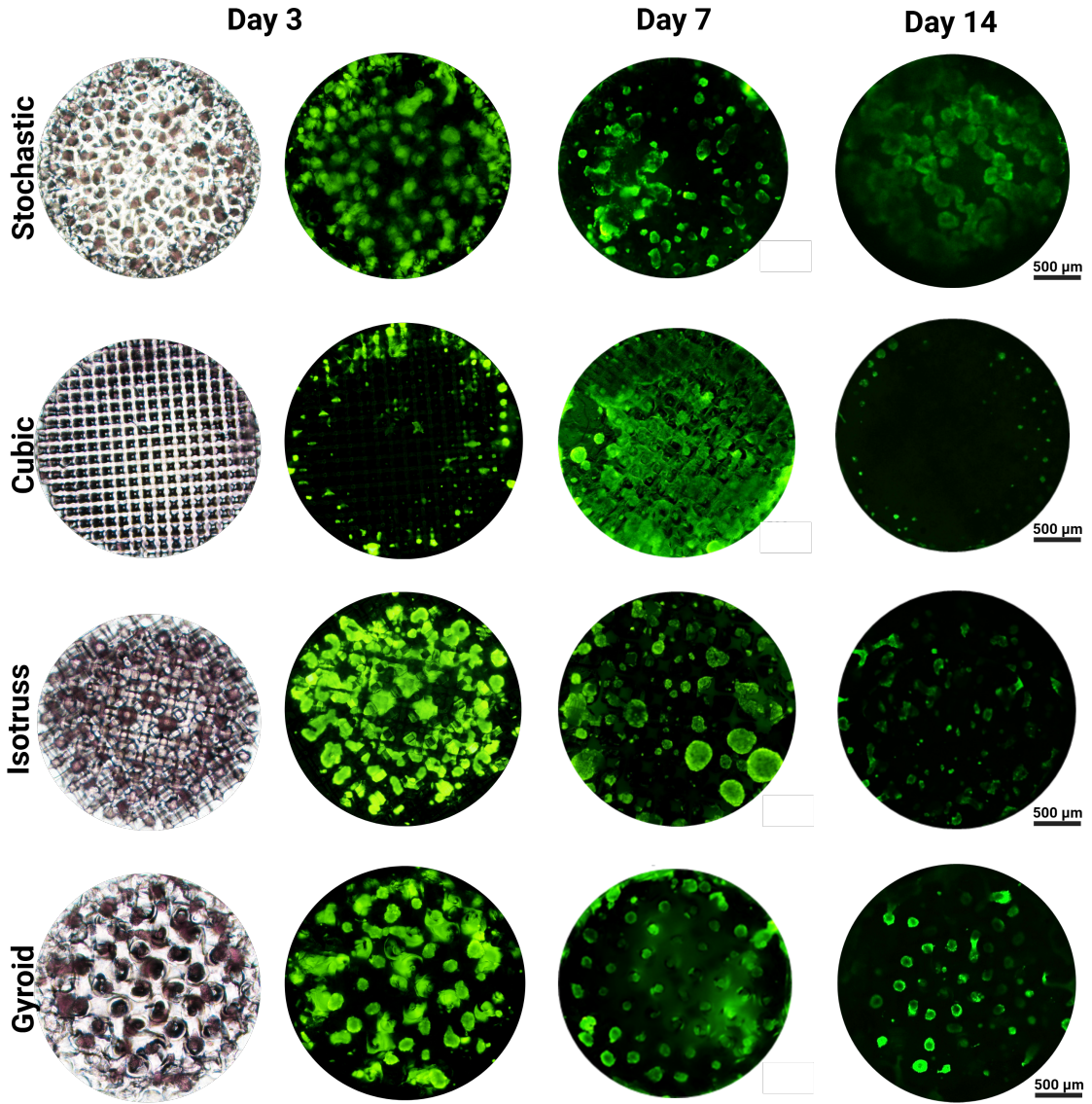


Figure 5.6: Live Dead microscopy Images of HepG2 cells in different geometry scaffolds

et al., 2017). This behaviour is particularly dominant in environments where strong, stable cell-matrix adhesion is geometrically or chemically discouraged. The concave surfaces and irregular pores of the Stochastic and Isotruss scaffolds, for instance, appear to favour these cell-cell interactions over extensive cell spreading, leading to the rapid formation of spheroids. This behaviour has been observed in other studies (Taymour et al., 2021; Wu et al., 2020; Tabatabaei Rezaei et al., 2025) and might be due to a combination of the stiffness of the material (Ma et al., 2018)

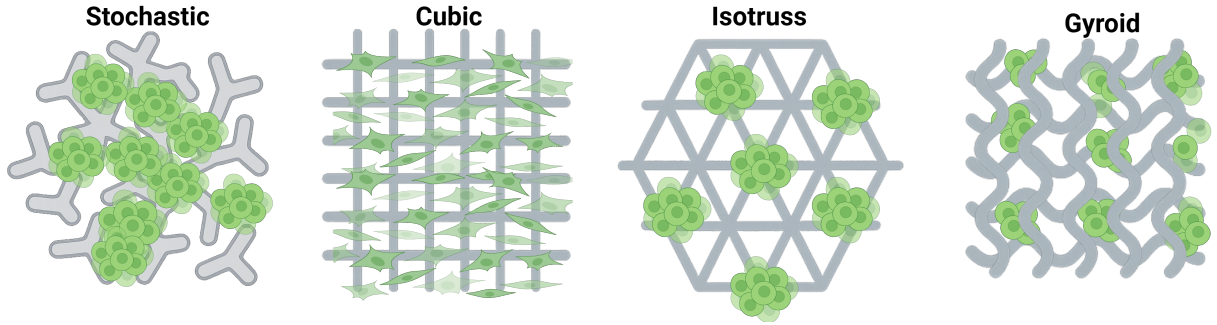


Figure 5.7: Schematic of cell distribution in different scaffolds geometries. Cells formed dense aggregates in Stochastic scaffolds, aligned along linear struts in Cubic scaffolds, concentrated around vertices in Isotruss scaffolds, and stayed confined in void spaces in Gyroid scaffolds

and the influence the geometry has on the cells' way of arranging according to Ingber's tensegrity principle (Ingber, 2003). This shift towards aggregation is significant, as spheroid models are known to better mimic the *in-vivo* state and exhibit more physiologically relevant functions compared to monolayers (Duval et al., 2017; Bokhari et al., 2007; Ramaiahgari et al., 2014).

This same principle explains the cell patterns in the Gyroid geometry, but with a different outcome due to its unique architecture. The Gyroid's smooth, continuous curvature also discourages the formation of strong cell-matrix adhesions, causing the cells' natural propensity to self-aggregate to become the dominant organisational force (Blanquer et al., 2017). However, a critical distinction emerges. In the Stochastic scaffolds, the highly interconnected pore network allowed these newly formed spheroids to make contact and merge over time, forming larger, continuous tissue-like aggregates. In contrast, the discrete and segmented nature of the Gyroid's cavities physically confined the spheroids, preventing them from interacting with neighbouring clusters. This architectural difference was crucial, as the interconnected aggregates are better positioned to establish the extensive cell-cell communication networks required for coordinated functional

maturation.

In contrast, the highly structured and mechanically anisotropic nature of the Cubic scaffold, with its flat and linear struts, promotes contact guidance (Thé et al., 2006). This phenomenon guides cells to elongate and align along the architectural paths, favouring strong cell-matrix adhesion and a dispersed, flattened morphology over the formation of spheroids. This alignment is visually confirmed in Figure 5.6, where the scaffold's layers appear to act as a series of 2D surfaces, encouraging monolayer formation.

Interestingly, this geometry also exhibited a higher cell count at day 7, likely due to this rapid 2D-like expansion (Figure 5.5). However, similar to the TCP control, this was followed by a decrease in cell number by day 14, ultimately resulting in the lowest cell population of all the groups. This suggests that while the Cubic architecture initially supports rapid proliferation, its simple geometry may lead to confluence-like effects that are not sustainable for long-term 3D culture.

These results demonstrate that scaffold geometry modulates cellular organisation and proliferation in HepG2-laden constructs without inducing cytotoxicity. The superior performance of the Stochastic scaffolds, particularly their ability to sustain the highest cell numbers by day 14, is likely attributed to their biomimetic microarchitecture. The high degree of pore interconnectivity in this design allows initially separate cell clusters to merge, forming larger, more cohesive tissue-like aggregates. This structure provides a high surface area for initial attachment and also features a highly interconnected porous network that not only sustains inter-hepatocyte cluster communication but also enhances the transport of nutrients and removal of metabolic waste, factors that are crucial for maintaining high cell densities in long-term 3D culture.

Together, these findings underscore the importance of scaffold design in sustaining not only cell numbers but also in guiding the self-organisation necessary for constructing functionally viable hepatic tissues. The LIVE/DEAD imaging validates the scaffold designs, particularly the Stochastic design, as supportive microenvironments for HepG2 cells, enabling both high viability and geometry-influenced spatial organisation, key attributes for the development of functional hepatic tissue models.

5.4 Liver gene expression

5.4.1 RT-qPCR

HNF6

The expression of HNF6 was assessed in HepG2 cells cultured on four scaffold geometries, Stochastic, Isotruss, Cubic, and Gyroid, over a 14-day period. Gene expression was normalized to TATA-binding protein (TBP) and reported as fold change relative to TCP culture. Tracking HNF6 expression provides insight into the temporal dynamics of hepatic differentiation and the extent to which 3D scaffold environments facilitate or delay maturation (Rowe et al., 2013; Zabulica et al., 2019). The typical expected temporal trend consists of relatively high expressions during immaturity and progressively downregulated as cells transition toward a more mature hepatic phenotype (Figure 5.8B).

At Day 3, the highest levels of HNF6 expression were observed in the Gyroid condition, followed by Isotruss suggesting hepatocyte immaturity (Figure 5.8). In contrast, Stochastic and Cubic scaffolds exhibited expression levels close to baseline and relatively lower to TCP.

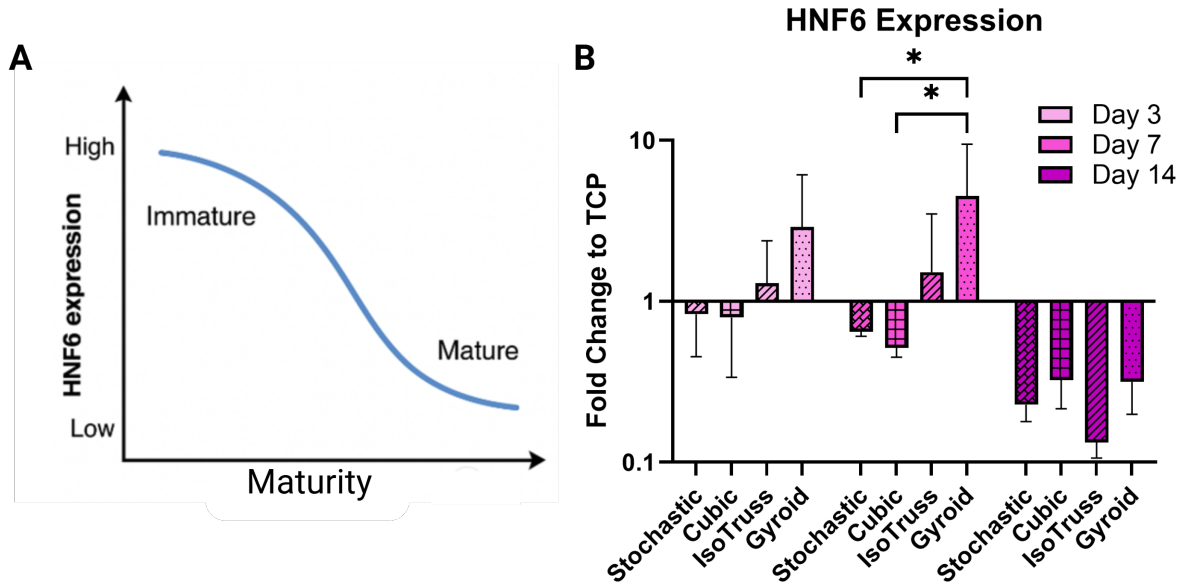


Figure 5.8: HNF6 expression as an indicator of hepatic maturity. A) A schematic illustrating the principle that HNF6 is a marker of cellular immaturity; its expression is high in immature cells and low in mature cells. B) Fold change of HNF6 to TBP as housekeeping gene and normalized to TCP expression of HepG2 cells in different geometry scaffolds throughout 14 days. Data are presented as mean $\pm$ SEM (*: $p < 0.05$).

Given that HNF6 is transiently upregulated during the very early stages of hepatic maturation, the downregulation of the gene found in Stochastic and Cubic geometries suggest an early maturation, even before day 3. The disordered nature of the Stochastic geometry allows for more immediate and dense cell aggregation promoting rapid signalling events and early hepatic specification (Ranucci et al., 2000; Ramaiahgari et al., 2014). As a result, cells within this environment could have passed the HNF6 peak expression window before Day 3, leading to relatively low levels at the measured time point. This hypothesis is supported by the known rapid activation and decline of HNF6 during hepatocyte lineage commitment, and could suggest that Stochastic scaffolds accelerate the maturation timeline, potentially priming cells for maturation earlier than IsoTruss and Gyroid geometries.

Conversely, the advanced maturation profile of HNF6 expression with the Stochastic geometry aligns with the large, interconnected cell aggregates observed in Figure 5.6. These aggregates promote the re-establishment of critical intercellular communication networks. These networks consist of both direct physical junctions and localized molecular signalling.

Figure 5.9 provides a schematic representation of the formation of intercellular junctions as hepatocytes aggregate. It is postulated that as hepatocytes aggregate, they form adherent junctions, mediated by E-cadherin, which are critical for establishing tissue integrity. Concurrently, it is believed they assemble tight junctions to establish the cell polarity required for functional bile canaliculi. Furthermore, the formation of gap junctions, channels that directly connect adjacent cells, is thought to allow the rapid exchange of ions and small molecules (Tostões et al., 2012). This direct intercellular communication is hypothesized to be essential for coordinating metabolic activity across the cell population, theoretically allowing the spheroid to function as a single, integrated unit.

In addition to physical connections, the 3D structure creates a localised microenvironment where secreted molecules can accumulate to physiologically relevant concentrations. This enables effective paracrine and autocrine signalling, as growth factors and cytokines secreted by the hepatocytes are not immediately diluted in culture media. This concentrated signalling, combined with the cells' ability to deposit their own extracellular matrix, creates a positive feedback loop that reinforces the mature hepatic phenotype and promotes long-term functional stability (Bell et al., 2016).

The Cubic scaffold also presents earlier maturation patterns, but this might be due through a distinct pathway to maturation. While it did not promote the formation of large aggregates, its highly directional and rigid architec-

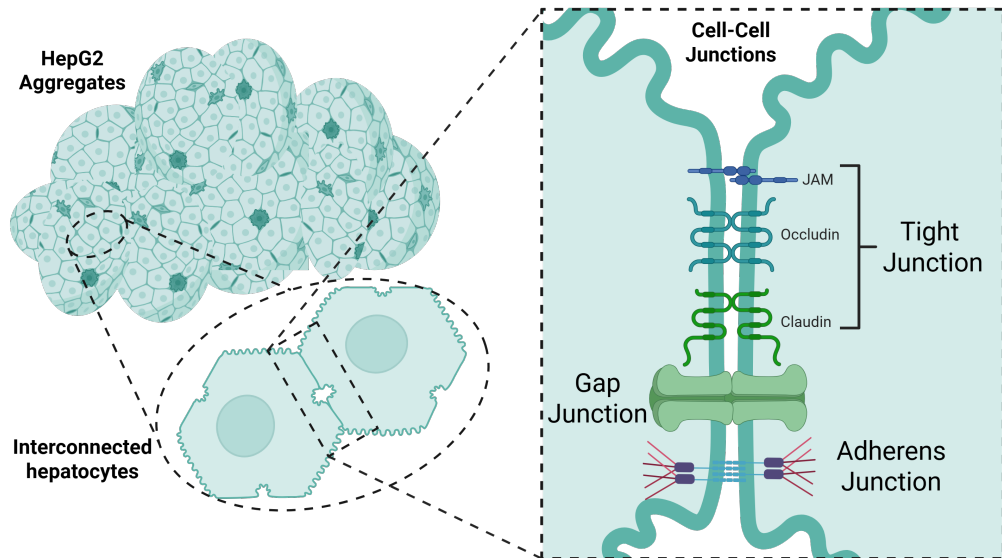


Figure 5.9: Intercellular Junctions in Hepatocyte Aggregates. This schematic illustrates the specialized cell-cell junctions that is hypothesised to re-established when hepatocytes self-aggregate in a 3D culture, especially in the Stochastic scaffold. These connections might include adherens junctions, which link the cytoskeletons to provide mechanical strength; tight junctions (composed of proteins like claudin and occludin), which form a seal to create polarized structures such as bile canaliculi; and gap junctions, which form channels for direct intercellular communication. The formation of this complex junctional architecture could be a hallmark of a mature, functional hepatic phenotype, enabling the aggregate to behave as a coordinated tissue.

ture compelled cells to adopt an elongated, flattened morphology through contact guidance (Thé et al., 2006). This enforced cell shape generates high internal cytoskeletal tension, which is a powerful mechanotransductive signal known to regulate gene expression and drive cellular maturity (Ingber, 2003; Janson and Putnam, 2015). Therefore, the Cubic geometry appears to induce a mature phenotype not through enhanced cell-cell communication, but by imposing a specific cell-matrix interaction that activates maturation pathways via cytoskeletal tension.

By Day 7, Isotruss retained modestly elevated levels, while Gyroid expression spiked, indicating a prolonged immaturity state. Stochastic and Cubic remained low and stable with both geometries being significantly lower than Gyroid. These patterns reinforce the idea that scaffold geometry can influence not only the magnitude but also the timing of hepatic gene activation (Lewis et al., 2018). When pairing this information with cell distributions seen earlier in Figure 5.6, the slower maturation in the Gyroid and Isotruss geometries corresponds to their tendency to form isolated cell clusters, which were either confined within voids or restricted to strut junctions. This spatial segregation may limit the extensive cell-cell communication required for coordinated maturation.

At Day 14, HNF6 expression was uniformly lower than control in all geometries, with fold changes ranging between 0.1 and 0.3. This downregulation aligns with the gene's reduced role in mature hepatocytes, and further supports the notion that scaffold-induced HNF6 activity is both transient and time-dependent. This downregulation in all geometries compared with TCP also pairs up with studies that claim that 3D cultures promote a more mature and physiologically relevant hepatocyte phenotype due to the promotion of 3D cell-cell interactions that better mimic the native liver environment (Ramaiahgari et al., 2014; Bokhari et al., 2007; Lewis et al.,

2018; Rowe et al., 2013).

Collectively, the temporal expression patterns of HNF6 reveal that scaffold architecture is a potent modulator of the early stages of hepatic maturation.

G6PC1

Evaluating G6PC1 transcription offers critical insight into the metabolic competence of hepatocyte cultures and the extent to which scaffold geometry supports or impairs terminal hepatic maturation.

The expression of G6PC1, as a key enzyme involved in gluconeogenesis and a marker of hepatocyte functional maturity, was analysed in HepG2 cells cultured on four scaffold geometries, Stochastic, Isotruss, Cubic, and Gyroid, over 14 days. Expression was normalized to TBP and represented as fold change relative to TCP. In contrast to the early-expressed HNF6, G6PC1 is expected to increase gradually over time, reflecting the progressive acquisition of metabolic hepatic functions as illustrated in Figure 5.10 A .

The highest expression levels of G6PC1 were unexpectedly observed as early as Day 3, with no statistically significant differences between scaffold geometries or discernible trends across groups (Figure 5.10 B). This early peak in G6PC1 expression aligns with the hypothesis of rapid early hepatic maturation, previously observed with HNF6, supporting the notion that HepG2 cells undergo an accelerated maturation process under 3D culture conditions. Since G6PC1 is considered a late liver marker associated with gluconeogenic capacity and glucose homeostasis, its strong induction at early times may reflect the establishment of metabolic competency soon after cell seeding in these 3D environments.

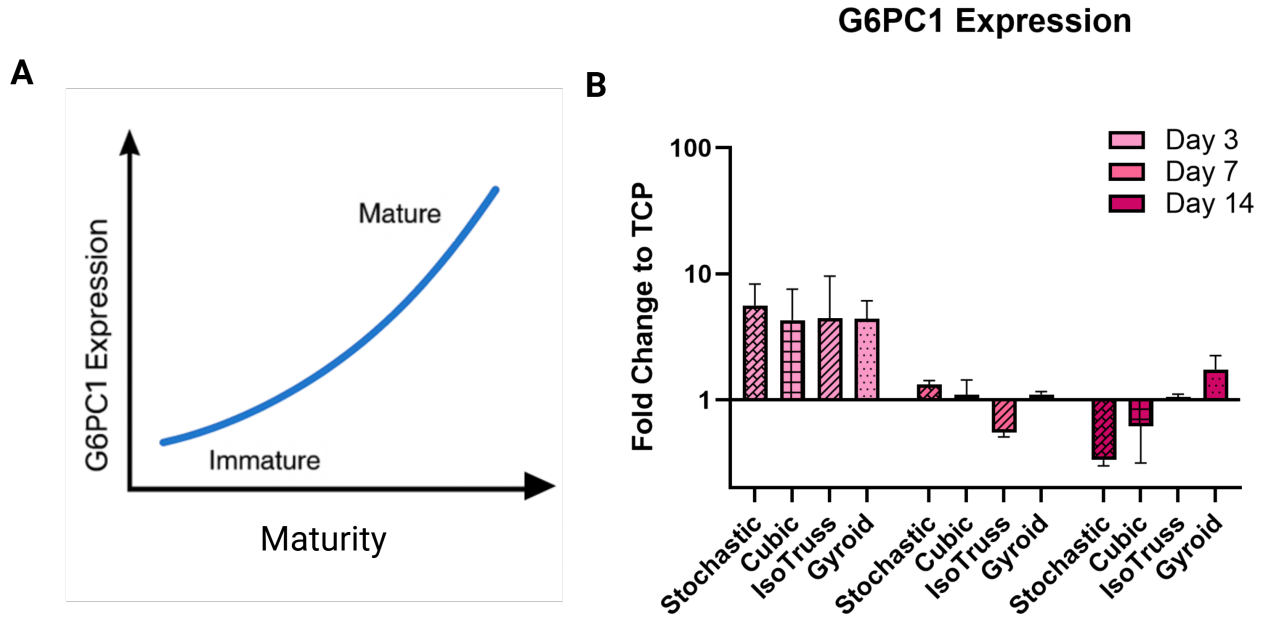


Figure 5.10: G6PC1 expression as an indicator of hepatic maturity. A) A schematic illustrating the principle that G6PC1 expression is positively correlated with cellular maturity. B) Fold change of G6PC1 to TBP as housekeeping gene and normalized to TCP expression of HepG2 cells in different geometry scaffolds throughout 14 days. Data are presented as mean $\pm$ SEM. One-way ANOVA showed no significant differences between groups

It is important to note that sustained G6PC1 expression typically requires metabolic stimulation, such as hormonal cues (e.g., glucagon or cAMP) or nutrient stress. In this study, no exogenous inducers were applied in order to avoid interfering with the basal functional readouts. This likely contributed to the progressive downregulation of G6PC1 observed at Days 7 and 14, suggesting that without continuous stimulation, metabolic gene expression declined *in-vitro* (Rowe et al., 2013). While this late reduction contrasts with expectations for mature hepatocytes, it aligns with observations in HepG2 cultures, where maturation plateaus or declines beyond 10–12 days, especially without specialized media (Elaut et al., 2006).

Together, HNF6 and G6PC1 delineate distinct but complementary axes of hepatic maturation. While HNF6 downregulation indicates progression

from an immature or fetal-like transcriptional state, G6PC1 upregulation reflects the acquisition of mature metabolic functionality. Their combined expression trajectories offer a robust framework for evaluating both developmental and functional fidelity in engineered liver models.

Interestingly, although HNF6 expression remained elevated in Isotruss and Gyroid scaffolds at later time points, indicating delayed developmental maturation, the early induction of G6PC1 was observed across all geometries. This might suggest that metabolic maturation may proceed independently and more rapidly than transcriptional developmental remodelling (Rowe et al., 2013). In other words, cells might attain metabolic competence in all scaffold types by Day 3, yet only in Stochastic and Cubic scaffolds do they proceed to further transcriptional refinement as evidenced by declining HNF6 levels. This divergence underscores the importance of assessing multiple layers of hepatic identity, as different scaffold designs may variably support functional versus developmental maturation trajectories.

5.5 Functional Assays

5.5.1 Urea Synthesis Evaluation

In this study, urea production was quantified at days 3, 7, and 14 across four scaffold geometries and a TCP control to evaluate how scaffold architecture influences hepatocyte-specific metabolic activity over time.

While all 3D scaffolds demonstrated significantly higher urea synthesis than the 2D TCP control at all time points, the Stochastic geometry consistently showed the most robust performance (Figure 5.11). By day 7, it exhibited significantly higher urea synthesis than all other 3D geometries. This result

indicates that while a 3D environment is generally beneficial, the specific micro-architecture of the scaffold is a critical determinant of the level of functional enhancement. TCP remained consistently lower than all scaffold conditions across time points and statistically significant differences in urea production were observed between all 3D scaffold geometries and the 2D TCP condition at every time point.

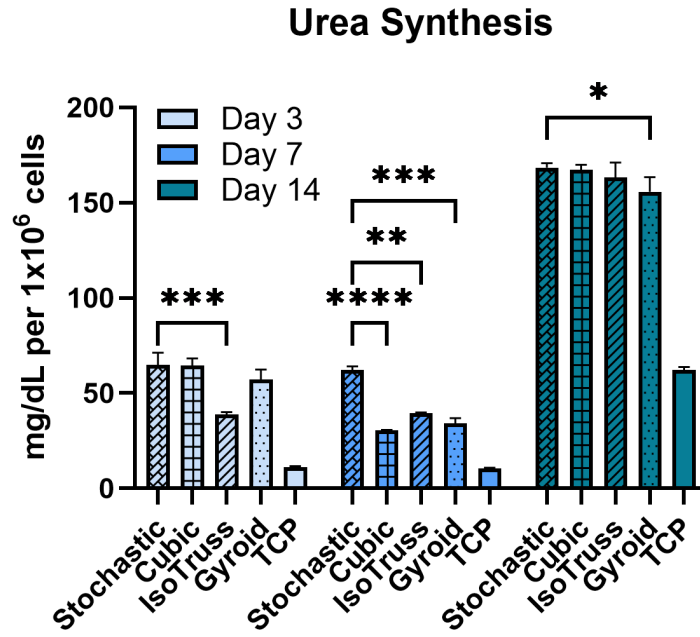


Figure 5.11: Comparative analysis of Urea synthesis on HepG2 cells cultured on different scaffold geometries versus TCP assessed at multiple time points. Tests were normalized to cell count with dsDNA content and measured at days 3, 7 and 14. All 3D geometries were significantly different to TCP ($p < 0.0001$) at all time points. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.00001$) between samples. $N=3$, $n=3$.

The superior performance of all 3D scaffolds over the TCP control is attributed to the restoration of a more physiologically relevant microenvironment. Unlike the rigid, flat surface of a 2D culture, 3D scaffolds provide the necessary architecture for complex cell-cell and cell-matrix interactions, which in turn activate the mechanotransductive signalling pathways that modulate metabolic activity (Ramaiahgari et al., 2014). The enhanced

performance of the Stochastic scaffold, in particular, is likely driven by its ability to accelerate maturation through a combination of structural and molecular cues, as suggested by the early downregulation of HNF6 and upregulation of G6PC1 previously shown.

The enhanced urea synthesis in the Stochastic scaffolds at days 3 and 7 can be attributed to an accelerated maturation timeline driven by both structural and mechanotransductive cues. The scaffold's disordered architecture promotes the rapid self-aggregation of hepatocytes into large, interconnected clusters, as shown in Figure 5.6. This 3D organisation is critical for re-establishing the cell-cell communication networks required for coordinated metabolic activity (Ranucci et al., 2000). This extensive physical contact is essential for the cells to form the dense network of gap junctions needed for a higher level of metabolic cooperation. This direct communication enables metabolic cooperation. For the urea cycle, which is a complex, multi-step process, this means that metabolic intermediates can be efficiently shared between cells (Tostões et al., 2012). A cell that is overwhelmed with ammonia can pass intermediates to its neighbours, effectively distributing the metabolic load across the entire aggregate. This makes the tissue as a whole far more efficient at detoxification than the sum of its individual, isolated cells (Tostões et al., 2012; Loh and Choong, 2013). In contrast, geometries that isolate cells or force them into linear patterns limit the number and quality of these critical junctions, thereby reducing the overall efficiency of the urea cycle.

At the molecular level, this structural complexity provides superior mechanotransductive signals that directly boost metabolic function. Urea cycle activity is modulated through transcription factors such as HNF-4 α and C/EBP α , whose expression is responsive to integrin-mediated mechanotransduction through FAK and ERK signalling. Studies have shown that

3D environments enhance these pathways, leading to increased expression of key urea cycle enzymes like CPS1 and ARG1 (Baze et al., 2018; Underhill and Khetani, 2018). The biomimetic architecture of the Stochastic scaffold likely provides spatial and mechanical cues that reinforce these signalling cascades, resulting in the observed increase in urea biosynthesis capacity.

5.5.2 Albumin Production Assessment

Albumin is the most abundant protein in blood plasma and is synthesized exclusively by hepatocytes, making its secretion a key functional indicator of hepatic biosynthetic capacity and cellular maturity (Kalra et al., 2023). As a constitutive function, albumin production serves as a robust metric for evaluating how well a scaffold supports long-term hepatocellular function *in-vitro*. Albumin secretion was therefore quantified at days 3, 7, and 14 across the four scaffold geometries and the TCP control.

As illustrated in Figure 5.12, the results mirrored the trends observed for urea synthesis and all 3D scaffolds supported significantly higher albumin secretion compared to the 2D TCP control at all time points, further confirming the superiority of 3D culture environments for enhancing liver-specific functionality.

Within the 3D groups, a time-dependent increase in albumin production was observed, with the Stochastic scaffolds consistently exhibiting significantly higher secretion levels than all periodic geometries at day 3 and day 7 and significantly higher than Cubic in day 14. This superior performance aligns directly with the findings from the previous sections. The biomimetic, irregular architecture of the Stochastic scaffold promotes a state of "low cytoskeletal tension" that is often associated with a mature,

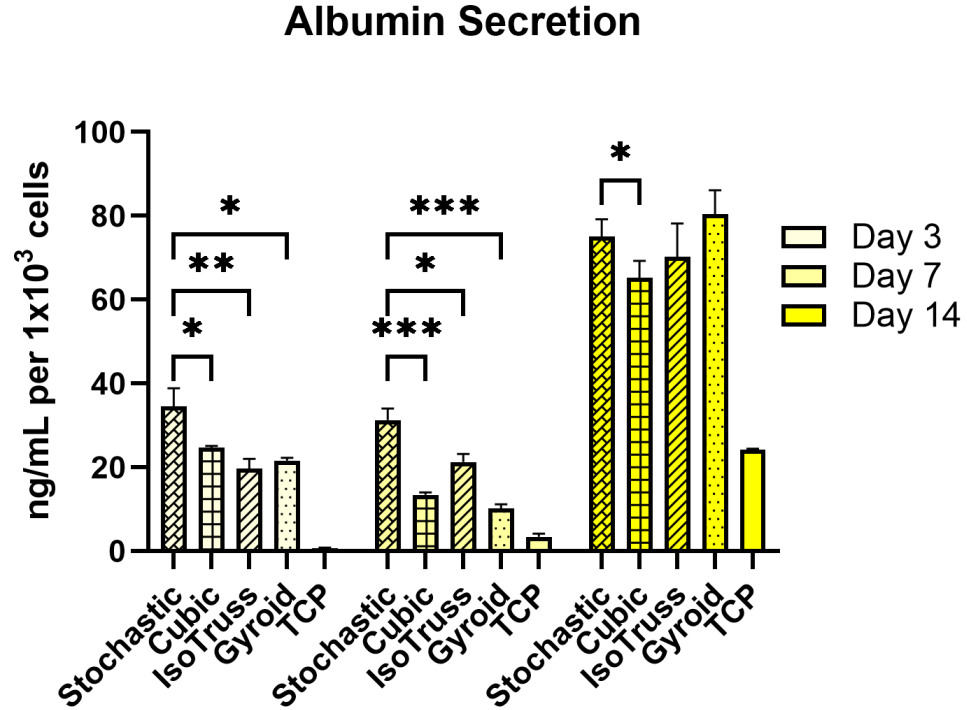


Figure 5.12: Albumin secretion by HepG2 cells cultured in 3D scaffolds of varying geometry over 14 days. Albumin levels were quantified at days 3, 7, and 14 using ELISA from culture supernatants. All 3D scaffolds showed increased albumin production over time, with Stochastic scaffolds exhibiting significant differences to all geometries at day 3 and 7. Although at day 14, hepatocytes in Isotruss seemly produce more urea than any other in day 4, only the Stochastic scaffolds presented a significant difference compared to Cubic at day 14. All 3D geometries were significantly different to TCP ($p < 0.0001$) at all time points. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$) between samples. $N=3$, $n=3$.

functional phenotype and serves as a signal to the cell that it is in a tissue-like environment and should focus on its specialised job, like making albumin, rather than migrating or dividing (Ingber, 2003; Tostões et al., 2012). This plus the formation of large, interconnected cell aggregates (as seen in Figure 5.6) and its morphology also correlated with accelerated maturation (via HNF6 downregulation) and enhanced metabolic activity (urea synthesis), suggesting that the same architectural features that facilitate superior cell organization and early maturation are also responsible for driving a more robust biosynthetic output earlier in time.

However, by day 14 the behaviour of the system diverges from the earlier time points and warrants further discussion. Specifically, while the Stochastic scaffolds maintained a high level of albumin secretion, they did not outperform all other geometries at the final time point. Both the Isotruss and Gyroid scaffolds showed substantial increases in albumin production by day 14, greater than their day 3 and day 7 levels, indicating a delayed but pronounced functional upregulation. This suggests that although these periodic architectures do not support accelerated early maturation, they may enable a more gradual establishment of hepatocellular function over prolonged culture durations.

The late rise in albumin secretion in Isotruss, in particular, may reflect the onset of stable cell–cell contacts within its longitudinal strut arrangement, while the Gyroid’s inherently high surface curvature is known to promote cell spreading and improved mass transport over time, potentially contributing to the delayed functional enhancement observed at day 14.

At the molecular level, albumin expression is transcriptionally controlled by hepatic-enriched factors such as HNF1 and HNF4 (Tachmatzidi et al., 2021). These factors are highly sensitive to ECM engagement and spatial cues that promote cell-cell junction formation. It is hypothesised that the heterogeneous topography of the Stochastic scaffold enhances the formation of stable focal adhesions and downstream signalling more effectively than the uniform periodic designs. In contrast, the limited performance of the periodic scaffolds suggests that while they support viability, their structural homogeneity may be insufficient to stimulate the complex mechanotransductive pathways required for robust, sustained protein production.

Taken together, these results highlight that the Stochastic geometry provides the most consistent and sustained functional performance across the

entire culture period, supporting both early and stable albumin production. In contrast, the Isotruss and Gyroid scaffolds exhibit a more delayed functional profile, with markedly increased albumin secretion emerging only at day 14. This pattern suggests that while the periodic architectures do not promote early hepatic maturation to the same extent as the Stochastic design, they nevertheless enable substantial functional upregulation over longer timescales. Overall, these findings underscore the importance of evaluating both early and late functional outputs when assessing scaffold performance, as geometries that initially appear less favourable may still support significant hepatocellular maturation with extended culture.

5.6 Drug Metabolism

5.6.1 CYP3A4 Activity Evaluation

CYP3A4 enzyme activity, indicative of hepatic metabolic competency, was measured using the P450-Glo™ CYP3A4 Assay and normalized relative to Cubic scaffolds, which is the most simple geometry tested. All geometries exhibited significantly higher CYP3A4 activity compared to the Cubic scaffold at day 14, with the Stochastic scaffold showing a tendency toward higher activity than Isotruss and Gyroid, suggesting enhanced metabolic functionality.

However, both the Isotruss and Gyroid scaffolds also displayed substantial increases in CYP3A4 activity at day 14, indicating that these periodic geometries likewise support late-stage metabolic maturation. This delayed rise mirrors the trend observed in albumin and urea secretion, where both geometries demonstrated improved performance at the later time point de-

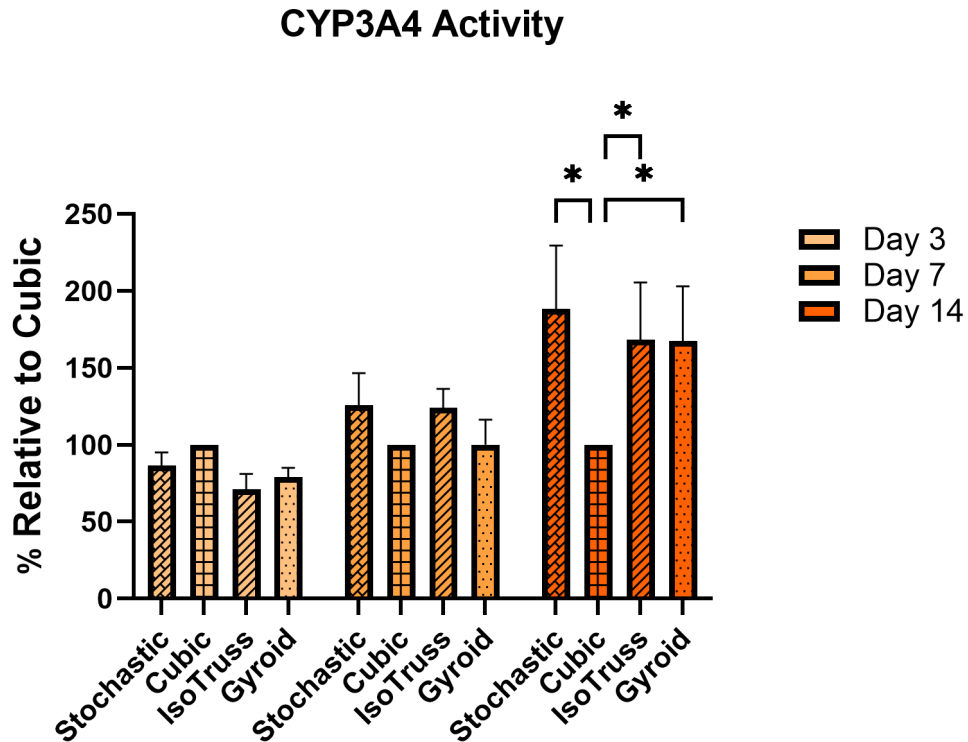


Figure 5.13: Assessment of CYP3A4 enzymatic activity in HepG2 cells cultured on different scaffold geometries. Data presented relative to tissue culture plastic (TCP). Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc test showed significant differences (*: $p < 0.05$) between samples. $N=3$, $n=3$.

spite more modest behaviour earlier in culture. The elevated CYP3A4 activity observed in Stochastic scaffolds compared to Cubic highlights the significance of scaffold complexity and structural biomimicry in promoting optimal hepatocyte function. The Stochastic scaffold, by providing a more physiologically relevant microenvironment with heterogeneous ECM characteristics, likely promotes enhanced cellular organization, signalling interactions, and metabolic maturity. Thus, scaffold complexity emerges as a critical parameter influencing hepatocyte metabolic functionality, reinforcing Stochastic scaffold designs as highly beneficial for advanced hepatic tissue engineering applications.

Nonetheless, the robust metabolic activity seen in the IsoTruss and Gyroid

geometries at day 14 indicates that these more ordered architectures retain the capacity to support functional hepatic maturation over longer culture durations. Their late-stage performance, when considered alongside the albumin data, suggests that periodic scaffolds may undergo a slower trajectory of functional enhancement that ultimately results in comparable metabolic activation.

CYP3A4 expression is regulated by nuclear receptors such as PXR and CAR, which are themselves responsive to hepatic differentiation status and HNF-4 α activity. Enhanced albumin and urea production suggest increased HNF-4 α levels, which in turn promote the transcription of CYP3A4 (Rodrigues et al., 2018). Studies have also demonstrated that ECM-rich and topographically complex environments enhance the nuclear localization and activation of PXR, further boosting CYP3A4 expression and enzymatic function (Dash et al., 2009; Lee et al., 2020). Therefore, the pronounced CYP3A4 activity in Stochastic scaffolds may stem from synergistic activation of mechanotransduction pathways and nuclear receptor signalling induced by the geometry’s biomimetic complexity.

5.7 Conclusions

The overarching aim of this chapter was to determine how scaffold lattice geometry controls hepatocyte function in 3D hydrogel constructs. By systematically comparing a bio-inspired Stochastic design against three distinct periodic geometries (Cubic, Isotruss, and Gyroid), this work has generated a comprehensive dataset linking scaffold architecture to cell viability, spatial organisation, and metabolic competence. The findings converge on a unifying conclusion: controlled architectural disorder, as embodied by the

Stochastic lattice, accelerates and amplifies hepatocellular maturation and function relative to ordered periodic designs and conventional 2D culture.

The investigation began by establishing a robust and reproducible seeding protocol that achieved high initial cell retention (>95%) across all geometries, ensuring that subsequent functional differences were driven by the scaffolds themselves and not by initial cell number bias. Despite this uniform start, the scaffolds immediately began to guide distinct cellular behaviours.

The Stochastic and Cubic geometries prompted an accelerated maturation timeline, as evidenced by the rapid downregulation of the early hepatic marker HNF6 by Day 3. In contrast, the Gyroid and Isotruss scaffolds exhibited prolonged HNF6 expression, indicating a delayed developmental progression.

This divergence in maturation appears correlated with two distinct mechanobiological pathways. The first pathway is driven by cell-cell communication. The disordered architecture of the Stochastic scaffold promoted the formation of large, cohesive, tissue-like aggregates, which is hypothesized to enhance the critical cell-cell communication networks (e.g. gap and adherent junctions) essential for coordinated metabolic activity. The second pathway, seen in the Cubic geometry, appears to achieve a similar outcome by enforcing a specific cell shape that generates high cytoskeletal tension.

Conversely, the Gyroid and Isotruss geometries, by confining cells to isolated clusters, appear to delay this process by limiting the establishment of these crucial networks. This suggests that while periodic structures maintain high cell viability, their structural homogeneity may slow the full functional maturation of hepatocyte cultures, highlighting the importance of biomimetic design

The functional consequences of these architectural differences are insightful. Across all key metabolic assays (urea synthesis, albumin secretion, and CYP3A4 activity) the Stochastic scaffold consistently and significantly outperformed the periodic designs, particularly at earlier time points. This demonstrates that the biomimetic irregularity of the Stochastic scaffold provides a superior microenvironment that not only supports cell viability but actively enhances the complex functions of mature hepatocytes.

In conclusion, this chapter demonstrates that introducing a controlled level of geometric disorder into scaffold design is a potent strategy for enhancing liver-specific behaviour *in-vitro*. The Stochastic scaffold achieved both rapid developmental maturation and superior, sustained metabolic function without compromising cell viability. These findings establish clear design rules for biofabrication and highlight the potential of biomimetic, Stochastic scaffolds as advanced platforms for creating more physiologically relevant liver models for drug screening and disease studies. Future work should focus on validating these findings with primary human hepatocytes and integrating perfusion to further leverage the benefits of this optimised micro-architecture.

Chapter 6

Conclusions and Future Work

6.1 Conclusions

This thesis successfully met its objectives through a multi-stage investigation that logically progressed from material engineering to biological validation.

The work presented in Chapter 3 focused on establishing the material foundation for this study. A novel GelMA-PEGDA composite hydrogel was developed and optimised for P μ SLA, successfully achieving the crucial balance between biological compatibility and the mechanical integrity required for high-resolution printing. Through comprehensive characterisation, the hydrogel's stiffness was tuned to match that of native liver tissue, its nanoscale mesh network was confirmed to be favourable for mass transport, and its cytocompatibility was validated to exceed 95% viability. The chapter concluded by establishing a robust and fabrication-ready hydrogel platform, thereby successfully meeting the first objective.

Chapter 4 addressed the architectural blueprint for the scaffolds. By performing a quantitative analysis of decellularised liver ECM, key architectural parameters such as pore and strut size and volume fraction were extracted. This data-driven approach informed the computational design of both periodic (Cubic, Isotruss, Gyroid) and biomimetic Stochastic (Voronoi-based) lattice geometries. The fabrication of these designs demonstrated high fidelity, while the Stochastic design successfully captured the architectural irregularity of native tissue. This chapter fulfilled the second objective by creating a library of precisely controlled and biologically relevant scaffolds and the third objective by faithfully reproducing the geometries with P μ SLA, setting the stage for a systematic comparison.

Finally, Chapter 5 presented the core biological investigation, determin-

ing how these different scaffold architectures affected hepatocyte function. Confirming the central hypothesis of this work, the key findings from this chapter demonstrate that controlled architectural disorder, embodied by the Stochastic lattice, accelerates and amplifies hepatocellular maturation and function when compared to all other periodic designs tested. The investigation revealed that the Stochastic geometry prompted an accelerated maturation timeline, evidenced by the rapid downregulation of the early marker HNF6. This was directly correlated with the spatial organization of the cells, where the interconnected, disordered architecture of the Stochastic scaffold promoted the formation of large, cohesive, tissue-like aggregates. The functional consequences were profound, with the Stochastic scaffold consistently and significantly outperforming all periodic designs in key metabolic assays, including urea synthesis, albumin secretion, and CYP3A4 activity.

6.2 Central Conclusion and Significance

The findings from this thesis provide strong support for the central hypothesis. The superior performance of the biomimetic Stochastic scaffold demonstrates that replicating the structural disorder of the native ECM is a potent strategy for enhancing liver-specific behaviour *in-vitro*. This work reveals that scaffold architecture dictates not only if maturation occurs, but how and when.

This research makes a significant contribution to the field by systematically demonstrating, for the first time in a hepatic context, the functional superiority of stochastic over periodic lattice designs. These findings establish clear design rules for the biofabrication of liver models and highlight

the importance of moving beyond simplistic, ordered geometries to create scaffolds that are truly biomimetic.

6.3 Limitations and Future Work

This study provides a strong foundation, but several key avenues remain for future investigation. The gene expression trends observed in this work must first be validated at the protein and functional levels. For instance, the unexpectedly high G6PC1 levels at Day 3 across several scaffolds suggest that HepG2 cells may express this marker earlier than expected, potentially due to their partially mature phenotype. Further analysis, alongside protein-level or functional validation like glucose production assays, would be required to determine whether these transcriptional trends translate into meaningful metabolic activity. Similarly, to confirm the hypothesis that accelerated HNF6 downregulation primes cells for advanced functionality, future work must assess structural maturation. This includes analyzing the formation of a functional bile canaliculi network, which would provide direct evidence of advanced cell polarity. A deeper mechanistic analysis is also required, and future studies should quantify the gene expression of key nuclear factors, such as HNF-4 α and C/EBP α , to directly validate the hypothesis that these transcriptional networks are being activated.

Other several limitations should also be acknowledged, which in turn suggest clear directions for future research. The biological evaluations in this thesis were conducted using the HepG2 cell line. Although a valuable and widely used model, HepG2 cells are a hepatoblastoma-derived line and may not fully recapitulate the complex responses of primary human hepatocytes (PHHs) or induced pluripotent stem cell (iPSC)-derived hep-

atocytes. Therefore, a crucial next step is to validate these findings with primary human hepatocytes or iPSC-derived hepatocyte-like cells. This would confirm whether the architectural principles established here translate to more clinically relevant cell sources.

Furthermore, the current study was conducted under static culture conditions. The integration of the optimised stochastic scaffolds into a perfusion bioreactor is a logical and promising future direction. Dynamic flow could further leverage the benefits of the interconnected micro-architecture by enhancing mass transport, providing physiologically relevant shear stress, and potentially unlocking an even higher level of metabolic function.

Finally, to deepen the mechanistic understanding, transcriptomic and proteomic analyses could be employed. These powerful tools could elucidate the specific signalling pathways that are differentially activated by stochastic versus periodic geometries, providing a more complete picture of how mechanical disorder is translated into hepatic lineage commitment and functional maturation. Such insights would guide the rational design of the next generation of biofabricated liver models for drug discovery and disease modelling.

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