

Bioengineered scaffolds as a tool to decode the complexity of
cancer cell interactions *in vivo*.

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Declaration

I, Tatiana Rizou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Following primary tumour removal, many cancer types undergo delayed relapse at distant sites, and this represents the leading cause of cancer related death. It is now apparent that cancer cells not only change their local tissue environment, but also the primary tumour can perturb distant tissues creating a more permissive environment for disseminating cancer cells. Towards a deeper understanding of metastatic growth, we set out to create an artificial system that would allow us to manipulate and dissect key aspects of cancer cell interactions *in vivo*. Here we successfully engineer an implantable biomaterial scaffold, with defined physicochemical properties. This scaffold integrates with the host to create a biocompatible microenvironment *in vivo*, incorporating stromal cells, endothelial cells, and immune cells, with macrophages as the predominant infiltrating population. In the presence of a primary fat pad tumour, our scaffold achieves to attract and capture spontaneously disseminated tumour cells from the circulation, which subsequently alter the surrounding cellular composition of the scaffold, by increasing the level of non-immune cells. Leveraging the full functionality of the scaffolds, we show that increasing the stiffness of the scaffold or functionalisation of the scaffold with the pro-angiogenic factor VEGF leads to a difference in their cellular infiltrate and subsequent increased cancer cell seeding. Collectively, this tool allows us to modify specific parameters of the disseminating cancer cell niche revealing this way their contribution to different aspects of cancer cell growth.

(233 words)

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Abbreviations

% (w/v)	Percent weight/volume
2D	Two dimensional
3D	Three dimensional
ANOVA	Analysis of variance
APC	Allophycocyanin
BSA	Bovine serum albumin
BUV	Brilliant Ultra Violet dyes
BV	Brilliant Violet dyes
CD	Cluster of differentiation
CTCs	Circulating tumour cells
DoF	Degree of Functionalisation
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DTCs	Disseminated tumour cells
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
EpCAM	Epithelial cellular adhesion molecule
FBGCs	Foreign body giant cells
FBR	Foreign body response
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GelMA	Methacrylated gelatin
GFOGER	Gly-Phe-Hyp-Gly-Glu-Arg
GFP	Green fluorescent protein
H&E	Haematoxylin and eosin
IL	Interleukin
kDa	Kilodalton
LAP	Lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate

LVR	Linear viscoelastic region
RGD	Arg-Gly-Asp
MAA	Methacrylic anhydride
NETs	Neutrophil extracellular traps
microCT	Microcomputed tomography
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MSCs	Mesenchymal stem cells
NMR	Nuclear magnetic resonance spectroscopy
NSG	NOD scid gamma
P/S	Penicillin/ Streptomycin
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PDMS	Polydimethylsiloxane
PE	Phycoerythrin
PLG	Polyethylene glycol
PLGA	Poly(lacride-co-glycolide)
PyMT	Polyoma middle T antigen
RT	Room temperature
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Scanning electron microscopy
SSC	Side scatter
TMF α	Tumour necrosis factor alpha
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

1.1 Cancer & Metastasis

1.1.1 Cancer beyond genetic mutations: the tumour microenvironment

Our understanding of cancer has significantly evolved over the last decades. For a long time, cancer research predominantly focused solely on the mutated cells, whereas contemporary evidence necessitated contextualising oncogenic mutations within a more tissue-centered framework. The survival of tumour cells within the healthy tissue is achieved by the ability of the tumour cells to recruit, reprogram the host cells and modify their surrounding components. If successful, this tissue perturbation will allow the formation of the tumour microenvironment (TME) or tumour niche which will unleash the tumorigenic potential of the mutate cancer cells (Yuan *et al.*, 2022). The communication between cancer cells and their niche is achieved through cell-cell contact and paracrine signalling (Dominiak *et al.*, 2020). Cell-cell contact is mediated via a variety of adhesion molecules, including integrins and paracrine signalling through the release of cytokine, chemokines, and extracellular vesicles including exosomes (Niel *et al.*, 2022).

The TME comprises of all the non-cancerous cells and extracellular components in which the tumour develops. It consists of a variety of immune cells, fibroblasts, endothelial cells, pericytes and tissue-resident cell types. Apart from the different cell types it includes non-cellular components like the extracellular matrix (ECM), secreted factors and extracellular vesicles. Depending on the tissue that the tumour arises, the composition of the TME can differ extensively with various cells having a tumour supporting or tumour suppressive role (**Figure 1**).

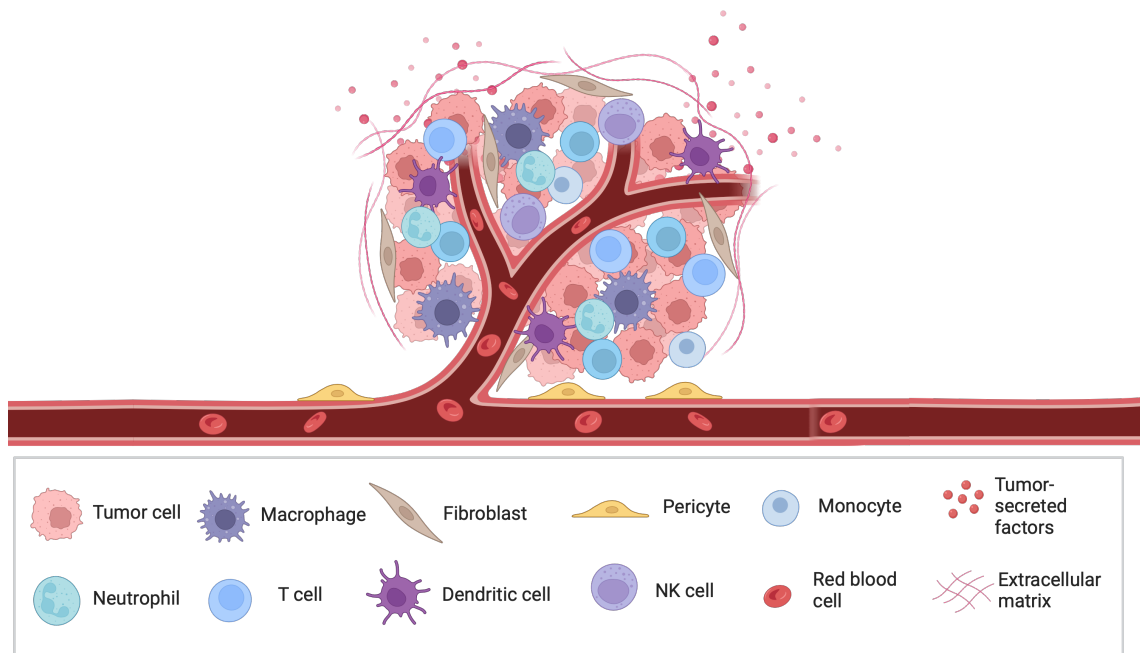


Figure 1. The tumour microenvironment. The tumour microenvironment consists not only of the tumour cells but also a variety of immune cells, fibroblasts, endothelial cells, and tissue-resident cells depending on the tissue that the tumour is growing. Apart from the different cell types, the tumour microenvironment consists also of other non-cellular components like the extracellular matrix, secreted factors, and extracellular vesicles. *Created using Biorender.*

1.1.2 Cancer metastasis: the search of a new home

Although cancer mortality has decreased over 30% in the past 30 years (Siegel *et al.*, 2023), metastatic disease represents the final and most lethal stage of cancer progression. Indeed, the vast majority of cancer patients die due to metastases. Despite the major improvements in health screening, cancer vaccines and treatment, the 5-year survival of cancer patients has barely improved, with 5-year survival rates ranging from 5–30% depending on the tumour type (Siegel, Miller and Jemal, 2020; Esposito, Ganesan and Kang, 2021). Therefore, it is imperative to identify the fundamental mechanisms driving metastatic spread and growth, as this knowledge has the potential to greatly enhance the survival rates of many patients with late-stage cancer.

Metastasis is a complex process, which consists of multiple steps and results in the colonisation of distant site from tumour cells. This process is often referred as “the

metastatic cascade” (**Figure 2**). The metastatic cascade initiates with the invasive growth of cancer cells to the surrounding tissues, by remodelling the ECM through the secretion of distant or local MMPs (Yan *et al.*, 2010). Cancer cells will then intravasate nearby blood or lymphatic vessels. Within the circulation, circulating cancer cells (CTCs) can be found either as single cells or clusters (Aceto *et al.*, 2014) or clusters with other immune cells, *e.g.* neutrophils (Szczerba *et al.*, 2019). One of the main challenges that CTCs encounter within the bloodstream is fluid shear stress and immune surveillance. During this process, CTCs modify their gene expression and physical properties in order to survive both from the physical challenges (Barnes, Nauseef and Henry, 2012; Cognart, Viovy and Villard, 2020) but also from the immune system, by exploiting a variety of immune-escape mechanisms such as alterations in the expression of MHC molecules, NK-cell ligands, FAS, FAS ligand (FASL), and immune-checkpoint molecules (Mohme, Riethdorf and Pantel, 2017). Once the disseminated tumour cells (DTCs) extravasate, they encounter the pre-metastatic niche, which is defined as the distant tissues that are systemically perturbed by the presence of the primary tumour growth. These changes are orchestrated by various factors and will be discussed in detail below. Cancer cells that have now colonised the distant tissue, will eventually start to proliferate forming distant metastases that will lead of metastatic colonisation either immediately or after a latency period. Latency is defined as the period during which the cancer cells start disseminating until they successfully outgrow (Steeg, 2006).

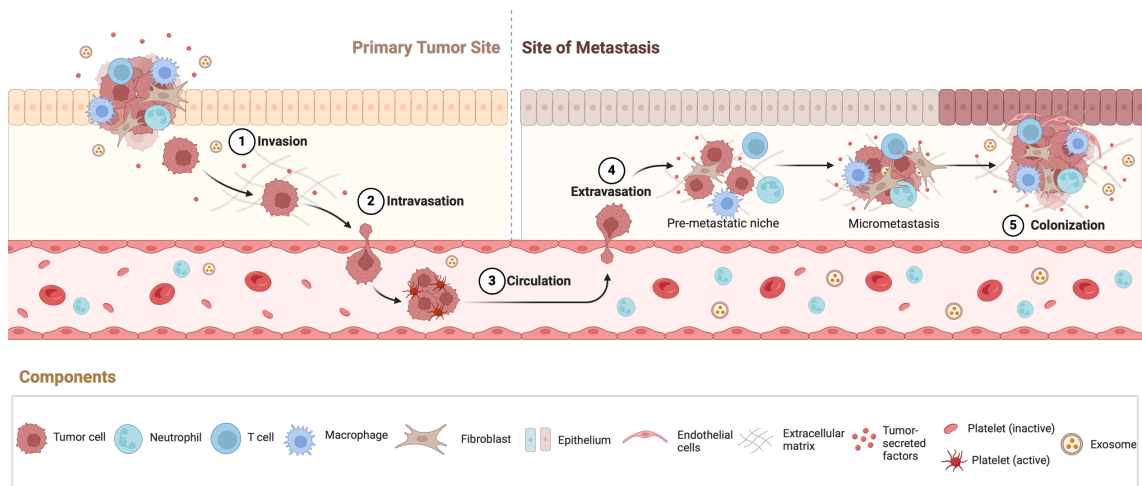


Figure 2. Overview of the metastatic cascade. The metastatic cascade is divided over 5 main steps. Disseminated cancer cells invade the surrounding tissue and migrate towards the blood vessels or lymphatics. Intravasation is the step when cancer cells enter the circulation. Circulating tumour cells (CTCs) can travel through the circulation either as single cells or clusters. Once reached the distant tissues, CTCs can now exit the circulation where they encounter a new microenvironment also known as the pre-metastatic niche. After being established in the new microenvironment, cancer cells with the potential to outgrowth start proliferating and progress from micro- to macro-metastases, leading to the final step of the metastatic cascade: the colonisation of the distant tissue. *Created using Biorender.*

Metastatic progression is not a linear process. Metastatic dissemination starts very early during primary tumour growth, (Hüsemann *et al.*, 2008; Eyles *et al.*, 2010; Hosseini *et al.*, 2016) however the number of metastases found in patients is a small proportion compared to the CTCs found in patients (Nagrath *et al.*, 2007; Gay and Malanchi, 2017). Additionally numerous of circulating cancer cells appear to be unable to initiate distant metastases (Meng *et al.*, 2004), indicating that the microenvironment in which cancer cells seed plays an important role.

1.1.3 Organotropic metastasis

Metastasis organotropism or organ-specific metastasis refers to the tendency of different cancer types to metastasise to particular organs. For example, breast and prostate cancer commonly recurs in the bone (Patanaphan, Salazar and Risco, 1988; Edlund, Sung and Chung, 2004), while melanoma predominantly spread to the liver (Triozi, Eng and Singh,

2008; Nguyen, Bos and Massagué, 2009). Although the steps of the metastatic cascade are the same for all tumour types, the requirements for metastatic cells seeding differ based on the distinct characteristics of each organ's composition as well as the barriers to infiltration.

The bone microenvironment consists of unique cell types including osteoblasts, osteocytes and osteoclasts, which have been implicated in promoting metastasis (Wang *et al.*, 2015). On the other hand, bone marrow NG2⁺/Nestin⁺ mesenchymal stem cells (MSCs) have been reported to instruct DTCs to enter dormancy (Nobre *et al.*, 2021), a state during cancer progression which is expanded within the next section. High vascularisation of the bone has also been implicated in cancer progression. In the work from Ghajar *et al.*, thrombospondin-1 (TSP-1) derived from the perivascular niche induces sustained breast cancer cell quiescence (Ghajar *et al.*, 2013), highlighting the fact that different cell types sustain different stages of metastasis.

The lung is one of the most frequent sites for metastasis of various types of primary tumours such as breast, melanoma and thyroid (Gao *et al.*, 2019). Its high vascularisation and large surface area makes it an ideal organ for the spread of metastatic cells. However, the endothelial layer formed between the endothelial cells and the basement membrane creates a barrier for circulating tumour cells. Within the lung microenvironment, lung resident cells can influence the proliferation of tumour cells through the secretion of various components. In the work from Kaplan *et al.*, lung fibroblasts were shown to upregulate fibronectin for the recruitment of VEGFR1 bone marrow-derived haematopoietic progenitor cells (HPCs), providing a permissive niche for tumour cells (Kaplan *et al.*, 2005). In contrast, monocytes attracted by endothelial cells within the lung, recruit and activate natural killer (NK) cells to reduce tumour metastasis (Hanna *et al.*, 2015), highlighting the complexity of the tumour microenvironment.

Most brain metastases originate from lung cancer, breast cancer and melanoma. The brain is distinguished from other organs, mainly because it is protected by the blood-brain barrier (BBB), a continuous layer formed by endothelial cells and supported by astrocytes, pericytes and the basement membrane, which creates a barrier for the

infiltration of circulating tumour cells, which need to acquire specific functions to infiltrate (Weil *et al.*, 2005). In the work from Valiente *et al.*, it was shown that plasmin released by astrocytes triggers cancer cell death through a FasL-dependent mechanism and breaks down L1 cell adhesion molecule (L1CAM), which is necessary for vascular co-option and metastatic growth (Valiente *et al.*, 2014).

In conclusion, organotropic metastasis refers to the tendency of certain cancers to metastasize to specific organs based on a combination of tumour cell characteristics, organ microenvironments, and systemic factors. Understanding these mechanisms is crucial for developing strategies to prevent or treat metastatic cancer.

1.1.4 The pre-metastatic niche: Preparing for the move.

Certain organs may create a supportive environment for future metastasis before cancer cells even arrive. Factors secreted by the primary tumour, such as exosomes and cytokines, can modify distant organ sites, making them more favourable for cancer cell colonization. This concept explains how some organs are ‘primed’ to become metastatic sites.

It was in 1889, when Stephen Paget proposed the “seed-and-soil” hypothesis, that metastatic spread in patients is not random, rather than a receptive microenvironment is required for the cancer cells to engraft tissues and successfully form metastases. In this study, he performed 700 autopsies on females with breast cancer and concluded that cancer cells show clear preferences for the colonisation of certain organs, breast cancer predominantly spreads to the lung, liver, bones, and brain (Paget, 1889).

In the 1930s, James Ewing challenged Paget’s theory by advocating that metastatic dissemination could be solely attributed to the mechanical forces and flow dynamics within the bloodstream across all tissues. Ewing's hypothesis emphasized the role of blood flow pressure in facilitating the spread of cancer cells throughout the body, suggesting an alternative perspective on the mechanisms underlying metastasis (“Neoplastic Diseases. A Treatise on Tumors,” 1941).

Later, Isaiah Fidler's research in the 1970s revealed that while the mechanical aspects of blood flow were indeed significant, effective metastatic colonization was restricted to specific organ sites. This was the first study to provide experimental evidence for organotropic metastasis (Fidler and Nicolson, 1976; Fidler, 2003).

Indeed, latest discoveries elucidated that tumours prompt the creation of microenvironments in distant organs that support the survival and proliferation of tumour cells even before their migration to these locations (Kaplan *et al.*, 2005; Hirakawa *et al.*, 2007; Psaila and Lyden, 2009; Wculek and Malanchi, 2015; Chin and Wang, 2016). These perturbed microenvironments are called “pre-metastatic niches”. The pre-metastatic niche is a complex microenvironment that consists of inflammatory immune cells, stromal cells, ECM proteins, tumour secreted exosomes and homing factors. Among the factors that are directly secreted by the TME are soluble factors such as cytokines, chemokines and extracellular vesicles and particles (EVPs) including exosomes which have been shown to contribute directly to immunosuppression (Chen *et al.*, 2018). The pre-metastatic niches rely on the interaction between tumour secreted factors with the local stroma, as tumour-derived factors mobilise and recruit bone-marrow-derived cells to niches. In fact, as shown in the work from Kaplan *et al.*, the arrival of vascular endothelial growth factor 1 (VEGFR1)-positive BMDCs to the premetastatic site preceded the arrival of metastatic cells (Kaplan *et al.*, 2005). Additionally, fibroblasts, endothelial cells and lung epithelial cells have been linked to the formation of the pre-metastatic niche through the secretion of inflammatory cytokines and chemokines (Hiratsuka *et al.*, 2006; Erler *et al.*, 2009; Liu *et al.*, 2016).

The priming of the distant sites consists of a stepwise process that perturb the homeostasis of the tissue to a more receptive microenvironment for the DTC colonisation and it includes vascular leakiness (Huang *et al.*, 2009; Psaila and Lyden, 2009), abnormal angiogenesis, ECM remodelling and the creation of an immunosuppressive microenvironment. The mediators of the pre-metastatic niche formation are either produced directly by the cancer cells and tumour associated cells in the TME or by the systemic inflammation that is activated during cancer (Greten and Grivennikov, 2019).

Steven Paget's theory is nowadays referred as organotropism, namely the propensity of cancer cells to disseminate to specific organs. Indeed, it has been shown that organs such as the lung, liver, brain, bone and lymph nodes (LNs) are more susceptible to metastasis compared to the skeletal muscle, intestine, spleen and kidneys, that are rarely targets for metastatic growth despite the increased blood flow (Crist and Ghajar, 2020). These organs are often called “anti-metastatic niches” and why these tissues are inhospitable for metastatic growth, is yet to be dissected.

1.1.5 The sleeping beauty wakes up: Outgrowth vs Dormancy

After successful dissemination within a distant site, DTCs are pressured to initiate metastatic colonisation. However, some of the DTCs that reach the target site do not form any metastatic lesions for months or even years (Ganesh and Massagué, 2021). Several of the DTCs do not die, but remain at a non-proliferative state, entering a viable state of reversible long-term dormancy, which is defined by a quiescent cycle arrest in G0-G1 (Naumov *et al.*, 2002; Aguirre-Ghiso, 2007; Giancotti, 2013). Alternatively, DTCs can start growing but with a high rate of cell death and therefore the equilibrium between cell proliferation and cell death does not allow for the successful outgrowth of cancer cells (Gimbrone *et al.*, 1972; Gay and Malanchi, 2017). This is usually induced by limited nutrient supply and can be reversed by the induction of angiogenesis. Therefore, it is not surprising that DTCs are often located near the vasculature (Carbonell *et al.*, 2009; Ghajar *et al.*, 2013; Yip *et al.*, 2021). Last, cancer cells may acquire a quiescent programme in order to avoid immune evasion (Malladi *et al.*, 2016; Sun *et al.*, 2022).

Metastatic dormancy is one of the most unexplored processes mainly to the difficulty in replicating the prolonged dormancy timeline effectively in mouse models. However, new strategies are starting to shed light in the transcriptional states that cancer cells acquire during dormancy (Malanchi *et al.*, 2023). A growing body of evidence shows that systemic inflammation, such as surgery, tobacco smoke or exposure to bacterial liposaccharides can activate proliferation in dormant cells (Albregues *et al.*, 2018; Risson *et al.*, 2020).

The lack of models to study cancer cell dormancy has hindered progress in understanding the precise mechanisms of dormancy and reactivation. Bioengineered scaffolds, aiming to recreate a specific metastatic tissue *e.g.* bone (Malanchi *et al.*, 2023) or artificial (Wang *et al.*, 2023) have offered a versatile platform to recreate the tumour microenvironment and explore the cell-microenvironment interactions that underlie dormancy.

1.2 Bioengineered scaffolds as a tool for tissue engineering

Tissue engineering (TE) was first introduced by Langer and Vacanti in 1993, where they described TE as ‘an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function’ (Langer and Vacanti, 1993). Tissue engineering employs a combination of biomaterial scaffolds and organ-specific cells, bridged with a variety of biochemical factors (*e.g.* growth factors) to create three-dimensional (3D) tissue-like structures, which aim to mimic the structure and the function of native tissues. These tissue-like structures can then be used to replace or repair damaged or diseased tissues in the body (Berthiaume, Maguire and Yarmush, 2011). Tissue engineering specifically addresses the critical shortage of donated organs available for transplantation by offering an alternative approach to organ replacement, an approach that holds promise for alleviating the burden on waiting lists for organ transplants and improving outcomes for patients in need of treatment (Furth and Atala, 2014).

In the field of tissue engineering, two approaches are primarily used to generate bioengineered tissues (Langer and Vacanti, 1993; Howard *et al.*, 2008). The first approach is based on scaffold-based tissue engineering, where scaffolds from biocompatible materials are used as a supportive structure onto which cells are seeded *in vitro*. The scaffolds provide an environment which enables cells to adhere, proliferate and differentiate within the scaffold, forming a tissue-like structure. The source of cells plays an important role, which imposes challenges, therefore, typically the use of stem cells for this approach has gained appreciable attention, including embryonic stem (ES) cells, bone marrow mesenchymal stem cells (BM-MSCs) and umbilical cord-derived mesenchymal

stem cells (UC-MSCs), due to their ability to differentiate into different cells lineages (Bianco and Robey, 2001). In this approach, once the tissue is developed, it can be transplanted into the patient.

The second approach relies on a scaffold-mediated growth factor/drug delivery. In this approach, the scaffold serves as a delivery vehicle for growth factors or drugs that stimulate tissue growth or regeneration. The scaffold is typically impregnated with bioactive molecules such as growth factors or cytokines. When implanted, the molecules can be released in a controlled manner from the scaffold, recruiting cells from the surrounding tissue. A key challenge with this approach is optimising the timing, concentration and combination of growth factors or cytokines to stimulate the desired regenerative response.

1.2.1 Essential characteristics for scaffolds in tissue engineering

Several factors influence the choice of the scaffold material based on the intended application. Essential requirements include biocompatibility, biodegradability, cell adhesion, proliferation and differentiation, consistent mechanical strength, controlled swelling, pore size and interconnectivity, easy fabrication and low cost (**Figure 3**).

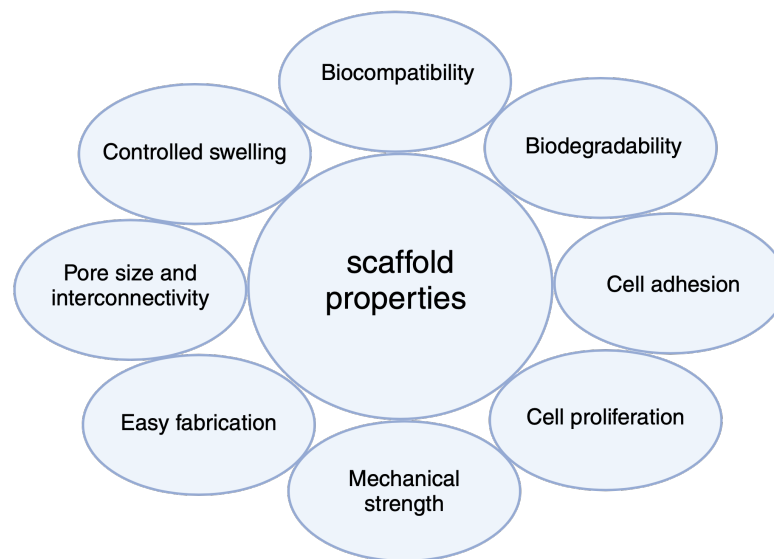


Figure 3. The essential parameters involved in scaffold design for tissue engineering. Among essential requirements while designing a scaffold are biocompatibility, biodegradability, cell adhesion, cell proliferation, mechanical strength, controlled swelling, pore size and interconnectivity as well as easy fabrication.

1.2.1.1 Cell adhesion, proliferation, and differentiation

Polymer based biomaterials can exhibit varying degrees of cell adhesion and proliferation, depending on factors such as surface chemistry, topography, mechanical properties, and degradation characteristics (Bacakova *et al.*, 2011). Surface chemistry refers to the chemical composition and structure of a biomaterial's surface, which influences protein adsorption and subsequently, cell adhesion. Proteins from the surrounding environment (such as fibronectin or vitronectin) absorb onto the biomaterial surface and mediate integrin binding, a key process in cell adhesion.

1.2.1.2 Biocompatibility

The term of biocompatibility has been extensively used within the field of biomaterial science, yet there still remains some ambiguity regarding its precise definition and the underlying mechanisms that encompass the various phenomena associated with the term. During the development of the first generation of implantable devices in 1940-1980s, it

became increasingly clear that the optimal biological performance was achieved with materials that were the least chemically active. However, the term evolved in order not to solely refer to the intrinsic property of the material rather than microenvironment that the scaffold is implanted into, that should not elicit an inflammatory response. In 1987, the definition of biocompatibility was re-defined as “the ability of a material to perform with an appropriate host response in a specific situation” (Donaruma, 1988). Although the term is very general, it highlights: 1) the fact that the material has to perform while not being inert after implantation within the tissue, 2) the response of the tissue has to be appropriate for its application and 3) the bidirectional interaction of the material and the tissue may vary depending on both the type of the material as well as the tissue itself (Williams, 2008).

1.2.1.3 Biodegradability

The term of biodegradability is closely linked with biocompatibility, as it refers the capability of a biomaterial to gradually decompose after fulfilling its intended function, without the production of toxic byproducts, which can be metabolized or excreted by the host organism. The degradation rate can be influenced by factors such as the material’s composition (natural or synthetic), porosity and scaffold architecture as well as the local environment upon implantation (*e.g.* pH, presence of enzymes).

1.2.1.4 Controlled swelling

Controlled swelling of biomaterials refers to the intentional and regulated increase in volume of a biomaterial when exposed to a specific environment or stimulus. This is a critical property for many applications, especially in the fields of tissue engineering, drug delivery, and wound healing (Chai, Jiao and Yu, 2017). Swelling of hydrophilic polymers and hydrogels in particular leads to volume expansion and therefore needs to be controlled in order to retain the mechanical properties, size stability and porosity of the scaffold. Selection of polymers with specific properties, such as the presence of functional groups or side chains or with the choice of anti-swelling hydrogels, the swelling behaviour of hydrogels can be tailored (Zhan *et al.*, 2021).

1.2.1.5 Porosity and pore interconnectivity

Porosity is defined as the percentage of void space in a solid (León, 1998). Scaffold pore size is a crucial factor for cell infiltration, as it affects how cells migrate within the scaffold. In tissue engineering, scaffolds are designed to provide a 3D structure that mimics the ECM, supporting cell growth and differentiation, therefore optimising the pore size is an essential requirement for enhancing cell infiltration, nutrient diffusion (Sharkawy *et al.*, 1997; Annabi *et al.*, 2013), angiogenesis (Galperin, Long and Ratner, 2010) and tissue development. Common techniques for the induction of porosity within a biomaterial include salt leaching, gas foaming, phase separation, freeze-drying and sintering (Hernandez and Woodrow, 2022). Control over the pore size and porosity greatly influences cell behaviour and the mechanical properties of the scaffold (Nam *et al.*, 2007).

1.2.1.6 Stiffness

Stiffness refers to a material's resistance to deformation when subjected to an external force. In general terms, it is the extent to which a material resists being deformed under an applied load, such as tension, compression, bending, or torsion (Oliver and Pharr, 2004).

Stiffness can be defined in different ways depending on the context of loading:

- Linear Stiffness (k): Stiffness is often defined as the ratio of applied force to the displacement it causes. For example, in a spring system, stiffness is expressed as:

$$k = F / \delta,$$

with k = stiffness (in N/m), F = force applied (in Newtons) and δ = displacement (in meters).

- Young's Modulus (E): When dealing with materials under tensile or compressive stress, stiffness is defined by Young's modulus, which is a measure of the material's intrinsic resistance to uniaxial deformation. It is expressed as:

$$E = \sigma / \varepsilon,$$

with E = Young's modulus (in pascals, Pa), σ = stress (force per unit area, in Pa) and ε = strain (dimensionless, the ratio of change in length to original length).

Young's modulus is a measure of material stiffness, and higher values indicate a stiffer material. It is an intrinsic material property, meaning it does not depend on the shape or size of the sample.

Stiffness is crucial in determining the suitability of biomaterials for various biomedical applications, as it affects their ability to support or mimic tissues, withstand mechanical loads and interact with cells and biological systems (Discher, Janmey and Wang, 2005). In tissue engineering, the stiffness of the biomaterials can be tuned to have specific properties. From a biological perspective, living tissues are also designed by nature to have diverse stiffness, which can range from a few pascals (brain) to gigapascals (bone) (Guimarães *et al.*, 2020) (**Figure 4**). Many pathologies including cancer, are characterised by stiffening of the tissue, hence stiffness is an important parameter to control (Butcher, Alliston and Weaver, 2009).

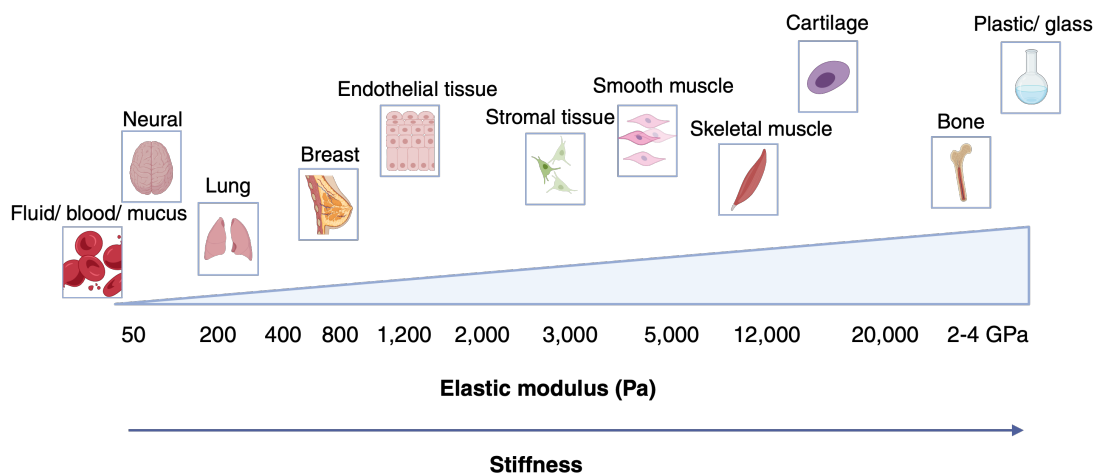


Figure 4. Variation in tissue stiffness spans from pascal to gigapascal range. Different cell types and tissues have different stiffness which is generated based on cell-cell or cell-extracellular matrix interactions. Central nervous system tissues are generally characterised as soft tissues,

whereas tissues such as the cartilage and bone are the stiffest tissues in the body. *Adapted from Butcher et al.* (Butcher, Alliston and Weaver, 2009).

1.2.2 Polymers as a biomaterial for scaffold engineering.

Polymers in tissue engineering can be divided into two categories based on their source. They are typically either naturally derived or made from synthetic polymers. Semi-synthetic polymers are a combination of both natural and synthetic polymers, where a naturally derived material has been modified to improve the properties of the material. Other classifications of polymers can be functional or structural, depending on whether they are hydrogels (Baroli, 2007), injectable (Kretlow, Klouda and Mikos, 2007) surface modified (Chung and Park, 2007) *etc.*

1.2.2.1 Natural polymers

Because of their abundance in the native extracellular matrix (ECM), naturally derived polymers generally allow for better interactions with cells (Pariante, Kim and Atala, 2001) and can show a minimal inflammatory response at the site of implantation compared with synthetic polymers. However, depending on the origin of the material the main disadvantages of naturally derived polymers include immunogenicity issues, poor mechanical strength, batch-to-batch variability and decreased tuneability of the material properties.

Based on their structures, natural polymers can be divided into three classes:

- Polypeptide- and protein based: *e.g.* collagen, gelatin, fibrin, fibrinogen, silk, elastin, keratin and actin.
- Polysaccharide- based: *e.g.* chitin, chitosan, alginate, hyaluronic acid, cellulose, agarose, dextran and glycosaminoglycans.
- Polynucleotide-based: *e.g.* DNA, linear plasmid DNA and RNA (Reddy *et al.*, 2021).

Collagen is the most abundant protein in the ECM and due to its fibrillar structure, largely contributes to the structural integrity of the ECM (Gelse, Pöschl and Aigner, 2003). To

date, there are more than 20 different isoforms identified within the collagen superfamily (Ricard-Blum, 2011), with type I collagen being the most abundant and found in skin, tendons and other connective tissues. Collagen type II is predominantly found in cartilage, whereas collagen type III is mainly present in the skin, blood vessels and internal organs (Burgeson and Nimni, 1992). Among all the isoforms, the right-handed triple helix, made up of three α -chains is one of the characteristic structural features of collagen. These may consist of three identical chains (homotrimers) as in collagen types II, III, VII, VIII, and X or of two or more different chains (heterotrimers) as in collagen types I, IV, V, VI, IX, and XI (Dong and Lv, 2016). In addition, collagen offers high biocompatibility, biodegradability and allows for cell adhesion, migration and the differentiation of cells (Chevallay and Herbage, 2000). However, as a natural polymer, collagen lacks mechanical strength without the pre-organisation typically found in nature, which limits its application. For practical usage, the collagen therefore needs to be either cross-linked or combined with other synthetic polymers in order to increase its mechanical strength.

Gelatin is a naturally derived polymer originating from the hydrolytic degradation of collagen, specifically from alkaline or acidic hydrolysis of type I collagen. Its distinctive structure of 19 amino acids (glycine, proline and hydroxyproline) possesses great benefits. Gelatin can be extracted from animal derived collagen, such as bovine, porcine or fish skin (Alipal *et al.*, 2021), either by partial acid (type A) or alkaline hydrolysis (type B). In some applications, gelatin is preferred over collagen since it does not cause an antigenic response due to its hydrophilic nature. Its main disadvantage is its high degradability due to enzymatic digestion by matrix metalloproteinases (MMP-2 and MMP-9) and its solubility in physiological environment, however, these properties can be tailored by chemical crosslinking due to the presence of active chemical functional groups (*e.g.* NH_2 and COOH) (Raucci *et al.*, 2019).

1.2.2.2 Synthetic polymers

Synthetic polymers offer greater flexibility compared to naturally derived polymers, mainly due to their tuneable properties, availability and low cost. Their chemically

defined structures allow for minimization of variability which have benefitted their usage in widespread applications (Place *et al.*, 2009). Typical examples of synthetic polymers used for scaffold development include: poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL), and polyethylene glycol (PEG) among others.

Poly(lactic acid) (PLA) is a gradually crystallizing semicrystalline polyester. PLA is a biocompatible and biodegradable polymer that supports cell adhesion and proliferation. PLA-based scaffolds can provide a temporary matrix for cell infiltration, tissue ingrowth, and regeneration.

Poly(lactic-co-glycolic acid) (PLGA) is copolymer of poly(lactic acid) and poly(glycolic acid) (PGA) (Alizadeh-Osgouei, Li and Wen, 2019). It is a biodegradable polymer known for its non-toxicity, cell adhesion and controlled degradation rate which can be fine-tuned by varying the ratio between the two polymers (Haghighat and Ravandi, 2014). PLGA is one of the most defined U.S. Food and Drug Administration (FDA) approved synthetic biomaterials for drug delivery (Makadia and Siegel, 2011).

Poly(ϵ -caprolactone) (PCL) is a semicrystalline and aliphatic polymer demonstrating good biocompatibility, biodegradability, structural stability and defined mechanical properties.

Poly(ethylene glycol) (PEG) is one of the most widely used synthetic polymer for bioengineered scaffolds. PEG is hydrophilic and therefore suitable for cell culture; however, it exhibits minimal or no intrinsic biological activity due to the nonadhesive nature of PEG chains (Zhu, 2010). Nevertheless, it can be modified with diverse functional groups and formed into hydrogels using various polymerization techniques (Tibbitt and Anseth, 2009). After implantation, PEG is typically immunogenic (Chen, Cheng and Roffler, 2021), however, PEG-conjugated drugs have been approved by the FDA for safe use in humans (Alconcel, Baas and Maynard, 2011).

1.2.2.3 *Semi-synthetic polymers*

Semi-synthetic polymers, as the name suggests, are a combination of polymers from both natural and synthetic sources. These polymers are created by modifying natural polymers through chemical or enzymatic reactions, incorporating synthetic components to enhance certain properties or functionalities. The advantages of these materials are that they can retain some of the desirable characteristics of natural polymers, such as biocompatibility and bioactivity, while offering improved mechanical properties, tunability, and stability.

1.2.2.4 *Hydrogels*

Hydrogels consist of a crosslinked network of hydrophilic polymers capable of expanding in water, swelling to multiple times their initial mass (Peppas *et al.*, 2006). The physical and biochemical characteristics of hydrogels are predominantly influenced by their compositions, polymerization methods, and crosslinking density. These materials offer a flexible platform to incorporate specific combinations of properties tailored for intended applications. Hydrogels can be divided in two categories including naturally derived hydrogels such as collagen, gelatin, alginate, and chitosan which are appealing for biological applications due to their cell signalling and cell-interactive properties and biodegradability. However, naturally derived hydrogels have drawbacks such as low mechanical properties, a lack of control over their degradation and structure and potential immunogenicity.

1.3 Foreign body response to biomaterials

1.3.1 The cascade of the foreign body response

One important aspect to consider when choosing a biomaterial for different biomedical applications, ranging from implants to drug delivery is the unwanted reaction from the host. Foreign body response or reaction (FBR) is the consequence of any material implanted into the body that triggers an inflammatory process. This process comes in different stages, that starts from the moment of implantation (acute FBR) and can take up to months after the initial implantation, a process that is defined as the chronic phase of

FBR and is a transition from an inflammatory to a fibrotic process (Carnicer-Lombarte *et al.*, 2021). When synthetic biomaterials are introduced into the body, they elicit a cascade of cellular and molecular events aiming to isolate the foreign material. The cascade of events following implantation of the biomaterial include injury, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction and fibrous capsule development, as described by Anderson *et al.* (Anderson, Rodriguez and Chang, 2008).

In the initial tissue injury phase, the implantation of the biomaterial causes tissue injury, which leads to the immediate release of proteins and blood components at the site. Additionally, the surface of the biomaterial quickly gets coated with a layer of proteins derived from the surrounding fluids, such as fibrinogen, albumin and immunoglobulins. This protein layer can influence subsequent cell attachment and activation, by activation of the coagulation and complement systems (Anderson, 2001).

The acute inflammation phase occurs in response to tissue injury and is the initial response involved the recruitment of immune cells, primarily neutrophils and monocytes. Neutrophils exit the bloodstream and migrate to the site of injury in response to various signals, such as cytokines, leukotrienes (LT) and histamine, which are released by platelets, endothelial cells and damaged cells surrounding the implanted biomaterial. IL-8 is highly produced in inflammatory sites and acts as a chemoattractant for neutrophils. Leukotriene B4 (LTB4) has also been shown to play an important role in neutrophil recruitment (Oliveira, Rosowski and Huttenlocher, 2016). Injured cells play a crucial role in attracting inflammatory cells to the site of implantation by releasing danger-associated molecular patterns (DAMPs) (Morris, Stamer and Kyriakides, 2017). The primary role of neutrophils is to eliminate the foreign implant with the following: 1) phagocytosis by secretion of proteolytic enzymes and reactive oxygen species (ROS) degrading the surface of the biomaterial, 2) degranulation by the release of proteases and anti-microbial molecules and 3) release of neutrophil extracellular traps (NETs). NETs are networks of extracellular fibers composed of DNA, histones and anti-microbial proteins. NETs have been associated with a fibrotic response as they contribute to the development of a thicker fibrous capsule around the biomaterial implant in order to isolate it from the surrounding

tissue and impair its intended function (Schauer *et al.*, 2014). By releasing factors that increase the vascular permeability, monocytes are also attracted to the site of implantation, which will then differentiate toward macrophages. These macrophages will then mediate the inflammatory response by releasing factors such as TNF α , IL-6 and IL-8 (Franz *et al.*, 2011). Macrophages usually attach to the surface of the implant, a process that poses a significant challenge to the degradability of the material. At this point in the process, the innate immune response is central to the FBR, with lymphocytes also considered to be significantly contributing to this process (Anderson and McNally, 2011; Kyriakides *et al.*, 2022).

As the acute inflammatory response subsides, a chronic inflammatory phase ensues, marked by the predominance of macrophages and foreign body giant cells (FBGCs) at the implant site. Foreign body giant cells are multinucleated cells formed upon the fusion of multiple macrophages together and are formed as a response to the biomaterial that cannot be degraded or phagocytosed by individual macrophages. Their function is the release of enzymes, ROS and other degradative substances to break down the material (Anderson, 2000; Anderson, Rodriguez and Chang, 2008). Anderson *et al.* showed that IL-4 plays a key role in the induction of macrophage fusion and is dependent on the activation of the mannose receptor, however multiple receptors are involved in the fusion phenomenon (McNally, DeFife and Anderson, 1996; Jay *et al.*, 2010). During this stage, macrophages usually switch from a pro-inflammatory phenotype to an anti-inflammatory and tissue regeneration phenotype (Mantovani *et al.*, 2004; Porcheray *et al.*, 2005). Macrophages and FBGCs attempt to encapsulate the foreign material by forming a fibrous capsule composed of collagen and other extracellular matrix components, in order to isolate the foreign material from the surrounding tissue and impede its integration and functionality. The formation of the granulation tissue includes the recruitment of fibroblasts and vascular endothelial cells, which start proliferating around the surface of the implant. This eventually leads to the formation of newly blood vessels which are formed by angiogenesis (neovascularisation) (Anderson, 2001). During this phase, the adaptive immune response, in particular T cells release factors such as IL-17 (Chung *et al.*, 2020).

The last step of this process is the regeneration process which includes the migration of monocytes to the inflammatory site and their differentiation to macrophages. This eventually leads to the degradation of the biomaterial and the release of cytokines for the anti-inflammatory healing process.

1.3.2 Effect of the biomaterial properties in the modulation of the immune response

All biomaterials, when implanted *in vivo*, induce cellular and tissue responses. The traditional approach for the design of implanted biomaterials focused specifically on the prevention of the inflammation with the use of inert biomaterials. However, latest approaches include the use of specific properties of biomaterials to modulate the immune response, for example by functionalisation of the physicochemical properties of biomaterial surfaces, release of cytokines and drugs can change the response of immune cells to implanted biomaterials (Franz *et al.*, 2011). For example, various studies have shown that the modulation of the biomaterial properties leads to macrophage polarization towards the desired phenotypes (Sridharan *et al.*, 2015). The modulation of the immune response is influenced by chemical cues on biomaterial surfaces, which include, but are not limited to, factors such as material type, the number of cell adhesion ligands (Spiller and Koh, 2017), surface chemistry (Zaveri *et al.*, 2014) and extracellular matrix (Kajahn *et al.*, 2012). In contrast, the impact of physical cues is determined by the biomaterial's stiffness (Patel *et al.*, 2012) and pore size (Sussman *et al.*, 2014).

1.3.2.1 Biomaterial type

As mentioned above, natural polymers release degradable byproducts in the body, which are mostly nontoxic as their characteristics closely mimic the biological molecules already present in the host. These compounds provide cell adhesion sites, which undergo degradation and trigger a less pronounced FBR compared to synthetic biomaterials. Synthetic biomaterials elicit a host response with the formation of a fibrous capsule within a few weeks of implantation (Li *et al.*, 2020). In synthetic biomaterials, such as PLA and PGA, one major concern is the acidity of the degraded byproducts, leading to the increase of inflammatory response (Kim *et al.*, 2007; Jones, 2008). The decreased pH of the

byproducts has been shown to affect the surface marker profile of cells infiltrating the implant. For example, a significantly lower amount of CD31 cells were found within a fast-degrading implant compared to slow-degrading PCL, leading to reduced angiogenesis, which was correlated with the acidic environment produced by the degradation of the biomaterial (Sung *et al.*, 2004). In a clinical study involving 2,528 patients comparing PGA with PLA implants, it was shown that PGA-based devices elicited a FBR within 11 weeks, in contrast with slow-degrading PLA where FBR occurred after 4.3 years of implantation (Böstman and Pihlajamäki, 2000).

1.3.2.2 *Surface chemistry*

In the work from Brodbeck *et al.*, it was shown that hydrophilic, anionic surfaces induced the highest levels of apoptosis in biomaterial-adherent FBGCs. Since FBGCs contribute negatively to biomaterials by promoting oxidative damage and attracting other types of inflammatory cells, the authors showed that triggering apoptosis in adherent FBGCs could mitigate these harmful effects and enhance tissue remodelling (William G. Brodbeck *et al.*, 2002). Additionally, in a different study the authors suggested that hydrophilic, anionic surfaces inhibited monocyte adhesion and IL-4-mediated macrophage fusion into FBGCs (W.G. Brodbeck *et al.*, 2002). These studies highlight the dependence of the FBR on biomaterial surface chemistry.

1.3.2.3 *Stiffness*

The role of mechanosensitivity in macrophage function has been extensively studied. Patel *et al.* showed higher cell elasticity and phagocytic ability on stiffer substrates indicating that substrate elasticity modulates macrophage elasticity and phagocytosis through actin polymerisation (Patel *et al.*, 2012). In the work from Blakney *et al.*, the authors showed that increasing the stiffness of 3D PEG-RGD hydrogels did not have an impact on macrophage attachment but elicited differences in cell morphology. Subcutaneous implantation of hydrogels *in vivo* led to a higher FBR in stiffer hydrogels, whereas hydrogels with lower stiffness led to reduced macrophage activation (Blakney, Swartzlander and Bryant, 2011).

1.3.2.4 Pore size

The effect of porosity in host responses including the FBR has been nicely summarised by Hernandez and Woodrow (Hernandez and Woodrow, 2022). During FBR, non-porous materials tend to induce the formation of a thick fibrous capsule formation. It has been extensively shown that materials with pores around 30-40 μm in diameter improve tissue healing responses and decrease scar tissue growth (Fukano *et al.*, 2010; Klopfleisch and Jung, 2017; Tylek *et al.*, 2020; Hady *et al.*, 2021). In detail, 40 μm size porosity has been demonstrated an ability to recapitulate key elements of the surrounding tissue, when porous implants were implanted in C57BL/6 mice, including infiltration of fibroblasts, endothelial cells and inflammatory cells and the presence of blood vessels and collagen fibers post 2-3 weeks of implantation (Fukano *et al.*, 2010).

Additionally in other studies, pore size has been linked to macrophage phenotype modulation. In the work of Sussman and colleagues, the authors utilized Poly-hydroxy-ethyl-methacrylate (p-HEMA) scaffolds with small (34 μm) and large (160 μm) pores to investigate macrophage responses in a subcutaneous mouse model three weeks post-implantation. It was observed that smaller pores led to increased vascular density, a higher presence of macrophages with a pro-inflammatory phenotype, and greater tissue remodelling. In contrast, on the outer part of the porous structure, macrophages showed a significant enrichment in anti-inflammatory markers (Sussman *et al.*, 2014), highlighting the fact that both states of macrophage activation can co-exist within the implant. In another study from Almeida *et al.*, the authors studied the role of pore size structure (orthogonal vs. diagonal) in macrophage behaviour *in vitro* and showed that macrophages in scaffolds with a diagonal porosity exhibited a more elongated shape and was linked with a pro-healing activation phenotype (McWhorter *et al.*, 2013).

1.4 Engineering the tumour niche

As discussed above, microenvironmental signals play an important role in cancer cell growth. Latest advances within the literature have shifted from conventional 2D cancer

cell cultures to more advanced 3D culture systems, including spheroid cultures and gel systems *in vitro*. These have improved our understanding regarding cancer cell growth and interactions of cancer cells with their microenvironment (Bissell and Radisky, 2001). However, because of the intrinsic heterogeneity of cancer microenvironments, it is important to create distinct tumour niches. For example, using synthetic scaffolds as a 3D cancer cell culture system has proven to better recreate the *in vivo* growth of OSCC-3 cancer cells compared to Matrigel (Fischbach *et al.*, 2007).

1.4.1 Natural biomaterials

Among the naturally derived biomaterials, Matrigel, is the most commonly used material to study cancer cell interactions both in *in vitro* cultures and *in vivo*. Matrigel is a reconstituted basement membrane, extracted from Engelbreth–Holm–Swarm murine sarcoma cells and consists of several ECM proteins, predominantly laminin (~60%) and collagen IV (~30%) (Kleinman and Martin, 2005; Lee *et al.*, 2007; LeSavage *et al.*, 2022). Matrigel is the gold standard since it represents native ECM components, allows for cell attachment and is degradable, due to cells remodelling the hydrogel. However, despite its ability to support cancer cell growth, as discussed above, Matrigel is an animal derived material that exhibits variation in the mechanical and biochemical properties which leads to substantial batch-to-batch variability and restricted modification possibilities (Pampaloni, Reynaud and Stelzer, 2007; Weigelt and Bissell, 2008; Aisenbrey and Murphy, 2020). The mechanical properties are not controlled due to their degradation over time and the low stiffness of the material (~100 Pa) makes it unsuitable to mimic the stiffness of stiff tumour tissues like the pancreas (5.5 ± 3.2 kPa) (Rubiano *et al.*, 2018). Additionally, in terms of manipulation of Matrigel, the viscoelastic properties of Matrigel impede it to be 3D printed in its pure form (Stefano *et al.*, 2021), therefore needs to be combined with other bioinks (Rawal *et al.*, 2021).

Despite the advantages of Matrigel, *in vitro* studies for cancer growth necessitate accurate replication of the biomechanical cues from the tumour microenvironment including matrix stiffness within 3D models. As highlighted by Micalet *et al.* Matrigel exhibits much lower stiffness compared to most organs and tissues (Micalet *et al.*, 2023; Micalet,

Moeendarbary and Cheema, 2023). In this work, the authors used collagen type I hydrogels with increased stiffness that compares with the stiffness of colorectal tumour tissue in order to create an *in vitro* colorectal cancer cell model to study cancer cell invasion. They showed that low invasive cancer cell lines stiffen their microenvironment, compared to highly invasive cancer cell lines that showed significant softening of the ECM.

1.4.2 Synthetic biomaterial-based scaffolds

Bioengineered scaffolds from different materials, such as polycaprolactone (PCL) (Aguado *et al.*, 2016; Rao *et al.*, 2016; Bushnell *et al.*, 2019) and poly(lactide-co-glycolide) (PLGA) (Fischbach *et al.*, 2007; Azarin *et al.*, 2015) have been successfully used as synthetic material to create *in vivo* models which mimic the pre-metastatic niche. Work from the Mooney group showed that cancer cells cultured in PLGA scaffolds better recreated the microenvironmental characteristics of tumour *in vivo*, which is related to angiogenic changes. Pre-cultured human oral squamous cell carcinoma (OSCC-3) cancer cells in PLGA scaffolds generated larger tumours *in vivo* compared to cancer cells previously cultured in 2D (Fischbach *et al.*, 2007).

Azarin *et al.* showed that PLGA scaffolds implanted either subcutaneously or peritoneally can successfully recruit metastatic cells from the primary tumour both in immunocompromised and immunocompetent models. Additionally, they found a correlation between disease exposure time and the relative abundance of Gr^{hi}CD11b⁺ cells found in both the scaffold implants as well as other metastatic sites as the lung, indicating the similarities between metastatic sites and the implanted scaffolds (Azarin *et al.*, 2015).

In a follow up study from the same group, the authors showed that implanted PCL scaffolds exhibited greater stability compared to PLGA scaffolds. PCL scaffolds were used to characterize the dynamic immune response in the implant spanning the acute and chronic foreign body response. Notably, after 30 days of stabilisation of the immune cell response to the scaffolds, the immune cell composition changed dramatically following

tumour inoculation. The implanted scaffolds recruited tumour cells from the primary tumour, while decreasing the metastatic burden from other metastatic sites and resultantly extended the lifespan of tumour-bearing mice (Rao *et al.*, 2016). Moreover, these engineered scaffolds have the ability to alter the microenvironment of the primary tumour. Following transcriptomics of cancer cells from the primary tumours after mock surgery or scaffold implantation in mice, the authors identified differentially regulated pathways relevant to invasion and metastasis progression, which resulted from scaffold mediated modulations of immune cell trafficking (Aguado *et al.*, 2018).

Additionally, Bushnell *et al.* showed that disseminated cancer cells from scaffolds can be isolated and expanded into a new cell line *in vitro*, and that scaffold-captured cancer cells are more aggressive compared to the primary tumour, revealing distinct features for gene expression signatures associated with metastasis (Bushnell *et al.*, 2019). In the latest work from the Shea group, Wang *et al.* showed that inflammatory response to biomaterials caused by foreign body response, successfully eradicates disseminated tumour cells within the scaffold and dissects the multifaceted role of neutrophils in different metastatic sites, *e.g.* lung versus scaffolds (Wang *et al.*, 2023).

Fibronectin and collagen IV-coated PCL scaffolds have been used to identify novel ECM proteins involved in cancer cell seeding *in vivo*. Utilising orthotopic breast cancer mouse models, Aguado *et al.* showed that fibronectin and collagen IV-coated scaffolds implanted subcutaneously captured significantly more cancer cells compared to uncoated scaffolds (Aguado *et al.*, 2016).

Other materials, like polyacrylamide (Lee *et al.*, 2012; Bersani *et al.*, 2014) or silk (Seib *et al.*, 2015) have been used to mimic the Young's modulus of a specific tissue, *e.g.* bone to identify key factors involved in metastasis.

Jaeghere *et al.* employed a hybrid hydrogel-poly(lactic acid) (PLA) scaffolds in order to recapitulate the microenvironment of peritoneal metastases. In this work, PLA scaffolds are coated with type I collagen in order to enhance the biocompatibility of the scaffold *in vivo*. By pre-seeding cancer associated fibroblasts (CAFs), known to be implicated in

peritoneal metastases (Zhao *et al.*, 2017) and different cancer cell lines, the authors successfully developed an *in vivo* technology platform for intraperitoneal xenografts, which recapitulates the importance of scaffolds used in order to dissect complex cell interactions within the tumour microenvironment (Jaeghere *et al.*, 2018).

In summary, bioengineered scaffolds such as PCL and PLGA have proven to be valuable tools for recreating the pre-metastatic niche and studying tumour interactions with the host *in vivo*. These scaffolds not only support tumour growth and metastasis but can also modulate the immune microenvironment, providing insights into the mechanisms of metastasis and offering potential avenues for therapeutic interventions by altering the tumour-immune dynamics to reduce metastatic spread and enhance survival.

1.5 Methacryloyl gelatin (GelMA) and its biomedical applications

1.5.1 GelMA properties

Naturally derived hydrogels have excellent cell biocompatibility, however, they lack sufficient mechanical properties and are characterised by batch-to-batch variability and lack of control of the hydrogel microarchitecture. In contrast, synthetic hydrogels such as polyethylene glycol (PEG) have stronger mechanical properties and decreased biodegradability compared to natural hydrogels, however, they lack cell-adhesive motifs which limit their application without further modifications in tissue engineering (Lutolf and Hubbell, 2005). To bridge the gap between biological and synthetic materials, semi-synthetic matrices combine the advantages of natural ECM components and tuneable properties, allowing for higher reproducibility (Wu *et al.*, 2021). Methacryloyl gelatin (GelMA) is a functionalised semi-synthetic material which shares the advantages of both naturally derived and synthetic hydrogels.

Methacryloyl gelatin (GelMA) is a versatile biomaterial with a broad range of applications. It is a semi-synthetic hydrogel which is synthesised after reaction between gelatin and methacrylic anhydride (MAA), first developed by Van Den Bulcke in 2000 (Bulcke *et al.*, 2000). Gelatin is denatured collagen that can be derived from various

different sources and includes binding motifs and MMP-sensitive degradation sites. The addition of methacryloyl groups to the amine- containing side groups of gelatin allows it to become light polymerizable into a hydrogel that remains stable at 37 °C. In the literature it is frequently referred to with different names such as gelatin methacrylate (Chen *et al.*, 2012), methacrylamide modified gelatin (Bulcke *et al.*, 2000), gelatin methacrylamide (Kaemmerer *et al.*, 2014; Visser *et al.*, 2015) or gelatin methacryloyl (Yue *et al.*, 2015; Loessner *et al.*, 2016; Pepelanova *et al.*, 2018; Sun *et al.*, 2018; Zhu *et al.*, 2019; Vigata *et al.*, 2020). Given the fact that GelMA is gelatin derivatised with methacrylamide and methacrylate groups, methacryloyl gelatin reflects the most suitable name and will therefore be used throughout this thesis. GelMA can be used in various forms based on its characteristics, for example 3D printed scaffold (extrusion-based or photomask-based), injectable hydrogels, 3D scaffolds by photo-crosslinking or electrospun fibrous membranes (EFM) (Piao *et al.*, 2021).

1.5.2 Synthesis of GelMA

Since the first report of GelMA by Van Den Bulcke *et al.* (Bulcke *et al.*, 2000), protocols have been modified with minor differences from the initial method for GelMA synthesis. Briefly GelMA is synthesised by the direct reaction of gelatin with methacrylic anhydride (MAA) in phosphate buffer (pH=7.4) at 50°C (**Figure 5A**). By controlling the stoichiometry of MAA added, different degrees of functionalisation can be achieved. After the reaction time, the reaction is stopped by diluting the solution with 4x phosphate buffer. The solution is then dialysed against deionised water through a dialysis membrane to remove any unreacted MAA and other by-products such as methacrylic acid. The final solution is then usually freeze dried and stored at -20°C until further use.

GelMA undergoes a photo-initiated free radical polymerisation under UV irradiation in the presence of photo-initiator forming a covalently crosslinked network (**Figure 5B**). Different choices of photo-initiators include hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959) (Benton *et al.*, 2009; Nichol *et al.*, 2010; Celikkin *et al.*, 2018; Nguyen, Watkins and Kishore, 2019) or lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate photo-initiator (LAP) (Ouyang *et al.*, 2022). LAP has a

higher solubility in water and higher molar extinction coefficient at 365 nm than Irgacure 2959, hence is often the choice of photo-initiator (Fairbanks *et al.*, 2009).

1.5.3 Characterisation of GelMA

The physical properties of GelMA can be modified by tuning various different parameters related to the crosslinking density of the hydrogel network. These include the degree of functionalisation (DoF), the concentration of GelMA, the type and concentration of the photo-initiator and the exposure time.

Gelatin can be derived from mammal or marine sources and due to economic, environmental and clinical factors, the preference of gelatin sources has shifted from mammalian to marine. Fish skin is a common byproduct of the fish-processing industry and therefore an inexpensive material (Badii and Howell, 2006) that has shown promising results in tissue engineering. Gelatin contains abundant Arg-Gly-Asp (RGD) sequences that enhance cell attachment (Liu and Chan-Park, 2010) as well as sequences of MMP-sensitive degradation sites that support cell remodelling (Vandooren, Steen and Opdenakker, 2013). Beyond the RGD motifs, collagen contains other integrin recognition sequences, including the GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg) motif that also interacts with integrins, particularly $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Knight *et al.*, 2000; Zeltz and Gullberg, 2016). Endothelial cells, express integrins like $\alpha 1\beta 1$ and $\alpha 2\beta 1$, which bind to collagen type I and IV, whereas fibroblasts express $\alpha 2\beta 1$ integrins, promoting their migration into the collagen matrix and enabling remodelling. Macrophages and immune cells also interact with collagen via specific integrins (*e.g.*, $\alpha v\beta 3$), supporting the immune-modulatory function of collagen-based scaffolds (Pang *et al.*, 2023).

When comparing the different sources of gelatin, fish gelatin has a lower melting point compared to porcine gelatin, therefore different physicochemical properties (Yoon *et al.*, 2016). In the work of Elkhoury *et al.*, the physicochemical and biological properties from porcine and fish GelMA were compared after preparation of porcine and fish GelMA with similar methacrylation levels (Elkhoury *et al.*, 2021). By assessing the rheological properties of the hydrogels, the authors showed that the elastic modulus G' is lower than

the G' of porcine GelMA, due to the higher amount of proline and hydroxyproline amino acids present in porcine GelMA (Haug, Draget and Smidsrød, 2004), leading to higher structural stability. The authors also assessed the biological properties of fish and porcine GelMA by evaluating the cell viability of C2C12 cells and showed no difference in cell viability between the two types of hydrogels. In a different study, Zaupa *et al.* demonstrated that fish GelMA showed similar Young's modulus compared to porcine GelMA, but improved capacity of cell remodelling both *in vitro* and *in vivo* (Zaupa *et al.*, 2019), highlighting that fish GelMA can be degraded by mouse metalloproteinases and collagenases. Indeed fish collagen, though structurally similar to mammalian collagen, has some compositional differences in its amino acids that may affect its integrin binding capacity. Despite these differences, fish collagen retains key motifs like GFOGER that can still interact with mammalian $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins (Yamada *et al.*, 2014).

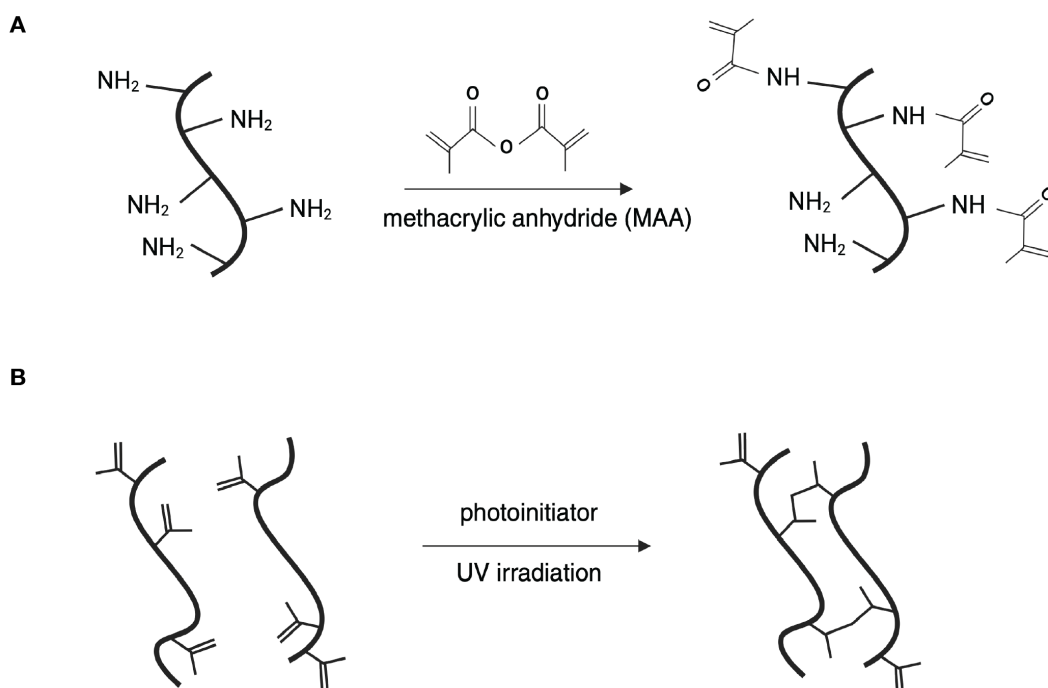


Figure 5. Synthesis of methacryloyl gelatin (GelMA). **A)** Gelatin containing primary amine groups react with methacrylic anhydride (MAA) to add methacryloyl functional groups. **B)** Methacryloyl gelatin is photo-crosslinked using UV irradiation in the presence of photo-initiator creating a covalently crosslinked hydrogel network.

1.5.4 GelMA and its use as a 3D cell culture system that mimic the ECM

After the reaction of gelatin with methacrylic anhydride (MAA), GelMA retains its biochemical properties when the chemical modification only involves a low percentage of the amino acid residues (Bulcke *et al.*, 2000). Specifically, for gelatin, Arg-Gly-Asp (RGD) is the most common peptide motif responsible for cell adhesion and is a minimal peptide sequence which targets integrin receptors that typically bind fibronectin or vitronectin. The RGD motifs do not contain functional groups that will react with MAA, ensuring the preservation of good cell adhesive properties.

Indeed, cell attachment and their proliferation in GelMA hydrogels has been widely characterised, for different types of cells such as, HUVECs (Nichol *et al.*, 2010), endothelial colony-forming cells (ECFCs), mesenchymal stem cells (Chen *et al.*, 2012; Zhao, Liu, *et al.*, 2016) as well as keratinocytes (Zhao, Lang, *et al.*, 2016). It has been shown that cells can efficiently attach to GelMA hydrogels and proliferate (Dubruel *et al.*, 2007), with their proliferation typically decreasing with higher DoFs (Chen *et al.*, 2012). In addition, it has been shown that cells can be either seeded on top of GelMA hydrogels (Dubruel *et al.*, 2007) or encapsulated in GelMA solution and UV photo-crosslinked to form cell laden 3D hydrogels, as short exposure to UV light or violet light (405 nm) does not affect cell viability (Benton *et al.*, 2009; Nichol *et al.*, 2010). As DNA has an absorption maximum at 260 nm, photo-crosslinking at 365 nm and subsequently 405 nm, greatly minimises the risk of direct DNA damage (Loessner *et al.*, 2016). In specific, Nichol *et al.* showed that successful encapsulation of HUVECs in GelMA hydrogels allowed cell migration, with the cells organising into elongated morphologies. HUVECs readily adhered, migrated and proliferated in 10% (w/v) GelMA with high DoF (81%). In contrast, cells failed to adhere to polyethylene glycol diacrylate (PEGDA) surfaces as PEG is not cell adhesive (Nichol *et al.*, 2010). In another study from Liu *et al.* showed that smooth muscle cells (SMCs) encapsulated in GelMA hydrogels remain viable 2 weeks post seeding and successfully formed 3D cellular networks in softer GelMA hydrogels, whereas cells successfully adhered and remained alive in stiffer hydrogels but without cellular network formation (Liu and Chan-Park, 2010). In a related study, MSCs encapsulated in GelMA hydrogels with a high DoF exhibited a round shape morphology, without cell contact formation indicating that whilst a high DoF can be used

to create hydrogels with increased stiffness to gelatin, it is a critical factor in terms of cell-material interactions (Pepelanova *et al.*, 2018). GelMA has also been used for cartilage tissue engineering applications by encapsulation of chondrocytes, which resulted in a rounded cell morphology of the cells after 8 week of *in vitro* culture, which subsequently enhanced chondrogenesis.

Apart from the RGD motifs, MMP sensitive motifs are also present in GelMA, since type I or type II collagenases accelerates the degradation of GelMA, allowing for the hydrogel matrix to be remodelled (Benton *et al.*, 2009; Xu *et al.*, 2012; Peerani, Candido and Loessner, 2019).

1.5.5 GelMA and its application in vascular regeneration

Because of the tuneable mechanical properties of GelMA, it has been widely used for supporting the development of 3D vascular networks. Nikkhah *et al.* produced endothelial cord structures with a circular cross-section using micropatterned GelMA constructs. GelMA constructs with different heights (50-150 μm) were created and differences in endothelial cell proliferation, alignment and cord formation were observed due to the different geometrical dimensions. GelMA constructs with 100 μm height were shown to provide the optimal microenvironment for endothelial cord structure formation (Nikkhah *et al.*, 2012). Chen *et al.* proved that GelMA hydrogels can support human progenitor cell-based vascular network formation, using GelMA hydrogels with different degrees of methacrylation (50%, 64% and 73%) using 1 M, 5 M and 10 M methacrylic anhydride stock solutions. Human endothelial colony-forming cells (ECFCs) and mesenchymal stem cells (MSCs) were shown to be successfully cultured in GelMA hydrogels with different DoFs in monoculture as well as in co-culture *in vitro*, with their proliferation rate decreased in hydrogels with a higher DoF, indicating that GelMA with 50% DoF possesses suitable mechanical properties and availability of peptide sequences for cell adhesion, creating a more favourable environment for promoting vascular formation compared to 73% DoF. Additionally, it was shown that co-culture of MSCs with ECFCs in GelMA hydrogels induced the formation of capillary-like networks, that was more profound in 50% DoF GelMA compared to 73%. Implantation of cell-laden GelMA

hydrogels *in vivo* in immunodeficient mice led to the successful formation of functional anastomoses between the bioengineered human vascular network and the mouse vasculature. However, the 50% DoF GelMA hydrogels were partially degraded after 7 days and mechanically collapsed after 28 days, in comparison to 73% DoF GelMA hydrogels that showed a slower degradation rate due to the higher stiffness (Chen *et al.*, 2012). In other work from the same group, the authors injected GelMA prepolymer solutions loaded with ECFCs and MSCs *in vivo* and the solution was polymerised *in situ* by transdermal UV exposure. Indeed after 7 days, bioengineered vascular networks were successfully developed with formation of functional anastomoses with the host vasculature (Lin *et al.*, 2013). Loading of GelMA with desferrioxamine (DFO) further improved the formation of vasculature. The results *in vitro* showed a significant increase of vascular tubule formation of HUVECs seeded in DFO-GelMA scaffolds compared to GelMA only. This was due to the upregulation of HIF-1 α within the microenvironment, a factor which significantly affects blood vessel formation (Chen *et al.*, 2016).

3D bioprinting of GelMA provides another technique for the fabrication of 3D vascularised constructs, mainly due to its controlled spatial deposition of different components. One approach includes the direct bioprinting of structures with interconnected channels. For example, endothelial cells can be directly bioprinted within microfibrillar GelMA hydrogel scaffolds. The endothelial cells have the ability to migrate to the periphery of the microfibers forming a layer of endothelial structure within GelMA (Colosi *et al.*, 2016; Zhang *et al.*, 2016). However, one of the main limitations of this strategy is the structural instability while printing, since the weight of the top layer causes the bottom layers to deform the underlying pores (Ouyang *et al.*, 2016). This limitation has led to the development of alternative printing-casting approaches, where the vascular template is 3D printed using fugitive inks (*e.g.* Pluronic F-127). The matrix is then formed by casting a second material around the structure, which is then removed in order to form channels followed by the post-seeding of endothelial cells (Kolesky *et al.*, 2016). Although this method has yielded promising results, its complexity loses many of the key advantages of fast, single-step 3D bioprinting. A study from Ouyang *et al.* overcomes this limitation, with the use of a combination of two bioinks, a gelatin based templating bioink and a photo-crosslinkable matrix bioink which are printed simultaneously. This method

relies on the properties of gelatin to liquify at 37 °C and therefore after photo-crosslinking, the structure is incubated at 37 °C, where the gelatin can be removed and create a network of channels which can be vascularised *in situ* (Ouyang *et al.*, 2020). An alternative method reported a 3D micromolding technique using bioprinted agarose template fibers to fabricate microchannel networks. The microchannels were successfully embedded within GelMA hydrogels. In this study, the printed agarose fibers were then easily aspirated with a light vacuum allowing the formation of microchannel networks within the photo-crosslinked GelMA hydrogels (Levett *et al.*, 2014). As a proof of concept, perfusion of the formed microchannels of different sizes (250 µm, 500 µm and 1000 µm) with HUVECs allowed the maturation of cells into fully perfusable microvascular networks.

1.5.6 The use of GelMA in recreating specific tissue microenvironments

1.5.6.1 Creating a bone microenvironment

One main use of GelMA has been to structurally and biologically mimic key aspects of the bone microenvironment. Celikkin *et al.* used porous GelMA scaffolds for seeding of bone marrow derived rat mesenchymal stem cells and showed that 5% (w/v) GelMA scaffolds supported homogeneous extracellular matrix calcification (Celikkin *et al.*, 2018). Functionalisation of GelMA with uniform layers of nanoceria (nCe) lead to increased proliferation of pre-seeded bone marrow derived rat mesenchymal cells, indicating the biocompatibility of the material (Kurian *et al.*, 2022). In a different study, Ravichandran *et al.* developed a 3D bone marrow adipose tissue (BMAT) model by resuspending human bone marrow cells in GelMA precursor solutions followed by UV photo-crosslinking and culturing in adipogenic differentiation media, generating a stable adipogenic cell-laden GelMA hydrogel (Ravichandran *et al.*, 2021). Osteoprogenitors (Choi *et al.*, 2021) and adipocytes (O'Donnell *et al.*, 2020) have also been used in GelMA hydrogels and showed high bone differentiation. Although the human bone presents high stiffness (>35 kPa) (Nelson and Roy, 2016), in 3D culture systems osteoprogenitors show higher viability and mineralisation in lower stiffnesses, possibly due to enhanced remodelling activity. In a different study, human osteoprogenitors were cultured in different concentrations of GelMA and it was shown that cell viability was not affected

at a stiffness range of 6.3-40.2 kPa, however lower stiffness hydrogels enabled enhanced osteoblast differentiation and increased mineralisation (Bessot *et al.*, 2023).

Within the same study, the authors implanted GelMA hydrogels pre-seeded with differentiated osteoblasts followed by the implantation of GelMA hydrogels pre-seeded with differentiated adipocyte cells subcutaneously in immunodeficient NSG mice. They showed that implants had the ability to get mineralised after 11 weeks *in vivo* and have a structure similar to the trabecular bone. However, the cortical shell of the bone construct was formed by host cells and not by pre-seeded human cells, whereas the centre of the construct stained specifically for human bone matrix proteins (Bessot *et al.*, 2023).

1.5.6.2 Use of GelMA in cardiovascular tissues

The engineering of cardiac constructs using GelMA-based hydrogels has been reported. Chen *et al.* implemented an alternative microfluidic configuration and observed that shear stress resulting from fluid flow significantly influenced cell-cell interactions. They noted that the presence of valvular endothelial cells, along with the shear stress induced by the flow, impeded the pathological differentiation of valvular interstitial cells (VICs) into α -smooth muscle actin-positive myofibroblasts (Chen *et al.*, 2013). In other work the authors found that cardiomyocytes (CMs) showed cellular alignment within the microchannels coated with GelMA hydrogels, driven by the flow direction (Annabi *et al.*, 2013).

1.5.7 GelMA and its use in studying cancer cell interactions *in vitro* and *in vivo*

In the context of cancer, hydrogels have been attractive candidates due to structural and chemical similarities of the native ECM proteins. GelMA has been used as 3D model to mimic the tumour microenvironment, as the interaction between cancer cells and their surrounding ECM has been proven critical in cancer progression and drug response (Loessner, Holzapfel and Clements, 2014). GelMA has been used as a platform that allowed the formation of multicellular spheroids from ovarian cancer cells. Leveraging the ability to fine tune its material properties, it was demonstrated that spheroid morphology and proliferation was dependent on the concentration of GelMA and

subsequently its stiffness. Additionally, when GelMA hydrogels pre-seeded with ovarian cancer cells were implanted *in vivo*, they induced tumour formation and metastasis (Kaemmerer *et al.*, 2014). Similarly, metastatic breast cancer cells have also been embedded in GelMA hydrogels and showed high cell viability when implanted in close proximity to a humanised tissue-engineered bone in order to mimic invasion of the humanised bone by the breast cancer cells (Loessner *et al.*, 2016). In other work, Arya *et al.* used GelMA hydrogels as a platform to study the growth of breast cancer cells by seeding human MDA-MB-231 cells on top of GelMA hydrogels, instead of embedding. The authors showed that GelMA hydrogels were able to support spheroid formation and cancer cells seeded in GelMA hydrogel showed increased invasiveness *in vitro* and increased metastatic potential *in vivo* compared to 2D seeded cells (Arya *et al.*, 2016). GelMA has also been successfully used for the study of prostate cancer in the work of Bessot *et al.*. Within the same study, the authors used GelMA hydrogels with 2-12 kPa stiffness to engineer prostate tumour microtissues *in vitro*. Next, the authors used their engineered bone model (GelMA hydrogels with pre-seeded adipocytes) with a tumour model (GelMA hydrogel with prostate cancer cells) and they showed that *in vivo* implantation of both hydrogels within the same subcutaneous pocket led to increased human prostate cancer growth *in vivo*, compared to implantation of GelMA hydrogels with prostate cancer cells alone, indicating that adipocytes supported tumour growth (Bessot *et al.*, 2023). In another study, GelMA was implemented to create a humanised prostate tumour microenvironment (McGovern *et al.*, 2018, 2021). The authors showed that humanized prostate TME decreases metastasis of PCa cells to a PCL-based tissue-engineered bone implant, but did not affect the metastatic potential of PCa cells to the endogenous murine bones or organs (McGovern *et al.*, 2021).

In a more complex setting, Tang *et al.* developed a 3D bioprinting system using GelMA to create a biomimetic 3D cancer microenvironment for glioblastoma. 3D printed GelMA was successfully used as a platform for culturing patient derived glioblastoma stem cells (GSCs), macrophages, astrocytes, and neural stem cells (NSCs). This platform was used to dissect cancer cell interactions and functional dependencies between all important components of the tumour microenvironment (Tang *et al.*, 2020). In a more simplified approach, Shah *et al.* used GelMA scaffolds for seeding and culturing glioma cells,

demonstrating that glioma cells within GelMA hydrogels resemble a more mesenchymal-like phenotype that is typically found around hypoxic and vascular regions of the tumour and are associated with poor survival (Shah *et al.*, 2021). This highlights the ability to create engineered microenvironments as cell specific culture platforms that are able to best replicate the clinical scenario.

GelMA has also been used as an *ex vivo* drug testing platform for patient-derived breast cancer organoids. The authors cultured breast cancer organoids in different hydrogels and showed that GelMA supported the growth and organoid formation of HR⁺/HER2⁺ primary cancer cells for at least 2–3 weeks. Additionally, it was shown that the response of cancer cells to drug treatment (doxorubicin, paclitaxel) differed between the different hydrogels and also compared to 2D, highlighting that mimicking the ECM and stiffness of breast cancer shows a differential drug response (Bock *et al.*, 2023).

In summary, GelMA hydrogels offer a versatile platform for studying various aspects of cancer biology. GelMA hydrogels can be used as 3D cancer models that mimic the tumour microenvironment more accurately than traditional 2D cell cultures. 3D GelMA-based cancer models can serve as valuable platforms for drug screening and evaluation, since it offers a better representation of the interaction of cancer cells with the ECM. Their tuneable properties and ability to recapitulate aspects of the tumour microenvironment make them valuable tools for advancing our understanding of cancer and developing novel therapeutic strategies.

1.6 Hypothesis and aims of the thesis

1.6.1 Project hypothesis

In the past decade, 3D scaffolds have been developed to recapitulate the key aspects of the metastasis microenvironment in order to dissect the complexity of the metastatic niche. Studying these processes through engineered ectopic sites *in vivo* has provided valuable information. Designing artificial biomaterials that replicate the pre-metastatic niche offers translational opportunities, such as redirecting metastatic cells away from

target organs and developing early-detection strategies that were previously unattainable with traditional methods (Azarin *et al.*, 2015; Rao *et al.*, 2016). In the context of pre- and metastatic niche, biomaterial scaffolds have been used to dissect complex cancer cell interactions. The majority of the studies have used synthetic biomaterials leveraging the ability of the biomaterials to induce an inflammatory response with the host. Focusing on the work of the Shea and Jeruss groups, PCL and PLGA biomaterial scaffolds have been shown to induce an immune cell response and subsequently cancer cell recruitment from the primary tumour within the scaffold (Azarin *et al.*, 2015; Rao *et al.*, 2016; Bushnell *et al.*, 2019; Wang *et al.*, 2023).

As a semi-synthetic biomaterial, GelMA has been extensively characterised since its discovery in 2000s. GelMA has shown great biocompatibility and cell adhesion *in vitro* and *in vivo*. In the context of cancer, GelMA has been used as a 3D model to mimic the tumour microenvironment, however studies have been limited to direct cancer cell seeding of various cancer cell types in GelMA hydrogels *in vitro* and *in vivo*.

Given the differences in host responses between natural and synthetic biomaterials, this project hypothesizes that GelMA-based bioengineered scaffolds can be used to create an *in vivo* microenvironment for the study of cancer cell interactions.

Furthermore, altering the physicochemical properties of GelMA scaffolds, a main advantage of this biomaterial, such as porosity and stiffness, will influence tumour-host interactions, cell infiltration, vascularization, and potentially the systemic metastatic burden. Specifically:

- GelMA scaffolds can be optimized to mimic the pre-metastatic niche *in vivo*, facilitating a deeper understanding of cancer cell behaviour in the host.
- The biocompatibility of GelMA will result in different tumour-host responses compared to synthetic biomaterials, which typically trigger a more substantial foreign body response (FBR).
- Implantation of GelMA scaffolds in a tumour-bearing host will modulate the systemic microenvironment, possibly influencing distant metastatic sites, such as the lung.

- Modifications to the physicochemical properties of GelMA, such as stiffness or functionalization with metastasis-related factors, will affect cancer cell dissemination and seeding.

1.6.2 Project aims

In order to address the above hypotheses, the main project aims include the following:

Aim 1: Engineering and mechanical characterisation of GelMA Scaffolds with different porosity.

- Develop GelMA-based scaffolds with tuneable porosity in order to explore how different size of pores within the scaffold can affect cell infiltration *in vivo*.
- Mechanical characterisation of porous GelMA scaffolds using rheology to ensure a physiologically relevant stiffness is achieved.

Aim 2: Characterisation of porous GelMA scaffolds for *in vivo* biocompatibility.

- Characterize cell infiltration and vascularization of the porous GelMA scaffolds upon subcutaneous implantation *in vivo* to ensure proper scaffold integration and function.
- Optimize the scaffold's properties (*e.g.*, porosity, stiffness) to enhance their performance as artificial tissues that mimic *in vivo* environments.

Aim 3: Study of GelMA scaffold interaction with the tumour-bearing host.

- Implant GelMA scaffolds in tumour-bearing mice to investigate whether the scaffolds interface differently with a tumour-bearing host compared to naive.
- Characterize the tumour-host interactions, including changes in the scaffold infiltrate, cancer cell infiltration, and tissue remodelling.
- Explore whether the presence of a primary tumour affects how the host responds to the scaffold, potentially altering the tumour microenvironment or systemic signals.

Aim 4: Investigation of GelMA Scaffold Impact on Lung Metastasis

- Investigate whether GelMA scaffold implantation affects the systemic metastatic burden, particularly in the lung of tumour-bearing mice.

- Explore if scaffold implantation modulates the lung microenvironment through systemic immune or biochemical responses, similar to observations made in previous studies with synthetic scaffolds.

Aim 5: Fine-Tuning GelMA Properties to modulate cancer cell seeding and metastasis

- Assess how different scaffold conditions affect metastatic dissemination, cancer cell seeding, and the dynamics of the tumour microenvironment *in vivo*.
- Investigate how alterations in the physicochemical properties of GelMA, such as changes in scaffold stiffness or functionalization with metastasis-related factors, influence cancer cell behaviour *ex vivo*.

Chapter 2. GelMA scaffold development

2.1 Introduction

Methacryloyl gelatin (GelMA) is a semi-synthetic gelatin-based material that has been proven to be versatile for tissue engineering, drug delivery, and 3D printing applications (Yue *et al.*, 2015). Important advantages of using GelMA as a material is its great biocompatibility, cell adhesion due to the presence of RGD sequences and mechanical properties that can be tailored according to the experimental needs.

Micropore formation is essential for 3D cell culture systems and tissue engineered scaffolds as it creates a large surface area for cell attachment and transportation of nutrients for cell survival. As seen in section 1.3.2.4, pore size of the biomaterial implant influences the type of cell infiltration. Indeed, it was observed that pore size of 40 μm is sufficient to induce cell infiltration and blood vessel formation (Fukano *et al.*, 2010). Additionally in the work of Hady *et al.*, it was shown that larger pores ($>160 \mu\text{m}$) promote macrophage polarisation towards an anti-inflammatory response and subsequent secretion of anti-inflammatory and pro-angiogenic cytokines (Yin *et al.*, 2020; Hady *et al.*, 2021). Pore sizes of 100-160 μm have been shown to induce unfavourable immune responses (Hernandez and Woodrow, 2022), and even larger pores ($>400 \mu\text{m}$) lead to inefficient cell-cell interactions due to excessive large pore size or the loss of ECM production (Y. Wang *et al.*, 2014; Z. Wang *et al.*, 2014)

In the past decades, tissue engineering has made considerable progress to create porous scaffolds. Methods include freeze drying, gas foaming, electrospinning and two-phase emulsion (Annabi *et al.*, 2010). 3D printing has emerged as the latest technology for simultaneous manipulation of cells and pore formation to directly engineer cell- laden tissue constructs with high spatial resolution. However, there is a trade-off between the size of the tissues and the size of the pores. Whilst high-resolution printing techniques such as two-photon 3D printing can achieve high spatial resolution (hundreds of microns), it is impractical to fabricate tissues larger than a few millimetres.

2.2 Aims & Objectives

This chapter seeks to develop a bioengineered GelMA scaffold, with tailored pore formation which can potentially be used as an artificial microenvironment *in vivo*. Given the fact that different pore size induces different types of cell infiltration, two different methods to create porosity were explored. The first method was by creating GelMA casted scaffolds using different fabrication methods, either using an emulsification method or using a microfluidics chip platform to produce the porogens. These methods aim to create a small pore size (<150 μm), however sufficient for cell infiltration, nutrient/oxygen transportation and vessel formation *in vivo*. An alternative method for creating porous scaffolds was also investigated by using SLA based 3D printing techniques, which can induce pore formation up to 250 μm .

Additionally, rheological analysis was used to assess the stiffness of the scaffolds. Achieving a physiologically relevant stiffness is crucial because the mechanical environment affects how cells infiltrate and function within the scaffold. *In vivo*, tissue stiffness influences cell differentiation, migration, and vascularization.

The expected outcome is the creation of a versatile and tuneable GelMA scaffold that can be further explored for its interaction with different cell types and tissues in future *in vivo* studies.

2.3 Material & Methods

2.3.1 GelMA synthesis

In brief, gelatin from fish skin (Sigma-Aldrich, G77041-500G) was dissolved in Ultrapure water to a final concentration of 10% (w/v) at room temperature in a round-bottom flask with a magnetic stirrer bar. After gelatin dissolution, the mixture was heated to 50°C to allow gelatin to be fully dissolved and the solution to become clear. Then, 0.6 g of methacrylic anhydride (Sigma-Aldrich, 276685-100mL) per 1 g of gelatin was

added for a high degree of methacryloyl functionalisation. After stirring for 3 hours the solution was transferred to 50 mL falcon tubes and unreacted methacrylic anhydride was removed by centrifugation at 3,500 g for 3 min at room temperature. The supernatant was decanted into a 500 mL glass beaker and diluted with two volumes of pre-heated Ultrapure water. The solution was transferred into a dialysis membrane with a 6-8 kDa MWCO (Spectrum Laboratories, 132665) and dialysed at 40°C, with the water changed five times over 5 days. After dialysis, the solution was transferred into 50 mL falcon tubes, freeze dried and lyophilised to give GelMA as a foam like solid.

2.3.2 Fluorescence labelled GelMA synthesis

Fluorescein-conjugated GelMA (Fluorescein-GelMA) and Rhodamine-conjugated GelMA (Rhod-GelMA) were synthesized by reacting GelMA with NHS-Fluorescein (ThermoFisher, 46410) and NHS-Rhodamine (ThermoFisher, 46406), respectively. GelMA was fully dissolved in phosphate buffer (pH ~8.1) at 37 °C at a concentration of 10 % (w/v), followed by the addition of NHS-Fluorescein or NHS-Rhodamine (30 mg per 1 g of GelMA). The reaction was performed for 3 to 8 hours at 37 °C in the dark and subsequently quenched by adding water (4 volume equivalents). The product was then dialyzed using a dialysis membrane 6-8 kDa (Spectrum Laboratories, 132665) against water at 40 °C, with the water changed five times over 5 days. All dialyzed solutions above were freeze-dried and stored at -20°C before use.

2.3.3 Porcine gelatin beads fabrication

To a round bottom flask with a magnetic stirrer bar was added 12.5 mL of 1:1 (v/v) ethanol: Ultrapure water, followed by the addition of 500 mg of porcine skin derived gelatin (Sigma-Aldrich, G1890-100G). The solution was heated to 50 °C to allow the gelatin to be fully dissolved. Then, 125 mg of pluronic acid (Sigma-Aldrich, P2443-250G) and 50 mg gum arabic (Sigma-Aldrich, 51198-250G) were added to the solution and left to spin at 400 rpm overnight at RT. The next day, the solution is transferred to a 50 mL falcon tube and diluted with 25 mL Ultrapure water and centrifuged at 500g for 5 minutes. The supernatant was discarded, and the pellet containing the gelatin beads was

washed with 50 mL phosphate buffer saline (PBS), followed by centrifugation at 1000 g, 4 °C for 5 minutes. The washing step was repeated three times to minimize the retention of ethanol and surfactants. The collected beads were finally resuspended in PBS and stored at 4 °C fridge until use.

2.3.4 Rhodamine labelled gelatin beads fabrication

To a round bottom flask with a magnetic stirrer bar was added 12.5 mL of 1:1 (v/v) ethanol:Ultrapure water, followed by the addition of 500 mg of porcine skin derived gelatin (Sigma-Aldrich, G1890-100G) mixed with 2 mg of NHS-Rhodamine (ThermoFisher, 46406). The solution was heated to 50°C to allow the gelatin to be fully dissolved. Then, 125 mg of pluronic acid (Sigma-Aldrich, P2443-250G) and 50 mg gum arabic (Sigma-Aldrich, 51198-250G) were added to the solution and left to spin at 400 rpm overnight at room temperature. The next day, the solution is transferred to a 50 mL falcon tube and diluted with 25 mL Ultrapure water and centrifuged at 500g for 5 minutes. The supernatant was discarded, and the pellet containing the gelatin beads was washed with 50 mL phosphate buffer saline (PBS), followed by centrifugation at 1000 g, 4 °C for 5 minutes. The washing step was repeated three times to minimize the retention of ethanol and surfactants. The collected beads were finally resuspended in PBS and stored at 4 °C fridge until use.

2.3.5 Casted scaffold preparation

Lyophilised GelMA was weighed and resuspended in PBS at a final concentration of 10% (w/v), mixed with 4×10^{-3} M lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate photo-initiator (LAP) (TCI, L0290). The GelMA solution was warmed to 37 °C and left until completely dissolved. The solution was then mixed with porcine gelatin beads in a volume ratio of 1 gelatin beads: 2 GelMA and applied in a 3D printed silicon mould (6 mm diameter, 3 mm height) and was then photo-crosslinked using *in situ* light irradiation (365 nm, 10 mW cm⁻², OmniCure S1500) for 5 minutes. The photo-crosslinked GelMA scaffolds containing gelatin beads were then placed in 1mL PBS for further use.

2.3.6 Fluoraldehyde assay

The fluoraldehyde reagent (Thermo Scientific, 26025) was aliquoted into a 15 mL falcon tube and warmed to room temperature. Stock solutions of fish skin derived gelatin (1, 0.5, 0.1 and 0.02 mg/mL) and fish-GelMA samples at 1 mg/mL were prepared in PBS. A 300 μ L of each sample was mixed with 600 μ L of the fluoraldehyde reagent and then 3 x 250 μ L of each mixture was transferred to a 96-well fluorescence plate. The plate was read using excitation wavelength = 350 nm and emission wavelength = 450 nm. A calibration curve was calculated from the standards. The degree of functionalisation (%) of the GelMA was determined using the equation $(1 - X)/1 \times 100$, where X = determined concentration of GelMA. The determined degree of functionalisation was 93%.

2.3.7 NMR spectroscopy

^1H NMR spectra was recorded on a JEOL ECZ400S NMR spectrometer operating on a frequency of 400 MHz. NMR spectra were recorded at 298 K in D_2O , with the chemical shifts being referenced to sodium 3-trimethylsilyl-propionate-2,2,3,3- d_4 (TMSP), at a concentration of 1 mg/mL. Results were analysed using MestRenova software (v14.3.3-33362).

2.3.8 Gelatin beads release

For the quantification of the gelatin beads release from the scaffolds, Rhodamine-labelled gelatin beads were fabricated and scaffolds were prepared as per the standard protocol. The scaffolds were placed in 1 mL of PBS in an Eppendorf tube and left stirring at 37 $^\circ\text{C}$. For the negative control, scaffolds were placed at 4 $^\circ\text{C}$. For the positive control, gelatin beads were mixed with GelMA and without photo-crosslinking, the solution was mixed with 1 mL of PBS. The fluorescence of the rhodamine-labelled gelatin beads was detected by absorbance at 546/567 nm with an EnVision® plate reader (PerkinElmer) over the period of 7 days, by pipetting 100 μ L of supernatant in a 96-well plate and after measuring the solution was returned to same Eppendorf respectively.

2.3.9 Imaging of scaffolds

The porous scaffolds were imaged using Light-sheet imaging (Lavision UMII) at day 5 and 3D imaging of scaffolds was reconstructed using Imaris 9.5.1 and ImarisFileConverter 9.5.1 software. *Ex vivo* imaging of the scaffolds was performed using SteREO Lumar.V12 stereomicroscope (Zeiss).

2.3.10 Rheological characterisation of porous GelMA scaffolds

Rheological measurements of the porous scaffolds were recorded using an Anton Paar MCR302 rheometer fitted with a 25 mm stainless steel parallel plate (PP25) and Peltier temperature-controlled hood (P-PTD200/80/I). To determine the thermal responsivity of different formulations, oscillatory temperature sweeps were carried out with temperature ramp change at a rate of $5^{\circ}\text{C minute}^{-1}$, while shear strain and frequency were fixed at 1% and 1.5 Hz, respectively. In addition, a sharp temperature change from 10 to 37°C was applied to the oscillatory time sweeps to demonstrate the successful melting of microgels under physiological temperature. Oscillatory strain and frequency sweeps were performed with a fixed frequency (1.5 Hz) and strain (1%), respectively. To determine the photo-crosslinking kinetics, time sweeps at 37°C were performed, during which in situ light irradiation (365 nm, 10 mW cm^{-2} , OmniCure S1500) was applied for 5 minutes. Unless otherwise stated, constant values of strain, frequency and temperature were 1%, 1.5 Hz, and 37°C , respectively.

2.3.11 Negative mould fabrication

Moulds containing six round cylinders (diameter 9 mm, height 4 mm) in a tray, was designed in SolidWorks® (Dassault Systèmes) and an STL file was generated. The mould was 3D printed using Prusament Resin (Tough Orange) using a Prusa SL1S using a layer height of 0.025 mm, and the mould CAD file positioned in the same plane as the printing platform.

2.3.12 Positive mould fabrication

Positive moulds were fabricated using soft lithography using SYLGARD™ 184 Silicone Elastomer Kit, a two-part polydimethylsiloxane (PDMS)-based elastomer (VWR, 634165S). A 10:1 (w/w) mixture was prepared with the base elastomer and curing agent and poured into the mould to form a layer covering the mould. The mould was placed in a heat-tolerant plastic tray. The layer was then degassed in a vacuum desiccator and cured in an oven (Mettler) at 60 °C for 90 minutes to form the patterned layer of the chips. The layer was peeled off the mould and the individual chips were cut to size.

2.3.13 Microfluidics chip fabrication using soft lithography

The microfluidics platform was designed and optimised by a former member of the Stevens Group (Dr. Camille Marijon). The microfluidic chip mould was designed on SolidWorks and 3D printed. Chips were fabricated by soft lithography using SYLGARD™ 184 Silicone Elastomer Kit, a two-part polydimethylsiloxane (PDMS)-based elastomer (Figure 13). A 10:1 (w/w) base elastomer: curing agent solution was prepared and poured into the mould to form a layer approximately 3 mm thick. The layer was then degassed in a vacuum desiccator and cured in an oven (Mettler) at 60 °C for 90 minutes to form the top patterned layer of the chips. The layer was peeled off the mould and the individual chips were cut to size and perforated to form the inlets and outlet using a biopsy punch (Miltex® 1 mm D6345 from Williams Medical Supplies for the inlets, Core sampling tool 0.5 mm 69039-05 from Electron Microscopy Sciences for the outlet). A 2 mm-thick bottom unpatterned layer was prepared by adding the same mixture to a large plastic dish. This layer was degassed in a vacuum desiccator and cured in an oven (Mettler) at 60 °C until slightly sticky (approximately 40 minutes). Chips were then assembled by placing the top patterned layers facing down onto the slightly sticky bottom unpatterned layer. The assembly was placed in an oven at 60 °C until fully cured and the two layers assembled. The individual chips consisting of the patterned and unpatterned layer were cut to size and peeled off the plastic dish.

2.3.14 Microgel fabrication using microfluidics platform

A 25 mL Hamilton Gastight glass syringe (26212, Sigma Aldrich) was filled mineral oil (Sigma Aldrich, M5310-1L) and surfactant SpanTM-80 (sigma Aldrich, S6760-250ML) and attached onto a first Fusion 100 Touch dual-channel syringe pump (KR Analytical). connected to the chip using a tubing connector and a 50 cm-long piece of tubing (15249134, Portex Fine Bore LDPE tubing 800/100/120, Smiths Medical, OD 1.09 mm, ID 0.38 mm, Fisher Scientific). The oil phase flow rate was set to 100 μ L/minute to fill and prime the chip channels, followed by a reduction at 15 μ L/minute. The outlet tubing (10793527, Portex Fine Bore LDPE tubing 800/100/100, Smiths Medical, OD 0.61 mm, ID 0.28 mm, 0.5 to 3.5 m length, Fisher Scientific), was wrapped around a 100 mL glass bottle (VWR) to keep it in place, and then connected and the system was run for approximately 5 minutes to allow the outlet tubing to be filled and the flow to stabilise. Two 500 μ L glass syringes with a 22G blunt needle (509914, Sigma Aldrich) containing the hydrogel precursor solutions were assembled on a second Fusion 100 Touch dual-channel syringe pump (KR Analytical) and connected to the chip using tubing connectors and 50 cm long pieces of tubing (15249134, Portex Fine Bore LDPE tubing 800/100/120, Smiths Medical, OD 1.09 mm, ID 0.38 mm, Fisher Scientific). The hydrogel precursor flow rate was set to 5 μ L/minute until microdroplets started forming at the FFJ, and then reduced to 2 μ L/minute and left for 10 minutes to allow the outlet tubing to be filled with the microemulsion and the flow to stabilise. The end of the outlet tubing was placed into a collection tube (Kimax centrifuge tube, Z254851, with polytetrafluoroethylene septa in cap, 27515, Sigma Aldrich) containing 500 μ L of oil phase. The collection tube was placed inside a beaker filled with ice and attached onto a shaking vortex (FisherbrandTM) using a foam tube holder, microgels were left under agitation for another 15 minutes. Microgels were then washed and stored at 4 °C until further use.

2.3.15 Microgels collection and wash

Photo-crosslinked microgels were collected as a suspension containing mineral oil and underwent a series of washes before use. To wash the microgels, a 9:1 (v/v) of pentane:microgels was added to the collection tube, which was shaken vigorously, then

left for 2 minutes to allow the microgels to settle at the bottom of the tube. The supernatant was then removed, and the process was repeated 3 times. After the last wash microgels were resuspended with PBS containing 100 U mL⁻¹ penicillin-streptomycin (ThermoFisher, 15070063), vigorously shaken and left static for 1 hour at room temperature to let the microgel phase separate from the oil phase. Most of the oil phase was removed after 1 hour using a pipette and the leftover residual oil was left to evaporate overnight under a chemical fume hood. The next day the microgels were centrifuged at 3000 rpm for 30 minutes and the supernatant was removed. The microgel pellet was resuspended in PBS with 100 U mL⁻¹ penicillin-streptomycin.

2.3.16 3D printed scaffolds

Scaffolds (8 mm diameter, 2 mm height) with pore sizes ranging from 100 µm-250 µm (**Figure 12B**) were designed in Solidworks® and an STL file was generated. A printing solution was prepared using 20% (w/v) GelMA prepared as previously, 1% (w/v) lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate photo-initiator (LAP) (TCI, L0290) and 0.5 mM tartrazine (Amazon) photo-absorber. The solution was loaded on the 3D printer (Prusa SL1). After printing, scaffolds were rinsed with PBS and were detached from the surface of the printer using a blade. Scaffolds were extensively washed with PBS and then cultured with PBS containing 100 U mL⁻¹ penicillin-streptomycin (ThermoFisher, 15070063).

2.3.17 Microcomputed tomography (microCT) scan of 3D printed scaffolds

A solution of barium sulphate was prepared by dissolving 21.5g of barium sulphate (Sigma Aldrich, 243353) was dissolved in 300 mL of H₂O. Scaffolds were incubated in the contrast agent solution overnight and scaffolds were imaged using microCT scanner (Zeiss) and data were analysed using ImageJ.

Table 1. Key resources table 1

Reagent	Source	Catalog Number
Chemicals		
Gelatin from fish skin	Sigma-Aldrich	G77041-500G
Gelatin from porcine skin	Sigma-Aldrich	G1890-100G
Methacrylic anhydride	Sigma-Aldrich	276685-100ML
Pluronic acid	Sigma-Aldrich	P2443-250G
Gum arabic	Sigma-Aldrich	51198-250G
NHS-Fluorescein	ThermoFisher	46410
NHS-Rhodamine	ThermoFisher	46406
Lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate	TCI	L0290
Fluoraldehyde™ o-Phthaldialdehyde Reagent Solution (OPA)	ThermoFisher	26025
SYLGARD™ 184 Silicone Elastomer Kit	VWR	634165S
Barrium Sulphate	Sigma-Aldrich	243353
Softwares		
MestRenova v.14.3.3	Mestrelab	https://mestrelab.com
Imaris v.9.5.1	Oxford Instruments	https://imaris.oxinst.com
Solidworks	Dassault Systemes	https://www.solidworks.com
ImageJ	Open source	https://imagej.net/ij/
Other		
Dialysis membrane 6-8 kDa MWCO	Spectrum Laboratories	132665

2.4 Results

2.4.1 GelMA synthesis

For the aim of this project, the first step in the development of GelMA scaffolds was GelMA synthesis. GelMA derives from the reaction between gelatin with methacrylic anhydride and contains both methacrylamide and methacrylate groups. This modification allows the formation of a covalently crosslinked network when exposed to ultraviolet (UV) light. GelMA was synthesised according to published protocols with the reaction of methacrylic anhydride with gelatin (Loessner *et al.*, 2016). After the reaction period GelMA was dialysed for 5 days and the solution was lyophilised. Methacryloyl groups were primarily introduced on lysine side-chains (amines) from the reaction with methacrylic anhydride. The degree of functionalisation (DoF), namely the percentage of functionalisation of the lysines to methacrylamide and methacrylate groups (via other hydroxyl functional groups) can be determined using two methods, namely nuclear magnetic resonance (^1H NMR) and the fluoraldehyde assay. The ^1H NMR analysis of lyophilised GelMA showed the protons integrated to determine the DoF (**Figure 6A**). The signals at $\delta = 5.4$ and $\delta = 5.7$ ppm in GelMA are the protons of the methacrylate vinyl group of MAA, namely the methacrylamide and methacrylate protons respectively. The signal at $\delta = 2.9$ ppm corresponds to the protons of the methylene group of lysine which is decreased compared to gelatin, as well characterised in other studies (Ma *et al.*, 2021; Villata *et al.*, 2023) however, due to the complex NMR spectra in this region, we opted to use an internal standard to characterise the degree of modification. Because of the variance in gelatin composition based on source, the mmol/g of methacryloyl groups per gram of gelatin can be determined as described previously by Claaßen and co-workers (Claaßen *et al.*, 2018). Their integration can be compared to the integration of the methacrylamide protons (including methacrylates). The signal at 0 ppm is from the internal standard, sodium 3-trimethylsilyl-propionate-2,2,3,3-d₄ (TMSP) and was integrated to the nine protons from the trimethylsilyl group. The ^1H NMR spectra shows the successful introduction of methacrylate groups in the lysine side-chains of gelatin.

A more common method to determine the DoF is by fluoraldehyde assay. The fluoraldehyde assay, also known as the OPA assay (o-phthalaldehyde assay), is a

sensitive chemical method used for detecting and quantifying primary amines. As after methacrylation, some of the amine groups are modified with methacrylate groups, the number of free primary amines is reduced. Using the fluoralddehyde assay, the extent of substitution can be assessed. Gelatin solutions of different concentrations were prepared (0.02, 0.1, 0.5, 1.0 and 2.0 mg/mL) in order to draw the standard curve. 0.5 mg/mL of GelMA solution dissolved in PBS was prepared. Gelatin/GelMA solutions and the fluoralddehyde reagent solution were then mixed in 1:2 ratio and the fluorescence intensity at 450 nm was determined. The fluorescence intensity of the GelMA sample corresponding to a gelatin concentration x mg/mL on the calibration curve and consecutively the residual amine concentration was determined. The DoF was then calculated according to the equation (Loessner *et al.*, 2016; Abraham *et al.*, 2024):

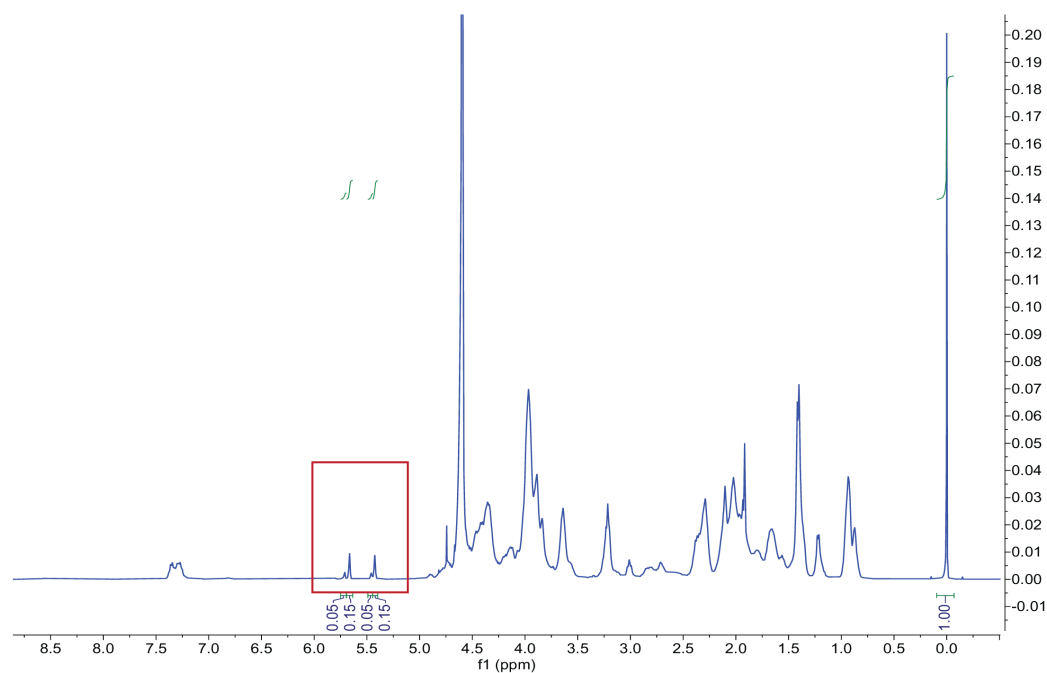
$$\text{DoF} = (1 - X) / 1 \times 100 \%$$

Where:

- 1 represents the original concentration of free amine groups in the unmodified gelatin (*e.g.*, 100% of gelatin's lysine residues are assumed to be reactive).
- X represents the fraction of free amines that remain unmodified after the reaction (as measured by the fluoralddehyde assay).

This formula assumes that prior to methacrylation, gelatin has a baseline of 1 moles of free amines per mole of gelatin (*e.g.*, 100% of the lysine residues are available to react). After modification, the fluoralddehyde assay measures the remaining free amine content (X), which is then subtracted from 1 to determine how many amines have been modified. In this experiment, the degree of functionalisation of fish GelMA was determined at DoF = 93% (**Figure 6B**).

A NMR analysis GelMA



B Fluoraldehyde assay GelMA

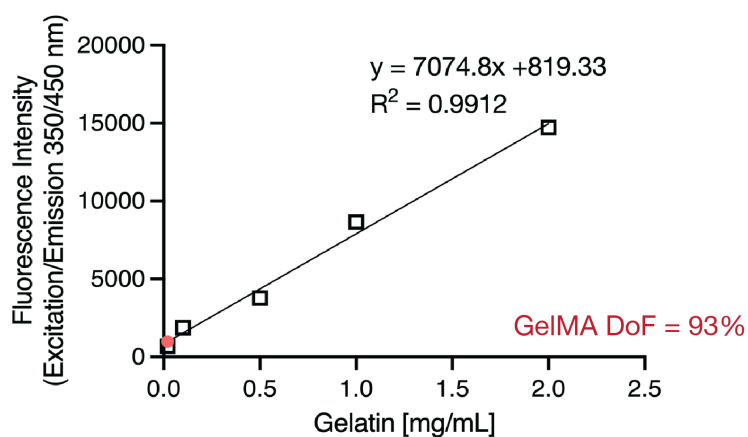


Figure 6. Fish GelMA characterisation. **A)** Representative ^1H NMR spectra of fish GelMA with sodium 3-trimethylsilyl-propionate-2,2,3,3- d_4 (TMSP) as an internal standard. **B)** Fluoraldehyde assay of fish GelMA to determine the degree of functionalisation based on amine modification. Standard curve was determined using gelatin samples in different concentrations (0.02, 0.1, 0.5, 1.0 and 2.0 mg/mL). Data shown as average from $n = 3$ (technical replicates).

2.4.2 Gelatin beads fabrication using the emulsification method

The next step of the study was to fabricate the gelatin beads as porogens for the hydrogels. For this purpose, two methods were tested, namely the emulsification method (**Figure 7A**) and using a microfluidics platform (**Figure 11**).

The emulsification method that was followed was modified from the approach of Lee *et al.* (Lee *et al.*, 2019) and used by Ouyang *et al.* (Ouyang *et al.*, 2022). In brief porcine gelatin was dissolved in a 1:1 (v/v) water/ethanol solution supplemented with surfactants (125 mg pluronic acid and 50 mg gum arabic) to form an initially miscible solution upon heating and stirring. The gelatin solution was then cooled to RT which allowed for the separation of the gelatin phase from ethanol and the formation of spherical size hydrogel beads, due to the reduced solubility in ethanol and thermal gelation in water. The reaction was typically left under constant stirring overnight and the next day the gelatin beads were collected and extensively washed with PBS for the removal of ethanol (**Figure 7A**).

Using rhodamine-labelled gelatin for the fabrication of gelatin beads allowed the visualisation of gelatin beads under confocal microscope at 546/567 nm. As seen in **Figure 7B** gelatin beads were nicely formed with a consistent spherical size between different preparations. The size of the porcine gelatin beads was measured and ranged between 50-100 μm , with average size at 70 μm (**Figure 7C**).

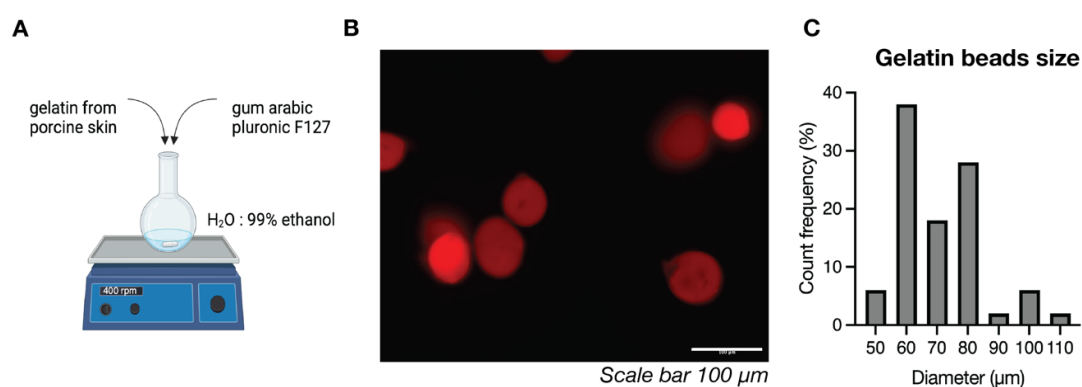


Figure 7. Gelatin beads fabrication using the emulsification method. **A)** Experimental design of gelatin beads fabrication illustrating the method followed. **B)** Representative fluorescence microscopy image of rhodamine labelled gelatin beads. Scale bar 100 μm . **C)** Diameter

distribution of gelatin beads at different size ranges. Total of 100 gelatin beads quantified from $n = 3$ gelatin bead preparations.

2.4.3 GelMA casted scaffold development

The next step was the introduction of porogen to create an interconnected porous hydrogel. The approach we followed was the incorporation of porcine gelatin beads within fish GelMA before UV photo-crosslinking. Porcine gelatin dissolves at 37 °C, therefore it was hypothesised that by incubating scaffolds at 37 °C, the gelatin beads would dissolve and provided there was a uniform distribution of gelatin beads within the scaffold, this would create an interconnected porous system within the scaffold. The experimental approach that was followed was mixing 10% (w/v) of fish GelMA with porcine gelatin beads at a volume ratio of 1:2 (v/v) followed by the addition of LAP photo-initiator that allows the photo-crosslinking of GelMA. The solution of GelMA with gelatin beads was applied in a PDMS casted mould of a specific size (6 mm diameter 4 mm height) and was then photo-crosslinked using light irradiation (365 nm, 10 mW cm⁻², OmniCure S1500) for 5 minutes. UV photo-crosslinking allowed the immediate gelation of GelMA within the mould, as seen in **Figure 8B**. The scaffold was carefully removed from the mould using a spatula and placed within a 24-well plate in PBS to prevent dehydration. The scaffolds were then placed at 37 °C to allow the dissolving of the porcine gelatin beads (**Figure 8A**).

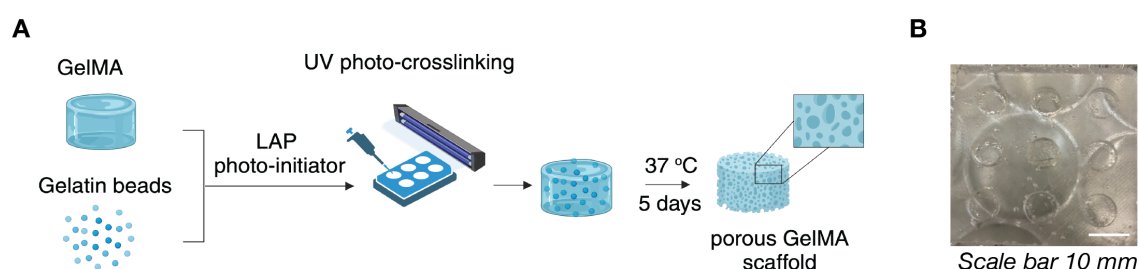


Figure 8. GelMA casted scaffold development. **A)** Schematics showing the experimental procedure used for the development of porous GelMA casted scaffolds. **B)** Image of casted scaffolds within the PDMS casted negative mould after UV photo-crosslinking.

2.4.4 Gelatin beads release from casted GelMA scaffolds

To understand whether the gelatin beads had the ability to dissolve at 37 °C and create a porous system within the GelMA scaffolds, the removal of the beads was monitored using Light-sheet microscopy which allowed for fluorescence imaging of the whole scaffold. For using this technique, FITC-labelled GelMA was synthesised, and Rhodamine-labelled gelatin beads were incorporated within the scaffold. After UV photo-crosslinking scaffolds were placed either at 4 °C or at 37 °C under constant agitation. After 4 days, scaffolds were imaged with Light-sheet microscopy at 488 nm and 555 nm for the imaging of FITC-labelled GelMA and Rhodamine-labelled gelatin beads respectively (**Figure 9A**). The 3D reconstruction of the scaffold showed a uniform distribution of gelatin beads within the scaffold after incubation at 4 °C in PBS. In contrast, after 4 days of incubation at 37 °C it was shown that the majority of the gelatin beads have dissolved, creating a porous system within the scaffold (**Figure 9B**). The orthogonal view of the generated scaffolds confirmed the absence of fluorescence at 546/567 nm from the Rhodamine-labelled gelatin beads (**Figure 9C**). Next, the timecourse of the release of gelatin beads from the scaffolds was quantified, in order to measure the amount of time required for gelatin to be dissolved and to generate the porous scaffolds. To assess this, gelatin beads were labelled with Rhodamine, then the scaffolds were incubated in PBS either at 37 °C or 4 °C. The release of the gelatin beads within the supernatant was quantified by measuring the absorbance of the supernatant at 546/567 nm every 24 hours (**Figure 9D**). To quantify the percentage of the gelatin beads released from the scaffold, as a positive control for the total amount of fluorescence of the supernatant was measured the signal from gelatin beads mixed with GelMA without any UV photo-crosslinking and incubated at 37 °C in order to allow full dissolution of the gelatin beads. Indeed, as depicted in **Figure 9E**, more than 50% of the gelatin beads were released within 24 hours, with 100% of the gelatin beads released by day 5. After day 5 a plateau in the Rhodamine absorbance was observed, indicating that the total amount of gelatin beads has dissolved within the supernatant.

Both imaging of GelMA scaffolds by Light-sheet microscopy as well as quantification of the gelatin beads release suggest that a porous system with an interconnected GelMA structure can successfully be engineered with the use of gelatin beads fabricated with the

emulsification method. Further experiments would be required to assess the interconnectivity of the pores by calculating the estimated number of gelatin beads per void space within the porous GelMA scaffolds. As seen in the work of Ouyang *et al.* (Ouyang *et al.*, 2022), the volume of a single gelatin bead can be determined by the estimated diameter, and the porosity can be calculated by dividing the total volume of space within the scaffold by the void space, data, which can be obtained by confocal analysis.

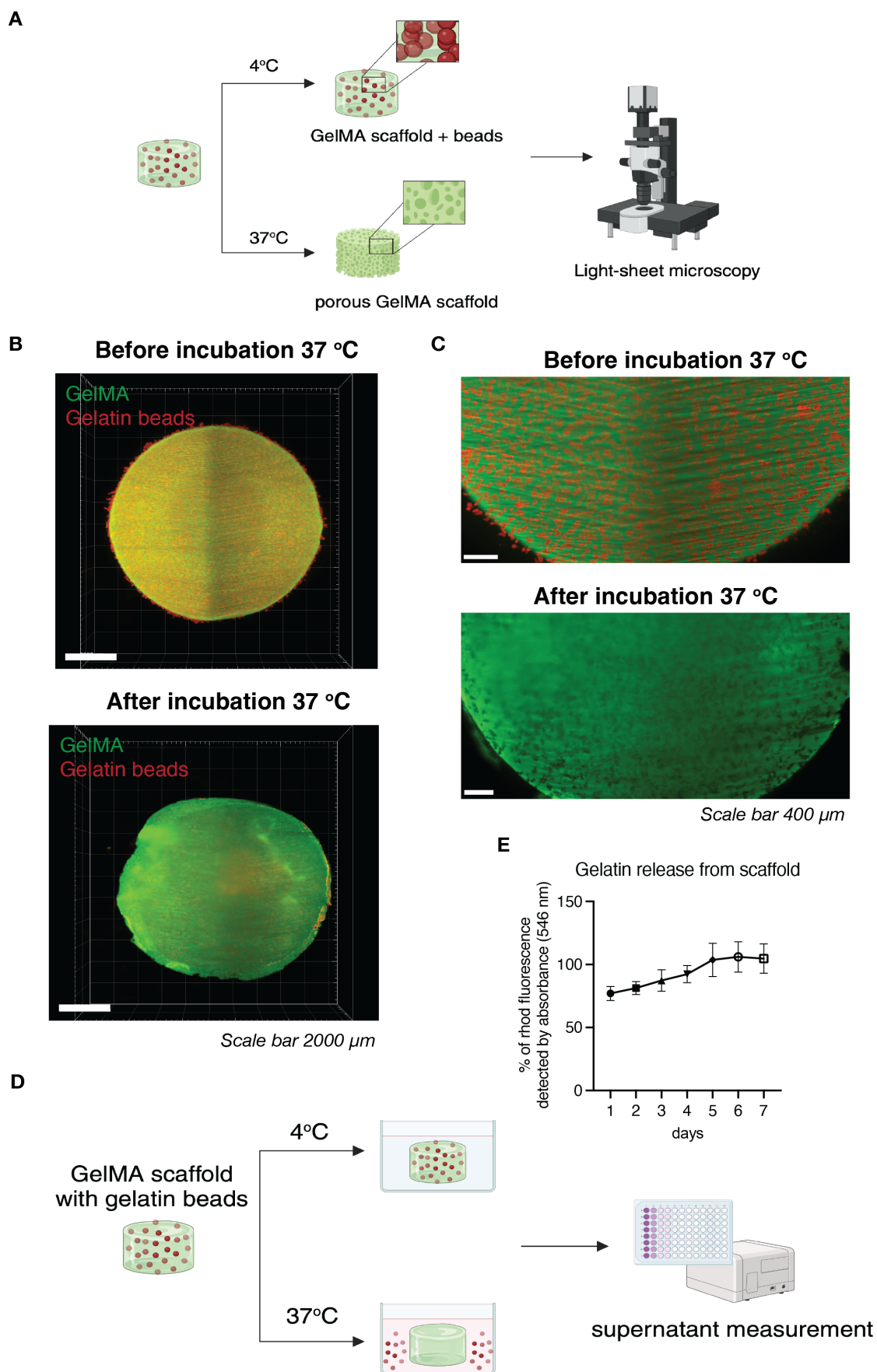


Figure 9. Gelatin beads effectively get released from GelMA casted scaffolds. **A)** Illustration indicating the experimental design for the study of gelatin beads release from the GelMA scaffold post UV photo-crosslinking. **B)** 3D view and **C)** Orthogonal view of generated FITC-labelled GelMA casted scaffolds mixed with Rhodamine-labelled gelatin beads before incubation at 37 °C (left) and after incubation at 37 °C (right) using Light-sheet microscopy. The red fluorescence indicates the rhodamine labelled gelatin beads and the green fluorescence indicates the FITC labelled GelMA. Scale bar B) 2000 μm C) 400 μm . **D)** Illustration indicating the experimental design for the quantification of gelatin beads release from GelMA casted scaffolds over time. **E)** Quantification of gelatin beads release as measured from the absorbance at 546/567 nm of the supernatant of cultured GelMA casted scaffolds mixed with Rhodamine labelled gelatin beads. Data shown as mean $\pm$ S.D. (n = 3).

2.4.5 Rheological properties of porous GelMA scaffolds

In order to achieve a physiologically relevant stiffness of the material, the next step of the study was to determine the rheological properties of the scaffolds. For this purpose, scaffolds were prepared as previous described. The scaffolds were incubated at 37 °C in order to create the porous system. Their rheological properties were characterised by measuring their frequency sweep and their shear sweep using rheology (**Figure 10A**).

A frequency sweep is used to measure the viscoelastic properties of a material as a function of the frequency of measurement, which ranges from low to high. The test is performed at constant strain or stress level (within the linear viscoelastic region (LVR) to ensure the material's structure isn't disrupted. A shear (or strain) sweep measures how a material responds to varying levels of applied strain (deformation) at a constant frequency. This test is particularly useful for determining the LVR, where the material behaves linearly and elastically before undergoing structural breakdown or yielding. The LVR is defined as the range of strain over which the material's storage modulus (G') and loss modulus (G'') remain constant, indicating that the material's internal structure is intact and responds elastically (Stojkov *et al.*, 2021). The storage modulus G' (G prime, in Pa) represents the elastic portion of the viscoelastic behaviour, which describes the solid-state behaviour of the sample. The loss modulus G'' (G double prime, in Pa) characterizes the viscous portion of the viscoelastic behaviour, which can be seen as the liquid-state behaviour of the sample.

As seen on **Figure 10B** and **10C** the storage modulus G' is significantly higher than the loss modulus G'' , indicating that the scaffolds show gel-like properties, and they remain stable over a range of frequencies between 0.1 and 1 Hz (**Figure 10B**) and shear strains between 1-10% (**Figure 10C**). The shear strain sweep shows a crossover point at approximately 50% strain and a deviation from the linear viscoelastic region at approximately 10% strain. The plateau modulus for the scaffolds is approximately 400 Pa.

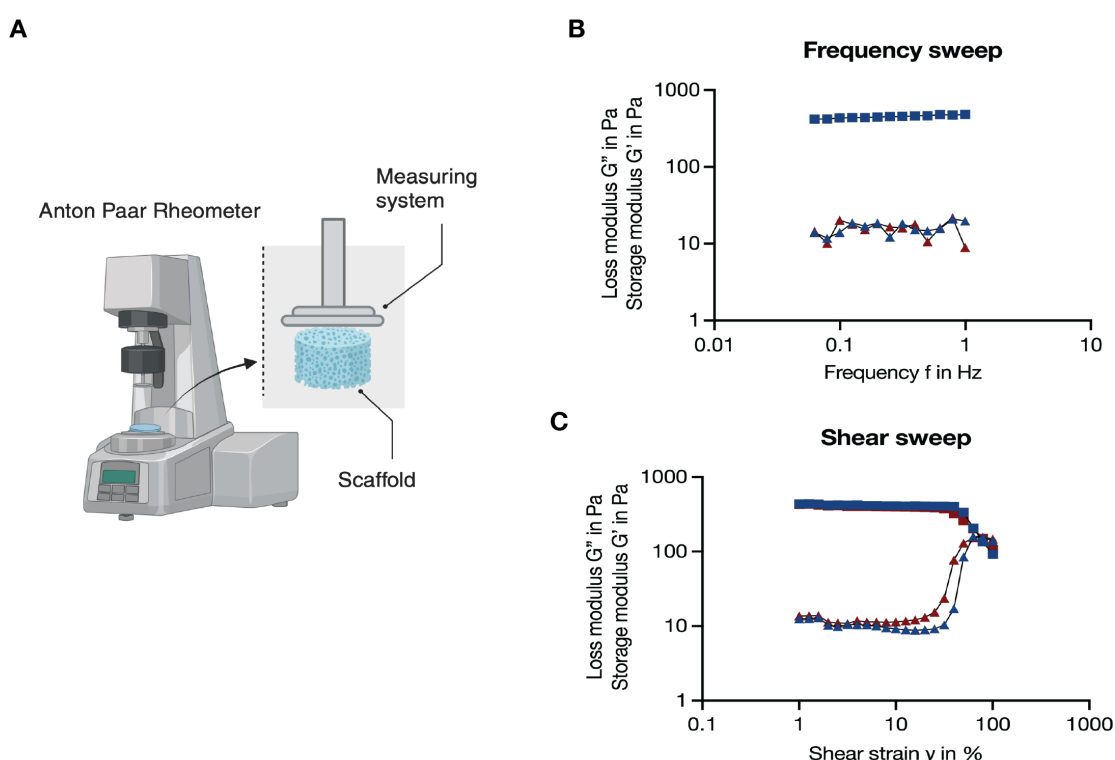


Figure 10. Rheological characterisation of GelMA casted scaffolds. **A)** Illustration of experimental design for the rheological characterisation of GelMA casted scaffolds using the Anton Paar Rheometer. **B)** Frequency sweep strain measured in Pa with stable shear strain at 0.5% in log scale. **C)** Shear sweep strain measured in Pa with stable frequency at 1 Hz in log scale. Blue and red colours indicate two different biological samples ($n = 2$).

2.4.6 Gelatin beads fabrication using a microfluidics platform

An alternative method of fabricating the gelatin beads is using an emulsion-polymerisation method, which consists of two phases: the aqueous phase containing the gelatin mixed with an oil phase to form water-in-oil microemulsion. In this system the

microdroplets are stabilised using a small amount of surfactant combined with the mineral oil (Peng *et al.*, 2011) before being photo-crosslinked into microgels.

For this purpose, the microfluidic chip was designed in Solidworks and 3D printed by a former member of the Stevens Group, Dr. Camille Marijion. The design is based on a water-in-oil microemulsion and consists of two phases, the oil phase, consisting of a mixture of mineral oil and surfactant SpanTM-80 merging with the aqueous phase consisting of porcine gelatin and forming microdroplets. The two phases are connected at the flow-focusing junction (FFJ), where the aqueous phase is squeezed within the oil phase from channels that are in a 90° angle. The size of the microdroplets is determined by the relative flow rates, as well as the channel dimensions, phase viscosities and surfactant concentration (Ward *et al.*, 2005; Peng *et al.*, 2011; Allazetta, Hausherr and Lutolf, 2013). The aqueous phase which includes the porcine gelatin is injected through two different inlets that meet the oil phase at the FFJ and the droplets that are formed exit through a wider channel as depicted in **Figure 11A**, in order to stabilise the size of the gelatin beads. The dimensions of input design of the chip are the following: $a = 120\ \mu\text{m}$, orifice width $b = 100\ \mu\text{m}$, outlet channel width $c = 300\ \mu\text{m}$, and have been optimised previously.

The microfluidic chip consists of two layers, one patterned and one unpatterned that are fabricated by soft lithography using PDMS as described in 2.3.13. The patterned layer is depicted in **Figure 11B**. The key steps for the microdroplet fabrication includes the oil phase (mineral oil + 10% (v/v) SpanTM-80) and the aqueous phase including the porcine gelatin. Both phases are injected via syringe pumps that control their flow rate and reach the microfluidics platform via separate inlet tubing. After the FFJ the microdroplets exit the platform via outlet tubing and are photo-crosslinked until they reach the collection tube which includes an oil solution. The experimental procedure for the microdroplet fabrication is illustrated in **Figure 11C** and was set up experimentally as seen in **Figure 11D**.

The parameters for the flow rate of the oil and aqueous phase have been optimised previously and for microdroplets fabrication of size $150\ \mu\text{m}$, the oil flow rate (Q_o) is set

at 15 $\mu\text{L}/\text{minute}$ and the gelatin flow rate (Q_w) at 2 $\mu\text{L}/\text{minute}$, with a ratio of $Q_w:Q_o=0.0133$. After fabrication microdroplets were collected in mineral oil solution and washed according to the optimised protocol described in 2.3.15. Microdroplets were imaged after wash under microscope and as seen in **Figure 11E**, their size was very consistent and ranged between 135-160 μm . (**Figure 11F**), twice the size of the gelatin beads fabricated with the emulsification method.

After successful production of microdroplets, GelMA scaffolds were prepared according to the previous protocol, by mixing pre-weighed microdroplets with GelMA solution in the presence of LAP photo-initiator before photo-crosslinking (**Figure 8A**). After photo-crosslinking the scaffolds were placed at 37 $^{\circ}\text{C}$ for 5 days in order to allow dissolving of the microdroplets and create a porous system within the scaffold. However, after imaging of the scaffolds post incubation, microdroplets remained still intact within the scaffold and did not form pores compared to gelatin beads fabricated with the emulsification method, as expected.

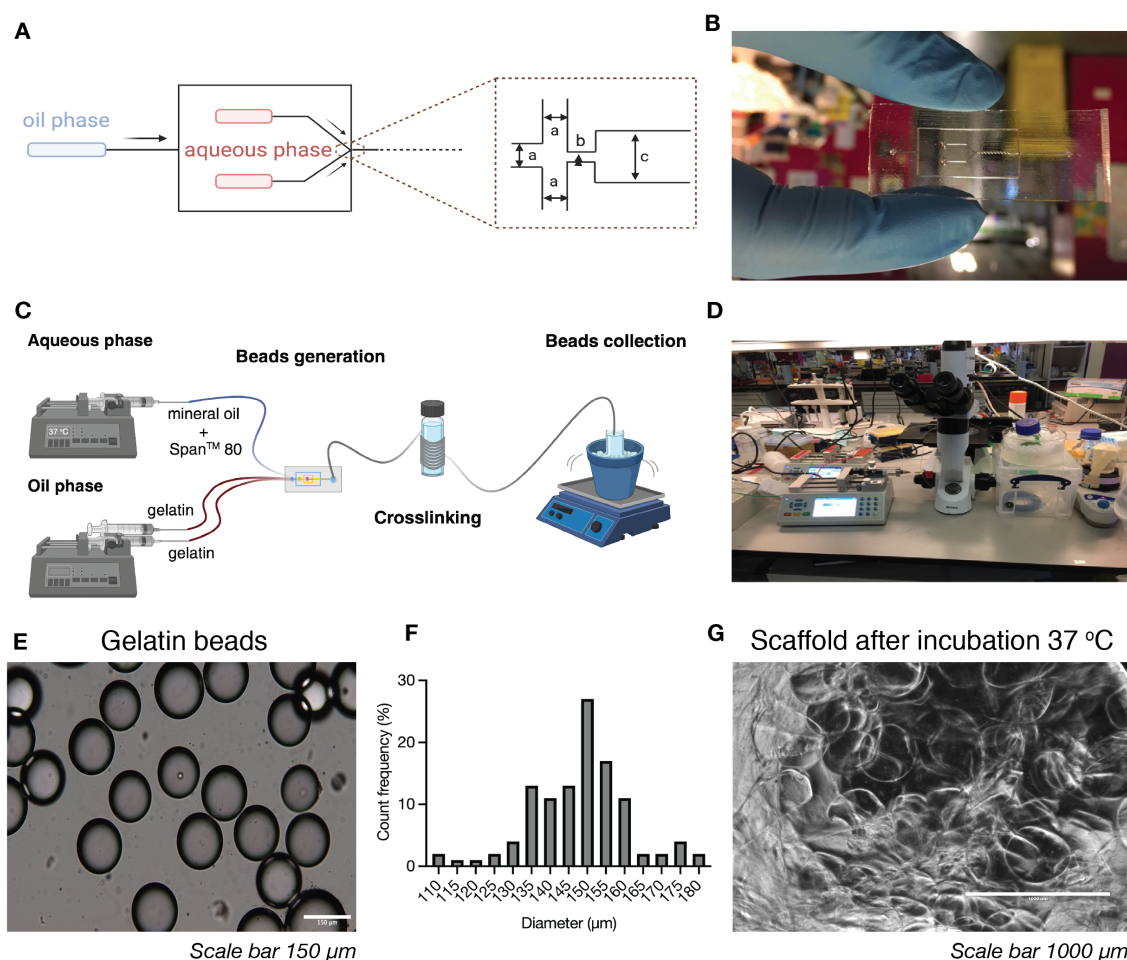


Figure 11. Gelatin beads fabrication using microfluidics platform. **A)** Microfluidics platform chip design based on a water-in-oil microemulsion, which consists of two phases, the aqueous phase and the oil phase merging and forming microdroplets. The dimensions of input design of the chip are the following: $a = 120 \mu\text{m}$, orifice width $b = 100 \mu\text{m}$, outlet channel width $c = 300 \mu\text{m}$. **B)** Fabricated chips based by soft lithography using PDMS based on the microfluidics platform design of A). **C)** Illustration of the microfluidics platform containing all the key steps involved in the microdroplets fabrication process. The aqueous dispersed phase contains the porcine gelatin heated at 37 $^{\circ}\text{C}$ and the continuous oil phase is composed of mineral oil and a stabilizing surfactant Span™-80. The two phases meet at the flow-focusing junction where they form microdroplets. The microdroplets are then frozen on ice and collected in cold oil under continuous stirring. **D)** Microfluidics platform experimental set up containing all the steps described in C). **E)** Imaging of gelatin beads formation using the microfluidics platform. Scale bar 150 μm . **F)** Quantification of the size of gelatin beads using the microfluidics platform. **G)** Imaging of GelMA scaffold development with gelatin beads fabricated with using the microfluidics platform post incubation at 37 $^{\circ}\text{C}$ indicating the presence of the beads within the scaffold. Scale bar 1000 μm .

2.4.7 3D printed porous GelMA scaffolds

In parallel it was investigated whether the development of the porous GelMA scaffolds using 3D printing could be achieved. 3D printing includes various advantages, including precise design of the desired structure using CAD software, and fast and simultaneous 3D printing of various scaffolds within one print. After designing the scaffold, the file was loaded onto the Pruca SL1 3D printer. Following this the printing solution for the scaffolds was prepared: 20 % (w/v) GelMA, 1% (w/v) LAP photo-initiator and 0.5 mM tartrazine photo-absorber and loaded to the printer (**Figure 12A**). The initial design included the fabrication of scaffolds as a grid with different pores sizes ranging from 100 μm to 250 μm with a 25 μm difference (**Figure 12B**). The scaffolds were successfully printed and removed from the printer followed by multiple washed with PBS for the removal of any excess material (**Figure 12C**). Notably 3D printed scaffolds using 10 % (w/v) GelMA mechanically collapsed during printing, therefore 20 % (w/v) GelMA solution was prepared. Next, it was investigated whether the pore formation was successful using 3D printing. For this, scaffolds were incubated with barium sulphate, a contrast agent typically used for imaging of commercial hydrogels, overnight and scaffolds were scanned with microCT scanner. As seen in **Figure 12D**, no pores were visible after 3D printing, even from scaffolds with the highest pore design, namely 250 μm as the scaffold shows a solid formation with no porosity. This indicates that the printing parameters need optimisation in order to achieve clear pore formation.

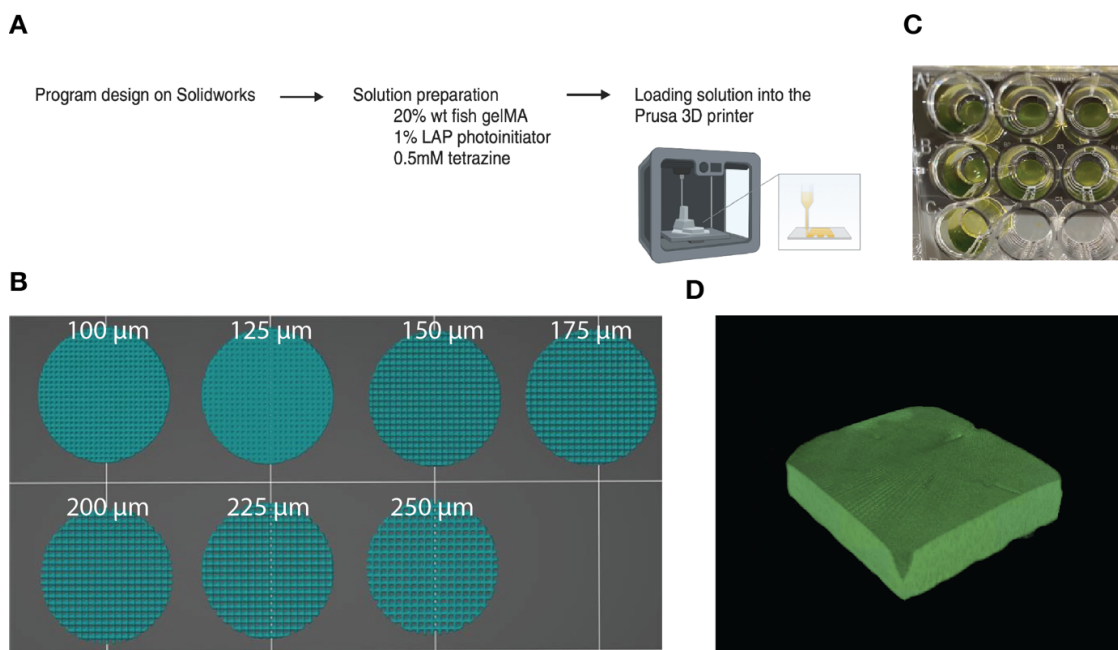


Figure 12. Development of porous 3D printed GelMA scaffolds. **A)** Illustration including the key steps for 3D printing of GelMA scaffolds. **B)** Printing design of scaffolds with different pore sizes ranging from 100 μm – 250 μm using Solidworks. **C)** Scaffolds with design indicated in B) after 3D printing. Top 100 μm to bottom 250 μm . **D)** MicroCT scan from scaffold with 250 μm properties.

Next, it was explored whether the rheological properties of the 3D printed scaffolds would differ from the properties of the GelMA casted scaffolds. For this purpose, scaffolds with 100 μm designed porosity were 3D printed as described previously. Their rheological properties were characterised by measuring their frequency sweep and their shear sweep using rheology (**Figure 13A**). The frequency sweep is the application of different frequencies at a constant amplitude and the shear sweep is the application of different shear stress at a specific frequency. The storage modulus G' (G prime, in Pa) represents the elastic portion of the viscoelastic behaviour, which describes the solid-state behaviour of the sample. The loss modulus G'' (G double prime, in Pa) characterizes the viscous portion of the viscoelastic behaviour, which can be seen as the liquid-state behaviour of the sample. As seen on **Figure 13B** and **13C** the storage modulus G' is significantly higher than the loss modulus G'' for the 3D printed scaffolds, indicating that the scaffolds show gel-like properties, and they remain stable over a range of frequencies between 0.1 and 1 Hz (**Figure 13B**) and shear strains between 1-10% (**Figure 13C**). The shear strain

sweep shows a crossover point at approximately 50% strain and a deviation from the linear viscoelastic region at approximately 10% strain. The plateau modulus for the scaffolds is approximately 4000 Pa, 10 times higher compared to the GelMA casted scaffolds.

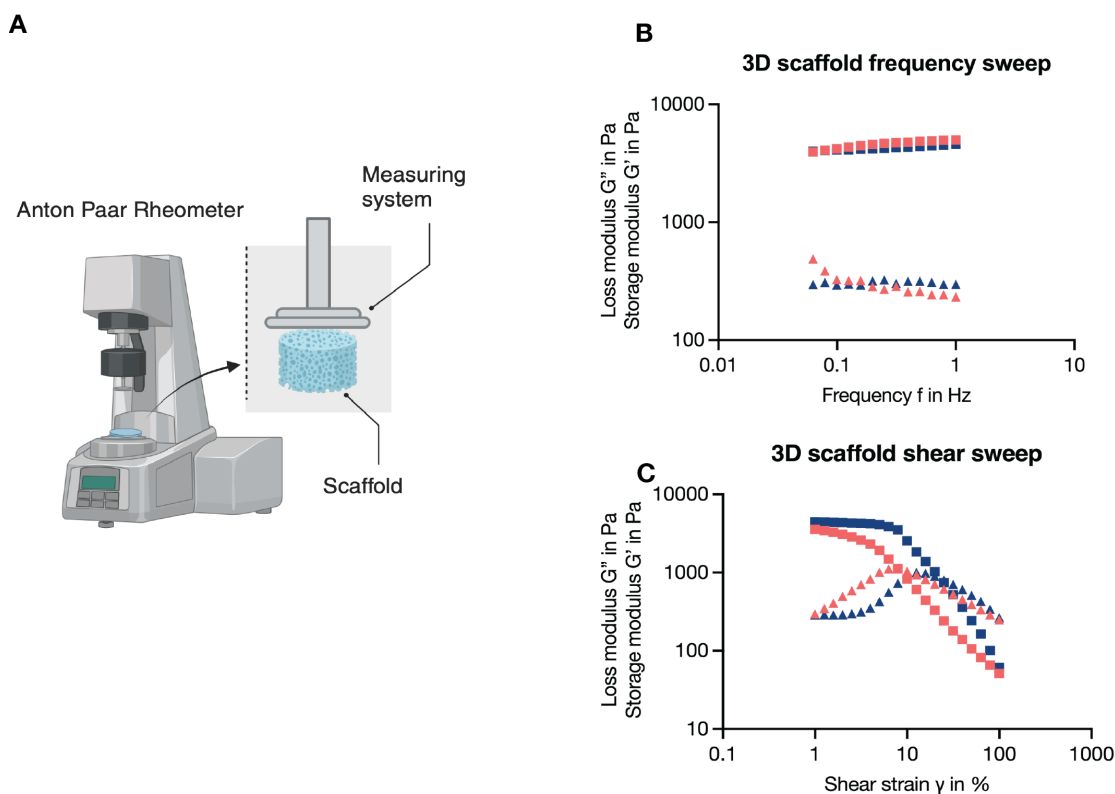


Figure 13. Rheological characterisation of 3D printed GelMA scaffolds with 100 μm designed porosity. **A)** Illustration of experimental design for the rheological characterisation of GelMA casted scaffolds using the Anton Paar Rheometer. **B)** Frequency sweep strain measured in Pa with stable shear strain at 0.5% in log scale. **C)** Shear sweep strain measured in Pa with stable frequency at 1 Hz in log scale. Blue and red colours indicate two different biological samples ($n = 2$).

2.5 Conclusions & future work

The aim of this chapter was to create a porous GelMA hydrogel scaffold, with its potential use *in vivo*. GelMA was successfully synthesised according to published protocols (Shirahama *et al.*, 2016) and by the addition of methacrylic anhydride, methacryloyl groups were added to gelatin on the lysine side-chains (amines). The degree of

functionalisation (DoF) was characterised both by ^1H NMR analysis and by the fluoroldehyde assay which determined the DoF= 93%.

Given the importance of porosity to support cellular infiltration, tissue integration, nutrient diffusion, and waste removal, porosity was next introduced within the scaffold. For the development of a porous scaffold two options were explored: GelMA casted scaffolds with the introduction of gelatin beads which have the ability to dissolve at 37 °C and 3D printed porous scaffolds.

The gelatin beads were fabricated using two methods. The first method was adapted from Lee *et al.* (Lee *et al.*, 2019) and modified by Ouyang *et al.* (Ouyang *et al.*, 2022). The second method utilised a microfluidic chip platform previously developed from the Stevens Group. For the emulsification method, porcine gelatin beads were successfully fabricated, and the average size was determined at 70 μm . The emulsification method includes various advantages including the maintenance of sterile conditions during the reaction and the production of a high amount of gelatin beads in a short step protocol which includes an overnight reaction. This method could be scaled to increase production, as more material could be added to the reaction without being more time-consuming. By Rhodamine-labelling of the gelatin beads it was shown that beads have the ability to dissolve at 37 °C and create a porous system within the GelMA scaffold after 5 days of incubation. This observation was supported both by Light-sheet microscopy by visualisation of the absence of gelatin beads post incubation and by quantification of the gelatin beads release over a period of 7 days by culturing the scaffolds in PBS and measuring the fluorescence released in the supernatant. Given the fact that the GelMA itself is porous, it is unclear from these data whether the removal of the dissolution of the gelatin beads from the scaffold is performed due to the interconnectivity of the pores or the microporosity of the scaffold itself. Additional experiments are required to assess the interconnectivity of the pores, including confocal analysis in order to calculate the estimated number of gelatin beads per void space in GelMA scaffolds. Finally, the GelMA porous scaffolds were characterised for their rheological properties, using frequency and shear sweeps and their stiffness was determined at 400 Pa. This indicates

a physiologically relevant stiffness which corresponds to the stiffness of the lung and breast tissue (**Figure 4**).

An alternative method of gelatin beads fabrication for the introduction of the porous system was also explored, namely by using a microfluidic chip platform. The microfluidics platform design was based on water-in-oil microemulsion and was previously optimised by a former member of the Stevens Group. By using this platform, the fabrication of porcine gelatin beads with higher size was indeed successful, namely the average diameter was determined at 150 μm . However, the gelatin beads fabricated with this method failed to dissolve during scaffold development post incubation at 37 °C. This method was not optimised further for three main reasons: The complicated experimental set up required, the lack of sterile conditions and the time-consuming protocol among them. In more detail, the microfluidics platform required constant supervision for potential blockages within the tubing and the production of gelatin beads after 4 hours of reaction within the microfluidics platform would have been enough for the development of two scaffolds. This was mainly because of two reasons; first the multiple washes required for the removal of the oil phase and because of the stickiness of the gelatin beads at the tip of the pipette during mixing with the GelMA, which resulted in the loss of material. For the above-mentioned reasons although the fabrication of gelatin beads of higher size was successful using the microfluidics platform, many drawbacks were observed during the experimental process and therefore was not suitable for this project.

An alternative option that was explored was the development of porous GelMA scaffolds. The method is fast, since several scaffolds can be printed simultaneously and controlled as the structure can be designed precisely beforehand. The disadvantages of this method include the requirement for high amount of material solution during 3D printing and the lack of maintenance of sterile conditions during 3D printing. However, the latter can be overcome, by incubating printed scaffolds with high amount of antibiotics in order to prevent any contaminations. For this, scaffolds with a grid structure and pore formation ranging from 100-250 μm were designed using Solidworks and 3D printed. Using 10% (w/v) of GelMA solution for 3D printing, scaffolds mechanically collapsed and therefore

concentration of GelMA was increased to 20% (w/v). MicroCT scanning of printed scaffolds with 250 μm pore size failed to show any pore formation indicating that further optimisation was required in order to achieve precise pores. Interestingly, the mechanical properties of the 3D printed scaffolds highlight a stiffness of 4,000 Pa, 10 times higher than the GelMA casted scaffolds. This can be explained by the increase in GelMA concentration for the 3D printing solution from 10% (w/v) to 20 (w/v). Additionally, GelMA casted scaffolds had a successful porosity by the use of gelatin beads, in contrast with the 3D printed scaffolds where porosity was not achieved during 3D printing.

Based on the results of this chapter, this study was proceeded using the GelMA casted scaffolds with porcine gelatin beads fabricated with the emulsification method, since using this method, pore formation was successful.

Chapter 3. GelMA scaffolds as an *in vivo* artificial tissue microenvironment

3.1 Introduction

The biocompatibility of GelMA has been extensively characterised, as it provides cells with an optimal biological environment, *e.g.* RGD motifs for cell attachment (Liu and Chan-Park, 2010; Zhu *et al.*, 2019) as well as target sequences for MMPs that are required for cell remodelling (Steen *et al.*, 2002). Therefore, GelMA hydrogels offer a versatile toolkit for 3D cell culture for studying cell-matrix and cell-cell interactions, cell proliferation (Dubruel *et al.*, 2007), migration (Nichol *et al.*, 2010) and differentiation (Chen *et al.*, 2012). GelMA has also been used to study cancer cell interactions *in vivo*. The majority of studies using GelMA rely on direct cancer seed seeding within the hydrogel scaffolds (Loessner *et al.*, 2016), rather than spontaneous dissemination of cancer cells from the primary tumour to the scaffold.

When GelMA scaffolds are implanted *in vivo*, they generally trigger a mild immune response in the early stages, which is a normal physiological reaction to foreign materials. Initially, immune cells like macrophages and neutrophils are recruited to the site of implantation. Studies have shown that GelMA induces a limited inflammatory response, primarily driven by macrophages, with low levels of pro-inflammatory cytokines being expressed (Ullm *et al.*, 2014). This is in contrast to other synthetic materials that may induce a more severe reaction, such as extensive neutrophil activation or the formation of FBGCs.

3.2 Aims & Objectives

In this chapter, it was hypothesised that porous GelMA scaffolds have the ability to interface with the host. As mentioned in Chapter 1, as a semi-synthetic biomaterial, GelMA provides good biocompatibility and therefore it was explored the level of foreign

body response from the host post implantation. A significant part of the study involves optimising methods to analyse cell infiltration into the scaffold post *in vivo* implantation. Flow cytometry analysis and histology were utilized to assess the immune and non-immune cell infiltrate of the scaffold.

Provided that porous GelMA scaffolds have the ability to interface with the host and dependent on the cellular infiltrate of the GelMA scaffold, for the next aim of this project it was investigated whether cancer cells would have the ability to disseminate from a primary tumour to the GelMA scaffold. One of the essential aims is to assess the vascularization within the GelMA scaffold after implantation. Investigating whether functional blood vessels form in the scaffold is crucial for understanding how the scaffold may support tumour metastasis. Blood vessel formation is not only an indicator of the scaffold's ability to integrate with the host but also serves as a potential route for tumour cell dissemination into the scaffold. The analysis includes determining the integrity of these blood vessels, possibly using endothelial markers and staining techniques.

In order to investigate whether cancer cells have the ability to disseminate from a primary tumour to the GelMA scaffold, a breast cancer model was employed. Breast cancer is one of the most well-studied cancers regarding its mechanisms of metastasis. As mentioned in Chapter 1, the dissemination of tumour cells from the primary site to secondary organs, such as the lungs, liver, bones and brain is a hallmark of breast cancer progression. Therefore, a subcutaneous scaffold offers a controlled, accessible environment for investigating how cancer cells might interact with artificial tissues mimicking these distant metastatic niches. Indeed, breast cancer cells, including the MDA-MB-231 (Azarin *et al.*, 2015; Rao *et al.*, 2016; Bushnell *et al.*, 2019) and the 4T1 model (Wang *et al.*, 2023) have been shown to disseminate to implanted bioengineered scaffolds, indicating the suitability of the model. In this project, the PyMT (Polyoma Middle T Antigen) or 4T1 model were used. The PyMT model allows for spontaneous dissemination and mimics the progression of human breast cancer. This model is well-characterised for spontaneous metastasis, which makes it a suitable model to track cancer cell dissemination (Regua *et al.*, 2021).

The capacity for tumour cells to disseminate to the scaffold from a distant primary tumour site is a central milestone of this project. This chapter examines whether cancer cells leave the primary site and colonize the scaffold, through the vascularisation, potentially mimicking how tumour cells colonize distant organs. The presence of tumour cells in the scaffold would support the notion that GelMA can serve as an artificial tissue that the primary tumour can condition, and cancer cells can spontaneously home. Based on the principles of the pre-metastatic niche (Patras *et al.*, 2023), the next aim of this chapter was to investigate whether porous GelMA scaffolds would have the ability to get remodelled from the primary tumour.

Given the work from Wang *et al.* (Wang *et al.*, 2023), which revealed the multifaceted role of neutrophils infiltrating synthetic scaffolds compared to neutrophils present in the lung, this chapter also aims to investigate whether the presence of the scaffold implant has any impact on lung metastasis. In detail, it aims to assess whether GelMA scaffold implantation affects the systemic metastatic burden in the lung of tumour-bearing mice.

3.3 Material & methods

3.3.1 Statistics and reproducibility

All statistical analyses were performed using Prism for Mac (v. 10.2.0, GraphPad Software). P values are indicated in the figures and data were presented as mean $\pm$ SD, unless otherwise indicated. For animal experiments, each mouse is considered as a biologically independent sample.

Normality of the data was tested using the Kolmogorov-Smirnov test subject to $\alpha = 0.05$ before any other statistical test. For normally distributed samples, the statistical significance of the differences between two experimental groups was assessed by two-tailed Student's t-test (paired or unpaired). When assessed statistics of three or more groups, one-way analysis of variance (ANOVA) controlling for multiple comparison using Tukey's test was performed. If any of the comparative groups failed normality (or

if the number was too low to estimate normality), a nonparametric Mann-Whitney test was performed.

Histological quantification of metastatic burden was performed blinded. Other data collections and analysis were not performed blinded due to the conditions of the experiment.

3.3.2 Mouse experiments

All mouse lines used in this study were bred and maintained under specific pathogen-free conditions by the Francis Crick Biological Research Facility (BRF). Only female mice between 6-14 weeks of age were used in all experiments. Breeding and all animal procedures were performed under the Animals (Scientific Procedures) Act 1986 and Animal Welfare Act 2006 at the Francis Crick Biological Research Facility in accordance with the UK Home Office project licenses P83B37B3C and PP5920580. Up to 5 mice were housed in individually ventilated cages with wood chip bedding and nestlets under a 12 h light/dark cycle at 21 ± 2 °C and $55\% \pm 10\%$ humidity. Food and water were provided ad libitum.

Wild type FVB/n from The Jackson Laboratory were provided by the Francis Crick Biological Research Facility. MMTV-PyMT mice (FVB/n background) were provided by The Francis Crick Biological Research Facility. Actin-GFP (FVB/n) mice (Okabe *et al.*, 1997) and RAG1 deficient FVB/n mice (Guy, Cardiff and Muller, 1992) were originally a gift from J. Huelsken laboratory (EPFL, Lausanne, Switzerland). Actin-GFP (FVB/n) and MMTV-PyMT (FVB/n) mice were crossed to generate actin-GFP MMTV-PyMT mice.

3.3.3 Isolation of endothelial progenitor cells (EPCs) from adult mice

The isolation of endothelial progenitor cells from adult mice was followed using an adapted protocol according to Wang *et al.* (Wang *et al.*, 2018). Femurs and tibias from 8-12 weeks of age immunocompetent FVB/n mice were separated, and muscles and

connective tissues were manually removed. The edges of the femurs and tibias were cut, and the bone marrow was flushed using a 30 G needle/syringe with HBSS. Cells were then lysed with Red Blood Cell Lysis Solution (Miltenyi Biotec 130-094-183) and washed twice with DMEM + 10% (v/v) FBS. Cells were then centrifuged and resuspended in EGM-2 media which contained endothelial cell basal medium-2, EGM™-2 MV SingleQuots™ (both Lonza Group, Ltd., Basel, Switzerland), containing 100 U mL⁻¹ penicillin-streptomycin (ThermoFisher, 15070063). Cells were then plated in a fibronectin coated 10 cm culture dish (Sigma-Aldrich). After 4 h the non-adherent cells were transferred in a new fibronectin coated culture dish and cultured with EGM-2 medium. The media was replaced every 3-4 days.

3.3.4 Isolation of MMTV-PyMT primary cells

MMTV-PyMT and actin-GFP MMTV-PyMT primary cells were obtained from tumours growing from transgenic mice. Tumours were dissected from all fat pads of the mice and noticeable necrotic areas were avoided. Tumours were manually minced using scalpel and scissors and then enzymatically digested with Liberase Tm (Roche Diagnostics, 5401127001), Liberase Th (Roche Diagnostics, 5401151001) and Deoxyribonuclease I (Merck Sigma-Aldrich, DN25-100MG) in HBSS solution for 60 minutes at 37 °C with 180 rpm agitation. The digested mixture was then mashed through 100-µm cell strainer (Corning, 431752) to break cell aggregates and washed with 10% (v/v) FBS in HBSS (ThermoFisher Scientific, 14025092) solution. The cells were centrifuged for 5 minutes, at 4 °C with 1250 rpm and supernatant was discarded. Cells were then passed through a 70-µm cell strainer (Corning, 352350) and washed once with HBSS before being plated on collagen coated plates (HBSS, 30 µg/mL PureCol collagen (advanced Biomatrix, 5005), 0.1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, A7906-500G), 20 mM HEPES (ThermoFisher, 35050061) overnight in MEM media (DMEM/F12 containing 2% (v/v) FBS, 100 U mL⁻¹ penicillin-streptomycin (ThermoFisher, 15070063), 20 ng EGF (ThermoFisher, PHF0313), 10 µg mL⁻¹ insulin (Sigma-Aldrich, 19278) and Glutamax™ (ThermoFisher, 3505061). On the next day, the plates were washed with PBS containing 1 mM EDTA and trypsinised (ThermoFisher, 25200056) to generate a single cell suspension. Viable cells were counted using 0.4% (w/w) trypan blue solution

and cryovials contained 3×10^6 or 10×10^6 were prepared so they could be thawed in 10 or 15 cm plates, respectively. MMTV-PyMT cells were always thawed on a collagen-coated plate one day prior to use. Cells were cultured in a humidified incubator at 37 °C and 5% CO₂.

3.3.5 Cancer cell lines and culture conditions

All cell lines were provided by the Cell Services Unit of The Francis Crick Institute where they were routinely screened for mycoplasma and authenticated using short tandem repeat profiling. The MMTV-PyMT-derived breast cancer cell line, TS1 (Shree *et al.*, 2011), was a gift from J. Joyce (University of Lausanne, Ludwig Institute for Cancer Research, Lausanne, Switzerland). TS1 cells were grown in Advanced DMEM/F12 containing 10% FBS, 100 U mL⁻¹ penicillin-streptomycin, 15 mM HEPES and GlutaMAX™. 4T1 GFP cells were grown in DMEM containing 10% (v/v) FBS and 100 U mL⁻¹ penicillin streptomycin. Cells were grown in a humidified incubator at 37 °C and 5% CO₂.

3.3.6 Primary tumour induction

PyMT cancer cells were trypsinised, washed with PBS, and filtered through a 20-µm strainer-capped tube to generate a single-cell suspension. Cells were counted using 0.4% (w/w) trypan blue solution and resuspended in Growth Factor Reduced Matrigel (Scientific Laboratory Supplies, 356231) before mammary fat pad injection (50 µL containing 0.5×10^6 tumour cells per mouse) using a 29 G needle. Mice were anaesthetised with IsoFlo (isoflurane, Abbott Animal Health) and tumour cells were transplanted within the fourth mammary fat pad on one flank only. Tumour growth was monitored (**Figure 14**) and the total superficial tumour burden didn't exceed a diameter of 1.5 cm.

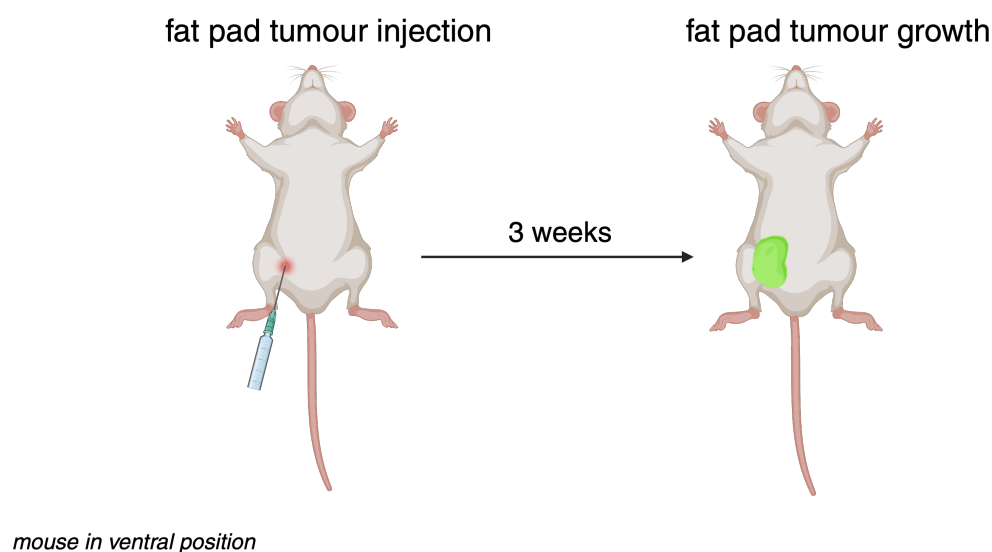


Figure 14. Schematics of primary fat pad tumour injection and tumour growth.

3.3.7 Subcutaneous scaffold implantation

Scaffolds were incubated with 100 U mL^{-1} penicillin-streptomycin in PBS for 3 days at 37°C prior to surgery. Mice were anaesthetised with IsoFlo following with subcutaneous injection of analgesic solution (10 mg/kg Meloxicam, 0.1 mg/kg Buprenorphine in sterile H_2O). Lubrithal eye drops was applied to the eyes of the mice to prevent them from drying. The lower back of the mice was shaved and disinfected with Chlorohexidine using sterile swabs. Animals underwent surgery using aseptic techniques. Two incisions were made using sharp scissors at the back of the mouse and the skin underneath was detached using round scissors to create a pocket. Scaffolds were implanted contralaterally underneath the skin incision on two different sides with at least 1 cm distance between them so that they don't attach (**Figure 15A**). After implantation each cut was sutured using absorbable sutures (6-0 Vicryl, Ethicon) with 3 sutures per cut. Sterile surgical gloves were worn throughout the surgery. Surgical tools were sterilised using a bead steriliser. EMLA numbing cream was applied after surgery at the incision sites and on the following day.

In experiments with primary tumour induction, the implanted scaffold was placed in distance of the mammary fat pad in order not to attach to the growing primary tumour (**Figure 15B**).

Given recent guidelines suggesting the use of both female and male mice, scaffold implantation was performed on both female and male FVB/n mice. Male mice showed signs of aggressiveness post-surgery, resulting in open wounds which required re-stitching. For this reason, only female mice were used for scaffold implantation, where the incisions remained intact.

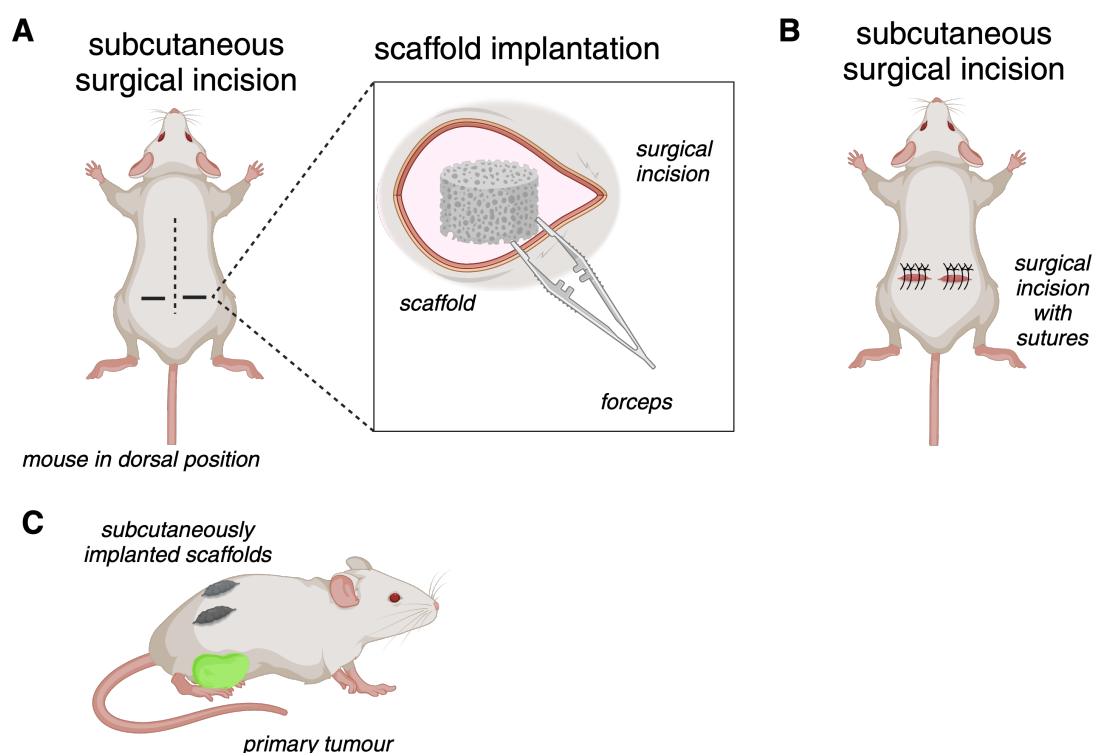


Figure 15. Schematics of subcutaneous scaffold implantation. **A)** Location of the two surgical incisions and scaffold implantation within the incision. **B)** Post surgical incisions with sutures. **C)** Location of scaffold implants in tumour bearing mouse.

3.3.8 Scaffold dissociation for flow cytometry analysis

Scaffolds were cleaned from any leftover residual soft tissue and manually cut into smaller pieces using scalpel and placed in a well of a 24-well plate. Scaffolds were enzymatically digested using 1 mg/mL (w/v) collagenase type I from *Clostridium histolyticum* (ThermoFisher, 17018029) according to previous published work (Peerani,

Candido and Loessner, 2019) in DMEM (ThermoFisher, 41965062) for 35 minutes at 37 °C with 150 rpm agitation. After digestion, 1mL of DMEM + 10% (v/v) FBS was added and then passed through a 20- μ m strainer capped tube to remove any leftover aggregates and generate a single-cell suspension. Cells were then centrifuged at 1250 rpm, 4 °C for 5 minutes. After centrifugation, cells were washed with MACS buffer (0.5% BSA and 2 mM EDTA in PBS) and then stained with fluorescent-conjugated antibodies for flow cytometry analysis.

3.3.9 Fluorescence labelled GelMA synthesis

Cyanine5.5 GelMA (Cy5.5-GelMA) was synthesized by reacting GelMA with sulfo-cyanine5.5 NHS ester (Lumiprobe, 27320). GelMA was fully dissolved in phosphate buffer (pH ~8.1) at 37 °C at a concentration of 10% (w/v), followed by the addition of cyanine5.5 NHS ester (30 mg per 1 g of GelMA). The reaction was run for 3 to 8 hours at 37 °C in the dark and subsequently quenched by adding water (4 volume equivalents). The product was then dialyzed using a dialysis membrane 6-8 kDa (Spectrum Laboratories, 132665) against water at 40°C, with the water changed five times over 5 days. All dialyzed solutions above were freeze-dried and stored at -20 °C before use.

3.3.10 Lung dissociation for flow cytometry analysis

Lungs were dissected and put into a tube containing cold PBS free of calcium and magnesium. Scalpel and scissors were used to mince the lungs, which were then transferred into appropriate tubes for enzymatic digestion with Liberase Tm (Roche Diagnostics, 5401127001), Liberase Th (Roche Diagnostics, 5401151001) and Deoxyribonuclease I (Merck Sigma-Aldrich, DN25-100MG) in HBSS solution for 30 minutes at 37 °C with 180 rpm agitation. The digested mixture was then passed through a 100- μ m strainer, mashed with a plunger to break cell aggregates, washed with 10% FBS HBSS solution and centrifuged for 8 minutes at 300 x g. Samples were then subjected to hypotonic lysis with Red Blood Cell Lysis Solution (Miltenyi Biotec, 130-094-183) for 5 minutes at room temperature for removal of erythrocytes and passed through a 40- μ m strainer. After centrifugation for 8 minutes at 300 $\times$ g, cells were washed with MACS

buffer (0.5% (w/v) BSA and 2 mM EDTA in PBS) and passed through a 20- μ m strainer-capped tube to generate a single-cell suspension. Cells were then stained with fluorescent-conjugated antibodies for flow cytometry analysis.

3.3.11 Scaffold flow cytometry analysis

For surface marker staining, prepared single cell suspensions were incubated with mouse FcR Blocking Reagent (Miltenyi Biotec, 130-092-575) at 1:50 dilution in MACS buffer for 5 minutes at room temperature and then incubated with fluorescently conjugated antibodies (1:200) for 30 minutes at 4 °C in the dark. Zombie NIR™ Fixable Viability Dye (1:300) (Biolegend, 423106) or 4',6-diamidino-2-phenylindole (DAPI) (Merck Sigma-Aldrich, D9542) were used to stain dead cells.

Flow cytometry analyses were performed on a BD LSR-Fortessa (BD Biosciences) and subsequently analysed using FlowJo v10.8.2 (FlowJo, LCC 2006-2018).

3.3.12 Immunohistochemistry of frozen tissue

Scaffolds were cleaned from any leftover residual soft tissue and fixed with 4% (w/v) paraformaldehyde (PFA) (Sigma, 28908) for 24 hours at 4 °C and extensively washed with PBS the following day. Scaffolds were then embedded in cryomolds 15 × 15 × 5 mm (Polysciences, 25375-500) using OCT embedding medium (ThermoFisher, 15212776) and snap frozen using dry ice. Blocks were sectioned for histology into 10 μ m sections using the cryostat (Leica, CM3050S) and adhered to glass slides. Haematoxylin and eosin (H&E) staining was performed as per standard protocols, using an automated tissue stainer (Tissue-Tek Prisma, Sakura). For immunohistochemistry, sections were blocked using endogenous peroxidase 1.65% (v/v) diluted in PBS (13.3 mL: 250 mL PBS) of 30% (v/v) H₂O₂ (Sigma, H1009-500ML) for 10 minutes and the reaction was terminated using dH₂O for 5 minutes. Sections were then blocked with a solution of 1% (w/v) BSA, 10% (v/v) FBS and 0.2% (v/v) Triton-X in PBS for 1 h at room temperature. Next, sections were incubated with primary antibody in a humidified chamber overnight at 4 °C according to the following dilutions. After

3 PBS washes, slides were stained with secondary antibody at a 1:250 dilution for 45 minutes at room temperature in the dark. Slides were washed with PBS 3 times for 3 minutes and incubated with Avidin-Biotin Complex (ABC) kit (Vector, PK-6100) for 30 minutes and washed with PBS 3 times for 3 minutes. Peroxidase substrate (DAB) chromogen (Vector, SK-4100) was then added and macroscopically monitored until sufficient signal strength was observed without high background. Sections were then washed with dH₂O to terminate the development reaction. Last, sections were then counterstained by staining with light haematoxylin for 1 minute, washed in water for 5 minutes to blue haematoxylin. Sections were then dehydrated through 70%, 100% IMS, cleared in xylene and cover slipped with DPX mountant. Stained slides were scanned using Zeiss Axio scan Z1 slide scanner at 20x magnification and images were analysed using Qupath software.

3.3.13 Immunofluorescence of frozen tissue

For immunofluorescence staining sections were thawed at room temperature for 10 minutes, then blocked with a solution of 1% (w/v) BSA, 10% (v/v) FBS and 0.2% (v/v) Triton-X in PBS for 1 h at room temperature. Next, sections were incubated with primary antibodies in a humidified chamber overnight at 4 °C according to the following dilutions. After 3 PBS washes, slides were stained with secondary antibodies at a 1:250 dilution, and DAPI at 1:500 dilution for 45 minutes at room temperature in the dark. Slides were washed with PBS 3 times for 5 minutes. Finally, slides were rinsed in distilled water and mounted using Vectashield Vibrance® antifade mounting medium (Vector Laboratories, H-1700) and #1 Menzel coverslips (Thermo Fisher Scientific, 15737592). Fluorescence images were taken with a Zeiss Upright 710 confocal microscope and further processed using Fiji software (v. 2.9.0/1.53t, ImageJ).

3.3.14 Immunohistochemistry of paraffin tissue

Lungs were dissected, fixed overnight in 10% (v/v) neutral-buffered formalin (NBF) at room temperature, and submitted to a specimen processing machine for dehydration followed by paraffin embedding. Paraffin blocks containing lung tissue were cut into

4 μm sections and adhered to glass slides. For deparaffinization, slides were incubated in xylene and then successively rehydrated in 100%, 95%, 70% ethanol. Slides were then stained with haematoxylin and eosin (H&E), or further processed for immunofluorescence staining.

For immunostaining, heat-mediated antigen retrieval was performed by incubating the slides in citric acid buffer (0.01 M, pH = 6.0) at 95 °C for 20 minutes. Sections were then blocked with a solution of 1% (w/v) BSA, 10% (v/v) FBS and 0.2% (v/v) Triton-X in PBS for 1 h at room temperature. Next, sections were incubated with primary antibodies in a humidified chamber overnight at 4 °C according to the following dilutions. Slides were washed with PBS 3 times for 3 minutes and incubated with Avidin-Biotin Complex (ABC) kit (Vector, PK-6100) for 30 minutes and washed with PBS 3 times for 3 minutes. Peroxidase substrate (DAB) chromogen (Vector, SK-4100) was then added and macroscopically monitored until sufficient signal strength was observed without high background. Sections were then washed with dH₂O to terminate the development reaction. Last, sections were then counterstained by staining with light haematoxylin for 1 minute, washed in water for 5 minutes to blue haematoxylin. Sections were then dehydrated through 70%, 100% IMS, cleared in xylene and cover slipped with DPX mountant.

3.3.15 *In vivo* vascularisation assay

Scaffolds were subcutaneously implanted *in vivo* for 3 weeks. For the assessment of presence of blood vessels within the scaffold, Lycopersicon esculentum (Tomato) Lectin, Texas Red (ThermoFisher, L32482) was diluted at a concentration of 1mg/ mL in PBS and 100 μg /100 μL of solution was intravenously injected according to previous published protocols (Robertson *et al.*, 2015). 15 minutes after injection, scaffolds were harvested and fixed with 4% (w/v) PFA and cryosectioned as previously. Following cryosectioning, sections were stained for DAPI and imaged for Texas Red with FITC filter set, Excitation/Emission: 595/615 nm to assess the presence of blood vessels.

For the assessment of integrity of the vessels, 70 kDa dextran (Texas Red) (Invitrogen, 11540236) was intravenously injected at a concentration of 1 mg/ 100 μL (Tsukada *et al.*,

2021). 15 minutes after injection, scaffolds were harvested and imaged using the IVIS® molecular imaging platform (Perkin Elmer). Imaging and quantification of signals were obtained using the acquisition and analysis software Living Image v. 4.7.3 (Xenogen Corp.). To measure the intensities of the emitted light, the regions of interest (ROI) were drawn over the emitted region of target signal (total p/s/cm²/sr). After imaging scaffolds were fixed with 4% (w/v) PFA and cryosectioned as previously. Following cryosectioning, sections were stained for DAPI and imaged for Texas Red using FITC filter set, Excitation /Emission: 595/615 nm to assess the integrity of blood vessels.

Table 2. Key resources table 2

Reagent	Clone	Source	Catalog Number
Antibodies			
Goat polyclonal anti-GFP (1:100)	Polyclonal	Abcam	Ab6673
Rabbit polyclonal anti-Ki67	Polyclonal	Abcam	Ab15580
Alexa Fluor 488 donkey anti-goat IgG (H+L)	Polyclonal	Invitrogen	A11055
Alexa Fluor 555 donkey anti-rabbit IgG (H+L)	Polyclonal	ThermoFisher	A-31572
Rabbit CD11b (1:250)	EPR1344	Abcam	Ab133357
Rabbit CD3 (1:300)	EPR4517	Abcam	Ab134096
Monoclonal mouse actin smooth muscle (1:300)	1A4	Agilent Dako	M0851
Rabbit Anti-Rat IgG, mouse absorbed (H+L), Biotinylated (1:250)	Polyclonal	Vector Laboratories	BA-4001
Anti-Endomucin	V.7C7	Santa Cruz	Sc-65495
Goat anti-Rat IgG (H+L), HRP (1:275)	Polyclonal	ThermoFisher	31471
CD45-BUV395	30-F11	BD-Horizon	564279

CD31-PE	390	eBioscience	12-0311-82
CD326-BV711	G8.8	Biolegend	118233
F4/80-eFluor647	F4/80	Invitrogen	MF48021
CD11b-FITC	M1/70	Biolegend	101206
CD31-FITC	390	Biolegend	102405
CD133-APC	13A4	eBioscience	17-1331-81
CD309-PE (VEGFR-2)	Avas12a1	eBioscience	12-5821-82

Softwares

FlowJo software v.10.8.2	BD Life Sciences	https://www.flowjo.com
GraphPad Prism	Graphpad	https://www.graphpad.com
Imaris v.9.5.1	Oxford Instruments	https://imaris.oxinst.com
Solidworks	Dassault Systemes	https://www.solidworks.com
ImageJ	Open source	https://imagej.net/ij/
Biorender	Biorender	https://app.biorender.com

Chemicals

Sulfo-cyanine5.5 NHS ester	Lumiprobe	27320
Liberase TM	Riche	5401127001
Liberase TH	Roche	5401151001
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	DN25-100MG
BSA	Sigma-Aldrich	A7906-500G
PureCol collagen	Advanced Biomatrix	5005
HBSS	ThermoFisher	14025092
Fetal bovine serum	Labtech	FB-1001/100-500
Trypsin-EDTA (0.25%), phenol red	ThermoFisher	25200056
DMEM	ThermoFisher	41965062
DMEM/F12	ThermoFisher	11330032

GlutaMAX Supplement	ThermoFisher	35050061
Insulin solution human	Sigma-Aldrich	19278
Red Blood Cell Lysis Solution	Miltenyi Biotec	130-094-183
Zombie NIR™ Fixable Viability Dye	Biolegend	433106
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542
Growth factor reduced (GFR) matrigel	Corning	356231
Collagenase type I	ThermoFisher	170180029
16% Paraformaldehyde	Sigma-Aldrich	28908
OCT embedding medium	ThermoFisher	15212776
30% Endogenous peroxidase	Sigma-Aldrich	H1009-500ML
Avidin-Biotin Complex (ABC)	Vector Laboratories	PK-6100
Peroxidase substrate	Vector Laboratories	SK-4100
Vectashield Vibrance® antifade mounting medium	Vector Laboratories	H-1700
70 kDa Dextran (Texas Red)	Invitrogen	11540236
Lycopersicon esculentum (Tomato) Lectin (Texas Red)	ThermoFisher	L32482

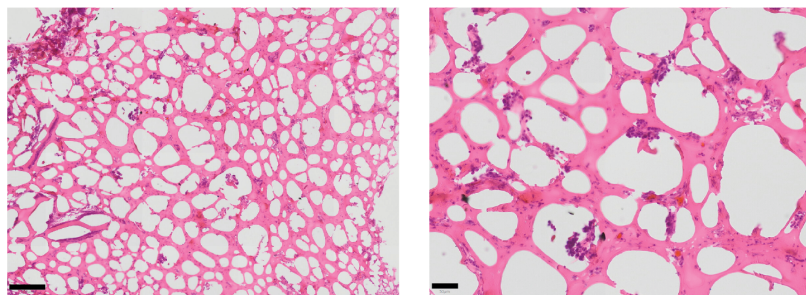
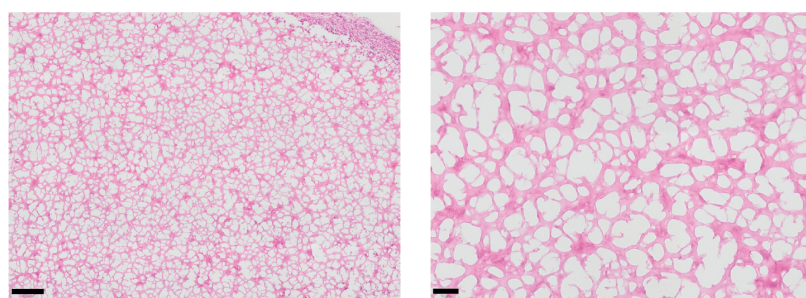
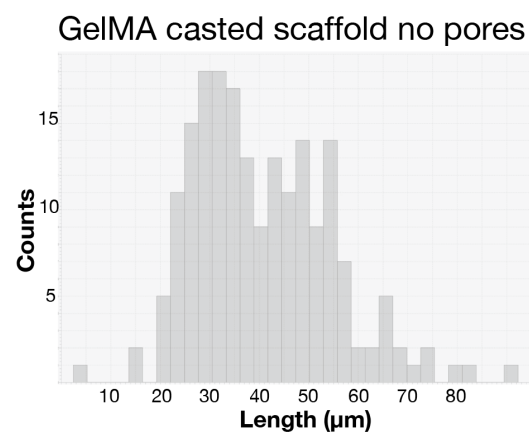
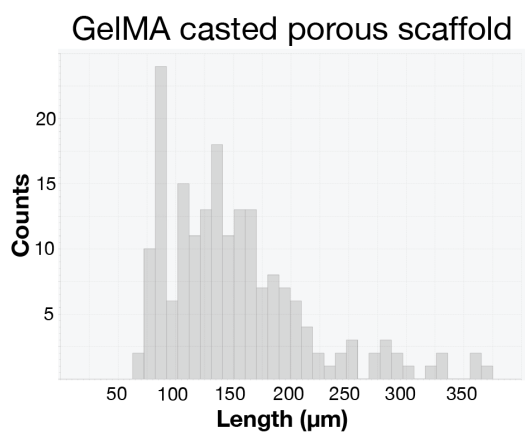
3.4 Results

3.4.1 *In vivo* biocompatibility of porous GelMA scaffolds.

Considering the results obtained in Chapter 1, the first aim was to check whether GelMA casted scaffolds have the ability to interface with the host *in vivo*. Specifically, the first step was to investigate the biocompatibility of GelMA casted scaffolds with and without pore formation, in order to assess whether pore formation induces different cellular responses. GelMA casted scaffolds were prepared as mentioned in previous chapter, namely by mixing 2:1 (v/v) ratio of GelMA:gelatin beads (prepared with the emulsification method) and UV photo-crosslinked for 5 minutes. For scaffolds without any added porogens, pure GelMA without any gelatin beads was used for UV photo-crosslinking. GelMA scaffolds with and without pores were cultured in PBS + 1% (v/v) P/S for 5 days and media was refreshed every day. Following this, scaffolds were implanted subcutaneously at the flank of immunocompetent FVB/n mice under sterile conditions. Mice were anaesthetised and analgesia (10 mg/kg Metacam + 0.1 mg/kg buprenorphine) was injected subcutaneously prior to the surgery. Two different incisions were made at the right and left flank and scaffolds were implanted underneath the skin and the cut was closed using absorbable sutures. The two incisions were made with 3 cm distance from each other, in order to prevent the scaffolds from attaching to each other, due to their stickiness. Scaffolds were left implanted *in vivo* for 3 weeks in order to: i) ensure the potential cell infiltration would be stabilised over time and ii) to check whether the scaffolds would degrade over time.

GelMA scaffolds with and without pores were implanted in the same animals, on the right and left flank respectively to reduce variability between samples. Scaffolds were harvested after 3 weeks of implantation were cleaned from any residual surrounding tissue. Noticeably, all scaffolds remained intact with no obvious degradation over time. Scaffolds were fixed with 4% (w/v) PFA overnight and extensively washed with PBS the following day. Scaffolds were embedded using two methods: 1) paraffin embedding or 2) embedded with OCT embedding medium and snap frozen for cryosectioning. The two methods were compared in order to optimise the best method to be used for immunostainings while retaining the scaffold integrity. Paraffin embedding mechanically

damaged the scaffolds, which then failed to retain their size and were shrunk, losing their original shape. Paraffin-embedding techniques require dehydration of hydrogels, resulting in substantial collapse and deformation. In contrast, freeze-drying and cryosectioning of GelMA scaffolds retained their structure. After cryosectioning, sections were stained for haematoxylin and eosin (H&E), a typical staining where haematoxylin stains cell nuclei in purple and eosin stains the extracellular matrix and cytoplasm in pink. As seen in **Figure 16**, porous GelMA scaffolds have a uniform cell infiltration throughout the scaffold section (**Figure 16A, left**) and especially in the middle of the section (**Figure 16A, right**). In contrast, H&E from GelMA scaffolds without pore formation failed to show any cell infiltration (**Figure 16B**), only a thin layer on the outer part of the scaffold (**Figure 16B, left**) which corresponds to the connective tissue surrounding the scaffold after harvest. Additionally, comparison of the two conditions shows that GelMA scaffolds without pores have a denser structure compared to GelMA scaffolds with pore formation, indicating that gelatin beads form wide porous structures within the scaffold. Quantification of the pores from three different sections of GelMA casted scaffolds with gelatin beads showed a mean diameter of 152 μm (**Figure 16C**). In contrast, GelMA scaffolds without gelatin beads as a porogen exhibited microporosity with 40 μm as mean diameter. It is important to note that GelMA itself presents microporosity after freeze-drying, which would be a possible explanation for the porosity of GelMA scaffolds without gelatin beads. In the work of Chen *et al.*, GelMA hydrogels were synthesised with different DoF (49.8, 63.8 and 73.2%) using 1,5 and 10M MAA respectively. The average pore size of the resulting GelMA hydrogels was 50 (49.8%), 30 (63.8%), and 25 μm (73.2%), as characterised by SEM after freeze-drying (Chen *et al.*, 2012). Another factor that should be considered is the duration of *in vivo* implantation of the scaffolds, which leads to some level of degradation and remodelling of the scaffold. Interestingly, it is observed that cells within the porous scaffold are present not only inside the pores, but also throughout the GelMA structure, indicating the successful cell attachment and infiltration within the porous GelMA scaffolds.

A**GelMA casted porous scaffold****B****GelMA casted scaffold no pores**Scale bar 200 μm (left) 50 μm (right)**C****Pore Diameter Quantification**

Diameter of pores	GelMA with gelatin beads	GelMA no gelatin beads
Count	190	194
Min	62.829	2.442
Mean	151.484	40.722
Max	375.11	92.304
Std Dev	62.946	13.952

Figure 16. Histological analysis of GelMA casted scaffolds with and without pore formation. Haematoxylin and eosin (H&E) staining of **A)** GelMA casted scaffold with pores **B)** GelMA only no pores. Scale bar 200 μm (left), 50 μm (right). **C)** Histogram of the pores from three different sections from GelMA scaffolds with gelatin beads and without indicating their diameter in μm (top) and details of total count of pores, minimum, mean and maximum diameter as well as standard deviation from pore diameter quantification (bottom).

3.4.2 Effect of endothelial progenitor cell (EPC) seeding in the biocompatibility of porous GelMA scaffolds *in vivo*.

Following the observation that porous GelMA scaffolds have the ability to become infiltrated with cells *in vivo*, it was investigated whether pre-seeding endothelial progenitor cells within the GelMA scaffolds would increase the infiltration or form functional vessels within the scaffold. For this experiment, endothelial progenitor cells (EPCs) from adult mice were successfully isolated and cultured *in vitro*, according to previously published protocols from Wang *et al* (Wang *et al.*, 2018). EPCs are precursors of endothelial cells and can mature into cells that line the lumen of blood vessels. In brief, bone marrow cells were flushed from the tibia and femur of adult GFP⁺ mice and cultured in a fibronectin coated culture dish. After 4 hours of incubation, floating cells were transferred to new coated plates and cultured with EGM-2 complete medium. The morphology of the cells after 7 days of culture was similar to the published protocol (**Figure 17A**). After 7 days, cells were analysed with flow cytometry for the following surface markers; vascular endothelial growth factor receptor 2 (VEGFR2), prominin-1 (CD133) and endothelial cell adhesion molecule (PECAM)-1 (CD31), typical endothelial markers for cells potent for endothelial vascular growth (**Figure 17B**). Flow cytometry analysis was performed on different biological samples from two different experiments (**Figure 17C**).

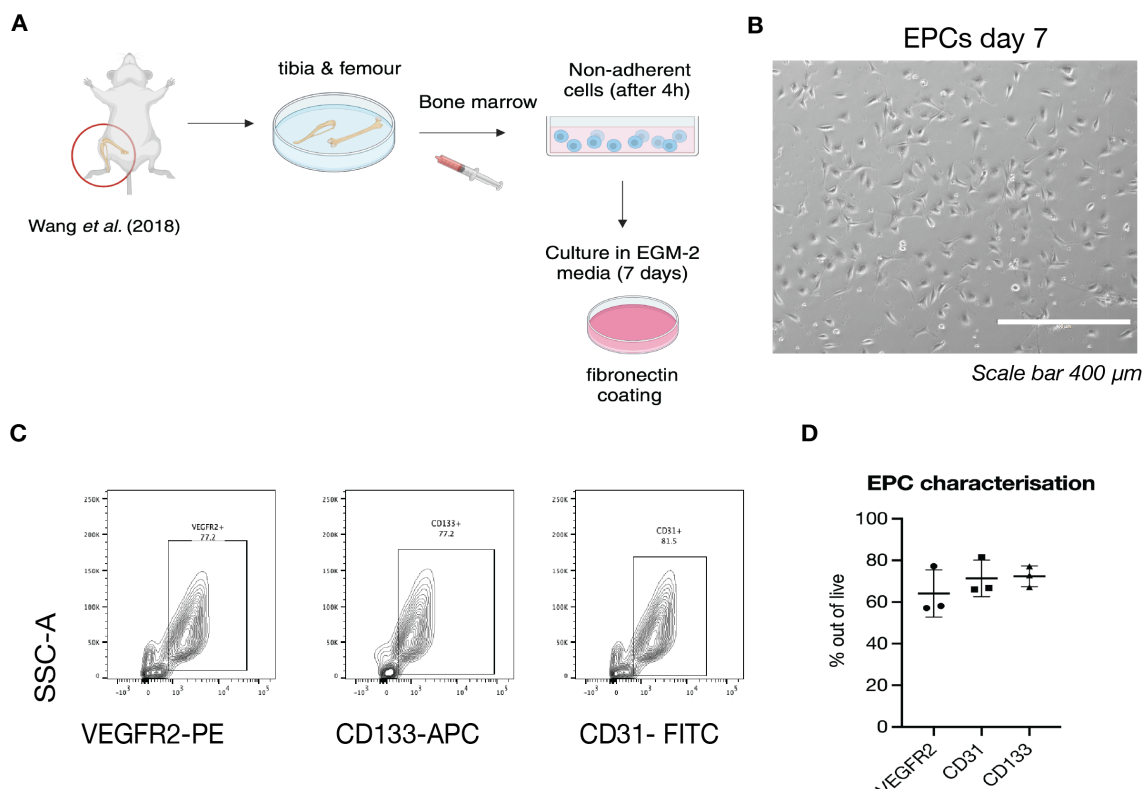


Figure 17. Endothelial progenitor cell (EPC) isolation and flow cytometry characterisation. **A)** Schematics of the protocol for isolation of endothelial progenitor cells (EPCs) based on the protocol from Wang *et al.* **B)** Imaging of isolated endothelial progenitor cells (EPCs) from tibia and femur of GFP⁺ mice and cultured in complete EGM-2 media for 7 days. Scale bar 400 μ m. **C)** Flow cytometry analysis of isolated EPCs after 7 days of culture. **D)** Quantification of markers (VEGFR2, CD31 and CD133) from flow cytometry analysis of cultured EPCs at day 7. Data shown as mean $\pm$ S.D. (n = 3).

Cells were first isolated from GFP⁺ immunocompetent mice and cultured as before, and GelMA scaffolds were prepared and incubated at 37 °C for 5 days in order to induce porosity. On the day of the seeding, scaffolds were coated with fibronectin and 200,000 EPCs were injected within the scaffold and left to attach overnight (**Figure 18A**). The day after, scaffolds were imaged for GFP and EPCs were shown to be successfully attached to the scaffolds (**Figure 18B**). GelMA scaffolds with and without pre-seeded EPCs were implanted in immunocompetent mice for 3 weeks and were then harvested and processed for histology. H&E staining of harvested scaffolds showed that in both conditions, GelMA scaffolds were infiltrated by cells (**Figure 18C**). Quantification of the number of cells infiltrating the scaffolds was performed using QuPath software. Given the interconnected structure of GelMA, the area of GelMA was drawn in order to exclude

the porous area, as seen in **Figure 18D**. The total area in μm^2 was quantified (**Figure 18E**) as well as the total number of cells within the total area. Quantification of the total amount of cells per 1000 μm^2 showed that pre-seeding scaffolds with EPCs did not increase the infiltration of the GelMA scaffolds *in vivo* compared to GelMA scaffold without prior EPC cell seeding (**Figure 18F**).

GelMA scaffolds with pre-seeded EPCs were negative for GFP following immunofluorescence staining (data not shown) indicating that pre-seeded EPCs either did not survive throughout the *in vivo* engraftment in order to form functional blood vessels or the number of cells seeded is not enough to induce this level of endothelial vascular growth. Given the fact that GelMA scaffolds without pre-seeding EPCs can successfully become infiltrated *in vivo*, the next experiments were proceeded without pre-seeding of cells in order to simplify the experimental procedure and reduce animal use.

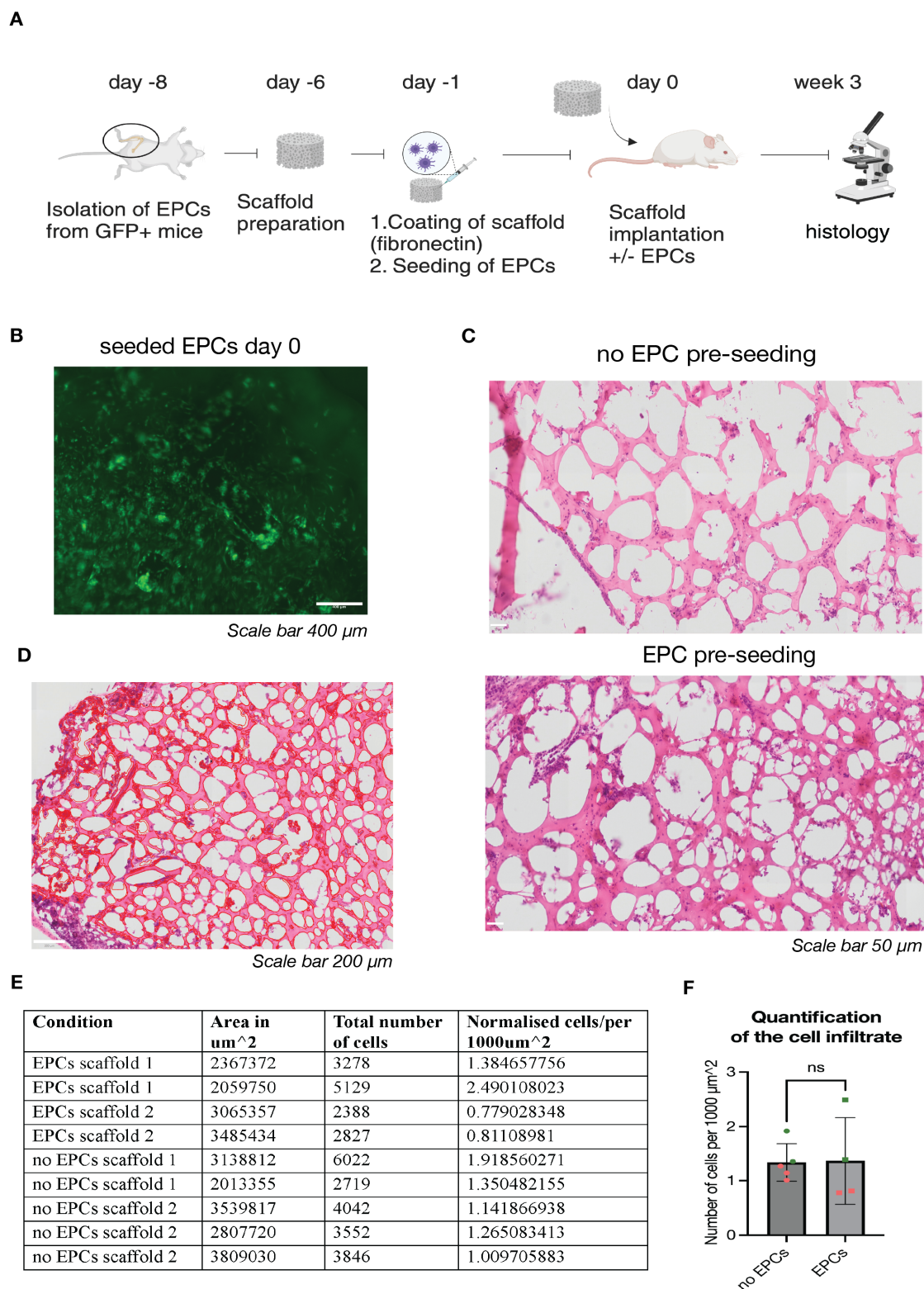


Figure 18. Endothelial progenitor cell (EPC) seeding in porous GelMA scaffolds. A) Experimental approach indicating the key steps for isolation of EPCs, seeding to GelMA scaffolds and implantation of seeded scaffolds *in vivo*. **B)** Imaging of GFP⁺ EPCs within GelMA scaffolds 1 day post seeding. Scale bar 400 μm . **C)** Haematoxylin and eosin (H&E) staining of scaffolds without seeded EPCs (left) and seeded EPCs (right). Scale bar 50 μm . **D)** Screenshot of the total

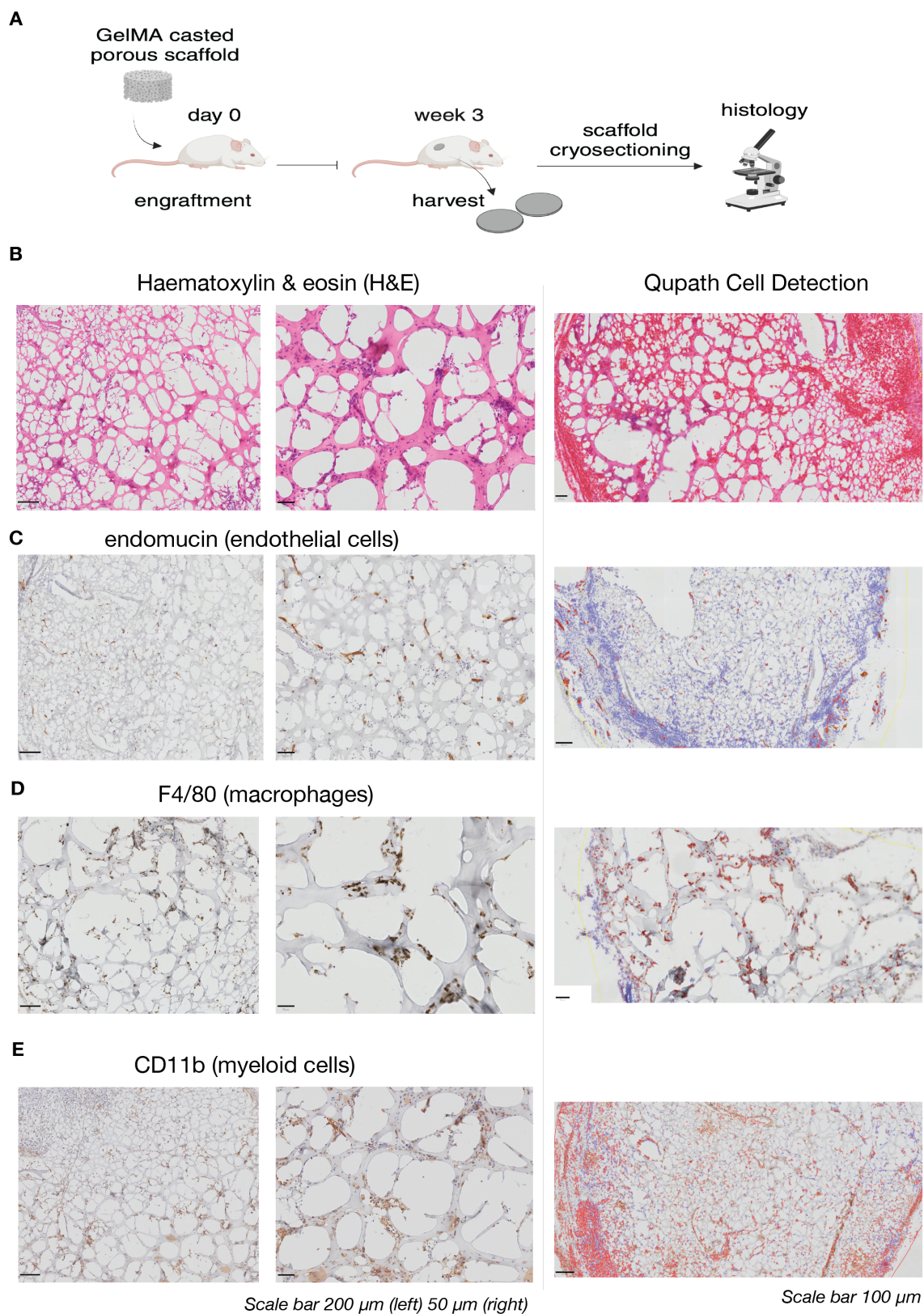
quantification of the area in μm^2 per section using QuPath software. Scale bar 200 μm . **E)** Table of analysed data from scaffolds pre-seeded with EPCs or without EPCs showing the total area in μm^2 the total number of cells per section and the number of cells per 1000 μm^2 . **F)** Quantification of the cell infiltrate per section from scaffolds pre-seeded with EPCs or without EPCs. Colour highlights the different biological samples. Data shown as mean $\pm$ S.D. ($n = 5$ no EPCs, $n = 4$ EPCs). Statistical analysis performed using unpaired t-test.

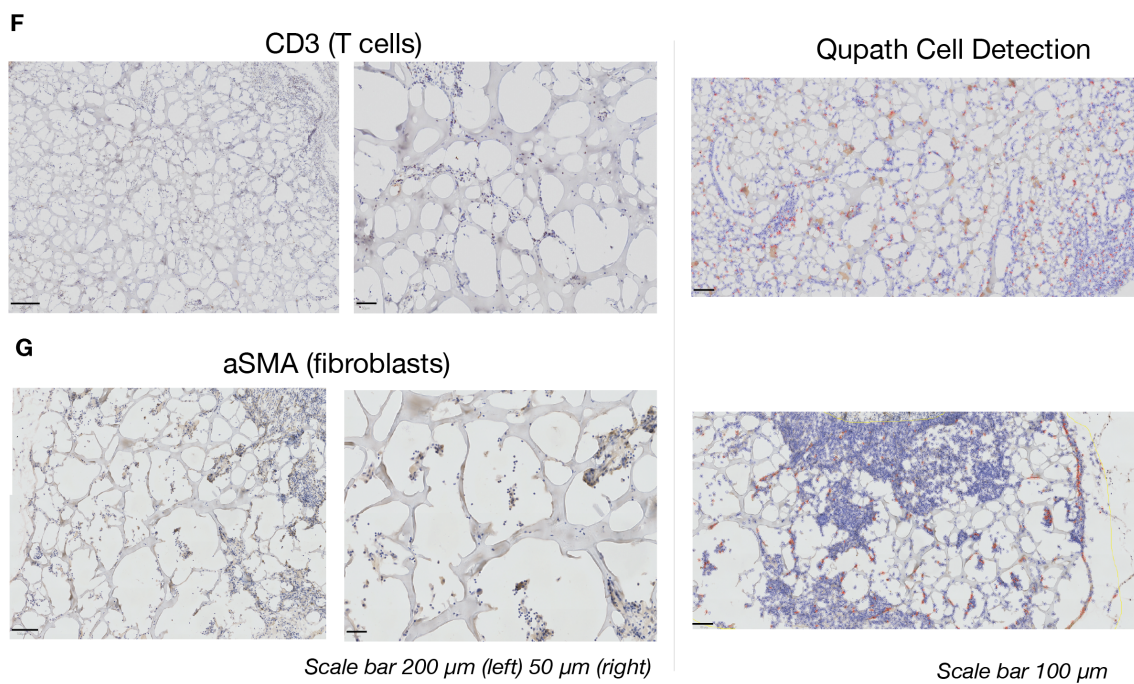
3.4.3 Characterisation of the cell infiltrate from porous GelMA scaffolds from immunocompetent mice.

Next, it was hypothesised that GelMA would have the ability to interface with the host, and the level of biocompatibility *in vivo* would differ from a synthetic biomaterial. To examine this, the next aim was to investigate the types of cells infiltrating the GelMA scaffolds. The same experimental procedure was followed, namely porous GelMA casted scaffolds were implanted *in vivo* in immunocompetent (FVB/n) mice and left for 3 weeks to ensure cell infiltration. Scaffolds were harvested after 3 weeks and processed for cryosectioning and histology staining (**Figure 19A**). Samples were stained for H&E (**Figure 19B**), endomucin as a marker for the presence of endothelial cells (**Figure 19C**), F4/80 as a marker for tissue-resident macrophages (**Figure 19D**), CD11b as a marker for myeloid cells (**Figure 19E**), CD3 as a marker for T cells (**Figure 19F**) and α SMA as a marker for fibroblasts (**Figure 19G**). The stainings and choice of antibodies were optimised for the GelMA scaffolds in order to achieve strong signal without high background.

Quantification of the stainings was performed by positive cell detection using Qupath software, as seen on the right column of the figure for every staining (positive cells were detected in red, negative cells were detected in blue). Detailed analysis of the total number of positive and negative cells stained as well as the total amount of cells and the percentage of positive cells per section is provided in **Figure 19H** for all the stainings performed. As seen in **Figure 19H**, the highest percentage of cells were stained for CD11b and F4/80, with the percentages varying from 48.6-71.6% for CD11b and 46-55% for F4/80 across different scaffolds. CD11b, as a marker of macrophages and other myeloid cells, indicates that macrophages or monocytes are recruited to the site of implantation. Macrophages are key mediators in the body's response to foreign materials, often playing a central role in both inflammation and tissue remodelling. This suggests

an active immune response around the implant. The presence of F4/80⁺ cells suggests the recruitment or differentiation of macrophages at the implant site, which could be involved in phagocytosis, orchestrating the immune response, or leading tissue repair. A significant presence of macrophages can be a sign of an ongoing foreign body response (FBR) to the implant. An average of 9-15% were stained for CD3, indicating the presence of T cells within the scaffolds, suggesting the activation of the adaptive immune response within the scaffold. Interestingly, the presence of endothelial cells and fibroblasts were also detected within the scaffolds, ranging at 12% and 4-10% respectively. The presence of α SMA⁺ fibroblasts suggests that a fibrotic capsule might be forming around the scaffold, one of the main principles of the foreign body response. Additionally, H&E staining showed the presence of cross sections of vessels present in the scaffolds, indicating that endothelial cells could form functional blood vessels within the scaffold. Collectively, these data show that different cell populations are infiltrating the scaffold at different rates.



**H**

Staining	Number of positive cells	Number of negative cells	Total number of cells	% of positive cells
CD11b scaffold 1	2452	1919	4371	56
CD11b scaffold 2	2781	1099	3880	71.6
CD11b scaffold 3	25047	12234	37281	67.1
CD11b scaffold 4	13175	13923	27098	48.6
CD3 scaffold 1	3873	21464	25337	15
CD3 scaffold 2	4176	40756	44932	9.2
CD3 scaffold 3	3478	18977	22455	15.4
CD3 scaffold 4	1336	10243	11579	11.5
Endomucin scaffold 1	2773	20292	23065	12
Endomucin scaffold 2	1362	9549	10911	12.5
Endomucin scaffold 3	3059	20478	23537	12.9
Endomucin scaffold 4	3066	19261	22327	13.7
F4/80 scaffold 1	4316	4312	8628	50
F4/80 scaffold 2	2628	2150	4778	55
F4/80 scaffold 3	2932	3449	6381	45.9
aSMA scaffold 1	446	10478	10924	4
aSMA scaffold 2	1221	11411	12632	9.6
aSMA scaffold 3	681	11279	11960	5.6

Figure 19. Histological analysis of porous GelMA casted scaffolds post *in vivo* engraftment. **A)** Illustration of experimental design for the *in vivo* engraftment of porous GelMA casted scaffolds and analysis by flow cytometry and histology post 3 weeks of implantation. **B)** Haematoxylin and eosin (H&E) staining of harvested porous GelMA scaffolds. Immunohistochemistry staining for **C)** endomucin for endothelial cells, **D)** F4/80 for

macrophages, **E**) CD11b for myeloid cells, **F**) CD3 for T cells and **G**) aSMA for staining of fibroblasts. Scale bar 200 μm (left columns) and 50 μm (middle columns). Right column indicates the quantification performed for positive (in red) and negative (in blue) cell detection using Qupath analysis. **H**) Table with quantification of stainings performed in Figures 19C-G, showing the number of positive and negative cells stained, the total number of cells and the % of positive cells detected per section.

Given the observations of the cellular infiltrate characterised by immunohistochemistry, the next aim was to optimise the cell infiltrate using flow cytometry. In order to investigate this, the same process was followed as previously described, namely porous GelMA casted scaffolds were engrafted in immunocompetent (FVB/n) mice for 3 weeks and then harvested (**Figure 20A**). Samples were then digested in order to generate a single cell suspension. Previous work on optimisation of enzymatic approaches for the degradation of cell containing GelMA scaffolds showed that collagenase type I from *Clostridium Histolyticum* showed the best viability from cells recovered (Peerani, Candido and Loessner, 2019). Therefore, after harvest, GelMA scaffolds were placed in a 24 well plate and digested with 1 mg/mL of collagenase type I for 45 minutes at 150 rpm. After 45 minutes, GelMA scaffolds were completely dissolved, and collagenase was quenched using complete media. After this, samples were processed according to the methods described in section 3.3.5. Flow cytometry analysis of the cells infiltrating the scaffolds showed high cell viability (~80%), with 60% of total live cells positive for CD45, indicating that both immune cells and non-immune cells were infiltrating the scaffolds. Noticeably, CD31⁺ cells were also present within the GelMA scaffolds (**Figure 20B**). Harvest of scaffolds at different timepoints (week 1, week 2 and week 3) showed a gradual increase of CD11b⁺F4/80⁺ macrophages at week 3 compared to previous timepoints week 1 and week 2 (**Figure 20C, 20D**). The gradual increase of CD11b⁺F4/80⁺ macrophages in the GelMA scaffolds at week 3 compared to earlier timepoints (week 1 and week 2) is likely linked to the different phases of the FBR. Upon implantation of the scaffold, early infiltration of inflammatory cells such as neutrophils and monocytes would occur, whereas after week 2 the host response to the scaffold would transition to a more chronic inflammation, with the recruitment of macrophages in higher numbers or the differentiation of the monocytes to tissue-resident macrophages. At week 3, macrophage infiltration would reach a peak, with their increased presence reflecting their potential involvement in foreign body giant cell formation, ECM remodelling and fibrosis.

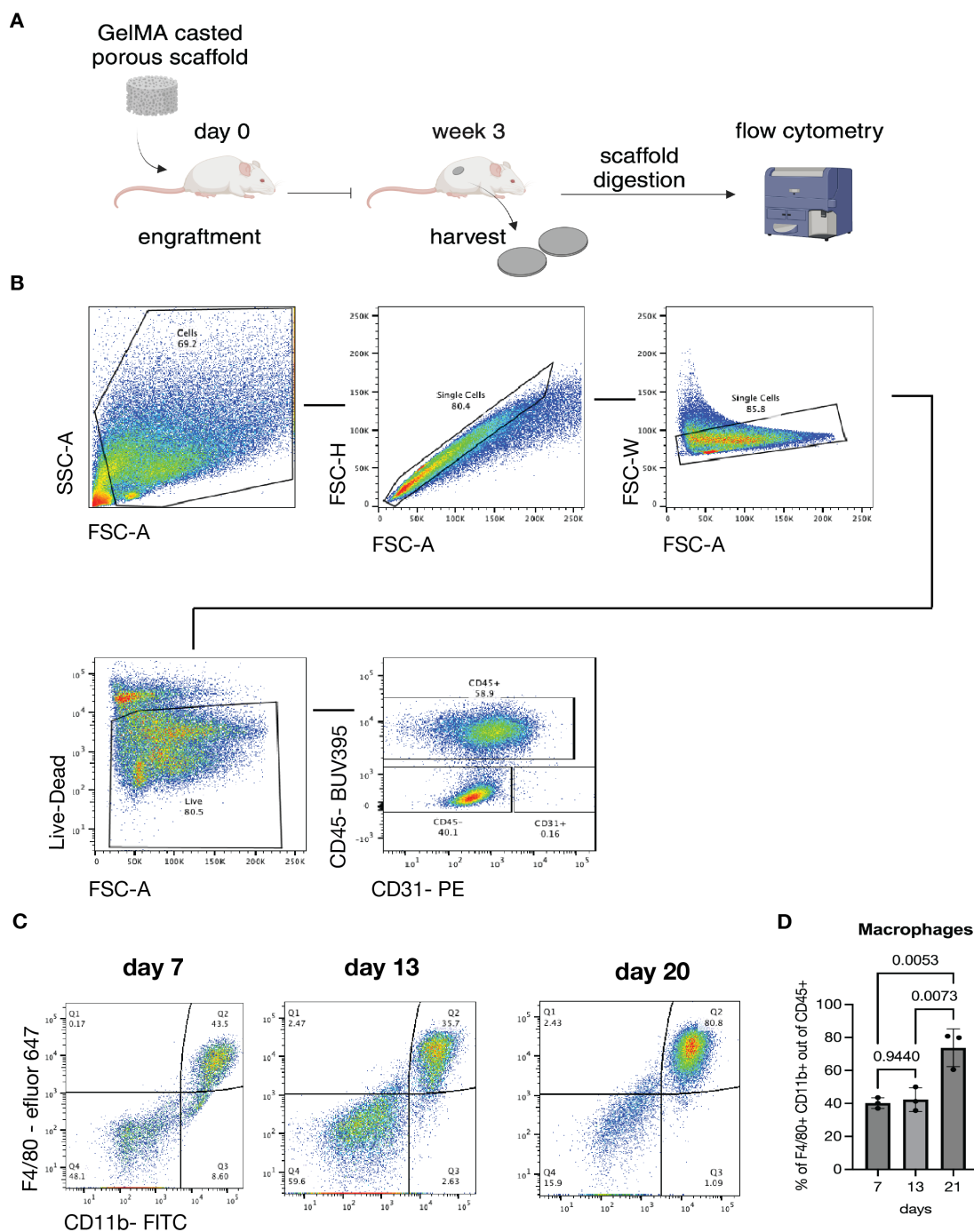


Figure 20. Flow cytometry analysis of porous GelMA casted scaffolds post *in vivo* engraftment. **A)** Illustration of experimental approach indicating the key steps for flow cytometry analysis of harvested GelMA scaffolds. **B)** Flow cytometric gating strategy of cells isolated from GelMA porous scaffolds. **C)** Representative flow cytometry plots of CD11b⁺F4/80⁺ cells gated out of CD45⁺ immune cell population of scaffolds harvested at different timepoints (day 7, day 14 and day 21). **D)** Quantification of CD11b⁺F4/80⁺ cells at days 7, 14 and 21. Data shown as mean $\pm$ S.D. (n = 3). Statistical analysis performed using Tukey's test for multiple comparisons.

CD11b⁺F4/80⁺ macrophages that infiltrate the scaffold are typically tissue-resident macrophages, specifically from the monocyte-derived macrophage lineage, which play a crucial role in the immune response to foreign materials. These cells are often associated with inflammation and tissue repair and are key players in the foreign body response (FBR). Although this flow cytometry analysis focused on staining for immune cells (CD45), tissue-resident macrophages (CD11b⁺F4/80⁺) and endothelial cells (CD31⁺), a full flow cytometry analysis to assess all the spectrum of the cellular infiltrate from the innate and adaptive immune response would be required for better characterisation of the scaffold. Detailed characterisation of the types of macrophages infiltrating the scaffold would be crucial to understand whether these cells are pro-inflammatory, by staining for inducible nitric oxide synthase (iNOS) or anti-inflammatory, by staining for CD206 and CD163. Additionally, cytokine analysis from the extracted scaffolds would be important in order to assess whether these macrophages are secreting pro-inflammatory cytokines (IL-1, IL-6, IL-8 and TNF- α) or anti-inflammatory cytokines such as IL-4, IL-10 and TGF- β (Antmen, Vrana and Hasirci, 2021). This would be essential in order to assess the type of macrophages present in the scaffold.

3.4.4 Characterisation of blood vessel formation within porous GelMA scaffolds.

Given the presence of endothelial cells within the GelMA scaffolds characterised with immunohistochemistry (**Figure 19C**) and flow cytometry (**Figure 20B**), it was questioned whether CD31⁺ endothelial cells have the ability to form blood vessels within the scaffolds. Previous work has shown that intravascular injection of fluorescent tomato lectin successfully labelled vascular elements *in vivo* (Tsukada *et al.*, 2021). In detail, tomato lectin (from *Lycopersicon esculentum*) is a carbohydrate-binding protein that binds specifically to endothelial cells surfaces and the basement membrane, making it a useful tool for visualisation of blood vessels. By injecting fluorescently labelled tomato lectin into the vascular system of the mouse, it gets circulated through the vasculature, binding to endothelial cells. For this experiment, GelMA scaffolds were implanted *in vivo* for 3 weeks. The day of the harvest, 100 μ g/ 100 μ L of *Lycopersicon esculentum* was intravenously injected and left for 10 minutes in order to reach the vasculature of the mouse. Scaffolds were then harvested and fixed with 4% (w/v) PFA and processed for cryosectioning (**Figure 21A**). Sections were then stained for DAPI for staining the nuclei

of the cells and imaged for tomato lectin. A positive fluorescent signal was observed within the scaffold section, indicating that blood vessels are formed within the scaffolds (**Figure 21B**). Lectin was detectable within sections of 5/5 scaffolds (**Figure 21C**), however further high-resolution imaging and reconstruction of the fluorescently labelled lectin within the scaffold would be required for quantification of the number of vessels formed. Additional co-staining with other vascular markers such as VE-cadherin and α -SMA would also be essential to confirm the maturity of the vessels.

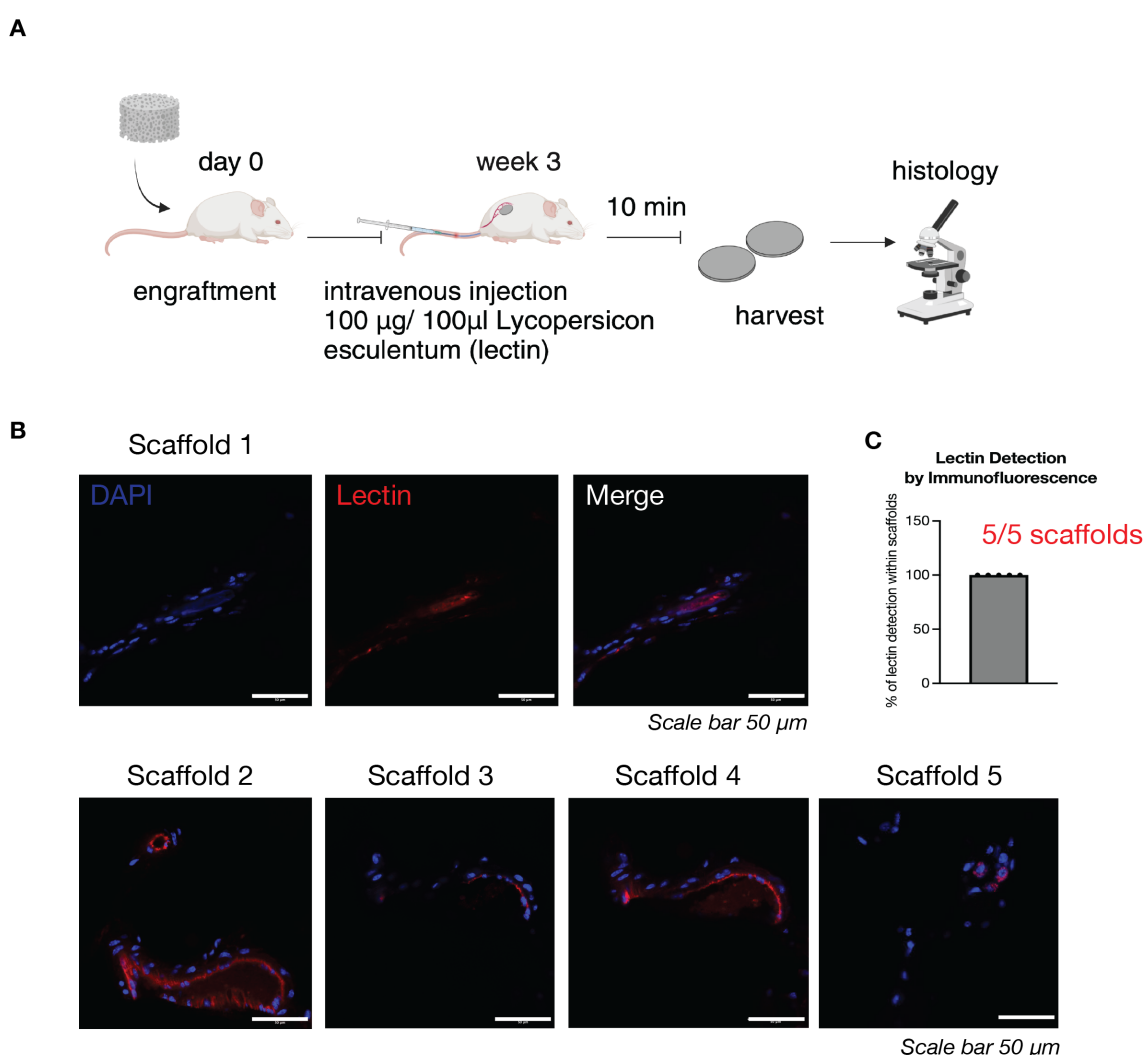


Figure 21. Detection of blood vessels *in vivo* by intravenous injection of lectin. **A)** Illustration for the experimental design of *in vivo* labelling of vessels by intravenous injection of fluorescently labelled lectin before harvest. **B)** Representative immunofluorescence image of labelled blood vessels within GelMA casted scaffolds. Separate channels for DAPI (blue), Texas Red-labelled lectin (red) and composite in top row, immunofluorescent images (merged) from different scaffolds in bottom row. Scale bar 50 µm. **C)** Quantification of the percentage of lectin detection within different scaffolds detected by immunofluorescent staining of Texas Red.

In order to ensure proper blood flow within the scaffold, functional testing of the vessels was required. In order to address this, the functionality of the blood vessels formed within the GelMA scaffolds was investigated using dextran as a vascular permeability tracer (Olsson *et al.*, 1975). For this experiment, the sample experimental procedure was followed as before, 70 kDa Texas Red fluorescent dextran was injected intravenously 10 minutes before the scaffold was harvested (**Figure 22A**). This molecular weight of dextran is ideal for assessing vascular permeability because it is large enough to stay in the vasculature unless there are functional blood vessels or leakage points within the scaffold. After harvesting of the GelMA scaffolds, the presence of dextran was visualised and quantified using fluorescence IVIS imaging. As seen in **Figure 22B**, scaffolds harvested from mice injected with dextran have a strong positive signal (top) compared to non-injected animals (bottom) indicating some level of perfusion of blood vessels within the scaffolds. As a control for this experiment, GelMA scaffolds without gelatin beads as a porogen were implanted. Indeed, quantification of fluorescence signal of dextran by IVIS showed that dextran failed to diffuse within the GelMA scaffolds to the same extent as within the porous GelMA scaffolds (**Figure 22C**), indicating that porosity is required for the formation of the blood vessels. Additionally, immunofluorescence staining of cryosections of GelMA scaffolds confirmed the presence of dextran throughout the scaffold from injected mice, with dextran⁺ cells (**Figure 22D, top**) compared scaffolds from non-injected mice (**Figure 22D, bottom**). This experiment indicates the ability of dextran to diffuse within the porous GelMA scaffolds, however, further experiments for the quantification of the amount of dextran present in the scaffolds would be required to determine the extent of vascular perfusion and functionality. One important aspect to address would be the timepoint of the formation of the blood vessels. In order to address this, scaffolds would need to be harvested at earlier as well as later timepoints in order to gain insight into the stability of the blood vessels formed.

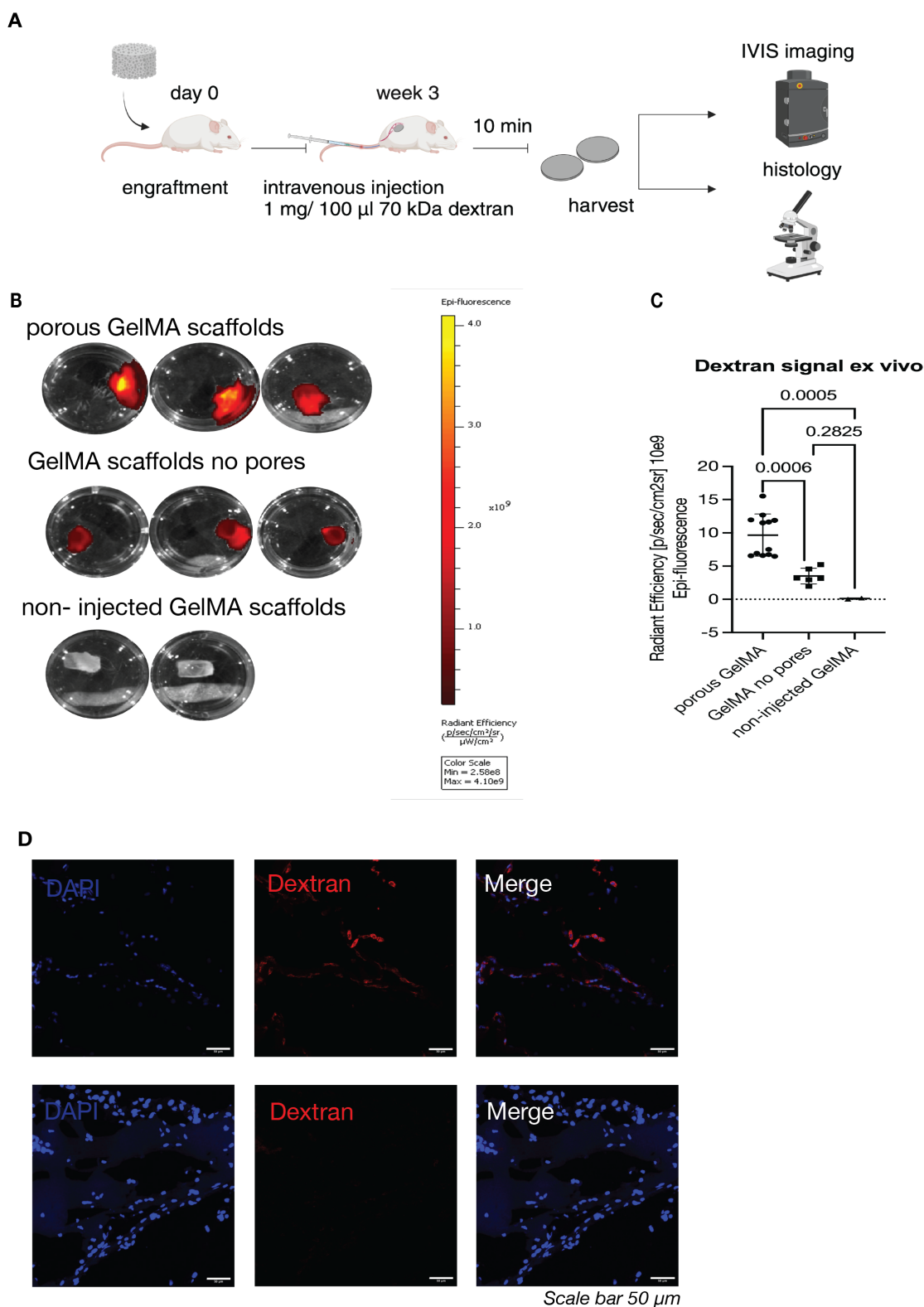


Figure 22. Characterisation of the integrity of blood vessels infiltrating the scaffold by intravenous injection of fluorescently labelled dextran. **A)** Illustration of the experimental design of assessing the integrity of blood vessels *in vivo* by intravenous injection of 70 kDa fluorescent labelled dextran. **B)** *Ex vivo* IVIS imaging of harvested scaffolds indicating the

fluorescence from the dextran (top) compared to scaffolds from non-injected animals. **C)** Quantification of *ex vivo* IVIS signal from extracted porous GelMA scaffolds, non-porous GelMA scaffolds. Non-injected GelMA scaffolds served as a negative control for the background signal. Data presented mean $\pm$ SD ($n = 12$ for porous GelMA scaffolds, $n = 6$ for non-porous GelMA scaffolds, $n = 2$ for non-injected scaffolds). Statistical analysis performed using Tukey's test for multiple comparisons. **D)** Representative immunofluorescence images from labelled blood vessels with GelMA scaffolds from injected animals (top) compared to scaffolds harvested from control (non-injected) animals. Scale bar 50 μm .

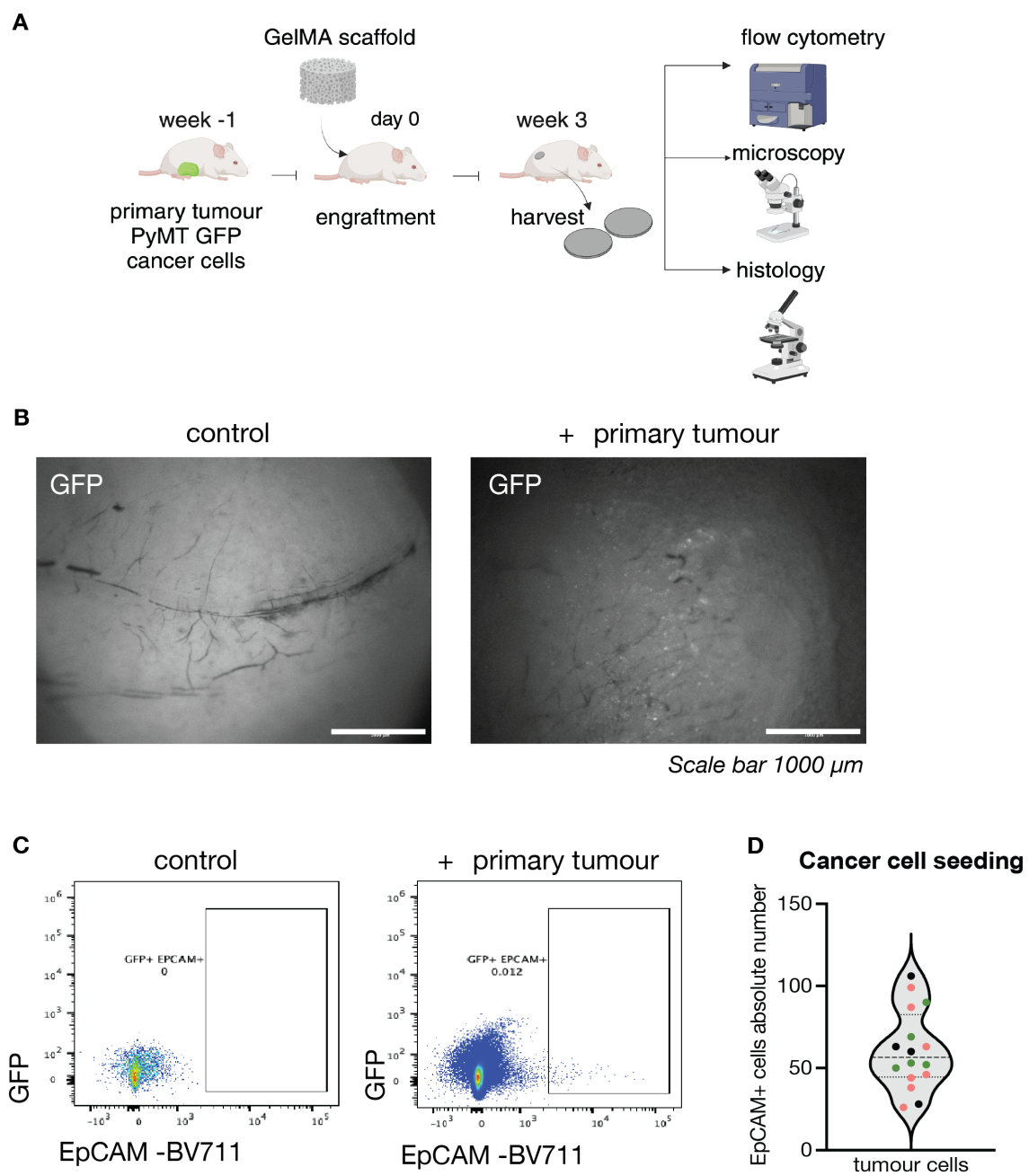
3.4.5 Cancer cells successfully disseminate to porous GelMA scaffolds from primary fat pad tumours.

Given that porous GelMA scaffolds have the ability to interface with the host, the next step was to investigate whether the porous GelMA scaffolds would have the ability to interact with the tumour bearing host. For this, MMTV-PyMT primary breast cancer cells were used, a model that recapitulates human breast cancer progression *in vivo* (Attalla *et al.*, 2021). Actin-GFP (FVB/n) and MMTV-PyMT (FVB/n) mice were crossed to generate actin-GFP MMTV-PyMT mice. In order to use PyMT GFP⁺ cells, Rag1 KO mice (FVB/n background) were used, that produce no mature T and B cells (Mombaerts *et al.*, 1992) in order to avoid PyMT GFP⁺ cancer cells to be rejected due to the expression of GFP. However, since the predominant population of the scaffolds are macrophages (CD11b⁺ F4/80⁺ cells), as seen in Figure 15 and Figure 16, no change in the infiltration was expected. The experimental process that was followed was to inject PyMT GFP⁺ cancer cells orthotopically in the fat pad of the mice one week before the implantation of porous GelMA scaffolds, and harvest after 3 weeks of implantation (**Figure 23A**). This timeline would be ideal as it would allow the infiltration of the scaffolds as well as the primary tumour growth at a level that would allow cancer cell dissemination and metastasis. Scaffolds were therefore implanted in naïve, and tumour bearing mice and harvested after 3 weeks of implantation. Scaffolds were imaged *ex vivo* under a stereoscope and the presence of GFP⁺ cancer cells was observed in GelMA scaffolds derived from tumour bearing mice compared to control (**Figure 23B**). This indicates the successful dissemination of PyMT GFP⁺ cancer cells to the GelMA scaffolds.

Next, it was checked whether we disseminated cancer cells could be detected by flow cytometry. For this, the same experimental protocol for the digestion was followed and

the single cell population obtained was stained with epithelial adhesion molecule (EpCAM), an epithelial marker highly expressed by cancer cells. As seen in **Figure 23C**, EpCAM is not expressed from cells derived from GelMA scaffolds from naïve mice, in contrast a population positive for EpCAM in GelMA scaffolds derived from tumour bearing mice was observed, indicating the presence of PyMT cells successfully disseminating in scaffolds and that EpCAM staining is specific for PyMT cells within the scaffold. Notably, GFP was not detected by flow cytometry, a typical feature of disseminated cancer cells, that are known to lose their GFP expression during dissemination (Grzelak *et al.*, 2022). Quantification of the total number of cancer cells per scaffolds indicated that only a few amount of DTCs are present within the scaffold (**Figure 23D**). In order to avoid bias of the location of the scaffolds, scaffolds either from the left or right side were collected for flow cytometry per each animal, and the other scaffold was kept for histology. As seen in **Figure 23D**, the location of the scaffold did not affect the amount of cancer cell seeding, indicating that cancer cells recruited from the circulation. However, further experiments including intravenous injection of cancer cells in mice with implanted scaffolds and quantification of the total amount of cancer cells from scaffolds harvested either from the left or right side would be essential in order to make this conclusion.

Immunofluorescence staining of cryosections from porous GelMA scaffolds from tumour bearing mice were also optimised (**Figure 23E**). As a positive control for staining, cryosections from primary tumours were used, indicating the strong signal for GFP and ki67. Indeed, as seen in **Figures 23E** and **23F**, PyMT GFP⁺ cancer cells were detected in cryosections of GelMA scaffolds from tumour bearing mice, with negative control cryosections of GelMA scaffolds from naïve mice. Notably, disseminated PyMT cells are negative for the proliferation marker, ki67, a protein expressed in all active states of the cell cycle except for the G0 phase. Indeed, given the total number of DTCs found within the scaffold, DTCs reaching the scaffold seem to not proliferate. However, additional analysis and quantification of multiple sections and scaffolds would be required in order to support this observation. These experiments would also shed light into the different states cancer cells would appear within the scaffold, namely whether they are found as single cells or clusters (as seen in **Figure 23E**).



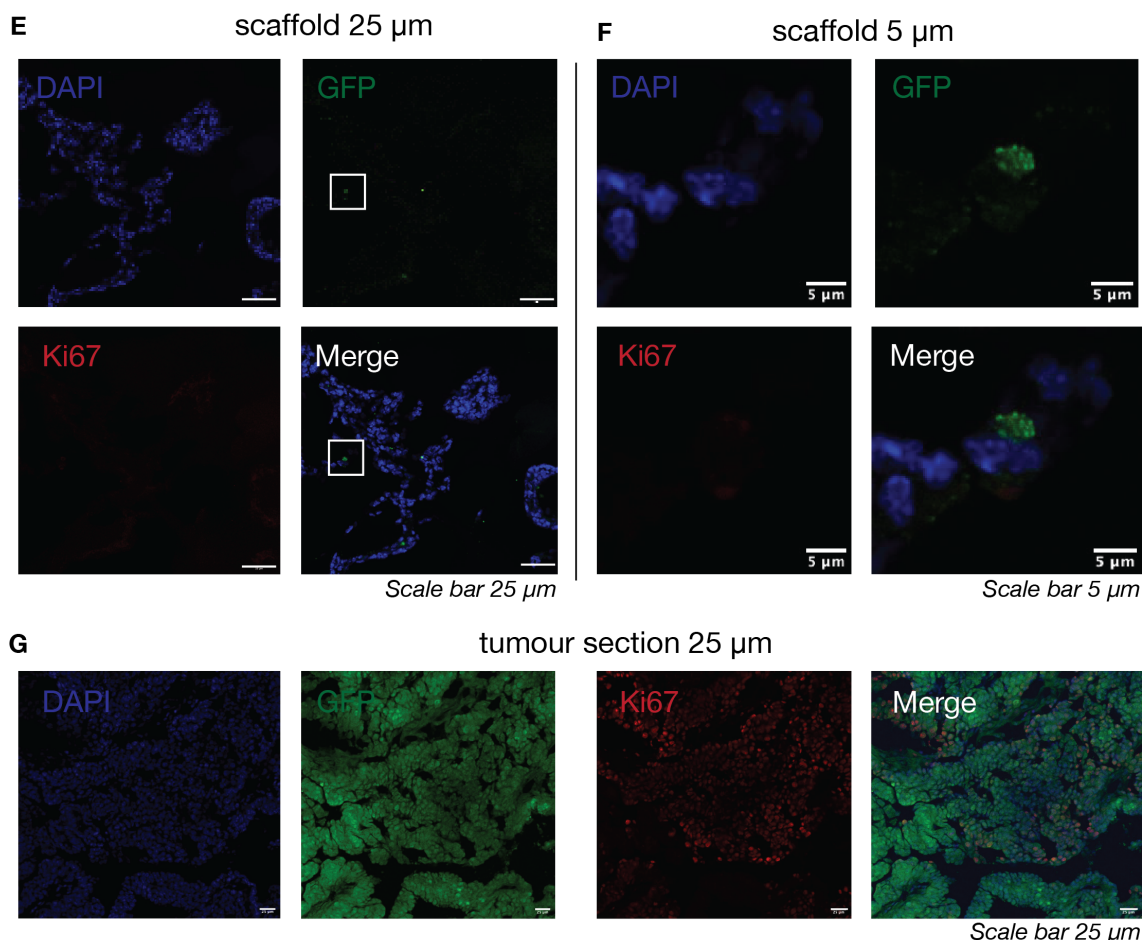


Figure 23. Dissemination of cancer cells from the primary fat pad tumour to implanted GelMA scaffold. **A)** Illustration of the experimental design of assessing tumour cell dissemination from a primary PyMT GFP⁺ fat pad tumour to implanted scaffolds. **B)** *Ex vivo* imaging of scaffolds using the GFP channel to image the scaffolds from control (no fat pad tumour) and scaffolds from primary tumour bearing mice. Scale bar 1000 µm. **C)** Flow cytometry analysis from digested scaffolds from control or primary tumour bearing mice, stained for epithelial cellular adhesion molecule (EpCAM), gated out of CD45⁻ population. **D)** Quantification of the absolute number of EpCAM⁺ cancer cells in scaffolds harvested from tumour bearing mice and analysed by flow cytometry. Data presented mean $\pm$ SD (n = 16, from different experiments color indicates data from other figures). **E), F)** Immunofluorescence staining of cryosections from scaffolds of tumour bearing mice stained for DAPI (blue), anti-GFP (green) and ki67 (red). Scale bar E) 25 µm, F) 5 µm. **G)** Immunofluorescence staining of tumour cryosections stained for DAPI (blue), anti-GFP (green) and ki67 (red). Scale bar 25 µm.

3.4.6 Interaction of cancer cells with macrophages within porous GelMA scaffolds.

Next, it was hypothesised that cancer cells disseminating to the scaffold, would interact with the scaffold infiltrate. Since macrophages were the predominant population of the GelMA scaffolds, the next step was to assess if macrophages in the scaffold come in to

contact with cancer cells within the scaffold. Co-staining for CD11b and GFP was used to detect both cancer cells as a cluster and macrophages surrounding the cancer cells within GelMA scaffolds (**Figure 24A**).

To investigate whether macrophages come to direct contact with cancer cells, a niche labelling tool previously developed in the Malanchi lab was applied (Ombrato *et al.*, 2019, 2020). This tool allows for the labelling of surrounding cells normal cells that have come in direct contact with cancer cells disseminating to a secondary site. TS1 breast cancer cells, a MMTV-PyMT-derived cell line (Shree *et al.*, 2011) were genetically engineered to constitutively express the Cherry niche tool, a cell membrane-permeable mCherry protein (sLP-mCherry) that is secreted and taken up by surrounding cells *in vivo*. TS1 mCherry cancer cells were orthotopically injected in the fat pad followed by GelMA scaffold implantation. Scaffolds were harvested after 3 weeks of engraftment and stained following flow cytometry protocol and analysed using the Imagestream system (**Figure 24B**). Imagestream analysis combines the features of flow cytometry and simultaneous fluorescence microscopy on a cellular level (Zuba-Surma, Kucia and Ratajczak, 2008). This method was used instead of conventional flow cytometry in order to avoid potential artefacts or debris which would not be visible. The analysis that was followed was based on gating for single cells, live cells, CD45⁺ cells for the total immune infiltrate, CD11b⁺F4/80⁺ cells for the macrophage population (**Figure 24C, top**). When gating for mCherry, it was noticeable that macrophages from scaffolds from naïve mice were not positive for this marker, in contrast with macrophages extracted from scaffolds of tumour-bearing mice (**Figure 24C, bottom**). As seen in the high-resolution imaging provided in **Figure 24D**, macrophages (CD45⁺CD11b⁺F4/80⁺) from GelMA scaffolds of tumour bearing mice were positive for mCherry in contrast with macrophages from mice without a primary tumour that were negative for mCherry.

It is also worth noting that this experiment indicates that TS1 mCherry cancer cells come into contact with macrophages, both present in the scaffold. Further experiments would be essential to provide insight as to whether cancer cells interact with the macrophages as soon as they disseminate to the scaffold or during circulation. The timeline of interaction between the two populations would be crucial to answer fundamental

questions such as the changes macrophages would undergo post interaction. Regarding the technical aspect of this experiment, although this method provides proof that macrophages come into contact with TS1 mCherry labelling cancer cells, the use of Imagestream creates complex datasets that require advanced analysis methods, due to the combination of imaging and flow cytometry data. Additionally, Imagestream is not recommended for the design of more complex panels, due to the potential for fluorescence overlap. Last, data analysis using these methods proved to be challenging and time-consuming therefore, more conventional methods would be beneficial for the quantification of macrophages interacting with cancer cells.

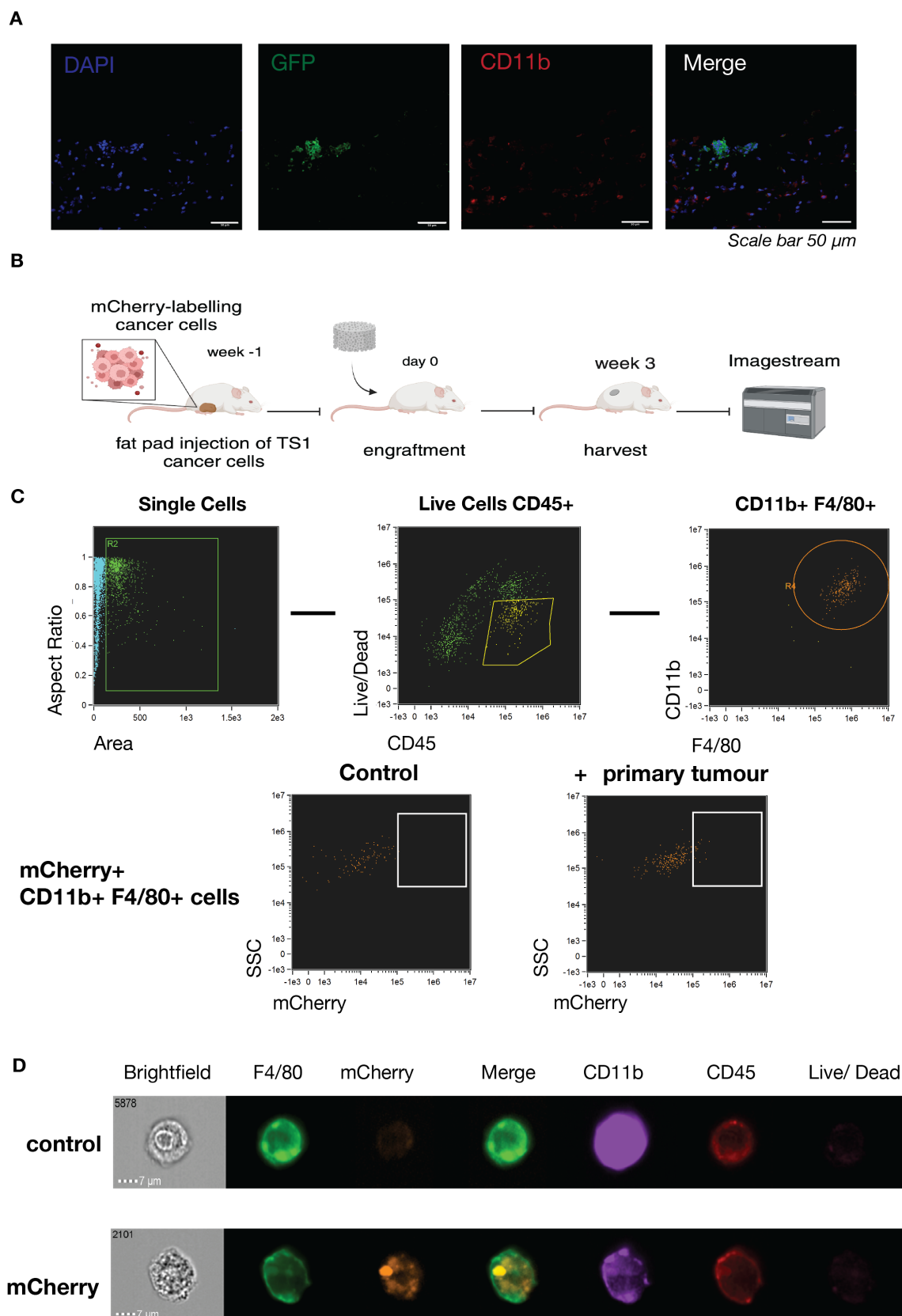


Figure 24. Interaction of disseminated cancer cells within porous GelMA scaffolds. A) Immunofluorescence staining of cryosections of GelMA scaffolds from tumour bearing mice

stained with DAPI (blue), anti-GFP (green), CD11b (red). Scale bar 25 μm . **B)** Illustration indicating the experimental design for assessing the dissemination of TS1 mCherry labelling cancer cells to implanted scaffolds and analysed using Imagestream. **C)** Gating strategy of Imagestream analysis followed, namely single cells, live cells, CD45⁺ cells, CD11b⁺F4/80⁺ cells (top). Bottom row shows the gating strategy of mCherry⁺CD11b⁺F4/80⁺ cells from scaffolds extracted from control and tumour-bearing mice. **D)** Imagestream analysis indicating the analysis of macrophages (Live, CD45⁺CD11b⁺F4/80⁺) from scaffolds either from control or tumour bearing (TS1 mCherry) mice.

3.4.7 Remodelling of porous GelMA scaffolds from the tumour bearing host.

Given the principles of the pre- and metastatic niche, discussed in Chapter 1, the next aim was to investigate whether there were any changes in the GelMA scaffolds from tumour bearing mice compared to scaffolds from mice without a primary tumour. H&E staining showed an increased cell infiltration of GelMA scaffolds in the presence of a primary tumour compared to the control (**Figures 25A and B**). Notably, flow cytometry analysis of CD45 positive cell populations from GelMA scaffolds from control and tumour bearing mice showed a decrease in the ratio of immune to non-immune cells in the GelMA scaffolds from tumour bearing mice compared to the control. This shows that porous GelMA interface differently with the tumour bearing host compared to naïve control mice, indicating that the primary tumour might perturb the scaffold in order to allow for cancer cell dissemination. Additionally, given the increase in the non-immune cell population, it was observed that CD31⁺ endothelial cell population was significantly increased in the GelMA scaffolds from tumour bearing mice compared to the control (**Figure 25C, 25E**).

The data would be in line with the systemic perturbation that the tumour causes to the distant tissues in order to allow cancer cells to successfully disseminate to a more favourable microenvironment. Indeed, the data show a pre-metastatic conditioning caused by the primary tumour which induces a different immune cell infiltration of the scaffold and an increased endothelial population compared to the non-tumour host. Further experiments would be required in order assess the differences in the infiltration of scaffolds between control and tumour-bearing mice, such as full characterisation of the immune cell populations in response to the primary tumour.

The next aim was to check whether the GelMA itself gets remodelled *in vivo*. To study this, GelMA was fluorescently labelled with cyanine5.5 (Cy5.5-labelled GelMA) and scaffolds were prepared same as in previous experiments. The experimental procedure that was followed was same as in **Figure 25A**, namely Cy5.5-labelled porous GelMA scaffolds were engrafted in naïve or PyMT GFP tumour bearing mice and harvested 3 weeks post implantation. Cy5.5-labelled porous GelMA scaffolds that were cultured *in vitro* in PBS for 3 weeks were used as a control. Scaffolds were fixed and cryosectioned according to protocol and sections were stained for DAPI. As seen in **Figure 25F**, fluorescence imaging for Cy5.5 shows that *in vitro* cultured GelMA scaffolds retain their GelMA structure, whereas GelMA scaffolds harvested from control or tumour bearing mice get remodelled *in vivo*. This remodelling is assessed by the loss of Cy5.5 signal in GelMA scaffolds from both control and tumour bearing mice (**Figure 25G**), indicating that GelMA gets remodelled *in vivo*. Further experiments would be essential to show whether there is a significant change in the remodelling of GelMA between control and tumour bearing mice as well as characterisation of the ECM changes present in the scaffold. Masson's trichrome staining would be essential to evaluate collagen deposition and fibrosis within the scaffold, as this would indicate changes in ECM remodelling. Picrosirius Red staining would specifically assess collagen fiber organization and density, which may change in response to tumour-induced remodelling.

These additional experiments will help clarify how the primary tumour influences the GelMA scaffold, leading to changes in immune cell infiltration, endothelial cell recruitment, and the potential creation of a pre-metastatic niche. Understanding these mechanisms will contribute to the broader understanding of tumour-host interactions and the role of artificial scaffolds in metastasis.

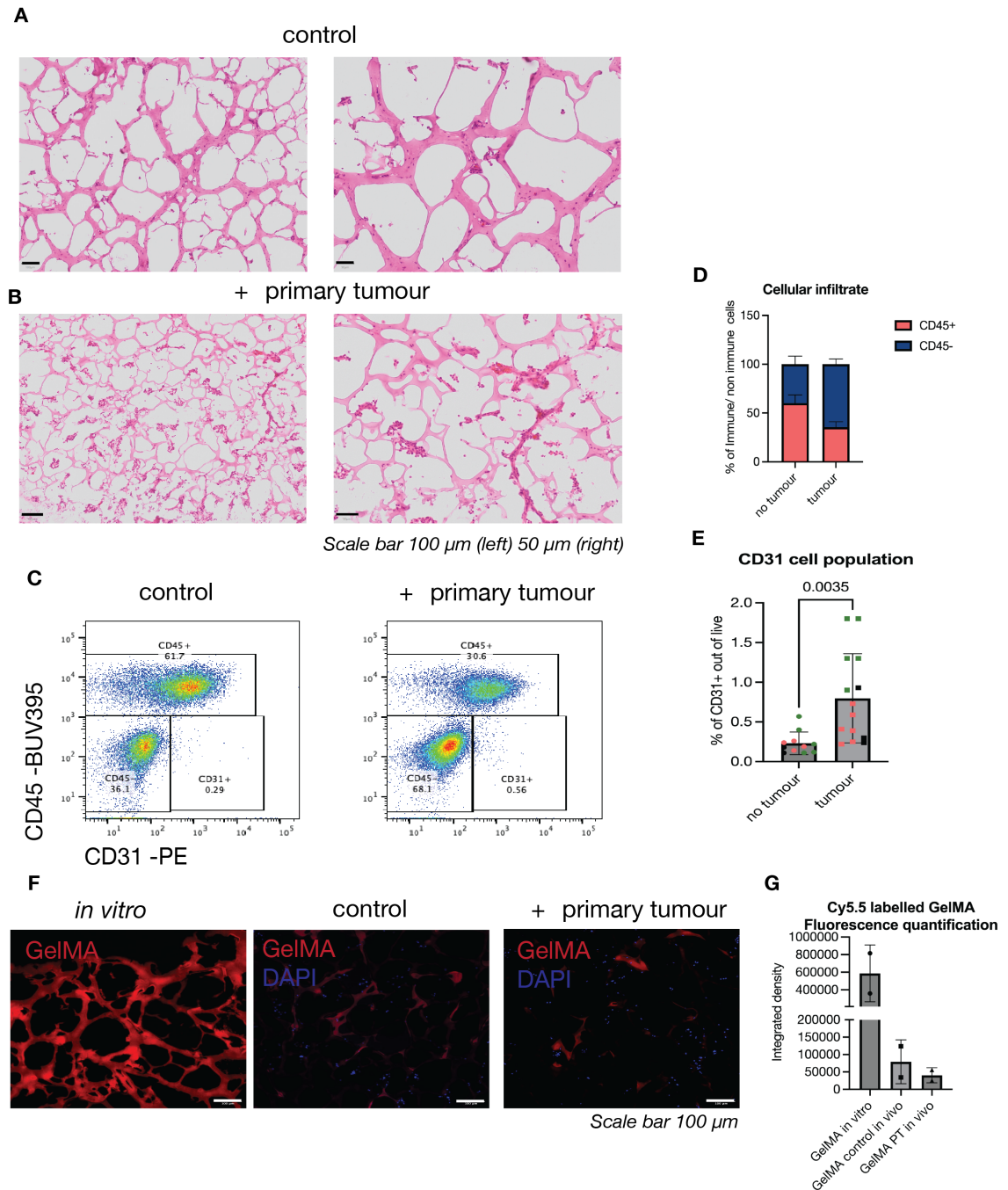


Figure 25. Remodelling of GelMA scaffolds from disseminated cancer cells. Haematoxylin and eosin (H&E) staining of cryosections of GelMA scaffolds from **A**) control and **B**) tumour bearing mice. Scale bar left 100 μ m, right 50 μ m. **C**) Representative flow cytometry plots of CD45 and CD31 populations from GelMA scaffolds from control or tumour bearing mice. **D**) Quantification of the ratio of CD45⁺ (immune) and CD45⁻ (non-immune) cell population gated out of live cell population from scaffolds derived from control (no tumour) and tumour-bearing mice (tumour). Data presented mean $\pm$ SD ($n = 8$ no tumour, $n = 12$ tumour). **E**) Quantification of the CD31⁺ cell population gated out of live cell population from scaffolds derived from control (no tumour) and tumour-bearing mice (tumour). Data presented mean $\pm$ SD ($n = 11$ no tumour, $n = 14$ tumour, colour indicates different experiments). Statistical analysis performed using unpaired t-test. **F**) Immunofluorescence staining of Cy5.5 labelled GelMA cryosections from *in*

vitro cultured scaffold (left), from naïve mice (middle) or tumour bearing mice (right), stained with DAPI (blue). Scale bar 100 μ m. **G**) Quantification of the integrated density of Cy5.5-labelled GelMA from cryosections of GelMA scaffolds cultured *in vitro* or after 3 weeks of implantation in control or tumour bearing mice. Cells were stained for DAPI. No statistical analysis performed due to the low number of analysed samples.

3.4.8 Changes in the lung environment upon GelMA scaffold engraftment.

Given the work of Lonnie Shea and Jacqueline Jeruss showing a reduction of the metastatic burden in different metastatic sites upon scaffold implantation, the next step of the study was to investigate whether the pre-metastatic conditioning of the scaffolds from the primary tumour as well as the recruitment of cancer cells to porous GelMA scaffolds would have any systemic effect in the metastatic dissemination of other tissues. In order to study this, the 4T1 cancer cell line, a highly tumorigenic and metastatic model, derived from the mammary gland tissue of a BALB/c mouse model, was chosen. This model is known to be highly metastatic to multiple distant sites, the lung among others.

Before employing the tumour model, the first step was to check whether there are any changes in the lung upon scaffold implantation. For this reason, GelMA scaffolds were implanted in immunocompetent BALB/c mice at day 0. For the control group, mice underwent mock surgery in order to exclude any effect from the surgical procedure itself. Lungs were harvested 3 weeks after scaffold implantation and analysed by flow cytometry (**Figure 26A**). As seen in **Figure 26B**, there was no change in the immune cell population (CD45⁺) from the lungs of mock surgery treated mice compared to scaffold bearing mice. To further characterise the immune cell composition, flow cytometry analysis of all the main immune cells of the lung was performed. Analysis included the main lymphoid cell populations; B cells (B220⁺), NK cells (NKp46⁺), T cells (CD3⁺), and in specific CD4 T cells (CD4⁺CD3⁺), CD8 T cells (CD8⁺ CD3⁺). In regards of the myeloid cell populations, neutrophils (F4/80^{med} and Ly6G⁺), alveolar macrophages (AMs) (Ly6G⁻SiglecF⁺), interstitial macrophages (IMs) (Ly6G⁻SiglecF⁻F4/80⁺Ly6C^{low}) and dendritic cells (DCs) (Ly6G⁻ SiglecF⁻F4/80⁻CD11c⁺MHC II⁺) were analysed. From non-immune cells, EpCAM cells (EpCAM⁺) were analysed. As seen in **Figure 26C**, there was no significant differences in any of the cell populations of the lungs from mock surgery treated mice compared to scaffold bearing mice. This indicates that there is not

any systemic effect caused by the engraftment of GelMA scaffolds in other tissues, such as the lung.

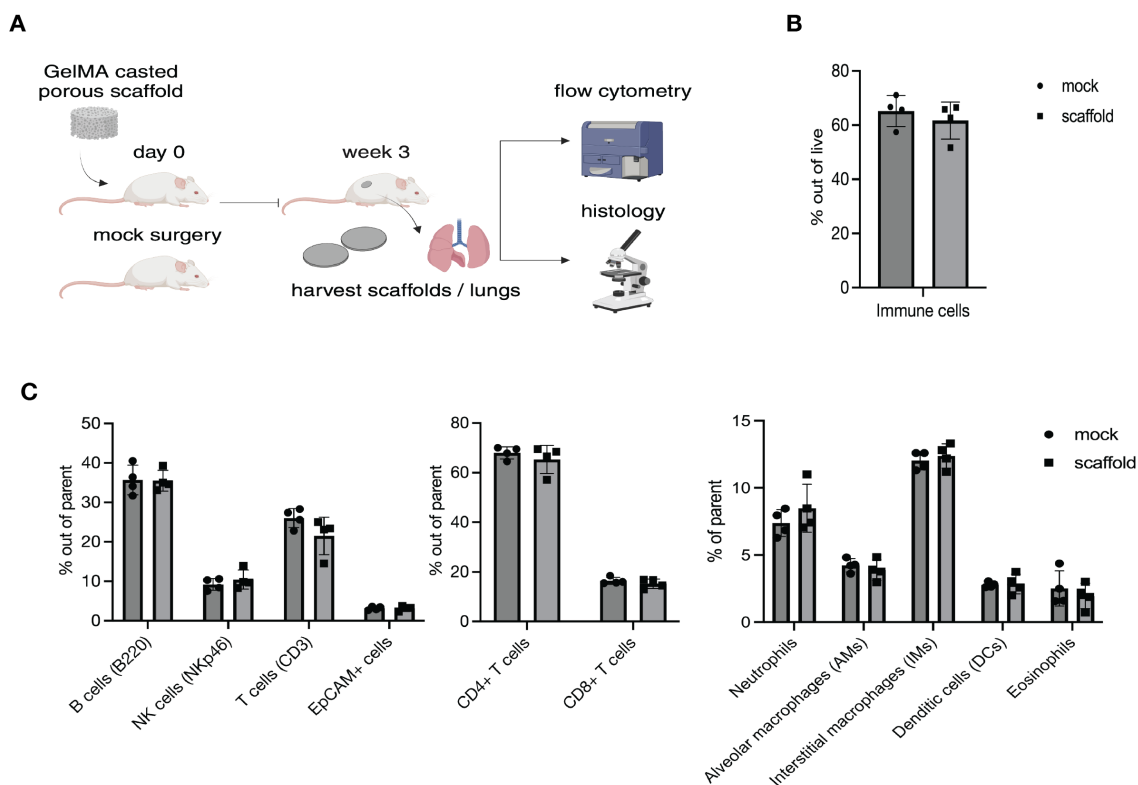


Figure 26. Immune cell characterisation of the lung upon scaffold engraftment. A) Illustration of the experimental design of scaffold engraftment and analysis of the lung after 3 weeks of scaffold implantation. **B)** Quantification of immune cells ($CD45^+$) in mock surgery treated or scaffold bearing mice. Data shown as mean $\pm$ S.D. ($n = 4$). **C)** Quantification of B cells, NK cells, EpCAM cells, T cells ($CD4^+$ and $CD8^+$) cells, and myeloid cells (neutrophils, alveolar macrophages (AM), interstitial macrophages, dendritic cells (DCs) and eosinophils) in mock surgery treated or scaffold bearing mice analysed by flow cytometry. Data shown as mean $\pm$ S.D. ($n = 4$).

3.4.9 Systemic effect of GelMA scaffold engraftment in cancer cell dissemination.

Following the observation that there were no changes in the lungs between mock surgery treated and scaffold bearing mice, the next question that was asked was whether scaffold implantation would reduce the metastatic burden in other metastatic sites such as the lung. For this, 4T1 GFP⁺ cancer cells were orthotopically injected in the fat pad of immunocompetent BALB/c mice followed by the implantation of GelMA scaffolds. As previously, mice treated with mock surgery were used as control. Lungs were harvested

after 3 weeks of scaffold implantation and their tumour burden was quantified based on H&E staining (**Figure 27A**). Metastatic lesions were easily observed by H&E staining, and as seen in **Figure 27B**, metastatic lesions from the lung of scaffold bearing mice were smaller compared to mock surgery treated mice. Quantification of the first experiment (**Figure 27C**) showed that that lung metastases was significantly lower in scaffold bearing mice compared to mock surgery treated mice, however with the addition of the second experiment, this difference was not significant (**Figure 27D**). Additionally, flow cytometry analysis of the lung from scaffold bearing mice compared to mock treated showed no changes in the number of CD45⁺ immune cell populations (**Figure 27E**) and among different lymphoid and myeloid cell populations there was no change between the two groups only a trend to an increase in T cells (CD3⁺) in the scaffolds engrafted mice compared to mock surgery.

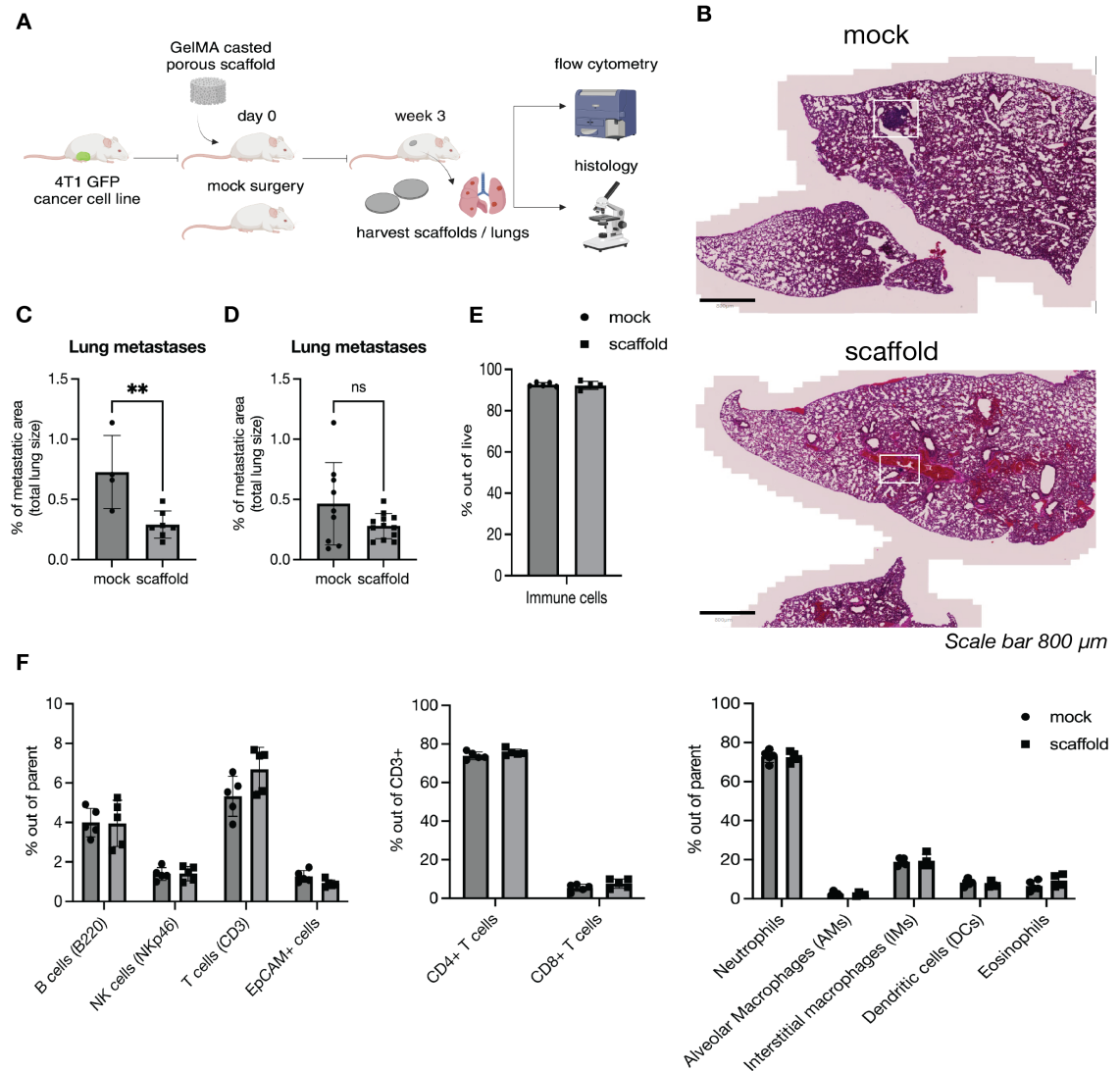


Figure 27. Analysis of the tumour burden and subsequent cell characterisation of the lung upon scaffold engraftment in a spontaneous dissemination model. **A)** Illustration of the experimental design of spontaneous dissemination model using 4T1 cancer cell line and characterisation of the tumour burden in the lung upon mock surgery or scaffold engraftment. **B)** Haematoxylin and eosin (H&E) images of lung sections from mock surgery treated mice (top) and scaffold engrafted mice (bottom). Scale bar 800 μm . **C)** Quantification of the percentage of metastatic area of the lung from mock surgery or scaffold engraftment. Data shown as mean $\pm$ S.D (n = 4 mock, n=7 scaffold). Statistical analysis performed using unpaired t-test. **D)** Quantification of the percentage of metastatic area of the lung from mock surgery or scaffold engraftment. Data shown as mean $\pm$ S.D (n = 9 mock, n=12 scaffold). Statistical analysis performed using unpaired t-test. **E)** Quantification of immune cells (CD45⁺) in mock surgery treated or scaffold bearing mice. Data shown as mean $\pm$ S.D. (n = 5). **F)** Quantification of B cells, NK cells, EpCAM cells, T cells (CD4⁺ and CD8⁺) cells, and myeloid cells (neutrophils, alveolar macrophages (AM), interstitial macrophages, dendritic cells (DCs) and eosinophils) in mock surgery treated or scaffold bearing mice analysed by flow cytometry. Data shown as mean $\pm$ S.D (n = 5).

3.5 Conclusions & Future work

The hypothesis of this chapter was that porous GelMA scaffolds would have the ability to interface with the host and act as an artificial metastatic microenvironment *in vivo*. In order to address this hypothesis, the first aim was to assess the biocompatibility of the porous GelMA scaffold *in vivo* and the immune response that gets triggered. Whether pore formation influences cell infiltration *in vivo* was investigated. Indeed, porous GelMA scaffolds get highly infiltrated in contrast to GelMA scaffolds without any porogen, indicating that porosity is required for cells to infiltrate and migrate throughout the scaffold. Although GelMA scaffolds without gelatin beads incorporated as a porogen did not show cell infiltration after 3 weeks of implantation, it cannot be excluded that cells could have been infiltrated at a former timepoint. Additional experiments, including proteomics analysis would verify the level of remodelling of GelMA scaffolds with and without gelatin beads as porogen. GelMA scaffolds without gelatin beads still exhibited some level of porosity, possibly due to the microporosity of the GelMA itself. Indeed, in the work of Chen *et al.*, GelMA hydrogels exhibited porosity as high as 50 μm , as characterised by SEM after freeze-drying (Chen *et al.*, 2012). Additional parameter would include the degradation rate of the GelMA scaffold post three weeks of implantation.

Several parameters would be essential for quantification after scaffold extraction, including the degradation of GelMA scaffolds, size of the scaffolds before and after implantation as well as variations of the residual surrounding tissue cleaned from the extracted scaffolds.

The next aim of this chapter was to assess whether cell infiltration within the scaffold can be modulated. For this aim, it was hypothesised that pre-seeding endothelial progenitor cells (EPCs) in the scaffolds before implantation would induce an increase in the cell infiltration within the porous GelMA scaffolds. For this, EPCs from adult mice were successfully isolated and characterised. Porous GelMA scaffolds were pre-seeded with EPCs before implantation. However, the results obtained showed that EPC pre seeding does not increase cell infiltration throughout the scaffolds, namely the cellular infiltration between the non-seeded and EPC seeded scaffolds was similar after 3 weeks

of implantation. However, further analysis would be required to assess the differences in the cell infiltration between the two conditions regardless of the number of cells infiltrated. Staining for endothelial markers would be essential to address the differences in the phenotype of cells infiltrating the scaffold, when pre-seeded with EPCs. Additionally, further experiments would be crucial to optimise the number of EPCs pre-seeded within the scaffolds in order to check for any differences in cell infiltration. From the experimental setting tested, one explanation would be due to cells not surviving throughout the engraftment period of the scaffolds or due to the low number of cells seeded. However, since porous GelMA scaffolds get highly infiltrated, the next experiments were performed without any cell pre-seeding in order to simplify the experimental procedure and reduce animal use.

Next, immunohistochemical staining for different markers was optimised and it was observed that the predominant population infiltrating GelMA scaffolds is macrophages, staining positive for CD11b and F4/80 markers. This is in line with flow cytometry analysis of GelMA scaffolds, which also showed that macrophages are highly increased at week 3 compared to previous timepoints, week 1 and week 2. CD11b⁺F4/80⁺ macrophages that infiltrate the scaffold are typically tissue-resident macrophages, specifically from the monocyte-derived macrophage lineage, which play a crucial role in the immune response to foreign materials. These cells are often associated with inflammation and tissue repair and are key players in the foreign body response (FBR). Additionally, endothelial cells are present throughout the scaffold, indicating their potential to form blood vessels within the scaffold. Quantification of the endomucin⁺ cells and additional analysis of the endothelial cell population for endothelial markers such as CD31 and VE-cadherin would be required to confirm the presence and distribution of endothelial cells within the scaffolds. Functional characterisation of the blood vessels was performed using *in vivo* perfusion assays with Texas-red fluorescently labelled dextran and tomato lectin, in order to visualise and confirm the presence of the blood vessels. Further experiments, such as staining for pericytes (by staining for α SMA) would help to confirm the recruitment of pericytes to the vessels, required for vessel maturation and stability.

One limitation of this study includes the limiting cell populations assessed by histology and flow cytometry. Characterisation of additional cell populations, such as neutrophils, lymphocytes, dendritic cells and mast cells, would be essential in order to fully characterise the biocompatibility of GelMA *in vivo* and assess its differences compared to other biomaterials. Each of these populations plays a unique role in the immune reaction, and the degree of their involvement can influence the success of the scaffold in tissue repair or regeneration. Therefore, it would be important to assess the timing of recruitment of these populations and their ratio at different timepoints in order to assess the FBR of the GelMA scaffold *in vivo*.

Following the characterisation of the cell infiltrate of the GelMA scaffold from the host, the next objective was to investigate whether GelMA scaffolds can interface effectively and differently with the tumour bearing host compared to the control. For this, a spontaneous metastasis model was used, namely by orthotopically injection of PyMT primary cancer cells in the fat pad of the mice. Indeed, using various methods, two observations were made. First, cancer cells can successfully disseminate to GelMA scaffolds through the vasculature, and they come into contact with the macrophages residing in the GelMA scaffolds. Further experiments would be required in order to assess the timepoint of interaction between macrophages and cancer cells, whether it occurs within the scaffold or from the circulation. Additionally, it would be important to highlight the changes that this interaction causes, which would be achieved by single-cell analysis of the macrophage population from scaffolds extracted from control and tumour-bearing mice. In regards to the cancer cells present within the scaffold, quantification of multiple sections from different scaffolds would help validate whether these DTCs are uniformly non-proliferative across the scaffold. This would allow a more comprehensive understanding of the spatial distribution of cancer cells and the state in which they are found, namely either as single disseminated cells or clusters or both. Additionally, it would be interesting to explore whether the number of cancer cells would increase at a later timepoint of harvest, and in combination with staining for the proliferative marker Ki67, to assess whether a pool of cancer cells within the scaffold is constantly being replenished by the circulation or if the DTCs within the scaffold actively proliferate to increase their number. Last but not least, other dormancy-associated markers such as p27

(Oki *et al.*, 2014) could add information of the stages of cell cycle of DTCs within the scaffold.

Second, the presence of a growing primary tumour induces changes in the cells infiltrating the scaffold, by increase of the non-immune cell population within the scaffold and specifically an increase in the endothelial cell population. This is in line with the phenomenon of cancer-dependent conditioning that can generate pre-metastatic conditioning of distant organs (Patras *et al.*, 2023). Given the increase in the endothelial cell population in scaffolds from tumour-bearing mice, further characterisation of these cells would be required in order to assess whether a change in number and phenotype of these cells occurs. Characterisation of the endothelial population using specific markers such as CD31, VEGFR-2 and VE-cadherin would be beneficial to identify and quantify the endothelial cells within the scaffolds between the two conditions (Grant *et al.*, 2021). Assessing the expression of angiogenic factors, such as VEGF using qPCR or ELISA would be crucial to show whether the primary tumour is able to induce angiogenesis at the scaffold site. Last, to further explore this phenomenon, key experiments, including proteomics analysis of scaffolds extracted from control and tumour bearing mice would be required to understand potential factors that may be responsible for the pre-metastatic conditioning.

Last, it was investigated whether the scaffold engraftment and cancer cells disseminating to the scaffold would have any effect in the tumour burden of other metastatic sites such as the lung. Scaffold engraftment seems to reduce tumour burden of the lung; however, this decrease was not statistically significant and therefore further experiments are required to validate this. Additionally, no major changes were observed in the lymphoid cell and myeloid cell populations of the lung between scaffold bearing mice compared to mock surgery treated mice. This suggests that although cancer cells disseminate to the scaffolds, either their number is not sufficient to reduce the tumour burden of other metastatic sites, or the scaffold engraftment does not cause any systemic effect that influences cancer cell dissemination and metastasis. Work of the Shea group has shown that the inflammatory response caused to the implanted synthetic biomaterials develops potent antitumour immunity within the scaffold causing cancer cells to stay dormant

(Wang *et al.*, 2023), which is mediated by the presence of Gr1⁺CD11b⁺Ly6G⁺ neutrophils, activated dendritic cells (DCs) and T cells within the scaffolds. In this study, the authors revealed the multifaceted roles of neutrophils within different metastatic sites, namely lung versus scaffold. This is based on the immune response developed towards the synthetic PCL scaffold, which recruited Gr1⁺CD11b⁺Ly6G⁺ and systemically affected the immune response of other metastatic sites, affecting cancer cell seeding. In this study, PCL scaffolds were highly infiltrated mainly by Gr1⁺CD11b⁺ myeloid cells and Gr1⁺CD11b⁺Ly6G⁺ cells, supporting the notion that the foreign body response towards synthetic biomaterials are more persistent compared to natural or semi-synthetic. The different cellular infiltrate of porous GelMA scaffolds compared to PCL could potentially explain why GelMA scaffold implantation does not seem to systemically affect distant metastatic sites such as the lung. However, further experiments including single-cell RNA sequencing of scaffolds and other metastatic sites from naïve or tumour -bearing mice would shed light on other systemic effects of the presence of GelMA scaffolds.

Chapter 4. Modulation of the physicochemical properties of porous GelMA scaffolds.

4.1 Introduction

As a semi-synthetic material, GelMA combines the properties of both naturally derived and synthetic materials. It provides higher reproducibility compared to naturally derived materials and allows the modification of its physicochemical properties by tuning various parameters, such as the DoF, the concentration of GelMA, the type and concentration of the photo-initiator and the exposure time under UV irradiation (Zhu *et al.*, 2019). Enhanced stiffness is often achieved by increasing the concentration of the hydrogel precursor solution. However, important considerations need to be acknowledged. First, increasing GelMA concentration, does not solely affect stiffness, as it also influences other parameters such as porosity, swelling capacity and degradation rate. Additionally, higher concentration of GelMA may lead to uneven crosslinking across the scaffold, resulting in regions of varying stiffness. Increased GelMA concentration can reduce the diffusion of nutrients, oxygen and waste products through the scaffold, influencing cell viability (Nichol *et al.*, 2010; Yue *et al.*, 2015). High GelMA concentration can restrict cell spreading and migration, as mentioned in section 1.5.4. Indeed, providing a physiologically relevant stiffness both for the normal as well as the malignant tissue is an important aspect for 3D cell culture systems (Ishihara and Haga, 2022; Micalet, Moeendarbary and Cheema, 2023).

Due to the photo-crosslinking, GelMA precursor solutions can also be mixed with different factors, which will be incorporated with GelMA after solidification. Vascular endothelial growth factor (VEGF) is a proangiogenic factor that induces angiogenesis and is implicated both in homeostasis and in cancer (Carmeliet, 2005).

4.2 Aims & Objectives

This chapter aims to leverage the tuneable physicochemical properties of GelMA. First, it was hypothesised that changing the stiffness of GelMA would lead to changes in the cell infiltrate *in vivo*. The change in infiltration is achieved in two ways. In order to decrease the stiffness of GelMA, the degree of methacrylation (DoF) was lowered, whereas an increase in the stiffness of GelMA was achieved by the increase in the concentration of GelMA from 10% (w/v) to 20% (w/v). The different stiffnesses were compared, and scaffolds were implanted *in vivo* to assess the potential differences observed in the cell infiltration of the scaffolds as well as in the subsequent cancer cell seeding.

Additionally, an *ex vivo* culture system of GelMA scaffolds was introduced, in order to assess whether the *in vivo* priming of the GelMA scaffolds plays a role in cancer cell growth. For this aim, scaffolds are implanted in control and tumour bearing mice. After harvest, primary PyMT cells were *ex vivo* injected and cultured in GelMA scaffolds from control or tumour bearing mice and their growth was assessed by imaging.

Given the differences observed in the previous chapter in endothelial cell population increasing in the scaffolds of tumour bearing host, the potential of GelMA to be functionalised with specific chemokine and growth factors before implantation in order to influence the type of cells infiltrating as desired, was explored. As a proof of concept, the possibility of increasing the degree of vascularisation of the GelMA scaffolds using the pro-angiogenic vascular endothelial growth factor, VEGF was assessed. The type of changes observed in the cell infiltration of the scaffolds as well as in the subsequent cancer cell seeding were assessed by flow cytometry.

4.3 Material & Methods

4.3.1 GelMA synthesis with different degree of DoF

In brief, gelatin from fish skin (Sigma-Aldrich, Cat. No. G77041-500G) was dissolved in Ultrapure water to a final concentration of 10% (w/v) at RT in a round-bottom flask with

a magnetic stir bar. After gelatin dissolution, the mixture was heated to 50 °C to allow the gelatin to be fully dissolved and the solution to become clear. Different amounts of methacrylic anhydride were added with respect to the gelatin, namely 0.05 mL/g, 0.0375 mL/g and 0.025 mL/g in order to achieve lower degree of functionalisation. After stirring for 3 hours the solution was transferred to 50 mL falcon tubes and unreacted methacrylic anhydride was removed by centrifugation at 3,500 g for 3 minutes at RT. The pH was adjusted to pH = 7, using 1 M HCl. The supernatant was decanted into a 500 mL glass beaker and diluted with two volumes of pre-heated Ultrapure water. The solution was transferred into a dialysis membrane with a 6-8 kDa MWCO (Spectrum Laboratories, 132665) and dialysed at 40 °C for 5 days. After dialysis, the solution was transferred into 50 mL falcon tubes, freeze dried and lyophilised to give GelMA as a foam like solid.

4.3.2 Rheological characterisation of GelMA with different DoF

Rheological measurements of GelMA were recorded using an Anton Paar MCR302 rheometer fitted with a 25 mm stainless steel parallel plate (PP25) and Peltier temperature-controlled hood (P-PTD200/80/I). To determine the thermal responsivity of different formulations, oscillatory temperature sweeps were carried out with temperature ramp change at a rate of 5 °C minute⁻¹, while shear strain and frequency were fixed at 1% and 1.5 Hz, respectively. In addition, a sharp temperature change from 10 to 37 °C was applied to the oscillatory time sweeps to demonstrate the successful melting of microgels under physiological temperature. Oscillatory strain and frequency sweeps were performed with a fixed frequency (1.5 Hz) and strain (1%), respectively. To determine the photo-crosslinking kinetics, time sweeps at 25 °C were performed, during which in situ light irradiation (365 nm, 10 mW cm⁻², OmniCure S1500) was applied for 5 minutes. Unless otherwise stated, constant values of strain, frequency and temperature were 1%, 1.5 Hz, and 37 °C, respectively.

4.3.3 Compression testing of porous GelMA scaffolds with different DoF

Unconfined, quasi-static uniaxial compression tests were performed on cylindrical scaffolds of 9 mm diameter and 4 mm height using a TA Electroforce 3200 mechanical

testing machine equipped with a 2.5 N load cell. Upon removal from the PBS buffer, the specimens were loaded between two parallel compression plates and compressed at a rate of 0.03 mm/s to 3.5 mm displacement, followed by a constant-deformation dwell of 10 seconds and unloading at 0.1 mm/s. During post-processing, a compressive pre-load of 0.01N was assumed, and the compression modulus was estimated as the slope of a linear curve between 2% and 5% strain.

4.3.4 VEGF loading in porous GelMA scaffolds

Lyophilised GelMA was weighed and resuspended in PBS at a final concentration of 10% (w/v), mixed with 4×10^{-3} M lithium phenyl (2,4,6-trimethylbenzoyl)phosphinate photo-initiator (LAP) (TCI, L0290). The GelMA solution was warmed to 37 °C and left until completely dissolved. The solution was then mixed with porcine gelatin beads to a ratio of 1 gelatin beads:2 GelMA (v/v). Mouse VEGF (R&D Systems, 493-MV-025/CF) was added to the final solution at a concentration of 0.1 mg/mL. The solution was then applied in a 3D printed silicon mould (6 mm diameter, 3 mm height) and was then photo-crosslinked using *in situ* light irradiation (365 nm, 10 mW cm⁻², OmniCure S1500) for 5 minutes.

4.3.5 ELISA for VEGF release from porous GelMA scaffolds

Samples were prepared as previously described. Scaffolds were placed in 0.5 mL PBS. A 50 µL aliquot of supernatant was collected at different timepoints and kept at 4 °C. Scaffolds were placed in fresh PBS after the collection of the aliquot, in order to assess the new release of VEGF between the timepoints. After collection, aliquots of the different timepoint were measured with mouse VEGF Quantikine ELISA kit (R&D Systems, MMV00) according to manufacturer's instructions. In brief, 50 µL of assay diluent was added to a 96-well microplate, followed by the addition of 50 µL of standard or sample to each well. The plate was covered with a plate sealer and was incubated at room temperature for 2 hours. After incubation time, the solution from each well was aspirated and the plate was washed with wash buffer for a total of 5 washed. 100 µL of conjugate was added to each well and the plate was covered with a new plate sealer and

incubated at room temperature for 2 hours. Following this, the supernatant was aspirated, and the plate was washed 5 times. A 100 μL aliquot of substrate solution was then added to each well and the plate was incubated at room temperature for 30 minutes covered in foil. Then, a 100 μL aliquot of stop solution were added to each well and the plate was read at 450 nm using a plate reader, with a wavelength correction of 570 nm.

4.3.6 *Ex vivo* culture of porous GelMA scaffolds with PyMT cancer cells

GelMA scaffolds were harvested from control or mice bearing GFP- PyMT primary tumour. 0.1×10^6 GFP⁺ PyMT cancer cells resuspended in Growth Factor Reduced Matrigel (Scientific Laboratory Supplies, 356231) before injection (50 μL) using a 29 G needle directly in the centre of the scaffold. Scaffolds were placed into a 24-well plate containing transwell inserts with a 0.4 μm pore polyester membrane (Corning, 3470 or Greiner, 662641). A final volume of 100 μL of MEM media (DMEM/F12 containing 2% FBS, 100 U mL^{-1} penicillin-streptomycin (ThermoFisher, 15070063), 20 ng EGF (ThermoFisher, PHF0313), 10 $\mu\text{g mL}^{-1}$ insulin (Sigma-Aldrich, 19278) and Glutamax (ThermoFisher, 3505061) was added within the well, surrounding the scaffolds. Bright-field or fluorescent images of PyMT seeded GelMA scaffolds were acquired at day 5 using an EVOS microscope.

Table 3. Key resources table 3

Reagent	Source	Catalog Number
Chemicals		
Mouse VEGF	R&D Systems	493-MV-025/CF
VEGF Quantikine ELISA kit	R&D Systems	MMV00
Softwares		
FlowJo software v.10.8.2	BD Life Sciences	https://www.flowjo.com
GraphPad Prism	Graphpad	https://www.graphpad.com
ImageJ	Open source	https://imagej.net/ij/
Biorender	Biorender	https://app.biorender.com

Other		
24-well transwell polyester membrane 0.4 μm	Corning	3470

4.4 Results

4.4.1 Characterisation of GelMA scaffolds with different stiffness.

Leveraging the ability to fine-tune the mechanical properties of GelMA, it was investigated whether changing the stiffness of the scaffold would have an effect in cancer cell seeding *in vivo*. In the previous chapters, 10% (w/v) GelMA were used as a standard for the scaffold preparation, and the aim was to increase and decrease the stiffness of the scaffold accordingly. In order to increase the stiffness, the concentration of GelMA was increased from 10% (w/v) (GelMA^{med}) to 20% (w/v) (GelMA^{high}) (**Figure 28A**). Decreasing the concentration of GelMA from 10% (w/v) to 5% (w/v) led to mechanical collapse of the scaffolds (data not shown), and therefore it was instead reasoned that decreasing the degree of functionalisation (DoF) of the GelMA would lead to lower mechanical stiffness (**Figure 28A**). By reducing the amount of methacrylic anhydride added to the GelMA reaction a reduction of DoF from 93% to 63% (GelMA^{low}) was achieved, as characterised by a fluoralddehyde assay (**Figure 28B**).

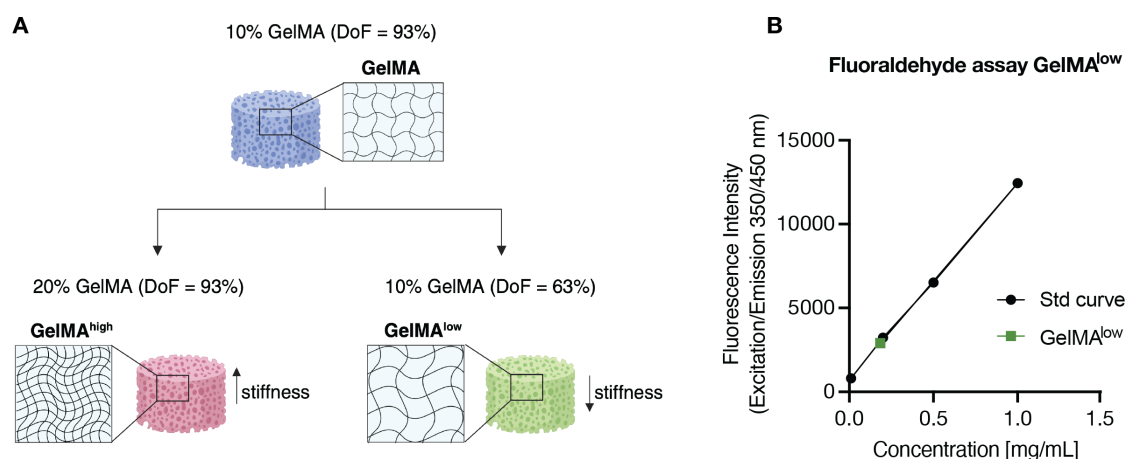


Figure 28. Modulation of the stiffness of GelMA. A) Illustration showing the increase of stiffness of GelMA by increasing the concentration from 10% (w/v) (GelMA^{med}) to 20% (w/v) (GelMA^{high}) and decrease of stiffness of GelMA by decreasing the DoF from 93% to 63% (GelMA^{low}). B) Fluoralddehyde assay of GelMA^{low} to determine the degree of functionalisation based on amine modification. Standard curve was determined using gelatin samples in different concentrations (0.02, 0.1, 0.5, 1.0 and 2.0 mg/mL). Data shown as mean $\pm$ S.D. (n = 3, technical replicates).

Next the stiffness of GelMA^{low}, GelMA^{med} and GelMA^{high} was characterised using two different methods, namely rheology for the measurement of the storage modulus (G') and using compression testing for the measurement of the elastic modulus. As mentioned in Chapter 2, rheology measures the material's resistance to shear deformation. In this case, the storage modulus (G) reflects the elastic part of the material's response to oscillatory stress. In contrast, the compression testing assesses how the material behaved under uniaxial compression, which provides the bulk stiffness of the material and reflects how the entire structure deforms when subjected to compressive forces. In this case, the stiffness (elastic modulus) is quantified by measuring the force applied to compress the sample and the resulting displacement (Richbourg, Rausch and Peppas, 2022).

In order to assess the differences of GelMA itself, GelMA was characterised without gelatin beads as a porogen. For the rheological characterisation, GelMA solutions of the three conditions, GelMA^{low}, GelMA^{med} and GelMA^{high} were prepared in addition of LAP photo-initiator as described previously and the solutions were then directly placed between the plates of the rheometer. Rheological analysis was performed from *in situ* photo-rheology measurements, which involves applying a rheological measurement while the material undergoes photopolymerization. This allows the measurement of how the viscoelastic properties evolve as the material cures. Rheological analysis from *in situ* photo-rheology measurements showed that the storage modulus (G') of GelMA^{med} was higher compared to GelMA^{low}, however, not significantly (**Figure 29A**). It was hypothesised that this is because a degree of functionalisation of 63% is close to the maximum cross-linking density achievable at 10% (w/v), meaning any additional methacryloyl groups are sterically hindered from cross-linking to contribute to an increased cross-linking density and hence increased G' . In contrast, the storage modulus of GelMA^{high} was significantly higher compared to GelMA^{med} (**Figure 29B**). For all different hydrogels G' remained stable over a range of frequencies and higher compared to the loss modulus (G''). Quantification of the storage modulus G' showed a notable increase in the stiffness of GelMA^{high} compared to GelMA^{low} and GelMA^{med} (**Figure 29C**). However, quantification of this experiment cannot be performed due to the limited number of samples, and therefore additional experiments need to be performed.

Results from compression testing showed similar stress over different strains for GelMA^{low} and GelMA^{high} scaffolds (**Figure 29D**) and a similar elastic modulus (**Figure 29F**). In contrast GelMA^{high} scaffolds exhibited a higher stress over different strains compared to GelMA^{med} (**Figure 29E**) and subsequently a higher elastic modulus (**Figure 29F**). This indicated that a significantly higher stiffness is achieved by increase of concentration of GelMA, however, the properties of GelMA^{med} and GelMA^{low} remained similar.

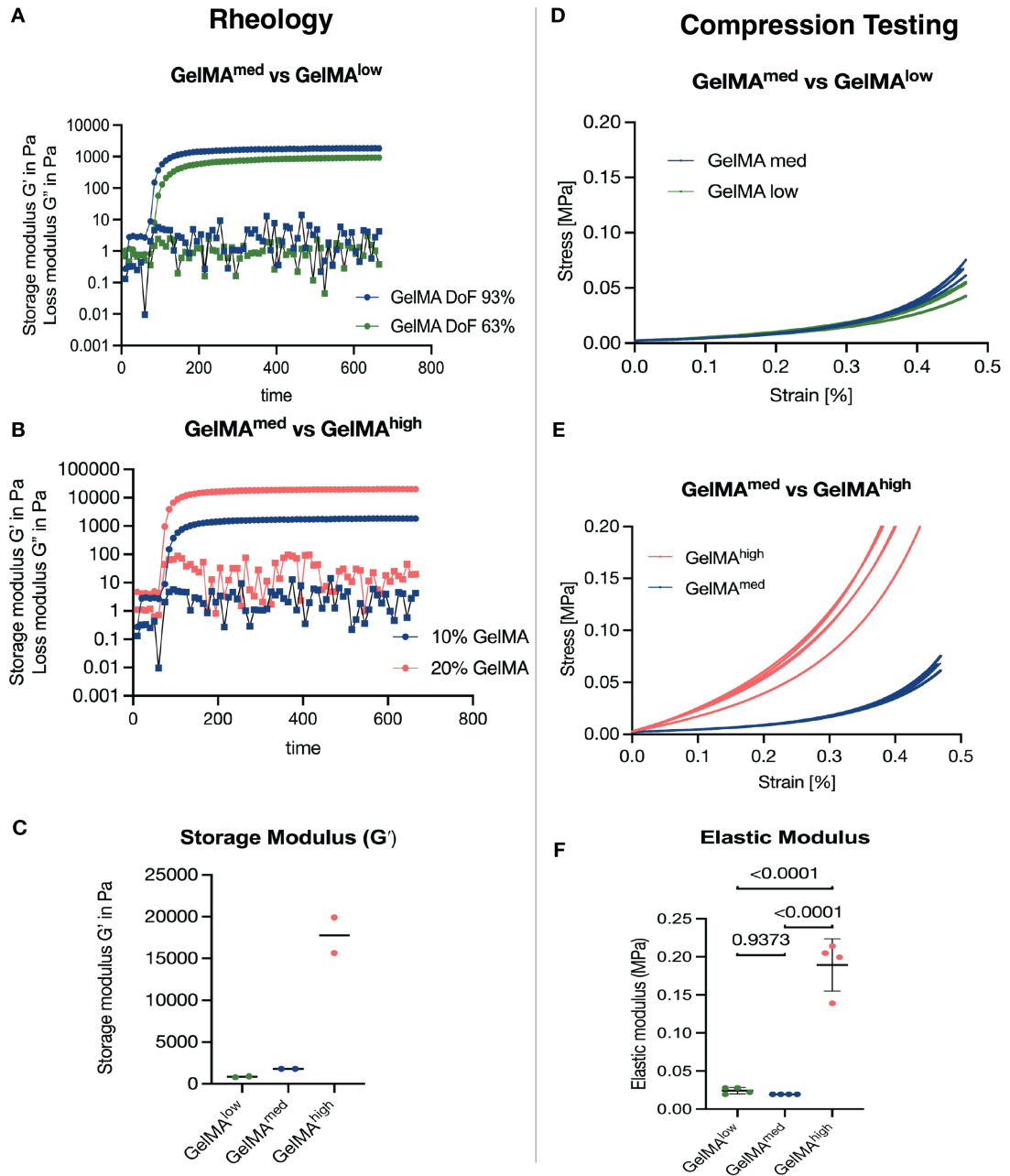


Figure 29. Characterisation of the stiffness of GelMA with different concentrations/ DoF. **A)** Rheological characterisation of *in situ* photo-rheology GelMA^{med} and GelMA^{low} from one representative sample. **B)** Rheological characterisation of *in situ* photo-rheology GelMA^{med} and GelMA^{high} from one representative sample. **C)** Storage modulus (G') from GelMA^{low}, GelMA^{med}, GelMA^{high}. No statistical analysis was performed due to the low number of samples. **D)** Compression testing analysis from GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds calculating the stress over different % of strain. **E)** Elastic modulus quantification from GelMA^{low}, GelMA^{med}, GelMA^{high} scaffolds (n = 4). **F)** Quantification of the elastic modulus from GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds performed with compression testing. Data shown as mean $\pm$ S.D (n = 4). Statistical analysis performed with Tukey's test for multiple comparisons test.

As mentioned previously, rheological stiffness typically refers to the storage modulus (G'), which is related to the elastic response of the material under oscillatory shear. For comparison with compression stiffness (elastic modulus), conversion formulas can be used based on the type of material and its mechanical behaviour. For example, for linear elastic materials, the relationship between the storage modulus G' and elastic modulus (E) is:

$$E = 2G' (1 + \nu)$$

where ν is Poisson's ratio (usually around 0.5 for soft biological materials) (Ramli *et al.*, 2022). However, as seen from the individual values of the storage modulus (G') and elastic modulus from **Figure 30**, this equation does not apply in these experiments. Key factors for this discrepancy could be explained mainly due to the differences in the measurement technique, as compression testing assesses the full bulk mechanical properties whereas rheology isolates the materials' resistance to shear. Additionally, GelMA, like other hydrogels, may exhibit anisotropic behaviour depending on the test environment. In compression testing, the material's bulk density, crosslinking and hydration level play a significant role in determining stiffness. However, in rheology, only the internal molecular network's response to sliding forces is being measured. Last, in rheological characterisation, GelMA was photo-crosslinked *in situ*, whereas in the compression testing experiment, the samples were photo-crosslinked prior to the testing. All these factors need to be considered and given that each test highlights different aspects of the material's mechanical properties, this may lead to variations in measuring stiffness.

A

Rheology					
GelMA ^{low}	Storage Modulus in Pa	GelMA ^{med}	Storage Modulus in Pa	GelMA ^{high}	Storage Modulus in Pa
1	792	1	1797	1	19916
2	906	2	1812	2	15651

B

Compression Testing					
GelMA ^{low}	Elastic Modulus in Pa	GelMA ^{med}	Elastic Modulus in Pa	GelMA ^{high}	Elastic Modulus in Pa
1	22173	1	19493	1	204994
2	27768	2	19438	2	214055
3	27764	3	19422	3	199506
4	19453	4	19458	4	138872

Figure 30. Quantification of storage modulus (G') and elastic modulus by rheology and Compression Testing analysis respectively. A) Table showing the individual values of storage modulus for GelMA^{low}, GelMA^{med}, GelMA^{high} from rheological characterisation. **B)** Table showing the individual values of elastic modulus for GelMA^{low}, GelMA^{med}, GelMA^{high} scaffolds from compression testing analysis.

4.4.2 *In vivo* assessment of changes of GelMA scaffolds with different stiffness in cell infiltration and cancer cell seeding.

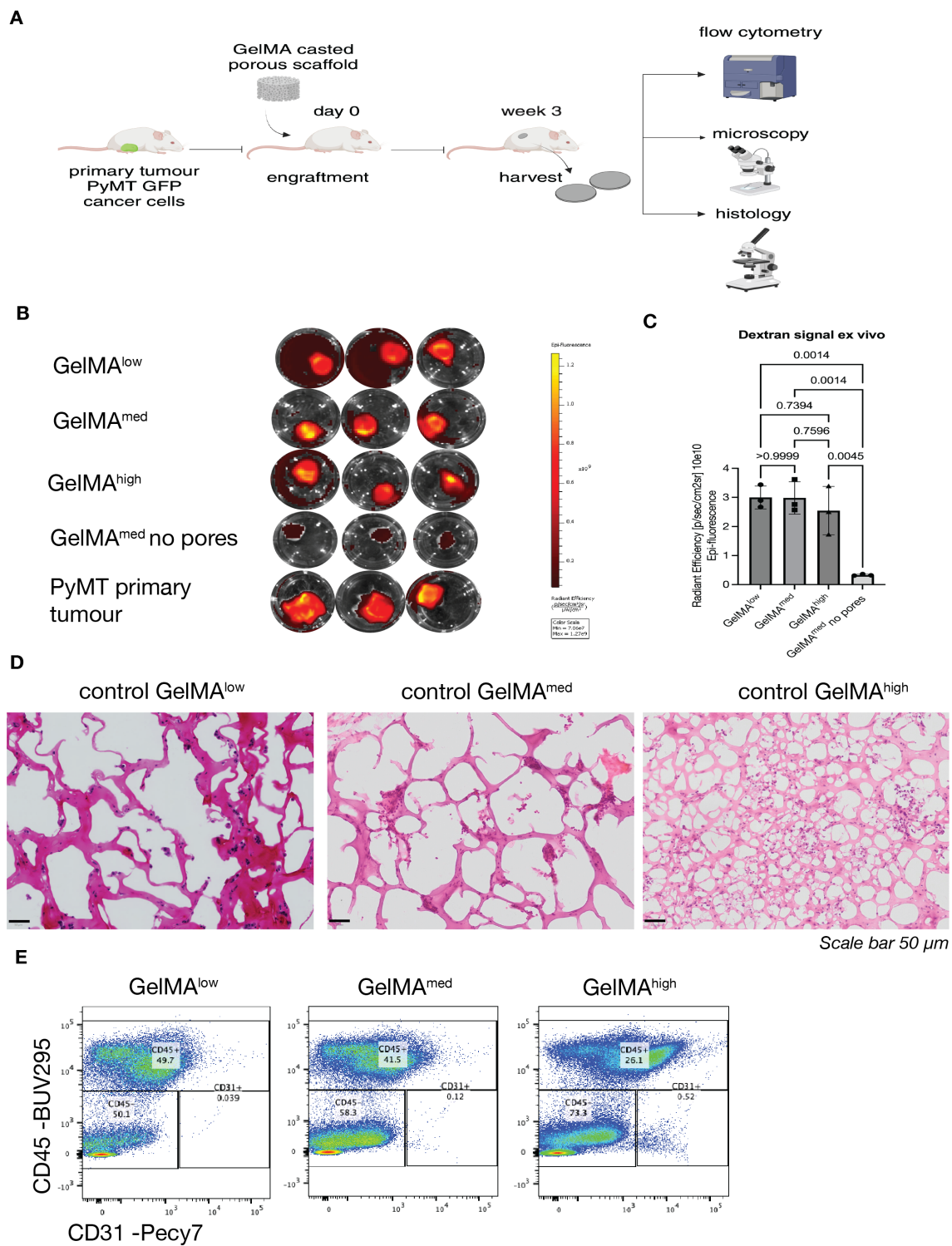
Next, it was hypothesised that the difference in the stiffness of the scaffolds would result in differences in cell infiltration *in vivo*. To test this, GelMA scaffolds were prepared as usual; using 10% (w/v) GelMA with lower DoF for GelMA^{low}, standard 10% (w/v) GelMA for GelMA^{med} and 20% (w/v) GelMA for GelMA^{high}. The ratio between GelMA and gelatin beads was consistent for all the hydrogels. First, it was assessed whether difference in the concentration of GelMA would affect the infiltration of cells within the scaffolds. For this, scaffolds were implanted subcutaneously in RagKO mice and harvested 3 weeks post implantation, according to previous experiments (**Figure 31A**). Before harvest, Texas Red-fluorescently labelled dextran (70 kDa) was intravenously injected as previously described and IVIS imaging showed that extracted scaffolds from different stiffness GelMA^{low} GelMA^{med} and GelMA^{high} were efficiently vascularised.

Notably, GelMA^{med} scaffolds without any gelatin beads as a porogen did not get equally diffused with dextran compared to porous GelMA scaffolds as seen in **Figure 31B**. Extracted PyMT primary tumours served as positive control for the diffusion of dextran. Quantification of dextran by IVIS from extracted scaffolds showed no significant difference between GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds, in contrast to GelMA^{med} scaffolds without porosity (**Figure 31C**). However, additional experiments including immunofluorescence staining for dextran and CD31 endothelial cell immunohistochemistry would be necessary in order to assess the effect of stiffness in cellular infiltration.

In order to investigate whether GelMA scaffolds with different stiffness were successfully infiltrated *in vivo*, scaffolds were then processed for cryosectioning as described in the experimental section. H&E staining from GelMA^{low} and GelMA^{med} scaffolds from control mice show similar cell infiltration, which is in line with the similar stiffness observed between GelMA^{low} and GelMA^{med} scaffolds previously. Efficient cell infiltration was also observed in GelMA^{high} scaffolds (**Figure 31D**). A denser structure was observed in GelMA^{high} scaffolds compared to GelMA^{med}, as expected because of its higher concentration, 20% (w/v) compared to 10% (w/v) (**Figure 31D**). Quantification of the total immune cell infiltrate would be essential to investigate whether GelMA^{high} scaffolds get infiltrated to the same extend as GelMA^{med} scaffolds.

Scaffolds from different stiffness were then implanted in mice bearing a fat pad primary PyMT GFP⁺ tumour and harvested 3 weeks post implantation according to **Figure 31A**. Scaffolds were then processed for flow cytometry analysis. As seen in **Figure 31E**, an increased non-immune cell infiltration (CD45⁺ population) was observed in the different stiffness conditions from scaffolds harvested from tumour bearing mice compared to scaffolds extracted from control mice without a primary tumour. This cancer-conditioning effect is more striking in the GelMA^{high} scaffolds from tumour bearing mice, where also a higher CD31⁺ population was observed. Quantification of the non-immune cell infiltrate of GelMA scaffolds from tumour bearing mice shows a significant increase, consistent in both GelMA^{med} and GelMA^{high} (**Figure 31E, 31F**). As seen previously, a higher CD31⁺ cell population was observed in GelMA^{med} scaffolds from tumour bearing

mice compared to control (**Figure 31G, left**). Noticeably, an increase in CD31⁺ cell population was observed in GelMA^{high} scaffolds compared to GelMA^{med} in mice without a primary tumour. The increased CD31⁺ cell population in GelMA^{high} without a primary tumour was to a similar level as in GelMA^{med} scaffolds from tumour bearing mice (**Figure 31G, right**). GelMA^{low} scaffolds showed similar changes as GelMA^{med} scaffolds, in support with their similar stiffness properties seen in **Figure 29**. Next, it was hypothesised that the changes in the cellular composition of the scaffold and particularly in the increased endothelial cell population would lead in changes in the cancer cell seeding. Flow cytometry analysis of GelMA^{med} and GelMA^{low} scaffolds from tumour bearing mice show similar cancer cell seeding as expected given its similar properties. Notably, GelMA^{high} scaffolds exhibited higher cancer cell seeding, when staining the samples with EpCAM and gating for GFP⁺ EpCAM⁺ population, out of CD45⁻ cells (**Figure 31H**). Quantification of the total number of EpCAM cancer cells within GelMA^{med} and GelMA^{high} scaffolds showed a significant increase of cancer cells with the GelMA scaffold with the higher stiffness (**Figure 31I**). This indicates that by changing the stiffness of GelMA, the cellular composition of the scaffold is different and subsequently a higher cancer cell seeding is achieved. Further experiments would be essential in order to assess whether the increased cancer cell seeding is solely due to the increased CD31⁺ endothelial cell infiltrate and subsequently the increased cancer cell recruitment from the vasculature or due to the increased scaffold stiffness leading to higher cancer cell adhesion and invasion. Direct co-culture of cancer cells in extracted GelMA scaffolds with different stiffness and RNA sequencing, Western blotting, or phosphorylation assays would help investigating pathways such as integrin, focal adhesion kinase (FAK), and YAP/TAZ signalling, which are mechanosensitive to assess if stiffness enhances the activation of these pathways.



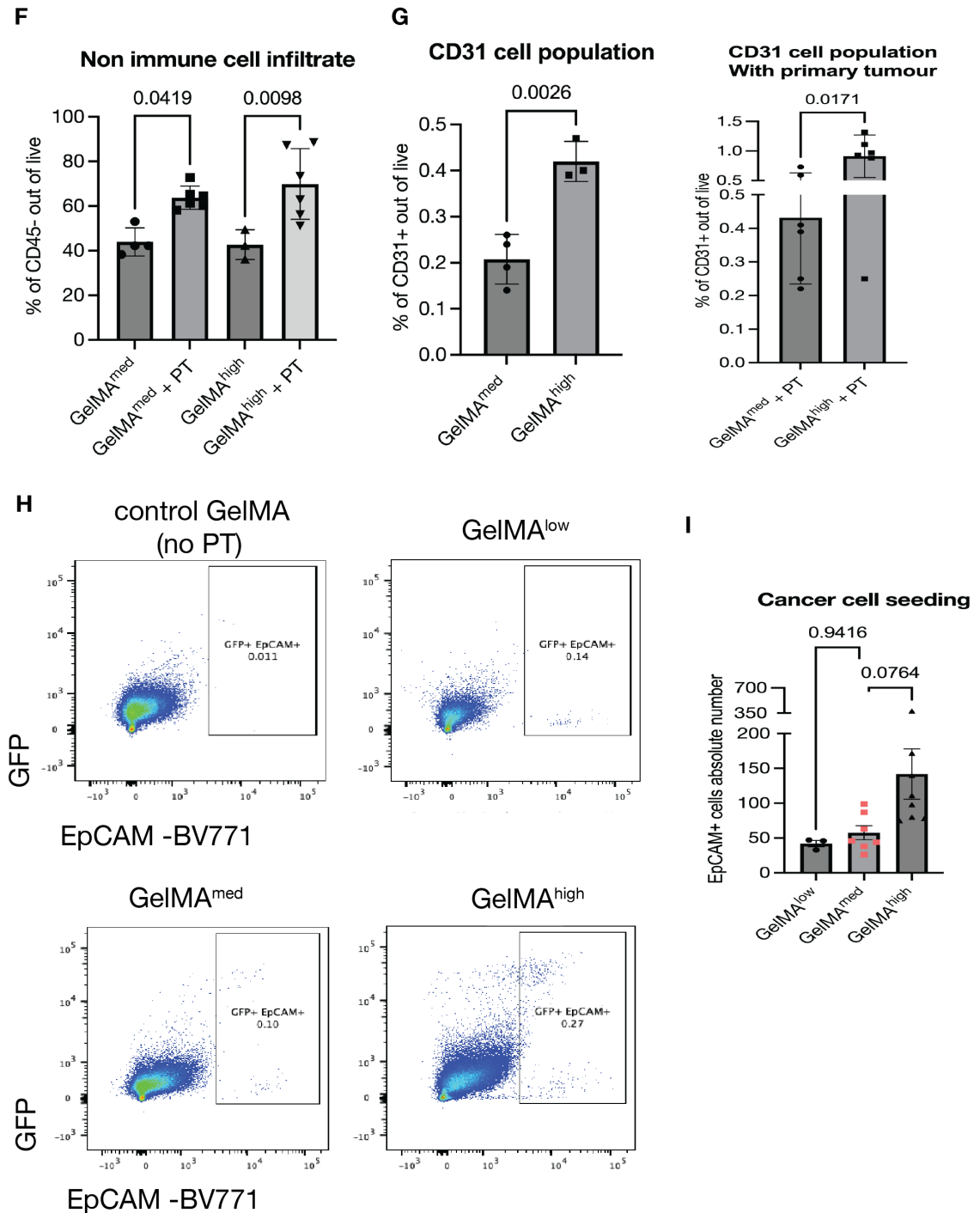


Figure 31. *In vivo* characterisation of GelMA scaffolds with different stiffness. A) Illustration of the experimental design of assessing tumour cell dissemination from a primary PyMT GFP⁺ fat pad tumour to implanted GelMA scaffolds with different stiffness. B) *Ex vivo* IVIS imaging of harvested scaffolds indicating the fluorescence from the dextran diffusion for GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds. GelMA^{med} scaffolds without gelatin beads as porogen served as a negative control for the background signal. Extracted PyMT primary tumours served as a positive control for the diffusion of dextran. C) Quantification of *ex vivo* IVIS signal from extracted porous GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds and non-porous GelMA

scaffolds. Data presented mean $\pm$ SD ($n = 3$ for all conditions). Statistical analysis performed using Tukey's test for multiple comparisons. **D)** Representative haematoxylin and eosin (H&E) staining of GelMA scaffolds with different stiffness after 3 weeks of engraftment. **E)** Representative flow cytometry plots of GelMA scaffolds with different stiffness (GelMA^{low}, GelMA^{med} and GelMA^{high}) stained for CD45 and CD31 populations gated out of live cells. **F)** Quantification of the non-immune cell infiltrate (CD45- population) of GelMA^{med} and GelMA^{high} scaffolds extracted from control or tumour bearing mice. Statistical analysis performed using Tukey's test for multiple comparisons test. **G)** Quantification of the CD31⁺ cell population of GelMA^{med} and GelMA^{high} scaffolds from control (left) and tumour bearing mice (right). Data shown as mean $\pm$ S.D. ($n = 4$ GelMA^{med}, $n = 3$ GelMA^{high}, $n = 6$ GelMA^{med} + PT, $n = 6$ GelMA^{high}). Statistical analysis performed using unpaired t-test. **H)** Representative flow cytometry plots of GelMA^{low}, GelMA^{med} and GelMA^{high} scaffolds from tumour bearing mice stained with EpCAM and gated for GFP⁺EpCAM⁺ populations. **I)** Quantification of EpCAM⁺ cells in GelMA scaffolds with different stiffness. Data shown as mean $\pm$ S.D. ($n = 3$ GelMA^{low}, $n = 7$ GelMA^{med}, $n = 6$ GelMA^{high}). Statistical analysis performed using Tukey's test for multiple comparisons test.

4.4.3 *Ex vivo* culture of GelMA scaffolds from different stiffness differentially supports cancer cell growth.

Next, it was hypothesised that porous GelMA scaffolds, after being vascularized and infiltrated with cells *in vivo*, could be used as a complex multicellular co-culture system to support cancer cell growth *ex vivo*. In order to address this, PyMT GFP⁻ cancer cells were engrafted in mice, then after one week of implantation, GelMA^{med} and GelMA^{high} scaffolds were subcutaneously implanted *in vivo*. These scaffolds were implanted in control mice, without the presence of a primary tumour. After harvesting the scaffolds, GFP⁺ PyMT cancer cells were then directly injected *ex vivo* within the scaffolds and their growth was assessed after one week in this complex *in vivo* generated co-culture system (**Figure 32A**). PyMT GFP⁻ cancer cells were used as for the primary tumour in order to distinguish them from PyMT GFP⁺ cells that were seeded after extraction of the scaffolds. In detail, 0.1×10^6 PyMT GFP⁺ cancer cells were injected in the centre of the scaffold using a 29G needle. Scaffolds were placed within a transwell insert with a 0.4- μ m pore polyester membrane in a 24-well plate. Scaffolds were cultured for 7 days in culture and the growth of PyMT GFP⁺ cancer cells was assessed with imaging. Scaffolds that were never implanted *in vivo*, and therefore only contained cancer cells were used as negative control. Indeed, it was observed that PyMT cancer cells alone within the scaffolds, grow very slow in GelMA^{med} without any cell infiltrate (*in vitro*). Strikingly, cancer cells seeded in GelMA^{med} scaffolds derived from tumour-bearing mice proliferate at a higher rate compared to GelMA^{med} control scaffolds, indicating that the scaffolds are primed *in*

vivo from the presence of the primary tumour to provide a better support for cancer cells growth. In contrast, GelMA^{high} scaffolds, which strongly increased the ability of spontaneous dissemination *in vivo* (**Figure 31I**), also promote higher cancer cell growth compared to the GelMA^{med} when explanted from naïve mice and tested for cancer cell growth *ex vivo*. It also allows cancer cells to acquire a more mesenchymal shape compared to GelMA^{med} (**Figure 32B**). However, when GelMA^{high} scaffolds explanted from mice harbouring primary tumour did not show the boost in cancer cells growth *ex vivo* observed for the cancer primed GelMA^{med}. GelMA^{high} scaffolds show a consistent high *ex vivo* cancer cells growth compared to GelMA^{med}, at a similar rate as GelMA^{med} from tumour bearing mice likely due to the increased stiffness (**Figure 32D and E**). Conversely, when using the less supportive GelMA^{med} scaffold, a strong pro-tumorigenic effect of *in vivo* cancer conditioning leading to a different cell infiltration can be measured when cancer cells growth is tested *ex vivo* (**Figure 32C**).

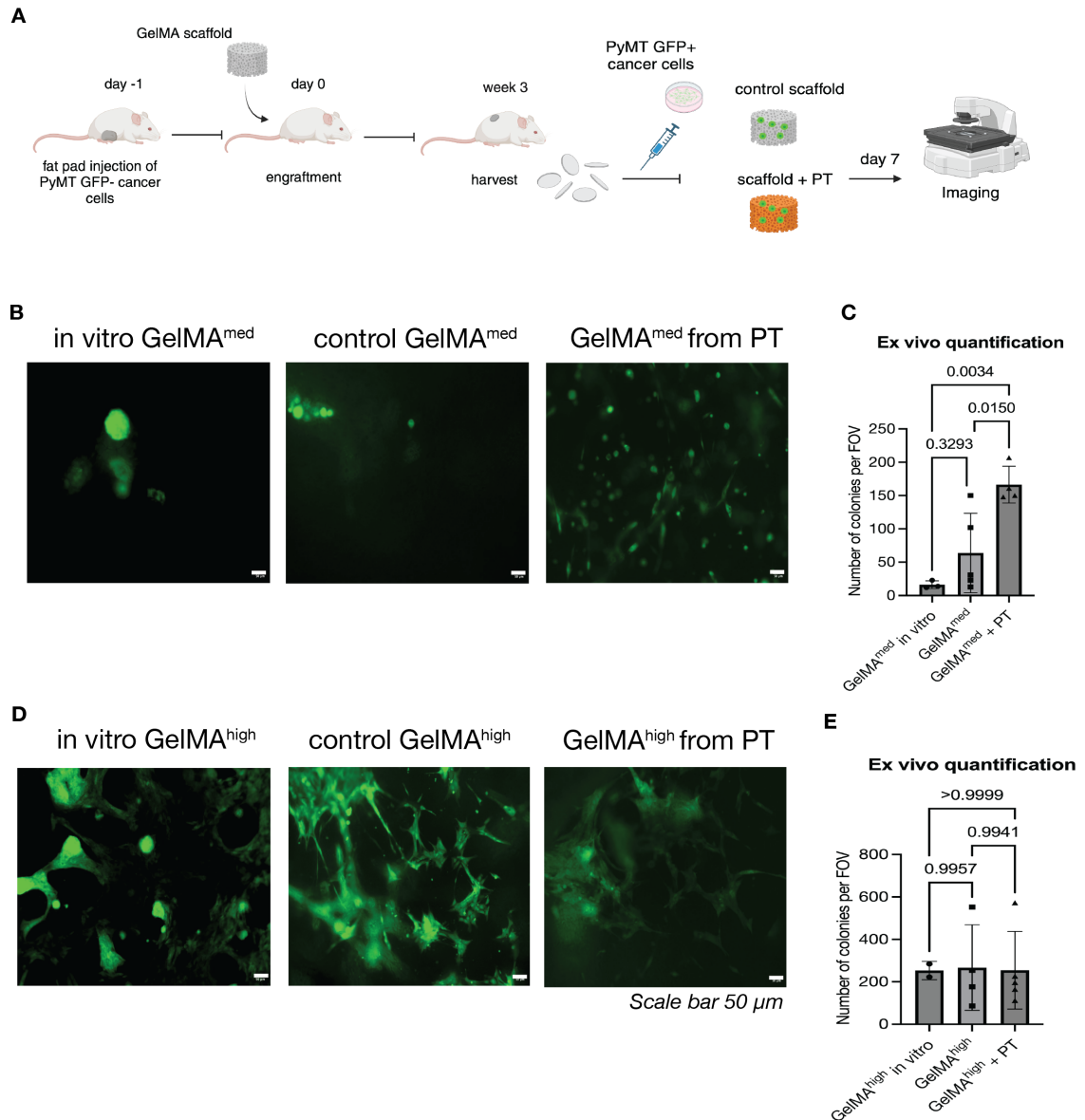


Figure 32. Ex vivo culture of GelMA primed scaffolds supports cancer cell growth.

A) Schematics illustrating the experimental approach for *in vitro* assessing the growth of PyMT GFP⁺ cancer cells in *ex vivo* GelMA^{high} and GelMA^{med} harvested scaffolds from control or tumour bearing mice. **B)** Representative images from *in vitro* seeding of GFP⁺ cancer cells in GelMA^{high} and GelMA^{med} harvested scaffolds from control and tumour-bearing mice *ex vivo* post 7 days of culture. *In vitro* scaffolds without any cell infiltrate were used as control. Scale bar 50 μ m. **C)** Quantification of PyMT GFP⁺ cancer cells seeded in *ex vivo* harvested from control GelMA^{med} and GelMA^{med} scaffolds from tumour bearing mice at day 5. Data shown as mean $\pm$ S.D. (GelMA^{med} *in vitro* n = 3, GelMA^{med} n = 5, GelMA^{med} + PT n = 4). Statistical analysis performed using Tukey's test for multiple comparisons test. **D)** Quantification of GFP⁺ cancer cells seeded in *ex vivo* harvested from control GelMA^{high} and GelMA^{high} scaffolds from tumour bearing mice at day 5. Data shown as mean $\pm$ S.D. (GelMA^{high} *in vitro* n = 2, GelMA^{high} n = 4, GelMA^{high} + PT n = 5). Statistical analysis performed using Tukey's test for multiple comparisons test.

Although this approach shows that porous GelMA scaffolds have the ability to support *ex vivo* growth of cancer cells and can also be used as an *ex vivo* culture system, several limitations need to be addressed. First, one of the main aspects that was not investigated is the degradation of GelMA *in vivo*. GelMA, as a hydrogel, is subject to degradation, which can be influenced by factors such as enzymatic activity and the local cellular environment. The presence of enzymes like matrix metalloproteinases (MMPs), produced by the cellular infiltrate (including immune cells and cancer cells), may influence scaffold breakdown. In this study, it is unclear how the GelMA scaffold might have degraded during the *in vivo* conditions, potentially creating more space for cancer cell growth during the *ex vivo* culture. It is essential to compare the degradation profiles of GelMA scaffolds from the different *in vivo* conditions (scaffolds from control mice vs. those with a primary tumour) to assess whether scaffold degradation influences *ex vivo* growth. Another key limitation is that the survival of the cellular infiltrate post-extraction and during the *ex vivo* culture was not assessed. Since scaffolds were kept in culture for 7 days after harvest, there is a possibility that a portion of the infiltrating cells may have undergone cell death. This could alter the interpretation of the observed cancer cell growth, as dead or dying infiltrate cells may not contribute to the process. Given that scaffolds were kept in culture for 7 days post harvesting in order to assess the *ex vivo* cancer cell growth, this period allows for the cellular infiltrate to undergo cell death. Immunohistochemistry and flow cytometry of the cellular infiltrate in both conditions would be essential in order to gain more insight on explaining the results.

4.4.4 *In vivo* assessment of VEGF loaded GelMA scaffolds in cell infiltration and cancer cell seeding.

Following the observation that increasing the stiffness of GelMA scaffolds, leads to higher vascularisation (*i.e.*, more CD31⁺ endothelial cells) and subsequent higher cancer cell seeding, the next step of the study was to investigate whether changes in the chemical properties of GelMA increase the presence of specific cells infiltrating the scaffold *in vivo*. Given that CD31⁺ cell population was increased in GelMA^{med} of tumour bearing mice compared to control, as well as in GelMA^{high} compared to GelMA^{med} scaffolds from control mice, it was investigated whether functionalizing GelMA scaffolds with VEGF

would lead to higher endothelial infiltration, as VEGF is known to be a pro-angiogenic factor. For this GelMA scaffolds were prepared with the same protocol as before. Within the GelMA solution, mouse VEGF was added at a concentration of 0.1 mg/mL. It was hypothesised that VEGF would gradually be released from the scaffolds (**Figure 33A**). GelMA scaffolds were incubated in 0.5 mL PBS at 37 °C after UV photo-crosslinking. A 50 μ L aliquot was collected at different timepoints, namely 30 minutes, 1h, 2h, 4h, 24h and 48h. Scaffolds were placed in fresh PBS after the collection of the aliquot, in order to assess the new release of VEGF between the timepoints. Samples were then analysed by ELISA to measure the release of VEGF at different timepoints. Indeed, it was observed that the release of VEGF was strikingly increased within the first 4h post incubation and then stabilised after 1 and 2 days (**Figure 33B**). This was expected, however, the amount of VEGF released within the first 2 days was significantly less compared to the amount of VEGF loaded within the scaffolds. As total of 85 μ L of GelMA solution was used for each scaffold and given the concentration of 0.1mg/mL of VEGF loading, 0.025 mg VEGF protein was added per scaffold. As seen in **Figure 33B**, ELISA results showed that approximately 3000 pg/mL of VEGF get released at 24h and 48h, indicating a high amount of VEGF remains within the scaffold. Scaffolds were then implanted *in vivo* and harvested 3 weeks post implantation. A significant increase of CD31⁺ cells between control and VEGF GelMA scaffolds (**Figure 33C, top**). Additionally, when scaffolds were implanted in tumour bearing mice, CD31⁺ population was increased in control GelMA scaffolds compared to when control GelMA scaffolds from control mice, which was also observed in the previous chapter. However, CD31⁺ cell population was also increased in VEGF GelMA scaffolds from tumour bearing mice compared to control GelMA scaffolds from tumour bearing mice (**Figure 33C, bottom**). Quantification showed an increase of CD31⁺ cells in VEGF scaffolds compared to control GelMA, in both control and tumour bearing mice, however only in control mice this difference was significant due to the strong increase of CD31 cells in the context of cancer (or in cancer-primed scaffolds) (**Figure 33D**). Nonetheless, when assessing whether this change in endothelial cell infiltration would have an effect in cancer cell seeding from the primary tumour, more EpCAM⁺ cancer cells were found seeded in VEGF GelMA scaffolds compared to control GelMA (**Figure 33E-F**). Quantification of the absolute number of EpCAM⁺ cancer cells showed a significant increase in the cancer cells seeded in VEGF

GelMA scaffolds compared to control indicating that the increased endothelial infiltration of GelMA scaffolds functionalised with VEGF can lead to increased spontaneous cancer cell dissemination from the primary tumour (**Figure 33F**).

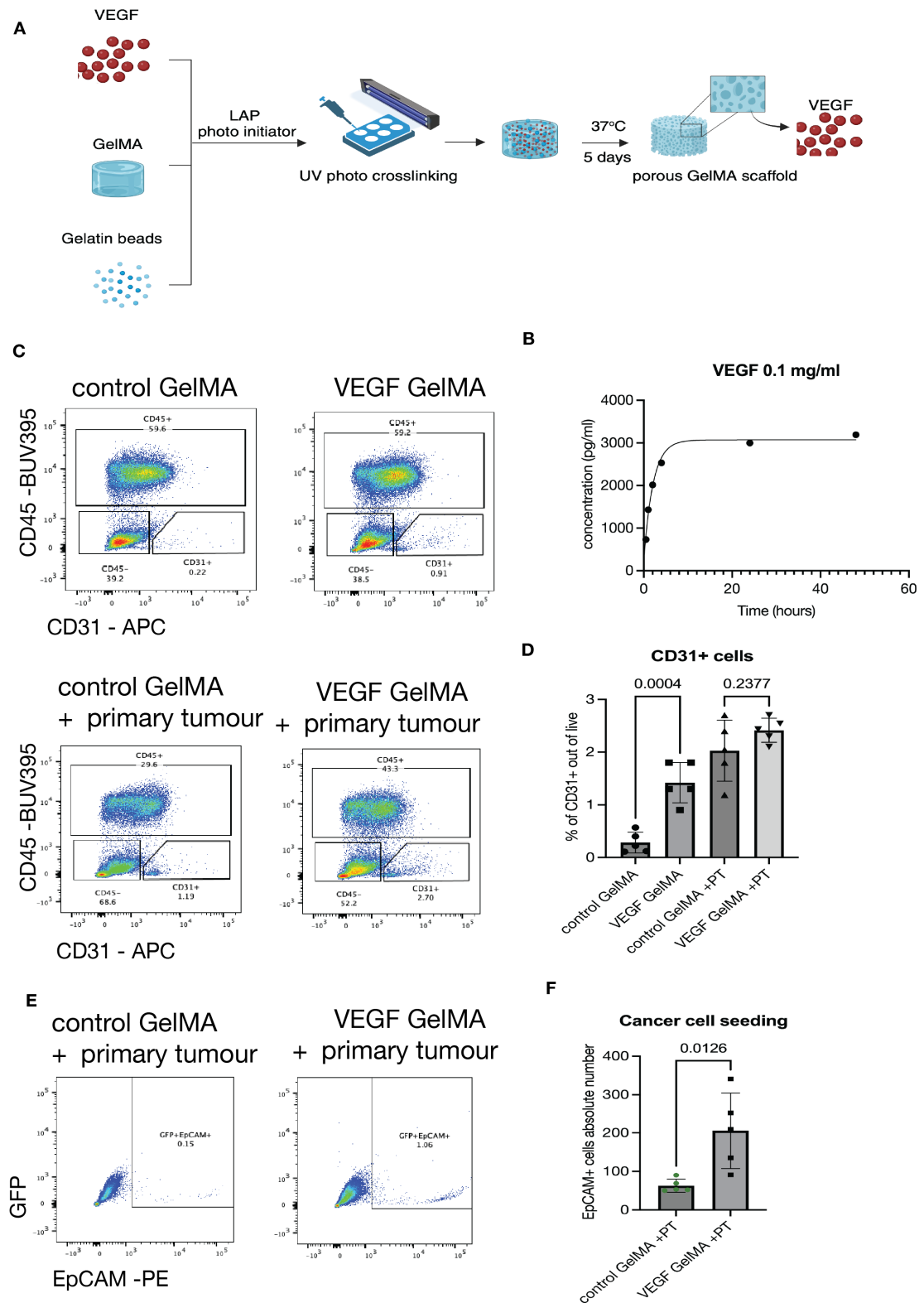


Figure 33. VEGF loading of GelMA scaffolds leads to higher vascularisation and increased cancer cell seeding. **A)** Illustration of experimental design of loading GelMA scaffolds with VEGF mouse protein. **B)** VEGF ELISA of the supernatant from GelMA scaffolds upon culture at 37 °C for different timepoints. Data shown as mean from three different biological replicates.

C) Representative flow cytometry plots from GelMA scaffolds after harvest from control mice (top) and tumour bearing mice (bottom) and gated for CD45 and CD31 cell populations. **D)** Quantification of CD31⁺ cell population in control and VEGF GelMA scaffolds from control and tumour bearing mice. Data shown as mean $\pm$ S.D (n = 5). Statistical analysis was performed using Tukey's test for multiple comparisons. **E)** Representative flow cytometry plots of GFP⁺ EpCAM⁺ cancer cells from control and VEGF GelMA scaffolds of tumour bearing mice. **F)** Quantification of EpCAM⁺ cancer cells in control and VEGF GelMA scaffolds from tumour bearing mice. Data shown as mean $\pm$ S.D (n = 5). Statistical analysis performed using unpaired t-test.

4.4.5 *Ex vivo* culture of VEGF loaded GelMA scaffolds support cancer cell growth.

In order to gain insight whether the VEGF loaded GelMA scaffolds could support cancer cell growth *ex vivo*, scaffolds were used as an *ex vivo* culture system as described previously. In detail, PyMT GFP⁻ cancer cells were engrafted in the fat pad of mice and one week later, control and VEGF loaded porous GelMA scaffolds were engrafted subcutaneously. Scaffolds were also implanted in mice without the presence of the primary tumour that served as control. After harvesting the scaffolds, PyMT GFP⁺ cancer cells were directly seeded within the harvested GelMA scaffolds, and their growth was assessed after 7 days (**Figure 34A**). Indeed, similar results were obtained as before; namely cancer cells seeded in control GelMA scaffolds derived from tumour bearing mice proliferate more compared to control GelMA scaffolds, showing that GelMA scaffolds are primed *in vivo* by the primary tumour to create a cellular environment favouring growth. Strikingly, higher cancer cell growth in VEGF scaffolds from naïve mice was observed, and their growth matches the growth of cancer cells from control GelMA scaffolds from tumour bearing mice. Last there was no difference between VEGF GelMA scaffolds from control and tumour bearing mice, indicating that the cellular infiltrate recruited from VEGF can recreate a cellular context similar to the one induced by the cancer-conditioning in supporting cancer cell growth (**Figure 34B, 34C**). However, as mentioned in section 4.4.3, the limitations of the GelMA degradation and remodelling over time as well as the survival of the cellular infiltrate would need to be addressed in order to better support these results. Last but not least, full characterisation of the differences in the cellular infiltrate and the interaction between cancer cells and their microenvironment would be essential in order to gain insight on the mechanistic aspect of this observation.

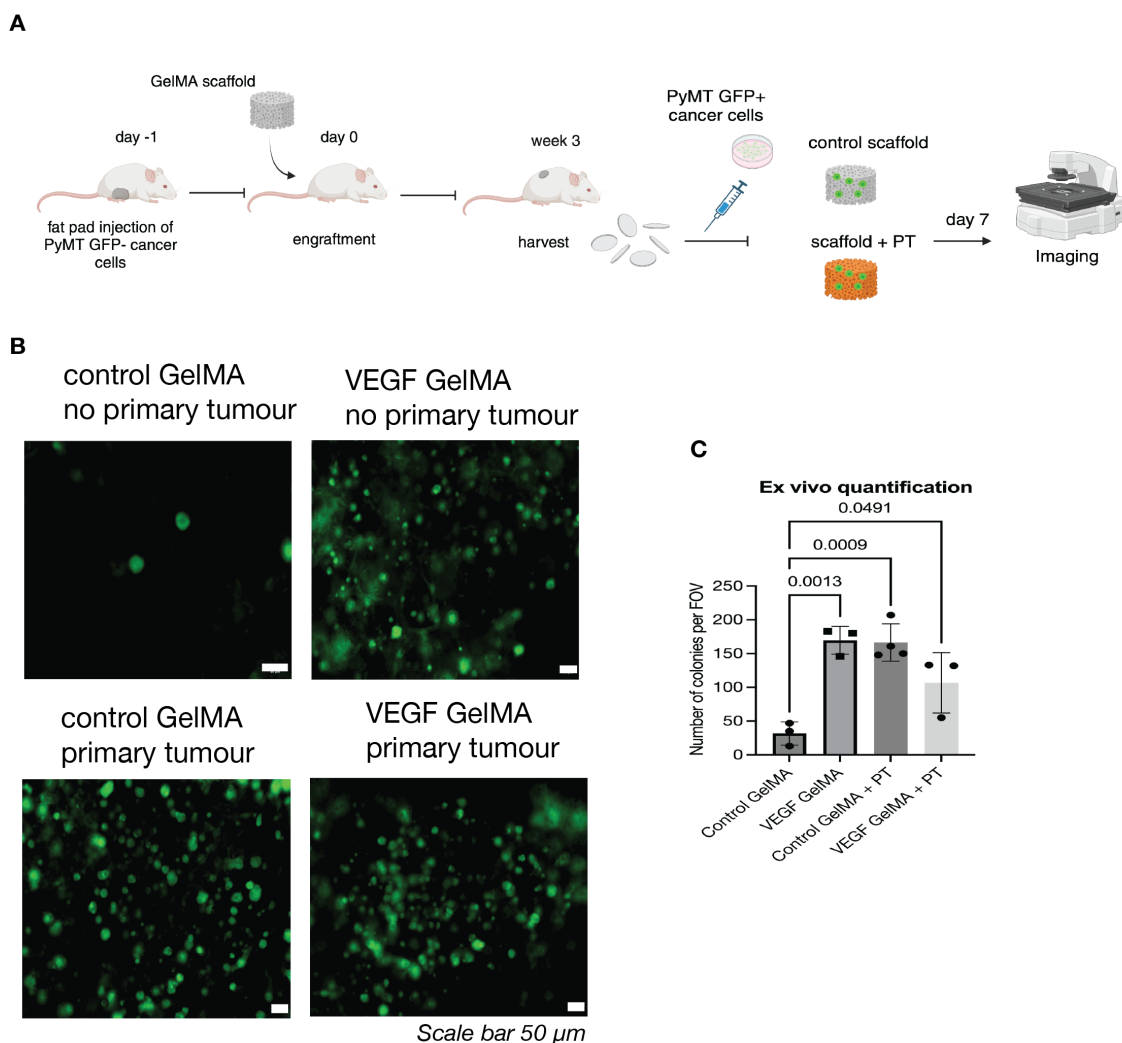


Figure 34. *Ex vivo* culture of VEGF loaded scaffolds supports cancer cell growth. **A)** Schematics illustrating the experimental approach for *in vitro* assessing the growth of PyMT GFP cancer cells in *ex vivo* GelMA and VEGF GelMA scaffolds from control or tumour bearing mice. **B)** Representative images from *in vitro* seeding of GFP⁺ cancer cells in control or VEGF GelMA harvested scaffolds from control and tumour-bearing mice *ex vivo* post 5 days of culture. Scale bar 50 μ m. **C)** Quantification of PyMT GFP⁺ cancer cells seeded in *ex vivo* harvested control and VEGF GelMA scaffolds from control or tumour bearing mice at day 5. Data shown as mean $\pm$ S.D (n = 3). Statistical analysis was performed using Tukey's test for multiple comparisons.

4.5 Conclusions & Future work

The aim of this chapter was to explore the effect of change in the physicochemical properties of GelMA as a material in the host response. One aspect is tuning the stiffness of GelMA, which can be modified with various methods, such as changing the degree of

methacrylation (DoF) or the concentration among others. The first question was whether the increase or decrease of the stiffness would have any effect in cell infiltration *in vivo*. A decrease of the stiffness of GelMA was achieved by lowering the degree of methacrylation from 93% (GelMA^{med}) to 63% (GelMA^{low}). However, the rheological properties of GelMA and compression testing showed no significant differences between the two conditions. One explanation would be because a degree of functionalisation of 63% is close to the maximum cross-linking density achievable at 10% (w/v), meaning any additional methacryloyl groups are sterically hindered from cross-linking to contribute to an increased cross-linking density, and hence increased G'. An *in vivo* assessment of GelMA^{low} scaffolds showed similar cell infiltration and subsequently similar cancer cell seeding from the primary tumour, which reflects the similar properties observed by rheology and compression testing.

Next, increase of the stiffness of GelMA was achieved by increase of the concentration of GelMA from 10% (w/v) (GelMA^{med}) to 20% (w/v) (GelMA^{high}). GelMA^{high} scaffolds showed a significant increase in storage modulus G' by rheology and significantly higher elastic modulus by compression testing. The overall stiffness of GelMA increased from 1 kPa to 10 kPa. Increasing stiffness by increasing GelMA's concentration may lead to decreased porosity, which can limit cell infiltration and nutrient transport. Additionally, increased concentration may reduce the material's biocompatibility, as the gel becomes less hydrated, and cells might encounter difficulties migrating and interacting with the stiffer hydrogel. An *in vivo* assessment of GelMA^{high} scaffolds denser structure of GelMA, which was evenly infiltrated by host cells showing that the porosity of GelMA^{high} scaffolds was not affected. Additionally, cellular infiltration was also observed within the GelMA^{high} scaffolds, both at the inner and outer part of the scaffold, indicating that increased GelMA concentration did not massively affect the material's biocompatibility. However, quantification of the total cell infiltrate would be required in order to make this conclusion. Although the immune cell infiltrate of the GelMA^{med} and GelMA^{high} scaffolds was not fully characterised, GelMA^{high} scaffolds showed increased endothelial cell infiltration and subsequent increased cancer cell seeding compared to GelMA^{med} scaffolds, indicating that the change in stiffness alters the cell infiltrate and cancer cell

seeding within the scaffolds. Whether this change is directly related to the increased stiffness or due to the increased endothelial cell population, it is yet to be addressed.

Use of GelMA scaffolds as a complex *ex vivo* culture system for cancer cells growth revealed that GelMA^{med} scaffolds, in presence of a primary tumour, strongly change its cell infiltrate composition and provide a better support for cancer cell growth, suggesting that this artificial environment is subject to similar cancer-conditioning reported at pre-metastatic sites (Patras *et al.*, 2023). In contrast, GelMA^{high} scaffolds promote higher cancer cell growth and show a more mesenchymal shape compared to GelMA^{med}, suggesting that stiffness affects cancer cell attachment within the scaffold. Given the complexity of this system, several limitations need to be addressed for the support of this claim. Comparative analysis of the cellular composition and GelMA integrity between scaffolds from control and tumour-bearing mice would clarify the contribution of these factors to the experimental outcome. By addressing these limitations and conducting further experiments, the study could offer a more comprehensive understanding of the interactions between GelMA scaffolds, cancer cells, and the host environment *in vivo*, as well as how these factors influence *ex vivo* growth.

Next, the potential of GelMA to be functionalised with specific factors was explored in order to influence the cell infiltration. Given the changes in the endothelial cell population in scaffolds in the presence of primary tumour, as well as the increase in endothelial cell infiltration in GelMA^{high} scaffolds compared to GelMA^{med}, scaffolds were loaded with VEGF protein, in order to increase the degree of vascularisation. Indeed, when VEGF scaffolds were implanted *in vivo*, a higher amount of CD31⁺ endothelial cells was observed compared to control GelMA scaffolds. Interestingly, increased vasculature by VEGF loading leads to a subsequent increase in DTC recruitment.

By using VEGF GelMA scaffolds as an *ex vivo* culture system to monitor cancer cell growth, it was observed that PyMT cancer cells grow more in VEGF GelMA scaffolds compared to control GelMA, to a similar level as control GelMA scaffolds primed from the primary tumour. This suggests that GelMA scaffolds represent a pre-metastatic microenvironment, and that the presence of the primary tumour can modify even artificial

microenvironment like implanted scaffolds. However, further experiments in order to address the limitations of this model would be required in order to support this claim.

Chapter 5. Perspective & Conclusions

Metastasis is a complex process which progresses through a stepwise cascade of events including invasive tumour growth, angiogenesis, stromal invasion, extravasation, and colonisation at distant sites. It is established that the tumour microenvironment plays an important role in the metastasis process through complex molecular mechanisms. Identifying the mechanisms has been an imperative for years. The last decades, the field of bioengineering has made significant contributions to study biology of metastasis in several ways. *In vitro* models that mimic the complex tumour microenvironment, including 3D cell culture systems, organ-on-a-chip platforms and tumour organoids have allowed researchers to study the interactions between cancer cells and their surrounding stroma, blood vessels and immune cells in a controlled microenvironment. Scaffolds, hydrogels, and different matrices have been proven to mimic the extracellular matrix and provide a physical support for cell growth and differentiation and have shifted the mindset from conventional 2D cultures to 3D models. Last, these systems allow the investigation of the mechanical forces and physical properties of the tumour microenvironment and their effects of cancer cell behaviour and shed light in way mechanical cues influence processes such as cell migration, invasion, and metastasis.

Apart from advances studying cancer cell interactions *in vitro*, scaffolds have played a significant role *in vivo*, either by mimicking a specific tissue or by creating an artificial microenvironment for cancer cell interactions with the host. A requirement for implantation of scaffolds *in vivo* is the non-toxicity and the biocompatibility of the chosen material. Synthetic materials have been used for the development of scaffolds due to their strong mechanical properties and the ability to fine-tune their properties, however they usually require further modifications with natural polymers in order to interact with the host. Semi-synthetic materials have provided a good alternative as they can retain some of the desirable characteristics of natural polymers, such as biocompatibility and bioactivity, while offering improved mechanical properties, tunability, and stability.

In this work, a bioengineered scaffold was developed using methacrylated gelatin (GelMA) with the aim to study cancer cell interactions *in vivo*. One aspect focused on the choice of material. GelMA is a hydrogel derived from gelatin, a natural protein which can be found in animal tissues, primary from skin and bones. It is a semi-synthetic material created by modifying gelatin through the addition of methacryloyl groups which allow for crosslinking of the gel under UV light in the presence of a photo-initiator. Through photo-crosslinking, GelMA forms a stable hydrogel network, combining the biocompatibility, tuneable properties and ability to support cell growth. The choice of a semi-synthetic material was to induce a less pronounced foreign body response that synthetic materials create when implanted *in vivo* (Dondossola *et al.*, 2016), while retaining the tuneable properties and mechanical strength that synthetic materials offer. GelMA hydrogels have been shown to have great biocompatibility *in vitro* and *in vivo*, due to the presence of RGD motifs that ensure the preservation of cell adhesion. Additionally, GelMA obtains MMP sensitive motifs that allows the hydrogel matrix to be remodelled. Work from various groups have shown that GelMA with different degree of methacrylation (DoF) and subsequently different stiffness allows for different morphology of cell attachment *in vitro* (Liu and Chan-Park, 2010; Pepelanova *et al.*, 2018). *In vivo*, Chen *et al.* has shown that seeding MSCs and human endothelial colony-forming cells (ECFCs) in GelMA hydrogels successfully induces formation of capillary-like networks, indicating that cells remain viable encapsulated in GelMA hydrogels and can differentiate *in vivo*. However, this work showed that GelMA hydrogels with lower DoF mechanically collapsed after 28 days compared to GelMA hydrogels with higher DoF. This highlights the importance of keeping a balance between maintaining an intact structure and cell infiltration.

For this study, fish GelMA was the material of choice. The work from Elkhoury *et al.* levels (Elkhoury *et al.*, 2021). and Zaupa *et al.* (Zaupa *et al.*, 2019) collectively demonstrate that while fish GelMA exhibits some lower mechanical strength compared to porcine GelMA (due to differences in amino acid composition like proline and hydroxyproline), it maintains similar cell viability and may have an enhanced capacity for matrix remodelling, which could be advantageous for certain biological applications. Furthermore, despite some differences in integrin binding due to amino acid variations,

fish collagen retains key binding motifs (such as GFOGER and RGD) that allow it to interact effectively with mammalian integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins (Yamada *et al.*, 2014). These findings suggest that fish GelMA could be a viable alternative to porcine GelMA, particularly in situations where the biodegradability or remodelling capacity of the matrix is crucial.

The second aspect focused on the porosity of the bioengineered scaffold. A porous scaffold is essential for creating a large surface area for cell attachment and facilitate nutrient and water exchange, cell infiltration and tissue growth. Small pores facilitate crucial molecular transport necessary for nutrition, waste removal, and signalling, while larger pores promote cell migration, capillary formation, and the integration of nerves and blood vessels (Fierz *et al.*, 2008; Rasoulianboroujeni *et al.*, 2017). Additionally, non-porous materials tend to elicit a more severe foreign body response, characterized by the formation of a thick fibrous capsule. This capsule acts as a barrier between the implant and surrounding tissue, limiting integration and function.

GelMA scaffolds were engineered with a high degree of methacrylation (DoF = 93%) and defined porosity using porcine gelatin beads (average size 70 μm). It was shown that gelatin beads can dissolve at 37 °C and create a porous system with an interconnected GelMA structure. This is in line with previously published work from the Stevens group (Ouyang *et al.*, 2022). Rheological characterisation of the bioengineered scaffolds showed a stiffness of 400 Pa, which is physiologically relevant compared to the range of healthy mouse and human tissues (Guimarães *et al.*, 2020; Micalet, Moeendarbary and Cheema, 2023). Based on the work of Micalet *et al.*, achieving a physiologically relevant stiffness is critical for accurately modelling cell behaviour, stem cell differentiation, tissue repair, and disease progression. Achieving the correct stiffness ensures that engineered tissues and *in vitro* models can more faithfully replicate the mechanical environment found in the body, leading to better outcomes in tissue engineering (Micalet, Moeendarbary and Cheema, 2023)

The next hypothesis of this project was that porous GelMA scaffolds would be able to trigger a host response following subcutaneous implantation *in vivo*. Indeed,

bioengineered scaffolds were able to get highly infiltrated after subcutaneous implantation in immunocompetent mice, incorporating stromal cells, endothelial cells, and immune cells, predominantly of myeloid origin. Importantly, implantation of porous GelMA scaffolds did not trigger a substantial-elicited foreign body response, as only a low number of immune cells of lymphoid origin were present after 3 weeks of implantation. The structure of the GelMA scaffolds remained intact, in contrast with implantation of GelMA scaffolds with lower concentration that mechanically collapsed *in vivo* after 3 weeks. One important aspect that was not investigated in this study is the degradation rate of the GelMA scaffolds following *in vivo* implantation. This is particularly crucial as the degradation rate can affect the long-term immune response. As the scaffold degrades, it may release degradation byproducts that could elicit an immune response or trigger additional inflammation. Evaluating the degradation rate would provide insight into whether biodegradation products contribute to any late-stage immune reactions.

Highlighting the importance of an interconnected structure with a porous system, it was shown that implantation of GelMA scaffolds without gelatin beads as a porogen failed to integrate with the host *in vivo*. Porous GelMA scaffolds successfully induced the formation of functional blood vessels *in vivo*, without prior cell seeding or encapsulation of cells. Previous studies using GelMA hydrogels required co-culture of MSCs and ECFCs in GelMA hydrogels prior to implantation for the successful formation of functional anastomoses between the bioengineered vascular network and the mouse vasculature (Chen *et al.*, 2012). This bioengineered model shows that gelatin beads of 70 μm was sufficient to induce cell infiltration and functional blood vessel formation *in vivo*. Quantification of the pore diameter from histology sections of extracted scaffolds showed an average size of 150 μm , in contrast to non-porous GelMA scaffolds which exhibited microporosity of average 40 μm . This negatively impacted the successful infiltration of host cells within the non-porous scaffolds.

The cellular infiltrate within GelMA scaffolds primarily consists of CD11b⁺F4/80⁺ macrophages, which play a pivotal role in the foreign body response. The host response to GelMA is in line with other studies showing that GelMA induces a limited

inflammatory response, primarily driven by macrophages, with low levels of pro-inflammatory cytokines being expressed (Ullm *et al.*, 2014). These macrophages, derived from the monocyte lineage, are crucial for mediating inflammation and facilitating tissue repair in response to the presence of foreign materials. Alongside macrophages, endothelial cells are present throughout the scaffolds, indicating active angiogenesis and the potential formation of blood vessels. The infiltration of these cells is essential for establishing a supportive microenvironment, which is necessary for both the healing process and the stabilization of the implant. Functional characterization through perfusion assays has demonstrated the presence of blood vessels, further underscoring the scaffolds' ability to integrate with host tissue. This interplay between macrophages, endothelial cells, and the resultant vascularization highlights the dynamic nature of the FBR, as it orchestrates a coordinated immune response while promoting healing and integration of the GelMA scaffolds into the surrounding tissue.

Although the biocompatibility of GelMA has been well characterised *in vivo*, the use of GelMA to study cancer cell interactions has been limited. Most of the studies have used GelMA for direct culture of cancer cells from different cell types, including ovarian cancer cells (Kaemmerer *et al.*, 2014), breast cancer cells (Arya *et al.*, 2016; Loessner *et al.*, 2016) or prostate cancer cells (Bessot *et al.*, 2023) *in vitro* followed by the implantation of pre-seeded hydrogels *in vivo*. Given the fact that porous GelMA bioengineered scaffolds have the ability to get highly infiltrated *in vivo*, it was investigated whether scaffolds would achieve to capture disseminated cancer cells (DTCs) from a primary tumour. Indeed, when porous GelMA scaffolds were implanted in tumour bearing mice, DTCs were found within the scaffold. Importantly porous GelMA scaffolds were broadly perturbed in the presence of the primary tumour, with higher stromal population compared to porous GelMA scaffolds implanted in naïve mice. In addition, it was observed the remodelling of GelMA scaffolds from tumour bearing mice, indicating that the composition of the extracellular matrix (ECM) of the hydrogel changes as consequence of the increased stromal presence. Further experiments would shed light into the type of remodelling occurring within the scaffolds. In conclusion, it was shown that the presence of a primary tumour can have a pre-metastatic effect even

an implanted porous GelMA scaffold and systemically perturb this artificial tissue in order to favour metastatic dissemination (Patras *et al.*, 2023).

Establishing a supportive local niche at a foreign site upon dissemination poses a significant bottleneck for the outgrowth of DTCs upon arrival in distant organs (Sleeman, 2012; Martin *et al.*, 2015; Visser and Joyce, 2023). Crucially, cancer can systematically disturb distant organs through the release of factors and inflammatory molecules, even prior to the dissemination of DTCs, thereby facilitating the process of metastatic colonization (Peinado *et al.*, 2017). The perturbation of the GelMA scaffolds in tumour bearing mice shows that cancer can strongly influence an artificial tissue similarly to any other endogenous organ and implanted scaffolds can act as an artificial pre-metastatic microenvironment *in vivo*. This is in line with the work from Rao *et al.*, which showed that the immune response within PCL implanted scaffolds, dramatically changed following primary tumour engraftment (Rao *et al.*, 2016).

Another aim of this project was to study whether perturbation of GelMA scaffolds from the primary tumour would have a systemic effect in metastasis of other sites. Latest work from the Shea group showed that the inflammatory response caused by the implantation of synthetic biomaterial scaffolds leads to eradication of DTCs within the scaffold and a systemic response that leads to reduced metastatic burden in the lung (Rao *et al.*, 2016; Wang *et al.*, 2023). The results obtained from this work showed that although there was a trend in reduced tumour burden in the lung, there was no difference in the cellular composition of the lung in the presence or absence of scaffolds in tumour bearing mice. One explanation could be that GelMA scaffolds do not induce an inflammatory response cause by foreign body reaction in the same level as a synthetic biomaterial. However, additional experiments would be required to investigate this.

Leveraging the ability to finetune the properties of GelMA, one aspect aimed to modulate cancer cell seeding of DTCs by increasing the stiffness of the scaffolds. The physical properties of GelMA can be modulated by various parameters including the degree of functionalisation (DoF), the concentration of GelMA, the type and concentration of the photo-initiator and the exposure time. The stiffness of the GelMA was increased by an

increase of its concentration. Rheological characterisation showed that the stiffness was increased from 0.8-1 kPa to 10 kPa. Implantation of GelMA scaffolds with higher stiffness showed that the infiltration and vascularisation is not affected, in contrast, an increased endothelial cell population was observed in scaffolds with higher stiffness. Additionally, it was noticeable that GelMA scaffolds with higher stiffness achieved a higher cancer cell seeding of DTCs compared to GelMA scaffolds with lower stiffness. Matrix stiffness is known to contribute to cancer progression and is associated with cancer cell adhesion, migration, and invasion (Nieberler *et al.*, 2017; Ishihara and Haga, 2022). It is known that cancer tissues are stiffer than normal tissues, for instance the mammary cancer tissue is stiffer (~4 kPa) than the normal mammary tissue (~0.2 kPa) (Paszek *et al.*, 2005), with altered integrins. Increased concentration of GelMA indicates higher amount of tripeptide motif Arg-Gly-Asp (RGD). Eight members of the integrin superfamily recognise the RGD motif within extracellular proteins (D'Souza, Ginsberg and Plow, 1991; Nieberler *et al.*, 2017). Therefore, the increased concentration of GelMA and subsequent higher amount of RGD motifs would explain the higher cancer cell seeding within the GelMA scaffold.

Additionally, it was hypothesised that primed GelMA scaffolds from the presence of the primary tumour could act as a tumour supportive environment. Infiltrated GelMA scaffolds were successfully used *ex vivo* for assessing the growth of cancer cells and it was shown that in GelMA scaffolds with lower stiffness, perturbed by the primary tumour scaffolds presented a supportive microenvironment for *ex vivo* cancer cell growth. This shows that GelMA scaffolds can be used as a complex *ex vivo* culture system for the study of tumour cell interaction with their microenvironment. Given the differences observed in cancer cell growth from *in vivo* primed scaffolds, this complex multicellular co-culture system can be used to gain insight into mechanisms of the pre-metastatic niche. Further use of this system would offer a valuable tool to dissect the interaction between cancer cells with their microenvironment, combining the benefits both from *in vivo* and *in vitro*.

Finally, another option for the use of GelMA scaffolds as an artificial microenvironment is by the incorporation of different factors during photo-crosslinking. Given the observation of an increased endothelial cell population in perturbed from the tumour host

scaffolds compared to the control, and in scaffolds with higher stiffness, it was hypothesised that functionalisation of GelMA scaffolds with the pro-angiogenic vascular endothelial growth factor (VEGF) would have an effect in the scaffold infiltration *in vivo*. VEGF plays an important role in angiogenesis and can bind two receptors (VEFR-1 and VEGF-2) which are expressed by vascular endothelial cells. Additionally, VEGF is a key mediator of angiogenesis in cancer as it facilitates tumour cell survival through its immunosuppressive functions. This is achieved by degrading the ECM and attracting tumour cells (Carmeliet, 2005). In this study it was shown that VEGF can get gradually released from GelMA scaffolds *in vivo*. This resulted in increased infiltration and vascularisation of VEGF loaded GelMA scaffolds compared to control GelMA, and, subsequently, increased cancer cell seeding in tumour bearing mice. *Ex vivo* culture of VEGF loaded GelMA scaffolds from naïve mice also showed differences in cancer cell growth indicating that the differences in the cellular infiltration due to the VEGF play a role in cancer cell growth. This highlights the advantage of GelMA scaffold as an artificial platform to study cancer cell interactions.

Overall, in this study, a GelMA scaffold with defined porosity and physicochemical properties was successfully developed. It was shown that porous GelMA scaffolds can successfully get infiltrated *in vivo*, with the incorporation of stromal cells, endothelial cells and immune cells, with macrophages as the predominant population. In the presence of a primary tumour, porous GelMA scaffolds get highly perturbed with changes in its cellular composition. Additionally, DTCs have the ability to spontaneously disseminate to the scaffolds. The cancer-primed GelMA scaffolds support the growth of cancer cell growth *ex vivo*, confirming that they functionally represent a pre-metastatic niche. Leveraging the potential of finetuning the physicochemical properties of GelMA, it was shown that increase of the stiffness of GelMA or functionalisation with VEGF leads to higher cancer cell seeding *in vivo* as well as changes in cancer cell growth *ex vivo*.

Chapter 6. Appendix

PhD publications

Rizou, T., Wojciechowski, J. P., Blasio, S. D., Callens, S., Ouyang, L., Echalier, C., Bridgeman, V. L., Rodrigues, F. S., Karoutas, A., Stevens, M. M., Malanchi I. (2024). Creating an artificial *in vivo* pre-metastatic microenvironment using porous GelMA scaffolds. (*In preparation*)

Blasio, S. D., **Rizou, T.**, Gay, L., Rabas, N., Chakravarty, P., Snoeks, T., Karoutas, A., Rodrigues, F., Bridgeman, V., Fan, X., Bonnet, D., Abarategi, A., & Malanchi, I. (2023). An extramedullary bone model to study metastatic dormancy reveals a piecemeal model for metastatic reactivation. <https://doi.org/10.21203/rs.3.rs-3173228/v1> (Preprint)

Cioce, A., Calle, B., **Rizou, T.** *et al.* Cell-specific bioorthogonal tagging of glycoproteins. *Nat Commun* **13**, 6237 (2022). <https://doi.org/10.1038/s41467-022-33854-0>

Rodrigues, F., Karoutas, A., Rabas, N., Ruhland, S., **Rizou, T.**, Blasio, S. D., Bridgeman, V. L., Ferreira, R. M. M., Goldstone, R., Sopena, M. L., Lee, J.-H., Ombrato, L., & Malanchi, I. (2023). Bidirectional Activation of Stem-Like Programs between Metastatic Cancer and Alveolar Type 2 Cells within the Niche. <https://dx.doi.org/10.2139/ssrn.4540765> (Preprint)

Conferences attended

March 2024 EACR – The Tumour Ecosystem: Cellular Interactions and Therapeutic Opportunities | Bergamo, Italy (Spotlight Presentation & Poster)

May 2023 Keystone Symposia – Metastasis | Vancouver, Canada (Short Talk & Poster)

Jun 2022 International PhD Student Cancer Conference (IPSCC) | Heidelberg, Germany (Poster)

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