Modular design and development of PEG-based hydrogel 3D systems for human intestinal organoid culture

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COVID-19 Statement

My research revolved around the manufacturing of a synthetic hydrogel to support 3D culture of human intestinal organoids. The main data output for this work included monitoring the growth of these organoids, their morphology and viability and assessing the cellular expression within them. This research heavily relied on regular access to wet labs in order to passage, feed and maintain the organoid cultures. In addition, regular use of lab and imaging facilities were required to perform biological assays and monitor the growth and performance of organoids. The project also required routine lab access for development and characterisation of hydrogel products.

My ability to access the lab was severely impacted by the COVID-19 pandemic. The 3-month national lockdown halted all ability to access the lab facilities at UEA meaning organoid cultures could not be established and maintained and new hydrogel products could not be developed and tested. During this period, data generation was not possible, halting progress in the project.

Post-lockdown, social distancing measures meant access to shared lab facilities were still greatly reduced with shift patterns restricting access to only a few hours per day. Furthermore, periods of self-isolation, both precautionary and due to contracting covid, totalled several weeks of limited/no lab access, incurring several disruptions and pauses to this research.

The ability to adapt the research in order to mitigate the impact of Covid-19 has been severely limited. This is due to the research relying directly on access to materials that are only available in a controlled lab environment. The central research proposition revolves around culture of human intestinal organoids within synthetic hydrogels. This requires access to fume hoods, cell culture hoods, incubators, imaging facilities as well as several biological assays and material characterisation techniques.

Due to time constraints caused by the limited lab access experienced as a result of the Covid pandemic, we have pivoted from our initial research proposition in order to focus on the primary objectives of constructing a synthetic hydrogel that supports organoid growth. Additional research questions of assessing organoid mechanotransduction pathways utilising our gels have had to be sacrificed in order to complete the research, within the timeframe, to a doctoral standard.

Acknowledgements

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I am filled with thanks towards my mother, father, grandparents and sister, who have been a solid foundation and source of continuous love and support in the face of overwhelming adversity. A special thanks to family friend Mike Luxford for his provision of a laptop in my time of need, on which the majority of this thesis was written on.

To my wife Dr. Hannah Staggs-Sandy, the completion of this work would be impossible without your love, guidance and support. I cannot thank you enough.

I dedicate this work to the memory of my brother Benjamin Edward Sandy MBBS MRes. To the most fiercely intelligent man I will ever know.

Proverbs 1:7 “The fear of the LORD

is the beginning of wisdom”

Abstract

Human intestinal organoids (HIO) are a powerful 3D, platform for use in research and clinical environments. Due to their organotypic morphology and resemblance to the native colonic epithelium in structure and function, HIO's have been utilized to model complex disease pathologies. The full potential of HIO remains unexplored, due to the reliance of 3D organoid growth within an ill-defined, tumor-derived basement membrane extract known commercially as Matrigel. Matrigel is derived from a rat-sarcoma cell line, which presents the risk of immunogen and pathogen transfer. This has provided the need for a chemically defined synthetic hydrogel which supports the 3D culture of HIO. Research has indicated that defined hydrogels can be optimised with various extracellular matrix (ECM) components and adhesive ligands which facilitate organoid culture to a similar standard as Matrigel. Additionally, the role of matrix environments such as stiffness have an impact on mechano-transduction pathways influencing stem cell biology and potentially organoid development. Growth of human colonic organoids has not been fully achieved in a defined environment and there remains the potential for a fully defined and optimised hydrogel designed specifically for optimal HIO culture. We demonstrate HIO growth can be achieved in a synthetic, fully defined hydrogel, based on the reaction between four-armed, thiol and maleimide-terminated poly(ethylene glycol) (PEG) macromers. We identify that PEG hydrogels are a suitable candidate for continued optimisation towards HIO culture due to their ability to support organoid growth and viability to a limited capacity in a non-functionalised state. the sub-optimal organoid morphology and overall change in organoid cross-sectional area over 7 days in non-functional PEG gels can be improved by the inclusion of the biologically relevant ECM proteins such as laminin and replicated with fully defined synthetic peptides. This work provides insight into the mechanical and biochemical requirements for colonic human intestinal organoid culture.

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1. Literature Review

1.1 The Adult Intestinal Epithelium

The large intestine, also known as the colon, forms part of the human gastrointestinal tract and has key roles in nutrient absorption and digestion. To protect the systemic circulation of the host from the often-hostile luminal contents of the gut, such as exogenous bacteria, fungi and viruses; the intestine is lined with a monolayer of polarised columnar epithelial cells known as the intestinal epithelium (Okumura and Takeda, 2017). This colonic epithelium contains a number of specialised cell types as seen in Figure 1.1, such as enterocytes (colonocytes) necessary for water absorption, and goblet cells, which are responsible for the secretion of mucus, and other proteins that are associated with the formation of the protective barrier (Allaire *et al.*, 2018). Both the protective and absorptive functions of the gut are dependent on maintaining an intact epithelium. Therefore, the colonic epithelium is capable of rapid tissue renewal, with homeostasis being maintained through the shedding of differentiated epithelial cells, and the rapid, continuous proliferation and migration of undifferentiated cells to replenish lost cells, or repair tissue following injury (Humphries and Wright, 2008). The intestinal epithelium is highly dynamic, with the entire epithelial lining being replenished every 5 to 7 days (van der Flier and Clevers, 2009).

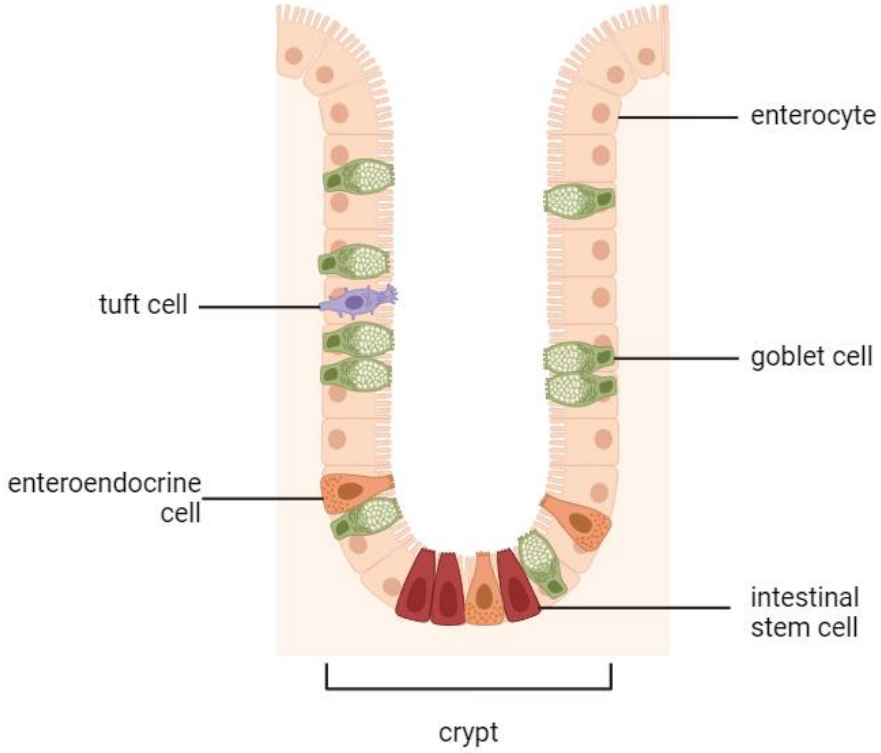


Figure 1.1 Cells of the large intestinal epithelium. Schematic diagram showing the organisation and various specialised cell types of the large intestinal epithelium. Made using BioRender.

1.2 Intestinal stem cells

The process of epithelial cell renewal is dependent on a subset of adult intestinal stem cells (ISC's) which give rise to progenitor cells, that are capable of differentiation into all cell types required for healthy gut function (Chee, Virshup and Madan, 2015). These stem cells are housed in specialised microenvironments which reside at the base of invaginations into the underlying mucosa, known as crypts of Lieberkühn. These crypts represent the single, unitary structure of self-renewal; where the base of each crypt serves as the intestinal stem cell niche, housing long-lived stem cells in a relatively sterile environment (Humphries and Wright, 2008). Here, ISC exposure to bacterial toxins and metabolites, dietary mutagens, immunological cytokines and oxidative stress is reduced, minimising the risk of accumulating molecular damage (Chee, Virshup and Madan, 2015). ISCs at the crypt base fuel the constant replenishment of shed gut epithelial cells, whose brief residency in the gut also aid in reducing the probability that any singular epithelial cell will accumulate the complement of genetic mutations required for tumour initiation (Humphries and Wright, 2008).

1.3 The Intestinal Stem Cell Niche

In the human gut epithelium, crypts are formed by epithelial invaginations into the extracellular matrix (ECM) which is supported by stromal cells (Meran, Baulies and Li, 2017). The intestinal stem cells, which are located at the crypt base, are maintained by their surrounding niche for regulation of both differentiation and self-renewal under homeostasis (van der Flier and Clevers, 2009). The ECM provides the physical niche and is comprised of a network of fibrous structural proteins including proteoglycans (PGs) and glycoproteins which act as a physical scaffold, maintaining a 3D morphology (Meran, Baulies and Li, 2017). The ISC

niche is also surrounded by other ECM components such as different laminin isoforms, collagens, glycosaminoglycans and fibronectins (Czerwinski and Spence, 2017) that provide structural support, as well as influencing stem cell behaviour through mediation of instructive cell signalling (Vazin and Schaffer, 2010). For example, 2 isoforms of the heterotrimeric ECM protein Laminin (α1 and α2) are enriched in the physiological crypt base (basal laminae) and have been seen to interact with ISCs via integrins – transmembrane heterodimeric receptors located on the cell surface that link the ECM with the intracellular cytoskeleton. This interaction mediates cell anchorage and mechanotransduction, as well as suggesting a potential role for integrin-mediated signalling in ISC regulation (Meran, Baulies and Li, 2017). In addition to the physical niche, cells embedded into the ECM form the cellular niche (Figure 1.2), secreting a range of matrix components and growth factors which are capable of altering ISC proliferation and differentiation (Chee, Virshup and Madan, 2015). The ECM is therefore vital to maintain the ISC niche, and both physical and cellular elements of the ECM are known to regulate both proliferation and differentiation through several pathways including the Wnt/β-catenin cascade, Notch signalling, TGF- β/BMP and Hedgehog pathways (Meran, Baulies and Li, 2017). In addition, the ECM is suggested to function as a reservoir for growth factors, through the ability of heparin sulfate proteoglycan, a common component of many ECM PGs, to bind growth factors such as FGF and VEGF allowing for the establishment of morphogen gradients – which plays a vital role in patterning, growth and development (Hynes, 2009). These growth factors may also be released during degradation of the ECM, highlighting how proteolytic remodelling of the surrounding ECM by secreted matrix metalloproteinases (MMPs) may enhance ISC proliferation (Yin *et al.*, 2016).

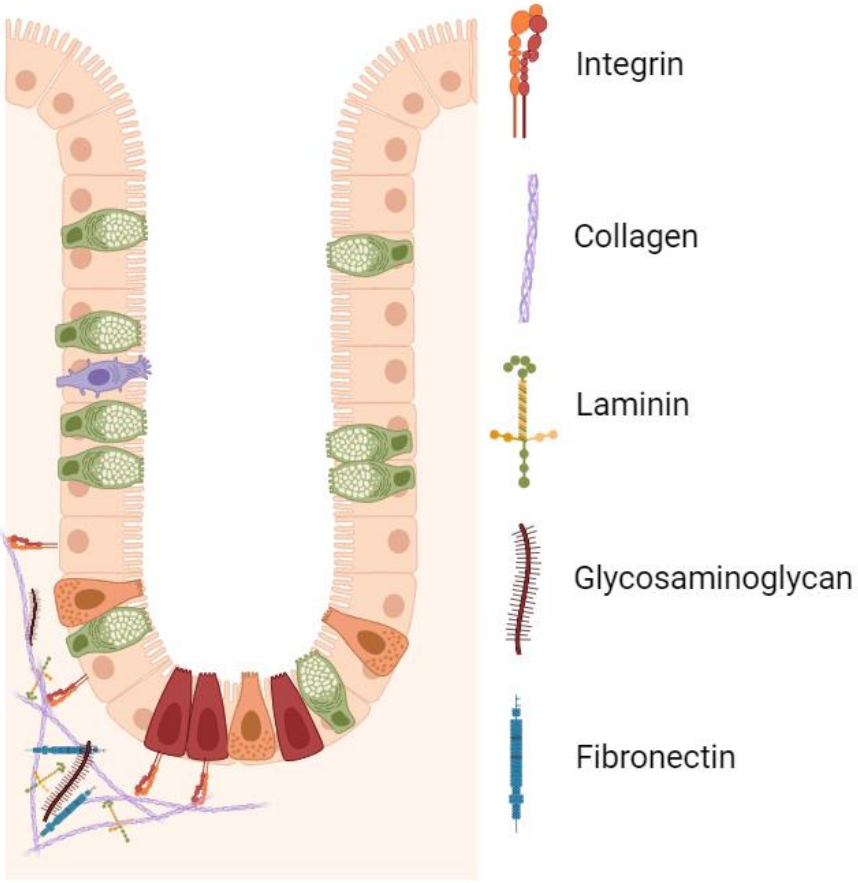


Figure 1.2 Schematic diagram of the interaction between intestinal epithelial crypts (the intestinal stem cell niche) and the surrounding microenvironment. Detailed on the left is the interaction between the highly structured ECM which is mediated through integrins and a key describing the representations of integrin, collagen, laminin, glycosaminoglycan, and fibronectin is shown on the right. Made using BioRender.

1.4 The role of Wnt signalling and LGR5

Wnt signalling was found to be a driving force behind physiological crypt proliferation, through its dysregulation being the primary driver of colorectal cancer (Cernat *et al.*, 2014). Overall, around 80% of bowel cancer cases are fuelled by mutated APC genes (CRUK, 2012) which results in the failure of the APC destruction complex to function, and subsequent build-up of β-catenin, the key effector of Wnt signalling (van der Flier and Clevers, 2009) (Figure 1.3). Accumulation of β-catenin was found to induce transcriptional activation of Tcf4 target genes resulting in uncontrollable cell proliferation (van der Flier and Clevers, 2009). This, coupled with evidence that Tcf4 knockout mice lack proliferative crypts (Korinek et al., 1998), highlights the importance of Wnt signals in regulating proliferation within the crypt and establishing the stem cell compartment.

Early microarray experiments on the Wnt-pathway-controlled genes, expressed in both colon cancers and healthy crypts, unveiled the prominence of the Leucine-rich, repeat-containing, G-protein coupled receptor 5 (LGR5) gene. LGR5 encodes for a receptor of Wnt pathway agonists, R-spondins ((Barker *et al.*, 2007). R-spondin signalling works to enhance Wnt signals in intestinal crypts by neutralising transmembrane ligases, that would otherwise remove Wnt receptors from the cell surface (de Lau *et al.*, 2014), and is a prominent driver of crypt cell renewal (Figure 1.3). Using an inducible Cre knock in allele and the Rosa26 lacZ reporter strain, lineage tracing experiments were performed in mice and demonstrated exclusive LGR5 expression in cycling crypt base columnar cells (CBC), which were capable of generating all epithelial cell lineages over a 60-day period (Barker et al., 2007). This data allowed the identification of the intestinal stem cell and demonstrated the significance of LGR5 as an

intestinal stem cell marker which has since been exploited to isolate adult intestinal stem cells and produce self-renewing 3D organoid culture models.

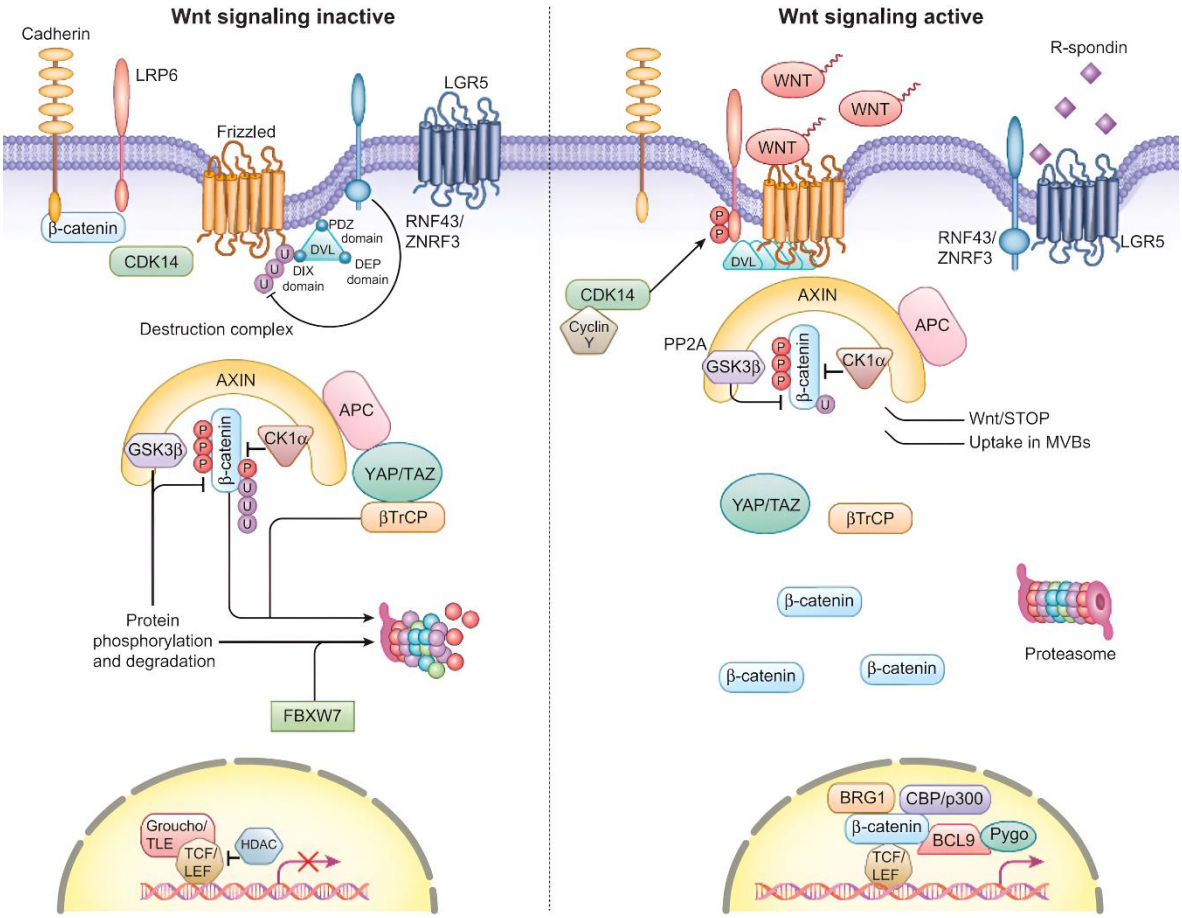


Figure 1.3 Schematic diagram of the canonical Wnt signalling pathway in active (right) and inactive (Left) state (Zhan, Rindtorff and Boutros, 2017). Canonical Wnt signalling is activated through binding of secreted Wnt ligands (Wnt signalling active, right side). This leads to inactivation of the destruction complex and the sequential accumulation of β-catenin, which translocates into the nucleus. β-catenin there forms an active complex resulting in transcriptional change, altering cellular processes. The absence of Wnt signals (Wnt signaling inactive, left side) leads to phosphorylation of β-catenin by the destruction complex, resulting in proteasomal degradation.

1.5 Organoid culture

Organoids are 3D structures grown from stem cells, that consist of organ specific cell types which self-assemble via cell sorting and spatially restricted lineage commitment (Clevers, 2016). Organoids can therefore be derived from several sources, including embryonic stem cells, inducible pluripotent stem cells and organ-specific adult stem cells giving rise to organoids from several different tissues. These organoids have a wide range of uses ranging from disease modelling to drug screening platforms. For example gastric organoids have been used to recapitulate the characteristic disease pathology of *Helicobacter pylori* bacterial infections (Bartfeld *et al.*, 2015), whilst Patient derived intestinal organoids have laid the foundation for organoid research in personalised medicine by being successfully used to screen for drugs to treat cystic fibrosis (CF), a genetic disease caused by mutations in the CFTR gene (Saini, 2016).

It was discovered that single LGR5+ intestinal stem cells could be expanded in culture to generate 3D gut epithelium cultures, otherwise known as organoids (Figure 1.4) (Sato *et al.*, 2009). These organoids maintain the LGR5(+) stem cell hierarchy, are self-organizing and do not require an epithelial cellular niche. Organoids can be cultured indefinitely in epithelial growth factor (EGF), Noggin and R-spondin enriched conditions, supported by a 3D matrix, and retain the hallmarks of the in vivo intestinal epithelium (Sato *et al.*, 2013), therefore these adult stem-cell-derived organoids have become promising models of disease development including CRC and IBD, potential drug screening platforms and a potential source of transplantable tissue (Rossi, Manfrin and Lutolf, 2018). This potential is highly dependent on 3D culture conditions which allow for highly reproducible generation of organoids, with a high level of functional and structural similarity to physical organs.

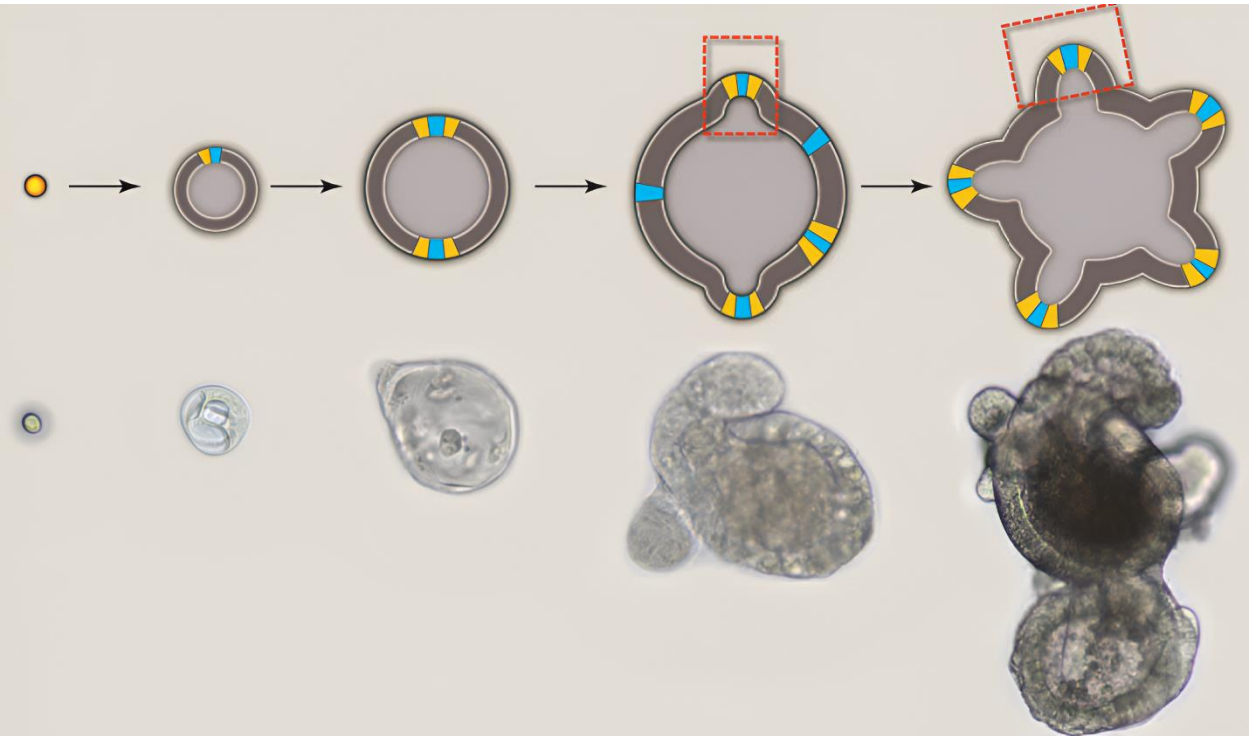


Figure 1.4 Organoid development from intestinal stem cells. Schematic and brightfield images highlight the stages of murine small intestinal organoid growth and development. Budding structures are highlighted (red rectangle) and demonstrate crypt-like morphology. CBC cells including LGR5+ ISCs are highlighted in blue (Sato et al., 2013)

1.6 Human intestinal organoid culture

One method of establishing routine 3D culture of human intestinal organoids is to embed isolated intestinal crypts within a growth factor enriched matrix gel. When cultured in the correct environment, this process establishes budding spheroid structures with a hollow central lumen encompassed by the varied intestinal epithelial cell types found in the native intestine. These structures mimic the organisational patterns of the intestine and have hence been named "mini-intestines" or organoids. The development of human intestinal organoid models have been largely of focus due the growing interest and concern around gut health. In the UK, colorectal cancer is currently the fourth most prevalent cancer with second highest mortality rate (Cancer research, UK). Additionally, it is estimated that 1 in every 123 people in the UK suffer from inflammatory bowel diseases (Caviglia et al., 2023). As intestinal tumours can be present within the large intestine and pose a significant threat to human health, the application of 3D organoid culture towards colonic human intestinal organoids has been significant. Colorectal organoids can be cultivated to retain the genetic characteristics of the primary tumour (Van De Wetering et al., 2015). When combined with presently established libraries of healthy patient derived organoids, there is the ability for organoids to serve as tools to investigate tissue functionality and development, alongside tumorigenesis and the pathology of colorectal cancers. Additionally, this innovation offers novel opportunities for individual and personalised approaches towards treatments for patients with intestinal tumours.

The use of HIO are however not without limitation, currently these models display a high level of heterogeneity, owing to the diversity between individuals and protocols. This can result in a high degree of variance between studies dependent on several different factors (Kim, Koo

and Knoblich, 2020). In addition, organoid studies can present a biased snapshot due to a lack of more varied cell types present in microenvironment, however co culture systems between HIO and other relevant cell types are becoming more prevalent (Van Neerven et al., 2022). Perhaps the largest limitation preventing access to the full potential that these colonic HIO models possess is scalability and standardisation. There is currently no standard protocol for the establishment of HIO cultures and with this, the absence of quality control (Kim, Koo and Knoblich, 2020). A vital step to overcoming this major limitation is the development of a suitably robust, cost effective and defined culture system that overcomes the key drawbacks of the current culture matrix used for research purposes, Matrigel.

1.7 Matrigel

Organoids have the potential to be a powerful tool for research and at a clinical level, it is of the utmost importance that their 3D culture conditions allow for highly reproducible generation of organoids, with a high level of functional and structural similarity to physical organs. Currently, routine 3D culture methods have relied on the commercially available, hydrogel matrix known as Matrigel, a basement membrane-like gel secreted by mouse sarcoma cells (Sato and Clevers, 2013). Proteomic analysis of Matrigel revealed its composition of a complex assortment of 1851 unique proteins, glycoproteins and proteoglycans, primarily consisting of ECM proteins such as laminin, collagen type IV and enactin (Hughes, Postovit and Lajoie, 2010). In addition, several growth factors were detected in varying quantities including epidermal growth factor, fibroblast growth factor, transforming growth factor beta and others (Hughes, Postovit and Lajoie, 2010). Although effective in promoting cell growth and self-organisation, Matrigel is limited in its ability to direct organoid morphogenesis and offers no control over the 3D self-organisation process

(Hughes, Postovit and Lajoie, 2010). This is owing to its poorly defined chemical composition, containing largely variable concentrations of growth factors and proteins from batch-to-batch, as well as its inability to be chemically or physically modified, preventing specific tailoring of biophysical or biochemical matrix properties for intestinal organoids (Gjorevski *et al.*, 2016).

With both chemical and mechanical/physical cues from the ECM driving organogenesis, it is important to be able to modulate matrix cues such as mechanical stiffness, adhesive ligand presentation and degradability in order to model the complex interplay between cell behaviour and the physical environment that is demonstrated in the intestinal epithelium (Raeber, Lutolf and Hubbell, 2005). Furthermore, Matrigel is an animal-derived product which confounds its use in a clinical environment due to the risk of immunogen and pathogen transfer (Gjorevski et al., 2016). There is therefore a requirement for a chemically defined 3D culture system, whose chemical and physical properties are subject to manipulation to fully optimise the growth and maturation of organoids embedded into the gel. By controlling the distribution of signalling molecules and ECM proteins, as well as manipulating mechanical properties such as porosity and stiffness, there is the potential for organoid cultures within this system to reproducibly surpass the growth and self-organising potential achieved in Matrigel.

1.8 Biomaterials

Biomaterials are any substance, natural or synthetic, that have been engineered to interact with biological systems and are a rapidly emerging tool with a broad range of potential applications ranging from use in healthcare materials to drug delivery systems. Included in these are hydrogels, a class of three-dimensional (3D), viscoelastic polymers that form network structures and can provide a suitable 3D cell culture platform for several cell types (Catoria *et al.,* 2019). Hydrogels can be derived from natural or synthetic polymers and their hydrophilic nature allows for absorption and retention of a significant amount of water, which enables these 3D viscoelastic hydrogels to swell, resembling the physical properties of biological tissue making them suitable for cell culture systems (Kratochvil *et al.*, 2019). Hydrogels in general have several tuneable characteristics which effect the health and behaviour of encapsulated cells, each of which can be manipulated by altering the gels chemical and physical properties, to design a gel that is tailor-made for its application. These characteristics include the mechanical stiffness, degradability and swelling capacity (Gjorevski *et al.*, 2016; Y. Wang *et al.*, 2018), among others, and are often dictated by the type of crosslinking used to form the gel, which can be chemical or physical (Peppas *et al.*, 2006). Both natural and synthetic hydrogel categories can be used in a cell culture environment and different advantages and disadvantages are associated with each type. It is therefore necessary to explore different hydrogel categories to identify a hydrogel platform that provides the best environment for human colonic organoids, taking into consideration the ease of use and manipulation.

1.9 Natural hydrogels

Natural hydrogels are often protein-based materials e.g. collagen or fibrin, or polysaccharide-based such as alginate. They are promising materials for cell culture applications due to their high level of biocompatibility owing to their natural presence in the extracellular matrix. Furthermore, natural-derived hydrogels possess the ability to provide structural support with inherent biodegradability whilst being bioactive, influencing intracellular biochemical signalling (Ahmed, 2015). Natural hydrogels are not without limitation, with often an increased difficulty to source. Collagen for example, is mainly extracted from murine tails, presenting problems when developing xeno-free cell culture environments for human tissue. Natural hydrogels also prove more difficult to manipulate, removing the ability to completely control the physical and chemical composition of the gel.

1.10 Collagen

Collagen is a universal component of the ECM in living organisms and has various roles including mechanical support as well as supporting cell division, migration and differentiation. Collagen consists of repeating tripeptides of glycine, proline and hydroxyproline which form a triple helix, with a higher order structure of self-assembling fibres. There are 29 categories of collagen, with collagen I being the most commonly used for biomedical applications. Collagen I hydrogels are biocompatible with low levels of antigenicity and inflammatory response; however, the mechanical properties of collagen hydrogels do not replicate the mechanical strength seen in native tissues due to the absence of covalent crosslinking. For this reason, physical and chemical crosslinking mechanisms have been employed in order to improve the mechanical performance (Achilli, Lagueux and Mantovani, 2010)

Collagen-based hydrogels have been successfully employed to culture murine and human-derived colonic organoids to a similar standard of those cultured in Matrigel (Jee *et al.*, 2019) which suggest collagen cultures may improve the efficacy of developing therapeutic organoids for epithelial wound repair

1.11 Fibrin

Fibrin is a naturally occurring protein involved in the tissue repair process and the coagulation cascade. The inactive form fibrinogen can be crosslinked in the presence of cells to form fibrin hydrogels by the addition of thrombin (Broguiere, Isenmann and Zenobi-Wong, 2016). It was found that soft fibrin matrices are capable of both providing the physical support necessary for organoid growth and contain naturally occurring RGD domains - a short tripeptide sequence which serves as the principal integrin-binding domain present within ECM proteins such as fibronectin, which support cellular adhesion and induce intracellular signalling (Peppas *et al.*, 2006). Fibrin hydrogels have been utilised as a basis for the long-term culture of murine small intestinal organoids that, with optimisation from laminins, produced comparable levels of organoid growth, survival and expression of characteristic cell markers as organoids cultured in Matrigel (Broguiere *et al.*, 2018).

1.12 Cellulose

Cellulose hydrogels are highly hydrated, porous materials of either bacterial or plant origin (Catoira *et al.*, 2019). Organised in hydrophilic nanofibrils, cellulose hydrogels fulfil many of the requirements for a useful biomaterial. Cellulose nanofibrils are biocompatible as well as

inherently biodegradable, in addition cellulose can be functionalised in order to enhance the physical and chemical properties to offer control over biological interactions within the gel (Curvello, Raghuwanshi and Garnier, 2019). Nanocellulose hydrogels have been used as ECM-mimetic platforms for 3D cell culture, mainly in liver cells, demonstrating their ability to serve as a highly engineerable platform which can be tailored for a specific function (Curvello, Raghuwanshi and Garnier, 2019).

1.13 Alginate

Alginate is a polysaccharide component derived from cell walls in algae. Alginate structure is based on two monomers β-D-mannuronate (M) and α-L-guluronate (G) organized into blocks (Catoira *et al.*, 2019). These G blocks form ionic bridges and the mechanical properties of alginate-based hydrogels can be modulated by altering the amount and disposition of these blocks (Catoira *et al.*, 2019). Alginate is a favourable biomaterial due to its FDA approval, biocompatibility and control of gelation offered via crosslinking with calcium (Peppas *et al.*, 2006). Despite the weaker adhesive properties and mechanical characteristics, alginate has been utilised as a culture system for human intestinal organoids derived from pluripotent stem cells, which resemble their Matrigel-grown counterparts on a molecular level (Capeling *et al.*, 2019).

1.14 Synthetic Hydrogels

Polymer networks can be synthesized by various chemical methods, such as thermal or photo-initiated polymerization, and recent research has been focused on gradually replacing natural hydrogels with synthetic counterparts (Peppas *et al.*, 2006). Synthetic hydrogels often have a longer service life, higher mechanical strength and a high capacity for water absorption making them useful alternatives to natural hydrogels. Synthetic polymers will generally have well defined structures and can be modified to achieve desired properties such as functionality or degradability (Ahmed, 2015). A summary of some key differences between natural and synthetic hydrogels are displayed in Table 1 (Liu et al., 2024).

Table 1. Comparison between several examples of natural and synthetic hydrogels

Hydrogel	Type	Advantages	Disadvantages	Application(s)	Cost
Alginate	Natural (Algae)	Rapid gelation speed, FDA approval, good stability	Poor cell attachment, weaker mechanical properties	Human neural stem cells, Human intestinal organoids	Low
Cellulose	Natural (Plant waste)	Biocompatibility, Biodegradability, possible functionalisation	Poor cell adhesion, fast degradation	Human Liver 3D Cell culture	Low
Fibrin	Natural (Animal or human plasma)	Possess natural occurring RGD domains, strong cell adhesion	Poor mechanical stability	Murine small intestinal organoids	Low
Collagen I	Natural (Rat tail tendons etc)	Biocompatibility, Call adhesion, strong support for printing	Poor mechanical performance	Epithelial wound repair, human/murine derived colonic organoids	Low
Hyaluronic Acid (HA)	Natural (Mesenchymal cells)	Promotes proliferation, rapid gelation	Rapid degradation, poor mechanical stability	Human cardiac progenitor cells	Low/moderate
Matrigel	Natural (Mouse sarcoma cells)	Supports proliferation and differentiation	Tumour- derived, batch variability, expensive	Human epithelial cells, HIO	High
Polyethylene glycol (PEG)	Synthetic	Tuneable properties, amenable to functionalisation	Low cell proliferation and adhesion	Organoid culture, drug delivery	Moderate
Poly(acrylic acid)	Synthetic	High adhesive strength, non-toxic, functionalisation with other polymers	Low durability, poor mechanical properties	Drug delivery, vascular tissue engineering	Moderate
Gelatin methacryloyl (GelMA)	Synthetic	Degradability, Mechanical strength	Slow gelation, requires UV light	Human BMSCs	Moderate

1.15 Poly(ethylene glycol) based hydrogels

Polyethylene glycol (PEG) is a readily available hydrophilic polymer, that when crosslinked into hydrogel networks, possess a high-water content, making them suitable to emulate soft tissues (Leach, 2011). A major advantage of PEG based hydrogels is their relative inertness and cytocompatibility, as evidenced by their failure to initiate an immune response in the cells they encounter (Mäder, 2011). Often referred to as a 'blank slate material' PEGs are amenable to functionalisation with user-defined crosslinking groups, allowing for control over the crosslinking properties of the gel (Caliari and Burdick, 2016). This is due to the basic structure of PEG, which is capped with hydroxyl end groups, allowing the polymer to partake in a variety of chemical reactions (Zhu, 2010). Raeber, Lutolf and Hubbel pioneered the use of PEGs functionalised with vinyl sulfone (VS) in hydrogel preparations, as PEG-VS reacts specifically with free thiols, such as peptides terminated with cysteine residues (Raeber, Lutolf and Hubbell, 2005). Cells and proteins within the gel rarely possess exposed cysteine residues on their surface and thus peptides can be incorporated that render the gel with biological activity, such as adding cell adhesive domains from ECM proteins and peptide substrates for matrix metalloproteinase degradation, allowing cells to mimic attachment and migration through the ECM (Leach, 2011).

A pivotal study identified that a chemically defined, synthetic PEG-based hydrogel could be used as a suitable Matrigel replacement to culture intestinal epithelial organoids (Gjorevski *et al.*, 2016). PEG as a biomaterial offers a highly amenable matrix that can be independently modulated to alter variables including matrix stiffness, degradability and adhesiveness, in order to assess their effects on ISC proliferation, organoid formation and cellular differentiation. This work observed that a mechanically dynamic matrix was required

providing an initial stiffness that favoured stem cell expansion, but was hydrolytically active and therefore able to soften, alleviating mechanical forces and favouring cell differentiation (Gjorevski et al., 2016). Hydrogel mechanical properties can be characterised using a technique known as rheology, in which a small amount of a sample is subject to rotational compressive forces, probing the stress-strain relationship of the gel and obtaining the samples equilibrium shear modulus (Zuidema *et al.*, 2014). This information can be used to reveal structural properties such as degree of crosslinking and mechanical stiffness. The hydrogels generated by Gjorevski et al., were optimised using a mouse intestinal epithelial organoid model, however experiments have also demonstrated that PEG hydrogels could include functionalisation with human-derived intestinal ECM components, as well as investigating how spatiotemporal control of mechanical cues and signalling gradients can enable hydrogels to better mimic the structure of the native human intestine (Wang *et al.*, 2017).

1.16 Michael type addition (crosslinking chemistry)

PEG hydrogels can utilise different methods of crosslinking, each with individual advantages and disadvantages, which can result in different hydrogel characteristics. The thiol-Michael type reaction is useful in hydrogel synthesis (Figure 1.5), which often occur in aqueous conditions, as it proceeds at room temperature without interference from water protons. PEG hydrogels can be generated based on the reaction between two functionalised PEGs, PEG-thiol (SH) and PEG-Maleimide (MAL). Maleimide reacts readily with free thiols under physiological conditions allowing for the formation of a high-water content hydrogel, with a rapid gelation time and in conditions optimal for organoid survival (Jansen *et al.*, 2018). PEG-

MAL was seen to exhibit fast reaction kinetics, and allow for the generation of hydrogels of lower polymer weight and across a larger range of Youngs moduli (Phelps *et al.*, 2012). Using a PEG hydrogel system as a foundation provides a platform for further modification. The interaction between thiol and maleimide functional groups provide chemical cross-linking with tuneable reaction kinetics, whilst varying the PEG molecular weight (PEG chain length) and overall % PEG content of the hydrogel alters the physical interactions caused by entanglement of the PEG chains. PEG hydrogels therefore provide adaptable hydrogel characteristics including, stiffness, mesh size and porosity, as well as enabling the inclusion of adhesive ligands and degradable peptides.

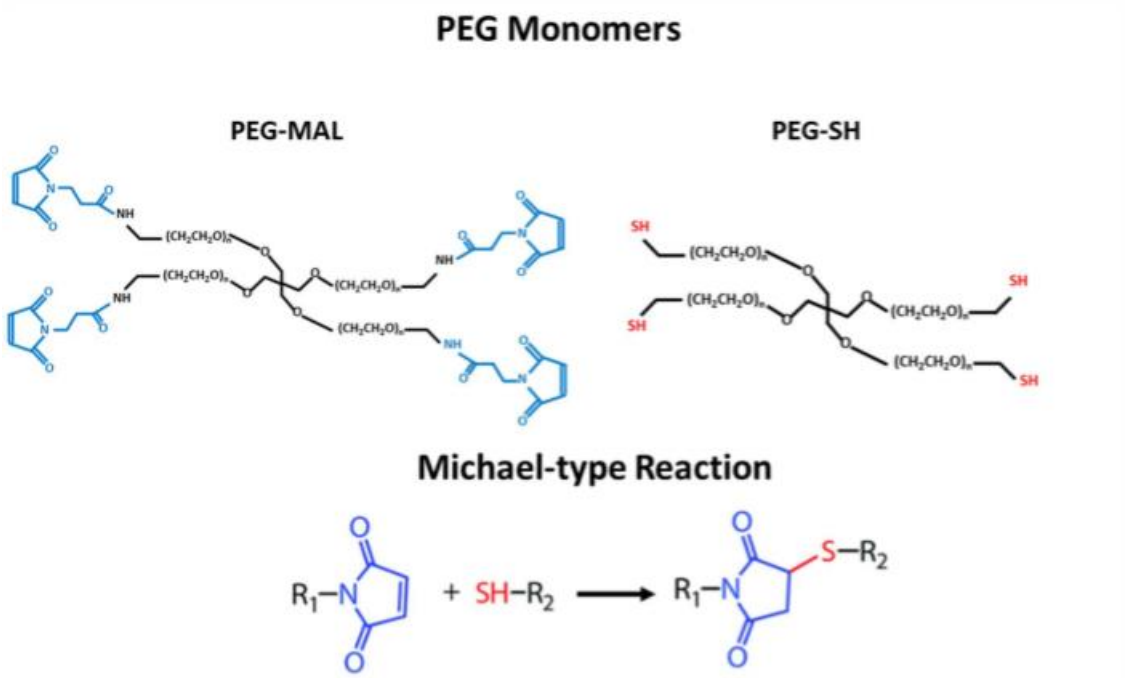


Figure 1.5 Michael type reaction between PEG monomers. Schematic diagram highlighting the structure of PEG Maleimide and Thiol, and overview of the Michael type reaction process.

1.17 Degradable Hydrogels

Degradability is an important consideration when identifying a suitable hydrogel for 3D organoid culture. The native intestinal ECM environment is highly dynamic with continued proteolytic remodelling. This process is pivotal for the restructuring of tissue architecture, serving as an important mechanism involved in regulating the intestinal stem cell niche and influencing intestinal epithelial cell differentiation. The replication of native degradation within hydrogels enables encapsulated cells to escape physical confinement, facilitating migration and morphological changes. In addition, a degradable environment provides dynamic mechanical properties whilst influencing the biochemical cues needed for organoid growth and development. Natural hydrogels including Matrigel are often inherently degradable, but have varied disadvantages including the lack of required mechanical strength or access to full optimisation that is offered by synthetic hydrogels (Zhu, 2010). Hydrogels for HIO culture need to have controlled degradation, that degrade in a predictable time frame which aligns with the mechanical needs of the cell or organoid.

There are several methods by which synthetic PEG based hydrogels can be made degradable, such as the attachment of degradable peptides (Raeber, Lutolf and Hubbell, 2005; Foster *et al* 2017), however certain crosslinking methods for the formation of PEG hydrogels are capable of providing the hydrogel with a degradable nature. Poly(ethylene glycol)-diacrylate (PEGDA) is a functionalised PEG that can be crosslinked with the dithiol molecule dithiothreitol (DTT) (Figure 1.6) in a step-growth polymerisation reaction (Raeber, Lutolf and Hubbell, 2005). The acrylate groups form ester bods with the thiol crosslinked, which are susceptible to hydrolysis, thus creating a hydrolytically degradable hydrogel network (Leach, 2011). Through the modulation of PEG molecular weight and total concentration, the number

of hydrolytically degradable sites can be controlled, providing control over the overall degradability of the hydrogel. Degradable PEG Acrylate based hydrogels have been used to successfully culture murine small intestinal organoids in a matrix that decreases in stiffness over time, owing to hydrolytic degradation (Leach, 2011).

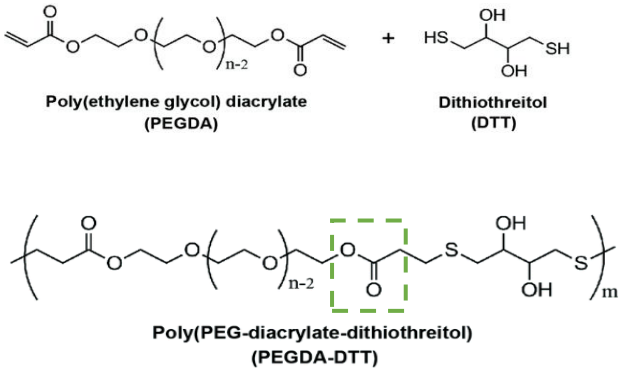


Figure 1.6 PEGDA, DTT and PEGDA-DTT structures. Hydrolytic degradation mechanism of PEG-DTT afforded by the ester linkage (Green box). A hydroxyl ion attacks the carbonyl carbon proximal to the thio-ether, and this yields a carboxylic acid and an alcohol. (D. Wang *et al.*, 2018).

1.18 Mechanotransduction

Mechanotransduction is the process by which cells and tissues translate mechanical cues from their environment into specific intracellular biochemical signals (Ingber, 2006). For mechanically sensitive tissues such as the gastrointestinal (GI) tract, mechanical strains associated with cell or tissue morphological changes, will have a role in regulating gene expression and protein activation (Broders-Bondon *et al.*, 2018) . In vitro studies using cultured stem cells have identified a role for different forces, cell size, and substrate stiffness in regulating cell fate and differentiation (Broders-Bondon *et al.*, 2018). These studies have helped highlight the complex interplay between the biomechanical structure of living tissues and their biochemical activities at a molecular level. The importance of the mechanotransduction process has been highlighted by the anomalous mechanical properties of tumour tissue, such as high matrix stiffness, directly influencing the activation of biochemical pathways that enhance the cell cycle, epithelial-mesenchymal transition and cell motility. Research has since begun to identify the mechanisms by which cells and tissues perceive their surrounding environment and influence biological signalling.

1.19 Mechanosensitive ion channels: Piezo 1

At a fundamental level, many cells have developed mechanosenitivity due to the need to sense normal physiological forces, and thus cells have developed several mechanisms of mechanosensation for example, the mechanical interaction of integrins with their substrates allow cells and tissues to interact with their ECM (Beaulieu, 1992). The mechanically active GI tract however, also contains several mechanosensitive cell types, which are all similar in their ability to respond to mechanical forces by altering transmembrane ionic currents to open

mechanosensitive ion channels in a process known as mechanoelectric coupling (Alcaino, Farrugia and Beyder, 2017). Mechanosensitive ion channels are transmembrane proteins that form ion conduction pores, with gates strongly altered by mechanical force. One example of mechanosensitive ion channels which are expressed throughout the GI tract is the Piezo family. These ion channels are characterised by distinct biophysical properties such as non-selective cationic permeability and distinct reaction kinetics (Alcaino, Farrugia and Beyder, 2017). The biochemical consequence of piezo activation in the GI epithelium is not fully characterised however Piezo1 activation is associated with an influx of intracellular calcium. Structure-function studies show that Piezo1 can be activated directly through membrane stretch, in the absence of other cellular components (Lacroix, Botello-Smith and Luo, 2018). This suggests Piezo channels directly sense transmitted forces through lipids, however the mechanism by which these forces are detected is unclear. It has been shown that Piezo1 can be activated by a synthetic small molecule called Yoda1, which functions through stabilizing the open conformation of the piezo channel, thus reducing the mechanical threshold for activation, and this has allowed for the role of mechanotransduction in biological stem cell niches to be explored (Botello-Smith *et al.*, 2019).

Recent research demonstrated that mechanical signals from the surrounding matrix can be 'overridden' through the inhibition of the mechanoresponsive ion channel Piezo1, in order to increase proliferation and differentiation of oligodendrocyte progenitor cells in the ageing central nervous system, whose stem cell niche stiffens with age (Segel *et al.*, 2019). As it has been demonstrated that the piezo channel opens upon direct, physical deformations of the lipid bilayer, such as increased membrane tension and remains closed in the absence of external mechanical force (Botello-Smith et al., 2019), It is conceivable that the stiffening of the stem cell niche may alter piezo 1 sensitivity. It is theorized that through Yoda 1 induced

inhibition, or activation of Piezo1 channels expressed in intestinal epithelial organoids, organoid growth and proliferation can be moderated, even in stiffer matrices, and this research will explore this.

1.20 Yes-associate protein influenced mechanotransduction

It must also be considered that niche and matrix stiffness influence intestinal stem cell expansion by influencing YAP (Yes-associated protein) activity and nuclear localization, with high matrix stiffness enhancing ISC survival and proliferation through a YAP-dependent mechanism (Gjorevski et al., 2016). The same study also identified that in stiff matrices, the proportion of cells with nuclear YAP activity rapidly decrease over time, whilst organoids cultured in Matrigel maintain intermediate levels of nuclear YAP activity. This indicated that whilst organoid culture within a stiff matrix initially increases YAP activity and supports organoid growth, continued exposure to a stiff matrix results in compression and YAP inactivation, preventing further growth (Gjorevski et al., 2016). The data produced in a 2019 collaborative study between the Williams and Saeed laboratory (Sandy et al., 2019) is concurrent with this published data, as we identified that 10K PEG, which offers increased matrix stiffness and therefore, higher predicted YAP activity, over 20 and 40K PEGs, provided the highest mean growth ratio. Increasing the stiffness further with a 5K PEG offered no growth benefit for the organoids and this may be due to the reduced mesh size and porosity, effecting the delivery of fresh nutrients to the organoids, enforcing the notion that optimal organoid growth occurs in a narrow window of niche stiffness. This, in combination with other literature, enforces the idea that the effect of matrix stiffness on mechanotransduction should be considered when optimising a hydrogel system.

1.21 Overall aims

1. Fabricate a synthetic PEG hydrogel that supports colonic human intestinal organoid growth.
2. Assess the growth and morphology of human intestinal organoids cultured in synthetic PEG hydrogels in comparison to organoids cultured in Matrigel.
3. Utilise synthetic PEG hydrogels to elucidate mechanical and biochemical requirements for colonic human intestinal organoid culture.

2. Materials & Methods

Overview

During this research, several techniques and methods were utilised and developed to assess and optimise the growth and morphological development of human colonic organoids, within a 3D hydrogel. All experiments utilised HIO generated from colonic crypts isolated from patients' tissue samples and cultured using Matrigel. Previous research conducted by the Williams lab demonstrates this model as a gold standard of colonic organoid culture, permitting the study of protein localisation with immunofluorescence techniques (table 2.2) and permitting DNA and RNA extraction for genomic and transcriptomic analysis (table 2.1).

HIO were passaged from Matrigel and placed into a relevant hydrogel where the culture was monitored using brightfield microscopy. Detailed imaging analysis was conducted to provide insight into overall organoid growth (monitored by changes in cross sectional area) and presence of key morphological traits. These studies, taken in combination with organoid performance in Matrigel, served as the basis for rounds of hydrogel optimisation.

Synthetic PEG hydrogels were optimised through the tuning of mechanical properties, assessed with rounds of rheological characterisation, and the addition of biologically relevant functional peptides. The best performing PEG hydrogels were utilised for in depth analysis of cell proliferation and protein localisation using immunofluorescent labelling.

2.1 Reagents and buffers

Table 2.1 RNA isolation reagents.

Reagent	**Supplier**
ReliaPrepTM RNA Miniprep System	Promega
Oligo-dT15 primers	Promega
M-MLV Reverse Transcriptase kit	Thermo Fisher Scientific
GOTaq® G2 DNA Polymerase	Promega
GOTaq® Buffer	Promega
RNasin® Plus RNase Inhibitor	Promega
Low Molecular Wight DNA Ladder	New England Biolabs

Table 2.2 Primary and secondary antibodies used for immunolabelling.

Antibody	**Antibody Type**	**Species**	**Working Concentration**	**Supplier**
Anti-Piezo	Primary	Rabbit	1:100	New East Biosciences
Anti-Chromogranin A	Primary	Mouse	1:100	Abcam
Anti-E-Cadherin	Primary	Goat	1:100	R&D systems
Anti-Muc2	Primary	Mouse	1:100	Santa Cruz
Anti-Rabbit Alexa Fluor 488	Secondary	Donkey	1:200	Invitrogen
Anti-Mouse Alexa Fluor 647	Secondary	Donkey	1:200	Invitrogen
Anti-Goat Alexa Fluor 568	Secondary	Donkey	1:200	Invitrogen

2.2 Methods

2.2.1 Human tissue sample collection

Biopsy samples of healthy colonic epithelial tissue were obtained from patients at the Norfolk & Norwich University Hospital (NNUH) that had undergone exploratory colonoscopy procedures. The samples were collected by members of the Williams laboratory, with consent, and in compliance with the Human Tissue Act of 2004 and NHS research and development approval. Biopsy samples were obtained from colorectal tissue from patients undergoing sigmoid endoscopy, right-hemicolectomy or anterior resection. The histologically-normal mucosa samples were obtained from at least 10 cm away from the tumour locations, and only used if there was no apparent intestinal pathology. Biopsy samples were kept cool within a sealed container and transported to the laboratory via a member of the Williams Lab.

2.2.2 Human intestinal crypt isolation

Human colonic crypts were isolated from fresh biopsy samples obtained from the NNUH following a protocol outlined by Parris and Williams (Parris and Williams, 2015), whereby whole crypts are liberated from the mucosal tissue into fresh crypt isolation solution. A 100 µL sample of isolated crypt suspension is added to 400 µL of chilled Matrigel® and mixed before dispensing 20 µL onto coverslips and placing at 37 ^{o}C for 15 minutes to allow polymerisation of the Matrigel. Once polymerised, the wells were flooded with 400 µL of human colonic crypt culture medium (hCCCM): advanced F12/DMEM containing B27, N2, n-acetylcysteine (1 mM), Hepes (10 mM), Pen/Strep (100U/mL), L-Glutamine (2 mM), Wnt-3A (100ng/mL), IGF-1 (50ng/mL), Noggin (100ng/mL), RSPO-1 (500ng/mL) and A83-01 (0.5 µM), and the plates returned to the incubator.

2.2.3 Human intestinal colonic organoid culture

Human colonic organoids were obtained by members of the Williams lab after the passage of colonic crypts grown for 7 days. The samples were detached from the bottom of the plate by scratching the surface, and mechanically dissociated into smaller fragments using a pipette. The suspension containing the crypt fragments was transferred into centrifuge tubes and pelleted at 4°C. The supernatant containing cell debris and Matrigel remnants was aspirated, and fresh media was added, and the crypt fragments resuspended. The final pellet was resuspended into Matrigel or the relevant PEG hydrogel. Organoids within the gels were flooded with hCCCM and cultured at 37 °C and 5% CO_2 and fed every 2-3 days.

2.2.4 RNA Isolation

RNA from freshly isolated colonic primary mucosa, freshly isolated crypts and human colonic organoids was isolated by the Williams lab using the ReliaPrep ™ RNA Miniprep System (Promega) according to the manufacturer's instructions. Total RNA concentration and purity was then measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Reverse-transcription PCR was used to generate cDNA from 500 nM of RNA using oligo-dT primers (Promega) and M-MLV Reverse Transcriptase kit (Thermo Fisher Scientific).

2.2.5 Reverse Transcription PCR (RT-PCR)

RT-PCR was performed by members of the Williams lab using 25ng/µl of cDNA in a final volume of 25µl. The reaction comprised 10 nM of PIEZO1 or GAPDH forward and reverse primers (Table 2.3), 200 µM of dNTPs, 0.04 U/µl of GOTaq® G2 DNA Polymerase (Promega), PCR buffer (Promega) and 2.5 mM of $MgCl_2$ and was carried out using a GStorm thermocycler. cDNA was amplified following 1 initial denaturation cycle at 94°C for 1 minute, 35 cycles comprising denaturation at 94°C for 30 seconds, annealing at a temperature ranging from 55

to 65° for 30 seconds, and extension at 72°C for 1 minute; finishing with 1 cycle of final extension at 72°C for 5 minutes. The recovered PCR products were run in 3% agarose gel containing 0.5 µg/ml of ethidium bromide and visualised under UV light with a gel imager.

Table 2.3 Outline of primers used for RT-PCR

Gene	**Forward**	**Reverse**	**Amplicon Size (bp)**	**Supplier**
PIEZO 1	5'-CTGCTGCCCAT GGTCATTTTC-3'	5'-GAAGATGTCCT GGCAGAGCTTG-3'	207	Fisher Scientific
GAPDH	5'-GTCAGTGGTGG ACCTGACCTG-3'	5'-TGCTGTAGCC AAATTCGTTG-3'	245	Fisher Scientific

2.2.6 RNA sequencing (RNAseq)

Following RNA isolation and purification by the Williams laboratory, an RNAseq library was prepared from isolated colonic primary mucosa, freshly isolated crypts and human colonic organoids of matching patients. Illumina RNAseq (175 bp PE HiSeq 4000; 100 million reads/sample) was performed at Earlham Institute Genome Centre. Data is expressed in reads per kilobase million (RPKM) which is a normalised value to correct differences in sample sequencing and gene length.

2.2.7 Hydrogel Components

Thiol-terminated 4-Arm PEG (PEG-SH) and Maleimide-terminated 4-Arm PEG (PEG-MAL) in various molecular weights 5, 10, 20 and 40 kDa (5K, 10K, 20K and 40K) were purchased from Jenkem Technology USA. Corning® Growth factor reduced Matrigel® basement membrane

matrix was purchased from VWR international. Collagen-I was purchased from Sigma and human recombinant laminin (LAM) isoforms 111,121 511 and 521 were purchased from BioLamina and further purified using a Vivaspin 500 100K MWCO spun at 12,000g for 10 minutes. The bis-cysteine, MMP-degradable peptide GCRDVPMSMRGGDRCG (VPM) (MW: 1696.97 Da) and adhesion promoting peptide GRDDSPC (RGD) (MW: 690.72 Da) were purchased from Lifetein. The adhesion promoting fibronectin binding (FN-Binder) GCRETLQPVYEYMVGV (MW: 1844.11 Da) and basement membrane binding (BM-binder) Ac-GCREISAFLGIPFAEPPMGPRRFLPPEPKKP-Am (MW: 3477.11 Da) were custom synthesised from Lifetein from the published sequences by Hernandez-Gordillo et al., 2020.

2.2.8 Hydrogel fabrication

Further details for fabrication of each iteration of PEG hydrogel are located in chapter-specific methodology sections. In brief, hydrogels were prepared using thiol-ene chemistry where both SH and MAL functionalised 4-Arm PEGs of the desired molecular weight (10K unless otherwise stated) were independently dissolved in filtered PBS 1X containing 5 mM EDTA, degassed with N_2(g), to achieve precursor solutions of a desired 5% w/v (unless otherwise stated). Following a brief vortex, PEG precursor solutions were kept on ice before equivalent volumes of PEG-MAL and PEG-SH were mixed under physiological conditions to generate hydrogel networks via Michael addition of a desired final PEG content, where the stochiometric ratio of SH:MAL was 1:1.

2.2.9 Hydrogel optimisation

In order to impart bio-functionality into PEG hydrogels Matrigel, Collagen-I and Laminin were integrated independently into the hydrogel network via physical blend. Matigel, Collagen-I or various laminin isoforms were added at the desired concentration to the PEG-SH suspension

to constitute 10 or 20% of the total w/v dependant on the experimental conditions. Following thorough mixing, the PEG-SH suspension containing Matrigel, collagen or laminin was crosslinked with PEG-MAL to form hydrogels. For the integration of bio-functional peptides into the PEG hydrogel network, thiol-ene chemistry was again exploited to pre-react PEG-MAL with thiol-containing cysteine residues found within the functional peptides. RGD, BM binding peptide and FN binding peptide were reacted with PEG-MAL to achieve final concentrations of 1.5 mM, 0.25mM and 0.25 mM respectively within the hydrogel. The VPM degradable peptide was also incorporated via thiol-ene chemistry to constitute either 10 or 20% of the final gel w/v% (chapter 3) or as a degradable crosslinker (chapter 4). Further details of hydrogel optimisation are located in chapter specific methodology sections.

2.2.10 Quantification of free thiol in PEG hydrogels

To identify hydrogel crosslinking efficiency, thiol conversion was quantified by measuring the unreacted thiols in PEG hydrogels using Ellman's reagent, in accordance with manufacturers guidelines (Thermo Scientific 22582). Briefly, PEG-4SH, PEG-4MAL solutions and the necessary functional or degradable peptide were mixed and incubated at 37 °C to allow gel formation of the relevant native, functional, degradable or non-degradable hydrogel. 30 µL of PEG hydrogels, PEG starting materials or mid-reaction product were immersed in 300 µL of Ellman's reagent solution and then incubated at room temperature on a shaker for 30 min. Absorbance of coloured products of thiols reacting with Ellman's reagent was measured at 412 nm on a plate reader (BMG Labtech), and the amount of free thiols in PEG hydrogels, starting materials or mid reaction product was quantified by comparison to a standard curve composed of known concentrations of L-cysteine.

2.2.11 Rheological characterisation of PEG Hydrogels

Rheological characterisation was performed on all hydrogel samples using the TA instruments Discovery HR 30 rheometer with the 20 mm diameter parallel plate test geometry (TA instruments). A hydrogel sample of 500 µL was dispensed in liquid state onto a pre-heated rheometer plate (37^{o}C) and the test geometry lowered to the desired gap height of 500 µm. the shear modulus (G') was determined by performing small-strain oscillatory measurements in the form of progressive time, amplitude and frequency sweeps with the parameters summarised in Table 2.4. Each hydrogel sample was utilised for one testing sequence, each test was performed in triplicate and data represents n=3 with corresponding standard deviation. As testing times were relatively short the humidified chamber was not utilised.

Table 2.4 Outline of parameters used for rheological characterisation.

Parameter	Amplitude Sweep	Frequency Sweep
Temperature (°C)	37	37
Plate Diameter (mm)	25	25
Gap Size (µM)	500	500
Equilibrium Time(min)	1	10
Frequency (rad/s)	1	0.1 - 100
Strain (%)	0.1-100	3

2.2.12 Organoid encapsulation

Organoids cultured in Matrigel were routinely passaged according to the procedure outlined in section 2.2.3 to yield a concentrated suspension of organoid fragments. The organoid fragment suspension was added to the PEG-MAL precursor solution and following sufficient mixing with a pipette stored on ice. For rapid gelation speed hydrogels, the organoid containing gels were formed directly onto a tissue culture plate through the distribution of 20 µL PEG-MAL droplets and subsequent addition and rapid mixing of 20 µL PEG-SH. For slower gelation speed hydrogels, the PEG-MAL suspension containing organoid fragments was mixed with the thiol containing pre functionalised PEG prior to the plating of 40 µL droplets of organoid containing gel. Following polymerisation of the hydrogel, each well was flooded with hCCCM.

2.2.13 Quantification of HIO growth

Organoid growth was monitored in real time by placing a multi-well plate into a climate-controlled chamber (5% CO_2, 37^{o}C) located on the stage of an inverted microscope. DIC illumination was established with a x10 objective and whole well images were obtained. Images were captured every other day over the period of 7 days. Organoid health and morphology were assessed visually and for viable organoids, the size was determined using the Nikon, NIS-elements software or Image J to trace organoid circumference, obtaining a value of cross-sectional area (CSA) as depicted in figure 2.X. Healthy organoids were monitored over the 7-day duration and CSA was measured on alternate days. Normalised growth was reported as fold increase to organoid CSA in relation to starting size.

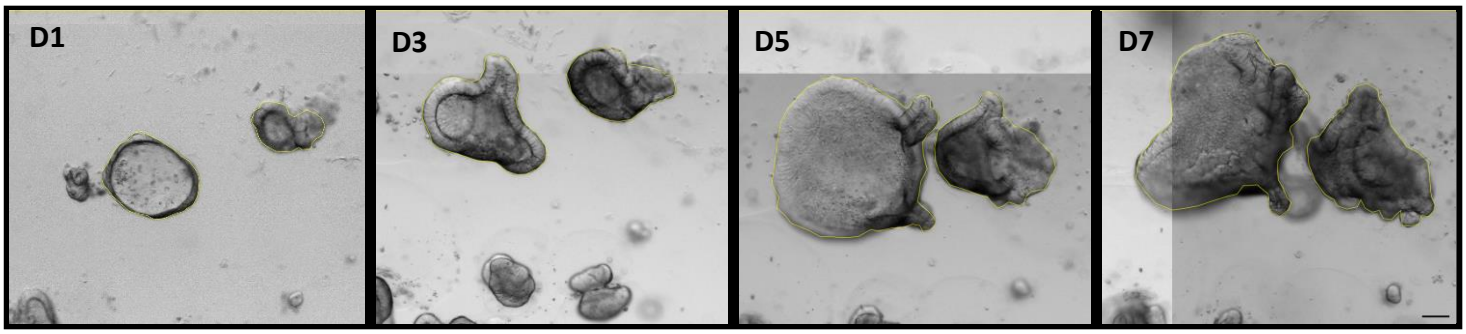


Figure 2.1 Organoid growth measurements. colonic organoids were imaged on the first day of culture, 24 hours after plating (D1) until day 7 (D7). Yellow lines indicate the cross-sectional area measurements. Scale bar 100 µm

2.2.14 Qualitative assessment of HIO morphology and survival

Organoid morphology within PEG hydrogels was visually assessed with the key morphological traits detailed in **Figure 2.2**. Where applicable organoid morphological assessment is reported as a percentage of viable organoids that possess each trait. For organoid survival, the number of viable organoids present within the gel are counted on the initial day of culture. By assessing the morphological characteristics to determine organoid viability, the percentage of viable organoid structures are reported on subsequent days of culture.

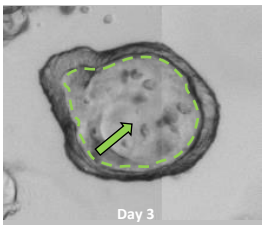
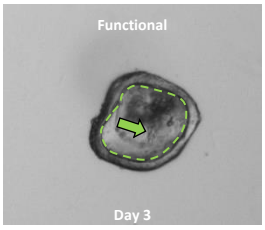
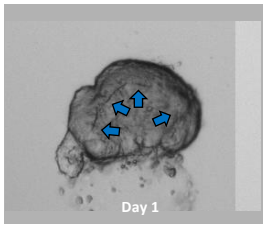
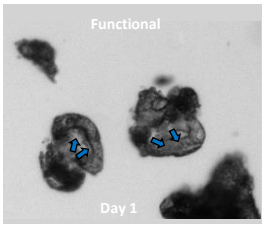
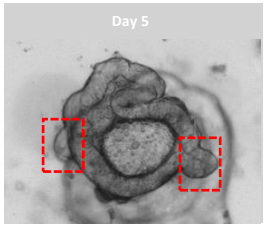
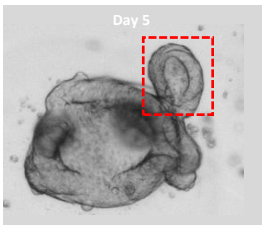
Morphological trait	Example		Description
	Matrigel	PEG	
Lumen			Healthy colonic organoids possess a layer of epithelial cells which form around a central, hollow lumen with a clear distinction between lumen and epithelium.
Epithelium			Even in the absence of a distinct lumen, the epithelium is somewhat visible and intact indicating some level of membrane polarisation.
Budding	 		New, budding 'crypt-like' domains protrude from the central organoid structure and are indicative of a population of adult stem cells

Figure 2.2. Representative images of morphological traits used for morphological assessment of HIO embedded in degradable PEG hydrogels. Individual brightfield images of HIO possessing relevant morphological traits (listed on left hand side) extracted from 7-day whole well images of organoids in the relevant culture conditions (Matrigel and degradable PEG) with the day of culture indicated on the image. Brief description of morphological trait is presented (right hand side).

2.2.15 Immunofluorescence analysis

HIO cultured in PEG hydrogels or Matrigel were fixed with 4% (PFA) for 1 hour at room temperature before washing twice with PBS. The samples were then treated with NH_4Cl_2 for 13 minutes to remove the excess of aldehyde bonds, after which 1% SDS and 1% Triton 100-X were added to permeabilise the membrane. The samples were later treated with donkey serum and 1% BSA for 2h to prevent non-specific binding of the antibodies. Samples were incubated over night with the primary antibodies at 37 ºC. Specific Alexa Fluor-conjugated secondary antibodies were added to the organoids for 2h, before applying Vectashield mounting solution containing cell nuclear stain Sytox Blue or Hoescht. Organoids when then mounted onto slides and the immunolabelling was imaged using laser confocal microscopy (Zeiss LSM) or epifluorescence microscopy (Nikon).

2.2.16 Edu labelling of human intestinal organoids to assess proliferation

HIO cultured in PEG hydrogels and Matrigel were incubated with Edu (Fischer Scientific) for 2 hours at 37 ºC. After fixing the organoids in PFA, the Click-IT reaction was prepared following the manufacturer's instructions (Fischer Scientific) and colonic organoids were incubated for 40 minutes in the dark at room temperature. The reaction was stopped with 3% BSA and the samples were then treated for 2h with 10% donkey serum and 1% BSA to avoid unspecific binding of the antibodies. HIO were incubated with primary antibodies overnight at 40ºC and then treated with the appropriate Alexa fluor-conjugated secondary antibodies for 2h at 40ºC. Samples were mounted with Vectashield with the nuclear stain Hoescht and imaged using an epifluorescence microscope (Nikon Ti) or laser confocal microscopy (Zeiss LSM).

2.2.17 Statistical Analysis

Data is presented as means ± standard error. Statistical analyses were performed using SPSS v28. Appropriate tests were selected as appropriate for the data. Statistical tests used were independent t-test, one-way ANOVA followed by Tukey's post-hoc testing (equal variances assumed by Levene's test) or Welch's ANOVA followed by Games-Howell post-hoc testing (equal variances not assumed by Levene's test). For ANOVA reporting the 'F' value refers to the variation between the sample means, relative to variation within the sample. For t-test reporting the 't' value measures the size of the difference in relation to variation in the data sample. 'P' values are used as indicator of statistical significance. 'N' refers to the number of subjects (i.e. patient derived organoid line) and 'n' to the number of experiments analysed. Individual n numbers for total number of organoids assessed are available in appendix 1 and 2.

3. Optimisation of a defined 3D-PEG hydrogel system for human intestinal organoid culture.

3.1 Introduction

Before the advent of robust ex vivo culture systems, research into the structure and physiological function of the human colonic epithelium has been over-reliant on the utility of adenocarcinoma cell lines such as Caco-2 (Sambuy et al., 2005) and T84 (Devriese et al., 2017). Grown as 2D monolayers, this type of *in vitro* cell culture is low cost and easy to maintain, whilst yielding highly reproducible results; however, like all 2D cell culture methods they fail to replicate complex cell-to-cell, cell-to-extracellular matrix interactions and wider cell polarity that is found in tissues. As an alternative, animal models have been utilised as more complex *in vivo* models which build upon *in vitro* studies and have enabled a deeper understanding of gut epithelial cell biology, disease pathology and cell physiology. There remains however, a translational limitation between the research conducted using animal models and human clinical trials due to the significant genetic differences which are present (Mak, Evaniew and Ghert, 2014). This has resulted in a rise in interest for the development of 3D culture systems which seek to recreate the *in vivo*-like architecture, genetic profile and cell polarisation of the tissues from which they are derived. (Kim, Koo and Knoblich, 2020)

Organoids are 3D, multicellular, self-organising structures that are derived from stem cells and recapitulate the structure and spatial organisation of the organ they're derived from (Lancaster and Knoblich, 2014). Organoid cultures are generated *in vitro* and develop free from undesired, interfering cell types (i.e., vascular or nervous). A wide variety of use cases for organoid cultures have been established including organ developmental studies,

modelling various diseases including cancer, and as pharmaceutical testing platforms to name a few. (Boj et al., 2015; Grapin-Botton, 2016; Van De Wetering et al., 2015). Despite the heterogeneity of organoid cultures, and the absence of routine and robust protocols for their establishment, the prospect of organoid cultures for clinical and research purposes have made them of vast importance.

Intestinal organoids were amongst the first organoid systems validated as a useful research tool and were developed using a mouse model by Sato, Clevers and colleagues (Sato et al., 2009). Dissociated small intestinal crypts, containing adult intestinal stem cells, were embedded into a complex mixture of extracellular matrix proteins known as Matrigel. With additional supplementation from matrix-derived growth factors it was possible to generate a 3D structure that resembled a crypt-like morphology, whilst successfully recapitulating the intestinal epithelial characteristics – with stem cells at the base of crypt-like, budding structures which proliferate to give rise to all types of differentiated cell within the intestinal epithelium. Subsequent research has resulted in the development of a 3D, human colonic organoid culture system derived from adult Lgr5-expressing intestinal stem cells, which when cultured in specific three-dimensional conditions, undergo continued cycles of self-renewal, differentiation and morphogenesis; all while self-organising to consist of a highly polarised epithelium around a central lumen, with the maintenance of stem cell housing, crypt-like domains projecting outwards. (Barker et al., 2007; Sato and Clevers, 2013; Fujii et al., 2018; Pleguezuelos-Manzano et al., 2020).

Fundamental research by the Williams lab has developed a well-established protocol for the routine culture of adult, human colonic, patient-matched organoids derived from healthy and disease tissue following colorectal surgery, which are utilised for the study of adult intestinal

stem cell renewal in both healthy and diseased state (Parris and Williams, 2015; Pelaez-Llaneza et al., 2022 pre-print)The translational potential of this human colonic organoid research to the clinic, however, is not yet fully realised, due to current routine 3D culture methods relying heavily on the commercially available, animal-derived hydrogel known as Matrigel. Matrigel, although serving as the gold standard for organoid growth and expansion in the laboratory, is an ill-defined, complex mixture of extracellular matrix proteins and diffusible growth factors that is prone to batch-to-batch variability. In addition, Matrigel is not conducive to further modification towards the culture of a specific tissues, whilst its animal, tumour-derived origin provides a risk of immunogen and pathogen transfer, confounding its use for downstream clinical applications. (Hughes, Postovit and Lajoie, 2010; Kozlowski, Crook and Ku, 2021).

Synthetic or semi-synthetic biomaterials have become a useful tool in the development of a suitable Matrigel replacement for 3D culture of human colonic organoids. This is owing to their modular nature allowing the fine tuning and systematic study of mechanical or biochemical properties, resulting in the identification of a defined, tailored 3D culture environment with high degree of cellular specificity for a desired organoid culture (Aisenbrey and Murphy, 2020; Kozlowski, Crook and Ku, 2021). One category of synthetic biomaterial that has been utilised for colonic organoid culture is Polyethylene glycol (PEG) based hydrogels. Formed via crosslinking of multi-arm PEG macromers, these hydrogels are biologically inert, mimicking the soft, hydrated physical properties of Matrigel (Toepke et al., 2013). Substantial research and optimisation have shown PEG hydrogels crosslinked via thiol-Michael addition reactions efficiently produce a well-defined network structure over a large range polymer content range which, when optimised for parameters such as bulk mechanical stiffness, degradability and bio-functionality, has been seen to support the growth and

expansion of mouse (Gjorevski et al., 2016)and Inducible pluripotent stem cell (IPS) derived (Spence et al., 2017) small intestinal epithelial organoids with relative efficiency. However, human tissue is naturally more fastidious to culture, and colonic tissue more demanding than small intestine. In addition, the mechanical, physical, and biochemical requirements for the culture of human, adult stem cell-derived organoids differ to that of mouse or IPS cells - (Sato et al., 2011)hence there remains the requirement for an effective, fully defined and optimised Matrigel alternative for colonic, human intestinal organoid (HIO) culture.

Utilising previous experience from the Saeed lab in developing 3D, stem cell culture systems with bio-responsive gels (Saeed et al., 2015), early collaborative efforts between the Saeed and Williams laboratory, conducted by Sandy and colleagues, began to employ a PEG-based hydrogel formed via thiol-Michael addition between 10 kDa 4-arm, thiol (SH) and maleimide (MAL) terminated PEG macromers (**Figure 3.1**) as a platform to assess the biochemical and biomechanical requirements necessary to support colonic HIO growth. The output of this collaboration serves as the foundation for a series of optimisation and modifications to a PEG-based hydrogel system that are explored throughout this chapter, with the aim of improving the growth and morphology of HIO cultured in a fully defined synthetic PEG hydrogel.

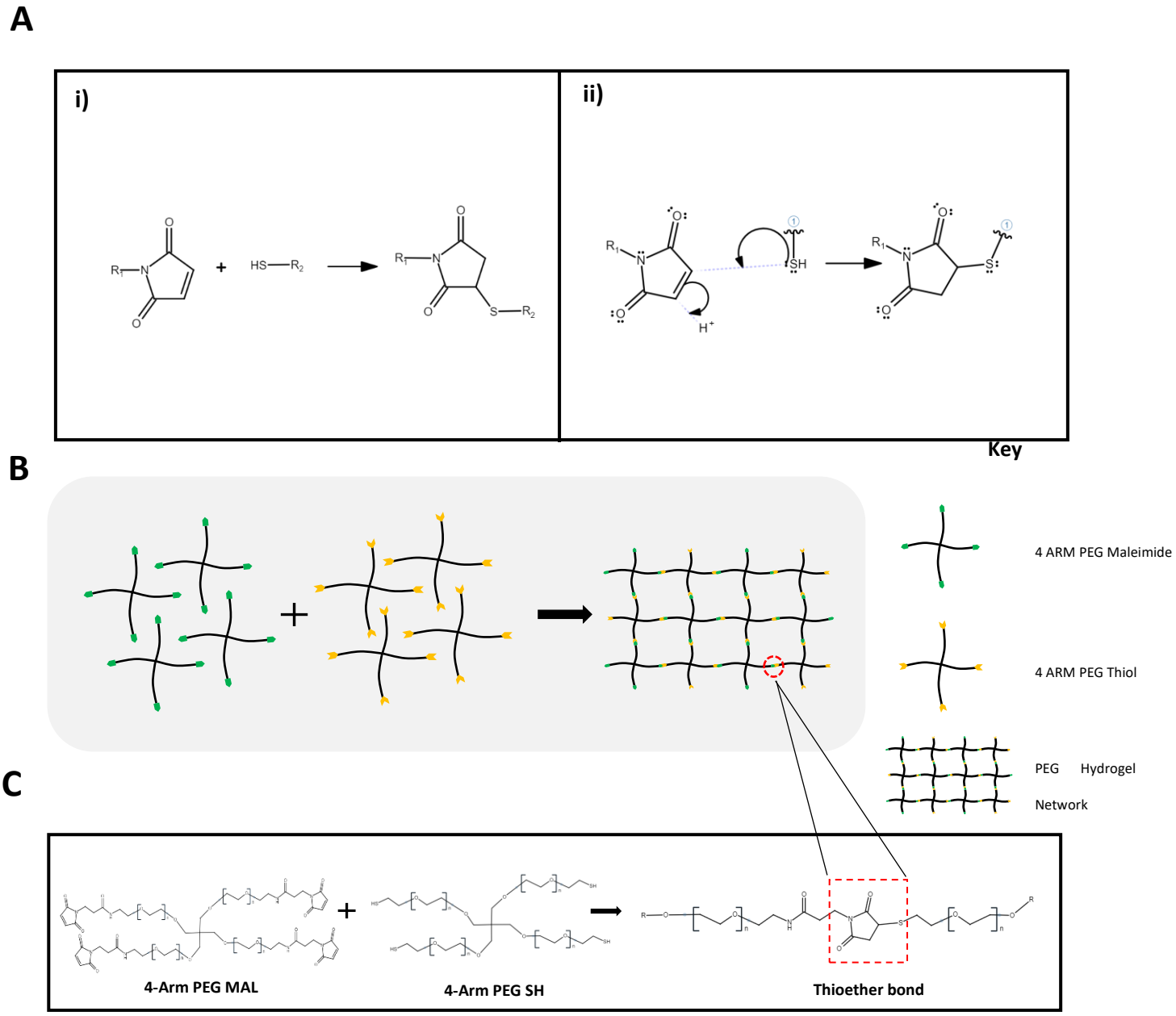


Figure 3.1 Overview of Thiol-Michael addition reaction for the crosslinking of PEG hydrogels. (Ai) Simplified general mechanism for the thiol-maleimide reaction. (Aii) detailed reaction mechanism for thiol-michael addition. (B) Gel crosslinking schematic for the formation of a PEG hydrogel network based on the reaction between 4 ARM PEG maleimide and 4 ARM PEG SH. (C) Chemical structures of 4 ARM PEG MAL and SH. Red box highlights thioether bond formation

3.2 Hypothesis, Aims & Objectives

Hypothesis

The mechanical properties of PEG hydrogels can be manipulated to improve organoid growth and morphology. Organoid growth and morphology will be improved with the inclusion of biological functionality.

Aims

The aim of this chapter is to establish a fully defined, PEG based hydrogel for the culture of colonic HIOs. This is achieved through modification to the PEG hydrogels physical and biochemical properties in attempt to optimise organoid growth and morphology to a comparable level of that achieved in Matrigel.

Objective I

To fabricate PEG hydrogels with bespoke mechanical properties

Objective II

To assess the suitability of native PEG hydrogels as a platform for further optimisation through physical blend with Matrigel, and to replicate this via integration of large recombinant extracellular matrix proteins.

Objective III

To further optimise PEG hydrogels with bio-functionality, replacing large human recombinant ECM proteins with well-defined biological peptides.

3.3 Materials and Methods

3.3.1 Hydrogel fabrication

hydrogels were prepared using thiol-ene chemistry where both SH and MAL functionalised 4-Arm PEGs of the desired molecular weight (10K unless otherwise stated) were independently dissolved in filtered PBS 1X containing 5 mM EDTA, degassed with N_2(g), to achieve precursor solutions of a desired 5% w/v (unless otherwise stated). Following a brief vortex, PEG precursor solutions were kept on ice before equivalent volumes of PEG-MAL and PEG-SH were mixed under physiological conditions to generate hydrogel networks via Michael addition of a desired final PEG content, where the stochiometric ratio of SH:MAL was 1:1.

3.3.2 Hydrogel functionalisation

To impart bio-functionality into PEG hydrogels Matrigel, Collagen-I and Laminin were integrated independently into the hydrogel network via physical blend. Matigel, Collagen-I or various laminin isoforms (see section 2.2.7) were added at the desired concentration to the PEG-SH suspension to constitute 10 or 20% of the total w/v dependant on the experimental conditions. Following thorough mixing, the PEG-SH suspension containing Matrigel, collagen or laminin was crosslinked with PEG-MAL to form hydrogels. For the integration of bio-functional peptides into the PEG hydrogel network, thiol-ene chemistry was again exploited to pre-react PEG-MAL with thiol-containing cysteine residues found within the functional peptides. RGD, BM binding peptide and FN binding peptide (see section 2.2.7 for sequence details) were reacted with PEG-MAL to achieve final concentrations of 1.5 mM, 0.25mM and 0.25 mM respectively within the hydrogel. The VPM degradable peptide was also incorporated via thiol-ene chemistry to constitute either 10 or 20% of the final gel w/v%

3.3.3 Organoid culture and analysis

Full details of organoid culture and analysis used throughout this chapter are presented in chapter 2

3.4 Results

In order to develop a 3D PEG hydrogel as a suitable Matrigel replacement in colonic HIO culture, gels were subject to a series of progressive stages of optimisation and characterisation, with the effect of these modifications on organoid growth and morphology assessed in relation to the performance of organoids during routine culture in Matrigel. Firstly, the biomechanical nature of the HIO cultures were explored utilising immunofluorescence, RT-PCR and mRNA sequencing to identify key mechano-sensing receptors. Then, non-functionalised, native PEG hydrogels constructed to assess the influence of gel mechanical properties on organoid growth. Next, a suitable candidate hydrogel was selected for further optimisation based on mechanical properties and organoid performance in the native environment. These early stages of optimisation involved creating physical blends between PEG hydrogels and various ECM proteins to identify necessary biochemical cues required to replicate organoid growth and morphology exhibited in Matrigel. Finally, the large recombinant ECM proteins incorporated via physical blend were replaced with simplified peptide sequences incorporated into the hydrogel network and their influence on organoid growth and morphology was assessed in relation to performance in Matrigel.

3.4.1 Assessment of colonic HIO culture in Matrigel

In accordance with the established Williams lab protocol, adult ISC containing colonic crypts were mechanically dissociated into small fragments and embedded into Matrigel (Reynolds et al., 2014; Parris and Williams, 2015; Pelaez-Llaneza et al., 2022 pre-print). When cultured in human colonic crypt culture media (hCCCM) and incubated at 37 °C, 5% CO_2, these fragments yield organoids which continue to develop new crypt-like budding structures in the first 2 – 3 days of culture, which possess a well-defined lumen serving as an indicator of defined apicobasal polarity (**Figure 3.2A and 3.2B**). These organoids continue to grow in size, as observed by an average 3.75 (± 0.5) fold increase in overall cross-sectional area. The continued formation and growth of new crypt-like structures, suggests the presence of maturing differentiated cell types. Peak growth and morphology were often observed between 5 and 7 days, where organoids were then passaged, and fragments embedded in Matrigel to achieve a routine colonic HIO culture in Matrigel.

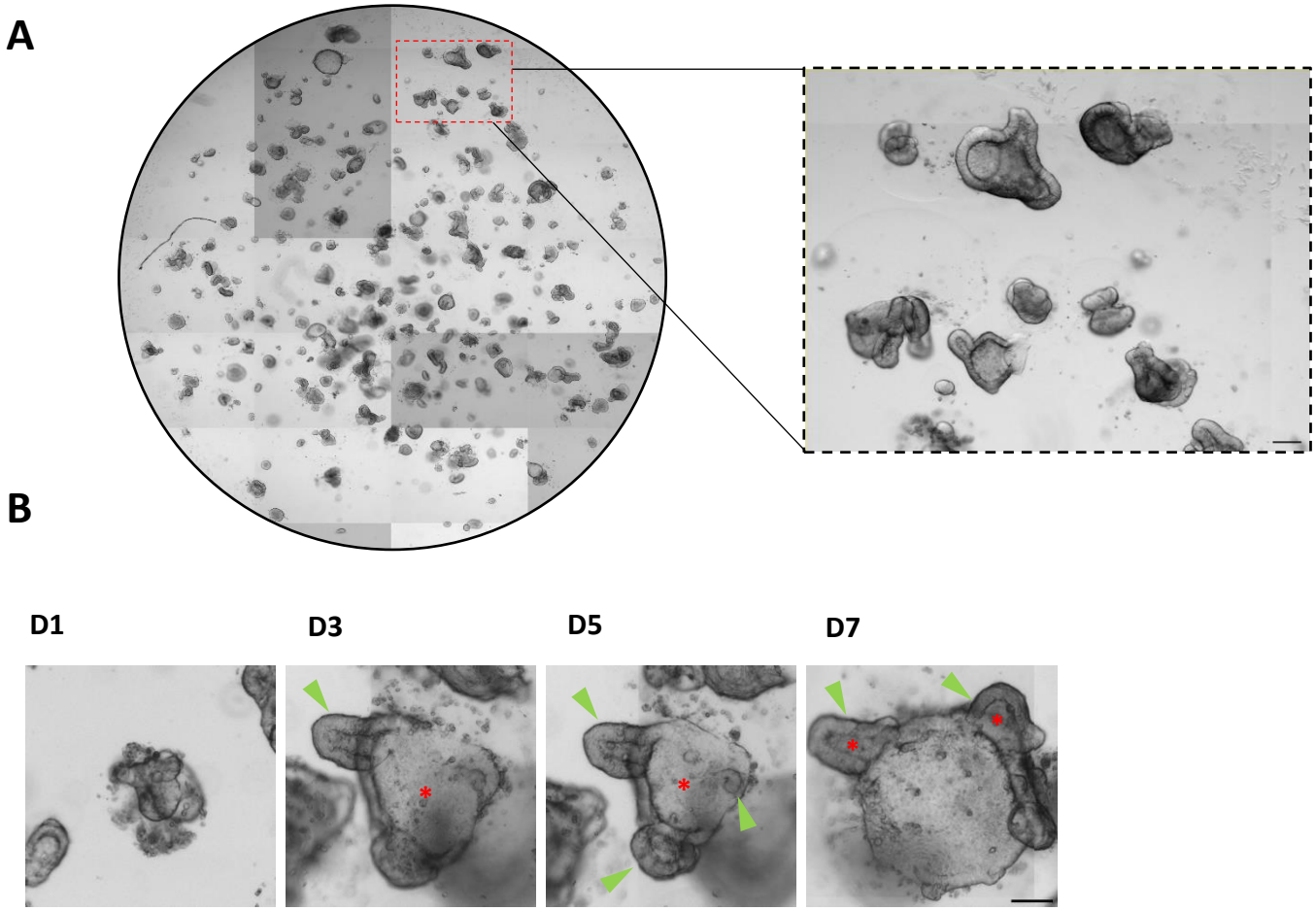


Figure 3.2 Colonic HIO 3D culture in Matrigel. Representative brightfield images of (A) budding colonic HIO cultured in Matrigel. The right-hand side depicting high magnification images of individual organoid structures depicting their morphology. Scale bar 100 µM. (B) Brightfield images depicting growth of colonic organoids on alternate days of a 7-day period. Red star indicates the presence of a defined lumen whilst arrowheads highlight budding structures. Scale bar 100 µM.

3.4.2 Identification of mechanosensing receptor in colonic HIO culture

Routine colonic HIO culture system in the Williams lab utilising Matrigel serves as a useful tool for assessing the potential requirements of a synthetic PEG hydrogel alternative. To evaluate the potential bio-mechanical needs of the HIO platform we decided to analyse organoid gene expression and localisation of the mechanically sensitive ion channel PIEZO1. Members of the Williams lab isolated and purified RNA from organoid cultures and Reverse transcription PCR amplification for specific transcripts (see materials and methods) revealed expression at the mRNA level for PIEZO1 (**Figure 3.3A**). This was confirmed by bulk RNA sequencing, of genetic material from organoids as well as isolated crypts and tumour material, also performed by members of the Williams lab, which revealed increased expression for PIEZO 1 in organoids (**Figure 3.3B**). Finally, organoids cultured in Matrigel were fixed 24 hours after passage before immunolabelling for Piezo-1 receptors, alongside an enteroendecrine cell marker chromogranin A (CHGA), the epithelial cell marker E-cadherin (ECAD) and the nuclear stain sytox blue (**Figure 3.3C**).

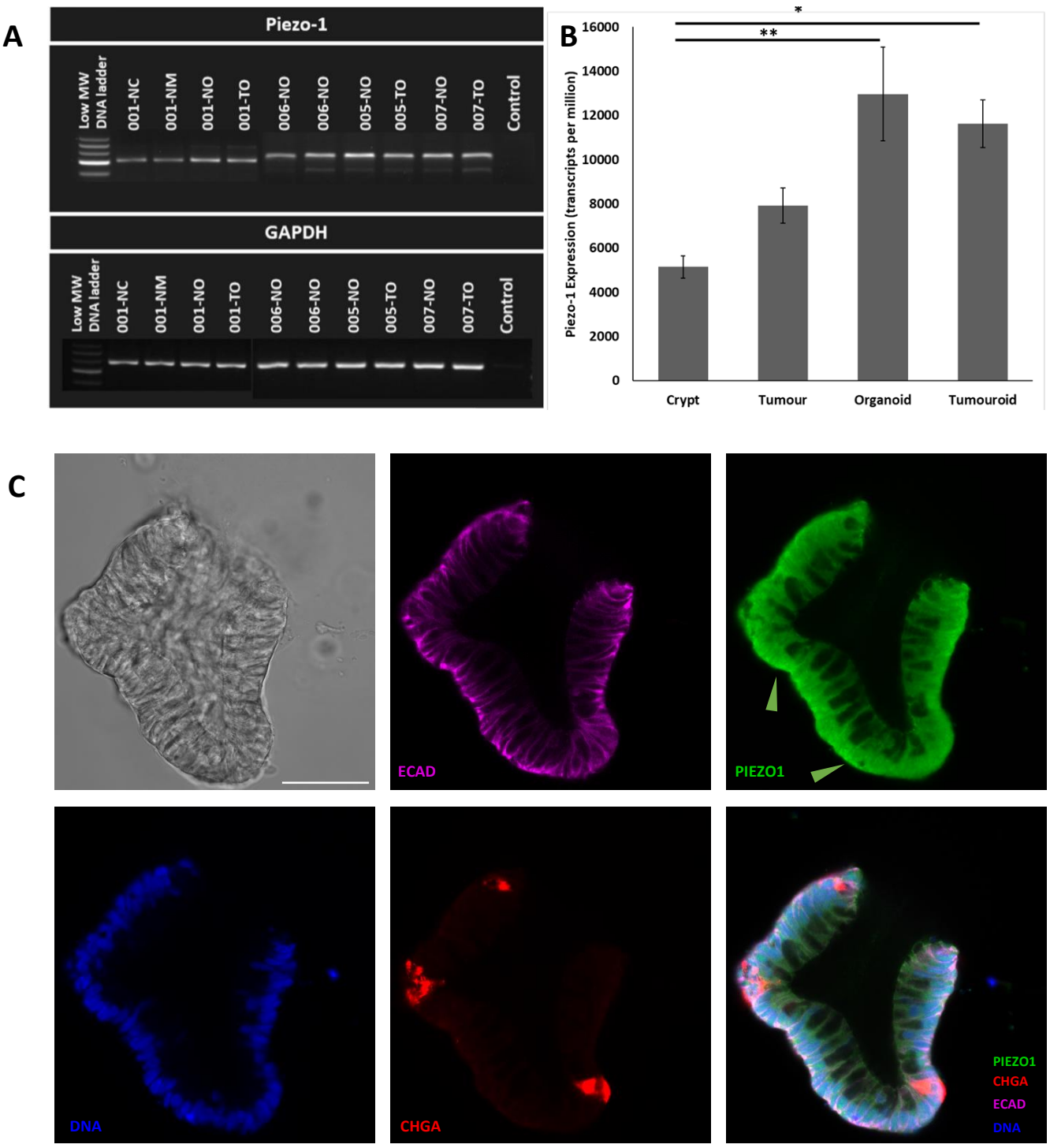


Figure 3.3 Identification of the mechano-sensitive PIEZO1 receptor in HIO culture. (A) Piezo1 and GAPDH in Crypt, Mucosa, Organoid, and Tumouroid, samples alongside Low Molecular Weight DNA ladder. 00X represents the patient, NC=healthy crypt, NM=healthy mucosa, NO= healthy organoid, TO=tumouroid (B) mRNA-seq of Piezo1 in Crypts, Mucosa, Organoids, and Tumouroids expressed in transcripts per million (n=6). ANOVA utilised to determine statistical

significance ($F_{3,19} = 7.471$, $p=0.002$). Statistical significance following a Bonferroni correction is displayed as such: =$p<0.05$, *=$p<0.01$. (C) Immunofluorescent labelling of colonic organoids cultured in Matrigel with Piezo 1 (green), Chromagranin A (red), sytoxblue (DNA-blue). Scale bars=50 µm. green arrows denote areas of Piezo-1 expression.

3.4.3 Mechanical optimisation of native PEG hydrogels for HIO growth

With the presence of the PIEZO1 channel strongly implicating our human organoid system as a mechanically sensitive culture, we deemed it necessary to characterise the mechanical profile of biologically inert PEG hydrogels, formed via crosslinking of 10 kDa 4-arm, thiol (SH) and maleimide (MAL) terminated PEG macromers as outlined in figure 3.1. Utilising rheological characterisation (see materials and methods section 2.2.11) we performed frequency sweeps at a fixed 10% strain for hydrogels ranging from 2–5% wt/vol total PEG content, identified as a suitable range through the literature alongside previous collaborative efforts between Saeed and Williams, conducted by Sandy (Sandy et al., 2019). We identified a storage modulus range for the generated PEG hydrogels of approximately 400 pa (2% PEG) – 6.5 kPa (5% PEG) (**Figure 3.4B**). Organoid fragments were then encapsulated within PEG hydrogels with ranging PEG content and cultured for a period of 7 days with their growth over the 7-day culture period and morphology on alternate culture days assessed. (**Figure 3.4A**) Organoids cultured in 5% PEG hydrogels displayed the highest increase to CSA whilst presenting viable, albeit irregular morphological properties.

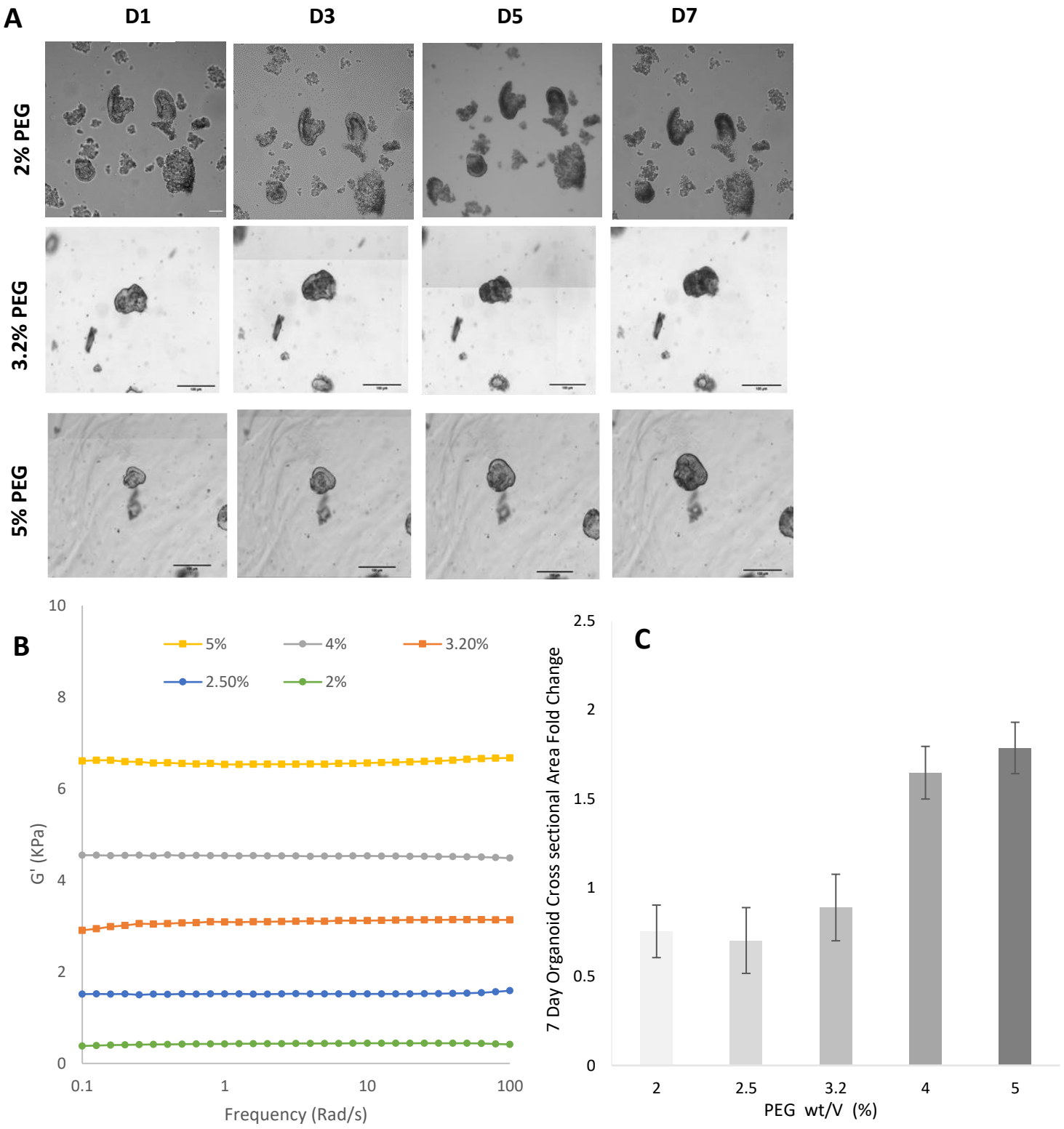
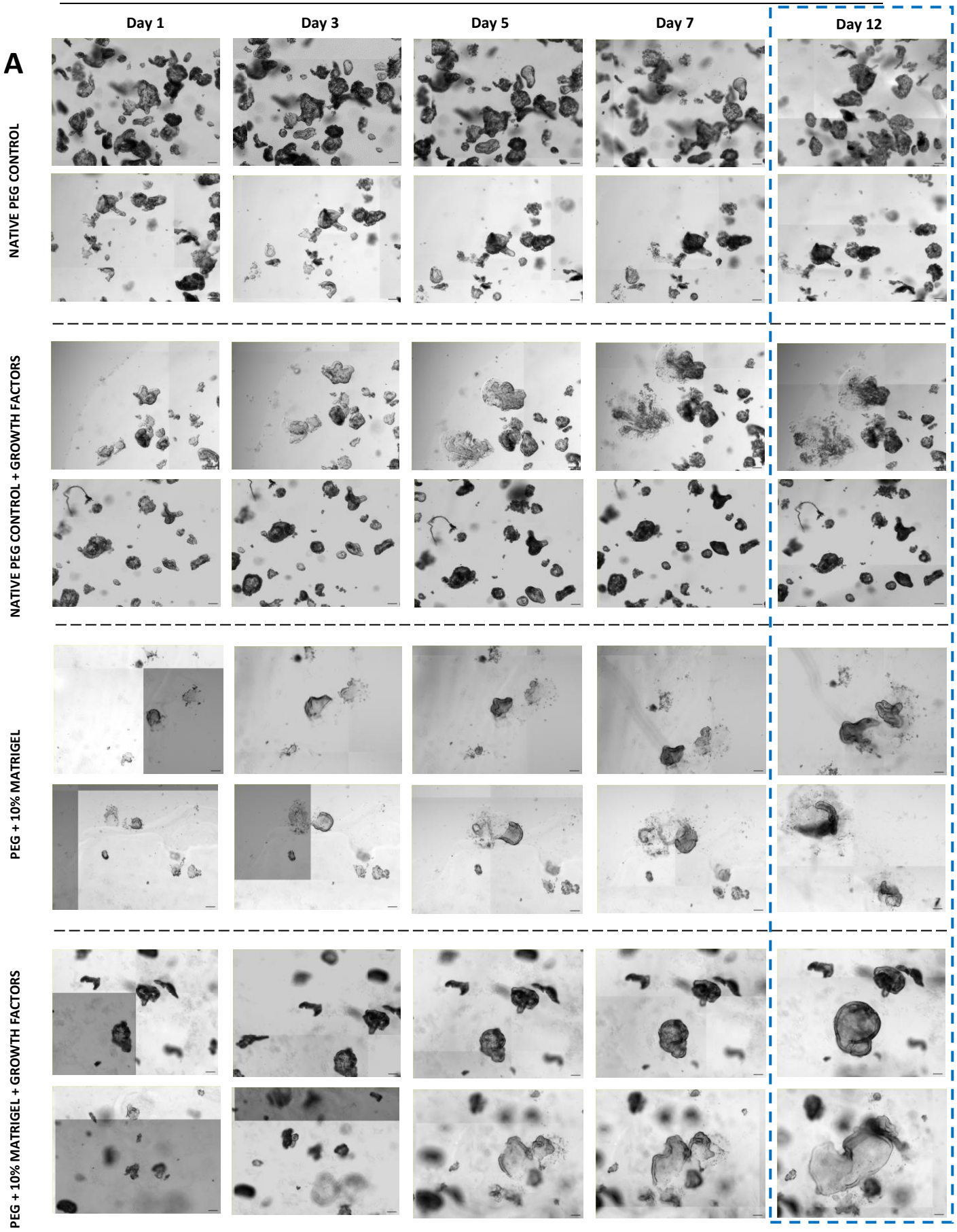


Figure 3.4 Mechanical optimisation of native PEG hydrogels towards HIO growth. (A) Rotational rheometric analysis of storage modulus (G') via frequency sweep for native PEG hydrogels of decreasing PEG wt/vol (%) n =3 (B) Representative brightfield images of organoids cultured in

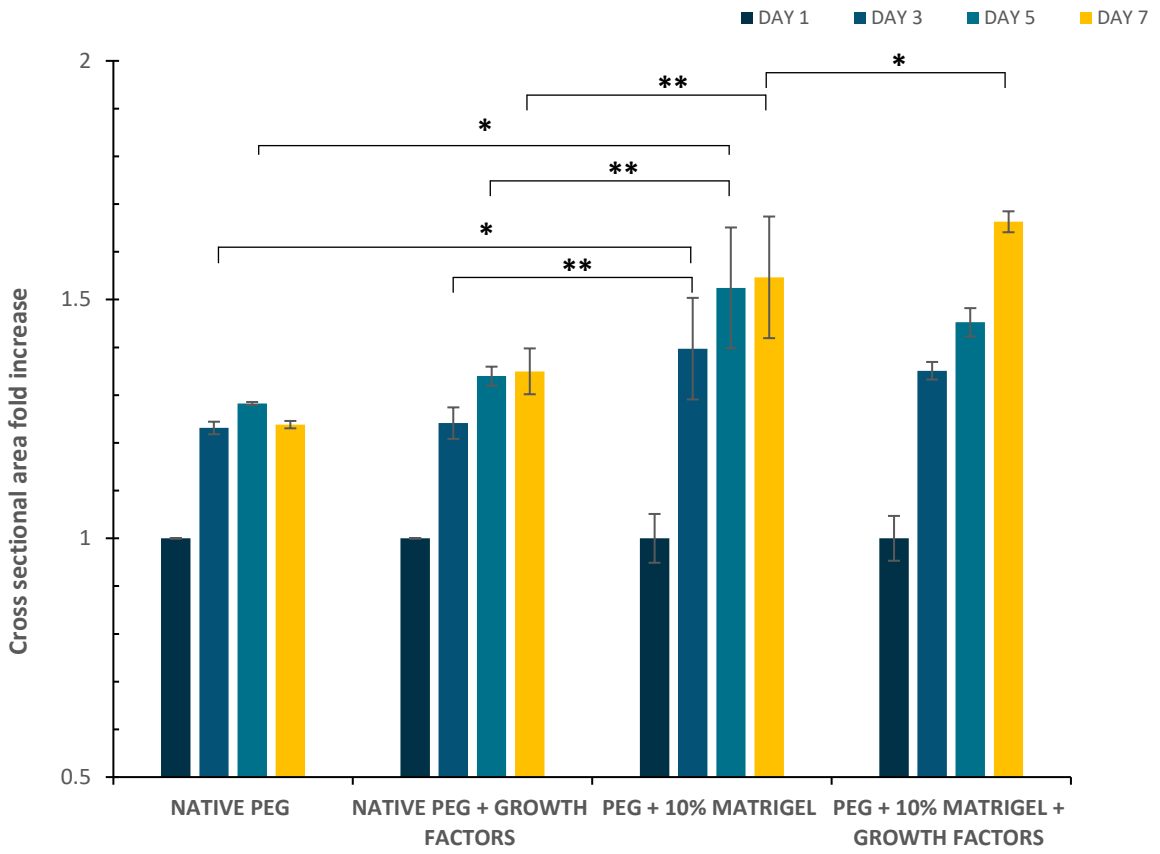
PEG hydrogels of the indicated wt/vol% tracked on alternate days of a 7 day culture. (C) Average increase to organoid cross-sectional area over a 7-day period reported as an average fold increase between starting size at day 1 and day 7 of culture.

3.4.4 Early PEG hydrogel optimisation: physical blends

Initial mechanical optimisation identified that PEG provides a tuneable environment by which the final PEG wt/vol% influences the mechanical stiffness of the gel. These bulk mechanical properties in turn effect the growth of encapsulated HIO's. Having observed the largest increase in CSA exhibited by organoids encapsulated within 5% PEG hydrogels (**Figure 3.4C)**, we chose to proceed with the 5% PEG hydrogel as a platform for further optimisation. We rationalised that imparting 5% PEG hydrogels (selected for their mechanical properties) with a biochemical stimulus, would improve overall organoid growth and morphology to a level comparable with organoids cultured in Matrigel. As a proof of concept, we generated 5% PEG hydrogels physically blended with low concentrations of Matrigel as to not influence the bulk mechanical properties of the gel. In addition, we investigated the potential benefits of including growth factors routinely used to supplement the organoid growth media, during the gelation phase (pre-addition of media) as an additional avenue to impart a biochemical stimulus into the gels. Organoid fragments were encapsulated and cultured within native 5% PEG hydrogels alongside 5% PEG hydrogels containing 10% Matrigel via physical blend either with or without growth factors and their growth and morphology observed (**Figure 3.5**).


A
Day 1
Day 3
Day 5
Day 7
Day 12
NATIVE PEG CONTROL
NATIVE PEG CONTROL + GROWTH FACTORS
PEG + 10% MATRIGEL
PEG + 10% MATRIGEL + GROWTH FACTORS

B


DAY 1
DAY 3
DAY 5
DAY 7
Cross sectional area fold increase
2
1.5
1
0.5
*
**
*
**
*
**
NATIVE PEG
NATIVE PEG + GROWTH FACTORS
PEG + 10% MATRIGEL
PEG + 10% MATRIGEL + GROWTH FACTORS

C

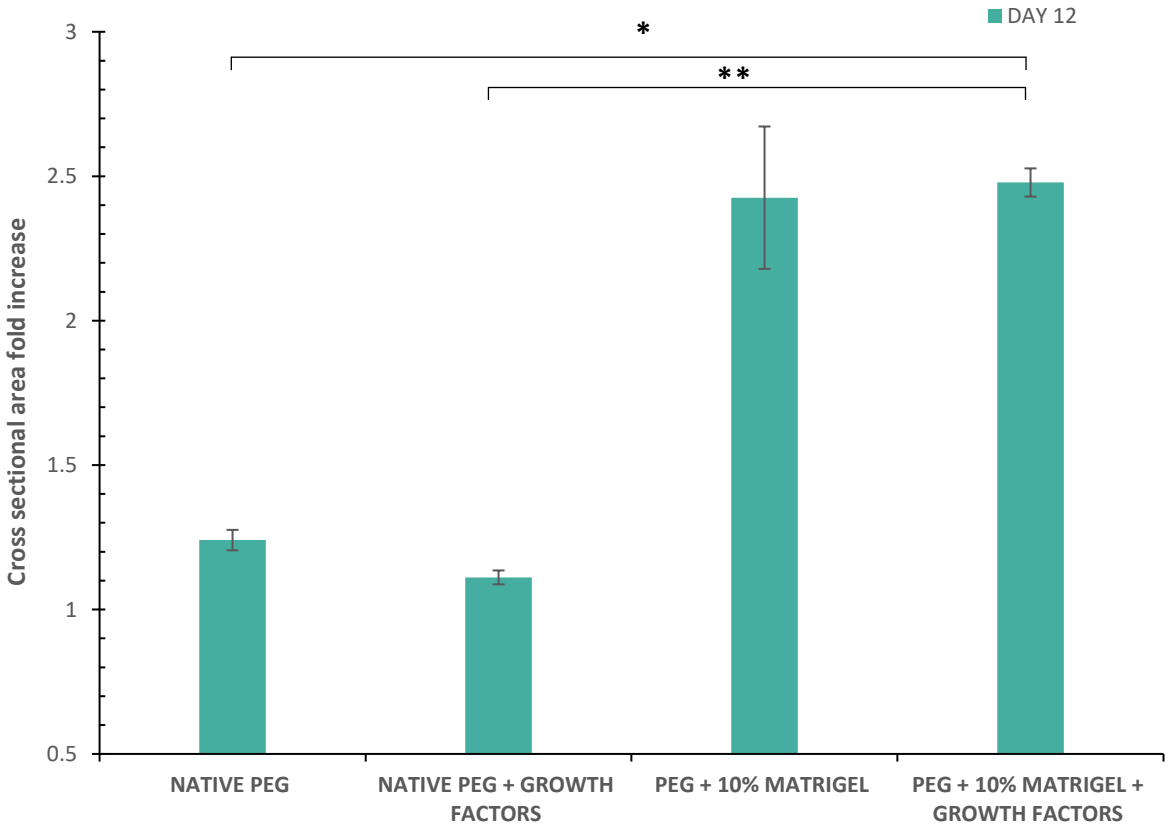

DAY 12
Cross sectional area fold increase
3
2.5
2
1.5
1
0.5
*
**
NATIVE PEG
NATIVE PEG + GROWTH FACTORS
PEG + 10% MATRIGEL
PEG + 10% MATRIGEL + GROWTH FACTORS

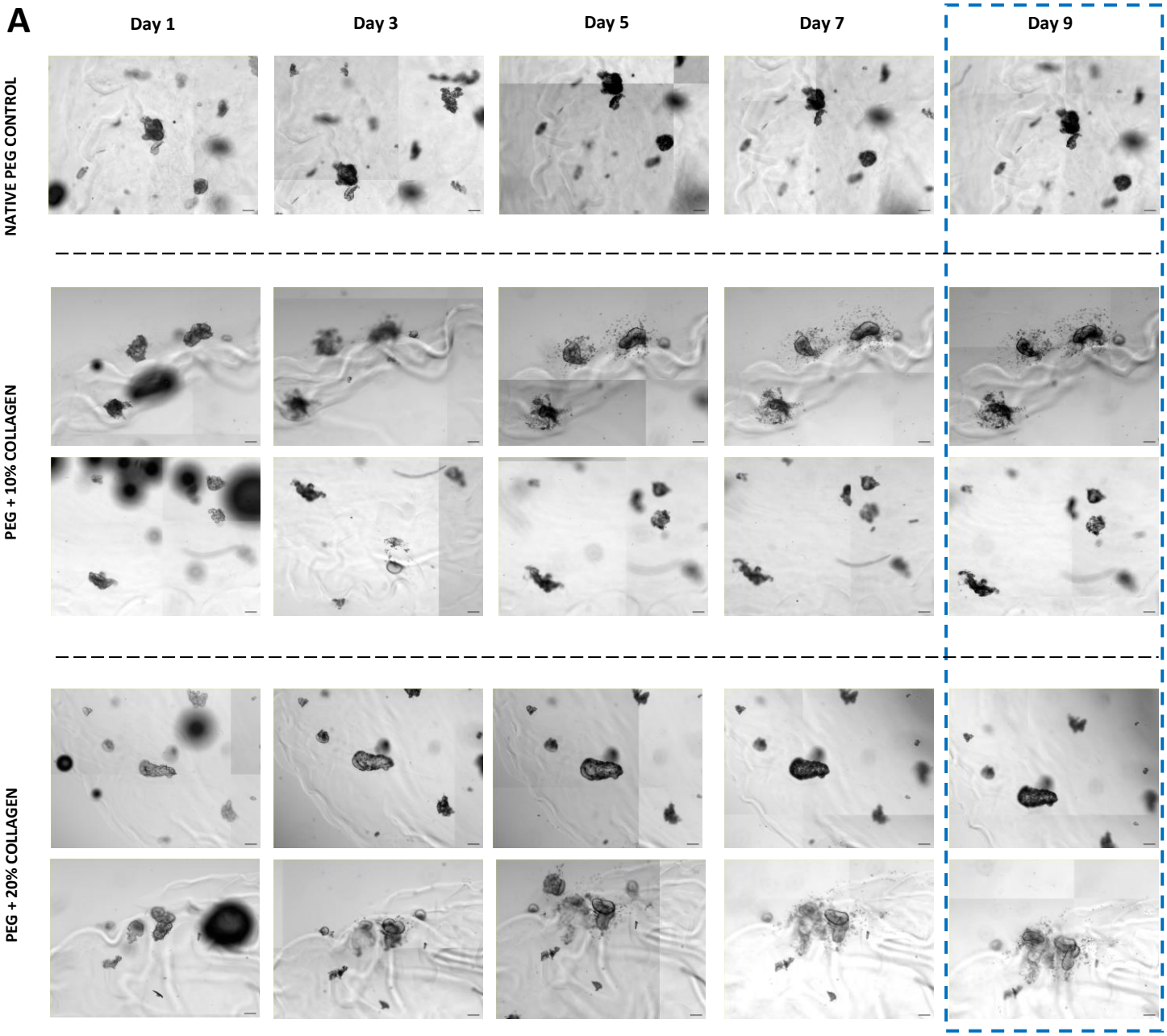
Figure 3.5 Colonic HIO culture in hybrid PEG hydrogels supplemented with biochemical cues

(A) Representative brightfield imagery of human colonic HIO cultured in Native PEG, PEG with additional growth factors, PEG supplemented with 10% Matrigel and PEG supplemented with 10% Matrigel and growth factors. Images are taken on alternate culture days as indicated. Viable organoid structures present on days beyond standard 7-day culture highlighted (blue dashed rectangle). Scale bar 100 µm. (B) Assessment of cross-sectional fold increase between alternate culture days. Data is reported as mean ± SE, Welch's ANOVA used to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($F_{3,130.972}$ =18.362 p <0.001, $F_{3,\ 126.423}$ =5.035 p = 0.002 $F_{3,131.141}$ =4.346 p = 0.006 and $F_{3,136.491}$ = 4.361 p = 0.006) (C) overall fold increase to cross sectional area on final day of culture. Data is reported as mean ± SE, Welches ANOVA used to determine statistical significance ($F_{3,110.648}$ = 5.754 p = 0.001). Statistical significance following Games-Howell post hoc test is displayed as *p < 0.05, **p <0.01 (N=1, n=2). Total n of organoids assessed per replica are available in appendix 1.

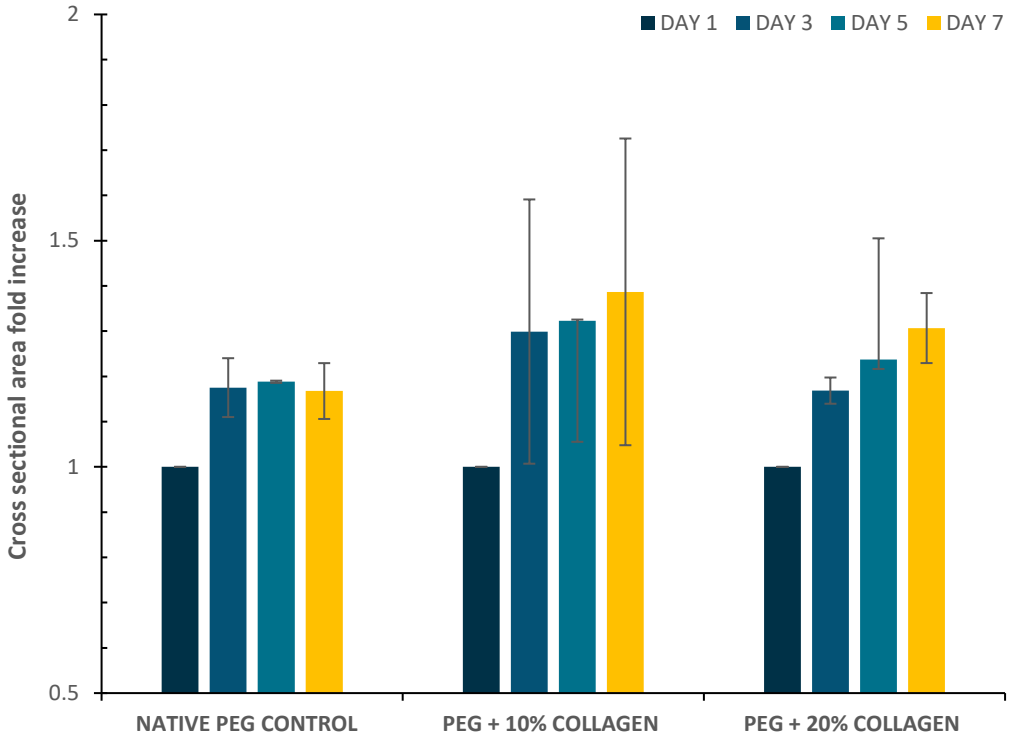
Both native PEG hydrogels and PEG hydrogels supplemented with Matrigel contained organoids displaying signs of viability and growth beyond the initial 7-day culture period (**Figure 3.5A**). After 12 days, organoids cultured in PEG gels supplemented with Matrigel and growth factors increased in cross sectional area significantly more (2.47-fold) than organoids in native PEG (1.24-fold p=0.024) and native PEG with growth factors (1.11-fold p=0.001) (**Figure 3.5C**). Within the initial 7-day culture period organoids cultured in PEG with 10% Matrigel increased in CSA significantly more than native PEG hydrogels (with and without growth factors) after 3 days and 5 days. There was so significant improvement from the addition of growth factors in either native PEG or PEG supplemented with Matrigel, until day 7 of organoid culture, where organoids in PEG with Matrigel and growth factors grew significantly more (1.66-fold increase against 1.55-fold increase p=0.028) than organoids without. Organoids cultured in native PEG conditions appeared static, some organoids with defined lumens and intact epithelium were present, however signs of deterioration (dark and dense morphology coupled with organoid fragmentation) began from day 5. Organoids cultured in PEG supplemented with Matrigel (with and without growth factors) appeared more dynamic with a greater increase in cross sectional area, defined lumens that experienced greater expansion and the formation of larger more complex structures (**Figure 3.5A**).

3.4.5 Mid-stage PEG hydrogel optimisation: human recombinant proteins

After demonstrating that the addition of biochemical input into the PEG hydrogels in the form of physical blends with Matrigel could improve the observed morphology and overall growth of the organoid structures, our next phase of optimisation sought to replace the Matrigel component within these proof-of-concept gels to create both a mechanically defined (section 3.4.3) and biochemically defined hydrogel. To achieve this, we generated 5% PEG hydrogels physically blended with Collagen-1, or Laminin - two biologically relevant ECM matrix proteins found in both the human intestinal ECM, and which contribute towards the bulk composition of Matrigel. Laminin isoforms were selected based on their biological significance as presented in the literature(Simo et al., 1991; Kikkawa et al., 2013; Barros et al., 2019). Included at similarly low concentrations, as to not influence the bulk mechanical properties of the gel, 5% PEG with collagen (**Figure 3.6**) at 10 and 20%, and 5% PEG with a range of human recombinant Laminin (**Figure 3.7**) including 111, 121, 511, 521, a combination of 511 and 521 and a combination of all aforementioned laminins at 10% concentration, were utilised for HIO culture and contrasted with the results in Native PEG.


A
Day 1
Day 3
Day 5
Day 7
Day 9
NATIVE PEG CONTROL
PEG + 10% COLLAGEN
PEG + 20% COLLAGEN

B



C

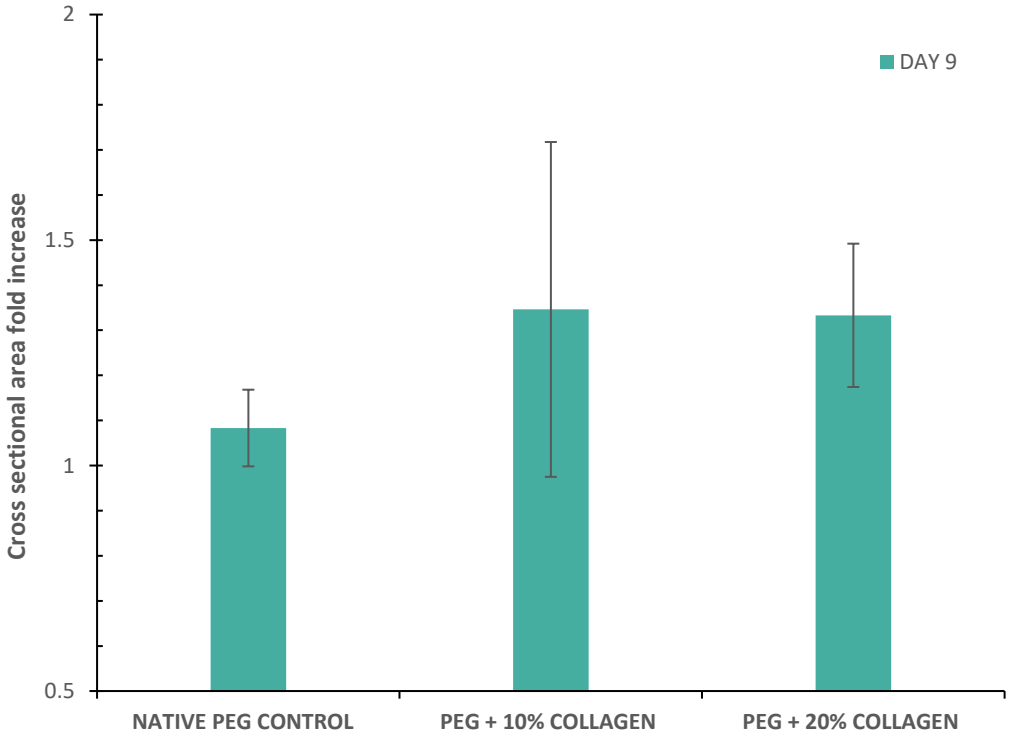
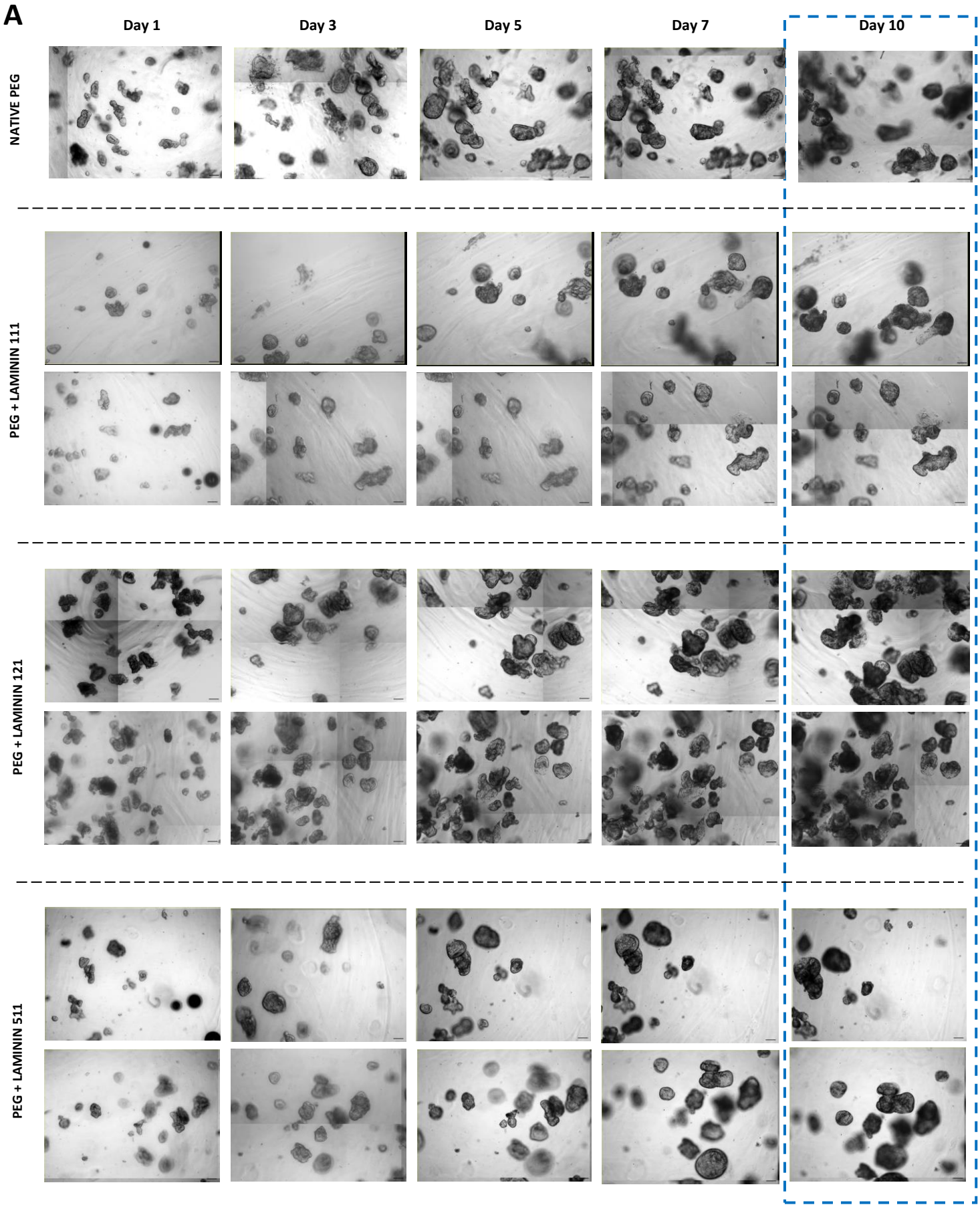


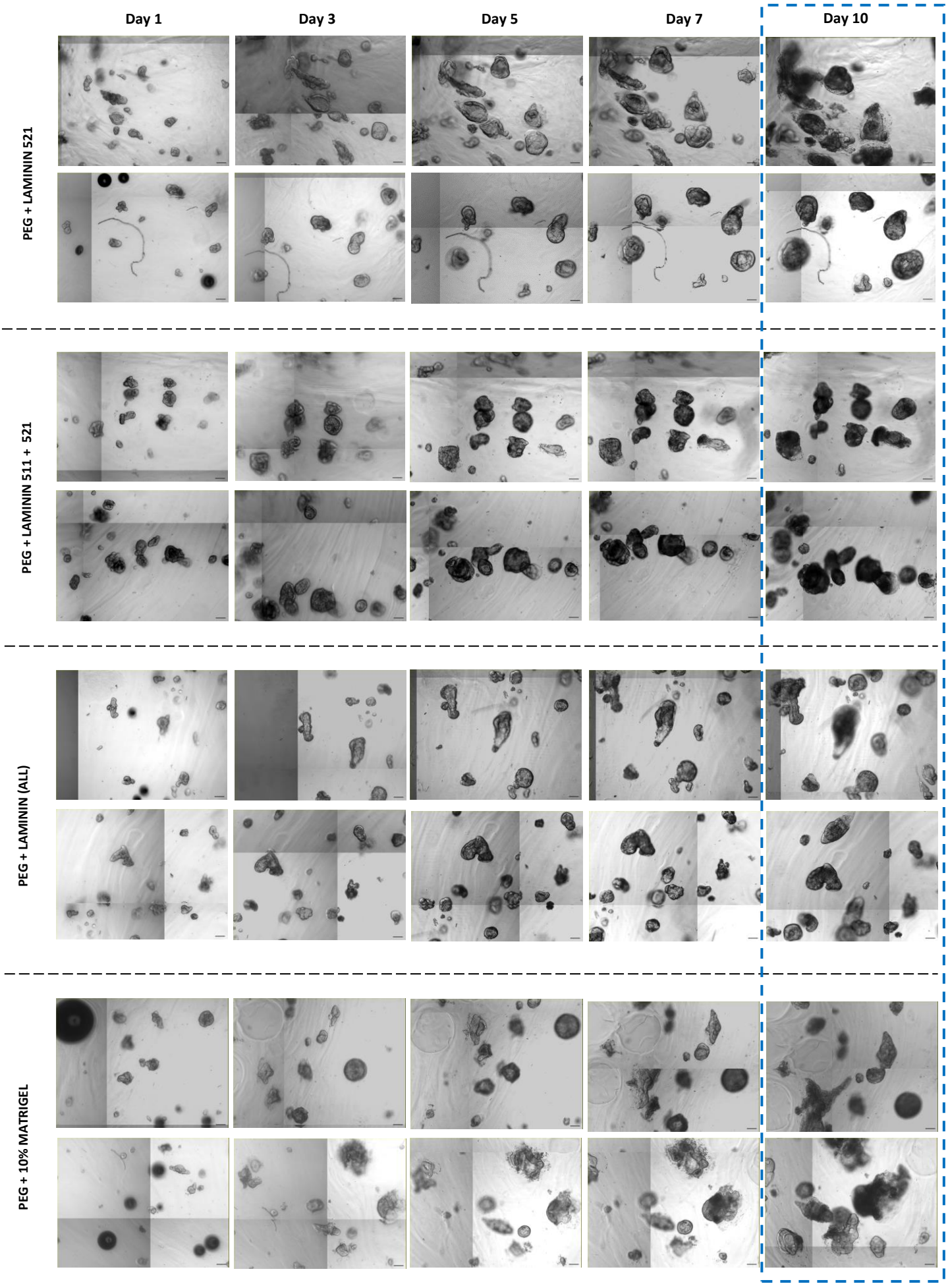
Figure 3.6 HIO growth in PEG hydrogels functionalised with Collagen-1. (A) Representative brightfield images of human colonic HIO cultured in Native PEG, PEG with 10% Collagen-1 and PEG supplemented with 20% Collagen-1. Images are taken on alternate culture days as indicated. Viable organoid structures present on days beyond standard 7-day culture

highlighted (blue dashed rectangle). Scale bar 100 µm. (B) Assessment of cross-sectional fold increase between alternate culture days. One-way ANOVA was used to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($F_{3,76.285}$=1.231 p =0.221, $F_{3,212}$ =1.437 p = 0.233 $F_{3,229}$ =0.381 p = 0.767 and $F_{3,234}$ = 0.608 p = 0.610). (C) overall fold increase to cross sectional area on final day of culture. Data is reported as mean ± SE, one-way ANOVA used to determine statistical significance ($F_{3,245}$= 5.754 p = 0.610). (N=1, native PEG n =, PEG+10% Collagen-1 n =2, PEG+20% Collagen-1 n=4). Total n of organoids assessed per replica are available in appendix 1.

Organoids cultured in both native PEG hydrogels and PEG hydrogels supplemented with collagen-1 displayed a dark and dense morphology indicating a reduced organoid viability within these gels. The organoids cultured under these conditions did not possess clearly defined lumens and displayed limited increase to their size (**Figure 3.6**). PEG hydrogels containing 20% collagen-1 contained better preforming organoids from a morphological perspective, however these failed to expand in cross-sectional area and there was no significant improvement from either 10% or 20% Collagen-1 over native PEG hydrogels (p=0.610).


A
Day 1
Day 3
Day 5
Day 7
Day 10
NATIVE PEG
PEG + LAMININ 111
PEG + LAMININ 121
PEG + LAMININ 511

3. Optimisation of a defined 3D-PEG hydrogel system for human intestinal organoid culture.

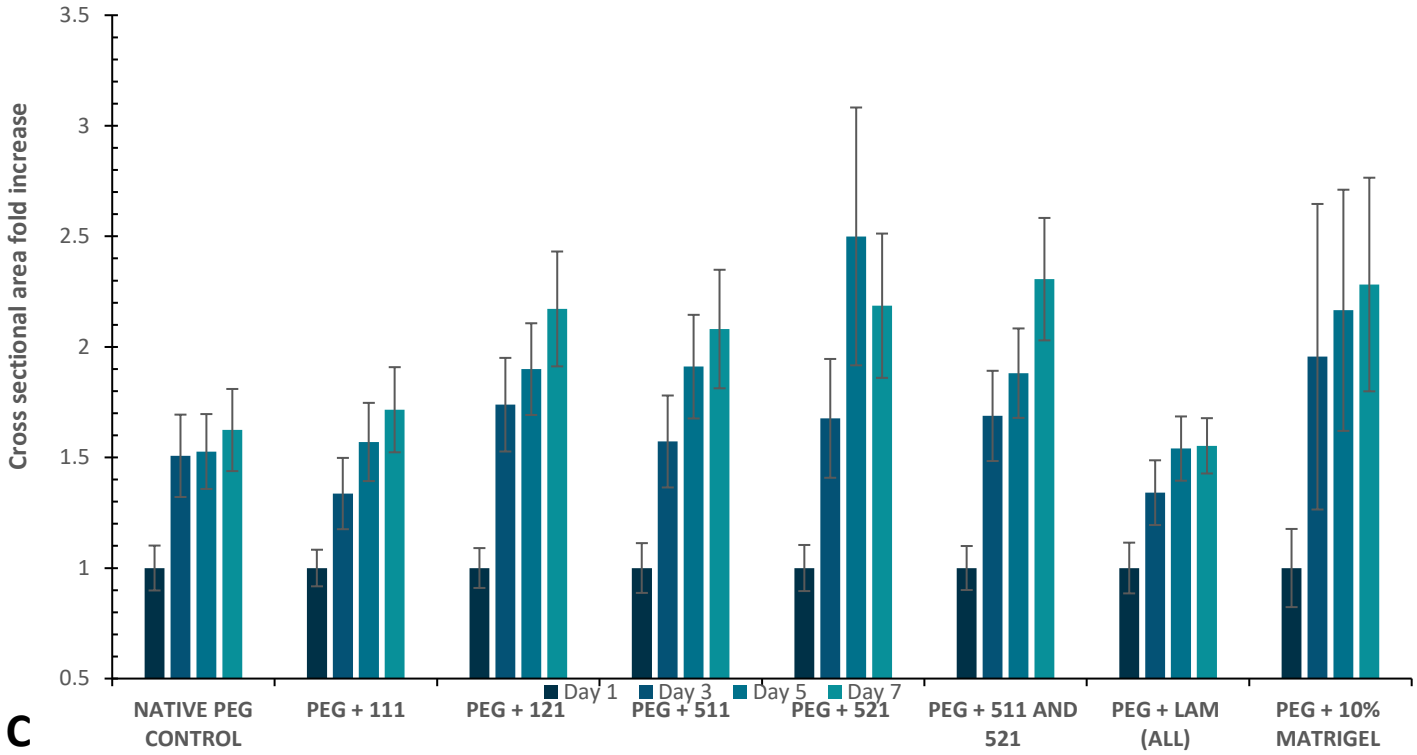


B

Day 3	PEG + 111	PEG + 121	PEG + 511	PEG + 511 + 521
NATIVE PEG	*		**	
PEG + 121	***		***	
PEG + 511				*
PEG + 521		*		

Day 5	PEG + 111	PEG + 121	PEG + 511	PEG +521
NATIVE PEG	*		**	
PEG + 111		***		*
PEG + 121				
PEG + 511				*

Day 7	PEG + 111	PEG + 511	PEG +521
NATIVE PEG	*	**	
PEG + 111			*
PEG + 511 AND 521		*	



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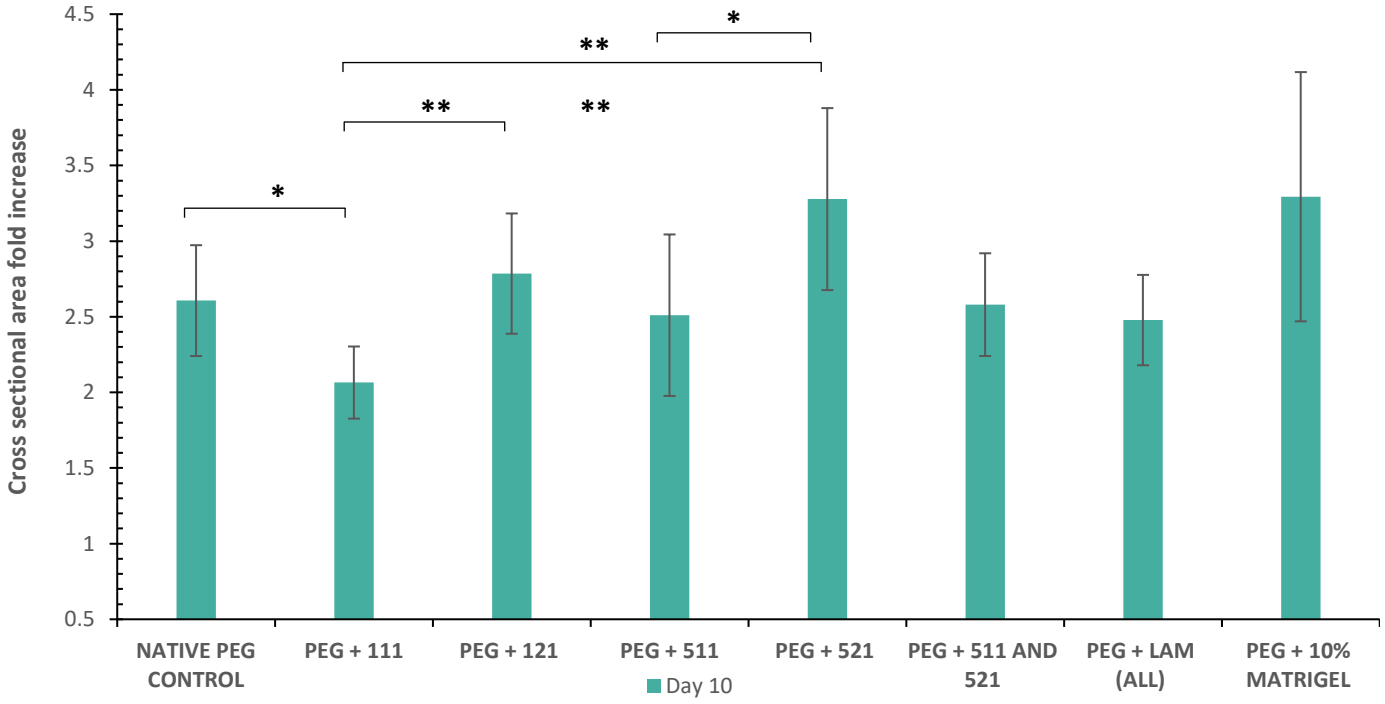


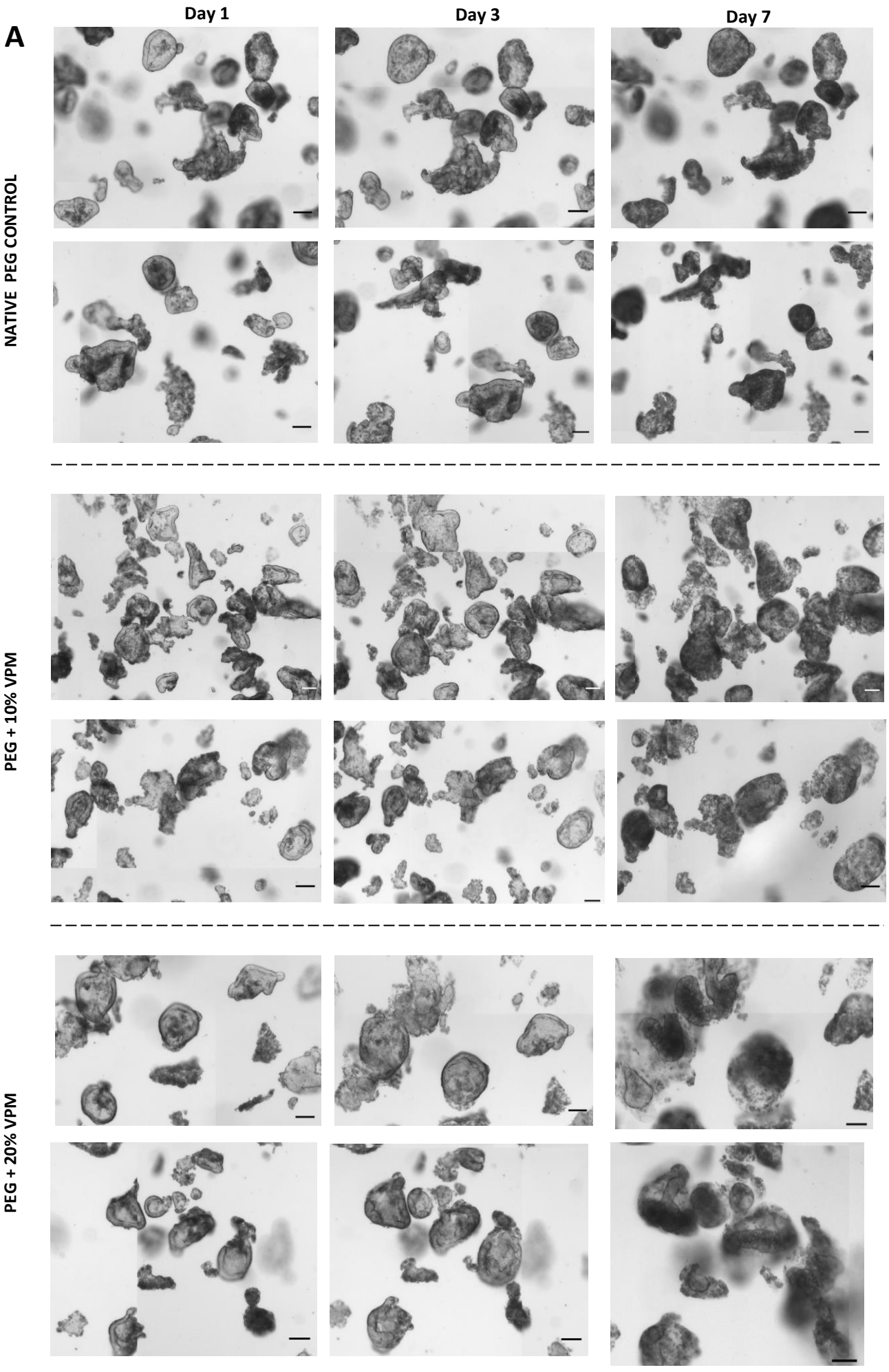
Figure 3.7 HIO culture in PEG physically blended with human recombinant Laminin. (A) Representative brightfield images of human colonic HIO cultured in Native PEG (Control), PEG + Laminin 111, PEG + Laminin 121, PEG + Laminin 511, PEG + Laminin 521, PEG + Laminin 511 and 521 together and PEG + all laminins (111, 121, 511 and 521). Images are taken on alternate culture days as indicated. Viable organoid structures present on days beyond standard 7-day culture highlighted (blue dashed rectangle). Scale bar 100 µm. (B) Assessment of cross-sectional fold increase between alternate culture days. Welch's ANOVA was used to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($F_{7,76.285}$=5.941 $p < 0.001$, $F_{7,212}$ =6.718 $p < 0.001$ $F_{7,229}$ =7.038 $p < 0.001$ and $F_{7,234}$ = 6.635 $p < 0.001$). For clarity, significant differences between groups are highlighted in tables above graph. (C) overall fold increase to cross sectional area on final day of culture. Data is reported as mean ± SE, Welches ANOVA used to determine statistical significance (F_7 = 6.635 $p < 0.001$). Statistical significance following Games-Howell post hoc test is displayed as *$p < 0.05$, **$p < 0.01$ *** $p < 0.001$. (N=1 n=1). Total n of organoids assessed per replica are available in appendix 1.

Organoids cultured in in native PEG hydrogels supplemented with different laminin isoforms displayed varied results when utilised for HIO culture. From a morphological perspective, organoids cultured in PEG + Lam 111 and PEG + Lam 521 appeared lighter, more translucent and less granulated which serve as a good indicator of organoid viability. General organoid viability within the native PEG environment was high, however the retention of epithelial membrane integrity and clear lumen definition appears the highest amongst Lam + 111, Lam + 521 and Lam + All, all of which demonstrated similar organoid morphological performance to PEG + 10% Matrigel (**Figure 3.7A**). This was reflected in the organoid growth as measured by the fold increase in cross sectional area, where over the initial 7-day culture organoids in PEG + Lam 521 displayed the greatest increase that significantly out-performed other PEG + Laminin groups (**Figure 3.7B**). Interestingly, there appears no cumulative benefit from combining laminin isoforms to organoid growth or morphology. Across the full 10 days of organoid culture there appears no statistically significant advantage in organoid growth provided by the inclusion of laminin over the native PEG environment, however the organoids displaying the largest fold increase in cross sectional area are located in PEG + Lam 521 or PEG supplemented with Matrigel (**Figure 3.7C**).

3.4.6 Mid-stage PEG hydrogel optimisation: degradability

With the goal of creating a defined PEG hydrogel for colonic HIO culture that reproduces the growth and morphological development of organoids displayed in Matrigel, we found it necessary to address another potential avenue for optimisation: degradability. In Matrigel, as organoids grow and expand over time their cells secrete enzymes known as matrix metalloproteinases (MMPs) which begin to breakdown the surrounding Matrigel until their necessary passage between days 5 and 7. Due to the covalent crosslinks formed by thiol-Michael addition between our PEG macromers, organoids are unable to degrade the PEG

hydrogels. As a possible result of this, we observed the organoid morphology begins to deteriorate towards the late stages of a seven-day culture. We therefore rationalised that the implementation of degradability in PEG hydrogels that have been optimised for their mechanical properties and imparted with necessary biochemical cues, would continue to improve organoid growth and morphology. To this end, we investigated the inclusion of the VPM peptide which contains a sequence susceptible to MMP degradation, into the hydrogel network. To identify what effect, if any, alongside the optimal concentration of VPM required to impart degradation, we investigated the inclusion of two VPM concentrations (10 and 20%) and monitored organoid growth and morphology in comparison to native 5% PEG hydrogels over a 7-day period.


A
Day 1
Day 3
Day 7
NATIVE PEG CONTROL
PEG + 10% VPM
PEG + 20% VPM

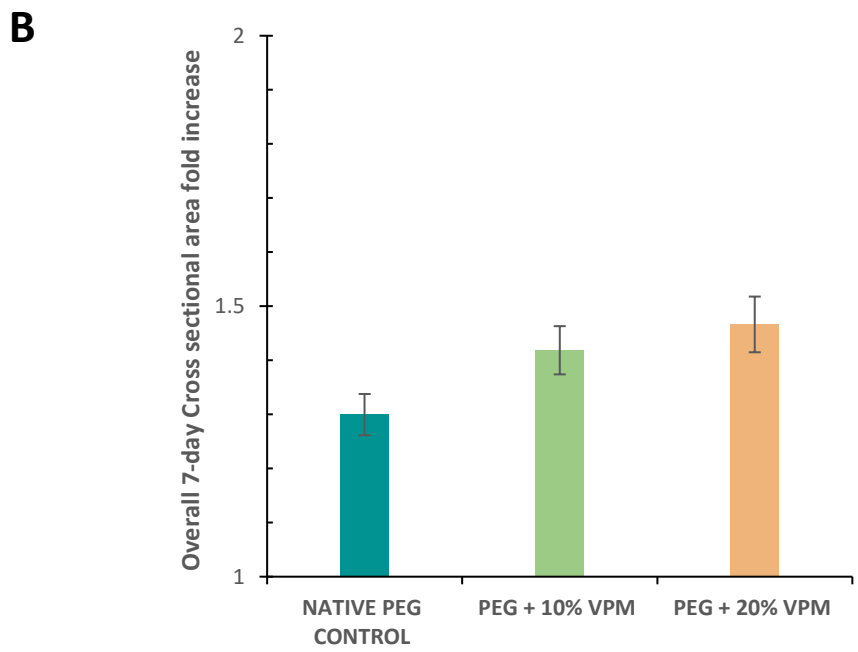


Figure 3.8 Assessment of HIO in partially degradable PEG hydrogels. (A) Representative brightfield images of human colonic HIO cultured in Native PEG, PEG with 10% VPM and PEG supplemented with 20% VPM. Images are taken on alternate culture days as indicated. Scale bar 100 µm. (B) Assessment of overall cross-sectional fold increase between starting and final day of culture.

Organoids cultured in native PEG hydrogels alongside PEG supplemented with 10 and 20% VPM all display similar morphologies, where similar quantities of organoids possessing defined lumens and apparent epithelial polarity are present between days 1 and 3, of which most are lost and replaced by dark, dense and granulated morphologies by day 7 (**Figure 3.8A**). Fold increase was seen to be increased in PEG gels where VPM was present in a concentration dependent manor (**Figure 3.8B**), however when assessing the organoid morphological performance these organoids appear more 'spread' with lots of cell debris and appearing less viable.

3.4.7 Late-stage PEG hydrogel optimisation: integration of biological peptides

Carrying the 5% 10K 4-arm PEG hydrogel forward through early and mid-stage rounds of optimisation, with evidence that organoid performance can be influenced via the addition of ECM protein (Collagen and Laminin) and a candidate concentration of the VPM peptide to further explore the requirements of degradability, our next priority was to replace the ECM protein components integrated via physical blend, with biologically functional peptides integrated into the PEG hydrogel network. By replicating the biochemical cues imparted through collagen and laminin with carefully selected functional peptides, we rationalised that the PEG hydrogel could be made fully synthetic and fully defined, with tuneable properties fully tailored towards colonic HIO culture.

We chose to functionalise 5% PEG gels with peptides that provide the biochemical queues located in the human physiological intestinal stem cell niche, with the rationale this will encourage greater stem cell proliferation resulting in improved organoid growth, morphology, and viability. As intestinal stem cells located at the crypt base express several integrins including α2β1 and α5β1(Kim, Turnbull and Guimond, 2011), and ligands of these integrins are attractive targets for developing a synthetic stem cell niche, we selected an RGD peptide which has high affinity for α5β1 integrin. In addition, organoids secrete a natural ECM consisting of various integrin ligands and other ECM-binding receptors, specifically sequencing data of patient matched HIO cultured in the Williams lab reveal organoids possess transcripts for collagen IV and fibronectin (data not shown) which we aimed to utilise via integration of two peptides with high affinity for these proteins known as basement membrane binding (BM binder) and Fibronectin binding (FN Binder) peptides. To incorporate this synthetic ECM into the PEG hydrogel network we utilised thiol-Michael addition to pre-functionalise 10K 4-arm PEG MAL with thiol-containing, cysteine terminated RGD, BM binding

peptide (with high affinity for collagen IV) and FN binding peptide, at concentrations previously seen in literature. The functional PEG MAL (fPEG MAL) was then crosslinked with PEG SH into hydrogel networks.

With the high degree of modification to the hydrogel network caused by integration of peptides via Michael type addition, we deemed it first necessary to characterise the resulting mechanical properties of the 5% fPEG hydrogels in comparison with their non-functional counterparts. Having observed a significant deviation from the previously optimised matrix stiffness of ~6.5 kPa to ~3.5 kPa when functional peptides are present (**figure 3.9A**) we found that consideration was required in order to more carefully balance the stoichiometric ratio of functional groups as outlined in **table 3.1**. This would be necessary to preserve the number of PEG MAL arms free for crosslinking with the PEG SH to form a hydrogel network. We identified that increasing the concentration of PEG MAL utilised to compensate for the additional thiol groups and provide more binding sites for functional peptides further decreased the overall bulk mechanical stiffness of our functionalised hydrogels to approximately 1.4 kPa (**figure 3.9B**). Despite this deviation from previously optimised value for mechanical stiffness, this value is closer to previously published mechanical properties of successful hydrogels (Gjorevski et al., 2016), whilst more accurately replicating the softer (>1kpa) property of Matrigel. We therefore proceeded with these hydrogels to the organoid encapsulation phase.

Table 3.1 Overview of hydrogel crosslinking chemistry for the integration of functional and degradable peptides

		No Compensation			**PEG MAL Compensation**		
		PEG + VPM	fPEG	fPEG+VPM	PEG + VPM	fPEG	fPEG+VPM
4 ARM 10K PEG SH	PEG SH (w/v%)				5		
	PEG SH (mM)				5		
	number of SH per 1 mol (Arms)				4		
Functional Peptides	RGD Concentration (mM)	-	1.5	1.5	-	1.5	1.5
	FN/BM Concentration (mM)	-	0.5	0.5	-	0.5	0.5
	VPM Concentration (mM)	2.5	-	2.5	2.5	-	2.5
	Number of SH per 1 mol RGD	-	1	1	-	1	1
	Number of SH per 1 mol BM/FN Binder	-	1	1	-	1	1
	Number of SH per 1 mol VPM	2	-	2	2	-	2
4 ARM 10K PEG MAL	PEG MAL (w/v%)				5		
	PEG MAL (mM)				5		
	number of MAL per 1 mol				4		
Compensation	Compensation amount		-		1.25	0.75	1.75
	New PEG MAL Concentration (mM)		-		6.25	5.75	6.75
SH:MAL ratio	Total SH (mM)	25	23	27	25	23	27
	Total MAL (mM)	20	20	20	25	23	27
	Total SH Excess (mM)	5	3	7	0	0	0
Free PEG Arms	PEG MAL Arms occupied by RGD (%)	0	7.5	7.5	0	6.52	5.56
	PEG MAL Arms occupied by Matrix binders (%)	0	2.5	2.5	0	2.17	1.85
	PEG MAL Arms occupied by VPM peptide (%)	25	0	25	20	0.00	18.52
	PEG MAL Arms free for gelation (%)	75	90	65	80	91.30	74.07

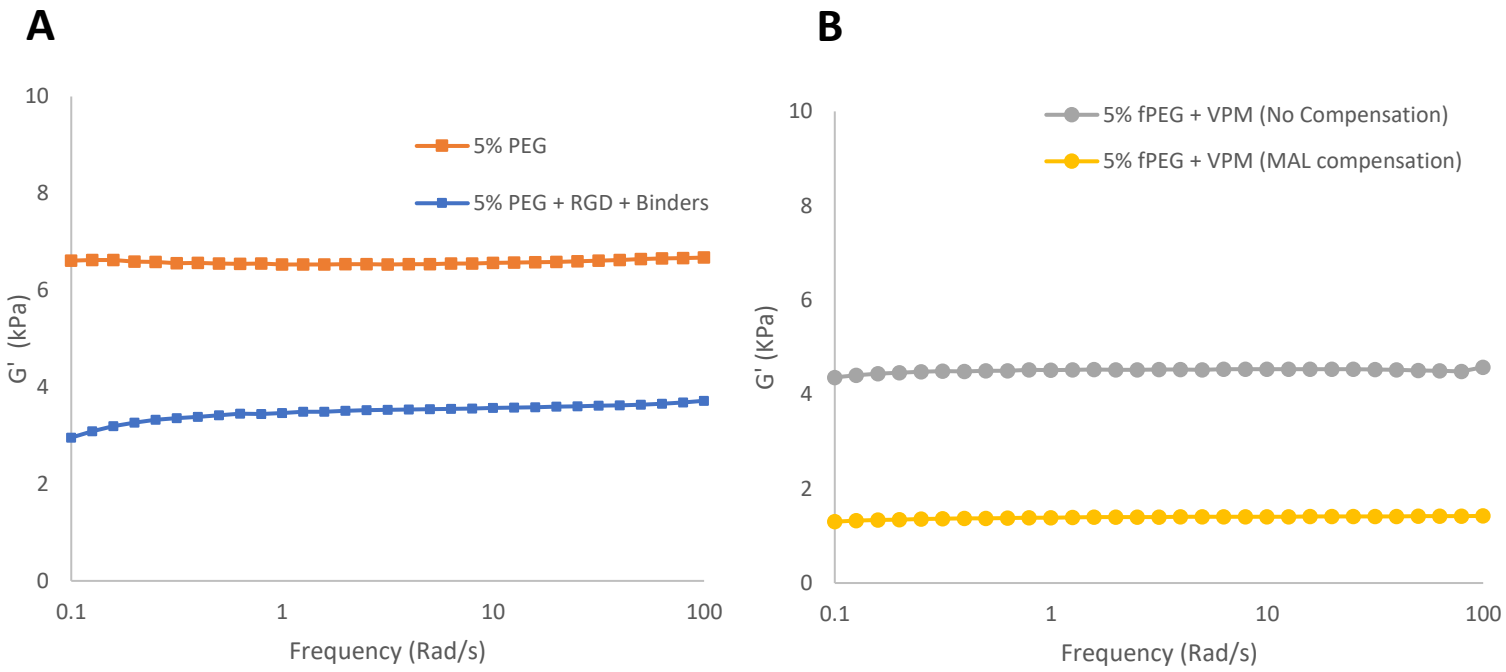
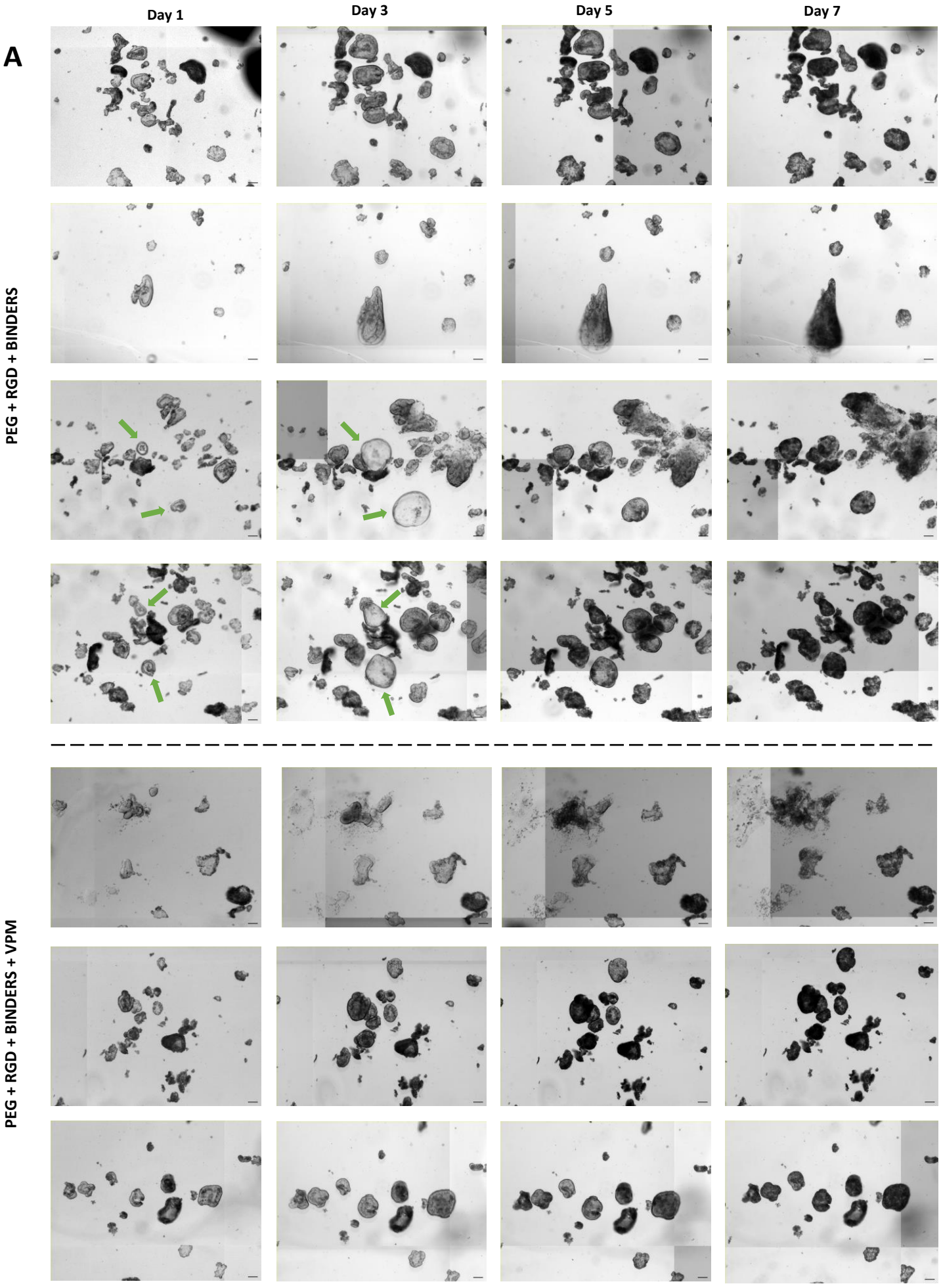


Figure 3.9 Assessment of functional PEG hydrogel mechanical properties. (A) Rotational rheometric analysis of storage modulus (G') via frequency sweep comparing 5% PEG native PEG hydrogels with 5% PEG + RGD + Binders. N=3 (B) Rheometric analysis of storage modulus (G') via frequency sweep for 5% PEG + RGD + Binders (fPEG) + VPM, with and without optimising hydrogel crosslinking chemistry. N=3

HIO fragments were cultured in functionalised PEG hydrogels with and without the presence of the VPM degradable peptide to assess the potential benefits of implementing degradability into a functional system with mechanical properties reflective of those found in published gels and more closely replicating the mechanical environment of Matrigel. Interestingly we observed organoids in the functionalised PEG hydrogels that displayed a marked increase in CSA within the first 3 days of culture. These formed as spherical cyst-like structures that appear as a single layer of epithelial cells (**Figure 3.10A** Green arrow), which are often present in Matrigel and mature into multi-lobular crypt-like structures, however within these functional PEG hydrogels deterioration begins from day 5. Within the functional an VPM degradable hydrogels the organoids to not display as great a size increase within the first 3 days as reflected in their lower fold increase in CSA at day 3 (**Figure 3.10B**) however the organoids do display some characteristic traits of healthy organoids including the defined lumen and regular spheroid structures. In both conditions the organoids begin to deteriorate towards the later stages of the culture, with organoid morphology becoming dark and dense indicative of cell death.


A
Day 1
Day 3
Day 5
Day 7
PEG + RGD + BINDERS
PEG + RGD + BINDERS + VPM

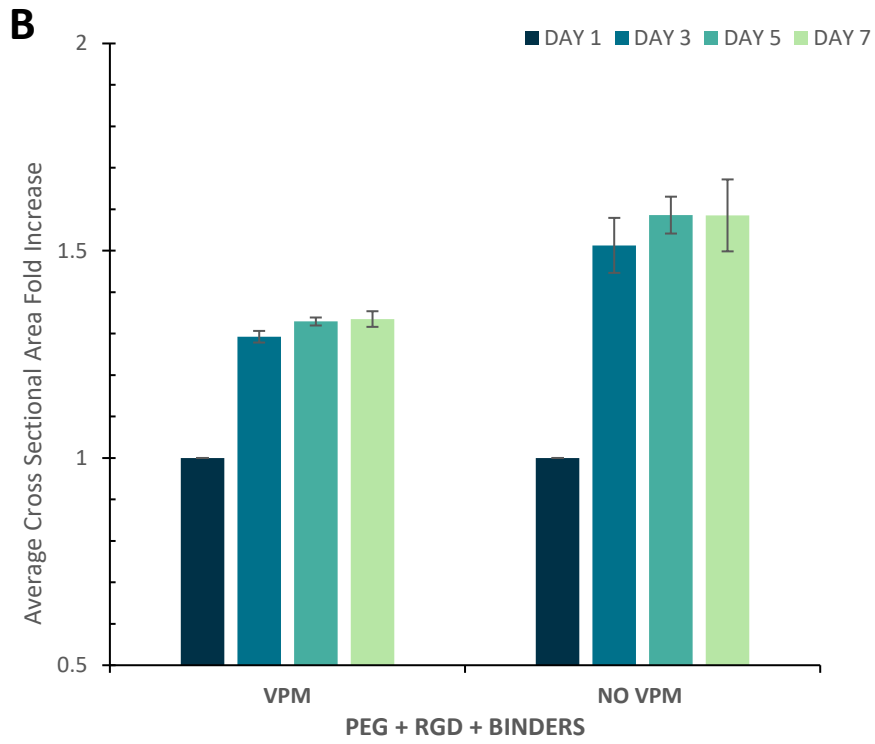
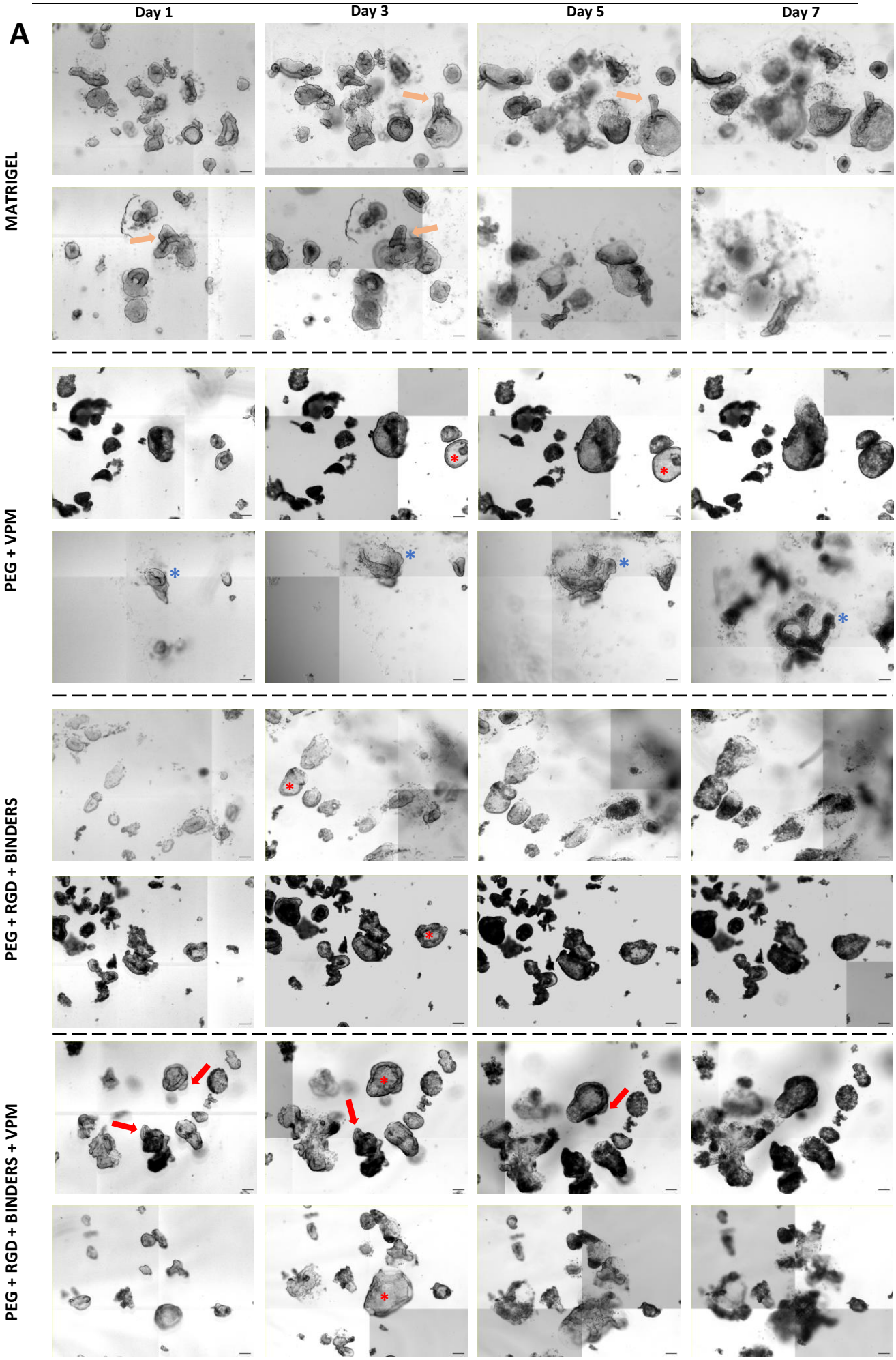


Figure 3.10 Assessment of partial degradability on HIO growth within functional PEG hydrogels.

(A) Representative brightfield images of human colonic HIO cultured in PEG + RGD, FN binder and BM binder with (bottom) and without (top) VPM degradable peptide. Images are taken on alternate culture days as indicated. Green arrow indicates key organoid morphologies. Scale bar 100 µm. (B) Assessment of cross-sectional fold increase between alternate days of culture. Independent samples T test was performed to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($t_{514} = 1.613$ $p = 0.107$, $t_{556} = -0.171$ $p = 0.864$, $t_{616} = -1.852$ $p = 0.064$ and $t_{615} = 1.836$ $p = 0.067$). (N=2, n=4)

We then encapsulated HIO organoids in functional PEG hydrogels and compared the organoid performance over a 7-day culture with organoids cultured in Matrigel, as well as non-functional PEG counterparts. Organoids remained viable and increased in CSA over a 7-day culture in all PEG hydrogels, however deterioration of organoid morphology between day 5 and 7 was worse in all PEG hydrogels than in Matrigel (**Figure 3.11A**). In functional PEG hydrogels, we identified some organoid structures with clearly defined lumens, that increased rapidly in CSA and displayed some early indicators of potential organoid budding (indicated with arrowheads) however these fail to mature and unlike organoids in Matrigel, there is the absence of new bud formation. Interestingly there was no significant improvement in growth measured as CSA fold increase when using functional PEG compared to native PEG with a VPM degradable peptide included, however when observing the morphology of organoids within the native PEG with degradable peptide, there were several irregular organoid structures (**Figure 3.11A blue star**) not observed in functional gels.

3. Optimisation of a defined 3D-PEG hydrogel system for human intestinal organoid culture.



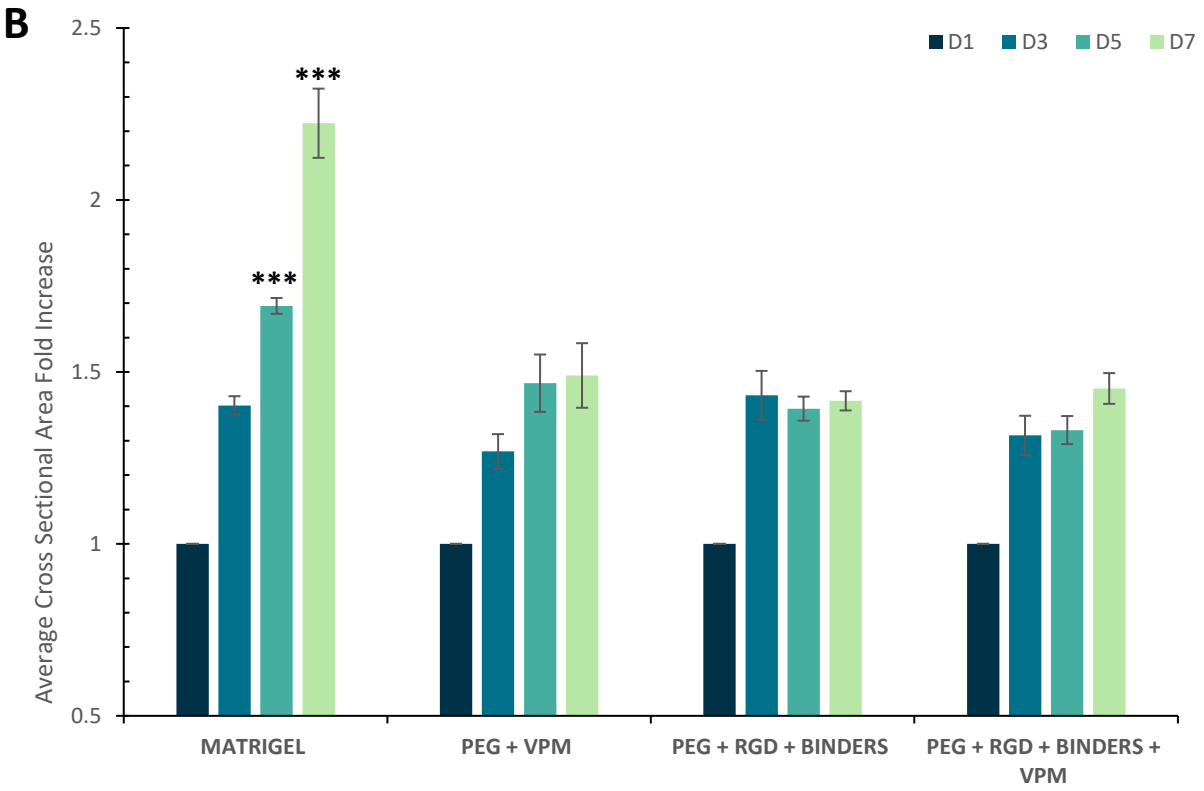


Figure 3.11 HIO culture in defined, functional PEG hydrogels. (A) Representative brightfield images of human colonic HIO cultured in Matrigel, PEG + 20% VPM, PEG + RGD, FN binder and BM binder and PEG + RGD, FN binder and BM binder and VPM degradable peptide. Images are taken on alternate culture days as indicated. Red star indicate presence of defined lumen. Orange arrow indicated bud formation. Red arrow indicates potential, failed bud formation. Blue star highlights irregular organoid morphology. Scale bar 100 µm. (B) Assessment of cross-sectional fold increase between alternate days of culture. One-way ANOVA was used to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($F_{3,1259}=3.520$ $p=0.015$, $F_{3,1325}=5.117$ $p=0.002$ $F_{3,610.754}=10.805$ $p<0.001$ and $F_{3,640.3}=12.498$ $p<0.001$). Statistical significance following Tukey post hoc test is displayed as $*p<0.05$, $**p<0.01$ (N=1, Matrigel n=3, PEG+VPM n=4, PEG + RGD + Binders n=4, PEG + RGD + Binders + VPM n=4). Total n of organoids assessed per replica are available in appendix 1.

3.5 Discussion

The results presented in this chapter explore the fabrication of a fully defined PEG based hydrogel for the culture of colonic HIOs and the use of this PEG hydrogel to begin to elucidate the requirements of successful colonic HIO culture from a mechanical and biochemical perspective. Using metrics of organoid growth in Matrigel ascertained by overall changes to the cross-sectional area, and characteristic morphological traits that are commonly displayed by HIO cultured in Matrigel; we progress through stages of PEG hydrogel optimisation with the goal of improving organoid growth and morphological performance, better replicating organoid culture in Matrigel.

3.5.1 Mechanical optimisation of native PEG hydrogels

A key benefit of using PEG hydrogels is the ability to influence the mechanical properties of the gel through consideration of the number of arms of a multi arm PEG, PEG molecular weight (MW) and final w/v % of PEG present in the final hydrogel samples. An increase in PEG MW increases the overall length of the PEG chains. This increased length reduces the density of cross-linking within the gel which in turn increases the mesh size (average distance between crosslinks), resulting in a softer gel with reduced matrix stiffness. Additionally, we can further alter the overall cross-linking density and gelation time by varying the final solid polymer content (w/v %) of the gel, with higher PEG content gels having an increased matrix stiffness (Toepke et al., 2013; Jansen et al., 2018). Assessment of the literature alongside prior collaboration between Saeed and Williams identified that 4-arm, 10 kDa PEG SH and MAL were capable of crosslinking to form PEG hydrogels at manageable gelation speeds under physiological conditions with a wide range of polymer contents, however the specific requirements of matrix stiffness required for colonic HIO culture were not yet clearly defined.

A landmark study conducted by Lutolf and colleagues with a mouse-derived intestinal organoid model (Gjorevski et al., 2016) identified an elevated shear modulus of ≈1.3 kPa for the expansion of ISC into epithelial organoids were required, whilst softer matrices, like that of Matrigel (reported mechanical stiffness ranges from 50-450 pa) favoured later stage organoid formation. Our studies show a greater fold increase to overall cross-sectional area when colonic HIO fragments were cultured in native PEG hydrogels between 4 and 5%, which possess a higher storage modulus (G') between 4.3 and 6.5 kPa (**Figure 3.4**). Despite the absence of morphological traits, including presence of a clearly defined lumen, and formation of new budding crypt-like structures (as observed in Matrigel), these organoids grew and remained viable after 7 days of culture, as suggested by the absence of breakdown and dissociation of the organoid structures. This data highlights the importance that mechanical properties such as matrix stiffness have in human colonic organoid development and outlines a potential use case by which a well-defined PEG hydrogel system can help deepen our understanding of this.

With colonic HIO displaying a differing growth response to their mechanical environment as we observe in native PEG hydrogels, the question can be raised of how organoids are able to perceive mechanical cues from their environment and translate this stimulus into biochemical signals which influence cellular behaviour such as growth and differentiation. This mechanical signal transduction (mechanotransduction) is a continual area of research, with the presence of mechanical sensors such as Yes-associated protein (YAP) and its downstream effector (TAZ) being seen in the literature to have a key role in regulating intestinal stem cells through the Hippo signalling pathway (Deng et al., 2022). Lutolf and colleagues identified using a mouse derived intestinal organoid culture that matrix stiffness influences intestinal stem cell expansion by influencing YAP activity and nuclear localization, with high matrix stiffness

enhancing ISC survival and proliferation through a YAP-dependant mechanism. The same study also identified that in stiff matrices, the proportion of cells with nuclear YAP activity rapidly decrease over time, whilst organoids cultured in Matrigel maintain intermediate levels of nuclear YAP activity. This indicated that whilst organoid culture within a stiff matrix initially increases YAP activity and supports organoid growth, continued exposure to a stiff matrix results in compression and YAP inactivation, preventing further growth. Data produced in this study (**figure 3.3**) identifies the presence of another mechanoresponsive calcium channel that is becoming more widely understood – Piezo1. The exact signalling mechanism of Piezo1 in the colonic epithelium is unknown, however it has been reported that piezo channels remain closed in the absence of necessary external mechanical force (Botello-Smith et al., 2019). A recent study has associated stem cell differentiation induces a mechanically responsive cell state that is characterised by upregulation of the Piezo1 channel in a mouse-derived intestinal organoid culture (Tallapragada et al., 2021). The development and optimisation of a defined PEG hydrogel with tuneable mechanical properties has a clear use case for the continued research into the altered expression levels and mechanism, if any, Piezo 1 receptors have in successful culture of colonic HIOs.

3.5.2 Replicating intestinal ECM in PEG hydrogels

As previously discussed, the initial establishment of intestinal organoid cultures from ISC containing crypts by Clevers and colleagues required the delivery of specific matrix derived growth factors during culture to ensure the development of mature organoid structures (Sato et al., 2009). Similarly for colonic HIO cultures a well-defined combination of growth factors including Wnt and Rspondin is delivered to colonic crypt cultures to achieve the formation of colonic organoid cultures. In addition to this, the routine culture system used for the generation of 3D intestinal organoid cultures, be it murine or human, has been Matrigel.

Although commercial Matrigel is poorly defined and prone to batch variability issues, the composition is well reported to contain ~ 60% laminin and ~30% collagen (Hughes, Postovit and Lajoie, 2010). It can therefore be seen that there is the requirement for biochemical signalling from native ECM proteins and growth factors that must be imparted into PEG hydrogels in order to closely mimic the growth and morphological developments of HIO in Matrigel.

The inclusion of diffusible growth factors alone during organoid encapsulation (prior to the addition of media) did not improve the cross sectional area expansion within the first 3 days of organoid culture, however after a 7 day period there was a reported 1.66 fold increase in organoid cross sectional area compared to a 1.54 fold increase in organoids encapsulated in native PEG alone – potentially conferring a longer term benefit to organoid development over time (**Figure 3.5**). There was however a marked improvement in cross sectional area and morphology in organoids cultured in PEG hydrogels blended with Matrigel, that progressed beyond a standard 7-day culture, with organoid growth continuing after 12 days. These PEG-Matrigel blended gels serve as a proof of concept that the physiological traits of organoids can be improved upon through the addition of ECM, within a majority PEG environment.

In addition to the large degree of collagen and laminin within Matrigel composition revealed by proteomic analysis, there has been several successful studies that have demonstrated that mouse-derived colonic crypts and isolated Lgr5+ progenitor cells embedded into collagen can be expanded for long term culture, and more recently the development of collagen-based 3D matrices for mouse intestinal organoids (Yui et al., 2012; Jee et al., 2019). Similarly, several laminin isoforms have been added to synthetic hydrogels to improve the growth and differentiation of different organoid cultures with relative success (Barros et al., 2019; Dobre

et al., 2021). We therefore identified collagen and laminin as suitable candidates to replace the Matrigel component in our physically blended proof-of-concept hydrogels and impart necessary ECM-derived biochemical cues. We observed limited success when culturing organoids in PEG hydrogels blended with collagen. Organoid morphology appeared largely dark and dense, lacking the characteristic lumen and defined epithelium. For organoids that did expand the overall increase in CSA was comparable to that of Native PEG hydrogels and there was no development of new budding structures. In contrast to this, organoids cultured with laminin isoforms demonstrated varying degrees of improvements to organoid growth and morphology. The performance of the mechanically optimised native 5% PEG control gels in these experiments (**Figure 3.7A**) highlights the variable nature of organoid culture which can vary based on factors such as the starting size of organoid fragments, with larger fragments reforming with less difficulty than smaller fragments. Despite this, PEG hydrogels supplemented with laminin 521 appear to confer the largest fold increase after 5 days, with continued cross-sectional area increase up to 10 days, comparable to that seen with PEG hydrogels blended with Matrigel, alongside clear and defined lumen and epithelium. Interestingly, various organoids cultured with laminin isoforms – including 521, appear to develop convoluted budding-like structures, however unlike within Matrigel, these do not fully develop and appear to struggle to protrude away from the central organoid structure **(Figure 3.7**). Laminin 521 is well expressed within Matrigel and known to play a key role in regulating pluripotency within the stem cell niche for various tissues (Hagbard et al., 2018) and recently full-length laminin proteins have been integrated into PEG hydrogels for 3D culture of neural cells (Dobre et al., 2021). Our data when, taken in combination with literature, demonstrate a potential pivotal role for laminin 521 in colonic HIO culture.

3.5.3 Development of an optimised, fully synthetic, defined PEG hydrogel for HIO culture

Having demonstrated the advantage towards overall organoid growth and morphology that is achieved via the inclusion of ECM proteins into PEG hydrogels first optimised for mechanical properties, but not fully replicating the CSA expansion and characteristic morphology established in Matrigel – we saw it necessary to replicate or improve upon these advantages through the replacement of full ECM proteins, with smaller, defined, synthetic peptide alternatives. A key reason for this is that as collagen and laminin were integrated into the hydrogel network via physical blend, there is no control over distribution within the hydrogel. By utilising the same Michael type addition chemistry that forms the PEG hydrogel network, we can occupy a small percentage of the PEG arms with functional peptides that become well integrated throughout the hydrogel network.

Towards better replicating the proteolytic turnover of the surrounding matrix that is displayed by HIOs cultured in Matrigel, we incorporated a degradable peptide (VPM) containing a sequence susceptible to cell secreted MMPs that has been used to successfully impart a degree of degradability in other hydrogels (Greg A. Foster, Devon M.Headen, Cristina Gonzalez-Garcia, Manuel Salmeron-Sanchez, Haval Shirwan, 2017; Dobre et al., 2021). In a native PEG environment, it appears that the inclusion of 20% VPM degradable peptide imparted a growth advantage over their native PEG counterparts (**Figure 3.8**) however the organoid morphology was consistent between degradable and non-degradable native PEG hydrogels, with defined lumens and epithelium at day one but showing clear signs of deterioration by day 7. Upon assessing the PEG hydrogel chemistry with the inclusion of the degradable peptide it becomes clear that the ability for the VPM peptide to impart degradation into the network is somewhat limited. As the VPM integrates into the hydrogel network via thiol-Michael addition between thiol-containing cysteine residues in the peptide

sequence and the 10K 4-arm PEG MAL, it only occupies arms of the PEG MAL, and not the PEG SH. Cleavage of the MMP binding site will therefore not break covalent links within the hydrogel network formed by the 4-arm PEG's, but instead may form 'pockets' within the hydrogel network that an organoid may be able to expand into. Furthering this, fundamental research from Metters and Hubbell identified for hydrogels formed via thiol-Michael addition between multi arm PEGs and bi-functional components (such as the bi-cysteine flanked VPM utilised in this study) are prone to a common form of network defect known as primary cycles or loops (Metters and Hubbell, 2004). This research identified for PEG hydrogels of polymer content > 10% (w/v), diluted bi-functional components are prone to reacting intramolecularly (**Figure 3.12**) which has an adverse effect on mechanical properties of the gel network along with excessive swelling. This limits the use of these hydrogels for cell culture applications due to the variable nature and undesirable network parameters caused by primary loop formation. Despite this, due to the perceived improvement to organoid growth potential, we continued investigation into the integration of VPM into functionalised PEG hydrogels, although it was noted that alterations to organoid performance may be due to changes to hydrogel network structure and bulk stiffness.

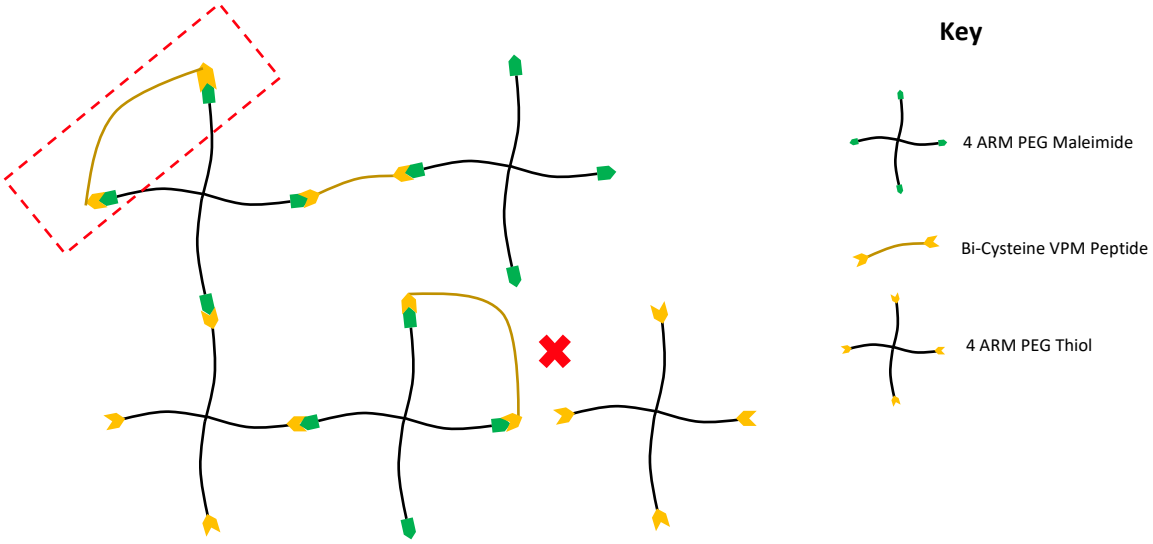


Figure 3.12 Primary loop formation disrupts PEG hydrogel networks. Simplified schematic highlighting the formation of primary loops (red dashed rectangle) formed if the bi-cysteine VPM peptide reacts intramolecularly with multiple arms of the same 4 Arm PEG Maleimide. Subsequent disruption to hydrogel crosslinking is noted by primary loop formation disrupting crosslinking between PEG SH and MAL (red cross)

A key study by Griffith and colleagues demonstrated the use of a multi-arm PEG based hydrogel that was functionalised with the integrin ligand RGD, along with peptides displaying high affinity for collagen and fibronectin capable of capturing secreted ECM by human intestinal organoids derived from single cells (Hernandez-Gordillo et al., 2020). The organoids cultured in this study, derived from single cells, display a spherical morphology composed of a thin layer of epithelial cells. This differs to the more mature, differentiated budding HIO phenotype that is routinely cultured in Matrigel in the Williams lab and is the focus of replication within our synthetic PEG hydrogel. Despite these differences the relative improvements gained in this published work by the inclusion of these functional peptides highlighted them as candidates to replace the collagen and laminin components of our physically blended PEG hydrogels.

When incorporating these functional peptides into the PEG hydrogel network it became necessary to consider the stochiometric ratio of thiol to maleimide functional groups, as the addition of a peptide via thiol-Michael addition occupies one of the available PEG MAL arms rendering it unavailable for crosslinking into the gel network. A tool to combat this (employed by Griffith and colleagues) is to use PEG macromers with a larger number of arms i.e an 8-arm PEG, which provides an ample number of arms available for crosslinking and functionalisation. In order to proceed with our optimised 4-arm PEG system we found it necessary to increase the concentration of the PEG MAL to provide an increased number of potential binding sites. This subsequently compensates for the additional thiol content from functional peptides and maintains a 1:1 stochiometric ratio between thiol and maleimide (**Table 3.1**). This strategy enabled the fabrication of hydrogels with reproducible gelation patterns that successfully encapsulated organoids and maintained their structural integrity throughout the organoid culture, traits that would not be expected if the hydrogel was unable to form due to crosslinking issues. There was however, a noticeable shift in the measured matrix stiffness of the functionalised PEG hydrogels that were produced with and without compensating for the additional peptides, with the compensated functional gels being noticeably softer at 1.4 kPa (**Figure 3.9**). Despite this deviating from the previously optimised relatively stiff native hydrogels, the new observed matrix stiffness falls more in line with literature published mechanical properties for successful culture of intestinal organoids and is closer in mechanical properties to replicating Matrigel.

Organoids cultured in these fully synthetic, functional PEG hydrogels displayed some of the most promising morphological properties observed within a PEG hydrogel in these studies. We demonstrated the ability of an organoid fragment cultured in PEG, RGD and binders to greatly increase in CSA over initial 3 days of culture, growing as a spherical cyst-like structure that is often observed in early Matrigel culture (**Figure 3.11A** green arrow). These organoids are believed to be stem cell rich, as budding does not occur until the differentiation of mature cell types with higher contractility. This results in invagination of the core domain which creates internal pressure, causing outward protrusion of less contractile, stem cell-rich budding domains (Broguiere et al., 2018). The failure of these organoids to form new budding structures, yet increase vastly in CSA over early days of culture and begin to deteriorate from day 5, is a potential indicator that these functional PEG hydrogels support early stem cell development and expansion, but different requirements are necessary to encourage differentiation of mature cell types.

Within these functionalised PEG hydrogels (**Figure 3.11**) there are numerous organoid structures which increase in size whilst possessing clear, defined lumens, an intact epithelium and consistent form reminiscent of early-stage organoids in Matrigel. When assessing the organoid growth over a routine 7-day culture, it was observed that organoids within functional PEG hydrogels increased in size by approximately 1.6-fold. This is amongst the highest fold increases in organoid size that we observed in PEG and is closer in comparison with the fold increase observed in organoids cultured in PEG physically blended with laminin or Matrigel (~2-fold increase) which had previously produced the best performing organoids. This, alongside the morphological improvements, further enforces the idea that a functional ECM-like environment can be recreated in a synthetic PEG hydrogel using peptides.

3.5.4 Limitations of the optimised, fully synthetic, defined PEG hydrogel

After several rounds of optimisation, we developed PEG hydrogels containing functional peptides with a defined mechanical profile, that has improved organoid growth and morphology over native PEG hydrogels. Despite this, our PEG system failed to reproduce the characteristic development of new budding crypt-like structures, and larger increase to cross sectional area that is routinely displayed in organoids cultured in Matrigel. Although there is further optimisation potential that could be realised through further mechanical fine tuning, alterations to peptide concentrations and development of semi-synthetic hydrogels containing peptides and a laminin blend, there appears to be fundamental limitations with this PEG hydrogel system that prevents the replacement of Matrigel for routine culture of HIO.

One such limitation is the absence of true degradability. Dynamic remodelling of the ECM has key roles in tissue renewal and stem cell development (Lu et al., 2011), which is replicated by organoids cultured in Matrigel. Several research groups have explored the use of enzymatic and hydrolytic degradation methods to impart PEG hydrogels with degradability (Gjorevski et al., 2016; Broguiere et al., 2018; Hernandez-Gordillo et al., 2020). As previously discussed, the integration of a bi-cysteine VPM peptide into 4 Arm PEG SH and MAL hydrogels via thiol-Michael addition does not act as a sufficient crosslinker, due to its ability to only interact with half of the PEG arms used for crosslinking (PEG MAL). Moreover, at the concentrations of PEG hydrogels used for these studies, VPM integration is more likely to form network defects known as primary loops. The PEG hydrogels used in these studies therefore remain covalently crosslinked and are unable to be enzymatically degraded MMP's produced by the organoids. This prevents simple, routine retrieval and passage of the organoids out of PEG hydrogels at the end of a 7-day culture period, which limits the potential of these organoid cultures to be

expanded indefinitely (a routine practice for organoids cultured in Matrigel). Moreover, this may have wider implications towards the organoids inability to form more differentiated cell types which results in growth of new budding structures. Research by Lutolf and colleagues identified a stiff matrix promotes intestinal stem cell expansion, whilst a softer matrix will support organoid formation and the development of mature cell types. (Gjorevski et al., 2016). The inability of our functional PEG hydrogels to be degraded and gradually soften over time, may contribute to the inability of these organoids to form new buds and truly replicate Matrigel.

To investigate the absence of budding structures in organoids cultured in PEG hydrogels, it would be necessary to characterise the cellular composition of these organoids to observe the presence and distribution of stem cell populations alongside differentiated cell types observed in HIO's. Key labelling techniques such as immunohistochemistry have been utilised for organoids cultured in Matrigel to validate colonic HIOs as a 3D culture system for the study of human gut physiology, however the use of these PEG hydrogels presented several issues when conducting these experiments. We experienced technical issues with antibody penetration of PEG hydrogels, alongside excessive swelling causing issues with mounting and imaging. Overcoming these issues may be possible through continued optimisation of the protocols utilised, however we deemed necessary changes could be made to the hydrogel network that would help mitigate several issues with this hydrogel design.

3.6 Conclusion

This chapter describes a guided optimisation process to produce a synthetic PEG hydrogel with defined mechanical parameters and bio-functionality tailored towards colonic HIO growth. Each round of optimisation is guided by monitoring changes to organoids CSA and characteristic morphological traits, with the use of organoids cultured in Matrigel as a benchmark of optimal organoid performance. We first validated the use of native PEG hydrogels as a platform for mechanical and biological optimisation towards colonic organoid growth. We then identify the ability of a fully defined, synthetic PEG hydrogel to create an ECM-like environment and support the growth of HIO fragments displaying a regular morphology, defined lumen and intact epithelium. However, organoids cultured in defined, functional PEG hydrogels display the absence of characteristic budding and large increases to CSA that is observed in Matrigel. Consideration of these findings, along with identification of key limitations of the crosslinking chemistry used in this PEG hydrogel system, highlight necessary avenues for improvement and continued optimisation that are subsequently explored in chapter 4.

4. Development of a degradable PEG hydrogel platform for human intestinal organoid culture

4.1 Introduction

One key component in the successful culture of human intestinal organoids (HIO) is an environment which promotes healthy maintenance of the intestinal stem cell niche. It is widely accepted that the ISC niche resides at the base of invaginations into the surrounding extracellular matrix (ECM) known as crypts (Leedham et al., 2005). The self-renewal or differentiating capacity of an ISC is carefully regulated within this niche via interactions with both the physical and cellular components of the ECM. This includes both the structural proteins vital to maintaining a 3D architecture, along with various cells embedded within the ECM, which secrete growth factors and matrix components aiding in the regulation of stem cell proliferation and differentiation (Yen and Wright, 2006). The fundamental nature of ECM interactions in HIO culture is demonstrated by the reliance upon the aforementioned, tumour-derived ECM Matrigel, which was utilised in early organoid studies to provide suitable structural and biochemical cues for the expansion of single intestinal stem cells into organoids (Sato et al., 2013) for which an adequate replacement remains the subject of vast research.

One method by which ISCs interact with and are in turn regulated by their niche, is through continuous and dynamic proteolytic degradation and remodelling of the ECM. Cleavage of ECM components are necessary in regulating the composition and structure of the ECM, but also enables the release of biologically active molecules i.e growth factors, capable of triggering biochemical queues within ISCs. The degradation of the ECM is afforded by a large family of proteases known as matrix metalloproteinases (MMPs), each with varying substrate

specificity which co-operate to cleave all components of the ECM (Bonnans, Chou and Werb, 2014). The use of Matrigel in organoid culture became the gold standard, and a key factor for this was sufficient replication of the ECM in the ISC niche. This enables HIO to interact with and degrade their surrounding environment, allowing for necessary matrix remodelling and alterations to the mechanical environment, alongside the release of necessary bio-active molecules (Hughes, Postovit and Lajoie, 2010). However, Matrigel is not without its limitations, the inability to finely tune or customise the properties of Matrigel results in uncontrolled and unregulated degradation from HIOs (Aisenbrey and Murphy, 2020). This presents difficulties when researching the role of parameters such as matrix degradation and stiffness in both regulation of stem cell fate and organoid growth and development.

In recent years, matrix degradation has been highlighted as a key parameter in regulating cellular biology and synthetic strategies to design biomaterials such as hydrogels which possess tuneable control over matrix properties such as degradation. Gjorevski and colleagues demonstrated the use of hydrolytically degradable chemical moieties to impart degradability into a defined PEG-based system for intestinal organoid culture. The stiffness and degradability had a major effect on the ability of induced pluripotent cells to differentiate into intestinal organoids and through varying the ratio of hydrolytically labile functional groups (dPEG) to stable functional groups (sPEG), the rate of degradation of the gel was controlled (Gjorevski et al., 2016; Gjorevski and Lutolf, 2017).

Although several methods to impart hydrogels with degradability have been utilised one method, which has previously been utilised for HIO culture is to embed enzymatically degradable peptides within the hydrogel network (Spence et al., 2017). Cruz-Acuña and colleagues demonstrated that hydrogels composed of maleimide-terminated 4-arm PEG

(PEG-4MAL) and MMP sensitive bis-cysteine peptides promote the generation of human pluripotent stem cell (hPSCs)-derived intestinal organoids.(Cruz-Acuña et al., 2017a). The results of said research provide a foundation for the inclusion of a suitable degradable peptide into a PEG-based hydrogel for the colonic, adult stem cell derived HIO culture that is explored throughout this study.

Consideration of the target protease enables the selection of an appropriate peptide crosslinker for use with HIO culture. The peptide sequence GCRDVPMSMRGGDRCG (VPM) has been shown to be degraded by proteases such as matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2) and collagenase (Patterson and Hubbell, 2010) and has been successfully embedded into PEG-based hydrogels for various use cases due to the exploitation of thiol -maleimide reactions, owing to the presence of thiol on the cysteine residues. (Greg A. Foster, Devon M.Headen, Cristina Gonzalez-Garcia, Manuel Salmeron-Sanchez, Haval Shirwan, 2017; Ahmad et al., 2019). Despite the suitability of VPM to serve as a degradability crosslinker within a PEG-based hydrogel system, this method has not been suitably utilised for HIO culture to date.

The inclusion of the bi-cysteine VPM peptide as a crosslinker within a PEG-based hydrogel system is explored in this chapter, not only for the implementation of degradability, but also for its alteration to the overall network structure of the hydrogel. As reported in the literature the fast gelation kinetics associated with thiol-Michael addition, PEG-based hydrogels utilised in chapter 3 are often related with various crosslinking heterogeneities (Jansen et al., 2018). As outlined in section 3.5.3, network defects can arise when intramolecular crosslinks occur between two functional groups of the same macromer, resulting in the formation of primary loops (**Figure 3.11**)(Metters and Hubbell, 2004). These network defects can be seen to reduce

the number of elastically active chains, which in turn have an adverse effect on the mechanical properties of the gel and can lead to excessive gel swelling (Metters and Hubbell, 2004; Zhou et al., 2012).

As discussed in section 3.5.3, it became apparent that in theory the network structure created through the direct crosslinking of 4-arm PEG Maleimide and 4-arm PEG thiol utilised throughout chapter 3, in both native but more-so in functionalised states, would be prone to a large degree of network defects. This in principle occurs through the vast reduction in number of elastically active, PEG-SH to PEG-MAL crosslinks. To reduce the number of network defects and combat primary loop formation caused by the introduction of a bis-cysteine crosslinker, Rezakhani and colleagues adapted the conventional crosslinking mechanism between PEG-co-peptide hydrogels formed by Michael addition of bis-cysteine peptides and vinyl sulfone (VS)-terminated PEG macromers (PEG-VS) (Lutolf and Hubbell, 2005) and were seen to achieve increased availability of bioactive ligands whilst generating PEG hydrogels at low polymer solid content with minimised structural imperfections and swelling (Rezakhani et al., 2020). These gels were seen to support largely murine and some HIO culture to standards comparable to Matrigel. We have adapted this crosslinking methodology presented by Rezakhani *et al* for the crosslinking of 4-Arm PEG Mal with the bis-cysteine VPM peptide, to impart degradability and reduce the occurrence of network defects within the hydrogel structure. Outlined in **figure 4.1** and **4.2**, this crosslinking strategy enables the generation of both native, biochemically inert VPM crosslinked PEG hydrogels, as well as biologically functionalised VPM crosslinked hydrogels, both of which are utilised for human colonic intestinal organoid culture and the suitability of which are explored in this chapter.

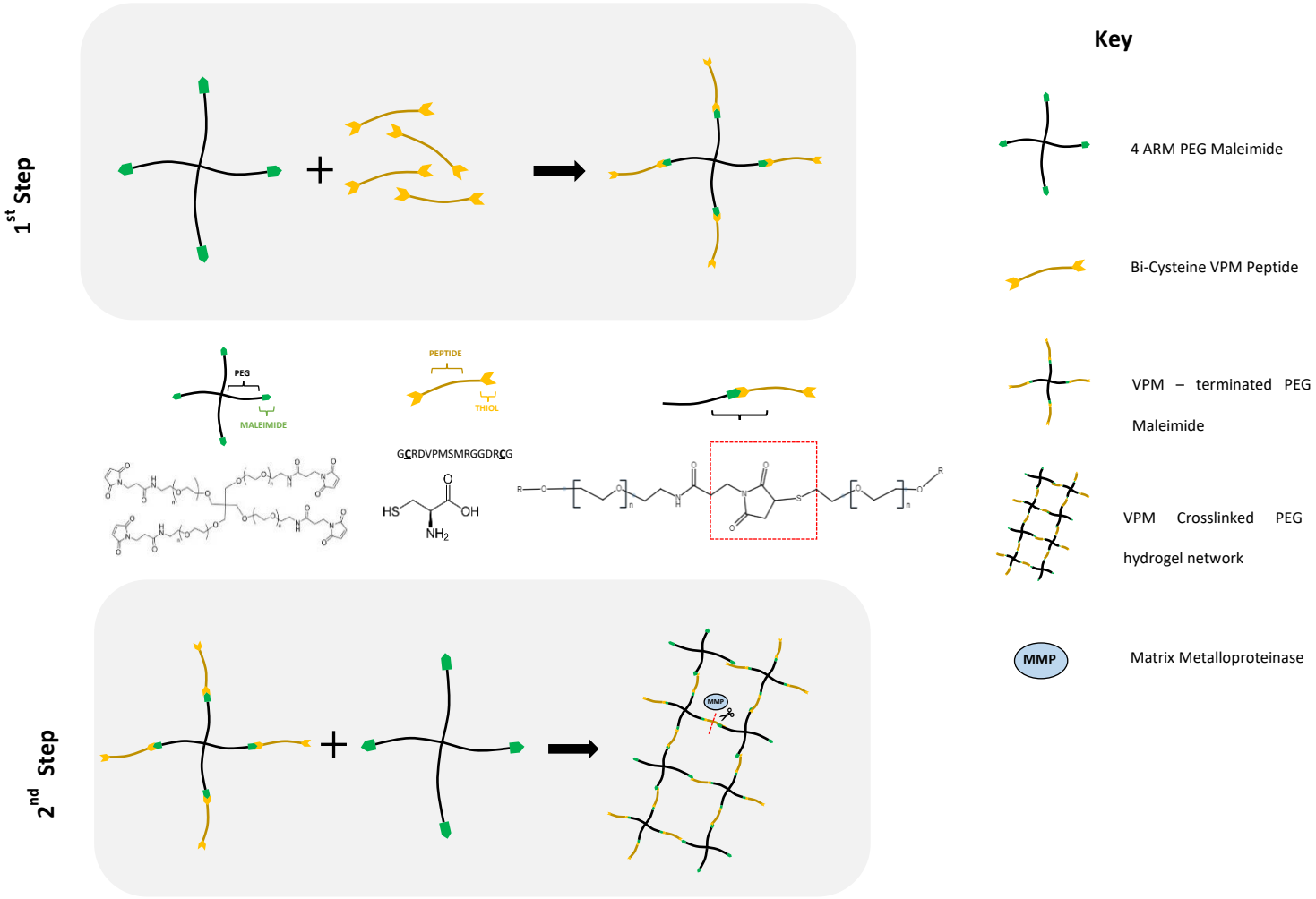


Figure 4.1. Hydrogel crosslinking mechanism for native and degradable PEG-based hydrogels. Stepwise reaction of 4 ARM PEG macromers with a bifunctional VPM peptide in excess at a 1:4 molar ratio to promote intermolecular, MMP-degradable VPM crosslinking and increase the number of elastically active chains.

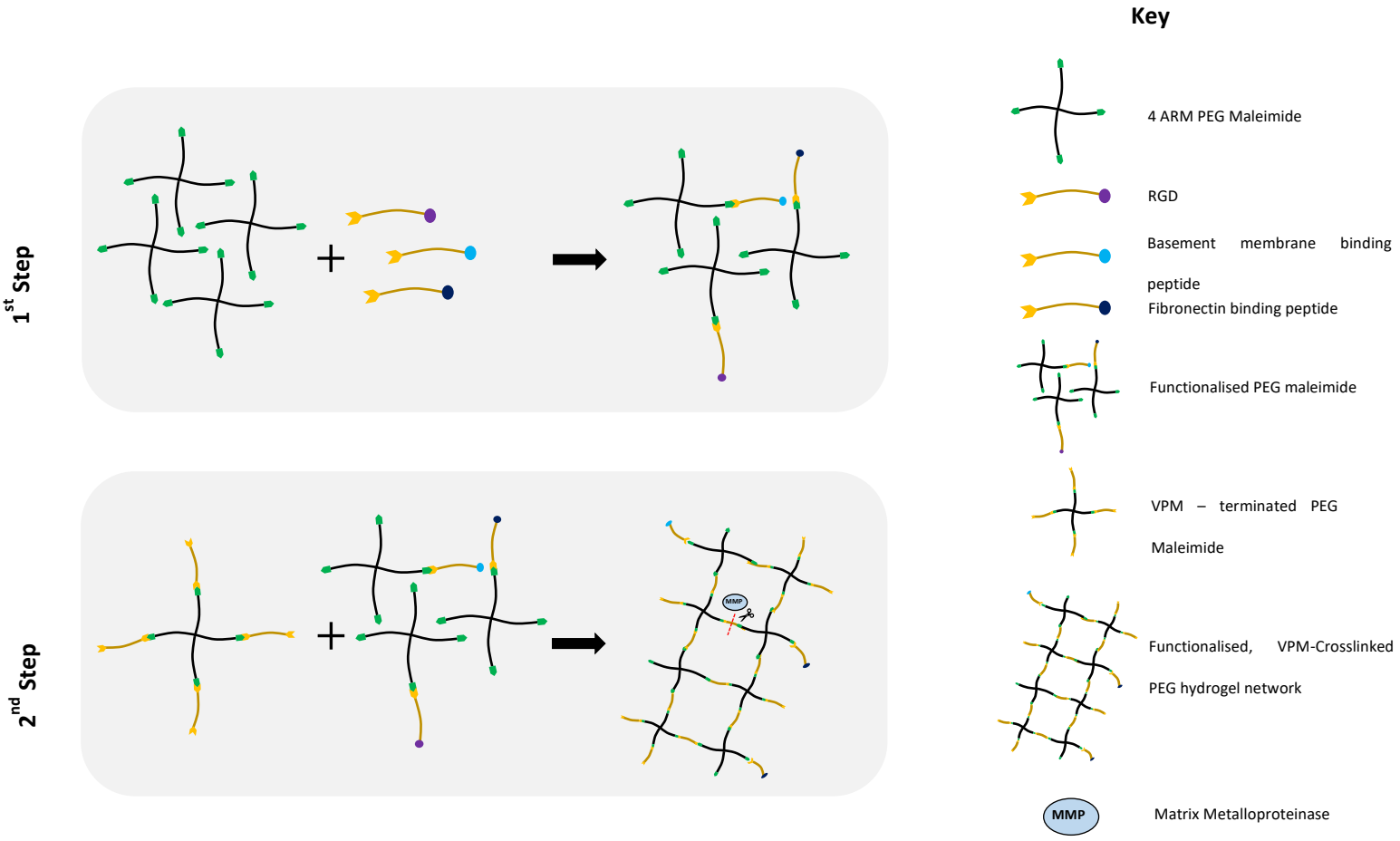


Figure 4.2. Hydrogel crosslinking mechanism for functionalised and degradable PEG-based hydrogels. Pre-functionalisation of 4 ARM PEG macromers with cysteine terminated adhesive peptides, followed by stepwise reaction with VPM peptide terminated 4 ARM PEG maleimide provides intermolecular, MMP-degradable VPM crosslinking with added adhesive functionalisation.

4.2 Hypothesis, Aims & Objectives

Hypothesis

The refined inclusion of degradable and functional peptides will improve the mechanical properties of the PEG hydrogels and result in improved organoid growth and morphology.

Aims

The aim of this chapter is to address the limitations of the PEG hydrogel system detailed in chapter 3. This is achieved through the development of a degradable, VPM crosslinked PEG hydrogel, which serves as a basis for further mechanical and biochemical optimisation to improve HIO growth and survival within a degradable PEG system.

Objective I

To characterise the mechanical properties of the VPM crosslinked PEG hydrogels (**Figure 4.1 and 4.2**) and ascertain their suitability for HIO culture.

Objective II

To encapsulate HIO within native (**Figure 4.1**) and functionalised (**Figure 4.2**) degradable PEG hydrogels and monitor organoid growth and morphological performance.

Objective III

Investigate the proliferative capacity and protein localisation within organoids encapsulated in optimised PEG hydrogels

4.3 Materials & Methods

4.3.1 Hydrogel components

Full details of hydrogel starting materials including functionalised multi-arm PEGs, degradable crosslinker and functional peptides and buffers are located in section 2.2.7

4.3.2 Native, VPM-crosslinked PEG hydrogel preparation

Degradable VPM crosslinked hydrogels were formed via stepwise Michael type addition (as outlined in **Figure 4.1**) between 10 kDa 4-Arm maleimide-terminated PEG (PEG-Mal) and the MMP-sensitive VPM peptide, followed by further reaction with 10 kDa 4-Arm PEG-Mal. To prepare native, degradable hydrogels of the required final PEG content i.e 5% (w/v) or 5 mM, the required mass of PEG-Mal was dissolved in buffer solution and reacted with the VPM peptide (freshly prepared and dissolved in buffer solution) with consideration to the stochiometric ratio of functional groups so that thiol was in excess (1:4). The reaction was allowed to persist for 2 minutes and following brief vortex the pre-reacted PEG-Mal-VPM solution was further reacted with 4-Arm PEG-Mal in a 1:1 stochiometric ratio of thiol to maleimide.

4.3.3 Functional VPM crosslinked PEG hydrogel preparation

The preparation of functionalised, VPM crosslinked hydrogels occurs as described above (section 4.3.2 and **Figure 4.2**) with the addition that pre-reacted PEG-Mal-VPM solution is further reacted with 4-Arm PEG Mal that is pre-functionalised with biological peptides. For this pre-functionalisation, PEG-Mal is freshly weighed and dissolved with consideration of the desired final PEG content and the addition of excess thiol from cysteine residues on functional peptides. For example, for a desired concentration of 5 mM PEG-Mal, with the addition of 1mM of functional peptides a 6mM solution of PEG-Mal is prepared. The functional peptides

as detailed and prepared in section 2.2.7 (main methodology) are added to the PEG-Mal solution, briefly mixed via gentle pipetting and the reaction is allowed to persist for 2 minutes. The pre functionalised PEG-Mal is further reacted with the pre-reacted PEG-Mal-VPM solution at a 1:1 stochiometric ratio of thiol to maleimide.

4.3.4 Quantification of thiols in PEG precursors and hydrogels.

Free thiol quantification via Ellman's reagent is detailed in section 2.2.10

4.3.5 Rheological assessment of PEG hydrogels

Details of parameters used for rheological gelation point, strain and frequency sweeps are detailed in section 2.2.11

4.3.6 Human intestinal organoid culture and analysis

Details surrounding HIO culture and analysis are presented in chapter 2

4.4 Results

To ascertain whether degradable and functional VPM-crosslinked PEG hydrogels (**Figure 4.1 and 4.2**) successfully address the limitations of the PEG-SH and PEG-MAL crosslinked hydrogels outlined in chapter 3 (**Figure 3.1B**), gels were subject to a series of chemical and physical characterisations before assessing organoid growth, morphology and survival withing the degradable PEG gels. Firstly, the chemical crosslinking of the new PEG crosslinking strategy employed herein was assessed in comparison to non-degradable gels using Ellman's reagent. Then, VPM-degradable PEG hydrogels were subject to a series of stepwise rheological characterisation to identify the bulk mechanical properties. Organoid morphological performance, growth and survival was then assessed within degradable PEG hydrogels, before employing Immunological and EdU labelling to characterise organoid performance at a cellular level.

4.4.1 Quantification of free thiol to determine gelation efficiency

The presence of free thiols was quantified using the robust and facile Ellman's reagent to serve as a measure of thiol consumption during the gelation process and as an indication of overall gelation efficiency. Pre-gelation starting materials, mid-gelation reactions and final gel products were incubated with the Ellman's reagent and the relevant thiol concentrations are presented in **figure 4.3**. In the pre-reacted, diluted, gel-starting materials the PEG Mal was free of any thiol whilst PEG SH contained an abundance of thiol registering 1.28 mM (± 0.04) or when accounting for the 1:50 dilution factor 64 mM. The media utilised in HIO cultured was seen to possess low quantities of free thiol at 0.084 mM (±0.009) whilst the bis-cysteine VPM peptide contained 0.82 mM (± 0.05) free thiol, or 82mM when accounting for the 1:100 dilution factor. For the mid-reaction products there was a negligible concentration of

0.017mM (±0.04) free thiols present in the pre-functionalised fPEG MAL and 0.61 mM (±0.03) in the PEG Mal-VPM mid gelation product. Finally in the fully formed degradable VPM crosslinked hydrogel products discussed within this chapter, 0.098 mM (± 0.024) and 0.11 mM (±0.025) concentrations of free thiols were present in the native and functional gels respectively. For the non-degradable PEG hydrogels discussed throughout chapter 3, 0.30 mM (±0.11) and 0.23 mM (±0.06) concentrations of free thiols were present in the native and functionalised gels respectively.

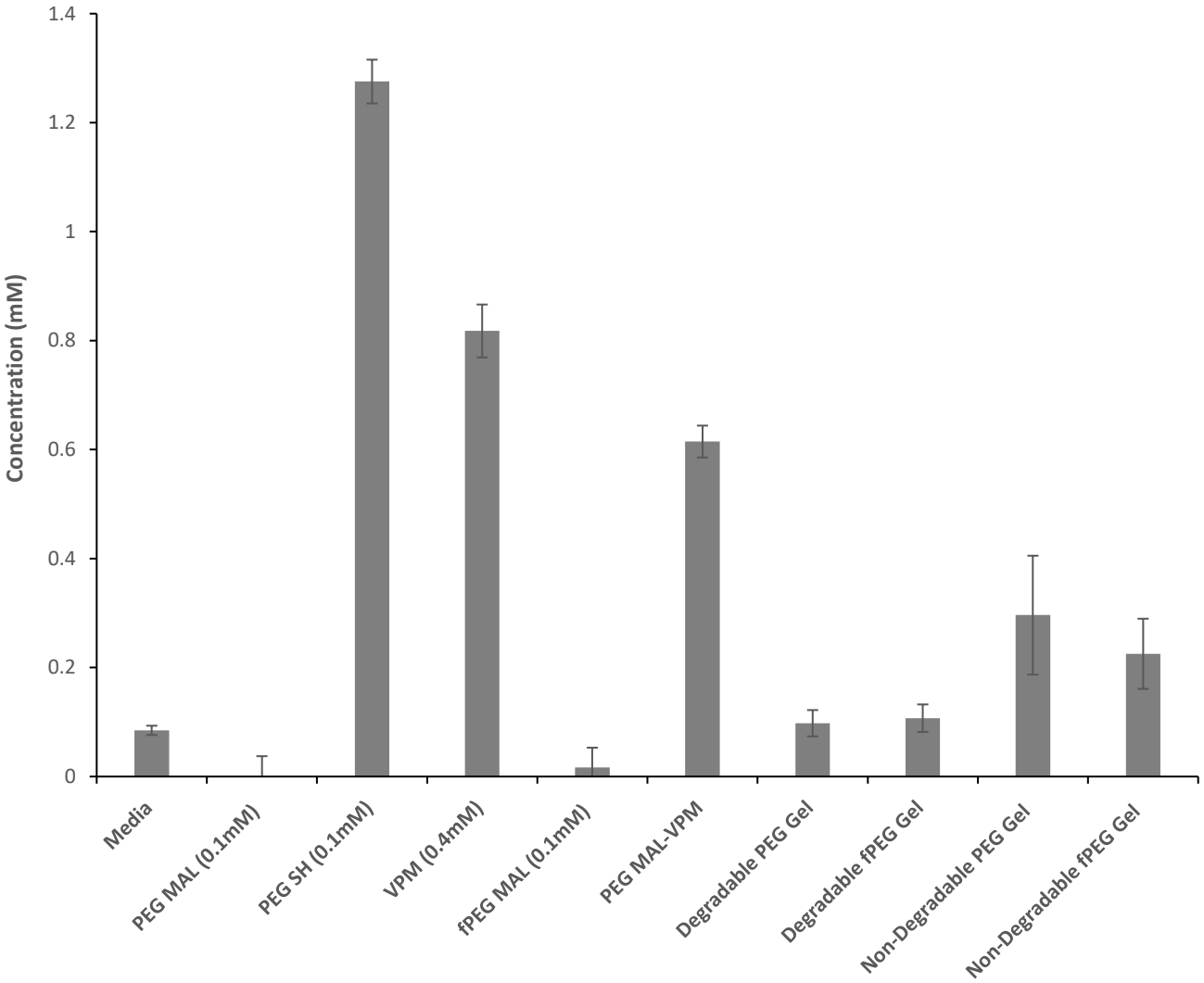


Figure 4.3 Quantitative Ellman's assay setup to evaluate consumption of free thiol in PEG hydrogel products. PEG hydrogels were mixed and incubated at 37 °C to allow gel formation, 30 µL gel droplets and PEG starting materials and mid-reaction products were incubated with 300 µL Ellman's reagent for 15 minutes before measuring the absorbance of reaction products at 412nm. Free thiol concentration was quantified by comparison to a standard curve and data shown illustrates the mean (n=3) ± standard error.

4.4.2 Rheological characterisation of degradable VPM crosslinked hydrogels

4.4.2.1 Gelation time

The initial characterisation of gelation time is necessary to identify parameters for subsequent characterisation. The degradable VPM crosslinked hydrogel samples were loaded onto the rheometer plate in liquid form before lowering the test geometry and initiating the experiment with the parameters identified from the literature. The gelation time for degradable VPM crosslinked PEG hydrogels is outlined in **Figure 4.4**. After approximately 100 seconds there is a clear separation between the storage modulus, G' and loss modulus, G'' in which G' > G'' as of that in elastic solids. At approximately 1000 seconds the storage modulus begins to plateau as the gelation reaches equilibrium.

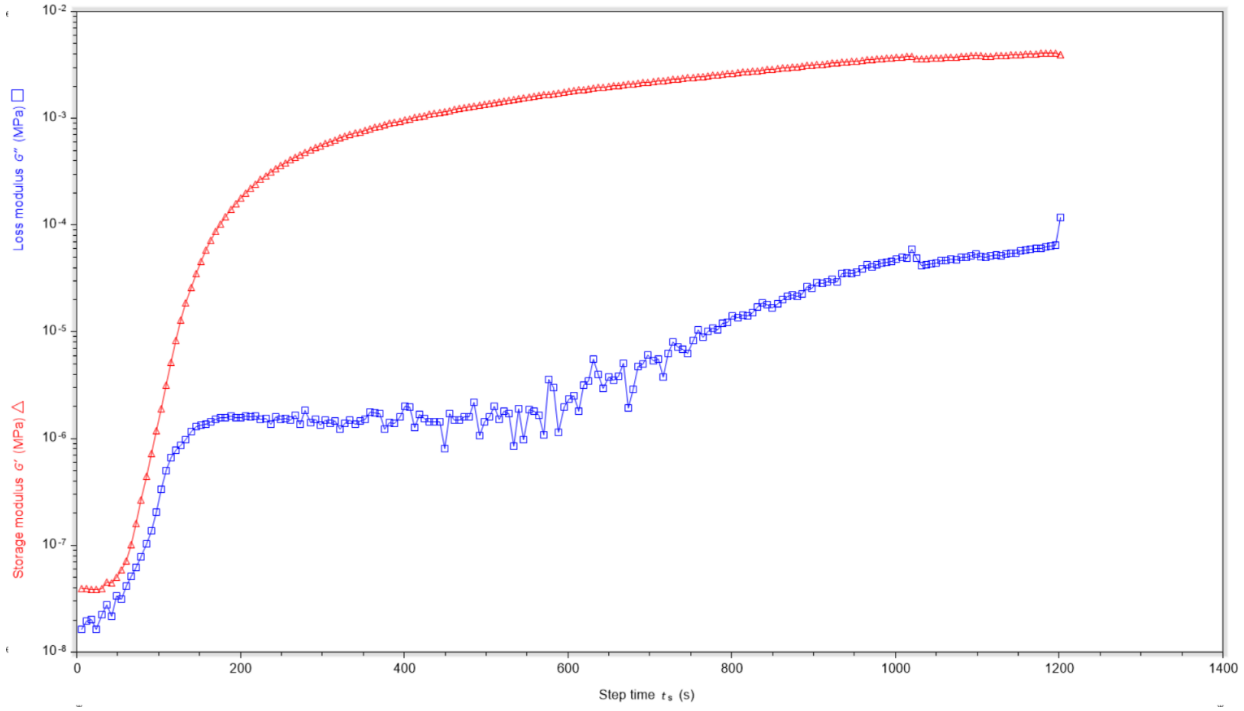


Figure 4.4. Rheological characterisation of gelation point in degradable VPM crosslinked hydrogels. Degradable VPM crosslinked PEG hydrogel samples were prepared directly onto the rheometer plate before lowering of the test geometry. Hydrogel samples were run at 37 °C with a fixed frequency of 1.0 Hz and 10% fixed strain for a duration of 1200 seconds (20 minutes). Storage modulus, G' and Loss modulus, G'' are reported in MPa.

4.4.2.2 Strain sweep

Strain sweeps were conducted on fully formed degradable VPM crosslinked PEG hydrogel samples to identify the linear viscoelastic (LVE) limit of the gel. Identification of the LVE allows for accurate selection of a fixed strain % to be applied in subsequent studies in order to identify bulk hydrogel stiffness. Gel samples were placed in liquid form on the rheometer test plate and the appropriate time for gelation equilibrium to be achieved, as established in the relevant time sweep (see section 4.4.2.1) was applied before conducting a strain sweep at fixed frequency. The results are presented in **figure 4.5**. The degradable VPM-crosslinked PEG hydrogels displayed linear behaviour, by which the G' remains constant with the increased oscillation strain (%) applied, up until values of 150% strain.

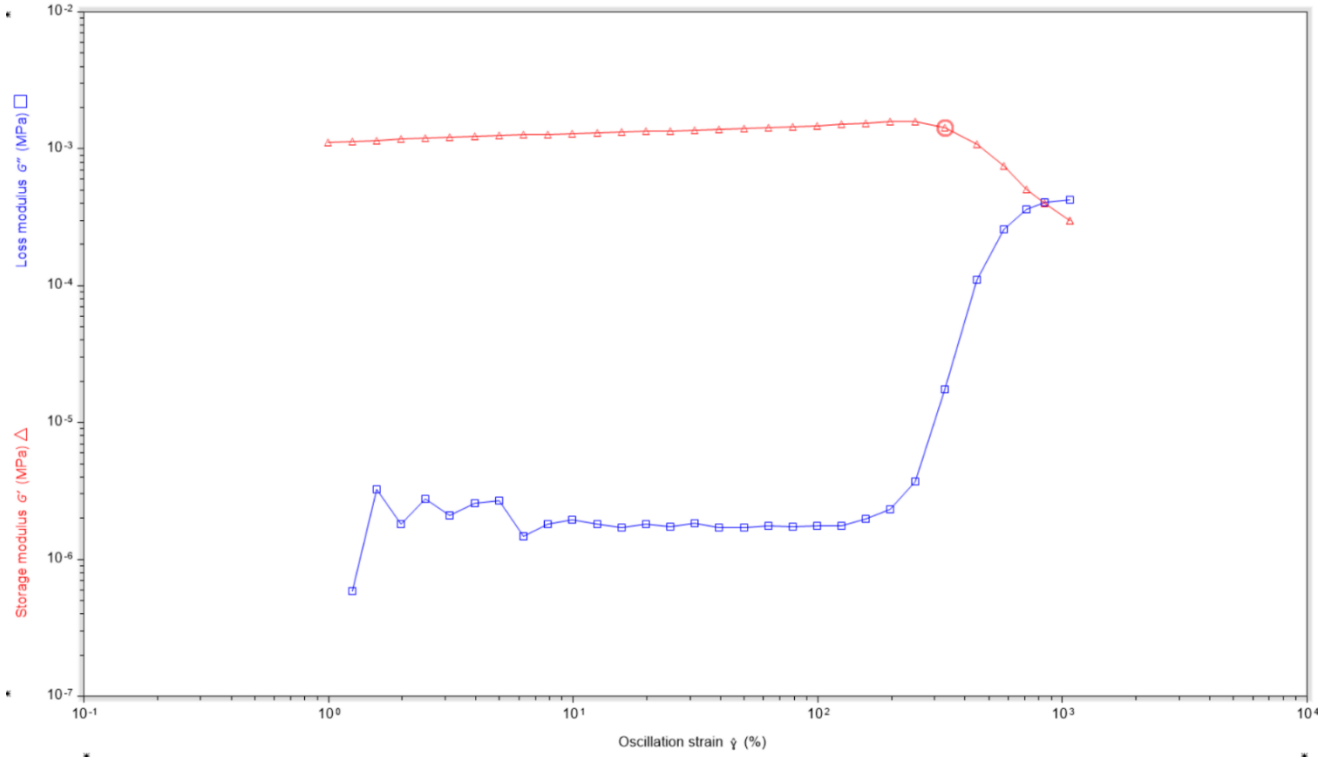


Figure 4.5. Rheological characterisation via strain sweep of degradable VPM crosslinked PEG hydrogels. Hydrogel samples were placed onto the rheometer test plate and allowed to reach gelation equilibrium before lowering the test geometry and applying increasing oscillation strain from 0.1 to 1000% at a constant 37 °C and fixed 1.0 Hz frequency

4.4.2.3 Frequency Sweep

With the identification of the LVE and gelation equilibrium point in the preceding sections it is possible to identify storage modulus, G', values for degradable, VPM crosslinked PEG hydrogels across a range of frequencies, with a fixed strain applied. Degradable, VPM crosslinked PEG hydrogels were formed across a range of total PEG content concentrations from 3mM to 6mM and the results of said frequency sweeps are detailed in **figure 4.6**. For all concentrations of degradable, VPM crosslinked PEG hydrogels monitored via frequency sweep, a constant gel behaviour was displayed across all frequencies (0.1 to 100 rad/s). The storage modulus, G', values were ascertained to be 1.05 kPa, 0.36 kPa ,0.20 kPa and 0.1 kPa for the 6mM, 5mM, 4 mM and 3 mM final PEG concentration, degradable hydrogels. The G' value returned from the frequency sweep of the degradable VPM crosslinked PEG hydrogels at 5mM final PEG concentration which are discussed throughout this chapter is presented alongside the G' returned from the frequency sweep of the equivalent concentration (5mM) non-degradable hydrogel utilised in chapter 3 (**Figure 4.7**)

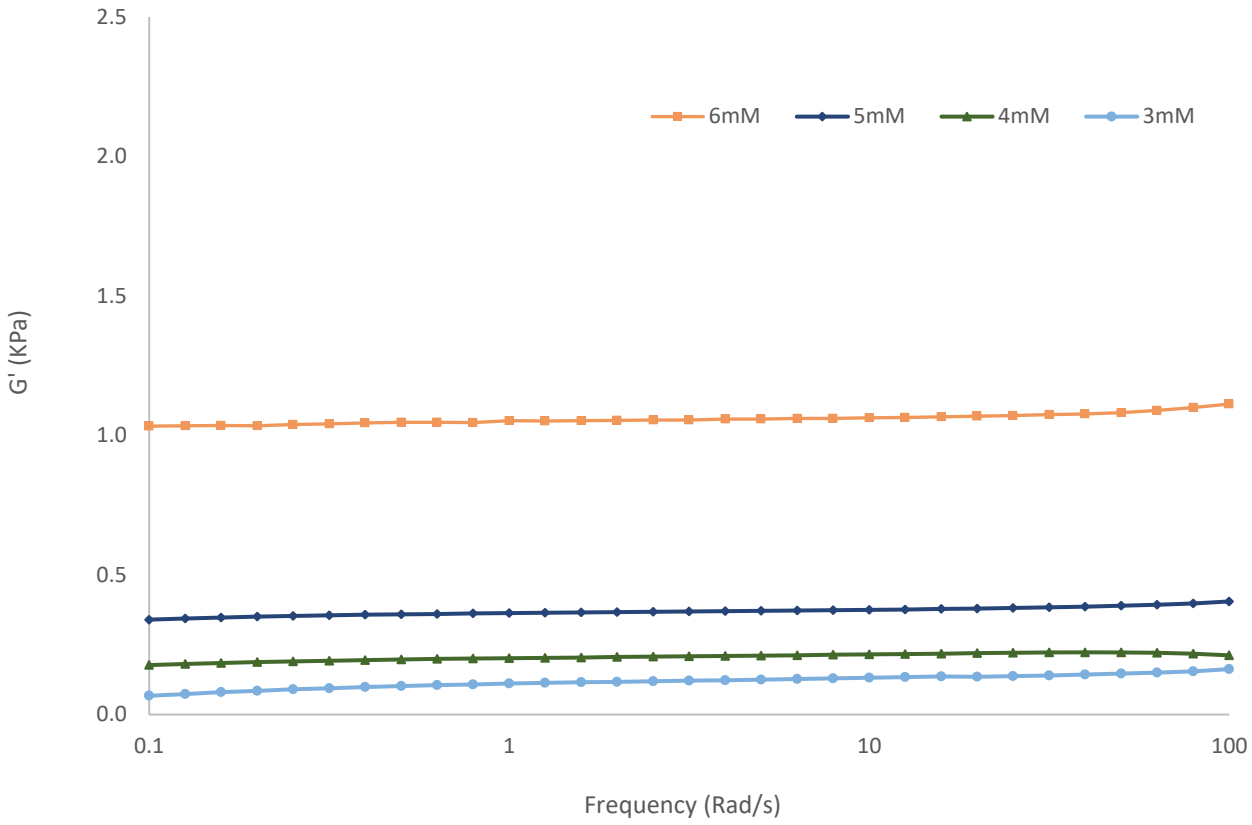


Figure 4.6. Frequency sweeps performed on Native, VPM-Crosslinked PEG hydrogels. The G' was determined at 37 °C from 0.1 to 100 rad/s at fixed 10% strain for gels containing 6mM (square), 5mM (diamond), 4mM (triangle) and 3mM (circle) final PEG concentrations.

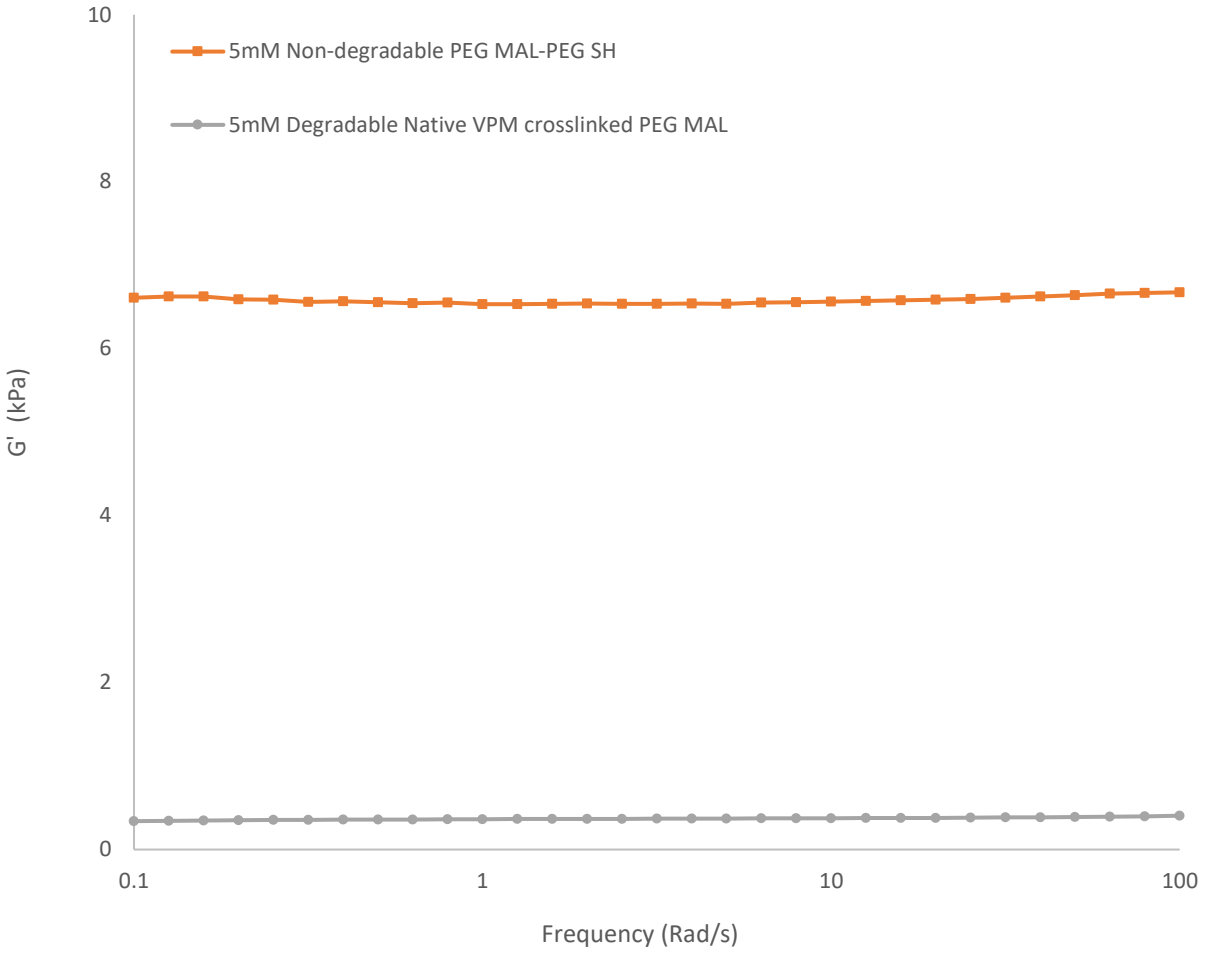


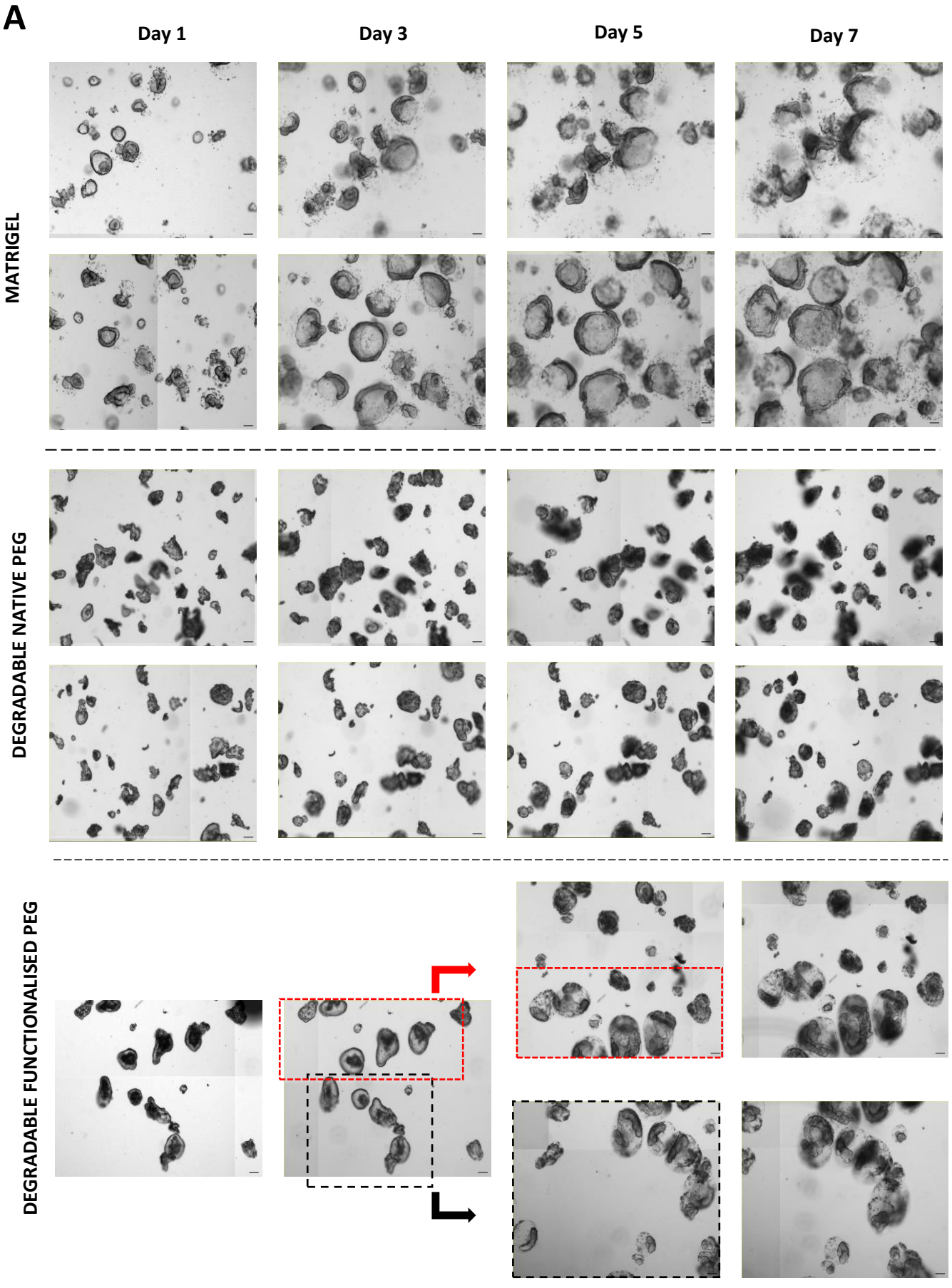
Figure 4.7. Frequency sweep performed on degradable VPM-crosslinked PEG hydrogels compared to a Non-degradable PEG SH – PEG MAL crosslinked hydrogels n=3. G' was determined from 0.1 to 100 rad/s at fixed 10% strain at 37 °C.

4.4.3 HIO culture in degradable VPM crosslinked PEG hydrogels

4.4.3.1 Assessment of organoid growth via cross sectional area analysis

To investigate the suitability of the degradable VPM crosslinked PEG hydrogels for HIO culture, organoids cultured in Matrigel were passaged and their fragments embedded into freshly prepared VPM crosslinked gels that are either free of biological peptides (native) or containing functional peptides at concentrations outlined in section 2.3.9. The organoids were monitored via brightfield microscopy and fed over a 7-day culture period (**Figure 4.8A**) and their growth, as measured by increase to their cross-sectional area is reported in **Figure 4.8B**. Organoids cultured in Matrigel display characteristic growth patterns over the 7-Day culture, expanding significantly higher in size (2.57-fold increase) in cross-sectional area than organoids cultured in degradable PEG ($p < .001$). These organoids remain healthy as indicated by their translucence (i.e non-granulated) and presence of apparently intact epithelial membranes. Additionally, organoids cultured in Matrigel continue to grow and expand in cross sectional area between days 5 and 7 of the culture period displaying an increase between 1.86-fold and 2.57-fold, a further 0.71-fold increase in area ($p <.001$). Conversely, organoids cultured within native (non-functional) degradable hydrogels show minimal fold increase (1.27x) over the course of 7 days, significantly less than in functional degradable PEG and Matrigel ($p < .001$). This is reflected in the representative brightfield images where organoid fragments embedded at day 1 show little to no expansion in their lumen size or area between days 1 and 3. Further to this, from culture day 5 HIO embedded in PEG degradable hydrogels display characteristic cell death traits of being dark and dense. It appears that any increase to organoid CSA within native gels occurs within the first 3 days of culture, with minimal fold increase between days 3-5 and 5-7. For organoids cultured in functionalised

degradable hydrogels there is a clear expansion of the lumen and increase in the cross-sectional area between culture days 1 and 3 (1.3x fold increase) which continues to rise to 1.61-fold on day 5 and 1.98-fold overall. This is significantly higher than organoids cultured in non-functional PEG but less than in Matrigel ($p < .001$), reflected in the brightfield images by which organoids embedded in the functionalised gels display a greater overall expansion than organoids in the native gels, however they appear less intact than those cultured in Matrigel.


A
Day 1
Day 3
Day 5
Day 7
MATRIGEL
DEGRADABLE NATIVE PEG
DEGRADABLE FUNCTIONALISED PEG

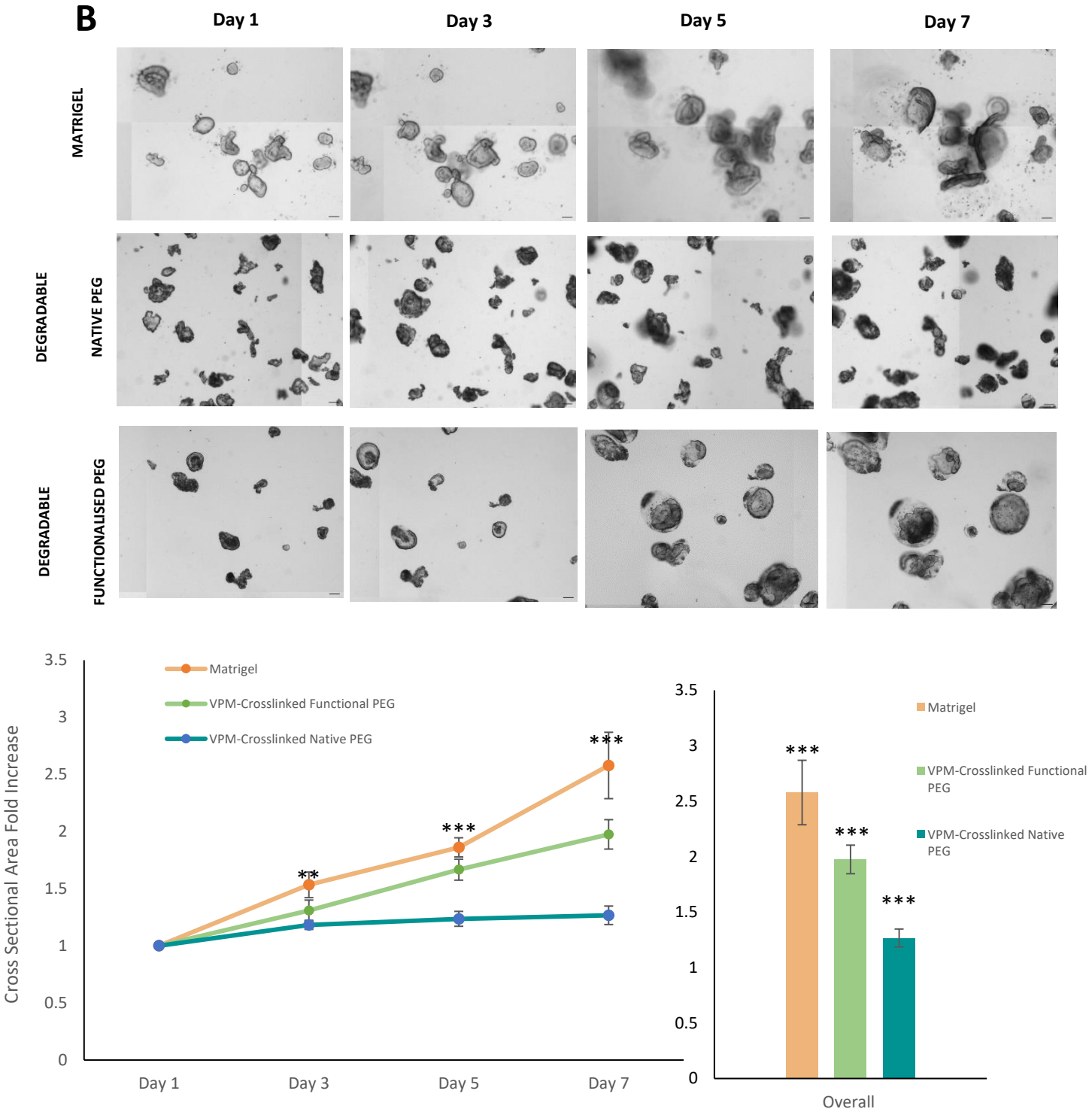
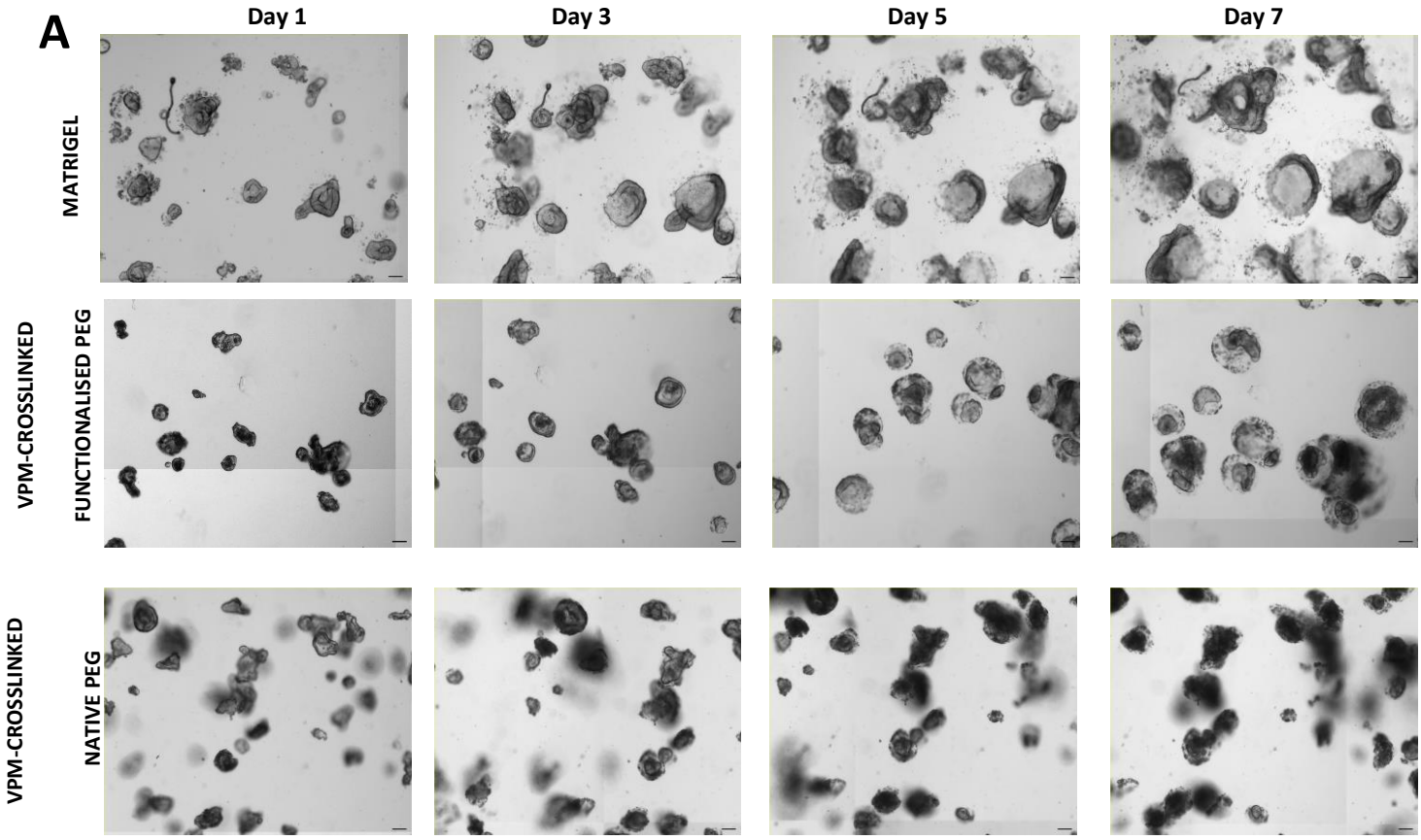


Figure 4.8 Adult HIO growth in native and functionalised VPM crosslinked, degradable hydrogels. (A) Representative brightfield images of adult human intestinal organoids cultured in Matrigel, VPM-Crosslinked, Native, Degradable PEG hydrogels and VPM crosslinked, functionalised hydrogels. Images are taken on alternate culture days, 1, 3 ,5 and 7 as indicated.

Scale bars, 100µm (B) Further representative images of HIO cultured in Matrigel, Native and Functionalised, VPM crosslinked degradable gels and the assessment of cross-sectional area fold increase between alternate culture days and overall. Data is plotted ± SE. Welch's ANOVA was used to determine statistical significance between separate culture conditions on day 1, 3, 5 and 7 respectively ($F_{2, 2195.315} = 61.355$ $p < .001$, $F_{2,2376.064} = 13.272$ $p < .001$, $F_{2, 2401.891} = 15.598$ $p < .001$ and $F_{2, 2183.702} = 53.129$ $p < .001$). Statistical significance following Games-Howell post hoc test is displayed as $*p < 0.05$, $**p < 0.01$ $***p < 0.001$ (N=2, Matrigel n=7, LDTM native n=10, LDTM functional n=8). Total n of organoids assessed per replica are available in appendix 2.

4.4.3.2 Assessment of viable organoid retention in degradable PEG hydrogels

In addition to monitoring the CSA increase of organoids, the number of viable organoid structures was monitored via morphological assessment. Organoids were cultured and monitored for 7 days via brightfield images (**Figure 4.9A**). The number of organoid structures that appear viable whilst cultured in Matrigel, native degradable PEG hydrogels and functionalised degradable PEG hydrogels are reported in **Figure 4.9B** as a percentage of overall number of organoid structures present within the gel on the first day of culture. Organoids cultured in Matrigel at each day of organoid culture display characteristic morphological traits of viable organoid structures, with a light, translucent (non-granulated) morphology, regular spherical structure, and intact epithelia. This is displayed in the 96.32% retention of viable organoid structures between day 1 and 7. For organoids cultured in the native degradable PEG environment we observe the emergence of a dark, dense and granulated morphology which becomes more prevalent with each day of culture. This is reflected in the 17.47% retention rate of viable organoids after 7 days of culture. Additionally, the steepest decline in overall retention of viable organoid structures occurs within the non-functional (native) degradable PEG, falling from 100% to 17.47% ($p < 0.001$). Organoids cultured in functionalised degradable hydrogels also display their biggest loss of viable organoid structures within the first 3 days of culture however the overall decline is less prominent falling from 100% to 58.22% ($p < .001$). The overall retention rate of viable organoids in functional degradable gels was significantly higher than in non-functional PEG, but significantly lower than in Matrigel ($p < .001$).



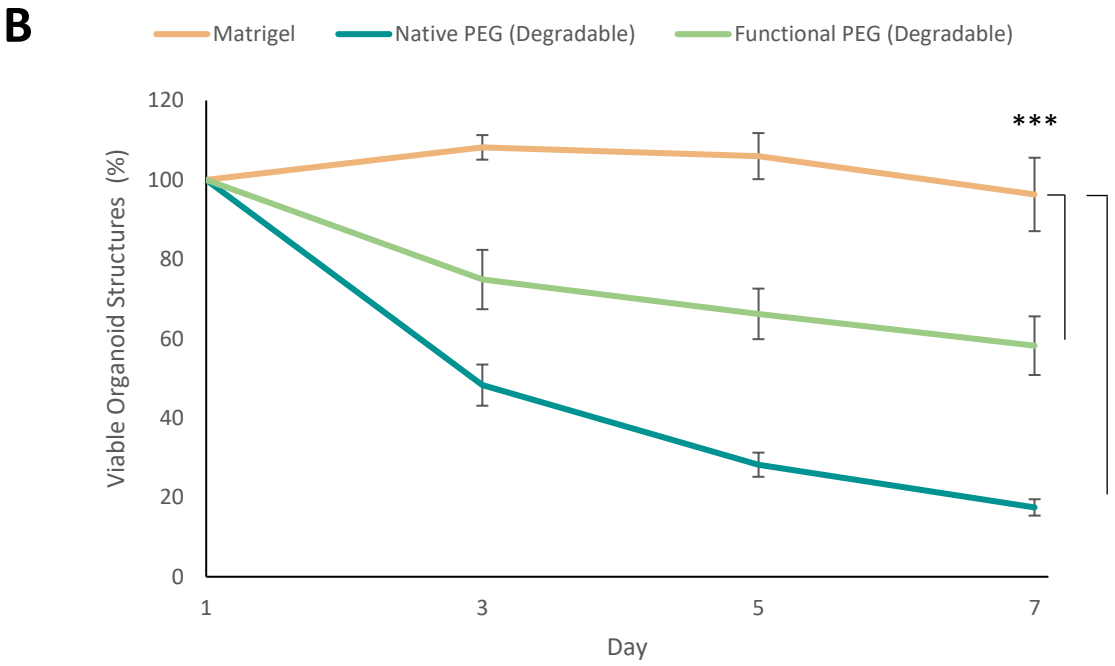


Figure 4.9 Assessment of viable organoid retention in native and functional degradable PEG hydrogels. (A) HIO fragments were cultured in Matrigel, functionalised and native degradable PEG hydrogels and monitored by brightfield images on alternate days of a 7 Day culture. Scale

bar 100 µM. (B) Viable organoid structures were identified via visual assessment and the number of remaining viable organoids is reported as a percentage (%) of viable organoids present on culture day 1. Data presented ± SE. One-way ANOVA was used to determine statistical significance between separate culture conditions and the rate of decrease of viable organoids by linear regression across days 1 to 7 ($F_{2, 18}$ =50.512 $p < 0.001$). Statistical significance following Tukey post hoc test is displayed as ***$p < 0.001$ (N=1, Matrigel n=7, native n=8, functional n=6). Total n of organoids assessed per replica are available in appendix 2.

4.4.3.3 Morphological assessment of HIO in degradable PEG hydrogels

Brightfield images obtained from alternate days of a 7-day culture of HIO encapsulated in Matrigel, native degradable PEG hydrogels and functionalised degradable PEG hydrogels were carefully analysed to assess the presence of characteristic traits of healthy organoids as outlined in Chapter 2. Materials and Methods **Figure 2.2**. The number of organoids possessing each morphological trait is reported as a percentage of the total organoid population present within the relevant gel at day 1 of the organoid culture. The changes in organoid morphology over the course of a 7-day culture can be assessed in more detail and is reported in **Figure 4.10**. For organoids cultured in Matrigel in can be seen that the proportion of organoids possessing an intact epithelium increases throughout the culture period, rising from 70% to 84% between day 1 and day 7. At day 7 there are significantly more intact epitheliums in Matrigel ($p < 0.001$) Similarly, when focusing on organoids possessing a distinct lumen, organoids in Matrigel rise from 47% to 57% within the 7 days to possess significantly more organoids with clear defined lumens than in degradable ($p < 0.001$). Organoids cultured in Matrigel are the only organoids out of those observed to possess characteristic budding structures with the % of budding structures remaining consistent around 8%. For organoids cultured in native degradable PEG the percentage of organoids possessing an intact epithelium or distinct lumen is initially lower than those within Matrigel at 51% and 38% respectively. In addition, the percentage of organoids with these morphological traits continue to fall steadily throughout the 7-day organoid culture with no organoids present with either an intact epithelium or prominent lumen at day 7 (0%). For organoids cultured in the functionalised degradable PEG there is an increase in the percentage of organoids that display an intact epithelium between culture day 1 and 3 rising from 40% to 52%. This trend is repeated for organoids possessing prominent lumens within the functional gels with the

percentage increasing from 29% to 46%. In spite of this initial increase in percentage of intact epithelium and prominent lumens, the presence of these traits begin to fall from day 5 onwards with final values being 40% and 31% respectively, which remains an overall increase of 0.3% and 2.7% respectively from the initial percentage of each trait observed at day 1. Functional PEG hydrogels, when compared to their non-functional (native) counterparts, were seen to contain organoids with significantly more intact epithelium after 7 days ($p<0.001$) and significantly more prominent lumens at day 3, 5 and 7 ($p=0.002$, $p=0.002$ and $p<0.001$ respectively).

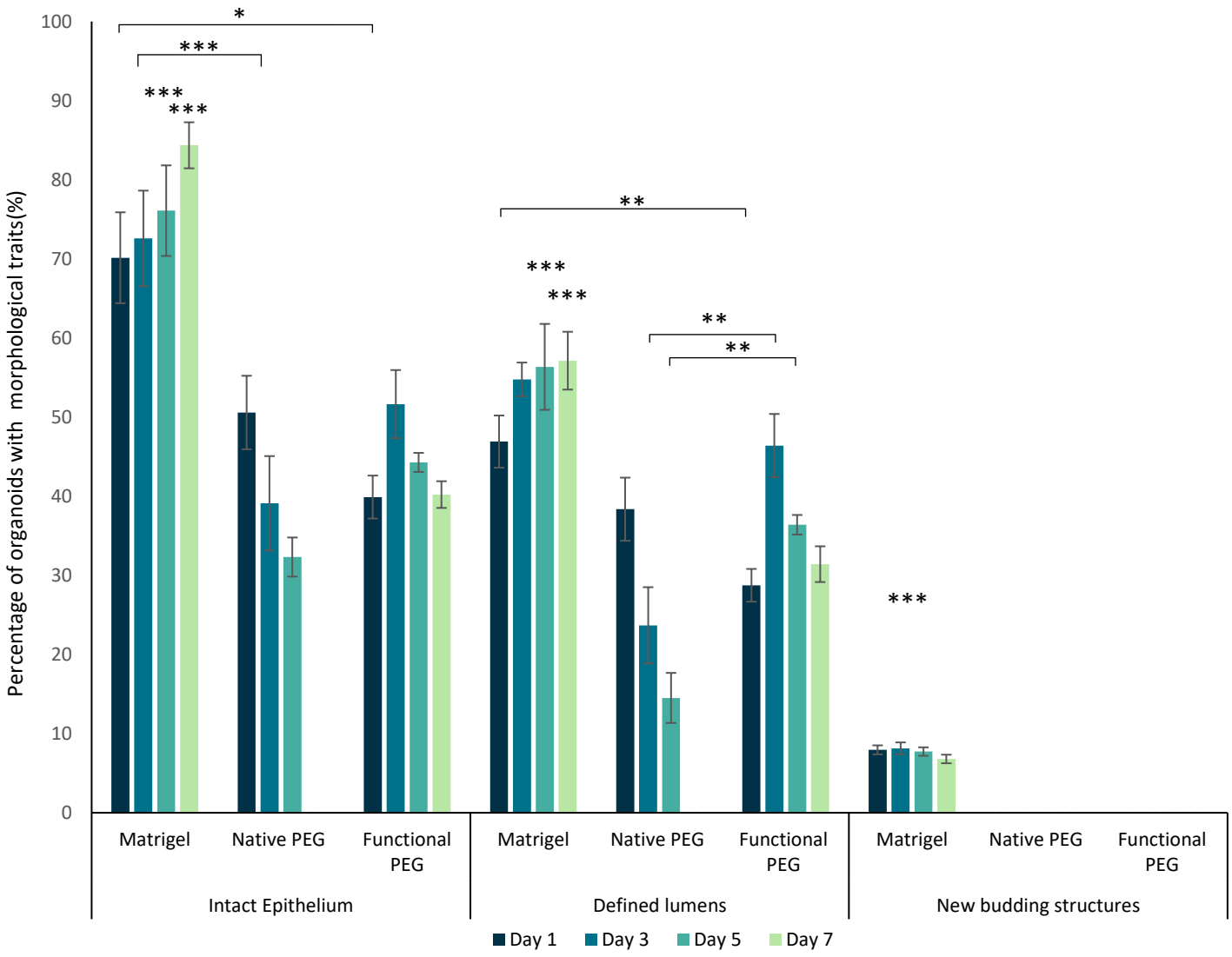


Figure 4.10. Morphological analysis of human intestinal organoids cultured in Matrigel, degradable native PEG and degradable functionalised PEG hydrogels. The number of organoids possessing key morphological traits including a distinct epithelium, prominent lumen and nascent budding structures were established on alternative days of organoid culture (day 1, day 3, day 5 and day 7). Data is presented as a percentage of total viable organoid structures present at each time point. One-way ANOVA/ Welch's ANOVA was used to determine statistical significance between presence of lumens, intact epithelium, or budding structures under separate culture conditions across days 1-7. Counts were relative to the number of viable organoid structures counted on day 1. For lumens observed on day 1, 3, 5 and 7 ($F_{2, 18}$ =6.503 p = 0.007, $F_{2, 18}$ =17.794 p < 0.001, $F_{2, 18}$ =32.001 p < 0.001 and $F_{2, 18}$ =154.81 p < 0.001).

For intact epithelium observed on day 1, 3, 5 and 7, ($F_{2, 18}$ =11.305 p = 0.002, $F_{2, 18}$=9.316 p = 0.002, $F_{2, 18}$ =38.134 p < 0.001 and $F_{2, 18}$ =542.138 p < 0.001). For budding structures observed on day 1, 3, 5 and 7, ($F_{2, 18}$ =137.156 p < 0.001, $F_{2,18}$ =238.237 p < 0.001, $F_{2, 18}$ =107.887 p < 0.001 and $F_{2, 18}$ =157.62 p < 0.001). Statistical significance following Tukey post hoc test is displayed as *p <0.05, **p < 0.01 ***p < 0.001. N=2, Matrigel n=7, native n=8, functional n=6). Total n of organoids assessed per replica are available in appendix 2.

4.4.4 Cellular characterisation of HIO cultured in degradable, VPM-crosslinked PEG

We identified there is an observable difference between morphological performance in organoids cultured in Matrigel and degradable PEG hydrogels that occurs from the initial day of culture (**Figure 4.8A**). To evaluate, and better understand organoid behaviour during the early phase (initial 24 hours) of organoid culture in VPM-crosslinked degradable PEG, organoids were incubated with fluorescently labelled EdU. Utilising fluorescent microscopy, newly proliferating cells were observed within organoids cultured in Matrigel, Degradable non-functional PEG hydrogels and fully functionalised, degradable PEG hydrogels and the percentage of newly proliferating cells compared (**Figure 4.11**). Newly proliferating cells were observed in all culture conditions. Organoids cultured for 24 hours in Matrigel contained significantly more EdU positive cells (31.68%, $p < .001$) (**Figure 4.11B**) than both VPM crosslinked native PEG (12.17%) and VPM-crosslinked functional PEG (9.16%). There was no significant difference observed between degradable gels with and without functional peptides.

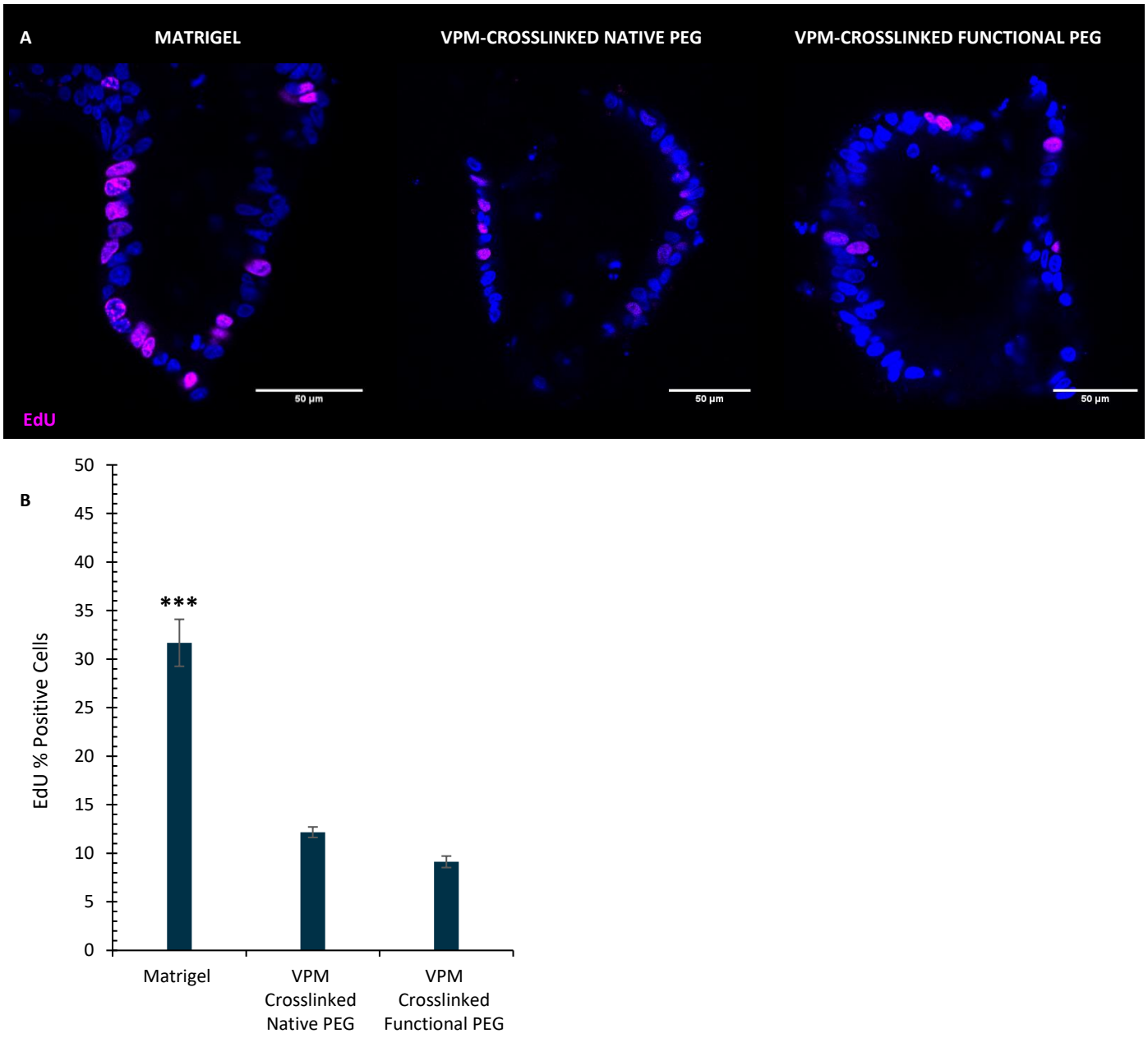


Figure 4.11 Assessment of cell proliferation in degradable PEG hydrogels. (A) Representative epifluorescence images of colonic HIO cultured for 24 hours in Matrigel (left), native degradable PEG (middle) and functional degradable PEG (right), labelled with EdU (magenta) and Hoescht (Blue). Scale bar 50 µm. (B) Bar chart indicates the percentage of positive EdU cells throughout the organoid structure compared to the total number of nuclei stained with Hoescht. One-way ANOVA was used to determine statistical significance between EDU positive cell counts under separate culture conditions ($F_{2, 6} = 69.102$ $p < 0.001$). Statistical significance

following Tukey post hoc test is displayed as ***p <.001 (N=1, for each condition n=3) Total n of cells assessed per replica are available in appendix 2.

4.4.5 Expression of intestinal stem cells in HIO cultured in degradable PEG

To further elucidate the contributing factors to the reduced growth, morphological performance and proliferation in organoids cultured in degradable PEG, we sought to identify the presence and localisation of the stem cell population within colonic HIO organoids within degradable PEG hydrogels. Early organoid expansion has been seen to require proper intestinal stem cell development (Gjorevski et al., 2016; Sato & Clevers, 2013). With this knowledge, we evaluated the expression of a key stem cell marker OLFM4 in organoids cultured for 24 hours in Matrigel, functional and native degradable PEG, alongside the epithelial marker E-cadherin (ECAD) and a nuclear stain (Sytox blue) (**Figure 4.12**). Organoids cultured in Matrigel displayed their characteristic morphology with crypt-like budding structures identified with OLFM4 labelled stem cells residing at the base of new budding structures (green arrows). Stem cells were also present in organoids cultured in both functional and native degradable PEG hydrogels. Although these organoids displayed more irregular morphologies (Brightfield images **Figure 4.12**), OLFM4 was expressed basally as typically observed in Matrigel.

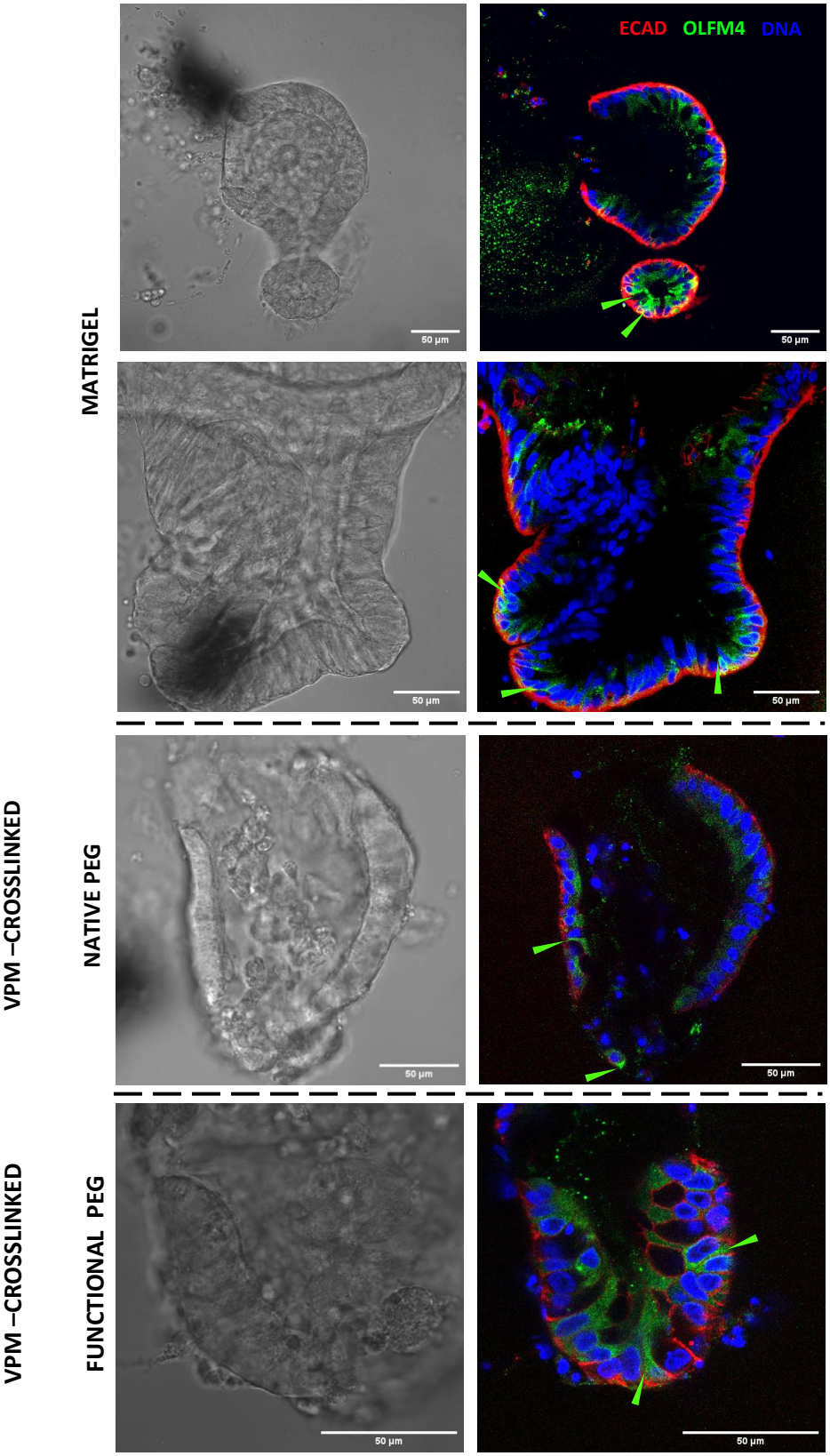


Figure 4.12 Identification of stem cells in degradable PEG hydrogels. Representative confocal images of organoids cultured for 24 hours in Matrigel (top), degradable native PEG (middle)

and degradable functional PEG (bottom). Brightfield images of overall organoid structure are presented on the left. LGR5+ cells are labelled with OLFM4 (green) and highlighted with green arrows. Scale bar 50 µm .

4.5 Discussion

The main goal of the experiments conducted herein, is the construction of a degradable PEG hydrogel possessing fewer network defects, resulting in a mechanically softer gel with improved gelation kinetics, that may serve as a basis for further optimisation towards HIO culture. Using our previously established metrics of organoid growth in Matrigel ascertained by overall changes to the cross-sectional area, and characteristic morphological traits that are commonly displayed by HIO cultured in Matrigel; we investigate the potential benefits of implementing true network degradability on HIO culture.

4.5.1 Assessment of hydrogel crosslinking chemistry

An initial step towards this was to assess the gel crosslinking chemistry via the consumption of thiol during the reaction to ensure the gelation reaction was proceeding as required. The hypothetical reaction process for the hydrogel gelation would see 50% of the thiol present from the VPM crosslinker consumed via reaction with the 4-arm PEG Mal, with 50% available for further crosslinking with PEG Mal to form the hydrogel. Through monitoring the presence of free thiols via Ellman's reagent (**Figure 4.3**) it was identified that of the 0.82 mM of total thiol present in the VPM crosslinker, 0.61mM remained unreacted, post-reaction with the PEG Mal. Although the reaction efficiency between thiol and maleimide is very high (Jansen et al., 2018), under the physiological conditions with the buffers used in this gelation it is not anticipated that 100% reaction efficiency would be achieved between the VPM peptide and PEG-Mal. In addition, the Ellman's assay although selected due to its ease and robustness, has been suggested to be interpreted with caution due to variance in sensitivity across a range of variables (Schmidt et al., 2023). This, amongst precision error associated with preparing low mass samples of PEG, may be a reason for the higher than anticipated quantity of thiol

amongst the PEG-SH and higher than expected quantity of thiol in the PEG Mal-VPM mid reaction product. When considering these observations, in combination with the low quantity of thiol detected in degradable hydrogel samples (0.1mM) the data here serves as a useful indication that the degradable peptide is reacting effectively with the PEG-Mal and becoming successfully incorporated into the PEG hydrogel network.

To further the aim of producing a degradable hydrogel platform with reduced network defects and favourable gelation kinetics it is necessary to assess the gelation efficiency data alongside the mechanical characterisation to make inferences towards the gel's suitability for HIO culture. In principle, the progression towards a stepwise gelation mechanism using the VPM as a crosslinker (**Figure 4.1**) reduces the prevalence by which the bis-cysteine VPM binds with and occupies two maleimide groups of the same 4-Arm PEG, enabling an improved reaction efficiency between the thiols present within the pre-reacted PEG Mal-VPM and 4-Arm PEG Mal. This, in theory provides all four PEG arms the freedom to crosslink into the hydrogel network, resulting in fewer network defects and more elastically active PEG-Peptide-PEG chains. These improvements not only manifest themselves in the more efficient gelation, identified using Ellman's reagent on fully reacted hydrogel samples, indicating this new, degradable crosslinking method, results in less free thiol present in the final gel products (0.3 mM in non-degradable gels and 0.1mM in VPM-crosslinked, degradable gels) (**Figure 4.3**), but in the mechanical properties of the gel probed via rheological characterisation.

4.5.2 Rheological characterisation & network properties of degradable PEG hydrogels

A key trait that defines a hydrogels suitability towards HIO culture applications is the rate of gelation. This must be carefully considered as it provides the limiting factor for the duration by which the gel can be successfully handled and manipulated for organoid encapsulation. For non-degradable PEG hydrogels formed between 4-Arm PEG-SH and PEG-Mal explored in chapter 3, the rate of gelation was extremely high with rheological characterisation not being successful in capturing the initial gelation point (**Figure 4.4B)**. In practice, this provided issues when encapsulating organoids, with gels being required to be prepared *in situ* on culture plastic, with minimal mixing permitted, thus inevitably contributing to heterogenous networks with defects present as described in Metters and Hubbell (2004). In contrast, the VPM-crosslinked gels prepared via stepwise reaction as discussed in this chapter possess slower and more favourable gelation kinetics. In progressing to the hydrogel crosslinking detailed in this chapter, we identified a more manageable gelation profile which takes place in a 30-minute window, where the elastic nature of the gel (G') overtakes the viscous gel nature (G'') at around 2 minutes (**Figure 4.4A**). In practice these gels behave more like the gold standard of Matrigel. Although their gelation is not temperature driven (like Matrigel), the slower gelation speed allows organoids to be encapsulated, mixed sufficiently, and plated, with full gelation occurring within an incubator. This provides favourable conditions for the organoids before media is added. It can be inferred that this more manageable gelation is a result of the VPM functioning as a crosslinker, which has a low molecular weight in comparison to PEG (1690 Da vs 10,000 Da), as studies have shown that hydrogel gelation kinetics can be manipulated and tuned using different molecular weight crosslinkers (Jain et al., 2017).

One further benefit of the improved crosslinking strategy is the effect it has on mechanical properties such as bulk stiffness. Following the necessary steps to identify parameters for rheological characterisation, we observed that the VPM degradable hydrogels have a lower stiffness, as identified by the equilibrium storage modulus, than the non-degradable gels prepared in chapter 3 (**Figure 4.7**), which was softer and closer in stiffness to that of the gold standard – Matrigel (<1kPa). This can be attributed to the improved gelation kinetics and increase in the number of elastically active chains that in principle is provided by the use of a VPM crosslinker. As reported when fabricating hydrogels via Michael type addition between 4-Arm PEG polymers, due to fast reaction kinetics there is the potential for multiple network defects to occur which can result in heterogenous networks containing primary loops, fewer elastically active chains, and abnormal swelling - with a higher storage modulus present as a result. This is a process that is only intensified when modification through the addition of functional peptides further reduces the number of binding sites available for network crosslinking (Jansen et al., 2018; Kim et al., 2016). The adaptation of the crosslinking strategy minimises such defects, with a favourable outcome on the storage modulus and overall gel stiffness. Furthermore, this crosslinking strategy provides a tuneable environment by which the bulk mechanical properties can be altered by changing the final PEG concentrations within the gel (**Figure 4.6**). As anticipated, in accordance with the literature, reducing the final PEG concentrations within the VPM-crosslinked degradable gels, reduces the overall crosslinking density which in turn lowers the overall stiffness of the gel (Lee et al., 2014). This is a useful tool in the continued optimisation of a degradable PEG hydrogel by which the mechanical properties can be further tweaked, in addition to the addition of biological function.

This data, although useful in inferring about the network properties of the produced degradable hydrogels and indicating their suitability towards HIO culture, would require additional supporting data to assess the full extent of the gels network structure. For example, the presence and extent of elastically active chains can be assessed using the Flory-Rhener equation (Kim et al., 2016) and Primary loop formation can be quantified as described by Zhou and colleagues (2012). These metrics would be useful to validate and describe how the hydrogel crosslinking varies in response to altered hydrogel crosslinking strategies. This would be necessary to fully define and optimise the network structure of a PEG hydrogel to better support HIO culture to levels consistent with Matrigel.

4.5.3 Degradable PEG hydrogels for colonic HIO culture

The initial characterisation led to the fabrication of VPM-crosslinked, degradable hydrogels possessing favourable and manageable gelation kinetics with a softer and tuneable stiffness more comparable to Matrigel, in two separate variations – native and functionalised (**Figure 4.1 & 4.2**). The native (non-functional) environment allows for observations around the organoids behaviour to be more associated towards the physical and mechanical conditions of the gels environment, whilst the functionalised gels assess if the organoids performance can be improved upon within the same mechanical environment by the addition of biological functionality, in the form of adhesive peptides. The growth, survivability, and morphology of HIO can then be compared and contrasted, between native and functional VPM-crosslinked degradable gels and the gold standard Matrigel.

The use of degradable, non-functional PEG hydrogels which have been manipulated to have a similar (G’ < 1 kPa) mechanical stiffness to Matrigel, allows us to identify that mechanical optimisation alone, with the addition of degradation, is not sufficient to replicate the key

parameters outlined for healthy organoid development over a 7-day culture (**Figure 4.8-4.10**). It is well documented that HIO's are mechanically sensitive possessing the molecular mechanisms of mechano-sensing and transduction through the presence of mechanically activated ion channels and their downstream effectors (Cellesi et al., 2020), and it was previously demonstrated in section 3.4.3 that varying the mechanical stiffness in a non-functional, non-degradable PEG environment, has a direct influence on HIO development. Additionally, numerous published hydrogel platforms have utilised degradation to recreate the matrix degradation experienced *in vivo* that is replicated in Matrigel. (Gjorevski & Lutolf, 2017; Leach, 2011; Zustiak & Leach, 2010). To this end, it was hypothesised that continued optimisation towards a low matrix stiffness environment, comparable to that of the current gold standard Matrigel, with the addition of degradability may provide an improved platform for organoid growth than the high stiffness, non-degradable gels produced in chapter 3.

In contrast to non-degradable PEG hydrogels described in chapter 3, where organoids appeared 'confined', VPM-crosslinked gels offer the organoids the advantageous ability to grow and expand into their environment via the MMP mediated degradation of the hydrogel. Despite this, within the native condition of degradable hydrogels the organoids show an overall limited capacity for growth. In stark contrast to Matrigel, The HIO fragments embedded into these native degradable hydrogels displayed an overall fold increase in cross sectional area of only 1.26 over a 7-day period ($p < .001$)(**Figure 4.8B)**, and when assessing the health and viability of said organoids at late stages of the culture, it appears that changes to CSA are more likely to occur as a result of fragmentation and the characteristic traits of cell death than the growth of a viable organoid (**Figure 4.8A**). In addition, although the organoids in the short term (< 24 hours) display traits associated with viability such as a viable epithelium

and a defined lumen, there is a distinct decline in presence of healthy organoids between days 1 and 3 of the culture, which begins a continuing trend of decline towards culture day 7 where there are significantly fewer organoids displaying healthy organoid morphological traits than in Matrigel ($p < .001$). It therefore appears, in agreement with the published literature, that in addition to the mechanical optimisation, a degree of biological functionality, like that of Matrigel, must be replicated for successful HIO culture.

4.5.4 Degradable and functional PEG hydrogels for colonic HIO culture

As discussed in chapter 3, analysis of the literature reveals biochemical functionality can be imparted into synthetic PEG hydrogels with the inclusion of functional peptides (Broguiere et al., 2018; Gjorevski et al., 2016; Hernandez-Gordillo et al., 2020). Griffith and colleagues demonstrated the incorporation of bio-functional peptides including the integrin ligand RGD, along with peptides displaying high affinity for collagen (BM-binder) and fibronectin (FN-binder) capable of capturing secreted ECM by human intestinal organoids, into a PEG hydrogel network (Hernandez-Gordillo et al., 2020). This was seen to improve the growth and development of single cell-derived epithelial organoids. Assessing the non-degradable PEG hydrogel chemical composition used throughout chapter 3 suggests that the full benefits of including functional peptides into that PEG hydrogel system may not have been fully realised, due to the increase in network defects owing to disruptions in hydrogel crosslinking. In addition, we rationalised that the absence of true network degradability could also counteract any advantages gained from the inclusion of biochemical input. To this end, we deemed it necessary to reintroduce bifunctionality in the form of these same peptides (RGD, BM binder and FN binder) to VPM-crosslinked degradable PEG hydrogels, and monitor the effect, if any, on organoid growth and morphology.

We observed that organoids cultured in VPM-crosslinked functional PEG hydrogels grew significantly more than in their non-functional (native) degradable counterparts. This was observed between all days of culture where organoids displayed a 1.30 (day 3), 1.67 (day 5) and 1.98-fold (overall, day 7) increase in cross sectional area ($p=0.002$, $p<.001$ and $p<.001$ respectively) (**Figure 4.8B**). When assessing the retention of viable organoid structures, we also observed that the rate of organoid retention was increased in functional PEG hydrogels over non-functional, degradable gels ($p < .001$) (**Figure 4.9B**). These improvements were further enforced when observing the morphology of organoids cultured in functional, degradable PEG hydrogels (**Figure 4.8A** and **Figure 4.10**). There was seen to be a significantly higher percentage of organoids displaying clearly defined lumens in degradable, functional PEG hydrogels over non-functional, on days 3 and 5 of culture ($p=0.002$). In addition, organoids in functional PEG hydrogels continued to display intact epitheliums until day 7, unlike organoids within non-functional (native gels). Despite these improvements to organoid growth, survival (retention of viability) and morphology, organoids cultured in Matrigel continued to provide a significant advantage in all categories. This is including the characteristic development of new budding structures which was not observed from organoids cultured in functional degradable hydrogels.

The improvements to key metrics of successful organoid culture that are displayed in degradable, functional PEG hydrogels provides promise and further highlights the ability of synthetic PEG hydrogels to serve as a platform for increased optimisation towards colonic HIO culture without Matrigel. Due to the control over mechanical stiffness, potential scaling of the rate of degradation and control over the selection of functional peptides, hydrogels can be

constructed with a spectrum of mechanical and biochemical properties and further fine-tuned in order to replicate organoid culture in Matrigel.

4.5.5 Early-stage colonic HIO characterisation in degradable PEG hydrogels

Notwithstanding the benefits observed from the inclusion of bio-functionality in VPM-crosslinked degradable hydrogels, there is a clear, significant improvement in all key metrics displayed by organoids within Matrigel. The morphological advantage and ability for organoid fragments to successfully reform is often observed and established from early stages of organoid culture, from their first assessment after 24 hours in culture (day 1). To this end, we found it necessary to assess changes to organoid behaviour at a cellular level between Matrigel and degradable PEG organoid cultures. It became apparent that degradable PEG hydrogels, whether functional or native, do not support cell proliferation to the same capacity as Matrigel (**Figure 4.11**). Although we identify the presence of stem cells within our organoid populations cultured in VPM-crosslinked degradable PEG (**Figure 4.12**), the conditions within PEG hydrogels may not be fully conducive to intestinal stem cell development which is known to be a key factor in early organoid development (Sato et al., 2013). Several avenues can potentially be explored to identify the cause of these differences. As Matrigel contains a vast number of ECM-derived proteins and diffusible growth factors, it stands to reason that our more defined and less complex hydrogel alternatives do not provide the necessary combination of ECM and growth factors that support the early expansion of these organoids. In addition, the access that organoids have to necessary diffusible growth factors in the media, as well as synthetic peptides within the hydrogel is not characterised. There remains then, the potential that access to the necessary biochemical stimuli and adhesion moieties

are restricted due to poor diffusive characteristics, which are inherently linked to gel crosslinking density (Weber et al., 2009)

4.6 Conclusion

This chapter introduces a new method for effective hydrogel crosslinking that addresses limitations with the hydrogel platform optimised in chapter 3. We explore the introduction of a degradable crosslinker to ensure network degradability whilst reducing the occurrence of network imperfections caused by crosslinking defects. Following characterisation and assessment of these new, VPM-crosslinked degradable PEG hydrogels for HIO culture, we explored the biological functionalisation of these gels. Using metrics of organoid growth, morphology and survival, we identify that the inclusion of a biochemical stimulus in the presence of network degradability provides a significant improvement over non-functional, degradable gels. These improvements were however, still significantly reduced in contrast to organoids cultured in Matrigel. The work produced in this chapter continues to lay the foundation for further avenues of hydrogel optimisation to replicate effective organoid growth, morphology and survival within a fully synthetic, defined system.

5. General Discussion

The work produced in this thesis has focused on the fabrication of a synthetic PEG hydrogel with the goal of creating a 3D culture environment for colonic HIO that replicates the growth and morphology of organoids cultured in Matrigel. The use of Matrigel has proved invaluable in establishing adult stem cell-derived colonic organoid cultures, however, has largely confounded the translational potential of HIO research into clinical environments due to its ill-defined nature and animal origin. Within this thesis, we demonstrate that alterations to the concentration of biologically inert PEG hydrogels can support limited, yet varied HIO growth (**Chapter 3**) with the suggestion that this serves as a fundamental starting point for gel optimisation, by which the mechanical properties of the gel are first selected for optimal biochemical signalling. We also develop the groundwork for an optimisation pipeline that tailors PEG hydrogels specifically towards colonic HIO, using organoid culture in Matrigel as the gold standard (**Chapter 3**). Our findings, when combined with understanding of the literature, lead to the redevelopment of our PEG hydrogel system – designed to impart degradability, and counteract the limitations of previous gel iterations (**Chapter 4**). We showed the ability that incorporation of a synthetic ECM, in the form of functional peptides has to improve organoid growth, morphology and survival in degradable PEG hydrogels that possess similar mechanical profiles to published hydrogels (**Chapter 4**). Comparisons of the hydrogels used in chapters 3 and 4 are summarised in **Figure 5.1.** The novel use of PEG hydrogels for adult stem cell-derived human colonic organoid culture, and the findings presented throughout this thesis, lay the foundations to produce an optimised synthetic hydrogel for routine organoid culture and the elucidation of biochemical and mechanical requirements of organoid development.

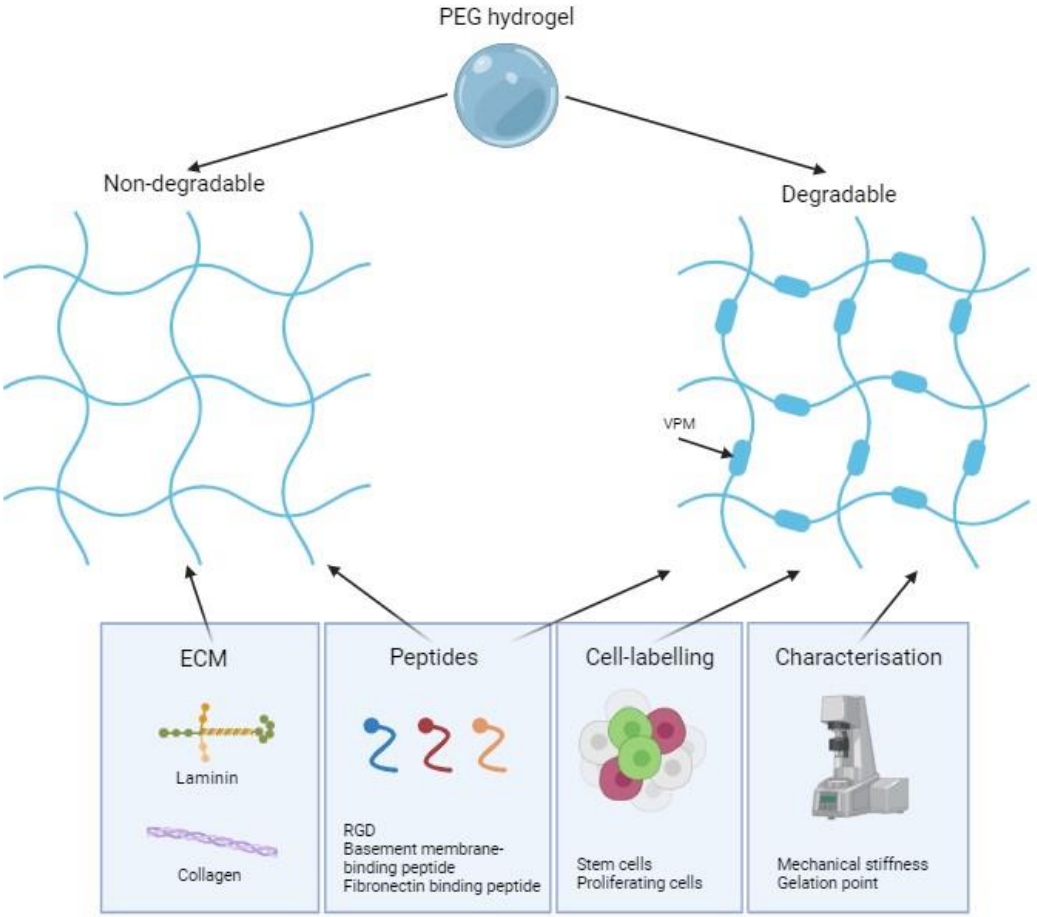


Figure 5.1 Summary of studies performed on PEG hydrogels. Non-degradable (chapter 3) and degradable (chapter 4) hydrogels were used for assessment of their performance as scaffolds for intestinal organoid growth. Degradable hydrogels contained VPM crosslinkers. In non-degradable hydrogels ECM components were assessed (laminin and Collagen-1). In degradable hydrogels, cell-labelling of stem cells and proliferative cells was performed along with characterisation of the hydrogels themselves determining their mechanical stiffness and gelation point. The peptides RGD, basement membrane-binding peptide and fibronectin binding peptide were used in both degradable and non-degradable hydrogels.

5.1 Comparison of non-degradable and degradable PEG hydrogels

It has become well understood that the ideal biomaterial for organoid culture in general should mimic the dynamic nature of the ECM in terms of its viscoelastic properties and susceptibility to degradation (Kozlowski et al., 2021). Whilst non-degradable hydrogels may serve as suitable platforms to assess the necessary biochemical and mechanical factors that contribute to healthy organoid culture, to replicate the continual routine culture of organoids observed in Matrigel a degradable environment is necessary. There have been several successful examples where making a PEG hydrogel susceptible to MMP-mediated degradation was necessary to support differentiation of intestinal organoids or was required for long-term prolonged survival of organoids (Cruz-Acuña et al., 2017b, 2018). We identified that the method for incorporating an MMP degradable peptide sequence in the PEG-SH and PEG-MAL hydrogels outlined in chapter 3 was not conducive to efficient crosslinking, nor did it impart global degradation throughout the entire hydrogel scaffold. We therefore addressed this limitation through an adaptation to the crosslinking method outlined in chapter 4. We found that due to a new stepwise protocol and longer gelation time, the handling and manipulation of the degradable gels were much more manageable for organoid cultures, instantly improving the ease of use and scalability potential of these hydrogels.

Although no direct comparison between organoid growth and morphology in non-degradable PEG hydrogels (**Chapter 3**) and VPM-crosslinked, degradable hydrogels (**Chapter 4**) were made under controlled experimental conditions, wider inferences to the performance of HIO in these gels can still be made. In our relatively stiff non-degradable hydrogels (**chapter 3**), improved organoid morphology and growth was observed first by the inclusion of human recombinant laminin and further seen with the use of functional peptides. These

improvements however were not always significant when compared to organoids cultured in PEG with the absence of biochemical stimulus from ECM proteins or synthetic peptides. In contrast to this, organoids cultured in our, relatively soft, VPM-crosslinked degradable PEG hydrogels (**Chapter 4**) displayed a significant improvement in long term growth, morphology, and survival from the inclusion of functional peptides, over non-functional, degradable hydrogels. These findings when taken together highlight the requirements for optimal bulk mechanical properties, biochemical input and degradation to be optimised in unison to replicate organoid culture in Matrigel. Furthermore, we highlight the suitability of a PEG hydrogel platform to be utilised to assess each of these requirements in colonic HIO culture.

5.2 Potential applications of PEG hydrogels

The hydrogels produced in this thesis although not sufficiently replicating the characteristic organoid growth and morphology displayed in Matrigel, have other potential applications that render them useful for the study of colonic HIO. Data produced in chapter 3 revealed that organoids possess mRNA transcripts for the mechanically sensitive ion channel PIEZO1 which was immunologically labelled and found to be present in colonic HIO culture. A potential application that was not explored throughout this thesis would be to utilise PEG hydrogels of varying stiffness to attempt to probe PIEZO mediated mechanotransduction within HIO cultures. Research showed the inhibition of PIEZO1 could override *in vivo* mechanical signals in adult stem cells in the central nervous system (Segel et al., 2019). The investigation of inhibition and activation of the PIEZO1 channel to HIO in the presence of a mechanically controlled PEG environment has the potential to generate interesting results.

In a wider scale the potential applications of organoid research are vast and have been increasing in recent years. The study of human tissue biology such as the mechanisms of tissue

renewal in the gut under healthy and diseased states, the study of disease mechanisms, patient-derived organoid biobanks and drug screening platforms and personalised, regenerative medicine techniques are constantly advancing through the generation of stable 3D organoid cultures (Rossi et al., 2018).Considering this, the replacement of Matrigel with a synthetic hydrogel with tuneable properties which can be optimised for specific organoid cultures are also vast. The studies produced in this thesis, although tailored towards human colonic organoid culture, serve as a potential blueprint for sequential phases of PEG hydrogel optimisation towards other specific organoid cultures (**Figure 5.2**).

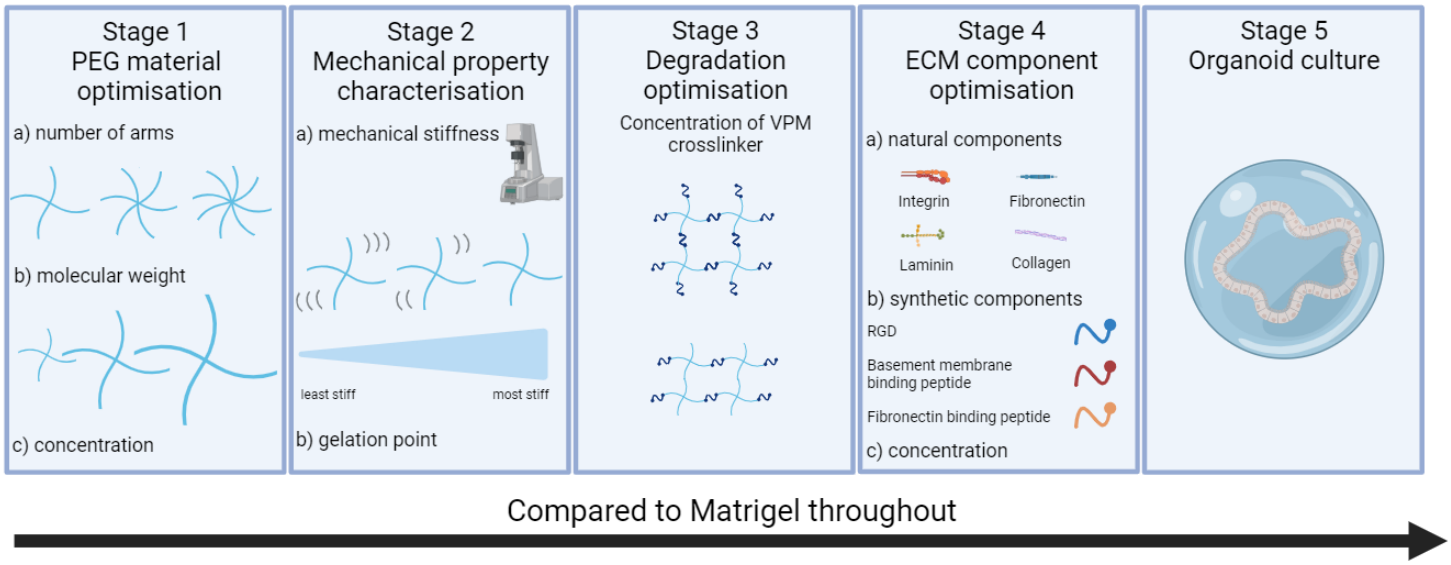


Figure 5.2 Optimisation of PEG hydrogels for organoid culture. Stage 1: PEG material optimisation. PEG materials were selected based on their properties, their number of arms and their molecular weights, and was further optimised through the concentrations used. Stage 2: Mechanical property characterisation. Rheology was performed on the PEG hydrogels to determine their mechanical stiffness and gelation point. Stage 3: Degradation optimisation. The concentration of the degradable crosslinker VPM was varied to optimise the degradation properties of the PEG hydrogel. Stage 4: ECM component optimisation. Natural and synthetic ECM components were selected, combined and their concentrations varied for optimisation. Stage 5: Organoid culture. Intestinal organoids were then cultured in the optimised PEG hydrogel. Throughout all stages comparisons to Matrigel were carried out.

5.3 Limitations of the study

One such limitation of the investigation of HIO culture within PEG hydrogels conducted throughout this thesis is the use of cross-sectional area as a metric of organoid growth. Whilst these measurements do reflect the total number of cells present and gives insight to overall growth of the organoid, they remain a two-dimensional output of 3D organoid culture. As organoid cross-sectional areas are only assessed in one plane of focus, there is the potential to miss out on nuances of overall growth or budding that occurs towards the Z-plane. High resolution 3D imaging of organoids are becoming more prominent (Dekkers et al., 2019) however as a simple, rapid and reliable means of measuring organoid growth to make inferences towards further optimisation requirements, CSA analysis is an effective tool.

Another limitation of the study comes from limited N numbers across certain studies, where N refers to the number of patient-derived organoid lines examined in the study. Although sufficient replicas of each experiment were available for statistical analysis ($n > 3$), in order to reliably make inferences into how changes to the hydrogels mechanical and chemical composition effect organoid performance, multiple organoid lines should be examined to see if changes are successfully replicated across all lines.

5.4 Future work

With the research conducted in this thesis providing groundwork for further optimisation of PEG hydrogels towards successful HIO culture there are several avenues of optimisation that due to time constraints were not carried out as part of this thesis. These include the characterisation and optimisation of porosity and swelling properties of PEG hydrogels. These are of interest due to the overall effect swelling (which is likely to be experienced by gels submerged in media) has on bulk mechanical properties such as gel stiffness, hydrogel

porosity will influence the availability of secreted growth factors and nutrients in the media to organoids.

To further this there is the potential for hybrid gels created from the inclusion of synthetic peptides and physical blend of recombinant ECM proteins into the hydrogel. As the use of human recombinant laminin showed early promise, the reintroduction of laminin isoforms into degradable and functional PEG hydrogels produced in chapter 3 would have been of great interest.

5.5 Concluding remarks

The work conducted in this thesis explores the development of a fully defined, synthetic and functional PEG hydrogel, with the aim of replicating characteristic growth and morphological development that is observed in 3D colonic organoid cultures in Matrigel. We identify that PEG hydrogels are a suitable candidate for continued optimisation towards HIO culture due to their ability to support organoid growth and viability to a limited capacity in a non-functionalised state. The inclusion of functional peptides was seen to improve organoid performance and was seen to be necessary for HIO in a degradable PEG environment. The replacement of Matrigel for human colonic organoid research is still a matter of great interest and the data produced in this thesis provides vital insights into the mechanical and biochemical requirements for colonic, stem cell derived, human intestinal organoid culture.

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Appendices

Appendix 1

Chapter 3 total organoid counts, given per condition by day.

Table 1. Organoid counts in native and PEG Matrigel with and without growth factors.

	Day 1	Day 3	Day 5	Day 7	Day 12
Native	190	190	203	203	186
Native and growth factors	164	174	166	163	172
PEG and Matrigel	21	22	26	28	33
PEG, Matrigel and growth factors	44	62	61	59	58

Table 2. Organoid counts in collagen and Matrigel conditions.

	Day 1	Day 3	Day 5	Day 7	Day 9
Native	49	60	69	64	73
PEG, 10% collagen	22	20	24	20	24
PEG, 20% collagen	68	63	57	70	69
PEG, 10% Matrigel	46	73	83	84	83

Table 3. Organoid counts in conditions of various laminins (L).

	Day 0	Day 3	Day 5	Day 7	Day 10
Control	127	99	107	108	98
L111	146	179	191	192	191
L121	123	116	124	119	113
L511	81	100	109	104	97

L521		96	89	74	102	89
L511+L521		101	111	116	111	119
All		76	64	82	93	82
PEG, Matrigel	10%	32	33	47	60	66

Table 4. Organoid counts with and without VPM.

	Day 1	Day 3	Day 5	Day 7
With VPM	262	256	309	312
Without VPM	254	302	309	305

Table 5. Organoid counts in conditions of PEG, RGD, binders and VPM.

	Day 1	Day 3	Day 5	Day 7
Matrigel	361	421	430	377
PEG, RGD and binders	204	202	215	223
PEG, RGD, binders and VPM	448	456	458	447
PEG and VPM	250	250	255	252

Appendix 2

Table 1. Total cell count and number of which were EDU positive in either Matrigel, VPM crosslinked native PEG or VPM crosslinked functional PEG.

	Total cell count	EDU+ cells
Matrigel	248	79
VPM Crosslinked Native PEG	170	21
VPM Crosslinked Functional PEG	142	13

Table 2. Organoid counts in Matrigel, LDTM native and LDTM functional gels.

	Day 1	Day 3	Day 5	Day 7
Matrigel	1232	1441	1445	1245
LDTM Native	1226	1289	1133	1077
LDTM Functional	930	993	1087	1056

Table 3. Viable organoid, lumen, intact epithelium and new budding structure counts in Matrigel, native and functional gels.

		Day 1	Day 3	Day 5	Day 7
Viable organoid structures	Matrigel	849	905	878	792
	Native	501	219	131	84
	Functional	490	361	316	277
Lumens	Matrigel	412	498	492	448
	Native	211	47	18	0
	Functional	139	175	115	87
Intact epithelium	Matrigel	615	672	680	676
	Native	272	78	42	0
	Functional	200	191	140	111
New budding structures	Matrigel	98	78	73	55
	Native	0	0	0	0
	Functional	0	0	0	0