Micro-engineering the biomechanical niche for brain organoids

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Contents

Abstract

Brain organoids represent a robust model system that offers various potential applications in drug screening and disease modeling. Experimental control over brain organoid cultures is in part limited by variability of the physical properties of the culture environment. Matrigel, a soluble basement membrane-rich extract is currently the gold standard material for organoid development. It supports organoid formation by providing both the structural scaffold and the source of signals influencing various biological functions, namely tissue polarity and cell migration. However, Matrigel suffers from considerable variability and poor mechanical properties prompting the search for more reproducible ECM-mimetics for brain organoid culture. To enhance the gel mechanics while keeping the source of adhesive signaling cues, we chose to add a mechanically tunable polymer Alginate to Matrigel. In this project, we demonstrated that adding Alginate to Matrigel enhances the microstructure and viscoelasticity of the resulting hybrid hydrogels. Our results suggest that Matrigel's high variability in composition is also depicted in its viscoelastic behavior. We have also shown that Alginate can have similar viscoelastic behavior to Matrigel with concentrations of 1% to 2% Alginate (w/v). Furthermore, our findings interestingly show that Matrigel 50%/Alginate 1% hybrid hydrogels are more viscoelastic than Matrigel 50% and Alginate 1% alone. This work highlights the potentials of Alginate as a simple-to-use and inexpensive polymer with the final goal of having more consistent brain organoid cultures.

Résumé

Les organoïdes cérébraux constituent un modèle de recherche robuste aux applications diverses telles que la modélisation de maladies et le criblage de médicaments potentiels. Le contrôle de l'expérimentateur sur les cultures d'organoïdes cérébraux est en partie limité par la variabilité des propriétés physiques de l'environnement de culture. Le Matrigel - un extrait soluble, riche en membrane basale- constitue le milieu de référence utilisé pour la culture d'organoïdes. Il fournit à la fois un échafaudage et une source de signaux influençant des fonctions biologiques telles que la polarité tissulaire et la migration cellulaire. Cependant, le Matrigel présente une grande variabilité et des propriétés mécaniques inadéquates, incitant à trouver de nouveaux milieux qui miment la matrice extra-cellulaire (MEC) de manière plus reproductible. Afin d'améliorer les propriétés mécaniques tout en conservant la source de signaux d'adhésion cellulaire, nous avons choisi d'ajouter un polymère aux propriétés mécaniques ajustables - l'Alginate - au Matrigel. Ici, nous démontrons que l'ajout d'Alginate au Matrigel améliore la microstructure et la viscoélasticité des hydrogels hybrides résultants. Nos résultats suggèrent que la forte variabilité de Matrigel dans la composition est également représentée dans son comportement viscoélastique. Nous avons également montré que l'Alginate peut avoir un comportement viscoélastique similaire au Matrigel avec des concentrations de 1% à 2% d'alginate (m / v). En outre, nos résultats montrent de manière intéressante que les hydrogels hybrides Matrigel 50% / Alginate 1% sont plus viscoélastiques que Matrigel 50% et Alginate 1% seuls. Ce travail met en évidence le potentiel de l'Alginate en tant que polymère simple d'utilisation et bon marché dans le but final d'obtenir des cultures organoïdes cérébrales plus cohérent.

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Contribution to original knowledge

In order to take a step in the advancement of the 3D hydrogels in which organoids can grow and develop differentiated structures, we have considered advantages and drawbacks of several proposed materials. Since Matrigel is currently the most commonly used hydrogel to support organoid growth, we were interested in keeping that and focused our attention on finding a material with complementary characteristics to Matrigel. Among our options, we decided to work with alginate [1]. Physically cross-linked hydrogels, such as calcium cross-linked alginate, are inherently viscoelastic due to the reversible nature of the cross-links which makes it favorable to be considered as a mimic of native extracellular matrix (ECM) [2]. Hence, we chose to work with alginate as it adds long term structural stability to the biologically permissive Matrigel matrices [1].

In this project, I aimed to capitalize on alginate's tunability to build a reproducible biomimetic support for developing brain organoids. To this end, I accomplished the following specific aims:

- Developing a workflow to characterize the viscoelastic properties of Matrigel and Alginate hydrogels having polyacrylamide as an elastic control.
- Generating hybrid Alginate/Matrigel hydrogels with varying polymer ratios to manipulate the viscoelastic properties of the support matrices in order to improve the structure of the brain organoids.

Contribution of Authors

All chapters were written by myself, Saba Aslani. The French abstract was translated by Ms. Jeanne Madranges. The thesis was reviewed and edited by Dr. Behrang Sharif, Dr. Timothy Kennedy and Dr. Thomas Durcan.

Introduction

The use of human induced pluripotent stem cells (iPSCs) to generate organoid model systems *in vitro* has been a pivotal breakthrough in the fields of developmental biology and regenerative medicine [3]. Patient-specific iPSC-derived organoids have vast potential clinical application. However, factors like high variability between and across batches, random tissue identity, and incomplete morphological differentiation still limit the utility of organoids. Brain organoids are 3D self-organizing tissues formed *in vitro* with various potential applications in drug screening and disease modeling. While inconsistent biochemical neural induction protocols are reported to be the main source of variability between brain organoids [4], physical properties of the culture environment have also been noted as a source of variation [5]. Indeed, it has been shown that other than bioreactor-related culture environment, the micro-niche of the organoids plays an important role in their development [2]. As an attempt to improve the biologic relevance of iPSC derived tissues, 3D organoids as *in vitro* models have recently emerged to better mimic the physiological human context. These systems present numerous potential advantages over conventional 2D models e.g. increased reproducibility, precise control over cultivation conditions, and incorporation of human cells [1].We take *in vivo* organogenesis as an ideal reproducible process. During development, every embryo receives the same collection of cell types organized into the same anatomical structure. The more accurate the in vitro model of virtually invariant process of organogenesis, the more reproducible the resulting organoids [6].

Mechanical interaction with the surrounding microenvironment is proven to regulate several phenomena such as tissue organization and cell proliferation. On this note, developing brain organoids such as the 3D models of the human brain lack a standard support structure during organ formation and reorganization resulting in inconsistent tissue morphology and

characteristics. These inconsistencies may result in an inaccurate assessment of developmental and physiological properties of the tissue, highlighting the need for more realistic environments and better tissue engineering to design more valid tissue characterization or disease mechanism studies, and pharmacological assays [1]. Another major barrier in the organoid field that has attracted the focus of researchers is insufficient oxygenation and nutrient transfer to the core of the growing organoids leading to necrosis. It has been reported that access for oxygen and small molecules to the center of the organoids is dramatically reduced in the absence of any form of vascularization. This lack of vascularization also prevents the delivery of certain patterning cues essential for progenitor differentiation. Developing brains passing early stages of organogenesis rely heavily on vascularization as the niche for progenitor cells are generally located in proximity of vessels. Therefore, the organoids are grown in spinning bioreactors to maximize oxygen and nutrient exchange through media stirring. Within bioreactors, it has been shown that brain organoids display a longevity of up to 1 year. However, it has been also reported that organoid growth becomes stationary after 5 months, with organoids shrinking in size in subsequent months due to neuronal loss and disappearance of progenitors [7].

Hydrogels have attracted attention due to their high biocompatibility and favorable gas and nutrient transportation. However, crucial features such as appropriate mechanical properties to best mimic developing human brain extracellular matrix (ECM) are still missing in current hydrogel-based organoid systems [1]. **Figure 1** depicts the developing human brain with different regions having differing mechanical properties [8]. Brain tissue has an elastic modulus of several hundreds of Pa to several tens of kPa depending on the brain region and also the measurement technique [9].

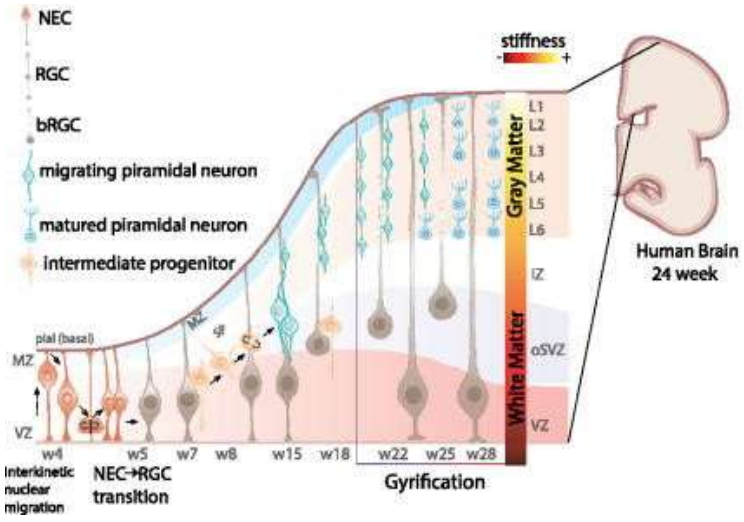

Figure 1 adapted from Oksdath et al.- Schematic of human brain development [8]. Representation of early brain cortical development. At week 22, the mechanical properties across different cortical regions change and the white matter becomes softer than the grey matter. NEC, neuroepithelial stem cell; RGC, radial glial cell; bRGC, basal radial glial cell; VZ, ventricular zone; MZ, marginal zone; SP, subplate; oSVZ, outer subventricular zone; IZ, intermediate zone; and L1-L6, neuronal layers 1 to 6.

The developing brain niche consists of a myriad of interacting components including the ECM. These components provide biophysical and biochemical inputs that regulate organoid formation and function [2]. The natural ECM is a network of proteins and polysaccharides that anchors cells within their specific microenvironment. The mechanical properties of ECM control the ability of cells to generate tension and therefor modulate cellular signaling pathways [2]. Although previous mechanobiological studies that have simply characterized native ECM as an elastic solid, this matrix is indeed inherently viscoelastic; meaning it shows time-dependent mechanical properties. Viscoelastic material exhibits stress-relaxation in response to constant stress. The polymer chains that make up the viscoelastic network rearrange in response to loads to dissipate the applied force. Thus, in order to better mimic the mechanical behavior of the native ECM, recent efforts have been directed toward designing hydrogels with tunable viscoelasticity [2]. In order to recapitulate the *in vivo* niche, embedding organoids in hydrogels is

an approach to control various aspects of organoid microenvironment [2]. Many brain organoid development protocols currently involve a step during which embryoid bodies are embedded in an ECM-derived hydrogel called Matrigel [10].

Matrigel is extracted from the basement membrane of Engelbreth–Holm–swarm tumor in mice and is composed of nearly 2000 unique proteins including major ECM components that include laminin, type IV collagen, and nidogen. Matrigel is the best-known natural mimic of the basement membrane that enhances the self-assembling capacity of pluripotent stem cells (PSCs). Matrigel offers advantages such as a built-in complex distribution of nutrients and protein gradients, fast gelling kinetics, and the availability of commercialized products with high quality control. These features help Matrigel far exceed other biomaterials, making it the most commonly used material for organoid cultures. On the other hand, Matrigel suffers from notable drawbacks for tissue engineering applications. First, the inherent compositional variability between batches limits control over specific micro-environmental parameters. Also, the cocktail of growth factors sequestered in Matrigel may interfere with the signal transduction required for organogenesis and could cause defective understanding of self-assembly mechanisms. Second, the fast gelling of Matrigel due to its temperature sensitivity makes precise control over gelation kinetics problematic and causes generation of unpredicted microstructure in the final network. Furthermore, the inability to tune the mechanical properties of Matrigel further limits mechanotransduction studies during development. Lastly, inherent compositional inconsistency of Matrigel gives rise to reproducibility issues within organoid cultures and limits its application in clinical research [10]. These limitations have prompted studies into fully defined synthetic matrices to support organoid cultures *in vitro* [3].

Recently, other hydrogel systems have been employed to facilitate 3D mechanobiology studies of encapsulated organoids [2]. Achieving an *in vitro* model for drug screening and personalized medicine requires a stable 3D structure over a prolonged period of time. Alginate-based 3D hydrogels which can be precisely tuned via ionic cross-linking are promising candidates for accomplishing this structural design [1]. A recent study has presented evidence that unmodified alginate can be used as a simple hydrogel system that supports human intestinal organoid growth and development *in vitro* and transplantation *in vivo* into immunocompromised mice [3].

Alginate is a biocompatible (FDA-approved), natural, linear, binary copolymer composed of covalently linked D-mannuronic acid (M) and L-guluronic acid (G) monosaccharide units. M and G produce heterogeneous alternating (MG) and homogeneous (MM or GG) sequences in the primary polymer structure with varying distribution ratios. In aqueous solutions, alginate exists as a negatively charged polyanion that forms a hydrogel through the physical association of polysaccharide chains by ionic crosslinking of G residues of divalent cations like Ca^{2+}. The density of the alginate fiber network within a hydrogel and its gel stiffness are regulated by the number of linked gelling sites; as a result, these properties are elevated with an increasing level of crosslinking ion saturation (e.g. Ca^{2+}) in the alginate and reduced with an increasing number of free G blocks [11]. Since unmodified alginate does not possess cell attachment ligands and its hydrophilic nature inhibits protein adsorption, it only provides mechanical support for organoids in a 3D environment [2].

Hybrid hydrogels with different concentrations of alginate and Matrigel have been also used as 3D materials for modeling breast cancer, where morphological and invasiveness characteristics observed in metastasis were successfully reproduced in a particular type of hydrogel (i.e. 50%

Alginate, 50% Matrigel). This approach is particularly effective as it combines the advantages of two bulk material (Alginate and Matrigel) for the final aim to achieve a biologically permissive and yet structurally performing 3D matrix [1].

In terms of stiffness, brain is one of the softest organs in the body. However, the mechanics of the brain such as its stiffness and viscoelasticity are often neglected when it comes to *in vitro* culture of brain cells. The consequence of this negligence would be different morphology and behavior of cells cultured *in vitro* compared to the brain *in vivo*. Cells in the brain such as neurons and microglia are mechanosensitive and have differing preferences in mechanical properties of their microenvironment [9]. The potential significance of microenvironment mechanics on brain organoid development has prompted us to first characterize Matrigel as the gold standard material used to embed brain organoids and then to develop Alginate/Matrigel hydrogels with varying mechanical properties to study the effect of the embedding hydrogel mechanical properties on the growing brain organoid.

As brain organoid research is growing rapidly, optimization of the organoid development protocol is becoming essential. Here, we would like to test the intriguing idea of using Alginate/Matrigel hybrid hydrogels as mechanically tunable support structures for developing brain organoids. Structural viscoelasticity and stability, as well as the microstructure of Alginate and Matrigel were investigated.

Literature Review

Induced Pluripotent Stem Cells (iPSCs)

The advent of induced pluripotent stem cells (iPSC) has introduced thrilling prospects in the field of translational neuroscience. Tremendous plasticity and salient *in vitro* replicative capacity renders iPSCs the ideal platform for clinical research. In particular, patient-derived iPSCs solve the issue of accessibility to neurons affected by a specific diseases and provide an unprecedented opportunity to study the very phenotype of these disorders in a dish [7]. As the genetic background of donors is retained in their iPSCs, these cells offer an enticing alternative for disease modeling and drug screening, especially considering the limitations of traditional models [12].

There are specific methylation and acetylation patterns that modulate gene expression and influence development in differentiated cells. Most gene promoters in differentiated cells are hypermethylated which is a sign of silenced chromatin, while the opposite occurs in stem cells having mostly hypomethylated promoters. The distinctive patterns of methylation, acetylation, and ubiquitination of genes and histones is known as epigenetics which involves transcription factors playing major roles to obtain pluripotency [13].

In ground breaking studies culminating in the Nobel prize for Dr. Shinya Yamanaka, it was demonstrated that mouse and human somatic cells including dermal fibroblasts and peripheral blood mononuclear cells (PBMC) can be reprogrammed into pluripotent cells and display an embryonic-like phenotype by ectopic overexpression of the transcription factors OCT3/4, KLF4, SOX2, L-MYC, and LIN28, known to be expressed in pluripotent embryonic stem cells (ESCs). Epigenetic reprogramming of cells via transfection with vectors expressing the transcription

factors enables the cells to endogenously express OCT4 and NANOG – determinant factors of pluripotency, self-renewal and proliferative capacity – through a series of stochastic events. In synergy, the ectopic overexpression of these genes results in DNA demethylation and chromatin changes through triggered epigenetic modifications which eventually gives rise to pluripotency in transfected cells [7].

In order to deliver the reprogramming factors to cells, numerous methods have been investigated that fall into non-viral and viral, and integrating and non-integrating categories of vectors. For instance, retroviruses, lentiviruses and more recently Sendai non-integrating virus are among viral vectors used for transfection of reprogramming cells. Alternatively, non-viral approaches include mRNA or protein delivery or transient expression achieved with episomal plasmids [7].

On one hand, using viral vectors has advantages such as high integration efficiency and their applicability in even non-dividing cells. On the other hand, there are also disadvantages regarding the biosafety protocols for handling viral vectors and the notoriety of using viral vectors in clinical trials [13].

Due to the known ethical issues regarding the integration of foreign genes into the human genome, new strategies such as the use of circular plasmids and mRNA was implemented with iPSC technology to induce the expression of pluripotency genes from unintegrated vectors. These molecules are easily handled and can enter cells through liposomes or cell pores that are generated by electroporation [13].

The presence of specific markers such as cell surface proteoglycans (TRA-1-60 and TRA-1-81) and glycosphingolipids (SSEA-3 and SSEA-4) and the expression of transcription factors (OCT4 and SOX2) demonstrate pluripotency in stem cells. iPSCs share the same degree of plasticity

with ESCs for differentiating into almost any tissue type of the three germ layers (endoderm, mesoderm and ectoderm) when stimulated by the appropriate cocktail of signaling cues and growth factors. Examples of cells derived from the germ layers include nervous and epidermal tissue from the ectoderm, hematopoietic and muscle cells from the mesoderm, and pancreatic cells from the endoderm [7].

The neuroectoderm formation in vivo has been reproduced with great fidelity by the differentiation of ESCs in vitro to embryoid bodies (EBs). EBs can be cultured in serum-free conditions to selectively promote the growth of neural cells, which self-organized to form progenitor zones in structures reminiscent of neural tubes, called the rosettes [7]. **Figure 2** shows a schematic of rosettes in organoids and their cellular composition [14].

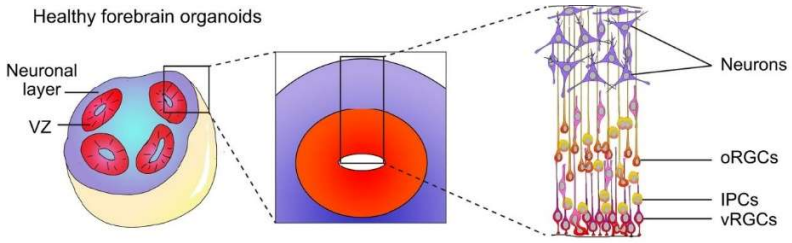

Figure 2 adapted from Qian et al. – Schematics of neural rosettes (red) in healthy forebrain organoids [14]. VZ, ventricular zone; oRGC, outer radial glial cell; vRGC, ventricular radial glial cell; IPC, intermediate progenitor cell.

Brain organoids; moving toward 3D culture protocols

Neural rosettes exhibit high levels of self-organization and recapitulate key features of early developmental stages in the brain including neurogenesis in a timely manner. However, these neurons structures are not capable of establishing specific layered structures that are observed with the brain, which highlights the need for more nuanced 3D culture systems to capture the structural features of the developing brain [15].

The potential of EBs to form primitive neuroectoderm in suspension was a determinative discovery. Implementing more reproducible serum-free methods, known as SFEB (serum-free, floating culture of EB-like aggregates) led to enhanced neural differentiation into telencephalic progenitors. Cortical tissue formation was further improved by adding signaling cues, such as the ROCK inhibitor to elicit faster reaggregation time of EBs [15].

The surface of floating EBs becomes the starting platform for a continuous polarized neuroepithelial sheet within the first week of culture which eventually converts into multiple small neural rosettes surrounding and growing around apical lumens. Following plating these aggregated, SFEB rosettes develop mimicking neural tube-like progenitor zones *in vivo* with temporal *neurogenesis.* SFEB aggregates made up of human ESCs were not completely flattened upon plating and formed a "dome-like" structure. Contrary to previous results using mouse-derived and human 2D rosette protocols, these rosettes developed continuous apical lumens that were much greater in size, which could possibly be an intriguing reflection of the widely expanded human cortex compared to mice. However, this spatiotemporally relevant model of early neurogenesis still requires improvements in terms of recapitulating layered cortical architecture [15].

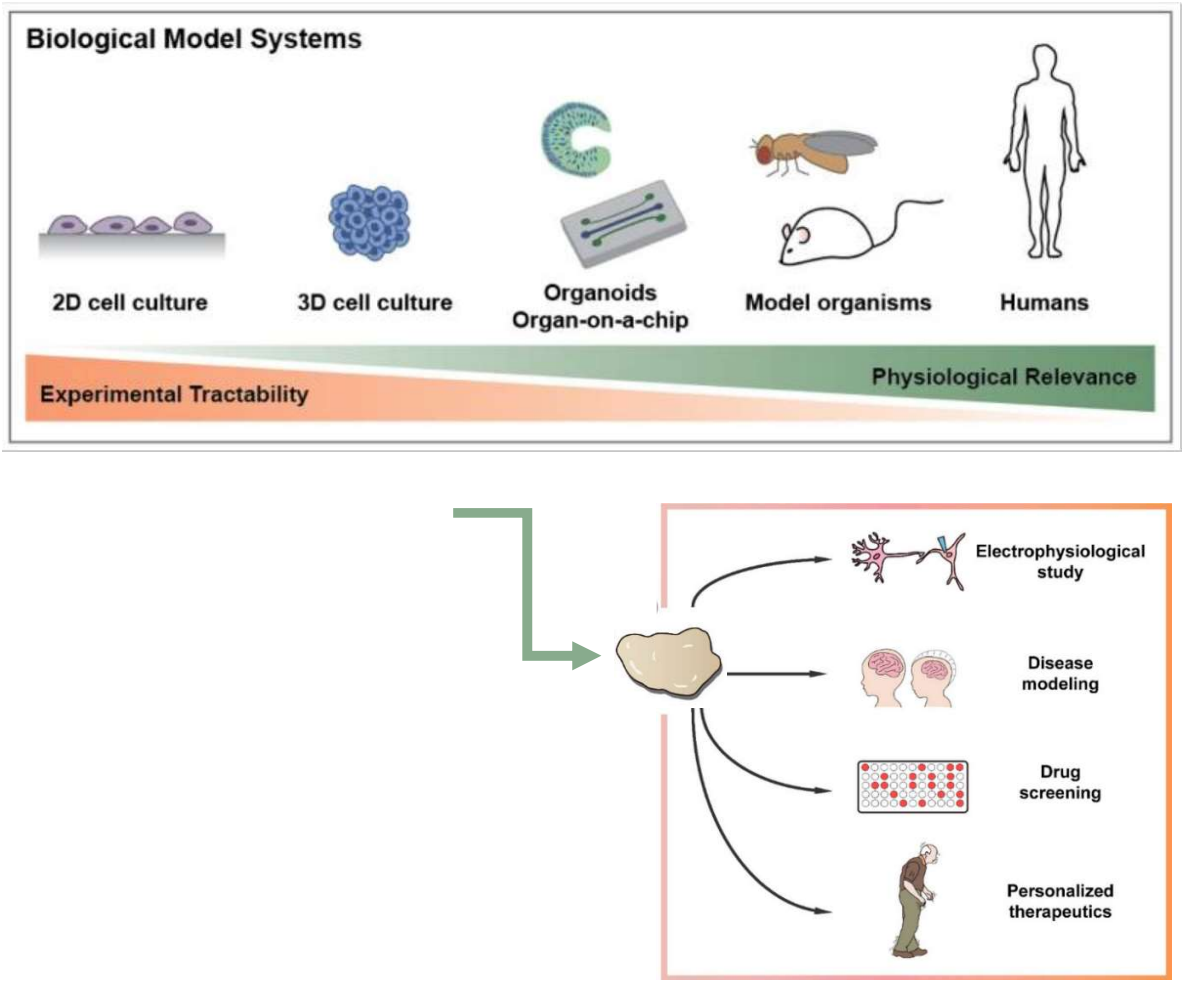

Figure 3 adapted from Jackson et al. and Sun et al- Model systems for studying human biology ranging from 2D cell cultures with high experimental tractability to model organisms with high physiological relevance. Human brain organoids lie in the middle of the spectrum with numerous clinical applications such as disease modeling and drug screening. [16] [17]

Formation of the first 3D brain organoid

ECM hydrogel such as Matrigel played an essential role in the progression of organoid field. It has been revealed that adding Matrigel at different steps of 3D brain organoid protocols contributed to rapid formation of polarized neural tube-like buds from neuroepithelial tissue. During cortex development in vivo, neurons are organized radially into a dense band called the cortical plate. While Matrigel-embedded organoids are capable of generating basally migrating cortical neurons organizing primitive layers, adding dissolved Matrigel to developing organoids at neurogenic stages has proven to be pivotal for cortical plate generation [15].

Self-Patterned Organoids

Neural identity is achieved by default when the external signaling cues are absent. Lancaster et al applied this concept in designing a simple culture condition for organoids in the absence of signaling molecules [18]. By not putting boundaries on the developmental landscape through guiding towards a specific cell fate, organoids self-organize and self-pattern on their own into broad regional identities within the same organoid [15].

Patterning Organoids with Small Molecules

Although it is remarkable to observe a wide range of identities among the brain regions generated in self-patterned organoids, developing specific brain areas of interest from forebrain to midbrain to hindbrain in a reproducible and efficient way is often desirable. Most of the protocols published to date apply distinct media compositions to assist in directing organoid developmental fate and minimize heterogeneity through the use of defined patterning factors [15].

Brain Organoid Applications

The emergence of brain organoid technology has triggered an exponential surge of research. Brain organoids introduce an amenable platform not only for neurodevelopment and neurological disorders, but also for novel neurotherapeutic discovery. It is noteworthy that brain organoid technology as new *in vitro* disease modeling tools is introducing a new chapter for potential stem cell applications in the clinic. Particularly, organoids are key candidates for drug discovery and testing due to their adaptability to genome editing techniques and gene therapy approaches [7].

The effect of prenatal exposure to alcohol and nicotine as well as illegal drugs such as cocaine on neurogenesis in brain organoids has been investigated. However, brain organoid application in studying the outcomes of drug exposure on development would not be limited to substances of abuse but could also be used in the context of neurodevelopmental toxicity; organoid systems can be used to assess toxicological profiles of compounds such as valproic acid or environmental chemicals on teratogenicity or neurotoxicity [7].

Organoids are powerful platforms for studying evolutionary developmental biology and comparing neurogenesis between species *in vitro*. Advancements in gene editing technologies have made the generation of Neanderthal brain organoids possible. It has been demonstrated that the similarities of these organoids to organoids derived from autistic patients may be linked to socialization capabilities [7].

Furthermore, the generation of region-specific organoids and their fusion have opened new windows for studies on cell-migration, cross-talk and circuit formation [7].

Studying effects of microgravity on neural development in brain organoids is an interesting example of how the application of organoid systems is substantially versatile. It has been shown that microgravitational changes affects axis formation patterning genes [7].

In terms of neurodevelopment, it was initially suggested that fully matured brain organoids could only correspond to the early embryonic cerebral development observed at 8–10 weeks gestation. Results of more recent studies using single cell sequencing technique, however, suggests the capability of brain organoids to replicate late-mid fetal periods of a 19–24 weeks gestational brain [7].

Caveats in current brain organoid model systems

Although brain organoids are the closest possible models for studying intercellular interactions during organogenesis, there are also some limitations associated with these culture systems. Batch variability for example, is one of the greatest limitations. In the absence of developmental axes *in vitro*, spontaneous events with a high degree of stochasticity run cell differentiation within organoids which results in regional identities that are different between organoids in terms of distribution, composition and density of cells. The high degree of heterogeneity and complexity observed in brain organoids causes morphological inconsistencies between and across organoid batches that raise concerns regarding inherent reproducibility, accuracy and scalability of organoid platforms [7].

In terms of disease modelling, drug screening or neurodevelopmental studies, organoid heterogeneity may severely affect the consistency of the phenotypes associated with disease/healthy or treated/non-treated states. In fact, organoid variability could potentially mask the differences rising from phenotypes linked to certain disorders or therapeutics [7].

Bioreactor-based effects could partly account for organoid variability and enhanced reproducibility could be achieved by controlling organoid microenvironment more closely. It has been also observed that the application of micro-filamentous scaffolds used to enhance neuroectoderm formation also contributes to generation of more homogeneous organoids [7].

Interestingly, it has been shown that using small molecules as patterning cues to produce region specific organoids has led to more consistent organoids with reduced batch variability. Meanwhile, it is important to consider that *in vitro* replication of developmental axes through generation of morphogen gradients in a spatiotemporal manner is challenging. Although morphogen containing microbeads have been suggested as a potential option, applying excessive amounts of signaling cues runs the risk of flattening the developmental landscape [7, 15].

The tradeoff between heterogeneity versus uniformity of the generated organoids requires more accurate consideration. While reproducible regional identities are achievable through the addition of signaling molecules to organoid culture, several of these patterning signals contribute to directing cell fates beyond simple patterning that does not fully reflect in vivo organogenesis [15].

Adding external signaling cues in excessive amounts may compromise the intrinsic developmental program of organoids. Also, this could potentially cover up the critical features of development that might be relevant in the context of diseases [15].

Conversely, organoids with increased consistency between and across batches in terms of reproducibility, size, growth and neural cell composition and maturity in the absence of some inductive and growth factors have been recently obtained through an optimized protocol; eminent neuronal zone and presence of general neuronal and astrocytic markers along with

significant upregulation of genes involved in synaptic function have been observed in these brain organoids [7].

Variability in EB preparation steps is also likely to play a part in organoid heterogeneity and if controlled, spatial disorganization and unsynchronized differentiation of EB aggregates could in turn be reduced leading to more reproducible organoids. Implementing centrifugal forced-aggregation and silicon micro-textured surfaces have resulted in enhanced size and symmetry and eventually differentiation in EBs through controlling aggregate size and uniformity by cellular confinement. Microfabrication technologies where organoids are cultured on a micro-pillar arrays are also among potential bioengineering solutions to enhance consistency in cortical organization [7].

As brain organoids are formed from uniform neural ectoderm, they lack some cells of the central nervous system (CNS) with non-ectodermal lineage including endothelial cells composing the cerebral vasculature, the blood–brain barrier (BBB), and microglia. These cells help neural cells including astrocyte and cortical neurons mature and differentiate via extrinsic signaling and contribute to central nervous system development [7].

The absence of vascularization – explained in the introduction – and BBB in brain organoids are the main issues limiting the spectrum of application of this platform. Additional structural complexities such as introducing iPSC-derived endothelial cells to organoids could possibly help overcome these limitations and improve the viability of organoid model systems [7].

Basement Membrane

Basement membranes are specialized, sheet-like extracellular matrices (ECMs) that are found adjacent to cells including nerve cells. A diverse number of structures and many complex mosaic macromolecules together form the ECM. Tissues get their tensile strength and elasticity from their ECMs. Furthermore, ECMs account for the maintenance of the tissue bulk and act as sheets separating planes of cells and filtering molecules. Importantly, ECMs sequester growth factors required for cellular processes and in some cases are crucial for cell growth and differentiation [19]. A schematic of unique brain ECM is exhibited in **Figure 4**. Compared to the fibrous protein networks observed in peripheral soft tissues, such as collagen type I, brain ECM is mainly composed of proteoglycans, hyaluronic acid and glycoproteins that results in the softness (low elastic modulus) of the brain [9, 20].

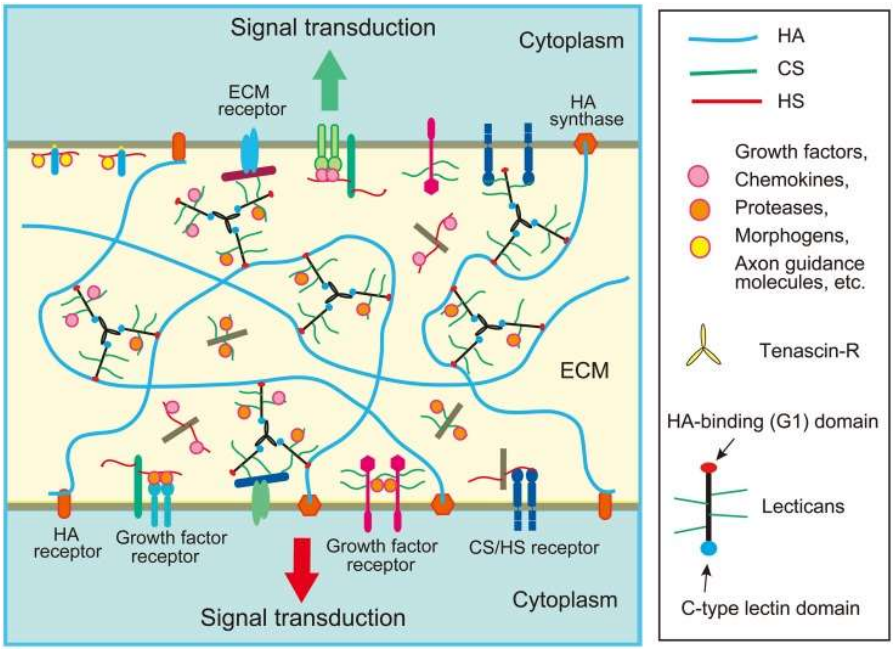

Figure 4 adapted from Maeda et al. - Schematic structure of the extracellular matrix (ECM) in the brain [20]. Basement membrane is composed of a thin layer of ECM and the ECM of the brain is mainly composed of chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans, hyaluronic acid (HA), and glycoproteins.

Composition of basement membranes

Basement membranes are composed of glycoproteins and proteoglycans [21]. Ubiquitous ECM components include laminins, type IV collagens, entactin (also known as nidogen) heparan sulphate proteoglycans (perlecan) and osteonectin [19, 21]

Type IV collagens belong to the network-forming collagens and represent the most abundant collagens of basement membranes [21].Type IV collagen is a complex trimeric molecule composed of at least five genetically distinct polypeptide chains. There are several interruptions in type IV collagen making it more flexible than typical collagen structures and giving this molecule the capability of forming a network [21]. The importance of the basement membrane minor components is exemplified by studies on mutations in tissue specific chains of type IV collagen [19].

Laminins are the very first ECM molecules in the mammalian embryo [22]. Laminin belongs to a heterotrimer family with each trimer being composed of three different chains [21]. Different types of each chain combine together and form up to fifteen laminin isoforms with the isoform extracted from the EHS tumor being the most studied one [19, 21].

Nidogens/entactins are small glycoproteins [21]. Entactin acts as a link molecule between type IV collagen and laminin and this linking function has been shown to play an important role in the deposition of laminin and type IV collagen into basement membranes [19].

Members of the heparan sulphate carrying proteoglycans are associated with either cells or basement membranes with the latter belonging to the most prominent proteoglycans of basement membranes. Perlecan and agrin are among the well-characterized heparan sulphate proteoglycans of basement membranes [21]. Perlecan is a heparan sulfate proteoglycan and one of the most

interesting components of the basement membrane. Perlecan shares an array of protein motifs with laminin through which it interacts with fibronectin. Thus, the molecular interactions of the proteoglycan may be dominated by its complex protein core rather than by its highly charged heparan sulfate side chains [19].

In addition to the abovementioned intermolecular interactions among basement membrane components, many other interactions have been demonstrated. Instances include interactions of heparan sulfate proteoglycan with itself, type IV collagen and laminin, interactions of entactin with itself and with fibronectin and interaction between type IV collagen and osteonectin [19].

Characterization of further components possibly leads to the discovery of more possible interactions. These interactions may contribute to the heterogeneity of basement membranes in terms of structure and function [19].

Basement membrane formation *in vivo*

It was initially thought that basement membranes were formed spontaneously by the self-assembly of their components. Later it became clear that cellular receptors are required to stabilize the basement membranes in specific locations in *vivo* [21].

Structure of basement membranes

Understanding the molecular architecture of basement membranes was made possible by studies on the basement membranes' self-assembly in vitro and their molecular organization and biochemical composition *in situ*. However, despite all the data available on protein–protein interactions *in vitro* and on their ultrastructural localization *in situ*, the supramolecular organization of basement membranes *in situ* still remains to be fully explained [21]. Studies suggest that under physiological conditions, basement membrane components interact in rather constant proportions and form supramolecular complexes in a gel format, which may be an intermediate state in the formation of the matrix. It is worth noting that each of these components appears to be essential for matrix reconstruction [23].

The workhorse system for studying basement membranes has been the Engelbreth-Holm-Swarm (EHS) sarcoma [19]. Basement membranes are generally composed of two major frameworks, the covalently stabilized type IV collagen network and the more flexible mesh of laminin molecules. Interestingly, when laminin is extracted from the basement membrane-like matrix deposited by the EHS sarcoma, a filamentous type IV collagen network is exposed, whereas the enzymatic removal of type IV collagen reveals a laminin mesh. The type IV collagen and laminin networks, can thus exist independently [19]. However, the two frameworks are thought to be linked together via two members of the nidogen family to form an integrated network.

Additionally, perlecan might contribute to the stabilization of this uniform network due to its capability of interacting with laminin, type IV collagen and nidogen molecules [21].

Function of basement membranes

Unique properties of basement membranes are their ability to influence diverse functions of the surrounding cells that include growth, migration or differentiation. For this reason, basement membranes are vital structures modulating several cell biological processes which are necessary for development and activity of most organs [21].

Basement membrane development is a time-sensitive process. *De novo* formation of basement membrane under certain circumstances such as in CNS development may become problematic which further stresses the critical role of basement membrane in regulating cell behavior [21].

Self-assembly of basement membrane components *in vitro*

It was previously thought that the simple presence of basement membrane components in the extracellular space was sufficient for its spontaneous formation. This idea was proposed based on the discovery of self-assembly capability of laminin and type IV collagen molecules under physiological pH and temperature *in vitro*. Also, based on electron microscopy results, *in vitro* co-incubation of laminin, type IV collagen and heparan sulphate proteoglycan molecules yields basement membrane-like structures [21].

Basement membranes consist of a three-dimensional network of 3-4 nm cords, visible on an electron microscope. Similar basement membrane-like structures form when a mixture of the major basement membrane components is incubated at physiological temperature and calcium ion concentration. This observation indicated that homophilic and heterophilic interactions

among the constituent molecules is sufficient for the basement membranes to form meaning that the information required for assembly is inherent in the structures of the components [19].

Since spontaneous formation of the basement membranes can happen without any catalytic activity of external factors *in vitro* it was hypothesized that the same would happen *in vivo* [21].

Basement membrane components in the extracellular space – Is their presence the only pre-requisite for basement membrane formation *in vivo*?

If the mere presence of basement membrane components was enough for polymer networks to assemble *in vivo*, the only regions where these components could be found were the basement membrane zone. However, a considerable body of research has shown the presence of basement membrane components in areas devoid of an actual basement membrane. This proves that, *in vivo*, basement membrane components may co-exist in the interstitial space and not only in basement membrane zones, where they then assemble into basement membranes. Collectively, the simple presence of basement membrane components is certainly not sufficient for basement membranes to form *in vivo* and additional factors are required to drive the interaction of components in a spatiotemporal manner [21].

While studies support the idea of cell receptors mediating basement membrane formation by binding to its components, the exact mechanism of basement membrane formation and stability is not fully clear yet. Potentially, cell receptors could regulate the production of laminin and possibly other components. Also, by binding and organizing basement membrane components at the cell surface, cell receptors might enhance the stability of the basement membranes [21].

In other words, by altering both the amount and structure of secreted basement membrane components, cells contribute to its assembly process. Upon ECM assembly, further alterations

may be introduced to stabilize the interactions. The interaction of one basement membrane component with cells may adjust the deposition of other components in the basement membrane [19].

Cell receptor facilitated basement membrane formation *in vivo* vs. spontaneous assembly of components *in vitro*

Laminin and type IV collagen self-assembly processes *in vitro* are concentration dependent, meaning that a critical concentration of the components is necessary for the self-assembly process to be triggered. For instance, compared with pure laminin environment, the presence of lipid bilayers reduces the critical concentration required for laminin self-assembly about 10 times [21].

Protein binding to lipid bilayers, although weak and non-specific, enhances laminin concentration at their surfaces. This indicates that basement membrane formation could be greatly facilitated by mechanisms that help concentrating basement membrane components locally. On the other hand, specific binding of cell surface receptors to basement membrane components and their catalytic function in turn renders the assembly of components more efficient. Additionally, other micro-environmental factors such as acidic pH and negatively charged groups – present on cell surfaces- further facilitate basement membrane assembly [21]. Previous studies have revealed that acidification of bulk pH can trigger the polymerization of ECM components where laminin concentration is low. This finding suggests the probability of acidic polymerization where the local pH is circumstantially reduced by neighboring negatively charged glycoproteins, glycolipids and proteoglycans [24].

The EHS tumor and history of Matrigel

In an attempt to study the role of ECM components in development and disease, an ECM-rich mouse tumor, originally identified as a poorly differentiated chondrosarcoma, was being characterized. Complementary studies such as ultrastructural and amino acid analyses suggested that the tumor matrix was distinct from cartilage and instead resembled basement membrane [25].

To acknowledge J. Engelbreth-Holm and Richard Swarm who discovered and characterized the tumor respectively, it was called the EHS tumor. In normal tissues, basement membranes are relatively insoluble structures present in insignificant amounts. Basement membranes are composed of a unique collagen, type IV collagen, regularly arrayed sulfated macromolecules, along with a high content of glycosylated molecules. Due to its rapid growth in mice and relatively benign nature, the EHS tumor was established as a source of basement membrane components. This facilitated access to minute basement membrane components for characterization which was previously hindered by the limited amounts available in normal tissues [25].

In a study on the cell interaction activity of EHS tumor extracts, the tumor tissue was washed free of cell- and serum-derived proteins with 20% NaCl and the residue, largely matrix, was extracted with 2 M urea to break up protein–protein interactions. After centrifugation, the viscous supernatant was dialyzed versus PBS in the cold. The resulting solution, when warmed to 37°C, formed a strong clear gel. This material was later named Matrigel by John Hassell [25].

Unexpectedly, successful outcomes in the use of Matrigel to support cell growth at the NIH prompted researchers to use Matrigel for their work. The increasing interest in Matrigel

applications resulted in the commercialization of its distribution via licensing through NIH Tech Transfer.

Furthermore, to tailor the material to specific research applications, some variants of standard Matrigel such as growth factor depleted and collagen IV-rich were made [25].

Polymers

Matrigel

As the self-assembling capacity of the cells drives organoid formation, creating a mimicry niche microenvironment is necessary. Although a variety of materials have been used for spheroid cultures, the primary choice for organoid culture are animal derived-ECM matrices such as Matrigel [10].

Relying on Matrigel hinders more precise studies of organoid– microenvironment interactions and makes culture reproducibility and clinical translation problematic [26]. Matrigel is made of several components: primarily laminin, collagen type IV and nidogen. Analysis of different batches of Matrigel has identified more than 14,000 unique peptides and nearly 2,000 unique proteins, making careful chemical characterization of the material impractical [27]. Additionally, batch-to-batch variability of Matrigel limits reproducibility. Within the complex mixture of proteins in Matrigel, only ~53% are found consistently in each lot [28]. The high cost of Matrigel often hinders scaling up production [3]. Thus, Matrigel offers no direct control over the concentration and identity of cell-binding ligands. Furthermore, this complex component mixture can influence cells in unexpected ways [26]. Inability to control biophysical and biochemical properties, and the potential for pathogen transfer limit biological control during experiments and hamper downstream clinical applications [3].

Alginate

Alginate supports cancer cell attachment, integration and growth. Major features of glandular epithelium *in vivo* such as acini, apical morphogenesis, and expression of stem cell-associated proteins has been recapitulated by 3D clusters of hepatocellular carcinoma cells cultured in an

alginate matrix. Under such treatments, cancer cell activity and resistance have reported to vary depending on 2D and 3D cultures [10].

As major integrin-binding sites are missing in alginate, it can be functionalized with peptide sequences like an RGD (Arg-Gly-Asp) present in main ECM proteins to enable ECM interaction and signaling. Independent of oxygen levels, cell-ECM interactions in RGD-conjugated Alginate increased proangiogenic molecules that promoted invasion of endothelial cells into the matrix [10]. Cancer cell confinement and spontaneous spheroid formation were facilitated by alginate microcapsules. Effective enrichment of cancer stem cell genes expressed in the spheroid is argued to be due to retention of autocrine factors of cells in close proximity when cells were encapsulated in the microcapsules. Harvesting alginate-embedded spheroids and implanting them in mouse models leads to significantly larger tumors compared to injection of cells cultured in 2D. This suggests that alginate 3D cultures potentially mimic the structure of early embryos (the native home of totipotent–pluripotent stem cells) and in this way enhances the content of cancer stem cells [10].

Biophysical Characterization of the ECM

Aside from the chemical cues that ECM provides for cells, it has also been considered a supportive scaffold. The impact of physical properties of ECM such as stiffness, viscoelasticity, porosity and pore size on stem cell proliferation and differentiation has been established. Cells respond to these physical cues through mechano-sensing. Cells detect slight stresses and deformations in the ECM providing cues to cells through cytoskeleton reorganization that lead to the activation of the corresponding pathways such as integrin signaling pathways. Dynamic changes such as strain or stress rate in the ECM have emerged in studies as cell fate determinant factors [22]. As embryoid bodies and organoids are reminiscent of early developmental stages,

we have hypothesized that the physical properties of their surrounding matrix can play a crucial role in stem cell differentiation within organoids.

The elastic modulus is a measure of stiffness and the effect of the ECM elastic modulus on cell behavior has been widely studied using hydrogels. Increased stiffness of the hydrogels in 2D cultures affects stemness of stem cells, cell migration, neuronal branching, cell spreading and malignancy and various other important mechanisms [29]. Although 2D cultures are convenient and have provided us with extensive information on biological processes, they fail to capture specific cell behavior. 3D microenvironments are more physiologically relevant which is critical for proper cell function. The effect of altered stiffness on biological processes has also been found with cells cultured in hydrogel-based 3D environments. Although the role of mechanics has been clearly demonstrated by these studies, measuring elastic modulus is not sufficient as native ECM is not only elastic, but viscoelastic, and the role the viscous portion of ECM plays in controlling cell behavior is not fully known. Substantial viscoelasticity has been found in many soft tissues including the brain and embryonic tissues. Viscoelasticity is a combination of the properties of elastic solids and viscous liquids and viscoelastic materials are recognized by two major features. First, unlike elastic material that store energy, they can dissipate energy through deformation. If the resulting deformation is permanent, the material is considered plastic. Second, viscoelastic material exhibits time dependent behavior that includes stress relaxation and creep in response to applied constant deformation (strain) and stress respectively [29].

Hydrogel viscoelasticity is strongly associated with its microstructure and could provide useful information for regulating its functional features. Rheometry can monitor the viscoelastic properties of materials with virtually no disturbance on their microstructure. In this study, rheological studies were performed to evaluate the viscoelastic profiles of alginate and Matrigel

gelation. The effects of gelation time and polymer concentration on gelation were tested. These findings provide us a better understanding into the gelation kinetics of hydrogels, thereby providing essential insight for designing hybrid *in situ* gelable hydrogels for future biomedical applications, and in particular for the growth and maintenance of neuronal organoids [30].

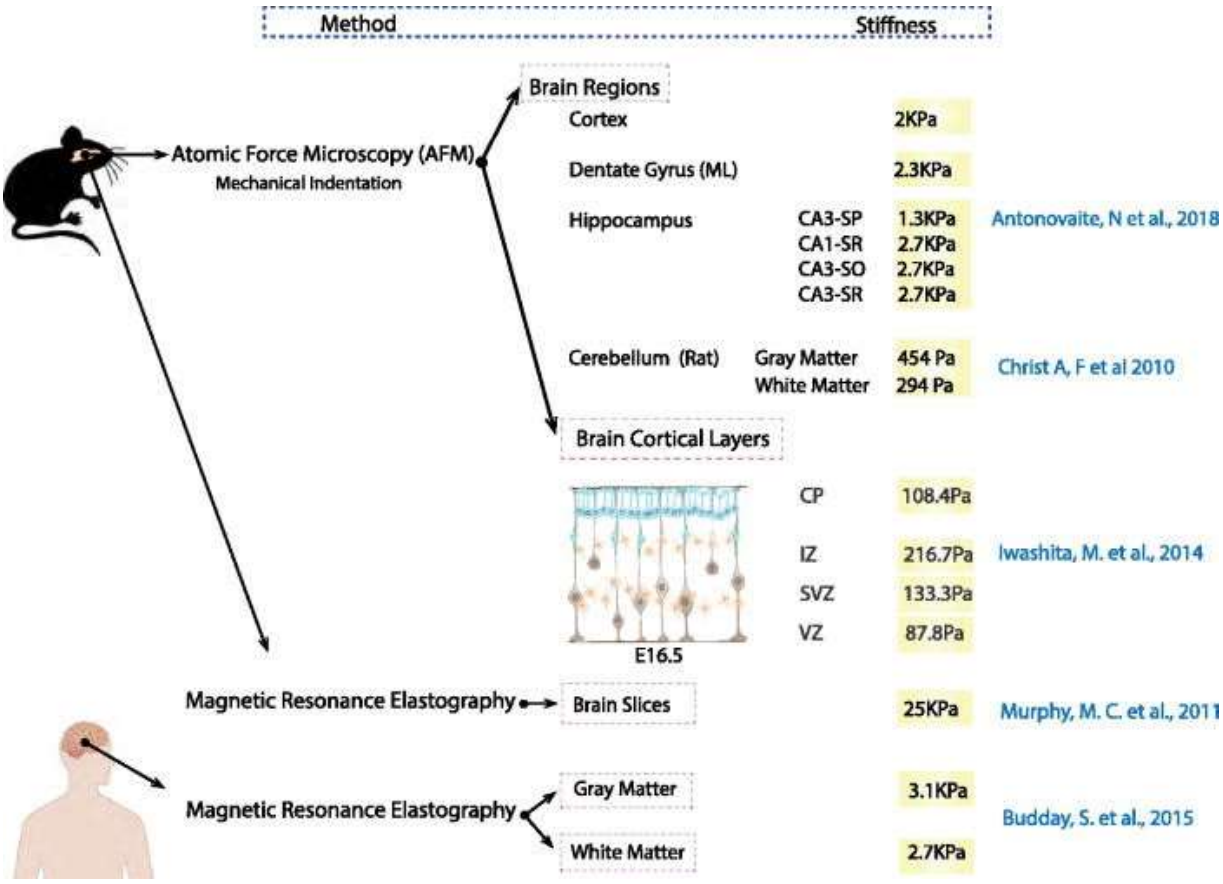

Figure 5 adapted from Oksdath et al.- Brain tissue stiffness in mice and human brain regions determined by atomic force microscopy and magnetic resonance imaging [8]. Note that the stiffness results depend on the specific technique of measurement.

Elasticity vs. Viscoelasticity

Elasticity describes the stiffness of a material that is, its resistance against deformation when subjected to a given stress. Since native tissue architecture is more complex than synthetic elastic

polymers, it is difficult to qualify elastic properties of many biological tissues in terms of mere stiffness (Young's modulus).

Most biological tissues consist of multiple molecular components; for instance, basement membranes contain two intertwining, independently cross-linked networks with very different mechanical properties, collagen IV-based and laminin-based. Certain tissues are multilayered, such as arterial wall, and each layer has its own elastic properties. In most ECMs present in these layers, there is also a viscous component that affects tissue behavior [22].

Viscoelastic material has both elastic and viscous (dissipative) structural elements. When a constant strain is applied, these materials exhibit stress-relaxation properties: material becomes less stressed with time due to a dissipation of elastic energy by its viscous part flow. When subjected to a constant stress, the viscoelastic materials undergoes creep, which is gradual increase in strain with time [22].

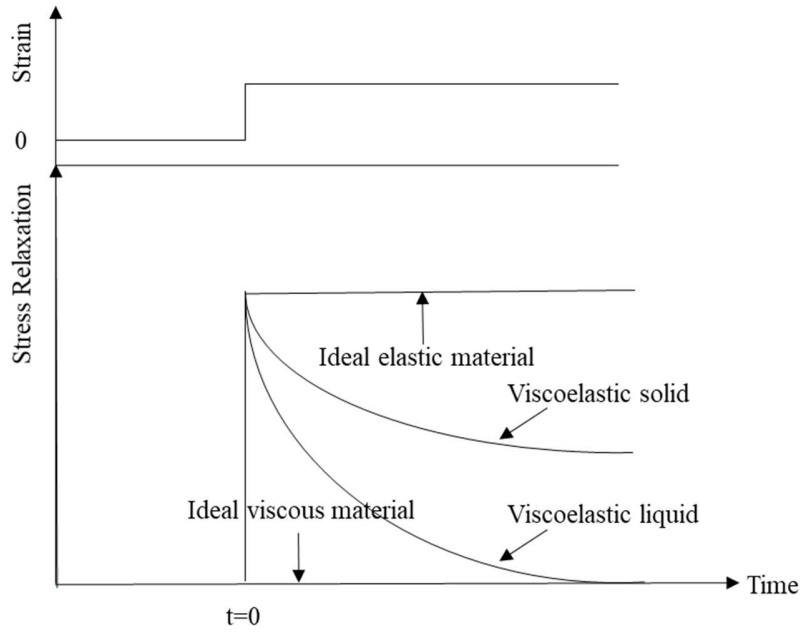

Figure 6 adapted from Steffe - Stress relaxation curves for elastic, viscous and viscoelastic materials [31].

Most biological tissues exhibit viscoelasticity due to either flow of the viscous fluids that occurs during deformation or the sliding of macromolecules of the ECM. Viscoelastic materials usually are described by complex shear modulus G, which consists of the elastic component, the storage modulus, and the dissipative or viscous component, the loss modulus. The higher the magnitude of the viscous component for a matrix, the more the substrate will creep with time under applied force. Values of loss modulus of the skin are in the range of 10 to 300 Pa, while for tendon it is in the order of 1 GPa [22].

The creep or the stress-relaxation process takes time; slope of relaxation kinetics curve is characterized by relaxation time. That is why viscoelastic measurements depend on the rate of stress or strain changes and, consequently, on the frequency of the applied dynamic forces [22].

Storage, Loss and Stress Relaxation Moduli

To characterize viscoelasticity of hydrogels, several approaches have been used to provide us with information regarding the time-dependent mechanics of the material. Measuring the viscoelasticity is typically done using a rheometer to apply and measure shear stresses and strains [1].

A standard test used to measure the viscoelastic properties of hydrogels is a stress relaxation test achieved through shear rheology. In this test, a constant strain is applied, and the responding stress is measured over time. An elastic material would maintain a constant stress, while viscoelastic materials relax the stress over time [1].

Either the stress, or the relaxation modulus can be reported. In this modality of measurement, the relaxation modulus, corresponding to the resistance in deformation, is linearly related to the

stress. The stress relaxation response can be experimentally defined, for example using the time at which it takes for the stress to relax to half its original value or $\tau 1/2$. A covalently crosslinked polyacrylamide hydrogel exhibits minimal stress relaxation under compression, while various soft tissues exhibit substantial stress relaxation with $\tau 1/2$ ranging from ~10 s–200 s [1].

Another common approach to measuring viscoelasticity in hydrogels is dynamic mechanical testing. This method involves application of a sinusoidal stress or strain to the material, and measurement of the responding strain or stress. By comparing the amplitude and phase shift of the response, the storage, or elastic, modulus and the loss, or viscous, modulus can be characterized as a function of frequency [1].

Rheometry

Cross-linking reaction kinetics can be monitored by a well-established technique called dynamic rheology. Changes in the rheological properties of the material reflects the formation and density of the crosslinks.

Material properties, such as the elastic and viscous moduli, can be detected using small amplitude oscillatory shear rheometry, with minimal disruptions to the chemical reaction. *In situ* rheometry is a convenient approach to examine biodegradable hydrogels with uses in regenerative medicine and bio-therapeutics. In order to monitor the gelation kinetics and tailor the mechanical properties of hydrogels to the desired applications, *in situ* rheology is advantageous. An *in situ* cross-linkable material can be characterized in a rheometer while the sample is exposed to the cross-linking agent which captures the transition from sol to gel in real time. The dynamic moduli of the curing material can accurately predict the gel point, signifying the time at which hydrogel networks span between the plates of the rheometer [32].

Pore Size, Porosity, and Permeability

The volume of voids around matrix scaffold molecules per unit volume of the gel is referred to as porosity. Pore size, unlike porosity, directly targets pore geometry. Cell motility and adhesion depends on pore size rather than porosity of the scaffold. Unlike natural polymers, mean pore size is correlated with porosity for many synthetic polymers [22].

The accessibility of small molecules such as nutrients and oxygen, large molecules, cell processes or cells in general is recognized by permeability. Cell survival requires permeability for fluid flow and molecular diffusion as low permeability may decrease nutrient transport and cause ischemia [22].

3D scaffolds act mostly as a mechanical barrier for fluid flow and diffusion. In general, using the same material, stiffer matrices exhibit decreased pore size and permeability. However, it is possible to produce matrices with the same stiffness but variable pore size. Solute permeability is notably enhanced under dynamic deformation because of the increased fluid flow [22].

The porosity of a hydrogel determines its swelling kinetics and the degree to which it can absorb water. Thus, hydrogel's porosity is regarded as one the most important features to be controlled through either physical or chemical manipulations [22].

Scanning Electron Microscopy (SEM)

One of the techniques that is used to portray a picture of surface morphology and topography of numerous hydrogels is scanning electron microscopy (SEM). SEM reveals the porosity and nature of hydrogel structure. Moreover, the morphological changes with hydrogel modification or even changes in polymer concentration can be detected within SEM images. Sample

preparation for SEM involves freezing the swelled hydrogels in liquid nitrogen, then freeze-drying and finally mounting prior to SEM observation [33].

Research Rationale

In this section, after touching on iPSCs, I reviewed the history of brain organoids highlighting the role of Matrigel in organoid development. As a substitute for the *in vivo* micro-niche, Matrigel acts as not only a compliant scaffold supporting organoid's growth but also a source of signaling molecules directing cell function and behavior.

To better understand how Matrigel –which is ECM-derived itself- is playing the role of basement membranes, I took a closer look at the composition, structure and function of the basement membranes. I focused on how basement membranes form *in vivo* vs. *in vitro* to have a more in-depth idea about the assembly of proteins within Matrigel, which is the underlying reason of Matrigel's high variability.

I overviewed the history of Matrigel and introduced another polymer, Alginate, as a companion to Matrigel offering tunable mechanics. I then went over the biophysical characteristics of the ECM and the concept of viscoelasticity. Finally, I introduced the techniques we selected to work with for measuring the mechanical properties of our hydrogels.

In the next section, I am going through the workflow of characterizing the biophysical properties of Matrigel and Alginate hydrogels in detail. Using the same workflow, I am going to study the mechanics and the microarchitecture of the hybrid Alginate/Matrigel hydrogels.

Methodology

Hydrogel Formation

Sodium alginate powder (W201502, Sigma Aldrich) was dissolved in 1 mL of deionized H2O to a dilution of 2% as stock solution. Different alginate concentrations were then obtained by further diluting the stock depending on the favorable conditions in specific experiments. Excess alginate solutions were stored at 4°C and used within two weeks of initial preparation.

Growth factor reduced Matrigel (356230, Corning) was aliquoted and stored in a -80°C freezer. Matrigel from two different batches were tested in the experiments. Batch #1 and #2 were assigned to Matrigel from Lot # 8062075 and Lot# 9049004, respectively. For each experiment, an aliquot was put in the cold room overnight to ensure a gentle thaw.

I studied *in situ* gelation of 100 µL droplets of gel. A 30 mm dish was attached to the bottom plate of the rheometer. After adjusting the rheometer, 100 µL droplets of Matrigel were placed at the center of the dish and then the top plate (8 mm) was lowered down until a gap of 1 mm was between the two plates, which was filled with gel. The temperature was set at 37°C and gelation tests were run to measure the gelation time for Matrigel. For Matrigel 50%, it was first diluted in DMEM (10-565-018, Thermo Fisher Scientific) at 4°C and then the same procedure was applied.

Rheological measurements

Rheological experiments were performed with an Anton Paar MCR 302 rheometer temperature adjustable bottom plate fixture (**Figure 7**). An 8 mm aluminum parallel plate was used for the top geometry. Three different gap sizes (0.5, 1, 2 mm) were initially tested and for the rest of the study, the gap was maintained at 1 mm. All measurements were made within two weeks after

preparing alginate solutions. Solution gelling behavior was monitored using dynamic oscillatory time and frequency sweep experiments. For Matrigel and Alginate/Matrigel hydrogels, samples were exposed to heat (37°C) for 10 minutes for Matrigel to polymerize at the start (t=0) of each time sweep. Under isothermal conditions, Matrigel gradually forms a gel and we could measure the gelation time by performing dynamic mechanical testing.

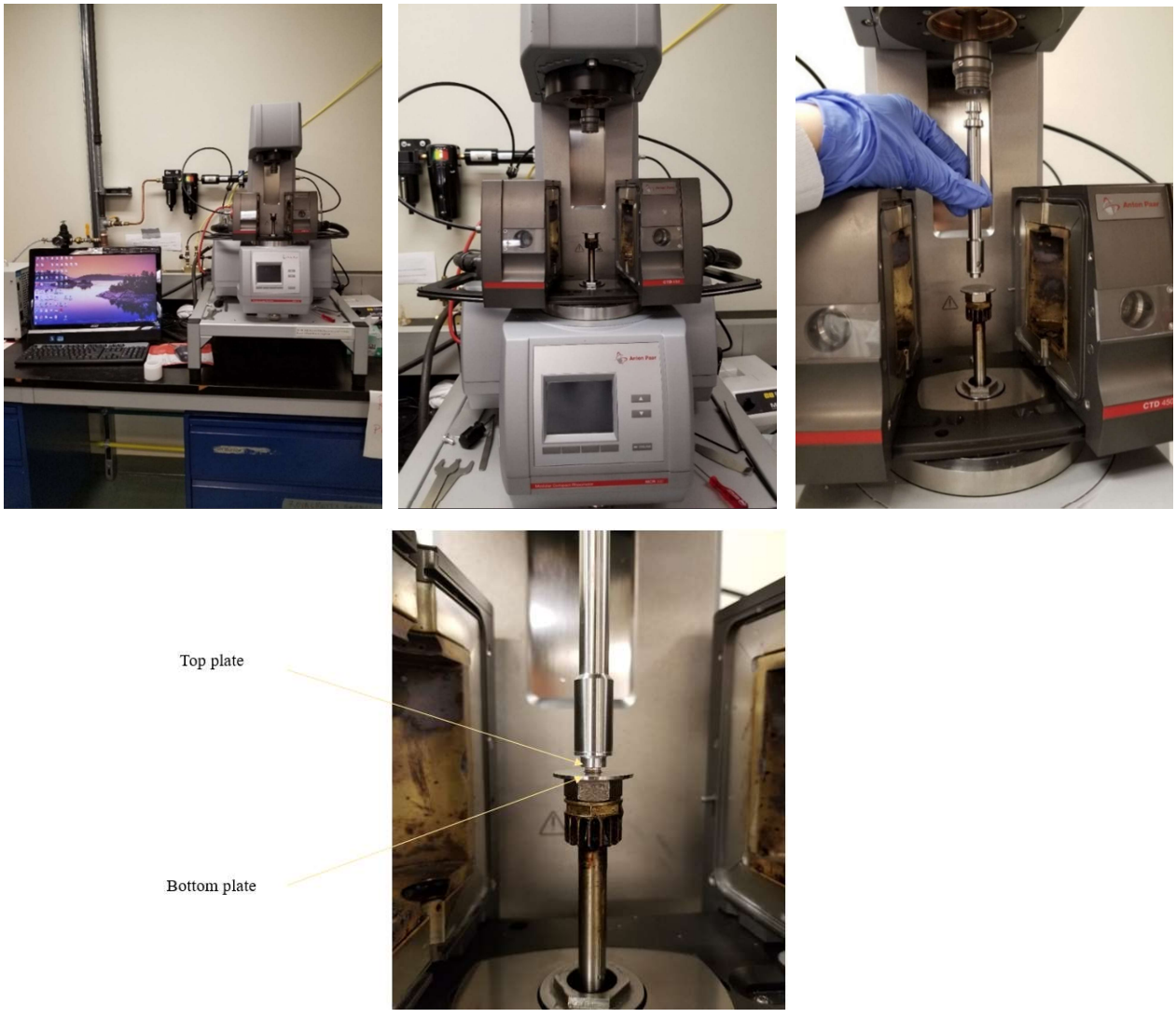

Figure 7- Anton Paar MCR 302 rheometer that was used for dynamic mechanical testing and stress relaxation measurements of this study.

For Alginate gelation, samples were exposed to calcium chloride either at the start (t=0) of each time sweep for pure alginate or after Matrigel gelation for hybrid hydrogels. Throughout the time sweep, constant oscillations were applied at a fixed frequency (1rad/s) with a strain of 2%, which was in the linear viscoelastic (LVE) regime of the material. The time sweep was continued until storage and loss moduli reached steady state indicating completion of gelation. After determining the gelation time for all types of hydrogels using dynamic mechanical testing, stress relaxation tests were performed on fresh hydrogels with a strain of 2%. After having the workflow set up, rheological measurements were performed in triplicate to ensure reproducibility [30, 32].

For hybrid hydrogels, Matrigel and Alginate were mixed together in varying concentrations (Matrigel 50%/Alginate 0.08%, Matrigel 50%/Alginate 0.16% and Matrigel 50%/Alginate 1%) on ice and then the gel droplet was deposited directly on the center of the dish. Next the bottom plate was heated to 37°C. Based on previous data on pure Matrigel and Alginate gelation, calcium chloride (Sigma-Aldrich, 449709) 1% (w/v) was added to the dish after 10 minutes. The gelation of alginate was instantaneously initiated so we started performing the measurements. The dish was filled with calcium chloride solution to avoid hydrogel evaporation. The gels polymerized at 37°C for 10 minutes.

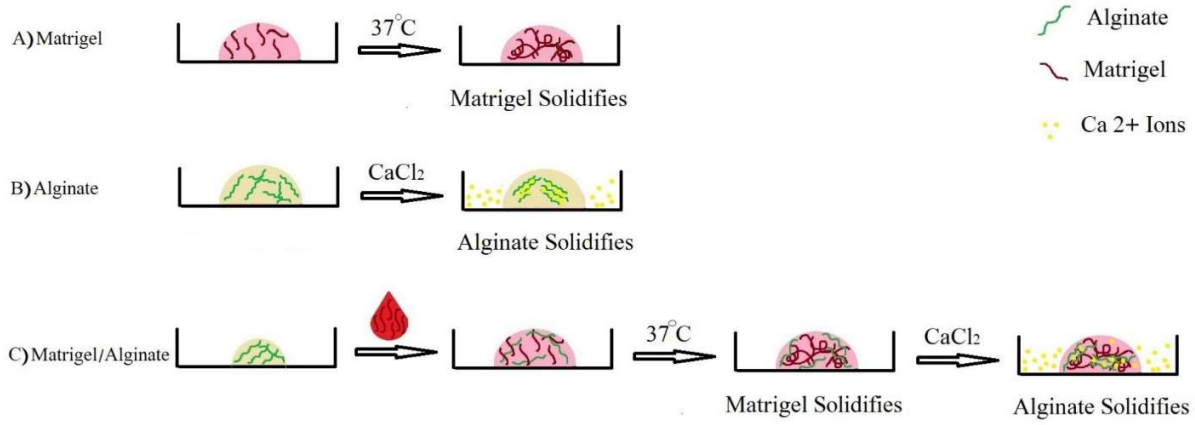

Figure 8 - Gel formation steps for Matrigel, Alginate and hybrid Matrigel/Alginate hydrogels. All process were done *in situ* on the rheometer to keep the hydrogels intact.

Sample preparation and analysis by SEM

For the morphological study of sodium alginate, Matrigel 100%, Matrigel 50%, Matrigel 50%/Alginate 1% and Alginate 1% hydrogels were formed on a dish as described previously. After the gelation was complete, hydrogels were immediately frozen in liquid nitrogen for 2 minutes and freeze-dried overnight. The dried samples were cut into halves and placed on aluminum stubs with electrically conductive carbon adhesive tape.

The pore architecture might be affected by the method of freeze-drying, specially by controlling the freezing rate [34]. However, freeze-drying remains one of the most liable methods of sample preparation for SEM when it comes to thermosensitive material such as Matrigel. This method preserves the structure of protein components of Matrigel.

On the other hand, fewer exchanges of liquids are required for freeze-drying and hence chemical fixation may not be absolutely required for samples prepared by freeze-drying [35]. For SEM

sample preparation, I benefited from this aspect of freeze-drying and did not apply any chemical fixation which helps preserving the native structure of hydrogel even more.

For the Image acquisition, I have been trained for using the microscope by the resident technician at McGill Material Services and the set up was confirmed by the technician at each imaging session.

Morphological analysis was conducted in a Hitachi SU3500 scanning electron microscope in the variable pressure mode at an accelerating voltage of 3–10 kV.

Image analysis by ImageJ

In this study, I used ImageJ an open source image processing package to calculate the pore size distribution of the freeze-dried hydrogels. The diameters of distinct pores within each image were measured for at least 20 pores per image and the average pore diameter was calculated for each image. For each type of hydrogel, three images were analyzed [36].

Results

Storage and Loss Moduli

There is a rising need to have independent control over the physical and chemical properties of hydrogels (e.g. matrix elasticity, ligand density, and porosity) as well as their micro-scale homogeneity [37]. Hence, the use of mechanically tunable polymers (e.g. Alginate) are often preferred over reconstituted ECM-derived ones. Various soft tissues, such as brain and reconstituted ECM-derived polymers (e.g. Matrigel) are all viscoelastic and exhibit partial stress relaxation when a constant strain is applied [37].

To examine the presence and extent of Matrigel variability in mechanical properties, I tested multiple aliquots of Matrigel from two separate batches. Storage and loss moduli of Matrigel 100% samples were measured and the results are shown in **Figures 9-12**. Based on the measurements, Matrigel inconsistency is clearly visible in the viscoelastic behavior of different samples of one batch, let alone different batches.

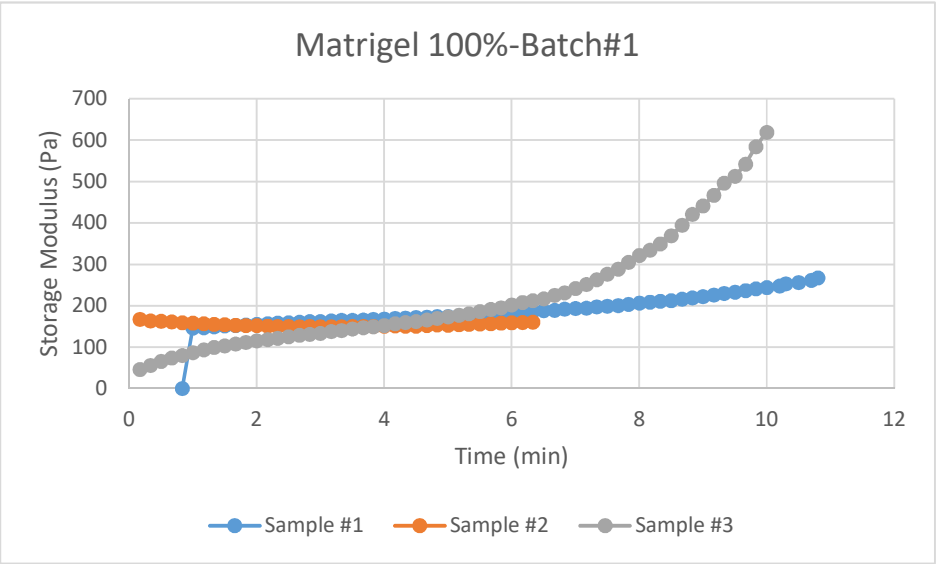

Figure 9 - Elasticity (storage modulus) of three distinct Matrigel 100% samples. Each sample represents one gelation experiment using one aliquot of the same Matrigel batch (#1).

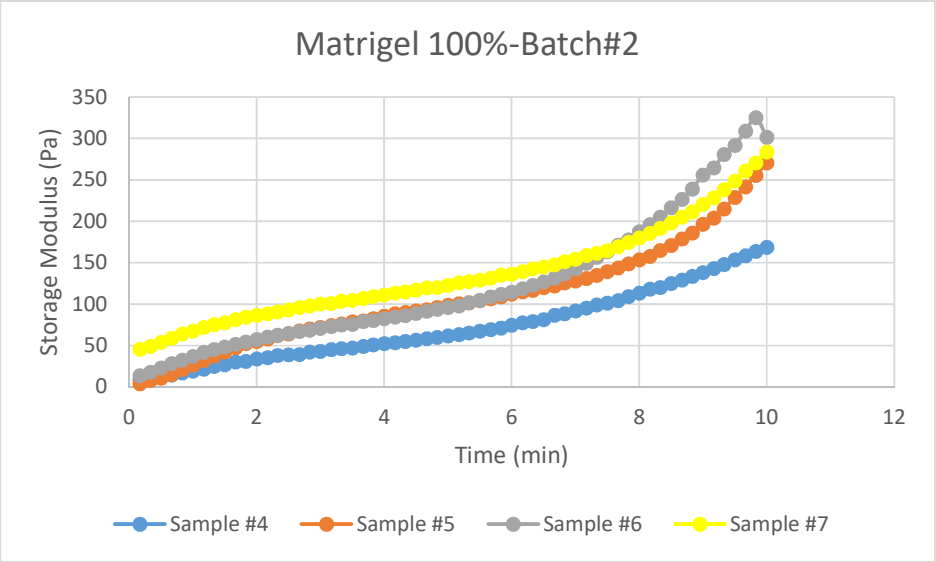

Figure 10 - Elasticity (storage modulus) of four distinct Matrigel 100% samples. Each sample represents one gelation experiment using one aliquot of the same Matrigel batch (#2).

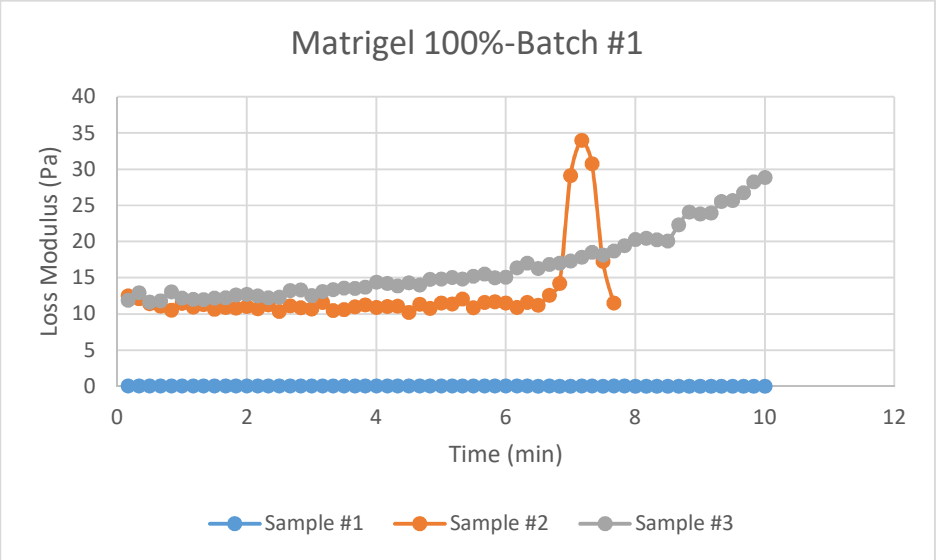

Figure 11- Viscosity (loss modulus) of three distinct Matrigel 100% samples. Each sample represents one gelation experiment using one aliquot of the same Matrigel batch (#1).

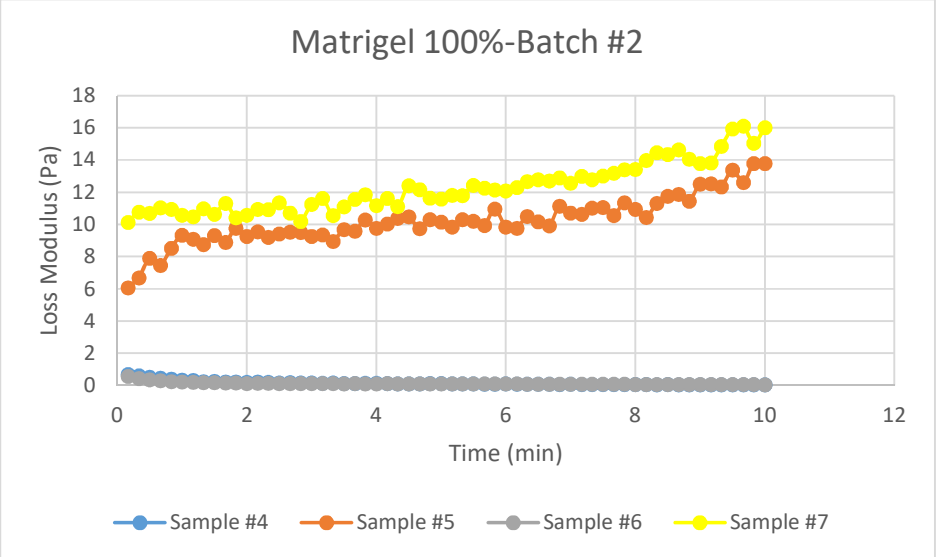

Figure 12 - Viscosity (loss modulus) of four distinct Matrigel 100% samples. Each sample represents one gelation experiment using one aliquot of the same Matrigel batch (#2).

In order to characterize and then compare the viscoelasticity of Alginate and Matrigel, I measured the storage, loss and relaxation moduli for Matrigel 100%, along with1 and 2% Alginate using polyacrylamide as a purely elastic control. To fine-tune both the structural performance and bio-fidelity, I also studied the viscoelastic behavior of Matrigel 50%/Alginate 0.08%, Matrigel 50%/Alginate 0.16% and Matrigel 50%/Alginate 1% hybrid hydrogels. Based on the results, Alginate 2% shows a similar initial elasticity to Matrigel 100% (**Figure 13**). I also observed similar range of elasticities for Matrigel 50% and Alginate 1% (**Figure 13**). Interestingly, the Matrigel 50%/Alginate 1% hybrid hydrogel shows a significantly higher degree of elasticity than both its components. It could be explained by the entanglement of alginate molecules within Matrigel protein network which may lead to a hydrogel with increased final stiffness (**Figure 13**). I tested Matrigel 50%/Alginate 0.08% as well but repeated measurements were problematic due to poor *in situ* gelation of the mix (**Figure 13**). As expected, the elasticity of polyacrylamide 40% as a control was significantly higher than all gel types (around 40 times higher than Matrigel 100% and Alginate 2% and almost two orders of magnitude higher than Matrigel 50% and Alginate 1%) (**Figure 13**).

In terms of viscosity, Matrigel 100% and 50% exhibited less energy dissipation than Alginate hydrogels (**Figure 14**). It is notable that Matrigel 50%/Alginate 1% hybrid hydrogel was significantly more viscous than both pure Matrigel concentrations and also Alginate 1% hydrogels (**Figure 14**). In fact, Matrigel 50%/Alginate 1% hybrid hydrogel exhibited the highest degree of viscoelasticity among all gel types based on dynamic mechanical testing results (**Figures 13-14**).

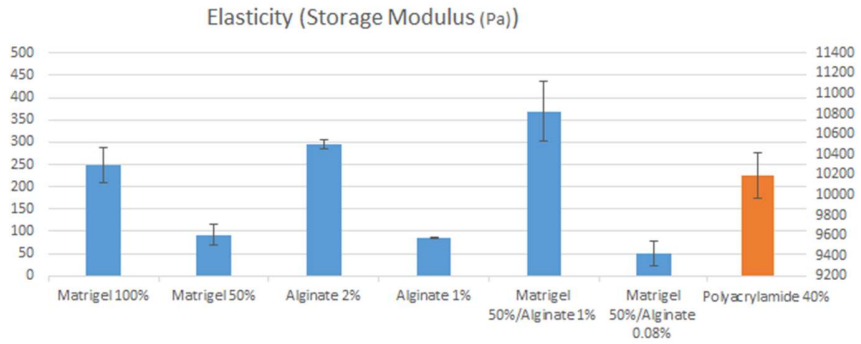

Figure 13 - Characterization of the stiffness of hydrogels through the storage modulus. The elasticity was measured via *in situ* rheometry for Matrigel 100%, Matrigel 50%, Alginate 2%, Alginate 1 %, Matrigel 50%/Alginate 1%,and Matrigel 50%/Alginate 0.08%, hybrid hydrogels (y-axis on left) along with Polyacrylamide 40% (y-axis on right) as an almost purely elastic control. (Data presented as mean ± standard error of mean; n = at least 3 gels per type of hydrogel.)

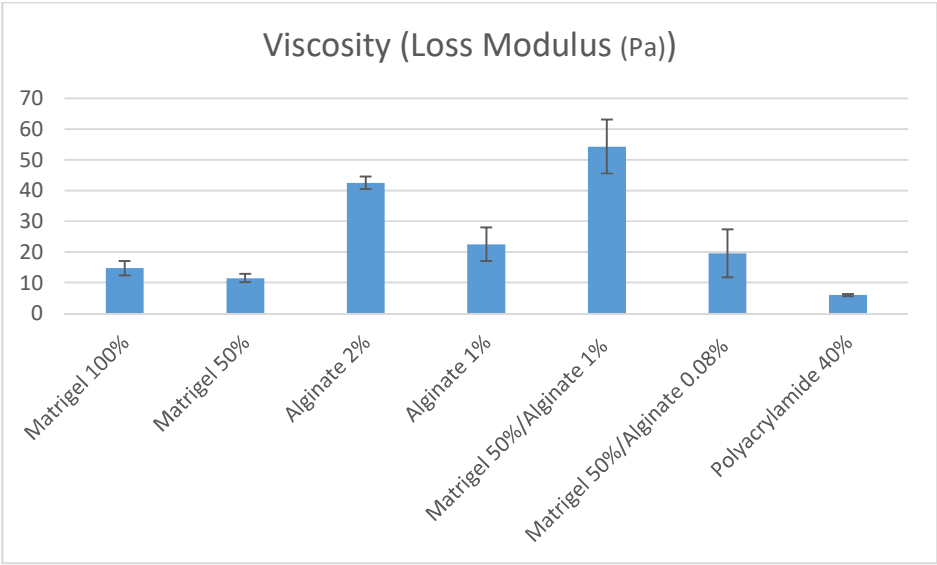

Figure 14 - Characterization of the viscosity of hydrogels through measuring the loss modulus. The viscosity was measured via *in situ* rheometry for Matrigel 100%, Matrigel 50%, Alginate 2%, Alginate 1 %, Matrigel 50%/Alginate 1% hybrid, and Matrigel 50%/Alginate 0.08%, and hydrogels as well as polyacrylamide 40% as a non-viscous control. (Data presented as mean ± standard error of mean; n = at least 3 gel per type of hydrogel.)

Stress Relaxation Half Time (τ1/2)

Stress relaxation experiments present straightforward methods for studying the time-dependent modulus of viscoelastic polymers. Through this method, a specimen is strained to a fixed level and the slow decay of stress is monitored. When polymers undergo stress relaxation, the modulus of the material typically decays from an initial value to a final stable value and the speed of this process, which has practical implications, is characterized in terms of a relaxation time constant τ [38]. Stress relaxation half time is defined as the time it takes for the polymer to relax the stress to half of its initial value.

To check whether the variability observed in the storage and loss moduli of different Matrigel samples from the same batch was also visible in their stress relaxation moduli, we measured ten different samples from one batch (**Figure 15**). Although samples #2, 4 and 9 share the same pattern of relaxation, the majority of the samples do not show similar relaxation moduli and do not share the same relaxation behavior, which further supports inconsistent viscoelastic behavior of Matrigel.

Unlike the initial relaxation modulus that was higher in Matrigel 100%, Matrigel 50% required a longer time period to relax the initial stress (**Figure 16**). This may be explained by Matrigel 50% being less viscous than Matrigel 100% (**Figure 14**). Matrigel 50%/Alginate 1% hybrid hydrogels exhibited the longest stress relaxation time. As this hybrid hydrogel has shown the highest viscosity among all gel types, the reason why it needs more time to for stress relaxation is unclear. However, this higher stress relaxation half time compared to Matrigel 100% and 50% could be partially explained by 50%/Alginate 1% hybrid hydrogels having the highest elasticity among all gel types.

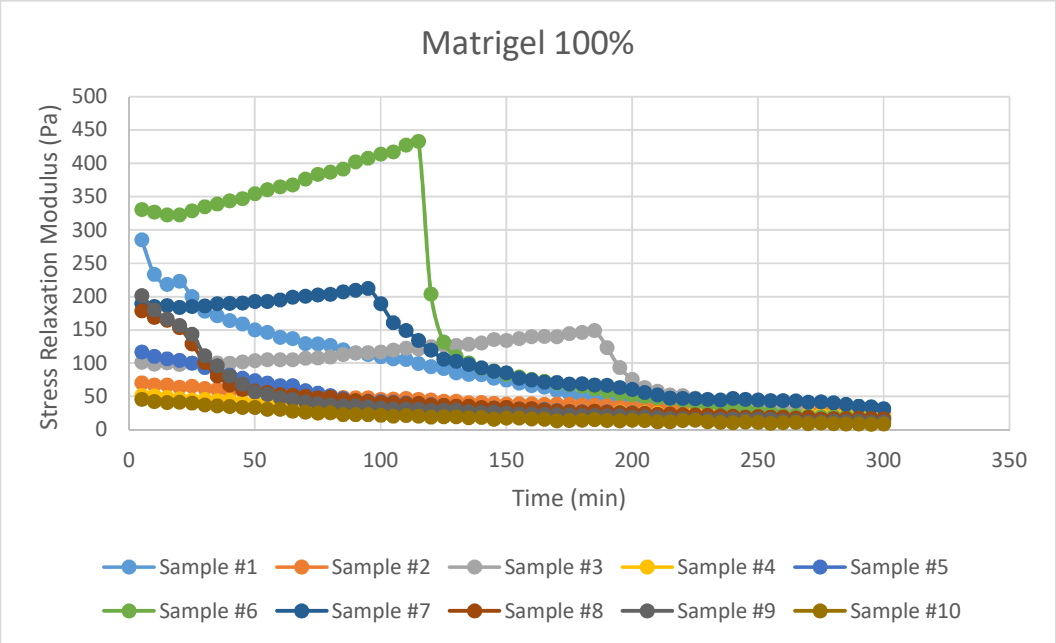

Figure 15 - Stress Relaxation moduli of ten different samples from one batch (#2) of Matrigel. The results show inconsistent behavior among separate samples even from one batch.

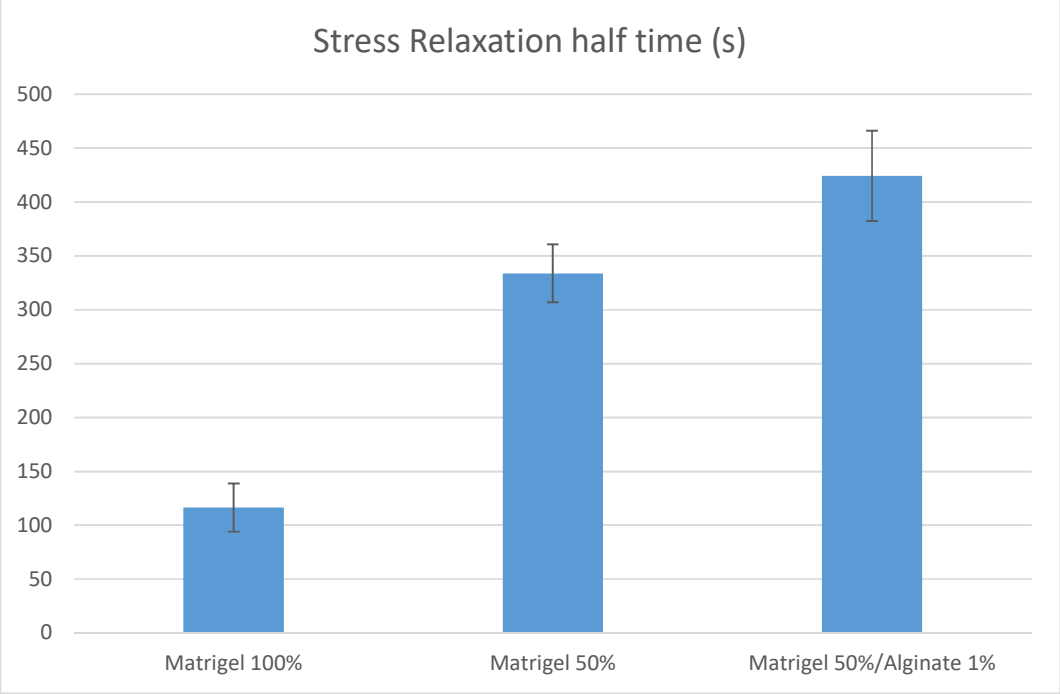

Figure 16 - Stress Relaxation half time measured in Matrigel 100%, Matrigel 50% and Matrigel 50%/Alginate 1%. Three hydrogel samples were tested for each condition. (Data presented as mean ± standard error of mean; n = 3 gels per type of hydrogel.)

SEM images

Pore structure is a term which describes the shape, size, distribution and connectivity of the pores inside a porous structure. Pore structure has obvious impact on the transport properties of a matrix and is critical to both encapsulation efficiency and release kinetics.

Too small a pore size can hamper timely release and too large a pore size will result in content leakage. Although Matrigel pore size distribution is not extensively researched, numerous studies have investigated Alginate pore size through various techniques such as imaging and diffusivity measurements. Pore size distribution in Ca^{2+} crosslinked alginate hydrogels depends on polymer and cross-linker concentration as well as the measurement technique. For instance, in experiments where SEM was used, a larger range of pore sizes have been observed compared to results obtained from atomic force microscopy (AFM) imaging [39]. Based on the SEM imaging results of this current study, average pore size of 15 μm was measured for Alginate 1% hydrogels (**Figures 19 and 21**). This is consistent with previous studies that have observed a range of pore sizes from 5 nm - 21 μm for Alginate concentrations between 1.5% and 3% Alginate. Sources of discrepancies include the range of variables associated with the gelation technique, the artifacts of sample preparation, and the resolution of the measurement technique.

According to the observed pore size distribution for different Matrigel concentrations, Matrigel 50% presented with pores that were almost double the size relative to Matrigel 100% (**Figures 17, 18 and 20**). This can be due to less polymer density available to form a network. Matrigel 50%/Alginate 1% has also demonstrated a significantly increased average pore size compared to its components alone which can be explained by relatively larger gaps between polymer molecules due to hybrid nature of the hydrogel (**Figures 20 and 21**).

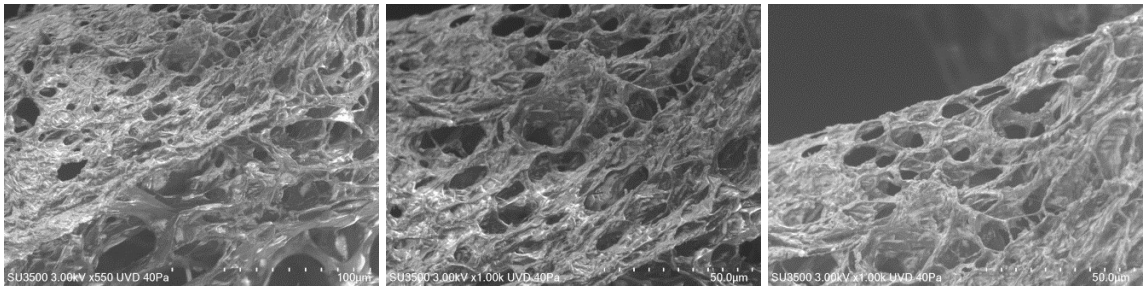

Figure 17 - SEM images of freeze-dried Matrigel 100% hydrogel.

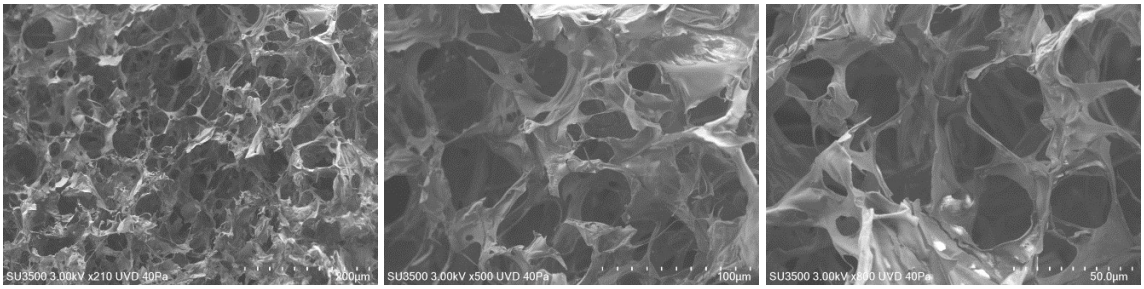

Figure 18 - SEM images of freeze-dried Matrigel 50% hydrogel.

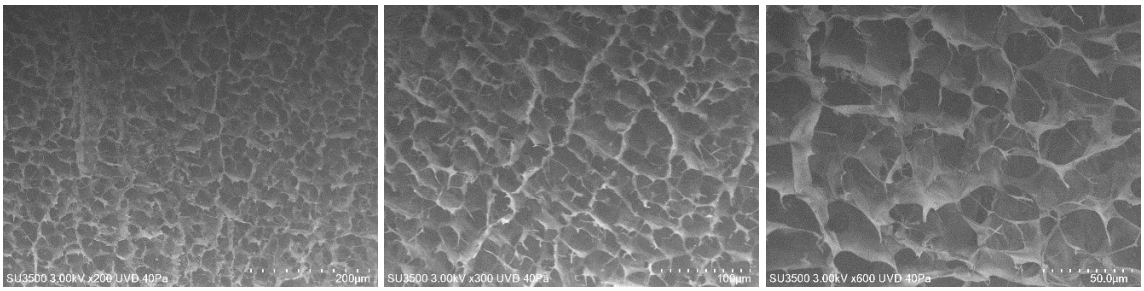

Figure 19 - SEM images of freeze-dried Alginate 1% hydrogel.

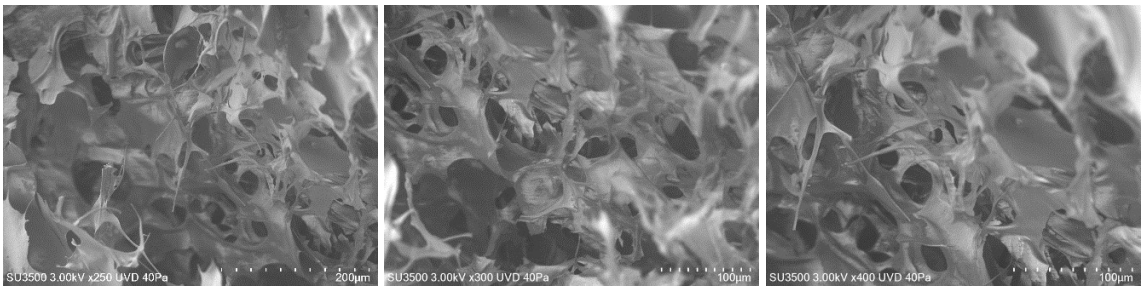

Figure 20 - SEM images of freeze-dried Matrigel 50%/Alginate 1% hydrogel.

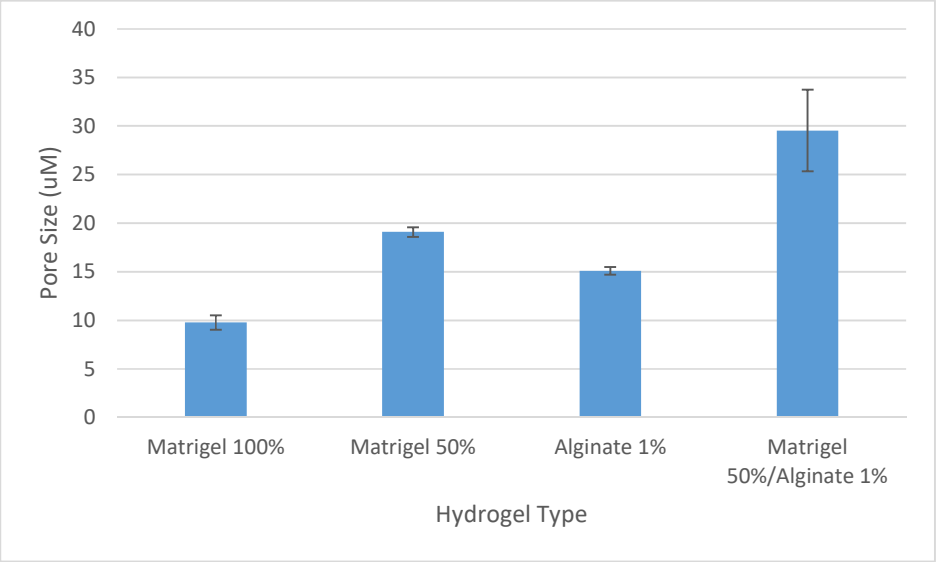

Figure 21- Pore size distribution of freeze-dried hydrogels. Pore sizes were measured in ImageJ (data presented as mean ± standard error of mean; n = 3 images per gel type, at least 10 pores measured per image.)

Discussion

Here, I developed a workflow to characterize the viscoelastic properties of Matrigel and Alginate hydrogels using polyacrylamide as an elastic control. Using this workflow, I generated hybrid Alginate/Matrigel hydrogels with varying polymer ratios to manipulate the viscoelastic properties of the support matrices with the final goal of improving the structure of the brain organoids.

The first and most important limitation of this study would be using Matrigel itself. As mentioned earlier throughout the thesis, due to Matrigel's thermosensitivity handling and characterizing Matrigel specially using in situ rheology is difficult. In addition, Matrigel inconsistent composition makes experiments' reproducibility challenging.

Matrigel's variable composition between and across batches is a major limitation for its clinical research application [3]. In this work, we observed inconsistent mechanical properties in Matrigel that further highlight the reproducibility issue in using Matrigel. The ECM components present in Matrigel interact with each other in numerous possible forms and these interactions may contribute to the heterogeneity of Matrigel hydrogels in terms of structure and mechanics [19]. The minor components of the ECM in Matrigel, e.g. agrin are also of great importance. Variable presence and participation of minor components in separate gel structures could potentially result in dissimilar mechanical behavior among hydrogels [19]. Furthermore, slight changes in micro-environmental factors such as pH, temperature and Ca^{2+} concentration also affect the assembly of the components into gels, thus influencing the mechanical properties of the resulting hydrogels [21].

Still, further research is required to find a substitute for Matrigel with defined composition to provide cells with the right mixture of attachment cues. On top of that, the presence of ECM components in Matrigel is critical for inducing tissue polarity within brain organoids, therefore their contribution needs to be addressed when replacing Matrigel [15]. Here, to make the brain organoid microenvironment more robust, we mainly focused on enhancing the mechanical properties of Matrigel by adding alginate.

According to previous studies, negatively charged lipid bilayers can act as cores that attract basement membrane components non-specifically [21]. Since alginate polymer chains are also negatively charged, they can potentially organize matrices through adsorption of Matrigel proteins. The observation of hybrid hydrogels having higher storage moduli could be due to the enhanced formation of matrix structures around the negatively charged alginate chains that could add to the rigidity of the hydrogels. Moreover, studies on mixing alginate with other polymers have shown increased elasticity in the resulting hydrogels due to the synergistic interactions between interpenetrating network of polymers which is also in line with our hybrid hydrogels being more elastic than Matrigel and Alginate hydrogels alone [40].

Our SEM data revealed increased pore size in hybrid Matrigel 50%/Alginate 1% hydrogels compared to Matrigel 50% or Alginate 1% alone. By increasing the temperature to 37°C, Matrigel gels first, trapping alginate polymer chains. Perhaps entangled alginate chains straighten up within the already formed Matrigel network following the addition of Ca^{2+}. Considering the affinity of Matrigel components to the negatively-charged alginate chains, alginate interpenetrating network formation leads to pore wall expansion within the hydrogel. This idea could help explain larger pores in hybrid Matrigel 50%/Alginate 1% hydrogels compared to Matrigel 50% and Alginate 1% gels.

Freeze-drying technique also has its own caveats. In the absence of suitable stabilizers, removal of the hydration shell from proteins during drying can cause destabilization of protein structure [41]. Furthermore, the pore structure and size relies heavily on the freezing rate which should be noted for reproducibility purposes [34].

Organoid variability severely limits the application of these model systems in clinical research and translational medicine. Achieving reproducible organoids in terms of size, shape, cellular composition and 3D architecture is of great importance. Lack of control over organoid generation process potentially gives rise to heterogeneity in organoids which can be addressed by using a tunable hydrogel like alginate. According to a recent study, multicellular aggregates self-organize into relatively reproducible bodies with tunable size and internal architecture [42]. Furthermore, gentle organoid recovery process is essential to keep organoids intact. Ionic crosslinking of Alginate chains makes Alginate hydrogels amenable for embedding organoids as well as to organoid recovery for downstream applications [43].

As organoids are becoming more useful models for high throughput studies such as drug discovery, the scalability of organoid generation will be invaluable. Using Alginate to embed organoids, a recent study has increased the capacity of organoid generation by more than 60 fold in the same area with a shorter protocol with the potential to be automated [42].

Another recent study has revealed that alginate hydrogels support human intestinal organoid growth *in vitro* with a nearly equivalent epithelium compared to Matrigel-grown organoids. In addition, when transplanted *in vivo*, maturation of alginate-embedded organoids was also similar to human fetal intestine. This study suggest that alginate hydrogels alone can support intestinal organoid development by providing pure mechanical support; and triggered the idea that organoids could create their own niche with their mesenchyme and epithelium secreting

basement membrane components and trophic molecules [3]. Since brain organoids lack mesenchymal cells, pure mechanical support might not be sufficient for organoid development and the addition of several molecules could help compensate the absence of mesenchyme [3, 4].

Neurons in the developing cortex *in vivo* form a dense band called the cortical plate (CP) and show radially aligned morphology. CP formation reflects neuronal organization into radial units which is a prerequisite for the formation of functional neuronal columns in the adult cortex. Non-neuronal mesenchyme, which is not present in organoids, is thought to generate a basement membrane that potentially acts a ground for the dense CP and radial unit formation observed *in vivo*. A recent study has reproduced data on how the addition of soluble Matrigel to culture medium of organoids derived from independent cell lines leads to the maintenance of a laminin-rich basement membrane with radialized basal layer consistent with CP morphology *in vivo*. The same group tested if adding laminin alone or in combination with nidogen and collagen IV – the three most abundant proteins in Matrigel - would be sufficient to reproduce the same effect in CP maintenance. The results showed that these treatments did not recapitulate the degree of CP formation seen with Matrigel and this potentially suggests the need for other ECM components [4].

As collateral anchorage of the laminin network is provided by the perlecan and agrin, we hypothesize that by adding these two proteoglycans we could potentially improve the maturation of the basement membrane which is essential in the brain development *in vivo* [44]. Since these protein components form networks together, further studies can illustrate whether their simultaneous presence with alginate's mechanical support could effectively promote brain organoid growth when Matrigel is absent.

Lower organoid yield was observed in alginate-embedded intestinal organoids as compared to Matrigel, potentially due to the absence of serum proteins, growth factors and in general cell interacting cues in alginate that allows cells to remodel their microenvironment [3]. The effect of Alginates modified with adhesive/degradable peptides such as adhesive peptide RGD or the protease degradable peptide GPQ-W could be investigated in organoid model systems.

In terms of cost, ease of handling and gentle maintenance of organoids, it is also noteworthy that alginate-grown human intestinal organoids could be cultured for up to 30 days while kept in the same hydrogel; this is due to the lack of alginate degrading enzymes in mammalian cells which leads to alginate structural stability as opposed to Matrigel that can be the subject of degradation by cells within the organoids [3].

Given the similarities between Matrigel and Matrigel/Alginate hydrogels, alginate is an effective complement to Matrigel-based culture systems, which reduces reliance on animal-derived materials and reduces cost, thereby increasing the translational potential. The alginate used in our experiments cost approximately 700–900 times less than Matrigel ($0.44 alginate versus $300-$400 Matrigel per 10 mL, depending on type), presenting a critical cost advantage for hybrid hydrogels [3].

Altogether, our results suggest that adding alginate to Matrigel successfully improved its microstructure and mechanical properties. Fine-tuning the biophysical properties of brain organoid environment opens up new avenues to further investigate the role of tissue mechanics in development and disease.

Conclusion

In this work, I have developed a workflow to characterize the biophysical properties of Matrigel, Alginate and Matrigel/Alginate hybrid hydrogels. I have shown that Matrigel's high variability in composition is also reflected in its viscoelastic behavior. Storage, loss and relaxation moduli measurements highlight inconsistency in Matrigel's time-dependent mechanical properties.

In line with a recent study [3], I have shown that Alginate can have similar viscoelastic behavior to Matrigel at concentrations of 1% to 2% alginate (w/v).

From a mechanobiological standpoint, perhaps the most interesting observation of this study was the increase in both viscosity and elasticity of Matrigel 50%/Alginate 1% hybrid hydrogels compared to Matrigel 50% or Alginate 1% alone. This increased elasticity probably led to increased stress relaxation half time.

The hybrid hydrogel system described here can likely be implemented to support long-term brain organoid culture systems in a more defined, cost-effective manner to advance personalized medicine and drug discovery.

References

1. Cavo, M., et al., *A new cell-laden 3D Alginate-Matrigel hydrogel resembles human breast cancer cell malignant morphology, spread and invasion capability observed "in vivo"*. Scientific reports, 2018. **8**(1): p. 1-12.
2. Madl, C.M. and S.C. Heilshorn, *Engineering hydrogel microenvironments to recapitulate the stem cell niche.* Annual review of biomedical engineering, 2018. **20**: p. 21-47.
3. Capeling, M.M., et al., *Nonadhesive alginate hydrogels support growth of pluripotent stem cell-derived intestinal organoids.* Stem cell reports, 2019. **12**(2): p. 381-394.
4. Lancaster, M.A., et al., *Guided self-organization and cortical plate formation in human brain organoids.* Nature biotechnology, 2017. **35**(7): p. 659-666.
5. Quadrato, G., et al., *Cell diversity and network dynamics in photosensitive human brain organoids.* Nature, 2017. **545**(7652): p. 48-53.
6. Velasco, S., et al., *Individual brain organoids reproducibly form cell diversity of the human cerebral cortex.* Nature, 2019. **570**(7762): p. 523-527.
7. Pacitti, D., R. Privolizzi, and B.E. Bax, *Organs to cells and cells to organoids: The evolution of in vitro central nervous system modelling.* Frontiers in cellular neuroscience, 2019. **13**.
8. Oksdath, M., et al., *Synthetic scaffolds to control the biochemical, mechanical, and geometrical environment of stem cell-derived brain organoids.* APL bioengineering, 2018. **2**(4): p. 041501.
9. Kim, H.N. and N. Choi, *Consideration of the Mechanical Properties of Hydrogels for Brain Tissue Engineering and Brain-on-a-chip.* BioChip Journal, 2019. **13**(1): p. 8-19.
10. Thakuri, P.S., et al., *Biomaterials-Based Approaches to Tumor Spheroid and Organoid Modeling.* Advanced healthcare materials, 2018. **7**(6): p. 1700980.
11. Matyash, M., et al., *Swelling and mechanical properties of alginate hydrogels with respect to promotion of neural growth.* Tissue Engineering Part C: Methods, 2014. **20**(5): p. 401-411.
12. Fiorotto, R., et al., *Liver diseases in the dish: iPSC and organoids as a new approach to modeling liver diseases.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2019. **1865**(5): p. 920-928.
13. Orqueda, A.J., C.A. Giménez, and F. Pereyra-Bonnet, *iPSCs: a minireview from bench to bed, including organoids and the CRISPR system.* Stem cells international, 2016. **2016**.
14. Qian, X., et al., *Using brain organoids to understand Zika virus-induced microcephaly.* Development, 2017. **144**(6): p. 952-957.
15. Benito-Kwiecinski, S. and M.A. Lancaster, *Brain Organoids: Human Neurodevelopment in a Dish.* Cold Spring Harbor perspectives in biology, 2019: p. a035709.
16. Jackson, E. and H. Lu, *Three-dimensional models for studying development and disease: moving on from organisms to organs-on-a-chip and organoids.* Integrative Biology, 2016. **8**(6): p. 672-683.
17. Sun, A.X., H.H. Ng, and E.K. Tan, *Translational potential of human brain organoids.* Annals of clinical and translational neurology, 2018. **5**(2): p. 226-235.
18. Lancaster, M.A., et al., *Cerebral organoids model human brain development and microcephaly.* Nature, 2013. **501**(7467): p. 373.

19. Mosher, D.F., et al., *Assembly of extracellular matrix.* Current opinion in cell biology, 1992. **4**(5): p. 810-818.
20. Maeda, N., *Proteoglycans and neuronal migration in the cerebral cortex during development and disease.* Frontiers in neuroscience, 2015. **9**: p. 98.
21. Quondamatteo, F., *Assembly, stability and integrity of basement membranes in vivo.* The Histochemical journal, 2002. **34**(8-9): p. 369-381.
22. Akhmanova, M., et al., *Physical, spatial, and molecular aspects of extracellular matrix of in vivo niches and artificial scaffolds relevant to stem cells research.* Stem cells international, 2015. **2015**.
23. Kleinman, H.K., et al., *Basement membrane complexes with biological activity.* Biochemistry, 1986. **25**(2): p. 312-318.
24. Freire, E., et al., *Structure of laminin substrate modulates cellular signaling for neuritogenesis.* Journal of cell science, 2002. **115**(24): p. 4867-4876.
25. Kleinman, H.K. and G.R. Martin. *Matrigel: basement membrane matrix with biological activity*. in *Seminars in cancer biology*. 2005. Elsevier.
26. Kratochvil, M.J., et al., *Engineered materials for organoid systems.* Nature Reviews Materials, 2019. **4**(9): p. 606-622.
27. Aisenbrey, E.A. and W.L. Murphy, *Synthetic alternatives to Matrigel.* Nature Reviews Materials, 2020: p. 1-13.
28. Hughes, C.S., L.M. Postovit, and G.A. Lajoie, *Matrigel: a complex protein mixture required for optimal growth of cell culture.* Proteomics, 2010. **10**(9): p. 1886-1890.
29. Chaudhuri, O., *Viscoelastic hydrogels for 3D cell culture.* Biomaterials science, 2017. **5**(8): p. 1480-1490.
30. Weng, L., X. Chen, and W. Chen, *Rheological characterization of in situ crosslinkable hydrogels formulated from oxidized dextran and N-carboxyethyl chitosan.* Biomacromolecules, 2007. **8**(4): p. 1109-1115.
31. Steffe, J.F., *Rheological methods in food process engineering*. 1996: Freeman press.
32. Bonino, C.A., et al., *Real-time in situ rheology of alginate hydrogel photocrosslinking.* Soft Matter, 2011. **7**(24): p. 11510-11517.
33. Rahman, M., et al., *Morphological Characterization of Hydrogels.* Polymers and Polymeric Composites: A Reference Series, 2019: p. 819-863.
34. O' Brien, F.J., et al., *Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds.* Biomaterials, 2004. **25**(6): p. 1077-1086.
35. Lee, J.T.Y. and K.L. Chow, *SEM sample preparation for cells on 3D scaffolds by freeze-drying and HMDS.* Scanning, 2012. **34**(1): p. 12-25.
36. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nature methods, 2012. **9**(7): p. 671-675.
37. Chaudhuri, O., et al., *Hydrogels with tunable stress relaxation regulate stem cell fate and activity.* Nature materials, 2016. **15**(3): p. 326-334.
38. Obaid, N., M.T. Kortschot, and M. Sain, *Understanding the stress relaxation behavior of polymers reinforced with short elastic fibers.* Materials, 2017. **10**(5): p. 472.
39. Simpliciano, C., et al., *Cross-linked alginate film pore size determination using atomic force microscopy and validation using diffusivity determinations.* Journal of Surface Engineered Materials and Advanced Technology, 2013. **3**(04): p. 1.
40. Pescosolido, L., et al., *In situ forming IPN hydrogels of calcium alginate and dextran-HEMA for biomedical applications.* Acta biomaterialia, 2011. **7**(4): p. 1627-1633.

41. Roy, I. and M.N. Gupta, *Freeze-drying of proteins: Some emerging concerns.* Biotechnology and applied biochemistry, 2004. **39**(2): p. 165-177.
42. Rossen, N.S., et al., *Injectable therapeutic organoids using sacrificial hydrogels.* iScience, 2020: p. 101052.
43. Caliari, S.R. and J.A. Burdick, *A practical guide to hydrogels for cell culture.* Nature methods, 2016. **13**(5): p. 405.
44. Hohenester, E. and P.D. Yurchenco, *Laminins in basement membrane assembly.* Cell adhesion & migration, 2013. **7**(1): p. 56-63.