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3D biomaterial models of human brain disease

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ABSTRACT

Inherent limitations of the traditional approaches to study brain function and disease, such as rodent models and 2D cell culture platforms, have led to the development of 3D *in vitro* cell culture systems. These systems, products of multidisciplinary efforts encompassing stem cell biology, materials engineering, and biofabrication, have quickly shown great potential to mimic biochemical composition, structural properties, and cellular morphology and diversity found in the native brain tissue. Crucial to these developments have been the advancements in stem cell technology and cell reprogramming protocols that allow reproducible generation of human subtype-specific neurons and glia in laboratory conditions. At the same time, biomaterials have been designed to provide cells in 3D with a microenvironment that mimics functional and structural aspects of the native extracellular matrix with increasing fidelity. In this article, we review the use of biomaterials in 3D *in vitro* models of neurological disorders with focus on hydrogel technology and with biochemical composition and physical properties of the *in vivo* environment as reference.

1. Introduction

The brain is the most complex organ in the human body with unique architectural and functional features across scales. Traditionally, common experimental tools used to study brain function and disease have been rodent models and two-dimensional (2D) cell culture platforms. Both approaches have led to numerous breakthroughs in neuroscience research and still remain indispensable. However, their inherent drawbacks have become increasingly acknowledged in recent years. While experiments with rodents provide behavioral output and full biological complexity of a living organism, findings made from these studies often fail to translate to the clinic due to interspecies differences (Zeiss et al., 2017). On the other hand, 2D cell culture platforms provide a well-established simplistic way to deconstructs the incomprehensible complexity of neural tissue into observable biological processes under highly controlled laboratory conditions. However, culturing cells on rigid surfaces such as plastic or glass poorly matches the physiological environment. Cells in 2D assume flattened shape with adhesion restricted to a single plane and limited connections to neighboring cells (Fig. 1 left). These morphological changes lead to significant alterations in gene expression profiles, epigenetic features, and network activity that often render 2D cultures inadequate to model complex neurological diseases (Frega et al., 2014; Luo et al., 2016; Tekin et al., 2018).

In the living brain, numerous types of neurons and glia are organized in a three-dimensional (3D) space. The *in vivo* environment allows simultaneous connections between multiple cells, but also functional interaction between cells and the adaptive 3D network of macromolecules called the extracellular matrix (ECM) that surrounds them. The molecular components of the brain ECM are synthesized by neurons and glia themselves. While the ECM used to be considered as merely a passive mechanical support for the cells, nowadays it is accepted that the ECM is a highly dynamic and sophisticated network that directly regulates cellular fate and function. The ECM plays a critical role in the maintenance of structural integrity and homeostasis in neural tissue by providing adhesion sites, gradients of signaling molecules, nutrients and gases, and a diffusion barrier that ensures efficient signal propagation (Franco and Müller, 2011; Jakeman et al., 2014). Furthermore, it is actively involved in neurogenesis, cellular migration, neurite pathfinding, synaptogenesis, and long-term survival of terminally differentiated neurons (Ferrer-Ferrer and Dityatev, 2018; Long and Huttner, 2019; Suttkus et al., 2016).

Unlike 2D cultures that are limited by fundamental dimensional constraints, 3D platforms have shown great potential to better mimic aspects of cellular heterogeneity, spatial organization, biochemical

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composition, and mechanical properties of the native tissue (Fig. 1 right) (Camp et al., 2015). The development of increasingly sophisticated 3D models has become possible due to the convergence of advancements across different disciplines. Breakthroughs in stem cell biology, genetic engineering, and cell reprogramming techniques have allowed *in vitro* generation of subtype-specific human neurons and glia from renewable cell sources (Dolmetsch and Geschwind, 2011). Together with the cutting edge tools for single cell sequencing and bioinformatic analysis of genomic, epigenomic, and transcriptomic data, this has paved the way to a deeper understanding of human-specific processes underpinning brain development, function, and disease (Atamian et al., 2020).

Simultaneously, a multitude of biomaterials have been developed to mimic the brain ECM and as such supply physiologically relevant structural and functional 3D support to cells cultured *in vitro* (Cembran et al., 2020). These materials, natural or synthetic, can be designed with tunable mechanical, topological, and biochemical cues in order to evoke cellular response analogous to those found *in vivo*. Additionaly, biomaterial scaffolds can provide a dynamic environment that can be remodeled by cells or serve as a substrate for temporally controlled release of biochemical cues or formation of gradients, an important aspect in the study of brain development and repair (Xu et al., 2018; Zheng et al., 2019). Another advantage of 3D platforms is the possibility to achieve long-term maturation and maintenance of sensitive neuronal populations that would otherwise spontaneously lose adhesion and degrade in planar cultures. This makes 3D cultures a particularly useful tool for the study of neuronal function and slow-developing human--specific pathological phenotypes related to neurodegenerative diseases.

In this review we will discuss the use of biomaterials, predominantly hydrogels, as ECM-mimicking scaffolds in 3D *in vitro* neural cultures with focus on neurological disease modeling. We will first provide an overview of the biochemical and physical properties of the native brain ECM. Then we will review how hydrogels with different origins (natural ECM-derived or synthetic) allow 3D neural disease modeling. Finally, we will discuss the future prospect of different bioengineering approaches that may enhance the handling, patterning, and implementation of 3D neural cultures.

2. Properties of the native brain ECM

In this section we will give a brief overview of the molecular and mechanical properties of the ECM in healthy and diseased brain. The grasp of the complexity discovered in the native tissue provides a reference point for the engineering of materials that attempt to mimic it. This information also reveals the striking gap between the *in vivo* environment and the most advanced *in vitro* models developed so far.

2.1. Biochemical heterogeneity

Molecular composition and organizational properties of the brain ECM has been studied for decades and reviewed extensively (Novak and Kaye, 2000; Rauti et al., 2019). Even though it shows similarities with the ECMs of other tissues, the matrix found in the brain contains distinct features and uniquely expressed components. On the one hand, adult brain tissue is characterized by low quantities of fibrous proteins such as collagen and fibronectin that are found in abundance in the rest of the body. On the other hand, the brain ECM is rich with glycosaminoglycans (GAGs), which are negatively charged polysaccharide compounds that form long linear chains. The only form of GAGs that is found in the unbound form are hyaluronic acid (HA) polymers. HA is one of the main components of the brain ECM that directs cell migrations through specific cell surface bound receptors and maintains the ECM integrity through interaction with other components of the matrix (Laurent et al., 1996).

All other GAGs are covalently linked to proteins to form proteoglycans (PGs). There are multiple types of PGs in the brain with the two most represented ones being heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs). Some HSPGs are cell-surface associated (e.g. membrane-anchored protein glypican and transmembrane protein syndecan). These HSPGs are known to be major regulators of developmental processes through their interaction with key molecules such as the fibroblast growth factors, WNT, and hedgehog (Condomitti and De Wit, 2018). Other HSPGs such as perlecan and agrin are excreted in the extracellular space where they provide mechanical reinforcement and facilitate diffusion of molecules (Bishop et al., 2007). The CSPGs consist largely of HA-binding proteins called lecticans (e.g. aggrecan, versican, neurocan, and brevican). These proteins are

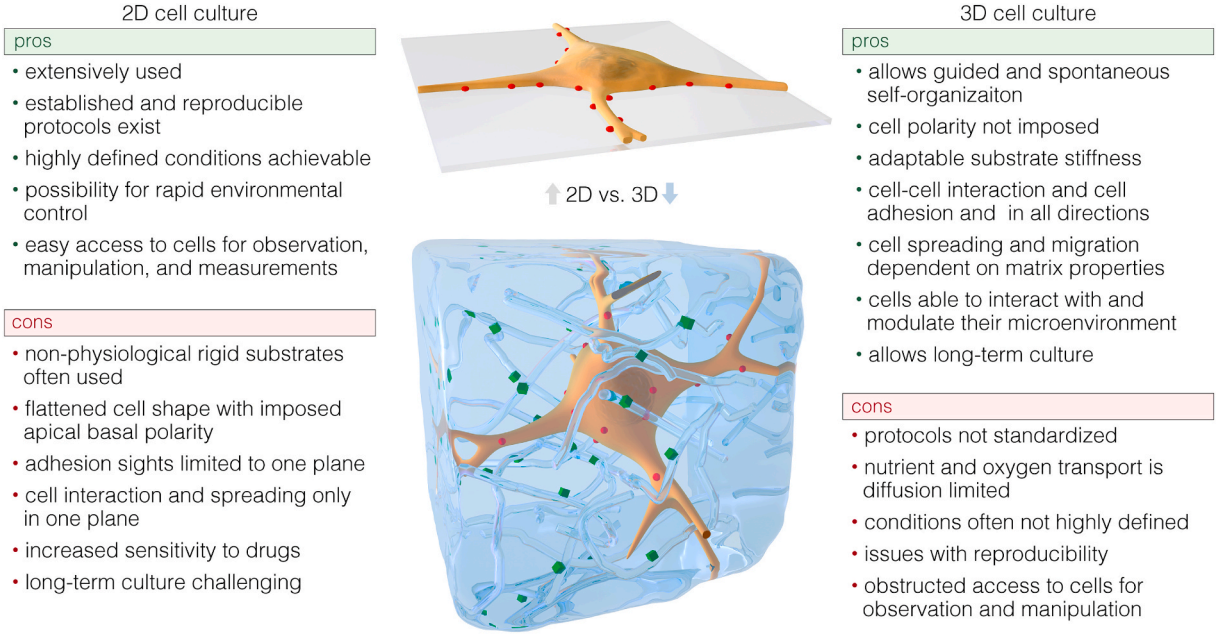

Fig. 1. Advantages and disadvantages of 2D and 3D cell culture: In the illustrations, red spheres represent cell adhesion sights, green cubes represent bioactive molecules, and transparent cylindrical structures represent the polymer network of the hydrogel.

involved in a variety of important processes such as cellular migration, axon guidance, gliogenesis, and neurite outgrowth (Avram et al., 2014).

In addition to HA and PGs, the ECM also contains other glycoproteins. For example, Hapln (hyaluronan and proteoglycan link protein) is a glycoprotein that facilitates connections between different components of the ECM (Oohashi et al., 2015). Tenascin-R is a brain-specific glycoprotein that interacts with collagens to promote or inhibit cell adhesion (Anlar and Gunel-Ozcan, 2012). A major family of fibrous glycoproteins in the brain is the laminin family. Laminins are associated with processes related to neural progenitor proliferation, neuronal migration and survival, and synaptic activity (Nirwane and Yao, 2019). Collagen IV and fibronectin are also fibrous glycoproteins that can be found in the brain ECM and, despite not being abundant, represent key structural elements that promote cell adhesion, chemotaxis, mechanical integrity, and direct developmental processes (Novak and Kaye, 2000).

The structural dynamics and functional activity of the extracellular space are further regulated by ECM-modulating enzymes and ECM-binding factors. The former (e.g. matrix metalloproteinase, hyaluronidase, heparanase) are essential for the regulation of the ECM topography through remodeling and degradation. The ability of the ECM to bind soluble components such as growth factors and cytokines limits their diffusion through the tissue to allow fine-tuning of their local concentration and formation of gradients.

During development, the brain ECM undergoes large-scale spatiotemporal transformations of its composition. For example, CSPG levels fluctuate throughout development with high expression detected during embryogenesis, but with reduced levels found in the mature brain (Bandtlow and Zimmermann, 2000). Also, relative abundance of GAGs and PGs in the stem cell niche precisely regulates neural stem cell differentiation and self-renewal (Reinhard et al., 2016). Moreover, common fibrous ECM proteins are highly expressed during brain development to serve as substrates for extensive cellular migration (Lau et al., 2013). The levels of these proteins are then drastically reduced through targeted enzymatic activity in the adult brain.

In contrast to the changes taking place during development, the ECM composition of the adult human brain under homeostasis is considered to be relatively stable. The ECM network is present throughout the whole brain and comprises approximately a fifth of its volume. Interestingly, the molecular composition of the matrix is different for different brain regions reflecting their distinctively specialized function (Dauth et al., 2016). Regardless of region-specific differences, the ECM microenvironment can be divided into three major components: the basement membrane (basal lamina) that surrounds blood vessels; the perineuronal nets (PNNs) that surround dendrites and neuronal cell bodies; and the interstitial matrix, a diffusely distributed matrix between brain parenchymal cells (Lau et al., 2013). Each of these compartments is comprised of different combination of ECM components (Fig. 2) that further contribute to the heterogeneity of the brain ECM.

2.2. Tissue mechanics and mechanotransduction

The importance of mechanical cues in neuronal development and pathology is increasingly recognized. These cues (e.g. shear stress, compression loading, ECM rigidity) affect the cells through mechanotransduction, a process where mechanical forces are translated into biochemical signals that can modulate cell proliferation, differentiation, migration, and survival (Barnes et al., 2017). Mediators of mechanotransduction are a family of transmembrane proteins called integrins that bind to the ECM and transmit the biophysical forces into the cell, subsequently initiating rapid responses in cellular mechanics through cytoskeletal remodeling and long term changes in the form of altered gene expression (Z. Sun et al., 2016). In the brain, integrins play a key role in the maintenance of dendritic spines, pathfinding and growth of neuronal projections, as well as in the formation and plasticity of synapses (Lilja and Ivaska, 2018). For example, abnormalities in β3 integrin molecule whose expression regulates serotonin levels in the brain, have been linked to autism spectrum disorder and schizophrenia (Carter et al., 2011; Wang et al., 2013).

Brain tissue is one of the softest tissues in the human body. This softness arises from the lack of fibrous proteins that directly correlate with stiffness of other tissues, and high water content (around 80% of total mass) arising from the abundance of highly polar PGs that attract water molecules (Dityatev et al., 2010). The elastic modulus of neural tissue has been reported to be in the range from 100 Pa to 3 kPa depending on the part of the brain tested and the measurement technique used, indicating its complex viscoelastic properties and heterogeneous mechanical constitution (Budday et al., 2020; Oksdath et al., 2018).

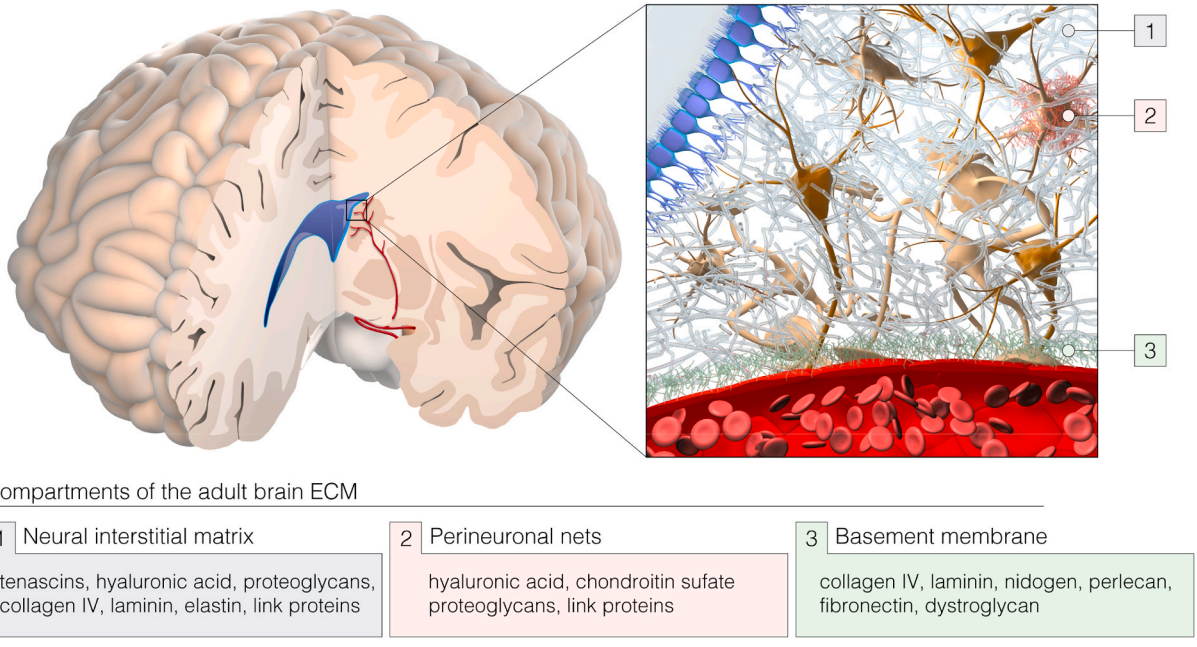

Fig. 2. Heterogeneity of brain tissue: Simplified illustration of the complexity found in native brain tissue including different cell types (e.g. astrocytes, microglia, neurons), a blood vessel, the ventricular zone, and multiple compartments of the ECM.

It has been shown that intrinsic mechanical properties of the ECM can independently guide stem cell differentiation in the absence of biochemical cues. The effect of the mechanical properties of cell culture substrates on stem cell differentiation and neural growth and maturation has been extensively studied (Kim and Choi, 2019). For example, undifferentiated stem cells grown on substrates mimicking the elasticity of a given tissue were found to express precursor proteins for the cell type typically present in that tissue (Reilly and Engler, 2010). Specifically, *in vitro* studies have confirmed that neural progenitor cells, neurons, and glia respond best to soft substrates (Cukierman et al., 2008). These substrates have shown to induce *in vivo*-like morphology in neurons with more elaborate branching. Furthermore, neurons grown on softer substrates formed more than three times as many branches as those grown on stiffer gels (Flanagan et al., 2002). These findings indicate that local matrix stiffness together with the availability of integrin-binding ligands plays an important factor in activation and inhibition of certain cellular functions and needs to be considered when designing biomimetic materials.

2.3. Disease-related changes in ECM composition

Considering the important role that the brain ECM plays in the control of developmental processes, maintenance of homeostasis, and propagation of neuronal signaling, it is not surprising that many pathological conditions are characterized by transformations in the ECM composition and structure (Bonneh Barkay and Wiley, 2009). For example, mutations in type IV collagen give rise to cerebral small vessel disease (Shi and Wardlaw, 2016). Altered expression profiles of several GAGs and PGs have been observed in schizophrenia and suggested to be linked to propagation and clearing of extracellular plaques and neurofibrillary tangles in Alzheimer's disease (AD), hallmark pathologies that arise from aggregates of amyloid β protein and abnormal tau proteins respectively (Lepelletier et al., 2017; Sethi and Zaia, 2017). Defective transformations of the ECM have also been reported in a mouse model of α-synuclein-induced dopaminergic neurodegeneration, a prominent neuropathology associated with Parkinson's disease (PD). These transformations included changes in nanoscale diffusion parameters and HA remodeling that could contribute to the spreading of neurodegeneration (Soria et al., 2020). ECM molecules have also been linked to epilepsy with observations that epileptic seizures cause drastic changes in the ECM structure and that the ECM reciprocally assists the spread of epileptic molecular pathologies (Dityatev, 2010). In multiple sclerosis (MS), active brain lesions show parenchymal accumulation of laminin, usually associated with the basal lamina, and upregulation of matrix metalloprotease-19 expressed by microglia (Van Horssen et al., 2007).

Moreover, disease-related modulation of the ECM structure has a direct effect on physical properties of neural tissue that in turn influences the activity of highly mechanoresponsive neurons and glia. An indication that mechanics play an important role is that the tissue can become diseased in response to abnormalities in mechanical cues, such as changes in ECM stiffness or an increase in interstitial pressure (Barnes et al., 2017). It has been shown, using magnetic resonance elastography measurements, that brain tissue becomes significantly softer in MS and AD patients, even though neural tissue generally stiffens with age (Streitberger et al., 2012). Likewise, changes in physical properties of brain tissue have been detected with the progression of PD. In particular, distinct hyperechogenicity patterns (increased response during ultrasound measurements) in substantia nigra have been observed in PD patients that likely reflect elevated presence of heavy metals such as iron (Berg, 2011). Another example of striking changes in physical properties caused by the perturbed matrix within the brain is the presence of tumors. Lower-grade glioma and glioblastoma show incrementally stiffer characteristics in comparison with non-tumorigenic gliotic tissue. Glioblastomas exhibit upregulated production of HA and tenascin-C, the ECM component that crosslinks lecticans bound to HA. Progressive formation of HA-lectican-tenascin-C complex restricts the ECM elasticity and increases the rigidity of the tissue (Goldbrunner et al., 1999).

3. Disease modeling in biomimetic hydrogels

Hydrogels are hydrated 3D networks of crosslinked hydrophilic polymer chains that can retain a large amount of water (over 90%) while maintaining solid form. They are the most common type of biomaterial used to support bioengineered 3D neural cultures as their physical and chemical properties can be tuned to mimic the native brain ECM (Yildirimer et al., 2019). Both natural (e.g. bulk ECM-extracts or individual ECM components) and synthetic polymers have been developed to form hydrogels that contain mechanical, architectural, and biochemical cues found *in vivo*. Furthermore, different mechanisms (e.g. enzymatic, ionic, crosslinker-based, light activated, or pH and temperature dependent) have been employed for the crosslinking of liquid hydrogel precursors into soft elastic solids. Depending on the starting cellular configuration, hydrogels can be used to provide support to a single-cell suspension in a more conventional 3D culture format or to be used as an embedding medium for aggregated stem cells for the generation of brain organoids (Fig. 3). In this section we will review the use of hydrogels, according to their origin, in 3D *in vitro* models of brain diseases.

3.1. Natural hydrogels from bulk ECM-extracts

3.1.1. Matrigel

Matrigel is one of the most common biomaterials used in cell culture systems, both as coating in 2D cultures and as 3D hydrogel scaffold. It is a solubilized basement membrane extract from Engelbreth-Holm-Swarm mouse tumor that forms a hydrogel at 37 °C. Matrigel is abundant with growth factors and ECM proteins such as laminin (~55%), collagen IV (~30%), entactin (~6%), and heparan sulfate proteoglycans, but contains also more than thousand other distinct components (Hughes et al., 2011; Kleinman et al., 1986). Together with the soft physical properties of the formed polymer network (450 Pa elastic modulus) (Soofi et al., 2009), this complex mixture of functional and structural molecules provides a favorable environment for 2D and 3D *in vitro* neural culture applications (Irons et al., 2008).

The advent of brain organoid technology has initiated a surge in the use of Matrigel for 3D cultures in the neuroscience field. Brain organoids are 3D *in vitro* systems that are able to recapitulate structural and functional processes of the developing brain by relying on the self-organization capability of differentiating human pluripotent stem cells, leading to a higher cellular diversity and an enhanced maturation profile compared to conventional cell culture systems (Nascimento et al., 2019). Brain organoids are formed from stem cell aggregates commonly encapsulated within a Matrigel drop that provides key biochemical cues for cellular reorganization, neural progenitor sheet polarization, and neuroepithelial bud formation and expansion (Lancaster et al., 2013).

When combined with genetic engineering, single-cell transcriptomics, and methods for recording of functional outputs, brain organoid technology amounts to a powerful tool that provides researchers with new opportunities to investigate fundamental questions regarding neural development and neurological disorders (Table 1) (Amin and Paşca, 2018; Wang, 2018). For example, the pioneering study that introduced the first protocol for brain organoid formation demonstrated their potential to model microcephaly (Lancaster et al., 2013). These organoids, termed cerebral brain organoids, rely on spontaneous intrinsic self-organization of stem cells and result in the formation of a heterogeneous structure with entities resembling several brain regions (e.g. forebrain, hindbrain, retina). In addition to microcephaly, cerebral organoids have been utilized to model aspects of Rett syndrome, brain tumor formation, Miller Dieker syndrome, AD, frontotemporal dementia, autism, and COVID-19 (Bershteyn et al., 2017; Bian et al., 2018; Ghatak et al., 2019; Mellios et al., 2018; Ramani et al., 2020; Seo et al., 2017; Wang et al., 2017).

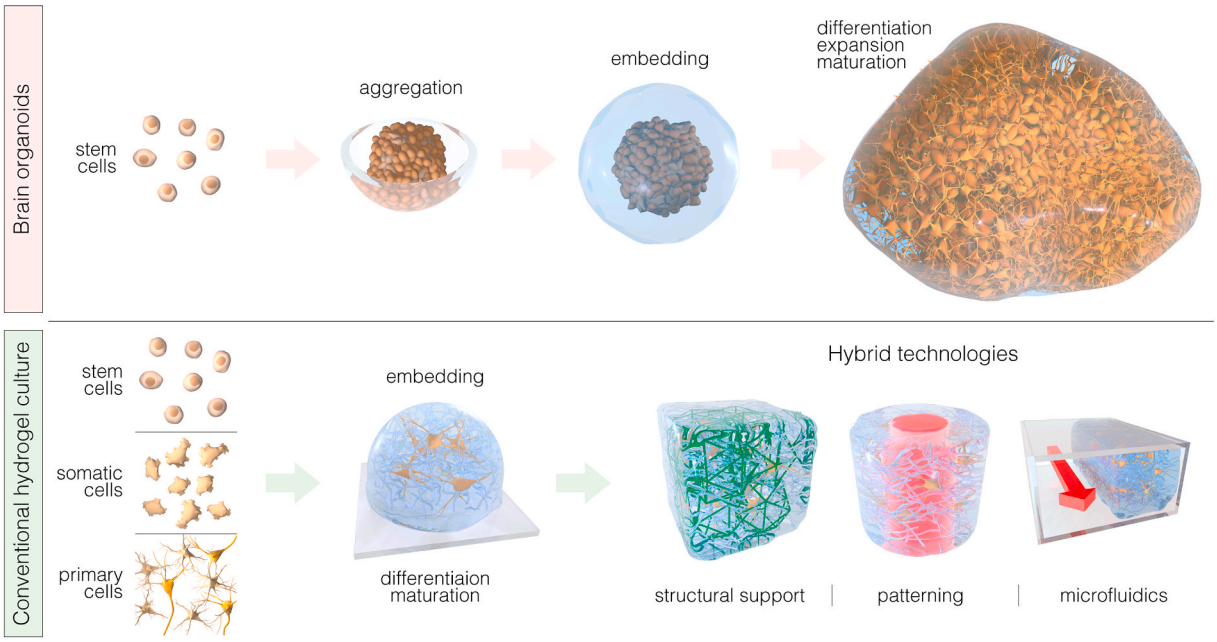

Fig. 3. Two major routes for the generation of 3D hydrogel cultures: Top sequence depicts the formation of brain organoids from self-assembled neurospheres embedded in hydrogels. The bottom part of the figure depicts formation of 3D neural cultures generated by embedding single-cell suspensions in hydrogel. Bottom right illustration represents different hybrid technologies used to enhance 3D hydrogel cultures by providing structural support, patterning, or perfused microfluidic channels.

Table 1
Examples of disease modeling in brain organoids.

Disease	Reference	Remark
Autism spectrum disorders	Mariani et al. (2015)	Neurogenic defects revealed in patient-derived forebrain organoids.
Glioblastoma	Bian et al. (2018)	Modeling tumorigenesis in cerebral organoids by introduction of oncogenic mutations
Alzheimer's disease	Ghatak et al. (2019)	Aberrant electrical activity and neurite morphology in cerebral organoids with AD-associated mutations
	Pavoni et al. (2018)	Chemical induction of Aβ accumulation in cerebral organoids
Rett syndrome	Mellios et al. (2018)	Defects in neurogenesis and neuronal differentiation in cerebral organoids
Parkinson's disease	Kim et al. (2019)	α-syn pathology in patient-derived midbrain organoids
	Smits et al. (2019)	Loss of number and complexity of dopamine neurons in midbrain-like organoids
	Ahfeldt et al. (2020)	Oxidative stress in midbrain organoid model of PD
	Kwak et al. (2020)	Dopamine neuron-specific toxicity to MPTP stress in midbrain-like organoids
Retinal dystrophy	Parfitt et al. (2016)	Dysfunction of ciliogenesis in retinal organoids
Microcephaly	Lancaster et al. (2013)	Neurogenic defects in cerebral organoid model of microcephaly
	Esk et al. (2020)	Screening technology enables identification of microcephaly genes and mechanisms involved in brain size control
Miller Dieker syndrome	Bershteyn et al. (2017)	Migration and mitotic defects in a neurodevelopmental model of lissencephaly
Schizophrenia	Ye et al. (2017)	Defective neurogenesis and new mechanism of actions for schizophrenia pathogenesis
ZIKA virus infection	Qian et al. (2016)	ZIKA virus promote death of neural progenitor in forebrain organoids
COVID-19	Ramani et al. (2020)	Virus exposure associated with changes in Tau distribution, hyperphosphorylation, and neuronal death

In addition to intrinsically specified brain organoids, directed organoids and region-specific brain organoids can be formed by the addition of extrinsic patterning molecules that mimic biochemical cues present during the development of the particular brain region of interest (Xiang et al., 2020). The application of exogenous patterning factors provides a way to increase reproducibility (one of the main pitfalls in the brain organoid field) and reduce tissue heterogeneity, thus allowing researchers to model diseases that affect distinct brain subdomains. PD, for instance, is characterized by targeted degeneration of dopaminergic neurons in substantia nigra pars compacta located in the midbrain. To this end, midbrain organoids have been developed to study several aspects of PD such as selective susceptibility of dopamine neurons to neurotoxins, α-synuclein pathology induced by LRRK2 mutation, oxidative stress damage, and disease-related decrease in subtype-specific neuronal development and complexity (Ahfeldt et al., 2020; Kim et al., 2019; Kwak et al., 2020; Smits et al., 2019). Organoid cultures that recapitulate forebrain development have been used to model Zika virus infection and dysregulation of inhibitory neurons in autism spectrum disorder (Mariani et al., 2015; Qian et al., 2016). Generation of retinal organoids has allowed the study of inherent blindness caused by Leber congenital amaurosis (Parfitt et al., 2016). Furthermore, an organoid platform mimicking hippocampal formation has been established with potential to study diseases such as AD and schizophrenia (Sakaguchi et al., 2015). Organoids also provide a platform for the maintenance of mixed cultures of glial cells and neurons, an important step towards the recapitulation of the physiological environment (Krencik et al., 2017; Sloan et al., 2017).

In a more traditional manner compared to brain organoid cultures, Matrigel has been used as 3D extracellular support of single cell suspensions (Table 2) (Dana et al., 2014). By providing cells with native tissue-mimicking microenvironment, it allows studies of disease pathologies not observable in 2D cultures, such as the presence of extracellular deposits of β-amyloid peptides, one of the hallmark pathologies of AD. In 2D, the secreted β-amyloid quickly diffuses in the cell culture media and is consequently removed with each media change. On the other hand, in 3D culture, Matrigel matrix provides a diffusion barrier

Table 2
Examples of disease modeling in non-organoid 3D hydrogel cultures.

Disease	Reference	Material	Cell type	Remark
Rett Syndrome	Zhang et al. (2016)	HA	iPSCs	3D culture reported to improve neuronal maturation and electrophysiological activity.
Glioblastoma	Yi et al. (2019)	brain dECM	glioblastoma cells	3D bioprinted model demonstrates patient-specific responses to chemoradiotherapy.
	Gomez-Roman et al. (2017)	Matrigel	glioblastoma cells	Better translational potential of cellular responses to drug and radiation exposure.
	Koh et al. (2018)	brain tumor dECM	glioblastoma cells	Mode and dynamics of glioblastoma cell invasion.
	Sood et al. (2019b)	silk, HA, collagen, dECM	primary brain tumor cells	Patient-derived pediatric and adult human brain tumor response.
	Lee et al. (2019)	fibrin	glioblastoma cells	More physiological response to drug screening compared to planar culture.
Alzheimer's disease	Zhang et al. (2014)	SAPs	iPSCs	Recapitulation of amyloid β pathology.
	Labour et al. (2016)	collagen	neuronal-like cells	Amyloid β pathology driven by hydrogel porosity.
	Choi et al. (2014)	Matrigel	neural precursor cells	Protein pathology in 3D model of AD.
	Rouleau et al. (2020)	silk, Matrigel	iPSCs	Long term 3D cultures reveal signs of neurodegeneration and AD-related pathology.
	Papadimitriou et al. (2018)	PEG-heparin	primary human fetal astrocytes, iPSCs	Impact of ECM on proliferation and neurogenic activity.
	Park et al. (2018)	Matrigel	neurons, microglia, astrocytes	Protein aggregation and neuroinflammatory response observed in 3D triculture.
Parkinson's disease	Bolognin et al. (2019)	Matrigel	iPSCs	More robust PD-related pathology observed in comparison to 2D culture.
	Moreno et al. (2015)	Matrigel	iPSCs	Microfluidic technology for automated, high-throughput screening for drug discovery.
	Taylor-Whiteley et al. (2019)	Matrigel	SH-SY5Y cells	3D culture recapitulates *in vivo*-like Lewy body pathology.
Ischemic stroke	Jin et al. (2018)	brain dECM	primary mouse fetal fibroblast	Direct reprograming in 3D hydrogels reported to improve neural conversion and subsequent motor recovery in animal model.

(one of the elementary roles of the native ECM) that promotes aggregation of β-amyloid peptides in the extracellular space. The 3D Matrigel culture promoted an increase in neuronal differentiation and dramatic elevation of misfolded filamentous tau protein (4R adult isoform), another AD pathology (Kim et al., 2015). Most importantly, the study showed that tauopathy can be reduced by inhibiting β-amyloid formation thus providing experimental validation of the hypothesis that toxic β-amyloid species are able to cause tauopathy in absence of degenerative tau mutations (Choi et al., 2014). A more recent study incorporated astrocytes and microglia in a 3D *in vitro* model of AD to successfully demonstrate elements of neuroinflammatory response (e.g. microglia recruitment and axonal cleavage) in addition to β-amyloid aggregation and phosphorylated tau accumulation (Park et al., 2018).

3D Matrigel cultures have also been explored as approaches for the enhanced generation of dopaminergic neurons as a mean to study the processes that underlie PD (Moreno et al., 2015). By coupling 3D culture with high content microscopy and image analysis algorithms, it has been demonstrated that a PD-specific mutation in the LRRK2 gene leads to decreased dopaminergic differentiation, reduced neurite complexity, changes in mitochondrial morphology, and increased cell death (Bolognin et al., 2019). Interestingly the study showed that it is the genetic background of PD patients rather than LRRK2 mutation that had the strongest contribution to the development of the described dopaminergic phenotype, which was successfully rescued after inhibition of LRRK2 inhibitor 2. In another study, fully differentiated human neuroblastoma cells grown in 3D rapidly developed Lewy body-like pathology (ubiquitin-positive nuclear aggregates of α-synuclein, characteristic for PD) when exposed to exogenous α-synuclein in absence of protein overexpression (Taylor-Whiteley et al., 2019). It is noteworthy that phosphorylated α-synuclein was not observed in these aggregates. Matrigel has also been a key component to form a 3D compartmentalized model of demyelination characteristic for neuropathy of the peripheral nervous system (Hyung et al., 2021).

Furthermore, models of glioblastoma have been developed in 3D and 2D Matrigel cultures to investigate drug and radiation responses (Gomez-Roman et al., 2017). Compared to planar cultures, the results from 3D experiments predicted more reliably the therapeutic outcomes observed in clinical trials thus indicating better translational potential of cells grown in a 3D microenvironment.

Despite the fact that Matrigel is a material of choice for many researchers, owing to its availability and ease of use, it has well known inherent limitations. Its composition remains poorly defined, is subject to batch-to-batch variability, and without the possibility of tuning neither mechanical nor biochemical properties (Aisenbrey and Murphy, 2020).

3.1.2. *Decellularized tissue hydrogels*

Decellularization is a process of selective removal of cellular and genetic material from tissue specimen. Acellular tissue largely preserves ECM-composition and organ-specific architecture. It can be further processed (through controlled mechanical and enzymatic digestion) and solubilized into viscous solution that gels at 37 °C. Decellularized ECM (dECM) from a variety of tissues has been shown to provide a physiologically relevant platform for cell culture (Saldin et al., 2017; Zhang et al., 2009). In particular, brain tissue-specific dECM accelerates network formation and neuronal maturation both in 2D and 3D *in vitro* systems (Dequach et al., 2011; Lam et al., 2019). Processed dECM is also a favorable environment for brain organoid formation (Simsa et al., 2021). It has also been observed that cells react differently to dECM hydrogels derived from different brain regions, indicating brain-region-specificity of ECM composition (Reginensi et al., 2020). Furthermore, compared to its adult counterpart, composition of fetal dECM displayed higher amount of GAGs and resulted in formation of denser axonal networks with spontaneous activity of embedded primary rat cortical neurons (Sood et al., 2016).

One study explored the potential of dECM derived from human specimen as a 3D substrate for direct non-viral conversion of mouse embryonic fibroblasts into therapeutic neurons for neural disorders (Jin et al., 2018). These dECM hydrogels boosted the conversion efficacy and enhanced the generation of neurons with mature phenotype. It is postulated in the study that a soft dECM environment stimulates mechanosensitive signaling and histone modification involved in neuronal conversion.

Brain dECM has also been utilized to generate 3D *in vitro* human brain tumor models. In one instance, patient-specific glioblastoma cells were encapsulated in brain dECM hydrogel and surrounded by vascular

endothelial cells in a concentric ring arrangement that recapitulates biochemical and structural properties of native tumors along with the radial oxygen gradient (Yi et al., 2019). This glioblastoma-on-a-chip system has reproduced clinically observed variability in treatment resistance between patients and therefore provides potential means for identification of effective treatment for glioblastoma patients. In another study, both patient-specific cells and patient-specific brain tumor dECM (providing both composition and microarchitecture of native brain tumor) were incorporated in 3D culture models used to investigate mode and dynamics of glioblastoma invasion (Koh et al., 2018). Utilizing this model, researchers showed that glioblastoma cells are able to rapidly adapt their migratory strategy to the specific local microenvironment in order to achieve higher invasiveness. These findings could lead to a better understanding of cell-ECM interactions in brain tumors and consequently to more efficient ECM-targeted treatments.

3.2. Natural hydrogels from purified and recombinant ECM-components

The complex heterogenous composition of cell culture microenvironments generated via top-down approaches described above cannot be fully defined and only minimally altered. Fortunately, individual components found in the native ECM can be purified from animal sources or produced as recombinant proteins. These components can then be used as building blocks in a bottom-up approach for the generation of hydrogel scaffolds with defined structural and functional elements (Murphy et al., 2017).

Collagen type I, owing to its availability, biocompatibility, and ease-of-use, has been the most common single-component natural hydrogel scaffold in neural cell culture applications. It is derived from animal sources and processed into an acidic liquid solution that gels with a pH change to the physiological range. Despite the fact that collagen is not abundant in the native brain ECM, it contains cell-binding sites that provide a favorable substrate for stem cell differentiation, neuronal growth and migration, axonal elongation, as well as for functional network formation (Bourke et al., 2018). To create hydrogels with the stiffness that resembles native tissue, mechanical properties of collagen hydrogels can be directly regulated via the manipulation of collagen concentration or degree of crosslinking (Iwashita et al., 2019). Furthermore, biological significance of collagen hydrogels can be enhanced by incorporation of glycans to the collagen backbone (Rebelo et al., 2020).

Adaptable porosity of collagen scaffolds provides a way to tune the diffusion rate of soluble extracellular components such as β-amyloid peptides. Taking advantage of this approach, researchers have been able to promote the formation of dense β-amyloid plaques within the hydrogel and more importantly to show that these aggregates become toxic only when the ECM microenvironment allows their rapid diffusion and access to differentiating cells (Papadimitriou et al., 2018). In another study focused on AD, collagen hydrogels were utilized to create an *in vitro* model of the blood-brain-barrier affected by the disease, which showed recapitulation of several key aspects, such as increased permeability of the barrier, increased expression of reactive oxygen species, and deposition of β-amyloid peptides at the vascular endothelium (Shin et al., 2019). 3D neural cultures in collagen hydrogels, when reinforced with silk fibers, have shown potential for long term studies of neurodegenerative diseases (Cantley et al., 2018). One study showed that neurons and glia, generated from AD patient-derived stem cells, could be maintained in these hydrogels for more than 2 years with stable cell retention and stress markers (Rouleau et al., 2020). Similar collagen hydrogel systems have been used to explore the microenvironmental role in pediatric and adult glioblastoma (Sood et al., 2019b). The use of collagen for brain organoid formation constitutes one of the first steps towards bioengineered brain organoids with tunable environment for disease modeling (Zafeiriou et al., 2020). It has also been instrumental in the generation of a microphysiological platform to model amyotrophic lateral sclerosis through interaction of optogenetic motor neurons and muscle cells (Osaki et al., 2018b).

In vivo experiments revealed that reactive astrocytes are able to switch their function from neuro-supportive to toxic within the progression of neurodegenerative diseases (Li et al., 2019). The study of astrocytic reactivity is limited in planar cultures since astrocytes assume a highly reactive state in 2D. On the other hand, 3D collagen hydrogels provide a physiological environment for maintenance of astrocytes in a state of reduced reactivity. Importantly, higher reactive state in these astrocytes could be triggered to allow real-time monitoring of astrocytic function (East et al., 2009).

HA, an ubiquitous structural backbone of the native brain ECM, has been extensively studied as a 3D substrate for neural cultures (Khaing and Seidlits, 2015). Since its unmodified form does not allow cell attachment, HA needs to be functionalized with cell binding motifs or mixed with other components (e.g. collagen, fibronectin, laminin) (Suri and Schmidt, 2010). Functionalized HA hydrogels have been shown to promote neural development, neurite elongation, and functional network formation (Broguiere et al., 2016; Tarus et al., 2016).

One study demonstrated a layered HA hydrogel system developed to investigate migration and maturation defects in Rett syndrome (Zhang et al., 2016). Their findings revealed aberrant migration behavior in human stem cell-derived neural progenitors containing a mutation in the MECP2 gene associated with the disease. Furthermore, neurons derived from these cells formed fewer synapses and had reduced neurite elongation.

A number of scientific publications have described the use of HA hydrogels in the development of glioblastoma models. For example, one group mixed HA hydrogels with a synthetic component and gelatin (denatured collagen still containing functional cell-binding motifs) to create a 3D platform with adoptable adhesive and microstructural environment to investigate the role of EGFR tyrosine kinase inhibitors in tumorigenesis (Pedron et al., 2013). They further showed that both crosslinked and soluble HA polymers impacts glioblastoma sensitivity and resistance to these inhibitors (Pedron et al., 2017). HA based hydrogels were used in other studies to recreate a ECM-mimicking system with tunable stiffness and ligand functionalization to elucidate mechanobiological effects in glioma cell morphology and invasion (Ananthanarayanan et al., 2011; Xiao et al., 2018).

Laminin is another key ECM component that has been used extensively in *in vitro* cell cultures. Individual isoforms of laminin are cell-type specific and are able to facilitate a variety of functions from maintenance of stem cell pluripotency to directing cell fate specification (Hagbard et al., 2018). As laminins are not able to form hydrogel scaffolds on their own, they serve as a complementary component to enhance the bioactivity of established hydrogel platforms (e.g. HA or collagen) by providing meaningful cell-matrix interaction without changes in the mechanical stiffness (Barros et al., 2019; Swindle-Reilly et al., 2012).

Fibrin, a fibrous protein that is formed from fibrinogen through the enzymatic activity of thrombin, occurs naturally at the sights of peripheral nerve damage. It has been shown that fibrin is able to promote growth of axonal projections both *in vitro* and *in vivo* (Man et al., 2011; Wilems et al., 2015). While it is mostly applied in the studies of nerve regeneration after spinal cord injury (Yu et al., 2020), fibrin holds promise to be used in *in vitro* neurological disease models. For example, fibrin has been used as a 3D scaffold for successful differentiation of human stem cells into neurons (Abelseth et al., 2019; Arulmoli et al., 2016; Bayat et al., 2016; Sharma et al., 2020). It has also been successfully utilized to create a 3D glioblastoma model for drug screening that shows increased resistance in comparison to 2D culture (Lee et al., 2019).

3.3. Synthetic hydrogels

While polymers of natural origin provide high physiological relevance, they are inherently limited with batch-to-batch variation,

constrained relationship between physical properties and functional groups, and insubstantial number of available components (compared to the native microenvironment). On the other hand, synthetic hydrogels are able to mimic prevalent elements of the brain ECM without these restrictions (Aisenbrey and Murphy, 2020). Engineering synthetic networks allows stringent, reproducible and, most importantly, fully independent control over physical and chemical properties of the hydrogel. With this approach, the density and the type of a large number of physiologically relevant functional ligands within the scaffold can be precisely controlled. Cell-degradable sites can be introduced in order to create pliable hydrogels susceptible to cell remodeling. Furthermore, hydrogel stiffness can be modulated independently of other parameters, providing a platform for direct study of physical cues.

Poly(ethylene glycol) (PEG) has been the most commonly used synthetic polymer scaffold for cell culture applications due to its availability and biocompatibility. Inherently inert, modulus-tunable PEG backbone can be biofunctionalized to, for example, promote induction of stem cell pluripotency, direct neural stem cell differentiation (e.g. to dopaminergic fate), or control neurite outgrowth (Adil et al., 2017; Caiazzo et al., 2016; Hynes et al., 2009; Mahoney and Anseth, 2006). Modeling developmental events in synthetic hydrogels has demonstrated that early neurogenesis can be precisely controlled by the extracellular cues, allowing observation of symmetry-breaking cellular reorganization that leads to neural tube development (Ranga et al., 2016). PEG hydrogels have also been utilized in 3D models of neurological disorders. One group developed a reductionist culture system that promotes human neural stem cell proliferation and neurogenesis in order to study how these cells lose their plasticity in AD. They found that administration of β-amyloid causes characteristic AD pathology and hampers neural stem cell plasticity by inducing kynurenic acid production that can be rescued by interleukin-4 cytokines (Papadimitriou et al., 2018). Another study used PEG-HA hydrogels with decoupled mechanical and biochemical properties to create an artificial niche for the study of glioblastoma cell fate in relation to matrix stiffness. They discovered that glioblastoma cells can employ different HA synthases and matrix metalloproteinases to remodel their environment as a response to varying mechanical cues (Wang et al., 2014). Synthetic hydrogels were also employed, for the first time *in vitro*, to control astrocyte quiescence and activation on demand, providing a tool to explore the functional role of astrocytes in healthy and diseased brain. Furthermore, this study showed that, in order to achieve the native star-like shape of astrocytes, they need to be able to cleave metalloproteinase-degradable peptides incorporated in the synthetic matrix (Galarza et al., 2020).

Self-assembling peptides (SAPs) provide another approach for rational design of synthetic hydrogels. When exposed to an aqueous environment, these amphiphilic co-polymers, synthesized from naturally occurring amino acids, self-assemble into 3D networks with an exposed hydrophilic sequence and sequestered hydrophobic core. RADA16, which is the most widely studied SAP for cell culture applications, spontaneously forms a network of interwoven β-sheets with more than 99% water content (Cormier et al., 2013). It has been used to support reprogramming and maturation of human neurons as well as to investigate β-amyloid pathology and the effect of F-actin proteins in AD (Francis et al., 2016; Marchini et al., 2020; Y. Sun et al., 2016).

4. Enhancing hydrogel cultures with hybrid technologies

4.1. Secondary polymers for the structural support

Hydrogels designed to mimic the exceptionally soft native brain tissue are inherently flawed due to structural instability, an effect that is further enhanced over long culturing periods due to cell-induced hydrogel degradation. To address this issue, a secondary polymeric network can be incorporated in the 3D culture to provide mechanical integrity. Silk, a natural polymer extensively used in tissue engineering due to its biocompatibility, processibility, and mechanical properties, has been successfully incorporated into collagen and dECM hydrogels in several studies of neuronal development, function, and disease (Cantley et al., 2018; Rouleau et al., 2020; Sood et al, 2016, 2019a, 2019b; Tang-Schomer et al., 2014). Furthermore, fibrous scaffolds prepared via electrospinning and highly porous scaffolds made via freeze-drying (cryogels) could be employed to enhance neuronal cultures as a structural support as well as a route towards more efficient tissue oxygenation and nutrient supply (Baiguera et al., 2014; Béduer et al., 2015; Jurga et al., 2011; Liu et al., 2016; Ucar et al., 2021). Recently, a 3D printed scaffold has been used as a support for the generation of planar brain organoids that feature gyrification (Rothenbücher et al., 2021).

4.2. Patterning methods

Defined spatial patterning of soft hydrogels is challenging due to the poor structural integrity of these materials. In the pre-gel stage, soft hydrogels are viscous liquids and as such are not able to hold the predefined architecture. Even after gelation takes place, the crosslinked hydrogels are affected by body forces, such as sagging and buckling, that prevent them from conforming with the defined shape. Several methods have been developed in order to overcome these issues such as cryogenic 3D printing, embedded printing in granular supports, bio-acoustic levitation, patterning of porous silk scaffolds, building-block assembly, molding, and printing of layered hydrogel structures (Bhattacharjee et al., 2015; Bouyer et al., 2016; Kato-Negishi et al., 2013; Lozano et al., 2015; Pagan-Diaz et al., 2019; Tan et al., 2017; Tang-Schomer et al., 2014). Furthermore, orientation of fibrous proteins, such as collagen, can be influenced by shear forces in order to create aligned networks able to direct growth of neurites (Kim et al., 2017; Lanfer et al., 2010).

4.3. Microfluidics

Incorporation of microfluidic channels into 3D cell culture models delivers several attractive benefits. Most notably, microfluidic technology allows oxygenation of thick tissue constructs, continuous delivery of nutrients, and removal of waste products. Leveraging the effects of capillary forces and laminar flow, defined 3D cell cultures and co-cultures with perfused channels can be engineered in microfluidic devices, enabling the study of stem cell differentiation, cellular crosstalk, paracrine signaling, and vascular network generation (Adriani et al., 2017; Osaki et al., 2018a; Yamada et al., 2016; Yang et al., 2015). These platform have further enhanced the study of neurological diseases such as PD, AD, and glioblastoma (Ayuso et al., 2017; Moreno et al., 2015; Park et al., 2018). Importantly, microfluidic approaches have been developed for robust generation and maintenance of molecular concentration gradients with defined steepness. These platforms provide an invaluable tool to study developmental processes (e.g. neural tube formation) as well as neurite guidance (Kothapalli et al., 2011; Rifes et al., 2020; Xu et al., 2018). Microfluidic systems have also been extensively explored as platforms for blood-brain-barrier models to facilitate high-throughput drug screening and to study the physiologically relevant role of the blood-brain-barrier in different neurological diseases (Oddo et al., 2019; Shin et al., 2019).

5. Concluding remarks

Recent breakthroughs in cell reprogramming, gene editing, and single cell transcriptomics have spurred a growing interest in 3D models of brain development and disease, with brain organoid technology at the forefront. In parallel, advances in biomaterials engineering have opened up new routes towards recapitulation of native ECM *in vitro*, thus providing cultured cells with a more physiologically relevant microenvironment. However, the functional use of biomaterials in these cell culture models is still in its infancy and afflicted by numerous limitations. One major problem is the lack of a bioengineering platform that is easily accessible and provides compositional complexity while

maintaining parametric space needed for the systematic investigation of individual components. For example, bulk ECM extracts such as Matrigel and dECM are highly complex but with fixed and poorly defined composition riddled with batch-to-batch variability. On the other hand, individual ECM components such as collagen and HA allow generation of compositionally defined hydrogels with finetuned physical properties. However, this reductionist approach lacks the complexity of the native tissue and provides only rudimentary functional moieties such as generic adhesion peptides. Synthetic hydrogels hold great promise to overcome limitations of natural polymers as their chemical and physical properties can be tuned independently with the possibility to include a large number of biofunctional groups found *in vivo*. Unfortunately, more widespread use of this approach is hampered by high cost and the need for specialized knowledge in chemistry and bioengineering. Due to its simplicity and effectiveness, most biology- and neuroscience-oriented research groups retreat to using Matrigel (or collagen) as a well-established off-the-shelf solution that does not require expertise in hydrogel technology. This leads to the creation of 3D models that, even though advantageous in comparison to 2D cultures, are characterized with high variability and do not allow exploration of the effects different aspects of the ECM elicit on cellular identity, maturation, and function. On the other hand, bioengineering- and materials science-oriented research groups that have access to more advanced hydrogel technologies are limited in their capacity for in-depth cellular studies necessary to address more relevant biological questions. Therefore, meaningful future advancements in 3D models of neurological disorders will require close interdisciplinary collaboration between material scientists, bioengineers, neuroscientists, and stem-cell biologists. Considering the progress made in each one of these fields independently, the opportunity to combine them holds great promise to yield 3D cultures with higher complexity, closer resemblance to native human tissue, and ability for high-throughput drug screening. Moreover, the application of microfluidic and biofabrication techniques will allow modeling of the interactions between different brain regions as well as between different organs in the human body. We anticipate that these multidisciplinary efforts will lead scientists to novel insights into brain function and disease, allowing discovery of novel treatments for patients suffering from neurological disorders.

Author statement

JK has determined the structure of the review and written most of the text. FN contributed to the section on natural hydrogels and put together the tables. AF contributed to the section on brain organoids. MP and JE provided critical input on cellular models and hydrogel systems respectively.

Decleration of competing interest

The authors declare no competing interest.

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