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Advances in 3D tissue models for neural engineering: self-assembled *versus* engineered tissue models

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Neural tissue engineering has emerged as a promising field that aims to create functional neural tissue for therapeutic applications, drug screening, and disease modelling. It is becoming evident in the literature that this goal requires development of three-dimensional (3D) constructs that can mimic the complex microenvironment of native neural tissue, including its biochemical, mechanical, physical, and electrical properties. These 3D models can be broadly classified as self-assembled models, which include spheroids, organoids, and assembloids, and engineered models, such as those based on decellularized or polymeric scaffolds. Self-assembled models offer advantages such as the ability to recapitulate neural development and disease processes *in vitro*, and the capacity to study the behaviour and interactions of different cell types in a more realistic environment. However, self-assembled constructs have limitations such as lack of standardised protocols, inability to control the cellular microenvironment, difficulty in controlling structural characteristics, reproducibility, scalability, and lengthy developmental timeframes. Integrating biomimetic materials and advanced manufacturing approaches to present cells with relevant biochemical, mechanical, physical, and electrical cues in a controlled tissue architecture requires alternate engineering approaches. Engineered scaffolds, and specifically 3D hydrogel-based constructs, have desirable properties, lower cost, higher reproducibility, long-term stability, and they can be rapidly tailored to mimic the native microenvironment and structure. This review explores 3D models in neural tissue engineering, with a particular focus on analysing the benefits and limitations of self-assembled organoids compared with hydrogel-based engineered 3D models. Moreover, this paper will focus on hydrogel based engineered models and probe their biomaterial components, tuneable properties, and fabrication techniques that allow them to mimic native neural tissue structures and environment. Finally, the current challenges and future research prospects of 3D neural models for both self-assembled and engineered models in neural tissue engineering will be discussed.

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1. Introduction

Neural function relies on systematic interactions between the external environment and the exquisite nervous system structures comprising more than 100 billion neuronal cells, extracellular matrix (ECM), signalling molecules and electrical signals.[1,2] Damage to neural tissue disrupts connections, leading to permanent loss of sensory and motor functions, isolating affected individuals.[3,4] Laboratory research often focuses on 2D cell culture systems, which have limitations such as lack of *in vivo*-like structures, limited cell–cell interaction, and cellular heterogeneity.[5,6] To better understand neural damage mechanisms and develop repair methods, studying biology on the bench needs to be more representative of human neural physiology. The emergence of *in vitro* 3D human cell culture techniques has great potential for overcoming the limitations of 2D approaches.[7] However, despite the advances in developing 3D culture approaches, the high complexity of neural tissues challenges the current capacity to accurately model neural tissue on the bench.

Neural tissue intricacy includes networks formed by neurons through dendrites and axons, crucial for connecting tissues and transmitting signals.[8] The network is highly spatially organized comprising complex and high fidelity features with axon diameters ranging from 1 to 100 micrometres with lengths that can extend to over 1 meter.[9,10] Given this complexity, studying nerve tissue regeneration or repair

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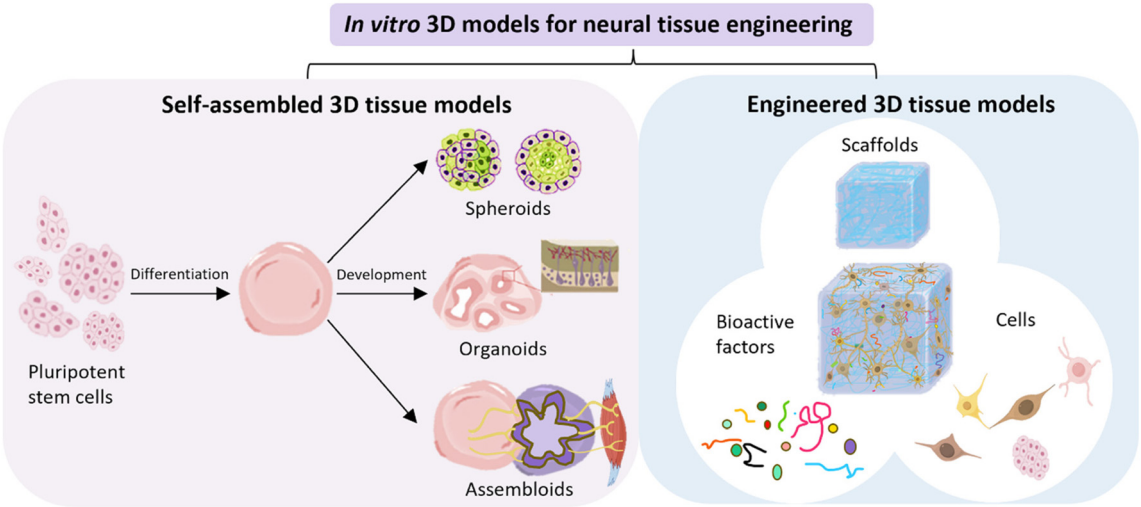

Fig. 1 Schematic overview of two main categories of 3D models for *in vitro* neural tissue engineering. Self-assembled 3D tissue models (left) and engineered 3D tissue models (right).

strategies can be greatly supported by replicating the neural system microenvironment *in vitro* integrating biochemical, mechanical, physical, and electrical cues.[11] This review explores 3D models in neural tissue engineering, emphasizing self-assembled organoids and hydrogel-based engineered 3D models. As summarised in Fig. 1, self-assembled models allow stem cells to organize into aggregates and tissue-like arrangements, while engineered models involve designing structures with relevant cell types and biomaterial scaffolds. However, both approaches have limitations in replicating tissue architectures and appropriate ECM and cellular signalling.

Self-assembled models are generated by providing appropriate environments for stem cells to organise and develop into 3D structures such as spheroids, organoids, or assembloids (Fig. 1).[12,13] These models are typically exploited to study the behaviour and interactions of differentiated cell types in environments that more accurately reflect the cellular and ECM composition of neural tissues than 2D cultures.[14] In contrast, engineered models are generated by designing the desired structures and fabricating them through the integration of relevant cell types with biomaterial scaffolds, fulfilling the tissue design requirements (Fig. 1).[15] Through manipulating the microenvironment surrounding the cells, engineered 3D tissues promote specific cellular behaviours, such as proliferation or differentiation.[16] However, both approaches have limitations. Structures in most self-assembled 3D neural tissue models do not replicate tissue architectures that occur in the nervous system,[17,18] whereas engineered models rely on fabricated structures that may not mimic the appropriate ECM and cellular signalling.[12,13]

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Both self-assembled and engineered tissue structures have a role in generation of new knowledge of neural cell and tissue functions. Self-assembled tissues have been applied to studying cell, tissue, and disease development and are ideal for evaluating interactions of drugs with tissues.[19,20] Engineered tissue models are typically centred on applications in regenerative medicine, including approaches to repair and replace tissues.[7,21,22] One of the applications of 3D engineered tissue models which attracts significant interest is the development of tissue and organ systems which can be used to test medical devices on the bench.[23,24] Such models require complex anatomical structures combined with living cells to study how devices impact on surrounding tissues and microenvironment as well as to support rapid throughput studies to accelerate product development.[25,26] Given that self-assembled models are not able to develop these complex architectures, researchers must consider how engineered 3D structures could be optimised to provide better models of neural tissue. Therefore, the selection of biomaterial components, manipulation of properties, and choice of fabrication techniques emerge as pivotal considerations during the development of engineered models.

Many different biomaterials have been developed and studied for fabricating tissue engineered scaffolds. However, soft neural tissue structures ideally require similarly soft ECM-like materials to provide appropriate biochemical and mechanical environment.[27,28] Neural ECM is a complex mixture of matrix molecules including large glycoproteins, laminin, collagen, and fibronectin that assembles into fibrils or other complex macromolecular structures.[29,30] ECM plays a crucial role in neural development *in vivo* and is a major niche element serving as a scaffold for cell support to regulate cell behaviours.[31,32] Hydrogels, comprising both biological and synthetic polymers, have potential to be engineered to mimic neural tissue ECM, thereby providing an ideal model for physiologically relevant ECM environments.[33,34] However, considering the microenvironment in neural tissue, the 3D scaffold is expected to meet biochemical, mechanical, and electrical requirements at the same time in a system to comprehensively reproduce the native tissue.[35]

Such 3D tissue-mimicking substrates can be fabricated into complex shapes and tailored to provide specific mechanical properties. With the capacity to integrate ECM and soluble biological molecules, hydrogels provide an ideal platform for developing functional and organised structures.[34,36] The combination of hydrogels with other fabrication techniques, such as mould casting and bioprinting, allows fabrication of highly complex and multi-functional neural tissue models that more accurately reflect the natural tissue microenvironment.[23,33] However, to represent the natural cellular environment comparable to that observed in native tissue, and to a degree in self-assembled models, engineered models require more advanced hydrogel based biomaterials and fabrication approaches.

Self-assembled and engineered models both offer unique advantages and limitations, and the choice of which model to use depends on the specific final application. While engineering materials and fabrication approaches have great promise for development of biomimetic neural tissue models, there remain significant challenges with recapitulating native ECM structures, situating cells in the appropriate niches and integrating chemical and electrical signalling. This review will systematically discuss the advantages, challenges, and future perspectives associated with self-assembled and engineered models, especially in hydrogel-based biomaterials, their properties, and fabrication for engineered models, shedding light on their contributions to our understanding of neural development and potential therapeutic applications for neurological disorders.

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2. Self-assembled 3D neural tissue models

Human self-assembled 3D tissue models are generated by human pluripotent stem cells (hPSCs), which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).[37] Self-assembled models are known for their remarkable self-organization when spontaneously aggregated into 3D structures. These can be classified based on their developmental complexity level as spheroids, organoids, or assembloids (Fig. 1).[14] With the development of stem cell research and advancement of techniques, more intricate organ-like models have been created, which provide researchers with promising platforms to study complex physiological processes and offer novel experimental models that, in part, bridge the gap between animal models and human biology.[38–40] This section will discuss each of these self-assembly models in more detail.

2.1. Spheroids, organoids, and assembloids

Spheroids are simple 3D spherical cellular aggregates that form primarily through cell-to-cell adhesion (Fig. 1). They offer a platform to study intricate cell–cell and cell–matrix interactions, as well as differentiation processes and drug responses.[38,41] While spheroids do not have complex tissue structures, they can overcome some of the limitations of traditional 2D cell culture approaches to replicate natural cellular responses *in vitro*, as shown in Fig. 2.[42] Generating spheroids can be achieved through various techniques that spatially concentrate monodispersed cells to promote cell–cell adhesion and impede interaction with other substrates.[43] In approximately two weeks cells aggregate into spherical-shaped, multilayered structures that range in size from several hundred micrometres to several millimetres,[44,45] which can be maintained over long-term culture.[20,46] With the implementation of diverse tissue culture techniques spheroids can be grown to display features of the neuronal tissue milieu that closely represent those *in vivo*.[20,45]

Spheroids have been used to mimic essential characteristics of neural structures like brain tissues, including diverse cell types, electrical activity, production of ECM, and mechanical properties. For instance, spheroids formed from neonatal rat cortex cells have been shown to develop electrically active neural networks, secrete relevant ECM molecules like laminin, and present an elastic modulus comparable to that of the native *in vivo* tissue.[47] Spheroids also allow manipulation of specific cell populations, which can be useful for studying the role of different cell types in tissue development and function.[48] Biopsy samples or surgical resections can isolate tumour cells to grow as spheroids, which can be used to model disease development.[49] For example, Seidel *et al.* used human neuroblastoma cell line (SH-SY5Y) which came from bone marrow of a patient with neuroblastoma, to generate spheroids with overexpress EGFP-fused tau as a model to study the pathologies of tau protein in Alzheimer Disease.[50]

The development and differentiation of spheroids can be promoted and regulated by adjusting cell types and cell ratios. Song *et al.*, investigated the effect of cell ratio on the expression of neural trophic factors, ECM molecules, and neural differentiation *in vitro*.[51] This study showed that co-culturing iPSC with human mesenchymal stem cells (hMSC) at a ratio of 25 : 75 supported a significantly higher expression of neuronal markers like β-III tubulin as compared to 50 : 50 and 75 : 25 ratios, and substantially higher than that of iPSC-only cultures. In addition, adjusting cell ratio and cell type can provide control of ECM deposition and thus influence cellular behaviour. For instance, the 25 : 75 iPSC-MSC group exhibited increased expression of collagen IV, while the expression of laminin appeared to rise with the proportion of hMSC. Also, the incorporation of hMSC can substantially upregulate the expression of proliferating and tissue forming cytokines like transforming growth factor-β1 and prostaglandin E2.[51]

While the cellular organization into 3D spheroids is largely defined by random cell interactions, the arrangement of cells into tissue structures can be influenced through incorporation of heterotypic cells prior and following formation of spheroid structure. For instance, Liqing *et al.*,[51] showed that in co-cultures where both hMSC and iPSC are monodispersed and mixed, cell organization is driven based on the relative degree of adhesiveness of each cell type. With hMSC showing stronger

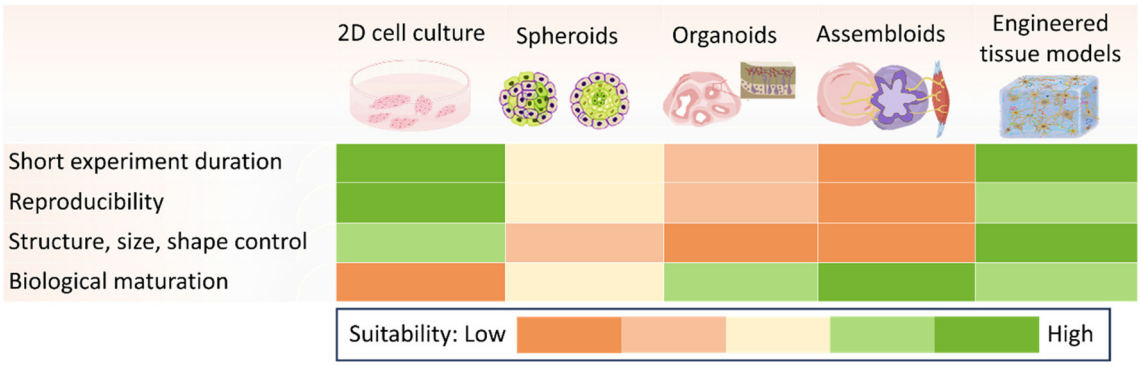

Fig. 2 A representation of the advantages and disadvantages of the common models in neural tissue engineering.

cell–cell interactions than iPSC, this co-culture generated spheroids with a core of hMSC and an outer shell of iPSC. Whereas culturing monodispersed hMSC with pre-formed iPSC spheroids allows for the formation of an iPSC core with a hMSC outer shell. While spheroids can better represent native tissue compared to traditional 2D counterparts, they are not able to mimic the *in vivo* structure and functionality of native tissues and organs.[38] More advanced tissue models are required to present the complex physiological structure and environment.

Under specific conditions, the hPSC self-organize into complex, multicellular structures resembling mini organs, called organoids (Fig. 1).[6,52,53] Spheroids and organoid models have overlapping and distinct purposes (Fig. 2). Both can develop tissue models to study neuronal cell development under healthy and pathological conditions in 3D, thus enabling the study of previously inaccessible aspects in a controlled laboratory setting. The key difference is that organoids with more complex structures can often better represent the cellular heterogeneity and physiological functionality of organs as illustrated in Fig. 2. Organoids more closely resemble the native tissue both histologically and genetically.[6] Brain organoids can model many features of the early development of the human brain and are a powerful *in vitro* system to model congenital brain disorders induced by neurotrophic viral infections, or inherited genetic mutations.[54] For example, Latour *et al.*, generated GLB1 knockout cerebral organoids from a isogenic iPSC lines with lysosomal β-gal deficiency by employing CRISPR/Cas9 genome editing to model human GM1 gangliosidosis in the central nervous system.[55] These features of organoids support a wide range of applications in neural development research, including the study of neural diseases, neural injury repair, and the establishment of fundamental knowledge in the field.

Although organoids have an important role as a research tool in medicine and biology, they do not recreate the body's interconnectedness and are only adapted for studies on a single organ type.[56] To address these limitations, studies have explored strategies to optimize the culture conditions for organoids, for example development of a vascularised system to provide a closer-to-ideal growth environment. Vascularisation can encourage the formation of structural units that partially resemble structure and function of organs, and promote maturity and support nutrient delivery.[57] Worsdorfer *et al.*, incorporated human mesodermal progenitor cells (MPCs) into neural organoids that resulted in a vascularised and well-structured neuroepithelium.[58]

Integration of organoids with other organoids as well as spheroids or engineered tissue models, known as assembloids, has allowed creation of more complex and physiologically relevant systems for neural tissue engineering.[52] Assembloids have emerged as an advanced tool that facilitate the spatial organization of multiple types of cells, tissues, or organoids (Fig. 1).[59] Their primary function is to replicate the complex microenvironment and intricate interactions between multiple organs and tissues, thereby enabling more accurate representation of tissue structure, function, and molecular mechanisms involved in development and disease modeling.[60]

By integrating different tissue types into a single 3D model, assembloids allow for the study of inter-organ and inter-tissue interactions, which are crucial for understanding physiological processes and disease pathogenesis.[61,62] These interactions can involve cell–cell communication, cell–ECM interactions, and the formation of functional units that resemble natural tissue architecture.[59] Assembloids have a potential to more closely mimic the complexity and heterogeneity of native neural tissue including cell–cell interactions and circuit formation, as compared with spheroids or organoids(Fig. 2).[60] For instance, to study the human cortico-striatal pathway and how its dysfunction leads to neuropsychiatric disease, Miura and colleagues developed a method to model long-range neuronal connectivity in human brain assembloids by generating 3D spheroids resembling specific domains of the nervous system and then integrating them physically to allow axonal projections and synaptic assembly.[63] Furthermore, assembloids can be constructed from multiple organoids to create "multi-region assembloids", which further enhances their capability to mimic the complexity of native tissue structures and interactions across different regions of the body.[64]

Despite their potential, one significant drawback of assembloids is the technical complexity involved in their construction.[65] Integrating multiple types of cells, tissues, or organoids into a single 3D model requires precise control over spatial organization and cell–cell interactions, which can be challenging to achieve consistently.[66] Moreover, assembloids may struggle to accurately replicate the dynamic and intricate physiological structures found *in vivo*, leading to limitations in their ability to fully recapitulate complex tissue behaviours.[59,67] Additionally, the scalability and reproducibility of assembloids across different experimental setups may be hindered by their intricate nature and the variability inherent in their construction process.[56] These challenges highlight the need for further refinement and optimization of assembloid technologies to fully harness their potential in neural tissue engineering and disease modelling.[68]

In summary, spheroids, organoids, and assembloids as self-assembling tissue models represent remarkable advancements in neural tissue engineering, offering versatile platforms for studying complex neural processes *in vitro*. These tissue structures, mimicking the architecture and functionality of native tissues to varying extents, have revolutionized our approach to understanding neural development, and disease mechanisms. However, despite their potential, challenges and limitations persist in effectively recapitulating *in vivo* tissue complexity with self-assembled models.

2.2. The challenges of self-assembled 3D neural tissue model

Self-assembled 3D systems go part of the way to bridging the gap between conventional 2D *in vitro* models and animal models. However, the potential for assembloids as *in vivo* tissue surrogates is still challenged. While limited access to primary tissue for comparison purposes has been one factor,[64]

the challenges extend further to encompass various tissue design elements, summarised in Fig. 2, which have not been successfully addressed. As discussed in this section, such challenges include long developmental times, poor reproducibility, uncontrolled structure, size and shape, and insufficient biological maturation.[69,70]

A significant challenge in self-assembled systems is the long developmental time to differentiate from stem cells to 3D organoids.[71] Reported development times for organoids and assembloids in the neural tissue engineering field are up to 6 months, depending on the system complexity.[72] For instance, assembloids modelling spinal cord multi-synaptic circuitry *in vitro* can take up to 50 days,[73] whereas brain assembloids require 3 to 4 months to develop.[63] Similarly, organoids targeting the formation of neuromuscular junctions[74] as well as cerebral cortex[75] and cortico-motor models[73] can require up to 5 months for development. Generating and maintaining assembloids throughout this time frames can be difficult and costly, often requiring precise timing in culture conditions and several iterations to adjust cell-type ratios.[64] Notably, the process requires complex multidisciplinary skills and know-how,[56] overall challenging their reproducibility.

While organoids produced from the same cellular batches or in replicate batches under similar experimental conditions are expected to be morphologically and phenotypically similar, there is notable variation in the shape and size of individual organoids within the same batch. Typically, these organoids are smaller than the actual size of the organ *in vivo*.[14] For instance, cerebral organoids modelling the human cerebral cortex have been reported to have a diameter of approximately 4 mm, substantially smaller than the target tissue with a diameter close to 5 cm.[17] Moreover, the culture of self-assembled models requires the use of animal-based ECM, such as Matrigel™ or Basement Membrane Extract.[6,76] These extracts have inherent batch-to-batch variability in their composition, which may affect the reproducibility of experiments.[77,78] For instance organoids grown on Matrigel substrates have variable architectures as compared to organoids grown on synthetic scaffolds with chemically defined composition that yield more reproducible structures.[70,79] Moreover, the inconsistent mechanical and biochemical properties observed both within individual batches and across different batches have resulted in ambiguity and reduced reproducibility in cell culture experiments.[80,81]

Incomplete biological maturation is another significant challenge faced by self-assembled systems in accurately modelling native tissue *in vitro*. This can manifest as a lack of specific cell types, an inability to replicate functional tissue structures, and undersized structures. For instance, current cortical spheroids have been shown to generate various cortical cell types, including neural progenitors, mature neuron subtypes, and astrocytes. These cells self-organize into distinct cortical layers and establish functional neural networks. However, they still lack oligodendrocytes, the myelinating glia of the central nervous system.[82]

Guiding the development of specific organ structures poses significant challenges, as these structures are vital for tissue functionality, encompassing ducts, canals, ventricles, and vascular networks crucial for facilitating mass transfer of physiological fluids.[17,83,84] In particular, the limited diffusion of nutrients and oxygen represents a well-documented constraint in organoid culture,[71] and self-assembling tissue models, impeding the formation of higher order tissue structures. As organoids grow in size, diffusion-dependent nutrient supply and waste removal become less efficient, often resulting in necrosis at the centre of organoids.[52,85] These mass transfer limitations are a key factor that affects the terminal size and maturation of organoids and typically results in a necrotic core in larger tissue masses. To overcome these limitations, organoid culture protocols often use dynamic systems like spinning bioreactors to enhance diffusion.[52] However, these dynamic culture processes can generate mechanical stress such as shear forces during rotation, which can affect the shape and structure of organoids.[86] Recent advancements in engineering technology have aimed to address this issue, through the development of sophisticated devices that facilitate nutrient transportation into the core of the organoid and by co-culturing blood vessels alongside the organoid.[87]

In addition, stem cells, in their spontaneous organization, conform to specific structures influenced by the complex composition of ECM and soluble factors. Orchestrating such complex microenvironment *in vitro* is a key challenge to address for enabling control on the organization and structure of organoids during the culture process.[88] It is expected that these challenges can be overcome when combining self-assembling systems with emerging advanced biomaterial-based cell supporting technologies. The next section discusses the potential to develop engineered tissues, crafted from a combination of cells, substrates, and bioactive factors. This holds significant promise in addressing several challenges of self-assembled models, such as structural control, reproducibility, and maturation, in a precise manner.[36]

3. Engineered 3D neural tissue model

Engineering neural tissue models involves integrating cells with biomaterials and culture media that provide supporting growth and developmental factors.[89] These models have emerged as a promising approach to overcome some of the challenges of self-assembled models.[90,91] Moreover, the engineered tissue models with isolated cells from patients can be applied on functional tissue regeneration and replacement, or disease modelling to optimize current disease treatments.[92] While differentiation and maturation of neural cells still requires biological processes that are difficult to direct, engineered models aim to present cells with a defined supporting milieu, theoretically improving on reproducibility and development time.[93] However, designing and developing functional nerve tissue models requires considering several factors and environmental conditions that support neuronal tissue grow

and function.[7,94] As described in this section, this involves biochemical, mechanical, physical, as well as electrical factors reported in literature that support grow, differentiation, survival, and function of neural cells.[95]

Selection of biomaterials is crucial for engineering neural tissue models *in vitro*.[33] Hydrogels, among various technologies, show great promise and are widely employed as scaffolds in nerve tissue engineering.[25,34,96] Table 1 summarises common uses of hydrogel biomaterials for neural tissue engineering. They exhibit high cyto-compatibility, permeability to nutrients, and mechanical properties closely resembling those of biological tissue.[97] Hydrogels, which are polymer systems extensively investigated for tissue engineering,[98] form networks through physical or chemical crosslinks.[99] The design of these networks can allow for incorporation of bioactive molecules in substrates with various degradation profiles or may be non-degradable.[100] Also, the structure and mechanical properties of hydrogel networks are controllable through the selection of fabrication techniques and chemical compositions.[101]

Hydrogels are commonly fabricated from natural materials such as decellularized tissue matrix, ECM proteins, animal or plant derivatives, polysaccharides and protein-engineered materials,[102–105] or synthetic materials like poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), and hybrid matrices.[106–108] While synthetic polymers enable the precise control of hydrogel mechanical properties, they often lack cellular activity due to the absence of bioactive elements.[97] On the contrary,

Table 1 Application of biomaterials in 3D hydrogel for neural tissue engineering

Biomaterials				
Natural	Synthetic	Cell types	Application for neural tissue engineering	Ref.
Fibrinogen	N/A	Transgenic mouse ESCs	Neurons and astrocytes differentiation	102
Collagen-1, gelatine, laminin, poly-D-lysine, fibronectin, Matrigel, hyaluronic acid	N/A	Mouse ESCs	Neural and glial cells differentiation and maturation	103
Fibrin, hyaluronic acid, laminin	N/A	Human neural stem/progenitor cells (SC27 and SC23)	Neurons and astrocytes differentiation; vascularization	111
Chitosan	N/A	Motor neuron-like neural stem cells-34 cell line	Cell survival and neuronal differentiation	105
Collagen	N/A	Mouse endogenous neural stem cells	Neurogenesis and inhibited astrogliogenesis	112
Matrigel	N/A	iPSC line HCS1–2	Neuronal survival, differentiation, and maturation	104
Geltrex, gelatin methacrylate (GelMA), arginine-glycine-aspartate acid (RGD)	N/A	hiPSCs, human neuroblastoma SH-SY5Y	Stemness maintain and neuronal differentiation support	113
Laminin 111 alginate	N/A	hiPSCs	Long-term survival, differentiation into neurons and astrocytes as well as synaptogenesis	114
Alginate functionalized with RGD	N/A	Human SH-SY5Y cells	Cell survival and neuronal differentiation	115
Alginate, gellan gum, laminin	N/A	hiPSC-IMR90 lines	Differentiate into neurons and astrocytes and display spontaneous intracellular calcium signals	116
GelMA	N/A	Bone mesenchymal stem cells (MSCs), neural stem cells	Survival, proliferation, neural stem cells differentiated more toward neurons and oligodendrocytes than toward astrocytes	117
PuraMatrix	N/A	Adipose derived stem cells	Proliferation, adhesion, and differentiation into motor neuron-like cells	118
N/A	PNIPAAm-PEG	Human ESCs lines H1 and H9	hPSCs expansion and differentiation	106
Peptide	PEG	Hb9:GFP mouse ESCs	Motor neuron differentiation and axonal outgrowth	107
Sericin, gelatin	PVA	PC12s Schwann cells (SCs)	SCs and PC12s developed neuronal networks	23
Velvet antler polypeptides, sodium alginate (SA)	PVA	iPSCs	iPSCs differentiated into neurons, astrocytes, and oligodendrocytes, with the presence of VAPs promoting oligodendrogenesis in a dose-dependent manner	108
GelMA	PEG diacrylate (PEGDA)	SH-SY5Y cell line	Cell survival in GelMA hydrogel, but not in PEGDA hydrogel	119
Collagen hyaluronic acid	PEG	Primary mouse microglial cells, cortical neurons, and astrocytes	Cell metabolic activity up to 21 days and synapse formation	120
Collagen	Polypyrrole (PPy)	PC12 cells	Cell extension and neuronal functional expression	121
Silk fibroin	Graphene	Rat SCs, PC12 cells, mouse embryonic stem cells	Regulating nerve cell behaviours	122
Poly (ethylene glycol) dimethacrylate (PEGDMA)	Multi-walled carbon nanotubes (CNTs)	PC12 cells	Neural differentiation	123

natural polymers support enhanced cellular interactions but tend to form constructs that are less mechanically and dimensionally stable compared to synthetic hydrogels.[109] Co-hydrogel or biosynthetic systems aim to combine the advantages of both natural and synthetic polymers, allowing for greater control over polymer properties.[110] The following sections will discuss the capability of hydrogels to incorporate and deliver cells with crucial growth cues which are essential for neural tissue modelling.

3.1. Tuneable properties of 3D hydrogel system

As illustrated in Fig. 3, cell-compatible hydrogels can be designed to integrate and control biochemical, mechanical, and electrical cues. This allows modulation of cell behaviours, such as proliferation, differentiation, adhesion, migration, and cell–cell and cell-microenvironment interaction.[35,124] However, the key challenge consists in tailoring hydrogel technologies to fulfil all of the design requirements for neural tissue modelling in a single system. This section discusses recent findings about integrating biochemical, mechanical, physical, and electrical cues into hydrogels to promote neural cells development, separately.

3.1.1. Biochemical cues. In neural tissue models supported by hydrogel substrates, extensive research has focused on integrating biochemical cues, including growth factors, metabolites, and peptides or proteins intrinsic to the native ECM.[96] These biochemical cues play essential roles in promoting adhesion, survival, growth, and differentiation of neural cells.[125] Effective integration of these biomolecules to hydrogel matrices is key to provide control on neuronal growth and development. As outlined in Table 2, to incorporate biochemical cues within hydrogels, one approach involves replicating the ECM microenvironment using ECM components,[102,111] while another entails providing bioactive cues like peptides[107,115,126] or soluble molecules[103,127,128] to facilitate cell adhesion and growth, either individually or in combination.[103,126] Therefore, hydrogels incorporating biochemical cues can be classified into ECM-integrating hydrogels, peptide-functioned hydrogels, and soluble molecule-encapsulating hydrogels. This customization allows for a broad spectrum of biochemical cues to intricately guide cell behaviours.

ECM-integrating hydrogels aim to mimic the composition the tissue matrix integrating single ECM components such as laminin,[114] collagen,[112] hyaluronic acid,[120] and fibronectin[70] or multiple components through decellularized matrix[129] or commercially available tissue-derived substances like Matrigel,[130] and Geltrex.[113] Notably, naturally sourced hydrogels can readily present ECM components, whereas synthetic variants necessitate exogenous incorporation through physical or chemical methods. It is essential to allow for spatiotemporal control of ECM components independent of the hydrogel material as their presence and relative distribution can influence neuronal outgrowth and behaviours.[131] For instance, in peripheral neurons a laminin-rich substrate supports elongation of neurites whereas collagen-IV rich substrates promote the growth of dendritic processes for innervation of target tissue.[132,133]

Hydrogels can also be crosslinked with designed peptides by physical or chemical bonding to optimise their biocompatibility. Li *et al.*, generated synthetic peptide hydrogels composed

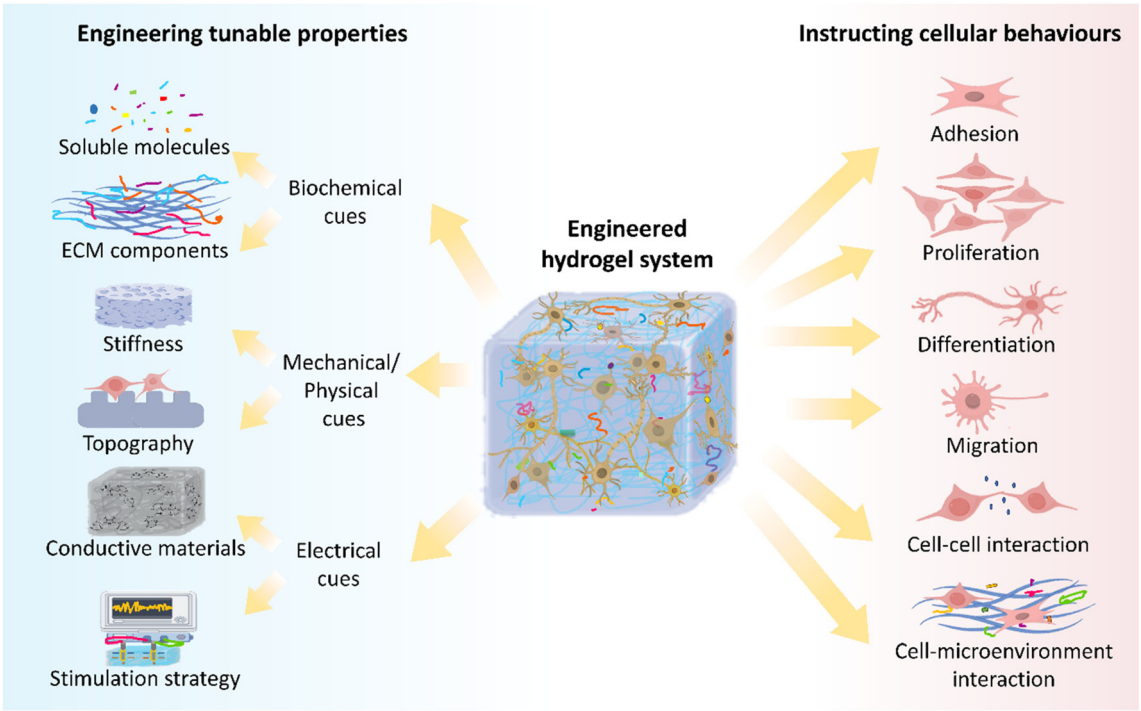

Fig. 3 Schematic of hydrogel systems with various tunable properties to instruct cellular behaviors in modeling 3D neural tissue *in vitro*.

Table 2 Hydrogel systems capable of providing biochemical guidance cues for neural tissue engineering

Hydrogel material	Biochemical cues	Cell/tissue type	Key findings	Ref.
Matrigel, hyaluronic acid	Retinoic acid (RA), sonic hedgehog (Shh), anti-a6, anti-b1	mESC	Blocking a6 or b1 integrin subunits inhibited effects on neural differentiation and neurite outgrowth; RA and Shh promote differentiation into neural and astrocyte lineages	103
Fibrin nanofiber hydrogel	AFG and fSAP	SCs	AFG/fSAP hydrogel exhibit significantly higher density of myelinated nerve fibres and innervated muscles and motor function, compared with AFG group	126
Acrylated PLA-*b*-PEG-*b*-PLA	CNTF	Mouse retinas	CNTF released from a degradable hydrogel above an explanted retina could stimulate outgrowth of neurites	127
Silk fibroin nanofibers	NGF	Rat SCs; PC12 cells; rat neural stem cells, implantation to rat spinal cord	NGF was optimized to regulate the neural/astroglial differentiation and to obtain the differentiation ratios	128
Matrigel	Basic fibroblast growth factor (bFGF)	Mouse neural stem cells	When combined with bFGF, electric field stimulation establishes neural tissue with a proper neuronal cell number, highly branched neurites, and a well-developed neuronal network	130
Collagen type I	EpoB-loaded PCL microspheres	hEnSCs	The collagen hydrogel containing the EpoB improves MN-like cell differentiation and maturation of hEnSCs	136
Silk fibroin/collagenhydrogel	Continuous spatial biochemical gradient, NGF	DRG neurons	Scaffold with nerve growth factor gradient along oriented microstructure promote nerve regeneration and directional elongation	137
AFG/fSAP	KLT and RGI peptides	Implantation to rat spinal cord	AFG/fSAP can facilitate spinal cord regeneration *via* guiding regenerated tissues, accelerating axonal regrowth and remyelination, and promoting angiogenesis	145
Elastin-like polypeptide hydrogels	RGD ligand	Chicks DRG	Increasing the cell-adhesive RGD ligand density led to a significant increase in the rate, length, and density of neurite out-growth	146
PCL microfiber	Oxymatrine (OMT) Decellularized spinal cord ECM	Neural stem cells, implantation to rat spinal cord	Composite scaffold could guide the directional growth of axons, reduce scarring, and promote the recovery of motor function in rats	147
Pentapeptide IKVAV-functionalized poly (lactide ethylene oxide fumarate) (PLEOF) hydrogel	Acrylated IKVAV(Ac-IKVAV) peptide and YIGSR (Ac-YIGSR) peptide	Rat neural stem cells, L929 (fibroblasts), rabbit red blood cells	Neural stem cells could be readily formed as spheroids that can attached and proliferated within the 3D hydrogel constructs; new blood vessels formed *in vivo*, and few inflammatory responses were observed in 4-week implantation study	148
Methacrylated hyaluronic acid (MeHA)/ collagen-I hydrogel	NGF, GDNF	SCs, dorsal root ganglia	MeHA/collagen-I bioink improved cell viability, and was conducive for cell adhesion, growth factor sequestration, and neural cell infiltration	138
Acellularized spinal cord matrix and gelatine-acrylated-β-cyclodextrin-polyethene glycol diacrylate hydrogel, aligned PCL microfibers	WAY-316606, an activator of Wnt/ β-catenin signalling	Rat neural stem cells, implantation to rat spinal cord	Composite hydrogel could significantly recover the motor function of rats after spinal cord injury	149

of collagen-like peptide (CLP) and CLP with an integrin-binding motif arginine-glycine-aspartate.[134] These hydrogels facilitated the spontaneous organization of primary cerebellar cells into tissue-like clusters which exhibited fast-rising calcium signals in the soma, indicative of action potential generation.[135]

Soluble molecule-encapsulating hydrogels can be engineered to release growth factors, which are important for the survival, proliferation, and differentiation of neural cells.[128,136] Nerve growth factor (NGF) is commonly used to support differentiation and neurite outgrowth of nerve cells along with brain derived neurotrophic factor (BDNF) and ciliary-neurotrophic factor (CNTF).[127,128,137] The incorporation of other factors like Glial cell line-derived neurotrophic factor (GDNF) supports the survival and growth of various types of neuronal and glial cells.[138]

The primary requirement for neural cells development hinges upon a biochemical environment that supplies adhesion sites and sustains crucial biological functions for cell survival, growth, migration, and differentiation.[139] Neural cells, in turn, perceive their environment through adhesion molecules known as integrins. These integrins engage with the cell cytoskeleton and modulate gene expression, proliferation, and survival *via* bidirectional signalling with the biochemical milieu. For instance, collagen, a key ECM component, is frequently employed *in vitro* for cell culture studies.[120] Epothilone B (EpoB), a microtubule-stabilizing agent known for its ability to penetrate the blood–brain barrier, exhibits promise in inducing axon regeneration and elongation in damaged nerve cells.[136] Mahmoodi *et al.* demonstrated this potential by fabricating a composite of 3D collagen hydrogel containing EpoB-loaded polycaprolactone (PCL) microspheres with varying concentrations of EpoB, as depicted in Fig. 4A.[136] Their findings indicated that EpoB-loaded PCL microspheres within the 3D collagen hydrogel could enhance the adhesion, proliferation, and motor neuron differentiation efficacy of human endometrial stem cells (hEnSCs).[136] Neural cells can sense the environment through adhesion molecules, termed integrins.[140] The integrins interact with the cell cytoskeleton and influence gene expression, proliferation, and survival through bidirectional signalling with the biochemical environment.[141]

Hydrogels can be modified to present spatial gradients of biochemical cues or developed to present specific structures designed to regulate cell behaviour aiming to recapitulate native microenvironment.[142] For example, 3D printed silk fibroin/collagen hydrogel displaying continuous spatial NGF gradient can direct the axonal outgrowth of dorsal root ganglia

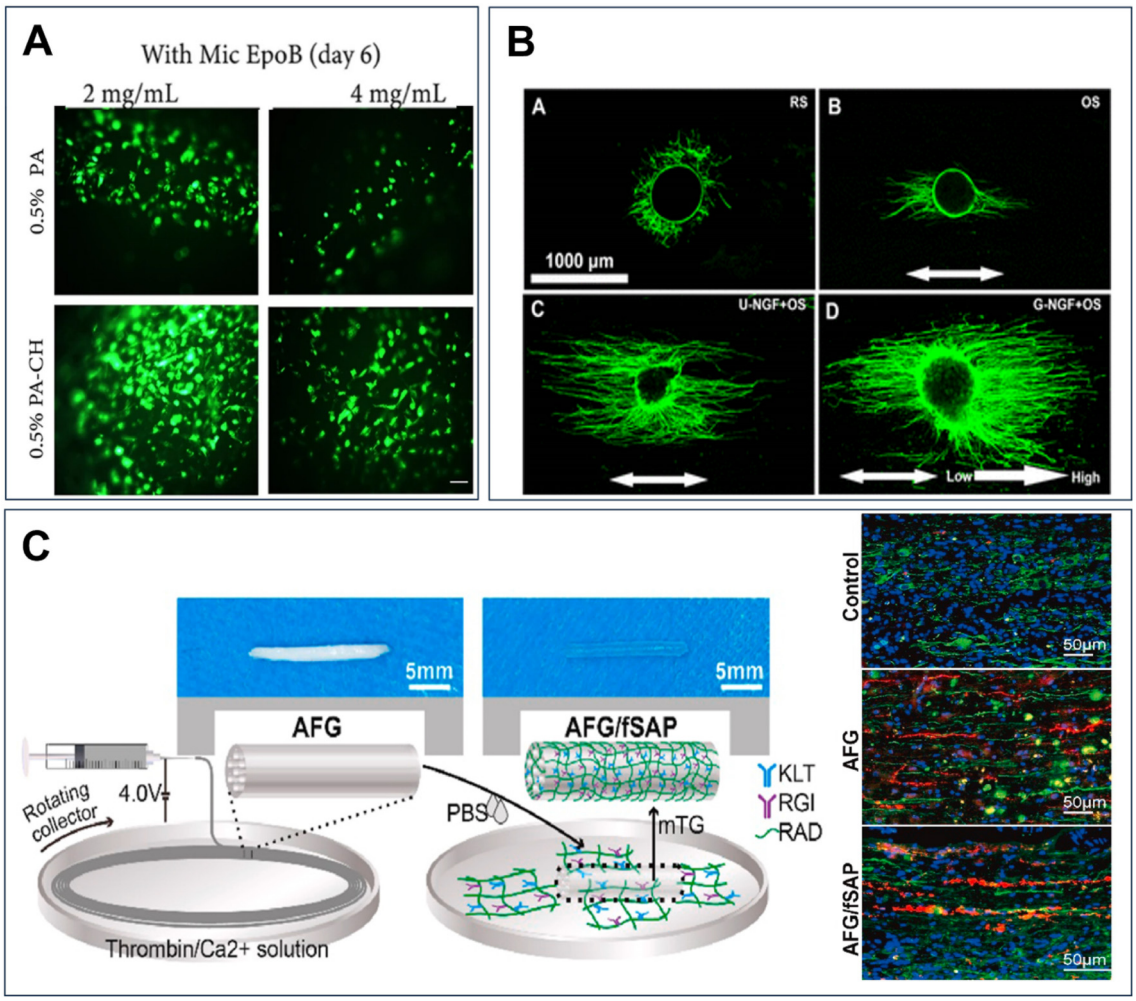

Fig. 4 Incorporating biochemical cues into 3D hydrogel system for neural tissue engineering. (A) Adhesion, and proliferation of human endometrial stem cells (hEnSCs) regulated by different concentrations of Proanthocyanidin (PA) and epothilone B (EpoB) in collagen hydrogels.[136] Reproduced from ref. 136 with permission from *Springer Nature*, copyright 2021. (B) Nerve growth factor gradients 3D microchannels directed axonal orientation of dorsal root ganglia on the longitudinal sections of silk fibroin/collagen hydrogel.[137] Reproduced from ref. 137 with permission from *American Chemical Society*, copyright 2020. (C) Aligned fibrin hydrogel (AFG) and functionalized self-assembling peptides (fSAP) nanofiber composite hydrogel facilitate spinal cord regeneration *via* guiding regenerated tissues, accelerating axonal regrowth and remyelination, and promoting angiogenesis.[145] Reproduced from ref. 145 with permission from *Biomaterials*, copyright 2021.

(DRG) toward high-NGF concentration side as shown in Fig. 4B.[137] Self-assembled 3D neural tissue models naturally generate a dynamic microenvironment through cell–cell interactions. In the process, signaling molecules or extracellular matrices are secreted spatiotemporally.[143] However, it is still necessary to offer regulations over the microenvironment in self-assembled models to guide cells behaviors.[144] By combining hierarchically aligned fibrin hydrogel (AFG) and functionalized self-assembling peptides (fSAP), nanofiber composite hydrogel AFG/fSAP can present both biochemical and topographical cues for SCs in the microenvironment, illustrated in Fig. 4C.[145] The addition of fSAP improved the water content of the interpenetrating hydrogel and supplied more adhesion sites for cells.[145] *In vivo* results showed that AFG/fSAP facilitated spinal cord regeneration *via* guiding tissue regeneration, accelerating axonal regrowth and remyelination, and promoting angiogenesis.[145]

Overall, hydrogels can be modified to support various biochemical composition, concentration, and structure, which can change hydrogel microenvironment to guide cell behaviors through bioactive signaling and cell–ECM interaction.[115] In addition to biochemical cues, mechanical and physical cues also exert significant influence on the regulation of neural cell behaviors.

3.1.2. Mechanical and physical cues. Mechanical and physical cues such as substrate stiffness, topography, and elasticity conferred by the surrounding ECM have been shown to play an important role in influencing the development and function of neural tissue.[80] Fig. 5 and 6 show representative examples of how mechanical and physical properties of these substrates can influence cell behaviours and tissue development and Table 3 compiles examples of how hydrogel biomaterials have been designed and tailored to meet diverse mechanical properties for promoting neuronal cell growth and development.[2,28,150,151] The stiffness of neural tissue varies depending on the location and type of tissue within the nervous system. In general, neural tissue is relatively soft and compliant, with Young's modulus ranging from 0.1 kPa to 10 kPa for brain tissue and 1.02 MPa to 1.37 MPa for spinal cord tissue.[152–154] The softness and compliance of neural tissue are essential for its proper function. Thus, changes in the mechanical properties like scaffold stiffness,[23] elasticity,[151] topography,[155–157] surface roughness[158] can significantly impact on the formation of healthy and diseased neuronal tissue models.[159,160]

Differentiation of stem cells towards a specific lineage can be influenced by external stimulus such as the mechanical stiffness of the supporting substrate.[161,162] This stiffness level

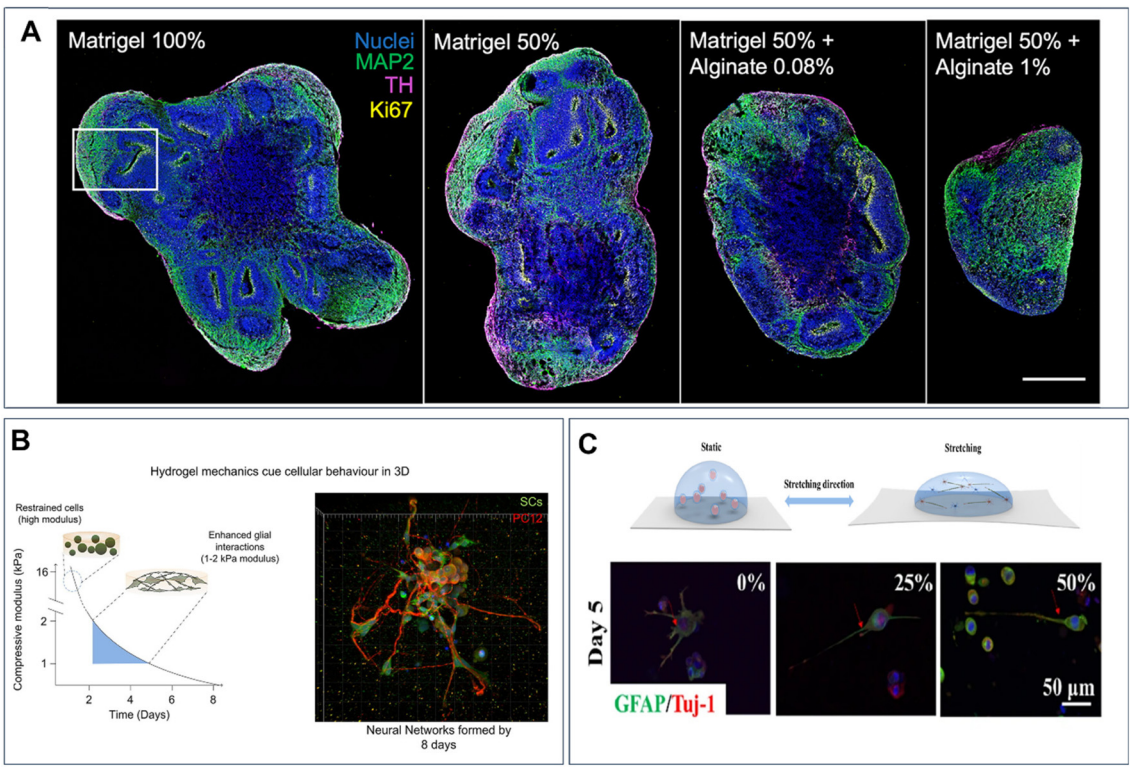

Fig. 5 The effects of stiffness of hydrogel on cellular behaviours for neural tissue engineering. (A) Brain organoids development and differentiation were significantly affected by matrix stiffness in Matrigel hydrogel modified with an interpenetrating network of alginate.[167] Reproduced from ref. 168 with permission from *American Chemical Society*, copyright 2022. (B) The higher modulus 16 kPa PVA-sericin/gelatine supports development of neuronal networks when Schwann cells were co-encapsulated with PC12s.[23] Reproduced from ref. 23 with permission from *Acta Biomaterialia*, copyright 2019. (C) Mechanical stretching enhance neurite extension and axon elongation of N2a cells in 3D GelMA-PEGDA hydrogel.[151] Reproduced from ref. 151 with permission from *Springer Nature*, copyright 2022.

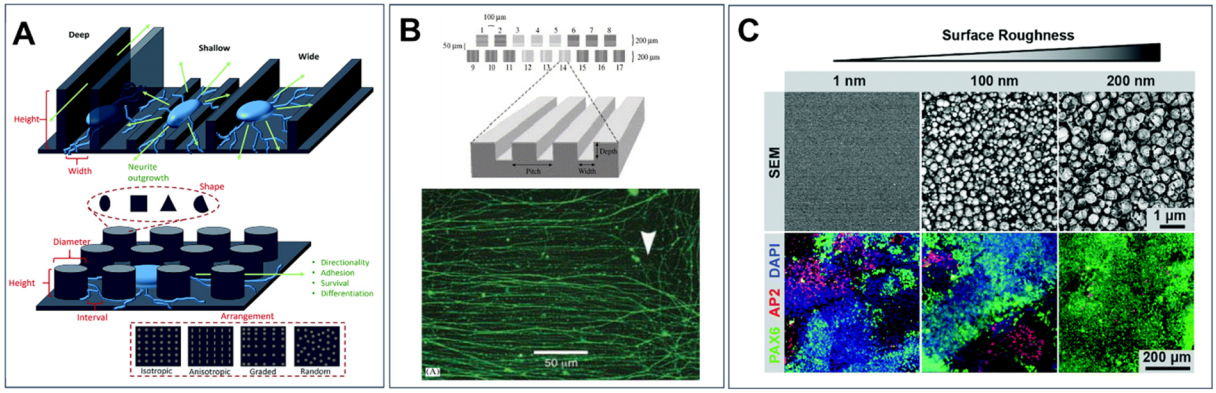

Fig. 6 The effects of topography of substrate on cellular behaviours for neural tissue engineering. (A) Schematic illustration of different featured topographies and their effects on cell behaviours.[150] Reproduced from ref. 150 with permission from *J Mater Chem B*, copyright 2021. (B) Axons aligning along a horizontally imprinted pattern (200 nm width and 400 nm pitch) in polymethylmethacrylate (PMMA)-covered silicon chips.[157] Reproduced from ref. 157 with permission from *Biomaterials*, copyright 2006. (C) Nanotopographic substrates promote human pluripotent stem cell neuroepithelial conversion on glass substrates functionalized with human vitronectin.[158] Reproduced from ref. 158 with permission from *Nanoscale*, copyright 2018.

has been shown to support neuronal differentiation of hMSCs and primary neural stem cells.[163–165] To investigate how cells sense matrix stiffness in 3D environments, Long *et al.* co-polymerised vinyl sulfone with RGD-functionalised dextran to develop a hydrogel in which matrix stiffness can be tuned independently.[166] Hydrogel stiffness was modulated by tuning the ratio of components, resulting in Young's moduli ranging from 0.1 kPa to 6 kPa. They found that hMSCs spread more extensively on stiffer hydrogels. Furthermore, as shown in Fig. 5A, Cassel de Camps *et al.*, found that the development of organoids is significantly affected by matrix stiffness.[167] They encapsulated iPSCs derived brain organoids in 3D modified Matrigel hydrogel. The Matrigel matrix was modified with an interpenetrating network of alginate to tune the mechanical properties with matrix stiffness ranged from 1 Pa to 1000 Pa.[167] Gross morphological differences developed with the stiffest hydrogel beyond 2 weeks, as organoids in the stiffest matrix grew to be rounder and significantly smaller than those in the softer variants.[167] Additionally, the number of neural rosette structures, indicative of ESCs differentiating into neuroprogenitors, decreased over threefold in stiff gels. While there were no notable differences in the proportion of dopaminergic neurons (as indicated by TH expression), a significant increase in the proportion of mature neurons (as indicated by MAP2 expression) was observed in the stiffest matrix formulation tested.[167] Overall, supporting that hydrogel mechanics can impact on self-organizing capacity and development of organoids.

Other studies have shown that careful tailoring of matrix stiffness can support neural cell development and survival in complex co-culture systems.[168] For example, Aregueta-Robles *et al.* co-cultured SCs and neuron-like PC12 cells within a degradable PVA-based hydrogel (Fig. 5B).[23] In this scenario, cells are expected to grow and extend a supporting network before the hydrogel completely degrades. To achieve this, the hydrogel stiffness was tailored to transiently support SCs growth, allowing cells to expand processes and form a supporting substrate for the PC12 cells. SCs and PC12 cells were able to grow neuronal networks when the initial hydrogel compressive modulus was 16 kPa.[23] As the hydrogel degraded SCs were presented with a substrate stiffness around 1kPa–2 kPa, which is a mechanical modulus reported to support development of cytoplasmic processes.[153] Hydrogels with a mechanical modulus lower than 1 kPa were not able to support effective growth and development of neural cells.[23] Indeed, SCs have been reported to express less adhesion proteins as the substrate stiffness decreases,[169] and to grow less processes when grown on soft substrates (<1 kPa).[23]

Mechanical stretching is another property reported to influence cell behaviour.[170] Mechanical stretching has been shown to enhance neurite extension and axon elongation (Fig. 5C).[151] Quanjing and colleagues studied the differentiation of mouse neuroblastoma cells Neuro2a (N2a) encapsulated in GelMA hydrogels co-polymerised with PEGDA. These cells were subjected to mechanical stretching at 0%, 25%, and 50% of the initial hydrogel length (Fig. 5C).[151] Results showed that stretching induced significant neurite outgrowth with longer neurites than the unstretched control group. Moreover, cells subjected to stretching exhibited up to a threefold increase in Tuj-1 (neuron-specific class III β-tubulin) expression and a twofold increase in GFAP (Glial fibrillary acidic protein, GFAP) expression compared to non-stretched controls.[151] Notably, cell cultures were not highly confluent, most likely due to the confinement of the non-degradable matrix.[151] However, these studies support hydrogel stretching as a potential strategy to promote neural differentiation towards specific lineages and to direct axon outgrowth.[151]

Providing topographical cues is a common approach to directly impacting cell adhesion, migration, morphology, and neurite outgrowth.[171] Fig. 6 shows different approaches to

Table 3 Hydrogel systems with mechanical and physical cues for neural tissue engineering

Hydrogel materials	Mechanical properties	Cell types	Key findings	Ref.
Poly (vinyl alcohol) copolymerised with sericin and gelatine (PVA-SG)	Compressive moduli of 16 kPa and 2 kPa	PC12 cells, SCs	The higher modulus PVA-SG supports development of neuronal networks when SCs were co-encapsulated with PC12s.	23
Glycosaminoglycan-binding polyacrylamide hydrogels	Gel stiffness (0.7 kPa, 3 kPa and 10 kPa)	hESC lines (H1, H7, H9, H9 Syn-GFP, and SA02)	Stiff substrates maintain hESC proliferation and pluripotency; compliant hydrogels facilitate hESC neuronal differentiation	182, 183
Laminin coated polyacrylamide (PA) gels	Shear modulus (200 Pa, 250 Pa, 2150 Pa, 9000 Pa)	Rat neuronal and glial cells	Laminin-coated soft gels encourage attachment and growth of neurons while suppressing astrocyte growth	153
Alginate hydrogels	Shear modulus (183 Pa, 1028 Pa, 1735 Pa, 19 700 Pa)	Rat neural stem cells	The rate of proliferation and differentiation of neural stem cells decreased with the increase in modulus	186
Collagen I-Matrigel hydrogel	Matrix stiffness (5.1 kPa to 9.8 kPa)	SCs	Collagen I-50% Matrigel hydrogel with 9.8 kPa matrix stiffness could support Schwann cell spread and grow over 14 days	187
Methacrylamide chitosan hydrogel	Young's elastic modulus (E(Y)) (1 kPa to 30 kPa)	Rat neural stem/progenitor cell (NSPC)	NSPCs exhibited maximal proliferation on 3.5 kPa surfaces; neuronal differentiation was favoured on the softest surfaces with (E(Y)) < 1kPa; oligodendrocyte differentiation was favoured on stiffer scaffolds (>7 kPa)	164
Elastin-like polypeptide hydrogels	Elastic moduli of 0.5, 1.5, or 2.1 kPa	Chicks DRG	Most compliant materials (0.5 kPa) led to the greatest out-growth, with some neurites extending over 1.8 mm by day 7	146
Poly-L-lysine and laminin coated polydimethylsiloxane (PDMS) and polyacrylamide	Young's modulus (5 kPa, 500 kPa)	Rat primary rat cortical neurons	Migration of cortical neurons is enhanced on soft substrates, leading to a faster formation of neuronal networks	188
Laminin coated polyacrylamide (PAA) hydrogel	Shear modulus (1 kPa, 0.1 kPa)	Xenopus eye primordia	Retinal ganglion cell axons grown on stiff substrates were significantly longer than those grown on soft substrates	189
Polyacrylamide gels	Young's modulus (170 Pa–3200 Pa)	Rat hippocampal cells	Soft substrates (170 Pa) promoted the formation of neurites than stiff substrates (3200 Pa)	190
Polyethylene glycol diacrylate-gelatine-methacryloyl (GelMA) hydrogel	Tensile modulus (60 kPa–10 kPa) and compressive modulus (6 kPa–0.8 kPa)	Mouse neuroblastoma cell line Neuro2a	Mechanical stretching could significantly enhance neurite extension and axon elongation and the neurites were more directionally oriented to the stretching direction	151
Vinyl sulfone and RGD functionalized dextran hydrogel	Young's moduli ranging from 0.1 kPa to 6 kPa	Human mesenchymal stromal cell	Matrix degradability regulates cell spreading kinetics, while matrix stiffness dictates the final spread area once cells achieve equilibrium spreading	166
Gelatin methacrylate and gelatin mixed with fibrin hydrogels	Single channel size: 150 × 300 × 5000 ($w \times h \times l$) μm;	iPSC – derived spinal neuronal progenitor cells (sNPCs) and oligodendrocyte progenitor cells (OPCs)	The bioprinted sNPCs differentiated and extended axons throughout microscale scaffold channels	155
Poly(lactide-*co*-glycolide)	A bridge mould (1.5 × 2.6 × 4 ($w \times h \times l$) mm) with channels of 250 or 150 μm diameter.	Bridge implantation in rat spinal cord lateral hemisection injury model	Bridges supported substantial cell infiltration, aligned cells within the channels, axon growth across the channels, and high levels of transgene expression at the implant site with decreasing levels on rostral and caudal portions	156

guide neurite outgrowth and promote neuronal differentiation into specialised lineage. Typical topographical factors include grooves and surface features with variable arrangement patterns (Fig. 6A).[150] Hydrogels in particular have been engineered to provide topographical cues, such as channels, grooves or ridges, and various degrees of surface roughness that can ultimately lead to alterations in the growth, differentiation, and proliferation of cells during neural development.[172] *In vivo*

studies have shown that tubular-shaped hydrogels can support regeneration of severed nerves.[173,174] In this scenario, the pathway for cell growth is constrained by the hydrogel substrate, thus forcing unidirectional growth. *In vitro* studies have also shown that directional growth of DRG neurons can be directed by spatially defining their 3D environment.[175] Notably, while hydrogels serve to guide neurite outgrowth, the degree of regeneration is substantially greater, comparable to autografts, when the substrate is supplemented with ECM or growth factors.[157] Autografts represent the gold standard approach for peripheral nerve regeneration but repairing gaps longer than 5 mm proves difficult,[176] in part because regenerating axons taper as they grow.[173] Hydrogel nerve guidance conduits have shown some success of nerve repair in greater gaps up to 40 mm, with larger defects of up to 5 cm requiring incorporation of living cells within the graft.[177] However, matching the regenerative level of autografts has not been accomplished.

Micropatterning growth substrates has also been used as a strategy to drive directed cell growth. Berkovitch and Seliktar encapsulated DRG neurons in PEG hydrogels conjugated with fibrinogen, gelatin, and albumin.[178] They showed that neurons grew and extended axonal processes through laser ablated microchannels of the order of 10 to 70 μm. Similarly, Li *et al.*, micropatterned a polyacrylamide hydrogels grafted with YIGSR peptides.[179] The patterning consisted of aligned ridge/groove structures, both of 30 μm, and tested its potential to align Schwann cells. Hydrogel micropatterns effectively controlled the aligned growth of Schwann cells and increased the expression of genes related to the cytoskeleton, indicating promising potential for their use in nerve regeneration applications.[179] Of note, cell growth over microfabricated materials is complex. Goldner *et al.* found an unusual capability of a subpopulation of neonatal rat DRG neurons to extend neurites that spanned across the grooves, with no underlying solid support, which is of interest to designing biomaterials for 3D axon guidance.[180] Johansson *et al.* fabricated nano-printed patterns in polymethylmethacrylate (PMMA)-covered silicon chips on which patterns consisted of parallel grooves with depths of 300 nm and varying widths of 100 to 400 nm to test the effect of nano-printed patterns on axonal outgrowth (Fig. 6B).[157] After 1 week of incubation, axons displayed contact guidance on all patterns and the nerve cell processes preferred to grow on ridge edges and elevations in the patterns rather than in grooves.[157]

Changes to substrate roughness can also impact on cell growth and behaviour. As shown in Fig. 6C, Chen *et al.* showed that conversion of hiPSCs and hESCs into neuroepithelial cells can be promoted by increasing the surface roughness of cell culture substrates.[158] Stem cells cultured on vitronectin-coated glasses with varied surface roughness differentiated into neuroepithelial cells as early as day 2 on substrates with local surface features of 200 nm, as evidenced through expression of PAX6, a marker of early neuroectodermal differentiation.[158] This was half the time required for start observing PAX6+ cells in smoother surfaces (1 nm).[158] In addition, the rougher substrates yielded over 88% PAX6+ neuroepithelial cells by day 8, compared to approximately 32% in the smooth variants.[158] This suggests that nano-topographic cues can provide potent regulatory signals to mediate adult stem cell behaviours, including self-renewal and differentiation.[158] However, there has been minimal research on application of such physical features to hydrogels. In a study by Hou *et al.*, the impact of surface roughness and stiffness on the mechanical response and osteogenesis of hMSCs was investigated.[181] GelMA hydrogels were patterned with nano- and micro-aggregates, resulting in variable surface roughness ranging from 200 nm to 1.2 μm and mechanical properties ranging from 3.8 kPa to 31.3 kPa.[181] It was observed that the mechano-response and osteogenesis of hMSCs were significantly enhanced on the rougher regions of soft hydrogels and on regions with intermediate roughness on stiff hydrogels.[181] This suggests a synergistic effect between roughness and stiffness in driving cellular mechano-response.[181]

Comprehensive studies are still required to fine-tune the mechanical microenvironment for neural tissue engineering.[182,183] However, tailoring the mechanical and physical cues can only address in part the guidance of neuronal growth and cell behaviors.[184] Combinations of biochemical, mechanical and physical factors are all critical contributors to nerve growth guidance.[185] While their relative contribution is not clear, it is likely all of these factors are interdependent.[185] In addition to these three factors, electrical signals also play a key role in neural tissue development and function.

3.1.3. Electrical cues. Electrical activity plays a fundamental role in the functioning of nerve cells, and electrical stimulation has demonstrated beneficial effects on paracrine activity, cellular alignment and migration, synapse formation, and recovery from peripheral nerve injuries.[191] Notably, the influence of electric stimulation on cell behaviour can extend to non-neuronal cell types.[192,193] Ideally, platforms designed to study neural tissue function should integrate components that promote conductivity of the matrix to enable electrical stimulation. However, hydrogel materials are not active electric conductors and need to be modified with conductive materials. As described in Table 4, the influence of electrical cues through hydrogel materials has been explored through the incorporation of conductive polymers,[194,195] carbon nanotubes,[196,197] or graphene,[198,199] to create a conductive hydrogel.[200] The conductive hydrogel can then be used to more effectively deliver electrical stimulation to neural cells within the hydrogel, promoting neural cell growth, migration, differentiation, and function. As shown in Fig. 7, there are representative approaches to incorporating electrical cues into constructs.

He *et al.* incorporated carbon nanotubes into self-assembling peptide hydrogels to study the effect of electrical stimulation on axon outgrowth and Schwann cell migration (Fig. 7A).[201] DRG spheres were encapsulated in the conductive hydrogel substrate and subjected to 1 mA at 10 Hz for 30 minutes per day over 30 days.[201] Electrical stimulation promoted axon outgrowth, Schwann cell migration away from DRG spheres, and promoted myelination of growing neur-

Table 4 Hydrogel systems capable of providing electrical guidance cues for neural tissue engineering

Hydrogel materials	Electrical components	Cell type	Key findings	Ref.
Silk/collagen hydrogel	Gold wires, alternating field of 80 mV mm^{-1}, 0.5 Hz–2 kHz	Rat primary cortical neurons	Alternating electric field stimulation could direct axon 3D length growth and orientation	203
PEGDMA	CNTs	PC12 cells	PEGDMA with randomly distributed CNTs promote neural differentiation	165
PEG-PEGDA hydrogel	Polyimide stimulating electrodes, biphasic electrical stimulation of 100, 500 and 1000 μA, with a 100 Hz frequency at 100 μs	Neural stem cells	Scaffolds promoted neural stem cell proliferation and early neuronal differentiation; 500 μA current promoted neuronal maturity	197
Silk fibroin and graphene oxide hydrogel	Graphene oxide nanosheets	SCs	Soft substrates supported SC survival, proliferation, spreading, and gene expression of neurotrophic factors, while the increased conductivity may also be beneficial to SC functional behaviours	206
Polydopamine functionalized reduced graphene oxide (rGO-PDA) with PVA	Graphene oxide	PC12 cells	Highly efficient neuronal differentiation was also observed both with and without electrical stimulation	198
mPEG-PLV polymer with nerve growth factor	Tetraniline	PC-12 cells, neural stem cells isolated from newborn Sprague–Dawley rat	Electrical stimulation promoted the neuronal differentiation of neural stem cells and axonal growth	204
Amino-functionalized graphene crosslinked collagen	Electric (3.8 ± 0.2 m Siemens per cm); electric stimulation (100 mV mm^{-1})	Raw 264.7 cells, bone marrow derived MSCs	Suppressing the neuro-inflammation and promoting the neuronal cellular migration and proliferation	199
Silk-gelatine/polylactic acid	NGF-incorporated Fe_3O_4-graphene nanoparticles	PC12	A biologically and electrically conductive cell passage with one-dimensional directionality was constructed to allow for a controllable constrained geometric effect on neuronal adhesion, differentiation, and neurite orientation	207
3D Matrigel with bFGF	Direct current electric field *via* 5% FBS agar bridges (150 mV mm^{-1} DC).	Neural stem cell from the fatal brain tissue of C57BL/6 mice at E12–14	EF-stimulated neural stem cells in 3D Matrigel mainly differentiated into neurons, bFGF promoted neural differentiation with highly branched neurites, and neuronal network development	130
PEG-based hydrogel	Poly(3,4-ethylenedioxythiophene) (PEDOT)	C2C12 myoblasts	Combination of topographical and electrical cues maximized the differentiation of C2C12 myoblasts into myotubes	208
Collagen–polypyrrole hybrid hydrogel	PPy nanoparticles, 100 mV cm^{-1}	PC12 cells	The oriented fibrous microstructures enhanced neuron-like cells aligning with fibres' axon; the matrix conductivity improved cell extension and upregulated neural related gene expression; external electrical stimulation further promoted the neuronal functional expression	121
Silk fibroin	Graphene	Rat SCs, PC12 cells, mouse embryonic stem cells	Multiple cues including nanofibrous structure, aligned topography, stiffness, bioactive graphene sheets and hydrogel state were successfully introduced into SF-based materials, resulting in synergistic effects on nerve cell behaviours	122
Poly (terephthalate)	PEDOT:PSS, indium tin oxide on poly (terephthalate) conductive back electrode, zinc oxide electron transfer layer, poly(3-hexylthiophene) photoactive layer	Primary hippocampal neuron, primary cardiac myocyte	These devices enable stimulation of individual hippocampal neurons and photocontrol of beating frequency of cardiac myocytes *via* safe charge-balanced capacitive currents	209
Matrigel	Electrotactic chamber *via* 5% FBS agar bridges, 150 mV mm^{-1} DC	Human neural stem cells, mice neural stem cells	Electrical stimulation could induce neuronal differentiation, the orderly growth of neurites and the maturation of neuron subtypes to construct a well-developed neuronal network with synapses and myelin sheaths	210

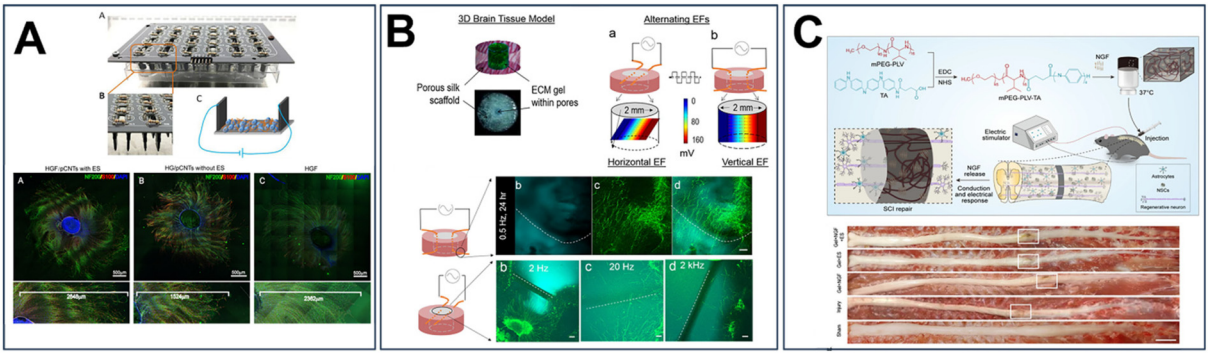

Fig. 7 Incorporating electrical cues into 3D hydrogel system for neural tissue engineering. (A) Electrical stimulation promotes axon outgrowth, Schwann cell migration and myelination in nanofiber hydrogels incorporated with carbon nanotubes.[201] Reproduced from ref. 201 with permission from *American Chemical Society,* copyright 2020. (B) Rat cortical neurite outgrowth at 24 h in a silk hydrogel after electrical stimulation of varying frequencies showed a preference of orientation towards the embedded electrodes, but not affected by AC field frequencies.[203] Reproduced from ref. 203 with permission from *Brain Research,* copyright 2018. (C) Thermo-sensitive electroactive hydrogel that made by poly (ethylene glycol)-*co*-polyvaline (mPEG-PLV) polymer grafted with tetraniline and loaded with nerve growth factor combined with electrical stimulation promoted endogenous neurogenesis and improved motor function.[204] Reproduced from ref. 204 with permission from *Springer Nature,* copyright 2021.

ites.[201] Additionally, the stimulation approximately doubled the expression of the myelin basic protein gene and the c-Jun gene,[201] the latter known for initiating a repair process in Schwann cells and facilitating the development of specialized cells that support regeneration.[202] Of note, the incorporation of carbon nanotubes had a negative impact on neurite outgrowth compared to a non-conductive hydrogel control.[201] Thus, while electrical stimulation has potential to enhance nerve regeneration, careful consideration of material properties is crucial for optimizing outcomes.

Lee *et al.* further elaborated on this concept, demonstrating how electroconductive scaffolds augmented neural stem cell proliferation and differentiation.[197] By integrating amine-functionalized multi-walled carbon nanotubes (MWCNTs) within PEGDA polymer, they observed that MWCNT-incorporated scaffolds facilitated neural stem cell proliferation and early neuronal differentiation compared to scaffolds lacking MWCNTs.[197] Moreover, biphasic pulse stimulation with 500 μA current at 100 Hz for 4 days promoted neuronal maturation, as evidenced by increased RNA expression of Tuj1, Nestin, and GFAP protein.[197]

Furthermore, Wu *et al.* emphasized the synergistic benefits of combining matrix orientation with electroconductivity to enhance neural function.[121] Electroconductive PPy nanoparticles were synthesized with modified hydrophilicity to enhance their uniform distribution within collagen hydrogels.[121] Findings showed that collagen–PPy hydrogel microfibers with aligned microstructures could effectively guide neurite growth of PC12 cells along the matrix-defined direction.[121] Additionally electrical stimulation further promoted neurogenesis, improved cell extension, and significantly upregulated the expression of tubulin-βIII, neurofilament protein, and voltage-gated calcium channels genes. Therefore, alongside electroconductivity, the oriented microstructure and desired bioactivity of the matrix should be considered in the design of biomimetic ECM for neurogenesis.[121]

While hydrogel materials inherently lack conductive elements, their hydrophilic nature enables them to act as passive conductors. Tang-Schomer *et al.*, for instance, introduced electrodes into a hydrogel system to stimulate axon growth in rat cortical neurons (Fig. 7B).[203] This system consisted of a 3D silk/collagen hydrogel with embedded gold wires to deliver an alternating field of 80 mV mm^{-1} at 0.5 Hz to 2 kHz for up to 4 days, in which rat cortical neurons were exposed.[203] Results showed that 0.5 to 20 Hz can promote axon growth, with 2 Hz producing the biggest effect of about 30% increase in axon length compared to control cultures.[203] Interestingly, neurite outgrowth showed a preference of orientation towards the embedded electrodes.[203]

Building upon these findings, Liu *et al.* took a comprehensive approach by combining biochemical, physical, and electrical cues within a thermosensitive polymer electroactive hydrogel (TPEH) to repair spinal cord injuries, shown in Fig. 7C.[204] This hydrogel consisted of poly (ethylene glycol)-*co*-polyvaline (mPEG-PLV) polymer grafted with tetraniline and loaded with NGF.[204] They found that electrical stimulation promoted the differentiation of neural stem cells and axonal growth *in vitro*.[204] The average neurite length in the TPEH + NGF + ES group was 136.30 μm, which was significantly longer than that in the control (25.48 μm), TPEH + NGF (38.64 μm), and TPEH + ES (45.12 μm) groups.[204] In a rat model of spinal cord injury, the rate of neurogenesis in the group of TPEH + NGF + ES was 1.2 and 3.1 times higher than that of the Gel + NGF, and Gel + ES groups, respectively.[204] Their study revealed that electrical stimulation, alongside the delivery of nerve growth factor, not only promoted neuronal differentiation and axonal growth *in vitro* but also stimulated endogenous neurogenesis, ultimately leading to improved motor function.[204]

Overall, this integration highlights the importance of incorporating multifaceted growing cues in the design of biomimetic extracellular matrices for neurogenesis and tissue repair.[205] The next section discusses common fabrication

technologies for engineering 3D hydrogels, offering insights into how these technologies can be leveraged to design cell supporting substrates with tailored properties conducive to neural tissue regeneration and repair.

3.2 The fabrication technologies for engineering 3D hydrogel

Identifying fabrication techniques that bridge the gap between the natural, uncontrolled microenvironment characteristic of self-assembled models and the controlled, fabricated synthetic scaffold used in engineered structures is critical for developing functional and representative 3D neural tissue models. A significant challenge in engineering nerve tissue involves creating nerve guiding conduits with supporting substrates that enable control over cell growth and organization into functional neural architectures.[211] Shaping hydrogels into nerve guiding conduits has been achieved through conventional methods like mould-casting and more recently, 3D printing techniques. However, each approach presents advantages and limitations, necessitating a balance between bioactivity and structural fidelity.

3.2.1. 3D-printing. 3D printing is an automated, high-throughput, and versatile process that allows fabrication of complex geometries to create functional, artificial tissues and organs with precise control over cell and biomaterial placement.[212,213] Rapid advancements in 3D printing technologies have led to various methods described in reviews for printing biomaterials, especially hydrogels, owing to their high-water content and low-viscosity.[214–216] The predominant approach involves constructing hydrogel-based 3D structures using "bioinks", which are precursor solutions containing cells, although it has also been used for solutions without cells.[213,217,218] The basic steps for 3D printing structures involve creating a 3D digital model, slicing it into thin cross-sectional layers, preparing of the bioink according to the printing technology criteria. Depending on the polymerization properties, the bioink can extruded as a filament, deposited in a supporting medium, or cured with digital light patterns to build the physical object.[219]

The ability to precisely manipulate the arrangement and combination of transplanted cells holds great potential for tissue engineering, whether it is for growing nerve tissue in the lab or regenerating tissue at injury sites. However, incorporating cells into hydrogel bioinks presents additional challenges due to their impact on ink rheology and the potential effects of ink components on cellular viability.[213,220] Fig. 8 illustrates the representative technologies of 3D bioprinting to fabricate hydrogel systems for neural tissue engineering. For instance, in techniques like stereolithography (SLA) or digital light processing (DLP) printing, the presence of photoinitiators[221] and photoabsorbers[222] may pose toxicity concerns for cells depending on their concentration and exposure.[222] Similarly, in extrusion-based printing, factors such as printing process parameters like speed, time and pressure, and bioink biocompatibility, can dramatically impact cell viability.[223] Additionally, cell density can influence the mechanical and rheological properties of the ink.[213]

Despite these challenges, recent advancements in printing techniques and materials offer promising solutions. Specialized bioinks, such as the one developed by Daikuara *et al.*, have been engineered to balance printing requirements with cell biocompatibility (Fig. 8A).[215] This bioink, specifically designed for extrusion-based printing, consisted of platelet lysate and GelMA, aiming to mimic native skin tissue *in vitro*.[215] This bioink exhibited favorable rheological properties and shape fidelity, along with tunable mechanical properties to the stiffness of match dermal skin.[215] Within the printed hydrogel, dermal fibroblasts exhibited high viability, good attachment, and enhanced proliferation. Consequently, developing bioinks involves multiple iterations to identify an optimal component ratio that aligns printing requirements with cell biocompatibility.[215] Of note, 3D printing large tissue structures can entail varying durations, ranging from minutes to hours. Prolonged printing and photopolymerization processes may potentially compromise cell health while they are suspended within the bioink.[217]

While 3D printing holding promise for creating nerve guiding-like structures, challenges persistent in simultaneously achieving biochemically supportive, mechanically robust tissue structures with finely resolved microscale features.[224] In extrusion-based platforms, aligning hydrogel formulations with the rheology criteria of the printer often yields soft and poorly resolved structures.[225] Recent advances in extrusion-based techniques, such as negative sacrificial templates[226] or supporting baths,[213] have facilitated the creation of highly intricate 3D hydrogels structures, yet achieving fine resolution remains a persistent challenge.[227] Fine resolution is crucial for seamless cell perfusion and the precise placement of cells within the tissue-supporting structure.

Alternatively, SLA or DLP printing methods can generate structures with higher resolution, but further characterisation is essential to match the dynamic ratios of crosslinker density, photo-absorber, and photo-initiator for precise control over features during polymerization.[228] These adjustments necessitate additional characterization steps to ensure that desired bioactivity and mechanical properties remain unaffected. For instance, Koffler *et al.* used a microscale continuous projection printing method (μCPP) to create a complex neural structures for spinal cord regenerative medicine applications (Fig. 8B).[229] This study showed that μCPP can print 3D biomimetic hydrogel scaffolds tailored to the dimensions of the rodent spinal cord in 1.6 seconds and is scalable to human spinal cord sizes and lesion geometries.[229] They depicted 3D biomimetic scaffolds fabricated from polyethylene glycol–gelatine methacrylate (PEG-GelMA) loaded with neural progenitor cells (NPCs) using DLP-based printing to construct a complex scaffold.[229] This 3D biomimetic scaffolds supported injured host axon regeneration and significantly improved functional outcomes, highlighting the potential therapeutic efficacy of 3D PEG-GelMA scaffolds in enhancing CNS regeneration through precision medicine.[229]

All in all, the remarkable capabilities of 3D printing technologies have revolutionized the landscape of tissue engineering,

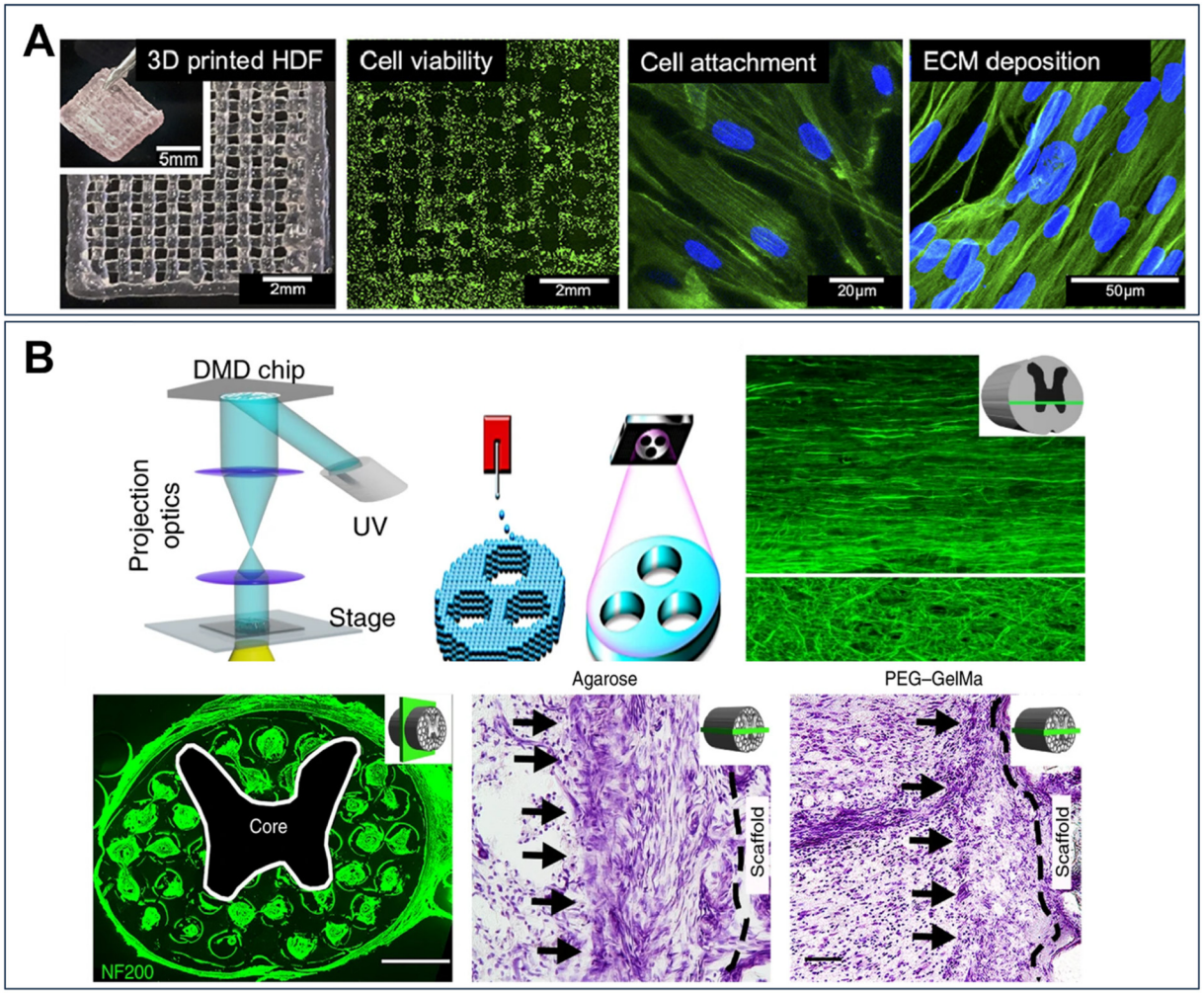

Fig. 8 3D bioprinting technologies for neural tissue engineering. (A) High cell viability, good cell attachment and improved proliferation of dermal fibroblasts in 3D printed platelet lysate and gelatine methacryloyl hydrogel.[215] Reproduced from ref. 215 with permission from *Acta Biomaterialia*, copyright 2021. (B) Biomimetic 3D-printed polyethylene glycol-gelatine methacrylate (PEG-GelMA) bioink loaded with neural progenitor cells (NPCs) by digital light processing (DLP) printing for spinal cord injury repair.[229] Reproduced from ref. 229 with permission from *Springer Nature*, copyright 2019.

particularly in the creation of highly customized and biomimetic constructs for neural tissue regeneration.[230,231] 3D bioprinting emerges as an appealing technology for fabricating intricate neural grafts, allowing for the seamless integration of diverse bioactive factors and cells to synergistically promote advanced neural regeneration.[232] The advancements underscore the transformative impact of 3D printing in regenerative medicine and neuroscience, supporting the development of advanced therapies and treatments tailored to individual patients. As research in this field continues to progress, the integration of biochemical, mechanical, physical, and electrical cues within printed constructs holds promise for addressing complex neurological disorders and advancing our understanding of the nervous system.[233]

3.2.2. Mould-casting. Mould-casting is a conventional fabrication technique for shaping hydrogels into complex 3D architectures and stands out for its simplicity and accessibility.[234] The process involves creating a mould or template with the desired shape and then casting the hydrogel precursor solution with or without cells into the mould and polymerize *via* light exposure or other curing methods.[235]

The key advantage of mould-casting is the potential for high-resolution architectures without compromising the application-intended properties of the hydrogel substrate, while ensuring uniformity and consistency.[236] Fig. 9 shows representative technologies of mould casting for neural tissue engineering. By carefully controlling component ratios, curing time and curing parameters, hydrogel architectures with consistent properties can be achieved seamlessly.[188] This process facilitates the integration of bioactive molecules or conductive materials into the hydrogel structure, enhancing its functionality without detracting from its intended properties.[237] Furthermore, this method ensures the uniform distribution of additives, thereby maintaining the overall integrity of the

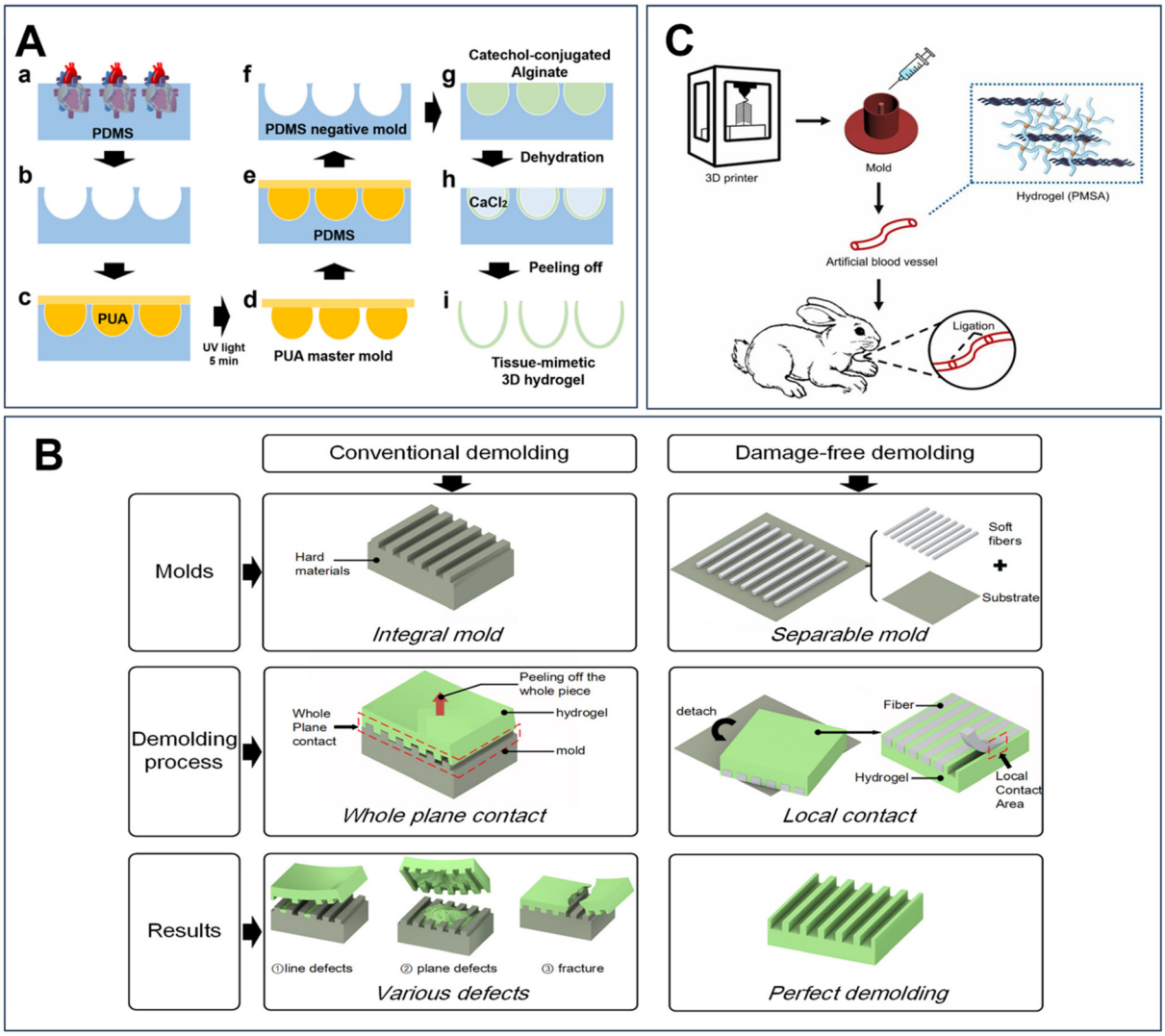

Fig. 9 Mould-casting technologies for neural tissue engineering. (A) A direct tissue casting method to fabricate catechol-conjugated alginate based hydrogel for reproducing complex-shaped target tissues.[238] Reproduced from ref. 238 with permission from *Bioengineering*, copyright 2021. (B) A new strategy for damage-free demolding is based on using the 3D printed soft ultrafine fibers as the molds and peeling off these fibers from the hydrogel softly.[240] Reproduced from ref. 240 with permission from *IOP Publishing*, copyright 2020. (C) A simple 3D mold fabrication technology to prepare tubular tissue grafts of different sizes by combination of 3D printing and mould-casting.[244] Reproduced from ref. 244 with permission from *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, copyright 2023.

hydrogel substrate.[236] As depicted in Fig. 9A, this versatility is exemplified by An *et al.*, where a simple and efficient method for fabricating 3D structures was proposed without resorting to complex processes such as soft lithography and 3D bioprinting.[238] Utilizing a direct tissue casting method, a complex U-shaped tissue structure was reconstructed using catechol-conjugated alginate-based hydrogels, rendering a non-toxic substrate with suitable stiffness biocompatibility for H9c2 cells.[238] Furthermore, the stable attachment of model drugs to the surface of the 3D film structure *via* the catechol group supports the potential of direct tissue casting as an alternative approach for implementing multi-scale tissue mimetic strategies.[238] This method can be further applied for neural tissue fabrication.

The primary challenge lies in devising non-toxic, easy-to-remove templates capable of accurately replicating the desired tissue geometry and topography. Common limitations to mould-casting are inherent to the moulding materials and to the complexity of the intended structure. First, moulding materials should not interact with the hydrogel solution to facilitate mould removal. For example, acrylic resin is highly versatile but it can stick to hydrogel materials like PVA,[239] which challenges removal and can damage the intended hydrogel structure.[240] Recently, there are some methods emerging to avoid brittle hydrogels damage while demolding. As shown in Fig. 9B, making soft removable moulds to decrease local contact between the moulds and the hydrogel, which can induce significantly less adhesive and frictional force.[240]

Second, the size and geometry of the mould can impact on the intended final structure. For instance, simple nerve conduit models are commonly achieved using tubular structures like needles, rods, or by first creating sheets and rolling them on a mandrel with a variety of materials.[235,241] However, this approach is limited when aiming to create nerve guiding conduits with channel size in the range of tens to few hundreds of microns, which are dimensions suggested to supporting axonal regeneration.[239] While there are non-toxic moulding materials available, such as stainless steel wires that meet this dimensional criteria, handling them to accommodate intended structures presents a challenge.[242] Stainless steel wires are inherently rigid, often thin and small in diameter which make it difficult to manipulate into complex shapes or configurations.[242]

Mould-casting is further limited when aiming to create non-regular 3D structures. While mould-casting can create 3D structures with specific patterns, channels, or gradients at the micro- and nanometre scale,[240,243] these are of moulds that only interface the hydrogels on the surface, ultimately not feasible for creating enclosed nerve conduits. Developing complex, three dimensionally irregular tissue structures *via* mould casting requires support of additive manufacturing technologies. For instance, a model of the scala tympani with characteristic free-form, non-uniform channels of cochlear structures, have been made possible by creating flexible silicone moulds enabled by 3D printing negative templates.[235] As shown in Fig. 9C, Zhou *et al.* developed an accessible, simple 3D mold fabrication technology to prepare tubular tissue grafts of different sizes by combination of 3D printing and mould-casting.[244] First, they used computer-aided design for additive manufacturing of 3D molds to fabricate PLA molds with different sizes and styles.[244] Then, the hydrogel precursor was poured into the PLA mold with a syringe to obtain hydrogel tubes with different wall thicknesses and layers.[244] Morever, this device can be used to prepare a hydrogel tube with a diameter less than 6 mm, which could be used as an artificial blood vessel for biological transplantation.[244] Other technologies like pre-cooling can be incorporated with mould-casting to advance the conventional methods and create higher resolution, more complicated structure. For example, He *et al.* found that pre-cooling before illumination made gelatin-based hydrogels resilient due to the partial regain of triple-helix structures, which could be demolded successfully and stably from a mold with a resolution of a feature size of 6–80 μm.[245] These methods can advance and expand the application scope of mould-casting from surface structure fabrication to inner or 3D architecture development.

In conclusion, mould-casting stands out as a versatile and cost-effective method for producing hydrogels with well-defined and precisely controlled structure, size, and mechanical properties. Its compatibility with cells, biochemical, and electrical cues make it suitable for a wide range of applications, spanning from *in vivo* transplantation and drug delivery to *in vitro* modeling of neural tissue.

3.3. The challenges of hydrogel-based neural tissue models

While hydrogels with their highly tuneable properties have emerged as promising tools for engineering 3D neural tissues, current models fall short of fully recapitulating the intricate complexity and organization of neural tissue at the human scale. There remain limitations in replicating intricate cell–cell and cell–ECM interactions inherent in biological tissue.[246] Achieving a high level of biomimicry in hydrogel-based neural models is a formidable challenge. It entails creating a dynamic physiological niche that replicates the normal *in vivo* environments produced through developmental processes. Challenges persist in integrating multiple cell types, ECM components, and growth factors with spatial and temporal control.[247] Addressing these complexities becomes more intricate when considering the diverse nutrient requirements of different cell types within a permeable hydrogel substrate.

Additionally, the absence of a vascular network is a key challenge for developing viable, long-term tissue cultures. Although hydrogels are inherently permeable to nutrients, the diffusion control is challenging and may lead to gradients of nutrients, oxygen, and signalling molecules that are not representative of the target tissue biology.[248] Moreover, growing cells over extended periods can bring issues like substrate degradation, cell overgrowth, and altered neural behaviours, challenging tissue culture stability and functionality.[249] This necessitates the development of hydrogels capable of mirroring the intricate mechanical properties of neural tissue while ensuring prolonged cell functionality.[33] Addressing these challenges demands the utilization of cutting-edge hydrogel biomaterials and fabrication methodologies.

As tissue models are scaled-up to meet human dimensions, the characterisation of tissue viability and function presents added technical challenges and may need customised solutions. While there are established imaging and biochemical techniques for assessing viability in cell cultures they have not been translated and validated for large-scale tissue models. Also, imaging becomes more challenging as hydrogels could be opaque requiring clearing techniques,[235] and may require more sophisticated approaches such as light-sheet microscopy or microtomography.[250] Where electrophysiological characterisation is needed, interfacing with external experimental systems, like microelectrode arrays is required and the tissue model needs to be sufficiently robust to withstand contact with stiff electrode materials.[251,252]

Finally, the scalability and reproducibility of neural tissue engineering approaches must be addressed for clinical translation. Developing consistent and reliable methods for fabricating hydrogel-based neural tissue models is essential, and validating findings against *in vivo* data remains elusive.[253] This task is further challenged by the lack of standardisation in the fabrication of hydrogel substrates, especially those with biological components. With cell-supporting biomolecules introducing natural variability and a multitude of hydrogel substrates, each with different formulations and fabrication approaches, the potential of hydrogels for engineering nerve

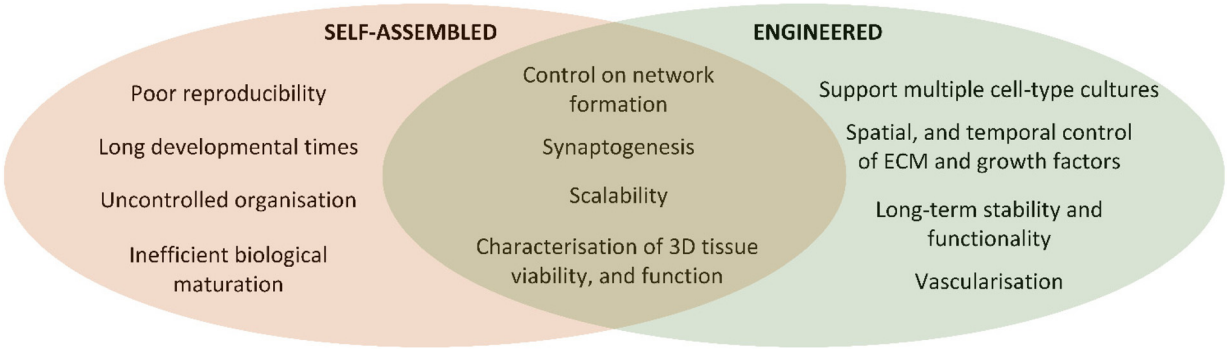

Fig. 10 Challenges of self-assembled and engineered 3D tissue models to recreate neural tissue in laboratory settings.

tissue is ultimately hindered. Overall, the development of engineered neural tissue models and hydrogel systems for neural tissue engineering is a complex and challenging task.[254] However, significant progress has been made in recent years, and the field holds great promise for the development of new therapies for neural injuries and diseases.[252] Through meticulous design and optimization, engineered tissues offer the potential to navigate these obstacles effectively, ensuring improved outcomes in tissue engineering and regenerative medicine endeavours.[255]

4. Conclusions and perspective

Neural tissues are characterised by environments with highly networked architectures that support rapid and efficient bi-directional flow of electrical and biochemical signals from the brain to the extremities. With our current technologies and techniques, it is not yet possible to replicate the exquisite organisation of human neural networks or represent the high complexity of neural pathways. This knowledge gap underscores the importance of accelerating research and developing innovative, advanced models for future advancement of the field.[256]

The growing field of neural tissue engineering has witnessed remarkable progress in advancing our comprehension of neural development and disease therapy[15] through the use of both self-assembled and engineered hydrogel-based approaches.[257] Such models have provided invaluable tools for investigating complex cellular behaviours and interactions within a context that, to an extent, mimics the native neural microenvironment.[258] The unique capability of self-assembled models to emulate the spontaneous formation of neural cells and tissue-like structures serves as a fundamental asset, enabling the recapitulation of some *in vivo* processes.[7] However, as shown in Fig. 10, challenges such as poor reproducibility, prolonged developmental times, uncontrolled organization, and inefficient biological maturation emphasize the need to refine protocols and methodologies.[69,70] Engineered models, particularly those utilizing hydrogels, while not ideal, offer controlled organization, a defined micro-environment, and tuneable properties that allow for the incorporation of crucial biochemical, mechanical, physical, and electrical cues.[22] Advanced fabrication techniques, like 3D printing and mould casting further enhance the precision and reproducibility of these models.[212,230,235]

The key challenges limiting development of any 3D tissue engineered models at the human scale are fabrication approaches, vascularisation, and long-term stability on the bench.[7,235,259,260] The fabrication of engineered tissues capable of supporting multiple cell types both structurally and functionally, while accurately reproducing the scale and extracellular environment in terms of its spatial and temporal dynamics, has not yet been achieved. The absence of a vascular network poses important challenges, highlighting the need for innovative solutions to deliver oxygen and nutrients and remove waste from tissue constructs. Additionally, maintaining stability and functionality over prolonged periods presents another obstacle, which necessitates ongoing research efforts to overcome these temporal limitations.[261] Additionally, maintaining stability and functionality over prolonged periods presents another obstacle, which necessitates ongoing research efforts to overcome these temporal limitations.[261]

In neural tissue engineering, alongside generic tissue engineering challenges, specific barriers remain unaddressed. These encompass guiding long axonal outgrowth, controlling network formation and synaptogenesis, and ensuring proper nerve fascicle development, which involves successful integration of glial and accessory cells. The persistent challenge of promoting long axonal outgrowth reflects the complexity of replicating *in vitro* the extensive connections formed by nerve cells *in vivo*, necessitating precise biochemical and topographical cues within engineered scaffolds. Similarly, achieving controlled network formation and synaptogenesis within an engineered construct demands meticulous regulation of cellular interactions and signalling pathways. Moreover, ensuring proper nerve fascicle development requires fine-tuning biochemical and mechanical cues within the hydrogel matrix. This requires integrating and develop glial and accessory cells, which adds another layer of complexity and demands a more comprehensive understanding of their interactions and roles in neural tissue physiology. Addressing these challenges is

vital for advancing neural tissue engineering and developing effective therapies for neurological disorders and injuries.

In the short term, integrating the strengths of self-assembled and engineered models is a promising strategy for mitigating individual limitations and enhancing overall model robustness.[262,263] The evolution of more advanced fabrication techniques with higher resolution and adaptability to cater to different materials is imperative for creating intricate tissue models with a structurally organised, biologically functional tissue.[264] This will contribute to achieving a higher degree of biomimicry and, subsequently, enhance the translational potential of these 3D models.

Of note, engineering neural tissue through hydrogel substrates necessitates alternative approaches, especially for the development or regeneration of long nerve fascicles. As discussed in section 3.1.1, the traditional approach involves presenting cells with a supporting substrate containing relevant growth factors for promoting axonal outgrowth. However, in biological tissue, this only represents the behaviour of cells of tissues in close proximity,[265] occurring during either developmental stages or through regeneration of injured tissue. Axonal outgrowth and innervation of tissue, such as skeletal muscle, can start developing in fetal stages and expands as the body grows. Tissue engineering such cellular behaviour would require a clear understanding of the environment of the developing tissue, as well as a substrate that can expand in parallel with cell growth. While stretching hydrogels have been proposed to support enhanced neurite extension and axon elongation,[151] the elasticity of current hydrogel matrices falls short of supporting the formation of a large nerve fascicle from cell cultures at the micro- and millimetre scale.

To effectively address such a multifaceted task, it is crucial to continue exploring hydrogel-based biomaterials, fabrication techniques, and cellular interactions. These efforts are essential for overcoming current limitations and unlocking the full potential of 3D models in understanding neural physiology and pathology and developing transformative applications for prevention, diagnosis, and treatment of neurological disorders. Moreover, it is vital to advance our understanding of neural tissue development processes. This understanding is crucial for guiding the precise delivery of physical and soluble factors necessary for growth and differentiation.

Given the extensive array of variables involved, leveraging bioinformatics, proteomics, and machine learning techniques may be indispensable in unravelling some of these challenges. The integration of these multidisciplinary approaches can help gaining deeper insights into the intricate mechanisms governing neural tissue development and enhance our ability to design tailored strategies for tissue engineering applications. Finally, to attain the ultimate goal of translating advanced 3D models into clinical applications requires not only scientific advancements but also collaboration with medical professionals, regulatory bodies and industry partners to revolutionise drug and device testing, and develop innovative, personalised interventions for neurological disorders.[266,267]

Author contributions

Shuqian Wan: conceptualization, writing – original draft, writing – review & editing, investigation. Ulises Aregueta Robles: conceptualization, supervision, investigation, writing – original draft, writing – review & editing. Laura Poole-Warren: conceptualization, supervision, project administration, writing – original draft, writing – review & editing. Dorna Esrafilzadeh: conceptualization, writing – original draft, supervision, writing – review & editing, project administration.

Conflicts of interest

There are no conflicts to declare.

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