Scaffolds for 3D In Vitro Culture of Neural Lineage Cells

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Abstract

Understanding how neurodegenerative disorders develop is not only a key challenge for researchers but also for the wider society, given the rapidly ageing populations in developed countries. Advances in this field require new tools with which to recreate neural tissue *in vitro* and produce realistic disease models. This in turn requires robust and reliable systems for performing 3D *in vitro* culture of neural lineage cells. This review provides a state of the art update on three-dimensional culture systems for *in vitro* development of neural tissue, employing a wide range of scaffold types including hydrogels, solid porous polymers, fibrous materials and decellularised tissues as well as microfluidic devices and lab-on-a-chip systems. To provide some context with in vivo development of the central nervous system (CNS), we also provide a brief overview of the neural stem cell niche, neural development and neural differentiation *in vitro*. We conclude with a discussion of future directions for this exciting and important field of biomaterials research.

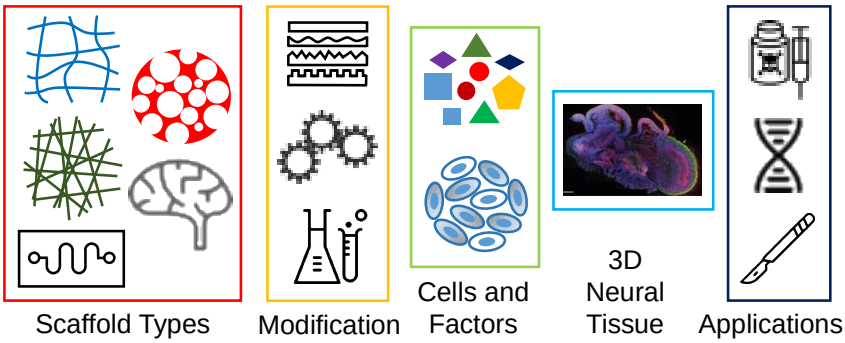
Keywords

Neural stem cells; scaffolds; 3D cell culture; polymers; hydrogels.

Statement of Significance

Neurodegenerative diseases, including dementia, Parkinson's and Alzheimer's diseases and motor neuron diseases, are a major societal challenge for ageing populations. Understanding these conditions and developing therapies against them will require the development of new physical models of healthy and diseased neural tissue. Cellular models resembling neural tissue can be cultured in the laboratory with the help of 3D scaffolds – materials that allow the organisation of neural cells into tissue-like structures. This review presents recent work on the development of different types of scaffolds for the 3D culture of neural lineage cells and the generation of functioning neural-like tissue. These *in vitro* culture systems are enabling the development of new approaches for modelling and tackling diseases of the brain and CNS.

Graphical Abstract



Abbreviations

2D	two dimensional
3D	three dimensional
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
d	days
ECM	extra cellular matrix
ECS	embryonic stem cell
EGF	epidermal growth factor
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGF8	fibroblast growth factor 8
GSK3βi	glycogen synthase kinase-3β inhibitor
hiPSC	human induced pluripotent stem cell
hPSC	human pluripotent stem cell
iPSC	induced pluripotent stem cell
NGF	nerve growth factor
NPC	neural progenitor cell
NS/PC	neural stem/progenitor cell
NSC	neural stem cell
PEG	poly(ethylene glycol)
PHEMA	poly(hydroxyethyl methacrylate)
PLA	poly(lactic acid)
PSC	pluripotent stem cell

RA	retinoic acid
RG	radial glial stem cell
SGZ	subgranular zone
SHH	sonic hedgehog
SVZ	subventricular zone
VEGF	vascular endothelial growth factor
vmIPN	variable moduli interpenetrating polymer network

Contents

1. Introduction

The brain is the least understood organ in the human body. It is difficult to access, highly susceptible to damage and complex in structure and function. The poor understanding of the human brain is reflected in the lack of effective treatments for various neurological disorders such as Parkinson's and Alzheimer's disease and motor neuron disorders. To address this research gap, new methods for the culture of human neural (neuronal and glial) lineage cells, particularly *in vitro* 3D culture, are being developed to more accurately reconstruct the complex *in vivo* structure and function of the human brain.

Human somatic cells cultured in flat, stiff, 2D environments typically display an irregular morphology and form unnatural cell-cell interactions [1]. Traditional monolayer cell cultures are simple and convenient to analyse, however tissue specific architecture, mechanical and biochemical cues and cell-cell communication are lost to various degrees. This can lead to physiological inaccuracies that can be extremely problematic for disease modelling and pre-clinical drug screening. In cancer research, it has been found that studies with animal models often do not result in successful translation to human trials because of the limited similarity to human physiology [2]. Both these scenarios can have considerable detrimental impacts on the progression of new drug candidates from pre-clinical trials to clinical trials, and can be particularly evident when modelling complex disease states such as those found in the central nervous system (CNS) [3].

Three dimensional (3D) cell culture systems aim to replicate the *in situ* functions of living tissue, by providing a more physiologically relevant environment for cell growth and function. Engineering neural tissue that is truly representative of that found in the human brain and central nervous system requires a scaffold to recreate the 3D *in vivo*

microenviornment. Various materials (natural and synthetic) in different formats (gels, porous solids and fibres) can be used as scaffolds to aid the 3D culture of replicating and terminally differentiated cells. To aid tissue growth and increase physiological relevance, scaffolds can be surface modified, mechanically tuned or chemically/biologically functionalised all of which have been shown to aid cell attachment, proliferation and differentiation. This review describes recent progress on developing scaffolds for the *in vitro* 3D culture of neural stem cells and their derivative neuronal and glial lineage cell types. To give the reader a deeper understanding of the topic, it also provides some background information on neurogenesis and the neural stem cell (NSC) niche in the embryonic and adult human brain.

2. The neural stem cell niche and neural differentiation *in vitro*

Somatic/adult stem cells reside in specialized microenvironments that provide specific extracellular conditions, primarily to maintain quiescence to prevent cell exhaustion, but also to induce differentiation and cell specialisation when required. Various stem cell niches exist within the adult human body including the bone marrow, the bulge of the hair follicle, the apex of the testis and the subventricular zone (SVZ) of the brain [4]. Much like any extracellular environment, the stem cell niche influences cell behaviour by a combination of signals from the extracellular matrix (ECM), various factors, nutrient and waste gradients, oxygen concentration, shear stress and temperature. The stem cell niche provides the ideal set of conditions for the maintenance and differentiation of stem cells. Understanding the makeup and function of the niche is critical in the construction of environments that mimic it, particularly in the field of tissue engineering. [5].

2.1 The embryonic neural stem cell niche and human brain development

In the developing embryo, four weeks post-conception, invaginating epithelial neural plate cells form what is known as the neural tube, a single layer of proliferating columnar neuroepithelial cells that eventually give rise to the CNS (brain and spinal cord). Through careful spatial and temporal environmental control, these primary neural stem cells can form neurons and glial cells, and organize themselves into the CNS. Neuroepithelial cells extend from ventricular (apical) to pial (basal) surfaces in an orientation known as apical-basal polarity. Neuroepithelial cells initially divide symmetrically producing two identical multipotent daughter cells [6]. Later in development they divide asymmetrically generating a self-renewing radial glial (progenitor) cell and a differentiating neuroblast [7] at the apical surface, and a basal progenitor and differentiating neuroblast at the basal surface [8, 9]. Outer radial glial cells, of the SVZ and beyond, act as a guide for the migration of newly formed neurons, which then is critical for the formation of the cortex layer (**Figure 1**) [10].

Figure 1. Cellular organization and differentiation during early development and neurogenesis in the human brain (time in weeks). See text for a description of processes of early development and neurogenesis. Figure reproduced from [11] © 2015 Budday, Steinmann and Kuhl.

2.2 The adult human neural stem cell niche

Neural stem cells reside in two defined niches, the SVZ of the lateral ventricles and the subgranular zone (SGZ) in the hippocampal formation. They are also theorised to reside in other less well characterised regions, including the neocortex, substantia nigra, amygdala and striatum [12].

The SVZ, which has been studied extensively, has been found to consist of four distinct layers (**Figure 2A**) [13]. A layer of ependymal cells acts as a barrier to the cerebrospinal fluid of the ventricle (**Figure 2A**). A layer of sparse neuronal cell bodies intertwined with ependymal processes then line the ependymal region (**Figure 2A**) [13]. On top of this layer sits a ribbon of proliferative astrocyte somata (**Figure 2A**), which have been shown to be primary neural progenitors in rodent brains, but not yet conclusively demonstrated as such in human brains [14, 15]. The fourth layer is then a transition between the third layer and the brain parenchyma, composed of some neuronal bodies and myelin tracts (**Figure 2A**) [16].

Figure 2. Schematic diagrams showing the human SVZ [17] (**A**) and the human SGZ [18] (**B**). See text for an explanation of zones in the SVZ. Abbreviations: SGZ, subgranular zone, GCL, granule cell layer, ML, molecular layer. Image in (**B**) reproduced with permission **©** Nature Publishing Group.

The SGZ is not as well characterised as the SVZ and is comparatively a much smaller niche (**Figure 2B**). The mammalian SGZ exists at the interface of the hilus and the granule cell layer within the hippocampus. SGZ progenitors, like the SVZ, exist as astrocyte-like cells. Radially oriented astrocytes, also referred to as type I progenitors or Type D cells (**Figure 2B** dark green), are oriented at the base of the SGZ and extend through the granule cell layer [19]. Horizontal astrocytes (**Figure 2B** purple), which have yet to be proven to act as precursor cells, also exist at the base of the SGZ, but lack the extension of radial astrocytes [20]. Radial astrocytes form type II progenitors (**Figure 2B** blue), which form tight clusters, migrate to the granule cell layer along the radial astrocytes, and differentiate to granule neurons [21].

2.3 Biological factors regulating/influencing neurogenesis in the neural stem cell niche

The neural stem cell (NSC) niche modulates a variety of biological factors to control NSC proliferation and differentiation. Both soluble and membrane bound factors of the neurogenic niches have been identified as participating in neurogenesis. Of these, the most important include epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) which promote NSC proliferation and β1-integrin expression at early and late stages of neurogenesis respectively [22].

ECM molecules and basal lamina components in the SVZ interact with NSCs to maintain quiescence, induce differentiation and aid migration [23]. Heparan and chondroitin sulphate proteoglycans present EGF and bFGF to neural stem cells to regulate proliferation and differentiation [24].

Morphogens such as bone morphogenic proteins (BMPs), Wnt, Notch and Sonic Hedgehog are all critical in the regulation of adult neurogenesis [25]. While the role of BMPs in

neurogenesis is extremely complex, and has been shown to exert a plethora of effects during development and maintenance of the nervous system, generally they are associated with the inhibition of neurogenesis. BMP antagonists such as Noggin, Chordin and Follistatin and others have been shown to play a key role in the induction of neurogenesis [26, 27]. For a further in depth analysis of the control of neurogenesis by morphogenic-signalling molecules the reader is directed to the review by Choe et al [25].

Vascularization to the NSC niche occurs simultaneously alongside its growth and expansion. Vascularization is critical for the growth, development and maintenance of the CNS. A critical factor in the development of vascularization and the neurovascular niche is vascular endothelial growth factor (VEGF). As well as being well known for controlling angiogenesis, VEFG has also recently been shown to be a critical factor in the direct promotion of neurogenesis [28-30]. VEGF has been shown to increase migration survival [31] and enhance proliferation and differentiation [32] of neural progenitor cells.

2.4 The extracellular matrix of the Central Nervous System

The extracellular matrix (ECM) in the brain, as in other organs, plays a defining role in regulating stem cell differentiation, migration and proliferation during development [33]. The brain ECM takes on many different forms and compositions throughout neurogenesis [34].

The ECM of the CNS can be divided into three main sections (**Figure 3**). The first is the basement membrane, a layer that surrounds the endothelial cells of blood vessels, which is mainly composed of laminin, fibronectin and heparin sulphate proteoglycan. The second section consists of perineuronal nets that surround neuronal cell bodies and smaller

neurons. These nets are composed of hyaluronic acid, proteoglycans, tenascin R and link proteins [35]. The final section is the neural interstitial matrix, a dense network of ECM components furthest away from the basement membrane and perineuronal nets. The ECM polymer network itself is primarily composed of a hyaluronic acid-based backbone [36] functionalised with ECM proteins.

Figure 3. The brain extracelluar matrix is arranged in three regions; the basement membrane, perineuronal nets and a neural interstitial matrix [37]. Image reproduced with permission © Nature Publishing Group.

Brain ECM proteins such as collagens, laminins and fibronectins provide mechanical support as well as important molecular cues for cell behaviour. Laminin, when compared to fibronectin and ECM-like matrices such as poly-L-ornithine and Matrigel™, has proven to be the most suitable 2D substrate for neural progenitor cell (NPC) migration, expansion, differentiation into neural cells and for the elongation of neurons [38]. During neurogenesis the ECM is constantly changing its morphology to modulate and guide axonal growth. Morphological changes are caused by cleavage of bonds by enzymes, particularly matrix metalloproteinases [39].

It is not only the chemical and biological cues from the extracellular environment that influence how neural stem and progenitor cells differentiate into mature neurons, astrocytes and oligodendrocytes; it is also physical, and more specifically mechanical, cues from the ECM that can determine stem cell fate. The elastic modulus of the human brain

varies across different regions. Different moduli are typically displayed by white and grey matter regions. The mammalian white matter, which is composed of the majority of glial cells and axons, is the stiffer of the two, with an elastic modulus of 1.9 kPa [40]. The mammalian grey matter, primarily composed of neuronal cell bodies, is elastically weaker with an elastic modulus of 1.4 kPa [40]. For a comprehensive review of mechanical property values of the brain the reader is directed to the review by *Aurand et al* [41].

2.5 Replicating neural differentiation pathways in vitro

Pluripotent stem cells (PSCs) can be expanded indefinitely, can potentially give rise to any cell type in the adult body, and are inclusive of embryonic stem cells (ESCs)[42] and induced pluripotent stem cells (iPSCs)[43, 44]. iPSCs are pluripotent stem cells produced via the genetic reprogramming of somatic cells to convert them back to a pluripotent-like state [45]. Significant work has been done to direct the differentiation of PSCs and neural stem cells to specific neural cell sub-types *in vitro* [46]. This has mainly been to study human neurobiology and interrogate neurological disease processes, which can be specific to certain neuronal or glial cell types, and to identify or test novel pharmacological therapeutic agents. The directed *in vitro* differentiation of hPSCs has been demonstrated for various neural lineages such as cortical neurons [47, 48], dopaminergic neurons [49], motor neurons [50], striatal neurons [51], and also for astrocyte and oligodendrocyte glial lineages [52]. This has been achieved by two approaches. The first is the introduction of growth factors or small molecules to hPSC culture to coax differentiation down a particular cell-type pathway (**Figure 4**). The second is by direct reprogramming of a somatic cell type via ectopic expression of genes that drive cells to a neural lineage fate, bypassing the pluripotent stage.

The ability to control and direct the differentiation of human pluripotent stem cells has huge potential impact for disease modelling and treatment strategies. As different neural cell types vary in their susceptibility to specific neurological diseases, patient-derived hiPSCs, and their derivative neuronal and glial lineage cells, may hold the key to accurately modelling an environment where these diseases can be studied in greater context and drug screening can identify effective therapeutics. This, coupled with the development of different types of scaffold materials in which to culture iPSC-derived neural precursor cells in a 3D format, represents a powerful technology platform for developing human neural tissue models, disease modelling and screening for new therapeutics. The majority of examples in this review explore the use of hPSC-derived NPCs and their progeny cultured in 3D formats.

Figure 4. Directed *in vitro* differentiation of pluripotent stem cells to different neuronal and glial cell subtypes. Induction factors highlighted in black and cellular markers highlighted in blue. Abbreviations: CNTF, ciliary neurotrophic factor; d, days of growth factor–driven differentiation; EGF, epidermal growth factor; FGF, fibroblast growth factor; FGF8, fibroblast growth factor 8; GSK3βi, glycogen synthase kinase-3β inhibitor; iPSC, induced pluripotent stem cell; RA, retinoic acid; SHH, sonic hedgehog; SMAD, intracellular proteins that transduce extracellular signals from TGFβ signaling; WNT, family of Wnt signaling pathways; WNTi, inhibitors of Wnt signaling pathways. [46]. Image re-used with permission © Annual Reviews.

3. Scaffolds for 3D *in vitro* NPC culture

Depending on the end application, numerous matrix materials have been used to support 3D cell culture. These include synthetic polymers, natural polymers, natural-synthetic hybrids, metals, ceramics, glass and carbon nanotubes. Synthetic polymers are preferred due to the ability to control their physical and chemical properties, but they lack the biological activity of natural polymers [53]. The format and manufacturing technique for different types of scaffolds varies depending on application. Some scaffold formats include polymer gels, solid porous scaffolds, fibrous scaffolds, and acellular scaffolds (**Figure 5**) [54, 55]. A comprehensive summary table of scaffold formats and materials used for 3D NPC and NPC-derivative cell culture is provided (**Table 1**).

Figure 5. Tissue engineering scaffold types and synthesis techniques. A) Hydrogel scaffolds formed via the crosslinking of polymers or macromers. B) Solid porous scaffolds formed via phase separation technique. C) Fibrous scaffolds formed via the electrospinning process [56]. Image reused with permission © John Wiley & Sons.

Table 1. A comprehensive summary of 3D scaffold formats and materials used for the culture and differentiation of neural stem and progenitor cells.

Scaffold Material	Cell Culture	Scope/Purpose	Reference
HYDROGELS: Natural Materials			
Type-I Collagen	Rat cortical neural precursor cells	Expansion and differentiation of NPCs to neurons and astrocytes in 3D	O'Connor SM, Stenger DA, Shaffer KM, Maric D, Barker JL, Ma W. Primary neural precursor cell expansion, differentiation and cytosolic Ca2+ response in three-dimensional collagen gel. Journal of Neuroscience

			Methods 2000;102:187-95. [57]
Type-I Collagen	Embryonic rat neural stem and progenitor cells	The first example of functional synapse and neuronal network formation in a 3D matrix	Ma W, Fitzgerald W, Liu QY, O'Shaughnessy TJ, Maric D, Lin HJ, et al. CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. Experimental Neurology 2004;190:276-88. [58]
Type-I Collagen	Embryonic rat neural stem cells and neural progenitor cells	Combined with a bioreactor system, scaffolds yield structures that are bigger with less necrosis	Lin HJ, O'Shaughnessy TJ, Kelly J, Ma W. Neural stem cell differentiation in a cell–collagen–bioreactor culture system. Developmental Brain Research 2004;153:163-73. [59]
Type-I Collagen	Embryonic rat neural stem and progenitor cells	When combined with a rotary wall bioreactor, structures had less necrosis and formed layered structures	Ma W, Chen S, Fitzgerald W, Maric D, Lin HJ, O'Shaughnessy TJ, et al. Three-Dimensional Collagen Gel Networks for Neural Stem Cell-Based Neural Tissue Engineering. Macromolecular Symposia 2005;227:327-34. [60]
Calcium-alginate	Mouse hippocampal neural stem cells	The calcium-alginate beads allowed for culture of large numbers of NSCs with high recovery rate	Li X, Liu T, Song K, Yao L, Ge D, Bao C, et al. Culture of neural stem cells in calcium alginate beads. Biotechnology progress 2006;22:1683-9. [61]
Type-1 Collagen/hyaluronan composite	Culture and differentiation of embryonic and adult mouse neural stem/progenitor cells (NS/PCs)	Enhanced the physiological relevance of NS/PC culture and differentiation compared to 2D	Brännvall K, Bergman K, Wallenquist U, Svahn S, Bowden T, Hilborn J, et al. Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. Journal of Neuroscience Research 2007;85:2138-46. [62]
Hyaluronic acid	Ventral midbrain mouse neural stem cells	Stiffer hydrogels cause mostly astrocyte differentiation and softer hydrogels neuronal differentiation	Seidlits SK, Khaing ZZ, Petersen RR, Nickels JD, Vanscoy JE, Shear JB, et al. The effects of hyaluronic acid hydrogels with tunable mechanical properties on neural progenitor cell differentiation. Biomaterials 2010;31:3930-40. [63]
Type-I Collagen	Human neural progenitor-derived astrocytes	Studying axon growth promoting effects in 3D on dorsal root ganglion	Führmann T, Hillen LM, Montzka K, Wöltje M, Brook GA. Cell–Cell interactions of human neural progenitor-derived astrocytes within a microstructured 3D-scaffold. Biomaterials 2010;31:7705-15. [64]
Type-I Collagen	Culture and differentiation of rat NS/PCs to neurons, astrocytes and oligodendrocytes	Applications as animal surrogates for drug discovery and toxicity testing	Ge D, Song K, Guan S, Qi Y, Guan B, Li W, et al. Culture and Differentiation of Rat Neural Stem/Progenitor Cells in a Three-Dimensional Collagen Scaffold. Applied Biochemistry and Biotechnology 2013;170:406-19. [65]
Hyaluronic acid-catechol	Human neural stem cells	Hydrogel displayed pH-dependent adhesive/cohesive properties for neural tissue engineering	Hong S, Yang K, Kang B, Lee C, Song IT, Byun E, et al. Hyaluronic Acid Catechol: A Biopolymer Exhibiting a pH-Dependent Adhesive or Cohesive Property for Human Neural Stem Cell Engineering.

			Advanced Functional Materials 2013;23:1774-80. [66]
Alginate	ReNcell VM human neural progenitor cells	A high throughput microarray platform for the growth, differentiation and toxicological study of NPCs	Meli L, Barbosa HSC, Hickey AM, Gasimli L, Nierode G, Diogo MM, et al. Three dimensional cellular microarray platform for human neural stem cell differentiation and toxicology. Stem Cell Research 2014;13:36-47. [67]
Type-I Collagen	Primary embryonic rat progenitor cells spheroids	Study of the proliferation and differentiation of corpus striatum progenitors in 3D	Cruz Gaitán AM, Torres-Ruíz NM, Carri NG. Embryonic neural stem cells in a 3D bioassay for trophic stimulation studies. Brain Research Bulletin 2015;115:37-44. [68]
Matrigel	ReNcell VM human neural progenitor cells	A 3D Alzheimer's disease model that exhibits key events in pathogenesis	Kim YH, Choi SH, D'Avanzo C, Hebisch M, Sliwinski C, Bylykbashi E, et al. A 3D human neural cell culture system for modeling Alzheimer's disease. Nat Protocols 2015;10:985-1006. [69]
Type-I Collagen	Embryonic murine neural stem cells	Investigates the sensitivity of NSCs in 3D to heavy metals	Tasneem S, Farrell K, Lee M-Y, Kothapalli CR. Sensitivity of neural stem cell survival, differentiation and neurite outgrowth within 3D hydrogels to environmental heavy metals. Toxicology Letters 2016;242:9-22. [70]
HYDROGELS: Synthetic Materials			
Poly(lactide-*co*-glycide) microspheres loaded in alginate hydrogel	Adult rat neural progenitor cells	NPCs can be cultured and expanded in this system and showed increased expansion in the degradable alginate compared to non-degradable systems	Ashton RS, Banerjee A, Punyani S, Schaffer DV, Kane RS. Scaffolds based on degradable alginate hydrogels and poly (lactide-co-glycolide) microspheres for stem cell culture. Biomaterials 2007;28:5518-25. [71]
IKVAV-incorporated RADA16 self-assembling peptide	Primary mouse neural stem cells	Self-assembling scaffold for neural tissue engineering applications	Zhang ZX, Zheng QX, Wu YC, Hao DJ. Compatibility of neural stem cells with functionalized self-assembling peptide scaffold in vitro. Biotechnology and Bioprocess Engineering 2010;15:545-51. [72]
Laminin-functionalised PuraMatrix	ReNcell VM human neural progenitor cells	Optimization of a 3D-scaffold NPC culture and differentiation protocol	Ortinau S, Schmich J, Block S, Liedmann A, Jonas L, Weiss DG, et al. Effect of 3D-scaffold formation on differentiation and survival in human neural progenitor cells. BioMedical Engineering OnLine 2010;9:70. [73]
$C_{16}H_{31}O$-$A_3G_4D_2$ IKVAV self-assembling peptide	Rat neural progenitor cells	A self-assembling peptide hydrogel for the encapsulation and differentiation of NPCs	Song Y, Li Y, Zheng Q, Wu K, Guo X, Wu Y, et al. Neural Progenitor Cells Survival and Neuronal Differentiation in Peptide-Based Hydrogels. Journal of Biomaterials Science, Polymer Edition 2011;22:475-87. [74]
Methacrylamide chitosan	Adult rat neural progenitor/stem cells	Macroporosity was shown to effectively promote NS/PC 3D differentiation	Li H, Wijekoon A, Leipzig ND. 3D differentiation of neural stem cells in macroporous photopolymerizable hydrogel scaffolds. PLoS One 2012;7:e48824. [75]

IKVAV-RADA16 self-assembling peptide	Rat neural stem cells	The IKVAV-functionalised peptide was shown both *In vivo* and *in vitro* to support the NSC growth and differentiation	Cheng T-Y, Chen M-H, Chang W-H, Huang M-Y, Wang T-W. Neural stem cells encapsulated in a functionalized self-assembling peptide hydrogel for brain tissue engineering. Biomaterials 2013;34:2005-16. [76]
Flourinated methacrylamide chitosan	Adult rat neural stem progenitor cells	The material enhanced oxygen exchange and neuronal differentiation when compared to the unflourinated material	Li H, Wijekoon A, Leipzig ND. Encapsulated Neural Stem Cell Neuronal Differentiation in Fluorinated Methacrylamide Chitosan Hydrogels. Ann Biomed Eng 2014;42:1456-69. [77]
Polyurethane	Adult mouse neural stem cells	Thermoresponsive biodegradable polyurethane bioink was used to print NSCs	Hsieh F-Y, Lin H-H, Hsu S-h. 3D bioprinting of neural stem cell-laden thermoresponsive biodegradable polyurethane hydrogel and potential in central nervous system repair. Biomaterials 2015;71:48-57. [78]
IKVAV/RGD functionalised RADA16-I self- assembling peptide	Embryonic rat neural progenitor and neural stem cells	A new self-assembling peptide with a more permissive environment for nerve regeneration	Sun Y, Li W, Wu X, Zhang N, Zhang Y, Ouyang S, et al. Functional Self-Assembling Peptide Nanofiber Hydrogels Designed for Nerve Degeneration. ACS Applied Materials & Interfaces 2016;8:2348-59. [79]
Methacrylate-modified hyaluronic acid	Human iPSC-derived NPCs	Layered hydrogel of different modulus influences migration and differentiation	Zhang Z-N, Freitas BC, Qian H, Lux J, Acab A, Trujillo CA, et al. Layered hydrogels accelerate iPSC-derived neuronal maturation and reveal migration defects caused by MeCP2 dysfunction. Proceedings of the National Academy of Sciences 2016;113:3185-90. [80]
RGD-functionalised strain-promoted azide-alkyne cycloaddition (SPAAC) covalently crosslinked elastin-like protein (ELP) gel	Murine neural progenitor cells	A versatile hydrogel capable of functionalization via crosslinking useful in bioprinting applications	Madl CM, Katz LM, Heilshorn SC. Bio-Orthogonally Crosslinked, Engineered Protein Hydrogels with Tunable Mechanics and Biochemistry for Cell Encapsulation. Advanced Functional Materials 2016;26:3612-20. [81]
PuraMatrix	Adult rat neural stem/progenitor cells	When compared, surface plating and injection culture methods gave better expansion and differentiation than an encapsulation method	Aligholi H, Rezayat SM, Azari H, Ejtemaei Mehr S, Akbari M, Modarres Mousavi SM, et al. Preparing neural stem/progenitor cells in PuraMatrix hydrogel for transplantation after brain injury in rats: A comparative methodological study. Brain Research 2016;1642:197-208. [82]
SOILD POROUS SCAFFOLDS			
Porous poly(lactic-*co*-glycolic acid)/Polylysine	Murine neural stem cells	Scaffolds were implanted with NSCs and used to mouse repair spinal cord	Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proceedings of the National Academy of

			Sciences 2002;99:3024-9. [83]
Poly(lactic-*co*-glycolic acid) porous polymer	Embryonic rat neural stem cells	Biodegradable scaffolds with aligned pores demonstrate ability to act as platforms for axonal regeneration	Olson HE, Rooney GE, Gross L, Nesbitt JJ, Galvin KE, Knight A, et al. Neural stem cell–and Schwann cell–loaded biodegradable polymer scaffolds support axonal regeneration in the transected spinal cord. Tissue Engineering Part A 2009;15:1797-805. [84]
Porous polystyrene	Human neural progenitor cells	Identification of 13 cytokines upregulated when cells are cultured in 3D compared to 2D	Lai Y, Asthana A, Cheng K, Kisaalita WS. Neural cell 3D microtissue formation is marked by cytokines' up-regulation. PloS one 2011;6:e26821. [85]
Porous polystyrene	Human neural progenitor cells	A new 3D scaffold for the culture of active NPCs	Lai Y, Kisaalita WS. Performance Evaluation of 3D Polystyrene 96-Well Plates with Human Neural Stem Cells in a Calcium Assay. Journal of Laboratory Automation 2012;17:284-92. [86]
Graphene foam	Adult mouse hippocampal NSCs	Conductive graphene foams gave good electrical stimulation of differentiated NSCs	Li N, Zhang Q, Gao S, Song Q, Huang R, Wang L, et al. Three-dimensional graphene foam as a biocompatible and conductive scaffold for neural stem cells. Scientific reports 2013;3. [87]
Multi-walled carbon nanotube functionalised porous chondroitin sulphate	Embryonic rat neural progenitor cells	Shows the formations of active neuronal cell cultures	Serrano MC, Nardecchia S, García-Rama C, Ferrer ML, Collazos-Castro JE, del Monte F, et al. Chondroitin sulphate-based 3D scaffolds containing MWCNTs for nervous tissue repair. Biomaterials 2014;35:1543-51. [88]
Alvetex porous polystyrene	Human neural stem cells	Identification of specific miRNA implicated in hNSC differentiation	Stevanato L, Sinden JD. The effects of microRNAs on human neural stem cell differentiation in two- and three-dimensional cultures. Stem Cell Research & Therapy 2014;5:49. [89]
Silk	Human neural progenitor cells	Enhanced cell viability and proliferation over 14 days showing potential for tissue engineering purposes	Subia B, Rao RR, Kundu SC. Silk 3D matrices incorporating human neural progenitor cells for neural tissue engineering applications. Polymer Journal 2015.[90]
FIBROUS SCAFFOLDS			
Polyglycolic acid microfibres	Murine neural stem cells	NSCs were seeded onto the polymer scaffold and implanted into injured mouse brains	Park KI, Teng YD, Snyder EY. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. Nature Biotechnology 2002;20:1111-7. [91]
Poly(L-lactic acid) nanofibres	Neonatal mouse cerebellum stem cells	NSCs differentiated and fibres acted as positive cues for neurite out-growth	Yang F, Murugan R, Ramakrishna S, Wang X, Ma Y-X, Wang S. Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering. Biomaterials 2004;25:1891-900. [92]
Self-assembled IKVAV functionalised peptide nanofibres	Murine neural progenitor cells	A high density of IKVAV functionalisation induced rapid neuronal differentiation	Silva GA, Czeisler C, Niece KL, Beniash E, Harrington DA, Kessler JA, et al. Selective

			Differentiation of Neural Progenitor Cells by High-Epitope Density Nanofibers. Science 2004;303:1352-5. [93]
Poly(L-lactic acid) nanofibres	Mouse cerebellum neural stem cells	Scaffold promotes NSC adhesion and supports differentiation and neurite outgrowth	Yang F, Xu C, Kotaki M, Wang S, Ramakrishna S. Characterization of neural stem cells on electrospun poly (L-lactic acid) nanofibrous scaffold. Journal of Biomaterials Science, Polymer Edition 2004;15:1483-97. [94]
Functionalised RADA16 self-assembling peptide	Adult mouse neural stem cells	The intrinsically defined scaffold gave similar NSC culture results to Matrigel	Gelain F, Bottai D, Vescovi A, Zhang S. Designer Self-Assembling Peptide Nanofiber Scaffolds for Adult Mouse Neural Stem Cell 3-Dimensional Cultures. PLOS ONE 2006;1:e119. [95]
Poly(ε-caprolactone) nanofibres	Rat brain-derived neural stem cells	Ethylenediamine functionalisation increased NSC cell adhesion	Nisbet DR, Yu LMY, Zahir T, Forsythe JS, Shoichet MS. Characterization of neural stem cells on electrospun poly(ε-caprolactone) submicron scaffolds: evaluating their potential in neural tissue engineering. Journal of Biomaterials Science, Polymer Edition 2008;19:623-34. [96]
BDNF-immobilized poly- ε-caprolactone nanofibres	Mouse neural stem cells	BDNF-functionalised nanofibers supports NSCs and their derivatives	Horne MK, Nisbet DR, Forsythe JS, Parish CL. Three-dimensional nanofibrous scaffolds incorporating immobilized BDNF promote proliferation and differentiation of cortical neural stem cells. Stem cells and development 2009;19:843-52. [97]
Polyhydroxylalkanoate nanofibres	Rat-derived neural stem cells	3D nanofibers display suitability for NSC attachment, synaptic outgrowth and synaptogenesis	Xu X-Y, Li X-T, Peng S-W, Xiao J-F, Liu C, Fang G, et al. The behaviour of neural stem cells on polyhydroxyalkanoate nanofiber scaffolds. Biomaterials 2010;31:3967-75. [98]
RGD and bone marrow homing peptide functionalised self-assembling peptide	Adult mouse neural stem cells	NSCs are viable, proliferate and differentiate on the material	Cunha C, Panseri S, Villa O, Silva D, Gelain F. 3D culture of adult mouse neural stem cells within functionalized self-assembling peptide scaffolds. Int J Nanomedicine 2011;6:943-55. [99]
Chitin-alginate microfibres	Human iPSCs and derived hNPCs and neurons	A platform for the generation of large number of targeted differentiated neural cells	Lu HF, Lim S-X, Leong MF, Narayanan K, Toh RPK, Gao S, et al. Efficient neuronal differentiation and maturation of human pluripotent stem cells encapsulated in 3D microfibrous scaffolds. Biomaterials 2012;33:9179-87. [100]
Poly-ε-caprolactone microfibers coated in BDNF immobilized polyelectrolyte multilayers	ESC-derived mouse neural progenitor cells	A biofunctionalised complex 3D scaffold for 3D neural cell culture	Zhou K, Thouas G, Bernard C, Forsythe JS. 3D presentation of a neurotrophic factor for the regulation of neural progenitor cells. Nanomedicine 2013;9:1239-51. [101]
ACELLULAR SCAFFOLDS			

Genepin crosslinked acellular rat brain matrix	Rat adult neural stem cells	Scaffolds showed high cytocompatibility and suitability as tissue engineering scaffolds for CNS tissue	He H, Li W, Su J, Wang H, Ye Z, Cai M, et al. In Vitro Evaluation of the Cytocompatibility of an Acellular Rat Brain Matrix Scaffold with Neural Stem Cells. Journal of Biomaterials and Tissue Engineering 2015;5:628-34. [102]
MICROFLUIDIC DEVICES AND LAB ON A CHIP SYSTEMS			
Matrigel microfluidic gradient generator	Human neural progenitor cells	Exponential FGF gradients were shown to be useful in generating asymmetrical neuronal cultures	Keenan TM, Grinager JR, Procak AA, Svendsen CN. In vitro localization of human neural stem cell neurogenesis by engineered FGF-2 gradients. Integrative Biology 2012;4:1522-31. [103]
Series of microchannels for nutrient diffusion	Immortalized mouse neural progenitor cells	The device controlled medium diffusion and prevented spontaneous differentiation caused by lack of nutrients	Wang B, Jedlicka S, Cheng X. Maintenance and Neuronal Cell Differentiation of Neural Stem Cells C17.2 Correlated to Medium Availability Sets Design Criteria in Microfluidic Systems. PLoS ONE 2014;9:e109815. [104]
Glass microtubes	Mouse neural stem cells	To study the influence of scaffolds dimensionality and confinement of NSC migration	Koch B, Meyer AK, Helbig L, Harazim SM, Storch A, Sanchez S, et al. Dimensionality of Rolled-up Nanomembranes Controls Neural Stem Cell Migration Mechanism. Nano Letters 2015;15:5530-8. [105]
Collagen multichannel device	Human foetal neural stem cells	The device mimics paracrine signalling in the body to study effects on NSC behaviour	Yang K, Park H-J, Han S, Lee J, Ko E, Kim J, et al. Recapitulation of in vivo-like paracrine signals of human mesenchymal stem cells for functional neuronal differentiation of human neural stem cells in a 3D microfluidic system. Biomaterials 2015;63:177-88. [106]
Poly(ethylene glycol) hydrogel microfluidic device	Human ESC-derived neural progenitor cells and other brain cell types	A self-assembled 3D neural construct capable of accurately predicting neural toxicity	Schwartz MP, Hou Z, Propson NE, Zhang J, Engstrom CJ, Costa VS, et al. Human pluripotent stem cell-derived neural constructs for predicting neural toxicity. Proceedings of the National Academy of Sciences 2015;112:12516-21. [107]
Poly(ε-caprolactone) microfibrous microfluidic channel	Rat adult hippocampal stem/progenitor cells	The scaffold supported the adhesion, survival and differentiation of NS/PCs and importantly allowed cell alignment important for reconnecting nerves	Sharifi F, Patel BB, Dzuilko AK, Montazami R, Sakaguchi DS, Hashemi N. Polycaprolactone Microfibrous Scaffolds to Navigate Neural Stem Cells. Biomacromolecules 2016;17:3287-97. [108]

3.1 Hydrogel Scaffolds

Hydrogels are used as tissue engineering scaffolds typically because their soft, hydrated form resembles that of naturally occurring living tissue. Hydrogels are 3D hydrated polymer

networks, held together by chemical and/or physical crosslinks, in a dispersant, typically water. The mechanical properties of hydrogels can be tuned in some cases to resemble a range of soft tissues [109]. The high water content and highly porous nature of hydrogels allow for facile transport of oxygen, nutrients and waste as well as effective transport of soluble factors [110]. An additional attraction of hydrogels is their ability to be administered via injection [111]. After injection, they can conform to the available space, allowing for uniform tissue regeneration. However, hydrogels have to be maintained in a hydrated state, therefore could possibly suffer from long term stability issues *in vitro* [112]. Also, densely crosslinked gels can inhibit the natural migration of cells [113].

Hydrogels can easily be made to mimic the mechanical properties of the CNS. The natural brain ECM is primarily composed of a hyaluronic acid-based hydrogel [36], functionalised with a myriad of biomolecules including laminins, fibronectin, collagens, vitronectin, tenacins and nidogens [10]. Hydrogels obviously therefore provide an excellent scaffold material for neural tissue engineering.

3.1.1 Natural polymer hydrogels

Natural hydrogels of macromolecules such as collagen, gelatin, laminin, alginate and hyaluronic acid possess biological properties, such as bioactive motifs and cell binding domains for cell-matrix interactions, which can be critical for the maintenance of natural tissue phenotype and function. A readily used example of a naturally derived mixture of ECM components is Matrigel^{TM}, an undefined and variable hydrogel mixture of ECM proteins. While natural hydrogels can retain their biological function *in vitro*, they do suffer from batch-to-batch variability, can have poorly defined compositions and are difficult to

modify biochemically [114, 115]. Natural hydrogels can be limited in their clinical application due to the risk of immune rejection and disease transfer [114]. While natural hydrogels often do not display the ideal set of characteristics for neural tissue engineering, and, unlike synthetic materials, cannot be designed from the bottom up, they are often modified to make them applicable as accurate 3D neural cell culture models.

Collagen type-I is by far the most popular natural hydrogel material, with numerous publications citing its use as a scaffold for neural cell culture [57-60, 62, 65, 116-120]. Some other interesting examples of natural hydrogels used for neural tissue engineering include chitosan/gelatin hybrid hydrogels. These novel composite hydrogels were shown to enhance neural stem and progenitor cell adhesion and long term expansion, as well as differentiation to neuronal and glial cells [121]. Cylindrical collagen channels have been used to mimic the stem cell niche migratory pathway of the CNS. Collagen hydrogel tubes of 180 µm were used as scaffolds to create aligned astrocyte bundles (**Figure 6**), which were demonstrated to direct the alignment of neurites, mimicking the glial tubes that direct the migration on NPCs *in vivo* [122]. These aligned bundles could have potential applications for directing cells to sites of neurodegeneration in the brain. A recent exciting class of natural hydrogels used for neural tissue engineering are gellan gums. Gellan is a highly versatile backbone material amenable to various physical and chemical functionalisation [123]. RGD-functionalised gellan gums have recently been 3D-printed in layers to mimic the human cortex [124]. Alternating cellular and acellular layers were composed to encourage cells to migrate between layers and form structures similar to that of the cortex (**Figure 7.B**). Cell viability was demonstrated after 5 days and some migration was observed (**Figure 7.C**) [124].

Figure 6. 0.1 mg/ml Collagen I cylinders support astrocyte bundle formation. Collagen I (green), glial cell marker GFAP (red) and Hoechst nuclear counterstain (blue) [122]. Scale bars 100 μm. Reproduced with permission © Elsevier.

Figure 7. 3D-printed layered RGD-functionalised gellan gum structures for neural tissue engineering. **A**. A 3D graphics representation of a six-layered structure. **B**. A three-layered gellan gum construct (dyes indicating different layers). **C**. β-III Tubulin immunostaining of murine cortical neurons in layers 1 and 3 migrating into the acellular layer 2 (colour represents migration in the z-direction) [124]. Reproduced with permission © Elsevier.

Some of the most recent remarkable examples of neural tissue engineering have come from the culture of neural cells using naturally derived hydrogels. Self-organized neural structures that resemble those present in an early developing brain (**Figure 8**), termed cerebral organoids, were created using Matrigel™ scaffolds and a spinning bioreactor. Neurospheres were derived from hiPSCs, then placed in Matrigel™ droplets. The droplets were then placed in a spinning bioreactor to yield an incredibly well-defined developing cortical-like structure [125]. This system has further been developed with the use of defined hydrogel scaffolds using hyaluronic acid-based materials as well as a defined culture medium [126]. Whilst organoid cultures can produce remarkable brain-like structures, they can suffer from hypoxia and areas of necrosis due to high cell density and lack of vasculature [112].

Figure 8. Matrigel™ cultured cerebral organoid cortical tissue shows layering similar to the ventricular zone in natural tissue [127]. Neurons shown in green (TUJ1) and radial glial stem cells (RGs) in red (PAX6). Arrowheads indicate PAX6 positive RGs outside the ventricular zone. Image re-used with permission © Nature Publishing Group.

3.1.2 Synthetic polymer hydrogels

Synthetic hydrogels are made from polymers such as poly(ethylene glycol) (PEG), poly(vinyl alcohol), poly(lactic acid) (PLA) and poly(hydroxyethyl methacrylate) (PHEMA) to name a few [128]. Synthetic hydrogels are able to be synthesised with controlled physical (elasticity and degradability) and chemical (surface functionalisation) properties to tailor the material to specific applications. They are typically highly reproducible and simple to manufacture.

Synthetic hydrogel scaffolds typically have two important features. Firstly, they are usually inert, but biodegradable. As cells grow, the scaffold degrades, making way for the cells to synthesise their own ECM and create their own scaffold [129, 130]. Secondly, they can contain immobilised biological components to better encourage natural cell-matrix interactions. PLA-PEG-dimethacrylate triblock macromer has been used to synthesise a degradable hydrogel for neural tissue engineering. By manipulating the degradation rate of the scaffold, embryonic rat forebrain cells were able to produce their own ECM at a rate similar to the degradation rate of the scaffold [130]. More recent examples of neural tissue engineering use ‘smart’ synthetic hydrogels and both primary and PSC-derived NSCs to generate neural tissue. Thermoresponsvie polyurethane hydrogels laden with primary adult mouse brain NSCs have been 3D printed, and shown to restore brain function in zebrafish with traumatic brain injury [78]. Self-assembling peptide hydrogels possess a fibrous

structure, similar to the native ECM. $RADA_{16}$-IKVAV functionalised peptide has been shown to self-assemble into nanofibrous hydrogel scaffolds, enhancing the survival of encapsulated primary rat NSCs and reducing the formation of glial cells [76]. Self-assembling peptides have also been shown to undergo gelation at physiological temperatures, making them ideal scaffolds for injectable applications [131].

3.2 Solid porous scaffolds

While hydrogels provide excellent scaffolds that mimic the natural ECM, they are limited in scale-up because of long term storage issues, stability and batch-to-batch variability. Highly porous solid scaffolds can be manufactured in a controlled and reproducible fashion, can be appropriately moulded, are inert in structure and have long term stability [132]. The mechanical stability of these scaffolds, along with their high porosity and pore interconnectivity make them ideal for highly interactive 3D cell cultures [55]. Porous scaffolds theoretically have some other key advantages. The high porosity of these scaffolds allows for deeper and more uniform nutrient transport and also allows cells to freely migrate throughout the structure without much resistance. The pores themselves can also limit the size of colonies, which when too large can cause cells to become necrotic [55]. The mechanical stability of solid porous scaffolds make them a more practical material for handling, which is difficult with soft hydrogels [112]. The key limitations of solid scaffolds however are, firstly, they are typically opaque and have poor light transmission properties, making *in situ* imaging difficult [133]. Secondly, recovering cells from the scaffold for analysis can be a difficult and tedious process [134]. Third, the Young's modulus of many

solid porous scaffolds is quite different to that of CNS tissue (0.1-16 kPa)[33] and so these scaffolds may not provide realistic tissue and disease models.

An array of techniques have been used to synthesise solid porous scaffolds, including salt leaching [135], phase separation [136], freeze drying [116], gas foaming [137], emulsion templating [138] and 3D printing [139]. The emulsion templating process has been shown to give good control over porosity, pore size and pore interconnect size, making it possible to produce highly porous structures with porosity up to around 90% [138]. When combined with 'click-chemistry' reactions and photocuring the preparation time of these porous polymer sponges is very short compared to other cell culture scaffolds [140], and can be used to polymerise emulsions of low stability.

Solid porous scaffolds produced by the salt leaching process were shown to be able to culture primary mice neuronal cells with characteristics more akin to the *in vivo* environment when compared to 2D culture. Voltage gated calcium channel functionality, which is known to be exaggerated in 2D cultures, was shown to decrease (compared to 2D) and behave more like that of *in vivo* tissue when cultured on solid porous poly-l-lactic acid scaffolds [141].

3D porous silk sponges are a recently discovered tissue engineering biomaterial that can be used in a range of formats including films, gels, sponges and mats [142]. Recently, silk sponges have been used in the 3D culture of primary rat cortical neurons. Carefully constructed silk-collagen composites have been used to create functional brain-like cortical tissue [143]. The compartmentalised structure of the silk-collagen scaffold (**Figure 9**) allows for spatial separation of neuronal cell bodies and neural projections, resemblant of the layered structure of the cerebral cortex, and grey and white matter [144].

Figure 9. Silk sponges of diameter 12 mm are constructed with 2 mm collagen centres (left) [143]. The composite, when cultured with primary rat hippocampus neurons, resembles structures similar to the white and grey matter of the brain (right) [143]. Images reused with permission © National Academy of Science.

3.3 Fibrous scaffolds

Fibrous scaffolds, and particularly nanofibrous scaffolds, hold great promise as tissue engineering scaffolds as their topography most resembles that of natural human ECM [145]. A range of techniques exist for the synthesis of nanofibers, including electrospinning, self-assembly, template synthesis and phase separation [146, 147]. Desirable properties of fibrous scaffolds include a high surface area-to-volume ratio and high porosity [146]. Fibrous scaffolds have been used in a range of tissue engineering applications including bone, cartilage, ligament, skeletal muscle, skin and many others. Like hydrogels, a range of both natural and synthetic polymers have been explored for use as fibrous scaffolds. Electrospun fibrous scaffolds typically do not allow infiltration of cells deep into the scaffold, mainly due to the fibre diameter [148, 149]. Recent research has focussed on reducing the fibre diameter to achieve better cell infiltration, to give 3D, and not 2D, cell growth, which has been the case for electrospun fibrous scaffolds previously [56].

Electrospun PLA fibrous scaffolds coated in electrically conductive polymers have been used for 3D neural progenitor cell culture. The scaffold was shown to support the attachment and migration of rat hippocampal neural progenitors, indicating biocompatibility [150]. Another example involved the culture of rat brain-derived NSCs on electrospun poly(ε-caprolactone)

scaffolds. Fibre surfaces were modified with an ethylenediamine coating, resulting in increased cell adherence [96]. Electrospun gelatin scaffolds were coated with decellularized rat brain ECM. The coating, which contains molecules such as glycosaminoglycans, collagen, laminin and fibronectin, was shown to induce the differentiation of mesenchymal stem cells to neural/glial precursor cells [151].

3.4 Acellular Scaffolds

Acellular tissue engineering scaffolds are typically prepared by removing the cellular components of a tissue or whole organ via chemical, biological or mechanical means, to produce a material that is predominantly natural ECM [152]. Upon tissue culture or implantation the acellular scaffold typically degrades, making way for the natural ECM secreted by ingrowing cells [55]. Acellular scaffolds have advantages over other tissue engineering scaffolds: they have similar if not the same chemical and biological composition as natural ECM; they retain native ECM architecture and mechanical properties; and considerably reduce immunological complications associated with whole tissue transplants. Products incorporating acellular scaffolds including heart valves, small intestine submucosa and urinary bladder matrix have all been approved for human medical use [153]. However, as acellular scaffolds are typically allogenic or xenogeneic, there is potential for immune rejection for implanted scaffolds [154].

Porcine brain tissue was successfully decellularized using sodium dodecyl sulphate detergent. The ECM was digested and turned into a liquid, and used as a cell culture coating for the culture of human iPSC-derived neurons. When compared with Matrigel^{TM} coating, the brain matrix coating was shown to enhance primary and secondary dendrite formation

as well as increase synapsin expression, a marker of synapse formation. The digested liquid ECM material was also shown to self-assemble, forming a gel upon injection, indicating suitability as a tissue engineering scaffold for injection [155]. However, due to the animal origin of the material, clinical implementation could be difficult to achieve.

3.5 Microfluidic Devices/Lab-on-a-chip systems

Most tissue engineering models suffer from one important drawback, an inability to incorporate vascularization. This introduces a drastic disparity between tissue engineered models and *in vivo* tissue in terms of access to nutrients and waste diffusion channels. Microfluidic systems have the potential to address such issues with the introduction of small channels which introduce fluid flow, nutrient/waste gradients, medium perfusion and subsequent shear stress to otherwise static tissue culture systems [156]. Microfluidic devices also offer the aspect of spatial control of cells. This can be done with the use of membranes to separate or segregate cells or, more recently, hydrogels for three dimensional spatial control.

A microfluidic device has been developed to prevent the spontaneous differentiation of NSCs, which can occur when the cells are provided with limited nutrients. The device, through a series of microchannels, supplies medium to the cells in a specifically controlled manner. The device was shown to harbour cultures of minimum spontaneous differentiation when compared with control experiments [104]. Other microfluidic devices have been engineered to investigate the influence of paracrine signalling on hNSC differentiation as seen in the brain. hNSCs confined in a 3D channel of collagen were flanked by channels of GDNF-overexpressing hMSCs, with signalling molecules able to diffuse

between the channels (**Figure 10**). The system yielded reduced glial differentiation and enhanced neuronal differentiation of hNSCs [106].

Figure 10. A microfluidic device specifically designed to mimic paracrine signalling that naturally occurs in the brain [106]. Reproduced with permission © Elsevier.

Even more sophisticated systems have been developed in an attempt to mimic parts, regions and functions of the brain, in what are being called 'brain-on-a-chip' models. These models have the potential to be used to screen therapeutic agents, predict toxicity or study neurodevelopmental processes. Examples include models of functional blood-brain-barriers [157, 158], neuromuscular junctions [159] and neuronal circuitry. A recent interesting example involved the construction of a device to study the migratory patterns of hNPCs, which are important for understanding chemotaxis in the brain. Gradients of the chemokine CXCL12 were constructed to measure chemotactic response, and results revealed that a shallow chemotactic gradient best enhances hNPC chemotaxis [160]. Another recent brain-on-a-chip example involved prediction of neural toxicity. A 3D co-culture of NPCs, mesenchymal stem cells, endothelial cells and microglia/macrophage precursors in a functionalised, degradable PEG hydrogel reportedly assembled into a 3D neural construct of neurons, glia and an interconnected vascular network. The construct was then used to screen toxic and non-toxic compounds with an impressive 90% prediction success rate in a blinded trial [107].

4. Scaffold modifications to support neural lineage differentiation

Modifications can be made to tissue engineering scaffolds to increase their physiological relevance and provide a more favourable environment for tissue growth. Modifications of tissue engineering scaffolds can be broken down into three different categories: morphological and topographical; chemical and biological functionalization; and mechanical. Morphological changes such as topographical modifications include introducing roughness, grooves and patterns to scaffold surfaces. Scaffolds can be chemically modified to alter surface energy, induce surface charge, create reactive functional groups or reduce cell adhesion. Biological modifications can be made by attaching ECM proteins, peptide sequences, growth factors and other biomolecules that aid cell function. Finally mechanical properties can be manipulated to influence the attachment, differentiation and migration of certain cell types.

4.1 Topography and morphology

Directed cell growth and migration is fundamental for the development and repair of tissue. Consequently, directed growth of neurites is required for the regeneration of neural tissue in both central and peripheral nervous systems. By altering surface morphology and topography it has been demonstrated that it is possible to guide the growth and migration of neurites.

Topographical cues such as grooves, ridges, pores and nodes can influence cell adhesion, migration, proliferation and differentiation [147]. The most heavily investigated surface morphology for neural cell guidance and migration are ridged/grooved surfaces. These surfaces are intended to guide axonal outgrowth of neurons for spinal cord regeneration.

Grooves of varying width and depth on a variety of different surfaces have been shown to induce alignment of neurons in certain directions and induce outgrowth on surfaces patterned with ECM or growth factor coatings [161-163].

Nanofibrillar structures have also been shown to provide a means for neurite cell attachment and guidance as they somewhat mimic the extracellular environment and the topographical nature of tightly aligned neurite bundles [164]. Fibre dimensions have been shown to improve differentiation of rat hippocampal-derived adult neural stem cells into both neurons and glial cells, when compared to tissue culture polystyrene surfaces [165].

4.2 Mechanical Properties

Various mechanical factors such as shear from fluid flow [166] and ECM elasticity [167] can affect stem cell differentiation in a process known as mechanotransduction. When designing a tissue engineering scaffold it is import to mimic, as closely as possible, the biomechanical environment of the *in vivo* tissue. Scaffold stiffness has been shown to affect the ability of neural precursor cells to attach, survive, proliferate and differentiate to neurons and glia on a range of different substrates.

The modulus of mammalian brain tissue varies throughout the brain [40, 168], with age and development [169] and is species dependent. Studies using magnetic resonance imaging have determined human white and grey matter moduli to be 2.37 and 2.28 kPa respectively [168]. Other studies found that 16 month old bovine white and grey matter had a modulus of 1.9 and 1.4 kPa respectively [40]. Linear deformation experiments and computer simulations have found the Young's modulus of swine brain to be 3.24 kPa [170]. This topic has previously been reviewed and has broadly classified the elastic modulus of brain tissue

to be in the range 0.1 - 16 kPa [33]. In order to engineer tissue as accurately as possible, it is important for scaffold mechanical properties to resemble those of natural tissue as much as possible.

One of the most powerful ways in which a substrate's mechanical properties can affect cell behaviour in 3D is through its impact on differentiation. Substrate stiffness has been shown to direct the differentiation of mesenchymal stem cells to definitive cells types on polyacrylamide gels of Young's modulus values reflective of particular tissue types [171]. Adult mouse NSCs were shown to proliferate best, and express β-tubulin III (a marker indicative of neuronal differentiation), when encapsulated in alginate hydrogels of 183 Pa elastic modulus compared to stiffer materials of up to 19.7 kPa [172]. One of the most interesting examples of mechanotransduction is the ability of different stiffness substrates to affect the differentiation of NSCs to neuronal or glial cells. Slight variation in elastic modulus has been shown to affect differentiation - glial differentiation was observed on stiffer scaffolds and neuronal differentiation on slightly softer scaffolds. Cell culture on variable moduli interpenetrating polymer network (vmIPN) hydrogel surfaces indicated that softer gels (100-500 Pa) favour neuronal differentiation and harder gels (1-10 kPa) favour glial differentiation [173]. Similar results were found with type I collagen-hyaluronic acid hydrogels with varying stiffness, shown to induce neuronal differentiation for weaker scaffolds of 1 kPa modulus and glial differentiation for stiffer scaffolds of 10 kPa modulus [174]. Substrate stiffness has also been shown to influence cell attachment and proliferation. Attachment of rat NPCs on gelatin-based hydrogels was shown to be maximised for softer surfaces of 2 kPa stiffness compared to stiffer surfaces of the same material up to 35 kPa, though expansion of cells was shown not to be affected by substrate stiffness [175].

Materials softer than 100 Pa were shown to inhibit NSC dispersion and attachment indicating materials softer than brain tissue can also be detrimental to cell culture [173].

4.3 Surface Chemistry

The presence of basement membrane proteins such as collagen IV, laminin, fibronectin and others in 3D scaffolds is essential for the growth and adhesion of particular mammalian cell types. Human PSCs are extremely reliant on the extracellular environment for survival and for differentiation into particular cell types [176]. In an attempt to mimic the neural stem cell niche, tissue engineers are incorporating biological molecules onto and within scaffolds to provide a more realistic environment that can aid neural cell growth and differentiation.

The most common form of scaffold surface functionalization is the attachment of cell adhesion molecules. These molecules are typically short peptide binding motif sequences of ECM proteins such as collagen, laminin, fibronectin and vitronectin. The advantage of short peptide sequences over whole proteins is that they can be anchored to a surface, present the active binding site in the desired orientation and provide a high concentration of active binding sites to adhesion receptors [177, 178]. Some of the most commonly used short peptide sequences for human neural cell attachment are RGD [179-181], IKVAV [117, 182, 183] and YIGSR [120, 184-186].

The RGD sequence (Arg-Gly-Asp) [187] is one of the most physiologically ubiquitous binding motifs, and is present in adhesive proteins such as fibronectin, vitronectin, laminin and collagen I [14]. It is also the most widely studied adhesive peptide in the biomaterial field [177]. RGD is highly effective at promoting attachment of numerous cell types including hPSCs [177, 188]. RGD has been used as a purely adsorbed peptide and a chemically bound

peptide. RGD is recognized as a primary sequence by integrin receptors, which makes it robust, maintaining conformation throughout processing and sterilization [177]. RGD has been attached to the surface of many biomaterials and been shown to be successful in the culture and maintenance of mesenchymal stem cells [179-181]. However, its ability to provide continued support for these cultures over a long term has been limited [189]. This could possibly be due to the low affinity of linear RGD peptides, providing insufficiently strong adhesion [189]. Conformationally restricted RGD, in particular cyclic-RGD, increases integrin binding affinity and cell attachment [189, 190].

For the differentiation and maintenance of neural stem cells and neural cells in culture, the most widely used cell attachment molecule is laminin [38, 191], the primary component of the basement membrane in the brain. The IKVAV sequence (Ile-Lys-Val-Ala-Val) has been identified as an active site of laminin for cell adhesion, migration and neurite outgrowth [192]. IKVAV is specifically located on the α1 chain of laminin-1. Variations of the IKVAV peptide have been chemically bound to the surfaces of PEG hydrogels [182], PHEMA hydrogels [183] and collagen matrices [117] to varying degrees of success. Interestingly, it was found that immobilising an IKVAV peptide at each end in a PEG hydrogel produced a conformationally restricted, cyclic version of the peptide, similar to cyclic RGD. This novel cyclic IKVAV peptide outperformed the linear laminin IKVAV in attachment, proliferation, differentiation and migration of NSCs [182].

A less commonly studied laminin peptide is YIGSR (Tyr-Ile-Gly-Ser-Arg). Like IKVAV, YIGSR is an active laminin peptide, located on the β1 chain [193]. YIGSR-functionalized scaffolds have been shown to have similar cellular effects as laminin-coated and IKVAV-functionalised scaffolds [120, 184-186].

Other biological molecules such as growth factors have been identified as surface functionalization molecules. By immobilizing molecules such as growth factors on surfaces, their activity is prolonged, as their attachment prevents endocytosis [194]. Molecules such as bFGF and EGF have been covalently attached to surfaces to promote proliferation and control migration of embryonic stem cells and neural stem cells [195-197]. In terms of aiding neural cell culture, growth factors such as brain-derived neurotrophic factor (BDNF), which promotes survival of cortical neurons [198], and nerve growth factor (NGF), which is involved in maintenance and proliferation of neurons [199], have been attached to hydrogels [200], macroporous polymers [201] and nanofibrous scaffolds [202], and in the majority of cases shown to enhance neuronal survival.

5. Application of 3D *in vitro* neural lineage cell culture in disease modelling

Neurodegenerative diseases are a world-wide leading cause of death, yet in many cases, limited cures exist for these debilitating disorders. Current treatments can alleviate some symptoms, but typically do not stop disease progression. The lack of therapies for neurodegenerative diseases is attributed to poor results from clinical trials, owing to the complexity of these disease states and lack of knowledge of the CNS. This has led to a decline in the funding of such endeavours, which poses huge future problems for aging populations [203]. Accurate and predictive disease models are pivotal to finding treatments to neurodegenerative diseases.

Neurodegenerative diseases include Alzheimer's disease and other dementias, motor neuron diseases, Huntington's disease, Parkinson's disease and others. They are typically characterised by the loss of function, then death of specific neuronal subtypes.

Neurodegenerative diseases can be caused by genetic, epigenetic or environmental factors. Current models of these diseases rely on humanized transgenic animals which can give poor or misleading outcomes when screening for treatments [46].

The ease with which induced pluripotent stem cells (iPSCs) can now be generated from patient and normal human somatic cells has created new approaches to model diseases. HiPSCs have the advantage in that they provide a model with a completely human genome, bypass ethical concerns associated with embryonic stem cells, provide an unlimited supply and avoid cross-species issues with animal-derived products.

Somatic cells can be obtained from a patient with a particular neurological disease phenotype and used to generate hiPSC-derived neurons and glial cells of that particular phenotype. Previous methods of studying mutated ES cells, rodent brains and post-mortem brain tissue has had little success in identifying new therapeutic targets. HiPSC-derived neurons and glial cells offer a way of studying live, developing, disease-relevant cell types. While it is still a relatively new concept, hiPSC-derived neural cells of particular neurological disease types have been created from the somatic cells of patients with particular diseases such as dysautonomia [204], Rett syndrome [205, 206], Parkinson's disease [207], Huntington's disease [208], epilepsy [209] and others. For a review of neurological disease models using patient-derived iPSCs the reader is directed to the work of *Avior et al* [210].

Upon first thought it may seem far-fetched to model these diseases, which can take 50-70 years to appear symptomatically in a patient, in reprogrammed stem cell cultures only a few weeks old. Particular neurodegenerative diseases such as Huntington's disease can express genetic mutations even before birth [211], however most neurodegenerative disorders do not have known early genetic mutations. For this reason, research is being carried out into

the premature aging of iPSC-derived neural cells by increasing oxidative stress through chemical treatment [207] and the addition of progerin [212], which has been shown to accelerate the aging of iPSC-derived dopaminergic neurons in Parkinson's disease studies [213].

There are however some concerns or challenges associated with using PSCs to model diseases. PSCs are used most readily to model monogenic early onset disorders, and are less useful, as discussed above, at modelling complex disorders with late onset symptoms [210]. The potential to model complex disorders most likely exists in patient-derived iPSCs rather than ESCs. When modelling cell-specific disorders it is necessary to have reliable, robust and reproducible differentiation techniques, which do not yet exist for some cell types. Non-cell autonomous effects have been identified as factors in numerous conditions [214]. On this basis there is a need to form complex co-culture systems of multiple target cells types. Control PSC cell lines run alongside assays also have shown potential to develop mutations over extended culture periods [215]. ESCs and iPSCs used to generate target neural lineage cell types for study have the potential to contaminate populations with residual PSCs, which have the potential to introduce non-target cell types into assays [214]. Also, comparisons of disease-derived iPSCs with healthy control cell lines can be a challenge due to transcriptome variability between individuals [216]. When comparing the differentiation of iPSCs and ESCs to neuroepithelial cell types, iPSCs were shown to produce the same cell types with the same transcriptional network but with significantly reduced efficiency and increased variability [217]. The differentiation efficiency of iPSCs is possibly affected by the viral reprogramming techniques with which they are generated[218]. While there are challenges associated with the use of PSCs and their derivatives for the modelling of diseases, none look to be insurmountable with multiple research teams currently investigating solutions.

6. Outlook

6.1 Disease Modelling

The next logical step in neurological disease modelling is co-cultures of different types of iPSC-derived neurons and glia, and generating spatially accurate three-dimensional cultures. Only a few co-cultures of hPSC-derived neurons and astrocytes have been reported [219, 220]. The development of 3D cultures has demonstrated the ability of hPSCs to self-organize during differentiation [127, 221, 222], and are beginning to be used to model certain neurological conditions [223] such as Zika virus [224, 225], microcephaly [127] and Alzheimer's Disease [226].

6.2 Drug Screening

Drug development is a long, tedious and expensive process with a path to market typically taking up to 20 years. It is divided into five phases: discovery and development, preclinical research, clinical research, administrative review and post-market safety monitoring [227]. The progression of new drug candidates from therapeutic discovery to clinical use is currently at an unacceptably low rate [228] and this can largely be attributed to poor pharmacological and toxicological data obtained from preclinical 2D *in vitro* cell-based and *in vivo* animal-based assays [229]. Unexpected toxicity is one of the main factors for post-marketing product withdrawals, accounting for a staggering 90% of market withdrawals from 1992-2002 [230], and has been an area of investigation that has seen little improvement over the past 20 years. This extremely high failure rate highlights the need for improved models for preclinical drug toxicity screening.

Preclinical research is typically where 2D cell cultures or animal models are used to test efficacy and toxicity of new drug candidates. Both 2D cell culture [1] and animal models [2] are limited in their ability to mimic human physiology. Furthermore, immortalized cell lines with genetic modifications that are typically used in 2D cell culture models [231] raise further questions of physiological accuracy. HiPSCs generated using non-integrative strategies and cultured in 3D could be used to reduce the level of doubt associated with genetically modified cells [232], and may in the future lead to more reliable *in vitro* toxicity data.

6.3 Surgical Implants

Secondary injury that comes from trauma or stroke causes permanent damage to the brain, as mature axons cannot be repaired or regenerated [233]. These injuries induce the proliferation of astrocytes, fibroblasts and oligodendrocyte precursors, which form glial scar. Inhibitory molecules located within the glial scar such as chondroitin sulphate proteoglycans further prevent neurogenesis [234].

After brain trauma, widespread neuronal cell death typically occurs and a cavity forms in the brain [235]. An implant that induces the formation of new functional neural tissue is required for repair. Tissue engineering scaffolds offer a possible way to restore function to the brain. Scaffolds can be bare [118], or possess molecules that induce regeneration [119]. They could contain a mixture of neural cells ready to network with the existing brain tissue, or they could be seeded with neural stem cells, which upon implantation could differentiate and form new neuronal and glial tissue. Although there is promising research being undertaken in the area of 3D neural tissue engineering, as highlighted above, there are

currently no FDA approved cell-based neural implant products for cellular or tissue therapy [236].

7. Conclusions

The need for more accurate *in vitro* models for tissues of the human nervous system is clearly highlighted by the current lack of treatments for neurological disorders. A variety of materials with various modifications have been investigated for the 3D culture of neural tissue, but it is clear that even the most advanced materials struggle to mimic the complexity of natural tissue. This is reflected in the lack of 3D neural tissue products on the market. However, giant strides have been made with organoid and hydrogel cultures that have produced tissue with complexity that has not been seen previously *in vitro*. These cultures do however suffer from scale-up issues as they have poor nutrient diffusion abilities due to lack of vascularisation. There are still developments to be made before 3D *in vitro* functional neural tissue cultures can be used for disease modelling, drug screening and surgical implantations.

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