Tissue Engineering 3D Neurovascular Units: A Biomaterials and Bioprinting Perspective

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Abstract

Neurovascular dysfunction is a central process in the pathogenesis of stroke and most neurodegenerative diseases, including Alzheimer’s disease. The multicellular neurovascular unit (NVU) combines the neural, vascular and extracellular matrix (ECM) components in an important interface whose correct functioning is critical to maintain brain health. Tissue
engineering is now offering new tools and insights to advance our understanding of NVU function. Here, we review how the use of novel biomaterials to mimic the mechanical and functional cues of the ECM, coupled with precise layered deposition of the different cells of the NVU through 3D bioprinting, is revolutionising the study of neurovascular function and dysfunction.
Modelling the neurovascular unit in health and disease

The neurovascular unit (NVU) (see Glossary) is an organised multicellular and multicomponent network that is important to brain health [1]. This brain-localised unit is comprised of neural and vascular components, with the interface and interactions between these components being crucial to regulation of cerebral blood flow through neurovascular coupling, blood-brain barrier (BBB) function, neuroinflammation and neuronal function [1–3]. Damage to the NVU can lead to restricted transport of oxygen and nutrients to the brain, impaired ability to clear toxic compounds from the brain and/or allow for the infiltration of harmful molecules and immune cells across the BBB [4], resulting in both onset and progression of neurovascular dysfunction and neurodegeneration (Figure 1). The importance of vascular contributions to a range of brain diseases, including Alzheimer’s disease, vascular dementia, Parkinson’s disease, as well as stroke, is becoming increasingly recognised [5,6].

The neural component of the NVU consists of neurons and glial cells (microglia, astrocytes, oligodendrocytes), while the vascular component consists of endothelial cells, pericytes, and vascular smooth muscle cells (Figure 1A). The vascular component forms the BBB, a critical interface enabling selective permeability of molecules between the brain and systemic blood circulation. Specialised junctional protein complexes between adjacent endothelial cells help to maintain the physical and functional integrity of the BBB, tightly regulating permeability through the BBB and transferring extracellular mechanical signals to the surrounding cells (Figure 1A). Another critical component of the NVU is the extracellular matrix (ECM); a complex, acellular structure which provides a physical support for the various cells, as well as biochemical and mechanical cues that influence their behaviour.
These biochemical and physical interactions between neural and vascular NVU components provide the necessary stimuli to aid neurogenesis, angiogenesis and maintenance of brain homeostasis, while the ECM provides structural support and a platform for cellular migration and interaction. More information regarding the NVU ECM is provided in Text Box 1.

In the diseases mentioned above the integrity of the NVU is compromised. Degradation of junction protein complexes and the basement membrane [7] allows infiltration of systemic erythrocytes, leukocytes and antibodies into the brain and the subsequent induction of a pro-inflammatory activated microglial and astrocyte response [2] (Figure 1B). The pro-inflammatory response causes further BBB damage and neuron death as a result of demyelination of axons and direct damage to neurons (Figure 1B). This destruction of the NVU contributes to the neurodegeneration evident in Alzheimer’s disease and other forms of dementia [8,9].

Thus modelling NVU function and dysfunction is critical to understanding normal physiology, as well as revealing the molecular and cellular mechanisms underlying a range of neurovascular and neurodegenerative diseases. Accurately replicating the interactions between the different NVU cell types and ECM components within the brain is important to ensure the effectiveness of an NVU model. Despite the advances in brain imaging and the availability of animal models, there is an urgent need to develop human cell-based in vitro models of the NVU to provide new insight into neurovascular function, and its dysfunction in disease. Current cell-based models of the NVU commonly integrate 2 cell types separated by a porous physical layer (e.g. a transwell filter). Such models, however, fail to replicate the
3D complexity of the NVU. A promising area for development of NVU models is their **biofabrication** through **3D bioprinting** to produce a multi-component NVU in which the contribution of the different cell types to neurovascular function and dysfunction can be studied at the molecular and cellular level. In this review we discuss (i) how this can be achieved by combining the different cell types with appropriate biomaterials to mimic the ECM and (ii) the various advantages and disadvantages of utilising the advances in 3D bioprinting to construct functional 3D NVU models (Figure 2, Key figure).

**Cell types used for neurovascular unit engineering in vitro**

An important parameter when designing a NVU model is the selection of the different cell components. Endothelial cells and pericytes are the basic building blocks of the vascular counterpart in the NVU. The endothelial cells in the brain microvasculature are morphologically, biochemically and functionally distinct from non-brain endothelial cells. Although they express conventional adherens junction proteins such as VE-cadherin, the brain endothelial cells are also linked to each other by tight junctions, which reduce paracellular transport between neighbouring cells [10]. The tight junctions are formed by interactions between proteins such as occludins, claudins and junctional adhesion molecules. Pericytes, along with the vascular smooth muscle cells, are mural cells located directly on the capillary wall. In the brain, pericytes play a vital role within the NVU by supporting angiogenesis, regulating capillary function and participating in the formation and maintenance of the BBB [11]. The brain endothelium has significantly higher pericyte coverage than peripheral tissues [12,13], suggesting a specific brain function for this cell type. Recently, pericyte degeneration was shown to lead to neurovascular uncoupling and neurodegenerative changes [14]. The microvasculature creates a perivascular niche giving
neural stem cells (NSCs) the permissive cues for neurogenesis [15]. Factors secreted from endothelial cells, such as vascular endothelial growth factor and chemokines, support NSC expansion and differentiation as well as neural recruitment and migration [15,16]. In the NVU, astrocytes are the key cell type that mediate neurovascular coupling [17]. Astrocytes are the most abundant glial cells in the brain. They possess projections called astrocytic endfeet through which they interact with both endothelial cells and synapses on neurons, physically linking neighbouring neurons with their surrounding capillaries, sensing changes in the neuroenvironment and adjusting microvascular function accordingly. Astrocytic endfeet cover about 99% of the abluminal surface of cerebral vessels critically involved in autoregulation of cerebral blood flow by responding to local vasoactive factors [17]. Immune cells, such as microglia, although not a structural component of the BBB, are often included in the NVU as they influence barrier function in response to injury and disease [10] and are of particular relevance to modelling the NVU in stroke and neurodegenerative diseases where inflammation plays a major role in the initiation and/or progression of neurodegeneration [18].

There are many different factors which will dictate the source of the above cells for use in NVU models. These include availability, cost, ease of use and the ability to replicate the (patho)physiology required. To date the major sources of cells used for NVU research are immortalised cell lines (e.g. mouse brain microvascular endothelial bEnd.3 cells, human neuroblastoma SH-SY5Y cells) and primary cells obtained from rodent brain (e.g. neurons) or human umbilical vein (e.g. endothelial cells) [10]. Immortalised cell lines offer advantages including fast growth, robustness, ability to be passaged multiple times, and a relatively low cost to acquire and maintain, and are extremely useful for initial optimisation of a 3D NVU.
model [19]. However, immortalised cells often yield lower outputs in functional tests and do not always express the same transporters and tight junction proteins as *in vivo* and generally exhibit poor barrier function [19,20]. In BBB models primary cells have been shown to have higher functional output results than immortalised cell lines [21] and may better reflect the *in vivo* situation. However, primary cells are difficult to purify, may grow slowly and/or lose their phenotype in culture. Species differences also may impact on an NVU model if, for example, human endothelial cells are combined with rodent neurons.

The availability of human induced-pluripotent stem cells (iPSC) is now revolutionising our approach to NVU models, allowing the opportunity to use previously unattainable human cells. Advances in stem cell technology have made it possible to generate iPSCs from adults and differentiate them into the various cell types (endothelial cells, pericytes, astrocytes, neurons and microglia) that form the NVU [22–24]. The availability of disease-specific iPSCs carrying the exact DNA mutation and other genetic information present in the patient donor without overexpressing any mutant gene products, allows the effect of the disease mutation on NVU structure and function to be investigated in precise detail. Isogenic controls can be generated in which the disease-specific mutation is changed to the wild type sequence in a particular gene (or vice versa where the wild type sequence is changed to the disease mutation) allowing for the effect of mutations within individual cell types to be assessed on NVU function [20]. Indeed, the potential utility of iPSC cell models engineered to mimic the NVU was recently identified as a key new research tool for studying neurovascular dysfunction [25].
Biofabrication of a tissue engineered 3D NVU model, utilising hydrogel-based bioinks with specific spatial distribution of the different cell types and with different combinations of the normal and diseased NVU cells will allow precise dissection of the contribution of each cell type to the disease-specific dysfunction, such as neurovascular mis-coupling, abnormal expression patterns and/or secretions of trophic factors from different cell types, as well as facilitating high throughput drug screening.

**Biomaterials for neurovascular unit scaffolds: bioinks and hydrogel matrices**

A *bioink* is a pre-gelled biomaterial, commonly with cells encapsulated, which can be bioprinted and successfully gelled to form a solid construct. As the majority of bioinks suitable for NVU tissue engineering are hydrogel-based, unless otherwise stated this review will refer to hydrogel-based bioinks, and the pre-gelation formulation of hydrogels, as a bioink. There are many different biopolymers which can be developed into a bioink to form a *hydrogel matrix* for cells. These range from natural proteins and polysaccharides which are either constituents of the ECM or mimic the native ECM properties, or synthetic biopolymers and peptides which can be tuned to mimic the native NVU ECM. Ideally the hydrogel matrix will allow cells to synthesise and deposit their own native ECM, thus potentially replicating the physiological roles of the native tissue ECM [26]. A list of natural and synthetic biopolymer based bioinks which have suitable properties for tissue engineering a NVU, and have been utilised for bioprinting or 3D culture of neural or vascular cells previously are provided in Table 1, with a description of their properties in relation to neurovascular cell culture and bioprinting.
For a NVU model the bioink should be able to facilitate cellular migration and adhesion, vascularisation/angiogenesis and neurogenesis, as well as interactions between vascular and neural interfaces. Physical properties (determined by rheology) are also extremely important in dictating the effectiveness of the bioink for developing a hierarchal NVU structure, as well as providing the appropriate physical and mechanical cues required for the NVU cells. When developing a 3D-bioprinted NVU, rheological parameters need to be balanced with the functional output required from the model. For example, bioinks with a higher yield stress (and typically a higher stiffness) tend to print with better resolution than those with lower viscosities, yet the NVU exists within a soft in vivo environment of approximately 1 kPa (Text box 1) [27–31]. Bioprinting and gelation in a layered manner provides the opportunity for tailoring the interfacial interaction between different vascular and neural components in the NVU. Although a faster gelation rate correlates positively to the definition and resolution of a printed bioink, it is inversely correlated with angiogenesis and neurogenesis [32–35]. The key architectural properties for NVU appropriate bioinks can be divided into four subcategories: crosslinking, mechanical, porosity and cell adhesion.

**Crosslinking**

The transition from solution to gel requires intermolecular associations through either physical or chemical crosslinking. Physical crosslinking occurs through non-covalent interactions between biopolymer chains [36,37], whereas chemical crosslinking requires the covalent crosslinking between polymer chains to induce a solution to gel transition [26,28,38–41]. For NVU modelling it is important to consider the potential issues caused by the crosslinking strategy, with a risk from chemical crosslinking being cellular overexposure to the free radicals produced in the redox reactions required to facilitate gelation [42,43].
Mechanical Properties

Although the brain and NVU tissue is exposed to a relatively small amount of external physical stress – unlike bone, for example – there is a crucial role for mechanical stressors within the NVU for translating extracellular signals into intracellular transcriptional responses; referred to as mechanotransduction [44]. Blood flow and the surrounding ECM provide shear stress and mechanical cues to the NVU which regulate the expression of key junction proteins and promote functional NVU cellular phenotypes [45]. These physical and mechanical stimuli can be replicated in vitro by selecting bioinks which have similar physical and architectural characteristics to the native NVU ECM, and by introducing shear stress through flow-based bioreactor platforms; exemplified in the following studies [46–50]. The combination of bioprinting to attain specific NVU spatiotemporal distribution and the stimuli introduced through the material can promote bioassembly of NVU cell types into interactive interfaces between neural and vascular components.

Porosity

The porosity of a bioink can affect model permeability dramatically, with a low pore size potentially resulting in poor delivery of nutrients and oxygen to cells within the middle of the model leading to necrotic regions [51,52]. Pore size is also a crucial parameter for the growth of neural and vascular cells within a NVU model, with larger pore sizes promoting vascularisation and neuronal differentiation [40,51].
**Cell adhesive domains**

Cell adhesion to the surrounding microenvironment – whether it be the ECM or a hydrogel biopolymer – is important within the NVU where this interaction allows for mechanotransduction. Functional adhesion domains are specific amino acid sequences present within the ECM polymers that allow cells to bind and interact with their surrounding environment through cell membrane receptors. Two of the most common amino acid sequences present in functional adhesion domains that are relevant for the NVU are the tripeptide sequence Arg-Gly-Asp (RGD) and the pentapeptide sequence Ile-Lys-Val-Ala-Val (IKVAV). RGD domains are the key binding sites for integrins within multiple different ECM proteins [53] and IKVAV domains are present within laminin glycoproteins and have been shown to promote neurite outgrowth, synapse stabilisation and BBB performance [54–57]. Such peptide-based adhesion domains can be synthetically produced and integrated into a polymer chain to provide the intended physical attachment site. This has been manipulated to produce binding domains present on native ECM polymers, but has also been used to create biomimetic alternative peptide sequences to induce a similar effect; for example, the QK-peptide domain sequence is biomimetic for the receptor adhesive domain of vascular endothelial growth factor (VEGF), providing angiogenic stimuli to cells seeded with hydrogels containing this domain [38]. Bioinks with relevant physical and biochemical properties for incorporation into NVU models are listed in table 1, along with their inherent suitability for cellular adherence or the possibility for adaption to enable cellular adherence.

**Hydrogel-bioinks for development of neurovascular unit matrices**

*Natural polymers*
A common strategy for functionalisation of hydrogels for NVU-bioprinting purposes is through physical combination of two biopolymers (rather than chemical modification) to produce a blended hydrogel-based bioink with the advantageous properties of each polymer contributing to the favourable NVU properties required. This technique has been utilised commonly to create collagen based bioinks; as although collagen has an inherent RGD adhesion domain, collagen hydrogels typically lack the physical properties to be used as a bioink, with slow gelation times [58]. Collagen has been used to create mixed hydrogel-based bioinks – with various other matrices – to successfully bioprint neural and vascular cells; including agarose [34], gelatin methacrylate [41], matrigel [59] or alginate [57,59] altering its shear thinning properties to create a bioink effective for NVU cell culture.

Another strategy is the combination of multiple (more than two) polymers to produce a hydrogel with multiple advantageous properties for the tissue engineering of an NVU model. An example of this is the combination of fibrin, hyaluronan (hyaluronic acid) and laminin to produce a 3D model for both neural and vascular stem cells [60]. This hydrogel utilised the beneficial properties of fibrin for neural stem cells, with interpenetrating networks of hyaluronan to aid crosslinking and enhance the similarities of the gel with the native neural ECM. Hyaluronan biopolymers promote cell migration and proliferation and – crucial to the development of a NVU model – also promote angiogenesis and neurogenesis [24,61,62]. The addition of laminin with its own IKVAV sequence provided the cells with functional adhesive domains. Although the hydrogel was not optimised for bioprinting, the rheological properties of the hydrogel could be tailored to produce a bioink and utilise the NVU favourable components within the hydrogel.
Conversely, some hydrogels are bioprintable without modification or mixing but lack the inherent cell adhesive domain necessary for development of effective NVU models. Gellan gum is an example of a polysaccharide used commonly for producing hydrogels, which when unmodified does not have the functional domain for cellular adhesion, but can be 3D-printed with a crosslinker to form layered NVU structures [37,63]. The chemical modification of gellan gum to contain the IKVAV cell adhesive domain has allowed for the use of the polysaccharide for bioprinting layered cortical neural models [64].

*Synthetic biopolymers*

Synthetic hydrogels enable full control over production and functional parameters, by designing the exact structural composition and functionalities of the hydrogel to the intended application. This presents opportunities for developing NVU-suitable bioinks, as the exact bioprinting properties and cellular adhesive domains can be designed into the biopolymer structure. A common class of synthetic biopolymer based bioinks are self-assembling peptides (SAPs), which form ordered nano-fibrous β-sheets that replicate the structure of the native ECM [36]. SAPs can be designed to assemble quickly and autonomously through physical crosslinking interactions; or alternatively designed to be crosslinked through induction of physical or chemical interactions [38,65]. This biomolecular and cellular self-assembly is common place in vivo and can be replicated in hydrogel constructs, where cell migration through the pores in the hydrogel enables cell-cell interactions [66]. Due to their synthetic design, bioprintable properties can be tailored into the synthetic hydrogel to develop a fully functional bioink [67], and such materials have been utilised for NVU cell studies previously [56,68–70]. Synthetic peptides can also be developed to replicate different aspects of tissues, with elastin-like polypeptides (ELPs)
being designed to incorporate angiogenic peptide domains for utilisation in vascular cell 3D culture studies to promote vascular growth [38].

**Neurovascular unit biofabrication**

The NVU is complex with multiple cells and cell-cell interactions which present particular challenges to *in vitro* modelling. To simplify a NVU model – or to focus on the vasculature – several models compartmentalise the NVU by developing a BBB only model. Other approaches are to use a partial component of the BBB, i.e. endothelial cells and astrocytes/pericytes/vascular smooth muscle cells, with neural cells. These approaches do not give the effect of modelling a whole NVU but can be very useful for investigating the BBB and circumvent the difficulty of co-culturing up to 5 cell types in one model.

Within the context of tissue engineering and regenerative medicine applications, the definition of biofabrication as a research field has been clarified as ‘the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes’ [71,72]. Bioassembly utilises the self-assembling nature of cells to form interactive networks and tissues, where 3D bioprinting can be utilised to place cells in an appropriate spatiotemporal location for assembly. This can be utilised for the development of bioassembled vasculature through patterned 3D bioprinting [73], or for the development of hierarchal structures by using a thermoplastic material as a structural support in-between the layered deposition of bioink-cell formulations [74]. For development of NVU models
bioassembly is an important design strategy, where automated deposition of cells containing units (in the form of hydrogels) can initiate the bioassembly of neural-vascular interfaces and cell-cell interactions which are crucial to the development of hierarchal NVU models and the production of a BBB with functional phenotype.

There are many different bioprinting techniques, which have been clearly described in other reviews [72,75]. For bioprinting of a NVU model two main strategies have been employed: indirect additive manufacturing (indirect bioprinting) where an additive manufactured negative sacrificial mould is used to enclose and shape biomaterials and cells (Figure 4A-B) and bioplotting (direct bioprinting) where bioink and cells are printed directly into a 3D structure (Figure 4C-D). Both of these approaches to bioprinting have the potential to produce multicellular 3D NVU models, with defined cellular compartments, critical cellular interactions and vasculature.

Bioprinting also offers the potential to introduce channels within the NVU model (Figure 4A, B, D) – through use of sacrificial biomaterials in direct bioprinting – allowing for enhanced diffusion of media, nutrients and oxygen throughout the model; mimicking capillary networks within the in vivo NVU and avoiding the development of necrotic regions seen in organoid cultures and some particularly fibrous hydrogel constructs [76,77]. This channelled-network can then be seeded with endothelial cells to produce a vascular network. By integrating a peristaltic or microfluidic pump for perfusion, shear stress can be applied to the model, thus creating a bioreactor culture platform [46–50]. This incorporates bioassembly to the model, with shear stress promoting the formation of junction proteins and improving the functionality of the BBB and accompanying NVU cells [45]. This also
allows for the introduction of potentially harmful or protective compounds and drugs to combat NVU dysfunction through a physiologically relevant ‘systemic’ process; mimicking the introduction of drugs through intravenous or oral administration.

**Indirect Bioprinting**

There are different approaches to creating NVU models from indirect bioprinting. The two most common methods include additive manufacture of a ‘master’ mould, in which the subsequent biocompatible negative mould is created and removed; the negative mould is then seeded with cell-encapsulated hydrogel creating a 3D model of the NVU which can be crosslinked with another cured hydrogel to create vascular channels within the model (Figure 4A) [47,78]. The alternative method uses additive manufacture of a sacrificial biomaterial to create a channelled network, into which cell-laden hydrogel is then poured and cured around, enabling extraction of the sacrificial material to produce channels and without damaging the surrounding NVU construct (Figure 4B) [77,79].

Indirect bioprinting permits the construction of a 3D hydrogel construct without physically printing cells, thus avoiding printing shear stress which has been shown to affect cell viability [80]. A disadvantage of manufacturing an NVU model in this way arises when there is a need to deposit multiple cell types with different spatial distributions, as the mechanism requires crude ‘pouring’ of hydrogel rather than specific deposition. A further potential disadvantage to creating two hydrogel constructs to piece together is that the crosslinking process could damage encapsulated cells, as chemical or UV-crosslinking may have to be employed to join the pre-crosslinked individual layers [42,43]. Due to the crude aliquotting of cell-laden bioinks employed in this method of bioprinting the integration of neural and
vascular components is typically through either two biofabricated layers (one containing neural cells, one containing vascular cells) or through the encapsulation of neural cells within the bioink (set in a negative mould or around a sacrificial biomaterial), and subsequent seeding of vascular cells within a fabricated vascular channel.

**Direct Bioprinting**

Direct bioprinting utilises the direct deposition of a cell-laden bioink with the option of a sacrificial biomaterial to create the vasculature (Figure 4C-D) [81]. A further possibility is to bioprint a cell-laden hydrogel into a structure that incorporates pores that vascular cells can be seeded into, avoiding the use of sacrificial biomaterials. The direct bioprinting of cell-laden hydrogels and sacrificial channel network allows for the manufacture of very complex structures, with precise spatiotemporal cell deposition to ensure the desired cell-cell interaction and neural-vascular interface is achieved. The use of multiple bioprinting cartridges permits biofabrication of complex and precise multicellular NVU models through cell-specific bioink encapsulation that contain appropriate supplements for growth and optimal physical properties (i.e. rheology and stiffness) to represent the different ECM structures of the NVU [75]. This approach is ideal for the manufacture of NVU models, where an optimal model requires both neural and vascular cell-laden ECM, with an interface for cell-cell interactions. Bioprinting also enables the development of a reproducible model for comparative experiments between healthy and diseased cell types.

A common issue with direct bioprinting of NVU models is the difficulty achieving a defined resolution from bioinks post-printing. This is why the development and optimisation of bioinks with high shear thinning properties to create clearly defined post-print resolution is
an important step in designing a 3D NVU model. A method to circumvent this issue and enable the bioprintability of previously non-printable hydrogels is through the incorporation of a suspended manufacture technique which allows for the controlled gelation of hydrogels over time, rather than relying upon bioinks with high shear thinning properties and fast crosslinking rates. This technique utilises the visco-elasticity and shear thinning properties of a self-healing fluid gel to support the structure of gels during deposition, avoiding gel flow and enabling time for crosslinking to occur, after which the fluid gel can be removed, leaving a solid crosslinked 3D hydrogel construct (Figure 4E) [82].

**Outcome measures for 3D neurovascular unit models**

In order to assess the functionality of 3D NVU models a range of outcomes can be measured. BBB function can be measured by trans-endothelial electrical resistance (TEER), which uses a current between two electrodes as a measure of BBB permeability, or by movement of a coloured or fluorescent dye (e.g. FITC dextran) [10,50,83]. Although TEER values measured *in vivo* in rat brain range between 1200-1900 Ω cm$^2$, in cultured cells the permeability barrier is considered effective when the TEER value is above a threshold (typically approx. 250 Ω cm$^2$) [10]. The integrity of cell-cell interactions can be measured by immunofluorescent microscopy using antibodies against specific proteins, such as against VE-cadherin for endothelial cell adherens junctions, claudin, occludin and junctional adhesion molecule for tight junctions, N-cadherin for endothelial cell-pericyte adherin junctions and Connexin 43 for gap junctions. If required, the biofabricated 3D NVU models can be subjected to normal tissue histology approaches, such as paraffin embedding and tissue slicing, prior to immunohistochemistry [50]. Specific proteins can be quantified using a variety of protein quantification techniques, including Western blot and enzyme-linked
immunosorbent assay (ELISA) [60,84,85], while mRNA can be quantified by RT-qPCR [24].

Single cell analyses can be utilised to characterise particular cell types within the NVU, by digesting the matrix to release the cells, purifying an individual cell type from the rest by flow cytometry and then using RNA sequencing or proteomics to identify changes of interest under different conditions (e.g. disease specific mutation versus non-disease control). Other outcome measures will depend on the question being addressed of an NVU model. For example, the deposition of amyloid-β and phosphorylation of tau, and the ability of drugs to reverse these Alzheimer’s-like pathologies [86,87], could be measured in NVU models. The transport of amyloid-β, which was shown to be facilitated by apolipoprotein E across bioengineered human cerebral vessels [50], could be monitored across an NVU model.

**Concluding remarks and future perspectives**

Biofabrication of an *in vitro* 3D NVU model through bioinks and bioprinting enables the development of a multicellular model that more closely mimics the *in vivo* situation (see outstanding questions box); with the introduction of bioprinting enabling the production of interactive interfaces between the various cellular components. A tissue engineered model which can replicate the 3D tissue microenvironment of the NVU is imperative to elucidating cell-cell and neural-vascular interactions that are important to function and disease within an *in vivo* NVU. Both direct and indirect printing techniques are able to create channels within the 3D construct, with subsequent seeding of endothelial cells enabling the fabrication of a BBB or vascular network. Central to the effective execution of bioprinting a 3D NVU are the bioinks selected to support cells before, during and after bioprinting. By utilising bioinks and 3D bioprinting, the quality of the NVU model is increased significantly, which will lead to a better understanding of the molecular and cellular interactions between
the different cell types in the NVU and how these go wrong in diseases associated with neurovascular dysfunction. The availability of 3D NVU models may also facilitate screening for drugs to correct the neurovascular dysfunction underlying stroke, Alzheimer’s disease and other dementias.
Figure 1. The neurovascular unit (NVU) in health and disease

The NVU is composed of neural (neurons, microglia, astrocytes, oligodendrocytes) and vascular (endothelial cells, pericytes, smooth muscle cells) components, which interact to regulate cerebral blood flow. (A) In a healthy NVU, endothelial cells, pericytes, astrocytes and the basement membrane form the blood-brain barrier (BBB), with myelinated neurons, and glial cells surrounding the BBB in the brain extracellular matrix (ECM). Pericytes and astrocyte endfeet maintain contact with the basement membrane, with endothelial junctional protein complexes intact and functional. (B) In a dysfunctional NVU junctional protein complexes and the basement membrane degrades, in addition to induction of a pro-inflammatory activated microglial and astrocytic response; leading to demyelination and death of neurons. This dysfunction contributes to neurovascular damage and neurodegeneration.
Figure 2, Key Figure. Utilising different cells, biomaterials and bioprinting to construct a multi-component 3D-neurovascular unit
Different neurovascular unit (NVU) cell types can be combined with specialised biomaterials, known as bioinks, and bioprinting to manufacture multi-component 3D-neurovascular unit models. Neural and vascular cells are encapsulated within bioinks suitable for their viability and growth. Bioprinting allows for precise positioning of the neural and vascular cells to form appropriate interactive interfaces that mimic the *in vivo* situation.

**Figure 3. Cell-cell interactions in the neurovascular unit**

The individual cell types of the NVU interact with the other cellular components of the NVU through biochemical and physical cues, with both direct and indirect interactions between neural and vascular components. Adapted from [88] with permission from Elsevier.
The combination of cells and biomaterials enables the production of 3D NVU models with hydrogels and hydrogel-based bioinks encapsulating the cells within a 3D environment.

Hydrogels with physical properties suited to vascular and neural cells can be used to create
models with direct interface interaction. (A) Indirect bioprinting of a stamp from a ‘master’ mould allows for cell-laden hydrogels to be poured into the mould to create a construct. The hydrogel can then be crosslinked through photo-initiation or chemical reticulation (network formation) and two layers can be further crosslinked to create a channel between the two manufactured hydrogels. Additional vascular cell seeding and a flow based microfluidic pump can be incorporated. (B) Bioprinting of a vascular network from a sacrificial biomaterial enables cell-laden hydrogel to be poured around the sacrificial element, which is subsequently flushed away to leave the channel free for vascular cell seeding and introduction of a microfluidic pump. (C) Vascular and neural cells can be encapsulated within a vascular and a neural bioink, respectively, enabling the production of a layered 3D NVU construct with direct interaction through the layers of the hydrogel. (D) By using direct bioprinting of cell-laden hydrogel and a sacrificial biomaterial, a channel can be manufactured in the construct. Printing the sacrificial biomaterial within the bioprinted hydrogel allows for channel flushing once the gel has set and subsequent seeding of vascular cells within the channel to create a blood-brain barrier; addition of a microfluidic pump can introduce shear flow to the model. (E) Direct bioprinting of bioink into a self-healing fluid gel enables suspended manufacture of 3D NVU models through an extended and controlled gelation of bioinks. Removal of fluid gel leaves a solid post-print construct, ready for cell culture.
Text Box 1: The Neurovascular Unit Extracellular Matrix

The ECM is a complex yet well-organised network of molecules and proteins, which provides a dynamic platform for cell to cell signalling and enables the formation and remodelling of tissue. The neurovascular specific ECM is diverse, with distinct differences in both the neural cell ECM and vascular ECM; both of which can be altered structurally by proteases that degrade certain ECM components [89]. Receptors on the surface of cells interact with the ECM which leads to regulation of cytoskeletal coupling and allows cell migration [90]. For example, integrin receptors attach to the actin cytoskeleton promoting changes in the ECM mechanical environment that affect gene expression and activate multiple signalling pathways through mechanotransduction [91].

Brain ECM
A relatively large proportion (17-20%) of the brain consists of ECM [92]. The brain ECM has an architecture distinct from that of systemic tissues, with fibronectin and type I collagen being practically absent, and an abundant expression of proteoglycans and type IV collagen, with a high expression of lecticans [93,94]. Additionally the brain ECM has a different composition of growth factors, receptors, and proteases [95]. The brain is an extremely soft organ with a low elastic modulus (0.1-1 kPa), reflected by the low proportion of type I collagen [30,91].

Vascular Basement Membrane
A thin fibrous segment of ECM surrounds the brain vasculature, referred to as the basement membrane. This ECM component differs from typical neural tissue, with different protein expression and mechanical characteristics. The vascular ECM is primarily composed of collagen type IV and laminin, with fibronectin and proteoglycans integrated in the network
These basement membrane components have been used *in vitro* to grow endothelial layers with increased functionality compared to those without these components [96,97].

**Physicochemical hydrogel properties required for building a successful NVU**

The physical properties of the ECM are important when developing a model of the NVU. Studies have shown that physical stiffness of the extracellular environment is optimal when it mimics that of the native tissue, with neural and vascular tissues favouring ~1 kPa environments [27,29–31]. The physical stiffness and rheological properties of the hydrogel used also affects the capability of a biomaterial to be bioprinted, adding additional variables to the implementation of a bioink to develop a 3D NVU model.

**Outstanding Questions Box**

- Can the complex interactions between multiple cell types in the NVU be faithfully reproduced in 3D models?
- Will novel biomaterials replicate the mechanical and functional cues of the natural ECM?
- Will automated biofabrication enable high throughput manufacture of 3D NVU models?
- Will 3D NVU models improve our understanding of neurovascular function?
- How can the use of 3D NVU models help the discovery of new therapeutic and diagnostic agents for Alzheimer’s disease and other dementias with a vascular component?
Will ‘humanized’ 3D NVU models accelerate the translation of neurovascular and/or neurodegenerative disease therapies from bench to bedside?

**Trends Box**

- Advances in 3D biomaterials known as hydrogels, with the structural properties of a solid and the capability to contain a high water content (~99%) allow for 3D culture of neurovascular unit cells.

- Development of hydrogels with bioprintable properties, referred to as bioinks, allow for deposition of layered 3D neurovascular unit (NVU) models.

- Biofabrication strategies utilising bioprinting and bioassembly can be used to develop 3D NVU models.

- NVU models could be to incorporate channels seeded with vascular cells to mimic the blood-brain barrier (BBB).

- Attachment of fluidic pumps to a vascular channel can aid functionalisation of endothelial cells and the BBB as a whole.
**Glossary**

**Alzheimer’s disease**: A type of dementia characterised by deposition in the brain of tau protein and amyloid-β peptide which leads to progressive neurodegeneration

**Angiogenesis**: The formation of new vasculature from pre-existing vessels

**Basement membrane**: The extracellular matrix component which surrounds the vasculature and is degraded during neurovascular unit dysfunction

**Bioassembly**: The self-assembly of biological constructs following automated distribution of cell-laden units to form a construct

**Biofabrication**: In the context of tissue engineering and regenerative medicine – automated manufacture of tissues and organs

**Bioink**: A biomaterial which can be combined with cells or biological matter and subsequently be 3D bioprinted into a defined structure.

**Biopolymer**: A polymeric unit used for biological applications like the development of hydrogels and bioinks

**Bioprinting**: The additive manufacture of tissue and organs through layered deposition of biological material and cells ensuring precise spatiotemporal distribution of cell types, thereby building a 3D construct

**Blood-brain barrier (BBB)**: A cellular barrier of endothelial, smooth muscle and pericyte cells which form the brain capillaries and arterioles that separate the brain tissue from the blood

**Extracellular matrix (ECM)**: Tissue component surrounding cells and providing biomechanical and biochemical cues.

**Hydrogel matrix**: A highly water saturated (~99%) 3D matrix which cells can be encapsulated within
**Junctional protein complex**: Inter-cellular protein junctions which control selective permeability across the blood-brain barrier and include tight junctions, adherens junctions and gap junctions.

**Mechanotransduction**: Translation of extracellular mechanical cues through the cytoskeleton to induce changes within the cell.

**Neural component**: The neural cells of the brain, including neurons and glial cells (microglia, astrocytes and oligodendrocytes).

**Neurodegeneration**: The degeneration of neurons caused by a variety of pathological influences, e.g. hypoxia, oxidative stress.

**Neurogenesis**: The growth of new neurons from neural stem cells.

**Neurovascular unit (NVU)**: The interface between the neural and vascular components of the brain, crucial for neurovascular coupling and delivery of key nutrients and oxygen from the circulatory system.

**Rheology**: The science of flow – study of flow of a material in response to stress allowing for determination of mechanical properties.

**Stroke**: Ischemic or haemorrhagic brain injury from an infarct which blocks cerebral blood flow; often leads to vascular dementia due to the neurovascular damage caused.

**Vascular component**: The cells (endothelial cells, pericytes, smooth muscle cells) which form the blood vessels of the brain, the blood-brain barrier and the overall vascular microstructure.

**Vascular Dementia**: A form of dementia where reduced cerebral blood flow and neurovascular dysfunction leads to neurodegeneration.

**Vascularisation**: The formation of a capillary network within a model or tissue.
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<tbody>
<tr>
<td>Collagen (Natural; Protein)</td>
<td>Thermal; pH</td>
<td>~0.1 – 0.8</td>
<td>Inherent</td>
<td>Commonly combined with other materials or crosslinkers to print</td>
<td>Cell-mediated remodelling through collagenases</td>
<td>[34,41,5,8,98,99]</td>
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<tr>
<td>Gelatin (Natural; Protein)</td>
<td>Thermal; Photo-crosslinking</td>
<td>~0.1 – 1</td>
<td>Inherent</td>
<td>Print close to gelation temperature</td>
<td>As with collagen; caveat: requires modification for 37°C cell culture</td>
<td>[28,41,47]</td>
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<tr>
<td>Fibrin (Natural; Protein)</td>
<td>Fibrinogen crosslinking with thrombin</td>
<td>~0.3 – 4</td>
<td>Inherent</td>
<td>Combine with other material to print</td>
<td>Widely used for neural tissue engineering</td>
<td>[60,99–103]</td>
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<tr>
<td>Gellan gum (Natural; Polysaccharide)</td>
<td>Thermal; ionotropic; photo-crosslinking</td>
<td>~0.1 – 186</td>
<td>Requires chemical modification or combining with other material</td>
<td>Print with crosslinker; print into support matrix</td>
<td>Tuneable; NVU promoting cell adhesion domains chemically added</td>
<td>[28,64,104,105]</td>
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<tr>
<td>Hyaluronan (Natural; Polysaccharide)</td>
<td>Photo-crosslinking</td>
<td>~0.01 – 3.5</td>
<td>Inherent</td>
<td>Combine with other material to print or printed under UV exposure</td>
<td>Promote cell migration, proliferation, angiogenesis and neurogenesis</td>
<td>[24,60,106–108]</td>
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<tr>
<td>Self-assembling peptide (SAP) (Synthetic)</td>
<td>Self-Assembly</td>
<td>~1 - &gt;50</td>
<td>Require chemical modification or combining with other material</td>
<td>No modification required</td>
<td>Tuneability; customisable bioprinting and cell adhesion domains</td>
<td>[36,56,67–70,84,109]</td>
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<tr>
<td>Elastin-like polypeptide (Synthetic)</td>
<td>Self-assembly; photo-crosslinking; amine-reactive crosslinking</td>
<td>~1 - &gt;50</td>
<td>Require chemical modification or combining with other material</td>
<td>If not self-assembling: require combination or printing with crosslinker</td>
<td>As with SAP; similar physical properties to vascular basement membrane</td>
<td>[38,110,85,109,111]</td>
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<tr>
<td>Poly Ethylene Glycol (Synthetic)</td>
<td>Photo-crosslinking; thermal; ionotrophic</td>
<td>~0.9 – 132</td>
<td>Require chemical modification or combining with other material</td>
<td>No modification required</td>
<td>As with SAP; well documented usage</td>
<td>[65,112–115]</td>
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