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Three-dimensional neuronal cell culture: in pursuit of novel treatments for neurodegenerative disease

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Abstract

To gain a better understanding of the underlying mechanisms of neurological disease, relevant tissue models are imperative. Over the years, this realization has fuelled the development of novel tools and platforms, which aim at capturing *in vivo* complexity. One example is the field of biofabrication, which focuses on fabrication of three-dimensional (3D) biologically functional products in a controlled and automated manner. Herein, we provide a general overview of classical 3D cell culture platforms, particularly in the context of neurodegenerative disease. Subsequently, the focus is put on bioprinting-based biofabrication; its potential to advance 3D neuronal cell culture and, to conclude, the relevant translational bottlenecks, which will need to be considered as the field evolves.

Keywords

Biofabrication, bioprinting, 3D neuronal cell culture

Introduction

The vast majority of our current understanding of biological phenomena comes from routine classical cell culture experiments; growing of cells onto flat and rigid two-dimensional (2D) substrates. Even though these efforts have provided the research community with valuable insights into the mechanisms underlying a variety of biological processes, it is nowadays widely accepted that knowledge obtained from these studies might be too reductionist to accurately translate to the human situation.[1,2] Growing cells onto 2D substrates deviates significantly from the dynamic three-dimensional (3D) *in vivo* situation; cells lack tissue-specific polarity, have limited contact with neighbouring cells, and are exposed to non-physiologically uniform diffusion kinetics, which together alter how cells perceive and respond to their surrounding microenvironment (Fig. 1). [3-5] This discrepancy between traditional *in vitro* culture conditions and the *in vivo* environment has been recognized among a variety of research areas, including the area of neuroscience and more specifically neurological disease, the latter presenting a great challenge in modern medicine. [6]

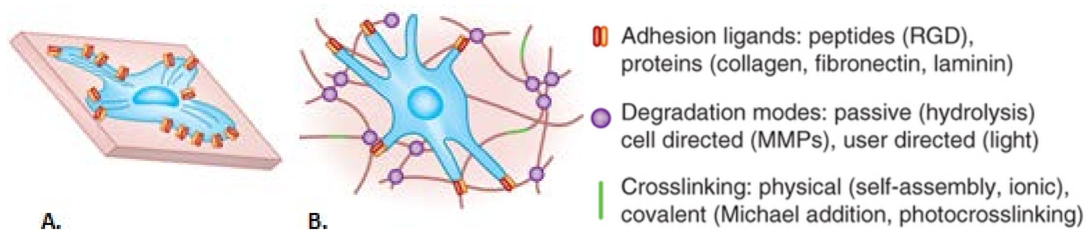

Figure 1. Schematic representation of cells (a) cultured on a stiff 2D tissue culture substrate and (b) embedded in a more physiologically relevant 3D hydrogel environment, which has unique design variables (*e.g.* degradation sites and crosslinking mechanisms) that direct cell behaviour. *In vivo*, cells reside in a complex and dynamic environment, which provides binding ligands for cell adhesion and directs processes such as cell adhesion, proliferation, migration, and morphology. Cells grown onto classical tissue culture substrates are confined to a planar surface, which leads to abnormal integrin binding and (mechano) signalling cascades, resulting in aberrant phenotypes. In the transition towards the third dimension, hydrogel-based strategies play a pivotal role. Hydrogel matrices can be tuned to meet application-specific requirements (*e.g.* degradation rate, porosity, and mechanical properties. Adapted by permission from Macmillan Publishers Ltd: [NATURE METHODS] (7), copyright (2016).

A glance at our nervous system

Our nervous system encompasses two main cell types, neurons and glial cells, which both have a crucial role in nervous system functioning.[8,9] Neurons are highly polarized cells, responsible for the transfer and processing of electrical and chemical signals that regulate body function. According to their function, neurons can be classified into sensory neurons, motor neurons, and interneurons, which can be excitatory, inhibitory or modulatory in their effect. Typically, neurons consist of a cell body with projections, known as axons and dendrites. These projections can vary in terms of number and position, which is highly linked to neuronal function.[10] The non-neuronal glial cells come in a variety of subtypes and have different roles in the development, maintenance, and functioning of the nervous system.[11] For a long time, glial cells were regarded to have a relatively simple supportive function.

However, research over the past years has uncovered their vital role and active participation in the development and functioning of the nervous system. Beyond these cellular components and their intricate communication, appropriate nervous system functioning requires a highly regulated extracellular environment.[12] This dynamic mechanical and biochemical interplay plays a key role in many essential processes, including spatio-temporal cell signalling, cell identity, and cell function.[13-17] To gain more insights into neurological disorders and advance current health care, it is essential to recapitulate this complex relationship between neurons and their interaction with the surrounding environment, which requires more accurate *in vitro* nervous systems models.

The rise of the third dimension

Over the years, various strategies aiming at capturing 3D tissue physiology have been developed. Examples range from relatively simple approaches, such as the formation of cellular spheroids, to more sophisticated scaffold-based approaches.[18-21] Whereas scaffold-free approaches mostly rely on the inherent tendency of adherent cells to form aggregates, scaffold-based approaches require a thorough consideration of a plethora of factors. Clearly, the scaffold-based environment should mimic the native niche as closely as possible, which calls for multidisciplinary expertise from biologist, engineers, and material scientists. Unfortunately, accurately recapitulating the complex nature of tissues and/or organs by means of traditional scaffold-based approaches remains challenging, especially when it comes to achieving precise architectural configurations and spatial positioning of (multiple) cells and scaffolding materials. To this end, the growing field of biofabrication offers an exciting toolbox.[22,23] As described by Groll *et al.*, the term biofabrication refers to the automated generation of products with biological function by means of bioprinting or bioassembly and subsequent maturation processes.[24] More specifically, biofabrication approaches encompass

living cells, cell aggregates, bioactive molecules, and biomaterials to generate biologically functional products in an automated and highly organized manner.

In this review, we focus on bioprinting-based biofabrication approaches and the potential to advance currently available 3D cell culture platforms for nervous system applications, particularly in the context of neurodegenerative diseases using Alzheimer's disease as an example. Noteworthy, here we refer to bioprinting as the use of printing technology to pattern and/or organize biological entities in 2D or 3D as described by Derby.[25] After a brief description of the general concept of 3D cell culture platforms, an introduction into the field of bioprinting is provided. Subsequently, the potential of bioprinting technology and its emerging applications for the nervous system will be discussed. To conclude, we highlight possible translational bottlenecks, which will need to be addressed as the technology matures. Key aspects include the necessary advances in converging disciplines, the need for standardization in the field, and ethical regulations.

Neural tissue engineering: the need for relevant 3D cell culture platforms

Neurodegenerative disease is a collective term for a group of disorders which primarily affect neurons.[26] Whereas a variety of disorders (*e.g.* Alzheimer's disease, Parkinson's disease, Huntington's disease) are covered by this umbrella term, in this review the focus is put on Alzheimer's disease, which represents the most common cause of dementia among adults and affects millions of people worldwide. As reported by the World Health Organization (WHO), the number of people living with dementia is currently estimated at over 47 million, which is expected to almost triple by 2050.

Alzheimer's disease is clinically characterized by gradual deterioration in cognitive function, including progressive memory loss, impaired judgment, and changes in personality and

behaviour, which can become incapacitating at later stage of illness.[27-28] Besides the impact on patients' quality of life, dementia is a tremendous social and economic challenge; the disease takes heavy toll on caregivers and has been identified as a major economic burden. [29-31]

At the cellular level, Alzheimer's disease is characterized by damage to cortical neurons, particularly in the associative neocortex and hippocampus.[28,32] This neuronal damage is correlated to deposition and accumulation of abnormal proteins; extracellular amyloid-β peptides and intracellular filamentous hyperphosphorylated tau proteins, which are the core hallmarks of Alzheimer's disease and also known as plaques and tangles respectively.[33] Whereas research was initially focused on neuronal cells and protein-mediated neuronal damage, it has become increasingly apparent that Alzheimer's pathogenesis has a more complex nature and is not restricted to these events. Over the years research has evidenced that other factors, such as immunological mechanisms, have an accompanying role in the pathway leading to progression of the disease.[34] Despite progress in understanding the underlying mechanisms and aspects of neurodegenerative disease, there are still many unknowns. This is reflected by the lack of effective therapeutic options for the vast majority of these disorders, including Alzheimer's disease, which are nowadays limited to symptomatic relief.[30,35] In order to advance the treatment of these disorders it is critical to gain a deeper understanding of processes such as neural network formation, organization, and functioning.

Classical 3D cell culture strategies

In response to the growing demand for more physiologically relevant models, a number of 3D cell culture platforms have been developed.[36,37] Examples range from cell-self-assembly-based approaches, to scaffold-based approaches, and on-chip biomimicry. In the next section

we aim to sketch and illustrate the importance of these classical 3D cell culture platforms and capturing the third dimension.

Cell-self-assembly-based approaches

One of the most common cell-self-assembly-based approaches is the formation of cellular spheroids (Fig. 2). Spheroid formation is generally considered to be a relatively simple 3D cell culture method and has shown to be a convenient means towards more accurate tissue models. The main benefit of using spheroids over 2D monolayer cultures is the presence of more physiologically relevant diffusion gradients (*e.g.* oxygen and nutrients) and cell-cell and cell-ECM interactions.[36,38,39] With time, a variety of methods have been developed to produce spheroids, one well-known example being the hanging drop method.[36,38] In this approach, aliquots of cell suspensions are seeded on an adherent surface, which is subsequently reverted, yet allowing the droplets to stay attached due to surface tension, resulting in gravity-enforced cell aggregation at the bottom of the droplets. Alternative popular approaches are based on dynamic culture conditions (*e.g.* the spinner flask method), low-adhesive surfaces (*e.g.* forced-floating technique), and more recently, micro-/nano-patterned surfaces and micromolding, the latter providing defined regions for cell immobilization and subsequent spheroid formation.[38] All these different methods are associated with their own advantages and disadvantages, resulting in spheroids of different sizes and shapes, and should be chosen depending on the experimental needs and final application.[36,39]

To date, spheroids have been successfully generated from a variety of cell types, including neuronal cells.[40] For example, Dingle *et al.* generated neural spheroids from primary rat cortical tissues by means of micromolding, which were reproducible in size and cellular

composition.[41] Spheroid characterization revealed resemblance to *in vivo* cortical tissue in terms of stiffness, neuronal electrophysiology, neural cell types, and morphology. Following on from this study, Boutin *et al*. assessed the potential of this heterogeneous spheroid model to form capillary-like networks, the later playing an important role in protecting the neuronal environment.[42] Immunohistochemical analysis revealed that within three days, cortical endothelial cells seemed to form networks in the spheroids, which were surrounded by basement membrane components and relevant cell types. In future studies, this relatively simple model could offer valuable insights in understanding disorders linked to neurovascular dysfunction.

A more advanced cell-self-assembly-based 3D cell culture platform that has been gaining increasing interest is the organoid model (Fig. 2).[43] Whereas various definitions are reported in the literature, here we refer to organoids as self-organizing 3D cell clusters, which can be established from different cell sources (*e.g.* primary cells, cell lines, stem cells), resembling the tissue architecture of origin.[37,44,45] Over the last decade, organoid technology has made tremendous progress and several *in vitro* organoids have been established.[37] Whereas the relatively scarce amount of literature on neuronal organoids is mainly focussing on neurodevelopmental phenomena, Raja *et al.* have shown its potential to study neurodegeneration.[45] In this study, neural organoids were formed from induced pluripotent stem cells derived from Alzheimer’s disease patients. This organoid model has shown to be robust, able to recapitulate both hallmarks of Alzheimer’s disease, and has been shown to be amenable to experimental manipulation in terms of drug treatment.

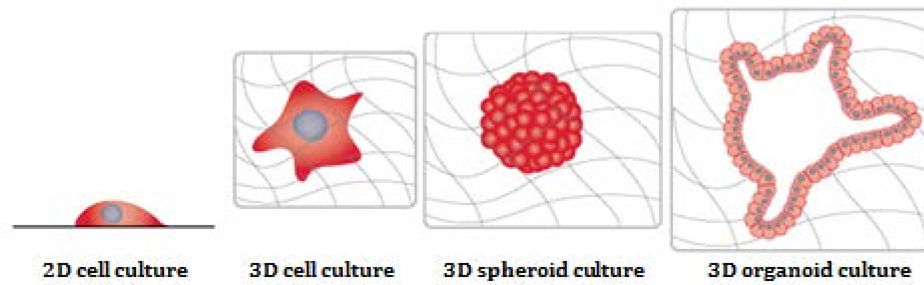

Figure 2. Cell culture platforms: monolayer culture on conventional tissue culture substrates, single cell in 3D matrix, spheroid culture, and organoid culture. Obtained and modified from[42] with permission from Elsevier.

Biomaterial-aided 3D cell culture platforms

In this section, we only focus on the non-biofabrication biomaterial-aided platforms that involve either pre-fabricated scaffolds for cell cultivation or polymer hydrogels for cell encapsulation. In both cases, the biomaterials do not only provide structural and mechanical support, but also guide the cells via acting as a template for tissue formation.[20,47,48] Intuitively, these biomaterials should resemble the dynamic native microenvironment of the specific target tissue as closely as possible, a task that can be extremely challenging; it does not only require the biological and functional integration of the cells with the surrounding biomaterial(s), but also careful consideration of biodegradability, mechanical properties, scaffold architecture, and manufacturing technology.[47,49]

Over the years, a myriad of materials have been investigated for their potential to support neuronal 3D cell culture.[50] These range from natural polymers (*e.g.* collagen, hyaluronic acid, alginate, Matrigel, and silk) and synthetic polymers (*e.g.* poly(lactide-co-glycolide (PLGA), poly-L-lactic acid (PLLA), and polyurethane (PU)) to hybrid materials, metals, ceramics, glass, and carbon nanotubes. For example, Frampton *et al.* described a 3D culture

system based on cell entrapment in peptide-functionalized alginate hydrogels.[51] Cell viability and functional analysis of primary rat glial and neuronal cells demonstrated appropriate cell viability and metabolic function within the constructs over a period of 14 days. Interestingly, cell morphology, particularly of astrocytes differed from cells cultured on 2D substrates. Analysis of the functional activity of primary rat neurons showed formation of functional synaptic elements and electrical activity. Together, the results showed that this system could offer a viable cell culture platform to (co-)culture a variety of relevant cell types for use in neural cell culture, supporting cell type-specific function. Lai *et al*. compared neuronal voltage gated calcium channel functionality in 2D to 3D setting.[52] Primary neuronal cells were harvested from mice and either cultured onto 2D flat surfaces or in 3D synthetic polymer scaffolds, the latter consisting of PLLA and polystyrene. It was found that cells in 3D scaffolds resembled native intact tissue more closely in terms of cell morphology and ion channel functioning. Another striking example that illustrates the importance of recapitulating an *in vivo*-like 3D cellular environment is described by Choi *et al*. who studied the pathogenic mechanisms of Alzheimer's disease in a 3D Matrigel-based brain tissue-like environment.[53] Whereas amyloid-β is thought to diffuse into the culture medium in 2D systems and to be removed during regular media changes, it was found that this closed 3D culture model system promoted amyloid-β aggregation, thereby more closely recapitulating Alzheimer's disease pathology. More recently, Kraus *et al*. developed a spheroidal co-culture model to examine neurite formation.[54] In this study, spheroids were formed from glial cells (*i.e.* Schwann cells) and neurons and subsequently encapsulated in a 3D collagen matrix. Evaluation of neurite lengths demonstrated a significant increase in neurite length after a period of seven days in the 3D set-up compared to 2D culture.

Organ-on-a-chip technology

Organ-on-a-chip technology refers to microfluidic cell culture devices, which are designed to reconstitute critical tissue- and organ-level functions and rely on a sophisticated interplay between engineering and biology.[55,56] These systems are used for and offer control over a variety of key system parameters, including the choice of cells and their spatial positioning. However, the added value of on-chip biomimicry lies within its potential to control parameters that are not easily controlled in 3D static cultures or bioreactors (*e.g.* fluid flow, physical and chemical gradients), which makes them promising tools to address fundamental questions in the area of tissue development, organ physiology, and disease etiology.[56]

Over the years, various microfluidic devices have been developed to provide insights into the pathophysiology of neurodegenerative disorders.[57] For example, Choi *et al*. and Cho *et al*. utilized a microfluidic-based approach to investigate the effects of amyloid-β (aggregation) on neurons and microglia respectively.[58,59] In their study, Choi *et al.* used a low-flow microfluidic system to assess the neurotoxicity of time-dependent amyloid-β aggregation and the effect of physiological flow on neuronal cell survival.[58] With their microfluidic platform, Cho *et al*. studied the effect of amyloid-β on microglial accumulation, which demonstrated a different role for soluble and bound amyloid-β during microglia recruitment and localization.[59] Another well-reported topic involves amyloid-β and tau protein propagation. Kunze *et al*. have utilized a microfluidic device to study the neurodegenerative propagation processes of Alzheimer's disease.[60] Primary rat cortical neurons were cultured in two separated cell compartments, one housing diseased neurons (*i.e.* showing tau-hyperphosporylation) and the other healthy neurons. By designing the microfluidic device in such a manner that connectivity was favoured through neurite outgrowth from both compartments over time, a co-pathological state within the same neural cell culture was established. More recent studies regarding amyloid-β and tau protein propagation are

presented by Song *et al.* who demonstrated amyloid-β spreading via neuronal connections along axonal membranes[61], Deleglise *et al.* who investigated the distant effects of local amyloid-β stress on neuronal subcompartments and networks[33], and Dujardin *et al.* and Calafate *et al.* who offered novel insights into tau protein transfer.[62,63]

Bioprinting

3D cell culture platforms provide the target tissues with a (temporary) microenvironment and thereby contribute to spatio-temporal cell signalling, cell identity, and cell function. Although these 3D cell culture platforms have been demonstrated to offer a promising route towards more physiologically relevant neuronal models and in-depth understanding of cellular behaviour and pathological conditions, there are still a number of limitations. One of the primary general challenges is related to the lack of adequate control. Regardless of the platform, issues such as irregular geometry (*e.g.* external shape and internal architecture of the scaffolds) and size, poor reproducibility, and difficulties regarding scaling-up impede their widespread use. To this end, bioprinting offers an exciting opportunity. By combining biomaterials, cells, and/or growth factors (composites referred to as bioink) with automated fabrication processes such as additive manufacturing (the generation of 3D constructs in a layer-by-layer manner based on a computer-aided design (CAD)), it facilitates the generation of 3D bio-engineered constructs with superior organization and more closely resembling native tissues.[22,23,24] This computer-controlled deposition of biologically relevant materials allows the fabrication of (multi)-cellular constructs. Besides, the layer-by-layer architecture allows tailoring beyond the cellular level, namely in terms of materials, and biochemical cues, which could be optimized to meet cell-specific requirements. In this manner, multiple cell

types could be contained throughout a single 3D construct, yet in an environment optimized for each of the individual components.

Bioprinting technologies

Although a wide variety of bioprinting technologies have been described in the literature, three main categories can be distinguished; inkjet printing, laser-assisted printing, and extrusion-based printing (Fig. 3).[64,65]

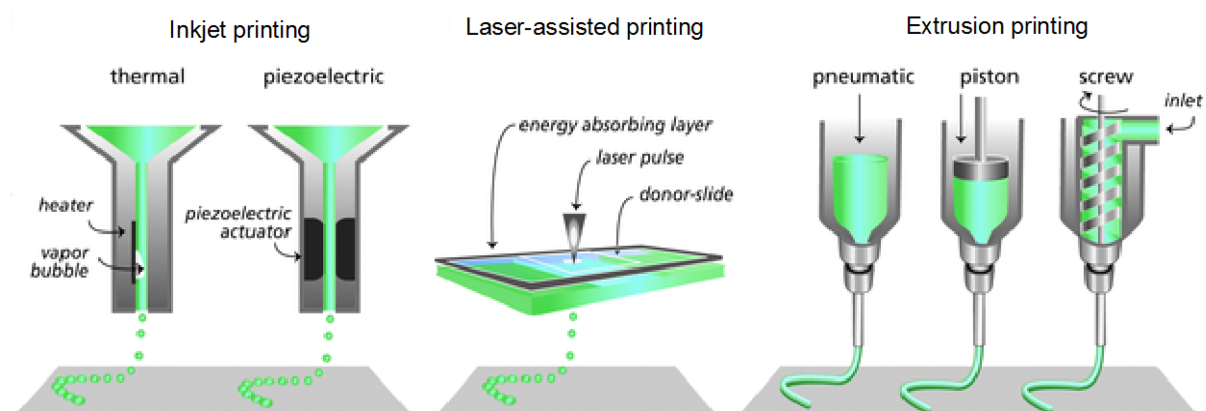

Figure 3. Main bioprinting technologies: (a) Inkjet printing, (b) Laser-assisted printing (c) Extrusion printing. Figure obtained and modified from [23] with permission from John Wiley and Sons.

Inkjet printing can be described as a noncontact printing process in which controlled volumes of (bio)ink (1–100 picolitres; 10–50 μm diameter) are dispensed on predefined locations, driven by thermal or piezoelectric forces (Fig. 3(a)). Both in thermal inkjet printing and piezoelectric inkjet printing, (bio)ink droplets are forced from the nozzle by creating a pressure pulse. In thermal inkjet printing, this pressure pulse is generated by heating. This heating results in vaporization of small volumes of the (bio)ink, producing the pulse required

to expel material from the nozzle. Piezoelectric inkjet printing involves the application of a direct mechanical pulse to the (bio)ink, which on its turn generates the pressure needed to force material though the nozzle. Regardless of the high resolution, relatively low cost, and wide availability of inkjet printing, several factors impede its successful widespread application.[23,65] These include the challenges regarding cell viability due to the small orifices, the potential risks of exposing the (bio)ink to thermal and mechanical stress, inconsistency in droplet size, nozzle clogging when using biologically relevant cell densities (>10^6 cells/ml), and the low upper viscosity limit of the (bio)ink (ideally < 10 mPa/s), which although considered to be beneficial for cell migration, proliferation, and cell viability, influences printing fidelity.[23,65]

As the name indicates, laser-assisted printing encompasses a plethora of manufacturing technologies which utilize a laser to deposit (biological) materials on a substrate. Laser-assisted bioprinting stems from the principles of a subclass of laser-assisted printing, namely laser-induced forward transfer (LIFT) (Fig. 3(b)). In LIFT approaches, a focused laser-source is used to transfer material from a so-called donor slide towards the receiving substrate. Typically the donor slide is covered with a laser energy absorbing layer and the desired (bio)ink. The focused laser pulse causes evaporation of the absorbing layer, leading to the formation a high-pressure bubble, which induces propulsion of the material. As this approach is nozzle free, the clogging issue associated with inkjet-based printing or extrusion-based printing is avoided. In addition, laser-assisted printing is compatible with materials with a broad range of viscosities (1–300 mPa/s) and able to deposit cells at medium cell density (*i.e.* 10^8 cells/ml) without detrimental effects on cell viability.[23,64,65] Despite these advantages, the presence of metallic residues in the final construct, the challenging and time-consuming

nature of preparing individual ribbons, and relatively high costs are several examples of hurdles yet to clear.

Nowadays, most of the commercially available bioprinters are based on extrusion-based dispensing (Fig. 3(c)). These systems function by robotically controlled extrusion of the (bio)ink, which is usually driven by mechanical action (piston or screw) or a pneumatic system, each posing their own (dis)advantages. Whereas mechanically driven deposition usually provides more control over the material flow, pneumatic systems have simpler drive-mechanism components, the latter allowing for higher maximum force capabilities. In contrast to inkjet printers, which yield single droplets of material, extrusion printers yield continuous filaments of the (bio)ink, usually with a resolution in the order of 200 µm. Besides, extrusion printing is compatible with materials with a wide range of viscosities (30 – 6×10^7 mPa/s). Even though the resolution is substantially lower when compared to laser- or inkjet-based systems, the fabrication speed, clinically relevant sizes, and ability to deposit high cell densities (>10^8 cells/ml) with high cell viability make this technology very promising for tissue engineering and regenerative medicine (TERM) applications.[23,65]

Bioprinting in 3D neuronal cell culture

Given that the field of bioprinting in general, and even more for this specific application, is still in its infancy, early studies of bioprinting in 3D neuronal culture have been focused mostly on healthy tissue. However, even though these studies do not directly concern diseased tissue(-like) constructs, these could be used as tool for a variety of applications, ranging from cell behaviour studies to understanding pathological conditions and drug testing, which is related to and relevant for neurodegenerative disease.

Various studies relating to this topic have been reported. Among these, several have been focussing on the effect of printing processes on cell viability. For example, Xu *et al*. and Xu *et al*. studied the delivery of neuronal cells by a modified thermal inkjet printer, and demonstrated no significant effect on cell survival.[66,67] More recently, Lorber *et al*. studied the potential of inkjet printing of adult rat retinal ganglion cells and retinal glia, demonstrating no significant difference in cell survival compared to non-printed controls.[68] In a similar approach, Tse *et al*. printed primary porcine Schwann cells and neuronal analogue NG108-15 cells with a piezoelectric inkjet printer.[69] This study showed that a higher range of experimental voltages has no adverse effects over a period of seven days. Whereas these studies could be considered relatively simplistic in nature (*i.e.* printing of cells to explore compatibility with the printing modality) these efforts have illustrated that relevant cells can be delivered and positioned by means of inkjet printing approaches without substantial damage.

A major advantage of bioprinting approaches is the ability to deliver and distribute cells and other biological factors in a very precisely controlled manner. An example is given by Lee *et al*. who fabricated cell–hydrogel composites for the purpose of *in vitro* neural culture.[70] Murine neural stem cells were deposited on top of a layer of printed collagen, subsequently crosslinked to immobilize the cells, and covered with an additional layer of collagen. Standard cell viability and proliferation assays showed that the printing technique did not affect cell viability. To explore the effect of growth factor release, a VEGF-releasing fibrin gel was added to the system. Whereas further studies should be conducted, initial experiments have shown that cellular proliferation and migration was supported over time. In a more advanced approach, Hsieh *et al.* explored bioprinting of cell-laden thermo-responsive biodegradable PU hydrogels.[71] Murine neural stem cells were harvested from adult mouse brain, encapsulated in different PU hydrogels, and subsequently bioprinted, forming a grid-

like structure. Cell viability, proliferation, and gene expression analysis demonstrated its potential as a suitable niche for neuronal stem cell proliferation and differentiation. Furthermore, the potential of this strategy in central nervous repair was evaluated in zebrafish and embryo neural injury models, which demonstrated its potential to rescue central nervous system functioning. Whereas the above-mentioned approaches illustrate the potential of bioprinting technology for neuronal applications, these fabricated constructs are mainly constricted to relatively thin layers of cells within cell culture media or on top of a hydrogel.

Given that the utility of actual bioprinting platforms for this particular application is at early stage, only a few studies have been reported in literature. One example is presented by our group and involves the development of a novel bioprinting-based method to recapitulate the layered structure of the brain and thereby fabricate 3D brain-like structures.[72] This study was based on previous work by Tang-Schomer *et al.*, who developed compartmentalized 3D brain-like cortical tissue with silk fibroin-based biomaterials.[17] Our group developed an Arg-Gly-Asp (RGD) peptide-modified gellan gum-based bioink (RGD-GG), which was assessed in terms of neural cytocompatibility, network formation, and printability. Discrete layers of cell-encapsulating (*i.e.* mouse primary neurons) RGD-GG were deposited using a simple hand-held printing device, resulting in layered constructs (Fig. 4(a-e)). Standard calcein/propodium iodide assays revealed acceptable cortical neuron and glial cell viability, both after encapsulation and printing RGD-GG. Immuno-staining seven days after encapsulation demonstrated neuronal network formation throughout the RGD-GG structures. To assess the potential of this strategy to create more complex multi-layered brain-like structures, six-layer structures were printed. Visual analysis revealed presence of distinct layers, within the solid structure. To assess the neural cytocompatibility of these larger structures, a three-layer construct was printed, consisting of two cell-laden RGD-GG layers,

separated by an acellular RGD-GG layer. Neuronal network formation was observed after five days of culture, with axons starting to penetrate into the acellular middle layer (Fig. 4(f-g)). These multi-layer brain-like structures have shown to provide an environment in which the dynamic process of neural growth can be observed and quantified in 3D and thereby offer a promising tool for a variety of applications ranging from cell behaviour studies to understanding pathological conditions and drug testing.

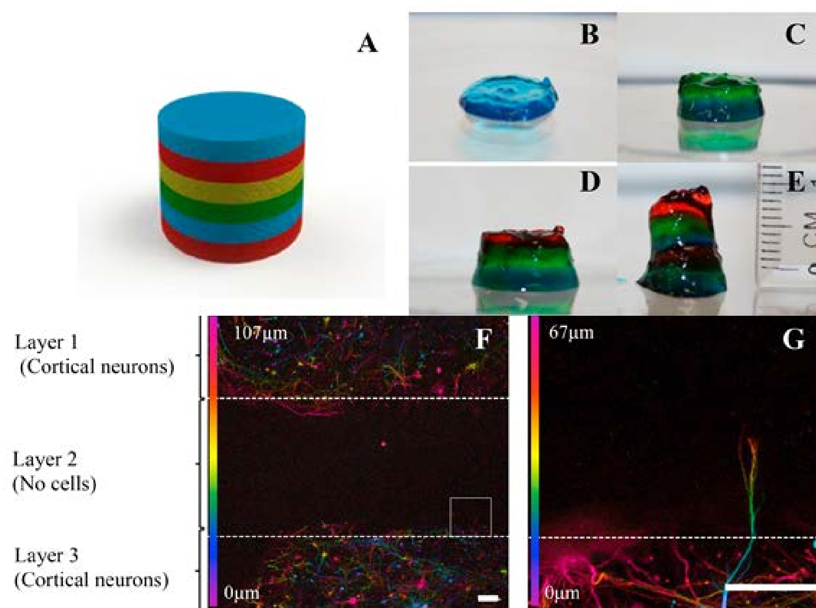

Figure 4. Layered brain-like structure: (a) SolidWorks schematic of the envisioned six-layered construct, (b-e) construct fabrication process, in which each colour represents a distinct layer, (f) confocal microscope images of the neurons coloured for their z-axis distribution after 5 days of culture, (g) showing neurite penetration through the gel into the acellular RGD-GG layer. Scale bars represent 100 µm. Figure obtained and adapted from [72] with permission from Elsevier.

In another approach towards establishing more accurate neuronal tissue-like structures, our group investigated *in situ* differentiation of human neural stem cells in bioprinted constructs.[73] Frontal cortical human neural stem cells were encapsulated in an optimized polysaccharide bioink consisting of alginate, carboxymethyl-chitosan, and agarose, and followed by a micro-extrusion approach. Characterization of the cells revealed homogenous cell distribution and high viability throughout the printed construct. Immunophenotyping and reverse-transcription quantitative PCR (RT-qPCR) analysis demonstrated successful differentiation into functional neurons and supporting neuroglia, illustrating the potential of this platform to be used in translational studies.

Following on from this study, Gu *et al*. described the first example of bioprinted human induced pluripotent stem cell constructs, which were successfully differentiated into homogenous neural tissues.[74] Human induced pluripotent stem cells were encapsulated in a bioink consisting of alginate (5% w/v), carboxymethyl-chitosan (5% w/v), and agarose (1.5% w/v) and subsequently bioprinted (Fig. 5(a)). Extrusion printing of the cell-laden bioink resulted in homogenous cell distribution throughout the constructs, with negligible cell death over a period of seven days, which was determined by standard calcein/propodium iodide assays (Fig. 5(b)).[74] Flow cytometry studies, performed after ten days in culture, revealed expression of pluripotency markers, which was further confirmed by induction of embryoid bodies. The bioprinted stem cells were then differentiated into cells with phenotypes that represent neuronal subtypes and microglia, as confirmed by immunophenotyping and RT-qPCR. This study has successfully demonstrated the ability to produce human induced pluripotent stem cell-laden constructs and differentiate these into relevant cell types, which is an important step towards bioprinting-based neuronal tissue models.

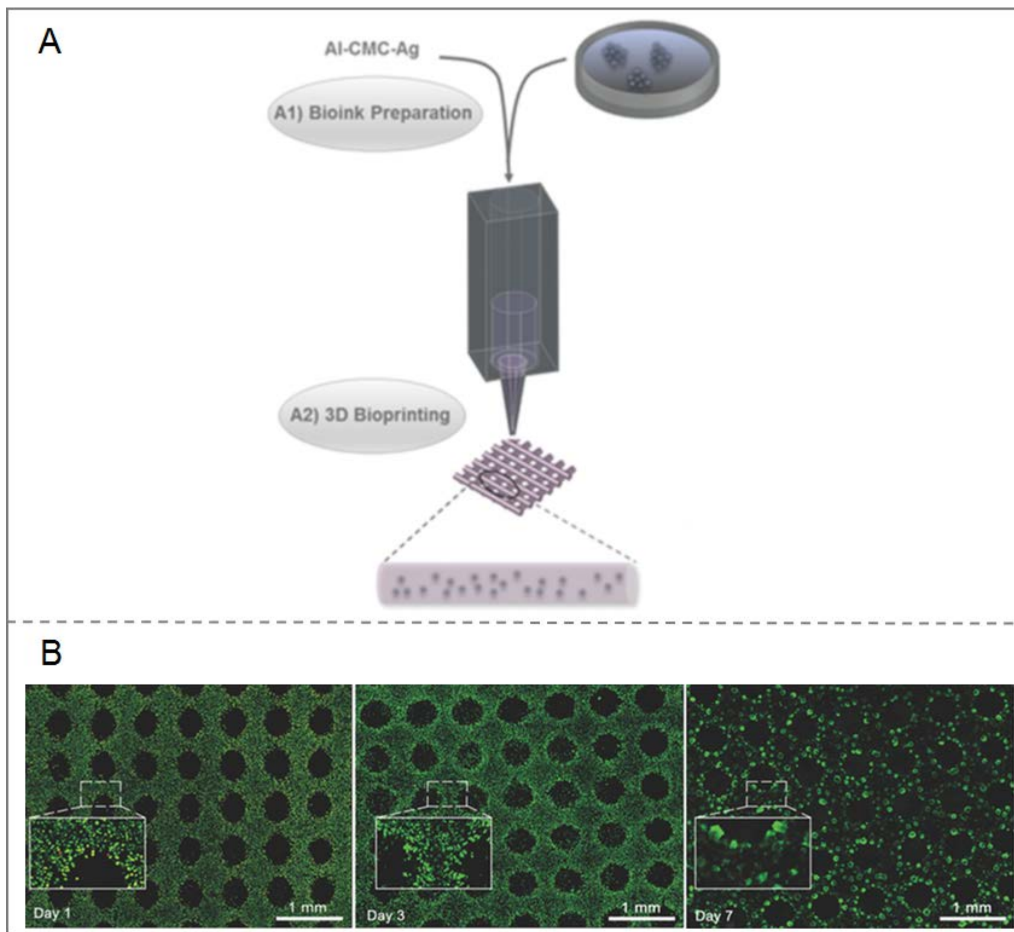

Figure 5. Bioprinting of human induced pluripotent stem cells: (a) schematic representation of the approach, in which the bioink is prepared by adding the cells to an optimized alginate, carboxymethyl-chitosan, and agarose (Al-CMC-Ag) mixture and subsequently bioprinted. Cell viability studies by means of calcein/propidium iodide staining have shown (b) homogenous cell distributions and high cell viability over a period of seven days post-printing. Scale bars represent 1 mm. Figure obtained and adapted from [74] with permission from John Wiley and Sons.

Bioprinting, a complementary approach

The true power of bioprinting technology lies in its superior potential to offer spatial and temporal control in a reproducible manner. However, it is important to realize that different

applications may require a different approach, which could be offered by other 3D cell culture platforms. Therefore, rather than being considered as superior, it is noteworthy to mention the complementary role of bioprinting technology, when being integrated with other existing 3D cell culture platforms.[75] Several papers have been reported regarding the integration of scaffold-free 3D cell culture platforms in combination with 3D printing technology. For example, Mironov *et al*. have demonstrated the potential of bioprinting to precisely deposit tissue spheroids formed from human dermal fibroblasts according to predefined patterns.[76] Very recently, Schneeberger *et al*. have reviewed the convergence of organoids and bioprinting technologies, highlighting the benefits bioprinting could have in terms of increasing the (clinical) application potential of organoids.[45]

Besides its added value for scaffold fabrication, additive manufacturing could facilitate the development of custom-made microfluidics devices.[77] For example Johnson *et al.* used additive manufacturing technology to fabricate a nervous system-on-a-chip.[78] This biomimetic chip consisted of three chambers with separate fluid environments and microchannels, the latter providing axonal guidance and alignment (Fig. 6). The different compartments were loaded with appropriate cell types and the complete chip was studied over time in terms of cell organization and its potential to be applied in viral infection assays. Whereas spatial separation was indeed achieved, axonal networks were able to penetrate into the chambers, allowing axon-to-cell integration between compartments. In line with this, analysis of axon-to-cell spreading of viral particles from one compartment to another demonstrated biological connectivity between the compartments. In this approach, relevant cell types were successfully integrated on their chip, illustrating that additive manufacturing could be an effective fabrication approach for the development of tailor-made on-a-chip biomimicry devices.

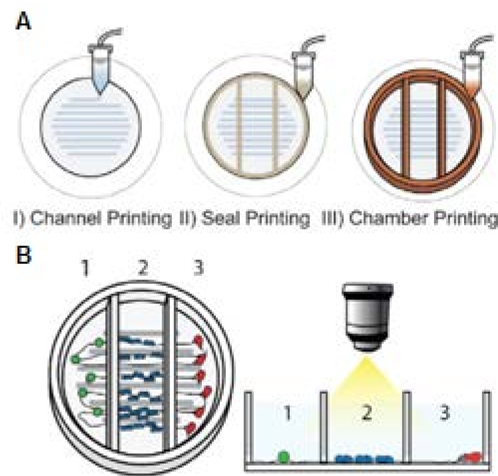

Figure 6. 3D printed nervous-system-on-chip: (a) schematic of the printing approach, which consisted of three consecutive steps. First, microchannels providing axonal guidance were printed, subsequently, a sealant layer was printed, after which the individual chambers were fabricated. The chip was completed by (b) functionalizing the chambers with the relevant cell types. Obtained and modified from [78] with permission of The Royal Society of Chemistry.

Future perspectives: challenges and opportunities

Over the years, bioprinting technology has shown to offer great potential to establish 3D tissue-like constructs and thereby approximate *in vivo* like conditions. Even though bioprinting technology and its implications for the nervous system are still in its infancy, several important challenges that remain to be addressed are already identified: 1) systematic studies of cell-material and cell-ECM interactions in 3D to enable optimization of bioink formulations and scaffold design, 2) improved characterization methods to monitor the dynamic process of neural growth in 3D, such as tracking of cell migration and

differentiation, 3) coupling of high throughput screening methodologies with 3D printed neural systems for fundamental studies and pharmacological tests.

Given its potential, research is increasingly turning to biofabrication technology to fabricate 3D functional constructs. However, it is essential to bear in mind that biofabrication represents an integrated group of enabling technologies and disciplines, which together have as a common goal to develop biologically functional products.[24,79] As the biofabrication field matures, developments within all these converging technologies and disciplines become equally important and will need to be streamlined in order for the field to advance.

The importance of converging disciplines

Central to the biofabrication approach is the software, which is the starting point for construct design and drives the transition from this design phase to actual printing.[80] The software forms the basis for the quality and usability of the final construct, and should cover construct design and machine control in a user-friendly manner. It is essential to develop the software as well as the hardware, which can keep up with our enhanced understanding and appreciation of biofabrication-based tissue engineering and associated disciplines (*e.g.* the need for multiscale geometric complexity and multimaterial management).

Another crucial pillar for the development of the biofabrication arena is the field of materials science. Key in the biofabrication discipline is the choice of the bioink. Regardless of the final application, this bioink should meet various general requirements, two crucial ones being its biocompatibility and printability. On the one hand, the bioink should resemble the native *in vivo* niche as closely as possible, which requires careful consideration of cellular behaviour, cellular functions, and -depending on the application- host integration. On the other hand, the bioink should exhibit appropriate viscous and mechanical properties, which enable accurate

deposition and shape-maintenance post-printing. Even though the scope of printable materials is expanding, to date, meeting both the requirements is hard to achieve, and usually involves a suboptimal, yet tolerable balance between both factors.[81] In order to advance the field, it is essential to develop more advanced biomaterials, which meet application-specific requirements. A relevant and exciting development is the emerging field of four-dimensional (4D) bioprinting, which is based on 3D bioprinting, but incorporates an extra fourth dimension in the form of 'time' to the system. 4D bioprinting goes hand-in-hand with developments in the area of smart stimuli-responsive materials.[82,83] These materials can be defined as materials that undergo a change in shape and/or function in response to an external stimulus such as temperature. Together, this facilitates the fabrication of constructs that can evolve over time after being printed, which makes it possible to add another level of complexity to the fabricated constructs, more closely resembling dynamic tissue conformations.

Whereas the above-mentioned challenges are mostly centred on the pre-printing and printing phase, it is important to anticipate on post-printing issues. One important, yet limiting aspect is related to the lack of optimized methods to analyse and assess the more complex 3D structures. For decades, research was predominantly focussing on thin, optically transparent 2D culture platforms, not only in terms of experimental design, but also in terms of subsequent data analysis. Whereas the increasing *in vivo* relevance that is offered by 3D cell culture platforms has its benefits, data interpretation becomes more challenging and requires developments in imaging technologies and establishing standard protocols.

In order to bring biofabrication-based innovation from the bench to the clinic, it is not only necessary to look into the scientific and technical aspects, but also to look beyond. Key aspects to consider are the need for standardization in the field and the ethical considerations.

The need for standardization

Among the abundant literature describing biofabrication and biofabrication approaches, a variety of ambiguous terms exist. [84,85] For example, terminology to describe the technology ranges from 3D printing, to bioprinting, additive manufacturing, additive biomanufacturing, and biofabrication, which are used interchangeably, often without being appropriately defined. This way of reporting could lead to potential confusion and makes it challenging to find relevant literature and compare results, which hinders knowledge transfer.

If the field is to move forward, it is not only essential to establish standards in terms of terminology, but also in terms of experimental design, procedures, and performance. In this context, it is essential to develop a regulatory framework, which accounts for the potential of biofabrication technology to create tailor-made products that are integrated with living cells. As described in detail by Hourd *et al*. points of consideration do not only arise around testing and validation of these customizable products, but already at the stage of experimental design.[84] For example, successful implementation requires sufficient knowledge of the materials in terms of cytocompatibility, which indeed influences performance, but also on its reaction when subjected to the printing process and to its post-printing conditions/environment. One should take into account that it is not only about a relatively novel manner of manufacturing, but also about a complex product, with the potential to be placed in a dynamic environment. The true challenge lies in developing a regulatory environment in which products pass a set of standards and regulations, while retaining the customizable nature and ability to fabricate patient-specific products.

Ethical considerations

Whereas the biofabrication arena has substantial similarities with the fields of regenerative medicine and tissue engineering, the unique character of biofabrication technology and its

potential applications necessitates thorough biofabrication-specific ethical considerations.[86-88] These involve questions related to the novelty of the technology itself, but also regarding its integration with biological products (*e.g.* stem cell technology). In addition, seen from a translational-application point of view, the great benefits that 3D biofabricated tissue products could offer in terms of personalised treatments raise their own ethical questions. From a preclinical 'bench-side' perspective, questions mainly arise around the use of animal and human materials. Several topics of debate are the choice and use of animals, use of cells sourced from embryonic and fetal tissues, and the privacy-related concerns associated with biobanking.[87,88] Other factors worth to consider include data integrity and appropriate study design, which could prevent premature transition from the bench to the bedside stage.[86] This bedside stage, which involves clinical trials, predominantly addresses questions related to the participants; selection of participants, and most importantly, the risks for the participant, which should be appropriate compared to the potential benefits on the larger scale. Lastly, there are society-related ethical considerations, which include the public's perception of the biofabrication field. As with any emerging technology, this perception is highly influenced by the public media. To make scientific developments more accessible to the general audience, complex findings are usually presented with excessive enthusiasm, giving weight to particular aspects of the topic. Given the multidisciplinary character and unknown risks of biofabrication technology, biofabricated products, and their possible applications, it may be difficult to look beyond the hype. However, it is crucial to realize that the manner of reporting affects the public's view on science. Overselling could easily lead to false expectations, disappointment, and eventually distrust in researchers and the technology. Sufficiently informing the public is essential for appropriate general understanding of science and could help when leading discussions on related topics (*e.g.* the role of biofabrication on human enhancement).

Conclusion

The vast majority of our understanding of neurological disorders is based on simplified 2D cell culture experiments; an environment that profoundly differs from the dynamic native *in vivo* situation. To gain a better understanding of our nervous system, the associated disorders, and to develop effective therapeutics, more accurate and meaningful models are imperative. As discussed, bioprinting-based biofabrication approaches offer the potential to more accurately recapitulate tissue features. These models are envisioned to shed new light on the causes and the mechanisms underlying (neurodegenerative) diseases and improve and accelerate the translation of *in vitro* findings to clinically relevant applications. However, one should realize that biofabrication encompasses a variety of disciplines, requiring constructive collaboration between engineers, scientists, and clinicians. Successful implementation of a novel biotechnology such as biofabrication requires scientists to look beyond experimental design, keep the final application in mind, and consider the interplay between their research and associated (potential) ethical and regulatory issues.

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