A Parkinson’s disease model composed of 3D bioprinted dopaminergic neurons within a biomimetic peptide scaffold

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Abstract

Parkinson’s disease (PD) is a progressive neurological disorder that affects movement. It is associated with lost dopaminergic (DA) neurons in the substantia nigra, a process that is not yet fully understood. To understand this deleterious disorder, there is an immense need to develop efficient *in vitro* three-dimensional (3D) models that can recapitulate complex organs such as the brain. However, due to the complexity of neurons, selecting suitable biomaterials to accommodate them is challenging. Here, we report on the fabrication of functional DA neuronal 3D models using ultrashort self-assembling tetrapeptide scaffolds. Our peptide-based models demonstrate biocompatibility both for primary mouse embryonic DA neurons and for human DA neurons derived from human embryonic stem cells. DA neurons encapsulated in these scaffolds responded to 6-hydroxydopamine, a neurotoxin that selectively induces loss of DA neurons. Using multi-electrode arrays, we recorded spontaneous activity in DA neurons encapsulated within these 3D peptide scaffolds for more than 1 month without decrease of signal intensity. Additionally, vascularization of our 3D models in a co-culture with endothelial cells greatly promoted neurite outgrowth, leading to denser network formation. This increase of neuronal networks through vascularization was observed for both primary mouse DA and cortical neurons. Furthermore, we present a 3D bioprinted model of DA neurons inspired by the mouse brain and created with an extrusion-based 3D robotic bioprinting system that was developed during previous studies and is optimized with time-dependent pulsing by microfluidic pumps. We employed a hybrid fabrication strategy that relies on an external mold of the mouse brain construct that complements the shape and size of the desired bioprinted model to offer better support during printing. We hope that our 3D model provides a platform for studies of the pathogenesis of PD and other neurodegenerative disorders that may lead to better understanding and more efficient treatment strategies.
1. Introduction

The development of the central nervous system (CNS) relies on highly structured processes involving the appropriate spatial and temporal presentation of physical supports and chemical cues. While the same processes work to repair the CNS in cases of disease, they are either inhibited or significantly reduced, with the result that only limited repair is possible. Current therapies for the treatment of CNS trauma or neurological disorders such as Parkinson’s disease (PD) are nonexistent, minimally effective, or associated with undesirable side effects, indicating the need for novel treatments. Developing such treatments requires an understanding of neurological disorders and their associated pathogeneses. Since it is difficult to access diseased brains, it is crucial to develop innovative techniques for disease modeling in vitro. These techniques must mimic the cues that occur in the normal developing brain and the changes caused by the disease. Doing so requires the use of advanced materials to support the environment and in vitro culture.

In this regard, the manufacturing and functionalization of biomaterials at the nanoscale, so-called nanobiomaterials, are of growing interest. In recent years, the importance of three-dimensionality for achieving superior in vitro models for PD has become evident [1–3].

Advancements in three-dimensional (3D) cell culture technologies, such as 3D bioprinting, have enabled printing of different cell types in a 3D environment. Currently available biomaterials or bioinks are based on either synthetic polymers or natural materials from non-human sources (e.g. Matrigel, collagen, and alginate), and they have significant batch-to-batch variations, which can affect the sustainability and immunogeneity of 3D scaffolds [4, 5]. Natural biomaterials such as collagen or Matrigel cannot be readily modified due to their complex and variable compositions. Their nonhuman origin introduces the risk of undesired immunogenic and pathogenic transfer, making them unsuitable for tissue and organ development in subsequent clinical applications [4, 5]. Bioinks that recapitulate the natural extracellular matrix, allow control over the composition, and provide flexibility for biophysical and biochemical modifications have already been shown to improve and revolutionize 3D cell culture [6]. Synthetic peptide-based materials that can self-assemble to generate 3D nanofibrous scaffolds are promising candidates for 3D cell culture applications due to their controllable composition and ease of chemical modification. Ultrashort peptides containing three to seven amino acids that self-assemble to form 3D hydrogel scaffolds resembling collagen fibers of the extracellular matrix and are capable of trapping 99.9% of water, enabling a wide variety of applications in tissue engineering and regenerative medicine [7–9]. Standard sterilization techniques such as UV irradiation and autoclaving did not result in breakdown of peptide compounds, as determined by nuclear magnetic resonance analysis. This observation supported the large-scale development of clinical-grade 3D scaffolds [10]. In this study, we used two N- and C-terminus-protected tetrameric peptide sequences, Ac-Ile-Val-Cha-Lys-NH2 (IVZK) and Ac-Ile-Val-Phe-Lys-NH2 (IVFK), for the development of 3D functional neuronal models within which ventral midbrain dopaminergic (vm DA) and vm non-DA neurons were encapsulated. IVFK and IVZK were previously found to be biocompatible and could be used to support the development of several in vitro 3D models [11–15]. Both peptides were previously used as suitable bioinks for in situ 3D bioprinting [11]. This encouraged us to explore their potential in the development of functional 3D vm DA models that could be used to model PD and to bioprint stable complex constructs suitable for long-term studies.

Additive manufacturing, widely known as 3D printing, is a promising technique used in tissue engineering and regenerative medicine. While 3D printing methods vary widely depending on the movement type and extrusion method, which is determined based on the materials to be used in the printing process, the primary method used in tissue engineering is extrusion-based 3D printing [16]. In this method, the printing resolution relies on the properties of the material used and is often enhanced by the incorporation of a support material. Due to its property of instantaneous gelation, the use of peptide bioink without the use of a support material can present challenges [17], especially when the aim is to develop complex multilayer constructs. However, incorporating support material during 3D bioprinting of cells can be undesirable, as it may require a multi-material extruder or increase printing time. Therefore, we suggest a hybrid method in which an external mold is created that complements the shape and size of the desired construct to offer support during printing. This process uses a commercially available non-cytotoxic liquid elastic resin and a stereolithography (SLA) printer [18]. The advantages of rapid prototyping mean that the proposed method is affordable and scalable. It is also time-efficient, as it does not impact the 3D bioprinting process; additionally, it improves printing quality and mechanical stability.

For further optimization and efficiency of syringe-based 3D bioprinting systems, automated time-pulsing methods can be implemented to maintain consistent gelation and extrusion of the desired bioink. Microfluidic pumps paired with control software, such as the Exigo pump offered by Cellix®, allow for various time-pulsing methods, including sinusoidal, square, and ramp pulses. Parameter optimization of duty cycles and pulse periods can improve the 3D bioprinting process by reducing flow interruptions and nozzle blockage. A previous study
reported optimized parameters for time-dependent pulsing of microfluidic pumps for 3D bioprinting of simple grid structures [17]. In this study, we aimed to implement these parameters for the first time to print mouse brain-inspired 3D vm DA neuron constructs at a scale of 1:1 with high precision and resolution.

With this work, we aimed to exploit the potential of 3D bioprinting using ultrashort peptide bioinks for creating PD models. We demonstrate the suitability of ultrashort peptide scaffolds for the 3D growth of both mouse and human vm DA. Our data show that peptide scaffolds promoted dendritic branching, a morphological feature that is directly related to synapses and thus has implications for the functioning of neurons [19]. As shown in the microelectrode arrays (MEAs) recordings, vm DA and vm non-DA neurons in our models maintained neuronal activity for over a month. Furthermore, we demonstrated the potential of our 3D systems to be used as disease models for PD by exposing vm DA neurons to 6-OHDA, a neurotoxin used to induce PD in vivo and in vitro. Finally, we discuss the importance of a suitable combination of nozzle design, 3D printing parameters, techniques, and bioinks to achieve 3D-printed neuronal models with high resolution that can be utilized to further study neurological disease. In this study, we 3D bioprinted a full-sized mouse brain model using IVFK and IVZK as bioinks and primary mouse VM neurons comprising vm DA and vm non-DA. We are confident that the 3D models developed in this study will provide a platform for further studies and will lead to the development of future efficient 3D neuronal models.

2. Materials and methods

2.1. Materials sources

Both peptide sequences Ac-Ile-Val-Phe-Lys-NH$_2$ (IVFK) and Ac-Ile-Val-Cha-Lys-NH$_2$ (IVZK) were synthesized manually by solid phase peptide synthesis. The 9-fluorenlymethoxy carbonyl (Fmoc) protected amino acids, MBHA Rink Amide resin, (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (TBTU)), and hydroxy benzotriazole (HOBr) were purchased from GL Biochem, Shanghai, China. N, N-diisopropylethylamine (DIPEA), piperidine, acetic anhydride, trifluoroacetic acid (TFA), triisopropylsilane, N, N-dimethyl formamide (DMF), dichloromethane (DCM), diethyl ether, ethanol, and phosphate-buffered saline (PBS, 10× concentration) were purchased from Sigma-Aldrich. Most of the cell culture reagents were purchased from Gibco, R&D Systems and STEMCELL Technologies. Antibodies were purchased from Promega, Abcam and Thermo Fisher Scientific. Ribonucleic acid (RNA) isolation kit was purchased from QIAGEN. Qualitative polymerase chain reaction (PCR) reagents were purchased from Promega and Applied biosystems. H9 human embryonic stem cell (H9hESC) line was derived from low passage (P# 43) WA-09 hESC (established by WiCell Research Institute Inc., Wisconsin). Primary mouse embryonic (E12.5) VM and cortical neurons were isolated from embryos from time-mated Swiss mice (Animal Resources Core Lab, King Abdullah University of Science and Technology (KAUST), Saudi Arabia and Animal House Center, King Fahad Medical Research Center, King Abdulaziz University (KAU), Saudi Arabia). Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC, USA.

2.2. Methods

2.2.1. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Fiber morphology for both IVFK and IVZK was visualized using SEM (FEI Magellan XHR Scanning Electron Microscope): acceleration voltage 3 kV and Quanta 3D FEG with an accelerating voltage of 10 kV. Peptide hydrogel samples were prepared for SEM by dehydrating them using increasing concentrations of ethanol and then drying them at 31.1 °C and 1072 Pa using a critical point dryer. Finally, 3 nm Au/Pd were used to coat the dried hydrogel samples before imaging.

FEI Titan G2 80–300 CT with a 300 kV emission gun was used for the TEM imaging. Peptide hydrogels were diluted in water for TEM imaging. A carbon-coated copper grid was treated with glow discharge plasma before a drop of the prepared peptide solution was placed on its surface. This was kept at room temperature for 10 min before the drop was blotted with filter paper. The grid was stained with 2% uranyl acetate for 1 min and then dried for at least 1 day before imaging to enhance the contrast.

2.2.2. Peptide synthesis

IVFK and IVZK peptides synthesis were done using the Fmoc-based solid phase peptide synthetic method on rink amide resin (1 mmol), as previously described [11]. Prior to amino acid coupling, the resin was swelled with DCM. The 20% (v/v) piperidine/DMF was then used to deprotect the Fmoc group on the resin followed by washing with DMF and DCM. A mixture of the coupling reagent TBTU (3 eq.), HOBr (3 eq.) used to produce activated esters, diisopropylethylamine (6 eq.), and Fmoc-protected amino acid (3 eq.) was then added to the reaction vessel and shaken for 90 min. To assess the efficiency of each coupling and deprotection step, a Kaiser test was performed. Capping of the N-terminal of the peptide with an acetyl group was subsequently done by adding a mixture of acetic anhydride[thin space (1/6-em)]::[thin space (1/6-em)]::[thin space (1/6-em)] diisopropylethylamine [thin space (1/6-em)]:[thin space (1/6-em)]:[thin space (1/6-em)]: DMF in the ratio of 2[thin space (1/6-em)]:[thin space (1/6-em)]:[thin space (1/6-em)]:[thin space (1/6-em)]:1 (v/v). The N-terminal of the peptide sequence was later capped with an acetyl group.
by adding a mixture of acetic anhydride: dimethylformamide (1:4). The solution was then allowed to stand at room temperature for 15 min. The reaction mixture was then filtered through a short pad of silica gel and the filtrate was evaporated to dryness. The residue was dissolved in methanol and lyophilized. The crude product was then purified by reverse phase HPLC on a C18 column (2%–98% ACN in 15 min) at a flow rate of 20 ml min−1.

2.2.3. Raman spectroscopy

The samples for Raman spectroscopy were prepared as follows: The peptide (10 mg) was dissolved in 900 µl of MilliQ water and stirred until completely dissolved. The 100 µl of PBS 1× pH 7.4 was then added to allow hydrogel formation. The sample was left undisturbed in a glass vial for 1 h and then measured using Raman spectroscopy following methods similar to those described in a previous report [11]. A Witec Alpha 300 A equipped with a 633 nm wavelength laser was used in backscattered configuration. A sample aliquot was positioned on top of the CaF2 slide and immediately measured. Three acquisitions, consisting of ten accumulations of 10 s each, were performed with a laser power below 1 mW in different areas of each sample. The spectra were then background-subtracted with a polynomial function of grade 5, normalized to the 1450 cm−1 Raman shift, and averaged. For the identification of secondary structure components, the Amide I band was considered in the range 1640–1690 cm−1. Three Voigt functions were fitted to identify the peaks, their areas, and the full width at half maximum (FWHM). The function fitting builder allowed the range of peak search to be set as follows: α-helix, 1650–1658 cm−1; β-turns, 1660–1666 cm−1; and unordered structures, 1680–1690 cm−1. The FWHM was constrained below 25 cm−1. Additional peaks related to water (approximately 1640 cm−1) and aromatic residues (approximately 1580 and 1610 cm−1) were introduced. The fit converged when a reduced χ2 value of 1 × 10−9 was reached.

2.2.4. Atomic force microscopy (AFM) imaging

A solution of each peptide was prepared in MilliQ water by completely dissolving the peptide at a concentration of 10 mg ml−1. The solution was then left undisturbed for 2 h before being diluted 20 times in MilliQ water. One 50 µl drop was positioned at the center of a freshly cleaved mica sheet and left to equilibrate for 15 min. The sample was then rinsed with 500 µl of MilliQ water and dried under N2 stream. AFM measurements were performed in a JPK Nanowizard III using AC_240TS_R3 probes from Olympus, characterized by a nominal resonance frequency of 70 kHz and a spring constant of 0.1 nN. The cantilever was calibrated using the contact-free thermal noise method. The samples were measured in tapping mode at 25 °C and 50% relative humidity. Scan areas of 2 × 2 µm (512 × 512 pixels) were considered for the analysis of the fiber profiles.

2.2.5. Primary mouse embryonic VM DA and cortical neurons isolation

This study followed international guidelines for the use of animals in research. All experimental protocols were approved by the ethics committees of KAUST and KAU (7-CEGMR-Bioeth-2018, 211ACUC06, and 211BEC023). Primary mouse embryonic (E12.5) VM and cortical neurons were isolated from time-mated Swiss mice (Animal House Center, King Fahad Medical Research Center, KAU, Saudi Arabia). Primary mouse embryonic (E12.5) VM and cortical neurons were isolated from embryos from time-mated Swiss mice (Animal Resources Core Lab, KAUST, Saudi Arabia and Animal House Center, King Fahad Medical Research Center, KAU, Saudi Arabia), applying the standards described in the Guide for the Care and Use of Laboratory (8th edition) [20]. Animals were time mated overnight, and visualization of a vaginal plug the following morning was considered embryonic day (E) 0.5. The VMs of E12.5-mouse embryos were dissected in ice-cold L15 media (Thermofisher, USA). The telencephalon–mesencephalon boundary and isthmic organizer were cut to isolate the midbrain and cortical tissues. The ventral third of the midbrain tissue was dissected and used to enrich the DA population in culture. The dorsal anterior region of telencephalic tissue was cut to enrich the cortical population. The isolated VM and cortical tissues were incubated in 0.05% trypin (Thermo Fisher Scientific, USA) and 0.1% DNase (Stem Cell Technologies, USA) diluted in Ca/Mg-free Hank’s balanced salt solution (HBSS; Thermo Fisher Scientific, USA) for 15 min at 37 °C. The tissues were washed three times in HBSS media and resuspended in N2 media consisting of a 1:1 mixture of F12 medium and minimum essential medium supplemented with 1 mM glutamine, 1 mg ml−1 bovine serum albumin, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 6 mg ml−1 glucose, 1% penicillin/streptomycin, and 1% N2 supplement (all N2 media components from Thermofisher, USA). Primary neurons were cultured in vitro prior to assessments, with the specific number of days cultured dependent on the experiments performed (demonstrated in the sections below).

2.2.6. Monolayer and 3D neuronal cell culture

Freshly isolated primary embryonic mouse E12.5 VM and cortical neurons were seeded at a density of...
60 K/well in 96 well cell culture plates on poly-d-lysine (PDL) coated plates. To prepare PDL, 5 mg of PDL was resuspended in 5 ml of 1× PBS, and the stock was stored at −20 °C until use. For coating, the previously prepared PDL was diluted 1:100 (10 µg ml−1) in 1× PBS and then added to the cell culture plates in variable volumes depending on the surface area. The plates were incubated in a CO2 incubator for 24 h. The PDL was then removed and discarded, and each well was washed twice with 1× PBS before the cells were seeded.

The first step in preparing the 3D culture was determining the minimum concentration at which IVZK and IVFK formed a stable hydrogel within minutes or less. Varying concentrations of the peptides were prepared in 1× Dulbecco’s PBS (DPBS), and the time required for a stable gel to form was recorded. The selected gelation concentration, 4 mg ml−1 for IVFK and 3 mg ml−1 for IVZK, was used in subsequent experiments. The weighed peptide was first resuspended in a volume of nuclease-free sterile water equal to half of the required final volume. A peptide base was first added to each well to prevent the cells from attaching to the plastic surface. A suitable volume of the resuspended peptide in water was added to the culture well, and an equivalent volume of 2× DPBS was added to enhance the gelation process. The plates were incubated for 5 min to ensure complete gelation. As gelation was almost instantaneous once DPBS was added, DPBS was only added inside the cell culture well. The 3D construct was prepared on top of the peptide base. The suitable volume of peptide was deposited, and the desired number of cells in 2× DPBS were added to an equivalent volume and mixed briefly with the peptide. The plates were incubated again for 2–3 min before adding the cell culture media. The N2 media was then carefully added to the culture plates. The cells were incubated at 37 °C and 5% CO2 until further analysis was performed.

2.2.7. Differentiation of human embryonic stem cells (hESCs) into DA neurons

hESCs were differentiated into DA neurons [21] and 3D cultured in IVZK and IVFK peptide scaffolds. Cell culture plates were coated with 5 µg ml−1 Laminin-521 (Gibco) diluted in PBS+/+/ (Gibco) and left in a CO2 incubator for ≥2 h. The excess laminin solution was removed and discarded, and the cells were plated. mTeSR media (STEMCELL Technologies) supplemented with 10 µM Y27632 (R&D Systems) was added to the cells. At day 0 (24 h after plating), the cells were inspected under a microscope to ensure that an even distribution of cells had been achieved and that cells had reached 95% confluence as a single monolayer.

Induction media was prepared by adding 47.75 ml of DMEM/F12 (GIBCO), 47.75 ml of Neurobasal media, 2 ml B27-Vitamin A (GIBCO), 1 ml N2 (GIBCO), 1 ml Glutamax, and 0.5 ml penicillin/streptomycin (GIBCO). The induction medium was then supplemented with 10 µM SB431542 (R&D Systems), 200 nM LDN193189 (R&D Systems), SMAD inhibitors of the transforming growth factor-β, and bone morphogenetic protein pathways. Media was removed and cells were washed twice with PBS−/− (GIBCO) to remove residual mTeSR before the previously prepared induction media was added. At day 1, media was removed and replaced with induction media supplemented with 10 µM SB431542 and 200 nM LDN193189 (R&D Systems), as well as ventralizing factors 100 ng ml−1 C25II SHH (R&D Systems) and 2 µM purmorphamine (R&D Systems). On days 2 and 3, media was replaced with induction media supplemented with 10 µM SB431542, 200 nM LDN193189, 100 ng ml−1 C25II SHH, 2 µM purmorphamine, and 2.5 µM of the Wnt agonist CHIR99021 to caudalize developing progenitors. From day 3 onward, media was changed once every other day, and the volume was increased. On day 5, media was changed to induction media supplemented with 200 nM LDN193189, 100 ng ml−1 C25II SHH (R&D Systems), 2 µM purmorphamine, and 2.5 µM CHIR99021 (R&D Systems). At days 7 and 9, media was changed to induction media supplemented with 200 nM LDN193189 and 2.5 µM CHIR99021 (R&D Systems). The 100 ml of maturation media was prepared by adding 46.75 DMEM/F12, 46.75 Neurobasal media, 2 ml B27 + Vitamin A (1×), 1 ml N2 (1×), 1 ml non-essential amino acids (1×), 1 ml ITS-A (1×), 1 ml Glutamax (1×), and 0.5 µl penicillin/streptomycin (0.5×). On day 11, cells were washed twice with PBS−/− to remove residual induction media and replaced with maturation media with 20 ng ml−1 rhBDNF, 20 ng ml−1 rhGDNF, 0.1 mM dibutyryl cAMP (R&D Systems), 200 mM ascorbic acid (Sigma Aldrich), 1 ng ml−1 rhTGF/β3, and 10 µM DAPT (R&D Systems) (Maturation Media + BGCATD), supplemented with 2.5 µM CHIR99021. From day 13–30 maturation media + BGCATD was added every other day. Differentiation of hESCs into vm DA neurons can be observed by the formation of axonal projections and branches from day 18–20. At day 25, the plates were fixed and immunostained with antibodies against TUJ1 and TH.

2.2.8. Ventral midbrain (VM) neurons viability and metabolic activity assessment

To compare the cytotoxicity for vm DA and vm non-DA neurons of IVFK and IVZK scaffolds with that of PDL, the cells were assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, L3224). The kit uses a fluorescence-based method to determine cell viability using two probes: calcein AM, which detects esterase activity in live intact cells through its ability to hydrolyze calcein AM to calcein (494/517 nm) and emits green fluorescence, and ethidium homodimer-1 (528/617 nm), which binds to the nucleic acids
of damaged cells and emits a detectable red fluorescence. Freshly isolated primary mouse VM neurons, comprising vm DA and vm non-DA neurons, were plated on PDL and encapsulated in IVFK and IVZK based scaffolds and cultured in N2 media. Cell viability was assessed at day 1, 2, and 3 of cell culture. Negative control for the LIVE/DEAD assay was also included. Mouse vm DA and vm non-DA neurons were treated with ethanol on day 3 of culture and incubated for 1 h before staining with calcein AM and ethidium bromide to assess the efficiency of the assay reagents (figure S6). A solution containing 2 µM calcein AM and 4 µM EthD-1 in 1× DPBS was prepared. The cell culture media was carefully replaced with the previously prepared calcein AM/EthD-1 solution. The cells were then incubated at room temperature for 20–40 min before imaging with a ZEISS inverted fluorescent microscope (Germany) using fluorescent isothiocyanate (FITC) and red fluorescent protein (RFP) filters.

Additionally, the metabolic activity of vm DA and vm non-DA neurons was assessed at days 2 and 3 of cell culture using the CellTiter-Glo® 3D Cell Viability Assay (Promega, G9681). This assay relies on measuring the adenosine triphosphate (ATP) release as an indicator of cellular metabolic activity. CellTiter-Glo® Reagent was added in a volume equal to the volume of the cell culture medium and mixed thoroughly by pipetting up and down ten times to break down the 3D construct comprised of cells and hydrogels. The plates were then incubated at room temperature for 25 min, and the luminescent signal was read using a PHERAstar FS plate reader. There were linear relationships between the luminescent signal, cell number, and ATP release.

2.2.9. Immunocytochemistry
Mouse embryonic VM neurons, comprising vm DA and vm non-DA neurons, and cortical mouse neurons were fixed using 4% paraformaldehyde (Santa Cruz, sc-281692) and stored at 4 °C until staining was performed. Primary antibodies against mouse neuron-specific class III beta-tubulin (TUJ1; Promega, G7121) and rabbit tyrosine hydroxylase (TH; Abcam, ab112) were used for vm DA and vm non-DA neurons staining. Cortical neurons immunostaining was done using primary antibodies against mouse TUJ1 and rabbit T-box brain protein 1 (TBR1; Abcam, ab31940). TUJ1 reacts with neuron-specific class III beta-tubulin. This protein is a component of the microtubules in the cytoskeleton and is known to be expressed primarily in neuronal cells. Because it plays a vital role in neural development and axonal transport, it is considered an important marker of neuronal cell differentiation [22]. Antibodies against TH are typically used to detect DA neurons. TH catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) through a series of enzymatic reactions to form the neurotransmitter dopamine [23]. TBR1 is a marker for the differentiation of preplate, early born glutamatergic neocortical neurons [24], and thus it was used as a marker for primary cortical neurons in this experiment. The fixed cells were incubated with primary antibodies diluted as follows: TUJ1 (1:1500), TH (1:500), and TBR1 (1:500) in a blocking buffer (5% FBS, 0.3% Triton-X, and 0.2% sodium azide) overnight at room temperature. After removing and discarding the primary antibody, the cells were incubated for 1 h at room temperature in a blocking buffer. This was followed by the addition of anti-mouse Alexa 488 and goat anti-rabbit IgG H&L (Alexa Fluor® 555; Abcam, ab150078) or goat anti-rabbit IgG H&L (Alexa Fluor® 647; Abcam, ab150079) secondary antibodies diluted in blocking buffer (1:200) and incubated for 2 h at room temperature. Subsequently, the cells were incubated for 5 min in 1× 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, D1306) diluted in water, and the wells were covered with 1× PBS. Imaging was performed using a ZEISS fluorescence microscope, Zen blue v 2.6 software, and/or a laser scanning confocal microscope (Zeiss LSM 800 Inverted Confocal Microscope, Germany). The same immunocytochemistry procedure using the same type of antibodies as described above was applied for human VM neurons.

2.2.10. Neurite outgrowth analysis
The number of neurites, total length of neurites, length of dominant neurites, and number of branches were analyzed for ten neurites per technical replicate, comparing TUJ1+/TH− vm non-DA and TUJ1−/TH+ vm DA primary mouse embryonic neurons that were 3D cultured in IVFK- and IVZK-based scaffolds with those that were cultured on PDL coated plates, according to a method described in previous studies [25, 26]. Three technical replicates were analyzed for each treatment, and three biological replicates from different batches of primary mouse embryonic vm DA and vm non-DA neurons were included in this experiment.

Neurite tracing was performed using the Neuron plugin in the ImageJ software v 1.51k; a public domain Java image processing program. Overlapping neurites and those shorter than 20 µm were excluded to avoid bias. Data obtained from TUJ1+/TH− vm non-DA and TUJ1−/TH+ vm DA primary mouse embryonic neurons encapsulated and cultured in IVFK- and IVZK-based scaffolds were normalized to those of the neurons plated on PDL (control group). Subsequently, the data were expressed as a percentage change from the control, which was considered 100%. Images used for this analysis were obtained using a ZEISS fluorescence microscope (Zen blue v 2.6 software on a 20× objective). Student’s t-test was performed using GraphPad Prism v 8.1.2, and all quantitative data were expressed as mean ± SEM with the significance set at p < 0.05.
2.2.11. Neurosphere migration assay
To test the migration ability of 3D-cultured vm DA and vm non-DA neurons encapsulated in IVFK and IVZK peptide scaffolds, freshly isolated embryonic mouse vm DA and vm non-DA neurons were cultured over a period of 5 d to generate neurospheres. Five million cells were seeded in 100 mm non-treated cell culture Petri dishes and 10 ml of N2 media supplemented with 11,000 Epidermal growth factor (EFG) and 1,500 basic fibroblast growth factor (FGF). The spheres were then transferred to the PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds. Cells were cultured in N2 media then fixed at day 3. Neurons were immunostaining against TUJ1, TH, and DAPI. A ZEISS 710 confocal microscope was used to image the neurons for qualitative assessment of the migration of vm DA and vm non-DA neurons out of the neurospheres.

2.2.12. Recording of extracellular spontaneous neuronal activity using microelectrode arrays (MEAs)
Mouse primary embryonic VM neurons were 2D and 3D cultured as described above in 60-6well MEA200/30IR-Ti-rcr or 60MEA200/30IR-Ti-pr MEAs. The cell culture medium used for this experiment was B-27™ Electrophysiology Kit (A1413701; Gibco). Recording was performed once every 3 d for 5 min using the following parameters in the MEA2100 data acquisition software: sample rate, 25,000 Hz; Butterworth high pass filter; and 100 Hz cutoff. The MEAs were cleaned after each experiment using 1% Terg-a-zyme® enzyme detergent and sterile water, as per the manufacturer’s recommendations. Subsequently, the MEAs were exposed to UV light for 30 min to ensure proper sterilization.

2.2.13. 6-hydroxydopamine (6-OHDA) hydrochloride treatment
The 6-OHDA hydrochloride is a neurotoxin that is commonly used to selectively induce apoptosis in DA neurons. It is widely used to induce PD in rodents. The 6-OHDA exerts cytotoxicity by generating reactive oxygen species, initiating cellular stress and cell death [27–29]. DA neurons 3D cultured in IVFK- and IVZK-derived peptide scaffolds and 2D cultures on PDL-coated plates were treated with 6-OHDA to mimic the DA loss that occurs in PD. A stock solution of 100 mM 6-OHDA in 0.2% ascorbic acid was prepared using oxygen-free water. Cells were treated at 0 days in vitro (DIV) with different concentrations of 6-OHDA, ranging from 300 nM to 50 nM. Non-treated controls were included in each experiment. Cell viability and metabolic activity were assessed after treatment by live/dead and ATP assays as described above. Immunostaining was also performed, and the number of TH+ vm DA was assessed in the treated and non-treated samples. Prism GraphPad was then used to normalize data to the non-treated control, and the results are shown as percentages of the control.

2.2.14. Co-culture of mouse embryonic vm DA and vm non-DA neurons with endothelial cells (HUVECs)
For the vascularization of vm DA and vm non-DA neurons 2D cultured on the PDL-coated plates and encapsulated in IVFK- and IVZK-derived peptide scaffolds; primary neurons were co-cultured with endothelial cells (HUVECs) at a ratio of 3:1. The 60,000 vm DA and vm non-DA neurons were co-cultured with 20,000 HUVECs for 3 d. The cells were fixed and immunostained. Vm DA and vm non-DA neurons were immunostained with antibody against TUJ1, and HUVECs were immunostained with antibody against CD31. The nuclei were counterstained with DAPI. Images were obtained using a ZEISS 710 confocal microscope.

2.2.15. Co-culture of mouse embryonic cortical and VM DA neurons
To evaluate the suitability of our 3D neuronal model to accommodate more than one type of neuronal cells without compromising viability, mouse embryonic cortical neurons were co-cultured with vm DA and vm non-DA neurons in a 1:1 ratio in N2 media (i.e. 30,000 vm DA and vm non-DA neurons were seeded with 30,000 cortical neurons). Cell metabolic activity was assessed by measuring the ATP release from cortical neurons monocultures, vm DA and vm non-DA neurons monocultures, and co-cultures of vm DA and vm non-DA and cortical neurons. Cells were also immunostained with anti-TUJ1, anti-TH, and anti-TBR1 primary antibodies to evaluate the formation of connections between TH+ and TBR1+ neurons. In addition, vm DA and vm non-DA neurons were stained with Tracker Green dye and seeded with unstained cortical neurons to assess the formation of connections between cells isolated from the VM and those isolated from the cortex of mouse embryos.

2.2.16. Quantitative PCR
RNA was isolated on day 3 of culture using the RNeasy Plus Universal Mini Kit (QIAGEN, Cat No. 73404) following the manufacturer’s recommendations. To ensure efficient homogenization of cells, TissueLyser II (Qiangen) was used, as recommended in the RNeasy kit protocol. RNA was isolated from vm DA and vm non-DA neurons 3D cultured in IVFK- and IVZK-derived peptide scaffolds and 2D cultured on PDL-coated plates. RNA from uncultured primary mouse vm DA and vm non-DA neurons was isolated as a control. As a negative control, RNA was isolated from tissues other than the brain. Complementary DNA (cDNA) synthesis was performed using an ImProm-II Reverse Transcription System (Promega). Subsequently, Platinum™ SYBR™ Green quantitative real-time PCR (qPCR) SuperMix (Thermo Fisher) was used for real-time PCR following the manufacturer’s recommendations. qPCR experiments were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems).
Table 1. Primers sequences.

| Gene symbol | Primer sequence |
|-------------|-----------------|
| En1         | Forward: 5'-TACAGCAACACCTAGTG-3' Reverse: 5'-CCGTCCTTCTTCTTCTTT-3' |
| Lmxa        | Forward: 5'-GGAGCCTCGCTCTTACCCG-3' Reverse: 5'-GCACCCATGACAAAACCTT-3' |
| Pitx3       | Forward: 5'-CTAGGCTTGGGGGTGCTTG-3' Reverse: 5'-CCTTCTCCGAGTCACTGTGC-3' |
| Th          | Forward: 5'-GAAGGAAGCAGCGACTGGCTTC-3' Reverse: 5'-GAGTGCGATAGGGTGAGGAAC-3' |
| Nurr1       | Forward: 5'-GACCCAGACCTGCTTTTGA-3' Reverse: 5'-ACCCCAATTGCAGAAGATGAG-3' |

RT-PCR StepOne System and Data Assist software were used to generate raw threshold cycle (CT) data for the negative control (negative tissue) and experimental groups (uncultured and cultured primary vm DA and vm non-DA neurons on PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds) for both the housekeeping reference gene (GAPDH) and the target genes (Th, Nurr1, Lmxa, En1, and Pitx3) in triplicate. The primer sequences are listed in Table 1. We normalized the CT values of the target gene to the CT values of the reference gene and the ∆CT values of the test sample to the ∆CT values of the control sample before analysis. The ∆∆CT method was used. The ∆CT (CT target gene − CT reference gene) value was calculated for each sample, ∆∆CT (ΔCT test sample − ΔCT control sample) value was calculated for comparative groups, and, finally, the relative quantification (Rq = 2 − ∆∆CT) and fold change (Log2FC) were calculated to evaluate the expression of target genes under different experimental conditions. The Rq values of each gene were compared across all samples of the five groups, and p values were calculated for identification of significantly expressed genes. Microsoft Excel functions were used to perform the analysis and generate statistical plots of the data.

2.2.17. 3D bioprinting

2.2.17.1. 3D bioprinting using a mold

We created a 3D-printed mold on a lab printer from an STL file, a standard format for 3D printing, using elastic biocompatible resin. Since the material does not exhibit the desired flexibility, the mold base thickness was minimized during the computer-aided design (CAD) process to achieve an easy release of the printed/molded brain. This technique allows the material to settle and take shape before nozzle extrusion.

2.2.17.2. Pre-printing preparation

An open-source CAD model of the mouse brain was obtained and modified on a 1:1 scale [30]. A G-code file was extracted from the CAD model, and parameters, including printing speed, infill density, and layer height, were set. Additionally, a VM model was designed in Solidworks® in standard triangle language (STL) format and sliced into a G-code.

For additional printing support, two molds were created by cross sectioning the mouse brain CAD model representing the upper and the lower halves of the brain. The molds were 3D printed as described above.

A robotic 3D bioprinter developed in house was prepared to carry out the experiments. A custom-made dual-coaxial nozzle was mounted for triple-inlet extrusion. Microfluidic pumps were set with automated pulsing parameters for each solution to maintain a continuous flow. Square pulse flow profiles were created for each peptide: for IVZK, a 50% duty cycle with a flow rate range of 55–60 µl min⁻¹ was used, while, for IVFK, a 50% duty cycle with a flow rate range of 50–55 µl min⁻¹ was used. For PBS, the duty cycle was set to 50%, with a flow rate range of 15–20 µl min⁻¹. The cell line flow rate was constant at 10 µl min⁻¹ and then increased to 20 µl min⁻¹ for the top two layers (figure 1).

2.2.17.3. 3D bioprinting of cell-laden constructs

The 10 mg of IVZK and IVFK peptides were weighed in glass vials and sterilized under UV light for 30 min. The peptides were then dissolved in 1 ml of MilliQ water, vortexed, and sonicated to obtain a homogeneous solution.

The in-house developed 3D bioprinter was set up as described in 2.2.17.2, and the microfluidic pumps were loaded with three solutions. Pumps 1 and 2 were loaded with the peptide solution and PBS, respectively, and both were set to their optimized square pulse flow profiles. Pump 3 was loaded with vm DA and vm non-DA neurons suspended in 1× PBS at a flow rate of 10 µl min⁻¹. A heated bed was set at 37 °C to create a suitable environment for the cells after extrusion. The same procedure was used for both peptides.

2.2.17.4. 3D bioprinting of mouse brain model

Three methods were used and compared for the biofabrication of the mouse brain model. In the first method, the sliced G-code file of a mouse brain model was 3D bioprinted using the pumping parameters mentioned above. No support material was used. In the second method, the bottom half of the cross-sectional mold was used as the printing support. The 3D bioprinter start point was oriented and set to be extruded inside the mold. Once the bottom half of the model had taken shape within the mold, the remaining layers were built up on top of the mold to complete the structure. The same pumping parameters reported previously were used. In the third method, a cross-sectional mold was used to create the top and bottom halves of the structure, and the structure was entirely molded. A concentration of 13 mg ml⁻¹ was used for both peptides, IVZK and IVFK. Cells were...
extruded in the top two layers of the constructs for all three methods.

3. Results

3.1. Physical-chemical structural characterization of IVZK and IVFK

In this section, we describe the structural and chemical properties of the peptides under investigation. To reveal the nanofibrous structure and 3D organization of IVFK and IVZK, both SEM (figures 2(a) and (c)) and TEM imaging (figures 2(b) and (d)) were performed. Both peptides generate scaffolds consisting of nanoscale fibers, thus creating the basis for the formation of hydrogels. To better depict the nanoscale structure of the fibers, AFM topography was also performed (figures 2(e)–(h)). In addition, the secondary structure characterizing the nanofibers was investigated by Raman spectroscopy (figures 2(i) and (j)).

3.1.1. AFM topography

AFM topography was performed in air on the fibers formed by IVFK and IVZK in water. Prior to imaging, the fibers were allowed to form for 2 h. The fibers from IVFK have a more homogeneous supramolecular structure (figure 2(e)), whereas the fibers from IVZK have a greater variety of features (figure 2(g)). These differences are evident from the measurement of the longitudinal profiles of the IVFK fibers in figure 2(f) and IVZK fibers in figure 2(h). Indeed, as reported in table 2, we observed that the critical dimensions for IVFK revealed only one type of helical supramolecular arrangement (with a helical pitch of approximately 70 nm), whereas three were observed in IVZK (with helical pitches of approximately 50, 70, and 100 nm). This distinction can be explained by the more pronounced tendency of IVFK to assemble laterally. The average diameters of the fibers were comparable between the two peptides. Notably, the IVFK helical structure is left-handed, whereas the IVZK helical structure is right-handed.

3.1.2. Raman spectroscopy

As can be seen in figures 2(i) and (j), the peak deconvolution of the Amide I band reveals that the secondary structures of IVFK and IVZK contain mostly β-turns. These peaks are found at Raman shifts of 1663–1666 cm⁻¹. Interestingly, the presence of α-helix was more significant in IVFK than IVZK.
Figure 2. Structural and chemical characterization of IVFK and IVZK peptides. (a) SEM micrograph on IVFK hydrogel; (b) TEM image on IVFK nanofibers; (c) SEM micrograph on IVZK hydrogel; (d) TEM image of IVZK nanofibers; (e) AFM topography performed in air of IVFK; (f) zoom-in on two representative IVFK fibers and their respective longitudinal profiles (scale bar 200 nm); (g) AFM topography performed in air of IVZK; (h) zoom-in on two representative IVZK fibers and their respective longitudinal profiles (scale bar 300 nm). Raman spectra of the Amide I region of the two peptides (i) IVFK and (j) IVZK, with the most contributing secondary structure indicated (Raman shift peak in cm$^{-1}$).

Table 2. Critical dimensions of IVFK and IVZK fibers as measured by AFM topography.

| Pitch (nm) | Average diameter (nm) |
|-----------|-----------------------|
| IVFK      |                       |
| 70.44 ± 2.10 | 8.26 ± 0.37;          |
|           | 10.23 ± 0.49;         |
|           | 11.52 ± 0.13          |
| IVZK      |                       |
| 51.94 ± 2.74 | 7.83 ± 0.46;          |
| 73.61 ± 0.70 | 11.06 ± 0.43;         |
| 100.28 ± 3.47 | 11.38 ± 0.34        |
| 154.44 ± 22.99 |

(figures 2(i) and (j), table 3). As shown in previous publications [31], short peptides form β-turn secondary structures after moving through several conformations. The higher α-helix content in the IVFK hydrogel might indicate slower formation kinetics (table 3). In contrast, the FWHM values of the peaks at approximately 1663 cm$^{-1}$, which are related to the packing of molecules in the β-turn secondary structure of the two peptides [32], seem to indicate a slightly more ordered conformation for IVFK than for IVZK.

3.2. Cytotoxicity, morphogenesis studies and characterization of 2D and 3D-cultured neurons

3.2.1. Viability and metabolic activity assessment of 2D- and 3D-cultured vm DA and vm non-DA neurons

The live/dead assay performed on E12.5 mouse embryonic vm DA and vm non-DA neurons 2D cultured on PDL-coated plates or 3D cultured in both IVFK and IVZK peptide scaffolds showed that, in both peptide scaffolds, the majority of neurons were viable over a period of 3 d. However, in vm DA and vm non-DA neurons cultured on PDL-coated plates, a noticeable decrease in the density of viable neurons was observed on day 3 (figure 3(a)). This decrease
Table 3. Raman spectroscopy typical values of hydrogels formed from IVFK and IVZK at 10 mg ml\(^{-1}\).

|          | α-helix | β-turn | Unord. Str. |
|----------|---------|--------|-------------|
| **IVFK** |         |        |             |
| Shift (cm\(^{-1}\)) | 1654    | 1665   | 1681        |
| FWHM (cm\(^{-1}\)) | 24      | 11     | 15          |
| Area (%) | 15%     | 65%    | 20%         |
| **IVZK** |         |        |             |
| Shift (cm\(^{-1}\)) | 1653    | 1662   | 1681        |
| FWHM (cm\(^{-1}\)) | 16      | 12     | 16          |
| Area (%) | 7%      | 68%    | 25%         |

Figure 3. Viability assays. Effect of PDL, IVFK, and IVZK peptides on (a) cells viability of vm DA and vm non-DA neurons assessed using live (green)/dead (red) staining over a period of 3 d (scale bar 100 µm) and (b) on cells proliferation at day 2 and day 3 of culture based on the ATP release. Error bars represents mean ± SD.

in viability was also confirmed from the ATP release results measured at day 2 and day 3 of culture, as ATP increased over time in vm DA and vm non-DA neurons cultured in IVFK and IVZK peptide scaffolds, while ATP was slightly decreased on day 3 in neurons cultured on PDL-coated plates (figure 3(b)). Additionally, a time-lapse video of primary mouse vm DA and vm non-DA neurons encapsulated in the IVFK-derived scaffold recorded over a period of 2 days showed cell attachment, motility, and neurite
outgrowth (video S1). This finding supports the enhanced viability of vm DA and vm non-DA neurons in the 3D construct.

3.2.2. Effect of IVFK and IVZK on neurite outgrowth of VM DA and non-DA neurons

To assess the effect of the selected peptide scaffolds on the morphology and maturation of vm DA and vm non-DA neurons, which have direct implications for their functionality [33], neurite outgrowth was quantified for TUJ1+/TH− and TUJ1−/TH+ neurons (figure 4(a)). TUJ1 is a marker for neurons in the peripheral and CNS and TH is a specific marker for vm DA neurons. We found no significant difference between TUJ1+ and TH+ neurons cultured on PDL, encapsulated in IVFK, or IVZK in terms of total neurite length, length of dominant neurites, and number of neurites (figures 4(b) and (c)). However, a considerable increase in the total number of dendritic branches was observed in the vm DA neurons encapsulated in our peptide scaffolds when compared to those cultured on PDL-coated plates (figures 4(b) and (c)). The increase in the number of branches was highly significant in the case of TH+ neurons 3D cultured in IVFK based scaffolds (figure 4(c)). A z-stack video of vm DA and vm non-DA neurons in an IVFK-derived scaffold also shows neuronal growth in multiple layers of the 3D construct (video S2). Additionally, human vm DA neurons differentiated from ESCs (hESCs) cultured in laminin-coated plates and encapsulated in IVFK and IVZK peptide scaffolds expressed TUJ1 and TH, confirming successful differentiation in our models (figure 5).

3.3.3. Gene expression analysis for vm DA neurons cultured on PDL and encapsulated in ultrashort self-assembling peptide-based 3D models

qPCR was performed to determine the temporal expression of five genes (Th, Nurr1, En1, Lmx1a, and Pitx3). These five genes are known to play major roles in the development of vm DA neurons. Fold-change values revealed the upregulation of Th, Nurr1, Lmx1a, and En1 in all conditions compared to negative tissues. Our data showed upregulation of the Th gene in primary vm DA neurons cultured on PDL, and encapsulated in IVFK-, and IVZK-based scaffolds in comparison to the uncultured primary vm DA neurons. Generally, our data show that all five genes were expressed in primary mouse vm DA neurons, both those 2D cultured on PDL-coated plates and those 3D cultured in IVFK and IVZK peptide scaffolds (figure 6(a)–(e)). A significant upregulation in Th expression was observed in the 3D-cultured vm DA neurons in IVZK scaffolds compared to the uncultured primary DA neurons (figure 6(a)). A significant upregulation was found in Nurr1 expression in primary vm DA neurons compared to the 3D-cultured neurons. The expression of Nurr1 was also significantly upregulated in vm DA neurons cultured on the PDL-coated plates compared to those 3D cultured in both peptide scaffolds and between vm DA neurons 3D cultured in IVFK based scaffold in comparison to those cultured in IVZK based scaffold (figure 6(b)). As for Lmx1a, the expression was found significantly upregulated in uncultured primary vm DA neurons compared to 3D-cultured vm DA neurons in IVFK- and IVZK-based scaffolds. In addition, Lmx1a was found to be significantly upregulated in neurons cultured on PDL compared to those 3D cultured in IVZK-based scaffolds (figure 6(c)). A significant upregulation in the expression of En1 was observed in uncultured vm DA neurons compared with those 3D cultured in IVZK-based scaffolds. Additionally, a significant upregulation in En1 expression was observed in neurons 3D cultured within IVFK-based scaffolds in comparison to those cultured in IVFK-based scaffolds (figure 6(d)).

4. Neurosphere migration assay

We assessed the effect of IVFK and IVZK on the migration of vm DA and vm non-DA neurons out of neurospheres that we developed for 5 d before seeding on the surface of PDL, or encapsulating in IVFK, and IVZK based scaffolds. Confocal images taken on day 3 for neurospheres stained with antibodies against TUJ1 and TH and nuclei counterstained with DAPI showed successful migration of the vm DA and vm non-DA neurons out of the neurospheres towards PDL surface, and within IVFK, and IVZK scaffolds (figures 7(a) and (b)).

4.1. Functionality assessment of vm DA and vm non-DA neurons in the newly developed 3D in vitro models

4.1.1. Extracellular spontaneous activity recording from 2D- and 3D-cultured vm DA and vm non-DA neurons using MEAs

Spontaneous activity of mouse primary embryonic vm DA and vm non-DA neurons cultured on PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds was recorded using MEAs (figure 8). In the long term, vm DA and vm non-DA neurons showed more stimulated activity overall in the 3D models in comparison to PDL. By comparing recordings at day 15, we found that the average numbers of both spikes and bursts were greater in 3D-cultured vm DA and vm non-DA neurons, especially those cultured in IVZK, when compared to those cultured on PDL-coated plates (figures 8(a) and (e)). In addition, an assessment of the distribution of the average number of spikes from the three most active electrodes over 1000 ms showed differences in the patterns of activity among the three different treatments (figure 8(b)). Spontaneous activity could be maintained without loss of signal in vm DA and vm...
non-DA neurons cultured on PDL for 15 d, encapsulated in IVFK scaffolds for 34 d, and in IVZK scaffolds for 42 d (figures 8(c), S5, and videos S3–S5). Additionally, burst activity was analyzed for primary mouse vm DA and vm non-DA neurons cultured on PDL, or encapsulated in IVFK, and IVZK based scaffolds (figures 8(d)–(f) and S4). Our data show differences in the burst pattern, with the highest

Figure 4. Analysis of vm DA and vm non-DA neurons’ morphological changes modulated by peptide scaffolds in comparison to PDL. (a) Confocal images of TUJ1+ (green) and TH+ (red) vm DA and vm non-DA neurons. Nuclei was counterstained with DAPI (blue). (b) Histograms of analysis of neurite outgrowth of TUJ1+ vm non-DA neurons and (c) TH+ vm DA neurons. Data are represented as mean ± SD, n = 3 experiments. Scale bar: 50 µm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
number of spikes per burst and the longest burst duration recorded in vm DA and vm non-DA neurons 3D cultured in IVZK based scaffold at day 15 (figures 8(e) and (f)), and the longest interburst interval observed in neurons encapsulated in IVFK based scaffold (figure 8(d)).

4.2. Effect of 6-OHDA on 2D- and 3D-cultured vm DA neurons
The 6-OHDA is a neurotoxin used to induce PD in vitro and in vivo. We treated E12.5 primary mouse vm DA neurons 2D cultured on PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds with 6-OHDA and measured the number of viable neurons and neurites. We found that 6-OHDA significantly reduced the number of viable neurons and neurites in both 2D and 3D cultures. However, 3D culture in peptide scaffolds protected against 6-OHDA toxicity compared to 2D culture on PDL-coated plates.
scaffolds with different concentrations of 6-OHDA and assessed the effect on both ATP release and the number of TH+ mature vm DA neurons after 48 h. Our results showed a dose-dependent decrease in the ATP levels of vm DA neurons 2D cultured on the PDL-coated plates and the ones encapsulated in IVFK and IVZK peptide scaffolds (figure 9(a)). Additionally, a decrease in the number of TH+...
neurons compared to non-treated vm DA neurons was observed, as expected (figure 9(b)).

4.3. Vascularization and co-culturing of vm DA and vm non-DA neurons

The vascularization of engineered tissues is crucial for the long-term survival of cells, especially in complex 3D constructs [36]. To address this, we co-cultured primary vm DA and vm non-DA neurons as well as cortical neurons with HUVECs. We also aimed to assess the potential of IVFK and IVZK peptide scaffolds to maintain both neuronal and endothelial cells in a co-culture. Confocal images of vm DA and vm non-DA and cortical neurons co-cultured with HUVECs showed neurons expressing TUJ1 and HUVECs expressing CD31. Interestingly, neurons tended to form dense networks of neurites in the presence of HUVECs (figures 10 and S1).

To confirm that the stimulation effect of the neurite network density was induced by HUVECs and was not due to other factors or to co-culture with other cell types, we focused on the interactions of vm DA neurons with cortical neurons. Cortical neurons are one of the targets with which vm DA neurons...
communicate in the brain [37, 38]. Thus, studying the ability of vm DA neurons to establish connections with cortical neurons in our 3D model is valuable for establishing multicellular functional 3D models. Confocal images taken on day 3 of immunostained primary mouse vm DA and cortical neurons showed no visible effect on neurite formation or density (figures S3(A) and (B)). However, we observed...
the formation of neurite connections between TH+vm DA neurons and TBR1+ cortical neurons (figure S3(B)). In addition, measurement of ATP release on day 3 from vm DA and cortical primary neurons monocultures and the co-culture showed increased ATP release in the co-culture when compared to the vm DA neurons monocultures (figure S3(C)). Spontaneous activity was recorded in the co-culture of vm DA and cortical neurons on PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds on day 7. An increase in neuronal activity was generally observed in vm DA and cortical primary mouse neurons co-cultured in IVFK- and IVZK-based scaffolds when compared to those cultured on PDL-coated plates. This increase in activity was indicated by a higher number of spikes and bursts from 3D-cultured neurons, with IVFK showing the highest numbers of spikes. However, spikes from neurons 3D cultured in IVZK-derived scaffolds had the highest average frequency, reaching more than 150 Hz, in comparison to less than 50 Hz in the case of vm DA neurons in IVFK scaffolds and in PDL-coated plates (figures S3(D)–(G)).

4.4. 3D bioprinting of a vm DA and vm non-DA neuronal model

One of the main objectives of our study was to achieve mechanical stability of peptide-based constructs using our custom 3D bioprinting process. We 3D bioprinted various structures inspired by mouse brain anatomy to assess shape fidelity, optimize printing parameters, and observe cell viability. We implemented three different approaches to develop the 3D in vitro mouse brain construct: 3D bioprinting using a dual coaxial nozzle (figure 11(c, II)), 3D bioprinting with mold support (figure 11(c, I)), and 3D constructs using two cross-sectional molds to create the full brain structure (figure 11(c, III)).

Low-complexity 3D constructs, including cylindrical rings, mouse VM models, and semi-filled cubes, were 3D bioprinted with cells using both IVZK and IVFK peptides (figure 11(c, IV)). IVZK peptide hydrogel was found to have mechanically stiffer properties, which corroborates the results of previous studies [11, 39], and to thus demonstrate better shape fidelity. The flow rates differed slightly for each peptide to accommodate their gelation rate. IVZK peptide hydrogel was pumped at a slightly higher flow rate to compensate for its faster gelation and to maintain continuous flow. When 3D bioprinting cell-laden constructs, the concentration of both peptides was reduced from 13 mg ml$^{-1}$ to 10 mg ml$^{-1}$ to decrease the overall peptide content in the 3D construct and allow sufficient nutrient diffusion for the cells.

Three-dimensional bioprinting of a mouse brain model was initially attempted at a full-size scale using optimized printing parameters. However, the printing resolution and shape fidelity were visibly reduced due to the intricate nature and size of the mouse brain. The CAD model was then enlarged to a 2:1 scale (figure 11(c, II)), and, while this considerably improved the print quality, further improvement was desired at a 1:1 scale. To this end, a support mold was designed and fabricated using an SLA 3D printer to act as a base scaffold during 3D bioprinting (figure 11(b)). A biocompatible elastic resin was used to create a mold to allow easy removal of the construct after printing. The mold offered support to the bottom half of the 24-layer structure to shape its crevices and guide the bioink flow for the remaining half of the construct. This remarkably improved shape fidelity and mechanical stiffness, highlighting the different regions of the mouse brain model and producing a consistent solid model scaled to the actual size (figure 11(c, I)).

In the third approach, mouse brain constructs were molded using two cross-sectional molds to create a completely molded structure. The cells were extruded on the top of each half of the scaffold (figure 11(c, III)). This method proved to be effective in creating stable peptide-based scaffolds. It is also recommended as a cost-effective alternative in situations in which 3D bioprinting is not feasible or when cell-laden structures are not needed. A mold can be easily designed and created using a high-end 3D printer and can be used to create a bioink scaffold. Rapid prototyping can ensure easy repeatability and customization.

Another key factor in 3D bioprinting of the mouse brain model to scale was maintaining seamless flow of peptide bioink through the nozzle without excess gelation or inconsistencies. To achieve this, a protocol involving automated time-dependent pulsing was implemented with microfluidic pumps; this protocol had previously been optimized for printing lines and basic grid shapes [17]. Pumping with a square pulse flow profile was found to assist in maintaining continuous flow by alternately reducing peptide and PBS flow rates at specific time points while maintaining the overall volume in the nozzle at any given time constant at 80 µl min$^{-1}$. Using automated pulsing when printing a complex construct such as a mouse brain enabled continuous pumping for longer periods of time and avoided manual interruptions due to clogging or excess gelation. Cell viability was assessed using a live/dead assay on a number of 3D-bioprinted constructs on day 3, and the cells were found to be viable. In addition, immunostained 3D bioprinted vm DA and vm non-DA neurons were found to express TUJ1 on day 3 (figure 11(d)).

5. Discussion

The study of neurodegenerative diseases such as PD presents many challenges due to the complexity of the human brain and the difficulty in obtaining
specimens. Therefore, there is an immense need to develop efficient *in vitro* 3D models that can recapitulate both the disease and the healthy states of the specific organs or tissues under study. Advancements in bioengineering and the emergence of 3D bioprinting technologies in recent years have led to
the successful development of multiple in vitro 3D models that represent effective tools to study cellular systems and complex disease [12–14]. However, the availability of efficient 3D neuronal models is limited, and the majority rely on organoids [40–42]. This can be attributed to the lack of suitable biomaterials that can create 3D environments resembling the extracellular matrix without adversely affecting the neurons. In this study, we aimed to address the obstacles in obtaining healthy and diseased 3D in vitro PD models with the use of newly developed approaches and biomaterials. We used ultrashort peptides that self-assemble into hydrogels, sustain the 3D growth of neuronal cells, and can be 3D bioprinted. Two peptide sequences that showed promise in other applications were selected for this study: Ac-Ile-Val-Cha-Lys-NH₂ (IVZK) and Ac-Ile-Val-Phe-Lys-NH₂ (IVFK) [12–14].

Firstly, we evaluated the chemical-physical properties of the peptides using several characterization techniques. The fibers that allow the peptides to form hydrogels are clearly visible in SEM, TEM, and AFM. The AFM topography of the IVFK and IVZK fibers showed that IVFK formed a more homogenous supramolecular structure. Additionally, the IVFK helical structure was left-handed, while that of IVZK was right-handed. This result recalls that of a previous study [39], where it was observed that the peptide containing Phe forms left-handed supramolecular helical fibers, while the peptide containing Cha forms right-handed supramolecular helical fibers. The results of Raman spectroscopy for IVFK and IVZK showed that the secondary structures of both peptides contained mostly β-turns. In IVFK, the presence of α-helix was emphasized, which confirms data from previous studies [31]. The presence of α-helix to a greater degree leads to slower kinetics of IVFK peptides when compared to IVZK peptides. Overall, these results support the rheology measurements and the molecular dynamic simulations performed previously for the two peptides [11]. These findings lead to the observation that IVZK peptide is more rigid than IVFK peptide. While both peptides showed comparable results in terms of manual 3D culturing, IVZK is expected to perform better in 3D bioprinting applications. It was previously demonstrated that Cha-containing peptides perform better in 3D bioprinting [39].

Assessment of cell viability using the live/dead assay at DIV1, 2, and 3 showed that the majority of 3D-cultured vm DA and vm non-DA neurons in IVFK- and IVZK-derived scaffolds were viable. Interestingly, a decrease in the viability of vm DA and vm non-DA neurons cultured on the PDL-coated plates was observed on day 3 (figure 3(a)). This finding supports the assumption that neither IVFK nor IVZK peptides are cytotoxic to this type of cell. To support these data, we also measured ATP release from primary mouse vm DA and vm non-DA neurons 2D cultured on PDL-coated plates and encapsulated in IVFK and IVZK peptide scaffolds on days 2 and 3 of culture. ATP acts as a neurotransmitter or co-transmitter in many regions of the central and peripheral nervous systems [43]. One of the known efflux mechanisms includes ATP accumulation in synaptic vesicles and calcium-dependent exocytosis [44–46]. It has been shown that energy starvation leads to deviations in cytoplasmic dopamine and intracellular calcium levels from the normal concentrations. A significant increase in dopamine and intracellular calcium levels leads to the formation of alpha-synuclein aggregates and thus, plays an important role in the pathogenesis of PD [47]. This makes ATP release an important measure for DA neurons’ health in vitro, especially when exposed to different chemical and mechanical cues, owing to its interaction with the scaffolds. Our data show that the ATP level was increased at day 3 compared to day 2 in the case of 3D-cultured vm DA and vm non-DA neurons in both peptide-based scaffolds, while it was slightly decreased over time in the case of PDL-coated plates (figure 3(b)).

Quantification of neurite outgrowth demonstrated that, compared to neurons cultured in PDL-coated plates, 3D-cultured vm DA and vm non-DA neurons showed an overall increase in the number of branches. In the case of IVFK based scaffolds, the observed increase in the number of branches of TH+ vm DA neurons was highly significant when compared to PDL-coated plates. Interestingly, it has been shown that selegiline (1-desprenyl), which is used in the treatment of PD, primarily leads to an increase in the neurites’ outgrowth, especially the number of branches. The increase in the number of branches induced by selegiline may play a role in its mechanism of action against neurodegenerative diseases [48].

Furthermore, as cell migration is an important process during tissue development, repair, and disease, neurosphere assays are key in vitro tests for developmental neurotoxicity [49]. Our data show that primary mouse vm DA and vm non-DA neurons successfully migrated out of the neurospheres towards the surface of PDL-coated and within IVFK and IVZK scaffolds in which they were encapsulated (figure 7). This finding confirms that both IVFK and IVZK support the development of vm DA and vm non-DA neurons and suggests the absence of any chemical or mechanical factors that may have an influence on radial migration away from adherent neurospheres in our developed 3D model [50].

To assess the functionality of neurons in our newly developed 3D models, we measured the spontaneous action potential at different time points using the MEAs, which simultaneously capture the field extracellular activity of a population of neurons [51]. Here, we report, for the first time, the use of MEAs
to record primary vm DA and vm non-DA neurons activity when encapsulated in ultrashort peptide-based 3D scaffolds. Our data show stimulation of the spontaneous activity of vm DA and vm non-DA neurons in our 3D models in comparison to what was observed from neurons cultured on PDL-coated wells. We also recorded spontaneous activity from vm DA and vm non-DA neurons encapsulated in IVZK and IVFK peptide scaffolds for more than 1 month without signal degradation. Interestingly, differences in the firing pattern between mouse primary vm DA and vm non-DA neurons cultured on the PDL-coated wells and the ones encapsulated in IVFK and IVZK peptide scaffolds were observed, as shown by the mean burst duration, interbursts intervals, and the average number of spikes. This finding highlights the influence of the mechanical and chemical cues induced by the culture strategy on the function of vm DA and vm non-DA neurons, and thus the efficiency of the developed in vitro model or its potential to recapitulate the in vivo situation.

Quantitative PCR was performed solely on vm DA neurons to assess the expression levels of Th, Nurrl, En1, Lmx1a, and Ptix3 in uncultured primary mouse vm DA neurons and in those 2D cultured on PDL-coated plates and 3D-cultured in IVFK- and IVZK-derived scaffolds. All the genes included in this study have pivotal roles in the development, maintenance, and differentiation of vm DA neurons TH is the rate-limiting enzyme for the synthesis of DA neurons and is thus, the first indication of the development of the DA neuronal phenotype [52-54]. Differentiation of DA neurons into post-mitotic neurons is promoted by the expression of Nurrl and Ptix3. Both genes cooperatively regulate neurogenesis in DA neurons [55, 56]. Lmx1a plays a major role in regulating the expression of Nurrl and Ptix3 [57, 58] and thus plays an integral role in the specification, differentiation, and maintenance of DA neurons [59]. It has been shown that in Engrailed-1 and 2 (En1/2) knockout mice, neurons die shortly after birth [60]. Additionally, previous studies have shown interactions between DA development and the expression of Lmx1a, Nurrl, and En1 [61-63]. Our data showed that Th, Nurrl, En1, Lmx1a, and Ptix3 were expressed under all conditions, including in the 3D models. This further demonstrates the successful development and maturation of vm DA neurons in the newly developed 3D models.

One of our main aims was to provide an efficient 3D system for in vitro modeling of PD [64]. To test whether our 3D models could mimic the histological or biochemical characteristics of PD in vitro, primary neurons were treated with 6-OHDA. The 6-OHDA is a classical neurotoxin used to induce PD in vitro and in vivo by dopamine depletion, leading to DA neuronal degeneration [27, 29]. Our data showed a dose-dependent decrease in ATP levels in vm DA neurons cultured on the PDL-coated plates and the ones encapsulated in the IVFK- and IVZK-based scaffolds (figure 9(a)). In addition, the number of TH+vm DA neurons was lower than that in the untreated controls (figure 9(b)). This response of vm DA neurons to 6-OHDA was expected and has been observed in previous studies [65, 66]. Interestingly, in IVZK-derived scaffolds, the decrease in the number of TH+vm DA neurons treated with different concentrations of 6-OHDA was slightly less than in untreated cells (figure 9(b)).

Several studies have discussed the role of vascularization in the long-term survival of cells in complex and multicellular 3D in vitro models. Vascularization allows for efficient nutrient exchange and waste disposal, leading to the development of in vitro bioengineered 3D constructs that mimic the physiology of natural tissues. These features make in vitro 3D models a promising tool for biomedical applications such as in vitro drug testing or, in some cases, organ transplantation [36, 67, 68]. To test the ability of IVFK and IVZK peptide scaffolds to accommodate endothelial cells in our 3D neuronal models, HUVECs were co-cultured with vm DA and vm non-DA neurons. Confocal images on day 3 showed the formation of highly dense networks of neurites in the presence of HUVECs when compared to images taken in the absence of HUVECs on day 3 and even on day 8 of culture. The same observation was made when primary mouse cortical neurons were co-cultured with HUVECs. A recently published study reported similar observations of increased stimulation of neuritogenesis in dorsal root ganglia explants in the presence of HUVECs and adipose-derived stem cells [69].

To further confirm that the formation of denser neurite networks could be attributed to the presence of HUVECs and was not induced by co-culturing vm DA and vm non-DA neurons with any other type of cells, we also co-cultured primary mouse cortical and vm DA neurons. No enhancement of neurite outgrowth was observed in the confocal images taken on day 3; however, we observed neurite overlaps between TH+ and TBR1+ neurons. We also observed a homogeneous distribution of vm DA and vm non-DA neurons labeled with tracker green dye and cortical neurons labeled with tracker red in the PDL-coated plates and in the IVFK and IVZK peptide scaffolds (figure S3(A)). These results support the assumption that the stimulation of neurite outgrowth was most likely induced by HUVECs. Additionally, communication between vm DA and cortical neurons in the brain has been shown in previous studies [37, 38], which makes the development of a 3D model that can accommodate both vm DA and cortical neurons in a functional state a task of high importance. Generally, these data demonstrate the potential of our developed 3D models to successfully maintain more than one type of neuronal cell, which may eventually lead to the creation of complex multicellular 3D systems to model neurological disorders.
In this study, we aimed to demonstrate the suitability of the selected tetrameric self-assembling peptide bioinks to create mechanically stable 3D bioprinted constructs that can be used to model PD disease in vitro. Our goal was to test the potentials of IVFK and IVZK bioinks for the 3D bioprinting of simple and complex structures.

To facilitate 3D bioprinting of more complex constructs such as the mouse brain, newly developed techniques were incorporated to achieve higher print resolution and a smoother printing process. For better shape fidelity, a mold was created from the negative imprint of the desired model and 3D printed with elastic biocompatible resin to promote an easy release of the printed/molded brain. This technique allows the bioink material to settle and take shape before the intricate details of the model are constructed. The mold was used as a base support structure when printing the model and was also used as a two-way complete mold without printing. Both techniques proved effective in maintaining actual size scale and quality print resolution without compromising fidelity or cell viability. The mold technique is recommended as a cost-effective alternative in situations in which 3D bioprinting is not feasible or when cell-laden structures are not needed. A mold can be easily designed and created using a high-end 3D printer and can act as a bioink scaffold. Rapid prototyping ensures easy repeatability and customization. It is also a time-efficient approach when a print model requires support structures, as it does not require the loading of additional support material. Since it can be prepared in advance, it does not prolong the printing process.

Another technique employed to better facilitate 3D bioprinting was automated pulsing using the 3D bioprinting system’s microfluidic pumps. As reported in [17], this technique allows for smoother printing by regulating the flowrate at different time intervals. By observing the user’s manual adjustments to the pump flow during printing, a square pulse wave profile was created to mimic the adjustments through automation. At intervals of 45 s, the flow rate was adjusted for each pump within a preset range. It was found that when printing a complex construct such as a mouse brain, automated pulsing enabled continuous pumping for longer periods of time without manual interruptions due to clogging or excess gelation. This approach is highly valuable for bioinks with instantaneous gelation properties and for less viscous solutions, as it avoids clumping and clogging within the nozzle and thus allows for a smoother printing process. Cell viability testing using a live/dead assay on day 3 showed that the cells were viable after bioprinting.

Generally, VM neurons did express TUJ1, which indicates that the development of neuronal cells was not affected throughout the printing process. Notably, the constructs maintained their shape and stability for over 3 months in the 4% paraformaldehyde fixed constructs with vm DA and vm non-DA neurons, indicating the potential for long-term shape fidelity.

6. Conclusion

In this study, we developed functional 3D DA and non-DA neuronal models using nanofibrous scaffolds derived from two ultrashort self-assembling tetrapeptide compounds, IVFK and IVZK. Both peptide scaffolds induced the formation of an increased number of neurite branches in vm DA and vm non-DA neurons compared to PDL-coated plates. Interestingly, 3D co-cultures of vm DA and vm non-DA neurons and HUVECs within the same peptide scaffolds demonstrated dense 3D neural network formation. We believe that this effect is induced or supported through vascularization. We observed a similar increase in neuronal network formation using cortical neurons in the presence of HUVECs. When following up on potential neuronal activities by electrophysiological recordings, we observed prominent action potentials caused by the spontaneous activity of vm DA and vm non-DA neurons encapsulated in the fabricated 3D peptide scaffolds. We monitored the action potential of the vm DA and vm non-DA neurons in the 3D peptide scaffolds for more than four weeks. Using the peptide biomaterials as bioinks, we successfully bioprinted a mouse brain-inspired 3D vm DA and vm non-DA neuronal model. We propose that the 3D in vitro models presented in this study represent an efficient platform for PD modeling for future studies in which long-term survival of vm DA neurons is required, and we hope that they will lead to a better understanding of the pathogenesis of PD and potentially other neurodegenerative disorders, and, thus, to better treatment options.

Ethics statement

This study followed international guidelines for the use of animals in research, applying the standards described in the Guide for the Care and Use of Laboratory (8th edition), ‘National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals.’ (2011), 62-147. All experimental protocols were approved by the ethics committees of King Abdullah University of Science and Technology (KAUST) and King Abdulaziz University (KAAU) (7-CEGMR-Bioeth-2018, 21ACUC06, and 21IBEC023).

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).
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Conflict of interest

The authors declare no competing financial interest.

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References

[1] Adl M M, Rodrigues G M, Kulkarni R U, Rao A T, Chernavsky N E, Miller E W and Schaffer D V 2017 Efficient generation of HPSC-derived midbrain dopaminergic neurons in a fully defined, scalable, 3D biomaterial platform Sci. Rep. 7 1–11
[2] Brito C, Simão D, Costa I, Malpique R, Pereira C I, Fernandes P, Serra M, Schwarz S C, Schwarz J and Kremer E J 2012 Generation and genetic modification of 3D cultures of human dopaminergic neurons derived from neural progenitor cells Methods 56 452–60
[3] Moreno E L, Hachi S, Hemmer K, Trietsch S J, Baumuratov A S, Hankemeier T, Vulto P, Schwamborn J C and Fleming R M 2015 Differentiation of neuroepithelial stem cells into functional dopaminergic neurons in 3D microfluidic cell culture Lab Chip 15 2419–28
[4] Gjorevski N, Sachs N, Manfrin A, Giger S, Bragina M E, Ordóñez-Moran P, Clevers H and Lutolf M P 2016 Designer matrices for intestinal stem cell and organoid culture Nature 539 560–4
[5] Caliari S R and Burdick J A 2016 A practical guide to hydrogels for cell culture Nat. Methods 13 405–14
[6] Alge D L, Azagarsamy M A, Donohue D F and Anseth K S 2013 Synthetically tractable click hydrogels for three-dimensional cell culture formed using tetrazine–norbornene chemistry Biomacromolecules 14 949–53
[7] Chapman H N, Fromme P, Barty A, White T A, Kirian R A, Aquila A, Hunter M S, Schulz J, DePonte D P and Wei et al 2011 Femtosecond x-ray protein nanocrystallography Nature 470 73–77
[8] Zhao X, Pan F, Xu H, Yaseen M, Shan H, Hauser C A, Zhang S and Lu J R 2010 Molecular self-assembly and applications of designer peptide amphiphiles Chem. Soc. Rev. 39 3840–98
[9] Loo Y, Golias M, Tekinay A B, Guler M O, Hauser C A and Mitraki A 2015 Self-assembled proteins and peptides as scaffolds for tissue regeneration Adv. Healthcare Mater. 4 2557–86
[10] Mishra A, Loo Y, Deng R, Chuah Y J, Hee H T, Ying Y J and Hauser C A 2011 Ultrasmall natural peptides self-assemble to strong temperature-resistant helical fibers in scaffolds suitable for tissue engineering Nano Today 6 232–9
[11] Rauf S et al 2021 Self-assembling tetramer peptides allow in situ 3D bioprinting under physiological conditions J. Mater. Chem. B 9 1069–81
[12] Ramirez-Calderon G, Susapto H H and Hauser C A 2021 Delivery of endothelial cell-laden microgel elicits angiogenesis in self-assembling ultrashort peptide hydrogels in vitro ACS Appl. Mater. Interfaces 13 29281–92
[13] Arab W T, Niyas A M, Seferji K, Susapto H H and Hauser C 2018 Evaluation of peptide nanogels for accelerated wound healing in normal microgrips Front. Nanosci. Nanotechnol. 4 1–9
[14] Alshehri S, Susapto H H and Hauser C A 2021 Scaffolds from self-assembling tetrapeptides support 3D spreading, osteogenic differentiation, and angiogenesis of mesenchymal stem cells Biomacromolecules 22 2094–106
[15] Seferji K A, Susapto H H, Khan B K, Rehman Z U, Abbas M, Emwas A H and Hauser C A 2021 Green synthesis of silver-plate nanoparticles generated by the photoionization process for anti-biofilm application ACS Appl. Bio Mater. 4 8522–35
[16] Oskui S M, Diamante G, Liao C, Shi W, Gan J, Schlenk D and Grover W H 2016 Assessing and reducing the toxicity of 3D-printed parts Environ. Sci. Technol. Lett. 3 1–6
[17] Khan Z, Kahin K and Hauser C A 2021 Time-dependent pulsing of microfluidic pumps to enhance 3D bioprinting of peptide bioinks Biomaterials, BioMEMS, and Medical Microsystems XIX (Bellingham, WA: International Society for Optics and Photonics) (https://doi.org/10.1117/12.2578830)
[18] Albalawi H I et al 2021 Sustainable and eco-friendly coral restoration through 3D printing and fabrication ACS Sustain. Chem. Eng. 9 12634–45
[19] Jan Y-N and Jan L Y 2010 Branching out: mechanisms of dendritic arborization Nat. Rev. Neurosci. 11 316–28
[20] Guide for the Care and Use of Laboratory Animals National research council (US) committee for the update of the guide for the care and use of laboratory animals 62–147
[21] Gantner C W, Cota-Coronado A, Thompson I H and Parish C I 2020 An optimized protocol for the generation of midbrain dopamine neurons under defined conditions STAR Protocols 1 100065
[22] Bédard A and Parent A 2004 Evidence of newly generated neurons in the human olfactory bulb Dev. Brain Res. 151 159–68
[23] Lüdecke B, Knapskog P M, Clayton P T, Surtees R A, Clelland J D, Heales S J, Brand M F, Bartholomé K and Flattmark T 1996 Recessively inherited L-DOPA-responsive Parkinsonism in infancy caused by a point mutation (L203P) in the tyrosine hydroxylase gene Hum. Mol. Genet. 5 1023–8
[24] Alsanie W F, Bahri O A, Habeeballah H H, Alhomrani M, Almehmadi M M, Alsharif K, Feltbamn E M, Alhboibati Y S, Almalki A H and Alsaaab H O 2020 Generating homogenous cortical preplate and deep-layer neurons using a combination of 2D and 3D differentiation cultures Sci. Rep. 10 1–11
[25] Alsanie W, Penna V, Schachner M, Thompson L and Parish C 2017 Homophilic binding of the neural cell adhesion molecule CHL1 regulates development of ventral midbrain dopaminergic pathways Sci. Rep. 7 1–10
[26] Blakely B D, Bye C R, Fernando C V, Horne M K, Oskui S M, Diamante G, Liao C, Shi W, Gan J, Schlenk D and Grover W H 2016 Assessing and reducing the toxicity of 3D-printed parts Environ. Sci. Technol. Lett. 3 1–6
[27] Blum D, Torch S, Lambeng N, Nissou M-F, Benabid A-L, Sadoul R and Verna J-M 2001 Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease Prog. Neurobiol. 65 135–72
[28] Deumens R, Blokland A and Prickaerts J 2002 Modeling Parkinson’s disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway Exp. Neurol. 175 303–17
[29] Gomez-Lazaro M, Bonekamp N A, Galindo M F, Jordán J and Schrader M 2008 6-Hydroxydopamine (6-OHDA) induces Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells Free Radics. Biol. Med. 44 1960–9
McInturff S 2018 Mouse Skull and Brain (available at: www.thiingverse.com/things/3079327)

Hauser C A et al 2011 Natural tri- to hexapeptides self-assemble in water to amyloid beta-type fiber aggregates by unexpected alpha-helical intermediate structures Proc. Natl Acad. Sci. USA 108 1361–6

Zhang P et al 2020 A droplet reactor on a super-hydrophobic surface allows control and characterization of amyloid fibril growth Commun. Biol. 3 857

Müller J P and Jacobs G A 1984 Relationships between neuronal structure and function J. Exp. Biol. 112 129–45

Lee S, Choi K, Ahn H, Song K, Choe J and Lee I 2005TuJ1 (class III β-tubulin) expression suggests dynamic redistribution of follicular dendritic cells in lymphoid tissue Eur. J. Cell Biol. 84 453–9

Tedjakumala S R, Rouquette J, Boizeau M-L, Mesce K A, Hoter L, Masso I and Giurfa M 2017 A tyrosine-hydroxylase characterization of dopaminergic neurons in the honey bee brain Front. Syst. Neurosci. 11 47

Chen E P, Toksoy Z, Davis B A and Geibel J P 2021 3D bioprinting of vascularized tissues for in vitro and in vivo applications Front. Bioeng. Biotechnol. 9 526

Bertram C, Dahlan I, Boorman L W, Harris S, Vaastrelle N, Leriche M, Redgrave P and Overton P G 2014 Cortical regulation of dopaminergic neurons: role of the midbrain superior colliculus J. Neurophysiol. 111 755–67

Bouarab C, Thompson B and Polter A M 2019 VTA GABA neurons at the interface of stress and reward Front. Neural Circuits 13 78

Susapto H H, Alhattab D, Abdelrahman S, Khan Z, Alshehri S, Kahin K, Ge R, Moretti M, Emwas A-H and Hauser C A 2021 Ultrashort peptide biosinks support automated printing of large-scale constructs assuring long-term survival of printed tissue constructs Nano Lett. 21 2719–29

Chia S J, Tan E-K and Choy Y X 2020 Historical perspective: models of Parkinson's disease Int. J. Mol. Sci. 21 2464

Rai S N and Singh P 2020 Advancement in the modelling and therapeutics of Parkinson’s disease J. Chem. Neuroanat. 104 101752

Smit M L and Schwamborn J C 2020 Midbrain organoids: a new tool to investigate Parkinson’s disease Front. Dev. Biol. 8 359

Choi Y, Jang J, Jang M, Kim S, Kang Y, Cho H, Chung S and Park M 2009 Modulation of firing activity by ATP in dopamine neurons of the rat substantia nigra pars compacta Neuroscience 160 587–95

Lazarowski E R 2012 Vesicular and conductive mechanisms of nucleotide release Purinergic Signal. 8 359–73

Mutafova-Yambolieva V N and Durnin L 2014 The purinergic neurotransmitter revisited: a single substance or multiple players? Pharmacol. Ther. 144 162–91

Ho T, Jobling A I, Grefeather U, Chuang T, Ramesh A, Fletcher E L and Vessey K A 2015 Vesicular expression and release of ATP from dopaminergic neurons of the mouse retina and midbrain Front. Cell Neurosci. 9 389

Post M R, Liebermann O J and Mosharab H F 2018 Can interactions between α-synuclein, dopamine and calcium explain selective neurodegeneration in Parkinson's disease? Front. Neurosci. 12 161

Kontkanen O and Castrén E 1999 Trojan effects of selegiline on cultured dopaminergic neurons Brain Res. 829 190–2

Nimtz L, Kloie J, Manjosthusmann S, Barenys M and Frischke E 2019 The neurosphere assay as an in vitro method for developmental neurotoxicity (DNT) evaluation Cell Culture Techniques (Berlin: Springer) pp 141–68

Durbec P, Franceschini I, Lazarini F and Dubois-Dalcq M 2008 In vitro migration assays of neural stem cells Neural Stem Cells (Berlin: Springer) pp 213–25

Spira M E and Hae A 2013 Multi-electrode array technologies for neuroscience and cardiology Nat. Nanotechnol. 8 83–94

Puelles L and Verney C 1998 Early neurogenic distribution of tyrosine-hydroxylase-immunoreactive neurons in human embryos J. Comp. Neurol. 394 283–308

Specht L A, Pickel V M, Joh T H and Reis D J 1981 Light-microscopic immunocytochemical localization of tyrosine hydroxylase in prenatatal rat brain. I. Early ontogeny J. Comp. Neurol. 199 233–53

Specht L A, Pickel V M, Joh T H and Reis D J 1981 Light-microscopic immunocytochemical localization of tyrosine hydroxylase in prenatatal rat brain. II. Late ontogeny J. Comp. Neurol. 199 255–76

Onoidei D, Acamopara D, Mancuso P, Prakash N, di Giovannantonio L G, Wurst W and Simeone A 2008 Anterior-posterior graded response to Otx2 controls proliferation and differentiation of dopaminergic progenitors in the ventral mesencephalon Development 135 3459–70

Hegarty S V, Sullivan A M and O'keeffe G W 2013 Midbrain dopaminergic neurons: a review of the molecular circuitry that regulates their development Dev. Biol. 379 123–38

Chung S, Leung A, Han B-S, Chang M-Y, Moon J-I, Kim C-H, Hong S, Pruszak J, Isacson O and Kim K-S 2009 Wnt1-lmx1a forms a novel autoregulatory loop and controls midbrain dopaminergic differentiation synergistically with the Shh-Ptc pathway Cell Stem Cell 5 646–58

Tan C Y, Levesque M, Claxton S, Johnson R L and Ang S-L 2011 Lmx1a and lmx1b function cooperatively to regulate proliferation, specification and differentiation of midbrain dopaminergic progenitors J. Neurosci. 31 12413–25

Anderson E, Trgygsvason U, Deng Q, Frieling S, Alekseenko Z, Robert B, Perlmann T and Ericson J 2006 Identification of intrinsic determinants of midbrain dopamine neurons Cell 124 395–405

Simon H H, Sauressig H, Wurst W, Goulding M D and O’Leary D D 2001 Fate of midbrain dopaminergic neurons controlled by the engrailed genes J. Neurosci. 21 3126–34

Sakurada K, Ohshima-Sakurada M, Palmer T D and Gage F H 1999 Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain Development 126 4017–26

Nakatani T, Kumai M, Mizuhara E, Minaki Y and Ono Y 2010 Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon Dev. Biol. 339 101–13

Simon H H, Thuret S and Alberi L 2004 Midbrain dopaminergic neurons: control of their cell fate by the engrailed transcription factors Cell Tissue Res. 318 53–61

Choeber A 2004 Classic toxin-induced animal models of Parkinson’s disease: 6-OHDA and MPTP Cell Tissue Res. 318 215–24

Ding Y M, Jaumotte J D, Signore A P and Zignmond M J 2004 Effects of 6-hydroxyp Parkinson on primary cultures of substantia nigra: specific damage to dopamine neurons and the impact of glial cell line-derived neurotrophic factor J. Neurochem. 89 776–89

Mochizuki T and Mohsenar H F 2003 Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture J. Neurosci. 23 428–35

Tomasina C, Bodet T, Mota C, Moroni L and Camarero-Espinosa S 2019 Bioprinting vasculature: materials, cells and emergent techniques Materials 12 2701

Sasmal P, Datta P, Wu Y and Oxbolat I T 2018 3D Bioprinting for Modelling Vasculature vol 2 (Hong Kong: Microphysiological Systems) (https://doi.org/10.21037/mpx.2018.10.02)

Chagas J S, Gomes E D, Afonso J L, Grana J, Silva N A, Shoichet M S, Sousa R A, Learmonth D A and Salgado A J 2020 In vitro evaluation of ASCs and HUVECs co-cultures in 3D biodegradable hydrogels on neurite outgrowth and vascularization Front. Cell Dev. Biol. 8 489